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An investigation into the relationship between process kinetics and microbial community dynamics in a lactate-fed sulphidogenic CSTR as a function of residence time and sulphate loading

By

Oluwaseun Oyekanmi Oyekola

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Dedicated to my family (THE “RUBIES”)…

This work would not have been possible without you.

My wonderful parents Olupappy and Olumummy Oyekola, my lovely siblings Kunle, Nike and Jummy

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An investigation into the relationship between process kinetics and microbial community dynamics in a lactate-fed sulphidogenic CSTR as a function of residence time and sulphate loading

Oluwaseun O. Oyekola

ABSTRACT

The treatment of acid mine drainage and other sulphate-laden wastewaters using biological sulphate reduction technology has been shown to offer several advantages. This process effectively removes the following toxicants: sulphate, heavy metals and acidity. Previous studies have also revealed that the sub-process, sulphate reduction, is influenced by the choice of electron donor, constituents of the microbial population mediating the process, the feed sulphate concentration and other operational parameters. The current work investigated the potential of an ecological approach in the treatment of sulphate-laden wastewaters. This was based on the relationship between the structure and function of the sulphate-reducing bacterial population. The main objective was to examine the effects of feed sulphate concentration and residence time on the kinetics of biological sulphate reduction and the community structure of the microbial system involved. Chemostat studies using a mixed microbial consortium of sulphate-reducing bacteria fed with lactate as the sole carbon-source and electron donor were carried out. Steady-state concentrations of sulphate, lactate, acetate, propionate and bicarbonate were measured to estimate the reaction stoichiometry and kinetics. 16S rRNA-based molecular techniques (fluorescence *in situ* hybridisation and restriction enzyme digestion analyses) were used to analyse the community structure and the relative abundance of microbial species involved in the microbial consortia. The results obtained were used to examine the link between the process kinetics and the community dynamics of the mixed culture of sulphate-reducing bacteria involved in the biological sulphate reduction treatment process.

Results obtained from this study showed that the volumetric sulphate loading rate, mediated through both sulphate concentration in the feed and dilution rate, significantly influenced the kinetics and the stoichiometry of biological sulphate reduction. The profiles obtained revealed that the process kinetics of a biological

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sulphate reduction system fed with lactate is dependent on the lactate metabolic pathway. At feed sulphate concentrations in the range 1.0 to 10.0 g l^{-1}, at low dilution rates (0.0083 to 0.014 h^{-1}) where incomplete lactate oxidation and concomitant sulphate reduction were dominant, the volumetric sulphate reduction rate was influenced positively by the volumetric sulphate loading rate. Substrate toxicity and the inhibition of bacterial growth were evident in the reactor fed with 15.0 g l^{-1} sulphate. At the higher dilution rates (0.021 to 0.014 h^{-1}), the utilisation of lactate via the oxidative pathway remained predominant in the reactors fed with sulphate concentrations of 1.0 and 10.0 g l^{-1}. In contrast, within the same range of dilution rates, lactate fermentation was pronounced at feed sulphate concentrations of 2.5, 5.0 and 15.0 g l^{-1}.

The molecular techniques, fluorescence *in situ* hybridisation and restriction enzyme digestion analyses, allowed the characterisation of the microbial populations present in the bioreactors employed. They revealed the presence of a starting inoculum composed of five sulphate-reducing bacteria, namely, *Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* and *Desulfococcus multivorans*. The occurrence of non-SRB lactate fermenter(s) (not identified) congruent with the propionate production was also established in the sulphidogenic system. Increased feed sulphate concentration and dilution rate led to reduced SRB microbial diversity. This suggested the selection of robust community members under extreme operating conditions. It was established that the metabolic shifts observed in this study was a consequence of changes in the microbial community structure. Further, the preservation of multi-component populations in the chemostat studies reported in the current work, despite varying experimental conditions, suggest the occurrence of cooperative interactions among the SRB strains. On the other hand, there was evidence suggesting a competitive interaction between the lactate fermenters and lactate oxidisers (SRB).

Using previously developed mathematical models, microbial growth constants (μ_{max} and K_s) and energetic coefficients (m_s and $Y_{x/s}$) were determined under lactate limiting conditions. These constants were incorporated into the Contois and Monod expressions to describe the kinetics of lactate oxidation and lactate fermentation

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respectively. There was also evidence to show that high sulphide concentration (0.5 g l^{-1}) is inhibitory to lactate fermenters while not significantly affecting the lactate oxidisers. Further, simulations were conducted to describe the competitive interaction between the lactate oxidisers (SRB) and lactate fermenters. Based on the kinetic properties of these two groups of bacteria, the lactate oxidisers were shown to out-compete the lactate fermenters under conditions of limiting lactate concentrations while the lactate fermenters dominated in the presence of excess lactate concentrations. In the current study, a multi-stage chemostat system operated under limiting lactate availability was shown to perform better than single-stage chemostat systems fed in stoichiometric ratio with respect to lactate and sulphate.

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NOMENCLATURE

ABBREVIATIONS

AcH	Acetic acid
AMBR	Anaerobic migrating blanket reactors
AMD	Acid mine drainage
APHA	American Public Health Association
APS	Adenosine phosphosulphate
APR	Acetate production rate
ARDRA	Amplified rDNA restriction analysis
AS	Assayed sulphide
ATP	Adenosine triphosphate
BSR	Biological sulphate reduction
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
D	Dilution rate
CV	Coefficient of variance
DAPI	4′, 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DS	Dissolved sulphide
ES	Expected sulphide
FAD	Flavin adenine dinuclotide (reduced)
FBR	Fluidised-bed reactor
FISH	Fluorescence *in situ* hybridisation
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
LC	Lactate conversion
LF	Lactate fermenters or fermentation
LO	Lactate oxidisers or oxidation
LUR	Lactate utilisation rate
MPA	Methanogens
n	Number of observations
NADH	Nicotinamide adenine dinucleotide (reduced)

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OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PHC	Petroleum hydrocarbon
PPR	Propionate production rate
PCR	Polymer chain reaction
PP	Pyrophosphate
rDNA	Ribosomal DNA
rRNA	Ribosomal ribonucleic acid
SC	Sulphate conversion
RT	Residence time
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SR	Sulphate reduction
SRA	Sulphate reducing archaea
SRB	Sulphate-reducing bacteria
SRR	Sulphate reduction rate
SSE	Sum of squared errors
T-RFLP	Terminal restriction fragment length polymorphism
TR	Trench reactor
TS	Total sulphide
UASB	Upflow anaerobic sludge blanket reactor
UPBR	Upflow packed bed reactor
VFA	Volatile fatty acids
VLUR	Volumetric lactate utilisation rate
VSLR	Volumetric sulphate loading rate
VSRR	Volumetric sulphate reduction rate
w/v	Weight by volume

ROMAN SYMBOLS		UNITS
D	Dilution rate	h^{-1}
e^-	Electron	
I	Concentration of the inhibitory compound	$g\ l^{-1}$

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$\mathbf{K_{e\text{-}d}}$	Half saturation constant for electron donor	$g\ l^{-1}$
$\mathbf{K_I}$	Half saturation constant of the inhibitory compound	$g\ l^{-1}$
$\mathbf{K_{SO_4^{2-}}}$	Half saturation constant for sulphate	$g\ l^{-1}$
$\mathbf{m_s}$	Maintenance coefficient	$g\ g^{-1}\ h^{-1}$
$\mathbf{q_s}$	Specific substrate utilisation rate based on substrate S	$g\ g^{-1}\ h^{-1}$
$\mathbf{r_A}$	Volumetric acetate production rate	$g\ l^{-1}\ h^{-1}$
$\mathbf{r_P}$	Volumetric propionate production rate	$g\ l^{-1}\ h^{-1}$
RL	Residual lactate concentration	$g\ l^{-1}$
$\mathbf{r_L}$	Volumetric lactate utilisation rate	$g\ l^{-1}\ h^{-1}$
$\mathbf{r_S}$	Volumetric sulphate reduction rate	$g\ l^{-1}\ h^{-1}$
$\mathbf{r_x}$	Rate of biomass formation	$g\ l^{-1}\ h^{-1}$
S	Substrate concentration	$g\ l^{-1}$
$\mathbf{S_i}$	Concentration of substrate produced	$g\ l^{-1}$
$\mathbf{S_o}$	Feed substrate concentration	$g\ l^{-1}$
T	Temperature	°C
X	Bacterial concentration	$g\ l^{-1}$
$\mathbf{Y_{x/s}}$	Yield coefficient of bacteria X based on substrate S	$g\ g^{-1}$
$\Delta \mathbf{G_{rxn}}$	Gibbs free energy of reaction	$kJ\ mol^{-1}$
$\Delta \mathbf{G_0'}$	Gibbs free energy of formation	$kJ\ mol^{-1}$

GREEK SYMBOLS

$\boldsymbol{\mu}$	Specific microbial growth rate	h^{-1}
$\boldsymbol{\mu_{max}}$	Maximum specific microbial growth rate	h^{-1}

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GLOSSARY

Abiotic	Describes a reaction condition in the absence of microorganisms.
Acetogenesis	The reaction that degrades short chain fatty acids such as propionic acid, butyric acid, or longer chain fatty acids, as well as other intermediates such as ethanol, to acetic acid and hydrogen.
Acidophilic	Describes an organism that has its growth optimum between about pH 1.0 and 5.5.
Acidogenesis	The process in which long chain soluble monomers or dimers, such as carbohydrates and amino acids, are reduced to short chain volatile fatty acids, such as acetic acid, propionic acid, butyric acid, lactic acid and ethanol, or longer chain fatty acids.
Anaerobic digestion	This is the process by which organic wastes are biologically transformed in the absence of oxygen.
Archaea	A group of microorganisms, genetically and metabolically different from bacteria. They appear to be survivors of an ancient group of organisms.
Autotrophic	Describes a microorganism that utilises carbon-dioxide as its sole carbon-source.
Batch culture	Microbial culture produced by inoculating a closed culture vessel containing a single batch of medium. Conditions within batch culture change dynamically with time.
Biosulphidogenesis	The generation of sulphide from biological sulphate reduction.
Chemical Oxygen Demand	A measure of the total amount of organic material in the waste stream.

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Chemostat	A continuous bioreactor culture into which medium is fed at the same rate as it is removed by reaction and flow out of the reactor, resulting in a constant chemical concentration as a function of time under steady-state.
Citric acid cycle (TCA cycle)	The metabolic cycle that oxidises acetyl coenzyme A to carbon dioxide and generates NADH and $FADH_2$ for oxidation in the electron transport chain with concomitant energy storage as ATP.
Competitive inhibitor	A molecule that inhibits enzyme activity by competing with the substrate at the enzyme's active site.
Eukaryotes	Organisms with cells having chromosomes with nucleosomal structure separated from the cytoplasm by a two membrane nuclear envelope and compartmentalisation of a function in distinct cytoplasmic organelles.
Heterotrophic	Describes a microorganism that utilises simple or complex organic molecules as its sole carbon-source.
Hybridisation	The reaction by which the pairing of complementary strands of nucleic acid occurs.
Inhibition	The suppression of bacterial function.
Inoculum	Microbial cells added to start a culture.
Mesophilic	Describes an organism that can grow in the temperature range 20 to 45°C.
Metabolism	The physiochemical transformations through which simple substrates are either synthesised into complex elements or complex substances are converted into simple ones and energy is made available for use by the organism.
Methanogenesis	The process through which low molecular weight substrates are degraded to form methane. It is a terminal stage of the anaerobic digestion involving methane-producing microorganisms called methanogens.

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Mixed culture	Microbial population consisting of two or more types of microorganisms.
Non competitive inhibitor	Inhibition of enzyme activity that results from the inhibitor binding at a site other than the active site and altering the enzyme's shape to make it less active.
Phylogenetic	Related to the evolutionary development and history of microorganisms or higher taxonomic grouping of organisms.
Pollution	An adverse alteration of the environment.
Syntrophic	A syntrophic reaction is one in which two (or more) organisms interact metabolically to consume a substrate that neither can consume independently.
Thermophilic	Describes an organism that can grow at high temperatures ≥45°C.

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CHAPTER 1
GENERAL INTRODUCTION

1.1. BACKGROUND

The exploitation of coal and metals from ores containing sulphidic minerals is responsible for the generation of substantial quantities of acid mine drainage (AMD). Acid mine drainage is generated on subsequent weathering of waste rock, tailings and surfaces exposed on mining. AMD is characterised by a low pH and elevated concentrations of heavy metals and sulphate. When discharged untreated, it poses a threat to the quality of fresh water resources and consequently the well-being of humans and the environment at large. AMD is a major concern in semi-arid South Africa, where water is a limited natural resource. The AMD generated causes the acidification and contamination of the ground water with heavy metals (Naicker *et al*., 2003).

Sulphate is the major pollutant in the wastewaters, both AMD and effluent streams, emanating from South African mining activities. Sulphate increases the salinity of receiving water bodies, which in turn reduces the availability of potable and usable water (Pulles *et al*., 1995). Therefore, one of the major aims of developing technology for AMD treatment in South Africa is to reduce the sulphate concentration to acceptable levels. Various approaches and technologies have been developed. These technologies show differing efficiencies, advantages and disadvantages (Feng *et al*., 2000; Burgess and Stuetz, 2002; Matlock *et al*., 2002; Doye and Duchesne, 2003).

Anaerobic biological sulphate reduction has attracted intensive research in recent years due to the advantages it offers over the other AMD treatment technologies. In this treatment process, sulphide and bicarbonate are produced by sulphate-reducing bacteria (SRB) in the presence of a suitable electron donor and carbon-source. The bicarbonate alkalinity neutralises acidity while dissolved metals are precipitated by the sulphide. These reactions are summarised in Reactions 1.1 to 1.3 (Drury, 1999).

$$Electron\ donor + SO_4^{2-} \rightarrow HS^- + HCO_3^- \qquad (1.1)$$

$$Me^{2+} + HS^- \rightarrow MeS \downarrow + H^+ \qquad (1.2)$$

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$$HCO_3^- + H^+ \rightarrow H_2O + CO_2 \quad (1.3)$$

where Me^{2+} represents the metal ion, such as Zn^{2+}, Cu^{2+}, Pb^{2+} and Ni^{2+}.

The anaerobic biological sulphate reduction (BSR) technology has been employed effectively under controlled and optimised conditions at both laboratory and full-scale. The use of two full-scale sulphidogenic technologies, namely, BioSURE® and Thiopaq® processes has been widely reported (Boonstra *et al*. 1999; Corbett, 2000; Rose *et al*., 2000; Hulshoff Pol *et al*., 2001; van Houten *et al*., 2006; Whittington-Jones *et al*., 2006). The provision of a suitable carbon-source and electron donor for this process remains a challenge. Lactate supports the growth of a wide spectrum of sulphate-reducing bacteria, encouraging microbial diversity and consequent treatment system resilience (Widdel and Pfennig, 1981; Ingvorsen *et al*., 1984; Widdel, 1987; Okabe and Characklis, 1992; Reis *et al*., 1992; Konishi *et al*., 1996; O'Flaherty and Colleran, 1998; Kaksonen *et al*., 2003).

Several studies have been undertaken to improve the overall performance of BSR for the treatment of AMD by focusing on the contributions of physicochemical factors such as the concentrations of sulphate, sulphide, dissolved oxygen, pH, choice of electron donor and operational parameters (Okabe and Characklis, 1992; Okabe *et al.,* 1992; 1995; Reis *et al*., 1992; van Houten *et al*., 1996; Chang *et al*., 2000; Sahinkaya *et al*., 2007). However, there is a dearth of literature concerning the influence of these factors on the active players in the consortium of SRB involved in BSR. Specifically the response of SRB to the perturbations and the subsequent effect on the overall performance of BSR has received little attention. There is a need to understand the biological response of the component species in the SRB consortia to physicochemical and operational parameters. This is in order to gain a better understanding of the community structure or dynamics of the sulphate-reducing consortia and the sulphate reduction process. Understanding these factors gives insight into how to maximise the efficiency of biological treatment of AMD using SRB.

The provision of a suitable carbon-source and electron donor for this process remains a challenge. Lactate is a potential carbon-source and electron donor, offering

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advantages in the biological sulphate reduction process. It supports the growth of a wide spectrum of SRB, encouraging microbial diversity and consequent treatment system resilience (Kaksonen *et al*., 2003). The exposure of a chemostat culture to changes in operational conditions selects for certain population members that can perform well under extreme conditions (Shuler and Kargi, 1992). Using lactate as a substrate for BSR has been reported to decrease sulphide toxicity (an impediment to anaerobic digestion). Its utilisation offers a better energy yield in comparison to other electron donors (H_2, ethanol, propionate and acetate). A survey of literature, based on the growth kinetic and thermodynamic properties of SRB grown on different electron donors, also substantiates the preferential utilisation of lactate as the sole electron donor.

A quantitative assessment of the BSR process is equally essential in gaining a better understanding of this treatment technology. In the present work, studies of the kinetics of biological sulphate reduction and the dynamics of the SRB community structure using lactate as the sole carbon-source are presented. In order to provide a comprehensive approach to assessing the BSR process, the inter-relatedness of the process kinetics, reaction stoichiometry and the microbial community dynamics was investigated.

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1.2. THESIS STRUCTURE

An extensive review of literature on previous investigations on acid mine drainage, and in the areas of biological sulphate reduction, sulphate reduction kinetics and microbial population analyses are presented in **Chapter 2**. Section 2.1 elucidates the current status of knowledge on acid mine drainage with respect to impact and treatment technologies. Emphasis is placed on the BSR process in the following sections. Sections 2.2 to 2.6 describe the BSR kinetics, motivation for the use of lactate as the electron donor for the current study, SRB community and the interaction of this group with other anaerobic microbial consortia. Thereafter, the relationship between microbial dynamics and reaction kinetics, and how this phenomenon can be investigated by combining both culture-dependent and –independent procedures are evaluated (Sections 2.7 to 2.8). The concluding part of this Chapter (Sections 2.9 to 2.11) describes the research motivation, hypotheses and the objectives of the study.

The procedures, experimental apparatus and the experimental programme employed in this thesis are described in detail in **Chapter 3**.

Results from the chemostat study of BSR kinetics are presented in **Chapter 4**. The effect of the volumetric sulphate loading rate, mediated through the feed sulphate concentration and the dilution rate, on the BSR kinetics is presented and discussed. Lactate, at 120% of the theoretical stoichiometric amount required for sulphate reduction, was added to the sulphidogenic CSTRs fed with media containing sulphate concentrations of 1.0 to 15.0 g l^{-1}.

Similar to the investigation presented in **Chapter 4**, the effect of the volumetric sulphate loading rate on both the lactate metabolism and microbial community dynamics are discussed in **Chapters 5 and 6** respectively. **Chapter 6** highlights the link between the biological sulphate reduction kinetics, stoichiometry of lactate utilisation and the microbial community dynamics. Using the kinetic data obtained in **Chapter 4**, a mathematical model describing the effect of feed substrate concentration on the BSR kinetics was explored. Details of this are presented in **Chapter 7**. This modelling approach was used to predict the relative dominance of competing populations, thereby proposing and validating appropriate experimental configurations.

Based on the findings of this study, conclusions are drawn and recommendations for further studies in this field of research are presented in **Chapter 8**.

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CHAPTER 2
LITERATURE REVIEW

This literature review provides a detailed background to previous work and reviews the theory on which this study is based. It highlights the environmental, economic and social impacts of acid mine drainage (AMD). Widely applied treatment technologies are discussed briefly while emphasis is placed on the biological sulphate reduction (BSR) process and its mediators (SRB) (Sections 2.1, 2.2 and 2.5). The efficiencies of the technologies available for the treatment of AMD are compared. BSR processes are potentially the most sustainable of all the treatment technologies. There have been several approaches aimed at improving the BSR efficiency. The need for a novel approach in the bioremediation of AMD, focusing on the study of the linkage between BSR process kinetics and the structure of the microbial population mediating the process is also presented herein (Sections 2.6 and 2.7). The use of lactate as the sole electron donor in this study is justified based on a comparative analysis with other simple electron donors. The underlying principles of the BSR kinetics are briefly discussed and the need for an extension of previous investigations on BSR kinetics at the Department of Chemical Engineering, University of Cape Town, through this study was identified (Section 2.3 and 2.4). Current molecular procedures employed in microbial ecology are outlined. An overview of these techniques, presented in Sections 2.8, shows that the 16S rRNA-based techniques are efficient culture-independent tools in microbial ecology. Against this literature background, the motivation for the current study is presented (Sections 2.9 and 2.10) and its objectives highlighted (Section 2.11).

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2.1. ACID MINE DRAINAGE

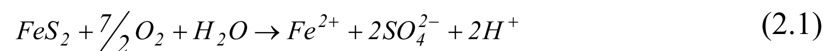
With increasing global population and industrial activities, generation of wastewaters is rapidly increasing (Cenni *et al*., 2001). Examples of such wastewaters posing a great danger to the ecosystem include those emanating from the following industries: pulp and paper (Thompson *et al.,* 2001; Hulshoff Pol *et al*., 1998), chemical, metallurgical and mining (Baker and Banfield, 2003; Johnson and Hallberg, 2003; Kuo and Shu, 2004) and fertilizer (Ghigliazza *et al*., 2000). The effluents from these industries are sulphate-rich or heavy metal-laden. The mining industry is a major part of the industrial sector in South Africa and the activities of this industry are

accompanied by the generation of acid mine drainage (AMD). Its management thus requires attention (Naicker *et al*., 2003).

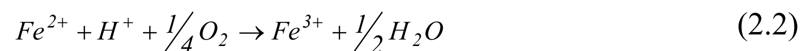
2.1.1. Generation of Acid Mine Drainage

Acid mine drainage arises from oxidation following the exposure of sulphide minerals such as pyrite, contained in waste rock, closed mining sites and coal tailings, to atmospheric oxygen and moisture (Reactions 2.1 to 2.4). The formation of AMD can occur either abiotically or biotically. The presence of microorganisms increases the process kinetics. Iron (dominant metal) and other metals (e.g. Zn, Cu and Mn) (Table 2.1) are generated from the solubilisation of their corresponding sulphide minerals by the acidic stream produced by oxidation (Reactions 2.1 to 2.4) (Baker and Banfield, 2003; Johnson and Hallberg, 2003).

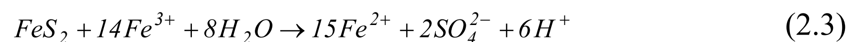
Pyrite (FeS_2) is responsible for starting acid generation and metals solubilisation. Prior to pyrite oxidation, ferrous ions are oxidised to ferric ions in the presence of acidophilic mesophilic bacteria such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* or thermophilic archaea such as *Sulfolobus metallicus* and *Metallosphaera hakonensis* (Reaction 2.1) (Christensen *et al*., 1996; Benner *et al*., 2000; DeNicola and Stapleton, 2002; Doye and Duchesne, 2003). This reaction can also proceed abiotically. Ferric ions and the atmospheric oxygen act as the oxidising agents (Reactions 2.1 to 2.3). Further hydrolysis of ferric ion leads to the production of more hydrogen ions and the red-orange precipitate [$Fe(OH)_3$] seen in AMD discharges (Reaction 2.4). Alternatively, ferric ions can react with more pyrite as in Reaction 2.3. In the presence of sufficient dissolved oxygen, a continuous cycle of Reactions 2.2 and 2.4 is maintained (Johnson and Hallberg, 2003).



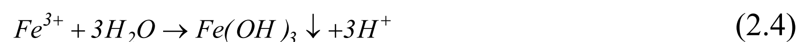
$$FeS_2 + \frac{7}{2}O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (2.1)$$



$$Fe^{2+} + H^+ + \frac{1}{4}O_2 \rightarrow Fe^{3+} + \frac{1}{2}H_2O \quad (2.2)$$



$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 6H^+ \quad (2.3)$$



$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 \downarrow + 3H^+ \quad (2.4)$$

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Table 2.1 Chemical composition of typical AMD streams. All concentrations are in mg l^{-1}

AMD Stream	pH	Fe	Zn	Cu	Mg	Na	K	SO_4^{2-}	NO_3^-	Cd	As	Al	Mn
Tinto river, Spain[a]	2.2	2.3×10^3	225	109	3.4×10^2	2.6	7.42	10.1×10^3	9.33	nd	nd	nd	nd
Falu mine, Sweden[b]	2.5	7.0×10^3	2.2×10^3	8	3.0×10^3	8.0×10^2	nd	3.5×10^4	nd	1.0	nd	3.0×10^2	2.0×10^2
Wallenberg mine, Norway[c]	5.5	1.40×10^2	0.34×10^2	1.45	3.5×10^2	nd	nd	2.9×10^3	nd	nd	nd	1.4	13.6
Gammelgruva mine, Norway[c]	2.4	1.2×10^3	0.39×10^2	0.1×10^2	2.1×10^2	nd	nd	6.6×10^3	nd	nd	nd	2.6×10^2	11.7
Ynysarwed, Wales[d]	6.2	3.0×10^2	nd	nd	nd	nd	nd	2×10^3	nd	nd	nd	nd	nd
Wheal Jane, England[d]	3.4	2.5×10^2	nd	nd	nd	nd	nd	4×10^2	nd	nd	nd	nd	nd
Highveld, South Africa[e]	1.15-2.91	0.25-6.3×10^3	nd	nd	0.4-2.8×10^3	0.4-4.1×10^3	11-52.6	6.16-14.9×10^3	0.4-1.6×10^2	nd	nd	nd	0.3-2.4×10^2
Grootvlei, South Africa[f]	6.3	1.9×10^2	nd	nd	nd	2.9×10^2	0.13×10^2	2.2×10^3	nd	nd	nd	nd	nd
West Rand, South Africa[f]	2.4	6.7×10^3	nd	nd	nd	0.8×10^2	nd	2.3×10^4	nd	nd	nd	nd	nd
Klipspruit, South Africa[f]	2.6	nd	nd	nd	nd	1.9×10^3	nd	8.1×10^3	nd	nd	nd	nd	nd
Iron Duke mine, Zimbabwe[g]	0.52	1.3×10^5	0.6×10^2	0.2×10^2	6.7×10^3	2.7×10^2	nd	3.6×10^5	0.13×10^2	4	0.72×10^2	12.0×10^3	1.2×10^2

a: López-Archilla *et al*., 2001.
b: Sandström and Mattson, 2001.
c: Christensen *et al*., 1996.
d: Johnson and Hallberg, 2003.
e: Gitari *et al*., 2005.
f: Department of Mineral and Energy Affairs, 1995.
g: Williams and Smith, 2000. nd: not determined

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Through the combination of ferric ion and acid leaching with microbial regeneration of the leach agents through ferrous ion and sulphur oxidation, soluble metal ions, acidity and sulphate species accumulate in solution (Reactions 2.4 and 2.5). Hence, acid mine drainage is characterised by low pH (<4), elevated heavy metals, suspended solids, low carbon nutrient and high sulphate concentration. In contrast to pyrite, the leaching of some of the metal ions from their corresponding sulphide minerals does not lead to acidity production (Younger *et al*., 2002) (Reaction 2.6).

$$MeS + 2O_2 \rightarrow Me^{2+} + SO_4^{2-} \quad (2.6)$$

where Me represents the metals, such as Zn, Cu, Cd, Pb and Ni.

Table 2.1 demonstrates the variability in composition of AMD streams from eight different countries. In all the cases, iron and sulphate are the dominant species. The characteristic heavy metal composition of the AMD streams (Table 2.1) are significantly above the limits recommended by USEPA and WHO guidelines for domestic water and aquatic habitats (Table 2.2). The characteristic pH values of these streams are in the range pH 0.5 to 6.3. These characteristics show the potential toxicological effects of AMD generation on the ecosystem.

Table 2.2: Environmentally accepted limits of heavy metals based on water legislations of USEPA and WHO. (1) in potable water and (2) aquatic habitat. All concentrations are in mg l^{-1}. Adapted from Williams and Smith, 2000.

Metal	1	2
Al	0.01-1.0	nd
Pb	0.05	0.004-0.05
Cr	0.01	0.004-0.4
Cd	0.005	0.05-0.1
Cu	1.0	0.01-0.1
Fe	1.0	nd
Mn	0.5	nd
Zn	5.0	0.01-5

nd: not determined

2.1.2. Environmental Impact of AMD and Mining Activities

AMD is a global environmental issue for both closed and operational mines. Sources of AMD persist for centuries even after the closure of mines (Razowska, 2001; Kalin *et al*., 2006). Hence, the environmental impact of AMD is perennial. The

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characteristic extreme composition of AMD (low pH, high heavy metal concentration, low nutrients and salinity) is detrimental to the environment and its resident organisms (flora and fauna) if discharged untreated (Ledin and Pedersen, 1996). AMD causes remarkable environmental degradation. It is of concern to human health and it adversely affects socio-economic well-being (Tables 2.2, 2.3 and 2.4). It has been noted that the groundwater and soil in the areas surrounding mining activities are often highly polluted with heavy metals and characterised by acidic pH, leading to the death of resident organisms, consequent reduction in biodiversity, reduction of water potability and availability for human use (Tables 2.3 and 2.4).

A comprehensive approximation of the worldwide pollution of water bodies by AMD in 1989 were approximately 19 300 km of streams and rivers, and 73 000 ha of lakes and reservoirs (Johnson, 2006). Another report (USEPA, 1997) indicated that coal mines in the Appalachian region of the North-eastern USA have destroyed more than 12 000 km of streams. A countrywide estimation of AMD environmental pollution in USA was documented as follows: 7000 km of streams and rivers, 23 000 ha of lakes and reservoirs (Kleinmann, 1989). In a report prepared by Rossman *et al*. (1997) for the Department of Environmental Protection, Pennsylvania, USA, it was stated that more than 5 billion USD was required for the rehabilitation of lands and streams polluted by AMD in Pennsylvania alone. Coal mining in the Witbank Coalfield of South Africa, has led to the extensive contamination of ground and surface water. This has subsequently resulted in the destruction of flora over an expanse of 3 ha where the seepage takes place (Bell *et al*., 2001). Termination of mining and dewatering activities is often associated with the restoration of the water table to its natural level, a phenomenon known as groundwater rebound. This may lead to uncontrolled AMD seepage into the surrounding areas that are topographically lower (Younger *et al*., 2002).

Younger *et al*. (2002) identified six key areas in the generation of AMD that lead to environmental damage or pollution. These are summarised in Table 2.3. These impacts result from different processes involved in mining and emphasise the need to intensify research in the area of AMD treatment.

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Table 2.3: Environmental effects of mining activity and AMD generation. Adapted from Younger *et al*. (2002).

Source	Effect
Excavation	Disruption of groundwater hydrology
Mineral processing e.g. bioleaching, milling and gold-mercury amalgamation	Polluted runoff
Dewatering i.e. draining water out of mining shafts	Water-table depression, reduction of groundwater resources, groundwater catchment modifications
Generation of AMD from pyrite	Discharge of contaminated leachate from mine tailings
Flooding of abandoned mine workings and water table rebound	Erosion, subsidence (sinkhole formation), accumulation of acid-generating salts,
Discharge of untreated mine water after flooding	Contamination of surface water or overlying aquifers

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2.1.2.1. Effects on Human Health

The presence of sulphate in potable water can cause a bitter and salty taste. The acceptable limit for taste is <250 mg l^{-1} (WHO, 2006). General water legislations allow for sulphate content of potable water in the range of 250 to 500 mg l^{-1} (WHO, 1996; USEPA, 1999). High sulphate concentration leads to increased water salinity. Ingestion of sulphate concentrations ≥600 mg l^{-1} is known to cause disturbance in the human gastrointestinal tract. As shown in Table 2.1, sulphate content of AMD far exceeds this value. The toxic heavy metals dissolved by the AMD, including manganese, aluminium, iron, nickel, zinc, cobalt, copper, radium and uranium (Table 2.1) are all characterised by varying degrees of toxicity and radioactivity. This is of great threat to human health. Uranium and radium can cause cancer (Fernandes *et al*., 1995). AMD pollution also reduces available farmlands for agricultural activities owing to salinity and acidity (Antunes *et al*., 2002). Other potential problems resulting from AMD include: (i) deterioration of waterways leading to their reduced application for activities such as recreation and irrigation and (ii) destruction of vegetation (Lupankwa *et al*., 2006).

2.1.2.2. Impact on the Aquatic Ecosystem

Sulphate build up in an aquatic environment may induce sulphide production, a compound that is lethal to several life forms. The malodorous characteristic of sulphide is also an environmental nuisance (Ghigliazza *et al*., 2000). Hence, sulphate removal in the treatment of AMD is imperative. Sediments of AMD-polluted water

bodies are often covered with metal precipitates which hamper macroscopic life cycles and in turn impair the food chain resulting in the eventual loss of biodiversity (Ledin and Pedersen, 1996; Gray, 1997; Feng *et al*., 2000; Naicker *et al*., 2003) (Table 2.4).

Table 2.4: Effects of AMD pollution on aquatic habitats. Adapted from Gray (1997).

Chemical	Physical	Ecological and Biological
Imbalance in the bicarbonate buffering system	Precipitation	Population shifts
Mineral solubilisation	Assimilation of metals onto sediment surfaces	Acid-base stability breakdown in organisms
Crust formation in the sediment	Decreased turbulence	Obliteration of vulnerable species
Lowered pH	Reduction in light diffusion	Habitat wipe out
	Substrate alteration	Decline in habitat diversity
	Elevated stream flow rate	Alteration of the food chain

Despite the numerous undesirable effects associated with AMD, it is a potential source of extremophilic microorganisms and industrial enzymes which are stable under extreme conditions of low pH. SRB isolated from AMD are well adapted for its treatment using bioengineered systems (García *et al*., 2001; Baker and Banfield, 2003; Hallberg and Johnson, 2003; Johnson and Hallberg, 2003).

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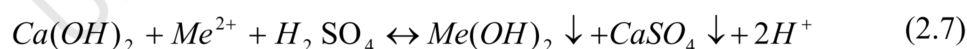
2.1.3. Treatment Technologies

Diverse technologies have been developed for the treatment of AMD to reduce its potential health risks and environmental hazards. Further, some technologies are implemented for the purpose of water recycle and reuse. These methods are targeted at water neutralisation, sulphate removal and metal removal. Since the toxic metal content attracts the greatest concern, most treatment methods are aimed at metal removal (Burgess and Stuetz, 2002). However, the reduction or removal of sulphate from AMD is also crucial in preventing environmental pollution. The choice of AMD treatment is dependent on the nature and extent of contamination, and the proposed use of the treated water (Gazea *et al*., 1996; Ledin and Pedersen, 1996).

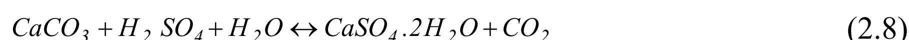
Acid mine drainage treatment can be classified broadly into passive and active processes. Active processes require constant replenishment of process requirements and constituents, and ongoing energy input e.g. aeration, mixing, chemical additives etc. These processes are typified by more rapid kinetics and enhanced control in comparison with the passive processes. Passive treatment methods are relatively less intensive, but require large treatment areas and are not as controllable owing to lower kinetics. Less defined conditions result in less control and predictability. Wetland is a typical example of passive treatment process (Johnson, 2006).

2.1.3.1. Chemical Treatment

Chemical treatment methods include flocculation, precipitation, coagulation, and ion-exchange (Boshoff *et al*., 2004a). The most widely used chemical approach is the addition of lime [$Ca(OH)_2$] (Reaction 2.7) or limestone ($CaCO_3$) (Reaction 2.8) for metal precipitation and neutralisation. This removes both acidity and metal toxicity of the wastewater (García *et al*., 2001). Liming also precipitates out some sulphate by the formation of gypsum ($CaSO_4.2H_2O$) from Ca and dissolved sulphate (Reaction 2.9) (Elliott *et al*., 1998). The sludge (gypsum and metal hydroxides) generated is unstable at low pH leading to the resolubilisation of its metal content. This generates a secondary waste (Kalyuzhnyi *et al*., 1997; Widerlund *et al*., 2005). Furthermore, this treatment approach leaves a significant amount of residual sulphate (2.0 g l^{-1}) in solution owing to the saturation concentration of gypsum. The solubility of gypsum depending on the ionic strength of the solution is 1.5 to 2.0 g l^{-1} (Lorax, 2003; Potgieter-Vermaak, *et al*., 2005).



$$Ca(OH)_2 + Me^{2+} + H_2SO_4 \leftrightarrow Me(OH)_2 \downarrow + CaSO_4 \downarrow + 2H^+ \quad (2.7)$$



$$CaCO_3 + H_2SO_4 + H_2O \leftrightarrow CaSO_4.2H_2O + CO_2 \quad (2.8)$$

$$Ca(OH)_2 + H_2SO_4 \leftrightarrow CaSO_4.2H_2O \downarrow \quad (2.9)$$

The hydrolysis and/ or oxidation of Fe, Al and Mn generate less soluble oxides, oxyhydroxides and hydroxides. The application of this step prior to the lime neutralisation procedure reduces reagent requirement (Wieder, 1989; Hedin *et al*., 1994; Batty *et al*., 2002; Woulds and Ngwenya, 2004; Herrera *et al*., 2007). Drawbacks of these techniques include the inability to remove metals when present in

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trace quantities (Boshoff *et al*., 2004a), production of voluminous sludge with low solid content, requirement of chemical addition and the high cost of handling (Chang *et al*., 2000). The generated sludge requires further treatment by dewatering and results in secondary wastes (Chang *et al*., 2000; Matlock *et al*., 2002). Owing to these facts, the chemical treatment technology is unsustainable in the long run.

2.1.3.2. Membrane technology

Membrane technology is often used as a secondary treatment after neutralisation and precipitation. It is exemplified by reverse osmosis and electrical dialysis. In reverse osmosis AMD is pumped at high pressures into a closed reactor containing a series of semi-permeable membranes. The ions are retained in the membrane while pure water permeates the void spaces. In contrast, electrical dialysis relies on electric potential to force dissolved ions through the membrane, while the purified water is retained. Membrane technology is hampered by membrane fouling. This treatment technology has led to the development of the SPARRO (Slurry Precipitation and Recycle Reverse Osmosis) process (Juby *et al*., 1996).

2.1.3.3. Ion-exchange

Ion-exchange technology relies on the electrostatic attraction of heavy metals to oppositely charged surfaces. Untreated AMD is pumped through columns containing a strong acid cation resin (R-H) and a weak base anion resin (R-OH). R-H removes cations such as Ca^{2+} and Mg^{2+}, while the R-OH removes anions including SO_4^{2-} (Ahmed *et al*., 1998; Lorax, 2003).

2.1.3.4. Biological Sulphate Reduction (BSR)

Sulphate-reducing bacteria play a major role in bioremediation. Sulphate reducers have been identified in mining environments (Ledin and Pedersen, 1996; Benner *et al*., 2000). Biological sulphate reduction in the management of AMD is attracting increasing attention owing to the advantages it offers. The following products of sulphate reduction, HS^-, S^{2-}, HCO_3^- and CO_3^{2-} neutralise the excess protons in AMD, therefore buffering the system at pH 6 to 8 (Dvorak *et al*., 1992; Kaksonen, 2004) (Reactions 1.1 to 1.3, Chapter 1). The H_2S produced by BSR can precipitate certain metals as sulphides (Reaction 1.2). The metal sulphide precipitates generated by BSR

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in the treatment of AMD are formed rapidly, even at low pH. These are less soluble than their hydroxide equivalents allowing lower residual metal concentration in solution (Hammack *et al*., 1994).

In the bioreactor design for BSR, to reduce metal toxicity to SRB, the sulphate reduction stage is separated from the metal precipitation step. The sulphide formed is contacted with the AMD prior to it entering the bioreactor to facilitate metal removal. Anaerobic BSR technology has been applied in different ways and carried out successfully under controlled and optimised conditions at both laboratory and full-scale. The reactor types employed include fluidised-bed reactor (FBR) (Kaksonen *et al*., 2003), up-flow anaerobic sludge blanket (UASB) (Elliot *et al*., 1998), sequencing batch reactor (Herrera *et al*., 1997), hybrid reactor (Steed *et al*., 2000), gas lift reactor (van Houten and Lettinga, 1995; van Houten *et al*., 2006), trench reactor (TR) (Boshoff *et al*., 2004b), upflow packed bed reactor (Baskaran and Nemati, 2006) and stirred tank reactor (Moosa, 2000; Boshoff *et al*., 2004b) (Table 2.5). Reactors such as UASB, FBR and upflow packed reactor have been used due to their biomass retention ability (Kaksonen *et al*., 2003; Baskaran and Nemati, 2006). On the other hand, the CSTR has been employed as a research tool in the study of BSR kinetics and microbial structure dynamics (Moosa, 2000; Icgen and Harrison, 2006a; 2006b; Zhao *et al*., 2007).

It has been suggested that the *in situ* treatment of AMD at the point of generation, employing mine-pits as bioreactors, is feasible and sustainable (Christensen *et al*., 1996). AMD can also be channelled into wetlands where BSR process takes place passively (Kalin *et al*., 2006).

The use of two full scale sulphidogenic technologies has been reported widely. These are the BioSURE® and Thiopaq® processes. The BioSURE® technology was developed at Rhodes University, South Africa. This technology links AMD treatment and sewage sludge disposal (Corbett, 2000; Rose *et al*., 2000; Whittington-Jones *et al*., 2006). This system consists of a falling sludge bed reactor (FSBR) in which the macromolecules of the sewage sludge are degraded to provide electrons for the SRB. This technology is being used on a pilot-scale at Grootvlei Mine (South Africa) (Lorax, 2003). The Thiopaq® system was developed in Netherlands by Paques B.V. It

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is based on a gas-lift reactor fed with hydrogen gas as the electron donor and CO_2 as the carbon source. Sulphate is reduced into sulphide which can be partially re-oxidised into sulphur in two separate compartments of the Thiopaq® system (Boonstra *et al*. 1999; Hulshoff Pol *et al*., 2001; van Houten *et al*., 2006). The sulphur produced can be recycled to sulphuric acid (Hulshoff Pol *et al*., 2001). This technology has been implemented on a full-scale at the Kennecott Bingham copper mine, USA, the Budelco zinc refinery, Netherlands (van Houten *et al*., 2006), and the synthetic fiber production plant of Akzo Nobel, Netherlands (Hulshoff Pol *et al*., 2001).

As shown in Table 2.5, reactor design, feed sulphate concentration and choice of electron donor are critical factors that influence performance of biological sulphate reduction systems. Immobilised-cell reactor systems encourage high biomass concentration at short residence times, thus giving a better performance in comparison to suspended-cell systems (Baskaran and Nemati, 2006; Kaksonen *et al*., 2006). In CSTR, Moosa *et al*. (2002) reported increasing sulphate removal rate with increasing feed sulphate concentration in the range 1.0 to 10.0 g l^{-1}. Sulphate inhibition was reported above this range.

Simple carbon-sources such as lactate, acetate and methanol are readily available for SRB. In contrast, the use of complex carbon-sources requires the presence of fermentative microbial groups to facilitate degradation into easily assimilable substrates. Operation at higher residence times will thus be required for reactors fed with complex carbon-sources (Christensen *et al*., 1996; Chang *et al*., 2000; Boshoff *et al*., 2004b).

Results from a recent study by Pruden *et al*. (2007) showed that the reactors inoculated with a culture from an active sulphidogenic reactor exhibited a better performance in terms of sulphate and metal removal relative to the ones inoculated with manure. A shorter start-up period of the former was attributed to the adaptation to the inoculum from the sulphidogenic environment to the presence o metals and sulphate.

The abovementioned highlights the need to select carefully for a mixed culture composed of population members that are well-adapted to the BSR treatment

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conditions. As envisaged in this study, this can be achieved through the study of the response of a mixed SRB consortium to the volumetric sulphate loading rate. The volumetric sulphate loading rate is mediated through both the feed sulphate concentration and the dilution rate.

Table 2.5: Performance of various bioreactors used to treat sulphate-containing and heavy metal-laden waste streams.

Reactor type	T (°C)	Electron donor/ Carbon-source	Source of inoculums	Feed sulphate concentration (g l^{-1})	Sulphate reduction rate (g $l^{-1}d^{-1}$)	Sulphate conversion (%)	Metal removal (%)	Acidity removal Influent pH	Effluent pH	Reference
Column	nd	compost, limestone, sheep manure and limestone	creek sediment	0.5	0.016-0.044	18-27	nd	2	7.7	Gilbert *et al*., 2004
CSTR	35	Acetate	Wastewater treatment plant	1.0-10.0	0.168-4.08	60-93	nd	nd	nd	Moosa *et al*., 2002
CSTR	nd	Tannery effluent	Anaerobic digester	1.8	0.25	60-80	nd	nd	nd	Boshoff *et al*., 2004b
Gas lift	30-35	synthesis gas/ ethanol	Anaerobic digester	5-10	15	88	95	nd	nd	van Houten *et al*., 2006
Packed bed	18-24	spent mushroom compost	Spent mushroom	1.0	0.021-0.032	17-20	>95	3.2	6.4	Dvorak *et al*., 1992
Packed bed	14-24	Whey	cow manure and whey	nd	nd	80-98	40-98	2	6.5	Drury, 1999
Packed bed	nd	cow manure, whey	AMD contaminated sediments	0.9	0.003-0.0044	19-27	nd	4.5	6.0	Christensen *et al*., 1996
Flask	35	Lactate	Mine	2.8	0.18-3.3	35-87	>90	nd	nd	Jalali and Baldwin, 2000
FBR	35	Lactate	Anaerobic digester	2.3	2.2	65	99.9	2.5	7.8	Kaksonen, 2004
FBR	35	Ethanol	Anaerobic digester	2.0	2.3	81	99.9	2.5	7.7	Kaksonen, 2004
UASB	nd	Tannery effluent	Anaerobic digester	1.8	0.6	60-80	nd	nd	nd	Boshoff *et al*., 2004b
TR	nd	Tannery effluent	Anaerobic digester	1.8	0.4-0.5	60-80	nd	nd	nd	Boshoff *et al*., 2004b
UPBR	22	Lactate	oil reservoir	1.0-5.0	2.4	17-100	nd	nd	nd	Baskaran and Nemati., 2006

nd: not determined

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Table 2.6: Summary of the process performance of full- and pilot-scale technologies employed in the treatment of AMD.

Description of method	**Efficiency**				**References**
	% Metal removal	Acidity removal	% SO_4^{2-} removed	SO_4^{2-} reduction rate($g\ l^{-1}d^{-1}$)	
(1) Chemical technology					
(a) SAVMIN process: Involves calcium/aluminium precipitation	-	-	75	-	Smit, 1999
(b) CESR (Cost Effective Sulphate Removal) process: Involves calcium/aluminium precipitation and pH neutralisation using recarbonation	24-99	-	99	-	Hydrometrics, 2001
(2) Membrane technology					
(a) SPARRO (Slurry Precipitation and Recycle Reverse osmosis	-	-	98	-	Pulles *et al*., 1995; Juby *et al*., 1996
(3) Ion-exchange					
(a) GYP-CIX technology composed of a series of ion resins	50-91	increase in pH from 4.5 to 8-9	96-98	-	Schoeman and Steyn, 2001
(4) Microbial sulphate reduction					
(a) Pilot-scale continuous reactors using spent mushroom compost as a carbon-source	95	increase in pH from 3.7 to 6.9	20	0.021-0.032	Dvorak *et al*., 1992
(b) Rhodes BioSURE® process using sewage sludge as the carbon-source	-		>95		Corbett, 2000; Rose *et al*., 2000
(c) Paques Thiopaq®-process (gas-lift reactor fed with hydrogen/synthesis gas as the electron donor)	95-99	increase in pH from 2.5 to 8.5	75-88	15	Boonstra *et al*. 1999; Hulshoff Pol *et al*., 2001; van Houten *et al*., 2006
(5) Wetlands/ Passive treatment					
(a) ARUM (acid reduction using microbiology) ponds. System comprises of oxidation cells, sulphide and alkali generation cells	87-98	increase in pH from 1.4 to 7.2	-	-	Kalin and Chaves, 2003
(b) Constructed wetlands	-	-	>90	0.0075-0.058	Lloyd *et al*., 2004; Sheoran and Sheoran, 2006

nd: not determined

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2.1.3.5. Wetlands

The use of wetlands in the treatment of AMD is regarded as a relatively low-cost , passive treatment technology. However, it usually requires a large land area for implementation (Gazea *et al*., 1996). Natural or constructed wetlands with growing macrophytes are employed. This simple technology is dependent on the interactions among the residing biotic community of this environment, and myriad biological, chemical and physical reactions (Gazea *et al*., 1996; Ledin and Pedersen, 1996; Ayaz and Akça, 2001). The easiest process in wetland treatment is mine water dilution. Due to the anaerobic condition provided by the surrounding water in deep wetlands, biological sulphate reduction may take place if there is ample organic content to sustain SRB growth. Some growing plants may also remove metals via adsorption and assimilation, increase pH and also supply oxygen for metal removal by oxidation. Putrefied plant matter is a good carbon-source for other microbial processes. The plant cellulose when fermented provides electron donors for sulphate reduction (Gazea *et al*., 1996).

2.1.3.6. Comparison of the Performance of AMD Treatment Processes

Table 2.6 shows the efficiencies of the treatment technologies discussed in the forgoing sections. Since these processes have been well implemented and commercialised, their performances on the full- and pilot-scale are compared. The biological process shows the greatest potential for AMD treatment. It addresses the three main toxicants of AMD: sulphate, heavy-metals and acidity (Table 2.6).

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Case studies carried out by Lorax (2003) showed that trace metals are efficiently removed in this process and fewer stages are involved in the BSR as compared to the other processes. Further, with the utilisation of less-expensive carbon-sources the operating cost will be reduced. Economical sources of carbon and electron for the BSR include the following: sewage sludge (Rose *et al*., 2000), micro-algal biomass (Boshoff *et al*., 2004a), tannery effluent (Boshoff *et al*., 2004b) and activated sludge (Burgess and Stuetz, 2002). A study by Schoeman and Steyn (2001) on the process economics of the mine water (80 to 100 megalitre per day) treatment from the Grootvlei Proprietary, South Africa, predicted the annual operating costs for reverse osmosis (RO) and GYP-CIX treatment technologies to be 17.2 and USD 8.6 million respectively. Further, the potable water recovery based on the removal of total solids

was 85 and 54% respectively for RO and GYP-CIX (Schoeman and Steyn, 2001). Biological sulphate reduction in bioreactors requires less land space for operation in comparison to the passive treatment in wetlands. Application of wetlands is also limited to acid waters with low metal content (Gazea *et al*., 1996).

2.2. SULPHATE-REDUCING BACTERIA

2.2.1. The Role and Application of SRB

Sulphate, a stable sulphur compound, is reduced by most bacteria, fungi, and plants into sulphide, for the synthesis of certain amino acids and subsequent metabolism. This process is called assimilatory sulphate reduction and is a biosynthetic process (Widdel, 1988; Cooney *et al*., 1996). Conversely, during dissimilatory sulphate reduction catalysed by sulphate reducers, sulphate acts as an electron acceptor for the degradation of organic substrates (electron donor). There is concurrent carbon transformation and sulphide liberation (Reaction 1.1, Chapter 1).

$$Electron\ donor + SO_4^{2-} \rightarrow HS^- + HCO_3^- \quad (1.1)$$

This process provides the bacteria with energy for growth and maintenance (Postgate, 1984; Widdel, 1988) and produces a greater amount of sulphide as compared with the assimilatory process (Cooney *et al*., 1996). Sulphate-reducing bacteria play a critical role in the environmental sulphur cycle and contribute to the carbon cycle (Devereux *et al*., 1996). Sulphate reducers are of economic importance through their involvement in the bioremediation of several wastewaters especially sulphate-rich mine, pulp and paper, and textile industrial effluents (Moosa *et al*., 2002; Boshoff *et al*., 2004a; 2004b; Maree *et al*., 2004; Albuquerque *et al*., 2005). SRB activity has been reported to enhance primary sewage sludge hydrolysis (Whittington-Jones, 2000; Whiteley *et al*., 2002a; 2002b; 2003; Pletschke *et al*., 2002; Oyekola *et al*., 2007a).

SRB are employed in the development of the microbial fuel cells which transform chemical energy into electrical energy. Through sulphate reduction and the oxidation of sulphide produced (which involves electron transfer), electrical current can be generated (Cooney *et al*., 1996). Sulphate reducers have also been implicated in the following: air pollution from sulphide emission, paper pulp blackening in the paper

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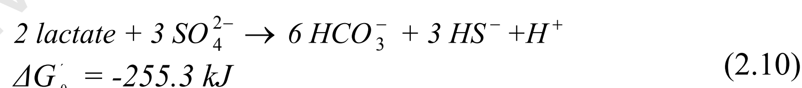
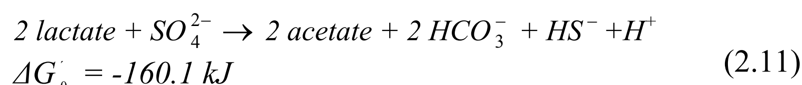
and pulp industry, oil souring and pipe corrosion (Postgate, 1984; Miranda *et al*., 2006).

2.2.2. Characterisation of Sulphate Reducers

Sulphate reducers are highly diversified physiologically and nutritionally (Wagner *et al*., 1998; Castro *et al*., 2000; Shen and Buick, 2004). They are either autotrophs or heterotrophs. The heterotrophic SRB utilise simple organic molecules while the autotrophic SRB rely on H_2 and CO_2 as their electron donor and carbon-source respectively (Nagpal *et al*., 2000; Lens and Kuenen, 2001). SRB can be divided into two major groups, based on their mode of oxidisation of organic matter: (i) complete oxidisers and (ii) incomplete oxidisers. The complete oxidisers can completely oxidise organic matter to bicarbonate or carbon dioxide with concomitant reduction of sulphate to sulphide (Reaction 2.10). They include members of the following genera: *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, *Desulfonema* and *Desulfobacterium*. The second group carry out an incomplete oxidation, usually to acetate (Reaction 2.11) (Widdel, 1988). Incomplete oxidisers are postulated to lack the citric acid cycle required for the oxidation of acetate. In general, these SRB use a smaller range of substrates in comparison to the complete oxidisers. They are exemplified by lactate-oxidising species of *Desulfotomaculum, Desulfobulbus* and *Desulfovibrio* genera, and *Desulfomonas pigra* (O'Flaherty *et al*., 1998; Widdel, 1988; Chang *et al*., 2000; Scheid and Stubner, 2001; Sass *et al*., 2002).



$$2\ lactate + 3\ SO_4^{2-} \rightarrow 6\ HCO_3^- + 3\ HS^- + H^+ \quad \Delta G_0' = -255.3\ kJ \qquad (2.10)$$



$$2\ lactate + SO_4^{2-} \rightarrow 2\ acetate + 2\ HCO_3^- + HS^- + H^+ \quad \Delta G_0' = -160.1\ kJ \qquad (2.11)$$

With the aid of molecular tools, such as 16S rRNA gene sequence analysis, SRB have been well characterised and classified into four groups. These are as follows: (i) Gram negative mesophilic SRB, constituting the largest number of genera, (ii) Gram positive spore forming SRB of which the genus *Desulfotomaculum* is the most prevalent member, (iii) thermophilic bacterial SRB, and (iv) sulphate reducing archaea (SRA). Gram negative sulphate reducers have moderate temperature optima, while the other groups thrive at higher temperatures with thermophilic archaea having

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the highest temperature optima, often in excess of 80ºC. Novel SRB are being discovered continually and many are yet to be classified (Castro *et al*., 2000).

2.2.2.1. Occurrence

Sulphate reducers are known for their occurrence in several anaerobic environments, especially sulphate-rich ones (Castro *et al*., 2000). Sulphate-reducing bacteria have been isolated from petroleum reservoirs, anaerobic sludge, terrestrial hot springs, digestive tracts of humans and animals, soils, fresh, marine and brackish waters, and acidophilic heavy metal-laden ecosystems (Colleran *et al.,* 1994; Briglia and Verstraete, 1995; Manz *et al*., 1998; Castro *et al*., 2000; Jeathon *et al.,* 2002; Kleikemper *et al*., 2002a; González-Toril *et al*., 2005).

2.2.2.2. Dissimilatory Sulphate Reduction

Dissimilatory sulphate reduction is a form of anaerobic respiration in utilising the electrons obtained from the oxidation of organic compounds or hydrogen (electron donors) in the reduction of other compounds (electron acceptors) (Singleton, 1993). The principal goal for the reduction of sulphate by the SRB is energy gain. Dissimilatory sulphate reduction is responsible for a major percentage of the flux through the carbon cycle occurring in anoxic sulphate-rich environments (Ledin and Pedersen, 1996; Santegoeds *et al*., 1998). This process plays a major role in Earth's lithosphere. It accounts for the formation of minerals such as pyrite (Shen and Buick, 2004; Natarajan *et al*., 2006).

2.2.2.3. Mechanism of Dissimilatory Sulphate Reduction

Several enzymes are involved in sulphate reduction. These include pyrophosphatase, ATP sulphurylase, APS reductase and sulphite reductase (Gibson, 1990; Sass *et al*., 1992; Visscher *et al*., 1992; Mudryk *et al*., 2000). The biochemical dissimilatory sulphate reduction pathway for sulphate-reducing bacteria and archaea commences with the active transportation of exogenous sulphate across the bacterial cell membrane into the cell (step 1) (Figure 2.1). This is aided by an active symport with $2H^+$ or Na^+. The intracellular sulphate is then reduced in sequential stages to sulphide (Figure 2.1) (Lengeler *et al*., 1999; Shen and Buick, 2004). Sulphate is highly stable and therefore requires activation before it is subsequently reduced (step 2) (Figure 2.1) (Matias *et al*., 2005).

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ATP sulphurylase catalyses the first reaction between the intracellular sulphate and adenosine triphosphate (ATP) to produce the highly activated molecule adenosine phosphosulphate (APS), as well as pyrophosphate (PPi) (Reaction 2.12) which can be cleaved subsequently to yield inorganic phosphate (Pi) (Reaction 2.13). PPi hydrolysis in the presence of pyrophosphatase drives the endergonic sulphate activation step. APS is the actual electron acceptor that is subsequently converted to AMP and sulphite by the enzyme APS reductase (step 3) (Figure 2.1) (Ullrich and Huber, 2001; Pletschke *et al.*, 2002) (Reaction 2.14).

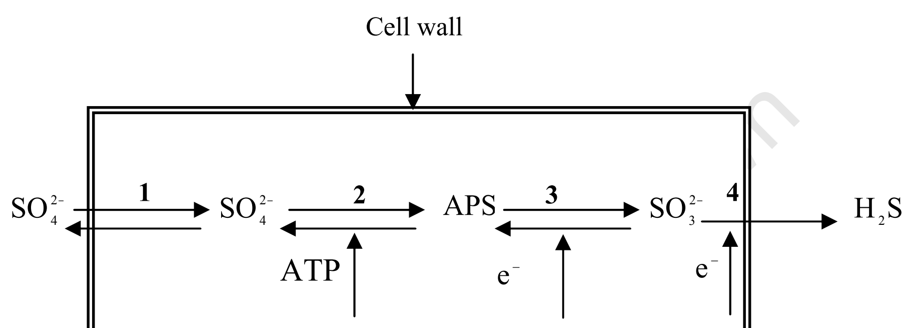
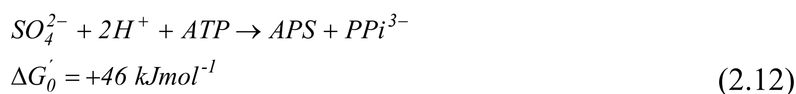


Figure 2.1: The pathway of dissimilatory sulphate reduction (e^-= electron). 1-4 represent the steps involved in the dissimilatory sulphate reduction process (Shen and Buick, 2004).

The sulphite formed is either reduced through a series of intermediates such as metabisulphite ($S_2O_5^{2-}$), dithionite ($S_2O_4^{2-}$), trithionate ($S_3O_6^{2-}$) and thiosulphate ($S_2O_3^{2-}$) to form sulphide (step 4) (Postgate, 1984; Lengeler *et al.*, 1999) or directly through a single step involving transfer of six-electrons in the presence of sulphite reductase (Reaction 2.15). The sulphide generated is then excreted into the environment (Shen and Buick, 2004; Menert *et al.*, 2004).



$$SO_4^{2-} + 2H^+ + ATP \rightarrow APS + PPi^{3-}$$
$$\Delta G_0' = +46\ kJmol^{-1} \quad (2.12)$$

$$PPi^{3-} + H_2O \rightarrow 2Pi^{2-} + H^+$$
$$\Delta G_0' = -33\ kJmol^{-1} \quad (2.13)$$

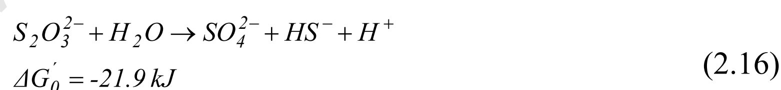
$$APS \rightarrow HSO_3^- + AMP + H^+$$
$$\Delta G_0' = -69\ kJmol^{-1} \quad (2.14)$$

$$HSO_3^- \xrightarrow{3H_2(6e^-)} HS^- + 3H_2O$$
$$\Delta G_0' = -171\ kJmol^{-1} \quad (2.15)$$

2.2.3. Metabolic Requirements

Nutritional requirements for SRB are relatively simple and the major ones include: inorganic electron acceptor, carbon-source/electron donor, nitrogen source and metals.

(1) Inorganic electron acceptor: Sulphate-reducing bacteria reduce several electron acceptors, even though they derive their name from one electron acceptor (sulphate) (Cypionka, 2000). Some SRB carry out dissimilatory reduction of nitrate to nitrite to ammonia (Widdel and Pfennig, 1982). A number of sulphate reducers have also been recognised to grow fermentatively or via metal reduction independently of sulphate (Raskin *et al.*, 1996; Amend and Teske, 2005). These shifts of metabolic systems encourage their survival and competition in the presence of other microorganisms (O'Flaherty and Colleran, 1998). Dissimilatory sulphate reduction intermediates such as sulphite, dithionite, tetrathionate and thiosulphate can also serve as electron acceptors for SRB. Unlike sulphate, these compounds do not require prior activation (Widdel, 1988; Sass *et al.*, 1997). In addition, some sulphate reducers are known to first convert sulphite and thiosulphate to sulphate, which can be subsequently utilised in the oxidation of some electron donors (Reactions 2.16 and 2.17) (Postgate, 1984). Some *Desulfovibrio* strains have also been reported to utilise elemental sulphur as electron acceptor in the presence of certain electron donors such as lactate (Mogensen *et al.*, 2005).



$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+ \quad \Delta G_0' = -21.9\ kJ \tag{2.16}$$

$$4SO_3^{2-} + H^+ \rightarrow 3SO_4^{2-} + HS^{--} \quad \Delta G_0' = -235.6\ kJ \tag{2.17}$$

(2) Carbon-source/electron donor: The SRB are able to utilise a wide spectrum of substrates as their carbon-sources and electron donors. Mostly, SRB use the same compound as the carbon-source and electron donor. They do not degrade polymeric compounds such as polysaccharides, proteins or lipids but rely syntrophically on fermentative bacteria for the supply of carbon-sources from these compounds (Ravenschlag *et al.*, 2000). Essentially, the substrates utilised are volatile fatty acids

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e.g. acetate, propionate, butyrate; C_3 and C_4 fatty acids (e.g. lactate, pyruvate, malate); alcohol (e.g. ethanol and propanol) (Gibson, 1990).

Energy sources such as butyrate, propionate and higher fatty acids which cannot be fermented by other anaerobic microorganisms are referred to as "low-energy substrates", while lactate and ethanol which can be anaerobically degraded by other microorganisms exemplify "high-energy substrates" (Widdel, 1988). It thus follows that SRB in mixed cultures fed with "high-energy substrates" are prone to competition with these other anaerobes (Laanbroek *et al*., 1982). Only a minute number of SRB genera are known to readily degrade a broad range of organic acids (Hanselmann *et al*., 1995; Nagpal *et al*., 2000; Kuever *et al*., 2001), e.g. *Desulforhabdus amnigenus* is able to consume lactate, acetate, butyrate and propionate (Oude Elferink *et al*., 1995). Other carbon-sources and electron-donors include glucose for which SRB have an affinity comparable with that of acetate and lactate (Song *et al.*, 1998; Sass and Cypionka, 2004), molecular hydrogen (as electron donor only) (Jeathon *et al.*, 2002), choline, amino acids (Widdel, 1988), synthesis gas (van Houten *et al*., 1995; 1996) and phthalate esters (Chang *et al*., 2005).

Certain SRB are known to utilise environmental contaminants such as petroleum hydrocarbon (PHC) constituents (e.g. benzene, toluene, ethylbenzene, xylenes, polycyclic aromatic hydrocarbons and alkanes) or halogenated compounds directly as a source of carbon and energy (Ensley and Suflita, 1995; Zhang and Young, 1997; Kleikemper *et al*., 2002a). The ability of SRB to use several environmental contaminants as carbon-sources has made them widely applicable in bioremediation. A diverse group of complex wastes have also been utilised as carbon-sources for SRB metabolism and growth. These include, primary sewage sludge (Whittington-Jones, 2000), micro-algal biomass (Boshoff *et al*., 2004a), tannery effluent (Boshoff *et al*., 2004b), rice husk filtrate (Chockalingam *et al*., 2005) and whey (Drury, 1999).

As mentioned earlier, SRB rely on fermentative microorganisms to degrade the polymeric compounds present in these complex energy sources. Selection of electron donor is influenced by cost and potential formation of secondary pollutants. Owing to possible generation of secondary pollutants resulting from partial oxidation of

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complex electron donors and the precise chemical composition of simple substrates, simple electron donors are often preferred (van Houten *et al*., 1996).

(3) Nitrogen-source: Ammonium ions serve as a nitrogen source for the growth of most sulphate-reducing bacteria (Gibson, 1990). Several marine strains of *Desulfovibrio* can use a number of amino acids as carbon and nitrogen sources (Stamps *et al*., 1986). Some *Desulfovibrio* and *Desulfotomaculum* strains have also been reported to fix gaseous nitrogen (Postgate, 1984). Other nitrogen sources include nitrate, peptone or tryptone (Jeathon *et al.*, 2002).

(4) Metals: Selenium and nickel are essential co-factors for hydrogenase activity, while iron is required for both cytochrome and hydrogenase production (Barton and Tomei, 1995).

2.2.4. Environmental Conditions and Sulphate Reduction

The important environmental factors that influence SRB activity include: pH, temperature, substrate availability and presence of inhibitors such as undissociated sulphide and metal ions (Rintala and Lettinga, 1992; Soto *et al*., 1993; Hao, 2000).

(1) Temperature: Sulphate reducers grow well over a wide range of temperatures, exhibiting high flexibility to temperature changes (Postgate, 1984). While some thrive at temperatures as low as 5°C, the spore-forming thermophilic species grow well at temperatures from 65 to 80°C (Gibson, 1990). Sulphate reduction has also been indicated at temperatures ≥100°C (Jeathon *et al*., 2002; Amend and Teske, 2005). Most SRB are mesophiles, metabolising optimally at temperatures of 25 to 40ºC (Hao *et al*., 1996).

(2) pH: The ideal pH for sulphate reducers is in the region of pH 7. Typically SRB are inhibited at acidic (<6) and very alkaline (>9) pH ranges. However, SRB have been isolated from acidic environments where they are believed to exist in microhabitats of suitable pH within these environments (Elliot *et al*., 1998; Johnson, 2000). These microorganisms buffer their surrounding pH by consuming hydrogen (Reaction 2.18)

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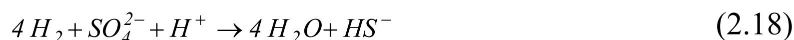
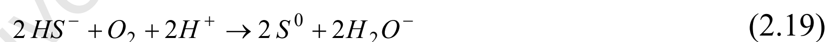
or via the production of hydrogen-consuming, HS^- and HCO_3^- ions (Reaction 1.1, Chapter 1) (Widdel, 1988).

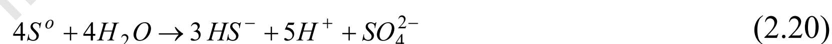


$$4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^- \quad (2.18)$$

(3) Oxygen: Sulphate-reducing bacteria require a low redox potential (<-200 mV) for growth, thus limiting their existence to reducing environment. The presence of sulphide produced from sulphate reduction and the absence of oxygen are responsible for the low redox potential observed (Postgate, 1984). Although this group of bacteria has been regarded as strictly anaerobic, thus adversely affected by oxygen, some SRB have been noted to tolerate and respire in the presence of minute oxygen concentrations (~5%) (Sass *et al*., 2002). Members of the *Desulfobulbus*, *Desulfonema, Desulfovibrio, Desulfococcus* and *Desulfomicrobium* genera, most of which are incomplete oxidisers, have been isolated from oxic habitats where they are exposed to oxygen stress (Teske *et al*., 1996; Krekeler *et al*., 1997; Sass *et al*., 1997; Bade *et al*., 2000; Nagpal *et al.,* 2000; Fournier *et al*., 2006; Mogensen *et al*., 2005). Lobo *et al*. (2007) reported the growth of *Desulfovibrio desulfuricans* ATCC 27774 in the presence of an oxygen concentration of 18%. Some of these aerotolerant strains are believed to reduce oxygen by reoxidising sulphide to sulphate which can subsequently serve as an electron acceptor (Reactions 2.19 and 2.20). This mechanism is used in removing oxygen and creating an anoxic environment (Dannenberg *et al*., 1992; Fuseler *et al*., 1996; Sass *et al*., 2002).



$$2HS^- + O_2 + 2H^+ \rightarrow 2S^0 + 2H_2O^- \quad (2.19)$$



$$4S^o + 4H_2O \rightarrow 3HS^- + 5H^+ + SO_4^{2-} \quad (2.20)$$

This facilitates the ability of these aerotolerant SRB to thrive in their natural environments (Fareleira *et al*., 2003). Furthermore, sulphate-reducing bacteria respond to oxygen stress via formation of aggregates, thus forming anoxic microniches, and migration to oxygen-free regions (Krekeler *et al*., 1998; Cypionka, 2000). These responses allow SRB to participate in aerobic wastewater treatment processes such as activated sludge (Manz *et al*., 1998).

(4) Inhibitors: Sulphide and acetate are known to inhibit sulphate reducers; their degree of inhibition is also known to be pH-dependent (O'Flaherty *et al*., 1998; Reis *et al*., 1992). Inhibition of SRB activity by sulphide is widely reported (O' Flaherty *et*

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al., 1998; Konishi *et al*., 1996; Icgen and Harrison, 2006a; Moosa and Harrison, 2006). This compound is believed to be toxic in its undissociated state (H_2S) because of its ease in traversing the cell hence hampering intracellular pH. Formation of this sulphide species is favoured by low pH and temperature (Reis *et al*., 1992; Percheron *et* al., 1997; Nagpal *et al*., 2000; Hulshoff Pol *et al*., 2001). The pH-dependent hydrogen sulphide dissociation in aqueous medium can be represented as follows (Garrels and Christ, 1965; Dean 1999):

$$H_2S \leftrightarrow H^+ + HS^- \quad pK_{a1} = 6.97\,(25^\circ C) \qquad (2.21)$$

$$HS^- \leftrightarrow H^+ + S^{2-} \quad pK_{a2} = 12.9\,(25^\circ C) \qquad (2.22)$$

At a pH above pH 7, dissolved sulphide is predominantly in the ionised form, while the formation of non-ionised species is favoured at lower pH (Vallero, 2003).

Other inhibitors of SRB activity are molybdate (Yadav and Archer, 1989; O'Flaherty *et al*., 1998; Isa and Anderson, 2005) and heavy metals (Clancy *et al*., 1992). Molybdate is a selective inhibitor of SRB activity. It functions by preventing the active transportation of sulphate into the cell (Menert *et al*., 2004). This sulphate analogue enters the cell via the sulphate transport mechanism, consequently preventing the formation of APS (Patidar and Tare, 2005).

High concentrations of certain heavy metals (Cr, Cu, Mn, Ni, Zn) are inhibitory to SRB (Hao *et al*., 1994; Poulson *et al*., 1997; Cabrera *et al*., 2006). These metals have been reported to inhibit SRB activity in the concentration range 25 to 60 mg l^{-1} (Morton *et al*., 1991; Ueki *et al*., 1991; Hao *et al*., 1994). Heavy metals can inhibit SRB activity by deactivating certain enzymes through the masking of the catalytically active groups, denaturing protein conformation or complexing with metal ions involved in the enzyme-substrate complexes (Cabrera *et al*., 2006).

(5) Salinity: Halophilic sulphate reducers such as members of *Desulfobacterium*, *Desulfonema, Desulfovibrio, Desulfosarcina, Desulfococcus* and *Desulfobacter* genera, have been isolated from hypersaline environments, with salinities in the range of 20 to 30%. Most of the SRB strains from these habitats are believed to be

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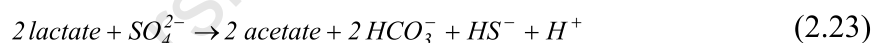
incomplete oxidisers (Ollivier *et al*., 1994; Krekeler *et al*., 1997; Oren, 1999; Brandt *et al*., 2001).

2.3. LACTATE AS A CARBON-SOURCE AND ELECTRON DONOR

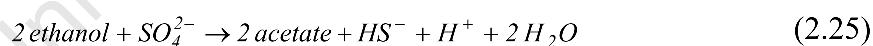
Lactate is used as carbon-source and electron donor by many SRB strains (Dvorak *et al*., 1992; Okabe and Characklis, 1992; Okabe *et al*., 1992; 1995; Kaksonen *et al*., 2003). Using lactate as a substrate for BSR has been reported to decrease sulphide toxicity (a major threat to biological treatment technology). As reported by Kuo and Shu (2004), acetate- and butyrate-fed anaerobic systems showed inhibition by dissolved sulphide (DS) concentrations in the range 150 to 200 mg l^{-1} and undissociated hydrogen sulphide concentrations in the range 60 to 75 mg l^{-1}, while the system utilising lactate operated steadily at elevated concentration ranges of 200 to 400 mg l^{-1} and 100 to 150 mg l^{-1} of DS and undissociated hydrogen sulphide respectively.

The complete oxidation of one mole of lactate results in the production of three moles of bicarbonate alkalinity (Reactions 2.23 and 2.24) in comparison to other simple organic molecules such as ethanol, which yield two moles of bicarbonate on complete oxidation, with concomitant reduction of 1.5 moles sulphate in both cases (Reaction 2.25) (Nagpal *et al*., 2000; Kaksonen *et al*., 2004).



$$2\,lactate + SO_4^{2-} \rightarrow 2\,acetate + 2\,HCO_3^- + HS^- + H^+ \quad (2.23)$$

$$acetate + SO_4^{2-} \rightarrow 2\,HCO_3^- + HS^- \quad (2.24)$$



$$2\,ethanol + SO_4^{2-} \rightarrow 2\,acetate + HS^- + H^+ + 2\,H_2O \quad (2.25)$$

As reported by Brandt *et al*. (2001), in a study where hypersaline sediments obtained from Great Salt Lake were incubated with butyrate, propionate, acetate, lactate and H_2, the highest sulphate reduction rate was exhibited with lactate and H_2 as electron donors. Purdy *et al*. (1997) amended slurries obtained from freshwater and marine sediments with a variety of short chain fatty acids (lactate, butyrate, acetate and propionate). The highest sulphate reduction rate (SRR) and electron donor utilisation of approximately 1.67 to 3.0 mM d^{-1} and 10 mM d^{-1} respectively, were observed in slurries using lactate. This was confirmed by Kleikemper *et al* (2002a); lactate fed samples from sediments contaminated with petroleum hydrocarbon (PHC) exhibited the highest sulphate consumption and carbon-source mineralisation (Table 2.7).

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Postgate (1984) reported a doubling time for SRB growing on lactate in the range of 3 to 6 h, in contrast to doubling times not less than 20 h for acetate utilisers. In a study by Qatibi *et al*. (1990), the cell counts with mixed cultures amended with electron donors lactate, acetate and propionate were 450×10^6, 0.025×10^6 and 1.5×10^6 viable cells ml^{-1}, respectively. This is in agreement with the results of Kleikemper *et al*. (2002a) (Table 2.7).

Table 2.7: Comparison of sulphate consumption, carbon-source mineralisation and total cell counts in PHC-contaminated aquifer sediment. Adapted from Kleikemper *et al*. (2002a).

Substrate amended	**Total sulphate consumed (Starting SO_4^{2-} concentration = 3 mM)** (mmol)	**Total carbon-source mineralised** (mmol)	**Total numbers of DAPI-stained cells** (10^9 g^{-1} dry sediment)
Lactate	1.80 ± 0.00	1.45 ± 0.00	4.10 ± 1.00
Acetate	1.19 ± 0.01	1.38 ± 0.00	2.80 ± 0.40
Butyrate	1.80 ± 0.15	0.80 ± 0.00	2.80 ± 0.60
Propionate	1.66 ± 0.37	1.27 ± 0.30	3.30 ± 0.60

Despite the many advantages described for the use of lactate in BSR, its application is restricted in large scale BSR processes by its cost (Kaksonen *et al.,* 2004). Dairy waste, rich in lactic acid and lactose (which can be fermented further to yield lactic acid) (Mostafa, 2001), provides a potential carbon-source and electron donor for BSR. Drury (1999) reported that whey addition in AMD treatment increased the efficiency of metal and sulphate removal as well as neutralisation.

Although the ranges of collected data are subject to varying experimental conditions, a general comparison of the kinetic parameters generated using different carbon-sources shows lower $K_{SO_4^{2-}}$ and $K_{electron\ donor}$ values with lactate, signifying higher substrate affinity by lactate utilisers. A higher negative Gibb's free energy of the lactate oxidation reaction further substantiates preference for lactate as the carbon-source (Table 2.8). In comparison with other electron donors (H_2, ethanol, propionate and acetate), growth of SRB on lactate gives a better energy yield (Table 2.8).

2.3.1. Lactate Metabolism

Lactate is a common and easily degradable substrate utilised by many microorganisms via different metabolic pathways (Zellner *et al*., 1994). Lactate is oxidised either completely or incompletely in the presence of sulphate by a diverse range of SRB strains (Reactions 2.10 and 2.11) (Widdel, 1988; Okabe *et al*., 1995;

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Kaksonen *et al*., 2003). Thus, its utilisation is expected to encourage high microbial diversity and consequently resilience of the system to environmental challenges. Postgate (1984) proposed that *Desulfovibrio*, the most studied SRB genus, oxidises lactate to acetate and bicarbonate via pyruvate and acetyl phosphate, coupled with sulphate reduction. This is catalysed by the enzyme lactate dehydrogenase (Figure 2.2).

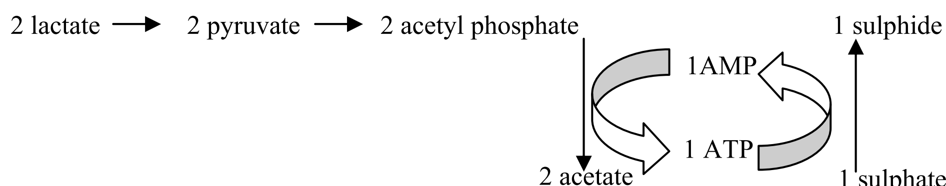
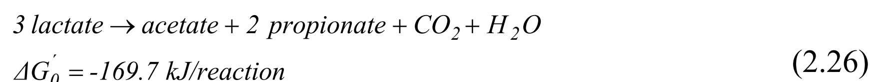
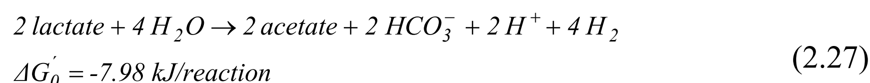


Figure 2.2: Metabolic pathway of lactate to acetate via pyruvate (Postgate, 1984).

Other strains belonging to this same genus are able to oxidise lactate using nitrate as the electron acceptor (Postgate, 1984). Sulphate reducers such as *Desulfobulbus propionicus* are able to ferment lactate in the absence of sulphate, resulting in the production of propionate and acetate (Reaction 2.26) (Widdel and Pfennig, 1982; Heimann *et al*., 2005). Bryant *et al*. (1977) showed that *Desulfovibrio* sp. co-cultured with hydrogen-utilising methanogens, in the absence of sulphate, converted lactate via a thermodynamically unfavourable fermentative pathway to acetate (Reaction 2.27). This reaction was made thermodynamically feasible by inter-species hydrogen transfer. The fermentative growth rate of *Desulfovibrio* strains on lactate is slower and produces lower growth yields than lactate oxidation coupled to sulphate reduction (Bryant *et al*., 1977). This is a consequence of positive or low negative Gibb's free energy of the lactate fermentation reaction resulting from H_2 production (Menert *et al*., 2004).



$$3\ lactate \rightarrow acetate + 2\ propionate + CO_2 + H_2O \quad \Delta G_0^{'} = -169.7\ kJ/reaction \qquad (2.26)$$



$$2\ lactate + 4\ H_2O \rightarrow 2\ acetate + 2\ HCO_3^- + 2\ H^+ + 4\ H_2 \quad \Delta G_0^{'} = -7.98\ kJ/reaction \qquad (2.27)$$

Kinetic properties are key factors in determining the preferred lactate metabolic pathway. In an investigation of a full-scale anaerobic digester by Zellner *et al*. (1994), *Desulfovibrio* sp. (a lactate oxidiser) was shown to have lower K_s and μ_{max} values than

Clostridium sp. (a lactate fermenter). In a non-sterile continuous system, composed of a mixed culture of microorganisms, characterised by low lactate concentration, lactate degradation by *Desulfovibrio* sp. was thus the preferred metabolic pathway (Zellner *et al*., 1994).

Table 2.8: Comparison of growth kinetic and thermodynamic parameters of SRB grown on different electron donors.

Investigator	Electron donor (e^-d)	Organism	T (°C)	pH	Reactor	ΔG_{rxn} (k cal mol $^{-1}_{e^-d}$)	Biomass yield (g $_{DW}$ g $^{-1}_{SO_4^{2-}}$)	Biomass yield (g $_{DW}$ g $^{-1}_{e^-d}$)	μ_{max} (h^{-1})	Energy yield (g_{DW} $kcal^{-1}$)	K_{e-d} (mg l^{-1})	$K_{SO_4^{2-}}$ (mg l^{-1})
Ingvorsen and Jorgensen, 1984	lactate	*Desulfovibrio vulgaris*	30	7.2	B	_	0.074	_	0.011	_	_	0.5
		D. sapovorans	30	7.2	B	_	0.091	_	0.007	_	_	0.7
		D. salexigens	30	7.2	B	_	0.083	_	0.021	_	_	7.4
Okabe and Characklis, 1992	lactate	*Desulfovibrio desulfuricans*	35/43	7	C	_	_	0.036/0.032	0.46/0.55	_	3.6/10	_
Reis *et al*., 1992	lactate	*Desulfovibrio* strains	37	6.2-6.6	B	_	_	0.028-0.079	0.336	0.13-0.37	_	_
Okabe *et al*., 1992	lactate	*Desulfovibrio desulfuricans*	35/43	7	C	_		0.02/0.017	0.34/0.35	_	_	1.8/1
Okabe *et al*., 1995	lactate	*Desulfovibrio desulfuricans*	35	7	B	_	_	_	0.058-0.2 0.21-0.33	_	_	_
	lactate	*D.desulfuricans*	35	7	C	_	_	0.011-0.036		_	_	_
Cooney *et al*., 1996	lactate	*Desulfovibrio desulfuricans*	37	7.8	B	_	0.07-0.09	0.04-0.06	0.029-0.056	0.37	_	_
Konishi *et al*. , 1996	lactate	*Desulfovibrio desulfuricans*	37	7	B	_	_	_	0.37	_	_	_
Nagpal *et al*., 2000	lactate	*Desulfovibrio desulfuricans*	_	_	_	-18.87	_	0.03-0.032	_	0.14-0.15	_	_
Kaksonen *et al*., 2003	lactate	Mixed culture	35	6.8	C	_	0.035-0.074	0.039-0.054	_	_	_	_
O'Flaherty and Colleran,1998	ethanol	*Desulfovibrio multivorans*	37	7.5-7.8	B	_	_	_	0.039	_	70	22

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Table 2.8 (contd): Comparison of growth kinetic and thermodynamic parameters of SRB grown on different electron donors.

Investigator	Electron donor (e^-d)	Organism	T (°C)	pH	Reactor	ΔG_{rxn} (kcal.mol$^{-1}_{e^-d}$)	Biomass yield (g_{DW} $g^{-1}_{SO_4^{2-}}$)	Biomass yield (g_{DW} $g^{-1}_{e^-d}$)	μ_{max} (h^{-1})	Energy yield (g_{DW} $kcal^{-1}$)	K_{e-d} (mg l^{-1})	$K_{SO_4^{2-}}$ (mg l^{-1})
Erasmus, 2000	ethanol	Mixed culture	35	7.4	C	_	_	_	0.0114	_	5.5-6.4	284
	ethanol	Mixed culture	35	7.8	B	_	_	_	_	_	9.84	6.81
Nagpal *et al*., 2000	ethanol	*Desulfovibrio desulfuricans*	_	_	_	-15.91	_	0.0084-0.01	_	0. 047-0.059	_	_
Widdel and Pfennig, 1981	acetate	*Desulfobacter potsgatei*	32	_	B	-11.35	_	0.074	0.035	_	_	_
Ingvorsen *et al*., 1984	acetate	*Desulfobacter postgatei*	30	_	B	_	0.158	_	0.03	_	_	4.2
Widdel, 1987	acetate	*Desulfobacter curvatus*	25-28	_	B	_	_	_	0.033	_	_	_
O'Flaherty *et al*., 1998	acetate	*Desulfonema magnum*	30	7.5-8	B	_	_	_	0.018	_	120	45
	acetate	*Desulfobacter postgatei*	30	7.5-8	B	_	_	_	0.039	_	12	20
Moosa , 2000	acetate	Mixed SRB culture	35	7.8	C	_	0.58	0.56	0.05-0.065	_	71	27-231
Cooney *et al*., 1996	pyruvate	*Desulfovibrio desulfuricans*	37	7.8	B	_	0.13	0.09	0.068	_	_	_
O'Flaherty and Colleran,1998	propionate	*Desulfobulbus propionicus*	37	7.5-8	B	_	_	_	0.115	_	50	3

Reactor types: B, batch; C, continuous.

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In an investigation by Krekeler *et al*. (1998), it was reported that lactate was oxidised by certain *Desulfovibrio* strains, isolated from an oxic-anoxic environment, using oxygen as the electron acceptor. However, this respiration only resulted in an increase in cell size but not cell number, indicating the inhibition of cell division by oxygen.

2.4. MICROBIAL GROWTH KINETIC MODELS

The rate of microbial reaction is dependent on the concentration of substrates. This can be described using the empirical Monod kinetics model (Equation 2.28) (Monod, 1949; Widdel, 1988) which relates specific growth rate of the bacterial population to the concentration of the limiting substrate.

$$\mu = \frac{\mu_{max} S}{K + S} \tag{2.28}$$

where μ and μ_{max} are specific and maximum specific growth rates (h^{-1}) of the microorganism respectively; S and K are the concentration and half-saturation constant of the limiting substrate. At low limiting substrate concentration, μ is directly proportional to S, resulting in first-order reaction kinetics. At high S, μ becomes independent of the substrate concentration, thus resulting in zero-order kinetics.

Dependence of specific growth rate on two limiting substrates is typically described using a multiplicative approach, represented as:

$$\mu = \mu_{max} \frac{S_1}{K_1 + S_1} \frac{S_2}{K_2 + S_2} \tag{2.29}$$

where S_1 and S_2 are concentrations of the limiting substrates; K_1 and K_2 are half-saturation constants for the two limiting substrates respectively (Bailey and Ollis, 1986).

Although the Monod kinetic model has been used most commonly, other mathematical models used for the prediction of microbial growth rate include the Chen and Hashimoto (Equation 2.30) and Contois models (Equation 2.31). These two models have been used to describe specific microbial growth rate as a function of residual substrate concentration at both high feed substrate and biomass concentrations (Chen and Hashimoto, 1980). In the Contois model, the specific growth rate was shown to be dependent on the growth limiting nutrient in both the effluent and feed substrate concentrations using an empirical constant which was

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related to the bacterial concentration (Contois, 1959). The Chen and Hashimoto model is a modification of the Contois model. It describes the K_s term as the product of the yield coefficient and the K_s term in the Contois expression (Chen and Hashimoto, 1980).

$$\mu = \frac{\mu_{max} S}{K_s S_o + (1 - K_s) S} \tag{2.30}$$

$$\mu = \frac{\mu_{max} S}{K_s'' X + S} \tag{2.31}$$

where S_o is the initial substrate concentration (mg l^{-1}) and X is the biomass concentration (mg l^{-1}).

2.4.1. Microbial Energetics

In the absence of extracellular product formation, the energy obtained from microbial substrate utilisation is channelled via two main routes. The proportion that is utilised for functions essential for the cell's integrity is known as the maintenance energy. These functions are non-growth associated processes such as cell material turnover, motility requirement, regulation of cell membrane potential, osmotic pressure and internal pH. The other fraction of the energy is used for cell growth (Pirt, 1965). Biomass yield from a substrate is often described by Pirt equation (Equation 2.32). This expression shows that the apparent or observed biomass yield is dependent on the specific growth rate and the maintenance coefficient under substrate-limited conditions. It thus follows that, the slower the growth rate the larger the percentage of the total substrate utilised for maintenance and subsequent decrease substrate availability for biomass production.

$$\frac{1}{Y_{app}} = \frac{1}{Y_{max}} + \frac{m_s}{\mu} \tag{2.32}$$

where Y_{app} ($g_{biomass}$ $g^{-1}_{substrate\ consumed}$) is the observed growth yield, Y_{max} ($g_{biomass}$ $g^{-1}_{substrate\ consumed}$) is the maximum theoretical yield equivalent to the stoichiometric growth yield, μ (h^{-1}) is the observed growth rate, and m_s ($g_{substrate\ consumed}$ $g^{-1}_{biomass}$ h^{-1}) is the maintenance coefficient.

The observed growth yield, Y_{app}, describes the total amount of biomass production as a result of total substrate utilisation by both growth and maintenance activities. In

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contrast, the maximum theoretical growth yield, $Y_{max,}$ accounts for the substrate assimilated directly into cell material only (Nielsen *et al*., 2005).

2.4.2. Environmental Effects on Growth Kinetics

Bacterial growth and metabolism are dependent on temperature, with bacterial activity increasing with increasing temperature in temperature ranges below an optimum (Bailey and Ollis, 1986). The kinetic constants, μ_{max}, K_s and Y_{app} which characterise microbial growth for given growth conditions are also known to be temperature dependent (Okabe and Characklis, 1992). A previous kinetics study showed that μ_{max} and Y_{app} were negatively affected by high total sulphide concentrations (108 to 437.5 mg l^{-1}) (Okabe *et al*., 1995).

Undissociated forms of acetic acid and hydrogen sulphide were shown to have toxic effects on SRB growth kinetics, and these effects were described to be pH-dependent. At relatively lower pH (~6.2), acetic acid inhibition was profound, but at higher pH (6.6 to 7.0) hydrogen sulphide inhibition was prevalent. The combined effect of these products of sulphate reduction and lactate oxidation by *Desulfovibrio* strains can be expressed by Equation 2.33 (Reis *et al*., 1992):

$$\frac{\mu}{\mu_{max}} = \left(1 - \frac{H_2S}{547}\right)^{0.401} \times \left(1 + \left(\frac{AcH}{54}\right)^{1.08}\right)^{-1} \tag{2.33}$$

where AcH and H_2S represent acetic acid and hydrogen sulphide concentrations respectively.

Specific growth rate and cell yield have also been shown to be pH-dependent, increasing with increase in pH below the optimum pH. This can be described by the following kinetic model of non-competitive inhibition (Reis *et al*., 1992):

$$\mu = \frac{\mu_{max}}{1 + \frac{H^+}{K_H} + \frac{K_{OH}}{H^+}} \tag{2.34}$$

where K_H and K_{OH} are inhibition constants.

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A model has been previously developed, based on kinetics investigations of SRB growth on acetate as carbon-source and electron donor (Moosa 2000; Moosa *et al*., 2002; 2006). A kinetic study using ethanol as a limiting substrate for SRB mixed culture has also been undertaken (Erasmus, 2000; Hansford *et al*., 2007). In the investigation by Moosa *et al*. (2002), the Contois equation was the preferred equation describing bacterial growth based on goodness of fit. Arrhenius dependence on temperature was included. The derived model is shown in Equation 2.35 (Moosa *et al*., 2002; 2006). This expresses the dependence of the rate of sulphate reduction on inlet sulphate concentration, bacterial concentration, temperature and residual sulphate concentration:

$$r_S = \left(\frac{(0.061)S}{6.52\times 10^{-35} e^{198/RT} S_o X + S} - 8.8\times 10^{11} e^{-78.7/RT} \right) \frac{X}{0.567} \quad (2.35)$$

where r_s is the rate of sulphate reduction (kg l^{-1} h^{-1}), R is the universal gas constant (atm 1 mmol^{-1} K^{-1}), S and S_o are the residual and feed sulphate concentrations (kgl^{-1}) respectively and T is the absolute temperature (K).

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Using the kinetic model previously developed by Moosa *et al*. (2002) (Equation 2.36), Hansford *et al*. (2007) showed that the expression developed using acetate as carbon-source fitted the experimental data obtained from the kinetic study of biological sulphate reduction when ethanol served as the electron donor. This expresses the dependence of the rate of sulphate reduction on inlet sulphate concentration, bacterial concentration and residual sulphate concentration:

$$r_S = \left(\frac{\mu_{max} S}{K_s' S_o X + S} - K_d \right) \frac{X}{Y} \quad (2.36)$$

where K_s' is the apparent saturation constant (kg dry weight^{-1} m^3), K_d is the decay coefficient (h^{-1}). The other terms are as described in Equation 2.35.

2.5. INTERACTIONS OF SRB WITH OTHER MICROBIAL GROUPS

Sulphate reducers demonstrate diverse types of inter-relationships with other groups of microorganisms in nature (Figure 2.3). These enable them to play several roles in the environment. Mixed consortia of bacterial populations carry out biological wastewater treatment. The ultimate performance of this treatment process is

dependent on the interactions occurring between the microorganisms (Lens *et al*., 1995). It is known that the degradation process of complex organic compounds in anaerobic treatment of wastewater is significantly affected by the presence of sulphate (Oude Elferink *et al*., 1998a).

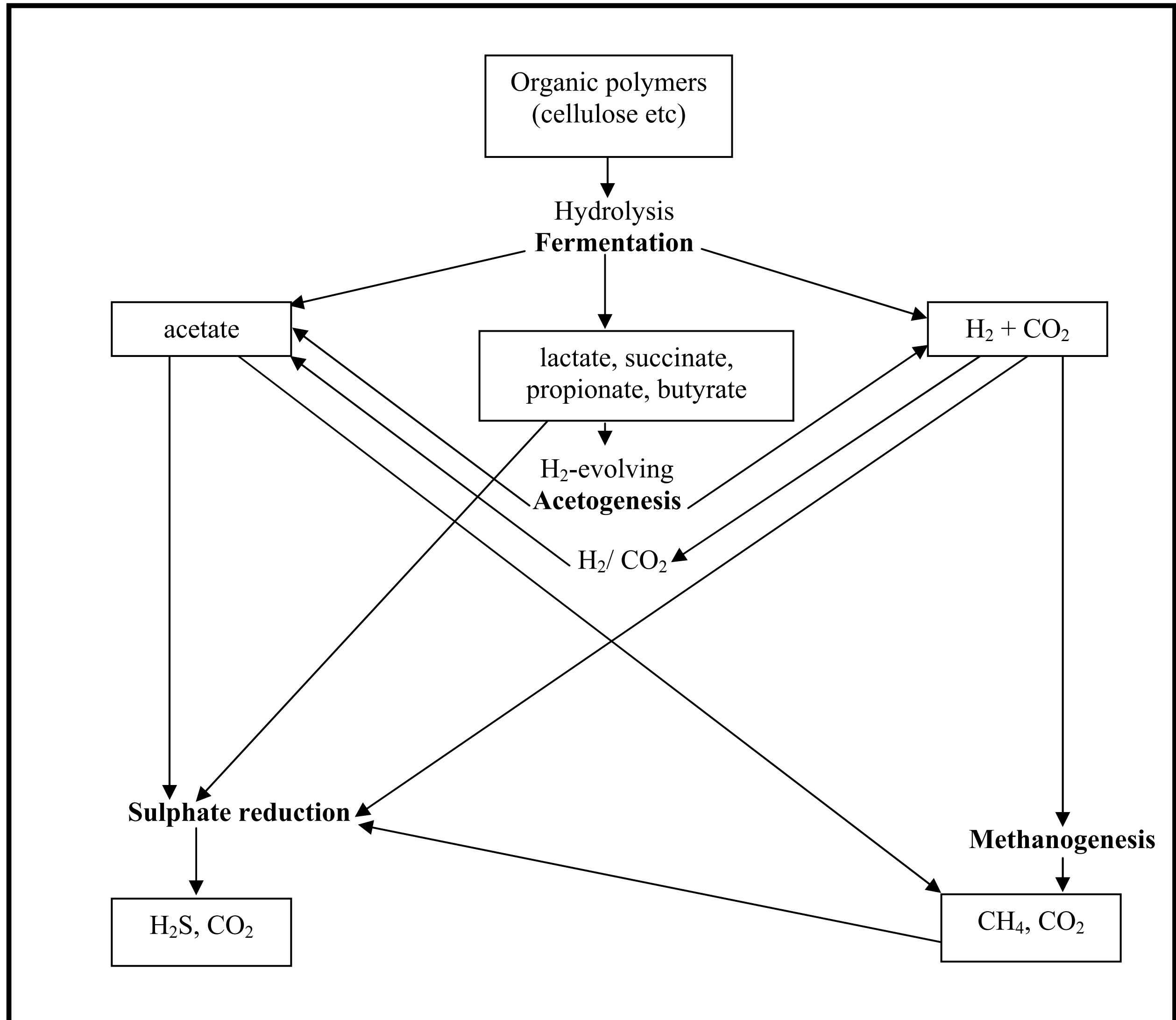

Figure 2.3: Metabolic pathways involved in anaerobic digestion (Lorax, 2003).

The anaerobic degradation process of sulphate-containing wastewaters occurs through the synergistic interaction of five different classes of microorganisms: hydrolytic, fermentative, acetogenic, sulphate-reducing and methanogenic in a multi-step process, with methanogenesis and sulphate reduction acting as the terminal processes (Figure 2.3) (Menert *et al*., 2004; Patidar and Tare, 2005).

Sulphate-reducing bacteria rely syntrophically on fermentative bacteria for the supply of carbon-sources from polymeric compounds (Ravenschlag *et al*., 2000) whilst the former also aid the activity of the acetogenic bacterial group when co-cultured (Harada *et al*., 1994). Coexistence of SRB with methane producing archaea (MPA or methanogens) has been the most widely studied interaction of SRB with other microorganisms in anaerobic digestion. Differing reports have been made on the effect of sulphate reduction on the anaerobic process. Many reported competition between the sulphate-reducing bacteria and methane producing archaea while others indicated a syntrophic relationship between these two groups of bacteria (Vossoughi *et al*., 2003). Essentially, competition between different microbial groups is dependent on the kinetic properties of the interacting microorganisms as defined by maximum specific growth rate (μ_{max}) and substrate affinity (K) (Weijma, *et al*., 2002).

Acetate and hydrogen (products of acidogenesis and fermentation) are substrates common to both MPA and SRB (Tang *et al*., 2004). Hence, their relative affinities for these substrates influence their interaction. Sulphate reducers have higher affinities for acetate and hydrogen, consequently out-competing MPA at low substrate concentrations (Gupta *et al*., 1994; Koizumi *et al*., 2003). The MPA-SRB relationship is also a function of COD/Sulphate ratio. Sulphate reduction is favoured when this ratio is less than 0.66 ($g\ g^{-1}$) (Oude Elferink *et al*., 1998a). SRB are the predominant group when sulphate is in excess, while methanogens dominate under limiting sulphate concentrations (Raskin *et al*., 1996). Work by Raskin *et al*. (1996) showed that a fixed-bed methanogenic reactor was composed of 25% methanogens and 15% SRB when fed with glucose and in the presence of 0.02 mM sulphate. When 0.313 mM sulphate was included in the feed stream, the abundances of populations in the reactor were 30 to 40% SRB and 8% MPA. This shift in microbial population was accompanied by the predominance of sulphidogenesis in the anaerobic reactor. Hydrogen sulphide, a product of sulphate reduction, is known to be inhibitory to MPA (Gupta *et al*., 1994). Sulphide accumulation in anaerobic digestion is known to lead to digester failure (Hulshoff Pol *et al*., 2001).

Bryant *et al*. (1977) reported a synergistic relationship between *Desulfovibrio* strains and H_2-consuming methanogens. Hydrogen produced by sulphate reduction acts as the electron donor for the methanogens. Consumption of hydrogen by these

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microorganisms reduces the Gibb's free energy to a more negative value, consequently encouraging concomitant growth of the two microbial groups. Other factors influencing the SRB-MPA relationship include pH, microbial concentration, reactor configuration and operational conditions, and temperature (Oude Elferink *et al.,* 1994). It is also noteworthy that competitive relationships for sulphate exist within groups of sulphate reducers, in accordance with varying μ_{max}, $K_{e\text{-}d}$, $K_{SO_4^{2-}}$, yield and maintenance requirements (Oude Elferink *et al*., 1998a).

2.6. ENVIRONMENTAL EFFECTS ON SRB COMMUNITY STRUCTURE

It is well known that optimum cultivation conditions differ across pure SRB cultures. Existence of SRB in extreme (low temperature, hypersaline, geothermal, contaminated etc.) environments exhibits their wide diversity and adaptability. Population changes and relative composition of members in a heterogeneous community are induced by environmental perturbations of both physical and chemical nature. These include pH, temperature, substrate availability and metabolic activities of the community constituents. These changes often lead to population instability, and consequently, reduced biodiversity (von Wintzingerode *et al*., 1997; Amann and Kühl, 1998). Microbial community dynamics refers to the versatility of population members to physicochemical changes. This phenomenon describes the ability of a microbial system to react and adapt to environmental perturbations. Microbial population stability as induced by biodiversity, and community dynamics in response to physicochemical changes, are vital in maintaining a robust biological wastewater treatment (Miura *et al*., 2007). In mixed microbial environments, the existing microorganisms are the robust members resulting from the "survival of the fittest" due to evolution and adaptation over time in response to nutritional requirements and influence from persisting environmental conditions (Roychoudhury, 2004). This sort of selectivity of robust and resilient groups of microorganisms can be realised in microbially-mediated systems by using continuous culturing with exposure to varying physicochemical challenges to engineer the population. Of these environmental conditions, substrate limitation is crucial as this is characteristic of natural mixed communities. Hence, dynamic bacterial populations are required for survival as many population groups compete for the limited available nutrients. The ability of certain groups of microorganisms to thrive in these environments is dependent on their

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coexistence with other groups and interactions with these groups. Analysis of microbial population distribution gives a better understanding of a mixed system, the biological processes involved and the roles of the microorganisms undertaking the processes (Bowman and McCuaig, 2003). Increased diversity encourages adaptation and resistance to perturbations such as metal and acid toxicity experienced in BSR treatment of acidic wastewaters rich in heavy metals. Ito *et al*. (2002a) reported that the distribution of population members of a mixed microbial community (biofilm), and the link between the SRB community dynamics and sulphate reducing activity were dependent on substrate limitation.

2.6.1. SRB Community Structure Response to Substrate Amendment

Studies have shown that all SRB genera preferentially oxidise specific electron donors (Hanselmann *et al*., 1995; Purdy *et al*., 1997; Nagpal *et al*., 2000; Kuever *et al*., 2001; Ingvorsen, *et al*., 2003). Very few strains of SRB are known to oxidise a broad range of electron donors readily (Oude Elferink *et al*., 1995). Thus, there will be a predominance of certain strains of SRB when mixed cultures are amended with a particular substrate. The understanding of the link between shifts in microbial community structure in response to substrate utilisation is critical in the development of biological treatment technology (Rogers *et al*., 2000).

The community dynamics, interactions and activity distribution of the complex consortia of microorganisms involved in the anaerobic digestion of organic matter have been well studied and characterised (Raskin *et al*., 1995a; 1996; Oude Elferink *et al*., 1998a; Santegoeds *et al*., 1999). However, the population analysis, interactions and dynamics of the SRB communities involved in BSR with respect to different substrate have not received much attention. One of the few studies reported was by Kaksonen *et al*. (2004). Greater bacterial diversity was observed in the ethanol-fed reactor relative to the lactate-fed reactor. Using clone library and DGGE techniques, operational taxonomic units of 23 and 15 were observed in the ethanol- and lactate-fed reactors respectively. In addition, a major distinction was observed between SRB population groups growing on the different substrates. The dominant sequences obtained from the ethanol-fed reactor were affiliated to the *Proteobacterium* group while the majority of the clones obtained from the lactate-fed reactor belonged to the

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Nitrospira phylogenetic group. In another study, the highest number of SRB cells was detected in lactate-amended sediments samples, in comparison with the sediments amended with acetate, butyrate, and propionate (Table 2.3) (Kleikemper *et al*., 2002a). Lactate-utilising SRB include, among others, *Desulfobacca acetoxidans*, *Desulforhabdus amnigenus*, *Desulfovibrio desulfuricans*, *Desulfovibrio vulgaris* and *Desulfobulbus* spp. (Okabe and Characklis, 1992; Nagpal *et al*., 2000; Kleikemper *et al*., 2002a; Kaksonen *et al*., 2004). Knowledge of microbial group-substrate specificity is fundamental in exploring the relationship between community dynamics and process kinetics (Purdy *et al.,* 2002).

2.7. RELATIONSHIP BETWEEN SULPHATE REDUCTION KINETICS AND SRB COMMUNITY DYNAMICS

Previous investigations have focused on the effects of operating variables on the rate of microbial sulphate reduction and SRB growth kinetics. Okabe and Characklis (1992) investigated the effects of temperature and phosphorus; while Konishi *et al*. (1996) studied the correlation between rates of anaerobic growth of *Desulfovibrio desulfuricans*, pH and inoculum size. Okabe *et al*. (1992; 1995) and Reis *et al*. (1992) investigated kinetics of sulphide inhibition on *D. desulfuricans* and *Desulfovibrio* species activities respectively. There are reports of population shifts based on substrate amendments and specificity of SRB groups for substrates in natural environments, as discussed earlier (Section 2.6). Even though the efficiency of BSR is dependent on SRB consortium composition, physicochemical characteristics and the operating conditions (Geets *et al*., 2006), studies of the correlation between sulphate reduction kinetics and community dynamics, and the quantitative estimation of SRB growth or metabolic activities using community analysis in BSR processes are rare (Ito *et al*., 2002a). Kinetic properties often indicate microbial community dynamics in response to environmental perturbations (Roychoudhury, 2004).

Investigation of the relationship of population diversity and dynamics with the performance of microbially-mediated processes has been attracting intensive research due to the potential of this link in the optimisation and control of bioremediation processes (Boon *et al*., 2002; von Canstein *et al*., 2002; Reinthaler *et al*., 2005). Several studies have shown that there is a link between microbial community structure and reactor performance (Collins *et al*., 2003; Pender *et al*., 2004). These

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investigations provide better understanding of bioreactor ecosystems and opportunity for process optimisation through the manipulation of the community structure. Most of the studies that have addressed the effect of perturbations on microbial ecology have focused on natural ecosystems (soil and aquatic habitats). They include the effects of disturbances of physicochemical or anthropogenic nature, seasonal cycles and contamination on the microbial community structure in these natural environments. These investigations have also described the relationship between microbial community composition and the processes they mediate (Ferris and Ward, 1997; Ferris *et al*., 1997; Tuomi *et al*., 1997; Li *et al*., 1999; Griffiths *et al*., 2005; Reinthaler *et al*., 2005). Other systems that have been studied include methanogenic systems (Fernández *et al*., 1999; 2000) and activated sludge reactors (Kaewpipat and Grady, 2002). The stability of a microbial system depends on consistency in both the microbial community structure of the system and the process they mediate, when subjected to external changes of a physicochemical nature (Fernández *et al*., 1999). Of these two characteristics of an ecosystem, the population stability plays a major role. This feature is defined by the robustness of the constituent members of the microbial community.

The relationship between the microbial community structure and metabolic activity of heterogeneous communities consisting of SRB and methanogens has been well established. SRB are the predominant group when sulphate is in excess, while methanogens dominate under limiting sulphate concentrations (Raskin *et al*., 1995a; 1996). In the investigation by Raskin *et al*. (1996), shifts between sulphidogenesis and methanogenesis with coincident population shifts were observed under perturbation of sulphate concentration in anaerobic biofilms (Figure 2.4).

There is a positive link between functional and structural dynamics of SRB populations (Santegoeds *et al*., 1998). Li *et al*. (1999) reported a significant direct relationship between *Desulfobulbus* predominance and sulphate reduction rate ($P<0.001$, $r = 0.827$). Poulsen *et al*. (1993) demonstrated that there was a positive link between SRB growth rates and their rRNA content in anaerobic biofilms, using FISH intensity as a measure. This observation was further corroborated by Ravenschlag *et al*. (2000), whose study investigated the community composition of a marine ecosystem with the aid of FISH and rRNA slot blot hybridisation. The vertical

variation of the dominant group, *Desulfosarcina-Desulfococcus* was congruent with the sulphate reduction rate.

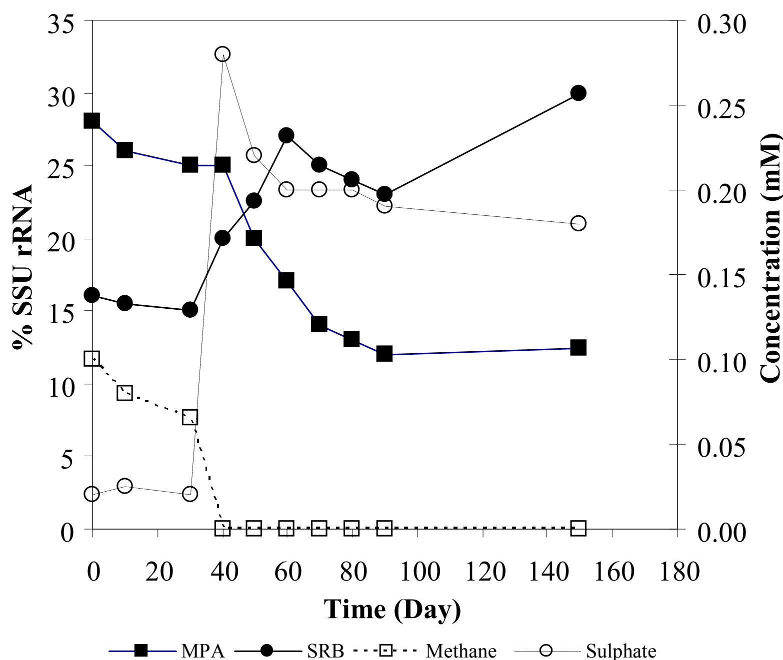

Figure 2.4: Microbial population and activity shifts in anaerobic biofilms. Sulphate concentration perturbation on day 40 with congruent increase in SRB population and SRR. (Adapted from Raskin *et al*., 1996).

Most of the previous correlation studies (SRB distribution and metabolic activities) have been aimed at characterisation and stratification of natural environments or gaining insight into the survival of sulphate-reducers in these environments (Santegoeds *et al* 1998; Li *et al*., 1999; Okabe *et al*., 1999; Ravenschlag *et al*., 2000; Ito *et al*., 2002a; 2002b).

The application of the knowledge of the correlation studies to active bioremediation processes is not extensively reported (Ito *et al*., 2002a). Prevalence of bacterial groups is found to correspond to the levels of their activities and significance in a mixed community (Santegoeds *et al*., 1999). Hence, changes in microbial distribution or community dynamics affect processes undertaken by these microorganisms. Growth rates of microorganisms and stoichiometry of a biological process are influenced by the type of microbial species present in a mixed consortium (Chiu *et al*., 1972).

The simultaneous application of culture-dependent procedures and molecular tools establishes the link between the existence of different microbial community members

and their individual roles in the consortium (Ravenschlag *et al*., 2000). In addition, this provides a more explicit insight into improving bioreactor performance (Pender *et al*., 2004).

Raskin and co-workers (1995b) showed a direct and quantitative link between anaerobic bioreactor efficiency and microbial population composition by employing rRNA-based hybridisation in conjunction with traditional metabolic assays involving substrate-product concentration estimation. They were also able to determine the relative contributions and importance of the different community members (i.e. SRB and methanogens) to the net performance of different anaerobic digesters. The relationship between operational factors involved in wastewater treatment processes and the relative dominance of different microbial groups in the consortium responsible for the process is also crucial in influencing the ultimate performance of the system. The operating parameters can be selected or varied to favour the groups of microorganisms required for the process (Wilderer *et al*., 2002).

Microbial population diversity and dynamic community structure are required for the stable performance of biological wastewater treatment systems (Miura *et al*., 2007). While biodiversity preserves the microbial community structure, a flexible community is important for a robust system performance (Fernández *et al*., 2000). Additionally, optimal reactor performance is not necessarily a consequence of a large population, but the presence of active population members (Merkel *et al*., 1999). However, it is imperative to preserve as much biodiversity as possible in a bioremediation system. This is because a more diverse community is more likely to consist of a greater number of active members in comparison to a less diverse community (Cardinale *et al*., 2006).

2.8. CHARACTERISATION OF SULPHATE-REDUCING BACTERIA COMMUNITIES

The diversity of SRB is influenced by their interaction with the environment and their response to the physicochemical properties characterising these ecosystems (Amann and Kühl, 1998; Hunter-Cevera, 1998; Hallberg and Johnson, 2005). In wastewater treatment processes, microbial diversity is also influenced by the operational parameters (Wilderer *et al*., 2002). There are limitations to characterisation,

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phylogenetic and physiological studies of microbial consortia in natural environments due to the small size of some microorganisms and the inability to culture a large number of them. Hence, only a relatively small proportion of all microorganisms has been identified and characterised (Olsen *et al*., 1986; Ward *et al*., 1992; Simon *et al*., 1995; Muyzer and Smalla, 1998; Santegoeds *et al*., 1998). Culture-dependent procedures do not account for a wide spectrum of constituents of microbial communities, hence, underestimating population diversity (Amann *et al*., 1995; Giraffa, 2004; Tamaki *et al*., 2005). This approach often does not give details on the predominance and significance of microbial species in a microbial consortium (Brune *et al*., 2000). The advent of culture-independent molecular tools, such as denaturing gradient gel electrophoresis (DGGE), 16S gene rRNA cloning and sequencing, terminal restriction fragment length polymorphism (T-RFLP) and fluorescence *in situ* hybridisation (FISH) has resulted in a better understanding of SRB microbial communities and how the constituent members interact (Amann *et al*., 1990; 1995; Phelps *et al*., 1998; Besemer *et al*., 2005; Pérez-Jiménez and Kerkhof, 2005). Both molecular and culture-based techniques can be used in concert to provide a more extensive picture of the community structure of mixed microbial culture environments (Muyzer and Smalla, 1998; Santegoeds *et al*., 1998; Kimura *et al*., 2005).

Although natural environments inhabited by sulphate-reducing bacteria have been well characterised, characterisation of the SRB community structure employed in the treatment of AMD at the bioreactor level has not received much attention (Raskin *et al*., 1995b; Kaksonen *et al*., 2006).

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2.8.1. Ribosomal RNA (rRNA), an Important Molecular Marker

Ribosomal RNA (rRNA) plays a key role in protein synthesis. These molecules occur in every organism (with the exception of viruses) and are highly conserved through billions of years of evolution. This accounts for its important role in the analysis of microbial diversity (Ward *et al*., 1992). Some regions within the primary and secondary structures of rRNA between different species are highly conserved. These regions play key roles in the function and structure of rRNA. This molecule provides a consistent link between all organisms, consequently providing prospects of establishing phylogenetic relationships among them (Olsen *et al*., 1986). In addition,

rRNA-based techniques are able to detect novel microorganisms in natural environments (Spring *et al*., 2000).

Most prokaryotes have three types of rRNA molecules: 5S, 16S and 23S rRNA molecules. These are characterised by nucleotide sequences of approximately 120, 1 500 and 3 000 nucleotides respectively. Owing to the small size of the 5S molecule, it does not have sufficient information for phylogenetic inferences as compared to 16S and 23S (Olsen *et al*., 1986; Amann *et al.*, 1995; 1997). Larger-sized rRNA molecules (16S and 23S) have been used to analyse unculturable and small size microorganisms which could not be previously studied (Simon *et al*., 1995). Using 16S rRNA to analyse microorganisms provides more specific information than is provided by morphology. For example, *Fibrobacter* strains which were initially classified by phenotypic similarity and grouped into the genus *Bacteroides* were found to possess a different 16S rRNA gene sequence from the other members of this genus, thus necessitating their reclassification (Amann *et al*., 1990). In addition, microorganisms that were formerly grouped into different genera and species based on their morphology were discovered to be closely related e.g. *E. coli* and *Proteus vulgaris* previously regarded as distant relatives were both found to possess a common 16S RNA nucleotide sequence with a high degree of conservation (Ward *et al*., 1992).

2.8.1.1. Ribosomal RNA (rRNA) Population Analyses

Earlier developments in the utilisation of ribosomal RNA for the characterisation of mixed microbial communities involved extraction of 5S rRNA from the constituent members and their comparative analyses with the existing database (Stahl *et al*., 1984; 1985). More recent approaches employ larger molecules; 16S in prokaryotes and 18S in eukaryotes (Olsen *et al*., 1986; Ward *et al*., 1992). Exploitation of 16S rRNA allows for more accurate determination of phylogenetic relatedness. It is not limited by community complexity as opposed to the 5S rRNA molecule.

Ribosomal RNA-based analyses are attracting more attention and are employed in environmental studies (Olsen *et al*., 1986; Amann *et al*., 1990; Simon *et al*., 1995). The utilisation of these molecular techniques in population characterisation can be achieved via cloning and sequencing of the primary nucleotide sequence, followed by a comparative analysis against an extensive assemblage of known sequences from

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pure cultures (database). RNA molecules can be directly isolated, and the DNA coding for the production of the sequence can be indirectly used via shotgun cloning, amplification using PCR (polymerase chain reaction) or rcDNA (ribosomal complementary DNA) (Olsen *et al*., 1986; Amann *et al*., 1992; 1995; Ward *et al*., 1992; Brown, 1995). Also, rDNA (ribosomal DNA) in conjunction with culture-dependent methods can be employed in investigating microorganisms with low growth rates (MacGregor, 1999).

These methods cannot be used as absolute quantitative measures, but provide an extensive picture of microbial diversity (Wobus *et al*., 2003). Moreover, the PCR-based approach and the use of rcDNA are known to have some shortcomings including the formation of imperfect hybridisation of the primer to rRNA nucleotide sequence and the production of chimeric molecules. Chimeric molecules are formed by the hybridisation of two heterologous nucleotide sequences from different species during the amplification process. The exploitation of DNA molecules has other disadvantages, such as the inability to subject DNA molecules to the rigorous extraction treatments which are often required for environmental samples (Amann *et al*., 1992; Ward *et al*., 1992), and their relative structural complexity in comparison with rRNA molecules (Edgcomb *et al*., 1999). Intact DNA molecules can still be obtained from dead cells. Hence, DNA analysis is not viable for indicating cell function or activity in a consortium (von Wintzingerode *et al*., 1997; Spring *et al*., 2000). The other problems associated with PCR-based analyses, as reviewed by von Wintzingerode *et al*. (1997) include: inhibition of PCR by contaminants from environmental samples, biased amplification of different molecules and the generation of mutants and DNA contamination.

By contrast, in the hybridisation probing procedure, complementary sequence or hybridisation probes which anneal to rRNAs based on the degree of sequence homology are used. This approach prevents some of the aforementioned drawbacks of PCR-based approaches (Spring *et al*., 2000). Ribosomal RNA sequence is said to be a *target* nucleotide if its primary nucleotide sequence is required for identification in hybridisation. Conversely, if the rRNA gene sequence is used in locating or identifying the sequence of another molecule it is called a *probe*. Hence,

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complementary oligonucleotides can be used to identify and quantify an individual or a specific group of organisms in a mixed population.

2.8.1.2. Fluorescence *in situ* hybridisation (FISH)

This method has been used effectively for the quantitative analysis of mixed communities, the distribution of SRB, their response to environmental changes and relationships between population members in these communities, e.g. biofilms (Amann *et al*., 1992; Santegoeds *et al*., 1998; Okabe *et al*., 1999; Bade *et al*., 2000; Wimpenny *et al*., 2000; Ito *et al*., 2002b), anaerobic granular sludge (Santegoeds *et al*., 1999), activated sludge (Wagner *et al*., 1993; Manz *et al*., 1998; Ingvorsen *et al*., 2003), laboratory CSTR (Icgen and Harrison, 2006a; 2006b) and marine environment (Ramsing *et al*., 1996). Many SRB are not amenable to laboratory cultivation (Brandt *et al*., 2001; Pérez-Jiménez *et al*., 2001), hence *in situ* probing, being a culture-independent procedure, has found wide application in assessing population shifts at different stages involved in BSR (Manz *et al*., 1998; Wobus *et al*., 2003; Icgen and Harrison, 2006a; 2006b).

As opposed to most culture-dependent procedures, FISH quantitatively links community structure or composition to activities of the population members (Amann *et al*., 1992; Rogers *et al*., 2000; Gillan *et al*., 2005; Wagner *et al*., 2006). Signals from the probes represent cellular rRNA content; these are indicative of the relative activities of the target community members. Being *in situ*, not involving nucleic acid extraction or subsequent amplification, FISH eliminates some potential biases (Wilderer *et al*., 2002). Ribosomal RNA-targeted FISH is often used, owing to rRNA's ubiquity, high genetic conservation and copious availability, just to mention a few (Ward *et al*., 1992: Amann *et al*., 1995; Amann and Ludwig, 2000). Moreover, it is well known that there is a direct relationship between rRNA quantity and growth rate of SRB (Raskin *et al*., 1996; MacGregor, 1999; Spring *et al*., 2000; MacGregor *et al*., 2001). This tool is also applicable for quantifying microorganisms with slow growth rates (Poulsen *et al*., 1993), but the sensitivity might be limited (Wallner *et al*., 1993).

Several rRNA-targeted oligonucleotide probes of varying specificities: group-specific; genus-specific; and species-specific, and comprehensive databases have been

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developed for SRB phylogenetic studies (Devereux *et al.,* 1992; Manz *et al*., 1998; Ito *et al*., 2002a).

In view of the fact that probes are designed to specifically hybridise with target microbial members of a mixed population, the degree of hybridisation can be used to quantify growth rates of the target strains (Poulsen *et al*., 1993). In an investigation by Simon *et al*. (1995), which employed rRNA-targeted FISH to study oceanic picophytoplanktonic cells (size 0.2 to 2 µm), there was a positive correlation between the magnitude of cell-bound probes and the growth rate.

The probe design is determined by the variability of the sequence of the target rRNA molecule. The degree of conservation of the target sequence determines the level of microbial group identification or relatedness (e.g. kingdom, genus and species) (Olsen *et al*., 1986; Amann *et al*., 1992; 1995; Devereux *et al.,* 1992; Ward *et al*., 1992). Use of *in situ* hybridisation is associated with some pitfalls. These include the following: (i) detection of little or no signal in the presence of low cell concentrations or low ribosomal content of inactive cells, (ii) specificity problems arising from the hybridisation of unknown sequences to a specific probe, (iii) the inability of sequences of closely related organisms to hybridise with a probe meant for all organisms of a particular population group, and (iv) hindered accessibility of target cells by probes because of some cells' surface structures (e.g. spore coat) retard probe diffusion (Fischer *et al*., 1995).

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In some instances, after the probe has gained access to the cell, it has to compete with rRNA molecules with complementary sequences which form double-stranded rRNA molecules with the target molecule (Ward *et al*., 1992; Amann *et al*., 1995; Zarda *et al*., 1997; Head *et al*., 1998). The sensitivity problem can be circumvented by employing multiple-labelled or enzyme-labelled (e.g. horseradish peroxidase) probes and improved visualisation instrumentation (Amann *et al*., 1995; Ishii *et al*., 2004; Demergasso, *et al*., 2005).

2.8.1.3. 16S rRNA gene restriction enzyme analysis

The 16S rRNA gene restriction enzyme analysis represents a rapid PCR-based method for the assessment of a microbial community. PCR products generated by the selective amplification of bacterial 16S rRNA genes are utilised in this analysis. This

method is qualitative (Coram and Rawlings, 2002; Coram-Uliana *et al*., 2006). Some of the methods available for the assessment of microbial community structure including, terminal restriction fragment length polymorphysim and thermal gradient gel electrophoresis provide very detailed information, but are known to be costly and time-consuming. A time-efficient and cost-effective analysis is essential for routine monitoring of the ecology of a wastewater treatment system.

Although this method has not been extensively described in the literature, it was successfully employed in the microbial characterisation of samples obtained from bioleaching environments, to the species-level (Coram-Uliana *et al*., 2006). A detection limit of 5% of the total microbial community was noted in the study by Coram-Uliana *et al*. (2006). Cook and Meyers (2003) employed a similar method in the identification of filamentous actinomycetes in soil isolates to the genus-level. Similar to the 16S rRNA gene restriction enzyme analysis is the amplified rDNA restriction analysis (ARDRA). The amplified rDNA restriction analysis has been found to be suitable for the extensive characterisation of microbial communities (Gurtler *et al*., 1991; Kohler *et al*., 1991). ARDRA has been widely employed in the microbial characterisation of anaerobic reactors and natural environments where methanogens and SRB thrive (Liu *et al*., 1997; Massol-Deyá *et al*., 1997; Smit *et al*., 2001; Ravenschlag *et al*., 2000; Pender *et al*., 2004; Musat *et al*., 2006). Amplified rDNA restriction analysis is often used in conjunction with DGGE, cloning and sequencing (Roest *et al.,* 2005; Geets *et al*., 2006). Additionally, few restriction enzymes (2 to 4) are employed, which implies that the technique results in a limited and broad classification of community members (Massol-Deyá *et al*., 1997). The application of a simple and rapid 16S rRNA gene restriction enzyme analysis in the characterisation of mixed SRB communities or anaerobic digestion under biosulphidogenic conditions has not been reported, except for the one recently presented from data obtained from this current work (Oyekola *et al*., 2007b). This technique thus represents a novel approach to the rapid assessment of microbial structure and dynamics in bioremediation. This method can also be carried out using simple and routine molecular-biology equipment. The application of an extensive suite of restriction enzymes (21), as described in the current study (Section 3.6.4, Chapter 3) provides significant discriminatory power. This facilitates the

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characterisation of the mixed consortium by positively identifying and excluding some species from a wide range of target groups.

Other common analytical tools that have found application in microbial community assessments include rRNA slot blot hybridisation (Ravenschlag *et al*., 2000), use of microelectrodes (Okabe *et al*., 1999), microautoradiography (Ito *et al*., 2002b) and denaturing gradient gel electrophoresis (DGGE) (Ito *et al*., 2002a). In several instances these tools are used in combination so as to exclude biases involved with individual techniques (Snaidr *et al*., 1997; Amann and Kühl, 1998).

2.9. RESEARCH MOTIVATION

Earlier investigations aimed at improving the efficiency and effectiveness of BSR include the following studies: choice of substrate (van Houten *et al*., 1996; Chang *et al*., 2000), effect of sulphide concentration (Reis *et al*., 1992), choice of bioreactor and effect of pH (Elliott *et al*., 1998). Knowing the role of the physicochemical factors in BSR, it is imperative to understand how the catalysts of the process, the SRB consortium, respond to these factors in order to achieve the net outcome of sulphate reduction. Interactions between SRB and other microbial population groups involved in anaerobic mineralisation and the effect of these interactions on the efficiency of the anaerobic digestion process have been well studied (Widdel, 1988; Harada *et al*., 1994; Lens *et al*., 1995; Oude Elferink *et al*., 1998; Ravenschlag *et al*., 2000; Menert *et al*., 2004; Patidar and Tare, 2005). Investigating the interactions of a mixed population of SRB in the catalysis of anaerobic sulphate reduction will be useful in the optimisation of this process.

Generally, mixed microbial consortia are utilised in the biosulphidogenic treatment of AMD, with no attention given to the characterisation of the microbial community structure or tailoring their response to optimise operational conditions i.e the exploitation of the knowledge of community dynamics to achieve the desired end effect. The implementation of the knowledge of the link between sulphate reduction kinetics and the SRB community dynamics in bioremediation has not been widely reported. van Houten *et al*. (2006) studied the ecology of a full-scale synthesis gas-fed sulphidogenic reactor. Their study described the dynamics of the microbial consortia composed of homoacetogens, methanogens and SRB. The relative dominance of each

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microbial group was congruent with the substrate utilisation profiles and metal loading. The extreme operational conditions imposed and low solid retention time selected for a limited number of well adapted population members. Recent studies by Kaksonen *et al*. (2004; 2006) have shown that simple carbon-sources (lactate and ethanol) support the growth of a diverse SRB community in the fluidised-bed treatment of AMD. Results from their study revealed that the performance of the biosulphidogenic system was dependent on the loadings of the carbon-source and metal with varying hydraulic retention time (HRT). Utilisation of ethanol as a carbon-source supported a more diverse community in comparison with lactate (Kaksonen *et al*., 2006). While these studies (Kaksonen *et al*., 2004; 2006) characterised the microbiology of the reactors, the response of the mixed culture to the operating conditions, in terms of the reactor performance, was not a priority.

There is a need for the simultaneous investigation of reactor performance and microbial consortium dynamics in response to changes in operational conditions. This will enable the establishment of the relationship between the BSR process and the SRB community dynamics.

Most kinetic data available in the literature for microbial sulphate reduction using lactate as carbon-source are incomplete and are for pure cultures (Table 2.8). However, mixed cultures are generally employed in BSR for wastewater treatment. Hence, this research focuses on the kinetics of growth and sulphate reduction of mixed SRB cultures on lactate. In contribution to the research initiative on BSR and AMD treatment at the Department of Chemical Engineering, UCT, centred to date on kinetics studies using acetate and ethanol, this study extends previous work to investigate the use of lactate as a carbon-source and electron donor. Lactate is employed owing to reports of its augmentation of culture diversity and improved tolerance of sulphide (Kaksonen *et al.,* 2003; Kuo and Shu, 2004). Specifically, the study addresses BSR using lactate in terms of process performance, BSR kinetics and community dynamics of mixed culture.

Against the background of the review of literature, the overall objective of the present study was to investigate the link between community dynamics and reactor

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performance in terms of reaction kinetics, extent of reaction and yield, using lactate as the electron donor and carbon-source.

2.10. RESEARCH HYPOTHESES

The study of the link between sulphate reduction kinetics and SRB community dynamics with respect to the responses of these factors to changing operating conditions is necessary for the understanding and optimisation of the process of biological sulphate reduction. This approach will provide a new avenue for biological wastewater treatment technology, maximising system performance and robustness. This study will address two broad hypotheses.

- The investigation of BSR process kinetics, stoichiometry and microbial community dynamics will provide sufficient understanding and a comprehensive means of managing wastewaters.
- The use of lactate as an electron donor for biological sulphate reduction supports a diverse SRB community, augmenting process robustness.

2.11. RESEARCH OBJECTIVES AND KEY QUESTIONS

Based on the previous research reviewed in the foregoing sections and the hypotheses described above, the following objectives were developed for the current work.

1. To investigate the performance of BSR, kinetics of sulphate reduction and stoichiometry of the reactions involved in a mixed SRB culture, using lactate as a carbon-source and electron donor, as a function of sulphate concentration, specific growth rate and sulphate loading.
2. To determine change in population with change in growth rate, sulphate loading and lactate loading imposed.
3. To investigate the link between kinetics and stoichiometry of biological sulphate reduction, and the dynamics of the mixed culture mediating the process.
4. To use the existing mathematical models to explore the kinetics of BSR and lactate metabolism in mixed culture under biosulphidogenic conditions, enabling the outcome of these models to propose optimal reactor configuration to maximise sulphate reduction with minimal requirement for carbon-source.

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CHAPTER 3
MATERIALS AND METHODS

Chapter 3 presents the detailed experimental methodologies and the materials employed in this study. These were used to achieve the objectives of this study, detailed in Section 2.11 of Chapter 2. The experimental approach includes investigation of the process kinetics, the reaction stoichiometry and metabolism, and the microbial community structure.

3.1. MICROORGANISMS AND GROWTH MEDIUM

A mixed culture of SRB, adapted to growth on lactate, was obtained from the laboratory of Prof. John Duncan (Rhodes University, South Africa). Modified Postgate B medium, in which lactate formed the sole carbon-source and electron donor, was used as the growth medium (Postgate, 1984). Bromo-ethane-sulphonic-acid (BESA) (3.2 g l^{-1}) was added to the culture at the enrichment stage, prior to the culturing of the continuous reactors, to inhibit methanogenic activity (Visser, 1995). The medium containing 1.0 g l^{-1} of sulphate ions had the following composition in 1 litre deionised water: 0.5 g KH_2PO_4; 1.0 g NH_4Cl; 2.0 g $MgSO_4.7H_2O$; 1.0 g Na_2SO_4; 1.0 g yeast extract; 2.2 g lactate; 0.3 g sodium citrate. All reagents were analytical grade. Magnessium sulphate and sodium sulphate were used as the sulphate source, while soium lactate acted as the lactate source. Lactate was added at 120% of the stoichiometric requirement based on the feed sulphate concentration, unless otherwise specified. The medium was sterilised by autoclaving at 121°C and 103 kPa for 20 min.

3.2. EXPERIMENTAL SET-UP AND PROCEDURES

Studies were carried out using identical 1 litre anaerobic stirred tank bioreactors, equipped with overhead stirrers driving Rushton impellers for continuous mixing at 400 rpm (tip speed of 1.05 m s^{-1}). The bioreactors were initiated in batch culture using a 10% inoculum. Constant temperature was maintained at 35°C by placing the reactors in a constant temperature water bath while pH was kept constant at pH 8 ± 0.2 by manually adding concentrated NaOH or HCl as required. Figure 3.1 shows a schematic representation of the continuous bioreactor set-up. Following the

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establishment of a viable microbial population and stable sulphate reduction, the reactors were converted to continuous operation. In the continuous mode, the medium was introduced into the bioreactor using a variable speed peristaltic pump, while the effluent was discharged by gravity through a U-shaped overflow tube. Steady-state was achieved at each flow rate to provide data to estimate the kinetics of sulphate reduction and bacterial growth. Steady-state conditions were assumed to be established when both the residual sulphate and bacterial concentrations varied by <10% during a period of operation equal to three retention times. Vacuum grease was applied to seal glass lids of reactors and other glass fittings to maintain strict anaerobic conditions. In order to investigate the effects of sulphate concentration and its volumetric loading on the kinetics, community structure and the stoichiometry of biological sulphate reduction, five separate experimental runs were carried out. The feed sulphate concentrations used were 1.0, 2.5, 5.0, 10.0 and 15.0 g l^{-1} at a constant excess lactate concentration of 20%. The reactors were operated at dilution rates in the range from 0.0076 to 0.083 h^{-1} (residence times of 0.5 to 5.5 days). The inoculum was well adapted for each feed concentration before the continuous operation. For experiments with feed sulphate concentrations 1.0 and 2.5 g l^{-1}, the stock culture was used as the inoculum.

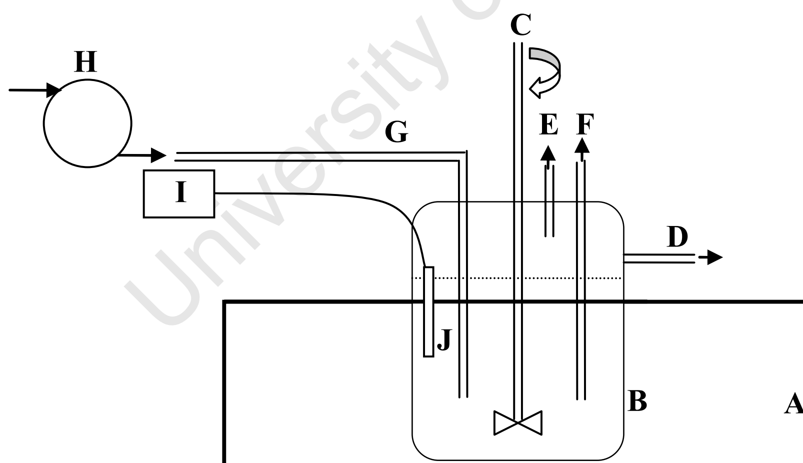

Figure 3.1: Schematic diagram of experimental set-up: (A) thermoregulated waterbath; (B) anaerobic bioreactor; (C) overhead stirrer; (D) overflow port; (E) gas vent; (F) sampling port; (G) feed inlet; (H) feed pump; (I) pH meter; (J) pH probe.

The bioreactor operating at 5.0 g l^{-1} of feed sulphate was inoculated with culture from the reactor fed with 2.5 g l^{-1} of sulphate, while the 10.0 and 15.0 g l^{-1} feed sulphate experiments were inoculated with culture taken from experiment with media containing 5.0 g l^{-1} sulphate. Samples for analyses were collected on alternate days

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(three times) after the establishment of steady state, from these reactors using disposable syringes under air-tight conditions.

3.3. ANALYTICAL METHODS

3.3.1. Sulphate assay

Sulphate concentration was quantified as barium sulphate using a turbidimetric method (APHA, 1975). Analysis was performed on the crude supernatant obtained after centrifuging the samples obtained from the reactors (13 000 rpm, 15 min). To 5ml of sufficiently diluted sample, 0.25 ml conditioning reagent was added, followed by the addition of an excess amount of finely ground $BaCl_2$. This reaction mixture was stirred for 1 min using a vortex mixer, after which absorbance was measured at a wavelength of 420 nm using a UV spectrophotometer (Thermo Spectronic, Model GENESYS 10 UV). The absorbance values were translated to sulphate concentrations using a sulphate standard curve. Standard sulphate solutions (5 to 40 mg l^{-1}) were treated in the same way as the samples. The standard curve and reagents preparation are given in *Appendix A*.

3.3.2. Sulphide assay

Total dissolved sulphide was estimated spectrophotometrically at 670 nm, following the colour development of methylene blue resulting from the reaction between the sulphide and the colorimetric reagent, *N,N*-dimethyl-*p*-phenylenediamine sulphate in acid medium. Ferric chloride acts as a catalyst for this reaction (Cline, 1969). An appropriate volume sample was added to 200 µl of 1% zinc acetate immediately after sample collection. This was further diluted up to a volume of 5 ml using deoxygenated dH_2O. A volume of 500 µl *N,N*-dimethyl-*p*-phenylenediamine hydrochloride solution, followed by 500 µl of ferric chloride solution were added to the sample. This was mixed thoroughly and allowed to react for 15 min. Absorbance values were translated to sulphide concentration using a sulphide standard curve. Standard sulphide solutions (0.2 to 1.0 mg l^{-1}) were treated in the same way as the samples. Standard curve and reagents preparation are shown in *Appendix B*.

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3.3.3. Alkalinity assay

Bicarbonate alkalinity was assayed by titrating 5 ml crude samples with 0.1 N H_2SO_4 to pH 4.5 according to the standard method by APHA (1975) (APHA method number 2320). H_2SO_4 was continuously added using a burette while the pH was concurrently monitored. The volume of acid added was recorded at the pH end points, 8 ± 0.2 and 4.5 respectively. Values of the volume of H_2SO_4 utilised in titration were used to calculate the concentration of bicarbonate alkalinity. See *Appendix C* for calculation details.

3.3.4. Fatty acids assay

Acetate, propionate and lactate concentrations in the bulk supernatant were determined using high performance liquid chromatography (HPLC, Beckman, System Gold), with a UV detector (Detector no. 168) and a glass lined Wakosil column, as adapted from Moosa *et al*., (2002). Phosphoric acid (20 mM, pH 2.5) acted as the mobile phase at a flow rate of 0.5 ml min^{-1}. Prior to injection, diluted samples were acidified using 4 N H_2SO_4 and filtered using sterilised 0.22 μm (Express® PES membrane) (Millipore, Corp.). Standard solutions (0.05, 0.1, 0.5 and 1.0 g l^{-1}) were prepared by performing serial dilutions on HPLC grade acetate, propionate and lactate using deionised water.

3.3.5. Determination of bacterial dry mass

Bacterial dry mass was estimated as dry mass. 4.5 ml samples from the bioreactors were centrifuged (16 000 ×g, 15 min), using eppendorf® centrifuge (model 5415 D) in pre-dried (80°C, 48 h) and pre-weighed microfuge tubes. The supernatant was decanted. This was followed by the washing of pellets obtained twice with distilled water and drying of washed sample pellets (80°C, 48 h). Cooled tubes (in a desiccator) with the pellet were then weighed to determine the dry biomass.

3.4. SCANNING ELECTRON MICROSCOPY (SEM)

The pellet obtained after centrifuging (16 000 ×g, 15 min) a 4.5 ml sample, was fixed by resuspension in 500 μl cold 2.5% glutaraldehyde in 1× phosphate-buffered saline

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(PBS) solution (pH 7.2) for 12 hours at 4°C. After this primary fixation, the specimens were washed twice with 1× PBS solution. This was followed by a second fixation in 1% osmium tetroxide (500 µl), prepared in PBS solution, for 1 hour. Fixed cells were washed once in PBS solution, followed by a second wash with distilled water. The supernatant was removed and replaced with 30% ethanol to start a sequence of dehydration using increasing concentrations of ethanol of 30, 50, 70, 80, 90, 95% and twice in 100% with 10 minute incubation at each step. This was followed by filtering the specimens through a spi pore poly membrane filters (13 mm, 0.05 µm PK/ 100, West Chester, PA 19381, USA). The filters were then mounted onto SEM stubs with silver paint. A few drops of hexamethyldisilazane (HMDS) were instantly added onto the mounted filters. More HMDS was added after 5 minutes to ensure a well-dried sample. The dried samples were coated with gold-palladium (60:40) and observed using a Leo S440 SEM. This procedure was adapted from Botes *et al*. (2002).

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3.5. GRAM STAINING

Gram stains were performed using the standard technique. Samples of cell suspension were heat fixed on microscope slides, followed by primary staining with crystal violet (60s). Smears were washed with water, followed by iodine staining (60s). Subsequent destaining of the slides with 95% ethanol was carried out. Slides were then counterstained by adding safrarin (60s), followed by washing with water. Light microscopy was performed using an Olympus model BX40 camera-microscope.

3.6. MOLECULAR ANALYSES

3.6.1. Oligonucleotide Probes

16S rRNA-targeted oligonucleotide probe sequences were accessed from probeBase (Loy *et al*., 2003). The 16S rRNA-targeted oligonucleotide probes used were: (i) one domain-specific (ARCH915) and (ii) a group-specific probe (SRB385). The oligonucleotide probes employed in this study were commercially synthesised and labeled with fluorescein isothiocyanate (FITC) at the 5′ end (Department of Molecular and Cell Biology, University of Cape Town, South Africa). Their specificities, hybridisation conditions, and references are shown in Table 3.1.

3.6.2. Nucleic Acid Extraction

Cells were harvested via centrifugation (10 000 ×g, 10 min) of 40 ml samples using a Beckman centrifuge (AVANTI® J-25 Model). The pellet recovered was washed with 1×PBS solution (pH 7.2), centrifuged (8 000 rpm, 5 min) followed by resuspension in appropriate volume of the same buffer. Total DNA was extracted using a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions.

3.6.3. Polymerase Chain Reaction (PCR) For 16S rRNA gene Analysis

Bacterial 16S rRNA genes were selectively amplified using universal forward primer Uni-F (5′-CCGGATCCGTCGACGTGCCAGCXGCCGCGGTAA-3′) and reverse primer rDD2 (5′-CAAAGCTTCTAGACGGXTACCTTGTTACGACTT-3′) to generate amplicons of approximately 1.0 kb. Genomic DNA was subjected to amplification in a PCR mix of total volume 50 µl, containing 2 µl (~100 ng) of template DNA and the following in their final concentrations: 3 µl $MgCl_2$ (1.5 mM); 1 µl working stock (200 µM each) deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 µl (0.25 µM) of each primer (Uni-F and rDD_2); 5 µl of 10 × PCR buffer; 33.6 µl of sterile Millipore water and 0.4 µl FastStart Taq DNA Polymerase (5 U/µl) (Roche). Amplification conditions included denaturation step for 10 min at 94°C followed by 25 amplification cycles of 30 s at 94°C, 30 s at 52°C, and 90 s at 72°C. A final step for 120 s at 72°C and a cooling step at 4°C for 60 s completed the reaction. PCR amplification was performed in a PCR Sprint thermal cycler (Thermo Hybaid, UK). 0.8% (w/v) agarose gel electrophoresis and ethidium bromide (0.05 µg ml^{-1}) staining were used to validate amplification and size of the products prior to restriction enzyme analysis, using 5 µl aliquots of PCR product.

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3.6.4. 16S rRNA gene Restriction Enzyme Analysis

3.6.4.1. Principle of 16S rRNA gene restriction enzyme analysis

This method is based on the fact that an individual restriction endonuclease identifies specific nucleotide sequences within the DNA and cuts the DNA between specific bases within the recognition site. A restriction enzyme digestion will produce different DNA fragments of different sizes based on the target nucleotide sequence of

the enzymes used (Brown, 1995). Applying a combination of carefully selected enzymes to amplified DNA samples from a mixed microbial consortium, a mixture of differing fragments peculiar to each member of the community is generated. Separation of this mixture into discriminatory banding patterns can be achieved using gel electrophoresis. Fragment sizes are then determined by comparing them with standard molecular weight markers (Brown, 1995). Organisms are identified by comparing the discriminatory patterns generated from the experiment with the ones obtained theoretically using the DNAMAN software for Windows program, version 4.13 (1994-99) (Coram-Uliana *et al*., 2006; Oyekola *et al*., 2007b).

Enzymatic digestions of the PCR amplicons were performed using a suite of 21 restriction enzymes, namely, *Apa*I, *Asp*700I, *BbrP* I, *Bfr*, *BpuA*I, *Bse*AI, *Dra*I, *EclX*I, *Eco*RI, *Eco*RV, *Hpa*I, *Ksp*I, *Mlu*I, *Nco*I, *Pst*I, *Pvu*I, *SgrA*I, *Sma*I, *Sph*I, *Xba*I and *Xma*CI restriction enzymes (Roche Diagnostics GmbH). These restriction enzymes were selected such that specific enzymes generated unique DNA fragment banding patterns for each species targeted. These enzymes were selected primarily to identify SRB species. The enzymes chosen are also readily available commercially. The corresponding buffer used for each enzyme was in accordance to the manufacturer's instructions. 16S rRNA sequences of the target microorganisms were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). To generate the banding patterns experimentally, the reaction mixture contained 3 μl of the PCR product; 0.5 μl of each restriction enzyme; 2 μl of the equivalent enzyme buffer. This mixture was made up to 20 μl with sterile Millipore water and then incubated at the respective optimal temperature of each enzyme for 1 hour. After the reaction was completed, 3 × tracking dye (2 μl) was added to the reaction mixture to monitor the electrophoresis. Discriminatory banding patterns were visualised, after restriction fragments were separated on 1.5% agarose gels (containing 0.05 μg ml^{-1} ethidium bromide) in 1 × Tris-borate-EDTA buffer at 100 V and 4°C. The fragment sizes were determined using 100-bp gene ladder (Fermentas). Preparation of reagents, a list of target microorganisms and the theoretical banding patterns obtained are provided in *Appendix D*.

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3.6.5. Fluorescence *In Situ* Hybridisation (FISH)

3.6.5.1. Principle of FISH

Fluorescence *in situ* hybridisation is based on the fact that two single-stranded nucleic acid molecules can react together through their complementary base-pairs to form a hybrid. This hybrid can be RNA-DNA, DNA-DNA or RNA-RNA. The detection of the nucleotide sequence on a nucleic acid is carried out indirectly by hybridising a single-stranded nucleic acid fragment containing a sequence complementary (probe) to the target sequence of another nucleic acid (Brown, 1995). The probe is usually labelled for direct and easy microscopic visualisation. Different methods of labelling have been used, including enzymatic (e.g. horseradish peroxidase) and fluorescent markers. Radioactive labelling is however becoming unpopular due to risk in handling and low stability. Fluorescent-dye-conjugated probes are used in the FISH technique (Höfler *et al*., 1998). Signals from probes represent cellular rRNA content (Amann *et al*., 1995; Simon *et al*., 1995; Llobet-Brossa *et al*., 1998).

Cell fixation: Pellet from harvested cells (10 000 ×g, 10 min) was washed (8 000 rpm, 5 min) in 1× PBS and resuspended in an appropriate volume (375 µl) of fresh 1× PBS. three volumes (1.125 ml) of freshly prepared paraformaldehyde solution (4% [w/v] in 1× PBS) was added, followed by incubation (4°C, 3 h or overnight). This was subsequently washed in fresh 1× PBS then stored in 1× PBS: absolute ethanol (ice-cold) (1:1 [v/v], -20°C) until hybridisation (Amann, 1995).

Slide preparation: Resuspended fixed cells (5 µl) were mounted onto glass slides and oven-dried (46°C, 20 min). 5µl lysozyme (10 mg ml^{-1}) was added on to the cell spot followed by incubation on ice for 20 min. Lysozyme was added to increase the permeability of the target cells fro the oligonucleotide probes. Lysozyme was washed off using ddH_2O, prior to dehydration of the sample slides, using an increasing ethanol series (3 min each in 50, 80 and 98% ethanol). The slides were allowed to air dry and then stored in at -20 °C prior to hybridisation (Li *et al*., 1997).

Hybridisation: 2 ml fresh hybridisation buffer was prepared by adding the following stock solutions consecutively 36 µl NaCl (5 M); 40 µl Tris-HCl (1 M, pH 7.2); 2 µl SDS (10%); varying concentrations of formamide concentrations for different probes

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(Table 3.1). Sterilised ddH_2O was added to make up to 2 ml. Nine μl of the hybridisation and 1 μl of each probe were added to the cell spots, resulting in a probe concentration of ~ 50 ng μl^{-1}. Cover slips were placed on top of the slides to ensure that the entire sample was exposed to the hybridisation mix. Slides were placed in 50 ml tubes containing the excess hybridisation buffer. These were sealed and laid horizontally in the oven for incubation (46°C, 2 h).

Washing: Slides were immersed in pre-warmed (48°C) washing buffer (varying NaCl concentrations (Table 3.1); 1 ml Tris-HCl (1 M, pH 7.2); 50 μl SDS (10%), made up to 50 ml with sterilised ddH_2O) followed by incubation (48°C, 20 min). The slides were rinsed in a beaker containing ice-cold ddH_2O, and then air-dried in the dark. Samples were then counterstained by adding 10 μl DAPI (1 μg ml^{-1} in ddH_2O) on to the slides. Cover slips were placed on top of the slides. This was followed by incubation (ambient temperature, 5 min), rinsing with ddH_2O and air drying. Aliquots of antifade solution, AFI (Citiflour Ltd., London UK) were added to the wells, with the cover slips pressed down gently to remove excess antifade, prior to microscopy.

Microscopy: This was performed using an Olympus model BX40 epifluorescence microscope.

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Table 3.1: 16S rRNA-targeted oligonucleotides probes used in this study.

Probe[a]	Target group	Sequence of probe (5′→3′)	Target site[b]	[FA][c] (%)	[NaCl][d] (M)	Reference
ARCH915	*Archaea*	GTGCTCCCCCGCCAATTCCT	915-934	25	0.15	Schrenk *et al*., 1998
SRB385	Gram-negative[e] mesophilic SRB	CGGCGTCGCTGCGTCAGG	385-402	30	0.10	Amann *et al*., 1992

[a]nomenclature according to Alm *et al*. (1996).
[b]16S rRNA position according to *E.coli* numbering (Brosius *et al*., 1981).
[c]formamide concentration in the hybridisation buffer.
[d]sodium chloride concentration in the washing buffer.
[e] the universal probe for Gram-negative SRB was tested on widely different SRB species. Alignment analyses were carried out on 250 partial and complete 16S rRNA sequences of SRB species (Amann *et al*., 1992).

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3.7. RESEARCH STRATEGY

The objectives of the present study were addressed through discrete stages, illustrated in Figure 3.2. The experimental stages included the investigation of the biological sulphate reduction kinetics, microbial community dynamics and stoichiometry of lactate metabolism under biosulphidogenic conditions. The data obtained from the kinetics study were utilised to investigate the relevance of the existing kinetic models developed for BSR (Moosa *et al*., 2002; 2005).

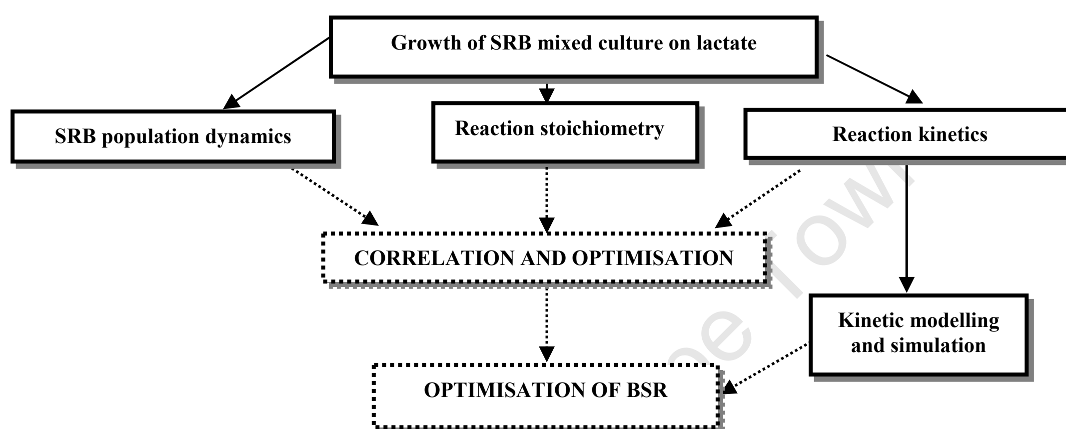

Figure 3.2: Diagrammatical description of the present work.

Continuous mixed planktonic cultures cultivated in CSTR were employed in the experimental stages. The effect of the feed sulphate concentration in the presence of a 20% stoichiometric excess of lactate, on the kinetics of biological sulphate reduction (BSR), the stoichiometry of the reactions involved in the BSR based on lactate utilisation and the community dynamics of the reaction mediators (SRB) was investigated. This was achieved by setting up CSTRs to receive sulphate feed concentrations in the range 1.0 to 15.0 g l^{-1}. The bioreactors were maintained at previously determined optimal conditions of temperature (35°C) and pH (8.0 ± 0.2). Residence times were varied through 0.5 to 5.5 days to vary the specific growth rate and the volumetric sulphate loading rate. Data were collected on residual substrate concentrations, product concentrations, conversion rates and the microbial population. The stoichiometry of the kinetic data obtained was further analysed. Conventional molecular techniques were employed to characterise the microbial population involved in the BSR process. The relationship between the reaction stoichiometry, community structure and the reaction kinetics was explored using the data obtained.

The data obtained from the experimental studies were used to investigate the applicability of the mathematical models previously developed to describe microbial sulphate reduction kinetics in the presence of acetate and ethanol, using lactate as a carbon-source and electron donor. Based on findings, these models would be developed further.

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CHAPTER 4

KINETIC STUDY OF BIOLOGICAL SULPHATE REDUCTION

4.1. INTRODUCTION

In the bioremediation of acid mine drainage and other sulphate-laden wastewaters mediated by SRB, sulphate reduction is a critical process (Reaction 1.1) (Baskaran and Nemati, 2006).

$$Electron\ donor + SO_4^{2-} \rightarrow HS^- + HCO_3^- \qquad (1.1)$$

Previous kinetic studies using acetate (Moosa *et al*., 2002) and ethanol (Erasmus, 2000; Hansford *et al*., 2007) as carbon-sources for SRB have shown that the biological sulphate reduction (BSR) reaction kinetics are influenced by residual sulphate concentration and its volumetric loading, feed sulphate concentration, biomass concentration and residence time. These studies were based on the research initiative on biological sulphate reduction in the treatment of acid mine drainage, at the Department of Chemical Engineering, UCT. In this current work, the kinetic studies were extended to the use of lactate as the sole carbon-source. Other reports have also shown that the kinetics of biological sulphate reduction is influenced by volumetric loading rate, residual and feed sulphate concentration (Mohanty *et al*., 2000; Habicht *et al*., 2005; Baskaran and Nemati, 2006).

As previously discussed in Chapter 2, lactate is a favourable substrate with respect to its thermodynamic and kinetic properties, relative to acetate and ethanol (Widdel and Pfennig, 1981; Ingvorsen *et al*., 1984; Ingvorsen and Jorgensen, 1984; Widdel, 1987; Okabe and Characklis, 1992; Reis *et al*., 1992; Okabe *et al*., 1992; 1995; Konishi *et al*., 1996; O'Flaherty and Colleran, 1998; Erasmus, 2000; Moosa , 2000; Nagpal *et al*., 2000; Kaksonen *et al*., 2003). Kuo and Shuo (2004) also reported that lactate supports BSR operation at high sulphide concentration. Lactate is known to support the growth of a wide spectrum of SRB. Its utilisation is expected to support a robust BSR system in the treatment of AMD.

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The specific objectives of the kinetic study presented in this Chapter were as follows:

i. To investigate the effect of sulphate concentration and its volumetric loading rate, and residence time on the kinetics of anaerobic sulphate reduction by a lactate-fed, mixed SRB culture.

ii. To examine the ability of reactor performance to return to initial levels after the restoration of less extreme operating conditions, thereby investigating robustness of the catalyst i.e. the microbial community structure.

4.2. EXPERIMENTAL APPROACH

All analytical measurements were carried out in triplicate from samples obtained when each bioreactor reached a steady-state at the specified operating conditions. At each steady-state samples were taken at regular intervals. The data plots represent mean values of the steady-state measurements. All raw data obtained in these experiments are given in *Appendix E*.

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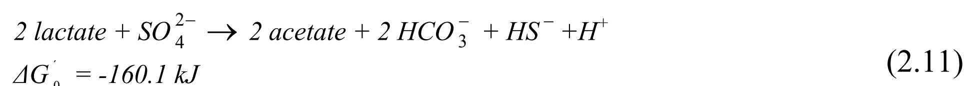
4.2.1. Effect of Feed Sulphate Concentration on Biological Sulphate Reduction

For this study, laboratory scale chemostat cultures at different residence times (0.5 to 5.5 d) and feed sulphate concentrations 1.0, 2.5, 5.0, 10.0 and 15.0 g l^{-1} were employed. For experiments with feed sulphate concentrations 2.5 to 15.0 g l^{-1}, the steady-state at residence time of 5 d was maintained for five retention times before reducing the residence time to 4 days to allow for culture adaptation to higher substrate concentrations. A 20% stoichiometric excess of lactate was added according to Reaction 2.11. The detailed experimental approach is provided in Chapter 3.



$$2\ lactate + SO_4^{2-} \rightarrow 2\ acetate + 2\ HCO_3^- + HS^- + H^+$$
$$\Delta G_0^{'} = -160.1\ kJ \qquad (2.11)$$

4.2.2. Test of Reactor Resilience

To investigate the ability of the bioreactors to recover after a major perturbation (high dilution rate), operation of the bioreactors receiving sulphate feed concentrations of 2.5, 5.0 and 10.0 g l^{-1} were restored to a residence time of 5 d from a steady-state at residence time of 1 d. The bioreactor with a feed containing 1.0 g l^{-1} sulphate was restored to operate at a residence time of 5 d after reaching a steady-state at 0.5 d.

Sulphate conversion and volumetric sulphate reduction rate at residence time 5 d were monitored in these experiments over a period of 60 to 100 days.

4.2.3. Data Handling

4.2.3.1. Kinetic Calculations

Sulphate conversion (SC) and lactate conversion (LC): These were calculated as follows:

$$SC = \frac{S_o - S}{S_o} \times 100\% \tag{4.1}$$

where S_o and S represent the feed and residual substrate (sulphate or lactate) concentrations (g l^{-1}) respectively. LC accounts for both acetate and propionate production.

Expected sulphide (ES): This was calculated based on the amount of sulphate removed. The molecular weights of sulphur, sulphide (HS^{-1}) and sulphate are 32, 33 and 96 g mol^{-1} respectively. The sulphur content of sulphate ($Sulphur_S$) and sulphide ($Sulphur_{HS}$) can be calculated as follows:

$$Sulphur_S = \frac{32}{96} \times Sulphate\ concentration\ ,\ Sulphur_{HS} = \frac{32}{33} \times Sulphide\ concentration\ .$$

Therefore

$$ES = \frac{S_o - S}{3} \tag{4.2}$$

where S_o and S represent the feed and residual sulphate concentrations (g l^{-1}) respectively.

Volumetric substrate utilisation and production rates: The volumetric sulphate reduction and volumetric lactate utilisation rates, r_s and r_L respectively (g l^{-1} h^{-1}) were calculated as follows:

$$r_s \text{ or } r_L = (S_o - S)\, D \tag{4.3}$$

where S_o and S represent the feed and residual substrate (sulphate or lactate) concentrations (g l^{-1}) respectively and D is the dilution rate (h^{-1}) (inverse of residence time) of the medium in the bioreactor.

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Similarly, the volumetric acetate (r_A) and propionate (r_P) production rates were estimated using the concentrations of the substrate produced, S_i (g l^{-1}), according to Equation 4.4.

$$r_A \text{ or } r_P = S_i \times D \quad (4.4)$$

Volumetric sulphate loading rate (*VSLR*): This is the product of the feed sulphate concentration and the dilution rate, given in Equation 4.5:

$$VLR = S_o \times D \quad (4.5)$$

Specific substrate utilisation rate:

The specific substrate utilisation rate (q_s) is defined as the volumetric rate of substrate utilisation (r_s) (g l^{-1} h^{-1}) per unit biomass (X) (g l^{-1}), given in Equation 4.6:

$$q_s = \frac{r_s}{X} \quad (4.6)$$

where X accounts for all the bacterial groups present under the experimental conditions.

Biomass yield and maintenance

The bacterial yield ($Y_{x/s}$) and maintenance (m_s) coefficients were obtained using the Pirt Equation (Equation 4.7a) (Pirt, 1965). Specific substrate utilisation rate as a function of the dilution rate (equivalent to the specific growth rate μ in steady-state continuous culture) (Equation 4.7c) yield a straight line with slope $1/Y_{x/s}$ and an intercept of m_s. These values were obtained within the range of dilution rates where Pirt Equation is applicable i.e. the following assumptions hold:

(i) Cell death is negligible.

(ii) The energy derived from substrate utilisation is utilised both for microbial growth and for non-growth associated functions, otherwise referred to as maintenance processes which are vital for the cells' integrity (Pirt, 1965).

$$r_s = r_x \frac{1}{Y_{x/s}} + m_s X \quad (4.7a)$$

$$\frac{r_s}{X} = \frac{r_x}{X} \frac{1}{Y_{x/s}} + m_s \quad (4.7b)$$

since $r_x = \mu X$

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$$\frac{r_s}{X} = \mu \frac{1}{Y_{x/s}} + m_s \tag{4.7c}$$

where r_x (g l^{-1} h^{-1}) is the rate of biomass formation

4.2.4. Reproducibility of Experiments

Reproducibility of the kinetic experiments was assessed by repeating the experiment at the feed sulphate concentration of 1.0 g l^{-1} under identical conditions. Results under steady-state conditions at a feed sulphate concentration of 1.0 g l^{-1} are typical of the set of kinetics experiments and show a good reproducibility (Figure 4.1). Two sets of data for the volumetric sulphate reduction, sulphate conversion and bacterial dry mass measurements are presented.

Reproducibility was calculated as the coefficient of variance (CV), which is the standard deviation expressed as a percentage of the mean. Average CV values for repeated datasets are shown in Table 4.1. Standard deviations of duplicate experimental runs for the measurements of volumetric sulphate reduction rate, sulphate conversion and bacterial dry mass were less than 15% of their respective mean values. This illustrates acceptable reproducibility of the kinetics experiments.

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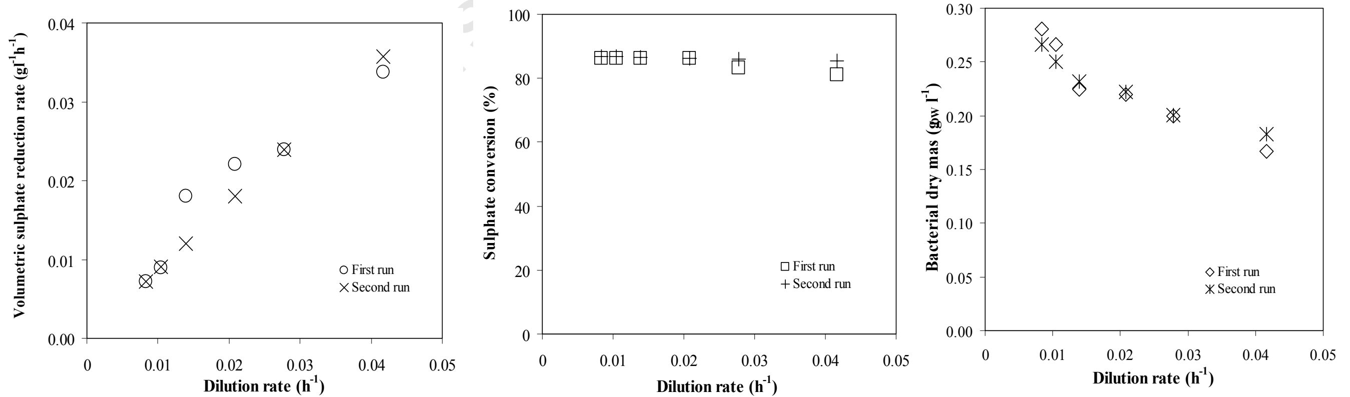

Figure 4.1: The steady-state profiles of volumetric sulphate reduction rate, sulphate conversion, and bacterial dry mass at feed sulphate concentration 1.0 g l^{-1} for two datasets obtained from two replicate experimental runs.

Table 4.1: Test of experimental reproducibility as measured by coefficient of variance.

Datasets	Coefficient of variance (%)	
	Average	Range
Volumetric sulphate reduction rate	3.92	0.12-14.53
Sulphate conversion	1.21	0.14-3.73
Bacterial dry mass	2.93	0.2-6.52

4.3. RESULTS AND DISCUSSION

4.3.1. Steady-State Kinetics Profiles

The steady-state profiles of bacterial dry mass, sulphate conversion, lactate conversion and volumetric sulphate reduction rate as a function of volumetric sulphate loading rate and dilution rate for the different feed sulphate concentrations (1.0 to 15.0 g l^{-1}) are shown in Figures 4.2a to 4.6a. Steady-state conditions were assumed to be established when both the residual sulphate and bacterial dry mass varied by <10% after a period of operation equal to three retention times since system perturbation. The corresponding concentrations of residual lactate, residual sulphate, acetate produced and propionate produced are shown in Figures 4.2b to 4.6b. Figures 4.2c to 4.6c show the change of dissolved sulphide concentration and bicarbonate alkalinity for the five different feed concentrations over the range of dilution rates studied.

4.3.1.1. Feed Sulphate Concentration of 1.0 g l^{-1}

The steady-state profiles at a feed sulphate concentration of 1.0 g l^{-1} are shown in Figures 4.2a to 4.2c. As shown in Figure 4.2a, within the range of dilution rates 0.0083 to 0.042 h^{-1} (residence time: 5.0 to 1.0 d), the volumetric sulphate reduction rate increased linearly with an increase in the volumetric sulphate reduction rate up to a value of 0.036 g l^{-1} h^{-1}, while sulphate conversion to sulphide remained relatively stable at 86 ± 0.5%. The corresponding lactate conversion across this range of dilution rates was 99 ± 0.6%. Figure 4.2a shows that the bacterial dry mass was low and gradually decreased with increasing dilution rate. An average value of 0.226 ± 0.031 g l^{-1} bacterial dry mass was maintained over the operation range of this study. This suggests an efficient microbial sulphate utilisation.

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On reduction of the residence time to 0.5 d (volumetric loading rate = 0.083 g l^{-1} h^{-1}), the bacterial dry mass was slightly reduced to 0.169 g l^{-1}. This was accompanied by a slight increase in the volumetric sulphate reduction rate to a maximum value of 0.041 g l^{-1} h^{-1} and a sharp decrease in sulphate conversion to 50% (Figure 4.2a). Despite the reduction in the sulphate conversion, the conversion of lactate remained at 99%. Based on stoichiometric estimation and the amount of sulphate reduced, the expected residual lactate concentration at this residence time was 1.31 g l^{-1}. However, a lower concentration of 0.029 g l^{-1} was detected. This indicates that the sulphate conversion was not limited by lactate concentration. This observation also suggests that while sulphate reducers oxidised lactate at a rate near their maximum, another group of microorganisms, characterised by higher μ_{max} and K_s values for lactate utilisation, was able to proliferate due to increased lactate availability at high volumetric loading rate of lactate (sulphate). Consequently, there was no build-up in the residual lactate. Hence, the decline in sulphate conversion was rather a consequence of some SRB species wash out as the reactor was operated at a dilution rate greater than their μ_{max} and selection of resilient SRB strains.

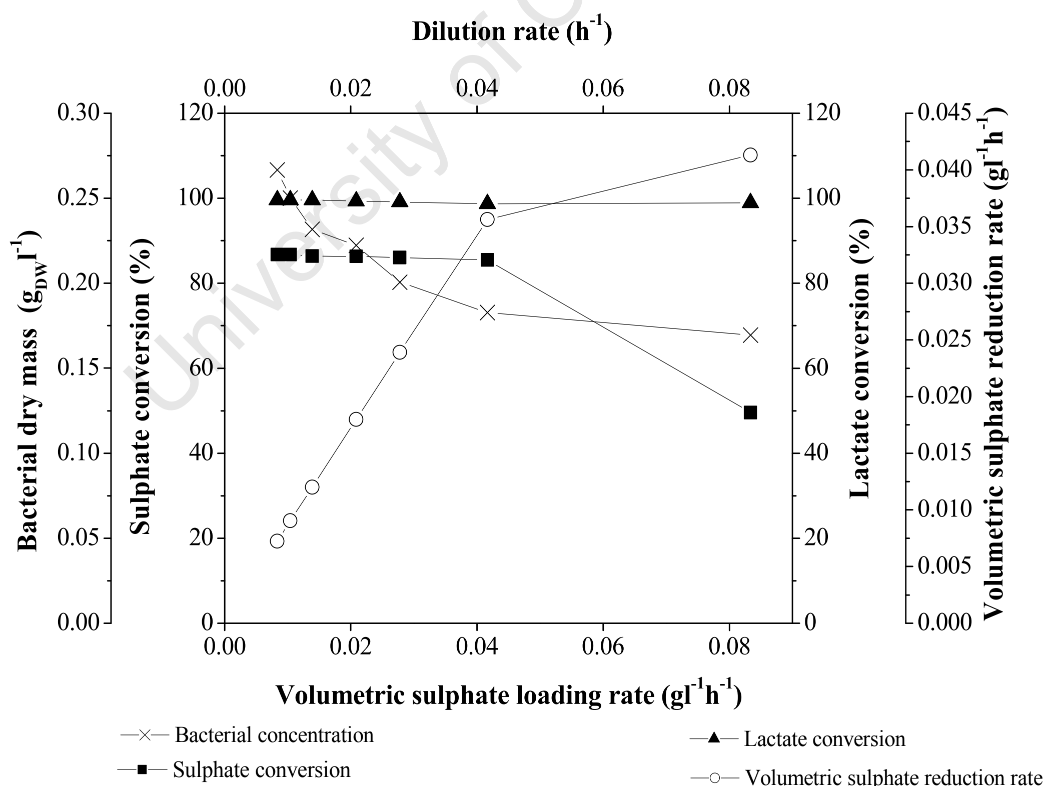

Figure 4.2a: Steady-state kinetics of continuous reactor with a feed sulphate concentration of 1.0 gl^{-1}. Kinetic data presented are bacterial dry mass, sulphate conversion, lactate conversion and volumetric sulphate reduction rate.

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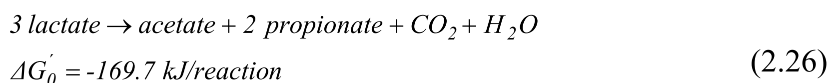
Complete utilisation of lactate was observed at dilution rates 0.0083 to 0.014 h^{-1} (residence time: 5 to 3 d). Beyond this range, low residual lactate levels (0.0014 to 0.029 g l^{-1}) were recorded (Figure 4.2b). Further, decreasing acetate production, an indication of decline in lactate oxidation, was congruent with increased levels of propionate and residual sulphate (Figure 4.2b). Propionate production is an indication of lactate fermentation (Reaction 2.26) (Heimann *et al*., 2005).



$$3\ lactate \rightarrow acetate + 2\ propionate + CO_2 + H_2O$$
$$\Delta G_0' = -169.7\ kJ/reaction \quad (2.26)$$

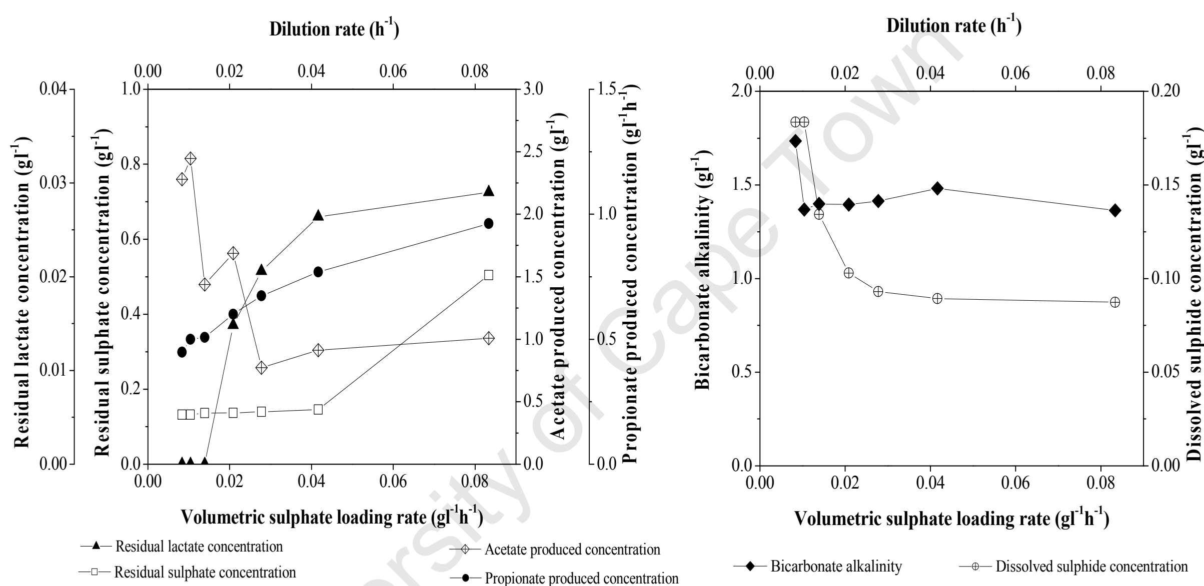


Figure 4.2b: Steady-state profiles of residual lactate, residual sulphate, acetate produced and propionate concentrations of continuous reactor with a feed sulphate concentration of 1.0 g l^{-1}.

Figure 4.2c: Steady-state profiles of bicarbonate alkalinity and dissolved sulphide concentration of continuous reactor with a feed sulphate concentration of 1.0 g l^{-1}.

These trends suggest that lactate oxidation was predominant at low dilution rates while increasing dilution rate favoured the lactate metabolism via the fermentative pathway. The phenomenon of metabolic shift with increasing dilution rate is described in detail in Chapter 5. This result is consistent with the report by Baskaran and Nemati (2006). They showed that sulphate conversion of a lactate-fed chemostat culture, receiving feed sulphate concentration of 1.0 g l^{-1}, decreased from 100% to 58% as the sulphate volumetric loading rate was increased from 0.001 to 2.85 g l^{-1} h^{-1}. This was attributed to reduced lactate oxidation.

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Consistent low levels of residual sulphate (0.13 to 0.14 g l^{-1}) were maintained in the volumetric loading rate range of 0.0083 to 0.042 g l^{-1} h^{-1}. Beyond this range, at the highest volumetric loading rate imposed (0.083 g l^{-1} h^{-1}), a significant increase of residual sulphate concentration to 0.5 g l^{-1} (50% sulphate conversion) was observed. As previously discussed, increase in residual sulphate concentration is attributable to reduced lactate oxidation. Bicarbonate alkalinity, acetate produced and dissolved sulphide levels show similar trends (Figures 4.2b and 4.2c). The concentrations of these products of biological sulphate reduction steadily declined with increasing volumetric sulphate loading rate at dilution rates 0.0083 to 0.021 (residence time: 5.0 to 2.0 d). Beyond this range, the concentrations of bicarbonate alkalinity, acetate produced and dissolved sulphide remained relatively constant at 1.41 ± 0.05 g l^{-1}, 0.9 ± 0.1 g l^{-1} and 0.090 ± 0.0029 g l^{-1} respectively.

Despite the high sulphate conversion maintained at dilution rates 0.0083 to 0.042 h^{-1}, there was a consistent decrease in sulphide levels within this range. This can be attributed to the loss of some of the sulphide produced. The assayed sulphide concentration (AS) was the total amount of sulphide in the liquid phase. This underestimated the equivalent sulphide (ES) that would be expected from the amount of sulphate reduced (Table 4.2).

Table 4.2: Comparison of expected sulphide (ES) and assayed sulphide (AS) concentrations across the experimental conditions investigated for BSR in chemostat culture.

RT (day)	Dilution rate (h^{-1})	Expected sulphide (ES) and assayed sulphide (AS) concentrations (g l^{-1}) at different feed sulphate concentrations (S_o)									
		S_o (g l^{-1})									
		1.0		2.5		5.0		10.0		15.0	
		AS	ES	AS	ES	AS	ES	AS	ES	AS	ES
0.5	0.083	0.087	0.165	nd	nd	nd	nd	nd	nd	nd	nd
1.0	0.042	0.089	0.285	0.014	0.067	0.022	0.099	0.312	0.72	nd	nd
1.5	0.028	0.093	0.287	0.027	0.096	0.088	0.254	0.312	0.90	nd	nd
2.0	0.021	0.103	0.288	0.033	0.162	0.232	0.513	0.352	1.04	nd	nd
3.0	0.014	0.134	0.288	0.059	0.348	0.369	0.665	0.557	1.10	0.200	0.327
3.5	0.012	nd	nd	nd	nd	nd	nd	nd	nd	0.300	0..329
4.0	0.010	0.184	0.289	0.131	0.453	0.519	0.924	0.559	1.29	0.292	0.332
5.0	0.0083	0.184	0.289	0.189	0.452	0.529	0.970	0.622	1.34	0.401	0.670
5.5	0.0076	nd	nd	nd	nd	nd	nd	nd	nd	0.356	0.670

nd: not determined.

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The observation of sulphur biofilms on the inner walls of some of the reactors and the volatilisation of sulphide from the gas vent in the reactor set-up used in this study is associated with the significant loss of volatile sulphide. Baskaran and Nemati (2006) suggested that a reactor set-up having a large head space and continuously mixed at 400 rpm, as in the current study, would facilitate the evolution of sulphide produced from the liquid phase (Baskaran and Nemati, 2006).

4.3.1.2. Feed sulphate concentration of 2.5 g l^{-1}

At a feed sulphate concentration of 2.5 g l^{-1}, the volumetric sulphate reduction rate increased to an optimum value of 0.015 g l^{-1} h^{-1} at a low dilution rate of 0.014 h^{-1} (residence time: 3d) (Figure 4.3a). The corresponding sulphate conversion was 42%. The maximum values of the bacterial dry mass and sulphate conversion achieved were 0.35 g l^{-1} and 54% respectively. These values were obtained at the residence time of 5 d. A sharp decline in the volumetric sulphate reduction rate was accompanied by a gradual decrease in the bacterial dry mass with the increasing volumetric loading rate (residence time: 3 to 1 d).

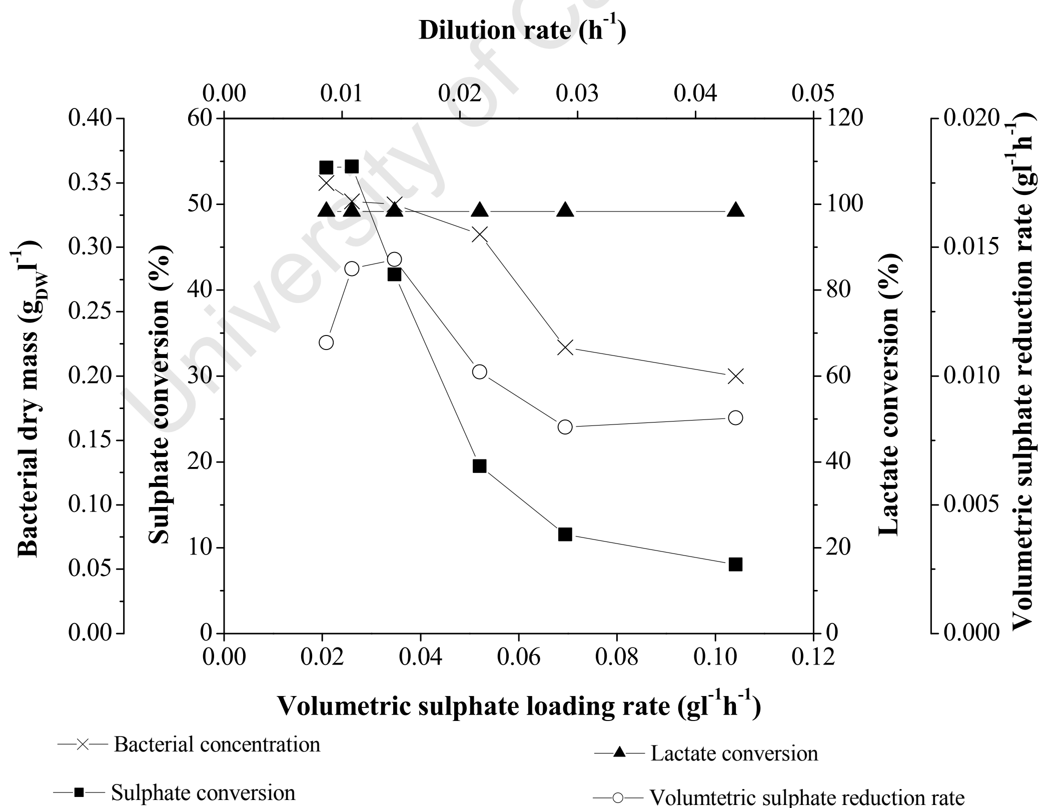

Figure 4.3a: Steady-state kinetics of continuous reactor with a feed sulphate concentration of 2.5 g l^{-1}. Kinetic data presented are bacterial dry mass, sulphate conversion, lactate conversion and volumetric sulphate reduction rate.

Across the range of dilution rates studied, the lactate conversion was maintained at 100% despite the significant decline in the sulphate conversion as the volumetric loading rate was increased. These observations suggest the presence of non-SRB lactate utilisers which became predominant as the dilution rate was increased.

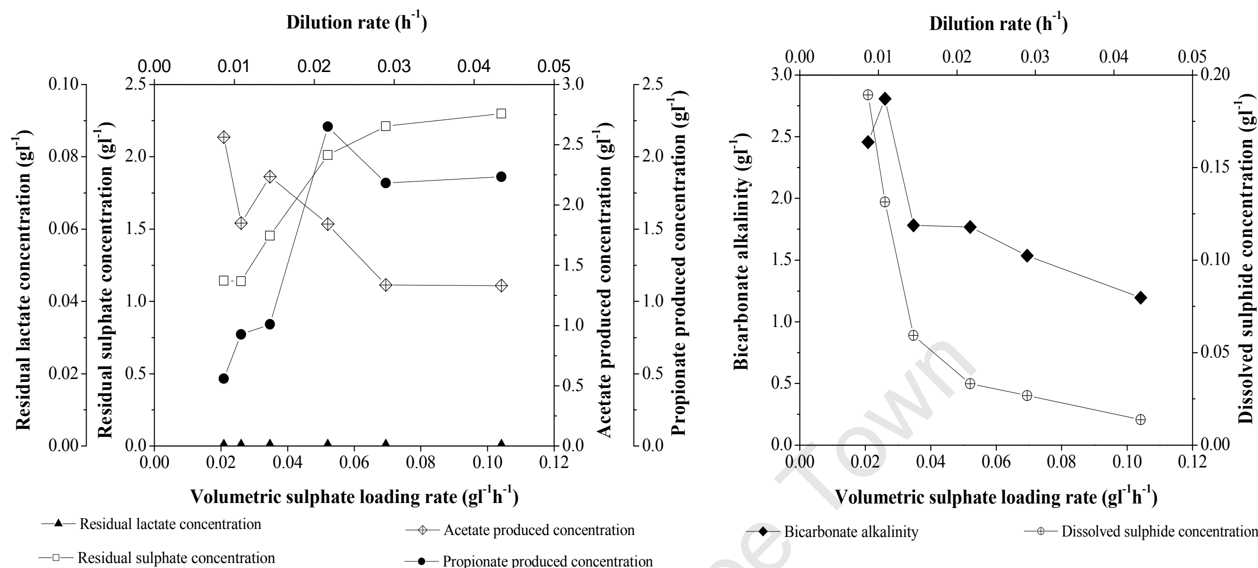

Figure 4.3b: Steady-state profiles of residual lactate, residual sulphate, acetate produced and propionate concentrations of continuous reactor with a feed sulphate concentration of 2.5 g l^{-1}.

Figure 4.3c: Steady-state profiles of bicarbonate alkalinity and dissolved sulphide concentration of continuous reactor with a feed sulphate concentration of 2.5 g l^{-1}.

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Competition by these microorganisms could have led to the reduced availability of lactate for SRB growth; hence, decline in the volumetric sulphate reduction rate (Figure 4.3a). This observation was consistent with the concomitant sharp increase in the concentrations of propionate and residual sulphate observed with increasing volumetric sulphate loading rate (Figure 4.3b). These observations indicate that lactate metabolism via oxidation, which is coupled to sulphate reduction, was only prevalent at the range of low dilution rates of 0.0083 to 0.014 h^{-1} (residence time: 5 to 3 d). Further, there was an apparent shift to lactate metabolism via fermentation over the operation range in this reactor, suggested by decreasing sulphate reduction and increasing propionate formation. At residence time 1 d (*D*= 0.042) minimum values of 0.0084 g l^{-1} h^{-1}, 8% and 0.2 g l^{-1} were observed for the volumetric sulphate reduction rate, sulphate conversion and bacterial dry mass respectively.

Even though the stoichiometric amount of lactate in the medium containing 2.5 g l^{-1} sulphate was 2.5 fold that present in the reactor receiving a feed sulphate

concentration of 1.0 g l^{-1}, the acetate production in these two reactors was comparable (Figures 4.2b and 4.3b). Furthermore, similar lactate conversion was recorded for these two reactors, yet the sulphate conversion was lower in the reactor fed with 2.5 g l^{-1} sulphate (Figures 4.2a and 4.3a). This indicates a lower fractional lactate conversion by oxidation in the reactor fed with 2.5 g l^{-1} sulphate. These observations further suggest that lactate was utilised via a secondary metabolic pathway.

As shown in Figures 4.2b and 4.3b, in spite of higher bacterial dry mass observed in the bioreactor operating with 2.5 g l^{-1} sulphate, the sulphate reduction rate was lower than at 1.0 g l^{-1}. This suggests a possible shift in the microbial population of the mixed consortia i.e. a large proportion of the lactate-utilisers in the bioreactor operating with 2.5 g l^{-1} sulphate were non-SRB. The shift in lactate metabolic pathway and the response of the mixed population to varying operating conditions are discussed in detail in Chapters 5 and 6 respectively.

As depicted in Figure 4.3c, there was a steady decrease in both the bicarbonate alkalinity and sulphide concentrations. These trends are closely related to the progressive increase in the sulphate and propionate concentrations (Figure 4.3b), and the significant decrease in acetate production and sulphate conversion. This observation reinforces the coupling of sulphate conversion to sulphide and lactate oxidation to acetate and bicarbonate (Reaction 2.11, Chapter 2).

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4.3.1.3. Feed sulphate concentration of 5.0 g l^{-1}

The trends observed in this experiment were similar to those observed for the reactor with a feed sulphate concentration of 2.5 g l^{-1}. Maximum sulphate conversion of 58% was observed at a volumetric sulphate loading rate of 0.042 g l^{-1} h^{-1} (residence time: 5 d) (Figure 4.4a). The corresponding bacterial dry mass was 0.458 g l^{-1}. The sulphate conversion, lactate conversion and bacterial dry mass decreased with increasing volumetric sulphate loading rate.

Increasing the volumetric sulphate loading rate in the range 0.042 to 0.104 g l^{-1} h^{-1} resulted in a gradual increase of the volumetric sulphate reduction rate to a maximum of 0.032 g l^{-1} h^{-1} at the residence time of 2 days (volumetric loading rate: 0.104 g l^{-1} h^{-1}). The corresponding sulphate conversion was 31%. Further increase in

the sulphate loading rate beyond this range led to a significant decline in the volumetric sulphate reduction rate. The reactor fed with a feed sulphate concentration of 5.0 g l^{-1} exhibited lower lactate conversion in comparison to the previous reactors receiving feed sulphate concentrations of 1.0 and 2.5 g l^{-1}.

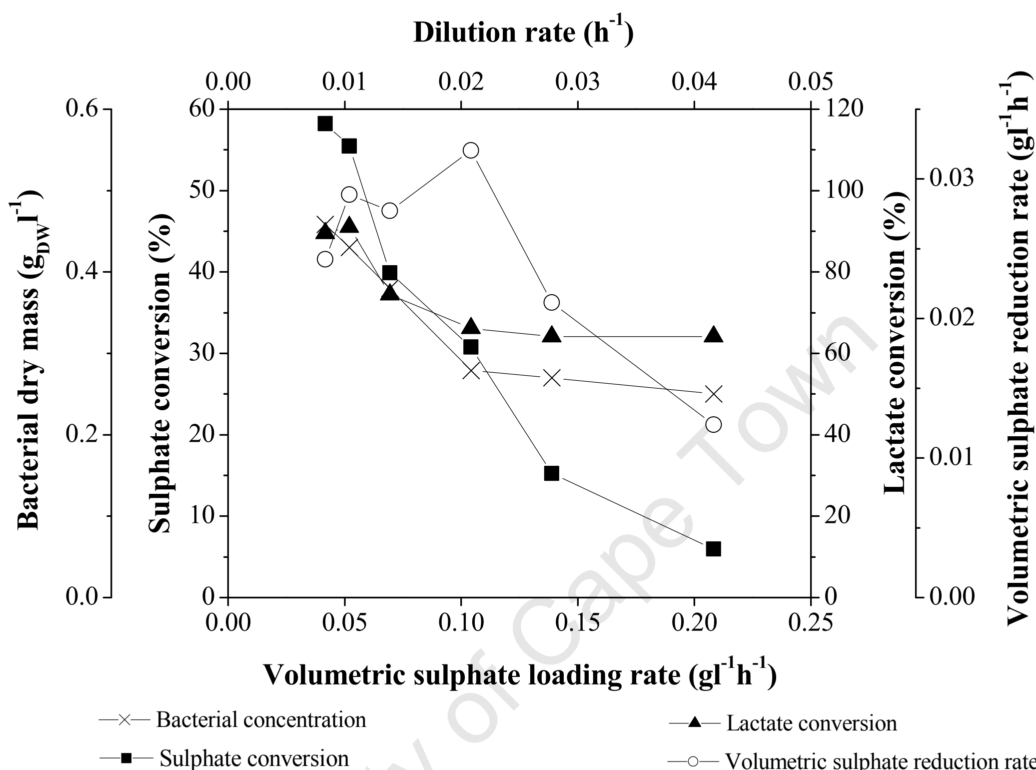

Figure 4.4a: Steady-state kinetics of continuous reactor with a feed sulphate concentration of 5.0 g l^{-1}. Kinetic data presented are bacterial dry mass, sulphate conversion, lactate conversion and volumetric sulphate reduction rate.

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At residence times 3 to 5 d, the lactate conversion varied between 74 and 91%. Beyond this range, 65% lactate conversion was maintained. Hence, significant amount of lactate (4.2 ± 0.7 g l^{-1}) was detected at high dilution rates (0.021 to 0.042 h^{-1}) (Figure 4.4a). Despite this disparity, the sulphate conversion recorded in the reactors receiving feed sulphate concentrations of 2.5 and 5.0 g l^{-1} was similar (Figures 4.3a and 4.4a). The decline in sulphate conversion was consistent with the decrease in sulphide concentration (Figure 4.4c). Decrease in both the bicarbonate and acetate concentrations was congruent with a steady increase in the concentrations of residual lactate, residual sulphate and propionate (Figures 4.4b and 4.4c).

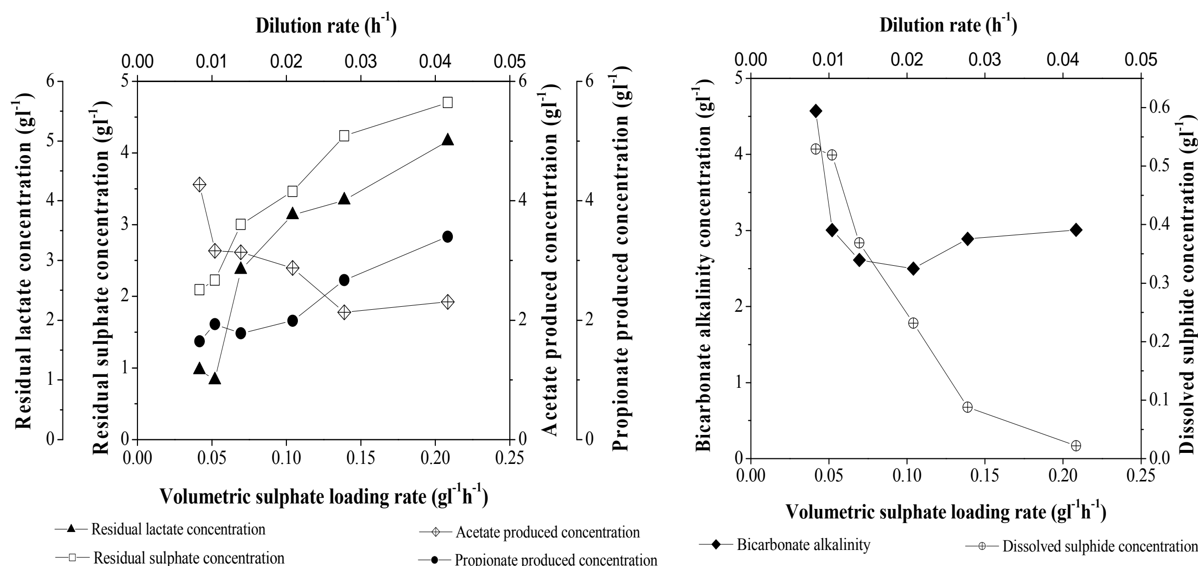

Figure 4.4b: Steady-state profiles of residual lactate, residual sulphate, acetate produced and propionate concentrations of continuous reactor with a feed sulphate concentration of 5.0 g l^{-1}.

Figure 4.4c: Steady-state profiles of bicarbonate alkalinity and dissolved sulphide concentration of continuous reactor with a feed sulphate concentration of 5.0 g l^{-1}.

4.3.1.4. Feed sulphate concentration of 10.0 g l^{-1}

The experiment at a feed sulphate concentration of 10.0 g l^{-1} was chracterised by low sulphate conversions. The highest percentage conversion of 40% was observed at the lowest volumetric loading rate of 0.0417 g l^{-1} h^{-1} (residence time: 5 d) (Figure 4.5a). Nevertheless, this relatively low sulphate conversion translated into a significant removal of sulphate (2.2 to 4.0 g l^{-1}) and production of sulphide (0.31 to 0.62 g l^{-1}) (Table 4.2 and Figure 4.5c). The gradual decline in the sulphate conversion with increasing loading rate was accompanied by a steady lactate conversion of 60 ± 1.8%. This indicated that a portion of the electrons released from lactate metabolism was channelled via other courses apart from sulphate reduction. This was consistent with a steady increase in the concentrations of propionate and the residual sulphate (Figure 4.5b).

Similar to the trend exhibited by the reactor receiving a feed medium of 1.0 g l^{-1} sulphate, the volumetric sulphate reduction rate increased linearly across the range of dilution rates studied, with the maximum value of 0.090 g $l^{-1}h^{-1}$ recorded at the residence time of 1 day. The corresponding sulphate removal efficiency was 22%. High bacterial dry mass (0.44 to 0.59 g l^{-1}) was maintained over the operation range of this experiment.

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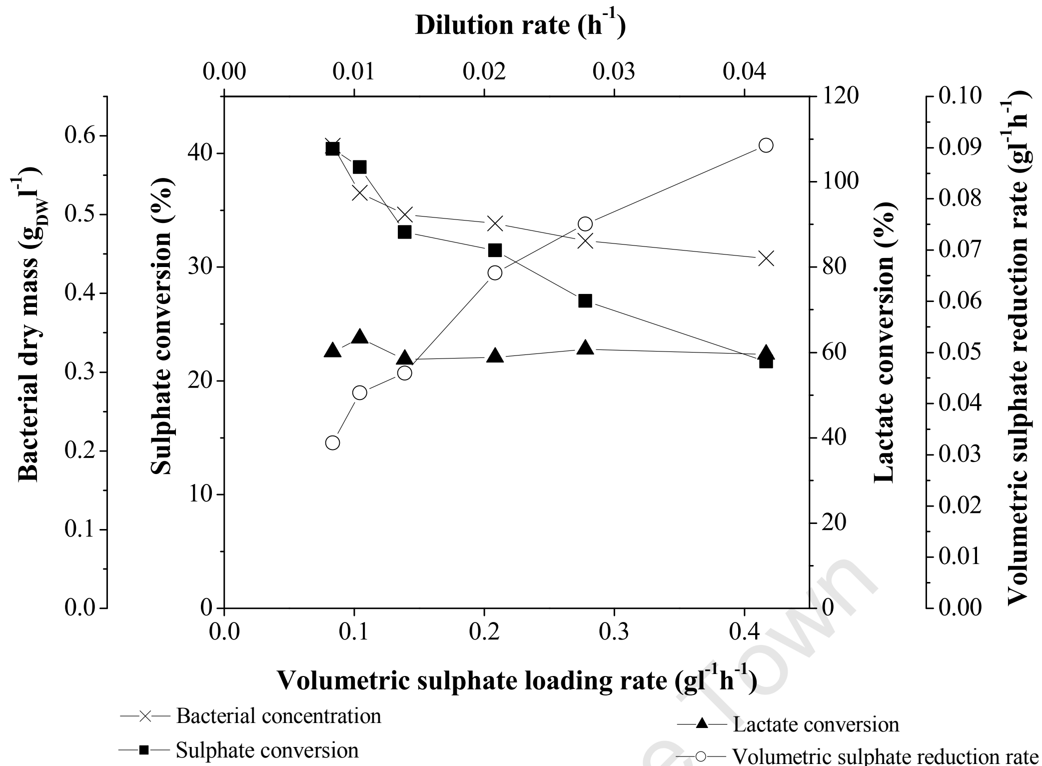

Figure 4.5a: Steady-state kinetics of continuous reactor with a feed sulphate concentration of 10.0 g l^{-1}. Kinetics data presented are bacterial dry mass, sulphate conversion, lactate conversion and volumetric sulphate reduction rate.

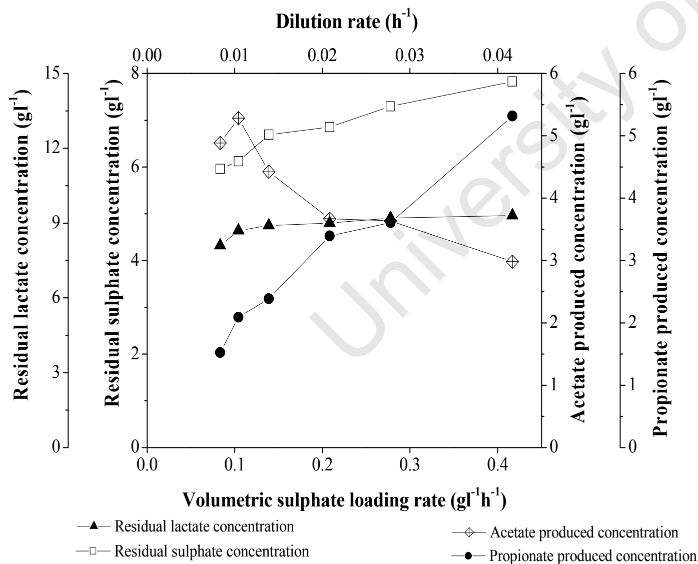

Figure 4.5b: Steady-state profiles of residual lactate, residual sulphate, acetate produced and propionate concentrations of continuous reactor with a feed sulphate concentration of 10.0 g l^{-1}.

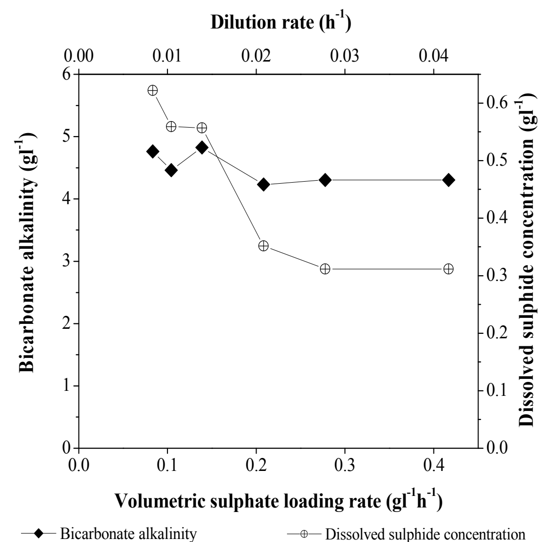

Figure 4.5c: Steady-state profiles of bicarbonate alkalinity and dissolved sulphide concentration of continuous reactor with a feed sulphate concentration of 10.0 g l^{-1}.

This could have been responsible for the sustained enhancement of the volumetric rate of sulphate reduction and the gradual decrease in the sulphate conversion observed

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over the entire period of bioreactor operation. There was a slight increase in the residual lactate over the entire period of bioreactor operation. High lactate concentration (8.9 ± 0.4 g l^{-1}) was maintained. Decline in the acetate concentration with increasing volumetric loading rate was similar to the trend exhibited by the sulphate conversion (Figures 4.5a and 4.5 b). There was a consistent increase in both the concentrations of residual sulphate and propionate produced. On the other hand, the stable concentration of bicarbonate alkalinity over the entire period of bioreactor operation (Figure 4.5c) indicated that lactate oxidation was predominant in this experiment.

4.3.1.5. Feed sulphate concentration of 15.0 g l^{-1}

The reactor receiving a sulphate concentration of 15.0 g l^{-1} exhibited a poor performance as reflected by the low volumetric sulphate reduction rate (Figure 4.6a). The volumetric sulphate reduction rate varied between 0.010 and 0.017 g l^{-1} h^{-1}, compared to the maximum of 0.090 g l^{-1} h^{-1} recorded at 10.0 g l^{-1} feed sulphate. The highest volumetric sulphate reduction rate (0.017 g$l^{-1}h^{-1}$) was achieved at the dilution rate of 0.0083 h^{-1} (residence time: 5 d). The corresponding sulphate removal efficiency was 13%. Lower bacterial dry mass (0.26 to 0.38 g l^{-1}) was observed in this experiment as compared with the bioreactor receiving 10.0 g l^{-1} sulphate. These were comparable with the values observed at lower feed sulphate concentrations (2.5 and 5.0 g l^{-1}). Both the sulphate conversion and bacterial dry mass declined steadily with increasing dilution rate in the range 0.0076 to 0.014 h^{-1} (volumetric sulphate loading rate: 0.11 to 0.21 g l^{-1} h^{-1}; residence time: 5.5 to 3 d). The maximum volumetric loading rate (0.21 g l^{-1} h^{-1}) achievable in this experiment was significantly lower than the maximum value (0.42 g l^{-1} h^{-1}) obtained at a feed sulphate concentration of 10.0 g l^{-1}.

In addition, the sulphate removal efficiency at the low (0.11 to 0.13 g l^{-1} h^{-1}) and high volumetric sulphate loading rates (0.16 to 0.21 g l^{-1} h^{-1}) remained constant at 13 and 6.5% respectively. The lactate conversion decreased with increasing volumetric loading rate (Figure 4.6a). As a result of the limited sulphate conversion achieved, substantial levels of residual sulphate (11.3 to 12.1 g l^{-1}), residual lactate (17.7 to 25.4 g l^{-1}) and propionate (3.5 to 3.9 g l^{-1}) were maintained throughout this experimental run (Figure 4.6b). Conversely, the concentrations of acetate, dissolved sulphide and

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bicarbonate alkalinity (Figures 4b and c) were significantly reduced as the dilution rate was increased.

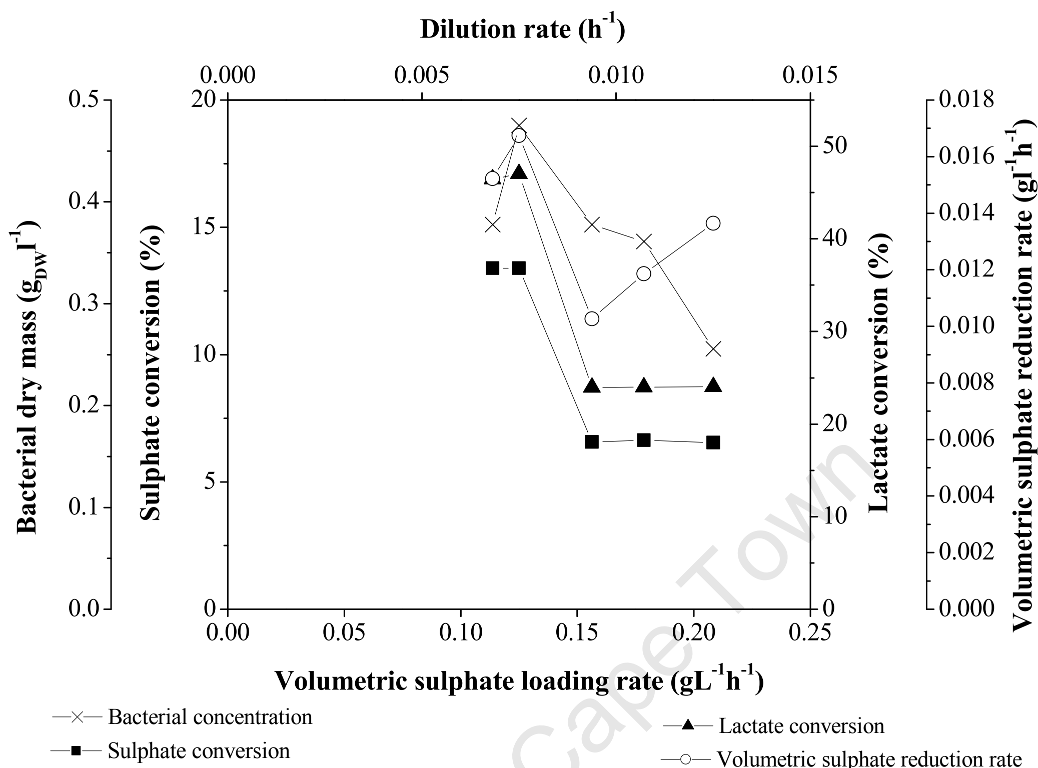

Figure 4.6a: Steady-state kinetics of continuous reactor with feed sulphate concentration of 15.0 g l^{-1}. Kinetic data presented are bacterial dry mass, sulphate conversion, lactate conversion and volumetric sulphate reduction rate.

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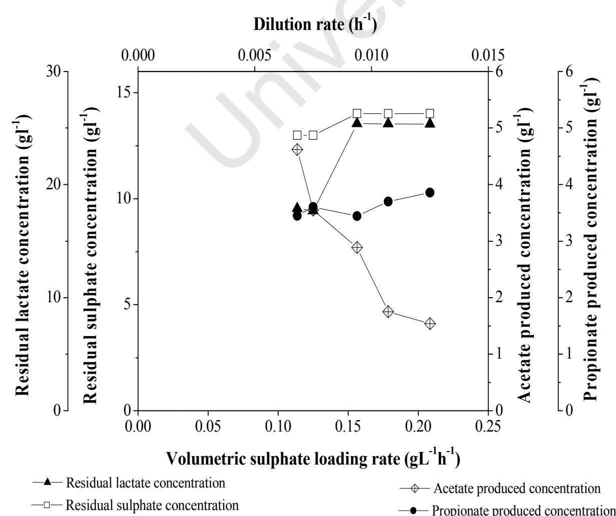

Figure 4.6b: Steady-state profiles of residual lactate, residual sulphate, acetate produced and propionate concentrations of continuous reactor with feed sulphate concentration of 15.0 g l^{-1}.

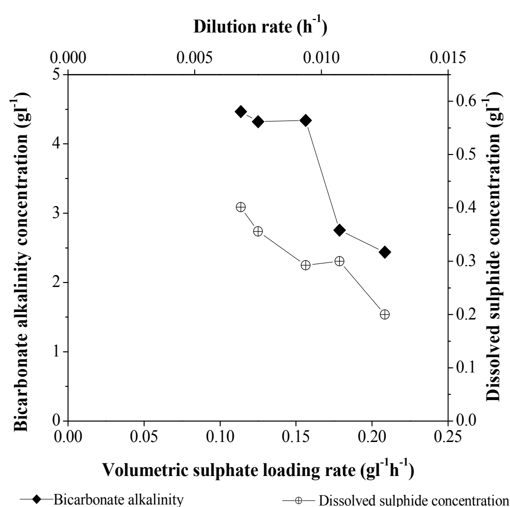

Figure 4.6c: Steady-state profiles of bicarbonate alkalinity and dissolved sulphide concentrations of continuous reactor with a feed sulphate concentration of 15.0 g l^{-1}.

4.3.2. Dependency of Biological Sulphate Reduction Kinetics on Feed Sulphate Concentration, Residence Time and Sulphate Loading Rate

4.3.2.1 Volumetric Sulphate Reduction Rate

Although the results shown in Table 4.3 suggest that the maximum volumetric sulphate reduction rate may be influenced by the feed sulphate concentration, a consistent trend is not found. In Figure 4.7, the volumetric sulphate reduction rate (VSRR) is reported as a function of the dilution rate and the volumetric sulphate loading rate (VSLR) across the feed sulphate concentrations used. Similar trends of the response of the volumetric sulphate reduction rate to the loading rate of sulphate were exhibited by the reactors receiving 1.0 and 10.0 g l^{-1} sulphate (Figure 4.7).

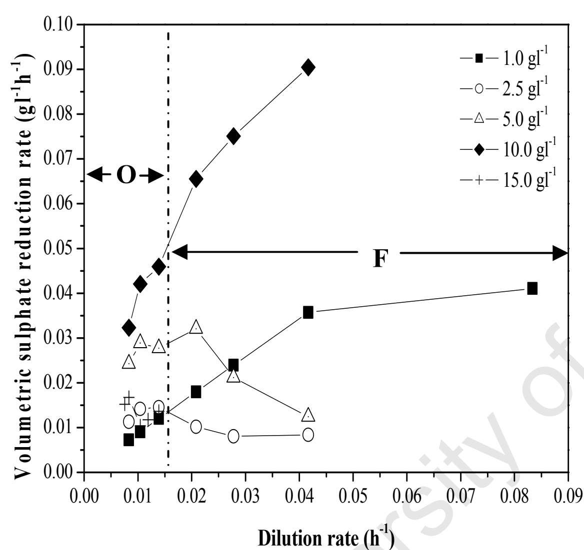

Figure 4.7a : Effect of feed sulphate concentration and dilution rate on volumetric sulphate reduction rate. Dotted line distinguishes between regions of the predominance of lactate oxidation (O) and fermentation (F) when S_o= 2.5 and 5.0 g l^{-1}.

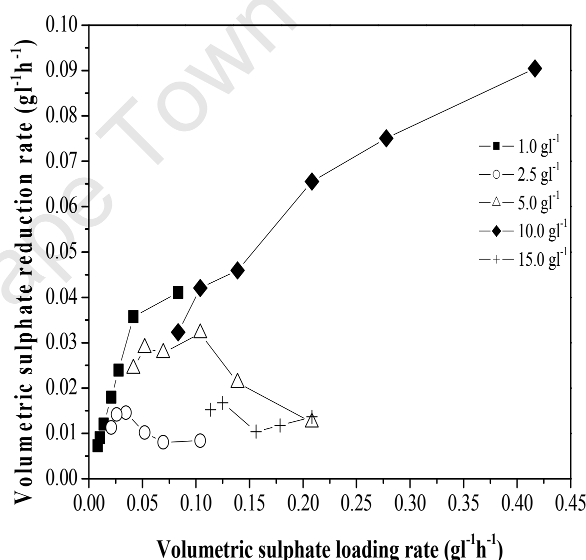

Figure 4.7b: Effect of feed sulphate concentration and volumetric sulphate loading rate on volumetric sulphate reduction rate.

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Across the range of the dilution rates studied, the VSRR increased with increasing VSLR in these two experiments. On the contrary, in the reactors receiving media containing 2.5 and 5.0 g l^{-1} sulphate concentration, the volumetric sulphate reduction rate increased to a maximum at low dilution rates (0.0083 to 0.014 h^{-1}, residence time: 5 to 3 d) (Figure 4.7a). Further increase of the loading rate beyond 0.035 and 0.104 g l^{-1} h^{-1} in the reactors fed with 2.5 and 5.0 g l^{-1} respectively led to a decline in the sulphate reduction rate (Figure 4.7b). In addition, the maximum volumetric sulphate reduction rates achieved in the reactors receiving feed sulphate

concentrations of 1.0 and 10.0 g l^{-1} were higher than those obtained at 2.5 and 5.0 g l^{-1} (Table 4.3).

Table 4.3: Effect of feed concentration and volumetric sulphate loading rate on maximum volumetric sulphate reduction rate.

Feed sulphate concentration (g l^{-1})	Maximum volumetric sulphate reduction rate (g l^{-1} h^{-1})	Corresponding volumetric sulphate loading rate (g l^{-1} h^{-1})	Corresponding residence time (d)	Corresponding sulphate conversion (%)
1.0	0.041	0.083	0.5	50
2.5	0.015	0.035	3	42
5.0	0.032	0.104	2	31
10.0	0.090	0.417	1	22
15.0	0.017	0.114	5	13

There was an early onset of reactor failure in the experiment with a 15.0 g l^{-1} feed sulphate concentration, as demonstrated by the lowest maximum sulphate reduction rate of 0.017 g l^{-1} h^{-1} (Table 4.3) in comparison with the lower feed concentrations 1.0 to 10.0 g l^{-1}. This value was observed at a very low dilution rate of 0.0076 h^{-1}. This can be attributed to metabolic inhibition. This is consistent with the observations by Moosa (2000). On studying the effect of feed sulphate concentration in an acetate-fed chemostat, it was revealed that VSRR increased with increasing feed sulphate concentration in the range 1.0 to 10.0 g l^{-1}. Inhibition of the microbial growth was observed when the feed medium contained 15.0 g l^{-1} sulphate. Further, Hansford *et al*. (2007) reported that increasing the feed sulphate concentration in the range 1.0 to 10.0 g l^{-1} enhanced the volumetric sulphate reduction rate in an ethanol-fed system. A further increase of the feed concentration to 15.0 g l^{-1} resulted in an unstable system.

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Contrary to the observations reported in the suspended-cell systems discussed above, in immobilised cell-systems fed with lactate, increasing feed sulphate concentrations, in the range 1.0 to 5.0 g l^{-1}, negatively influenced the reactor performance as measured by the volumetric sulphate reduction rate. This was attributed to sulphide toxicity. Although similar feed sulphate concentrations were employed the higher sulphate reduction efficiency associated with the immobilised cell-systems resulted in sulphide concentrations inhibitory to SRB growth (Baskaran and Nemati, 2006). The maximum VSRR was reduced from 1.7 g l^{-1} h^{-1} at a feed sulphate concentration of 1.0 g l^{-1} to 0.7 g l^{-1} h^{-1} at a feed sulphate concentration of 5.0 g l^{-1}. The corresponding

maximum sulphide concentrations were 0.6 g l^{-1} and 1.1 g l^{-1} at feed sulphate concentrations 1.0 and 5.0 g l^{-1} respectively.

A batch study by Mohanty *et al*. (2000) showed that sulphate reduction rate decreased with increase in feed sulphate concentration from 1.3 to 3.6 g l^{-1}. This was associated with sulphate toxicity. On the contrary, our study did not follow a particular trend with respect to the dependency of maximum volumetric sulphate reduction rate on feed sulphate concentration (Table 4.2). This can be attributed to the fact that lactate supports the growth of a wide variety of anaerobic microorganisms (SRB and non-SRB). Hence, varying operational conditions may alter the predominance of different reactions and different microbial population members.

The three possible effects of substrate on the systems investigated in the current study are discussed in the following order: sulphate toxicity, sulphide toxicity and volatile acid toxicity.

(1) Sulphate toxicity: According to White and Gadd (1996), the inhibitory effect of residual sulphate on the biological sulphate reduction in a lactate-fed sulphidogenic system was attributable to its effect on the operating pH and redox potential. An increasing concentration of residual sulphate increased the redox potential and reduced the operating pH. An increased redox potential would select for non-SRB lactate utilisers in the mixed consortia used since SRB are known to thrive successfully at low negative redox potentials (Postgate, 1984; White and Gadd 1996). In the current study, residual sulphate was recorded in all the reactors across the operating conditions studied (Figures 4.2b to 4.6b). This indicated that the microbial activity was not limited by the feed sulphate concentrations (1.0 to 15.0 g l^{-1}). Additionally, the reactors were maintained at pH 8.0 ± 0.2. Hence, possible sulphate toxicity by lowering the reactor pH was eliminated. Sulphide, known to result in negative redox potential (Postgate, 1984), was detectable across the operation range except at the range of high dilution rates in the reactors receiving 2.5 and 5.0 g l^{-1} sulphate (Table 4.2). It can therefore be postulated that in the reactors with feed sulphate concentrations 2.5 and 5.0 g l^{-1}, low sulphide levels (0.014 to 0.088 g l^{-1}) at high dilution rates (0.021 to 0.042 h^{-1}) could not counteract the positive effect of residual sulphate on redox potential. It thus follows that the residual sulphate might

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have raised the redox potential subsequently encouraging the prevalence of non-SRB lactate utilisers at high dilution rates in these two reactors. Since redox potential was not monitored in the current study, the effect of residual sulphate on the redox potential could not be substantiated.

At feed sulphate concentration of 15.0 g l^{-1}, dissolved sulphide concentration was maintained in the range 0.2 to 0.4 g l^{-1} (Table 4.2), this maintained a negative redox potential in the bioreactor (-350 to -475 mV). Therefore sulphate toxicity through increased redox potential can be excluded in the reactor receiving 15.0 g l^{-1} sulphate in the feed. Previous studies (Soto *et al*., 1993; Visser, 1995; Lens *et al*., 1998) have shown that sodium ion at concentrations 15.0 to 17.5 g l^{-1} is inhibitory to SRB. The sodium concentration present in the media fed into the 15.0 g l^{-1} sulphate-fed reactor contained 11.0 g l^{-1}. Hence, the sodium ion would not be expected to be inhibitory in the current study. Barton and Tomei (1995) reported the occurrence of two mechanisms of sulphate reduction by SRB, namely: a low sulphate and a high sulphate concentration system. The low sulphate concentration system is turned off in the presence of high sulphate concentrations to prevent flooding of the cell with sulphate. Consequently, the bacterial activity is reduced. This mechanism could have resulted in the substrate inhibition observed at feed concentration of 15.0 g l^{-1} sulphate.

(2) Sulphide toxicity: Previous investigations of acetate-fed sulphidogenic reactors have shown that 50% growth rate inhibition for a mixed consortium of SRB occurred at HS^- concentrations between 0.4 and 1.04 g l^{-1} (total sulphide= 0.57 to 1.11 g l^{-1}) as the pH was varied between pH 7.2 and 8.5 (Visser, 1995; O'Flaherty *et al*., 1998). Results from an investigation by Kuo and Shu (2004) revealed that acetate- and butyrate-fed anaerobic systems exhibited inhibition by dissolved sulphide (DS) concentrations of 0.15 to 0.2 g l^{-1} (0.06 to 0.075 g l^{-1} of undissociated hydrogen sulphide), while the system depending on lactate operated steadily at elevated concentration ranges of 0.2 to 0.4 g l^{-1} of DS (0.1 to 0.15 g l^{-1} undissociated hydrogen sulphide). Additionally, a chemostat study of acetate-sulphate systems by Moosa and Harrison (2006) showed that the VSRR decreased with increasing total soluble sulphide concentration in the range 0.75 to 1.45 g l^{-1}, corresponding to an undissociated sulphide concentration of 0.07 to 0.16 g l^{-1}.

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At pH 8.0 ± 0.2 maintained in the current study, HS^- was expected to be the predominant sulphide species. As shown in Table 4.2, concentrations of sulphide detected (0.014 to 0.23 g l^{-1}) within the range of dilution rates 0.021 to 0.042 h^{-1} (residence times: 2 to 1 d) in the reactors with feed sulphate concentrations 2.5 g l^{-1} and 5.0 g l^{-1} were below the concentrations reported to result in inhibition. This indicated that the SRB growth rate was probably not inhibited by sulphide at the high dilution rates for these experiments. High sulphide concentrations which fall within the range at which 50% sulphide inhibition occurs, reported by O'Flaherty *et al*. (1998), were only detected at low dilution rates 0.0083 to 0.010 h^{-1} for the reactor receiving feed sulphate concentration of 5.0 g l^{-1} and throughout the operation periods for the reactors operated with media containing 10.0 and 15.0 g l^{-1} sulphate. The investigation by O'Flaherty *et al*. (1998) showed that sulphide inhibition is species-specific. Hence, level of inhibition experienced by each system also depends on the dominant group(s) under different operating conditions.

(3) VFA toxicity

The inhibitory effect of lactate on SRB growth kinetics has not been reported in the available literature. However, acetate in its undissociated form is known to inhibit SRB activity at low pH values (≤6) (Reis *et al*., 1992). In the current study, the reactor pH was maintained at pH 8.0 ± 0.2. Owing to this, it is hypothesised that the VFA concentrations in the bioreactors were not responsible for the decline in the metabolic activity of the SRB. As discussed in Section 4.3.1 the SRB metabolic activity could have been limited by a shift in the lactate metabolic pathway from oxidation to fermentation.

Against this background, the following hypotheses are made to give possible reasons for the contrasting trends in the profiles of the VSRR in response to VSLR across the feed sulphate concentrations studied.

(1) Shift in lactate metabolism: It may be hypothesised that lactate oxidation which occurs concomitantly with sulphate reduction was the predominant reaction throughout the operation periods in both reactors receiving feed sulphate concentrations of 1.0 and 10.0 g l^{-1}. On the contrary, this reaction was only prevalent at residence times of 5 to 3 d (D= 0.0083 to 0.014 h^{-1}) in experiments with media

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sulphate concentrations of 2.5 and 5.0 g l^{-1} (Figure 4.7a). Within this range, the volumetric sulphate reduction rate was positively influenced by the volumetric sulphate loading rate, in the range 1.0 to 10.0 g l^{-1}. This is consistent with previous studies described above (Moosa *et al*., 2002; Hansford *et al*., 2007) (Figure 4.7a and Table 4.4). This can be supported by the sulphide levels and the concentration of acetate and bicarbonate alkalinity produced relative to the inlet sulphate and lactate concentrations. Shifts in the stoichiometry of sulphate reduction and lactate metabolism with increasing volumetric sulphate loading rate are presented in detail in Chapter 5.

Table 4.4: Effect of feed concentration on the volumetric sulphate reduction rate at the residence time of 3 d (D= 0.014).

Feed sulphate concentration (g l^{-1})	Volumetric sulphate reduction rate (g l^{-1} h^{-1})	Corresponding volumetric sulphate loading rate (g l^{-1} h^{-1})	Corresponding sulphate conversion (%)
1.0	0.012	0.0139	86
2.5	0.015	0.0347	42
5.0	0.028	0.0694	31
10.0	0.046	0.139	22
15.0	0.010	0.208	13

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(2) Influence of sulphide concentration on microbial populations: High levels of sulphide could have selected for active lactate-oxidising SRB in experiments with media concentrations of 10.0 g l^{-1} sulphate, while the non-SRB lactate fermenters were inhibited. An investigation by Icgen and Harrison (2006b) showed that the growth of certain groups of SRB (*Desulfobacter*, *Desulfotomaculum* and the *Desulfovibrionaceae* group) was enhanced by increased sulphide concentrations up to 0.75 g l^{-1} added in the feed medium. At pH 8.0 ± 0.2 maintained in this study, HS^- is the predominant sulphide species. The effect of sulphide inhibition on SRB at this working pH should be minimal (Kaksonen, 2004). On the contrary, other anaerobic microorganisms are known to be strongly inhibited by sulphide (Kalyuzhnyi *et al*., 1997; O' Flaherty *et al*., 1998b).

As shown in Figures 4.3a and 4.4a at feed sulphate concentrations of 2.5 and 5.0 g l^{-1}, decrease in bacterial dry mass was gradual despite the marked decline in VSRR with

increase in the volumetric sulphate loading rate. Significant propionate production, an indication of the activity lactate fermenters was also evident (Figures 4.3b and 4.4b).

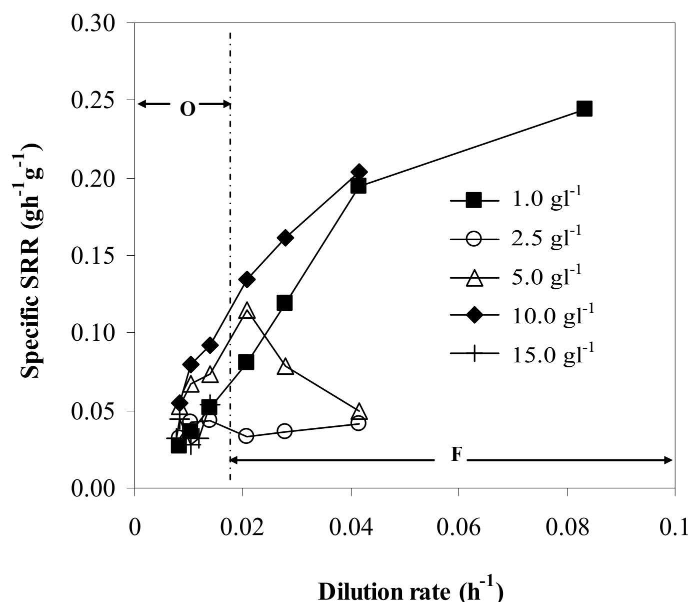

Figure 4.8: Dependency of specific sulphate reduction rate on dilution rate. Dotted line distinguishes between regions of the predominance of lactate oxidation (O) and fermentation (F) when S_o= 2.5 and 5.0 g l^{-1}.

This suggests that while SRB metabolic activity was reduced or lost, a significant microbial population of non-SRB was maintained by the metabolism of lactate. This observation is supported by the profiles of the specific sulphate reduction rate as a function of dilution rate (Figure 4.8). Specific SRR was reduced in the region where fermentation was prevalent for the experiments with feed sulphate concentrations of 2.5 and 5.0 g l^{-1}. This further supports the speculation of significant shifts in the dominant microbial population from lactate oxidisers to lactate fermenters as the dilution rate is increased for these two chemostat cultures. On the other hand, there was a consistent increase in specific SRR throughout the operation periods in experiments with media concentrations of 1.0 and 10.0 g l^{-1} sulphate. This implies that despite the decrease in the total microbial concentration, significant SRB activity was retained. Details of shifts in microbial populations in response to increasing volumetric sulphate loading rate are presented in Chapter 6.

4.3.2.2. Sulphate conversion

As depicted in Table 4.5 and Figure 4.10a, an increase in feed sulphate concentration has a negative influence on the sulphate conversion. The maximum sulphate

conversion achieved at each feed sulphate concentration was at the lowest dilution rate applied (Table 4.5). In contrast to the other reactors, in the 1.0 g l^{-1} sulphate-fed reactor, high sulphate conversion of 86 ± 0.5 % was maintained over a wide range of dilution rates (0.0083 to 0.042 h^{-1}). As the feed sulphate concentration was increased in the range 2.5 to 15.0 g l^{-1} decreasing sulphate conversion with increasing loading rate of sulphate was evident.

Table 4.5: Effect of feed sulphate concentration on sulphate conversion.

Feed sulphate concentration (g l^{-1})	Maximum sulphate conversion (%)	Corresponding volumetric sulphate loading rate (g l^{-1} h^{-1})	Corresponding residence time (d)
1.0	87	0.0083-0.010	5-4
2.5	54	0.021-0.026	5-4
5.0	58	0.042	5
10.0	40	0.083	5
15.0	13	0.110	5.5

Additionally, at high loading rates, where low sulphate conversions were recorded, there were concomitant increases in propionate, residual sulphate and residual lactate concentrations (Figures 4.2 to 4.6; Figure 4.10c). This suggests the presence of lactate fermenters (LF) and a consequent shift in lactate metabolism to lactate fermentation at high loading rates. Figure 4.9a indicates that there is no consistent relationship between lactate conversion and sulphate conversion as the feed sulphate concentration was varied between 1.0 and 10.0 g l^{-1}. Alternatively, across the feed sulphate concentrations studied, at low dilution rates, the sulphate removed remained relatively constant despite increasing acetate production. At high dilution rates there was a marked decrease in sulphate removal accompanied by a slight decrease in acetate production (Figure 4.9b). This pattern is congruent with increasing propionate production within this range of dilution rates (Figure 4.9c).This phenomenon is attributable to the production of acetate from other non-SRB microorganisms. Lactate is known to be fermented to acetate and propionate by some non-SRB lactate fermenters such as *Veillonella parvula* and *Propionibacterium* spp. (Reaction 2.26) (Seeliger *et al.,* 2007).

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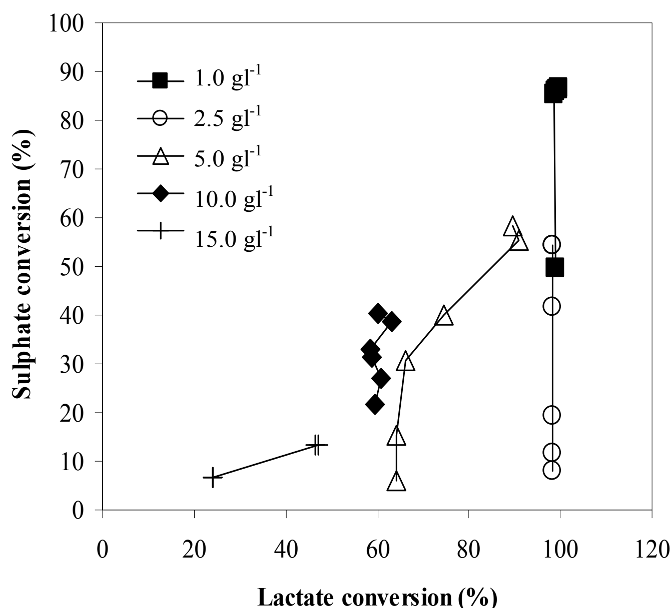


Figure 4.9a: Relationship between sulphate conversion and lactate conversion.

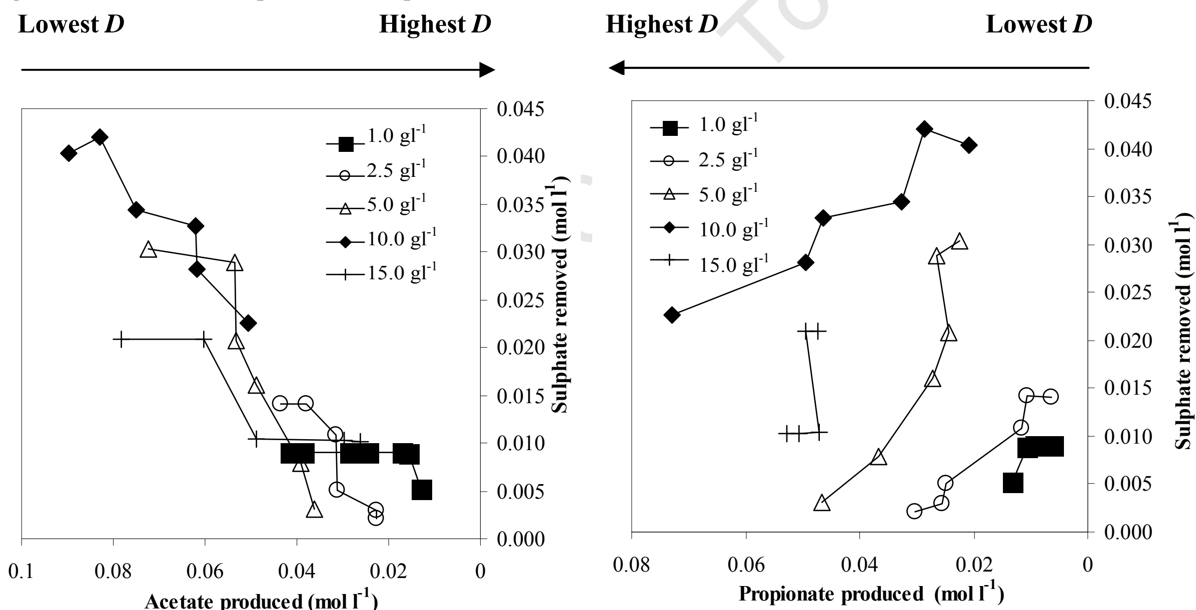
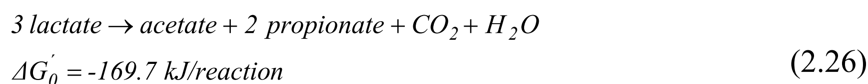


Figure 4.9b: Dependency of sulphate removed on acetate produced. ***D***= dilution rate

Figure 4.9c: Dependency of sulphate removed on propionate produced. ***D***= dilution rate



$$3\ lactate \rightarrow acetate + 2\ propionate + CO_2 + H_2O$$
$$\Delta G_0' = -169.7\ kJ/reaction \qquad (2.26)$$

Similar to the results obtained in the current study, Baskaran and Nemati (2006) demonstrated that maximum sulphate conversion of 100% was achieved when the feed sulphate concentrations were 1.0 and 2.5 g l^{-1} in lactate-fed immobilised-cell systems. Increasing the feed sulphate concentration to 5.0 g l^{-1} led to a decline in the sulphate conversion to 90%. Furthermore, a study by Kalyuzhnyi *et al.* (1997) showed that sulphate conversion was maintained at 80% when the influent sulphate

concentration of an ethanol-fed UASB reactor was below 1.34 g l^{-1} at a constant hydraulic retention time. Above this feed sulphate concentration, the sulphate removal efficiency was reduced to 62%. This was associated with decrease in pH to 6.8 and the resulting increased levels of undissociated sulphide. Since the pH was kept constant at pH 8 ± 0.2 in this present study, the effect of undissociated sulphide on the sulphate conversion was negligible. It can thus be speculated that the sulphate conversion was limited by the competition between SRB and lactate fermenters.

4.3.2.3. Bacterial dry mass

As shown in Table 4.6, increased feed sulphate concentration in the range 1.0 to 10.0 g l^{-1} resulted in increased bacterial dry mass. Substrate inhibition of bacterial growth was significant in the experiment receiving 15.0 g l^{-1} sulphate. Mohanty *et al*. (2000) reported that the volumetric sulphate reduction rate was positively influenced by biomass concentration in a batch culture. A four fold increase of the biomass concentration resulted in a 1.5 fold increase of the sulphate reduction rate. Results from the current study concur with a previous investigation by Moosa (2000). Increase in sulphate utilisation with increasing feed sulphate concentration was hypothesised to be responsible for the increase in biomass concentration with increasing feed sulphate concentration in the range 1.0 to 10.0 gl^{-1} for acetate-fed reactors. In the current study, maximum bacterial dry mass values were obtained at the lowest dilution rate in all experiments. This indicates that biomass concentration was negatively influenced by increasing volumetric sulphate loading rate in each experiment (Table. 4.6).

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Table 4.6: Effect of feed sulphate concentration on bacterial dry mass.

Feed sulphate concentration (g l^{-1})	Maximum bacterial dry mass (g_{DW} l^{-1})	Corresponding volumetric sulphate loading rate (g l^{-1} h^{-1})
1.0	0.267	0.083
2.5	0.350	0.10
5.0	0.458	0.21
10.0	0.588	0.42
15.0	0.475	0.21

Dilution rates at which a greater proportion of the starting bacterial dry mass was lost were specific for each feed sulphate concentration. For experiments with media

sulphate concentrations of 1.0 and 10.0 g l^{-1}, 75 and 80% of the initial biomass concentration was maintained up to a dilution rate 0.028 h^{-1} (Table 4.7). This is consistent with the corresponding increase in the volumetric sulphate reduction rate with increasing volumetric sulphate loading rate (Figures 4.2a and 4.5). On the other hand, 65 and 59% of the maximum biomass concentrations were observed at the same dilution rate for feed sulphate concentrations of 2.5 and 5.0 g l^{-1} respectively (Table. 4.7). These trends coincided with the pronounced decline in the volumetric sulphate reduction rates observed for these experiments (Figures 4.3a and 4.4a). These observations suggest that the energy obtained from the lactate fermentation reaction is lower than that obtained from lactate oxidation. Hence a smaller microbial population was sustained in the reactors receiving sulphate concentrations of 2.5 and 5.0 g l^{-1} in comparison to the experiments with media sulphate concentrations of 1.0 and 10.0 g l^{-1}.

Table 4.7: Effect of feed sulphate concentration and dilution rate on bacterial retainment.

RT (day)	**Dilution rate (h^{-1})**	**Relative biomass concentration (%) at different feed sulphate concentrations (S_o)**				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	64	nd	nd	nd	nd
1.0	0.042	69	57	55	77	nd
1.5	0.028	75	65	59	79	nd
2.0	0.021	83	89	61	83	nd
3.0	0.014	87	95	83	85	54
3.5	0.012	nd	nd	nd	nd	76
4.0	0.010	94	96	94	90	80
5.0	0.0083	100	100	100	100	80
5.5	0.0076	nd	nd	nd	nd	100

nd: not determined. RT= residence time. Relative biomass concentration expressed as the percentage of the maximum value at each feed sulphate concentration (S_o).

4.3.2.4. Biological Sulphate Reduction Energetics

The Pirt equation, given in Equation 4.7c, was used to determine the biomass-based yield coefficient and maintenance coefficient at the feed sulphate concentration of 1.0 g l^{-1}. This analysis was carried out at this feed sulphate concentration only, due to the deviations from a typical biological sulphate reduction reaction observed at feed sulphate concentrations 2.5 to 15.0 g l^{-1}. High propionate production suggested the proliferation of non-SRB lactate fermenters in these experimental runs. It has been

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suggested that lactate-fed SRB's are prone to competition from other microorganisms when present in mixed cultures (Laanbroek *et al*., 1982). Should competition occur under the experimental conditions used, the bacterial dry mass measured would account for both SRB and non-SRB. Hence, energetic analyses based on sulphate utilisation are inappropriate for this range of feed sulphate concentration as they do not describe a specific population.

The maintenance coefficient of the lactate-grown SRB population was negligible in the 1.0 g l^{-1} sulphate fed-reactor, indicating an efficient sulphate reduction at low feed sulphate concentration. Using the Pirt expression (Pirt, 1965) in Equation 4.7, when m_s is negligible or equal to zero, the observed growth yield, Y_{app} equals the maximum theoretical growth yield, Y_{max}.

$$\frac{1}{Y_{app}} = \frac{1}{Y_{max}} + \frac{m_s}{\mu} \qquad (4.7)$$

It thus follows that under this condition the substrate consumed is channeled to cell growth only.

4.3.3. Reactor Resilience

In the reactors with feed sulphate concentrations of 1.0 to 10.0 g l^{-1}, sulphate conversion efficiencies were studied following operation at very low residence times. Full recovery of the reactor performance as measured by the sulphate conversion and the volumetric sulphate reduction rate was achieved 35 d and 14 d respectively after their operation was restored to 5 d residence time (Table 4.8) (Figures 4.10a and 4.10d). In contrast, for the experiment with a 2.5 g l^{-1} feed sulphate concentration, reactor failure was apparent. In spite of the restoration of a mild operating condition (residence time: 5 d), the sulphate conversion achieved after 30 days was only two-fold higher than that obtained at the residence time of 1 day (Figure 4.10b). There was a corresponding decrease in the volumetric sulphate reduction rate from 0.0083 to 0.0032 g l^{-1} h^{-1}. Despite the complete conversion of lactate observed, the volumetric sulphate reduction rate decreased.

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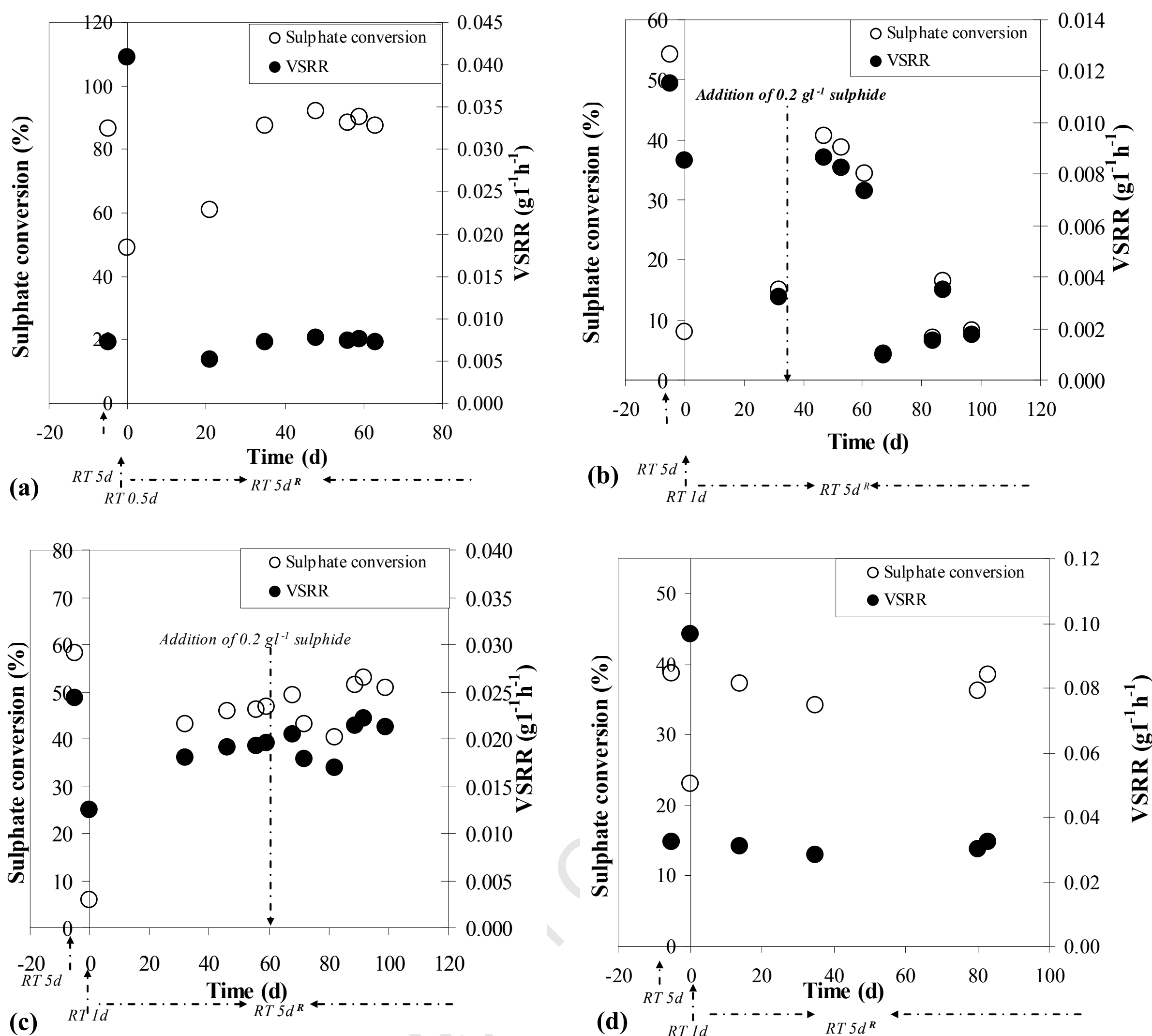

Figure 4.10: Investigation of the effect of feed sulphate concentration on reactor resilience. Less extreme operating conditions were restored after major perturbations (high dilution rates). (a) Feed sulphate concentration 1.0 g l^{-1}; (b) Feed sulphate concentration 2.5 g l^{-1}; (c) Feed sulphate concentration 5.0 g l^{-1}; (d) Feed sulphate concentration 10.0 g l^{-1}. **RT 0.5 d**, **RT 1d** and **RT 5d**: Residence times employed in the experiments investigating the effect of feed sulphate concentration kinetics. **RT 5d^{R}**: Residence time of 5 days during test of resilience experiment. VSRR: Volumetric sulphate reduction rate.

These findings support the hypothesis of a change in community structure from a consortium predominantly composed of sulphate reducers to one with a higher proportion of lactate fermenters on increasing the dilution rate.

The system with a feed concentration of 2.5 g l^{-1} sulphate concentration was spiked with 0.2 g l^{-1} sulphide on day 32. This was done based on the hypothesis that the presence of significant sulphide concentration in the mixed culture employed in the current study discouraged the growth of lactate fermenters, thus favouring the lactate oxidation pathway.

The reactor responded by a pronounced increase in both the sulphate conversion and the volumetric sulphate reduction rate from 15 to 41% and 0.0032 to 0.0083 g l^{-1} h^{-1} respectively on day 47. This was followed by a sharp decline in both the percentage of sulphate removal and the volumetric rate of sulphate reduction to 8% and 0.018 g $l^{-1}h^{-1}$ respectively on day 97 of reactor operation at the residence time of 5 days. This can be attributed to the wash out of sulphide.

As depicted in Figure 4.10c, for the experiment with 5.0 g l^{-1} feed sulphate concentration, there was a gradual increase in the sulphate conversion and the volumetric sulphate reduction rate from day 0 to day 59 of operation at 5 d residence time resulting in sulphate conversion of 47% and a VSRR of 0.020 at 59 d. Addition of 0.2 g l^{-1} sulphide on day 60 was associated with a slight increase in sulphate conversion to 49% and the volumetric sulphate reduction rate of 0.021 g l^{-1} h^{-1} on day 68. This was followed by a slight drop in the reactor performance. The final sulphate conversion and volumetric sulphate reduction rate obtained on day 99 were 51% and 0.021 g l^{-1} h^{-1} respectively. These values were only slightly lower than the ones obtained at the initial 5d residence time employed in the kinetic studies, as summarised in Table 4.8.

Table 4.8: Comparison of reactor performance during perturbations (residence time: 1d, RT 1d and residence time: 0.5 d, RT 0.5 d) and restoration periods (residence time 5 d, RT 5dR). RT 5d= residence time of 5 days employed in the experiments investigating the effect of feed sulphate concentration kinetics. VSRR= Volumetric sulphate reduction rate.

Feed sulphate concentration (g l^{-1})	RT 5d		RT 0.5d/ 1.0 d		RT 5d^R	
	VSRR (g l^{-1} h^{-1})	Sulphate conversion (%)	VSRR (g l^{-1} h^{-1})	Sulphate conversion (%)	VSRR (g l^{-1} h^{-1})	Sulphate conversion (%)
1.0	0.0072	86	0.041	51	0.0073	87
2.5	0.011	54	0.0084	8	0.0018	8
5.0	0.024	58	0.012	6	0.021	51
10.0	0.032	39	0.090	22	0.032	39

These results suggest that the reactors receiving feed sulphate concentrations of 1.0 and 10.0 g l^{-1} were more robust than the ones receiving sulphate concentrations of 2.5 and 5.0 g l^{-1}. These observations were postulated to be a consequence of the predominance of lactate oxidation over lactate fermentation owing to the presence of low lactate concentration and consistently high sulphide concentration which

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influenced the microbial community structures at feed sulphate concentrations of 1.0 and 10.0 g l^{-1} respectively.

As discussed in Sections 4.3.1.2 and 4.3.1.3, the inability of the 2.5 g l^{-1} sulphate-fed reactor to recover its previous performance after the restoration of a less extreme condition is attributable to the out-competition by lactate fermenters and a consequent wash out of SRB cells in the presence of low sulphide and high residual lactate concentrations.

At a feed sulphate concentration of 5.0 g l^{-1}, SRB cells may have been present in a higher proportion in this reactor as compared with the one receiving 2.5 g l^{-1} sulphate concentration. A recent investigation by Sahinkaya *et al*. (2007) reported that delayed process recovery could be attributed to the loss of critical population members under extreme operational conditions. Results from the current study suggest that specific members of the population were not completely washed out at the highest dilution rate imposed in reactors receiving 1.0, 5.0 and 10.0 g l^{-1} sulphate, but were rather present in small quantities (Ayala-Del-Río *et al.,* 2004). Further, the period it took these reactors to recover their previous performance was dependent on the proportion of SRB cells in the biomass concentration.

4.4. SUMMARY

Similar to previous investigations on biological sulphate reduction (Erasmus 2000; Moosa *et al*., 2002; Hansford *et al*. 2007; Baskaran and Nemati, 2006), it was evident from this study that volumetric sulphate loading rate, mediated through both sulphate concentration in the feed and dilution rate significantly influenced the kinetics of biological sulphate reduction. Similar phenomena were observed for experiments with feed sulphate concentrations of 1.0 and 10.0 g l^{-1}. Patterns obtained at feed sulphate concentrations of 2.5 and 5.0 g l^{-1} were similar to each other and deviated from trends obtained in the reactors receiving 1.0 and 10.0 g l^{-1} of sulphate. Inhibition of bacterial growth and substrate toxicity were pronounced in the reactor fed with 15.0 g l^{-1} sulphate. There was evidence to suggest that the unexpected trends reported in this study, with increasing sulphate feed concentration, were as a result of the predominance of different population members and different lactate utilisation pathways. To further substantiate the observations and hypotheses developed in this

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section, the effects of volumetric loading rates on the reaction stoichiometry and the microbial community structures involved in this study were investigated. These are reported in Chapters 5 and 6 respectively.

CHAPTER 5
LACTATE METABOLISM UNDER BIOSULPHIDOGENIC CONDITIONS

5.1. INTRODUCTION

In spite of the rigorous investigations aimed at improving the overall performance of biological sulphate reduction (BSR) (Reis *et al*., 1992; van Houten *et al*., 1996; Chang *et al*., 2000). Rigorous kinetic studies of electron donor utilisation, required for process optimisation are limited. Specifically, with respect to using lactate as the carbon-source. Lactate may be metabolised via fermentation or oxidation or both by a wide range of microorganisms. When lactate is used as an electron donor and carbon-source by sulphate reducers, sulphate reduction occurs concurrently with lactate oxidation. Hence, optimisation of lactate utilisation via the oxidative pathway in preference to fermentation would aid in maximising the efficiency of biological sulphate reduction.

Habicht *et al*. (2005) showed that the stoichiometry of lactate utilisation at low sulphate concentrations (0.06 to 1.4 g l^{-1}) is independent of sulphate concentration. In this present study, the effects of sulphate concentration, feed lactate concentration and volumetric loading rate on the stoichiometry of biological sulphate reduction under biosulphidogenic conditions over a wide range of feed sulphate concentrations (1.0 to 15.0 g l^{-1}) are described. Further, the hypotheses described in Sections 4.3.2 and 4.3.3 to explain the dependency of the kinetics of BSR on stoichiometry is further elucidated.

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5.2. EXPERIMENTAL APPROACH

5.2.1. Effect of Feed Sulphate Concentration on the Stoichiometry of Biological Sulphate Reduction

Experiments were carried out as described in Section 4.2.1. The stoichiometry of the kinetic data presented in Section 4.3.1 were analysed. Continuous reactors were operated at increasing feed sulphate concentrations (1.0 to 15.0 g l^{-1}) in the presence of a 20% stoichiometric excess of lactate calculated according to Reaction 5.1 (Table 5.1). These reactors were maintained at dilution rates in the range 0.0076 to 0.083 h^{-1}

(residence time: 5.5 to 0.5 d). Steady-state concentrations of sulphate, sulphide, bicarbonate alkalinity, lactate, acetate and propionate at each residence time studied were used to estimate the stoichiometry of the reactions involved in biological sulphate reduction and lactate metabolism. All analytical procedures were carried out as described in Section 3.3.

5.2.2. Stoichiometric Calculations

The stoichiometric ratios were quantified as moles of lactate utilised per mole of sulphate reduced (L:S), moles of lactate utilised per mole of acetate produced (L:A) and moles of lactate utilised per mole of bicarbonate produced (L:HC). The L:HC and L:A ratios can be combined as indices of both lactate oxidation and lactate fermentation, while the L:S ratio is representative of lactate oxidation only (Tables 5.1 and 5.2a). To quantify the fate of lactate metabolised by fermentation, excess lactate (^{e}L) was estimated as the difference between the lactate utilised and the expected amount of lactate oxidised for sulphate reduction, which was based on the theoretical ratio of the incomplete lactate oxidation reaction (Reaction 5.1, Table 5.1) i.e. a ratio of moles lactate utilised per unit mole sulphate reduced = 2. Excess lactate utilised per mole of propionate produced (^{e}L:P) represents lactate fermentation (Reaction 5.3, Table 5.1).

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Table 5.1: Reactions and free-energy changes for reactions involving anaerobic metabolism of lactate (Bryant *et al*., 1977; Zellner *et al*., 1994; Heimann *et al*., 2005).

$\Delta G_0'$ (kJ/ reaction)	Lactate metabolic pathway	Reaction	Reaction number
-160.1	Incomplete oxidation	2 lactate + $SO_4^{2-} \rightarrow$ 2 acetate + 2 $HCO_3^- + HS^- + H^+$	5.1
-255.3	Complete oxidation	2 lactate + 3 $SO_4^{2-} \rightarrow H^+ + 6\ HCO_3^- + 3\ HS^-$	5.2
-169.7	Lactate fermentation	3 lactate $\rightarrow$ acetate + 2 propionate + $HCO_3^- + H^+$	5.3
-7.98	Lactate fermentation	2 lactate + 4 $H_2O \rightarrow$ 2 acetate + 2 $HCO_3^- + 4\ H_2$	5.4

5.3. RESULTS AND DISCUSSION

5.3.1. Stoichiometry of Biological Sulphate Reduction

At a low feed sulphate concentration of 1.0 g l^{-1}, high sulphate conversion was accompanied by high oxidation of lactate to acetate and relatively negligible formation of propionate from lactate fermentation (Figures 5.1a to 5.1d). This is an

indication of the dominance of incomplete oxidation of lactate with concomitant sulphate reduction (Reaction 5.1) over lactate fermentation via reaction 5.3 (Table 5.1). Owing to the detection of propionate in the experiments conducted in this study and the low Gibb's free energy associated with Reaction 5.4, lactate fermentation was assumed to proceed via Reaction 5.3 only.

Table 5.2: Dependency of molar ratio of lactate utilised to the other substrates involved in biological sulphate reduction on feed sulphate concentration, using lactate as the sole carbon-source and electron donor. Average values of experimental stoichiometric ratios (Table 5.2b) are compared with the theoretical ratios (Table 5.2a).

Table 5.2a: Theoretical ratios.

Reaction number	L:A	L:S	L:HC	L:P
5.1	1.0	2.0	1.0	
5.2		0.67	0.33	
5.3	3.0		3.0	1.5
5.4	1.0		1.0	

Table 5.2b: Experimental ratios.

Feed Sulphate concentration (g l^{-1})	[a] Total moles lactate used/mole acetate produced (L:A)	[a] Total moles lactate used/mole sulphate reduced (L:S)	[a] Total moles lactate used/mol bicarbonate produced (L:HC)	[a] Excess moles lactate used/mole propionate produced ([e]L:P)
1.0	1.15 ± 0.50	2.8 ± 0.005	1.05 ± 0.083	0.92 ± 0.18
2.5	2.06 ± 0.55	15.02 ± 0.84	2.11 ± 0.65	2.89 ± 1.61
5.0	1.90 ± 0.30	8.81 ± 8.69	1.89 ± 0.33	1.92 ± 0.23
10.0	2.32 ± 0.70	4.70 ± 1.11	2.05 ± 0.13	2.30 ± 0.68
15.0	2.70 ± 0.65	8.68 ± 0.16	2.08 ± 0.50	1.94 ± 0.76

[a] Average values for all experimental runs at different dilution rates.

At the feed sulphate concentration of 1.0 g l^{-1}, the experimental stoichiometric ratio values (L:A, L:S and L:HC) given in Table 5.2 concur largely with the theoretical values of incomplete lactate oxidation via reaction 5.1 (Table 5.1). Furthermore, these ratios were relatively constant as the dilution rate was increased from 0.0083 to 0.083 h^{-1} (residence time: 5.0 to 0.5 d) (Figures 5.1a to 5.1c). The high conversion of sulphate (86 ± 0.5%) observed in the reactor fed with 1.0 g l^{-1} sulphate at residence times of 1 to 5 days agrees well with the stoichiometry profiles (Figure 4.2a, Section 4.3.1.1). In contrast, at higher sulphate concentrations of 2.5 to 15.0 g l^{-1} the observed stoichiometric ratios (Table 5.2b) deviated significantly from the theoretical ratios (Table 5.2a) for incomplete lactate oxidation. This was most evident for the moles of lactate utilised per mole of sulphate reduced.

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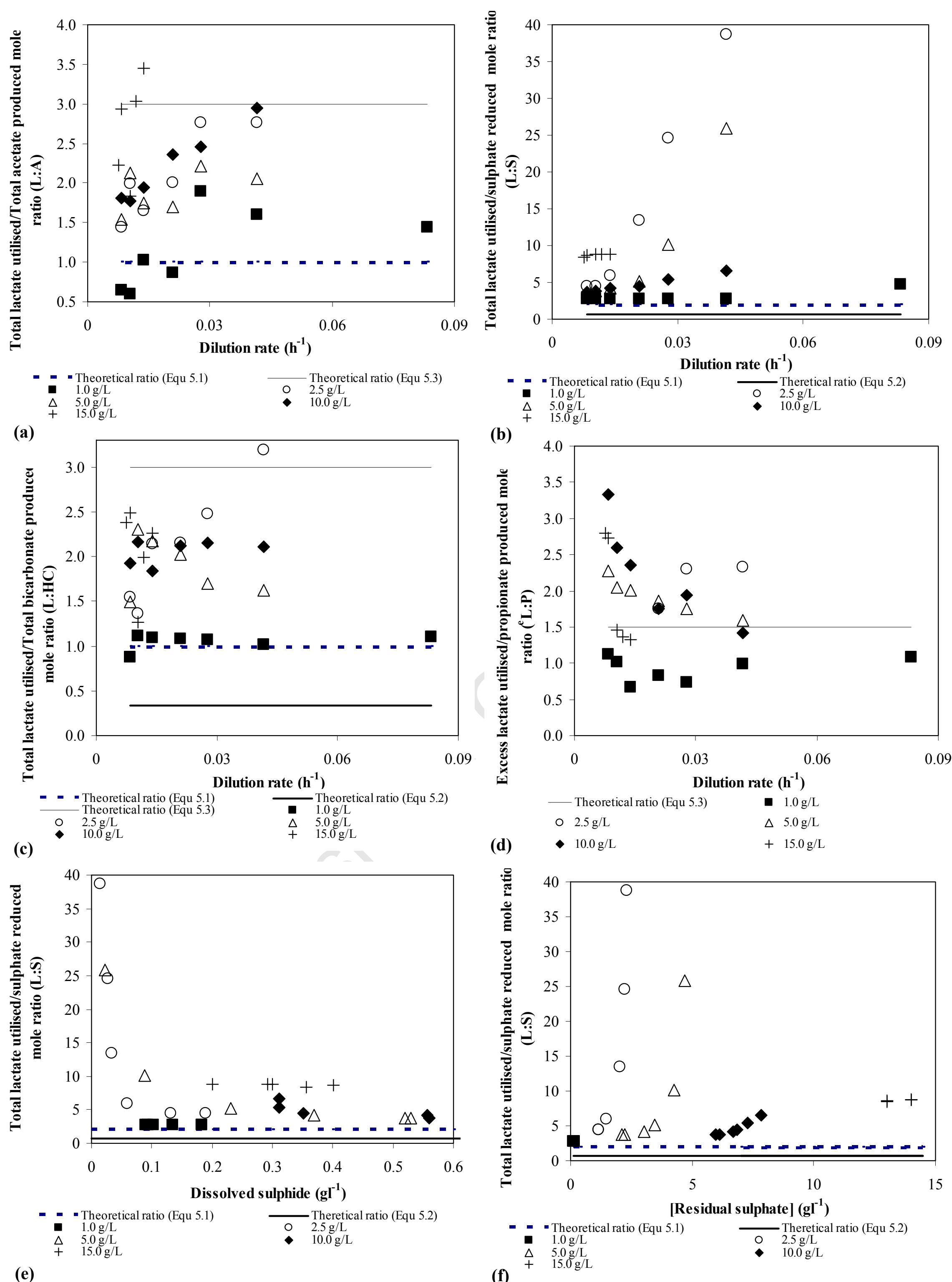

Figure 5.1: Steady-state data of continuous reactors investigating the effect of feed sulphate concentration (1.0 to 15.0 g l^{-1}) and dilution rate (0.0083 to 0.083 h^{-1}) on biological sulphate reduction stoichiometry. (a) Moles of lactate utilised per mole total acetate produced; (b) Moles of lactate utilised per mole sulphate reduced; (c) Moles of lactate utilised per moles total bicarbonate produced; (d) Moles of excess lactate utilised per mole propionate produced; (e) Dependence of moles of lactate utilised per mole reduced sulphate on dissolved sulphide concentration; (f) Dependence of moles of lactate utilised per mole reduced sulphate on residual sulphate concentration.

The increase in feed sulphate concentration in the range of 1.0 to 15.0 g l^{-1} led to an increase in the average L:S ratio. The highest value of the L:S ratio was at the feed sulphate concentration of 2.5 g l^{-1}. This observation suggests the predominance of lactate fermentation in the experiment with feed sulphate concentration 2.5 g l^{-1}. The L:S ratio observed at higher feed sulphate concentrations was significantly higher than the theoretical ratio of 2. This implies that a lower fraction of lactate available was oxidised with concomitant sulphate reduction at higher feed sulphate concentrations (2.5 to 15.0 g l^{-1}) relative to a feed sulphate concentration of 1.0 g l^{-1}. This corroborates the results described in Section 4.3.2.2, of decline in fractional sulphate conversion with increase in the feed sulphate concentration, owing to limited availability of lactate for oxidation resulting from its metabolism through competing reactions. Moreover, deviation of these ratios from the corresponding theoretical stoichiometric ratios increased with the increasing dilution rate, for each feed sulphate concentration in this range. Sulphate reduction, in concurrence with lactate oxidation, was major at low dilution rates of 0.0083 to 0.014 h^{-1} (residence time: 5.0 to 3.0 d) (Figures 5.1a and 5.1b, Table 5.3), in experiments with feed sulphate concentrations 1.0 to 10.0 g l^{-1}.

Table 5.3: Fractional lactate oxidised as a function of feed sulphate concentration (S_o) and dilution rate. RT= residence time.

RT (day)	**Dilution rate (h^{-1})**	**Fractional lactate oxidised (%) at different feed sulphate concentrations (So)**				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	41.5	nd	nd	nd	nd
1.0	0.042	72.4	5.3	7.7	30.3	nd
1.5	0.028	72.4	8.3	19.8	37.1	nd
2.0	0.021	72.4	15.2	38.7	44.5	nd
3.0	0.014	72.3	34.0	47.4	47.3	22.7
3.5	0.012	nd	nd	nd	nd	22.8
4.0	0.010	72.6	45.3	52.4	53.2	23.1
5.0	0.0083	72.6	45.2	54.2	53.8	23.7
5.5	0.0076	nd	nd	nd	nd	24.0

[e] Lactate oxidised was expressed as a percentage of the total lactate utilised. Lactate oxidised was estimated from the sulphate reduction data, based on the theoretical ratio of the incomplete lactate oxidation reaction (Reaction 5.1, Table 5.1) i.e. moles of lactate utilised per unit mole of sulphate reduced = 2.
nd: not determined.

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At a 15.0 g l^{-1} sulphate feed concentration, lactate oxidation and sulphate reduction were minimal even at these low dilution rates (Figures 5.1a and 5.1b, Table 5.3). This implies significant substrate inhibition. As the feed sulphate concentration was increased in the range of 2.5 to 15.0 g l^{-1}, excess lactate (lactate metabolised via other reactions apart from sulphate reduction) of approximately 2 to 8 fold, was channelled into providing electrons for other activities such as lactate fermentation or cell maintenance (Table 5.2b). At a 1.0 g l^{-1} sulphate feed concentration, lactate was primarily utilised for sulphate reduction (Table 5.3), while sulphide levels were consistently maintained at 0.123 ± 0.039 g l^{-1} over the range of dilution rates studied (Table 4.2, Section 4.31.1).

It is also noteworthy that the experimental L:S ratio observed at a feed sulphate concentration of 10.0 g l^{-1} did not vary from the theoretical ratio as much as the experimental L:S ratios obtained at 2.5, 5.0 and 15.0 g l^{-1}. As shown in Table 5.3, more lactate was incompletely oxidised at a sulphate concentration of 10.0 g l^{-1} in comparison with 2.5, 5.0 and 15.0 g l^{-1}. This indicates that sulphate reduction and lactate oxidation were also significant at a feed concentration of 10.0 g l^{-1}. This was attributed to the high dissolved sulphide concentrations (0.3 to 0.6 g l^{-1}) observed in the reactor fed with 10.0 g l^{-1} media, which was maintained over the range of dilution rates studied (Sections 4.3.1.4 and 4.3.2.1). The high sulphide concentration observed was postulated to have repressed the metabolic activity of the lactate fermenters, consequently allowing out-competition by the lactate oxidisers.

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Hydrogen sulphide has been reported to be inhibitory to many fermentative bacterial activities (Manilal *et al*., 2000). Recently, an investigation by Moosa *et al*. (2006) of acetate-fed continuous SRB culture maintained at pH 7.8 demonstrated that there was a maximum sulphide concentration (1.5 g l^{-1} total soluble sulphide concentration) beyond which reduction in sulphate conversion occurred. A chemostat study of *Desulfovibrio desulfuricans* by Okabe *et al*. (1995) gave evidence to show that total dissolved sulphide concentrations in the range 0.1 to 0.44 g l^{-1} enhanced the specific lactate oxidation rate. Beyond this range, the activity of the SRB strain was inhibited. These results show that lactate fermenters are more sensitive to sulphide relative to the sulphate reducers.

There was a pronounced decline in dissolved sulphide concentration with increasing dilution rates, for chemostats with feed concentrations of 2.5 and 5.0 g l^{-1} sulphate. As the dilution rates increased in the range 0.021 to 0.042 h^{-1} (residence time: 2.0 to 1.0 d), low dissolved sulphide concentrations (0.0138 to 0.088 g l^{-1}) (Figure 5.1e) were observed in the reactors fed 2.5 and 5.0 g l^{-1} sulphate. The relationship between lactate oxidation and concomitant sulphate reduction, as signified by L:S ratio, on residual sulphate and dissolved sulphide concentrations are shown in Figures 5.1e and 5.1f. For the feed sulphate concentrations in the range 1.0 to 10.0 g l^{-1}, the regions where the observed stoichiometric ratios are comparable with the theoretical values are consistent with the regions where low residual sulphate and high dissolved sulphide concentrations were observed. Further, increased propionate production at high dilution rates, as indicated by the lower ^{e}L:P ratio values observed, suggests the predominance of lactate fermentation at high dilution rates.

It thus follows that as the lactate metabolic pathway shifted towards fermentation with increasing dilution rate, as the feed sulphate concentration was increased in the range 1.0 to 10.0 g l^{-1}, the availability of lactate for sulphate removal declined. This must have resulted in the increasing residual sulphate and decreasing sulphide concentrations.

High sulphide concentrations (0.2 to 0.4 g l^{-1}) were observed in the reactor fed with 15.0 g l^{-1} sulphate across the dilution rates studied. On the contrary, the observed stoichiometric ratios were consistently higher than the theoretical values for incomplete lactate oxidation (Figures 5.1a to 5.1e, Table 5.2). Results presented in Chapter 4 showed inhibition of the microbial activity (Section 4.3.2). These two facts suggest the possibility of additional energy being required by the cells to combat the inhibitory pressure, hence the higher than expected lactate utilisation. The high sulphide concentrations observed are direct consequence of the amount of sulphate removed (0.98 to 2.0 g l^{-1}), despite the low sulphate conversion observed.

The results of the present study at feed sulphate concentration of 1.0 g l^{-1}, are consistent with previous observations by Habicht *et al*. (2005) where sulphate was supplied in limited amounts to a chemostat culture of *Achaeoglobus fulgidus* strain Z.

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It was shown that the stoichiometry of lactate utilisation was not affected by feed sulphate concentrations in the range 0.06 to 1.4 g l^{-1}.

5.3.2. Lactate Oxidation and Bioreactor Performance

Bioreactor performance was assessed by volumetric sulphate reduction rate. Incomplete lactate oxidation to acetate which occurs simultaneously with sulphate conversion to sulphide, was higher at the initial sulphate concentration of 1.0 g l^{-1} (i.e. lower L:A ratio) relative to the higher feed sulphate concentration range (2.5 to 15.0 g l^{-1}) (Tables 5.2 and 5.3). The utilisation of lactate per unit acetate produced increased by a factor of approximately 2 at higher range of feed sulphate concentrations relative to that at a feed sulphate concentration of 1.0 g l^{-1} (Table 5.2b). As the dilution rate increased, there was a concomitant increase in the L:A and L:S ratios across all the feed sulphate concentrations (Figures 5.1a and 5.1b). This was evidence of a decline in total acetate production and a possible shift towards acetate production via lactate fermentation (Reaction 5.3, Table 5.1) as the volumetric loading rate of sulphate increased (Figure 5.1a). A decrease in acetate production by incomplete oxidation (L:A >1) as the feed sulphate concentration increased in the range 2.5 to 10.0 gl^{-1}, could also suggest the presence of complete oxidisers (Widdel, 1988). Under these conditions a higher production of bicarbonate alkalinity is expected, as illustrated in Reaction 5.2 (Table 5.1) representing complete lactate oxidation. On the contrary, lower concentrations of bicarbonate were measured, as indicated by the higher L:HC ratio values (Table 5.2b). Hence, the occurrence of complete lactate oxidation may not explain the decreasing trend in acetate production with increasing sulphate concentration in the current work. Alternatively, if complete lactate oxidation occurred (Reaction 5.2), or the acetate produced from incomplete lactate oxidation was further oxidised (Reaction 2.24), the excess bicarbonate molecules could have been utilised in the consumption of hydrogen produced via other reactions to buffer the pH of the SRB.

As shown in Table 5.4, an increase in feed sulphate concentration across the range 1.0 to 10.0 g l^{-1} positively influenced the volumetric sulphate reduction rate, at low dilution rate (0.0083 h^{-1}), where lactate oxidation was predominant in all the experiments (Table 5.3). This was accompanied by an increase in specific sulphate

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reduction rate (Table 5.4) to a maximum observed of 0.53 to 0.55 g l^{-1} g_{DW}^{-1} at feed sulphate concentrations of 5.0 and 10.0 gl^{-1}.

Table 5.4: Effect of feed sulphate concentration on the bioreactor performance (Values at residence time: 5.0 d; dilution rate= 0.0083).

Feed Sulphate concentration (g l^{-1})	Bacterial dry mass (g_{DW} l^{-1})	Volumetric sulphate reduction rate (g l^{-1} h^{-1})	Specific sulphate reduction rate (g h^{-1} g_{DW}^{-1})
1.0	0.267	0.0072	0.0271
2.5	0.350	0.011	0.0322
5.0	0.458	0.024	0.0529
10.0	0.588	0.032	0.0550
15.0	0.378	0.017	0.0443

A similar phenomenon was observed in an investigation by Moosa *et al*. (2002). As in the current study, both the specific and the volumetric sulphate reduction rates of acetate-fed chemostat, inoculated with a mixed SRB consortium, increased with increasing sulphate concentration in the range of 1.0 to 10.0 g l^{-1}. The increasing volumetric sulphate reduction rate was attributed to an increase in biomass concentration over this range of sulphate concentrations 1.0 to 10 g l^{-1}. The maximum specific sulphate reduction rate observed at feed sulphate concentrations of 5.0 and 10.0 gl^{-1} was associated with a maximum metabolic rate of the cells, despite increasing supply of sulphate for reduction.

5.3.3. Lactate Fermentation

At all sulphate concentrations studied, it was evident that lactate was utilised in excess of that required for oxidation. Propionate production from lactate fermentation (Reaction 5.3, Table 5.1) is expected to occur under sulphate deficient conditions and when SRB are co-cultured with methane producing archaea (MPA) (Bryant *et al*., 1977; Menert *et al*., 2004; Heimann *et al*., 2005). Propionate production was noticeable in all experiments (Figure 5.1d, Table 5.2b), indicating some level of fermentation, even though growth of MPA was inhibited at the culture enrichment stage (Section 3.1). Residual sulphate concentrations were also observed in all reactors, indicating a sulphate sufficient condition.

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Further, at feed sulphate concentrations of 5.0 to 15.0 g l^{-1}, residual lactate was present throughout the operating conditions, indicating that the SRB were not carbon-source limited in these cases. Data presented in Sections 4.3.1 and 4.3.2 show that the decline in the volumetric sulphate reduction rate with increasing volumetric sulphate loading rate beyond an optimal value at each feed sulphate concentration was not due to a decreased biomass concentration.

The experimental ^{e}L:P ratio, providing a measure of lactate utilised through pathways other than lactate oxidation, was lower at a feed sulphate concentration of 1.0 g l^{-1} than at higher feed sulphate concentrations (2.5 to 15.0 g l^{-1}) (Table 5.2b). This can be attributed to a reduced amount of lactate (27% equivalent to 0.6 g l^{-1}) available for metabolism via other routes at this feed concentration at dilution rates of 0.0083 to 0.042 h^{-1} (Table 5.3). At higher feed sulphate concentrations (2.5 to 15.0 g l^{-1}), 45 to 95% of the lactate consumed (3.0 to 26 gl^{-1}) could not be attributed to sulphate reduction (Table 5.3). As shown in Figure 5.1d, there was an increased production of propionate (lower L:P ratio) as the dilution rate increased. This is in agreement with the decline in acetate production observed with increasing dilution rate (Figure 5.1a).

Negligible residual lactate concentrations in the range of 0.009 to 0.029 g l^{-1} were measured in reactors with feed sulphate concentrations of 1.0 g l^{-1}, while higher lactate concentrations (0.09 to 25 g l^{-1}) were observed in the reactors receiving feed sulphate concentrations of 5.0 to 15.0 g l^{-1}. Further, the residual lactate concentrations steadily increased with increasing dilution rate (Figures 4.2b to 4.6b, Section 4.3.1) across the range of feed sulphate concentrations studied. It thus follows that lactate fermenters were favoured in the presence of high lactate concentration. Similarly, in an investigation by Purdy *et al*. (1997), it was reported that in high sulphate sediment slurries (3.0 g l^{-1} sulphate concentration) fed with lactate (20 mM, 1.78 g l^{-1}), sulphate reduction was accompanied by the production of acetate and propionate from lactate degradation. This indicated the utilisation of lactate via both the fermentative and the oxidative pathways. This was supported by the results of Laanbroek and Pfennig (1981). The addition of lactate (20 mM) and 1.92 g l^{-1} sulphate (10 mM) to marine sediment samples was shown to encourage the proliferation of lactate fermenters at the expense of SRB growth.

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5.3.4. Overall Lactate Metabolism

At higher feed sulphate concentrations (2.5 to 10.0 g l^{-1}), there were considerable changes in the reaction stoichiometry with varying dilution rates, as shown in the trends represented in Figures 5.1a to 5.1f. This suggests shifting metabolic pathways (Habicht *et al*., 2005). Furthermore, it was possible that microbial population shift, in which a particular group predominate on the basis of their kinetic properties, as the dilution rate increased, was responsible for the marked changes in metabolism at these concentrations. Mixed consortia of sulphate reducers are known to exhibit varying distributions with changes in volumetric sulphate loading. This in turn leads to changes in continuous bioreactor system performance (Icgen and Harrison, 2006b).

As shown in Figure 5.2, similar volumetric sulphate reduction rate profiles, as a function of the volumetric lactate utilisation rate, were observed at feed sulphate concentrations of 1.0 and 10.0 g l^{-1}. These trends concur with the theoretical profile representing the assumed condition whereby lactate was utilised via incomplete oxidation only (Reaction 5.1, Table 5.1). There was a steady increase in the volumetric sulphate reduction rates (VSRR) with increasing volumetric lactate utilisation rate (VLUR) throughout the operation periods in both reactors. These trends are attributable to the channelling of a major fraction of lactate utilised to sulphate reduction in these experiments (Table 5.3). A more pronounced increase was demonstrated in the 1.0 g l^{-1} sulphate-fed reactor as the dilution rates varied between 0.0083 and 0.042 h^{-1} (residence time: 5.0 to 1.0 d), relative to the 10.0 g l^{-1} sulphate-fed reactor. This was as a result of the higher oxidation of lactate at feed sulphate concentration of 1.0 g l^{-1} (72 ± 0.1%) relative to that observed at feed sulphate concentration of 10.0 g l^{-1} (44 ± 9%) (Table 5.3).

Even though a similar trend to what was observed in the reactors receiving feed sulphate concentrations of 1.0 and 10.0 gl^{-1} was exhibited by the reactor receiving feed sulphate concentration of 15.0 gl^{-1}, lower rates (VSRR and VLUR) were observed in this experiment owing to the inhibition of bacterial growth and substrate toxicity.

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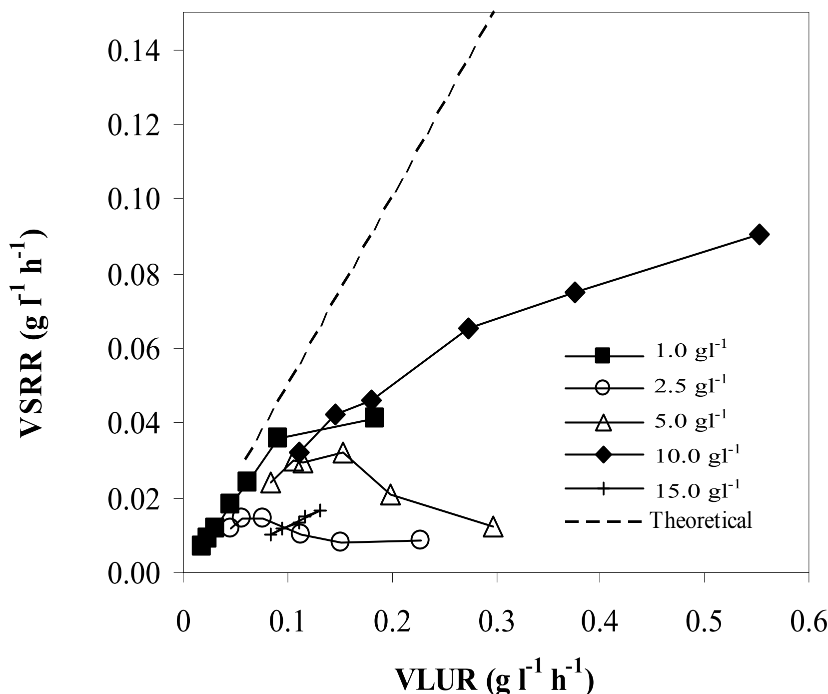

Figure 5.2: The relationship of sulphate reduction to lactate utilisation rate. Theoretical VSRR was calculated using the theoretical stoichiometric ratio, assuming lactate was utilised via the incomplete oxidation pathway only (Reaction 5.1, Tables 5.1 and 5.2a).

For feed sulphate concentrations of 2.5 and 5.0 gl^{-1}, a linear relationship between sulphate reduction rate and lactate utilisation rate, demonstrating a similar utilisation pattern to the1.0 and 10.0 gl^{-1} feed sulphate, was exhibited at lower dilution rates in the range 0.0083 to 0.014 h^{-1} (residence time: 5.0 to 3.0 d). Further increase of dilution rate beyond this range shows a decreasing trend despite the increasing lactate utilisation rate for feed concentrations 2.5 and 5.0 g l^{-1}. Corresponding low sulphide and high residual sulphate concentrations were recorded across the range of higher dilution rates (0.021 to 0.042 h^{-1}) for these feed concentrations (Figure 5.1e). These changes in the dependency profiles of VSRR of VLUR further substantiate significant shifts in lactate metabolism in these two reactors.

The volumetric lactate utilisation rate increased with increasing feed sulphate concentration in range 1.0 to 10.0 g l^{-1} and increase in the dilution rate (0.0083 to 0.083 h^{-1}) despite lower lactate oxidation observed at higher feed sulphate concentrations (Figures 5.3a and 5.3b). Under the same conditions, the sulphate reduction rate showed different responses to both the dilution rate and feed sulphate concentration.

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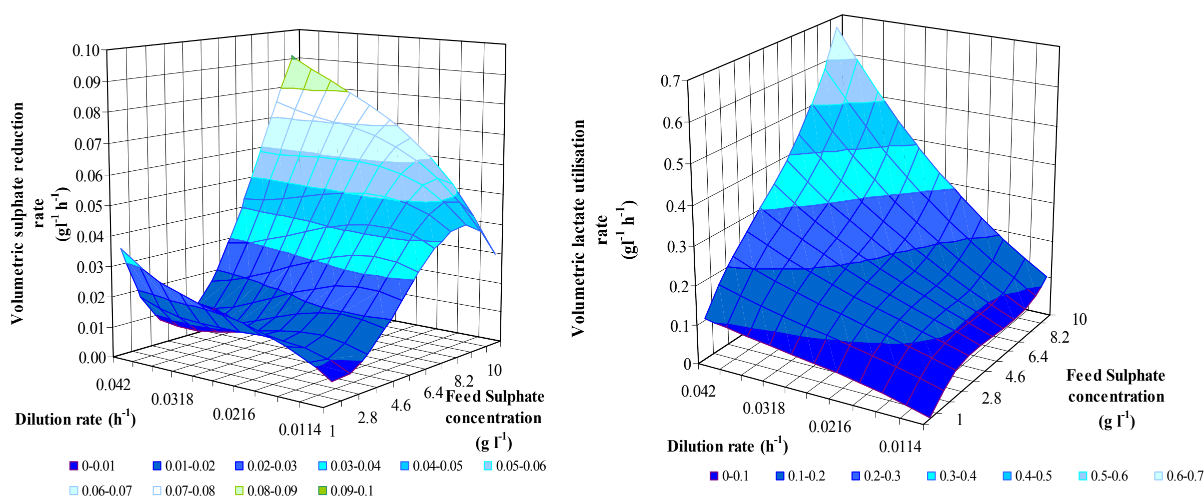

Figure 5.3: Effect of feed sulphate concentration and dilution rate on biological sulphate reduction kinetics. (a) Dependency of volumetric sulphate reduction rate; (b) Dependency of volumetric total lactate utilisation rate.

The response of the volumetric lactate utilisation rate observed in this study is in agreement with an earlier report by Baskaran and Nemati (2006) who investigated biological sulphate reduction in immobilised-cell systems. Their study showed that lactate utilisation was positively influenced by the volumetric sulphate loading rate until an optimum value was reached. Similarly, results obtained from batch studies by other authors (Purdy *et al*., 1997; Qatibi *et al*., 1990) revealed that lactate was rapidly and optimally metabolised via both fermentation and oxidation within 48 hours.

5.4. SUMMARY

It was evident from the results obtained in this study that the stoichiometry of biological sulphate reduction and lactate utilisation, and their kinetics, are influenced by volumetric loading rate as mediated through both the feed sulphate concentration and dilution rate. Incomplete lactate oxidation was favoured at feed sulphate concentrations of 1.0 and 10.0 g l^{-1}, and at low dilution rates (0.0083 to 0.014 h^{-1}). The stoichiometry of lactate utilisation was consistent with the theoretical expectations in this range. Further, at 10.0 g l^{-1}, high sulphide concentrations repressed the activity of lactate fermenters, which led to the predominance of the incomplete lactate oxidative pathway. These speculations were exploited and reported in Chapter 7.

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Data obtained in the current study show that the stoichiometry of biological sulphate reduction is dominantly coupled to incomplete lactate oxidation at low sulphate concentration (1.0 g l^{-1}). The observed stoichiometric ratios of L:S, L:A and L:HC were consistent with the theoretical values at this feed sulphate concentration. At higher feed sulphate concentrations (2.5 to 15.0 g l^{-1}), deviation from the theoretical stoichiometric ratios for biological sulphate reduction reactions increased significantly with increasing volumetric loading rate of sulphate.

At feed sulphate concentrations of 2.5, 5.0 and 15.0 g l^{-1}, lactate fermentation was predominant as evidenced by the increased production of propionate. This was congruent with the higher values of the L:S, L:A and L:HC. These ratios deviated significantly from the theoretical values of incomplete lactate oxidation and tended towards those of the lactate fermentation reaction, with increasing dilution rate. The volumetric lactate utilisation rate increased with increasing dilution rate and feed sulphate concentrations across the feed sulphate concentrations studied (1.0 to 15.0 g l^{-1}). It was noted that these increasing feed sulphate concentrations were associated with increasing feed lactate concentration owing to stoichiometry consistency.

Results of this study can also be supported by deductions from previous investigations. Haack and co-workers (1995) and Garland (1996) noted that differing substrate utilisation and production profiles were closely linked to microbial diversity in a mixed microbial culture. These previous studies also suggest that substrate utilisation patterns provide a rapid assessment of the structure and dynamics of microbial communities in comparison with molecular tools. Molecular studies for microbial community characterisation were undertaken to validate observations presented herein and investigate the postulations developed. Findings from this study suggest that the influence of sulphate concentration, dissolved sulphide and the dilution rate, on the stoichiometry of biological sulphate reduction and the marked shift in lactate metabolism at higher lactate concentrations could have resulted from changes in the microbial community structure. These are investigated explicitly in Chapter 6.

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CHAPTER 6

MICROBIAL COMMUNITY DYNAMICS UNDER BIOSULPHIDOGENIC CONDITIONS

6.1. INTRODUCTION

As discussed in Chapters 4 and 5, the changes reflected in the kinetics and stoichiometry of biological sulphate reduction with dilution rate and the feed sulphate concentration were possible consequences of shifts in microbial community structure or microbial metabolism or both. Previous chemostat studies have shown that the volumetric sulphate loading rate, influences both the dynamics of mixed consortia of sulphate reducers and the kinetics of biological sulphate reduction (Icgen and Harrison, 2006b; Oyekola *et al*., 2007b). Similarly, Icgen and Harrison, (2006a) showed that increasing sulphide loading influences SRB community structure.

A recent study by Zhao *et al*. (2007), using a CSTR, demonstrated a close link between variation in alkalinity and shifts in a mixed culture of SRB. These changes were reflected in the bioreactor performance. These aforementioned studies have established the fact that there is a link between the response of microbial cultures to changes of a physicochemical nature, the resulting community structure dynamics and the kinetics of the reaction (BSR).

The main objective of this Chapter was to confirm the postulation resulting from Chapters 4 and 5 that changes in kinetics and stoichiometry of biological sulphate reduction are consequences of microbial community dynamics. The link between community dynamics, diversity and bioreactor performance was investigated.

6.2. EXPERIMENTAL APPROACH

6.2.1. Examining the Effect of Feed Sulphate Concentration on the Microbial Community structure in Biological Sulphate Reduction

In order to understand the microbial community dynamics as influenced by sulphate concentration and dilution rate, steady-state samples from the experiments described in Sections 4.2.1 were analysed. The characterisation of the microbial consortia was carried out using FISH (fluorescence *in situ* hybridisation) and restriction enzyme

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digestion analyses. The restriction endonuclease fingerprints and FISH were used qualitatively and quantitatively, respectively, to characterise the microbial consortia. The restriction enzyme digestion analysis was used to identify microorganisms at the species level while the FISH technique was used to identify major microbial groups, viz: Archaea and Gram-negative mesophilic SRB.

Total DNA was extracted using a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. A portion of the 16S rRNA gene, of approximately 1 kb was amplified as described in Section 3.6.3. Microbial characterisation was carried out using restriction enzyme digestion analyses of the PCR products. A total of 16 SRB species, three lactate fermenters and four methanogenic species were targeted in this study. These are putatively typical anaerobic lactate utilisers and degraders of lactate metabolic products as previously described in the literature. The complete 16S rRNA gene sequences for these target organisms were obtained from the GenBank database. As shown in *Appendix D*, the restriction enzymes could not provide comprehensive discriminatory patterns for the lactate fermenters and the methane producing archaea that were targeted because they were primarily selected to identify the SRB strains.

The list of target microorganisms is given in Table 6.1. This restricted selection was justified based on the usage of sulphate and lactate. Restriction band patterns obtained by digesting PCR amplified products of bacterial 16S rRNA genes with a suite of restriction enzymes were used to monitor the changes in SRB microbial diversity across the concentrations and residence times studied. Using the discriminatory patterns obtained from these analyses, microbial groups that were present and absent under each operating condition were identified concurrently. Though qualitative, this method maps changes within a microbial community. The detailed experimental approach is provided in Section 3.6.4. The theoretical banding patterns are given in *Appendix D*.

For the FISH analysis, oligonucleotide probes (15 to 20 bases) used were domain- and group-specific. Microbial samples were fixed using paraformaldehyde solution and hybridised with the probes selected. The hybridised samples were observed by fluorescent microscopy. In the current study, the FISH probes used and their

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specificities were as follows: ARCH915 (domain Archaea) and SRB385 (Gram-negative mesophilic SRB). Details of these probes are provided in Table 3.1. The microscopic data of positive signals obtained from the hybridised samples were compared with the results from DAPI staining. While data from the FISH analysis represent the target microbial groups, DAPI staining targets all microorganisms present in the consortia. The cells stained with DAPI but not hybridised by the general SRB probe (SRB385) are representative of the non-SRB cells or other SRB that were not targeted by the probe. The detailed experimental approach is provided in Section 3.6.5

6.2.2. Examination of Bacterial Morphology

In order to examine the morphology of the microorganisms present in the mixed consortia utilised in this study, scanning electron microscopy (SEM) and Gram staining were employed. Samples obtained from the experiments carried out in Section 4.2.1, investigating the effect of feed sulphate on the BSR kinetics, were fixed and viewed by SEM as described in Section 3.4. The detailed procedure of Gram staining is provided in Section 3.5.

6.3. DEFINITIONS

To focus the discussion of this chapter, some of the terms used are highlighted in this section as previously described in the literature. These definitions are adhered to throughout this thesis.

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Microbial Community Dynamics: This refers to the change in relative dominance of community members in response to physicochemical changes. This phenomenon describes the ability of a microbial system to react and adapt to environmental perturbations (Miura *et al*., 2007).

SRB Microbial diversity: Microbial diversity is a measure of the abundance and the uniformity of distribution of different microbial members in a microbial consortium (Øvreås and Torsvik, 1988; Dunbar *et al*., 2000; Saikaly *et al*., 2005). In the current study, diversity was estimated as the number of different SRB species identified in the mixed culture according to the protocols used.

Community structure: This refers to the numerical composition different species in a mixed communnity. This influences a system's net stability (Miura *et al*., 2007).

Community Stability: This is defined as resistance to perturbation or physicochemical changes (McGrady-Steed *et al*., 1997; Naeem and Li, 1997; Yachi and Loreau, 1999).

Community Resilience: This is the ability of a heterogeneous community to recover from perturbation (McGrady-Steed *et al*., 1997; Naeem and Li, 1997; Yachi and Loreau, 1999).

Reactor or Treatment Technology Performance: This has been described by many authors as the rate of substrate removal or degradation in a bioremediation system (Ayala-Del-Río *et al*., 2004; Baskaran and Nemati, 2006). Herein, reactor performance was measured as both the fractional sulphate conversion and the volumetric sulphate reduction rate.

6.4. RESULTS AND DISCUSSION

6.4.1. Microbial Characterisation

6.4.1.1. Restriction enzyme digestion analyses

To establish the reliability of the experimental protocols, the continuous experiment at a feed sulphate concentration 1.0 g l^{-1} was repeated twice under identical conditions. A stable replicated community structure was obtained. This indicated that the threshold conditions for the restriction enzyme digestion analyses had a good confidence and provides a baseline for comparison using other conditions. Using the restriction enzyme digestion analyses, a total of five SRB species out of the 16 targeted species detailed in Table 6.1 were identified in this study. These were under feed conditions of 1.0 g l^{-1} sulphate and 2.25 g l^{-1} lactate at a dilution rate of 5 d. The SRB species are as follows: *Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* and *Desulfococcus multivorans* (Tables 6.1, 6.2 and 6.3).

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Table 6.1: Microbial characterisation results using restriction enzyme digestion analyses of 16S rRNA PCR products. List of organisms targeted. Organisms identified in the current study are indicated by the symbol "√".

Target organisms	Identification	Target organisms	Identification
SRB		**Lactate fermenters**	
Desulfovibrio gigas	√	*Clostridium homopropionicum*	
Desulfococcus multivorans	√	*Veillonella parvula*	
Desulfomonas pigra		*Pelobacter propionicus*	
Desulfonema limicola		**Methanogens**	
Desulfobacter postgatei	√	*Methanosarcina barkeri*	
Desulforhabdus amnigenus		*Methanosarcina lacustris*	
Desulfosarcina variabilis	√	*Methanosarcina acetivorans*	
Desulfotomaculum nigrificans		*Methanogenium cariaci*	
Desulfotomaculum orientis			
Desulfotomaculum ruminis			
Desulfovibriogigas	√		
Desulfovibrio africanus			
Desulfovibrio vulgaris			
Desulfovibrio sapovorans			
Desulfovibrio salexigens			
Desulfovibrio desulfuricans			
Desulfobulbus propionicus	√		

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Figure 6.1 shows the result of a multiple digestion (21 enzymes) performed on the PCR product of 16S rRNA gene of a steady-state sample obtained from the reactor receiving 1.0 g l^{-1} sulphate operated at the residence time of 5 d. Table 6.2 shows the theoretical band sizes of the fragments obtained for the microorganisms identified in this study using the DNAMAN software for Windows program, version 4.13 (1994-99). This result demonstrates the high discriminatory power of the method employed in the characterisation of the microbial community. Hence the data presented herein represent the critical sulphate reducers present in these experiments, as the target microorganisms employed in this study are the common ones described in literature to

grow under the operating conditions employed in this study (Postgate, 1984; O'Flaherty *et al*., 1998; Widdel, 1988).

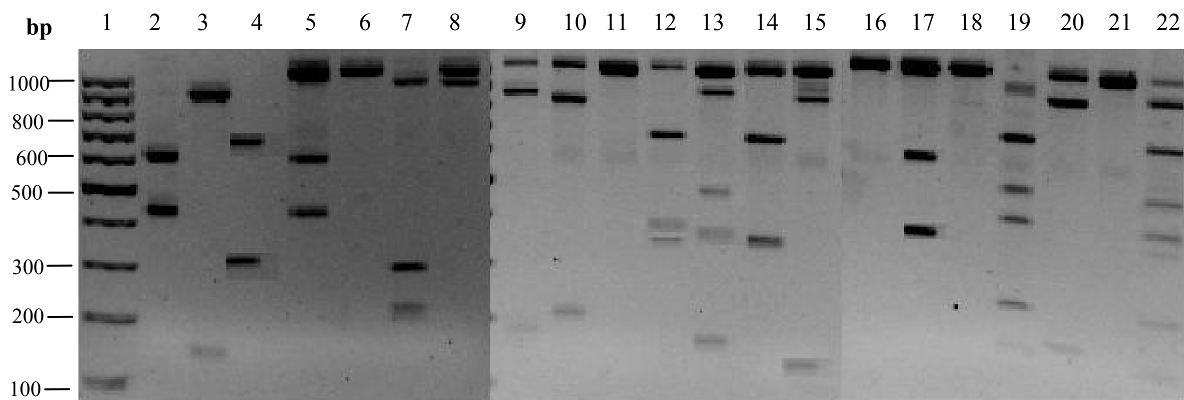

Figure 6.1: Restriction enzyme digestion analyses of PCR product from reactor fed with 1.0 gl^{-1} sulphate at residence time of 5 d, using 21 endonucleases. Lane 1: Molecular size marker. Lanes 2 to 22 are the following consecutive endonucleases, *Apa*I, *Asp*700I, *BbrP* I, *Dra*I, *Bfr*, *BpuA*I, *Bse*AI, *Eco*RI, *Eco*RV, *Ec*lXI, *Hpa*I, *Ksp*I, *Mlu*I, *Nco*I, *Pst*I, *Pvu*I, *SgrA*I, *Sma*I, *Sph*I, *Xba*I and *Xma*CI.

The distribution of these organisms as influenced by the feed sulphate concentration and the dilution rate is shown in Table 6.3. Unknown species, indicated by some undigested bands and digested bands for non-targeted microorganisms (Figure 6.1) in these analyses, would require sequencing for identification. Morphological examination of the lactate fermentation experiment (Section 7.2.1) showed the occurrence of other microorganisms not targeted in this study. Additionally, the FISH analysis (Section 6.4.1.3) revealed the absence of methanogens. It thus follows that the undigested bands most likely represent the lactate fermenters. Alternatively, they may represent previously unknown or untargetted SRB species. Characterisation of these unknown species was not investigated in the current study.

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Table 6.2: List of identified SRB and the theoretical banding patterns obtained from the DNAMAN software for Windows program, version 4.13 (1994-99).

THEORETICAL FRAGMENT SIZES (bp) OBTAINED WITH 21 RESTRICTION ENZYMES																					
ORGANISM	***Apa*I**	***Asp* 700I**	***BbrP*I**	***Dra*I**	***Bfr***	***BpuA*I**	***BseA*I**	***Eco*RI**	***Eco*RV**	***Ecl*XI**	***Hpa*I**	***Ksp*I**	***Mlu*I**	***Nco*I**	***Pst*I**	***Pvu*I**	***SgrA*I**	***Sma*I**	***Sph*I**	***Xba*I**	***Xma*CI**
Desulfobacter postgatei	595, 432	878, 149	292, 732	637, 390	-	-	-	-	820, 205	-	-	870, 155	-	912, 115	-	-	-	665, 220, 140	159, 866	-	-
Desulfococcus multivorans	595, 432	879, 148	292, 732	-	-	-	-	-	820, 205	-	364, 355, 306,	502, 369, 154	-	-	-	628, 397	-	886, 139	158, 867	-	-
Desulfosarcina variabilis	595, 432	880, 147	292, 733	-	-	-	-	-	820, 205	-	660, 365	872, 153	-	-	-	-	-	502, 385, 140	157, 868	-	-
Desulfovibrio gigas	-	-	-	-	-	-	-	-	820, 205	-	-	-	363, 662	-	-	-	-	498, 527		-	-
Desulfobulbus propionicus	595, 432	884, 143	-	-	-	-	-	-	820, 205	-		876, 149	-	-	-	-	-	891, 134	155, 872	-	-

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Table 6.3: Distribution of SRB strains across different operating conditions. Total number of strains identified under each operating condition in parentheses.

RT (d)	Dilution rate (h^{-1})	Microbes identified at different feed sulphate concentrations (S_o)				
		S_o (gl^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, (2)	nd	nd	nd	nd
1.0	0.042	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* (4)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, (2)	*Desulfobulbus propionicus*, *Desulfobacter postgatei* (2)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	nd
1.5	0.028	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* (4)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, (2)	*Desulfobulbus propionicus*, *Desulfobacter postgatei* (2)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	nd
2.0	0.021	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* (4)	*Desulfobulbus propionicus*, *Desulfobacter postgatei* (2)	*Desulfobulbus propionicus*, *Desulfobacter postgatei* (2)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	nd
3.0	0.014	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)

nd: not determined.

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Table 6.3 (contd): Distribution of SRB strains across different operating conditions. Total number of strains identified under each operating condition in parentheses.

RT (d)	Dilution rate (h^{-1})	Microbes identified at different feed sulphate concentrations (S_o)				
		S_o (gl^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
3.5	0.012	nd	nd	nd	nd	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)
4.0	0.010	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)
5.0	0.0083	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis*, *Desulfococcus multivorans* (5)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)
5.5	0.0076	nd	nd	nd	nd	*Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfococcus multivorans* (3)

nd: not determined.

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6.4.1.2. Morphology examination

The microbial characterisation results are corroborated by the morphology data. The SEM and Gram staining analyses using light microscopy (Figures 6.2a, 6.2b, 6.3a and 6.3b) revealed different cell morphotypes (short rod, coccoid, oval shape, ovoid and vibrio [curved rod]).

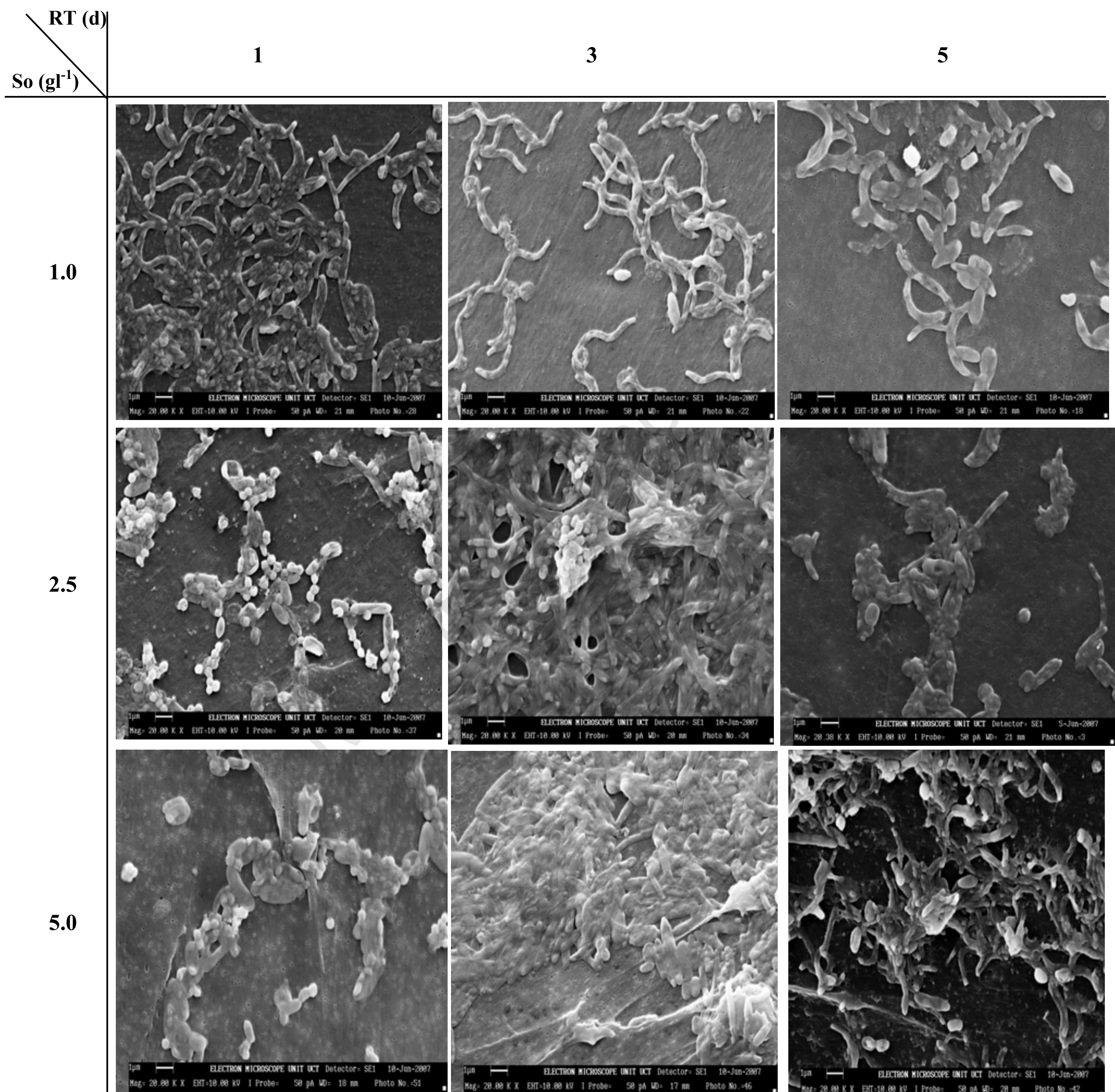

Figure 6.2a: SEM micrographs showing the microbial morphology under varying conditions of feed sulphate concentration (1.0 to 5.0 g l^{-1}) and dilution rate (0.0083 to 0.042 h^{-1}, residence time: 5 to 1 d). Magnification was 20 000 ×.

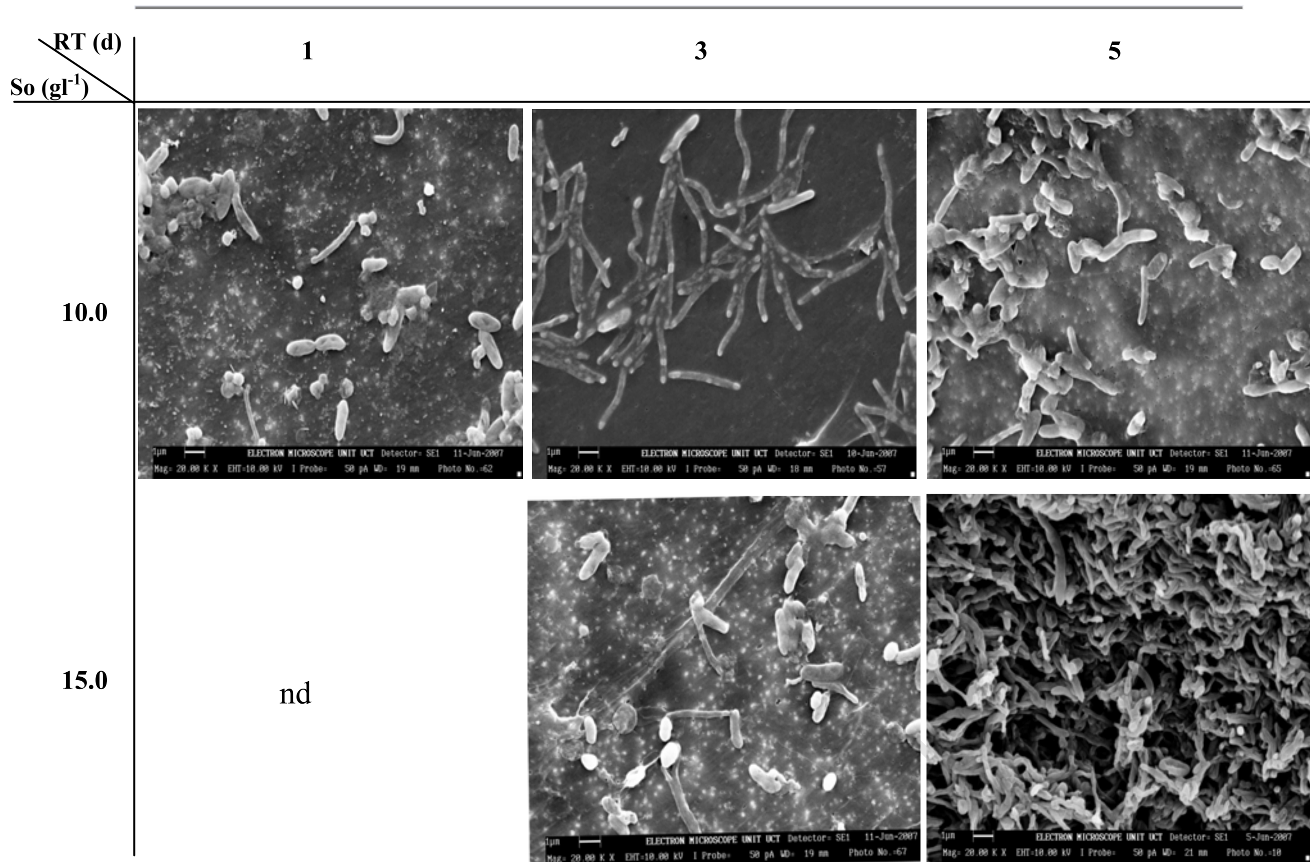

Figure 6.2b: SEM micrographs showing the microbial morphology under varying conditions of feed sulphate concentration (10.0 and 15.0 g l^{-1}) and dilution rate (0.0083 to 0.042 h^{-1}, residence time: 5 to 1 d). Magnification was 20 000 ×. nd= not determined.

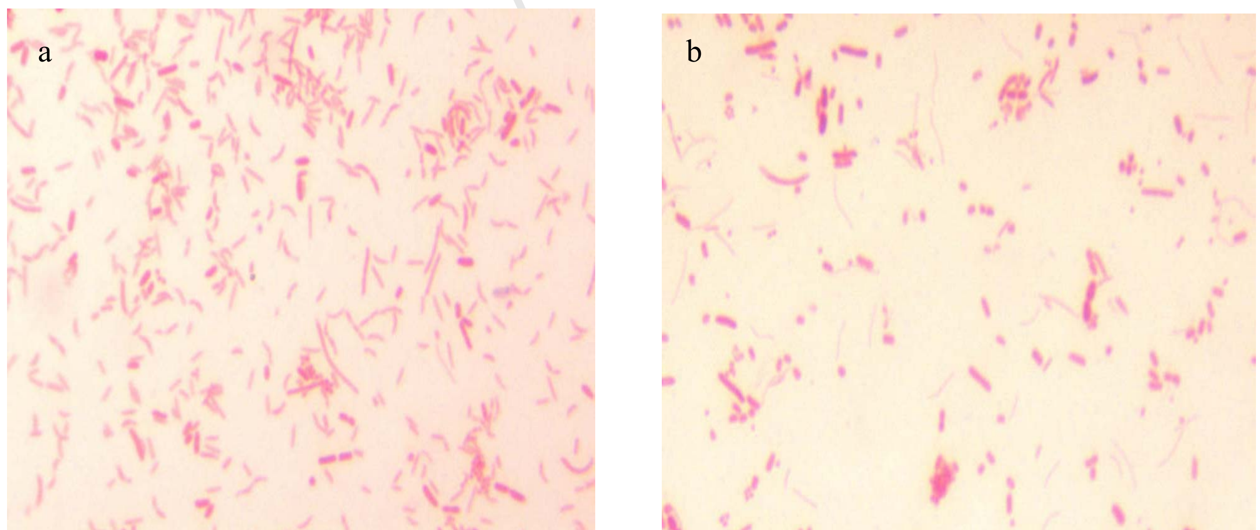

Figure 6.3a: Light microscope preparation of Gram stained samples showing the microbial morphology under varying feed sulphate concentration (a) 1.0 g l^{-1}; (b) 2.5 g l^{-1} and constant dilution rate (0.0083 h^{-1}, residence time: 5 d). All cells stained were Gram negative. Magnification was 1000 ×.

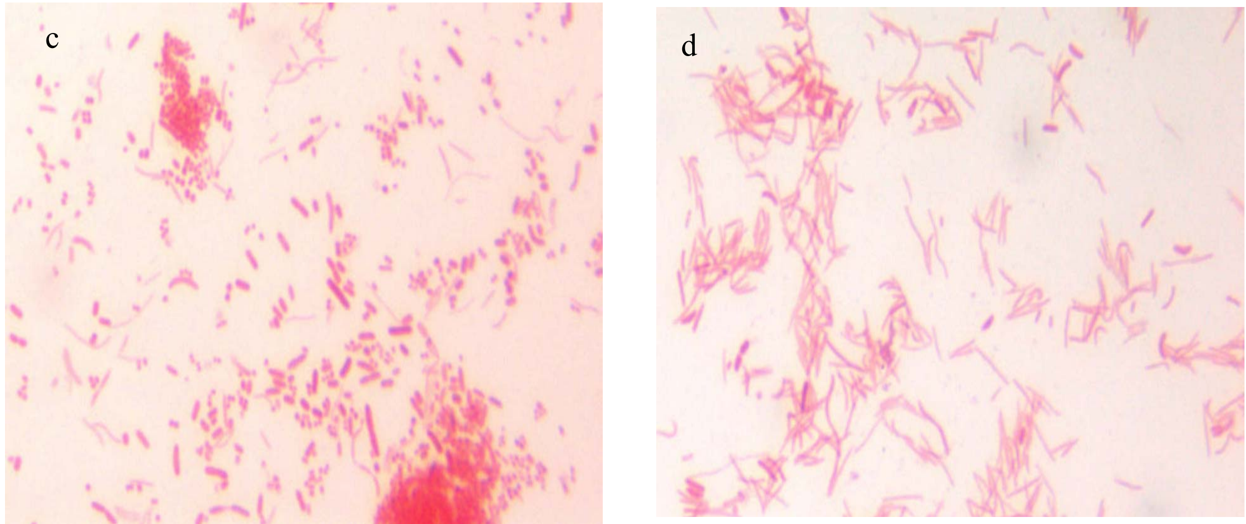

Figure 6.3b: Light microscope preparation of Gram stained samples showing the microbial morphology under varying feed sulphate concentration (c) 10.0 g l^{-1}; (d) 15.0 g l^{-1} and constant dilution rate (0.0083 h^{-1}, residence time: 5 d). All cells stained were Gram negative. Magnification was 1000 ×.

Table 6.4: Characteristics of SRB identified in this study (Laanbroek and Pfennig, 1981; Laanbroek *et al.*, 1983; Postgate, 1984; Schink, 1984; Widdel, 1988; Devereux *et al.*, 1989; O'Flaherty *et al.*, 1998; Seeliger *et al.*, 2007).

Organism	Morphology and size (length × width[µm])	Oxidation[a]	Electron donor[b] ($_{e-d}$)	Sulphide inhibition: IC50 at pH 8[c] (g l^{-1})
Desulfobulbus propionicus	ovoid (1.8-2)×(1-1.3)	i	lactate (+), propionate (+), ethanol (+), hydrogen (+)	0.5
Desulfobacter postgatei	short rod (1.7-2.5)×(1-1.5)	c	lactate (±), acetate (+), ethanol (±),	1.1
Desulfovibrio gigas	spirilloid/ large vibrio (6-11)×(0.8-1)	i	lactate (+), ethanol (±), hydrogen (+)	-
Desulfosarcina variabilis	oval rod, packages (1.5-2.5)×(1-1.5)	c	lactate (+) , acetate (±), ethanol (+), hydrogen (±)	-
Desulfococcus multivorans	coccoid na× (1.5-2.2)	c	lactate (+), acetate (±), ethanol (+)	1.5

a: i, incomplete; c, complete
b: +, utilised; ±, poorly utilised.
c: IC50 value = the total sulphide concentration giving 50% inhibition of the growth rate
na= not applicable

The SEM micrographs of the 1.0 g l^{-1} sulphate–fed reactor revealed the presence of all of these morphotypes, indicating the heterogeneity of the microbial community present in this experiment. These correspond to the morphological properties of the SRB strains identified in this study (Table 6.4). Rods were predominant across the

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experimental conditions investigated. At feed sulphate concentrations 2.5 to 15.0 g l^{-1} (Figures 6.2a and 6.2b) and residence time of 1 d, the predominant bacterial cells exhibited stubby rod and lemon shape (Figures 6.2a and 6.2b) which correspond well with the species identified under these conditions (*Desulfobacter postgatei* and *Desulfobulbus propionicus*) (Tables 6.3 and 6.4). Clumping, which could indicate bacterial stress (Brock and Madigan, 1991), was evident at high feed sulphate concentrations (2.5 to 15.0 g l^{-1}) (Figures 6.2a and 6.2b). Large vibrio, characteristic of *Desulfovibrio gigas*, were absent at the feed sulphate concentrations 10.0 and 15.0 g l^{-1} (Figures 6.3b) while very common at a feed sulphate concentration of 1.0 g l^{-1}. The Gram stained micrographs revealed the dominance of spherical cells (*Desulfococcus multivorans*) and short rods (*Desulfobacter postgatei*) in the 10.0 g l^{-1} sulphate-fed reactor. All the cells appear Gram negative (Figures 6.3a and 6.3b). This is characteristic of the SRB strains identified in the current study (Castro *et al*., 2000). The absence of Gram positive cells is indicative of the absence of members of the genera *Desulfotomaculum* and *Clostridium* (Castro *et al*., 2000; Sass and Cypionka, 2004). Further, decreasing variation of cell morphology with increasing feed sulphate concentration was evident. These observations corroborate the results from the restriction enzyme digestion analyses.

6.4.1.3. FISH analyses

Negative signals were detected with the ARCH915 probe in all the samples. This indicated the absence of the target methanogens. This observation can be attributed to the inhibition of this microbial group by BESA at the culture enrichment stage (Section 3.1). Comparison of the micrographs obtained from the DAPI stain and hybridisation with general probe for Gram negative SRB (SRB385), shown in Figure 6.4, revealed the presence of non-SRB bacteria. These non-SRB cells may be assumed to be responsible for the lactate fermentation observed in this study. FISH analyses of steady-state samples obtained from reactors receiving feed sulphate concentrations of 1.0 and 2.5 g l^{-1} are used as representative micrographs (Figure 6.4). The non-SRB cells were present in low proportion at a feed sulphate concentration of 1.0 g l^{-1}, under conditions of low and high dilution rates. Nonetheless, more of these cells were identified at the residence time of 0.5 d relative to the residence time of 5 d.

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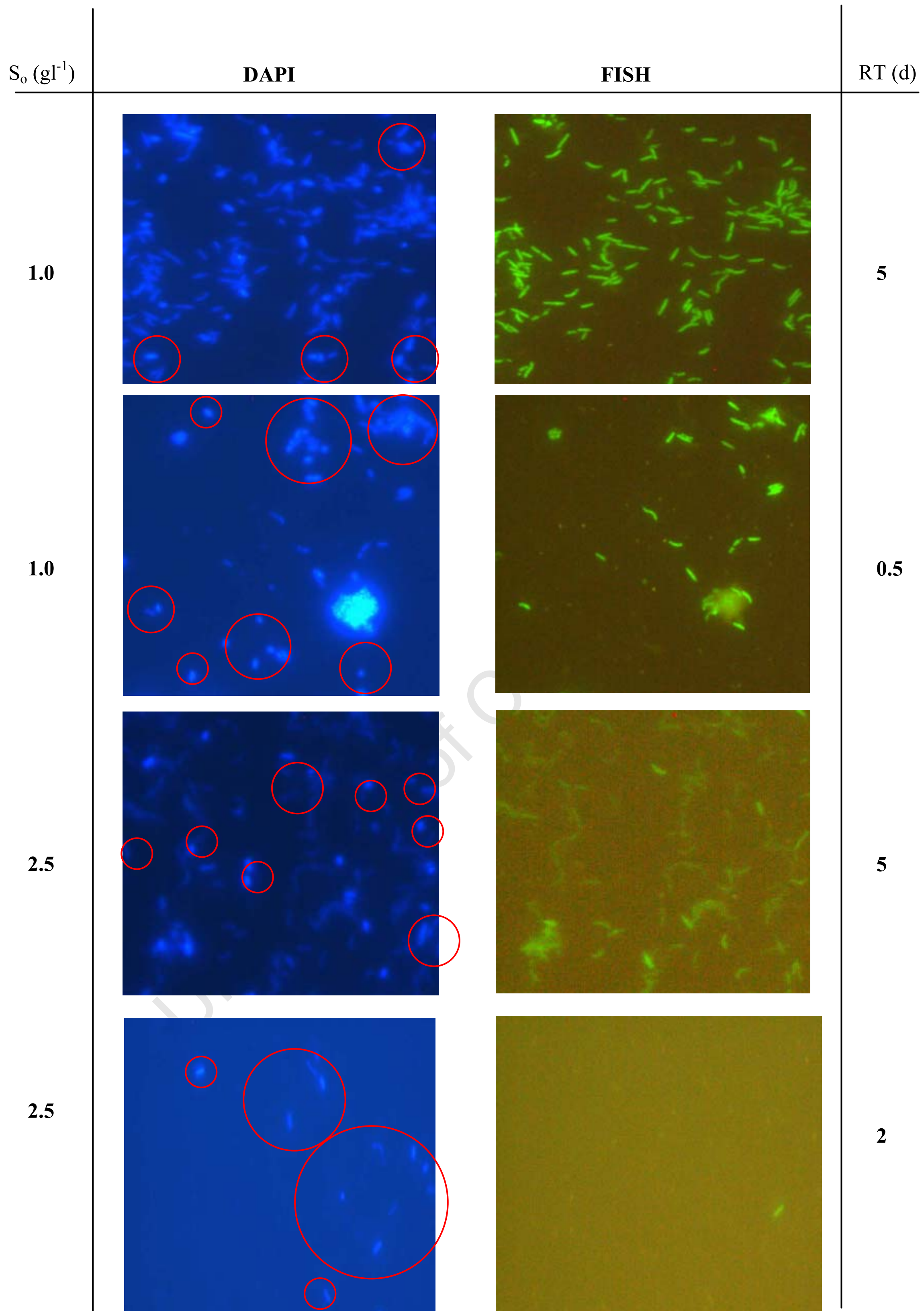

Figure 6.4: Epifluorescence micrographs of samples obtained from reactors receiving feed sulphate (S_o) concentrations of 1.0 (RT = 0.5 and 5 d) and 2.5 g l^{-1} (RT= 2 and 5 d). DAPI staining on the left column is compared with FISH performed with general SRB probe SRB385 on the right column (same microscopic field). Examples of non-SRB encircled in red.

On the other hand as the residence time was reduced from 5 to 2 d (increased dilution rate) in the reactor receiving a sulphate concentration of 2.5 g l^{-1}, a shift in the microbial population was evident with a decrease in SRB. As shown in Figure 6.4, more of the cells stained by DAPI were hybridised by the SRB probe under operating conditions of 2.5 g l^{-1} feed sulphate concentration and residence time of 5 d. As the dilution rate was increased at the low residence time of 2 d non-SRB cells became predominant.

The results obtained from this study are consistent with the operating conditions supporting the growth of a wide variety of SRB, both incomplete and complete oxidisers (Dvorak *et al*., 1992; Okabe and Characklis, 1992; Okabe *et al*., 1992; 1995; Kaksonen *et al*., 2003). This observation is supported by the results of stoichiometry presented in Section 5.3.2. There was evidence indicating the metabolism of lactate via the incomplete oxidation and the possible complete oxidation of the acetate produced. The utilisation of simple carbon-sources (ethanol and lactate) is known to support the growth of a diverse consortium of SRB (Kaksonen *et al*. 2004; Dar *et al*., 2007). A recent investigation by Kaksonen *et al*. (2004) demonstrated that an ethanol-fed sulphidogenic FBR, treating acidic metal-containing wastewater, supported the growth of a more diverse SRB community (23 operational taxonomic units [OTU]), in comparison with the lactate-fed reactors (15 OTU). Despite the difference in SRB microbial diversity, the performance of the two reactors was similar. This suggested that increased bacterial diversity is not necessarily associated with an improved biological treatment process.

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Characteristics of the strains identified in the current study and the possible reasons for their relative dominance are discussed in the following sections.

6.4.1.4. Characteristics of microorganisms identified In This Study

1. ***Desulfobacter postgatei***: This SRB species is prevalent in marine environments where sulphate is present in excess amounts (Ingvorsen *et al*., 1984; Purdy *et al*., 2003). Although reported to be acetotrophic (Widdel, 1988), growth of this species on lactate has been reported (Table 6.4) (Widdel and Pfennig, 1981). Its ability to utilise both lactate and acetate

which were both detected in all experiments could be responsible for the presence of this microbe under all experimental conditions.

2. ***Desulfobulbus propionicus***: This microbe has been identified in both marine and freshwater habitats (Laanbroek and Pfennig, 1981). It has been linked to H_2 consumption and lactate fermentation in the absence of sulphate as well as lactate oxidation and a concomitant sulphate reduction in its presence (Purdy *et al*., 2003; Heimann *et al*., 2005). *Desulfobulbus propionicus* is able to respire in oxic regions of natural environments by employing a wide variety of substrates including sulphide, nitrate, lactate and thiosulphate (Dannenberg *et al*., 1992; Teske *et al*., 1996; Santegoeds *et al*., 1998; Sass *et al*., 2002). Additionally, *Desulfobulbus propionicus* can oxidise propionate to acetate incompletely (Postgate, 1984). The nutritional versatility (Table 6.4) and physiological robustness of this strain can be associated with its presence in all operating conditions in this study (Table 6.3). This observation is in agreement with the report by Li *et al*. (1999) in which *Desulfobulbus propionicus* were the most dominant active SRB in a freshwater lake throughout the study period of 15 months, when compared to members of the *Desulfobacterium* and *Desulfovibrio* genera, despite seasonal changes.
3. ***Desulfovibrio gigas***: Members of the *Desulfovibrio* genus are the most widely studied sulphate reducers (Bryant *et al*., 1977; Okabe and Characklis, 1992; Zellner *et al*., 1994; Okabe *et al*., 1995; Nagpal *et al*., 2000). They have been shown to be good scavengers of both hydrogen and lactate in natural environments (Widdel, 1988). Growth of the members of the *Desulfovibrio* genus is favoured by high substrate concentrations and a limited variety of electron donors (Laanbroek *et al*., 1984; Ravenschlag *et al*., 2000) (Table 6.4).

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Bryant and co-workers (1977) showed that *Desulfovibrio* strains are able to ferment lactate and ethanol under sulphate-deficient conditions when co-cultured with hydrogen-consuming methanogens. Recent studies have reported the physiological characteristics and the ability of *Desulfovibrio gigas* to tolerate oxic conditions (Lemos *et al*., 2001; Fareleira *et al*., 2003).

4. ***Desulfosarcina variabilis*** and ***Desulfococcus multivorans***: Similar to *Desulfovibrio gigas* and *Desulfobulbus propionicus*, *Desulfococcus multivorans* are able to grow under highly oxic conditions (Minz *et al*., 1999). Ravenschlag and co-workers (2000), in a study aimed at characterising the community structure of marine sediments using *in situ* hybridisation analyses, showed that members of the *Desulfosarcina* and *Desulfococcus* genera are robust strains. These microorganisms are characterised by the ability to utilise several electron donors (lactate, acetate, ethanol, hydrogen, formate and benzoate) (Devereux *et al*., 1989). They also thrive in sulphate-deficient environments. These properties are responsible for their predominance under extreme conditions of substrate limitation. *Desulfosarcina variabilis* and *Desulfococcus multivorans* can utilise fatty acids with chain lengths $\leq$16 carbon atoms (Widdel, 1988). As noted by Musat *et al.* (2006), in a study aimed at characterising the microbial community of a marine environment, members of the *Desulfosarcina* and *Desulfococcus* groups were detected under conditions of low sulphate reduction rates. Their investigation (Musat *et al*., 2006) also revealed that the activities of these SRB population members remained stable despite challenging seasonal changes. These investigators also suggested that members of these genera could utilise Fe (III) and oxygen as electron acceptors.
5. **"Non-SRB lactate fermenters"**: The coexistence between non-SRB lactate fermenters and SRB in the presence of both lactate and sulphate has been reported. This occurs both in the natural environment (Laanbroek *et al*., 1981; Purdy *et al*., 1997) and anaerobic digesters (Zellner *et al*., 1994). While lactate fermentation is not sulphate dependent, the oxidation of lactate is linked to sulphate reduction. Due to their kinetic properties, high levels of lactate encourage the growth of fermentative bacteria. In contrast, lactate

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oxidation becomes dominant under conditions of lactate limitation and excess sulphate (Laanbroek *et al*., 1981; Zellner *et al*., 1994). The mixed culture employed in the present study was enriched from the anaerobic pit of a facultative pond treating sewage. A complex microbiological population is present in any biological sewage treatment process, including SRB and non-SRB lactate fermenters (Murray *et al*., 1984; Lester, 1988; Houghton and Quarmby, 1999; Wagner and Loy, 2002). Hence, the presence of a non-SRB microbe in the mixed culture can also be attributed to the inoculum source. Recent study revealed that bacterial strains related to *Clostridium* were isolated from laboratory-scale sulphidogenic fluidised bed reactors. These lactate- and ethanol-fed reactors were employed in the treatment of synthetic AMD streams (Kaksonen *et al*., 2006).

6.4.2. Effect of Volumetric Loading Rate of Sulphate on Community structure

The community structure determined in this study, as reported in Table 6.3 was influenced by the volumetric loading rate of sulphate as mediated via both feed sulphate concentration and the dilution rate. SRB species diversity decreased with increasing feed sulphate concentration (Figure 6.5). A decrease in species richness with increasing dilution rate was observed at feed sulphate concentrations 1.0 to 5.0 g l^{-1} (Figure 6.5). On the other hand, at the feed sulphate concentrations 10.0 and 15.0 g l^{-1}, species number became independent of the volumetric loading rate of sulphate. This observation suggests the selection of active or robust population members and the exclusion of slow growers with increasing dilution rate (Figure 6.5 and Table 6.5). The same community composition was detected at the lowest dilution rate imposed (0.0083 h^{-1}: residence time 5d) for reactors receiving feed sulphate concentrations in the range 1.0 to 5.0 g l^{-1} (Table 6.3). This further shows the reproducibility of the restriction enzyme digestion analyses.

A similar study investigating the effect of reactor operating conditions on microbial structure (Briones *et al*., 2007), revealed a strong effect of sulphate loading on population diversity, as assessed by terminal restriction fragment length polymorphism (T-RFLP) analysis in the upflow anaerobic sludge blanket (UASB) and anaerobic migrating blanket reactors (AMBR).

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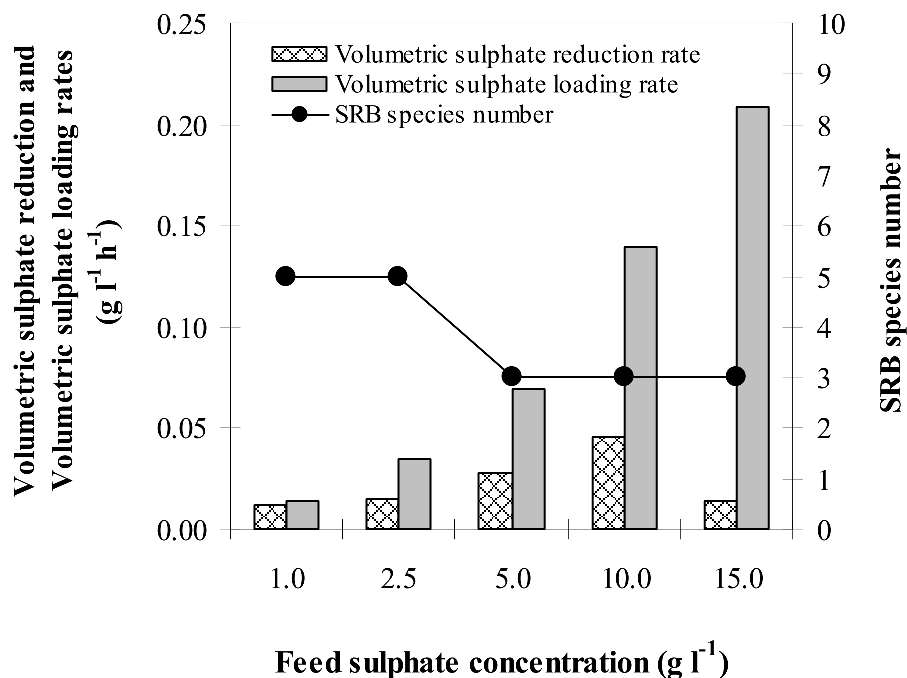

Figure 6.5: Dependence of SR kinetics and SRB dynamics on feed sulphate concentration (S_o), (RT= 3 d).

Increasing the sulphate load from 0.3 g l^{-1} (COD:SO_4^{2-} = 24) to 1.4 g l^{-1} (COD:SO_4^{2-} = 5) led to a decreased bacterial diversity in the two reactor types that were started with the same inoculum. Higher biodiversity at COD:SO_4^{2-} = 24 can be attributed to the higher COD content and low sulphide production, conditions which could sustain the growth of a wide range of non-SRB anaerobic organisms. The decrease in microbial diversity was accompanied by decreased soluble COD (SCOD) removal which is consistent with the loss of non-SRB community members. Nevertheless, greater biodiversity was maintained in the UASB. In their study (Briones *et al*., 2007), the UASB reactor exhibited a better performance in comparison with the AMBR. The SCOD removal declined from 88% to 64% in the UASB while the AMBR was associated with a decrease in SCOD removal from 77% to 20% with the increasing sulphate loading. This was speculated to have resulted from a steady community structure, presence of a predominant population member (*Thermotogales*-like) tolerant to sulphide and also able to utilise a wide variety of substrates, interspecies competition and syntrophic interactions (Briones *et al*., 2007).

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Table 6.5: Effect of feed sulphate concentration and dilution rate on SRB species exclusion and relative lactate oxidation. Fractional amount of lactate oxidised (%) in parentheses.

RT (day)	Dilution rate (h^{-1})	Lost SRB species at different and relative lactate oxidised at feed sulphate concentrations (S_o)				
		S_o ($g\ l^{-1}$)				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.0833	m, g, v (41.5)	nd	nd	nd	nd
1.0	0.042	m (72.4)	m, g, v (5.3)	m, g, v (7.7)	g, v (30.3)	nd
1.5	0.028	m (72.4)	m, g, v (8.3)	m, g, v (19.8)	g, v (37.1)	nd
2.0	0.021	m (72.4)	m, g, v (15.2)	m, g, v (38.7)	g, v (44.5)	nd
3.0	0.014	none (72.3)	none (34.0)	g, v (47.4)	g, v (47.3)	g, v (22.7)
3.5	0.012	nd	nd	nd	nd	g, v (23.8)
4.0	0.010	none (72.6)	none (45.3)	g, v (52.4)	g, v (53.2)	g, v (23.1)
5.0	0.0083	none (72.6)	none (45.2)	none (54.2)	g, v (53.8)	g, v (23.7)
5.5	0.0076	nd	nd	nd	nd	g, v (24.0)

m= *Desulfococcus multivorans*
g= *Desulfovibrio gigas*
v= *Desulfosarcina variabilis*

Colour code indicating the loss of different number of strains

1 strain	2 strains	3 strains

The reactor design may also play a crucial role in the different observations. In the investigation by Briones *et al*. (2007), the AMBR consisted of multiple compartments which were designed to create population and activity gradients. In contrast, the UASB had a single compartment which would encourage interactions among different microbial groups.

In contrast to the observations presented herein, Saikaly *et al*. (2005) showed that activated sludge sequencing batch reactors (SBR) operated at solids retention time (SRT) of 2 days were characterised by higher microbial diversity than equivalent reactors at an SRT of 8 days. Despite differing diversity, reactors demonstrated similar COD removal (90.81 ± 1.11 to 96.84 ± 0.4%).

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Possible reasons for the profiles observed in the current study are described in the following sections. In order to understand the responses of the identified strains to the operating conditions, a comparative analysis of these microorganisms was carried out based on the available literature. The published literature on the kinetic data and the growth parameters for the microorganisms identified in this study are based on different substrates and are subject to varying experimental conditions (Table 6.6). Consequently it is difficult to draw consistent conclusions on the relative growth properties of these microorganisms based on lactate utilisation. However, according to Chiu *et al*. (1972), the phenomenon whereby cell washout of slower growing species occur at different stages for the various reactors as the dilution rate was increased, is peculiar to mixed microbial populations. Any strain will be excluded under conditions where the imposed dilution rate exceeds its characteristic maximum specific growth rate.

The description of the interactions among SRB species in bioreactors and how their intrinsic kinetic properties influence their relative dominance are not extensively reported in literature. In a chemostat study by Laanbroek *et al*. (1984), *Desulfobulbus propionicus* was predominant under limiting conditions of sulphate and ethanol, while *Desulfobacter postgatei* was poorly competitive. This implied that *Desulfobulbus propionicus* is characterised by a high affinity for sulphate (Table 6.6); this might explain the occurrence of this strain at all operating conditions in the current study (Table 6.3). Further, low affinity for sulphate that characterise *Desulfobacter postgatei* and *Desulfococcus multivorans*, as indicated by high K_s values (Table 6.6), suggest their predominance at high sulphate concentrations. This is consistent with the results presented in Table 6.3.

The effect of other factors on the interactions of the SRB strains identified in the current study were carried out based on previous experiments in which these strains were cultured under similar conditions. These are highlighted as follows:

(1) <u>Effect of Alkalinity</u>: The effect of bicarbonate alkalinity levels on SRB community structure in an acidogenic sulphate reducing bioreactor was described in a recent study by Zhao *et al*. (2007). They showed that the *Desulfobacter* strain was

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more sensitive to a decrease in alkalinity than *Desulfobulbus* and *Desulfovibrio* strains. During the start-up of the bioreactor, the alkalinity concentration and sulphate conversion were maintained at 3.0 g l^{-1} and 80% respectively. Throughout the experiment, constant sulphate loading rate, pH and hydraulic retention time were maintained at 24 g l^{-1} d^{-1}, 6.7 and 10 h respectively. Under these conditions, the predominant group was the *Desulfobacter* spp. This was attributed to the production of acetate from the incomplete oxidation of the corresponding electron donors of *Desulfobulbus* and *Desulfovibrio* strains. This implied a syntrophic relationship among these strains. Conversely, when the feed alkalinity was reduced to 1.0 g l^{-1}, the growth rate of the *Desulfobacter* strain was reduced by 3 to 4 fold, while the *Desulfovibrio* spp. became the predominant group. During this period, the *Desulfobulbus* spp. was not negatively affected as much as the *Desulfobacter* strain.

Data obtained from the current study show that *Desulfobacter postgatei* and *Desulfobulbus propionicus* were not as sensitive to changes in alkalinity as *Desulfovibrio gigas*. *D. gigas* was not identified at high feed sulphate concentrations of 10.0 and 15.0 g l^{-1} (Tables 6.3 and 6.5). *D. postgatei* and *D. propionicus* persisted at varying concentrations of alkalinity. The corresponding bicarbonate alkalinity concentrations at lower feed sulphate concentrations of 1.0 to 5.0 g l^{-1} and in the higher range of 10.0 to 15.0 g l^{-1} were 1.3 to 4.6 g l^{-1} and 2.4 to 4.8 g l^{-1} respectively. High acetate levels in the range 0.8 to 4.6 g l^{-1} were observed in all the reactors. This might be responsible for the prevalence of *Desulfobacter postgatei* in this study (Tables 6.2 and 6.4).

(2) <u>Sulphide Inhibition</u>: Recent chemostat studies of acetate-fed mixed culture (pH 7.8, 35 °C) revealed that the *Desulfococcus* group was prevalent at high sulphate concentration (15.0 g l^{-1}), while *Desulfobacter* was a predominant group at lower sulphate concentration (10.0 g l^{-1}). In contrast, at both feed sulphate concentrations (10.0 and 15.0 g l^{-1}), the *Desulfobulbus* spp. was inhibited (Icgen and Harrison, 2006b). These observations were corroborated by a similar investigation (Icgen and Harrison, 2006a); the *Desulfococcus* group could thrive in the presence of high concentrations of sulphide up to 1.5 g l^{-1} before the onset of inhibition.

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Table 6.6: Reported energetic and kinetic properties of the SRB identified in the current study

Organism	Electron donor ($e^{-}d$)	T (°C)	pH	K_{e-d} (mg l^{-1})	$K_{SO_4^{2-}}$ (mg l^{-1})	μ_{max} (h^{-1})	Biomass yield ($g_{DW}\ g^{-1}_{SO_4^{2-}}$)	Biomass yield ($g_{DW}\ g^{-1}_{e^{-}d}$)	Reference
Desulfobulbus propionicus	propionate	37	7.5-8	50	3	0.115	nd	-	O'Flaherty *et al*., 1998
Desulfobacter postgatei	acetate	32	nd	nd	nd	0.035	nd	0.074	Widdel and Pfennig, 1981
	acetate	30	nd	nd	4.2	0.03	0.158	nd	Ingvorsen *et al*., 1984
	acetate	30	7.5-8	12	20	0.039	nd	nd	O'Flaherty *et al*., 1998
Desulfovibrio gigas	lactate	nd	nd	nd	nd	0.092	nd	0.042	Traore *et al*., 1982
Desulfovibrio strains	lactate	37	6.2-6.6	-	-	0.336	nd	0.028-0.079	Reis *et al*., 1992
Desulfosarcina variabilis	-	-	-	-	-	-	nd	nd	-
Desulfococcus multivorans	ethanol	37	7.5-7.8	70	22	0.039	nd	nd	O'Flaherty *et al*., 1998

nd: not determined.

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On the other hand, the *Desulfobulbus* spp. were very sensitive to sulphide. As depicted in Table 6.4, O'Flaherty *et al*. (1998) determined the sulphide toxicity threshold of different SRB strains subjected to the same growth conditions. The concentration of total sulphide which resulted in 50% inhibition was highest for *Desulfococcus multivorans*; this further substantiates the report of Icgen and Harrison (2006a). In the current study, dissolved sulphide levels $\geq$0.5 g l^{-1} were only recorded at dilution rates 0.0083 to 0.014 h^{-1} (residence time: 5 to 3 d) for feed sulphate of concentrations of 5.0 and 10.0 g l^{-1}. This compares with the value reported by O'Flaherty *et al*. (1998) (Table 6.4) to cause 50% inhibition of *Desulfobulbus propionicus* growth at pH 8. Hence, *Desulfobulbus propionicus* would not be expected to be completely inhibited in the current study. It can be deduced from these observations that at high concentrations of sulphate and sulphide, *Desulfococcus multivorans* will have a competitive advantage over the other SRB groups (Tables 6.4 and 6.6). This might explain the persistence of this strain in this study (Table 6.3).

6.4.3. Correlation of Microbial Community Structure to the BSR Kinetics and Stoichiometry

The observations from the present study substantiate the hypothesis that there is a close relationship between the kinetics of biological sulphate reduction and the community structure of SRB. From the foregoing discussion, the following can be highlighted:

1. The response of the microbial structure to the volumetric loading rate of sulphate is linked to observations of the stoichiometry and kinetics of biological sulphate reduction described in Chapters 4 and 5. At low feed sulphate concentrations of 1.0 to 5.0 g l^{-1}, the disappearance of *Desulfococcus multivorans*, *Desulfovibrio gigas* and *Desulfosarcina variabilis* at high dilution rates (residence time: 0.5 to 2 d) was congruent with low relative fraction of lactate oxidised and low sulphate conversion (Table 6.5 and Figure 6.6). The predominance of lactate fermenter(s) might have resulted in the low SRB diversity in this range.
2. At feed sulphate concentration of 1.0 g l^{-1}, as the dilution rate increased in the range 0.021 to 0.042 h^{-1}, *Desulfococcus multivorans* was lost from the SRB

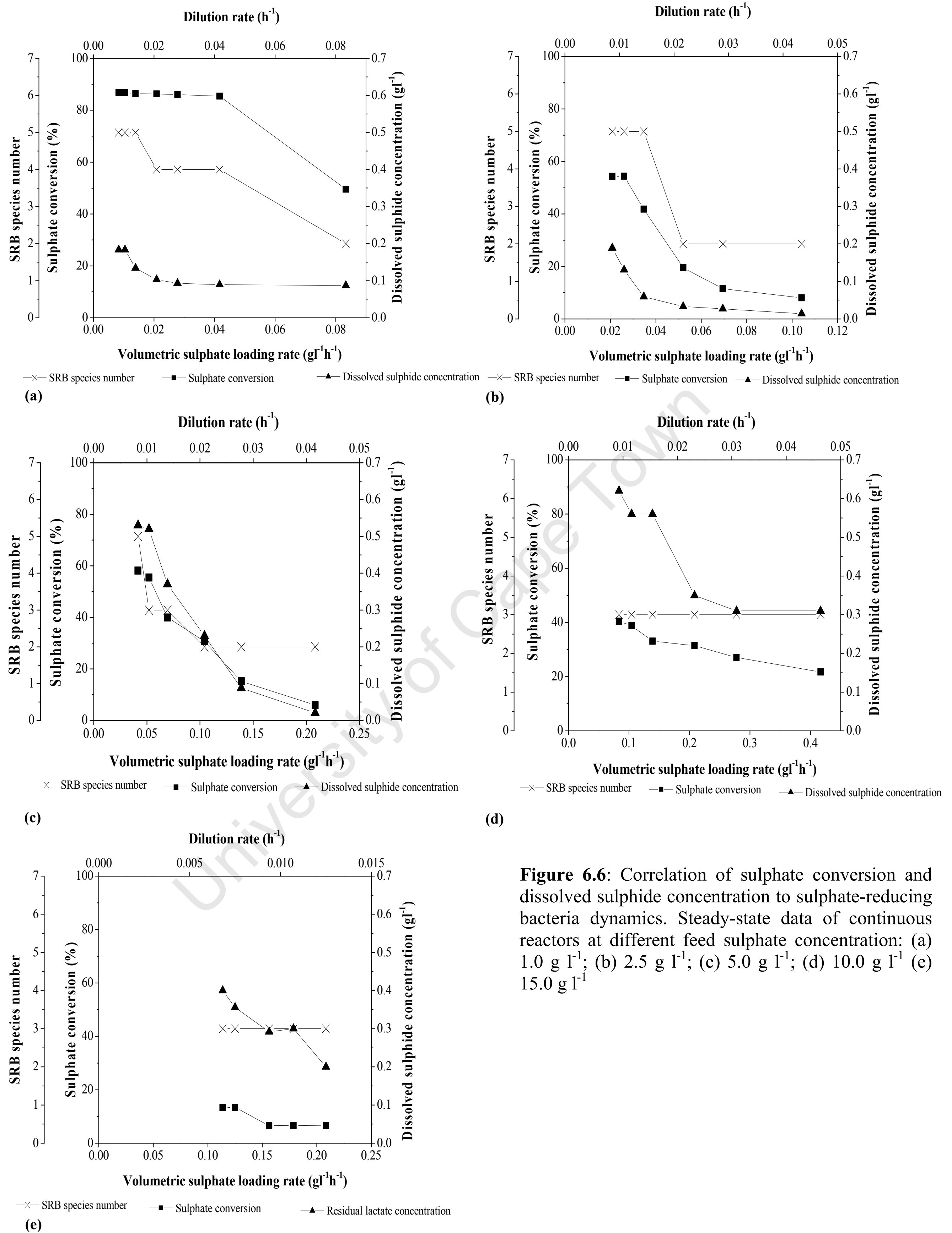

Figure 6.6: Correlation of sulphate conversion and dissolved sulphide concentration to sulphate-reducing bacteria dynamics. Steady-state data of continuous reactors at different feed sulphate concentration: (a) 1.0 g l^{-1}; (b) 2.5 g l^{-1}; (c) 5.0 g l^{-1}; (d) 10.0 g l^{-1} (e) 15.0 g l^{-1}

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consortium (Table 6.5). Nevertheless, the sulphate conversion remained constant at 86.3 ± 0.5 (Figure 4.2a). In contrast, this strain persisted at high feed sulphate concentrations 10.0 and 15.0 g l^{-1}. This suggests that *Desulfococcus multivorans* is characterised by high K_s for sulphate and its presence in the consortium is critical at high feed sulphate concentration. Possible characteristic high K_s and low μ_{max} of *Desulfococcus multivorans* results in this species being easily washed out or out-competed at low substrate concentration. Further, this SRB strain may be more tolerant to sulphate and sulphide in solution than other SRBs despite its characteristic lower μ_{max}.

3. At feed sulphate concentrations of 10.0 and 15.0 g l^{-1}, similar microbial community structures were observed across the range of dilution rates applied. *Desulfovibrio gigas* and *Desulfosarcina variabilis* were absent at these sulphate concentrations (Table 6.5). At feed sulphate concentration range 1.0 to 5.0 g l^{-1}, the loss of these two strains corresponded to high volumetric loading rates (≥0.052 g l^{-1} h^{-1}) at which a concomitant reduction in sulphate conversion occurred (Table 6.5 and Figure 6.6). These observations suggest that these two strains are sensitive to high sulphate and sulphide levels. Additionally, these strains might be characterised by lower growth rates relative to the other groups identified in this study under the operating conditions imposed. Thus their disappearance at high dilution rates.

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Data obtained from this study suggest that the persistent SRB population members identified throughout the operating conditions imposed in this study, *Desulfobulbus propionicus* and *Desulfobacter postgatei*, were able to thrive under different extreme conditions. Previous studies have shown that these strains are well adapted to physicochemical challenges (Tebo and Obraztsova, 1998; Chang *et al*., 2001; Nakagawa *et al*., 2002; Kaksonen *et al*., 2004; 2006; Geets *et al*., 2006; Icgen and Harrison, 2006a; 2006b; Zhao *et al*., 2007).

6.4.4. Effect of Population Diversity on Bioreactor Performance

The predominance of lactate fermentation at feed concentrations of 2.5 and 5.0 g l^{-1} (Sections 4.3.1.2, 4.3.1.3, 5.3.1 and 5.3.3) and the early onset of wash out of slow

growing cells observed at dilution rates 0.021 to 0.042 h^{-1} (residence time: 2 to 1 d) are congruent with the loss of SRB species richness (Table 6.3, Figures 6.5 and 6.6). Furthermore, the predominance of sulphate reduction and lactate oxidation at low dilution rates (0.0083 to 0.014 h^{-1}, residence time: 5 to 3 d) correspond to a relatively more diverse group, observed in the reactors receiving these feed sulphate concentrations. Conversely, at feed sulphate concentrations of 1.0 and 10.0 g l^{-1} more stable SRB biodiversity can be associated with the high volumetric sulphate reduction rate and the predominance of lactate oxidation. This is also consistent with the observation described in Section 4.3.3, where reactors receiving feed sulphate concentrations 1.0 and 10.0 g l^{-1} exhibited an early recovery after the restoration of a mild operational condition (residence time: 5 d). System resilience is known to be dependent on species richness, both in SRB populations, and as a general ecological principle (Saikaly *et al*., 2005; Miura *et al*., 2007).

The observations reported herein are similar to the result of a recent investigation by von Canstein *et al*. (2002) who demonstrated that a diverse consortium of mercury-reducing biofilms was resistant to mercury toxicity in comparison with monoculture biofilms. The multispecies biofilms included the monoculture and other population members. While biodiversity decreased with increased mercury load, the original microbial composition was fully restored after the inlet mercury concentration into the bioreactor was reduced. The increased mercury load led to a system failure in the monoculture biofilms.

Fernández *et al*. (2000) reported that substrate perturbation by the addition of glucose to two sets of methanogenic bioreactors with different population composition and diversity resulted in different responses. The community structure of the reactors with higher diversity overcame the perturbation imposed more readily, relative to the reactors characterised by lower microbial diversity. In the study by Fernández *et al*. (2000), microbial species that were lost in the less diverse reactors during the substrate perturbation persisted in the more diverse community. Individual members play distinct roles in an ecosystem and the interactions between these members result in the net outcome of the process they mediate. An investigation of methanogenic reactors by Sekiguchi *et al*. (1998) corroborated these findings. A mesophilic

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methanogenic granular sludge characterised by a higher microbial diversity was less prone to be affected by environmental perturbations relative to the thermophilic community with a lower microbial diversity.

These studies have demonstrated that there is a positive link between microbial population diversity and the ability of a microbial consortium to resist changes in physicochemical properties (system robustness) or to recover when exposed to these changes. In a mixed microbial community, increasing biodiversity results in strong species interactions, increasing resistance of the whole community to external perturbations (Yachi and Loreau, 1999; Tilman, 1999). This phenomenon is summarised by the "insurance hypothesis" which suggests that a massive consortium of species offers a buffering effect on the process as a result of varying response of individual members to perturbations. Even though this theory has been mostly based on experimental and theoretical studies on a macro scale of plant and animal ecosystems (McGrady-Steed *et al*., 1997; Naeem and Li, 1997; Tilman, 1999; 2000), and the influence of human activities on the environment, it is relevant at the microbial level (Loreau *et al*., 2001; von Canstein *et al*., 2002; Cardinale *et al*., 2006).

In plant studies, it was shown that resistance of grasslands to drought was strongly influenced by biodiversity when the community composition was ≤9 species. Grasslands composed of ≤4 species were prone to drought and exhibited biomass production of 0 to 25% of what was produced prior to the perturbation. On the other hand, plots composed of 10 to 15 plant species were able to produce up to 50% of their pre-perturbation period production during the drought (Tilman and Downing, 1994). Similar trends were found in recovering their full productivity post-perturbation. This study concluded that the more diverse communities contain more drought–resistant plants relative to the less diverse communities. Biodiversity offers a pool of species with complementary properties to maintain ecosystem integrity under varying environmental conditions (von Canstein *et al*., 2002). The influence of biodiversity on an ecosystem's function can also be described by the phenomenon known as "sampling effect". In a more diverse community there is a greater possibility of predominance by the very active members (Cardinale *et al*., 2006).

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6.4.4.1. Presence of Active Population Members Necessary for Efficient Bioreactor Performance

The occurrence of a more diverse microbial community does not necessarily result in a higher reactor performance. The crucial factor is the presence of active members. Fewer population members were identified in the starting culture at the feed sulphate concentration of 10.0 g l^{-1}, relative to the feed sulphate concentration of 2.5 g l^{-1}. Nevertheless, lactate oxidation and sulphate reduction reactions were predominant in the former. Furthermore, the same microbial community composition was observed at residence time of 5 d in the reactors fed with 1.0 to 5.0 g l^{-1} sulphate concentration, yet sulphate removal decreased with increasing feed sulphate concentration in this range (Section 4.3.2.2). Additionally, the same population composition was observed in experiments with feed sulphate concentrations of 10.0 and 15.0 g l^{-1}. However, lower reactor performance was recorded at feed sulphate concentration of 15.0 g l^{-1} (Sections 4.3.2.1 and 4.3.2.2).

Merkel *et al*. (1999) reported that despite increasing biomass concentration of an anaerobic digester composed of a heterogeneous microbial community, by three-fold, there was no corresponding increase in the reactor performance. This reiterates the need to select for a diverse microbial community constituted by versatile members, in the design of bioremediation processes. This can be achieved through the investigation of the linkage between microbial population and function.

6.4.5. Effect of Community Dynamics on Bioreactor Performance

As recorded in Section 5.3.1, a shift in the lactate metabolism pathway was observed with increasing feed sulphate concentrations and dilution rate. This can be associated with changes in the community structure (Figure 6.4 and Table 6.3).

Community dynamics is required for a robust bioremediation system (Fernández *et al*., 1999; 2000). Fernández *et al*. (1999) studied a continuous methanogenic reactor, maintained at constant physicochemical conditions, temperature and dilution rate for a period of 605 days. It was shown that continuing shifts in the microbial population accompanied by varying metabolic pathways were necessary for the maintenance of a robust methanogenic system. Changes in the microbial population did not result in

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any change in the reactor performance throughout the experimental study. This suggested that a stable bioreactor performance is linked to a dynamic microbial community.

6.4.6. Possible Interactions among the Microbial Groups Identified In This Study

In CSTR systems, based on the competitive exclusion principle, a monoculture can be selected from a mixed microbial consortium on exposure to varying physicochemical challenges over a long period of operation (Speece, 1983; Shuler and Kargi, 1992). In the present study, chemostat cultures were selected to provide defined and steady-state reactor conditions, and are recognised as the optimal vehicle for kinetics studies. It is therefore well suited to relate biokinetics, reaction stoichiometry and community dynamics. In the current study, even though SRB species richness decreased with increasing dilution rate and feed sulphate concentration, multi-component populations of SRB species were observed in all experiments (Table 6.3). The stratification and interactions of sulphate-reducing bacteria in natural mixed communities have been well documented (Santegoeds *et al* 1998; Li *et al*., 1999; Okabe *et al*., 1999; Ravenschlag *et al*., 2000; Ito *et al*., 2002a; 2002b). The description of the interactions among SRB species in bioreactors are not extensively reported in literature. A chemostat study by Zhao *et al*. (2007) reported commensalism among members of the genera *Desulfobacter*, *Desulfobulbus* and *Desulfovibrio*. The acetate produced from the incomplete oxidation of the corresponding electron donors of *Desulfobulbus* and *Desulfovibrio* strains supported the growth of *Desulfobacter* spp. Investigation by Laanbroek *et al*. (1984) revealed competitive relationships for sulphate and ethanol among *Desulfobacter*, *Desulfobulbus* and *Desulfovibrio* species. *Desulfovibrio baculatus* was shown to be the best scavenger of limiting concentrations of sulphate and ethanol in the presence of excess iron concentration (7.0 mg l^{-1}). Even though the kinetic parameters of these strains were not determined in the experiment by Laanbroek and co-workers (1984), the observations presented suggested that *Desulfovibrio baculatus* was characterised by the lowest K_s value for both sulphate and ethanol as compared to the competing species.

The shifts in the community structure from lactate oxidisers (SRB) to lactate fermenters with increasing feed sulphate concentrations and dilution rate, as described

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in Sections 6.4.1, 6.4.2 and 6.4.3 suggest both competition and amensalism. These two types of microbial interactions are suggested by the hypotheses developed in Section 4.3.2.1, viz:

i. High lactate concentration favours lactate fermenters while the proliferation of SRB is favoured under conditions of lower lactate concentrations.
ii. The sulphide produced by the SRB inhibits the metabolic activity of lactate fermenters at lower concentrations than the lactate oxidisers.

These hypotheses are tested and the interaction between the lactate fermenters and lactate oxidisers are demonstrated explicitly in Chapter 7.

6.6. SUMMARY

This study demonstrated the development of a simple and rapid method for the assessment of a microbial community in a CSTR under sulphidogenic conditions. This revealed the presence of a starting inoculum composed of at least five sulphate-reducing bacteria, namely, *Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* and *Desulfococcus multivorans*. The identified SRB strains are different with respect to morphology, electron donor utilisation and response to substrate inhibition. These strains have also been identified previously in mines (Chang *et al*., 2001; Nakagawa *et al*., 2002) and heavy-metal contaminated sites (Tebo and Obraztsova, 1998; Geets *et al*., 2006). There are reports of the isolation of the members of this consortium in sulphidogenic reactors including the FBR (Kaksonen *et al*., 2004; 2006), UASB (Dar *et al*., 2007) and CSTR (Icgen and Harrison, 2006a; 2006b; Zhao *et al*., 2007). These findings suggest the suitability of these organisms for the treatment of AMD streams. Similar to the investigation by Kaksonen *et al*. (2006), there was evidence indicating the presence of non-SRB lactate fermenter(s) under biosulphidogenic conditions in the current study.

The close link between microbial community dynamics, biological sulphate reduction kinetics and stoichiometry was established. It can be inferred from the results presented herein, that microbial population diversity and dynamic community structure are required for the stable performance of biological wastewater treatment systems (Miura *et al*., 2007). While biodiversity preserves the microbial community structure, a flexible community is important for a robust system performance (Fernández *et al*., 2000). Additionally, efficient reactor performance is not necessarily

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a consequence of a large population, but the presence of active population members (Merkel *et al*., 1999). However, it is imperative to preserve as much biodiversity as possible in a bioremediation system to enhance its robustness and resilience (Cardinale *et al*., 2006).

This chapter highlights the need to develop a system in which a diverse microbial community constituted by active and versatile population members is maintained. There is also a need for the selection of a stable community through adaptation to prevent compromising of reactor performance on system perturbation. This could aid in the optimisation of the BSR process. This can be achieved by establishing the proper population diversity through the combination of "ideal" and dominant microbial groups adapted to function efficiently within the operating conditions found in wastewater treatment processes.

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CHAPTER 7
OPERATING CONSIDERATIONS FOR LACTATE-FED SULPHIDOGENIC REACTORS

7.1. INTRODUCTION

Results presented in Section 4.3.1 showed that the feed sulphate concentration affects both the sulphate reduction rate and the size of the mixed microbial population supported. Further, results presented in Chapters 4, 5 and 6 indicate the presence of two competing metabolic pathways of lactate utilisation mediated by a heterogeneous bacterial community. The BSR kinetics were shown to be strongly influenced by the shift in reaction stoichiometry between the oxidative and fermentative pathways of lactate metabolism. Shifts in the reaction stoichiometry and community structure were also suggested to be dependent on the availability of lactate, sulphate and dissolved sulphide concentration (Chapters 4, 5 and 6). The objectives of the current Chapter were as follows:

i. To test the hypotheses of competing lactate oxidation and lactate fermentation developed in Section 4.3.2 by investigating the influence of culture conditions on metabolic pathway of lactate metabolism, viz: effects of lactate and dissolved sulphide concentrations on shifts in reaction stoichiometry and community structure.
ii. To determine the maximum specific growth rate (μ_{max}) and saturation constant (K_s) under conditions dominated by either lactate oxidation or lactate fermentation using the experimental data.
iii. To demonstrate the competition between lactate fermentation and oxidation using simulations based on the kinetic constants obtained and the existing kinetic models. To explore whether this mimics experimental findings.
iv. To investigate the effect of reactor configuration and lactate availability on BSR kinetics using a two-stage chemostat system.

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7.2. INFLUENCE OF CULTURE CONDITIONS ON REACTOR PERFORMANCE THROUGH LACTATE METABOLISM

Results from the current study indicate that lactate is consumed with the concomitant reduction of sulphate and production of acetate, propionate, bicarbonate and sulphide. Similar observations have been documented previously (Purdy *et al*., 1997; Baskaran and Nemati, 2006). In all experiments conducted in the present study, the marked emergence of propionate as the reaction product of lactate metabolism when acetate production declined coincided with an increase in residual sulphate concentration, hence decreased sulphate conversion (Figures 4.2b to 4.5b). This observation suggests that lactate oxidising SRB were less active under conditions of high lactate concentrations and dilution rates. These trends are associated with low sulphate conversion and low sulphide production. Under these conditions, lactate fermenters which are known to be characterised by high K_s values (Zellner *et al.,* 1994) are expected to be dominant. This phenomenon is postulated to have been responsible for the majority of lactate utilisation observed at low sulphate conversion. The effect of feed sulphate concentration and dilution rate on shifts in lactate metabolic pathway is described in detail in Chapter 5.

The aim of this section was to validate the hypotheses proposed in Sections 4.3.2 and 4.3.3. These hypotheses attribute the trends observed in the kinetics of biological sulphate reduction, based on the effect of the volumetric sulphate loading rate to be a consequence of shift in lactate metabolism. This shift was postulated to be driven by sulphide and lactate concentrations, such that lactate fermentation was favoured by high lactate and low sulphide concentrations. On the other hand, under conditions of low lactate and high sulphide concentrations, SRB out-compete the lactate fermenters. Thus, lactate oxidation and a concomitant reduction of sulphate can be expected to become predominant.

The following sections describe the experimental approach and the results obtained from the investigations carried out to test these hypotheses.

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7.2.1. Kinetics of Lactate Fermentation

The kinetics of lactate fermentation were investigated in the absence of sulphate using the same standard experimental conditions described in Sections 3.2 and 4.2.1, with the temperature and pH maintained at 35°C and pH 8.0 ± 0.2 respectively. The feed media contained the same lactate concentration (5.6 g l^{-1}) as experiments at the feed sulphate concentration of 2.5 g l^{-1}. Magnesium sulphate was replaced with magnesium chloride to completely eliminate sulphate. Other media components remained the same as utilised with a feed sulphate concentration of 2.5 g l^{-1}. The bioreactor was inoculated with culture taken from the continuous culture treating a 2.5 g l^{-1} feed sulphate concentration. Residence times were varied in the range 0.5 to 3.0 d. The steady-state at residence time 3 d was maintained for four retention times before reducing the residence time to 2 days to allow a complete washout of sulphate and sulphide (Figure 7.1).

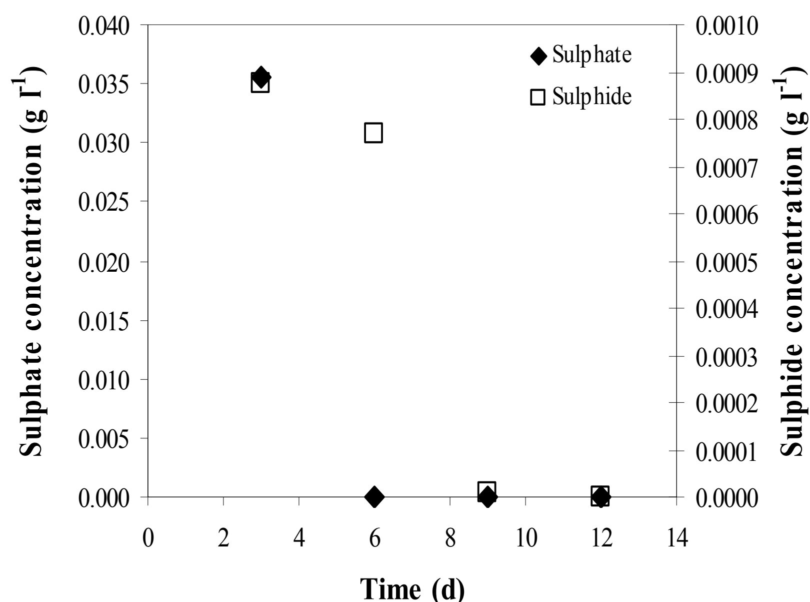

Figure 7.1: Dynamic data of sulphate and sulphide concentrations from the lactate fermentation experiment. Reactor was maintained at residence time of 3 d for four retention times before reducing residence time to 2 d.

The results obtained were compared with the data from the 2.5 g l^{-1} sulphate-fed reactor. As depicted in Figures 7.2a to 7.2f, the presence of sulphate did not effect any considerable change in both the volumetric lactate utilisation and acetate production rates. The volumetric propionate production rate was slightly higher in the fermentation experiment.

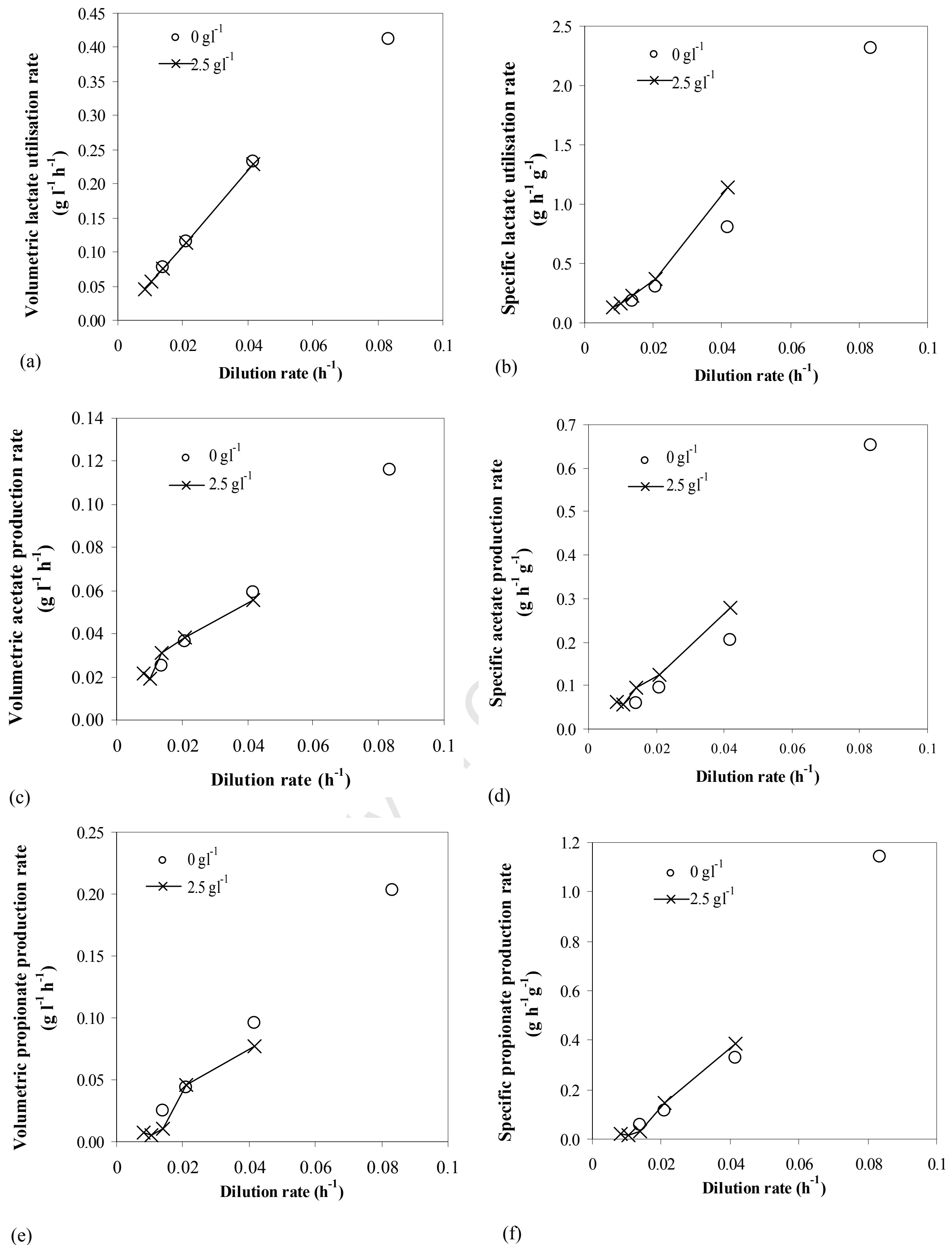

Figure 7.2: Effect of sulphate on lactate metabolism investigated at 2.5 g l^{-1} sulphate in feed (×) and in the absence of sulphate in feed (○) (a) Volumetric lactate utilisation rate; (b) Specific lactate utilisation rate; (c) Volumetric acetate production rate; (d) Specific acetate production rate; (e) Volumetric propionate production rate; (f) Specific propionate production rate.

This difference was more apparent at high dilution rates (greater than or equal to 0.042 h^{-1}). This observation supports the postulation that lactate fermentation was

prevalent in the 2.5 g l^{-1} sulphate-fed reactor and this metabolic pathway was dominant at high dilution rates. Additionally, the lactate fermenters were not inhibited in the presence of sulphate. As reflected by the specific rates of lactate utilisation, acetate and propionate production, higher biomass concentrations were observed in the fermentation experiment in comparison with the sulphate-fed reactor (Figure 7.2).

7.2.2. Sulphide Inhibition of Lactate Fermentation

This investigation was carried out based on the observation exhibited by the reactor receiving 10.0 g l^{-1} feed sulphate (Sections 4.3.1.4 and 5.3.1). The result suggested that at high sulphide concentrations (0.3 to 0.6 g l^{-1}) lactate fermenters were inhibited resulting in the predominance of lactate oxidation in this experiment. In order to substantiate this observation, a fermentative experiment was set-up using the inoculum from the 5.0 g l^{-1} sulphate-fed reactor. The feed media used for this experiment contained the same lactate concentration (11.1 g l^{-1}) as was utilised in the 5.0 g l^{-1} sulphate-fed reactor. Magnesium sulphate was replaced with magnesium chloride as described in Section 7.2.1. The chemostat culture was maintained at a constant residence time of 3 d. After 3 retention times (day 9), sulphide was added to the feed media at concentration of 0.5 g l^{-1}, using sodium sulphide. The pH of the media was adjusted to pH 8 by adding aliquots of HCl (32%).

Figure 7.3 shows that the addition of sulphide in the media stream resulted in the inhibition of the activity of the microorganism(s) involved in lactate fermentation. The profiles observed suggest that the fermentative microorganism(s) are sensitive to high concentrations of sulphide. In the presence of sulphide, the bacterial dry mass was reduced from its optimum (0.36 g l^{-1}) by 85%. The maximum rates of lactate utilisation and propionate production were reduced by 70%, while the acetate production rate declined by 65% of the maximum observed (Figure 7.3).

This result substantiates the observation of the predominance of lactate oxidation in the 10.0 g l^{-1} sulphate-fed reactor. The inhibition of the lactate fermenting microorganisms results in their out-competition by the SRB. This observation is supported by the report of Laanbroek *et al*. (1983). In their study, a lactate oxidiser (*Desulfovibrio* spp.) and a lactate fermenting microbe (*Veillonella* spp.) were cultured

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under the same conditions, the μ_{max} value of *Veillonella* spp. was decreased by 50% as the sulphide concentration increased from 0.0165 to 0.165 g l^{-1} while that of the oxidiser remained unchanged.

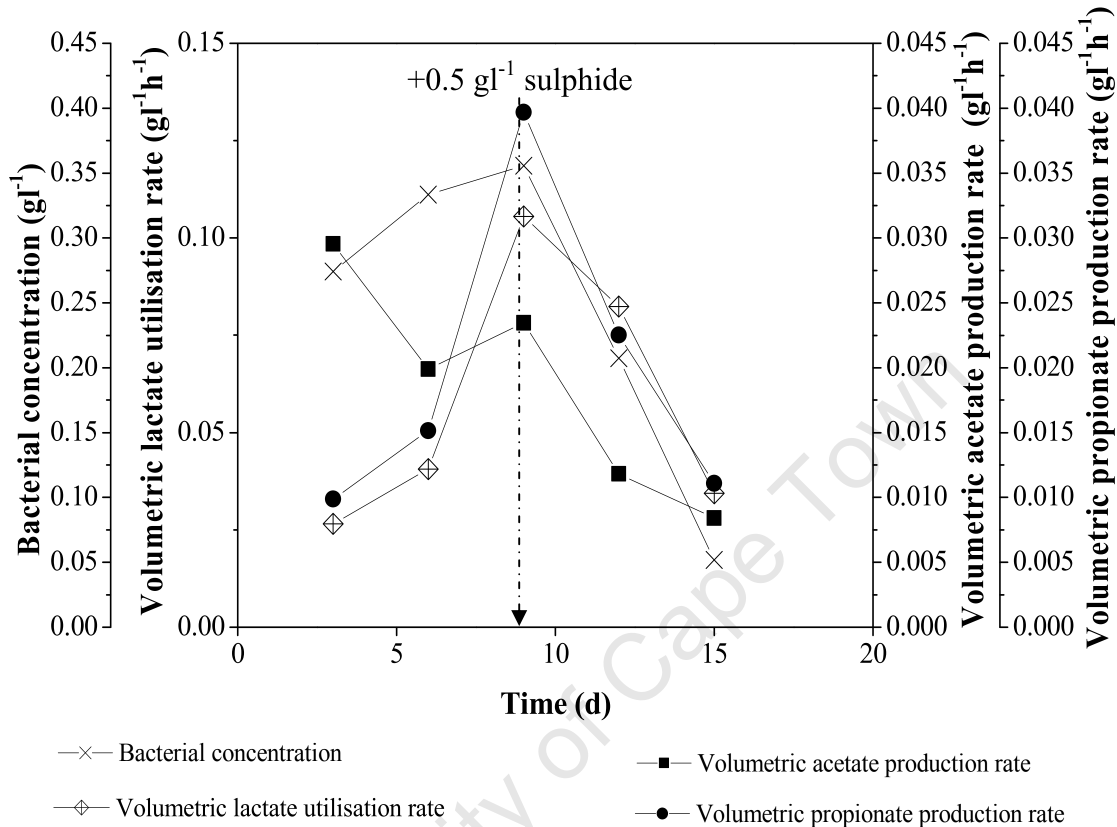

Figure 7.3: Effect of sulphide on lactate fermentation. Sulphide was added at concentration of 0.5 g l^{-1} to media stream on day 9. Chemostat culture was operated at residence time of 3 d.

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A microorganism's kinetic properties of μ_{max} and K_s are the critical determinants of its predominance under a single substrate-limited competitive condition (Veldkamp *et al*., 1984). It thus follows that, due to lower specific growth rate, lactate fermenters wash out under conditions of high sulphide concentrations.

7.3. DETERMINATION OF KINETIC CONSTANTS

Modelling is a crucial tool to assist in gaining a comprehensive understanding of a process (Kalyuzhnyi *et al*., 1998). Mathematical models are employed in bioremediation process research to describe the sub-processes (of physicochemical and biological nature) involved, their interactions and how they influence the reaction kinetics (McCarty and Mosey, 1991; Kalyuzhnyi *et al*., 1998). They are also used to

validate experimental data, to test mechanistic frameworks and to predict the treatment technology performance (Işik and Sponza, 2005).

First order kinetic model expressions of Monod (Equation 2.28), Chen and Hashimoto (Equation 2.30), Contois (Equation 2.31) and the one developed by Moosa *et al*. (2002) based on the Contois approach (Equation 2.3.6a), described in Section 2.4, were used to fit the data presented in Section 4.3.1. These models describe the dependency of specific bacterial growth rate, μ, on the residual limiting substrate concentration, S, feed substrate, S_o, and the bacterial concentration, X.

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{2.28}$$

$$\mu = \frac{\mu_{max} S}{K_s S_o + (1 - K_s) S} \tag{2.30}$$

$$\mu = \frac{\mu_{\max} S}{K_s^{"} X + S} \tag{2.31}$$

$$\mu = \frac{\mu_{max} S}{K_s^{'} S_o X + S} \tag{2.36a}$$

For a continuous culture at steady-state, where cell death is negligible and the feed is sterile, $\mu = D$.

In the current study, the inverse plots of the Monod, Chen and Hashimoto, Contois and the model proposed by Moosa *et al*. (2002) (Table 7.1) were used to determine the microbial growth constants (μ_{max} and K_s) using the experimental steady-state data obtained for feed sulphate concentrations, 1.0, 2.5, 5.0 and 10.0 g l^{-1} (Section 4.3.1). These three models were tested by Moosa *et al*. (2002) to describe the kinetics of biological sulphate reduction using acetate as the electron donor. In their study sulphate was the dominant limiting substrate.

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Table 7.1: Summary of plots used to determine the kinetic constants.

Model	Plot	Slope	Intercept
Monod	$\frac{1}{\mu}$ versus $\frac{1}{S}$	$\frac{K_s}{\mu_{max}}$	$\frac{1}{\mu_{max}}$
Chen and Hashimoto	$\frac{1}{\mu}$ versus $\frac{S_o}{S}$	$\frac{K_s}{\mu_{max}}$	$\frac{1-K_s}{\mu_{max}}$
Contois	$\frac{1}{\mu}$ versus $\frac{X}{S}$	$\frac{K_s}{\mu_{max}}$	$\frac{1}{\mu_{max}}$
Moosa *et al*. (2002)	$\frac{1}{\mu}$ versus $\frac{S_o X}{S}$	$\frac{K_s'}{\mu_{max}}$	$\frac{1}{\mu_{max}}$

The Contois equation was the preferred equation describing bacterial growth based on goodness of fit. The modified form of Contois model developed by Moosa *et al*. (2002), shown in Equation 2.36, describes the effect of feed sulphate concentration on the performance of the sulphate reduction process. It has also been used to describe the kinetics of biological sulphate reduction using ethanol as the carbon-source and electron donor (Hansford *et al*., 2007).

$$r_S = \left(\frac{\mu_{max} S}{K_s' S_o X + S} - K_d \right) \frac{X}{Y} \tag{2.36b}$$

The Monod, Chen and Hashimoto, and Contois models have been used extensively to describe the kinetics of other microbially-mediated reactions in chemostats, under conditions where one substrate was limiting (Aspé *et al*., 1997; Gadekar *et al*., 2006). In describing the kinetics of the anaerobic digestion of ice-cream wastewater, using a CSTR, Hu *et al*. (2002) showed that the Contois model fitted the experimental data well when the effect of the feed substrate concentration was incorporated. Similarly, Moosa *et al*. (2002) reported the dependence of the K_s term on the feed sulphate concentration, which led to the modification of the Contois model to include the feed sulphate concentration (S_o) (Equation 2.36).

7.3.1. Assuming Sulphate Is the Dominant Limiting Reactant

Under the experimental conditions described in Section 4.2.1, sulphate was assumed to be the only limiting substrate owing to lactate being added in stoichiometric excess. Steady-state experimental data obtained for feed sulphate concentrations of 1.0, 2.5, 5.0 and 10.0 g l^{-1} were analysed using the inverse plots described in Table 7.1 to determine the kinetic constants. As shown in Table 7.2, all the models tested in the

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current study gave negative values for the biokinetic constants. This indicates that sulphate was not limiting in the kinetic studies reported in Chapter 4. Unlike the observations presented in the current study, in the investigations by Moosa *et al*. (2002) and Hansford *et al* (2007) sulphate was the only limiting substrate because the reaction stoichiometry was unaffected by the feed substrate concentration and there were no alternative metabolic pathways for the electron donors (acetate and ethanol) employed.

Table 7.2: Kinetic constants obtained using Monod, and Chen and Hashimoto kinetic models (including the correlation coefficient values from the linearised plots). Assuming sulphate is the dominant limiting substrate.

S_o (g l^{-1})	Monod			Chen and Hashimoto		
	μ_{max}	K_s	R^2	μ_{max}	K_s	R^2
1.0	-0.0540	-0.63	0.364	-0.0013	-0.17	0.835
2.5	-0.0220	-3.86	0.942	0.037	2.22	0.964
5.0	-0.0220	-7.44	0.976	0.042	2.54	0.959
10.0	-0.0037	-8.36	0.882	-0.026	-5.40	0.954

Table 7.2 (contd): Kinetic constants obtained using Moosa *et al*. (2002) and Contois models (including the correlation coefficient values from the linearised plots). Assuming sulphate is the dominant limiting substrate.

S_o (g l^{-1})	Contois			Moosa *et al*. (2002)		
	μ_{max}	K_s	R^2	μ_{max}	K_s	R^2
1.0	-0.035	-2.08	0.699	-0.0068	-0.88	0.949
2.5	-0.126	-47.76	0.958	-0.126	-19.06	0.958
5.0	-0.134	-95.96	0.973	0.623	65.5	0.982
10.0	-0.008	-20.63	0.977	-0.0086	-2.10	0.977

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The possible reasons why the BSR in the current study cannot be described effectively by these expressions are as follows:

(i) The microbial community employed in this study is composed of versatile strains which are able to utilise lactate and the products of lactate metabolism (acetate and propionate) as electron donors and carbon-sources. Deviations of the observed stoichiometric ratios from the theoretical values suggest possible utilisation of acetate and propionate in the current study (Section 5.3.1).

(ii) There was evidence suggesting lactate limitation. There were shifts in the BSR stoichiometry and microbial populations with varying experimental

conditions. Hence, there was reduced availability of lactate for SRB growth under conditions where lactate fermentation prevailed (Sections 4.3.2.1 and 5.3.1).

7.3.2 Describing the Competition between Lactate Oxidisers and Fermenters

In the following sections lactate was assumed to be the dominant limiting substrate and the steady-state experimental data were used to determine the kinetic constants. The microbial growth parameters obtained were then used to model the interaction between lactate fermenters and lactate oxidisers under different experimental conditions.

7.3.2.1. Approach

Based on the postulations in Section 7.3.1, the observations recorded in Sections 4.3.1 and 4.3.2, and the dependency of the BSR kinetics on lactate utilisation, the microbial kinetic parameters were determined assuming lactate as the dominant limiting substrate. The steady-state data were analysed using the Chen and Hashimoto, Contois and Monod kinetic expressions to determine the kinetic constants. Data from the experiment using 1.0 g l^{-1} feed sulphate were used to describe lactate utilisation under conditions where the biological sulphate reduction reaction and concomitant lactate oxidation reaction were dominant. Data obtained from the experiment in the absence of sulphate, described in Section 7.2.1, were used to describe the kinetics of lactate utilisation under conditions where lactate fermentation was the dominant metabolic pathway. The model description was based on the relationship between the kinetics of bacterial growth and the lactate utilisation rate (LUR) (r_L) as described by the Pirt equation (Equation 4.7c) (Pirt, 1965) (Section 4.2.3.1). Analysis led to values of bacterial yield ($Y_{x/s}$) and maintenance (m_s) coefficients.

$$\frac{r_L}{X} = \mu \frac{1}{Y_{x/s}} + m_s \qquad (4.7c)$$

The microbial growth constants (μ_{max} and K_s), obtained by using the three models (Chen and Hashimoto, Contois and Monod), describing the dependence of microbial growth rate on the limiting substrate concentration, were used to calculate the specific growth rate (μ) values. The values obtained from these mathematical expressions were then compared with the actual values (i.e. experimental dilution rates) in a parity

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chart. The calculated μ values and the energetic constants ($Y_{x/s}, m_s$) were fitted into the Pirt equation to calculate the theoretical specific lactate utilisation rate (r_L/X). Using a parity chart, the calculated r_L/X values were then compared with the actual values obtained from the experiments. The parity charts are used to show the goodness of fit of these models. The sum of squared errors (SSE) (Equation 7.1) was used to further estimate the accuracy of these expressions to predict the experimental data. The sum of squares due to error (SSE) is defined as the sum of the square of the difference between the observed (y_i) and the predicted ($\hat{y}_i$) values as shown in Equation 7.1.

$$SSE = \sum (y_i - \hat{y}_i)^2 \tag{7.1}$$

The model characterised with the smallest value of this quantity is the one with the highest consistency to fit the experimental data.

7.3.2.2. Modelling Competition across Feed Sulphate Concentrations Studied

As shown in Table 7.3, the biomass yield estimated based on lactate utilisation was higher for the oxidative metabolism of lactate ($Y_{x/s}$ = 0.08 g g^{-1}) than for the fermentative pathway ($Y_{x/s}$= 0.05 g g^{-1}). This indicates a more efficient lactate utilisation via the oxidative pathway. This is in agreement with the observation by Bryant *et al*. (1977) which showed that the fermentative growth rate of *Desulfovibrio* strains on lactate was slower and produced lower growth yields compared with lactate oxidation coupled to sulphate reduction. Negative values were obtained for the maintenance coefficient (Table 7.3) and this term was significantly smaller than $\frac{\mu}{Y_{x/s}}$, hence it was approximated as negligible and excluded in the calculation of the lactate utilisation rate using Equation 4.7c.

As shown in Table 7.4, Chen and Hashimoto and the Monod models gave negative values for the kinetic constants under conditions where the biological sulphate reduction reaction and concomitant lactate oxidation reaction were dominant. Hence, the Contois model was the preferred expression to describe the lactate oxidation kinetics. The appropriateness of this model in representing the experimental data is shown in Figure 7.4.

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Table 7.3: The values of biomass yield and maintenance coefficients based on lactate utilisation (including the correlation coefficient values from the linear plots).

Lactate metabolism	Pirt		
	$Y_{x/s}$ ($g_{DW} \cdot g^{-1}_{SO_4^{2-}}$)	m_s ($g_{Lactate}\, g^{-1}_{DW}\, h^{-1}$)	R^2
Oxidation	0.08	-0.05	0.997
Fermentation	0.05	-0.20	0.990

Table 7.4: Kinetic constants obtained under conditions where lactate oxidation was dominant (including the correlation coefficient values from the linearised plots) assuming lactate is the dominant limiting substrate.

Model	μ_{max}	K_s	R^2
Monod	-0.18	-0.15	0.961
Chen and Hashimoto	-0.19	-0.07	0.961
Contois	0.20	0.60	0.960

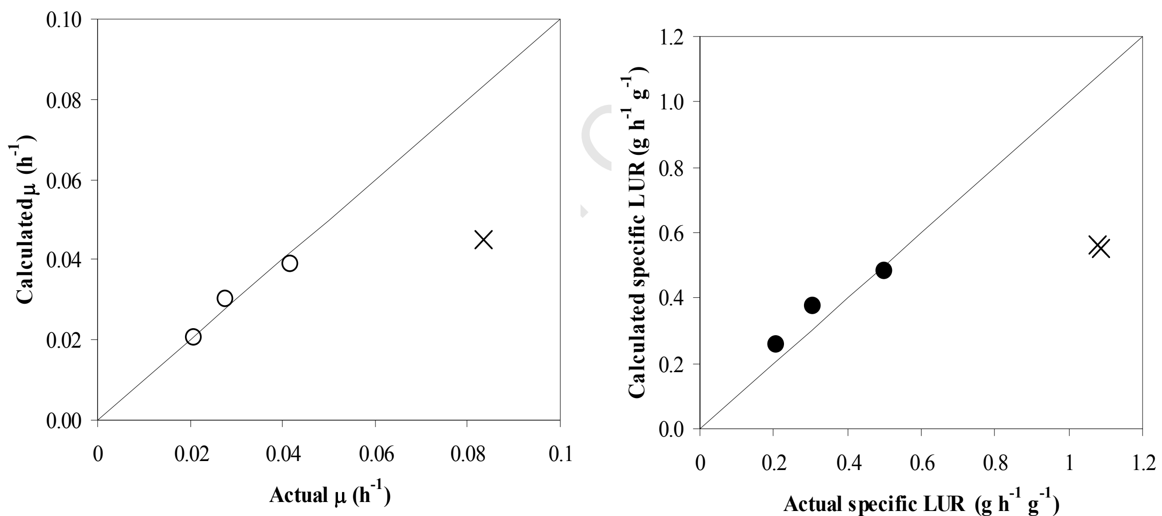

Figure 7.4: Parity chart for the Contois model under conditions where the biological sulphate reduction reaction and concomitant lactate oxidation reaction were dominant (S_o =1.0 g l^{-1}). Comparison of the actual and the predicted values. (a) Specific growth rate; (b) Specific lactate utilisation rate. (o) and (●) represent conditions when the fractional lactate oxidised is greater than 70%; (×) represent the condition when the fractional lactate oxidised is 41%.

The data points representing the 1.0 g l^{-1} sulphate-fed experiment (Figure 7.4) were obtained at the high dilution rates (0.021 to 0.083 h^{-1}) where residual lactate concentrations were detected (Section 4.3.1.1).

The Contois model fits the data reasonably well over the range of dilution rates 0.021 to 0.042 h^{-1}. Across this range of dilution rates, the fractional lactate oxidised was

greater than 70% (Table 5.3). Beyond this range, at the highest dilution rate imposed (0.083 h^{-1}), there was a disparity between the actual values and the calculated values as predicted by the Contois model (Figure 7.4). This is in agreement with the observation presented in Section 4.3.1.1. At this dilution rate (0.083 h^{-1}), a remarkable decline in the sulphate conversion, attributable to shift from the oxidative pathway towards the fermentative metabolic utilisation of lactate was recorded (fractional lactate oxidised = 41%) (Figures 4.2a, 4.2b, 5.1 and Table 5.3).

For all the models employed in the description of lactate fermentation, the data points are scattered on either sides of the parity line suggesting fluctuations in the data obtained due to experimental error. All the models tested gave positive values for the kinetic constants and R^2 values above 0.96 (Table 7.5). However, at higher dilution rates the experimental data points deviate from the parity line as the utilisation of lactate decreased with increasing dilution rate. Based on the goodness of fit, as indicated by the parity charts (Figure 7.5) and the values of SSE (Table 7.5), the Monod equation was preferred for describing lactate fermentation kinetics.

Table 7.5: Kinetic constants obtained under conditions where lactate fermentation was dominant (including the correlation coefficient values from the linearised plots and SSE values based on both μ and specific LUR) assuming lactate is the dominant limiting substrate.

Model	μ_{max}	K_s	R^2	SSE based on μ	SSE based on specific LUR
Monod	0.30	3.30	0.985	0.0010	0.51
Chen and Hashimoto	0.10	0.07	0.967	0.0026	1.30
Contois	0.09	1.00	0.973	0.0026	1.16

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Using the kinetic equations selected, simulations were carried out to investigate competition between lactate oxidation and lactate fermentation. The maximum specific growth rate was higher for the fermentative metabolism of lactate (μ_{max}= 0.3 h^{-1}) than for the lactate oxidative pathway (μ_{max}= 0.2 h^{-1}) (Table 7.6). The K_s value, which is defined as the residual lactate concentration at which the specific microbial growth rate is half the maximum specific growth rate, obtained using the Monod expression, was 3.3 g l^{-1} for the lactate fermentation reaction.

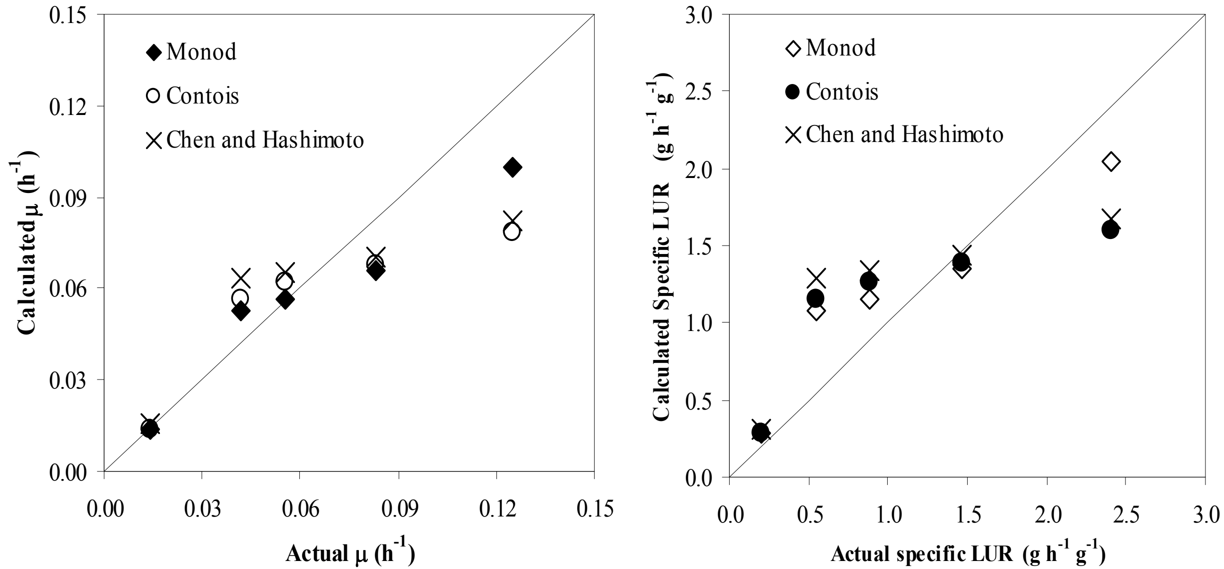

Figure 7.5: Comparison of the actual and the predicted values using parity charts. Values under conditions where the lactate fermentation, independent of sulphate reduction reaction, was dominant. (a) Specific growth rate; (b) Specific lactate utilisation rate. The feed media contained the same lactate concentration (5.6 g l^{-1}) as experiments at the feed sulphate concentration of 2.5 g l^{-1}.

The equivalent value, obtained using the Contois model, ($K_s^{"}X$), was lower for the lactate oxidation reaction ($K_s^{"}X$ = 0.12 g l^{-1}, estimated at the mean biomass concentration).

Using the Monod and Contois expressions for the fermentative and oxidative lactate metabolic pathways respectively, the competition between lactate fermenters (LF) and oxidisers (LO) was modelled by incorporating the kinetic constants estimated above and varying the residual lactate concentration in the range 0.0 to 25.0 g l^{-1} (as detected in the current study) (Section 4.3.1).

Table 7.6: Kinetic constants based on lactate utilisation using the Contois and Monod expressions (including the correlation coefficient values from the linearised plots).

Model	Lactate Metabolic pathways	μ_{max} (h^{-1})	K_s (g l^{-1})	R^2
Monod	Fermentation	0.3	3.30	0.96
Contois	Oxidation	0.2	*0.12	0.99

*The K_s value obtained using the Monod equation (Equation 2.28) is equivalent to $K_s^{"}X$ in the Contois expression (Equation 2.31). $K_s^{"}$= 0.6. The mean value of the biomass observed when S_o= 1.0 g l^{-1} was used, X= 0.2 g l^{-1}.

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For the simulation of lactate oxidation, using the Contois model, the minimum and the maximum values of the bacterial concentrations observed when S_o= 1.0 g l^{-1}, i.e. 0.17 and 0.27 g l^{-1} respectively, were tested. There was a marginal difference in the trends when the biomass was varied at these two values, hence the average value of the bacterial concentration was assumed in this study, i.e. X= 0.2 g l^{-1}.

As depicted in Figure 7.6, the dominant utilisers of lactate were the lactate oxidisers at low lactate concentrations increasing in the range 0.0 to 5.0 g l^{-1}. Beyond this range, as the lactate concentration was varied between 6.0 and 25.0 g l^{-1}, the lactate fermenters became a better competitor for lactate utilisation. This explains the observations stated in Sections 4.3.1 and 5.3.1, and the hypotheses drawn from these observations as stated in Section 4.3.2.1. Lactate oxidation and the concurrent reduction of sulphate was the dominant reaction in the 1.0 g l^{-1} sulphate-fed experiment (Section 4.3.1.1). In this experiment, the feed lactate concentration was 2.22 g l^{-1} while the detected residual lactate concentrations were in the range 0.015 to 0.029 g l^{-1}. On the contrary, as the feed sulphate concentration increased from 2.5 to 15.0 g l^{-1} the corresponding feed and residual lactate concentrations were 5.56 to 33.3 g l^{-1} and 4.0 to 25.0 g l^{-1} respectively. The dominance of lactate fermentation in the experiments with feed sulphate concentrations 2.5 to 15.0 g l^{-1} described in Sections 4.3.2.1 and 5.3.1 are attributable to high lactate concentrations.

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This observation is supported by reports from previous studies describing the competition between sulphate-reducing bacteria and fermentative bacteria (Veldkamp *et al.*, 1984; Szewzyk and Pfennig, 1990; Zellner *et al.*, 1994) (Table 7.7). These studies showed that sulphate-reducing bacteria out-competed the fermenting bacteria under limiting conditions of the electron donor, owing to the higher affinity (lower K_s value) for the electron donor by the sulphate reducers (Szewzyk and Pfennig, 1990; Zellner *et al.*, 1994) (Table 7.7).

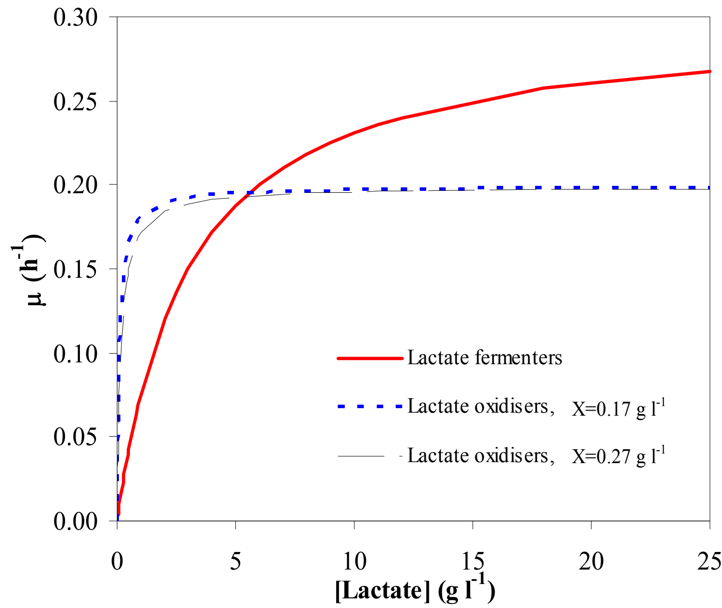

Figure 7.6: Model description of competition for lactate between lactate fermenters and oxidisers in the current study. Specific growth rate (μ) as a function of lactate concentration.

Table 7.7: Comparison of growth kinetic parameters of SRB and lactate fermenters grown on different electron donors.

Reference	Electron donor	*Microorganism	μ_{max} (h^{-1})	K_s (g l^{-1})
Szewzyk and Pfennig (1990)	Ethanol	*Pelobacter propionicus* (EF)	0.096	0.0025
		Desulfobulbus propionicus (EO)	0.033	0.00064
Zellner *et al*. (1994)	Lactate	*Clostridium* spp. (EF)	0.71	0.22
		Desulfovibrio sp. (EO)	0.25	0.13

*: EF, electron donor fermenting bacteria; EO, electron donor oxidising bacteria

The study by Zellner *et al*. (1994) demonstrated that the growth of *Clostridium* spp. was favoured by the high initial concentration of lactate (3.56 g l^{-1}) while the growth of a *Desulfovibrio* strain increased following the degradation of 50% of this lactate. This phenomenon was attributed to the kinetic properties of these microorganisms. *Clostridium* spp. were characterised by high μ_{max} (0.71 h^{-1}) and K_s (0.22 g l^{-1}) values while the μ_{max} and K_s values for the SRB species were 0.25 h^{-1} and 0.13 g l^{-1} respectively. Zellner *et al*. (1994) concluded that these kinetic parameters were the critical determinants of the shift in the lactate metabolic pathway.

Validation of the Simulation

The experimental data supporting the simulation provided above is represented using the steady-state data for 1.0 and 5.0 g l^{-1} (Figure 7.7). Data from the experiment using

a 1.0 g l^{-1} feed sulphate concentration show the dependence of volumetric sulphate reduction rate (VSRR) on the dilution rate under conditions where the biological sulphate reduction reaction and concomitant lactate oxidation reaction were dominant. As depicted in Figure 7.7, the volumetric sulphate reduction rate in the experiment receiving 1.0 g l^{-1} consistently increased with increasing dilution rate. In this experiment, over the range of dilution rates studied (0.0083 to 0.042 h^{-1}), low residual lactate concentration was maintained, in the range 0.005 to 0.026 g l^{-1}. On the contrary, in the 5.0 g l^{-1} sulphate-fed experiment, increasing VSRR with an increase in dilution rate was observed within the range at which residual lactate concentrations 0.9 to 3.9 g l^{-1} were detected (D= 0.0083 to 0.021 h^{-1}).

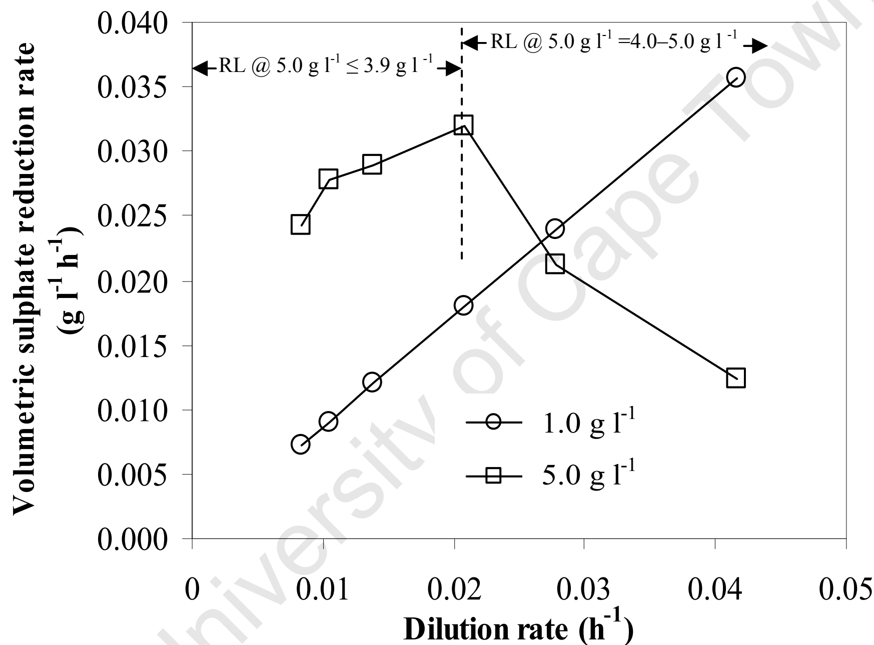

Figure 7.7: Validation of the simulation describing the competition between lactate oxidation and fermentation. Dependency of volumetric sulphate reduction rate on dilution rate. Dotted line distinguishes between regions of the predominance of lactate oxidation at low residual lactate (RL) concentrations (0.9 to 3.9 g l^{-1}) and fermentation at high residual lactate (RL) concentrations (4.0 to 5.0 g l^{-1}) when S_o= 5.0 g l^{-1}. At S_o= 1.0 g l^{-1} low RL (0.005 to 0.029 g l^{-1}) was maintained over the range of dilution rates studied (0.0083 to 0.042 h^{-1}).

Further increase of dilution rate beyond this range shows a decreasing trend in VSRR. The corresponding residual lactate concentrations within this range of dilution rates (D= 0.028 to 0.042 h^{-1}) were 4.0 to 5.0 g l^{-1} (Figure 7.7). This range is congruent with the region in which lactate fermentation was the predominant reaction (Figures 4.7 and 5.1). The corresponding feed lactate concentrations in the reactors fed with 1.0

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and 5.0 g l^{-1} sulphate were 2.2 and 11.1 g l^{-1} respectively. These results are in agreement with the simulation presented in Figure 7.6.

7.3.2.3. Model Description of the Effect of Sulphide on the Competitive Interaction

Based on the observation reported in the current study (Section 7.2.2) and the report of Laanbroek *et al.* (1983) described above, the estimated specific growth rate of the lactate fermenting culture (0.3 h^{-1}) was reduced to 0.15 h^{-1} to simulate sulphide inhibition of lactate fermentation. The μ_{max} value of LO, and the K_s values for LO and LF were kept constant (Table 7.8).

Table 7.8: Assumed kinetic constants in the 10.0 gl^{-1} sulphate-fed experiment.

Model	Lactate Metabolic pathways	μ_{max} (h^{-1})	K_s ($g\ l^{-1}$)
Monod	Fermentation	0.15	3.30
Contois	Oxidation	0.20	*0.12

*The K_s value obtained using the Monod equation (Equation 2.28) is equivalent to $K_s^{''}X$ in the Contois expression (Equation 2.31). $K_s^{''}$ = 0.6. The mean value of the biomass observed when S_o= 1.0 g l^{-1} was used, X= 0.2 g l^{-1}.

The profile presented in Figure 7.8 describes the continued dominance of lactate oxidation at feed sulphate concentration of 10.0 g l^{-1} with increasing dilution rate despite the high feed (22.3 g l^{-1}) and residual (8.0 to 9.0 g l^{-1}) lactate concentrations in this experiment (Sections 4.3.2.1 and 5.3.1). The trend was attributable to high sulphide concentrations (0.3 to 0.6 g l^{-1}) detected in the reactor receiving media containing 10.0 g l^{-1} of sulphate which inhibited the lactate fermenters. This results in the feed lactate being available for the reduction of sulphate.

As shown in Figure 7.9, the results obtained from the experiment describing the kinetics of BSR at a feed sulphate concentration of 10.0 g l^{-1} presented in Section 4.3.1.4 are in good agreement with the simulation presented in Figure 7.8.

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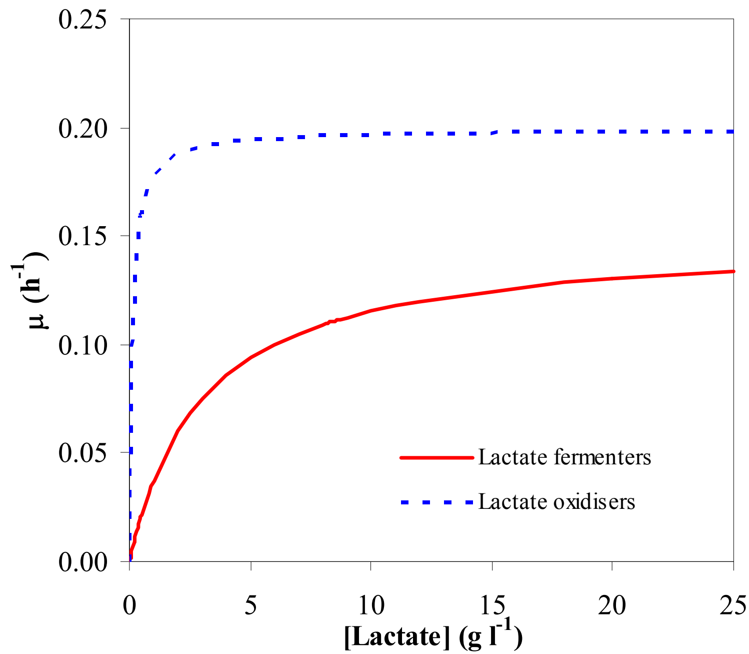

Figure 7.8: Model description of the observation at 10.0 gl^{-1} sulphate in the presence of high sulphide concentration (0.3 to 0.6 g l^{-1}) at which the specific growth rate (μ) is estimated to be reduced by 50%. Specific growth rate (μ) as a function of lactate concentration.

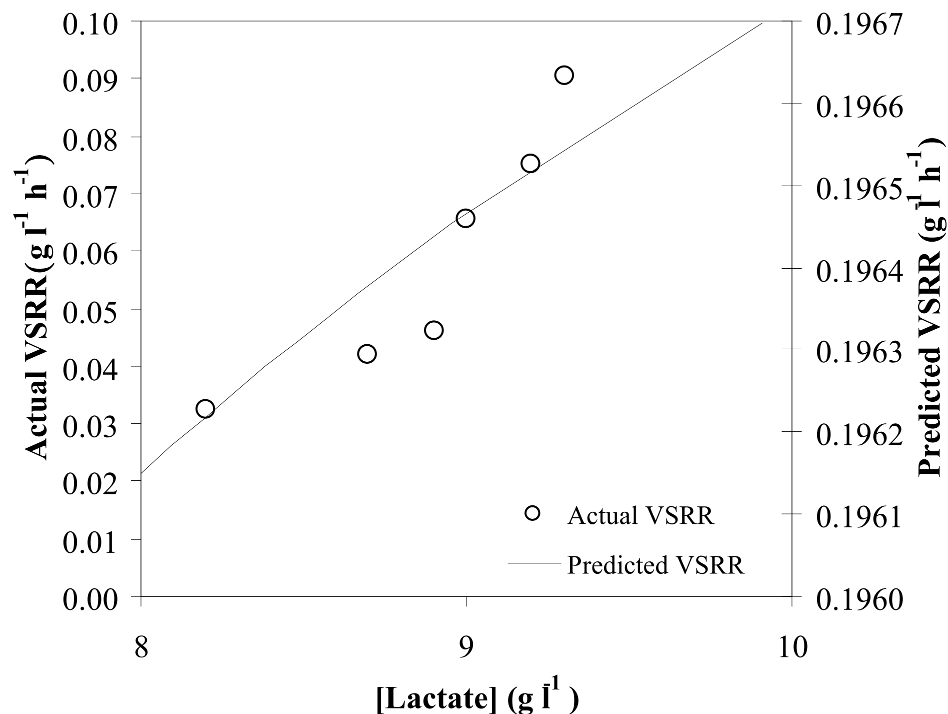

Figure 7.9: Assessing simulation of the competition between lactate oxidation and fermentation at feed sulphate concentrations 10.0 g l^{-1} in the presence of high sulphide concentrations (0.3 to 0.6 g -1). Comparison of the predicted VSRR under conditions where lactate oxidation was dominant (S_o= 1.0 g l^{-1}) with the actual VSRR when S_o= 10.0 g l^{-1}. Dependency of volumetric sulphate reduction rate on residual lactate concentration.

The predicted VSRR was estimated using the specific growth rate values presented in Figure 7.8 and the biomass yield based on sulphate reduction ($Y_{x/s}$= 0.20 g g^{-1}), when S_o= 1.0 g l^{-1}, i.e. under conditions where lactate oxidation was dominant. The trends representing the model description developed using S_o= 1.0 g l^{-1} and the experimental volumetric sulphate reduction rate collected at S_o= 10.0 g l^{-1} reveal that the VSRR increased with increasing residual lactate concentration in the range 8.0 to 10.0 g l^{-1} (Figure 7.9).

7.4. EFFECT OF FEED LACTATE CONCENTRATION ON BSR KINETICS AND THE COMMUNITY STRUCTURE

According to the simulation shown in Figure 7.6, decreasing the residual lactate concentration by reducing the feed lactate concentration should encourage the proliferation of lactate oxidisers (SRB) over lactate fermenters. Sulphate reducers are better scavengers of lactate under limiting conditions of the substrate in comparison to lactate fermenters (Figure 7.6). In order to investigate the effect of feed lactate concentration on the kinetics of BSR, two reactors were set-up. The feed sulphate concentration was maintained at 10.0 g l^{-1}, while the feed lactate concentration was varied from 36 to 63% of the stoichiometric lactate requirement for sulphate reduction, calculated according to Reaction 5.1 (Table 5.1). These were compared with results reported in Section 4.3.1.4 for the kinetics studies of 10.0 g l^{-1} sulphate-fed reactor receiving a lactate concentration of 120% of the stoichiometric requirement across residence times in the range 1 to 5 d to yield steady-state data.

As shown in Tables 7.9a and 7.9b the volumetric sulphate conversion and sulphate reduction rate were typically enhanced by decreasing the feed lactate concentration from 120% to 63% of stoichiometric requirement. Conversely, Tables 7.9c to 7.9e illustrate that the volumetric rates of lactate utilisation, acetate production and propionate production were reduced when the lactate feed concentration was reduced from 120 to 36% of the stoichiometric requirement. This was a consequence of the electron donor limitation. As shown in Table 7.9f, the enhanced reactor performance can be attributed to the more effective use of lactate for sulphate reduction when less lactate was made available to the bacteria consortium.

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Table 7.9: Steady-state data of continuous reactors investigating the effect of feed lactate concentration (36 to 120 % of the stoichiometric requirement for BSR) on biological sulphate reduction kinetics. (a) Sulphate conversion; (b) Volumetric sulphate reduction rate; (c) Volumetric lactate utilisation rate; (d) Volumetric acetate production rate; (e) Volumetric propionate production rate; (f) Relative lactate oxidised; (g) Bacterial dry mass.

(a)

Residence time (d)	Dilution rate (h^{-1})	Sulphate conversion		
		120%	63%	36%
5	0.0083	39	49	nd
4	0.010	40	nd	30
3	0.014	33	37	nd
1	0.042	22	21	nd

(b)

Residence time (d)	Dilution rate (h^{-1})	Volumetric sulphate reduction rate ($g\ l^{-1}\ h^{-1}$)		
		120%	63%	36%
5	0.0083	0.032	0.047	nd
4	0.010	0.042	nd	0.031
3	0.014	0.046	0.057	nd
1	0.042	0.090	0.090	nd

(c)

Residence time (d)	Dilution rate (h^{-1})	Volumetric lactate utilisation rate ($g\ l^{-1}\ h^{-1}$)		
		120%	63%	36%
5	0.0083	0.11	0.085	nd
4	0.010	0.15	nd	0.091
3	0.014	0.18	0.12	nd
1	0.042	0.55	0.44	nd

(d)

Residence time (d)	Dilution rate (h^{-1})	Volumetric acetate production rate ($g\ l^{-1}\ h^{-1}$)		
		120%	63%	36%
5	0.0083	0.041	0.034	nd
4	0.010	0.055	nd	0.036
3	0.014	0.061	0.056	nd
1	0.042	0.124	0.12	nd

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(e)

Residence time (d)	Dilution rate (h^{-1})	Volumetric propionate production rate ($g\ l^{-1}\ h^{-1}$)		
		120%	**63%**	**36%**
5	0.0083	0.013	0.011	nd
4	0.010	0.022	nd	0.0060
3	0.014	0.033	0.025	nd
1	0.042	0.220	0.140	nd

(f)

Residence time (d)	Dilution rate (h^{-1})	Relative lactate oxidised (%)		
		120%	**63%**	**36%**
5	0.0083	54	83	Nd
4	0.010	53	nd	85
3	0.014	47	80	nd
1	0.042	30	38	nd

Lactate oxidised was expressed as a percentage of the total lactate utilised. Lactate oxidised was estimated from the sulphate reduction data, based on the theoretical ratio of the incomplete lactate oxidation reaction (Reaction 5.1, Table 5.1) i.e. moles of lactate utilised per unit mole of sulphate reduced = 2.
nd: not determined.

(g)

Residence time (d)	Dilution rate (h^{-1})	Bacterial dry mass ($g_{DW}\ l^{-1}$)		
		120%	**63%**	**36%**
5	0.0083	0.588	0.411	nd
4	0.010	0.528	nd	0.248
3	0.014	0.500	0.367	nd
1	0.042	0.444	0.300	nd

As discussed previously, lactate fermenters are characterised by a lower affinity for lactate (Section 7.3.2.2). It thus follows that lactate oxidisers will be the predominant group at lower feed lactate concentration. Lower biomass concentrations were observed at the reduced feed lactate concentrations (Table 7.9g). Therefore, the specific sulphate reduction rate was also positively influenced by the reduced feed lactate concentration (Figure 7.10). This suggests that fewer non-SRB bacteria were present in the reactors where lactate supply was lower than the stoichiometric requirement for the sulphate reduction. It does not however necessarily suggest a change in the specific rate of metabolism of sulphate by the sulphate reducers.

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The microbial community analyses revealed that the same SRB community structure was maintained under conditions when 120 and 63% feed lactate concentration of its stoichiometric requirement were used. The enhanced volumetric sulphate reduction rate observed when the feed lactate concentration was reduced implies that improved reactor performance did not necessarily result from increased SRB biodiversity but as a consequence of increased activity of the SRB community members which was coincident with the shift of lactate metabolism towards the oxidative pathway.

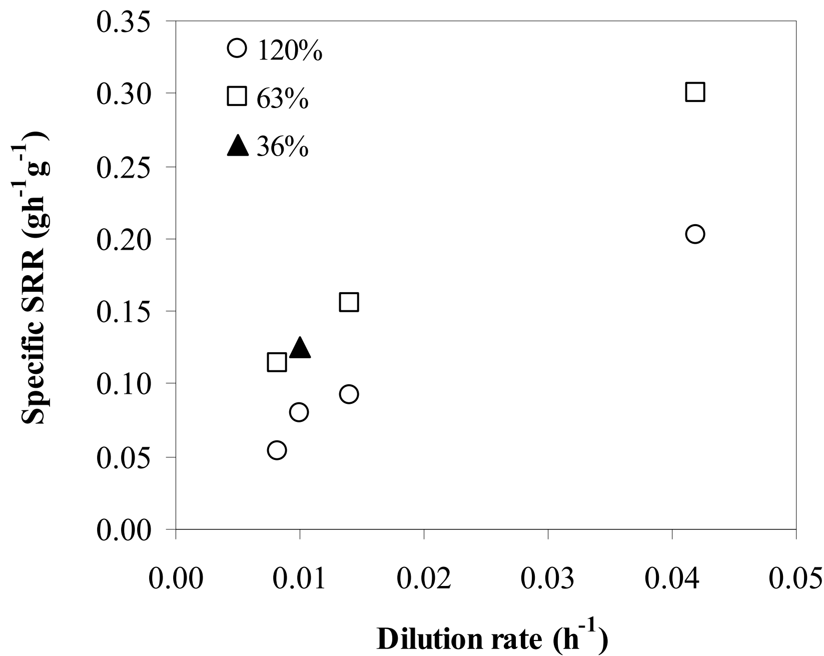

Figure 7.10: Investigation of the effect of feed lactate concentration on specific sulphate reduction rate. Feed sulphate concentration was kept constant at 10.0 g l^{-1} while the feed lactate concentration was varied in the range 36 to 120% of the stoichiometric requirement.

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7.5. CONSIDERATION OF REACTOR CONFIGURATION

To exploit the observation that SRB are better competitors for lactate under conditions of limiting lactate concentrations, it can be expected that sulphate reduction can be enhanced by operation of reactors in series, to which the total lactate feed is divided. Two reactors were set up in series, such that the first reactor (R_1) received 10.0 g l^{-1} feed sulphate concentration and feed lactate concentration of 36% of the stoichiometric lactate requirement for sulphate reduction (S_o). The second reactor (R_2) was fed with the effluent stream from R_1 (S_1 and X_1), such that the residual sulphate and lactate from R_1 served as the feed sulphate and lactate for R_2 (Figure 7.11).

Reactor R_2 received an additional lactate feed (63% of the stoichiometric lactate requirement for sulphate reduction at 10.0 g l^{-1} feed sulphate concentration) (S'_o), at a flow rate of 3.5 ml h^{-1}. Both reactors were maintained at a constant residence time of 4 days (D= 0.010 h^{-1}). Other operating conditions were as described in Sections 3.2 and 4.2.1, with the temperature and pH maintained at 35°C and pH 8.0 ± 0.2 respectively.

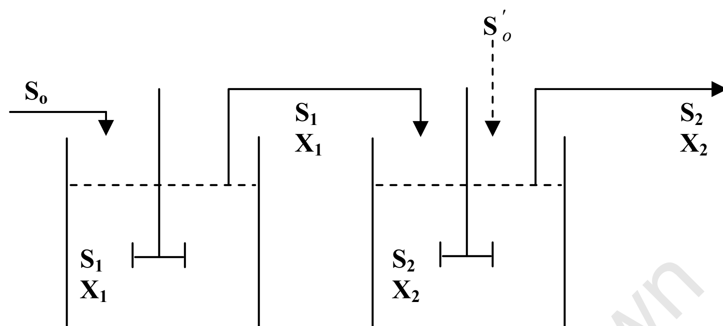

Figure 7.11: Schematic diagram of experimental set-up for Section 7.5 experiment. A multi-stage chemostat system composed of reactors R_1 and R_2 in series: S_o, feed substrates (10.0 g l^{-1} sulphate and 6.6 g l^{-1} lactate), S'_o = feed lactate concentration (11.1 g l^{-1}), S_1 and S_2 = residual substrate concentration (lactate and sulphate) in reactors R_1 and R_2 respectively, X_1 and X_2 = biomass in reactors R_1 and R_2 respectively.

Table 7.10: Steady-state data of continuous reactors in series (R_1 and R_2) investigating the effect of reactor configuration on biological sulphate reduction kinetics.

Reactor	Biomass (g l^{-1})	Sulphate conversion (%)	VSRR (g l^{-1} h^{-1})	Sulphide concentration (g l^{-1})	Relative lactate oxidised (%)	Average stoichiometric ratio (L:A)
R_1	0.39	26	0.027	0.25	73	1.7
R_2	0.29	63	0.056	0.60	53	1.1
*R_{Net}	-	69	0.042	-	60	1.3

Lactate oxidised was expressed as a percentage of the total lactate utilised. Lactate oxidised was estimated from the sulphate reduction data, based on the theoretical ratio of the incomplete lactate oxidation reaction (Reaction 5.1, Table 5.1) i.e. moles of lactate utilised per unit mole of sulphate reduced = 2.
*R_{Net}: Refers to the entire unit i.e. R_1 and R_2. Values describe the overall performance of the multi-stage reactor.

As illustrated in Table 7.10, the reactor performance as measured by the sulphate conversion, volumetric sulphate reduction rate and relative lactate oxidised was enhanced by the two-stage reactor design. Higher overall sulphate conversion and volumetric sulphate reduction rate of 69% and 0.042 g l^{-1} h^{-1} respectively were achieved in this system in comparison with the values recorded when the reactor receiving were operated in a single-stage. Single-stage reactors receiving 120 and

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36% of the stoichiometric lactate requirement for sulphate reduction were characterised by sulphate conversions of 40 and 30% and VSRR values of 0.042 and 0.031 g l^{-1} h^{-1} respectively (Tables 7.9a and 7.9b). This improvement in reactor performance can be attributed to a phenomenon whereby the reactor R_1 encourages dominance by the SRB group owing to the limiting feed concentration of lactate (36% of the stoichiometric lactate requirement for sulphate reduction). It thus follows that the second reactor R_2 will be augmented with a biomass constituted of a high proportion of SRB.

Further, a higher fractional lactate conversion by oxidation was observed in reactor R_2, indicating a more effective utilisation of lactate for the conversion of sulphate to sulphide (Table 7.10). This observation is corroborated by a shift in the reaction stoichiometry towards incomplete lactate oxidation as indicated by the decrease in the L:A ratio from 1.7 to 1.0 (Table 7.10). These results reiterate the need to consider culture conditions and reactor configuration that encourage the optimisation of lactate utilisation via the oxidative pathway in preference to fermentation. This will aid in maximising the efficiency of biological treatment of AMD using SRB.

7.6. SUMMARY

In this chapter, it was shown that culture conditions and reactor configuration influenced the kinetics of biological sulphate reduction, reaction stoichiometry and microbial growth. Further, the responses of the BSR kinetics were consequences of the effects of these parameters (sulphate, lactate and sulphide concentrations) on the reaction stoichiometry and the microbial consortia involved. Using previously developed mathematical expressions, sulphate was shown not to limit the microbial growth of the mixed culture employed in the current study. In contrast, the BSR kinetics were shown to be dependent on lactate limitation. Assuming lactate limitation, using the Monod model, determination of the growth parameters K_s and μ_{max} of the mixed culture employed in the current study revealed a higher maximal growth rate μ_{max} of 0.3 h^{-1} and a lower affinity for lactate with K_s of 3.3 g l^{-1} for the lactate fermenters. On the contrary, the lactate oxidisers (SRB) were characterised by lower values of μ_{max} (0.2 h^{-1}) and $K_s'' X$ (equivalent to the K_s obtained from the Monod model) (0.12 g l^{-1}), using the Contois expression. By incorporating these biokinetic

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constants into the Contois and Monod expressions, the competition between lactate fermenters and lactate oxidisers was demonstrated. The latter are better scavengers of lactate under limiting lactate conditions while lactate fermenters prevail under conditions of higher lactate concentrations. At a high feed sulphate concentration (10.0 g l^{-1}), stoichiometrically limiting feed lactate concentrations (36 and 63% of the stoichiometric requirement for sulphate reduction) enhanced the sulphate conversion, volumetric sulphate reduction rate and specific sulphate reduction rate. This implies that, in the biological sulphate reduction treatment of sulphate-rich wastewaters, the staggered addition of lactate may improve the efficiency of the treatment process.

Further, lactate fermentation was found to be strongly inhibited at a sulphide concentration of 0.5 g l^{-1}. This phenomenon prevents limitation of the biological sulphate reduction process by lactate fermentation. By incorporating the result of the inhibition of lactate fermentation and assuming a reduction of the maximal growth rate of lactate fermenters by 50% in the presence of high concentrations of sulphide, lactate oxidation was shown to be the preferred pathway of lactate metabolism despite the presence of high lactate concentrations in the 10.0 g l^{-1} sulphate-fed reactor. Sulphide stripping, a common practice in acetate- and ethanol-fed BSR reactors, may be unnecessary and unbeneficial for lactate-fed reactors, except at sulphide concentrations inhibitory to the SRB. Inhibitory sulphide concentrations have been shown to be higher for lactate oxidisers than acetate or ethanol oxidisers (Kuo and Shu, 2004).

A two-stage chemostat, composed of two reactors linked in series, was shown to favour the selective enrichment of lactate oxidisers in preference to lactate fermenters. Improvement in BSR kinetics was achieved as the effluent stream from the first reactor, constituted of a higher proportion of SRB, was fed into the subsequent reactor. In the second reactor, lactate supplementation was used effectively for lactate oxidation owing to the lactate concentration in the reactor being maintained in the range favouring its oxidation. High sulphide concentration (0.6 g l^{-1}), inhibitory to lactate fermenters was also detected in this reactor. These factors led to a further reduction of the residual sulphate fed from the preceding reactor, resulting in an overall higher sulphate conversion and fractional use of lactate to support sulphate reduction.

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CHAPTER 8
GENERAL CONCLUSIONS AND RECOMMENDATIONS

8.1. INTRODUCTION

Acid mine drainage (AMD) is a detrimental by-product of mining activities. It emanates from surface and underground mine workings, waste and development rock, and tailings. AMD poses a potential problem owing to its characteristic composition of acidity, sulphate and heavy metals. The management of this effluent has been the subject of intensive research. Anaerobic sulphate reduction, mediated by sulphate-reducing bacteria (SRB), has been successfully employed in the treatment of AMD.

The current study was carried out to investigate the effect of volumetric sulphate loading rate, controlled by the feed sulphate concentration and the dilution rate, on the kinetics and stoichiometry of the sulphate reduction process, as well as the community dynamics of the microbial consortia mediating this process. Owing to the potential of lactate to support a wide range of sulphate reducers, its utilisation was hypothesised to confer system resilience and resistance. Hence it was used as the carbon-source and electron donor in the current study. The study of the relationship between biokinetics, reaction stoichiometry and community dynamics was also postulated to provide a holistic approach in the optimisation of the process of biological sulphate reduction. One litre chemostat cultures (pH 8 ± 0.2, 35°C) were monitored under varying conditions of feed substrate concentration (sulphate and lactate) and dilution rates. The major findings from this work are presented in this chapter. Further, areas of prospective investigations in this field of study are also highlighted.

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8.2. CONCLUSIONS

Chemostat reactors were operated at feed sulphate concentrations of 1.0 to 15.0 g l^{-1}. These reactors received lactate concentrations of 120% of the stoichiometric requirement across residence times in the range 0.5 to 5.5 d to yield steady-state data. At residence times of 5 to 3 d (D= 0.0083 to 0.014 h^{-1}), the volumetric sulphate reduction rate was enhanced by the volumetric sulphate loading rate, in the feed sulphate concentration range of 1.0 to 10.0 g l^{-1}. This trend was accompanied by increased bacterial dry mass. The maximum bacterial dry mass values were 0.267 and 0.588 gl^{-1} for feed sulphate concentrations 1.0 and 10.0 g l^{-1} respectively. At the

residence time of 3 d (D= 0.014 h^{-1}), a VSRR of 0.012 and 0.046 g l^{-1} h^{-1} were obtained for feed sulphate concentrations 1.0 and 10.0 g l^{-1} respectively. The reaction stoichiometry was consistent with the process kinetics. Incomplete oxidation of lactate, which supports sulphate reduction, was dominant in the range of feed sulphate concentrations of 1.0 to 10.0 g l^{-1} and residence times of 5 to 3 d. As the dilution rate was increased in the range of 0.021 to 0.042 h^{-1} (residence times of 2 to 1 d), the volumetric sulphate reduction rates decreased with increasing volumetric loading rate (VLR) for feed sulphate concentrations 2.5 and 5.0 g l^{-1}. On the other hand, the VSRR steadily increased with increasing VLR across all dilution rates studied (D= 0.0083 to 0.042 h^{-1}) in the 1.0 and 10.0 g l^{-1} sulphate-fed reactors as hypothesised.

The significant decrease in VSRR observed for feed sulphate concentrations of 2.5 and 5.0 g l^{-1} was not commensurate with a decrease in bacterial dry mass. This observation implied a shift in the primary metabolic pathway. The analyses of the reaction stoichiometry supported this observation. The yield of propionate from lactate metabolism increased at feed sulphate concentrations of 2.5 to 10.0 g l^{-1}, indicating the occurrence of lactate fermentation. At each feed concentration of sulphate, in the range 2.5 to 10.0 g l^{-1}, varying dilution rates resulted in significant shifts in metabolic pathways. Lactate oxidation was higher at a feed sulphate concentration of 10.0 g l^{-1} relative to 2.5 and 5.0 g l^{-1}. Results indicated that increased lactate concentration as observed in the 2.5 and 5.0 g l^{-1} sulphate-fed reactors encouraged the proliferation of lactate fermenting microorganisms, owing to their characteristic high K_s. Metabolic inhibition was evident at the feed sulphate concentration of 15.0 g l^{-1}. This was revealed by the poor reactor performance and lower bacterial dry mass as compared to what was observed in the 10.0 gl^{-1} sulphate-fed reactor.

The shifts in the lactate utilisation pathways with varying experimental conditions made determining the kinetic parameters describing the SRB growth difficult. Hence, the kinetics of lactate fermentation, in the absence of sulphate was investigated. Residence times were varied in the range 0.5 to 3.0 d. In this experiment, conventional techniques including optical microscopy and Gram staining revealed the presence of 5 to 6 morphotypes composed of both Gram positive and Gram negative bacterial strains. The study also revealed that lactate fermentation was significantly

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inhibited by high sulphide loading (0.5 g l^{-1}), both in terms of lactate utilisation and bacterial dry mass. This observation implied that lactate fermenters were inhibited by high sulphide concentrations (0.3 to 0.6 g l^{-1}) obtained during operation at a feed sulphate concentration of 10.0 g l^{-1}. Consequently the oxidative pathway for lactate utilisation dominated.

Using the steady-state experimental data and the Monod model, assuming lactate limitation, the biokinetic constants of the mixed culture employed in the current study revealed a higher maximum specific growth rate μ_{max} of 0.3 h^{-1} and a lower affinity for lactate with K_s of 3.3 g l^{-1} for the lactate fermenters. On the contrary, the lactate oxidisers (SRB) were characterised by lower values of μ_{max} (0.2 h^{-1}) and $K''_s X$ (equivalent to the K_s obtained from the Monod model) (0.12 g l^{-1}), determined using the Contois expression. Modelling of the competition between lactate fermenters and lactate oxidisers illustrated that lactate oxidisers compete more effectively for lactate under conditions of low lactate concentrations (≤5 g l^{-1}) while lactate fermenters out-compete the oxidisers under conditions of higher lactate concentration (>5 g l^{-1}). These simulations were consistent with the observations recorded at the feed sulphate concentrations 1.0 to 10.0 g l^{-1}. Further, the maximum specific growth value of the lactate fermenters was adjusted to account for sulphide inhibition based on a previous study. The simulation under these conditions showed that despite high lactate concentrations (>5 g l^{-1}), the lactate oxidation reaction was the predominant metabolic pathway. This was attributed to the inhibition of lactate fermentation by high sulphide concentration, further corroborating the trends observed at a feed sulphate concentration of 10.0 gl^{-1} relative to 2.5 and 5.0 g l^{-1}.

Using the simulations as operating principles, a multi-stage chemostat system, composed of two reactors arranged in series, was shown to improve the BSR efficiency. This was achieved by selectively enriching the lactate oxidisers in preference over lactate fermenters. The first reactor in the series selected for lactate oxidisers under conditions of limiting lactate. The subsequent reactor was augmented by a biomass rich in lactate oxidisers from the effluent stream of the first reactor. The maintenance of a low lactate concentration favoured the effective utilisation of lactate via the oxidative pathway.

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Results showed that lactate, utilised as the sole electron donor in this study, supported the growth of a diverse microbial community. Due to the source of the mixed culture employed in this study, the pit of a facultative pond treating sewage, the starting inoculum was composed of lactate fermenters and lactate oxidising bacteria. Using the 16S rRNA gene restriction enzyme analyses, 5 of the possible 16 SRB species (previously described in the literature) were identified. These are as follows: *Desulfobulbus propionicus*, *Desulfobacter postgatei*, *Desulfovibrio gigas*, *Desulfosarcina variabilis* and *Desulfococcus multivorans.* Of these strains, *Desulfobulbus propionicus*, *Desulfobacter postgatei* and *Desulfococcus multivorans* persisted under most experimental conditions. This was in agreement with the kinetic properties of these microorganisms as identified in the literature. The analyses revealed that some DNA remained uncut. This was consistent with the presence of non-SRB lactate fermenters because the restriction enzymes were primarily selected to identify SRB strains. When the reactor operated in the absence of sulphate, to study the lactate fermentation kinetics, was converted to an oxidative reactor receiving 1.0 g l^{-1} feed sulphate concentration, lactate oxidation was restored. This implied the presence of lactate fermenting SRB which maintained high enough growth rates to prevent their washout under conditions where lactate fermentation was the predominant reaction. This was consistent with the presence of *Desulfobulbus propionicus*. This SRB strain is able to metabolise lactate in the absence of sulphate.

Further, the FISH analysis indicated the presence non-sulphate-reducing bacterial populations. In the reactors associated with metabolic shifts from predominnatly lactate oxidation to lactate fermentation, the proportion of SRB relative to the total cells decreased. Across the range of feed sulphate concentrations and dilution rates studied, species richness, quantified as the number of SRB strains detected under different operating conditions, declined with both increasing feed sulphate concentration and dilution rate. Low SRB diversity at feed concentrations of 2.5 and 5.0 g l^{-1} corresponded to the predominance of the lactate fermentative pathway and low reactor resilience. At feed sulphate concentrations of 1.0 and 10.0 gl^{-1}, a higher SRB diversity was associated with high volumetric sulphate reduction rates, predominance of lactate oxidation and high reactor resilience. Similar to the previous work using ethanol as the sole carbon-source and electron donor (Hansford *et al.*,

2007), the mixed culture employed in this study was not able to oxidise the substrate completely, resulting in the accumulation of acetate.

Results obtained highlight the need to develop a system in which a diverse microbial community, constituted by active and versatile population members is maintained.

8.3. RECOMMENDATIONS

Based on the general findings reported in the current study, investigations that would aid in the further improvement of the application of the anaerobic sulphate reduction process in the treatment of sulphate-laden streams are highlighted in the following sections:

1. **Investigation of the effect of biomass retention on SRB community structure**: The data obtained from the current study revealed that the removal of certain microbial population members (decrease in SRB population diversity) with increasing dilution rate and sulphate loading rate can jeopardise the performance of a biosulphidogenic reactor. Hence, operation at high residence times is imperative. Further, the study indicated that restoration of less extreme operating conditions does not always allow reactor performance to return to initial levels. This highlights a need to develop a system in which microbial diversity is maintained to prevent compromising of the reactor performance on system perturbation. The choice of chemostat as the research tool in the current study facilitated the collection of rigorous kinetic data and the demonstration of mixed population interactions such as competition. Immobilised cell reactor systems encourage high biomass concentration at short residence times, thus giving a better performance in comparison to suspended cell systems (Baskaran and Nemati, 2006). These systems also withstand high loading rates which would normally lead to substrate inhibition in the suspended systems (Kaksonen *et al*., 2006). A more diverse structure or a species-rich ecosystem is expected to confer process resilience to perturbations and enhanced performance to a heterogeneous system. An investigation of reactor design for biomass retention would be useful. Reactors such as the upflow anaerobic sludge blanket (UASB) facilitate the uncoupling of microbial growth rate and residence time. This allows operation of the wastewater treatment processes to be carried out over shorter periods.

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Microbial community structure with cell retention is more dominantly influenced by the tendency for retention than microbial growth rate. Moreover, a robust reactor performance that is associated with increased microbial diversity can be maintained during a continuous wastewater treatment process.

2. **Creating an optimum biodiversity through the combination of "ideal" and dominant microbial groups adapted to function efficiently within the operating conditions found in wastewater treatment processes**: Results from the present study indicated the selection of certain SRB strains adapted to function at extreme conditions of high dilution rates and feed sulphate concentrations. Based on this, a tailored, mixed consortium of microorganisms identified through microbial population studies may lead to a better resilience and manipulation of wastewater treatment processes.
3. **Further validation with a constructed mixed population**: Using a mixed population comprised of known organisms, a structure-function study can be carried out to validate the observations presented in the current study.
4. **Use of effluent from lactate-fed BSR as feed stream for acetate and propionate oxidising cultures**: Significant levels of the products of lactate metabolism (acetate and propionate) were observed in all the experiments carried out in the current study. These products can serve as electron donors for further sulphate reduction. Therefore, a multi-stage BSR system can be designed, such that the effluent stream from lactate-fed BSR reactors is fed to acetate and propionate oxidisers in other bioreactors. This will result in a more effective use of lactate as an electron donor and COD removal.
5. **Use of multi-stage reactor configuration with incremental feeding with lactate to maintain lactate oxidation**: The current study indicated that a multi-stage reactor configuration encourages BSR performance. Hence, operating a series of reactors to which increasing lactate is fed will selectively enrich lactate oxidisers and consequently improve sulphate removal.

It is envisaged that this present research approach will open up new ways for wastewater treatment and also provide a comprehensive means of managing acid mine drainage. The inter-relatedness of the process kinetics, reaction stoichiometry and the microbial community dynamics reported herein has potential for use as a diagnostic tool in the assessment of bioremediation process thus preventing a complete failure of

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the anaerobic treatment reactor. This phenomenon also provides insight into strategies to maximise the efficiency of biological treatment of AMD using SRB.

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APPENDICES

Appendix A

Determination of sulphate concentration. Details of method given in Section 3.3.1.

Standard curve

Mean values (n=3, R^2= 0.9934)

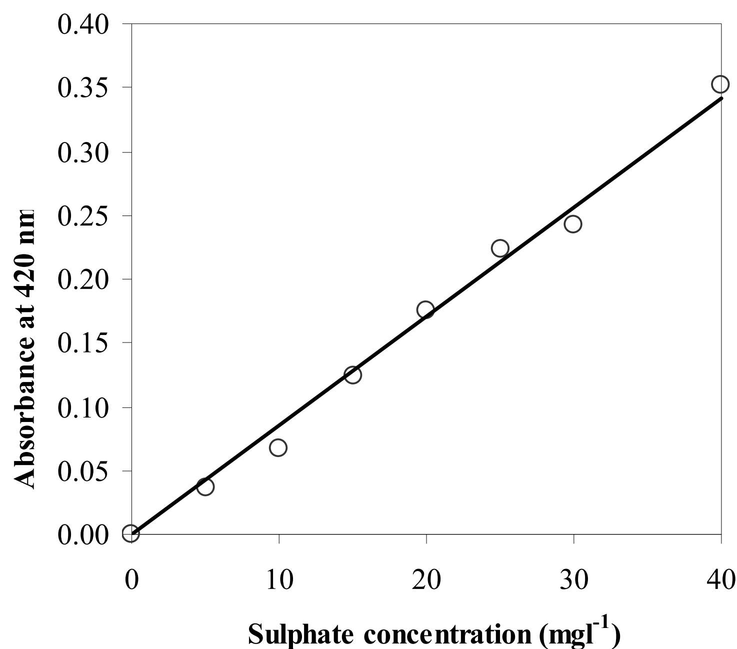

Figure A1: Sulphate standard curve.

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Reagents

1. Conditioning reagent: The following were dissolved 300 ml deionised water, 50 ml glycerol, 30 ml concentrated HCl (32%), 75 g NaCl, 100 ml ethanol.
2. Sulphate stock solution (100 mg l^{-1}): 0.148 g of Na_2SO_4 was dissolved in 1 litre of dH_2O.

Appendix B

Determination of sulphide concentration. Details of method given in Section 3.3.2.

Reagents

1. Amide-sulphuric acid solution: 2 g N-N dimethyl-p-phenylene diamine dihydrochloride was dissolved in 500 ml of 6 M hydrochloric acid. Solution was stored in a dark bottle at room temperature.

2. Ferric chloride: 8 g $FeCl_3$ was added to 500 ml of 6 M hydrochloric acid. Solution was stored in a dark bottle at room temperature.
3. Hydrochloric acid (6 M): 574.2 ml of commercial HCl (32%) was made to a final volume of 1litre.
4. Sulphide stock solution (100 mg l^{-1}): 0.75 g $Na_2S.9H_2O$ was dissolved in oxygen-free dH_2O.
5. 1% Zinc acetate: 10 g of zinc acetate was dissolved in dH_2O and made up to 1 litre.

<u>Standard curve</u>

Mean values (n=3, R^2= 0.9948)

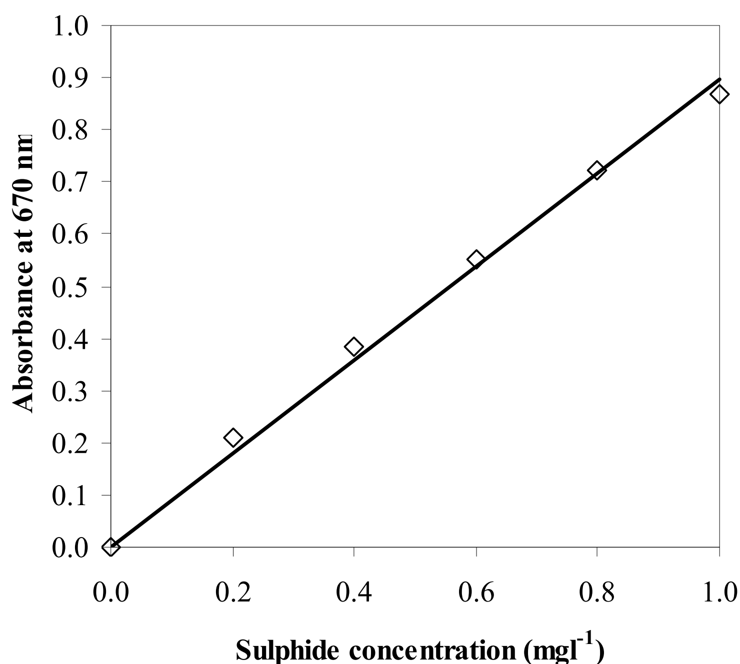

Figure B1: Sulphide standard curve.

Appendix C

Calculation of alkalinity (APHA, 1975). Details of method given in Section 3.3.3.

Total alkalinity as mg l^{-1} $CaCO_3$ equivalents, $C = \frac{A \times N \times 50\,000}{\text{ml sample}}$

where:

A = ml standard acid used and N = normality of standard acid

Total alkalinity as mg l^{-1} $NaHCO_3$ equivalents, $D = C \times \frac{84}{50}$

Molar concentration of bicarbonate = Molar concentration of *D* – Molar concentration of sulphide estimated from analysis.

Reagent

0.1 N H_2SO_4: Commercially available (Analytical grade, Merck Chemicals [Pty], Ltd)

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Appendix D

Table D1: List of target microorganisms and the theoretical banding patterns obtained from the DNAMAN software for Windows program, version 4.13 (1994-99).

ORGANISM	THEORETICAL FRAGMENT SIZES (bp) OBTAINED WITH 21 RESTRICTION ENZYMES																				
	***Apa*I**	***Asp* 700I**	***BbrP* I**	***Bfr***	***Bpu* AI**	***Bse*A I**	***Dra*I**	***Ecl*X I**	***Eco* RI**	***Eco* RV**	***Hpa*I**	***Ksp*I**	***Mlu*I**	***Nco*I**	***Pst*I**	***Pvu*I**	***Sgr*A I**	***Sma*I**	***Sph*I**	***Xba*I**	***Xma* CI**
	SULPHATE-REDUCING BACTERIA																				
Desulfobacter postgatei	595, 432	878, 149	292, 732	-	-	-	637, 390	-	-	820, 205		870, 155	-	912, 115	-	-	-	665, 220, 140	159, 866	-	-
Desulfococcus multivorans	595, 432	879, 148	292, 732	-	-	-	-	-	-	820, 205	364, 355, 306,	502, 369, 154	-	-	-	628, 397	-	886, 139	158, 867	-	-
Desulfomonas pigra	599, 428	877, 150	-	-	-	-	-	-	-	820, 205	360, 667	-	362, 663	-	-	-	-	663, 141, 221	-	-	-
Desulfonema limicola	595, 432	880, 147	292, 733	-	-	-	-	-	-	820, 205	364, 356, 307	872, 153	-	-	-	-	-	887, 140	157, 868	-	-
Desulforhabdus amnigenus	595, 432	879, 148	-	-	-	-	-	-	522, 503	-	-	871, 154	366, 659	-	-	-	-	886, 141	158, 867	-	-
Desulfosarcina variabilis	595, 432	880, 147	292, 733	-	-	-	-	-	-	820, 205	660, 365	872, 153	-	-	-	-	-	502, 385, 140	157, 868	-	-
Desulfotomaculum nigrificans	595, 432	883, 144	-	-	-	-	-	-	1027		661 364	-	-	-	353, 674	-	-	890, 135	-	517, 510	-
Desulfotomaculum orientis	-	-	452, 575	-	-	-	-	-	-			-	-	-	-	-	-	-	-	657, 370	-
Desulfotomaculum ruminis	595, 432	883, 144	-	-	-	-	-	-	-		661, 364	-	-	-	-	-	-	890, 135	-	-	-
Desulfovibrio africanus	-	-	-	-	-	-	-	-	-			-	582, 445	-	-	-	-	-	-	-	-
Desulfovibrio vulgaris	-	504, 374, 149	-	-	-	-	-	-	534, 497		313, 356, 362	-	667, 364	-	-	-	-	-	159, 866	-	-
Desulfovibrio sapovorans	-	-	292, 732	-	-	-	-	-		822, 203		871, 154	-	-	351, 676	632, 395	-	-	158, 867	-	-
Desulfovibrio salexigens	-	-	-	-	-	-	-	-	535, 490	820, 205	363, 672	-	-	-	-	-	-	665, 221, 139	158, 867	516, 509	-
Desulfovibrio desulfuricans	598, 429	878, 149	-	-	-	-	-	-	-	820, 205	355, 360, 310	-	429, 362, 234	-	-	-	-	664, 221, 140	159, 866	-	-
Desulfovibrio gigas	-	-	-	-	-	-	-	-	-	820, 205		-	363, 662	-	-	-	-	498, 527		-	-
Desulfobulbus propionicus	595, 432	884, 143	-	-	-	-	-	-	-	820, 205		876, 149	-	-	-	-	-	891, 134	155, 872	-	-

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Table D1 contd: List of target microorganisms and the theoretical banding patterns obtained from the DNAMAN software for Windows program, version 4.13 (1994-99).

ORGANISM	THEORETICAL FRAGMENT SIZES (bp) OBTAINED WITH 21 RESTRICTION ENZYMES																				
	***Apa*I**	***Asp* 700I**	***BbrP* I**	***Bfr***	***Bpu* AI**	***Bse*A I**	***Dra*I**	***Ec*IX I**	***Eco* RI**	***Eco* RV**	***Hpa*I**	***Ksp*I**	***Mlu*I**	***Nco*I**	***Pst*I**	***Pvu*I**	**SgrA I**	***Sma*I**	***Sph*I**	***Xba*I**	***Xma* CI**
	METHANOGENS																				
Methanosarcina barkeri	762, 268	775, 245	-		-	-	-	624, 396	-	-	-	-	-	-	-	-	-	724, 208	711, 309	-	-
Methanosarcina lacustris	-	775, 245	-	608, 412	-	-	-	624, 396	-	-	-	-	-	-	-	-	628, 358	425 296, 299	-	-	-
Methanosarcina acetivorans	-	774, 246	-		-	-	-	524, 496	-	-	-	-	-	-	-	-	-	425 296, 299	-	-	-
Methanogenium cariaci	-	-	-		-	536, 484	-	-	-	-	-	-	-	-	-	537, 483	-	724, 208	-	-	-
NON-SRB LACTATE FERMENTERS																					
Clostridium homopropionicum	-	867, 147	721, 293		541, 473	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	762 142, 110
Veillonella parvula	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	882, 132
Pelobacter propionicus	-	877, 149	730, 296	-	-	-	-	-	-	-	-	-	-	-	675, 351	-	-	-	-	-	881, 133

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Reagents

1. Tracking dye (3 ×): 62.5 mg bromophenol blue and 10 g sucrose were dissolved in 15 ml dH_2O, 1 ml of 0.5 M EDTA was added. This was then made up to a final volume of 25 ml with dH_2O. The solution was sterilised by autoclaving.
2. Tris-borate-EDTA (TBE) (1 ×) buffer: 10.8 g of Tris (HCl/ base), 5.5 g of boric acid and 0.67 g of EDTA were dissolved in 1litre of dH_2O. pH of solution was adjusted to pH 8.
3. Phosphate buffered saline (PBS) (1 ×): 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were dissolved in 800 ml. pH of solution was adjusted to 7.4. This was then made up to a final volume of 1 litre with dH_2O. The solution was sterilised by autoclaving.
4. 4% paraformaldehyde: 33 ml of dH_2O was heated to 60°C, 2 g of paraformaldehyde was added while stirring. 10 N NaOH was added drop wise until paraformaldehyde was completely dissolved. This was followed by the addition of 16.5 ml PBS buffer (3 ×). pH of solution was adjusted to 7.4. Solution was then filtered through a sterilised 0.45 μm membrane.

Appendix E

Raw data of experiments reported in Chapter 4

Table E1: Steady-state data of sulphate conversion across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Sulphate conversion (%) at different feed sulphate concentrations (S_o)				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	49.6	nd	Nd	nd	nd
1.0	0.042	85.5	8.0	6.0	21.7	nd
1.5	0.028	86.0	11.5	15.2	27.0	nd
2.0	0.021	86.3	19.5	30.8	31.5	nd
3.0	0.014	86.4	41.8	39.9	33.1	6.5
3.5	0.012	nd	nd	nd	nd	6.6
4.0	0.010	86.6	54.4	55.4	40.4	6.6
5.0	0.0083	86.3	54.3	58.2	38.8	13.4
5.5	0.0076	nd	nd	nd	nd	13.4

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Table E2: Steady-state data of lactate conversion across the experimental conditions investigated in the current study.

RT (d))	Dilution rate (h^{-1})	Lactate conversion (%) at different feed sulphate concentrations (S_o)				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	98.9	nd	nd	nd	nd
1.0	0.042	98.7	100	64.2	59.6	nd
1.5	0.028	99.1	100	64.2	60.7	nd
2.0	0.021	99.3	100	66.2	58.9	nd
3.0	0.014	100	100	74.4	58.3	24.0
3.5	0.012	nd	nd	nd	nd	24.0
4.0	0.010	100	100	91.0	63.3	23.9
5.0	0.0083	100	100	89.5	60.1	47.0
5.5	0.0076	nd	nd	nd	nd	46.5

Table E3: Steady-state data of VSRR across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Volumetric sulphate reduction rate (g l^{-1} h^{-1}) at different feed sulphate concentrations (S_o)				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	0.041	nd	nd	nd	nd
1.0	0.042	0.036	0.0084	0.012	0.090	nd
1.5	0.028	0.024	0.0080	0.021	0.075	nd
2.0	0.021	0.018	0.010	0.032	0.066	nd
3.0	0.014	0.012	0.015	0.028	0.046	0.014
3.5	0.012	nd	nd	nd	nd	0.012
4.0	0.010	0.0090	0.014	0.029	0.042	0.010
5.0	0.0083	0.0072	0.011	0.024	0.032	0.017
5.5	0.0076	nd	nd	nd	nd	0.015

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Table E4: Steady-state data of bacterial dry mass across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Bacterial dry mass (g_{DW} l^{-1}) at different feed sulphate concentrations (S_o)				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	0.169	nd	nd	nd	nd
1.0	0.042	0.183	0.20	0.250	0.444	nd
1.5	0.028	0.200	0.22	0.270	0.467	nd
2.0	0.021	0.222	0.31	0.279	0.489	nd
3.0	0.014	0.232	0.33	0.380	0.500	0.256
3.5	0.012	nd	nd	nd	nd	0.361
4.0	0.010	0.250	0.34	0.430	0.528	0.378
5.0	0.0083	0.267	0.35	0.458	0.588	0.475
5.5	0.0076	nd	nd	nd	nd	0.378

Table E5: Steady-state data of residual sulphate concentrations across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Residual sulphate concentration (g l^{-1}) at different feed sulphate concentrations (S_o)				
		S_o (g l^{-1})				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	0.50	nd	nd	nd	nd
1.0	0.042	0.15	2.30	4.70	7.83	nd
1.5	0.028	0.14	2.21	4.24	7.30	nd
2.0	0.021	0.14	2.01	3.46	6.85	nd
3.0	0.014	0.14	1.45	3.00	6.69	14.0
3.5	0.012	nd	nd	Nd	nd	14.0
4.0	0.010	0.13	1.14	2.23	6.12	14.0
5.0	0.0083	0.13	1.14	2.09	5.96	13.0
5.5	0.0076	nd	nd	nd	nd	13.0

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Table E6: Steady-state data of residual lactate concentrations across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Residual lactate concentration ($g\ l^{-1}$) at different feed sulphate concentrations (S_o)				
		S_o ($g\ l^{-1}$)				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	0.029	nd	nd	nd	nd
1.0	0.042	0.026	0.00	5.00	9.3	nd
1.5	0.028	0.021	0.00	4.01	9.2	nd
2.0	0.021	0.015	0.00	3.76	9.0	nd
3.0	0.014	0.000	0.00	2.84	8.9	25.4
3.5	0.012	nd	nd	nd	nd	25.4
4.0	0.010	0.000	0.00	1.00	8.7	25.4
5.0	0.0083	0.000	0.00	1.17	8.2	17.6
5.5	0.0076	nd	nd	nd	nd	17.9

Table E7: Steady-state data of acetate concentrations across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Acetate produced concentration ($g\ l^{-1}$) at different feed sulphate concentrations (S_o)				
		S_o ($g\ l^{-1}$)				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	1.00	nd	nd	nd	nd
1.0	0.042	0.91	1.33	2.30	2.98	nd
1.5	0.028	0.77	1.34	2.13	3.64	nd
2.0	0.021	1.69	1.84	2.87	3.67	nd
3.0	0.014	1.44	2.24	3.14	4.42	1.5
3.5	0.012	nd	nd	nd	nd	1.8
4.0	0.010	2.45	1.85	3.16	5.28	2.9
5.0	0.0083	2.28	2.56	4.28	4.89	3.5
5.5	0.0076	nd	nd	nd	nd	4.6

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Table E8: Steady-state data of propionate concentrations across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Propionate produced concentration ($g\ l^{-1}$) at different feed sulphate concentrations (S_o)				
		S_o ($g\ l^{-1}$)				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	0.963	nd	nd	nd	nd
1.0	0.042	0.769	1.86	3.40	5.32	nd
1.5	0.028	0.673	1.82	2.67	3.61	nd
2.0	0.021	0.600	2.21	1.99	3.39	nd
3.0	0.014	0.508	0.84	1.78	2.39	3.9
3.5	0.012	nd	nd	nd	nd	3.7
4.0	0.010	0.500	0.77	1.93	2.09	3.4
5.0	0.0083	0.448	0.47	1.65	1.52	3.6
5.5	0.0076	nd	nd	nd	nd	3.4

Table E9: Steady-state data of bicarbonate concentrations across the experimental conditions investigated in the current study.

RT (d)	Dilution rate (h^{-1})	Bicarbonate concentration ($g\ l^{-1}$) at different feed sulphate concentrations (S_o)				
		S_o ($g\ l^{-1}$)				
		1.0	**2.5**	**5.0**	**10.0**	**15.0**
0.5	0.083	1.36	nd	nd	nd	nd
1.0	0.042	1.48	1.20	3.01	4.30	nd
1.5	0.028	1.41	1.54	2.89	4.30	nd
2.0	0.021	1.40	1.77	2.50	4.23	nd
3.0	0.014	1.40	1.78	2.61	4.83	2.44
3.5	0.012	nd	nd	nd	nd	2.75
4.0	0.010	1.37	2.81	3.01	4.46	4.34
5.0	0.0083	1.74	2.46	4.57	4.76	4.32
5.5	0.0076	nd	nd	nd	nd	4.47

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