Isolation and characterisation of esterases from thermophilic *Actinomyces*

By

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DECLARATION

I declare that **Isolation and characterisation of esterases from thermophilic *Actinomyces*** is my own work and has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references

Ms. Megan Oldale November 2010

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ABSTRACT

Alternative sources of fuel are required worldwide, and bio-ethanol is the leading candidate. Lignocellulosic biomass, a waste component of the agricultural industry, is a promising renewable source. Due to its complex structure it is highly recalcitrant, requiring the synergistic action of a battery of enzymes to achieve complete digestion. These enzymes include cellulases, hemicellulase and the accessory enzymes acetyl xylan esterase (AXE) and ferulic acid esterase (FAE). Thermpohilic *Actinomyces* isolates with the ability to hydrolyze xylan were screened for esterase activity. Two isolates (ORS10 and GSIV1), identified as *Streptomyces* spp, were positive for AXE activity. A cosmid library representative of isolate ORS10 was composed and screened for AXE activity using β-naphthyl acetate as substrate. An 18 kb cosmid clone, 18D7, tested positive for AXE activity. Intracellular fractions extracted from ORS10 were precipitated with ammonium sulphate and partially purified 161-fold. Specific activity was measured after dialysis and ion-exchange chromatography. Overall yield of the partially purified enzyme was 34 %. Two protein bands of molecular masses 40 kDa and 60 kDa have been subjected to trypsin digestion and MALDI-TOF mass spectrometry analysis. The partially purified AXE displayed optimum activity at pH 9 and at 50°C. AXE activity was stable for at least 1.5 hours between 30°C and 40°C, and for 24 hours between pH 6-9. The k_M and V_{max} values were 16.93 mg/ml and 1645 units/mg enzyme, respectively. The stability of the partially purified AXE at 30˚C-40˚C suggests potential for industrial applications that utilise mesophilic fermentations.

This thesis is dedicated to my family and Ryan for their continuous support

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PREFACE

This thesis is presented as a compilation of 4 chapters. The first 4 chapters comprise the thesis, followed by the reference list.

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CHAPTER 1
GENERAL INTRODUCTION

CHAPTER 1

1.1. Introduction

It is generally acknowledged that alternative sources of fuel are needed worldwide, and that bio-ethanol is the leading candidate. Until recently, starch and sucrose-based material were used as the main source of fermentable sugars. However, lignocellulosic biomass, a waste component of the agricultural industry, is now the most promising renewable source, breaking the reliance on precious food crops (Saxena *et al,* 2009).

Lignocellulosic material is composed of cellulose, hemicellulose, lignin and ester linkages, linked to form a complex, fibrous and insoluble matrix. Pre-treatment coupled with the synergistic action of hemicellulases, cellulases and esterases (accessory enzymes) are an absolute requirement to break down the highly recalcitrant lignocellulose (Kumar, 2009).

Ferulic acid esterases (FAEs) are a subclass of the carboxylic ester hydrolase family that liberate phenolic acids such as ferulic acid and its according dimers from hemicellulose and pectins. They are involved in breaking the bonds between arabinose and ferulic acid, thereby releasing the covalently bound lignin from hemicellulose

Much like FAEs, acetyl xylan esterases (AXEs) also belong to the carboxylic ester hydrolase family, and act on carboxylic ester bonds. The role of AXEs is to hydrolyze the release of acetyl groups at position -2 and/or -3 of xylose moieties in xylan (Tsujiyama and Nakano, 1996). Some AXEs are capable of releasing ferulic acid from plant cell wall-derived substrates, like FAEs, and therefore the two enzymes share a similar function.

Thermophilic enzymes offer significant advantages in industry, such as an increased rate of hydrolysis and reduced risk of mesophilic- microbial contamination (Sun and Cheng, 2002). Substrates are more readily digested and a rich variety of degradative enzymes are expressed at elevated temperatures.

1.2. Project Aims

This project primarily focuses on the side-chain cleaving thermophilic esterases, acetyl xylan esterase and ferulic acid esterase that degrade the components of lignocellulose material. The project aims to isolate and characterise thermophilic esterases from *Actinomyces* for potential use in lignocellulose digestion. The objectives are summarised below:

1. To screen the thermophilic *Actinomyces* for AXE and FAE activity using β-naphthyl acetate and ethyl ferulate, respectively, as substrates
2. Carry out phylogenetic studies including comparative 16S rRNA gene analysis on single isolates displaying high specific activity.
3. Construct cosmid libraries for screening of each enzyme.
4. Screen cosmid libraries for general esterase/lipase activity using tributyrin as the substrate.
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CHAPTER 2

2.1. The Energy Crisis and Global Warming

One of the great challenges facing modern society is meeting the growing demand for energy which is vital for transportation, industrial processes and heating (Hahn-Hagerdal *et al*, 2006). The ever increasing demand for energy can be attributed to developments in industry, a growing population in most countries, and escalating levels of consumption without the development of sufficient renewable energy resources to support it. This is a global problem affecting developing and developed countries. For the past 30 years, petroleum and its derivatives have dominated energy markets all over the world as they are vital commodities in the global economy. However, such a high level of dependency on these products has saturated the market and placed economies, energy security, homeland security and the environment under significant risk (Demain, 2008).

The major source of the world's energy comes from conventional sources such as oil, coal and natural gas. In 2006, the world used 85 million barrels of oil per day and this is expected to increase dramatically by 2015. Seventy-one percent of the energy we use comes from fossil sources: 25% from crude oil, 23% from natural gas and 21% from coal whilst the remaining 21% is from hydraulic and nuclear energy (9%), and from solar and wind energy (12%). The estimates place total energy provided by renewable resources at only 2.5% (Demain, 2008), which clearly must be remedied.

Without exception, every country faces the same two problems associated with the use of conventional fuel. Firstly, the world's oil reserves may be depleted as soon as 2050, meaning that significant progress must now be made into renewable energy sources. Secondly, burning these conventional energy sources causes widespread air pollution through greenhouse gas emissions. Sulphur dioxide (SO_2) is one of the major causes of acid rain. Moreover, excess carbon dioxide (CO_2) in the atmosphere is a threat to the environment as it exacerbates global warming. In 2000 it was estimated that over 20 million metric tons of CO_2 are released into the atmosphere each year. If

the trend is not altered, natural disasters such as excessive rainfall, flood and droughts are likely to be much more frequent (Saxena *et al*, 2009).

The onus is therefore on science and biotechnology to simultaneously find renewable solutions for the planet's future energy needs whilst reducing greenhouse gas emissions.

2.1.1. Biofuels as a solution

In political and economical circles the debate continues with respect to alternative renewable energy sources. The most promising solution to curb our energy addiction from conventional sources is believed to be the development of biofuels. (Demain, 2008). Biofuels refer to the following products: biodiesel, bioalcohol (bio-ethanol, bio-butanol), biogas and syngas.

In theory, biofuels can be produced from any biological carbon source; however, the most common sources for biofuel production are photosynthetic plants. The global fuel market has been established, and biofuels are now used in many countries to power vehicles and to warm homes. In particular, the biofuel industries in Europe, Asia and America, are rapidly expanding (Demain, 2005).

Biofuels are produced either by 1st generation, 2nd generation or 3rd generation processes (bio-fuels from algae). In 1st generation processes biofuels are made from sugar, starch or animal fats using conventional technology. However, this is not considered a viable approach in the production of biofuels as the starting materials are food crops. With food safety already a major issue (particular in Africa), the 2nd generation process seems more valid, as here biofuels are generated from non food crops such as lignocellulosic material.

2.1.1.1. Bio-ethanol

Bio-ethanol is an attractive alternative fuel as it is bio-based, renewable, and better for the environment than conventionally generated fuels. The oxygenated nature of the fuel improves combustion and thereby reduces hydrocarbon, carbon monoxide and particulate emissions. It has a number of advantages over gasoline which are summarised in **Table 1**.

Table 1: The advantages and disadvantages that bio-ethanol hold over gasoline (Adapted from Demain, 2008)

Advantages
Offers favourable trade balance
Cleaner and more efficient burning
Higher octane rating
Decreases smog formation due to low volatility
Decreases greenhouse gas emissions due to recycling by growth of plants
Decreases particles and toxic emissions
Decreases level of ozone pre-cursors omitted, thereby averting air pollution
Contains no sulphur, therefore less toxic to humans
Disadvantages

Only 2/3 of the energy content
Requires engine modification when mixed with gasoline and over 15% of total fuel
Cannot be shipped via pipelines

In 2006, global bio-ethanol production stood at 13.5 million gallons, 60% from sugar cane whilst 40% is from other crops. The leading countries for the production of bio-ethanol are America and Brazil, which combined, account for 70% of world bio-ethanol production (Balat *et al,* 2008).

As stated above, almost all bio-ethanol is produced by the fermentation of corn glucose (US) or the fermentation of sucrose (Brazil). However, it is possible for any country with a considerable agronomic-based economy to use current technologies for biofuel production. This is because significant progress in the past two decades has indicated that non-food crops can also be used to produce bio-ethanol, and this will become both a reality and a necessity (Lin and Tanaka, 2006). **Table 2** highlights the top ten bio-ethanol producers in the world (in million litres ethyl alcohol produced).

Table 2: The list of the top ten bio-ethanol producers in the world (in million litres ethyl alcohol produced) (Adapted from Sanchez and Cardona, 2007)

Country	**2006**	**2005**
USA	18 376	16 139
Brazil	16 998	15 999
China	3 849	3 800
India	1 900	1 699

France	950	908
Germany	765	431
Russia	647	749
Canada	579	231
Spain	462	352
South Africa	386	390

2.2. Production of bio-ethanol

2.2.1. Feedstocks for bio-ethanol production

Any biological feed-stock containing vast amounts of sugar, or materials that can be converted to sugar can be fermented to produce bio-ethanol. There are three types of bio-ethanol feed-stocks: sucrose-containing (sugar cane, sorghum), starchy material (wheat, corn, barley) and lignocellulosic biomass (wood, straw, grasses). Each type of feed-stock has advantages and disadvantages (Balat *et al,* 2008), which will be discussed further.

2.2.1.1. Sucrose-containing feed-stock

Brazil is the largest producer of sugar cane, accounting for 27% of global production (Balat *et al,* 2008). In Brazil, the production of bio-ethanol from sugar cane is an extremely economical process due, in part to the government supported mandates which propose blending bio-ethanol with gasoline for use in cars (Demain, 2008). Consequently, the price of sugar cane has dropped which supports the continued production of, and creates a demand for, biofuels.

In Europe, beet molasses and sugar beet are used for the same purpose; sugar beet is preferred over wheat because it has a lower cycle of crop production, gives higher yields, a higher tolerance to climatic variation and has low water and fertilisation requirements. Additionally, particularly in

developed countries, sweet sorghum is emerging as promising candidate as it has the ability to remain dormant in dry periods and contains a vast amount of sugar (Balat *et al,* 2008).

2.2.1.2. Starchy material

Starch is a homopolymer consisting of one monomer, D-glucose. To produce bio-ethanol from starch, the chain of carbohydrates must be broken down to obtain glucose syrup, which can then be converted into bio-ethanol by yeasts. Starch contains large chains of glucose molecules and so can be converted to fermentable sugars by hydrolysis, either acid hydrolysis or enzyme hydrolysis. The need for enzyme hydrolysis has posed great problems for the starch-based bio-ethanol industry due to low enzyme efficiency, high costs (due to the high temperatures required and large amounts of amylolytic enzymes that need to be added) and low yields. However, much work has been devoted to circumvent these problems and the market is gradually becoming more economically viable (Balat *et al,* 2008).

On an international scale, bio-ethanol production has relied on corn and wheat. In the US in particular, 90% of all bio-ethanol is produced from corn (Demain, 2008). In spite of this it is expected to remain the predominant feed-stock due to its high availability. Moreover, as a result of improved corn yields, the US corn sector can supply the bio-ethanol industry without major price increases, leading to enhanced profitability (Balat *et al,* 2008).

2.3. Lignocellulosic biomass as an emerging alternative feedstock

Lignocellulose is a major component of woody and non-woody plants (e.g. grass) and represents a key source of renewable organic matter. Due to its chemical properties and components, lignocellulose is an ideal and very important substrate in biotechnology (Kim and Dale, 2004). Lignocellulosic biomass is converted to useful forms of energy using various processes depending on the type, quantity and property of biomass feed-stock as well as the desired form of energy including power/heat generation, transportation fuel and chemical feedstocks (Lee, 1997).

The use of biomass to produce energy at present contributes 2.5% of the world's energy supply. Biomass usage is a preferred energy resource because it is renewable, sustainable and has a limited environmental impact (as it contains only a negligible amount of sulphur and therefore does not contribute to sulphur dioxide emissions). As biomass is a domestic resource it is not subject to price fluctuations or supply uncertainties that can face imported fuels (Saxena *et al*, 2009).

Biomass can be used for bio-ethanol production from various sources, such as waste (from agricultural production, crop residues), standing forests and energy crops. Growing crops for bio-ethanol production leads to issues such as food scarcity and price increases, therefore lignocellulosic biomass has become the focus of current research (Kim and Dale, 2004) including this study.

2.3.1. Components of lignocellulosics

Lignocellulosic biomass is made up of three basic polymers: cellulose ($C_6H_{10}O_8$), hemicellulose ($C_5H_8O_4$) and lignin (Balat *et al*, 2008). **Figure 1** illustrates the chemical structures of cellulose, hemicellulose and lignin.

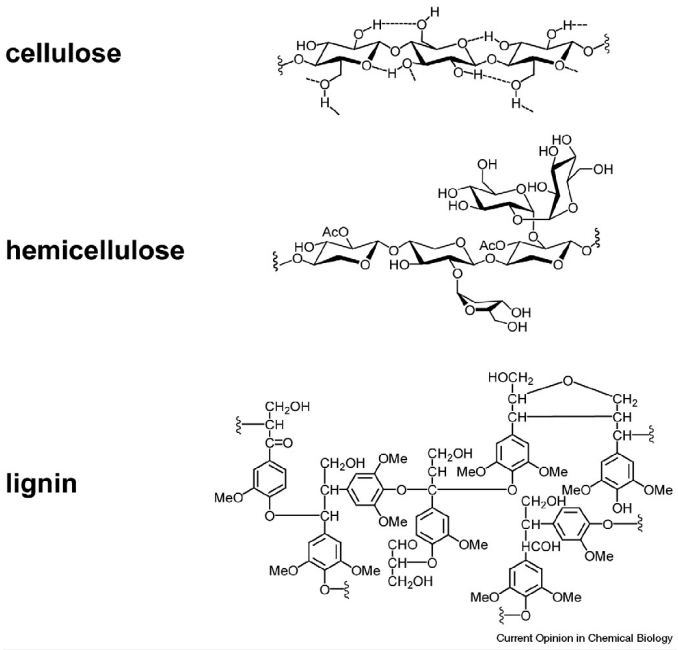


Figure 1: The three biopolymers that are present in lignocellulosic material (Adapted from Chang, 2007*)*

The combination of hemicellulose and lignin provides a protective sheath around the cellulose, making it extremely recalcitrant to enzymatic degradation. This layer needs to be modified or removed before the hydrolysis of cellulose can occur (Hamelinck *et al*, 2005).

Cellulose (40-60% of dry biomass) is a homopolysaccharide composed of β-D-glucopyranose and is linked by glycosidic bonds. Cellobiose is a repetitive unit of cellulose and can be converted to glucose residues (Kumar *et al*, 2008). The orientation of the glycosidic linkages as well as additional hydrogen bonding, make cellulose very difficult to degrade. In order to utilise the cellulose as a biofuel, the carbohydrate potential of cellulose must be developed, namely by converting it into sugars such as glucose (van Wyk, 2001). This is done during the process of hydrolysis, which breaks the polysaccharide down to free sugar molecules by the addition of water – this process is known as saccharification (Hamelinck *et al*, 2008).

Hemicellulose accounts for 20-40% of dry lignocellulosic biomass and is the second most abundant form of biomass. Hemicelluloses form heterogeneous polymers that are made up of pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and acids (Kumar *et al*, 2008). The biopolymers bind bundles of cellulose fibrils to form microfibrils which enhance the stability of the cell wall. Lignin cross-linking creates a complex web of bonds that further promotes structural strength (van Wyk, 2001). Due to their amorphous (unstructured) nature, hemicelluloses are relatively easy to hydrolyse, especially when compared to cellulose (Hamelinck *et al*, 2005). The most abundant hemicelluose is xylan and is β-1,4-linked D-xylose polymer with arabinofuranose, glucuronic acid, methylglucuronic acid, ferulic acid and acetyl side chains (Degrassi *et al*, 1998).

Lignin is composed of highly branched, substituted, mononuclear aromatic polymers that are found in the cell walls of plant cells. Along with lignin are phenylpropane and methoxy groups as well as polyphenolic substances that aid in stabilising cell walls and cementing them together. They often bind to adjacent cellulose fibers and so form a lignocellulosic complex (Bald *et al*, 2008). What adds to the complexity of lignin is the level of cross-linking via a variety of chemical bonds, making the whole complex extremely difficult to degrade (Hamelinck *et al*, 2008). Only a few organisms are capable of degrading lignin and dedicated research is being carried out to find out more about this property.

In addition to hemicellulose, cellulose and lignin, lignocellulosics contain generous amounts of cell-wall bound hydroxycinnamate esters that are linked to polysaccharides (Bunzel *et al*, 2005). Hydroxycinnamic acids (ferulic acid, p-coumaric acids) are phenolic acids that are ester linked to arabinoxylan and lignin, or cross-linked to lignin and polysaccharide through ether and ester bonds, respectively (McSweeney *et al,* 1999). The functional roles of hydroxycinnamates in plant cell walls have been extensively studied and accordingly have been implicated in regulating cellular expansion and plant defence. Hydroxycinnamates reduce the digestability of the cell wall by restricting the accessibility to carbohydrates (Benoit *et al*, 2006).

Ferulic acid is the most abundant hydroxycinnamic acid in plant cell walls. Ferulic acids are covalently linked to the polysaccharide by ester bonds as well as to the components of lignin through ester linkages (Mathew and Abraham, 2005). In wheat bran, ferulic acid is quite abundant and linked at different positions on the arabinose sugar. Additionally ferulic acid can be cross-linked through diferule bridges to heteroxylan and pectin tissues indicating a possible pivotal role in the structure of non-lignified cell walls (Benoit *et al*, 2006).

2.3.2. Problems associated with lignocellulosics

Lignocellulosic biomass is converted to ethanol via the process of hydrolysis coupled with downstream fermentation processing. Unlike the fermentation of C_6, this process is much more complicated. Additionally, it is not as cost-effective and does not produce very high yields especially when compared to the production of bio-ethanol from starch/sugar (Kumar *et al*, 2008).

Whereas starch is a storage compound composed of glucose linked by α-1,4 and α-1,6 glycosidic bonds, cellulose is a structural component composed of only glucose linked via β-1,4 glycosidic bonds. As a result of this β-1,4 linkage, cellulose has a crystalline and compact structure, thereby making it more resistant to biological attack (Gray *et al*, 2006).

Lignin, which makes up 10-25% of lignocellulosic biomass, contains no sugar and is impossible to convert into sugars. Consequently lignin is rather a residue in ethanol production and is a huge challenge to convert it into a valuable product (Kumar *et al*, 2008).

Generally hemicellulose consists of a chain of xylan backbone residues (β-1,4 linkages) with varying branches of mannose, arabinose, galactose and glucuranic acid. The extent of branching and the make-up of the minor sugars in hemicellulose are highly variable and dependent on the type of plant. Lignin is in some instances covalently linked to hemicellulose via ferulic acid ester linkages. This compact and complex nature makes lignocellulose more difficult to enzymatically degrade to

fermentable sugars when compared to starch. The cost of producing a gallon of ethanol from lignocellulose is higher than that from starch due to these additional treatments needed to degrade components (Gray *et al*, 2006).

The question therefore begs: If the process is so arduous and costly, why is all emphasis placed on using lignocellulosics to produce bio-ethanol as opposed to starch? Firstly, reports indicate that ethanol from lignocellulosic biomass reduces greenhouse gas emissions by 80% as compared to ethanol from corn that reduces them by 20-30% (Demain, 2008). Secondly, bioconversion from lignocellulosics creates an opportunity for a biorefinery which could produce a valuable co-product as well as fuel bio-ethanol. As an example, the sugars may be bacterially fermented under aerobic/anaerobic conditions to produce products such as lactic acid which can be used in the plastics industry (Gray *et al*, 2006).

2.3.3. Production of bio-ethanol from lignocellulosics

There are three major steps in the conversion process: Pre-treatment, enzymatic saccharification and the fermentation of released sugars by specialist organisms (Gray *et al*, 2006). **Figure 2** illustrates the relevant steps in producing ethanol from biomass.

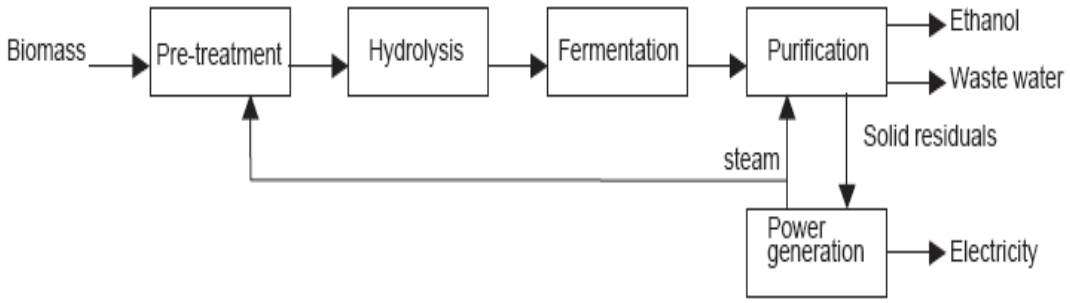


Figure 2: The process of producing ethanol from biomass

2.3.3.1. Pre-treatment

When cellulose is ensnared in lignin, cellulolytic enzymes cannot access it, thus limiting the hydrolysis rate. Various pre-treatment options are available which act to accelerate the hydrolysis rate (Fernandes *et al*, 2009) by separating carbohydrates from the lignin matrix whilst minimising the chemical destruction of the fermentable sugars needed for ethanol production (Mielenz, 2001). The most common physico-chemical pre-treatment method for lignocellulosic material is steam explosion where chipped biomass is treated with high pressure saturated steam followed by a swift reduction in pressure. This combination causes hemicellulose degradation and lignin transformation, thereby increasing the likelihood of cellulose hydrolysis. Chemical pre-treatment includes alkaline hydrolysis where the mechanism of action is saponification of intermolecular ester bonds which crosslink xylan hemicelluloses and other components, and acid hydrolysis. Acids are powerful tools for cellulose hydrolysis but are toxic, hazardous and corrosive (Sun and Cheng, 2002). Biological pre-treatment is also an option and involves brown, white and soft-rot fungi which naturally degrade lignin and hemicellulose. Brown rots predominantly attack cellulose whilst white and soft-rot attack cellulose and lignin. White-rot fungi have been found to be the most effective basidomycete for the biological pre-treatment of lignocellulosic material. The main advantages for adopting the biological approach include low energy requirement and mild environmental conditions. However, the rate of hydrolysis is extremely low (Sun and Cheng, 2002).

2.4. Enzymatic Hydrolysis

In general, enzymatic hydrolysis of cellulose is carried out by highly specific cellulase enzymes. The resulting products are usually reducing sugars such as glucose. The cost of enzymatic hydrolysis is low in comparison to acid or alkaline hydrolysis, as enzyme hydrolysis is conducted using milder conditions and with no corrosion problems. Bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosics. The micro-organisms involved may be anaerobic or aerobic, and mesophilic or thermophilic – included are *Clostridium, Cellulomonas, Bacillus, Streptomyces.* Two organisms in particular, *Clostridium thermocellan* and *Bacteroides cellulosolvens* produce cellulases with high specific activity but with very low enzyme titres. As anaerobes have low growth rates and require anaerobic conditions coupled with low enzyme titre, most research is focused on fungi such as *Trichoderma* (Sun and Cheng, 2002).

Many factors affect enzyme hydrolysis, such as substrate concentration, cellulase activity and native conditions (temperature, pH). In order to improve the yield and rate of enzyme hydrolysis, research focuses on optimizing hydrolysis and enhancing cellulase activity. Substrate concentration is one of the main factors affecting yield and initial rate of enzyme hydrolysis of cellulases. At low substrate concentrations, there tends to be a decrease in yield and hydrolysis rate, whereas high substrate concentration causes substrate inhibition which lowers efficiency of hydrolysis.

2.4.1. Cellulases

Cellulases are essentially a mixture of several enzymes. They belong to two groups of enzymes known as the endoglucanases (EGs) and cellobiohydrolases (CBHs) – the two work synergistically in cellulose hydrolysis (Sukumaran *et al*, 2009). Endoglucanases initiate random attacks at multiple internal sites in the amorphous regions (fibril regions) of cellulose, thereby creating free-chain ends and opening them up for subsequent attack by the cellobiohydrolases (Lee, 1997). Cellobiohydrolases remove mono and dimmers from the end of the glucose chain and in doing so release short chain cello-oligosaccharides (cellobiose) (Howard et al, 2003). A third type of enzyme,

β-glucosidase hydrolyses cellobiose into glucose molecules. If β-glucosidase is absent, end-product inhibition from cellobiose will occur (Keshwani *et al*, 2009).

Cellulase activity is inhibited by cellobiose and glucose in some instances. Many methods have been established to overcome this inhibition such as increasing the concentration of the enzyme, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (SSF). In SSF, reducing sugars produced via hydrolysis or saccharification are simultaneously fermented to ethanol thereby reducing product inhibition. The micro-organisms *Trichoderma reesei* and *Saccharomyces cerevisiae* are commonly used in SSF at temperatures of about 38°C, which is a compromise between the optimal temperature for hydrolysis (45°C-50°C) and the optimal temperature for fermentation (30°C). Compared to the two-stage hydrolysis and fermentation process, SSF holds many advantages such as: increased hydrolysis rate, lower enzyme requirement, high product yields and shorter process times (Zaldivar *et al*, 2001).

2.4.2. Hemicellulases

As hemicellulases have a complex chemical composition, diverse mixtures of hemicellulases are needed for complete degradation. In general, for each structural feature in hemicellulase an associated enzyme exists that can hydrolyse or chemically modify the feature. Therefore like cellulose hydrolysis, hemicellulose degradation requires the co-ordinated action of different enzymes. As a result hemicellulase include enzymes that break down both β-1,4-xylan (xylanases, β-xylosidase) and various side chains (α-l-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, ferulic acid esterases) (Gray *et al*, 2006). **Table 3** illustrates the major classes of hemicellulases.

Hemicellulases are multi-domain proteins containing structurally discrete catalytic and non-catalytic modules. The most important non-catalytic module consists of carbohydrate binding domains (CBDs), interdomain linkers and dockerin molecules. CBDs facilitate enzyme targeting to the polysaccharide while dockerin molecules mediate binding of the catalytic domain via cohesion-

dockerin interactions. Based on the amino acid or nucleic acid sequence of their catalytic modules, hemicellulases are either glycoside hydrolases (GHs) which hydrolyses glycosidic bonds or carbohydrate esterases (CEs) that hydrolyze ester linkages of acetate or ferulic acid side chains (Howard *et al*, 2003). Another way of categorising hemicellulases is to group them as: endo-acting enzymes, exo-acting enzymes or accessory (ancillary) enzymes. Endo-acting enzymes attack polysaccharide chains internally with little activity on short oligomers, while exo-acting enzymes act progressively from either the reducing or non-reducing termini. Accessory enzymes are required for the hydrolysis of hemicellulose in native plant tissues. These accessory enzymes include a variety of acetylesterases and esterases that hydrolyze lignin glycoside bonds, such as ferulic acid esterases. Additionally there are numerous enzymes for the removal of specific substituents such as arabinosidases and fucaosidases (Taylor II *et al*, 2008).

Table 3: The major hemicellulases (Adapted from Howard *et al, 2003*)

Enzymes	**Substrates**	**EC Number**	**Family**
Exo-β-1,4-xylosidase	β-1,4-xylooligomers xylobiose	3.2.1.37	GH 3, 39,43, 52, 54
Endo-β-1,4-xylanase	β-1,4-xylan	3.2.1.8	GH 5, 8, 10, 11, 43
Exo-β-1,4-mannosidase	β-1,4-mannooligers mannobiose	3.2.1.25	GH1, 2, 5
Endo-β-1,4-mannanase	β-1,4-mannan	3.2.1.78	GH , 26
Endo-α-1.5-arabinanase	α-1,5-arabinan	3.2.1.99	GH 43

α-L-arabinofuranosidase	α-arabinofuranosyl xylooligomers α-1.5 arabinan	3.2.1.55	GH 3, 43, 51, 54, 62
α-glucuronidase	4-O-methyl- α-glucuronic acid xylooligomers	3.2.1.139	GH 67
α-galactosidase	α-galatopyranose mannooligomers	3.2.1.22	GH 4, 27, 36, 57
Endo-galactanase	β-1,4-galactan	3.2.1.89	GH 53
β-glucosidase	β-glucopyranose mannopyranose	3.2.1.21	GH 1, 3
Acetyl xylan esterase	2- or 3-O-acetyl xylan	3.1.1.72	CE 1-6
Acetylmannan esterase	2- or 3-O-acetyl mannan	3.1.1.6	CE 1
Ferulic and p-coumaric acid esterase	2- or 3-O- acetyl mannan	3.2.1.73	CE 1

2.4.2.1. Esterases

Broadly speaking, esterases catalyze the hydrolysis of aliphatic and aromatic esters and have been widely studied due to their metabolic functions. Esterases are hydrolase enzymes that split esters into an acid and alcohol in a chemical reaction known as hydrolysis. Recently, they have become recognized as important enzymes in the breakdown of lignocellulosic matter as it was found that the synergistic action of some esterases (accessory enzymes) are required in conjunction with the core hemicellulase enzymes for the complete degradation of xylan. (Kumar *et al,* 2009).

Esterases are low molecular weight enzymes and this makes them ideal for the effective penetration of lignocellulose. In lignocellulose they are pivotal in removing the bound acetic and phenolic acids allowing for complete hydrolysis. Most esterases possess highest activities and isoelectric points in

the neutral or low-acidic range. Morevoer, most show maximal activity and good thermostability between 40°C-50°C (Kuhad *et al*, 1994), indicating that they are moderately thermostable and these are the enzymes that are of most importance in industry.

The class of enzymes that are of interest in this study are the esterases (belonging to hemicellulases), in particular ferulic acid esterases and acetylxylan esterases.

2.4.2.1.1. Ferulic acid esterase

Complete degradation of plant cell wall polymers requires an array of enzymes with different activities. The hydrolysis of hydroxycinnamate esters is catalysed by cinnamoyl esterases (CEs) such as cinnamoyl ester hydrolases, feruloyl esterases (ferulic acid esterases) and *p*-coumaric esterases. CEs are a subclass of carboxylesterases and are characterised by high activity on various hydroxycinnamate esters (Fazary and Ju, 2007).

Ferulic acid esterases (FAEs) falls in a subclass of the carboxylic ester hydrolase family that liberate phenolic acids such as ferulic acid and *p*-coumaric acid, as well as their dimers from hemicellulose and pectins (Topakas *et al,* 2007). In the case of ferulic acid linked to L-arabinofuranose-containing polysaccharides such as L-arabino-D-xylans, the ferulic acid that is esterified can either be unsubstituted or linked to another esterified ferulic acid to form several types of diferuloyl bridges involved in inter- and intra- cross-linking of polymers within the cell wall (Ghatora *et al,* 2006). Consequently they are involved in breaking the bond between the arabinose and ferulic acid, thereby releasing the covalently bound lignin from hemicellulose. Hence they function as accessory enzymes needed for the complete saccharification of hemicelluloses (Mathew and Abraham, 2005).

FAEs represent an exceptionally diverse set of enzymes with little unifying sequence and physical features to link them. Based on multiple sequence alignment studies, FAEs can be divided into four groups: A, B, C and D. Additionally, there is a strong evolutionary relationship between FAEs and

acetylxyan esterases based on their specificity toward mono and diferulates. It is well documented that esterases act on a broad range of substrate and this is confirmed when delving into the specificities of each group within the FAE class. Type A displays preference for phenolic moieties as well as hydrophobic substrates with bulky components on the benzene ring. Although Type A and Type B are similar in activity, Type B prefers substrates with one or two hydroxyl substitutions such as *p*-coumaric acid (Topakas *et al*, 2007).

The first purified FAE was reported in 1991 (Fazary and Ju, 2007). Since this time, more than 30 enzymes have been purified and characterised from several micro-organisms including: *Clostridium thermocellum, Lactobacillus acidophilus* and *Aspergillis niger.* **Figure 3** illustrates the crystal structure of FAE from *Aspergillus niger.* This FAE displays a α/β hydrolase fold similar to that found in fungal lipases. Site-directed mutagenesis as well as crystallographic studies allowed the identification of a catalytic triad (Ser133-His247-Asp194) that forms the catalytic machinery of the enzyme (Hermoso *et al,* 2004).

Purified FAEs show huge amounts of variation in physical characteristics such as molecular weight, isoelectric point (pI), and optimal hydrolysis conditions. Molecular weights are generally in the range of 11 -220 kDa and pI from 3-9.9. The optimal temperature and pH for activity ranges from 30-65°C and pH 4-8 respectively (Topakas *et al*, 2007).

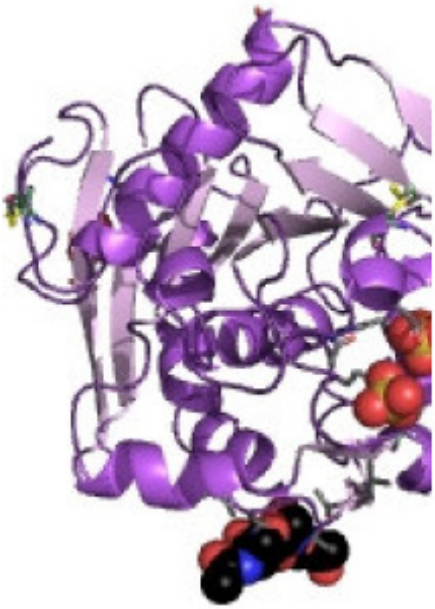


Figure 3: Crystal structure of ferulic acid esterase from *Aspergillus niger* (Adapted from Hermoso *et al*, 2004)

2.4.2.1.2. Acetylxylan esterase

Arabinoxylans are the most extensive hemicellulose in cereals and consist of a chain of β-1,4 linked xylopyranosyl residues which form the backbone. In some cases the xylose residues are substituted with acetyl groups (Ghatora *et al*, 2006). AXE hydrolyzes the release of acetyl groups at position -2 and/or -3 of xylose moieties in xylan. It was found that the contents of acetylated hemicellulose can reach 20-30% in wood component materials; hence acetyl xylan esterases play an important role in the biodegradation of acetyl xylan (Tsujiyama and Nakano, 1996).

Similar to ferulic acid esterases, acetyl xylan esterases belong to the carboxylic ester hydrolase family, i.e. they act on carboxylic ester bonds. Acetylxylan esterases have been categorised into seven sub-families on the basis of their amino acid sequence similarity and their affinity to polymeric and oligomeric substrates (Ghatora *et al,* 2006). Ferulic acid esterases show overlapping activity to acetylxylan esterases in terms of acetyl groups. Similarly some acetylxylan esterases are also capable of releasing ferulic acid from plant cell wall-derived substrates, much like FAEs.

Hence one can imply that acetylxylan esterases have a similar function to FAEs. In comparison to FAEs, acetyl xylan esterases are able to remove acetyl ester groups from positions C-2 or C-3 of D-xylopyranosyl residues in xylan chains (Ghatora *et al*, 2006).

Studies involving the substrate specificity of acetylxylan esterases suggest that some remove the acetyl group from C-2 or C-3. On the other hand, some perform sequential deacetylation by removing the acetyl group from position 2 and then rapidly from position 3. In other instances double deacetylation has been observed in which an acetyl group is removed simultaneously from both positions (Ghatora *et al,* 2006). As a result of the occurrence of acetyl groups at both positions, it is believed this could be important in the substrate specificity of AXE.

The acetyl xylan esterase that liberates acetyl groups from the backbone of xylan have been studied in several fungi (such as *Aspergillus niger, Schizophyllum commune, Trichoderma reesei* and *Penicillium purpurogenum)* and bacteria (such as *Fibrobacter succinogenes*, *Pseudomonas fluorescens, Streptomyces lividans* and *Thermoanaerobacterium* sp) (Degrassi *et al*, 1998). To date most research has focused on the isolation, purification and characterization of AXE from these microorganisms. AXE consists of a C-terminal substrate binding domain and an N-terminal catalytic domain that is separated by a glycine-rch linker region (Tsujibo *et al,* 1997; Taylor *et al,* 2006; DuPont *et al,* 1996 and Nisole *et al,* 2006). The AXE gene is located downstream of the xylanase B gene from *Streptomyces lividans.* (Nisole *et al,* 2006; Shareck *et al,* 1995). This is consistent with the role that AXE plays in the xylanolytic enzyme system that leads to the complete hydrolysis of xylan (Nisole *et al,* 2006).

2.5. Ethanol fermentation from lignocellulosic biomass

Supernatants from the enzymatic hydrolysis of lignocelluloses contain hexose and pentose sugars as by-products. Due to the lignocellulose sources, the hydrosylate could contain glucose, xylose, arabinose, galactose, mannose, fucose and rhamnose, with glucose and xylose the most dominant

sugars (Keshwani and Cheng, 2009). Essentially fermentation is carried out by micro-organisms that use fermentable sugars for food and in this process ethyl alcohol, and other by-products are produced. Typically they use 6-carbon sugars, glucose being the most common. As a result cellulosic biomass material that contains high levels of glucose (or precursors to glucose) are the easiest to convert to bio-ethanol and the yeast *Saccharomyces cerevisiae* is commonly used to achieve this (Balat *et al*, 2008). However, biomass containing mainly xylose is more problematic to digest and recent research aims to improve this process. Cellulose-ethanol biotransformation is conducted using various anaerobic thermophilic bacteria such as *Clostridium thermocellum* (Ingram *et al,* 1997) as well as *Manilia* sp. (Saddler and Chan, 1982). However, subsequent studies have indicated that the process is long and produces vast amounts of unwanted by-products (Wu *et al*, 1986).

2.5.1. Actimomycetes used for fermentation

Fungi are generally regarded as the most important primary lignocellulose degraders (McCarthy and Williams, 1992). As a result, much of the research in this area was and still is concentrating on fungal isolates. However, *Actinomyces* and other bacteria are now recognised as active lignocellulose degraders.

Actinomyces are Gram+, high GC bacteria that inhabit a vast number of habitats, some of which are extreme (Williams *et al,* 1983). These organisms typically grow as branching hyphae and are highly adapted to penetrate and degrade insoluble substrates such as lignocellulose (McCarthy, 1987). Primary carbon sources in soil are insoluble and polymeric, therefore a range of enzymes that will allow hyphae to penetrate and colonise the substrates are secreted (McCarthy and Williams, 1992).

Thermophilic *Actinomycetes* are well known and a number of genera are included, such as: *Sacharomonospora, Sacharopolyspora, Streptomyces, Thermoactinomycetes, Thermobifidia* and *Thermomonospora*. The occurrence and isolation of thermophilic *Actinomycetes* has been

extensively studied and present a potential source of novel, thermophilic esterases (Song *et al,* 2001).

2.5.2. Thermostable enzymes for use in ethanol fermentation

As scientist's knowledge of enzymes has increased, coupled with the discovery of thermostable enzymes and their inherent benefits, many possibilities for new industrial processes have emerged (Demirjian *et al*, 2001). Thermophiles have optimum growth temperatures of 45°C to 85°C in comparison to mesophiles which have an optimum of 20°C to 35°C (Demirjian *et al*, 2001). Thermostable enzymes are mainly isolated from thermophilic organisms and are preferential to mesophilic enzymes due to their inherent stability in commercial processes. The utilisation of thermostable enzymes was made possible due to the isolation of thermophilic organisms from extreme ecological zones and the subsequent extraction of the enzymes (Bharat and Hoondall, 1998; Burrows, 1973; Kohli *et al*, 2003).

Thermophilic organisms adapt to extreme environments in a variety of ways. 'Chaperonins' may be produced to assist other proteins in refolding, thereby restoring function following denaturation (Everly and Alberto, 2000). The cell membranes of thermophiles are composed of saturated fatty acids that provide a hydrophobic environment which maintains cell rigidity at elevated temperatures (Herbert and Sharp, 1992). Another method of adaptation is that the DNA of thermophiles contains reverse DNA gyrase. This enzyme results in DNA becoming supercoiled, thereby elevating the DNA melting point to at least the maximum growth temperature of the organism. Thermophiles tolerate high temperature by increasing interactions such as: electrostatic, hydrophobic interaction and disulphide bridges (Kumar and Nussinov, 2001).

2.5.2.1. Advantages of thermostable enzymes in ethanol fermentation

The use of thermophilic bacteria such as *Actinomyces,* in large-scale commercial processes can confer a number of advantages. Biotechnological processes may be run at higher temperatures reducing the risk of contamination by mesophiles. High temperature also influences the

bioavailablity and solubility of organic compounds to a great extent, thereby allowing efficient bioremediation (Becker and Botes, 2003). Reaction rates are increased at higher temperatures due to decreased viscosity. Higher process yields are obtained due to the increase in solubility of the substrate and products (Mozhaev, 1993; Krahe *et al,* 1996).

CHAPTER 3

RESEARCH PAPER

CHAPTER 3

Isolation and characterisation of esterases from thermophilic *Actinomyces*

ABSTRACT

Thermpohilic Actinomycete isolates with the ability to hydrolyze xylan were screened for esterase activity. Two isolates (ORS10 and GSIV1), identified as *Streptomyces* spp, were positive for acetyl xylan esterase (AXE) activity. A cosmid library representative of isolate ORS10 was composed and screened for AXE activity using β-naphthyl acetate as substrate. An 18 kb cosmid clone, 18D7, tested positive for AXE activity. Intracellular fractions extracted from ORS10 were precipitated with ammonium sulphate and partially purified 161-fold. Specific activity was measured after dialysis and ion-exchange chromatography. Overall yield of the partially purified enzyme was 34 %. A molecular mass of 40 kDa was estimated by SDS-PAGE. The partially purified AXE displayed optimum activity at pH 9 and at 50°C. AXE activity was stable for at least 1.5 hours between 30°C and 40°C, and for 24 hours between pH 6-9. The K_M and V_{max} values were 16.93 mg/ml and 1645 units/mg enzyme, respectively.

INTRODUCTION

Due to the depletion of worldwide oil reserves and the ubiquitous emission of high levels of greenhouse gasses, the identification of renewable energy sources has never been so important. So far, the most promising and economically viable development is biofuels, particularly bioethanol (Demain, 2008). Bio-ethanol is a liquid biofuel that can be produced from 1st generation (sugar cane and beet sugar in particular) or 2nd generation (lignocellulosic matter) processes. It is an attractive alternative fuel as it is bio-based, renewable and better for the environment compared to current fuel derivatives (Balat *et al,* 2008).

Any biological feed-stock high in sugar, starch or lignocellulosic biomass can be used as the starting point for bio-ethanol production. Because it is not ideal to divert food crops towards fuel production, especially in Africa, this study was focused on lignocellulosic biomass. Lignocellulose is the major component of woody and non-woody plants (e.g. grass) and represents a key source of renewable organic matter (Kim and Dale, 2004). Lignocellulosic biomass consists of three basic polymers: cellulose ($C_6H_{10}O_8$), hemicellulose ($C_5H_8O_4$) and lignin (Balat *et al*, 2008). The combination of hemicellulose and lignin provides a protective sheath around the cellulose, conferring it extremely recalcitrant to enzymatic degradation, and therefore this layer needs to be modified or removed before the hydrolysis of cellulose can commence (Hamelinck et al, 2005) The synergistic action of a battery of enzymes, such as cellulases, hemicellulaes and side-chain cleaving esterases are required for complete degradation.

This study focuses on the side-chain cleaving esterases, in particular, ferulic acid esterases (FAEs) and acetyl xylan esterases (AXEs) produced by thermophilic Actinomycetes. Thermophilic enzymes offer significant process advantages, such as increased rate of hydrolysis. Both enzymes hydrolyze ester linkages between phenolic acids, xylan, and polysaccharides in plant cell walls. FAEs and AXEs are considered as 'accessory enzymes' in the degradation of lignocellulose (Mozhaev, 1993).

FAEs (EC 3.1.1.73) fall in a subclass of the carboxylic ester hydrolase family that liberates phenolic acids, such as ferulic acid and its according dimers, from hemicellulose and pectins. These enzymes are involved in breaking the bond between arabinose and ferulic acid, thereby releasing the covalently bound lignin from hemicellulose (Tsujiyama and Nakano, 1996).

AXEs (EC 3.1.1.72) also belongs to the carboxylic ester hydrolase family, and act on carboxylic ester bonds. The role of AXEs is to hydrolyze the release of acetyl groups at position -2 and/or -3 of xylose moieties in xylan (Tsujiyama and Nakano, 1996). AXEs have predominantly been isolated from yeast (Blum *et al,* 1999; Colombres *et al,* 2008), but have been recently isolated from bacteria, such as *T. reesei* (Sundberg and Poutanen, 1991), *S. rubiginosus* (Keller, 1992), *S. commune* (Biely

et al, 1987) and others (Blair *et al,* 2004; Chung *et al,* 2002; Tsujibo *et al*, 1997). In 2006, Nisole *et al* reported that the AXE gene of *Streptomyces lividans* is located downstream of the xylB gene of *S. lividans* which encodes xylanase B. They also noted that the AXE consists of a C-terminal substrate binding domain and an N-terminal catalytic binding domain that is separated by a glycine-rich linker. This C-terminal domain shares a high sequence similarity with the C-terminal of xylB. The N-terminal catalytic domain contains a catalytically competent NodB homology domain that is universally conserved amoungst enzymes from CE family 4 (Nisole *et al,* 2006). The work reported by Nisole *et al* is consistent with previous studies and reinforces the theory of synergistic action of hemicellulase in the xylanolytic enzyme system (Tsujibo *et al,* 1997; Taylor *et al,* 2006 and DuPont *et al,* 1996).

MATERIALS AND METHODS

3.1. Maintenance of *Actinomyces* organism

3.1.1. Organisms and growth medium

Twenty-one thermophilic Actinomycete organisms, isolated from Namibian and Zambian hot springs, were maintained in either 172F medium or Desert Minimal Media (DMM). Namibian isolates were grown for 1-2 weeks at 45°C in DMM media containing 0.05% glucose (Merck), 0.05% yeast extract (Merck), 0.05% NaCl (Merck), 0.05% $MgSO_4$. $7H_2O$ (Fluka), and 0.1% KPO_4 (Sigma) pH 7. After autoclaving, trace elements (0.1% Iron sulphate $FeSO_4$. $7H_2O$ (Merck), 0.1% $MnCl_2$. $4H_2O$ (Merck), 0.1% $ZnSO_4.7H_20$ (Merck)), 1 mg/ml Nalidixic acid (Sigma) and 1mg/ml Cycloheximide (Sigma) were added. Zambian isolates were grown at 45°C in 172F Media containing 1% glucose, 0.5% yeast extract, 1.5% Sodium NaCl, 0.25% $MgSO_4$. $7H_2O$, 1% Soluble starch (Merck), 0.25% Casamino acids (MP Biomedicals LLC), 0.1% $CaCl_2$. $2H_2O$ (Sigma), pH 7. After autoclaving, trace elements (0.1% $FESO_4$. $7H_2O$, 0.1% $MnCl_2$. $4H_2O$, 0.1% $ZnSO_4.7H_20$), 1mg/ml Nalidixic acid and 1 mg/ml Cycloheximide were added.

3.2. Screening of *Actinomyces* for ferulic acid esterase (FAE) and acetyl xylan esterase (AXE) activity

3.2.1. Xylan hydrolysis

All organisms were tested for their abilityl to degrade xylan (Shirling and Gottlieb, 1966). Organisms were grown on either DMM or 172F agar with glucose substituted for 1% Birchwood xylan (Sigma). After 1-2 weeks of growth, plates were flooded with Gram's iodine (Sigma) to observe zones of clearance.

3.2.2. Esterase/Lipase screen

Isolates/clones were plated onto LB agar (0.5% Yeast extract, 1% Tryptone (Merck) 1% NaCl and 1.5% agar (Merck)) containing 1% Gum Arabic (Sigma) and 1% (v/v) Tributyrin (Merck) and appropriate antibiotic (Sigma). These constituents were sonicated in 100 ml volumes (3 x 30 sec cycles) at half power. After incubation at 37°C/45°C for 1-2 days, the plates were observed for zones of clearance around the wells.

3.2.3. Ferulic acid esterase (FAE) assay

Organisms positive for xylan hydrolysis were plated on either DMM or 172F plates substituted with 0.4% Ethyl ferulate (EF) (Sigma) in dimethyl sulphoxide (DMSO) (Merck). Isolates were either placed on media by punching holes and dropping in supernatant, or colonies picked and pricked onto the surface of the agar. The formation of clearing zones around the point of inoculum indicated FAE activity (Donaghy *et al*, 1998 with modifications as described by Rashamuse *et al,* 2007).

3.2.4. Acetyl xylan esterase (AXE) assay

Organisms positive for xylan hydrolysis were assayed for acetyl xylan esterase activity spectrophomometrically. The assay contained 0.17 ml of 50 mM Sodium phosphate buffer ($NaPO_4$) (Riedel-de Hahn) pH 7, 0.1 ml enzyme solution (culture supernatant) and 0.02 ml of 40 mM β-

naphthyl acetate (Sigma) (in methanol (Kimix)) in an eppendorf tube, and was incubated at 37°C for 5 minutes. After the incubation, 0.1 ml Fast Garnet GBC sulphate salt (Sigma) (60 mg/ml solution in 10% SDS (Sigma)) was added and the absorbance determined at 560 nm (BioMate 3 Spectrophotometer) after diluting 0.02 ml of the reaction mixture with 0.98 ml of water. The calibration curve was constructed using β-naphthol (Fluka) in the concentration range of 0 - 9 mM. The amount of β-naphthol produced and specific activity for each isolate was determined (Krastanova et al, 2005). Unit of enzyme activity was defined as μg/μl/min.

3.2.5. Bradford assay

For each isolate, to determine protein concentration, a Bradford assay (Bradford, 1976) was conducted using Bovine Serum Albumin (BSA as standard).

3.3. Phylogenetic analysis of organisms positive for FAE and AXE activity

3.3.1. 16S rRNA analysis

DNA was extracted from cultures using the method by described by Wang *et al,* (1999). The 16S rRNA gene was amplified using primers 5' AGAGTTTGATCTGGCTCAG (F1) and 5' ACGGTACCTTGTTACGACTT (R5), based on highly conserved regions of the 16S rRNA gene (Felske *et al,* 1997). Amplification involved an initial 2 minute denaturation step at 96°C followed by 30 cycles of 45 seconds at 96°C, 30 seconds at 56°C and 2 minutes at 72°C, and a final 5 minute extension step at 72°C using the GeneAmp^{R} PCR System 2700 (Applied Biosystems). The PCR products were analysed by electrophoresis in a 0.8% agraose (Sigma) gel containing ethidium bromide (Sigma). The PCR products were purified using the GFX PCR DNA and gel band quantification kit (Illustra), and sequenced at Stellenbosch University using the F1, R5 and F3 (5' GCCAGCAGCCGCGGTAATAC 3') primers. Isolates were identified by using the BLAST program (www.blast.ncbi.nlm.nih.gov/) to align the 16S rRNA gene sequence with other identified organisms.

3.4. Cosmid Libraries

3.4.1. Cosmid Library construction

Two cosmid libraries were constructed. Metagenomic DNA was isolated according to the method described by Wang *et al,* (1999). Library ONWG was constructed with genomic DNA isolated from isolates ORS3, NDS4, WBDS9 and GSIV1. Library ONO represented strains ORS10, NDS9 and ORS13. High molecular weight genomic DNA from the Actinomycete isolates was end-repaired using the End-It DNA End-repair kit (EPICENTRE Biotechnologies), ligated into the cosmid *E. coli-Streptomyces* shuttle vector pFD666 (Denis and Brzezinski, 1992), and the resulting DNA packaged into MazPlax Lambda (EPICENTRE Biotechnologies). Packaged DNA was transfected into the *E. coli* Genehog ($recA^-$) cells and cosmid clones selected on LB agar using kanamycin (Sigma) at 50 μg/ml.

3.5. Positive cosmid clones

Positive clones were screened for FAE and AXE activity**.** Cosmid DNA was extracted using the Qiagen Plasmid Midi Kit (Qiagen) and analysed by electrophoresis in a 0.8% agarose gel stained with ethidium bromide. Concentration of cosmid DNA was determined by including lambda DNA (Fermentas) in the concentration range of 10 ng/μl – 100 ng/μl. The cosmid was digested using *Hind*III (Fermentas) and *Pst*I (Fermentas) restriction endonucleases. Clones were end-sequenced at Stellenbosch University using primers, 5' TAATACGACTCACTATAGGG (T7 promoter) and 5' GCTAGTTATTGCTCAGCGG (T7 terminator)

3.6. (AXE) gene sequence determination

3.6.1. Transposon Mutagenesis

AXE positive clones identified from library ONO, was exposed to transposon mutagenesis with the HyperMu™ CHL-1 Insertion Kit (EPICENTRE) according to the manufacturers instructions. Mutants that lost AXE activity were propagated for cosmid isolation and sequenced.

3.6.2. Subcloning

Cosmid DNA was extracted according to the method previously described (Section 5.2.1). A bulk digestion using *BamH*1 (Fermentas) restriction endonuclease was used to release the insert DNA, and the appropriate bands excised and extracted using the Qiagen Gel Extraction kit (Qiagen). A multi-functional *E. coli-Streptomyces* shuttle vector pUWL219 was isolated and digested with *Bam*H1. The plasmid was dephosphorylated using FastAP (Fermentas) as per manufacturers instruction and ligated to DNA fragments (2 kb, 3 kb and 5 kb) using T4 DNA ligase (Fermentas) at 4°C overnight. The ligation reaction was transformed with electrocompetent *E.coli* genehog cells and plated on LB agar containing 50 μg/ml ampicillin (Sigma).

3.6.3. Primer-walking

The 18 kb insert was subjected to a number of rounds of primer-walking. Sequencing primers were designed using Genscript (www.genscript.com/cgi-bin/tools/sequencing_primer_design), synthesised at Inqaba Biotech, and sequenced at the University of Stellenbosch.

3.7. ORS10 growth, and AXE activity

3.7.1. Growth

Am single freshly grown ORS10 colony was picked from a DMM agar plate, inoculated into 10 ml of DMM media containing 1% Birchwood xylan, and cultured at 45°C. An overnight culture (100 μl) was inoculated into 100 ml of DMM media supplemented with 1% Birchwood xylan. Overnight culture (50 ml) was centrifuged at 5000 g for 10 minutes and the supernatant retained the (extracellular fraction). The pellet was sonicated at half power (5 x 30 sec cycles), cell debris removed by centrifugation and the supernatant retained (intracellular fraction). Protein concentration was determined using the Bradford assay and both fractions were assayed for AXE activity.

3.7.2. Activity

3.7.2.1. Enzyme Recovery

Isolate ORS10 was grown in DMM media (supplemented with 1% Birchwood xylan) at 45°C for 2 days. A 1 ml spore suspension was used to inoculate 1 L of media. The fermentation broth was centrifuged at 4 000g for 15 minutes at 4°C. The pellets were retained, resuspended in 50 mM sodium phosphate buffer and sonicated on ice for 30 s at half-power until the solution was homogenous, followed by centrifugation (4 000g for 45 minutes at 4°C). The resulting culture was transferred to a beaker and placed on a magnetic stirrer at 4°C. Within 20 minutes, ammonium sulphate powder (Merck) was added to obtain 80% saturation followed by 30 minutes of continuous stirring. The precipitate was recovered by centrifugation at 4 000g for 45 minutes at 4°C. The cell pellet was resuspended in 50 mM sodium phosphate buffer and this constituted the crude enzyme preparation. This preparation was dialysed against 1L of 50 mM sodium phosphate buffer overnight. The dialysed protein was filter-sterilsed. Protein concentration and AXE activity were determined as previously discussed (Section 3.2.4 and 3.2.5).

3.7.2.2. Enzyme purification

The dialysed ammonium sulphate precipitate was subjected to ion-exchange chromatography using the AKTA FPLC P-920 (Amersham Biosciences) and the Hi-Load™ 26/10 Q-sepharose column. The column was equilibrated with 25 mM potassium-phosphate buffer and elution performed with a linear gradient of 0-1M Sodium-chloride. Protein concentration was monitored with a UV recorder set to 280 nm. Fractions were collected and active fractions assayed for AXE activity. Protein fractions conferring AXE activity were pooled and concentrated using the Vivaspin 20 Concentration Columns – 3, 000 MWCO PES membranes (Sartorius Biotech).

SDS/PAGE was performed according to the method described by Laemmli (1970), in 12% (w/v) polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue (Sigma). Bands correlating to the AXE protein were sequenced at the Proteomics Research group at the University of the Western Cape using MALDI-TOF MS.

3.8. Enzyme characterisation

3.8.1. Temperature

The temperature optimum was established by assaying for AXE activity at 30°C to 80°C at 10°C intervals. Thermostability was established by incubating the dialysed enzyme at 30°C-80°C. At 30 minute intervals the AXE activity was assayed for a 2 hour period and then assayed again after 7 hours.

3.8.2. pH

pH optima was established by assaying for AXE activity in the pH range of 3-10. pH stability was established by incubating the assay reaction mixture (using buffers in the range of pH 3-10) at 4°C for 24 hours. After 24 hours the reactions were assayed for AXE activity. Protein concentrations were determined by the Bradford assay, and specific activites were determined and converted to relative Activity (%).

3.8.3. Enzyme Kinetics

To determine the kinetic constants**,** the assay was performed under optimal conditions (temperature and pH) with a concentration of β-naphthyl acetate ranging from 5-60 mM. The graph was constructed in GraphPad Prism and the K_M and V_{max} determined.

RESULTS

Xylan hydrolysis

Thermophilic Actinomycete strains, isolated from Namibian and Zambian hot springs, were screened for the ability to hydrolyze xylan. Of the 21 isolates, 18 were able to utilise xylan as a carbon source (**Table 1**).

Table 1: Actinomycete organisms tested for ability to utilise xylan, produce AXE and FAE.

Actinomycete Strains	Positive (+) for xylan xydrolysis with addition of 1 % xylan	+/- AXE	+/- FAE
ORS 15			
ORS 13	+		
ORS 2	+		
ORS 3	+		
ORS 1	+		
ORS 10	+	+	
NDS 10	+		+
NDS 11	+		
NDS 15	+		
NDS' 14	+		
NDS 9	+		+
NDS 5	+		
NDS 4	+		
NDS 14			
WBDS 1			
WBDS 9	+		+
WBDS 6	+		
WBDS 10	+		
WBDS 8	+		
WBDS 11	+		
GSIV 1	+	+	

Enzyme screening

(i) Ferulic acid esterase screen

Strains with the ability to degrade xylan were screened for FAE activity. Three organisms were positive for FAE activity: WBDS9, NDS9 and NDS10 **(Table 1)**. Isolate WBDS9 displayed the highest FAE activity as judged by the production of the biggest halo in relation to colony size.

(ii) Acetyl xylan esterase screen

A qualitative liquid assay was used to detect AXE activity based on the amount of β-naphthol produced. A calibration curve using β-naphthol in the concentration range 0 – 9 mM was constructed in order to determine AXE specific activity.

Table 2: Acetyl xylan esterase (AXE) screen production by Actinomycete strains

Isolate	Extracellular protein concentration (µg/µl)	β-naphthol produced after 5 minutes (µM)	Specific activity(mM/min.mg)
ORS 10	0.061	0.13	4.26
GSIV 1	0.059	0.91	30.9

Of the 18 organisms identified with the ability to degrade xylan, isolates, ORS10 and GSIV1 displayed significant AXE activity **(Table 2)**.

(iii) 16S rRNA gene analysis

16S rRNA gene sequences of 1322 bp and 1260 bp were obtained for WBDS9 (FAE producer) and ORS10 (AXE producer), respectively **(Table 3)**. A BLAST search of the GENBANK database revealed that isolate ORS10 shared a 94.0% sequence similarity to its closest neighbour *Streptomyces* Ruj7-1 (Accession number: GQ222219.1). Isolate WBDS9 shared a 98.0% sequence similarity to its closest neighbour *Streptomyces radiopugnans* (Accession number: DQ912930).

Table 3: 16S rRNA gene sequence analysis

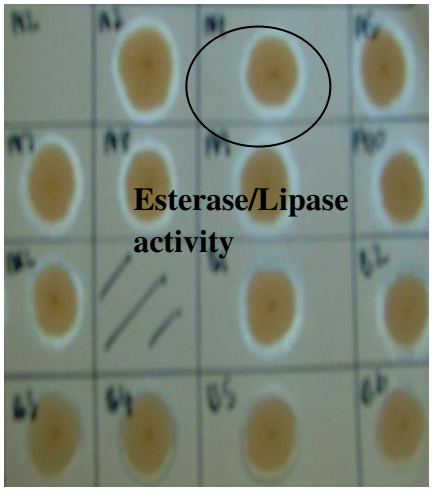
Strain	Nearest neighbour	Sequence obtained (bp)	% Identity
WBDS9	*Streptomyces radiopugnans*	1322	98%
ORS10	*Streptomyces* Ruj7-1	1260	94%

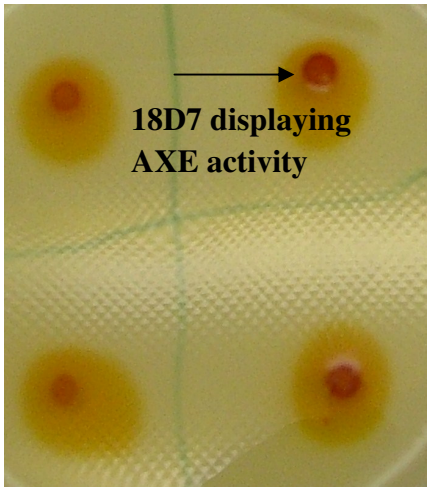
Cosmid library construction and screening

(i) Screening of cosmid libraries for FAE and AXE activity

Two cosmid libraries were constructed to screen for FAE and AXE activity. Library ONWG was representative of WBDS9 DNA, conferring FAE activity. Library ONO was representative of ORS10 DNA, conferring AXE activity. Average insert size corresponded to 32 kb and 35 kb for library ONO and ONWG, respectively. Both libraries were estimated to fully cover the genomes present (Bentley *et al,* 2002).

Cosmid libraries ONWG and ONO were first screened for general esterase/lipase activity. Seven clones from library ONO, and three clones from library ONWG were positive as seen by the formation of a halo of clearance when cultured on tributyrin-containing media (**Figure 1 a).** Esterase positive clones were screened for FAE and AXE activity. Two ONO library clones, 18D7 and 32D3, displayed AXE activity, visualised by the formation of deep red coloured colonies on a LB plate flooded with a solution containing β-naphthyl acetate and Fast Garnet GBC sulphate salt **(Figure 1 b).** Cosmid clone 11A9 from library ONWG tested positive for FAE activity **(Figure 1 c)**.

a) 

b) 

c) 

Figure 1: a) Clones from Library ONWG plated on 1% Tributyrin agar displaying esterase/lipase activity. b) Clones 18D7 and 32D3 (from library ONO) on LB plate flooded with β-naphthyl acetate and Fast Garnet GBC Sulphate salt in Sodium Phosphate buffer, displaying AXE activity. c) Clone 11A9 from library ONO plated on LB plate supplemented with ethyl ferulate, displaying FAE activity

(ii) Determination of insert size and origin of library clones conferring AXE activity

AXE positive cosmid clones, 18D7 and 32D3, were digested with *Hind*III and *Pst*I restriction endonucleases to release the 5.25 kb cosmid vector and insert size was calculated by analysing the digestion profile **(Figure 2).**

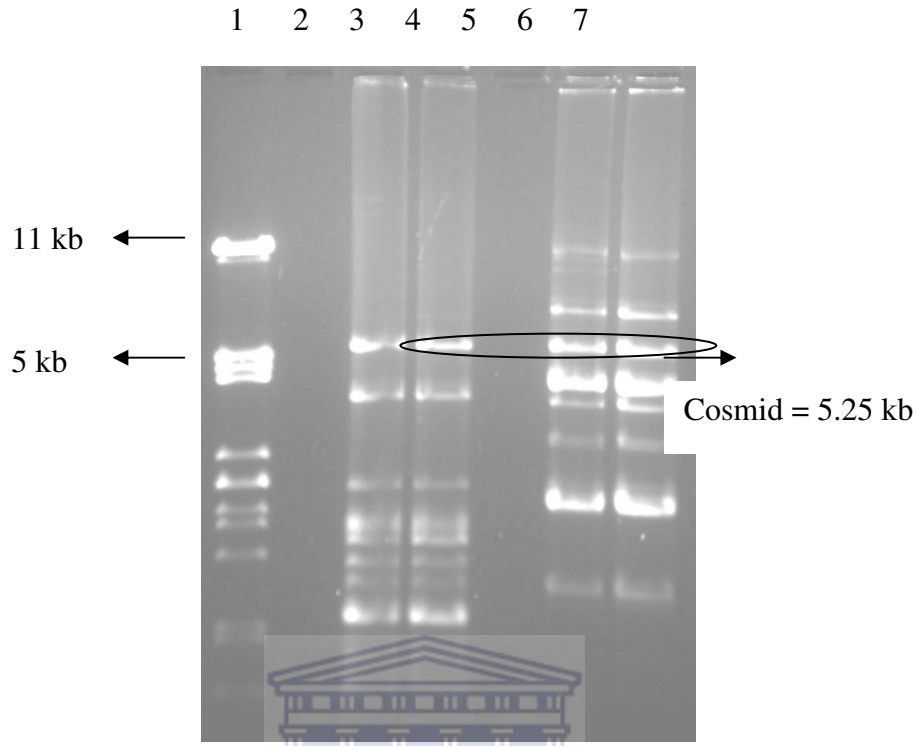


Figure 2: Cosmid digestion profile of clones 18D7 and 32D3, digested with *Hind*III and *Pst*I restriction endonucleases, visualised on a 0.8% agarose gel stained with ethidium bromide (lane 1 = Molecular weight marker, lanes 3,4 = Clone 18D7, lanes 6,7 = Clone 32D3)

Cosmid end-sequences suggested *Streptomycete* origin (**Table 4**) after a BLAST search of the GENBANK. Clone 18D7, with the smallest insert size (18 kb), was chosen for further studies.

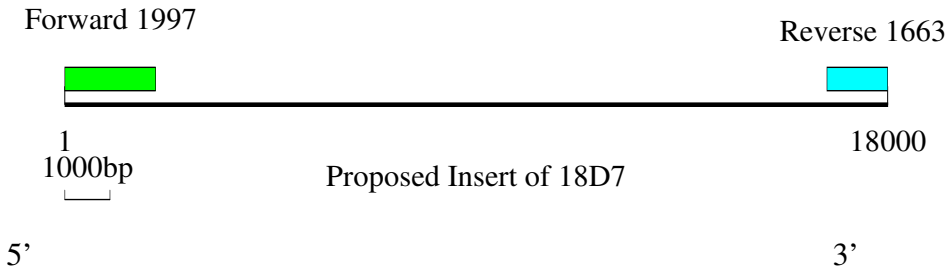
Table 4: Insert size and origin of the clones, 18D7 and 32D3.

Clone	Insert size	Sequence obtained (bp)	Origin	% Identity
18D7	18 kb	692	*Streptomyces sp. SPB74*	83%
32D3	32 kb	405	*Streptomyces sp. Mg1*	79%

Primer walking

The 18D7 clone was subjected to primer-walking. A 1997 bp and 1663 bp stretch of nucleotide sequence **(Figure 3 a)** was obtained from the C and N terminal ends of the insert, respectively. An excess of 14 000 bp remains unsequenced. After translating the sequence, an esterase consensus sequence 'GXSXG' was found near the end of the 18 kb insert as illustrated in **Figure 3 (b).**

(a)



(b)

E A Q A G S L A G F N A S S A G V P ML V S G A W P T T S Q A G G A A G S I W L A R Q L P K R Z Y A A A S
A I D T T E P S G K L H H T A S A P I V R P S H Q A N G R R T T H I D T N S T A S G A I T A P V A R T V P N S
T MV K P N R M N D H S T I A L M C W A I S I A P P S S G R N R P S E C R L N R Z T R P T S A P V I A R L N
A S P M P V M A P D A I P A A G A D V L R D H R R D A G A D G Q R R H L H V G P Z L R G D A V R R R G L
R A V L V D Q R H H H D R G R R D H D H L Q A H R Q S L G H Q R A Q D A E V G A Q I A Q L A P MQ R Q E
M L A A I E Q P H H C R Q A D Q L R D Q R C Q R R A R D V H A R N R S E A E D Q Q R V Q R D V E H H R Q
Q H E I E R R A R V A G P T Q R H H H E V V E V H E G Q R Q E D D P Q I A D R Q R P R V R R R A H R A E D
V R R E Q I A Q R R G D H Z Q Q H E E R G A G A D H A P G L V E V A R A D R L S D H D G V G H A D A E H
H A E Q E E Q H D I G V R R R R Q R R L A E I M A H P D R I D R G V Q R L Q D V A A E H G Q R K Q Q Q V

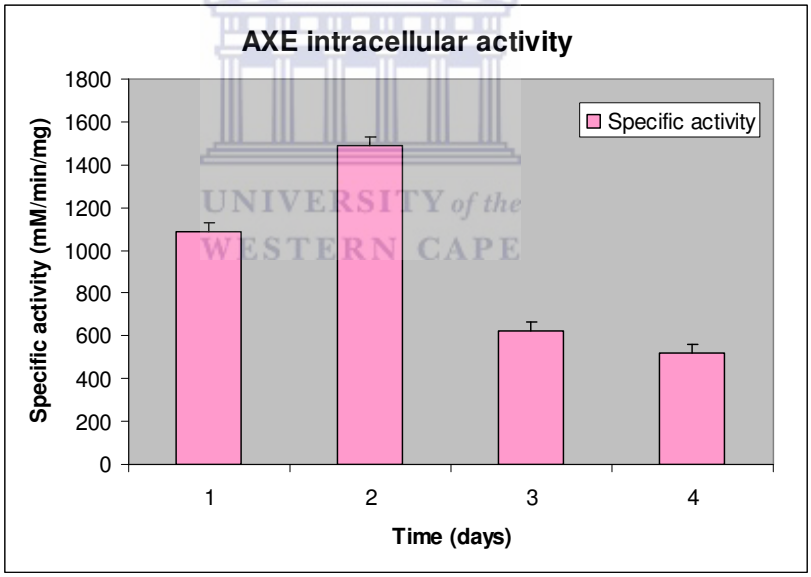
R P I G P V T S L A S G F I V I G S V S R G **G G S R G** A S S Y S I P A G P K R Z S A P D R H R P A P D R H P V P P Z S R R P A P Q A P T

Figure 3: (a) Clone 18D7 and the amount of sequence data obtained using primer-walking (b) Amino acid sequence of 18D7 containing the Esterase consensus sequence (underlined)

Obtaining the AXE protein sequence

(i) Growth and Activity curve

In order to recover and purify the AXE protein, a curve displaying ORS10 growth and AXE specific activity was constructed. Both intracellular and extracellular proteins were assayed daily for AXE activity. Significant levels of activity were detected in the intracellular protein fraction. Enzyme specific activity is illustrated in **Figure 4 (a)**. Protein concentration (calculated as µg)was highest on day three **(Figure 4 b)**, while specific activity of AXE was highest on day two.



(a)

(b)

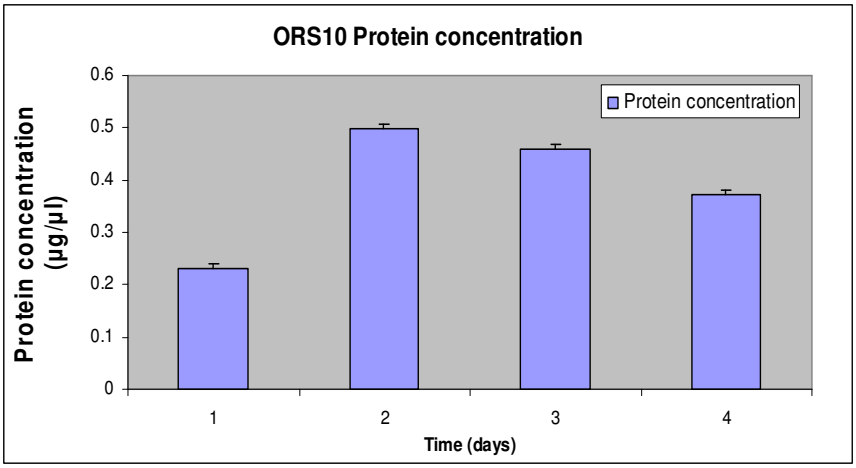


Figure 4: (a) Activity assay of the ORS10 intracellular proteins using ß-naphthyl acetate as substrate. (b) Protein production of isolate ORS10

(ii) Protein recovery and purification

On day 2, the 1L culture of ORS10 was sonicated to obtain the intracellular fraction and dialysed overnight against 1L of buffer. Proteins subjected to ion-exchange chromatography using Fast Performance Liquid Chromatogaphy (FPLC) revealed two peaks **(Figure 5)**. AXE activity was detected within peak 1 in 4x2ml fractions. No AXE activity was detected in the second peak.

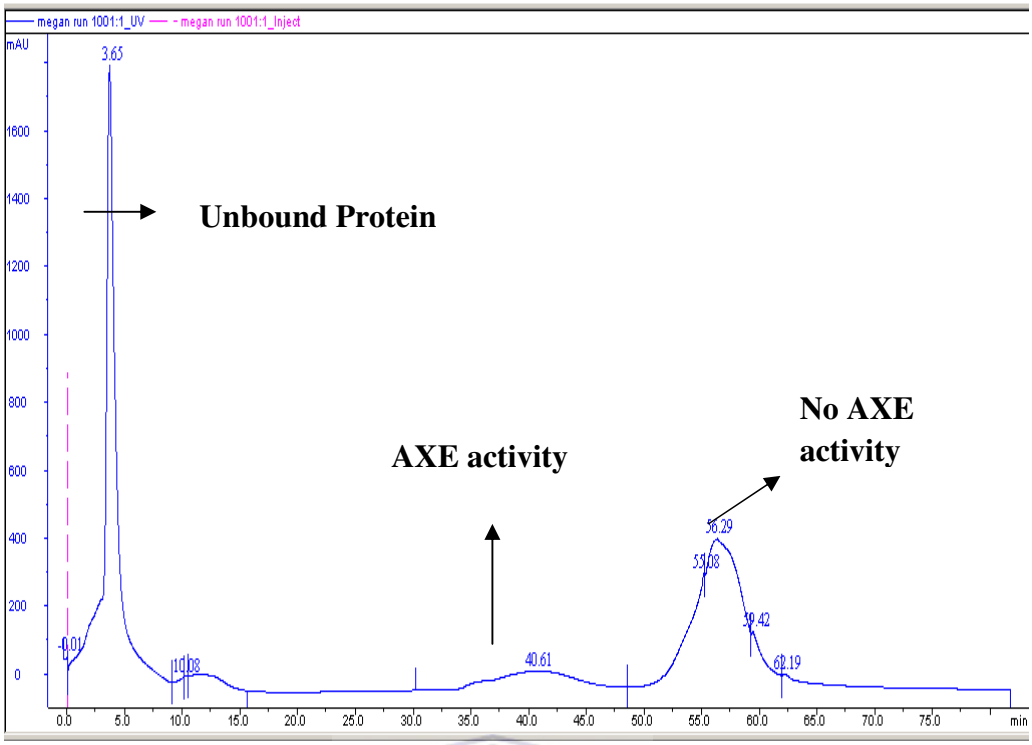


Figure 5: FPLC ion-exchange chromatogram of ORS10

At each stage of purification (crude extract, dialysed ammonium sulphate precipitate, ion-exchange, and concentration) the total protein concentration, total activity, specific activity and activity yield were determined (**Table 5**). Purifications resulted in a 161 fold increase in specific activity.

Table 5: Purification table of AXE from ORS10

Purification stage	Volume (ml)	Total protein concentration (µg/ml)	Total activity (AU)	Specific activity (mM/min/mg)	Increase in specific activity (%)	Act yield (%)
Intracellular (crude extract)	27	301.86	18213	60	1	100
Dialysed	4	1.652	12434	7526	125	68
Ion-exchange	8	0.832	7265	8731	145	40
Concentrated after Ion-exchange	1.2	0.631	6115	9690	161	34

Protein fractions were subjected to 1D SDS-PAGE analysis **(Figure 6).** Lane 3 contains the active AXE fraction (peak 2 on **Figure 5**). One intense band at 40 kDa is observed, correlating to the reported sizes of AXE (Koseki *et al,* 2006; Kam *et al,* 2005; Blum *et al,* 1999). However, MALDI-TOF MS identified the 40 kDa protein band as a 43 kDa xylose isomerase. The 60 kDa band was also sent for sequencing, and MALDI-TOF MS results are pending. Numerous faint bands at higher molecular weight masses were observed.

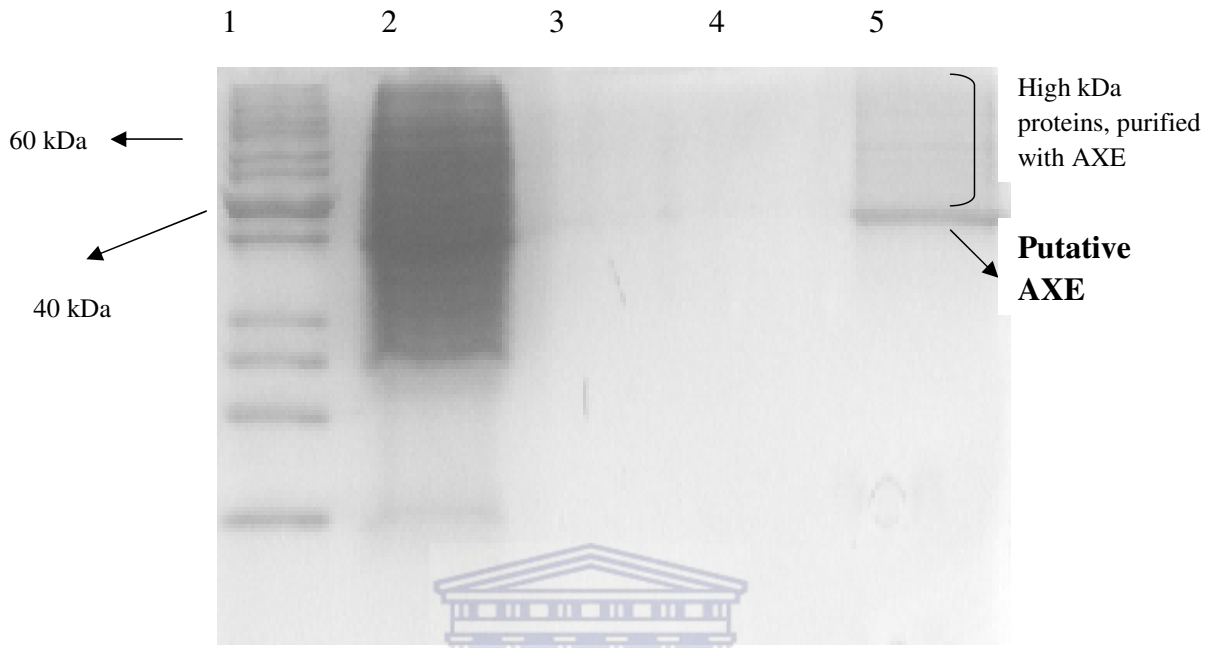
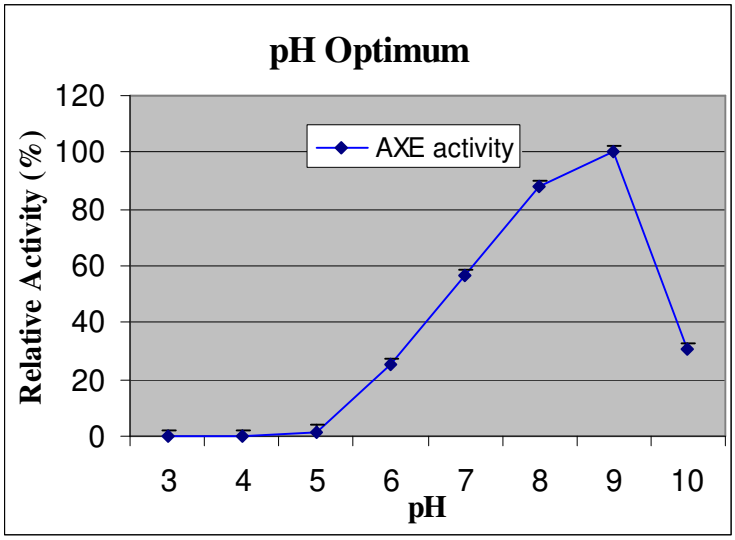


Figure 6: 1D SDS-PAGE of eluted fractions of ORS10, subjected to ion-exchange chromatography (lane 1=Protein ladder, lane 2= dialysed ammonium sulphate precipitate, lane 5= active AXE fraction after ion-exchange chromatography and concentration)

(iii) AXE characterisation

The AXE protein was characterised in terms of pH, temperature and enzyme kinetics. The AXE from ORS10 was active between pH 6-10 and displayed optimum activity at pH 9 **(Figure 7 a).**

(a)



(b)

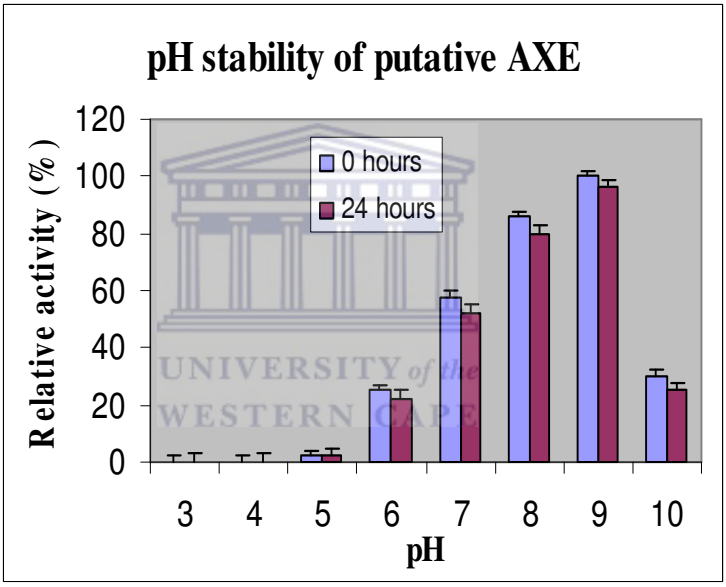
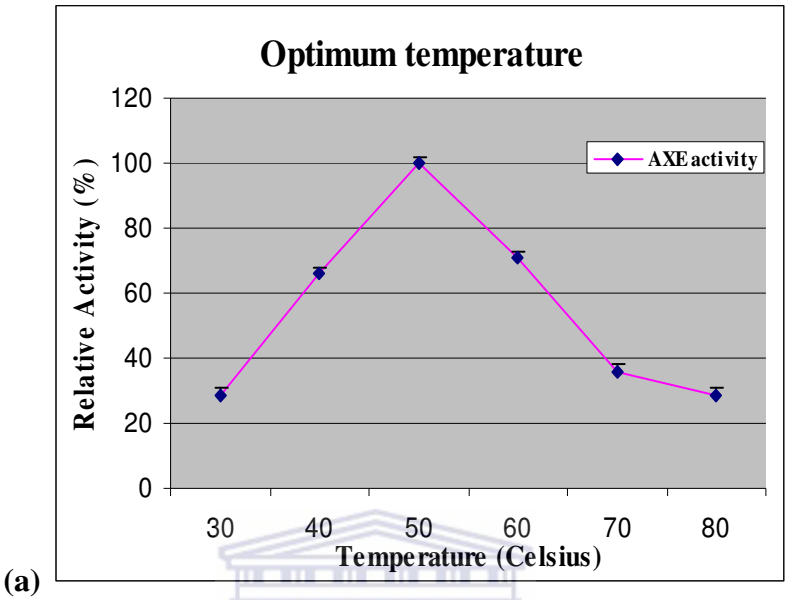


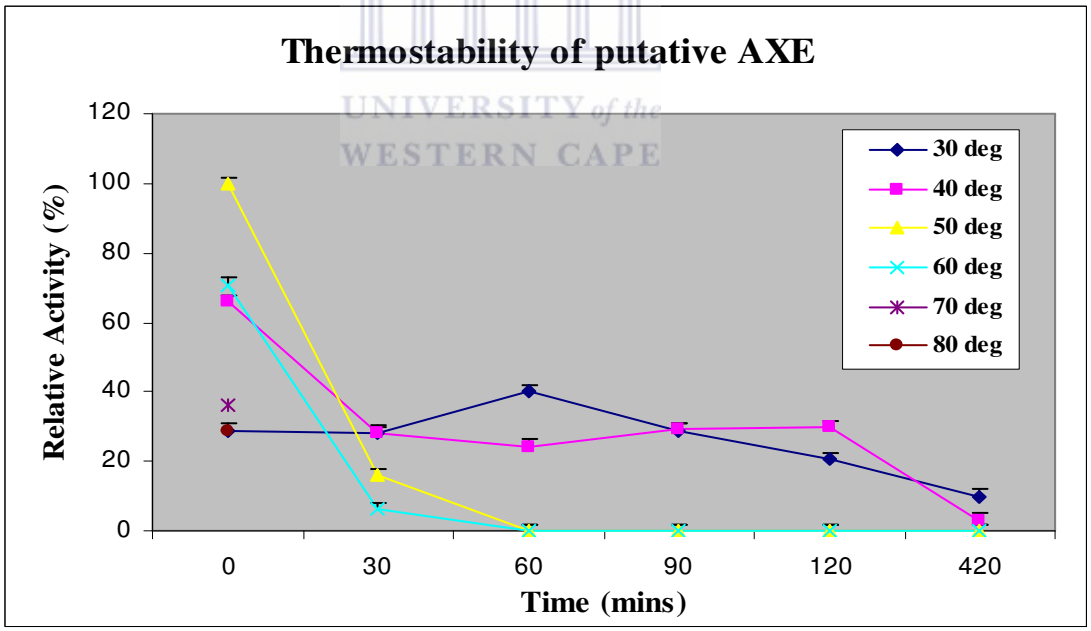
Figure 7: (a) Optimal pH of partially purified AXE from ORS10. (b) pH Stability of partially purified AXE from ORS10

Between pH 6-10 the AXE enzyme had no major decline in activity over a period of 24 hours (**Figure 7 b**). The enzyme displayed optimum activity at 50°C (**Figure 8 a**). Activity steadily increased from 30°C up to 50°C, and then steadily decreased as temperature increased. At 80°C, the

enzyme retained only 16% activity. At 30°C and 40°C the enzyme displayed most thermostability **(Figure 8 b).**



(a)



(b)

Figure 8: (a) Optimal temperature of partially purified AXE from ORS10 (b) Thermostability of partially purified AXE from ORS10

The Michaelis Menten constants were determined with 205 ng of dialysed ORS 10 protein and β-naphthyl acetate in the concentration range of 5-60 mM **(Figure 9)**. GraphPad Prism Non-linear regression software was used to determine K_M and V_{max} as 16.93 mM and 1645 units/mg of enzyme respectively.

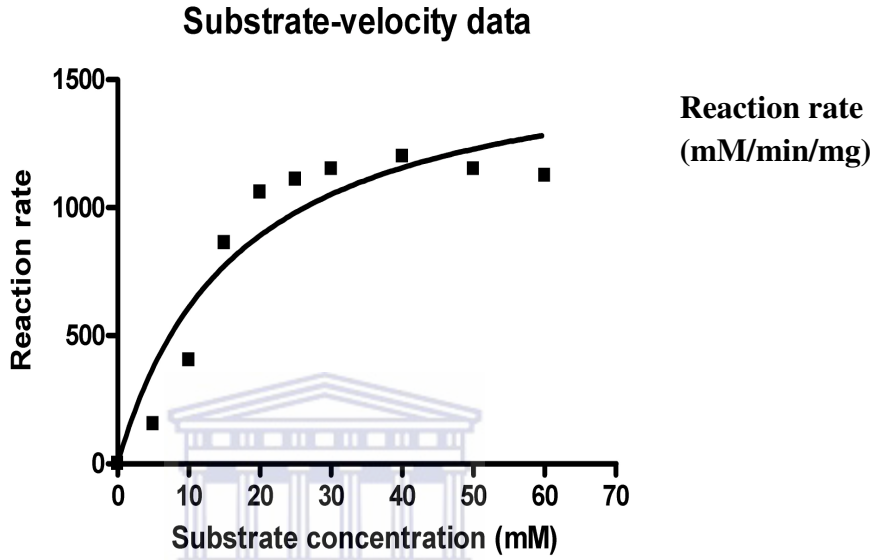


Figure 9: Michaelis-Menten graph of substrate concentration (mM) vs. Reaction rate (mM/min/mg) of AXE from ORS10

DISCUSSION

The world's oil reserves may be depleted as soon as 2050, meaning that significant progress must now be made into renewable energy sources. Moreover, burning these conventional energy sources causes widespread air pollution through greenhouse gas emissions (Saxena *et al*, 2009). Lignocellulose represents a key source of renewable organic matter. As a result of their chemical properties and components, lignocellulose is an ideal and very important substrate in biotechnology (Kim and Dale, 2004) and can be converted to useful forms of energy (Lee, 1997). Due to a highly recalcitrant structure, lignocellulose is not easily degraded and a battery of enzymes is required for complete hydrolysis, such as cellulases, hemicellulases and accessory enzymes, including acetyl

xylan esterase and ferulic acid esterase. This study hopes to provide insight into this ever-expanding area of research.

The 21 *Actinomycetes* screened for specific AXE and FAE activity were not previously characterised and were potentially a good source of novel enzymes. Two isolates displayed specific AXE activity, GSIV1 and ORS10. Although the enzyme produced by isolate GSIV1 showed higher specific activity, ORS10 offered uniqueness. A BLAST search using the 16S rRNA amplified gene of ORS10 revealed 94% identity to its closest neighbour. Since the threshold identity value delineating bacterial species is considered to be 97.5% (Wayne *et al,* 1987), ORS10 may be a novel species. Moreover, the strain displayed an array of other enzyme activities, such as cellulase and xylanase. ORS10 was therefore the focus of this study. Three strains were able to hydrolyze ethyl ferulate, of which isolate WBDS9 displayed highest FAE activity. *Streptomyces* strain WBDS9 was thus chosen for further studies.

In order to find the genes responsible for esterase activity, two cosmid libraries were constructed. Library ONO was composed of 6000 clones and represented full coverage of three genomes (ORS13, NDS9, ORS10) (Bentley *et al,* 2002). As a plate assay for AXE activity has not been reported in literature, a liquid screen was modified to a plate assay. Two AXE positive cosmid clones (18D7 and 32D3) were identified from library ONO, clearly visualised as red-coloured colonies as opposed to the negatives which remain orange in colour. The screen was however subjective in nature as the staining solution stains not only stains the colony, but also the surrounding media. The ambiguity of the screen was exposed when screening of knock-out mutants. Distinguishing between AXE positive and negative mutants was difficult.

Three approaches were adopted to obtain the AXE gene sequence of isolate 18D7: transposon mutagenesis, subcloning and primer walking. Of the 92 transposon mutagenesis clones, 11 lacked activity, and therefore were expected to contain the transposon within the AXE gene. However, all 11 were 99% homologous to the shuttle cosmid vector, pFD666, indicating that the transposon had

inserted in the vector and not the insert DNA. This result might not be due to the failure of transposon mutagenesis, but rather to the ambiguous AXE plate screen. The screen is open to interpretation, and therefore false negatives are generated. With subcloning, three bands, with a combined size of 10 kb, were cloned. The subclones were all negative for AXE activity. The remaining 8 kb may contain the AXE gene and may be the focus of subsequent studies. Primer-walking was also used to determine the DNA sequence of the AXE conferring cosmid clone. A total of 3.6 kb of nucleotide sequence was assembled **(Figure 3 a)**. The remaining 14 kb of sequence was unaccounted for. The forward and reverse nucleotide sequences of clone 18D7 were translated and analysed for any motifs relating to AXE enzymes. An esterase consensus sequence 'GXSXG' was located 120 bp before the end of the 18 kb fragment. Koseki *et al* (1995) cloned and expressed the AXE gene from *A. niger* into *Pichia pastoris.* The recombinantly expressed AXE contained the consensus sequence 'GXSXG' as well as an Asp202 and His252 that is highly conserved among AXEs (Koseki *et al,* 1997). In all previous studies, this consensus sequence has been found over mid-way through the AXE gene, toward the C-terminal **(Figure 10).** The presence of an esterase/AXE could however not be confirmed with a BLAST search of the GENBANK database.

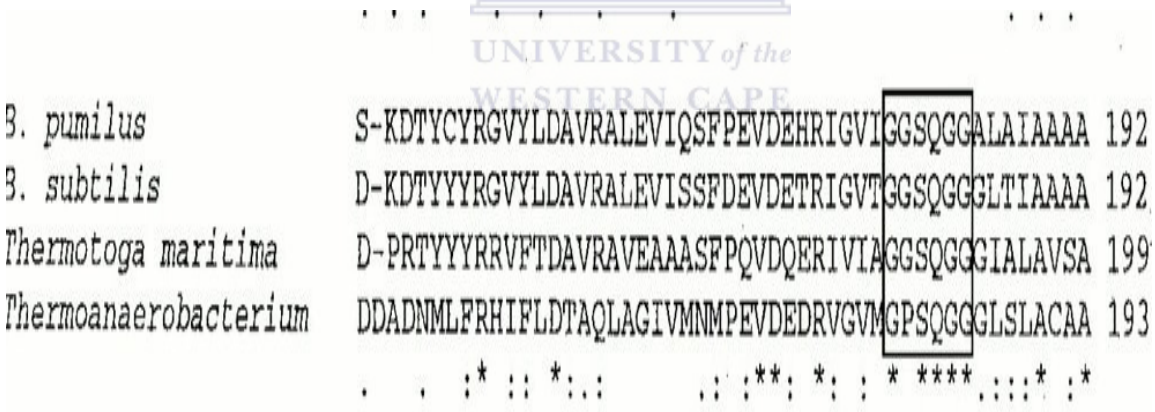


Figure 10: Esterase consensus sequence 'GXSXG' (Adapted from Degrassi *et al,* 2000)

Both intracellular and secreted proteins of ORS10 were assayed for AXE activity. The highest specific activity was detected on day two of growth (mid log phase), within the intracellular fraction. Accordingly, the intracellular proteins were ammonium-sulphate precipitated on day two, dialysed

and purified by ion-exchange chromatography. AXE active elution peaks **(Figure 5)** were subjected to SDS-PAGE analysis (**Figure 6**) which revealed one intense band at 40 kDa, along with numerous other faint bands at higher molecular weights. It appeared likely that the 40 kDa band conforms to the AXE as it falls within the expected molecular weight range (30-40 kDa) for reported AXE (Koseki *et al,* 2006; Kam *et al,* 2005; Blum *et al,* 1999). However, MALDI-TOF MS identified the protein as a xylose isomerase (data not shown). AXEs have have been isolated from *Strepyomyces* species, such *S. rubiginosus* (Keller, 1992), *S. commune* (Biely *et al,* 1988), and *S. lividans* (DuPont *et al,* 1996). As ORS10 may be a novel species in the *Streptomyces* genera, this study may provide additional information on the highly relevent AXE enzymes that are able to degrade lignocellulose.

Ion exchange was used to purify the AXE protein. Purifications did not result in a major increase in specific activity **(Table 5).** As seen by multiple protein bands revealed with SDS-PAGE analysis, the protein could not be purified to homogeneity by ion-exchange chromatography alone. Attempts to decrease the flow rate of ion-exchange chromatography, which aids better separation of proteins, proved ineffective as the same elution profile was obtained. Further purification steps would be useful, such as hydrophobic chromatography (Halgasova *et al*, 1994) and size-exclusion chromatography (Degrassi *et al,* 2000; DuPont *et al,* 1996).

When an enzyme with industrial application from a novel bacterium is identified, it is important to characterise the enzyme in terms of temperature and pH. The AXE from ORS10 had a pH optimum of 9 and was active in a pH range of 6-9. This value is not surprising, as AXEs have a been reported to display activity within a broad pH range (Yang and Lui, 2008; Koseki *et al,* 2006; Halgasova *et al,* 1994, DuPont *et al,* 1996; Degrassi *et al,* 2000). The enzyme was stable between pH 6-9 for at least 24 hours. The pH optima and stability of AXE from ORS10 resembled characterisation data obtained for the rAXE from *Bacillus pumilis* (Degrassi *et al,* 2000). The temperature optimum of the AXE produced by ORS10 was 50°C and showed thermostability at 30°C and 40°C. Most activity (84%) was lost after 30 minutes at elevated temperatures. The temperature parameters are in close proximity to those of the rAXE from *Bacillus pumilis* (DuPont *et al,* 1996) and the AXE from *Schizophyllum commune* (Halgasova *et al,* 1994). AXEs have been reported to optimally function

within a broad temperature range, ranging from 30°C-80°C (Yang and Lui, 2008; Koseki *et al,* 2006; Halgasova *et al,* 1994, DuPont *et al,* 1996; Degrassi *et al,* 2000). Due to its high stability at 30°C-40°C, the partially purified AXE in this study is ideal for application in mesophilic fermentation processes employed in the pharmaceutical (Martinez-Martinez *et al,* 2007), paper and pulp, and the animal feedstock industry (Satyanarayana and Johri, 2005).

Another parameter of interest for enzymes with commercial applications is kinetic data. In this study, the K_M and V_{max} values of the AXE were determined as 16.93 mg/ml and 1645 units/mg of enzyme, respectively. Both parameters were compared to values in literature. The V_{max} value is slightly lower than that obtained from *S. lividans* using acetylated birchwood xylan as substrate but much higher than other reported values for AXE (Yang and Lui, 2008; Koseki *et al,* 2005; Halgasova *et al,* 1994). The K_M value of 16.93 mg/ml is considerably higher than that obtained from the *S. lividans,* which has been shown to be 7.94 mg/ml (DuPont *et al,* 1996). The relatively high K_M in comparison to the K_M of other AXEs, suggests that the substrate used in this study, β-naphthyl acetate, is probably not a particularly 'optimal' substrate for this enzyme. AXEs generally display broad substrate specificity. Considering possible positional specificity affecting AXE activity (Biely *et al,* 1996), and as it is not known which carbohydrate esterase (CE) family the AXE belongs to, β-naphthyl acetate might not be the most suitable substrate for the partially purified AXE reported in this study.

FUTURE WORK

This paper has identified, partially purified, and charcterised an acetyl xylan esterase (AXE) from a *Streptomyces sps* (isolate ORS10). Future work includes purification of the enzyme to homogeneity by incorporating hydrophobic interaction chromatography and size-exclusion chromatography. As the 40 kDa protein was identified as a xylose isomerase, the identity and sequence of the 60 kDa protein should be determined by MALDI-TOF MS. To obtain the AXE gene sequence, primer-walking on the 18D7 AXE positive cosmid clone should continue. For ferulic acid esterase (FAE), the small-insert library ligation of the FAE positive isolate, WDBS9, should be optimised. Once the

correct amounts of clones are generated for full-coverage of the genome, the library may be screened for FAE activity and positive clones sequenced.

CHAPTER 4

DISCUSSION

CHAPTER 4

4.1. Cultivation and screening of *Actinomyces* for AXE and FAE

Thermophilic Actinomycete organisms have been isolated from Namibian/Zambian hotsprings. Actinomycetes are high GC, Gram-positive bacteria that populate a wide range of habitats (Williams *et al,* 1983). They are recognised as leading xylanolytic species during biomass transformation, and as such their enzymes may be imperitive in the recovery of fermentable sugars in lignocellulose (Belfaquih and Penninckx, 2000). Therefore they are an ideal source of lignocelluloytic enzymes such as acetyl xylan esterase (AXE) and ferulic acid esterase (FAE).

To increase the chance of finding these enzymes, the cultivation media used for *Actinomyces* maintenance was enriched with xylan as a source of glucose. Xylan is a polymer composed of 1,4-linked β-D xylopyrosyl residues and is the most abundant hemicellulosic polysaccharide in plants. Plants are structurally mixed in composition and are highly decorated with non-xylose compounds such as ferulic acid and O-acetyl (Chung *et al,* 2002). The hypothesis is that because of the abundance of non-xylose components, it is a pre-requisite that organisms producing both FAE and AXE should grow in, and have the ability to, hydrolyze xylan. Eighteen of the 21 organisms initially tested were capable of hydrolyzing xylan and were therefore taken forward for further studies. These strains were thermophiles, growing optimally at 45˚C. Enzymes produced under thermostable conditions are expected to function at elevated temperatures. Thermophilic enzymes have steadily become more important in industrial processes due to the process advantages they offer such as increased reaction rates (Mozhaev, 1993; Krahe *et al,* 1996).

4.2. Positional Specificity of AXE and its possible effect on choice of substrate

Biely *et al,* (1996) suggested that AXE removes the acetyl groups at position 2 and/or 3 of xylose moieties in xylan. The acetyl groups are fairly distributed between these positions and in some cases there is migration of acetyl groups between the positions so that one position is favoured (Biely *et al,*

1996). This statement raises questions regarding the positional specificity of AXE and how this may affect the substrate specificity and their allocation to the carbohydrate esterase (CE) family. AXEs can belong to one of seven CE families based on sequence homology and substrate specificity. In terms of their allocation into various CE families, literature is very much unclear regarding this matter. Tenkanen *et al,* (2003) suggest that CE family 4 performs double deacetylation whilst Altaner *et al,* (2003) considers cleavage at position 3 only. Moreover, Tenkanen *et al,* (2003) suggests CE family 1 and 5 deacetylate at position 3 and then 2, while Altaner *et al,* (2003) propose that CE family 5 cleave acetyl groups at position 2 only. AXEs have a broad range of substrate specificity. Substrates include 4-methylumbelliferyl acetate (Tsujibo *et al,* 1996), acetylated oat spelt xylan (Tsujibo *et al,* 1996; DuPont *et al,* 1996), xylan (Caufrier *et al,* 2003), chitinous substrates (Caufrier *et al,* 2003), xylose tetra-acetate (Degrassi *et al,* 2000; DuPont *et al,* 1996), cellulose acetate (Altaner *et al,* 2003), p-Nitrophenyl acetate (DuPont *et al,* 1996) and α/β-naphthyl acetate (Koseki *et al,* 2005). β-naphthyl acetate was chosen as substrate in the current study based on comprehensive studies by Degrassi *et al* (1998, 2000). Numerous substrates have been compared and highest specific activity was obtained with β-naphthyl acetate.

4.3. ORS10 and WBDS9 as choice of thermophilic strains for FAE and AXE gene discovery

Due to the increased industrial demands for biocatalysts that can function efficiently under industrial conditions, considerable effort has been dedicated to the search for these enzymes (Rozzell, 1999). Although great strides have been made to identify these enzymes, it is still not sufficient to meet industry's demands (Herbert, 1992; Madigan and Mars, 1997). Therefore, emphasis is placed on the discovery of novel enzymes from novel organisms. For this reason, although isolate ORS10 displayed lower specific AXE activity than isolate GSIV1, it was chosen as the isolate to continue further studies as it offered novelty. The BLAST search of the GENBANK revealed that isolate ORS10 shared a 94.0% sequence similarity to its closest neighbour, *Streptomyces* Ruj7-1, implying that it may be a novel species of the *Streptomyces* genera. Thus, an AXE isolated from this isolate might be novel. Isolate WBDS9, positive for FAE activity, shared a 98.0% sequence similarity to its closest neighbour *Streptomyces radiopugnans*. A FAE has never been isolated from this species.

4.4. Cosmid library construction as a source of FAE and AXE

Two cosmid libraries were constructed: Library ONWG to screen for FAE (contained WBDS9 DNA) and library ONO to screen for AXE (contained ORS10 DNA). Libraries ONWG and ONO were firstly screened for general esterase/lipase activity. While screening library ONWG, the library became contaminated. This approach was therefore aborted. Library ONO yielded 7 esterase/lipase positives. As no plate assay has been reported for AXE, a liquid screen was adapted to a plate screen. The liquid assay is a qualitative and quantitative one; quantitative because an absorbance reading is taken to determine the amount of β-naphthol produced, and qualitative because a colour change is clearly visible if AXE activity is present. Because of the qualitative nature of the assay it was adapted to a plate-assay which clearly distinguishes between a red-coloured positive colony and an orange-coloured negative colony. Following the plate assay, two very distinctive AXE positive clones were identified.

4.5. Obtaining the FAE gene

Ferulic acid esterase is an inducible extracellular enzyme that hydrolyses the ester linkage between ferulic acid and xylan resuting in increased accessibility for enzymatic attack on hemicellulose, hence its importance in the complete degradation of lignocellulose (Garcia, 1998; Mackenzie *et al,* 1987). Due to this important role in biotechnological processes, the amount of research directed to FAEs has increased tremendously since 1990. As an example, during 1991-2000 and 2001-2010, the average number of refereed publications involving FAE research was 24 and 61, respectively. This increase coincides with recent discoveries in isolation, purification and characterisation of fungal and bacterial FAEs (Fazary and Ju, 2007; Topakas *et al,* 2007).

To obtain the gene sequence of FAE, a small-insert library of the FAE positive organism, WBDS9, was attempted. A small-insert library offers the advantage of small-insert clones which makes sequencing more rapid (Ostrander *et al,* 1992). An excess of 12 000 clones were estimated for complete coverage of the *Streptomyces* genome. After plating the transformed ligation mix, the library contained only 2 000 clones. Obtaining high quality and high yields of *Streptomyces*

genomic DNA is a challenge as the typical growth of the organisms is observed as 'balls' in liquid media. Because the extracellular polysaccharides make the cells clump together, lysing the cells proved to be particularly inefficient. Additionally, the isolate was a slow grower, taking two weeks before the cells were ready to harvest. Due to time constraints the aim of obtaining the FAE gene was aborted.

4.6. Obtaining the AXE gene

In 2002, the Chung group designed degenerate primers specific to a consensus nucleotide sequence from the most conserved regions within the catalytic domain of serine esterases for *Aspergillus niger* and *Aspergillus ficuum.* Genomic libraries of the two organisms were screened using the PCR product as a probe. This approach was not feasible for this project because there is no significant homology between known *Streptomyces* AXE protein sequences to allow degenerate primer design. Three strategies were followed to obtain the AXE gene sequence: Transposon mutagenesis, Subcloning and Primer-walking.

4.6.1. Transposon mutagenesis and ambiguity of the AXE plate screen

Transposon mutageneis is a powerful tool for creating knock-out mutants of enzymes. In transposon mutagenesis the active gene is only knocked out if the transposon is inserted in the active region (Goryshin *et al,* 2000). The corresponding gene sequence may be obtained by a primer-walking event with sequencing primers integral to the transposon. While screening mutants for loss of AXE function, the modified AXE plate-assay proved to be ambiguous in the sense that the assay was not able to clearly distinguish between positive and negative clones. Analysis of 11 'AXE negative' clones showed 99% homology to the cosmid vector indicating the transposon had inserted in the vector and not the insert DNA. This negative result was not because transposon mutagenesis failed but rather that the AXE plate-assay was too ambiguous. It is likely that this ambiguity was due to the stain itself which stained not only the colony but the surrounding media, making differentiation of positives and negatives difficult. The stain had to be made fresh before each use. As the colour

indicator (Fast Garnet GBC Sulphate salt) dissolved inconsistently, the concentration in the stain was therefore not the same after each preparation

4.6.2. Primer-walking

Due to the high GC content of clone insert DNA, primer design for sequencing was complicated and the sequencing process slow. Only 3.6 kb has been sequenced to date. The 3.6 kb sequence was translated and the esterase consensus sequence 'GXSXG' sought within the translated protein sequence. Although found, the presence of an esterase/AXE could not be confirmed. A BLAST search of the GENBANK database using the DNA sequence was performed to find homology with a published AXE or xylanase. Homology to a xylanase may infer that an AXE gene is in close proximity as past studies have found the AXE gene in close proximity to the xylanase gene (Nisole *et al,* 2006). BLAST results however revealed no homology to an AXE or a xylanase. This could mean that either there is no AXE gene within the 3.6 kb insert, or that the sequence is so novel that it is not in the NCBI database. For future study this clone may be fully sequenced to obtain the AXE gene sequence. The remaining 14.4 kb may contain the AXE gene.

4.7. ORS10 as a source of AXE enzyme production

In the current study, the growth and activity curve of ORS10 determined the highest specific AXE activity, with β-naphthyl acetate as substrate, after two days of growth. Specific activity (1423 mM/mg/min) was higher than the values reported for other AXE enzymes. These studies used α/β-naphthyl acetate as substrate (Kam *et al,* 2005; Koseki *et al,* 2005) or other substrates (Egena *et al,* 1996; Kosugi *et al,* 2002). This study confirms that β-naphthyl acetate is a good substrate for AXE production. Surprisingly, higher AXE activity was measured in intracellular fractions. To date, in excess of 95% of AXEs are expressed as secreted proteins. Only *Clostridium cellulovorans* (Kosugi *et al,* 2002) and *Caldocellum saccharolyticum* (Luthi *et al,* 1990) have been reported to produce AXE intacellularly. As AXE was detected in both intracellular and extracellular fractions, ORS10 may produce two forms of the same enzyme.

The first step for the purification of AXE from *Streptomyces* isolate ORS10 was dialysis of an ammonium sulphate precipitate followed by ion-exchange chromatography. As expected, a decrease in total protein concentration and total activity was noted after ion-exchange chromatography. Specific activity increased as expected, however the increase was not drastic. Proteins eluted at two different sodium chloride concentrations as seen by two distinct protein peaks. Following SDS-PAGE two prominent bands at 40 kDa and 60 kDa were visualised as well as many more faint bands at higher molecular weight masses. As this band seemed likely to be the AXE enzyme, as literature suggests, it was sent for sequencing. However, the protein band was identified as a xylose isomerase. Although there are no studies which report bifunctionality of AXE and xylose isomerase, it could be the case in this study. Currently only three reported AXE enzymes have molecular masses in excess of 40 kDa, the AXE from *Fibrobacter succinogenes* (58 kDa) (Kam *et al,* 2005), *Sulfolobus solfactaricus* (57 kDa) (Maurelli *et al,* 2008) and *Clostridium cellulovorans* (57 kDa) (Kosugi *et al,* 2002). Therefore the 60 kDa protein band was also sent for sequencing, results are pending.

In order to purify the protein to homogeneity, more purification steps are required. Literature suggests that in addition to ion-exchange chromatography, hydrophobic chromatography and size-exclusion chromatography (gel filtration) is required to purify AXE to homogeneity (Halgasova, 1994; Degrassi *et al,* 2000; DuPont *et al,* 1996). AXE enzymes have been purified and characterised from several microorganisms such as fungi (Egena *et al,* 1996; Komelink *et al,* 1993) and bacteria (Degrassi *et al,* 1998; Shao and Wiegel, 1995). DuPont *et al* (1996) cloned AXE from *S. lividans* and purified the enzyme from the culture supernatant. The enzyme had a molecular mass of 34 kDa. Degrassi *et al* (1998) and company purified an AXE from the culture supernatant of *B. pumilus.* The molecular mass was determined by SDS-PAGE as 40 kDa (similar to the AXE in the current study), but gel filtration suggested a mass of 190 kDa that implies a homotetrameric or homopentameric structure of the enzyme (Degrassi *et al*, 1998).

4.8. Characterisation of AXE from ORS10

AXE enzymes have an exceptionally broad substrate range of pH optimum, pH stability, temperature optimum and thermostability (Yang and Lui, 2008; Koseki *et al,* 2005; Halgasova *et al,* 1994,

DuPont *et al,* 1996; Degrassi *et al,* 2000). The AXE from ORS10 has a pH optimum of 9. The pH optimum is higher than the optima of other reported *Streptomyces,* but is similar to the AXE from *Bacilus pumilis.* The temperature optimum of AXE from ORS10 is 50°C, not surprising considering that the *Streptomyces* isolate is a thermophile. The enzyme is however not stable above 40°C, losing 82% of its activity after 30 minutes, suggesting that the enzyme is not an ideal candidate for industrial thermophilic fermentations. High stability at 30°C-40°C is ideal for application in mesophilic fermentation processes employed in the pharmaceutical industry, for example in the chemoenzymatic deacetylation of cephalosporins for the synthesis of antibiotics (Martinez-Martinez *et al,* 2007). AXE also has applications in the paper and pulp industry (in the bio-bleaching of pulp), and the animal feedstock industry (Satyanarayana and Johri, 2005).

The K_m and V_{max} were determined as 16.93 mg/ml and 1645 units/mg of enzyme respectively. The V_{max} is comparable to the AXE of *S. lividans* (DuPont *et al,* 1996). The K_m is however is substantially higher than the values obtained for other AXE enzymes (Kam *et al,* 2005; Koseki *et al,* 2005). A high K_m value implies that the substrate does not have a high affinity for the enzyme and therefore a higher concentration of substrate is required to half-saturate the AXE from ORS10. Although a good substrate for AXE production as observed by the high specific activity, there could be another substrate that is better suited (as observed by the high K_m), in direct contradiction of results obtained by Degrassi *et al* (1998, 2000). Of course, this can only be determined once the enzyme is tested with different substrates. The high specific activity and V_{max} obtained when using β-naphthyl acetate, indicates a high initial rate of reaction. Therefore, when β-naphthyl acetate half-saturates the enzyme, the rate at which the substrate converts to product is very high.

4.9. Conclusion

This study has identified a novel *Streptomyces* species, isolate ORS10, that produces AXE. Although attempts were made to obtain the AXE gene sequence, it was not possible. A cosmid clone (18 kb insert size), positive for AXE activity, has been identified and 3.6 kb sequenced thus

far. The AXE gene was not found, suggesting that the remaining 14.4 kb may contain the AXE gene. In addition, a putative AXE has been partially purified from *Strepmoyces* isolate ORS10. The temperature optimum of the enzyme was determined as 50˚C. As the AXE was not stable at this temperature, but was stable between 30°C and 40°C, it has possible industrial applications utilising mesophilic fermentations.

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