THE ENZYMOLOGY OF SLUDGE SOLUBILISATION UNDER BIOSULPHIDOGENIC CONDITIONS: ISOLATION, CHARACTERISATION AND PARTIAL PURIFICATION OF ENDOGLUCANASES

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

MASTER OF SCIENCE

Of

RHODES UNIVERSITY

in the

Department of Biochemistry, Microbiology and Biotechnology

Faculty of Science

By

OLUWASEUN OYEKANMI OYEKOLA

November 2003

ABSTRACT

Endoglucanases play an important function in cellulose hydrolysis and catalyse the initial attack on the polymer by randomly hydrolysing the β-1,4 glucosidic bonds within the amorphous regions of cellulose chains. Cellulolytic bacteria have been isolated and characterised from the sewage sludge and the activation of several hydrolytic enzymes under biosulphidogenic conditions of sewage hydrolysis has been reported. The aims of this study were to: identify, induce production, locate and isolate, characterise (physico-chemical and kinetic) and purify endoglucanases from anaerobic biosulphidogenic sludge.

The endoglucanase activities were shown to be associated with the pellet particulate matter and exhibited a pH optimum of 6 and temperature optimum of 50 ^{o}C. The enzymes were thermally more stable when immobilised to the floc matrix of the sludge than when they were released into the aqueous solution via sonication. For both immobilised and released enzymes, sulphate was slightly inhibitory; activity was reduced to 84 % and 77.5 % of the initial activity at sulphate concentrations between 200 and 1000 mg/l, respectively. Sulphite was stimulatory to the immobilised enzymes between 200 and 1000 mg/l. Sulphide stimulated the activities of the immobilised endoglucanases, but inhibited activities of the soluble enzymes above 200 mg/l. The enzyme fraction did not hydrolyse avicel (a crystalline substrate), indicating the absence of any exocellulase activity. For CMC (carboxymethylcellulose) and HEC (hydroxylethylcellulose) the enzyme had $K_{m,app}$ values of 4 and 5.1 mg/ml respectively and $V_{max,app}$ values of 0.297 and 0.185 μmol/min/ml respectively. Divalent ions (Cu^{2+}, Ni^{2+} and Zn^{2+}) proved to be inhibitory while Fe^{2+}, Mg^{2+} and Ca^{2+} stimulated the enzyme at concentrations between 200 and 1000 mg/l. All the volatile fatty acids studied (acetic acid, butyric acid, propionic acid and valeric acid) inhibited the enzymes, with acetic acid eliciting the highest degree of inhibition.

Sonication released ~74.9 % of the total enzyme activities into solution and this was partially purified by PEG 20 000 concentration followed by DEAE-Cellulose ion exchange chromatography, which resulted in an appreciable purity as measured by the purification factor, 25.4 fold.

Keywords: Anaerobic biosulphidogenic sludge; biosulphidogenic conditions; cellulose; endoglucanases

TABLE OF CONTENTS

Title page………………………………………………………………………………….i

Abstract…………………………………………………………………………………..ii

Table of contents………………………………………………………………………...iv

List of figures…………………………………………………………………………….x

List of tables…………………………………………………………………………xiii

List of abbreviations……………………………………………………………………...xiv

Acknowledgements……………………………………………..………………..xvi

CHAPTER 1: Literature Review…...………………………………………………1

1.1 Anaerobic digestion……………………………………………….………..1

1.1.1 Synergism in anaerobic digestion……...……………………………………...1

1.1.2 Biological hydrolysis of primary sewage sludge…..……………………..…..3

1.1.3 Anaerobic microbes and enzymes involved in sewage sludge hydrolysis……4

1.2 Sulphate reducing bacteria……………...……………………………….……5

1.2.1 Occurrence………………………......…………………………..……….....5

1.2.2 Metabolic requirements………………………………………...…………..6

1.2.3 Mechanism of sulphate reduction……………………………...…………...7

1.3 Sewage sludge as carbon source for SRB...……………………………..………7

1.3.1 Enhanced solubilisation of primary sewage sludge…………………………...8

1.3.2 Possible mechanism of enhanced PSS hydrolysis under biosulphidogenic conditions…………………………………………………………………………10

1.4 Sewage sludge; a useful waste……………………………………………...10

1.5 Cellulose………………………………………………………………………12

1.5.1 Occurrence in nature……………………………………………………….12

1.5.2 Structure………………………………………………………..………...13

1.5.3 Enzymatic hydrolysis of cellulose.....……………………………...……………14

1.5.3.1 Cellulases and their modes of action………………………...……………..15

1.5.3.2 Synergism…………………………………………………...………………17

1.5.3.3 Processes preceding enzymatic hydrolysis of cellulose………...……………18

1.5.3.4 Rate limiting factors in enzymatic hydrolysis……………………………….. 18
1.5.3.5 Cellulosome……………………………………………………………………...18
1.5.3.6 Cellulases in sewage sludge……………………………………………………...19
1.5.3.7 Uses of cellulases………………………….…………………………………….20
1.6 Research hypothesis…………………………..…………………………… 22
1.7 Research objectives…………………………..…………………………… 23

CHAPTER 2: Production and induction of endoglucanases under biosulphidogenic conditions…………………………………………………………………….... 24
2.1 Introduction………………………………………………………………… 24
2.2 Materials and methods………………………………………………….26
2.2.1 Materials……………………………………………………………......26
2.2.2 Methods………………………………………………………............. 26
2.2.2.1 Reactor set-up and sludge source……………………………………........26
2.2.2.2 Enzyme preparation……………………………………………………... 27
2.2.2.3 Time course study…………………………..…………………………...….. 27
2.2.2.3.1 Endo-β-1, 4-D-glucanase activity assay……………………………..... 28
2.2.2.3.2 Protein determination.…………………………………………………… 28
2.2.2.3.3 Reducing sugar estimation………………………………………………...28
2.2.2.3.4 Analytical procedures………………………………………………….29
2.2.2.4 Induction studies……………………………………………………...…. 29
2.3 Results and discussion………………………………………………….30
2.3.1 Endoglucanases production under biosulphidogenic and methanogenic conditions……………... 30
2.3.2 Sulphate removal and sulphide production……………………………........ 31
2.3.3 Protein distribution………………………………………………………. 32
2.3.4 Production of reducing sugars…………..…………………………………... 33
2.3.5 pH and temperature fluctuations over time………………………..……... 34
2.3.6 Induction studies………………………………...…………………….. 35
2.4 Summary……………………..…………………………………………...37

CHAPTER 3: Enzyme location and extraction... 39
3.1 Introduction.. 39
3.2 Materials and methods..41
3.2.1 Materials...41
3.2.2 Reactor set-up and sludge source...42
3.2.3 Methods..42
3.2.3.1 Endo-β-1, 4-D-glucanase activity assay.......................................42
3.2.3.2 Protein determination...43
3.2.3.3 Enzyme location..43
3.2.3.4 Enzyme extraction..43
3.2.3.4.1 Sonication...43
3.2.3.4.2 Detergent...43
3.2.3.4.3 Organic solvent...44
3.3 Results and discussion...44
3.3.1 Location...44
3.3.2 Extraction...45
3.3.2.1 Sonication...45
3.3.2.2 Detergent..46
3.3.2.3 Organic solvent...48
3.4 Summary.. 48

CHAPTER 4: Characterisation of endoglucanases from biosulphidogenic anaerobic digested sludge.. 51
4.1 Introduction... 51
4.2 Materials and methods..53
4.2.1 Materials...53
4.2.2 Methods..54
4.2.2.1 Sludge sample source...54
4.2.2.2 Enzyme preparation... 54
4.2.2.3 pH.. 54

4.2.2.4 Temperature……..…………………………...55
4.2.2.5 Thermal stability……..……………………………………………………..........55
4.2.2.6 Metal ions……………………………………………………….................... 55
4.2.2.7 Volatile fatty acids…………………………………………….................... 55
4.2.2.8 Sulphur-containing compounds…..……………………………….................56
4.2.2.9 Protease inhibitors……………..………………………………………………… 56
4.2.2.10 Catalytic properties and substrate specificity…………….............................. 56
4.3 Results and discussion.…………………………………………….............. 56
4.3.1 pH…………………………………………………………………........ 56
4.3.2 Temperature…………………………..…………………………..…..... 57
4.3.3 Thermal stability……………………..…………………………………...........58
4.3.4 Metal ions…………………………………………………………….........59
4.3.5 Volatile fatty acids……………………………………..………….........60
4.3.6 Sulphur-containing compounds…….…………………………………..........61
4.3.7 Protease inhibitors……………………………………………………….....62
4.3.8 Catalytic properties and substrate specificity of endoglucanase…………......63
4.4 Summary……………………………………………………………………….64

CHAPTER 5: Purification of endoglucanase from biosulphidogenic anaerobic digested sludge.. 67
5.1 Introduction………………………………….....……………………….....67
5.1.1 Extract concentration.. 67
5.1.1.1 Ultrafiltration…....………………………………………………………… 67
5.1.1.2 Freeze-drying……………………….....……………………………………68
5.1.1.3 Precipitation by salting out……………………………………………..... 68
5.1.2 Chromatography…………………………………………...............................69
5.1.2.1 Ion exchange chromatography………………...……………………………......69
5.1.2.2 Size exclusion chromatography………………………………………………...70
5.1.2.3 Affinity chromatography…………………………………………………...…..71
5.1.2.4 Adsorption chromatography………………………………………………….... 72
5.1.3 Electrophoresis…………………………………………………………………73

5.1.3.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)........................73
5.2 Materials and methods........................73
5.2.1 Materials........................73
5.2.2 Methods........................74
5.2.2.1 Sludge sample source........................74
5.2.2.2 Endoglucanase activity and protein determination........................74
5.2.2.3 Endoglucanase purification........................74
5.2.2.3.1 Preparation of cell free extract........................74
5.2.2.3.2 Ammonium sulphate precipitation........................74
5.2.2.3.3 Freeze-drying........................75
5.2.2.3.4 Extract concentration with PEG........................75
5.2.2.3.5 DEAE-Cellulose ion exchange chromatography........................75
5.2.2.3.6 Gel filtration with Sephadex G-100........................76
5.2.2.3.7 Adsorption chromatography using hydroxylapatite........................76
5.2.2.3.8 Affinity Chromatography using phosphoric acid-swollen cellulose........................76
5.2.2.4 SDS-PAGE........................77
5.3 Results and discussion........................77
5.3.1 Extract concentration........................77
5.3.2 Chromatography........................78
5.3.3 SDS-PAGE........................80
5.4 Summary........................80

CHAPTER 6: General discussion and conclusions........................82
6.1 Introduction........................82
6.2 Production of endoglucanases under biosulphidogenic conditions........................82
6.3 Location and physico-chemical characterisation of endoglucanases........................83
6.4 Purification of endoglucanase........................84
6.5 Future work........................84

REFERENCES:........................86

APPENDICES:……………………………………………………………………...,.102
Appendix 1: Cellulose……………………………………………………………………102
Appendix 2: Preparation of DNS reagent... 104
Appendix 3 Glucose standard curve………………………………………………….. 104
Appendix 4: Protein standard curve…………………………………………………...105
Appendix 5: Molecular weight determination……………………..………………….. 106
Appendix 6: Regeneration and equilibration of the column…………………………...106
Appendix 7: Calculation of the number of grams of ammonium sulphate (g)………..106
Appendix 8: Preparation of phosphoric acid-swollen cellulose ……….......................107
Appendix 9: Preparation of SDS-PAGE ....…………………….......................... 107

LIST OF FIGURES

Figure 1.1: Metabolic steps and microbial groups involved in anaerobic digestion; 1) Fermentative bacteria, 2) H_2-producing acetogenic bacteria, 3) H_2-consuming acetogenic or homoacetogenic bacteria, 4) CO_2-reducing methanogenic bacteria, 5) Acetoclastic methanogenic bacteria.. 2

Figure 1.2: The pathway of dissimilatory sulphate reduction................................ 7

Figure 1.3: Diagram showing the β –1,4–linkages in a cellulose chain……………….. 13

Figure 1.4: Diagram showing the cellulose microfibrils in layers.......................... 14

Figure 1.5: Schematic representation of the morphology and organisation of crystalline cellulose.. 14

Figure 1.6: Diagram showing enzymatic hydrolysis of cellulose........................... 15

Figure 1.7: The two mechanisms of enzymatic hydrolysis of the glycosidic bonds... 16

Figure 1.8: Simplified schematic view of interaction between the *Clostridium thermocellum* cellulosome and its substrate, and its connection to the cell surface via an associated anchoring protein................................. 20

Figure 2.1: Schematic diagram of the laboratory-scale (closed/single stage) stirred tank reactor, used for monitoring the production of endoglucanases during anaerobic degradation: (A) 10 % (w/v) zinc acetate solution as hydrogen sulphide trap; (B) rubber stopper; (C) 10 l glass tank; (D) sampling port; (E) metal clamp; (F) magnetic stirrer bar; (G) magnetic stirrer; (H and H_1) Oxygen impermeable Tygon® tubing... 27

Figure 2.2: Production of endoglucanase under biosulphidogenic and methanogenic conditions.. 31

Figure 2.3: Sulphate reduction and concurrent sulphide production in the bioreactors.. 32

Figure 2.4: Distribution of proteins in the biosulphidogenic and methanogenic reactors over time.. 33

Figure 2.5: Production of reducing sugar in the biosulphidogenic and methanogenic bioreactors.. 34

Figure 2.6: pH and temperature fluctuations during the time course...................... 35

Figure 2.7: Production of endoglucanase in the presence of different inducers..... 36

Figure 2.8: Dependence of endoglucanase production on the concentration of α-cellulose as inducer.. 37

Figure 3.1: Action of detergent in solubilising the membrane bound proteins....... 41

Figure 3.2: Diagram of laboratory scale biosulphidogenic reactor: (A) feed vessel containing PSS (COD and SO_4^{2-} 2000mg/l); (B) experimental biosulphidogenic bioreactor containing 10 % (v/v) SRB mixture culture; (C) overflow vessel to receive passive sludge effluent from the biosulphidogenic bioreactor; (D) magnetic stirrers; (E) peristaltic pump set to allow HRT for two days; (F) plastic support; (G) zinc acetate solution as hydrogen sulphide trap; (H) sampling port; (I) vent and (J) overflow port.. 42

Figure 3.3: Enzyme activity associated with different fractions in sludge from the biosulphidogenic reactor.. 45

Figure 3.4: Endoglucanase release from the pellet particulate via sonication: (a) Optimising enzyme release by sonication, (b) Relative activity present in different fractions.. 46

Figure 3.5a: Endoglucanase release from the pellet particulate using detergent (Triton X-100). Optimising enzyme release using detergent............... 47

Figure 3.5b: Endoglucanase release from the pellet particulate using detergent (Triton X-100). Relative activity present in different fractions............ 48

Figure 3.6: Endoglucanase release from the pellet particulate using organic solvent (n-Butanol): (a) Optimising enzyme release using organic solvent (b) Relative activity present in different fractions.................. 49

Figure 3.7: Ultrastructure of the *Clostridium thermocellum* cell surface (a) Transmission of a resting polycellulosomal protuberance. (b) Transmission electron micrograph of a protracted polycellulosomal. The cellulosome is mainly associated with the cellulose surface and connected to the cell via extended fibrous material, believed to comprise the anchoring proteins. Scale bars = 100nm........................ 50

Figure 4.1: Effect of pH on endoglucanase activity.. 57

Figure 4.2: Effect of reaction temperature on endoglucanase activity................. 58

Figure 4.3: Thermal stability profile of the endoglucanase: (a) Soluble enzyme extract (b) Immobilised enzyme extract... 59

Figure 4.4: Effect of metal ions on endoglucanase activity.................................. 60

Figure 4.5: Effect of volatile fatty acids on endoglucanase activity...................... 61

Figure 4.6: Effect of sulphur-containing compounds on endoglucanase activity: (a) Soluble enzyme extract (b) Immobilised enzyme extract.............. 62

Figure 4.7: Effect of protease inhibitors on endoglucanase activity...................... 63

Figure 4.8: Dependence of endoglucanase activity on different substrate types. (a) Michaelis-Menten curve. Double reciprocal (Lineweaver-Burk) plot with CMC as substrate (inset) (b) Michaelis-Menten curve. Double reciprocal (Lineweaver-Burk) plot with HEC as substrate (inset).. 65

Figure 5.1: Separation of different sized molecules by size exclusion chromatography... 71

Figure 5.2: Purification of an enzyme by affinity chromatography....................... 72

Figure 5.3: DEAE-Cellulose ion exchange chromatography. Column dimension: 1 × 25 cm. Flow rate:1ml/min. Endoglucanases were eluted with 0-1 M NaCl in sodium phosphate buffer (0.05 M, pH 6).......................... 79

Figure 5.4: SDS-PAGE (12 % resolving gel). (a) Coomasie blue staining (b) Silver staining. Lane 1: Prestained molecular weight markers (Myosin, 200 kDa; β-galactosidase, 116.3 kDa; Phosphorylase b, 97.4 kDa; Bovine serum albumin, 66.2 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 31 kDa; Soybean trypsin inhibitor, 21.5 kDa; Lysozyme, 14.6 kDa; and Aprotinin, 6.5 kDa). Lane 2: Commercial cellulase (control). Lanes 3a and b: PEG concentrated extract. Lanes 4a and b: sonic extract. Lanes 5a and b – 9a and b: Peaks 1-5 from DEAE-Cellulose.. 81

LIST OF TABLES

Table 1.1: Cellulolytic bacteria from sewage sludge and anaerobic digesters...………..20

Table 4.1: Substrate specificity and kinetic parameters of endoglucanase……………..66

Table 5.1: Comparison of extract concentration methods……………….…………...78

Table 5.2: Purification table of endoglucanase…………..……………………………79

LIST OF ABBREVIATIONS

AMP = Adenosine monophosphate

AMD = Acid mine drainage

APS = Adenosine phosphosulphate

ATP = Adenosine triphosphate

BSA = Bovine serum albumin

CCR = Carbon catabolite repression

CMC = Carboxymethylcellulose

COD = Chemical oxygen demand

CSTR = Continuous stirred tank reactor

ddH_2O = Double deionised water

EPS = Extracellular polymeric substance

EDTA = Ethylene diamine tetraacetic acid

HEC = Hydroxyethylcellulose

HRT = Hydraulic retention time

l = Litre

MPB = Methane producing bacteria

MR = Methanogenic bioreactor

NMWC = Nominal molecular weight cut-off

PAGE = Polyacrylamide gel electrophoresis

PEG = Polyethylene glycol

PHC = Petroleum hydrocarbon

Pi = Inorganic phosphate

PMSF = Phenylmethanesulfonyl fluoride

PPi = Pyrophosphate

PSS = Primary sewage sludge

SDS = Sodium dodecyl sulphate

SR = Biosulphidogenic bioreactor

SRB = Sulphate reducing bacteria

U = Units

VFA = Volatile fatty acids

v/v = Volume per volume

w/v = Weight per volume

ACKNOWLEDGEMENTS

It is my sincere pleasure to appreciate those who aided in the actualisation of this thesis. These people have contributed immensely to bring into reality this “dream”:

- My most profound gratitude goes to God almighty that gave me strength through it all and gave me reasons to laugh at the end of it all.
- Professor C.G. Whiteley, thank you so much for the opportunity you gave me to be a part of this challenging field of research.
- Dr. B.I. Pletschke, I appreciate the help and guidance offered especially with respect to my purification studies.
- Special thanks to Dr. Jo Burgess who relentlessly took me through the nitty-gritties of wastewater treatment and helped in proof reading this thesis.
- Henry Roman, I owe you my sincere appreciation for always keeping me abreast with information and providing many of the “raw materials” used in “constructing” this thesis.
- Victor Wutor and Jabu Mthombeni, thanks for your friendship and support.
- Lab 309 crew (Enos, Nonthy, Vuyo and Xolisa), thanks for bearing with me at all times and keeping the team spirit alive to steer this boat to a wonderful harbour.
- My family, you have been my source of inspiration. I am very grateful for the love and moral support given me without measure.
- Titi, special thanks to you for being there for me and never giving up on me.
- The Odunugas, thanks for being a channel of blessings to me. You are godsend.
- The Water Research Commission (WRC), thanks for the financial support throughout the course of this project.

CHAPTER 1
LITERATURE REVIEW

1.1 ANAEROBIC DIGESTION

This may be defined as the process by which organic wastes are biologically transformed in the absence of oxygen. Different bacterial populations are involved in this process, which degrade organic compounds to produce a valuable high energy mixture of gases (mainly methane and carbondioxide) (Lastella *et al*., 2002). Apart from treating the wastewater and energy generation, anaerobic digestion of biodegradable waste also results in the reduction of greenhouse gas emissions. It would not only substitute the use of fossil fuels but would also use methane generated from the waste (Kansal *et al*., 1998). Some other advantages of anaerobic degradation include: low energy requirement, low nutrient requirement, small land requirement, a high efficiency in reducing COD (chemical oxygen demand) in soluble and insoluble forms and no chemical handling (Forday and Greenfield, 1983; Massé and Masse, 2000). This method of waste treatment is therefore practical and economical.

1.1.1 Synergism In Anaerobic Digestion.

A consortium of microbes exists in the environment and these microbes individually secrete enzymes needed for anaerobic digestion. Synergism involves the improved effect of the microorganisms involved in this process when acting jointly as compared to the sum of their individual effects. The anaerobic degradation process occurs through the synergistic interaction of four different classes of microorganisms; hydrolytic, fermentative, acidogenic and methanogenic bacteria in a multi-step process (Adney *et al*., 1991) (Figure 1.1). During the first stage (hydrolysis), insoluble complex organic compounds are decomposed by extracellular enzymes to form soluble products; this step is therefore called liquefaction (Lastella *et al*., 2002). Cellulose and starches are hydrolysed to simple sugars; proteins are broken down into the amino acids and lipids to fatty acids and glycerol. The soluble compounds resulting from this process are transported into the bacterial cells to be used for their metabolic activities (Cadoret *et al*., 2002). The facultative and anaerobic bacteria involved in this hydrolysis are also responsible for the formation of volatile acids (Perot *et al*., 1988). The second stage,

acidogenesis occurs with the formation of CO_2 and H_2, acetate and higher organic acids. With increasing concentration of acids the pH of the anaerobic digester decreases.

During the third stage, acetogenesis (generation of acetate) occurs and the organic acids produced during acidogenesis are converted into H_2 and acetate by the acetogenic bacteria. These conversions of volatile fatty acids are important as these acids are mainly lethal to the methanogenic bacteria (Forday and Greenfield, 1983). During anaerobic digestion, hydrolytic reactions tend to slow down the production of methane and so can be referred to as the rate-limiting step of the overall anaerobic microbial digestion. Methanogenesis is the last stage of the anaerobic digestion involving methane-producing microbes called methanogens. Methanogens are found in several environments where they require strict anaerobic conditions for growth and can grow over a wide range of temperature with an optimum temperature of 35 ^{o}C (Mutkowska, 1997). They live at a pH range of 7-8 (Lastella *et al*., 2002). The acetoclastic methanogens convert acetate to methane while the CO_2-reducing methanogens convert CO_2 to methane (Novaes, 1986).

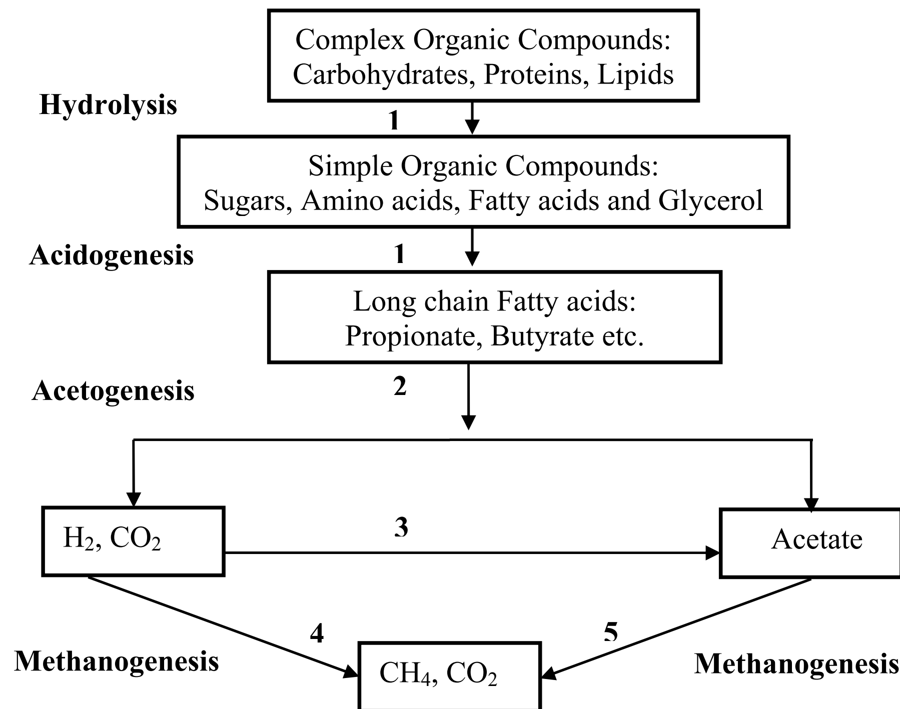


Figure 1.1: Metabolic steps and microbial groups involved in anaerobic digestion; 1) Fermentative bacteria, 2) H_2-producing acetogenic bacteria, 3) H_2-consuming acetogenic or homoacetogenic bacteria, 4) CO_2-reducing methanogenic bacteria, 5) Acetoclastic methanogenic bacteria (Novaes, 1986).

1.1.2 Biological Hydrolysis Of Primary Sewage Sludge

Land fillings and incineration have been recognised as economical and easy alternatives for the disposal of sewage sludge, but these methods will cause likely environmental risks that affect the well-being and living environment of man (Cenni *et al*., 2001). As a result of these dangers imposed, a better and environmentally friendly way of disposing domestic wastewater is therefore required. Anaerobic digestion of sewage sludge provides some of the solutions to these problems. The process decreases bad odour, kills aerobic pathogens, stabilises the organic matter, minimises the water content of the sediments and decreases the availability of free forms of heavy metals due to their immobilisation (Selivanovskaya *et al*., 2001). Another advantage that gives this process preference is its gas production (Mikkelsen and Keiding, 2002). Anaerobic digestion of municipal sewage sludge is an essential method of converting organic materials into CO_2 and methane (biogas) through successive anaerobic microbial processes (Fukui *et al*., 2000). This treatment is used to stabilise concentrated sludge at the municipal waste treatment plants (Selivanovskaya *et al*., 2001). Therefore, sewage sludge is not actually sewage but one of the final products of the treatment of sewage at a sewage treatment plant.

Typically, domestic wastewater has a very complex mixture of both organic and inorganic compounds. The make-up of wastewater depends on age; the one that is few minutes or a few hours old may be different from the one that has been conveyed for several hours. This can be accounted for by some factors such as: microbial growth and respiration that take place in both bulk water and in biofilms, solubilisation, enzymatic hydrolysis of macromolecules and hydraulic shear forces (Nielsen *et al*., 1992). Sewage sludge is carbonaceous and rich in organic matter (Chen *et al*., 2002). Considering the high percentage of organic matter, the process of enzymatic hydrolysis in biological wastewater treatment is vital for total solubilisation of these organics (Goel *et al*., 1998).

Organic matter in urban wastewater includes soluble carbohydrates, amino acids, alcohol, and volatile acids with polymers and heteropolymers including proteins, polysaccharides, lipids and sometimes lignin (Raunkjǽr, *et al*., 1994; Zeeman and Sanders, 2001). Many of these particles are large, preventing them from being transported into the bacterial cells. Before bacteria can assimilate high molecular weight compounds, the compounds

are usually broken down by extracellular hydrolytic enzymes into soluble products which are used as carbon sources and energy for subsequent metabolic processes (Cadoret *et al*., 2002). The hydrolysis of wastewater polymers to monomers seems to be the rate-limiting step of the biodegradation process due to the fact that these polymers are hydrolysed slowly (Ubukata, 1999). This is most probably a consequence of limited availability of extracellular enzymes from the microbial cells involved in the process (Jung *et al*., 2002).

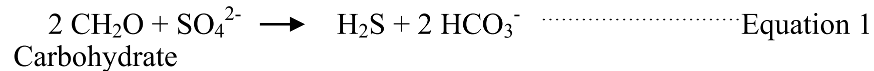
1.1.3 Anaerobic Microbes And Enzymes Involved In Sewage Sludge Hydrolysis.

A complex microbiological population is present in any biological sewage treatment process and these microorganisms are responsible for most of the carbon and nutrient utilisation (Wagner and Loy, 2002). They decompose the organic matter and transform the resultant products into microbial biomass, humic material and waste gases such as carbon dioxide and methane (Houghton and Quarmby, 1999). The make-up of the community is dependent on the influent to the sewage treatment plant, as different substrate constituents of the influent are needed for the growth of different bacteria (Yuan and Blackall, 2002). Possible sources of anaerobic microbes in wastewater are land drainage and storm water, faecal matter and biological processes. In sewage, faecal bacteria will be expected to be the largest community of microorganisms present. Although the typical bacteria that are used as indices for faecal pollution are the coliforms (*Escherichia coli* I, *Enterococci* and *Clostridium welchii*) these may not be the major bacteria in view of number (Crowther and Harkness, 1975). Clostridia and micrococci appear to produce most of the lipases for the hydrolysis of lipid in sewage sludge and cellulolytic species of *Bacteriodes* was isolated from a methanogenic cellulose enrichment culture of municipal sewage sludge which produces cellulases for cellulose degradation (Lester, 1988; Murray *et al*., 1984).

Due to the complexity of the composition of the wastewater entering the wastewater treatment plant, it is quite likely that a wide range of extracellular enzymes is essential for the mineralisation of the different substrates (Goel *et al*., 1998). Extracellular enzymes responsible for the hydrolysis of organic polymers in the sewage sludge are either found attached to the cell surface of the microbes producing them or free in soluble form in the aqueous medium or adsorbed on surfaces apart from that of the organisms secreting them (Chróst, 1991).

1.2 SULPHATE REDUCING BACTERIA

Sulphate is reduced by most bacteria, fungi, and plants into sulphide, which is used in the synthesis of certain amino acids. This process is called assimilatory sulphate reduction and is a biosynthetic process. On the other hand, during dissimilatory sulphate reduction, sulphate acts as an electron acceptor for the degradation of organic substrates (Equation 1) (Knobel and Lewis, 2002).



$$\underset{\text{Carbohydrate}}{2\,CH_2O} + SO_4^{2-} \longrightarrow H_2S + 2\,HCO_3^- \quad \text{Equation 1}$$

Sulphate reducing bacteria (SRB) are anaerobic microbes involved in dissimilatory sulphate reduction to acquire energy, resulting in the liberation of sulphide (Loubinoux *et al*., 2002). When the oxidation of the organic compounds is coupled to the sulphate reduction by the SRB, the energy released from the degradation of the organic compounds is used for growth and metabolism of these bacteria. This group of bacteria has found application in the management of a range of sulphate-rich industrial effluents (Moosa *et al*., 2002). They are the only bacteria so far recognised to be involved in sulphate reduction and thus the sulphur cycle in natural environments can only be initiated by reduction of sulphate to sulphide (McLeod, *et al*., 2002; Gavel *et al*., 1998). This group of bacteria are physiologically diverse (Castro *et al*., 2000). They can be divided into two main groups: those that can completely oxidise organic matter to H_2S and CO_2 and those that can carry out an incomplete oxidation, usually to acetate (O'Flaherty *et al*., 1998).

1.2.1 Occurrence

SRB comprise a wide range of microorganisms taking part in several crucial reactions in many anaerobic environments (Castro *et al*., 2000). These microorganisms are recognised for their ubiquity in natural aqueous environments, especially sulphate-rich ones, they are found in deep offshore petroleum reservoirs (Jeathon *et al*., 2002), petroleum hydrocarbon (PHC) contaminated aquifers (Kleikemper *et al*., 2002), terrestrial hot springs (Castro *et al*., 2000) and anaerobic sludge (Manz *et al*., 1998), to mention a few. They have also been identified in the digestive tracts of humans and animals (Loubinoux *et al*., 2002).

1.2.2 Metabolic Requirements

Some SRB are able to thrive at temperatures below 5 ^{o}C while at the opposite extreme; the spore-forming thermophilic species grow well at temperatures from 65 ^{o}C to 80 ^{o}C (Gibson, 1990). The preferred pH for their growth is around 7 and they are typically inhibited at pH values lower than 6 and higher than 9 (Widdel, 1988). Nutritional requirements for SRB are relatively simple and the major ones include:

(1) Inorganic electron acceptor: SRB seem to be the organisms that reduce the highest number of different electron acceptors, although they are named after a single electron acceptor (sulphate) which is used by most strains. Some SRB carry out dissimilatory reduction of nitrate to nitrite to ammonia (Widdel and Pfennig, 1982).

(2) Carbon sources/electron donors: Numerous substrates have been reported as energy sources for SRB. These vary with different genera. SRB do not degrade polysaccharides, proteins or lipids but depend on the activity of fermentative bacteria for the supply of energy sources (Knobel and Lewis, 2002). Basically the electron donors used are volatile fatty acids e.g. acetate, propionate, butyrate; C_3 and C_4 fatty acids, e.g. lactate, pyruvate, malate; alcohol e.g. ethanol and propanol (Gibson, 1990). Studies have shown that all SRB genera preferentially degrade a wide range of organic acids (Hanselmann *et al*., 1995; Kuever *et al*., 2001). Only a small number of SRB genera are known to readily degrade a broad range of organic acids; e.g. *Desulforhabdus amnigenus* is able to consume lactate, acetate, butyrate and propionate (Oude Elferink *et al*., 1995). In general, lactate seems to be the most common carbon source for SRB (Kleikemper *et al*., 2002). Others include glucose, with an affinity to the SRB that is comparable to that of acetate and lactate (Song *et al.*, 1998); and molecular hydrogen (Jeathon *et al.*, 2002). Certain SRB are known to utilise environmental contaminants such as petroleum hydrocarbon (PHC) constituents (e.g. benzene, toluene, ethylbenzene, xylenes, polycyclic aromatic hydrocarbons, alkanes) or halogenated compounds directly as a source of carbon and energy (Ensley and Suflita, 1995; Zhang and Young, 1997; Kleikemper *et al*., 2002). Primary sewage sludge as later discussed can also serve as a carbon source.

(3) Nitrogen source: Ammonium ions serve as a nitrogen source for the growth of most sulphate reducing bacteria (Gibson, 1990). Several marine strains of *Desulfovibrio* can use a number of amino acids as carbon and nitrogen sources (Stamps *et al*., 1986). Others include nitrate, peptone or tryptone (Jeathon *et al.*, 2002).

1.2.3 Mechanism Of Sulphate Reduction

The principal aim for the reduction of sulphate by the SRB is for energy gain. SRB synthesise numerous enzymes that catalyse sulphate reduction (Mudryk *et al*., 2000) for example; pyrophosphatase, ATP sulphurylase, bisulphate reductase, desulphoviridin and desulphofuscidin (Gibson, 1990; Saas *et al*., 1992; Visscher *et al*., 1992). The biochemical sulphate reduction starts by the transportation of extracellular sulphate across the bacterial cell membrane into the cell (Lengeler *et al*., 1999). ATP sulphurylase catalyses the reaction between the intracellular sulphate and ATP to produce the highly activated molecule adenosine phosphosulphate (APS), as well as pyrophosphate (PPi) which can be subsequently cleaved to yield inorganic phosphate (Figure 1.2). APS is the actual electron acceptor that is subsequently converted to AMP and bisulphite by the enzyme APS reductase (Ullrich and Huber, 2001; Pletschke *et al*., 2002). The bisulphite formed is reduced through a number of intermediates such as metabisulphite ($S_2O_5^{2-}$), dithionite ($S_2O_4^{2-}$), trithionite ($S_3O_6^{2-}$) and thiosulphate ($S_2O_3^{2-}$), to form the sulphide ion (Lengeler *et al.*, 1999).

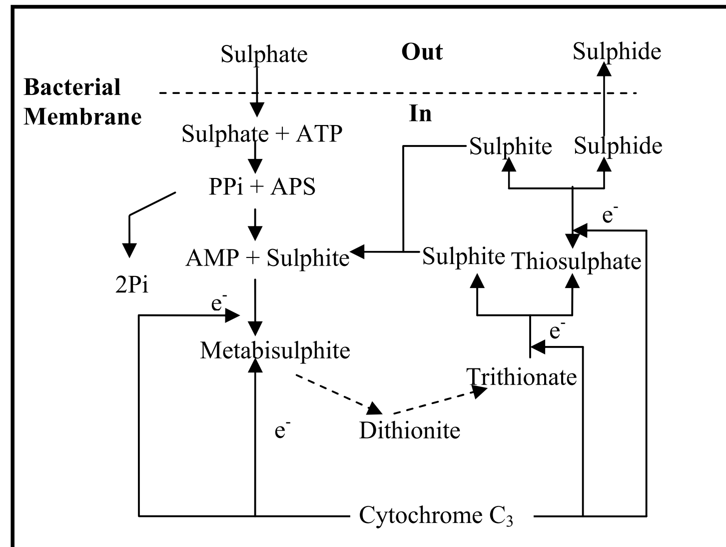


Figure 1.2: The pathway of dissimilatory sulphate reduction (e^-=electron) (Gibson, 1990).

1.3 SEWAGE SLUDGE AS CARBON SOURCE FOR SRB

Several waste carbon sources have been considered in a bid to develop an economical process involving sulphate reduction process (Chang *et al*., 2000), some of which include straw and hay (Béchard *et al*., 1994), sawdust (Wakao *et al*., 1979), peat (Eger and

Lapakko, 1988), spent mushroom compost (Dvorak *et al*., 1992) whey (Christensen *et al*., 1996) and sewage sludge (Butlin *et al*., 1956). Primary sewage sludge has been reported as a low cost, readily available and suitable carbon source for SRB (Pletschke *et al*., 2002). Complex organic compounds (proteins, carbohydrates and lipids), which are present in the sewage sludge, are hydrolysed and further converted to acetate and higher organic acid than acetate, hydrogen and CO_2 (Whiteley *et al.*, 2002a). The organic acids formed during this anaerobic digestion of primary sewage sludge such as, propionate, butyrate and acetate become electron donors for SRB. In sulphate-rich environments, interaction between the bacteria involved in the anaerobic degradation of the organic matter and SRB play a crucial role in the metabolism of organic matter (Gibson, 1990).

1.3.1 Enhanced Solubilisation Of Primary Sewage Sludge

The enzymatic hydrolysis of the organic content of wastewater is a very important step in anaerobic degradation and sludge digestion (Whiteley *et al*., 2003). A major part of the organic matter in municipal wastewater is insoluble and will be partially hydrolysed during the biological treatment processes (Henze and Mladenovski, 1991). The hydrolysis of polymers to their respective monomers is regarded as the rate-limiting step in anaerobic digestion (Penaud *et al*., 1997) as shown by the fact that high molecular weight compounds are hydrolysed slowly and the yield of conventional anaerobic hydrolysis of primary sewage sludge carried out under methanogenic conditions results in low solubilisation (Banister and Pretorius, 1998; Ubukata, 1999).

The rate-limiting hydrolytic step in anaerobic digestion of sewage sludge has been shown to be dependent mainly on the concentration of the hydrolytic enzymes and the contact between these enzymes and their substrates (Jain *et al*., 1992). Particle size has also been shown to have an immense contribution on the rate of anaerobic digestion of complex substrates (Müller *et al*., 1998).

There has not been much information about the location of the extracellular enzymes involved in anaerobic primary sewage sludge (PSS) hydrolysis, apart from previous investigations done in our research group (Whiteley *et al*., 2002a; 2002b; 2003). The findings are similar to observations reported about the hydrolytic enzymes involved in degradation of activated sludge. A large proportion of the extracellular enzymes involved

in activated sludge hydrolysis are immobilised in the extracellular polymeric substances (EPS) (Frølund *et al*., 1995). In general, primary sludge and anaerobic digested sludge have similar properties to the activated sludge with EPS from the activated sludge having larger size (Mikkelsen and Keiding, 2002). Most of the enzymes (lipases, proteases and β-glucosidases) involved in PSS hydrolysis have been shown to be immobilised to the organic particulate matter, which might imply adsorption to either the bacterial cell walls or the floc matrix (Whiteley *et al*., 2002a; 2002b; 2003). Consequently, a little surface of the enzyme is available for the substrate to bind (Goel *et al*., 1998). Moreso, if mass transport to and from the floc matrix is decreased, build-up of product within the floc may cause product inhibition of the produced enzyme and a severe reduction or obstruction of hydrolysis (Whittington-Jones, 2000).

It is known that the degradation process of complex organic compounds in anaerobic treatment of wastewater is significantly affected by the presence of sulphate in the wastewater (Oude Elferink *et al*., 1998). Contradictory reports have been made on the effect of sulphate reduction on the anaerobic process. Some observed competition between the methane producing bacteria (MPB) and SRB while others observed syntropic relations between these two groups of bacteria (Vossoughi *et al*., 2003). The hydrogen sulphide produced during sulphate reduction has also been said to be inhibitory to many bacterial activities (Manilal *et al*., 2000). Under sulphate-limitation conditions, the degradation process of biological compounds can be very complicated (Oude Elferink *et al*., 1998).

The enhanced solubilisation of primary sewage sludge and stimulated enzyme activities under biosulphidogenic conditions as a result of increased hydrolysis of macromolecular polymeric constituents have been reported (Whittington-Jones, 2000; Whiteley *et al*., 2002a; 2002b; 2003; Pletschke *et al*., 2002). An increase in the hydrolytic rate should be a consequence of increase in enzyme activity (Goel *et al*., 1998). Under anaerobic conditions, degradation of organic substrates can be tied to sulphate reduction by the SRB, as the SRB use the energy produced for growth and maintenance (Moosa *et al*., 2002). Fukui *et al*. (2000) observed that sulphate reduction was one of the crucial carbon hydrolysis processes as well as methanogenesis in municipal anaerobic digesters, signifying joint occurrence of SRB and MPB. Vossoughi *et al*. (2002) has also reported

that increasing sulphate concentration leads to a higher removal of COD (chemical oxygen demand). Acetate and hydrogen are substrates common to both SRB and MPB (Koizumi *et al*., 2003).

1.3.2 Possible Mechanism Of Enhanced PSS Hydrolysis Under Biosulphidogenic Conditions

Even though hydrolysis of PSS in the presence of SRB (using the end-products of the process as carbon-source and energy gain) is accelerated, the actual mechanism of this process has not been fully understood. The structure of the sludge floc matrix is as a result of the interaction between the biopolymers (carbohydrates, lipids, proteins, polysulphates and polyphosphates) produced by microbes and a range of cations in the sludge, alterations in ionic concentrations and ionic composition can therefore change the structure of the sludge floc (Keiding and Nielsen, 1997). Elimination of these cations from the sludge must reduce the bound biopolymer content of the microbes present. There is accelerated solubilisation of the sludge when the interactions stabilising the floc matrix are broken up (defloculation). As the flocs disintegrate, macromolecules that are previously secluded from enzyme attack are released and may be degraded by hydrolytic enzymes (Whiteley *et al*., 2002a). An increase in the sulphide concentration (as sulphate is reduced by the SRB) may lead to a reaction with some of the cations on the surface of the floc leading to neutralisation and subsequent disintegration of the floc. With increased surface area and enzyme release into the medium there will be increased solubilisation of the primary sewage sludge and increased hydrolytic enzyme activities (Pletschke *et al*., 2002).

1.4 SEWAGE SLUDGE; A USEFUL WASTE

Management of both natural resources and energy, as well as a possible recycling of wastes are necessary (Pèlach *et al*., 2003). With a growing global population, the annual production of sewage sludge is rising and this increase is deplorable (Cenni *et al*., 2001). The disposal and storage of large quantities of untreated sludge is of environmental concern requiring an urgent way out. Recently, there have been search for possible ways of utilising or reducing disposal quantities of sewage sludge (Onyeche *et al*., 2002). Since all sludges contain potentially risky contaminants, beneficial uses must be even-handed against tolerable risks for human well-being and environmental impacts (Wong *et al*.,

2001). The extent of risk depends directly on the initial sludge quality and the way the sludges are managed (heat drying, composting or anaerobic digestion) as heat dried pellets of sewage sludge are pathogen-free (Poverly and Siaba, 1996).

Some of the applications of sewage sludge are discussed below:

(1) Fuel: Sewage sludge is a potentially important, practically underutilised energy source (Fermor, 1993). Heat dried sludge has approximately equal heat value as low grade coal and can be burned to produce steam used as a fuel to generate electricity (Poverly and Siaba, 1996). As a reusable waste fuel, as cited by Cenni *et al*. (2001) sewage sludge can be burned with zero CO_2 release, thus reducing greenhouse gases. Research is still continuing on the bioconversion of sludge into other biofuels like methane, acetate, ethanol (Poverly and Siaba, 1996).

(2) Agriculture: In recent times, the utilisation of sewage sludge as an organic fertiliser has become a usual practice (Selivanovskaya *et al*., 2001). Since municipal sludges are derivatives of the foods we eat, they contain essential elements such as nitrogen, phosphorus and potassium (Poverly and Siaba, 1996). Proper land application is a means of reprocessing these nutrients and returning them to the soil (Fjällborg and Dave, 2003). Sewage sludge provides organic carbon which improves soil physical and chemical properties (Wong and Su, 1997). Sewage sludge has also been observed to increase microbial biomass and soil enzyme activities (Banerjee *et al*., 1997). Spreading sewage sludge on ferrallitic soils (acidic soils with $pH \leq 5$), which are poor in organic matter and nutrients, increases their agricultural usability, thus increasing crop production. (Cornu *et al*., 2001). With respect to agricultural use, the aspect of greatest concern is the high concentration of heavy metals such as Cd, Co, Cu, Pb, Hg, Ni and Zn in sludge, as they can be toxic to microbes and plants (Wong *et al*., 2001). However, natural zeolite (clinoptiolite) can be composted with the sewage sludge to reduce this risk, as it acts as a cation exchanger (Zorpas *et al*., 2003)

(3) Bioremediation of contaminated soil and acid mine drainage (AMD): Adding organics including sewage sludge, to contaminated soils can aid degradation of organic contaminants as they can provide the carbon source and a variety of microorganisms required for bioremediation. Additions of sewage sludge and compost have been used as an economical treatment technique in remediating diesel–contaminated (Namkoong *et al*., 2002) and limestone–contaminated soils (Heal and Salt, 1999). AMD is characterised

by high concentrations of sulphate, heavy metals, low pH and suspended solids. Sulphate reduction by the SRB has been identified as a practical process for AMD treatment as the process leads to the precipitation of sulphur and co–precipitation of metals including iron, zinc, lead and cadmium found in AMD (O' Sullivan *et al*., 1999; Boshoff, 1999). Sewage sludge can provide the carbon source for SRB utilisation with respect to this (Pletschke *et al*., 2002).

(4) Construction works: Cement and concrete can be supplemented with fly ash from the incineration mixture of bituminous coal and municipal sewage sludge which is then used in open–air construction works (roads, landscape and landfills) based on the chemical composition and characteristics of the ash extract (Cenni *et al*., 2001).

(5) Enzyme source: Several enzymes have been characterised from sewage sludge under biosulphidogenic conditions. These enzymes (proteases, phosphatases, β–glucosidases, lipases and ATP–sulphurylases) as discussed earlier have been shown to be stimulated by sulphide (Pletschke *et al*., 2002; Whiteley *et al*., 2002a; 2002b; 2003). With respect to this, sewage sludge can provide a cheap and readily available source of enzymes, as use of enzymes is increasing in the bid to use environmentally friendly processes in different industrial activities. Moreover, enzyme activities can be closely controlled, are very specific and can also be maintained under mild conditions. This research focuses on the use of sewage sludge as a source of the enzyme, endoglucanase, as this enzyme has several applications as discussed later in this chapter.

1.5 CELLULOSE

1.5.1 Occurrence In Nature

Cellulose is one of the most abundant biological polymers on earth (Karlsson *et al*., 2002). It has been approximated that 7.2 x 10^{11} tonnes of cellulose is reserved in plants and that the yearly production of cellulose is 4 x 10^{10} tonnes (Coughlan, 1985). It is found in nature almost entirely in plant cell walls maintaining the cell wall integrity, although it is also produced by some animals (e.g. truncates) (Lynd *et al*., 2002) a few bacteria and marine algae (Schwarz, 2001). In rare cases (remarkably cotton bolls), cellulose is present in a nearly pure state (Lynd *et al*., 2002). Generally in nature, the cellulose fibres are tightly surrounded by other polymers such as xylan, other hemicellulose components and lignin in a matrix (Pohlschröder *et al*., 1994). This interaction is a critical structural characteristic preventing the rate and level of utilisation

of intact, untreated biomass materials (Lynd *et al*., 2002). Cellulose of organic origin has a highly recalcitrant crystalline structure (Gan *et al*., 2003) which never occurs as a single chain, rather as a complex of many chains, termed microfibrils. Cellulose is synthesised as single molecules (linear chains of glucosyl residues) which further undergo self-assembly at the site of biosynthesis (Brown *et al*., 2000).

1.5.2 Structure

Cellulose is chemically simple because it contains simple repeating units of glucose, but has a complex structure because of the long chains of glucose subunits joined together by β–1,4–linkages (Figure 1.3) (Lynd *et al*., 2002). Cellulose is stabilised by some interactions, these stabilising factors are weak individually but collectively form strong bonds. The chains are in layers held jointly by van der Waals forces and hydrogen–bonds (intramolecular and intermolecular) (Gan *et al*., 2003). About thirty individual cellulose molecules are arranged into units called protofibrils, which are further arranged into larger units called microfibrils. These in turn assemble into cellulose fibres. As shown in Figure 1.4, chains are arranged in layered sheets (Mosier *et al*., 1999).

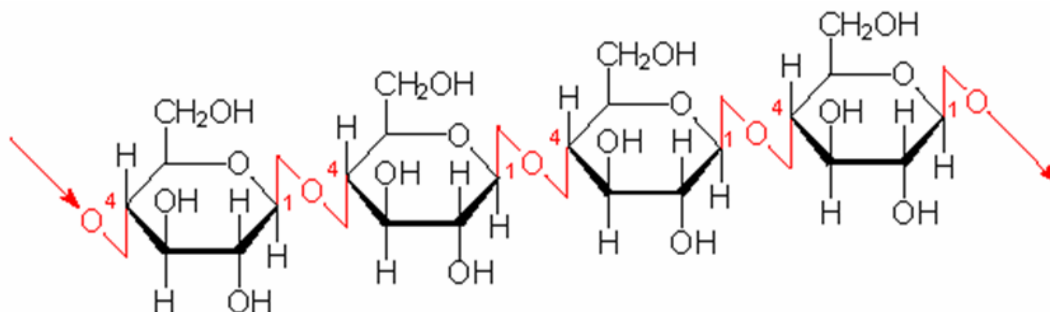


Figure 1.3: Diagram showing the β –1,4–linkages in a cellulose chain. Adapted from Samejima *et al*., 1998.

Although microfibrils are tightly packed to form cellulose crystalline structure, the polymer is not entirely crystalline in nature; the tightly packed and well ordered sites are spaced by loosely arranged ones called amorphous regions (Levy *et al*., 2002) (Figure 1.5). The amorphous region is the easily hydrolysable portion of the cellulose.

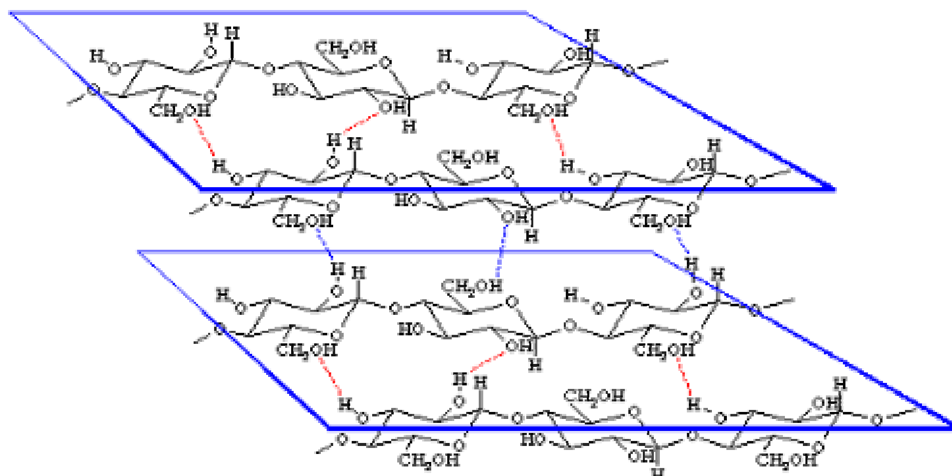


Figure 1.4: Diagram showing the cellulose microfibrils in layers. The red dotted lines are the intermolecular H–bonds while the blue dotted lines represent the intramolecular H– bonds. (©Newton, 2001) (*Appendix 1*).

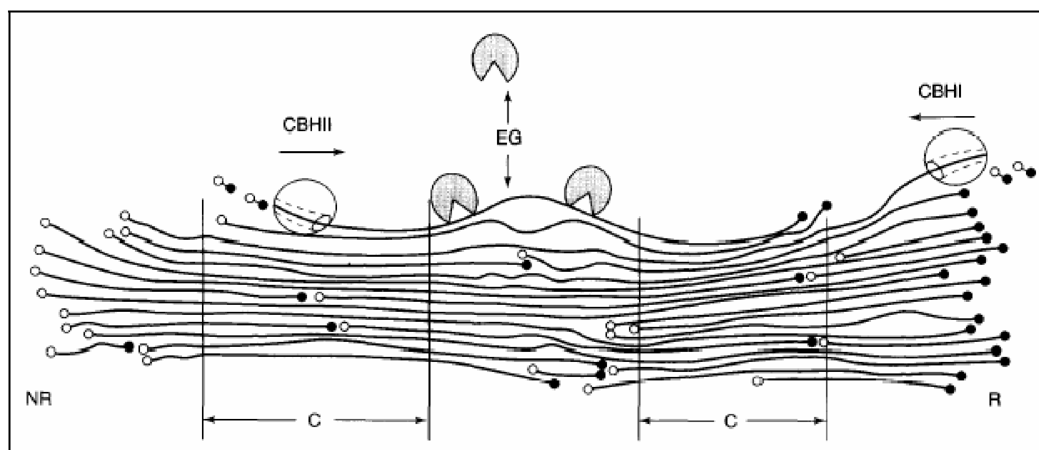


Figure 1.5: Schematic representation of the morphology and organisation of crystalline cellulose. Amorphous regions occur in particular near the crystal surfaces but many also transverse the entire width of the crystalline. Different exoglucanases or cellobiohydrolases (CBH) are currently thought to attack the crystalline areas at the opposite chain ends and the endoglucanases (EG) in the middle of the more disordered regions of cellulose. The filled circles, denoted R, represent the reducing ends and the open circles, denoted NR, represent the nonreducing ends. C defines the highly crystalline regions (Teeri, 1997).

1.5.3 Enzymatic Hydrolysis Of Cellulose

As a result of the insolubility and heterogeneity of native cellulose, it is recalcitrant to enzymatic hydrolysis. The degradation of crystalline cellulose is a complex process requiring the participation of many enzymes (Schwarz, 2001). As cellulose can be

regarded as the most abundant and biologically renewable resource for bioconversion, its exploitation can be maximised on hydrolysis to glucose and other soluble sugars which can be further fermented into ethanol for use as liquid fuel (Eriksson *et al*., 2002).

Cellulases are the enzymes responsible for the cleavage of the β–1,4–glycosidic linkages in cellulose (Figure 1.6). They are members of the glycoside hydrolase families of enzymes that hydrolyse oligosaccharides and / or polysaccharides (Schülein, 2000).

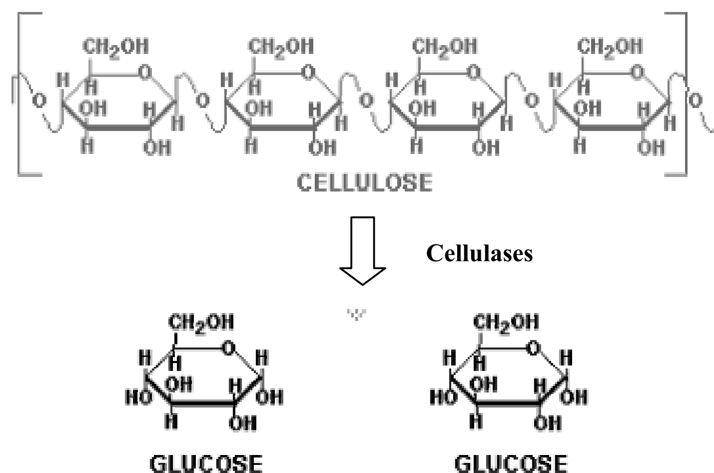


Figure 1.6: Diagram showing enzymatic hydrolysis of cellulose. Adapted from Samejima *et al*., 1998.

1.5.3.1 Cellulases And Their Modes Of Action

Cellulases include three main types of enzymes, endoglucanases (EC 3.2.1.4), cellobiohydrolases or exoglucanases (EC 3.2.1.91) (Figure 1.5) and β-glucosidases (EC 3.2.1.21). These enzymes can either be free (mostly in aerobic microbes) or grouped in a multicomponent enzyme complex (cellulosome) found in anaerobic cellulolytic bacteria (Bayer *et al*., 1998). Cellulases from different sources have also been reported to show similar modes of action (Mosier *et al*., 1999).

The enzymatic hydrolysis of the glycosidic bonds takes place through general acid catalysis involving two carboxylic acids (glutamate or aspartate) (Mosier *et al*., 1999). This hydrolysis occurs through two major pathways which give rise to either retention or inversion of the anomeric configuration of the substrate (Figure 1.7). Inversion is a simple single displacement reaction while the retention mechanism involves two steps.

During inversion mechanism, an acidic amino acid residue donates a proton while another charged acidic amino acid residue opposite the proton donor "activates" a water molecule for subsequent nucleophilic attack, and the proton donor then becomes the charged residue while the charged residue becomes protonated. It is as a result of this alternation of the protonation of the catalytic residues that this mechanism is called inversion. The retention mechanism involves the formation of a covalent glycosyl-enzyme intermediate which is subsequently hydrolysed through oxocarbonium ion-like transition states (Figure 1.7) (Schülein, 2000).

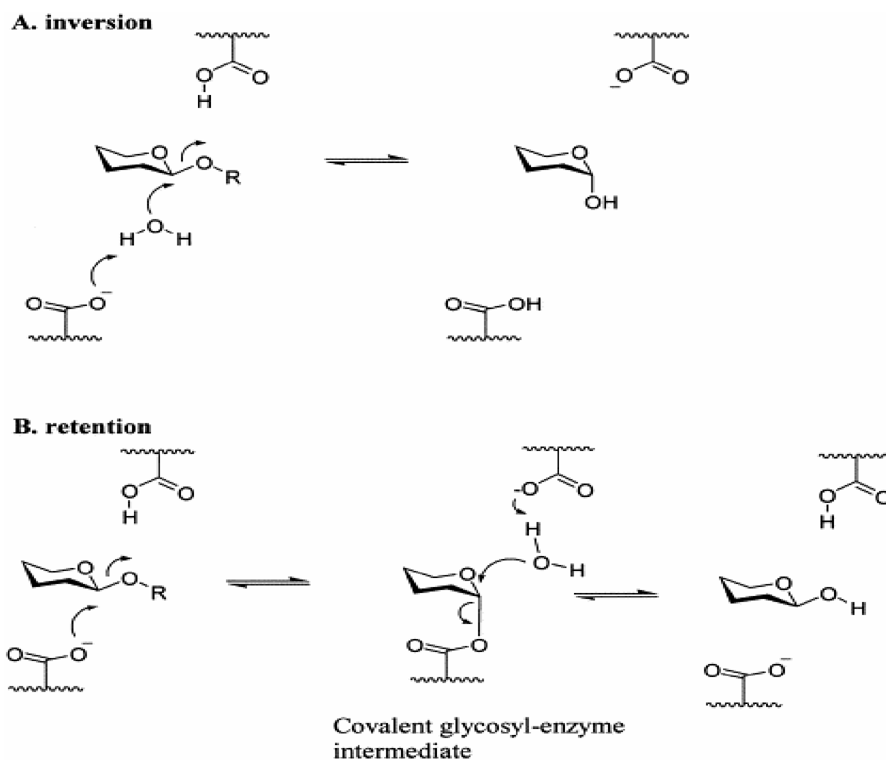


Figure 1.7: The two mechanisms of enzymatic hydrolysis of the glycosidic bonds (Schülein, 2000).

(1) Endoglucanases: These cellulases play an important role in the cellulose hydrolysis by cleaving cellulose chains randomly and thus encouraging strong degradation (Cao and Huimin, 2002). Generally, the indiscriminate action of endoglucanases progressively increases the accessibility of cellulose chain ends, in this manner increasing the specific surface area of the substrate for exocellulase activity (Ramos *et al.*, 1999). Endoglucanase attacks the β-1,4 glycosidic bonds within the amorphous regions of

cellulose chains (Mosier *et al*., 1999). The products of this attack are oligosaccharides of various lengths and subsequently new chain reducing ends (Lynd *et al*., 2002).
(2) Exoglucanases: These degrade crystalline cellulose most efficiently and act in a processive mode on the reducing or non–reducing ends of cellulose polysaccharide chains, releasing either glucose (glucohydrolases) or cellobiose (cellobiohydrolases) as major products (Bhat and Bhat, 1997; Lynd *et al*., 2002).
(3) β-glucosidases: These complete the hydrolysis of cellulose. They hydrolyse cellobiose, a potential inhibitor of cellobiohydrolases (Lemos *et al*., 2003). The catalytic activity of β-glucosidase is inversely proportional to the degree of substrate polymerisation. Though a cellulase, it does not attack cellulose. These enzymes can be grouped as aryl β-D-glucosidases (hydrolysing exclusively aryl-β-glycosides), cellobiases (hydrolysing diglycosides and cellooligosaccharides) or β-glucosidases with wide range of substrate specificities (Bhat and Hazlewood, 2001).

1.5.3.2 Synergism

Hydrolysis of cellulose requires interaction of numerous cellulase constituents in a mixed reaction system (Gan *et al*., 2003). Cellulase systems consisting of the three cellulase groups (endoglucanases, exoglucanases and β-glucosidases) act in concert to efficiently degrade cellulose. Cellulase systems demonstrate better joint activity than the addition of the individuals' activities, a phenomenon known as *synergism* (Lynd *et al*., 2002). Four forms of synergism have been reported, and these are highlighted below:
(1) Endo–exo synergism between exoglucanases and endoglucanases, and this is as a result of the generation of more chain ends on the cellulose surface by endoglucanases for cellobiohydrolases to attack (Lemos *et al*., 2003).
(2)Exo-exo synergism between exoglucanases processing from the reducing ends and the ones processing from the non–reducing ends of cellulose chains.
(3) Synergism between exoglucanases and β–glucosidases that gets rid of cellobiose (and cellodextrins) as end products of the first two enzymes (Lynd *et al*., 2002).
(4) Intramolecular synergism between catalytic domains and cellulose binding domains (which effects binding of the enzymes to cellulose surface). This synergism is dependent on enzyme structure (Mosier *et al*., 1999).

1.5.3.3 Processes Preceding Enzymatic Hydrolysis Of Cellulose

It is known that there are three processes that occur before the inception of the enzymatic hydrolysis of cellulose, these are: (a) diffusion of cellulase in the aqueous medium, (b) the movement of cellulase from the liquid to the surface of the substrate and (c) the surface assimilation of cellulose to the enzyme resulting in an enzyme–substrate complex. When the substrate concentration is high, the transfer of cellulase to the substrate surface is impeded (Cao and Huimin, 2002).

1.5.3.4 Rate Limiting Factors In Enzymatic Hydrolysis

The rate of conversion of cellulose fibres to individual, easily hydrolysable shorter chains is dependent on some factors. For enzymatic hydrolysis of natural cellulose, as cited by Lynd *et al.*, (2002), a number of determining factors of hydrolysis rate have been postulated, including:

(1) Crystallinity: This is generally regarded as a key factor influencing cellulose hydrolysis at both enzymatic and microbial levels. The highly crystalline regions of cellulose chains are recalcitrant to hydrolysis, as a result of their tightly packed nature which prevents accessibility of the enzymes. The more of these regions present the slower the rate of hydrolysis.

(2) Degree of polymerisation: The longer the cellulose chain, the lower the rate of hydrolysis (Walker *et al.*, 1990).

(3) Particle size: Within any given cellulose sample, there is a great measure of unevenness of the size and shape of individual particles, which thus affects the rate of hydrolysis.

(4) Pore volume: The pore structure of cellulosic materials must be able to accommodate particles of the size of a cellulolytic enzyme. The greater the availability, the more the enzymes that are adsorbed (Mosier *et al*., 1999).

(5) Accessible surface area: Most cellulose chains are hidden within the microfibrils, which prevents exposure to enzymes and thus limiting the rate of hydrolysis.

1.5.3.5 Cellulosome

This is an extracellular, multicomponent enzyme complex of high molecular weight that is attached to the cellulolytic microbe's cell surface and encourages both cellular attachment to cellulose and effective hydrolysis of crystalline cellulose. (Forsberg *et al.*,

2000). These structures are found mainly in anaerobic systems where stringent control of metabolism and metabolic products is vital. In contrast, aerobic bacteria produce numerous free, extracellular enzymes with binding modules for different cellulose configurations (Shoham *et al.*, 1999; Schwarz, 2001). Cellulosomes must have evolved as a result of the inability of anaerobic bacteria to gain access to cellulosic materials and perhaps had to find another mechanism for degrading cellulose and gaining access to the products of cellulose hydrolysis in the presence of competition from other microbes for limited nutrients for their growth and maintenance (Lynd *et al.*, 2002). The cellulosome of *Clostridium thermocellum*, a moderately thermophilic bacterium is the most studied so far 55-65 °C) (Schwarz, 2001).

The specific activity of cellulosome for cellulosic substrates is greater than that of the free enzyme systems because it encourages synergism. The assembly of enzymes into cellulosomes increases their catalytic activities and arranges them in a proper position both in relation to each other and the cellulosic substrate (Figure 1.8) (Shoham *et al.*, 1999). The cellulosome also reduces the distance over which products of cellulose hydrolysis must pass through thus facilitating assimilation of these sugars by the bacterial cell (Schwarz, 2001). The other advantages conferred by the cellulosome include: (a) prevention of stoppage of hydrolysis on reduction of one structural type of cellulase at the site of adsorption by the presence of other enzymes with different specificity; (b) in the presence of an inadequate number of binding sites the whole complex is bound to a single site through a strong binding domain with low specificity (Schwarz, 2001).

1.5.3.6 Cellulases In Sewage Sludge

Sewage sludge has been identified as one of the anaerobic environments in which microbial cellulose degradation takes place (Khan and Patel, 1991). The microbes involved in sewage sludge degradation produce cellulolytic enzymes to degrade cellulose (which is one of the polymers present in sewage sludge) into glucose as energy source for their metabolism. However, it has been reported that extremely low activities of cellulases were found in anaerobic digesters (Adney *et al.*, 1991). Some of the cellulolytic bacteria identified and isolated from sewage sludge and anaerobic digesters and their properties are listed below (Table 1.1).

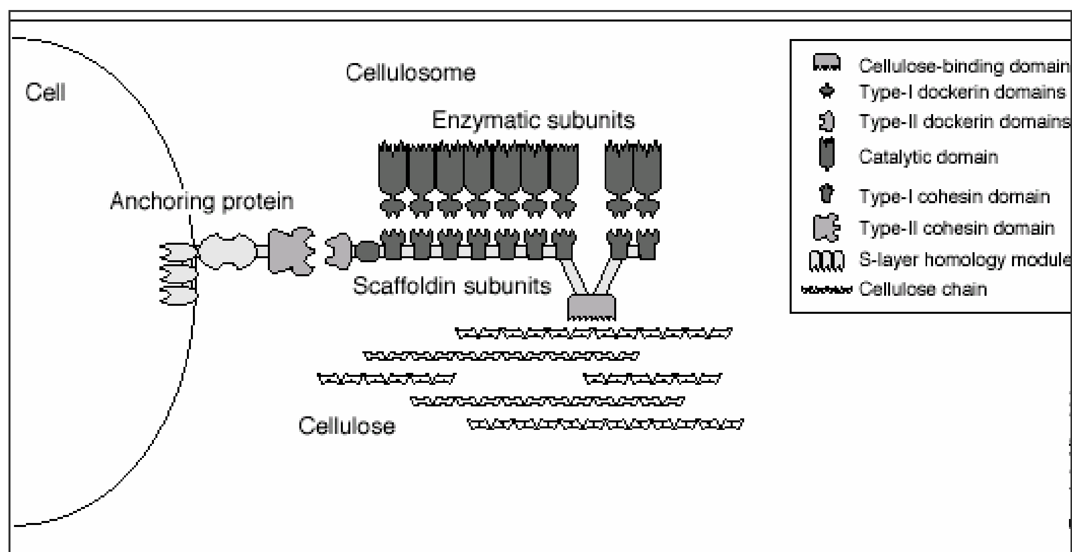


Figure 1.8: Simplified schematic view of the interaction between the *Clostridium thermocellum* cellulosome and its substrate, and its connection to the cell surface via an associated anchoring protein (Shoham *et al*., 1999).

Table 1.1: Cellulolytic bacteria isolated from sewage sludge and anaerobic digesters. Adapted from Leschine, 1995.

Source	Organism	Growth substrates	Fermentation products
Sewage sludge	*Acetivibrio cellulolyticus*	Cellulose, cellobiose, salicin	Acetate and trace amount of ethanol
	Bacterioides cellulosolvens	Cellulose and cellobiose	Acetate, ethanol and lactate
Anaerobic digester	*Clostridium aldrichii*	Cellulose, cellobiose and xylan	Acetate, isobutyrate, butyrate, isovalerate, lactate and succinate
	Clostridium celerecrescens	Broad	Acetate, ethanol, formate, butyrate, caproate etc.
	Clostridium cellulovorans	Broad	Acetate, butyrate, lactate and formate

1.5.3.7 Uses Of Cellulases

Owing to its biodegradability and physical strength, cellulose has been used as starting materials in many industries (Teeri, 1997). The usage of cellulose actually depends on the products of its degradation and this is done under mild, easily controlled and specific conditions by the cellulases. Recently, cellulases are used in many industrial applications and the demand for more thermostable, highly active and specific enzymes is on the increase (Bhat, 2000). Some of the uses of cellulases are discussed below:

<u>Pulp and Paper Industry</u>: Cellulases have several uses in this industry as the raw materials used are lignocellulosics (Tengerdy and Szakacs, 2003) in which cellulose is sequestered within a matrix of other components. Cellulases therefore are used at both the pre–production and post-production stages in pulp and paper making, and these include:

(1) Treatment of waste paper: Detachment of ink (bio–deinking) is improved by using cellulases in waste paper treatment and this waste paper can be subsequently biotransformed to fermentable sugars (van Wyk and Mohulatsi, 2003). Glucose released from cellulose is fermented into ethanol, which shows a potential alternative for fossil fuels due to its low net emission of CO_2 upon combustion (Levy *et al*., 2002). The main advantage of enzymatic deinking is the prevention of the use of an alkali, it is therefore environmentally friendly (Bhat, 2000).

(2) Treatment of recycled pulps: Endoglucanase facilitates pulp drainage by removing amorphous cellulosic materials such as fines and surface elements (peeling off of individual fibrils) (Ramos *et al*., 1999).

<u>Feed and Food Industry</u>: Cellulases hydrolyse plant cell wall, thus finding usage in animal feeds and the food industry. Applications of cellulases in this area are:

(1) Increase in juice production by decreasing the viscosity of the raw juice slurry from fruits during the production of fruit drinks (Levy *et al*., 2002).

(2) Cellulases in combination with hemicellulases are added to animal feeds to supplement the animal's own digestive enzymes, thus increasing the feed digestibility and vitamin assimilation in the animal's gut as a result of the partial hydrolysis of the lignocellulosic materials (Gilbert and Hazlewood, 1993).

<u>Textile and Laundry Industry</u>: Cellulases have been widely used in textile and laundry due to their ability to alter cellulosic fibres in a controlled and mild manner, thus increasing the quality of the fabrics (Bhat, 2000). The textile industry uses cellulases for finishing textiles during manufacture and washing (Teeri, 1997). Of recent, the application of these enzymes in the textile industry represents the major market for cellulase enzymes (Cavaco-Paulo, 1998).

(1) Cellulases are used for stone washing (by removing excessive dye without damaging the fibre) and defuzzing (depiling) fabrics (Levy *et al*., 2002). The softness caused by

cellulase treatment also reduces the need for cationic fabric softeners (Galante *et al*., 1998).

(2) Cellulase preparations are used as additives to laundry detergents to improve the colour brightness, texture and dirt removal from cotton and cotton garments (Bhat, 2000).

Research, Development and Agriculture: Applications of cellulases in these aspects include:

(1) Cellulases and related enzymes (hemicellulases and pectinases) are used as biological control of plant pathogens and infections as a result of their ability to degrade the cell wall of plant pathogens, inhibit spore germination, germ tube elongation, and fungal growth (Bhat, 2000).

(2) Cellulases and related enzymes can be used in biotechnology for developing new breeds of plant or fungal protoplasts with desired characteristics by solubilising the plant or fungal cell walls which can be merged to form hybrids (Bhat, 2000).

1.6 RESEARCH HYPOTHESIS

Anaerobic digested biosulphidogenic sludge is a potential, attractive, cheap and readily available source of endoglucanases.

Enzymatic hydrolysis of cellulosic materials to produce reducing sugars has long been investigated for its possibility of providing abundant food and energy resources. Commercial application of enzyme cellulose hydrolysis however has been deterred by the high cost of enzymes (Gan *et al*., 2003). With this limitation, it is therefore of great necessity to find a cheap and readily available source of cellulases. Some cellulolytic bacteria have been isolated and characterised from sewage sludge and there have been several reports of the stimulation of enzyme activities by sulphide during the solubilisation of sewage sludge in a biosulphidogenic anaerobic environment (Pletschke *et al*., 2002; Whiteley *et al*., 2002a; 2002b; 2003). One of the enzymes that form part of the cellulolytic system, β-glucosidases have actually been previously studied in our research group and found to be stimulated by sulphur metabolites (Whiteley *et al*., 2003). This research focuses on another cellulase, endoglucanase, which plays a key role in cellulose degradation and has a wide range of industrial applications.

1.7 RESEARCH OBJECTIVES

1. To identify the presence and the distribution of endoglucanases in sewage sludge under biosulphidogenic conditions of hydrolysis.
2. To induce the production of this enzyme.
3. To locate, isolate and purify the enzyme.
4. To characterise the enzyme with respect to pH, temperature and thermal stability.
5. To investigate some kinetic parameters; $K_{m,app}$ *and* $V_{max,app}$.
6. To study the effects of sulphur-containing compounds (sulphate, sulphide and sulphite); volatile fatty acids (acetic, butyric, propionic and valeric acids) and metals (Ca, Cu, Fe, Mg, Ni and Zn) on the enzyme.

CHAPTER 2

PRODUCTION AND INDUCTION OF ENDOGLUCANASES UNDER BIOSULPHIDOGENIC CONDITIONS

2.1 INTRODUCTION

Production and use of enzymes are crucial in the bioprocessing industry (Vlaev *et al*., 1997). Enzyme costs can be reduced by finding optimum conditions for their production (Ponce-Noyola and de la Torre, 2001). If endoglucanases can be obtained cheaply, readily and easily it will be of a great advantage to the bioprocessing industry. Due to the fact that enzymes present in anaerobic degradation of PSS have been reported to be stimulated under sulphate-reducing conditions (Whittington-Jones, 2000; Whiteley *et al*., 2002a; 2002b; 2003), biosulphidogenic digestion might provide a solution to the problem faced in enzymatic cellulose degradation.

The production of hydrolytic enzymes is directly related to the available substrates (Nybroe *et al*., 1992), therefore an increase in the concentration of a particular substrate should stimulate the microorganisms to produce enzymes specific for the utilisation of that substrate. As discussed in Chapter 1 (section 1.1.2), organic matter carried by urban wastewater is a complex mixture of polymeric compounds and these compounds are hydrolysed by extracellular enzymes (Cadoret *et al*., 2002). Microbes produce enzymes such as endoglucanases in response to their carbon and energy needs as the soluble hydrolysis products are needed for further metabolic activities (Sutherland, 1999). Sewage sludge contains a considerable amount of cellulose (Fermor, 1993) and cellulolytic microbes in this sewage sludge therefore produce cellulases to break down the cellulose which cannot transverse the cell membrane (Dror *et al*., 2003). Enzyme activities therefore reflect microbial activities in wastewater (Nybroe *et al*., 1992). In light of the previous observations that PSS hydrolysis is accelerated under biosulphidogenic conditions (consequence of enzyme activation), cellulases being one of the major groups of enzymes required for hydrolysis, would be expected to be activated or over produced also. Optimal conditions of pH, temperature etc. are required for enzyme production. Thus, these factors have to be monitored during enzyme production.

It is required to investigate how the production of endoglucanases is related to the liberation of sulphide and how its synthesis is regulated in the presence of metabolites (such as glucose produced from cellulose hydrolysis) under biosulphidogenic conditions. Further induction of endoglucanase by supplementing the inherent cellulose in the PSS with commercial cellulose will be examined.

Organisms control the stimulation and repression of groups of genes by intricate combinations of restrictive signals. Catabolite control has been shown to be the general regulatory system in many organisms. It has also been established that the synthesis of cellulases is regulated by this mechanism (Fernández-Abalos *et al*., 1997). Carbon catabolite repression (CCR) can be defined as the regulatory system by which the expression of genes necessary for the use of secondary carbon sources is prohibited in the presence of a favoured or easily metabolisable substrate. This mechanism is used by bacteria in their natural environments to increase their continued existence by optimising growth rates when complex mixtures of nutrients are available (Stülke and Hillen, 1999). These microbes accomplish this by synthesising only enzymes necessary for the preferred nutrients. Carbon catabolite repression is also used by the bacteria to control carbon assimilation and utilisation (Brückner and Titgemeyer, 2002).

Catabolite repression will prevent the microbe from producing a surplus quantity of cellulase under conditions where there is a sufficient quantity of easily metabolisable carbon sources (Suto and Tomita, 2001). It has been established that cellulolytic enzymes are inhibited by cellobiose, glucose, or both products (Gan *et al*., 2003), while the synthesis is induced in the presence of cellulose (Béguin and Aubert, 1994). The end product inhibition and repression do not only prevent enzyme production but also inhibit the activity (Zaldívar *et al*., 2001).

2.2 MATERIALS AND METHODS

2.2.1 Materials

Alpha (α)-cellulose was purchased from Sigma (South Africa, (Pty) Ltd), while Solutions A (containing ferrous ammonium sulphate) and B (containing potassium dichromate and sulphuric acid), carboxymethylcellulose (CMC) (sodium salt, medium viscosity), sulphate and hydrogen sulphide test kits, were obtained from Merck (South Africa, (Pty) Ltd). Dinitrosalicylic acid, n-Butanol, Bradford reagent and other analytical-grade reagents were purchased from Sigma (South Africa, (Pty) Ltd), or Merck (South Africa, (Pty) Ltd). Absorbances were measured using Power Wave$_X$ (Bio-Tek Instrument Inc, South Africa). Centrifugation was performed with Beckman J2-21 centrifuge with a JA-14 rotor and sonication with a Virsonic-100 sonicator (VirTis, Co., Inc, USA).

2.2.2 Methods

2.2.2.1 Reactor set-up and Sludge source: Primary sludge, SRB and methanogens were collected from the Grahamstown Municipal Sewage works, Grahamstown, South Africa. Reactors were set up in 10 l glass tanks (Figure 2.1) at room temperature. The methanogenic reactor (MR) (where methane is produced as one of the final products) was seeded with a 10 % (v/v) (1000 ml) mixed culture of methanogenic bacteria. This served as a control reactor and was used to monitor the conventional hydrolysis of polymeric compounds and endoglucanase production in anaerobic sludge in the absence of SRB and sulphate. The 10 l biosulphidogenic (experimental) bioreactor (SR) was used to monitor the production of endoglucanases under biosulphidogenic conditions. This was seeded with 10 % (v/v) (1000 ml) inoculum of SRB. Both SR and MR were fed with the primary sludge (sieved through 2 mm mesh sieve and diluted to a COD of 2000 mg/l). In addition, to the SR was added sulphate to a final concentration of 2000 mg/l (to obtain a COD:SO_4^{2-} ratio of 1:1). Both bioreactors were sealed with rubber stoppers and covered with aluminium foil to maintain strict anaerobic conditions and to prevent any photosynthesis respectively. The contents were continuously agitated gently to maintain homogeneity. To further maintain strict anaerobic conditions during sampling, high rate

N_2 gas was spurged through oxygen impermeable Tygon® tubing (H) (Swiss Lab, South Africa), connected to glass tubing into the reactor. The mixture of N_2 and H_2S flows out of the reactor through port H_1. The reactors were maintained at room temperature.

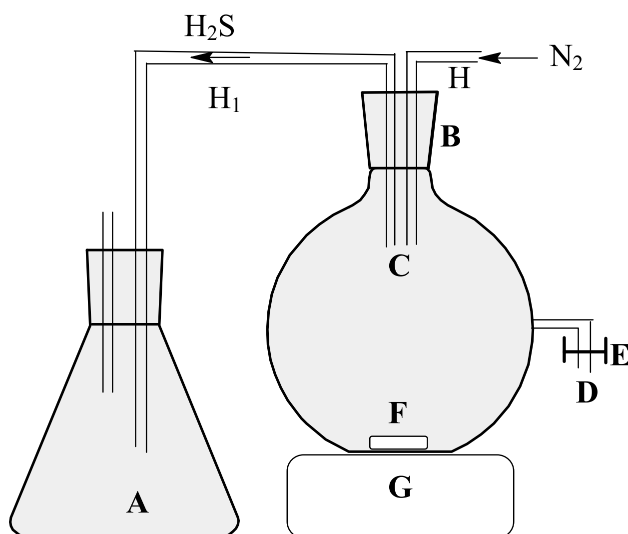


Figure 2.1: Schematic diagram of the laboratory-scale (closed/single stage) stirred tank reactor, used for monitoring the production of endoglucanases during anaerobic degradation: (A) 10 % (w/v) zinc acetate solution as hydrogen sulphide trap; (B) rubber stopper; (C) 10 l glass tank; (D) sampling port; (E) metal clamp; (F) magnetic stirrer bar; (G) magnetic stirrer; (H and H_1) Oxygen impermeable Tygon® tubing.

2.2.2.2 Enzyme Preparation : Crude samples obtained from the bioreactor were centrifuged (10 000 ×g, 30 min, 4 °C) and separated into pellet and crude supernatant, the pellet was washed three times with distilled water (1:5 w/v), followed by resuspension in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v). The resuspended pellet was subjected to sonication (30 s intervals, 10 W, 4 min) on ice followed by centrifugation (10 000 ×g, 10 min, 4 °C). The supernatant obtained was used to assay for both the enzyme activity and the immobilised proteins (protein that would normally be adsorbed to the floc matrix but released via cell disruption).

2.2.2.3 Time course study : A time course study was employed to monitor the levels of endoglucanase enzyme, sulphate, sulphide, reducing sugars (glucose), protein and pH in a closed methanogenic and biosulphidogenic bioreactor systems over a period of 35 days;

under the same conditions as other researchers from this research group (Ngesi, 2001 and Tshivunge, 2001). Moreover, according to Sterritt and Lester (1988) mesophilic digesters typically run at mean sludge retention times in the range of 25-35 days. Samples for analysis were collected on alternate days from these reactors using disposable syringes.

2.2.2.3.1 Endo-β-1, 4-D-glucanase activity assay: Endoglucanase activity was determined spectrophotometrically as adapted from the recommendations of the Commission of Biotechnology (Wood and Bhat, 1988). An enzyme sample (1 ml) was added to a 50 ml vial and warmed in a water bath (50 °C, 5 min), 1 ml substrate (CMC) solution (1 % (w/v)) was added, and the solution mixed well and maintained at 50 °C, 30 min. Dinitrosalicylic acid (DNS) reagent (3 ml) was added and the solution was boiled for 5 min, followed by the addition of distilled water (20 ml) and the solution mixed completely. The absorbance was then read at 540 nm and after subtraction of enzyme blank (boiled extract) and control (DNS added to the reaction mixture before incubation), translated using the glucose standard curve, into micrograms of glucose produced during the enzyme reaction. DNS reagent was prepared as described in *Appendix 2*. One unit of enzyme activity is defined as the amount of enzyme producing 1 μmol of glucose per minute. A glucose standard curve was constructed as shown in *Appendix 3*.

2.2.2.3.2 Protein determination: Both soluble (present in the crude supernatant) and the immobilised proteins were estimated using the Bradford method (1976), with bovine serum albumin (BSA) as a standard. The reaction was carried out by placing Bradford reagent (250 μl) with 5 μl sample into a microtiter plate. The absorbance was then read at 595 nm. Complete reaction mixture containing ddH_2O instead of the sample served as blank. The standard curve is shown in *Appendix 4*.

2.2.2.3.3 Reducing sugar estimation: Free reducing sugars present in the bioreactors were measured as glucose in the crude supernatant obtained after centrifuging the sludge samples (10 000 ×g, 30 min, 4 °C) using the DNS method (Miller, 1959). To 1 ml sample was added DNS reagent (3 ml). This was then boiled (5 min), followed by the addition of 20 ml distilled water. Mixture containing ddH_2O instead of the sample served as blank

and a control (sample was acidified to remove sulphide) was as well run to check for the interference of sulphide on this assay. The absorbance was read (540nm) and translated as described above for endoglucanase activity estimation.

2.2.2.3.4 Analytical procedures

Sulphide and sulphate estimation: To monitor the growth of SRB in the bioreactors dissolved sulphide and sulphate were quantified using the Merck® spectroquant test kits. The sludge samples from the reactors was centrifuged (10 000 ×g, 30 min, 4 °C) and the crude supernatant obtained was further filtered through cellulose acetate membrane filters with pore size 0.45 µm (Osmonics, Inc, South Africa) so as to remove all suspensions which might interfere with the assays. The sulphate and sulphide concentrations in the filtrate were then read with the Spectroquant® (NOVA 60, Merck).

pH analysis: 2 ml samples for pH analysis were placed in the test tube and pH readings were taken using a inoLab pH Level 1 pH meter (Merck, (South Africa, (Pty) Ltd).

COD determination: To 0.3 ml of solution A and 2.3 ml of solution B were added 3 ml sludge sample, mixed and incubated in a thermoreactor cell (TR-420, Spectroquant®, Merck) (148°C, 2 h). The samples were then cooled to room temperature for 30 min and the COD measured using the Spectroquant® (NOVA 60, Merck). For the blank, 3 ml of water was added instead of the sludge sample.

2.2.2.4 Induction studies

Effects of various inducers: The two substrates that were employed were α-cellulose (insoluble cellulose form) and CMC (soluble cellulose substituent). Single doses of these inducers were added to two different SR's to a final concentration of 0.1 % (w/v) on day one. No cellulose was added to the control.

Optimisation of endoglucanase induction at varying substrate concentration: For a maximal yield of endoglucanases, based on the previous results obtained from above, endoglucanases production at final concentrations of 0.1, 0.2 and 0.3 % (w/v) of α-cellulose were studied. Single dose of substrates were added on the 1st day.

2.3 RESULTS AND DISCUSSION

2.3.1 Endoglucanase production under methanogenic and biosulphidogenic conditions: In the SR there was a slow production of endoglucanases during the first five days which reached its maximum (0.323 ± 0.016 μmol/min/ml) by day 7, after which there was a marked decline that was maintained until day 35 (Figure 2.2). In a similar investigation β-glucosidases were found to reach maximum activity from day 13 to17 under biosulphidogenic conditions (Ngesi, 2001). This indicates the action of endoglucanases in cellulose hydrolysis prior to that from β-glucosidases. On the other hand, no enzyme activity was detected in the MR from day one till day 17, though from day 19 to 29 there was an increase in enzyme activity after which it steadily decreased (Figure 2.2). Since enzymes are directly involved in the hydrolytic process, this result implies both an accelerated rate of PSS hydrolysis and enzyme activation under biosulphidogenic conditions as reflected by the earlier and greater enzyme production in the SR compared to the MR. These findings further confirm previous reports that hydrolysis of cellulosic substrates in the sulphidogenic environment is more rapid, with percentage and rate of paper solubilisation approximately twice of that occurring under methanogenic conditions (Pareek *et al*., 1998) as well as activated enzyme activity during sulphidogenic hydrolysis of PSS (Whittington-Jones, 2000).

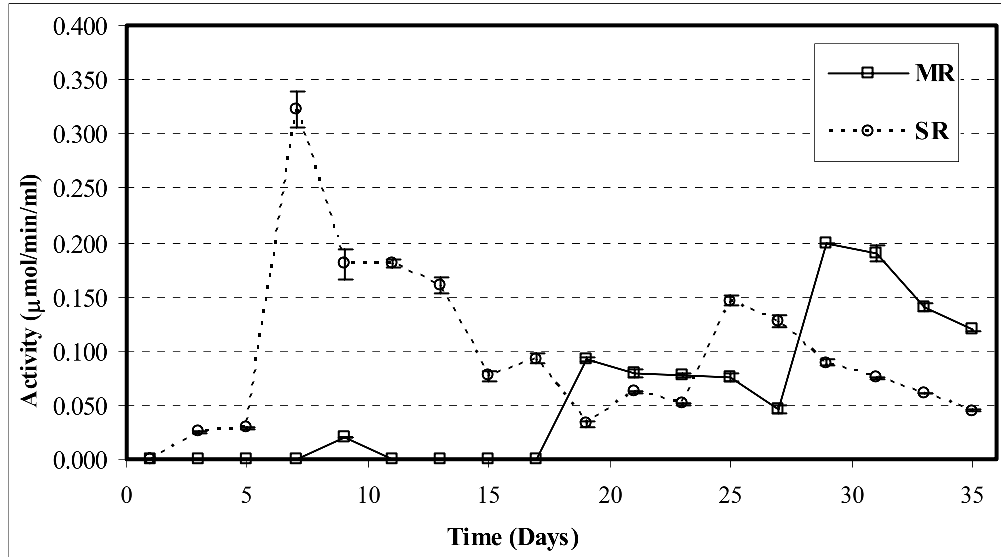


Figure 2.2: Production of endoglucanases under biosulphidogenic and methanogenic conditions.

2.3.2 Sulphate removal and sulphide production: In all of the biosulphidogenic bioreactors studied (with or without substrate supplementation) in this section, no significant difference was observed in the sulphate reduction and sulphide production rates. Increase in cellulose concentration did not cause increase in sulphate reduction, this is as a result of the inability of the SRB to utilise polymers as carbon sources (Knobel and Lewis, 2002). In the biosulphidogenic bioreactors, sulphate concentration steadily declined from ~2400 mg/l on day one to ~345 mg/l on day 33 (Figure 2.3). There was a consequent sulphide production with concentration increasing from ~0.09 mg/l on day one to 426.7 ± 27.3 mg/l on day 33. On the other hand in the MR, the sulphate detected (400 mg/l) was constant throughout the time-course; as a result of the absence of SRB. In addition, minimal amount of sulphide (~0.14-5.62 ± 0.05 mg/l) was detected which could have been generated from the degradation of sulphur-containing amino-acid residues of proteins (cysteine and methionine). The sulphate reduction and resultant sulphide generation is an indication of SRB growth or metabolism with the sewage sludge acting as the carbon source.

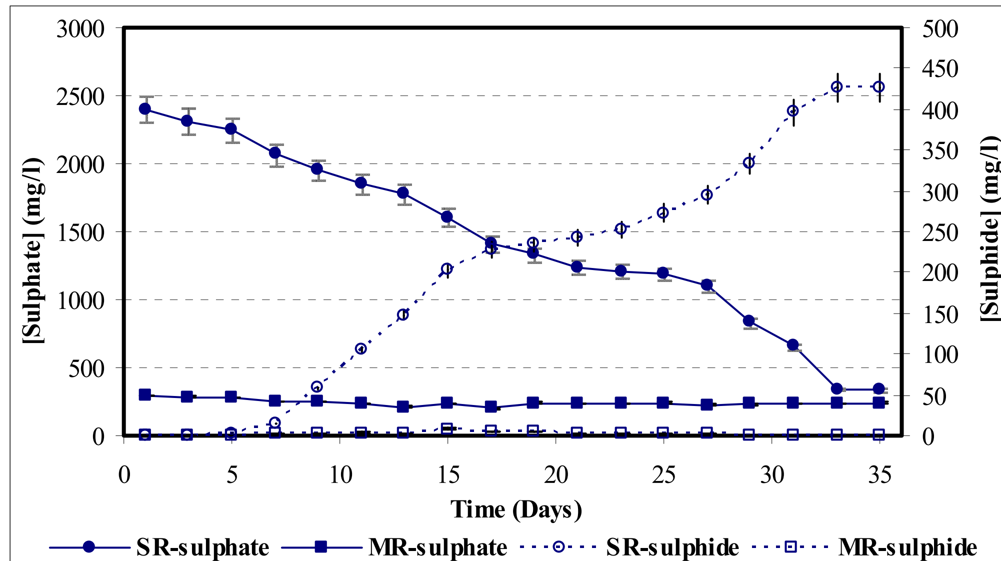


Figure 2.3: Sulphate reduction and concurrent sulphide production in the bioreactors.

2.3.3 Protein distribution: Immobilised proteins in the MR steadily increased from day one throughout the time-course while there was a fluctuation in its emergence in the SR (Figure 2.4). It is interesting to note that trends exhibited by the concentration of the immobilised (insoluble) protein in both the SR and MR corresponded to that of endoglucanase production in these bioreactors. One more point worthy of note is that, despite the lower enzyme activities there was greater insoluble protein concentration present in the MR as compared to the SR. This further supports the observation of stimulated enzyme activities in the SR. There were corresponding peaks of protein production within the same periods that there were maximal enzyme production, days 27-31 in MR and days 7-13 in SR. The implication of this relationship is that the enzyme is produced in the particulate matter in which the cellulolytic bacteria are probably embedded. There was a marked increase in soluble protein detected in the raw supernatant (surrounding aqueous medium) of the SR from day 19 to day 31. This increase could be as a consequence of deflocculation in the SR which is linked to sulphide production (Whittington-Jones, 2000) as the soluble protein was detected during the same period of maximum sulphide production (19th day to the 31st day) (Figure 2.3). Proteins are one of the major constituents of EPS which interact to form flocs in sludge (Mahmoud *et al*., 2003). No enzyme endoglucanase activity was detected in this soluble

protein, pointing to the possibility of inactivation of the enzyme when released into the aqueous medium. The increase in soluble protein could also be due to cell-lysis. Conversely, in the control reactor (MR), no soluble protein was detected during the full time-course.

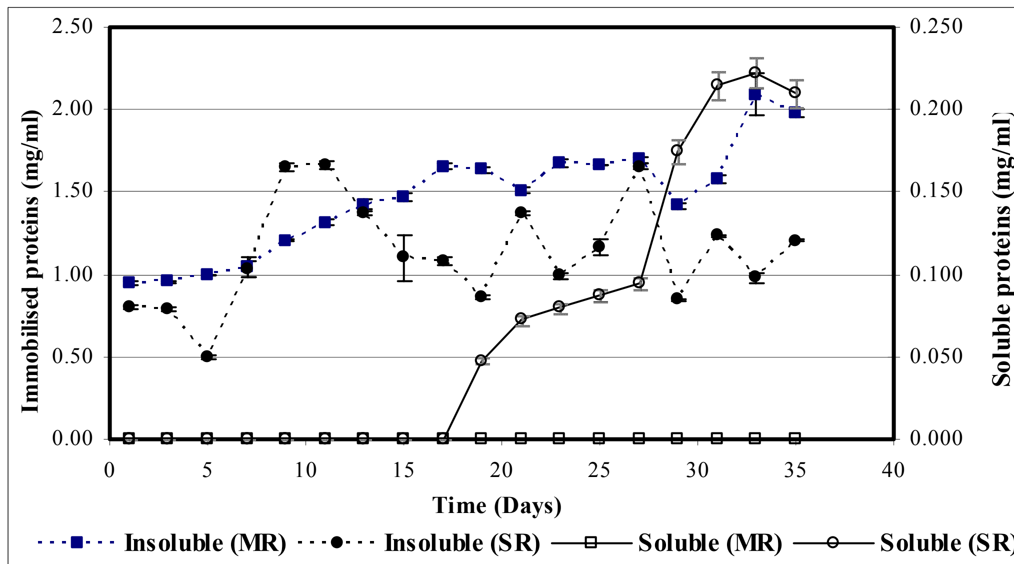


Figure 2.4: Distribution of proteins in the biosulphidogenic and methanogenic bioreactors over time

<u>2.3.4 Production of reducing sugars</u>: In the SR from day one to 21 when little or no glucose was detected (Figure 2.5); the soluble sugars released (which are the actual substrates for cell growth) may have been taken up immediately by the microbial cells (Desvaux *et al*., 2001a). It was actually within this same period that high endoglucanases' activities were measured. On the other hand, in the MR, even when no glucose was detected there was still no enzyme production during this period and the reason for this is not well understood. The production of these enzymes is an indication of cell growth (Spiridonov and Wilson, 1998). In the SR there was a marked increase in glucose concentration from day 21 to day 35 (~0.071-4.43 ± 0.09 mg/ml), whereas minute amounts (~0.047-0.182 ± 0.005 mg/ml) were detected in the MR within the same period. The accumulation of the reducing sugars could have resulted in catabolite repression as well as enzyme inhibition leading to the marked decline in endoglucanases activities (Gan *et al*., 2003) (Figure 2.2). A previous report showed that there was inhibition of cell

growth when reducing sugar accumulated in the growth media of *Clostridium thermocellum* (Desvaux *et al*., 2000).

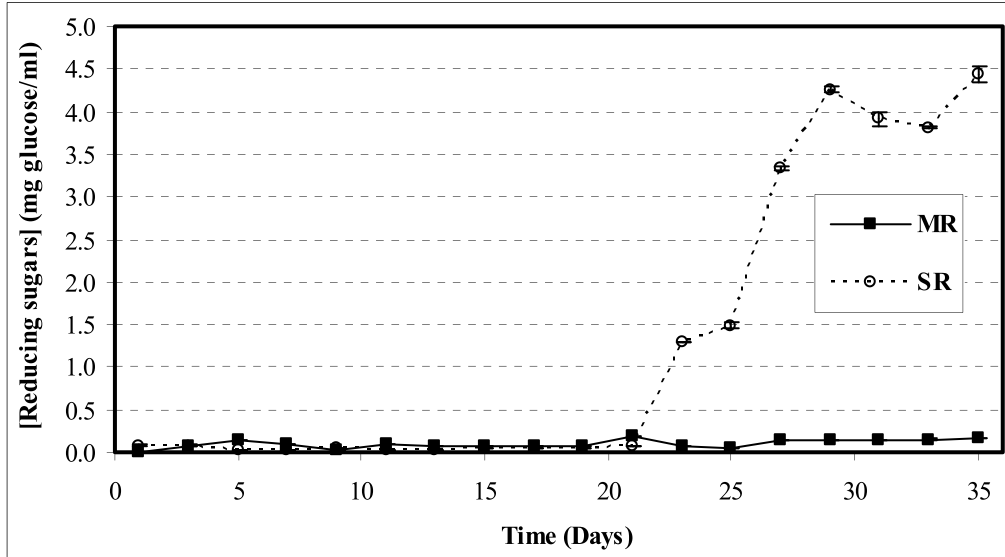
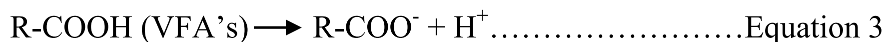


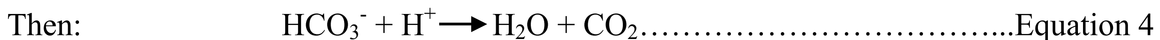
Figure 2.5: Production of reducing sugar in the biosulphidogenic and methanogenic bioreactors.

2.3.5 pH and Temperature fluctuations over time

pH: The somewhat stable pH observed (7.76-8.67) in the SR (Figure 2.6) is within the pH range that SRB require for growth (6-9) (Widdel, 1988). This pH stability could be accounted for by the buffering mechanisms taking place in the biosulphidogenic system. During sulphate reduction pH of the system becomes more alkaline as a result of the increase in concentrations of HCO_3^-, OH^- and HS^- ions (Whiteley *et al*., 2003). This alkalinity could probably neutralise acidity conferred by VFA's (formed during acidogenic stage of anaerobic digestion) resulting in the formation of water and carbon dioxide (Whiteley *et al*., 2002a).

$3\ SO_4^{2-} + \text{Carbon-source} \rightarrow 6\ HCO_3^- + 3\ H_2S$ ....Equation 2


$\text{R-COOH (VFA's)} \rightarrow \text{R-COO}^- + H^+$ ....Equation 3


Then: $HCO_3^- + H^+ \rightarrow H_2O + CO_2$ ....Equation 4

The growth of *Clostridium cellulolyticum* and consequent enzyme (cellulases) production was reported to increase in a pH-controlled fermentation (Desvaux *et al*., 2001b) indicating the need for a constant pH for cellulase production. Many studies have shown that anaerobic cellulolytic microbes do not survive when the pH drops below 6.0 which imply their development in environments where acid tolerance was not a requirement for existense (Desvaux *et al*., 2001b).

Temperature: The bioreactors operated at ambient temperatures (20-25 °C) (Figure 2.6). Anaerobic digestion is well developed at temperatures above 18-20 °C (Araújo *et al*., 1998), as enzymes are required for this process, the observed temperature trend should be appropriate for enzyme production. Although some of the cellulolytic bacteria are mesophilic while others are thermophilic, the ones present in the bioreactors are expected to be mesophiles.

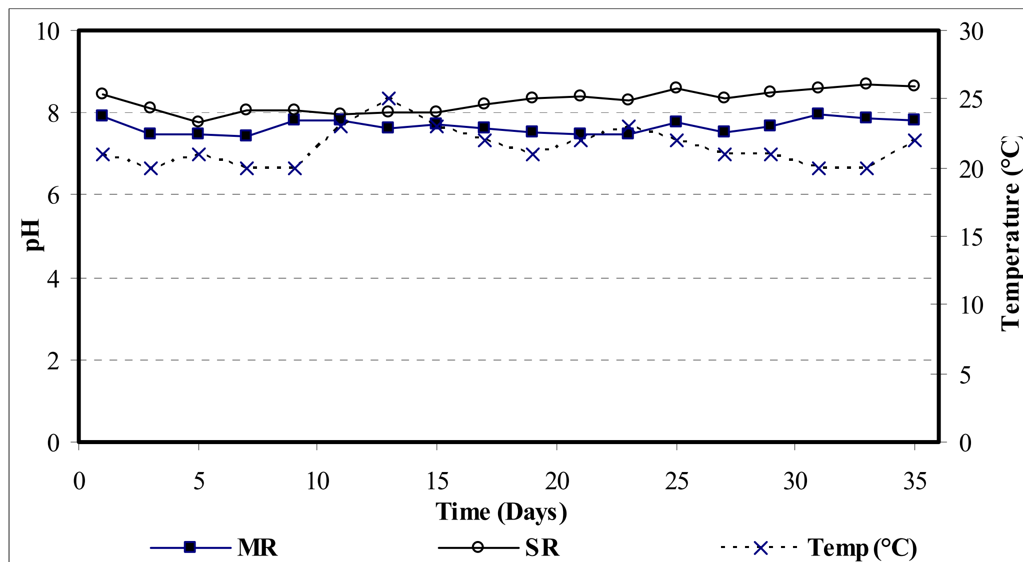


Figure 2.6: pH and temperature fluctuations during the time course.

2.3.6 Induction studies

Effects of various inducers: The endoglucanases activities in the SR supplemented with CMC increased more rapidly (day 1-9) than the other fed with α-cellulose (day 7-15)

(Figure 2.7). This can be attributed to the solubility and the availability of CMC to the microbes as compared to the α-cellulose. Although it would have been expected for CMC, as the sole carbon source for cellulolytic microbes, to effect higher yield of endoglucanases due to its solubility, α-cellulose-supplemented SR exhibited better enzyme production with 0.34 ± 0.014 μmol/min/ml enzyme activity produced on day 15 as compared to the optimal enzyme activity (0.26 ± 0.004 μmol/min/ml) in the CMC-supplemented SR on day 9. There was also a corresponding increase in glucose concentration in the bioreactors during the period of decline in enzyme activity, day 25-35, in α-cellulose-supplemented SR and day 9-17 in the CMC-supplemented SR. The faster glucose production in the CMC-supplemented SR implies more favourable utilisation of CMC by the cellulolytic bacteria. Similar observations have been made with respect to enzyme induction with different substrates. Enzyme activity unit in solka floc- and sigmacell- (insoluble cellulose substrates) grown cultures were found to be 1.1-13 times higher than activity exhibited by CMC-grown cultures (Spiridonov *et al*., 1998; Rajoka and Malik, 1997; Cai *et al*., 1999). The α-cellulose-supplemented SR showing higher enzyme production can be attributed to its closer resemblance of the natural substrate (Desvaux and Petitdemange, 2001). In addition, the presence of crystalline cellulose in growth media of *Clostridium cellulovorans* was reported to promote the assembly of cellulosomes from the free major subunits (Matano *et al*., 1994).

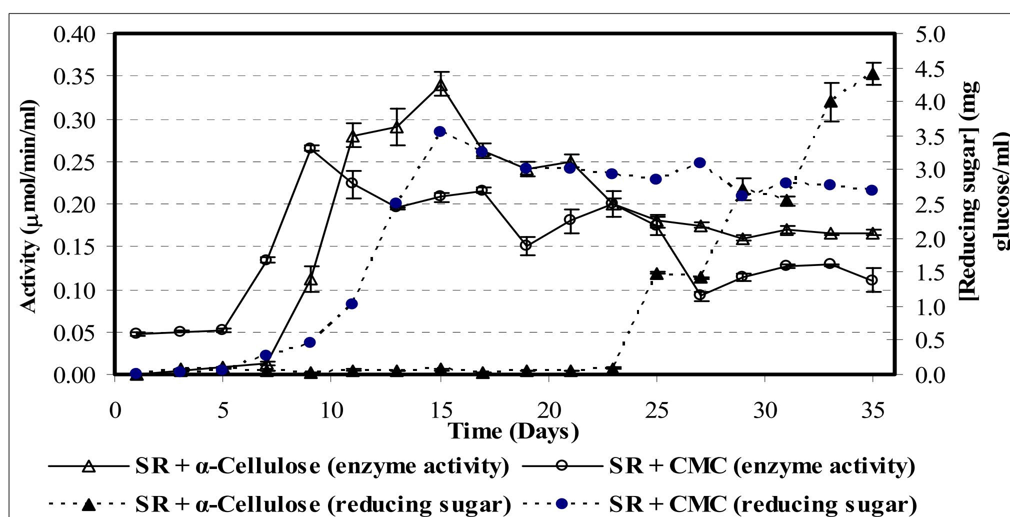


Figure 2.7: Production of endoglucanases in the presence of different inducers.

Optimisation of endoglucanases induction at varying substrate concentration: As enzymes are produced by microbes in response to substrate availability, it should follow that more enzymes would be produced in the presence of higher substrate concentration. Following the previous result obtained above, α-cellulose being a better inducer as compared to CMC, its final concentration in the SR was varied from 0.1 to 0.3 % (w/v). The highest production of endoglucanases occurred at substrate concentration 0.2 % (w/v). At this concentration 0.63 ± 0.003 μmol/min/ml enzyme activity was detected on day 15 as compared to 0.32 ± 0.016 μmol/min/ml on day 7 when no substrate was added (0 % (w/v)) (Figure 2.8). In natural environments where cellulolytic microorganisms thrive, nutrients are not always found in excess amounts. Complex media with high substrate concentration was reported to be unfavourable to *Clostridium cellulolyticum* and so the microbe was unable to utilise excessive substrate (Guedon *et al*., 2002). In a similar investigation, α-cellulose at concentration 1 mg/ml resulted in high formation of endoglucanase by *Sclerotium rolfsii* (Sachslehner *et al*., 1998).

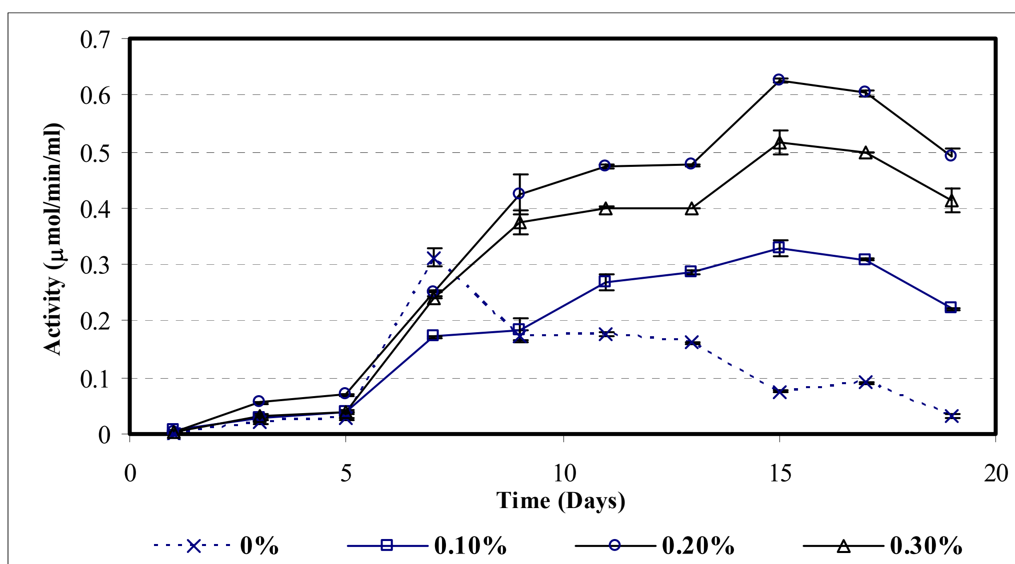


Figure 2.8: Dependence of endoglucanases production on the concentration of α-cellulose as inducer.

2.4 SUMMARY

Results from this study have shown that the production of endoglucanase is accelerated under biosulphidogenic conditions. Further addition of crystalline cellulose at a

concentration of 0.2 % (w/v) as an inducer improves production by two-fold. Depletion of the endoglucanase activities after some time could probably be as a result of the following factors: (a) inhibition of the cellulolytic microbes at high sulphide concentration, as sulphide has been shown to be inhibitory to many bacterial activities (Manilal *et al*., 2000) or inhibition of the enzyme activities; (b) catabolite repression from the increased glucose concentration, (c) proteases, as these enzymes are stimulated under biosulphidogenic conditions of PSS hydrolysis as well (Whiteley *et al*., 2002a). Therefore for a greater yield the system might be challenged with more substrate with a concomitant removal of the metabolites (glucose and sulphide). Cellulosic wastes could also have been used as cheaper inducers in the SR, as this would have been more cost effective.

pH and temperature are two crucial environmental factors influencing enzyme activity and the rate of solubilisation in reactors (Penaud *et al*., 1997). Most reactors are mesophilic and increasing the temperature should increase enzyme activity (Whiteley *et al*., 2003). However, in increasing the reactor temperature and maintaining the pH of the bioreactors for optimal enzyme production, the optimum temperature and pH of the enzyme as well as its thermal stability should be considered. Since production of SR was higher and faster in the SR, endoglucanases from the SR were used in the subsequent chapters.

CHAPTER 3
ENZYME LOCATION AND EXTRACTION

3.1 INTRODUCTION

The purpose of this section is to locate the enzyme (endoglucanase) and optimise its release into solution, if it is not already present in the aqueous medium. Extraction is the first step in the purification of an enzyme as it leads to the separation of an enzyme from its source and its conversion into a soluble form for ease in subsequent procedures.

Several extraction methods are employed to obtain target proteins from different sources, but the selection of a suitable method is dependent on several factors, for example the nature of the source (animal tissue, plant tissue, bacterial cells etc.), scale of operation and stability of the enzyme (Trevan *et al*., 1987). Extraction methods are for the disruption of cells, but in instances when the enzyme is present in an organelle (e.g. anaerobic bacterial endoglucanase present in the cellulosome), the methods are still appropriate as they also disrupt the organelles (Scopes, 1982). Cellulosomes are not easy to disrupt without some loss in total activity and individual constituents of the complex (Bayer *et al*., 1998). When considering enzyme extraction methods that require some degree of cell disruption, the method with minimal disruption with a corresponding release of the major proportion of the target protein should be used, thus decreasing the level of contamination, decrease degradation of the required protein and simplifying subsequent purification steps (Roe, 2001a).

Enzyme extraction starts with an evaluation of the exact cellular location of the enzymes, i.e. extracellular, intracellular or membrane bound (Trevan *et al*., 1987). Extracellular proteins secreted from cells are simple to purify since there is minimum level of other protein contaminants as compared to intracellular proteins, which require preparation of an extract (Cumming and Iceton, 2001). Isolation of intracellular proteins usually involves a difficult series of operations (Agrawal and Pandit, 2003). Extracellular proteins are more stable since they already exist in a relatively harsh environment. Intracellular proteins are generally less stable when released from their natural

environments. Many of the disruption procedures impose stresses, such as shear, heat or gas bubbles which can denature the enzymes (Cumming and Iceton, 2001).

It has been suggested that sludge enzymes are either associated with the cell surface of the cell or are in free form, in solution or adsorbed to the surfaces of the substrate (Chróst, 1991).

The principles of the cell disruption procedures used in this study are briefly highlighted below.

(1) Sonication: This method is common on a small scale and is used with less resistant cell walls such as bacteria and fungi (Trevan *et al*., 1987). This method works by a principle called cavitation. When cellular suspensions are subjected to ultrasonic waves (with frequency 20 kHz and above), there is a rapid formation of microbubbles which grow and coalesce until they reach their resonant size, vibrate violently and eventually collapse, thereby disrupting the cell (Onyeche, 2002). At sufficient periods of exposure of the suspension to ultrasonic waves, membrane bound and cytoplasmic proteins are released (Cumming and Iceton, 2001). The disadvantages of this method include heat generation and the difficulty of transmitting sufficient power to large volumes of suspension (Scawen, 1985).

(2) Detergents: Most membrane bound proteins can be released from their host membranes by employing detergents which have lipophilic chains that bind to the protein at its hydrophobic surface in lieu of the normal membrane (Figure 3.1). Detergents are able to dislodge a protein, firstly, by dissolving the membrane followed by substituting the membrane with aliphatic or aromatic chains that form the lipophilic part of the detergent (Neugebauer, 1990). The reversible displacement of the natural lipid by the detergent could lead to loss of activity (Scopes, 1982).

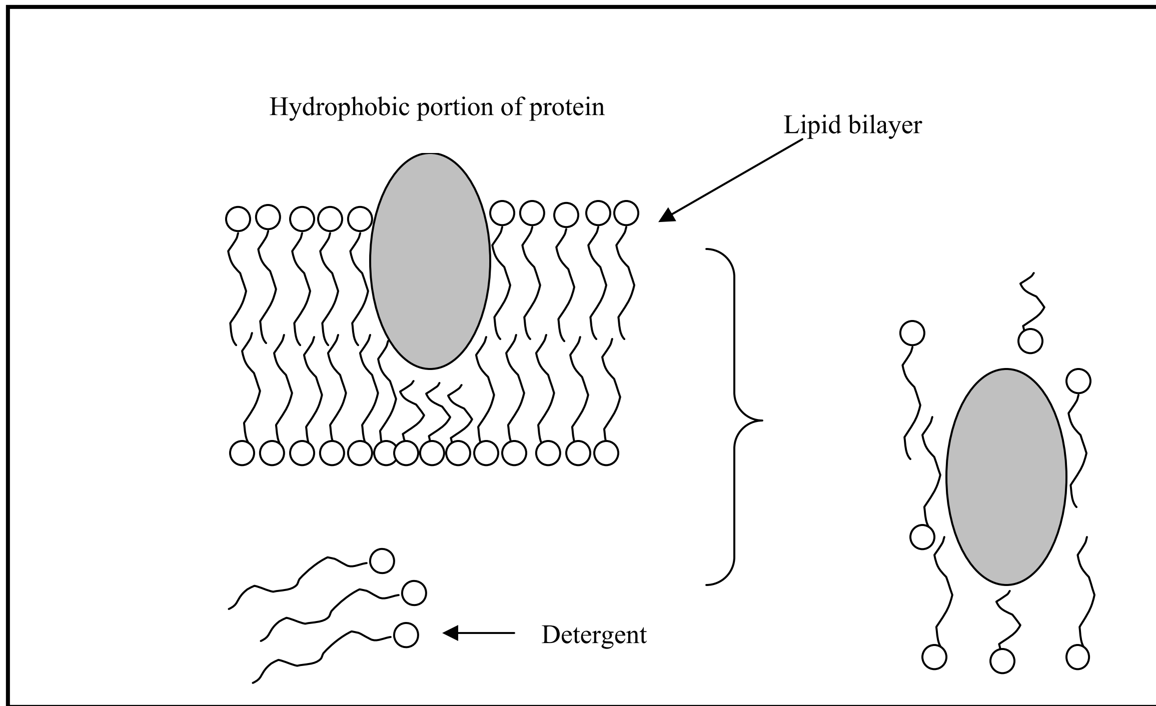


Figure 3.1: Action of detergent in solubilising the membrane bound proteins. Adapted from Scopes, 1982.

(3) Organic solvents: Organic solvents such as toluene, isopropanol and ethanol can facilitate formation of perforations on the cell wall, thus resulting in the cell liberating its contents. Organic solvents are also used in protein concentration because they reduce the solubility of the proteins by reducing the dielectric constant of the medium (Harris, 2001). At low concentrations of solvent (<5 % (v/v)) there is release of small molecular weight materials from cells while higher concentrations can lead to the release of intracellular proteins (Cumming and Iceton, 2001). Although organic solvents are effective in disrupting numerous cell types and are conventional methods of cell disruption, they are not used on a large-scale, because of their toxicity, protein-denaturating ability and flammability (Trevan *et al*., 1987).

3.2 MATERIALS AND METHODS

3.2.1 Materials

Triton X-100 (t-Octylphenoxypolyethoxyethanol) was obtained from Sigma (South Africa, (Pty) Ltd). Absorbances were measured using Power Wave$_X$ (Bio-Tek Instrument

Inc, South Africa). Centrifugation was performed with Beckman J2-21 centrifuge with a JA-14 rotor and sonication with a Virsonic-100 sonicator (VirTis, Co., Inc, USA). Hotplate magnetic stirrer from Snidjers, South Africa, was used to stir samples. Other analytical-grade reagents were purchased from Sigma (South Africa, (Pty) Ltd), or Merck (South Africa, (Pty) Ltd)

3.2.2 Reactor set-up and Sludge source

The lab-scale continuous stirred tank reactor (CSTR) used for these studies is shown in Figure 3.2. The same feed source used for the SR in chapter 2 (2.2.2.1) was used in this study. Unlike the closed reactor employed in chapter 2, this continuous reactor was fed every third day. Samples were obtained from the anaerobic reactor (B) through the sampling port (H).

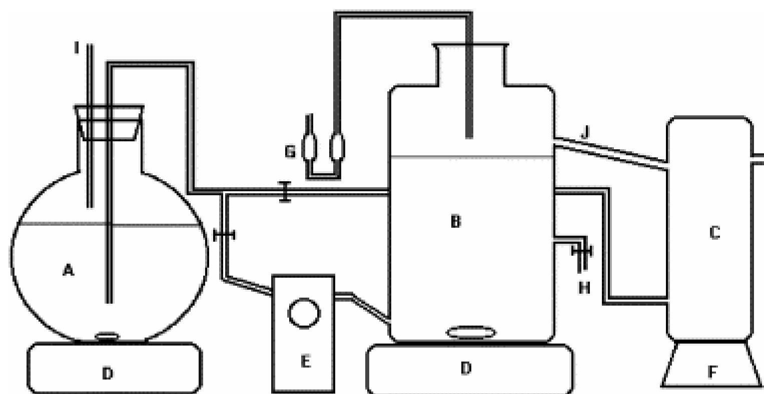


Figure 3.2: Diagram of laboratory scale biosulphidogenic reactor: (A) feed vessel containing PSS (COD and SO_4^{2-} 2000mg/l); (B) experimental biosulphidogenic bioreactor containing 10 % (v/v) SRB mixture culture; (C) overflow vessel to receive passive sludge effluent from the biosulphidogenic bioreactor; (D) magnetic stirrers; (E) peristaltic pump set to allow HRT for two days; (F) plastic support; (G) zinc acetate solution as hydrogen sulphide trap; (H) sampling port; (I) vent and (J) overflow port.

3.2.3 Methods

3.2.3.1 Endo-β-1, 4-D-glucanase activity assay: Endoglucanase activity was determined spectrophotometrically as described in Chapter 2 (section 2.2.2.3.1).

3.2.3.2 Protein determination: Protein concentration was estimated using the Bradford method as described in Chapter 2 (section 2.2.2.3.2)

3.2.3.3 Enzyme location: The sewage sludge sample from the continuous reactor was divided into three fractions in order to identify the location of the enzyme activity: a) intact sludge samples were used to measure the whole-cell enzyme activity that would normally be available in the sludge, this was named fraction "W"; b) pellets from centrifuged sludge (10 000 × g, 30 min, 4 °C) were washed three times with distilled water (1:5 w/v) , followed by resuspension in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v). This represented the immobilised enzyme on the sewage sludge particulate matter or bacterial cell wall and was labelled fraction "P"; c) raw supernatant obtained after centrifugation of the whole sludge represented the soluble free enzyme present in the aqueous medium or secreted extracellularly and was labelled fraction "S".

3.2.3.4 Enzyme extraction

3.2.3.4.1 Sonication: The sludge samples collected from the continuous reactor were centrifuged (10 000 × g, 30 min, 4 °C), the pellet was washed three times with distilled water (1:5 w/v) , followed by resuspension in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v). Separate samples were subjected to sonication at 4 °C on ice at (30s intervals, 10 W) for a period of 0-8 min, followed by centrifugation (10 000 × g, 10 min, 4 °C). Both pellet and supernatant obtained were assayed for protein and endoglucanase activity.

3.2.3.4.2 Detergent: The sludge samples collected from the continuous reactor were centrifuged (10 000 × g, 30 min, 4 °C) washed three times with distilled water (1:5 w/v) , followed by resuspension in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v), containing Triton X-100 in varying concentrations of 0-2.5 % (v/v) (0.5 % increments). This slurry was stirred gently overnight at 4 °C, centrifuged (10 000 × g, 10 min, 4 °C) and both pellet (resuspended as before) and supernatant fractions were dialysed (12 h,

ddH_2O) to remove the detergent and finally assayed for protein and endoglucanase activity.

3.2.3.4.3 Organic solvent: The sludge samples collected from the continuous reactor were centrifuged (10 000 × g, 30 min, 4 °C) washed three times with distilled water (1:5 w/v) , followed by resuspension in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v), n-Butanol was added in varying concentrations (0-20 % (v/v) to separate suspensions. The mixtures were gently stirred (30 min) and then centrifuged (10 000 × g, 10 min, 4 °C), the excess butanol was removed by suction and the solution was filtered to obtain a clear extract. The endoglucanase activity and protein concentration were determined.

3.3 RESULTS AND DISCUSSION

3.3.1 Location: No activity was detected in the raw supernatant (S) fraction (Figure 3.3), almost all (95 ± 7.07 %) of the endoglucanase activity was associated with the pellet (P) as compared with the whole cells (W) (100 % activity = 0.18 ± 0.02 μmol/min/ml). This indicates that the enzyme is immobilised to the sludge particulate matter, which could be intracellular, membrane or cell-wall bound (Whiteley *et al*., 2003). The deviation in the percentage (±7.07 %) could be as a result of the dilution of the pellet when suspended in the intact whole sludge fraction as compared to when resuspended in the buffer. The bacteria producing these enzymes themselves might be associated with or immobilised within the particulate matter, synthesising the enzyme close to its substrate (Goel *et al*., 1998). Polysaccharases such as endoglucanases are produced by microbes for the degradation of EPS and EPS provide an extracellular covering for these microbes, it would therefore be expected to have the enzyme close to the extracellular surface of the microbes (Sutherland, 1999). Considering the location of the enzyme, some disruptions would be required for the release of this enzyme into the soluble medium so as to facilitate purification.

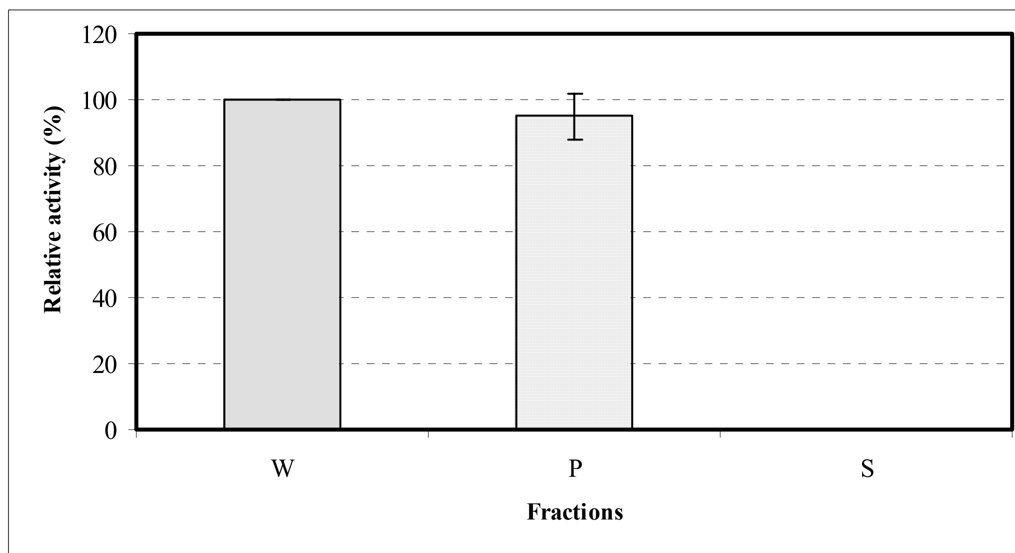
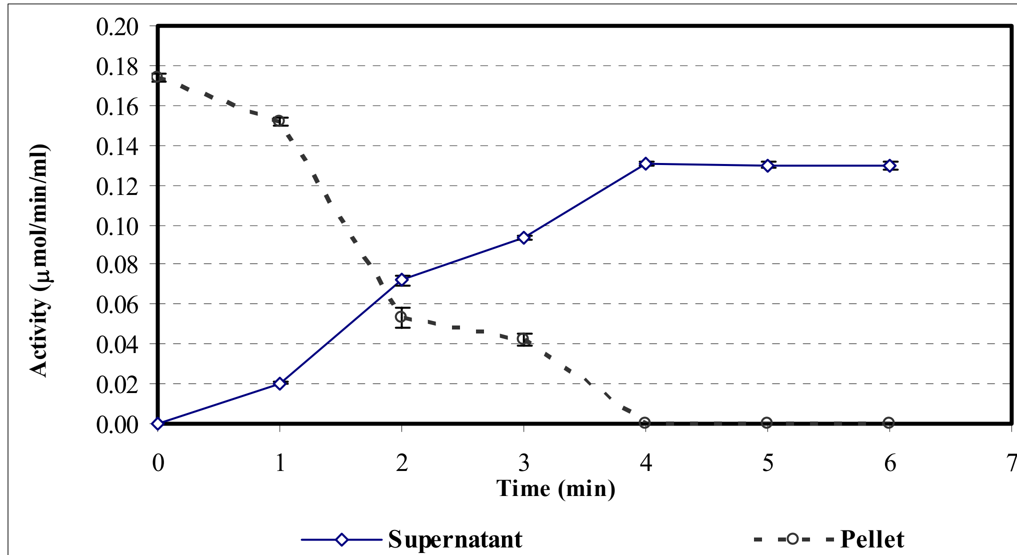


Figure 3.3: Enzyme activity associated with different fractions in sludge from the biosulphidogenic reactor. Relative activity expressed as percentage of the total enzyme activity, 100 % activity = 0.18 ± 0.02 μmol/min/ml.

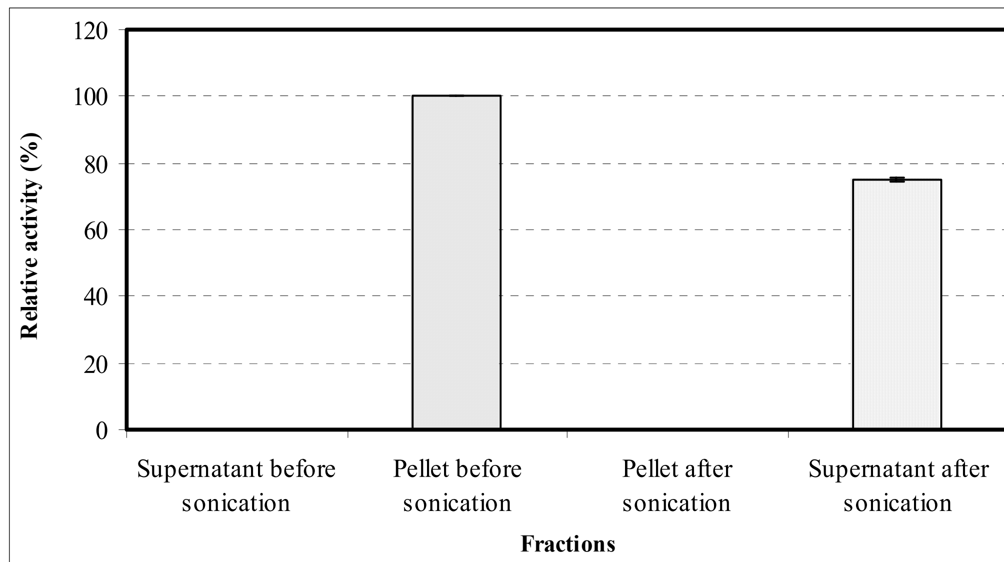
3.3.2 Extraction

The results obtained were expressed in relative percentage of the control (intact resuspended pellet) for comparison of amount of enzyme extracted by the different techniques employed.

3.3.2.1 Sonication: A major proportion (74.9 ± 0.64 %) of the total enzyme activity present in the sludge was released into the supernatant after 4 min of sonication before starting to level off (Figure 3.4). There was no activity remaining in the pellet after the optimum period of suspension exposure to the ultrasonic waves, showing that the remaining ~25.1 % of the activity was destroyed. Though the method was carried out in ice to minimise heat generation, denaturation of the enzyme still occurred.



(a)



(b)

Figure 3.4: Endoglucanase release from the pellet particulate fraction via sonication: (a) Optimising enzyme release by sonication, (b) Relative activity present in different fractions. Relative activity expressed as percentage of the total enzyme activity present in the resuspended pellet, 100 % activity = 0.174 ± 0.002 μmol/min/ml.

3.3.2.2 Detergent

The maximum enzyme release by Triton X-100 occurred at concentration of 0.1 % (v/v) with the activity dropping off after that (Figure 3.5a). At the optimum concentration (0.1

%) 66.2 % of the total enzyme activity was detected in the supernatant and 30.2 % was found retained in the pellet, indicating that 3.6 % of the total activity was denatured (Figure 3.5b). This shows that this technique, even with increasing detergent concentration, could not release the entire enzyme associated with the pellet into the supernatant.

Triton X-100 was used because it is zwitterionic in nature, mild and can be tolerated by many proteins. In a similar investigation of extraction of protease with Triton X-100 from activated sludge, it was observed that after the highest activity of enzyme was released at 0.1 % Triton X-100 concentration, with increasing detergent concentrations the levels of enzyme activity recovered was decreased which could be attributed to enzyme inactivation (Gessesse *et al*., 2003).

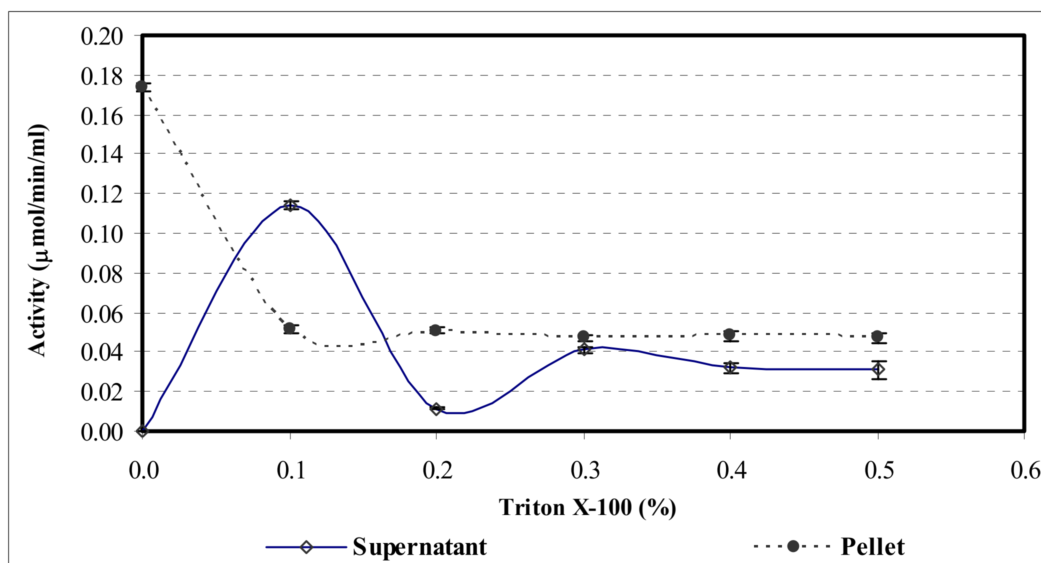


Figure 3.5a: Endoglucanase release from the pellet particulate using detergent (Triton X-100): Optimising enzyme release using detergent. Relative activity expressed as percentage of the total enzyme activity in present in resuspended pellet, 100 % activity = 0.174 ± 0.002 µmol/min/ml.

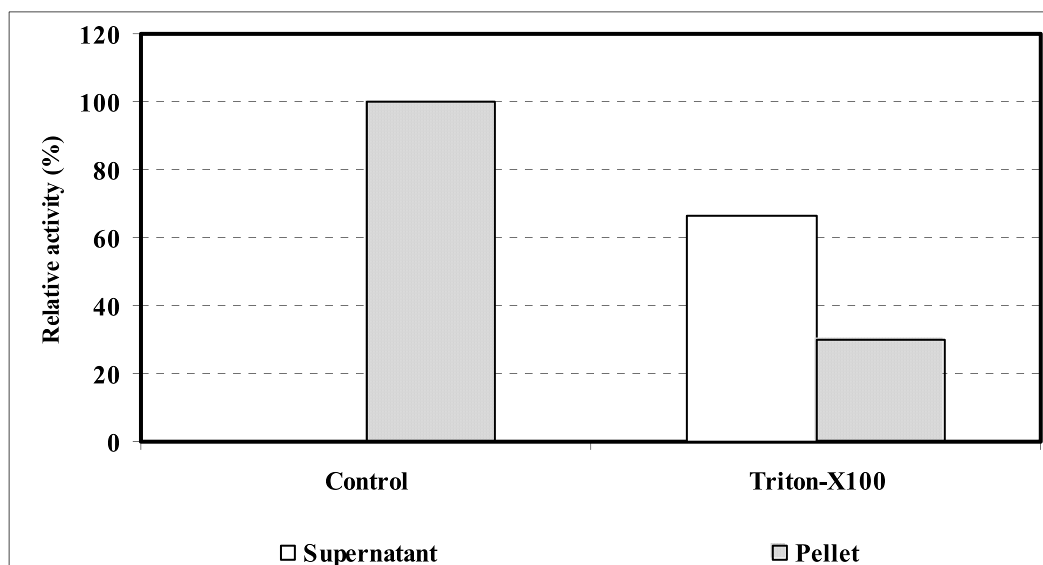


Figure 3.5b: Endoglucanase release from the pellet particulate using detergent (Triton X-100): Relative activity present in different fractions. Relative activity expressed as percentage of the total enzyme activity in present in resuspended pellet, 100 % activity = 0.174 ± 0.002 µmol/min/ml.

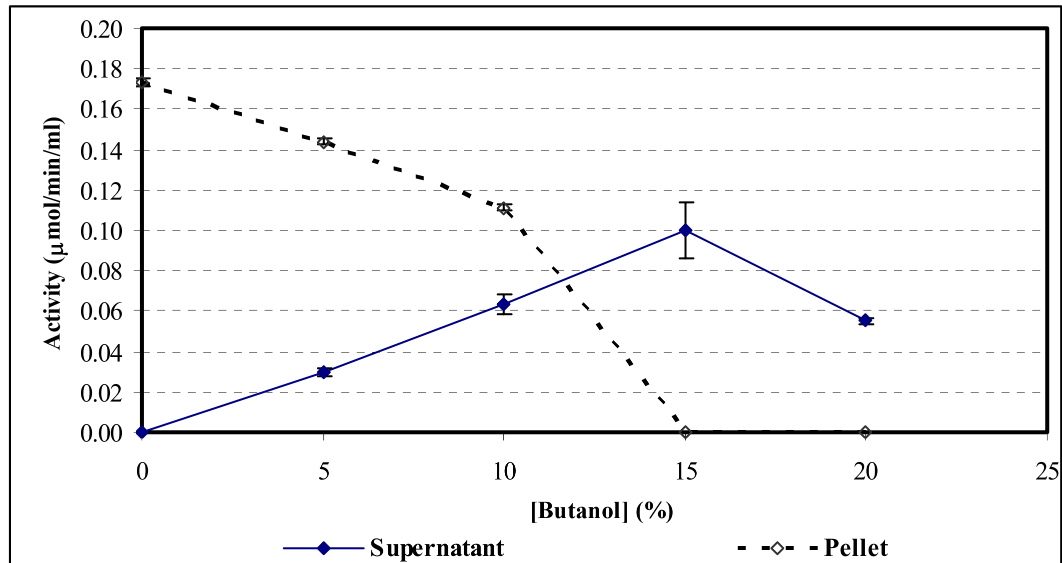
3.3.2.3 Organic solvent

Though a reasonable amount of enzyme (57.6 ± 7.6 %) was released into the supernatant at 15 % (v/v) concentration of n-Butanol, a large amount of enzyme activity (42.4 ± 7.5 %) was lost in this procedure (Figure 3.6) in comparison to the two other techniques. No residual activity was found in the pellet after treatment with the solvent, indicating that the total enzyme was released and partially deactivated or denatured afterwards. Long-chain alcohols have been observed to cause high denaturation of enzymes (Harris, 2001). n-Butanol was the choice because of its ability to precipitate proteins that are lipid-bound which are found on the cell membrane.

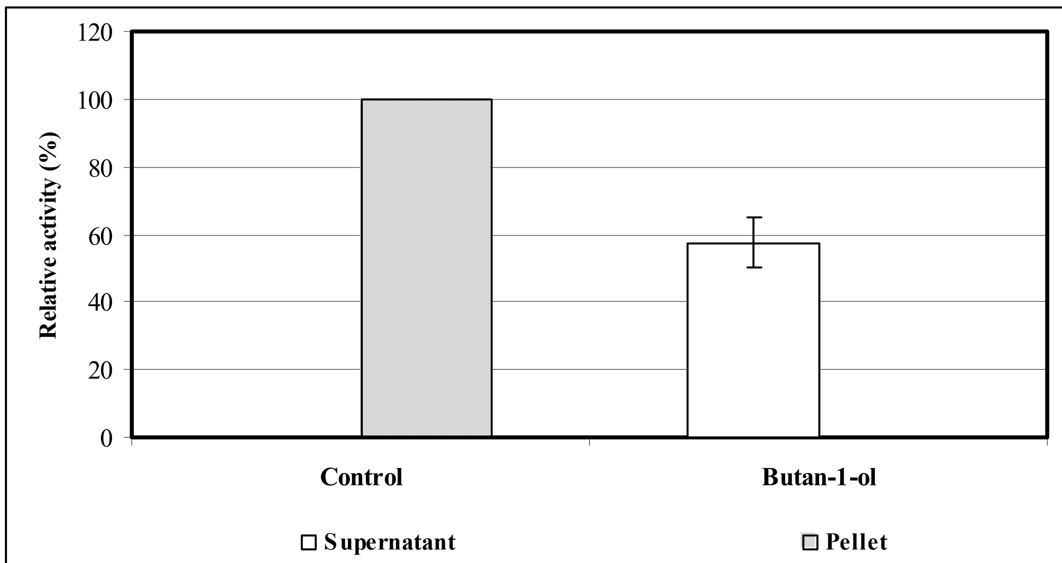
3.4 **SUMMARY**

The observed total activity associated with the pellet indicated that the enzyme was immobilised to the organic particulate matter or bound to the cell-wall or intracellular membrane. This result further confirms location of enzymes previously studied under biosulphidogenic conditions of sewage hydrolysis. (Pletschke *et al.*, 2002; Whiteley *et al.*, 2002a; 2002b; 2003). In addition, cellulosomes found in anaerobic cellulolytic

bacteria are arranged on the cell surface as protuberance-like organelles (Figure: 3.7) (Shoham *et al*., 1999).

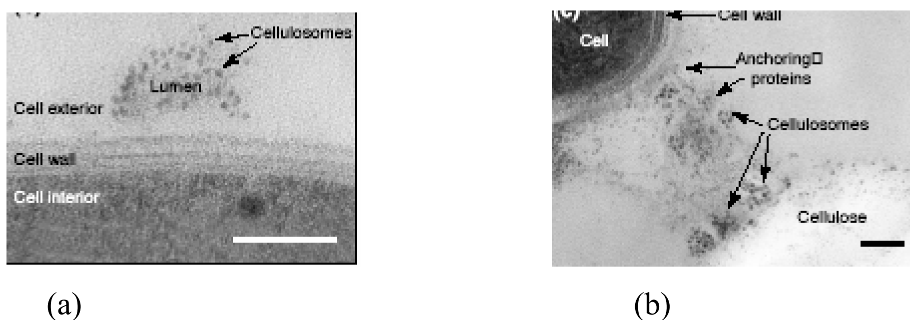


(a)



(b)

Figure 3.6: Endoglucanase release from the pellet particulate using organic solvent (n-Butanol): (a) Optimising enzyme release using organic solvent (b) Relative activity present in different fractions. Relative activity expressed as percentage of the total enzyme activity present in the resuspended pellet, 100 % activity = 0.174 ± 0.002 μmol/min/ml.



(a) (b)

Figure 3.7: Ultrastructure of the *Clostridium thermocellum* cell surface (a) Transmission of a resting polycellulosomal protuberance. (b) Transmission electron micrograph of a protracted polycellulosomal. The cellulosome is mainly associated with the cellulose surface and connected to the cell via extended fibrous material, believed to comprise the anchoring proteins. Scale bars = 100nm (Shoham *et al*., 1999).

From all the extraction methods used sonication released the greatest amount of enzyme into the supernatant, although there was nearly 25 % denaturation of enzyme. Previous works (Goel *et al*., 1997; Cadoret *et al*., 2002) have shown that proteins are released from sewage sludge particulates into solution via sonication due to the disintegration of the floc matrices, cell-cell interactions and cellular membrane. Although, enzyme extraction using detergents showed the lowest degree of denatured enzyme (3.6 %), the total “loss” (amount of enzyme retained + amount of enzyme denatured = 33.8 %) in this method is large and also very time-consuming (~ 22 h) compared to 4 min used for sonication. In the case of organic solvent, the greatest loss of enzyme activity (42.4 ± 7.5 %) was observed. Sonication proved to be the most effective (degree of extraction) and efficient extraction method and was therefore used in all subsequent experiments in order to prepare the crude cell-free extract. As mentioned earlier, the least disruptive method consistent with the release of a major proportion of the target enzyme should be employed (Roe, 2001b). On a lab scale, sonication is easily applicable while on a large industrial scale it might be uneconomical as a result of the energy cost involved (Onyeche *et al*., 2002).

CHAPTER 4

CHARACTERISATION OF ENDOGLUCANASES FROM BIOSULPHIDOGENIC ANAEROBIC DIGESTED SLUDGE

4.1 INTRODUCTION

An understanding of the structure, role and characteristics of the target protein is essential and plays a vital role in manipulating the purification approach (Roe, 2001a). Knowledge of the molecular weight, stability of the proteins at varying pH and temperature, affinity for substrates and sensitivity to metal ions can help in designing the crucial steps involved in purification (Willson, 1999). Since there are concerted reactions taking place in the anaerobic digestion of sewage sludge under biosulphidogenic conditions, it is of great importance to investigate *in vitro* the interactions occurring among these reactions. Products of certain reactions might be needed for the progress of some others, while the presence of certain products might impose adverse effects on other reactions. As enzymes are specific and sensitive to changes in their environment, an investigation of the influence of some substances produced and present in the sewage sludge during anaerobic degradation under biosulphidogenic conditions on endoglucanases will be highly beneficial.

The objectives of this section are highlighted below:

(1) pH: The ionisation state of amino-acid residues in the active site of an enzyme is dependent on pH. Since catalytic activity is dependent on the state of ionisation of these residues, enzyme activity is consequently pH dependent (Wilson, 2000a). Usually, enzymes are active only over a narrow range of pH with a particular pH (pH optimum) at which their catalytic activity is maximal.

(2) Temperature: The rates of enzyme-catalysed reactions are usually directly proportional to temperature. On the other hand, at temperature above 50 to 60 °C, some mesophilic enzymes characteristically show a decrease in activity. The loss of enzyme activity at elevated temperatures is a consequence of thermal denaturation of protein (Garrett and Grisham, 1999a). At the characteristic temperature optimum of an enzyme, there is maximum activity beyond which denaturation sets in leading to loss of activity.

(3) Thermal stability: The loss of enzyme activity depends both on time and temperature, it is therefore important to investigate the stability of the enzyme with respect to these factors (Roe, 2001a). After examining the enzyme temperature optimum, the thermal stability of the enzyme over a particular period must be studied. Most, but not all enzymes are stable at lower temperatures (Garrett and Grisham, 1999a).

(4) Metal ions: The presence of metals such as Fe, Ni, Zn, Pb etc. in sewage sludge has been reported (Zorpas *et al*., 2001; Cornu *et al*., 2001) and sometimes Al, Ca and Fe are added during the clarification process in sewage treatment (Cenni *et al*., 2001; Pletschke *et al*., 2002). There is also a great possibility of Cu deposits from canned foods, which pass undigested through the gastrointestinal tract into the faeces. The occurrence of considerable amounts of heavy metals in urban sewage sludge reflects the origin of the sludge material, either industrial or domestic (Banerjee *et al*., 1997). Although the metal content of municipal sewage sludge increases with an increase in industrial activity, metals are still found as trace elements in domestic sewage sludge (Wong *et al*., 2001; Linden *et al*., 1995). As some enzymes require metal ions for the stabilisation of their protein structure and for the regulation of their activities, it will be significant to look at the effects of some of these metal ions found in the sewage sludge on the enzyme, endoglucanase.

(5) Volatile fatty acids (VFA's): Some of the VFA's produced during the acidogenic stage of anaerobic digestion of primary sewage sludge include, acetic, propionic, butyric, iso-butyric, valeric, 3-methyl butyric and 2-methyl butyric acids. Acetic acid is the most common product, as it is formed directly from the fermentation of carbohydrates and proteins, as well as during the anaerobic oxidation of lipids (Elefsiniotis and Oldham, 1994). Consequently it is relevant to investigate the influence of these compounds on endoglucanase activity.

(6) Sulphur-containing compounds: Since this research investigates the production of endoglucanase in sewage sludge under biosulphidogenic conditions it is important to study the influence of sulphide, sulphate and sulphite on endoglucanases. Sulphate is reduced by the sulphate reducing bacteria to sulphide with sulphite ions as intermediate.

(7) Inhibition of proteases: It has been previously shown that proteases are stimulated under biosulphidogenic conditions of sewage sludge hydrolysis (Whiteley *et al*., 2002a).

The presence of these enzymes is disadvantageous to other enzymes, as they become substrates for the proteases. Furthermore, during protein purifications which may involve cell disruption the target protein (endoglucanase) will be exposed to such proteases. So as to minimise the loss of the target proteins, the activity of the proteases should be decreased. A common method is to add inhibitors to the original extract to prohibit the hydrolytic action of proteases. The most frequently used chemical is Phenylmethanesulfonyl fluoride (PMSF) in a final concentration of 0.5-1.0 mM, which inhibits serine, thiol, and some carboxypeptidase protease activity (Roe, 2001a). It is necessary to investigate the specific concentration at which the protease inhibitor affects the protease and not the target protein (endoglucanase) so as to have more available endoglucanases for purification.

(8) Catalytic properties and substrate specificity of endoglucanases: At low substrate concentrations the rate of the enzyme-catalysed reaction (measure of enzyme activity) has a direct proportional relationship to the substrate concentration, resulting in a first-order reaction. Conversely, at high substrate concentrations, the rate of reaction becomes independent of the substrate concentration, hence yielding zero-order kinetics. The rate of the enzyme-catalysed reaction reaches is maximal at this point and this limit is referred to as V_{max}, this indicates enzyme saturation with the substrate. The *Michaelis constant* (K_m) is associated with the strength of binding of substrate to enzyme (affinity) (Garrett and Grisham, 1999a). The *Michaelis constant* is inversely proportional to the affinity of the enzyme for its substrate, therefore as K_m decreases, affinity of an enzyme for its substrate increases (Mathews and van Holde, 1990). Under certain conditions, K_m is the substrate concentration that would produce 50 % V_{max}.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Avicel, acetic acid, butyric acid, propionic acid and valeric acid, hydroxyethylcellulose (HEC) and PMSF (Phenylmethanesulfonyl fluoride; α-Toluenesulfonyl fluoride) were purchased from Sigma (South Africa (Pty) Ltd, Purum grade) while EDTA (Ethylene diamine tetraacetic acid di-sodium salt) was from Merck (South Africa (Pty) Ltd, Analar

grade). Absorbances were measured using Power Wave$_X$ (Bio-Tek Instrument Inc, South Africa). Centrifugation was performed with Beckman J2-21 centrifuge with a JA-14 rotor and sonication with a Virsonic-100 sonicator (VirTis, Co., Inc, USA). Other analytical-grade reagents were purchased from Sigma (South Africa, (Pty) Ltd), or Merck (South Africa, (Pty) Ltd)

4.2.2 Methods

The range of concentration studied (0-1000 mg/l) for the sulphur-containing compounds falls within the range of inherent sulphide and sulphate detected in the sewage sludge (Figure 2.3). For all the effectors studied (metal ions, volatile fatty acids, sulphur-containing compounds and protease inhibitors), controls were run to ensure that they do not interfere with the enzyme assay. Reaction mixture was incubated with the final concentrations of the effectors; water was used instead of the enzyme extract. These served as controls. The assay conditions described in section 2.2.2.3.1 was followed.

4.2.2.1 Sludge sample source: The sludge samples were collected from a continuous biosulphidogenic digester (Figure 3.1) described in Chapter 3 (section 3.2.2).

4.2.2.2 Enzyme preparation: Crude samples obtained from the continuous biosulphidogenic reactor were centrifuged (10 000 ×g, 30 min, 4 °C), the pellet was washed three times with distilled water (1:5 w/v) in order to completely remove all the water-soluble inherent effectors (VFA's, sulphur-containing compounds and metals) studied, and then resuspended in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v). During the determination of the pH optimum, the pellet was resuspended in different buffers at varying pH. The resuspended pellet (immobilised enzyme sample) was further subjected to sonication (30 s intervals, 10 W, 4 min) on ice followed by centrifugation (10 000 ×g, 10 min, 4 °C). The supernatant represented the soluble enzyme sample.

4.2.2.3 pH: The different buffers used at varying pH were: 0.05 M sodium acetate (pH 3-4.5), 0.05 M sodium citrate (pH 5-5.5) and 0.05 M sodium phosphate buffer (pH 6-8). Since carboxymethyl cellulose (CMC) is an ionic substrate, its properties would change

with pH, therefore a non-ionic substrate, hydroxyethylcellulose (HEC), was used in this study. The substrate and the soluble enzyme extract were prepared in the different buffers. The activity of the endoglucanase was determined according to the assay described in Chapter 2 (2.2.2.3.1).

4.2.2.4 Temperature: The temperature profile for endoglucanase between 10 and 70 °C at the pH optimum was determined. The soluble enzyme extract was incubated with the substrate (CMC) at the varying temperatures (10-70 °C) and pH optimum. The endoglucanase activity was determined as described in Chapter 2 (2.2.2.3.1).

4.2.2.5 Thermal stability: Enzyme extracts (immobilised and soluble) were heated at 30, 50, and 70 °C for 1 h at the pH optimum, 3 ml aliquots were withdrawn from each extract at 10 min intervals. Samples were prepared in triplicate and residual enzyme activity was measured as described in Chapter 2 (section 2.2.2.3.1) by starting the reaction with the addition of the substrate to the samples. Estimation of enzyme activity in the immobilised extract was determined using the same method above, but the reaction mixture was centrifuged after assay so as to remove the suspended solids that could cause interference during the absorbance reading.

4.2.2.6 Metal ions: A range of metal chlorides (Ca, Mg, Fe, Cu, Ni and Zn) at varying concentrations (0-1000 mg/l) were prepared using 0-0.4 ml solutions and incubated (15 min, 37° C) with enzyme extract (0.8 ml), sodium phosphate buffer (0.05 M, pH 6) was added to give a final volume of 1.2 ml. The assay was initiated by the addition of 0.8 ml substrate (1 % w/v, CMC). The residual enzyme activity was measured as before.

4.2.2.7 Volatile fatty acids: The volatile fatty acids used were, acetic acid, butyric acid, propionic acid and valeric acid. They were prepared at varying concentrations (0-1000 mg/l) using 0-0.4 ml solutions (VFA's) and incubated (15 min, 37° C) with enzyme extract (0.8 ml), sodium phosphate buffer (0.05 M, pH 6) was added to give a final volume of 1.2 ml. The assay was initiated by the addition of 0.8 ml substrate (1 % w/v, CMC). The residual enzyme activity was measured.

4.2.2.8 Sulphur-containing compounds: Sulphate, sulphide and sulphite at varying concentrations (0-1000 mg/l) were prepared using 0-0.4 ml of their sodium salt solutions and incubated (15 min, 37° C) with enzyme extract (0.8 ml), sodium phosphate buffer (0.05 M, pH 6) was added to give a final volume of 1.2 ml. The assay was initiated by the addition of 0.8 ml substrate (1 % w/v, CMC). The residual enzyme activity was measured as described in Chapter 2 (2.2.2.3.1).

4.2.2.9 Protease inhibitors: Ethylene diamine tetraacetic acid (EDTA) (inhibitor of metalloproteases) and PMSF were added to the enzyme extract to establish any change in the endoglucanase activity by protease inhibition. PMSF (dissolved in acetone) and EDTA were separately added to the resuspended pellet to final concentrations of 0-2.5 mM. At the different concentrations in different tubes, each suspension was sonicated (30 s intervals, 10 W, 4 min) then pre-incubated at room temperature for 15 min to allow for total inhibition of the proteases, followed by centrifugation (10 000 ×g, 30 min, 4 °C). The reaction was then initiated by adding 1 ml substrate (1 % w/v, CMC) to the supernatant (1 ml) obtained. The residual enzyme activity was measured as before.

4.2.2.10 Catalytic properties and substrate specificity: The substrates used were CMC, HEC and avicel. Kinetic studies were carried out using CMC and HEC prepared in sodium phosphate buffer (0.05 M, pH 6) at a concentration range 0.1-1 % (w/v). The enzyme specificity for the different substrates (using soluble enzyme extract) was determined by using 1 % (w/v) CMC, HEC and avicel as substrates and following the assay conditions described in Chapter 2 (section 2.2.2.3.1).

4.3 RESULTS AND DISCUSSION

All the effectors (metal ions, VFA's, sulphur-containing compounds and protease inhibitors) studied did not interfere with the assay.

4.3.1 pH: The pH optimum of endoglucanases from the biosulphidogenic reactor was found to be 6 (Figure 4.1). This finding is in accordance with previous observations of pH optima for other endoglucanases from: *Reticulitermes speratus* (pH 6) (Watanabe *et*

al., 1997); *Aspergillus niger* (pH 6) (Akiba *et al*., 1995). Also, the enzyme's pH optimum falls within the range in which SRB grow (pH 6-9).

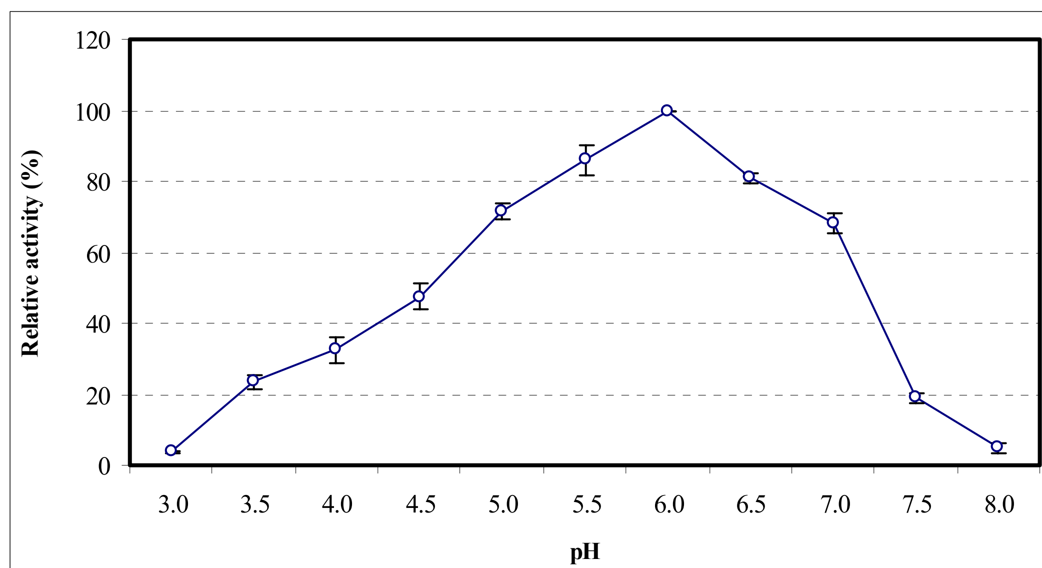


Figure 4.1: Effect of pH on endoglucanase activity. Relative activity expressed as percentage of the maximum enzyme activity, 100 % activity = 0.29 μmol/min/ml.

4.3.2 Temperature: The optimal temperature for endoglucanases under biosulphidogenic conditions, at pH 6 was 50 °C (Figure 4.2). This observation is similar to previous findings of temperature optima of other endoglucanases; 50 °C (Lee and Kim, 1999); 50-60 °C Marques *et al*., 2003) and 55 °C (Murashima *et al*., 2002) from *Bacillus cellulolyticus* K-12*, Aspergillus terreus* and *Rhizopus oryzae* respectively. The characteristic high temperature optimum exhibited by this enzyme is a characteristic that is common to all enzymes previously identified in biosulphidogenic digesters (Pletschke *et al*, 2002; Whiteley *et al*., 2002a; 2002b; 2003).

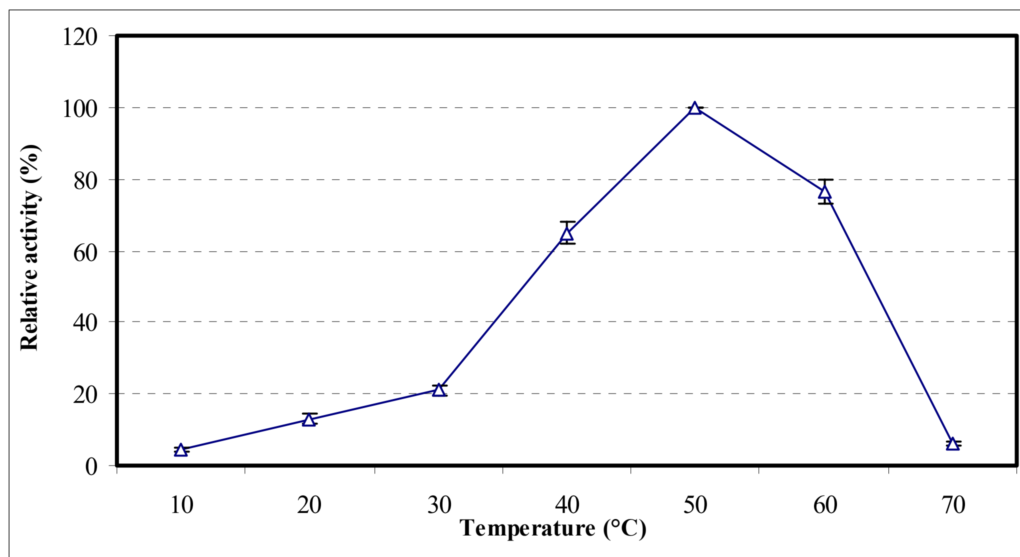
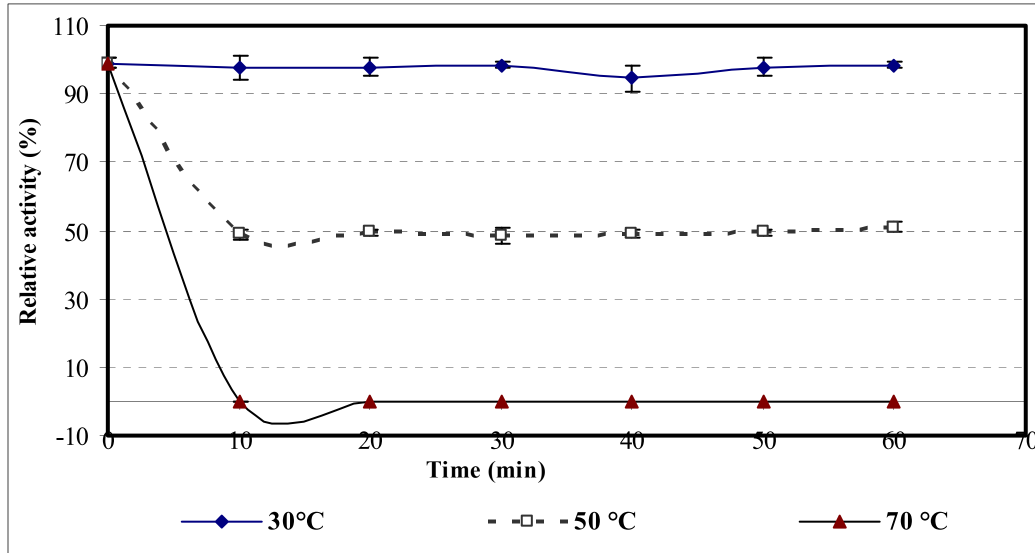
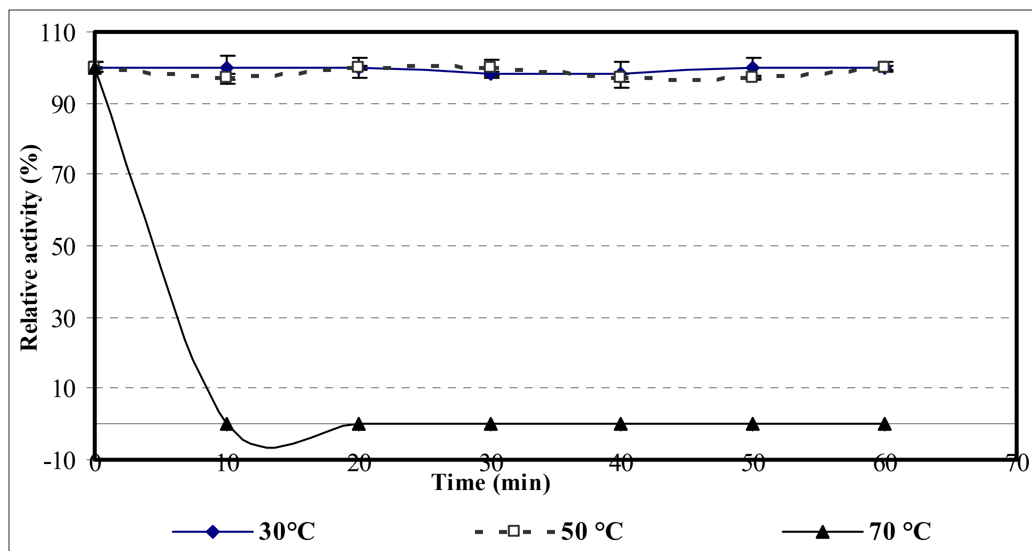


Figure 4.2: Effect of reaction temperature on endoglucanase activity. Relative activity expressed as percentage of the maximum enzyme activity, 100 % activity = 0.29 μmol/min/ml.

4.3.3 Thermal stability: The enzyme remained 100 ± 1.29 % active at 30 °C for 1 h both in its soluble and immobilised forms (Figure 4.3). At the optimum temperature (50 °C) however, the enzyme retained full activity for 1 h only when immobilised to the floc matrix. In the soluble form, the enzyme lost 50 ± 0.84 % of this activity after 10 min, maintaining this for the next 50 min. At 70 °C, there was a complete loss of activity in both soluble and immobilised forms after 10 min. These results show that the enzyme is thermally more stable when immobilised to the floc matrix of the sludge in its native state than when it is soluble. This observation is in accordance with the fact that immobilised enzymes are well protected and stabilised by the matrices that provide external support for the enzyme (Trevan *et al*, 1987). Release of the enzyme into solution is an alteration of its native state, which exposed it to the extraneous environment and confers stress that could consequently lead to a change in conformation and denaturation. The result obtained is similar to previous observation by Ding *et al*. (2002), with investigations on endoglucanase from recombinant *Volvariella volvacea* where the native form of the enzyme was more thermally stable than the recombinant form. These enzymes were also shown to be stable at 30 °C but lost all activity at 70 °C after 10 min (ibid).



(a)



(b)

Figure 4.3: Thermal stability profile of the endoglucanase: (a) Soluble enzymes extract (b) Immobilised enzyme extract. Relative activity expressed as percentage of the maximum enzyme activity, 100 % activity = 0.29 µmol/min/ml.

4.3.4 Metal ions: Results obtained with metal ions showed that endoglucanase was inhibited by all the heavy metals studied except Fe^{2+}, which appeared to activate the enzyme at concentrations, 200-800 mg/l (Figure 4.4). Iron has been identified as an essential cofactor for the *Clostridium thermocellum* complex (Coughlan and Ljungdahl,

1988). Heavy metals can inhibit enzyme activities in different ways; by masking the catalytically active groups, denaturing protein conformation or complexing with metal ions involved in the enzyme-substrate complexes (Giafreda and Bollac, 1996). These findings further support the earlier observations about the effects of heavy metals on endoglucanase with Cu^{2+}, Zn^{2+} and Ni^{2+} reducing the endoglucanase activity by 3-100 % total enzyme activity (Ding *et al*., 2002; Akiba *et al*., 1995; Murashima *et al*., 2002; Lee and Kim, 1999); iron causing enzyme activation (Sanwal, 1999). Ca^{2+} and Mg^{2+} were stimulatory to the enzyme, as $CaCl_2$ and $MgCl_2$ have been reported to be required by cellulases and Ca^{2+} has been shown to enhance the substrate binding affinity of the enzyme and also stabilise the conformation of the catalytic site (Mansfield *et al*., 1998; Chauvaux *et al*., 1995).

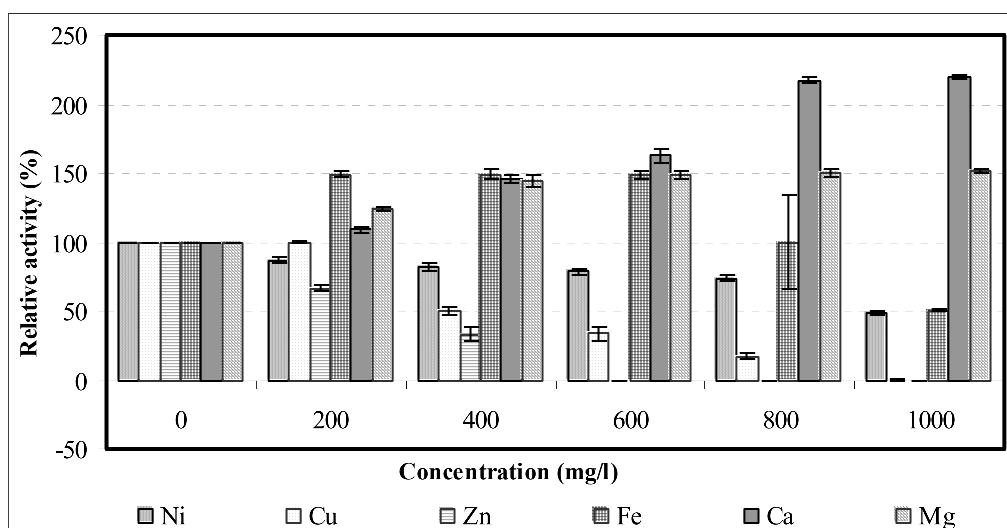


Figure 4.4: Effect of metal ions on endoglucanase activity. Relative activity expressed as percentage of the enzyme activity in the control without the effectors, 100 % activity = 0.29 μmol/min/ml.

<u>4.3.5 Volatile fatty acids</u>: All the VFA's studied proved inhibitory to the enzyme, with acetic acid showing the most profound effect (Figure 4.5). Although it has been reported that fermentation products inhibit enzyme-mediated hydrolysis of cellulose (Lynd *et al*., 2002), how these VFA's inhibit endoglucanases is unknown. The reaction mixture was buffered; the inhibitory effect should therefore not be as a result of drop in pH with the addition of the VFA's.

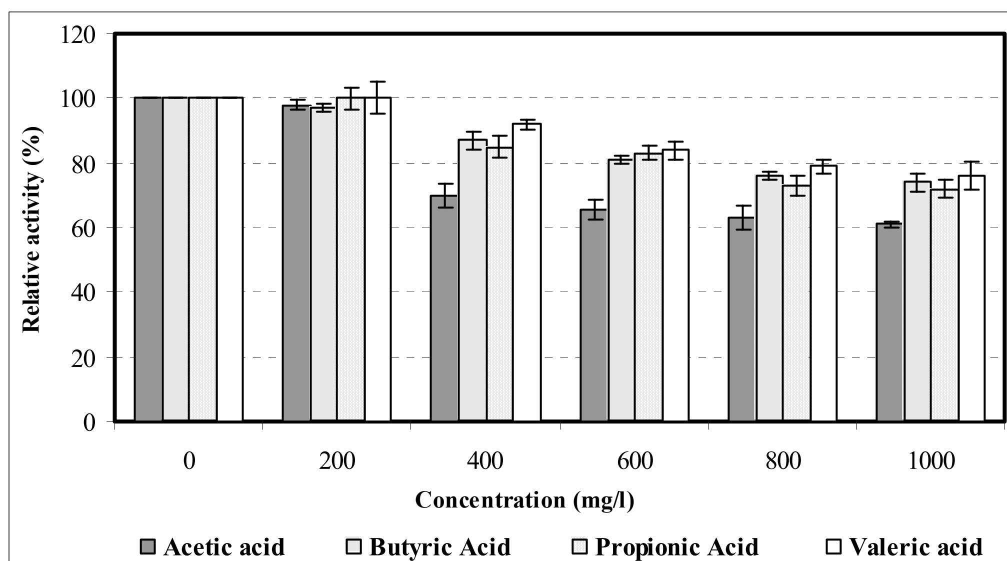
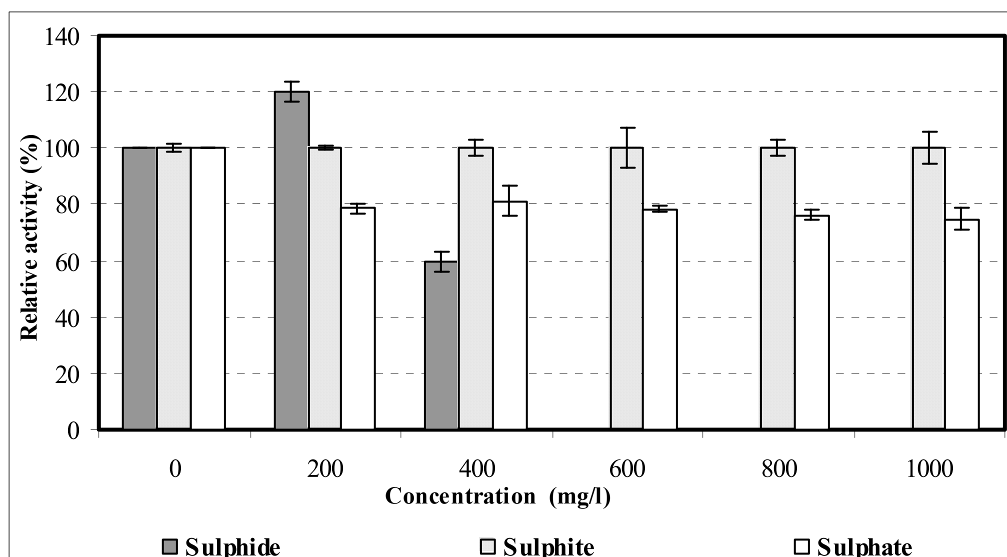
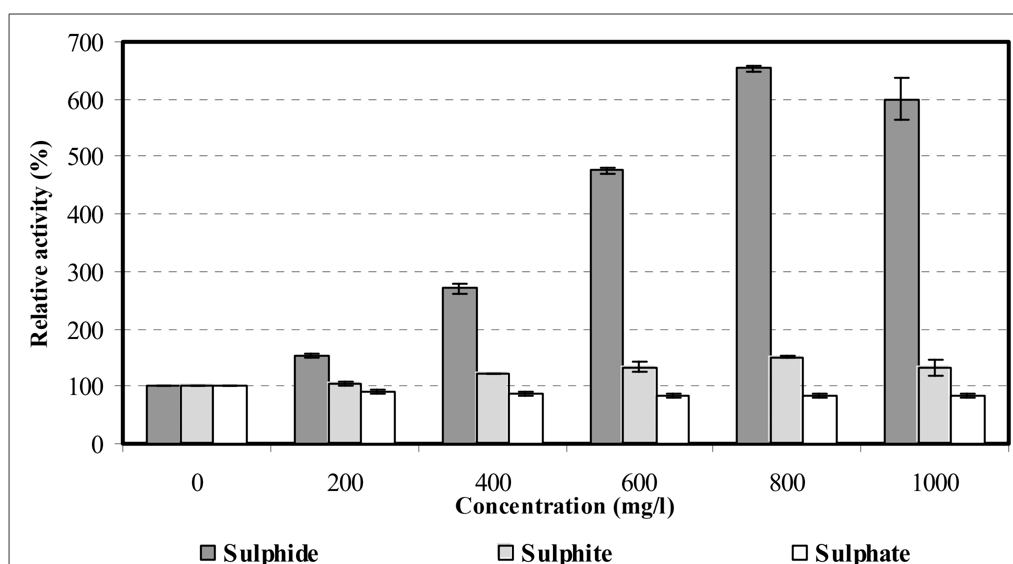


Figure 4.5: Effect of volatile fatty acids on endoglucanase activity. Relative activity expressed as percentage of the enzyme activity in the control without the effectors, 100 % activity = 0.29 µmol/min/ml.

4.3.6 Sulphur-containing compounds: Sulphate at concentrations from 200-1000 mg/l showed slight inhibitory effects on endoglucanase in the immobilised and soluble forms, indicating a reduction in enzymatic activity by 16-22.5 % (Figure 4.6). Sulphite the intermediate compound of sulphate reduction however, had no effect on the soluble form of the enzyme but was slightly stimulatory to the immobilised enzyme, increasing the enzymatic activity by ~1-1.5 fold at 0-1000 mg/l concentrations (Figure 4.6). Sulphide stimulated the enzyme in its immobilised state by increasing its activity by 1.5-6.5 fold as its concentration increased from 200-1000 mg/l. The stimulatory effect of sulphide on the enzyme was not the case when released from the floc matrix, the activity showed 20 ± 3.53 % increase at sulphide concentration 200 mg/l, beyond which it was toxic as it totally decreased the enzyme activity to zero at 600 mg/l. These findings are similar to previous observations made of stimulation of other enzyme activities (proteases, lipases and β-glucosidases) under the biosulphidogenic conditions of PSS hydrolysis within the range of 1.5-12.5 fold (Whittington-Jones, 2000; Whiteley *et al.*, 2002a; 2002b; 2003; Pletschke *et al.*, 2002).



(a)



(b)

Figure 4.6: Effect of sulphur-containing compounds on endoglucanase activity: (a) Soluble enzyme extract (b) Immobilised enzyme extract. Relative activity expressed as percentage of the enzyme activity in the control without the effectors, 100 % activity = 0.29 μmol/min/ml.

<u>4.3.7 Protease inhibitors</u>: As shown in Figure 4.7, there was an increased activity of the enzyme by ~19 % with PMSF (0.5 mM) and ~10-29 % EDTA (0.5-1.5 mM), which implied the inhibition of proteases, thus minimising exposure of the endoglucanases to proteolysis. Beyond these concentrations there was decrease in endoglucanases activities.

The reason for the decrease in endoglucanases activities in the presence of PMSF is not fully understood, but we cannot rule out the possibility of modification of an important serine residue in the active site of the endoglucanases by PMSF (Kambourova *et al.*, 2003). As for EDTA it was reported to reduce the enzyme activity to zero at 10mM (Ding *et al*. 2002). The reduction in endoglucanases activity by EDTA could probably be a result of its metal chelating ability as previous study (section 4.3.4) showed that endoglucanases might require metal ions as cofactors.

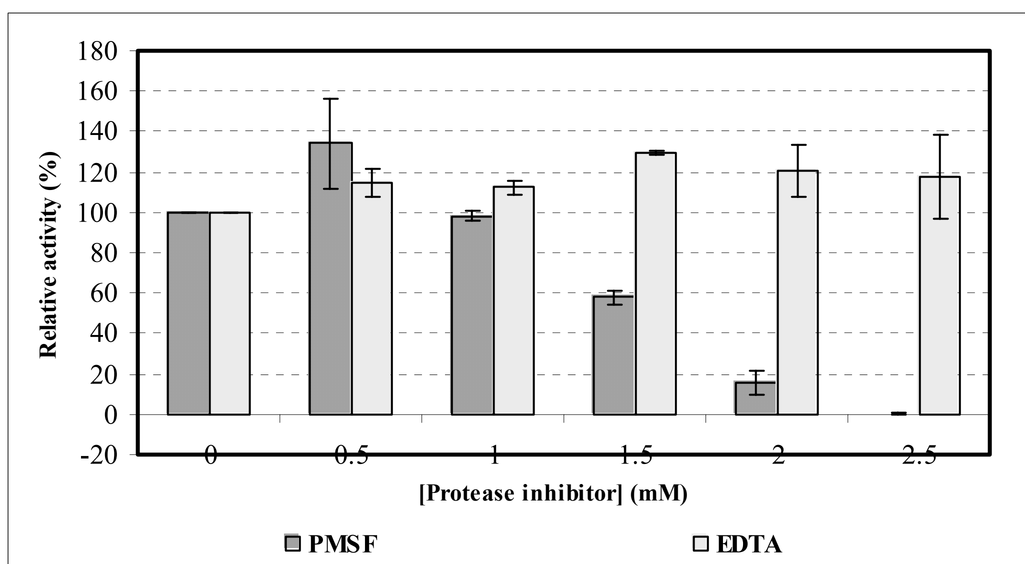


Figure 4.7: Effect of protease inhibitors on endoglucanase activity. 100 % activity = 0.29 µmol/min/ml.

<u>4.3.8 Catalytic properties and substrate specificity of endoglucanase</u>: The activity of endoglucanase with CMC as the substrate was significantly higher as compared to that with HEC. The reason for this is not clear, but the inability of the enzyme extract to hydrolyse avicel is an indication of lack of exocellulase activity. The kinetic parameters $K_{m,app}$ and $V_{max,app}$ of the enzyme were determined by Lineweaver-Burk double reciprocal plot of enzyme activity against substrate concentration. The substrate saturation was hyperbolic in nature (Figure 4.8). Graphs were drawn using the *Enzyme Kinetic Module 1.1* program (SPSS Inc.). For CMC and HEC at pH 6, the enzyme had $K_{m,app}$ values of 4 and 5.1 mg/ml respectively and $V_{max,app}$ values of 0.297 and 0.185 µmol /min/ml (Table 4.1). Lower $K_{m,app}$ when CMC was used as the substrate indicates the endoglucanase's

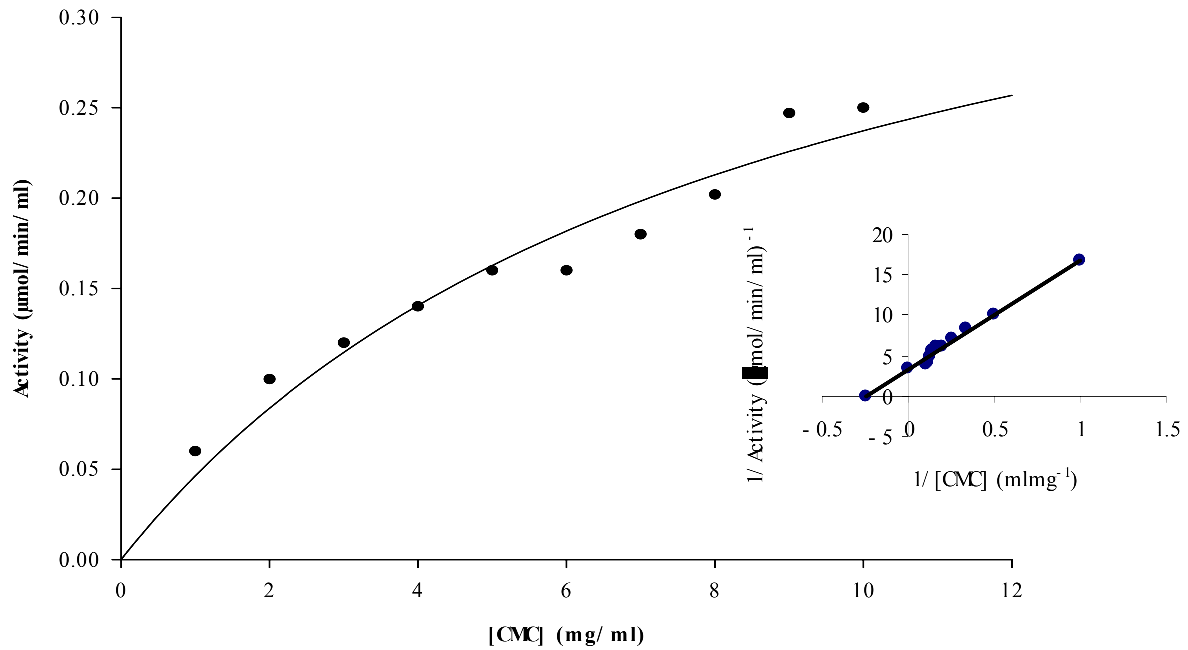
higher affinity for this substrate as compared to HEC. Similar results indicating higher affinity of endoglucanase for CMC and $K_{m,app}$ values with CMC as substrate have been previously obtained (Mawadza *et al*., 2000; Mansfield *et al*., 1998). The $K_{m,app}$ value when CMC was used as substrate is in the order of magnitude of K_m values in previous reports; 3.5 mg/ml (Maclachan, 1988); 7.6 and 6.3 mg/ml (Mansfield, *et al*., 1998).

4.4 SUMMARY

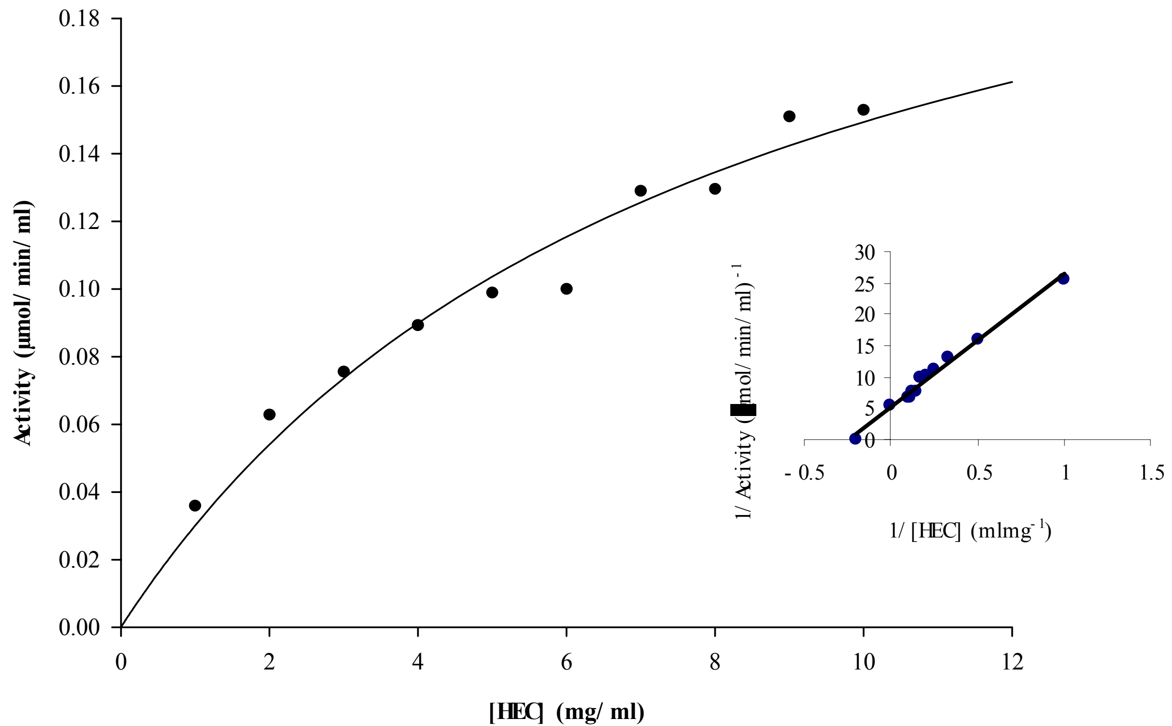
The high temperature optimum compared with the low thermal stability of the enzyme might infer that the substrate confers a stabilising effect on the enzyme as the enzyme was incubated with substrate during temperature optimum investigation (Mawadza, *et al*., 2000). The findings showing the more thermal stability of the endoglucanase when immobilised to the floc matrix suggests that immobilisation might be required before the enzyme is utilised.

With the different effects of sulphide on the enzyme in its soluble and immobilised states, it is possible that the sulphide disrupted the floc matrix, thus exposing more enzymes to the substrate (Whittington-Jones, 2000), but after the enzyme had been completely released, continuous direct exposure to the sulphide was toxic and inhibitory to the enzyme. This might be the reason why soluble protein concentration increased in the aqueous medium with increasing sulphide production while no enzyme activity was detected during the time course study (Figure 2.4). The stimulatory effects of calcium and magnesium ions on endoglucanase activity might suggest that they are cofactors for endoglucanases from biosulphidogenic sludge.

The presence of some the substances (sulphur-containing compounds, VFA's and heavy metals) studied in the biosulphidogenic sludge may present limitations to the availability of endoglucanases. These substances must therefore be removed before enzyme extraction and purification. To minimise proteolysis before and during enzyme purification, all procedures should be carried out as quickly as possible at low temperatures (0-5 °C).



(a)



(b)

Figure 4.8: Dependence of endoglucanase activity on different substrate types. (a) Michaelis-Menten curve. Double reciprocal (Lineweaver-Burk) plot with CMC as substrate (inset) (b) Michaelis-Menten curve. Double reciprocal (Lineweaver-Burk) plot with HEC as substrate (inset).

Table 4.1: substrate specificity and kinetic parameters of endoglucanase

SUBSTRATE	RELATIVE ACTIVITY	$K_{m,app}$ (mg/ml)	$V_{max,app}$ (μmol/min/ml)
CMC	100	4	0.297
HEC	61	5.1	0.185
Avicel	0	-	-

CHAPTER 5

PURIFICATION OF ENDOGLUCANASE FROM BIOSULPHIDOGENIC ANAEROBIC SLUDGE

5.1 INTRODUCTION

Enzyme purification involves a series of fractionating procedures to remove contaminants from the target protein. The types of contaminating molecules or substances present in a crude extract depend on the source of the target protein. In this case endoglucanase was isolated and purified from the biosulphidogenic sludge, which is a complex mixture of several substances (e.g. carbohydrates, proteins, sulphide, sulphate, VFA's etc.), both organic and inorganic. Purifying endoglucanase from this source can therefore be tasking and might require several steps so as to eliminate many of the unwanted substances. Aside from removing contaminants, purification also optimises the yield and specific activity of the target protein. Enzyme loses activity with increased exposure to denaturants and other factors such as increased temperature and proteases. Though it is unavoidable to totally prevent loss of activity as purification progresses, purification steps should be few, rapid and performed at low temperatures (0-4 °C) (Roe, 2001b). The different methods exploited in this section and their underlying principles are briefly discussed in the following subsections.

5.1.1 Extract Concentration

Prior to protein separation using chromatographic techniques, the crude extract obtained requires concentration so as to increase the protein content and decrease the volume. Three extract concentration methods were employed in this study and they are briefly described below.

5.1.1.1 Ultrafiltration: This involves the removal of water or solutes through a membrane impervious to proteins. The pore size of the membrane is defined by the nominal molecular weight cut-off (NMWC), which is the minimum molecular weight globular molecule that cannot traverse the membrane. When the dialysis tubing containing the extract is surrounded by polyethylene glycol (PEG), low osmotic pressure

is maintained outside the tubing and high inside, thus driving the water and other solutes by osmosis into the PEG. Use of polyethylene glycol (PEG) for concentration is not absolutely an ultrafiltration method. Unlike other methods requiring centrifugation or high pressure to drive the water and other solutes out, this method removes water and other solutes by osmotic forces. The merits of this method include: minimum time of processing and the ability to remove salts which could become concentrated by other methods leading to denaturation of the target enzyme (Scopes, 1982; Harris, 2001).

5.1.1.2 Freeze-drying: Freeze drying, otherwise called lyophilisation involves the removal of water and other solvents from a frozen material by sublimation under a high vacuum. Sublimation is a process by which solid substance moves to the gaseous state without passing through the liquid state (Labconco Corporation, 1998). Using freeze-drying, dry powdered extract can be obtained and enzyme in a dry powder form is more stable than an aqueous preparation since the former lacks water required as medium for many biological activities leading to protein denaturation. Though this method is used in the large-scale production of many commercial enzymes, it has disadvantages which include: longer processing time, hence increased exposure period to denaturing elements and that salts are concentrated along with the proteins (Harris, 2001).

5.1.1.3 Precipitation by salting out: Solubility of a protein in an aqueous environment depends on the arrangement of the hydrophobic and hydrophilic groups on its surface (Harris, 2001). Charged and polar residues on protein surface are solvated by water molecule resulting in the solubility of a protein molecule (Walker, 2000a). Proteins can be precipitated out of solution by adding a neutral salt. The most commonly used, ammonium sulphate, is cheap, sufficiently soluble and has been indicated to stabilise proteins. This method relies on the hydrophobicity of the protein. When salt is increasingly added to an aqueous solution of protein, the salt is solvated by water consequently removing water from the protein surface. As water is removed, hydrophobic patches on different proteins are revealed which can interact with one another causing aggregation and precipitation. The rate of precipitation of proteins therefore increases according to the number hydrophobic patches (Harris, 2001).

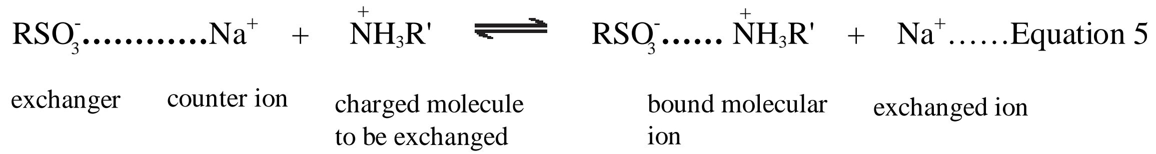
5.1.2 Chromatography

Chromatography is a common method used for the preparation of highly pure and active proteins (Willson, 1999). It usually involves two phases; the stationary phase, which may be a solid, liquid, or a solid/liquid mixture and is immobilised, and a liquid or gas mobile phase that flows over or through the stationary phase. Column chromatography is the most widely used form of chromatography, with the stationary phase attached to a suitable matrix and packed into a glass column, while the mobile phase is passed through the column under the influence of either gravitational force or using a pumping system (Wilson, 2000b). This technique generally involves selective adsorption of proteins on the surface of porous particles through different interactions (Willson, 1999) which confer retarding forces on the solutes so that each component in a mixture will separate into different bands as they migrate at different rates (Voet and Voet, 1995).

5.1.2.1 Ion exchange chromatography: This common method of separation is based on the electrostatic interaction between two oppositely charged particles. Since most proteins contain positively or negatively charged amino acid residues on their surface they can be separated by ion exchange chromatography, using reversible adsorption of charged molecules to oppositely charged immobilised ion exchange groups. The charges on amino acid residues change with pH, thus providing means of adjusting the extent of adsorption of the solute. This method can be further classified either into cation exchange (Equation 5), where the adsorbent is negatively charged and attract positively charged ions or anion exchange (Equation 6) where the adsorbent is positively charged, attracting negatively charged ions (Hagel, 2001).

Ion exchange takes place through four stages: (1) application of the sample to the adsorbent with the starting buffer, (2) the rate-limiting step including the movement of the sample through the matrix structure of the exchanger, (3) an immediate exchange of ions at the exchange site (Equations 5 and 6).

Cation exchanger



Anion exchanger

(4) the ion is desorbed from the adsorbent surface by changing the pH or ionic concentration of the eluent (Wilson, 2000b).

5.1.2.2 *Size exclusion chromatography*: This method, otherwise called gel filtration chromatography or molecular sieve chromatography, exploits the diversity of the molecular sizes of proteins. The stationary phase consists fine porous beads (with pore sizes approximately the same as that of macromolecules) packed into a column. When an aqueous solution containing molecules of different sizes like proteins and salts is applied onto the packed column, the smaller molecules enter the pores while the larger ones are excluded (Figure 5.1). Molecules emerge in the eluent according to decreasing size. This method is mild as it does not require harsh elution conditions such as varying pH or concentration of elution buffer and unlike many other chromatographic methods it is not an adsorption method (Cutler, 2001).

As shown in equation 7, V_t represents the total bed volume of the column and equals the sum of the volume outside the beads or void volume, V_o and V_i, which is the volume occupied by the beads.

$$V_t = V_i + V_o \quad \text{......Equation 7}$$

The volume required to elute a given solute with molecular size within the fractionating range of the beads is defined as the elution volume, V_e. This volume lies between V_t and V_o. Any particular solute that enters the pores of the beads has a distribution defined by the *distribution coefficient*, K_D (Garrett and Grisham, 1999b).

$$K_D = (V_e - V_o)/V_i \quad \text{Equation 8}$$

K_D (Equation 8) is a function of the molecular size of solutes. A solute with $K_D = 0$ is large and excluded from the mobile phase while for a small molecule that can have a complete access to the inner mobile phase $K_D = 1$. Other applications of this technique include relative mass determination, solution concentration and protein-binding studies (Wilson, 2000b).

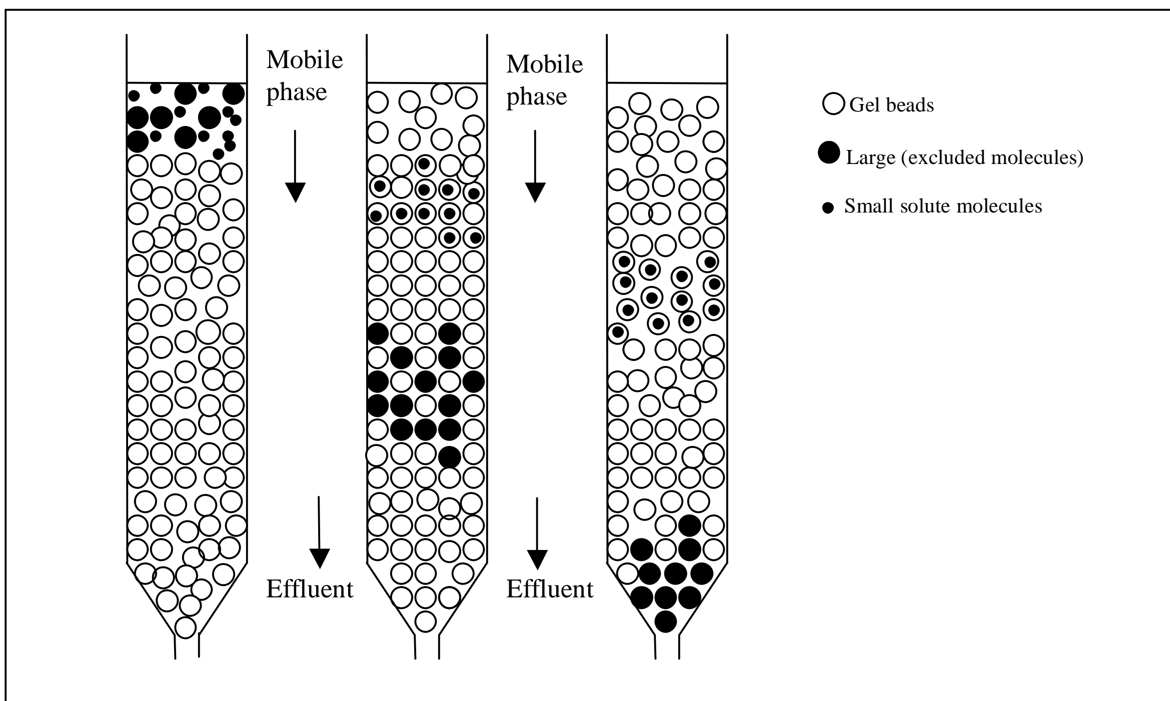


Figure 5.1: Separation of different sized molecules by size exclusion chromatography. Adapted from Wilson , 2000b.

5.1.2.3 Affinity chromatography: The principle of this separation method is based on the specificity of biological function of the target protein by binding with another biomolecule to form a complex, for example, enzymes with their specific substrates. In light of this, the biomolecule (ligand) specific for the target protein can be immobilised to an insoluble matrix like cellulose or polyacrylamide. When the mixture of proteins is passed over the immobilised ligand, the protein of interest will be bound while the contaminating constituents will be excluded. The bound protein can be subsequently

removed or dissociated by the addition of free ligand in solution (Figure 5.2) (Garrett and Grisham, 1999b).

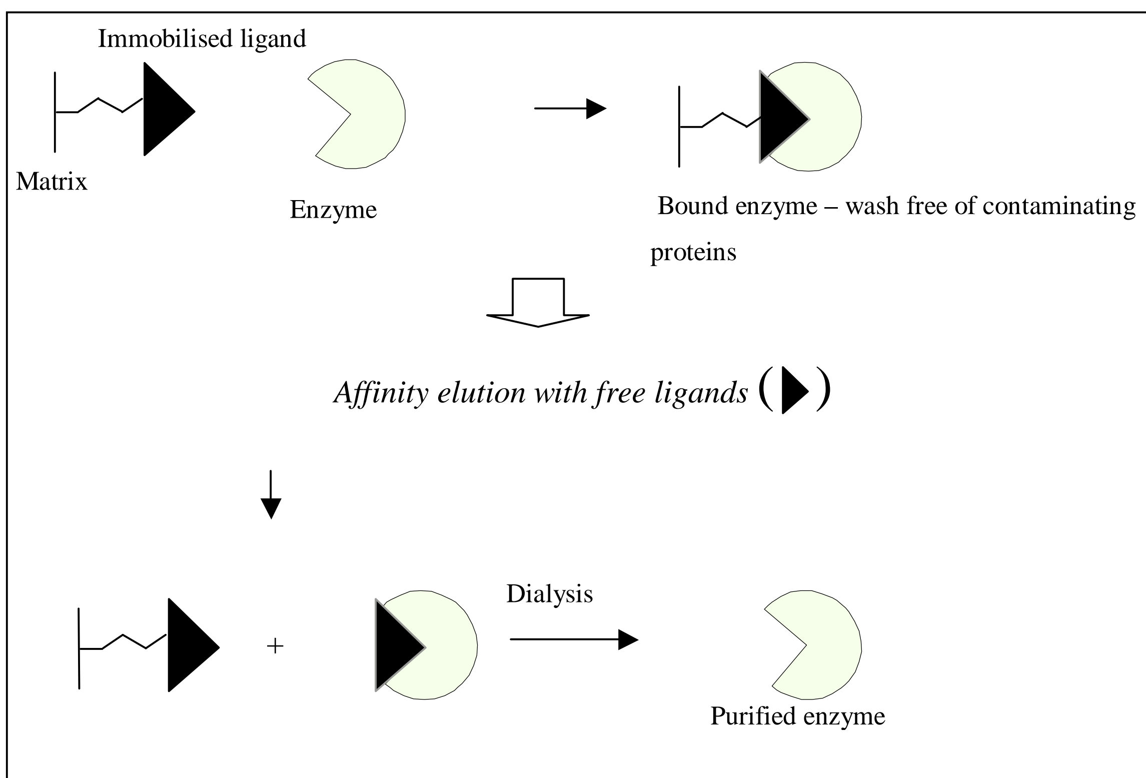


Figure 5.2: Purification of an enzyme by affinity chromatography. Adapted from Wilson, 2000b.

5.1.2.4 Adsorption chromatography: Adsorption chromatography is based on the ability of some materials called adsorbents to hold molecules to their surface using weak, non-ionic forces such as van der Waals and hydrogen-bonding. The strength of a solute or analyte to the adsorbent is a function of the functional groups present in its structure. As eluent is passed through the column, the analyte can be desorbed based on the relative strengths of the interactions of the analyte and eluent with the adsorbent (Wilson, 2000b). Previous studies showed that calcium might be required by endoglucanases for stability (section 4.3.4). Crystalline hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) being a calcium compound was therefore used as an adsorbent in this technique. Its mode of adsorption though not well understood is thought to involve dipole-dipole interactions and electrostatic attractions. Adsorption capacity of this adsorbent is highest in the region of neutral pH using 0.02 M phosphate buffer (Wilson, 2000b).

5.1.3 Electrophoresis

Electrophoresis is the movement of charged ions in an electric field. The electric force, *F*, on an ion with a charge, *q*, when voltage, *E*, is applied as the ion moves within the distance, *d*, between the electrodes is given by the expression;

$$F = Eq/d \quad \text{Equation 9}$$

In practice, the porous support matrix in which the method is carried out retards the movement of molecules according to their dimensions relative to the size of the pores in the matrix. The matrices in common use are agarose and polyacrylamide (Garrett and Grisham, 1999b).

5.1.3.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE): This is probably the most widely used method for determining the molecular weights of proteins as well as the assessment of purity. Sodium dodecyl sulphate (SDS) is a detergent having a 12-carbon hydrophobic tail which unfolds polypeptide chains, thereby disrupting its tertiary structure. The amount of SDS bound by a polypeptide directly depends on the number of amino-acid residues present in the polypeptide chain. SDS contributes negative charges which mask the inherent charges present in the protein, thus conferring identical charge to mass ratios and similar shapes on the SDS-bound polypeptides. These bound polypeptides thus migrate in the electrical field. This migration is independent of amino-acid composition since the SDS charges outweigh those of the amino-acid residues and also inversely varies with the logarithm of the protein's molecular weight (*Appendix 5*). Beta (β)-mercaptoethanol may be added to the SDS-PAGE so as to break disulphide links and release the protein subunits (Garrett and Grisham, 1999b).

5.2 MATERIALS AND METHODS

5.2.1 Materials

Dialysis tubing (cellulose membrane, 12 kDa MWC), prestained SDS-PAGE standard markers (6.5-200 kDa), brilliant blue R-staining solution (B-6529), silver stain kit (AG-

25), Coomasie dye protein reagent kit for protein determination, acrylamide, N, N′-methylene-bis-acrylamide were purchased from Sigma (South Africa (Pty) Ltd), while DEAE-Cellulose, β-mercaptoethanol (Purum grade) and polyethylene glycol 20 000 (PEG) were from Merck (South Africa (Pty) Ltd). Hydroxylapatite (Boehringer Mannheim GmbH, Germany), sodium dodecyl sulphate (BDH, England). Sephadex G-100 was obtained from Pharmacia Fine Chemicals and fraction collector purchased from Pharmacia Biotech. Other analytical-grade reagents were purchased from Sigma (South Africa, (Pty) Ltd), or Merck (South Africa, (Pty) Ltd).

5.2.2 Methods

5.2.2.1 Sludge sample source: The sludge samples were collected from a closed biosulphidogenic digester supplemented with α-cellulose on day 15 based on the result obtained in Chapter 2 (section 2.3.6) i.e. maximum induction and yield of enzyme.

5.2.2.2 Endoglucanase activity and protein determination: Endoglucanase activity was estimated as described in Chapter 2 (section 2.2.2.3.1). Protein concentration was measured using Bradford method as described in Chapter 2 (section 2.2.2.3.2).

5.2.2.3 Endoglucanase purification: Several methods were attempted so as to optimise endoglucanase purification. All procedures were performed in the cold (4 °C). Procedures for column regeneration and equilibration for ion exchange chromatography are outlined in *Appendix 6*.

5.2.2.3.1 Preparation of cell free extract: The cell free extract was prepared as described in section 4.2.2.2.

5.2.2.3.2 Ammonium sulphate precipitation: Solid ammonium sulphate (78 g) (as calculated using the equation shown in *Appendix 7*) was slowly added to the cell free extract (200 ml) over a period of 30 min to give final saturation of 60 %. The mixture was stirred (60 min) then centrifuged (10 000 ×g, 30 min, 4 °C), the supernatant removed

and the precipitated proteins resuspended in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v) followed by a final dialysis against the same buffer. The dialysate was analysed for protein concentration and endoglucanase activity.

5.2.2.3.3 Freeze-drying: The cell free extract was paced in a freeze-drying flask and frozen by slowly swirling the flask in a bath of liquid nitrogen. The freeze-drying flask was then attached to the vacuum pump of the freeze-dryer (Edwards). The extract was completely dried (-40 °C, 6 h). The recovered powder was weighed and dissolved in sodium phosphate buffer (0.05 M, pH 6) (1:2 w/v), followed by centrifugation (10 000 ×g, 10 min, 4 °C) so as to remove debris. Supernatant obtained was analysed for protein concentration and endoglucanase activity.

5.2.2.3.4 Extract concentration with PEG: Cell free extract (200 ml) was placed in a dialysis bag placed in a beaker containing PEG 20 000 powder enough to surround the bag (4 °C). The volume in the bag was checked every 30 min for 3 h until the desired volume was attained (34.8 ml). The concentrated extract was removed from the dialysis bag, centrifuged (10 000 ×g, 10 min, 4 °C) so as to remove debris and the supernatant analysed for protein concentration and endoglucanase activity.

5.2.2.3.5 DEAE-Cellulose ion exchange chromatography: Concentrated cell free extract (6 ml) was loaded onto a DEAE-Cellulose ion exchanger column (1 × 25 cm) equilibrated with sodium phosphate buffer (0.05 M, pH 6). The column was washed with the same buffer until the absorbance at 280 nm (A_{280nm}) of the eluate reached a base line. This was followed by a stepwise elution using 0-1 M NaCl in sodium phosphate buffer (0.05 M, pH 6); at a flow rate of 1ml/min. Fractions (5 ml) were collected and monitored for protein (A_{280nm}) and endoglucanase activity. The enzyme-containing fractions from different peaks were pooled and analysed for protein concentration and endoglucanase activity, followed by concentration using PEG 20 000. The concentrated samples were then subjected to SDS-PAGE to assess purity of the enzyme fractions.

5.2.2.3.6 Gel filtration with Sephadex G-100: Concentrated cell free extract (10 ml) was applied onto a Sephadex G-100 column (1.1 × 26.3 cm) equilibrated with sodium phosphate buffer (0.05 M, pH 6). The column was washed with the same buffer until the absorbance at 280 nm (A_{280nm}) of the eluate reached a base line. The samples were eluted with the same buffer and 5ml fractions monitored for protein (A_{280nm}) and endoglucanase activity were collected at a flow rate of 1 ml/min. The enzyme-containing fractions from different peaks were pooled and analysed for protein concentration and endoglucanase activity, followed by concentration using PEG 20 000.

5.2.2.3.7 Adsorption chromatography using hydroxylapatite: Concentrated cell-free extract (5 ml) in potassium phosphate buffer (0.02 M, pH 6.8) was applied onto a column (1 × 25 cm) of hydroxylapatite equilibrated with potassium phosphate buffer (0.02 M, pH 6.8). Potassium phosphate buffer is favoured for this procedure as compared to sodium phosphate buffer as a result of its better solubility (Roe, 1994). The column was washed with the same buffer until the absorbance at 280 nm (A_{280nm}) of the eluate reached a base line. The samples were eluted with the same buffer at increasing stepwise concentrations of NaCl (0.02-0.3M), fractions (5ml) collected at a flow rate of 1ml/min and monitored for protein (A_{280nm}) and endoglucanase activity. The enzyme-containing fractions from different peaks were pooled and analysed for protein concentration and endoglucanase activity, followed by concentration using PEG 20 000.

5.2.2.3.8 Affinity chromatography using phosphoric acid-swollen cellulose: This method exploits the ability of cellulases to bind to their substrate cellulose and has been modified into a new method called "affinity digestion". The enzymes bind to the substrate leading to subsequent digestion (Morag *et al.*, 1992). Concentrated cell-free extract (20 ml) was added to amorphous (phosphoric acid-swollen) (2.5 mg) cellulose followed by an incubation period (2 h, 4 °C). The adsorbent was removed by centrifugation (10 000 ×g, 10 min, 4 °C). The pellet recovered was resuspended in stabilising buffer (12 mM $CaCl_2$, 2mM EDTA, 50 mM tris buffer, pH 7.5) (720 µl), introduced into a dialysis bag and dialysed against the stabilising buffer (3 h, 4°C) so as to remove the accumulating

digestion products. Preparation of phosphoric acid-swollen cellulose is outlined in *Appendix 8*.

5.2.2.4 SDS-PAGE: In order to determine the molecular weight of endoglucanase after the partial purification procedures, the SDS-PAGE method by Laemmli (1970) was used. Samples (10 µl) and molecular weight markers (10 µl) of known weights (6.5-200 kDa) were applied on a 12 % SDS-PAGE (*Appendix 9*) at 250 V. The gels were stained using Coomasie brilliant blue R-250 staining solution (*Appendix 9*) followed by destaining in methanol-acetic acid-water (1:1:8 v/v/v) destaining solution (*Appendix 9*). The distance moved by the purified protein was measured and its corresponding molecular size calculated from the calibration curve of log molecular weight versus distance migrated (*Appendix 5*).

5.3 RESULTS AND DISCUSSION

5.3.1 Extract concentration

In contrast to previous report that ammonium sulphate stabilises most enzymes (Scopes, 1982), it was observed to totally deactivate endoglucanases in this study (Table 5.1), the reason for this is not well understood. The low recovery observed during freeze-drying could either be due to the long period of procedure leading to increased exposure to denaturants or increase in salt concentration alongside protein concentration which might lead to denaturation as well (Harris, 2001). Although the pellet was washed several times to remove all soluble salts before enzyme extraction, the possibility that water-insoluble salts were left behind after washing cannot be ruled out. However, since extract concentration using PEG showed the highest recovery (Table 5.1) it was therefore adopted as the extract concentration procedure used in the purification of endoglucanases in this study. PEG concentration also was more rapid, thus exposing the enzymes to denaturants for a shorter time. The use of PEG also allows small solutes (which would be retained by the other methods) to be removed through the pores of the dialysis tubing.

Table 5.1: Comparison of extract concentration methods

Method	Volume (ml)	Total protein (mg)	Total activity (U)	Recovery (%)
Sonication	200	242	120	100
PEG	34.8	54.3	44.9	37.4
Freeze-drying	9.5	49.5	20.71	17.3
Ammonium sulphate	2	13.9	0	0

5.3.2 Chromatography

Even though three different column chromatographic techniques were used in the purification of endoglucanases in this study, poor recovery and separation were still observed even after optimisation. The most successful of these techniques in terms of separation and recovery is presented and discussed here. Endoglucanases purification with PEG and DEAE-Cellulose ion exchange is summarised in Table 5.2. The crude cell free extract (200 ml) obtained by sonication, containing 242 mg total protein and 120 U total endoglucanase activity was concentrated by PEG 20 000. This resulted in increased specific activity from 0.5 to 0.83 U/mg protein with 37.5 % recovery of activity. There was also a resultant marked reduction of total protein concentration from 242 to 54.3 mg and total activity from 120 to 44.9 U during this purification step. The elimination of a major percentage (77.6 %) of the total starting protein by PEG concentration could partly be due to the removal of contaminating proteins and degradation by proteases as the method progressed. There was no considerable purification at this stage as indicated by the fold purification which slightly increased from 1 to 1.66 fold. The concentrated extract was applied onto DEAE-Cellulose ion exchange column chromatography for further purification. As shown in the chromatogram (Figure 5.3) fifteen protein peaks (measured at A_{280}) emerged, showing that the extract is a complex mixture of proteins, they could as well be proteolysis products. Of all these peaks, endoglucanases activities were only detected in five peaks (1-5) which eluted at different NaCl concentrations. The samples representing the peaks were pooled and exhibited specific activities of 1.79, 6.5, 12.72, 0.3 and 0.53 U/mg protein with increase in purification fold to 3.58, 13, 25.4, 0.6 and 1.06 respectively. Aliquots of the pooled fractions containing endoglucanases activities from DEAE-Cellulose column were kept while the rest were concentrated by

PEG for further purification. Concentration by PEG resulted in a complete loss of enzyme activity (this purification process therefore remained a partial purification process as the loss of activity prevented further purification). To verify the purity of these pooled fractions (before concentration), samples were subjected to SDS-PAGE.

Table 5.2: Purification table of endoglucanase

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification factor (fold)
Sonication	200	120	242	0.5	100	1
PEG	34.8	44.9	54.3	0.83	37.5	1.66
DEAE-Cellulose						
Peak 1	30	2.1	1.17	1.79	1.75	3.58
Peak 2	20	2.86	0.44	6.5	2.38	13
Peak 3	30	1.59	0.125	12.72	1.33	25.4
Peak 4	30	6	19.7	0.3	5	0.6
Peak 5	10	0.9	1.7	0.53	0.75	1.06

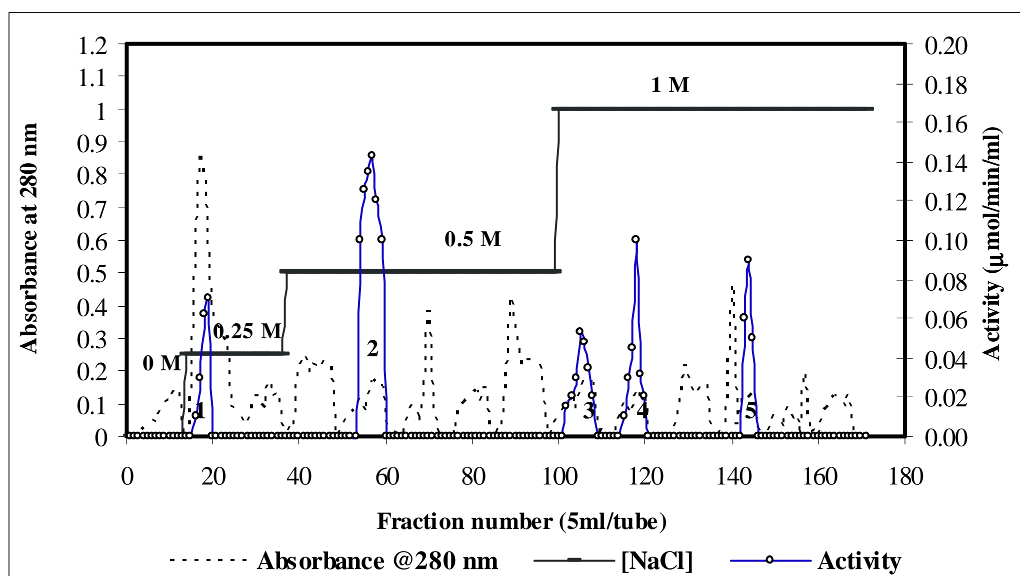


Figure 5.3: DEAE-Cellulose ion exchange chromatography. Column dimensions: 1 × 25 cm. Flow rate: 1ml/min. Endoglucanases were eluted with 0-1 M NaCl in sodium phosphate buffer (0.05 M, pH 6).

5.3.3 SDS-PAGE

The molecular weight markers, commercial cellulase from *Aspergillus niger* (Merck South Africa, (Pty) Ltd), PEG concentrated extract and cell free extract obtained by sonication (sonic extract) were loaded in lanes 1, 2, 3 and 4 respectively, while lanes 5-9 were loaded with pooled fractions (peaks 1-5) obtained from the DEAE-Cellulose chromatography. No bands were seen (Figure 5.4a) with the samples from the ion exchange chromatography, while very faint bands were observed in the crude extract obtained by sonication. The molecular weight values of these subunits are; 66.2, 52.4, 37.9 and 30.3 kDa and fall within the range of values of the polypeptide molecular weights of the cellulosome from *Clostridium thermocellum* reported by Bayer and Lamed (1988). Since no distinct subunits bands were identified on the SDS-PAGE after staining with brilliant blue R staining solution as a result of the low protein concentration, another gel was loaded with all the samples (with the exception of molecular weight markers and the commercial cellulase) as with the first gel and stained using silver stain. Silver stain was used because of its sensitivity and ability to detect trace amounts of proteins (Walker, 2000b). Nonetheless, similar result was obtained with no subunits bands detected from the pooled fractions (Figure 5.4b). Silver staining was carried out as described in the kit. This suggests that the minute amounts of proteins with enzyme activities resulting from DEAE-Cellulose chromatography were completely degraded during PAGE. An activity gel (no SDS included in the procedure) was run as well but to no avail, no bands were observed.

5.4 SUMMARY

Endoglucanase purification from biosulphidogenic sludge proved exceptionally difficult. For every purification process, a raw starting material having a high quantity of target protein is required (Willson, 1999). In contrast to this, there was low endoglucanase activity (0.6 µmol/min/ml) in the crude extract even after induction of enzyme production in the biosulphidogenic bioreactor. Total protein and enzyme activity decrease with each step in enzyme purification, starting with low levels of endoglucanase activity therefore left trace amounts of the target protein after few steps. Other likely reason for the low

yield or recovery in this purification includes the complex nature of the source and proteolysis.

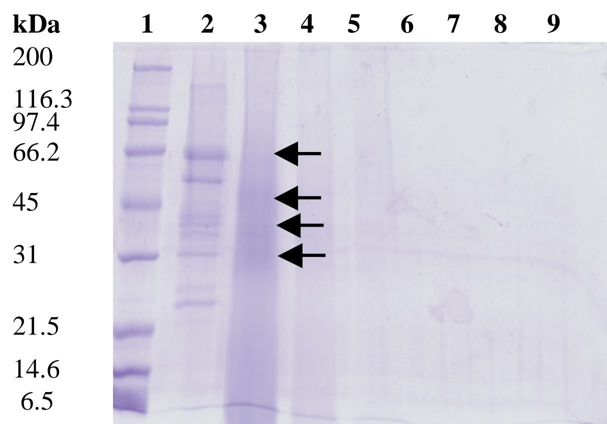


Figure 5.4: SDS-PAGE (12 % resolving gel). Coomasie blue staining. Lane 1: Prestained molecular weight markers (Myosin, 200 kDa; β-galactosidase, 116.3 kDa; Phosphorylase b, 97.4 kDa; Bovine serum albumin, 66.2 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 31 kDa; Soybean trypsin inhibitor, 21.5 kDa; Lysozyme, 14.6 kDa; and Aprotinin, 6.5 kDa). Lane 2: Commercial cellulase (control). Lane 3: PEG concentrated extract. Lane 4: sonic extract. Lanes 5–9: Peaks 1-5 from DEAE-Cellulose.

Trials to reduce proteolysis by including some protease inhibitors in the crude extract were unsuccessful as shown in Chapter 4 (section 4.3.7). Although all the purification procedures were performed at low temperatures there is still possibility of degradation of the target protein by proteases as they were not chemically inhibited. Notwithstanding all these obstacles, an appreciable level of purification was attained with a purification factor of 25.4 fold. Similar problems were encountered by previous investigators during the purification process of other enzymes (β-glucosidases and proteases) from the biosulphidogenic bioreactor (Ngesi, 2001; Tshivunge, 2001).

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSIONS

6.1 INTRODUCTION

Sulphate reduction coupled to primary sewage sludge hydrolysis has been an area of research intensely studied recently. Primary sewage sludge (PSS) has proved to be a readily available and cheap carbon source required for growth and metabolism of sulphate reducing bacteria (SRB). The hydrolysis of PSS has also been reported to be accelerated in the presence of SRB as a consequence of the activation of hydrolytic enzymes under biosulphidogenic conditions. Hydrolytic enzymes involved in PSS hydrolysis include proteases, lipases and cellulases which are required for the breakdown of the major polymers present in this complex mixture. Based on this, this study was aimed at exploring the possibility of using biosulphidogenic anaerobic digested sludge as a potentially inexpensive source of endoglucanases. Endoglucanases have several industrial applications, but these have been hampered by the high cost of enzymes and other factors. In order to fully understand endoglucanases in biosulphidogenic sludge and exploit these enzymes, endoglucanase production, induction, characterisation and purification were investigated in this study.

6.2 PRODUCTION OF ENDOGLUCANASES UNDER BIOSULPHIDOGENIC CONDITIONS

A greater and more rapid level of enzyme production was observed in the biosulphidogenic bioreactor as compared to the methanogenic bioreactor (conventional anaerobic digestion), confirming previous observations in literature (Whittington-Jones, 2000; Whiteley *et al*., 2002a; 2002b; 2003). Endoglucanase production was observed to decrease with the accumulation of reducing sugars and sulphide, thus suggesting product inhibition and possible enzyme or microbial deactivation with increased reducing sugars and sulphide content respectively. This result further supports the previous findings of stimulation of enzyme activity under biosulphidogenic conditions of PSS hydrolysis (ibid). The postulation of enhanced activities of enzymes under biosulphidogenic conditions is that, the sulphide produced during sulphate reduction is involved in the

defloculation of the sludge matrix, thus releasing previously entrapped enzymes and exposing them to increased levels of substrate (Whittington-Jones, 2000). There was a corresponding increase in soluble proteins and decrease in immobilised proteins with elevated sulphide production. The soluble proteins were found to lack enzyme activity, which might imply enzyme release from the immobilised state followed by deactivation by exposure to sulphide. The increased enzyme activity coincident with increase in immobilised proteins (in pellet particulate matter) at the early stage of enzyme synthesis was an indication of the location of endoglucanases in the biosulphidogenic sludge.

6.3 LOCATION AND PHYSICO-CHEMICAL CHARACTERISATION OF ENDOGLUCANASES

Endoglucanases were found to be associated with the pellet particulate matter reflecting the immobilisation of these enzymes to the floc matrix of the sludge. These enzymes were thermally more stable when immobilised to the floc matrix of the sludge than when released into the aqueous medium by sonication. Sonication proved to be the most successful extraction method as compared to treatment with detergent and organic solvent. The ultrasonic waves discharged during sonication led to the disruption of the floc matrix, thus releasing the entrapped enzymes into solution. Endoglucanases in the biosulphidogenic sludge have an optimum activity at pH 6 and temperature 50 °C. These characteristics are similar to the characteristics of the other enzymes (lipases, proteases and β-glucosidases) previously studied under the same conditions (Whiteley *et al.*, 2002a; 2002b; 2003). For carboxymethyl cellulose (CMC) and hydroxylethylcellulose (HEC) endoglucanases had $K_{m,app}$ values of 4 and 5.1 mg/ml respectively and $V_{max,app}$ values of 0.297 and 0.185 μmol/min/ml. Calcium, magnesium and iron are possible cofactors of endoglucanases. Calcium and magnesium exhibited stimulatory effects at concentrations 200-1000 mg/l, while iron activated the enzymes at concentrations 200-800 mg/l. On the other hand, other heavy metals studied (copper, iron and nickel) proved to be inhibitory. Similar observations were reported by Ngesi (2001) on the effects of these metals on β-glucosidases. All the volatile acids studied (acetic acid, butyric acid, propionic acid and valeric acid) inhibited the enzymes. The reason for this is not well understood, however Lynd *et al.* (2002) reported that these fermentative products are

inhibitory to cellulose degradation. Sulphate demonstrated slight inhibitory effects on the endoglucanases, while sulphite was stimulatory. Sulphide stimulated the activities of endoglucanases when immobilised to the floc matrix but proved inhibitory to the soluble enzymes above 200 mg/l. This further confirms the possible deactivation of these enzymes by sulphide after release from the floc matrix.

6.4 PURIFICATION OF ENDOGLUCANASE

Although endoglucanase purification was not as successful as anticipated, partial purification was achieved by PEG 20 000 concentration followed by DEAE-Cellulose ion exchange column chromatography. The highest purification factor resulting from one of the different peaks that were obtained from the ion exchange chromatography was 25.4 fold. The relatively stable immobilised enzyme (attached to the floc matrix) would have been a better choice as the starting material for the purification procedure, but due to its insolubility it could not be used.

Based on the results obtained in this study, biosulphidogenic anaerobic digested sludge can serve as a cheap source of endoglucanases. In application, it might be necessary to use these enzymes in their native state when immobilised to the pellet particulate or re-immobilise them onto an artificial matrix after release from the pellet. The major problem faced in this research was the low initial enzyme activity.

6.5 FUTURE WORK

(1) *Treatment of wastepaper*: Wastepaper is a major constituent of solid waste. The bioconversion of this cellulose-rich waste into fermentable sugars (glucose) will limit environmental pollution and encourage reuse and sustainability (van Wyk and Mohulatsi, 2003). Moreso, endoglucanases can be used in biodeinking of old newspapers before recycling. This gives better outcome as compared to the chemical method (Pèlach *et al.*, 2003). Besides wastepaper treatment, endoglucanases from biosulphidogenic sludge could also be used for the bioremediation of effluents from industries such as textiles and dyes, and paper and pulp that use cellulosic raw materials. Since enzyme cost hinders

bioprocesses such as these, biosulphidogenic sludge could be used as a readily available enzyme source for waste management and recycling processes.

(2) *Cellulosic wastes as carbon sources*: Supplementing inherent cellulose in the PSS with purified cellulosics as sole-carbon sources for cellulolytic microbes (as used in this study) is uneconomical on a large-scale. Previous reports have shown agricultural wastes to be strong inducers of cellulolytic enzymes (Rajoka and Malik, 1997; Vlaev *et al*., 1997). Natural agricultural residues such as baggase, cotton stalks and corn stover could therefore be added to the biosulphidogenic reactor, under optimal conditions of operation (pH, temperature, hydraulic retention time, mixing etc.) to induce overproduction of endoglucanases. This would reduce the cost of endoglucanase production and at the same time help in waste management.

(3) *Endoglucanase production in PSS hydrolysis under biosulphidogenic conditions in continuous and closed bioreactors*: This research focused on the time-course production of endoglucanases in a closed bioreactor and it was observed that decline in endoglucanase production coincided with accumulation of sulphide and reducing sugars. Since the system was closed there was no allowance for substrate replenishing or metabolites (sulphide, reducing sugars etc.) removal. Employing a continuous bioreactor under similar operation parameters (pH, temperature, mixing etc) as the closed bioreactor might provide some solutions to the limitations encountered with the closed bioreactor. The continuous bioreactor could be run for a 7-day hydraulic retention time (day on which maximum production of endoglucanases was observed in the closed biosulphidogenic bioreactor) after which it could be fed again (COD:SO_4^{2-} ratio 1:1). This would replenish the system of its used carbon source (cellulose) for the cellulolytic microbes and the electron acceptor (SO_4^{2-}) for the SRB. Furthermore, as the passive sludge effluent flows out of the bioreactor (Figure 3.2) it would relieve the system of some accumulated sulphide and reducing sugars.

REFERENCES

Adney, W.S., Rivard, C.J., Shiang, M., and Himmel, M.E. (1991). Anaerobic digestion of lignocellulosic biomass and wastes. Cellulases and related enzymes. *Applied Biochemistry and Biotechnology*. **30**: 165-183.

Agrawal, P.B. and Pandit, A.B. (2003). Isolation of α-glucosidase from *Saccharomyces cerevisiae*: cell disruption and adsorption. *Biochemical Engineering Journal*. **15**: 37-45.

Akiba, S., Kimura, Y., Yamamoto, K., and Kumagai, H. (1995). Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. *Journal of Fermentation and Bioengineering*. **79**: 125-130.

Araújo, L.d.S., Catunda, P.F.C., and van Haandel, A.C. (1998). Biological sludge stabilisation. Part 2: Influence of the composition of waste activated sludge on anaerobic stabilisation. *Water South Africa*. **24**(3): 231-236.

Banister, S.S. and Pretorius, W.A. (1998). Optimisation of sludge acidogenic fermentation for biological nutrient removal. *Water South Africa*. **24**(1): 35-41.

Banerjee, M.R., Burton, D.L., and Depoe, S. (1997). Impact of sewage sludge application on soil biological characteristics. *Agriculture Ecosystems and Environment*. **66**: 241-249.

Bayer, E.A., Shimon, L.J.W., Shoham, Y., and Lamed, R. (1998). Cellulosomes-structure and ultrastructure. *Journal of Structural Biology*. **124**: 221-234.

Bayer, E.A. and Lamed, R. (1988). The cellulosome concept: exocellular/extracellular enzyme reactor centers for efficient binding and cellulolysis. Biochemistry and genetics of cellulose degradation. *FEMS symposium*. No. 43. Academic Press. New York.

Béchard, G., Yamazaki, H., Gould, W.D., and Bédard, P. (1994). Use of cellulosic substrates for the microbial treatment of acid mine drainage. *Journal of Environmental Quality*. **23**: 111-116.

Béguin, P. and Aubert, J-P. (1994). The biological degradation of cellulose. *FEMS Microbiogy Reviews*. **13**(1): 25-58.

Bhat, M.K and Hazlewood, G.P. (2001). Enzymology and other characteristics of cellulases and xylanases. In: Enzymes in farm animal nutrition. Bedford, M.R. and Patridge, G.G. (eds). CAB International. pp.11-60.

Bhat, M.K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances*. **18**: 355-383.

Bhat, M.K. and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances*. **15**: 583-620.

Boshoff, G.A. (1999). Development of integrated biological processing for the biodesalination of sulphate and metal-rich wastewaters. PhD, thesis. Department of Biochemistry and Microbiology. Rhodes University.

Bradford, M.M. (1976). A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248-251.

Brown Jr., R.M. and Saxena, I.M. (2000). Cellulose biosynthesis: a model for understanding the assembly of biopolymers. *Plant Physiology and Biochemistry*. **38**: 57-67.

Brückner, R. and Titgemeyer, F. (2002). Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiology Letters*. **209**: 141-148.

Butlin, K.R., Selwyn, S.C., and Wakerby, D.S. (1956). Sulphide production from sulphate enriched sewage sludges. *Journal of Applied Bacteriology*. **19**: 3-15.

Cadoret, A., Conrad, A., and Block, J. (2002). Availability of low and high molecular weight substrates to extracellular enzymes in whole and dispersed activated sludges. *Enzyme and Microbial Technology*. **31**: 179-186.

Cai, Y.J., Chapman, S.J., Buswell, J.A., and Chang, S.T. (1999). Production and distribution of endoglucanase, cellobiohydrolase, and β-glucosidase components of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom. *Applied and Environmental Microbiology*. **65**(2): 553-559.

Cao, Y. and Huimin, T. (2002). Effects of cellulase on the modification of cellulose. *Carbohydrate Research*. **337**: 1291-1296.

Castro, H.F., Williams, N.H., and Ogram, A. (2000). Phylogeny of sulphate-reducing bacteria. *FEMS Microbiology Ecology*. **31**: 1-9.

Cavaco-Paulo, A. (1998). Mechanism of cellulase action in textile processes. *Carbohydrate Polymers*. **37**: 273-277.

Cenni, R., Janisch, B., Spliethoff, H., and Hein, K.R.G. (2001). Legislative and environmental issues on the use of ash from coal and municipal sewage sludge co-firing as construction material. *Waste Management*. **21**:17-31.

Chang, I.S., Shin, P.K., and Kim, B.H. (2000). Biological treatment of acid mine drainage under sulphate reducing conditions with solid waste materials as substrate. *Water Research*. **34**(4): 1269-1277.

Chauvaux, S., Souchon, H., Alzari, P.M., Chariot, P., and Beguin, P. (1995). Structural and functional analysis of the metal-binding sites of *Clostridium thermocellum* endoglucanase CelD. *Journal of Biological Chemistry*. **270** (17): 9757-9762.

Chen, X., Jeyaseelan, S., and Graham., N. (2002). Physical and chemical properties study of the activated carbon made from sewage sludge. *Waste Management*. **22**: 755-760.

Christensen, B., Laake, M., and Lien, T. (1996). Treatment of acid mine water by sulfate-reducing bacteria: results from a bench-scale experiment. *Water Research*. **30**: 1617-1624.

Chróst, R.J. (1991). Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: Chróst R.J. (ed). Microbial enzymes in aquatic environments. Springer, New York. pp. 29.

Cornu, S., Neal, C., Ambrosi, J-P., Whitehead, P., Neal, M., Sigolo, J., and Vachier, P. (2001). The environmental impacts of heavy metals from sewage sludge in ferralsols (São-Paulo, Brazil). *The Science of the Total Environment*. **271**: 27-48.

Coughlan, M. (1985). Cellulases: production, properties and applications. *Biochemical Society Transactions*. **13**: 405-406.

Coughlan, M.P. and Ljungdahl, G.L. (1988). Comparative biochemistry of fungal and bacterial cellulolytic systems. Biochemistry and genetics of cellulose degradation. *FEMS symposium*. No 43. Academic Press. New York.

Crowther, R.F. and Harkness, N. (1975). Ecological aspect of used water treatment. Academic press. London. pp. 65-89.

Cumming, R.H. and Iceton, G. (2001). Cell disintegration and extraction. In: Protein purification techniques. Roe, S.D (ed). Second edition. Oxford University Press Inc., New York. pp. 83-110.

Cutler, P. (2001). Chromatography on the basis of size. In: Protein purification techniques. Roe, S.D (ed). Second edition. Oxford University Press Inc., New York. pp. 187-190.

Desvaux, M., Guedon, E., and Petitdemange, H. (2000). Cellulose catabolism by *Clostridium cellulolyticum* growing in batch culture on defined medium. *Applied and Environmental Microbiology*. **66**(6): 2461-2470.

Desvaux, M., Guedon, E., and Petitdemange, H. (2001a). Carbon flux distribution and kinetics of cellulose fermentation in steady-state continuous cultures of *Clostridium cellulolyticum* on a chemically defined medium. *Journal of Bacteriology*. **183**: 119-130.

Desvaux, M., Guedon, E., and Petitdemange, H. (2001b). Metabolic flux in cellulose-fed continuous cultures of *Clostridium cellulolyticum* in response to acidic environment. *Microbiology*. **147**: 1461-1471.

Desvaux, M. and Petitdemange, H. (2001). Flux analysis of the metabolism of *Clostridium cellulolyticum* grown in cellulose-fed continuous cultures on a chemically defined medium under ammonium-limited conditions. *Applied and Environmental Microbiology*. **67**: 3846-3851.

Ding, S.-j., Ge, W., and Buswell, J.A. (2002). Secretion, purification, and characterisation of a recombinant *Volvariella volvacea* endoglucanase expressed in the yeast *Pichia pastoris*. *Enzyme and Microbial Technology*. **31**:621-626.

Dror, T.W., Morag, E., Rolider, A., Bayer, E.A., Lamed, R., and Shoham, Y. (2003). Regulation of the cellulosomal *celS* (*cel*48 A) gene of *Clostridium thermocellum* is growth rate dependent. *Journal of Bacteriology*. **185**(10): 3042-3048.

Dvorak, D.H., Hedin, R.S., Edenborn, H.M., and McIntire, P.E. (1992). Treatment of metal contaminated water using bacterial sulfate reduction: results from a pilot-scale reactor. *Biotechnology and Bioengineering*. **40**:609-616.

Eger, P. and Lapakko, K. (1988). Nickel and copper removal from mine drainage by a natural wetland. In: Mine drainage and surface mine reclamation. IC 9183, Bureau of Mines, US Department of the interior, Pittsburg, P.A. Volume 1. pp 301-309.

Elefsiniotis, P. and Oldham, W.K. (1994). Influence of pH on the acid-phase anaerobic digestion of primary sludge. *Journal of Chemical Technology and Biotechnology*. **60**: 89-96.

Ensley, B.D. and Suflita, J.M. (1995). Metabolism of environmental contaminants by pure and mixed cultures of sulfate-reducing bacteria. In: Sulfate-reducing bacteria. Barton, L.L. (ed). Plenum press, New York. pp. 339.

Eriksson, T., Börjesson, J., and Tjerneld, F. (2002). Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme and Microbial Technology*. **31**: 353-364.

Fermor, T.R. (1993). Applied aspects of composting and bioconversion of lignocellulosic materials: An overview. *International Biodeterioration and Biodegradation*. **31**(2): 87-106.

Fernández-Abalos, J.M., Ruiz-Arribas, A., Garda, A.L., and Santamaría, R.I. (1997). Effect of carbon source on the expression of *celA*$_1$, a cellulase-encoding gene from *Streptomyces halstedii* JM8. *FEMS Microbiology Letters*. **153**: 97-103.

Fjällborg, B. and Dave, G. (2003). Toxicity of copper in sewage sludge. *Environment International*. **28**: 761-769.

Forday, W. and Greenfield, P.F. (1983). Anaerobic digestion. *Effluent and Water treatment Journal*. 405-413.

Forsberg, C.W., Forano, E., and Chesson, A. (2000). Microbial adherence to the plant cell wall and enzymatic hydrolysis. In: Ruminant physiology: Digestion, metabolism, growth and reproduction. Cronje, P.B. (ed). CAB International.

Frølund, B., Griebe, T., and Nielsen, P.H. (1995). Enzymatic activity in the activated sludge matrix. *Applied Microbiology and Biotechnology*. **43**: 755-761.

Fukui, M., Suh, J-I., and Urushigawa, Y. (2000). *In situ* substrates for sulfidogens and methanogens in municipal anaerobic sewage digesters with different levels of sulfate. *Water Research*. **34**(5): 1515-1524.

Galante, Y.M., De Conti, A., Monteverdi, R. (1998). Application of *Trichoderma* enzymes in textile industry. In: *Trichodrerma* and *Gliocladium*-enzymes, biological control and commercial applications. Harman, G.F. and Kubicek, C.P. (eds). Volume 2. pp. 311-326. Taylor and Francis, London.

Gan, Q., Allen, S.J., and Taylor, G. (2003). Kinetic dynamics in heterogeneous enzymatic hydrolysis of cellulose: an overview, an experimental study and mathematical modeling. *Process Biochemistry*. **38**(7): 1003-1018.

Garrett, R.H. and Grisham, C.M. (1999a). Enzyme kinetics. In: Biochemistry. Riley, L.B., Ahrens, B., and Fitz-Hugh, S. (eds). Second edition. Saunders College Publishing. pp. 434-443.

Garrett, R.H. and Grisham, C.M. (1999b). Protein techniques. In: Biochemistry. Riley, L.B., Ahrens, B., Fitz-Hugh, S. (eds). Second edition. Saunders College Publishing. pp. 153-157.

Gavel, O Y., Bursakov, S.A., Calvete, J.J., George, G.N., Moura, J.J.G., and Moura, I. (1998). ATP sulfurylases from sulfate-reducing bacteria of the genus *Desulfovibrio*. A novel metalloprotein containing cobalt and zinc. *Biochemistry*. **37**: 16225-16232.

Gessesse, A., Dueholm, T., Petersen, S.B., and Nielsen, P.H. (2003). Lipase and protease extraction from activated sludge. *Water Research*. **37**: 3652-3657.

Giafreda, L. and Bollac, J.M. (1996). Influence of natural and anthropogenic factors on enzyme activity in soil. In: Soil biochemistry. Stotzky, G. and Bollac, J.M. (eds). Marcel Dekker, Inc., New York. pp. 123-193.

Gibson, G.R. (1990). Physiology and ecology of sulphate reducing bacteria. *Journal of Applied Bacteriology*. **59**: 769-797.

Gilbert, H.J. and Hazlewood, G.P. (1993). Bacterial cellulases and xylanases. *Journal of General Microbiology*. **139**: 187-194.

Goel, R., Mino, T., Satoh, H., and Matsuo, T. (1998). Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. *Water Research*. **32**(7): 2081-2088.

Guedon, E., Desvaux, M., and Petitdemange, H. (2002). Improvement of cellulolytic properties of *Clostridium cellulolyticum* by metabolic engineering. *Applied and Environmental Microbiology*. **68**(1): 53-58.

Hagel, L. (2001). Separation on the basis of chemistry. In: Protein purification techniques. Roe, S.D. (ed). Second edition. Oxford University Press Inc., New York. pp. 166-172.

Hanselmann, K.W., Kaiser, J.P., Wenk, M., Schön, R., and Bachofen, R. (1995). Growth on methanol and conversion of methoxylated aromatic substrates by *Desulfotomaculum orientis* in the presence and absence of sulfate. *Microbiological Research*. **150**: 387-401.

Harris, E.L.V. (2001). Concentration of the extract. In: Protein purification techniques. Roe, S.D. (ed). Second edition. Oxford University Press Inc., New York. pp. 111-143.

Heal, K.V. and Salt, C.A. (1999). Treatment of acidic metal rich drainage from reclaimed ironstone mine spoil. *Water Science and Technology*. **39**(12): 141-148.

Henze, M. and Mladenovski, C. (1991). Hydrolysis of particulate substrate by activated sludge under aerobic, anoxic and anaerobic conditions. *Water Research*. **25**: 61-64.

Houghton, J.I. and Quarmby, J. (1999). Biopolymers in wastewater treatment. *Current Opinion in Biotechnology*. **10**: 259-262.

Jain, S., Lala, A.K., Bhatia, S.K., and Kudchadker, A.P. (1992) Modelling of hydrolysis controlled anaerobic digestion. *Journal of Chemical Technology and Biotechnology*. **53**: 337-344.

Jeathon, C., L'Haridon, S., Cueff, V., Banta, A., Reysenbach, A-L., and Prieur, D. (2002). *Thermosulfobacterium hydrogeniphilum* sp. nov., a thermophilic chemolithoautotrophic, sulfate-reducing bacterium isolated from a deep sea hydrothermal vent at Guaymas Basin, and emendation of the genus *Thermodesulfobacterium*. *International Journal of Systematic and Evolutionary Microbiology*. **52**: 765-772.

Jung, J., Xing, X-H., and Matsumoto, K. (2002). Recoverability of protease released from disrupted excess sludge and its potential application to enhanced hydrolysis of proteins in wastewater. *Biochemical Engineering*. **10**: 67-72.

Kambourova, M., Kirilova, N., Mandeva, R., and Derekova, A. (2003). Purification and properties of thermostable *Bacillus stearothermophilus* MC 7. *Journal of Molecular Catalysis*. **22**: 307-313.

Kansal, A., Rajeshwari, M.B., Balakrishnan, M., Lata, K., and Kishore, V.V.N. (1998). Anaerobic digestion technologies for energy recovery from industrial wastewater- a study in Indian context. *TERI Information Monitor on Environmental Science*. **32**(2): 67-75.

Karlsson, J., Siika-aho, M., Tenkanen, M., and Tjerneld, F. (2002). Enzymatic properties of the low molecular mass endoglucanases Cell2A (EGIII) and Cel45A (EGV) of *Trichoderma* reesei. *Journal of Biotechnology*. **99**:63-78.

Keiding, K. and Nielsen, P.H. (1997). Desorption of organic macromolecules from activated sludge: effect of ionic composition. *Water Research*. **31**(7): 1665-1672.

Khan, A.W. and Patel, G.B. (1991). In: Biosynthesis and biodegradation of cellulose. Haigler, C.H. and Weiner, C.J. (eds). Marcel Dekker, New York. pp. 355-375.

Kleikemper, J., Schroth, M.H., Sigler, W.V., Schmucki, M., Bernasconi, S.M., and Zeyer, J. (2002). Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. *Applied and Environmental Microbiology*. **68**(4): 1516-1523.

Knobel, A.N. and Lewis, A.E (2002). A mathematical model of a high sulphate wastewater anaerobic treatment system. *Water Research*. **36**: 257-265

Koizumi, Y., Takii, S., Nishino, M., and Nakajima, T. (2003). Vertical distribution of sulfate-reducing bacteria and methane-producing achaea quantified by oligonucleotide probe hybridization in the profundal sediment of a mesotrophic lake. *FEMS Microbiology Ecology*. **44**:101-108

Kuever, J., Konneke, M., Galushko, A., and Drzyzga, O. (2001). Reclassification of *Desulfobacterium phenolicum* as *Desulfobacula phenolica* comb. nov and description of strain Sax (T) as *Desulfotignum balticum* gen. nov., sp nov. *International Journal of Systematic Evolutionary Microbiology*. **51**: 171-177.

Labconco Corporation. (1998). A guide to freeze drying for the laboratory. An industry service publication. Labconco Corporation 8811 Prospect Avenue, Kansas City, Missouri, USA.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of a bacteriophage. *Nature*. **227**: 680-685.

Lastella, G., Testa, C., Cornacchia, G., Notornicola, M., Voltasio, F., and Sharma, V.K. (2002). Anaerobic digestion of semi-solid organic waste: biogas production and its purification. *Energy Conversion Management*. **43**: 63-75.

Lee, T.-K. and Kim, C-H. (1999). Molecular cloning and expression of an endo-beta-1,4-D-glucanase I (avicelase I) gene from *Bacillus cellulolyticus* K-12 and characterization of the recombinant enzyme. *Applied Biochemistry and Biotechnology*. **80**: 121-125.

Lemos, M.A., Teixeira, J.A., Domingues, M.R.M., Mota, M., and Gama, F.M. (2003). The enhancement of the cellulolytic activity of cellobiohydrolases I and endoglucanase by the addition of cellulose binding domains derived from *Trichoderma reesei*. *Enzyme and Microbial Technology*. **32**: 35-40.

Lengeler, J.W., Drews G., and Schelgel, H.G. (1999). Biology of the Prokaryotes. Blackwell Science, Stuttgart. pp. 292-294. Cited in: McLeod, E.S., MacDonald, R., and Brözel, V.S. (2002). Distribution of *Shewanella putrefaciens* and *Desulfovibrio vulgaris* in sulphidogenic biofilms of industrial cooling water systems determined by fluorescent *in situ* hybridization. *Water South Africa*. **28**(2): 123-128.

Leschine, S.B. (1995). Cellulose degradation in anaerobic environments. *Annual Review of Microbiology*. **49**: 399-426.

Lester, J.N. (1988). Anaerobic wastewater treatment. Microbiology for environmental and public health engineers. E and F.N. Spin Ltd., London.

Levy, I., Shani, Z., and Shoseyov, O. (2002). Modification of polysaccharides and plant cell wall by endo-1, 4-β-glucanase and cellulose-binding domains. *Biomolecular Engineering*. **19**: 17-30.

Linden, D.R., Larson, W.E., Dowdy, R.H., and Clapp, C.E. (1995). Agricultural utilization of sewage sludge. University of Minnesota, Agricultural experiment bulletin 606-1995. University of MN, St. Paul.

Loubinoux, J., Bronowicki, J-P., Pereira, I.A.C., Mougenel, J-L., and Le Faou, A.E. (2002). Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. *FEMS Microbiology Ecology*. **40**(2): 107-112.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., and Pretorius, I.S. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular biology Reviews*. **66**(3): 506-577.

Maclachlan, G. (1988). β-Glucanases from *Pisum sativum*. In: Methods in Enzymology. Wood, W.A. and Kellogg, S.T. (eds). Academic press. Volume 160: pp. 382-391.

Mahmoud, N., Zeeman, G., Gijzen, H., and Lettinga, G. (2003). Solids removal in upflow anaerobic reactors, a review. *Bioresource Technology*. **90**: 1-9.

Manilal, V.B., Litvin-scramm, S.B., and Suidan, M.T. (2000). Effect of sulphidogenesis on acid-phase digestion of waste activated sludge. *Bioprocess Engineering*. **23**: 595-597.

Mansfield, S.D., Saddler, J.N., and Gübitz, G.M. (1998). Characterization of endoglucanases from the brown rot fungi *Gloeophyllum sepiarium* and *Gloeophyllum traberum*. *Enzyme and Microbial Technology*. **23**: 133-140.

Manz, W., Eisenbrencher, M., Neu, T.R., and Szewzyk, U. (1998). Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiology Ecology*. **25**: 43-61.

Marques, S., Pala, H., Alves, L., Amaral-Collaço, M.T., Gama, F.M., and Gírio, F.M. (2003). Characterisation and application of glycanases secreted by *Aspergillus terreus* CCM1 498 and *Trichoderma viridie* CCM1 84 for enzymatic deinking of mixed office wastepaper. *Journal of Biotechnology*. **100**: 209-219.

Massé, D.I. and Masse L. (2000). Characterization of wastewater from hog slaughterhouses in Eastern Canada and evaluation of their in-plant wastewater treatment systems. *Canadian Agricultural Engineering*. **42**(3): 139-146.

Matano, Y., Park, J-S., Goldstein, M.A., and Doi, R.H. (1994). Cellulose promotes extracellular assembly of *Clostridium cellulovorans* cellulosomes. *Journal of Bacteriology*. **176**(22): 6952-6956.

Mathews, C.K. and van Holde, K.E. (1990). Enzymes: biological catalysts. In: Biochemistry. Benjamin/Cummings Publishing Company, Inc., Redwood City. pp. 358-360.

Mawadza, C., Hatti-Kaul, R., Zvauya, R., and Mattiasson, B. (2000). Purification and characterization of cellulases produced by two *Bacillus* strains. *Journal of Biotechnology*. **83**: 177-187.

McLeod, E.S., MacDonald, R., and Brözel, V.S. (2002). Distribution of *Shewanella putrefaciens* and *Desulfovibrio vulgaris* in sulphidogenic biofilms of industrial cooling water systems determined by fluorescent *in situ* hybridization. *Water South Africa*. **28**(2): 123-128.

Mikkelsen, L.H. and Keiding, K. (2002). Physico-chemical characteristics of full scale sewage sludges with implications to dewatering. *Water Research*. **36**: 2451-2462.

Miller,G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry*. **31**: 426-428.

Moosa, S., Nemati, M., and Harrison, S.T.L. (2002). A kinetic study on the anaerobic reduction of sulphate, Part I: Effect of sulphate concentration. *Chemical Engineering Science*. **57**: 2773-2780.

Morag, E., Bayer, E.A., and Lamed, R. (1992). Affinity digestion for the near-total recovery of purified cellulososme from *Clostridium thermocellum*. *Enzyme and Microbial Technology*. **14**: 289-292.

Mosier, N.S., Hall, P., Ladisch, C.M., and Ladisch, M.R. (1999). Reaction kinetics, molecular action, and mechanisms of cellulolytic proteins. *Advances in Biochemical Engineering/Biotechnology*. **65**: 23-40.

Mudryk, Z.J., Podgórska, B., Ameryk, A., and Bolalek, J. (2000). The occurrence and the activity of sulphate reducing bacteria in the bottom sediments of Gulf of Gdánsk. *Oceanologia*. **42**(1): 105-117.

Müller, J., Lehne, G., Schwedes, J., Battenberg, S., Näveke, R., Kopp, J., Dichtl, N., Scheminski, A., Krull, R., and Hempel, D.C. (1998). Disintegration of sewage sludges and influence on anaerobic digestion. *Water Science and Technology*. **38**(8-9): 425-433.

Murashima, K., Nishimura, T., Nakamura, Y., Kuga, J., Moriya, T., Sumida, N., Yaguchi, T., and Kono, T. (2002). Purification and characterization of new endo-1,4-β-D-glucanases from *Rhizopus oryzae*. *Enzyme and Microbial Technology*. **30**: 319-326.

Murray, W.D., Sowden, L.C., and Colvin, R. (1984). *Bacteriodes cellulosolvens* sp. nov., a cellulolytic species from sewage sludge. *International Journal of Systematic Bacteriology*. **34**(2): 185-187.

Mutkowska, E. (1997). Heavy metals removal in anaerobic treatment. Department of Ecological Engineering, Stensund University Thesis.

Namkoong, W., Hwang, E-Y., Park, J-S., and Choi J-Y. (2002). Bioremediation of diesel-contaminated soil with composting. *Environmental Pollution*. **119**(1): 23-31.

Neugebauer, J.M. (1990). In: Methods in enzymology. Deutscher, M.P. (ed). Volume182, pp.182. academic Press, London.

Newton, T.A. (2001). http://www.biologie.uni-hamburg.de/b-online/e26/26a.htm. (accessed 9th March 2003).

Ngesi, N. (2001). The role of cellulases and glucohydrolases in the solubilisation of primary sewage sludge. MSc thesis. Department of Biochemistry and Microbiology. Rhodes University.

Nielsen, P.H., Raunkjær, K., Norsker, N.H., Jensen, N.A., and Hvitved–Jacobsen, T. (1992). Transformation of wastewater in sewer systems. A review. *Water Science and Technology*. **25**: 17-31.

Novaes, R.F.V. (1986). Microbiology of anaerobic digestion. *Water Science and Technology*. **18** (12): 1-14.

Nybroe, O., Jørgensen, P.E., and Henze, M. (1992). Enzyme activities in wastewater and activated sludge. *Water Research*. **26**:579-584.

O'Flaherty, V., Lens, P., Leahy, B., and Colleran, E. (1998). Long term competition between sulphate-reducing bacteria and methane-producing bacteria during full-scale anaerobic treatment of citric acid production wastewater. *Water Research*. **32**(3): 815-825.

Onyeche, T.I., Schläfer, O., Bormann, H., Schröder, C., and Sievers, M. (2002). Ultrasonic cell disruption of stabilised sludge with subsequent digestion. *Ultrasonics*. **40**: 31-35.

O'Sullivan, A.D., McCabe, O.M., Murray, D.A., and Otte, M.L. (1999). Wetlands for rehabilitation of metal mine wastes. *Biology and Environment: Proceedings of the Royal Irish Academy*. **99B** (1): 11-17.

Oude Elferink, S.J.W.H., Maas, R.N., Harmsen, H.J.M., and Stams, A.J.M. (1995). *Desulforhabdus amnigenus* gen-nov, sp-nov., sulfate reducer isolated from anaerobic granular sludge. *Archives of Microbiology*. **164**: 119-124.

Oude Elferink, S.J.W.H., Vorstman, W.J.C., Sopjes, A., and Stams, A.J.M. (1998). Characterization of the sulfate-reducing and syntrophic population in granular sludge from a full-scale anaerobic reactor treating papermill wastewater. *FEMS Microbiology Ecology*. **27**: 185-194.

Pareek, S., Kim, S.K., Matsui, S., and Shimizu, Y. (1998). Hydrolysis of ligno(cellulosic) materials under sulphidogenic and methanogenic conditions. *Water Science and Technology*. **38**:193-200.

Pèlach, M.A., Pastor, F.J., Puig, J., Vilaseca, F., and Mutjé, P. (2003). Enzymic deinking of old newspapers with cellulase. *Process Biochemistry*. **38**:1063-1067.

Penaud, V., Delgenes, J.P., Torrijos, M., Moletta, R., Vanhoutte, B., and Cans, P. (1997). Definition of optimal conditions for the hydrolysis and acidogenesis of pharmaceutical biomass. *Process Biochemistry*. **32**: 515-521.

Perot, C., Sergent, M., Richard, P., Phan Tan Luu, R., and Millot, N. (1988). The effect of pH, temperature and agitation speed on sludge anaerobic hydrolysis-acidification. *Environmental Technology Letters*. **9**: 141-752.

Pletschke, B.I., Rose, P.D., and Whiteley, C.G. (2002). The enzymology of sludge solubilisation utilising sulphate reducing systems: Identification of ATP-sulphurylases. *Enzyme and Microbial Technology*. **31**: 329-336.

Pohlschröder, M., Leschine, S.B., and Canale-Parola, E. (1994). Multicomplex cellulase-xylanase system of *Clostridium papyrosolvens* C7. *Journal of Bacteriology*. **176**(1): 70-76.

Ponce-Noyola, T. and de la Torre, M. (2001). Regulation of cellulases and xylanases from a depressed mutant of *Cellulomonas flavigena* growing on sugar-cane bagasse in continuous culture. *Bioresource Technology*. **78**: 285-291.

Poverly, J. and Siaba, L. (1996). Beneficial uses of sludge. Fact Sheet 6. Cornell Waste Management Institute. pp. 1-3.

Rajoka, M.I. and Malik, K.A. (1997). Cellulase production by *Cellulomonas biazotea* cultured in media containing different cellulosic substrates. *Bioresource Technology*. **59**: 21-27.

Ramos, L.P., Filho, A.Z., Deschamps, F.C., and Saddler, J.N. (1999). The effect of *Trichoderma* cellulases on the fine structure of a bleached softwood kraft pulp. *Enzyme and Microbial Technology*. **24**: 371-380.

Roe, S.D. (1994). Separation based on structure. In: Protein purification method. Harris, E.L.V. and Angal, S. Oxford : IRL Press at Oxford University Press. pp. 239-242.

Raunkjær, K., Norsker, Hvitved–Jacobsen, T., and Nielsen, P.H. (1994). Measurement of pool of proteins, carbohydrates and lipids in domestic water. *Water Research*. **28**(2): 251-262.

Roe, S.D. (2001a). Getting started. In: Protein purification techniques. Roe, S.D. (ed). Second edition. Oxford University Press Inc., New York. pp. 11-26.

Roe, S.D. (2001b). Purification strategy. In: Protein purification techniques. Roe, S.D. (ed). Second edition. Oxford University Press Inc., New York. pp. 1-9.

Saas, H., Steuber, J., Kroder, M., Kroneck, P.M., and Cypionka, H. (1992). Formation of thionates by freshwater and marine water strains of sulphate-reducing bacteria. *Archives of Microbiology*. **158**: 418-421.

Sachslehner, A., Nidetzky, B., Kulbe, K.D., and Haltrich, D. (1998). Induction of mannanase, xylanase, and endoglucanase in *Sclerotium rolfsii*. *Applied and Environmental Microbiology*. **64**: 594-600.

Samejima, M., Sugiyama, J., and Igarashi, K. (1998). Enzymatic hydrolysis of bacterial cellulose. *Carbohydrate Research*. **305**: 281-288.

Sanwal, S.G.G. (1999). Purification and characterization of a cellulase from *Catharanthus roseus* stems. *Phytochemistry*. **52**: 7-13.

Scawen, M.D. and Melling, J. (1985). Large-scale extraction and purification of enzymes and other proteins. In: Handbook of enzyme biotechnology. Wiseman, A. (ed). Second edition. Ellis Horwood Ltd., England. pp. 15-53.

Schülein, M. (2000). Protein engineering of cellulases. *Biochimica et Biophysica Acta*. **1543**: 239-252.

Schwarz, W. H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. A mini-review. *Applied Microbiology and Biotechnology*. **56**: 634-649.

Scopes, R.K. (1982). Protein Purification: Principle and Practice. Spriger-Verlag, New York. pp.21-38, 52-59, 67-100, 151-163.

Selivanovskaya, S.Yu., Latypova, V. Z., Kiyamova, S. N., and Alimova, F. K. (2001). Use of microbial parameters to assess treatment methods of municipal sewage sludge applied to grey forest soils of Tatarstan. *Agriculture Ecosystems and Environment*. **86**(2): 145-153.

Shoham, Y., Lamed, R., and Bayer, E.A. (1999). The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. *Trends in Microbiology*. **7**(7): 275-281.

Song, Y-C., Piak, B-C., Shin, H-K., and La, S-J. (1998). Influence of electron donor and toxic materials on the activity sulfate-reducing bacteria for the treatment of electroplating wastewater. *Water Science and Technology*. **38**(4-5): 187-194.

Spiridonov, N.A. and Wilson, D.B. (1998). Regulation of biosynthesis of individual cellulases. *Journal of Bacteriology*. **180**(14): 3529-3532.

Stamps, A.J.M. and Hansen, T.A. (1986). Metabolism of L-alanine in *Desulfotomaculum ruminis* and two marine *Desolfovibrio* strains. *Archives of Microbiology*. **145**: 277-279.

Sterritt, R.M. and Lester, J.N. (1988). Microbiology for environmental and public health engineers. London: E and F.N. pp. 181-194. Cited in: Pletschke, B.I., Rose, P.D., and Whiteley, C.G. (2002). The enzymology of sludge solubilisation utilising sulphate reducing systems: Identification of ATP-sulphurylases. *Enzyme and Microbial Technology*. **31**: 329-336.

Stülke, J. and Hillen, W. (1999). Carbon catabolite repression in bacteria. *Current Opinion in Microbiology*. **2**: 195-201.

Sutherland, I.W. (1999). Polysaccharases for microbial exopoysaccharides. *Carbohydrate Polymers*. **38**: 319-328.

Suto, M. and Tomita, F. (2001). Review. Induction and catabolite repression mechanisms of cellulase in fungi. *Journal of Bioscience and Bioengineering*. **92**(4): 305-311.

Teeri, T.T. (1997). Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends in Biotechnology*.**15**: 160-167.

Tengerdy, R.P. and Szakacs, G. (2003). Bioconversion of lignocellulose in solid substrate fermentation. *Biochemical Engineering Journal*. **13**: 169-179.

Trevan, M.D. (1987). Enzyme technology. In: Biotechnology: The biological principles. Trevan, M.D., Boffey, S., Goulding, K.H., and Stanbury, P. (eds). Open University Press, Milton Keynes, England and Taylor and Francis, New York, USA. pp. 155-177.

Tshivhunge, A.S. (2001). Enzymology of activated sewage sludge during anaerobic treatment of wastewaters: Identification, characterisation, isolation and partial purification of proteases. MSc thesis. Department of Biochemistry and Microbiology. Rhodes University.

Ubukata, Y. (1999). Kinetics and mechanisms of starch removal by activated sludge: hydrolysis of starch to maltose and maltotriose is the rate determining step. *Water Science and Technology*. **40**(1): 61-68.

Ullrich, T.C. and Huber, R. (2001). The complex structures of ATP sulfurylase with thiosulfate, ADP and chlorate reveal new insights in inhibitory effects and the catalytic cycle. *Journal of Molecular Biology*. **313**: 1117-1125.

van Wyk, J.P.H. and Mohulatsi, M. (2003). Biodegradation of wastepaper by cellulase from *Trichoderma viride*. *Bioresource Technology*. **86**: 21-23.

Visscher, P.T., Prins, R.A., and Germerden, H. (1992). Rates of sulfate reduction and thiosulfate consumption in marine microbial mat. *FEMS Microbiology Ecology*. **86**: 283-294.

Vlaev, S.D., Djejeva, G., Raykovska, V., and Schügerl, K. (1997). Cellulase production by *Trichoderma* sp. grown on corn fibre substrate. *Process Biochemistry*. **32**(7): 561-565.

Voet, D. and Voet, J.G. (1995). Techniques of protein purification. In: Biochemistry. Second edition. John Wiley and Sons, Inc., USA.

Vossoughi, M., Shakeri, M., and Alemzadeh, I. (2003). Performance of anaerobic baffled reactor treating synthetic wastewater influenced by decreasing COD/SO_4 ratios. *Chemical Engineering and Processing*. **42**: 811-816.

Wagner, M. and Loy, A. (2002). Bacterial composition and function in sewage treatment systems. *Current Opinion in Biotechnology*. **13**:218-227.

Wakao, N., Takahashi, T., Sakurai, Y., and Shiota, H. (1979). A treatment of mine water using sulfate-reducing bacteria. *Journal of Fermentation Technology*. **57**: 445-452.

Walker, J.M. (2000a). Protein structure, purification and characterization. In: Practical biochemistry, principles and techniques. Wilson, K. and Walker, J. (eds). Fifth edition. Cambridge University Press. pp. 318-339.

Walker, J.M. (2000b). Electrophoretic techniques. In: Practical biochemistry, principles and techniques. Wilson, K. and Walker, J. (eds). Fifth edition. Cambridge University Press. pp. 602-603.

Walker, L.P., Wilson, D.B., and Irwin, D.C. (1990). Measuring fragmentation of cellulose by *Thermomonspora fusca* cellulase. *Enzyme Microbial Technology*. **12**: 378-386. Cited in: Lynd, L.R., Weimer, P.J., van Zyl, W.H., and Pretorius, I.S. (2002). Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular biology Reviews*. **66**(3): 506-577.

Watanabe, H., Nakamura, M., Tokuda, G., Yamaoka, I., Scrivener, A.M., and Noda, H. (1997). Site of secretion and properties of endogenous endo-beta-1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. *Insect Biochemistry and Molecular Biology*. **27**: 305-313.

Whiteley, C.G., Burgess, J.E., Melamane, X., Pletschke, B., and Rose, P.D. (2003). The enzymology of sludge solubilisation utilising sulphate-reducing systems: the properties of lipases. *Water Research*. **37**: 289-296.

Whiteley, C.G., Heron, P., Pletschke, B.I., Rose, P.D., Tshivhunge, S., and Van Jaarsveld, F.P., and Whittington-Jones, K. (2002a). The enzymology of sludge solubilisation utilising sulphate-reducing systems. Properties of proteases and phosphatases. *Enzyme Microbial Technology*. **31**: 419-424.

Whiteley, C.G., Pletschke, B.I., Rose, P.D., and Ngesi, N. (2002b). Specific sulphur metabolites stimulate β-glucosidase activity in an anaerobic sulphidogenic bioreactor. *Biotechnology Letters*. **24**: 1509-1513.

Whittington-Jones, K. (2000). Enhanced hydrolysis of primary settled sewage under sulphide rich conditions. PhD thesis. Department of Biochemistry and Microbiology. Rhodes University.

Widdel, F. (1988). Microbiology and ecology of sulfate and sulfur reducing bacteria. In: Biology of anaerobic microorganisms. Zehnder J.B. (ed). Wiley. New York. pp. 469-485.

Widdel, F. and Pfennig, N. (1982). Studies on dissimilatory sulphate reducing bacteria that decompose fatty acids. II. In: complex oxidation of propionate by *Desulobulbus propionicus* gen. nov., spp. nov. *Archives of Microbiology*. **131**: 360-365.

Willson R.C. (1999). Purification and characterization of proteins. In: Manual of industrial and microbial biotechnology. Demain, A.L., Davies, J.E., Atlas, R.M., Cohen, G., Hershberger, C.L., Hu, W-S., Sherman, D.H., Wilson, R.C., and Wu, J.H.D. (eds). Second edition. ASM Press. Washington D.C. pp. 226.

Wilson, K. (2000a). Biomolecular interactions: 1. In: Practical biochemistry, principles and techniques. Wilson, K. and Walker, J. (eds). Fifth edition. Cambridge University Press. pp. 373.

Wilson, K. (2000b). Chromatographic techniques. In: Practical biochemistry, principles and techniques. Wilson, K. and Walker, J. (eds). Fifth edition. Cambridge University Press. pp. 647-668.

Wong, J.W.C. and Su, D.C. (1997). The growth of *Agropyron elongatum* in an artificial soil mix from coal fly ash and sewage sludge. *Bioresource Technology*. **59**: 57-62.

Wong, J.W.C., Li, K., Fang, M., and Su, D.C. (2001). Toxicity of sewage sludge in Hong-Kong. *Environment International*. **27**:373-380.

Wood, T.M. (1988). Preparation of crystalline, amorphous and dyed cellulase substrates. In: Methods in Enzymology. Wood, W.A. and Kellogg, S.T. (eds). Academic press. Volume 160. pp. 23.

Wood, T.M. and Bhat, K.M. (1988). Methods for measuring cellulase activities . In: Methods in Enzymology. Wood, W.A. and Kellogg, S.T. (eds). Academic press. Volume 160. pp. 87-112.

Yuan, Z. and Blackall, L.L. (2002). Sludge population optimisation: a new dimension for the control of biological wastewater treatment systems. *Water Research*. **36**: 482-490.

Zaldívar, M., Velásquez, J.C., Contreras, I., and Pérez, L.M. (2001). *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. *Electronic journal of Biotechnology*. **4**(30): 161-168

Zeeman, G. and Sanders, W. (2001). Potential of anaerobic digestion of complex waste(water). *Water Science and Technology*. **44**(8): 115-122.

Zhang, X. and Young, L.Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenathrene by sulphidogenic consortia. *Applied and Environmental Microbiology*. **63**: 4759-4764.

Zorpas, A.A., Arapoglou, D., and Panagiotis, K. (2003). Waste paper and clinoptilolite as a bulking material with dewatered anaerobically stabilized primary sewage sludge (DAPSS) for compost production. *Waste Management*. **23**: 27-35.

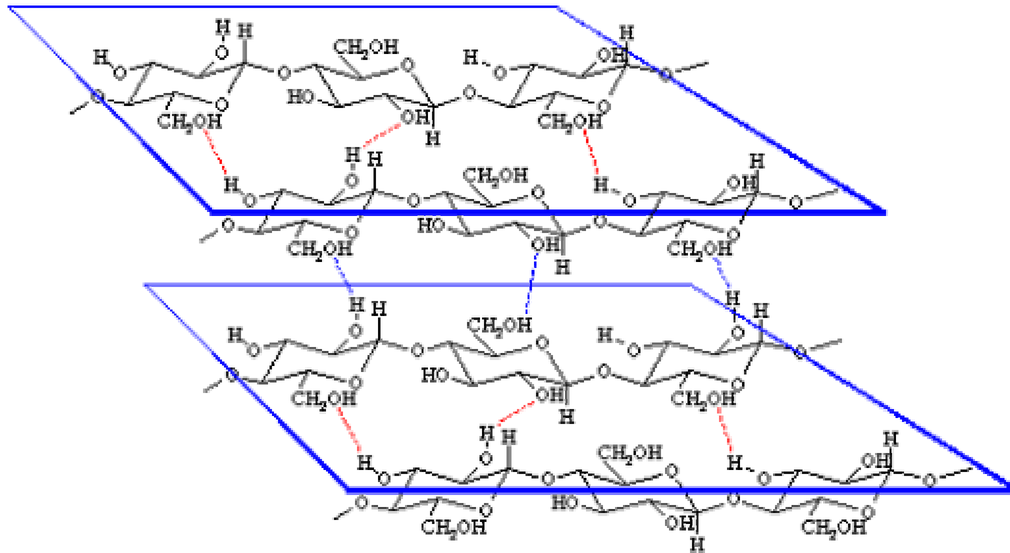
Appendix 1. Cellulose

http://www.biologie.uni-hamburg.de/b-online/e26/26a.htm last change: 07/02/2003 11:09:11

Cellulose

Cellulose is composed of linear chains of covalently linked glucose residues. It is very stable chemically and extremely insoluble. In the primary cell wall consists one glucose polymer of roughly 6000 glucose units, in the secondary wall is their number increased to 13 - 16000 units. Cellulose chains form crystalline structures called **microfibrils**. A microfibril with a diameter of 20 - 30 nm contains about 2000 molecules.

Crystalline and non-crystalline sections alternate. In crystalline ones forms the cellulose three-dimensional lattices due to the formation of the highest possible number of hydrogen bonds. This high degree of organization is not achieved in the other sections, called paracrystalline. Crystals polarize light. By studying cellulose between crossed polarisators can the main orientation of the microfibrils be determined. In the primary wall do they occur in every possible orientation (disperse texture). During the development of the secondary wall are they deposited in layers (as lamellas). The microfibrills of each layer are parallel to each other (parallel texture). Their orientation changes from layer to layer. Often, especially in very strong cell walls (like those of cotton) are the microfibrills arranged screw-like around the cell's axis. In such cases changes the turning angle from layer to layer (screw-like texture).



© Thomas A. NEWTON

Although cellulose is by far the most common macromolecule - nature synthesizes roughly 10^{11} tons per year and breaks most of it down again - is astonishingly little known about its biosynthesis. The enzyme (or the enzyme complex?) cellulose synthase is still a largely hypothetical quantity.

Since the preparation techniques (freeze etching) were optimized several years ago have directed particles (or particle complexes) been observed at the outer side of the plasma membrane. It is assumed that they take part in the synthesis of cellulose. In some algae (*Micrasterias, Spirogyra* and also in cells of higher plants) do the complexes form hexagonal rosettes (R. M. BROWN, D. MONTEZINOS, 1976; O. KIERMAYER and U. B. SLEYR, 1979; W. HERTH, 1983).

A striking correlation of the orientation of cortical microtubuli and the neighbouring microfibrills (spatially separated by the plasma membrane) exists. Despite of existing criticism does it look as if the microtubuli would take part in the orientation of cellulose synthase (directed arrangement of the complexes seen in the electron microscope) and would thus exert an indirect influence on the orientation of the microfibrills. Experiments with microtubuli-disrupting agents have an influence on the orientation pattern of newly developing microfibrills.

© *Peter v. Sengbusch - b-online@botanik.uni-hamburg.de*

Appendix 2. Preparation of DNS Reagent

Dinitrosalicylic acid (10 g); phenol (2 g); sodium sulphite (0.5 g) and sodium potassium tartrate tetrahydrate (200 g) were dissolved in 2 % (w/v) sodium hydroxide solution (500 ml). This was then made up to 1 l with distilled water.

Appendix 3. Glucose standard curve

Glucose stock solution: 0.1 g of anhydrous glucose was dissolved in 10 ml of ddH_2O to prepare stock solution of concentration 10 mg/ml.

Table A3.1. Preparation of glucose standard curve

Glucose Conc. (mg/ml)	Glucose stock solution (ml)	ddH_2O (μl)	Dilution
2.0	1	4.0	1:5
3.3	1	2.0	1:3
5.0	1	1.0	1:2
6.7	1	0.5	1:1.5

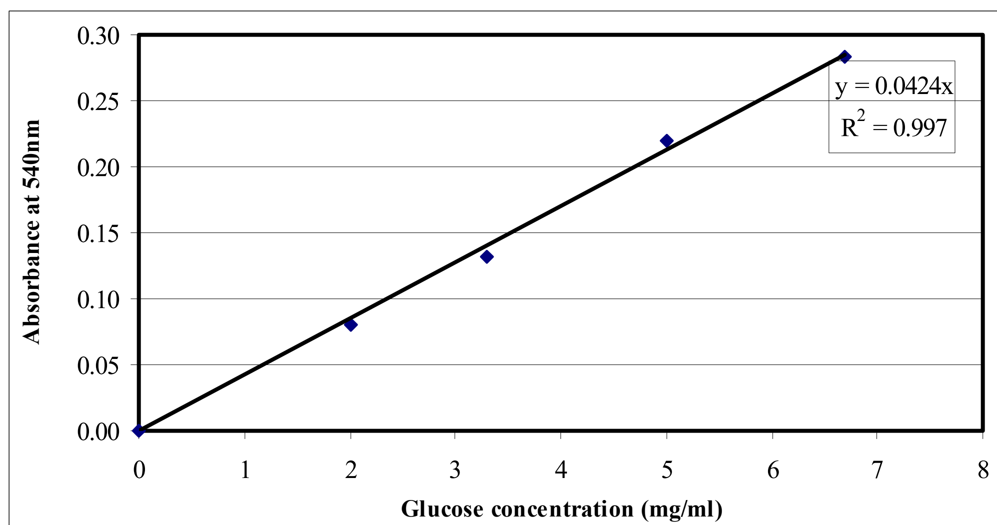


Figure A3.1: Glucose standard curve

Appendix 4. Protein standard curve

Protein stock solution: 0.02 g of Bovine serum albumin (BSA) was dissolved in 10 ml of ddH_2O to prepare stock solution of concentration 2 mg/ml.

Table A4.1. Preparation of protein standard curve

Protein Conc. (mg/ml)	BSA stock solution (µl)	ddH_2O (µl)	Bradford reagent
0	0	5	250
0.4	1	4	250
0.8	2	3	250
1.2	3	2	250
0.6	4	1	250
2	5	0	250

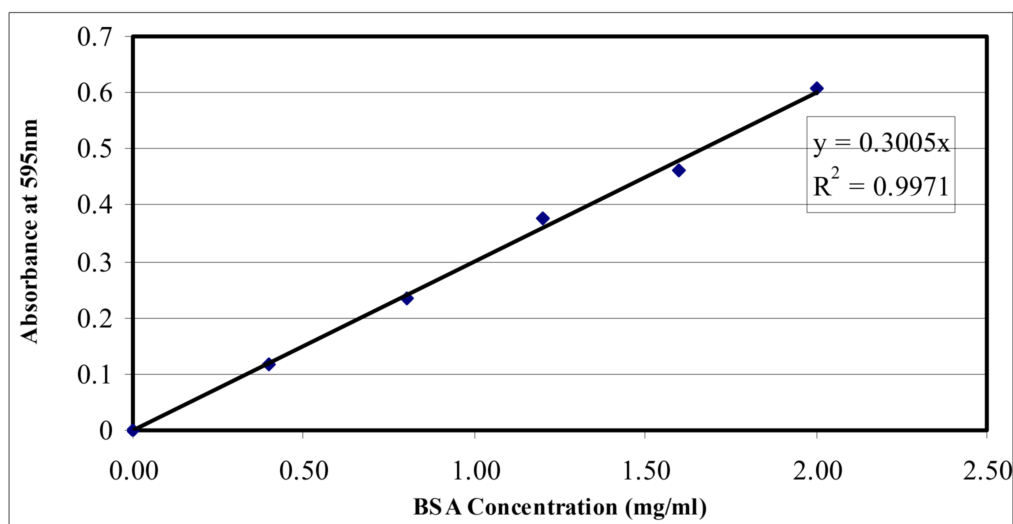


Figure A4.1: Protein standard curve

Appendix 5. Molecular weight determination

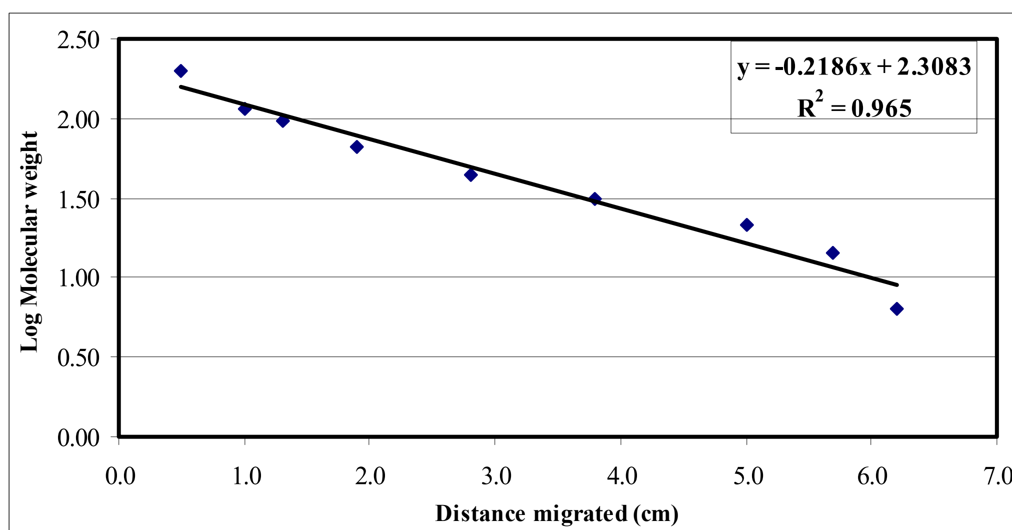


Figure A5.1: Calibration curve of the log of molecular weight markers (Myosin, 200 kDa; β-galactosidase, 116.3 kDa; Phosphorylase b, 97.4 kDa; Bovine serum albumin, 66.2 kDa; Ovalbumin, 45 kDa; Carbonic anhydrase, 31 kDa; Soybean trypsin inhibitor, 21.5 kDa; Lysozyme, 14.6 kDa; and Aprotinin, 6.5 kDa) versus distance migrated.

Appendix 6. Regeneration and equilibration of the column

(i) Wash approximately 50 g resin in 100 ml, 1 M HCl for 30 min and decant supernatant

(ii) Wash the resin in 500 ml, ddH_2O for 30 min three times, after each step decant supernatant

(iii) Wash the resin in 100 ml, 1 M NaOH for 30 min and decant supernatant

(iv) Wash the resin in 500 ml, ddH_2O for 30 min three times, after each step decant supernatant

(v) Equilibrate with the starting buffer and check if pH of the washing buffer until is equal to that of the starting buffer.

Appendix 7. Calculation of the number of grams of ammonium sulphate (g) to add to 1 litre sample to give desired concentration (per cent saturation)

$$g = \frac{533\,(S_2 - S_1)}{100 - 0.3\,S_2}$$

S_1: Starting concentration

S_2: Final concentration

Appendix 8. Preparation of Phosphoric acid-swollen cellulose (Wood, 1988)

(i) Suspend 10 g of avicel in phosphoric acid (85 % (w/v)) and gently stir (1 h, 4 °C)

(ii) Pour slurry into ice-cold water and leave to stand (30 min).

(iii) Wash the swollen cellulose several times with cold water followed by washing with 1 % (w/v) $NaHCO_3$ solution

(iv) Dialyse suspension against water until dialysate becomes neutral (4 °C)

(v) Store in aqueous suspension (5 m M with respect to NaN_3) at 4 °C

(vi) Homogenise using Waring blender (2 min) and remove lumps by decantation.

Appendix 9. Preparation of SDS-PAGE (Laemmli, 1970) Recipes

Acrylamide/Bis: (30 % T, 2.6 % C): Dissolve 87.6 g of acrylamide and 2.9 g of N'N-bis-methylene-acrylamide in 300 ml. The solution was filtered and stored in the dark. Stable for maximum of 30 days.

10 % Ammonium per sulphate (APS) (w/v): 0.1 g of APS was dissolved in 1 ml of ddH_2O. The solution was prepared fresh daily.

10 % SDS (w/v): Dissolve 10 g of SDS in 90 ml ddH_2O with gentle stirring and make up to 100 ml with ddH_2O.

0.5 % (w/v) Bromophenol blue: Dissolve 0.5 g of bromophenol blue in 90 ml ddH_2O with gentle stirring and make up to 100 ml with ddH_2O.

Resolving Gel Buffer: (1.5 M Tris-HCl, pH 8.8): Tris base (27.23g) was dissolved in 80 ml of ddH_2O; the pH was adjusted to 8.8 with 6 M HCl. The total volume was then made up to 100 ml with ddH_2O and stored at 4 °C.

Stacking Gel Buffer: (0.5 M Tris-HCl, pH 6.8): Tris base (6 g) was dissolved in 60 ml of ddH_2O and adjusted to pH 6.8 with 6 M HCl. The total volume was brought up to 100 ml with ddH_2O and stored at 4 °C.

Sample Buffer: Double deionised water (3.55 ml), 1.25 ml of stacking gel buffer, 2.5 ml of glycerol, 2.0 ml 10 % (w/v) SDS and 0.2 ml of 0.5 % (w/v) bromophenol blue were all mixed to make solution A. Beta-mercaptoethanol (5 µl) was added to 950 µl solution A prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95 °C for 5 min.

10 × Electrode (Running) Buffer, pH 8.3: Dissolve 30.3 g of Tris base, 144 g of glycine and 10 g of SDS in ddH_2O and make up to 1 l with ddH_2O. Store at 4 °C. Dilute (1:10) before use.

Staining Solution: Brilliant blue R-staining solution (Sigma, B6529).

Coomasie Gel Destain: Mix 100 ml of methanol and glacial acetic acid and make the total volume up to 1 l with ddH_2O.

12% Resolving Gel

Reagent	Volume (ml)
ddH_2O	3.4
Acrylamide/Bis	4
Resolving gel buffer	2.5
10 % (w/v) SDS	0.1
10 % (w/v) APS	0.05
TEMED	0.005

4 % Stacking Gel

Reagent	Volume (ml)
ddH_2O	6.1
Acrylamide/Bis	1.3
Resolving gel buffer	2.5
10 % (w/v) SDS	0.1
10 % (w/v) APS	0.05
TEMED	0.01