An investigation into the synergistic association between the major *Clostridium cellulovorans* cellulosomal endoglucanase and two hemicellulases on plant cell wall degradation.

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Abstract

The cellulosome is a multimeric enzyme complex that has the ability to metabolise a wide variety of carbonaceous compounds. Cellulosomal composition may vary according to the microbe's nutritional requirement and allows for the anaerobic degradation of complex substrates. The complex substrates of interest in this research study were sugarcane bagasse and pineapple fibre waste, as they represent two important lignocellulosic, South African agricultural crops. The effective degradation of complex plant biomass wastes may present a valuable source of renewable compounds for the production of a variety of biofuels, for example bioethanol, and a variety of biocomposites of industrial importance. The identification of renewable energy sources for the production of biofuels is becoming increasingly important, as a result of the rapid depletion of the fossil fuels that are traditionally used as energy sources. An effective means of completely degrading lignocellulose biomass still remains elusive due to the complex heterogeneity of the substrate structure, and the fact that the effective degradation of the substrate requires a consortium of enzymes.

The cellulosome not only provides a variety of enzymes with varying specificities, but also promote a close proximity between the catalytic components (enzymes). The close proximity between the enzymes promotes the synergistic degradation of complex plant biomass for the production of valuable energy products. Previous synergy studies have focused predominantly on the synergistic associations between cellulases; however, the synergy between hemicellulases has occasionally been documented. This research project established the synergistic associations between two *Clostridium cellulovorans* hemicellulases that may be incorporated into the cellulosome and a cellulosomal endoglucanase that is conserved in all cellulosomes.

This research study indicated that there was indeed a synergistic degradation of the complex plant biomass (sugarcane bagasse and pineapple fibre). The degrees of synergy and the ratio of the enzymes varied between the two complex substrates. The initial degradation of the bagasse required the presence of all the enzymes and proceeded at an

enhanced rate under sulphidogenic conditions; however, there was a low production of fermentable sugars. The low quantity of fermentable sugars produced by the degradation of the bagasse may be related to the chemical composition of the substrate. The sugarcane contains a high percentage of lignin forming a protective layer around the holocellulose, thus the glycosidic bonds are shielded extensively from enzymatic attack. In comparison, the initial degradation of the pineapple fibre required the action of hemicellulases, and proceeded at an enhanced rate under sulphidogenic conditions. The initial degradation of the pineapple fibre produced a substantially larger quantity of fermentable sugars in comparison to the bagasse. The higher production of fermentable sugars from the degradation of the pineapple fibre may be explained by the fact that this substrate may have a lower percentage of lignin than the bagasse, thus allowing a larger percentage of the glycosidic bonds to be exposed to enzymatic attack. The data obtained also indicated that the glycosidic bonds from the hemicellulosic components of the pineapple fibre shielded the glycosidic bonds of the cellulose component.

The identification of the chemical components of the different substrates may allow for the initial development of an ideal enzyme complex (designer cellulosome) with enzymes in an ideal ratio with optimal synergy that will effectively degrade the complex plant biomass substrate.

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List of Abbreviations

µl	Microlitre
2 × YT	2 × Yeast-Tryptone
APS	Ammonium persulphate
APS	Adenosine phosphosulphate
BSA	Bovine serum albumin
BWX	Birchwood xylan
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
DNS	3, 4-dinitrosalicylic acid
E. coli	*Escherichia coli*
EngE	Endoglucanase E
GH	Glycosyl hydrolase
IPTG	Isopropylthiogalactoside
LBG	Locust Bean gum
ManA	Mannanase A
MES	morpholineethanesulfonic acid
mg	Milligram
ml	Millilitre
mM	millimolar
MM	Molecular marker
Ni-NTA	Nickel nitrilotriacetic
OD	Optical density
PAPS	Phosphoadenosine 5' phosphosulphate
PIPES	piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sodium docecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine

Tris	Tris(Hydroxymethyl)Aminomethane
XynA	Xylanase A

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Appendices

Appendix 1: Chemicals and suppliers

(D)-Glucose	Saarchem (Cat. No. 2676020)
(D)-Xylose	Sigma (Cat No. X3877)
2-mercaptoethanol	Fluka (Cat. No. 63700)
3, 4-dinitrosalicylic acid	Sigma (Cat No. D0550)
Acrylamide	Sigma (Cat No. A8887)
Ammonium persulphate	Sigma Aldrich (Cat no A3678)
Ampicillin	Roche (Cat. No. 835269)
Bacteriological agar	Biolab (Cat. No. BX1)
Birchwood xylan	Fluka (Cat. No. 95588)
Bradfords reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Calcium chloride	Saarchem (Cat. No.1524900)
Carboxymethyl cellulose	Calbiochem (Cat. No. 217277)
Citric acid	Merck (Cat. No. 1.00244)
Coomassie brilliant blue R250	Merck (Cat. No. 1.12553)
Di-sodium hydrogen orthophosphate	Saarchem (Cat. No. 5822860)
Ethanol	Merck (Cat. No. 8.18700)
Gaunidinium chloride	Merck (Cat. No. 1.04219)
Glacial acetic acid	Merck (Cat No. 1.00063)
Glycerol	Saarchem (Cat. No.2676520)
Glycine	Merck (Cat. No. 1.04169)
Hyrochloric acid	Saarchem (Cat. No. 3063040)
Imidazole	Merck (Cat. No. 1.04716)
IPTG	Calbiochem (Cat. No. 420322)
Kanamycin	Roche (Cat. No. 106801)
Locust bean gum	Fluka (Cat. No. 62631)
Lysozyme	Fluka (Cat. No. 62971)

Magnesium chloride	ACE
Mannose	Sigma (Cat. No. M2069)
Methanol	Merck (Cat. No. 8.22283)
N,N,N',N'-tetramethylethylene diamine	Sigma Aldrich (Cat. No. T9281)
N,N-methylenebisacrylamide	Sigma (Cat. No.M7279)
Nickel (II) sulphate	Merck (Cat. No. 1.06727)
NiNTA agarose	Qiagen (Cat. No. 30210)
peqGold protein marker (II)	peqLab (Cat. No. 27-2010)
Phenol	Sigma (Cat.No. P3653)
Polyethylene glycol 20 000	Merck (Cat. No. 8.18897)
SDS	BDH biochemicals (Cat. No. 301754)
SDS-PAGE protein markers	Sigma (Cat. No. SDS6H2)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium chloride	Saarchem (Cat. No. 5822320)
Sodium dihydrogen phosphate	Saarchem (Cat. No. 3822650)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium metasulfite	Sigma Aldrich (Cat. No. 255556)
Sodium potassium tartrate	Merck (Cat. No. 1.08087)
Sodium sulphate	Saarchem (Cat. No. 5825200)
Sodium sulphite	Saarchem (Cat. No. 5825400)
Tris (hydroxymethyl) aminomethane	Merck (Cat. No. 1.08382)
Tri-sodium citrate dehydrate	Merck (Cat. No. 1.06448)
Tryptone	Fluka (Cat. No. 70169)
Yeast extract	Biolab (Cat. No. BX6)

Appendix 2: Preparation and transformation of *E. coli* BL21 (DE3) cells

2.1 Preparation of competent E. coli BL21(DE3) cells

Prior to the transformation of the *E. coli* strain BL21(DE3), the cells were made chemically competent. Using 20 µl of *E. coli* BL21 (DE3) cells, 5 ml of yeast-tryptone (2×YT) broth was inoculated. Incubating at 37°C, the inoculum was shaken at 200 rpm on a Labcon bench top shaker for approximately 12 hours. The overnight culture was diluted with fresh 2×YT broth (50ml) in the respective ratio of 1 to 200. At 37°C, and shaking at 200 rpm, the new inoculum was incubated for approximately 3 hours, until an optical density at 260 (OD_{260}) of between 0.3 and 0.6 was reached. The cells were harvested in sterile centrifugation tubes, at 5, 000×g for 5 minutes at 4°C in a Beckman Avanti centrifuge. The *E. coli* BL21 (DE3) cells were kept on ice for the remainder of the protocol. The cell pellet was resuspended in 50 ml ice cold, sterile 0.1 M $MgCl_2$, and kept on ice for 20 minutes. The cells were harvested in sterile centrifugation tubes, at 5, 000×g for 5 minutes at 4°C, and the pellet was resuspended in 25 ml ice cold, sterile 0.1 M $CaCl_2$. The cells remained on ice for a period of 2 hours. The cells were harvested as before, and the pellet resuspended in 5 ml ice cold, sterile 0.1 M $CaCl_2$, and the cells were mixed with 5 ml 30 % sterile glycerol, and stored at -70°C in suitable aliquots.

2.2 Transformation of competent *E. coli* BL21(DE3) cells

Growth of *E. coli* BL 21(DE3) harbouring the gene construct, for the protein of interest was performed at 30°C in 2×Yeast-Tryptone (2×YT) broth.

1. *2 ×YT broth*

    10 g Yeast extract

    16 g Tryptone (Pancreatic digest of casein)

    5 g Sodium chloride

The components were dissolved in 1 L distilled MilliQ water, and autoclaved at 121°C for 20 minutes. The medium was allowed to cool and using sterile techniques, the

appropriate antibiotic was added prior to inoculation with the *E. coli* BL 21(DE3) harbouring the gene construct for the protein of interest. The genes of interest were kindly donated by Prof. R.H. Doi in the form of plasmids. The mannanase (ManA) and the xylanase (XynA) genes had been cloned into pET29a(+) vectors (Figure 41), whereas the endoglucanase (EngE) gene had been cloned into a pET22b(+) vector (Figure 42).

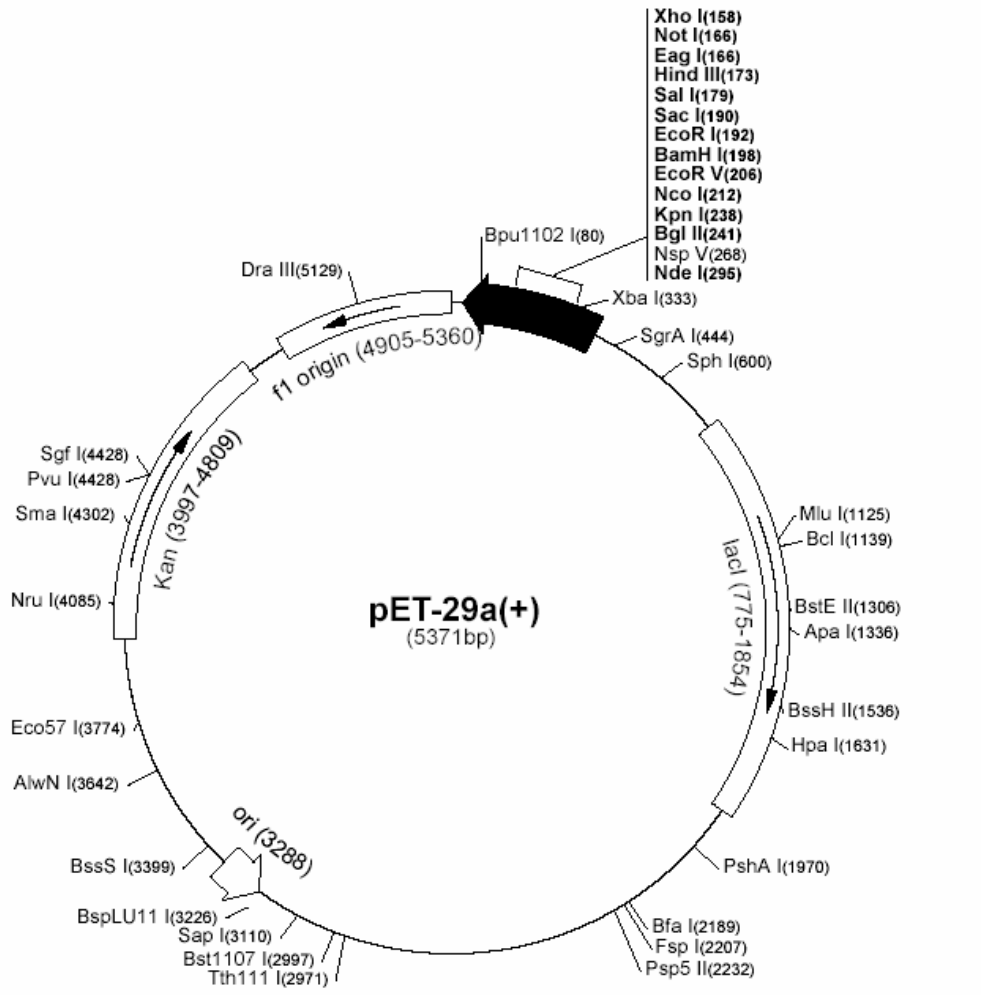


Figure 41: pET-29a(+) expression vector. The mannanase and xylanase genes were inserted between the EcoRI and BamHI.

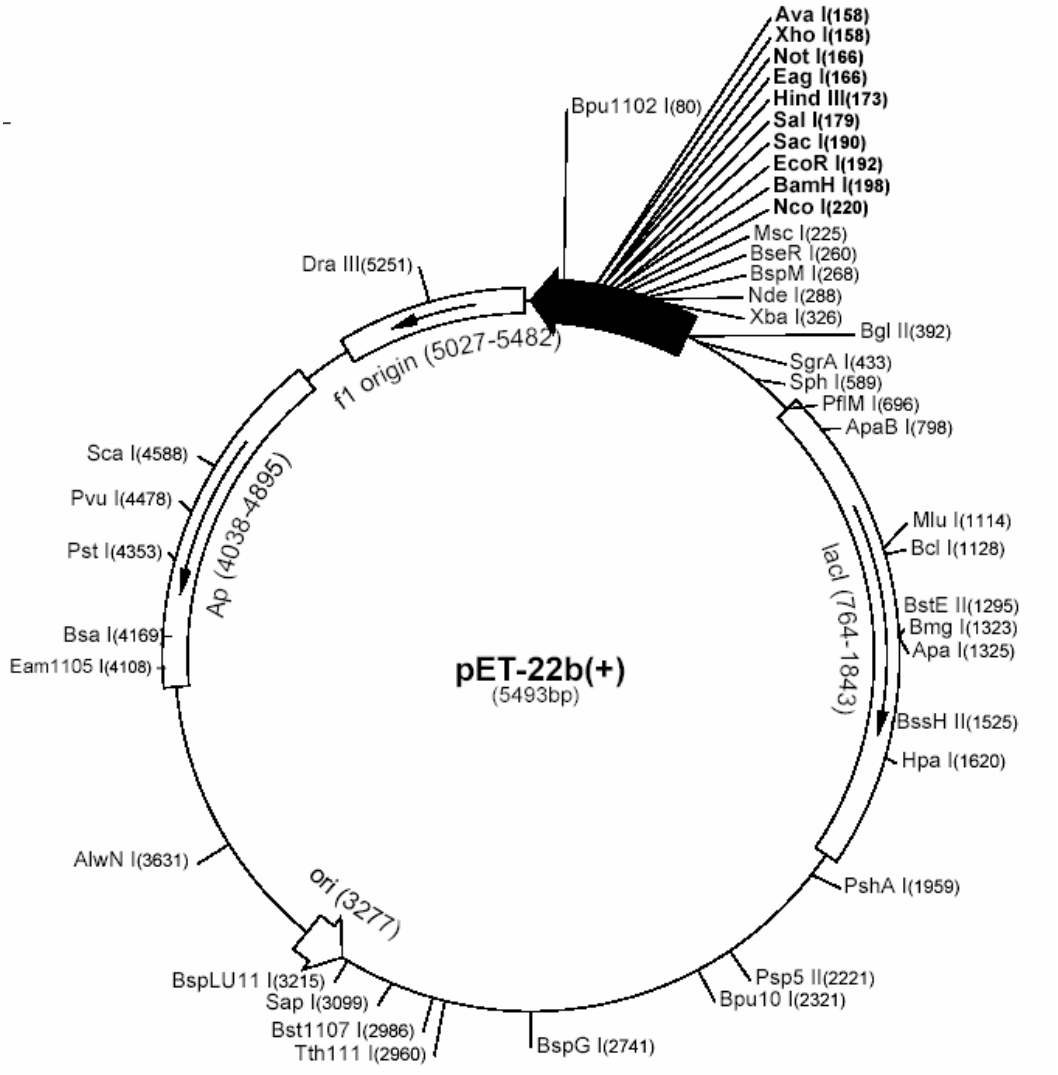


Figure 42: pET22b expression vector. The endoglucanase had been inserted between the restriction enzymes BamHI and NcoI.

Appendix 3: Purification of proteins

1. *Lysis Buffer (pH 7.0)*

    50 mM Sodium dihydrogen phosphate

    300 mM Sodium chloride

    10 mM Imidazole

    8 mg/ml Lysozyme

Once the pH of the solution had been corrected, the buffer was stored at 4°C.

2. *Wash Buffer (pH 7.0)*

    50 mM Sodium dihydrogen phosphate

    300 mM Sodium chloride

    10 mM Imidazole

Once the pH of the solution had been corrected, the buffer was stored at 4°C.

3. *Elution Buffer (pH 7.0)*

    50 mM Sodium dihydrogen phosphate

    300 mM Sodium chloride

    250 mM Imidazole

Once the pH of the solution had been corrected, the buffer was stored at 4°C.

The recombinant proteins were expressed in an *E. coli* BL21 (DE3) strain. The *E. coli* cells containing the appropriate gene construct were used to inoculate 250 ml of 2 × YT broth containing either ampicillin in the case of pET22b vector or kanamycin in the case on the pET29a vector. The inoculum was incubated at 30°C, shaking at 200 rpm, until the optical density at 600nm (OD_{600nm}) was between 0.5 and 0.6. During the growth of the *E. coli* harbouring the ampicillin based vector, the medium was spiked with ampicillin every 2 hours. Once the correct OD_{600nm} was obtained, the expression of the recombinant enzymes were initiated by the addition of IPTG to produce a final concentration of 400 μg/ml for the pET 22b vector and 100 μg/ml for the pET29a vector. Subsequent to the addition of the inducer, the cultures were incubated at 19°C for 14 hours.

The cells were harvested by centrifugation at 8 000 × g using a Beckman Avanti JA 14 rotor for 20 minutes. The cell pellet was resuspended in 10 ml Lysis buffer (pH7) and incubated at 4°C for 20 minutes. The sample was sonicated at 50 Hz six times for 10 seconds with 10 second rest intervals. The post sonicated sample was centrifuged at 10 000 × g using a Beckman Avanti JA 14 rotor for 20 minutes to produce a clear enzyme lysate. The lysate was mixed with nickel charged resin (Qiagen), shaking at 150 rpm at 4°C. The lysate-resin slurry was poured into a 5 ml polypropylene column (Qiagen) and allowed to settle. The column was washed twice with 10 ml of the wash buffer (pH 7.0) and the protein was eluted with the addition of three 10 ml washes with elution buffer (pH 7.0). The purified recombinant protein was desalted through over night (O/N) dialysis in a 50 mM sodium phosphate buffer (pH 7.0). Protein fractions were subsequently analysed for purity using SDS-PAGE, enzyme activity assays and protein assays.

Appendix 4: Protein Characterisation – Discontinuous Gel Electrophoresis

The purity of the recombinant proteins, which were purified using nickel-affinity chromatography were analysed using sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the Mini-Protean® 3 Cell instruction manual (modified according to Laemmli, 1970).
The SDS-PAGE required the following components:

1. *10 x SDS-PAGE Running buffer:*

   30.3 g Tris(Hydroxymethyl)Aminomethane
   144.0 g Glycine
   10.0 g sodium dodecyl sulphate (SDS)

The three chemicals were dissolved in distilled MilliQ water to 1 L, the pH was not adjusted. Once dissolved the 10 × running buffer was stores at 4°C. Prior to use, the 10 × running buffer was diluted to a 1 × running buffer by diluting 50 ml 10 × running buffer with 450 ml distilled MilliQ water.

2. *10% SDS Stock Solution*

   1 g SDS was dissolved in 10 ml distilled water

3. *10% Ammonium Persulphate (APS) Stock solution*

   0.1 g APS was dissolved in 1ml distilled water (Prepared freshly)

4. *30% Acrylamide Stock Solution (100ml)*

   29.8 g Acrylamide
   0.2 g Bis-Acrylamide

Made up to 100ml with distilled MilliQ water in a dark bottle or a bottle covered with aluminium foil.

5. *Stacking gel buffer (0.5M Tris-HCl, pH 6.8)*

   Dissolved 6 g Tris(Hydroxymethyl)Aminomethane (Tris) in 100 ml of distilled MilliQ water and adjusted the pH of the buffer to 6.8 with hydrochloric acid (HCl).

6. *Resolving gel buffer (1.5M Tris-HCl, pH 8.8)*

   Dissolved 18.15 g Tris in 100 ml distilled MilliQ water, and adjusted the pH of the buffer to 8.8 with hydrochloric acid (HCl).

7. *SDS reducing buffer*

   7.10 ml distilled water
   2.50 ml 0.5M Tris-HCl buffer (pH 6.8)
   5.00 ml glycerol
   4.00 ml 10% SDS solution (w/v)
   0.4 ml 0.5% bromophenol blue (w/v)

The reducing buffer was stored at room temperature. Prior to use, 50 µl β-mercaptoethanol was added to 950 µl SDS reducing buffer. The protein samples were diluted in a 1:2 ratio with the sample buffer, and boiled for 5 minutes prior to loading the protein samples onto the SDS-PAGE gel. For long term storage, the boiled samples were stored at -20°C.

8. *Coomassie Brilliant Blue Protein Staining*

The Comassie brilliant blue protein stain was prepared by mixing 0.075 % (w/v) Coomassie brilliant blue R250 to a solution of 40% methanol and 0.7% glacial acetic acid. The polyacrylamide gels were stained for a minimum of one hour with shaking.

9. *Coomassie destain solution*

The destain solution was prepared by mixing 45% methanol, 45 % distilled MilliQ water and 10 % glacial acetic acid and mixed by inversion. The polyacrylamide gels were destained until the stained protein bands were visible against a clear background.

10. Gel drying solution

7% Glycerol

10% Ethanol

The solution was made up to 1 L by the addition of distilled MilliQ water and stored at room temperature. After the polyacrylamide gels have been destained, the gels were placed in enough gel drying solution to cover gels for a minimum of 30 minutes before the gels were dried.

11. Preparation of SDS-PAGE gels

The 12% SDS-PAGE resolving gel was prepared by the addition of the following solutions in the following sequence:

3.4 ml distilled MilliQ water

4.0 ml 30% Acrylamide stock solution

2.5 ml 1.5 M Tris-HCl buffer (pH 8.8)

0.1 ml 10% SDS solution

0.1 ml 10% APS solution

0.05 ml TEMED

Once the gel solution had been poured into the gel apparatus, the gel was covered with 0.3 ml isopropanol and allowed to set. Prior to pouring the stacking gel, the isopropanol was removed with filter paper.

The 4% stacking gel was prepared by the addition of the following solutions in the following sequence:

6.1 ml distilled MilliQ water

1.3 ml 30% Acrylamide stock solution

2.5 ml 1.5 M Tris-HCl buffer (pH 8.8)

0.1 ml 10% SDS solution

0.1 ml 10% APS solution

0.05 ml TEMED

Once the stacking gel solution had been poured, plastic combs were inserted into the gel to form the wells and the gel was allowed to set.

Appendix 5: Standard curves for the determination of enzyme activity and protein concentration

5.1 Protein standard curve

The protein standard curve was generated using a modified Bradford protein assay (Bradford, 1976) and bovine serum albumin (BSA) (Sigma) as the reference protein (Figure 43). Various concentrations of BSA were made ranging from 0 and 0.4 mg/ml. The standard curve was generated by mixing 5 µl of the protein standard to 225 µl of the Bradford's reagent and allowed to stand at room temperature for 5 minutes. The samples were gently shaken for 30 seconds, and the change in colour was determined at 595 nm in the Power Wave X microplate reader from Bio-Tek Instruments using Kcjunior software. The standard curve was generated in a Microsoft Excel® worksheet (Figure 43).

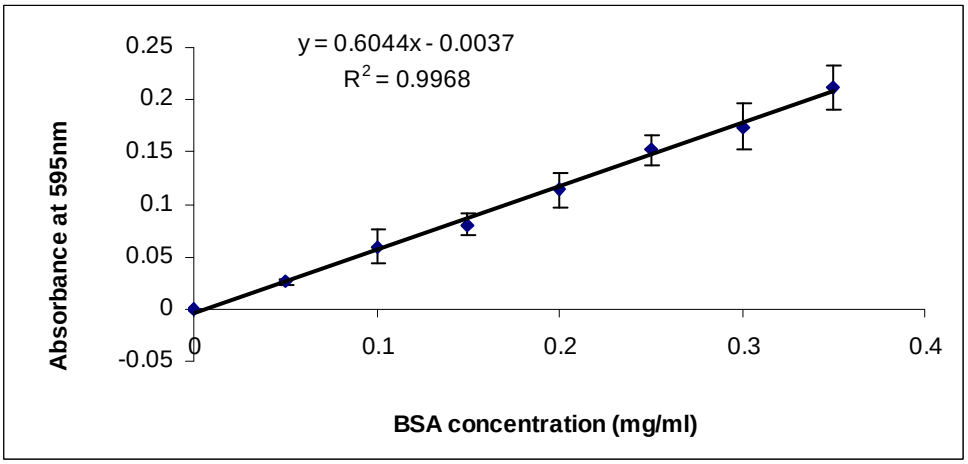


Figure 43: Protein standard curve generated using the Bradford protein assay using various concentrations of BSA as the reference protein (SD±3).

5.2 Sugar standard curves

DNS reagent

100 ml 2% (w/v) Sodium hydroxide
2 g Dinitrosalicylic acid (DNS)
40 g Sodium potassium tartrate (Roschelle salts)
0.1g Phenol
0.1 g Sodium Metasulfite
100 ml Distilled MilliQ water

Once all the components were dissolved, nitrogen gas (Afrox) was flushed through the solution for 30 minutes to remove any oxygen in solution.

The enzyme activities of the recombinant enzymes were determined by quantification of the reducing sugars liberated through the degradation of the various substrates. The quantity of reducing sugars was determined through the use of the DNS carbohydrate assay. Standard curves were generated using a modified scaled down assay based on the carbohydrate assay described by Miller (1959). Sugar standards (glucose, mannose and xylose) were prepared, ranging in concentration between 0 and 10 mM, of which, 300 µl of each was added to 600 µl DNS reagent. The sugar-DNS reagent mixture was incubated at 100°C for 5 minutes, followed by 5 minute incubation on ice. The change in colour was determined at 540nm in a Power Wave X microplate reader from Bio-Tek Instruments using Kcjunior software. Three sugar standard curves were generated using glucose, mannose and xylanose in a Microsoft Excel® worksheet (See Figures 44 – 46).

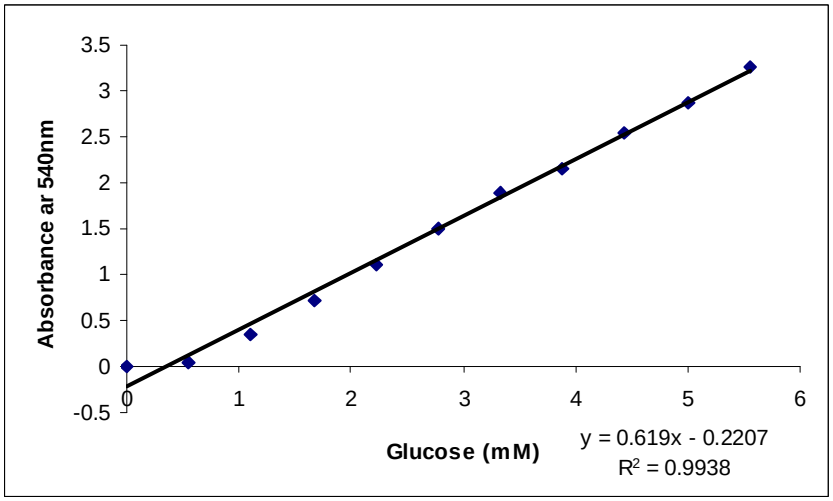


Figure 44: Glucose standard curve generated using the DNS carbohydrate assay. All the readings were performed in triplicate. (n = 3, SD were too small to be visualized).

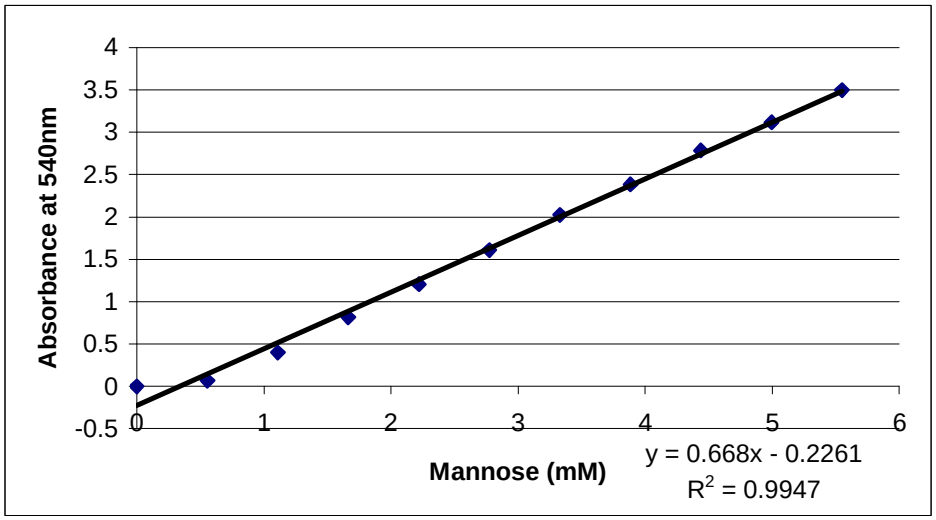


Figure 45: Mannose standard curve generated using the DNS carbohydrate assay. All the readings were performed in triplicate. (n = 3, SD were too small to be visualized).

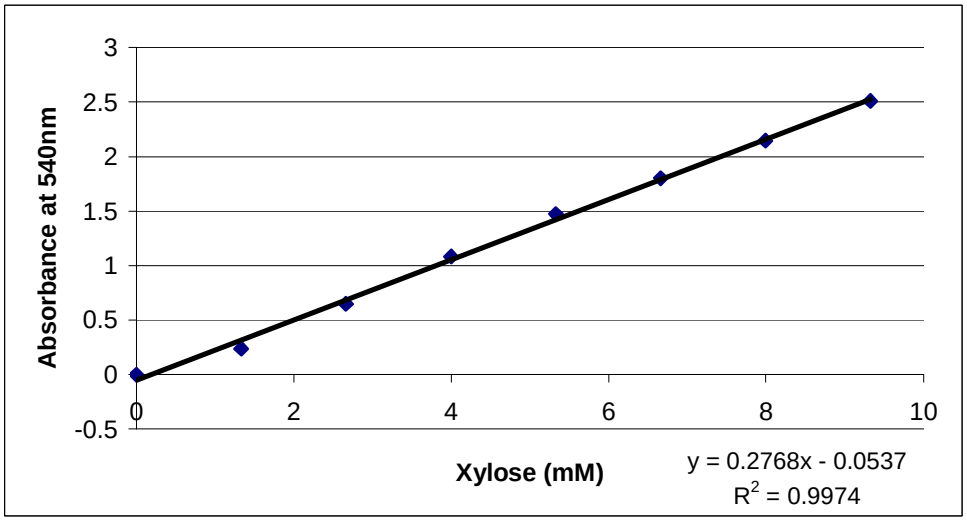


Figure 46: Xylose standard curve generated using the DNS carbohydrate assay. All the readings were performed in triplicate. (n = 3, SD are too small to be visualized).

Appendix 6: Assay components

6.1 Enzyme assays composition

40 µl of the purified enzyme

100 µl of the respective soluble substrate OR mixed substrate mixture OR complex. substrate

50 mM citrate buffer, pH 5.5 (660 µl of the buffer for the soluble substrate OR 760 µl of the buffer for the complex substrates)

The substrates and the buffer were incubated at 50ºC for 5 minutes prior to the addition of the respective purified enzymes. The reaction was performed at 50ºC for 30 minutes for the soluble substrates and for 120 hours for the complex substrates. The reactions were stopped by centrifugation at 16, 000 × g for 1 minute in a Heraeus Biofuge pica micro centrifuge. To 300 µl of the various concentrations of reducing sugar, 600 µl DNS reagent was added to stop the enzyme reaction. The DNS and reducing sugar mixture was incubated at 100°C on the Labnet dry bath for 5 minutes to develop the colour of the samples, and incubated on ice for 5 minute to stop the colour development. The standard curves were generated by measuring the development of the colour of the solutions at 540 nm with the Power Wave X spectrophotometer using KcJunior software. The standard curves were generated in Microsoft Excel®.

6.2 Synergy assays - Enzyme combinations

Table 14: Enzyme combination for the synergy studies

Combination number	Combination	ManA (µl)	XynA (µl)	EngE (µl)	Final volume (µl)
1	M100	40			40
2	M87.5 X12.5	35	5		40
3	M75 X25	30	10		40
4	M62.5 X37.5	25	15		40
5	M50 X50	20	20		40
6	M37.5 X62.5	15	25		40
7	M25 X75	10	30		40
8	M12.5 X87.5	5	35		40
9	X100		40		40
10	X87.5 E12.5		35	5	40
11	X75 E25		30	10	40
12	X62.5 E37.5		25	15	40
13	X50 E50		20	20	40
14	X37.5 E62.5		15	25	40
15	X25 E75		10	30	40
16	X12.5 E87.5		5	35	40
17	E100			40	40
18	E87.5 M12.5	5		35	40
19	E75 M25	10		30	40
20	E62.5 M37.5	15		25	40
21	E50 M50	20		20	40
22	E37.5 M62.5	25		15	40
23	E25 M75	30		10	40
24	E12.5 M87.5	35		5	40
25	M75 X12.5 E12.5	30	5	5	40
26	M62.5 X25 E12.5	25	10	5	40
27	M50 X37.5 E12.5	20	15	5	40
28	M37.5 X50 E12.5	15	20	5	40
29	M25 X62.5 E12.5	10	35	5	40
30	M12.5 X75 E12.5	5	30	5	40
31	M12.5 X62.5 E25	5	25	10	40
32	M12.5 X50 E37.5	5	20	15	40
33	M12.5 X37.5 E50	5	15	20	40
34	M12.5 X25 E62.5	5	10	25	40
35	M12.5 X12.5 E75	5	5	30	40
36	M25 X12.5 E62.5	10	5	25	40
37	M37.5 X12.5 E50	15	5	20	40
38	M50 X12.5 E37.5	20	5	15	40
39	M62.5 X12.5 E37.5	25	5	10	40
40	M50 X25 E25	20	10	10	40
41	M37.5 X37.5 E25	15	15	10	40
42	M25 X50 E25	10	20	10	40
43	M25 X37.5 E37.5	10	25	15	40
44	M25 X25 E50	10	10	20	40
45	M37.5 X25 E37.5	15	10	15	40

Chapter 1: Literature Review

1.1 Cellulosomes

Lamed *et al*. (1983) and Bayer *et al*. (1985) observed large protuberances on the cell surface of *Clostridium thermocellum*. These protuberances have become known as the cellulosomes. Since the discovery of cellulosomes, they have been recognised for their ability to hydrolyse insoluble polymeric substrates, such as crystalline cellulose. Cellulosomes may vary in size due to the formation of aggregates (Felix and Ljungdahl, 1993). In the case with *C. papyrosolvens*, the strain expresses several cellulase-xylanase complexes that aggregate to produce a combined molecular weight of up to 660 kDa (Pohlschrőder *et al.*, 1994). The activity of cellulosomes was initially thought to be limited to cellulose degradation. It has since been shown that these multi-enzyme complexes consist of a variety of degradative enzymes, and not only isomers of the same type of enzyme. Cellulosomes are also able to degrade other carbonaceous compounds, such as hemicellulose, chitin and pectin (Tamaru and Doi, 2001).

Cellulosomes are important to anaerobic microorganisms (bacteria and fungi) for several reasons:

i. The multi-enzyme complexes are assembled to achieve the closest possible proximity amongst the various catalytic components, thus promoting synergy between the enzymes (Shoham *et al*., 1999).
ii. The complexes permit the direct association of the substrate on the cell surface, for example, in the case of cellulose the association results in the formation of an intermediate cellulose-enzyme-microbe ternary complex (Lynd *et al.*, 2002). The insoluble substrates are localized at the interface of the cell through the interactions between the cohesion domains (which are subsequently attached to the substrate) and the dockerin domains that are, in turn, attached to the cell. The regions on the substrate that are solubilised become accessible to the cell for absorption (Shoham *et al.*, 1999).

iii. Organisms that utilise large polymers with high molecular weights produce extracellular enzymes to degrade the polymer into subunits. These subunits are accessible to the cell, via osmosis, endocytosis and other similar mechanisms. The problem with substrate degradation by the extracellular enzymes is that the diffusion of degradative products by the cell, results in the establishment of low cell densities. This is due to the low concentrations of the available soluble substrates. The cellulosome provides a solution to this problem, as it attaches the substrate to the cell (Figure 1A and B) (Shoham *et al.*, 1999).

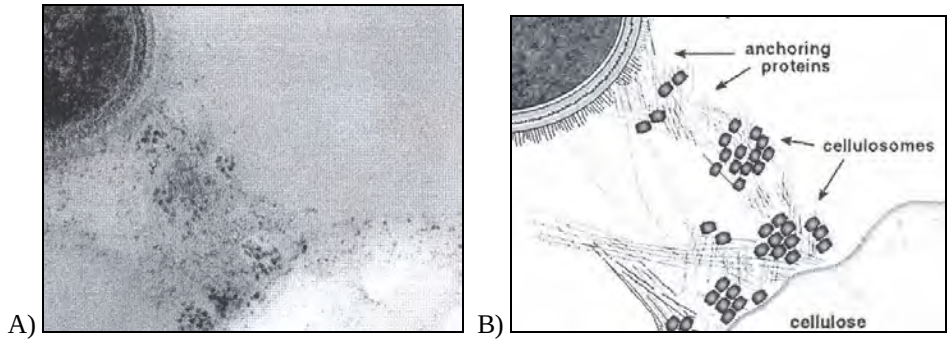


Figure 1: Formation of the ternary complex. A ternary complex is formed between the host cell, enzyme complex and substrate, in what seems to localise the substrate to the interface of the host cell. This localization ensures the released glycosyl moieties are accessible to the host cell (Bayer *et al.*, 1998b).

iv. The substrate degradation is regulated by feedback inhibition (Shoham *et al.*, 1999). The hydrolysis of carbonaceous compounds is not inhibited when the microorganism is present in a niche composed of a variety of carbonaceous compounds.

v. The presence of a diverse group of enzymes within the cellulosome, enables the microbes to degrade a variety of carbonaceous compounds in close proximity with varying degrees of specificity (Schwarz, 2001).

vi. The arrangement and subsequent interactions between cellulosomal components, and their respective interactions avoids the non-productive adsorption of various substrates on the cell surface (Schwarz, 2001).

vii. The attachment of the cellulosome to the cell surface eliminates competitive binding between the enzyme constituents (Schwarz, 2001).

viii. Cellulosomes isolated from different cellulosome-producing species are cross-reactive, thus they may serve as a tool for the identification of the cellulosome-associated species (Lamed *et al.*, 1987).

ix. Cellulosomes allow cellulolytic organisms to inhabit natural environments, where cellulose is present as the primary carbon source for example, in plant cell walls (Pohlschröder *et al*., 1994). This allows microorganisms to grow on cellulose and to play a crucial role in the anaerobic cycling of carbon (Hammel *et al.*, 2004).

Cellulosomes of bacterial origin are characterised into two groups: those that are composed of a single scaffoldin, as in the case of *C. cellulovorans*, and those that exhibit multiple scaffoldin proteins, as in the case of *C. thermocellum* (Bayer *et al*., 2004). The cellulosomal scaffoldin protein is essentially a large glycosylated protein, with several functional domains. The diversity of cellulosomes are established through the varying quantity of cohesion domains in a given scaffoldin, the expression of single or multiple scaffoldin proteins, as well as the presence/absence of carbohydrate binding modules (CBMs) or surface layer homology regions (SLHs). For example, the scaffoldin proteins of the mesophilic clostridia generally lack dockerin sites of SLH domains for anchoring the protein complex to the cell surface. They may contain a similar component that will facilitate binding of substrates to the cell. In the case of the mesophilic clostridium, *C. cellulovorans*, the endoglucanase E (EngE) is expressed as a conserved cellulosomal protein. This enzyme potentially mediates the binding between the cell surface and the cellulosome in a similar fashion to SLH domains (Tamaru and Doi, 1999; Kosugi *et al*., 2002a).

Cellulosomal enzymes are normally classified as β-1, 4-glucanases, as they degrade complex polymers into glucans (Mayer *et al*., 1987; Lamed and Bayer, 1988; Shoseyov and Doi, 1990). The non-cellulase cellulosomal enzymes possess the ability to degrade hemicellulose components like, xylan, mannan and lichenan. The protein complex

(cellulosome) randomly incorporates the cellulosomal enzymes that degrade both cellulose and hemicellulose (Perret *et al.*, 2004).

The cellulosomal enzymes have a characteristic modular nature, as they contain catalytic and dockerin domains (Mayer *et al.*, 1987; Lamed and Bayer, 1988; Shoseyov and Doi, 1990). These functional domains facilitate a wide variety of functions. For example, the adhesion of the catalytic domains of the enzymes occurs through Type I cohesion domains. The subsequent adhesion between the bacterial cell surface and the substrates occurs through the Type II dockerin domains (Bayer *et al.*, 1998b; 1999). The dockerin domains consist of highly conserved 22-amino acid repeats commonly known as the duplicated sequence (Tamaru and Doi, 2001). The calcium-dependent (Fierobe *et al.*, 2005) interactions between the cohesion and dockerin domains result in the selective characteristics of the cellulosome and has an effect on the stability of the cellulosome (Bayer *et al.*, 2004).

The carbohydrate-binding domain (CBD) on the scaffoldin protein facilitates the adhesion between the cellulosome and the substrate, e.g. cellulose (Bayer *et al.*, 1998b). To date, 180 CBDs (also known as CBMs), with a diverse range of properties, have been identified. CBDs may have different affinities for substrates, for example, some CBDs bind to crystalline celluloses, while others bind exclusively to amorphous cellulose (Carrard *et al.*, 2000). The CBD interacts with three glucose chains on the surface of cellulose, through a series of aromatic amino acids that are, aligned precisely along the cellulose surface during binding. This precise binding is considered to be the reason for the specific and strong binding which has been observed between the CBD and the crystalline cellulose fibres (Bayer *et al.*, 1998b). The ability of the CBD to disrupt the hydrogen bonds between the glucose chains, which make up the cellulose fibres, results in the cellulose fibres obtaining a more relaxed conformation that has been documented (Din *et al.*, 1991).

1.2 Clostridia

1.2.1 In general

Clostridia are ubiquitous in nature. These sporolating organisms (George and Finegold, 1985) have been isolated from soil and fresh and marine water. Clostridia may also be present in the lower intestinal tract in humans and animals and thus found in faecal matter (Miwa, 1975; George and Finegold, 1985; Kameyama *et al*., 1996; Borneman and Triplett, 1997; Nüsslein and Tiedje, 1998). The vast majority of species within the genus *Clostridium* produce rod shaped vegetative cells that may vary from coccoid to filamentous forms. In rare cases, the vegetative cells can join in to produce a spiral configuration. The vegetative cells that produce a spiral configuration have been seen for *C. cocleatum and C. spiroforme* (Cato *et al*., 1986). The genus *Clostridium* consists mainly of gram-positive organisms (Allen *et al*., 1999), Collins *et al*., 1994); however, in the strains *C. ramosum, C. clostridiiforme* and *C. tetani*, the cultures appear gram-negative after a 24-hour incubation period. In the case of *C. tetani* the culture generally appears to be gram-negative when the culture begins to sporolate (Brock and Madigan, 1988). There are species, e.g. *Clostridium cellulovorans* that are gram negative (Tamaru and Doi, 1999). Clostridia are characteristically obligate anaerobes; however, a few species that have been isolated in the past have had the ability to tolerate a short period of exposure to oxygen, for example *Clostridium perfringens* (Brock and Madigan, 1988; Shimiza *et al*., 2002). Clostridia obtain ATP at substrate-level phosphorylation because the genus does not possess a cytochrome system for electron transport phosphorylation (Brock and Madigan, 1988). The metabolic pathways by which clostridia obtain energy are diverse because of the wide variety of substrates that are utilised. Clostridia are fermentative and/or proteolytic in nature (Brock and Madigan, 1988).

1.2.2 *Clostridium cellulovorans* (ATCC 35296)

The cellulosome that was isolated from *C. cellulovorans* by Shoseyov and Doi (1990) had a molecular weight of approximately 900 kDa. *C. cellulovorans* is a solventogenic strain (Schwarz, 2001) that has the ability of utilising a wide variety of carbon sources for growth, e.g. cellulose, xylan and pectin (Sleat *et al*., 1984; Tamaru and Doi, 2000). *C. cellulovorans* has been documented to completely degrade crystalline cellulose (Avicel) as a substrate and utilises Avicel as the sole carbon source during growth (Hazelwood and Gilbert, 1993).If the *C. cellulovorans* cellulosome has been dissociated, the subunits are only active against soluble glucans, e.g. amorphous cellulose (Shoseyov *et al*., 1992). *C. cellulovorans* only has cellulolytic activity against insoluble crystalline cellulose, when the cellulosomal components have been assembled (Shoseyov *et al*., 1992). An interesting feature of the *C. cellulovorans* cellulosome is that it appeared to be devoid of xylanase activity, but was able to use xylan as a component to support growth (Hazelwood and Gilbert, 1993). Tamaru and Doi (1999) subsequently showed that EngE had xylanase activity, thus it was originally thought that the xylanase activity of the *C. cellulovorans* cellulosome was due to the presence of EngE. In contrast, Kosugi *et al*. (2001) found that *C. cellulovorans* had the ability to utilise various carbon sources as well as xylan and pectin. Xylanase activity was observed in *C. cellulovorans* cellulosomes, when the cells were grown on xylan-based media (Kosugi *et al*., 2001). Sleat *et al*. (1984) reported that *C. cellulovorans* was able to use pectin as the carbon source required for growth. In 2001, Tamaru and Doi proposed that *C. cellulovorans* had pectinase activity, even though there had been no previous reports of the presence of pectate lyase genes within the clostridial species. The pectate lyase gene *pelA* was identified in *C. cellulovorans*, and shown to possess the sequence for a cellulosomal dockerin domain at its C-terminus (Tamaru and Doi, 2001).

The three major subunits that have been identified on the *C. cellulovorans* cellulosome are the scaffoldin protein (CbpA) (Shoseyov *et al*., 1992) (Figure 2), endoglucanase EngE (Tamaru and Doi, 1999) and exoglucanase ExgS (Lui and Doi, 1998). The 189 kDa CbpA possesses a CBD (Hansen, 1992; Shoseyov *et al*., 1992), four hydrophilic regions

(HLDs), which are homologous to SLHs (Goldstein *et al*., 1993; Shoseyov and Doi, 1990; Tamaru and Doi, 1999), and nine hydrophobic regions (HBDs) (Figure 2) (Shoseyov *et al*., 1992; Takagi *et al*., 1993; Doi and Tamaru, 2000). The HBDs later became known as the catalytic domains (Doi and Tamaru, 2000).

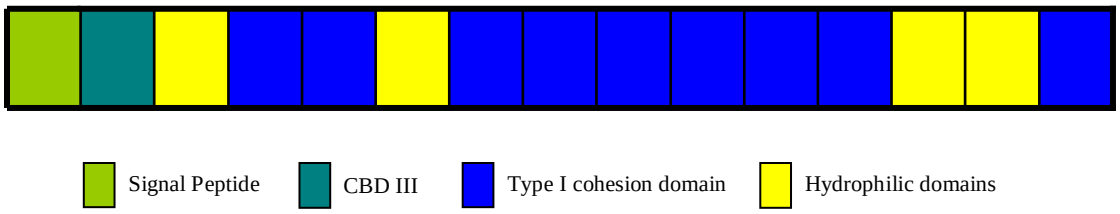


Figure 2: The proposed scaffoldin protein for *C. cellulovorans*. The *C. cellulovorans* scaffoldin protein contains a signal peptide sequence, a family III carbohydrate binding domain, nine hydrophobic type-I cohesion domains that will bind the dockerin domains present on the cellulosomal enzymes (CBD), and four hydrophilic domains (Doi *et al*., 2003).

The *C. cellulovorans* cellulosome consists of up to nine catalytic subunits, which bind to a noncatalytic scaffold protein (Figure 3) (Shoseyov *et al*., 1992). The catalytic composition of the cellulosome may vary depending on the substrate that is added to the medium used to culture *C. cellulovorans* (Han *et al*., 2003a, b, 2004).; however, three major subunits, CbpA, EngE and ExgS are always expressed in the different cellulosome subpopulations (Han *et al*., 2005). Assembly of the *C. cellulovorans* cellulosome requires the presence of a scaffoldin protein (CbpA) (Lamed *et al*., 1987), which has a strong binding affinity for crystalline cellulose (Shoseyov *et al*., 1992).

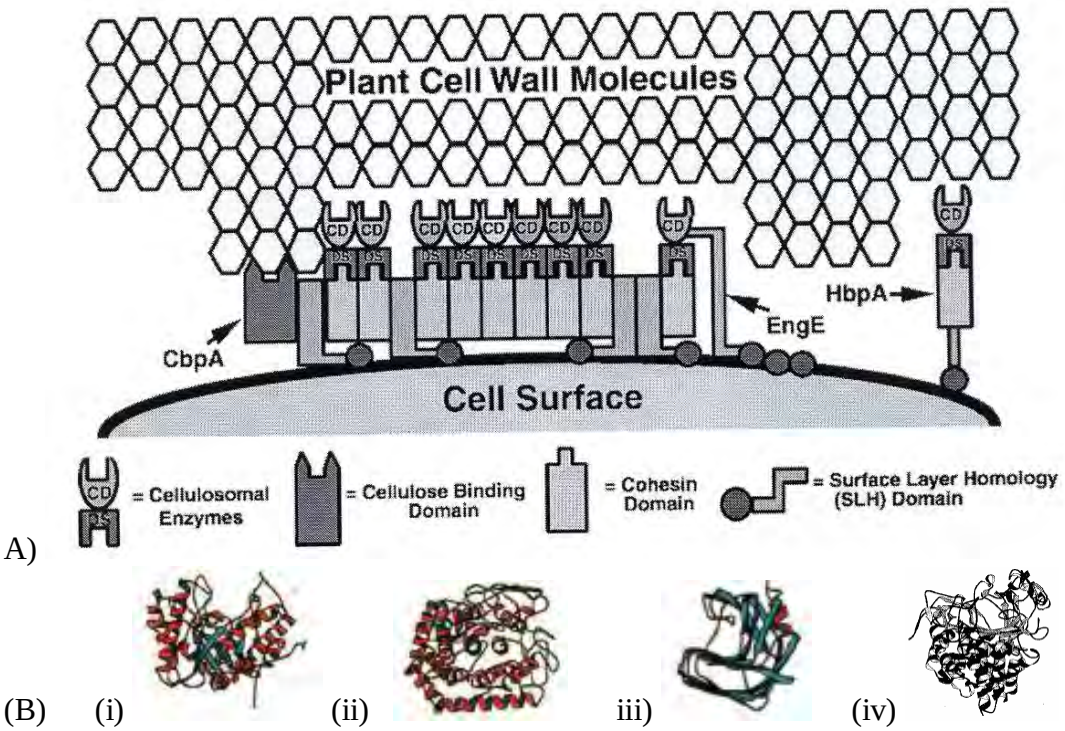


Figure 3: (A) The general cellulosome structure of *Clostridium cellulovorans.* The scaffoldin is depicted in light grey, the enzyme components in dark grey, the endoglucanase catalytic sites as open bowls and the catalytic sites of the exoglucanases as ellipsoids, X 1-4 (hydrophilic domains), 1-9 are the cohesion modules, and SLH is depicted as triple squares (Schwarz, 2001). (B) The crystal structures for the glycosyl hydrolase family 5 (i) is an example of mannanases and some endoglucanases, which has the general $(\beta/\alpha)_8$ barrel structure; for the glycosyl hydrolase family 9 (ii) is an example of endoglucanases E has the general $(\alpha/\alpha)_6$ barrel; for the glycosyl hydrolase family 11 (iii) is an example of xylanases has the general β-jelly roll structure; and family 48 (iv) is an example of exoglucanases. In this case, ExgS has the general $(\alpha/\alpha)_6$ barrel structure (Henrissat and Bairoch, 1993; Davies and Henrissat, 1995; Bayer *et al.*, 1998a; Tamaru and Doi, 1999; Doi and Tamaru, 2000; Kosugi *et al* 2002a).

The best studied *C. cellulovorans* cellulosomal enzymes are the endoglucanases, three hemicellulases and an exoglucanase (Table 1).

Table 1: *C. cellulovorans* cellulosomal components (Tamaru *et al.*, 2000; Tamaru and Doi, 2001; Kosugi *et al*., 2002b; Doi *et al*., 2003).

	Structure*	kDa	GenBank Accession number
Non-catalytic components			
CbpA	CBD-SLH-$(HBD)_2$-SLH-$(HBD)_6$-$(SLH)_2$-HBD	189	M73817
HbpA	SLH-HBD	25	AF123735
Exoglucanase			
ExgS	GH48-DS1	80	U34793
Endoglucanases			
EngB	GH5-DS1	49	M37456
EngE	$(SLH)_3$-GH5-X-DS1	112	AF105331
EngH	GH9-CBM3-DS1	79	U34793
EngK	CBM4-Ig-GH9-DS1	97	AF132735
EngL	GH9-DS1	58	AF132735
EngM	CBM4-Ig-GH9-DS1	96	AF132735
EngY	CBM2-GH9-DS1	80	AAG59608
Hemicellulases			
ManA	DS1-GH5	47	AF132735
PelA	X-CBD2-GPL9-DS1	94	AF105330
XynA	GH11-DS1-CE4	57	AF435978

*Abbreviations for the molecular structure: GH (glycosyl hydrolase), Ig (immunoglobulin-like module), DS1 (Dockerin domain type I), X (unknown domain), CE4 (carbohydrate family 4, acetyl xylan esterase), GPL (polysaccharide lyase family 9, pectate lyase)

The *C. cellulovorans* CBD has the ability to bind to chitin, because of the similarity between the chitin and cellulose backbones (Goldstein *et al*., 1993). The homology between the SLHs and the HLDs present on the scaffoldin (CbpA) (Doi and Tamaru, 2000) indicates that the HLDs may play a role in the attachment of the cellulosome to the surface of cell walls (Tamaru and Doi, 1999). SLH domains are present in the major cellulosomal component EngE, thus in the case of mesophilic clostridia, e.g. *C.*

cellulovorans, the SLH domains are often recognized as HLD domains (Kosugi *et al*., 2002a; Tamaru and Doi, 1999). The presence of nine hydrophobic repeats makes the cellulosome very hydrophobic in nature (Lamed *et al*., 1987). The assembly of the cellulosome is thought to be mediated by the interactions that occur between the HBDs and the endoglucanase subunits (Lamed *et al*., 1987; Mayer *et al*., 1987; Shoseyov and Doi, 1990; Fujino *et al*., 1992; Funjino *et al*., 1993).

The promoter region for *C. cellulovorans* is highly conserved and shares a strong similarity to the promoter sequence from other gram-positive bacteria, i.e. the σ^A promoter (Han *et al*., 2003a). The cellulosomal genes of *C. cellulovorans* are clustered in such a way that the scaffoldin gene is succeeded by the dockerin-borne enzymatic genes (Figure 4), some of which are transcribed as polycistronic mRNA units (Bagnara-Tardif *et al*., 1992; Tamaru *et al*., 2000; Doi *et al*., 2003), and others as monocistronic mRNAs (Tamaru *et al*., 2000; Han *et al*., 2003 a and b).

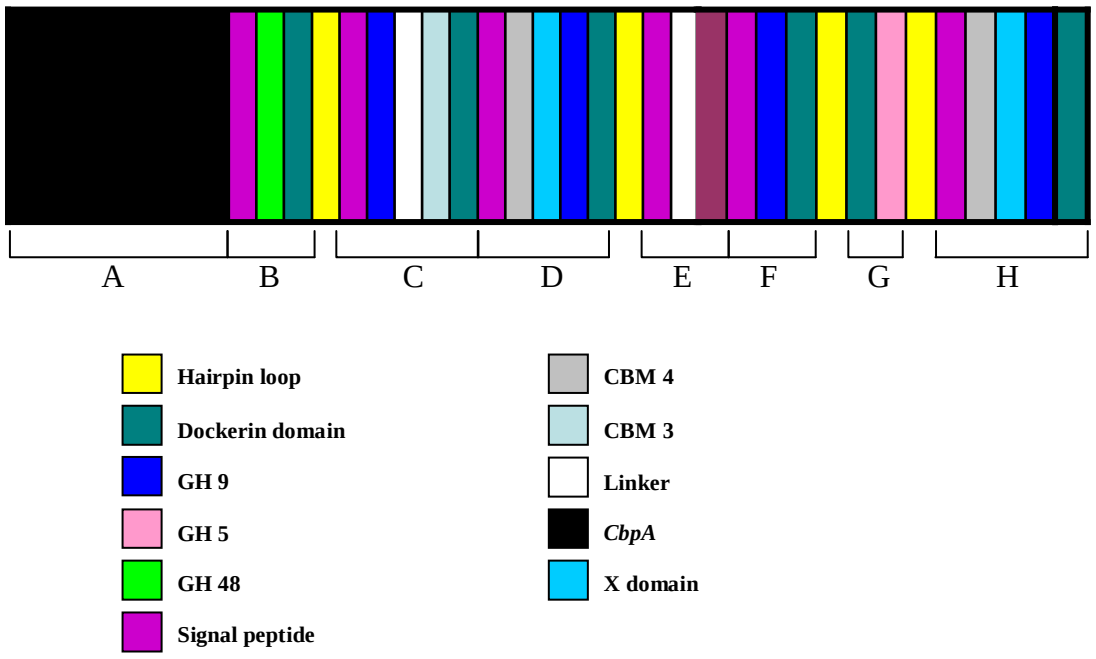


Figure 4: *C. cellulovorans* cellulosomal gene cluster of some of the enzyme genes (A) *CbpA*, (B) *exgS*, (C) *engH* (D) *engK* (E) *hbpA* (F) *engL* (G) *manA* (H) *engM* (Bayer *et al*., 2004; Tamaru *et al*., 2000).

The polycistronic operons that have been observed in *C. cellulovorans* are the transcripts encoding the mRNA for: (1) *cbpA*, *exgS*, *engH* and *engK*, (2) *cbpA* and *exgS*, and (3) *engK*, *hbpA* and *engL* (Bayer *et al*., 2004). HbpA is a hydrophobic (Shoseyov *et al*., 1992) surface anchoring protein, which contains a SLH domain and a Type I cohesion domain, and is thought to play a role in mediating binding of the dockerin domains of cellulosomal proteins to the cohesion domains (Schwarz, 2001). The enzymes, which are transcribed by the genes *manA* and *engM* are the monocistronic genes linked to the large gene cluster. The monocistronic mRNA genes *engE*, *xynA* and *pelA* are not linked to the main cellulosomal gene cluster (Bayer *et al*., 2003). The dominant *C. cellulovorans* cellulosomal gene cluster appears to have a tranposase gene present at its 3' end, suggesting the possibility that horizontal gene transfer (Tamaru *et al*., 2000) may have introduced the cluster. The majority of the cellulosomal genes have been efficiently expressed when the culture is grown on high molecular weight substrates, such as cellulose, xylan and pectin. However, the expression level is greatly reduced when *C. cellulovorans* is grown on smaller compounds such as cellobiose, fructose, mannose, lactose, glucose, galactose, maltose and sucrose, which may be the products of plant cell wall degradation. This suggests that the expression of cellulosomal genes may be subject to catabolite repression. An anti-termination mechanism is also thought to play a role in regulating the expression of some of the cellulosomal genes (Bayer *et al*., 2004).

1.3 Cellulose and cellulases

1.3.1 Cellulose

Cellulose and hemicellulose (xylan and galactomannan), comprise approximately 40-50% of plant cell wall components. They are also considered to be the largest component of the earth's biomass (Doi *et al*., 2003).

Cellulose is a linear insoluble biopolymer that is composed of the repeated units of β-D-glucopyranose/glucose monomers linked via β-glycosidic bonds (Pereira *et al*., 1988). Several cellulose macromolecules associate in a parallel fashion to form a microfibril

(Ljungdahl and Eriksson, 1985) and these microfibrils in turn associates to form fibres (Figure 5) (Atalla, 1993).

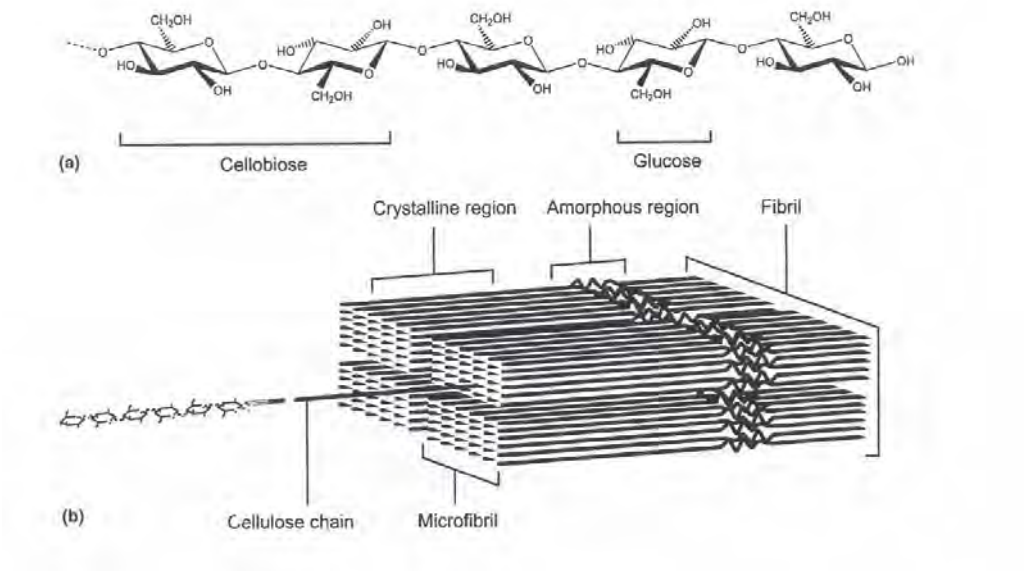


Figure 5: Structure of paracrystalline cellulose commonly found in nature (Desvaux, 2004).
(a) Represents the fundamental composition of cellulose, namely adjacent glucose monomers that produce cellobiose. (b) Represents paracrystalline cellulose, which is commonly known as natural cellulose, and is composed of both crystalline and amorphous cellulose

There are three forms of cellulose: amorphous, crystalline and paracrystalline. Amorphous cellulose is soluble and thus it is easily degradable. The solubility of amorphous cellulose is due to the loose association between the microfibrils, which result in the β-glycosidic bonds having an increased exposure to a variety of hydrolases that may be present in the surrounding environment (Young and Rowell, 1986). Crystalline cellulose is considered to be insoluble, because of the chemical homology of its composition, which induces crystallization (Schwarz, 2001). Crystallization increases the polymers' resilience to the enzymatic degradation by several β-1,4-glucanases (Young and Rowell, 1986). The tightly packed cellulose chains are stabilised by hydrogen bonding, aiding in the formation of tight, regular arrays that shield the glycosidic bonds from enzymatic attack (Young and Rowell, 1986; Rose and Bennett, 1999). Natural

cellulose is paracrystalline in composition. This is reflected in the presence of alternate amorphous and crystalline regions (Figure 5b) (Desvaux, 2004).

The variability in cellulose structure is thought to contribute to the polymer's recalcitrance to complete hydrolysis by individual enzymes. The most efficient means of cellulose degradation is through the concerted action of a variety of enzymes present in complex microbial enzymatic systems (McLean *et al.*, 2000). A possible reason for the resistance to the action of individual enzymes is the composition of natural biomass, where cellulose and hemicellulose are often tightly connected and intertwined, thus limiting the degradation by individual enzymes (Murashima *et al*., 2003).

1.3.2 Cellulases

The degradation of crystalline cellulose to produce reducing sugars such as glucose requires the synergistic action of at least three enzymes that possess endoglucanase, exoglucanase and β-glucosidase activities (Kim, 1995; Teeri, 1997; Schwarz, 2001). Endoglucanases, exoglucanases and β-glucosidases are the dominant types of cellulases that are present in the cellulosome. Exoglucanases or cellobiohydrolases are responsible for the cleavage of cellulose chains from the non-reducing or reducing ends of the cellulose chain to release cellobiose residues. This results in the progressive degradation of crystalline cellulose. Endoglucanases cleave cellulose chains in a random fashion. Endoglucanases degrade amorphous cellulose more readily than crystalline cellulose (Carrard *et al*., 2000, Lenting and Warmoeskerken, 2001). The third type of cellulases, β-glucosidases or cellobiases are responsible for the conversion of cellobiose to glucose (Lenting and Warmoeskerken, 2001). The synergistic associations between the enzymes mentioned above are responsible for the hydrolysis of the β-1,4-glycosidic bonds. Via general acid-base catalysis, glycosyl moieties are generated.

The catabolism of cellulose is initiated by structural depolymerisation through the synergistic activity of the cellulosome's catalytic components. The depolymerisation results in the subsequent release of various reducing sugars, i.e. glucose and soluble

cellodextrins (Pereira *et al*., 1988). These are absorbed by the host cell by diffusion. Glycosyl moieties that are taken up by the bacteria and are subjected to further degradation (Schwarz, 2001). The complete degradation of cellulose, and the subsequent metabolism of the glycosyl moieties, result in the formation of water and carbon dioxide as the final products in aerobic conditions and methane (CH_4) in anaerobiosis (Ljungdahl and Eriksson, 1985; Wolin and Miller, 1987).

The cello-oligosaccharides and the glucose moieties that are released remain in close proximity to the host cell, namely in the stagnant region, between the cell surface and the cellulosome (Bayer *et al*., 1998a; Lynd *et al*., 2002).

Cellulases are generally modular in nature, consisting of a catalytic core that is linked to one or more non-hydrolytic domains (Gilkes *et al*., 1991). These non-hydrolytic domains are required as a means of attachment between the catalytic domains and the polymeric substrate, thus they are able to moderate the degradation of insoluble substrates through catalysis (Bayer *et al*., 1994).

EngE consists of 1,030 amino acids, and is encoded by a gene consisting of 3,090 nucleotides. EngE belongs to family five of the glycosyl hydrolases (Henrissat and Bairoch, 1993). EngE is considered one of the three major subunits of the cellulosome isolated form *C. cellulovorans*, and was previously designated as P100 (Doi *et al*, 1994; Shoseyov and Doi, 1990), as a result of the enzymes molecular weight being approximately 100 kDa (Tamaru and Doi, 1999). EngE has a surface layer homology (SLH) domain, which is repeated three times at the N-terminus and a dockerin domain at the C-terminus of the protein (Tamaru and Doi, 1999). SLH domains show a degree of homology to HLDs of CbpA, thus indicating that EngE not only binds to the CbpA through the DS, but also has the capability of binding directly to the cell surface through the SLH domains (Tamaru and Doi, 1999).

In a 100 mM sodium acetate buffer, the enzyme has a pH and temperature optimum of 5.0 and 50°C respectively (Tamaru and Doi, 1999). EngE has very little activity against

acid swollen cellulose, Avicel or xylan; however, it is able to hydrolyse the β-(1-4)-cellulosidic bonds of CMC and lichenan to produce cellobiose as the main degradation product and is capable of transglycosylation (Tamaru and Doi, 1999).

1.4 Hemicellulose and hemicellulases

1.4.1 Xylan

In general, the heteropolysaccharide in hemicellulose has a lower degree of polymerisation (100-2000 units) compared to that of cellulose (1000-14000 units) (Howard *et al.*, 2003).

Xylan is considered the second most abundant renewable carbon source, as it is a major component of plant cell walls (Puls, 1997; Bissoon *et al.*, 2002). Xylan may be up to 30% of the dry weight of monocot and hardwood cell walls (Weinstein and Albersheim, 1979). This hemicellulose is composed of heteropolysaccharides, linked via β-1,4-glycosidic bonds. There is a large degree of diversity amongst xylan from different sources. This is a result of which various substituents groups are covalently bound to the xylan backbone (Biely, 1985; Puls and Poutanen, 1989). Acetyl, 4-*O*-methyl-D-glucuronosyl and α-arabinofuranosyl residues (Whistler and Richards, 1970; Puls, 1997) may be linked to the xylopyranose backbone via β-1, 4-glycosidic bonds (Biely, 1985; Thomson, 1993; Bissoon *et al.*, 2002; Subramaniyan and Prema, 2002). Xylan forms covalent and non-covalent interactions with various other components, such as lignin and cellulose, which increases the recalcitrance of biomass (Subramaniyan and Prema, 2002). The hydrolysis of xylan results in the release of small molecular weight sugars, namely xylose, xylobiose and xylotriose (Dekker and Richards, 1976; Wong *et al*, 1988; Debeire-Gosselin *et al*, 1992a &b) and various other oligosaccharides depending on the substituent groups (Zhao *et al.*, 1997).

Xylose is an economically important sugar due to its wide variety of applications. The most common application is in the production of xylitol, which is a sweetener, with anti-carcinogenic, and low caloric properties (Parajó *et al*., 1998).

Xylans have been described as alkali-soluble, thus these compounds can be precipitated from aqueous solution via alcohols (ethanol, methanol, ethylene glycol, triethylene glycol) and organic solvents (acetone) (Rezzoug and Capart, 1996; Sun and Cheng, 2002; Lynd *et al*., 2002; Pan *et al*., 2005). These have a higher solubility in mineral acids than cellulose (Wilkie, 1983). Xylans are generally situated between lignin and cellulose fibres; however, xylans are intertwined and covalently linked at various points within the overlying sheaths of lignin, resulting in a coat around the underlying cellulose strands (Biely, 1985; Jeffries, 1990; Joseleau *et al*., 1992). Heteroxylans have a complex structure, and as a result, not all xylosidic linkages are equally accessible to hydrolytic enzymes (Wong *et al*, 1988). The degradation of complex heteroxylans requires a consortium of enzymes, namely: xylanases (E.C. 3.2.1.8), xylosidases (E.C. 3.2.1.37), arabinofuranosidases, glucoronidases, galactosidases and acetyl xylan esterases. Xylanases are responsible for the depolymerisation of xylan, through the random hydrolysis of the xylanolytic backbone. Xylosidases cleave off the smaller oligosaccharides, and arabinofuranosidases, glucoronidases, galactosidases and acetyl xylan esterases are responsible for the liberation of side chains from the xylanolytic backbone (Subramaniyan and Prema, 2002) (Figure 6). Xylan chains have been reported to be linked to rhamnose and galacturonic acid on the reducing ends of the xylan chain's backbone rendering the end groups of xylan chains as alkali resistant (Kulkarni *et al.*, 1999). The acetyl groups on the xylan chains are responsible for xylan's partial solubility in water (Whistler and Richards, 1970); however, the acetylated substituents are readily removed when the xylan is subjected to alkali extraction (Dekker, 1989). Terrestrial plant xylans are characterised by a β-1, 4-linked xylopyranosyl main chain carrying a variable number of neutral or uronic monosaccharides (Kulkarni *et al.*, 1999). Hardwood xylans are highly acetylated, e.g. birchwood contains more than one mole of acetic acid per 2 moles of xylose (Dekker, 1989). In contrast, the softwood plant's xylan is mainly arabino-4-*O*-methyl glucuronoxylan, which in addition to 4-*O*-methyl glucuronic acid is also substituted by α-arabinofuranosyl residues (Kulkarni *et al.*, 1999).

1.4.2 Xylanases

Hemicellulases are multi-domain hydrolytic proteins (Henrissat and Davies, 2000; Prates *et al*., 2001). The multi-domain components consist of catalytic and non-catalytic components. The non-catalytic domain consists of a carbohydrate binding module (CBD) that facilitates the binding of the hydrolytic enzymes to the substrate through the binding of dockerin and cohesion domains either to the cell surface or to enzyme complexes, and in this case the cellulosome (Prates *et al*., 2001; Shallom and Shoham, 2003). In general, hemicellulases are either glycoside hydrolases, which catalyse the hydrolysis of glycosidic bonds, or carbohydrate esterases, which catalyse the hydrolysis of the ester linkage of acetate or ferulic acid side chains (Henrissat and Bairoch, 1996; Rabinovich *et al.*, 2002a and b).

Xylanases are economically important due to the wide range of industrial processes that the enzymes are associated with, e.g. the paper and pulp industry (Shoham *et al.*, 1992), bioconversion of lignocellulosic material and agro-wastes to fermentative products (Wong *et al.*, 1988), clarification of juices (Wong *et al.*, 1988), the extraction of coffee, plant oils and starch (Wong and Sadler, 1993) production of fuel (Linko *et al.*, 1989) and the improvement of the consistency of beer and digestible animal feed (Wong *et al.*, 1988; Linko *et al.*, 1989).

Xylanases belong to the glycoside hydrolase families 10 and 11 (Howard *et al.*, 2003). Industrially, xylanases are utilised for a wide range of processes, for example the bleaching of pulp (Bocchini *et al.*, 2003; Damiano *et al.*, 2003), increasing the digestibility of animal feed (Bedford and Classen, 1992), clarification of fruit juices and the degumming of vegetable fibres (Kapoor *et al*., 2001). The molecular weight of xylanase isomers range between 12 to 145 kDa, some isomers may form heterodimers depending on the conditions under which they are expressed (Petrosyan *et al.*, 2002). High molecular weight endoxylanases with low pI values belong to glycanase family 10,

while the low molecular weight endoxylanases with high pI values belong to the glycanase family 11 (Kuno *et al.*, 2000)

Due to the complexity and variability of xylan, the degradation requires synergy between several enzymes that are required for the complex hydrolysis of xylan (Figure 6), namely: 1,4-β-endoxylanases (E.C. 3.2.1.8) which are responsible for the random cleavage of the backbone, and 1,4-β-xylosidases (E.C. 3.2.1.37), which are responsible for the hydrolysis of the smaller oligosaccharides (Subramaniyan and Prema, 2002), α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterases and phenolic acid esterases (Figure 6) (Coughlan and Hazlewood, 1993).

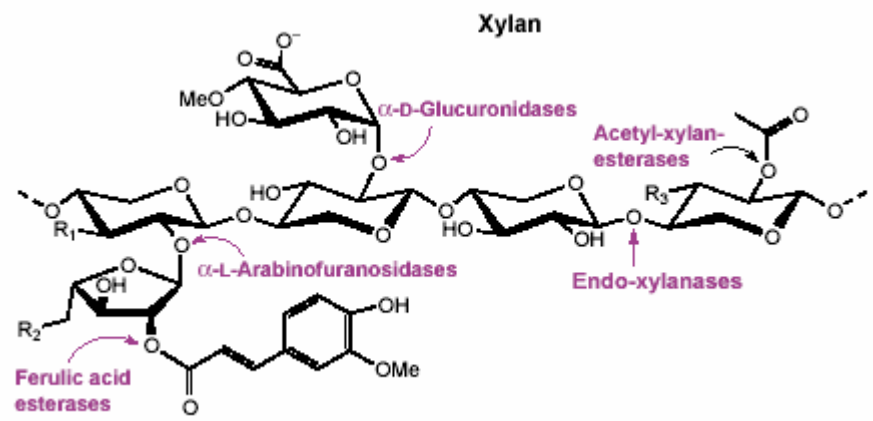


Figure 6: Enzymes involved in the hydrolysis of xylan (Shallom and Shoham, 2003).

Due to the heterogeneity of xylan, not all of the xylanosidic linkages are accessible to hydrolysis. The degradation of xylan not only requires various enzymes, but also the hydrolytic activity of xylanase isomers, which possess overlapping but different specificities (Wong *et al.*, 1988). Thus, the expression of multiple xylanolytic enzymes by the microorganism ensures cooperative action through xylanase synergy (Kelett *et al.*, 1990).

Many xylanases are multi-domain enzymes, i.e. they comprise several discrete domains such as the cellulose binding domain in addition to a catalytic domain (Tomme *et al.*, 1995). They generally have an acidic or neutral pH optimum. Those xylanases that have an alkaline pH optimum are of great interest in the paper and pulp industry as xylans are more readily soluble in alkaline solutions (Onysko, 1993; Tull *et al*., 1991). Similarly to cellulases, xylanases may also exist as endo- and exoxylanases. Endoxylanase activity was differentiated from general xylanolytic activity by using a carboxymethylated derivative of xylan (CMX) which is specific for endoxylanases (Khasin *et al*., 1988), as they are responsible for the cleavage of the internal glycosidic linkages of the backbone (Reilly, 1981). The depolymerising activity of endoxylanases, results in the conversion of complex polymeric substances into xylooligosaccharides and xylose (Shoham *et al.*, 1992).

The multiplicity of xylanases is a result of a variety of factors such as differential mRNA processing, post-secretion modification through proteolytic cleavage and various post-translational modifications such as glycosylation and auto-aggregation (Biely, 1985; Coughlan *et al*., 1993).

The expression of xylanases is constitutive and is controlled by catabolite repression (Brodel *et al.*, 1990). Products of xylan hydrolysis are small molecular weight sugars, e.g. xylose, xylobiose, xylotriose and other oligosaccharides (Wang *et al.*, 1993; Zhao *et al.*, 1997). These molecules enter cells and sustain growth by acting as energy and carbon sources (Biely, 1985; Zhao *et al.*, 1997). Xylose also induces the production of xylanases (Zhao *et al.*, 1997; Biely, 1985).

The cellulosomal xylanases in *C. cellulovorans* have not been well studied; however, Kosugi *et al.* (2001) have indicated that despite the fact that the majority of xylanases are produced independently of the cellulosome, some possess a dockerin domain, which is essential for binding to the cohesion domains on the scaffoldin protein. Cellulosomal xylanase activity has also been reported in *C. acetobutylicum, C. stercorarium and C. papyrosolvens* C7 (Nielsen *et al*., 1993; Wong *et al.*, 1988).

Hydrolysis of xylan-based substrates may result in either retention or inversion of the anomeric centre of the reducing sugar monomer of the carbohydrate, suggesting one or two chemical transitional states (Clarke *et al.*, 1993). The glycosyl transfer, that occurs, results in the nucleophilic substitution at the saturated carbon of the anomeric centre and takes place with either retention or inversion of the anomeric configuration (Figure 7) (Clarke *et al.*, 1993). The general double displacement mechanism for the anomeric-retention of the products occurs as follows (Figure 7b):

1. acid catalysis results in the protonation of the substrate (Clarke *et al.*, 1993),
2. carboxyl group of the enzyme is positioned on a covalent glycosyl enzyme intermediate with the carboxylate in which the anomeric configuration of the sugar is opposite that of the substrate (Clarke *et al.*, 1993),
3. the covalent intermediate is reached from both directions through transition states involving oxo-carbonium ions (Clarke *et al.*, 1993),
4. various non-covalent interactions provide most of the rate enhancement (Clarke *et al.*, 1993).

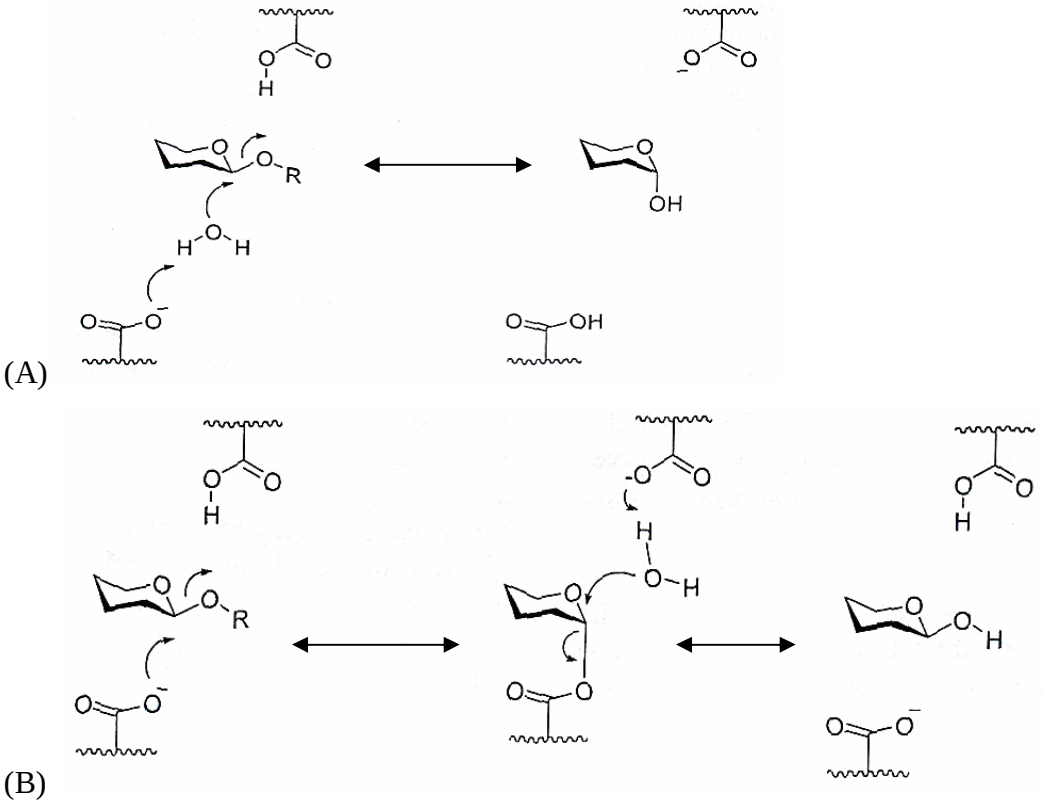


Figure 7: The general catalytic mechanisms of glycosyl hydrolases. (A) Illustrates the general inversion mechanism. (B) Illustrates the general retention mechanism (Schlülein, 2000).

Thus, the proposed mechanism for xylanase activity occurs in a similar fashion, where:

1. xylan is recognized and bound by xylanases,
2. the xylosyl residue becomes distorted and is pulled down toward the catalytic residues. This results in the glycosidic bonds becoming strained, and the bonds break to form the substrate-enzyme covalent intermediate,
3. an activated water molecule, following the classic retaining glycosyl hydrolase mechanism, attacks the intermediate. The products are released (Leggio *et al.*, 2000).

Clostridium cellulovorans constitutively expresses several xylanases ranging from 28 – 57 kDa (Han *et al*., 2003a); however, the major xylanases are 47 and 57 kDa (Kosugi *et al*., 2001). Kosugi *et al*. (2001) illustrated that the expression of xylanases varied when *Clostridium cellulovorans* was grown on different carbon sources, namely glucose, cellobiose, cellulose, Avicel, birchwood and oat spelt xylan. However, the highest xylanase activity was observed when the bacterium was grown on birchwood xylan, using a sodium phosphate buffer (pH 7) at 37°C. The 2.7kb *xynA* (Han *et al*., 2003a) encodes a 57 kDa protein with the signal sequence ATKTITXNETGNF (Kosugi *et al*., 2001).

1.4.3 Mannan

Mannan forms a significant part of the hemicellulolytic component of softwoods, legumes, nuts and beans (Puls and Schuseil, 1996). Galactomannan is a mannopyranose chain with galactopyranose side chains (Figure 8) (Kusakabe *et al*., 1986).

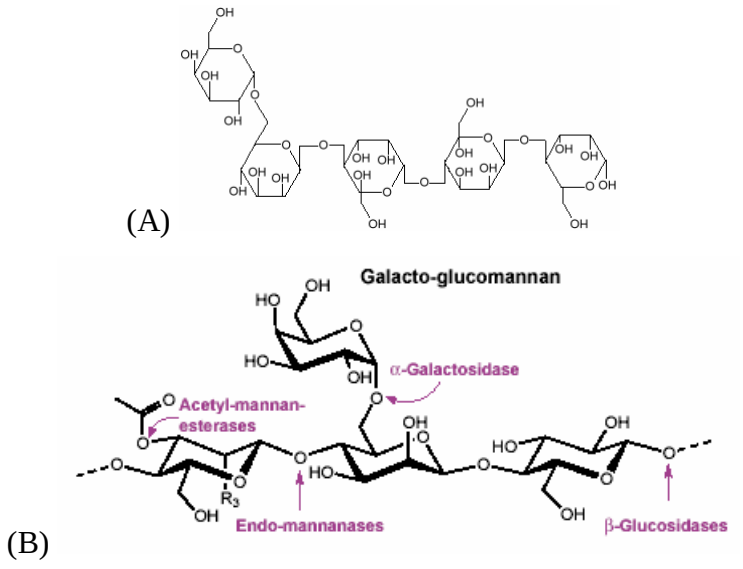


Figure 8: (A) Locust bean gum (*Ceratonia siliqua*) galactomannan, (B) Enzymes responsible for the degradation of galactomannans (Shallom and Shoham, 2003).

1.4.4 Mannanases (E.C. 3.2.1.78)

Mannanase are members of family five of the glycosyl hydrolases (GH5) (Tamaru and Doi, 2001). Like other hemicellulases, mannanases are secreted by microorganisms as multiple enzyme isomers (Johnson *et al*., 1990)**.** Endo-β-mannanases depolymerise the mannan backbone through random cleavage to release mannobiose units as well as a variety of other manno-oligosaccharides. The cleavage by exo-β-mannosidases releases mannose (Halstead *et al*., 1999). Random hydrolysis of the 1,4-β-D-mannopyranosyl linkages in 1,4-β-mannans, glucomannans, and galactomannans (McCleary, 1988) occurs via a retaining mechanism the glycosyl hydrolase family 5 releases mannose, glucose and galactose (Figure 9) (Davies and Henrissat, 1995). The GH 5 mannanases have eight amino acids, which are strictly conserved within the sixty GH5 cellulases (Sakon *et al*., 1996; Wang *et al*., 1993). The eight highly conserved amino acids are located in or near the active site. They are therefore believed to either aid in substrate binding (Davies *et al*., 1997) or stabilise and protonate the catalytic glutamate residue (Dominguez *et al*., 1995 & 1996; Ducros *et al*., 1995; Sakon *et al*., 1996).

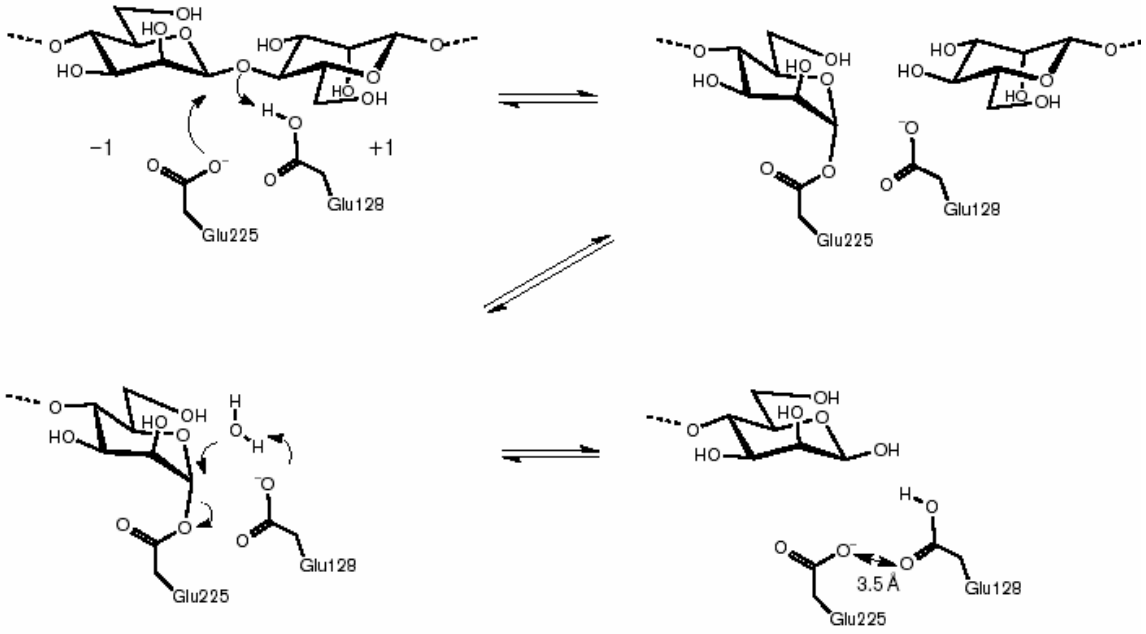


Figure 9: Enzymatic mechanism for glycosyl hydrolases family 5. The retaining mechanism of the β-mannanase (glycosyl hydrolase family 5) from *Thermomonospora fusca* explains the basic principles for the hydrolysis of β-glycosidic bonds for glycosyl hydralases in family 5. Glu128 acts as a proton donor by protonating the glycosidic oxygen of the mannanase. The anomeric carbon is attacked by the nucleophile Glu225, resulting in the formation of the mannosyl-mannanse intermediate. The intermediate is subsequently hydrolysed by water, releasing the product (Davies and Henrissat, 1995).

β-Mannanases liberate β-1, 4-manno-oligosaccharides through the hydrolysis of mannan. The manno-oligosaccharides are subsequently hydrolysed by β-mannosidases. Approximately 50 mannanases are found within the glycoside hydrolase families 5 and 26 (Howard *et al*, 2003). Endo-1,4-β-mannanases are used in combination with 1,4-β-endoxylanases in the paper and pulp industry to bio-bleach softwood pulps (Viikari *et al*., 1994).

The ManA dockerin (duplicated sequence/DS) sequence is present at the N–terminus of the *Clostridium cellulovorans manA* gene (Tamaru and Doi, 2000). The ManA catalytic domain shows a high degree of similarity to fungal mannanases, whereas its dockerin domain shows a high degree of similarity to other dockerin domains within the *Clostridial* genus (Tamaru and Doi, 2000).

The cellulosomal mannanase (ManA) from *C. cellulovorans*, which is encoded by a 1,275 nucleotide gene, is approximately 45 kDa in size and consists of 425 amino acids. The predicted molecular weight of ManA is 47 kDa (Tamaru and Doi, 2000). The predicted molecular weight of 47 kDa includes a signal sequence (Ala-X-Ala-Ala), the removal of which produces a protein with a molecular weight of approximately 44.5 kDa (von Heijne, 1985). ManA is a member of family five of the glycosyl hydrolases, which includes some endoglucanases (Ethier and Doi, 1998). ManA has a pH and temperature optimum of 7.0 and 45°C, respectively, using a piperazine-*N*,*N*'-bis(2-ethanesulfonic acid (PIPES) buffer at pH 7.0 (Tamaru and Doi, 2000). ManA was able to degrade glucomannan and locust bean gum effectively and was unable to degrade carboxymethylcellulose (CMC), thus ManA is unable to hydrolyze β-(1-4)-cellulosidic linkages, and is only effective on β-(1-4)-mannosidic linkages (Tamaru and Doi, 2000).

1.5 Natural biomass

The 1970s saw the development of an energy crisis. This increased the focus on the utilisation of the biological wastes (biomass) as potential energy sources. Previous studies were focused on the production of energy from agricultural wastes and by-products as a renewable energy source. In contrast to the usage of traditional fossil fuels, the use of biomass as a fuel source does not emit additional carbon dioxide into the atmosphere, because the carbon dioxide is absorbed by the crops that are replanted after biomass as been harvested (McKendry, 2002).

Biomass is defined as the total organic mass from any biological material that is economically important renewable carbon source (Howard *et al.*, 2003). Natural biomass is composed of a wide variety of polymers, namely cellulose (approximately 35-50%), hemicellulose (approximately 20-30%), and lignin (approximately 20-30%) (Puls, 1997; Bissoon *et al.*, 2002; Subramaniyan and Prema, 2002; Tamaru and Doi, 2001). As a result of the cellulose composition of biomass, it is the dominant carbon source in nature (Taiz and Zeiger, 1991). The degradation of plant biomass in anaerobic environments occurs through the catalytic activity of various lignocellulolytic bacteria and fungi. The best

established lignocellulolytic microorganisms belong to the *Clostridial* species (Leschine, 1995).

Lignocellulose is defined as a complex lattice structure with close associations between cellulose, hemicellulose and lignin. The lignin is associated to the holocellulose (combination of cellulose and hemicellulose), in such a way that the lignin forms a protective barrier around the holocellulolytic component of the plant biomass. Thus, the cellulose and hemicellulose polymers are protected from enzymatic and microbial attack (Deobald and Crawford, 1997; Crawford and Crawford, 1988; Paneek *et al*., 1998; Wood and Saddler, 1988). Lignocellulose degradation is recalcitrant to anaerobic biodegradation due to the presence of lignin (Breen and Singleton, 1999; Zeikus *et al*., 1982).

Research has focused on the degradation of lignocellulose; however, the exact mechanisms of degradation are not well established. Lignocellulose has always been considered recalcitrant to anaerobic degradation. Benner *et al*. (1985) demonstrated that the pH of the degradative environment may play a role in the degradation of lignocellulose by partially uncoupling the linkages between the lignin polymers and the holocellulose polymers. In the acidic and high alkaline pH range, there is less lignocellulolytic degradation in comparison to that obtained in neutral pH environment. A few years earlier Khan and Trottier (1978) found that the removal of sulphate from an anaerobic system involved in the biodegradation of lignocellulose resulted in a decrease in the cellulolytic activity by the bacterial consortium. Paneek *et al*. (1998) demonstrated that the hydrolysis of complex substrates by the bacterial consortium in anaerobic digestion occurred at an enhanced rate under sulphidogenic conditions similar to that under methanogenic conditions. A few years later, Paneek *et al*. (2001) demonstrated that the bonds linking the aromatic compounds in lignin could be cleaved under biosulphidogenic conditions. Research performed in the last decade has shown that the release of aromatic compounds from complex substrates and the rate of holocellulose hydrolysis is enhanced under conditions where sulphate is reduced (Whittington-Jones, 2000; Roman, 2004).

Lignin is an important component to consider in lignocellulolytic biomass as the degradation of lignin is one of primary rate limiting steps in the degradation of complex plant biomass and plant waste products (Breen and Singleton, 1999; Zeikus *et al*., 1982). Lignin is a structural polymer required to maintain plant cell wall rigidity (Deobald and Crawford, 1997; Crawford and Crawford, 1988; Paneek *et al*., 1998; Wood and Saddler, 1988), and is composed of a variety of polyaromatic compounds. The lignin's aromatic components are bound together via free radical coupling derivatives of *p*-hydroxycinnamyl alcohols (Benner *et al*., 1984; Crawford and Crawford, 1988; Zeikus, 1982). Millet and Baker (1975) indicated that lignin might be depolymerised to soluble products by treating the lignin with sulphur dioxide.

1.5.1 Sugarcane bagasse

Sugarcane (*Saccharum officinarum*) is an important commodity to the local (South African) economy. In addition to being economically important to developing international regions/countries such as Brazil and India, they are two of the world's largest sugarcane cultivators (KaDam, 2002). Sugarcane bagasse is the fibrous organic biomass that remains after sugar liquor has been extracted from the sugarcane. Approximately 70% of bagasse is utilised as a fuel source.

Bagasse is utilised in a variety of processes, ranging from the production of steam to generate and maintain the internal power supply required in small sugar mills to crystallise the sugarcane liquor (Khan *et al.* 2004) or may be utilised as a substrate for the production of bioethanol (KaDam, 2002); however, large quantities bagasse is required in order for it to be utilised by sugar mills as an energy source. Despite the economic importance of the sugar liquor obtained from sugarcane, bagasse is currently of low economic importance (Dawson *et al.*, 1990). The storage of bagasse poses environmental problems due to stockpiles being spontaneously combustible (Dawson *et al.*, 1990). Bagasse is lignocellulolytic in nature. Bagasse is composed of intertwined cellulose and hemicellulose components (Lavarack *et al.*, 2002). The hemicellulolytic component of bagasse is dominantly composed of xylan backbone, with the most common substituents being glucuronic acid and arabinose (Saavedra *et al*, 1989). Sugarcane bagasse was the

first complex plant biomass used in this study. Bagasse is lignocellulolytic in nature, thus it is composed of intertwined cellulose, hemicellulose and lignin components (Lavarack *et al.*, 2002). According to Ouensanga and Picard (1988), sugarcane bagasse has a dry weight composition of approximately 42% alpha-cellulose; 22% lignin; 28% hemicellulose, and cane wax and organic acids compose approximately 8%. The hemicellulolytic component of sugarcane bagasse is predominantly composed of xylan backbone, with the most common substituents being glucuronic acid and arabinose (Saavedra *et al*, 1989). Thus, it would be expected that XynA and EngE would have the ability to partially degrade the holocellulolytic components of sugarcane bagasse.

Previous studies have focused on the degradation of bagasse through the acid hydrolysis of the linkage between the cellulose and hemicellulose components to produce a variety of monosaccharides (Lavarack *et al.*, 2002). The acid hydrolysis of bagasse makes use of low concentrations of mineral acids, such as sulphuric and hydrochloric acid (Torget *et al.*, 1990; Youssef *et al.*, 1991; Lavarack *et al.*, 2002).

1.5.2 Pineapple fibre

The pineapple industry is one of the largest agricultural sectors in many countries and regions around the world (Bartolomé *et al*., 1995). Pineapples are exploited as a food crop but have recently also been linked to the production of course textiles (Van Tran 2006) and bio-energy in the form of biogas (Rani and Nand, 2004; Wang *et al*., 2006).

Van Tran (2006) analysed the chemical composition of the pineapple crown leaves. The results indicated that the leaves consisted of: 7 % ash, 23 % acetyl bromide lignin, 53 % holocellulose and 19 % alpha-cellulose. From this it was concluded that the dominant component of the pineapple crown leaves was hemicellulose, as it composes approximately 34 % of the holocellulose component. The composition of the ash was predominately potassium (4 % of the total ash), however silica, aluminium, magnesium, phosphate, sulphur, chloride and iron were also detected (Van Tran, 2006).

The chemical composition of pineapple leaf fibres appears to vary with age and type of the pineapple leaves. The chemical composition of pineapple leaf fibre is predominantly cellulose, with a total percentage ranging from 70% to 82%. The hemicellulose content is approximately 18% and the lignin content may vary between 5% – 12%. A previous study has indicated that pineapple crown leaves have a chemical composition of approximately 19% cellulose, 34% hemicallulose, 7% ash and 23% acetyl bromide lignin (Satyanarayana 1986; Doraiswamy and Chellamani, 1993; Majumdar and Chandas, 2001; Reddy and Yang, 2005, Van Tran, 2006).

1.6 Synergistic Relationships

Synergism is defined as the interactions that occur between two or more components that when combined, produces a total effect that is greater than the sum of the effects of the individual components. Previous synergy studies, focusing on the synergistic relationships between cellulosomal and non-cellulosomal cellulase isozymes, are well established (Beldman *et al*., 1988; Gaudin *et al*., 2000; Henrissat *et al*., 1985; Irwin *et al*., 1993, 2000; Jeoh *et al*., 2006; Murashima *et al*., 2002a; Nidetzky *et al*., 1994, Watson *et al*., 2002; Wood and McCrae, 1972, 1979; Wood *et al*., 1989, Woodward *et al*., 1988). With regard to the synergistic association between cellulosomal cellulases and other cellulosomal enzymes, the relationships have not been studied extensively. Ciruela *et al*., (1998) observed the first synergistic association of this type when EngE from *C. thermocellulum* was added to CipA (scaffoldin protein). Murashima *et al*. (2003) established the synergistic relationship between the cellulosomal cellulases (EngE, EngH and ExgS) and xylanase A (XynA) on the degradation of corn cell walls through the construction of recombinant xylanase cellulosomes and cellulase cellulosomes (EngE, EngH, ExgS and CbpA). The presence of synergistic relationships between enzymes other than cellulase based systems has been documented during the last decade. A synergistic effect between a xylanase and an acetyl xylan esterase has been demonstrated for the degradation of hemicellulose fibres (Blum *et al*., 2000). Koukiekolo *et al*. (2005) observed an increase in the degradation of corn fibre due to the synergistic association between XynA and ArfA (α-arabinofuranosidase) when bound to CbpA. A similar

synergistic association was observed with XynA and EngL on the degradation of cellulose and hemicellulose (Koukiekolo *et al.*, 2005). However, Koukiekolo *et al*. (2005) observed that there was no synergistic relationship between EngE and EngL. Kosugi *et al*. (2002c) showed a synergistic relationship between XynA and ArfA on the degradation of arabinoxylan, corn fibre gum and corn stem powder. This relationship had also been seen between ArfA, XynA and BgaA (β-galactosidase) on the same substrates as well as on the degradation of arabinogalactan using a 50 mM phosphate buffer (pH 6) at 37°C. However, BgaA and XynA did not show a synergistic association on the degradation of any of the substrates used in their study (Kosugi *et al*., 2002c).

As can be seen, the synergistic relationships, between different enzymes on the degradation of a variety of substrates have been documented; however, the potential synergistic associations between mannanase, pectate lyase, and different esterases etc., have not been well established. This is an important factor to consider, since lignocellulosic biomass constitutes a wide variety of compounds and not just cellulose and hemicellulose. Thus, in order to design an ideal enzymatic system that will promote the complete hydrolysis of lignocellulosic biomass, the substrate composition and subsequently the ratio of different enzymes required to promote the synergistic degradation of the biomass is important. Therefore, an in depth study into the synergistic relationships between several enzymes is essential.

1.7 Importance of sulphur-containing compounds

Sulphur and various sulphur derivatives are ubiquitous and are involved in a variety of biological processes. Sulphur and sulphur derivatives, especially sulphide, are produced during biosulphidogenesis, as a result of sulphate reduction (Postgate, 1984).

Most of the sulphur in the environment is in an oxidized state and therefore it is inaccessible to microbes. Sulphate is reduced to hydrogen sulphide (H_2S) prior to becoming biochemically useful to organisms. The reduction of sulphate in microbial systems requires energy, in the form of ATP, in order to produce sulphide (Postgate,

1984). There are two forms of sulphate reduction, assimilatory and dissimilatory sulphate reduction. Assimilatory sulphate reduction involves the reduction of sulphate to sulphide, which is subsequently incorporated into various sulphur-containing organic molecules (Roy and Trudinger, 1970). Dissimilatory sulphur reduction is an energy yielding process performed to obtain the energy required for microbial growth and maintenance (Postgate, 1984). Sulphate ions are used in anaerobic respiration as electron acceptors (Widdel, 1988; Stetter, 1996).

The initial step in the bacterial reduction of sulphate is the importation of sulphate into the cell. This process occurs in conjunction with the movement of sodium ions across the cell membrane. The transport of sulphur into a cell is proportionate to the electrochemical potential that is created by the flow of sodium ions into and out of the cell (Cypionka, 1989). The main problem associated with the usage of sulphur in bacteriological systems is that bacteria are incapable of using sulphates directly because of the high-energy requirement for the conversion of sulphate to sulphide. To solve this problem, microbes initially phosphorylate sulphate to produce adenosine phosphosulphate (APS) and pyrophosphate (PP_i) (Brüchert, 2004) using ATP sulphurylases (Shen and Buick, 2004). The formation of APS through ATP sulphurylase activity is common to both the assimilatory and dissimilatory pathways (Shen and Buick, 2004).

The reduction of sulphate in the assimilatory pathway may occur via two mechanisms subsequent to the formation of APS, namely the APS or phosphoadenosine 5' phosphosulphate (PAPS) mechanism. In the PAPS pathway, APS is phosphorylated to PAPS by APS kinase, and is subsequently reduced to sulphite by PAPS reductase. The sulphite is converted to sulphide by sulphite reductase, which is now incorporated into cysteine by *O*-acetylserine sulfhydrylases. The APS pathway varies from the PAPS pathway in that the formation of thiosulfonate is formed from APS, which in turn is reduced to cysteine (Figure 10) (Shen and Buick, 2004).

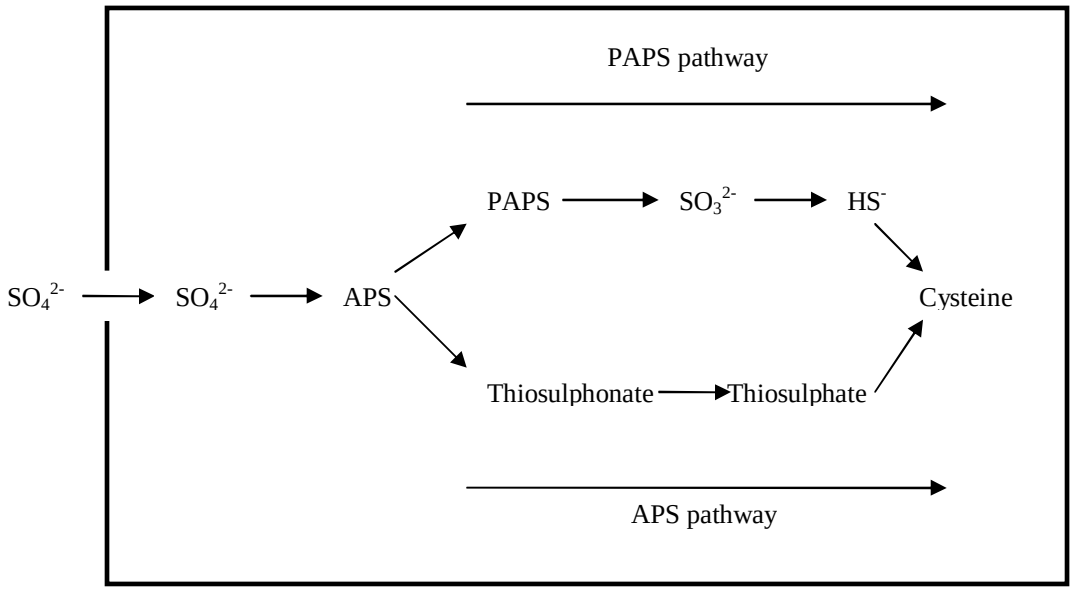


Figure 10: Assimilatory reduction of sulphate (Shen and Buick, 2004).

The dissimilatory pathway is carried out in two steps, (1) endergonically (whereby sulphate is reduced to sulphate by enzymes present in the cytoplasm) and (2) exergonically (where sulphide is converted to sulphite by membrane bound sulphite reductases, as depicted in Figure 11) (Brüchert, 2004, Shen and Buick, 2004).

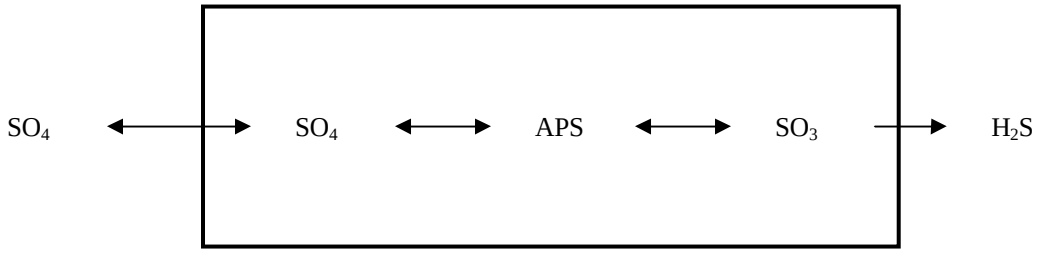


Figure 11: Dissimilatory reduction of sulphate (Shen and Buick, 2004).

It is important to note that clostridia are unable to carry out dissimilatory sulphate reduction (Collins *et al*., 1994).

The sulphide that is produced in both the assimilatory and dissimilatory pathways, similarily with the thiosulphate that is produced in the dissimilatory pathway, may be reoxidised to produce sulphate. The thiosulphate-oxidizing-multienzyme system (TOMES), commonly known as the Sox mechanism (Figure 12) is well characterised (Kelly *et al.*, 1997; Friedrich *et al.*, 2001). Each SoxYZ complex possesses a cysteine residue, which forms a disulphide bond with the sulphite/thiosulphate. The SoxXA complex initiates an oxidation reaction to produce the intermediate complex SoxY-thiocysteine-S-sulphate. The sulphate is removed through the SoxB catalysed hydrolysis to yield thiocysteine-S. The outer sulphur atom of thiocysteine is oxidized to SoxY-cysteine-S-sulphate, through the catalysis of SoxCD. The second sulphate is subsequently removed through the hydrolysis of SoxB, resulting in the regeneration of the SoxY cysteine residue (Friedrich *et al.*, 2001). The released sulphate molecules may then be taken up and subsequently reduced by the various sulphate-reducing prokaryotes present in the microhabitat.

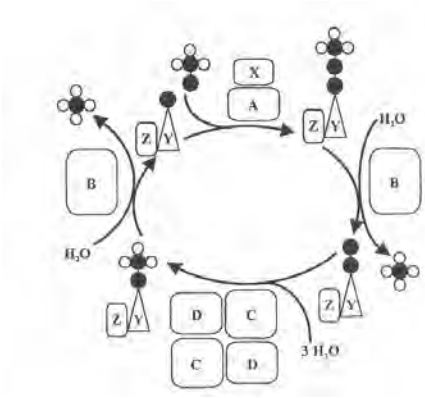


Figure 12: The Friedrich-Kelly model depicting the Sox mechanism. The Sox mechanism is commonly employed amongst various sulphur-oxidizing bacteria to oxidize thiosulphate to sulphate (Friedrich *et al.*, 2001).

1.8 Anaerobic digestion

Anaerobic degradation occurs in a wide variety of aquatic and terrestrial habitats (Novaes, 1986). Anaerobic digestion (Figure 13) is the conversion of a variety of compounds, ranging from insoluble compounds (cellulose) to soluble monomers, and the subsequent degradation of these compounds to produce methane and carbon dioxide. In 1906, Omelianski described the anaerobic digestion of cellulose to produce methane as a process that occurs through bacterial action (Novaes, 1986). The initial step in anaerobic digestion is the hydrolysis of complex insoluble compounds to soluble compounds (Novaes, 1986), for example the hydrolysis of cellulose to glucose monomers. The hydrolysis occurs predominantly through the action of extracellular enzymes like cellulases, lipases and proteases. Thus members of the genus *Bacillus, Clostridium, Butyrivibrio* and other facultative/ obligate anaerobes are utilized in the initial step (Novaes, 1986). The second step is the fermentation of the soluble monomers to produce fatty acids, acetate, carbon dioxide and hydrogen, by acetogenesis or methanogenisis. The third step is the conversion of the fatty acids to acetate, hydrogen and carbon dioxide. These compounds are made accessible to various methanogens to produce carbon dioxide and methane in the final stage (Novaes, 1986).

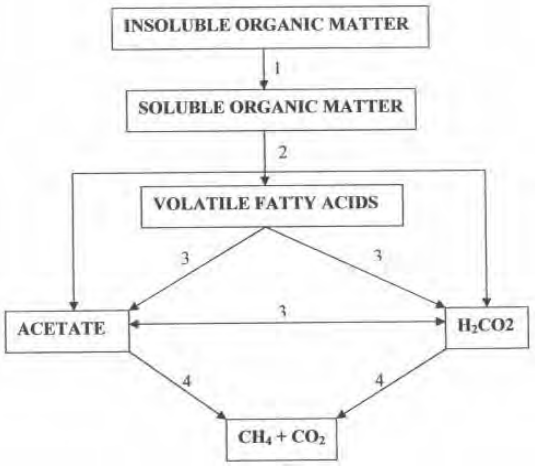


Figure 13: A schematic representation of the metabolic steps involved in anaerobic digestion of organic wastes. Anaerobic digestion takes place in four steps. 1) hydrolysis, 2) fermentation, 3) acetogenesis and 4) methanogenesis (Novaes, 1986).

Sulphate reducing bacteria (SRB) are often associated with methanogens in anaerobic environments as a result of sulphate inhibition observed in methanogenesis. Sulphur and sulphur derivatives, for example sulphide, are important because they potentially enhance the cellulolytic activity during the hydrolysis step. This enhancement of cellulolytic activity was demonstrated by Johnson *et al*. (1982) and Lamed *et al.* (1984) with regards to both the crude extract and purified sample. Lamed *et al.* (1984) observed that a thiol-enzymatic system produced a nine-fold increase in enzymatic activity for both crude and purified samples. The increase in the enzymatic activity under anaerobic conditions is potentially important with regards to achieving an accelerated anaerobic digestion of organic wastes (Figure 13).

This research project focuses on the enzymatic hydrolysis of sugarcane bagasse, specifically on the synergistic association of the cellulosomal hemicellulases mannanase (ManA) and xylanase (XynA) and one of the cellulosomal cellulases (EngE). The research will also investigate the effect of sulphide and sulphate on the synergistic associations, which may be obtained between EngE, ManA and XynA.

Chapter 2: Research motivation and hypothesis

Problem statement

A large component of plant biomass is lignocellulose. The chemical properties of lignocellulose have high biotechnological importance, as a result of the wide variety of industrial applications. A high cellulose content is preferred for the production of textiles and paper and a high hemicellulose content is preferred for the production of sugars and subsequently biofuels. Despite the industrial importance of lignocellulose, it is currently more economically viable to combust the lignocellulose as an energy source rather than to process the lignocellulose for the subsequent production industrially important products. The main problem associated with the use of lignocellulose as a renewable energy source is its recalcitrance to complete enzymatic and microbial degradation.

Microbes secrete a battery of enzymes that may form free enzyme systems where the enzymes act independently to degrade plant cell walls or multi-enzyme complexes that facilitates a close association and subsequently a potential synergistic relationship between the enzymes to degrade plant cell walls. These enzyme systems predominantly consist of cellulases, therefore the synergy studies that have been performed predominantly involved cellulases, thus only partial hydrolysis of lignocellulose substrates have been achieved.

Hypothesis

Cellulosomal enzymes (EngE, ManA and XynA) act synergistically to degrade both the cellulose and hemicellulose components of plant biomass, which will proceed at an enhanced rate in the presence of sulphide and sulphate, to produce valuable products.

Objectives

- To express cloned constructs containing the genes encoding a xylanase (XynA), mannanase (ManA) and an endoglucanase (EngE),
- To establish the effect of synergistic associations on the degradation of complex substrates,
- To establish the synergistic effects of the three *C. cellulovorans* enzymes under biosulphidogenic conditions for the degradation of complex substrates.

Chapter 3: Methodology

3.1 Protein expression

The *Escherichia coli* (*E. coli*) strain used as an expression host was BL21 (DE3). The *E. coli* BL21 mutant strains do not posses *lon* proteases that degrade proteins during purification. In addition, the BL21(DE3) strain does not posses *ompT* outer membrane protease, that are involved in lysis of the cell walls (Grodberg, *et al*., 1988). The strain BL21 (DE3) is a lysogen of the protease deficient bacteriophage DE3. As such, this strain contains a copy of the chromosomal gene encoding the T7 RNA polymerase, *lacI* gene and the *lacUV5* promoter (Novy *et al*., 2001; Studier and Moffat, 1986).

The hemicellulase and endoglucanase genes were cloned into pET-22b(+) (*engE*) and pET-29a(+) (*manA* and *xynA*). The pET-29a(+) vector has two fusions, an S-Tag™ on the N-terminus and a His-Tag® on the C-terminus. The vector also has a T7 promoter, transcription start and terminator sequence, an f1 *ori* and a pBR322 origin, a multiple cloning site and a *lacI* coding sequence. The pET-29a(+) vector also confers kanamycin resistance. The pET-22b(+) vector is similar to pET-29a(+) vector, due to pET-22b(+) vector having a His-Tag® on the C-terminus, the T7 promoter, transcription start sites and terminator sequences; an f1 *ori* and a pBR322 origin, a multiple cloning site and a *lacI* coding sequence. This vector varies form pET-29a(+) vector, in that it has ampicillin resistance and has a *pelB* coding region.

The expression of the target genes in both vectors is induced by isopropylthiogalactoside (IPTG). IPTG is a lactose analogue used for the induction of the *lac* operon. The *E. coli* lac operon consists of a promoter sequence of the lac repressor (*lacI*), followed by the single regulatory gene *lacI*. Downstream of the *lacI*, a single promoter and operating sequence, for the three structural genes (*lac Z, lacy* and *lacA*) are present on the *lac* operon (Turner *et al*., 2000). The most important feature of the *lac* operon, with regard to the expression of the target genes in the pET vectors is the lac repressor. The inducer

(IPTG) binds to the lac repressor, forcing a conformational change, thus reducing the repressors ability to bind to the *lac* operator. The inability of the repressor to bind to the operator site allows the polymerase to transcribe the target genes. Thus in the presence of an inducer, the transcription of genes downstream of the operator site is induced and the protein is expressed (Turner *et al*., 2000). The list of chemicals and their respective suppliers are listed in appendix 1.

3.1.1 Preparation of competent *E. coli* BL21 (DE3) cells

Prior to the transformation, the *E. coli* strain BL21 (DE3) cells were treated chemically to increase their competency (Appendix 2). Yeast-tryptone (2×YT) broth (5 ml) was inoculated with 20 µl of *E. coli* BL21 (DE3) cells. The inoculum was shaken at 200 rpm at 37ºC on a Labcon bench top shaker for approximately 12 hours. The overnight culture was diluted with fresh 2×YT broth (50 ml) to a ratio of 1 to 200. At 37°C, and shaking at 200 rpm, the new inoculum was incubated for approximately 3 hours, until an optical density at 260 (OD_{260}) of between 0.3 and 0.6 was reached. The cells were harvested in sterile centrifugation tubes, by centrifugation at 5, 000×g for 5 minutes at 4°C in a Beckman Avanti centrifuge. The *E. coli* BL21 (DE3) cells were kept on ice for the remainder of the protocol. The harvested cells were resuspended in 50 ml ice-cold, sterile 0.1 M $MgCl_2$ and kept on ice for 20 minutes. The cells were harvested in sterile centrifugation tubes, by centrifugation at 5, 000×g for 5 minutes at 4°C. The pellet was subsequently resuspended in 25 ml ice-cold, sterile 0.1 M $CaCl_2$. The cells were incubated on ice for 2 hours. The cells were harvested as before, and the pellet resuspended in 5 ml ice-cold, sterile 0.1 M $CaCl_2$, in a 1 to 1 ratio, the cells were mixed with 30 % sterile glycerol, and stored at -70°C in suitable aliquots.

3.1.2 Transformation of competent *E. coli* BL21 (DE3) cells

Prof. R.H. Doi, kindly provided the plasmid constructs (Tamaru and Doi, 1999 and 2000; Kosugi *et al*., 2002b). The plasmid constructs obtained included a pET-22b(+) vector with an *engE* insert and two pET-29a(+) vectors with either *manA* or *xynA*. The transformation of competent *E. coli* BL21 (DE3) cells with plasmid constructs occurred as follows: 2 µl of the constructs was added to 200 µl of competent cells and incubated on ice for 20 minutes. The cells were heat shocked at 42˚C for 50 seconds and subsequently the cells were incubated on ice for 2 minutes. To the cells 950 µl 2×YT broth was added and the cells were incubated at 37˚C, shaking at 200 rpm on a Labcon bench shaker, for 2 hours. The cells were harvested by centrifugation at 16, 000×g for 2 minutes in a Heraeus Biofuge pica micro centrifuge. The cell pellets were resuspended in 150 µl of 2×YT broth, and grown on 2×YT agar containing ampicillin (100 µg/ml) for the cells that had been transformed with the pET-22a(+) construct and kanamycin (50 µg/ml) for the cells that had been transformed with the pET-29b(+) constructs.

3.1.3 Expression of *engE*, *manA* and *xynA*

The transformed cells were grown in 250 ml 2×YT broth containing the appropriate antibiotic at 30˚C, shaking at 200 rpm in a Labcon shaking incubator until the OD_{600nm} of the cells using a Powerwave Spectroquant from Bio-Tek Instruments on Kc Junior software, was approximately 0.5. Once the cells reached an $OD_{600\ nm}$ of 0.5, the expression of the proteins was induced by the addition isopropylthiogalactoside (IPTG) to a final concentration of 100 µg/ml for the cells transformed with the pET-29b(+) constructs and 400 µg/ml for the cells transformed with the pET-22a(+) construct. Once IPTG was added, the cells were grown at 19˚C, shaking at 200 rpm in a Labcon shaking incubator. In order to establish the time required for the optimal expression of the enzymes, induction studies were performed.

3.2 Purification of EngE, ManA and XynA

The purification of the endoglucanase and two hemicellulases occurred via a one-step purification protocol (Appendix 3). The purification involved the use of affinity chromatography (that did not rely on the specific physical properties e.g. pI and size of the protein) as a means to separate the protein of interest from contaminating proteins. As the name implies, affinity chromatography exploits specific interactions that may occur between the proteins of interest and various biological compounds (ligand). For example, the protein concanavalin A has a strong binding affinity for glucose. Thus in order to purify concanavalin A, the protein is passed through column containing glucose as the ligand. The concanavalin A remains bound to the column's glucose matrix and contaminating proteins are eluted during washing steps. A solution with a high glucose concentration is applied to the column, to elute concanavalin (Walker, 2000). In this case, *E. coli* BL21 (DE3) expressed the enzymes, EngE, ManA and XynA as His-Tag® proteins thus the presence of the His-Tag® was exploited in the purification of these three enzymes. Nitrilotriacetic (NTA) acid agarose was the resin of choice for the purification of the three enzymes. NTA is a tetradentate chelating adsorbent; therefore it chelates to ligand's binding sites. Nickel ions acted as the ligand in the purification of the His-Tag® proteins. The resin used in the purifications was, Ni-NTA agarose. In Ni-NTA, the NTA resin is chelated to four of the six binding sites present on a nickel ion, allowing the His-Tag® on the expressed proteins to bind to the available binding sites (Hochuli, 1989). His-Tag® proteins are eluted from the Ni-NTA agarose columns with a solution containing a high concentration of imidazole.

3.2.1 Preparation of clear enzyme lysates

Prior to the preparation of the clear lysate, 2 ml of the culture was removed and stored at 4°C for further analysis of the crude enzyme samples. The 250 ml *E. coli* BL21(DE3) cells were harvested by centrifugation in a Beckman Avanti centrifuge at 10, 000×g for 20 minutes. The cells were resuspended in 10 ml lysis buffer (10 mM imidazole, 50 mM disodium hydrogen orthophosphate, 300 mM sodium chloride and lysozyme to a final concentration of 8 mg/ml, pH 7.0), incubated at 4°C for 30 minutes, sonicated at an amplitude of 40 Hz six times for 10 seconds with 10 second rest intervals. The cell debris collected by centrifugation at 8, 000×g for 20 minutes and discarded. The clear supernatant (lysate) was kept for the protein purification.

3.2.2 Affinity chromatography

The clear lysate was added to Ni-NTA)in a 2 to 1 ratio, i.e. 5 ml of the Ni-NTA resin was added to 10 ml enzyme lysate to produce the Ni-NTA-lysate slurry. The Ni-NTA and lysate slurry was incubated at 4°C, shaking at 200 rpm on a Labcon bench top shaker for an hour to allow binding between the His-Tag® present on the over-expressed protein and the nickel resin. The slurry was poured into 5 ml polypropylene columns (Qiagen) and allowed to settle. The flow through was collected, and the contaminating proteins that did not potentially contain a stretch of histidine residues (His-Tag®) removed by the addition of the two 10 ml washing steps with wash buffer (10 mM imidazole, 50 mM disodium hydrogen orthophosphate, 300 mM sodium chloride, pH 7.0). The recombinant enzymes were eluted with 10 ml elution buffer (250 mM imidazole, 50 mM disodium hydrogen orthophosphate, 300 mM sodium chloride, pH 7.0).

3.3 Characterisation of EngE, ManA and XynA

The purified enzymes were characterised using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine purity and molecular weight of the proteins purified by Ni-NTA affinity chromatography. The purified enzymes were characterised according to the activities obtained against a variety of substrates, temperature and pH optima was determined. The effect of the sulphur derivatives, sulphide and sulphate on the hydrolytic activities of the enzymes against different substrates was also studied. Once the individual enzymes analyses were completed, synergy assays were performed to determine the presence or absence of synergistic associations between the cellulase and the two hemicellulases against a variety of substrates. In the presence of a synergistic association, the enzyme molar ratio producing the highest degree of synergy was used to test the effects of the sulphur derivatives.

3.3.1 Discontinuous gel electrophoresis

Protein samples collected throughout the purification were resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine purity according to the protocol outlined in Mini-Protean® 3 Cell instruction manual (Bio-Rad, U.S.A.). This protocol is a modification of that described by Laemmli (1970). Cell pellet from 1 ml of the crude protein sample was harvested by centrifugation at 13, 000 rpm for 1 minute in a Heraeus Biofuge pica micro centrifuge. The cell pellet was resuspended in 50 µl SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, 10 % (v/v) glycerol, 2% (v/v) SDS solution, 5% (v/v) β-mercaptoethanol, 0.05% (v/v) bromophenol blue). The samples collected throughout the purification, were added to the SDS sample buffer in a 2 to 1 ratio. Subsequently the samples were incubated at 100°C for 5 minutes on a Labnet dry bath. The protein samples were resolved using a 12 % acrylamide resolving gel. The resolving gel (0.375 M Tris-HCl, pH 8.8, 0.1 % (v/v) SDS solution, 12% acrylamide solution), was polymerised by the addition of 0.1 % (v/v) ammonium persulphate (APS) and 0.025 % (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED). The gels were resolved for approximately 40 minutes at a constant voltage of 150 V using a Bio-Rad

Power Pac™ Basic, in 500 ml ice cold, 1 × SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 1% (w/v) SDS). The SDS-PAGE gels were stained with Coomassie protein stain (0.1% (w/v) Coomassie brilliant blue G 250 in 45% methanol and 10% glacial acetic acid) for a minimum of 2 hours. The excess and unbound Coomassie stain was removed with destain solution (45% methanol, 45% distilled water, 10% glacial acetic acid).

3.3.2 Protein determination

The protein concentrations of the different samples obtained during the purification were determined using a protocol based on the one described by Bradford (1976). A protein standard curve (Appendix 5) generated used the modified Bradford assay to detect various concentration of the reference protein, bovine serum albumin (BSA). The standard curve was generated by mixing 5 µl of the various concentrations of the BSA with 225 µl of the Bradford reagent. The protein standards stood for five minutes at room temperature to facilitate the binding between the Coomassie brilliant blue G250 present in the Bradford reagent to the basic amino acids, arginine and lysine. The samples were shaken gently for 30 seconds in the Power wave X spectroquant, and the change in colour was determined by readings at 595 nm with Kc Junior software. The standard curve generated with Microsoft Excel® software allowed the direct correlation between the increasing protein concentration with the increasing intensity of the colour obtained through the binding between the protein and the Coomassie brilliant blue dye. The protein concentration of the different samples obtained during the purification was determined using the modified Bradford assay described in the generation of the BSA standard curve.

3.3.3 Enzyme Assays

The quantification of the reducing sugars obtained by the hydrolysis of birchwood xylan (BWX), locust bean gum (LBG), CMC, sugarcane bagasse and pineapple fibre was detected using a modified 3, 4-dinitrosalicylic acid (DNS) protocol originally described by Miller (1959). Sugar standard curves were generated using sugar (glucose, mannose and xylose) concentrations between 0 and 10 mM. To 300 µl of the various concentrations of reducing sugars, 600 µl DNS reagent was added to stop the enzyme reaction. The DNS and reducing sugar mixture was incubated at 100°C on the Labnet dry bath for 5 minutes to develop the colour of the samples, and incubated on ice for 5 minute to stop the colour development. Standard curves were generated by measuring the development of the colour of the solutions at 540 nm with the Power Wave X spectrophotometer, using KcJunior software. The standard curve generated in a Microsoft Excel® software allowed for the direct correlation between the increasing reducing sugar concentrations and the increasing intensity of the colour. In order to allow a direct comparison between the enzymes' activities obtained, the protein concentration (mg/ml) of the enzymes used in the enzyme assays was standardised. The concentrations of the enzymes in the assays were as follows: 0.189 mM of EngE, 0.42 mM for ManA and 0.33 mM for XynA. Enzyme assays were performed at 50˚C using a 50 mM citrate buffer (pH 5.5) for 30 minutes for the 2% (w/v) BWX, LBG and CMC and for 120 hours for the sugarcane bagasse and the pineapple fibre (Appendix 6). After the appropriate time has lapsed for the enzyme assays, the reaction mixtures were centrifuged at 16, 000×g for 1 minute in a Heraeus Biofuge pica micro centrifuge. The reducing sugars released by the enzymatic hydrolysis of the various substrates were measured using the modified DNS protocol. The enzyme activities were expressed in units (U), where 1 unit was defined as the quantity of enzyme required to release 1 µmol of reducing sugar per min under the assay conditions specified.

3.3.4 Activation/Inhibition studies in the presence of sulphur metabolites

Activation and inhibition assays were preformed using sulphide and sulphate over a concentration range of 0 - 15.59 mM. The pH of the buffers containing the sulphur derivatives was adjusted to ensure that the pH of the different buffers remained constant. The reactions proceeded for 30 minutes for the hydrolysis of the soluble substrates (BWX, LBG and CMC) and 120 hours for the complex insoluble substrates (sugarcane bagasse and pineapple fibre). The enzyme activity was determined through the detection of the reducing sugars using the DNS reagent as previously described. After the appropriate time had lapsed for the enzyme assays, the reactions were centrifuged at 16, 000×g for 1 minute in a Heraeus Biofuge pica micro centrifuge. The reducing sugars released by the enzymatic hydrolysis of the various substrates were measured using the modified DNS protocol. The enzyme activities were expressed in units (U), where 1 unit was defined as the quantity of enzyme required to release 1 µmol of reducing sugar per min under the assay conditions previously specified.

3.3.5 Synergy studies

The degree of synergy between the recombinant enzymes was determined as described by Murashima *et al*. (2003) with varying molar ratios of the recombinant enzymes (Appendix 6). Four recombinant enzyme combinations were assayed, namely ManA and XynA, ManA and EngE, EngE and XynA, as well as a combination of the three enzymes. The reactions were performed at 50˚C using a 50 mM citrate buffer (pH 5.5) for 30 minutes for the 2% (w/v) soluble substrate combinations containing the appropriate commercially available substrates for the various enzyme combinations. The reactions that were performed with the sugarcane bagasse and the pineapple fibre were incubated at 50ºC using a 50 mM citrate buffer (pH 5.5) for 120 hours. After the appropriate time has lapsed for the enzyme assays, the reactions were centrifuged at 16, 000×g for 1 minute in a Heraeus Biofuge pica micro centrifuge. The reducing sugars released by the enzymatic hydrolysis of the various substrates were measured using the modified DNS protocol. The enzyme activities were expressed in units (U), where 1 unit was defined as the

quantity of enzyme required to release 1 μmol of reducing sugar per min under the assay conditions previously described.

Activation and inhibition assays were performed using sulphide and sulphate over a concentration range of 0 - 15.59 mM on the enzyme combinations that produced the greatest degree of synergy (as described previously).

Chapter 4: Purification and partial characterisation of the individual enzymes

Three *C. cellulovorans* enzymes that are expressed either as a cellulosomal enzyme (EngE) or as free enzymes that can be incorporated into the cellulosome (ManA and XynA) were studied. The *engE* was cloned into the Novagen pET-22b(+) vector and *manA* and *xynA* were cloned into pET-29a(+) vectors. The IPTG-induced proteins were expressed in *E. coli* BL21(DE3) cells and purified using nickel affinity chromatography. The purified enzymes were partially characterised to determine bioinformatic characteristics, purity (SDS-PAGE), temperature and pH optima. To analyse effect of sulphur derivatives on enzyme activity, sulphide and sulphate were used.

Bioinformatics is a relatively new field of research. Bioinformatics allows a researcher to use computers and specialised software programs to perform *in silico* research to analyse both protein and nucleic acid sequences. This *in silico* research allows the prediction of properties, ranging from peptide function through to the tertiary folding, size and physico-chemical characteristics of proteins (Rapley, 2000).

4.1 *C. cellulovorans* xylanase (XynA)

4.1.1 Bioinformatic analysis of XynA

Bioinformatic analysis, performed on *xynA* sequence (Accession no. AAN32825) was submitted to Genbank by Kosugi *et al*., (2001).

Using the databases Compute/pI and Protparam to analyse, the XynA protein, the xylanases's molecular weight and pI were predicted to be approximately 57 kDa and 8.01, respectively. Amino acid analysis of XynA (Table 2) using Protparam indicated that the xylanase is marginally positively charged and hydrophilic in nature.

Table 2: Amino acid composition of XynA.

Amino acid	Abbreviation	Quantity in the sequence	Percentage of the sequence (%)
Ala	A	30	5.8
Arg	R	9	1.7
Asn	N	32	6.2
Asp	D	29	5.6
Cys	C	6	1.2
Gln	Q	27	5.2
Glu	E	15	2.9
Gly	G	48	9.2
His	H	6	1.2
Ile	I	35	6.7
Leu	L	40	7.7
Lys	K	37	7.1
Met	M	22	4.2
Phe	F	17	3.3
Pro	P	21	4.0
Ser	S	40	7.7
Thr	T	50	9.6
Trp	W	8	1.5
Tyr	Y	23	4.4
Val	V	25	4.8

Using Interpro and My Hits search engine/database, 13 conserved domains (Table 3) and motifs (Table 4) were identified.

Table 3: Putative domains identified in XynA using the database Interpro scan.

Interpro accession number	Domain	Biological function
IPR001137	Glycoside hydrolase, family 11	~ Carbohydrate metabolic process
IPR008985	Calcium-binding EF hand	~ Binds calcium ions
IPR002105	Dockerin type I	~ Polysaccharide catabolic process
IPR002509	Polysaccharide deacetylase	~ Carbohydrate metabolic process
IPR008985	Concanavalin A-likelectin/glucanase	
IPR013319	Glycoside hydrolase, families 11 and 12, catalytic core	

Table 4: Putative motifs identified in XynA using the database My hits.

Position in sequence	Motif information	Legend
25-30	*N*-myristoylation site	Mystryl
34-37	Casein kinase II phosphorylation site	Phosphorylation
36-39	*N*-glycosylation site	Carbohydrate
39-226	Glycosyl hydrolase family 11	
79-82	Amilation site	
79-81	Protein kinase phosphroylation site	Phosphorylation
120-130	Glycosyl hydrolase family 11 active site signature I	
181-184	cAMP- and cGMP dependent protein kinase phosphorylation site	Phosphorylation
211-222	Glycosyl hydrolase family 11 active site signature II	
250-262	EF-hand calcium binding domain	Calcium binding
250-270	Dockerin type I repeat	Binds cohesion domains
314-435	Polysaccharide deacetylase	

There is a 99.1% probability of a signal peptide being present on the *N*-terminus of the sequence, with the potential cleavage site between the two Alanine residues at positions 28 and 29. The database SOSUI predicted that XynA has a transmembrane region between the Valine residue at position 42 of the sequence and Threonine residue at position 64 of the sequence.

4.1.2 Expression and purification of XynA

The pET-29a(+) vector containing the *xynA* gene was used to transform the *E. coli* BL21(DE3) cells. The expression of *xynA* was induced by the addition of IPTG to a final concentration of 100 μg/ml. *E. coli* BL21(DE3) cells were cultured in 2×YT broth at 30°C, shaking at 200 rpm. Once the optical density at 600nm (OD_{600nm}) for *E. coli* BL21(DE3) cells reached 0.5, IPTG was added and cells were grown at 19°C, shaking at 200 rpm overnight (approximately 16 hours). The induced expression of *xynA* was illustrated by the induction of the XynA protein on SDS-PAGE (12% gel) (Figure 14). From the induction of *xynA* expression (Figure 14) it was observed that the greatest quantity of XynA was expressed after 6 hours from the IPTG induction.

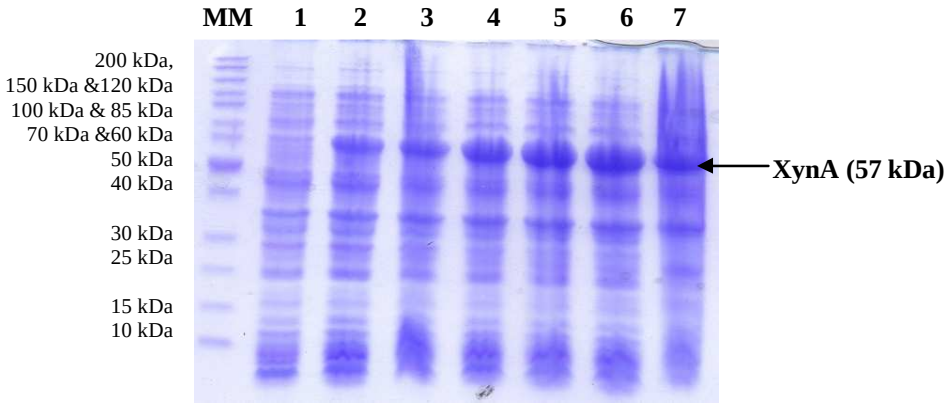


Figure 14: Expression of *xynA*. XynA was induced with the addition of IPTG to a final concentration of 100 µg/ml. SDS-PAGE patterns showing the molecular markers (MM), pre-induction sample (lane 1), post-induction sample after 1 hour (lane 2), post-induction sample after 2 hours (lane 3), post-induction sample after 3 hours (lane 4), post-induction sample after 5 hours (lane 5), post-induction sample after 6 hours (lane 6) and post-induction sample after 17 hours (lane 7).

Once the time required for maximum expression of XynA was established, fresh cultures were grown until the cultures reached an OD_{600nm} of 0.5. The cultures were then induced for 6 hours. The enzyme was purified using nickel affinity chromatography under native conditions. The purification was visualized using a 12% SDS-PAGE gel (Figure 15).

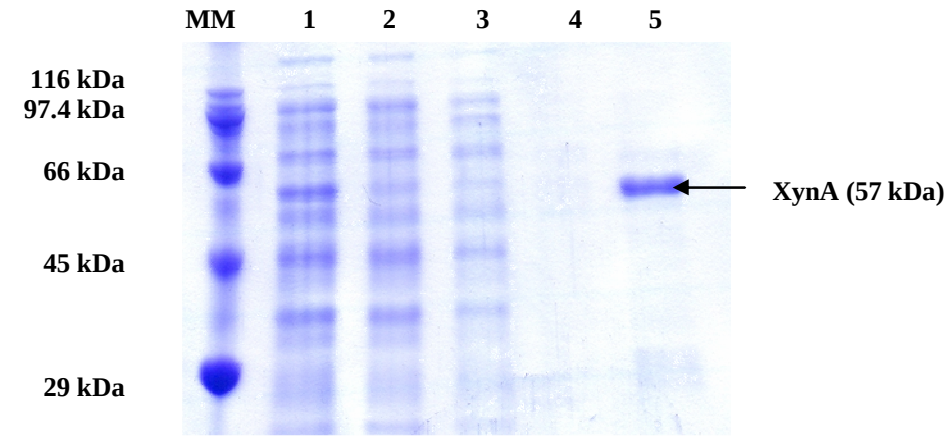


Figure 15: Purification of XynA using nickel affinity chromatography. The SDS-PAGE pattern showing the molecular markers (MM), enzyme lysate sample (lane 1), Ni-NTA column flow through sample (lane 2), wash 1 sample (lane 3), wash 2 sample (lane 4) and eluate (lane 5).

A purification table (Table 5) was produced with data collected at the different purification steps. It was determined that the native purification protocol used was successful in purifying the xylanase. A 42-fold purification was reached after the single purification step, and a yield of 35% indicated that the xylanase constituted a reasonably high percentage of the total protein expressed by *E. coli* BL21(DE3) cells.

Table 5: Purification table for XynA

Step	Volume (ml)	Protein concentration (mg/ml)	Total Protein (mg)	Total enzyme Activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	250	19.86	4965	807.88	0.163	100	1
Lysate	250	5.60	1400	172.33	0.123	21.33	0.75
Flow Through	10	2.89	28.90	37.78	1.307	4.68	8.02
Wash 1	10	1.71	17.10	33.91	1.983	4.20	12.17
Wash 2	10	0.86	8.60	2.03	0.236	0.25	1.45
Eluate 1	10	4.14	41.40	283.41	6.846	35.08	42

* U is defined as µmol xylose released per minute

4.1.3 Characterisation of XynA

The initial characterisation of XynA was performed with a temperature profile ranging from 30°C to 90°C, from which it was established that XynA was active between 30°C and 80°C. The highest activity was obtained at 50°C against birchwood xylan (BWX); however, the enzyme activity that was observed at 30°C to 50°C was similar to that of 50°C (Figure 16). Thus, it was seen that the xylanase was optimally active over a wide temperature range of 30°C to 50°C.

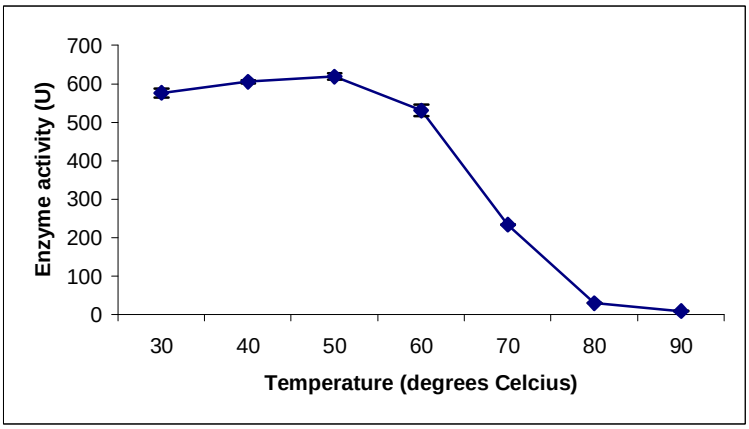


Figure 16: Temperature optimum for XynA using BWX as substrate. The enzyme activity was determined over a temperature range from 30°C to 90°C. Values are presented as mean values ± SD (*n* = 3), which are too small to be visible.

Once the temperature optimum was determined, a variety of 50 mM buffers were used to determine the pH optimum of XynA, covering a pH range of 3.0 to 11.0 (Figure 17). XynA appeared to be stable over a pH range of 4.0 to 9.0, however the highest activity against BWX was obtained at pH 6.0.

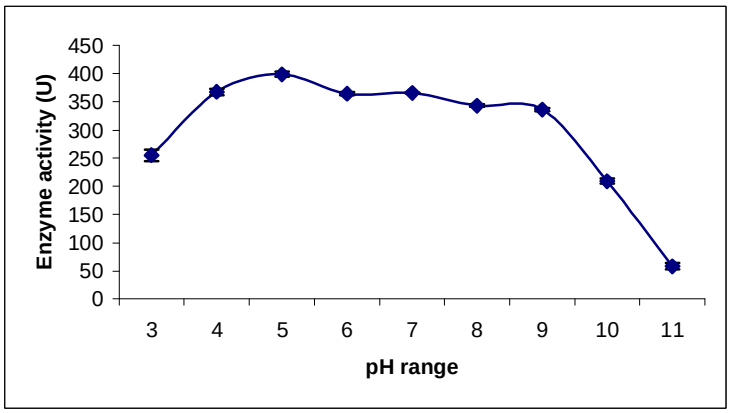
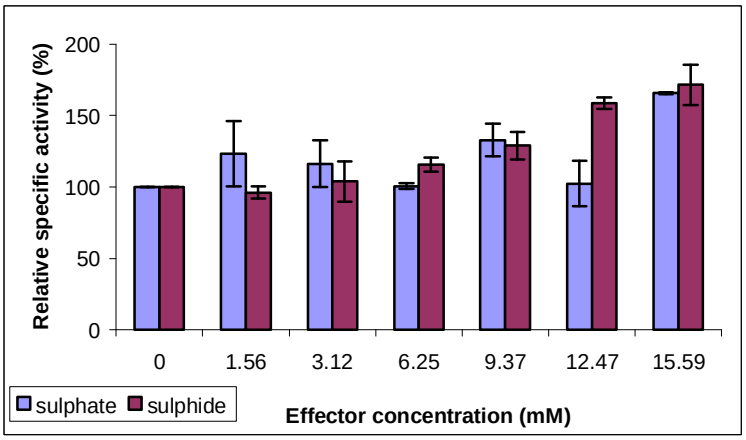
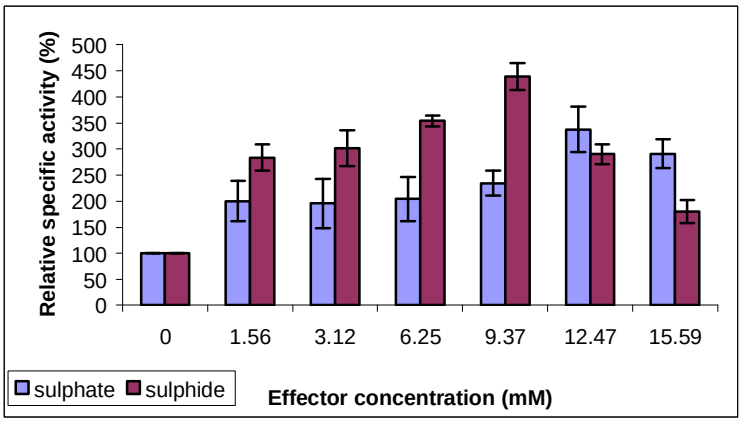


Figure 17: Determination of pH optimum for XynA using BWX as the substrate. A sodium citrate buffer was used for the pH range between 3.0 - 5.0, phosphate buffers were used for the pH range of 6.0 - 8.0 and glycine buffers were used between the pH range of 9.0 - 11.0. Values are presented as mean values ± SD (*n* = 3).

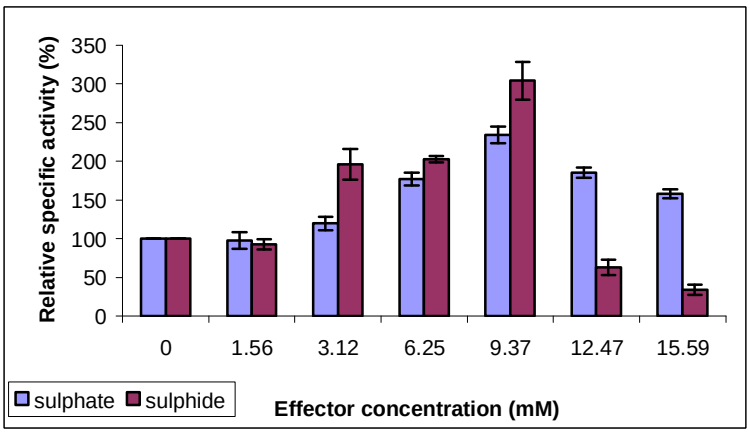
Once the initial characterisation with respect to temperature and pH was performed, the enzyme activity in the presence of the sulphur derivatives, sulphide and sulphate, was determined on BWX, sugarcane bagasse and pineapple fibre (Figure 18). Figure 18a illustrates the effect of the two sulphur derivatives on XynA activity against BWX. It was observed that both sulphur derivatives enhanced the xylanase activity relative to the enzyme assay in the absence of sulphide or sulphate. The effect of sulphur containing compounds on XynA activity against the complex substrates produced a slightly different trend. With the sugarcane bagasse (Figure 18b) there was a general increase in the enzyme activity with increasing concentrations of sulphate, with the greatest activity obtained with the addition of 12.47 mM sulphate in the reaction mixture. Sulphide produced a general increase in the enzyme activity; with the optimum activity obtained with the addition of 9.37 mM sulphide. However, there was a decrease in the enzyme activity with sulphide concentration exceeding 9.37 mM. With the pineapple fibre (Figure 18c), there was a general increase in the enzyme activity; however, the trend is similar to that obtained for the sulphate against the bagasse. The presence of sulphide enhanced the XynA activity against the pineapple fibre until the sulphide concentration reached 9.37 mM, and subsequently there was a drastic decrease in the enzyme activity (Figure 18c).



(a)



(b)



(c)

Figure 18: The effect of sulphide and sulphate on the specific activity of XynA on the degradation of BWX (a) sugarcane bagasse (b) and pineapple fibre (c) as represented by the specific activity relative to the specific activity obtained against the various substrates in the absence of the sulphur derivatives. Values are presented as mean values ± SD (n = 3).

4.2 *C. cellulovorans* mannanase (ManA)

4.2.1 Bioinformatic analysis of ManA

Bioinformatics analysis of *manA* sequence was performed from the sequence (Accession no. AAF06110) published by Tamaru and Doi, (2000).

Bioinformatics analysis of the *manA* predicted that the protein is soluble and does not have any transmembrane regions. Compute pI/Mw and Protparam predicted ManA to have a theoretical pI of 5.62 and that the protein was composed predominantly of hydrophobic amino acids and is negatively charged (Table 6). The predicted molecular weight for ManA, with and without the signal sequence is approximately 47 kDA and 45 kDa, respectively (Tamaru and Doi, 2000).

Table 6: Amino acid composition of ManA.

Amino acid	Abbreviation	Quantity in the sequence	Percentage in the sequence (%)
Ala	A	36	8.5
Arg	R	8	1.9
Asn	N	27	6.4
Asp	D	34	8
Cys	C	5	1.2
Gln	Q	9	2.1
Glu	E	9	2.1
Gly	G	34	8
His	H	8	1.9
Ile	I	26	6.1
Leu	L	27	6.4
Lys	K	28	6.6
Met	M	15	3.5
Phe	F	19	4.5
Pro	P	15	3.5
Ser	S	32	7.5
Thr	T	32	7.5
Trp	W	18	4.2
Tyr	Y	16	3.8
Val	V	27	6.4

Using the databases Interpro (Table 7) and My hits (Table 8), seven putative domains and motifs were identified in the protein sequence for ManA.

Table 7: Putative domains for ManA.

InterPro Accession no.	Domain	Biological Function
IPR002048	Calcium binding EF-hand	~ Binds calcium ions
IPR002105	Dockerin type I	~ Catabolism of polysaccharides
IPR013781	Glycoside hydrolase catalytic core	~ Catalytic activity ~ Carbohydrate metabolic process ~ Cation binding

Table 8: Putative motifs for ManA.

Position in sequence	Motif information	Legend
28-31	Casein kinase II phosphorylation site	Phosphorylation
31-51	Dockerin type I repeat	Cohesion domain binding
65-77	Calcium binding EF-hand	Binds calcium ions
96-101	*N*-myristoylation site	Myristyl
100-103	*N*-glycosylation site	Carbohydrate
158-160	Protein kinase C phosphorylation site	Phosphorylation

According to literature and bioinformatic analysis, the *N*-terminus of the cellulosomal subunit, ManA contains a signal peptide and the conserved sequence (Ala-X-Ala-Ala), with the predicted cleavage site between the two alanine residues at positions 25 and 26 (Tamaru and Doi, 2000; Von Heijne, 1985).

4.2.2 Expression and purification of ManA

A pET-29a(+) vector containing the *manA* gene was used to transform *E. coli* strain BL21(DE3) cells. The expression of *manA* was induced by the addition of IPTG to a final concentration of 100 μg/ml. The *E. coli* BL21(DE3) was cultured in 2×YT broth at 30°C, shaking at 200 rpm. Once the OD_{600nm} for the *E. coli* BL21(DE3) cells reached 0.5, IPTG was added and the cells were grown at 19°C. The induction of ManA (Figure 19)

indicated that the optimal time required for the expression of the protein was 3 hours, after which the quantity of protein appears to be constant.

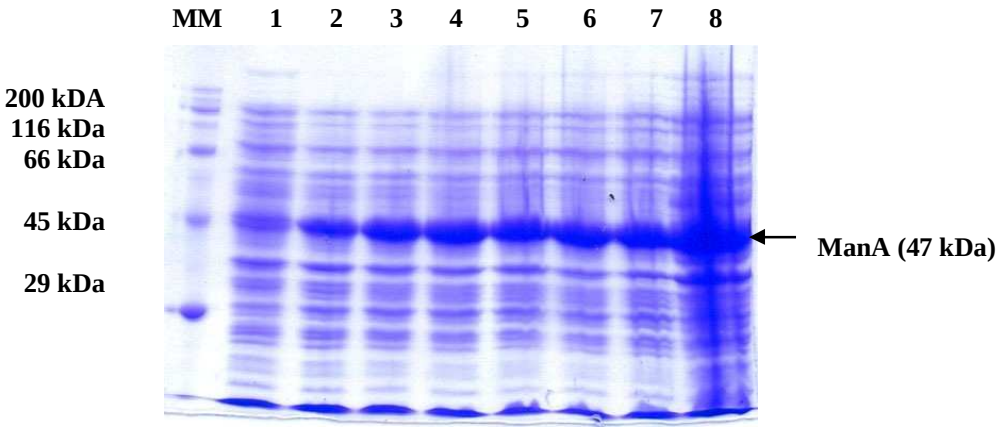


Figure 19: Expression of m*anA*. ManA was induced with the addition of IPTG to give a final concentration of 100 µg/ml. The SDS-PAGE patterns showing the molecular markers (MM), pre-induction sample (lane 1), post-induction sample after 1 hour (lane 2), post-induction sample after 2 hours (lane 3), post-induction sample after 3 hours (lane 4), post-induction sample after 4 hours (lane 5), post-induction sample after 5 hours (lane 6), post-induction sample after 6 hours (lane 7), and post-induction sample after 17 hours (lane 8).

Once the time required for the maximum expression of ManA was established, fresh cultures were grown until the cultures reached an OD_{600nm} of 0.5, and the cultures were then induced for the 3 hours. The enzyme was purified using nickel affinity chromatography under native conditions. The purification was visualized using a SDS-PAGE (12% gel) (Figure 20).

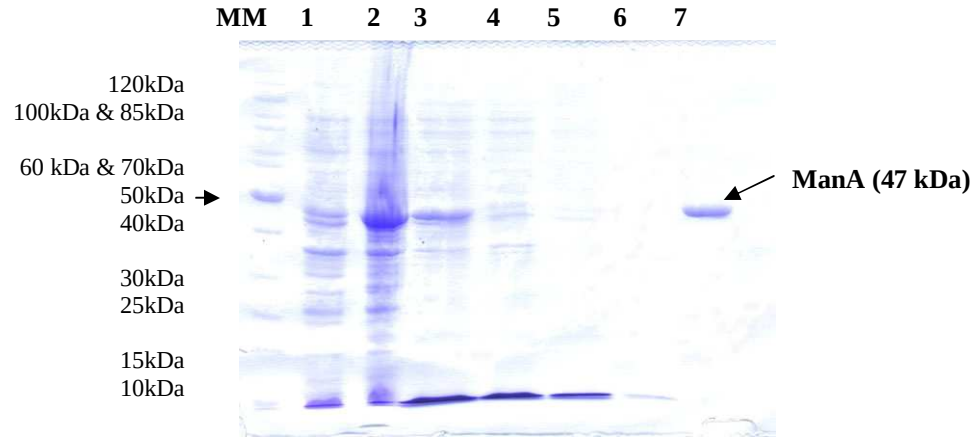


Figure 20: Purification of ManA using nickel affinity chromatography. The SDS-PAGE patterns showing the molecular markers (MM), pre-induction sample (lane 1), crude sample (lane 2), enzyme lysate sample (lane 3), Ni-NTA column flow through sample (lane 4), wash 1 sample (lane 5), wash 2 sample (lane 6) and eluate (lane 7).

A purification table (Table 9) was produced with data collected throughout the various purification steps of ManA, from which it was determined that the native purification protocol used was successful in purifying the manannase. The purification fold obtained was only 4.14. The yield of ManA was similar to that of XynA, and constituted approximately 40% of the total enzyme activity of the total protein content of the crude sample.

Table 9: Purification of ManA

Step	Volume	Protein concentration (mg/ml)	Total Protein (mg)	Enzyme Activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	250	0.09	22.50	126.25	5.610	100	1
Lysate	10	0.51	5.11	42.97	8.410	34	1.50
Flow Through	10	0.26	2.60	17.29	6.650	14	1.19
Wash 1	10	0.19	1.89	28.24	14.942	22	2.66
Wash 2	10	0.01	0.10	0.24	2.400	0.19	0.43
Eluate 1	10	0.22	2.20	51.04	23.200	40	4.14

* U is defined as the µmol reducing sugar, released per minute

4.2.3 Characterisation of ManA

The initial characterization of ManA was performed with a temperature profile ranging from 20°C to 90°C (Figure 21), from which it was established that ManA was active against locust bean gum (LBG) between 20°C and 50°C. The temperature optimum was at 50°C.

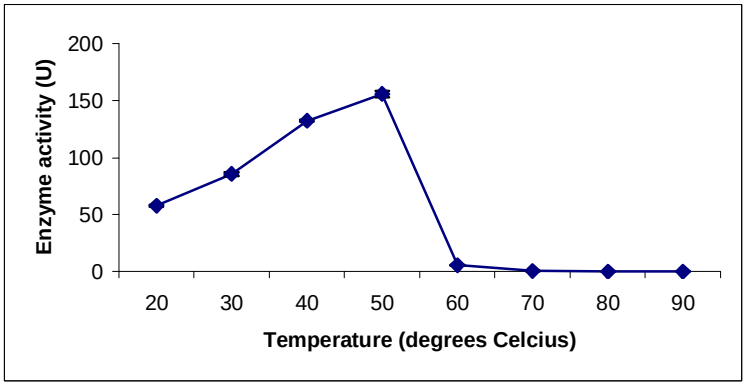


Figure 21: Determination of temperature optimum for ManA using LBG as substrate. The enzyme activity was determined over a temperature range from 20°C to 90°C. Values are presented as mean values ± SD (*n* = 3).

Once the temperature optimum was determined, a range of 50 mM buffers were used to determine the pH optimum of ManA over a pH range of 4.0 to 11.0 (Figure 22). ManA appeared to be stable between pH 5.0 and 6.0; however, the highest activity was obtained at pH 5.5 against LBG.

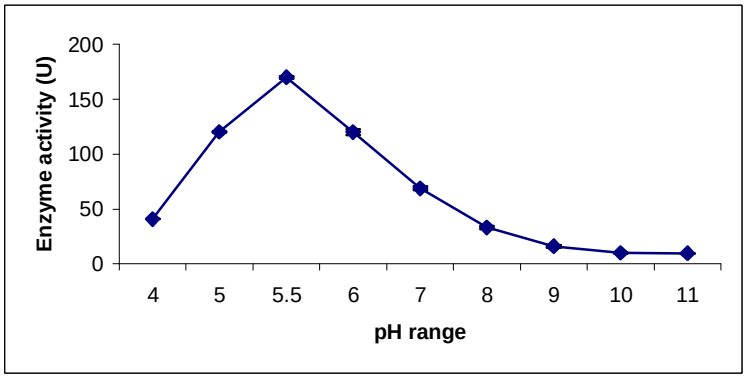
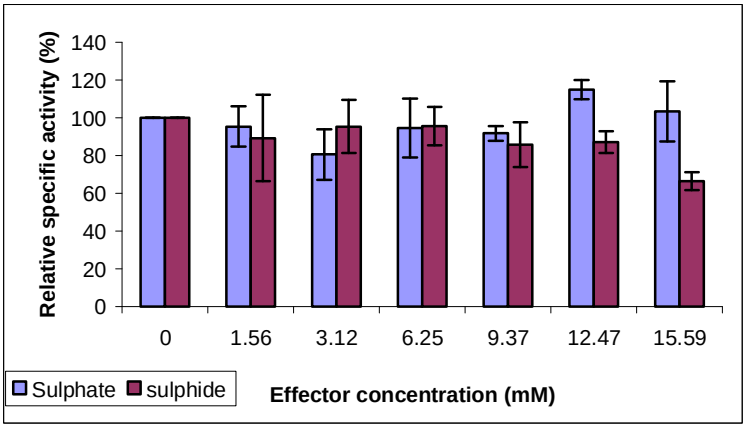
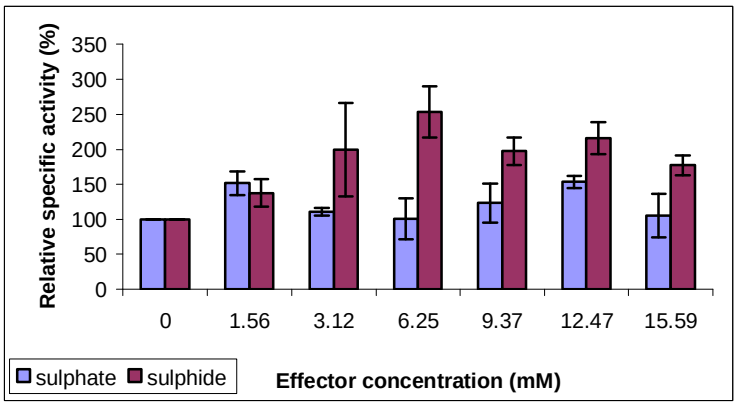


Figure 22: Determination of pH optimum for ManA using Locust Bean Gum as the substrate. A sodium citrate buffer was used for the pH range between 4.0 & 5.0, phosphate buffers were used for the pH range of 6.0 -8.0 and glycine buffers were used between the pH range of 9.0-11.0.

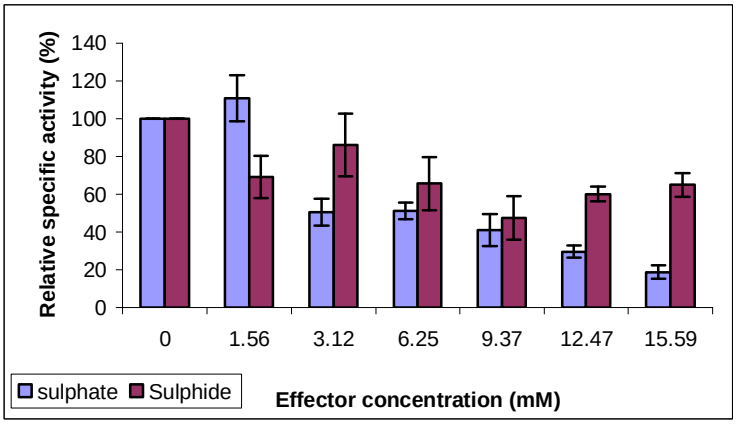
The effect of sulphide and sulphate on ManA activity against LBG, sugarcane bagasse and pineapple fibre was determined (Figure 23). Increasing concentrations of sulphide in the reactions showed an inhibitory effect of approximately 40% of the ManA activity with LBG at 15.59 mM. Increasing sulphate concentrations marginally decreased the mannanase activity up to 9.37 mM, until the sulphate concentration exceeded the concentration of 12.47 mM, at which point there was a slight increase in the general mannanase activity (Figure 23a). The effect of the sulphur derivatives on the mannanase activity against the complex substrates was different to the effect obtained with sulphide and sulphate on the enzyme activity against LBG. ManA showed a drastic enhancement in activity against the sugarcane bagasse in the presence of sulphide (Figure 23b), with the greatest mannanase activity being obtained at a sulphide concentration of 6.25 mM. In the case of sulphate, there was a slight increase in the ManA activity against the sugarcane bagasse; however, the enhanced activity showed no correlation to increasing sulphate concentration. The ManA activity against the pineapple fibre showed the same trend for both sulphur derivatives (Figure 23c). Both sulphide and sulphate had an inhibitory effect on the mannanase activity. With increasing sulphate concentrations, approximately 80% of the enzyme activity was lost. In the presence of increasing sulphide concentrations, approximately 35% of the enzyme activity was lost (Figure 23c).



(a)



(b)



(c)

Figure 23: The effect sulphide and sulphate on the specific activity of ManA on the degradation of LBG (a), sugarcane bagasse (b) and pineapple fibre (c) as represented by the specific activity relative to the specific activity obtained against the various substrates in the absence of the sulphur derivatives. Values are presented as mean values ± SD (n = 3).

4.3 *C. cellulovorans* endoglucanase E (EngE)

4.3.1 Bioinformatic analysis of EngE

Bioinformatic analysis was performed on the *engE* sequence (Accession no. AAD39739) published by Tamaru and Doi (1999).

The bioinformatic analysis of the gene sequence for protein EngE predicted that the enzyme would have a molecular weight of approximately 112 kDa and an isoelectric point of 4.91. Amino acid analysis, performed using Protoparam, indicated that the enzyme is dominantly composed of negatively charged and hydrophobic amino acids (Table 10).

Table 10: Amino acid composition of EngE.

Amino acid	Abbreviation	Quantity in the sequence	Percentage in the sequence (%)
Ala	A	116	11.3
Arg	R	13	1.3
Asn	N	84	8.2
Asp	D	72	7
Cys	C	4	0.4
Gln	Q	25	2.4
Glu	E	31	3.0
Gly	G	67	6.5
His	H	9	0.9
Ile	I	56	5.4
Leu	L	76	7.4
Lys	K	65	6.3
Met	M	22	2.1
Phe	F	41	4.0
Pro	P	34	3.3
Ser	S	58	5.6
Thr	T	109	10.6
Trp	W	18	1.7
Tyr	Y	49	4.8
Val	V	81	7.9

The databases Interpro and My Hits motif scan predicted that EngE had a total of 14 conserved domains (Table 11) and motifs (Table 12).

Table 11: Putative domains identified in the EngE protein sequence

InterPro Accession no.	Domain	Biological Function
IPR002048	Calcium binding EF-hand	~ Binds calcium ions
IPR002105	Dockerin type I	~ Catabolism of polysaccharides
IPR013781	Glycoside hydrolase catalytic core	~ Catalytic activity ~ Carbohydrate metabolic process ~ Cation binding
IPR001547	Glycoside hydrolase, family 5	~ Carbohydrate metablic process

Table 12: Putative motifs identified for EngE.

Position in sequence	Motif information	Legend
1-20	Prokaryotic membrane lipoprotein lipid attachment profile	
30-33	Casein kinase II phosphorylation site	Phosphorylation
35-38	ASN N-glycosylation site	Carbohydrates
37-39	Protein kinase C phosphorylation site	Phosphorylation
101-106	*N*-myristoylation site	Myristyl
234-263	Staphylocoagulase repeat domain	
305-318	Haemagglutinin	
526-810	Cellulase (Glycosyl hydrolase family 5)	
641-650	Glycosyl hydrolase family 5 signature	
742-754	HSF-type DNA binding domain	
920-945	Pseudomurein-binding domain	
979-998	Clostridium cellulosome enzyme repeated domain signature	
979-991	Calcium binding EF-hand	Binds calcium ions
979-999	Dockerin type I repeat	
1011-1030	Calcium binding EF-hand domain profile	
1018-1030	Protein prenyltransferase alpha subunit repeat profile	

There is a 72% probability that EngE has a signal peptide sequence, which has a cleavage site between residues 23 (serine) and 24 (lysine). EngE is also predicted to contain a

primary transmembrane helical region between residues 49 (serine) on the *N*-terminus and 71 (alanine) on the *C*-terminus.

4.3.2 Expression and purification of EngE

A pET-22b(+) vector containing the *engE* gene was used to transform *E. coli* BL21(DE3) cells. The expression of *engE* was induced by the addition of IPTG to a final concentration on 400 µg/ml. The *E. coli* BL21(DE3) was cultured in 2 × YT broth at 30°C, shaking at 200 rpm. Once the OD_{600nm} for the *E. coli* BL21(DE3) cells reached 0.5, IPTG was added and the cells were grown at 19°C, shaking at 200 rpm overnight (approximately 16 hours). The expression of *engE* was illustrated by the induction of the EngE protein (Figure 24). There was a slight induction of the endoglucanase, with the maximum yield of the enzyme occurring after 6 hours from induction. A small quantity of the endoglucanase was expressed prior to the IPTG induction. In addition to the expression of the endoglucanase, two additional unidentified proteins were expressed after 4 hours of IPTG induction

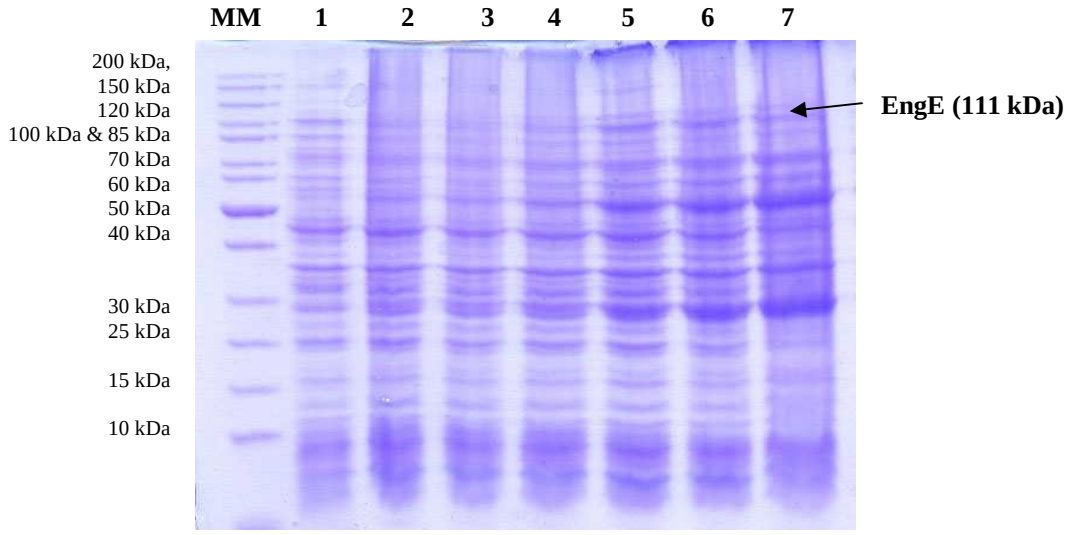


Figure 24: Expression of *engE*. EngE was induced with the addition of IPTG to give a final concentration of 400 µg/ml. The SDS-PAGE patterns showing the molecular markers (MM), pre-induction sample (lane 1), post-induction sample after 1 hour (lane 2), post-induction sample after 2 hours (lane 3), post-induction sample after 3 hours (lane 4), post-induction sample after 4 hours (lane 5), post-induction sample after 6 hours (lane 6) and post-induction sample after 17 hours (lane 7).

The endoglucanase was purified using nickel affinity chromatography under native conditions, as described previously for the mannanase and xylanase. The purification of EngE was successful (Figure 25); however, the quantity of protein obtained during the purification procedure was low.

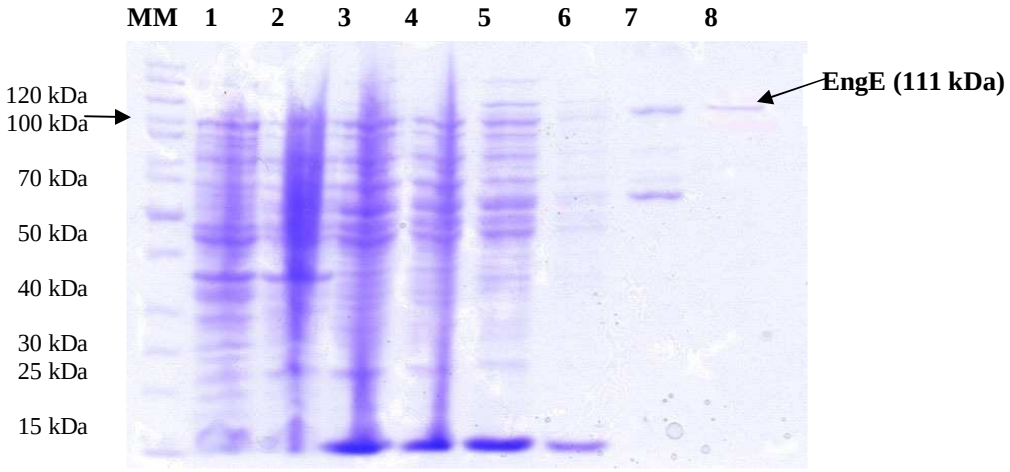


Figure 25: SDS-PAGE of the purification of EngE. The SDS-PAGE patterns showing the molecular markers (MM), pre-induction sample (lane 1), crude sample (lane 2), enzyme lysate sample (lane 3), Ni-NTA column flow through sample (lane 4), wash 1 sample (lane 5), wash 2 sample (lane 6) and eluate 1 (lane 7), eluate 2 (lane 8).

The endoglucanase was purified successfully; however, a fair amount of the protein was lost due to the presence of contaminating proteins, which bound non-specifically to the affinity column. As a result, a 2.27 fold purification was obtained with a low protein yield (Table 13).

Table 1: Purification table for EngE

Step	Volume (ml)	Protein concentration (mg/ml)	Total Protein (mg)	Enzyme Activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	250	1.2	300	363.49	1.212	100	1
Lysate	10	1.39	13.90	12.05	0.867	3.32	0.72
Flow Through	10	0.87	8.70	9.98	1.147	2.75	0.95
Wash 1	10	0.17	1.70	4.23	2.488	1.16	2.05
Wash 2	10	0.11	1.10	0.78	0.709	0.21	0.59
Eluate 1	10	0.05	0.50	2.31	4.620	0.64	3.81
Eluate 2	10	0.04	0.40	1.1	2.750	0.30	2.27

* One unit (U) is defined as the µmol glucose released per minute

4.3.3 Characterisation of EngE

Once the EngE was purified, initial characterisation was performed to determine its temperature and pH optima. The temperature profile for EngE (Figure 26) showed that EngE exhibited activity between 20°C and 50°C, with 30°C being the optimum temperature against carboxymethylcellulose (CMC).

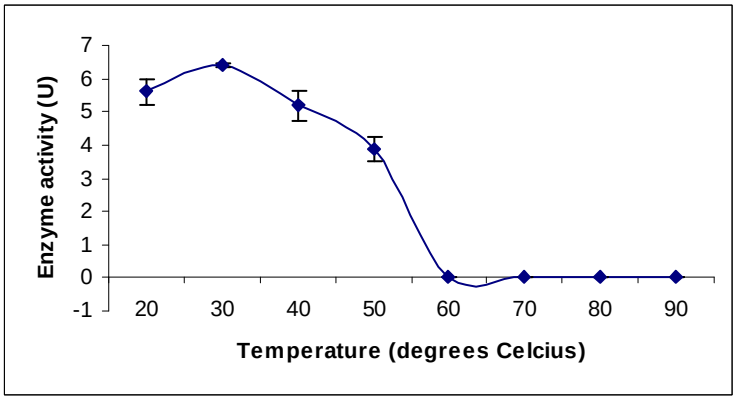


Figure 26: Determination of temperature optimum for EngE using CMC as substrate. The enzyme activity was determined over a temperature range from 30°C to 90°C. Values are presented as mean values ± SD (*n* = 3).

Once the temperature optimum was determined, a pH profile for the endoglucanase activity was established. A range of buffers (50 mM) covering pH values from 4.0 to 11.0 were used (Figure 27). From these it was observed that the pH optimum of EngE was 5.0 against CMC.

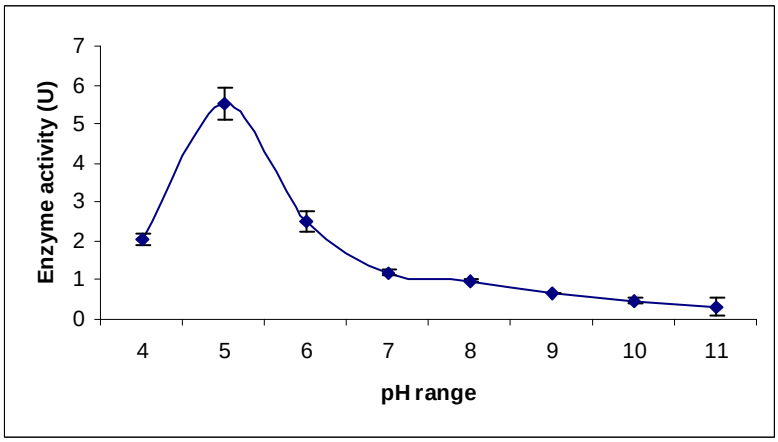
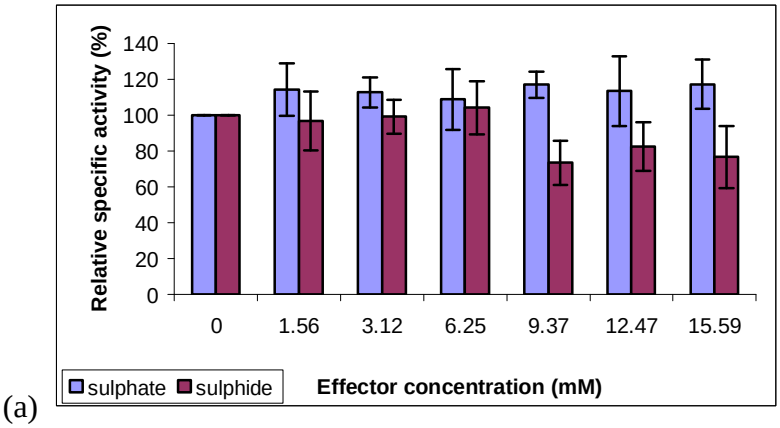
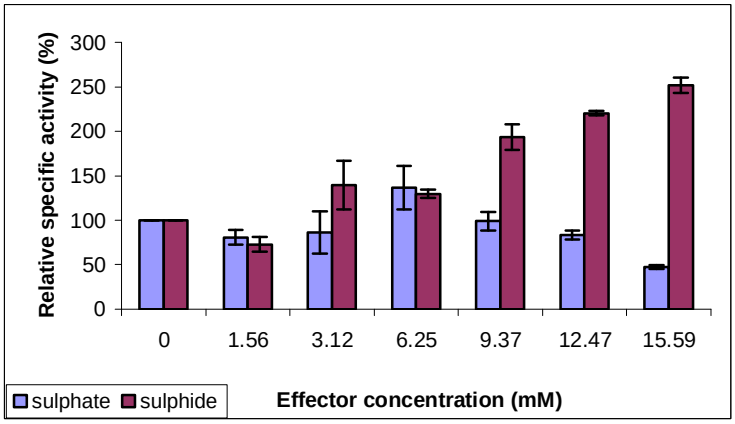


Figure 27: Determination of pH optimum for EngE. A sodium citrate buffer was used for the pH range between 4.0-5.0, phosphate buffers were used for the pH range of 6.0-8.0 and glycine buffers were used between the pH range of 9.0-11.0. Values are presented as mean values ± SD (n = 3).

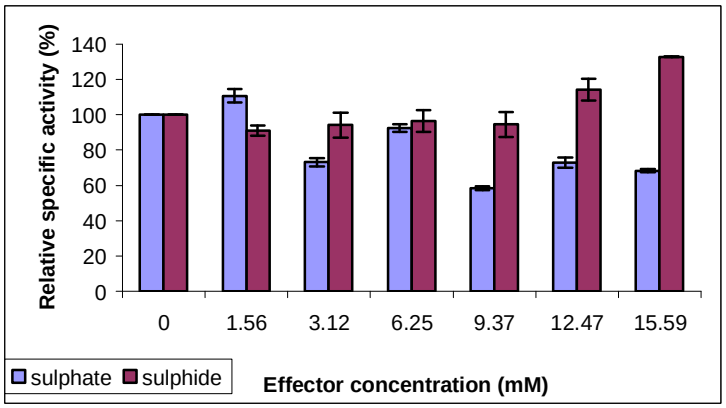
Once the temperature and pH optima of EngE were determined, the effect of sulphur derivatives on the enzyme activity against CMC, sugarcane bagasse and pineapple fibre was examined (Figure 28). The sulphide and sulphate had different effects on the soluble and complex substrates. In the presence of sulphate there was a general increase in endoglucanase activity against CMC with increasing sulphate concentrations, but the opposite was true for the sulphide (Figure 28a). With respect to the complex substrates, there was enhanced activity with increasing concentrations of sulphide (Figure 28b & c). With the sugarcane bagasse there was an initial increase in the enzyme activity; however, there was a decrease in the enzyme activity with sulphate concentrations exceeding 6.25 mM (Figure 28b). In the case of the pineapple fibre there was a general decrease in enzyme activity with increasing sulphate concentration (Figure 28c).



(a)



(b)



(c)

Figure 28: Effect of sulphate and sulphide on EngE activity against CMC (a) sugarcane bagasse, (b) and pineapple fiber (c), as represented by the specific activity relative to the specific activity obtained against the various substrates in the absence of the sulphur derivatives. Values are presented as mean values ± SD (n = 3).

4.4 Discussion

Xylan is considered the second most abundant renewable carbon source as it is a major component of plant cell walls (Puls, 1997; Bissoon *et al.*, 2002). A variety of enzymes are required for the complete degradation of its dominant hemicellulose, due to the complexity and variability of xylan (Subramaniyan and Prema, 2002). Only one of the enzymes associated with the degradation of xylan was analysed during this research. The xylan substrate that was chosen had a lower degree of complexity with regard to the presence and type of side chain present on the xylose backbone. BWX was chosen as the soluble xylan substrate as it is composed of a chain of xylose residues linked via β-(1→4)-xylosidic bonds**.** Xylanases are responsible for the depolymerisation of xylan via the random hydrolysis of the xylanolytic backbone (Subramaniyan and Prema, 2002). Leggio *et al*. (2000) proposed that xylanases hydrolyse substrates through the double displacement mechanism where the retention of the anomeric carbon of the monosaccharide unit of the xylan backbone undergoes a conformational change. The conformational change weakens the β-(1→4)-xylosidic bonds and increases the substrates susceptibility to hydrolysis.

Xylanases belong to the glycoside hydrolase families 10 and 11 (Howard *et al.*, 2003). The molecular weight of xylanase isomers range between 12 and 145 kDa, some of which may form heterodimers depending on the conditions under which they are expressed (Petrosyan *et al*, 2002). High molecular weight endoxylanases with low pI values belong to glycosyl hydrolase (GH) family 10, while the low molecular weight endoxylanases with high pI values belong to the glycanase family 11 (Kuno *et al.*, 2000). From the results obtained for the partial characterisation of XynA using bioinformatics and SDS-PAGE it was observed that the enzyme has a reasonably high pI of 8.01 and a low molecular weight of approximately 57 kDa, indicating that XynA belongs to the GH family 11. The high pI value of 8.01 is also indicative of XynA being positively charged, with regard to amino acid composition. This was supported using bioinformatics.

Initial biochemical characterisation of XynA was performed. XynA appeared to be robust, as it was active in a pH range of 4.0 to 9.0, and between 30°C and 60°C against BWX. Koukiekolo *et al*. (2005) assayed for XynA activity at 37°C, using a 50 mM acetate buffer (pH 6.0) against corn fibre and corn stalk fibre. They obtained a maximum release of 50 µM reducing sugar from the corn stem fibre. Murashima *et al*. (2002b) tested for XynA activity against oat spelt xylan using a 0.5 M morpholineethanesulfonic acid (MES) buffer titrated with sodium hydroxide to pH 6 at 37°C. Murashima *et al*. (2003) also used a sodium acetate buffer (pH 6) at 37°C against BWX, oat spelt xylan and corn stalk, to test the XynA activity. The greatest XynA activity against the oat spelt xylan was obtained with the MES buffer (pH 6). The XynA activity obtained against the oat spelt xylan was also greater than the activity against the corn stalk fibre. In this study, XynA activity against BWX was assayed at 50°C using a 50 mM citrate buffer (pH 5.5). The quantity of the reducing sugars released under these conditions was higher than that reported in literature.

Two factors (temperature and substrate composition) may have caused the increased XynA activity against BWX in comparison to literature. Temperature tends to increase the solubility of substrates. Temperature may have increased the solubility of the substrates by weakening the β-(1→4) xylosidic bonds allowing the substrate to be "opened", exposing the β-(1→4) xylosidic bonds to hydrolytic enzyme attack. Substrate composition is an important factor to consider when testing for the activity of a specific enzyme. Corn stalk fibre is an insoluble complex substrate, composed of varying percentages of cellulose, hemicellulose, lignin and pectin, depending on the age the plants are harvested. Corn stalk fibre consists of approximately 40 % - 60 % cellulose, 28 % hemicellulose, and 7 % - 20 % lignin (Ali *et al*., 2000; Reddy and Yang, 2005). The low activity obtained against corn stalk fibre would have been primarily due to the presence of lignin, which forms a protective layer around the holocellulose components of the substrate. Oat spelt xylan is partially soluble and has a high side chain consistence in comparison to BWX. The presence of the side chains may partially shield the β-(1→4)-xylosidic bonds when the xylose subunits undergo a conformational change due

to the retention generated from the double displacement mechanism associated with the hydrolysis of the xylan.

As previously mentioned, mannan is a hemicellulolytic polymer present in softwoods, legumes, nuts and beans (Puls and Schuseil, 1996); however, mannan is present in a variety of other plant species. The mannan that was used in the studies for the characterisation of ManA, and the synergy studies against a mixture of soluble substrates was locust bean gum (LBG), which is essentially a galactomannan (Figure 29).

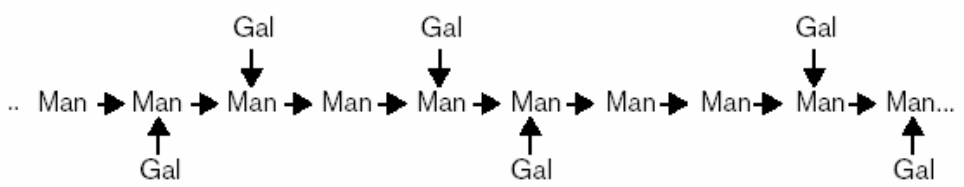


Figure 29: The chemical structure of galactomannan. The galactomannan backbone consists of a chain of D-mannose residues via β-(1→4) mannosidic bonds with D-galactosyl substituents, which are linked to the mannan backbone via α-(1→6) mannosidic bonds (Reid, 2000).

Mannanases are members of the GH family 5 (Tamaru and Doi, 2001). Endo-β-mannanases depolymerise the mannan backbone through random cleavage to release mannobiose units as well as a variety of other manno-oligosaccharides. The cleavage by exo-β-mannosidases releases mannose (Halstead *et al*., 1999). Random hydrolysis of the β-(1→4)-D-mannopyranosyl linkages in β-(1→4)-mannans, glucomannans, and galactomannans (McCleary, 1988) releases mannose, glucose and galactose via a retaining mechanism in glycosyl hydrolase family 5 (Davies and Henrissat, 1995).

From the results for the partial characterisation of ManA using bioinformatics and SDS-PAGE, it was predicted that the ManA had both a low molecular weight (47 kDa) and pI (5.62). The low pI indicated that ManA was composed predominantly of negatively charged amino acids, which was supported by bioinformatic analysis.

Initial characterisation of ManA was performed to determine the temperature and pH optima. ManA appeared to be active between 20°C and 50°C, with optimum activity

observed at 50°C. ManA was active between pH 5.0 and 6.0, with the optimum activity observed at pH 5.5. Tamaru and Doi (2000) indicated that ManA was able to degrade LBG and a variety of other galactomannans efficiently. They also found that the purified ManA that was assayed at 45°C with piperazine-*N*,*N'*-bis(2)-ethanesulfonic acid (PIPES) buffer at pH 7.0 was unable to degrade CMC. The ManA that was purified in this study, assayed at 50°C with a citrate buffer at pH 5.5, had activity against CMC. A possible explanation for the ManA activity against CMC (Figure 30) may be due to the use of a buffer with a lower pH.

Cellulose is a linear insoluble biopolymer that is composed of repeated units of β-D-glucopyranose/glucose monomers linked via β-glucosidic bonds (Pereira *et al*., 1988). Several cellulose macromolecules associate in a parallel fashion to form a microfibril (Ljungdahl and Eriksson, 1985), which in turn associates to form fibres (Figure 5) (Atalla, 1993). CMC is the commercially available cellulose macromolecule composed of repeated cellulose macromolecules with methylated side chains (Figure 30).

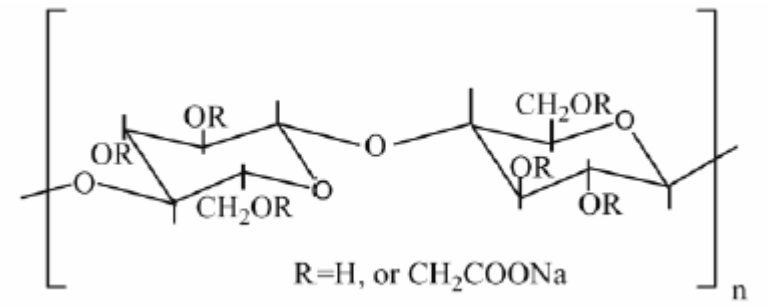


Figure 30: Chemical composition of the CMC used in the analysis of EngE (Mitsumata *et al*., 2003).

The degradation of crystalline cellulose to produce reducing sugars such as glucose requires the synergistic action of at least three enzymes that possess endoglucanase, exoglucanase and β-glucosidase activities (Kim, 1995; Teeri, 1997; Schwartz, 2001). Endoglucanases cleave cellulose chains in a random fashion. Endoglucanases generally degrade amorphous cellulose more readily than crystalline cellulose (Carrad *et al*., 2000,

Lenting and Warmoeskerken, 2001). EngE belongs to GH family 5 (Henrissat and Bairoch, 1993) thus belonging to the same family of hydrolases as ManA. As mentioned previously, glycosyl hydrolases in the GH families 5 and 11 degrade substrates by the double displacement retention mechanism. Then EngE will hydrolyse CMC through the double displacement mechanism. The retention of the anomeric carbon of the glucose unit of the CMC backbone induces a conformational change. The conformational change weakens the β-glycosidic bonds and increases the substrate's susceptibility to hydrolysis.

From the results for the partial characterisation of EngE using bioinformatics and SDS-PAGE, it was predicted that the EngE had both a high molecular weight (111 kDa) and low pI (4.91). The low pI indicated that ManA was composed predominantly of negatively charged amino acid; this was confirmed with bioinformatic analyses.

Initial characterisation of EngE was performed to determine the temperature and pH optima. From these results it was observed the EngE was active against CMC between 20˚C and 50˚C, and between a pH of 4.0 and 6.0. The optimum enzyme activity was obtained using a sodium citrate buffer at pH 5.0 at 30˚C. The optimum temperature and pH for EngE activity in literature varies. Tamaru and Doi (1999) tested for EngE activity against CMC using a 100 mM sodium acetate buffer (pH 5.0) at 50˚C. Murashima *et al.* (2002b) tested for EngE activity against CMC using a 500 mM MES buffer (pH 6.0) at 37˚C; however, Murashima and co-workers (2003) used a 50 mM sodium acetate buffer (pH 6.0) at 37˚C to test EngE activity. Koukiekolo *et al*. (2005) tested for EngE activity against corn stalk fibre and corn fibre gum, using 50 mM sodium acetate (pH 6.0) at 37˚C. Journal articles report that, EngE activity is generally tested using a buffer with a pH between 5.0 and 6.0, at 37°C, which is similar to the temperature and pH optima obtained in the partial characterisation of EngE.

Once the temperature and pH optima were determined, the effect of sulphide and sulphate on the activities of XynA, ManA and EngE against BWX, LBG, CMC, sugarcane bagasse and pineapple fibre was investigated. Thus far, literature does not indicate how sulphide and sulphate affects various enzymes. Literature mentioned that the presence of

sulphide and sulphate may enhance cellulolytic activity against complex plant-based substrates (Whiteley *et al.*, 2002, 2003**)**. Thus the theories presented in this study, with respect to enhancement and inhibitory trends observed in the presence of sulphide and sulphate, are based on previous studies using cellulases.

Increasing concentrations of sulphide and sulphate enhanced the activity of XynA against BWX. Increasing concentrations of sulphate enhanced the ManA and EngE activity against LBG and CMC respectively. The increasing sulphide concentrations had an inhibitory effect on ManA and EngE against LBG and CMC respectively. The main structural differences between the different soluble substrates are the presence of side chains in LBG and CMC. Thus, the unexpected inhibition of ManA and EngE activity on the respective substrates may be due to the sulphide affecting the solubility of the substrates by affecting the side chains. The assays were performed at 50°C, which would weaken the substrate backbones causing the substrate structures to "open", increasing the exposure of the β-(1→4)-glycosidic bonds to enzyme attack. There was an increase in the inhibition with increasing concentrations of sulphide, thus the sulphide may have caused the side chains to aggregate towards the backbone and shielding the β-(1→4)-glycosidic bonds.

Increasing concentrations of sulphide and sulphate enhanced the activity of XynA and ManA activity against sugarcane bagasse. Increasing concentrations of sulphate had an inhibitory effect on EngE activity against bagasse and the pineapple fibre; however, the increasing sulphide concentrations enhanced the cellulase activity against the bagasse and pineapple fibre as expected. The increase in sulphate concentration enhanced the xylanase activity against the pineapple fibre; however, the increasing sulphide concentration had both an enhanced and inhibitory effect on XynA activity against the pineapple fibre. Increasing concentrations of both sulphur derivatives had an inhibitory effect on ManA activity against the pineapple fibre.

Lignocellulose is generally defined as having complex lattice structure with holocellulose (combination of cellulose and hemicellulose) and lignin being closely associated, in such

a way, that the lignin forms a protective barrier around the holocellulolytic component of the plant biomass. Thus, the cellulose and hemicellulose polymers are protected from enzyme and microbial attack (Deobald and Crawford, 1997; Crawford and Crawford, 1988; Paneek *et al*., 1998; Wood and Saddler, 1988). Lignocellulose degradation is recalcitrant to anaerobic biodegradation due to the presence of lignin products (Breen and Singleton, 1999; Zeikus *et al*., 1982). Paneek *et al*. (2001) showed that the bonds linking the aromatic compounds in lignin could be cleaved under biosulphidogenic conditions. As mentioned previously, the release of lignin associated aromatic compounds and the enhanced degradation of holocellulose under sulphate reducing conditions have been observed (Whittington-Jones, 2000; Roman, 2004). Thus, the enhancement and inhibition of the enzyme activities against the complex substrates (bagasse and the pineapple fibre) may potentially be associated with the chemical composition, and specifically the lignin content, of the substrate. The enhancement of the activity may result from the separation of the lignin and the holocellulose fractions of the substrates. The inhibition of the enzyme activities may be due to increased concentrations of aromatic, phenolic compounds that are released from the lignin.

4.5 Conclusions

The results obtained for the partial characterisation of XynA, ManA and EngE indicated the following:

- temperature and pH optima for XynA were 50°C and 6.
- temperature and pH optima for ManA were 50°C and 5.5.
- temperature and pH optima for EngE were 30°C and 5.
- sulphide and sulphate generally enhanced XynA activity against the different substrates.
- sulphide and sulphate generally inhibited ManA activity against LBG and the pineapple fibre; however these compounds enhanced the activity against the sugarcane bagasse.
- sulphide enhanced the cellulase activity against the complex substrates; however these compounds inhibited enzyme activity against CMC.

- sulphate inhibited EngE activity against the complex substrates; however, these compounds enhanced EngE activity against CMC.

Chapter 5: Synergistic analysis of EngE, ManA and XynA

Previous synergy studies have focused on the synergistic relationships between cellulase isozymes (Gaudin *et al*., 2000; Henrissat *et al*., 1985; Irwin *et al*., 2000; Murashima *et al*., 2002a; Nidetzky *et al*., 1994; Wood and McCrae, 1972, 1979; Wood *et al*., 1989). However, the synergistic association between cellulosomal cellulases and other cellulosomal enzymes, has not been studied extensively.

The enzyme activities of the individual enzymes, as well as the activities of the various combinations of *C. cellulovorans* enzymes, were determined through the quantification of the reducing sugars present at the end of the reactions using DNS as a reagent. The synergistic associations between the different enzymes were determined by the dividing the experimental enzyme activities obtained by the theoretical activity that would be obtained from the sum of the combined enzyme activities as described by Murashima *et al* (2003). The recombinant enzyme combinations used were:

a) XynA and ManA;
b) ManA and EngE;
c) XynA and EngE,
d) XynA, ManA and EngE.

Specific activities of various combinations of the enzymes (EngE, ManA and XynA) were calculated and defined as µmol sugar released per minute per mg protein.

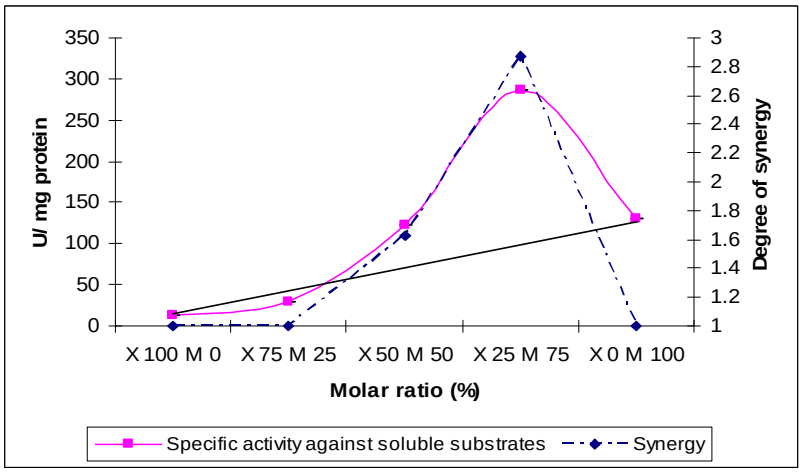
5.1 Combinations of two enzymes

5.1.1 Degree of synergy obtained with two enzymes against soluble substrates

The first batch of assays that was performed to establish the potential synergies between enzymes were combinations of two enzymes with molar ratios ranging between 0 % and 100 % (Appendix 5).

Figure 31 illustrates the resultant synergistic relationships between (a) XynA and ManA (b) ManA and EngE and (c) XynA and EngE against the soluble substrate solutions of 2% (w/v) BWX and 2% (w/v) LBG; 2% (w/v) LBG and 2% (w/v) CMC; and 2% (w/v) BWX and 2% (w/v) CMC, respectively. The XynA and ManA enzyme combination that displayed a degree of synergy of 2.87 (i.e. an enzyme molar ratio of XynA to ManA of 25%:75%) and also produced the greatest quantity of reducing sugar of 278.30 U/mg protein against the substrate mixture of BWX and LBG (Figure 31a) The ManA and EngE combination, in a molar ratio of 50% ManA:50% EngE, produced the highest quantity of reducing sugar (28.62 U/mg protein) with a degree of synergy of 1.76 (Figure 31b). The XynA to EngE combination produced the highest degree of synergy (3.59) at a molar ratio of 25%:75% (Figure 31c).

(a)



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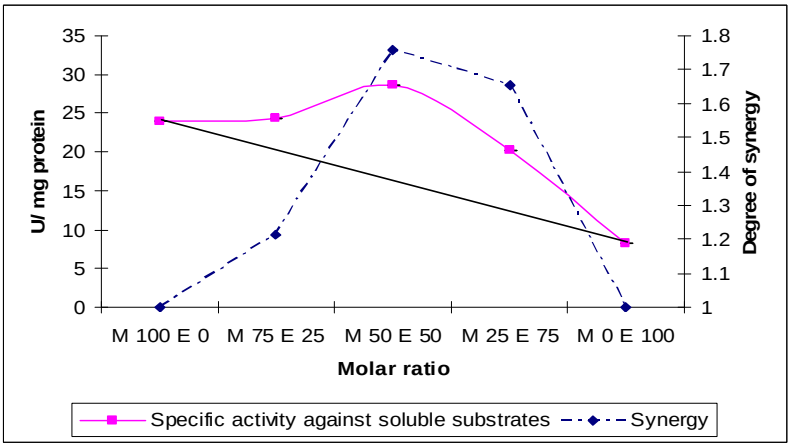


(c)

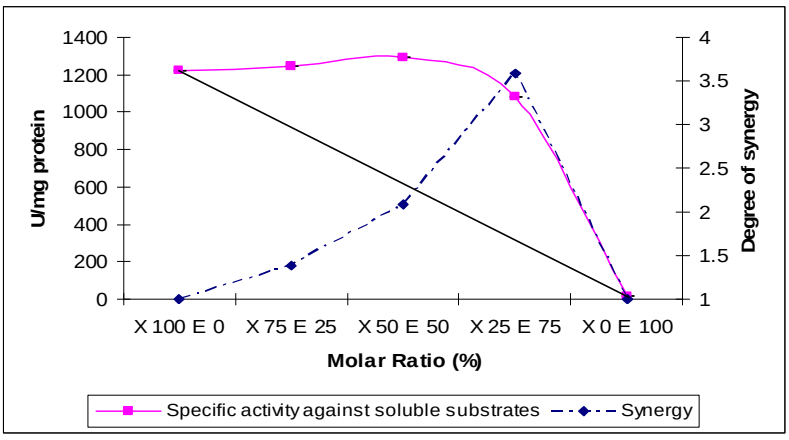
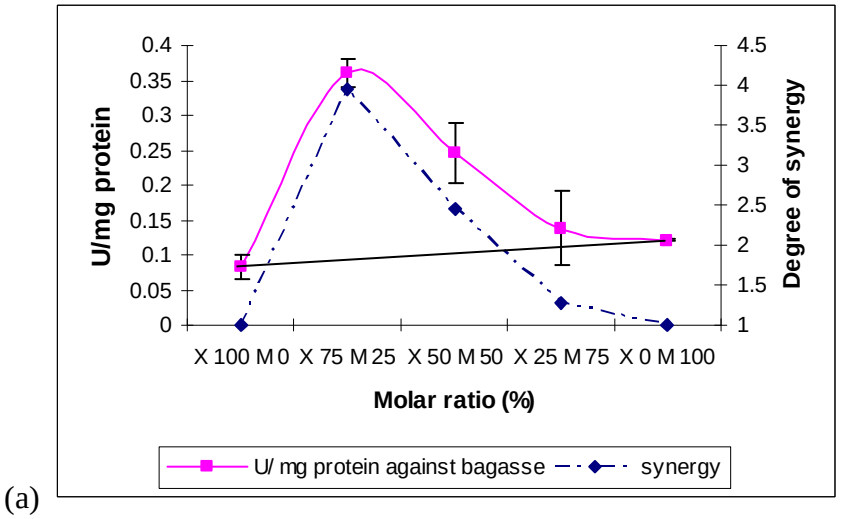


Figure 31: Specific activities and the respective synergistic associations with mixed substrates for various combinations of the enzymes. (a) XynA (X) and ManA (M) against the substrate mixture of 2% (w/v) birchwood xylan and 2% (w/v) locust bean gum, (b) ManA (M) and EngE (E) against the substrate mixture of 2% (w/v) locust bean gum and 2% (w/v) CMC, (c) XynA (X) and EngE (E) against the substrate mixture of 2% (w/v) birchwood xylan and 2% (w/v) CMC. Values are presented as mean values ± SD (n=3), but are too small to be visualised .

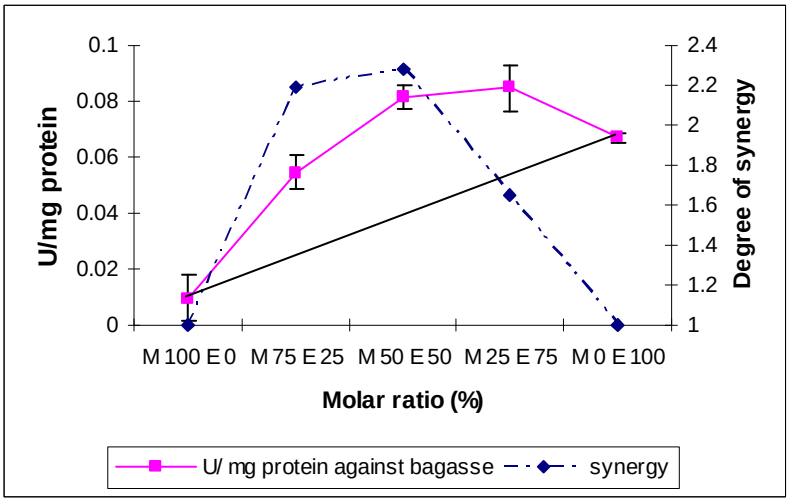
5.1.2 Degrees of synergy obtained with two enzymes against complex plant biomass

Bagasse is lignocellulolytic in nature and, therefore it is composed of intertwined cellulose and hemicellulose components (Lavarack *et al.,* 2002). The hemicellulolytic component of bagasse is dominantly composed of xylan backbone, with the most common substituents being glucuronic acid and arabinose (Saavedra *et al*, 1989). Previous studies have focused on the degradation of bagasse through the acid hydrolysis of the linkages between the cellulose and hemicellulose components, to produce a variety of monosaccharides (Lavarack *et al.,* 2002). Sugarcane bagasse has a dry weight composition of approximately 42% cellulose, 22% lignin, 28% hemicellulose, and cane wax and organic acids comprise approximately 8% (Ouensanga and Picard, 1988).

Figure 32 illustrates the synergistic relationships between (a) XynA and ManA, (b) ManA and EngE and (c) XynA and EngE against sugarcane bagasse. The molar ratio of XynA to ManA of 75%:25% produced the highest synergistic association of 3.95 and had a specific activity of 3.63×10^{-1} U/ mg protein (Figure 32a). The combination of ManA and EngE exhibited the greatest synergistic association (2.28) at a molar ratio of ManA to EngE of 50%:50% against sugarcane bagasse (Figure 32b) and produced the greatest quantity of reducing sugar (8.17×10^{-2} U/ mg protein) against sugarcane bagasse (Figure 32b). The combination of XynA and EngE at a molar ratio of 75%:25% (Figure 32c) produced the highest degree of synergy (4.65) and exhibited a specific activity of 9.50×10^{-2} U/mg protein.



(a)



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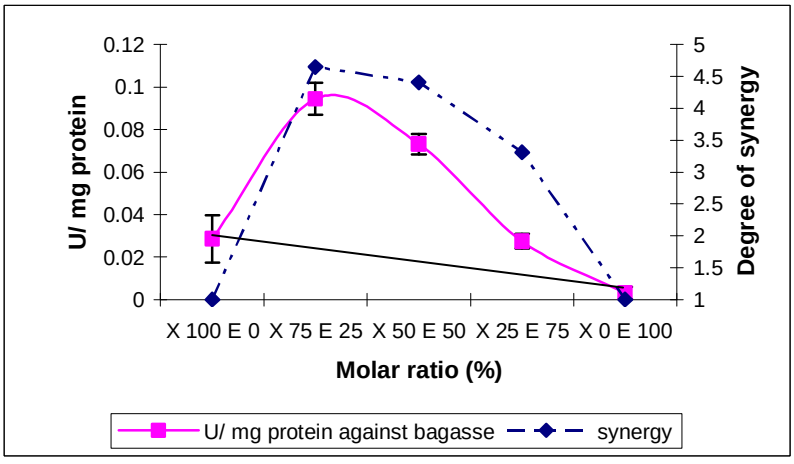
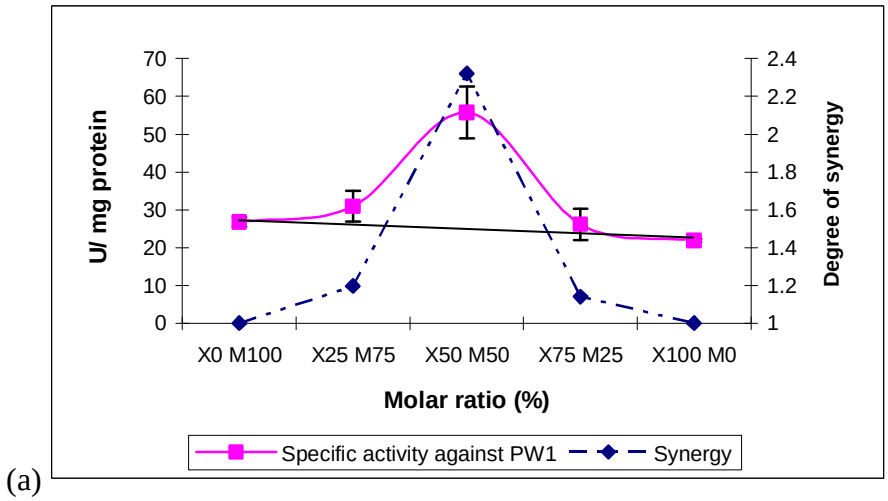


(c)

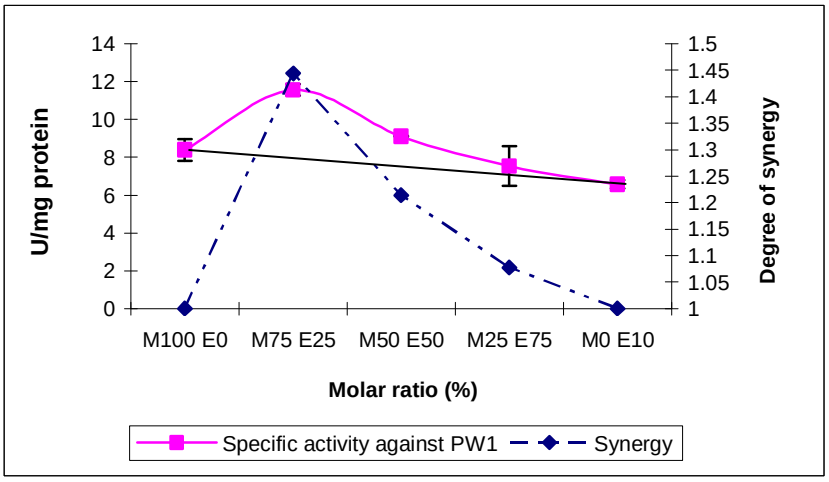
Figure 32: Specific activities and the respective synergistic associations against sugarcane bagasse for the various combinations of the enzymes. (a) XynA (X) and ManA (M), (b) ManA (M) and EngE (E), and (c) XynA (X) and EngE (E). Values are presented as mean values ± SD (n=3)

The enzyme activities and thus the degrees of synergy were determined on a second complex substrate. The pineapple fibre that was used was the waste product obtained from The Pineapple Growers Association (PGA), Bathurst, Eastern Cape, R.S.A. The pineapple fibre was washed with distilled MilliQ water to remove excess pineapple liquor and subsequently autoclaved and dried in an oven at 100˚C. The pineapple fibre was ground to a fine powder prior to use. The enzyme combinations used were the same as previously described for both the simple substrates and the sugarcane bagasse (Appendix 6).

An enzyme ratio of 50% XynA to 50% ManA provided both the highest specific activity (55.71 U/ mg protein) and degree of synergy (2.32) for the reactions containing various quantities of XynA and ManA (Figure 33a). The same trend was observed for the combinations containing XynA and EngE, where both the highest specific activity and degree of synergy was obtained for a combination of 50% to 50% (Figure 33c). However, with regard to the reactions containing various quantities of ManA and EngE, the optimal specific activity and degree of synergy was obtained for the molar combination of 75% ManA to 25% EngE (Figure 33b).



(a)



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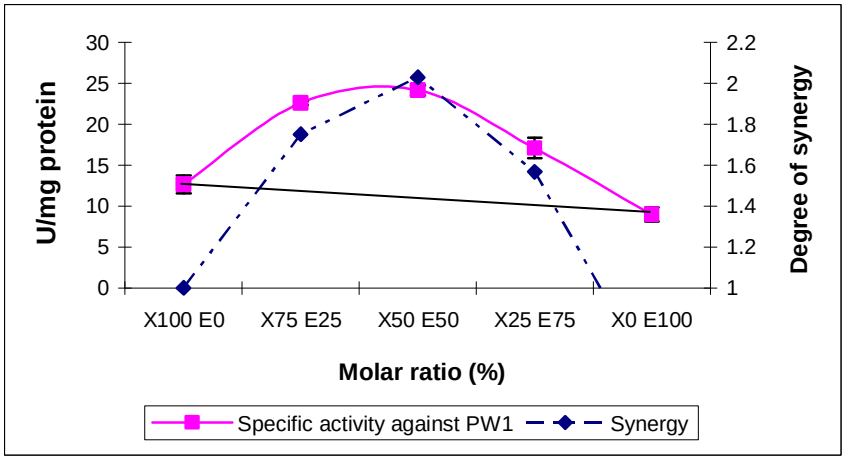


(c)

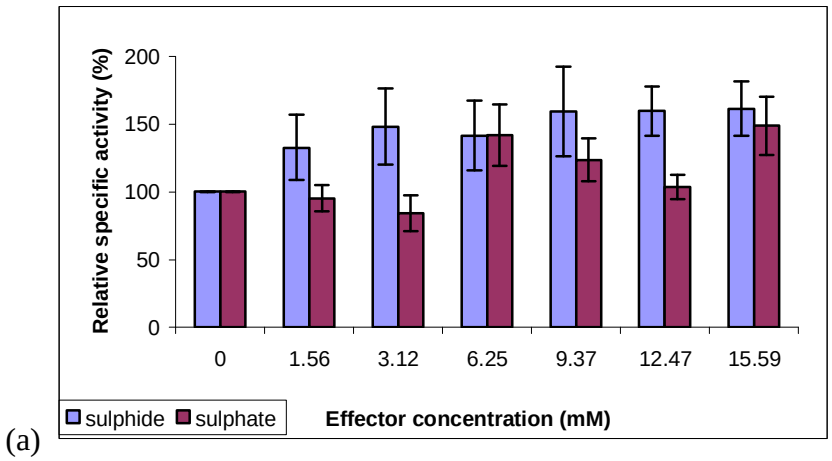
Figure 33: Specific activities and the respective synergistic associations against the pineapple fibre (PW1) for the various combinations of the enzymes. (a) XynA (X) and ManA (M), (b) ManA (M) and EngE (E), and (c) XynA (X) and EngE (E). Values are presented as mean values ± SD (n=3).

5.1.3 Effect of sulphur derivatives on the degradation of soluble substrates by two enzyme combinations

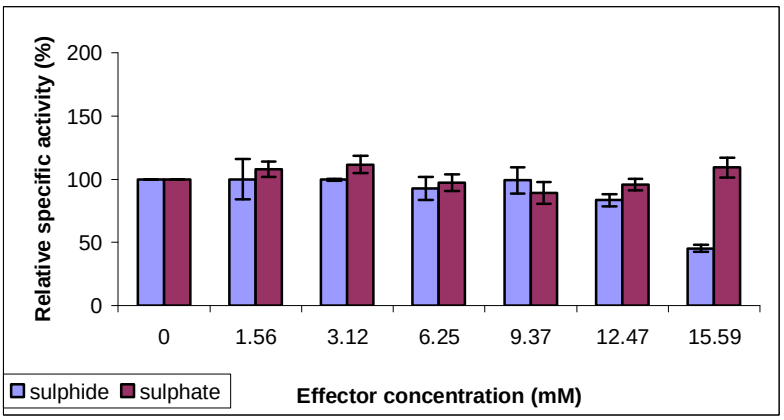
Sulphur and sulphur derivatives are ubiquitous in nature and, therefore, these derivatives are involved in a wide variety of natural processes (Postgate, 1984). An important process occurring in both aquatic and terrestrial environments is anaerobic digestion (Novaes, 1986). Anaerobic digestion has been defined as an anoxic process where a variety of insoluble compounds are converted e.g. cellulose to its monomeric components, through bacterial action, and subsequently reduced to methane and carbon dioxide (Novaes, 1986). Anaerobic digestion is essentially a four-step process, namely: (1) hydrolysis of organic matter to produce soluble products, which undergo (2) fermentation to produce volatile fatty acids (VFAs), and (3) the VFAs undergo acetogenesis, and the final step in anaerobic digestion is methanogenesis (Novaes, 1986). Sulphur and sulphur derivatives may be present in anaerobic digestion because of the possible association which occurs between sulphate reducing bacteria and methanogens (Novaes, 1986). Sulphur and sulphur derivatives, especially sulphide, that are produced during biosulphidogenesis, as a result of the reduction of sulphate (Postgate, 1984), may have an effect not only on the various steps in anaerobic digestion, but on the enzymes involved in the hydrolysis of the insoluble organic matter. Previous studies by Johnson *et al*. (1982) and Lamed *et al.* (1984), have demonstrated that the presence of sulphide increases cellulolytic activity, and thus increases the rate at which cellulose degradation occurs.

Once the optimal molar ratios for the degradation of the simple, soluble substrates were determined, inhibition/activation studies were performed using various concentrations of sulphide and sulphate. The enzyme combinations that were tested were the following: 25% XynA to 75% EngE (Figure 34a), 50% ManA to 50% EngE (Figure 34b), and 25% XynA to 75% ManA (Figure 34c). The sulphur derivatives, sulphide and sulphate, showed a general enhancement in the hydrolysis of the mixed substrate containing 2% (w/v) birchwood xylan and 2% (w/v) CMC using the molar ratio of 25% XynA to 75% EngE (Figure 34a). However, the hydrolysis of mixed substrates containing locust bean gum (Figure 34b & c) both show inhibition with an increasing concentration of sulphide.

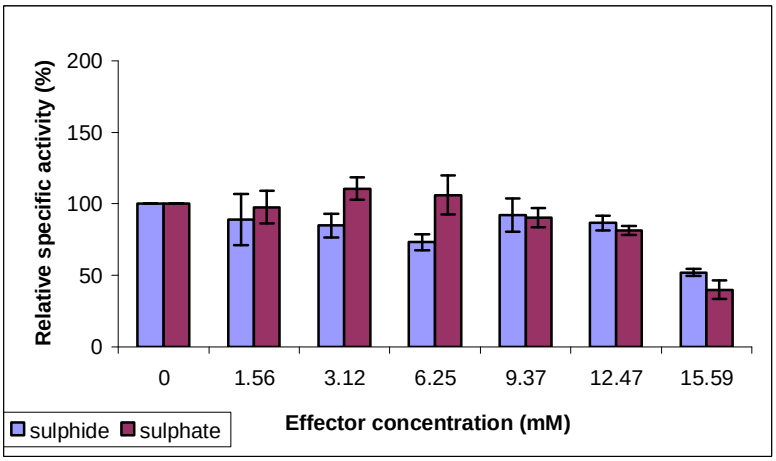
With regard to the effect on the enzymatic hydrolysis of the substrates, it was observed that the sulphate did not appear to have any noticeable effect of the hydrolysis of the mixed substrate containing 2% (w/v) locust bean gum and 2% CMC (Figure 34b). There was an inhibitory effect with an increasing concentration of sulphate on the hydrolysis of the substrate containing 2% birchwood xylan and 2% locust bean gum, with the enzyme molar ratio of 25% XynA to 75% ManA (Figure 34c).



(a)



(b)



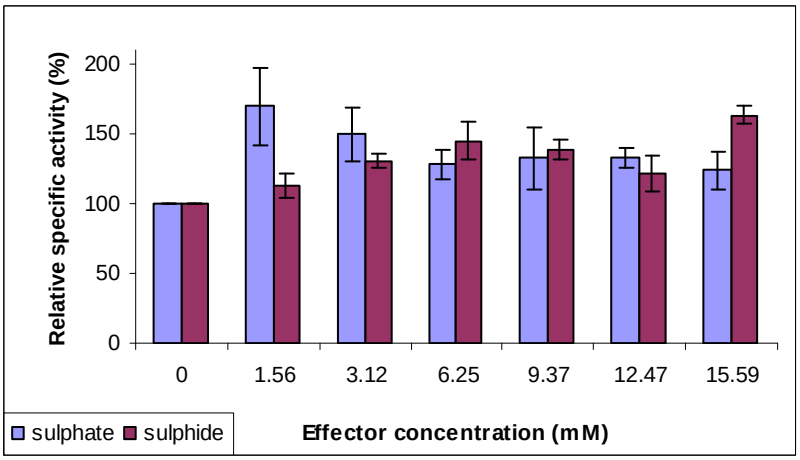
(c)

Figure 34: Effect of sulphur derivatives on the degree of synergy against the soluble substrates. Degradation of the substrate mixture containing 2 % (w/v) BWX and 2 % (w/v) CMC using the molar ratio of 25 % XynA to 75 % EngE (a), degradation of the substrate mixture containing 2% (w/v) LBG and 2 % (w/v) CMC using the molar ratio of 50 % ManA to 50 % EngE (b) and degradation of the substrate mixture containing 2 % (w/v) BWX and 2 % (w/v) LBG using the molar ratio of 25 % XynA to 75 % ManA (c), as represented by the specific activity relative to the specific activity obtained against the various substrates in the absence of the sulphur derivatives. Values are presented as mean values ± SD (n=3).

5.1.4 Effect of sulphur derivatives on the degradation of complex plant biomass by two enzyme combinations

As mentioned previously, sulphide and potentially other sulphur derivatives may increase the rate of cellulose degradation, which is important industrially with regard to the anaerobic digestion of plant biomass to fermentable sugars. An effective means to degrade plant biomass waste may act as an important renewable carbon source for the production of a variety of biofuels, such as ethanol, acetone and butanol. An important agricultural plant biomass waste product is sugarcane bagasse. Figure 35 illustrates the effect of sulphide and sulphate on the enzymatic degradation of bagasse using three combinations of enzymes, namely 75% XynA to 25% EngE (Figure 35a), 50% ManA to 50% EngE (Figure 35b) and 75% XynA to 25% ManA (Figure 35c). Both sulphide and sulphate had a stimulatory effect on the molar ratio combination of 75% XynA to 25% EngE; however, the greatest enhancement of enzymatic activity occurred at a concentration of 1.56 mM sulphate and 15.59 mM sulphide (Figure 35a). With regard to the effect of sulphur derivatives on the molar ratio of 50% ManA to 50% EngE, sulphide did not have a notable effect on the enzyme activity below a concentration of 12.47 mM, after which the activity increased by approximately 50%. On the other hand, sulphate stimulated an increase in enzyme activity; however, there was no correlation between an increase in sulphate concentration and enzyme activity (Figure 35b). The degradation of bagasse with the molar ratio of 75% XynA to 25% ManA showed a different trend to the other enzyme combinations, in the presence of sulphate. The sulphate appeared to have an inhibitory effect on the enzyme activity with increasing concentration (Figure 35c). With regards to the sulphide, a general increase in enzyme activity was observed with an increase in sulphide concentration (Figure 35c).

(a)



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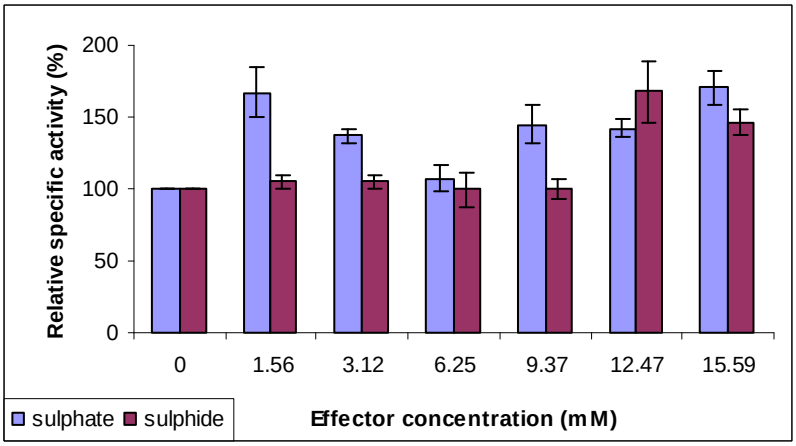

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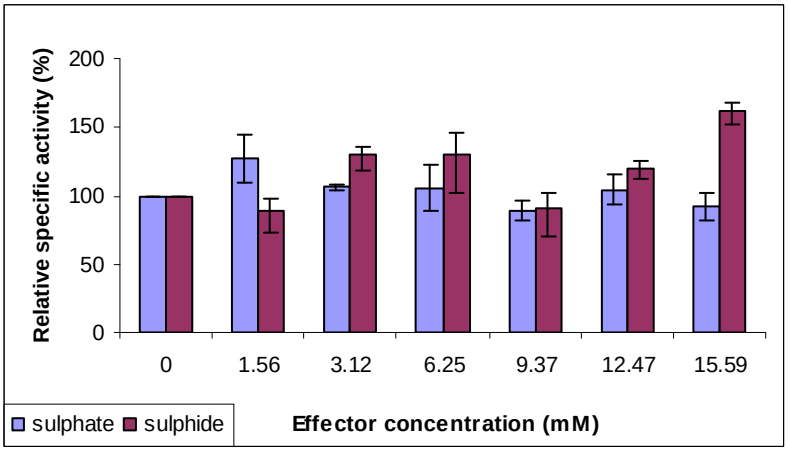
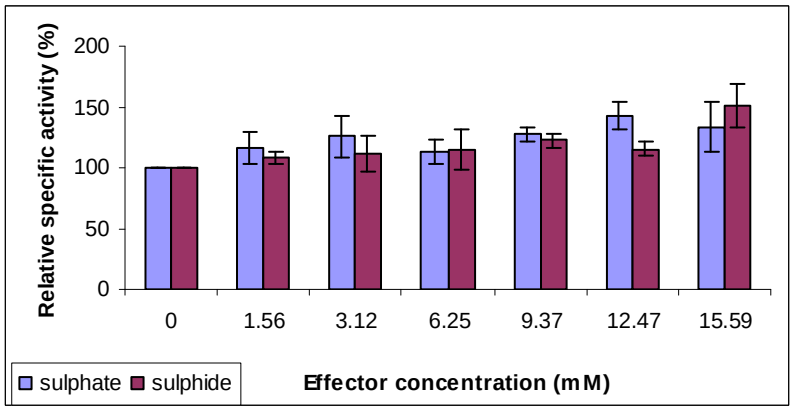


Figure 35: Effect of sulphide and sulphate on the synergistic associations between the various combinations enzymes against the sugarcane bagasse. The first enzyme combination was 75% XynA to 25% EngE (a), followed by 50% ManA to 50% EngE (b), and finally 75% XynA to 25% ManA (c), as represented by the specific activity relative to the specific activity obtained against the various substrates in the absence of the sulphur derivatives. Values are presented as mean values ± SD (n=3).

The second agricultural waste product used in the synergy and inhibition or activation studies was pineapple fibre. The molar ratios that produced the greatest of synergy against the pineapple fibre were used previously to determine the effect of sulphide and sulphate on the degradation of the pineapple substrate. The molar ratios used in the study were as follows: 50% XynA to 50% EngE (Figure 36a), 75% ManA to 25% EngE (Figure 36b) and 50% XynA to 50% ManA (Figure 36c). Both the sulphur derivatives enhanced the enzyme activities as there was a direct correlation between the enzyme activities and the concentration of the sulphur derivatives, i.e. an increase in the effector's concentration led to an increase in the enzyme activity against the pineapple fibre (Figure 36a). This was similar to the results obtained with the molar ratio of 50% XynA to 50% ManA (Figure 36c). Unlike the molar combinations of 50% XynA to 50% EngE (Figure 36a) and 50% XynA to 50% ManA (Figure 36c), the molar ratio of 75% ManA to 25% EngE showed a decrease in the enzyme activity in the presence of an increasing concentrations of sulphate. The opposite effect was observed with increasing concentrations of sulphide (Figure 36b).

(a)



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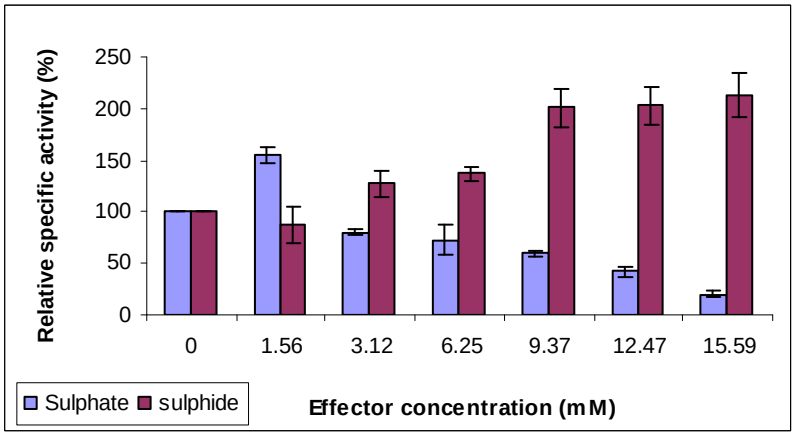

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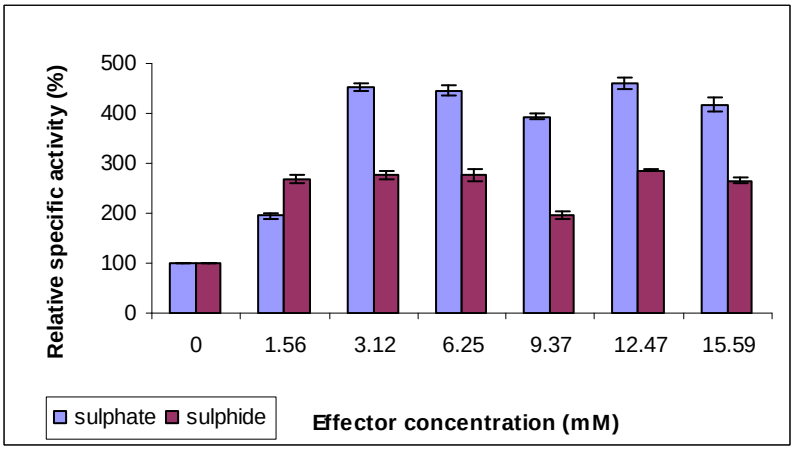


Figure 36: Effect of sulphide and sulphate on the synergistic association in the degradation against pineapple fibre. The molar ratios that produced the greatest degrees of synergy against the pineapple fibre were used in the inhibition/activation studies. The first enzyme combination had a molar ratio of 50% XynA to 50% EngE (a), the second combination had a molar ratio of 75% ManA to 25% EngE (b), and the final combination had a molar ratio of 50% ManA to 50% XynA (c) as represented by the specific activity relative to the specific activity obtained against the various substrates in the absence of the sulphur derivatives. Values are presented as mean values ± SD (n=3).

5.2 A combination of all three enzymes

5.2.1 Degree of synergy obtained with all three enzymes against the soluble substrates

Once the synergistic relationships between combinations of two of the three enzymes were elucidated, the relationship between combinations of all three enzymes was determined. The specific activities and degrees of synergy against 2% (w/v) soluble substrate (BWX, CMC and LBG) (Figure 37), sugarcane bagasse (Figure 38) and pineapple fibre (Figure 39) were determined for different enzyme combinations. The synergy studies involving three enzymes were performed in an identical approach to the synergy studies involving two enzymes. Various combinations containing different percentage of the three enzymes were assayed against the different substrates as depicted in Figures 37 – 39. Similarly, with the degrees of synergy, which were calculated by dividing the experimental activities obtained by theoretical activities of the individual enzymes. A degree of synergy greater than one indicated that the experimental activities obtained were greater than the sum of the activities for the individual activities.

The enzyme combination that consisted of a molar ratio of 12.5% XynA to 87.5% EngE produced the highest specific activity against the soluble substrates (Figure 37a); however, the greatest degree of synergy was obtained at a molar ratio of 25% XynA to 50% ManA to 25% EngE (Figure 37b).

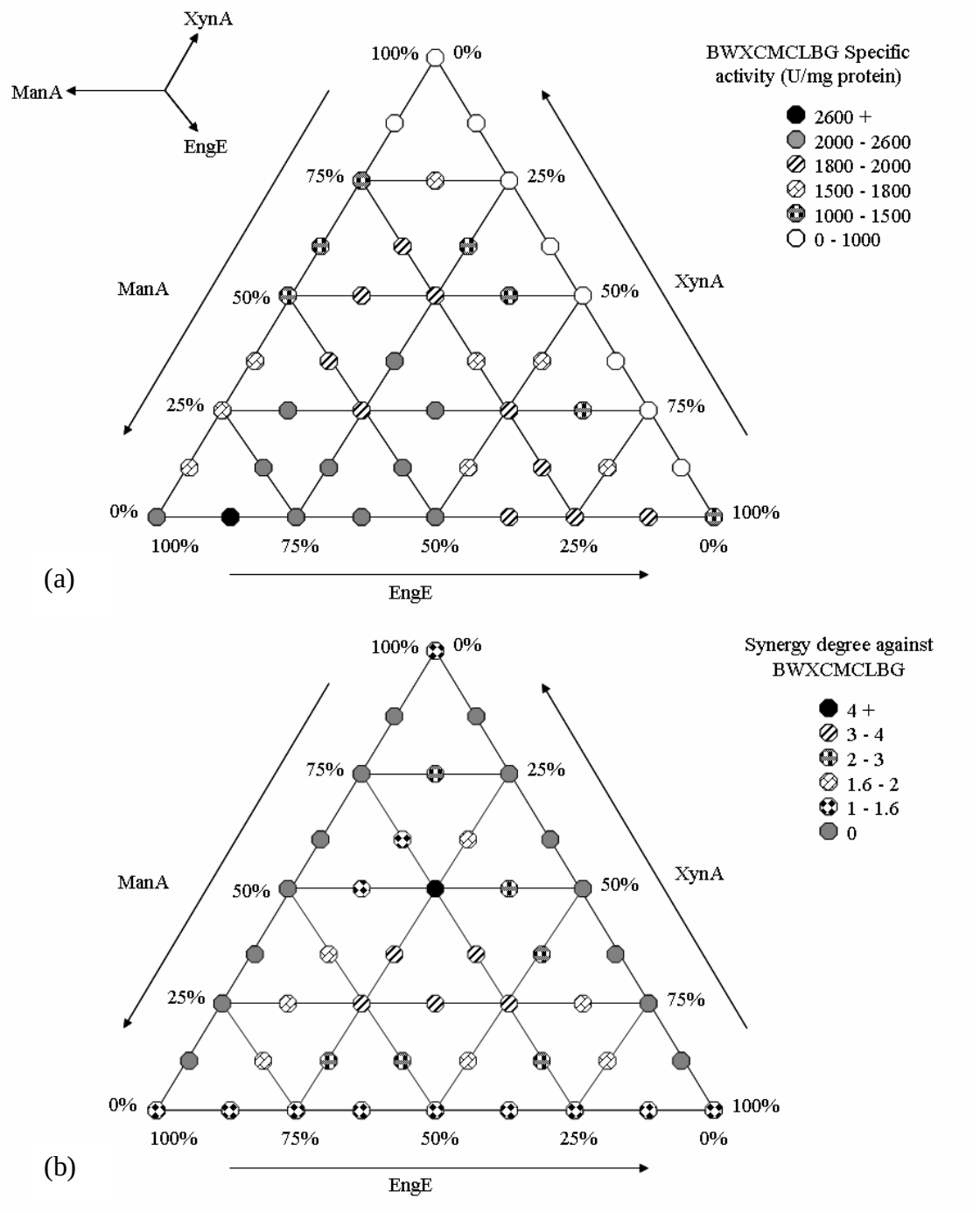
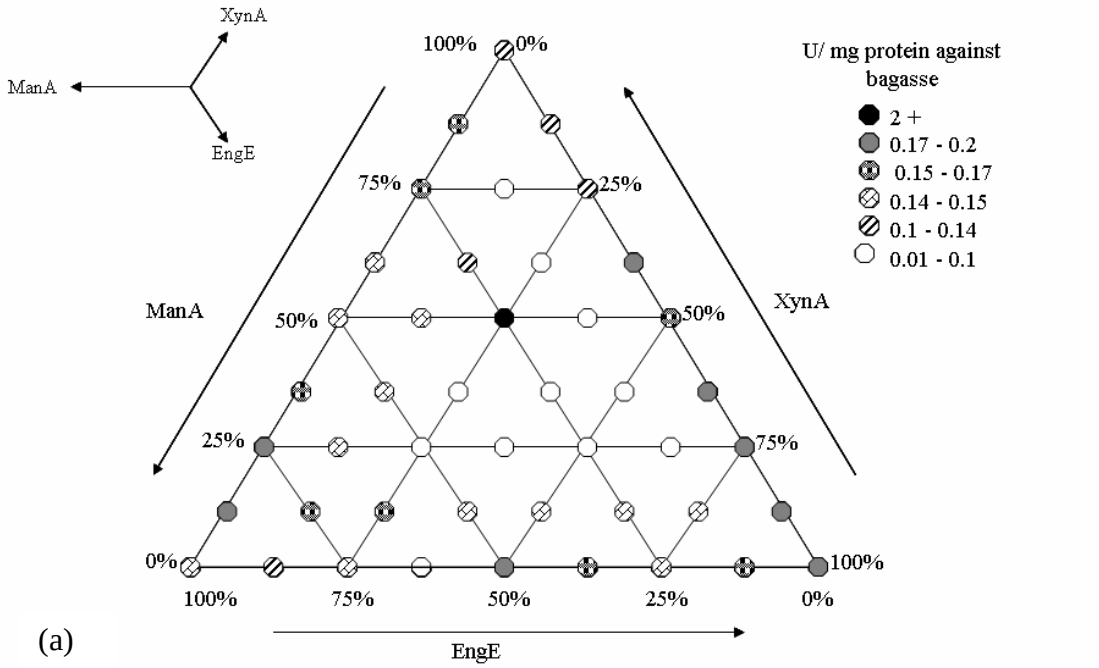


Figure 37: A schematic representation of the specific activities and degrees of synergy obtained for the three enzymes against the soluble substrate mixture. The specific activities (a) and degree of synergy (b) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a molar percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

5.2.2 Degrees of synergy obtained for combinations of all three enzymes against complex plant biomass

As previously mentioned, natural biomass consists of a wide variety of compounds, the most predominant constituent is cellulose, which is intertwined with hemicellulose. Thus, it would be expected that the most effective biomass conversion into various monomeric components would occur in the presence of a consortium of enzymes. The enzyme activities and the subsequent degrees of synergy were determined, between the three enzymes of interest, against sugarcane bagasse and pineapple fibre. In the case of the sugarcane bagasse, the optimum activity and degree of synergy was obtained for the molar ratio of 25% EngE to 50% ManA to 25% XynA (Figure 38 a and b). Interestingly, the optimum synergy and activity against pineapple fibre was obtained at a molar ratio of 50% ManA to 50% XynA (Figure 39 a and b).



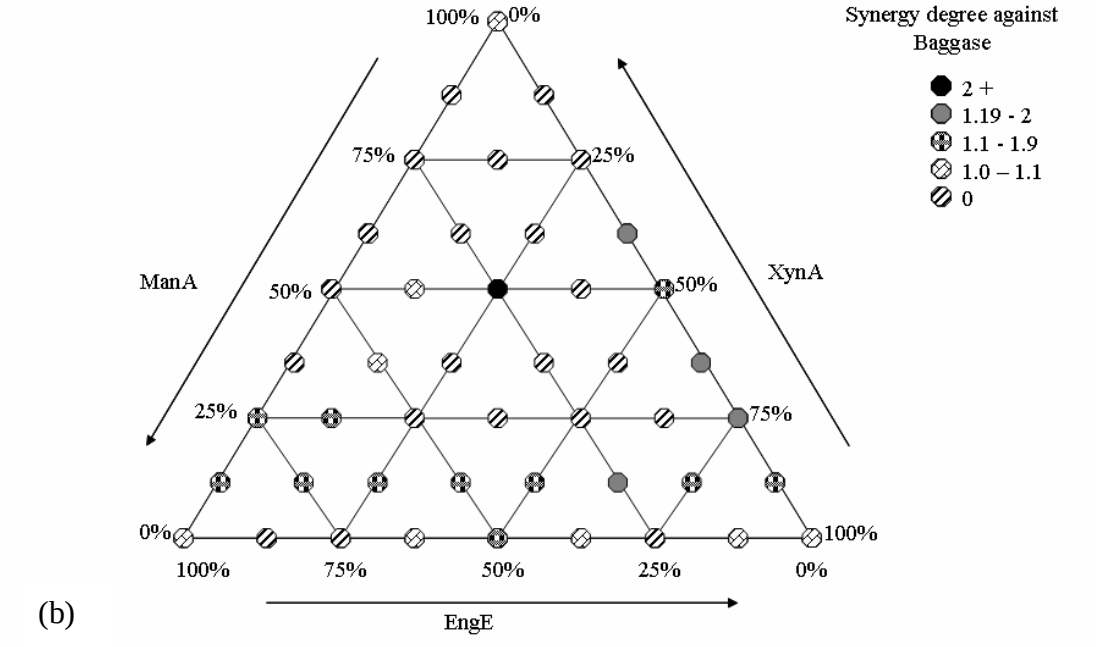
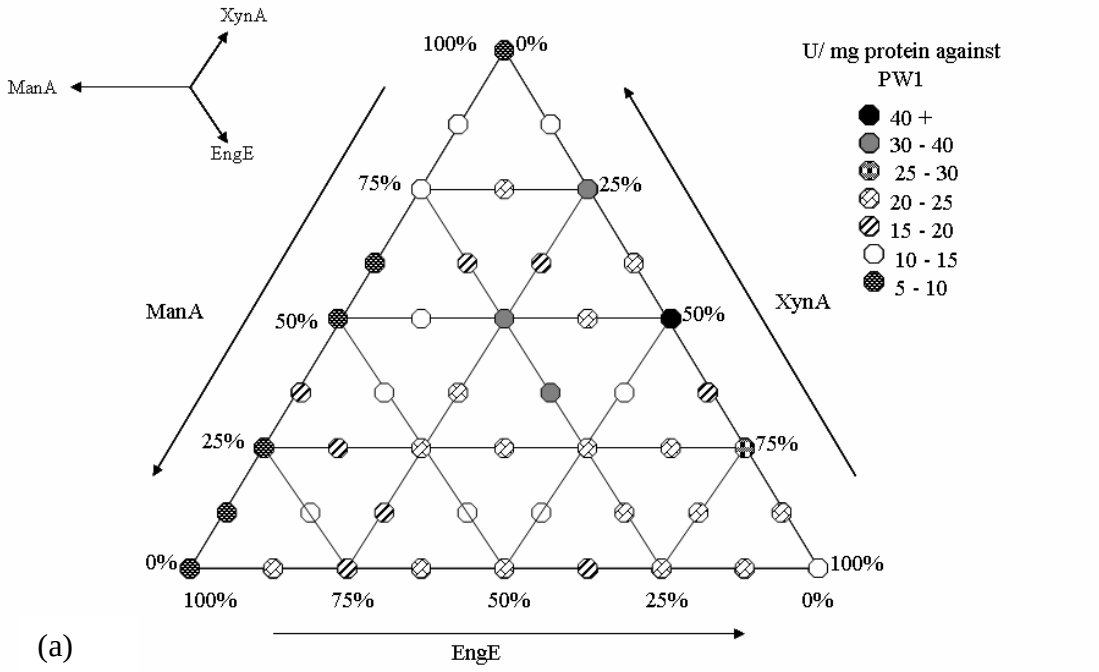


Figure 38: A schematic representation of the specific activities and degrees of synergy obtained for the three enzymes against the sugarcane bagasse. The specific activities (a) and degree of synergy (b) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a molar percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.



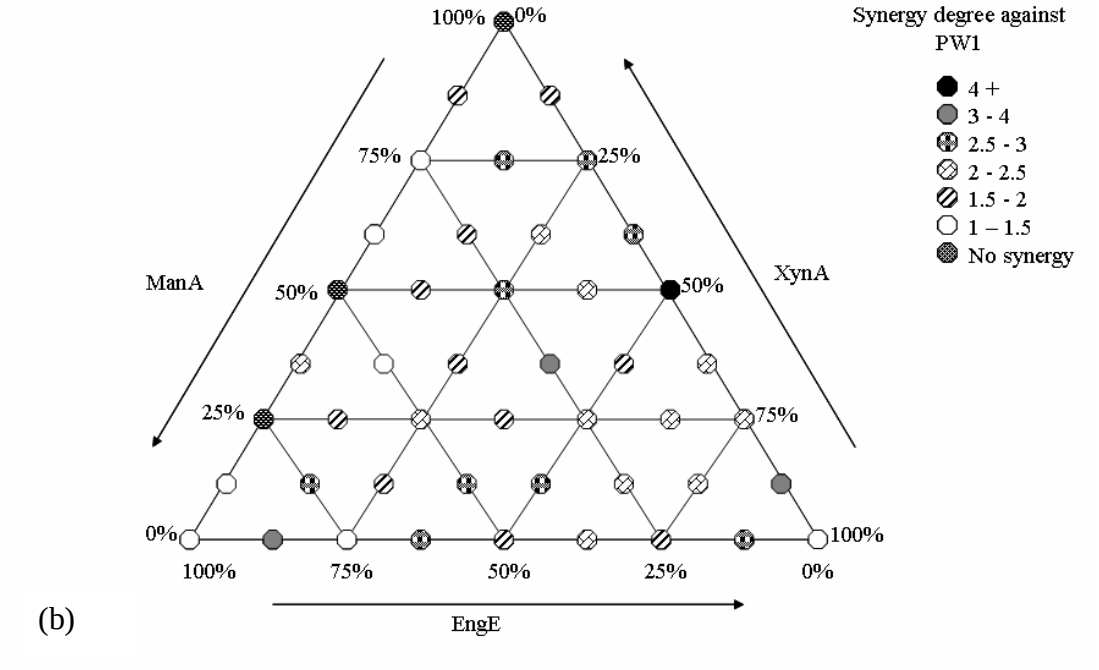
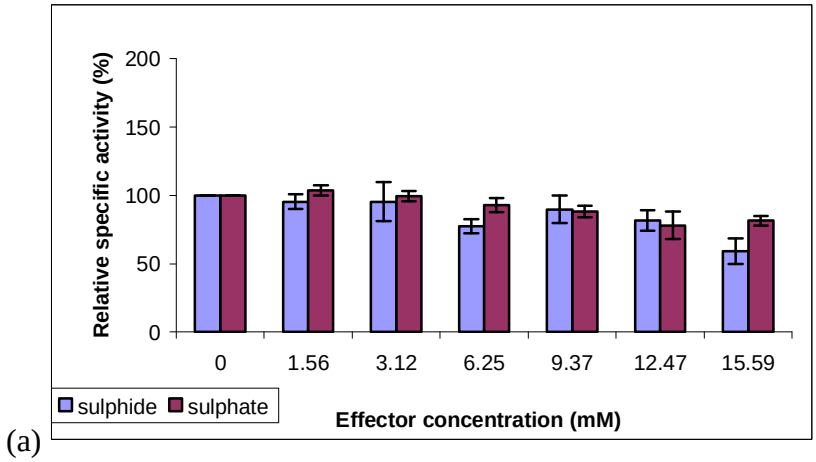


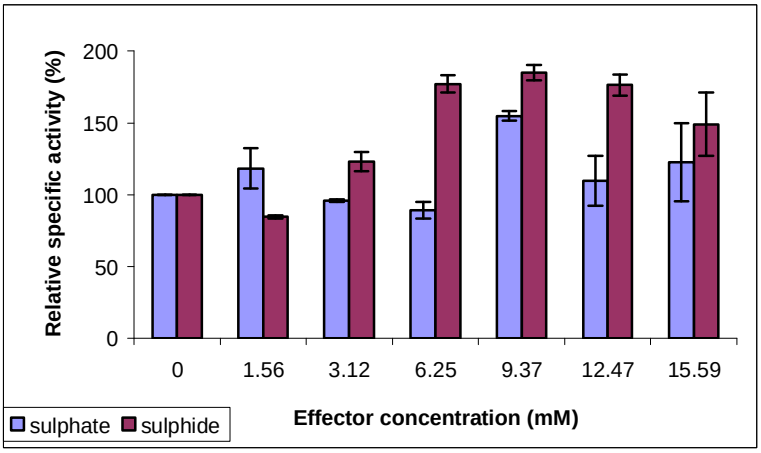
Figure 39: A schematic representation of the specific activities and degrees of synergy obtained for the three enzymes against the pineapple fibre (PW1). The specific activities (a) and degree of synergy (b) are represented as the different shaded circles, as shown in the figure. The enzymes were mixed in varying compositions shown according to the axis as a molar percentage. The arrows indicate the directions of axis for each enzyme. The degrees of synergy are shown as the actual activities divided by the theoretical activities.

5.2.3 Effect of sulphur derivatives on the degradation of the complex plant biomass

Once the optimum degrees of synergy were determined for the combinations depicted in figures 38 and 39, the potential inhibition and activation by the sulphur derivatives against the bagasse and pineapple fibre was determined (Figure 40). The soluble substrate mixture contained 2% (w/v) BWX, 2% (w/v) CMC and 2% (w/v) LBG. Increasing concentrations of both sulphide and sulphate had an inhibitory effect on the enzyme activities against soluble substrate mixture, with the enzyme molar ratio of 25 % EngE to 50 % ManA to 25 % XynA (Figure 40a). The optimum degree of synergy for the degradation of bagasse was 25% EngE to 50% ManA to 25% XynA. Unlike the effect seen in Figure 40a, an increase in sulphate concentration up until 9.37 mM increased enzyme activity, after which there was an inhibitory effect on the enzyme activity (Figure 40b). Sulphide studies, on the other hand, showed no correlation between an increase in sulphide concentration and enzyme activity (Figure 40b). The degradation of the pineapple fibre produced unexpected results. The optimum activity and degree of synergy was observed with a mixture of enzymes devoid of EngE. The maximum activity and degree of synergy was obtained with a molar ratio of 50% ManA to 50% XynA (Figure 39a and b). Thus, the effect of sulphate and sulphide was identical to that in Figure 36c, which is shown again in Figure 40c.



(a)



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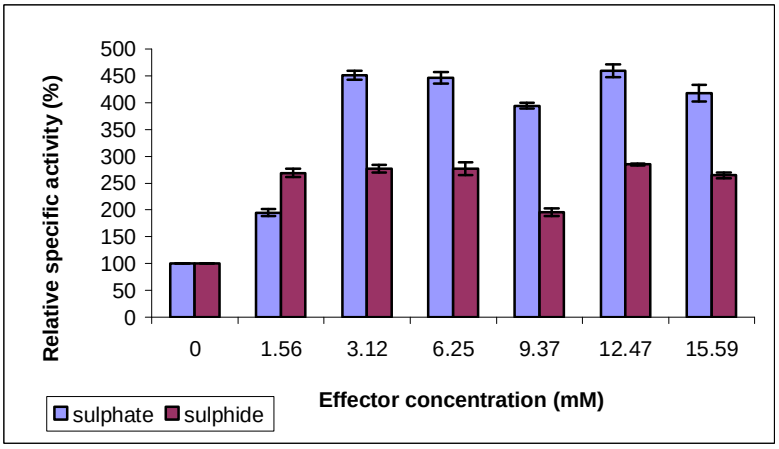

(c)

Figure 40: Effect of sulphur derivatives on the synergistic degradation of various substrates by three recombinant enzymes. Hydrolysis of the simple substrates using the molar ratio of 25% XynA to 25% EngE to 50% ManA (a), hydrolysis of sugarcane bagasse using the molar ratio of 25% XynA to 25% EngE to 50% ManA (b) and hydrolysis of pineapple fibre using the molar ratio of 50% XynA to 50% ManA.

5.3 Discussion

Once the initial characterisation, and the effect of sulphur derivatives on the hydrolytic activities, of the individual enzymes against various substrates was performed on the individual enzymes, the presence of synergistic associations between the cellulase and the two hemicellulases were investigated. As mentioned before, previous synergy studies have focused on the synergistic associations that exist between cellulase isozymes. The same organism may express the cellulase isozymes. Cellulases isolated from different sources have also been used in the synergy studies (Wood and McCrae, 1972, 1979; Henrissat *et al.*, 1985; Wood *et al.*, 1989; Nidetzky *et al.*, 1994; Gaudin *et al.*, 2000; Irwin *et al.*, 2000; Murashima *et al.*, 2002a). The synergistic associations that exist between cellulosomal cellulases and other cellulosomal or cellulosome associated enzymes have not been extensively studied. This chapter investigated the potential synergistic relationship that existed between one of the cellulosome's prominent endoglucanases (EngE) and two hemicellulases (ManA and XynA) that may be expressed in association with conserved cellulosomal enzymes.

The synergistic associations between the different enzymes were determined by dividing the actual specific enzyme activity obtained by the sum of the specific activity that would theoretically be obtained for the combined enzyme activities as described previously by Murashima *et al* (2002a). The recombinant enzyme combinations used were: (a) XynA and ManA, (b) ManA and EngE, (c) XynA and EngE, and (d) XynA, ManA and EngE.

The first assays were set up to determine the presence of synergistic associations, and were between two out of three enzymes using varying molar ratios of the enzymes, ranging between 100% of enzyme A to 0% of enzyme B, through to 0% of enzyme A to 100% of enzyme B. As expected, the results obtained for the synergy studies between two out of the three enzymes varied between the substrates, indicating the importance of substrate chemical compositions in designing the ideal enzyme mixture for the hydrolysis of complex plant biomass.

As mentioned previously, BWX is essentially composed of a chain of xylose residues linked via β-(1→4)-xylosidic bonds **and** LBG is composed of mannose residues linked via β-(1→4)-mannosidic bonds with galactose side chains. The effective degradation of the mixed substrate (BWX and LBG) required a higher percentage of ManA, potentially indicating that BWX is hydrolysed to a greater extent than LBG. This may be due to LBG having a more complex chemical structure due to the presence of the side chains that may interfere with recognition and binding of ManA to LBG. The other mixed substrate combinations required equal quantities of the respective enzymes for the maximal production of reducing sugar thus indicating that these enzymes have similar affinities for the respective substrates.

As previously mentioned sugarcane bagasse has a dry weight composition of approximately 42% alpha-cellulose, 22% lignin, 28% hemicellulose, and approximately 8% cane wax and organic acids (Ouensanga and Picard, 1988). The higher percentage of XynA to ManA required for the optimal degradation of bagasse suggests two possible scenarios.

Firstly, the xylan component of the hemicellulolytic fraction of bagasse is greater than the mannan component or the regions on the mannan backbone that are hydrolysed by ManA may not be accessible to the enzyme unless the hemicellulose structure is “opened” by the hydrolysis of the β-1,4-xylosidic bonds on the xylan backbone. It is generally accepted that the hemicellulose and cellulose fibres are closely associated. However, the associations between different hemicellulose fibres are not well established, i.e. if there is a consistent distribution of the different types of hemicellulose throughout the lignocellulose substrates, or if certain regions within the lignocellulose fibres have a higher percentage of the one form of hemicellulose, e.g. mannan in comparison to xylan. However, various forms of hemicellulose fibres are cross-linked, increasing the complexity of the hemicellulolytic fraction and the requirement for a consortium of enzymes to facilitate degradation (Rowell *et al*., 1997). If this is the case, the mannanase and the endoglucanase may have a higher specificity for those regions; thus the degradation of the β-mannosidic bonds between the mannose residues allows for a greater

percentage of the β-glucosidic bonds present on the cellulosic fibres to be exposed to the endoglucanase.

Another possible explanation for the equal quantity of the enzymes required for the degradation of the bagasse is the similarity between the mannan and cellulose backbone (Hilge *et al*., 1998). The similarity between the cellulose and mannan backbones may allow for decreased specificities of the mannanase and endoglucanase activity. This allows for the synergistic degradation of the mannose and cellulose fibres to occur through the activity of both enzymes instead of the individual enzymes recognising and hydrolysing soley their respective substrates. The XynA to EngE ratio is once again similar to that obtained with the ratio between the xylanase and mannanase, with the dominant enzyme in the combination being the xylanase. This potentially indicates that the xylan backbone is associated with the cellulose fibres in such a way that the xylan fibres form a protective barrier around the majority of the amorphous regions on the cellulose chains. This limits the exposure of the β-glucosidic bonds to the endoglucanase and therefore a higher percentage of xylanases would be required for the effective degradation of the complex substrates.

As previously mentioned, pineapple leaf fibre has a holocellulose content of approximately 70% to 82%; however the hemicellulose content is approximately 18%, and lignin content 5% – 12% (Satyanarayana 1986; Doraiswamy and Chellamani, 1993; Majumdar and Chanda, 2001; Reddy and Yang, 2005). These percentages may vary depending on the age of the plant when harvested. The XynA to ManA and XynA to EngE ratios required equal ratios of the enzymes for the degradation of the pineapple fibre potentially for different reasons. The requirement for equal ratios of the hemicellulases may indicate that the mannan and xylan components of the hemicellulolytic fraction of the pineapple fibre may be similar. The requirement of equal ratios of XynA and EngE, on the other hand, may indicate that the enzymes act simultaneously at equal rates to degrade the pineapple fibre. The ManA to EngE results obtained for the pineapple fibre indicate that the hydrolysis of the cellulose may be dependent on the degradation of the mannan backbone to increase the exposure of the β-

1, 4-glycosidic bonds to attack. These results were unexpected. The pineapple fibre consists predominantly of cellulose; however, the results indicate that the hemicellulose components may act to protect the cellulose fibres from degradation.

The molar ratio of 1:2:1 for the combination of XynA to ManA to EngE (that produced the greatest synergistic degradation of the soluble substrates and the bagasse) was expected, as the soluble substrate mixture consisting predominantly of commercial forms of hemicellulose. Hemicellulose and lignin partially protect cellulose from degradation; thus in order to effectively degrade cellulose fibres, the hemicellulose fibres need to be hydrolysed from both the bagasse and the pineapple fibre.

Murashima *et al*. (2002a) studied the synergistic associations between three cellulosomal enzymes (EngE, EngH and ExgS), from which they concluded that the optimal degradation of crystalline cellulose (Avicel®) occurred with a ratio of 1:2:1. In 2003, Murashima and co-workers used the cellulase ratio (1:2:1) to produce mini-cellulosomes, which they used to study the synergistic association between the mini-cellulosome and XynA against corn stalk fibre. According to literature, corn is not only an important international agricultural source of food, it has also been studied extensively as a potential renewable biomass for the production of various energy yielding compounds, like ethanol. Corn stalk fibre is an insoluble complex substrate, composed of varying percentages of cellulose, hemicellulose, lignin and pectin, depending on the age when the plants are harvested. Corn stalk fibre consists of approximately 40% - 60% cellulose, 28% hemicellulose and 7% - 20% lignin (Ali *et al.*, 2000; Reddy and Yang, 2005). The results obtained by Murashima and co-workers (2003) indicated that corn stalk fibre was effectively degraded with EngE to EngH to ExgS to XynA in a ratio of 2:4:2:1. This ratio was expected due to the chemical composition of corn stalk fibre, which is predominantly cellulose. Thus, for the optimal degradation of corn stalk fibre, cellulases are essential.

Once the optimal degrees of synergy had been established, the effect of sulphur derivatives on the synergistic activities was investigated. With the soluble substrate mixtures, there was a general decrease in the enzyme activity when ManA constituted

50% or more of the enzyme mixture. Sulphate had no effect on the ManA and EngE combination. As mentioned previously, the inhibition of the enzyme activity against the soluble substrates may be due to the presence of side chains. The presence of increasing concentrations of the sulphur derivatives enhanced the activity of the combinations containing XynA and EngE against bagasse. The presence of sulphide generally had no effect on the degradation of the bagasse with the ManA to XynA combination. Against bagasse; however, the presence of sulphide caused a slight enhancement in the activity. With the enzyme combination consisting of two out of three enzymes against the pineapple fibre, the increasing concentrations of sulphur derivatives enhanced the enzyme activities, except 75% ManA. In this case, increasing concentrations of sulphide enhanced the enzyme activities. However, increasing concentration of sulphate drastically decreased enzyme activity in the presence of 75% ManA. Thus, the results against the pineapple fibre may be indicative of two possible scenarios: sulphate may not only enhance the degradation of the pineapple fibre (possibly by the separation of the lignin and holocellulose components, increasing the exposure of the β-1, 4-glycosidic bonds) but may also affect ManA directly.

The synergistic degradation of the complex enzymes appears to focus predominantly on the hemicellulases, indicating that, in order to hydrolyse the cellulose fibres, the protective layers provided by the hemicellulose fraction and lignin need to be either partially removed (in the degradation of bagasse) or completely removed as in the case of the pineapple fibre. These results also indicate that the hemicellulose fractions are more easily hydrolysed in comparison to the cellulose, and that the presence of sulphide and sulphate enhance the degradation of the complex substrates in a bioreactor.

5.4 Conclusions

The results obtained for the synergism studies with XynA, ManA and EngE against different substrates indicated the following:

- the chemical composition of a substrate affected the synergistic associations between the different enzymes,
- hemicellulases played an essential role in the degradation of complex plant biomass, as the degradation of the hemicellulose fractions of biomass allowed for the exposure of the cellulose β-1, 4-glucosidic bonds to enzyme hydrolysis,
- enzyme activity was generally enhanced under sulphidogenic and sulphatogenic conditions,
- the presence of sulphide and sulphate may have induced separation of the lignin and holocellulose fraction of the complex substrates, and
- the inhibitory effects obtained may be due to the production and release of phenolic compounds from lignin.

Chapter 6: General discussion and conclusions

The most commonly studied agricultural by-products for the formation of valuable products is from corn, which is possibly the largest American agricultural product. The main problem associated with the use of corn as a renewable carbon source is the direct competition that will occur between research in the biofuel technology sectors and production of food products from corn. The two complex substrates of interest in this report were sugarcane bagasse and pineapple fibre, as these are the by-products of large South African agricultural sectors.

The results reported in this research show that hemicellulases are essential for the degradation of bagasse. The rate of enzyme hydrolysis against the bagasse was low; however, this indicates that the high lignin content has a direct effect on the enzymatic hydrolysis of the substrate as it forms a protective layer. The requirement of a high hemicellulase content in a mixture of enzymes suggests that the β-1,4-glycosidic bonds are partially shielded from degradation. The shielding of the cellulose glucosidic bonds by the hemicellulose fibres and lignin indicates that the hemicellulose fibres are twisted and wrapped around the cellulose chain, and that the cellulose-hemicellulose complex and protected by a lignin sheath, increasing the sugarcane bagasse's recalcitrance to hydrolysis. The low activity obtained against the bagasse (due to the substrates recalcitrance) indicates that untreated it would not be a good lignocellulose source for the production of biofuel, e.g. ethanol.

An interesting observation was made with the synergistic degradation of the pineapple fibre, is that the optimal synergy and degradation of the pineapple fibre was obtained with a ratio of 1:1 of XynA to ManA, despite the pineapple fibre consisting predominantly of cellulose. One potential reason for the sole requirement of hemicellulases in the degradation of the pineapple fibre, are that hemicellulases are required for the initial stages of degradation. Thus, once the hemicellulosic fraction of the pineapple fibres has been partially degraded, the composition of the enzymes required for the synergistic degradation of the pineapple may change to incorporate cellulases. In

comparison with the bagasse, the synergistic degradation of the pineapple fibre had a higher efficiency. Thus the pineapple fibre was potentially better suited as a lignocellulosic source for the production of sugars, which may be fermented to produce alcohols.

As mentioned previously, the presence of lignin appeared to increase the recalcitrance of plant biomass to enzyme and microbial degradation. Thus the removal of hydrolysis of lignin would decrease biomass recalcitrance and increase the degradation of the substrate. Studies have shown that the presence of chemical sulphur derivatives, especially sulphide enhances the degradation of lignocellulose by cellulolytic systems. This increases the production of reducing sugars and aromatic compounds. The same trend has been observed in biologically generated sulphidogenic conditions in the presence of a microbial consortium. Thus the presence of sulphide and sulphate in bioreactors may increase the efficiency of the degradation of the lignocellulose. The results obtained for the synergistic degradation of the complex substrates, in the presence of the sulphur derivatives, indicated that increasing concentrations of sulphide and sulphate dramatically enhanced the enzyme activities in comparison to the enzyme activities in the absence of the sulphur derivatives. The enhanced activity obtained in the presence of the sulphur derivatives indicated that the lignin present in the substrates was being affected by the presence of sulphide and sulphate. These results indicated the potential importance of sulphur derivatives in the degradation of complex substrates and thus the presence of sulphide and sulphate may enhance the anaerobic digestion of plant waste products to produce valuable energy yielding products. The only sulphur derivative that is used industrially is sulphide in the Kraft process. The Kraft process employs sulphide to enhance the chemical delignification of lignocellulose as sulphide inhibits the repolymerisation of lignin. It has been proposed that sulphide inhibits the repolymerisation of lignin by stabilising the phenolic compounds present in lignin, thus preventing lignin condensation (Clayton 1969).

Anaerobic degradation is an important natural process, due to its ubiquity; thus the effective conversion of insoluble compounds e.g. cellulose to soluble monomers, and the

subsequent degradation of these compounds to produce methane and carbon dioxide is important (Novaes, 1986). The anaerobic degradation of complex plant biomass occurs through the action of extracellular enzymes such as cellulases, lipases and proteases. Members of the genera *Bacillus*, *Clostridium*, *Butyrivibrio* and other facultative/obligate anaerobes and sulphate reducing bacteria/ fungi (Novaes 1986) are able to produce these enzymes. Thus, it would be expected to find sulphur derivatives in various metabolic processes within an anaerobic system. In the case of the genera *Bacillus* (which is associated with the initial degradation of complex plant biomass under anaerobic conditions), Beukes and Pletschke (2006) indicated that the sulphide and sulphate respectively enhance and inhibit the cellulosome associated cellulolytic activity against Avicel® and CMC. In relation to the results obtained, increasing the concentrations of sulphur derivatives in a bioreactor containing a consortium of microbes associated with the anaerobic degradation of plant biomass may potentially enhance both the growth of the micro-organisms present in the consortium and the degradation of the biomass to produce biotechnologically important products. These products include a variety of sugars, bioethanol, and various biocomposites (e.g. sinapyl and ρ-coumaryl alcohol) (Charkar and Ragauskas, 2004).

Future recommendations

This research study indicated the need to establish the synergistic association between different cellulosomal and non-cellulosomal enzymes that are associated or incorporated into the cellulosome. One of recommendations for future research is to establish the presence of synergistic associations between a wide variety of enzymes that are typically associated with the degradation of complex plant biomass, e.g. feruloyl esterase and other hemicellulases, in combination with the cellulosomal enzymes. This research will allow the design of the perfect multi-enzyme complex or designer cellulosome for the effective degradation of plant biomass or agricultural waste products, such as sugarcane bagasse and pineapple fibre.

As previously mentioned, biomass is considered recalcitrant to enzymatic and microbial degradation. This recalcitrance may be decreased with various pre-treatments. The partial enzymatic degradation of lignocellulose under conditions, where the sulphur derivatives, sulphide and sulphate were present was established in this research study. The degradation of the biomass may be further enhanced through various physicochemical pre-treatments (thermal, dilute acids and alkaline solutions).

The degradation of complex plant biomass also resulted in the production of various aromatic phenolic products, which may affect the enzyme activities. The identification of these potential inhibitors derived from the degradation of biomass may therefore allow the manipulation of the biomass degradation by preventing the accumulation of these inhibitors.

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