Isolation and characterisation of a xylanase producing isolate from straw-based compost

By

Rudzani Ruth Mutengwe

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae (M.Sc.) in the Department of Biotechnology, University of the Western Cape

Supervisor: Prof. D.A. Cowan

Co-supervisors: Dr. R. Bauer, Dr. M. Tuffin and Dr. M. Smart

February 2012

ABSTRACT

Lignocellulosic biomass, a waste component of the agricultural industry, is a promising source for use in bioethanol production. Due to a complex structure, the synergistic action of lignocellulosic enzymes is required to achieve complete digestion to fermentable sugars. This study aimed to isolate, identify and characterise novel lignocellulase producing bacteria from thermophilic straw-based compost (71°C). Colonies with different morphological characteristics were isolated and screened for lignocellulosic activity. A facultative aerobic isolate RZ1 showed xylanase, cellulase and lipase/esterase activity. In addition to these activities, it was also able to produce proteases, catalases, amylases and gelatinases. RZ1 cells were motile, rod-shaped, Gram positive and endospore forming. The growth temperature of isolate RZ1 ranged from 25-55°C with optimal growth at 37°C. The 16S rRNA gene sequence was 99% identical to that of *Bacillus subtilis* strain MSB10. Based on the biochemical and physiological characteristics and 16S rRNA gene sequence, isolate RZ1 is considered a member of the species *B. subtilis*. A small insert genomic library with an average insert size of 5 kb was constructed and screened for lignocellulosic activity. An *E. coli* plasmid clone harbouring a 4.9 kb gDNA fragment tested positive for xylanase activity. The *xyl* R gene was identified with the aid of transposon mutagenesis and the deduced amino acid sequence showed 99% similarity to an endo-1-4-β-xylanase from *B. pumilus*. High levels of xylanases were produced when isolate RZ1 was cultured (37°C) with beechwood xylan as a carbon source. On the other hand, the production of xylanases was inhibited in the presence of xylose. Marked xylanase activity was measured in the presence of sugarcane bagasse, a natural lignocellulosic substrate. While active at 50°C, higher xylanase activity was detected at 37°C. Isolate RZ1 also produced accessory enzymes such as β-xylosidases and α-L-arabinofuranosidases, able to hydrolyse hemicellulose.

DECLARATION

I declare that *Isolation and characterisation of a xylanase producing isolate from straw-based compost* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Full name: Rudzani Ruth Mutengwe Date: February 2012

Signed.....

ACKNOWLEDGEMENTS

- My heavenly father for providing guidance, strength and wisdom.
- My supervisor Professor Don Cowan for providing the opportunity and necessary guidance to complete my project.
- Dr Marla Tuffin and Dr Rolene Bauer for their professional supervision, guidance, sound judgement and enthusiasm.
- My co-supervisors Dr. M. Smart and Dr. I. Mulako for their extreme patience assistance and support.
- Dr Mark Taylor, Dr Rob Huddy, Dr Browyn Kirby, Natasha Mavengere, William Mavengere and Thulani Makhalanyane for their supervision.
- My friends at IMBM Munaka, Rhulani, Oratilwe, Freedom, Shoni and Timna for their encouragement and support.
- Dr Heidi Goodman for her encouraging words.
- My parents for their love, support and understanding through all my years of study. May God continue to richly and abundantly bless you.
- The National Research Foundation for providing the necessary funding.

CONTENTS

CHAPTER 3: MATERIALS AND METHODS

LIST OF FIGURES

CHAPTER 2

CHAPTER 4

LIST OF TABLES

CHAPTER 2

CHAPTER 4

LIST OF ABBREVIATIONS

bp	Base pair
BLAST	Basic Local Alignment Search Tool
CAZY	Carbohydrate-Active Enzymes database
CE	Carbohydrate esterase
CMC	Carboxymethylcellulose
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide tri-phosphates
°C	Degrees Celsius
EDTA	Ethylendiaminetetraacetatic acid
×g	Centrifugal force
g	Gram
GH	Glycoside hydrolase
gDNA	Genomic DNA
GeneBank	Nucleotise sequence database
HPLC	High performance liquid chromatography
IPTG	Isopropyl-D-thiogalactoside
kb	Kilobase
LB	Luria Bertani
mg	Microgram
ml	Milliliter
min	Minute
mM	Millimolar
µl	Microliter

NCBI	National Center for Biotechnology Information
ng	Nanogram
O.D	Optical Density
Ω	Ohm
PCR	Polymerase Chain Reaction
*p*NPA	*p*-nitrophenyl-α-L-arabinofuranoside
*p*NPX	*p*-nitrophenyl-β-D-xylopyranoside
RBB	Remazol Brilliant Blue
s	Seconds
SCB	Sugarcane bagasse
SCB(l)	Sugarcane bagasse liquid
SCB(s)	Sugarcane bagasse solid
sp.	Species (single)
spp.	Species (plural)
TAE	Tris-acetate-EDTA
Tris HCl	Tris (hydroxymethyl) methylamine hydroxychloride
TSB	Tryptic Soy Broth
TE	Tris-EDTA
U	Units
v/w	Volume per weight
w/w	Weight per weight
X-Gal	5-Bromo-4-Chloro-3-indolyl-D-galactopyranoside

CHAPTER 1: GENERAL INTRODUCTION AND PROJECT AIMS

The disadvantages associated with the use of fossil fuels, such as pollution and resource depletion, has led to a shift towards alternative energy sources such as biofuels (Rogner, 2000). Bioethanol is the most produced biofuel worldwide. Currently, bioethanol is produced from starch or sugar crops which are used as food crops for human consumption. The sugar yield from these sources is very low compared to the sugars retained in plant cell wall polysaccharides, also referred to as lignocellulosic biomass (Taherzadeh, 1999). Lignocellulose is the major structural component of plant biomass typically found in agricultural and municipal waste. It is comprised of cellulose, lignin and hemicellulose which are linked together to form a complex, fibrous and insoluble matrix. Producing bioethanol from lignocellulose is considered more sustainable than using first generation food crops or fossil fuels for energy production.

The production of bioethanol from plant biomass involves two processes. Firstly, cellulose and hemicellulose, the two main polysaccharides present in plant derived waste, are hydrolysed into fermentable sugars. These sugars are thereafter fermented to ethanol by microorganisms such as yeast and bacteria. Hydrolysis involves the combined action of a number of enzymes including hemicellulases, cellulases and esterases to break down the highly recalcitrant lignocellulose structure (Kumar, 2009). To increase the sugar yield and accessibility of enzymes to the lignocellulosic substrate, pre-treatment of the fibrous material is required preceding hydrolysis. This softens the biomass and breaks down the cell structure.

Hemicellulose is the second most abundant polysaccharide present in lignocellulosic biomass and is closely associated with cellulose in the plant cell wall. Xylans are the most abundant hemicelluloses and are composed of heteropolysaccharides with homopolymeric backbones consisting of β-1,4 linked D-xylopyranose chains (Saha, 2003). Heteropolysaccharide branches of xylan may contain arabinose, glucuronic acid and acetic, ferulic, and *p*-coumaric acids. The composition of hemicellulose, however, differs between plant sources from which the xylan is derived. Due to the heterologous nature of xylan, several xylan degrading enzymes are required for the complete hydrolysis of hemicellulose (Betts *et al*., 1991). Among these enzymes, xylanases are responsible for the hydrolysis of the largest proportion of hemicellulose as they hydrolyse the β-1,4 bonds of the xylan backbone. Xylanases belong to the glycosyl hydrolase family of enzymes as they act on the glycosidic bonds (Collins *et al*., 2004). Xylanases have broad industrial application. As discussed here, they may be used in improving the utilisation of hemicellulosic sugars of lignocellulose materials for conversion into bioethanol. Some of the other uses of xylanases are in the manufacturing of bread to increase bread volume (Maat *et al*., 1992) and in the textile industry to improve fabric softening (Prade, 1995). The major sources of commercial xylanase are from bacteria, fungi, yeast and plants (Kuhad *et al*., 1997; Sunna and Antranikian, 1997).

Lignocellulytic enzymes that function at elevated temperatures are desirable for thermophilic industrial processes, as their catalytic activity is unaltered by the exposure to high temperatures (Blumer-Schutte *et al*., 2008). Thermophilic enzymes offer significant advantages in industry, such as an increased rate of hydrolysis and reduced risk of mesophilic-microbial contamination (Sun and Cheng, 2002). Employing a high temperature process may result in more readily digested substrates, and a number of enzymes have already been identified that can be included in processes at elevated temperatures.

Thermostable enzymes can be isolated from organisms living in various hot environments through direct culturing and characterisation (Blumer-Schuette *et al*., 2008).

The main aim of this study was to identify a xylanase producing microorganism from straw-based compost (71°C) and characterise its xylanase activity. Specific objectives of this study are listed below:

- Isolate and identify a xylanase producing thermophilic bacterial strain from compost
- Construct a small insert genomic DNA library of this strain
- Screen the library for clones displaying lignocellulosic activity
- Determine the sequence identity of a gene displaying xylanase activity and assess novelty
- Investigate the production of xylanases by the bacterial strain for potential application in hydrolysis of lignocellulosic biomass

CHAPTER 2: LITERATURE REVIEW

Lignocellulose depolymerisation for bioethanol production

2.1 Biofuels as alternative fuel sources

Fossil fuels are non renewable energy resources and have been the major source used for energy production since the early twentieth century (Wyman, 1996). Increased industrialisation and the expanding human population have created a growing demand for energy. In the USA, one of the largest energy utilising countries, vehicles alone consume in excess of 450 billion litres of fossil fuel per year (EIA, 1998). All over the world it is currently acknowledged that fossil fuels will not be able to meet the ever-increasing high energy demand (Ballesteros *et al.*, 2006). Additionally, the processing of fossil fuels has contributed to the generation of high levels of air, water and soil pollution which are detrimental to the environment. Together, these factors have led to an increase in research for alternative renewable fuel sources such as biofuels (Wyman, 1996). Using plant material for fuel production may be considered an environmentally friendly process, as the growth of plant biomass utilises carbon dioxide from the air (Licht, 2006). This results in a reduction of air pollution and carbon emmission.

Biofuels are solid, liquid or gaseous fuels produced from plant biomass using direct or indirect fermentation (Chhetri and Islam, 2008; Demirbas, 2008). Direct fermentation involves the breakdown of biomass into fermentable sugars which are in turn converted to alcohol by microbial fermentation (Collins *et al.*, 2005). Indirect fermentation involves the burning of plant material resulting in the production of gas which is converted to biofuels by microorganisms (Klasson *et al.*, 1992).

Examples of biofuels are bioethanol, biobutanol, biohydrogen, biodiesel, biogas and biomethanol. Currently bioethanol is the most used biofuel in the world comprising more than 90% of the biofuel market (Dermirbas, 2008). In countries such as the USA, Brazil, Spain, Russia and Germany, concerns regarding the depletion of fossil fuel reserves have resulted in increased bioethanol production (Hahn-Hägerdal *et al.*, 2006). In Brazil, 14, 383 million litres of bioethanol were produced in 2005 which represented 40% of the fuel consumed in Brazil (Baez and Demain, 2008). Smaller countries have also developed smaller scale bioethanol industries to boost their economy (Sánchez and Cardona, 2008). Worldwide, the production of bioethanol has increased over the last 25 years with a sharp increase between the years 2000 to 2006 (Figure 2.1) (Licht, 2006).

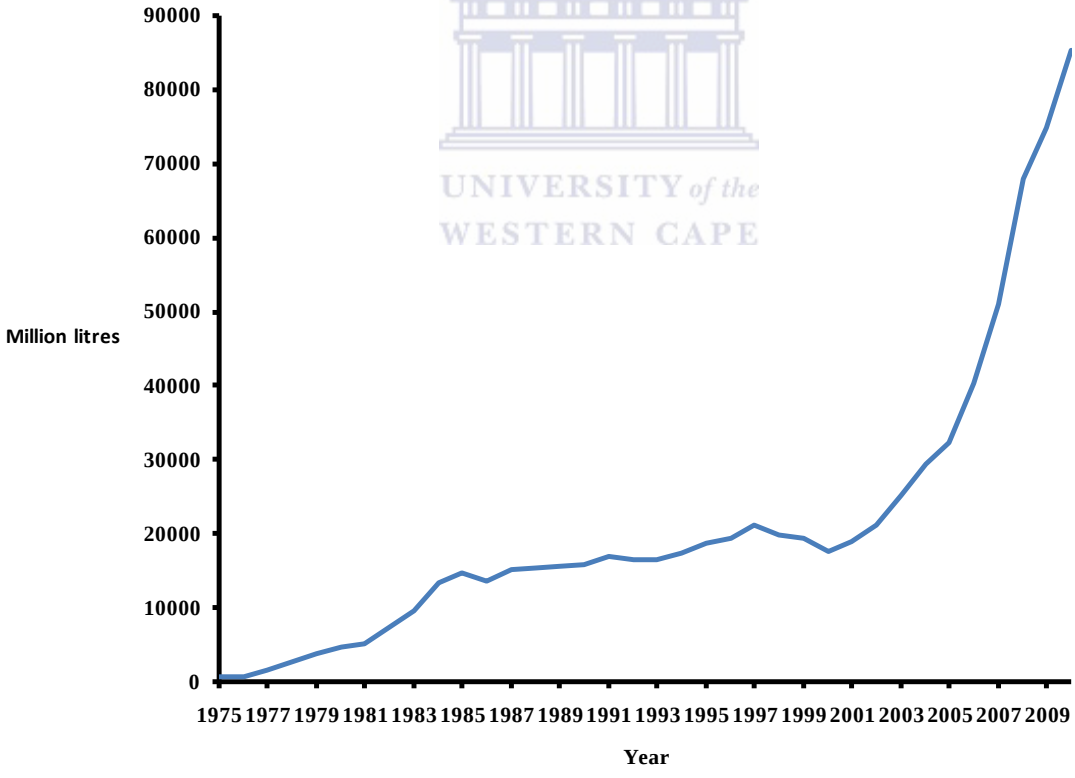

Figure 2.1 Global bioethanol production from 1975 to 2010. Quantities are given in million litres (http://www.earthpolicy.org/Updates/2006/Update55 data.htm).

The leading countries for the production of bioethanol are America and Brazil, which combined account for approximately 70% of the world bioethanol production (Balat *et al*., 2008). In developing countries, such as South Africa, the production of bioethanol is suppressed by high production costs (Von Sivers and Zacchi, 1996). These costs are due to the processing of bioethanol and the feedstocks required for its production. In total, the world bioethanol production reached 51,000 million litres in 2007 (Table 2.1) (Sánchez and Cardana, 2008).

Table 2.1 Bioethanol production for 2007 in the world in millions of litres produced (Sánchez and Cardana, 2008).

Ranking	Country	Ethanol production (million litres)
1	USA	18,376
2	Brazil	16,998
3	China	3,849
4	India	1,900
5	France	950
6	Germany	765
7	Russia	647
8	Canada	579
9	Spain	462
10	South Africa	386
Total		**51,000**

2.2 Plant biomass for bioethanol production

Bioethanol may be produced from either first or second generation processes (Mitchell, 2008). First generation bioethanol refers to fuels produced from food crops such as corn, sugar cane juice, starch and vegetable oil (Von Sivers *et al*., 1994). First generation biofuel

production has a negative social impact, particularly within the developing world, as the feedstock crops could be utilised for human consumption (Leung *et al.,* 2009). Furthermore, the desire to plant fuel-crops has contributed to increasing deforestation (Gomez *et al*., 2008a).

Recent efforts are concentrating on second generation biofuel production, which refers to fuels produced from agricultural waste known as lignocellulose biomass (Hahn-Hägerdal *et al.,* 1991; Hahn-Hägerdal *et al.,* 1993). It is predicted that second generation biofuel production will double global bioethanol yields without affecting the production and availability of food crops since it is produced from lignocellulose materials (Jeczmionek *et al*., 2006). Bioethanol production may also assist in job creation within rural areas (Koh *et al*., 2009).

Lignocellulose is the main constituent of plant cell walls and accounts for approximately 50% of the biomass in the world (Claassen *et al*., 1999). Lignocellulose is comprised of cellulose, hemicellulose and lignin as its major components (Lynd *et al*., 1999). Cellulose and hemicellulose bundles are coated by lignin forming a lignin-carbohydrate complex which acts as a physical component and barrier in the plant cell wall (Figure 2.2) (Mosier *et al*., 2005). Minor components such as proteins, oils and ash are also present within the lignocellulose structure (Wyman, 1994).

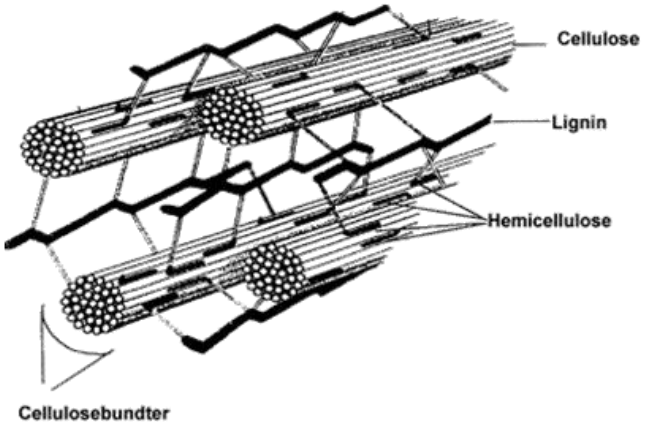

Figure 2.2 Diagramatic representation of the lignocellulose structure showing the three major components, namely cellulose, hemicellulose and lignin (Felby, 2009).

The composition of the major lignocellulosic components varies depending on the source of biomass (Table 2.2). On average cellulose and hemicellulose constitutes approximately 65-75% of the total lignocellulosic biomass, while lignin comprises about 15-25% (Wyman, 1996). Examples of lignocellulosic biomass include wheat straw, sugar cane bagasse, forestry waste, cornstalks and other organic material (Hahn-Hägerdal *et al*., 2006).

Table 2.2 Percentage of cellulose, hemicellulose and lignin composition for various lignocellulose materials (Jorgensen *et al*., 2007).

Lignocellulosic materials	% Composition		
	Cellulose	Hemicellulose	Lignin
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	25-35
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32	24	18
Leaves	15-20	80-85	0
Waste paper (from chemical pulps)	60-70	10-20	5-10
Fresh bagasse	33.4	30	18.9
Grasses	25-40	25-50	10-30

2.3 Production of bioethanol from lignocellulosic biomass

There are four major steps involved in the conversion of lignocelluloses into bioethanol, namely (i) pre-treatment, (ii) enzymatic saccharification, (iii) fermentation of released sugars by microorganisms and (iv) purification by distillation (Figure 2.3) (Gray *et al*., 2006).

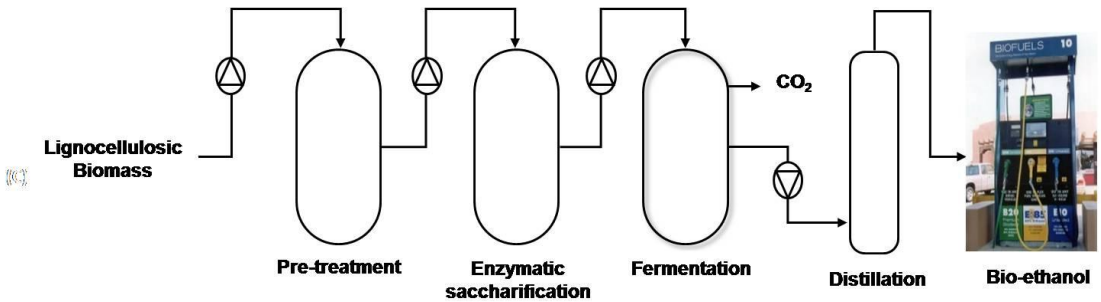

Figure 2.3 The process of producing bioethanol from lignocellulosic biomass.

2.3.1 Pre-treatment

Due to the complex and highly variable composition of lignocellulosic materials it is highly recalcitrant to enzymatic digestion and the subsequent release of free sugars (Ragauskas *et al*., 2006). Hydrolysis can be facilitated by pre-treatment into cellulose, hemicellulose and lignin fractions (Mosier *et al*., 2005). Pre-treatment strategies include physical (milling and grinding), physicochemical (steam autohydrolysis, hydrothermolysis and wet oxidation), chemical (alkali, dilute acid, oxidizing agents and organic solvents) and biological enzymatic processes (Kumar *et al*., 2009). The pre-treatment methods may be used in conjunction with each other and should ideally prevent complete degradation of fermentable carbohydrates (McMillan, 1994; Hsu, 1996). Pre-treatment steps result in improved accessibility of crystalline substrates for the subsequent enzymatic steps, as well as the release of cellulose, hemicellulose and lignin (Figure 2.4) for hydrolysis into pentose and hexose sugar monomers (Wyman *et al*., 2005b; Zhang *et al*., 2006b).

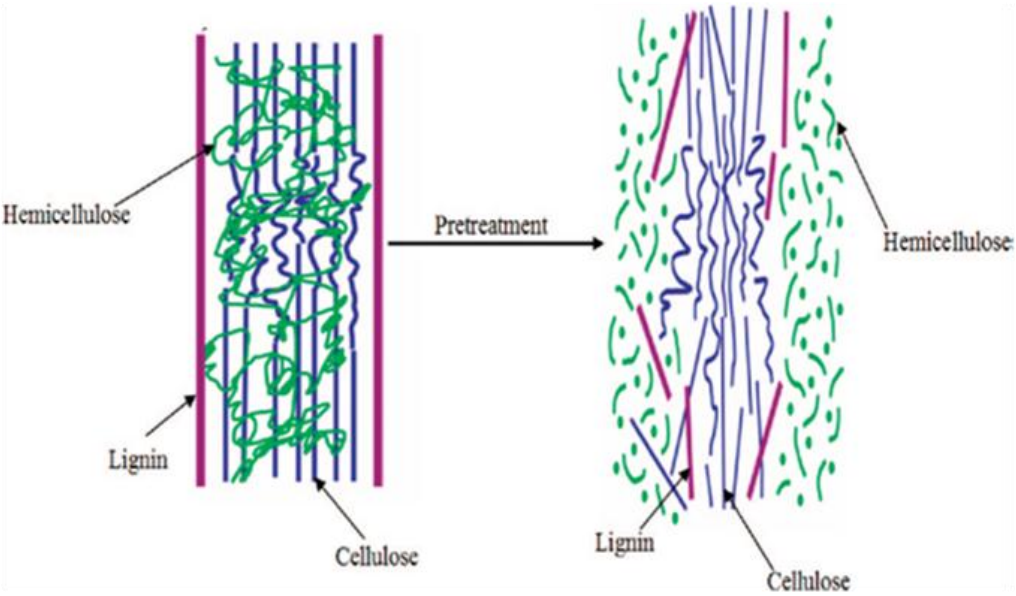

Figure 2.4 Diagramatic representation of the breakdown of lignocellulose into cellulose, hemicellulose and lignin by pre-treatment methods (Hsu *et al*., 1980).

2.3.2 Enzymatic hydrolysis of lignocellulosic biomass

Three main classes of lignocellulosic enzymes have been described on the basis of their respective substrates, namely ligninases, cellulases and hemicellulases (Howard *et al*., 2003).

2.3.2.1 Ligninases

Lignin is a large and complex polymeric structure comprised of 8,000-11,000 monomers. It is a polymer of phenylpropane, guaiacyl propanol and *p*-hydroxyphenyl linked by ether bonds (Kuhad *et al*., 1997). Lignin is a constituent of the primary cell wall of plants (Leonowicz *et al*., 1999) where it strengthens and acts as a water-proof sheath that protects the plant against pathogen invasion (Hinman *et al*., 1989; Hsu *et al*., 1996). Softwood plants have relatively high lignin contents, whereas herbaceous plants and grasses have relatively low lignin contents (Perez *et al*., 2002). Lignin lacks sites which can be readily cleaved by enzymes and within the lignin-carbohydrate complex it helps to prevent the enzymatic hydrolysis of cellulose and hemicellulose (Ragauskas *et al*., 2006; Zhang and Lynd, 2006). To enable hydrolytic enzymes to gain better access to the cellulose and hemicellulose portions, it is necessary to disrupt the lignin structure.

Oxidative extracellular enzymes have been implicated in the degradation of lignin, including lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13) and laccases (1.10.3.2.) (Borneman *et al.*, 1990). Lignin peroxidases degrade non-phenolic rings by removing an electron and producing cation radicals which are decomposed (Kirk *et al*., 1976). Manganese peroxidases degrade phenolic lignin by oxidising manganese II to manganese III which further oxidises phenolic rings to phenoxyl radicals and leads to the

degradation of lignin (Kirk and Farell, 1987). Laccases, a group of copper containing oxidases, oxidise phenolic rings to phenoxyl radicals (Bourbonnais and Paice, 1988; Thurston, 1994). A small number of microorganisms can break down lignin into organic acids, phenols and vanillin. An example is the white rot fungus, *Phanerochaete chyrsosporium*, which produces lignin peroxidases and manganese-dependent peroxidases during secondary metabolism in response to carbon and nitrogen limitation (Lee, 1997).

2.3.2.2 Cellulose saccharification by cellulases

Cellulose is a linear polymer of D-glucose monomer units linked together by 1,4-β-glucosidic bonds and can comprise chain-lengths of 4,000 to 8,000 monomers (Wilson, 2008). The molecule is composed of crystalline and amorphous regions, and forms a stable structure which is highly resistant to enzymatic hydrolysis (Marchessault and Sundarajan, 1993; Lynd, 1999). As many biomass sources contain a large percentage of cellulose (Table 2.2), cellulases are seen as the core enzymes required for efficient lignocellulose saccharification. Crystalline cellulose is hydrolysed through the action of endo- and exo-cellulases and β-glucosidases into oligosaccharides, cellobiose and glucose molecules, respectively (Philippids, 1996; Bath and Bath, 1997). Cellobiohydrolase acts on the sugar moieties at the free chain end and releases cellobiose units (Yi *et al*., 1999). These β-1-4 glucose dimers are subsequently hydrolysed to glucose by the action of β-glucosidase (Figure 2.5; Table 2.3). These glucose molecules can then be easily fermented to bioethanol (Bhat and Bhat, 1997).

Cellulases typically contain a catalytic core linked to a carbohydrate binding domain (CBD) which facilitates association with the substrate (Gilkes *et al.*, 1991). Adsorption of cellulases

onto the insoluble substrate is essential for the initiation of hydrolysis (Divne *et al*., 1998). Hydrolysis of cellulose to glucose requires the combined action of a range of cellulases (Goyal *et al*., 1991). Endo-β-1,4-glucanase starts the hydrolysis of cellulose by randomly cleaving internal β-1-4 glycosidic bonds within the amorphous regions of cellulose, opening free ends for cellobiohydrolase (exo-β-1,4-glucanase) attack (Wood, 1991).

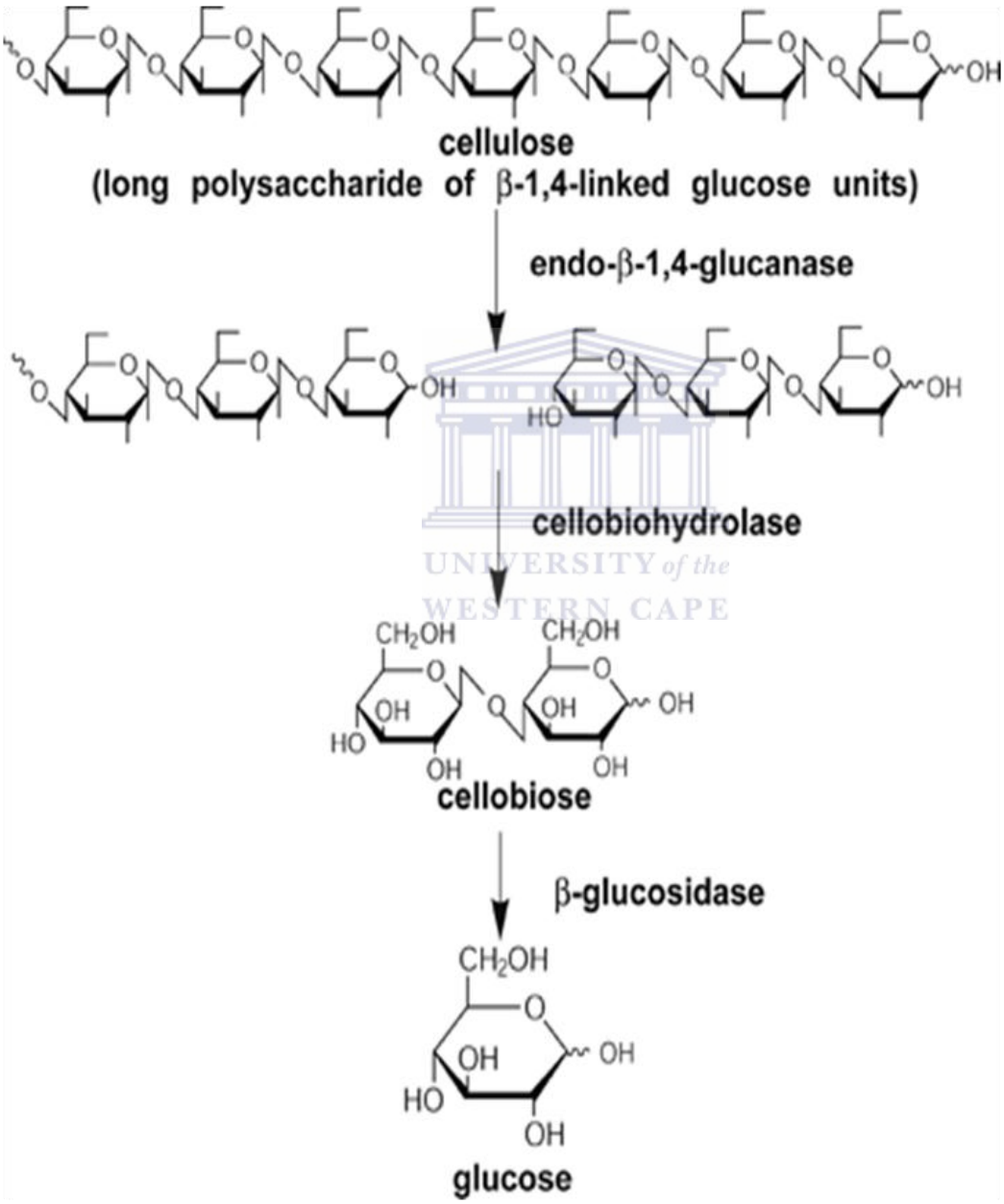

Figure 2.5 The hydrolytic action of endo-β-1,4-glucanase, cellobiohydrolase and β-glucosidase on the conversion of cellulose to glucose (Wilson, 2008).

Table 2.3 A comprehensive table showing enzymes involved in cellulose degradation (Zhou and Ingram, 2000).

Systematic name	Synonym	EC no	Substrate	Product	References
Endo-1,4-D-glucan 4-glucanohydralase	Endoglucanase	EC 3.2.1.4	Carboxymethyl Cellulose	Oligosaccharide	Wood, 1991
1,4-D-glucan Cellobiohydrolase	Exoglucanase cellobiohydralase	EC 3.2.1.91	P-nitrophenyl-β-D-cellobioside	Cellobiose	Yi *et al*., 1999
β-Glucohydralase	β-glucosidase	EC 3.2.1.21	Cellobiose	Glucose	Bhat and Bhat, 1997

The search for cellulolytic enzymes of bacterial and fungal origin for efficient bioconversion of lignocellulosic biomass into feedstocks for bioethanol production is ongoing. Only organisms with the ability to produce appropriate levels of endoglucanases, exoglucanases and β-glucosidases would be capable of degrading native cellulose effectively (Wojtczak *et al*., 1987). Several strains of the fungus *Trichoderma reesei* produce extracellular cellulases capable of efficiently degrading native cellulose (Nakari-Setäla and Pentilä, 1995). Many bacterial strains of the species *Rhodospirillum rubrum*, *Clostridium stercorarium*, *Bacillus polymyxa*, *Pyrococcus furiosus*, *Acidothermus cellulytics* and *Saccharophagus degradans* produce cellulases (Weber, 2001; Kato *et al*., 2005; Taylor *et al*., 2006; Das *et al*., 2007). A few microbial species (e.g. members of the genera *Neorosposa*, *Manilia*, *Paecilomyces* and *Fusarium*) are able to ferment cellulose to ethanol by simultaneous saccharification and fermentation (Lynd *et al*., 2005).

2.3.2.3 Hemicellulases

Hemicellulose is the second largest fraction of lignocellulose and is a polymer comprising xylan, mannan, galactan and arabinan in varying proportions (Bastawde, 1992). Hemicellulose comprises of approximately 30-35% xylan, with the polysacharide backbone made up of xylose monomers linked by β-1-4 glycosidic bonds (Whistler and Richards, 1970). Due to their unstructured nature, hemicelluloses are relatively easy to hydrolyse, especially when compared to cellulose (Hamelinck *et al*., 2005).

Hemicellulases are multi-domain proteins with catalytic and non-catalytic modules (Henrissat and Davies, 2000). Most non-catalytic modules consist of $CBD_{[S]}$ which assist in the access of the enzyme to the hemicellulose polysaccharide. Hemicellulases that hydrolyse ester linkages are grouped into the carbohydrate esterase (CE) family (Table 2.4) (Henrissat and Bairoch, 1996). Enzymes that hydrolyse glycosidic linkages to release monosugars, e.g. xylanases, belong to the glycoside hydrolase (GH) family (Prates *et al*., 2001; Shallom and Shoham, 2003).

Numerous studies have focused on the isolation, identification and characterisation of xylanases with potential application in biofuel production. Other industrially important applications include use in laundry detergents for improved fabric softness and brightness (Cavaco-Paulo, 1998), processing of fruit juices where enzymes act in juice clarification (Gong *et al*., 1999) and in the treatment of agricultural and paper wastes (Maat *et al*., 1992).

2.3.2.3.1 Glycoside hydrolase family

A new classification system for not only xylanases, but glycosidases in general was introduced by Henrissat (1991). This system is based on primary structure comparisons of the catalytic domains only, and groups enzymes in families of related sequences. Currently, 118 GH families have been described within the Carbohydrate Active Enzymes Database (Cantarel *et al*., 2009). Enzymes are further grouped into clans which are higher hierarchical levels (Bourne and Henrissat, 2001). There are 14 clans (GH-A to GH-N), and each clan contains two to three families except for clan GH-A which contains 17 families. Xylanases are grouped into GH families 5, 8, 10, 11 and 43 (Table 2.4). These enzymes contain one distinct catalytic domain with demonstrated endo-1,4-β-xylanase activity. Most reported xylanases belong to GH families 10 and 11 (Rabinovich *et al*., 2002a).

Table 2.4 Classification of hemicellulases into glycoside hydrolase and carbohydrate esterase families (Adapted from Saha, 2000; Lee *et al*., 2003).

Enzymes	EC number	Family*	
Endo-β-1,4 xylanase	3.2.1.8	GH	5, 8, 10, 11, 43
Exo-β-1,4-xylosidase	3.21.37	GH	3, 39, 43, 52, 54
Exo-β-1,4 mannosidase	3.2.1.25	GH	1, 2, 5
Endo-β-1,4-mannanase	3.2.1.78	GH	5, 26
α-Glucuronidase	3.2.1.139	GH	67
α-Galactosidase	3.2.1.22	GH	4, 27, 36, 57
Endo-galactanase	3.2.1.89	GH	53
α-L-arabinofuranosidase	3.2.1.55	GH	3, 43, 51, 54, 62
Acetyl xylan esterases	3.1.1.72	CE	1, 2, 3, 4, 5, 6
Acetyl mannan esterase	3.1.1.6	CE	1
Ferulic and p-cumaric acid esterases	3.2.1.73	CE	1

* GH, glycoside hydrolase and CE, carbohydrate esterase

2.3.2.3.1.1 Glycoside hydrolase family 5

Glycoside hydrolase family 5 (GH 5) is the largest family consisting of approximately 500 enzymes (Larson *et al*., 2003). This family comprises of several enzyme activities, including cellulase (EC 3.2.1.4), licheninase (EC 3.2.1.73), β-mannosidase (EC 3.2.1.25), cellulose 1,4-β-cellobiosidase (EC 3.2.1.91) and endo-1,4-β-xylanase (EC 3.2.1.8) (Coutinho and Henrissat, 1999). Few members with demonstrated activity on xylan have been reported, as reviewed by Collins *et al*. (2005). However, a number of possible xylanases have been identified by comparing sequence similarities in genome database including *Bacillus subtilis* strain 168 (Kunst *et al*., 1997), *Clostridium acetobutylicum* ATCC 824 (Nolling *et al*., 2001) and *Bacteroides thetaiotaomicron* VP-5482 (Xu *et al*., 2003). Only seven amino acid residues which include the nucleophile and the acid/base residues are strictly conserved amongst all members of GH5. These enzymes are different from one another with structural alignments which indicate root mean square (rms) deviations of 1.25 ± 0.12 Å between equivalent residues in its members. The typical structure of GH 5 enzymes includes a catalytic domain which displays a $(\beta/\alpha)_6$ barrel fold and belongs to clan GH-A (Larson *et al*., 2003).

2.3.2.3.1.2 Glycoside hydrolase family 8

Glycoside hydrolase family 8 (GH 8) is mainly composed of cellulases (EC 3.2.1.4), although enzymes such as chitosanases (EC 3.2.1.132), lichenases (EC 3.2.1.73) and endo-1,4-β-xylanases (EC 3.2.1.8) are also present (Coutinho and Henrissat, 1999). Three of the xylanases in this group have been isolated from *Bacillus* species and were found to be active at pH 6.5 (Takami *et al*., 2000). These enzymes showed higher activity on birchwood xylan in comparison to oat spelt xylan substrate, and were inactive on cellulose, carboxymethylcellulose (CMC), starch, lichenan and chitosan (Collins *et al*., 2002). Enzymes

belonging to GH 8 are characterised as having a high molecular mass, high pI and $(\alpha/\alpha)_6$ barrel fold. These enzymes function with inversion of the anomeric configuration with aspartic acid as proton acceptor and glutamic acid as proton donor and they are grouped into clan GH-M (Collins *et al*., 2003).

2.3.2.3.1.3 Glycoside hydrolase family 10

Glycoside hydrolase family 10 (GH 10) is comprised of mainly endo-1,4-β-xylanases (EC 3.2.1.8). In addition, endo-1,3-β-xylanases (EC 3.2.1.32) and cellobiohydrolases (EC 3.2.1.91) are also present (Coutinho and Henrissat, 1999). These enzymes are active on xylan, although a number of studies have shown them to also be active on cellulose substrates, such as aryl cellobiosides and cello-oligosaccharides (Claeyssens and Henrissat, 1992; Biely, 1993). Members of this family hydrolyse aryl β-glucosides of xylobiose and xylotriose at the agly-conic bond. They typically have a high molecular weight, low pI are grouped into clan GH-A and have an $(\alpha/\beta)_8$ fold structure, which is similar to that of GH 5 enzymes (Biely *et al*., 1997).

2.3.2.3.1.4 Glycoside hydrolase family 11

Glycoside hydrolase family 11 (GH 11) consists of true xylanases only which are active on substrates containing D-xylose (Biely *et al*., 1997). Similar to GH 8, members of GH 11 prefer long chain xylo-oligosaccharide. As with GH 10 enzymes, members hydrolyse aryl β-glycosides of xylobiose and xylotriose at the agly-conin bond, but they are inactive on aryl cellobiosides. The products of the action of GH 11 enzymes are further hydrolysed by GH 10 enzymes (Biely, 1993). Members of GH 11 have low molecular weight, high pI and a double

catalytic mechanism which contains two glutamates that act as catalytic residues (Fushinobu *et al*., 1998). Their structure is β-jelly roll fold which consists of β-sheets that surround the catalytic site. Two or three β-sheets are present and are packed to form a hydrophobic protein. One α-helix is present which is packed against the second β-sheet. Members of this family are grouped into clan GH-C (Harris *et al.,* 1997).

2.3.2.3.1.5 Glycoside hydrolase families 7 and 43

Only one enzyme with xylanase activity has been found in each of the GH 7 and 43 families. The importance of these enzymes in the xylanase family is still not clear since they are not true xylanases. The GH 7 enzyme, EGI (Cel7B) from *Trichoderma reesei,* is a non-specific endo-β-1,4-glucanase (EC 3.21.4). The enzyme displays more activity on cellulose than on beechwood xylan, and activity on cello-oligosaccharides was very high as compared to its activity on xylo-oligosaccharides (Biely, 1991). It has characteristics which are common to those of GH 10 and 11 xylanases. They have high molecular weight, low pI and small substrate binding sites and contain four subsites with a catalytic site in the middle. Their structure is a β-jelly roll fold. However differences exist in the length and orientation of the structural elements, these results in GH 7 enzymes being grouped in clan GH-B. The GH 43 XYND enzyme from *Paenibacillus polymyxa* displays both xylanase and α-L-arabinofuranosidase activities (Gosalves *et al*., 1991). Their members catalyse hydrolysis using a single displacement mechanism. The enzyme has a low molecular weight of 64 kDa, a 5-blade propeller fold and is grouped into clan GH-F (Kleywegt *et al*., 1997; Morris *et al*., 1999).

2.3.2.3.2. Hemicellulose saccharification by hemicellulases

The breakdown of xylan into xylose requires the action of several enzymes including β-1-4-endoxylanases, β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases and ferulic esterases (Figure 2.6) (Biely, 1985; Belancic *et al*., 1995). β-1-4-endoxylanases cleaves the glycosidic bonds of the xylan backbone randomly at the internal bonds into xylo-oligosaccharide and xylobiose which are cleaved by β-D-xylosidases into β-xylapyranosyl residues, reducing the depolymerisation of xylan and releasing xylose. β-xylosidases are classified as xylobiases and exo-1,4-β-xylanases and are able to cleave substrates such as p-nitrophenyl and o-nitrophenyl-β-D-xylopyranoside (Biely, 1993). β-xylosidases also play a major role in the degradation of xylan by removing the end products which inhibits endo-xylanase activity (Sunna and Antranikian, 1997). The side chain groups of the xylan backbone are cleaved by α-arabinofuranosidases, α-glucuronidases, p-coumaric acid and ferulic acid esterases (Biely, 1985; Puls *et al*., 1987; Subramaniyan and Prema, 2002). α-L-arabinofuranosidase is an auxiliary enzyme that cleaves α-L-arabinofuranosyl linkages from arabinose-rich polysaccharides such as arabinan, arabinoxylans, arabinogalactan and pectin (Margolles-Clark *et al.,* 1996). It displays synergism with other hemicellulases, accelerating the rate at which their glycosidic bonds are hydrolysed (Shallom *et al*., 2002).

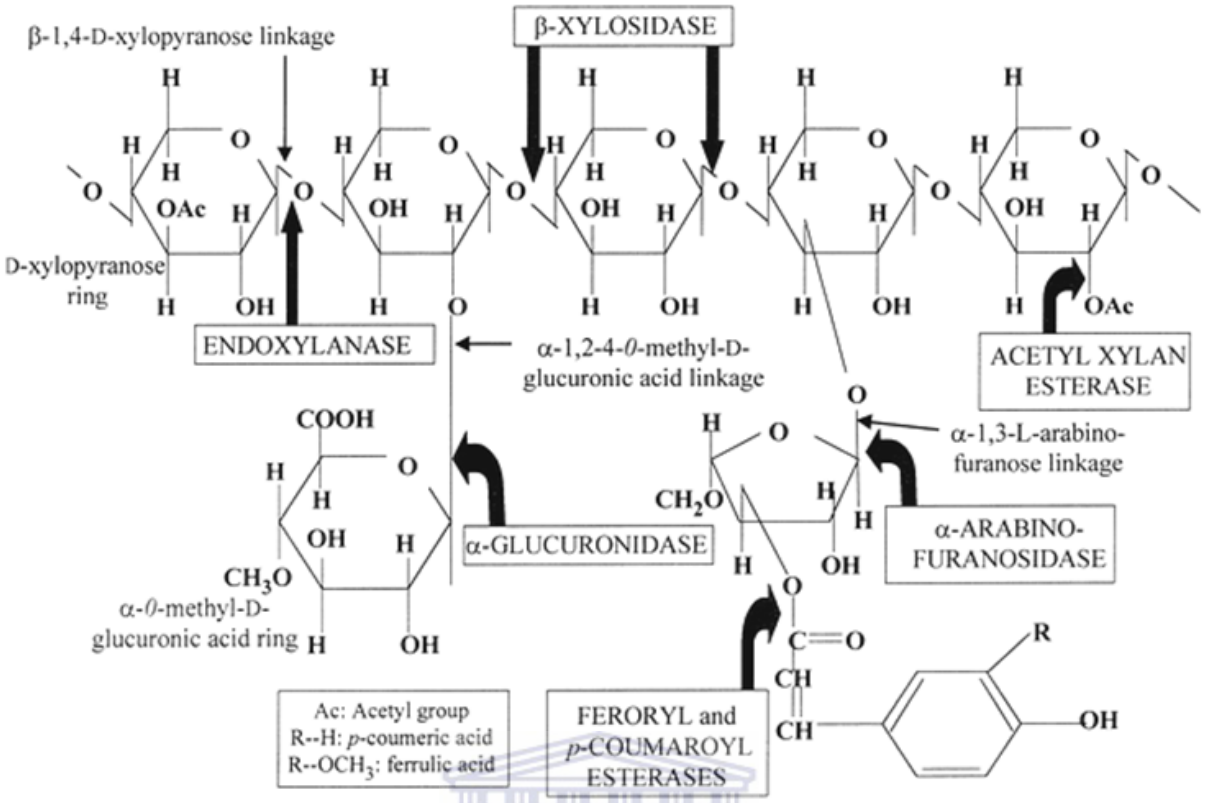

Figure 2.6 The structure of xylan showing the sites cleaved by the various core and accessory enzymes involved in the degradation of xylan (Beg *et al*., 2001).

2.3.2.4 Microorganisms producing hemicellulases

Bacteria, fungi, yeasts and plants have been shown to produce xylanases (Haki and Rakshit, 2003; Mitreva-Dautova *et al*., 2006; Kuhad *et al*., 1997). The nature of xylanases varies amongst the different microorganisms (Polizeli *et al*., 2005). A number of xylan degrading bacterial strains, including members of *Aeromonas* (Kudo *et al*., 1985), *Bacillus* (Dhillon and Khanna, 2000), *Cellulomonas* (Bhalerao *et al*., 1990), *Ruminococcus* (Romanec *et al*., 1989) and *Streptomyces* (Georis *et al*., 2000) have been reported. In addition, numerous thermophilic bacterial isolates have been reported to produce xylanases, including *Clostridium stercorarium* (Donaghy *et al*., 2000), *Bacillus subtilis* (Sakamoto *et al*., 1997)

and *Pyrococcus furiosus* (Gibbs *et al*., 1995) (Table 2.5). *Bacillus halodurans* have been reported to produce alkali tolerant xylanases (Yi *et al.,* 2006).

Table 2.5 Microorganisms producing hemicellulases enzymes and their optimum temperatures (Adapted from Howard *et al*., 2003).

Enzyme	**Organism**	**Substrate**	**Optimum Temperature (°C)**	**References**
Feruloyl esterase	*Clostridium stercorarium*	Ethyl ferulate	65	Donaghy *et al.*, 2000
Endo-1,4-β-xylanase	*Bacillus pumilus*	β-1-4-D-xylan	40	Monisha *et al*., 2009
Exo-β-1,4-mannosidase	*Pyrococcus furiosus*	p-nitrophenyl-β-D-galactoside	105	Gibbs *et al*., 1995
Endo-α-1,5-arabinanase	*Bacillus subtilis*	1,5-α-L-arabinan	60	Sakamoto *et al*., 1997
α-L-arabinofuranosidase	*Clostridium stercoarium*	alkyl-α-rabinofuranoside	70	Emi and Yamamoto, 1972
α-Galactosidase	*Escherichia coli*	raffinose	60	Won and Hyun, 1983

2.4 Thermostable enzymes from microorganisms

Extremophiles are microorganisms that survive at extreme conditions such as high or low temperatures, high or low pH and high salt concentration (Herbert, 1992). Thermophiles have an optimum growth temperature of 45-80°C (Stetter, 1996). Thermophilic bacteria with the ability to degrade lignocellulosic biomass have been isolated from hot springs (typically 70 to 100°C). For example, three cellulase producing bacterial isolates (EHP1, EHP2 and EHP3), displaying optimum growth at 75°C, were isolated from an Egyptian hot spring (Ibrahim and Ahmed, 2007). These isolates were shown to be closely related to *Anoxybacillus flavithermus, Geobacillus thermodenitrificans* and *Geobacillus sterothermophilus,* respectively. Furthermore, cellulase activity was demonstrated on CMC, avicel and cellobiose for all the isolates. Another good source of thermophilic bacteria is compost at temperatures above 50°C. The compost process undergoes several stages which include rapid increase in temperature, a time of sustained high temperatures and a period of cooling (Dees and Ghiorse, 2001). Microorganisms are involved in the composting process leading to the breakdown of organic matter into carbon dioxide and biomass. Numerous isolates producing thermophilic lignocellulosic enzymes have been identified from thermophilic compost (Tuomela *et al*., 2001). For example, a bacterial isolate displaying cellulase activity was isolated from compost soil at Rhodes University (Grahamstown, South Africa). The cellulase displayed optimum activity at a temperature of approximately 70°C (Mayende *et al*., 2006). The closest relative of the isolate was identified as *Bacillus subtilis*. In addition to cellulases, several studies have reported the discovery of novel thermostable xylanases (Pantazaki *et al*., 2002; Van den Burg, 2003). Xylanases have been isolated from bacteria such as *Thermonospora fusca* (McCarthy, 1987) thermophilic *Bacillus* spp. (Gruninger and Fiechter, 1986) and *Bacillus stearothermophilus* (Khasin *et al*., 1993). These xylanases displayed optimum activity in a temperature range of 65-80°C. A thermostable xylanase isolated from a

thermotolerant *Aspergillus* strain showed the highest activity at 80°C (Mendicuti *et al*., 1997). A xylanase from a thermophilic *Clostridium stercorarium* isolate displayed optimum activity at 70°C and a half-life of 90 min at 80°C, whereas a xylanase from *Thermotoga* sp. functioned optimally at 105°C and had a half-life of 90 min at 95°C (Simpson *et al*., 1991).

2.4.1 Features of thermostable enzymes

Thermostable enzymes, or thermozymes (Briuns *et al*., 2001), are mainly isolated from thermophilic microorganisms (Herbert, 1992), and are capable of resisting denaturation and proteolysis under conditions of high temperature (Kumar and Nussinov, 2001). Thermostable enzymes use electrostatic, disulphide bridges and hydrophobic interactions to enable tolerance to high reaction temperatures (Haki and Rakshit, 2003). Thermozymes may even be active at temperatures higher than the optimum growth temperature of their host microorganisms (Saboto *et al*., 1999).

2.4.2 Application of thermostable enzymes in bioethanol production

The fermentation of lignocellulose hydrolysates is dependent on temperature. *Saccharomyces cerevisiae* is often used in the fermentation of lignocellulose hydrolysate, however this organism is unable to ferment at temperature above 30°C (Becker and Boles, 2003). The temperature of the fermentation process should be maintained to allow microbial growth. Ethanol production processes at high temperatures requires thermophilic enzymes for efficient ethanol conversion. Such processes exploit the high efficiency and specificity of enzyme catalysis to synthesis ethanol from lignocellulose (Thomas *et al*., 1981). Bioethanol production at high temperature minimises the energy output required for process cooling,

while high vapour pressure of ethanol facilitates removal by gas stripping (Taylor, 2007). Microbial contamination by mesophiles is furthermore of little concern at temperatures above 60°C (Gong *et al*., 1999). Thermostable enzymes facilitate enhanced enzymatic hydrolysis of lignocellulosic biomass at high temperature by increasing the solubility of the polymeric substrates (Van den Burg, 2003). In addition, an increase in process temperature results in increased reaction rates and a decrease in the amount of enzyme that has to be added (Hanazawa *et al*., 1996).

Chapter 3: Materials and methods

3.1 General chemicals and methodology

Unless otherwise specified, chemicals were obtained from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, Missouri, USA) and Kimix (Cape Town, South Africa). Culture media was obtained from Oxoid (Cambridge, England) and Biolabs (Madison, USA). Media were prepared and autoclaved at 121°C for 20 min. Aseptic microbiological techniques were employed throughout this study. All DNA modifying enzymes (polymerase and restriction endonucleases) were purchased from Fermentas (Burlington, Canada). Oligonucleotides for Polymerase Chain Reaction (PCR) used in this study were synthesized by Inqaba Biotech (Johannesburg, South Africa). Glycerol stocks of isolates and clones of interest were prepared in 50% glycerol and stored at -80°C.

3.2 Sampling procedure

Thermophilic straw-based compost was obtained from the Medallion mushroom farm in Stellenbosch, South Africa (Medallion Mushrooms (Pty) Ltd.). The compost was collected on the 30th of November in 2009 by Dr R Bauer. Sample properties were as follows; the temperature was 71 -81°C and pH 7.2. All samples were stored at 4°C in the laboratory until analysed.

3.3 Bacterial isolation from straw-based compost

Bacteria were isolated from 1 g compost after the addition of 10 ml sterile distilled water. The compost sample was serially diluted 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 fold in distilled water. An aliquot of each dilution (0.1 ml) was plated on medium (pH7) containing compost extract (5 g L^{-1} yeast extract, 8 g L^{-1} glucose, 4.5 g L^{-1} K_2HPO_4, 15 g L^{-1} agar, 40% (v/v) compost extract). Compost extract was prepared by preparing a 0.33% (w/v) solution of compost in distilled water. The mixture was boiled for 30 min and the supernatant was used as compost extract. Plates were incubated at 45°C and 55°C for 24 hrs. Bacterial colonies visible after incubation from 10^2 fold dilution were cultured on compost extract medium until pure cultures were obtained for further characterisation.

3.4 Screening for lignocellulosic and lipase activity

Isolates were cultured on compost extract agar plates containing 1% carboxymethyl cellulose (CMC) and incubated at 45°C for 24 hrs. Isolates were screened for cellulase activity by staining plates with 0.1% (w/v) Congo red for 30 min (Voget *et al.,* 2006). After discarding the Congo red, the plates were destained with 1 M NaCl for 30 min (Kasana *et al*., 2008) and visually inspected for zones of clearing around colonies. All further screening was performed on compost extract agar plates supplemented with specific substrates mentioned below. Isolates were screened for xylanase activity by culturing single colonies on 0.7% (w/v) Remazol Brilliant Blue (RBB) xylan and incubated for 24 hrs at 45°C. For the functional screening of lipase/esterase activity, isolates were cultured on plates containing 1% (w/v) gum arabic and 0.1% (v/v) glycerol tributyrate. Screening plates were incubated at 45°C for 24 hrs and visually inspected for zones of clearing around colonies.

3.5 Identification of isolate RZ1 by 16S rRNA gene sequencing

3.5.1 gDNA isolation

Bacterial isolates were cultured overnight on TSB agar plates. A single colony was inoculated into 5 ml TSB (30 g L^{-1} TSB) and incubated overnight at 45°C with aeration. Genomic DNA was isolated from the cells using the method described by Wang *et al*. (1996) and stored at -20°C. DNA concentrations were determined spectrophotometrically on a NanoDrop^{R} ND-1000 spectrophotometer (Roche, Berlin, Germany). DNA integrity was determined by agarose gel electrophoresis on 0.8% (w/v) agarose gels prepared in 1 × TAE buffer (0.4 M Tris base, 5 M EDTA, pH 8) and supplemented with 0.5 µg ml^{-1} ethidium bromide. Samples were mixed with 10× standard loading dye [60% (v/v) glycerol, 0.25% (w/v) Orange G] and loaded into the wells of cast gels. Gels were electrophoresed at 10 V/cm for 2 hrs and subsequently visualised under ultraviolet light (UV) and photographed with a digital imaging system (AlphaImager 2000, Alpha Innotech, San Leandro, California) DNA was sized by comparing migration in the gel to that of known DNA molecular markers [Lambda DNA restricted with H*ind*III (λ H*ind*III) or Lambda DNA restricted with P*st*I (λ P*st*I)].

3.5.2 PCR amplification and cloning of the 16S rRNA gene

Amplification of the 16S rRNA gene was performed with universal primers: E9F (5' GAGTTTGATCCTGGCTCAG 3') and U1510R (5' GGTTACCTTGTTACGACTT 3'). PCR reactions (20 µl) contained 50 ng template gDNA, 1 × PCR buffer (200 mM Tris-HCl pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% (v/v) Triton X- 100), 0.2 µM of each dNTP, 0.5 µM of each primer and 1 U of lab DNA polymerase. PCR parameters were as follows: initial denaturation for 1 min at 94^{o}C, 35 cycles of 30 s at 94^{o}C, annealing for 30 s

at 52^{o}C, and an extension step for 1 min at 72^{o}C followed by a final extension step for 7 min at 72^{o}C. PCR reactions were performed in an automated thermal cycler (Thermo Hybaid system, Ashford, USA). A negative control, containing all reagents except template, was routinely included. An aliquot of each mixture was analysed on 0.8% agarose gel as described above (section 3.5.1). Bands of interest were excised from the agarose gels under long wavelength UV light (365 nm) and extracted from the agarose using the GFX^{TM} PCR and Gel Band Purification Kit (GE Healthcare limited, Buckinghamshire, USA) as per manufacturer's specifications. Extracted DNA was subsequently cloned into the pGEMT easy vector (Promega, Madison, USA) as described in the manufacturer's manual.

3.5.3 Transformation of competent *E. coli* (GeneHogs), plasmid extraction and sequencing

All *E. coli* cultures were incubated at 37°C and if cultured on broth incubation was accompanied by agitation at approximately 200 rpm. *E. coli* GeneHogs (Invitrogen, USA) cells were used to prepare electro-competent cells for transformations. These cells were cultured on LB agar (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, 15 g L^{-1} agar) at pH 7.0. A single colony of *E. coli* was used to inoculate 5 ml of SOB (20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl, 250 mM KCl, 2M Mg Cl_2) and cultured overnight. A 5 ml overnight culture was used to inoculate one litre of SOB media and cultured at 37°C until an OD of 0.6-0.9 at 600 nm was reached. Cells were kept on ice and 250 ml aliquots were transferred to chilled corning centrifuge bottles and centrifuged at 4, 000 × *g* for 10 min at 4°C. The supernatant was poured off and the cell pellet gently re-suspended in 200 ml ice-cold 10% (v/v) glycerol preceding centrifugation at 4,000 × *g* for 15 min at 4°C. The cells were re-suspended in 2 ml ice-cold sterile 15% (v/v) glycerol containing 2% (w/v) sorbitol

per litre initial culture and centrifuged at 4, 000 × *g* for 10 min. After the supernatant was removed, each cell pellet was gently re-suspended in 1 ml solution containing 15% (v/v) glycerol and 2% (w/v) sorbitol. The cell suspension was kept on ice and 50 µl aliquoted into chilled 1.5 ml eppendorf tubes and stored at -80°C until required for transformation. Aliquots of electro-competent cells were thawed on ice preceding electroporation. One microlitre of the ligation mixture (16S rRNA gene in pGEMT easy) was added directly to cells and electroporation was performed using the following conditions: An electric pulse was applied at a voltage of 1.8 kV with a capacitance of 25 µF and a resistance of 200 Ω. One ml of SOC (1 L SOB, 2 M $MgCl_2$) and 20 ml of a 1 M glucose solution were immediately added to the cuvette, mixed once and transferred to sterile tubes. Transformed cells were incubated for 1 hr at 37°C with agitation. Cells were plated onto LB agar plates supplemented with ampicillin (100 µg ml^{-1}), IPTG (20 µg ml^{-1}) and X-Gal (30 µg ml^{-1}). Recombinant transformants were selected by blue/white selection based on insertional inactivation of the lacZ gene. Six white colonies were picked from overnight culture plates using sterile toothpicks and inoculated into 5 ml LB broth supplemented with ampicillin (100 µg ml^{-1}). Cultures were incubated at 37°C overnight and plasmid extraction from cells was performed using a plasmid mini prep kit (Qiagen, Maryland, USA) according to the manufacturer's specification. DNA concentrations were determined by the NanoDrop®. Recombinant plasmids were screened for the presence of insert DNA using the M13 PCR primers. PCR reaction conditions and parameters were performed as described above with the following changes.

The vector specific M13 primers, M13 F 5' CCCAGTCACGACGTTGTAAAACG 3' and

M13 R 5' AGCGGATAACAATTTCACACAGG 3', were used to confirm the presence of the insert. The annealing temperature was raised to 64°C, the extension step during cycling extended to 90 s and the final extension step lengthened to 10 min. Products were analysed using gel electrophoresis (1% (w/v) agarose gel) as described previously in section 3.5.1.

Plasmids containing inserts were sequenced using M13 vector primers. Sequencing was carried out by the University of Cape Town. Sequences were analysed using the software packages Bioedit Version 7.0 (Hall, 1999) and DNAMAN Version 4.13. The Genbank database was used for homology searches utilising the Basic Local Alignment Search Tools (BLASTn) (Altschul *et al*., 1997) to determine sequence similarity and identity. For the construction of phylogenetic trees, Bio-Edit was used for editing sequences. Edited sequences were aligned using Bio-Edit with MEGA 4 (Tamura *et al*., 2007). The tree was constructed based on the maximum composite likelihood method and substitution model using Neighbour-Joining. The test of phylogeny used was based on 1000 bootstraps of replication and a pair wise deletion of gaps. Substitutions included transitions and transversions, with the pattern among lineages being assumed to be homogeneous (Saitou and Nei, 1987; Tamura *et al*., 2007).

3.6 Characterisation of the isolate RZ1

Colony morphology was examined by streaking single colonies on TSB agar and noting colour and shape after 24 hrs incubation at 45°C. Gram stain was performed by the method described by Dussault (1955). Motility of the isolate was tested after resuspending cells in water. A few drops were placed on a glass slide and covered with a cover slip. The slide was heated gently on a low flame for a few seconds and then examined immediately under the microscope for signs of cell motility. Spore formation was tested with the Schaeffer-Fulton spore stain (Cowan, 1974). The starch degradation ability of RZ1 was tested after culturing bacterial colonies on starch agar (0.5 g L^{-1} KNO_3, 1 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.1 g L^{-1} $CaCl_2$, traces $FeCl_3$, 10 g L^{-1} potato starch, 15 g L^{-1} agar, pH 7.2). After 48 hrs of incubation, plates were flooded with 0.7% (w/v) iodine solution and colonies observed for zones of hydrolysis. Caseinase activity was tested by streaking single colonies on skim milk

agar [1% (w/v) agar, 5% (w/v) skim milk powder, pH 7.2]. After an incubation period of 48 hrs, colonies were observed for zones of hydrolysis. The isolate was tested for catalase activity by adding a drop of 30% (w/v) hydrogen peroxide solution to a single colony placed on a glass slide. The ability to grow anaerobically was evaluated by streaking the isolate on TSB agar plates and observing growth after incubation in anaerobic jars for 48 hrs (Gaspak Anaerobic System, BBL Microbiology Systems, Cockeysville, Md).

The API CHB 50 and API 20 E kits (bioMerieux, Quebec, Canada) were used for the characterisation of the isolate as described in the manufacturer's manual. A single colony of RZ1 was resuspended in 5 ml of 0.85% NaCl. The API 20 strips were inoculated with the bacterial suspension then incubated at 37°C for 24 hrs then observed for color change. Several colonies of RZ1 were resuspended in 10 ml of the API 50 CHB medium. The bacterial suspension was inoculated into the API 50 CH strips and incubated at 37°C then observed for color change after 24 hrs and 48 hrs. The optimum growth temperature of the isolate was determined by measuring relative growth rate ($\frac{\Delta\ Abs\ 600\ nm}{\Delta\ time\ (hrs)}$) during the logarithmic growth phase when grown on TSB at 10°C, 25°C, 37°C, 42°C, 50°C and 60°C. A thermometer was placed in the incubator to monitor the temperature during incubations. Growth was observed by measuring the absorbance at 600 nm of three 100 ml cultures for 8 hrs at 30 min intervals. Readings were taken in triplicate.

3.7. Small insert library construction and screening

The pUWL219 (Wehmeier, 1995) vector was restricted to completion with H*ind*III and heat inactivated at 65°C for 10-20 min. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the tube was centrifuged at 13, 000 × g for 2 min. The aqueous phase was transferred to a sterile eppendorf tube, and an equal volume of chloroform was added. After centrifugation at 13, 000 × g for 2 min, the DNA was precipitated with 1/10 volume of 3 M sodium acetate (pH 7.2) and 2.5 volume of 100% ethanol and incubated at -80°C for 1 hr. The DNA pellet was obtained by centrifugation at 16, 000 × g for 30 min at 10°C and subsequently washed with 70% ice-cold ethanol. The pellet was air dried in a laminar flow hood and resuspended in 10 µl of 1 × TE (pH 8) and quantified using a Qubit fluorometer (Invitrogen, Oregon, USA). Preceding ligation the vector was dephosphorylated using Fast AP™ Thermosensitive Alkaline phosphatase (Fermentas, Burlington, Canada). Each reaction mixture was prepared by mixing plasmid DNA 100 ng, 2U Fast AP™ buffer and 1U of Fast AP™ to make up a 20 µl final volume. The reaction was incubated at 37°C for 10 min, followed by an incubation step at 75°C for 5 min in order to inactivate Fast AP ™. Insert DNA was prepared by partially restricting gDNA, isolated as described previously (section 3.5.1), with H*ind*III to generate fragments between 5-10 kb and separated by electrophoresis in low melting point 1% (w/v) agarose. The agarose portions containing DNA fragments of the desired size were melted at 65°C for 10 min, and transferred to a 42°C water bath for 5 min. One unit of agarase for every 100 mg of LMP agarose was added to each tube and the tubes were incubated at 42°C for 1 hr. The enzyme was inactivated by incubation for 10 min at 70°C. Five hundred microlitres aliquots were transferred to 1.5 ml sterile eppendorf tubes chilled on ice for 5 min and centrifuged at 10, 000 × g for 10 min. The supernatant was transferred to clean tubes and the DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 7) and 2.5 volumes of ice cold absolute ethanol and then incubated at -80°C for 1

hr. The DNA was pelleted by centrifugation at 16, 000 × g for 30 min at 10°C and washed with 70% ice-cold ethanol. The pellet was air dried in a laminar flow hood and re-suspended in 10 µl TE (pH 8) and quantified by using a Qubit fluorometer according to the manufacturer's specification.

Ligations were carried out in 10 µl volumes. Genomic DNA in the 5-10 kb range and vector pUWL219 were mixed in a 3:1 molar ratio. Ligation was performed using T4 DNA ligase (Fermentas) as per manufacturer's protocol. Reactions were incubated at room temperature overnight and used to transform competent *E. coli* cells as described in section 3.5.3. Lignocellulosic and lipase activity were screened as in section 3.4 with the following changes. Library clones were spot inoculated on LB agar supplemented with ampicillin and incubation was extended to 5 days at 37°C. The plates were incubated at 37°C and inspected daily for lignocellulosic and lipase activity. Clones displaying positive activity were subjected to secondary screening to confirm activity.

3.7.1 Identification of a xylanase gene using transposon mutagenesis

A xylan hydrolysing clone was mutated at random locations by transposon mutagenesis using the Hypermutation chloramphenicol Insertion kit (Epicentre®, Madison, USA) as per manufacturer's protocol. One microlitre of the mutagenesis reaction was transformed as described previously into electro-competent *E. coli* cells and 100 µl was plated on LB agar plates supplemented with 100 µg/ml ampicillin and 47µg/ml chloromphenicol. Positive transformants were spot inoculated on RBB xylan LB agar plates and incubated overnight in order to select knock-out mutants. Five transformants with reduced or no xylanase activity were inoculated on 5 ml LB supplemented with the appropriate antibiotics and incubated at 37°C overnight. Plasmid extraction (Macrogen Inc.) was performed as previously described

using the transposon-specific primers provided in the kit. The sequencing was done by the University of Cape Town. Xylanase gene sequences were identified and aligned using known transposon sequences. Homology to known xylanase sequences were determined by BLAST as described in section 3.5.3.

3.8 Xylanase production by RZ1 on sugarcane bagasse (SCB) and beechwood xylan

3.8.1 Pre-treatment of SCB

Sugarcane bagasse was pre-treated by steam explosion at 195°C for 11 min using a steam gun (supplied by Prof J Görgens, Process Engineering, University of Stellenbosch, South Africa). Steam exploded SCB was manually pressed using a 4 ton hydraulic jack to separate the liquid from the solid fraction. The liquid fraction was filtered through Whatman MM3 paper to remove any remaining solid material and stored at -80°C until use. The SCB solid fraction was washed with 200 volumes of tap water to remove residual sugars and contaminants. Remaining water was removed by low speed centrifugation and the SCB solid manually broken into smaller pieces and dried in an oven at 30-40°C until it reached a relative moisture content of approx. 10%. The dried bagasse was thoroughly mixed and milled to a particle size of less than 1 mm using a ultracentrifugal mill ZM200 (Retch, Haan, Germany). All composition analyses of the sugarcane bagasse were performed as outlined by the National Renewable Energy Laboratory (www.nrel.gov/biomass/analytical procedures.html) and performed by Dr M Garcia Aparicio (Process Engineering, University of Stellenbosch, South Africa). The solid SCB fraction consisting of glucan, xylan, arabinan and lignin was analysed by the standard methods as described by Sluiter *et al*. (2008). Sugars present were further analysed with HPLC. Samples were diluted to suit HPLC range, centrifuged at 16, 000 x g for 5 min and filtered using 0.22 µm nylon filters. HPLC analysis of glucose, xylose and

arabinose was performed on a Dionex Ultimate® 3000 system equipped with a CarboPac PA1 column operated at 25°C with a mobile phase of 30 mM sodium hydroxide and a flow rate of 1 ml/min. Sugars present in the liquid fraction were determined using HPLC. A 1/10 dilution of the liquid fraction and monosaccharide standards (fructose, xylose, glucose, sucrose and arabinose) at 40 mM were resolved on a Rezex R50 oligosaccharide column (Phonomenex). Sugars were analysed before and after hydrolysis with 4% sulphuric acid to measure both monomeric and oligomeric sugars. Approx. 20 µl per sample was injected onto the column and a run time of 40 min in a mobile phase of double distilled water was employed, while the column was maintained at 48°C throughout.

3.8.2 Enzyme production and assays

A 5 ml overnight culture of isolate RZ1 was inoculated on 100 ml TSB. After another overnight cultivation step, minimal salt medium (0.2 g/L^{-1} magnesium sulphate, 0.5 g/L^{-1} dipotassium hydrogen phosphate, 0.5 g/L^{-1} potassium dihydrogen phosphate, 0.1 g/L^{-1} calcium chloride, 2 g/L^{-1} yeast extract) was inoculated to an OD_{600} of 0.2 in a total volume of 200 ml. The minimal salt media was supplemented with one of the following carbon sources: 2% (w/v) SCB solid, 0.2% (v/v) SCB liquid, 2% (w/v) xylose or 2% (w/v) beechwood xylan. Non-supplemented minimal salt medium was used as the control. Fermentations were performed in triplicate in a 37°C incubator with shaking (150 rpm). A 2 ml aliquot from each culture was sampled at hourly intervals. Bacterial growth (absorbance at 600 nm) was measured for all treatments except the SCB solid supplemented cultures, as the presence of the solid material would interfere with the absorbance readings. One ml culture was centrifuged at 15, 700 x g for 10 min and the supernatant was assayed for extracellular xylanase activity. The resulting cell pellets were resuspended in 500 µl 0.2 M sodium

phosphate buffer (pH 7.0) and the cells lysed by addition of 10 µl BugBuster™ Protein Extraction Reagent (Novagen, USA). Following incubation at 37°C for 30 min, the lysed cells were centrifuged at 15, 700 x g for 10 min to separate the soluble cell extract from the insoluble cell debris. Pellets were resupended in 500 µl 0.2 M phosphate buffer (pH 7.0). Xylanase activity in both the soluble and insoluble cell fractions was assayed. Three points were sampled in the kinetic analysis. The graphs were plotted using y-intercept as the absorbance at 600 nm and x-intercept as the incubation time in hours.

3.8.3 Xylanase assays

Xylanase activity was measured in a total reaction volume of 50 µl containing 35 µl culture supernatant, soluble cell extract or insoluble cell extract and 0.3% (w/v) beechwood xylan in 50 mM sodium phosphate buffer (pH 7.0). Assays were performed at 37°C and 50°C for 12.5 hrs. The amount of reducing sugar released was measured by dinitrosalicylic acid (DNS) assay as described by Miller (1959). DNS (150ul) was added and the mixture boiled for 5 min. After cooling on ice, 800µl of distilled water was added and the absorbance measured at 560 nm. D-xylose was used to generate a standard curve. The amount of reducing sugar liberated was calculated using the equation $y = 0.303x - 0.024$, where (y) is the absorbance at 560 nm and (x) is the amount of reducing sugar. The background reducing sugar present in each sample preceding the xylanase assay was also measured. Enzyme activity was expressed as mg xylose released h^{-1} ml^{-1} cell culture supernatant, solubilised cell extract and insoluble cell extract.

3.8.4. β-xylosidase and α-L-arabinofuranosidase assays

β-xylosidase and α-L-arabinofuranosidase activity was measured using *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX) and *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA) as substrates. Assays were performed in 50 mM sodium phosphate buffer (pH 7) containing 100 µl culture supernatant and 2 mM *p*NPX or *p*NPA in a final volume of 250 µl. Incubation was performed at 37°C for 5 hrs and the reaction stopped by the addition of 750 µl 0.4 M sodium bicarbonate. The release of *p*-nitrophenol was measured spectrophotometrically at 410 nm. Assays were performed on supernatants from cultures supplemented with beechwood xylan and xylose after 8 hrs of fermentation, while non-supplemented minimal medium cultures were used as controls. Enzyme activity is expressed as µmol *p*-nitrophenol released h^{-1} ml^{-1} culture, based on the extinction coefficient for *p*-nitrophenol (6.925 mM^{-1} cm^{-1}) as calculated from a standard curve.

Chapter 4: Results and Discussion

Isolation of a xylanase producing isolate from straw-based compost

4.1 Screening of composts isolates for lignocellulosic activity

In a search for producers of thermostable lignocellulosic enzymes, bacteria were isolated from thermophilic straw-based compost sampled at 71°C using a culture dependent technique. Bacteria were cultured and screened for lignocellulosic degradation in compost extract media. Isolate RZ1 displayed zones of clearance on agar plates supplemented with CMC, RBB xylan and tributyrin, suggesting the production of cellulases, xylanases and lipases/esterases, respectively (Figure 4.1).

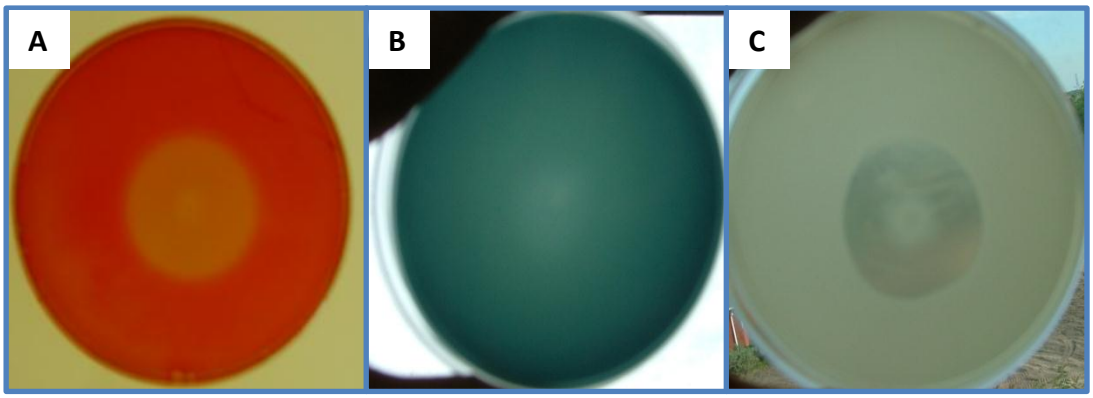

Figure 4.1 Functional screening of isolate RZ1 for cellulase (A), xylanase (B) and lipase/esterase (C) activity. Hydrolysis was visualized as zones of clearing on CMC, RBB xylan and tributyrin substrates, respectively.

4.2 Identification of isolate RZ1 by 16S rRNA gene sequencing

High molecular weight genomic DNA was isolated from isolate RZ1 (Figure 4.2) and used as template for 16S rRNA gene sequence analysis.

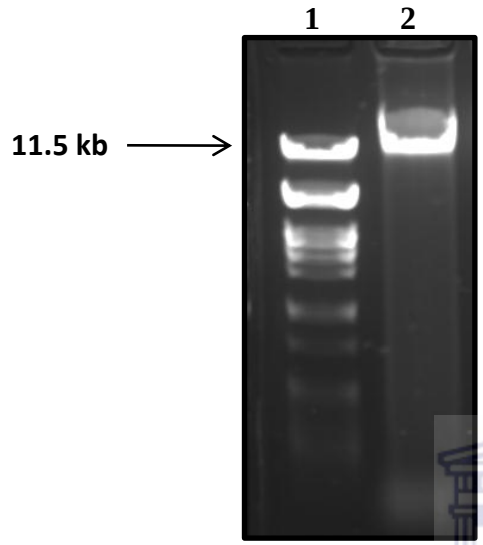

Figure 4.2 Genomic DNA isolated from RZ1, an isolate which demonstrated lignocellulosic activity. Lane 1, Lambda-P*st*I ladder; Lane 2, genomic DNA.

The 16S rRNA gene was amplified with universal primers E9F and U1015R. Amplicons (1.6 kb products) were cloned into the pGEMT cloning vector and transformed into *E. coli* GeneHog cells. The presence of an insert was confirmed by PCR amplification using vector specific M13 primers (Figure 4.3).

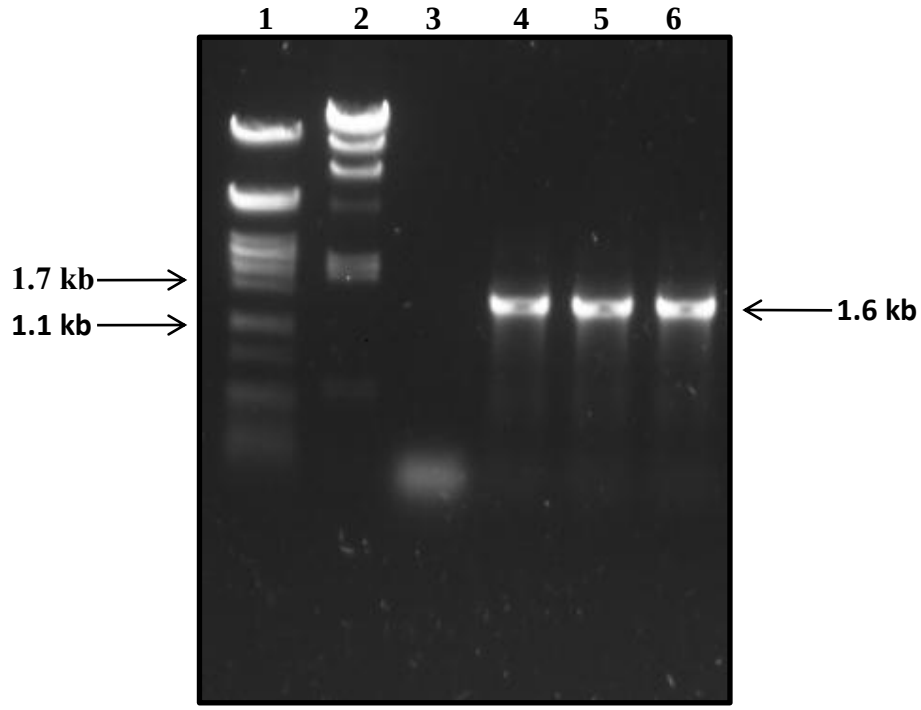

Figure 4.3 PCR amplification of bacterial 16S rRNA gene using primers E9F and U1510R. PCR products were visualised on 0.8% agarose gel. Lane 1, Lambda-P*st*I ladder; Lane 2, Lambda H*ind*III ladder; Lane 3, negative control; Lane 4-6, isolate RZ1 16S rRNA gene.

Because of its ubiquity and conservation of function, the 16S rRNA gene is the most common housekeeping gene employed in determining bacterial phylogeny (Janda and Abbott, 2007).

BLASTn analysis showed that isolate RZ1 was 99% identical to *B. subtilis* MSB10 (accession no FJ189764.1) isolated from a rice paddy in Korea (Math *et al*., 2007). A phylogenetic tree was constructed (Figure 4.4) and cluster analysis, supported by a 98% bootstrap value, grouped isolate RZ1 into the *B. subtilis* clade. *B. licheniformis*, a close relative, grouped into a separate clade. The presence of *Bacillus* sp. in compost has previously been demonstrated (Mayende *et al*., 2006).

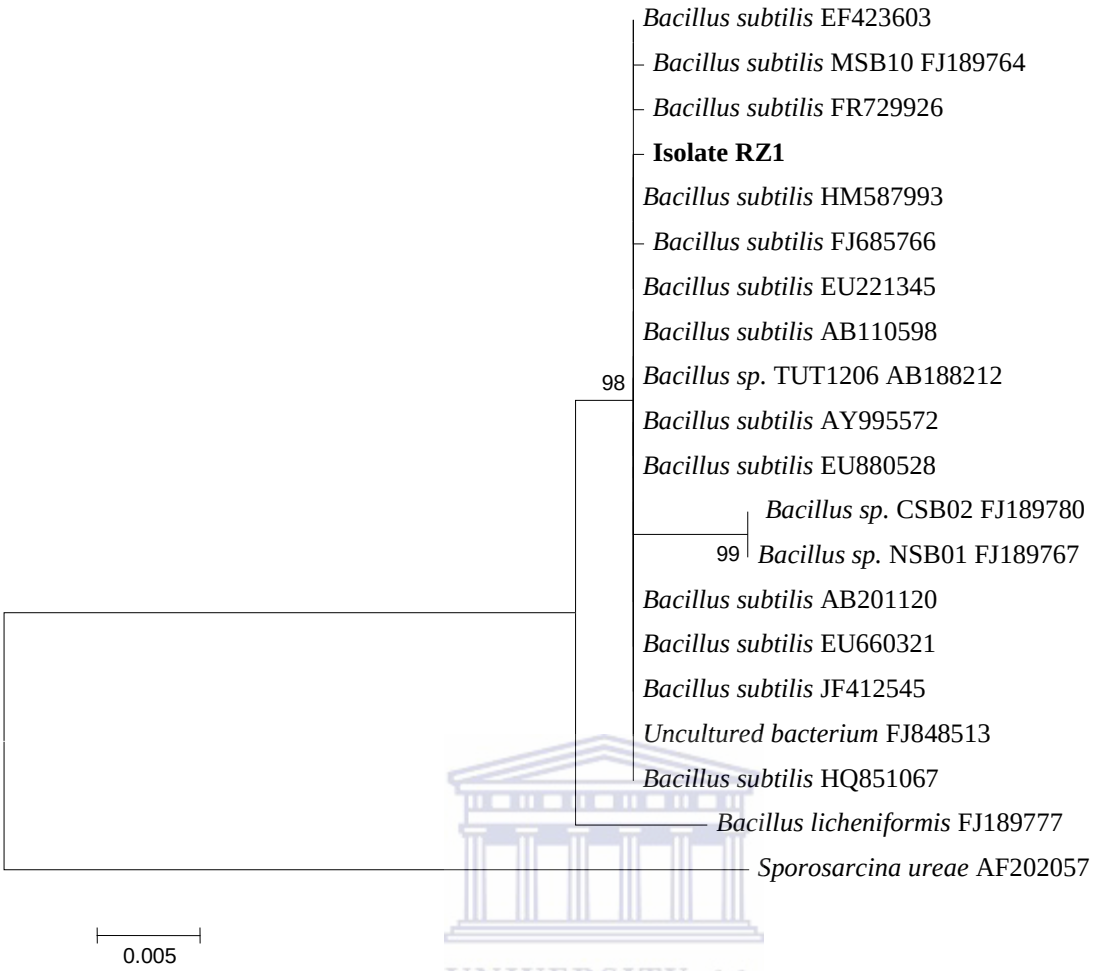

Figure 4.4 Phylogenetic tree constructed using Mega 4.0 neighbouring joining algorithm, showing the relationship of isolate RZ1 with known *Bacillus* strains. Sequences were obtained from Genbank and *Sporosarcina ureae* was used as an outlier species. The scale bar of all trees represents a 0.1% difference in nucleotide sequences. Bootstrap values provide a measure of the reliability of the phylogenetic analysis.

4.3 Physiological and biochemical characterisation of isolate RZ1

Isolate RZ1 was characterized by creamy white colonies with smooth edges and a shiny (slimy) character state. Colonies produced a brown pigment on TSB agar. Light microscopy revealed Gram positive rods (Figure 4.5) and motility was observed in liquid culture. Cells were able to produce oval shaped endospores (results not shown).

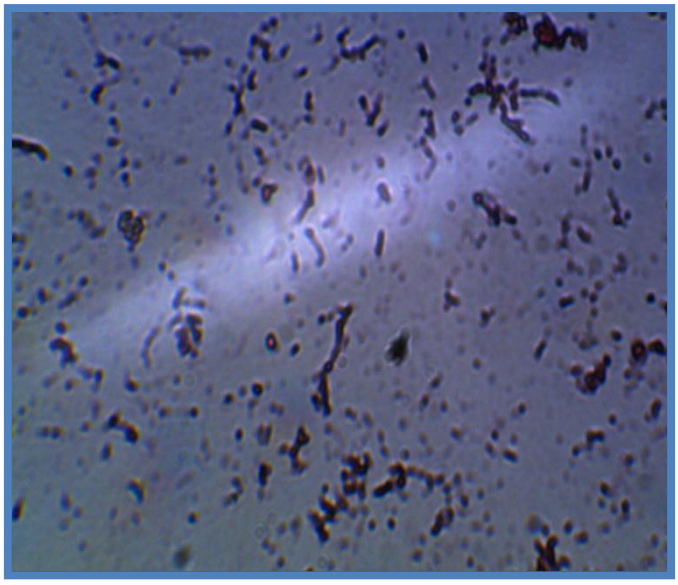

Figure 4.5 Photomicrograph of isolate RZ1 (Gram stained) as seen under the light microscope (magnification 1000×).

Isolate RZ1 was able to hydrolyse starch as seen by a zone of clearance on starch agar after iodine staining (Figure 4.6). This result suggests the production of exoenzymes able to hydrolyse α-1,4-glycosidic bonds such as α-amylase and oligo-1,6-glucosidase. These enzymes endoglycolytically degrade polysaccharides composed of glucose (Stülke and Hillen, 2000). RZ1 also hydrolysed casein in milk, indicating the production of proteases that cleave the peptide bonds in casein with the release of amino acids (Ageitos *et al*., 2006). Isolate RZ1 tested positive for catalase. Catalase is an enzyme that catalyses the decomposition of hydrogen peroxide into water and oxygen (Chelikani *et al*., 2004). Isolate RZ1 was able to grow under aerobic and anaerobic conditions, characterising it as a facultative aerobe. The morphological characteristics of this isolate conform to those described for *Bacillus* in Bergey's manual of determinative bacteriology (Bergey, 1957). Members of the *Bacillus* spp. are characterised by Gram positive rod shaped cells which form endospores and are catalase positive (Sonenshein, 2005).

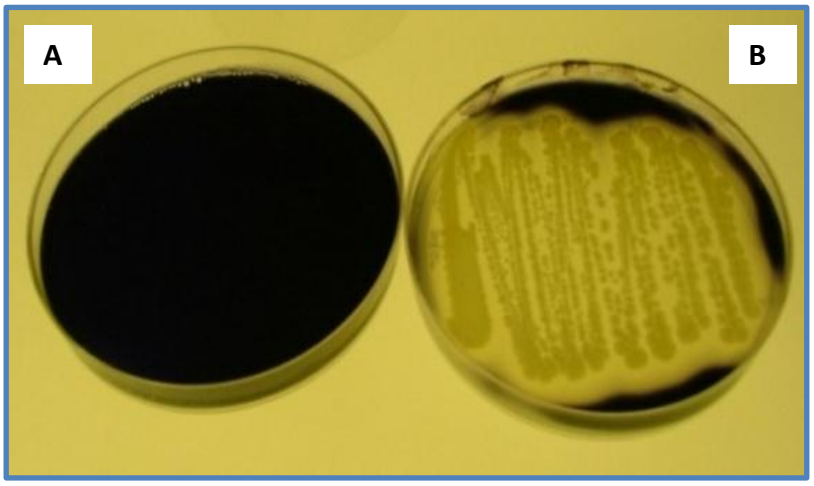

Figure 4.6 Amylase activity of RZ1 on starch agar. Plate A, negative control; Plate (B), starch hydrolysis as seen by a zone of clearance.

Isolate RZ1 was further characterised using the API CHB 50 and API 20 E kits. The API 50 CHB kit was used to determine the carbohydrates utilised by isolate RZ1 (Table 4.1). *B. subtilis* is known to utilise many carbon sources including unusual sugar derivatives such as β-arylglucosides, salin and arbutin (Krüger *et al*., 1996). Carbon utilisation was compared with the well characterized *B. subtilis* strain 168 (Min *et al*., 2009). Isolate RZ1 was able to utilise xylitol, starch, raffinose and D-trehalose as carbon sources, unlike *B. subtilis* strain 168. RZ1 was unable to utilise mannitol, sorbitol, methyl-D-glucoside and N-acetyl glucosamine, carbon sources readily utilised by *B. subtilis* 168. Both strains failed to utilise D-xylose and D-galactose, sugars commonly found in nature (Lindner *et al*., 1994). This is suprising because *B. subtilis* synthesizes proteins necessary to utilise these sugars. This is due to the fact that *B. subtilis* is unable to import these two sugars. Importation of these sugars has been shown to be dependent on the presence of Ara E protein synthesized by L-arabinose (Krispin and Allmansberger, 1998).

Table 4.1 The carbon sources of API 50 kit utilised by isolate RZ1

No	substrate	isolate RZ1
0	Control	-
1	Glycerol	+
2	Erythritol	-
3	D-Arabinose	-
4	L-Arabinose	+
5	D-Ribose	-
6	D-Xylose	-
7	L-Xylose	-
8	D-Adonitol	-
9	Methyl-β-D-xylopyranoside	-
10	D-Galactose	-
11	D-Glucose	+
12	D-Fructose	+
13	D-Mannose	+
14	L-Sorbose	-
15	L-Rhamnose	-
16	Dulcitol	-
17	Inositol	+
18	D-Mannitol	-
19	D-Sorbitol	-
20	Methyl-α-D-mannopyranoside	-
21	Methyl-α-D-glucopyranoside	-
22	N-Acetyl glucosamine	-
23	Amygdalin	+
24	Arbutin	+
25	Esculin	+
26	Salicin	+
27	D-Cellobiose	+
28	D-Maltose	-
29	D-Lactose	-
30	D-Melibiose	-
31	D-Saccharose	+
32	D-Trehalose	+
33	Inulin	-
34	D-Melezitose	-
35	D-Raffinose	+
36	Starch	+
37	Glycogen	+
38	Xylitol	+
39	Gentiobiose	+
40	D-Turanose	-
41	D-Lyxose	-
42	D-Tagatose	-
43	D-Fucose	-
44	L-Fucose	-
45	D-Arabitol	-
46	L-Arabitol	-
47	Gluconate	-
48	2-Ketogluconate	-
49	5-Ketogluconate	-

+: utilised; -: not utilised.

Further physiological characteristics of isolate RZ1 were determined using an API 20 E kit consisting of 20 biochemical tests. The isolate degraded gelatin and utilised L-arginine and L-tryptophane as nitrogen sources (Table 4.2). RZ1 utilised D-glucose, inositol, D-sucrose, amygdalin and also L-arabinose as carbon sources, confirming the API 50 results (Table 4.1).

Table 4.2 Biochemical test of API 20 E kit utilised by isolate RZ1

Tests	Active ingredients	Reaction enzymes	Results
ONPG	2-nitrophenyl-β-D-β-galactosidase Galactopyranoside	(OrthoNitrophenyl-βD-Galactopyranosidase)	-
ADH	L-arginine	Arginine DiHydrolase	+
LDC	L-lysine	Lysine Decarboxylase	-
ODC	L-ornithine	Ornithine Decarboxylase	-
CIT	trisodium citrate	CITrate utilization	-
H2S	Sodium thiosulfate	H_2S production	-
URE	Urea	URease	-
TDA	L-tryptophane	Tryptophan DeAminase	+
IND	L-tryptophane	Indole production	-
VP	Sodium pyruvate	Acetoin production	-
Gel	Gelatin	Gelatinase	+
Glu	D-glucose	Glucose fermentation	+
Man	D-mannitol	Mannitol fermentation	-
Ino	Inositol	Inositol fermentation	+
Sor	D-sorbitol	Sorbitol fermentation	-
RHA	L-rhamnose	Rhamnose fermentation	-
SAC	D-sucrose	Saccharose fermentation	+
MEL	D-melibiose	Melibiose fermentation	-
AMY	Amygdalin	Amygdalin fermentation	+
ARA	L-arabinose	Arabinose fermentation	+

+: utilised; -: not utilised.

The period used to obtain the growth rate was during the logarithmic phase. Isolate RZ1 displayed growth from 25°C to 55°C with optimum growth at 37°C (Figure 4.7) and therefore can be classified as a mesophile (Goldstein, 2007). The majority of *B. subtilis* spp. are mesophiles characterised by optimum growth temperatures of between 25°C and 40°C and a maximum growth temperature of 50°C (Droffner and Yamamoto, 1985). *B. subtilis* 168, for example, showed growth between 10°C and 40°C with an optimum growth temperature of 30°C (Jalal *et al*., 2009). RZ1 could sustain growth at 55°C suggesting that it

may have adapted to higher growth temperatures. This may be an environmental adaption as it was isolated from thermophilic compost.

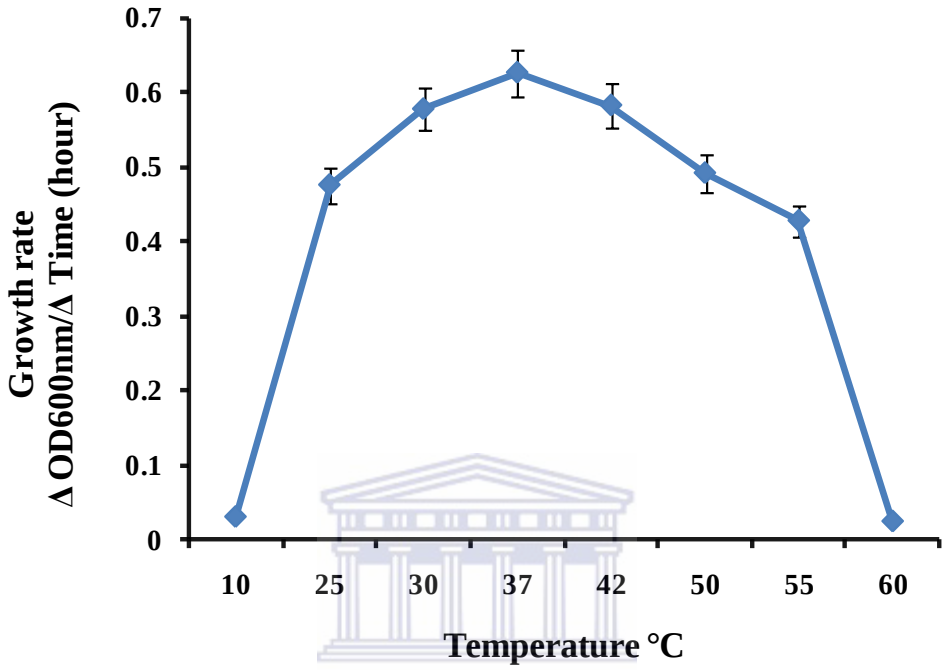

Figure 4.7 Temperature growth curve of isolate RZ1 in TSB medium.

4.4 Isolation of a xylanase from a small insert genomic library constructed from isolate RZ1

The emphasis of our research was to determine whether isolate RZ1 contained lignocellulosic enzymes with thermophilic properties which can be used in the hydrolysis of lignocellulose for the production of bioethanol. Since isolate RZ1 appeared to be adapted to growth at higher temperatures than those generally described for *B. subtilis* strains, the possibility of thermal adaption of the enzymes responsible for observed lignocellulosic activity was considered. A small insert genomic library was constructed for the discovery and

identification of coding sequences. Genomic DNA was partially digested with H*ind*III to generate fragments ranging from 5-10 kb (Figure 4.8). These fragments were cloned into the pUWL219 vector (Wehmeier, 1995). The library generated only 170 clones representing 30% coverage of the *B. subtilis* genome, estimated at 4.1 Mb based on sequence data (Kobayashi *et al*., 2003).

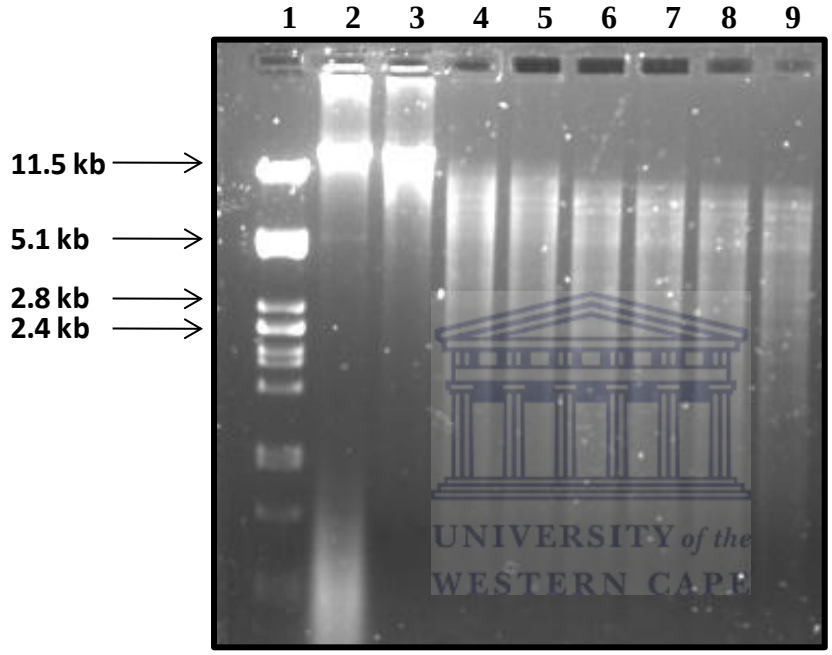

Figure 4.8 Genomic DNA from isolate RZ1 (1µg per sample) digested with H*ind*III. Lane 1, Lambda P*st*I ladder; Lane 2, undigested genomic DNA; Lane 3-9, digestion profile at 3 min intervals.

Plasmid DNA was isolated from 12 randomly selected library clones and the average insert size was assessed by restriction endonuclease digestion (Figure 4.9). Clones showed the presence of insert DNA ranging in size from 3-10 kb with an average insert size of 5 kb.

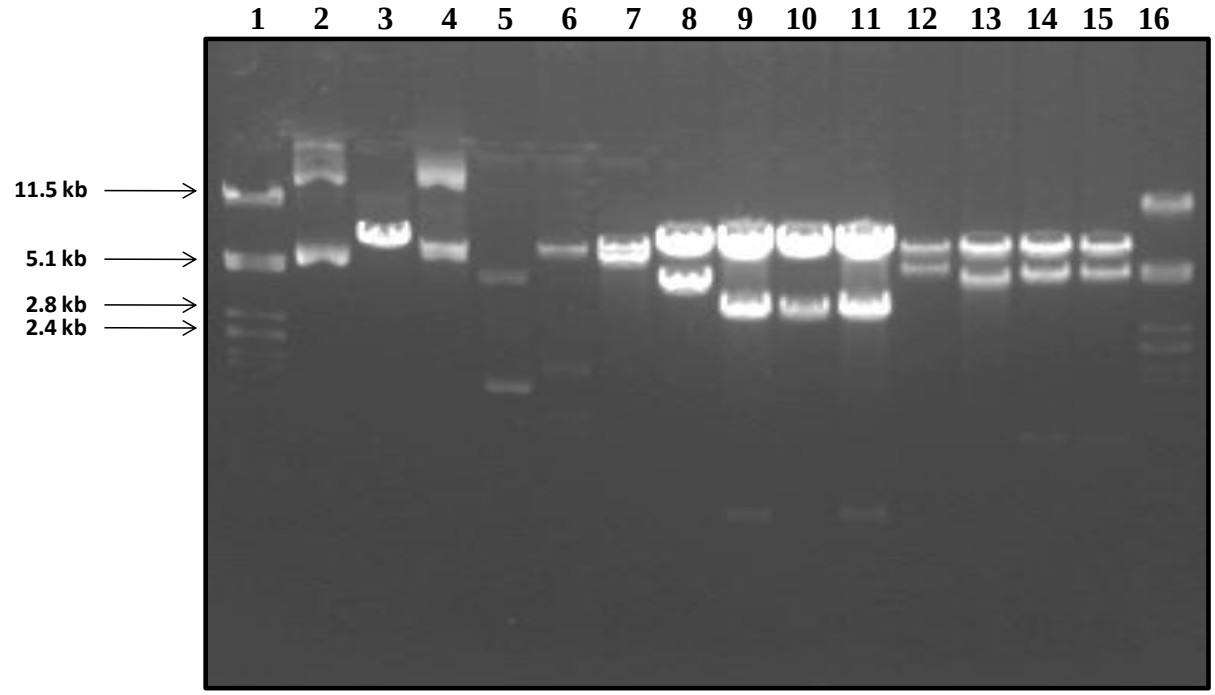

Figure 4.9 Analysis of 12 randomly selected recombinant plasmid clones from isolate RZ1 small insert genomic library. The clones were digested with H*ind*III to estimate the average insert size. Lane 1 and 16, Lambda P*st*I ladder; Lane 2, undigested plasmid; Lane 3, digested plasmid; Lane 4-15, recombinant plasmids clones digested with H*ind*III.

Although the library only represented 30% of the *B. subtilis* genome, at least one clone with xylanase activity (Figure 4.10) and another with esterase/lipase activity were identified during the screening for lignocellulosic enzymes. No clones with cellulase activity were identified. The clone displaying xylanase activity was named xyl R and further studied.

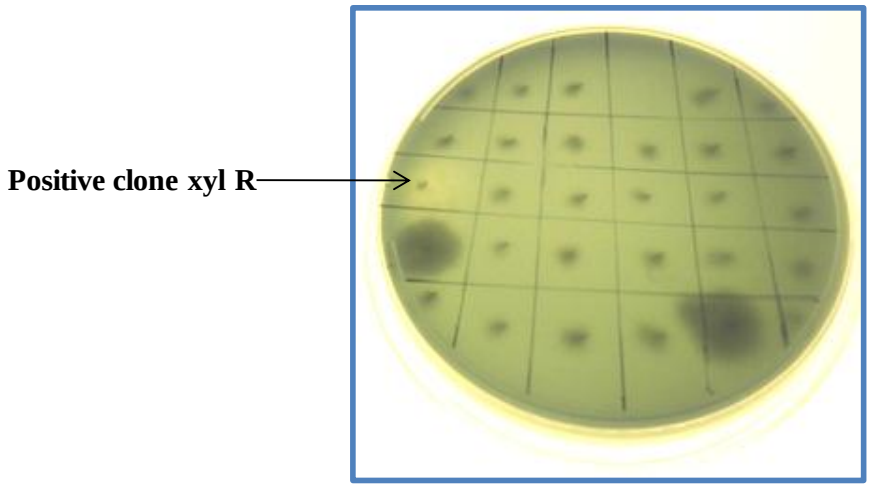

Figure 4.10 A positive xylanase clone, xyl R, from the library shown by a zone of clearance on LB agar enriched with RBB xylan.

Xylanase activity displayed by xyl R was confirmed by comparing activity with an *E. coli* clone harbouring the pUWL219 vector without insert. The transformant containing the control plasmid did not produce any halos on RBB xylan, whereas clone xyl R formed a clear zone around the bacterial streak (Figure 4.11).

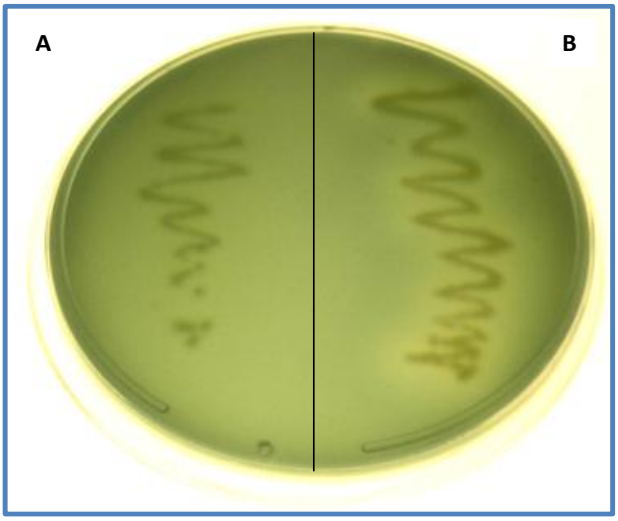

Figure 4.11 Growth of recombinant *E. coli* pUWL219 (without an insert) on RBB xylan agar (A). Clearing around recombinant *E. coli* pUWL219-xyl R colonies indicates hydrolysis of RBB xylan substrate (B).

The xyl R plasmid clone was digested with H*ind*III to release the plasmid vector and an insert size of 4.9 kb was calculated by analysing the digestion profile (Figure 4.12).

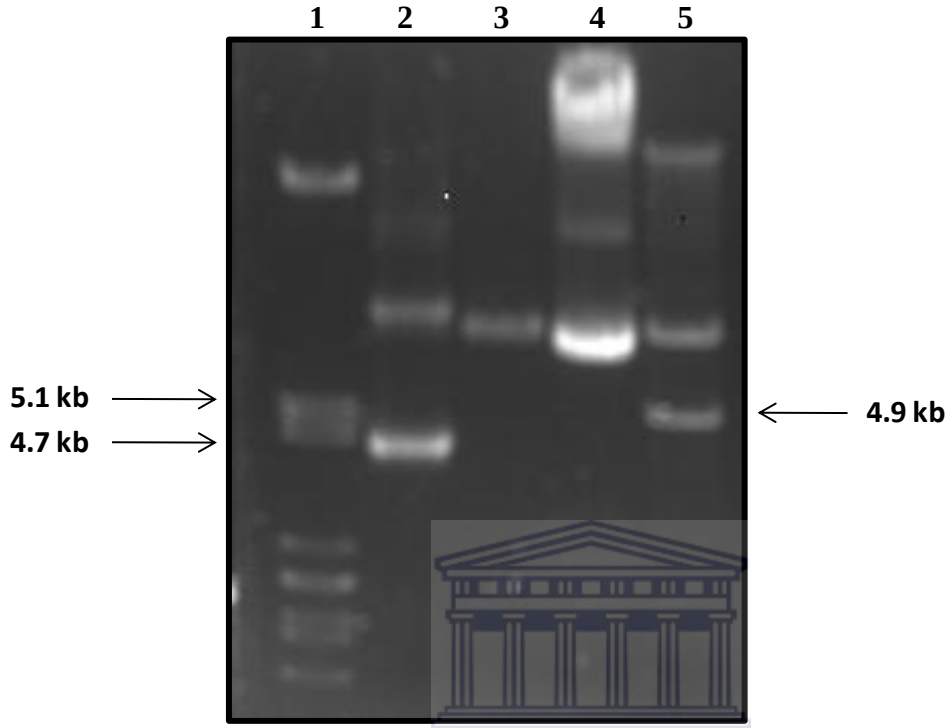

Figure 4.12 Restriction profiles of the RBB xylan hydrolysing clone that formed halos during activity based screening of the small insert genomic library. Lane 1, Lambda p*st*I DNA marker; Lane 2, undigested plasmid; Lane 3, digested plasmid with H*ind*III; Lane 4, undigested xyl R clone; Lane 5, xyl R clone digested with H*ind*III.

The xyl R clone was subjected to transposon mutagenesis to identify the gene conferring xylanolytic activity. Five mutants with loss-of-function or reduced activity (xylR-TM1-5) on RBB xylan as substrate were obtained (Figure 4.13). An ORF was identified by sequencing the regions flanking the transposon inserts.

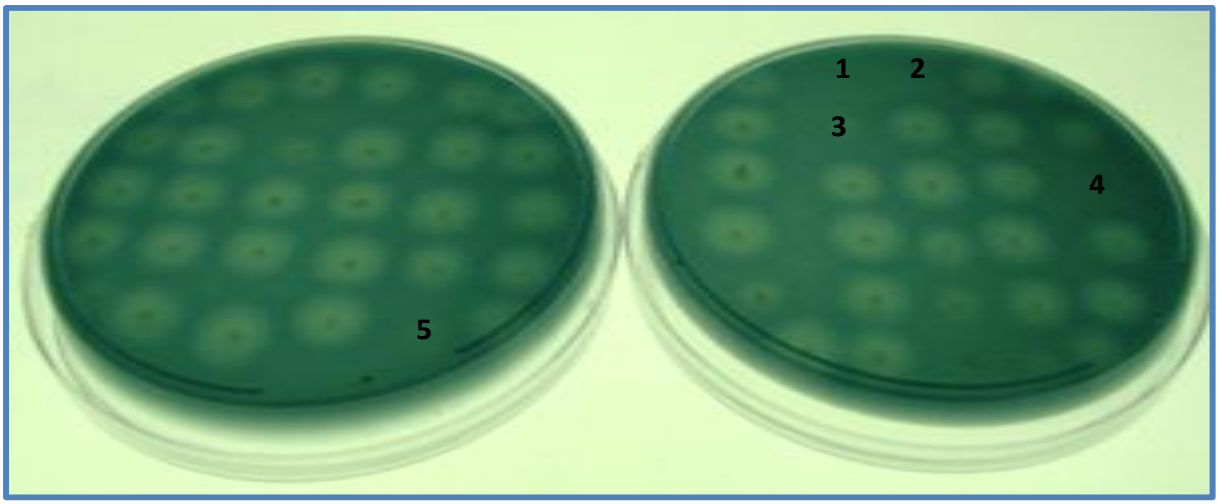

Figure 4.13 Transposon mutagenesis of a xyl R clone to identify the gene sequence. Knock out mutants (1-5) show no hydrolysis of RBB as seen by absence of halos.

Transposon insertion sites as generated by 5 individual mutation events are indicated in the nucleotide sequence of xyl R. Xyl R TM5 was inserted 14 nucleotides upstream of the start codon, possibly in regulatory elements thus interfering with xylanase activity.

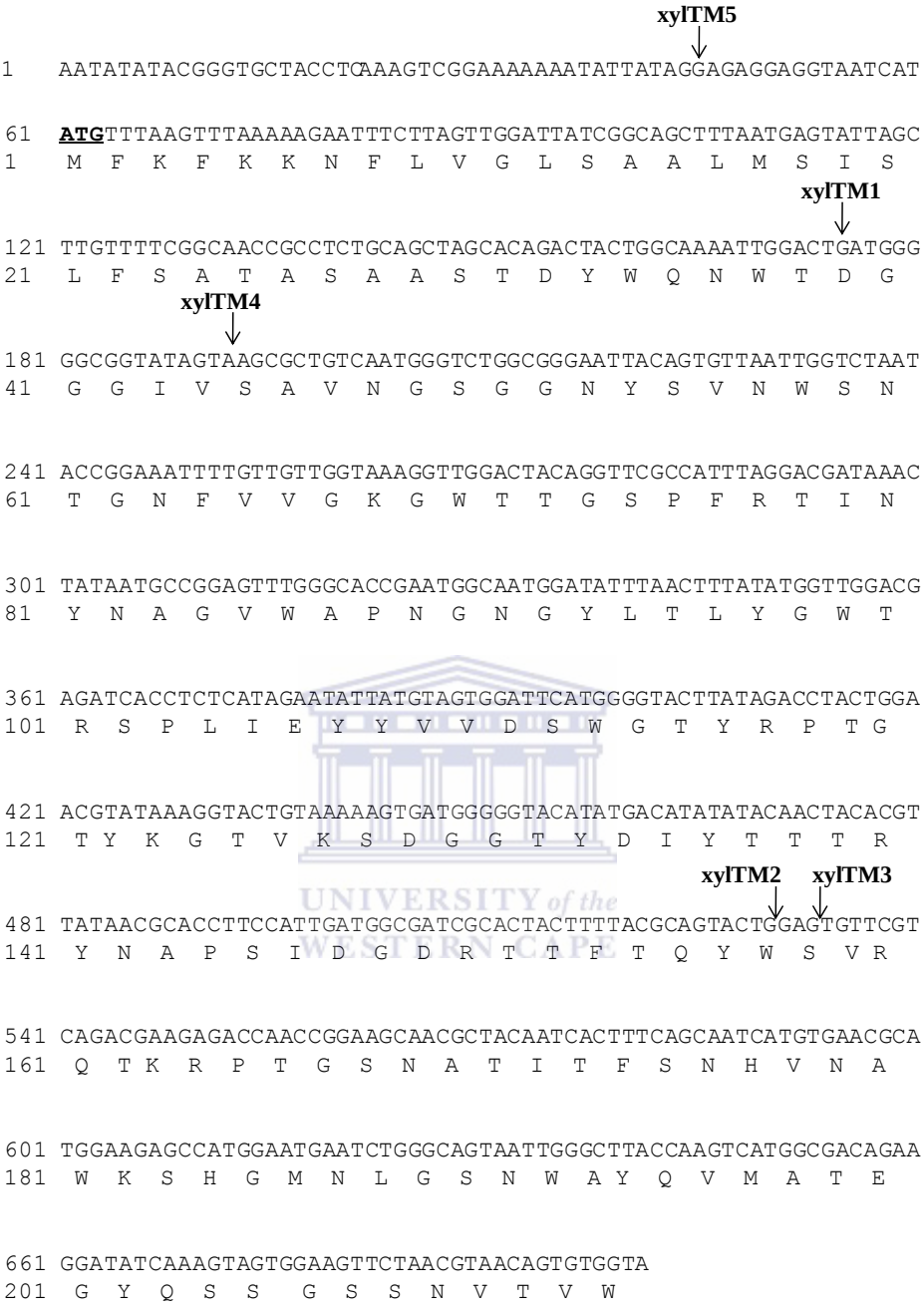

Figure 4.14 Nucleotide and deduced amino acid sequences of *xyl* R gene. The start codon translating the first amino acid of the deduced peptide sequence is underlined. Transposon insertion sites xylTM1-5 are indicated.

A protein-protein Blast search against Genbank sequences indicated that the xyl R deduced amino acid sequence was 99% similar to that of an endo-1,4-β-xylanase belonging to *Bacillus pumilus* (accession no AAZ17390). This enzyme belongs to the glycoside hydrolase family 11 (Cantarel *et al*., 2009). Phylogenetic analysis was performed using the *xyl* R gene sequence and other endo-1,4-β-xylanase gene sequences from *Bacillus* spp. (Figure 4.15). Genbank accession numbers of these proteins are given in brackets. The amino acid sequence of xyl R clustered with the sequence of a *B. pumilus* enzyme, a result supported by a bootstrap value of 61%. This compares to a study where the amino acid sequence of an endo-1,4-β-xylanase from *B. subtilis* was shown to be 100% identical to that of *B. pumilus* and *B. cereus* (Paice *et al*., 1986). These results suggest that different *Bacillus* spp. may contain enzymes with identical deduced amino acid sequences (Jalal *et al*., 2009).

A

B

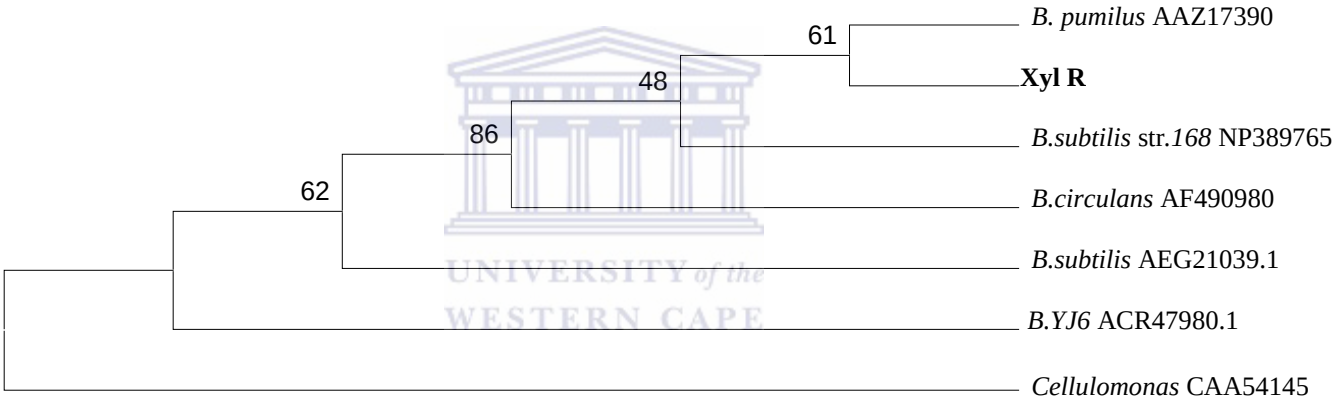

Figure 4.15 Phylogenetic tree showing the relationship between xyl R and other endo-1,4-β-xylanase sequences from *Bacillus* spp. *Cellulomonas fimi* was used as an outgroup (A). Scale bar of 0.2 represents the number of nucleotide substitutions per site. The topology of the tree is shown in (B). Bootstrap values are indicated.

4.5 Composition analyses of pre-treated sugarcane bagasse (SCB).

This study aimed to identify lignocellulosic enzymes for the saccharification of lignocellulosic material such as sugarcane bagasse (SCB). The SCB used in this study was pre-treated by steam expolosion to disrupt the lignocellulose structure thus releasing high sugar yields which can be used as substrates for xylanase production (Galbe and Zacchi, 2002). After pre-treatment of SCB, the solid fraction was separated from the liquid fraction, referred to hereafter as SCB(s) and SCB(l). SCB(s) contained a higher carbohydrate content compared to SCB(l)(Table 4.3). SCB(s) contained more than 50% glucan and only 6.5% xylan, while lignin constituted the remaining percentage. Due to the crystalline structure of cellulose, glucan is mostly protected from hydrolysis during pre-treatment (Arato *et al*., 2005). It is therefore not surprising that SCB(s) is comprised of mostly glucan. The SCB(l) contained mostly mono- and oligomeric xylose (Table 4.3), not surprising considering the instability of hemicellulose under harsh pre-treatment conditions (Buffiere *et al*., 2006). Martin *et al*. (2008) have reported similar results with highest xylose content in the liquid fraction, and highest glucan content in the solid fraction. By-products of pre-treated SCB such as acetic acid, formic acid, furfural and humic acid were also present in the liquid fraction, with acetic being the most abundant acid (Table 4.3).

Table 4.3 Composition analysis of SCB liquid and solid fractions after pre-treatment.

SCB(l)

Sugar constituents	Monomeric ($g.L^{-1}$)	Oligomeric*($g.L^{-1}$)	By-products	($g.L^{-1}$)
Glucose	2.9 ± 0.1	4.04 ± 0.04	Acetic acid	9.9 ± 0.1
Xylose	8.2 ± 0.1	25.4 ± 0.1	Formic acid	5.1 ± 0.05
Arabinose	0.48 ± 0.02	0.53 ± 0.15	Furfural	2.0 ± 0.03
			Humic acid	0.4 ± 0.01

SCB(s)

Component	Glucan	Xylan	Acid soluble lignin	Acid insoluble lignin
% dry weight	54.3 ± 2.7	6.5 ± 0.3	1.6 ± 0.09	29.2 ± 0.2

* Oligomeric sugars were measured after acid hydrolysis with 4% H_2SO_4 for 1 h.

4.5.1 The effect of carbon source on the growth of isolate RZ1 and xylanase production

Growth of isolate RZ1 was studied in minimal media (control) supplemented with 2% D-xylose, 2% beechwood xylan, or pre-treated 0.2% SCB(l) (Figure 4.16). Compared with the control culture, no significant difference in growth was observed when supplemented with 2% D-xylose as a carbon source, in agreement with results from the API50 kit which demonstrated that RZ1 failed to utilise D-xylose as a carbon source (Table 4.1). In nature, *B. subtilis* hardly encounters a situation where D-xylose is present as the only carbon source. It is more likely that a mixture of different sugars are utilised as carbon sources (Steinmetz, 1993). In the presence of 0.2% pre-treated SCB(l), the growth rate of RZ1 was slightly enhanced (Figure 4.16). SCB(l) contained a mixture of the sugars xylose, glucose and arabinose in both mono- and oligomeric forms (Table 4.3), a closer reflection of a situation that bacteria may encounter in the environment. Krispin and Allmansberger (1998) reported

the utilisation of D-xylose and D-galactose by *B. subtilis* strain 168 only when grown in the presence of L-arabinose. L-arabinose induces the Ara-E protein required for the transport of D-xylose, D-galactose and L-arabinose across the cell membrane. Interestingly, beechwood xylan appeared to inhibit the growth of isolate RZ1 in the lag phase (Figure 4.16). Utilisation of beechwood xylan was actively initiated after 6 hrs incubation. Due to the presence of particulate matter, the growth of RZ1 could not be assessed in SCB(s).

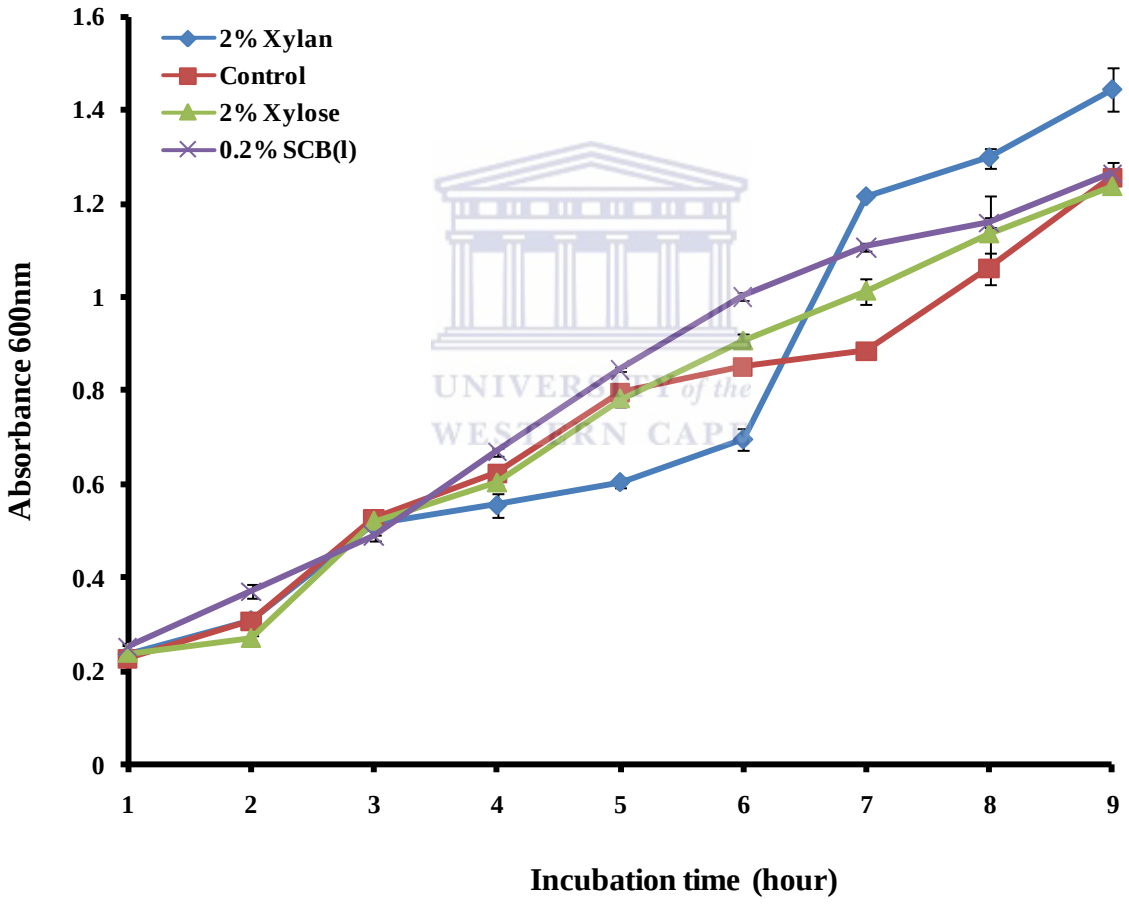

Figure 4.16 Growth of isolate RZ1 at 37°C on minimal medium supplemented with 2% beechwood xylan, 2% D-xylose and 0.2% pre-treated SCB(l) fraction as carbon sources. Minimal medium was used as control. The absorbance at 600 nm is plotted against time (hrs).

Xylanase activity was measured in culture supernatant of RZ1 cells grown in the presence of 2% D-xylose, 2% beechwood xylan, 2% SCB(s) or 0.2% SCB(l). Release of reducing sugars was measured with the Dinitrosalicylic (DNS) assay using a calibration curve generated with D-xylose as standard (Figure 4.17). Xylanase activity was determined with beechwood xylan as substrate.

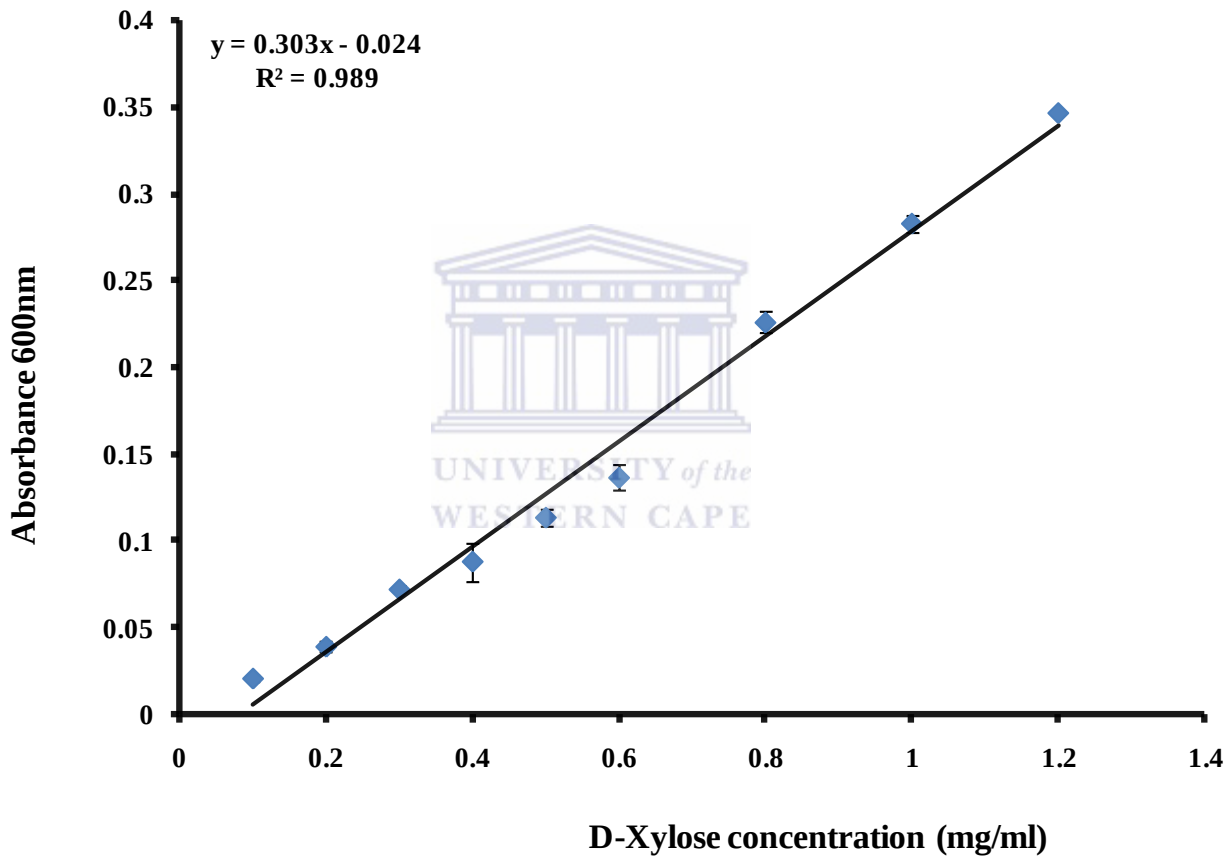

Figure 4.17 Xylose standard curve for DNS assay.

Intracellular xylanase activity was determined in cell free extracts prepared from cells collected at the various time points. Very low activity was detected (data not shown), suggesting that xylanases production by RZ1 was mainly extracellular. Extracellular xylanase

activity in cultures grown in the presence of either SCB(s) or SCB(l) was higher compared to activity measured in control cultures, suggesting that SCB might induce the production of xylanases (Figure 4.18).

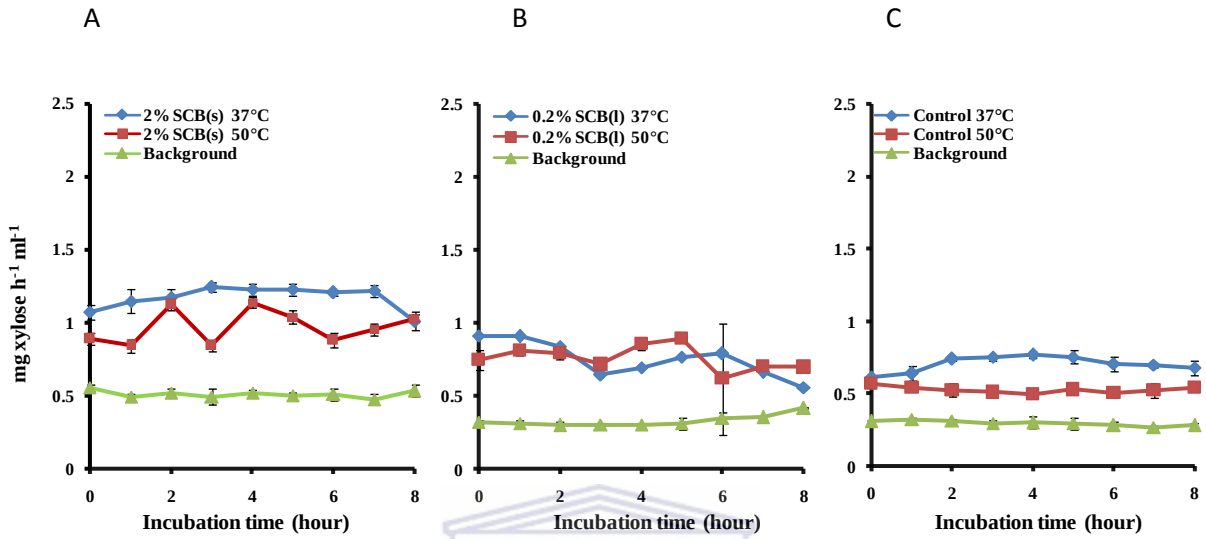

Figure 4.18 The effect of 2% SCB(s) (A) and 0.2% SCB(l) (B) on xylanase activity measured at 37°C and 50°C. Minimal medium was used as control (C). The background sugars present in the media at the various time points were also measured.

In the presence of 2% xylose, xylanase activity was not significantly changed suggesting repression by this carbon source (Figure 4.19B). There is a small increase in control media which is similar to that of the media supplemented with 2% xylose which may suggest that the increase in xylanase activity in media supplemented with 2% xylose may not be due the presence of xylose. Sugarcane bagasse has been shown to be a more suitable inducer compared to D-xylose for xylanase production (Battan *et al*., 2006). Xylanase activity was dramatically increased over a period of approximately 8 hrs of growth in the presence of 2% beechwood xylan (Figure 4.19 A), suggesting the induction of xylanase activity by xylan. Similar results have been reported by Saleem *et al*. (2002). Xylanase activity has been shown to increase during growth of a *B. subtilis* strain in the presence of xylan, while activity was repressed when D-xylose was used as a carbon source. Xylanase activity was higher at a

cultivation temperature of 37°C compared to activity measured at 50°C in the presence of SCB(s) (Figure 4.18A) or xylan (Figure 4.19A). Activity peaks, particularly obvious in the presence of SCB(s), suggest the presence of more than one xylanase. The *B. subtilis* genome may contain numerous xylanase genes (John *et al*., 2006). Accessory enzymes such as β-xylosidases and α-L-arabinofuranosidases are also required to release xylose from xylan (Gray *et al*., 2006).

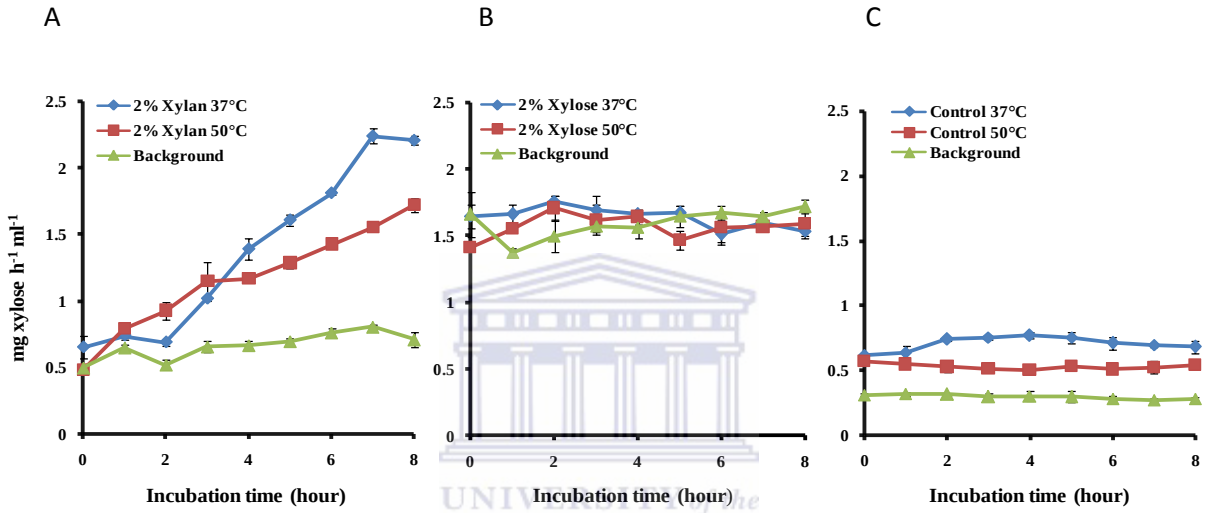

Figure 4.19 The effect of 2% beechwood xylan (A) and 2% D-xylose (B) on xylanase activity at 37°C and 50°C. Minimal medium was used as the control (C). The background sugars present in the media at the various time points were also measured.

4.6 β-xylosidases and α-L-arabinofuranosidase activity

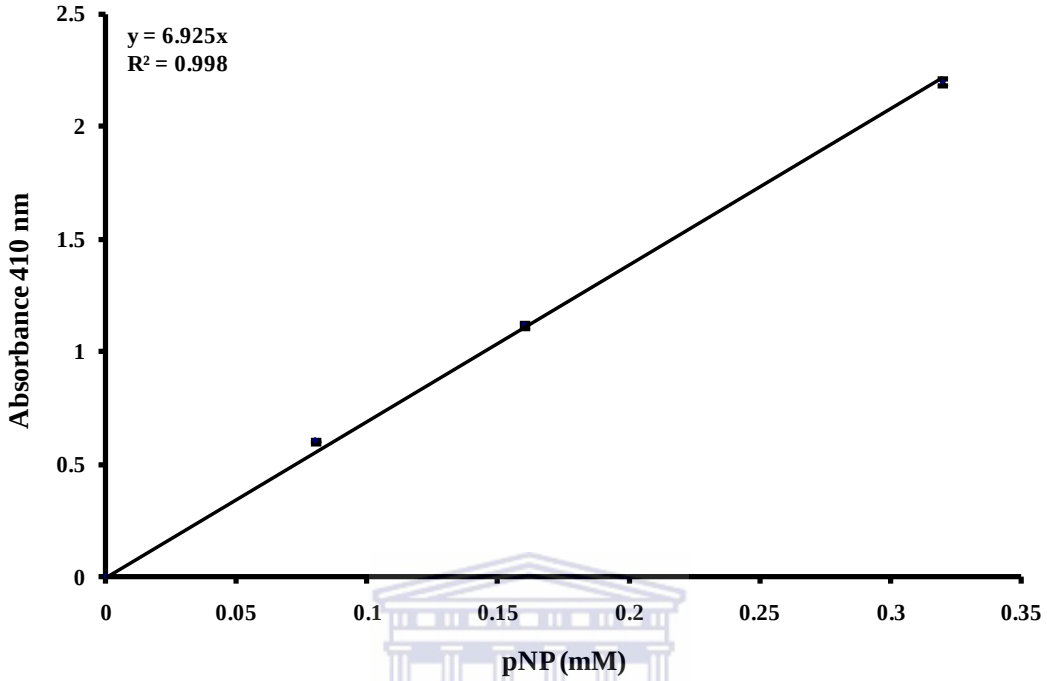

Figure 4.20 *p*-nitrophenol standard curve used to determine β-xylosidases and α-L-arabinofuranosidase activity.

The enzyme activities of two accessory hemicellulosic enzymes, β-xylosidases and α-L-arabinofuranosidases, were determined with p-nitrophenol (pNP) linked substrates, pNP-β-D-xylopyranoside and pNP-α-L-arabinofuranoside, respectively. Enzyme activities were measured in the supernatant of RZ1 cultures after 8 hrs of growth. The effect of 2% beechwood xylan or 2% D-xylose as carbon source on enzyme activity was evaluated. Significantly less β-xylosidase activity was measured in the presence of 2% D-xylose compared to cultures supplemented with 2% beechwood xylan (Table 4.4). β-D-xylosidases removes D-xylose residues from short xylo-oligosaccharides (Biely, 1985). Numerous sites in beechwood xylan may be cleaved by β-D-xylosidases whereas the monomer D-xylose is not hydrolysed and may act as an inhibitor of xylosidase activity. Lowest β-xylosidase activity

was measured in control media this may suggest that the β-xylosidase activity was overexpressed. Highest α-L-arabinofuranosidase activity was measured in media supplemented with 2% D-xylose (Table 4.4). α-L-arabinofuranosidase is an auxiliary enzyme that cleaves α-L-arabinofuranosyl linkages on the xylose subunit from arabinoses-rich polysaccharides such as arabinan, arabinoxylans, arabinogalactan and pectin (Saha, 2000). The presence of arabinose has been reported as a prerequisite for the uptake of xylose by *B. subtilis* cells (Krispin and Allmansberger, 1998). D-xylose may have induced the production of α-L-arabinofuranosidases by RZ1, releasing arabinose. α-L-arabinofuranosidase activity was also higher in the beechwood supplemented samples compared to the control (Figure 4.19A).

Table 4.4 Extracellular β-xylosidases and α-L-arabinofuranosidase activity (μ mol pnp $h^{-1}.ml^{-1}$) as measured after growth of isolate RZ1 in the presence of different carbon sources. Minimal medium was used for the control.

Carbon sources	**β-xylosidase activity** **μ mol pnp h^{-1} ml^{-1}**	**α-L-arabinofuranosidase** **μ mol pnp h^{-1} ml^{-1}**
2% Beechwood xylan	16.8 ± 0.6	132.1 ± 9.5
2% D-Xylose	5.9 ± 1.4	145.9 ± 7.9
Control	27.7 ± 0.5	122.5 ± 2.9

Chapter 5: Conclusion

A lignocellulase producing bacterium was isolated from thermophilic straw-based compost. Isolate RZ1 showed xylanase, cellulase and lipase/esterase activity. Phylogenetic analysis of the 16S rRNA gene sequence of RZ1 identified the isolate as a *B. subtilis* strain. Isolate RZ1 was able to grow at 55°C, higher than reported for most *Bacillus* strains. Considering that RZ1 was isolated from a thermophilic (71°C) compost source, the strain might have adapted to high temperatures. The isolate could utilise many carbohydrate sources for growth including glucose, mannose, fructose, starch, cellobiose, xylitol and glycogen. This suggested that it may harbour enzymes useful in bioethanol production.

A xylanase gene (*xyl* R) was identified after functional screening of a small insert genomic library generated from RZ1 gDNA. The gene product shared 99% homology with a previously characterised endo-1,4-β-xylanase produced by both *B. pumilus* and *B. cereus*. Due to high homology between xyl R and the previously characterised proteins, xyl R was not characterised in this study. Jalal *et al*. (2009) have shown that the enzyme exhibits highest activity in a temperature range of 40-50°C and at pH 6.0.

Xylanolytic activity was induced by culturing RZ1 in the presence of beechwood xylan and sugar cane bagasse. The activities of the produced xylanases were determined at both 50°C and 37°C using beechwood xylan as a substrate and a hydrolysis period of 12.5 hrs. Higher xylanolytic activity was measured when the activity was assayed at 37°C compared to 50°C. Numerous xylanases may be produced by *Bacillus* strains and these may be less stable at

50°C, thus resulting in a loss of activity during the incubation period. To determine the temperature at which the highest xylanase activity is achieved, activity will have to be measured over a time course.

B. subtilis RZ1 appears to harbour numerous lignocellulosic enzymes such as xylanases, cellulases and lipases/esterases. This study suggest that the isolate or its enzymes may have potential use in the process of degrading lignocellulosic biomass, such as sugarcane bagasse, for biofuel production. To further investigate the suitability of these enzymes in a thermogenic process, they will need to be purified and fully characterised.

References

Ageitos, J.M., Vallejo, J.A., Poza, M. and Villa, T.G. (2006). Fluorescein thiocarbamoyl-kappa-casein assay for the specific testing of milk-clotting proteases. *J Dairy Sci* **89**: 3770-3777.

Altschul, S.F., Madden, T.S., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.

Arato, C., Pye, E.K. and Gjennestad, G. (2005). The lignol approach to biorefining of woody biomass to produce ethanol and chemicals. *Appl Biochem Biotechnol* **121/124**: 871-882.

Baez, V.M.A. and Demain, A.L. (2008). Ethanol, biomass and *clostridia*. Harwood, C.S., Demain, A.L. and Wall, J.D. (eds). *Bioenergy*. Washington DC: ASM Press.

Balat, M., Balat, H. and Oz, C. (2008). Progress in bioethanol processing. *Progr Energ Combust Sci* **34**: 551-573.

Ballesteros, I., Ballesteros, M., Cabanas, A., Manzanares, P., Negro, M.J. and Oliva, J.M. (2006). Ethanol production from steam-explosion pre-treated wheat straw. *Appl Biochem Biotechnol* **130**: 496-508.

Bastawde, K.B. (1992). Xylan structure, microbial xylanases, and their mode of action. *World J Microbiol Biotechnol* **8**: 353-368.

Bath, M.K. and Bath, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnol Adv* **15**: 583-620.

Battan, B., Sharma, J. and Kuhad, R.C. (2006). High-level xylanase production by alkaliphilic *Bacillus pumilus* ASH under solid state fermentation. *World J Microbiol Biotechnol* **22**: 1281-1287.

Becker, J. and Botes, E. (2003). A modified *Saccharomyces cerevisiae* strain that consumes L arabinose and produces ethanol. *Appl Environ Microbiol* **69**: 4144-4150.

Beg, Q.K., Kapoor, M., Mahajan, L. and Hoondal, G.S. (2001). Microbial xylanases and their industrial applications: A review. *Appl Microbiol Biotechnol* **56**: 326-338.

Belancic, A., Scarpa, J., Peirano, A., Diaz, R., Steiner, J. and Eyzayuirre, J. (1995). *Penicillium purpurogenum* produces several xylanases: purification and properties of two of the enzymes. *J Biotechnol* **41**: 71-79.

Bergey, D.H. (1957). Bergey`s manual of determinative bacteriology. *Am Soc Microbiol*: 1860-1937.

Betts, W.B., Dart, R.K., Ball, A.S. and Pedlar, S.L. (1991). Biosynthesis and structure of lignocellulose. Berlin, Germany. Springer-Verlag.

Bhalerao, J., Patki, A.H., Bhave, M., Khurana, I. and Deobagkar, D.N. (1990). Molecular cloning and expression of a xylanase gene from *Cellulomonas* sp. into *Escherichia coli*. *Appl Microbiol Biotechnol* **34**: 71-76.

Biely, P. (1985). Microbial xylanolytic enzymes. *Trends Biotechnol* **3**: 286-290.

Biely, P. (1991). Biotechnological potential and production of xylanolytic systems free of cellulases. *ACS Symp Ser* **460**: 408-416.

Biely, P. (1993). Biochemical aspects of the production of microbial hemicellulases. In: Hemicellulose and Hemicellulases. Coughlan, M.P. and Hazlewood, G.P. (eds). Cambridge: Portland Press: 29-51.

Biely, P., Vrsanska, M., Tenkanen, M. and Kluepfel, D. (1997). Endo-beta-1,4-xylanase families: differences in catalytic properties. *J. Biotechnol* **57**: 151-166.

Blumer-Schutte, S.E., Kataeva, I., Westphiling, J., Adams, M.W.W. and Kelly, R.M. (2008). Extreme thermophilic microorganisms for biomass conversion: status and prospects. *Curr Opin Biotechnol* **19**: 210-217.

Borneman, W.S., Hartley, R.D, Morrison, W.H., Akin, D.E. and Ljungdahl, L.G. (1990). Feruloyl and p-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation. *Appl Microbiol Biotechnol* **33**: 345-351.

Bourbonnais, R. and Paice, M.G. (1988). Veratryl alcohol oxidases from the lignin degrading *basidiomycete Pleurotus sajor-caju*. *Biochem J* **255**: 445-450.

Bourne, Y. and Henrissat, B. (2001). Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr Opin Struct Biol* **11**: 593-600.

Briuns, M.E., Janssen, A.E. and Boom, R.M. (2001). Thermozymes and their applications: a review of recent literature and patents. *Appl Biochem Biotechnol* **90**: 155-186.

Buffiere, P., Loisel, D., Bernet, N. and Delgenes, J.P. (2006). Towards new indicators for the prediction of solid waste anaerobic digestion properties. *Water Sci Technol* **53**: 233-241.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* **37**: 233-238.

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

Chelikani, P., Fita, I. and Loewen, P.C. (2004). "Diversity of structures and properties among catalases." *Cell Mol Life Sci* **61**: 192-208.

Chhetri, A.B. and Islam, M.R. (2008). Towards producing a truly green biodiesel. Recovery utilization and environmental effects. *Energ Source Part A* **30**: 754-764.

Claeyssens, M. and Henrissat, B. (1992). Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. *Protein Sci* **1**: 1293-1297.

Claassen, P.A.M., Van Lier, J.B., Contreras, A.M.L., Van Niel, E.W.J., Sijtsma, L., Stams, A.J.M., De Vries, S.S. and Weusthuis, R.A. (1999). Utilisation of biomass for the supply of energy carriers. *Appl Microbiol Biotechnol* **52**:741-755.

Collins, T., Meuwis, M.A., Stals, I., Claeyssens, M., Feller, G. and Gerday, C. (2002). A novel family 8 xylanase: functional and physico-chemical characterization. *J Biol Chem* **277**: 35133-35139.

Collins, T., Meuwis, M.A., Gerday, C. and Feller, G. (2003). Activity, stability and flexibility in glycosidases adapted to extreme thermal environments. *J Mol Biol* **328**: 419-428.

Collins, T., Gerday, C. and Feller, G. (2004). Xylanases, xylanase families and extremophilic xylanases. Laboratory of Biochemistry, Institute of Chemistry B6, University of Liège, B-4000 Liège, Belgium.

Collins, T., Gerday, C. and Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol Rev* **29**: 3-23.

Coutinho, P.M. and Henrissat, B. (1999). Carbohydrate-active enzyme server (CAZY). [Online]. Available http://afmb.cnrs-mrs.fr/~cazy/CA

Cowan, S.T. (1974). Cowan and Steel's Manual for the Identification of Medical Bacteria, 2nd edition. Cambridge: Cambridge University Press.

Das, M., Royer, T.V. and Lev, L.G. (2007). Diversity of fungi, bacteria, and *actinomycetes* on leaves decomposing in a stream. *Appl Environ Microbiol* **73**: 756-767.

Dees, P.M. and Ghiorse, W.C. (2001). Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolates and extracted DNA. *FEMS Microbiol Ecol* **35**: 207-216.

Demirbas, A. (2008). The Importance of bioethanol and biodiesel from biomass. *Energ Source Part B* **3**: 177-185.

Dhillon, A. and Khanna, S. (2000). Production of a thermostable alkali-tolerant xylanase from *Bacillus circulans* AB16 grown on wheat straw. *World J Microbiol Biotechnol* **27**: 325-327.

Divne, C., Stahlberg, J., Teeri, T. and Jones, T.A. (1998). High-resolution crystal structures reveal how a cellulose chain is bound in the 50Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. *J Mol Biol* **275**: 309-325.

Donaghy, J.A., Bronnenmeier, K., Soto-Kelly, P.F. and McKay, A.M. (2000). Purification and characterisation of an extracellular feruloyl esterase from the thermophilic anaerobe *Clostridium stercorarium*. *J Appl Microbiol* **88**: 458-466.

Droffner, M.L. and Yamamoto, N. (1985). Isolation of thermophilic mutants of *B. subtilis* and *B. pumilus* and transformation of the thermophilic trait to mesophilic strains. *J Gen Microbiol* **131**: 2791-2794.

Dussault, H.P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**: 484-485.

Earth police institute. (2006). Supermakets and Service Stations for grain. Brown, L.R. In: Plan B updates. [Online]. Available http://www.earthpolicy.org/Updates/2006/Update55 data.htm

EIA. (1998). Annual energy outlook 1999, with projections to 2020 **99**. Energy Information Administration USA, Department of Energy, Washington DC.

Emi, S. and Yamamoto, T. (1972). Purification and properties of several galactanase of *Bacillus subtilis* var. *Amylosacchariticus Agr Biol Chem* **36**: 1945-1955.

Felby, C. (2009). *Marken - en stor solfanger - Det Biovidenskabelige Fakultet -Københavns Universitet.* [Online]. Available: http://www.life.ku.dk/forskning/online

Fushinobu, S., Ito, K., Konno, M., Wakagi, T. And Matsuzawa, H. (1998). Crystallographic and mutational analyses of an extremely acidophilic and acid-stable xylanase: biased distribution of acidic residues and importance of Asp37 for catalysis at low pH. *Protein Eng* **11**: 1121-1128.

Galbe, M. and Zacchi, G. (2002). A review of the production of ethanol from softwood. *Appl Microbiol Biotechnol* **59**: 618-628.

Georis, J., Giannotta, F., De Buyl, E., Granier, B. and Frere, J. (2000). Purification and properties of three endo-beta-1,4-xylanases produced by *Streptomyces* sp. strain S38 which differ in their capacity to enhance the bleaching of kraft pulp. *Enzyme Microbiol Technol* **26**: 177-183.

Gibbs, M.D., Reeves, R.A. and Bergquist, P.L. (1995). Cloning, sequencing and expression of a xylanase gene from the extreme thermophile *Dictyoglomus thermophilum* Rt46B. 1 and activity of the enzyme on fiber-bound substrate. *Appl Environ Microbiol* **61**: 4403-4408.

Gilkes, N.R., Claeyssens, M., Aebersold, R., Henrissat, B., Meinke, A., Morrison, H.D., Kilburn, D.G., Warren, R.A. and Miller, R.C. (1991). Structural and functional relationships in two families of beta-1,4-glycanases. *Eur J Biochem* **202**: 367-377.

Goldstein, R.A. (2007). Amino-acid interactions in psycrophiles, mesophiles, thermophilus and hyperthermophiles: Insight from the quasi-chemical approximation. *Protein sci* **16**: 1887-1895.

Gomez, L.D., Steele-King, C.G. and McQueen-Mason, S.J. (2008a). Sustainable liquid biofuels from biomass: the writing's on the walls. *New Phytol* **178**: 473-485.

Gong, C.S., Cao, N.J., Du, J. and Tsao, G.T. (1999). Ethanol production for renewable resources. *Adv Biochem Eng Biotecnol* **65**: 207-241.

Gosalves, M.J., Perez-Gonzalez, J.A., Gonzalez, R. and Navarro, A. (1991). Two L-glycanase genes are clustered in *Bacillus polymyxa*: Molecular cloning expression and sequence analysis of genes encoding a xylanase and an endo-L-(1,3)-(1,4)-glucanase. *J Bacteriol* **173**: 7705-7710.

Goyal, A., Ghosh, B. and Eveleigh, D. (1991). Characteristics of fungal cellulases. *Bioresour Technol* **36**: 37-50.

Gray, K.A., Zhao, L. and Emptage, M. (2006). Bioethanol. *Curr Opin Chem Biol* **10**:141-146.

Gruninger, H. and Fiechter, A. (1986). A novel, highly thermostable D-xylanase. *Enzyme Microb Technol* **8**: 309-314.

Hahn-Hägerdal, B., Linden, T., Senac, T. and Skoog, K. (1991). Ethanolic Fermentation of Pentoses in Lignocellulose Hydrolysates. *Appl Biochem Biotechnol* **28/29**: 131-144.

Hahn-Hägerdal, B., Hallborn, J. and Jeppsson, H. (1993). Pentose fermentation to alcohol: Saddler, J.N. (ed). In: *Bioconversion of Forest and Agricultural Plant Residues*, CAB International, Wallingford, UK: 231-290.

Hahn-Hägerdal, B., Galbe, M., Gorwa-Grausland, M.F., Liden, G. and Zacchi, G. (2006). Bioethanol the fuel of tomorrow from the residues of today. *Trends in Biotechnol* **24**: 549-556.

Haki, G.O. and Rakshit, S.K. (2003). Developments in industrially important thermostable enzymes: A review. *Bioresour Technol* **89**: 17-34.

Hall, T.A. (1999). Bioedit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symp Ser* **95/98**.

Hamelinck, C.N., Van Hooijdonk, G. and Faaij, A.P.C. (2005). Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long term. *Biomass Bioenerg* **28**: 384-410.

Hanazawa, S., Hoaki, T., Jannasch, H. and Maruyama, T. (1996). An extremely thermostable serine protease from hyperthermophilic archaeum, *Desulfurococcus* strain sy. Isolated from a deep sea hydrothermal vent. *J Mar Biotechnol* **4**: 121-126.

Harris, G.W., Pickersgill, R.W., Connerton, I., Debeire, P., Touzel, J.P., Breton, C. and Perez, S. (1997). Structural basis of the properties of an industrially relevant thermophilic xylanase. *Proteins* **29**: 77-86.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**: 309-316.

Henrissat, B. and Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem J* **316**: 695-696.

Henrissat, B. and Davies, G.J. (2000). Glycoside hydrolases and glycosyltransferases. Families, modules and implications for genomics. *Plant Physiol* **124**: 1515-1519.

Henrissat, B. and Coutinho, P.M. (2001). Classification of glycoside hydrolases and glycosyltransferases from hyperthermophiles. *Methods Enzymol* **330**: 183-201.

Herbert, R.A. (1992). Thermophilic cellulases producing bacteria have been isolated from an Egyptian. A perspective on the biotechnological potential of extremophiles. *Trends Biotechnol* **10**: 395-401.

Hinman, N.D., Wright, J.D., Hoagland, W. and Wyman, C.E. (1989). Xylose fermentation: an economic analysis. *Appl Biochem Biotechnol* **20/21**: 391-410.

Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L. and Howard, S. (2003). Lignocellulose bioetechnology: issues of bioconversion and enzyme production. *Afr J Biotecnol* **2**: 602-619.

Hsu, T.A., Ladisch, M.R. and Tsao, G.T. (1980). Alcohol from cellulose. *Chem Technol* **10**: 315-319.

Hsu, T. (1996). Pre-treatment of biomass**.** In Handbook on Bioethanol*:* Production and Utilization. Wyman, C. (ed). Washington DC: Taylor and Francis: 179-212.

Ibrahim, A.S.S. and Ahmed, I.E. (2007). Isolation and Identification of New Cellulases Producing Thermophilic Bacteria from an Egyptian Hot Spring and Some Properties of the Crude Enzyme. Department of Chemistry and Natural Microbial Products, National Research Centre. Dokki, Cairo, Egypt.

Jalal, A., Rashid, N., Rosooi, N. and Akhtar, M. (2009). Gene cloning and characterisation of a xylanase from a newly isolated *Bacillus subtillus* strain R5. *J Biosci and Bioeng* **107**: 360-365.

Janda, M.J. and Abbott, S.L. (2007). 16S rRNA gene sequence for bacterial identification in the diagnostic laboratory. *J Clin Microbiol* **45**: 2761-2764.

Jeczmionek, L., Oleksiak, S., Skret, I. and Marchut, A. (2006). The second-generation biofuels. *Przemysl Chem* **85**: 1570-1574.

John, F.J., Rice, J.D. and Preston, J.F. (2006). Characterization of XynC from *Bacillus subtilis* subsp. *subtilis* Strain 168 and Analysis of Its Role in Depolymerization of Glucuronoxylan. *J Bacteriol* **188**: 8617-8626.

Jorgensen, H., Kristensen, J.B. and Felby, C. (2007**).** Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities. *Biofuels Bioprod Bioref* **1**: 119-134.

Kasana, R.C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A. (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Curr Microbiol* **57**: 503-507.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M. and Igarashi, Y. (2005). Stable coexistence of Wve bacterial strains as a cellulose-degrading community. *Appl Environ Microbiol* **71**: 7099-7106.

Khasin, A., Alchanati, I. and Shoham, Y. (1993). Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Appl Environ Microbiol* **59**: 1725-1730.

Kirk, T.K., Connors, W.J. and Zelkus, J.G. (1976). Requirements for a growth substrate during lignin decomposition by two-wood-rotting fungi. *Appl Environ Microbiol* **32**: 192-194.

Kirk, T.K and Farell, R.L. (1987). Enzymatic "combustion": the microbial degradation of lignin. *Annu Rev Microbiol* **41**: 465-505.

Klasson, K.T., Ackerson, M.D., Clausen, E.C. and Gaddy, J.L. (1992). Bioconversion of synthesis gas into liquid or gaseous fuels. *Enzyme Microb Technol* **14**: 602-618.

Kleywegt, G.J., Zou, J.Y., Divne, C., Davies, G.J, Sinning, I., Stahlberg, J., Reinikainen, T., Sridodsuk, M., Teeri, T.T. and Jones, T.A. (1997). *Mol Biol* **272**: 383-397.

Kobayashi, K., Ehrlich, S.D., Albertini, A., Amati, G., Andersen, K.K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S.C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L.C., Danchin, A., Débarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S.K., Dreesen, O., Errington, J., Fillinger, S., Foster, S.J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C.R., Hecker, M., Hosoya, D., Hullo, M.F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauël, C., Meima, R., Mellado, R.P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H.M., Rapoport, G., Rawlins, J.P., Rivas, L.A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H.H., Scanlan, E., Schumann, W., Seegers, J.F., Sekiguchi, J., Sekowska, A., Séror, S.J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H.B., Vagner, V., Van Dijl, J.M., Watabe, K., Wipat, A., Yamamoto, H. and Yamamoto, M. (2003). Essential *Bacillus subtilis* genes. (2003). *Proc Natl Acad* **100**: 4678-4683.

Koh, L.P., Levang, P. and Ghazoul, J. (2009). Designer landscapes for sustainable biofuels. *Trends Ecol Evol* **24**: 431-438.

Krispin, O. and Allmansberger, R. (1998). The *Bacillus subtilis* AraE Protein Displays a Broad Substrate Specificity for Several Different Sugars. *J Bacteriol* **180**: 3250-3252.

Krüger, K.R., Gertz, S.S. and Hecker, M. **(**1996). Transcriptional analysis of *bglPH* expression in *Bacillus subtilis*: evidence for two distinct pathways mediating carbon catabolite repression. *J Bacteriol* **178**: 2637-2644.

Kudo, J., Ohkoshi, A. and Horikoshi, K. (1985). Molecular cloning and expression of a xylanase gene of alkalophilic *Aeromonas* sp. No. 212 in *Escherichia coli*. *J Gen Microbiol* **13**: 2825-2830.

Kuhad, R.C., Singh, A. and Eriksson, K.E.L. (1997). Microorganisms and enzymes involved in the degradation of plant fiber cell walls. *Adv Biochem Eng Biotechnol* **57**: 45-125.

Kumar, S. and Nussinov, R. (2001). How do thermophilic proteins deal with heat*? Cell Mol Life Sci* **58**: 1216-1233.

Kumar, D.M., Barrett, M.J. and Delwiche, P.S. (2009). Methods for Pre-treatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production Parveen. *Ind Eng Chem Res* **48**: 3713-3720.

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessières, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F. and Danchin, A. (1997)."The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*". *Nature* **390**: 249-256.

Larson, S.B., Day, J., Barba de la Rosa, A.P., Keen, N.T. and McPherson, A. (2003). First crystallographic structure of a xylanase from glycoside hydrolase family 5: implications for catalysis. *Biochemistry* **42**: 8411-8422.

Lee, J. (1997). Biological conversion of lignocellulosic biomass to ethanol. *J Biotechnol* **56**: 1-24.

Lee, R.C., Hrmova, M.A. and Burton, R.A. (2003). Bifunctional family 3 glycoside hydrolases from Barley with alpha-L-Arabinofuranosidase and beta-D-Xyylosidase activity characterisation, primary structure and COOH-terminal processing. *J Biol Chem* **278**: 5377-5388.

Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N.S., Hofrichter, M. and Rogalski, J. (1999). Biodegradation of lignin by white rot fungi. *Fungal Genet Biol* **27**: 175-185.

Leung, K.T., Maki, M. and Qin, W. (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int J Biol Sci* **5**: 500-516.

Licht, F.O. (2006). World Ethanol Market: The Outlook to 2015, Tunbridge Wells, Agra Europe Special Report, UK.

Lin, Y. and Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. *Appl Microbiol Biotechnol* **69**: 627-642.

Lindner, C., Stülke, J. and Hecker, M. (1994). Regulation of xylanolytic enzymes in *Bacillus subtilis*. *Microbiology* **140**: 753-757.

Lynd, L.R., Wyman, C.E. and Gerngross, T.U. (1999). Biocommodity engineering. *Bioetchnol prog* **15**: 777-793.

Lynd, L.R., Van Zyl, W.H., McBride, J.E. and Laser, M. (2005). Consolidated bioprocessing of cellulosic biomass: An update. *Curr Opin Biotechnol* **16**: 577-583.

Maat, J., Roza, M., Verbakel, J., Stam, H., Santos da Silva, M.J., Bosse, M., Egmond, M.R., Hagemans, M.L.D., Van Gorcom, R.F.M., Hessing, J.G.M., Van den Hondel, C.A.M.J.J. and Van Rotterdam, C. (1992). Xylanases and their applications in bakery. Visser, J., Beldman, G., Someren Kusters-Van, M.A. and Voragem, A.G.J. (eds). In: Xylans and xylanases. Elsevier, Amsterdam: 349-360.

Madigan, M.T. and Martinko, J.M. (2006). Brock Biology of Microorganisms, 11th edition. Pearson Prentice Hall, Upper Saddle River.

Marchessault, R.H. and Sundararajan, P.R. (1993). In Cellulose. In the polysaccharides. New York: Academic Press: 11-95.

Margolles-Clark, E., Tenkanen, M., Nakari-Setäla, T. and Pentilä, M. (1996). Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **62**: 3840-3846.

Math, R.K., Cho, K.M., Islam, S.M.A., Cho, J.J., Kim, J.M. and Yun, M.G. (2007). Impact of organic matter treatment on bacterial community in paddy field. Unpublished Journal. Korea: Gyeongsang National University: 660-701

Martin, C., Marcet, M. and Thomsen, A.B. (2008). Comparison between wet oxidation and steam explosion as pre-treatment methods for enzymatic hydrolysis of sugarcane bagasse. ‘‘Bagasse pre-treatment, bioethanol.’’ *Bioresource* **3**: 670-683.

Mayende, L., Brendan, S.W. and Brett, I.P. (2006). Cellulases (CMCases) and polyphenol oxidases from thermophilic *Bacillus* spp. isolated from compost. *Soil Biol Biochem* **38:** 2963-2966.

McCarthy, A.J. (1987). Lignocellulose-degrading actinomycetes. *FEMS Microbiol* **46**: 145-163.

McMillan, J.D. (1994). Pre-treatment of lignocellulosic biomass. *In Enzymatic Conversion of Biomass for Fuels Production*. *Am Chem Soc* **566**: 292-324.

Mendicuti, C., Laura, P., Trejo-Aguilar, B.A. and Aguilar, O.G. (1997). Thermostable xylanases produced at 37°C and 45°C by a thermotolerant *Aspergillus* strain. *FEMS Microbiol* **146**: 97-102.

Monisha, M.V., Uma, V. and Murthy, K. (2009). Partial purification and characterization of *bacillus pumilus* xylanase from soil source. *J Sci Eng Technol* **5**: 137-148.

Miller, L. (1959). ‘‘Use of Dintrosalicylic Acid Reagent for Determination of Reducing Sugar.” *Anal Chem* **31**: 426-428.

Mitchell, D. (2008). A note of rising food prices. Policy research working paper 4682. Development Prospects Group. The World Bank: Washington DC, USA.

Mitreva-Dautova, M., Roze, E., Overmars, H., Graäff, L., Schots, A., Helder, J., Goverse, A., Bakker, J. and Smant, G. (2006). A Symbiont-Independent Endo-1,4-β-Xylanase from the Plant-Parasitic Nematode *Meloidogyne incognita*. *Am Phytopathol Soc* **19**: 521-529.

Morris, D.D., Gibbs, M.D., Ford, M., Thomas, J. and Bergquist, P.L. (1999). Family 10 and 11 xylanase genes from *Caldicellulosiruptor* sp. strain Rt69B.1. *Extremophiles* **3**: 103-111.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. (2005). Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresour Technol* **96**: 673-686.

Moszer, I., Glaser, P. and Danchin. A. (1995). SubtiList: a relational database for the *Bacillus subtilis* genome. *Microbiology* **141:** 261-268.

Nakari-Setäla, T. and Pentilä, M. (1995). Production of *Trichoderma reesei* cellulases on glucose-containing media. *Appl Enviro Microbiol* **61**: 3650-3655.

Nolling, J., Breton, G., Omelchenko, M.V., Makarova, K.S., Zeng, Q., Gibson, R., Lee, H.M., Dubois, J., Qiu, D., Hitti, J., Wolf, Y.I., Tatusov, R.L., Sabathe, F., Doucette-Stamm, L., Soucaille, P., Daly, M.J., Bennett, G.N., Koonin, E.V. and Smith, D.R. (2001). Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J Bacteriol* **183**: 4823-4838.

Paice, M.G., Bourbonnais, R., Desrochers, M., Jurasek, L. and Yaguchi, M.A. (1986). xylanase gene from *Bacillus subtilis*: nucleotide sequence and comparison with *B. pumilus* gene. *Arch Microbiol* **144**: 201-206.

Pantazaki, A., Prista, A. and Kyriakidis, D. (2002). Biotechnologically relevant enzymes from *Thermus thermophilus*. *Appl Microbiol Biotechnol* **58**: 1-12.

Perez, J., Dorado, J.M., Rubia, T.D. and Martinez, J. (2002). Biodegradation and biological treatment of cellulose, hemicellulose and lignin: An overview. *Int Microbiol* **5**: 53-63.

Philippids, G.P. (1996). Cellulose bioconversion technology. Wyman, C.E. (ed). In: Handbook on Bioethanol: Production and utilization. Taylor and Francis, USA: 253-285.

Polizeli, M.L.T., Rizatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A. and Amorim, D.S. (2005). Xylanases from fungi: properties and industrial applications. *Appl Microbiol Biotechnol* **67**: 577-591.

Prade, R.A. (1995). Xylanases, from biology to biotechnology. *Biotechnol Genet Eng Rev* **13**: 10-15.

Prates, J.A.M., Tarbouriech, N. and Charnock, S.J. (2001). The structure of the feruloyl esterase module of xylanases 10B from *Clostridium thermocellum* provides insight into substrate recognition. *Structure* **9**: 1183-1190.

Puls, J., Schmidt, O. and Granzow, C. (1987). Glucuronidase in two microbial xylanolytic systems. *Enzyme Microb Technol* **9**: 83-88.

Rabinovich, M.L., Melnik, M.S. and Bolobova, A.V. (2002a). Microbial cellulases: A review. *Appl Biochem Microbiol* **38**: 305-321.

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J. and Liotta, C.L. (2006). The path forward for biofuels and biomaterials. *Science* **311**: 484-489.

Rogner, H.H. (2000). World Energy Assessment; energy and the challenge of sustainability. United Nations Development Programme UNDP: 135-171.

Romanec, M.P.M., Davidson, K., White, B.A. and Hazlewood, G.P. (1989). Cloning of *Ruminocouccus albus* endo-L-1,4 glucanase and xylanase genes. *Appl Microbiol* **9**: 101-104.

Saboto, D., Nucci, R., Rossi, M., Gryczyniski, Z. and Lakowics, J. (1999). The β-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: enzyme activity and conformational dynamics at temperatures above 100°C. *Biophys Chem* **81**: 23-31.

Saha, B.C. (2000). α-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. *Biotechnol Adv* **18**: 403-423.

Saha, B.C. (2003). Hemicellulose bioconversion. *J Ind Microbiol Biotechnol* **30**: 279-291.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406-425.

Saleem, M., Akhtar, M.S. and Jamil, S. (2002). Production of Xylanase on Natural Substrates by *Bacillus subtilis*. *Int J Agr Biol* **2**: 211-213.

Sakamoto, T., Yamada, M., Kawasaki, H. and Sakai, T. (1997). Molecular cloning and nucleotide sequence of an endo-1,5-alpha-L-arabinase gene from *Bacillus subtilis*. *Eur J Biochem* **245**:708-714.

Sánchez, O.J. and Cardona, C.A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour Technol* **99**: 5270-5295.

Shallom, D., Belakhov, V., Solomon, D., Gilead-Gropper, S., Baasov, T., Shoham, G. and Shoham, Y. (2002). Identification of the acid-base catalyst of α-L-arabinofuranosidase from *Geobacillus stearothermophilus* T-6, a family 51 glycoside hydrolase. *FEBS Letters* **514**:163-167.

Shallom, D. and Shoham, Y. (2003). Microbial hemicellulases. *Curr Opin Microbiol* **6**: 219-228.

Simpson, H.D., Haufler, U.R. and Daniel, R.M. (1991). An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*. *Biochem J* **277**: 413-417.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J. and Templeton, D. (2008). Determination of Sugars, By products, and Degradation Products in Liquid Fraction Process Samples. [Online]. Available http://www.nrel.gov/biomass/pdfs/42623

Sonenshein, A.L. (2005). CodY, a global regulatory of stationary phase and virulence in Gram-positive bacteria. *Curr Opin Microbiol* **8**: 203-207.

Steinmetz, M. **(**1993). Carbohydrate catabolism: pathways, enzymes, genetic regulation, and evolution. In "*Bacillus subtilis"* and other Gram-positive bacteria. *Am Soc Microbiol*: 157-170.

Stetter, K.O. (1996). Hyperthermophiles in the history of life. *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)* **202**: 1-18.

Stülke, J., and Hillen, W. (2000). Regulation of carbon catabolism in *Bacillus* species. *Lehrstuhl für Mikrobiologie, Institut für Mikrobiologie, Biochemie und Genetik der Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany*.

Subramaniyan, S. and Prema, P. (2002). Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Crit Rev Biotechnol* **22**: 33-64.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic material from ethanol production: A review. *Biores Technol* **83**: 1-11.

Sunna, A. and Antranikian, G. (1997). Xylanolytic enzymes from fungi and bacteria. *Crit Rev Biotechnol* **17**: 39-67.

Taherzadeh, M.J. (1999). Ethanol from lignocellulose: Physiological effects of inhibitors and fermentation strategies. *J Biotechnol* **56**: 1-24.

Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogasawara, N., Kuhara, S. and Horikoshi, K. (2000). Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Res* **28**: 4317-4331.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007)**.** MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596-1599.

Taylor, L.E., Henrissat, B., Coutinho, P.M., Ekborg, N.A., Hutcheson, S.W. and Weiner, R.M. (2006). complete cellulase system in the marine bacterium *Saccharophagus degradans* strain 2-40T. *J Bacteriol* **188**: 3849-3861.

Taylor, M.P. (2007). Metabolic engineering of *Geobacillus* species for enhanced ethanol production. Unpublished doctoral dissertation. UK: University of London

Thomas, K.N.G., Arie, B.B. and Zeikus, J.G. (1981). Ethanol production by thermophilic Bacteria: fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. *Appl Environ Microbiol* **41:** 1337-1343.

Thurston, C.F. (1994). The structure and function of fungal laccases. *Microbiology* **140**:19-26.

Tuomela, M., Hatakka, A., Raiskila, S., Vikman, M. and Itävara, M. (2001). Biodegradation of radiolabelled synthetic lignin (14C-DHP) and mechanical pulp in compost environment. *Appl Microbiol Biotechnol* **55:** 492-499.

Van den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Curr Opin Microbiol* **6**: 213-218.

Voget, S., Steele, H.L. and Streit, W.R. (2006). Characterization of a metagenome-derived halotolerant cellulase. *J Biotechnol* **126**: 26-36.

Von Sivers, M., Zacchi, G., Olsson, L. and Hahn-Härgerdal, B. (1994). Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. *Bioethannol Progr* **10**: 555-560.

Von Sivers, M. and Zacchi, G. (1996). Ethanol from Lignocellulosics; a review of the economy. *Bioresour Technol* **56**: 131-140.

Wang, Y., Zhang, Z. and Ruan, J. (1996). A proposal to transfer *Microbispora bispora* (Lechevalier 1965) to a new genus, *Thermobispora* gen. nov., as *Thermobispora bisporacomb.* nov. *Int J Sys Bacteriol* **46**: 933-938.

Weber, S., Stubner, S. and Conrad, R. (2001). Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. *Appl Environ Microbiol* **67**: 1318-1327.

Wehmeier, U.F. (1995). New multifunctional *Escherichia coli–Streptomyces* shuttle vectors allowing blue-white screening on X-gal plates. *Gene* **165**: 149-150.

Whistler, R.L. and Richards, E.L. (1970). Hemicelluloses. In: Pigman, W., Horton, D. (eds). The carbohydrates. Academic Press, New York: 447-469.

Wojtczak, G., Breuil, C., Yamuda, J. and Saddler, J.N. (1987). A comparision of the thermostability of cellulose from various thermophilic fungi. *Appl Miocrobiol Biotechnol* **27**: 82-87.

Won, K. and Hyun, J.L. (1983). Purification & characterization of alphagalactosidase from *E. coli*. *Korean Biochem J* **16**: 1-12.

Wood, T.M. (1991). Fungal cellulases. Haigler, L. (ed). In: Biosynthesis and Biodegradation of cellulose. New York: 491-534.

Wilson, D.B. (2008). Three microbial strategies for plant cell wall degradation. *Ann N Y Acad Sci* **1125**: 289-297.

Wyman, C.E. (1994*).* Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. *Bioresour Technol* **50**: 3-16.

Wyman, C.E. (1996). In *Handbook on Bioethanol: Production and Optimization*, Wyman, C. E. (ed). Taylor & Francis, Washington DC: 1-18.

Wyman, C.E., Dale, B.E., Elander, R.T., Holizapple, M., Ladish, M.R. and Lee, Y.Y. (2005b). Comparative sugar recovery data from laboratory scale application of leading pre-treatment technologies to corn stover. *Bioresour Technol* **96**: 2026-2032.

Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper, L.V. and Gordon, J.I. (2003). A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* **299**: 2074-2076.

Yi, J.C., Sandra, J.C. John, A.B. and Shuting, C. (1999). Production and distribution of endoglucanase, cellobiohydrolase and β-glucosidase components of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom. *Applied Environ Microbiol* **65**: 553-559.

Yi, X., Xie, Z.J., Deng, A.H., Wang, N. and Erkin, R. (2006). Isolation and identification of two xylanase-producing extremely alkali-tolerant strains of *Bacillus halodurans* from Turpan in China. *Acta Microbiol Sin* **46**: 951-955.

Yu, Z.S. and Zhang, H.X. (2004). Ethanol fermentation of acid hydrolysed cellulosic pyrolysate with *Saccharomyces cerevisiae*. *Biores Technol* **93**: 199-204

Zhang, Y.H.P., Himmel, M.E. and Mielenz, J.R. (2006b). Outlook for cellulase improvement: screening and selection strategies. *Biotechnol Adv* **24**: 452-481.

Zhang, Y.H.P. and Lynd, L.R. (2006). A functionally based model for hydrolysis of cellulose by fungal cellulase. *Biotechnol Bioeng* **94**: 888-898.

Zhou, S. and Ingram, L.O. (2000). Synergistic Hydrolysis of Carboxymethyl Cellulose and Acid-Swollen Cellulose by Two Endoglucanases (CelZ and CelY) from *Erwinia chrysanthemi*. *Am Soc Microbiol* **182**: 5676-5682.