INVESTIGATION INTO THE ENZYMOLOGY OF ACCELERATED PRIMARY SEWAGE SLUDGE SOLUBILISATION AND DIGESTION IN SULPHATE REDUCING SYSTEMS

CG Whiteley · BI Pletschke · JE Burgess · AS Tshivhunge · N Ngesi · K Whittington-Jones · G Enongene · F van Jaarsveld · P Heron · K Rashamuse · PD Rose

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Report to the
WATER RESEARCH COMMISSION

by

CG Whiteley, BI Pletschke, JE Burgess, AS Tshivhunge, N Ngesi,
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TABLE OF CONTENTS

Executive Summary

1. BACKGROUND

The treatment of municipal wastewater and sewage creates the single most complex and costly problem of the whole wastewater treatment process - that of the disposal of the by-products of the process. During municipal wastewater treatment, sludges are generated as by-products of the physical, chemical and biological processes which should be disposed of without creating health problems or further hindrance. Amongst the many treatment technologies available, anaerobic treatment process has proved to be unique and the most beneficial stabilisation technique as it optimizes cost effectiveness, it is environmentally sound, minimises the amount of final sludge disposal and has an ability to produce a net energy gain in the form of methane gas (De Baere, 2000). Anaerobic digestion of solid waste is a biochemical multi-step process that mineralises complex organic matter (such as carbohydrates, proteins and lipids) to methane and carbon dioxide through a series of reactions mediated by several groups of microorganisms (Nopharatana *et al*., 2003). The anaerobic treatment technology currently available, however, is only capable of partially treating waste in a conventional wastewater treatment system with high levels of degradation requiring longer retention times and/or further treatment methods, which add to the cost of the treatment (Parker *et al*., 1998).

Wastewater treatment bioreactors are complex ecosystems that contain a wide variety of organic substances and a mixed culture of heterogeneous microbial populations which effect sequential substrate removal when complex substrates are degraded. In such mixed cultures, sulphate-reducing bacteria (SRB) will compete in the presence of sulphate with methanogenic bacteria (MB) and acetogenic bacteria (AD) for the substrates available, the importance of which increases with a decrease in COD/SO_4 ratio (Oude Elferink *et al*., 1994; Colleran *et al*., 1995; Omil *et al*., 1996). Biological anaerobic wastewater treatment systems in which complex organic matter is completely degraded by SRB (i.e., sulphidogenic bioreactor systems) are a promising alternative for the methanogenic wastewater treatment systems (Lens *et al*., 1998) and the complex physico-chemical sulphate removal methods (Maree *et al*., 1991).

Sulphate-rich wastewater is produced by many industries such as the sulphuric acid in food processes, thiosulphate in the photographic industry, sulphite in tanneries, the sea-food processing industry, the leaching of sulphur rich soils in land fills and mines and by power-plant flue gases from the combustion of sulphur containing fuel. Biological anaerobic reduction offers a highly successful and efficient process for the removal of sulphate from these effluents as sulphide (Weijima *et al*., 2000). The anaerobic stage requires the presence of an organic substrate that serves as an electron donor or carbon source for this sulphate reduction. Previous work from the Environmental Biotechnology Group of Rhodes University has developed the use of raw municipal sewage sludge as a relatively cheap and readily available carbon source for anaerobic sulphate reduction (Rose *et al*., 1998). The SRB have the ability to utilise inorganic sulphate as a terminal electron acceptor during an ATP-requiring reaction, but require the supply of a low molecular weight carbon source as an electron donor for sulphate reduction. The SRB present in sulphate-reducing systems have, perhaps indirectly, been shown to stimulate the rate of primary sewage hydrolysis and solubilisation. Further to an investigation of the microbial

ecology of a tannery waste ponding system it was noted that a complete biological sulphur cycle prevailed in the water column after the sulphate enriched environment received high loads of complex organic particulate matter. (Rose *et al.*, 1998; Dunn 1998)

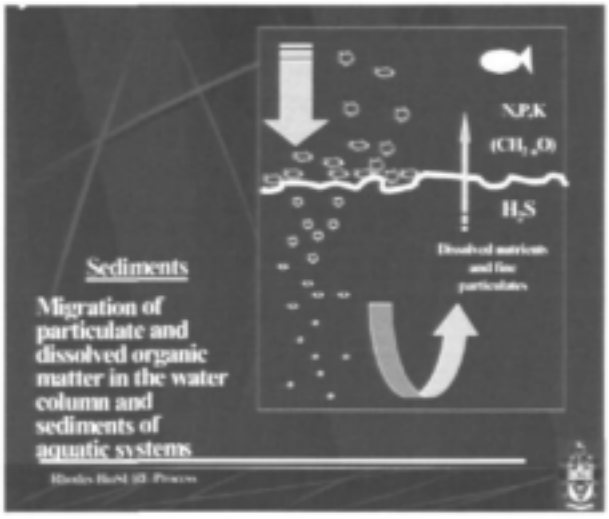

Figure 1. Migration of particulate matter.

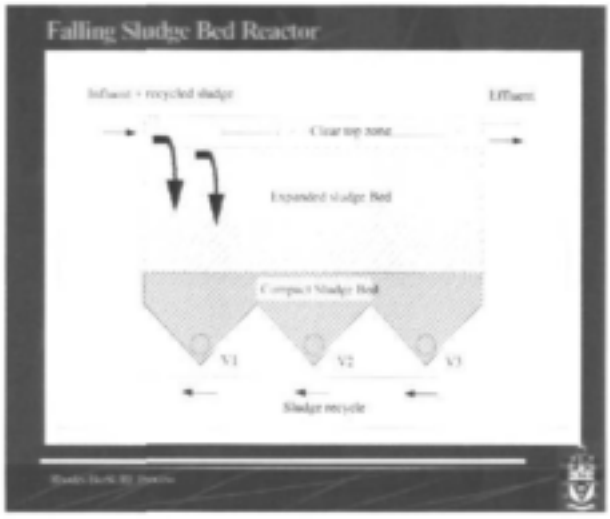

Figure 2. Reciprocating Sludge Bed Reactor

This was associated with an effective degradation of particulates and a subsequent settling towards the lower level anaerobic sulphidogenic zone within the ponds. Subsequent upwelling of dissolved and residual suspended organic matter supported an aerobic upper zone. Residual undegraded particulates settled once again to within the sulphidogenic zone and underwent a further cycle of degradation, eventually, over a period of time, achieving a surprisingly high level of solids removal in a system notoriously prone to sludge build-up (Figure 1). The mechanisms of this initial observation of enhanced degradation of organic particulate matter in the sulphidogenic environment has been investigated in a laboratory-scale Reciprocating Sludge Bed Reactor (RSBR). (Whittington-Jones, 2000) designed to mimic the proposed mechanism of degradation within the ponds (Figure 2).

While process development studies have made significant progress, the underlying enzymatic mechanisms for the enhanced solubilisation in the sulphate reducing system remain obscure. The rate at which hydrolysis proceeds is best described by first order kinetics and may be strongly influenced by environmental and operational parameters such as pH, temperature, biomass, particle/floc size, type and concentration of particulate substrate and production concentration. Any increase in the enzyme concentration in an anaerobic digester, or a reduction in mass transfer limitation or particle size will result in an increase in the rate of hydrolysis of complex particulate organics. Solubilisation of primary sewage sludge is enhanced under sulphate reducing conditions, because of a decrease in both particle and floc size, as a result of enhanced hydrolysis of macromolecular carbohydrates, proteins and lipids. Furthermore hydrolysis of these particulate organics may also be enhanced in the presence of sulphide since the latter is a strong reducing agent and is capable of reducing disulphide linkages that are essential for maintaining the three dimensional conformation structure.

With a necessity of improvement in biological remediation techniques, enzyme technology has been receiving increased attention. According to Aitken (1993), enzymes were first proposed for the treatment of waste in the 1930's, but it was not until the 1970's that enzymes were used to target specific pollutants in waste. The degradation of either soluble or insoluble substrates in municipal solid waste is thought to be mediated by bacterial groups with the degradation of insoluble substrate reported to involve an additional enzymatic reaction to catalyse the hydrolysis step, which converts the solid to soluble substrates (Nopharatana *et al.*, 2003).

Previous researchers have used enzymes in activated sludge systems as indicators of specific populations (Hankin and Sands, 1974), measure of active biomass (Teuber and Brodish, 1977; Richards *et al.*, 1984) and as indicators of processes such as chemical oxygen demand and phosphorous removal (Richards *et al.*, 1984; Lotter and van der Merwe, 1987). Hydrolytic enzymes such as lipases, proteases, glucosidases and sulphatases are ubiquitous in the environment and are able to act on a wide variety of substrates. These enzymes which are easily assayed using model substrates can be used to measure microbial abundance and activity in wastewater and sludge samples (Chróst, 1989; Boczar *et al.*, 1992; Nybroe *et al.*, 1992).

Sonication of the sludge released most of the enzyme activity (protease, phosphatase, sulphatase, glucosidase, lipase and ATP sulphurylase) into the supernatant indicating that these enzymes were, therefore, found either associated with or immobilised within the particulate matter. Since the biopolymers (proteins, carbohydrates and lipids) have been reported to be the major particulate organic fractions in municipal sewage sludge (Nielsen *et al.*, 1992; Raunkjær *et al.*, 1994; Metcalf and Eddy, 1991) the activities of α-glucosidase, β-glucosidase, protease and lipase should be very important in the hydrolysis process. Disruption of this network increases the susceptibility of the macromolecules, entrapped within the floc and hence protected from enzymatic degradation, to be attacked by the hydrolytic bacteria and their associated enzymes. This ultimately leads to enhanced solubilisation of the primary sewage sludge. High levels of α- and β-glucosidase as compared to other enzymes were observed in all three depths of the RSBR. These high activities observed may be explained by enzyme activation and induction resulting from the presence of cellobiose in sewage sludge which according to Chróst (1991) strongly induces the synthesis of glucosidases. This increase is likely to be associated with increase in microbial populations, sulphide concentration and concurrent increased production of hydrolytic extracellular enzymes that target specific polysaccharide cleavage sites.

The enzymes activities are a measure of the activity of the biomass in the digester and such activities reported in this study are the integrated result of the composition of the particulate organic matter which vary from 40-60% (Henze, 1992), the loading rate, the nature of the microbial population and the environmental conditions such as temperature, pH, alkalinity, sulphate and sulphide concentration, chemical oxygen demand and degree of anaerobiosis. Hattingh and Siebert (1967) reported that the intermediate enzyme activities revealed valuable information on the changes that took place in the biological pattern of the reactor. This study of the extracellular hydrolytic enzymes under biosulphidogenic conditions has thus proven to be a useful tool for understanding the anaerobic biological treatment systems.

Under anaerobic conditions, SRB hydrolyse simple organic compounds, hydrogen sulphide and bicarbonate ions are generated. The former reacts with many contaminant metals to remove them

from solution as insoluble metal sulphides while the latter combine with protons to form carbon dioxide and water thereby removing the acidity from the solutions as carbon dioxide gas. The hydrogen sulphide and the bicarbonate ions formed during sulphate reduction equilibrate into a mixture of H_2S, S^{2-}, CO_2, HCO_3^- and CO_3^- which will then buffer the solution to a particular value in the range of 6-7.5 if sufficient sulphate reduction occurs. 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 volatile fatty acids and the bicarbonates ions. In our reactor a decline in pH was not observed which indicated that there was no accumulation of volatiles fatty acids (VFA). VFA accumulation has been reported to lead to process failure due to the pH-drop they induce (Anderson and Yang, 1992) and their concentrations in anaerobic digesters have been monitored for a long time as process performance indicators (Vanrolleghem and Lee, 2003).

2. PHYSICO-CHEMICAL PARAMETERS

To ensure optimal degradation of complex organic matter in anaerobic digestion systems two conditions that must be satisfied are to provide optimal pH conditions for both the slowest growing group of microorganisms and enzymatic activities and low redox potential which is normally maintained by the presence of sulphide ions (Barnes *et al.*, 1991). The pH stayed constant throughout the study period with a minimum of pH 7.15 recorded by day 48 (depth 1) and a maximum of pH 7.50 by day 24 (depth 3) with an overall mean pH in the entire RSBR system to be 7.32. These results were in accordance with previously published work, 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 (Elefsiniotis and Oldham, 1994; Penaud *et al.*, 1997), which coincidentally reflects the optimum pH at which most important hydrolytic enzymes operate (Penaud *et al.*, 1997; 2000).

The influent sulphate (1000 mg/l, 100%) removal efficiency increased from 52.03% for depth 1, to 97.12 and 96.35 % for depth 2 and depth 3 respectively, resulting in sulphide generation of 75.66, 253.50 and 247.39 mg/l respectively for depth 1, depth 2 and depth 3. The profiles obtained in these studies show a progressive decline of sulphate concentration and a mirror image increase of sulphide concentration in the RSBR as expected from the bacterial consumption of sulphate and concomitant hydrogen sulphide production during anaerobic sulphate reduction. Bacterial sulphate reduction is a complex biochemical process which entails the formation of sulphate-enzyme complexes, as well as sulphite intermediate products between external sulphate and sulphide. In this process, the bacterial cells assimilate the dissolved sulphate which then reacts with adenosine triphosphate (ATP) to form adenosine-5'-phosphosulphate (APS) which thereafter is reduced to sulphite (Habicht and Canfield, 1997) and finally this pathway may involve the direct reduction of sulphite to sulphide (Aharon and Fu, 2003). During the reduction of sulphate to hydrogen sulphide by the SRB alkalinity is increased by two equivalent moles per mole of sulphate reduced (van Langerak *et al.*, 1997; Kim *et al.*, 2003).

Chemical Oxygen Demand (COD) fluctuated in all depths throughout the experimental period emphasizing that the reactor continuously adjusts to COD loading. The total COD had a maximum of 677.33 mgl^{-1} by day 40 to a low of 488.67 mgl^{-1} by day 28 for depth 1. The

maximum COD concentrations for depth 2 was at day 4 with values at 19233.33 mgl^{-1} and a minimum of 8666.67 mgl^{-1} in day 32, thus showing COD removal. With depth 3 the minimum was at day 12 with total COD concentration of 10433 mgl^{-1} and a maximum of 18433 mgl^{-1} in day 24. The mean total COD concentration for depth 1, depth 2 and depth 3 was 603.64 mgl^{-1}, 11735 mgl^{-1}, and 14988.89 mgl^{-1} respectively. This showed that the COD increased from depth 1 to depth 3. The $COD_{soluble}$ concentrations in all three depths were lower in comparison to COD_{total} with a mean of 230.33 mgl^{-1}, 1681.6 mgl^{-1} and 1552.73 mgl^{-1} for depth 1, depth 2 and depth 3 respectively. $COD_{particulate}$ was obtained as the difference between the COD_{total} and $COD_{soluble}$ with means being for depth 1, 373.31 mgl^{-1}; depth 2, 10054 mgl^{-1} and depth 3, 13436 mgl^{-1}.

The alkalinity (measured as mgl^{-1} $CaCO_3$) increased from 72.8 mgl^{-1} in the feed to 353.57 mgl^{-1}, (depth 1); 1453.77 mgl^{-1}, (depth 2) and 14774 mgl^{-1}, (depth 3) respectively, due to reduction of sulphate.

Coupled with the increased activity of the glucosidases is the relative increase in monosaccharide carbohydrates with increasing depth. At a depth of 16 cm the carbohydrate content was only 1.2 $mg.l^{-1}$ and this increased to 4.2 $mg.l^{-1}$ further down the RSBR. Changes in the levels of protein with depth were not as conclusive inferring proteolytic action. Though very little lipid was detected just below the surface of the RSBR (0.15$mg.l^{-1}$ at 16cm depth) its concentration was evident at 0.5 mg l^{-1} in a more anaerobic environment towards the middle and at the bottom of the reactor. These facts support the general observation that the top fraction of the reactor is always very low in concentration of carbohydrates, proteins and lipids.

3. ENZYMOLOGY

The variation of α-glucosidase and β-glucosidase activities respectively, in depth 1, depth 2 and depth 3 of the RSBR under steady state conditions during the experimental period are represented. Depth 3 showed a general decrease in specific activity of 123.65 to 70.94 μmol/min/mg protein for day 4 and day 60 respectively, whereas for depth 2, maximum specific activity was obtained in day 12 and minimum on day 28 with specific activities of 94.96 and 57.37 μmol/min/mg protein respectively. The α-glucosidase activity increased significantly from depth 1 to depth 3 (ANOVA, $p<0.005$, $df = 44$) while β-glucosidase activity increased progressively at varying rates during the 60-day experimental period for all three depths. A rise in protease activity was observed in depth 3 up to day 20 where the maximal protease specific activity was 3.75 μmol/min/mg protein. Depth 2 also showed a similar trend to depth 3 with the maximal protease specific activity of 3.37 μmol/min/mg protein occurring in day 24 of the study period. Protease specific activities were quite low for depth 1 with values of 1.55 μmol/min/mg protein seen in day 20. A variation in the lipase activity was observed between day 12 and 20 at depth 2 of the RSBR with activity increasing from 0.017 to 0.167 μmol/min/mg protein. The variation was also observed for lipase activity in depth 1 starting from day 20 and this trend was observed in day 36 and day 56. A progressive decrease in this enzymes activity was observed for depth 3 during the study period. Specific activity of sulphatases showed variation in all depths of the RSBR though no consistent patters could be established. Sulphatase specific activity of up to 26.22 μmol/min/mg protein was detected in depth 3 indicating that these enzymes are present in extracellular fractions of sludge samples and therefore could increase diffusion of substrates to the active site of enzymes. $ATPS_{SR}$ activity rapidly increased over the first few days in the bioreactor and reached a peak on day five (0.73 nmoles NADPH/min), followed by a gradual

decline in activity over the remainder of the time course. $ATPS_{SBR}$ activity, on the other hand,was reasonably low in the first two weeks, but then, surprisingly, an increase in activity was observed from day 16 onwards, reaching a peak on day 21 (0.82 nmoles NADPH/min) and remaining this high even after 31 days. The increased activity of $ATPS_{SBR}$ over the first five days correlated with the decrease in reactor sulphate concentration over the first nine days (from 1000 mgl^{-1} on day 1 to 440 mgl^{-1} on day 9).

All the inhibitors inhibited α-glucosidase significantly ($p < 0.005$) while only pCMB and $ZnCl_2$ inhibited β-glucosidase with $ZnCl_2$ being the most at 80% inhibition. EGTA and EDTA produced activation of β-glucosidase of between 37 and 42% probably due to the metal chelating capacity. Substantial reduction of proteolysis was observed with PMSF and pCMB suggesting that most of the degradation was as a result of serine proteases. $ZnCl_2$ and EDTA were not effective inhibitors of proteases indicating too that metalloproteases were not involved in the proteolysis. Lipase activity was positively influenced by all of the inhibitors with the most prominent being PMSF (128%) and pCMB (135.6%).

The results of experiments for the estimation of Km and Vmax for all enzymes studied as determined by *Hanes-Woolf* plot are represented in Table 1. The calculation of Km and Vmax were determined by fitting appropriate rate equations using SigmaPlot.

Table 1. Kinetic parameters, of α- and β-glucosidases, protease and lipase activities in the RSBR

Enzyme	Substrate	Km (μM)	Vmax (μmol /min/ml)
α-Glucosidase	p-Nitrophenyl α-D-glucopyranoside	0.161 ± 0.018	0.849 ± 0.024
β-Glucosidase	p-Nitrophenyl β-D-glucopyranoside	0.193 ± 0.066	0.491 ± 0.076
Protease	Azocasein	0.102 ± 0.013	2.310 ± 0.061
Lipase	Triacetin	0.218 ± 0.031	2.089 ± 0.019

4. FLOCS

The floc sizes were small with the bulk of the flocs diameter in the range of 0 – 59 μm for depth 1 within the first four days in the aerobic digester and up to 79 μm diameter for depth 2 and 3. The floc characteristics altered significantly from depth 1 to depth 3. The looseness factors of the flocs increased, showing that the flocs of depth 3 were the most dendritic and mesh-like while the flocs of depth 1 were more like pinpoint flocs than aggregated flocs. Particles from depth 1 were the smallest and most circular. Floc circularity decreased and volume increased moving down the reactor. Statistical analyses of floc measurement showed that mean floc volume was positively correlated with depth and negatively with circularity. Floc size distribution using feret volume measurements were generated for each sample and did not vary significantly over the course of the trial period. The majority of depth 1 particles were smaller than 20 μm^3, with progressively fewer flocs falling into increasing volume size categories. Most of the particles in depth 2 and depth 3 were in the range of 20 – 39 μm^3, with an asymmetrical distribution curve: depth 3 contained a number of particles over 200 μm^3. The size of the flocs and their tendency

towards open, dendritic structures increased moving down the RSBR from depth 1 to depth 3. These trends may be the results of concomitant increases in sulphide, alkalinity, COD, lipase, protease and α-glucosidase activity as well as the concomittant decrease in sulphate concentration.

5. STATISTICS

The Statistical Model presented shows a very high correlation between alkalinity, sulphide, sulphate, COD_{total} and lipase, protease, α-glucosidase, and β-glucosidase activities. Sulphatases showed generally weak correlations with the dependent variables (physico-chemical parameters) while temperature and pH had no effect on the enzyme activities. With simple linear regression analysis it was easy to note how a single dependent variable (enzyme activity) was affected by the values of one or more independent variables (physico-chemical parameters). From the results obtained with analysis of variance (ANOVA), it was found that the differences between the means for depth 1, depth 2 and depth 3 for the specific enzyme activities and physico-chemical parameters except pH in the RSBR were all statistically significant. A multiple comparison test was used to determine the significant difference between the depth 1 and depth 2, depth 1 and depth 3 and depth 2 and depth 3 groups (Table 2). Correlation and regression analyses were performed between the pairs of combinations of all the data at the three depths in the RSBR with the physico-chemical parameters (specific enzyme activities, alkalinity, sulphate and sulphide concentration and CODs, pH, temperature). Low correlations were obtained with individual depths of the reactor as compared to high correlations that were observed with the entire bioreactor. The analyses of correlation suggested that interaction between alkalinity, sulphide,

Table 2 Probabilities for Newman-Keuls multiple comparison 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)	+++	+++	+++
Sulphate (mg/l)	+++	+++	NS
Sulphide (mg/l)	+++	+++	NS
	Specific enzymatic activities (μmol/min/ml/mg protein)		
	1 and 2	1 and 3	2 and 3
Protease	+++	+++	++
Lipase	+++	+++	+++
α-Glucosidase	+++	+++	++
β-Glucosidase	++	+++	NS
Sulphatase	NS	++	++

sulphate and the CODs with the enzymes (lipase, protease, α- and β-glucosidase) appeared to be more adequate than those of pH, temperature and sulphatase. ($P < 0.05$ at 95% confidence level). Three dimensional surface plots for the response of enzymatic activity of proteases, lipases, α- and β-glucosidases versus sulphide and sulphate concentration and alkalinity were also recorded.

Statistically lipase, α-glucosidase, β-glucosidase and protease showed the best correlations with a 91.50% variance explained for alkalinity with lipase while sulphatases generally showed poor associations with the physicochemical parameters. Sulphate on the other hand showed significant negative correlations with the specific enzyme activities (R = -0.231 to -0.905). In contrast, the pH and temperature showed insignificant effect on the enzyme activities. To explore the response of the enzymes further with respect to depth of the RSBR, a Newman-Keuls multiple range test was performed for each enzyme (Table 2). The models indicated that up to 91% of the variability in the response could be explained by the model (95% confidence interval) and can be used to approximate the response of the hydrolytic enzyme activities with the physicochemical parameters. According to test statistic, P-value for the overall correlation is significant at 5% level and the lack of fit is insignificant, indicating that the model is very adequate in approximating the response of the hydrolytic enzymes with the physico-chemical parameters. This is further supported by the R^2 values which are very satisfactory.
The fact that sulphide and enzymes showed satisfactory correlations with corresponding high R^2 values is further supported by the fact that sulphide activates enzymes in sulphidogenic bioreactors (Whiteley *et al.*, 2002a, b, c, 2003a; Pletschke *et al.*, 2002).

6. PROJECT OBJECTIVES

❖To investigate the enzymology of accelerated primary sewage sludge solubilisation and disposal associated with sulphate reducing bacterial systems.

❖ To characterise the enzymology of the sludge solubilisation disposal process by identifying the major enzymes involved and determining the factors influencing their performance.

❖ To contribute to the development and optimisation of the primary sewage sludge solubilisation process and reactor design indicators by the maximisation of enzymatic performance.

❖ To construct a descriptive model of the physical mechanisms and the enzymatic role for the breakdown and digestion of the sewage sludge by investigating the action of the various enzymes.

This study presents an enzymological profile of enhanced solubilisation of PSS within a sulphidogenic RSBR at three different depths. An investigation of the activity of key enzymes (α- and β-glucosidases, proteases and lipases) greatly contributes to the current understanding of the enzymatic processes occurring in these bioreactor systems, aiding in the improvement of the overall design and performance of these bioreactors for the accelerated treatment of primary municipal sewage sludge. The study shows that changes in treatment plant operating parameters such as pH, alkalinity, temperature, COD, sulphate and sulphide concentrations as well as introducing specific enzyme inhibitors will be reflected in the enzymatic activities in the RSBR.

Furthermore the work shows a proper understanding of the mechanism involved in floc formation, distribution and disintegration within the RSBR and finally integrates a model of the interrelationship between the physiochemical parameters, environmental factors and the functioning of enzymes in enhancing the mineralisation of complex particulate organics in the RSBR. The results obtained in this investigation clearly establish the interrelationship between the physico-chemical parameters and the activities of the hydrolytic enzymes and makes an important contribution to the characterisation of the RSBR. This biochemical characterisation thus further contributes to understanding the process of enhanced hydrolysis process of primary sewage sludge by hydrolytic enzymes under biosulphidogenic conditions. In this work it is demonstrated that a spectrum of enzymatic activities is easily measurable in activated sludge samples.

In summary, this study has indicated that the enhanced mineralization of complex particulate organic matter in sewage sludge relies primarily on enzymatic hydrolysis of the macromolecules. Furthermore it provides a view of the enzymology of the RSBR with respect to depth of the reactor and concomitant effect of levels of sulphide, sulphate and alkalinity/pH of the overall reactor system.

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LIST OF FIGURES

Figure 18. Effect of sulphide on β-glucosidase from sulphidogenic □ and methanogenic ■ bioreactors: [100% β-glucosidase activity = 226.4 μmoles/min/mg dried sludge powder].

Figure 19 Time course concentrations of sulphate and sulphide within the RSBR

Figure 20. Time course values of sulphide with respect to depth within the RSBR

Figure 21. Specific activity of glucosidase, protease and lipase at depths within the RSBR

Figure 22. Concentration of carbohydrate, protein and lipid at different depths within the RSBR

Figure 23. The level of ATPS in the closed system bioreactors. $ATPS_{MR}$ (□) and $ATPS_{SR}$ (■).

Figure 24. Variation of COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$ during the 60 day period for a) depth 1, b) depth 2 and c) depth 3.

Figure 25 Variation of COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$ for depth 1, depth 2 and depth 3.

Figure 26 Variation of alkalinity with time during the experimental period.

Figure 27 Temperature and pH profile during the study period.

Figure 28. Variation of a) α-glucosidase and b) β-glucosidase activities with time in the RSBR.

Figure 29 Variation of a) α-glucosidase and b) β-glucosidase activities with RSBR depth.

Figure 30. Variation of protease specific activity in the RSBR a) with respect to time and b) with respect to depth.

Figure 31 Variation of lipase specific activity (mol/min/ml/mg protein) in the RSBR a) with respect to time and b) with respect to depth.

Figure 32 Variation of sulphatase specific activity (mol/min/ml/mg protein) in the RSBR a) with respect to time and b) with respect to depth.

Figure 33 Effect of inhibitors on a) α-glucosidase activity and b) β-glucosidase.

Figure 34 Effect of inhibitors on protease activity.

Figure 35 Effect of inhibitors on lipase activity.

Figure 36 Effect of $ZnCl_2$ and PMSF on α-glucosidase and β-glucosidase.

Figure 37 Effect of $ZnCl_2$ and PMSF on protease and lipase

Figure 38 Hanes-Woolf plot to determine kinetic parameters for proteases.

LIST OF TABLES

LIST OF SCHEMES

1. INTRODUCTION

The preservation and proper management of water as a natural resource has become increasingly difficult in the mineral mining regions of the world. The release of Acid Mine Drainage (AMD) into the aquatic environment affects the taste of drinking water, and exacerbates the already severe demand placed on suitable drinking water obtained from rivers and groundwater by increased domestic, agricultural, mining and industrial activity. AMD results from the oxidation of pyritic iron sulphide to sulphate and Fe^{2+} and is characterised by high concentrations of sulphate, heavy metals, low pH and suspended solids. Sulphate reducing bacteria (SRB) have attracted the attention of biotechnologists due to their fundamental properties and possible residual water treatment process application in acid mine drainage (AMD) remediation. Complex organic molecules are hydrolysed by enzymes from fermentative bacteria to simple organic compounds. These are acted upon by H_2 producing and consuming acetogenic bacteria to yield acetate, H_2 and CO_2 which in turn are acted upon by CO_2 consuming and acetoclastic methanogenic bacteria. Under anaerobic conditions the simple organic electron donor molecules, eg lactic acid, are used by the SRB's such as *Desulfovibrio* and *Desulfuromonas* to reduce sulphate to hydrogen sulphide and bicarbonate with a resultant increase in pH [equation 1]. Soluble metal salts react with H_2S *in situ* to produce insoluble metal sulphides, thereby reducing the concentration of metals and salts to acceptable levels [equation 2]. Bicarbonate ions react with protons to form CO_2 and water, thus removing the 'acidity' from solution as CO_2 gas [equation 3]

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

$$H_2S + M^{2+} \rightarrow MS(s) + 2H^+ \quad .....(2)$$

$$HCO_3^- + H^+ \rightarrow CO_2(g) + H_2O \quad ..... (3)$$

Sulphate is a stable non-reactive compound that must first be activated in order to participate in subsequent metabolic reactions such as reduction and sulphur transfer (Karomohamed and Nyren, 1999; Gavel, *et al.*, 1998). Dissimilatory sulphate reduction occurs in certain anaerobic bacteria such as SRB which use sulphate as the terminal electron acceptor. Adenosine 5'-phosphosulphate (APS) serves as the nucleoside sulphate donor in this process, and reduction results in the accumulation of hydrogen sulphide and in ATP synthesis through oxidative phosphorylation. The first step in dissimilatory sulphate reduction is catalysed by ATP-sulphurylase (ATP:sulphate adenylyltransferase; EC 2.7.7.4). This enzyme catalyses the formation of APS and inorganic pyrophosphate (PP_i) from ATP and inorganic sulphate (equation 4), and therefore plays a crucial role in sulphate activation, the key step for sulphate utilisation (Gavel, *et al.*, 1998; Li, *et al.*, 1991; Dahl, *et al.*, 1994).

$$ATP + SO_4^{2-} \xrightarrow{\text{ATP Sulphurylase}} APS + PP_i \quad (4)$$

APS is then catalysed to sulphite and adenosine monophosphate (AMP) by APS reductase in dissimilatory reduction. Sulphite is further reduced to sulphide by sulphite reductase (Sperling, *et al.*, 1998). ATP-sulphurylase (ATPS) is an enzyme widely distributed in nature and has been detected in SRB such as *Desulfovibrio* and *Desulfotomaculum* (Gavel, *et al.*, 1998).

When sulphate is removed biologically from an effluent such as AMD, the two major problems that are experienced are the availability and cost of the organic carbon substrate. One method of overcoming these problems and obtaining a potentially low-cost, readily available alternative energy carbon source for sulphate reduction, has been forthcoming with the use of municipal sewage sludge (Henzen and Pieterse, 1978). SRB could thus be used for the bioremediation of sulphate-rich industrial effluents, tannery effluents and acid mine drainage.

The SRB (obligate anaerobes) have the ability to utilise inorganic sulphate as a terminal electron acceptor during an ATP-requiring reaction, but require the supply of a low molecular weight carbon source as an electron donor for sulphate reduction. Sulphate reducers are able to oxidise a wide range of organic acids and more complex organic molecules (Widdel, 1988), including direct methanogenic substrates such as acetate, formate, H_2 and methanol, as well as propionate, butyrate, lactate, ethanol, fumarate, succinate and malate and higher branched fatty acids (Lens, *et al.*, 2000). As SRB are unable to degrade long polymeric molecules such as polysaccharides, proteins or lipids, they rely on the activities of hydrolytic and acidogenic bacteria to supply the monomeric carbon/energy source.
Sulphate-rich wastewater is produced by many industries such as the sulphuric acid in food processes, thiosulphate in the photographic industry, sulphite in tanneries, the sea-food processing industry, the leaching of sulphur rich soils in land fills and mines and by power-plant flue gases from the combustion of sulphur containing fuel. Biological anaerobic reduction offers a highly successful and efficient process for the removal of sulphate from these effluents as sulphide (Weijima *et al.*, 2000). The anaerobic stage requires the presence of an organic substrate that serves as an electron donor or carbon source for this sulphate reduction. Previous work from the Environmental Biotechnology Group of Rhodes University has developed the use of raw municipal sewage sludge as a relatively cheap and readily available carbon source for anaerobic sulphate reduction (Rose *et al.*, 1998). The SRB present in sulphate-reducing systems have, perhaps indirectly, been shown to stimulate the rate of primary sewage hydrolysis and solubilisation.

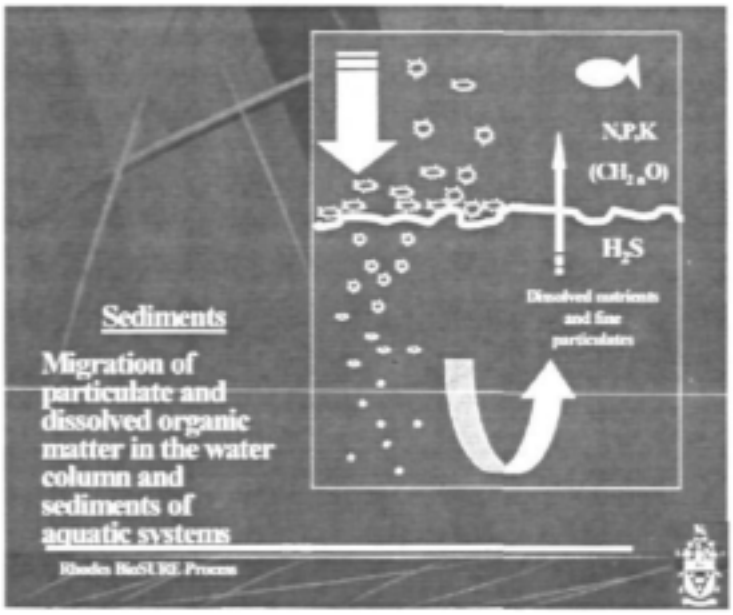
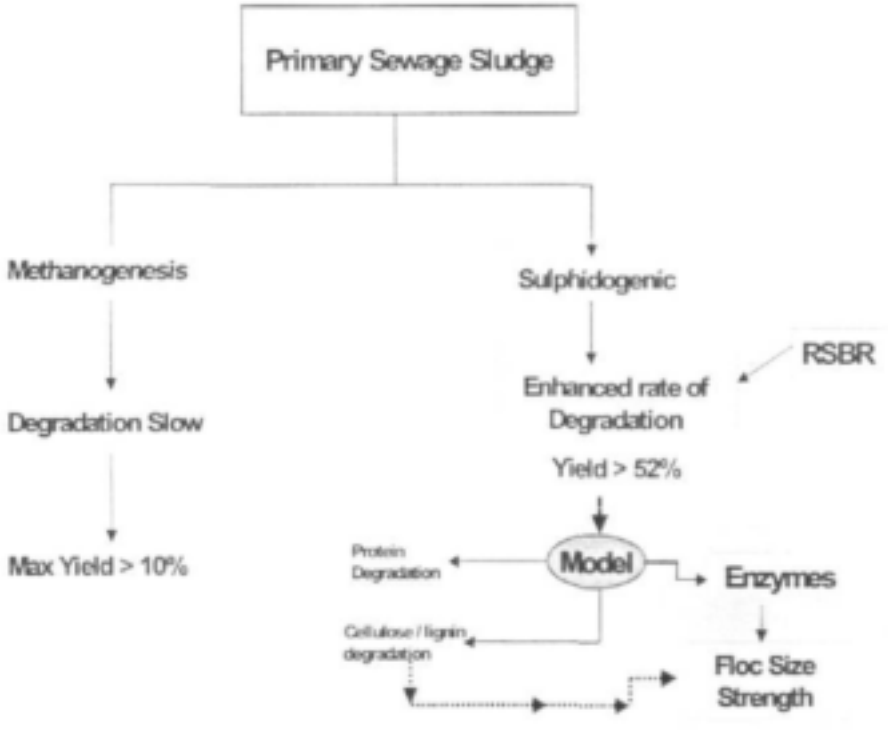


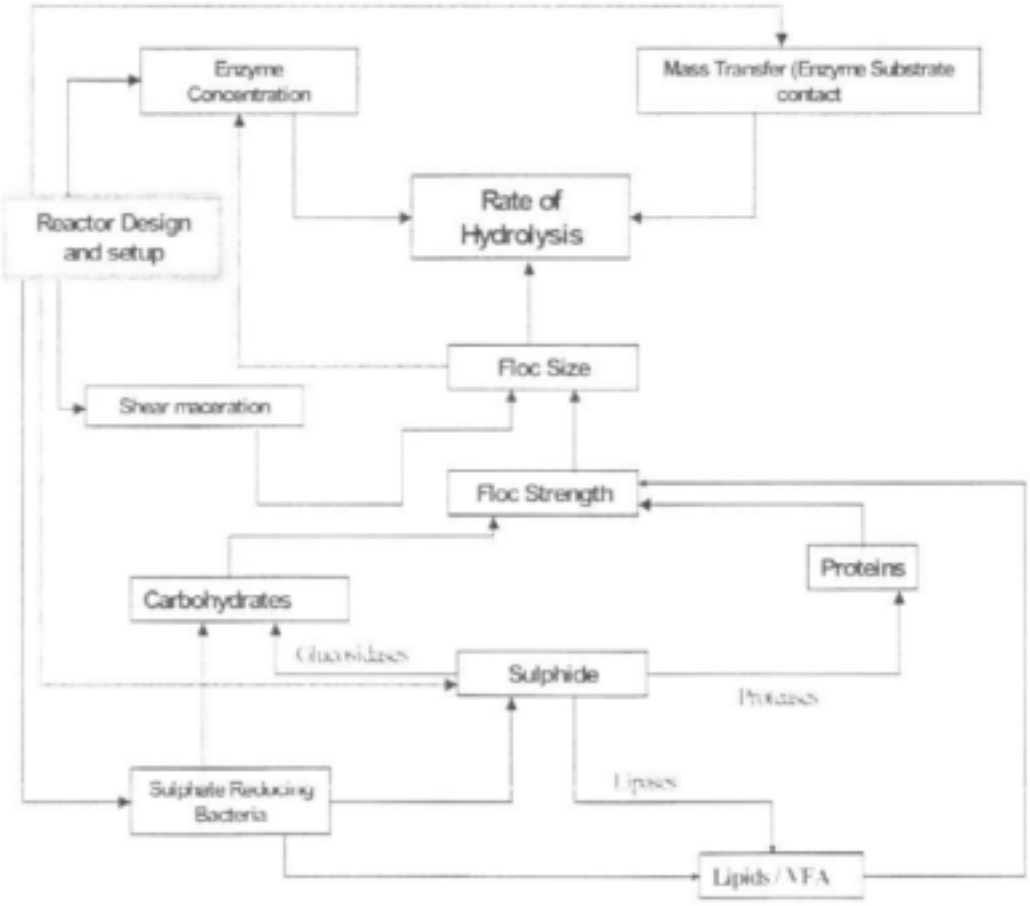
Figure 1: Migration of particulate and dissolved organic matter in water column.

Further to an investigation of the microbial ecology of a tannery waste ponding system it was noted that a complete biological sulphur cycle prevailed in the water column after the sulphate enriched environment received high loads of complex organic particulate matter. (Rose *et al.*, 1998; Dunn, 1998). This was associated with an effective degradation of particulates and a subsequent settling towards the lower level anaerobic sulphidogenic zone within the ponds. Subsequent upwelling of dissolved and residual suspended organic matter supported an aerobic upper zone. Residual undegraded particulates settled once again to within the sulphidogenic zone and underwent a further cycle of degradation, eventually, over a period of time, achieving a surprisingly high level of solids removal in a system notoriously prone to sludge build-up (Figure 1). The mechanisms of this initial observation of enhanced degradation of organic particulate matter in the sulphidogenic environment has been investigated in a Recycling Sludge Bed Reactor (RSBR). (Whittington-Jones, 2000). Based on the above observations, a laboratory-scale RSBR was designed to mimic the proposed mechanism of degradation in the ponds (Figure 2).
Solubilisation rates for primary sewage sludge (PSS) are slow in conventional anaerobic digestion systems (Pipyn and Verstraete, 1979) with maximum soluble product formation reported of about 10 days, and at yields of 5-10% in the mesophilic temperature range (Banister and Pretorius 1998; Canziani *et al.*, 1996; Elefsiniotis and Oldham 1994; Hatziconstantinou *et al.*, 1996; Shimizu *et al.*, 1993). By simulating the recycling of complex organic matter through a



Scheme 1 Conceptual schematic diagram showing the relationship between methanogenic and sulphate reducing environment in the solubilisation of primary sewage sludge.

sulphide gradient within the ponds, within a laboratory-scale RSBR, it was possible to demonstrate that the degradation of a complex carbon source, primary sludge, was enhanced significantly to 52% (Whittington-Jones, 2000). A conceptual diagram and flow events are represented in the model (Scheme 1) while the interrelationship of factors on the process of enhanced hydrolysis of PSS under sulphidogenic conditions is seen (Scheme 2). There is still no consensus on the mechanism of enhanced hydrolysis though the key might be the activation of the hydrolytic enzymes through a sulphide gradient within the RSBR. It was further proposed that reciprocal upwelling and settling events served to counteract mass transfer limitations between the water column and the sediment and that the sulphide gradients established in these systems play an important role in the biodegradation of particulate COD [COD_p]. Microbial aggregates generated in wastewater treatment using activated sludge provide an efficient organisation of bacterial communities. The bacteria are embedded in a matrix of exopolymeric substances which act as a trap for biodegradeable colloidal organic matter too large for direct assimilation by bacteria. Furthermore they act as a network confining extracellular enzymes exhibiting hydrolytic activity (Vavilin *et al.*, 1996). Models of anaerobic digestion of complex particulate substrates showed that both the concentration of the hydrolytic enzymes, and the



Scheme 2 Interrelationship of floc size and strength, protein, carbohydrates and lipids, proteases, glucosidases and lipases, and sulphide with the rate of enzymatic hydrolysis in the RSBR

contact between them and their substrates were crucial (Jain *et al.*, 1992).

Sludge components are cemented together by extracellular polymeric substances (EPS) produced from bacterial cellular metabolism, cell autolysis and the wastewater itself. Significant changes in sludge structure and microbial composition can result from the hydrolysis of the EPS and degradation of the sludge matrix (Nielsen *et al.*, 1996). Polysaccharides form the bulk of the extracellular material in the EPS, with up to 65% of the total extracellular material (Horan and Eccles, 1986) but other organic substances, such as proteins, lipids and nucleic acids are also present (Goodwin and Forster, 1985; Zhang *et al.*, 1998). Anaerobic storage of sludge is known to lead to rapid sludge deflocculation and several factors are believed to play a role during this process. It has been proposed (Starkey and Karr, 1984) that the production of EPS is inhibited as a result of the anaerobic processes taking place. Furthermore a significant degradation of the sludge floc matrix occurred during anaerobic storage (Nielsen *et al.*, 1996), and the reduction in sludge was mainly due to degradation of the sludge protein and carbohydrates. Since it has been found that the proteins may be involved in binding the floc together (Urbain *et al.*, 1993) the large reduction in proteins and some reduction in the carbohydrate content after a few days, which subsequently leads to deflocculation and reduced dewaterability, indicates that they affect the floc strength (Nielsen *et al.*, 1996). Associated with these sludge flocs is a certain amount of enzymatic activity, which is required in order to break down the complex molecules into their basic monomers. Proteolytic, lipolytic, glycolytic and cellulolytic enzymes synthesised within bacterial cells hydrolyse the adsorbed macromolecules into smaller subunits that can be transported across the cell membrane and then metabolised. These hydrolytic processes are recognised as being the overall rate-limiting step for the mineralisation of organic matter in the sludge treatment process (Levine *et al.*, 1985). The exoenzymes found in sludge originate from the influent sewage, from the sludge itself or even as actively secreted exoenzymes and may be either directly associated with the cell surface of the producer, or in the free form, dissolved in water, or immobilised in the sludge matrix within the EPS (Pletschke *et al.*, 2002).

While process development studies have made significant progress, the underlying enzymatic mechanisms for the enhanced solubilisation in the sulphate reducing system remain obscure. During the sulphate reduction process the pH of the system becomes more alkaline, due to the increase in concentration of HCO_3^-, OH^- and SH^- ions and it is suggested that there is a neutralisation of the acidic surface of the organic particulate floc. This, in turn, allows for a disruption of the ionic charges maintaining the integrity of the floc. Consequently there is an exponential increase in the rate at which polymeric substrate is released for further enzymatic degradation. Process optimisation studies are crucially dependent on an understanding of the enzymatic processes and hydrolysis in the sulphur reducing environment. The rate at which hydrolysis proceeds is best described by first order kinetics and may be strongly influenced by environmental and operational parameters such as pH, temperature, biomass, particle/floc size, type and concentration of particulate substrate and production concentration. Any increase in the enzyme concentration in an anaerobic digester, or a reduction in mass transfer limitation or particle size will result in an increase in the rate of hydrolysis of complex particulate organics. Solubilisation of primary sewage sludge is enhanced under sulphate reducing conditions, because of a decrease in both particle and floc size, as a result of enhanced hydrolysis of macromolecular carbohydrates, proteins and lipids. Furthermore hydrolysis of these particulate organics may also be enhanced in the presence of sulphide since the latter is a strong reducing agent and is capable of reducing disulphide linkages that are essential for maintaining the three dimensional

conformational structure. The aim of the present study is to present an enzymological profile of enhanced solubilisation of PSS within a sulphidogenic RSBR at three different depths. An investigation of the activity of key enzymes (α- and β-glucosidases, proteases and lipases) will greatly contribute to the current understanding of the enzymatic processes occurring in these bioreactor systems, aiding in the improvement of the overall design and performance of these bioreactors for the accelerated treatment of primary municipal sewage sludge. The study will show that changes in treatment plant operating parameters such as pH, alkalinity, temperature, COD, sulphate and sulphide concentrations as well as introducing specific enzyme inhibitors will be reflected in the enzymatic activities in the RSBR. The work will show a proper understanding of the mechanism involved in floc formation, distribution and disintegration within the RSBR and finally to integrate a model of the interrelationship between the physiochemical parameters, environmental factors and the functioning of enzymes in enhancing the mineralisation of complex particulate organics.

2. MATERIALS AND METHODS

2.1. Reactor Design and operation

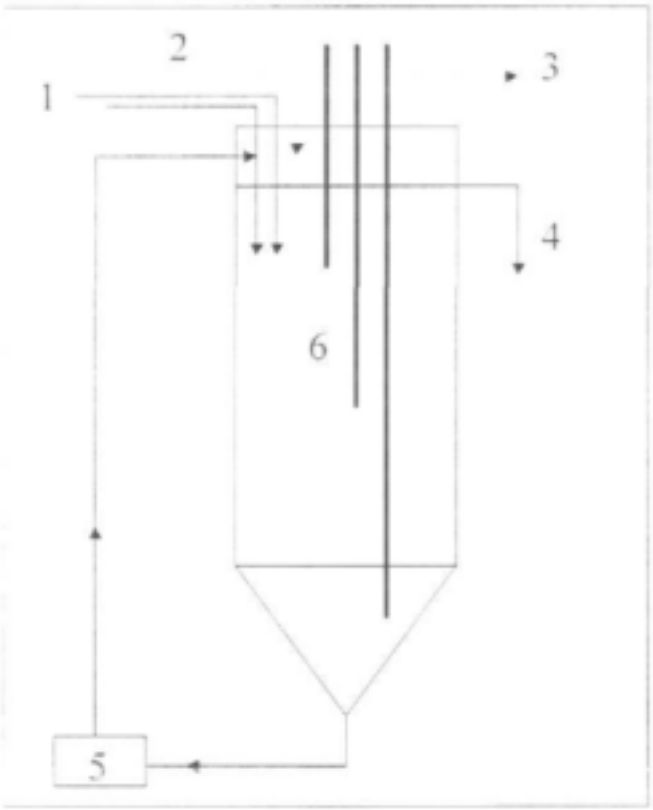


Figure 2. The design of the laboratory-scale Recycling Sludge Bed Reactor. 1 = separate influent tubes for the sulphate solution and primary sludge; 2 = inlet for nitrogen gas; 3 = outlet for nitrogen gas; 4 = effluent tube; 5 = peristaltic pump for recycling of settled sludge; 6 = three sample tubes inserted to different depths.

A laboratory-scale RSBR with a working volume of 5L was constructed from perspex (Figure 2). To facilitate recycling of settled sludge, the base of the reactor was cone-shaped with an angle of 60°. The 2 inlet tubes (for the sulphate solution and primary sludge) passed through the lid to a depth of 50mm below the surface of the reactor contents. Samples were drawn from three tubes (5mm i.d.) inserted to different depths (16, 32 and 50cm below the overflow pipe) through the lid. In order to limit introduction of oxygen into the system, all sample tubes where blocked with rubber stoppers when not in use and the effluent pipe contained an S-bend water trap. The headspace of the reactor was purged with nitrogen gas after sampling. The reactor was operated at a hydraulic retention time (HRT) of 2 days, at room temperature (22-25°C). It was initially seeded with 500ml of sludge from another laboratory-scale biosulphidogenic bioreactor that had been running for longer than a year. The system was then fed at a constant rate with a sulphate solution and fresh sieved (2mm mesh size) primary sludge obtained from the municipal sewage treatment plant in Grahamstown, South Africa. Sieved primary sludge was diluted with tap water to obtain a feed with a COD of 2000 $mg.L^{-1}$. The sulphate solution ($2000mg.L^{-1}$) was prepared by dissolving laboratory-grade Na_2SO_4 in tap water. The final concentration of COD and SO_4 in the combined effluent stream were both $1000mg.L^{-1}$ due to dilution of each stream by the other. The ratio of COD: SO_4 in the feed was thus 1:1. The sulphate solution and the primary sludge were refreshed every 48 hours and were stored in separate reservoirs to minimise degradation of the sludge and build-up of bacterial populations prior to it entering the RSBR. The glass feed reservoir containing the primary sludge was placed on a magnetic stirrer, and the contents stirred continuously, at low speed, to prevent settling of particulate organic matter. The neck of the feed reservoir was closed to limit oxygen transfer.

During operation, the particulate organic matter in the feed primary sludge settled into the base of the unit, and was then recirculated and re-entered the reactor adjacent to the influent stream, allowing a period of reaction. A portion of the influent water was drawn into the bed, while the remainder containing solubilized material flowed horizontally to exit the system via the overflow pipe. Although the retention of biomass is reliant on flocculation, the predominantly downward flow of liquid was thought to offer significant advantages, in terms of biomass retention, over the upward flow in the UASB-type reactors. The sludge that settled in the base of the RSBR was continuously recycled to the top of the unit at a rate of 20% reactor volume/hour, using a variable speed peristaltic pump (Watson-Marlow 504S). Turbulence created by the inflow of recycled sludge resulted in the formation of a suspended sludge bed within the reactor. The height of the bed could be regulated by altering the recycle rate, and was initially maintained at approximately 200mm below the overflow

2.2. Enzyme assays.

2.2.1. Proteases

Protease activity was measured according to the published procedures (Pin *et al.*, 1995; Goel *et al.*, 1998) using azocasein as substrate. The method is especially suitable for the determination of proteolytic activity in crude samples since it is very resistant to interference. The sludge (1.0ml) was incubated (37 °C, 1h) in suitable buffer at the optimum pH (Whiteley *et al.*, 2002b) with 2 %w/v azocasein (1.0ml). Ice-cold 10 % (w/v) trichloroacetic acid (TCA) (1.0ml) was added to stop the reaction and the tubes left at –20 °C to facilitate precipitation. The precipitated

protein was removed by centrifugation (4000 x g, 10 min) and the A_{440nm} of TCA-soluble peptides was measured. Specific activities were expressed as enzyme activity per unit biomass and were calculated by dividing the enzyme unit (U) by the mass of freeze dried sewage sludge. One unit of activity was defined as the activity in a 1ml volume of sample that produced an increase of 1.0 absorbance unit at 440 nm in 1h at 37°C.

2.2.2. Lipases

Lipase activity was measured by a slight modification of the procedure of Korn (1954) using the enzymatic cleavage of glycerol from a commercial lipid. The supernatant from the sonicated sludge (1.0 ml), sampled at each of the variable depths, was incubated at 25 °C for 10 min in 0.1 M sodium phosphate buffer, pH 7.5 with triacetin (1 %w/v, 1.0 ml). The reaction was stopped by the addition of sulphuric acid (5 M, 50 µl) and sodium periodate ($NaIO_4$) (0.1 M, 250 µl). The sample was mixed, $NaHSO_3$ (250 µl) added, then transferred to chromotropic acid reagent (2.5 ml) and heated (80 °C, 60 min). After cooling the glycerol released was measured by reading the absorbance at 570 nm and concentrations determined from a standard curve.

2.2.3. Glucosidases

α- and β-glucosidase activity was measured by a modification of the published procedure using the corresponding anomeric methylumbelliferyl-glucopyranoside as substrate and liberating the fluorogenic methylumbelliferone product which is measured fluorometrically at 455nm (Hattenberger *et al*., 2001). The sludge (1ml) was incubated at 50 °C for 5 min in glycine buffer (0.4 M, pH 10.8) with methylumbelliferyl-glucopyranoside (1.5 mM, 1 ml). Ice-cold ethanol (2.5 ml; 95 %) was added to stop the reaction and the tubes were centrifuged (2000 x g, 5 min) and the fluorescence measured in a spectrofluorimeter at an excitation wavelength of 365nm and an emission wavelength of 455nm. Glucosidase activities were calculated from a standard curve.

2.2.4. Sulphatases

The activity of these enzymes was measured by the enzymatic cleavage of ρ-nitrophenolsulphate liberating ρ-nitrophenol that generates an ion in alkaline solution which can be measured at 405nm (Bergmeyer, 1986). The sludge was incubated at 25°C for 20 min in acetate buffer (10mM) at the relevant pH with ρ-nitrophenolsulphate (30µl; 60µM). The reaction was stopped with the addition of NaOH (2.0ml, 0.5M) and the absorbance of the yellow colour measured in a spectrophotometer at 405nm. Specific sulphatase activities were expressed as enzyme activity per unit biomass and were calculated by dividing the enzyme unit (U) by the mass of freeze dried sewage sludge (mg). One unit of enzyme activity is defined as the amount of enzyme required to convert 1µM of ρ-nitrophenolsulphate to ρ-nitrophenol in 1 min at 25°C at 405nm.

2.2.5. Phosphatases

Phosphatase activity was determined by the enzymatic cleavage of ρ-nitrophenol phosphate liberating ρ-nitrophenol whose absorbance could be measured under alkaline conditions at 405nm (Garen and Levinthal, 1960). For these assays sulphidogenic sewage sludge (2ml), was incubated with substrate (25mM ρ-nitrophenol phosphate, 1ml) and 2ml of acetate buffer (pH

4.5). The reaction tubes were incubated at 55°C for 30 min, centrifuged (6000 x g, 2 min), 0.5ml supernatant removed, added to 5.5ml NaOH and the absorbance measured at 405nm. Specific phosphatase activity was expressed as enzyme activity per unit biomass and was calculated by dividing the enzyme unit (U) by the mass of freeze dried sewage sludge (mg). One unit of enzyme activity is defined as the amount of enzyme required to convert 1µM of ρ-nitrophenolphosphate to ρ-nitrophenol in 1 min at 25°C at 405nm.

2.2.6. ATP Sulphurylase coupled spectrophotometric assay

ATP sulphurylase activities in the methanogenic- and sulphidogenic reactor fractions were assayed by measuring the ATP generated by the reaction of APS with pyrophosphate according to the method of Dahl and Trüper, 1994). The rate of ATP production in the coupled spectrophotometric microassay was followed via the phosphorylation of glucose, and the subsequent oxidation of glucose-6-phosphate to D-gluconate-6-phosphate coupled to the reduction of NADP (equations 5,6)

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{Hexokinase}} \text{D-glucose-6-P} + \text{ADP} \quad (5)$$

$$\text{D-glucose-6-P} + \text{NADP} + H_2 \xrightarrow{\text{G-6-P Dehydrogenase.}} \text{Gluconate-6-P} + \text{NADPH}_2 \quad (6)$$

In a total volume of 190 µl, 100 mM Tris HCl (pH 8.0), 20 mM ß-D-glucose, 10 mM $MgCl_2$, 0.5 mM Na-NADP, 1 mM $Na_4P_2O_7$, 1 unit of glucose-6-phosphate dehydrogenase, 0.5 units of hexokinase and 50 µl of reactor sample was combined. The assay mixture was allowed to equilibrate at 37°C, and the reduction of NADP was followed at 340 nm after initiating the reaction with the addition of 10 µl 25 mM APS (final incubation concentration 1.25 mM). Blanks were prepared by replacing the reactor sample with 50 µl Tris-HCl buffer (pH 8.0). The background rate of ATP production due to the action of hexokinase and glucose-6-P-dehydrogenase was established by measuring the rate of ATP formation before the addition of APS. One unit of activity was defined as 1 nmol NADPH (ATP) produced per minute under the assay conditions described. All determinations were performed in duplicate (i.e. duplicate assays of 1 sample) in a microtiterplate reader (PowerWave$_X$, Bio-Tek Instruments, Inc).

2.2.7. ATP formation assay

The ATP sulphurylase coupled spectrophotometric assay is a rapid and convenient one as it is particularly useful for determining the activity of ATPS *in the absence* of the coupling enzymes hexokinase and glucose-6-phosphate-dehydrogenase, for example in determining the optimum pH and temperature of ATPS (Dahl and Truper, 1994). ATPS catalyses the generation of ATP from APS and pyrophosphate in the first step (reverse equation 4). The ATP generated is then spectrophotometrically determined in a second step in the presence of the two coupling enzymes hexokinase and glucose-6-phosphate dehydrogenase (Dahl and Truper, 1994).
The 200 µl reaction mixture contained the following: 100 mM Tris-HCl (pH 8.0, or pH to be tested), 10 mM $MgCl_2$, 1 mM PP_i and 50 µl reactor sample. The assay components were pre-incubated (at 30°C, or tested temperature) and the reaction was initiated by the addition of 1.25 mM APS and incubated at 30°C for 10 min. The reaction was terminated by boiling the samples at 100°C for 2 min, and the denatured protein removed by centrifugation at 3000 x g for 5 min at

4°C. The samples were placed in ice to protect the generated ATP against hydrolysis. An aliquot of 150 µl was removed for the qualitative determination of generated ATP by adding 4 µl 1 mM ß-D-glucose, 4 µl of 25 mM Na-NADP and 5 units hexokinase and 2.5 units of glucose-6-phosphate. The reaction mixture was followed spectrophotometrically at 340nm in the microtiterplate reader (PowerWave$_X$, Bio-Tek Instruments, Inc) at 37°C, and the difference in A_{340nm} (between start and end readings) was used to calculate the amount of ATP generated. Blanks were performed at each pH or temperature tested by replacing the 50 µl of reactor sample with 50 µl 100 mM Tris-HCl buffer (pH 8.0 or pH tested).

2.3. Preparation of anaerobic sewage sludge and cell-free extracts.

Methanogenic and sulphidogenic sludge was centrifuged (4000 x g, 30 min) and the supernatant and pellet fractions assayed for enzyme activity. Cell-free extracts were prepared using a slight modification from the method of Thiel and Hattingh (1967). A sample from the same sludge, precooled to 2°C in ice, was homogenised for 30 min using sonication, [energy setting at 220 kJ.sec^{-1}], followed by centrifugation (4000 x g, 30 min, 4°C). Sonicated samples as well as supernatant and pellet fractions (resuspended 2:1, v/w, in water) were assayed for enzyme activity.

2.4. Effect of sulphate, sulphite and sulphide.

The effect of different concentrations of sulphate and sulphite on enzyme activity in the sulphidogenic bioreactor at optimum pH (Whiteley *et al.*, 2002a; 2002b; 2003a) was studied by preincubation of sewage sludge with different concentrations (0–1000 mg l^{-1}) of effector in the reaction mixture. Incubation was started by the addition of substrate. It should be noted that the data represented is that for "total" effector and takes into account both inherent sulphate and sulphite as well as that added. The effect of sulphide on enzyme activity was also tested. Firstly, sludge was preincubated for an hour with an appropriately diluted sodium sulphide solution in the dark in a closed container with minimum "headspace" in order to avoid unnecessary oxidation. An aliquot of the above mixture was added to an appropriately buffered assay mixture to give final concentrations of sulphide of 0–1000 mg l^{-1}. All assay mixture components and conditions were optimised as described above. It must be noted that controls where substrate, sludge or buffer components of the assay mixture were replaced by water, in the presence and absence of sulphate, sulphite and sulphide proved that the observed increased enzyme activity was not due to the interaction of effector with any of the assay components but due to an effect on enzyme activity.

2.5. Enzyme properties

2.5.1. pH

The following buffers were used to establish the activity of phosphatases, and acidic, neutral and alkaline proteolytic activities: 0.2 M sodium acetate buffer, pH 4-5.5; 0.1 M sodium phosphate buffer, pH 6 and pH 6.5; 0.1 M Tris-HCl buffer, pH 7.0, 7.5, 8.0, 8.5, 9.0 and 0.1M carbonate/bicarbonate buffer, pH 9.5-11. The determination of the pH optimum for phosphatases was performed using the assays as described above at buffer pH values ranging

from pH 4 to pH 8. For lipases the pellet was incubated with triacetin (3 %) in 0.1 M sodium phosphate buffer in the pH range of 4.0 – 9.5. The pH optima of the ATPS fractions were determined using the ATP formation assay, and using a range of 100 mM Tris-HCl buffers in the pH range of 6.9 to 9.4.

2.5.2. Temperature.

The temperature profiles for proteases were performed, at pH 5, 7 and 10 between 20-70°C, and phosphatases, between 30-80°C from both methanogenic and sulphidogenic resuspended sewage sludge powder. Lipase activities of resuspended sewage powder were estimated after incubation with triacetin (3 %) at temperatures ranging between 40 - 90 °C and pH values at optimum from both methanogenic and sulphidogenic sewage sludge. The effect of temperature on ATPS activity was studied from 13°C to 70°C at pH 8.0

2.5.3. Stability.

The stabilities of all of the enzymes were investigated at respective optimum temperatures and pH over incubation times of up to 5h.

2.5.4. Kinetic parameters: K_m and V_{max}.

Ten different concentrations each of the substrates were chosen to give measurable reaction rates and the reactions were performed in triplicate. The affinity constant, K_m and the rate of reaction, V_{max} were determined using linear regression plots of Lineweaver-Burk (Lineweaver and Burk, 1934) and Hanes-Woolf (Cornish-Bowden, 1994).

2.5.5. Effect of Inhibitors

Stock solutions of each of the enzyme inhibitors were prepared: Zinc chloride [$ZnCl_2$] (1M); phenylmethylsulphonyl-fluoride [PMSF] 0.2M; p-chloromercuribenzoic acid [pCMB] (0.2M), ethylenediamine tetra-acetic acid di-sodium salt [EDTA] (0.1M) and ethyleneglycol-bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid [EGTA] (0.1M). $ZnCl_2$, EGTA and EDTA were dissolved in milli-Q water while pCMB and PMSF were dissolved in dimethyl sulphoxide (DMSO). The assay concentration of the inhibitors was 3.7×10^{-4}M $ZnCl_2$, 1 mM EDTA, 1mM EGTA, 1 mM PMSF and 1 mM pCMB. Sludge samples drawn from the RSBR were pre-incubated in flasks with the inhibitors for 30 minutes after which samples were removed and enzyme activities determined.

2.6. Time course and depth profiles

Levels of sulphate, sulphite, sulphide, proteins, lipids, carbohydrate concentrations and the activities of the enzymes glucohydrolases, proteases, lipases, phosphatases and sulphatases were monitored in the sulphidogenic bioreactor system at three depths (16cm; 32cm; 50cm) and over a period of 40 days. Samples were removed from these reactors on alternate days and stored at –20°C until analysis.

2.7. Floc size distribution

Flocs were prepared for microscopic examination using a technique adapted from Droppo et al. 1996. Sludge samples were settled in measuring cylinders and the supernatant discarded. The settled portions were then diluted sufficiently to allow clear views of the flocs by combining the settled solids with agarose (0.75%, 8ml) at 35°C. The settled sample volumes used were 3.0ml (depth 1); 1.5ml (depth 2) and 0.5ml (depth 3). The agarose plus samples were poured into 96mm diameter Petri dishes, left to cool and floc characteristics and size distributions determined by photography with an Olympus PM-C35DX digital camera mounted on a light microscope (Olympus BX50). Images were processed using SigmaScan Pro Version 5 to measure the feret volume (size of object), circularity (equation 7) and looseness (equation 8) of 100 flocs per sample.

$$\text{Circularity} = (4\pi \times \text{area})/(\text{perimeter})^2 \qquad (7)$$

$$\text{Looseness} = (\text{perimeter})^2/\text{area} \qquad (8)$$

2.8. Multivariate statistical analysis

All statistical analysis, including Pearson correlation coefficients, principal component analysis (PCA), linear regression, analysis of variance (ANOVA) and descriptive statistics were conducted using STATISTICA (data analysis software system), for Windows Version 6.0 (StatSoft, Inc. 2001, USA), Microsoft® Excel 2002 (Microsoft Corporation) or MATLAB Version 6.0 (Math Works). Mixed models repeated-measures analyses were used to describe enzymatic activities (Protease, lipase, α- and β-glucosidase and sulphatase) as a function of the various predictors such as temperature, pH, alkalinity, sulphide and sulphate concentrations, COD_{total}, $COD_{particulate}$ and $COD_{soluble}$ for each dependent variable (enzymes). The interaction between the enzymes and temperature was included in each model to analyse whether temperature modified association between the physicochemical parameters and the dependent variables (enzymes). Only significant factors where included in the final model.

2.9. Analytical procedures

COD concentration was determined using a Spectroquant® test kit (Merck # 14541). Filtered COD (COD_f) was determined by passing samples through a Glass Microfibre Filters (type GF/A: Whatman Ltd. # 1820 025), and calculating the COD of the filtrate. This method was modified from Lilley *et al.* (1990) and represented the soluble COD fraction. Prior to determination of COD, all samples were acidified with 32% HCl to pH<2 and shaken for 10 minutes, to remove any sulphide. Although the accuracy of COD quantification may have been improved if sludge samples were macerated prior to dilution and analysis, it was felt that this might have resulted in an artificial underestimation of the COD_p. Instead, determination of COD_t was carried out in triplicate and the mean value calculated, in an attempt to obtain accurate quantification of the COD_t of the highly particulate sludge samples. The difference between the COD_t and COD_f of a sample was used to calculate COD_p. Sulphide was determined as described by Rees *et al.*, 1971 and sulphate by Kolmert *et al.*, 2000. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976). Sulphide did not interfere with this assay which was in direct contrast to the more established Folin-Lowry protein assay

(Pletschke, personal communication). Carbohydrate concentration were determined using the anthrone reagent (Yemm and Willis, 1954) and lipid by the published procedure (Folch *et al.*, 1957).

3. RESULTS

3.1. Enzyme distribution.

Sonication, as expected, released proteases into the supernatant fraction with 1.27 units mg^{-1} protein, sulphidogenic and 1.74 units mg^{-1} protein, methanogenic for the pH 10 activity. Lower levels of specific proteolytic activity were present in the bulk solution before sonication, 0.06 units mg^{-1} protein (sulphidogenic) and 0.05 units mg^{-1} protein (methanogenic) for the pH 10 activity. Phosphatases followed the same pattern with activities in the methanogenic and sulphidogenic pellet fractions 167 and 226 times that of their respective supernatant fractions. Sonication of the pellet released most of the enzyme activity into the supernatant. Most proteolytic and phosphatase activities were, therefore, found either associated with or immobilised within the particulate matter. No β-glucosidase activity was found in the supernatant washings of the methanogenic and sulphidogenic sludge pellets so all subsequent results were realised from experiments with resuspended pellets. $ATPS_{MR}$ and $ATPS_{SR}$ activity (94% and 100% respectively) were released into the supernatant after sonication, indicating that the ATPS enzymes were located either intracellularly or immobilised in/on the floc. This is in agreement with the results of other studies (Li, *et al.*, 1991; Dahl, *et al.*, 1994, Dahl and Truper, 1994). $ATPS_{SR}$ had a significantly higher (approximately 15-20 fold) level of biocatalytic activity (approximately 5.94 nmoles NADPH produced min^{-1}, mg dry $mass^{-1}$), than $ATPS_{MR}$ (0.296-0.595 nmoles NADPH produced min^{-1}, mg dry $mass^{-1}$). The activity of the two ATPS enzymes was too low to be detected using the classical molybdolysis assay as outlined by Schiff and Saidha (1987). Less specific lipase activity is present in the solution before sonication - 0.036 units mg^{-1} protein (methanogenic) and 0.041 units mg^{-1} protein (sulphidogenic) at optimum pH. Sonication released lipases into the supernatant fraction with activity of 4.11 units mg^{-1} protein (methanogenic) and 5.06 units mg^{-1} protein (sulphidogenic). It was uncertain whether the specific lipase activity was immobilised on the organic particulate matter and/or intracellular membrane or cell wall bound.

3.2. pH

A whole pH range of proteases were present in the sewage sludge with the most prominent being the alkaline proteases at pH 10 while phosphatases from both methanogenic and sulphidogenic bioreactors show optimum activity in the acidic region between pH 4.5 and 5.5 (Figure 3). $ATPS_{MR}$ failed to exhibit a definite pH optimum ($ATPS_{MR}$ activity was very low and barely detectable), while the pH optimum of the $ATPS_{SR}$ was slightly higher at pH 9.0 (Figure 4). Li *et al.*, (1991) investigated the effect of pH on ATPS activity in *Euglena* as measured by the sulphate release assay (between pH 7.0 and 9.0), and reported a pH optimum of 8.8. pH optima reported in literature for ATPS from various organisms vary between 7.5 and 8.5 (Nozawa, 1980). A limited range of lipases are present in sewage sludge. It is quite evident from the profiles obtained that the lipases with the highest activities are those at pH 6.5 and 8 for both methanogenic and sulphidogenic bioreactors (Figure 5).

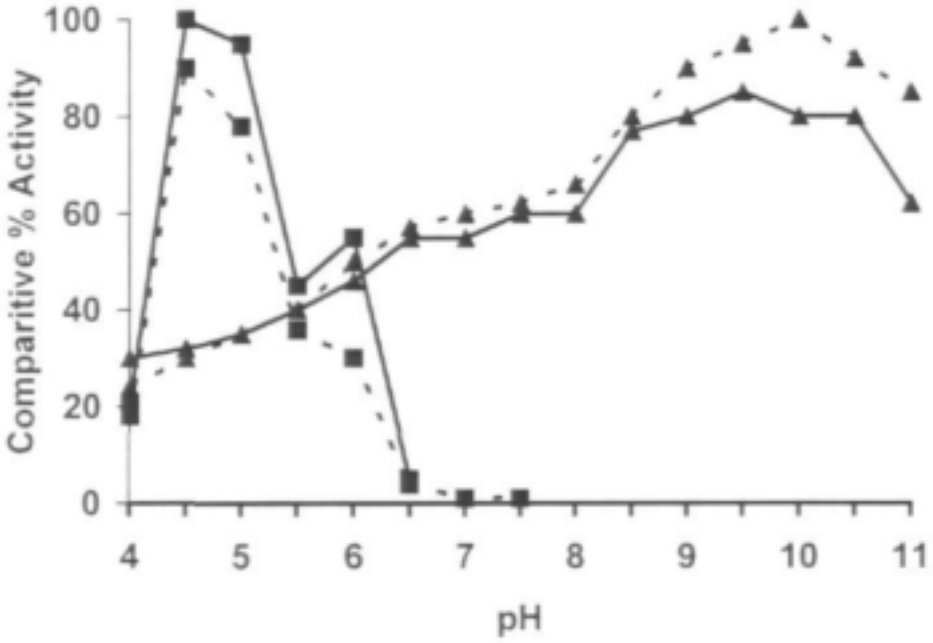


Figure 3. pH profiles of proteases(▲), phosphatases (■) from methanogenic (- -) and sulphidogenic (—) sewage sludge.

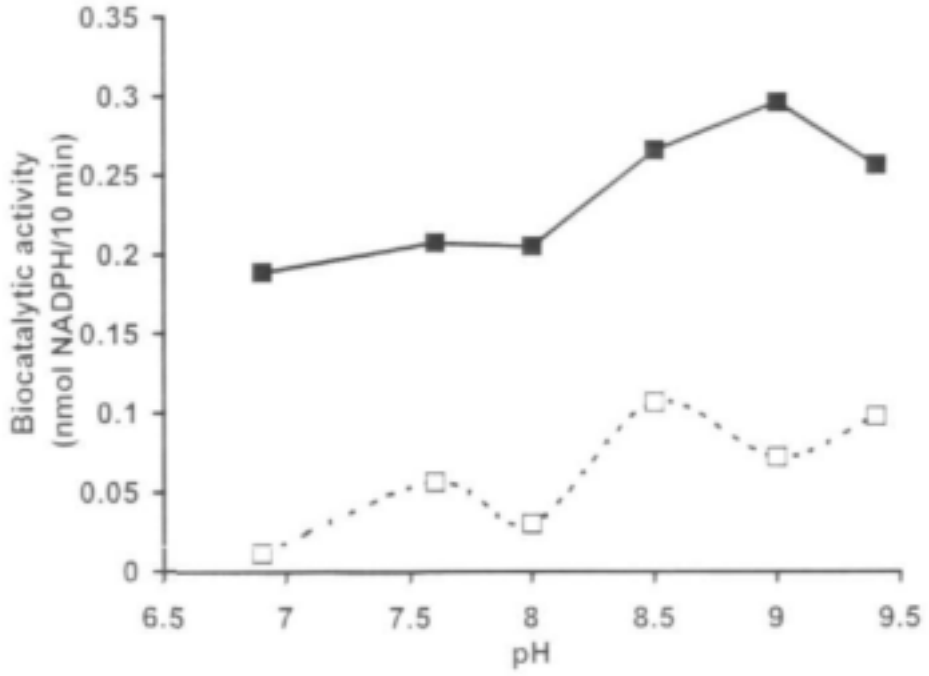


Figure 4. The effect of pH on ATPS biocatalytic activity. $ATPS_{MR}$ (□) and $ATPS_{SR}$(■).

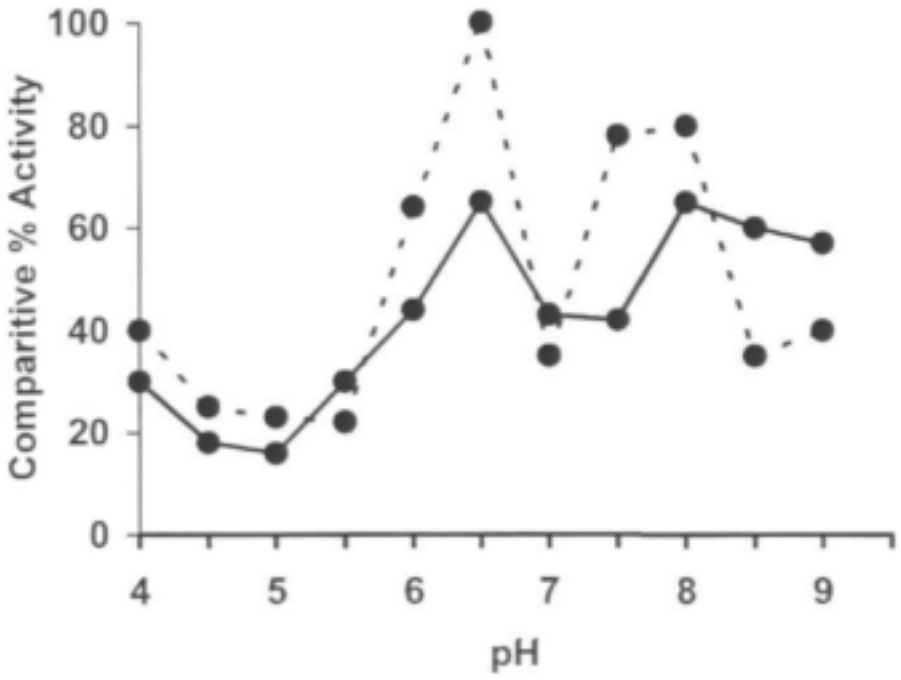


Figure 5. pH profiles of Lipase (●) activities in sewage sludge. Methanogenic (- -) and Sulphidogenic (—)

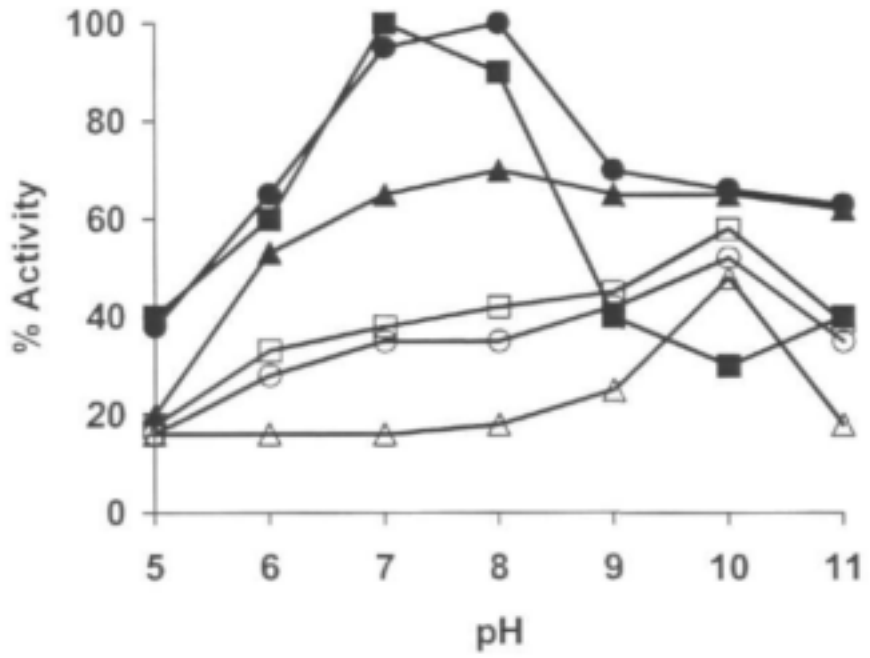


Figure 6. Combined pH and temperature profiles for proteases from sulphidogenic sludge. (■) 70°C; (●) 60°C; (▲) 50°C; (□) 40°C; (O) 30°C; (Δ) 20°C

3.3. Temperature.

The general pH profile with the higher alkaline and characteristic pH 10 proteolytic activity, stayed the same up to 60°C (Figure 6), with activities of all the proteases increasing with increased temperature. Above 50°C, however, the characteristic profile changed, with neutral proteases becoming more prominent, surviving temperatures of 70°C. The pH 7 and 10 proteases from the methanogenic bioreactor had temperature optima at 50°C, followed by a decrease at higher temperatures. Sulphidogenic and methanogenic protease enzymes at pH 5, on the other hand, had maximum activity at 30°C (Figure 7). The pH 7 and 10 proteolytic activities of sulphidogenic bioreactors showed temperature optima at 60°C, followed by a decrease in activity at higher temperatures (Figure 7) while both methanogenic and sulphidogenic phosphatases had optimum activity at 60°C. Authentic bacterial proteases [commercially available pronase E] showed a marked reduction in activity at these elevated temperatures (60°C and 70°C) when compared with the enzymes from the sewage sludge. This implied that the latter enzymes, apart from being stabilised by immobilization on the organic particulate matter, were in addition, more stable to the higher temperatures. $ATPS_{MR}$ was found to exhibit a temperature optimum of 50°C, while $ATPS_{SR}$ exhibited a temperature optimum at 55°C (Figure 8). Both enzymes rapidly lost activity above their temperature optima, and only retained 0-17% of their maximal activity above 70°C. Arrhenius plots were constructed for both ATPS enzymes using temperature optimum data and apparent activation energies (E_{aapp}) (apparent transition state energies) were calculated from the slope $-2.303E_a/R$ (Figure 9). E_{aapp} values for $ATPS_{MR}$ and $ATPS_{SR}$ were 7.37 $J.mol^{-1}$ and 11.94 $J.mol^{-1}$ respectively. The activity of the methanogenic and sulphidogenic lipases were maximal at 50 °C and 60 °C respectively (Figure 10). These relatively high temperatures reflect the fact that the enzymes are stabilized by being immobilized on the organic particulate matter.

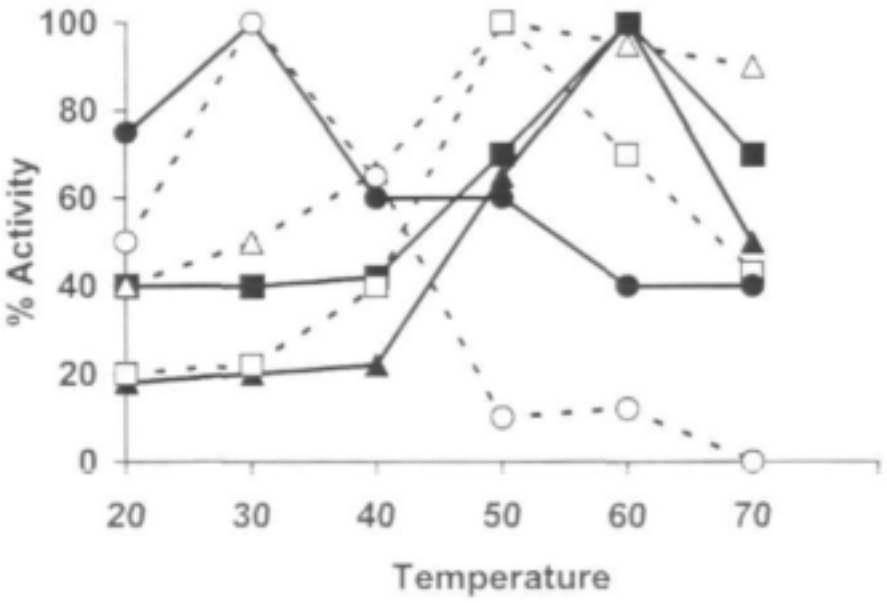


Figure 7. Temperature profiles of proteases from sulphidogenic (—) bioreactor : pH 5 (●), pH 7 (■), pH 10 (▲) and methanogenic (- - -) bioreactor : pH 5 (○), pH 7 (□), pH 10 (△).

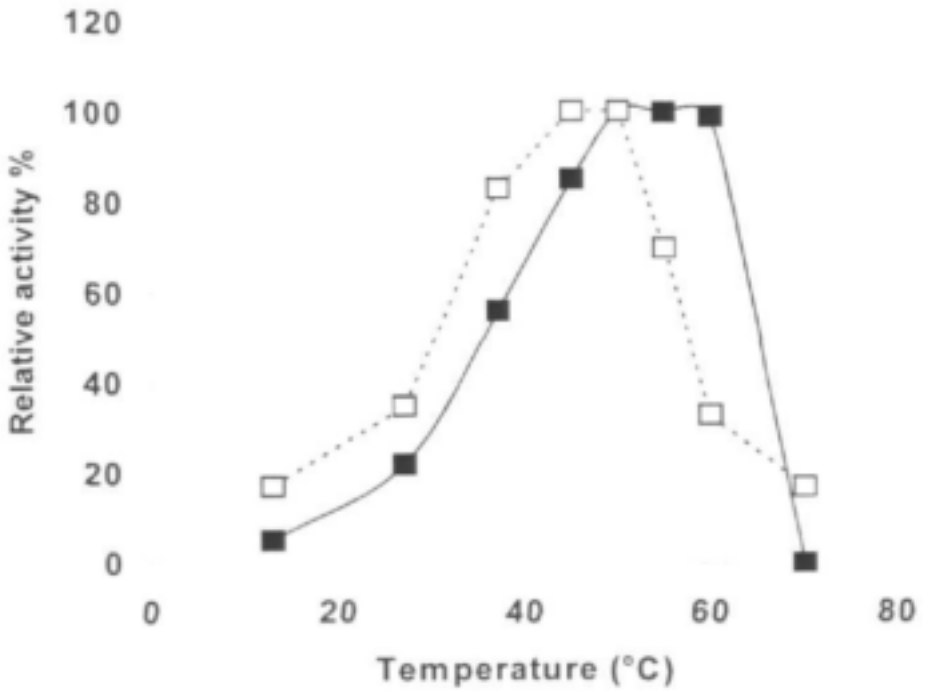


Figure 8 The effect of temperature on ATPS activity. $ATPS_{MR}$ (□) and $ATPS_{SR}$ (■).

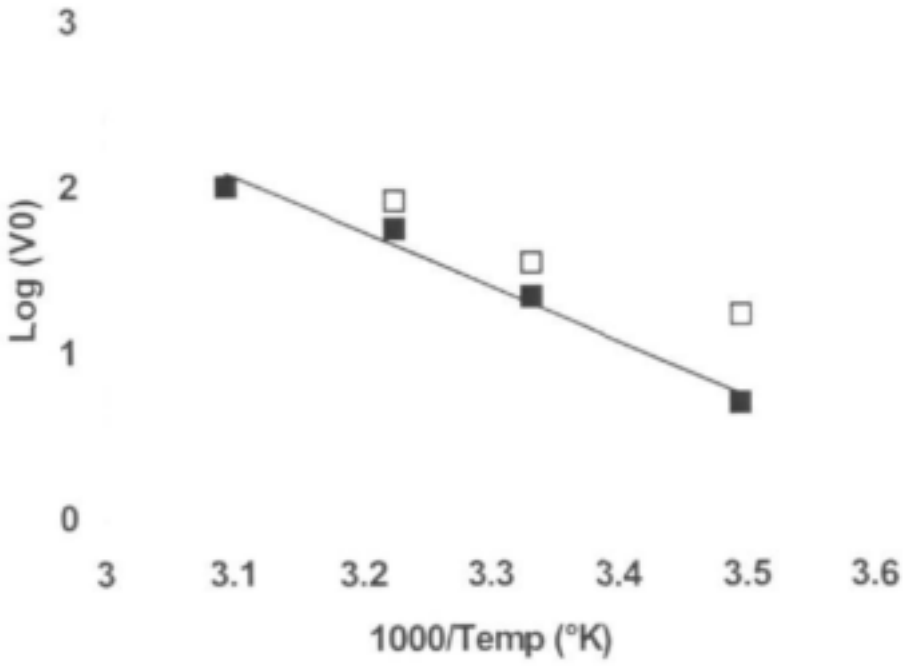


Figure 9 Arrhenius plots for the ATPS enzymes. $ATPS_{MR}$ (□) and $ATPS_{SR}$ (■).

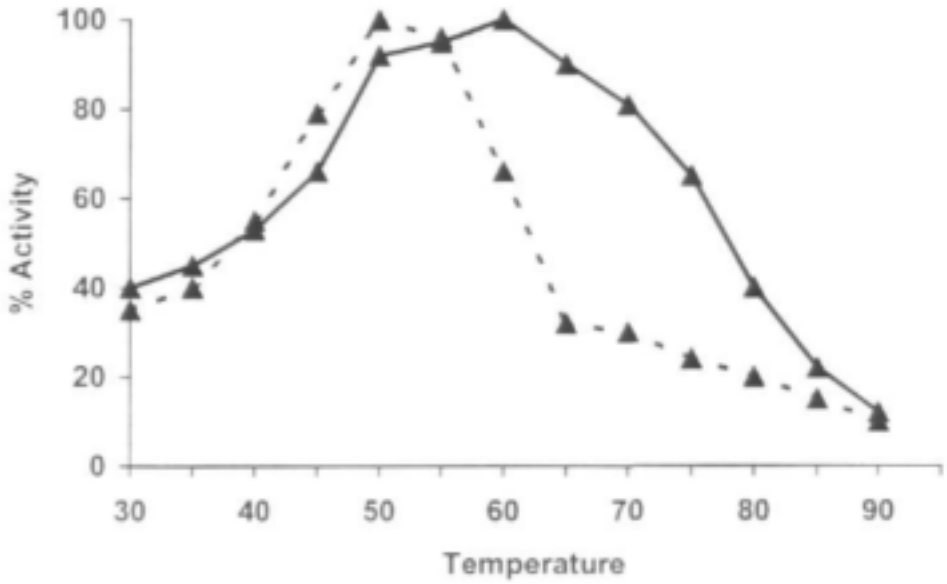


Figure 10. Temperature profiles for Lipases (▲) in sewage sludge Methanogenic: (- -) Sulphidogenic (—)

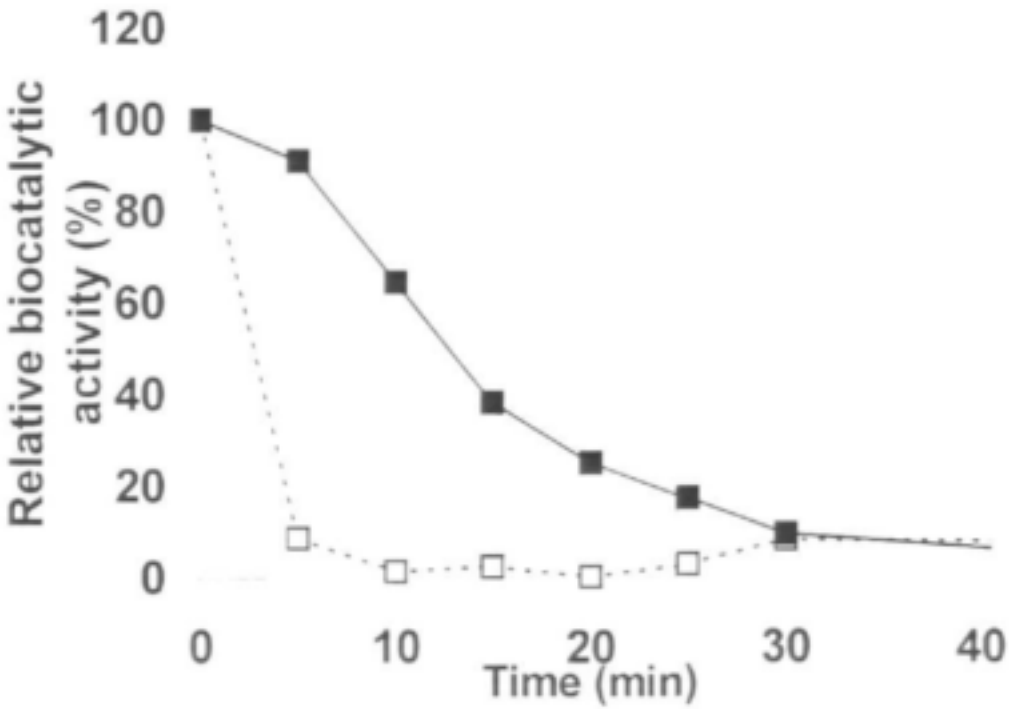


Figure 11 The stability profiles of the ATPS enzymes at their optimum temperatures. $ATPS_{MR}$ (□) and $ATPS_{SR}$ (■).

3.4. Stability.

The proteases from both the methanogenic and sulphidogenic bioreactors at all pH values had extended stability, at their respective optimum temperature, with only a slight decrease of less than 20% in activity after two hours, thereafter staying relatively constant for at least 5 hours. Phosphatase activity on the other hand decreased by 50% in activity after two hours for methanogenic enzymes and after 4 hours for sulphidogenic enzymes. $ATPS_{SR}$ exhibited a higher degree of temperature stability at its temperature optimum (55°C) compared to $ATPS_{MR}$ (50°C)(Figure 11). The relative biocatalytic activity of $ATPS_{SR}$ gradually decreased over 30 min, while $ATPS_{MR}$ was rapidly inactivated within the first 5 min (compared to 91% activity remaining after 5 min in the case of $ATPS_{SR}$). $ATPS_{SR}$ therefore exhibited not only a higher temperature optimum, but also a slightly higher degree of temperature stability. A reduction in relative biocatalytic activity of 98% and 35% over 10 min (normal time in standard assay conditions employed) was observed for $ATPS_{MR}$ and $ATPS_{SR}$, respectively. Both $ATPS_{MR}$ and $ATPS_{SR}$ lost all biocatalytic activity after 30 min at their respective optimum temperatures. The methanogenic and sulphidogenic lipase activities showed differing stability profiles, with only a slight drop in activity after one hour for methanogenic enzymes at 50°C (Figure 12). The sulphidogenic lipase enzymes, however, showed a 100 % decrease in activity within an hour at 60 °C.

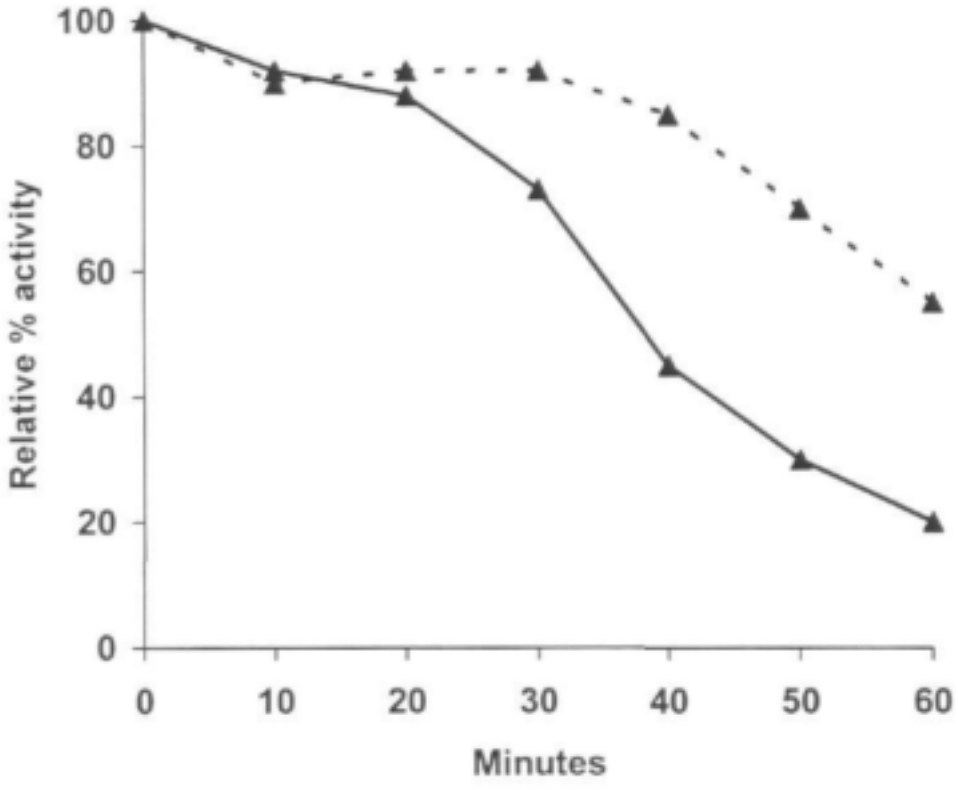


Figure 12. Lipase stability (▲) Methanogenic (- - -) and sulphidogenic (—) bioreactors

3.5. Effects of sulphate, sulphite and sulphide.

3.5.1. Proteases

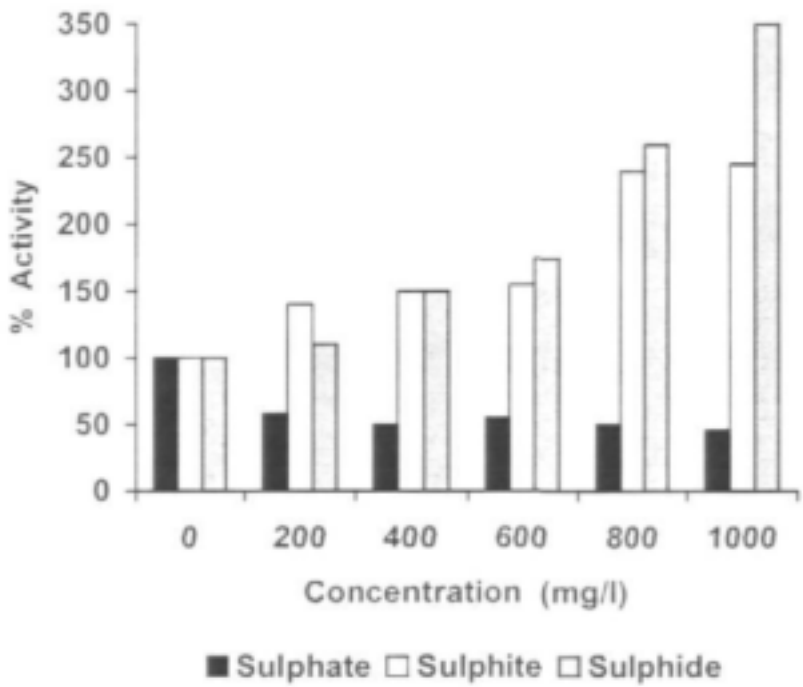


Figure 13. Effect of sulphate, sulphite and sulphide on sulphidogenic proteases.

Sulphate, showed a marked inhibition on protease activities from the sulphidogenic bioreactor at pH 7 with a 40 % reduction in activity at 200 mg l^{-1} sulphate extending to 50 % reduction in activity with 1000 mg l^{-1} sulphate (Figure 13). The enzyme at pH 7 showed a 3.5 fold increase in activity at 1000 mg l^{-1} sulphide and a gradual increase in activity with increasing concentration of sulphite, the most prominent effect being observed at a sulphite concentration of 800 to 1000 mg l^{-1} (Figure 13). All assays were in triplicate.

3.5.2. Lipases

A twelve-fold increase in lipase activity was observed with 400 mg sulphate l^{-1} present while nearly a 2-fold increase in activity was seen at 800 mg sulphite l^{-1} and dramatic changes in lipase activity was detected in the presence of sulphide (Figure 14). With only 400 mg sulphide l^{-1} a 5-fold increase in sulphidogenic lipase activity was observed increasing to 10-fold with 800 mg l^{-1}.

3.5.3. Sulphatases

There appeared only a slight change in activity of the sulphatases with 600mg sulphate l^{-1} (Figure 15). Increasing sulphide and sulphite concentrations showed a noticeable difference, however, with between 80 – 100% increase at concentrations of 600 – 800 mg l^{-1} (Figure 15).

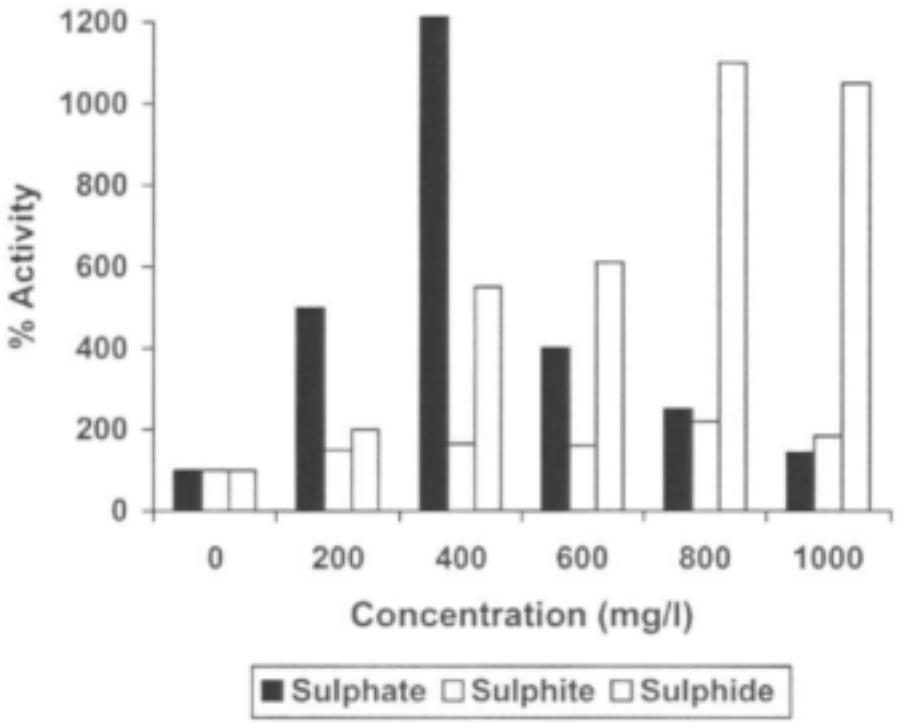


Figure 14. Effect of sulphate, sulphite and sulphide on sulphidogenic lipases

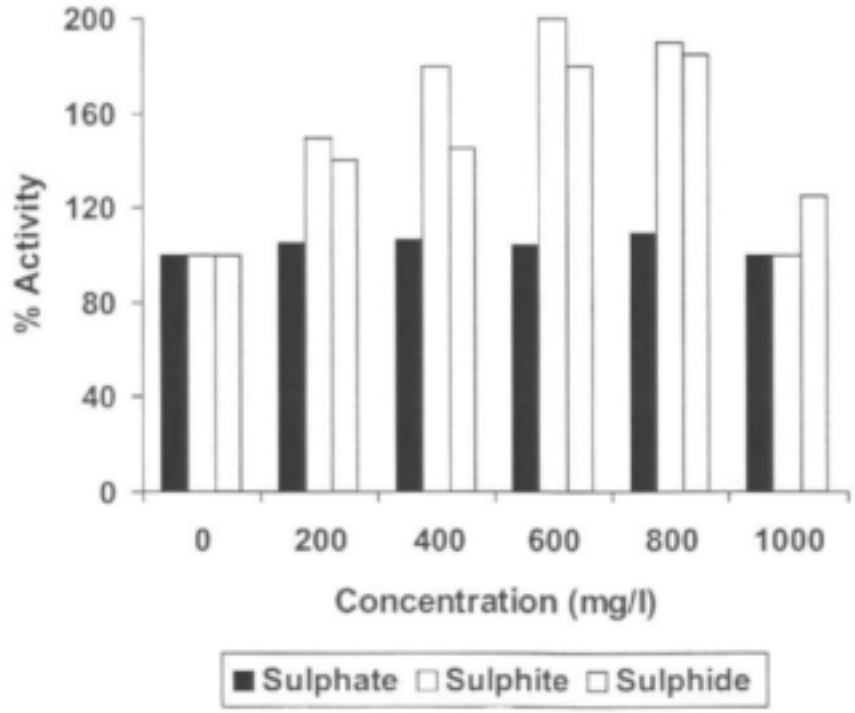


Figure 15. Effect of sulphate, sulphite and sulphide on sulphidogenic sulphatases

3.5.4. Glucosidases

Sulphate, showed very little effect on β-glucosidase activities from both the sulphidogenic- and methanogenic- (control) bioreactors. (Figure 16). However, more β-glucosidase activity was realised in the presence of sulphite and sulphide which is liberated as products during sulphate reduction. At between 200 – 600 mg sulphite l^{-1} there was a 2.5 fold increase in activity of methanogenic β-glucosidase compared to 1.7-2 fold increase in activity within the sulphidogenic bioreactor (Figure 17). There appeared a gradual decrease in enzyme activity at 600 – 1000 mg sulphite l^{-1} though values still remained higher than in the controls. It should immediately be noted that in the case of the sulphidogenic bioreactor the data represented is that for 'added' sulphite and would not take into account inherent sulphite already produced during the reduction process. The activation of the β-glucosidase enzymes from both reactors was even more pronounced in the presence of sulphide (Figure 18). At 200 mg sulphide l^{-1} there was a 1.7 fold increase in β-glucosidase activity in the methanogenic bioreactor compared to control values and

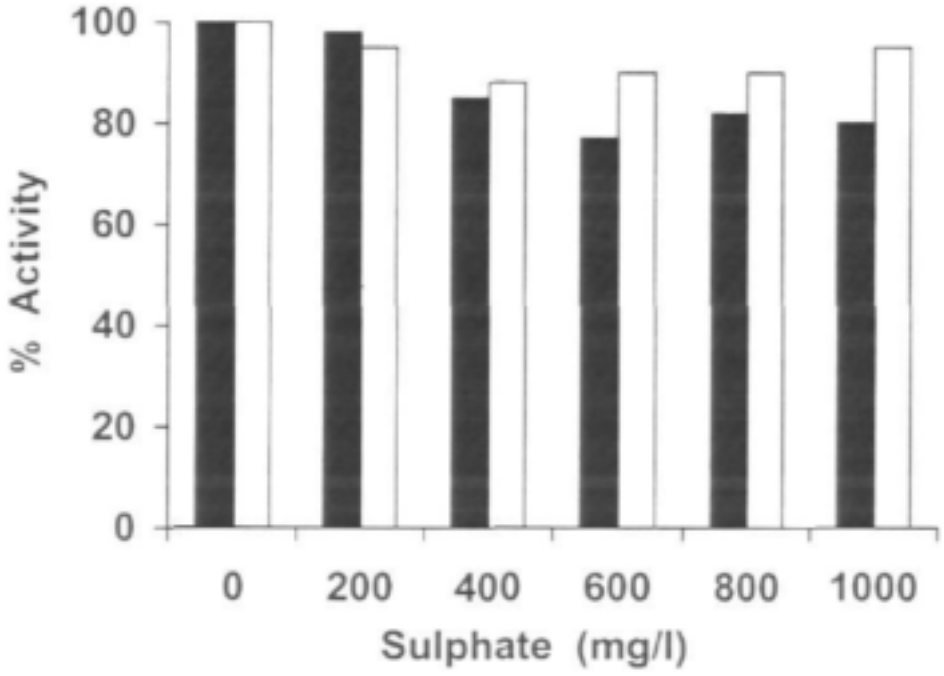


Figure 16. Effect of sulphate on β-glucosidase in sulphidogenic ⁻ and methanogenic ■ bioreactors: [100% β-glucosidase activity = 226.4 μmoles/min/mg dried sludge powder]

this increased to 3.6 and 6 fold respectively with 400 and 600-800 mg sulphide l^{-1}. Bearing in mind the inherent sulphide already present in the sulphidogenic bioreactor due to the sulphate reduction process, the effect on the β-glucosidase activity with added sulphide is less noticeable at lower concentrations of sulphide. Once again a six-fold increase of activity resulted at 600-800 mg sulphide l^{-1}. Sulphate and sulphide (0-1000 mg/l) had no effect on the activity on $ATPS_{SR}$, while sulphite had a slight inhibitory effect on $ATPS_{SR}$ activity. The absence of a strongly inhibitory effect of sulphate on $ATPS_{SR}$ activity was particularly surprising, as sulphate has been reported to have a strongly inhibitory effect on the activity of the reverse ATP-sulphurylase reaction (Segel, *et al.*, 1987).

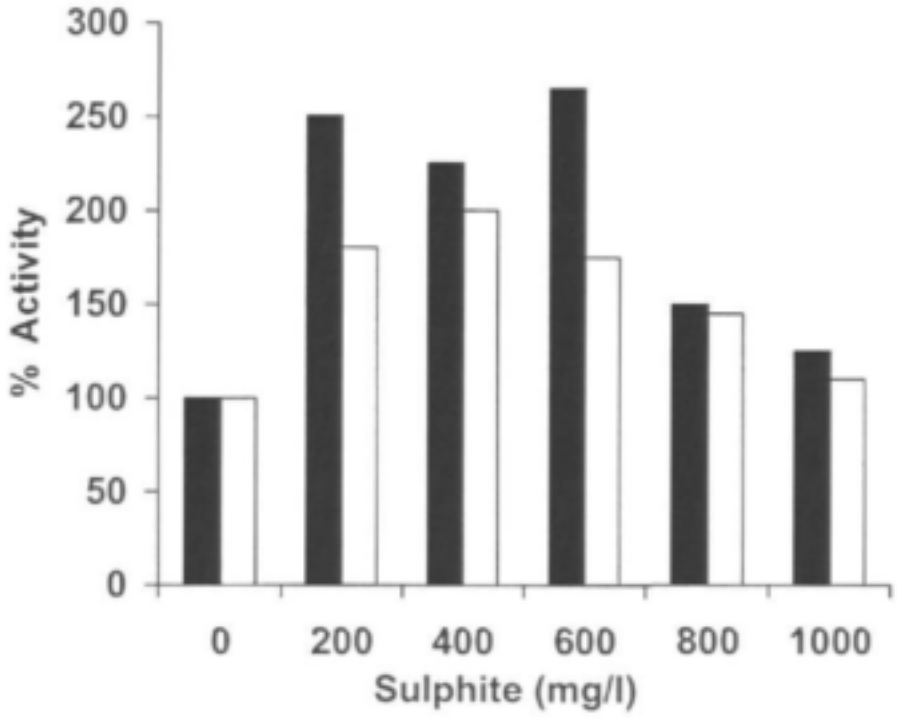


Figure 17. Effect of sulphite on β-glucosidase from sulphidogenic □ and methanogenic ■ bioreactors; [100% β-glucosidase activity = 226.4 μmoles/min/mg dried sludge powder]

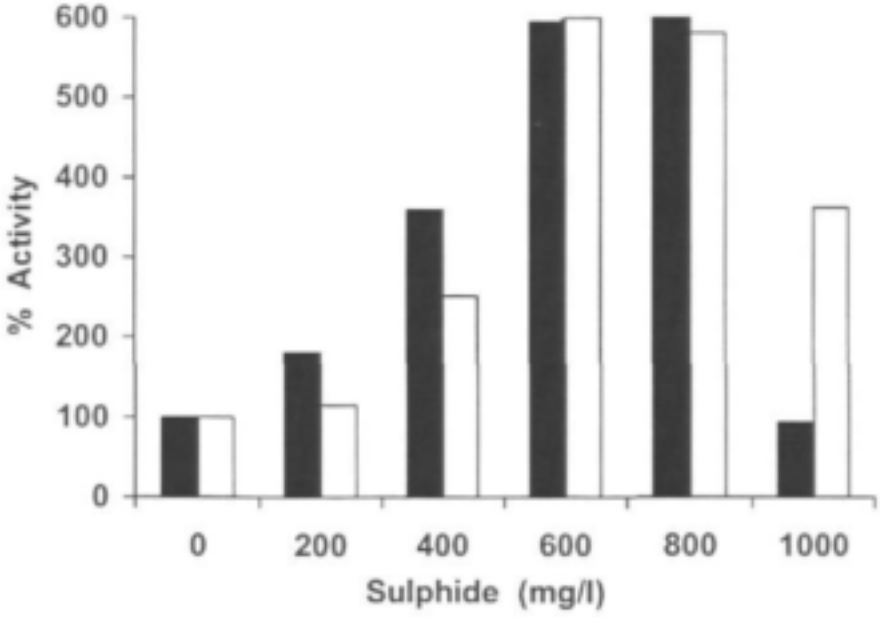


Figure 18. Effect of sulphide on β-glucosidase in sulphidogenic □ and methanogenic ■ bioreactors.

3.6. Time-course study

As expected the sulphate levels dropped over the first 5 days from 1000 mg.l^{-1} to 400 mg.l^{-1} with a concomitant increase in sulphide. Final concentrations of sulphide reached 160 mg.l^{-1} by day

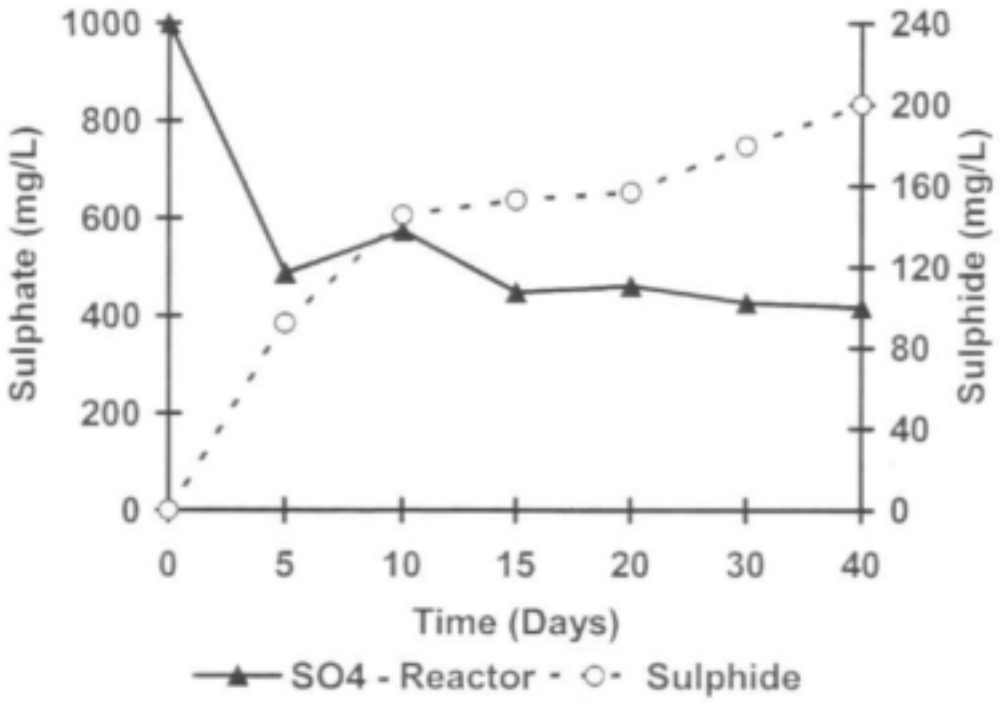


Figure 19 Time course concentrations of sulphate and sulphide within the RSBR

20 then gradually increasing to 200 mg.l^{-1} by day 40 (Figure 19). In agreement with these finding is the increase in sulphide concentration with depth of the RSBR (Figure 20). The specific activities of glucosidases, proteases and lipases showed similar trends with respect to depth within the RSBR. Glucosidase activity was 11 µmol/h/mg protein at the 16cm depth in the RSBR and this changed to 42 µmol/h/mg protein at 32cm and 65 µmol/h/mg protein at 50cm (Figure 21). Proteases, on the other hand, showed 2, 5 and 11 µmol/h/mg protein respectively at the three depths. There was very little lipase activity (0.2 µmol/h/ml/µg protein) at a depth of 16cm but this changed to 3.1 µmol/h/ml/µg protein and 4 µmol/h/ml/µg protein at depth 32cm and 50cm respectively.

Coupled with the increased activity of the glucosidases is the relative increase in monosaccharide carbohydrates with increasing depth. At a depth of 16 cm the carbohydrate content was only 1.2 mg.l^{-1} and this increased to 4.2 mg.l^{-1} further down the RSBR (Figure 22). Changes in the levels of protein with depth were not as conclusive inferring proteolytic action. Though very little lipid was detected just below the surface of the RSBR (0.15mg.l^{-1} at 16cm depth) its concentration was evident at 0.5 mg l^{-1} in a more anaerobic environment towards the middle and at the bottom of the reactor. These facts support the general observation that the top fraction of the reactor is always very low in concentration of carbohydrates, proteins and lipids.

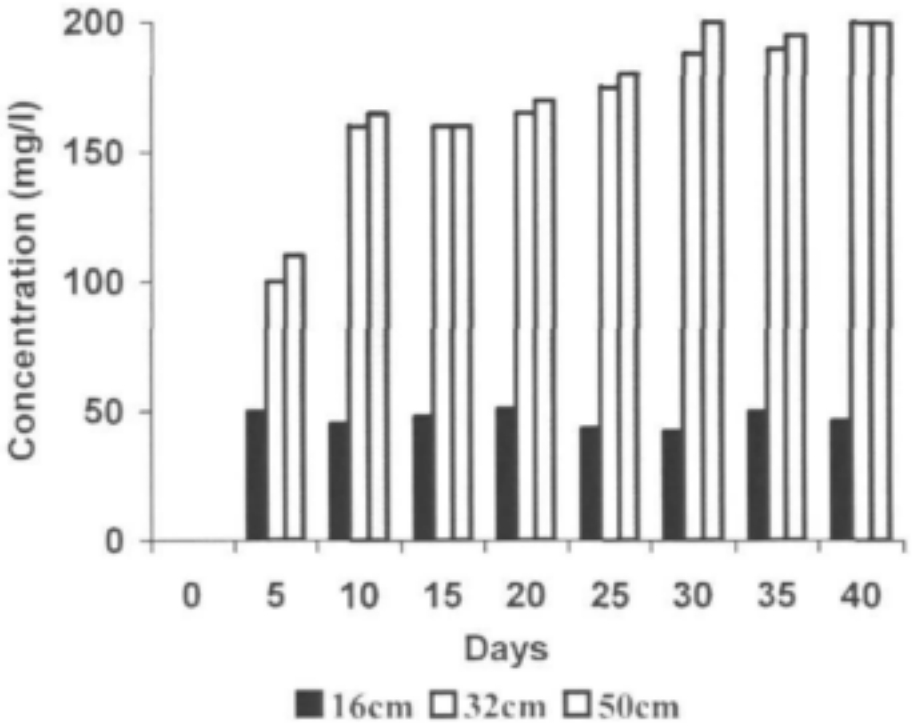


Figure 20. Time course values of sulphide with respect to depth within the RSBR

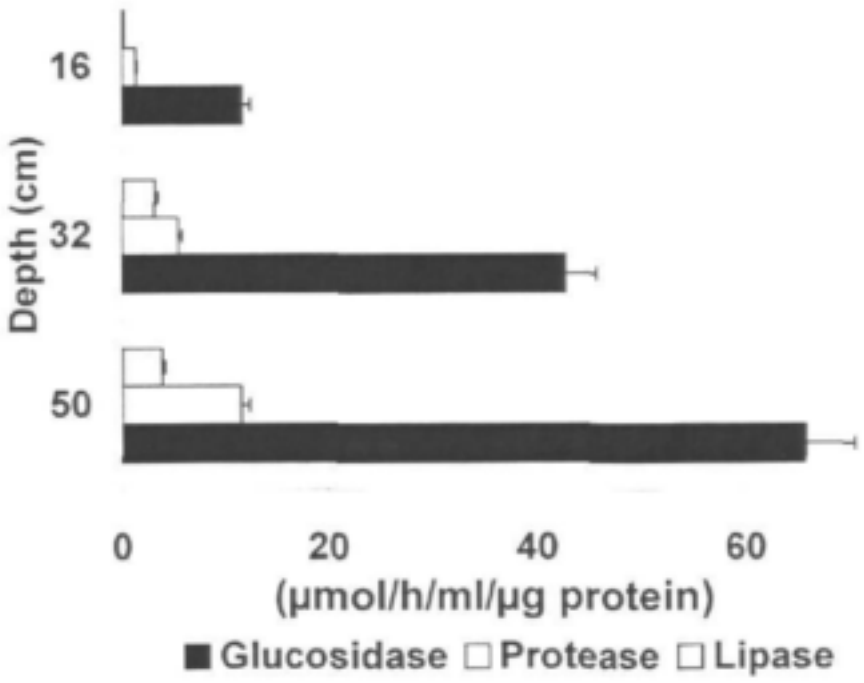


Figure 21. Specific activity of glucosidase, protease and lipase at different depths.

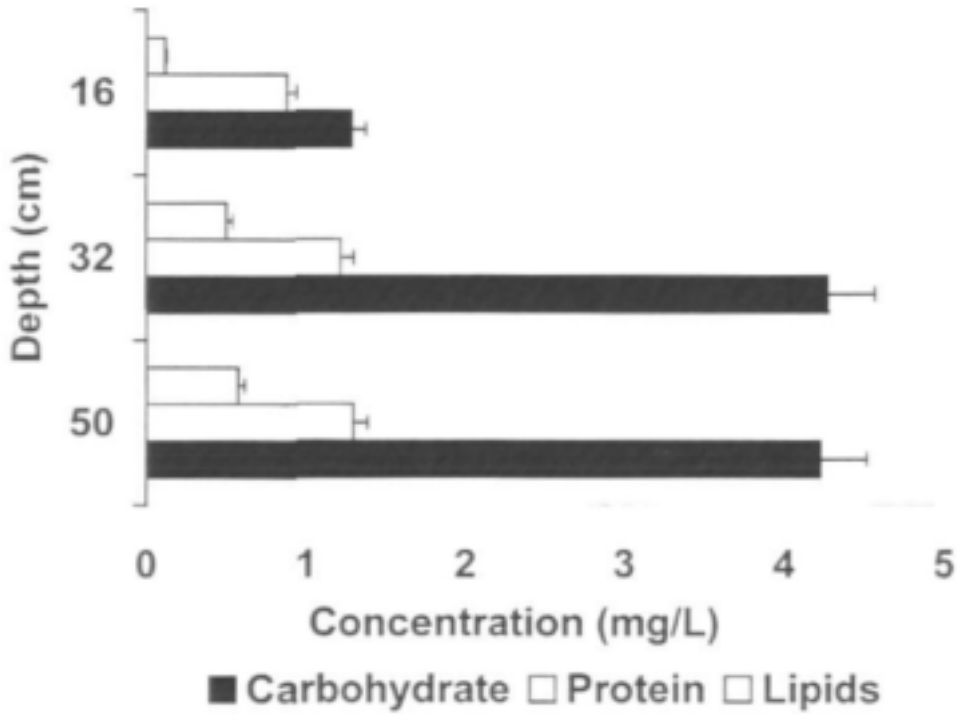


Figure 22. Concentration of carbohydrate, protein and lipid at different depths.

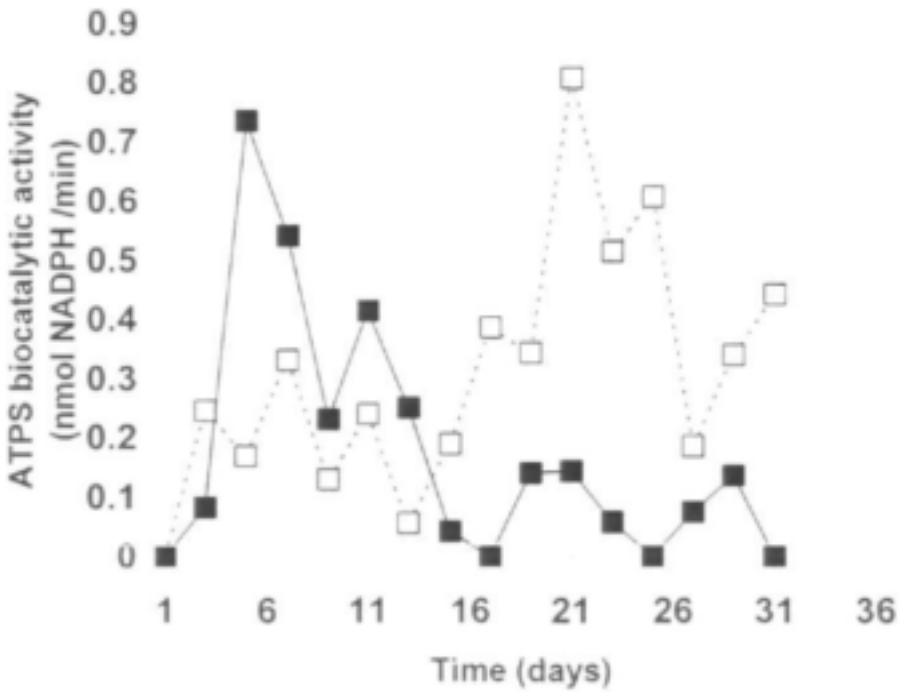
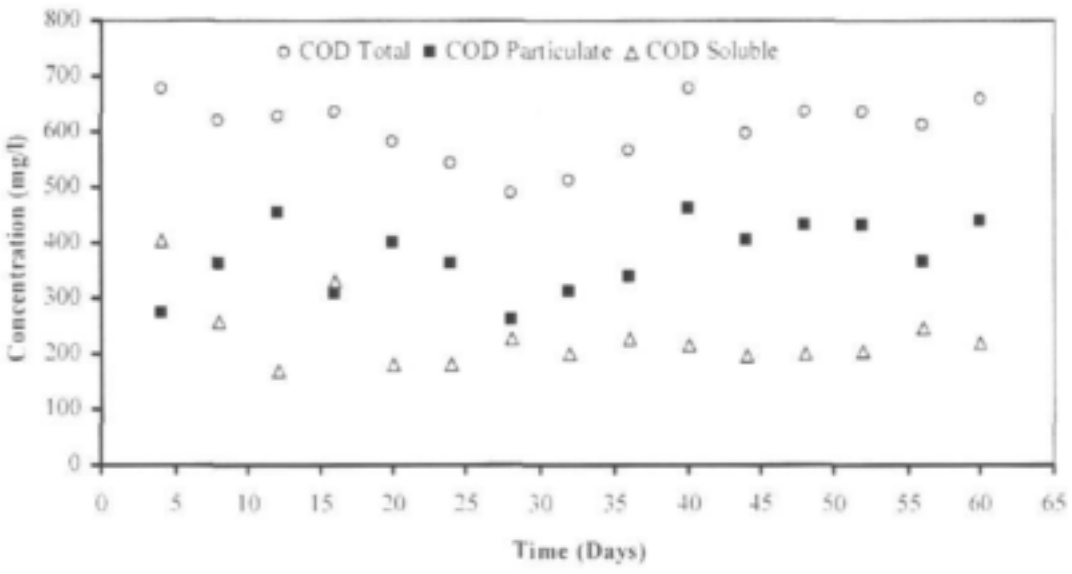


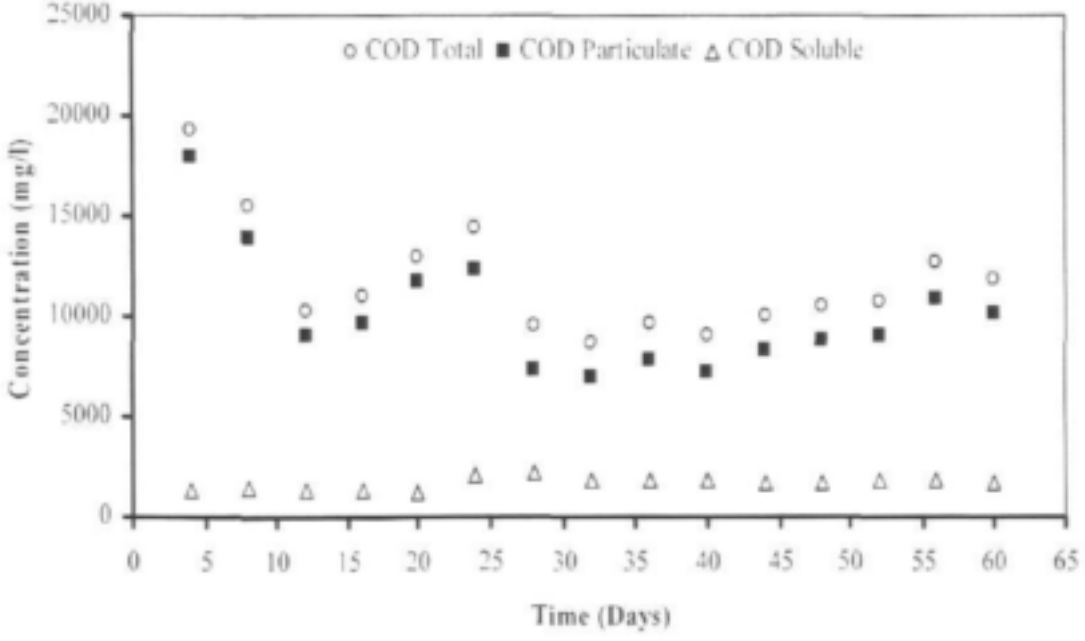
Figure 23. The level of ATPS activity in the closed system bioreactors. $ATPS_{MR}$ (□) and $ATPS_{SR}$ (■).

$ATPS_{SR}$ activity rapidly increased over the first few days in the closed system bioreactor and reached a peak on day five (0.73 nmoles NADPH/min), followed by a gradual decline in activity over the remainder of the time course (Figure 23). $ATPS_{MR}$ activity in the closed methanogenic reactor was reasonably low in the first two weeks, but then, surprisingly, an increase in activity was observed from day 16 onwards, reaching a peak on day 21 (0.82 nmoles NADPH/min). $ATPS_{MR}$ activity in the closed methanogenic reactor remained high, even after 31 days. The increased activity of $ATPS_{SR}$ over the first five days correlated with the decrease in reactor sulphate concentration over the first nine days (from 1000 mgl^{-1} on day 1 to 440 mgl^{-1} on day 9) (Figure 19)

a)



b)



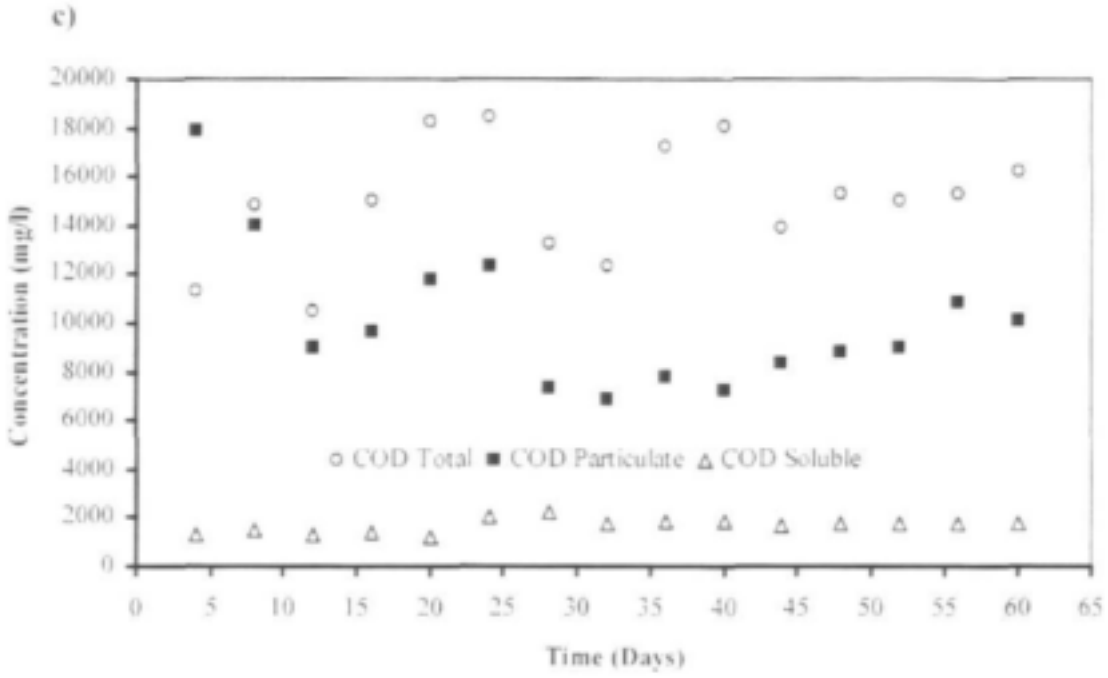


Figure 24. Variation of COD_{Total}, $COD_{Particulate}$) and $COD_{Soluble}$ during the 60 day period for a) depth 1, b) depth 2 and c) depth 3.

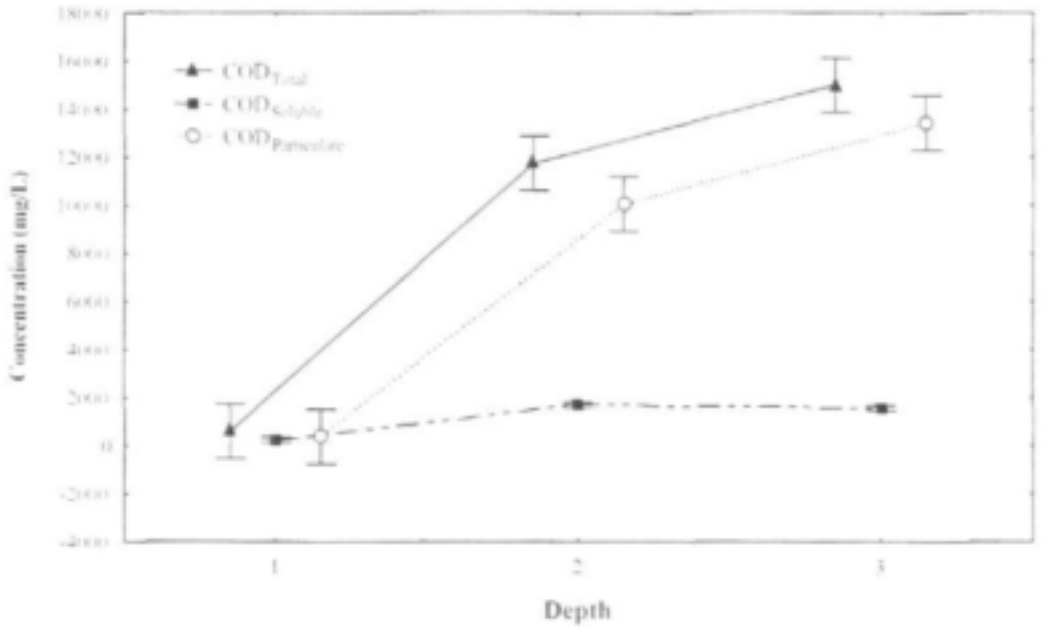


Figure 25 Variation of COD_{Total}, $COD_{Particulate}$ and $COD_{Soluble}$ for depth 1, depth 2 and depth 3 for RSBR.

The experimental results for COD_{total} $COD_{particulate}$ and $COD_{soluble}$ over time are presented (Figure 24), where as Figure 25 shows the variation with depth 1(16cm), depth 2 (32cm) and depth 3 (50cm). There was COD fluctuation in all depths throughout the experimental period

emphasizing that the reactor continuously adjusts to COD loading. The total COD had a maximum of 677.33 mgl^{-1} in day 40 to a low of 488.67 mgl^{-1} in day 28 for depth 1. The maximum COD concentrations for depth 2 was at day 4 with values at 19233.33 mgl^{-1} and a minimum of 8666.67 mgl^{-1} in day 32, thus showing COD removal. With depth 3 the minimum was at day 12 with total COD concentration of 10433 mgl^{-1} and a maximum of 18433 mgl^{-1} in day 24. The mean total COD concentration for depth 1, depth 2 and depth 3 was 603.64 mgl^{-1}, 11735 mgl^{-1}, and 14988.89 mgl^{-1} respectively. This showed that the COD increased from depth 1 to depth 3. The $COD_{soluble}$ concentrations in all three depths were lower in comparison to COD_{total} with a mean of 230.33 mgl^{-1} , 1681.6 mgl^{-1} and 1552.73 mgl^{-1} for depth 1, depth 2 and depth 3 respectively. $COD_{particulate}$ was obtained as the difference between the COD_{total} and $COD_{soluble}$ with means being for depth 1, 373.31 mgl^{-1}; depth 2, 10054 mgl^{-1} and depth 3, 13436 mgl^{-1}.

The variation of alkalinity, pH and temperature of the feed and reactor (depth 1, depth 2 and depth 3) over the period of study is presented in Figure 26 and 27. The alkalinity (measured as mgl^{-1} $CaCO_3$) increased from 72.83 mgl^{-1} in the feed to 353.57 mgl^{-1}, (depth 1); 1453.77 mgl^{-1}, (depth 2) and 14774 mgl^{-1}, (depth 3) respectively, due to reduction of sulphate to sulphide. The average pH for depth 1, depth 2 and depth 3 of the reactor was 7.32. Temperature profiles for the experimental period are shown in Figure 27. They stayed relatively constant during the treatment with a maximum at 21.8 °C and a minimum at 18 °C. Since hydrolysis is a biochemical reaction catalysed by enzymes, the hydrolysis is highly dependent on temperature.

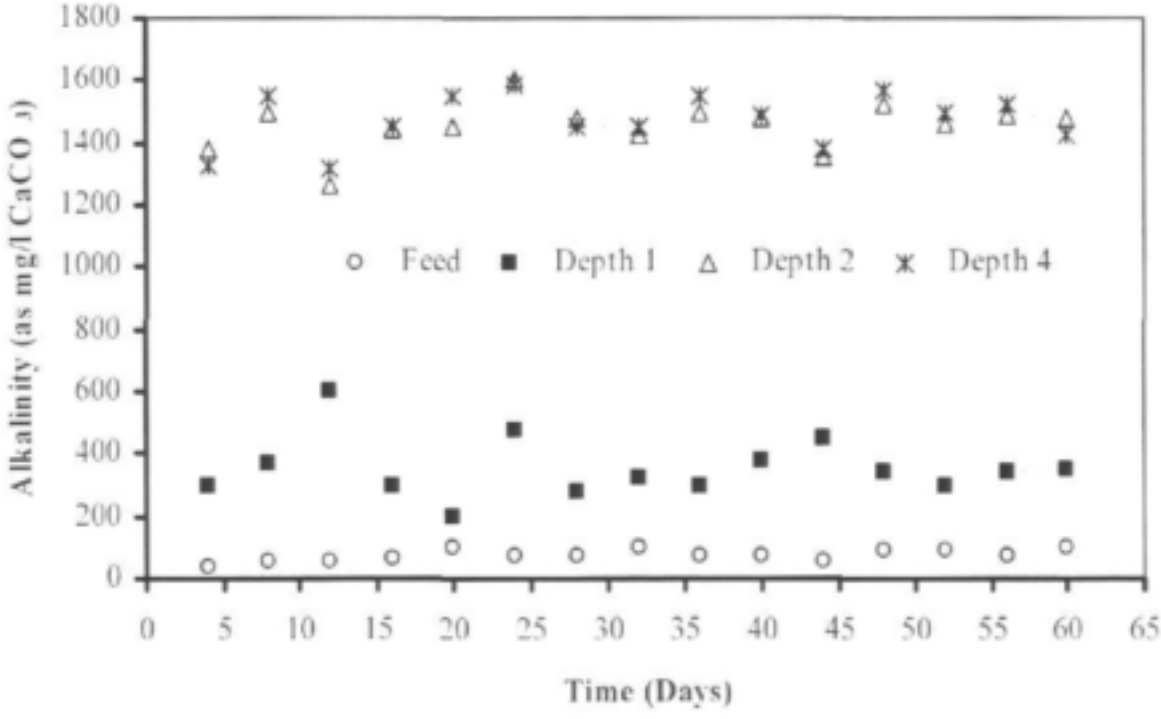


Figure 26 Variation of alkalinity with time during the experimental period. All points represent means of triplicate values

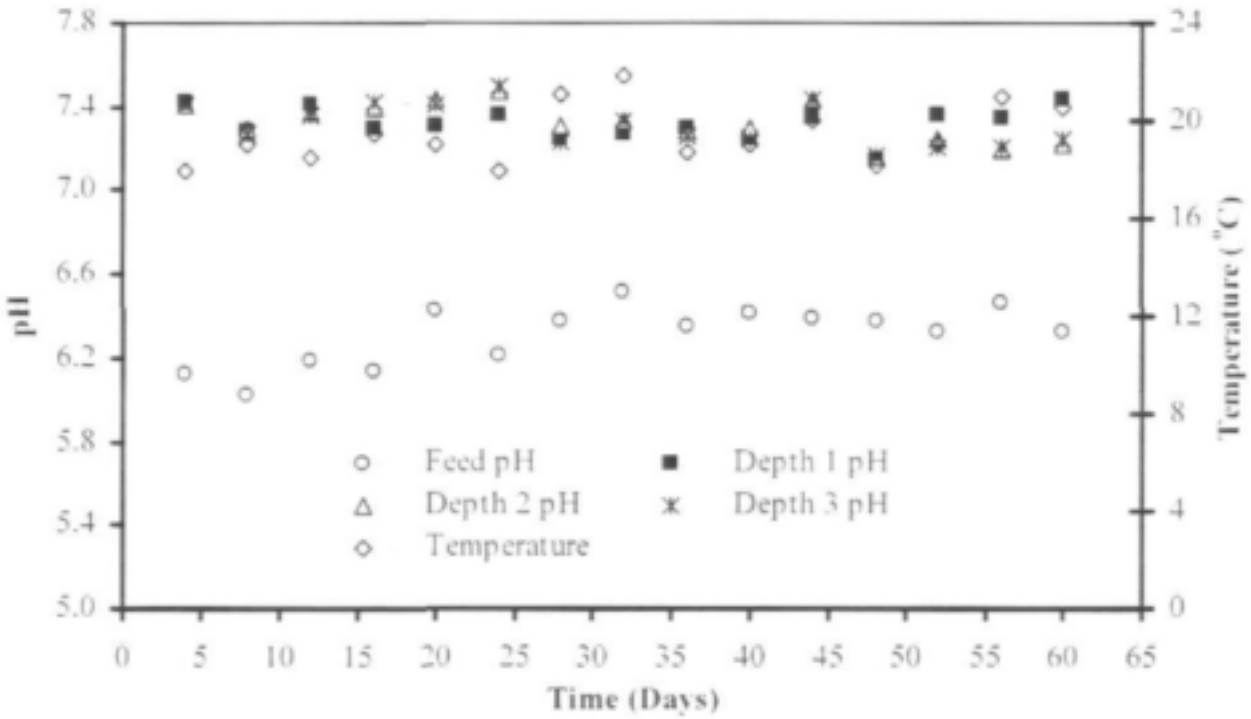


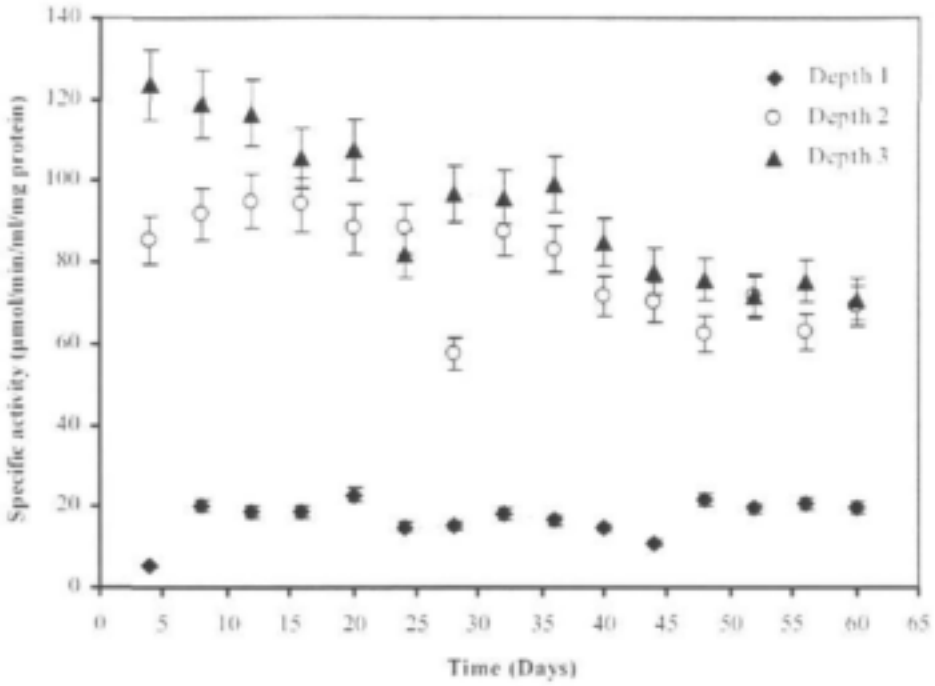
Figure 27 Temperature and pH profile during the study period. All points are means of three values

After the start-up period had elapsed the RSBR was run continuously and observations were made for 60 days. The RSBR was fed with primary sewage sludge (sieved through a 2mm mesh sieve, and diluted to a COD of 2000 mgl^{-1}, and sulphate was added at a concentration of 2000 mgl^{-1}, giving a 1:1 COD/SO_4 ratio. This was done with a loading rate of 0.01152 $kgCOD^{-1}$ per day with a hydraulic retention time (HRT) of 48 hours.

The variation of α-glucosidase and β-glucosidase activities respectively, in depth 1, depth 2 and depth 3 of the RSBR under steady state conditions during the experimental period are shown (Figure 28a & b). Depth 3 showed a general decrease in specific activity of 123.645 to 70.94 μmol/min/mg protein for day 4 and day 60 respectively, whereas for depth 2, maximum specific activity was obtained in day 12 and minimum on day 28 with specific activities of 94.96 and 57.37 μmol/min/mg protein respectively. The α-glucosidase activity increased significantly from depth 1 to depth 3 (ANOVA, $p<0.005$, $df = 44$). β-glucosidase activity increased progressively at varying rates during the 60-day experimental period for all the three depths. Increase in depth in the RSBR showed a significant increase in β-glucosidase activity (ANOVA, $p<0.005$, $df = 44$) with highest activity observed in depth 3 (Figure 29a & b).

The time course behaviour of the specific activity of the proteases over the 60-day study for depth 1, depth 2 and depth 3 is shown (Figure 30a & b). A rise in protease activity was observed in depth 3 up to day 20 where the maximal protease specific activity was 3.75 μmol/min/mg

a)



b)

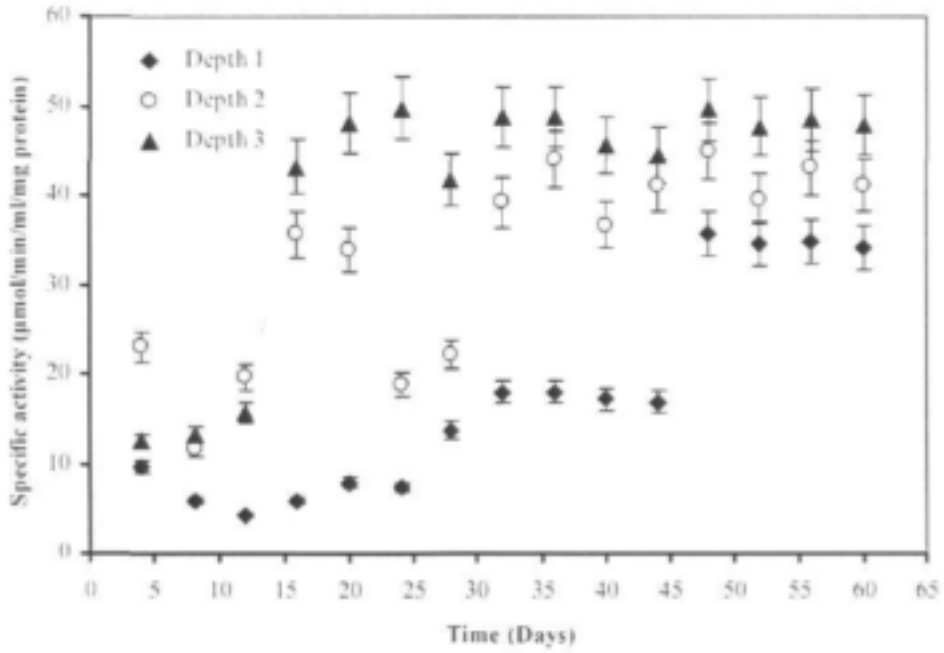


Figure 28. Variation of a) α-glucosidase and b) β-glucosidase activities with time in the RSBR. Each point represents the mean of determinations performed in triplicates. Standard deviations are represented by error bars

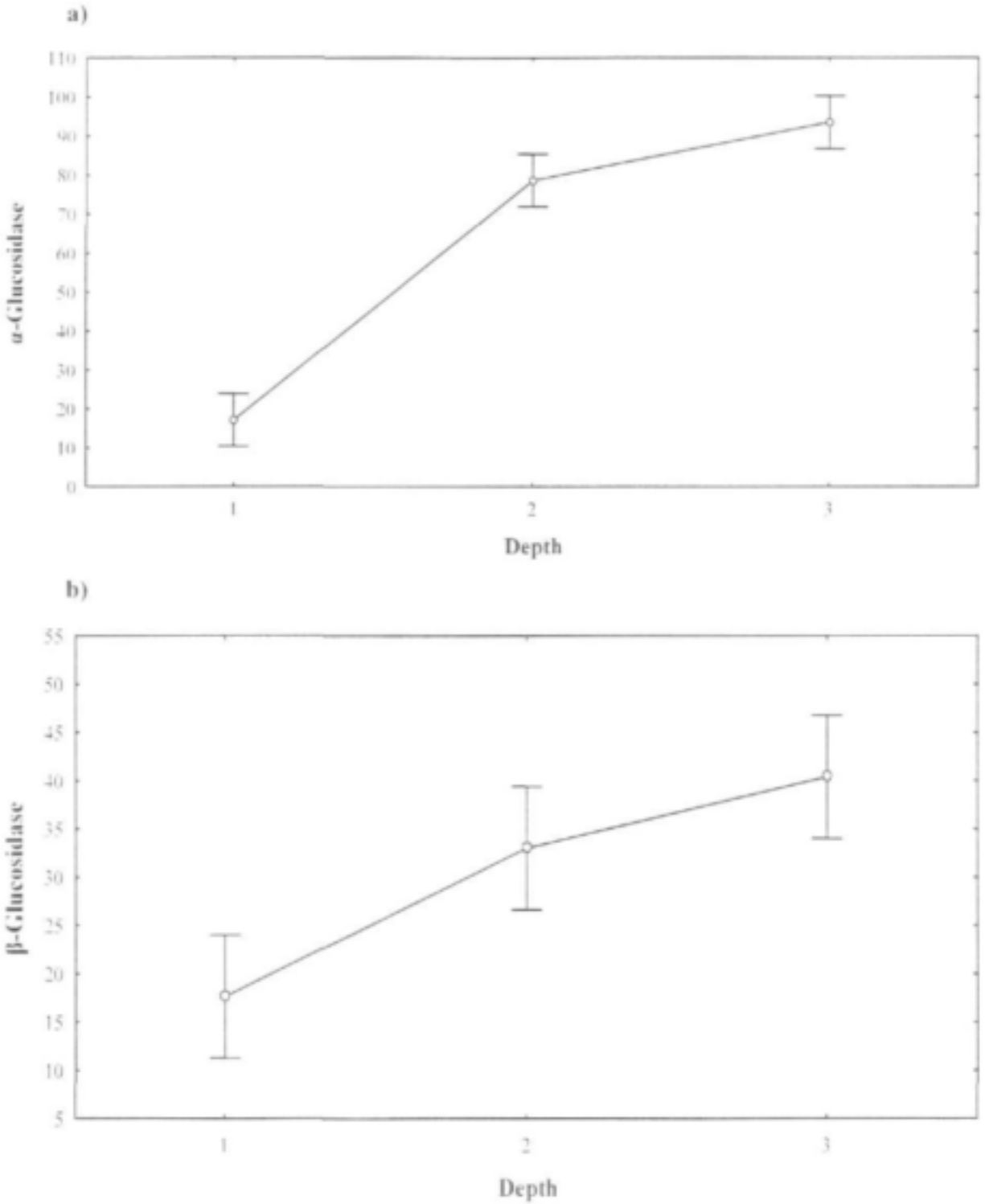


Figure 29 Variation of a) α-glucosidase and b) β-glucosidase activities with RSBR depth. Vertical bars denote 95% confidence intervals. $F(14, 72)=36.911$, $P < 0.001$. Enzymes are in specific activities with units of (μmol/min/ml/mg protein)

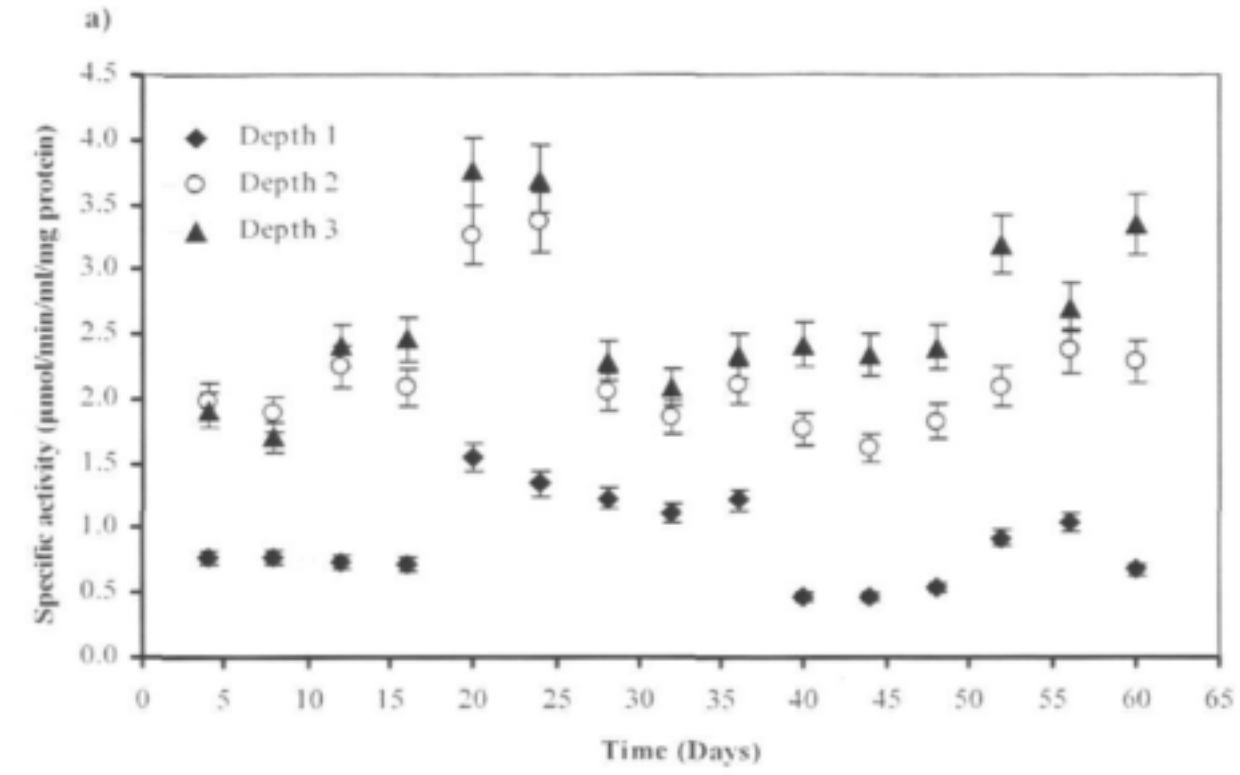
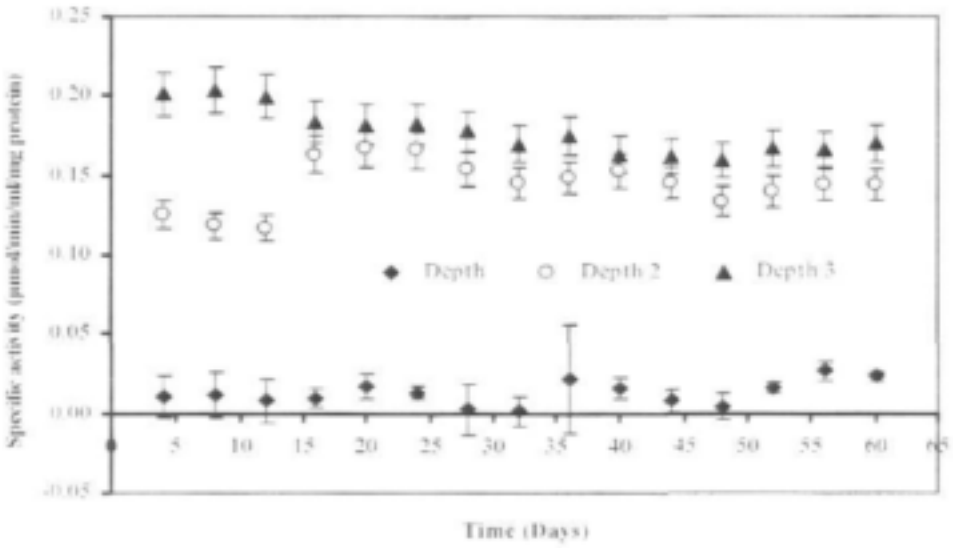


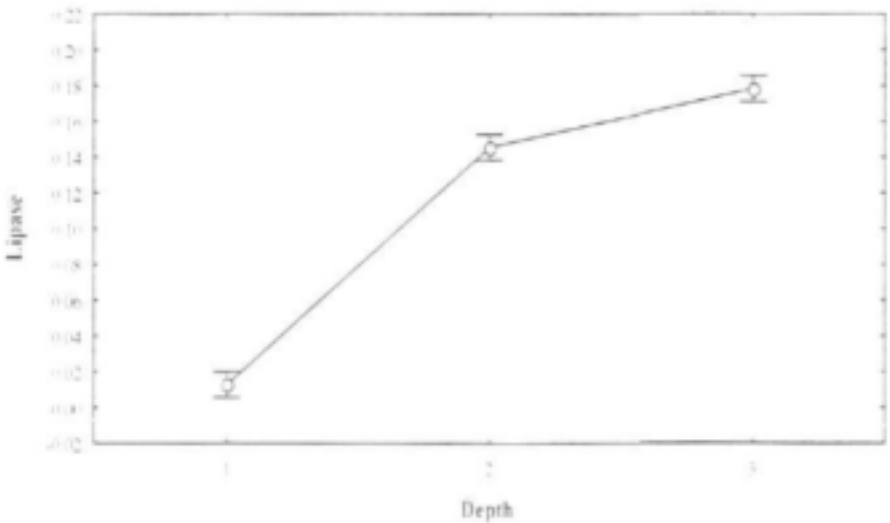
Figure 30. Variation of protease specific activity in the RSBR a) with respect to time and b) with respect to depth. Each point represents the mean of determinations performed in triplicate. Standard deviations are represented by error bars

protein. Depth 2 also showed a similar trend to depth 3 with the maximal protease specific activity of 3.37 µmol/min/mg protein occurring in day 24 of the study period. Protease specific activities were quite low for depth 1 with values of 1.55 µmol/min/mg protein seen in day 20.

A variation in the lipase activity was observed between day 12 and 20 at depth 2 of the RSBR with activity increasing from 0.017 to 0.167 µmol/min/mg protein. The variation was also observed for lipase activity in depth 1 starting from day 20 and this trend was observed in day 36 and day 56. A progressive decrease in enzyme activity was observed for depth 3 during the study period (Figure 31a & b)



a)

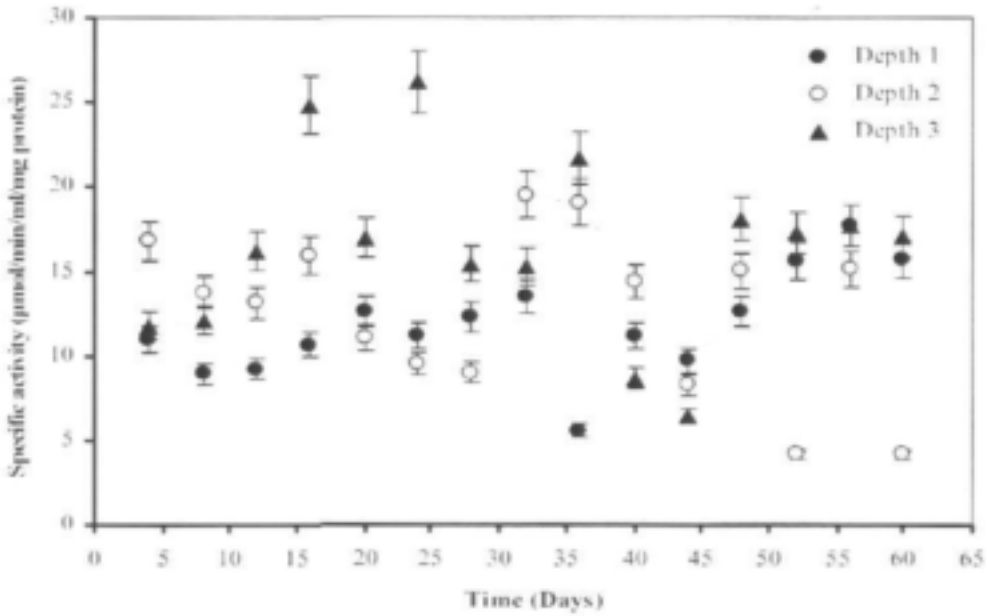


b)

Figure 31 Variation of lipase specific activity (mol/min/ml/mg protein) in the RSBR a) with respect to time and b) with respect to depth. Each point represents the mean of determinations performed in triplicates. Standard deviations are represented by error bars

Specific activity of sulphatases showed variation in all depths of the RSBR though no consistent patterns could be established. Sulphatase specific activity of up to 26.22 µmol/min/mg protein was detected in depth 3 indicating that these enzymes are present in extracellular fractions of sludge samples and therefore could increase diffusion of substrates to the active site of enzymes (Figure 32a & b).

a)



b)

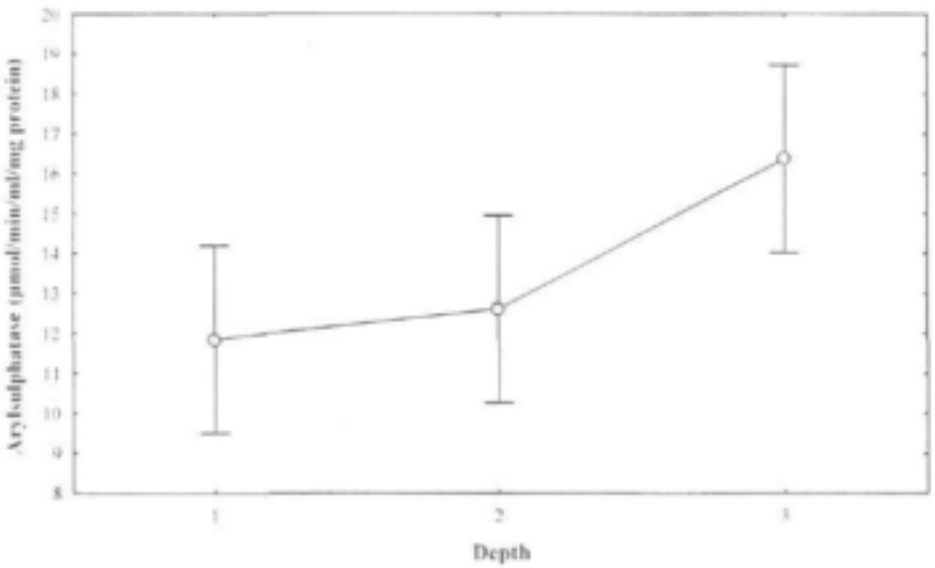
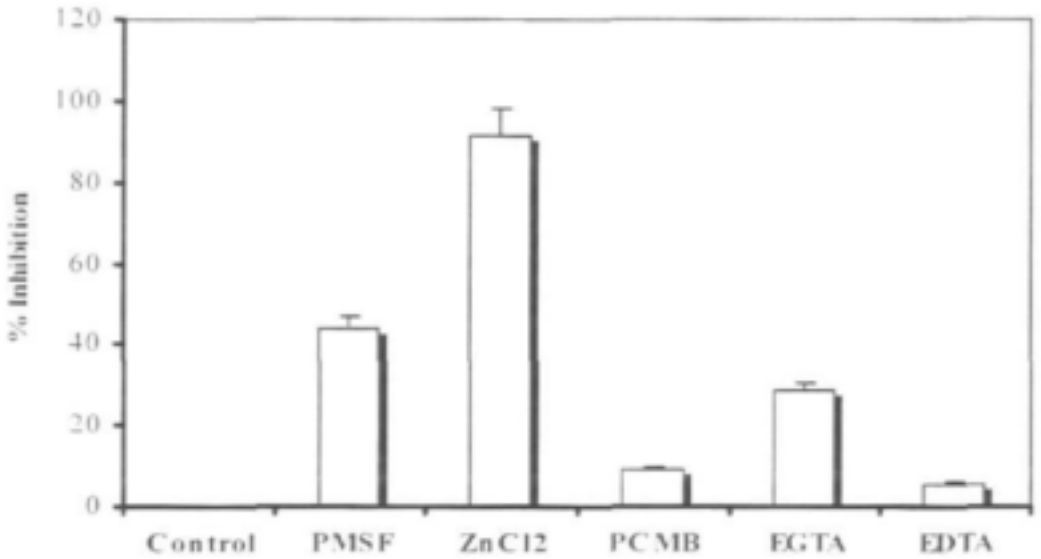


Figure 32 Variation of sulphatase specific activity (mol/min/ml/mg protein) in the RSBR a) with respect to time and b) with respect to depth. Each point represents the mean of triplicate determinations. Standard deviations are represented by error bars.

3.7. Effect of Inhibitors

All the inhibitors inhibited α-glucosidase significantly ($p < 0.005$) while only pCMB and $ZnCl_2$ inhibited β-glucosidase with $ZnCl_2$ being the most at 80% inhibition (Figure 33a & b). EGTA and EDTA produced activation of β-glucosidase of between 37 and 42% due to the metal chelating capacity.

a)



b)

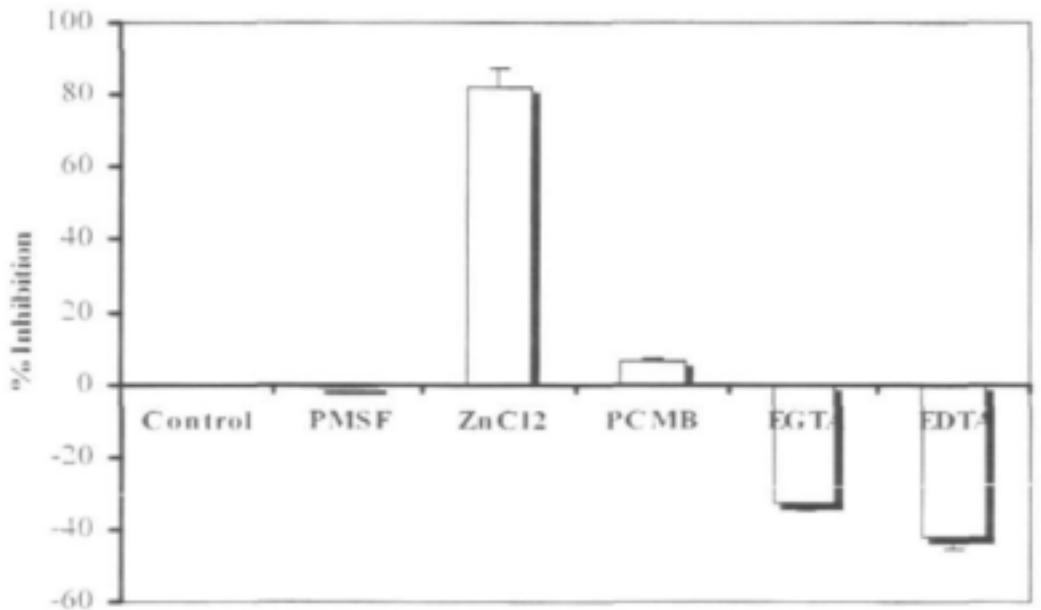


Figure 33 Effect of inhibitors on a) α-glucosidase activity and b) β-glucosidase. All values are means of triplicate determinations. Error bars represent standard deviations at 95% confidence level. Negative values indicate enzyme activation.

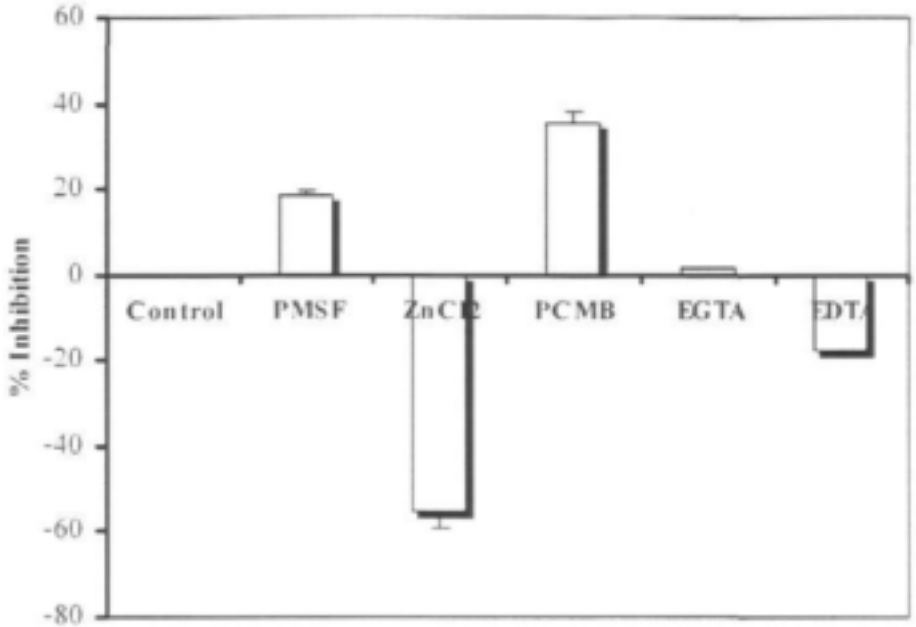


Figure 34 Effect of inhibitors on protease activity. All values are means of triplicate determinations. Error bars represent standard deviations at 95% confidence level. Negative values indicate enzyme activation.

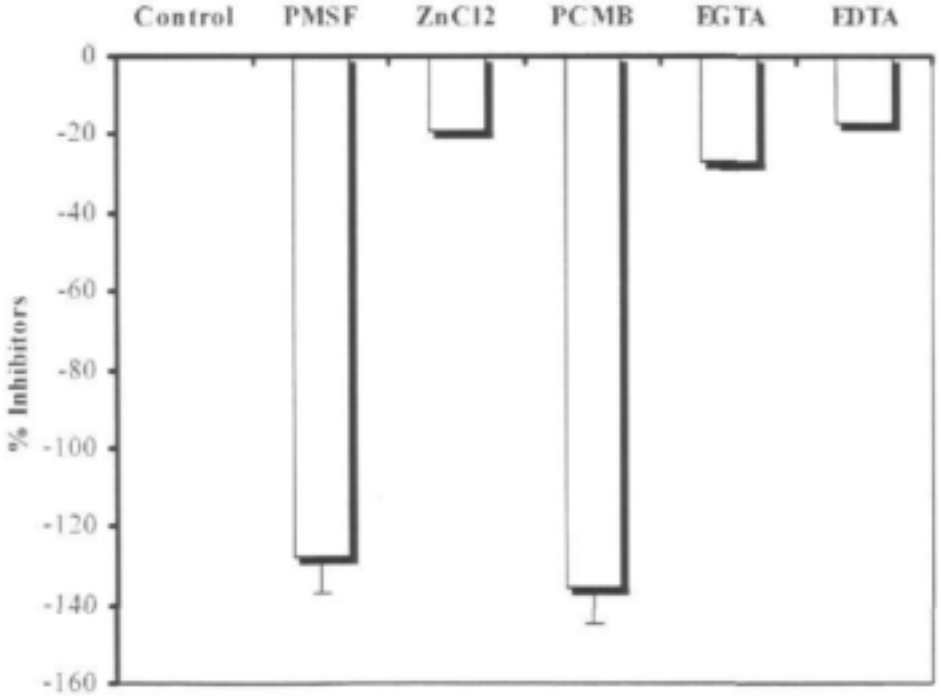


Figure 35 Effect of inhibitors on lipase activity. All values are means of triplicate determinations. Error bars represent standard deviations at 95% confidence level. Negative values indicate enzyme activation.

Substantial reduction of proteolysis was observed with PMSF and pCMB suggesting that most of the degradation was as a result of serine proteases. $ZnCl_2$ and EDTA were not effective inhibitors pf proteases indicating that metalloproteases were not involved in the proteolysis (Figure 34). Lipase activity was positively influenced by all of the inhibitors with the most prominent being PMSF (128%) and pCMB (135.6%) (Figure 35).

Attention was turned to the enzymes within the RSBR. $ZnCl_2$ had a greater effect on the activity of β-glucosidase with up to 75.13% inactivation compared to 43.4% observed for α-glucosidase. PMSF, however, caused deactivation of only 27% and 31% for β- and α-glucosidase respectively. Lipase activity of as much as 309.5% was observed in the flask that was preincubated with PMSF (protease specific inhibitor) and 133.5% relative lipase activity for the flask incubated with $ZnCl_2$ (Figure 36 and 37).

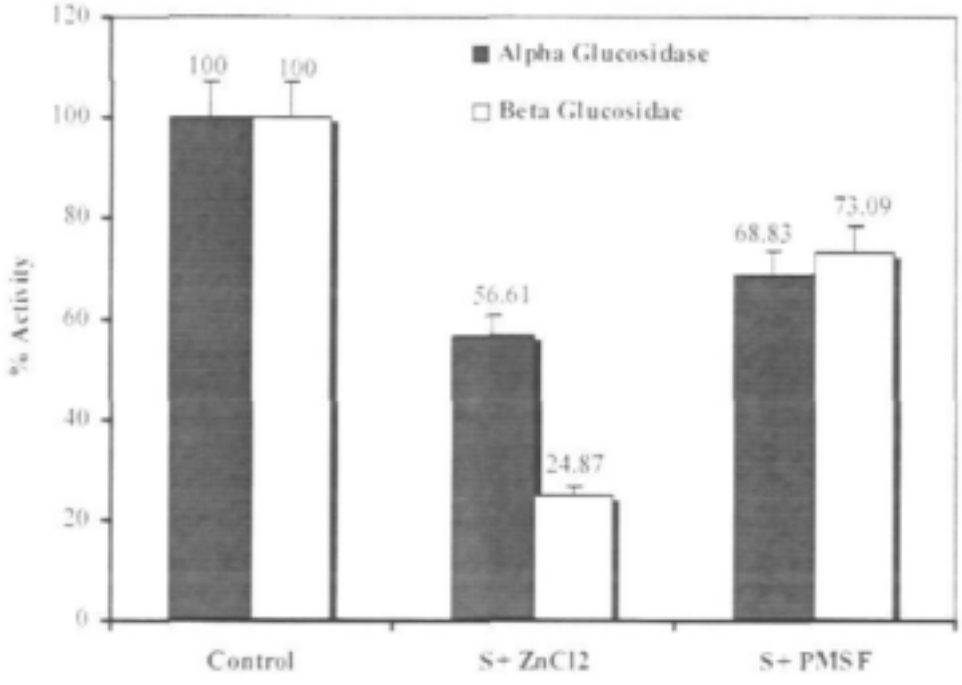


Figure 36 Effect of $ZnCl_2$ and PMSF on α-glucosidase and β-glucosidase.

3.8. Kinetic Parameters K_m and V_{max}

The results of experiments for the estimation of K_m and V_{max} as determined by Hanes-Woolf plot is represented in Table 1. The calculation of Km and Vmax were determined by fitting appropriate rate equations using SigmaPlot and a typical graphical representation is shown in Figures 38.

3.9. Floc size distribution

The frequency of the floc size distribution with respect to depth is shown (Figure 39). The floc sizes were small with the bulk of the flocs diameter in the range of 0 – 59 μm for depth 1 within the first four days in their aerobic digester and up to 79 μm diameter for depth 2 and 3.

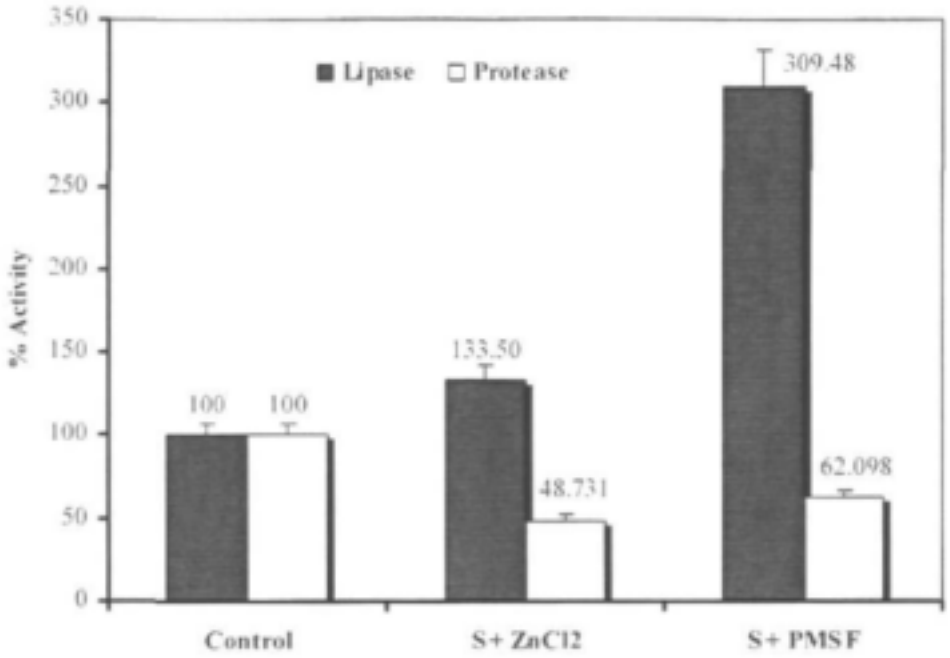


Figure 37 Effect of $ZnCl_2$ and PMSF on protease and lipase.

Table 1. Kinetic parameters, of α- and β-glucosidases, protease and lipase activities in the RSBR.

Enzyme	Substrate	Km (µM)	Vmax (µmol /min/ml)
α-Glucosidase	p-Nitrophenyl α-D-glucopyranoside	0.161 ± 0.018	0.849 ± 0.024
β-Glucosidase	p-Nitrophenyl β-D-glucopyranoside	0.193 ± 0.066	0.491 ± 0.076
Protease	Azocasein	0.102 ± 0.013	2.310 ± 0.061
Lipase	Triacetin	0.218 ± 0.031	2.089 ± 0.019

3.10. Multivariate statistics.

3.10.1. Physico-chemical and enzymatic activities

To establish the relationship between the group means (depth 1 and depth 2, depth 1 and depth 3 and depth 2 and depth 3), a multiple comparison test was used to determine the significant difference between the groups. Among the many multiple range test available the Duncan's and Newman-Keuls Multiple Range test were used since they are based on the range statistic, convenient and combines the ease of hypothesis testing with the power of testing each mean to each mean (Chew, 1980; Swallow, 1984; Day and Quinn, 1989; Montgomery, 1991; Winer *et al.*, 1991). The results obtained from the multiple range test are presented in Table 2. In multi-parameter systems like the RSBR, parameter relationship is achieved by carrying out a

multivariate data analysis (Shine *et al.*, 1995; Doherty *et al.*, 2000, Liu *et al.*, 2003). Some obstacles are however eminent in this type of system as the number of parameters are large.

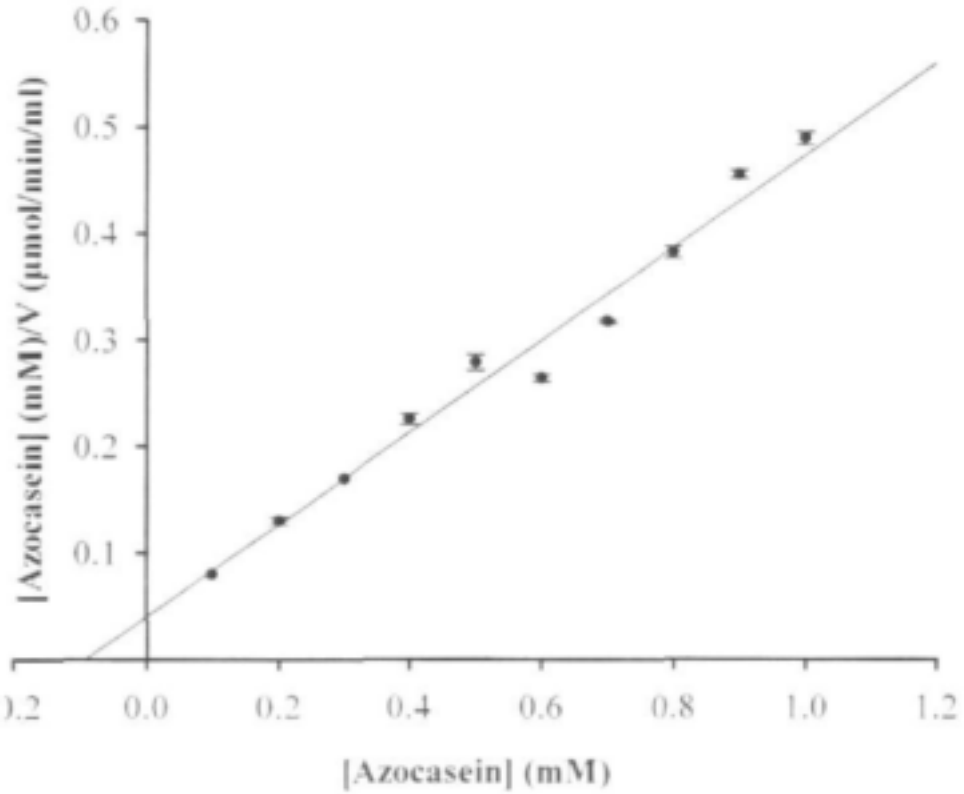
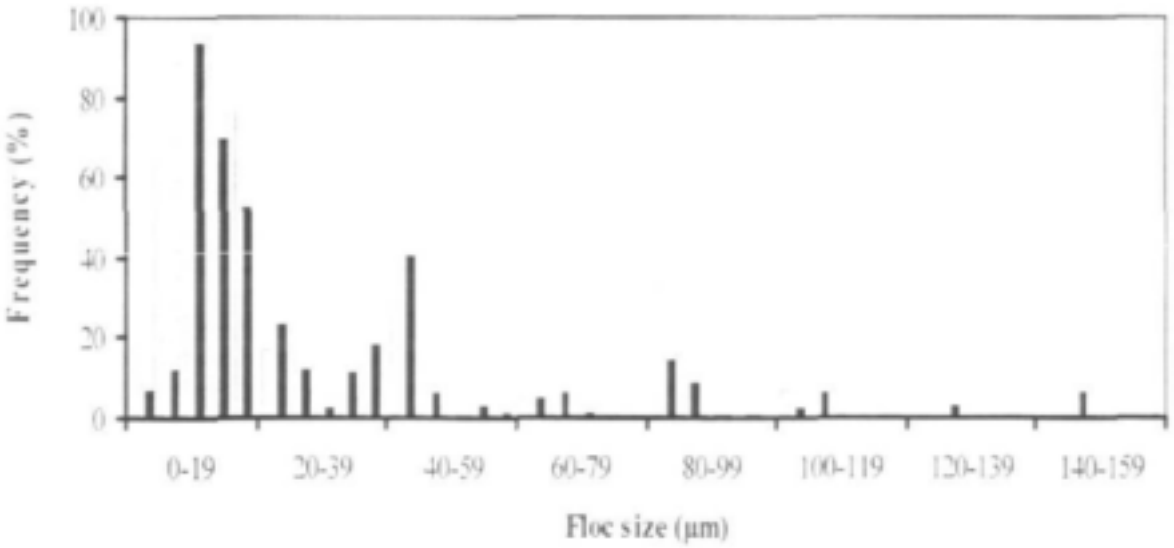
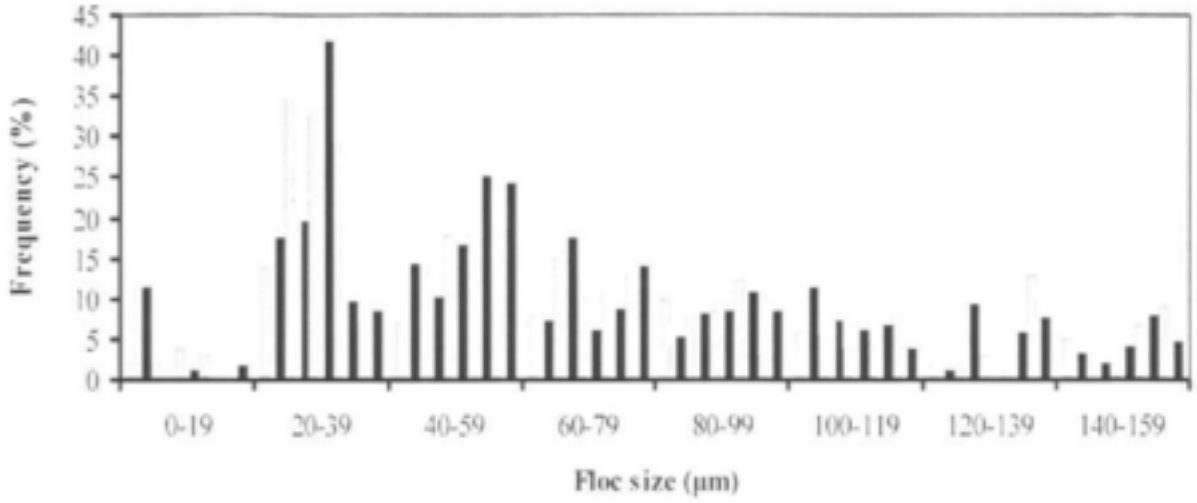


Figure 38 Hanes-Woolf plot to determine kinetic parameters for proteases.

a)



b)



c)

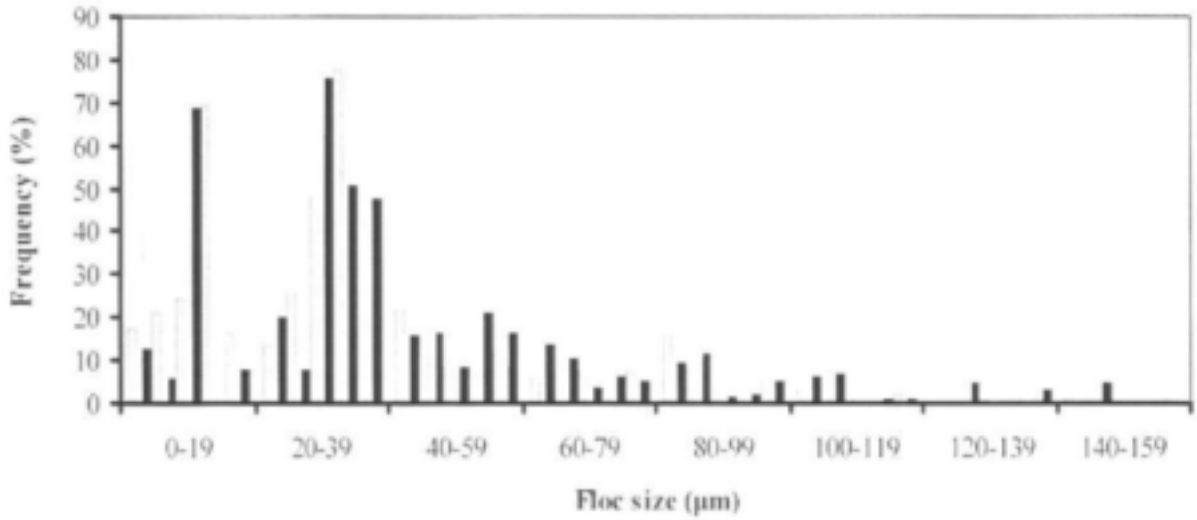


Figure 39 Distribution of floc size within the RSBR for a) depth 1 b) depth 2 and c) depth 3

Therefore, to reduce the amount of a data Pearson *R* correlation (Pearson, 1896), analysis and linear (Montgomery, 1991; Jaeckle and MacGregor, 1998) were performed between all pairs of combinations (Table 3). The correlation coefficient plays and 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. The population correlation calculation returns the covariance of two data sets divided by the product of their standard deviations. The correlation analysis

determines whether large values of physicochemical parameters are associated with large values of enzyme activities (positive correlation), whether small values of physicochemical parameters are associated with large values of enzyme activities (negative correlation), or whether values in both sets are unrelated (correlation near zero). The Statistical Model presented (Table 3), shows a very high correlation between, alkalinity sulphide, sulphate, total COD and lipase, protease, α-glucosidase, and β-glucosidase. Sulphatases showed generally weak correlations with the dependent variables (physico-chemical parameters) while temperature and pH had no effect on the enzyme activities.

Table 2 Probabilities for Newman-Keuls multiple comparison 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)	+++	+++	+++
Sulphate (mg/l)	+++	+++	NS
Sulphide (mg/l)	+++	+++	NS
	Specific enzymatic activities (μmol/min/ml/mg protein)		
	1 and 2	1 and 3	2 and 3
Protease	+++	+++	++
Lipase	+++	+++	+++
α-Glucosidase	+++	+++	++
β-Glucosidase	++	+++	NS
Sulphatase	NS	++	++

After careful study of the correlation table, a multiple regression (Pearson, 1908) model analysis was performed by using the "Least Squares" method to fit a line through the observations. The Least Square principle minimises the deviations form the actual data points to a hypothetical line. In this model the physico-chemical parameters (the explanatory variables or independent variables) jointly predict the outcome of the enzyme activities (response variable or dependent variables). All of the independent variables were specified simultaneously in the regression equation and the solution was obtained through the technique of Least Squares with the assumption that all the physico-chemical parameters together are necessary to explain the variation in the enzymatic activities. The correlation coefficient (R), the coefficient of determination (R^2) and the P-values are presented in Tables 3, 4 and 5. *R* square (R^2) provides a percentage of variance, while *R* provides a measure of the correlation between the two predictors combined and the dependent variable.

The regression analysis is equivalent to finding the best-fit model that describes the relation as shown in equation 9. In this model Y_i represented the specific enzyme activity and X_i was the physico-chemical parameters.

$$Y_i = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \ldots + \beta_{p-1} X_{i,\,p-1} + E_i \qquad (9)$$

where i indicates each observation, is the coefficient for parameter, is β_j the error term (deviation of measurement from prediction) and Y_i the dependent variable. Each β_1 was used to determine if there was a positive or negative relationship between the specific enzyme activity and the physico-chemical parameter. A positive slope, indicated by a positive value for β_1, showed that for every unit increase in X_i, the specific enzyme activity Y_i, had an increase of β_1 units. A negative slope indicated by a negative β_1 value showed that for every unit increase in X_i, the specific enzyme activity decreased by β_1 units. The results of these studies showing the multiple linear regression models of enzyme specific activity against the corresponding physico-chemical parameter are presented in Figures 42 and 43. It should be noted that only models that are significant at $P < 0.05$ and at 95% confidence interval are presented. A stepwise regression analysis was also carried out in which it was assumed that the physico-chemical parameters X_i are correlated. All the variables were simultaneously specified in the regression equation just like in Multiple Regression Model. The stepwise procedure (Himmelblau, 1970) systematically added physico-chemical parameters that made a systematic contribution to the explanation of the variation in enzyme activities. This procedure also eliminated the parameters that made no significant contribution to the variation in enzyme activities. It was however realised that the final prediction equation had very few physico-chemical parameters. The stepwise procedure did

Table 3. Pearson's (R) correlation coefficient between physico-chemical parameters and enzyme activities of RSBR for all depths

	Temp	*pH*	*Alkalinity*	*Sulphide*	*Sulphate*	COD_{Total}	COD_{Sol}	COD_{Part}	*Lipase*	*Protease*	*α-Glu*	*β-Glu*	*Sulfatase*
Temp oC	1												
pH	-0.195	1											
Alkalinity	-0.023	-0.007	1										
Sulphide	-0.033	0.092	0.951	1									
Sulphate	0.021	-0.020	-0.973	-0.947	1								
COD_{Total}	-0.087	0.036	0.932	0.882	-0.905	1							
$COD_{Soluble}$	0.054	-0.032	0.957	0.904	-0.944	0.862	1						
$COD_{Particulate}$	-0.101	0.041	0.913	0.864	-0.886	0.998	0.831	1					
Lipase	-0.011	0.060	0.957	0.916	-0.940	0.912	0.913	0.896	1				
Protease	-0.056	0.131	0.816	0.769	-0.801	0.863	0.744	0.860	0.827	1			
α-Glucosidase	-0.081	0.132	0.896	0.883	-0.899	0.875	0.823	0.866	0.947	0.747	1		
β-Glucosidase	0.242	-0.199	0.606	0.458	-0.583	0.605	0.585	0.597	0.581	0.580	0.438	1	
Arylsulphatase	0.022	0.083	0.274	0.135	-0.231	0.353	0.220	0.359	0.300	0.352	0.331	0.440	1

not address the model building requirements since it simply added and removed the parameters based on mathematical criteria. With simple linear regression analysis it was easy to note how a single dependent variable (enzyme activity) was affected by the values of one or more independent variables (physico-chemical parameters).

Table 4. Coefficient of determination for physico-chemical parameters and enzymatic activities.

	Coefficient of determination (R^2)						
Parameter	pH	Alkalinity	Sulphide	Sulphate	COD_{Total}	$COD_{Soluble}$	$COD_{Particulate}$
Lipase	0.0036	**0.9150**	**0.08351**	**0.8842**	**0.8321**	**0.8334**	**0.8034**
Protease	0.0173	**0.6655**	**0.5870**	**0.6411**	**0.7443**	**0.5540**	**0.7399**
α-Glucosidase	0.0173	**0.8031**	**0.7720**	**0.8080**	**0.7653**	**0.6773**	**0.7501**
β-Glucosidase	0.0396	0.3671	0.2040	0.3399	0.3665	0.3421	0.3559
Sulphatase	0.0068	0.0751	0.0175	0.0535	0.1244	0.0486	0.1291

Table 5 P-values of relationships between physico-chemical parameters and enzymatic activities

	P - values						
Parameter	pH	Alkalinity	Sulphide	Sulphate	COD_{Total}	$COD_{Soluble}$	$COD_{Particulate}$
Lipase	NS	+++	+++	+++	+++	+++	+++
Protease	NS	+++	+++	+++	+++	+++	+++
α-Glucosidase	NS	+++	+++	+++	+++	+++	+++
β-Glucosidase	NS	+++	++	+++	+++	+++	+++
Sulphatase	NS	NS	NS	NS	++	NS	NS

The ideal way of identifying parameter relations in a multi-parameter system such as this is to carryout a multivariate test of significance data analyses. Correlations and regressions analyses were performed between the pairs of combinations of all the data of the RSBR as this procedure provides a way of identifying parameter relationships. The specific enzymatic activities of the RSBR were correlated with the results of the physico-chemical parameters. Table 3 shows the correlation coefficients (R) of the specific enzymatic activities versus the physico-chemical parameters. The regression coefficients (R) and the corresponding coefficient of determination (Table 4) the F-values (Table 6), and the models were obtained from the analysis of data obtained from the three depths, viz. depth 1, depth 2 and depth 3. Three dimensional surface plots for the response of enzymatic activity of proteases, lipases, α- and β-glucosidases versus sulphide, sulphate concentration and alkalinity are represented in Figures 40 while the fitted

model with variables are shown on the scatter plots (Figure 41). Low correlations were obtained with individual depths of the reactor as compared to high correlations that were observed with the entire bioreactor. The analyses of correlation suggested that interaction between alkalinity, sulphide, sulphate and the CODs with the enzymes (lipase, protease, α- and β-glucosidase) appeared to be more adequate than those of pH, temperature and sulphatase. ($P < 0.05$ at 95% confidence level).

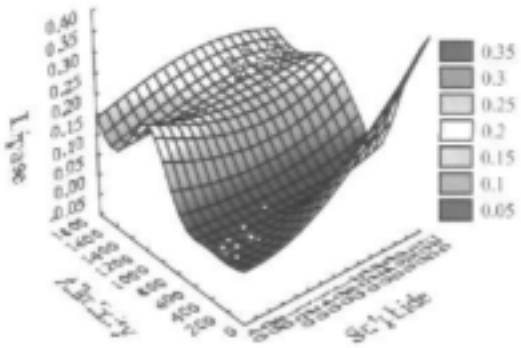
3.10.2. Descriptive ANOVA analysis.

Table 6 Descriptive statistics of physico-chemical parameters and specific enzyme activities for depth 1, 2 and 3 of the RSBR

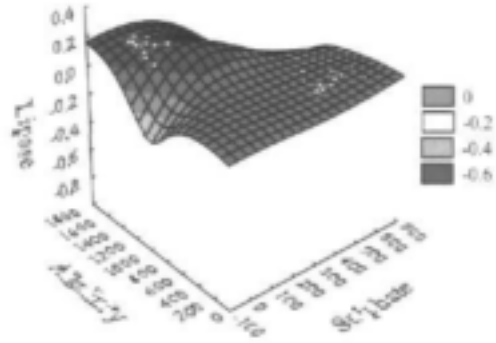
Parameter	Mean	*F*	*F* crit	*P*-value	95% CL	Median	Min	Max	Range	Std. Dev.	Std. Err
Temperature (°C)	19.42	-	-	-	19.77	19.0	18.00	21.80	3.80	1.17	0.174
pH	7.318	0.04	3.213	> 0.05	7.34	7.31	7.150	7.50	0.35	0.09	0.013
Alkalinity (mg/l)	1093.76	806.05	3.213	< 0.001	1254.89	1425.0	200.00	1600.00	1400.0	536.2	79.93
Sulphide (mg/l)	192.84	207.15	3.220	< 0.001	218.88	225.0	51.67	293.30	241.66	86.66	12.91
Sulphate (mg/l)	182.05	570.82	3.219	< 0.001	247.19	36.77	6.68	605.23	598.55	216.7	32.31
COD_{Total} (mg/l)	9104.98	181.95	3.220	< 0.001	11083.8	10533.3	419.33	19233.33	18814.0	6585.	981.6
$COD_{Soluble}$ (mg/l)	1154.89	229.68	3.219	< 0.001	1363.02	1450.00	170.67	2234.00	2063.3	692.9	103.2
$COD_{Particulate}$ (mg/l)	7943.26	145.26	3.220	< 0.001	9742.48	8980.67	160.00	17895.33	17735.3	5988.	892.7
Lipase	0.112	612.857	3.220	< 0.001	0.134	0.145	0.001	0.210	0.209	0.074	0.011
Protease	1.899	47.519	3.210	< 0.001	2.163	1.987	0.472	3.754	3.282	0.878	0.131
α- Glucosidase	63.091	146.245	3.220	< 0.001	73.831	71.686	5.265	123.64	118.38	35.75	5.329
β-Glucosidase	30.342	13.472	3.220	< 0.001	34.943	34.871	4.273	49.75	45.481	15.31	2.283
Sulphatase	13.610	4.342	3.220	< 0.050	15.064	13.506	4.182	26.21	22.036	4.839	0.721

Results on the overall descriptive statistics for the enzymatic activities and physico-chemical parameters are shown in Table 6 for depth 1, depth 2 and depth 3 of the RSBR. Specifically, the information shows the minimum and maximum values for the variable (range), mean (arithmetic average), median, confidence level, and the *P*-values. Detail statistical analysis was carried out with due consideration of variation within each depth, and the null hypothesis to be tested was that, there is no significant difference in parameters between and within the three depths of the RSBR. The physicochemical parameters and all enzymatic activities were significantly different in all depths ($P < 0.001$), except for pH where there was no significant difference ($P > 0.05$) depth 1, depth 2 and depth 3. This was however expected. Table 6 also shows the values of the

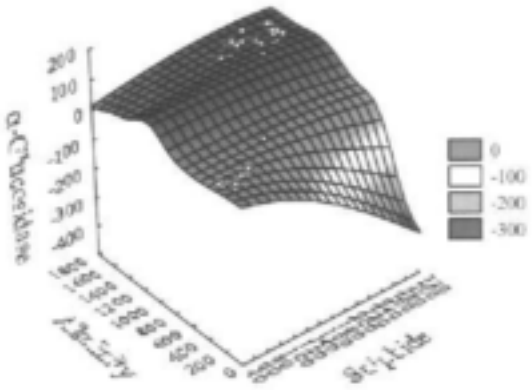
standard deviation (a measure of dispersion in the variable), standard error for all the depths of the RSBR. Also shown are the values of F statistic and F critical (F crit), with the rejection region defined by F critical. For $F < F$ crit there was no significant difference, whereas $F > F$ crit signified a significant mean difference. An individual group would fail to satisfy the null hypothesis at 95% confidence level if the F statistic is greater than F critical. Thus if the probability value P was greater than 95% the null hypothesis was rejected (Montgomery, 1991; Himmelblau, 1970), which meant that there was statistically significant differences between the physico-chemical parameters and the specific enzyme activities. The 95% confidence level was calculated for each group and is shown in Table 6. Data shown in Table 3 indicate that at 95% confidence level, most of the parameters measured showed $F > F$ crit and $P < 0.05$, thus failing to satisfy the null hypothesis except for pH where F < F crit and $P > 0.05$, signifying that the pH variations were minimal in the RSBR throughout the experimental period.



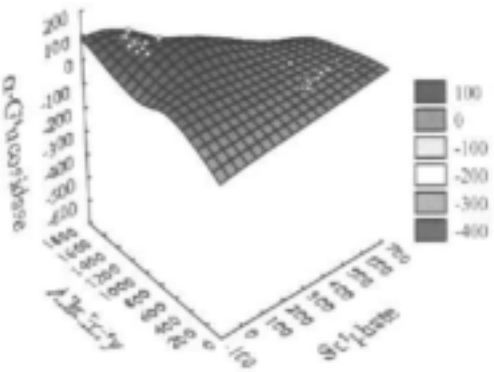
Sulphide vs. Alkalinity vs. Lipase



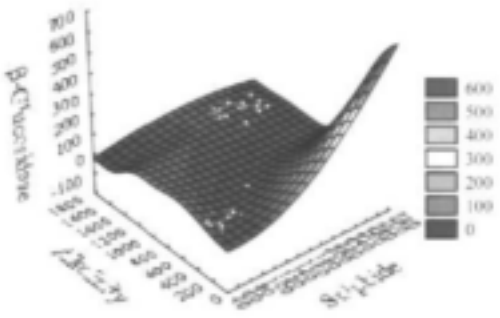
Sulphate vs. Alkalinity vs. Lipase



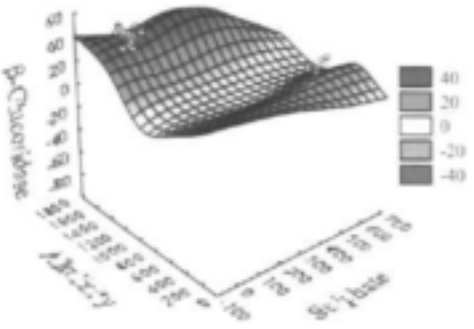
Sulphide vs. Alkalinity vs.α-Glucosidase



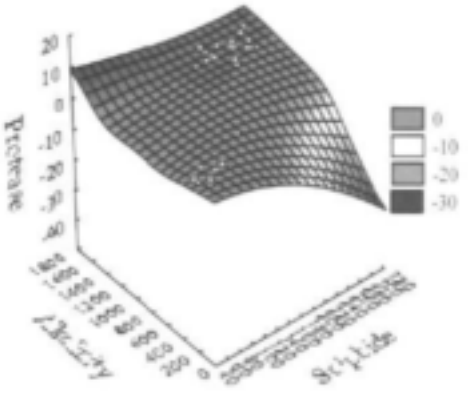
Sulphate vs. Alkalinity vs. α-Glucosidase



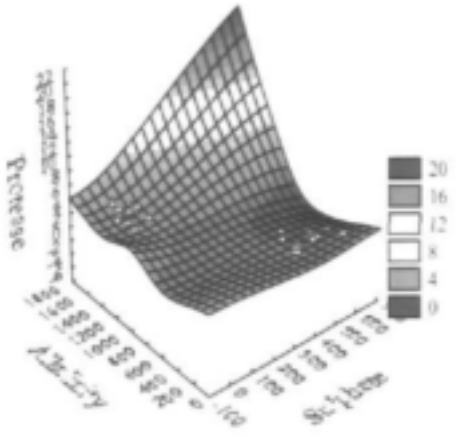
Sulphide vs. Alkalinity vs. β-Glucosidase



Sulphate vs. Alkalinity vs. β-Glucosidase

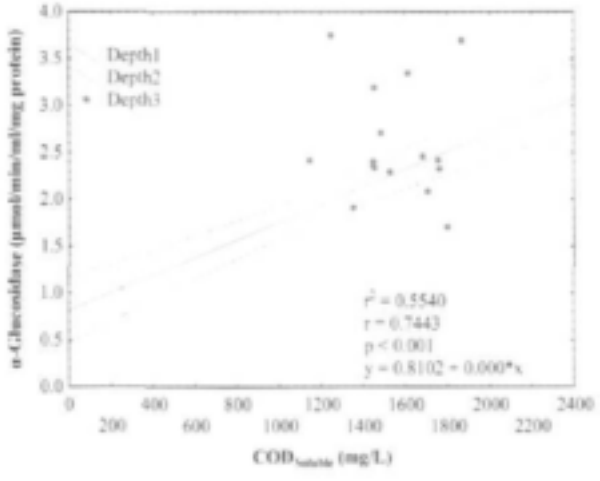


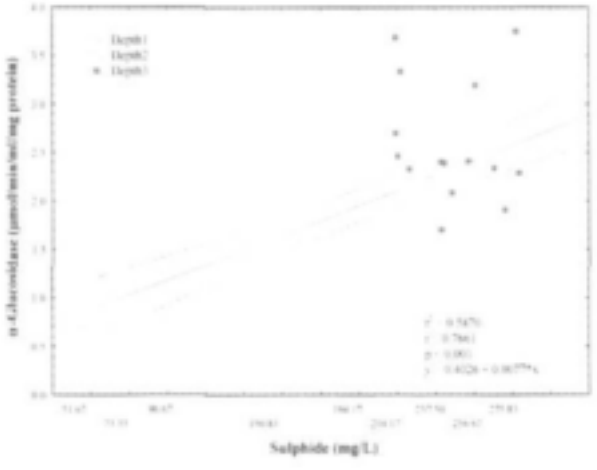
Sulphide vs. Alkalinity vs. Protease

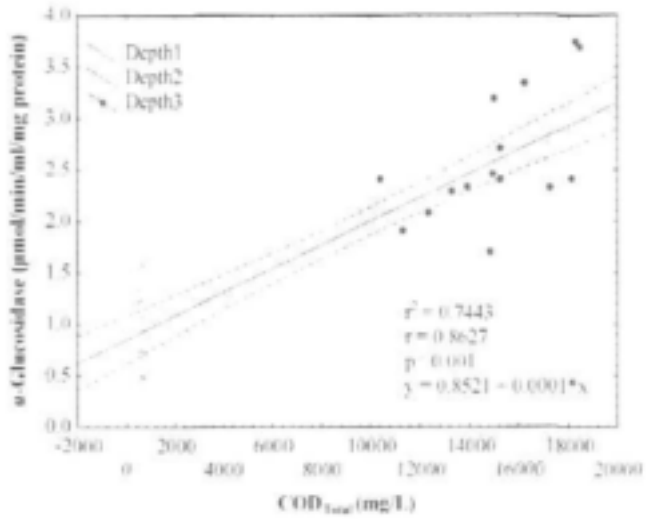


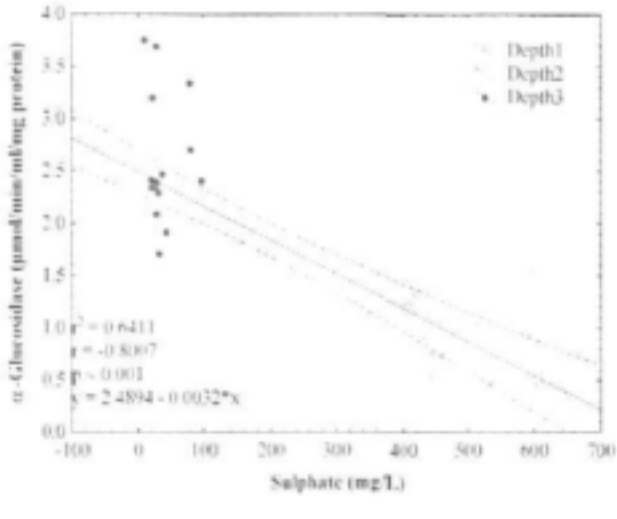
Sulphate vs. Alkalinity vs. Protease

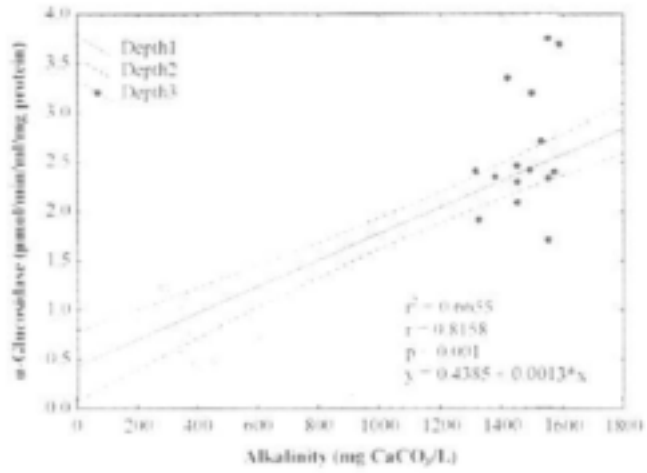
Figure 40. Three-dimensional Surface plots for the response of specific activity of lipase, protease, α- and β- glucosidase as a function of sulphide and sulphate with alkalinity

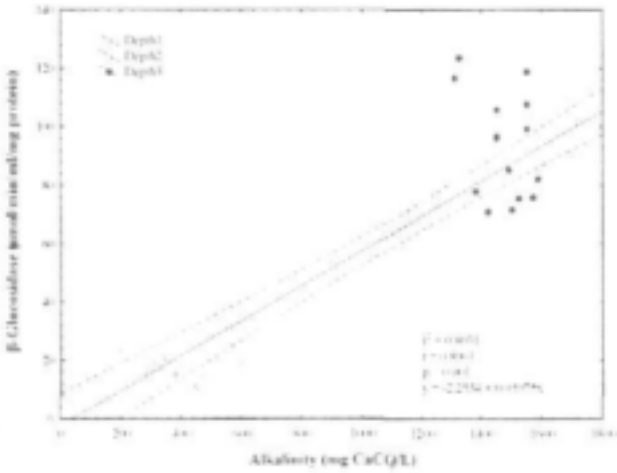


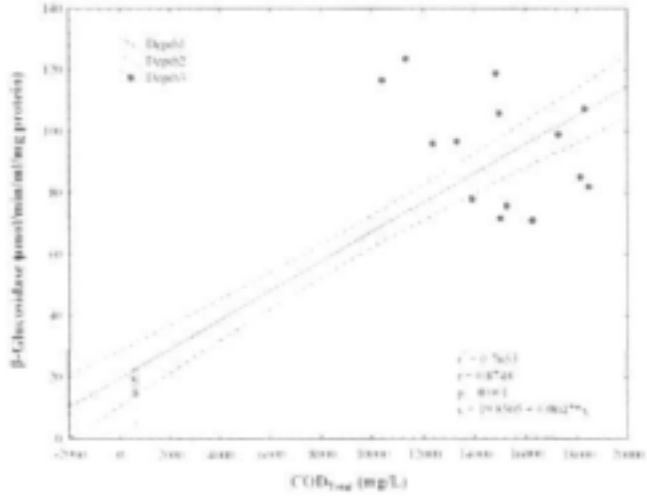


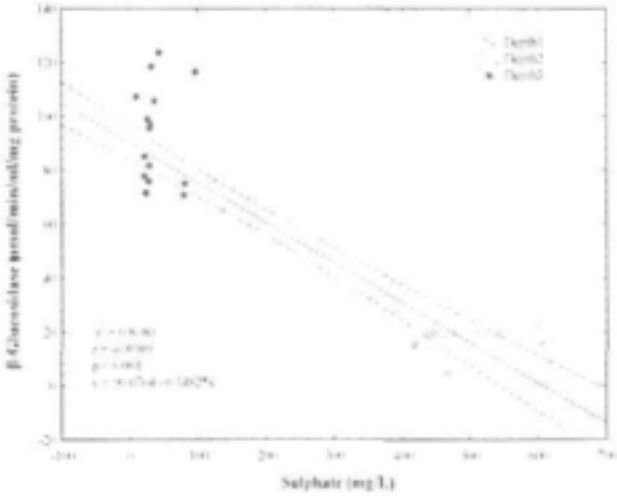

Depth1
Depth2
Depth3
r² = 0.7443
r = 0.8627
p = 0.001
y = 0.8521 + 0.0001*x
α-Glucosidase (pmol/min/ml/mg protein)
COD Total (mg/L)

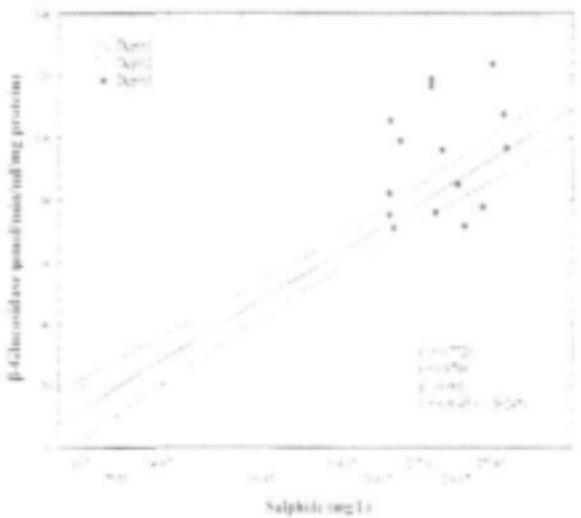

Depth1
Depth2
Depth3
r² = 0.6411
r = -0.8007
p = 0.001
y = 2.4894 - 0.0032*x
α-Glucosidase (pmol/min/ml/mg protein)
Sulphate (mg/L)

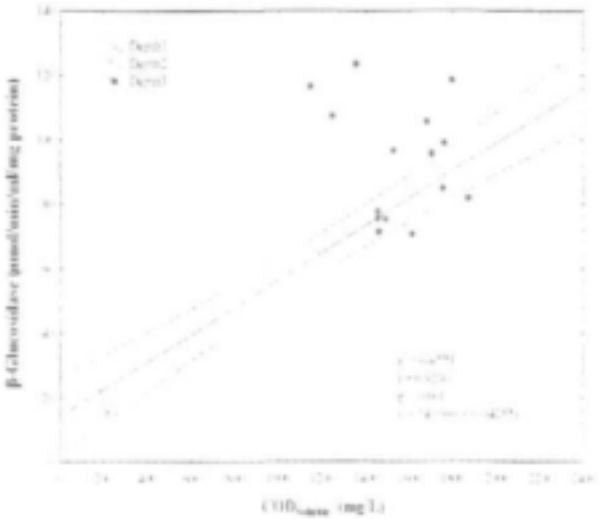

Depth1
Depth2
Depth3
r² = 0.6655
r = 0.8158
p = 0.001
y = 0.4385 + 0.0013*x
α-Glucosidase (pmol/min/ml/mg protein)
Alkalinity (mg CaCO3/L)

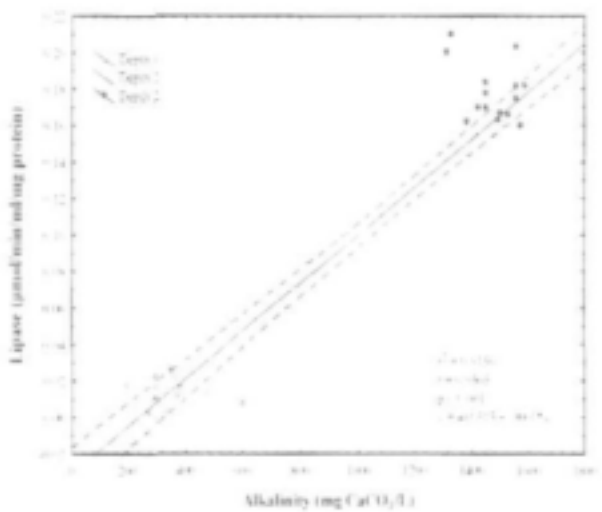

Depth1
Depth2
Depth3
β-Glucosidase (pmol/min/ml/mg protein)
Alkalinity (mg CaCO3/L)

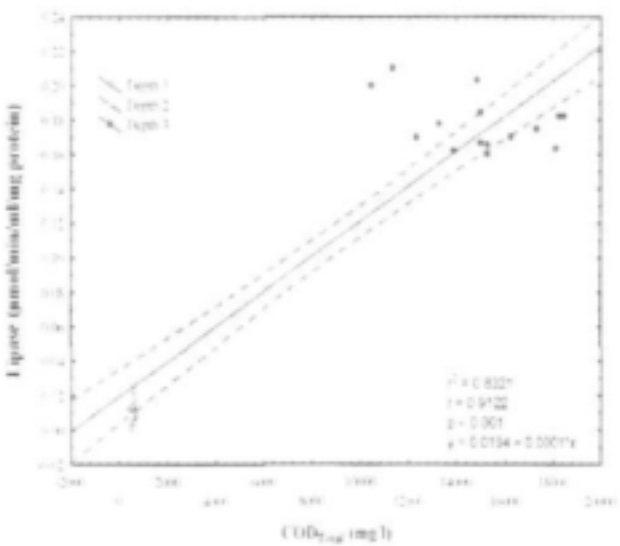

Depth1
Depth2
Depth3
β-Glucosidase (pmol/min/ml/mg protein)
COD Total (mg/L)

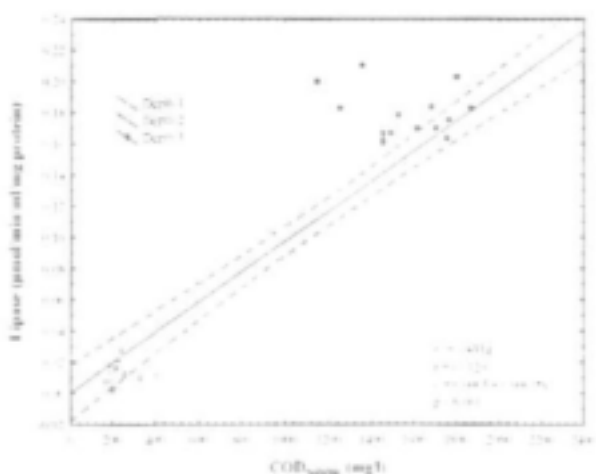

Depth1
Depth2
Depth3
β-Glucosidase (pmol/min/ml/mg protein)
Sulphate (mg/L)

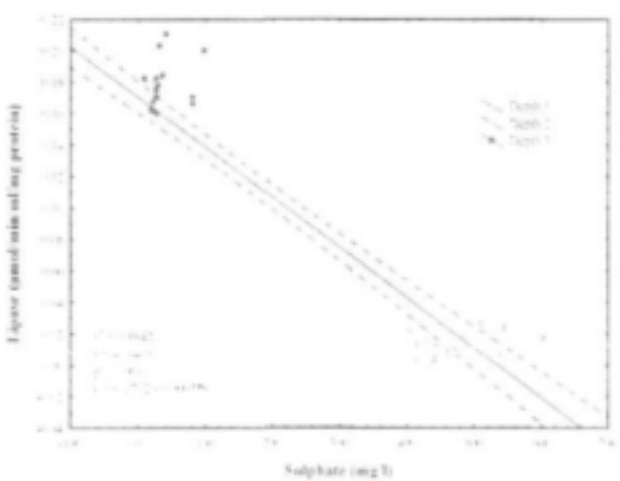





Alkalinity (mg CaCO3/l)






Sulphate (mg/l)

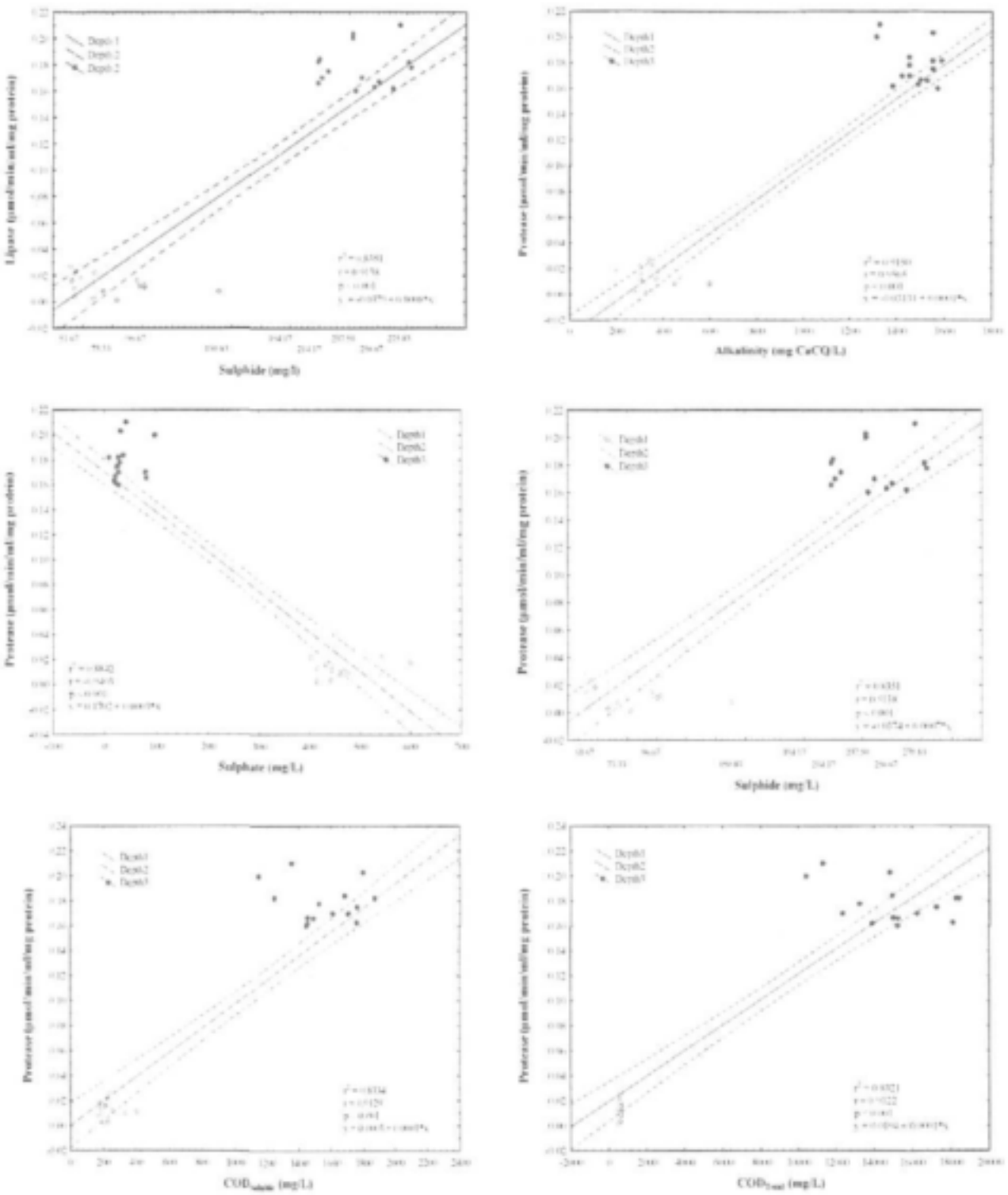


Figure 41 Scatterplots of the linear regression models (N=135) for α-, β-glucosidases, lipases and proteases with alkalinity, $COD_{soluble}$ COD_{total} sulphate and sulphide. The solid line is the linear regression equation, whereas the dashed lines indicate the upper 95% confidence interval based on the linear regression model of the enzyme. The incorporated equation represents the linear regression relationship with y representing the enzymatic activity.

4. DISCUSSION

Recent projects have demonstrated the utilisation of SRB systems for the accelerated hydrolysis of primary sewage sludge (Whiteley, *et al.*, 2003b). The underlying enzymatic mechanisms of the accelerated hydrolytic processes, however, remain obscure. Process optimisation studies is crucially dependent on an understanding of these underlying enzymatic mechanisms, especially those involved in anaerobic digestion and sulphate reduction. ATPS catalyses the first step in dissimilatory sulphate reduction in SRB such as in *Desulfovibrio* and *Desulfotomaculum* spp (Gavel, et al., 1998). Almost all the activity (94-100%) of the total ATPS activity was located in the supernatant samples after sonication (cell free extract). This is in agreement with other reports, that state that ATPS is located intracellularly Li, *et al*, 1991; Dahl and Truper, 1994; Schiff and Saidha, 1987). $ATPS_{MR}$ activity was significantly lower (10-20%) that the activity of $ATPS_{SR}$, which probably indicated that SRB require high levels of ATPS to efficiently utilise sulphate as a terminal electron acceptor. Both $ATPS_{MR}$ and $ATPS_{SR}$ exhibited high temperature optima (50-55°C), a characteristic common to all enzymes identified in our bioreactors thus far (Whiteley, 2003b). Both reactors were operated at room temperature (25°C), and it is not entirely certain as to why these ATPS enzymes exhibit such high temperature optima, reminiscent of those enzymes residing in thermophilic organisms. The fact that these enzymes were possibly immobilised on the sludge floc may perhaps explain their temperature stability at higher temperatures. Temperature and pH are two of the environmental factors that have been shown to influence both the rate and degree of solubilisation in reactors (Bannister and Pretorius, 1998; Elefsiniotis and Oldham, 1994; Gujer and Zehnder, 1983; Penaud, *et al.*, 1997). Most bioreactors are operated at ambient temperature (20 – 22°C). It is evident from Figure 3 that by increasing the temperature above ambient increased proteolysis can be attained, resulting in improved sludge solubilisation and an increase in the rate of formation of low-molecular-weight proton donors much needed by the SRB for sulphate reduction. Important information can also be derived from Figure 3 as to which proteolytic species would be the most prominent at a particular reactor temperature. Reactors run at higher temperatures up to 60°C and at pH 7 would be effective for hydrolysis and most suitable to the SRB. Widdel, 1988, reviewed the physiology of sulphate reducing bacteria extensively, reporting that these anaerobic organisms generally function best at pH values of 6-7 while a few are capable of operating maximally at temperatures of around 50°C. Further optimisation of hydrolysis of sewage sludge can be attained by separating the reactors for hydrolysis and sulphate reduction. Hydrolysis is considered to be a rate-limiting step in anaerobic digestion (Penaud, *et al.*, 1997; Ghosh, 1991; Nyns, et al., 1979) and by optimising environmental conditions such as pH and temperature to fit those of the bacterial systems and/or enzymes, it could be possible to attain a higher rate and more effective hydrolysis of complex organic molecules. In the hydrolysis reactor temperature can thus be regulated to 50°C and a pH of 10 for more effective and a higher rate of proteolysis of sewage sludge before being fed to the SRB. The relatively high temperature optima and stabilities observed for the proteases, and phosphatases in sludge from the methanogenic and sulphidogenic bioreactors are probably due to the multipoint attachment to the humus or solid particles preventing autolysis and to the protection by the particulate matter to which they are absorbed.

The effect of temperature on digestion has also been reported by other authors (Widdell, 1988) and it has been reported that thermal pre-treatment improved the production of biogas from

waste-activated sludge. Protease from activated sludge has been reported to have an optimum temperature of 60°C (Frϕlund, *et al.*, 1995; Boczar, *et al.*, 1992). Although the pH and temperature of digesters for the treatment of sewage for sulphate reduction can be readily adjusted, this may increase the running costs of the process and so are to be avoided if their use cannot be justified financially. An increase in especially the sulphidogenic bioreactor temperature to above 40-50°C would serve to improve the rate of sulphate activation (and subsequent sulphate reduction by SRB), as $ATPS_{SR}$ showed a high degree of activity and stability at higher temperatures. Of course, the optimum temperature for one enzymes does not imply that the whole organism grows best at this temperature. The physiology of SRB has been extensively examined, and it is evident that these anaerobic organisms function best at pH 6-7 and at temperatures around 28-30 or 30-36°C (Widdell, 1988).

The architecture of the sludge floc is dependent on the interaction between the produced biopolymers (carbohydrates, lipids, proteins, polysulphates, polyphosphates) and the various cations present in the sludge. Small changes in ionic strengths and ionic composition can alter the structural properties of the sludge floc (Keiding and Nielsen, 1997) as removal of these cations from the sludge decreases the bound biopolymer content. It has been suggested that the biopolymer network is stabilised by lectin like proteins binding polysaccharides that are cross linked to adjacent proteins. Divalent cations provide further structural stability by bridging negatively charged sites on the biopolymers (Higgins and Novak, 1997). Although hydrolytic bacteria would be incorporated into the sludge flocs, the majority of degradation of the floc components is likely to be from the outer surface where the concentration of bacteria is high. The higher enzyme activity of resuspended pellets before and after sonication, as compared to supernatant fractions supports our findings that the enzymes are found immobilised on the organic particulate matter thereby confirming that the microorganisms themselves are associated within the particulate matter. Molecules within the floc will be protected from enzymatic degradation and disruption of this network could lead to enhanced solubilisation of the sludge floc. As the flocs disintegrate, macromolecules that were previously protected from enzyme attack are exposed and may be degraded by hydrolytic enzymes. By increasing the frequency of floc cleavage, it is possible to facilitate hydrolysis by increasing the contact between enzyme and substrate (Whittington-Jones, 2000). Microbial biopolymers (extracellular polymeric substances) are mainly composed of polysaccharides and protein. This biopolymer matrix in wastewater treatment systems is extremely heterogeneous, and appears to play a crucial role in the flocculation of particulate matter and bacterial cells during treatment processes. Sludge flocs are dependent on the interaction between the microbially produced biopolymers and the cells present in the sludge (Widdell, 1988). It is therefore quite conceivable that small changes in the ionic composition may alter the structural integrity of the sludge flocs. Quite possibly then, an increase in the sulphide concentration may lead to interaction with some of this ionic composition, compromising the structural integrity of the floc and cause the consequent collapse of the floc, with increased surface area and enzyme release into the medium. In addition, sulphide has also been shown to increase the activity of the proteases present in the sludge in our laboratory (Whiteley, 2003b). The role of ATPS is therefore of crucial importance in determining the level of sulphide that is available to the medium, controlling the rate and extent of sludge deflocculation and degradation. It is proposed that the products of biological sulphate reduction both directly and indirectly facilitate this contact thereby enhancing overall enzyme activity. Studies on the degradation of complex organic material showed that the degree and rate

of solubilisation of complex organic material is higher under sulphidogenic than methanogenic conditions (Kim *et al.*, 1997; Pareek *et al.*, 1998). Sulphide and sulphite at high concentrations of between 800 and 1000 mg l^{-1} increased the activity of all of the enzymes. All of the enzymes in this study, except lipases, showed limited activity at the depth of 16cm where the levels of sulphate are relatively high and the reduction process to sulphide was in its infancy. It is unknown, at this point, why lipases should show such high activity (12-fold) in the presence of 400mg l^{-1} sulphate over control levels. Work is continuing with this and will be reported elsewhere. In fact it was established that a presence of sulphate reflected an inhibition of the proteases and only a marginal effect on the sulphatases, phosphatases and glucosidases at this depth. At the 32cm and 50cm depths, where the sulphate levels were diminishing and sulphide and sulphite levels increasing each of the enzymes showed the same relative increase in activity. Glucosidase activity changed from 42 µmol/h/mg protein at 32cm to 65 µmol/h/mg protein at 50cm (Figure. 8). Proteases, on the other hand, showed changes of 5 and 11 µmol/h/mg protein respectively at the two depths, while lipases altered from 3.1 µmol/h/ml/µg protein and 4 µmol/h/ml/µg protein at depth 32cm and 50cm respectively. Though the activity of sulphatases and phosphatases increased with concomittant increase in sulphide concentration and consequently with depth of the reactor their activity relative to proteases, glucosidases and (to a lesser extent) lipases was insignificant. Changes observed in these two enzymes at 32cm and 50cm depths were 0.1 to 0.2 and 0.05 to 0.1 µmol/h/ml/µg protein respectively.
Sulphate appeared to have no effect on the ATPS enzymes in both reactors. The absence of a strongly inhibitory effect of sulphate on ATPS activity is particularly interesting, as Segel *et al.* (1987) reported that this compound was strongly inhibitory to the reverse ATP synthesis reaction. A 400 mg l^{-1} sulphate concentration inhibits lipolytic activities of methanogenic anaerobically digesting sludge though very little change was observed with the lipases from the sulphidogenic bioreactor (Figure 5). It must be pointed out that the levels of sulphate concentration indicated are those for 'added' sulphate since with the SRB system high concentrations of sulphate are already present at the start of the process. The lower activity in the presence of sulphate could, at least in part, explain the improved solubilisation of complex particulate COD contained in primary municipal sewage sludge as observed in the novel Falling Sludge Bed Reactor (Whittington-Jones, 2000). With sulphite, inhibition of lipase activity from the methanogenic bioreactor (Figure 6) occurred with only 20 % activity remaining after the addition of 400 mg l^{-1}; a further 10 % decrease occurring at 800 mg l^{-1} (Figure 6). The lipase activity from sulphidogenic bioreactor proved slightly different with nearly a 2-fold increase at 800 mg l^{-1} (Figure 6). Dramatic changes in lipase activities were experienced in both bioreactors in the presence of sulphide (Figure 7). With only 400 mg l^{-1} sulphide a 5-fold increase in sulphidogenic lipase activity was seen (Figure 7) while 600 mg l^{-1} sulphide was required to show the same effect with the methanogenic lipases (Figure 7). At 800 mg l^{-1} there appeared a 5-fold increase in methanogenic lipase activity while at the same concentration the sulphidogenic lipase activity showed over 10-fold increase (Figure 7)

Sulphide and sulphite ions, on the other hand increased the lipase activity. This may be due to the neutralisation of the ions on the floc surface by sulphide, sulphite and associated bicarbonate and hydroxide ions, destroying the overall integrity of the floc structure exposing more substrate for enhanced enzyme activity. In order to test this hypothesis, the experiments carried out to investigate the effects of sulphide and sulphate were repeated, substituting hydroxide and bicarbonate as the effectors. The results showed that similar effects on lipase activity could be

obtained by the addition of hydroxide and bicarbonate (Figure 8), indicating that the action of the effectors may well be linked to their action on the floc structure. Further work to investigate the impacts of ion on flocs and on the enzymes is planned. Jung *et al* (2002) found that the activity of extracellular protease in activated sludge were extremely lower than that of intracellular protease, which suggested that one application of the enzymes released from the disrupted excess sludge is to improve the hydrolysis of high polymers present in wastewaters. Proteases can be used to enhance the hydrolysis of protein in wastewater, hopefully enhancing the whole anaerobic wastewater treatment process efficiency. The low protease activity indicated that the major activity of the hydrolytic enzyme lies within the extra cellular polymers of the floc. When considering the inhibition studies the high relative lipase activity observed in the presence of the protease specific inhibitor (PMSF) could be as a result of the fact that proteases are initially digesting lipases and other enzymes through hydrolysis. Inhibiting them cause their sudden rise in lipase activity. (Stoll and Blanchard, 1990; Roe, 2001; Price and Steven, 2002). Municipal wastewater contains a complex mixture of organic solids, the characteristics of which vary from location to location depending upon the population and the industrial sector served.

The determination of the kinetic constants, K_m and V_{max} was carried out to characterise the affinity of the respective substrates (p-nitrophenol-α-D-glucopyranoside; p-nitrophenol-β-D-glucopyranoside; azocasein and triacetin) for the enzymes (α-glucosidase, β-glucosidase, protease and lipase respectively) and to allow for the performance prediction of the bioreactor in which these enzymes are functioning. The hydrolytic rates which were the apparent values resulting from enzyme activities fitted with the Michaelis-Menten equation giving a rectangular hyperbola. This model suggests that hydrolysis rates can be shown to be proportional to enzyme

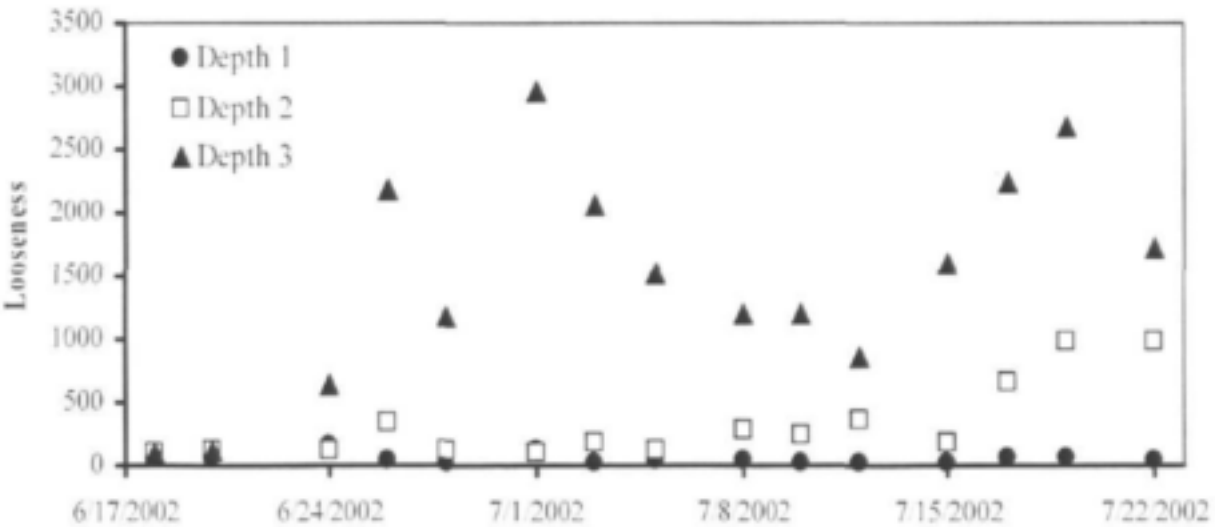


Figure 42 Floc looseness throughout the RSBR

activities (Chibata, *et al*, 1976). The K_m and V_{max} values should be represented as apparent values since sewage sludge actually consists of a group of each of these enzymes having specificity for these same substrates. The Km values reported here for the glucosidases compared well with those reported elsewhere (Spagna *et al*, 2002a, 2002b; Cadoret *et al*, 2002). The floc characteristics altered significantly from depth 1 to depth 3. The looseness factors of the flocs

increased, showing that the flocs of depth 3 were the most dendritic and mesh-like while the flocs of depth 1 were more like pinpoint flocs than aggregated flocs (Figure 42). Particles from depth 1 were the smallest and most circular. Floc circularity decreased (Figure 43) and volume increased moving down the reactor (Figure 44). Statistical analyses of floc measurement showed that mean floc volume was positively correlated with depth and negatively with circularity. Floc size distribution using feret volume measurements were generated for each sample and did not vary significantly over the course of the trial period. The majority of depth 1 particles were smaller than 20 μm^3, with progressively fewer flocs falling into increasing volume size categories. Most of the particles in depth 2 and depth 3 were in the range of 20 – 39 μm^3, with an asymmetrical distribution curve (Figure 45); depth 3 contained particles over 200 μm^3.

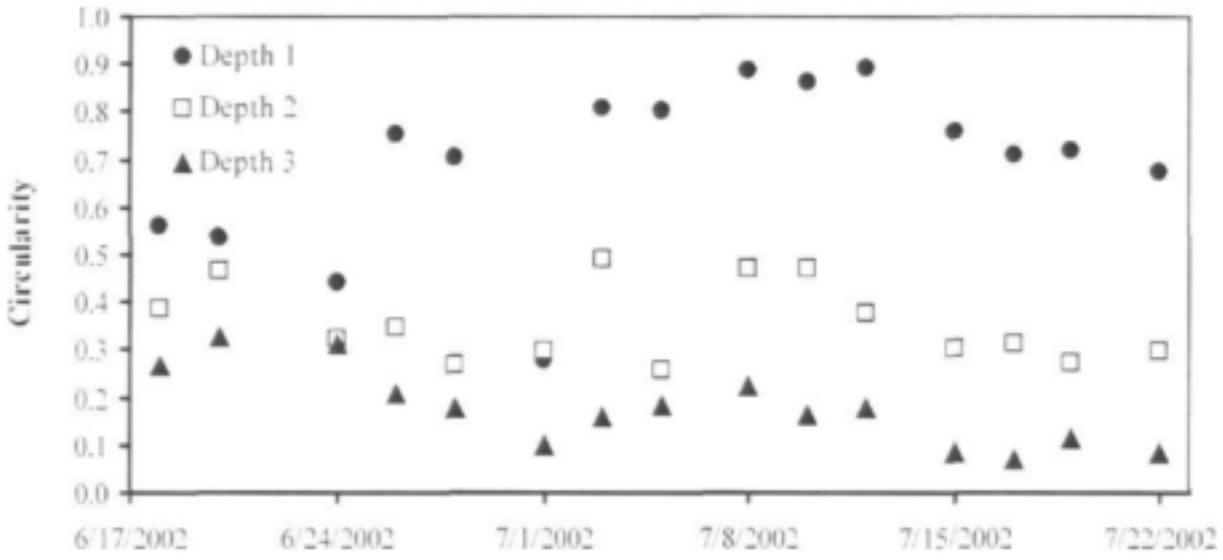


Figure 43 Floc circularity trends in the RSBR

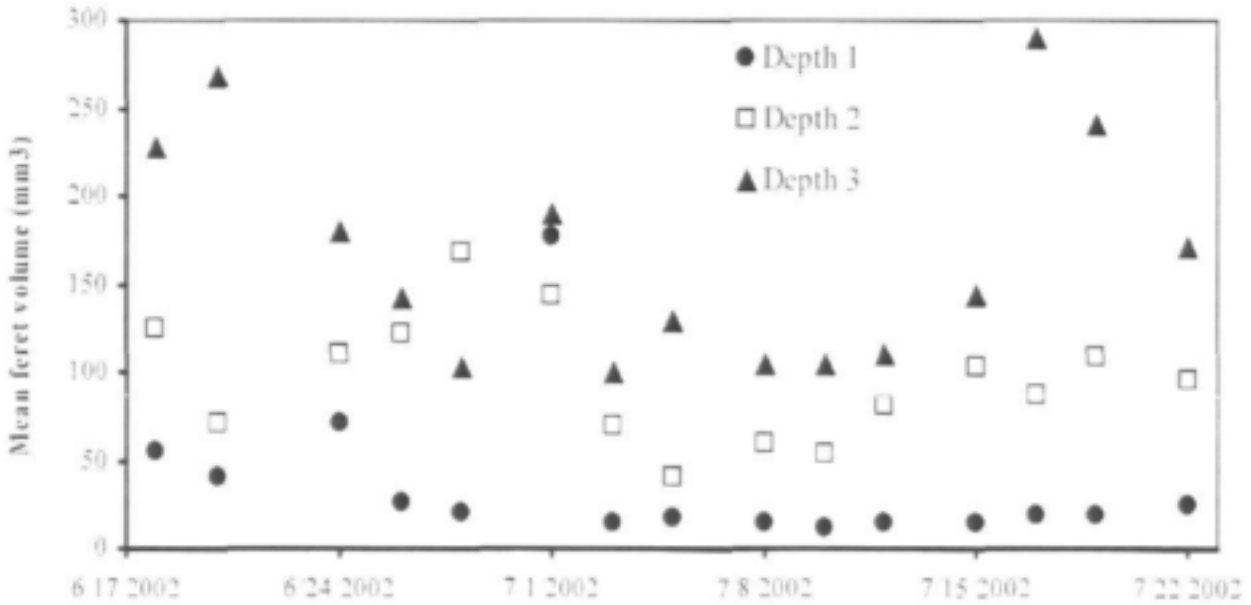


Figure 44 Mean floc feret volume

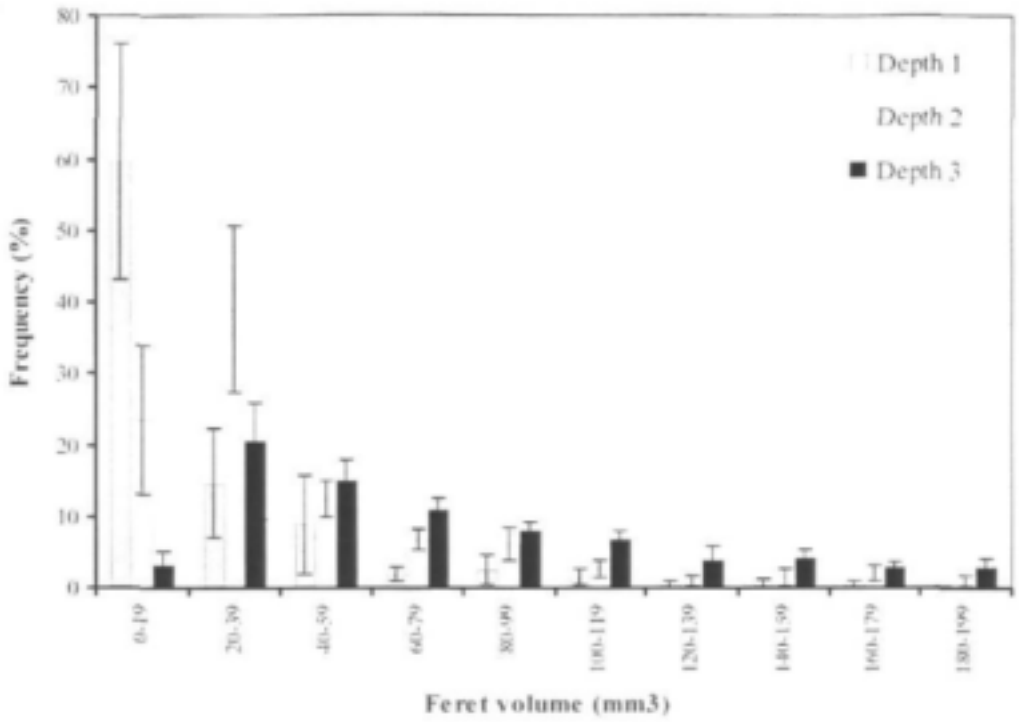


Figure 45. Mean particle size distribution up to 200 μm^3

The size of the flocs and their tendency towards open, dendritic structures increased moving down the RSBR from depth 1 to depth 3. These trends may be the results of concomitant increases in sulphide, alkalinity, COD, lipase, protease and α-glucosidase activity and the decrease in sulphate concentration.

Statistically lipase, α-glucosidase, β-glucosidase and protease showed the best correlations with a 91.50% variance explained for alkalinity with lipase while sulphatases generally showed poor associations with the physicochemical parameters [Table 4]. Sulphate on the other hand showed significant negative correlations with the specific enzyme activities (R = -0.231 to -0.905, Table 3). In contrast, the pH and temperature showed insignificant effect on the enzyme activities. To explore the response of the enzymes further with respect to depth of the RSBR, a post hoc test (Newman-Keuls multiple range test) was carried out for each of the enzymes [Table 2]. The models (equations shown on the scatter plots, (Figure 41) could be used to approximate the response of the hydrolytic enzyme activities with the physicochemical parameters. This fit of the model checked by the values of R^2 [Table 4] indicated that up to a 91 % of the variability in the response could be explained by the model at 95% confidence interval. According to test statistic, P-value for the overall correlation is significant at 5% level and the lack of fit is insignificant, indicating that the model is very adequate in approximating the response of the hydrolytic enzymes with the physico-chemical parameters. This is further supported by the R^2 values [Table 4] which are very satisfactory.

The fact that sulphide and enzymes showed satisfactory correlations with corresponding high R^2 values is further supported by the fact that sulphide activates enzymes in sulphidogenic bioreactors (Whiteley *et al.*, 2002a, b, c, 2003a; Pletschke *et al.*, 2002). It could therefore be

concluded that these analyses could be used to identify the optimum conditions under which the hydrolytic enzymes will enhance the solubilisation of primary sewage sludge under anaerobic sulphate reducing conditions. The models obtained in this study could be used to predict the trend of enzymatic activities in the RSBR with varying physico-chemical parameters. The results obtained in this work can be used as design parameters for a variety of bioreactor configurations used for the enhanced solubilisation of primary sewage sludge under sulphidogenic anaerobic conditions. In addition the models obtained could also reveal the key factors affecting enzyme activities, and thus could lead to optimisation strategies.
However, the complexity of sewage sludge could contribute to a majority of uncertainty and deviation in predictions using these models. Nevertheless, the approach demonstrated in this work is novel and seems to provide a tool for the better understanding of the enzymatic and biophysical characterisation of the anaerobic RSBR. One of the major goals of this research was to identify the factors and the corresponding enzymes that can produce enhanced hydrolysis of the primary sewage sludge.

Two hypothetical models can be suggested for the enhanced effect of sulphide and/or sulphite on enzymatic activity. First, as these sulphur species increased in concentrations during sulphate reduction they, and associated bicarbonate and hydroxide ions, neutralise the cations on the floc surface. This in turn destroys the overall integrity of the floc structure exposing more substrate leading to increased enzyme activity. Alternatively, the sulphite and sulphide species liberated during the sulphate reduction process, interact directly with the enzyme on the floc surface thereby enhancing their activity. Though both of these hypothetical models may have merit the second of these, is further substantiated by the data presented in this investigation. In order to establish unequivocally that the enzymes themselves are directly affected by the increase in concentration of sulphur species the resuspended pellet from the sulphidogenic bioreactor was boiled (80 °C, 60 min) to destroy any enzymatic material. After cooling a known amount of commercial enzyme was then added followed by sulphide and/or sulphite at concentrations of between 800 and 1000 mg l^{-1} resulting in a 3.6 – 8 fold increase in activity dependant on the enzyme studied.

The initial development of anaerobic treatment processes, over a century ago, was for the treatment of domestic wastewaters, using anaerobic filters and hybrid processes that are still of interest today. Its applications then expanded to include separate sludge digestion, then to industrial wastewater treatment. Several processes have been developed to quickly and efficiently treat wastewaters and sludges. Major contributions to the broad application of anaerobic treatment and the better understanding of this process have emphasised its importance for meeting the need for sustainable development in the future. Greater efforts are now needed for broader application of anaerobic treatment and for greater efficiency at lower energy costs. The volume of sludge produced from biological wastewater treatment processes has dramatically increased in recent decades as a result of the quantitative and qualitative expansion of wastewater treatment, and increasingly stringent environmental regulations. Biological stabilisation is widely considered to be one of the most attractive methods of reducing the major portion of the organic fraction in sludge, and anaerobic processes are favoured over aerobic digestion owing to the cost of aeration, the ability of anaerobic systems to maintain their temperature and the value of methane as a renewable energy source.

Considerable attention has been paid to the enzymatic hydrolysis of proteins for the improvement of sludge digestibility (Bomio *et al.*, 1989), as hydrolysis is regarded as the rate limiting step during sludge digestion (Häner *et al.*, 1994). In order to enhance the performance of the digestion process, it is essential that the activity of the microbial enzymes is maximised. Such enhancement of activity would allow enzymes to promote the lysis of bacterial cells in sludge via the cell wall decomposition, and result in the release of intracellular organic substances to the bulk liquid. These organic substrates are mainly proteins and carbohydrates, and they can be hydrolysed to unit molecules by further enzymatic activity. Enzymatic hydrolysis of organic particulate matter such as cellulose, carbohydrates, proteins and lipids in wastewater by anaerobic bacteria is an important step in prefermentation, anaerobic waste degradation and sludge digestion.

Despite the fact that hydrolysis is known to be the rate limiting step, as it is slow compared to others, it is not well understood. As a result, existing digestion process operation strategies may not be efficient in maximising solubilisation of sludge. It has been proposed that hydrolysis is either controlled by the reaction between enzyme and substrate to produce the solubilised products, or by the diffusion of enzymes from the micro-organisms to the bulk liquid. This research examines the activity of several enzymes under various conditions. It is of great importance that the enzymes involved in hydrolysis are identified and their optimal conditions determined, as such knowledge represents a key opportunity for the further development of the digestion process.

$ATPS_{SR}$ activity rapidly increased over the first three to five days days in the closed sulphidogenic bioreactor, followed by a gradual decline in activity over the remainder of the time course (Figure 6). We believe that this increase in $ATPS_{SR}$ activity on day five was coincident with the growth of the SRB populations present in the closed sulphidogenic bioreactor over the first five days. A possible explanation for the peak in $ATPS_{MR}$ activity observed on day 21 in the closed methanogenic reactor may be that $ATPS_{MR}$ has another role to play *in vivo* other than sulphate activation.

The rapid decrease in reactor sulphate concentration over the first nine days in the closed sulphidogenic reactor correlated well with the increased activity of $ATPS_{SR}$ on day five (Figure 7). It is unclear why the level of sulphate remained at a constant level of 400 mg/l after day 9. No sulphate was observed in the methanogenic reactor, but this was not surprising, as SRB were absent in the inoculum and sulphate was not added as feed to this bioreactor.

In addition, the reactor pH in both closed bioreactors was indicative of the proper functioning of these reactors, e.g. pH of 5.26± 0.26 for the methanogenic reactor, and pH 7.71 ± 0.25 for the sulphidogenic reactor. A deviation in these pH ranges would be indicative of gross reactor failure. The optimum pH range for anaerobic digestion has been determined to be between 6.5 and 8.0. The major controlling buffer at this pH would be the carbonate-bicarbonate system, with orthophosphoric acid, hydrosulphuric acid, volatile acids and ammonia all contributing to the stabilisation of pH. The sensitivity of anaerobic digestion to pH levels has been ascribed to the pH sensitivity of the methanogenic bacterial population (Forday and Greenfield, 1983).

Chemical oxygen demand is used to est imate the carbonaceous material content of the wastewater and typically divided into three main fractions – biodegradable (organic), unbiodegradable (inert) and heterotrophic active biomass which was due to the differential sludge particles in the reactor system (Figure 25). Variation of COD in the reactor was attributed to the amount of organic feed leaving the bioreactor as particulates, the amount leaving as $COD_{soluble}$, the amount of biomass produced, and the amount that has been used for a sulphate reduction and the fraction leaving as sulphide. COD leaves the system mainly as particulate and soluble fractions which also increase with reactor depth. The decrease in removal rate in day 55 for both depth 2 and depth 3 (Figure 24) was as a result of the complex carbon being degraded to provide simpler carbon units for the bacteria in the system, since SRB cannot utilise insoluble organic matter as their carbon and energy source.

In summary, the results of this investigation indicate that enhanced hydrolysis of complex carbon sources relies primarily on an enzymatic hydrolysis of biomacromolecules. Deflocculation and mixing are essential for the exposure of previously inaccessible macromolecules to cleavage by these enzymes as well as the release of trapped products thereby alleviating possible end-product inhibition. A reflocculation step, facilitated by the reciprocation of partially digested sludge, is also crucial and serves to increase contact between particle-bound enzymes, undigested substrates and biomass in a sulphate-rich micro-environment within newly formed flocs. Sulphide and sulphite released *in situ* during the sulphate reduction process increase the enzyme activity leading to enhanced floc breakdown and sludge solubilisation.

This study has investigated the overall enzymology within the Recycling Sludge Bed Reactor (RSBR) with respect to three different depths [16cm; 32cm; 50cm] and variations in sulphate, sulphite, sulphide, carbohydrates, protein and lipid.

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6. APPENDICES

6.1 STUDENTS

6.1.1 Post Doctoral Fellows

- F.vanJaarsveld: PhD: University Port Elizabeth: 1998
- B.Pletschke: PhD: University Port Elizabeth: 1999-2001
- J.Burgess: PhD: Cranfield University: 2002-2003.

6.1.2 PhD Students

- **G.Enongene** Enzymology within the Falling Sludge Bed Reactor

6.1.3. MSc Students

- **A.S Tshivhunge** Role of Proteases in solubilisation of sewage sludge
- **N.Ngesi** Effect of cellulases and glucosidases on sewage sludge solubilisation.
- **K.Rashamuse** Recovery of platinum from industrial effluents using a dehydrogenase enzyme from a sulphidogenic bioreactor.
- **N.Ngwenya** Dehydrogenase enzymes from sulphidogenic bioreactor and their role in bioremediation of rhodium from industrial effluents
- **X.Melamane** Enzymology of enhanced sludge solubilisation: Properties of lipases.
- **S.Oyekola** Role of the enzymes within the cellulosome in the bioremediation of industrial effluents.

6.2. PUBLICATIONS

6.2.3. Papers

- **C.G.Whiteley, B.Pletschke, P.Rose, G.Enongene, K.Whittington-Jones**
  Co-digestion of primary sewage sludge and industrial wastewater under anaerobic sulphate reducing conditions: Enzymatic profiles in a Reciprocating Sludge Bed Reactor.
  Water Science & Technology; 2003; Accepted

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

- **C.G.Whiteley, B.I.Pletschke, G.Enongene, P.Rose and K.Whittington-Jones**
  Enzymological profile within an anaerobic sulphidogenic bioreactor.
  Biotech and Bioeng., [Submitted]

- **C.G. Whiteley, P.Rose, B.Pletschke and X.Melamane**
  Enzymology of enhanced sludge solubilisation: Properties of lipases.
  Water Research, 37)(2), 289-296, 2003

- **C.Whiteley, B.Pletschke, P. Rose and N.Ngesi;**
  Specific Sulphur Metabolites Stimulate β-Glucosidase Activity in an Anaerobic Sulphidogenic Bioreactor. **Biotech. Letts., 24 (18), 1509-1513, 2002.**

- **C.G. Whiteley, P.Rose, and B.Pletschke;**
  Environmental enzymology: Enzymology of accelerated sludge solubilisation: Role of ATP Sulphurylases. **Enz. Microbiol. Tech. 31(3), 329-336, 2002.**

- **C.G. Whiteley*, P. Heron, B.Pletschke, P.D. Rose, S. Tshivhunge, F.P. van Jaarsveld and K.Whittington-Jones.** The Enzymology of Sludge Solubilisation Utilising Sulphate Reducing Systems. Properties of Proteases and Phosphatases; **Enz. Microbiol. Tech. 31(4), 419-425, 2002.**

6.2.4. Conferences

6.2.4.1. International

- **[KEY NOTE] C.G.Whiteley, P.Rose, B.Pletschke, F van Jaarsveld, P.Heron, N.Ngesi, S.Tshivhunge**: Environmental Enzymology: Enzymology of Accelerated Primary Sewage Sludge Solubilisation and Bioremediation of Acid Mine Drainage
  International Symposium Environmental Biotechnology, 5, 90, 2000, Kyoto, JAPAN

- **C.G.Whiteley, B.Pletschke, P.Rose, G.Enongene, K.Whittington-Jones**
  Co-digestion of primary sewage sludge and industrial wastewater under anaerobic sulphate reducing conditions: Enzymatic profiles in a Reciprocating Sludge Bed Reactor
  Anaerobic Digestion of Solid Wastes; IWA: Munich, GERMANY, 7a; 17-24; 2002

- **C.G.Whiteley, X.Melamane, B.Pletschke, P.Rose.**
  Effect of Lipases on the Acceleration of Solubilisation of Primary Sewage Sludge.
  Environmental Biotechnology 2002; IWA; NEW ZEALAND, B5, 379-386; 2002

- **C.G.Whiteley, B.I.Pletschke, P.D Rose and A.S Tshivhunge.**
  Environmental Enzymology: Enzymology of Accelerated Primary Sewage Sludge Solubilisation: Effect of sulphate, sulphite and sulphide on proteases:
  Asia Waterqual; IWA; Fukuoka, JAPAN, 2001.

- **C.G.Whiteley, B.I.Pletschke and P.D Rose:**
  Enzymology of Accelerated Primary Sewage Sludge Solubilisation: Effect of Sulphurylases: **Anaerobic Digestion; IWA; Antwerp, BELGIUM, 2001.**

- **C.G.Whiteley, B.I.Pletschke and P.D Rose;**
  Enzymology of Accelerated Primary Sewage Sludge Solubilisation: Effect of Proteases: **Sludge Management; IWA; Taipei; TAIWAN; 2001.**

6.2.4.2. Local

- **C.Whiteley, P.Rose, B.Pletschke and X.Melamane**:
  Environmental Enzymology: Role of lipases in sewage sludge solubilisation
  WISA Conference, 40, 2002, Durban, South Africa

- ***C.Whiteley, W.Leukes, B.Pletschke and X.Melamane***
  Defouling of membranes with sludge enzymes
  WISA Conference, 168, 2002, Durban, South Africa

- **C.Whiteley, D.Sanyahumbi, B.Pletschke and K Rashamuse**;
  Environmental Enzymology: Platinum recovery from industrial wastes using sludge enzymes; **WISA Conference, 162, 2002, Durban, South Africa**

- **C.Whiteley, B.Pletschke, T.Akhurst, S.Watson, and P.Rose.**
  Accelerated Sludge Solubilisation under Sulphate Reducing Conditions
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- **C.Whiteley, F.van Jaarsveld, P.Rose and N. Ngesi**:
  Environmental Enzymology: Role of cellulases and glycohydrolases in sewage sludge solubilisation. **WISA Conference, 26, 2000, Sun City, South Africa**
  BIOY2K, 85, 2000, Grahamstown, South Africa.

- **C.Whiteley, F.van Jaarsveld, P.Rose and S.Tshivhunge**: Environmental Enzymology: Identification, isolation, kinetics, characterisation and inhibition of proteases.
  WISA Conference, 32, 2000, Sun City, South Africa
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- **C.Whiteley, F.van Jaarsveld, P.Rose, N. Ngesi, P.Heron and S.Tshivhunge**;
  Environmental Enzymology: Enzymology of accelerated primary sewage sludge solubilisation.: **WISA Conference, 32, 2000, Sun City, South Africa**
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- **C.Whiteley, C.Corbett, K.Whittington-Jones, F.van Jaarsveld and P.Rose**: Enhanced Hydrolysis of Primary Sewage Sludge under Sulphate Reducing Conditions
  BIOY2K, 85, 2000, Grahamstown, South Africa.

6.2.5. Patents

- **RSA Patent 2003/2131 Whiteley, C.G., Pletschke, B.I., Burgess, J., Leukes, W.D**
  Enzyme additive for use in biological wastewater treatment

Other related WRC reports available:

Salinity, Sanitation and Sustainability: A Study in Environmental Biotechnology and Integrated Wastewater Beneficiation in South Africa (Report 1)

Rose PD

Saline effluents present intractable problems both in their treatment and disposal. Based on work in the field of algal biotechnology undertaken over a number of years at Rhodes University and LIRI Technologies, the objectives of this project were focused on the development of an integrated system for the treatment, the effective utilisation as a resource and ultimate disposal of saline effluent wastes.

Most desalination processing options produce a concentrated brine stream which must, nevertheless, also be dealt with. An additional complication is the accumulation of organics which cause odour problems and also the concentration of heavy metal pollutants. Membrane technology now offers cost-effective desalination but organics must be removed to prevent fouling, reduced fluxes, shorter membrane life and increased costs. In the tanning industry large quantities of water are used in the processing of hides and outflows contain highly elevated TDS values.
This project proposed a biotechnological approach to dealing with saline effluents and its purpose was to attempt to demonstrate the feasibility or otherwise, of the application of micro-algal technology to the problem.
The following deliverables, identified as desirable outcomes from the programme, were successfully demonstrated:

- It is a low-technology system for the economic disposal of saline effluents and possibly including the recovery of algal products of value
- It is a process for the co-disposal of refractory organic solids with saline effluents
- It provides a possible solution to the tannery effluent problem of "salting" as a method of preservation for green hides
- It is able to remove nutrients and heavy metals from brine effluents enabling their disposal by dilution where this is appropriate.

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