THE ENZYMOLOGY OF ENHANCED HYDROLYSIS WITHIN THE BIOSULPHIDOGENIC RECYCLING SLUDGE BED REACTOR (RSBR)

A thesis submitted in fulfilment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

(Biotechnology)

of

RHODES UNIVERSITY

by

Godlove Nkwelle Enongene

December 2003

Abstract

The hydrolysis of complex organic heteropolymers contained in municipal wastewater to simpler monomers by extracellular hydrolytic enzymes is generally considered the rate-limiting step of the biodegradation process. Previous studies of the Recycling Sludge Bed Reactor (RSBR) revealed that the hydrolysis of complex particulate organics, such as those contained in primary sludge (PS), was enhanced under anaerobic biosulphidogenic conditions. Although the mechanism was not fully understood, it appeared to involve the interaction of sulfide and sludge flocs.

The current study was conducted using a 3500 ml laboratory-scale RSBR fed sieved PS at a loading rate of 0.5 kg COD/m^3.day and an initial chemical oxygen demand (COD) to sulfate ratio (COD:SO_4) of 1:1. There was no significant accumulation of undigested sludge in the reactor over the 60-day experimental period and the quantity of SO_4 reduced indicated that the yield of soluble products from PS was at least as high as those reported previously for this system (> 50%).

In the current study, the specific activities of a range of extracellular hydrolytic enzymes (L-alanine aminopeptidase, L-leucine aminopeptidase, arylsulphatase, α-glucosidase, β-glucosidase, protease and lipase) were monitored in a sulfide gradient within a biosulphidogenic RSBR. Data obtained indicated that the specific enzymatic activities increased with the depth of the RSBR and also correlated with a number of the physico-chemical parameters including sulfide, alkalinity and sulfate. The activities of α-glucosidase and β-glucosidase were higher than that of the other enzymes studied. Lipase activity was relatively low and studies conducted on the enzyme-enzyme interaction using specific enzyme inhibitors indicated that lipases were probably being digested by the proteases.

Further studies to determine the impact of sulfide on the enzymes, showed an increase in the enzyme activity with increasing sulfide concentration. Possible direct affects were investigated by looking for changes in the Michaelis constant (K_m) and the maximal velocity (V_{max}) of the crude enzymes with varying sulfide concentrations (250, 400 and 500 mg/l) using natural and synthetic substrates. The results showed no significant difference in both the K_m and the V_{max} for any of the hydrolytic enzymes except for the protease. The latter showed a statistically significant increase in the K_m with increasing sulfide concentration. Although this indicated a direct interaction, this difference was not large enough to be of biochemical significance and was consequently not solely responsible for the enhanced hydrolysis observed in the RSBR.

Investigation into the floc characteristics indicated that the biosulphidogenic RSBR flocs were generally small in size and became more dendritic with the depth of the RSBR. Based on the above data, the previously proposed descriptive models of enhanced hydrolysis of particulate organic matter in a biosulphidogenic RSBR has been revised. It is thought that the effect of sulfide on the hydrolysis step is primarily indirect and that the reduction in floc size and alteration of the floc shape to a more dendritic form is central to the success of the process.

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List of Abbreviations

AMD	Acid mine drainage
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APHA	American Public Health Association
APS	Adenosine phosphosulfate
ASM	Activated sludge model
ATP	Adenosine triphosphate
BNR	Biological nutrient removal
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
BSR	Biological sulfate reduction
COD	Chemical oxygen demand (mg/l)
$COD_{Particulate}$	Particulate chemical oxygen demand (mg/l)
$COD_{Soluble}$	Soluble chemical oxygen demand (mg/l)
COD_{Total}	Total chemical oxygen demand (mg/l)
CSTR	Completely stirred tank reactor
ddH_2O	Double deionised water
dH_2O	Deionised water
DMSO	Dimethyl sulphoxide
EBRU	Environmental Biotechnology Research Unit
EDTA	Ethylene diamine tetra-acetic acid di-sodium salt
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid
EPS	Extracellular polymeric substances
H_2S	Hydrogen sulfide
HRAP	High rate algal pond

HRT	Hydraulic retention time
HUSB	Hydrolysis upflow sludge bed
IAPS	Integrated algal ponding systems
K_h	Hydrolysis rate flow constant
K_m	Affinity constant (Michaelis-Menten)
LCFA	Long chain fatty acids
MPB	Methane producing bacteria
MUF	Methylumbelliferone
M_{wt}	Molecular weight
N_2	Nitrogen gas
^oC	Degree Celsius
P	Probability
PCMB	p-Chloromercuric benzoic acid
pH	Logarithm of the reciprocal of the hydrogen ion concentration
pK_a	Dissociation constant
PMSF	Phenylmethylsulfonyl fluoride
p-NP	p-Nitrophenol (spectrophotometric grade)
PPi	Pyrophosphate
PS	Primary sludge
RSBR	Recycling Sludge Bed Reactor
SD	Standard deviation
SO_3^-	Sulphite
SO_4^{2-}	Sulfate
SRB	Sulfate reducing bacteria
SRT	Sludge retention time
SS	Suspended solids
STR	Stirred tank reactor
t	Time (days)
TCA	Trichloroacetic acid
TRIS	Tris (hydroxymethyl) aminomethane
UASB	Upflow anaerobic sludge blanket

UN	United Nations
v/v	Volume per volume
VFA	Volatile fatty acids (mg/l)
V_{max}	Maximal initial velocity
VSS	Volatile suspended solids
w/v	Weight per volume
WHO	World Health Organisation
WRC	Water Research Commission
WSSD	World Summit on Sustainable Development
$ZnCl_2$	Zinc chloride

Acknowledgements

This thesis is a result of the collaboration and support of many people. In particular, I would especially like to thank my supervisors, Professor Chris Whiteley and Dr. Kevin Whittington-Jones for their encouragement, enthusiasm, availability and tireless efforts in making this work a success. The support I received from them was truly first rate.

I would like to thank Professor Peter Rose, Dr Jo Burgess and Dr Brett Pletschke for suggesting improvements and for their great deal of expertise. I would also like to thank Dr Oliver Hart and the staff at EBRU for technical assistance.

I greatly appreciate the financial assistance from the National Research Foundation, the Water Research Commission of South Africa and the Rhodes University assistantship.

Thanks to all my lab mates, friends and fellow postgraduates especially Alphonsus Neba. I would like to offer my apologies to anyone whom I might have forgotten, and many thanks to the rest.

Finally, I would like to say a special thanks to my family especially my dad Mr Vincent Esambe, my mum Mrs Valentina Munge and my uncle Mr George Ngande who have been extremely patient and have provided tremendous support and love throughout the course of this project. Thanks to you all.

Godlove Enongene

December 2003

List of Publications

The following articles from the research outlined in this thesis has been published or presented in scientific journals and/or conferences.

- Whiteley, C., Pletschke, B., Rose, P., **Enongene, G.N** and Whittington-Jones, K. (2004). An investigation of the physicochemical parameters and flocs on hydrolytic enzymes of an anaerobic Recycling Sludge Bed Reactor (RSBR) *in situ*. In: *Waste Reticulation and Treatment, WISA Biennial Conference and exhibition*, Cape Town International Convention Centre, Cape Town, South Africa, May 2-6, 2004, Ref. No: 192. *Accepted for Oral Presentation.*

- Whiteley, C., Pletschke, B., Rose, P., **Enongene, G.N**. and Whittington-Jones, K. (2003). Co-digestion of primary sewage sludge and industrial wastewater under anaerobic sulfate reducing conditions: enzymatic profiles in a recycling sludge bed reactor (RSBR). *Water Science and Technology*, **48**(4):129-138.

- **Enongene, G.N**., Whiteley, C.G., Pletschke, B., Rose, P. and Whittington-Jones, K. (2003). Enhanced hydrolysis: an investigation of the kinetics of hydrolytic enzymes within a anaerobic sulfide gradient. In: *International Biofilm Symposium: IWA Specialist Group on Biofilms*, Cape Town International Convention Centre, Cape Town, South Africa, September 14-18, 2003, P-53.

- Whiteley, C., Pletschke, B., Rose, P., **Enongene, G.N**. and Whittington-Jones, K. (2003). Bioremediation of primary sewage sludge by enzymes under sulfate reducing conditions. In: *Enzymes in the Environment: Activity, Ecology and Applications*, Praha, Czech Republic, July 14-17, 2003.

- **Enongene, G.N**., Whiteley, C.G., Pletschke, B., Rose, P. and Whittington-Jones, K. (2003). Enzymology of enhanced hydrolysis of complex substrates under

biosulphidogenic conditions within a Recycling Sludge Bed Reactor (RSBR). In: *SASBMB 18th Congress: Molecules of Life*, UP-North, University of Pretoria, July 6-9, 2003.

- Whiteley, C., Pletschke, B., Rose, P., **Enongene, G**. and Whittington-Jones, K. (2003). Enzymological profile within an anaerobic sulphidogenic bioreactor: Implications for enhanced hydrolysis, *Submitted to Biotechnology and Bioengineering*.

- Whiteley, C.G., Pletschke, B.I., Burgess, J.E., Tshivhunge, A.S., Ngesi, N., Whittington-Jones, K., **Enongene, G**., van Jaarsveld, F., Heron, P., Rashamuse, K. and Rose, P.D. (2003). Investigation into the Enzymology of Accelerated Primary Sewage Sludge Solubilisation and Digestion in Sulfate Reducing Systems. Water Research Commission Report No: K5/1170/0/1, Pretoria, South Africa.

- Burgess, J.E., **Enongene, G.,** Whittington-Jones, K., Pletschke, B. and Whiteley, C. (2003). Floc morphology and size distributions of anaerobic sulphidogenic sludge flocs using immobilization for light microscopy and image analysis. *Submitted to Trans IchemE Part B.*

- Whiteley, C., Pletschke, B., Rose, P., **Enongene, G**. and Whittington-Jones, K. (2002). Co-digestion of primary sewage sludge and industrial wastewater under anaerobic sulfate reducing conditions. Enzymatic profiles in reciprocating sludge bed reactor (SBBR). In: *Proceedings of the 3rd International Symposium on Anaerobic Digestion on Solid Waste*, Munich / Garching, Germany, September 18 – 20, 2002.

- **Enongene, G.N**., Whiteley, C.G., Pletschke, B., Rose, P. and Whittington-Jones, K. (2002). The enzymology of accelerated sewage sludge: The role of aminopetidases in solubilization., In: *WISA Biennial WISA Conference and Exhibition*, ICC, Durban, South Africa, May 19 – 23, 2002. Paper Number 193.

Chapter 5

The Effect of Floc Characteristics on the Enhanced Hydrolysis of Primary Sludge within the Recycling Sludge Bed Reactor (RSBR)

5.1. Introduction

Anaerobic degradation of particulate organic matter is an important process in converting organic materials into carbon dioxide and methane and has been recognised as the most appropriate technique for the treatment of domestic sewage (Miron *et al*., 2000) and high strength municipal wastewaters (Vijayaraghaven and Ramanujam, 2000). During the anaerobic treatment of wastewater, organic particulates are broken down, resulting in a change in particle size distribution (Lawler *et al*., 1986; Nellenschulte and Kayser, 1997), which has been shown to influence the rates of diffusion, adsorption and sedimentation of these particles, as well as biochemical reactions such as enzymatic reactions (Levine *et al*., 1985; Kaminski *et al*., 1997). According to Biggs and Lant (2002), formation of flocs has been found to be crucial for the operation and efficiency of the treatment process and to achieve effluent of a desired standard. Failure of flocculation to occur may result in a loss of active biomass from the system, thereby resulting in a reduction of process efficiency and discharge of excess solids into the environment (Biggs and Lant, 2002). The retention of SRB in a digester also depends largely on the ability of the bacteria to adhere to the dense bacterial flocs which reduces the propensity for bacterial washout from the bioreactor system (Widdel, 1988; Kosaric and Blaszczyk, 1990). The physical characteristics of sludge flocs thus play an important part in the overall performance of the process and indeed all the basic principles of the process are related to or dependent on the physical characteristics of the flocs (Li and Ganczarczyk, 1990).

Snidaro *et al*. (1997) showed that the structure of activated sludge flocs consisted of three levels. The first level was made up of bacteria tightly bound together by a gel matrix to form the second level of structure, called the microcolonies. These microcolonies were found to have a median diameter of approximately 13 μm and were linked by polymers to make up the final activated sludge flocs. Li and Ganczarczyk (1990) also detailed similar conclusions about the presence of microcolonies in the floc structure. Activated sludge flocs are aggregates made up of diverse groups of microorganisms enmeshed in a polymeric network of extracellular polymeric substances (EPS) (Frølund *et al*., 1996; Biggs and Lant, 2002). The factors known to be responsible for flocculation in activated sludge floc systems were assumed to be equally essential in maintaining the integrity of

anaerobic flocs in the present study. The morphology (floc size distribution, fractal dimension, and filament index), physical (flocculating ability, viscosity, hydrophobicity and surface charge) and chemical (polymeric constituents and metal contents) properties of flocs can differ greatly due to differences in the environment within the bioreactor (Jin *et al.*, 2003). The floc size and structure of sludge produced by sulphidogenic reactors**,** in spite of the growing body of work on sulphidogenic upflow anaerobic sludge blankets, is still not well understood (Vallero *et al.*, 2003).

Previous studies on the RSBR suggested that floc dynamics play a major role in the phenomenon of enhanced solubilisation of complex organic biopolymers, particularly carbohydrates and proteins, in the presence of sulfide due to reduction of floc stability. In the present study, the initial expectations were that the activity of hydrolytic enzymes was enhanced directly by sulfide and that this was the mechanism underlying enhanced hydrolysis of complex carbon in the RSBR. The results have, however, shown that although enzyme activity is enhanced in the presence of sulfide, the effect is not a direct one as shown by the kinetic studies (Chapter 4). Consequently, it was hypothesised that there must be a correlation between the floc morphology (size, shape and floc looseness), enzymatic activities and the physico-chemical parameters (including the concentration of sulfide) and that these factors influence the activity of the key hydrolytic enzymes indirectly via changes in floc morphology.

5.2. Materials and Methods

4.2.1. Reactor set-up

All the studies were conducted in the laboratory-scale RSBR. The schematic diagram of the laboratory-scale RSBR system setup is shown in **Figure 2.1** and its operation was described in section 2.2.1 (page 41) of Chapter 2.

5.2.2. Analytical methods

All analyses of the physico-chemical parameters were conducted as described previously in section 2.2.3 (page 44) of Chapter 2. This included analysis of COD_{Total}, $COD_{Particulate}$, and $COD_{Soluble}$, sulfide, sulfate, alkalinity and pH.

5.2.3. Enzymatic assays

All specific enzymatic activities were carried out according to techniques described in section 3.2.8 (page 64) of Chapter 3.

5.2.4. Sample preparation and measurement

The well established technique of immobilisation and light microscopy followed by image analysis was used for the determination of the floc size distribution and shape characteristics. Samples used for this study were collected at regular intervals between days 58 and 86. The samples were carefully drawn from the RSBR using a syringe to avoid destabilisation of the floc particles. The procedure used to prepare flocs for microscopic examination was adapted from Droppo *et al*. (1996). This involved settling sludge samples (50 ml) in measuring cylinders for 30 min and carefully discarding the supernatant. The settled sludge was diluted sufficiently to allow clear views of the flocs by combining the settled solids with a solution of low-melting point electrophoresis grade agarose (0.75% w/v, 8.0 ml, 35^{o}C). The settled sample volumes of 3.0 ml for depth 1, 1.5 ml for depth 2 and 0.5 ml for depth 3 were found to provide clear views of the individual flocs. The floc characteristics and size distributions were determined by photography, using a light microscope (Olympus BX50) equipped with a digital camera (Olympus PM-C35DX). The images were captured at a magnification of x200 and processed using SigmaScan® Pro for Windows Version 5.0 (SPSS Inc. USA) to measure the flocs' feret diameter, circularity and looseness. A minimum of 100 flocs was measured for each sample and values reported as means.

The feret diameter, which describes the size of an object, gives the diameter of a fictitious spherical object that has the same area as the real object. A measurement demonstrating an object's two-dimensional shape is described by its circularity and this was calculated from equation (17). A circularity value of 1.0 describes a perfect circle whereas a line has a circularity value close to zero.

$$Circularity = \frac{4\pi \text{ x Area}}{(\text{Perimeter})^2} \qquad (17)$$

A numeric measurement of the shape and density of an object is described by its looseness which is defined using equation (18). A perfectly filled circular object gives a minimum looseness of 4π and this tends to infinity as the floc becomes more dendritic or open. The flocs' looseness was calculated using equation (18).

$$Looseness = \frac{(\text{Perimeter})^2}{\text{Area}} \quad (18)$$

5.2.5. Statistical analysis

Statistical analysis, including Pearson correlation coefficients, linear regression analysis, analysis of variance (ANOVA), descriptive statistics and the *T*-test were conducted using STATISTICA (data analysis software system), for Windows Version 6.0 (StatSoft, Inc. 2001, USA). The mean values were reported with standard deviation (±1 SD) at the 95% or 99% confidence level and correlations were considered as statistically significant at $P < 0.05$.

5.3. Results and Discussion

A total of 45 floc size distribution curves using feret diameter measurements of over 5000 flocs measured were generated for each sample for all the depths of the RSBR. The mean floc size distributions for each depth in the reactor did not vary significantly over the course of the trial as illustrated in **Figure 5.1**. The flocs were generally small with a mean feret diameter of 59 µm for depth 1, and 79 µm at depths 2 and 3. The majority of flocs (59%) at depth 1 were smaller than 20 µm, with progressively fewer flocs falling into increasing feret diameter categories. The flocs (39%) at depths 2 and 3 fell within the range of 20-39 µm, although the distribution curve was asymmetrical (**Figure 5.1**). The flocs feret diameters and their tendency towards open, dendritic structures increased with increasing depth of the RSBR (that is from depth 1 to depth 3). Depths 2 and 3 contained a small number of particles over 200 µm, whereas depth 1 did not. Paired T-tests carried out on the mean feret diameter distributions for each depth showed that at 95% confidence level, the differences between the distribution patterns in depth 1 and depth 3 were statistically significant ($T = 0.46$, $P < 0.05$; df = 50). The other pairwise tests for sample means for depth 1 versus depth 2 and depth 2 versus depth 3, however, showed

that there was no statistically significant difference between these depths of the RSBR. This suggests that there was a gradual change in the nature of the floc morphology with depth within the RSBR.

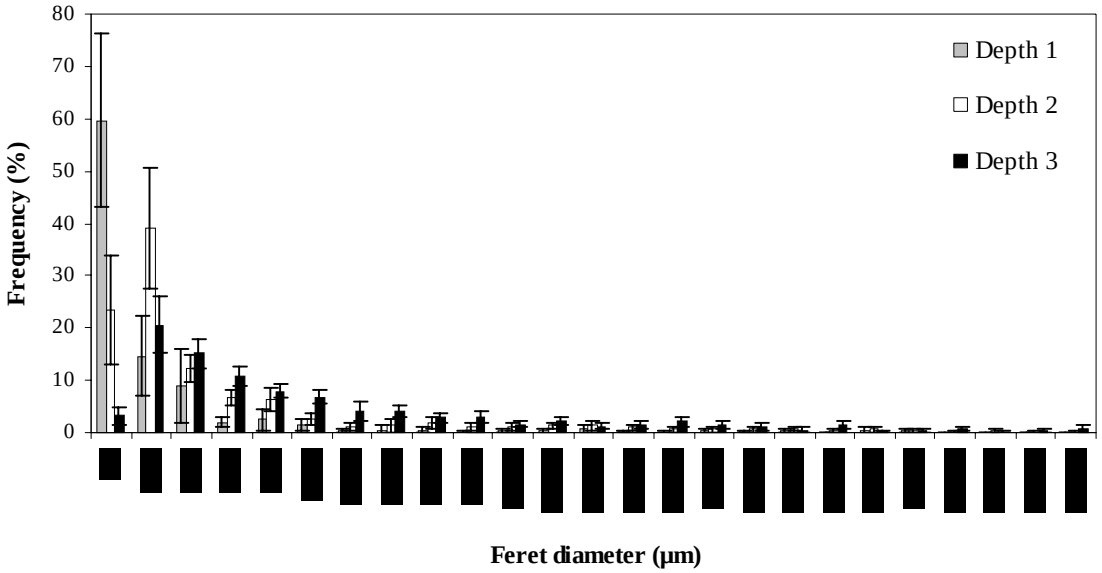

Figure 5.1 Mean floc size frequency distribution during the experimental period. Error bars represent standard deviation (± SD), n = 100

Other authors have reported floc sizes within the range observed in this study. Parker *et al*. (1971) reported a bimodal floc size distribution of 0.5 - 5 µm and 25 – 300 µm in activated sludge and Urbain *et al.* (1993) also reported a range of 20 - 200 µm. Barbusinski and Koscielniak (1997) noticed a significant drop in average floc size diameter from 125 - 65 µm within the first four days in their aerobic digester. The floc size distribution has an important influence in the reactor operation, as a high density and narrow size distribution allows a narrow settling distribution (Batstone and Keller, 2001).

The other floc characteristics measured in the RSBR altered significantly with depth and are summarised in **Table 5.1.** The floc looseness increased significantly (ANOVA, $P < 0.05$, df = 44) with depth of the RSBR (**Table 5.1**) and varied extensively with time at depth 3 (**Figure 5.2a**). The floc looseness at depth 3 increased up to day 68 and then dropped to about 71% on day 78. A sharp increase in the floc looseness of about 67% was observed again up to day 84. Floc looseness at depths 1 and 2, however, did not show any significant variability (**Figure 5.2a**).

Table 5.1 Mean floc characteristic of the 3 depths within the RSBR

	Depth 1	**Depth 2**	**Depth 3**	
	Mean	**Mean**	**Mean**	***P*-value**
Floc Size (Feret diameter (μm))	12.35 (8.26)	45.80 (16.74)	87.83 (25.80)	< 0.05
Floc shape (Circularity)	0.69 (0.17)	0.36 (0.08)	0.18 (0.08)	< 0.05
Floc looseness	52.41 (38.25)	324.64 (304.32)	1484.89 (850.57)	< 0.05

Values in brackets represent standard deviations (± SD), n = 100

While the flocs at depth 3 were the most dendritic and mesh-like, the flocs at depth 1 were more like pinpoints. Depth 1 flocs were the smallest and most circular and the mean floc shape (circularity) increased with the depth of the RSBR (**Table 5.1**). The floc circularity at each of the depths of the RSBR did not, however, show any significant variability with time during the study period (**Figure 5.2b**). Mean floc diameter (**Figure 5.2c**) increased and circularity decreased moving down in the reactor from depth 1, through depth 2 to depth 3.

Statistical analysis of the possible correlations between the floc characteristics with the hydrolytic enzymes and physico-chemical parameters are presented in **Table 5.2**. The mean floc feret diameter correlated positively with RSBR depth (R = 0.75) and negatively with circularity (R = -0.78), meaning that the flocs were more circular at depth 1. Circularity also showed a strong negative correlation with depth (R = -0.87). The looseness and the mean feret diameter of the flocs were not related to any of the other parameters measured except for lipase. The floc shape, however, showed significant correlations with a number of the enzymes (lipase, α-glucosidase, and protease) and other reactor parameters (alkalinity, sulfide, sulfate and the three forms of COD). These trends may be the results of concomitant increases in sulfide, alkalinity, COD, lipases, proteases and α-glucosidases activities and the decrease in sulfate concentration, or may be coincidental.

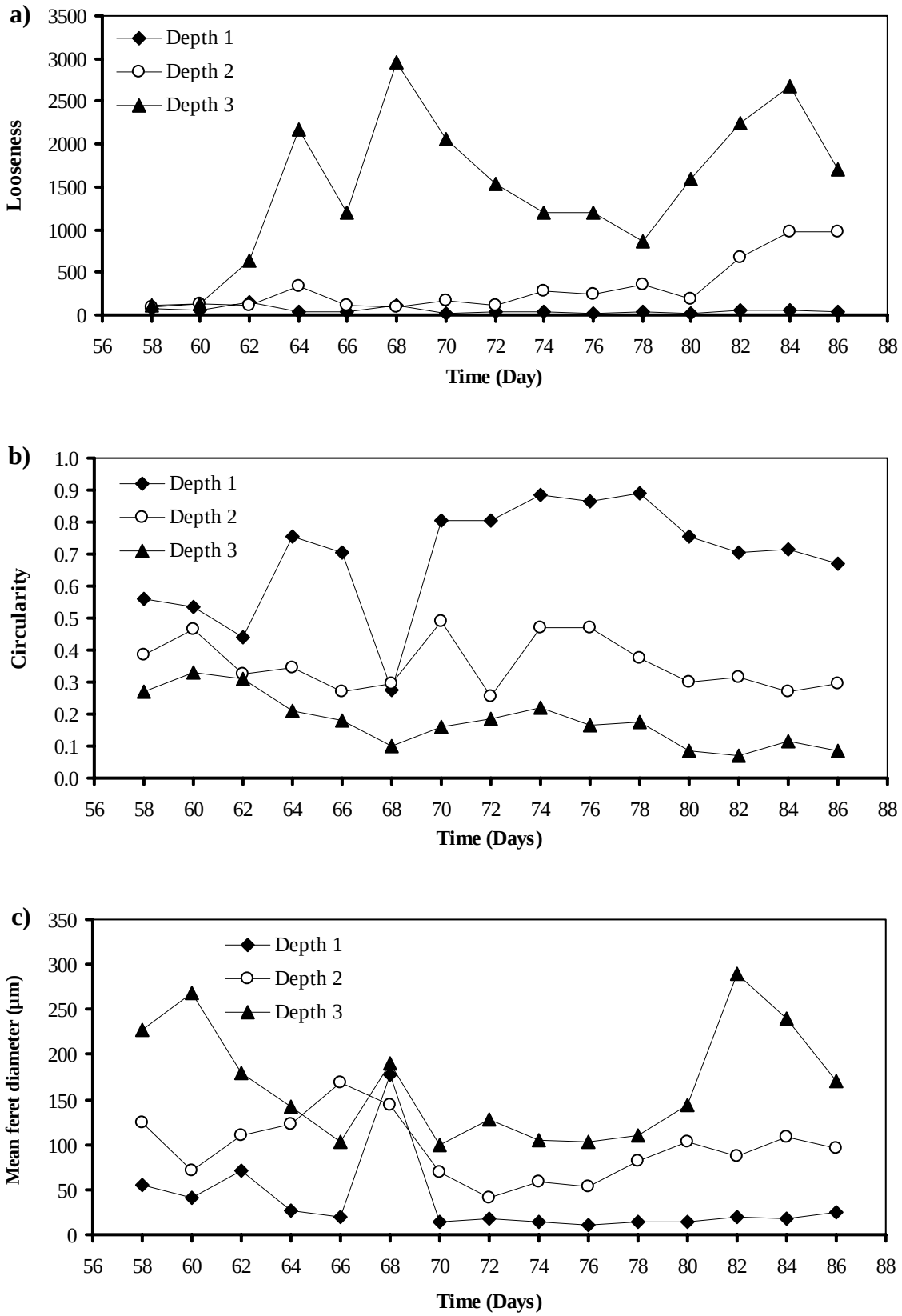

Figure 5.2 Mean floc a) looseness b) circularity trends c) feret diameter throughout the RSBR , n =100

Table 5.2 Correlation coefficient (*R*) for RSBR parameter with mean floc characteristics

	Depth	**Floc Looseness**	**Floc Shape (Circularity)**	**Floc Size (Feret diameter)**
Floc Looseness	0.67			
Floc Shape (Circularity)	**-0.87**	-0.65		
Floc Size (Feret diameter)	**0.75**	0.61	**-0.78**	
Arylsulphatase	0.39	0.46	-0.33	0.26
Lipase	**0.93**	0.51	**-0.84**	**0.71**
α-Glucosidase	**0.88**	0.37	**-0.77**	0.66
β-Glucosidase	0.61	0.63	-0.59	0.26
Protease	**0.78**	0.63	**-0.82**	0.67
L-Alanine aminopeptidase	0.49	0.23	-0.36	0.29
L-Leucine aminopeptidase	0.56	0.54	-0.58	0.40
pH	-0.02	-0.17	-0.05	0.07
Alkalinity	**0.86**	0.48	**-0.85**	0.65
Sulfide	**0.81**	0.38	**-0.80**	0.60
Sulfate	**-0.84**	0.44	**0.82**	-0.62
COD_{Total}	**0.90**	0.57	**-0.84**	0.68
$COD_{Particulate}$	**0.90**	0.58	**-0.84**	0.68
$COD_{Soluble}$	**0.79**	0.42	**-0.76**	0.54

Values in bold indicate significant correlations coefficients at 75 - 90% confidence level

Any increase in enzyme concentration in the bioreactor, or reduction in mass transfer limitation or particle size will result in an increase in the rate of hydrolysis of complex organic matter. Whittington-Jones (2000) showed that the phenomenon of enhanced hydrolysis is due to a rapid reduction in floc size which has been previously shown to change with depth in the Rhodes BioSURE® Recycling Sludge Bed Reactor (RSBR). Different sludges have different flocculation abilities (Jin *et al.*, 2003) and the physical properties of flocs are to a large degree defined by the extent of flocculation of the extracellular polymeric substances (EPS) (Nielsen and Keiding, 1998). EPS are high molecular weight organic molecules produced by microorganisms and have been reported to consist mainly of carbohydrates and proteins as the major compounds (Morgan *et al.*,

1990; Frølund *et al*., 1996). EPS act as a trap for these macromolecules (polysaccharides, proteins and lipids) too large for direct assimilation by the SRB and as a network confining hydrolytic enzymes to bacterial cell walls and floc matrix (Frølund *et al*., 1995; Vavilin *et al*., 1996; Confer and Logan, 1998; Goel *et al*., 1998). Disrupting these networks will lead to the exposure of macromolecules previously protected from enzymatic attacks for degradation. This in turn will lead to increased concentration of hydrolytic enzymes and consequently an enhanced solubilisation of sewage sludge. This flocculation may be ascribed to the properties of the EPS in the flocs and by the metal ions involved in neutralising the free charge of the organic polymers. It has been reported, however, that those factors responsible for flocculation such as non-covalent bonds between bacteria, metal ions and EPS are also essential for maintaining the integrity of the anaerobic flocs (Eriksson and Alm, 1991; Bruus *et al*., 1992; Urbain *et al*., 1993). Sutherland (1977) examined enzymes acting on bacterial surface carbohydrates and concluded that EPS molecules are so complex that there are several potential binding sites for hydrolytic enzymes. The action of enzymatic hydrolysis may remove EPS short side chains and attack the core structure. However, only the first mechanism was demonstrated.

During wastewater treatment microbial aggregates are generated and this provides an efficient organisation of bacterial communities which are embedded in the floc matrix of the EPS (Whiteley *et al*., 2003). It has also been shown that a combination of general carbohydrases, lipases and proteases can significantly reduce the EPS content of sludge and alter the floc structure (Thomas *et al*., 1993). Hydrolysis of PS is limited by mass transfer limitations due to floc size and structure, reduced contact between hydrolytic enzymes and substrates, poor retention of biomass and enzymes due to low immobilisation efficiency and inefficient separation of soluble products and undigested materials. Decrease in particle and floc sizes will thus ultimately lead to enhanced hydrolysis and solubilisation of sewage sludge in the RSBR.

Sulphidogenic flocs have been reported to be irregularly shaped and loose in structure compared to methanogenic and methanogenic-sulphidogenic flocs (Santegoeds *et al*., 1999). Methanogenic bacteria and SRB are generally present in a layered structure in

sulphidogenic and methanogenic-sulphidogenic aggregates, with corresponding regions of high sulfate reducing activity in the SRB-rich outer layers (Santegoeds *et al*., 1999). The cores of all three types of anaerobic flocs are made up of methanogenic bacteria, possibly owing to the diffusional limitations of sulfate allowing methanogens to outcompete SRB, even in flocs treating sulfate-rich wastewater (Overmeire *et al*., 1994) or possibly as a result of SRB colonising the surfaces of methanogenic bacterial flocs presents in the original inoculum (Santegoeds *et al*., 1999). The presence of methanogenic bacteria may even be essential to flocculation, as they have been shown to have better aggregating abilities than SRB and may initiate flocculation, being attached to later on by the SRB, during aggregate development (Santegoeds *et al*., 1999). This outer layer of sulfate-reducing activity often produces sulfide precipitates, especially in environments which are rich in cationic metals as well as sulfate, which impact on the structure of anaerobic flocs, enhancing sludge settleability (Yamaguchi *et al*., 2001).

High sulfide levels are known to destroy floc structures by removing the cationic metals required for flocculation via sulfide precipitation (Caccavo *et al*., 1996). Nevertheless, these observations have all been made on aerobically grown activated sludge, and it is clear from the coincidental increases in RSBR floc size and sulfide concentration that the same is not true for anaerobic sludge flocculation. The presence of sulfide in the RSBR, could also result in surface charge due to pH variations (Wilén *et al*., 2000), reduced floc stability, change in ionic strength and composition, and other biological aggregates which have been shown to promote deflocculation (Bruus *et al*., 1992; Zita and Hermansson, 1994; Keiding and Nielsen, 1997). Sulfate reduction by the production of sulfide, which reacts with Fe (III) to form FeS, has also been suggested to weaken floc stability leading to floc disintegration (Goel *et al*., 1998a; Nielsen and Keiding, 1998; Wilén *et al*., 2000).

Particle size distributions which are asymmetrical are typical of systems in which the particles are constantly aggregating and disaggregating (Cadoret *et al*., 2002). The cycle of flocculation-deflocculation suspected in the RSBR has been noted in previous work (Whittington-Jones *et al*., 2002). Such dynamic equilibria appear to be related to the sulfide gradient observed in the RSBR system. Deflocculation of activated sludge under anaerobic conditions affects approximately 1-2% of the sludge volume, and is due to the

attrition of smaller particles from the surfaces of larger aggregates (Wilén *et al*., 2000). The results of previous authors, who concluded that the breakdown of organic debris during anaerobic digestion resulted in a change in particle size distribution (Karr and Keinath, 1978; Lawler *et al*., 1986; Houghton and Stephenson, 2002; Whittington-Jones *et al*., 2002), corroborate the result obtained in these studies. Using a series of statistical analyses, this study has provided an insight into the relationship between the floc characteristics and both the specific enzyme activities and the physico-chemical parameters within the RSBR.

5.4. Conclusions

The results obtained in this work revealed the following:

- The mean particle size distributions within the RSBR showed that the flocs were of similar sizes to those in other digested sludges as reported in the literature.
- Floc morphology (size, circularity and feret diameter) changed with depth in the RSBR with flocs lower in the reactor being larger and more dendritic (less circular).
- There was a significant correlation between floc shape (circularity) and lipases, α-glucosidases and proteases as well as with alkalinity, sulfide, sulfate and the three forms of COD. However, of the enzymes tested in the current study, only lipase showed a significant correlation with floc size.
- Flocs became more dendritic with depth, that is, from depth 1 to depth 3, in the RSBR and this corresponded to an increased sulfide concentration and consequently the release of soluble products into the system.
- As the flocs become more dendritic in the presence of sulfide, the surface area of the flocs increases which, in turn, may lead to the exposure of macromolecules previously protected from enzymatic attack thereby resulting in enhanced hydrolysis within the RSBR system.

Chapter 1

General Introduction

1.1. Background

Mining remains the single most important industry in mineral-rich South Africa. Consequently, the formation of acid mine drainage (AMD) is one of the most serious environmental problems associated with these operations and the potential threat to the quality of fresh water resources is severe (Water Research Commission, 1982; Pulles *et al*., 1995; Pulles, 2000). AMD, which results from chemical and biological oxidation of sulfide ores, is characterised by low pH and high levels of sulfate, heavy metals and suspended solids (Wittman and Förstner, 1977). The biological and physico-chemical processes giving rise to pyrite oxidation, acid formation and heavy metal solubilisation have been reviewed comprehensively (Andrews, 1989; Silver, 1989; Kuenen and Robertson, 1992; Pronk and Johnson, 1992; Robb, 1994; Johnson; 1995). The remediation of acidic metal-rich wastewaters using natural or constructed wetlands is a passive low-cost approach that has found application worldwide (Johnson, 1995; Robbinson and Robb, 1995; Van Zyl, 1996; Younger *et al*., 1997), and provides a long term solution with relatively low operational cost. Often, mining operations that ceased many years ago lack funds for costly high-tech solutions required for the treatment of the remaining acidic drainage waters. Aerobic wetlands promote oxidation of AMD, thereby causing metals to oxidize and precipitate as oxides, while anaerobic wetlands rely on the reduction of sulfate and the subsequent precipitation of metal sulfides (Hulshoff Pol *et al*., 2001). This process can be fuelled by a wide range of electron donors (Hulshoff Pol *et al*., 2001).

Bacterial sulfate reduction (BSR) has been identified as a potentially valuable process for removing contaminant metals from coal and metal-mine drainage (Tuttle *et al*. 1969; Herlihy and Mills, 1985; Herlihy *et al*., 1987; Hendin *et al*., 1989). Under anaerobic conditions, sulfate-reducing bacteria (SRB) oxidise simple organic compounds, such as lactic acid, using sulfate as an electron acceptor thereby generating hydrogen sulfide and bicarbonate ions (equation (1)). These ions then react with protons to form carbon dioxide (CO_2) and water and remove the acidity from solution as CO_2 gas (equation (2)). The sulfide and bicarbonate ions formed during sulfate reduction equilibrate into a mixture of H_2S, HS^-, CO_2, HCO_3^- and CO_3^-. This mixture will buffer the solution pH to a

value in the range of 6-8 if sufficient sulfate reduction occurs. Raising the pH of acidic water will cause some metals to precipitate as insoluble hydroxides or oxides (Stumm and Morgan, 1981).

$$3SO_4^{2-} + \text{Lactic acid} \rightarrow 3H_2S + 6HCO_3^- \quad (1)$$

$$HCO_3^- + H^+ \rightarrow CO_2(g) + H_2O \quad (2)$$

The major problems encountered in using these reactions for the remediation of large volume flows of AMD is the availability and cost of organic carbon substrates (Rose *et al*., 2002a, 2002b). A potential solution to this problem is the use of municipal sewage sludge which provides a readily available low-cost carbon source to drive BSR (Maree *et al*., 1986). According to Andreasen *et al*. (1997), hydrolysis rates for primary sludge (PS) are slow in conventional anaerobic treatment systems with a maximum soluble product formation reported between 8 and 20 days. Yields vary between 5-10% under psychrophilic conditions (Shimizu *et al*., 1993; Elefsiniotis and Oldham, 1994; Canziani *et al*., 1996; Banister and Pretorius, 1998) to around 35% at 24^{o}C (Hatziconstantinou *et al*., 1996).

In 1990, the Rhodes University Environmental Biotechnology Research Unit (EBRU) developed a new low-cost process, the Rhodes BioSURE Process®, which links AMD treatment and PS disposal (Rose *et al*., 2002a, 2002b, 2002c; Whittington-Jones *et al*., 2002). In this process, PS serves as a source of electron donor and is simultaneously stabilized. Solubilisation of complex carbon substrates provides the primary reaction in the BioSURE Process®, and is effected in the Recycling Sludge Bed Reactor (RSBR). In the RSBR, suspended solids settle and are recycled back through the inlet port where large particles are hydrolysed within an increasing sulfide and alkalinity gradient. After being recycled, the hydrolysed compounds become available to sulfate reduction in a subsequent operation where residual solids settle and again go through another cycle of hydrolysis. Pilot-scale trials were conducted at Grootvlei Mine, (Gauteng, South Africa), and during the 18 months of operation, the process proved to be a reliable method for treating mine drainage wastewaters. In terms of acetate equivalents, the yield of soluble product exceeded 50% and further studies were conducted to gain a better understanding of the mechanism of enhanced hydrolysis (Whittington-Jones, 2000). Studies of sludge

solubilisation in the RSBR have suggested that sulfide and alkalinity, as well as other physico-chemical parameters, play a role in enhancing the hydrolysis process and accelerating the breakdown of proteins, carbohydrates and lignocellulose components in PS (Rose *et al*., 2002b). Enhanced solubilisation is thought to be the result of effective fracturing of settled organic particulate matter in an anaerobic sulphidogenic zone as observed in a tannery waste ponding system (Dunn, 1998; Rose *et al*., 1998). Ristow *et al*. (2002) and Whittington-Jones (2000) provided a descriptive model of this enhanced solubilisation under sulfate reducing conditions. Proof of the mechanism and an understanding of the role of hydrolytic enzymes are the main foci of the present study.

1.2. Municipal wastewater treatment in developing countries

1.2.1. The global sanitation and sustainability problem

According to the 1994 World Bank Annual Report "Adequate water supply and sanitary disposal of wastes are fundamental to a reasonable quality of life. Poor sanitation and lack of access to safe water contribute to more than two million deaths annually, while large economic and environmental costs are incurred to compensate for poor quality services" (WHO, 1996).

Between 1970 and 2000, just 30 years, the global population doubled from three to six billion people. The level of urbanization in developing countries in the same period doubled from less than 25% of the population to 50%, while the values of their combined economies grew from US$ 0.4 trillion to US$ 4 trillion. These countries are thus faced with the enormous challenge of promoting a good quality of life, although usually under adverse financial constraints. The items that have received less attention and investment in the developing world are amongst others, sanitation, environmental protection and natural resource conservation, which are all needed for the improvement of living conditions. For these countries, systems that provide sanitation associated with environmental protection and natural resource conservation at low cost are desirable. The worldwide explosion of urbanisation and industrialisation is the main cause of groundwater pollution, and also leads to the degradation of surface water quality by overloading with wastewater-borne organic material that cannot be assimilated naturally.

Developing countries are therefore faced with a great demand for improved water supply and sanitation services, yet the major constraints in achieving these goals have been the competition for development funds for other development sectors, and difficulties in managing the relatively few facilities that exist, followed closely by the high cost of building conventional treatment facilities (WHO, 1996).

Business and industry have been challenged by international concern for sustainable development over the past decade to 'clean up their act', with the King II Report (Institute of Directors, 2002) requiring business and industry to include a concern for the 'triple bottom line' and sustainability as part of their business reporting (UN Global Compact Network, 1999). The World Summit on Sustainable Development (WSSD) held in Johannesburg, South Africa in 2002 identified sustainability and the need for improved sanitation as areas of major concern and highlighted its impact on the lives of the poor, particularly in sub-Saharan Africa. The challenge to industry is therefore to achieve the goals of sustainable development and sanitation in ways that do not exacerbate social inequality or degrade the environment (Darroll, 2002).

1.2.2. Water availability

Worldwide, agriculture accounts for about 69% of water use, industry 23% and domestic consumption about 8%. In 1989 the World Health Organization (WHO) adopted a standard defining minimal biological quality standards for reuse of treated wastewater effluents in agriculture and aquaculture (Shuval 1990).

The amount of water available per person has been declining throughout the world as a result of increasing populations and environmental changes (Ayibotolo, 1992) (**Figure 1.1**). According to Ayibotolo (1992), water scarcity in many countries has led to irrigation of vegetable and other crops with untreated wastewater and subsequent exposure of the public to serious health risks. Water scarcity has also resulted in a series of problems such as the degradation and overuse of water resources and increasing competition and conflicts among user groups that are provoking policy makers to reconsider wastewater disposal practices. Water use patterns, however, vary from country to country and within countries. The demand for water, especially for irrigated agriculture

is high particularly in arid regions, such as the Middle East and North Africa and, on the other hand, there is increasing demand to satisfy urban needs (Engleman and LeRoy, 1993).

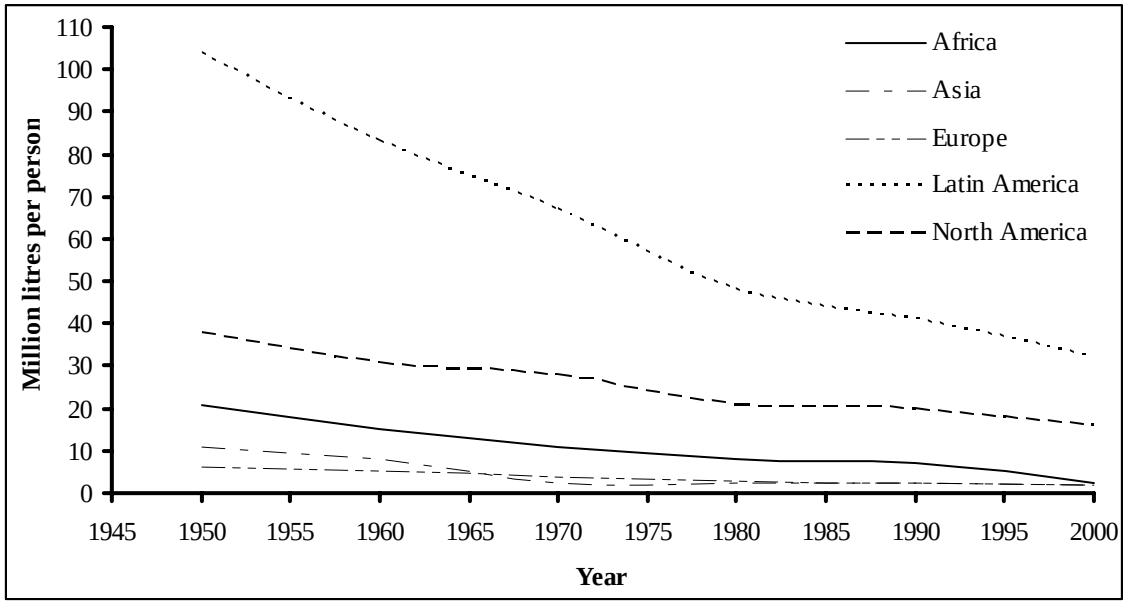

Figure 1.1 Water availability per person by region from 1950-2000

The total water demand in South Africa for agriculture, domestic use, industrialisation and mining has increased rapidly as a result of the growing population (Science Committee of the President's Council, 1983; Marais, 1984). In a mineral-rich country such as South Africa, the mining sector not only uses large volumes of water, but also contributes significantly to the contamination of fresh water resources. It is predicted that the demand for water will exceed the supply before the year 2020 (Bekker, 1982). Water has thus been identified as the most limiting natural resource of the country, and determines its human population carrying capacity (Science Committee of the President's Council, 1983). It is, therefore, desirable to treat wastewater correctly so that it doesn't contaminate the limited freshwater resources.

1.2.3. Steps for wastewater treatment

Municipal wastewater consists of a mixture of domestic wastewater, which includes typical waste from the kitchen, bathroom and laundry, as well as any other wastes that people may accidentally or intentionally pour down the drain. The characteristic of the wastewater will vary from location to location depending upon the population and industrial sector served, land uses, groundwater levels, and degrees of separation between storm water and sanitary waste (Tchobanoglous, 1987). Wastewater is usually characterized by a grey colour, musty odour and a solid content of 0.1% and 99.9% water content (Tchobanoglous, 1987). The solid can be suspended as well as dissolved. Wastewater is also composed of organic compounds (carbohydrates, proteins, fats and greases, surfactants, oils, pesticides, phenol, etc.) and inorganic compounds (such as heavy metals, nitrogen, phosphorous, sulfur, chloride, toxic compounds, etc.) compounds as well as various gases. It also contains various microorganisms, and may contain pathogenic organisms that originate from humans who are infected with diseases or are carriers of a particular infection.

Sewage sludge is any solid, semisolid, or liquid residue generated from the treatment of municipal wastewater (Linden *et al*., 1995; Meeroff and Bloetscher, 1998), and its agglomeration of pollutants, organic matter, and particular metal (Page and Chang, 1994). Sludge is the largest in volume amongst the by-products of wastewater treatment, and its processing and disposal is perhaps one of the most complex environmental problems (Werther and Ogada, 1999). The treatment of sludge is intended to reduce smell, the quantity of the organic solids, eliminate disease-causing bacteria, improve dewatering characteristics of the sludge, and reduce the water content so that the end product can be treated further or disposed of with fewer handling problems and environmental consequences.

The primary justification for wastewater treatment has been to prevent the spread of organisms that cause infectious diseases in humans, to remove the biodegradable organic material that pollutes the groundwater and upsets the ecological balance in surface waters and to be able to reuse the treated effluent directly and safely for economically productive

purposes. This is particularly important in water scarce regions. A series of steps involved in the complete wastewater treatment is presented (**Table 1.1**).

According to Toerien (1986), to protect the water quality of the receiving streams all effluents originating from domestic, municipal, industrial, agricultural and mining sources should be treated before disposal. Municipal wastewater in many developing countries tends to be more concentrated than in industrialised countries because less water on average is used in household activities (Toerien, 1986).

Table 1.1 Steps involved in the complete treatment of wastewater

- Preliminary treatment, removes large and heavy solids by screening and degritting, the screenings by-products are disposed of separately from the other wastewater sludges;
- Primary treatment, which involves the use of clarifiers and sedimentation tanks to settle 40-70% of settable solids that contain significant amounts of oxygen consuming substances (20-40%), but little or no removal of colloidal and dissolved organic matter;
- Secondary treatment consists of removal of about 85% of suspended solids and BOD_5/COD and partial stabilization of the latter and some destruction of pathogenic organisms, producing a sludge consisting of about 90% organic matter. This sludge is composed of approximately 2 to 3% solids and if not treated correctly becomes highly odiferous;
- Advanced or tertiary treatment is an added stage of biological, chemical and physical processes to treat wastewater beyond the secondary stage. At this stage, there is up to 99% removal of residual suspended solids and nutrients, mainly nitrogen and phosphorus. This process converts the organic solids to more inert forms so that they may be disposed of without causing health problems.

Source: (APHA 1981)

Municipal wastewater treatment depends on natural processes, such as gravity to clarify an effluent and bacterial action to stabilise the biodegradable organic fraction (Alaerts *et al*., 1990). Basic treatment mechanisms include screening, sedimentation and filtration to remove solid material, stabilization of biodegradable organic material by bacterial degradation and removal of inorganic nutrients, such as nitrogen and phosphorus. This treatment process involves several mechanisms, including bacterial nitrification, bioaccumulation by plants, adsorption or chemical precipitation. It is therefore essential that applicable water treatment technologies are available in order that treated effluents

can be reused directly, or be discharged back to the aquatic environment with minimal water quality deterioration, which also meets the sustainability criteria for environmental technologies (**Table 1.2**).

Table 1.2 Relevant sustainability criteria to be met for environmental technologies

- Little if any use of mineral resources and energy;
- Enabling production of resources/energy from waste;
- Pairing high efficiency with long-term of life;
- Applicable at any place and at any scale;
- Plain in construction, operation and maintenance.

Source: (Lettinga, 2001)

1.2.4. Effluent treatment technologies

Municipal wastewater treatment depends on natural processes such as gravity to clarify effluent and bacterial action to stabilise the biodegradable organic fractions of the wastewaters (Alaerts *et al*., 1990). Alternative treatment technologies that have been developed over the past 20 years place emphasis on cost reduction, integrated systems management, nominal mechanical operations, simple infrastructure that can be managed by non-specialists and, where feasible, water reclamation and nutrient conversion. Treatment systems currently in use in developing countries include passive or natural wastewater treatment systems, and conventional wastewater treatment systems.

The most commonly used wastewater treatment technology in developing countries is the waste stabilisation pond (USEPA, 1992). These may be used either in conjunction with other wastewater treatment technologies or alone. When used alone, they are usually designed as a series of three to five cells that hold the wastewater for at least 20-25 days to provide for adequate pathogen removal. Construction costs are low and pond systems are not difficult to operate and maintain when compared with mechanised facilities. They are, however, land intensive and the effluent often contains large quantities of suspended solid particles, mostly algae that transmit organic pollutants to receiving water bodies (USEPA, 1992).

Anaerobic and aerobic treatments constitute the two major processes for biological treatment of wastewater and biodegradable organic waste. Aerobic biodegradation involves the bacterial conversion of waste organic matter to new bacterial cell matter, which can subsequently be dewatered and disposed to land, and involves the use of oxygen as an electron acceptor during the degradation of the complex organic matter. Conventional wastewater treatment systems may use various types of mechanical equipment to supply air to aerobic bacteria that stabilise organic material and to mix the substrate with the bulk liquid. The conventional treatment systems used in developing countries include the aerobic activated sludge process, and more recently, variants including sequencing batch reactors, extended aeration and the oxidation ditch (carousel) (Shuval, 1990). Disadvantages of the conventional treatment technologies include high power consumption, high maintenance requirements and the need for close supervision by skilled operators (Meeroff and Bloetscher, 1998). In developing countries, conventional mechanical treatment facilities have had a sparse record of success as they frequently do not function as expected due to a variety of technical, financial and institutional reasons. With this in mind, it is timeous to review the role of anaerobic biological processes in wastewater treatment as these technologies have experienced significant technological advances in recent years.

1.3. The role of anaerobic treatment processes in the treatment of wastewater

1.3.1. A historical perspective

Anaerobic digestion, which is a process that involves the breakdown of complex organic materials in the absence of oxygen, is typically employed at many wastewater treatment plants to stabilise or treat sludges. This anaerobic biological process was first discovered in the 17th century by Jan Baptita Van Helmont and the recognition that the process resulted in methane production was attributed to Alessandro Volta. He concluded that a direct correlation existed between the amount of flammable gas produced and the amount of decaying organic matter (Barker, 1956). In 1808 Sir Humphry Davy demonstrated that methane was present in the gases produced during the anaerobic digestion of cattle manure and later in 1856, Reiset reported the liberation of methane from decomposing manure piles and proposed an in-depth study of the process to help explain the

decomposition of the organic matter. The first anaerobic treatment plant was built at a leper colony in Matunga-Bombay, India in 1859 (Meynell, 1976). The first full-scale application had a configuration similar to that of a septic tank and was called "Mouras' Automatic Scavenger." Anaerobic digestion reached England in 1895, where a "septic tank" modelled on the Automatic Scavenger was constructed in Exeter by Donald Cameron and the biogas recovered was used to fuel street lamps (McCabe and Eckenfelder, 1957; Metcalf and Eddy, 1915 cited in McCarty, 2001). The first anaerobic filter was a bed of sand at the Massachusetts experimental station (Reference Library, 1908 as cited by McCarty, 2001) whereas, the first anaerobic system was that described in the historical text on American sewerage practice in 1915 by Metcalf and Eddy. A two-stage process was put into operation in 1904 by William O. Travis in which the suspended solids settled into a separate chamber for digestion (Metcalf and Eddy, 1915 as cited in McCarty, 2001). The period 1920-1930 saw many studies on the anaerobic treatment process, and by the end of 1930 there was sufficient understanding of the process to allow for wide-scale practical application for industrial wastewater and agricultural residues treatment (McCarty, 2001) and the first large-scale application of the anaerobic filter was reported in 1972 (Taylor, 1972).

As reviewed by McCarty (1982), anaerobic processes for treatment of wastewater and sludges have existed as a technology for well over 100 years. The understanding of anaerobic digestion has grown steadily, from both a microbiological and chemical perspective. The environmental aspect and the need for renewable energy have been receiving interest and considerable financial support, thus expanding research and application work in these directions. Systems using anaerobic digestion have been erected in many developing and developed countries. Anaerobic digestion provides some exciting possibilities and solutions to such global concerns as alternative energy production, handling human, animal, municipal and industrial wastes safely, and controlling environmental pollution.

The statement by McCarty in 1964 that "Anaerobic wastewater treatment doesn't enjoy the popularity it truly deserves" convinced many researchers that anaerobic wastewater treatment should be their main field of research (McCarty 1964). However, during the

1980s and especially during the 1990s many research groups paid attention to the development of anaerobic digestion for application in the treatment of low strength effluents (Jewell, 1987; Sanz and Fdz-Polanco, 1990; Lettinga *et al*., 1993; Vieira *et al*., 1994; Kato, 1994). Some authors have reported that as a result of the introduction of one or more anaerobic steps in treatment systems of municipal wastewater, a 30 to 60% reduction in cost was achieved (Alaerts *et al*., 1993; Schelinkhout, 1993; Wang, 1994).

Different anaerobic wastewater treatment systems have been developed over the years around the world including the Anaerobic Filter (AT) (Young and McCarty, 1969), the Upflow Anaerobic Sludge Blanket (UASB) (Lettinga *et al*., 1980), the Fluidised and Expanded Bed Reactor (FEBR) (Schwitzenbaum and Jewell, 1980), the Down Flow Stationary Fixed Film Reactor (DFSFFR) (Murray and van den Berg, 1981) and the Baffled Reactors (BR) (Barber and Stuckey, 1999). Lettinga and co-workers introduced modified versions of the UASB viz. the UASB-septic tank reactor (Bogte *et al*., 1993), the Hydrolysis Upflow Sludge Blanket (HUSB) (Wang, 1994), the Staged Multi-Phase Anaerobic (SMPA) reactor (Lier, 1995) and the two stage-Anaerobic Filter (AF) - Anaerobic Hybrid (AH) system (Elmitwalli *et al*., 2002). Other interesting reactor configurations that have been investigated include: the Fluidised Bed Reactor (FBR), Anaerobic Baffled Reactor (ABR) (Foresti, 2001), Horizontal-Flow Anaerobic Immobilised Biomass (HAIB) (Zaiat *et al*., 2000) and Sequencing Batch Reactor (SBR) (Cybis and Pescado, 2000; Callado and Foresti, 2001). A summary of the history of technological developments in anaerobic treatment systems is presented in **Table 1.3**.

Lettinga's contributions to the development and applications of anaerobic treatment of wastewater have been considerable and he began reporting on this subject in the early 1970s. His first reported anaerobic treatment publications were directed towards dilute industrial wastewaters and beet wastewater (Lettinga *et al*., 1972; Lettinga and Stellema, 1974; Lettinga and van Velzen, 1974; Lettinga *et al*., 1976; Lettinga *et al*., 1977). To date, the most successful reactor design for the treatment of a variety of industrial and municipal wastewaters is the UASB process, (Lettinga *et al*., 1979a; 1979b; Lettinga *et al*., 1980; Lettinga *et al*., 1983; Van der Last and Lettinga, 1992; Bogte *et al*., 1993; Lettinga *et al*., 1993; Lettinga, 1995).

Table 1.3 Historical developments in anaerobic treatment systems technology

Investigator	Process description
• M. Louis Mouras (1881)	Mouras-Automatic Scavenger
• W.D. Scot-Moncrieff (1880) England	The first application of anaerobic filter
• Donald Cameron (1883) England	Septic tank
• At Matunga Bombay (1897)	Waste disposal tanks at leper colony with gas collector
• Harry W. Clark (1899)	Sludge was formed in a separate tank
• William O. Travis (1904)	Travis tank with hydrolyzing chamber
• Karl Imhoff (1905)	Modification of Travis tank
• Germany (1927)	The first sludge heating apparatus in a separate digestion tank was set up. The collected gas was delivered to municipal gas system
• Fair and More (1930)	Importance of seeding and pH control
• Morgan and Torpey (1950)	Mixing in digester and development of high rate digestion
• Stander (1950)	Development of Clarigester and anaerobic baffled reactor based on rotating biological contractor (RBC) concepts
• Young and McCarty (1969)	Anaerobic filter
• Lettinga (1979)	UASB
• Switzenbum and Jewell (1980)	Developed the further concept of anaerobic filters to fixed film reactors

Source: (Khanna, 1989 and Kansal *et al*., 1998)

1.3.2. The current perspective

The inception of the UASB led to the major breakthrough for anaerobic digestion as a high-rate anaerobic treatment system, and it is considered desirable for the treatment of high-strength organic wastewater at medium to large volumes (Lettinga *et al*., 1983; Liu *et al*., 2003). The UASB reactor system has been used in the treatment of several types of wastewaters (Foresti, 2001; Lettinga, 2001; McCarty, 2001) as well as the treatment of sewage (Haandel and Lettinga, 1994). The success of the UASB system lies in the establishment of a dense sludge bed formed by accumulation of incoming suspended

solids and a rich microbial diversity in the bottom of the reactor in which all biological processes take place (Seghezzo *et al*., 1998; Liu *et al*., 2003). Bacteria can naturally aggregate in flocs and granules (Hulshoff Pol *et al*., 1998; Hulshoff Pol *et al*., 2001) which have good settling properties, thereby reducing the susceptibility of bacterial biomass to washout. The retention of active sludge within the UASB enables good treatment performance at high loading rates. The major shortcoming of the UASB reactor is, however, the extremely long start-up period of about 2-8 months required for the development of the anaerobic granular sludge (Liu *et al*., 2003). A thorough description of the mechanisms and models as well as a discussion of the economics of anaerobic treatment in UASB has been presented elsewhere (Lettinga *et al*., 1983a; Seghezzo *et al*., 1998; Liu *et al*., 2003). Modern reactor systems such as the Expanded Granular Sludge Bed reactor (EGSB) have loading potentials that are significantly higher than the conventional UASB systems (Van Lier *et al*., 2001).

Research in South Africa has made a significant contribution to the development of anaerobic digestion. Full-scale experience with anaerobic wastewater treatment for concentrated industrial effluents using the reverse flow Dorr-Oliver Clarigester has been in existence since the 1950s (Stander, 1967). This system could be regarded as one of the precursors of sludge bed reactors including the UASB process (Lettinga *et al*., 1980) and the EGSB process (Lettinga *et al*., 1980, 1984, 1999; Lettinga, 1995, 1996; Rebac *et al*., 1998; Kato *et al*., 1999). Also, during the 60s in South Africa, interesting microbiological and biochemical research was conducted for characterising anaerobic sludge (Hattingh and Siebert, 1967; Siebert, 1967; Thiel and Hattingh, 1967; Thiel, 1969; Pretorius, 1972), and promising anaerobic wastewater treatment-plant feasibility studies were carried out to deal with the treatment of sewage (Pretorius, 1971; Simpson, 1971).

The Rhodes BioSURE Process®, developed at Rhodes University, South Africa, incorporates a Recycling Sludge Bed Reactor (RSBR). This novel low-cost process is an efficient anaerobic treatment process which links the remediation of acid mine drainage with PS serving as electron donor for SRB (Whittington-Jones 2000; Rose *et al*., 2002a, 2002b, 2002c). Preliminary results of sludge solubilisation in the RSBR indicated enhanced enzymatic hydrolysis of PS with yields of soluble products in excess of 50%.

The design, development and operation of the RSBR have been discussed elsewhere (Whittington-Jones, 2000; Whittington-Jones *et al.*, 2002; Rose *et al.*, 2002a, 2002b, 2002c).

1.4. Advantages and limitations of anaerobic wastewater treatment systems

Modern anaerobic treatment systems also offer major benefits over conventional aerobic systems (**Table 1.4**). The application of anaerobic wastewater treatment systems as primary and/or secondary treatment methods can also lead to a reduction in the release of greenhouses gases when properly designed and operated (Lexmond and Zeeman, 1994).

Table 1.4 Benefits of modern anaerobic systems over conventional aerobic systems

- Constitute relatively simple technology, like reactor systems such as UASB, AF, EGSB, or hybrid reactor types;
- Very appropriate everywhere and at any scale, because minimum, if any, energy demands, therefore high self-sufficiency;
- They are comprised of very compact systems in view of the very high applicable organic and hydraulic loading rates;
- Low consumption of resources but rather the production of energy carriers in the form of biogas, and fertilisers like ammonia and phosphates, and non biodegradable residues as soil conditioners;
- Feasible for a wide range of waste and wastewaters, that is, complex in composition, very low and very high strength, as well as low and high temperatures;
- Appropriate for champagne industries, because adapted anaerobic sludge can be preserved under unfed conditions for long period of time.

Source: (Lettinga, 2001)

Many authors have been engaged in discussions as to the choice between aerobic or anaerobic digestion systems for wastewater treatment. A comparison of these two systems has been presented in **Table 1.5**. Developments over the past 15 years have demonstrated that the anaerobic digestion system may be a viable and an attractive alternative for the treatment of a wide range of industrial wastewaters (Gijzen, 2001). To date many applications of anaerobic systems for the treatment of industrial wastewater

exist, and the application to domestic wastewater treatment has also been demonstrated in tropical regions (Gijzen, 2001).

Table 1.5 Summary of comparison of anaerobic and aerobic wastewater treatment

Comparison	**Anaerobic**	**Aerobic**
Mineralization of model substrate	• $C_6H_{12}O_6$ ($3CO_2 + 3CH_4$ (ΔGo = -393 kJ/mole glucose)	• $C_6H_{12}O_6$ + $6CO_2$ ($6CO_2$+ $6H_2O$ (ΔGo = -393 kJ/mole glucose)
Carbon balance	• 95% converted to biogas; 5% incorporated into microbial biomass	• 50-60% converted into CO_2; 40-50% incorporated into microbial biomass
Energy balance	• 95% retained as CH_4, 3-5% is wasted as heat, 5-7% is stored in new biomass	• 60% of energy is stored in new biomass, 40% lost as process heat
Energy requirements	• Low external energy input	• High energy input for aeration
Nutrient requirements	• Low nutrient requirement	• Nutrient addition sometimes required
Startup	• Long startup period	• Short startup period
State of development	• Recently established, still under development for specific applications	• Established technology
Nutrient removal	• Nutrient removal via post treatment	• Nutrient removal can be incorporated
Pathogen removal	• Low	• Low

Source: (Gijzen, 2001)

Although sewage treatment by conventional means is very efficient, this efficiency comes with high capital and running costs as well as technology requirements (El-Gohary *et al.*, 1995). These expensive systems do not offer a sustainable solution to sewage treatment in developing countries. Anaerobic treatment has been proven to be an admirable process and is considered by many authors as the core for sustainable waste management (Zeeman and Lettinga, 1999; Hammes *et al.*, 2000; Gijzen, 2001). Anaerobic treatment is

a natural process in which a variety of different species of bacteria and archea work together to convert organic wastes through a variety of intermediates to methane gas which is an excellent source of energy. While some may consider anaerobic treatment process as an archaic process, it is arguably the most promising wastewater treatment system for meeting the desired criteria for future technology in environmentally sustainable development (NRC, 1995).

The anaerobic process converts organic matter via a series of metabolic steps to gaseous ends products, mainly methane (which is a useful end product) and carbon dioxide. Anaerobic treatment can therefore be defined as the use of biological processes in the absence of oxygen to stabilize organic (carbonaceous) material by conversion to methane (CH_4) and inorganic products, including orthophosphate (ortho-PO_4^{-3}), carbon dioxide (CO_2), hydrogen sulfide gas (H_2S), nitrogen gas (N_2) and ammonia (NH_3) (McCarty, 1982). Also, while aerobic processes require an oxygen supply and relatively high nutrient levels, anaerobic systems function in the absence of oxygen and can operate without the addition of extra nutrients such as nitrogen and phosphorus. As a result, organic loadings to anaerobic reactors can be much higher than to aerobic reactors since oxygen mass transfer limitation is not involved, and energy requirements for mixing are greatly reduced. Anaerobic processes are largely self-controlled, depending almost entirely on the environmental conditions inside the reactors and not on sophisticated process control equipment (Foresti, 2001), thereby allowing in most cases, for the autonomy or self-sufficiency of the treatment plant (Ligero *et al*., 2001). In anaerobic processes, sludge is generated in much lower quantities than in aerobic processes. As a result, energy and sludge management costs are reduced, making anaerobic digestion the most frequently used biological treatment system for waste effluent with medium and high-organic loads (Lettinga *et al*., 1993). The main limitations or drawbacks of the anaerobic wastewater treatment systems are summarised (**Table 1.6**). Although anaerobic wastewater treatment has been around since the late 19th century, it has often been considered to be inefficient and slow (Gijzen, 2001), with a major limitation in the development of the high-rate anaerobic digester being the low yield and long doubling times of the microorganisms involved (**Table 1.6**).

Table 1.6 Limitations or drawbacks of anaerobic wastewater treatment

- Optimal reactor temperature is 20^{o}C and above;
- Longer startup time because of the slow growth rate of the anaerobic bacteria;
- Additional treatment is required to meet secondary quality standards in terms of oxygen consumption;
- Odour control measures are more important than for aerobic treatment;
- Methanogenic activity may be inhibited from the toxic effect of high concentrations of heavy metals, toxic organics, free ammonia and free H_2S;
- Chemical buffering may be required to maintain alkalinity in the reactor;
- Corrosion resistant materials, such as plastics and masonry coatings are required for the reactor vessels and pipes.

Source: (McCarty, 1981; Jewell, 1987; Alaerts, 1990; Giraldo and Eugenio, 1993; Orozco, 1993; Buitrago *et al*., 1994; Jewell, 1994)

One of the major advantages of anaerobic digestion over aerobic alternatives is that energy is produced during the degradation of pollutants. The use of anaerobic treatment biotechnology is therefore highly favourable in developing countries where sustainable technologies are required. Global concerns over energy shortages and green-house gasses have led to the need for more efforts towards renewable energy supplies. Consequently, anaerobic treatment processes as the future for the core of sustainable systems for natural resource conservation, have been increasingly recognised by many researchers in developing countries. They also appear to meet the sustainability development criteria (**Table 1.2**).

The disposal of both primary and waste activated sludge is a global environmental problem. However, this material may also be considered as one of the most readily available forms of carbon and if converted to a form that is usable by microbes, it could be used to drive a range of biological processes. Its application for driving biological nutrient removal has been well studied and more recently, soluble products have been used for the bioremediation of AMD (Whittington-Jones *et al*., 2002). Currently, the yields of soluble products are, however, low (**Table 1.7**, page 23). A better understanding of the biochemical processes involved in the conversion of complex organic matter to simple molecules is required before further optimisation can take place.

1.5. The biochemistry of anaerobic degradation of complex organic matter

In municipal wastewater, a significant fraction of biodegradable organic matter is comprised of dissolved and colloidal macromolecular material and particulate material. Prior to utilisation of this material by the microorganisms, extracellular hydrolytic and or solubilisation steps mediated by enzymes are necessary. In order to hydrolyse these particulate organics, which are mainly proteins, carbohydrates and lipids (Nielsen *et al*., 1992; Raunkjaer *et al*., 1994), microorganisms synthesise and secrete various hydrolysing enzymes. The degradation of complex organic matter has been described as a "multi-step process of a series of parallel reactions" (Pavlostathis and Giraldo-Gomez, 1991), which is accomplished by a complex microbial community involving hydrolytic, fermentating, homoacetogenic, syntrophic and methanogenic microorganisms (**Figure 1.2**) (Zinder, 1993; Stams, 1994; Schink, 1997). Biodegradation of carbohydrates, proteins, and lipids is carried out sequentially by several physiological groups of anaerobic bacteria that work together. The process can be described by the following four steps:

- **Hydrolysis**: extracellular enzymes produced by the inhabiting hydrolytic and fermentative bacteria hydrolyse the macromolecules into smaller and more digestible forms and ferment the resulting sugars to carboxylic acids and alcohols;
- **Acidogenesis**: includes fermentation and anaerobic oxidation which are executed by a large group of facultative and obligate anaerobes such as *Clostridium*, *Bifidobacterium*, *Desulphovibrio*, *Actinomyces*, and *Staphylococococcus*. Volatile fatty acids (VFA), such as propionic acid and butyric acid are produced along with carbon dioxide and hydrogen;
- **Acetogenesis**: acetogenic bacteria that breakdown volatile acids and alcohols to acetate, hydrogen and carbon dioxide;
- **Methanogenesis**: in this process, methanogenic bacteria such as *Methanobacillus*, *Methanococcus, Methanobacterium* and *Methanosarcina* are responsible for converting the end products of the acetogenic reactions to methane gas and carbon dioxide (Metcalf and Eddy, 1991).

The flow of carbon during anaerobic degradation of organic particulate matter and energy pathways that characterise methane fermentation of organic matter is shown in **Figure 1.2**.

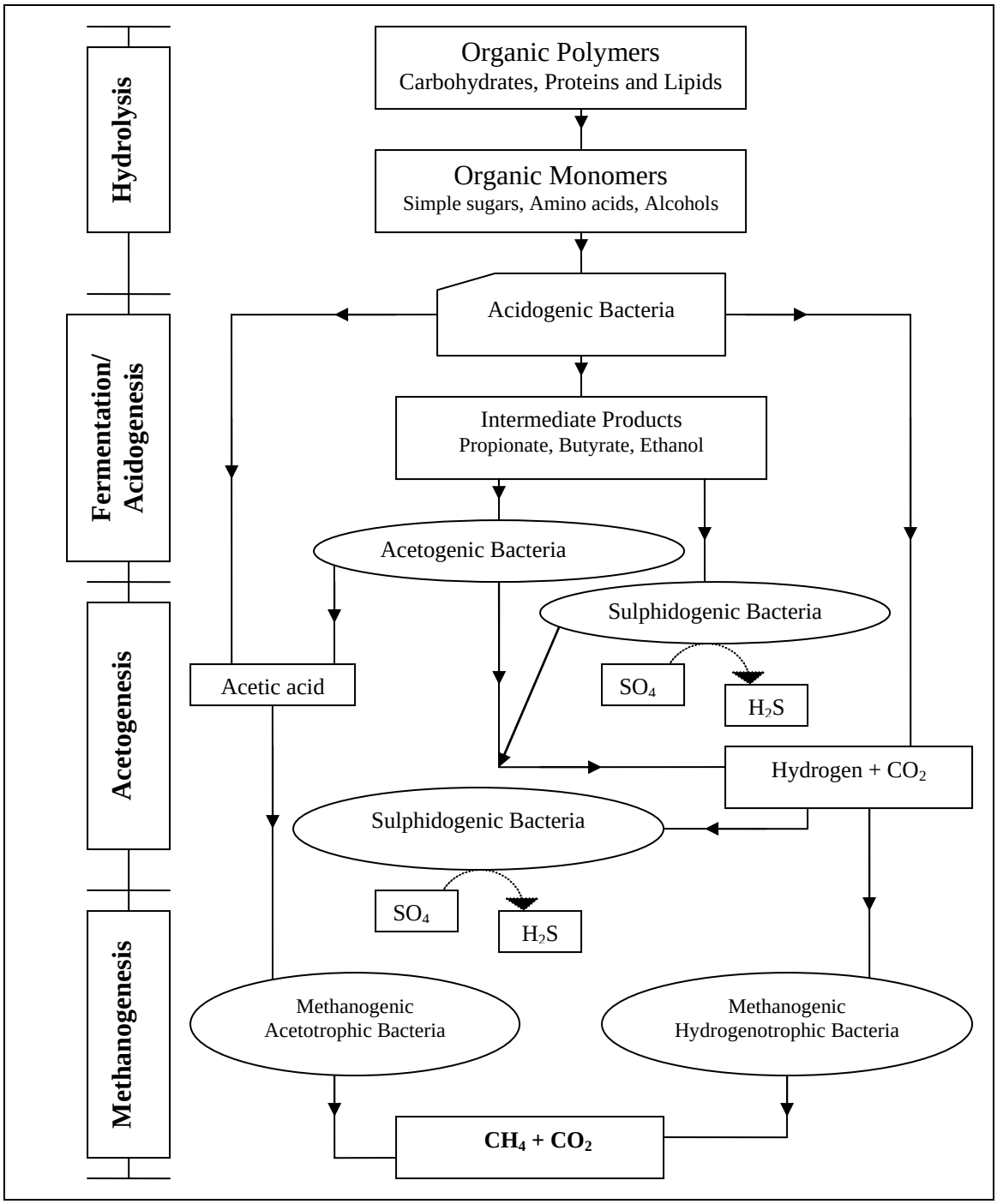

Figure 1.2 Multi-step nature of anaerobic degradation of complex organic matter. The energy yield is comparably low, since organic compounds are used both as electron donors and acceptors. Modified from Giraldo and Eugenio (1993)

The kinetics (first order) of the hydrolysis of complex organic matter under anaerobic conditions at constant pH and temperature has been described (Pavlostathis and Giraldo-Gomez, 1991) and is generally considered as the rate-limiting step. The first order kinetic

(equation 3) is an empirical relation and even when the reactor conditions and substrate are kept constant, changes in particle size distribution of the substrate can result in different k_h values (Hills and Nakano, 1984; Chyi and Dague, 1994).

$$\frac{dX_{degr.}}{dt} = -k_h . X_{degr.} \qquad (3)$$

where: $X_{degr.}$ = concentration of degradable substrate (kg/m^3); t = time (days); k_h = first order hydrolysis (/day).

Since the hydrolysis rate is a biochemical reaction catalysed by enzymes, it is highly dependent on temperature (Sanders, 2001) and can therefore be described by the Arrhenius equation (equation (4)) (Veeken and Hamelers, 1999).

$$K_h = Ae^{-Ea/RT} \qquad (4)$$

where: K_h = hydrolysis rate constant (/day); A = the Arrhenius constant (/day) for the particular reaction; Ea = activation energy (kJ/mole); T = the absolute temperature (^oK); R = the gas law constant (J/mole.^oK)

The operational temperature thus has a substantial effect on the conversion of organic matter and consequently the characteristics of the sludge bed. It has been reported by Morgan-Sagastume and Allen (2003), that biological treatment plants conventionally operate within the mesophilic temperature range of 25-35^{o}C. However, temperature shifts have been linked to decreased sludge metabolic activity (Barr *et al*., 1996; Koebitzsch *et al*., 1998), reduced treatment performance and system instability in full-scale biological plants treating pulp and paper mill effluent at temperature exceeding 38^{o}C (Carpenter *et al*., 1968; Cocci and McCarthy, 1998). Some authors have tried to develop a deterministic model for anaerobic hydrolysis in an effort to gain insight into the hydrolysis process (Hills and Nakano, 1984; Hobson, 1987, Vavilin *et al*., 1996). The assumption with this model is that the substrate particles are completely covered with bacteria that secrete an excess of hydrolytic exo-enzymes during the digestion process. The hydrolysis rate is therefore constant per unit area available and the hydrolysis

constant, K_{sbk}, is not affected by particle size of the substrate. The model is further referred to as the Surface Based Kinetics (SBK) model (equation 5).

$$\frac{dM}{dt} = -K_{sbk} . A \qquad (5)$$

where: M = mass of substrate (kg); t = time (days); K_{sbk} = surface based hydrolysis constant (kg/m^2.day); A = surface available for hydrolysis (m^2).

According to Zeeman and Sanders (2001), hydrolysis of particulate polymers can be described by SBK, but for practical use, the determination of the available surface is so complicated that the empirical first order relation is advised. The rates and yield of soluble products for the anaerobic degradation of PS and other complex organic macromolecules are summarised in **Table 1.7**. Reported values for the rates of hydrolysis and yields of PS are limited. However, a comparison of published data is often complicated by the fact that the criteria by which the rates were calculated differ. Eastman and Ferguson (1981) obtained 0.12/h which was significantly higher than 0.16/day reported by Lilley *et al.* (1990), although both rate calculations were based on soluble products. These differences may however, been due to temperatures differences for which the rates where calculated or due to differences in the composition of the sludge. Shimizu *et al.* (1993) showed that lipids and cellulose hydrolysis were relatively slower, (0.76 and 0.52/day) respectively, whereas the degradation of both proteins and carbohydrates were about 1.2/day. Canziani *et al.* (1996) and Hatziconstantinou *et al.* (1996) reported yields of PS hydrolysis in the range of 5% in psychrophilic conditions to about 35% at 24^{o}C.

Table 1.7 Summary of hydrolysis data obtained from reported studies

Feed	K (/day)	Yield (%)	Temp (^oC)	Sludge recycle	Scale	pH	*HRT (h)	**SRT (day)	Reference
Primary Sludge	-	15-35	150	None	Full	< 4	-	-	Karlsson and Göransson, 1993
Primary Sludge	-	5	150	None	Lab	6.7	15d	-	Canziani *et al*., 1996
Primary Sludge	-	Poor	16	Yes	Pilot	-	1.5	10	Canziani *et al*., 1996
Primary Sludge	-	10-15	25	None	Lab/ Pilot	-	3d	10	Brinch *et al*., 1994
Primary Sludge	0.16	17	20	None	Lab (Batch)	-	-	-	Lilley *et al*., 1990
Primary Sludge	-	9	18-20	None	Lab (Batch)	-	6	-	Banister and Pretorius, 1998
Primary Sludge	-	4.8-22.4	< 20	None	Lab	-	2	2	Hatziconstantinou *et al*., 1996
Primary Sludge	-	35	24	10%	Pilot	-	2	4-5	Hatziconstantinou *et al*., 1996
Primary Sludge	0.12/h	27	35	None	Lab	5.1	1.5	-	Eastman and Ferguson, 1981
Primary Sludge	-	9-16	20	None	Full	-	-	-	Andreasen *et al*., 1997
Activated Sludge	-	2.5	8-17	None	Full	-	-	-	Andreasen *et al*., 1997
Sludge Mix	0.25	-	35	None	Lab	-	10d	-	Siegrist *et al*., 1993
Raw Sewage	0.22	-	20	None	Lab	-	5d	-	Balmat, 1957
Waste Activated Sludge	0.16	65	37	Yes	Lab	7	-	-	Shimuzu *et al*., 1993
Released Organics	1.2	90	37	Yes	Lab	7	10d	-	Shimuzu *et al*., 1993
Starch	3.28	-	20	None	Lab (Batch)	7	-	-	San Pedro *et al*., 1994

Source: (Whittington-Jones *et al*., 2002). Abbreviations: HRT, Hydraulic Retention Time; **SRT, Sludge Retention Time

1.6. The role of enzymes in the hydrolysis of complex particulate organic matter under anaerobic conditions

The key to improving the performance of anaerobic digestion lies in understanding the biochemistry of enhanced hydrolysis, concentrating specifically on the role of hydrolytic enzymes. Due to the need for more efficient treatment of effluent streams, enzyme technology has received increased attention. According to Boczar *et al*. (1992), enzyme activities have received attention because of the following: (a) enzyme activities play a key role in the hydrolysis and mineralization of complex organic materials; (b) the

identification of microbial populations can be achieved by patterns of enzyme activities; (c) insights into the biochemical factors controlling the treatability of xenobiotics can be provided by characterisation of enzyme activities; and (d) overall treatment efficiencies can be improved by adding enzymes exogenously to the overall treatment process.

Enzymes were first proposed for the treatment of waste in the 1930s, but it was not until the 1970s that enzymes were used to target specific pollutants in waste (Aitken, 1993). Enzymes have the potential to be used for the destruction of compounds such as organophosphates (Copella *et al*., 1990), phenols and aromatic amines (Aitken, 1993), o-aminophenol and o-chlorophenol (Klibanov, 1980) and for enhanced hydrolysis of municipal solid waste under both acidogenic and methanogenic conditions (Lagerkvist and Chen, 1993). The degradation of both soluble and insoluble substrates in municipal solid waste is mediated by bacterial groups although the degradation of insoluble substrate is reported to involve an additional enzymatic reaction to catalyse the hydrolysis step, which converts the solid substrates to soluble products (Nopharatana *et al*., 2003). The hydrolysis of wastewater polymers to monomers appears to be a rate-limiting step of the biodegradation process as shown by the fact that high molecular weight compounds are hydrolyzed slowly (Eliosov and Argaman, 1995; Ohron and Çokgör, 1997; Ubakata, 1998; 1999).

Hydrolysis of complex organic substrates such as found in PS relies on a suite of enzymes, and these can be divided into distinct groups. Lipases convert lipids to long-chain fatty acids (LCFA) which are further degraded by β-oxidation to produce acetyl CoA. The microorganisms that appear to be responsible for most of the extracellular lipase producers are thought to be *Clostridia* and the micrococci (McCarty, 1982). Proteases secreted by *Bacteroides*, *Butyrivibrio*, *Clostridium*, *Fusobacterium*, *Selenomonas* and *Streptococcus*, hydrolyse proteins to amino acids, which are further degraded to fatty acids such as acetate, propionate, butyrate and eventually to ammonia (McCarty, 1982). Cellulases, amylases and pectinases hydrolyse polysaccharides such as cellulose, starch and pectin. The enzymatic hydrolysis of cellulose by cellulases is generally a slow and incomplete process (Schwarz, 2001). The majority of microbial cellulases are composed of three groups: (1) endoglucanases or 1,4-β-D-glucan 4-

glucunohydrolases (EC 3.2.1.4) acting randomly within the polymeric chain; (2) exoglucanases which include both 1,4-β-D-glucan glucunohydrolases (EC 3.2.1.74), liberating D-glucose from β-glucan and cellodextrins, and 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91) that liberate D-cellobiose from β-glucan in a processive manner, and (3) β-glucosidase or β-D-glucoside glucohydrolases (EC 3.2.1.21), which release D-glucose units from soluble cellodextrins and a variety of glycosides. α-Glucosidase (EC 3.2.1.20: α-D-glucoside glucohydrolase) is used as the final enzyme in the metabolism of starch, and attacks the α-1, 4 and/or α-1, 6 linkages of the non-reducing ends in short saccharides forming glucose as an end product (Giblin *et al*., 1987; Madi *et al*., 1987; Legin *et al*., 1997). Cellulolysis is performed by the synergistic action of these three enzymes on cellulose, effectively hydrolysing it to produce glucose. The hydrolysis of starch to glucose is performed in a two-step process, which employs a complex system of at least three enzymes: α-amylase, pullulanase and α-glucosidase (Legin *et al*., 1997; Fey and Conrad, 2003). In primary fermentation reactions, the resulting monosaccharides are fermented to fatty acids, alcohols, H_2 and CO_2 and subsequently, through secondary fermentation, to acetate, CO_2 and H_2, which are the immediate substrates of methanogenic archaea.

Activated sludge model number 2 (ASM No. 2) considers enzymatic hydrolysis under anaerobic, anoxic and aerobic conditions as the first step in complete metabolism of particulate substances. It is well known that before bacteria can assimilate high molecular weight compounds, the compounds are usually hydrolyzed by extracellular enzymes. These extracellular enzymes are either bound to the cell surface (ecto-enzymes) (Chróst, 1991) or released (exo-enzymes) into the medium in the free form (Vetter and Deming, 1999) before forming complexes with humic substances or other polymers (Wetzel, 1991). The activity of ecto-enzymes may be tightly coupled to substrate incorporation at low concentrations (Hoppe *et al*., 1988). These extracellular enzymes are involved in the biodegradation of high molecular weight compounds by sewage biofilms (Larsen and Harremoës, 1994; Confer and Logan, 1997, 1997a; Janning *et al*., 1997) and sludge flocs (McLoughlin and Crombie-Quilty, 1983; Henze and Mladenovski, 1991; Dold *et al*., 1995; Garcia *et al*., 1997; Sanders *et al*., 2000).

1.7. Acid mine drainage formation and biological treatment options

Acid mine drainage (AMD) is produced as a result of mining operations. It constitutes a worldwide environmental hazard and is the most well documented type of water pollution associated with mining activities (Johnson and Hallberg, 2003). AMD is characterised by high concentrations of sulfate, metal-enriched waters and low pH (pH < 3) originating from the chemical or biological oxidation of exposed sulfide minerals (Jong and Parry, 2003). A detailed description of the origin and nature of AMD has been reported elsewhere (Nordstrom, 2000; Johnson, 2003). AMD results from the oxidation of pyrite (FeS_2), the most abundant of sulfide minerals, with either molecular oxygen or ferric iron acting as the oxidant (equation 6, Davidson *et al*., 1989).

$$4FeS_2(s) + 15O_2(aq) + 10H_2O \rightarrow 4FeO(OH) + 8SO_4^{2-}(aq) + 16H^+(aq) \qquad (6)$$

Oxygen is only necessary for the microbially catalysed oxidation of ferrous to ferric iron once the acid generating reaction has begun. Pyrite will continue to be oxidised in the absence of oxygen by ferric iron and at this stage the process becomes largely unstoppable (McGinness and Johnson, 1993). AMD can be an extensive environmental problem since the oxidation process may continue for decades after the closure of a mine.

A range of options for the treatment of mine waters have been employed depending upon the volume, type and concentration of contaminants present. Chemical treatment processes are well established and usually involve the addition of lime (Thompson, 1980; Barnes and Romberg, 1986) to raise the pH and precipitate metals as hydroxides. Although chemical methods are quick and effective, they are, however, generally expensive due to the need for additional plants, the high cost of chemical reagents and the production of large volumes of sludge (Elliot *et al*., 1998; García *et al*., 2001).

The biological treatment of AMD by passive technology, such as natural and constructed wetlands has long been appreciated (Johnson, 1995; Gazea *et al*., 1996; Banks *et al*., 1997; Younger *et al*., 1997; Younger, 1998). The operation of this process uses both SRB and acidophilic iron bacteria (Hendin *et al*., 1989) and also depends on the provision of suitable organic substrates for alkali generation (Kalin *et al*., 1991; Johnson, 1995). The

use of SRB relies on their ability to convert sulfate to sulfide, which can be used to precipitate heavy metal ions. BSR also results in the generation of alkalinity and consequently, an increase in pH. The conversion of PS to soluble products, and the subsequent utilisation of these products for biological nutrient removal (BNR) has been well documented (Lee *et al*., 2003). More recently, researchers have attempted to use sewage sludge as a carbon source to drive biological remediation of AMD. SRB are usually viewed negatively due to odour formation and their association with corrosion. Nonetheless, they may also play a valuable role in the remediation of AMD. The use of wetlands provides a low cost approach to the long term management of the AMD problem. A drawback, however, includes the large surface area requirement for high AMD flows and long term stability of the deposited metals (Hendin *et al*., 1989).

1.8. Sulfate reducing bacteria

One of the oldest forms of life on Earth are the sulfate reducing bacteria (SRB), which may be traced back in the geological rock records to the early Archean (3900 to 2900 million years ago), when the oxygen concentration of the Earth's atmosphere was low (Vasconcelos and McKenzie, 2000). These microorganisms, discovered in 1895 by the Dutch microbiologist Beijerinck, can be defined as "a mixed group of morphologically and nutritionally diverse, strictly anaerobic bacteria which utilise sulfate and/or other oxidised sulfur compounds as electron acceptors for the dissimilation of organic compounds" (Widdel and Pfennig, 1984). SRB are obligate anaerobes which obtain energy for growth by the oxidation of organic substrates and use sulfate as the electron acceptor, resulting in the formation of sulfide (Postgate, 1984; Widdel, 1988). SRB are ubiquitous in anaerobic wastewater treatment sludges (Lens *et al*., 1995) and are ecologically important as terminal electron accepting microorganisms in environments where sulfate is present in sufficient amounts. They are also important in environmental biotechnology, such as, to remove sulfate or other sulfur oxyanions from a process wastewater, or for the precipitation of highly insoluble heavy metals as metal sulfides (Lens *et al*., 1995).

Biological sulfate reduction using SRB for the removal of contaminant metals from acidic wastewaters provides a valuable alternative to chemical treatment methods, given their role in the generation of insoluble metal sulfides and the neutralising effect of the sulfate reducing reaction (Postgate, 1984; Barton and Tomei, 1995). Increasing interest in the potential biotechnological applications of BSR as an alternative technique for the removal of sulfate and heavy metals from contaminated environments has been reported recently (Chang *et al*., 2000; Jong and Parry, 2003). Under anaerobic conditions SRB dissimilate sulfate for energy gain by transporting exogenous sulfate across the bacterial membrane into the cell (Cypionka, 1986). The sulphate, through the action of ATP (adenosine triphosphate) sulfurylase, is combined with ATP to produce a highly activated adenosine phosphosulfate (APS), which is the actual electron acceptor that is converted to bisulfide and adenosine monophosphate (AMP). The bisulfide formed is then rapidly converted to sulfide by APS reductase (Gibson, 1990). This enzyme also catalyzes the first step in the assimilatory sulfate reduction in bacteria and plants, or the biosynthesis of sulfate esters in plants and animals (Gibson, 1990). The biogenic sulfide produced reacts with dissolved metals in the medium to form metal sulfides, which precipitate, since their solubilities are generally low (Kim *et al*., 1999). A concomitant formation of bicarbonate ions increases the pH of the AMD solution.

1.8.1. Characteristics and physiology of sulfate reducing bacteria

The SRB can be considered a physiologically unified group of bacteria because of the dissimilatory sulfate reduction that they can undertake. Nevertheless, the SRB are morphologically diverse, with strong dissimilarities with respect to physiological and phylogenetic properties. This is exemplified by *Desulfovibrio* and *Desulfotomaculum*, which were the two first known genera of SRB. *Desulfovibrio* is 'comma'-shaped, and only grows at moderate (< 45^{o}C) temperatures. In contrast, *Desulfotomaculum*-species are spore-forming rods with some species growing at temperatures of 80^{o}C. *Desulfotomaculum*-species are more closely related to the non-sulfate reducing genus *Clostridium* than to *Desulfovibrio* (Widdel, 1988). A relatively wide range of genera of SRB has been identified, with at least 17 genera known to date. The SRB show a great variation in physiology which reflects the diversity of their habitats. This is exemplified

by the fact that sulfate reduction can make use of numerous electron donors. These include methanol, acetone, aniline, glycerol, benzoate, catechol, phenol, indole, and even alkenes (Widdel, 1988; Widdel and Hansen, 1992), amongst others. Almost all SRB can use hydrogen as electron donor, although for some species an organic carbon source is needed for growth. The biology, ecology, physiology and enzymology of these organisms have been reviewed comprehensively by Postgate (1984), Widdel (1988), Gibson (1990), Widdel and Bak (1991), Widdel and Hansen (1992), Odom and Singleton (1993) and Barton (1995). Some reviews focused specifically on the microbiology and the role of SRB in the anaerobic treatment of wastewater (Oude Elferink *et al*., 1994; Colleran *et al*., 1995) and corrosion (Hao *et al*., 1996).

SRB are also highly diverse with respect to the electron acceptors they use. Besides sulfate, the use of sulfite and thiosulfate as alternative electron acceptors is common, while some species also use elemental sulfur in addition. The use of alternative electron acceptors or the ability to grow fermentatively confers SRB a high degree of flexibility when challenged with fluctuating levels of electron acceptors (Postgate, 1984; Fitz and Cypionka, 1990; Lens *et al*., 1995). SRB usually produce H_2S as probably the most significant aspect of their metabolism. The excretion of minor amounts of sulphite and thiosulfate as end products has also been reported (Vainshtein *et al*., 1980; Fitz and Cypionka, 1990).

1.8.2. Environmental factors affecting sulfate reducing bacteria

The sulfate reduction rate strongly depends on temperature and may occur in a temperature range of 0 to 102^{o}C (Jørgensen *et al*., 1992; Isaken and Jørgensen, 1996). Most mesophilic SRB operate at temperature optima of between 28 and 32^{o}C (Hao *et al*., 1996), while the thermophilic species have an optimum of between 54 and 70^{o}C (Zeikus and Dawnson, 1983; Beeder *et al*., 1995; Rees *et al*., 1995; Nilsen *et al*., 1996). Studies on the effect of temperature on the performance of a mesophilic (30^{o}C) system using an upflow anaerobic sludge bed reactor by Visser *et al*. (1992), indicated that temperature shocks of 45^{o}C did not result in any detrimental effects, whereas temperature shocks of 55 and 65^{o}C led a significant reduction in treatment efficiency. Most of the known SRB

have optimum growth pH of 6-8 (Widdel and Pfennig, 1984), and are usually inhibited at pH values lower than 5.5 or higher than 9 (Pfennig *et al*., 1981; Widdel and Pfennig, 1984; Hao *et al*., 1996). For this reason, changing the pH of waste streams with acid or alkali has been suggested as a method for diminishing sulfate reduction in industrial plants. The treatment of a wide variety of organic wastes using the degradative capacity of the SRB is promising and has applications for environmental biotechnology (**Table 1.8**).

Table 1.8 Use of sulfate-reducing bacteria in biotechnological applications

Application	**Reference**
Biological sulfate removal	
Industrial wastewaters	Särner, 1990
Acid mine drainage	Maree *et al*., 1991
Spent sulfuric acid	Stucki *et al*., 1993
Scrubbing waters SO_2-rich gases	Kaufman *et al*., 1996
Heavy metal removal	
Extensive treatment (wetlands)	Hao *et al*., 1996
High rate reactors	Tichy *et al*., 1998
Process water	
Acid mine drainage	Barnes *et al*., 1991
Ground water	Scheeren *et al*., 1991
Micro aerobic treatment	
Treatment of domestic sewage	Takahashi and Kyosai, 1991
Reduction waste sludge production	Lens *et al*., 1995
Solid waste treatment	
Gypsum process	Deswaef *et al*., 1996

Source: (Hulshoff Pol *et al.*, 1998)

Since the distribution of sulfide species is determined by the system pH, a lower pH may play an important role in sulfide inhibition of the bacterial sulfate reduction because of the toxicity of H_2S species to the SRB, especially at high sulfide concentration (Postgate,

1984; Hilton and Oleszkiewicz, 1988; Rinzema and Lettinga, 1988; McCartney and Oleszkiewicz, 1993; Maillacheruvu *et al*., 1993). It is thought that sulfide inhibition of the SRB probably occurs when certain sulfide species (H_2S, HS^-, and S^{2-}) combine with ferredoxin and cytochrome C and other essential iron-containing compounds in the cell, thereby inhibiting the electron transport systems (Okabe *et al*., 1992). Though it was originally thought that sulfate reducing bacteria are obligate anaerobes, some strains, mainly from the *Desulfovibrio*, are able to survive exposure to oxygen, while others are even capable of limited aerobic respiration (Hardy and Hamilton, 1981; Cypionka *et al*., 1985; Canfield and Marias, 1991; Frund and Cohen, 1992; Cypionka, 2000). Their presence has also been demonstrated in oxidised environments, within reduced microniches (Jørgensen, 1977).

1.9. Electron donors for sulfate reduction

Biological sulfate reduction (BSR) is only possible if a suitable electron donor (carbon-source) is present. The preferred electron donors for SRB are usually low molecular weight compounds such as organic acids (e.g. acetate and lactate) (Middleton and Lawrence, 1977; Braun and Stolp, 1985; Nanninga and Gottschall, 1986). Ethanol, a substrate that supports fast growth of sulfate reducing bacteria (Postgate, 1984; Swezyk and Pfennig, 1990; Widdel and Hansen, 1992), is rapidly oxidized to acetate according the following equation:

$$2CH_3CH_2OH + SO_4^- \rightarrow 2CH_3COO^- + H^+ + HS^- + 2H_2O \quad (7)$$

Acetate is then used as a carbon source or is further oxidized to carbon dioxide:

$$2CH_3COOH + SO_4^- \rightarrow 2HCO_3^- + HS^- + H^+ \quad (8)$$

Methanol is widely used to drive denitrification in wastewater treatment processes but, Oremland and Polcin (1982) showed that methanogenic bacteria can out-compete sulfate reducing bacteria for methanol and therefore it is doubtful whether methanol is a suitable electron donor for sulfate reduction. Du Preez *et al*. (1992) showed that producer gas, also known as synthesis gas (gas mixture of H_2, CO and CO_2), is an effective energy source for the biological reduction of sulfate in industrial effluents. The advantage of

using synthesis gas is that hydrogen may be used directly as an electron donor for SRB. Oude Elferink *et al.* (1994) and Rinzema and Lettinga (1988) showed that SRB are able to out-compete methanogenic bacteria for hydrogen. More complex carbon sources such as molasses, sewage sludge and pulp and paper mill wastewater have also been used as electron donors (Maree and Strydon, 1985). The economic feasibility of using SRB for remediation thus depends on two factors: the cost of the electron donor per unit sulfate converted to the sulfide and the quality of the final product.

For the use of BSR to be feasible on a large scale, that is, for the remediation of AMD, low-cost electron donors have to be considered. Previous studies have shown that the performance of biological nutrient removal processes were enhanced by the addition of the products of hydrolysis of PS (Brinch *et al.*, 1994; Skalsky and Daigger, 1995; Canziani *et al.*, 1996; Hatziconstantinou *et al.*, 1996; Andreasen *et al.*, 1997; Banister and Pretorius, 1998). More recent studies conducted by Dunn (1998) and Whittington-Jones (2000) have shown that complex carbon-sources including tannery effluent and PS could be used as electron donors to drive biological sulfate reduction in both laboratory-scale and in 1.5 m^3 upflow anaerobic reactors, as well as in 25 m^3 reactors (Rose *et al.*, 2002c). An anaerobic reactor operated at a tannery was fed with a stream of the tannery mix effluent at a concentration of 2000 mg/l SO_4 and an 80% removal of sulfate was reported at a conversion rate of over 500 mg/l.day. PS is not only readily available, but it is also inexpensive; the only major cost being piping from the collection point to the reactor site. Sewage sludge, however, contains large quantities of organic matter which has to be broken into soluble polymers or monomers before it can be used by the SRB. As discussed previously, the yield of soluble products from PS is usually less than 36% (Hatziconstantinou *et al.*, 1996). Studies by Whittington-Jones (2000) and Rose *et al.* (2002b) resulted in the development of the Recycling Sludge Bed Reactor (RSBR) and demonstrated that yields in excess of 50% were possible at mesophylic temperatures.

1.10. The development of the Recycling Sludge Bed Reactor (RSBR)

Various reactor designs have been employed for the growth of SRBs including trench reactors (Younger *et al*., 1997), anaerobic filters (Chian and De Walle, 1983), mixed (Maree and Hill, 1989), anaerobic packed bed, fluidized bed (Umita *et al*., 1988), gas-lift (Du Preez and Maree, 1994) and baffled (Grobicki and Stukey, 1992) reactors. To date, the engineering of active biological wastewater treatment processes has concentrated primarily on comparatively high-cost bioreactor systems, and carbon sources such as ethanol (Johnson, 2003).

The development of the RSBR was motivated by the requirements for a technology that will be sustainable over a long period of time and provide a low-cost treatment of large volumes of sulfate-rich effluent. Research activity was thus focused on the development of relatively simple bioreactor designs and the use of complex particulate organic matter as electron donors. Dunn (1998) and Rose *et al*. (2002a) had shown that tannery effluent and PS could be used as sources of electron donors to drive biological sulfate reduction in laboratory-scale and in 1.0 m^3 reactors. The results obtained in the tannery Integrated Algal Ponding System (IAPS) was an efficient solubilisation and removal of organic particulates, and high sulfate reduction rates in their anaerobic compartments. This led to a series of follow-up studies on the phenomenon of enhanced hydrolysis of complex organic matter apparently occurring in these systems. Due to the importance of hydrolysis in the biodegradation of complex organic carbon structures into simpler monomeric substances for subsequent utilisation by microorganisms in anaerobic treatment processes, the apparent advantage of the recycling sedimentation was subjected to a more rigorous investigation in the RSBR. Results obtained from the laboratory-scale RSBR confirmed that PS can provide a cheap and abundant carbon source to drive biological sulfate reduction and that hydrolysis of PS was enhanced in a recycling biosulphidogenic environment. The results of these studies and the scale-up development of the RSBR which formed the basis of the Rhodes BioSURE Process® has been reported in detail elsewhere (Molipane, 1999; Corbett, 2001; Molwantwa, 2002; Rose *et al*., 2002a, 2002b, 2002c; Whittington-Jones *et al*., 2002).

1.11. The influence of biological sulfate reduction on the anaerobic digestion of complex organic matter

In the last 20 years, the anaerobic wastewater treatment process has gained substantial recognition as an established technology for the treatment of sewage sludge and a variety of low and high-strength industrial wastewaters (Franklin, 2001; Vallero *et al*., 2003; Vossoughi *et al*., 2003). With sulfate being a common constituent of many wastewaters, sulfate reduction may, however, cause several problems in this treatment process (Abram and Nedwell, 1978; Hilton and Archer, 1988), due to the production of odorous, toxic and corrosive hydrogen sulfide by sulfate reducing bacteria (**Table 1.6**).

The SRB play an important role in the degradation of organic matter under anaerobic conditions. The optimisation of the hydrolysis reactions controlling the anaerobic digestion of the particulate substrates from the sewage sludge could provide for a useful source of biodegradable carbon for efficient biological sulfate reduction. The development of treatment processes using the degradative capacity of the SRB opens promising perspectives for environmental biotechnology. SRB metabolise a far wider range of substrates and do not require balanced growth with acetogens, which implies less sensitivity to organic overloads. SRB are less sensitive to toxicants and can metabolise organic toxicants such aromatics (toluene, ethylbenzene), alkanes, chlorinated compounds (chloroform), and long chain fatty acids (Bollag and Kaiser, 1991; Gupta *et al*., 1996). BSR in addition to methanogenesis occurs in an anaerobic environment rich in oxidised sulfur compounds, as an end step in the anaerobic mineralization process (Lens *et al*., 1998) (**Figures 1.2** and **1.3**). There can thus be considerable alteration of the biodegradation pathways. Although the yield of soluble products in earlier studies of the RSBR system was higher than previously reported values in literature, the mechanism of "enhanced hydrolysis" was not understood.

It was thought that such an understanding would allow for further optimization of the RSBR system. As a result, two slightly different theories were proposed, which both involved the dynamic interaction between sludge flocs, sulfur species and hydrolytic enzymes in enhanced hydrolysis.

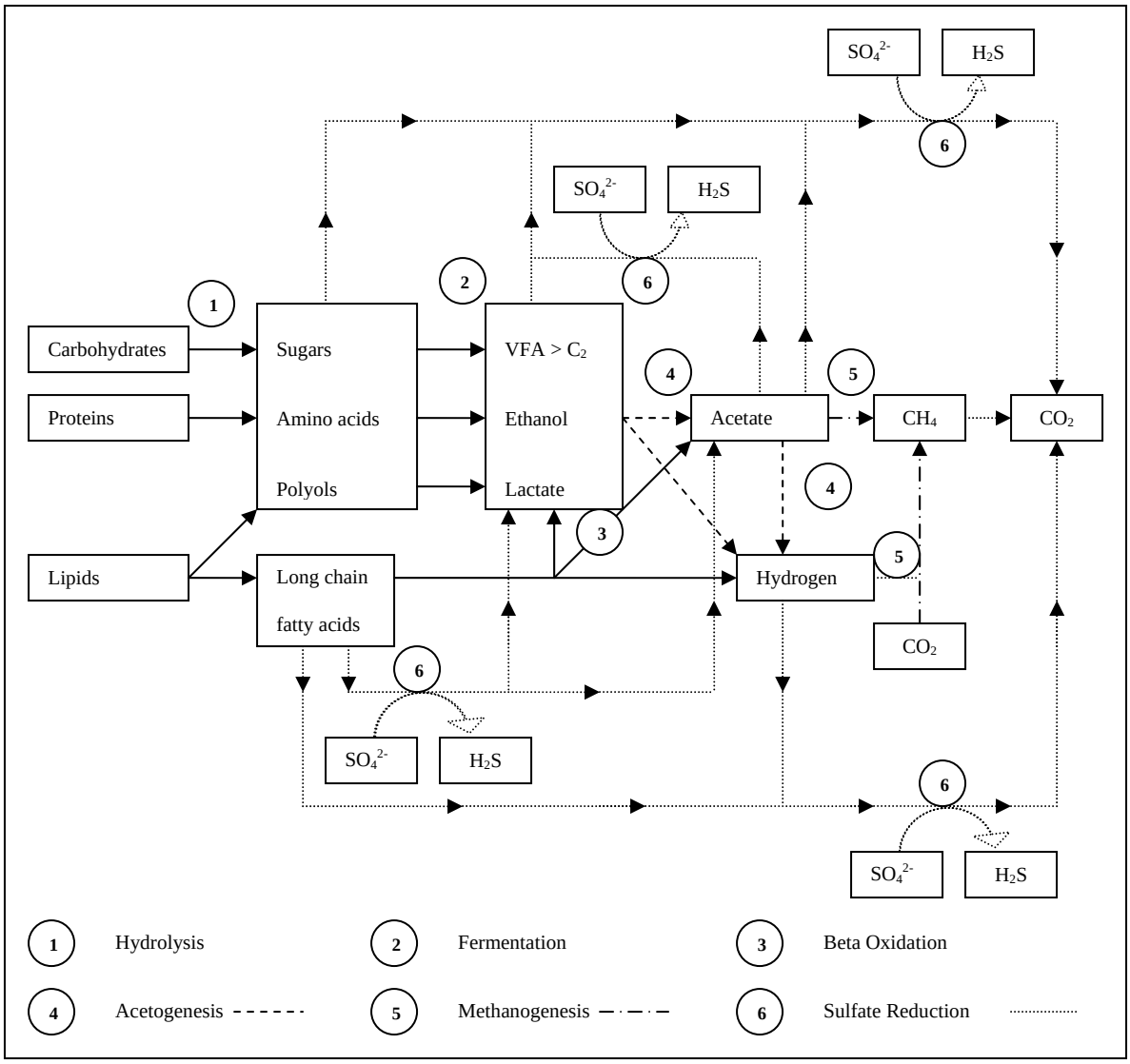

Figure 1.3 The degradation of complex organic matter in the presence of sulfate under anaerobic conditions (Source: Lens *et al*., 1998)

Firstly, Whittington-Jones (2000) proposed that as the sulfide concentration increased during sulfate reduction, the hydrolysis of sludge proteins was enhanced. The role of sulfide was, however, limited to the production of sufficiently high concentration of sulfide by the SRB. Secondly, it was proposed by Pletschke *et al*. (2002), that sulphite and sulfide species liberated during sulfate reduction interacted directly with the enzymes on the floc surface with a concomitant enhancement of enzymatic activities. This, in turn, results in enhanced degradation of complex macromolecules within the floc, resulting in the disruption of the floc. This is possible since molecules within the flocs are protected from enzymatic degradation and disruption of this network inevitably exposes those

macromolecules previously protected from enzymatic attack, which are then subsequently degraded. Currently, there is not enough evidence to conclusively support either model, and further investigation into the role of hydrolytic enzymes in the degradation of complex carbon in anaerobic environments is required.

Jain *et al*. (1992) reported in their model that the factors which had the greatest impact on the rate-limiting hydrolysis step comprised of the concentration of hydrolytic enzymes and the contact between these enzymes and their substrates. Other factors that have been reported to considerably affect the rate of hydrolysis in anaerobic digestion of complex substrates are particle size (Balmat, 1957; Levine *et al*., 1985; Szikriszt *et al*., 1988; Choi *et al*., 1997; Madhukara *et al*., 1997; Müller *et al*., 1998) and the ratio between the characteristic sizes of the hydrolytic bacteria and the substrate particles (Vavilin *et al*., 1996). Previous studies have reported an enhanced hydrolysis of complex particulate organic matter under biosulphidogenic conditions and speculate on the potential of the SRB to directly degrade these compounds (Kim *et al*., 1997; Pareek *et al*., 1998; Whittington-Jones, 2000). Other studies which may explain enhanced hydrolysis have indicated enhanced enzyme activity under biosulphidogenic conditions (Pletschke *et al*., 2002; Whiteley *et al*., 2002a, 2002b, 2002c, 2003). A further possibility proposed by Whittington-Jones (2000) was that the presence of sulfide in RSBR leads to the production of smaller sludge flocs thereby providing increased surface area for enzyme activity, which will result in an increase in the rate of hydrolysis of complex particulate organics. Other studies have shown that the rate and extent of the degradation of ligno-cellulose, a compound abundant in primary sludge (Elefsiniotis and Oldham, 1994), was enhanced in the presence of sulfur compounds (Khan and Trottier, 1978; Kim *et al*., 1997; Pareek *et al*., 1998). Also, hydrolysis of particulate organics (carbohydrates, proteins and lipids) may also be enhanced in the presence of sulfide, since sulfide is a strong reducing agent and is capable of reducing disulfide linkages that are essential for maintaining the three dimensional conformation structures.

1.12. Research hypothesis

The rate at which the hydrolysis of complex particulate organic matter proceeds is best described by first order kinetics and may be strongly influenced by environmental and operational parameters including pH, temperature, sulfide and sulfate concentrations, microbial biomass, particle size, type and concentration of particulate substrate and enzyme activities. Although reported rates of hydrolysis and yields of soluble products from PS under anaerobic conditions are generally low (**Table 1.7**), some studies have indicated that enzyme activity is enhanced in the presence of sulfide, thereby resulting in increased yields of soluble products from complex carbon sources such as PS. Furthermore, this process of solubilisation is facilitated by the design and the operation of the RSBR.

Based on the above, it is predicted that sulfide, whether derived chemically or biologically, has both a direct and indirect positive effect on the activity of key hydrolytic enzymes and thereby results in improved hydrolysis (as yield of soluble products) of complex carbon sources. Enhanced hydrolysis is further facilitated by the design of the RSBR, which ensures that undigested macromolecules pass through regions of high sulfide concentration and optimum pH levels and that contact between enzymes and their substrates is maximised.

1.13. Research objectives

The objectives of this research project were to:

- Gather baseline data to confirm enhanced hydrolysis of primary sludge within a biosulphidogenic RSBR;
- Establish an enzymological profile associated with the enhanced solubilisation of PS by examining spatial and temporal variability in the activity of key hydrolytic enzymes within a biosulphidogenic RSBR;
- Examine whether factors such as sulfide, sulfate, alkalinity and pH affect the activity of hydrolytic enzymes *in situ* within the RSBR and to examine correlations between changes in these factors and enzyme activities;

- Investigate the role of sulfide in enhanced hydrolysis and determine whether elevated sulfide concentrations have a direct influence on the activity of the key hydrolytic enzymes through kinetic studies;
- Investigate the role of enzyme-enzyme interactions in enhanced hydrolysis through the use of selective enzyme inhibitors;
- Investigate the relationship between biosulphidogenesis, morphology of sludge flocs and enhanced hydrolysis within the RSBR;
- Propose a descriptive model describing the relationship between enzymes and chemical and physical factors in enhanced hydrolysis of complex carbon within the biosulphidogenic RSBR.

Chapter 2

Characterisation of the Recycling Sludge Bed Reactor (RSBR): Physico-chemical Parameters

2.1. Introduction

The anaerobic digestion of complex organic matter is influenced by a range of factors including the composition of the substrate, the species of microorganisms present in the inoculum, the concentration of hydrolytic enzymes (Eastman and Ferguson, 1981; Levine *et al*., 1985), pH, temperature (Eastman and Ferguson, 1981; Gujer and Zehner, 1983; Perot *et al*., 1988; El-Fadel *et al*., 1996; Van Lier *et al*., 1997; Banerjee *et al*., 1998; Teichgräber, 2000), chemical oxygen demand (COD) (often divided into particulate and soluble) (Raunkjær *et al*., 1994), loading rate, hydraulic retention time, alkalinity, sludge retention time, mixing (Gujer and Zehner, 1983; Perot *et al*., 1988; Banister and Pretorius, 1998) and the reactor design. All of these parameters have been shown to influence the rate and degree of hydrolysis of components of municipal wastewater including lipid, protein fractions and carbohydrates (Metcalf and Eddy, 1991; Nielsen *et al*., 1992; Raunkjær *et al*., 1994).

Hydrolysis is generally considered as the rate-limiting phase of anaerobic sludge solubilisation. This might be due to the fact that certain organic molecules are more susceptible to degradation than others and less readily degradable fractions may reduce contact between hydrolytic enzymes and readily degradable substrates. Under certain conditions, products of hydrolysis and subsequent steps may be inhibitory to hydrolytic enzymes and therefore reduce the activity. This may be particularly true under methanogenic conditions where methane producing bacteria (MPB) are known to preferentially utilise acetate over other volatile fatty acids (VFA). This in turn may result in accumulation of VFAs and a reduction in the pH of the immediate environment.

A process for sludge digestion by sulfate reducing bacteria (SRB) was proposed by Butlin *et al*. (1956), where organic sludge was mixed with high sulfate wastes and allowed to digest anaerobically. Sulfate as sodium sulfate (Na_2SO_4) was added as electron acceptor in the reactor and the degradation of complex organic matter was carried out under sulfate reducing conditions by SRB. Kim *et al.* (1997) proposed that sulfate reduction can also enhance the stabilisation of waste, considering the wide range of organic substrates used by SRB along with thermodynamic and kinetics aspects of sulfate reduction.

The incorporation of the recycle of settled material within the biosulphidogenic RSBR was thought to facilitate the hydrolytic step by firstly separating undegraded substrates from soluble products upon the fracture of flocs as they move through a sulfide gradient. Secondly, a large proportion of total sulfate reduction occurs in the base of the RSBR, and based on previous studies, this would result in enhanced enzymatic activity in this region. Without recycle, there would be a separation between fresh undegraded substrates in the upper region and "activated" enzyme in the base. It was proposed that contact between a product of the process, that is, sulfide, and fresh substrate, was required for enhanced hydrolysis to occur. The principal objective of any biological reactor configuration should be to bring the substrate and enzymes into intimate contact for sufficient time to allow the reactions to occur.

Although the principal aim of this study was to interrogate the mechanism of enhanced hydrolysis from an enzymological perspective, it was first necessary to verify that the new laboratory-scale RSBR performed in a similar way and exhibited the same characteristics as the previously studied systems. The key parameters to be considered included solubilisation of particulate COD, pH, alkalinity, and sulfate reduction, with SRB using PS as a sole carbon source.

2.2. Materials and Methods

2.2.1. Reactor system and experimental set-up

The laboratory-scale RSBR was constructed from Perspex with internal dimensions of 500 mm (length) by 130 mm (diameter) and had a cone incorporated at the bottom to facilitate even settling and recycling of sludge (**Figure 2.1**). The reactor had a working volume of 3500 ml. The lid of the bioreactor and other glass fittings were sealed with vacuum grease to maintain anaerobic conditions. Oxygen impermeable Tygon® tubing was used for recycling, whereas, silicone tubing was used for feed influent and Marprene® tubing in the pump head. The particulate matter that settled into the base of the RSBR was re-circulated at a rate of 8.89 ml/min, equivalent of 15.2% of sludge volume per hour, using a peristaltic pump (Watson Marlow, 504S). This compared well with a recycle rate of 20% per hour used by Whittington-Jones (2000). The recycled

sludge re-entered the reactor at a position adjacent to the influent stream, thereby allowing reaction with fresh substrates.

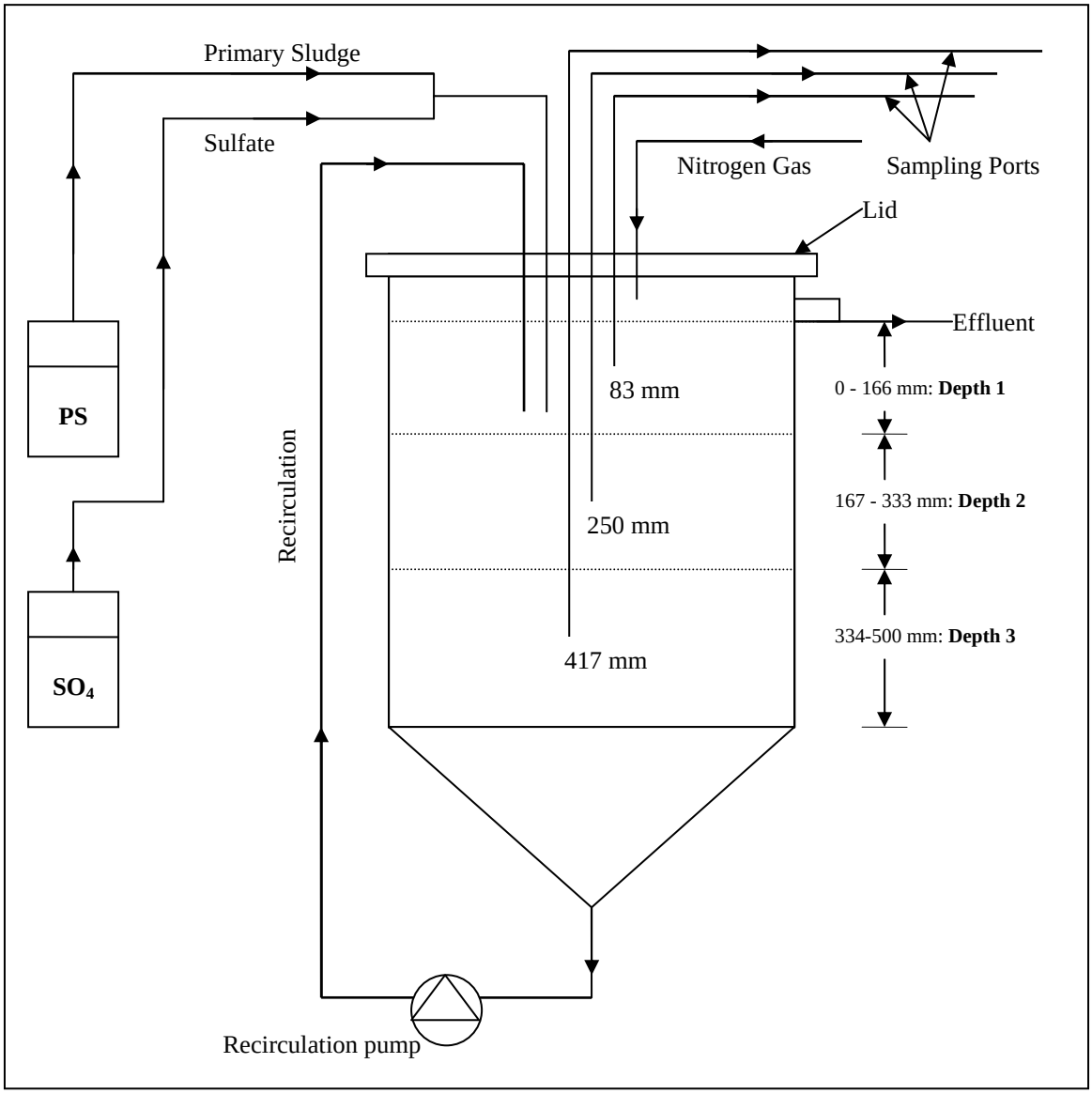

Figure 2.1 Schematic diagram of the laboratory-scale Recycling Sludge Bed Reactor (RSBR)

Fresh feed, consisting of PS and sulfate-rich mixture was fed into the bioreactor using variable speed peristaltic pumps (Model P1-19-4610-10, Pharmacia, Fine Chemicals) at a rate defined by the hydraulic retention time (HRT) of 48 h. A HRT of 48 h was determined as sufficient in earlier studies within these laboratories (Whittington-Jones, 2000), and this was used throughout the experimental period in this study. The system was run in the dark at ambient temperature (20-22^{o}C).

The mean organic loading rate was 0.5 kg COD/m^3.day for the duration of the studies and was calculated from equation (9):

$$OLR = \frac{Q.COD}{V} = \frac{COD}{HRT} \quad (9)$$

where:

OLR = organic loading rate (kg COD/m^3.day); Q = influent flow rate (m^3/day);

COD = chemical oxygen demand (kg/m^3); V = reactor volume (m^3);

HRT = hydraulic retention time (day).

Three glass sample tubes of varying length were inserted through the lid of the reactor and this allowed samples to be drawn daily with a syringe from 83 mm (depth 1), 250 mm (depth 2) and 417 mm (depth 3) for analysis (**Figure 2.1**).

2.2.2. Preparation of feed

Fresh primary sludge (PS), obtained from Grahamstown municipal works, was used as the carbon source for the SRB. The PS was collected from the underflow lines of the primary clarifiers, macerated, then passed through a sieve (2 mm mesh size) and stored at 4^oC until used. Fresh feed was made up and added to the system every 48 h to minimise the build up of large bacterial populations in the feed reservoir. The PS was diluted with tap water to obtain a final feed COD concentration of 2000 mg/l in a total volume of 2000 ml. A separate reservoir contained a 2000 mg/l sulfate solution made by dissolving Na_2SO_4 (Merck Chemical (Pty) Ltd) in distilled water. The COD:SO_4 ratio in the reactor feed was thus 1:1. Mixing of the feed was achieved using a magnetic stirrer (Model, Fried Electric) at a speed of 400 rpm to afford adequate mixing of the particulate organic matter and prevent any settling of heavier particulate matter prior to entering the RSBR. The effluent of the RSBR was discharged by gravity through an overflow tube.

The necks of the feed reservoirs were closed with rubber stoppers to minimize aerobic digestion of the fresh PS and to minimize any oxygen transfer into the reactor itself. Nitrogen gas was flushed into the headspace of the reactor system on a daily basis and before the collection of samples to maintain these anaerobic conditions.

The reactor was initially inoculated with 500 ml anaerobic sludge containing an active SRB population obtained from an existing laboratory-scale biosulphidogenic reactor (Molwantwa, 2002). A start-up period of 30 days was allowed after which the reactor was deemed to be operating at a steady-state condition. After the 30-day acclimation period, the reactor was run for a further 60 days.

2.2.3. Analytical Methods

The laboratory-scale RSBR was operated for a period of 60 days. During this period daily samples were drawn at regular intervals from the three depths of the RSBR (**Figure 2.1**, Depth 1 = 83 mm, Depth 2 = 250 mm, Depth 3 = 417 mm) using a syringe and analysed as described below. All analyses were carried out in triplicate and values reported as the means with standard deviations. All reagents and standards used were of analytical grade from Sigma-Aldrich Inc. (USA) or Merck Chemicals (Pty) Ltd. Samples used for sulfate, sulfide, alkalinity and $COD_{Soluble}$ were filtered through glass micro fibre filters (GF/A Circles 25 mmØ – Whatman® Int Ltd England) and the filtrate refiltered through 0.45 micron (Millipore AcetatePlus, # A04SP002500, Osmonics Inc). The prefiltration through the Glass Microfibre Filters was necessary to reduce blinding of the 0.45 micron filters.

Chemical Oxygen Demand

Chemical oxygen demand (COD) is defined as "the number of oxygen equivalents consumed in the oxidation of organic compounds by strong oxidizing agents, such as dichromate, permanganate or cerium" (Westbroek and Temmerman, 2001). COD is used to estimate the carbonaceous material content of the wastewater (WRC, 1984) and typically divided into three main fractions, biodegradable (organic), unbiodegradable (inert) and heterotrophic active biomass (HAB) (Mbewe *et al*., 1995).

Since sulfide contributes to COD, it had to be eliminated prior to COD analysis. This was achieved by reducing the pH of each sample to below 2 using concentrated H_2SO_4 and shaking for two minutes to facilitate the release of sulfide gas from the samples. Preliminary studies had indicated that this procedure had no adverse effects on the

determination of COD of the samples. Analysis for the determination of COD was conducted using the Merck Spectroquant® reagent kit, (COD Solution A, # 1.14538.0065 and COD Solution B, # 1.14539.0495, Merck KGaA, Germany) as per the manufacturer's instructions. The soluble fraction of COD ($COD_{Soluble}$) was determined by a modified method of Lilley *et al.* (1990), by filtering samples through Glass Microfibre Filters (GF/A-Whatman® Int # 1820025) as described above. All assays were read using Merck Nova 60 Spectroquant®. The particulate, that is, the non-soluble fraction of the COD ($COD_{Particulate}$) of the samples was calculated as the difference between the total COD of the sample (COD_{Total}) and $COD_{Soluble}$. Separation of COD into $COD_{Soluble}$ and $COD_{Particulate}$ is valuable when attempting to link hydrolysis of complex organic matter and biological sulfate reduction, as SRB can only utilise the soluble fraction. The complete procedure is described in **Appendix A**.

Acidity, Alkalinity and pH

Acidity and alkalinity measurements were carried out manually according to Standard Methods (APHA). Acidity was measured by titrating the sample solution to pH 8.3 using 0.1 N sodium hydroxide (NaOH). Alkalinity was measured by titrating the sample solution to pH 4.3 using 0.1 N sulfuric acid (H_2SO_4) (Merck, Titrisol® # 1.09981).The pH was measured with an electronic pH meter (InoLab Level 1, WTW Ltd).

Sulfate and Sulfide

Sulfate was analysed by the modified turbidimetric method described by Kolmert *et al*. (2000). In this method the precipitation of sulfate with barium ions is followed by a photometric monitoring of the resulting suspension. Standard sulfate solutions were made by dissolving potassium sulfate, K_2SO_4 (Sigma-Aldrich, Inc. USA), in deionized water to known concentrations in the range 0 to 5 mM. The calibration curve obtained using the standard solution in this range was fitted with a third degree polynomial curve over the range 0 to 5 mM sulfate (**Appendix B5**). The final sulfate concentration was calculated using MATLAB Version 6.0 (Math Works). Sulfide was analyzed with Merck Spectroquant® reagent kit, # 1.14779.001 (Merck KGaA, Germany) and the absorbance was read with Merck Nova 60 Spectroquant® at a wavelength of 665 nm.

2.2.3. Statistical procedures

All statistical analyses, including Pearson correlation coefficients, principal component analysis (PCA), linear regression analysis, analysis of variance (ANOVA) and descriptive statistics were conducted using STATISTICA (data analysis software system) for Windows, Version 6.0 (StatSoft, Inc. 2001, USA). The mean values were reported with standard deviation (±1 SD) at the 95% or 99% confidence level as well as the statistical levels of significance of the tests (*P* values). Where results obtained from ANOVA were significant for all depths, a Newman-Keuls multiple range post hoc test was used to establish relationships between the mean values at different depths within the RSBR.

2.3. Results and Discussion

2.3.1. General characterisation of the feed

The mean feed characteristics over the 60-day experimental period are summarised in **Table 2.1**. The influent sulfate and COD concentrations were both 1000 mg/l resulting in a COD:SO_4 ratio of 1:1. The mean $COD_{Soluble}$ was 261 mg/l with a corresponding $COD_{Particulate}$ of 738 mg/l. No sulfide was detected in the feed. The mean pH of the feed was 6.31, while alkalinity and acidity measured as mg $CaCO_3$/L were 73.13 and 573.26 respectively.

Table 2.1 Characteristics of the feed over the 60-day experimental period

Parameter	Mean values	Standard deviation
pH	6.31	0.14
Alkalinity (as mg $CaCO_3$/L)	73.13	10.87
COD_{Total} (mg/l)	1000	0
$COD_{Soluble}$ (mg/l)	261	24.20
$COD_{Particulate}$ (mg/l)	738	24.19
Sulfate (mg/l)	1000	0
Sulfide (mg/l)	0	0
Acidity (as mg $CaCO_3$/L)	573.26	6.23

The values are averages of triplicates with standard deviation (± SD) with n = 15

2.3.2. Chemical oxygen demand (COD)

The profiles of COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$ over the experimental period are presented in **Figure 2.2**, while **Figure 2.3** shows the mean COD concentration at each of the three depths within the RSBR. Over the 60 day experimental period there were fluctuations of COD_{Total} and $COD_{Particulate}$ at depths 2 and 3 but not at depth 1. At depths 2 and 3, an average of over 90% of the total COD concentration was in the particulate fraction. However, at depth 1, the soluble component made up approximately a third of the total COD over the 60 day period. This suggested rapid utilisation of the soluble fraction in the base of the reactor. While the concentration of $COD_{Particulate}$ and $COD_{Soluble}$ remained relatively constant at depth 1, depths 2 and 3 exhibited wide fluctuations. At depth 3, COD_{Total} concentrations peaked on day 20 and then decreased rapidly (**Figure 2.2**). The COD_{Total} reached a maximum of 677 mg/l on day 40 and a minimum of 488 mg/l on day 28 at depth 1. The COD_{Total} at depth 2 reached a maximum of 19233 mg/l on day 4 and exhibited a minimum of 8666 mg/l on day 32 representing a fluctuation of 55%. For depth 3, the trend was different with a minimum COD_{Total} of 10433 mg/l on day 12 and a maximum of 18433 mg/l on day 24 reflecting an increase of 44%. The standard error bars of some graphs reported where omitted for clarity.

The mean COD_{Total} concentration for depth 1, depth 2 and depth 3 were 603 mg/l, 11735 mg/l and 14988 mg/l respectively (**Figure 2.3**). This showed that there was a significant increase (Newman-Keuls test, $P < 0.001$, df = 42, n = 135) in both $COD_{Particulate}$ and COD_{Total} with depths, although the difference between depths 2 and 3 was not significant (Newman-Keuls test, $P > 0.05$, df = 42, n = 135) (**Table 2.2**). The cyclical accumulation and apparent solubilisation of $COD_{Particulate}$ at depth 3 in the current system was substantiated by the work of Whittington-Jones (2000).

If the periods of rapid COD removal at depth 3 (days 27-33 and 40-45) were due to solubilisation, then a brief accumulation of $COD_{Soluble}$ may have been expected. However, $COD_{Soluble}$ remained consistently low at all depths, suggesting that soluble products were utilised immediately. The $COD_{Soluble}$ concentrations at all three depths of the RSBR were low compared to COD_{Total} with a mean of 230 ± 64 mg/l, 1681 ± 347 mg/l and 1552 ± 324 mg/l for depth 1, depth 2 and depth 3, respectively.

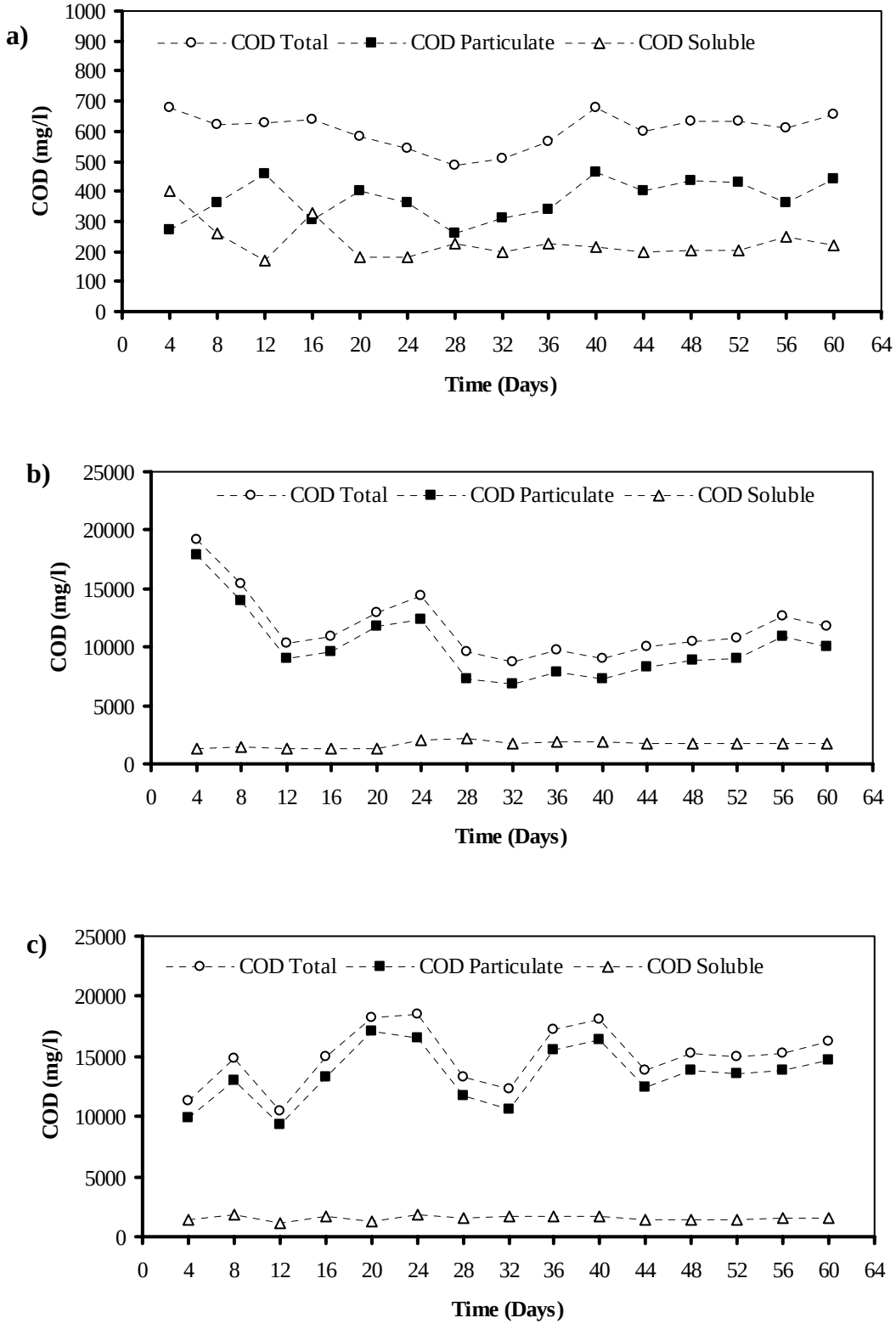

Figure 2.2 Variation of COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$ during the 60-day experimental period for a) depth 1, b) depth 2 and c) depth 3. Standard deviations are omitted for clarity and are presented in **Appendix C1**

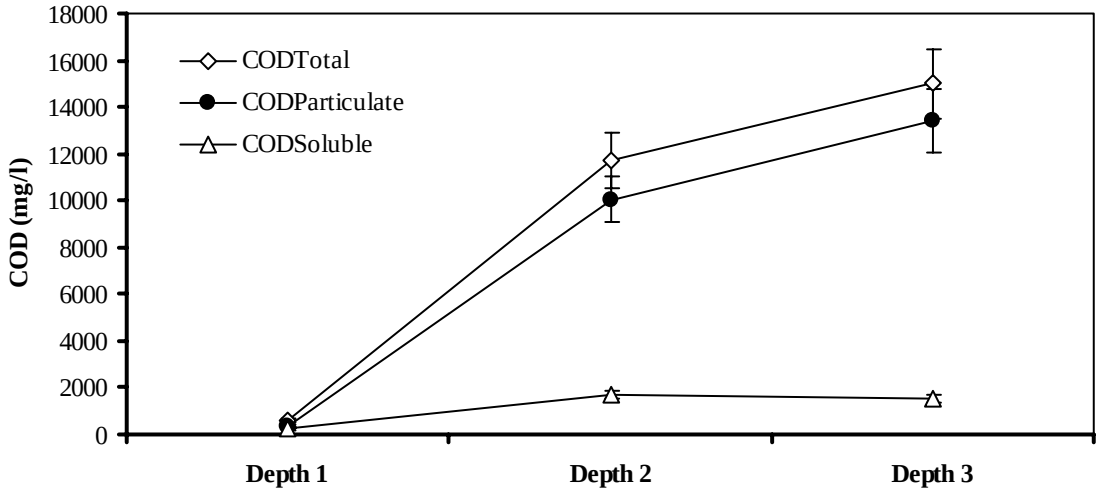

Figure 2.3 Variation of mean COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$ for depth 1, depth 2 and depth 3 for RSBR. Vertical bars represent ± SD at ± 95% confidence intervals, $P < 0.001$, F (14, 72) = 46.063

Table 2.2 Probabilities for Newman-Keuls multiple range Post Hoc test for RSBR parameters

	Depth		
Parameter	1 and 2	1 and 3	2 and 3
pH	NS	NS	NS
Alkalinity (as mg $CaCO_3$/L)	+++	+++	NS
COD_{Total} (mg/l)	+++	+++	+++
$COD_{Soluble}$ (mg/l)	+++	+++	NS
$COD_{Particulate}$ (mg/l)	+++	+++	+++
Sulfate (mg/l)	+++	+++	NS
Sulfide (mg/l)	+++	+++	NS

Marked differences are significant at $P < 0.001$, df = 42, at 95% confidence level. NS = Not significant, $P > 0.05$

Importantly, although the results showed periodic accumulation of particulate COD within the base of the RSBR system, the COD concentration at depth 3 did not increase substantially over the experimental period, indicating that, on average, the rate of solubilisation was close to the rate at which fresh material entered the system. Although there were periods of accumulation of particulate COD, these were short-lived. Over the 60-day period, there was a 25% increase in the $COD_{Particulate}$ in the base of the RSBR

(depth 3), indicating that at least a portion of the organic matter within the feed of the reactor was non-biodegradable. The reason for the sudden increase in $COD_{Particulate}$ between days 12-20 and 32-35 is not well understood. One possible explanation is the variability of the feed. The feed to the reactor during this period may have contained a larger proportion of the slowly degradable organic matter. This is possible as the actual organic composition of sewage may vary considerably. Thus, although degradation of this matter took place, it was slower than during other periods.

2.3.3. Sulfate reduction and sulfide production

The concentration of sulfate and sulfide was monitored during a 30-day start-up period to provide an indication of the activity of the SRB population (**Figure 2.4**). The start-up proceeded smoothly and sulfate reduction stabilised over the 30-day period.

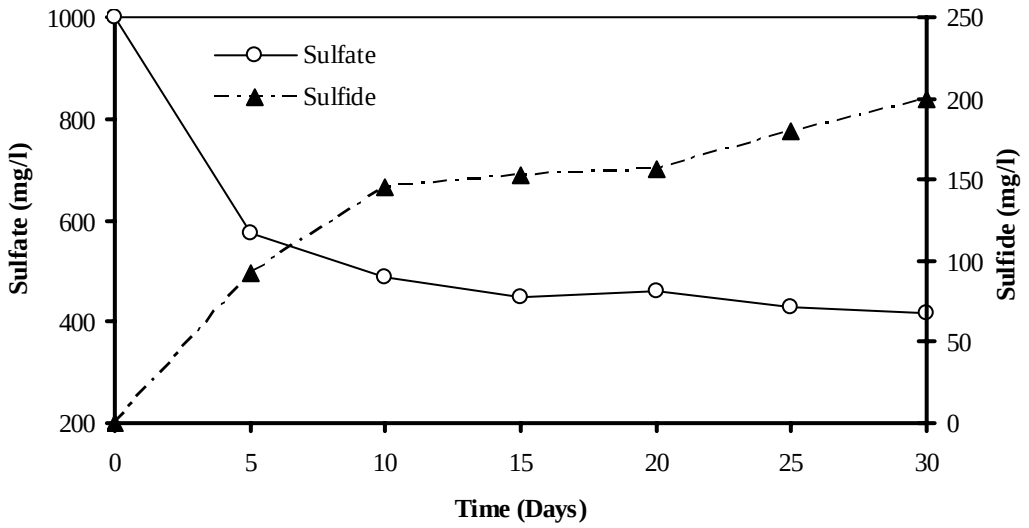

Figure 2.4 Removal of sulfate with concomitant production of sulfide during the 30-day start-up period of the RSBR

Examination of the start up data indicated that while sulfate was being removed from 1000 mg/l on day 0 to 416 mg/l on day 30, the concentration of sulfide in the reactor increased dramatically to about 200 mg/l on day 30 over the same period. Samples used for this study were drawn from depth 2, as it was assumed that this would provide a true representation of the reactor performance. Within 5 days, the sulfate concentration at depth 2 had decreased from 1000 mg/l to 573 mg/l, and sulfide concentration at this time

reached 92 mg/l. This indicated that the SRB population was active within a relatively short period. After 15 days, the concentration of sulfate remained stable at 449 mg/l, although the sulfide concentration continued to increase. At the end of acclimation period, the mean sulfate removal was 53%.

After the 30-day acclimation period the reactor performance was routinely assessed by determination of sulfate removal (**Figure 2.5**), and corresponding sulfide production (**Figure 2.6**) at the three depths of the reactor. Between days 15 to 53 of the experimental period, sulfate concentrations remained relatively constant at depth 2 and depth 3 and rarely exceeded 100 mg/l (**Figure 2.5**). However, at depth 1 the sulfate concentration varied between 402 mg/l (day 36) and 605 mg/l (day 40). The peaks in sulfate concentration observed on days 20 and 40 appeared to coincide with the peaks in COD in the base of the RSBR (**Figure 2.2c**).

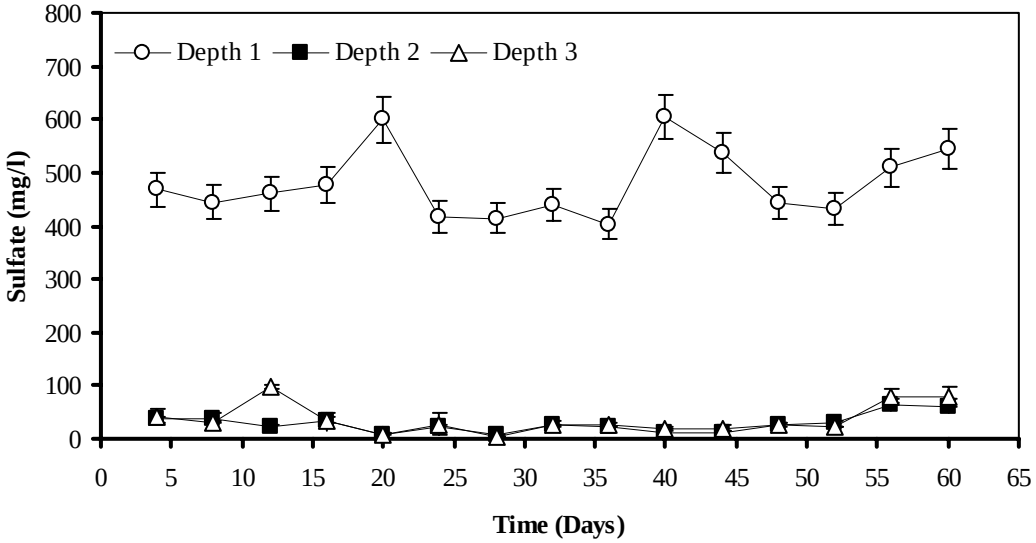

Figure 2.5 Profiles of sulfate during the experimental period for depth 1, depth 2 and depth 3. All points are means of triplicate values. Values of depth 1 were significantly different from depth 2 and depth 3 (ANOVA, $P < 0.05$, 95% confidence level, n = 135)

These observed peaks could be linked to the availability of electron donors, but then peaks in the sulfate concentration in the base of the reactor would have been expected. The mean sulfate and sulfide obtained at depth 1, depth 2 and depth 3 over the 60-day experimental period are presented in **Figure 2.7**. Very low sulfate concentrations were recorded at depths 2 and 3 in comparison to depth 1 for the duration of the experimental

period, suggesting that this was where active sulfate reduction took place. This was supported by high sulfide concentrations (**Figure 2.7**) and low $COD_{Soluble}$ at depths 2 and 3. Hulshoff Pol *et al.* (1998) pointed out that in the sulfate reducing stage in anaerobic digestion, a complete reduction of sulfate to sulfide is desired but this was not achieved in the current system where the mean sulfate removal was 52%.

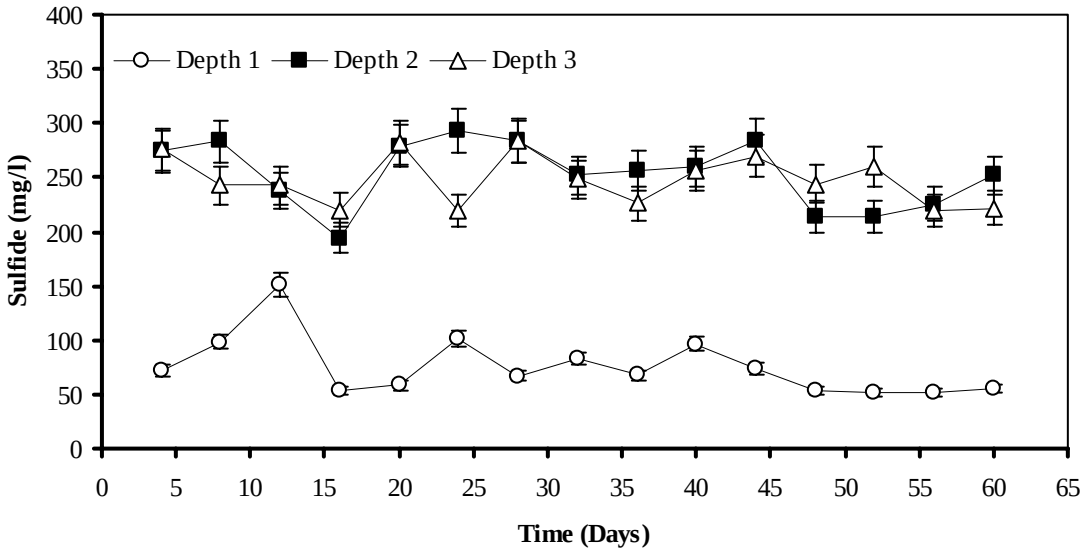

Figure 2.6 Profiles of sulfide during the experimental period for depth 1, depth 2 and depth 3. All points are means of triplicate values. Values of depth 2 and depth 3 were significantly different from depth 1 (ANOVA, $P < 0.05$, 95% confidence level, n = 135)

According to Hulshoff Pol *et al.* (1998), channelling of reducing equivalents towards the SRB is also enhanced by the ability of these organisms to effectively compete with other anaerobic bacteria for the available organic substrate and the sensitivity of the other bacteria to sulfide. Possible explanations for the relatively low sulfate removal include a limited supply of a suitable substrate or the competitive kinetics of H_2 utilisation by the sulphidogens and methanogens (Sam-Soon *et al.*, 1991). However, the formation of gas bubbles in the RSBR was not observed at any stage of the experimental period suggesting that methanogens were relatively inactive.

Considering the stoichiometric requirement of 2 g COD to reduce 1 g sulfate (Isa *et al.*, 1986; Lens *et al.*, 1995), and at influent COD of 1000 mg/l COD_{Total} and assuming that SRB were able to utilise 100% of the influent COD, a 50% sulfate removal would be expected. This value is close to the actual value of 52% sulphate removal obtained over

the 60 day study period. However, SRB are thought to be limited to using soluble organic matter, and this only made up 26% of the influent COD (261 mg/l) which translated to 130.5 mg sulfate removed (13%). Thus, in order to explain the sulphate removal observed, either SRB are able to utilise the complex carbon fraction ($COD_{Particulate}$) of the PS or the solubilisation of this fraction by the hydrolytic population within the biosulphidogenic RSBR is very high. This could result in the production of sufficient soluble products to explain higher levels of sulfate reduction. The high solubilisation level of $COD_{Particulate}$ is also supported by the slow accumulation of COD within the base of the RSBR

At the prevailing pH in the RSBR, it is expected that a certain percentage of the sulfide would exist as hydrogen sulfide gas and would therefore be able to volatilise and leave the reactor via the headspace. This would account for the consistently low sulfide concentration at depth 1. As seen in **Figure 2.7**, only 28 ± 17 and 36 ± 27 mg/l sulfate was detected in depth 2 and depth 3 respectively. These findings suggest that most of the sulfate reduction took place in the bed of the reactor (depth 2 and depth 3) and that PS served as a good carbon source for sulfate reduction.

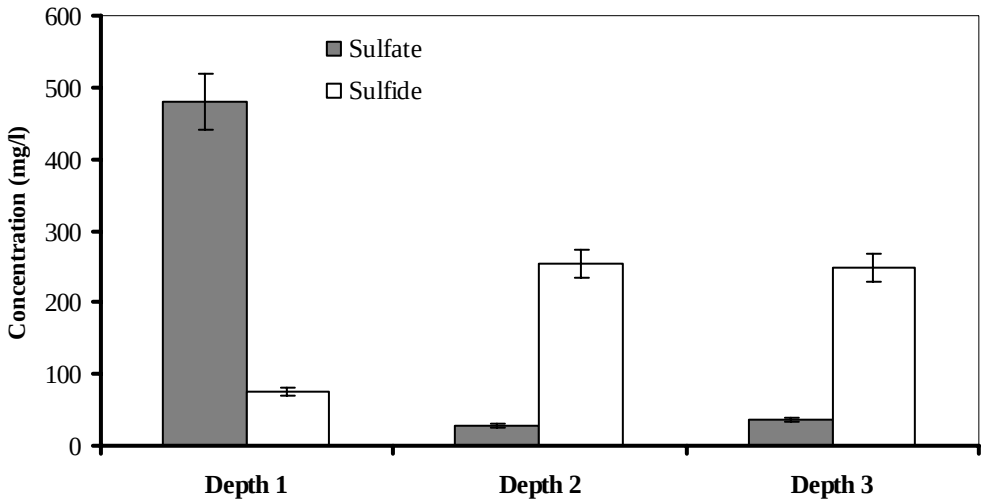

Figure 2.7 Variation of sulfate and sulfide with depth of the RSBR. Vertical bar denote ± SD at 95% confidence intervals, $P < .0001$ and $F(14, 72) = 46.063$

Interestingly, the peaks in sulfate concentration at depth 1 (days 20 and 40) coincided with peaks in COD in the bed of the reactor (**Figure 2.2c**). These fluctuations observed

could be attributed to the availability of soluble organic substrate and the rate of substrate (feed) utilisation by the SRB. The variation in performance of RSBR in terms of sulfate and COD removal efficiency are presented in **Figure 2.8** and **Figure 2.9** respectively. The percentage removal efficiency of both COD and sulfate were calculated according to the following equations:

$$\text{COD Removal Efficiency} = \left(\frac{\text{Influent COD}_{\text{Total}} - \text{Effluent COD}_{\text{Total}}}{\text{Influent COD}_{\text{Total}}} \right) * 100 \quad (10)$$

and

$$\text{Sulfate Removal Efficiency} = \left(\frac{\text{Influent Sulfate - Effluent Sulfate}}{\text{Influent Sulfate}} \right) * 100 \quad (11)$$

Over the 60-day experimental period, the mean sulfate removal efficiency was 52%. On days 20 and 40 a drop in sulfate removal efficiency (**Figure 2.8**) was observed which did not correspond to a decrease in sulfate.

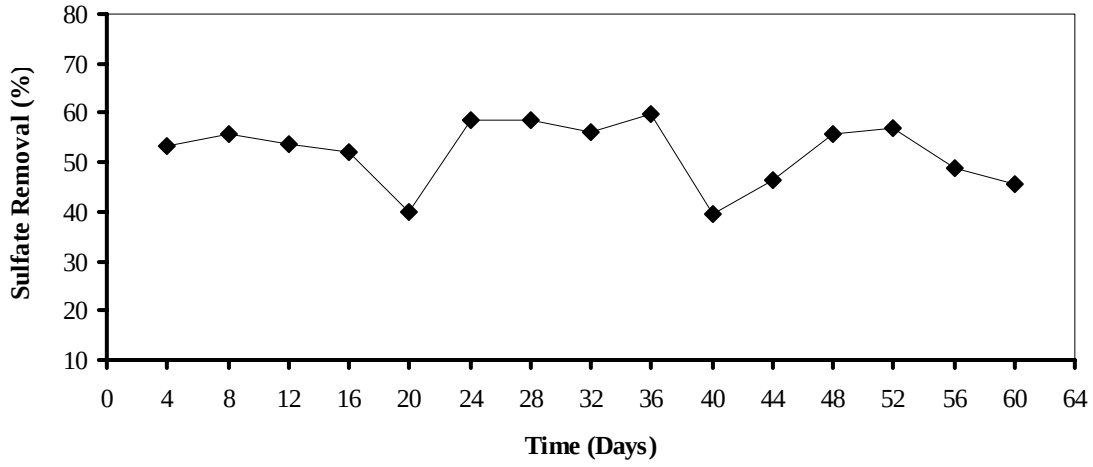

Figure 2.8 The performance of RSBR showing the removal efficiency of sulfate during the 60-day experimental period. All points represent averages of three values

The high COD removal efficiency observed may be related to elevated sedimentation capacity although, if this did occur, it would have resulted in a significant increase in $COD_{Particulate}$ in the base of the RSBR over the experimental period. The quantity of reduced sulfate and $COD_{Soluble}$ utilised may actually have been higher than reported, since re-oxidation of sulfide gas on the surface layer of the reactor may have led to the

formation of sulfate. This was further evidenced by the presence of elemental sulfur on the surface of the reactor contents. Also, any utilisation of $COD_{Soluble}$ by the methanogens would have resulted in an underestimation of the amount of $COD_{Particulate}$ solubilized in the system, although methanogenic bacteria are only expected to out-compete the SRB at a COD:SO_4 ratio greater than 2:1 (Li *et al.*, 1996).

Considering the hypothesis that elevated sulfide is required for enhanced solubilisation of complex carbons in the RSBR, it was decided to increase both the COD and sulfate concentrations of the feed in an attempt to increase the concentration of sulfide in the system. The impact on COD removal was then followed. A mean COD removal of 87% and a slightly elevated sulfide concentration (**Figure 2.10**) was observed at COD concentration of 2000 mg/l in comparison to 77% observed with COD concentration of 1000 mg/l (**Figure 2.9**). The 10% increase in COD removal observed during this phase of the study suggested an increase in the conversion rate of particulate organic matter to the soluble form by SRB in the RSBR.

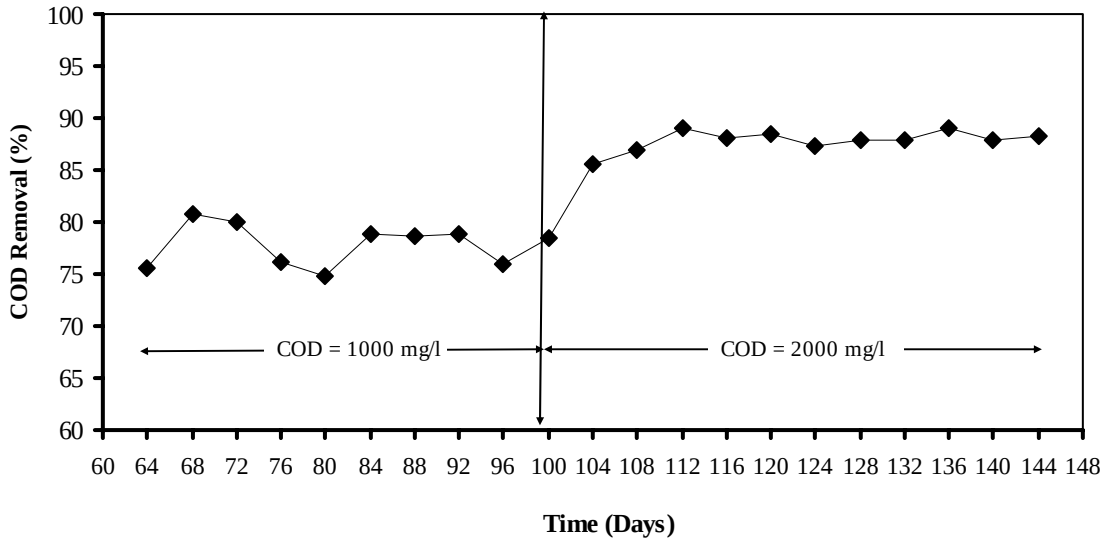

Figure 2.9 The performance of the RSBR with increased COD concentration

Although this high COD removal efficiency does not imply a high solubilisation rate, the solubilisation rate is expected to be higher than in non-biosulphidogenic systems. Another possible explanation for the high rate of solubilisation is evidenced by the reduced floc size (Chapter 5). Again, it should also be pointed out that the high COD

removal efficiency of the biosulphidogenic RSBR reported here might have been overestimated as some of the COD might have settled in the bed of the reactor.

Increasing the sulfate concentration in the RSBR from 1000 to 2000 mg/l did not, however, lead to any statistically significant difference (ANOVA, $P > 0.05$; df = 21) in the mean sulfide concentration during the experimental period. The reactor performance in terms of sulfide production during this study period for both effluent and depth 3 is shown in **Figure 2.10**. The SRB activities might have attained their "threshold" levels, therefore increasing sulfate concentration did not result to increased concentration of sulfide. A sulfide concentration of up to 352 mg/l was observed within the RSRB at depth 3, demonstrating that high sulfate-reducing activity took place at this depth. The sulfide concentration reported might have been substantially underestimated due to the highly volatile nature of hydrogen sulfide which might have been released from samples prior to analysis (that is, during sampling).

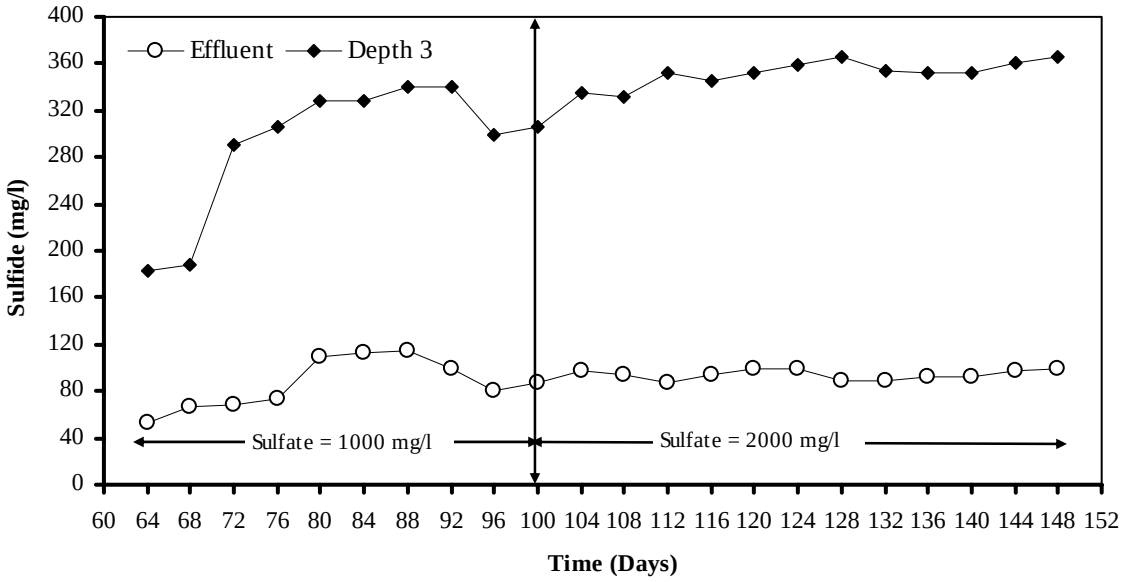

Figure 2.10 Sulfide production with increased sulfate concentration

Biological sulfate reduction under anaerobic conditions is known to cause an increase in alkalinity (Kim *et al.*, 2003). During reduction of sulfate to hydrogen sulfide, alkalinity increases by two equivalent moles per mole of sulfate according to equation (12) (Van Langerak *et al.*, 1997).

$$SO_4^{2-} + 2^{"}CH_2O^{"} + 2H^+ \rightarrow H_2S + 2H_2O + 2CO_2 \quad (12)$$

The variation of alkalinity and pH of the feed and within the RSBR (depth 1, depth 2 and depth 3) over the study period is presented in **Figures 2.11** and **2.12**. The alkalinity (measured as mg $CaCO_3$/L) increased on average from 72 ± 18 in the feed to 353 ± 92, 1453 ± 76 and 1477 ± 83 at depth 1, depth 2 and depth 3 respectively over the 60-day period. Similarly, although the mean pH of feed was about 6.5, average pH values at all depths were approximately 7.3. Although there were fluctuations in both alkalinity and pH over the 60-day experimental period, these parameters were similar and higher at depths 2 and 3 than in the feed and depth 1 (**Figures 2.11** and **2.12**). This corresponded to the regions of the RSBR where active biological sulfate reduction was observed.

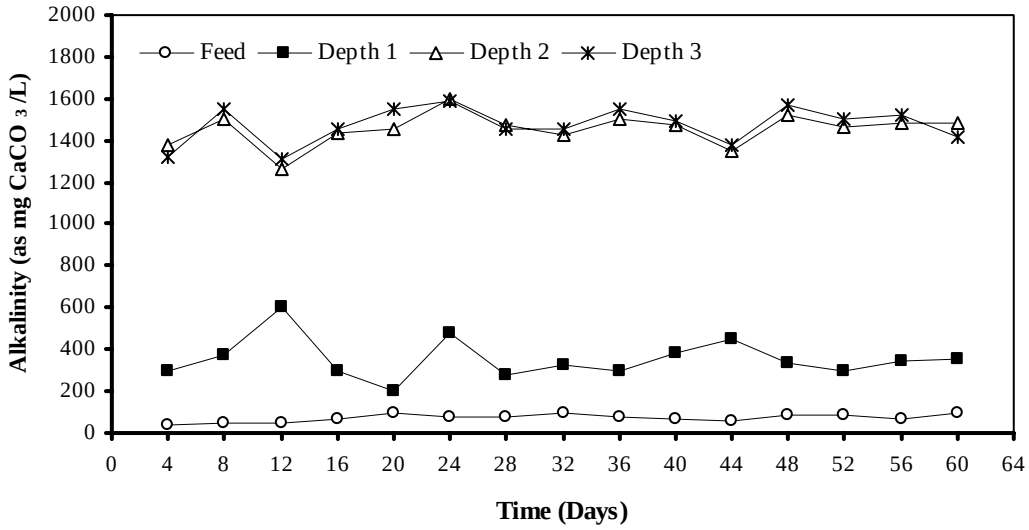

Figure 2.11 Variation of alkalinity during the 60-day experimental period. All points represent means of triplicate values

The close similarity between alkalinities at depth 2 and 3 during the 60-day experimental period provided a further indication that reduction of sulfate to sulfide was accomplished at these depths throughout the studies. Results obtained during the reactor operation for the three different depth shows that the pH and alkalinity remained within the optimal working range of anaerobic digesters, that is, 6.0-7.5 and above 1200 mg $CaCO_3$/L, respectively (Mukherjee and Levine, 1992).

The average pH for depth 1, depth 2 and depth 3 of the reactor was 7.32, 7.32 and 7.31, respectively (**Figure 2.12**), which remained well within the optimum range (6.8-7.4) for SRB (Yang *et al.*, 1990). An increase of pH beyond 7 is thought to result in a higher overall sulfate removal rate mainly due to the reduced toxicity of hydrogen sulfide (Okabe *et al.*, 1992; Reis *et al.*, 1992). It must be remembered that all biochemical reactions, including enzymatic hydrolysis, are affected by fluctuating pH levels.

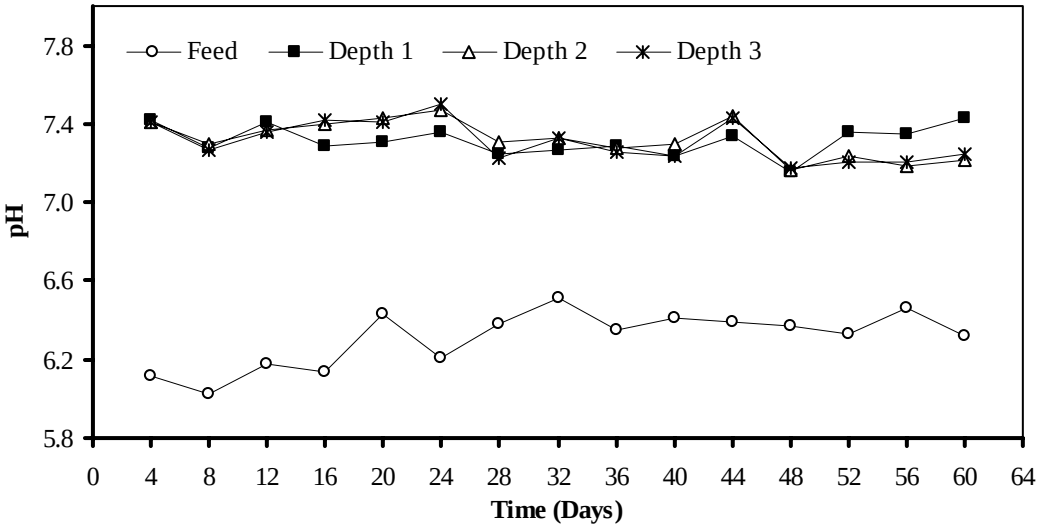

Figure 2.12 pH profiles during the 60-day study period. All points are means of three values

Alkalinity and pH are treated together because they are closely related. The incentive to measure the alkalinity originates from the fact that an imbalance of anaerobic digesters (due to accumulation of VFA) cannot easily be detected on the basis of pH measurements, especially in situations were the alkalinity of the reactor contents is high (Rozzi, 1991; Hawkes *et al.*, 1992; Bouvier *et al.*, 2002; Vanrolleghem and Lee, 2003). The high alkalinity level in the RSBR (**Figure 2.11**) is consistent with high levels of sulfate reduction (**Figure 2.7**). The pH change from the feed (pH 6.3) to the reactor (pH 7.32) is likely to be influenced by SRB activity (McIntire *et al.*, 1990; Machemer and Wildeman, 1992) because of the active biological sulfate reduction and subsequent production of bicarbonate ions, leading to increased biodegradation. Anaerobic digestion is a biological process and as such it depends on the successful operation of and the maintenance of an ideal environment for, the hydrolytic enzymes and the bacteria

involved. Temperature, pH, alkalinity, nutrient requirements and volatile fatty acids are among the important environmental factors (Moletta *et al*., 1994; Ahring *et al*., 1995; Vanrolleghem and Lee, 2003). However, under practical conditions, economic constraints may make it necessary to work under non-optimum biological conditions.

Although the pH and alkalinity of the RSBR is optimum for the majority of hydrolytic enzymes, the interaction between sulfur species and the activity of key hydrolytic enzymes requires further investigation. Initial studies into the relationship between enzymes and physical and chemical factors focused on the reactor environment. By comparing spatial profiles of enzymes with physico-chemical parameters (including sulfur species concentration) it may be possible to gain a deeper understanding of the mechanism underlying enhanced hydrolysis in the RSBR.

2.4. Conclusions

Based on the performance of the RSBR over a 60-day experimental period, a number of conclusions could be drawn:

- There was enhanced solubilisation of PS in the RSBR system, and the hydrolysis of the PS in the RSBR was improved by the recycling of the hydrolysable particulate organic materials.
- Biological sulfate reduction was taking place using PS as the sole carbon source.
- The results reported in this chapter clearly demonstrate that sulfate removal increased significantly with the depth in the RSBR with a concomitant increase in sulfide production.
- The pH within the RSBR remained relatively constant between 7.0 and 7.3 at all depths of the RSBR without any addition of alkali, demonstrating that the system had a substantial buffering capacity.

Chapter 3

The Enzymatic Profiles within the Recycling Sludge Bed Reactor (RSBR): Implications of Biosulphidogenic Conditions

3.1. Introduction

Both developed and developing countries are witnessing a rapid increase in the amount of sludge production as a result of a rapid population growth, and this trend is expected to continue up to the early part of the this century (Werther and Ogada, 1999). This increase is partly driven by tightening of pollution limits on the effluent discharges, and the availability of several technologies capable of achieving higher wastewater treatment efficiency (Ogada, 1995; Lungwitz and Werther, 1997; Peschen, 1997; Werther and Ogada, 1999). Amongst the available technologies, anaerobic digestion, which has been in use since 1881 (McCarty, 1982), is currently the most common process in sludge minimisation and stabilisation at municipal waste treatment plants (Hudson and Lowe, 1996; Houghton and Stephenson, 2002).

Municipal wastewater consists of different fractions of colloidal and suspended solids which contribute 60-70% of the organic load (Hunter and Heukelekian, 1965; Rickert and Hunter, 1967). A significant proportion of the total organic matter in the sewage sludge is in the form of complex organic macromolecules (Heukelekian and Balmat, 1959; Levine *et al*., 1985), viz. carbohydrates, proteins and lipids (Nielsen *et al*. 1992; Raunkjær *et al*., 1994). Degradation of these macromolecules can be accomplished under either anaerobic or aerobic conditions, in the presence of hydrolytic enzymes. Hydrolysis, which can be defined as the breakdown of complex organic biopolymers into simpler monomeric products for subsequent consumption and degradation by bacteria, is the first and often rate-limiting step during anaerobic digestion of complex organic biopolymers in wastewater (Morgenroth *et al*., 2002). The enzymatic hydrolysis of complex organic substrates during the anaerobic degradation is facilitated by three major mechanisms (Batstone, 2000). In the first step, the hydrolytic bacteria secrete hydrolytic enzymes into the bulk liquid, with a subsequent adsorption of these enzymes to particles or reaction with a soluble substrate (Jain *et al*., 1992). The second step involves the attachment of the hydrolytic bacteria to particles and the secretion of hydrolytic enzymes into the vicinity of the particle where the neighbouring hydrolytic bacteria will benefit from the release of the dissolved substrates (Vavilin *et al*., 1996). Finally, the hydrolytic bacteria have

attached hydrolytic enzymes that may possibly double as a transport receptor to the cell interior.

Certain aspects of the anaerobic treatment process, especially the enzymology, are still inadequately understood. The widespread application of enzymes in anaerobic digestion has been hampered by a lack of understanding of the factors associated with the stability and performance of the biological processes involved. In both anaerobic and aerobic systems, enzyme activities are known to be influenced by a range of factors including the composition of the substrate (organic matter), the loading rate, the nature of the microbial population and the environmental conditions such as temperature, pH, alkalinity and the degree of anaerobiosis (Björnsson *et al.*, 2000). Since the hydrolysis of complex particulate organics depends on the activity of hydrolytic enzymes, understanding the process requires a study of these enzymes in the system. By answering the following questions, it would be possible to make further predictions as to how the hydrolysis of complex organic matter by the hydrolytic enzymes is enhanced in the presence of sulfate reduction in a biosulphidogenic RSBR system.

- What are the relative concentrations of main organic fractions and what are the key hydrolytic enzymes in the RSBR?
- Does activity of these enzymes show spatial and temporal variability?
- Can variability be attributed to any physical, chemical or biological factors?
- As some of the above enzymes, that is, proteases, are able to act against other hydrolytic enzymes, are there any negative correlations?

The purpose of this aspect of the study was therefore aimed at gaining insight into how a range of hydrolytic enzymes (α-glucosidases, β-glucosidases, proteases, arylsulphatases, lipases, leucine aminopeptidases and alanine aminopeptidases) are influenced by a range of physico-chemical parameters within the sulphidogenic RSBR. With this information, it might be possible to further improve the overall performance of the RSBR in terms of conversion of complex organic matter into soluble products.

3.2. Materials and Methods

3.2.1. Reactor set-up

All data was collected from a laboratory-scale RSBR described in section 2.2.1 (page 41) of Chapter 2. A schematic diagram of the laboratory-scale RSBR system setup is shown in **Figure 2.1**.

3.2.2. Analytical methods

All reagents and chemicals used were of analytical or commercial grade. The analyses of the physico-chemical parameters were carried out as previously described in section 2.2.3 (page 44) of Chapter 2.

3.2.3. Sample collection

Samples for the determination of enzyme activities were collected every two days, over the 60-day experimental period, from the sulphidogenic bioreactor (RSBR) after the steady state conditions had been attained. Samples were taken from the RSBR from depth 1, depth 2 and depth 3 using a syringe with minimum disturbance to the system. All the samples were kept at 4^{o}C until analysed.

3.2.4. Physicochemical analysis

The analysis of COD_{Total}, $COD_{Particulate}$, and $COD_{Soluble}$, sulfide, sulfate concentrations, alkalinity, pH and temperature were conducted as described in section 2.2.3 (page 44) of Chapter 2.

3.2.5. Protein assay

Total protein concentration was determined using the method of Bradford (1976) with a commercially available reagent (Sigma, B6916). The absorbance of all samples was determined at 595 nm using a microtitre plate reader ($PowerWave_X$, Bio-Tek Instruments, Inc. USA). To obtain the protein concentrations, the absorbance of the samples was read against the absorbance value of a protein standard curve, prepared using bovine serum albumin (BSA) (**Appendix B1**).

3.2.6. Carbohydrate assay

Total carbohydrate concentration was estimated using the phenol sulfuric acid method (Dubois *et al.*, 1956). This method involved adding phenol solution (80% w/v, 50 µl) and concentrated sulfuric acid (2.0 ml) to the anaerobic sludge sample (100 µl) drawn from the RSBR and allowing the mixture to stand at room temperature for 10 min. The solution was vortexed and allowed to cool before reading the absorbance at 490 nm. Absorbance values obtained were translated into glucose equivalent using a glucose standard curve (**Appendix B4**).

3.2.7. Total lipid assay

A gravimetric method for total lipid determination was used (The Standard Methods, APHA *et al.*, 1998). This method involves the extraction of the lipids with chloroform and subsequent evaporation of the solvent and the determination of lipid by weight.

3.2.8. Enzymatic assays

All enzyme activity assays were carried out in triplicate. The control for each enzyme assay involved all of the respective reagents with the terminating solution added prior to the enzyme to ensure zero enzyme activity. The absorbances were measured on a Shimadzu UV-160A, UV-visible recording spectrophotometer. The substrate and buffer solutions were heated for 30 min at 37^{o}C prior to the addition of the sludge samples. For α-glucosidase, β-glucosidase, arylsulphatase, alanine and leucine aminopeptidase, the concentration of the stock solution was 1.0 mg per ml of the respective substrate. When necessary, samples were centrifuged in an Eppendorf 5810R centrifuge. The absorbance readings of controls were subtracted from the absorbances of the three samples and the mean value reported. Since it was not the intention to use purified enzymes, all enzymes assayed had to comply with the following criteria: linearity of readings with enzyme concentration; linearity within the incubation period and the dependence of enzyme on substrate concentration. As such, they could be assayed in whole sludge samples without having to extract and/or purify the enzymes. Enzymatic activities were determined by measuring specific enzymatic conversions of synthetic substrates to products that are quantified photometrically (Obst, 1985). Because sulfide is a strong reducing agent, it may interfere with certain colourimetric assays. In order to avoid artefacts, all enzyme

assays used were tested to ensure that a sulfide solution (50-1000 mg/l) containing no enzyme did not produce a positive result. That is, the presence of sulfide would not artificially elevate apparent enzyme activity.

3.2.8.1. Determination of protease activity

Protease activity was measured according to the procedure of Pin *et al*. (1995) using azocasein as the substrate. The reaction mixture contained an azocasein solution (1% w/v, 1.0 ml) in distilled water (2.0 ml) and the RSBR samples (3.0 ml) to give a total volume of 6.0 ml. After incubation at 37^{o}C for 30 min, the reaction was stopped by the addition of ice-cold trichloroacetic acid (TCA) (10% w/v, 2.0 ml). The mixture was centrifuged (3000 x g, 10 min, RT) using an Eppendorf 5810R centrifuge. The supernatant (2.0 ml) was removed and NaOH (2.0 M, 2.0 ml) added. A blank was prepared by replacing 3.0 ml of the anaerobic sludge fraction with 3.0 ml of distilled water. Controls were prepared by adding TCA to the sludge fraction at the start of the 30 min incubation period (and not at the end), vortexed well and the azocasein was added at the end of the 30 min incubation period. A single control was prepared for each triplicate set of assay. The precipitated protein was removed and the TCA-soluble peptides measured by absorbance at 440 nm using a Shimadzu UV-160A, UV-visible recording spectrophotometer. Enzyme activity was defined as one enzyme unit that hydrolysed one mg of azocasein per minute.

3.2.8.2. Determination of α-glucosidase and β-glucosidase activity

β-Glucosidase

The activity of β-glucosidase was measured by a modification of the procedure using methylumbelliferyl (MUF)-β-D-glucopyranoside (Sigma-Aldrich, Poole, England) as a substrate (Hattenberger *et al*., 2001). The fluorogenic methylumbelliferone product liberated was measured fluorometrically at a wavelength of 455 nm with a Hitachi, F-2500 Fluorescence spectrophotometer. RSBR sample (1.0 ml) was incubated (50^{o}C, 5 min) in glycine buffer (0.4 M, 1.0 ml, pH 10.8) with MUF- β-D-glucopyranoside (1.5 mM, 1.0 ml). Ice-cold ethanol (95%, 2.5 ml) was then added to stop the reaction and the tubes centrifuged (2500 x g, 5 min, RT). The fluorescence was measured using a spectrofluorometer with an excitation wavelength of 365 nm and an emission wavelength

of 455 nm. β-Glucosidase activity was calculated as µmol methylumbelliferone released per minute using the molar extinction coefficient of 18.30 $mol^{-1}cm^{-1}$ (Del Campillo and Shannon, 1982)

α-Glucosidase

The determination of α-glucosidase activity was carried out by incubating a reaction mixture containing *p*-nitrophenyl-α-D-glucopyranoside solution (0.1%, 1.0 ml), Tris (hydroxymethyl) aminomethane (Tris-HCl) GR buffer (0.2 M, pH 7.4, 2.0 ml) and sludge (1.0 ml) at 37^{o}C (Richards *et al*., 1984; Goel *et al*., 1998a). The enzyme reaction was stopped after 60 min by adding a terminating solution, NaOH (0.2 M, 2 ml). Absorbance of the resulting colour due to the release of *p*-nitrophenol ions was measured at 410 nm, after centrifugation (2500 x g, 5 min) to separate the sludge from the supernatant. Controls, in which the terminating solution, NaOH (0.2 M, 2.0 ml) was added before the addition of the sludge, were included to discount any non-enzymic activity. The hydrolysis product of α-glucosidase with the substrate used in the enzyme assay was *p*-nitrophenol, hence α-glucosidase activity was calculated as µmol *p*-nitrophenol formed per minute. To quantify the amount of *p*-nitrophenol formed in the reaction, a calibration curve was prepared using known amounts of *p*-nitrophenol (**Appendix B2)**.

3.2.8.3. Determination of arylsulphatase activity

Arylsulphatase activity was measured by adding *p*-nitrophenyl sulfate potassium salt solution (1 mg/ml, 1.0 ml) to carbonate-bicarbonate buffer (0.2 M, pH 9.6, 2.0 ml), and the RSBR sample (1.0 ml) (Richards *et al*., 1984). After incubation at 37^{o}C for 60 min, NaOH (0.2 M, 2.0 ml) was added to terminate the reaction. The amount of *p*-nitrophenol released due to enzymatic cleavage was measured using a UV-visible recording spectrophotometer, Shimadzu UV-160A, at a wavelength of 410 nm. A control was prepared in the same way as the experimental samples, except that the terminating solution was added before the sludge sample. To quantify the amount of *p*-nitrophenol released, a calibration curve was prepared using known amounts of *p*-nitrophenol (**Appendix B2**).

3.2.8.4. Determination of lipase activity

Lipase activity was determined according to the procedure of Korn (1954) with slight modifications. The assay reaction mixture consisted of sodium phosphate buffer (0.1 M, 500 µl, pH 7.5) containing triacetin (1% w/v, 1.0 ml) and 500 µl sludge sample and was incubated (37^{o}C, 15 min). The reaction was stopped by the addition of sulfuric acid (5.0 M, 50 µl) and sodium periodate (0.1 M, 250 µl) at 0^{o}C for 5 min. Sodium arsenate (10%, 250 µl) was added and the reaction mixture allowed to stand at 22^{o}C for 5 min. The reaction mixture (250 µl) was transferred into chromotropic acid reagent (2.5 ml) and heated (100^{o}C, 60 min). After cooling, the glycerol released was measured at 570 nm and the glycerol concentration was determined from a standard curve (**Appendix B3**). One unit of lipase activity was defined as the amount of enzyme releasing one µmol of glycerol per minute under the specified assay conditions.

3.2.8.5. Determination of L-alanine and L-leucine aminopeptidase activity

Both L-alanine aminopeptidase and L-leucine aminopeptidase were determined according to the procedures of Richards *et al.* (1984) and Goel *et al*. (1998a). RSBR samples (1.0 ml) were mixed with Tris-HCl buffer (0.2 M, 2.0 ml, pH 7.4). The enzymatic reaction was initiated by adding the respective substrate solution (1.0 mg/ml, 1.0 ml) (L-alanine-*p*-nitroanilide or L-leucine-*p*-nitroanilide dihydrochloride and was incubated at 37^{o}C for 30 min. The reaction was stopped by adding TCA (10% w/v, 2.0 ml). Samples were centrifuged (2600 x g, 5 min) and the absorbance of the supernatants was measured at a 410 nm. Controls were run in which TCA was added prior to the sludge samples. The L-alanine aminopeptidase and L-leucine aminopeptidase activities were calculated using the molar extinction coefficient of 1.6 $mol^{-1}cm^{-1}$ (Takeda and Hizukuri, 1969) and expressed as µmol of product formed per minute.

Calculation of specific enzyme activities

Unless otherwise stated, the enzyme activity was defined as the amount of enzyme required to convert one µmol of substrate per ml per minute. Specific enzyme activities were expressed as enzyme activity per mg protein.

3.2.9. Statistical analysis

Statistical analysis of all the data was performed using a one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc multiple range test where necessary (Winer *et al*., 1991) to determine significant differences of specific enzymatic activities between depths of the RSBR. The degree of confidence level used was 95% and 99% and the level of statistical significance was accepted at $P < 0.05$. All statistical analysis, including Pearson correlation coefficients, linear regression, ANOVA and descriptive statistics were conducted using STATISTICA (data analysis software system) for Windows Version 6.0 (StatSoft, Inc. 2001, USA), or SigmaPlot 2002 for Windows, Version 8.02 (SPSS Inc. USA). Mixed models repeated-measures analyses were used to describe enzymatic activities (arylsulphatases, alanine and leucine aminopeptidases, proteases, lipases, α-glucosidases, β-glucosidases) as a function of various predictors. The explanatory variables (predictors or physico-chemical parameters) used included sulfide and sulfate concentrations, COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$, temperature, pH and alkalinity. The dependent variables were specific enzyme activity.

3.3. Results and Discussion

3.3.1. Total lipids, proteins and carbohydrates

The characterisation of wastewater by direct measurement of organic constituents has been reported by Nielsen *et al*. (1992). To expand the knowledge about processes in wastewater treatments, characterisation at this level is therefore of importance. Although carbohydrates, proteins and lipids have been identified as the major constituents in PS, other constituents such as VFA, alcohols, amino acids and detergents are possibly present but were not included in the present measurements. These constituents may account for a considerable part of the remainder of organic fractions. The concentrations of the total carbohydrates, total proteins and total lipids for the feed and the 3 depths of the RSBR are presented in **Figure 3.1**. As expected, the feed exhibited the highest concentration of all the 3 macromolecules (carbohydrates, proteins and proteins) compared to those at the 3 depths of the RSBR. A decrease in concentration of up to 64% was observed for carbohydrates from the feed to the RSBR, whereas proteins and lipids showed a decrease in concentration of 38% and 39% respectively, suggesting that these fractions were being

degraded within the RSBR. The concentration of the total carbohydrates was significantly higher (ANOVA, $P < 0.05$) than that of the other two organic fractions (proteins and lipids) at all 3 depths of the RSBR.

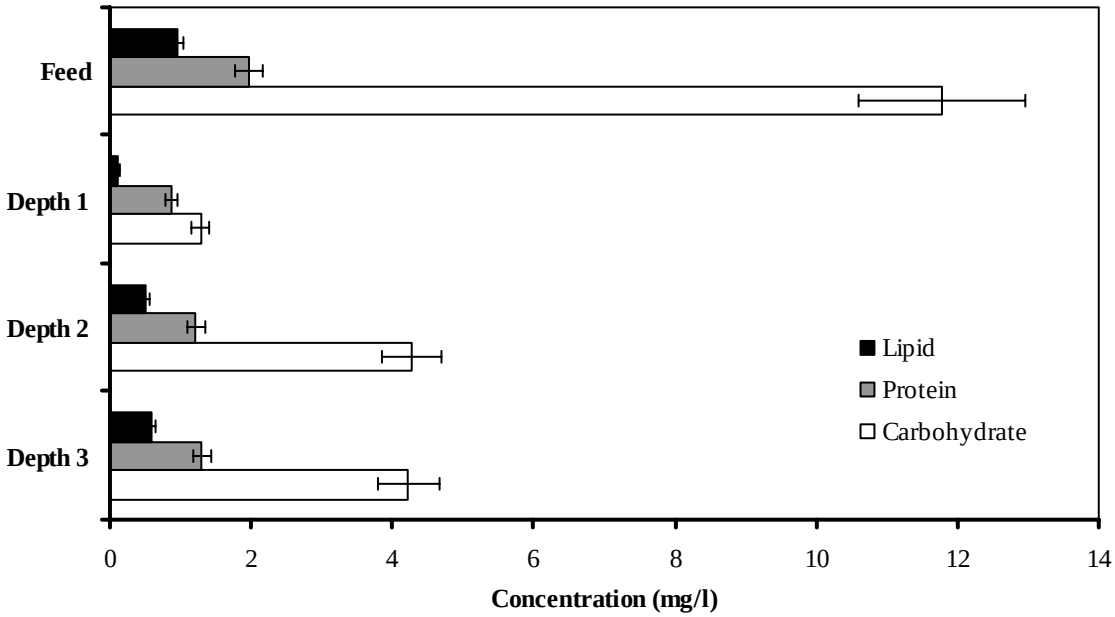

Figure 3.1 The concentration of total carbohydrate, protein and lipid for feed, depth 1, depth 2 and depth 3 in the RSBR. Error bars represent ± standard deviations at 95% confidence level (n = 5)

The concentrations of these macromolecules however, increased from depth 1 to depth 3 with lipids (0.12 to 0.58 mg/l) and proteins (0.85 to 1.30 mg/l). Carbohydrates showed a slightly different trend, with depth 2, having a concentration of 4.28 mg/l and depth 3 a concentration of 4.23 mg/l. The distribution of carbohydrates, proteins and lipids with the depth of the RSBR however, showed that there was no significant difference (ANOVA, $P > 0.05$) between depth 2 and depth 3. As a result, it would be expected that enzyme activities would also increase with depth of the RSBR. Enzymes degrading carbohydrates would also be expected in higher concentrations as compared to those degrading proteins and lipids.

Confer and Logan (1998) found that proteinaceous material made up only 8-12% of total dissolved organic carbon in a sulphidogenic anaerobic reactor while Raunkjær *et al*. (1994) reported 16-35% protein in dissolved organic carbon. Confer and Logan (1997) also found that the percentage of total dissolved organic carbon that accumulates as intermediate molecular weight protein hydrolytic fragments in domestic wastewater

treatment system was only 1-6%. These macromolecules have low diffusion coefficients, which can limit their transportation to unattached cells and aggregates in suspended growth reactors and to biofilm surfaces, and cells within the biofilm matrix, in bioreactors (Logan *et al*., 1987, 1987a; Confer and Logan, 1991). Thus if the hydrolysis of carbohydrates, proteins and lipids can be enhanced under biosulphidogenic conditions this could lead to enhanced hydrolysis of particulate organic matter such as PS. According to Fick's first law, the rate of mass transfer to cells in quiescent fluid is directly proportional to diffusivity. Therefore, low diffusivities limit the degradation of macromolecules by decreasing their transport to dispersed cells and aggregates in suspended cultures and within biofilms (Logan *et al*., 1987; Confer and Logan, 1991, 1997). Macromolecular compounds with large molecular weight cannot be assimilated directly by bacteria as these large molecules must be hydrolysed into monomers or smaller polymers by extracellular enzymes before they can be transported across the bacterial cell wall (Ferenci, 1980; Law, 1980). According to the current study, the amount of lipid and protein hydrolysis in RSBR is limited, suggesting low levels of extracellular enzymes responsible for the hydrolysis of these fractions. According to Yu and Fang (2000), the anaerobic degradation of lipids in most cases is slower than that of carbohydrates and proteins.

3.3.2. Time course study and enzymatic variation within the RSBR

3.3.2.1. α-Glucosidase (EC 3.2.1.20) and β-glucosidase (EC 3.2.1.21) activities

Glucosidases are involved in the degradation of starch and the hydrolysis of disaccharides originating from the degradation of polysaccharides. α-Glucosidase is involved in the degradation of starch (Nybroe *et al*., 1992), whereas β-glucosidase is one of the enzymes involved in the enzymatic degradation of cellulose (Turner *et al*., 2002). β-Glucosidases catalyse the hydrolysis of glucosides and as part of the cellulolytic enzyme system cleave cellobiose to release two moles of glucose per mole of cellobiose. This regulates the supply of an important energy source for microorganisms unable to assimilate cellobiose. The activity of this enzyme has been reported to be the rate-limiting step in cellulose degradation (Alef and Nannipieri, 1995) which might have a relevance to the hydrolysis step in the solubilisation of PS.

Table 3.1 Mean specific enzyme activities at various depths within the RSBR over the 60-day period

Enzyme	**Substrate**	**Depth 1**[a]	**Depth 2**[a]	**Depth 3**[a]
		Mean specific activity (μmol/min/mg protein)		
α-Glucosidase	*p*-Nitrophenyl α-D-glucopyranoside	17.18 (1.16)	78.63 (3.26)	93.51 (4.65)
β-Glucosidase	Methylumbelliferyl (MUF)- β-D-glucopyranoside	17.65 (3.02)	33.01 (2.81)	40.37 (3.60)
L-Alanine-aminopeptidase	L-Alanine-*p*-nitroanilide hydrochloride	0.67 (0.11)	0.94 (0.06)	1.18 (0.12)
L-Leucine-aminopeptidase	L-Leucine-*p*-nitroanilide hydrochloride	0.29 (0.02)	0.63 (0.05)	1.91 (0.17)
Protease	Azocasein (Sulfanilamide-azocasein)	0.91 (0.09)	2.19 (0.13)	2.60 (0.16)
Lipase	Triacetin	0.02 (0.02)	0.14 (0.01)	0.18 (0.04)
Arylsulphatase	*p*-Nitrophenylsulfate potassium salt	11.85 (0.79)	12.61 (1.23)	16.38 (1.38)

[a] Each preparation was assayed in triplicate and all values are reported as means with n =45. The values in brackets ( ) represent standard deviations (± SD)

The activity of the glucosidases was significantly higher (ANOVA, $P < 0.05$) than that of the other hydrolytic enzymes at all depths of the RSBR. α-Glucosidases exhibited higher mean specific activities at depth 2 (78.63 ±3.26 μmol/min/mg protein) and depth 3 (93.51 ± 4.65 μmol/min/mg protein) (**Table 3.1** and **Figure 3.2**) than β-glucosidase at depth 2 (33.01 ± 2.81 μmol/min/mg protein) and depth 3 (40.37 ± 3.60 μmol/min/mg protein) (**Figure 3.4**). Both α-glucosidase and β-glucosidase showed similar activity at depth 1 (**Table 3.1**). The activities of these enzymes were, however, significantly lower (ANOVA, $P < 0.05$, df = 44) at depth 1 than at any other depth of the RSBR system. The most likely explanation for this is that these enzymes are associated with the sludge flocs (Confer and Logan, 1998) and therefore the concentration is higher towards the base of the reactor where settled flocs accumulate (depth 2 and 3). However, the concentration of sulfide also increased with depth (chapter 2) and the possible stimulation of enzyme

activity by sulfide cannot be excluded. The relatively higher activities of glucosidases compared with that of the other hydrolytic enzymes studied in the RSBR suggests that glucosidases play an important role in the degradation of organic matter and consequently in the enhanced solubilisation of PS.

The variations of specific enzyme activities for α-glucosidase and β-glucosidase within the RSBR over the 60-day experimental period are depicted in **Figures 3.3** and **3.5** respectively. Depth 1 of the RSBR showed an initial increase in α-glucosidase specific activity from day 4 to 8 and then remained more or less constant with a slight increase from day 48 to 60. The specific activity of α-glucosidase at depth 1, however, remained consistently low compared to those of depths 2 and 3 during the 60-day experimental period. Over this period, there was a gradual decline in the specific activity of α-glucosidase with time at depth 2 from 94.96 µmol/min/mg protein (day 12) to 62.58 µmol/min/mg protein (day 48) and depth 3 from 123.65 µmol/min/mg protein (day 4) to 70.94 µmol/min/mg protein (day 60) (**Figure 3.3**). The maximum specific activity of α-glucosidase recorded at depth 2 was 94.96 µmol/min/mg protein (day 12) with a minimum of 57.37 (day 28), whereas, at depth 3, the maximum specific activity was 123.65 µmol/min/mg protein (day 4) and a minimum of 70.94 µmol/min/mg protein (day 60). An increase in the concentration of a particular substrate will result in the stimulation of the hydrolytic bacteria to produce enzymes that are specific to that substrate. This is logical since α-glucosidase is induced by microorganisms in response to availability of a suitable substrate (Turner *et al*., 2002).

The high enzyme activity observed initially could probably be due to the degradation of readily available substrates by free living microorganisms, resulting in a stimulation of metabolic activity. Since hydrolases are inducible enzymes (Burns, 1982; Nannipieri *et al*., 1990), the decrease in activity observed could be attributed to a decrease of enzyme synthesis by the microorganisms due to a decrease in the available organic substrates. The possibility of enzyme synthesis repression by particular metabolites or heavy metals can, however, not be ruled out completely (Burns, 1982). At depth 2 (day 28) and at depth 3 (day 24) sharp decreases in the specific activity of α-glucosidase were observed,

which is inexplicable. Nevertheless, this was most probably due to the introduction of fresh feed which might have differed in composition to the previously fed material.

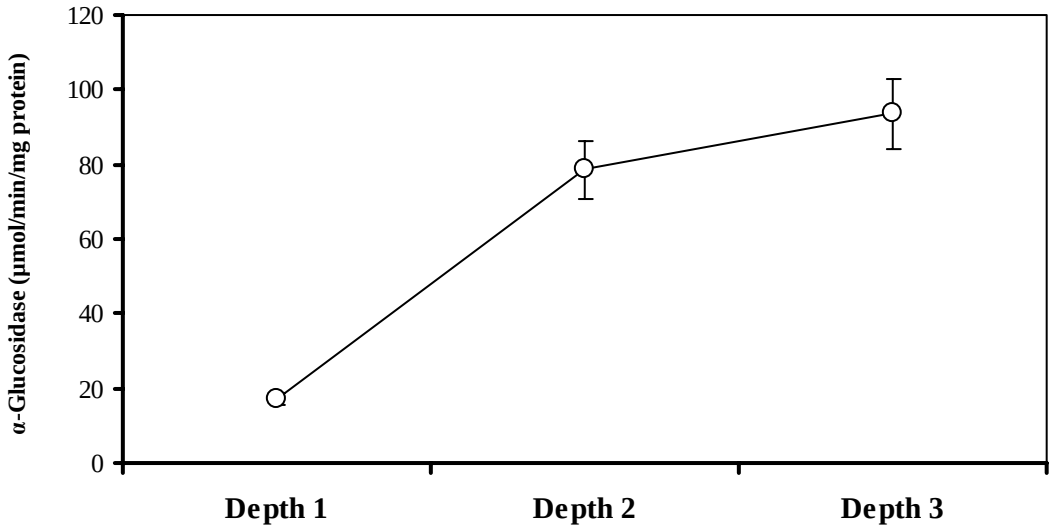

Figure 3.2 Profiles of specific α-glucosidase activity with RSBR depth. Values computed for covariates at their means. Error bars represent standard deviations (± SD) at 95% confidence interval, F (14, 72) =36.91, $P < 0.001$, n = 45

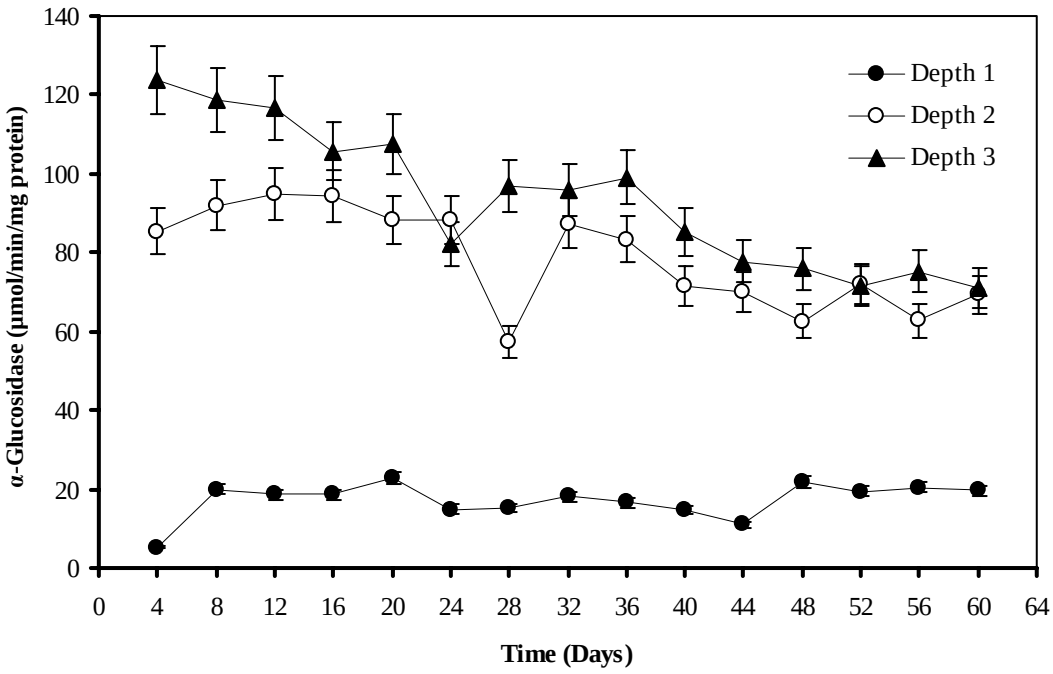

Figure 3.3 Variation in specific α-glucosidase activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

β-Glucosidase activity increased progressively at varying rates over the 60-day experimental period at all three depths (**Figure 3.5**). Low β-glucosidase specific activity was observed at all 3 depths of the RSBR for the first 10 days after which there was an increase in the specific activity. The β-glucosidase specific activity remained consistent after day 32 at depths 2 and 3 and after day 48 at depth 1. The activity of β-glucosidase in the RSBR was significantly lower (ANOVA, $P < 0.05$) than that of α-glucosidase (**Table 3.1**) at depths 2 and 3, although similar activity was observed at depth 1 for both enzymes. An increase in β-glucosidase specific activity was observed on day 16 at depths 2 and 3, however there is no obvious explanation for this enhanced enzyme activity. Nevertheless, a plausible suggestion could be attributed to an induction of enzyme expression in response to increased substrates or a change in composition of the feed (**Figure 2.2**). The activity of β-glucosidase did not appear to follow the trend characteristic of other inducible hydrolyases exhibiting higher activity as COD decreased. If the β-glucosidase was inducible, then the COD levels within the RSBR may not have provided an accurate reflection of the quantity of suitable substrates for this enzyme. Although the COD decreased, the suitable substrates for β-glucosidase may have increased due to the metabolic processes occurring within the RSBR system, although their concentration was not measured directly. Alternatively, β-glucosidase was not inducible under the biosulphidogenic conditions in the RSBR.

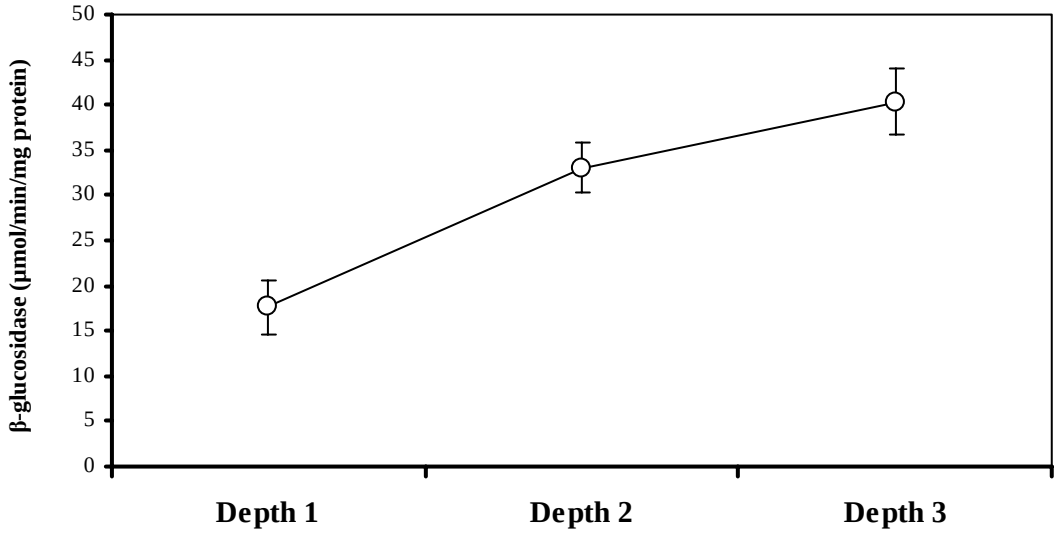

Figure 3.4 Profiles of specific β-glucosidase activity with RSBR depth. Values computed for covariates at their means. Error bars represent standard deviations (± SD) at 95% confidence interval, F (14, 72) =36.91, $P < 0.001$, n = 45

Based on the composition of the feed, a significant proportion of the accumulated COD was likely to have been carbohydrates and therefore more substrate stimulated β-glucosidase activity. A decrease in β-glucosidase activity of about 47% was observed on day 24 day followed by an increase from day 28. A possible explanation for this is the changing unavailability of organic matter. The β-glucosidase activity at depth 1 showed a completely different pattern. A progressive increase in activity was observed throughout the 60-day experimental period except between day 44 and 48 where an increase in activity of about 52.7% was observed.

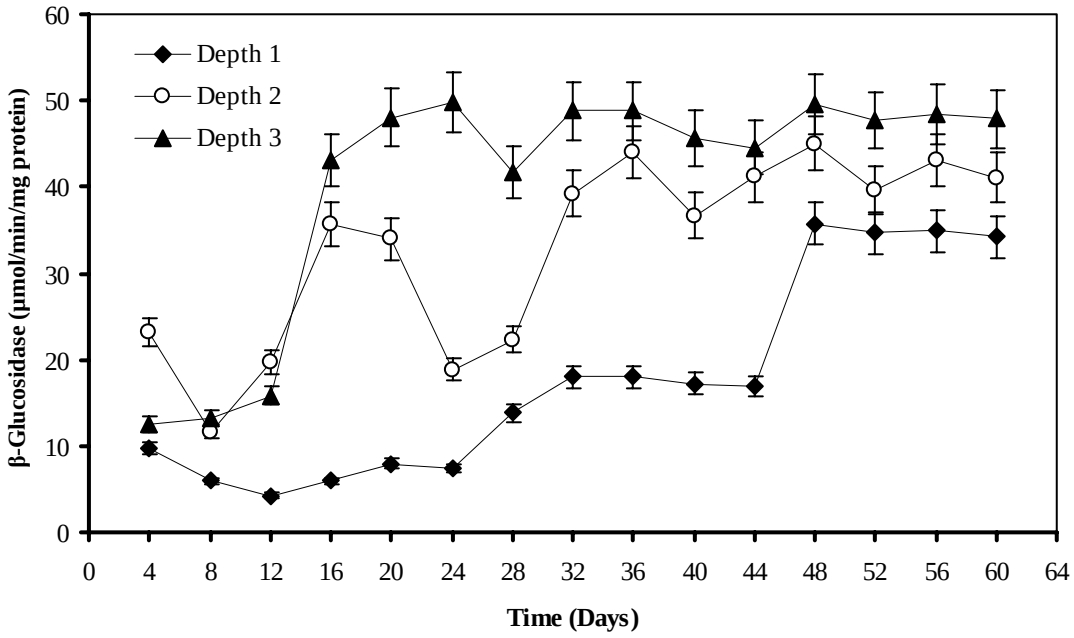

Figure 3.5 Variation in specific β-glucosidase activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

3.3.2.2. Protease activity

Proteases are essential for the degradation of particulate organics in wastewaters and sludges. One of the main enzyme activities in the sludge digestion process is the specific cleavage of peptide bonds from the C-terminal end by proteases (Drapeau *et al*., 1972). Considerable attention has been paid to the enzymatic hydrolysis of proteins for the improvement of sludge digestibility (Bomio *et al*., 1989), since protein hydrolysis is regarded as the rate limiting step (Ubukata, 1998, 1999) and the principal enzymatic reaction during waste sludge digestion (Häner *et al*., 1994; Kim *et al*. 2002).

The mean protease specific activity at each depth within the RSBR is shown in **Table 3.1** and **Figure 3.6**. The protease activities at the three depths of the RSBR were significantly different (Newman-Keuls, $P < 0.05$; df = 44), and increased with reactor depth. The possibility of sulfide involvement in the stimulation of the protease activity cannot be excluded, since this increase in activity corresponded with increase in sulfide concentration. However, there is no real evidence at this stage to support a casual relationship. The possibility of sulfide involvement can, however, be substantiated by the fact that hydrolysis of proteins was reported by Whittington-Jones *et al*. (2002) to be enhanced in the presence of sulfide, although the study did not examine the impact of sulfide on proteases. This is feasible as sulfide is a strong reducing agent that is capable of reducing disulfide linkages essential for maintaining the three dimensional conformation of large protein molecules. The smaller units of protein molecules produced by the enzymatic hydrolysis can be taken up directly by microorganisms for intracellular metabolism, thereby aiding hydrolysis of PS.

The time course behaviour of the protease specific activity over the 60-day study at various depths within the RSBR is shown in **Figure 3.7**. Protease activity at depth 3 increased to a maximum of 3.75 (± 0.01) μmol/min/mg protein on day 20 and then decreased and remained stable until day 60. Depth 2 also showed a similar trend with the maximal protease specific activity of 3.37 (± 0.02) μmol/min/mg protein on day 24 of the study period. Protease specific activities were low at depth 1 with the highest specific activity for protease being 1.548 (± 0.03) μmol/min/mg protein on day 20. A drop in protease activity was observed between days 25 and 28 at depths 2 and 3. The system recovered on day 30 and a steady rise in enzyme activity was seen through to day 60 at depth 3. Protease activity remained significantly different (ANOVA, $P < 0.001$; df = 26) with depth from day 28 to day 60. As with β-glucosidase, the increase in protease activity on day 20 coincided with an increase in COD at depths 2 and 3 of the RSBR. Consequently, the activity of proteases under sulphidogenic conditions may also be dependent on the substrate availability.

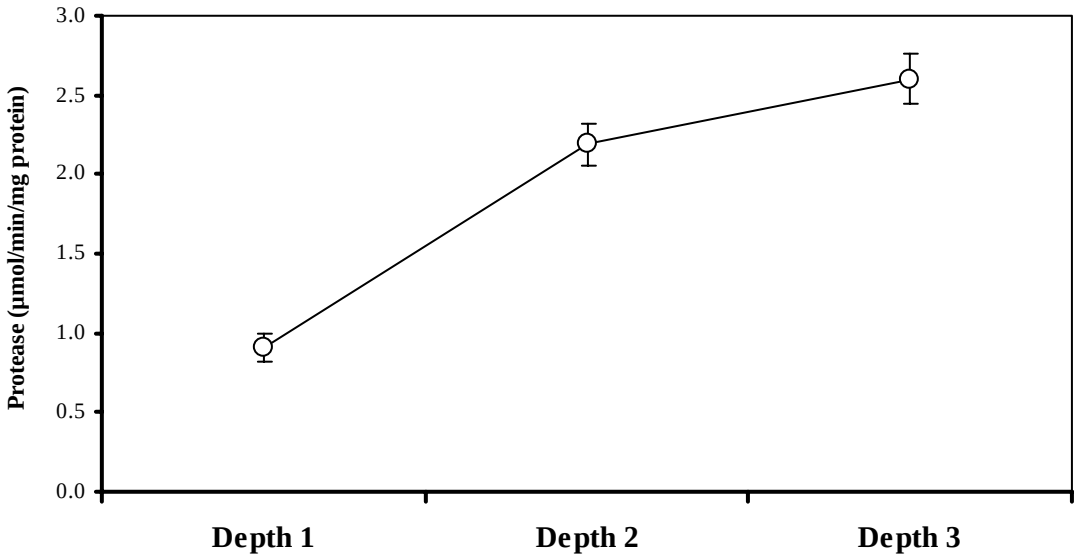

Figure 3.6 Profiles of specific protease activity with RSBR depth. Values computed for covariates at their means. Error bars represent standard deviations (± SD) at 95% confidence interval, F (14, 72) = 36.91, $P < 0.001$, n = 45

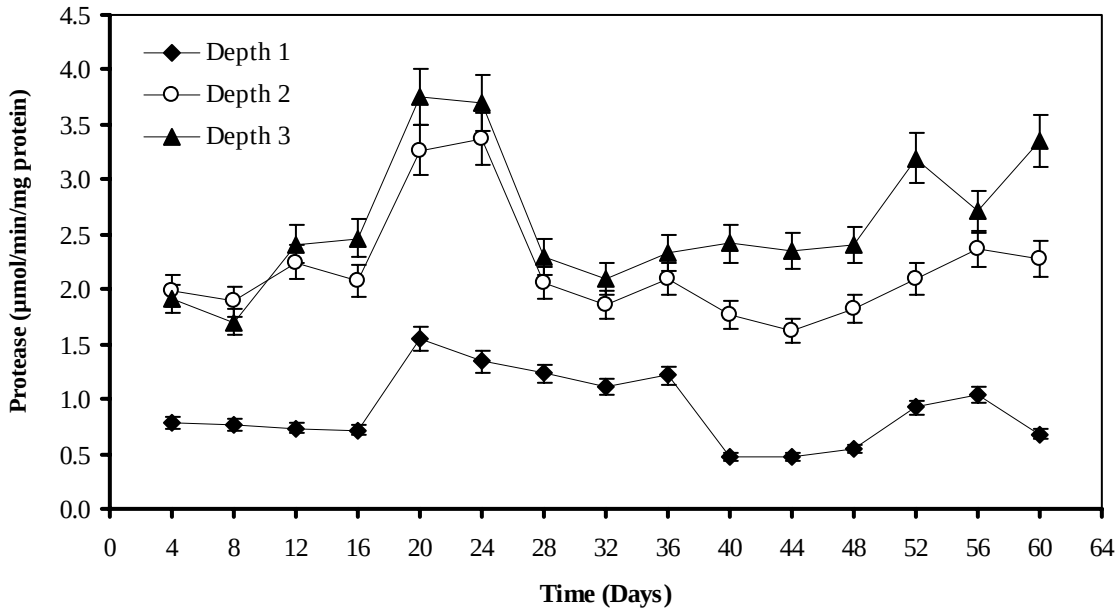

Figure 3.7 Variation in specific protease activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

An enzyme-adsorption based kinetic model was proposed by South *et al*. (1995) which predicts that the rate of hydrolysis for insoluble substrates increases with increasing concentrations of enzyme. Using the concept of this model, it is possible to describe the

hydrolysis of particulate organic matter and consequently the solubilisation rate of PS in the RSBR. Proteases can be used to enhance the hydrolysis of protein in wastewater, thereby enhancing the overall efficiency of the anaerobic wastewater treatment process. The low protease activity observed in this study compared to the activities of the α- and β-glucosidases suggested that the bulk of the activity of the protease enzymes lies within the extracellular polymers of the floc. Frølund *et al*. (1995) and Goel *et al*. (1998) have indicated that extracellular polymers could indeed hold a large pool of extracellular enzymes. Releasing these enzymes into solution within the RSBR will greatly contribute to enhancing the hydrolysis of PS within the RSBR.

3.3.2.3. Lipase activity

Lipases, defined as carboxylesterases which catalyse the hydrolysis of triglycerides and their synthesis from glycerol and long chain fatty acids, a process involving high specificity and enantioselectivity (Brockman, 1984; Ferrato *et al*., 1997; Jaeger *et al*., 1999), are of widespread occurrence throughout the earth's flora and fauna (Haki and Rakshit, 2003). Lipases are among the most widely used enzymes in industry and play an important role in a variety of biotechnological applications (Jaeger and Reetz, 1998). The most versatile enzymes are lipases of microbial origin and are known to bring about a range of bioconversion reactions (Vulfson, 1994), which includes hydrolysis, alcoholysis, acidolysis, aminolysis, transesterification and esterification (Jaeger *et al*., 1994; Pandey *et al*., 1999; Nagao *et al*., 2001, Kim *et al*., 2002a, 2002b).

Depth profiles of lipase specific activity within the RSBR are shown in **Figure 3.8**. Lipase activity did increase significantly (ANOVA, $P < 0.001$; df, 44) with depth. Depth 3 showed the highest activity and was closely followed by depth 2. Lipase activity at depth 1 was lowest in the RSBR and the increase in lipase activity from depth 1 to depth 3 represented about a 9 fold increase in activity. Again, the involvement of sulfide in this increased activity cannot be excluded, and can be substantiated by the findings of Whiteley *et al*. (2003) who reported a 10-fold increase in lipase activity with increasing sulfide concentration. The time course behaviour of specific lipase activity over the 60-day study period in the RSBR is shown in **Figure 3.9**. No statistically significant variation (ANOVA, $P > 0.05$; df = 14) was obtained for lipase activity during the

experimental period at depth 3. An increase in the lipase activity was observed between day 12 and 20 at depth 2 where there was an increase in activity from 0.02 (± 0.05) to 0.17 (± 0.06) µmol/min/mg protein, representing a 28.22% increase in activity.

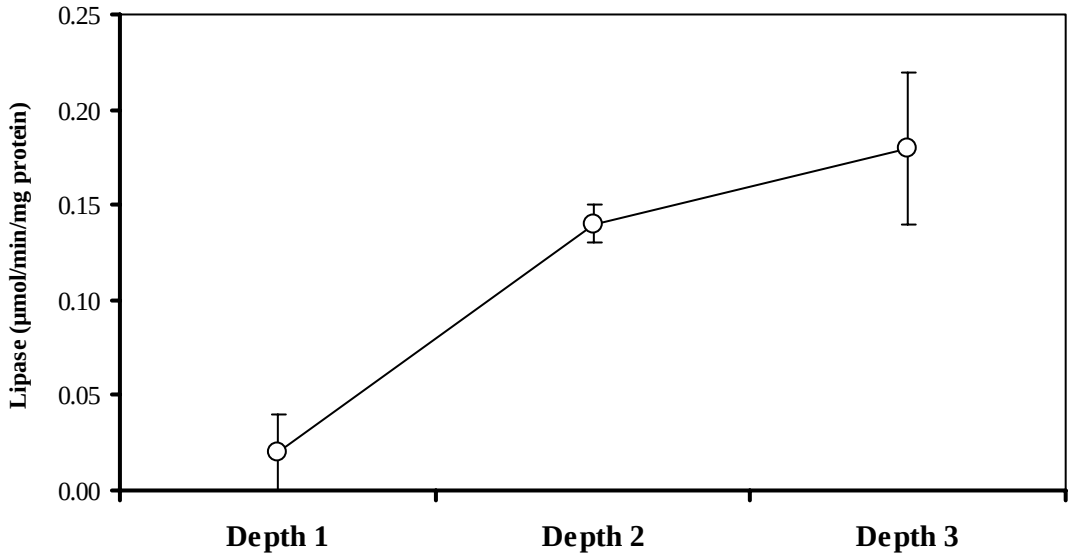

Figure 3.8 Profiles of specific lipase activity with RSBR depth. Values computed for covariates at their means. Error bars represent standard deviations (± SD) at 95% confidence interval, F (14, 72) = 36.91, $P < 0.001$, n = 45

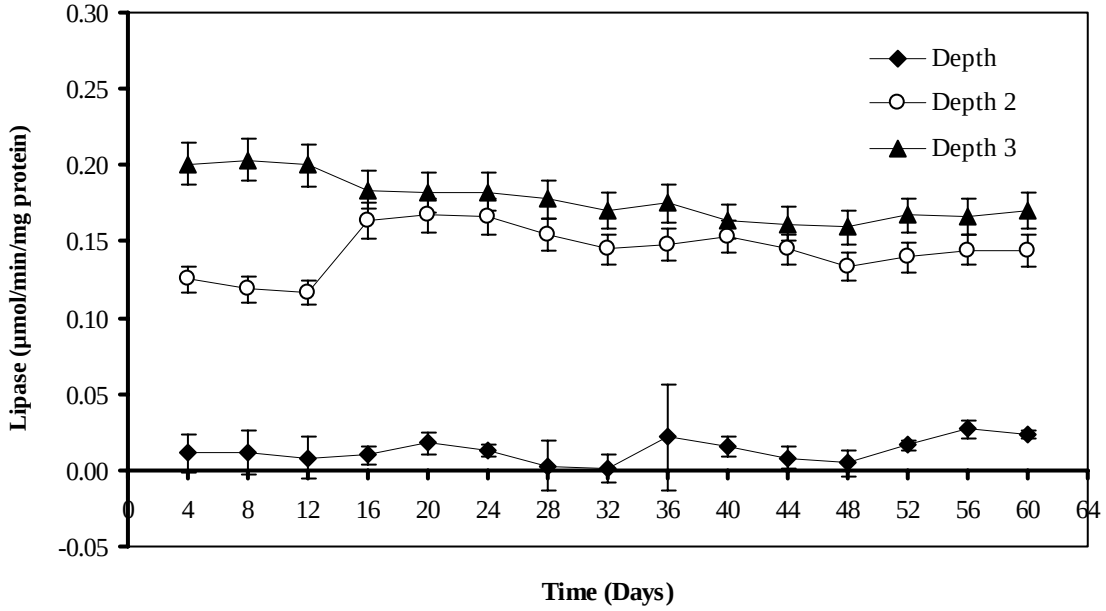

Figure 3.9 Variation in specific lipase activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

Thereafter, there was no significant difference (ANOVA, $P > 0.05$; df = 11) in lipase activity for the remainder of the study period. The specific lipase activities observed at all depths within the RSBR were generally very low compared to the specific activities of the other enzymes within the RSBR system (**Table 3.1**) for the duration of the study. Whiteley *et al.* (2003) also reported low lipase activity in sludge samples from a sulphidogenic bioreactor. A plausible explanation for the low lipase activity in the RSBR could be that lipase enzymes are either immobilised onto the organic matter or are intracellular membrane bound (Frølund *et al*., 1995; Whiteley *et al*., 2003).

3.3.2.3. L-Alanine aminopeptidase and L-leucine aminopeptidase activities

The aminopeptidases are involved in the cleavage of peptide bonds and in protein degradation. L-alanine aminopeptidases are specific for different amino acids, such as for alanine. Leucine aminopeptidase, an exopeptidase hydrolysing the peptide bond adjacent to a free amino group, is extensively used in the sequencing of proteins and peptides (Rover and Andrews, 1973). Protein metabolism plays an important role in microbial communities and most importantly, in the degradation of decaying biomass.

The mean specific activity of L-alanine aminopeptidase and L-leucine aminopeptidase at the various depths within the RSBR, are presented in **Figures 3.10** and **3.12** respectively. Significant increases (ANOVA, $P < 0.001$; df = 44) were observed in the activities of both L-alanine and L-leucine aminopeptidases with depth within the RSBR. At depths 2 and 3 of the RSBR, extensive fluctuations in both L-alanine aminopeptidase and L-leucine aminopeptidase were observed over the 60-day study period (**Figures 3.11** and **3.13**). The results and findings obtained here could be related to greater accessibility of easily degradable particulate organic matter to the microorganisms synthesizing and secreting these hydrolyzing enzymes. Roth and Lemmer (1994) determined that sewage system biofilms exhibited a high level of L-alanine aminopeptidase activity. The plant that these authors investigated, however, had different characteristics from the RSBR. The low activities reported in this study might be attributed to starvation effects induced by relatively low organic loads to the RSBR bioreactor.

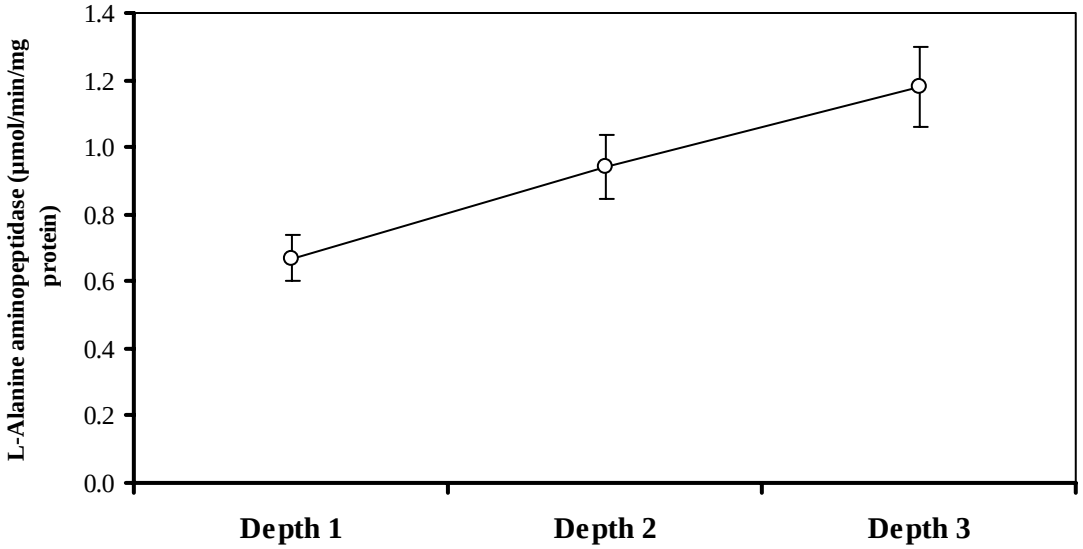

Figure 3.10 Profiles of specific L-alanine aminopeptidase activity with RSBR depth. Values computed for covariates at their means. Vertical bars denote standard deviations (± SD) at 95% confidence intervals, F (14, 56) =5.97, $P < 0.001$, n = 45

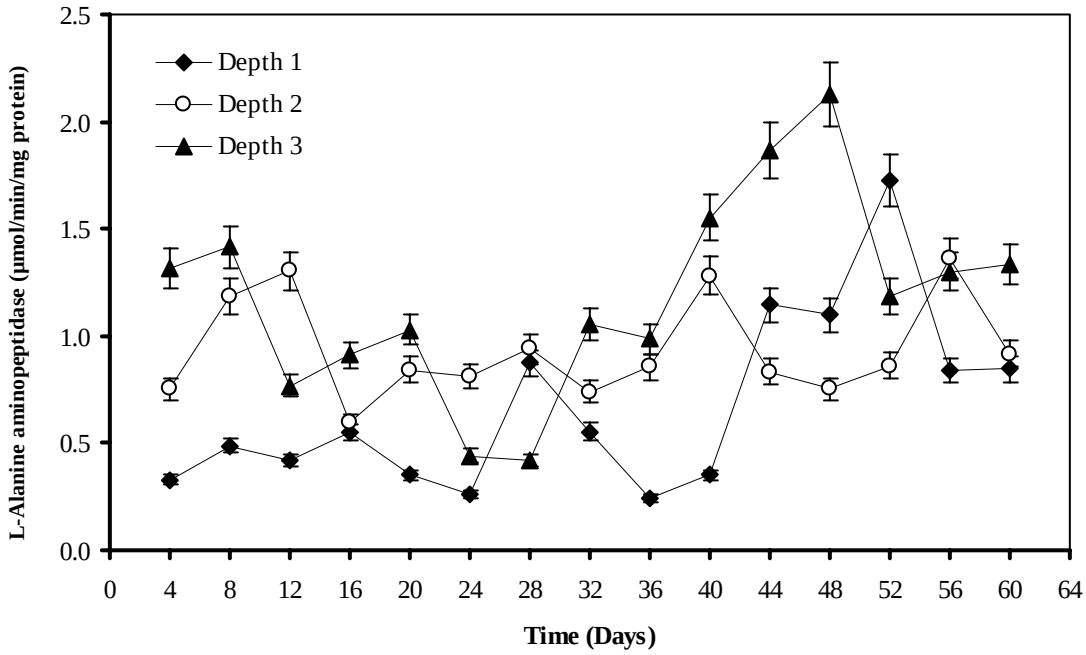

Figure 3.11 Variation in specific L-alanine aminopeptidase activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

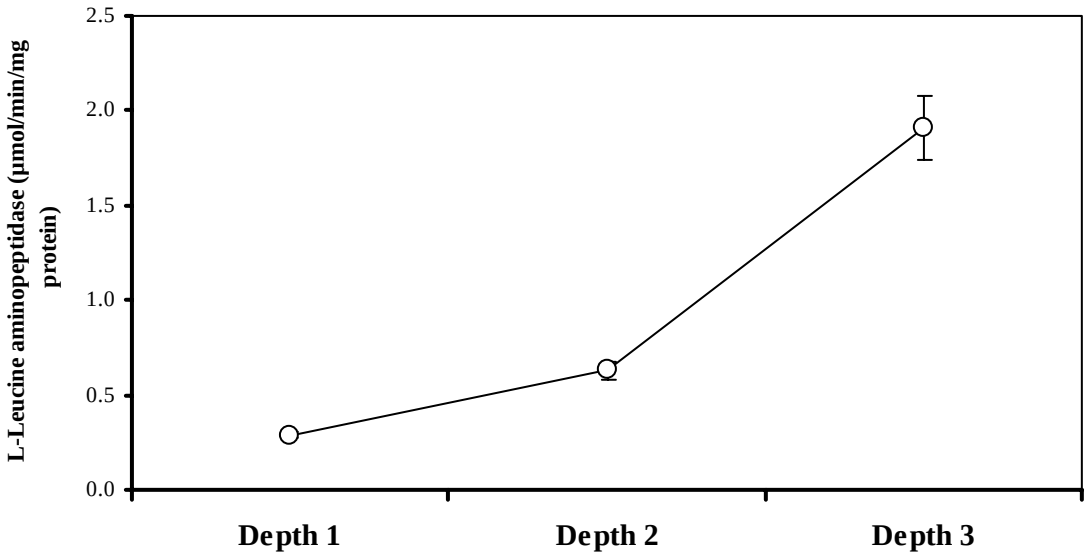

Figure 3.12 Profiles of specific L-leucine aminopeptidase activity with RSBR depth. Values computed for covariates at their means. Vertical bars denote standard deviations (± SD) at 95% confidence intervals, F (14, 56) =5.97, $P < 0.001$, n = 45

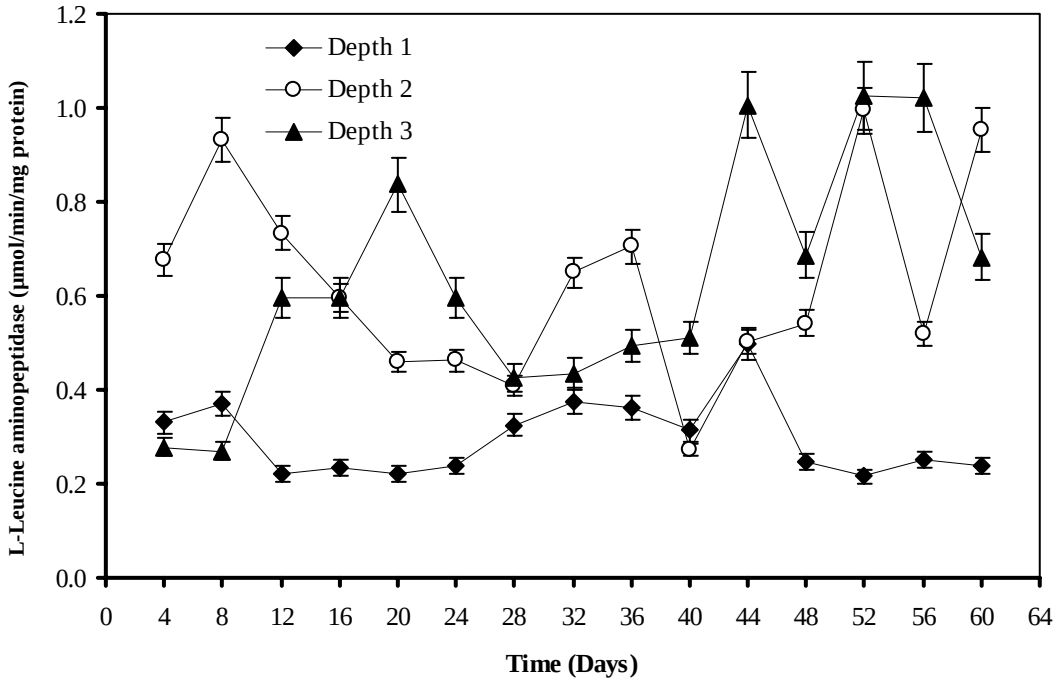

Figure 3.13 Variation in specific L-leucine aminopeptidase activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

3.3.2.4. Arylsulphatase activity

Arylsulphatase consist of a diverse group of enzymes (myrosulphatases, alkylsulphatases, glucosulphatases, steroidsulphatases, and chrondrosulphatases) catalysing the hydrolysis of ester sulfates by cleaving the organic moiety of the molecules to release sulfate (Dodgson *et al*., 1982; Knauff *et al*., 2003). Sulfate esters make up over 95% of sulfur in various soils and sediment environments and provide a readily source of sulfur for bacteria in anaerobic environments (Kertesz, 1999). Arylsulphatase activity is influenced by the presence of sulfur anions, pH (Dodgson *et al*., 1982) and the amount of organic matter present (Klose *et al*., 1999). Sulfur bacteria synthesise arylsulphatase enzymes, which hydrolytically cleave sulfate esters to release sulfate, which can be used by SRB as an energy source, during the hydrolysis of organic matter (Kertesz, 1999).

The mean arylsulphatase specific activity showed a significant increase (ANOVA, $P < 0.05$; df = 44) with depth in the RSBR (**Table 3.1** and **Figure 3.14**). Arylsulphatase specific activity showed variations at all depths over the 60-day study period although no consistent patterns were evident (**Figure 3.15**).

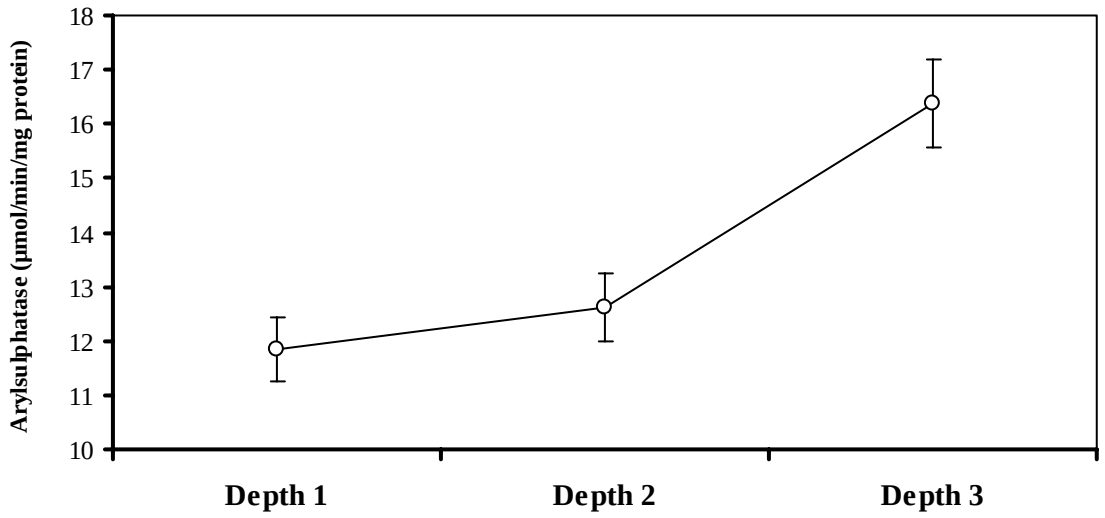

Figure 3.14 Profiles of specific arylsulphatase activity with RSBR depth. Values computed for covariates at their means. Vertical bars denote standard deviations (± SD) at 95% confidence intervals, F (14, 72) =36.91, $P < 0.001$, n = 45

The maximum specific activity of 24.85 (± 0.04) and 26.22 (± 0.15) µmol/min/mg protein was observed at depth 3 on day 16 and 24. This also corresponded to the period when high enzyme activity was recorded for the other hydrolytic enzymes studied. This high

activity could be attributed to the availability of substrates or easily biodegradable particulate organic matter during this period.

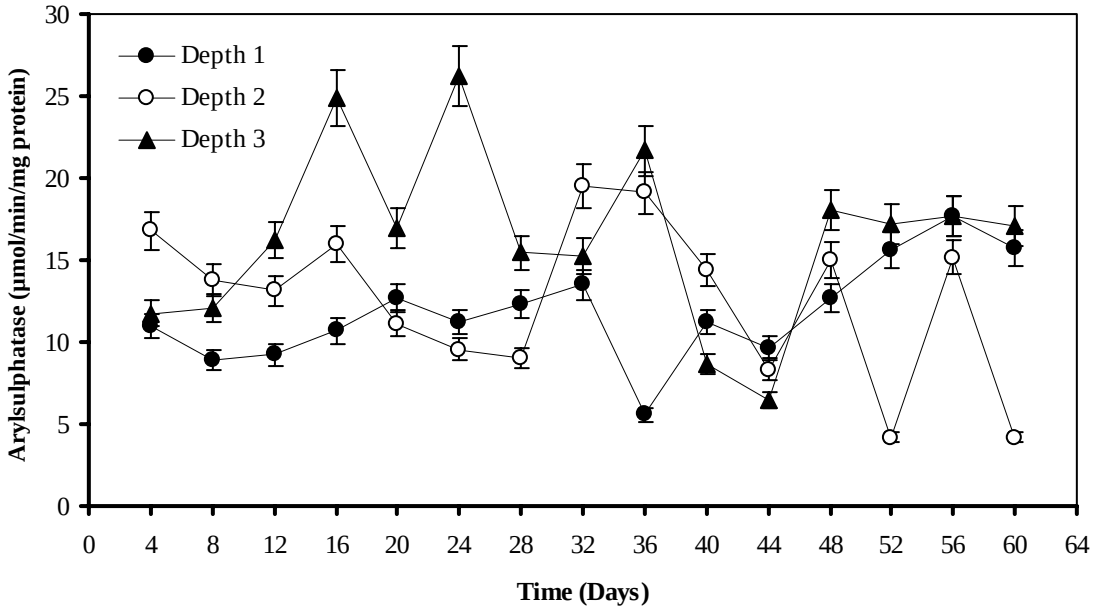

Figure 3.15 Variation in specific arylsulphatase activity at depth 1, depth 2 and depth 3 during the 60-day experimental period. Each point represents the mean of triplicate determinations

All enzymes tested in the current study within the RSBR showed spatial and temporal variability. This could be explained by substrate availability and also the variability in the physico-chemical parameters (sulfate, sulfide, pH and alkalinity) discribed in chapter 2. The high activities of the hydrolytic enzymes observed within the RSBR can also be linked to good retention of the organic matter with which the hydrolytic enzymes are most probably associated. The overall performance of the RSBR would benefit from the increased concentration of hydrolytic enzymes, since the initial velocity of enzymatic reaction is first order with respect to enzyme concentration.

Facilitating contact between the undigested substrates and the hydrolytic enzymes within the RSBR can furthermore be linked to the enhanced hydrolysis observed in the current study. Since enzyme activities are directly proportional to the concentration of enzyme (Goel *et al.*, 1997, 1998), an increase in enzyme activity should therefore reflect an increase in the hydrolysis rate and enhanced solubilisation of PS within the RSBR. With such a high percentage of organic particulates in biological wastewater, the process of

enzymatic hydrolysis is very important for their complete mineralization. The enzyme concentration within the RSBR can be increased considerably when suitable microbial substrates are at a premium, since for example, de-repression allows production of enzyme by microorganisms to liberate lower molecular weight compounds. Stimulating the growth of the microbial population will also result in production of more enzymes (Shackle *et al*., 2000) and consequently enhanced hydrolysis within the RSBR.

3.3.3. Statistical analysis of the RSBR parameters

In order to determine whether any of the variability in specific activity of the key hydrolytic enzymes correlated with the physicochemical or organic parameters, statistical analysis was performed on the data. In a multi-parameter system such as the RSBR, relationships between parameters can be investigated by carrying out a multivariate data analysis (Shine *et al*., 1995; Doherty *et al*., 2002, Liu *et al*., 2003a). Some obstacles, are however, encountered in this type of system, including the large number of parameters and the fact that not all parameters are dependent or independent. Therefore, to reduce the amount of data, Pearson *R* correlation (Pearson, 1896) analysis and linear regression analysis (Montgomery, 1991; Jaeckle and MacGregor, 1998) were performed between all pairs of combinations (**Tables 3.2** and **3.3**). The correlation coefficient plays an important role in measuring the strength of the linear relationship between two variables (Liu and Kao, 2002). These procedures measure the relationships between parameter sets and provide a way of identifying trends for subsequent multivariate analysis.

In this study, the correlation analysis was able to determine whether large values of physico-chemical parameters or distribution of organic macromolecules (carbohydrates, proteins and lipids) were associated with high specific enzyme activities (**Table 3.2** and **3.3**), whether small values of physico-chemical parameters were associated with high enzyme activities (negative correlation), or whether values in both sets were unrelated (correlation near zero). Proteins showed high correlations with all enzymes in contrast to carbohydrates and lipids which showed poor correlations with arylsulfatases and L-leucine aminopeptidases. However, considering the strong correlation between enzymes and non-substrate organic fractions, the validity of these correlations requires further investigation. The statistical analysis of the relationship between the physico-chemical

parameters presented in **Table 3.4** shows a very high correlation between, alkalinity, sulfide, sulfate, total COD and lipase, protease, α-glucosidase, β-glucosidase and L-leucine aminopeptidase. Alanine aminopeptidase and arylsulphatase showed generally weak correlations with the physico-chemical parameters. After careful study of the correlation table, a multiple regression (Pearson, 1908) model analysis was performed by using the "least squares" method. The least square principle minimises the deviations from the actual data points to a hypothetical line with the assumption that all the physico-chemical parameters together are necessary to explain the variation in the enzymatic activities. In this model the physico-chemical parameters jointly predicted the outcome of the enzyme activities.

Table 3.2 Pearson's (*R*) Correlation coefficient between carbohydrates, proteins, lipids and enzymatic activities of the RSBR

	Carbohydrates	Proteins	Lipids
α-Glucosidase	**0.980**	**0.998**	**0.999**
β-Glucosidase	**0.944**	**0.999**	**0.984**
L-Alanine aminopeptidase	**0.876**	**0.953**	**0.940**
L-Leucine aminopeptidase	0.651	**0.786**	**0.762**
Protease	**0.969**	**0.999**	**0.996**
Lipase	**0.967**	**0.998**	**0.995**
Arylsulfatase	0.618	**0.759**	0.733

Correlation coefficients (*R*) values are significant at 95% confidence level and $P < 0.05$

From the results obtained with these analyses, it was found that, the differences between the means of all the 3 depths of the RSBR for the specific enzyme activities and physico-chemical parameters except pH in the RSBR were all statistically significant. Weak correlations were obtained with the individual depths of the RSBR system as compared to strong correlations that were observed with the entire bioreactor.

Table 3.3 Parametric (Pearson's *R*) correlation coefficient between physico-chemical parameters and specific enzyme activities of the RSBR

	pH	Alkalinity	Sulfide	Sulfate	COD_{Total}	COD_{Sol}	COD_{Par}	Lipase	Protease	α-Gluc	β-Gluc	L-Ala am	L-Leu am
pH													
Alkalinity	-0.007												
Sulfide	0.092	**0.951**											
Sulfate	-0.020	**-0.973**	**-0.947**										
COD_{Total}	0.036	**0.932**	**0.882**	**-0.905**									
$COD_{Soluble}$	-0.032	**0.957**	**0.904**	**-0.944**	**0.862**								
$COD_{Particulate}$	0.041	**0.913**	**0.864**	**-0.886**	**0.998**	**0.831**							
Lipase	0.060	**0.957**	**0.916**	**-0.940**	**0.912**	**0.913**	**0.896**						
Protease	0.131	**0.816**	**0.769**	**-0.801**	**0.863**	**0.744**	**0.860**	**0.827**					
α-Glucosidase	0.132	**0.896**	**0.883**	**-0.899**	**0.875**	**0.823**	**0.866**	**0.947**	**0.747**				
β-Glucosidase	-0.199	0.606	**0.758**	-0.583	0.605	0.585	0.597	0.581	0.580	0.438			
L-Ala am	-0.215	0.417	0.370	-0.426	0.443	0.377	0.445	0.412	0.285	0.377	0.446		
L-Leu am	-0.126	0.637	0.586	-0.618	0.648	0.550	0.649	0.552	0.615	0.497	0.513	0.354	
Arylsulphatase	0.083	0.274	0.135	-0.231	0.353	0.220	0.359	0.300	0.352	0.331	0.440	0.077	0.080

The bold values denote significance level at $P < 0.05$, n =45. Positive or negative (-) prefix indicates slopes of the regression lines at 95% confidence level. Abbreviations: (α-Glu) α-glucosidase; (β-Glu) β-glucosidase; (L-Ala am) L-alanine aminopeptidase; (L-Leu am) L-leucine aminopeptidase; (Temp) temperature. Units: COD (mg/l); Alkalinity (as mg $CaCO_3$/L); sulfide and sulfate (mg/l); all enzymes (specific activities- µmol/min/mg protein

The analyses of the correlations indicated that the interaction between alkalinity, sulfide, sulfate and the CODs with the enzymes (lipase, protease, α-glucosidase and β-glucosidase) appeared to be more significant than those of pH, arylsulphatase and L-alanine and L-leucine aminopeptidase (Newman-Keuls, $P < 0.05$; df = 44).

Lipase, α-glucosidase, β-glucosidase and protease showed the better correlations with a 91.5% variance explained for alkalinity with lipase. Sulfate on the other hand showed significant negative correlations (**Table 3.2**) with the specific enzyme activities of most enzymes except arylsulphatase. In contrast, the pH and temperature correlations showed lower levels of statistical significance on the enzyme activities. The reason for this lack of correlation can be explained by the fact that the pH in the RSBR stayed between 7.17 and 7.50 during the entire duration of the study. This is essential since the optimum pH for the growth of SRB and the hydrolytic bacteria is between 6.5 and 7.5 (García *et al*., 2001) and low or high pH values will inhibit the activities of these bacteria (Dvorak *et al*., 1992). Inhibition of the activity of hydrolytic bacteria will directly impair the production of hydrolytic enzymes and consequently the hydrolysis of particulate organic matter.

The spatial studies of enzyme activities within the RSBR showed significant differences, and there were also fluctuations over time (although the latter could not be confirmed statistically). A number of factors (alkalinity, pH, sulfide, sulfate and COD) have been shown to influence enzyme activities and a statistically valid correlation may provide useful information on the potential link between the physico-chemical parameters and enzyme-enzyme activity. The *P*-values for the comprehensive post-hoc (Newman-Keuls multiple range) test for the effects of enzyme specific activities on each other at depth 1, depth 2 and depth 3 within the RSBR are presented in **Table 3.4**. Statistical analysis showed that throughout the RSBR there was no significant interaction between lipase and protease and L-alanine and L-leucine aminopeptidase.

The specific protease activity and the specific activities of L-alanine aminopeptidase and L-leucine aminopeptidase would be expected to correlate considering that they are all proteolytic in nature. This expected correlation between specific activities of enzymes

was evident between α-glucosidase and β-glucosidase at depth 2 and depth 3 where both α-glucosidase and β-glucosidase specific activities showed highly statistical significant interactions with all other enzymes except in depth 1, where the interaction between α-glucosidase and β-glucosidase was not statistically significant (Newman-Keuls, $P > 0.05$; df = 44).

Table 3.4 Probabilities for Newman-Keuls multiple range post hoc test for the RSBR parameters

	Depth		
	Specific enzymatic activities (μmol/min/mg protein)		
Enzyme	**1 and 2**	**1 and 3**	**2 and 3**
Protease	+++	+++	++
Lipase	+++	+++	+++
α-Glucosidase	+++	+++	++
β-Glucosidase	++	+++	NS
Arylsulphatase	NS	++	++
L-alanine aminopeptidase	NS	++	NS
L-leucine aminopeptidase	+++	+++	NS

All values are averages of 3 replicates. Marked differences are significant at $P < 0.05$, df = 44, at 95% confidence level. ++ = $P < 0.01$, +++ = $P < 0.001$, NS = Not significant. Abbreviation: *P* is short for probability. Results that yield $P < 0.05$ are considered borderline statistically significant; results that are significant at $P < 0.01$ are considered statistically significant, and $P < 0.001$ are highly significant

Both α-glucosidase and β-glucosidase are involved in the hydrolysis of polysaccharides. α-Glucosidase is used as the final enzyme in starch metabolism forming glucose as the end product (Giblin *et al.*, 1987; Madi *et al.*, 1987) while β-glucosidase completes the hydrolysis process of cellulose by catalysing the cleavage of cellobiose to release two molecules of glucose. The similar pattern shown by most of the enzyme activity interactions here might be due to the fact that all these enzymes showed an increase in activity with increasing depth of the RSBR. The highly significant interactions observed amongst most of the hydrolytic enzymes in this system could be of strategic importance in the sense that their combined actions not only enhance the rate at which the organic matter is being hydrolysed in the RSBR but also the overall process efficacy as well.

The complexity of sewage sludge contributes to the majority of the uncertainty and deviation in predictions using these statistical models. Nevertheless, the approach demonstrated in this work provides a better understanding of the interactions between the enzymes and biophysical factors within the anaerobic biosulphidogenic RSBR. The role of sulfide and other sulfur species is still not clear and possible modes of action are not understood. In order to answer some of the outstanding questions, it is necessary to consider the kinetics of the key hydrolytic enzymes.

3.4. Conclusions

From the results obtained in this chapter it can be concluded that:

- The carbohydrates were the most abundant organic component in the feed to the RSBR, followed by the proteins. The lipid concentration was generally very low in comparison to both carbohydrates and proteins.
- The activity of all the hydrolytic enzymes, except for L-leucine aminopeptidase and arylsulphatase exhibited strong correlation with the distribution of all three organic fractions and increased with the depth of the RSBR.
- The enzyme activities showed both spatial and temporal variation within the RSBR.
- The specific activities of α-glucosidase and β-glucosidase were significantly higher than those of the other hydrolytic enzymes studied.
- Strong correlations were obtained between the key hydrolytic enzymes and certain physico-chemical parameters (alkalinity, sulfide, sulfate) which suggested that these factors may be directly or indirectly involved in the enhanced hydrolysis of PS within the RSBR.
- The data obtained in this study has been used to identify the trend of enzymatic activities within the RSBR.

The Effect of pH, Sulfide and Enzyme-Enzyme Interactions on the Activity of Hydrolytic Enzymes and the Determination of Kinetic Parameters within a Biosulphidogenic Recycling Sludge Bed Reactor (RSBR)

4.1. Introduction

Anaerobic degradation of complex organic matter is an interrelated multistage microbial process of serial and parallel reactions (Gujer and Zehnder, 1983; Zinder, 1984) carried out by various enzymes (Jain *et al*., 1992). The intrinsic maximum hydrolysis rate is mainly dependent on the concentration and activity of hydrolytic enzymes which in turn are affected by the availability and concentration of substrates, activators, inhibitors (including accumulated products), pH and temperature (Jain *et al*., 1992). Sulfur producing compounds have also been employed in maintaining a reducing environment essential for the growth of hydrolytic bacteria responsible for hydrolytic enzyme production. Inorganic sulfur contained in sulfide thus appears to be an essential nutrient for the consortium of hydrolytic bacteria present in sewage sludge (Khan and Trottier, 1978).

Studies reported in this chapter, focused on the activities of the key hydrolytic enzymes (α-glucosidases, β-glucosidases, proteases and lipases) which are part of the important pathways for the biochemical degradation of the main macromolecules (carbohydrates, proteins and lipids) found in municipal wastewaters. The activities of these enzymes were also found to correlate well with a number of the physico-chemical parameters tested in this study (**Table 3.2**). These included sulfide, sulfate, alkalinity and COD and the presence of other enzymes. By conducting kinetic studies on the key hydrolytic enzymes, it may be possible to obtain a better understanding of the relationship between individual parameters, enzyme activity and enhanced hydrolysis. Previous studies have indicated an enhanced enzyme activity with an increase in sulfide concentration (Whiteley *et al*. 2002a). Therefore, changes in the kinetic parameters would be expected and as a result it would be possible to predict more about the nature of the interaction between the key hydrolytic enzymes and sulfide. The following questions, therefore, need to be answered in order to identify the parameters that could be used to optimize the hydrolysis of PS in the RSBR:

- What is the optimum pH at which the hydrolytic enzymes function in the RSBR and is there a pH range in which all the key hydrolytic enzymes exhibited high activity?

- Does sulfide stimulate enzymatic activities?
- What is the impact of sulfide on the kinetic parameters of the enzymes studied?
- What is the nature of interaction between the hydrolytic enzymes with each other?

The aim of this study was therefore to investigate the fundamental properties of the key hydrolytic enzymes within the RSBR. These would include: the pH optima, the effect of sulfur species, the enzyme kinetic parameters (K_m and V_{max}) and the effect of enzyme-enzyme interactions.

4.2. Materials and Methods

4.2.1. Reactor set-up

All samples for enzyme studies were obtained from the laboratory-scale RSBR system setup as shown in **Figure 2.1** and described in section 2.2.1 (page 41) of Chapter 2. The samples used for these studies were collected from depth 2 of the bioreactor, since this represented the zone where the mean values of the enzyme activities, sulfide, alkalinity and pH were optimum.

4.2.2. Enzymatic assays

All enzymatic assays were conducted according to the techniques described in section 3.2.8 (page 64) of Chapter 3. All the enzyme activities in this chapter were reported as percentage relative activity as defined in equation (13):

$$\text{Percentate Relative Activity} = \frac{\text{Activity (inhibited)}}{\text{Activity (no inhibition)}} * 100\% \qquad (13)$$

4.2.3. Determination of pH optima of the enzymes

All the enzyme assays were carried out as described in section 3.2.8 of Chapter 3, but the RSBR samples were incubated in assay solutions of different pH values. The pH of each of these solutions was controlled using the following buffer systems: pH 3.5-5.0, acetate-acetic acid; pH 5.5-6.5, sodium phosphate; pH 7.0-8.5, Tris-HCl and pH 9.0-12.5, carbonate-bicarbonate. All buffers in the assay solutions had a final concentration of 0.2 M.

4.2.4. Determination of the effect of sulfide on enzyme activities

Samples drawn from the RSBR were pre-incubated (30 min) in sulfide solution made from sodium sulfide (Merck Chemicals (Pty) Ltd) at concentrations of 100-600 mg/l. After the 30 min incubation period, enzyme activities (proteases, α-glucosidase, β-glucosidases and lipases) analyses were carried out according to the methods described in section 3.2.8 of Chapter 3. The desired sulfide concentrations were achieved by taking into account the initial sulfide concentration in the system and then elevating this to the desired final concentration using a sodium sulfide solution. The total sulfide concentration in the samples was determined as described in section 2.2.3 of Chapter 2. All the results were compared with that of a sulfide-free sludge mixture which served as the control. The relative enzyme activity measured in sulfide-free samples (control) was taken as 100% relative activity. The sulfide-free control was prepared by adding 500 µl concentrated sulfuric acid to 5.0 ml sludge sample and shaking for 2 min. In order to test if this acidification step had any impact on the enzyme assays, a preliminary experiment was carried out and this found no effect. A control for the effect of sulfide on each enzyme assay was conducted and was described in Chapter 3.

4.2.5. Determination of Kinetic parameters (K_m, V_{max})

The determination of the kinetic constants, the Michaelis constant (K_m) and the rate of reaction (V_{max}) was carried out to characterise the affinity of the synthetic substrates viz. methylumbelliferyl (MUF)- β-D-glucopyranoside, *p*-nitrophenyl α-D-glucopyranoside, azocasein and triacetin (Sigma-Aldrich Inc. USA) for the key hydrolytic enzymes (α-glucosidases, β-glucosidases, proteases and lipases respectively). Ten different concentrations of each of the substrates were chosen to give measurable reaction rates and the reactions were performed in triplicate. The substrate concentration range was carefully chosen as described by Cornish-Bowden (1979), since a very low substrate concentration range relative to the K_m will result in the plot being nearly horizontal while a high substrate concentration range relative to the K_m will result in the plot intersecting the axes very close to the origin. The K_m and the V_{max} were determined using linear regression plots of the Michaelis-Menten equation, the Lineweaver-Burk double reciprocal (Lineweaver and Burk, 1934) and the Hanes-Woolf (Cornish-Bowden, 1994)

plots. The calculation of the kinetic parameters K_m and V_{max} were performed by fitting to the appropriate rate equations using the SigmaPlot (2002) Enzyme Kinetics Module Version 1.1 for Windows (SPSS Inc. USA).

4.2.6. Effect of enzyme-enzyme interaction

The purpose of this experiment was to determine whether enzymes such as proteases had an impact on the other key hydrolytic enzymes. The only way this was to be achieved was to "knock out" the proteases and the glucosidases with selective inhibitors and then to determine whether this had an effect. This experiment was aimed at determining more carefully whether there were any enzyme-enzyme interactions within the RSBR system. Stock solutions of each of the enzyme inhibitors were prepared. $ZnCl_2$ (0.1 M) was used for the inhibition of α-glucosidase and β-glucosidase whereas phenylmethylsulfonyl fluoride (PMSF) (0.2 M) was used for protease inhibition. The RSBR samples (1.0 ml) for α-glucosidase and β-glucosidase, and (3.0 ml) for protease were drawn from the RSBR and pre-incubated (30 min, 37^oC) in the flask with the inhibiting solution (1.0 ml) and the buffer solution specific for the enzyme. The concentrations of the inhibiting solution in the assay mixture was $ZnCl_2$ (3.7×10^{-4} M) and PMSF (1.0×10^{-3} M) and were similar to those used by Klapwijk *et al*. (1974) and Price and Stevens (2002). The enzyme activities of the samples were determined according to the procedures described in section 3.2.8 (page 64) of Chapter 3.

4.3. Results and Discussion

4.3.1. pH optimum of the enzymes

The relative activities of the hydrolytic enzymes at different pH values for protease and lipase (**Figure 4.1**) and for α-glucosidase and β-glucosidase (**Figure 4.2**) indicated that all the enzymes exhibited activities over broad pH ranges and this might have contributed to the efficient solubilisation of the complex organics within the RSBR. The proteases showed several pH optima (**Figure 4.1**) which reflected that there were possibly several proteases with dissimilar characteristics. This was substantiated by other studies where it was reported that most bacterial alkaline proteases had their optimal activity in the pH range 7.5-11.5 (Gusek and Kinsella, 1987; Manachini *et al*., 1988; Peek *et al*., 1992; Aoyama *et al*., 2000; Bakhtiar *et al*., 2002; Gessesse *et al*., 2003). The broad pH ranges

shown by the proteases can increase their potential in biotechnological applications (Gessesse *et al*., 2003). These pH ranges also offers an advantage since it has been shown that these enzymes can operate at their suboptimal activity.

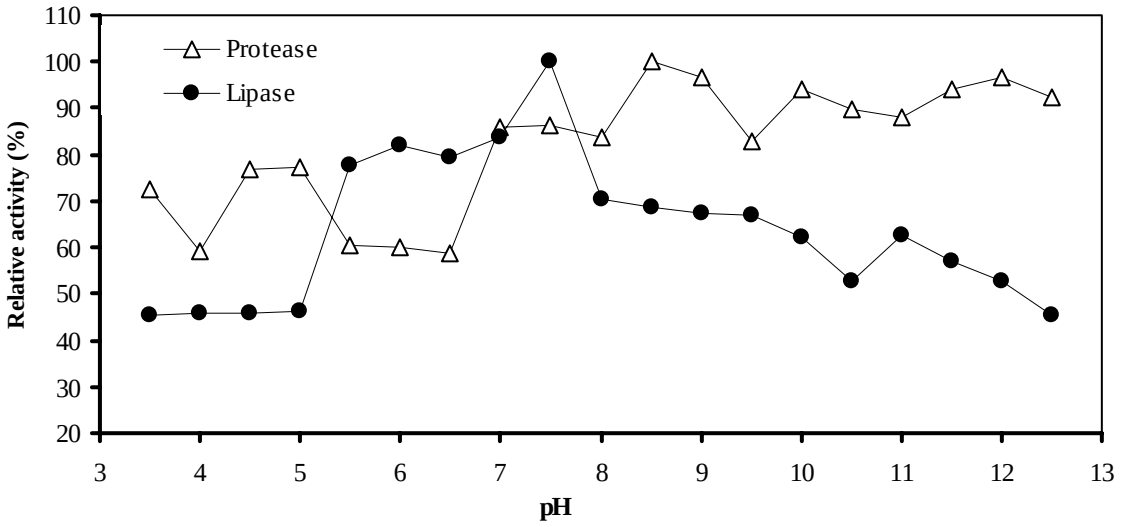

Figure 4.1 The optimal pH range for protease and lipase in the RSBR. All points are means of values obtained in triplicate

Lipases exhibited a pH optimum at pH 7.5 (**Figure 4.1**) and these findings agreed with those of Whiteley *et al*. (2003) who reported maximum lipase activities between pH 6.5 and 8.0 in both methanogenic and sulphidogenic bioreactors.

Optimal pH for α-glucosidase activity was 7.0 (**Figure 4.2**). This enzyme showed activity peaks in a broad pH range (pH 7.0-9.0). Other researchers found α-glucosidase activity to be optimal at pH 6.0 (Eivazi and Tabatabai, 1990). β-Glucosidase showed a rather lower pH optimum, pH 6.5, which was very close to published reports (Batistic *et al.*, 1980; Eivazi and Tabatabai, 1990; Eivazi and Zakaria, 1993).

In the current study, most enzymes in the RSBR showed a broad pH range in which relative activity is high. This suggests that these enzymes may be made up of suites of enzymes, each with a specific optimum, which as a whole are able to function over a wide pH range. The implication for this on the enhanced hydrolysis observed within the RSBR is that as long as the pH remains between 6.5 and 10 in the system, all the enzymes will function optimally.

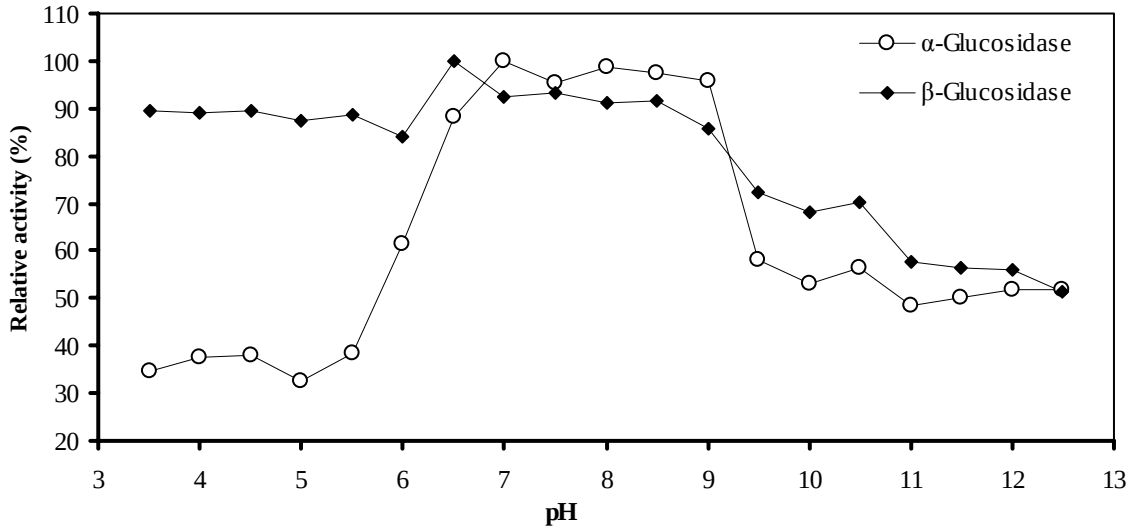

Figure 4.2 The optimal pH range for α-glucosidase and β-glucosidase in the RSBR. All points are means of values obtained in triplicate

Sewage sludge exhibits a broad range of pH values which may be attributed to a variety of natural and biological processes. Consequently, due to their biological nature, changes in pH values within the RSBR may have a strong impact on the biodegradation of the organic particulates and on the overall solubilisation process. The pH optimum in measurement of enzyme activity provides a measure of the maximum potential activity of the enzyme under natural conditions (Malcolm, 1983). It is also known that enzymes are irreversibly denatured by extremes of acidity and alkalinity and are most stable in the vicinity of pH optimum (Tabatabai, 1994). Consequently, enzyme-substrate recognition and catalytic activities *in situ* are reliant on the pH of the microenvironment. The pH dependence may alter the conformation of the enzyme, its active site as well as the binding of the substrate (Dixon and Webb, 1979, Günther *et al*., 2003). The overall effect on the enzyme catalytic activity as well as the stability in the RSBR can therefore be represented by a pH profile. Maximum hydrolysis and solubilisation rates of complex organic waste under anaerobic conditions have been reported at pH 6.0-9.0 (Eastman and Ferguson, 1981; Perot *et al*., 1988; Elefsiniotis and Oldham, 1994; Penaud *et al*., 1997) and agree with the results obtained in this study. The pH at which optimum solubilisation of organic particulates occurs is thought to reflect the pH at which most hydrolytic

enzymes, involved in the hydrolysis of organic substrates to smaller products in anaerobic digestion, operate (Morgenroth *et al*., 2002).

4.3.2. Effect of varying concentrations of sulfide on enzyme activities

The impact of varying concentrations of sulfide on the activity of key hydrolytic enzymes is presented in **Figure 4.3**. There was a general increase in the relative enzyme activity with increasing sulfide concentration. At depths 2 (188.79%) and 3 (164.75%), protease indicated approximately a 3-fold increase in relative activity at a sulfide concentration of 600 mg/l. This increase in the relative activity of protease at depths 2 and 3 with increasing sulfide concentration was significantly higher (ANOVA, $P < 0.01$, df = 13) than the relative activities of lipase, α-glucosidase and β-glucosidase at the same sulfide concentration. The effect of sulfide on lipase activity was low with a maximum of 43.63% stimulation observed at depth 3 at a sulfide concentration of 300 mg/l. A maximum stimulation (56.74%) of β-glucosidase relative activity at a sulfide concentration of 400 mg/l was observed. The highest α-glucosidase relative activity (56.44%) was seen at depth 2 at a sulfide concentration of 400 mg/l. These results were, however, not surprising as the trend of enzyme activities within the RSBR showed an increase from depth 1 to depth 3, that is, with increasing sulfide concentration (Chapter 3). The data obtained in the present study lends further evidence that sulfide enhances the activities of hydrolytic enzymes in the RSBR system, although the mechanism is not fully understood. Stimulation of enzymatic activities could be linked to the involvement of inorganic sulfur in the synthesis of sulfur containing amino acids, which are incorporated into microbial enzymes (Khan and Trottier, 1978), and may be relevant over a long period but does not explain the instantaneous enhancement observed in the present study. Coughlan and Ljungdahl (1988) reported that thiols stimulate the enzyme activity of *B. cellulosolvens* systems as well as the activity of exo-acting enzymes of the cellulolytic complex, but no explanation was provided.

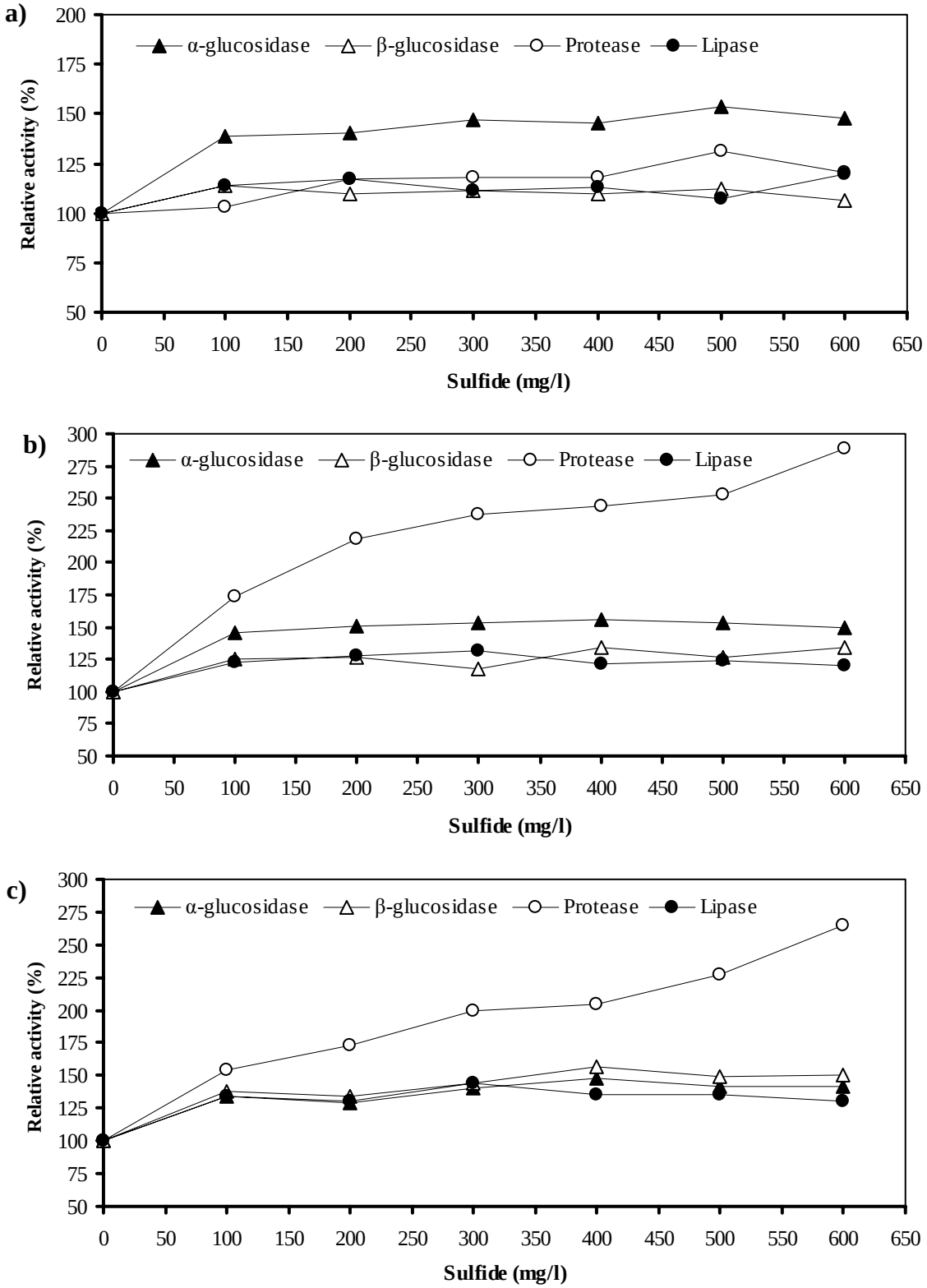

Figure 4.3 Effect of varying concentrations of sulfide on the relative enzyme activities in sludge sample from a) depth 1, b) depth 2 and c) depth 3 of the RSBR. All values reported are averages of three replicates. The relative enzyme activity measured in samples without sulfide (control) was taken as 100% relative activity

Whiteley *et al.* (2002b) reported a 1.7-fold increase in β-glucosidase at sulfide concentration of 200 mg/l, a 6-fold increase at sulfide concentration of 600-800 mg/l and a 5-fold to 10-fold increase in lipase activity at sulfide concentrations of 400 and 800 mg/l, respectively, which agrees with the increased relative activities observed in the present study. Although it is widely reported that the anaerobic degradation of complex organic matter is adversely affected by sulfide, this is limited to the gasification step (Maillacheruvu *et al.*, 1993). In the RSBR system, the presence of sulfide could, therefore, lead to the stimulation of enzyme activity and consequently enhanced hydrolysis and/or solubilisation of PS.

4.3.3. Estimation of the kinetic parameters

The apparent K_m and V_{max} of the key hydrolytic enzymes and the artificial (synthetic) substrates (Sigma-Aldrich Inc. USA) used in this study are presented in **Table 4.1**. These apparent values (K_m and V_{max}) resulting from the enzyme activities fitted the Michaelis-Menten equation (equation 14) giving a rectangular hyperbola. The Michaelis-Menten model suggests that hydrolysis rates can be shown to be proportional to enzymatic activities (Chibata *et al.*, 1976).

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (14)$$

In equation (14) v represents the initial velocity of the enzyme-catalysed reaction, with V_{max} being the maximum initial velocity achieved at substrate saturation. The maximal rate, V_{max}, reveals the turnover number of an enzyme measured by saturating all the active sites with substrate. The K_m value for an enzyme depends on the particular substrate, pH, temperature and ionic strength and is different for each enzyme. A high K_m indicates weak binding whereas a low K_m indicates strong binding of an enzyme with its substrate. The apparent Michaelis constant (K_m), represents the affinity of the enzyme for substrate and the apparent maximal velocity (V_{max},), the maximum velocity achieved by the enzyme at maximum substrate concentration

Table 4.1 The apparent *Km* and V_{max} of the key hydrolytic enzymes within the RSBR

Enzyme	**Substrate**	**Apparent K_m (µM)**	**Apparent V_{max} (µmol /min/ml)**
α-Glucosidase	*p*-Nitrophenyl α-D-glucopyranoside	0.16 ± 0.02	0.85 ± 0.02
β-Glucosidase	Methylumbelliferyl (MUF)- β-D-glucopyranoside	0.19 ± 0.07	0.49 ± 0.08
Protease	Azocasein	0.10 ± 0.02	2.31 ± 0.06
Lipase	Triacetin	0.22 ± 0.03	2.10 ± 0.02

The reported values for K_m and V_{max} represents the mean of 3 replicates with standard error (± SE) at 95% confidence interval, degrees of freedom = 86, $P < 0.05$, n = 30.

The plots of Lineweaver-Burk and Hanes-Woolf are presented in **Figures 4.4** and **4.5** respectively. The Hanes-Woolf plot is a rearrangement of the Lineweaver-Burk equation (equation 15) obtained from the direct transformation of the Michaelis-Menten equation (equation 14).

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad (15)$$

The Hanes-Woolf representation gives K_m and V_{max} with a good accuracy compared to other methods (Cornish-Bowden, 1979). The linear equation for Hanes-Woolf can be represented as follows (equation 16):

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_M}{V_{max}} \qquad (16)$$

A plot of $[S]/v$ versus [S] is thus linear with a slope of $1/V_{max}$ (**Figure 4.5**). The intercept on the ordinate axis gives K_m/V_{max}, (and when $[S]/v = 0$), the intercept on the abscissa gives $-K_m$. Both K_m and V_{max} were estimated using linear regression analysis suggested by Lineweaver and Burk (1934). According to Whiteley (2000), the Lineweaver-Burk (double reciprocal) plot provides the worst estimation of the kinetic constants because of the crowding of high data points close to the ordinate axis with further magnification of

errors in determining kinetics parameters when estimated from the slope and/or the intercept.

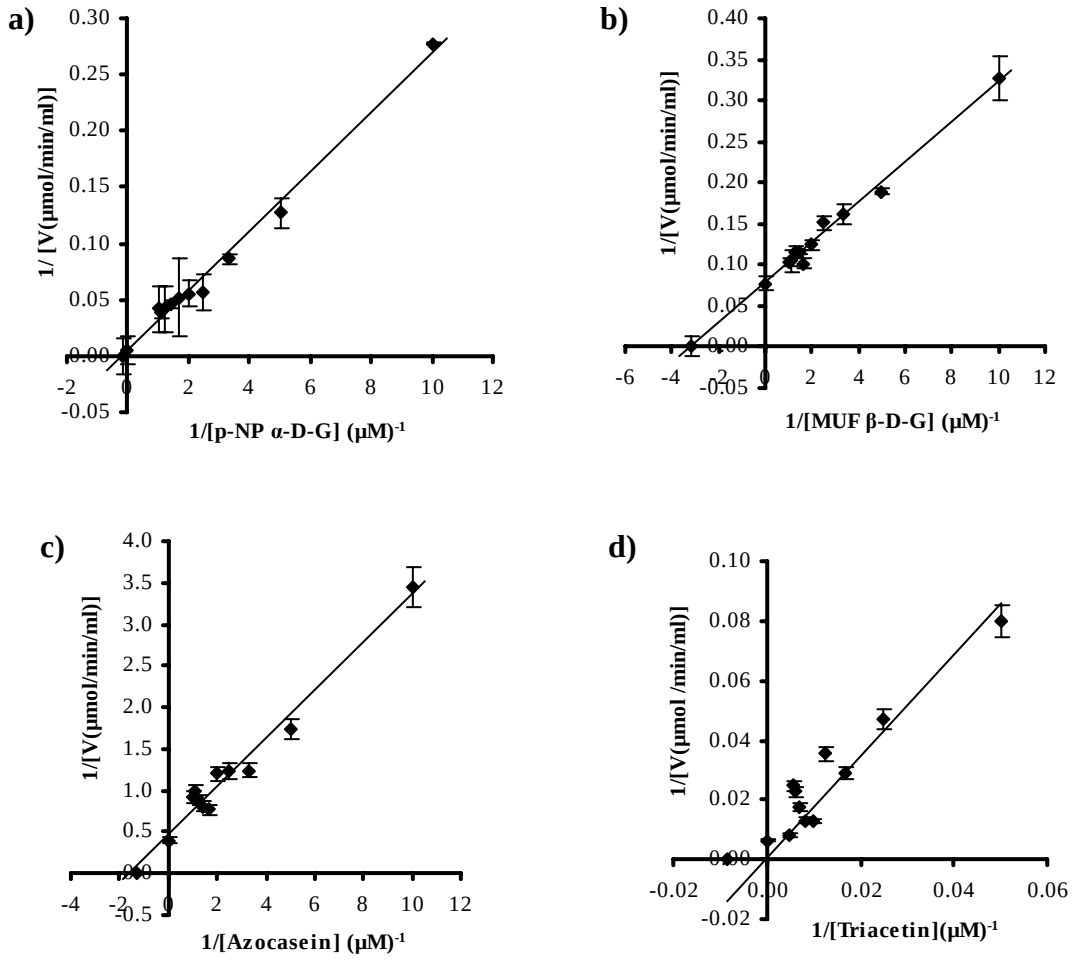

Figure 4.4 Lineweaver-Burk double reciprocal plots of a) α-glucosidase b) β-glucosidase c) protease d) lipase. Each point on the graph represents the mean ± SD of three replicate determinations. Abbreviations: *p*-NP- α-D-G, *p*-Nitrophenyl α-D-glucopyranoside; MUF β-D-G, methylumbelliferyl (MUF)- β-D-glucopyranoside

It must be stressed that the K_m and V_{max} values reported here are represented as apparent values since sewage sludge actually consist of a consortium of each of these enzymes and other enzymes which may apparently have specificity for these same substrates, and indeed the same V_{max} at a given enzyme concentration (Garrett and Grishman, 1999). Extreme caution should be taken when interpreting these values and it is, therefore, not possible to compare the data obtained from the literature which, are mainly those of purified enzymes, with those obtained in these studies.

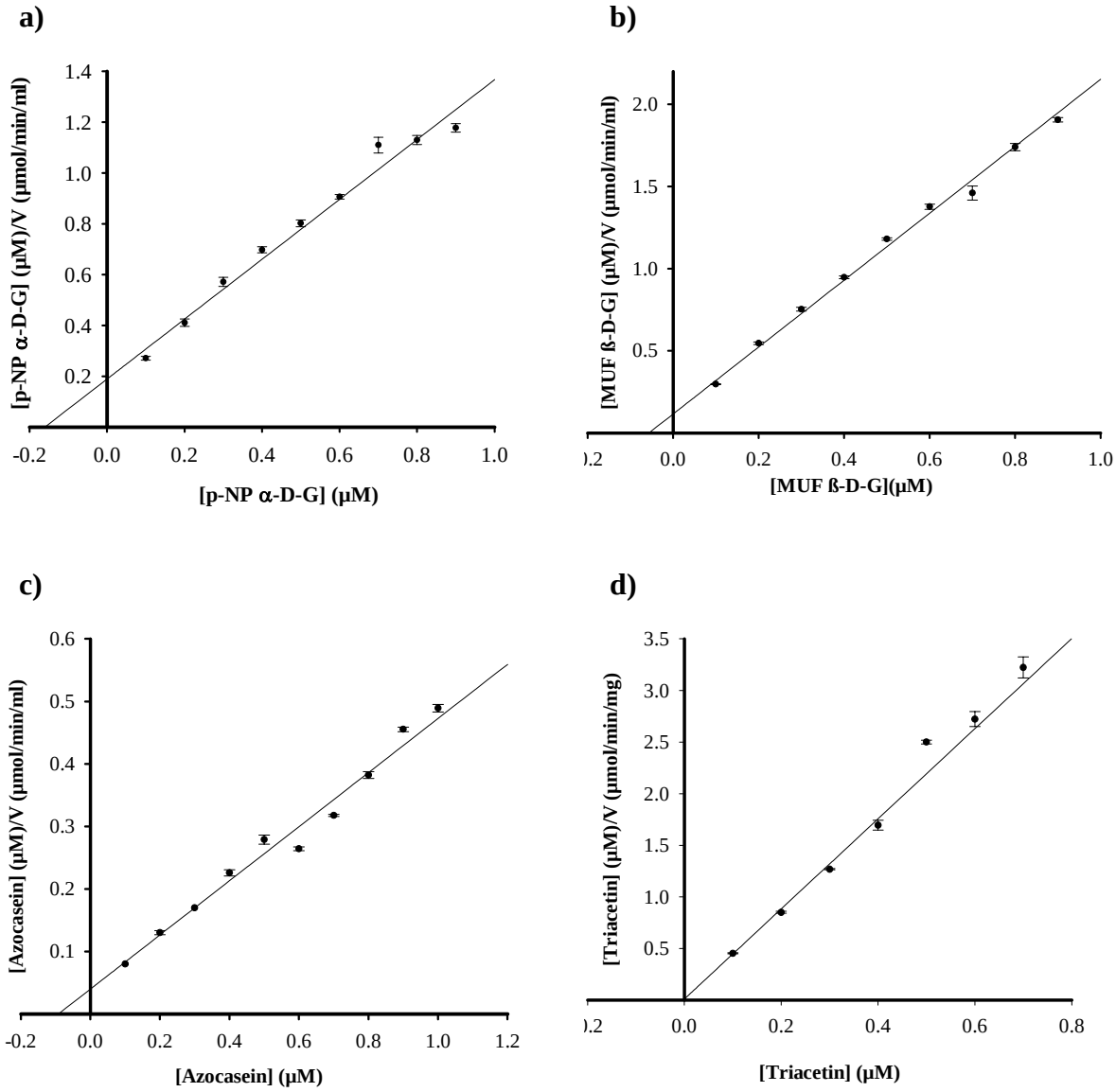

Figure 4.5 Hanes-Woolf plots of a) α-glucosidase b) β-glucosidase c) protease d) lipase. Each point on the graph represents the mean ± SD of three replicate determinations. Abbreviations: *p*-NP- α-D-G, *p*-Nitrophenyl α-D-glucopyranoside; MUF β-D-G, methylumbelliferyl (MUF)- β-D-glucopyranoside

Nevertheless, results obtained by Cadoret *et al.* (2002) for α-glucosidase on activated sludges showed a V_{max} of 0.90 ± 0.2 µmol/min/ml which compared well with the 0.85 ± 0.03 µmol/min/ml obtained in these studies. This comparison might be possible according to Mikkelsen and Keiding (2002) who reported that primary and anaerobically digested sludges generally showed comparable characteristics to the activated sludges.

In view of the fact that it has been established that sulfide stimulated the activity of the hydrolytic enzymes (section 4.3.2) it was decided to represent this further by exploring the apparent kinetic parameters (K_m and V_{max}) at varying sulfide concentrations in flask studies. The affinity constants (apparent K_m) and reaction rates (apparent V_{max}) obtained from the incubation with different sulfide concentrations (250, 400 and 500 mg/l) are presented (**Table 4.2**). Values for both the apparent K_m and V_{max} obtained at these sulfide concentrations for lipase, α-glucosidase and β-glucosidase were not significantly different (ANOVA, $P > 0.05$; df = 28). Although sulfide has been linked to the stimulation of enzyme activity (Chapter 3), it does not appear to have any direct effect neither on the K_m value nor the V_{max}, except for proteases. This probably suggests that any stimulation of enzyme activity by sulfide is due to an indirect effect.

Table 4.2 Kinetic parameters, apparent V_{max} and K_m of α-glucosidase, β-glucosidases, protease and lipase activities in the RSBR at sulfide concentrations of 250, 400 and 500 mg/l

	Apparent K_m (µM)				**Apparent V_{max} (µmol /min/ml)**			
Sulfide (mg/l)	250	400	500	*P*-Value	250	400	500	*P*-Value
α-glucosidase	0.16 (± 0.02)	0.16 (± 0.02)	0.17 (± 0.02)	> 0.05	0.88 (± 0.02)	0.87 (± 0.02)	0. 89 (± 0.02)	> 0.05
β-glucosidase	0.05 (± 0.06)	0.04 (0.04)	0.04 (± 0.04)	> 0.05	0.48 (± 0.02)	0.49 (± 0.08)	0.49 (± 0.06)	> 0.05
Protease	0.06 (± 0.03)	0.07 (± 0.01)	0.09 (± 0.01)	< 0.05	0.21 (± 0.01)	0.23 (± 0.06)	0.24 (± 0.08)	> 0.05
Lipase	2.021 (± 0.04)	2.057 (± 0.05)	2.043 (± 0.06)	> 0.05	0.23 (± 0.04)	0.24 (± 0.04)	0.26 (± 0.05)	> 0.05

The reported values for apparent K_m and V_{max} represents the mean of triplicate determinations with standard error (± SE) at 95% confidence interval, df = 28, $P < 0.05$, n = 30. The apparent K_m represents the affinity of enzymes for substrate and the apparent V_{max}, the maximum velocity achieved by the enzymes at maximum substrates concentration. K_m and V_{max} define the kinetic behaviour of an enzyme as a function of the substrate concentration

Since the protease activity was significantly stimulated with increasing concentration of sulfide (**Figure 4.3**), it was expected that this would have an effect on the kinetic parameters, K_m and V_{max}. However, only the K_m for protease showed a significant increase (ANOVA, $P < 0.05$, df = 29) with increasing sulfide concentration. The implication for this is that the increase in sulfide concentration resulted in a decrease in

the substrate affinity for the enzyme and this could have resulted in a decrease in V_{max}. The apparent V_{max} did not, however, change with increasing sulfide concentration. Pletschke *et al*. (2002) reported an increase in the enzyme activity with increasing sulfide concentration on pure synthetic enzymes which suggested a direct effect on the enzyme activity. The present study was carried out on a consortium of unpurified enzymes from different organisms unlike Pletschke *et al*. (2002) who used a purified enzyme from the same source. The results of these studies contradict their model of direct enzyme stimulation by sulfide. The increased enzyme activity with increasing sulfide concentration reported in the present study might be a result of a sulfide effect on the micro-environment rather than directly on the enzymes within the RSBR. This could possibly be due to the sulfide opening up the floc matrix, and in so doing exposing and releasing the enzymes which are entrapped within, thereby resulting in an increase in protease concentration and activity. This is crucial to the hydrolysis within the RSBR as a high concentration of enzymes could lead to an overall increase in the hydrolysis and solubilisation of the complex organic matter.

4.3.4. Effect of enzyme-enzyme interaction

The effect of inhibitors on the relative activities of the enzymes (protease, lipase, α-glucosidase and β-glucosidase) is shown (**Figure 4.6**). $ZnCl_2$ was used as an inhibitor for both α-glucosidase and β-glucosidase whereas PMSF was used as a protease inhibitor. Protease was inhibited by PMSF (38%), a serine protease inhibitor, (Thangam and Rajkumar, 2002). The limited reduction of protease relative activity observed with PMSF suggests that while some of the degradation was as a result of the presence of serine proteases (Gessesse *et al*., 2003) other proteases were also present in the RSBR. This limited reduction in protease activity resulted in a significant increase in the activity of lipases. Relative lipase activity of as much as 310% (3-fold) was observed in the flask pre-incubated with PMSF. The high relative lipase activity observed with RSBR samples incubated with PMSF suggested that proteases were hydrolysing the lipases and other enzymes, thereby reducing their activity. Inhibition of protease activity therefore resulted in a sudden rise in the activities of lipases and hence enhancement of autolysis (Stoll and Blanchard, 1990; Roe, 2001; Price and Stevens, 2002). Further control of

proteolysis could be obtained by including commercially available protease inhibitors in various buffer solutions. This can, however, be hampered greatly by the cost especially in large scale. Interestingly, the relative activity of both the α-glucosidase and the β-glucosidase was inhibited in the presence of PMSF with a deactivation of 27% and 31% respectively.

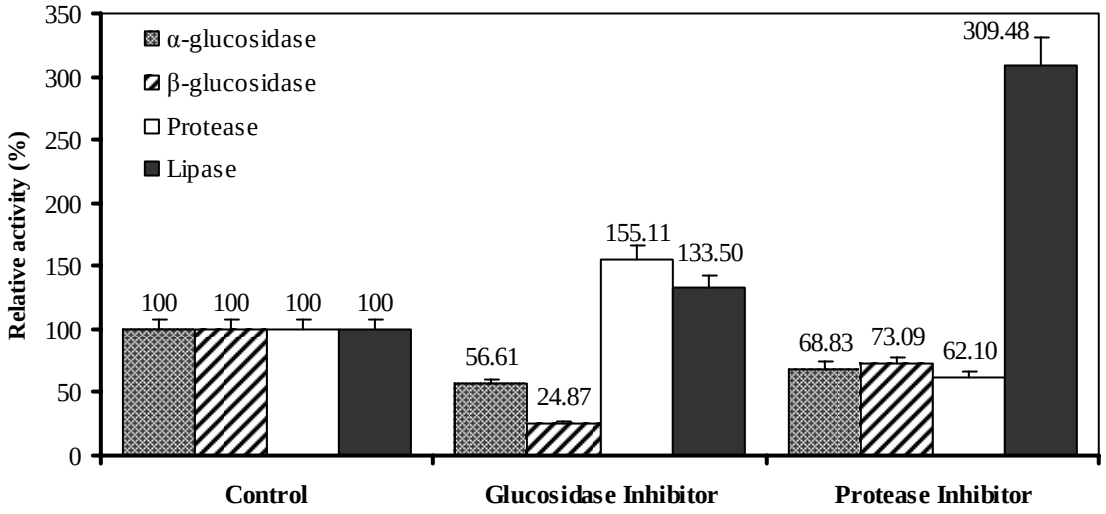

Figure 4.6 Effect of glucosidase inhibitor ($ZnCl_2$) and protease inhibitor (PMSF) on relative activities of the key hydrolytic enzymes. Values are means of three replicate readings. Error bars represent standard deviations (± SD). The relative enzyme activity measured in samples without the inhibiting solution (control) was taken as 100% relative activity. A positive control was also carried out using 0.1% commercial enzyme (results not shown) to verify the performance of assay

$ZnCl_2$ inhibited β-glucosidase by 75% and α-glucosidase by 43% (**Figure 4.6**). This inactivation may be the result of covalent modification of the active site of the enzyme. Since some of the glucosidases are metalloenzymes, that contain a metal ion involved in their catalytic activity; Zn ions may inactivate the enzyme reactions by reacting with the enzyme-substrate complex and with the protein-active groups of the enzymes (Deng and Tabatabai, 1995). Alternatively, the inhibition could be as a result of the competition between the protein-associated cation and the exogenous cations, thus resulting in the decrease of metalloenzyme activity (Lin *et al.*, 1998). Inhibition of α- and β-glucosidases resulted in an increase in the relative activity of both proteases and lipases. However, direct links between the decrease in glucosidase activity and increases in protease and

lipase cannot be explained. An alternative explanation of the results was that the glucosidase inhibitor, $ZnCl_2$, had a direct stimulatory effect on the other enzymes.

Enhancement of protease activity by $ZnCl_2$ might be as a result of Zn-binding proteases being stable in the system. The increased proteolysis by $ZnCl_2$ could as well suggest that the extra-cellular enzymes involved in this proteolysis were stimulated by the metal ions (Sierecka, 1998). Divalent ions such as Zn^{2+} affect the state of the microbial communities in digestion systems. Zinc ions were reported to inhibit protease activity at high concentrations but had a stimulatory effect at low concentrations (Kim *et al*., 2003).

Considering that the activity of protease enzymes was enhanced in the presence of sulfide and that proteases appeared to have a negative impact on the activity of lipases, it would appear that lipases play little or no role in the enhanced solubilisation of complex carbon in the biosulphidogenic RSBR. The results of these experiments show that enzymatic activities are affected by process parameters such as sulfide, pH and enzyme-enzyme interactions. It is therefore evident that manipulating these parameters to optimal conditions for enzymatic activities will enable enhanced hydrolysis of complex organic matter with a concomitant maximisation of sludge solubilisation in the RSBR. This information provides a key opportunity to the further development of the RSBR and the anaerobic degradation process in its entirety.

However, although a number of factors influence enzymatic activities in the RSBR, the affect of sulfide does not appear to be direct. Indirect affects, such as changes of floc structure, require further investigation.

4.4. Conclusions

The interactions between the hydrolytic enzymes and environmental factors within a biosulphidogenic RSBR are extremely complex. The outcome of these interactions can significantly influence the hydrolysis of the complex organic matters by the hydrolytic enzymes within the RSBR system. The optimum conditions for the enhanced hydrolysis and/or solubilisation of the complex organic matter by the hydrolytic enzymes within the biosulphidogenic RSBR were identified. In the current study it was observed that:

- All enzymes studied showed optimum activity in the pH range of 6.5-8.5, which was within the pH range at which the reactor was operated.
- α-Glucosidase, β-glucosidase and proteases were active over a wide pH range which could facilitate efficient mineralization.
- Enzymatic activities of the key hydrolytic enzymes, particularly proteases from depths 2 and 3 in the RSBR, were significantly stimulated by sulfide. A direct impact of sulfide on the enzymatic activities could not, however, be established.
- Sulfide concentrations of 250, 400 and 500 mg/l did not have any significant effect on the V_{max} and the K_m for lipase, α-glucosidase and β-glucosidase. A statistically significant increase in the protease K_m was, however, observed with increasing sulfide concentration, but the increase was not sufficient to be of biochemical significance.
- Inhibiting proteases in flask studies led to a significant increase in the activity of lipases. This suggested that the low lipase activity observed may, at least partially, have been as a result of digestion of the lipases by the proteases, and that lipases play little or no role in enhanced hydrolysis of primary sludge.

Chapter 6

General Discussion and Conclusions

General Discussion and Conclusions

During municipal wastewater treatment, sludges are generated as by-products of the physical, chemical and biological processes and should be disposed of without creating health problems or further hindrance. The anaerobic treatment process has proved to be the most beneficial stabilisation technique as it optimizes cost effectiveness, minimises the amount of final sludge disposal and has the ability to produce a net energy gain in the form of methane gas (De Baere, 2000). The anaerobic treatment technology currently available is only capable of partially treating waste in a conventional wastewater treatment system with high levels of degradation requiring longer retention times or further treatment methods, which add to the cost of the treatment (Parker *et al*., 1998).

Biological anaerobic wastewater treatment systems, in which complex organic matter is degraded in a sulphidogenic bioreactor, provides a promising alternative to methanogenic treatment systems (Lens *et al*., 1998), as well as an effective alternative to the complex physico-chemical methods for the removal of sulfate from wastewaters (Maree *et al*., 1991). The high rate of sulfate removal observed in the present study can be attributed to the suitability of primary sludge (PS) to serve as a carbon source for the sulfate reducing bacteria (SRB). Active biological sulfate reduction within the RSBR may also result in improved physico-chemical conditions, such as pH, for the hydrolytic enzymes. Such changes may play a role in the phenomenon of enhanced hydrolysis in the RSBR. The exact mechanism of this enhanced hydrolysis within the RSBR has, to date, not been fully understood and it was proposed that an examination of the enzymology of the system would provide further clues.

In the present study enhanced hydrolysis was confirmed and a range of studies on enzyme activities, physico-chemical parameters and floc morphology were carried out. This, combined with the information obtained in previous works on the RSBR, has led to the development of a more detailed conceptual model describing enhanced hydrolysis within the RSBR **(Figure 6.1)**. The schematic diagram shows a sequence of events from the introduction of fresh substrate (PS) into the RSBR through to the enhanced hydrolysis highlighting the parameters and processes of importance.

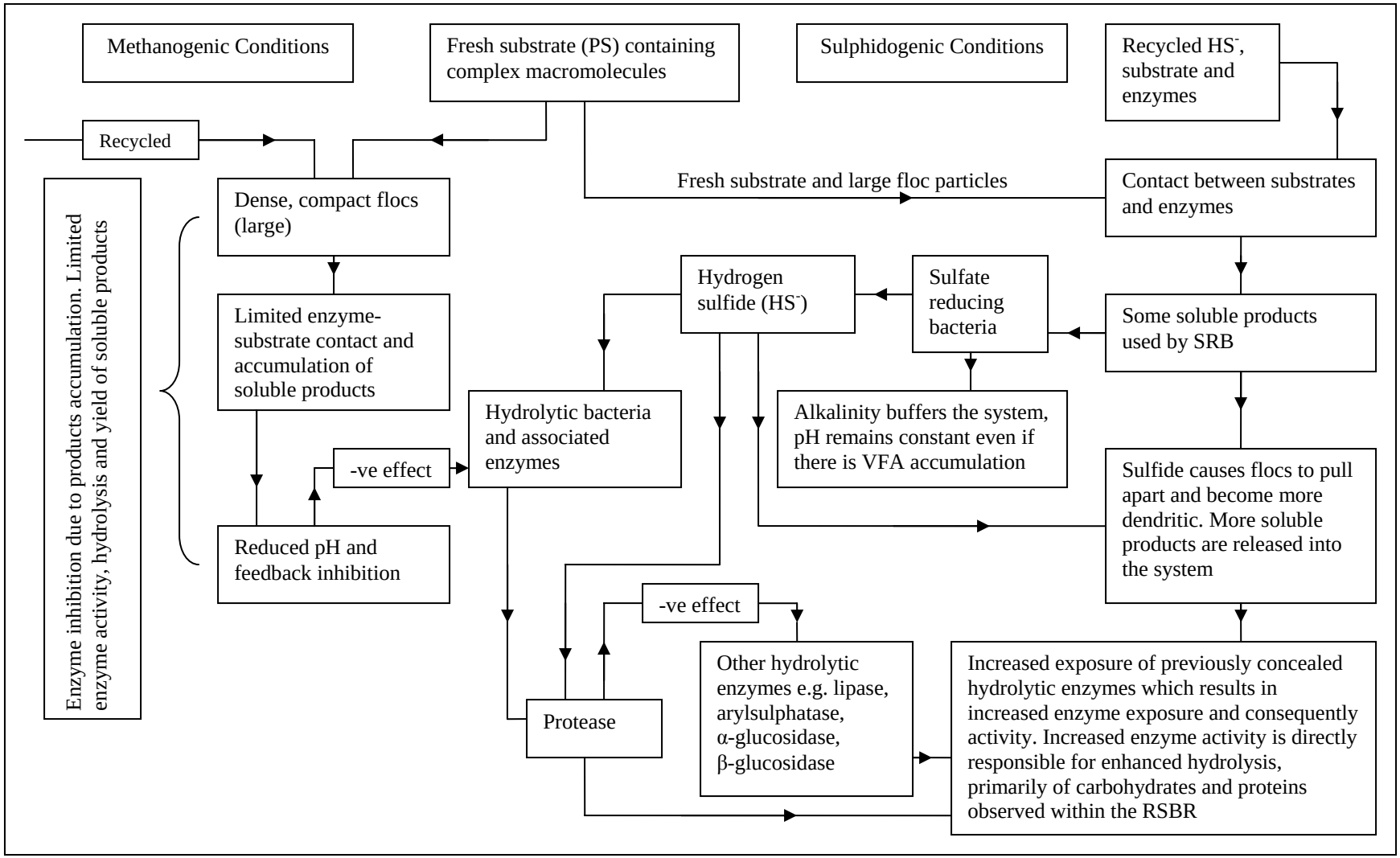

Figure 6.1 Proposed conceptual model for the mechanism of enhanced enzymatic activity and PS hydrolysis under biosulphidogenic conditions within the RSBR

Fresh PS substrates, made up of particulate and soluble COD, are introduced into the RSBR with the feed. The substrate is brought into contact with recycled sludge from the base of the RSBR containing high levels of enzymatic activity and combines to form flocs that settle towards the base of the reactor. It is proposed that the fate of undegraded macromolecules contained in the feed is different under methanogenic and sulphidogenic conditions.

The concentration of volatile fatty acids (VFA) in anaerobic digesters has been monitored as a process performance indicator (Vanrolleghem and Lee, 2003). The methanogenic bacteria preferentially use acetate over other VFA, therefore VFA such as propionate and butyrate are more likely to accumulate in the methanogenic system (Molwantwa, 2002). The methanogens are very sensitive to the accumulation of VFA and the corresponding pH drop which might result in irrevocable acidification of the digester system and a complete digester failure (Anderson and Yang, 1992; Veeken and Hamelers, 1999). The accumulation of VFA and other soluble products can take place on two scales. This can take place in the digester as a whole or perhaps on a micro-scale within the matrix of larger flocs. Consequently, hydrolysis may be inhibited either by a decreased pH or feedback inhibition due to accumulation of soluble products such as VFA. The result will be an accumulation of undegraded organic matter in the form of large stable flocs with the release of very little soluble products.

Based on the findings of the current study and of other authors, enhanced hydrolysis under sulphidogenic conditions is related to a number of factors, such as the activity of hydrolytic enzymes and the pH of the reactor system. The present study showed that optimum pH for the enzyme activities was in the neutral range. One of the advantages of the sulphidogenic system over the methanogenic system is that the pH is maintained in this range. The pH stability is maintained by two factors which are the utilisation of a wider range of VFAs by the SRB and the production of alkalinity. The SRB are more versatile than methanogens and can degrade propionate and butyrate directly which require syntrophic consortia in methanogenic environments (Hulfshoff Pol *et al.*, 2001). SRB have been reported to consume mainly acetate in marine and fresh water sediments when sufficient sulfate is present (Banat *et al.*, 1981; Smith and Klug, 1981; Isa *et al.*,

1986). In the RSBR system, optimal degradation of complex organic matter may have been facilitated by the maintenance of optimal pH conditions and alkalinity for both the microorganisms and the hydrolytic enzymes. According to Ahring *et al.* (1995), pH as a process indicator is strongly dependent on the buffering capacity or alkalinity of the system, with the main buffering species in anaerobic digesters being VFAs and the bicarbonate ions. The overall mean pH in the biosulphidogenic RSBR system was found to be 7.32. These results were in accordance with previously published works, where it was reported that under anaerobic conditions the rate and degree of solubilisation of complex particulate matter is highest at a pH range of 6.5-8.0 (Eastman and Ferguson, 1981; Perot *et al*., 1988; Elefsiniotis and Oldham, 1994; Penaud *et al*., 1997; 2000), which coincidentally reflected the optimum pH at which most key hydrolytic enzymes operated in the RSBR. A high alkalinity of 1600 as mg $CaCO_3$/L was reported in the RSBR system which was presumed to maintain the pH within neutral range by resisting any sudden lowering of the pH. During the reduction of sulfate to sulfide by SRB, alkalinity is increased by two equivalent moles per mole of sulfate reduced (van Langerak *et al*., 1997; Kim *et al*., 2003). The high alkalinity observed in the RSBR provided an indication that the system was safeguarded against erratic pH fluctuations.

Alkalinity and sulfide ions are involved in lignin solubilisation, a process which will result in the exposure of the underlying cellulose to enzymatic attack. PS is thought to contain a significant amount of lignocellulose (Heukelekian and Balmat, 1959; Hunter and Heukelekian, 1965; Elefsiniotis and Oldham, 1994) and enhanced solubilisation of these organic fractions will result in improved mineralization of the PS and the overall hydrolysis process.

Results from the flask studies and the RSBR indicated that there was some interaction between the hydrolytic enzymes and the sulfide concentrations. In the present study, the specific enzyme activities increased with the depth of the RSBR, and corresponded to increase in the sulfide concentration. Flask studies carried out on the key hydrolytic enzymes also indicated a relative increase in enzyme activity with increasing sulfide concentration. Proteases showed the greatest increase in activity with sulfide, with an increase in activity of about 2-fold at sulfide concentration of 600 mg/l. Kinetic data

indicated that the sulfide or other sulfide species did not interact directly with any of the key enzymes, therefore another indirect mechanism must have been involved in the enhanced enzyme activity observed within the RSBR and in the flask in the presence of sulfide. These kinetic data contradict the model proposed by Pletschke *et al*. (2002), who postulated that the enhanced enzymatic activities under biosulphidogenic conditions was a result of a direct interaction of the enzymes and sulfide. If this was the case, a significant decrease in the apparent Michaelis constant (K_m) and a significant increase in the apparent maximal velocity (V_{max}) would have been expected with increasing sulfide concentration, which was not observed in the present study. The K_m and V_{max} for all the key hydrolytic enzymes tested showed no significant difference with increasing sulfide concentration, except for proteases where a statistically significant increase in K_m was reported. Nevertheless, the biochemical significance of K_m, in this particular case could not be stated with absolute certainty in the absence of other data concerning the relative magnitudes of the various rate constants and due to the fact that the enzymes used in this study were not purified prior to the kinetic studies. The K_m nevertheless, represents a valuable constant that relates the velocity of an enzyme-catalysed reaction to the substrate concentration (Segel, 1976). For the K_m value to be of any significant impact it must be at least 5-fold (Pletschke, personal communication), and this was not the case for the proteases in this study. As a result, no evidence was obtained in the present study to support the concept of 'direct' stimulation of the hydrolytic enzymes by sulfide in the enhanced hydrolysis of PS in the RSBR. There was, however, strong evidence to support the notion of indirect enhancement of the enzymatic activities in the presence of sulfide.

Sulfide was shown to cause a change in the size and shape of the flocs. The flocs within the RSBR became more dendritic with increasing sulfide concentration and depth in the reactor. Although flocs in the upper zone of the RSBR were smaller than the flocs in the base of the RSBR, the flocs in the base of the RSBR were still smaller than the flocs in non-sulphidogenic systems (Santegoeds *et al*., 1999, Whittington-Jones, 2000). Nielsen and Keiding (1998) observed disintegration and increased shear sensitivity of the activated sludge flocs after the addition of sulfide under anaerobic conditions which led to the release of particles and major floc components such as EPS to the bulk water. The

disruption of flocs observed by Nielsen and Keiding (1998) in the presence of sulfide is expected to be true for the anaerobic sludge flocs in the sulphidogenic RSBR. This was further substantiated by smaller mean floc sizes (**Table 5.1**) observed in the anaerobic sulphidogenic RSBR, which indicated that disintegration of floc particles was enhanced and re-flocculation may have been impeded in the presence of sulfide.

The effect of reducing floc size or changing to a more dendritic form are thought to have similar effects on the activity of the hydrolytic enzymes and this effect is two fold. The susceptibility of the macromolecules previously concealed from enzymatic degradation within the flocs increases with an increase in the floc surface area as they can be accessed by the hydrolytic bacteria and their associated enzymes. The increase in the enzyme-substrate contact will therefore result in an increase in enzyme activity, given that enzymatic activities are in direct proportion to the amount and action of the hydrolytic enzymes (Goel *et al.*, 1997). The exposure results in increased and enhanced hydrolysis of the macromolecules by the hydrolytic enzymes and consequently the hydrolysis step in the solubilisation of sludge. In the RSBR, an increase in enzyme activity would result in enhanced hydrolysis of PS. Furthermore, the release of soluble products was facilitated and therefore reduced the probability of decreased pH within the floc, that is, on a micro-scale. De-flocculation, that is, the fracturing of sludge flocs, has been reported to promote the release of soluble products from the floc matrix (Jin *et al.*, 2003). This also results in increased enzyme concentration and contact between enzymes and substrates. These observations corroborate the findings of Whittington-Jones (2000) who demonstrated that sulfide had an impact on the cleavage of sludge proteins. As these macromolecules form part of the floc matrix, this may also contribute to the change of floc structure in the RSBR.

The initial expectation was that the key hydrolytic enzymes would be active in the RSBR and significant enzyme activity would be observed. However, very low specific activity of lipases, compared to other enzymes, was observed at all depths in the RSBR. These results could be explained by the fact that experiments carried out using protease inhibitor (PSMF), showed a 3-fold increase in lipase activity, suggesting that most of the lipases were being digested by the proteases. The low activity of these enzymes was not

expected considering they are actively involved in the degradation of complex organic substances to simple organic monomers utilisable by the microbial consortia in the RSBR.

Although floc fracture is thought to be essential for enhanced hydrolysis in the RSBR, smaller flocs are more susceptible to washout, which is accompanied by the washout of hydrolytic bacteria and enzymes closely associated with the floc matrix (Boczar *et al*., 1992; Frølund *et al*., 1995; Confer and Logan, 1998; Goel *et al*., 1998a). Floc particles are forced to re-flocculate at the base of the RSBR system, due to settling and accumulation, and hydrolytic enzymes are recycled and re-enter the reactor at the inlet. During recirculation, re-flocculated associated hydrolytic enzymes, bacteria and undigested material come into contact with fresh substrates and sulfate in the upper zone of the reactor. Recirculation causes large floc particles to be fractured, releasing soluble unused hydrolysis products and sulfide into the system, which aids in the deflocculation process and enhanced hydrolysis within the RSBR.

Enzyme activities within the biosulphidogenic anaerobic RSBR are the integrated result of the composition of the particulate organic substrates, the loading rate, the nature of the microbial population and the environmental and micro-environmental conditions such as pH, alkalinity, sulfate, sulfide and the floc characteristics. The results obtained in this investigation clearly established the interrelationship between the assortment of physico-chemical parameters, the floc characteristics and the hydrolytic enzyme activities and makes an important contribution to the characterisation of the RSBR. This biochemical characterisation thus further contributes to the understanding of the process of enhanced hydrolysis of complex organic matter by extracellular hydrolytic enzymes in anaerobic biosulphidogenic conditions and in the natural systems, such as marine and freshwater sediments and in the RSBR in particular. A deeper understanding of the mechanism underpinning enhanced hydrolysis within the biosulphidogenic RSBR has been provided and will enable further optimisation of the sludge digestion process. Since complex biopolymers such as proteins, carbohydrates and lipids form a significant component of municipal sewage sludge (Metcalf and Eddy, 1991; Nielsen *et al*., 1992; Raunkjær *et al*., 1994) the activities of α-glucosidases, β-glucosidases, proteases and lipases are thought to

be important in the hydrolysis process of these materials in the RSBR, but the impact of sulfide on the kinetics of these enzymes still requires further study.

In summary, the present study has provided an in depth view of the enzymology of the RSBR with respect to depth of the reactor, and the associated effect of levels and concentrations of sulfide, sulfate, pH and alkalinity of the overall reactor system. An understanding of the spatial and temporal variation of enzymes within the RSBR system, and the physico-chemical parameters affecting the enzymatic activities obtained in this study will help in the optimisation of the degradation of PS in the RSBR and anaerobic wastewater treatment plants in general. It has been demonstrated in this study that the enhanced mineralization of complex particulate organic matter, such as PS in the biosulphidogenic RSBR system by hydrolytic enzymes, can be enhanced indirectly by the sulfide in the system as well as the nature of the surrounding micro-environment of the system. Furthermore, it is proposed that the design and operational mode of the RSBR anaerobic biosulphidogenic system facilitated the build up of the hydrolytic enzymes and improved the contact between the substrates, the biomass and the enzymes, which is a precondition to the hydrolysis.

To date, no enzymatic studies have been carried out on a pilot-scale RSBR system and it would be of interest to investigate if the behaviour of the laboratory-scale RSBR applies on a larger scale. Using the understanding gained in the present study, it may be possible to further optimise the RSBR system for the improved conversion of complex substrates to soluble products thus contributing to reducing the global problem of sludge disposal. Increasing the sulphide concentration would lead to increased activity of certain key hydrolytic enzymes such as glucosidases, lipases and proteases. This will then result in increased hydrolysis of the target macromolecules within the biosulphidogenic RSBR and ultimately improved solubilisation of the PS.

Appendices

Appendix A: Determination of chemical oxygen demand (COD)

The COD determination measures the equivalent quantity of oxygen utilised during oxidation of organic and inorganic matter in wastewater under the conditions of the COD test described in Standard Methods (APHA *et al*., 1998), with units of milligrams per litre (mg/l). The sludge sample is oxidised by boiling a mixture of hot solution of dichromate and sulfuric acid. A sample is refluxed in a strong acid with a known excess of potassium dichromate (K_2CrO_7). The remaining K_2CrO_7 is titrated with ferrous ammonium sulfate after digestion, and the amount of K_2CrO_7 consumed is determined. The amount of oxidisable organic matter is calculated in terms of oxygen equivalent (APHA *et al*., 1998). The extent to which organic material is consumed is related to the COD.

Into a COD reaction cell was added 0.3 ml COD solution A # 1.14538.0065 and 2.3 ml COD solution B # 1.14539.0495 (Merck, KGaA, Germany) per determination. RSBR samples (3.0 ml) were added into the cells, mixed and incubated in a thermoreactor (Merck Spectroquant® TR 420) at 148^{o}C for 2 hours. The samples were then cooled at room temperature for 5 min. The blank was prepared by adding 3.0 ml of water instead of the sludge sample. The COD concentration (mg/l) was measured using the Merck Spectroquant® Nova 60.

Appendix B: Determination of standard curves

B1. Standard curve for protein determination

Protein concentration was determined using the Sigma Bradford (1976) reagent for protein determination (B6916). The method is rapid, convenient and produces an equivalent absorbance change for many proteins. When mixed with protein solution a protein-dye complex is formed which causes a shift in the dye absorption maximum from 465 to 595 nm. The dye is called Brilliant blue G-250, and the amount of absorption produced is proportional to the protein concentration. The standard curve based on Bovine Serum Albumin (BSA) gives a linear response (**Figure B.1**)

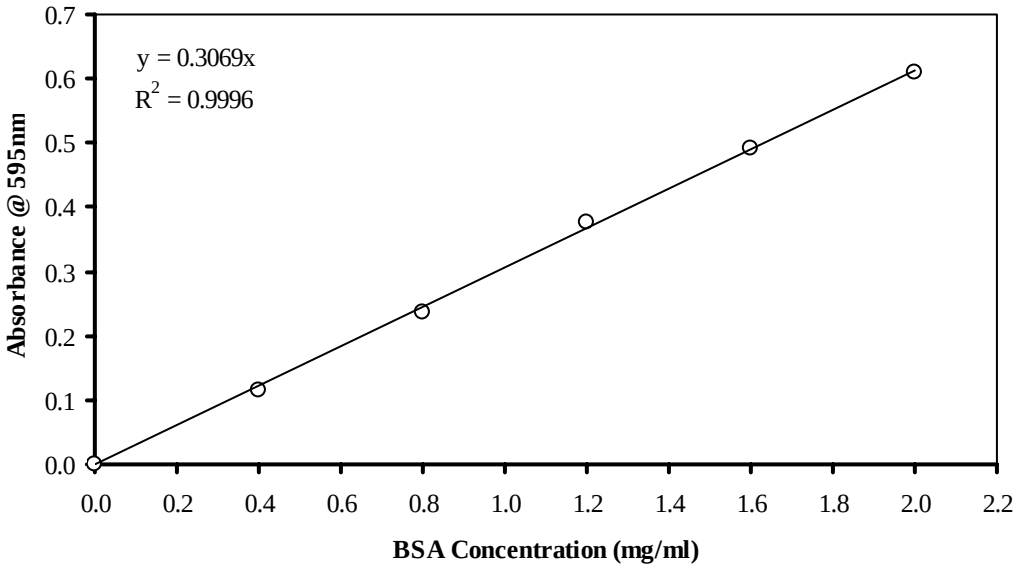

Figure B.1 Protein standard curve using BSA as a protein standard solution

B2. *p*-Nitrophenol standard curve

Principle: Sodium hydroxide (NaOH), added to end the reaction after 20 min, reacts with the *p*-nitrophenol to remove the phenolic proton leading to the production of yellow *p*-nitrophenolate ions whose absorbance is determined at 410 nm (Clark and Switzer, 1977). A standard curve (**Figure B.2**) was constructed by preparing 6 tubes containing 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 ml of 60 µM *p*-nitrophenol solution and each tube was brought to a total volume of 6.0 ml by addition of 0.02 M NaOH (0.08 g NaOH pellets dissolved in 100ml distilled water). The 60 µM *p*-nitrophenol was prepared by dissolving 83.5 mg *p*-nitrophenol in 100 ml of 0.02 M NaOH to yield a 6 mM solution which was further diluted 1/100 with distilled water to yield a 60 µM solution. The extinction coefficient (ε) for *p*-nitrophenol in 0.02 M NaOH is 18.8 x 10^3 at 410 nm (Colowicks and Kaplan, 1987) and was used to calculate the µmol of product formed from the absorbance reading according to the Beer-Lambert's Law (equation 18):

$$A = \varepsilon.c.l \qquad (18)$$

where: A = the absorbance; ε = the extinction coefficient; c = the concentration in moles per litre; l = length of light path (in cm).

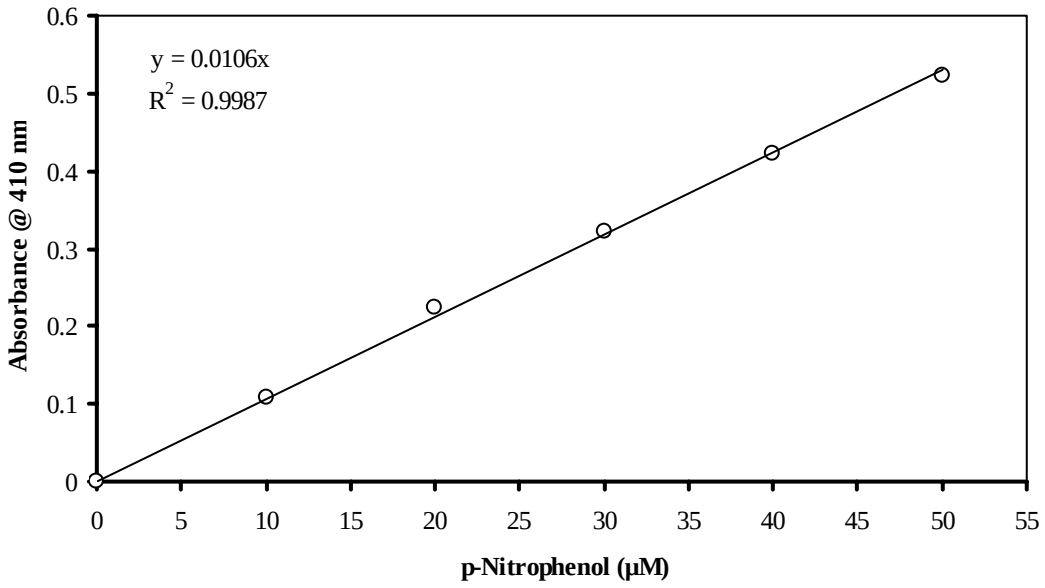

Figure B.2 *p*-Nitrophenol standard curve for the determination of enzymatic activities

B3. Preparation of glycerol standard curve for lipase determination

Glycerol standard curve (**Figure B.3**) was prepared by measuring 0.1 – 0.5 µmol/ml from 250 µM glycerol stock solution. The sample volumes were made up to 2.0 ml with distilled water. The reaction was stopped by the addition of sulfuric acid (5.0 M, 50 µl) and sodium periodate (0.1 M, 250 µl). Sodium arsenate (10%, 250 µl) was added and the whole vortexed and incubated at room temperature for 5 min (Korn, 1954). To a series of tubes containing 2.5 ml chromotropic acid was added 250 µl aliquot and incubated at 100 ^{o}C for 60 min. Glycerol levels were then determined by reading the absorbance at 570 nm. Lipase activity was then determined as per amount of glycerol released on incubation of the 1% triacetin and activity of lipase calculated as follows:

Actual glycerol = glycerol reaction – glycerol enzyme control (units of µmol/ml)

Lipase activity = actual glycerol x 2.805

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{mg/ml protein}} (\text{units of } \mu\text{mol/min/mgprotein}) \qquad (19)$$

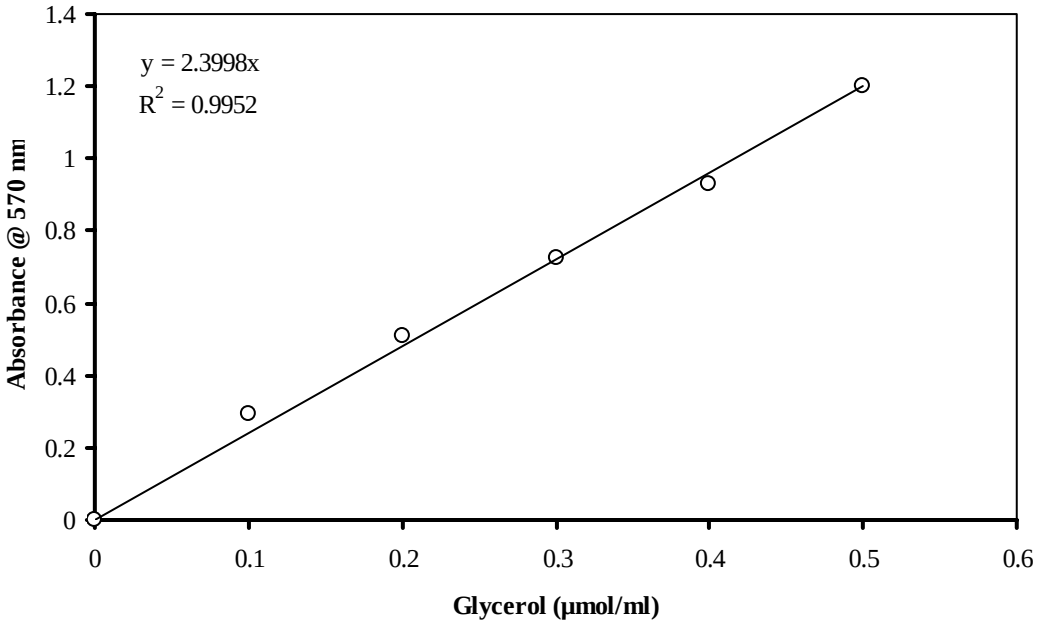

Figure B.3 Glycerol standard curve for determination of lipase activity

B4. Preparation of glucose standard curve for total carbohydrate determination

The phenol sulfuric acid carbohydrate assay as described by Dubois *et al*. (1956) was used. This method is based on the colour reaction between carbohydrate and phenol reagent in concentrated sulfuric acid (H_2SO_4). Polysaccharides are hydrolysed during heating by the acid to monosaccharide with the formation of an orange colour measured spectrophotometrically at absorbance of 490 nm. 100 mg glucose was dissolved into 100 ml (w/v) benzoic acid and standards prepared in the range of 0 - 15 µg/ml to give the standard curve shown in **Figure B.4**.

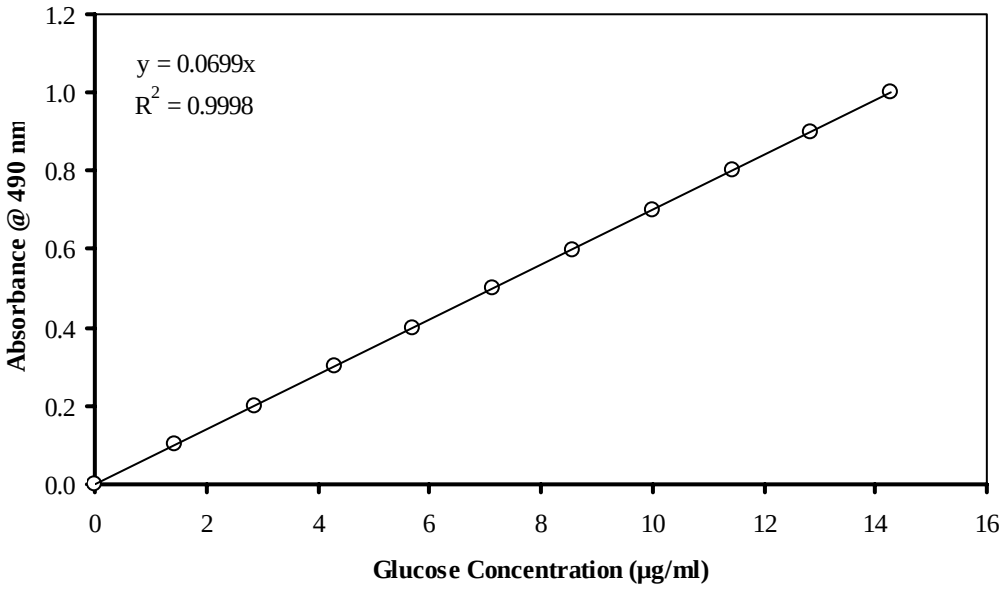

Figure B.4 Glucose standard curve for total carbohydrate determination

B5. Sulfate standard curve

The sulfate calibration curve was prepared by dissolving potassium sulfate (K_2SO_4) in deionised water to known concentrations in the range of 0 to 5 mM to give a standard sulfate solution. The calibration curve obtained (**Figure B.5**) using the solution in this range was fitted with a third polynomial curve over the range 0 to 5 mM sulfate. The polynomial equation obtained was solved with MATLAB® version 6.0 (The MathWorks, Inc. USA). The fitted curve described the equation:

$$y = 7E\text{-}09x^3 - 6E\text{-}06x^2 + 0.0025x \quad (20)$$

where: y is absorbance at 420 nm of the sample and x the corresponding sulfate concentration in mg/l.

One millilitre (1.0 ml) of sample was added to 1.0 ml conditioning reagent (150 g NaCl, 100 ml of 126 g glycerol, 60 ml concentrated HCl and 200 ml 95% ethanol made up to 1:1 with deionised water (APHA *et al.*, 1998)) in a test tube and mixed thoroughly. Crushed barium chloride, 60 mg was added and the solution vortexed for 60 sec at constant speed and absorbance determined at 420 nm.

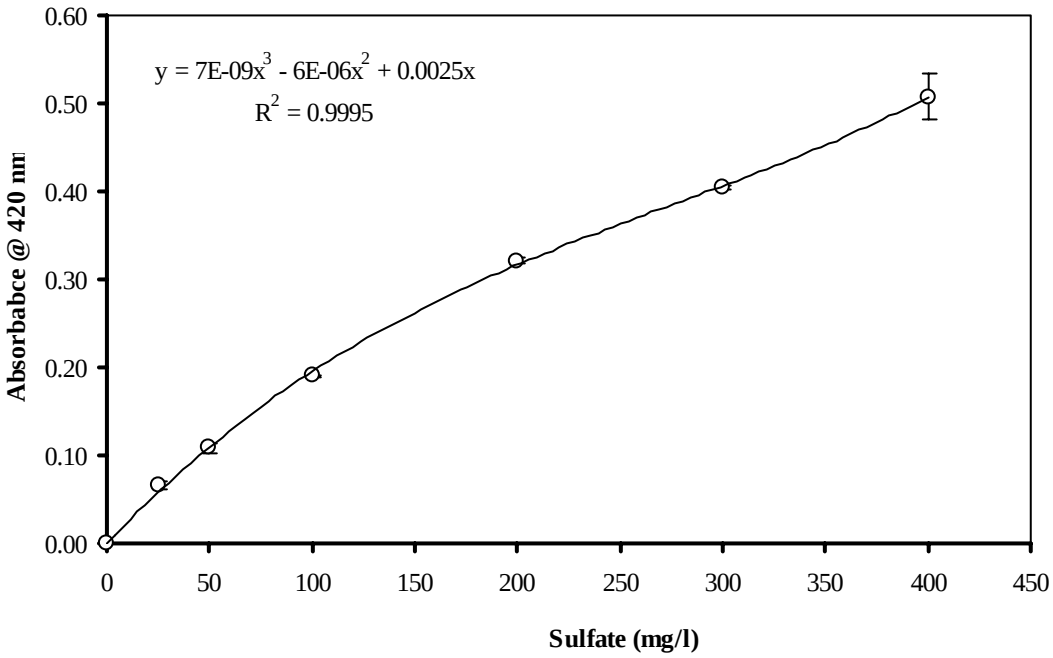

Figure B.5 Sulfate standard curve fitted with a third-order polynomial

C1. Chemical oxygen demand (COD) standard deviation

Table C1 Minimum and maximum standard deviation (± SD) for COD over the experimental period

	Standard Deviation (±SD)					
	Minimum			Maximum		
	Depth 1	**Depth 2**	**Depth 3**	**Depth 1**	**Depth 2**	**Depth 3**
COD_{Total} (mg/l)	28.73	503.32	550.57	223.02	7619.93	6703.98
$COD_{Soluble}$ (mg/l)	20.88	100.62	70.31	134.67	502.58	662.77
$COD_{Particulate}$ (mg/l)	23.35	698.72	536.91	259.93	7361.06	7212.30

The values are averages of triplicates with standard deviation (± SD) with n = 135

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