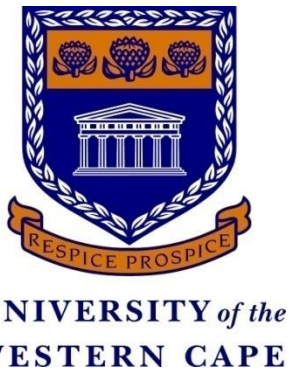

ISOLATION, EXPRESSION, PURIFICATION AND CHARACTERISATION OF A NOVEL ACETYL XYLAN ESTERASE FROM *STREPTOMYCES* SPECIES ORS10

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

I, Yu Gao, hereby declare that "**Isolation, expression, purification and characterisation of a novel acetyl xylan esterase from *Streptomyces* species ORS10**" is my own original work and that I have accurately reported and acknowledged all sources, and that this document has not previously, in its entirety or in part been submitted at any university for the purpose of obtaining an academic qualification.

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ABSTRACT

Lignocellulosic biomass represents an important renewable resource for biofuels production. Lignocellulosic biomass is comprised of cellulose, hemicellulose and lignin. Lignocellulosics are highly recalcitrant to enzymatic degradation and due to its complex nature a range of enzymes are required to synergistically hydrolyse biomass. Many microorganisms are capable of producing these enzymes as part of their hemicellulolytic hydrolysis system(s). The aim of this study was the characterisation of a thermophilic actinobacterial isolate (ORS10), capable of producing hemicellulosic enzymes, and the cloning and characterization of a hemicellulosic enzyme produced by the isolate. Phylogenetic analyses clustered ORS10 with species of the genus *Streptomyces.* BLAST analysis revealed that ORS10 was most closely related to *Streptomyces achromogenes* (99% identity). A small-insert genomic library was constructed and a putative acetylxylan esterase (AXEase) gene, *axe10*, was identified. The enzyme, Axe10, has moderate similarity to α/β hydrolase proteins, and contains an esterase/lipase superfamily conserved domain and a typical AXEase catalytic triad. The *axe*10 gene was sub-cloned into an expression vector [pET21a(+)] and a 28.7 kDa protein with demonstrated AXE activity was purified from *E. coli* Rosetta (DE3) pLysS. Axe10 displayed optimum activity at 37^{o}C and pH 7.0. Despite being derived from a thermophilic *Streptomyces* species Axe10 was not thermostable. However, given the relative novelty of Axe10, further characterisation and assessment of this enzyme is warranted.

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This thesis is dedicated to my lovely wife, my soulmate,

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for her continuous support and love,

and my parents for the encouragement from

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谨以此论文感谢在我身边永远支持我的学业和事业， 与

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Chapter 1

General Introduction and Project Aims

Globally, fossil fuels continue to be the major energy source (Licht, 2008). The relative contributions to global energy consumption were estimated to be 40, 28 and 20% for crude oil, coal and natural gas, respectively (Antoni *et al.,* 2007). A rapid depletion of the world's limited fossil fuel reserves has prompted the search for alternative and renewable energy sources including biofuels, wind, solar and water energy. Liquid biofuels, including bioethanol and biodiesel, are considered to be renewable, and offer alternatives to liquid fossil fuels (Taylor, 2008). In particular, bioethanol has attracted attention in recent years as a renewable transportation fuel, due to its application as a petrol additive. The use of bioethanol provides several advantages over the use of fossil fuels, including lower cost of production and enhanced petrol combustion efficiencies.

Concerns relating to the environment and food security, particularly within the developing world, have driven the development of 2nd generation biofuel production based upon the use of lignocellulosic biomass as a sustainable and renewable feedstock. Lignocellulosic biomass is primarily composed of cellulose, hemicellulose and lignin (Kumar *et al.,* 2008). Due to the complex and recalcitrant nature of lignocellulosic materials, their efficient deconstruction requires the synergistic action of a number of enzymes. Acetyl xylan esterases (AXEases) are members of the hemicellulosic degrading enzymes. They function in synergy with enzymes such as β-1, 4-xylanase and β-xylosidase to hydrolyse hemicellulose polymers, which are mainly composed of xylan, to

fermentable sugar monomers such as xylose and arabinose. A number of AXEases have been identified from various bacteria (Shao and Wiegel, 1995; Degrassi *et al.,* 1998) and fungi (Halgasova *et al.,* 1994; Egana *et al.,* 1996). However, relatively few examples of AXEases isolated from *Streptomyces* species have been described (Dupont *et al.,* 1996) and as actinomycetes are ubiquitous soil microorganisms and play important roles in carbon cycling, it is worth attempting to identify and characterise lignocellulosic enzymes from actinobacterial isolates.

The present study reports the preliminary characterisation of a *Streptomyces* isolate, ORS10, with hemicellulase activity. The study aimed at cloning a key hemicellulosic accessory enzyme, acetyl xylan esterase, for potential use in the enzymatic saccharification of lignocellulosic biomass.

The specific objectives of this study included:

- Screening an actinobacterial isolate (ORS10) for the production of hemicellulosic enzymes;
- Construction of a small-insert genomic library in *E. coli*;
- Functional screening of the genomic library for the identification of clone(s) expressing hemicellulase(s);
- Cloning and expression of the gene coding for an acetyl xylan esterase in *E. coli*;

- Purification and characterisation of the cloned gene product.

Chapter 2

Enzymatic saccharification of hemicellulose for biofuel production

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2.1 Biofuels as alternative energy

South Africa, like many other developing countries, is strongly reliant on oil imports to supplement its energy demand (EIA report, 2010). The establishment of a sustainable South African biofuels industry is a feasible alternative to reducing the countries long-term dependency on fossil fuel imports. However, issues surrounding food security need to be addresses and the use of non-food based feedstocks for bioethanol production may help to address and resolve concerns. The South African national biofuels strategy (2007), states that the development of biofuel programs has the potential to bring agricultural and economical benefits to sub-Saharan Africa and other developing regions. These benefits include attracting investment into rural areas, promoting agricultural development, decreasing the reliance on oil imports and overcoming the trade distorting effects of South Africa from the world.

2.1.1 First generation biofuels

Conventional industrial production of ethanol has been mainly based on the fermentation of food crops, such as sugar cane, corn and starch, by commercial yeast strains (e.g. *Saccharomyces cerevisae* and *Schizosaccharomyces pombe*) (Sánchez and Cardona, 2008). This process of converting food-based feedstocks to biofuels is known as 1st generation biofuel production. Approximately 79% of bioethanol produced in Brazil is the result of the fermentation of fresh sugar cane juice, while the remaining bioethanol is

produced from molasses feedstocks (Wilkie *et al.,* 2000). Similarly in the USA, bioethanol is produced almost exclusively from corn, which is converted to starch and in turn this is used as the primary carbon source for microbial ethanol fermentations (Pimentel and Patzek, 2005). In France bioethanol is mainly produced from surplus wheat (Sánchez and Cardona, 2008). The over-use of arable land to cultivate these crops for fuel may have a negative impaction food security (FAO report, 2011) and may contribute to a rise in food commodity prices, especially in developing countries (Fig. 2.1). For example, the USA has increased the use of corn for bioethanol production from 6% in 2006 to 23% in 2008 of the total production (Rahman *et al.,* 2008).

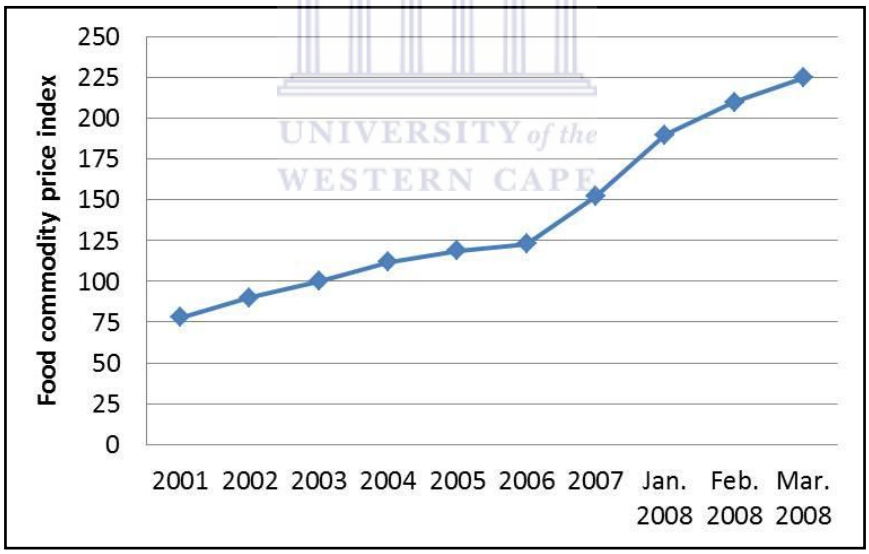

Figure 2.1 Global food commodity price indices (adapted from FAO report). It summarises the average of five commodity group price indices (representing 55 quotations), weighted with the average export shares of each of the groups.

2.1.2 Second generation biofuels

Second generation biofuels are based on non-food grade feedstocks,

such as lignocellulosic plant biomass (Hamelinck *et al.,* 2005). Plant biomass can be considered a renewable resource as it is produced in massive quantities world-wide as a result of normal agricultural practices. Examples of agricultural biomass include sugarcane baggase, wheat stalks and forestry waste. Utilization of these materials for biofuels production would not only generate energy, but may help to resolve the environmental issues associated with their disposal (Demain *et al.,* 2005; Kumar *et al.,* 2008).

2.2 Conversion of lignocellulosic materials

Lignocellulosic biomass is composed of a mixture of carbohydrate biopolymers comprising cellulose and hemicelluloses bound together by covalent hydrogen bonds with lignin (Fig. 2.2) (Kumar *et al.,* 2008). The relative composition of these three major components within lignocellulosic materials varies depending on the source of the plant biomass (Table 2.1).

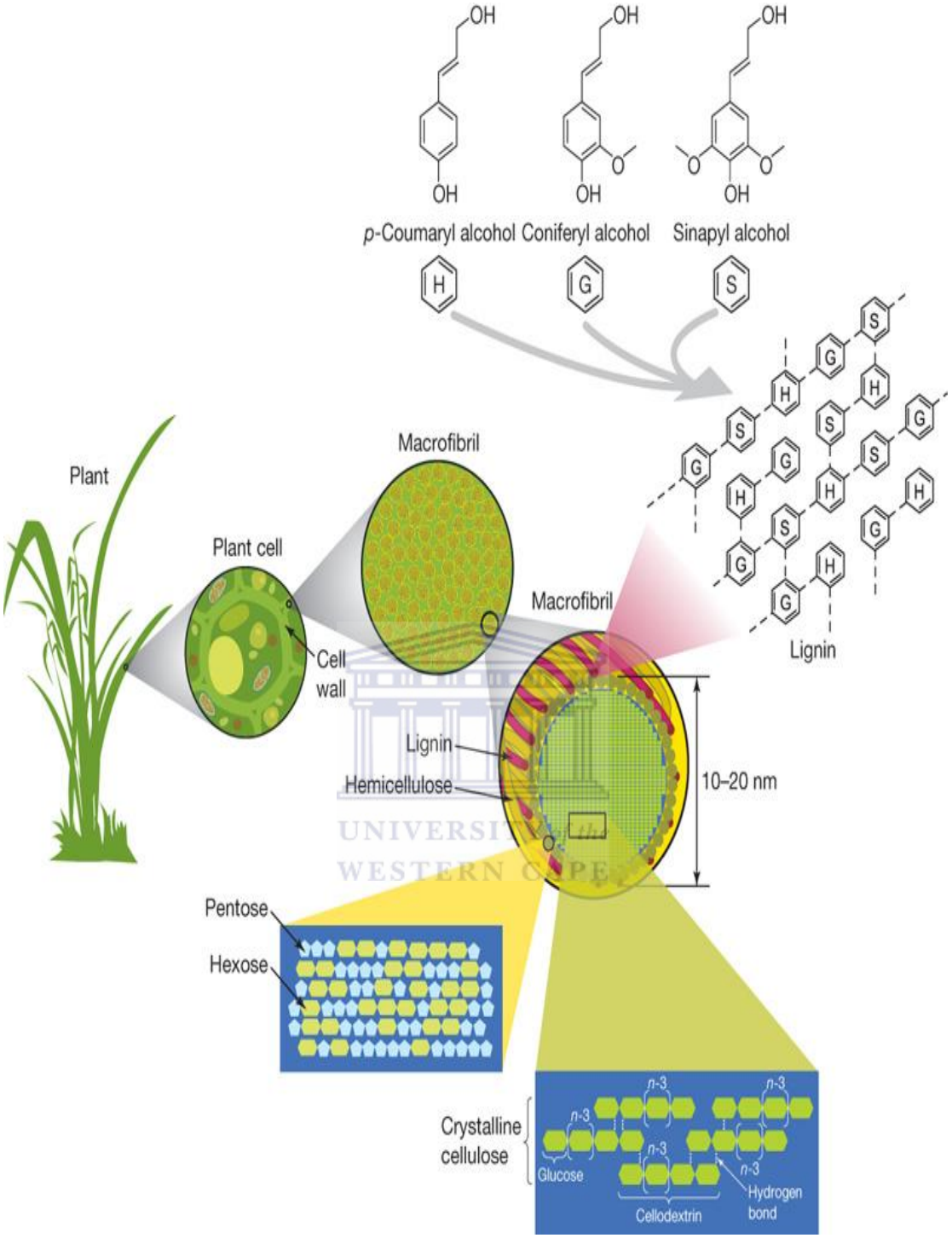

Figure 2.2 Schematic diagram illustrating the composition and interactions between the three main components, namely cellulose, hemicellulose and lignin, of lignocellulosic plant biomass. The lignin portion encases the cellulose microfibrils bound together with hemicellulose polymers (Rubin, 2008).

Due to its complex polymeric nature, the conversion of lignocellulosic biomass to fermentable sugars, and finally to bioethanol, requires several distinct steps. Typically lignocellulosic biomass is initially pre-treated before being subjected to enzymatic hydrolysis to release fermentable sugars, which are finally fermented to ethanol by an appropriate microbial system (Kumar *et al.,* 2008).

Table 2.1 Composition of cellulose, hemicellulose and lignin in common agricultural residues and wastes (adapted from Sun and Cheng, 2002).

Lignocellulosic materials	Percentage composition (%)		
	Cellulose	**Hemicellulose**	**Lignin**
Hardwood stems	40-55	34-40	18-25
Softwood stems	45-50	25-35	25-35
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Corn fibre *	15	35	8
Corn stover	40	25	17
Sugarcane bagasse	40	24	25
Switch grass	45	30	12
Wheat straw	30	50	20

***** - Corn fibre contains an additional 20% starch.

2.2.1 Pre-treatment of lignocellulosic biomass

Several pre-treatment steps need to be performed on lignocellulosic biomass to decrease its crystallinity and increase cellulose solubility (Sánchez and Cardona, 2008). These pre-treatment steps include physical (chipping, grinding, milling and/or steam explosion) and/or chemical methods (acid or

alkaline treatment) (Figure 2.3). Most industrial lignocellulosic pre-treatment processes combine more than one pre-treatment step, such as steam explosion and alkaline pre-treatment (Sun and Cheng, 2002).

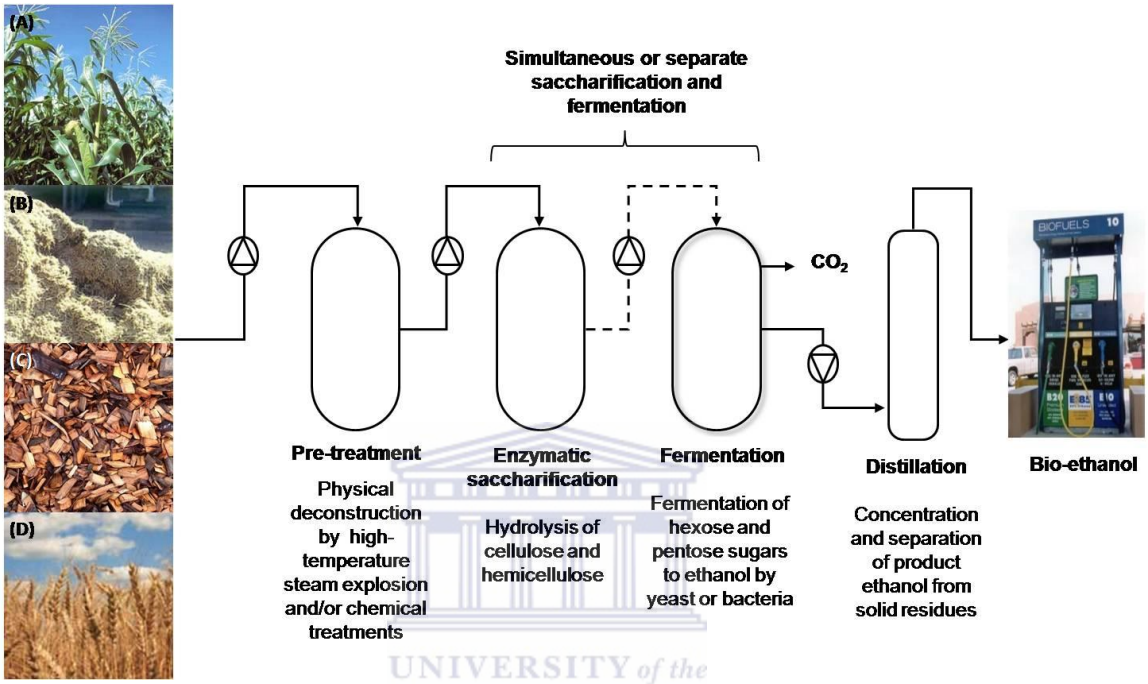

Figure 2.3 Graphical representation of a typical process converting lignocellulosic biomass [(A) maize husks, (B) sugar cane bagasse, (C) wood chips, and (D) wheat husks] to bioethanol (adapted from Viikari, 2007).

Within industrial processes, the choice of pre-treatment method(s) affects the cost and performance of downstream hydrolysis, fermentation and purification steps (Kumar *et al.*, 2008). Ideally, pre-treatment of lignocellulosic biomass should result in high yields of digestible cellulose and hemicellulose, while avoiding the loss of fermentable reducing sugars and the formation of compounds inhibiting fermentation (Sun and Cheng, 2002).

2.2.2 Enzymatic saccharification of lignocellulosic biomass

Enzyme saccharification usually follows pre-treatment of biomass (Fig. 2.3). The complex structure of lignocellulosic biomass is highly recalcitrant to enzymatic hydrolysis. Therefore, the efficient enzymatic saccharification of biomass requires the synergistic action of a number of enzymes (Table 2.2) to efficiently degrade the substrate. Three main classes of enzymes are required for efficient hydrolysis of lignocellulosic biomass and are broadly classified as cellulases, hemicellulases and ligninases (Kumar *et al.*, 2008).

Table 2.2 The main enzymes involved in lignocellulosic degradation.

Enzymes		EC number
Cellulases	β-glucosidases	EC 3.2.1.21
	Exoglucanases	EC 3.2.1.91
	Endoglucanases	EC 3.2.1.4
Hemicellulases	Endo-β-1,4-xylanases	EC 3.2.1.8
	β-xylosidases	EC 3.2.1.37
	Acetyl xylan esterases	EC 3.1.1.72
	Ferulic acid esterases	EC 3.1.1.73

The production costs of bioethanol from non-food based materials are higher than the production from food-based feedstocks, such as starch (Demain, 2005). However, the development of lignocellulosic bioethanol refineries could provide several additional benefits, including creation of jobs and the production of high value bi-products such as acetic acid and lactic

acid (Gray *et al.,* 2006). The identification and characterisation of novel lignocellulosic enzymes suitable for inclusion in industrial process may help to improve 2nd generation biofuels production processes

2.2.2.1 Cellulose and cellulolytic enzymes

Cellulose, the most abundant organic biopolymer in nature, is the primary structural polysaccharide of the plant cell wall (Fig. 2.2) (O'Sullivan, 1997). This linear polymer is composed of glucose monomers linked by β-1, 4-glycosidic bonds with a polymerization grade of up to 15,000 glucose units (Kumar *et al.,* 2008). Naturally occurring cellulose compounds are structurally heterogeneous, having both amorphous and highly ordered crystalline portions. They are completely insoluble in water (O'Sullivan, 1997). Numerous cellulases have been isolated and characterised (Feng *et al*., 2007; Wang *et al.,* 2008). Recent significant advances have been in the development of fungal cellulases (Kumar *et al.,* 2008).

Endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) are the main cellulolytic enzymes involved in the hydrolysis of the cellulose polymer to glucose monosaccharide units (Fig. 2.4) (Sun and Cheng, 2002). Complete degradation of cellulose requires the synergistic action of all three cellulolytic enzymes. Most mesophilic fungi secrete the different cellulases classes in varying concentration levels, which negatively impacts on the efficiency of crystalline cellulose degradation. For

example, *Aspergillus* sp. produces high levels of β-glucosidases and low levels of endoglucanase (Singh *et al.*, 1990). In comparison bacteria may harbor a controlled system, called the cellulosome, which is a multi-domain or enzyme complex (Carvalho *et al.,* 2003; Adams *et al.,* 2010). The concept of a cellulosome may be exploited to adjust the relative production levels of cellulolytic enzymes to feedstock composition (Schwarz, 2001).

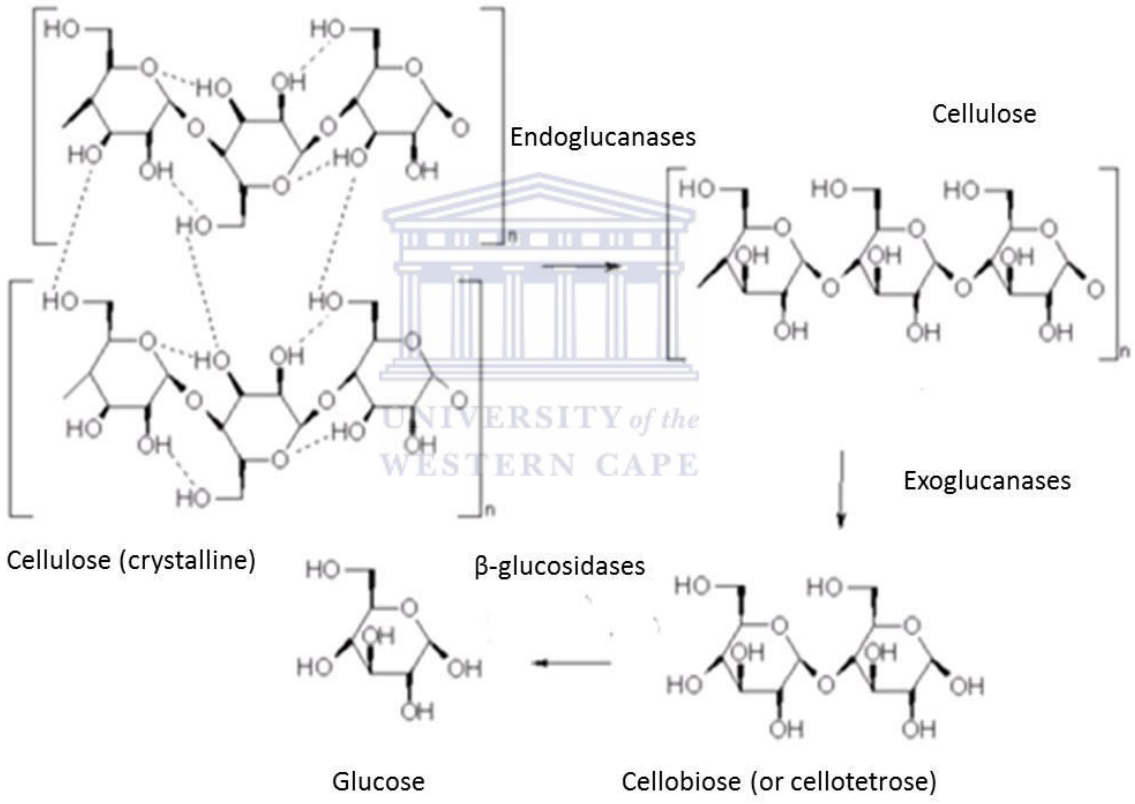

Figure 2.4 The enzymatic hydrolysis of cellulose. Endoglucanases de-crystallized crystalline cellulose to single chain cellulose polymers. These are further hydrolysed by the action of exoglucanases. Finally, β-glucosidases hydrolyse cellobiose to single glucose monosaccharide units (adapted from Kumar *et al.,* 2008).

2.2.2.2 Hemicellulose and hemicellulolytic enzymes

Hemicellulose is the second most abundant biopolymer on earth (Puls, 1997), representing approximately 25-35% of total lignocellulosic biomass (Kumar *et al.*, 2008). Unlike cellulose, which only consists of glucose monomers, hemicellulose has a far more complex structure (Ren *et al.,* 2009). It is primarily composed of polymeric pentose sugar monosaccharides (D-xylose, L-arabinose and D-galactose) (Puls, 1997) and a number of ester-linked side-chain groups (Vries *et al.,* 2000). These side-chain groups enable the hemicellulose polymers to interact and bind with each other, and in turn these hemicellulose polymers bind to the cellulose microfibrils. Aside from releasing hexose and pentose sugar monomers, complete degradation of hemicellulose could also release the hemicellulose-bound cellulose microfibrils and thereby may enhance the accessibility of cellulolytic enzymes to the cellulose polymers (Sunna and Antranikian, 1997).

Xylan is the most abundant component of hemicellulose (Subramaniyan and Prema, 2002). Similar to other polysaccharides of plant origin, xylan is present in a variety of plant species and is distributed throughout several types of tissues and cells (Puls, 1997). Complete hydrolysis of xylan to its substituent groups requires the synergistic action of a number of hemicellulolytic enzymes (Coughlan and Hazlewood, 1993). The most important enzymes involved in hemicellulose hydrolysis are endo-β-1, 4-xylanases (EC 3.2.1.8), which hydrolyse the xylan backbone to shorter di-saccharide chains (Fig. 2.4).

Thereafter, the xylobiose di-saccharides are further degraded to xylose monosaccharides by the action of β-xylosidases (EC 3.2.1.37) (Kulkarni *et al.,* 1999).

Depending on the extent and type of accessory side-chain groups present on the xylan backbone, a number of additional enzymes, known as accessory enzymes, may be required for the efficient and complete hydrolysis of hemicellulose (Vries *et al.,* 2000). The participation of accessory enzymes is a strategy to achieve effective xylan hydrolysis and increase enzyme synergy (Subramaniyan and Prema, 2002). It has been shown that acetylxylan esterases (AXEases) could enhance the release of acetic acid from acetylated xylan chains (Biely *et al.*, 1985), thereby increasing the accessibility of the xylan backbone to endo-xylanase attack (Puls, 1997). The action of endoxylanases may furthermore result in shorter acetylated xylan chains, which are the preferred substrate for AXEase activity (Biely *et al*., 1986). Therefore, the identification and characterisation of novel accessory enzymes is important for hemicellulosic degradation studies. Depending on the different substitute side chains on the xylan backbone a number of different accessory enzymes will be required (Beg *et al.*, 2001). For example, hardwood hemicellulose contains mainly acetic acid esterified xylose units. AXEase (EC 3.1.1.72) is the key enzyme involved in the hydrolysis of these side-chains, and its presence is required for the complete digestion of hardwood hemicelluloses (Subramaniyan and Prema, 2002). In grasses, arabinfuranoses

esterifed with p-coumaric and ferulic acid require the participation of ferulic acid esterase (FAEase) (EC 3.1.1.73) and α-L-arabinofuranosidases (EC 3.2.1.55) (Fig. 2.5).

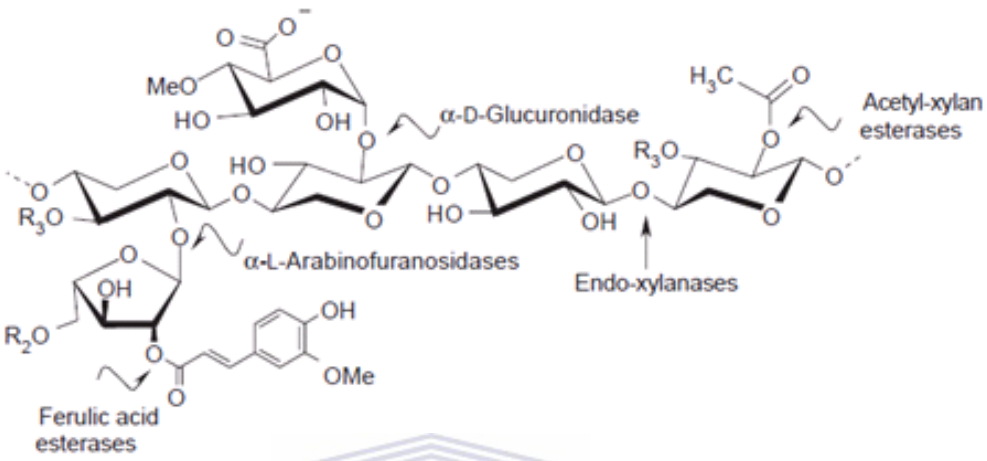

Figure 2.5 The complete hydrolysis of hemicellulose requires the synergetic action of several enzymes, including endo-xylanase and accessory enzymes such as acetyl xylan and ferulic acid esterases (Krastanova *et al.*, 2005).

Microorganisms may possess specific xylanolytic enzyme systems, for the hydrolysis of different xylan feedstocks, known as xylanosome (Sunna and Antranikian, 1997). Xylanosome are multi-enzyme complexes, comprising several cellulases and xylanases, found on the microbial cell surface (Beg *et al.*, 2001). These xylanosomes are multifunctional, enabling the breakdown different xylan sources (Sunna and Antranikian, 1997). *Butyvibrio fibrisolvens* H17c has an extracellular xylanosome complex shown to display both xylanase and endoglucanase activities (Lin and Thomson, 1991).

2.2.2.3 Lignin and ligninases

Lignin is composed of highly branched, aromatic polymers substituted with several side chains and provides structural rigidly and water impermeability to plant cell walls (Kumar *et al.,* 2008). Chemical changes in lignin degradation include a reduction in the aromaticity of the polymer and an increase in the number of carboxyl, carbonyl and phenolic hydroxyl groups in the modified lignin. There is also a significant amount of aromatic ring demethylation and cleavage of aromatic acid-lignin ester linkages (Borgmeyer and Crawford, 1985). Lignolytic enzymes, also known as lignin-modifying enzymes, include peroxidases (lignin, manganese and versatile peroxidases) and phenol-oxidases of the laccase type (Kumar *et al.*, 2008). The main producers of these enzymes are generally fungi such *Pleurotus eryngii* (Caramelo, 1999), and some filamentous bacteria, including *Streptomyces viridosporus* and *Clostridium stercorarium* (Ren *et al.,* 2009).

2.2.2.4 Esterases

Esterases belong to a diverse group of enzymes catalysing the cleavage of ester bonds to release acidic and/or alcoholic groups (Park *et al.*, 2007). These enzymes are widely used in industrial processes, including lignocellulosic biomass degradation to remove the ester bound side-chain groups from hemicellulose (Vries *et al.,* 2000). Examples of esterases involved in hemicellulose degradation are AXEase and FAEase, which hydrolyse the acetylxylan and ferulic acid side chain groups, respectively (Puls, 1997).

Advantages of these enzymes for use in biomass pre-treatment include high specific activity at neutral pH and no known co-factor requirements (Park *et al.*, 2007).

2.3 Acetyl xylan esterases

AXEases are key accessory enzymes required for the complete hydrolysis of xylan; particularly hardwood derived lignocellulosic biomass (Krastanova *et al.*, 2005). AXEases are widely distributed amongst seven carbohydrate esterase (CE) sub-families (Coutinho and Henrissat, 1999). The different families vary in terms of substrate specificity and molecular mechanisms. For example, CE1 AXEases initially de-acetylate positions 2 and 3 on xylose, while AXEase CE4 sub-family members perform a rapid double de-acetylation at both positions (Kafetzopoulos *et al.*, 1993). CE7 AXEases display activity toward broad acetylated substrates including p-nitrophenyl acetate, α-naphthyl acetate, 7-aminocephalosporanic acid, cephalosporin-C, xylose tetra acetate and glucose penta acetate (Coutinbo and Henrissat, 1999).

2.3.1 Microbial AXEases

AXEases have been identified and characterised from several groups of microorganisms, including fungi and bacteria. Fungal examples include *Penicillium purpurogenum* (Egana *et al.,* 1996), *Schizophyllum commune* (Halgasova *et al.*, 1994) and *Asperigillus awamori* (Kormelink *et al.,* 1993). Several AXEases have been identified from *Bacillus pumilus* and a

Thermoanarobacterium sp. (Shao and Wiegel, 1995; Degrassi *et al.,* 1998). However, very few AXEases have been studied from actinobacterial isolates, with the exception of one from *Streptomyces lividans* (Dupont *et al.*, 1996). Considering the important role that Actinobateria play in carbon cycling and hemicellulosic degradation, there are likely to be numerous AXEases produced by Actinobacteria that to date have not been identified.

Members of the genus *Streptomyces* are characterised as being Gram-positive with a high G+C content chromosomal DNA (Hopwood, 2006). Their cells have branched hyphae, and are well adapted to the penetration and degradation of lignocellulosic biomass (McCarthy, 1987). *Streptomyces* isolates are well characterised in terms of their ability to produce and secrete extracellular lignocellulosic degrading enzymes (Wachinger *et al.,* 1989; Lumba and Pennickx, 1992; Elegir *et al.,* 1994; Kosugi *et al.,* 2002; Jang and Chen, 2003). Hiroshi *et al.* (1992) identified two thermostable endo-xylanases produced by *Streptomyces thermoviolaceus*, while Nascimento (2002) partially characterised a xylanase produced by *Streptomyces* sp. strain AMT-3. Fujimoto *et al.* (2004) determined the crystal structure of a glycoside hydrolase (GH) family 10 xylanase produced by *Streptomyces olivaceoviridis* E-86. As these microorganisms require a suite of lignocellulosic enzymes for the conversion of lignocelluloses, there is a high possibility that they would also produce accessory lignocellulosic enzymes, such as AXEases.

2.3.2 Synergistic action of AXEases with xylanases

There are several studies focusing on the synergistic action of AXEases with other lignocellulosic degrading enzymes, include xylanases. One such study by Dupont *et al.* (1996) investigated AxeA together with the xylanases XlnA and XlnB. The acetylated xylan was fully degraded by XlnA or XlnB only when pre-treated with AxeA. Another AXEase isolated from a thermophilic actinomycete, *Thermobifida fusca*, liberates acetic acid from oat-spelt if combined with xylanases (Yang and Liu, 2008). AXEase also acts synergistically with xylanase against birchwood xylan in the *Aspergillus oryzae* system (Koseki *et al.,* 2006). Production of acetic acid was estimated to be 15-fold greater when rAoAXE (recombinant *Aspergillus oryzae* acetylxylan esterase) acted together with xylanase AgXYN.

2.4 Thermophilic microorganisms and thermostable enzymes

To meet the requirements of thermophilic industrial processes, novel and high efficiency thermostable biocatalysis need to be discovered and their suitability for inclusion in the processes assessed. Extremophilic microorganisms such as thermopiles represent an excellent microbial resource to be screened for thermostable enzymes (Van den Berg, 2003). Thermophilic microorganisms have an optimum growth temperature of 45-80^{o}C (Stetter, 1996). However, thermophiles can be divided into three groups on the basis of their optimal growth temperature, namely (1) thermotolerant microorganisms, which are characterized as having a similar optimum growth temperature as

mesophilic microorganisms, but can survive a maximum growth temperature of approximately 50^{o}C; (2) thermophiles have an optimum temperature of 50-70^{o}C and; (3) extreme thermophiles, which have an optimum growth temperature above 70^{o}C (Wiegel, 1998).

Extremophilic microorganisms, including thermophiles, are important sources of enzymes and metabolites for a wide variety of biotechnological applications (Van den Berg, 2003). High-temperature industrial lignocellulosic hydrolysis has numerous advantages over mesophilic processes (Turner *et al.,* 2007). The most important of these are that under thermophilic conditions the solubility of polymeric substrates is significantly enhanced, which increases the enzyme(s) penetration and substrate accessibility. In addition, the choice and use of thermostable enzymes in industrial processes also contributes a number of advantages. These include extended storage at mesophilic temperatures, enhanced tolerance to organic solvents, reduced risk of microbial and phage contamination, and minimal loss of activity during processing (Turner *et al.,* 2007).

2.5 Ethanol production following enzymatic saccharification

Following the pre-treatment and enzymatic saccharification of lignocellulosic feedstocks, more than one type of simple sugar is released including hexose sugars, such as glucose and sucrose, and pentose sugars, such as xylose and arabinose (Kumar *et al.,* 2008). The release of different

sugar monomers represents a significant challenge for standard microbial fermentations, as generally pentose sugars cannot be fermented by the same microorganisms that are capable of fermenting hexose sugars (Verho *et al.,* 2003). Industrial microbial fermentation strains include *Sacchromyces cerevisiae* and *Zymomonas mobilis* (Hahn-Hägerdahl *et al.*, 2007). Although microorganisms with the ability to ferment xylose have been identified, their slow fermentation rates and low ethanol tolerances mean that they are not suitable for industrial bioethanol production without genetic and metabolic manipulations (Du Preez, 1994).

A long-term goal in the optimization of bioethanol production from lignocellulosic feedstocks is the generation of a consolidated bioprocess (CBP) (Lynd *et al.*, 2005). The purpose of CBP is to generate an engineered microbial strain capable of producing all the necessary enzymes for digestion and fermentation of lignocellulosic biomass for bioethanol production. Therefore, a CBP aims to achieve the efficient simultaneous hydrolysis of the feedstock and fermentation of simple sugars released during the enzymatic saccharification to ethanol (Lynd *et al.,* 2002)

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Chapter 3

Materials and Methods

3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used during the course of this study are listed in Tables 3.1 and 3.2, respectively.

Table 3.1 Strains used in this study.

Strains	Genotype/Relevant features [a]	Reference
ORS10	Isolated from Omaruru River sand, Namibia.	Dr. Le Roes-Hill, CPUT, SA
E. coli Genehog	F- mcrA ∆(mrr-hsdRMS-mcrBC) Φ80lacZM15 ∆lacX74recA1 araD13 Invitrogen (USA)9 ∆(ara-leu7697 galUgalKrpsL (StrR) endA1 nupG	Invitrogen (USA)
E. coli Epi300	F- mcrA D(*mrr-hsd*RMS-*mcr*BC) f80d*lac*ZDM15 D*lac*X74 *rec*A1 *end*A1 *ara*D139 D(*ara*, *leu*)7697 *gal*U*gal*K l- *rps*L *nup*G*trf*A*ton*A*dhfr*	Epicentre Biotechnologies
E. coli Rosetta pLysS	F- ompThsdSB(rB- mB-) gal dcm (DE3)pLysSRARE (Cm^r)	Novagen (USA)

[a] Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

Table 3.2 Plasmids used in this study

Plasmid	Genotype/Relevant features [a]	Reference
pGEM-T-Easy	β-galacosidase, Amp^r	Promega, USA
pUWL219	LacZ, β-galacosidase; Amp^r, *Ter*	Wehmeier (1995)
pJet1.2/blunt	Suicide vector (*eco47IR*), blunt DNA ends for ligation with insert; Amp^r	Fermentas, USA
pET21a(+)	C-terminal His-tag, Amp^r	Novagen, USA
pFos_IB2	pCC1Fos containing ~22 kb metagenomic DNA insert with AXEase activity; Cm^r	Dr. Kirby, IMBM, SA
pUWL_AXE10	pUWL219 containing ~10 kb ORS10 genomic DNA; Amp^r	This study
pUWL_AXE10_TM	pUWL_AXE10 containing the HyperMu<CHL-1> transposon; Amp^r, Cm^r	This study
pJET_AXE10	pJet1.2/blunt containing *axe*10 gene; Amp^r	This study
pET_AXE10	pET21a containing *axe*10; Amp^r	This study

[a] Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

3.1.1 Media and culture conditions

ORS10 (Table 3.1) was isolated from Omaruru River dessert sand, Namibia, by Dr. Le-Roes-Hill (CPUT, South Africa). ORS10 was cultured at 45ºC in Desert Minimal Medium (DMM) [g/l: glucose 0.5, yeast extract 0.5, NaCl 0.5, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 1.0, Trace element solution 1ml, (Trace element solution, g/l: $FeSO_4.7H_2O$ 1.0, $MnCl_2.4H_2O$ 1.0, $ZnSO_4.7H_2O$ 1.0), pH 7.0] and maintained on Desert Minimal Medium Agar (DMMA; DMM

supplemented with 15 g/l bacteriological agar), unless otherwise stated. *E. coli* Genehog and Rosetta strains (Table 3.1) were either grown in Luria-Bertani broth (LB; g/l: tryptone 10.0, yeast extract 5.0 and NaCl 5.0, pH7.0) or on Luria-Bertani agar (LA; LB containing 15 g/l bacteriological agar) at 37ºC, unless otherwise stated. *E. coli* strains harboring pUWL219 (Wehmeier, 1995; Table 3.2) and pET21a(+) (Table 3.2) and, pUWL219, pJet1.2/blunt and pET21a(+) recombinant constructs (Table 3.2) were cultured in LB or on LA containing 100 µg/ml ampicillin (Amp; Sigma), unless otherwise stated. For long-term storage bacterial strains were maintained in 16% (v/v) glycerol (Merck) at -80^{o}C.

3.1.2 Preparation of *E. coli* electrocompetent cells

E. coli electrocompetent cells were prepared according to the method of Sambrook and Russell (2001). Briefly, *E. coli* Genehog and Rosetta strains were streaked from a glycerol stock onto LA solid medium, and incubated overnight at 37^{o}C. A pre-culture was prepared by inoculating a single *E. coli* colony into 10 ml LB and incubated overnight at 37^{o}C with shaking (100 rpm). The pre-culture was inoculated into 1,000 ml 2XYT liquid medium (g/l: tryptone 16.0, yeast extract 10.0, NaCl 5.0, pH 7.0) and incubated at 37^{o}C with shaking (100 rpm) until it reached an optical density at 600 nm (OD_{600}; BioMate 3 UV/VIS spectrophotometer, Thermo) between 0.6-0.9. Thereafter, the culture was incubated on ice for 10 min, before the cells were harvested by

centrifugation (25 min at 4,000 rpm and 4^{o}C). The supernatant was discarded and the cell pellet resuspended in 1/5th culture volume of ice-cold sterile 10% (v/v) glycerol. The cells were washed, as before with 1/10th culture volume of ice-cold sterile 10% (v/v) glycerol. The supernatant was discarded and the cell pellet was gently resuspended in 1/50th culture volume of ice-cold 15% (v/v) glycerol and 2% (w/v) sorbital. The cells were harvested by centrifugation and the cell pellet was resuspended in 1 ml 15% (v/v) glycerol and 2% (w/v) sorbital, aliquoted into sterile microfuge tubes and stored at -80^{o}C.

3.2 Strain characterisation

3.2.1 Hemicellulolytic enzyme activity screening

Extracellular xylanolytic activity screening was performed using a plate-based technique (Teather and Wood, 1982). Briefly, ORS10 was streaked from a glycerol stock onto DMMA and incubated at 45^{o}C for 5 days, to allow sufficient time for ORS10 to sporulate. A spore suspension was prepared, according to Hopwood *et al.* (1985), by gently scraping off the spores from the aerial mycelium of a single ORS10 colony with a metal inoculating loop. The spores were transferred to a sterile microfuge tube containing 100 µl sterile distilled water and thoroughly mixed by vortexing for approximately 30 sec. Approximately 10 µl of the resulting spore suspension was used to spot inoculate a DMMA agar plate (Section 3.1.1) supplemented

with 1% (w/v) birchwood xylan (Sigma). The plate was incubate at 45^{o}C for 4 days and screened for extracellular xylanolytic activity using Congo red staining, according to Teather and Wood (1982). Briefly, the agar plate was flooded with 0.01% (w/v) Congo red solution (Merck) and incubated at 22°C for approximately 20 min. Thereafter, the Congo red solution was discarded and the medium destained by flooding with 1 M NaCl for a further 20 minutes at 22°C. The destain was discarded, before the plates were flooded with 7% (v/v) glacial acetic acid (Kimix) to fix the colour of the growth medium and improve the visual contrast between a zone of xylan hydrolysis and the background colour of the growth medium. An extracellular xylanolytic phenotype was identified by visual detection of zones of clearing surrounding the bacterial colony on the solid agar growth medium.

ORS10 was inoculated into 50 ml DMM supplemented with 1% (w/v) birchwood xylan and incubate at 45^{o}C for 4 day. The cells were harvested by centrifugation (16,000 rpm for 5 min at 4°C) and resuspended in 100 mM NaH_2PO_4 buffer (pH 7.0), before being sonicated (Bandelin Sonopuls) (6 x 30 sec) on ice. Thereafter, the insoluble cell debris was pelleted by centrifugation (16,000 rpm for 5 min at 4ºC) and the supernatant containing the soluble intracellular proteins retained as the crude cell-free enzyme extract. The ORS10 cell-free extract was used as a crude enzyme preparation to test for AXEase activity in the Fast Garnet liquid assay (Koseki *et al.,* 2005). Briefly,

the 200 µl assay consisted of 170 µl 50 mM NaH_2PO_4 buffer (pH 7.0), 0.8 µmol α-naphthyl acetate (Fluka) and 10 µl ORS10 crude enzyme extract, the assay was performed at 37^{o}C for 10 min and stopped by the addition of 110 µl Fast Garnet [Sigma; 6 mg/ml stock solution in 10% (w/v) SDS]. A concentration series (0.25-5 mM) of the final released product (α-naphthol; Sigma) was used to generate a standard curve. Briefly, 50 µl of the assay product was mixed with 950 µl sterile distilled water and the absorbance determined at 560 nm. The absorbance was compared with the standard curve of α-naphthol to determine the concentration of released product and therefore AXEase activity. One unit of AXEase activity was defined as the amount of enzyme required to produce 1 µmol of product (α-naphthol) per min under the assay conditions. *E. coli* Epi300 harbouring the recombinant fosmid pFos_IB2 (Table 3.1), containing an AXE gene identified from a compost metagenomic library, was cultivated for 16 hours at 37°C in 5 ml LB containing 34 µg/ml Cm and 0.1% (w/v) L-arabinose. A cell-free extract was prepared by sonication, as described above, and used as a positive control of AXEase activity. Similarly, a cell-free extract of *E. coli* Genehog, cultivated for 16 hours at 37°C in 5 ml LB, was isolated as negative control.

3.2.2 Effect of temperature and pH on xylanase activity

Xylanase activity was determined by measuring the release of reducing sugar, as described by Chen *et al.* (1986). A standard curve was generated by

using four selected concentrations of D-xylose (Sigma) to determine the absorbance at 540 nm. A volume of 50 µl of culture supernatant was added to 950 µl of 1% (w/v) birchwood xylan in 50 mM sodium acetate buffer (pH 4.5), and incubated at 30^{o}C for 10 min. The reaction was stopped by the addition of 1 ml copper solution (16 mM $CuSO_4$, 1.3 M Na_2SO_4, 226 mM Na_2CO_3, 190 mM $NaHCO_3$ and 43 mM $NaKC_4H_4O_6$) and boiling the reaction tubes for 10 min. The absorbance at 540 nm was determined after adding 1 ml Ars-Mol solution (40 mM $O_3Mo.3H_2O$, 19 mM H_3AsO_4 and 756 mM H_2SO_4). One unit of xylanase activity was defined as the amount of enzyme required to catalyze the production of 1 µmol xylose/min under the described assay condition.

In order to determine the effect of incubation temperature on xylanase activity the assay was performed, as described above, across a range of incubation temperatures (37, 45, 55, 65 and 75^{o}C). In order to assess the effect of pH on ORS10 xylanase activity, the assay was performed, as described above, across a pH range of 1.0 to 11.0. A control reaction was performed at each pH and temperature, in order to determine the effects of temperature and pH on the assay reagents.

3.2.3 Physiological and biochemical characterisation of ORS10

In addition to 16S rRNA gene phylogenetic analysis, physiological and biochemical tests are important for the identification and characterisation of

microorganisms. The morphological and physiological growth characteristics, namely optimum growth temperature and pH, and salt (NaCl) tolerance of ORS10, were determined according to the guidelines of the International *Streptomyces* Project (ISP; Shirling and Gottlieb, 1966).

Carbohydrate utilisation was determined by growth of ORS10 on carbon utilization medium ISP9 [g/l: 15.0 bacteriological agar, 2.64 $(NH_4)_2SO_4$, 2.38 KH_2PO_4, 5.65 $K_2HPO_4.3H_2O$, 1.0 $MgSO_4.7H_2O$, 1.0 ml Trace salts solution (g/100ml: 0.64 $CuSO_4.5H_2O$, 0.11 $FeSO_4.7H_2O$, 0.79 $MnCl_2.4H_2O$ and 0.15 $ZnSO_4.7H_2O$) pH 7.0] supplemented with 1% filter serilised (w/v) carbon sources (glucose, fructose, sucrose, xylose, mannitol, lactose and arabinose) (Shirling and Gottlieb, 1966). These growth media were inoculated with ORS10 washed cells (section 3.2.1) and incubated at 45^oC for 8 days. The level of bacterial growth was considered to be representative of the carbon source utilisation of ORS10. Melanin production was determined on peptone yeast iron agar (ISP6, g/l: 15.0 peptone, 15.0 bacteriological agar, 5.0 proteose peptone, 1.0 K_2HPO_4, 0.5 ferric ammonium citrate, 1.0 yeast extract, 0.08 $Na_2S_2O_3$, pH7.0) and tyrosine asparagine agar [ISP7, g/l: 15.0 glycerol, 15.0 bacteriological agar, 0.5 L-Tyrosine, 1.0 L-Asparagine monohydrate, 0.5 K_2HPO_4, 0.5 $MgSO_4.7H_2O$, 0.5 NaCl, 0.01 $FeSO_4.7H_2O$, 1.0 ml Trace salts solution (g/100ml: 0.1 $FeSO_4.7H_2O$, 0.1 $MnCl_2.4H_2O$, 0.1 $ZnSO_4.7H_2O$), pH 7.0] (Shirling and Gottlieb, 1966). Degradation of adenine, guanine, casein,

starch and L-tyrosine were determinate using Bennet's medium (g/l: 10.0 glycerol, 2.0 casitone, 1.0 beef extract, 1.0 yeast extract, 15.0 bacteriological agar, pH 7.0) supplemented with the appropriate amount of substrate, according to Shirling and Gottlieb (1966). Spore colour was determined visually following growth of ORS10 on ISP5 [g/l: 1.0 L-Asparagine monohydrate, 15.0 bacteriological agar, 10.0 glycerol, 1.0 K_2HPO_4, 1.0 ml trace salt solution (g/100ml: 0.1 $FeSO_4.7H_2O$, 0.1 $MnCl_2.4H_2O$, 0.1 $ZnSO_4.7H_2O$), pH 7.0]. These growth media were inoculated with ORS10 (as described in section 3.2.1) and incubated at 45^oC for 8 days.

3.3 DNA analytical methods

3.3.1 Genomic DNA extraction

An ORS10 spore suspension was prepared (as described in section 3.2.1) and 20 µl of this was used to inoculate 10 ml DMM supplemented with 1% (w/v) birchwood xylan. The culture was incubated at 45^oC for 2 days with shaking (100 rpm), after which the purity of the culture was assessed by Gram staining (Gram, 1884) and visual inspection under Olympus CX21 light microscope (200x magnification). The whole volume of the starter culture was used to inoculate 100 ml DMM supplemented with 1% (w/v) birchwood xylan and incubated for 72 hours at 45^oC with shaking (100 rpm).

Genomic DNA (gDNA) was extracted from the ORS10 liquid culture using

a modified method of Wang *et al.* (1999). Briefly, the bacterial cells were harvested by centrifugation (4,000 rpm for 10 min) to achieve a cell pellet volume of approximately 0.2 ml. The cells were resuspended in a volume of 500 µl lysis buffer [50 mM D-glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mg/ml lysozyme (Fluka)] and incubated for approximately 16 hours at 37°C. Thereafter, SDS was added to a final concentration of 2% (w/v) and the mixture was incubated at 65^{o}C for a further 2 hours. An equal volume of pH 8.0 equilibrated phenol (Sigma) was added, gently mixed by inversion, and the phases separated by centrifugation (16,000 rpm at 22^{o}C for 1 min). The aqueous phase was subsequently transferred to a sterile microfuge tube, and extracted with an equal volume of chloroform:iso-amyl alcohol (24:1). The phases were separated by centrifugation (16,000 rpm at 22^{o}C for 1 min) and the aqueous phase transferred to a clean microfuge tube. An equal volume of ice-cold isopropanol (Merck) was added, to precipitate the nucleic acid, and the solution was incubated at 22°C for 10 min. The gDNA was collected by centrifugation (16,000 rpm for 10 min), washed with 70% (v/v) ethanol and resuspended in 100 µl Tris-EDTA (TE; 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) buffer containing 100 µg/ml RNAseA (Fermentas). ORS10 gDNA concentrations were determined using the NanoDrop ND-1000.

3.3.2 16S rRNA gene amplification and phylogenetic analysis

ORS10 gDNA (section 3.3.1) was used as the template for the polymerase

chain reaction (PCR) amplification of the 16S rRNA gene using the oligonucleotide primers F1 and R5 (Table 3.3). The primers were used in the following combinations: F1/R5 (Fig. 3.1). Each PCR reaction (final volume of 50 µl) contained 1x Dream*Taq* buffer (Fermentas), 2 mM $MgCl_2$, 0.2 U Dream*Taq* DNA polymerase (Fermentas), 200 µM of each dNTP (Bioline), 5 µM of each oligonucleotide primer and 50 ng of template DNA. The PCR cycles included an initial denaturation (96^{o}C for 2 min), and then 30 cycles of denaturation (96^{o}C for 45 sec), primer annealing (56^{o}C for 30 sec) and extension (72^{o}C for 2 min), followed by a final extension (72^{o}C for 10 min).

Table 3.3 Oligonucleotide primers used in this study.

Primer	Primer sequence (5'-3')[a]	Reference
F1	AGAGTTTGATCITGGCTCAG	Weisburg *et al*. (1991)
F3	GCCAGCAGCCGCGGTAATAC	Weisburg *et al*. (1991)
R5	ACGGITACCTTGTTACGACTT	Weisburg *et al*. (1991)
M13 Forward	CGCCAGGGTTTTCCCAGTCACGAC	Yanisch-Perron *et al.* (1985)
M13 Reverse	GAGCGGATAACAATTTCACACAGG	Yanisch-Perron *et al.* (1985)
T7 Promoter	CCATATGAATACAGATAAACGCAG	Novagen, USA
T7 Terminator	GCTCGAGTTTAATAAATTTTTCGCTC	Novagen, USA
MUCHL-1 FP-1	CACAGGTATTTATTCGGTCGA	Epicentre Biotechnologies
MUCHL-1 RP-1	TGGAGGTAATAATTGACGATA	Epicentre Biotechnologies
AXE10_Fwd	GACA**CATATG**TCCACTGCTCTGC	This study
AXE10_Rev	G**CTCGAG**GGCGCGGACGTCG	This study

[a] The restriction endonuclease sites incorporated into the PCR primers, AXE10_Fwd and AXE10_Rev, are indicated in coloured bold type. Red indicates *Nde*I and green indicates *Xho*I.

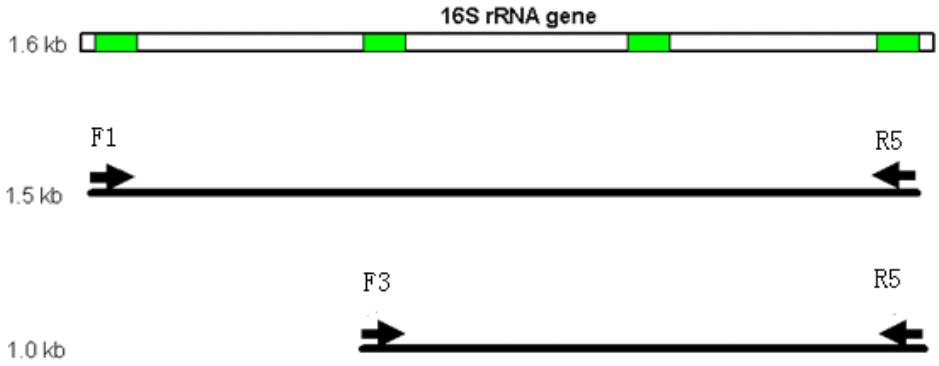

Figure 3.1 Diagram showing the PCR strategy employed to amplify the ORS10 16S rRNA gene using the oligonucleotide primer pair F1/R5. F1 and F3 represent the forward primers and R5 represents the reverse primer. The green areas represent highly conserved regions within the 16S rRNA gene. The sizes depict the approximate length of the 16S rRNA gene and the expected length of the amplified PCR product (adapted from Macey, 2005).

The amplified PCR product was separated on 1% (w/v) TAE agarose gel to confirm the amplification of the expected 16S rDNA fragment. The amplified PCR products were purified using a PCR product purification kit (GE healthcare, UK) according to the manufacturer's instructions. The purified PCR product was cloned into the pGEM-T-Easy cloning vector (Table 3.2), according to the manufacturer's instructions and transformed into electrocompetent *E. coli* Genehog cells (section 3.1.2). Briefly, 1 µl ligation product was mixed gently with electrocompetent *E. coli* Genehog cells, and the mixture transferred into a 0.1 cm pre-chilled electroporation cuvette (Bio-Rad). Electroporation was carried out under the following conditions: 1.8 KV, 25µF

and 200Ω on a Bio-Rad Gene Pulser. Following electroporation, the cells were mixed with 950 ml SOC (g/l: 20.0 tryptone, 5.0 yeast extract, 0.5 NaCl, 20 mM glucose and 2.5 mM KCl) incubated at 37^{o}C for 1 hour with shaking (100 rpm). Approximately 50µl of the transformation mixture was spread plated onto LA supplemented with 100 µg/ml Amp, 80 µg/ml X-gal (5-bromo-4-chloro-indolyl-galactopyranoside; Fermentas) and 100 µg/ml IPTG (Isopropyl β-D-1-thiogalactopyranoside; Fermentas), and incubated overnight at 37°C.

E. coli Genehog cells capable of growth on Amp as 'white' colonies were presumed to be harbouring recombinant pGEM-T-Easy vector constructs. These clones were further screened by colony PCR to confirm that they contained the expected insert DNA fragment. Briefly, individual *E. coli* Genehog colonies were picked directly from the transformation plates with a sterile toothpick and vigorously mixed into 25 µl of TE buffer in a sterile microfuge tube. The tube containing an individually resuspended *E. coli* Genehog clone was incubated on a heating block at 90^{o}C for 2 min. Approximately 1 µl of the resulting mixture was used as the template in a PCR reaction (0.2 mM dNTP, 0.5 M of each oligonucleotide primer and 0.2 U Dream*Taq*). The PCR cycles included an initial denaturation (94^{o}C for 2 min), and then 30 cycles of denaturation (94^{o}C for 45 sec), primer annealing (56^{o}C for 30 sec) and extension (72^{o}C for 2 min), followed by a final extension (72^{o}C

for 10 min). The oligonucleotide primers M13 Forward and M13 Reverse (Table 2.3) were used in the colony PCR. Amplified PCR products were separated on 1% (w/v) TAE agarose gel and visualized under long wavelength (365 nm) UV light (Alphaimager, Innotech). A single 'blue' colony, containing the pGEM-T-Easy vector without an insert, was used as a negative control for the colony PCR.

E. coli Genehog transformants shown to be harbouring the desired insert were picked from the plates, inoculated into LB containing 100 µg/ml Amp and incubated at 37^{o}C for 16 hours with shaking (100 rpm). Plasmid DNA was extracted from the overnight cultures using the QIAGEN mini-prep kit according to the manufacturer's instructions. The extracted plasmid DNA was sequenced using F1, F3, R5, M13 Forward and M13 Reverse sequencing primers (Table 3.3; Macrogen, Korea).

Sequences were edited using Chromas version 2.01 (Technelysium, Australia) and assembled using DNAMAN version 4.13 (Lynnon Biosoft). Local alignments were conducted for each consensus sequence using the nucleotide-nucleotide basic local alignment search tool (BLAST*n*) supplied by the GenBank database (www.ncbi.nlm.nih.gov). For phylogenetic analysis of ORS10, 16S rRNA sequences of the 10 most related organisms were aligned. Multiple sequence alignments were performed using ClustalW. Phylogenetic

analysis was conducted using MEGA version 4.0 (Tamura *et al.,* 2007). A neighbour-joining tree (Saltou and Nei, 1987) was constructed with bootstrap analysis based upon 1,000 re-sampled data sets (Felsenstein, 1985). Only bootstrap values greater than 40% were indicated.

3.3.3 Preparation of plasmid vector pUWL219

A *Streptomyces-E. coli* shuttle vector, pUWL219 (Table 3.2 and Fig 3.2; Wehmeier, 1995), was chosen for the construction of a small-insert ORS10 genomic library. This vector is based on the *E. coli* cloning vector pUC19 (Vieira and Messing, 1982) and the *Streptomyces* vector pUWL199 (Wehmeier, 1995). It contains the *Lac*Z gene, which enables 'blue/white' selection of *E. coli* clones, and origins of replication for both *E. coli* and *Streptomyces* (Wehmeier, 1995). Therefore, a genomic library constructed in this vector could potentially be screened in both *E. coli* and *Streptomyces* host systems.

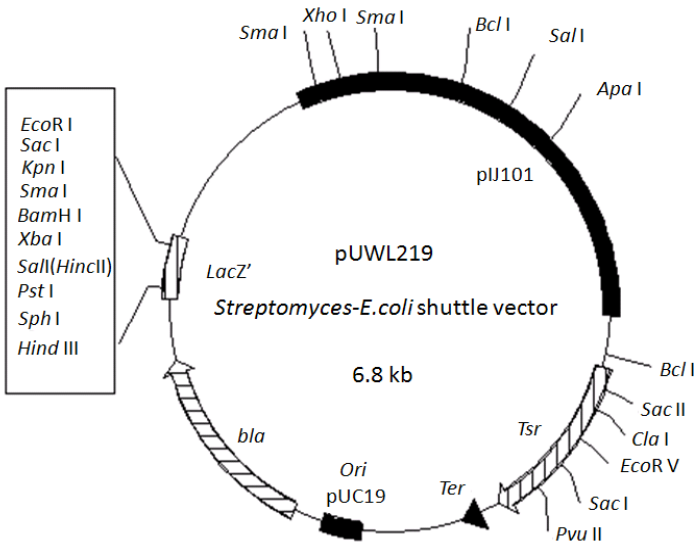

Figure 3.2 The *Streptomyces-E. coli* cloning vector pUWL219. The position of the *bla* (Amp) and *Tsr* (Thiostrepton) antibiotic resistance genes, and restriction enzyme recognition sequences are indicated (Adapted from Wehmeier, 1995).

Plasmid DNA was extracted from *E. coli* JM109 harbouring pUWL219 using the QIAGEN midi-prep kit according to the manufacturer's instructions. The vector was linearised by restriction enzyme digestion with *Bam*HI (Fermentas). Thereafter, the restriction enzyme was heat inactivated (80^{o}C for 20 min), before the linearised vector was dephosphorylated (Rapid Alkaline Phosphatase, Roche) according to the manufacturer's instructions. The linearised and dephosphorylated vector DNA was resolved on a 0.8% (w/v) TAE agarose gel. The linearised vector was excised from the agarose gel, under long wavelength UV light (365 nm), before being gel purified using the QIAGEN Gel Purification kit according to the manufacturer's instructions.

3.3.4 Partial ORS10 genomic DNA restriction enzyme digestion

In order to generate suitably sized ORS10 gDNA fragments (4-10 kb) for the construction of a small-insert genomic library, ORS10 gDNA (section 3.3.1) was partially digested with the restriction enzyme *Sau*3A (Fermentas).The partial *Sau*3A digestion was optimized on a small scale, in terms of the ratio of units of *Sau*3A to quantity of gDNA and incubation time, in order to generate the desired size distribution of gDNA fragments.

For the small scale optimization of the partial *Sau*3A restriction enzyme digestion, approximately 100 ng of ORS10 gDNA was digested with a range of *Sau*3A concentrations (0.000005, 0.00005, 0.0005, 0.005 and 0.05 units/µl) for 10 min at 37^{o}C. Thereafter, the restriction enzyme was rapidly inactivated by the addition of 2µlof 10x loading dye [1.25 mg/ml xylene cyanol, 1.25 mg/ml bromophenol blue, 62.5% (v/v) glycerol and 0.625% (w/v) SDS] and heat inactivated at 65^{o}C for 20 min. The resulting ORS10 gDNA restriction fragments were subsequently loaded and separated on a 1% (w/v) TAE agarose gel.

The optimal ratio of genomic DNA to *Sau*3A restriction enzyme determined from the preliminary small scale digestion trials, approximately 5 µg of ORS10 gDNA and 0.5 U *Sau*3A were used in five separate restriction enzyme

reactions incubated at 37°C for 8, 9, 10, 11 and 12 min, respectively. The resulting ORS10 gDNA restriction digestions were rapidly inactivated, as described above, and the resulting DNA fragments resolved on a 1% (w/v) TAE agarose gel. The gel was visualized and gDNA fragments between 4-10 kb excised using a sterile scalpel blade and purified using the QIAGEN gel extraction kit. The volume of purified DNA fragments was reduced (SpeediVac, Savant), prior to cloning into pUWL219.

3.3.5 Small-insert genomic library construction

In order to generate a small-insert ORS10 genomic library, a 1:1 (vector:insert) picomole-end ratio of *Sau*3A partially digested ORS10 gDNA fragments and de-phosphorylated pUWL219 (section 3.3.3) were ligated overnight at 4°C using T4 DNA ligase (Fermentas) according to manufacturer's instruction. Thereafter, the ligase was heat inactivated at 80°C for 10 min, and 1 µl of the ligation reaction transformed into 50 µl of electro-competent (section 3.1.2) *E. coli* Genehog cells (section 3.3.2). Following electroporation, the cells were immediately mixed with 950 µl SOC and incubated at 37°C for 1 hr with shaking. Approximately 50 µl of the mixture was subsequently spread plated onto LA containing 100 µg/ml Amp, 80 µg/ml X-gal and 1 mM IPTG, and incubated at 37°C for 16 hours. The transformation plates containing 'white' *E. coli* Genehog colonies, representing the ORS10 genomic library, were flooded with 3 ml ice-cold LB and the colonies gently scraped off with a glass

spreader. The mixtures of colonies were pooled together and transferred into a new tube. Sterile glycerol was added to achieve a final concentration of approximately 15% (v/v) glycerol and the genomic library stored at -80^{o}C.

3.3.6 Plasmid extraction and library verification

The library titer was tested by preparing a serial dilution (10^{o}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) of the pooled ORS10 genomic library in ice-cold LB and plating it onto LA containing 100 µg/ml Amp. This was done in order to identify the dilution that would provide approximately 100 colonies per 10 cm agar plate. The appropriate dilution was spread plated on LA containing 100 µg/ml Amp, 80 µg/ml X-gal and 1 mM IPTG, and incubated at 37^{o}C for 16 hours. A total of twenty 'white' *E. coli* Genehog colonies and two 'blue' colonies were randomly selected, individually picked and cultured in 5 ml LB supplemented with 100 µg/ml Amp for 16 hours at 37°C with shaking (100 rpm). Plasmid DNA was extracted from each *E. coli* clone using the small-scale alkaline lysis mini-prep method (Sambrook and Russell, 2001). Briefly, 2 ml of the overnight culture was centrifuged (16,000 rpm for 5 min at 22°C) to harvest the cell pellet. The cells were resuspended in 200µl of solution 1 [50mM glucose, 25 mM Tris-HCl (pH8.0) and 10mM EDTA (pH8.0)] containing 20 µg/ml RNAseA (Fermentas). A volume of 200 µl solution 2 [1% (w/v) SDS and 0.2M NaOH] was added; the mixture was mixed by gentle inversion and incubated at room temperature for 5 min. Following the addition

of 200 µl solution 3 [7.5M CH_3COONH_4 (pH 5.5)], the tubes were incubated on ice for 10 minutes and centrifuged at 16,000 rpm for 15 min at 4^oC to pellet cellular debris. The addition of 0.7 volumes isopropanol (Merck) to the supernatant was used to precipitate the DNA. The plasmid DNA was collected by centrifugation, washed with 70% (v/v) ethanol and collected by centrifugation. The DNA pellet was air-dried and resuspended in 1xTE buffer.

The insert size of the plasmids was determined by restriction enzyme digestion with *Eco*RI and *Hin*dIII (Fermentas), and separation of the digestion products on a 0.8% (w/v) TAE agarose gel. The approximate sizes of each DNA fragment was determined using DNAFRAG version 3.03 (Schaffer and Sederoff, 1981).

3.4 Library screening for hemicellulosic enzymes

The ORS10 small-insert genomic library was expression screened for hemicellulosic enzymes using solid-phase screens.

3.4.1 Preparation of RBB-xylan

Remazol Brilliant Blue (RBB)-xylan was prepared according to the method outlined by Biely (1985). Twenty-five grams of birchwood xylan was dissolved in 625 ml water while heating to 40-60^oC. Approximately 25 g RBB (Sigma) was added and dissolved by stirring. Thereafter, approximately 125 ml 0.4 M

NaOAc (Merck) was added and the heating turned off, before 250 ml 6% (w/v) NaOH (Saarchem) was added and the mixture stirred at room temperature for 60 minutes. The dyed product was precipitated by the addition of two volumes of 96% ethanol (Merck) and collected by repeated washing and centrifugation (5,000 rpm for 5 min at 22°C). The supernatant fraction was discarded and the precipitate washed repeatedly with a 2:1 (v:v) solution of 96% ethanol and 0.05 M NaOAc, until the supernatant was practically colourless. Thereafter, the dyed product was dissolved in 1,000 ml 0.05 M NaOAc, precipitated and washed as before until the supernatant was practically colourless. The dyed product was precipitated again with two volumes of ethanol, before the product was desalted by the addition of a 4:1 (v:v) solution of 96% ethanol and distilled water. The dyed xylan substrate was dissolved in distilled water, frozen at -80°C and freeze-dried (Vacutec, Labconco). The dried RBB-xylan was stored at room temperature and used at a final concentration of 0.7% (w/v) in solid growth medium for xylanase expression screening.

3.4.2 Library screening

In order to identify possible recombinant clones containing the xylanase gene within the ORS10 genomic library (sections 3.3.5 and 3.3.6); the library was plated onto LA containing 0.7% (w/v) RBB-xylan (section 3.4.1), 0.3% (w/v) birchwood xylan and 100 µg/ml Amp. In order to screen for general esterases, the ORS10 genomic library was plated onto LA supplemented with 1% (w/v)

gum arabic (Sigma), 0.1% (v/v) glycerol tributryate (Sigma) and 100 µg/ml Amp. These screening plates were incubated at 37^{o}C for 16 hours. An *E. coli* colony with zone of clearing surrounding it on either screening media would indicate possible enzyme activity due to substrate degradation.

3.4.3 Activity-based screening for accessory hemicellulosic enzymes

E. coli Genehog library clones shown to be positive for general esterases by primary trybutyrin agar screening (section 3.4.2) were further screened to identify possible AXE, FAE and/or ρ-coumaric acid esterase activities.

3.4.3.1 Acetylxylan esterase activity screening

E. coli Genehog clones harbouring recombinant pUWL219 constructs with general esterase activity (section 3.4.2) were screened for AXE activity using the Fast Garnet liquid assay (Koseki *et al.*, 2005), as described in section 3.2.1. *E. coli* Genehog clones harbouring recombinant pUWL219 constructs were inoculated into5 ml LB containing 100 µg/ml Amp and incubated overnight with shaking (100 rpm). The cells from the overnight cultures were harvested by centrifugation (16,000 rpm for 5 min at 4°C) and resuspended in 100 mM NaH_2PO_4 buffer (pH 7), before being sonicated (6x 30 sec) on ice. Thereafter, the insoluble cell debris was pelleted by centrifugation (16,000 rpm for 5 min at 4ºC) and the supernatant containing the soluble intracellular proteins retained as the crude enzyme containing cell-free extract.

3.4.3.2 Ferulic acid esterase activity screening

E. coli Genehog clones harbouring recombinant pUWL219 constructs with general esterase activity (section 3.4.2) were screened for FAE activity by streaking them onto LA containing 18mMethyl ferulate (Sigma) and 100 µg/ml Amp (Koseki *et al.*, 2005), and incubating at 37^{o}C for 4-5 days. A zone of clearing surrounding an *E. coli* Genehog colony indicated positive FAE activity.

3.4.3.3 ρ-Coumaric acid esterase activity screening

E. coli Genehog clones harbouring recombinant pUWL219 constructs with general esterase activity (section 3.4.2) were screened for ρ-coumaric acid esterase activity using a solid-phase assay, as described by Donaghy and Mckay (1994). This assay employs ethyl cinnamate as the substrate, and bromocresol green as a stain to identify clone(s) with ρ-coumaric acid esterase activity. Clones capable of expressing an active ρ-coumaric acid esterase would have a yellow/green zone surrounding the bacterial colony on a blue background due to a pH shift to below pH 4.The library clones were inoculated on LA supplemented with 100 µg/ml Amp and incubated for 16 hours at 37^{o}C. Thereafter, a soft-agar overlay comprising 0.7% (w/v) bacteriological agar, 0.5% (w/v) ethyl cinnamate (Sigma) and 0.01% (w/v) bromocresol green (Sigma) was prepared, autoclaved, and once cool poured over the LA plate on which the library clones had been cultured. Subsequently, the agar plate was

incubated for 48 hours and visually inspected on a daily basis for evidence of yellow/green zone(s) surrounding the bacterial colonies.

3.4.4 Plasmid extraction and transposon mutagenesis

The recombinant clone, pUWL_AXE10 (Table 3.2), was cultivated overnight with shaking (100 rpm) in 10 ml LB containing 100 µg/ml Amp at 37°C. Plasmid DNA was extracted from the overnight culture using the QIAGEN Midi-prep kit and subjected to transposon mutagenesis using the HyperMu™ CHL-1 insertion kit (Epicentre Biotechnologies) according to the manufacturer's instructions (Fig. 3.3).Transposon-treated plasmid DNA was transformed into electrocompetent *E. coli* Genehog cells, spread plated onto LA trybutyrin screening media (section 3.4.2) supplemented with 100 µg/ml Amp and 34 µg/ml Cm, and incubated overnight at 37°C. *E. coli* Genehog colonies capable of growth on the double antibiotic selection together with a loss of the extracellular esterase phenotype on trybutyrin agar may indicate inactivation and/or interruption of the gene encoding the esterase by integration of the HyperMu™<CHL-1> transposon into the plasmid. In order to confirm that the loss of extracellular general esterase phenotype observed on trybutyrin agar plates was linked to a loss of AXEase activity several *E. coli* Genehog clones, with various sized trybutyrin-hydrolysis zones, were randomly picked from the transformation plate and incubated overnight at 37°C with shaking (100 rpm) in 5 ml LB containing 100 µg/ml Amp and 34

µg/ml Cm. In addition, *E. coli* Genehog harbouring either pUWL219 or pUWL_AXE10 were inoculated into 5 ml LB containing100 µg/ml Amp and incubated overnight with shaking (100 rpm). The AXE activity of a cell-free extract (section 3.4.3.1) of each of the clones and the control cultures was assessed using the Fast Garnet liquid assay, as described in section 3.2.1.In addition, plasmid DNA was extracted from the 4 mutants without zones of hydrolysis on trybutyrin using the QIAGEN mini-prep kit, according to the manufacturer's instructions, and sequenced using the sequencing oligonucleotide primers MUCHL-1 FP-1 and MUCHL-1 RP-1 (Table 3.3 and Fig.3.3).

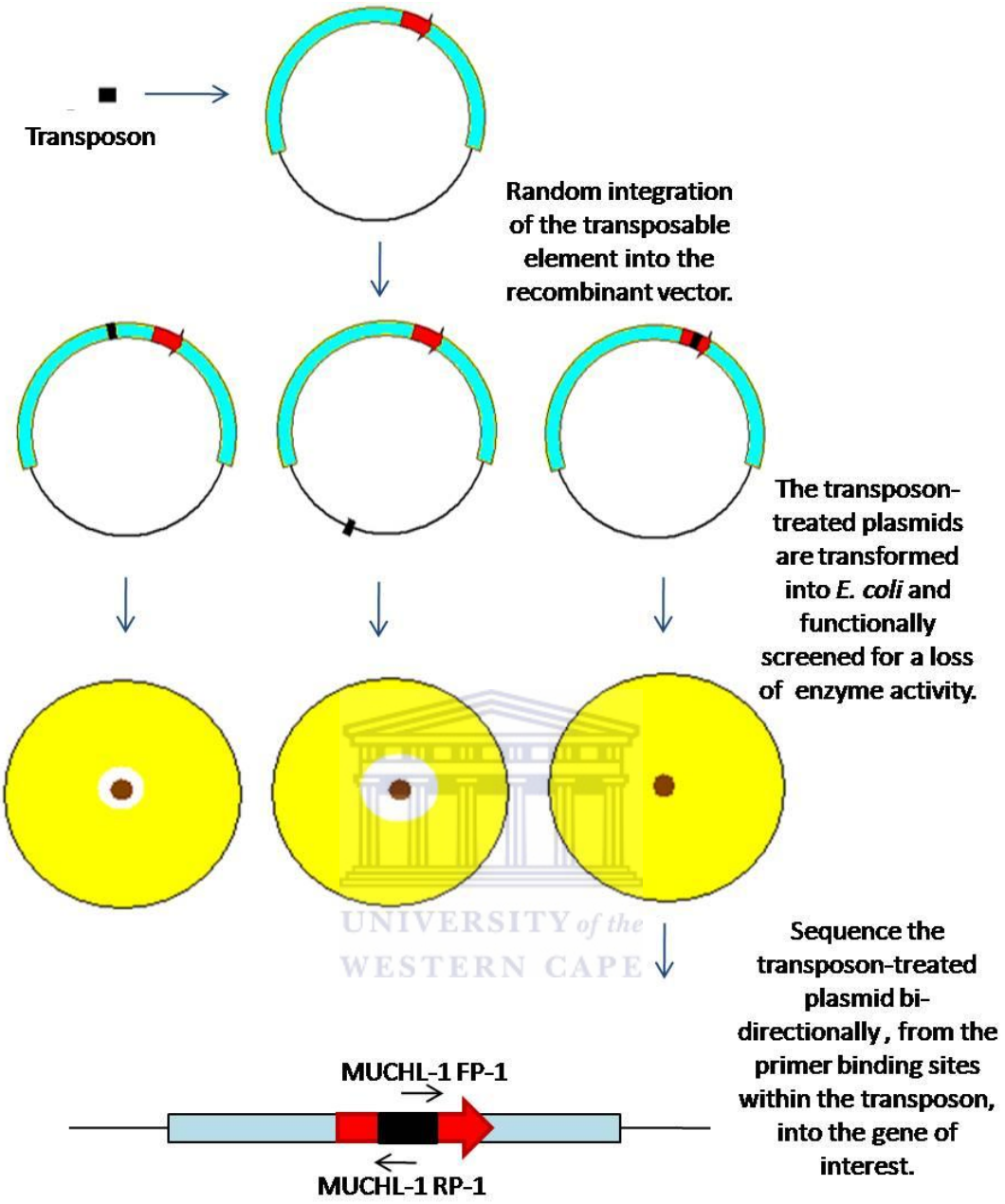

Figure 3.3 Schematic diagram indicating the transposon mutagenesis approach adopted in this study to sequence and identify the gene of interest from an ORS10 genomic library clone. The thin black line indicates the vector backbone, the black (■) box indicates the HyperMu<CHL-1> transposon, the turquoise (■) block indicates the cloned insert and the red (■) arrow indicates the gene of interest. The approximate positions of the transposon-based sequencing primers are indicated.

3.4.5 Sequence alignment and BLAST analyses

The sequences generated by sequencing the pUWL_AXE10 knock-out mutants with the MUCHL-1 forward and reverse primers were edited and assembled using Chromas version 2.01 and DNAMAN version 4.13, respectively. The consensus sequence was translated in DNAMAN to identify putative ORFs contained within this fragment of ORS10 genomic DNA. The predicted ORFs were subsequently compared to known protein sequences (BLAST*p*) within the GenBank database (www.ncbi.nlm.nih.gov). In addition, conserved domains and the catalytic site of this putative protein were identified using Interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

3.5 Gene cloning and protein expression

3.5.1 Primer design and amplification of AXE encoding gene

A pair of oligonucleotide primers (AXE10_Fwd and AXE10_Rev; Table 3.3) were designed to the cloned *axe*10 gene. *Nde*I and *Xho*I restriction sites were included within the primers at the 5′- and 3′-ends, respectively, to enable the amplified PCR product to be cloned in-frame with the vector encoded His-tag, into the protein expression vector pET21a(+) (Table 3.2 and Fig. 3.4).

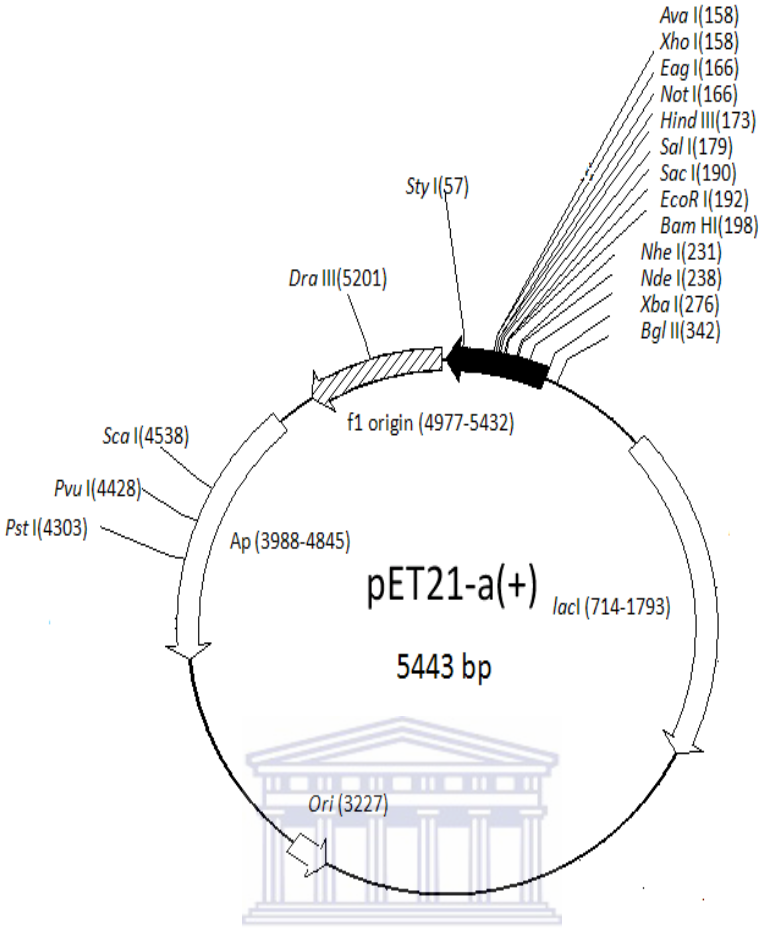

Figure 3.4 Plasmid map of the pET21a(+) expression vector used to sub-clone and express the *axe*10 gene. The positions of the restriction enzyme recognition sequences, in bp, are indicated in brackets. The position of the Amp (Ap) antibiotic resistance gene is indicated (Novagen).

Each PCR reaction (30 µl) contained 10x *Pfu* polymerase buffer (Fermentas) with 2 mM $MgSO_4$, 1.25 U *Pfu* DNA polymerase (Fermentas), 200 µM of each dNTP, 5µM of each oligonucleotide primer (AXE10_Fwd and AXE10_Rev) and 10 ng of pUWL_AXE10template DNA. The PCR cycles include an initial denaturation (95^{o}C for 3 min), and then 25 cycles of denaturation (95^{o}C for 30 sec), annealing (66^{o}C for 30 sec) and extension (72^{o}C for 1 min), followed by a final extension (72^{o}C for 10 min).

3.5.2 *Axe*10 gene cloning

The amplified PCR product was separated on a 1% (w/v) TAE agarose gel. The amplificon was excised from the gel and purified using QIAGEN gel extraction kit according to the manufacturer's instructions. The purified DNA fragment was ligated with the linear suicide vector pJET1.2/blunt (Table 3.2; Fermentas) according to the manufacturer's instruction. The ligation reaction was performed at 4^{o}C for 16 hours, and the ligase was inactivated subsequently by heating at 80^{o}C for 10 min. The ligation reaction was transformed into pre-chilled electro-competent *E. coli* Genehog cells (section 3.1.2) as described in section 3.3.2. Approximately 50 µl of the transformation reaction was spread plated onto LA containing 100 µg/ml Amp, and incubated overnight at 37^{o}C. A few colonies from the transformation plate were randomly selected, picked and inoculated into a 5 ml LB broth supplemented with 100 µg/ml Amp, and incubated at 37°C for 16 hours. Plasmid DNA was extracted from the overnight cultures using the small-scale alkaline lysis mini-prep method (section 3.3.6) and screened for the presence of the desired approximately 800 bp insert by *Xho*I and *Nde*I (Fermentas) restriction enzyme analysis. A recombinant construct containing the approximately 800 bp fragment was identified and designated pJET_AXE10 (Table 3.2).

3.5.3 *Axe*10 gene sub-cloning

The recombinant plasmid pJET_AXE10 was digested with the restriction

enzymes *Xho*I and *Nde*I (Fermentas) to release the approximately 800 bp DNA fragment containing the amplified *axe*10 gene. The resulting DNA fragments were resolved on a 1% (w/v) TAE agarose gel, before the fragment of interest was excised and purified using the QIAGEN gel extraction kit according to the manufacturer's instructions.

The protein expression vector pET21a(+) (Fig. 3.4; Table 3.2) was isolated from an overnight culture of *E. coli* Genehog using the QIAGEN midi-prep kit, according to the manufacturer's instructions. The vector was linearised by restriction enzyme digestion with *Xho*I and *Nde*I, and the resulting DNA fragments resolved on a 0.8% (w/v) TAE agarose gel. The 6.3 kb DNA fragment corresponding to linearised pET21a(+) was excised from the gel and purified using the QIAGEN gel extraction kit.

The *Xho*I and *Nde*I digested insert DNA fragment, comprising the *axe*10gene (section 3.5.2),and linearised pET21a(+) vector were ligated together at a 1:1 molar ratio using T4 DNA ligase (Fermentas) according to the manufacturer's instructions. The ligation reaction was subsequently transformed into electrocompetent *E. coli* Genehog cells (section 3.1.2), spread plated onto LA containing 100 µg/ml Amp and incubated overnight at 37°C. *E. coli* Genehog cells capable of growth on Amp were inoculated into 5 ml LB supplemented with 100 µg/ml Amp and incubated overnight with

shaking at 37ºC. Plasmid DNA was isolated from the overnight cultures using the small-scale alkaline lysis mini-prep method (section 3.3.6), and screened for the presence of the *axe*10 gene insert by restriction enzyme analysis with *Xho*I and *Nde*I. A recombinant construct containing the *axe*10 gene was identified and designated pET_AXE10 (Table 3.2). This construct was sequenced (Macrogene, Korea) using the T7 promoter and T7 terminator primers (Table 3.3), in order to confirm the sequence of the cloned gene and that the cloned gene was in-frame with the vector encoded His-tag. The plasmid pET_AXE10 was transformed into electro-competent *E. coli* Rosetta (Table 3.1) cells and spread plated onto LA containing 100µg/ml Amp and 34 µg/ml Cm, and incubated at 37°C for 16 hours.

3.6 *Axe*10 gene expression

3.6.1 Confirmation of Axe10 activity

E. coli Rosetta harbouring pET_AXE10 (section 3.5.3) was streaked onto LA supplemented with 1% (w/v) gum Arabic, 0.1% (v/v) glycerol tributryate, 100 µg/ml Amp and 34 µg/ml Cm, and LA supplemented with 1% (w/v) Gum Arabic, 0.1% (v/v) glycerol tributryate, 100 µg/ml Amp, 34 µg/ml Cm and 0.1 mM IPTG. These plates were incubated at 37^{o}C for 16 hours and visually examined for esterase activity, as indicated by zones of clearing, surrounding the bacterial colonies. To confirm that esterase activity detected for *E. coli* Rosetta clones harbouring pET_AXE10was AXE activity, and not due to

another esterase, *E. coli* Rosetta [pET21a(+)] and *E. coli* Rosetta (pET_AXE10) were inoculated separately into 10 ml LB containing 100 µg/ml Amp, 34 µg/ml Cm and 0.1 mM IPTG, and incubated at 37^{o}C for 16 hours. The cells were harvested by centrifugation (16,000 rpm for 5 min at 4°C) and a cell-free soluble protein extract prepared, as described in section 3.4.4. A Bradford assay (Bradford, 1976) was used to determine the total protein concentration of the cell-free extracts. Briefly, a set concentration range (100-900 µg/ml) of Bovine Serine Albumin (BSA; Sigma) was used to generate a standard curve of absorbance (A_{595}) against protein concentration. The sample was prepared as follows: 950 µl of Bradford reagent (Sigma) was mixed with 20 µl of suitably diluted protein sample, and incubated at room temperature for 15 min. Thereafter, the absorbance was determined at 595 nm and the concentration of protein determined by comparing the absorbance to the standard curve of BSA standards. An equal quantity of total protein (approximately 4 µg) from all the cell-free extract samples were used in each Fast Garnet liquid assay, as described in section 3.2.1.

3.6.2 Optimization of *axe10* expression

A series of small scale induction experiments were performed in order to optimize the over-expression of *axe*10 from *E. coli* Rosetta pET_AXE10. Briefly, a number of 5 ml LB pre-cultures supplemented with 100 µg/ml Amp and 34 µg/ml Cm were inoculated with *E. coli* Rosetta harbouring pET_AXE10

and incubated at 37°C for 16 hours, before being inoculated into 100 ml LB supplemented with 100 µg/ml Amp and 34 µg/ml Cm. These 100 ml cultures were subsequently incubated at 37°C with shaking (100 rpm) until the OD_{600} reached 0.6. Thereafter, each culture was divided into two equal volumes in two sterile 500 ml Erlenmeyer flasks. One of each pair was induced with a different final concentration of IPTG (0.2, 0.3, 0.4, 0.5, 0.6 or 1 mM), while the other was not. Each pair of flasks was then incubated at 37°C for 4 hours with shaking. In addition, incubation temperatures of 22 and 30°C were also investigated after induction; and the incubation time following IPTG induction was also increased to 16 hours in order to optimize the over expressed of Axe10. Thereafter, the cells from each experimental condition were harvested by centrifugation (4,000 rpm for 10 min).

The cell pellets from each induction experiment were sonicated (as described in section 3.2.1) in 1x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.0). As a negative control *E. coli* Rosetta harbouring pET21a(+) was also cultured as for the experimental cultures. Approximately 20 µl of cell free extract and insoluble fraction (cell debris) from each induction condition was mixed with 20 µl 2xloading buffer [20% (v/v) glycerol, 200 mM Dithiothreitol (DTT),100 mM Tris-HCl, 4% (w/v) SDS, and 0.2% (w/v) Bromophenol blue], denatured by incubation at 95°C for 10 min and loaded onto a SDS-PAGE gel [12% (w/v) separating gel and 4%

(w/v) stacking gel], which was prepared according to Laemmli (1970). The protein samples were separated under a constant voltage at 90 volts through the stacking gel and 120 volts through the separating gel. Following electrophoresis, the protein gel was stained with Coomassie staining solution [45% (v/v) methanol, 1% (v/v) glacial acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R250] for 2 hours, and then destained with destaining solution [20% (v/v) methanol and 10% (v/v) glacial acetic acid] overnight (Sambrook and Russell, 2001).

3.6.3 Large-scale over-expression of *axe*10

In order to generate sufficient *axe*10 protein for purification and analysis, the expression was scaled up. Firstly, 5 ml of LB supplemented with 100 µg/ml Amp and 34 µg/ml Cm was inoculated with *E. coli* Rosetta (pET_AXE10) and incubated at 37^{o}C for 16 hours with shaking (100 rpm). The whole volume of the starter culture was used to inoculate 200 ml LB supplemented with 100 µg/ml Amp and 34 µg/ml Cm, and incubated with shaking at 37^{o}C for approximately 7 hours until the OD_{600} reached approximately 0.6. Overexpression of *axe*10 was induced by the addition of 0.2 mM IPTG and the culture was incubated for an additional 16 hours at 37^{o}C with shaking (100 rpm). Thereafter, the cells were harvested by centrifugation (4,000 rpm for 10 min at 4°C), the supernatant discarded, while the cell pellet was retained and stored at -20^{o}C overnight.

3.7 Axe10 protein purification

3.7.1 His-tag purification of Axe10

Axe10 was purified using Ni-chelating chromatography prepared with His-Bind resin and buffer kit (Novagen) according to the manufacturers' instructions. Briefly, the column was charged and equilibrated by running three column volumes of sterile distilled water through, five column volumes of charge buffer (50 mM $NiSO_4$) and 3 column volumes of binding buffer (0.5 M NaCl, 5 mM imidazole and 20 mM Tris-HCl, pH7.9). *E. coli* Rosetta (pET_AXE10; Section 3.6.3) was sonicated in 1x binding buffer and centrifuged to pellet the cell debris. The enzyme containing cell-free extract was retained and filtered through a 0.45 µm syringe filter (Millipore). This was allowed to pass through the column under gravity flow. Thereafter, the column was washed with 10 volumes 1x binding buffer, 6 volumes 1x washing buffer (0.5 mM NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH7.9) and 6 volumes 1x elution buffer (0.5 mM NaCl, 20 mM Tris-HCl, 62.5 mM imidazole, pH7.9). The eluted protein fraction was dialysed against 200x volumes of 50 mM NaH_2PO_4 buffer (pH 7.0) containing 100 mM NaCl at 4^{o}C for 16 hours. A volume of approximately 20 µl of every fraction from each step of the column purification was prepared, separated and visualized on a 12% (w/v) SDS-PAGE gel, as described in section 3.6.2.

3.8 Axe10 protein characterisation

3.8.1 Protein quantification

Purified Axe10 protein was quantitated using the Bradford protein assay (as described in Section 3.6.1).

3.8.2 Substrate specificity

In order to determine the substrate specificity of Axe10, the activity of the purified enzyme was assessed using p-nitrophenyl esters (Sigma), including C2 (acetate), C3 (propionate), C8 (caproate), C10 (decanoate), C12 (laurate) and C16 (palmitate) chain lengths. The enzyme assays were performed as follows: 980 µl of buffer (100 mM NaH_2PO_4, 100 mM NaCl, pH7.5), 0.5 µM of substrate and 2 µg enzyme were thoroughly mixed in 1 ml cuvette and the absorbance (405 nm) determined for 1 min at 25^{o}C using a Cary 50 Bio spectrophotometer (Varian, CA, USA). A reaction to determine the blank rate was performed for each measurement.

3.8.3 α-naphthol standard curve

Additional AXE activity assays (effect of temperature and pH) were performed using α-naphthyl acetate as substrate, as described in section 3.2.1.

3.8.4 Effect of pH and temperature on Axe10 activity

To determine the optimum pH of Axe10, the AXE assay was performed, as described in section 3.2.1, across a pH range of 1.0-11.0.

Purified Axe10 was assayed as described previously (section 3.2.1) across a range of incubation temperatures (22, 30, 37, 40, 45, 50 and 60^{o}C) to assess the effect of incubation temperature on enzyme activity.

3.8.5 Axe10 thermostability

Purified Axe10 was incubated at various temperatures (30, 37, 45 and 50^{o}C) for up to 60 minutes. Aliquots were taken every 10 min and immediately placed on ice. To determine the residual activity, a sample of Axe10 was placed on the ice for the duration of the experiment, as an untreated control. Thereafter, the AXE assay was performed on these samples and the effect of thermal pre-incubation determined by comparing the residual activity of the heated samples to the untreated control.

3.8.6 Axe10 steady state enzyme kinetics

Initially AXE enzyme assay was performed using 1mM α-naphthyl acetate for a series of time intervals (1, 2, 3, 4, 5, 7.5, 10, 15, 20, 25 and 30 min) in order to determine the linear range of Axe10 activity. A curve was generated by plotting the product concentration and a time point within the linear portion of

the curve was selected for use in determining the initial rates and kinetic data.

For kinetic data, initial rates were measured over the substrate (α-naphthyl acetate) range of 0.1 to 5 mM, as described in section 3.2.1. The data was analysed using the direct linear method provided by the Enzpack programme (Biosoft Software for Science, UK) and K_M and V_{max} values were determined. Kinetic parameters were determined by non-linear fitting of data to hyperbolic curves according to Michaelis-Menten (GraphPad Prism v. 4.00, GraphPad Software, San Diego, CA, USA).

Chapter 4

Results and Discussion

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Chapter 4

Results and Discussion

4.1 Strain isolation and characterisation

ORS10 was isolated from Namibian desert soil by Dr. M. Le-Rose Hill (Biocatalyst and Technical Biology Research Group, CPUT, SA). The sampling site is considered to be thermophilic and oligotrophic. ORS10 grew under thermophilic conditions at 45°C and showed typical actinobacterial colony morphology (data not shown).

4.1.1 Hemicellulosic enzyme screening

A series of solid and liquid assays were performed to identify potential hemicellulosic degrading enzymes produced by ORS10. Xylanase solid-phase plate assays indicated that ORS10 had extracellular xylanolytic activity (Fig. 4.1). Actinobacterial isolates have a lot of potential for the production of a variety of biocatalysts (Arisawa *et al.,* 2002; Molinari *et al.,* 2005), including lignocellulosic degrading enzymes (Ladjama *et al.,* 2007; Rifaat *et al.,* 2008). Previous studies have isolated streptomycetes by solid-phase plate assays supplemented with xylan. *Streptomyces* sp. CD3 was shown to produce extracellular xylanase(s) by solid-phase screening on agar plates containing oat spelt xylan (Shama and Bajaj, 2005). Similarly, Sousa *et al.* (2008) isolated xylanolytic *Streptomyces* isolates using xylan as the sole carbon source.

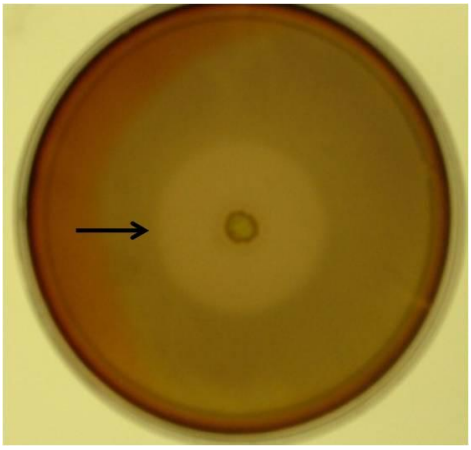

Figure 4.1 Extracellular xylanase activity of ORS10 when cultured on 1% (w/v) birchwood xylan supplemented growth media. Extracellular xylanase activity was clearly visible as a zone of xylan clearing (indicated by the black arrow) surrounding the ORS10 colony.

The optimum reaction temperature of the ORS10 crude extracellular xylanase enzyme preparation was 65^{o}C (Fig. 4.2), suggesting that this enzyme(s) may be thermophilic and/or thermostable. Similar results were found in recent studies. An extracellular xylanase produced by *Streptomyces cyaneus* SN32 was determined to have an optimal temperature at 60-65^{o}C (Ninawe *et al.,* 2008), while a xylanase isolated from *Streptomyces sp.* Ab106 has optimal activity at 60^{o}C (Techapan *et al.,* 2002).

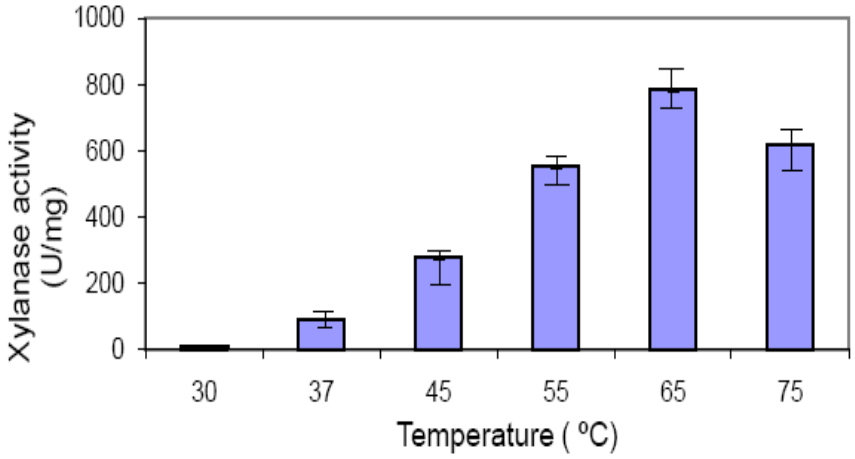

Figure 4.2 The effect of reaction temperature on ORS10 crude xylanase activity. 1 U Xylanase activity represents the amount of enzyme needed to produce 1 µmol xylose per min under the specified assay conditions. Data represents the mean ± standard error (n=3).

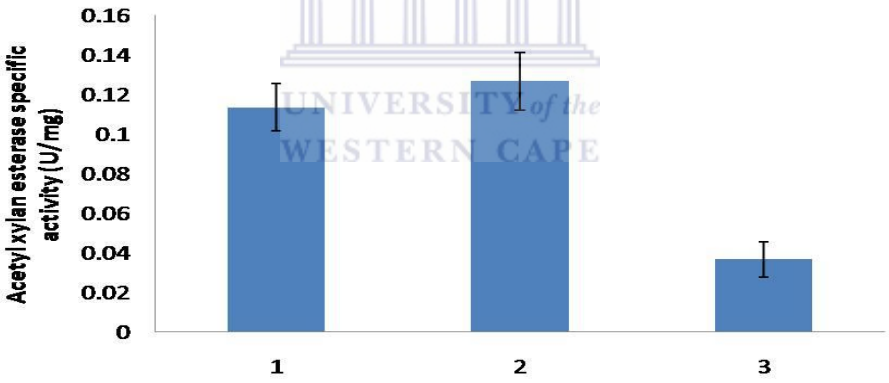

Figure 4.3 AXEase activity of ORS10 (1), *E. coli* Epi300 (pFOS_IB2) (2). *E. coli* Epi300 (3). 1 U of AXEase activity is the amount of enzyme needed to produce 1 µmol á-Naphthol per min under the specified assay conditions. Data represents the mean ± standard error (n=3).

In addition to extracellular xylanase activity, isolate ORS10 displayed acetyl xylan esterase (AXEase) activity (Fig. 4.3). In *Streptomyces lividans*, the *axe*A gene, encodes AxeA protein, is located downstream from *xln*B, which

encodes xylanase B (Shareck *et al.,* 1995). Both of these enzymes have been shown to be involved in the *S. lividans* xylanolytic system. In this study, an AXEase was induced and expressed by ORS10 in xylan supplemented liquid culture. A possible explanation for this finding is that birchwood xylan may contain some acetylated xylan, which could induce the expression of the AXEase. A study on the *Penicillium purpurogenum axe*II gene demonstrated that *axe*II was differentially expressed in response to different carbon sources and showed particularly high expression levels in xylan-containing growth medium (Chavez *et al.,* 2004). Furthermore, a study of *Aspergillus niger axe*A showed that this gene was also expressed in the presence of birchwood xylan (Van Peij *et al.,* 1998). The ORS10 AXEase may also be induced by xylose. ORS10 xylanase expression may result in the hydrolysis of birchwood xylan to xylose, which in turn may induce the ORS10 AXEase gene. Dupont *et al.* (1996) demonstrated that the *S. lividans axe*A gene was expressed when D-xylose was used as the sole carbon source.

4.1.2 ORS10 genomic DNA extraction

Chromosomal DNA extraction from actinobacteria, especially *Streptomyces* species, is considered to be very challenging (Cotârlet *et al.,* 2010). Some of the key issues in this regard are the manner in which many actinobacterial isolates grow in liquid cultures and the nature of their cell walls which makes them highly recalcitrant to enzymatic and chemical lysis (Lee

et al., 2003). When cultivated in shake flasks, ORS10 mycelia intertwined and formed “fluffy” balls (data not shown), a growth phenomenon characteristic of many actinobacteria (Whitaker, 1992), protecting cells against cell lysis (Lee *et al.,* 2003). In an attempt to reduce the formation and relative size of these balls, ORS10 was pre-cultured in a small volume (10 ml) of liquid broth for 24 hours and then transferred into a larger volume (100 ml) of growth medium.

The yields and quality of the chromosomal DNA isolated from ORS10 cultures using the routine Wang *et al.* (1999) method were not sufficient for the construction of a small-insert genomic library. Optimization of the Wang extraction protocol, by increasing the lysozyme concentration and incubation time, resulted in a significant improvement in ORS10 chromosomal DNA yields and quality (Table 4.1 and Fig. 4.4). When the two extraction protocols were compared (Table 4.1), the modified method consistently yielded ORS10 chromosomal DNA with higher 260/280 ratios, in comparison to those obtained for the Wang method. A high 260/280 ratio (>1.7) indicates pure DNA, while a low ratio (<1.7) is indicative of protein contamination (Yeates *et al.,* 1998). In addition, the modified method resulted in significantly less shearing of ORS10 chromosomal DNA (Fig. 4.4).

Table 4.1 Comparison of ORS-10 chromosomal DNA yield and quality obtained using the original Wang *et al.* (1999) and modified Wang *et al.* (1999) extraction protocols. Data represents the mean ± standard error (n=3).

Extraction Protocol	Concentration (ng/ìl)	260/280 ratio
Wang *et al.* (1999)	68 ± 25	1.43 ± 0.16
Modified method	1053 ± 120	1.93 ± 0.24

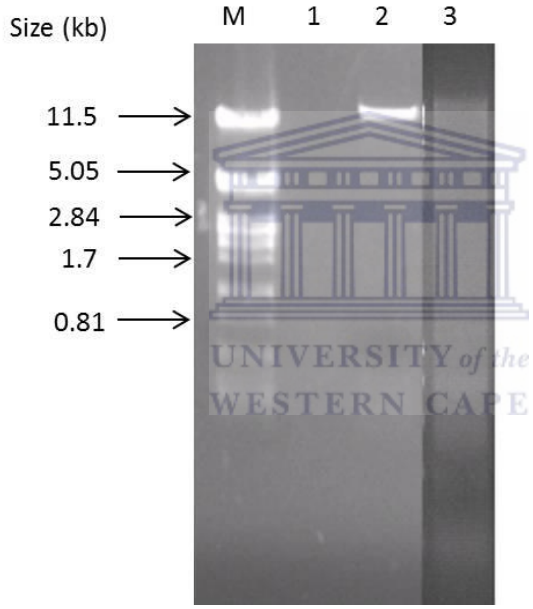

Figure 4.4 Comparison of ORS10 chromosomal DNA extracted using the original and modified Wang *et al.* (1999) methods, respectively. Lane M, λ-*Pst*I DNA molecular weight marker; lane 1, empty; lane 2, approximately 200 ng of ORS10 chromosomal DNA extracted using the modified method and; lane 3, approximately 200 ng ORS10 chromosomal DNA extracted using the original method. The arrows indicate the approximate sizes of the bands in kilobase pairs (kb).

The class Actinobacteria includes a diverse group of microbes and there is

no one DNA extraction protocol which works efficiently for all genera. A degree of optimization of existing protocols is required for all isolates. A recent study by Cotârlet *et al.* (2010) compared different chromosomal DNA extraction methods, in terms of chromosomal DNA yield and purity, for a cold-adapted *Streptomyces* strain. They found that the Powersoil™ DNA isolation kit (MO BIO Laboratories Inc., USA) yielded sufficient quantities and purity of DNA for their purposes. In addition to the use of commercial kits, some researchers employ chemical and enzymatic reagents such as CTAB to optimize the extraction protocol (Tripathi and Rawal, 1998). The modifications that we made to the existing protocol enabled us to isolate high molecular weight (HMW) genomic DNA (≥50 kb) at high yield and purity, with limited of RNA and/or protein contamination.

4.1.3 16S rRNA phylogenetic analysis of ORS10

For phylogenetic analysis, a 1,444 bp fragment of the ORS10 16S rRNA gene was amplified (Fig. 4.5), sequenced and assembled. A BLAST search of the GenBank database revealed that the ORS10 16S rRNA gene sequence has high similarity to a number of *Streptomyces* species, with the highest similarity (99%) to *Streptomyces achromogenes* subsp. *achromogenes* (Table 4.2). A neighbor-joining tree, containing related 16S rRNA sequences, confirmed that ORS10 clustered within the genus *Streptomyces* (Fig. 4.6).

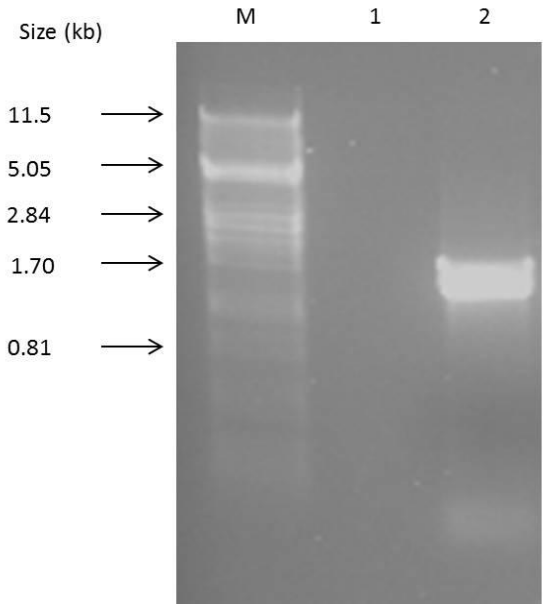

Figure 4.5 PCR amplification of ORS10 16SrRNA gene. Lane M, λ-*Pst*I DNA molecular weight marker; lane 1, negative (no template) control; lane 2, ORS10. The single band (approximately 1.5 kb) in lane 2 indicates the amplification of the ORS10 16S rRNA gene. The arrows indicate the approximate sizes of the bands in kilo base pairs (kb).

Phylogenetic analysis using the 16S rRNA gene is a powerful tool for the identification of microorganisms (Weisburg *et al.,* 1991). Conventional identification of actinobacterial isolates is based upon a combination of phylogenetic tools, together with the biochemical and physiological characteristics of the strain. Zhong *et al.* (2011) identified a novel *Streptomyces* species, isolate Eir11, from the Rhizosphere of *Rhizoma curcumae longae* by comparison of its 16S rRNA sequence against the GenBank database. Similarly a novel *Streptomyces koyangensis* sp. nov*.,* was identified using 16S rRNA gene analysis (Lee *et al.,* 2005).

Table 4.2 Identities of the closest related 16S rRNA sequences, obtained from a BLAST search of the NCBI database, to the ORS10 16S rRNA sequence (Database accessed 01/06/2011).

Accession no.	Description	Query coverage	Max identity
AB184109.1	*Streptomyces achromogenes subsp. achromogenes*	100%	99%
AB184503.1	*Streptomyces diastatochromogenes subsp. Luteus*	100%	98%
AB184486.1	*Streptomyces olivogriseus*	100%	98%
AB184192.1	*Streptomyces cellostaticus*	100%	98%
NR_043352.1	*Streptomyces durhamensis* strain NRRL B-3309	100%	98%
AB184387.1	*Streptomyces griseochromogenes*	99%	98%
NR_041083.1	*Streptomyces filipinensis* strain NBRC 12860	99%	98%
X79325.1	*Streptomyces galbus*	100%	98%
AB184444.1	*Streptomyces fimbriatus*	100%	98%
AB184074.1	*Streptomyces viridochromogenes*	100%	98%
AB045868.1	*Streptomyces fimbriatus*	100%	98%
AB184473.2	*Streptomyces griseochromogenes subsp. suitaensis*	100%	98%
AB184472.2	*Streptomyces globifer*	100%	98%
NR_043855.1	*Streptomyces niveoruber*strain NRRL B-2724	100%	98%

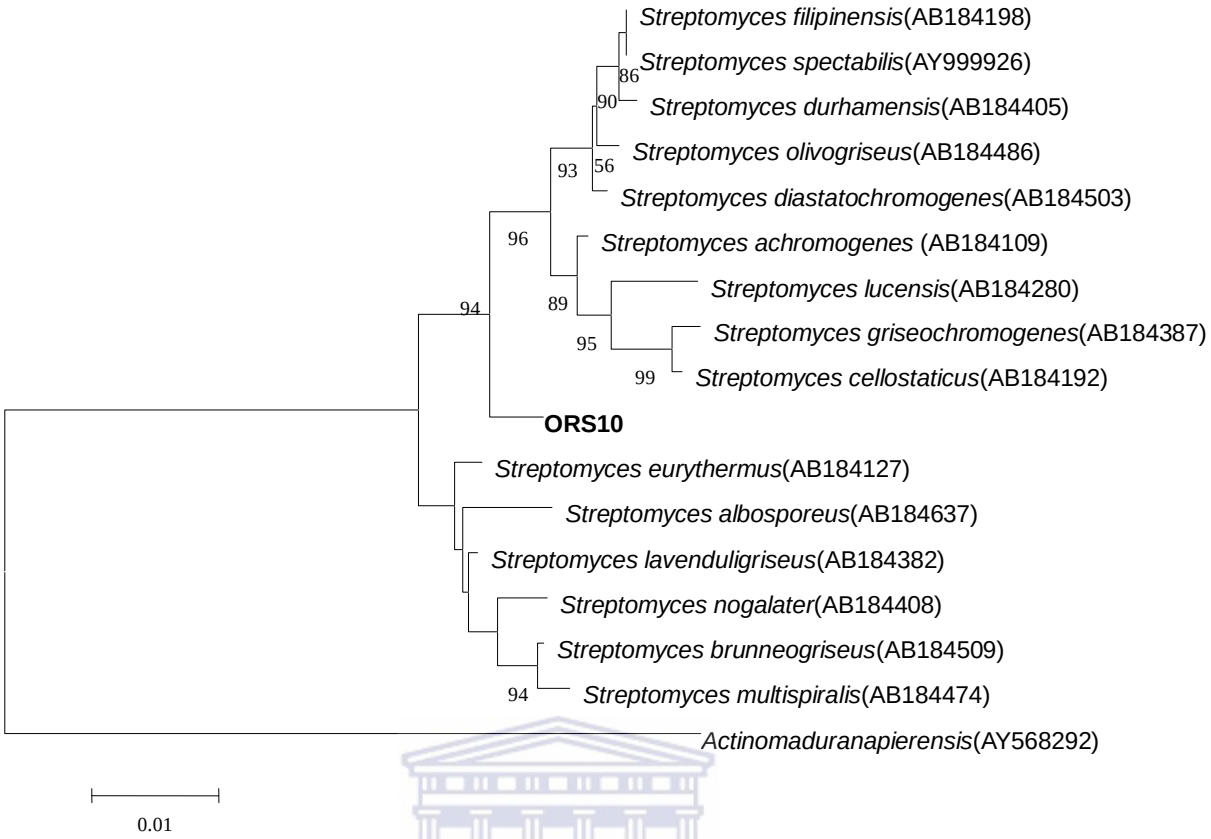

Figure 4.6 Neighbor joining phylogenetic tree, based on a partial 16S rRNA gene sequences showing the relatedness of isolate ORS10 to its closest phylogenetic neighbors within the genus *Streptomyces*. The robustness of the tree is based on 1,000 replicates and only bootstrap values greater than 40 are shown. The number shown in brackets represents the accession number of each species. *Actinomadura napierensis* was used as an outlier group.

4.1.4 Biochemical and physiological tests

In order to obtain a more accurate idea of ORS10 and its phylogenetic relatedness, a comparison of its biochemical and physiological characteristics to that of *S. achromogenes* was performed (Table 4.3). Several biochemical and physiological tests were performed on ORS10 and compared to published data for *S. achromogenes* (Garrity *et al.,* 2007).

Table 4.3 Physiological and biochemical characteristics of ORS10, in comparison to *S. achromogenes* (Garrity *et al.,* 2007).

		ORS10	***S. achromogenes***
Carbon utilization	Glucose	+	+
	Fructose	+	+
	Sucrose	+	-
	Xylose	+	-
	Lactose	+	-
	Mannitol	+	+
	Arabinose	+	+
Melanin production	ISP 6	+	+
	ISP 7	+	+
Degradation activity	Adenine	+	+
	Guanine	-	+
	Starch	+	+
	L-tyrosine	-	+
	Tween-80	-	+
Optimum growth temperature		45°C	28°C

ORS10 was capable of growth on most ISP media (Table 4.3) and when grown on ISP 5 the colony appeared to be covered with yellow aerial mycelium. ORS10 was capable of growth between 30-55^{o}C, with an optimum growth temperature of 45^{o}C (Table 4.3), but failed to grow at 65^{o}C (data not shown). A pH profile growth test showed the optimum pH for growth of ORS10 was 7.0, although ORS10 was capable of growth across a pH range of pH4.5-9. Furthermore, ORS10 produced spores with different colours, depending on the

pH of the media, with the colour getting darker as the pH increased (data not shown).

Although 16S rRNA analysis (section 4.1.3) showed that ORS10 has the highest similarity to *S. achromogenes* subsp*. achromogenes*, these two strains have several significant differences with respect to their biochemical and physiological characteristics. These differences include the optimum temperature for growth and utilization of carbon source. Furthermore, these differences may suggest that ORS10 belongs to a novel subspecies of *S. achromogenes* or even a novel *Streptomyces* species*.* In addition to biochemical and physiological tests, DNA-DNA hybridization is valuable tool for the identification of novel strains (Cho and Tiedje, 2001). Lee *et al.* (2005) showed that despite a 16S rRNA analysis of 99%, similarity to the top related strain DNA-DNA hybridization was less than 70% homology, suggesting novelty. Therefore, DNA-DNA hybridization should be considered in a future investigation of ORS10 to further analyse the relatedness of this isolate to *S. achromogenes*.

4.2 Construction of an ORS10 small-insert genomic library

ORS10 genomic DNA was used to construct a small insert library in a *Streptomyces-E. coli* shuttle vector. The optimum ratio for ORS10 *Sau*3A restriction enzyme digestion was determined to be 0.1 unit/μl to 1 μg genomic

DNA with a reaction time of approximately 10 min at 37°C (data not shown). Large-scale restriction enzyme digestions were performed on five ORS10 chromosomal DNA samples for 8, 9, 10, 11 and 12 minutes, respectively, yielding genomic DNA fragments within the distribution range of 4-10 kb (Fig. 4.7).

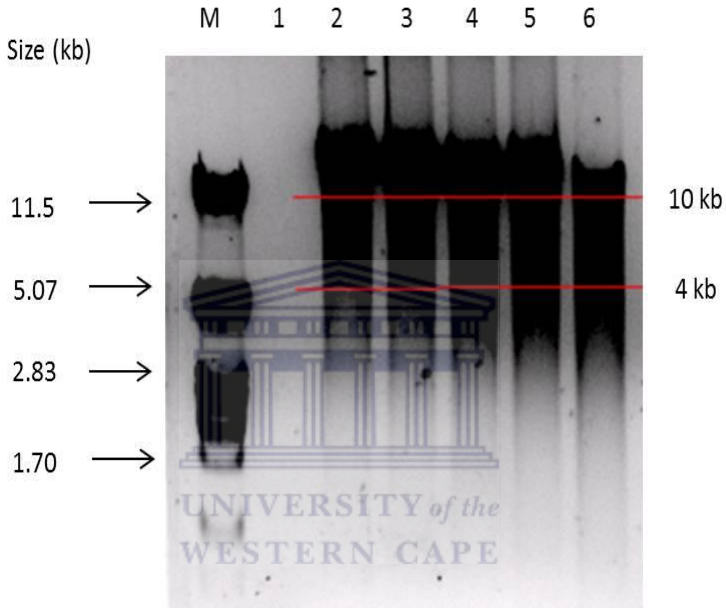

Figure 4.7 Large-scale partial *Sau*3A restriction enzyme digestion of ORS10 chromosomal DNA. Lane M, λ-*Pst*I DNA molecular weight marker; lane 1, empty; lanes 2-6, ORS10 genomic DNA digested with *Sau*3A for 8, 9, 10, 11 and 12 minutes, respectively. The arrows indicate the approximate sizes of the bands in kilo base pairs (kb). The DNA fragments between 4 and 10 kb (indicated with red lines) were excised.

Functional- or expression-based screening of a genomic library is an approach that can be adopted to identify gene(s) of interest. Genomic libraries include both small- (less than 10 kb) and large-insert libraries (up to 40 kb). The choice of approach is based upon several factors, including the desired

average insert size, copy number required and the quality of the sample DNA (Simon and Daniel, 2010). In this study, the aim was to identify hemicellulosic enzymes, such as xylanases and AXEases. The genes that encode these two enzyme classes are generally less than 2 kb in size (Dupont *et al.*, 1996; Fialho and Carmona, 2004; Collins *et al.*, 2005; Mo *et al.*, 2010). A small-insert metagenomic library with an average insert size of 4-9 kb has been successfully used to identify a xylanase gene (Hu *et al.,* 2008). In another study, *Streptomyces thermoviolaceus* genomic DNA has been used to construct a small insert library, with insert size range from 3-10 kb. This library was functionally screened and yielded two xylanases and a single acetyl xylan esterase (Tsujibo *et al.*, 1997). Therefore, the construction and screening of a small-insert library was considered suitable for the identification these enzymes from ORS10.

Three molar ratios [of insert DNA to prepared vector (1:2, 1:1 and 2:1)] for ligation were assessed to determine the optimal ratio for the genomic library construction. The 1:1 ligation ratio resulted in the best library coverage (data not shown), and the resulting genomic library comprised of approximately 8,000 *E. coli* clones. The average insert size of the library was estimated to be approximately 7 kb by restriction enzyme digestion of plasmid DNA extracted from randomly selected *E. coli* library clones (Fig. 4.8).

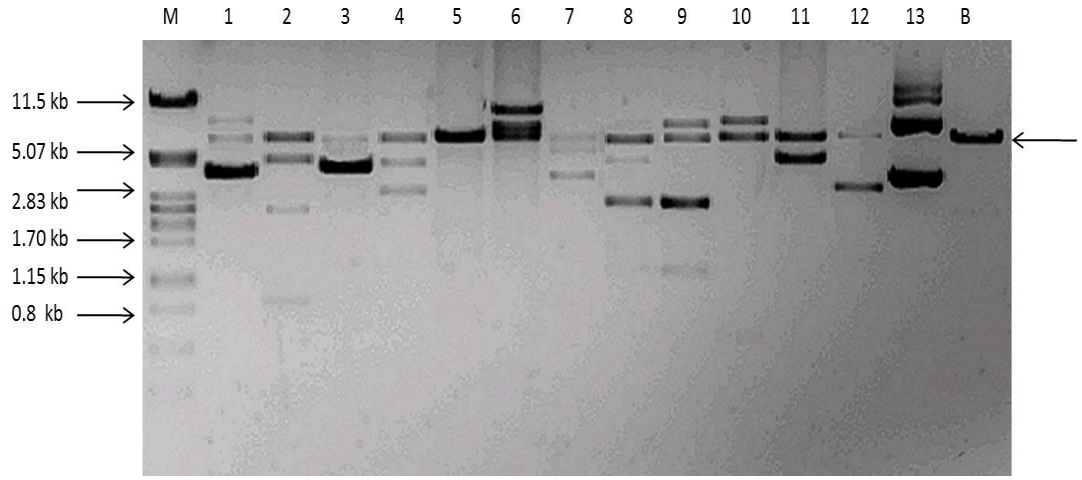

Figure 4.8 *Eco*RI and *Hin*dIII restriction enzyme profiles of 14 randomly selected ORS10 genomic library clones. Lane M: λ-*Pst*I DNA molecular weight marker; lane 1-13, plasmid DNA from selected recombinant pUWL219 constructs; lane B, Negative control of pUWL219. The arrows of the left hand side indicate the approximate sizes of the bands in kilo base pairs (kb). The arrow on the right hand side indicates the pUWL219 backbone (approximately 6.8 kb).

Using the genome coverage formula (W=Nl/G), it was estimated that the likelihood of finding a particular gene within the library, with 3-fold genome coverage (W), is approximately 95%. A 5-fold coverage would be required to increase the probability to 99% (Paterson, 1996). In this study, the full genome size of ORS10 is not currently known. Therefore, a streptomycete with a large genome (approximately 8.6 mega bases), *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002), was used as a conservative estimate of the ORS10 genome size. The genome coverage of the ORS10 library was calculated to be approximately 7-fold, which represents a probability of greater than 99% of finding a particular gene of interest within the genomic library. Therefore, the

ORS10 small insert genomic library constructed during the course of this study should be sufficient for expression-based screening for hemicellulosic enzymes.

4.3 Expression-based screening for hemicellulosic enzymes

4.3.1 Xylanolytic activity screening

Approximately 8,000 clones were screened on RBB-xylan to identify putative ORS10 xylanase(s). Despite an approximately 7-fold coverage of the ORS10 genome, no positive clones were identified (data not shown). This study relied on the *E. coli* cellular machinery for the successful expression, translation, folding and secretion of a gram-positive *Streptomyces* gene. It may be that *E. coli* lacks some of the necessary regulatory elements, contained within the *Streptomyces* cellular system, for this gene to be expressed. There is experimental evidence that suggests that there are numerous challenges relating to the successful expression of Gram-positive genes in Gram-negative hosts, like *E. coli*. In particular, researchers have reported similar issues when attempting to express *Streptomyces* genes. Vigal *et al.* (2006) reported that a *Streptomyces* amylase gene could not be expressed in *E. coli* and the gene product was detected within inclusion bodies. A comparison of *Streptomyces reticuli cel*-1 (cellulase) gene expression in different host systems found that this gene, together with its original upstream regulatory region, could not be expressed in *E. coli* (Walter and Schrempf, 1995).

The ORS10 small-insert genomic library was constructed in a *Streptomyces-E. coli* shuttle vector (pUWL219) and the library was maintained and screened in *E. coli*. Shuttle vectors such as pUWL219 (Wehmeier, 1995) have been constructed in order to study *Streptomyces* genes that otherwise cannot be expressed in *E. coli* alone. The use of these plasmid vectors could help to increase the likelihood of successfully expressing *Streptomyces* genes in either *E. coli* or established *Streptomyces* host systems, such as *S. lividans*. Shuttle vectors have been used successfully in several studies to express genes from different microorganisms. For example, Wang and Li (2002) successfully expressed a CTLA-4 gene in *S. lividans* using the shuttle vector pUWL219. In another study, researchers cloned the *Vitreoscillia* hemoglobin gene (*vhb*) into *S. lividans* and successfully expressed the protein (Yang *et al.*, 1998). The ORS10 library constructed in this study was not screened in a *Streptomyces* host system. The reason for not attempting this during the course of this investigation was primarily due to the numerous challenges associated with *Streptomyces* transformation, the most important being the very low transformation efficiencies that are generally obtained for *Streptomyces* (Kwak *et al.,* 2002). This makes it very difficult to achieve sufficient genome coverage for efficient library screening. Research is currently being done to enhance *Streptomyces* transformation efficiencies (Suzuki *et al.*, 2011). However, current efficiencies are still not high enough to

utilize *Streptomyces* as a genomic library host system.

4.3.2 Acetylxylan esterase activity screening

As no xylanase positive clones were identified from the ORS10 library by RBB-xylan plate screening (data not shown), the library of 8,000 clones was further screened for putative esterase activities. This primary screen yielded four clones positive for general esterase activities as observed by a zone of clearing on trybutyrin agar (Fig. 4.9). These clones were subsequently screened for accessory enzyme activities.

Secondary screening of these four clones for hemicellulosic accessory enzymes indicated that all four clones were negative when screened for p-coumaric acid esterase and ferulic acid esterase activities. However, a single clone harbouring the plasmid named pUWL_AXE10 was shown to produce a putative AXEase (Fig. 4.10). A crude enzyme preparation from *E. coli* Genehog (pUWL_AXE10) displayed comparable AXEase activity to an AXEase positive control, *E. coli* Ep300 (pFOS_IB2) (Fig. 4.10). Furthermore, there was negligible AXEase activity detected for the cell-free extract of the negative control strain, *E. coli* Genehog (pUWL219). These results suggest that an AXEase is encoded by the cloned ORS10 genomic DNA fragment within pUWL_AXE10.

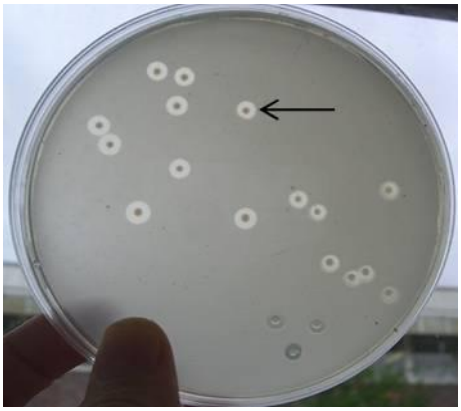

Figure 4.9 ORS10 genomic library clones displaying an extracellular esterase phenotype when cultured on tributryin agar plates. Extracellular esterase activity was identified by visual detection of clear zones of tributyrin hydrolysis surrounding the *E. coli* colony (indicated by the arrow).

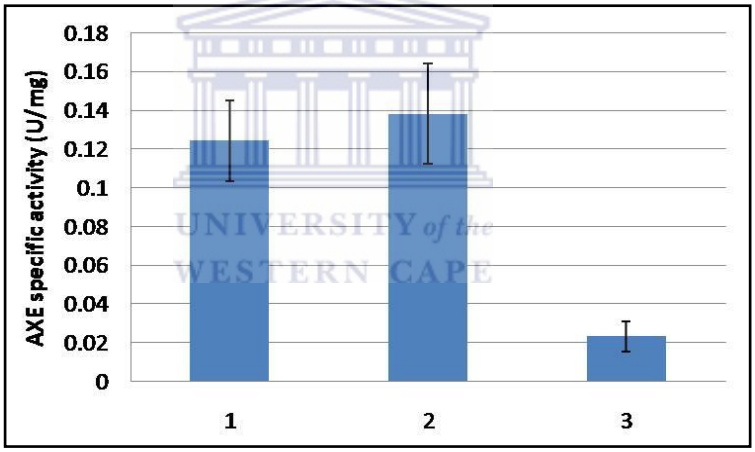

Figure 4.10 AXEase specific activity profile of (1) *E. coli* Genehog (pUWL_AXE10), (2) *E. coli* Ep300 (pFOS_IB2) and (3) *E. coli* Genehog with pUWL219. Data represents the mean ± standard error (n=3).

4.4 Identification and sequencing of the cloned AXEase gene

4.4.1 Plasmid DNA extraction and transposon mutagenesis

The insert size of the recombinant plasmid pUWL_AXE10 was determined to be approximately 10 kb by restriction enzyme analysis (data not shown). To

date, most of the ORFs encoding AXEase genes studied have been approximately 1,000 bp or less in size (Dupont *et al.*, 1996; Degrassi *et al.*, 1998; Koseki *et al.*, 2006). In order to avoid primer walking to identify the gene encoding the AXEase, we made use of a commercial transposon mutagenesis kit. Transposon mutagenesis has been successfully employed in a number of studies (Codling *et al.,* 2004; Hasselbring *et al.,* 2006; Kamisaka *et al.,* 2006). Approximately 200 transposon-mutated clones were identified. Preliminary analyses indicated that approximately 20% of these clones had lost, or had an altered, extracellular esterase activity phenotype. Confirmation of putative 'knock-out' mutants was performed as some of the mutants had no esterase activity (Fig. 4.11), while others were observed to produce variably sized zones of clearing on tributyrin agar plates, in comparison to an un-mutated pUWL_AXE10 control.

Enzyme-containing cell-free extracts of selected *E. coli* (pUWL_AXE10) transposon mutants were used to confirm that the loss of an esterase phenotype correlated with a loss of AXEase activity (Fig. 4.12). As had been observed on the trybutyrin screening plates, transposon-treated pUWL_AXE10 mutants displayed variable levels of AXEase activity (Fig. 4.12). The relative AXEase activity levels of these mutants correlated with the size of the zones of trybutyrin clearing observed for those mutants (data not shown).

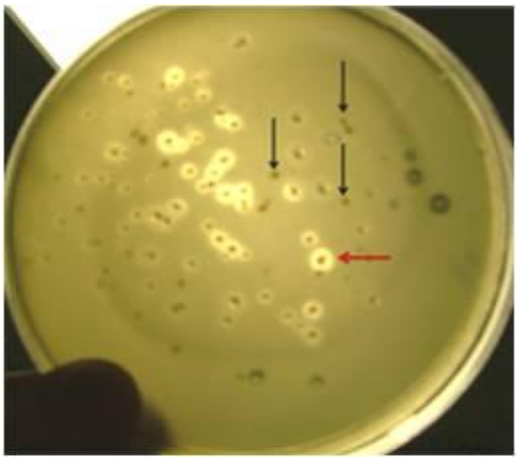

Figure 4.11 A tributryin agar plate on which HyperMu™ (CHL-1) transposon treated pUWL_AXE10 *E. coli* Genehog clones were cultivated. A number of *E. coli* clones lost their esterase activity phenotype, due to the integration of the transposon into the recombinant plasmid (indicated by black arrows), while other clones retained their extracellular esterase activity despite transposon integration (indicated by red arrow).

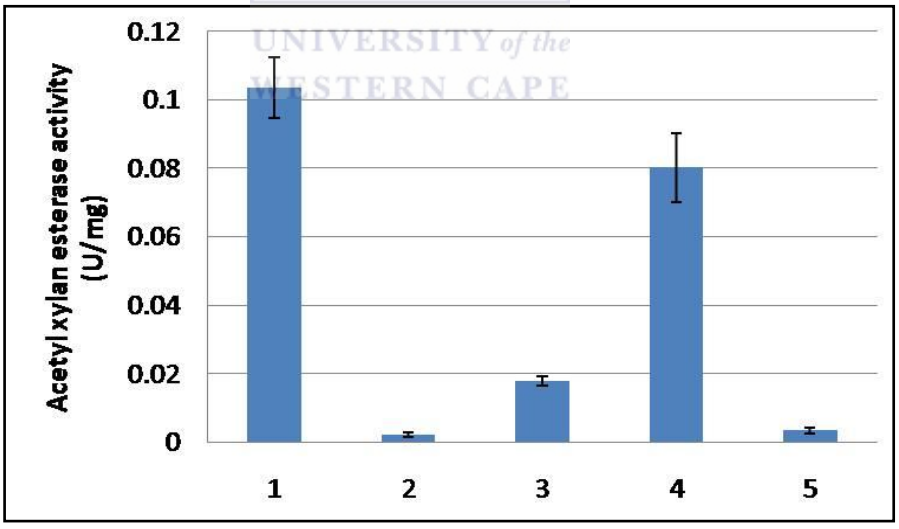

Figure4.12 AXEase activity of (1) *E. coli* Genehog (pUWL_AXE10), (2) *E. coli* Genehog (pUWL_AXE219_TM-1), (3) *E. coli* Genehog (pUWL_AXE219_TM-6), (4) *E. coli* Genehog (pUWL_AXE219_TM-8) and (5) *E. coli* Genehog (pUWL219). Data represents the mean ± standard error (n=3).

Plasmid mutants appeared to lose AXEase activity to different levels following HyperMu™ (CHL-1) transposon treatment (Fig. 4.12). The variation

observed in AXEase and esterase activities may be due to the different locations of transposon integration into pUWL_AXE10. The *axe* gene was expressed under the control of the native ORS10 promoter element. The promoter element could have been interrupted, to varying degrees, by the integration of the transposable element. The degree of interference may in turn affect the ability of the promoter, potentially leading to the variable levels of AXEase activity that were observed for the mutants. Similar result were shown by Hu and Coates (2005), when they performed transposon mutagenesis on 5,000 clones to identify a antimicrobial gene and found nine clones which become sensitive to selected antibiotics at different levels. In this study, four transposon mutants (Fig. 4.12) with a complete loss of AXEase activity were subsequently used to identify the cloned ORS10 *axe* gene.

4.4.2 Sequence assembly and ORF predication

The sequences of four 'knock-out' mutants (pUWL_AXE10-TM1, pUWL_AXE10-TM2, pUWL_AXE10-TM3 and pUWL_AXE10-TM4) were edited and assembled (Fig. 4.13) in order to obtain the full length sequence of the putative ORS10 AXEase gene, *axe*10 (Fig. 4.14). An ORF of 795 bp encoding a 265 amino acid protein was identified (Fig. 4.14). Protein BLAST of the deduced amino acid sequence revealed a conserved esterase/lipase superfamily domain (Fig. 4.15). The molecular weight of Axe10 was estimated to be approximately 27.7 kDa (DNAMAN), which is comparable to an AXEase

produced by *Thermobifida fusca* (Yang *et al.,* 2010).

Four α/β hydrolase fold-1 domains were identified within the Axe10 deduced amino acid sequence (Fig. 4.14). α/β Hydrolase fold domains are commonly found within a number of hydrolytic enzymes, including carboxyl esterases (CE) (Ollis *et al.,* 1992). More specifically, α/β hydrolase fold domains have been identified within AXEases from various microorganisms (Blum *et al.,* 1999; Ghosh *et al.,* 1999; Hakulinen *et al.,* 2000; Gordillo *et al.,* 2006; Taylor *et al.,* 2006).

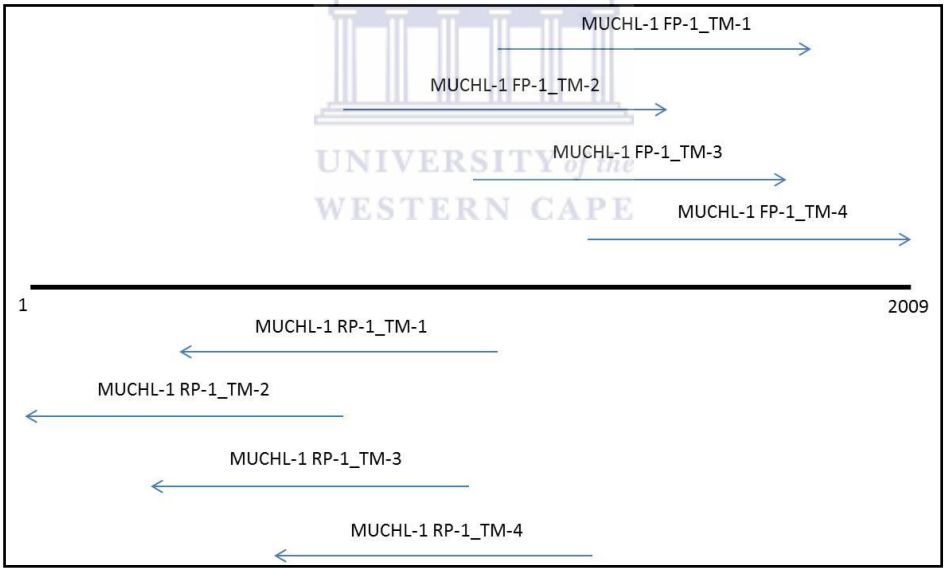

Figure 4.13 Diagramatic representation of the consensus sequence (2,009 bp) obtained by assembling the edited MUCHL-1 FP-1 and MUCHL-1 RP-1 from four pUWL_AXE10 'knock-out' mutants TM-1 to TM-4.

```
1        CCACAGCCACCACCCCGCAGTGATTCCCACTGAGAAACCGAAGGAGAGACACGTATGTCC
1                                                                M  S
61       ACTGCTCTGCCCACCTCAAGCCTCGGCCGTATCGTGCGCGGCACCGGCCCGGGCCTGCTG
3         T  A  L  P  T  S  S  L  G  R  I  V  R  G  T  G  P  G  L  L
121      CTCGCCCACGGCGCCGGCGGCGGCATAGACGCCAATTACGGCCCCGTCCTGGACACTCTG
23        L  A  H  G  A  G  G  G  I  D  A  N  Y  G  P  V  L  D  T  L
181      GCCGCCCAGCACACCGTCGTCGGGCCCGACTACCCCGGCACCGGCCGCACTCCGCGCGCA
43        A  A  Q  H  T  V  V  G  P  D  Y  P  G  T  G  R  T  P  R  A
241      AACGCTCCCCTTTCCCTGGACGGCCTGGCCGACGAGCTTGTCGCCACCGCCGTGGAGGAA
63        N  A  P  L  S  L  D  G  L  A  D  E  L  V  A  T  A  V  E  E
301      GGCGTGGAGACCTTCGCCATCGCGGGCTACTCGCTCGGCAGCCCGGTCGCCGTCCGGGCC
83        G  V  E  T  F  A  I  A  G  Y  S  L  G  S  P  V  A  V  R  A
361      GCCACCCGGCACCCCGAACGCGTCACCGCTCTCGTCCTCACCGCGGGTTTCGCCCACCCC
103       A  T  R  H  P  E  R  V  T  A  L  V  L  T  A  G  F  A  H  P
421      AACCCGCGGTTCCTGCTGGCGGCCCGGATGTGGCGCGACTTCCTCCGCTCGAACGACCTG
123       N  P  R  F  L  L  A  A  R  M  W  R  D  F  L  R  S  N  D  L
481      GAGGGCCTGGCCGGCTTCGTGTCCCTGATGGGCCTCAGCGCTCCCGTCCTGGACGCCGTA
143       E  G  L  A  G  F  V  S  L  M  G  L  S  A  P  V  L  D  A  V
541      GGCCAGGACGACCTGGACACGGCCATGAAGAACACCGCAGCCACGGTCCCGCCCGGCACG
163       G  Q  D  D  L  D  T  A  M  K  N  T  A  A  T  V  P  P  G  T
601      CCGGAACACGTGGACCTGCTCATCAACCACGTCGACGTACGAGCCGACCTCTCCACGGTC
183       P  E  H  V  D  L  L  I  N  H  V  D  V  R  A  D  L  S  T  V
661      ACCGTGCCGACCCTCGTCATTTCCACCACGCTCGACCAGCTCGTCACACCGCACCACCAC
203       T  V  P  T  L  V  I  S  T  T  L  D  Q  L  V  T  P  H  H  H
721      CGGCAACTCGCCGACGGCATCCCCGGCGCGCGATATGCCGAGATCCCCACCGGGCACCTG
223       R  Q  L  A  D  G  I  P  G  A  R  Y  A  E  I  P  T  G  H  L
781      CCGTTCATCGAGCAGCCCGAGCAGTGGGGCGCCCTCATGCGCGACTTCCTCCGCGACGTC
243       P  F  I  E  Q  P  E  Q  W  G  A  L  M  R  D  F  L  R  D  V
841      CGCGCCTGACTCCGGCCCCGCACCCGTCTCGGGCACTTCCCCGGCCGGGCTGTCGCCGAC
263       R  A  *
```

Figure 4.14 Nucleotide sequence of *axe10* and the deduced amino acid sequence of the AXEase protein, AXE10. The ATG start codon is highlighted in green (■), while an asterisk (*****) and red highlighting (■) indicates the TGA stop codon. The four alpha/beta hydrolase fold-1 domains (Q^{45}-P^{60}, I^{89}-A^{102}, A^{103}-T^{116} and T^{206}-H^{220}) predicted using Interproscan (www.ebi.ac.uk/interproscan/) are highlighted in turquoise (■), purple (■), yellow (■) and grey (■), respectively. The alpha/beta hydrolase_6 domain (L^{21}-W^{251}) is underlined. The esterase consensus sequence (Gly-x-Ser-x-Gly) is indicated with a black border, and the conserved active site residues (S^{93}, H^{106} and D^{214}) are in bold.

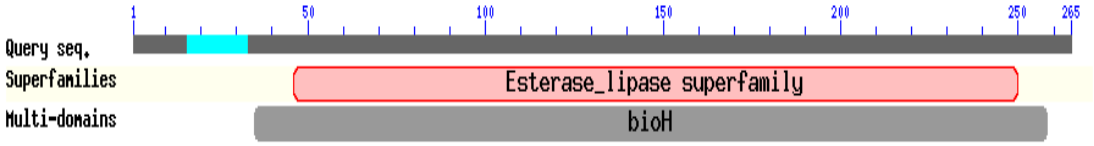

Figure 4.15 Conserved domains architecture within the deduced Axe10 protein sequence. Amino acids 45 to 251 have homology to the esterase/lipase protein superfamily (www.ncbi.nlm.noh.gov).

The predicted ORF identified in this study has the consensus sequence (Gly-X-Ser-X-Gly) within the second alpha/beta hydrolase fold-1 domain, I^{89}-A^{102} (Fig. 4.14). The consensus sequence (Gly-x-Ser-x-Gly) is commonly found in esterases (Gutiérrez *et al.,* 1998; Bornscheuer, 2002; Gordillo *et al.,* 2006). The serine in the centre of this sequence is an active site shared by most AXEases and forms part of a typical AXEase catalytic triad (Ser-His-Asp) (Gutiérrez *et al.,* 1998; Bornscheuer, 2002; Gordillo *et al.,* 2006; Puchart *et al.,* 2006; Taylor *et al.,* 2006). Based on a multiple sequence alignment of related proteins, the serine residue (S^{93}) at the centre of the motif identified in this study may represent the conserved serine residue with the active site catalytic triad (Fig. 4.16). Furthermore, the conserved catalytic residues, Ser^{93}, His^{106} and Asp^{214}, within Axe10 were identified from that of known and related AXEase sequences (Fig 4.16).

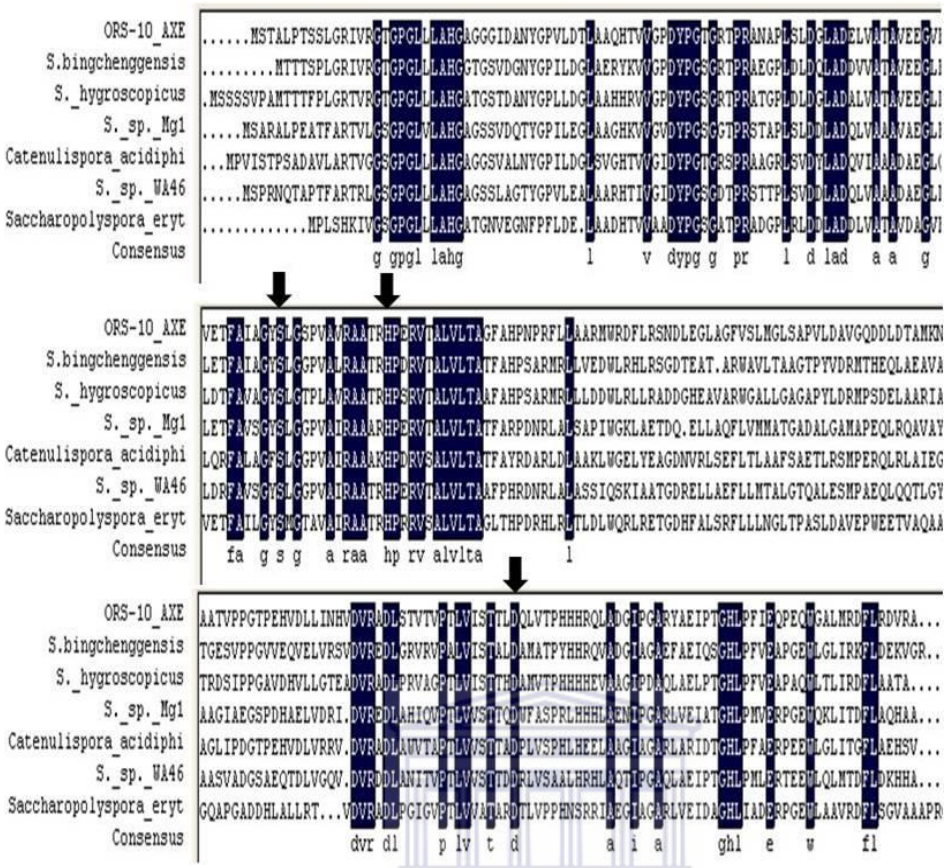

Figure 4.16 Multiple sequence alignment of axe10 with six top related protein sequences. The three putative catalytic residues (Ser[93], His[106] and Asp[214]) are indicated by the black arrows.

Table 4.4 Identities of the closest related protein sequences, obtained from a BLAST search of the GenBank database (accessed 03/06/2011), to the deduced Axe10 amino acid sequence.

Organism	Accession no.	Description	% Identity	*E*-value
Streptomyces bingchenggensis BCW-1	ADI10823.1	α/β hydrolase fold protein	62%	4e-81
Streptomyces hygroscopicus ATCC 53653	ZP_07298304.1	Putative hydrolase/decarboxylase	63%	3e-78
Streptomyces sp. Mg1	ZP_04999430.1	α/β hydrolase	56%	6e-72
Catenulisporaacidiphila	YP_003119315.1	α/β hydrolase fold protein	55%	5e-71
Streptomyces sp. WA46	BAC78382.1	putative hydrolase	55%	5e-68
Saccharopolysporaerythraea	YP_001103223.1	3-oxoadipate enol-lactonehydrolase/4-carboxymuconolactone decarboxylase	53%	2e-55
Salinisporaarenicola	YP_001537956.1	α/β hydrolase fold	46%	3e-48
Kineococcusradiotolerans	YP_001362051.1	α/β hydrolase fold	39%	2e-37
Streptosporangiumroseum	YP_003344184.1	Hydrolase	40%	1e-33
Streptomyces sviceus	ZP_06920598.1	esterase/lipase/thioesterase	38%	2e-33
Rhodobactersphaeroides	YP_001041841.1	esterase/lipase/thioesterase	39%	1e-27

4.5 PCR amplification and cloning of the *axe*10 gene

The *axe*10 gene was successfully PCR amplified (Fig. 4.17) from pUWL_AXE10 and cloned into pJet1.2/blunt. The resulting recombinant construct (pJET_AXE10) was isolated from *E. coli* Genehog and digested with *Nde*I and *Xho*I to release the 800 bp fragment, comprising the *axe*10 gene

(Fig. 4.18). The 800 bp DNA fragment was subsequently sub-cloned into pET21a(+). Colony PCR of *E. coli* clones presumably harbouring recombinant pET21a(+) constructs confirmed that *axe*10 had been successfully sub-cloned into pET21a(+) (Fig. 4.19), and the resulting construct was designated pET_AXE10. In addition, sequencing the recombinant construct pET_AXE10 showed that the *axe*10 gene had been cloned in-frame with the pET21a(+) vector encoded His-tag (Fig. 4.20).

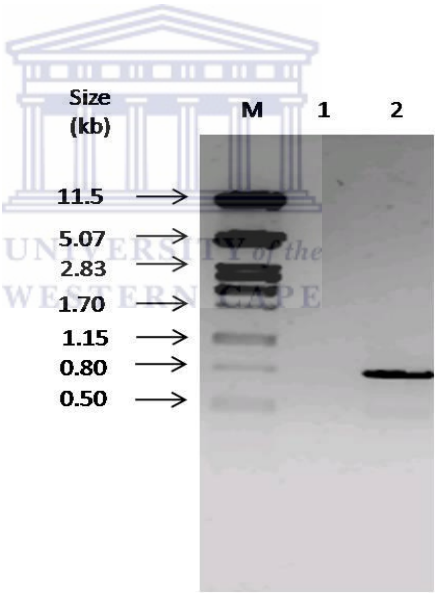

Figure 4.17 PCR amplification of *axe*10 gene from pUWL_AXE10. Lane 1: λ-*Pst*I DNA maker; lane 2: negative control (pUWL219); lane 3: pUWL_AXE10.

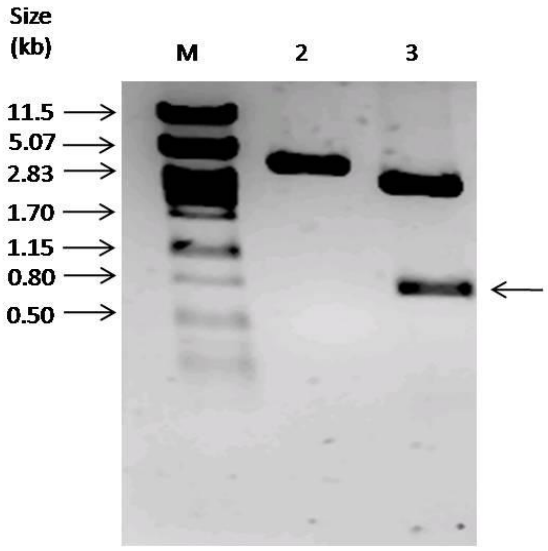

Figure 4.18 Restriction digestion of pJET_AXE10. M: λ-*Pst*I DNA maker; lane 2: pJET_AXE10 digested with *Nde*I; lane 3: pJET_AXE10 digested with *Nde*I and *Xho*I. The arrow on the right hand side indicates the *axe*10 gene fragment (approximately 800 bps).

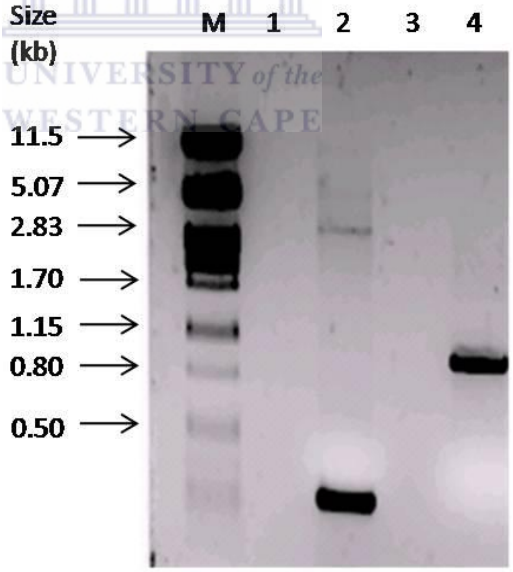

Figure 4.19 Colony PCR using T7 promoter and terminator primers. Lane M: λ-*Pst*I DNA maker; lane 2: pET21a(+) as negative control; lane 4: pET_AXE10; lane 1 and 3: Blank.

4.5.1 Activity of the cloned *axe*10 gene

The recombinant expression vector pET_AXE10 was transformed into

E. coli (DE3) Rosetta PlysS. The resulting transfomants were shown to have general esterase activity on tributryin agar plates (Fig. 4.21). Furthermore, *E. coli* Rosetta (DE3) PlysS (pET_AXE10) was shown to have AXEase activity when compared to *E. coli* Rosetta (DE3) PlysS [pET21a(+)] negative control (Fig. 4.22). From Figure 4.22, it is evident that even though the same amount of protein was used in each assay, *E. coli* Rosetta (DE3) PlysS (pET_AXE10) has significantly more AXEase activity than cells of the negative control, *E. coli* Rosetta (DE3) PlysS [pET21-a(+)]. In addition, *E. coli* Rosetta (DE3) PlysS (pET_AXE10) and *E. coli* Genehog (pUWL_AXE10) had comparable AXEase activity. Therefore, we have successfully cloned and expressed a functional AXEase gene.

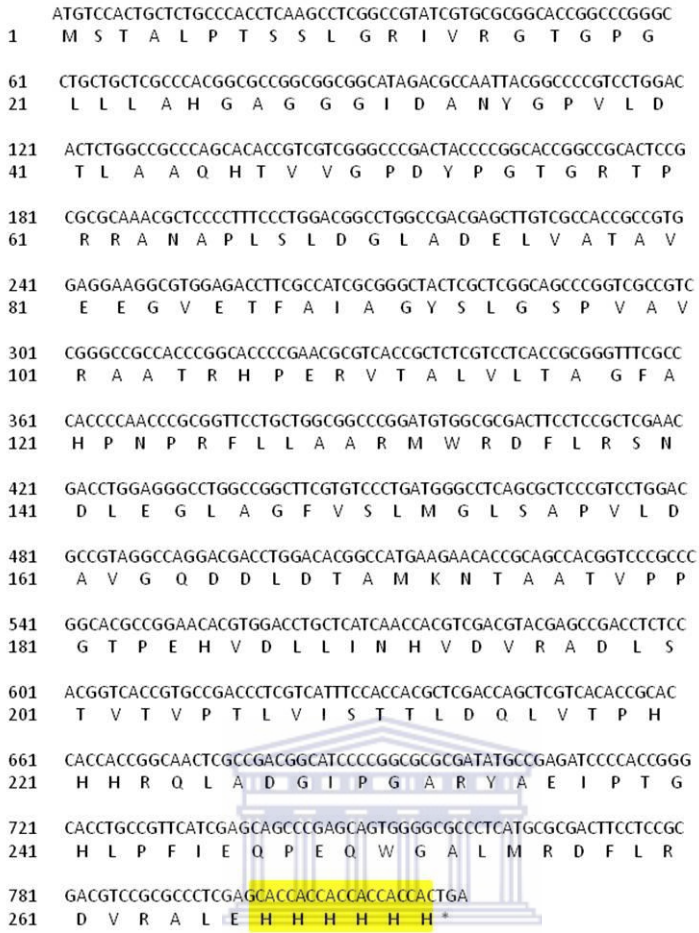

Figure 4.20 Translation of the *axe*10 gene sub-cloned in the protein expression vector pET21a(+). The deduced Axe10 amino acid sequence, containing a C-terminal six residue His-tag (highlighted in yellow) within the recombinant construct pET_AXE10 in shown together with the gene sequence. An asterisk (*****) indicates the TGA stop codon.

4.6 Over expression of *axe*10 and Axe10 purification

The recombinant Axe10 with a C-terminal 6X His-tag was heterologously expressed using pET21a(+) and purified using nickel-affinity chromatography. Analysis of the eluted fractions on an SDS-PAGE gel showed a single protein band of approximately 29kDa (Fig. 4.23). This apparently single protein species correlates with the predicted size of Axe10 including the addition of a six Histidine residue C-terminal tag.

Figure 4.21 Tributryin agar plate with *E. coli* Rosetta (DE3) PlysS harbouring pET_AXE10. Clear zone (indicated by the white arrow) surrounding *E. coli* (pET_AXE10) indicates esterase activity encoded by the cloned *axe*10.

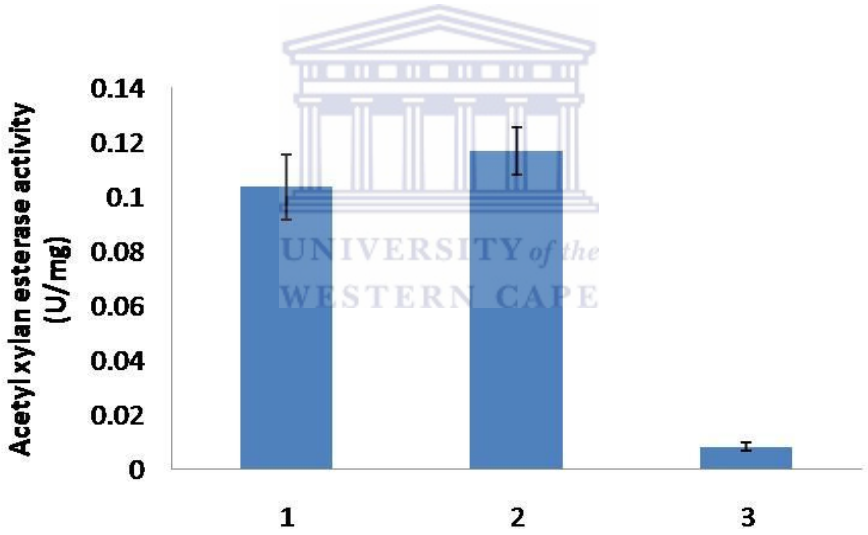

Figure 4.22 Acetylxylan esterase activity of (1) *E. coli* Rosetta (DE3) PlysS (pET_AXE10), (2) *E. coli* Genehog (pUWL_AXE10) and (3) *E. coli* Rosetta (DE3) PlysS [pET21a(+)]. One unit of activity defined as the amount of enzyme required to produce 1 ìmol á-Naphthol per minute under the assay conditions. Data represents the mean (n=3) ± standard error.

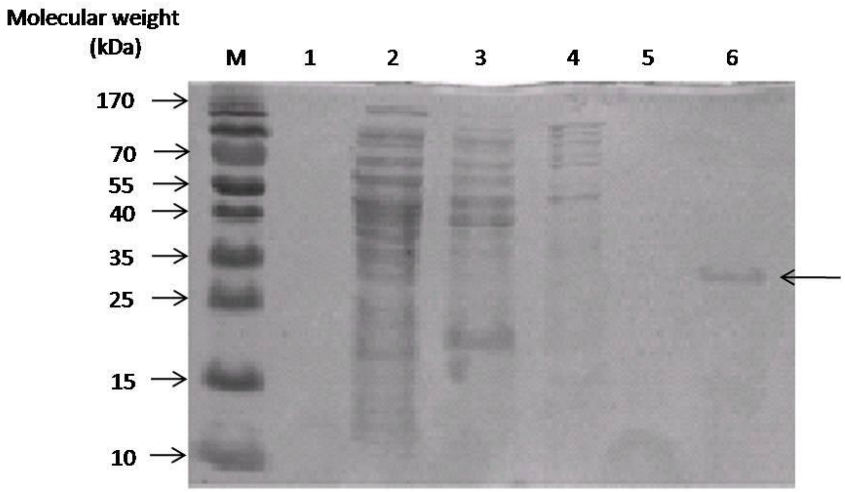

Figure 4.23 SDS-PAGE analysis of His-tag column purification of Axe10 from *E. coli* Rosetta (DE3) PlysS harbouring pET_AXE10. Overexpression of Axe10 was induced with 0.2 mM IPTG. Lane M: Protein maker (PageRuler protein ladder, Fermentas); lane 1, empty; lane 2, flow-through fraction; lane 3, binding buffer flow-through fraction; lane 4, wash buffer flow-through fraction; lane 5, strip buffer fraction and; lane 6,62.5mM Imidazole eluted Axe10. The arrows indicate the approximate sizes, in kDa, and positions of the molecular weight marker. The protein band corresponding to a size of approximately 29kDa is indicated by the black arrow.

4.7 Axe10 biochemical characterisation

In order to employ Axe10 in industrial processes, the biochemical characteristics of the enzyme need to be determined. Critical characteristics that were assessed include substrate specificity, optimum reaction temperature and pH, thermostability and enzyme kinetics.

4.7.1 Substrate specificity

Axe10 displayed activity against α-naphthyl acetate, reported before for rAwAXEA (Koseki *et al.,* 2005). Therefore, α-naphthyl acetate was utilised as

the substrate for further analysis of Axe10 activity. No detectable activity against the *p*-nitrophenyl esters was observed (data not shown). Inability to hydrolyse *p*-nitrophenyl esters has been reported previously for enzymes such as AxeA from *S. lividans* (Dupont *et al.,* 1996) and rAoAXE from *Aspergillus oryzae* (Koseki *et al.,* 2006).

4.7.2 Effect of pH and temperature on Axe10 activity

Axe10 displayed optimum activity at approximately pH 7.0 (Fig. 4.24). A number of studies have reported on AXEases with optimal activity under neutrophilic conditions. For example, Chungool *et al.* (2008) demonstrated that an AXEase from *Streptomyces sp.* PC22 displayed optimum activity in a pH range between 6.5 and 7.0. Similarly, a *S. lividans* AXE was shown function optimally at pH 7.5 (Dupont *et al.,* 1996).

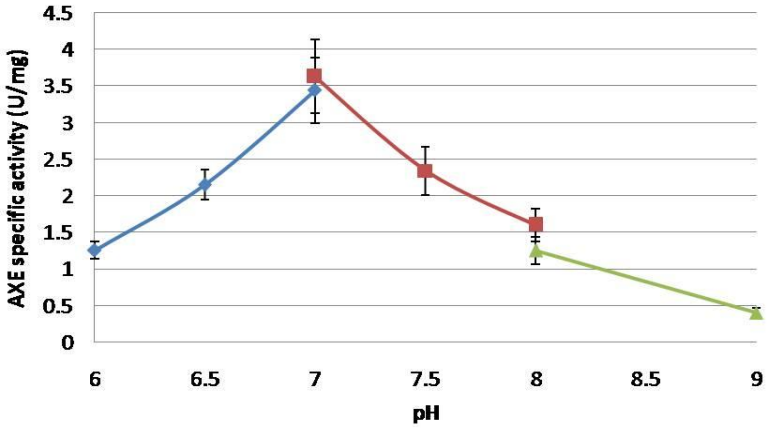

Figure 4.24 Effect of pH on purified Axe10 activity at 37°C. Buffers used were MES buffer (pH 6-7) (■), Phosphate buffer (pH 7-8) (■) and Tris buffer (pH 8-9) (■). Data represents the mean (n=3) ± standard error.

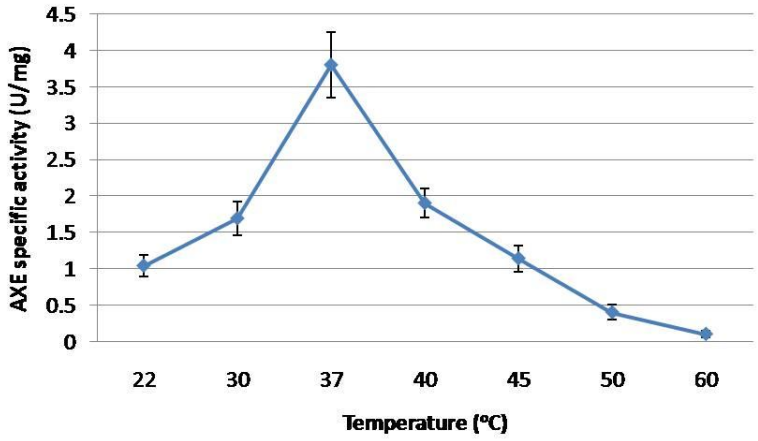

Figure 4.25 Effect of temperature on Axe10 activity using α-naphthyl acetate as substrate at pH 7.0 (50 mM phosphate buffer). Data represents the mean ± standard error (n=3).

The optimum temperature of recombinant Axe10 was determined to be 37^{o}C, and it retained about 50% activity around 40^{o}C (Fig. 4.25). This value is lower than a number of characterized AXEaes. AXE from *Streptomyces. sp.* PC22 has maximum activity at 50^{o}C (Chungool *et al.,* 2008), while AXE from *Bacillus pumilus* has an optimal reaction temperature at 55^{o}C (Degrassi *et al.,* 1998). AxeA from *S. lividans* has an optimal reaction temperature of 70^{o}C (Dupont *et al.,* 1996). In this study, recombinant Axe10 protein was temperature-sensitive and only retained 30% of maximum activity at 45^{o}C. This observation was surprising because the ORS10 strain was determined to grow optimally at 45^{o}C. As there is no report of expression of AXE genes from any *Streptomyces* species in *E. coli*, it may be worthwhile to perform a comparative study of the native purified AXE from ORS10 with recombinant AXE10, including the biochemical characteristics and structural studies.

4.7.3 Thermostability

Thermostability tests were performed on purified Axe10 (Fig. 4.26). Axe10 was found to be stable at 30°C, maintaining more than 95% residual activity after 60 min incubation at 30°C. The enzyme retained approximately 76% activity after 60 min incubation at 37°C. Axe10 was not stable above 40°C, as the enzyme lost more than 50% activity after 10 min incubation at 42°C and about 80% activity after 5 min incubation at 50°C.

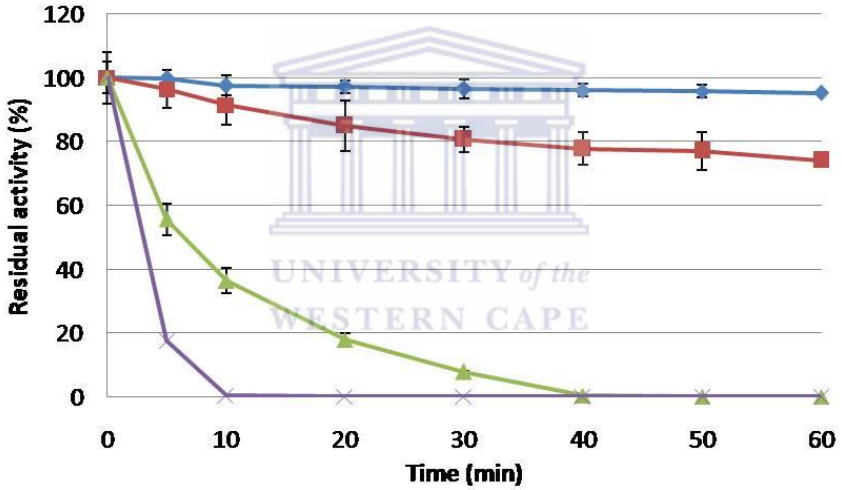

Figure 4.26 Thermostability profiles of purified Axe10 at 30 (◆), 37 (■), 42 (▲) and 50°C (×). Residual activity was determined as a percentage of an untreated control sample incubated on ice for the duration of the experimental period. Data represents the mean (n=3) ± standard error.

This result is similar to rAoAXE from *A. oryzae*, which is not stable at 40°C with a half life less than 60 min at 40°C, and 10 min at 50°C (Koseki *et al.,* 2006). The thermostability feature suggested Axe10 protein is not thermostable.

4.7.4 Axe10 steady state kinetics

Kinetic data of Axe10 was determined using á-naphthyl acetate as substrate. The first test on constant enzyme concentration and substrate concentration with a range of reaction times indicated that 10 min should be the most suitable reaction time for a kinetic study (data not shown). The Vmax and Km values were determined using the direct linear method in the Enzpack programme (Biosoft Software for Science UK). K_M and V_{max} values were determined as 3.17 mM and 18.17 U/mg, respectively (Fig. 4.27).

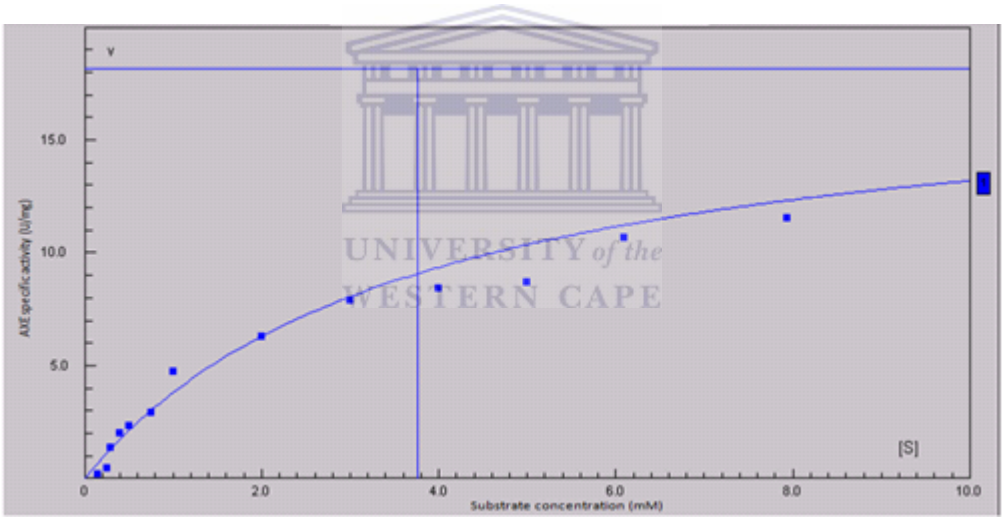

Figure 4.27 Michaelis-Menten Plots of rate (Vmax, U/mg) vs substrate concentration (mM).

The Vmax of Axe10 is similar to the reported value of 24.8 U/mg from *A. oryzae* rAoAXE (Koseki *et al.,* 2006) and lower than the value of 118.80 U/mg previously reported for *B. pumilus* AXE (Degrassi *et al.,* 1998). The Km of axe10 is higher than a number of other AXEs. For example, Km values of 1.54, 2.63, and 2.7 mM have been reported for AXEs isolated from *B. pumilus*

(Degrassi *et al.,* 1998), *Candida guilliermondii* (Basaran and Hang, 2000), and *Fibrobacter succinogenes* S85 (McDermid *et al.,* 1990), respectively. á-Naphthyl acetate may not the most suitable substrate for Axe10 as suggested by low affinity (1/Km). In future work, chemical synthesized substrates, such as acetylated xylan, should be tested. High performance liquid chromatography (HPLC) may be a useful technique to investigate the potential role of Axe10 in the hydrolysis of lignocellulosic substrates.

Chapter 5

General Conclusion

Liquid biofuels are considered to be an important alternative choice to meet rapidly increased global energy requirements. Lignocellulosic biomass represents a key sustainable and renewable resource for its production. The efficient deconstruction of biomass requires a suite of enzymes operating synergically to release fermentable sugars that can be subsequently fermented to bioethanol. Hemicellulose, as the second most abundant component of lignocellulosic matter, needs a number of accessory enzymes, including acetylxylan esterases (AXEase), together with core enzymes, such as endo-â-1,4-xylanase and â-xylosidase, to be completely degraded.

In this study, a thermophilic actinobacterial isolate (ORS10) was shown to display endo-β-1, 4-xylanase and AXEase activities. Phylogenetic analyses clustered isolate ORS10 with species of the genus *Streptomyces*, closest related to *Streptomyces achromogenes* subsp. *achromogenes* (99% identity) However, comparative biochemical and physiological characterisation of ORS10 with its closest relative suggest that ORS10 may represent a novel *Streptomyces* species or sub-species.

During the course of this study a small-insert genomic library of ORS10 was constructed and screened for hemicellulosic enzymes. Expression-based screening identified a gene encoding a putative AXEase. Based on the deduced protein sequence, the enzyme contains conserved amino acid

residues and domains indicative of an AXEase. On a protein level, Axe10 shows low similarity (62%) when compared with closest related proteins. In order to further classify Axe10 into a CE family, it will be necessary to investigate its substrate preference and specific mode of action on a greater number of substrates.

The Axe10 gene was heterologously expressed and purified from *E. coli*. To the best of our knowledge this is the first report describing the heterologous expression and purification of a *Streptomyces* AXEase in *E. coli*. The recombinant protein displayed optimal activity under neutral and mesophilic conditions. The enzyme was not stable at incubation temperatures above 40^{o}C. Expression and purification of this enzyme in a heterologous host system may have contributed to the lack of thermostability.

Collectively, the results obtained during the course of this study suggest that Axe10 may be suitable for inclusion in mesophilic neutrophilic industrial processes. To determine the value of Axe10 in lignocellulosic degradation industrial process, future work may include assessing the synergetic effect of Axe10 and other mesophilic lignocellulosic enzymes on the hydrolysis of industrially important lignocellulosic feedstocks.

References

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