Identification and characterisation of hemicellulases from thermophilic *Actinomycetes*

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DECLARATION

I, the undersigned, hereby declare that "Identification and characterisation of hemicellulases from thermophilic actinomycetes**"** is my own work that has not been submitted for any degree or examination at any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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Lesley-Ann A. Matthews Date

ABSTRACT

To ensure the sustainability of bioethanol production, major attention has been directed to develop feedstocks which provide an alternative to food-crop biomass. Lignocellulosic (LC) biomass, which is chiefly composed of industrial plant residues, is a carbon-rich reservoir that is presently attracting much attention. However LC material is highly recalcitrant to bioprocessing and requires a mixture of physical and enzymatic pretreatment in order to liberate fermentable sugars. Thermostable enzymes are extremely desirable for use in thermophilic fermentations due to their inherent stability. Hemicellulose, a core constituent of LC, requires a cascade of hemicellulases to stimulate the depolymerisation of its xylan backbone. α-L-arabinofuranosidase (AFase) increases the rate of lignocellulose biodegradation by cleaving arabinofuranosyl residues from xylan thereby increasing the accessibility of other hemicellulases. Twenty thermophilic *Actinomycete* isolates were screened for AFase activity using *p*np-arabinofuranoside as the substrate. Three strains (ORS #1, NDS #4 and WBDS #9) displayed significant AFase activity and were identified as *Streptomyces* species with 16S rRNA gene sequence analysis. Genomic DNA was isolated from these strains and a cosmid library constructed in the shuttle vector pDF666. Subsequent functional and PCR-based screening revealed no positive clones. Strain ORS #1 was analysed for its AFase activity by determining the rate of enzyme production when cultured in the presence of 1 % Birchwood xylan. Optimal production was obtained after three days of growth and more than 90 % of AFase activity was detected in the cell lysate. Cellular proteins were precipitated to 80 % ammonium sulphate saturation and separated via ion exchange chromatography. SDS-PAGE analysis revealed seven prominent protein bands in the active protein fraction, five of which correspond to the reported sizes of AFases as described in literature. Trypsin digestion and MALDI-TOF Mass spectrometry separation identified a 42 kDa protein as xylose isomerase with homologues in *Streptomyces*. Partially purified AFase displayed optimum activity at pH7 and at 50 ºC. The enzyme was stable at pH7 for at least 24 hours at 4 °C, and for one hour at 40 ºC. V_{max} and K_m values were found to be 3958U.mg^{-1} and 8.223 mM, respectively. Preliminary enzyme characterization data suggests that the AFase isolated in this study is suitable for use in mesophilic rather than thermophilic fermentations.

Keywords: *Bioethanol, lignocellulose, thermophilic, Streptomyces, α-L-arabinofuranosidase, sequencing, purification*

This thesis is dedicated to my Mommy for the selfless sacrifices, endless determination and enduring courage.

"The only people who achieve much are those who want knowledge so badly that they seek it while the conditions are still unfavourable. Favourable conditions never come."

- *C.S Lewis*

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PREFACE

This thesis is presented as a compilation of five chapters. All research conducted is represented in a single chapter.

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION

1.1 Introduction

In recent years a trend has developed which aims to make bioethanol production less reliant on food crops such as maize and cassava and redirects its focus onto more renewable, abundant feedstocks. Of these feedstocks lignocellulose has generated the most interest despite the fact that it is a highly recalcitrant polymer that requires extensive pretreatment in order to liberate the simple sugars required for bioethanol fermentation (Perez *et al*., 2002). Methodologies for the enzymatic hydrolysis of the core polymers, cellulose, lignin and hemicellulose, have been developed for the liberation of these simple sugars. Enzymatic hydrolysis is advantageous as it avoids the formation of by-products that may inhibit downstream processing (Hahn-Hagerdal, 2006). Lignocellulytic enzymes that function at elevated temperatures are extremely desirable for thermophilic industrial fermentations as their catalytic activity is unaltered by the high temperatures employed (Blumer -Schutte *et al*., 2008).

Hemicelluloses and xylan in particular represent 20-35% of lignocellulosic biomass. Several xylan degrading enzymes are required for the complete biodegradation of hemicelluloses (Betts *et al*., 1991). Xylanolytic enzymes have frequently been isolated from *Actinobacteria* genera including *Streptomyces, Rhodococus, Bifidobacterium* and *Arthrobacter (*Numan and Bhosle, 2006). Among these enzymes, α-L-arabinofuranosidases (AFases) hydrolyse arabinose residues present in arabino-glucuronoxylan and other xylo-oligomers. In addition, AFases have been shown to have a synergistic effect in the enzymatic hydrolysis process and are therefore of commercial value (De Vries *et al*., 2000).

1.2 Project Aims

The objectives of this study are summarized below:

i. Screening thermophilic *Actinobacteria* isolates for AFase activity
ii. 16S rRNA gene analysis of isolates that display the highest AFase activity
iii. Construction and screening of a cosmid library for positive clones
iv. Determination of the gene and protein sequence of the enzyme
v. Preliminary characterisation of the AFase enzyme

CHAPTER 2

LITERATURE REVIEW

BIOETHANOL PRODUCTION THROUGH LIGNOCELLULOSE DEPOLYMERSATION

CHAPTER 2

2.1 Bioethanol

As sources of fossil fuels near depletion and fuel prices fluctuate, biofuels have regained popularity as an alternative fuel source. Biofuels are derived from the fermentation of biological matter, such as starch and sucrose, to produce secondary energy carriers, which may then be used to fuel the industrial and commercial processes that rely solely on fossil fuels (Monique *et al.*, 2003; Saha, 2003; Gray *et al*., 2006; Lin and Tanaka, 2006; Antoni *et al*., 2007). Biofuels are an attractive substitute for fossil fuels as they not only reduce greenhouse emissions produced by biomass waste (the main source material for biofuels), but also contribute to sustainable development whilst minimizing the global dependence on non-renewable resources (Lynd, 1996; Gray *et al*., 2006). A variety of biofuels such as biomethanol, biobutanol and biodiesel are commercially available, although the most commonly used fuel is bioethanol (Balat *et al*., 2008).

The ethanol produced from biomass, also referred to as bioethanol, is an oxygenated fuel source that is conventionally produced through the fermentation of various food crops such as corn, sugar cane, wheat, barley rice and cassava. Microorganisms such as *Saccharomyces cerevisae* and *Zymomonas mobilis* are used to ferment these carbon sources into bioethanol (Sanchez and Cardona, 2008). Since the first commercial production of bioethanol in the 1860's its popularity oscillated, peaking in the 1970's as a direct result of the global oil crisis. This crisis sparked a renewed interest in the use of bioethanol as a fuel source (Zaldivar *et al*., 2001; Galbe and Zacchi, 2002; Antoni *et al*., 2007; Balat *et al*., 2008). The appealing characteristics of bioethanol include renewability of the substrate as well as the catalyst, enhanced automobile performance and an estimated 80% reduction in CO_2 emissions when compared to fossil fuel combustion (Wyman, 1996; Brown *et al*., 1998). The commercial value of bioethanol requires an increase in the supply to meet future demand.

2.1.1 Fuel ethanol production

In the United States of America, Brazil and some European countries such as Spain, Russia and Germany, concerns regarding diminishing fossil fuel reserves resulted in an increase in bioethanol production (von Sivers and Zacchi, 1996; Hahn-Hagerdal *et al*., 2006). Smaller countries, enticed by the concept of reduced oil imports, a boost in the rural economy and an increase in overall air quality, also developed smaller scale bioethanol industries (Galbe and Zacchi, 2002; Gray *et al*., 2006; Sanchez and Cardona, 2008). By 2007, global bioethanol production had reached 51 000 million litres per annum (Table 2.1) and was projected to increase substantially by 2010 (Gray *et al*., 2006; Sanchez and Cardona, 2008).

Table 2.1: Global bioethanol production for 2007 (Sanchez and Cardona, 2008).

Ranking	Country	(Ethanol in million litres)
1	United States of America	18 376
2	Brazil	16 998
3	China	3 849
4	India	1 900
5	France	950
6	Germany	765
7	Russia	647
8	Canada	579
9	Spain	462
10	South Africa	386
Total		51 056

For developing countries such as South Africa, national bioethanol production is constrained by the high production costs. These costs are due to the initial investment in feedstock development as well as in further downstream processing (von Sivers and Zacchi, 1996; Balat *et al*., 2008). Aside from the cost associated with using sugar and starch-containing crops, ethical issues take precedence as many crops used for bioethanol production are also used in livestock feed and/or consumed by the human population (Gray *et al*., 2006, Sanchez and Cardona, 2008). Furthermore, growing crops seperately for bioethanol production displaces land and water resources for growing food crops leading to inflated food prices as well as possible food shortages (Zaldivar *et al*., 2001; Rosegrant, 2008). Therefore, the use of crop feedstocks such as sugar cane, corn, wheat and rice (Figure 2.1 A-D) is likely to be limited.

In an attempt to make the process of bioethanol production more cost effective, the use of lignocellulosic biomass such as agricultural and forestry residues, fast growing trees, grasses and aquatic plants (Figure 2.1 E-H) was proposed (Wiselogel *et al*., 1996; Sun and Chen, 2002). The utilization of crop residues and other waste products was projected to produce 442 billion litres of bioethanol per annum, sparking wide-spread interest (Wheals *et al*., 1999; Bohlmann, 2006; Lin and Tanaka, 2006).

Figure 2.1: Conventional feedstocks versus lignocellulosic feedstocks. The conventional biomass used to produce bioethanol: corn [(*A*)(http://www.moosecrossingardencenter.com)], sugar cane (*B*), wheat and rice [(*C* and *D*)(http://www.khalipakistan.com)]. Lignocellulose feedstock encompass a broad range of material that include fast growing grass (*E*), corn stover [(*F*)(http://ww.sciencedaily.com)], agricultural residues (*G*) and sugar cane bagasse [(*H*)(http://www.biosmartpackaging.com)]

2.2 Lignocellulose

Lignocellulose is an important structural feature present in plant cell walls and represents approximately 50% of the plant matter produced during photosynthesis (Sanchez, 2009). The chemical composition of lignocellulose is influenced by both genetic and environmental factors, which generate interspecies variance in the core constituents, lignin, cellulose and hemicellulose(Figure 2.2) (Deobald and Crawford, 1997; Steward *et al*., 1997; Malherbe and Cloete, 2002). These three polymers are entwined and chemically bound through a series of non- covalent forces and covalent linkages, forming a structure highly recalcitrant to processing (Perez *et al*., 2002).

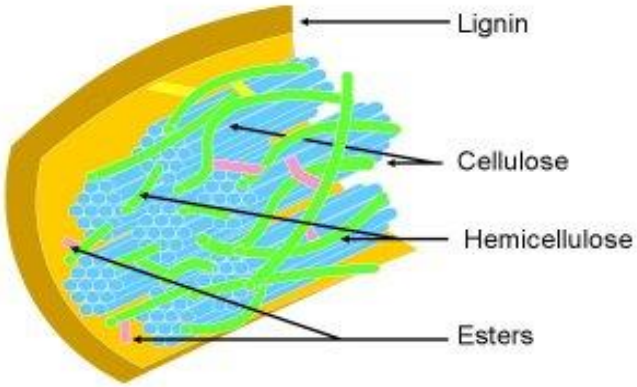

Figure 2.2: The composition of lignocellulose. A layer of lignin (*yellow*) surrounds the cellulose (*blue*) and hemicellulose (*green*) portions. These three structures are bound within the cell by ester (*pink*) linkages (Morrison, 2008).

Initial estimates in 1999 suggested that harvesting lignocellulosic biomass may result in a 255 million ton per year reduction in global CO_2 output by the year 2010 (Grassi, 1999). The use of lignocellulosic material circumvents the conflict surrounding the diversion of agricultural land for feedstock production (Hahn-Hagerdal *et al*., 2006; Sanchez and Cardona, 2008). Lignocellulosic biomass is less expensive to produce than conventional feedstocks and also requires less energy, pesticides and fertilizers (Chum and Overend, 2001; Balat *et al*., 2008).

2.2.1 Structural composition of lignocellulose

Angiosperms, gymnosperms and grasses have been identified as ideal substrates for bioethanol production (Zaldivar *et al*., 2001). Variance in composition between lignin, cellulose and hemicellulose in subsets of flora representative of these classes is presented in Table 2.2. When considering the angiosperms (flowering plants) and gymnosperms (plants that produced unenclosed seeds), it is interesting to note that these plants are fundamentally composed of cellulose with hemicellulose as the second most common polymer (McKendry, 2002). The class of plants referred to as grasses are primarily composed of hemicellulose (approximately 50%) followed closely by cellulose and much lower quantities of lignin (Betts *et al*., 1991; Thomas, 1993).

Table 2.2: Chemical composition of various lignocellulosic materials (Betts *et al*., 1991)

Plant material	Lignin %	Cellulose %	Hemicellulose %
Grasses	10-30	25-40	25-50
Angiosperms	25- 35	45- 50	25-35
Gymnosperms	18- 25	45- 55	24- 40

Biomass containing high quantities of cellulose is the primary candidate for bioethanol production as cellulose is comprised purely of the hexose sugar, glucose (Zaldivar *et al*., 2001). Biomass rich in hemicellulose is an attractive alternative because of its higher sugar content.

2.2.2 Hemicellulose

Hemicellulose is a matrix polysaccharide that constitutes up to 50% of lignocellulosic biomass depending on the source (Table 2.2). Structurally, this heteropolymer is comprised of various sugar residues (Figure 2.3) including pentose sugars (D-xylose and L-arabinose), hexose sugars (D-galactose, L-galactose, D-mannose, L-fructose and L-rhamnose) and uronic acids such as D- glucuronic acid, linked through β-1, 4 glycosidic bonds (Zaldivar *et al*., 2001; Malherbe and Cloete, 2002; Polizeli *et al*., 2005). Hemicellulose is a structurally diverse polymer that is classified according to the nature of the sugar forming its backbone. Xylan has been identified as an integral morphological feature that is structurally fundamental to all hemicellulose polymers (Heredia *et al*., 1995; Polizeli *et al*., 2005; Numan and Bhosle, 2006; Moreira and Filho, 2008).

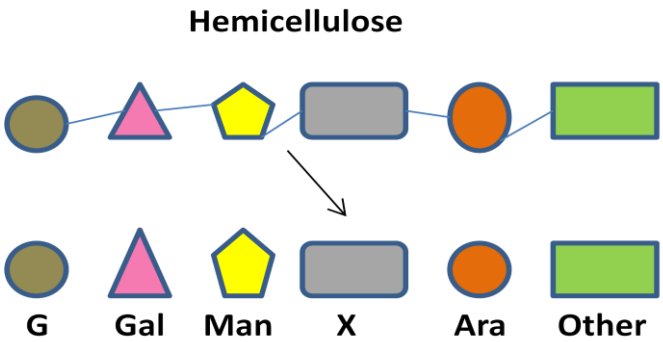

Figure 2.3: Hemicellulose constituents. Upon hydrolysis hemicellulose yields: galactose (*Gal*), mannose (*Man*), Xylose (*X*), arabinose (*Ara)*, L-rhamnose, L-fructose and uronic acids (*Other*) (Zaldivar *et al*., 2001).

Xylans

Xylans are heteropolysaccharides, composed of *D*-xylopyranose units bound by β1, 4 - linkages in the homopolymeric backbone (De Vries and Visser, 2001; Saha, 2003). Arabinoxylans, prominent in graminaceous plants, contain α-L-arabinofuranose side chains on the second and third oxygen molecules in the xylan backbone (Izydroczyk and Biliaderis, 1995; Adams *et al*., 2004; Numan and Bhosle, 2006). Further branch substitutions (Figure 2.4) include the β1,2 and β1,4 xylose linked D-galactose and glucuronic acid, while the arabinose portion is bound by 4- *O*- methyl ether, *p*- coumaric, ferulic and acetic acids joined by β1,5 linkages (Smith and Hartley, 1983; Adams *et al*., 2004). The frequency and occurrence of side-chain substitutions vary between xylans and are therefore designated according to the sugar most frequently incorporated into its backbone (Saha, 2000; Saha, 2003).

Figure 2.4: The structure of arabinoxylan. Represented here is the xylan backbone and prominent arabinose (*Ara*) substitutions. All other sugar side chains are clearly visible: xylose (*Xyl*), galactose (*Gal*), glucuronic acid (*Glca*), 2-o-acetyl ester (*AcE*) and ferulic acid (*FeA*) linked through their relative β-glycosidic bonds. Each number represents the site where xylanolytic enzymes will cleave their substrates. Numbers *1* (xylanase) and *2* (xylanosidase) act on xylose-xylose linkages to produce xylose monomers. Number *3* (ferulic acid esterase) hydrolyses ferulic acid from arabinofuranoside chains while number *4* (acetyl xylan esterase) liberates 2-o-acetyl ester from the xylan backbone. The arabinofuranosidase, number *5*, exclusively cleaves arabinofuranose from the xylan backbone (Adams *et al*., 2004; Numan and Bhosle, 2006).

The function of xylan has been determined via *in situ* studies. The covalent linkage of xylan to lignin as well as its non-covalent association with cellulose plays an important role in

sustaining the structural integrity of cellulose by defending the fibres against microbial cellulase degradation (Uffen, 1997; Beg *et al*., 2001).

2.3 From lignocellulose to fermentable sugars

The recalcitrant nature of lignocellulose biomass means that it requires significant processing to relinquish sugars sufficient for fermentation to bioethanol (Mosier *et al*., 2005). These processes involve polymer deconstruction through physical/ chemical or physiochemical pretreatment methods. This step is succeeded by a chemical or enzymatic hydrolysis step wherein all polysaccharides present are depolymerised to liberate simple fermentable sugars (Galbe and Zacchi, 2002; Balat *et al*., 2008).

Polysaccharide depolymerisation through acidic hydrolysis is a highly effective technique but produces inhibitory by-products that affect downstream processing, thus affecting commercial viability (Hahn-Hagerdal *et al*., 2006). Enzymatic hydrolysis of polysaccharide substrates renders substrate-specific conversions, which eliminate the risk of by-product formation and ensures the complete conversion of lignocellulose (Hahn-Hagerdal *et al*., 2006). The use of thermostable enzymes ensures total hydrolysis of the substrate as its catalytic activity is unaffected by the high temperatures employed during industrial fermentation (Blumer-Schutte *et al*., 2008; Sanchez and Cardona, 2008). These enzymes are produced by thermophilic bacteria (active at 45˚C- 80˚C) including *Clostridium, Thermoanaerobacterium* and multiple members of the *Actinobacteria* family (Sommer *et al*., 2004). *Actinobacteria* are known for their ability to degrade recalcitrant polymers such as lignocellulose. Species such as *Streptomyces, Nocardia, Arthrobacter* and *Rhodococus* produce multiple thermostable hemicellulases, cellulases and lignases that play a pivotal role in lignocellulose degradation (Goodfellow and William, 1983; MacKenzie *et al*., 1987; Tuncer *et al*., 1999; Khanderparker *et al*., 2008).

The hydrolysis of hemicellulose by hemicellulases is of utmost importance in biofuel production as this polysaccharide is widely distributed throughout the lignocellulosic

structure and contains a large amount of fermentable sugars. The complete depolymerisation of hemicellulose requires a cascade of hemicellulases (Table 2.3) that either display glycoside hydrolase (GH) activity or carbohydrate esterase (CE) activity. Due to its particular importance in the degradation of xylan, the hemicellulase α-L-arabinofuranosidase (AFase) will be the focus of this study.

Table 2.3: Classification of hemicellulolytic enzymes (adapted from Shallom and Shoham, 2003).

EC number	Enzyme	Substrate	Family
3.2.1.8	Endo- β 1-4-xylanase	β 1-4-xylan	GH 5,8,10,43
3.2.1.37	Exo-β 1-4-xylosidase	B1-4-xylooligomers xylobiose	GH 3,39,43,52,54
3.1.2.55	α-L-arabinofuranosidase	α-L-arabinofuranosyl (1,2) or (1,3) xylooligomers α-L-arabinan	GH 3,43,51,54,62
3.2.1.99	Endo α-1,5-arabinase	1,5-arabinan	GH 43
3.2.1.139	α-glucuronidase	4-*O*-methyl-α-glucuronic acid, (1,2) xylooligomers	GH 67
3.2.1.78	Endo-β1,4-mannase	β1,4-mannan	GH 5, 26
3.2.1.25	Exo-β1,4- mannosidase	β1,4-manooligomers mannobiose	GH 1,2,5
3.2.1.22	α-galactosidase	α-galactopyranoside (1,6) mannoloigomers	GH 4,27,36,57
3.2.1.21	β-glucosidase	β glucosidase	GH 1, 3
3.2.1.89	Endo- galactanase	β-1,4 galactan	GH 53
3.1.1.72	Acetyl xylan esterase	2- or 3-*O*-acetyl xylan	CE 1,2,3,4,5,6,7.
3.1.1.6	Acetyl mannan esterase	2- or 3-*O*-acetyl mannan	
3.1.1.73	Ferulic and *p*-cumaric acid esterases	Ferulic acid, *p*-cumaric acid	CE 1.

2.4 α-L-arabinofuranosidase

α-L-arabinofuranosidase (AFase) is an auxiliary enzyme that cleaves α-L-arabinofuranosyl linkages from arabinose-rich polysaccharides such as arabinan, arabinoxylans arabinogalactan and pectin (Figure 2.5) (Margolles-Clark *et al*., 1996). AFases display synergism with other hemicellulases, accelerating the rate at which their glycosidic bonds are hydrolysed by 10^{17} fold (Rye and Withers, 2000; Shallom *et al*., 2002). For this reason AFases have been described as one of the most efficient catalysts available (Numan and Bhosle, 2006). Synergy ensures the rapid and efficient hydrolysis of hemicellulose to render soluble substrates that are easily assimilated by the AFase producer (Saha, 2000; Takao *et al*., 2002; Numan and Bhosle, 2006).

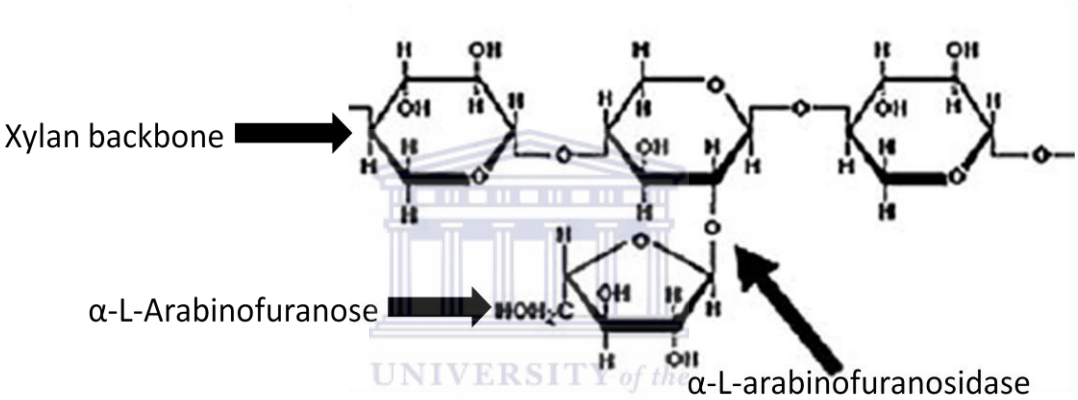

Figure 2.5: Enzymatic action of α-L-arabinofuranosidase. α-L-arabinofuranosidase acts on the xylan backbone, cleaving α-L-arabinofuranose residues from the second oxygen molecule on the xylose subunit (adapted from Kumar *et al.*, 2008).

Industrial applications of α-L-arabinofuranosidases include enhancements in the aroma of wine, the quality of bread, the efficacy of juice clarification and pulp treatment, synthesis of anti-metastatic and anti-carcinogenic compounds, bioethanol and oligosaccharides (Numan and Bhosle, 2006).

2.4.1 Classification systems for α-L-arabinofuranosidase activity

AFase was first described in 1928 by Ehrlich and Schubert as an enzyme that was capable of liberating arabinose from arabinan beet (Uesaka *et al*., 1978). Subsequent purification and

crystallisation studies on the enzyme isolated from *Aspergillus niger* demonstrated that the enzyme was an α-L-arabinofuranosidase (Kaji and Tagawa, 1964, 1970; Kaji *et al*., 1967; Kaji *et al*., 1969).

The classification system developed by Kaji (1984), grouped AFases according to their origin and substrate specificity. Beldman *et al* (1997) elaborated on Kaji's system by including the mode of action of the enzyme in the classification criteria. According to this scheme, two types of AFase exist. The first group, Arafur A, displayed little or no activity toward arabinose-containing polysaccharides while the second group, Arafur B, was capable of hydrolysing L-arabinofuranosyl residues from polymers. This classification system proved to be insufficient as its criteria were too broad to define the substrate specificity of all AFases. The problem was further compounded by the isolation of AFases that displayed novel mechanisms of action. These factors lead to the subdivision of group Arafur B into three subclasses: (I) AXHB-md 2, 3 (II) AXHB-m 2, 3 and (III) AXHd3 (Numan and Bhosle, 2006).

The primary subclass (AXBH-md 2, 3) is capable of liberating arabinose from single as well as di-substituted xylose residues. In addition, this enzyme is able to cleave α-L-arabinofuranoside from *p*-nitrophenyl (*p*np) α-L-arabinofuranoside at rates that parallel those obtained for oligosaccharide substrates (Ferre *et al*., 2000). The second (AXHBm 2, 3) and third (AXHd3) subclasses were isolated from *Bifidobacterium adolescentis* (Van Laere *et al*., 1997; Van Laere *et al*., 1999). AXHB-m 2, 3 describes a class of enzymes that are capable of hydrolysing arabinose residues with α-1, 2 as well as α-1, 3 linkages to mono-substituted xylose residues. AXHd3 are α-L-arabinofuranosidases that exclusively cleave α-1, 3 glycosidic bonds in order to liberate arabinose. Neither of these two classes is able to hydrolyse *p*np α-L-arabinofuranoside. This implies that the *p*np assays (the most common screening tool for the detection of AFase activity) alone may be insufficient for the detection of α-L-arabinofuranosidases.

Despite all attempts to classify AFases, the current classification does not accommodate the more recently characterised AFases. Birgisson *et al* (2004) and Miyazaki (2005) described an AFase that hydrolyses not only the internal α-1, 5 backbone but also the α-1, 3 side chains of arabinan and debranched arabinan. This enzyme also actively hydrolyses *p*np-α-L-arabinofuranoside. Wagschal *et al* (2009) described a novel AFase isolated from a compost starter mixture that displays dual xylosidase activity on a number of natural and artificial substrates. Instead of revising the current classification system, AFase may alternatively be grouped according to GH families as this system is less complicated and can accommodate all known AFases according to their mechanism of action.

2.4.2 Physio-chemical characteristics of α-L-arabinofuranosidases

The biochemical properties of AFases vary greatly between species. The interspecies differences in enzyme structure and tolerance to environmental stresses ensure α-L-arabinofuranosidase production and activity in the harshest of environments. AFases function over a pH range of 2 – 9 and temperature range of 30˚C – 80˚C (Table 2.4).

Table 2.4: Comparative characteristics of microbial α-L-arabinofuranosidases

Organism	Molecular Weight	Optimal Temperature	Optimal pH	Reference
Anoxybacillus kestanbolensis	57 kDa	65˚C	5.5	Canakci *et al* (2008).
Arthrobacter sp. MTCC5214	97 kDa	80˚C	8	Khanderparker *et al* (2008)
Aureobasidium pullans	210 kDa	75˚C	4	Saha and Bothast (1998)
***Bacillus pumilus* ARA**	56 kDa	60˚C	6.4	Pei and Shao (2008)
***B. stearothermophilus* T-6**	256 kDa	70˚C	5.5	Gilead and Shoham (1995)
Corticium rolfsii	-	-	2.5	Kaji and Yoshihara (1971)
Penicillium chrysogenum	79 kDa and 52 kDa	50˚C	4- 6.6 3.3- 5	Sakamoto and Kawasaki (2003)
Rhodotorula flava	-	30˚C	2	Uesaka et *al* (1978)
***Streptomyces thermoviolaceus* OPC- 520**	37 kDa	60˚C	5	Tsujibo *et al* (2002)
Streptomyces avermitilis NBRC 14893	-	30 ˚C	5- 6.5	Ichinose *et al* (2008)
Thermomonospora fusca	92 kDa	65 ˚C	9	Tuncer and Ball (2003)

2.4.3 α-L-arabinofuranosidase production: location, isozymes and enzyme variation

The majority of AFases produced by microbes are secreted into the culture media and therefore play an active role in polysaccharide degradation (Matuso *et al*., 2000). Some AFases, such as those found in *S.lividans, S.avermitilis* NBRC 14893 and *Pseudomonas flourescens* are strictly intracellular enzymes (Kellett *et al*., 1990; Manin *et al*., 1994;

Ichinose *et al*., 2008). These enzymes lack the gene associated with the production of the signalling peptide that is required for exportation to the extracellular environment (Manin *et al*., 1994). Matsumura *et al* (2004) noticed that AFases secreted in high quantities are predominantly found in monomeric configurations. Most organisms struggle to produce and export high concentrations of multimeric AFases which results in total or partial enzymatic inactivity.

Isozymes of AFases have been identified in *S.diastaticus* and *Penicillium chrysogenum* (Tajana *et al*., 1992; Sakamoto and Kawasaki 2003). These isozymes have the same catalytic mechanism but differ in their physical response to environmental stresses such as pH and temperature variations, thus ensuring the proficient hydrolysis of arabinose- rich structures (Ahmed *et al*., 2001). *Aspergillus awamori IFO4033* and *Streptomyces chartreusis* GS901 have been shown to simultaneously produce both AFase A and B, as classified according to the Beldman *et al.* (1997) system (Kaneko *et al*., 1998; Matsuo *et al*., 2000). This imparts on the microbe the ability to assimilate both arabinose containing oligosaccharides and polysaccharides, and results in the total hydrolysis of arabinofuranosyl linked structures. Organisms that co-produce α-L-arabinofuranosidase A and B are therefore of more commercial potential than organisms that produce only one form of the enzyme.

CHAPTER 3

RESEARCH RESULTS

DETECTION, ISOLATION AND PARTIAL CHARACTERISATION OF α-L-ARABINOFURANOSIDASES FROM THERMOPHILIC ACTINOMYCETE ISOLATES

CHAPTER 3

Detection, isolation and partial characterisation of α-L-arabinofuranosidases from thermophilic *Actinomycete* isolates

Introduction

The degradation of xylan is a key step in the production of bioethanol from lignocellulosic (LC) material, as xylan constitutes 25-50% of LC biomass (Betts *et al.,* 1991). Structurally, xylan is composed of a xylose backbone that is linked to various sugar moieties, the most important of which is arabinose. Arabinose residues stabilize the plant cell wall through cross–linkages with lignin and cellulose, while simultaneously inhibiting the action of xylanolytic enzymes (Aristidou and Penttila, 2000; De Vries *et al.,* 2000; Saha B.C. 2000). The cleavage of arabinose moieties from the xylan backbone is thus a pivotal step in xylan hydrolysis. AFase (E.C 3.2.1.55) is an auxillary enzyme that cleaves terminal non-reducing α-L-arabinofuranosyl linkages to liberate terminal monosaccharide units (Shallom and Shoham, 2003). AFases have been shown to have a synergistic effect on the catalytic activity of other lignocellulolytic enzymes including xylanase, ferulic acid esterase, acetyl xylan esterase and xylosidase, and are therefore widely employed in various industrial processes (Tuncer and Ball, 2003; Numan and Bhosle, 2006). Moreover, thermostable AFases are of greater commercial value as their high degree of specificity ensures efficient catalysis at elevated temperatures (Haki and Rakshit, 2003; Pei and Shao, 2008).

AFases have been isolated from *Aspergillus spp.* (De Vries *et al*., 2000; Matsumura *et al*., 2004), *Trichoderma reesei* (Nogawa *et al.,* 1999), *Bacillus stearothermophilis* (Gilead and Shoham, 1995), *Bacillus subtilis* (Inacio *et al*., 2008), *Thermomonospora fusca* (Buchmannand McCarthy, 1991; Tuncer, 2000), and *Actinobacteria* such as *Arthrobacter spp*. (Khanderparker *et al*., 2008), *Streptomyces spp*. (Kaji *et al*., 1981; Vincent *et al*., 1997; Matsuo *et al*., 2000; Tsujibo *et al*., 2003) and *Bifidobacterium spp*.(Shin *et al*., 2003). A number of techniques, ranging from genomic library construction to enzyme purification are used to isolate AFases (Margolles-Clark *et al*., 1996; Sakamoto and Kawasaki, 2003). In this manuscript we report on an α-L-arabinofuranosidase produced by a *Streptomyces strain ORS #1*. The enzyme was partially purified and preliminary characterisation data provided.

3.1 MATERIALS AND METHODS

3.1.1 Screening for α-L-arabinofuranosidase activity

3.1.1.1 Strain acquisition and growth conditions

Thermophilic *Actinomycetes* were isolated from environmental samples obtained from the Namib (NDS), Omaruru (ORS) and Walvis Bay (WBDS) regions of Namibia as well as the Gwisho (GS) and Bwanda (BS) hot springs in Zambia. Isolates were cultivated on Desert minimal agar [0.5g glucose, 0.5g yeast extract, 0.5g NaCl, 0.5g $MgSO_4.7H_2O$, 1g K_2HPO_4 15g agar and 1ml Trace elements solution (0,1g $FeSO_4.7H_2O$, 0.1g $MnCl_2.4H_2O$ and 0.1g $ZnSO_4.7H_2$ in 100ml dH_2O)] and 172F agar (10g glucose, 5g yeast extract, 10g soluble starch, 2.5g tryptone, 2.5g casamino acids, 2.5g $MgSO_4.7H_2O$, 1g $CaCl_2.2H_2O$, 15g NaCl), respectively and incubated at 45˚C for four days. The isolates were then Gram stained, transferred to the corresponding broth and grown for a further four days at 45˚C, with subsequent maintenance in 20% (v/v) glycerol.

3.1.1.2 Screening for xylanolytic activity

The presence of xylanolytic activity was determined through carbon utilization assessment (Shirling and Gottlieb, 1966) using the basal media ISP9 supplemented with 1% (w/v) Birchwood xylan (Sigma). Inocula were incubated at 45˚C for four days.

3.1.1.3 Biomass production and preparation of cellular proteins

The isolates were cultivated in 10 ml cultures of ISP9 medium supplemented with 0.1% Birchwood xylan at 45˚C for three days, and centrifuged for 10 minutes at 10 000 rpm (Eppendorf 5810R). The supernatant was retained and the pellets washed in 50 mM potassium phosphate buffer (pH 7). Cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7) and sonicated at 50% power for six cycles of 30 seconds each. Intracellular proteins were collected via centrifugation as previously described for supernatant isolation. Both intracellular and supernatant extracts were stored on ice.

3.1.1.4 Screening for crude α-L-arabinofuranosidase activity

AFase activity was determined by spectrophotometric measurement (410 nm) as *p*ara-nitrophenyl (*p*np) released from *p*np-α-L-arabinofuranoside (Carbosynth) and *p*np-α-L-arabinopyranoside (Sigma). The AFase assay was adapted from Birigisson *et al*. (2004) and Pei and Shao (2008). The 200 µl reaction contained 50 mM potassium phosphate buffer (pH 7), 10 mM *p*np-α-L-arabinofuranoside (*p*npAraf) or *p*np-α-L-arabinopyranoside (*p*npArap) and 10 µl of crude enzyme. The reaction was incubated at 49˚C for 10 minutes, stopped with the addition of 550 mM Na_2CO_3 and an absorbance reading taken. All samples that rendered an absorbance reading higher than 0.15 were re-assayed at 60˚C to check thermodurability. Protein concentrations were determined via the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as the standard and AFase activity expressed as µmol *p*-nitrophenol released per mg of protein in one minute (U.mg).

3.1.2 Genebank assembly and screening

3.1.2.1 Cosmid library construction

A cosmid library was constructed with high molecular weight genomic DNA extracted from strains ORS #1, NDS #4 and WBDS #9. The DNA was end–repaired (End-It, Epicentre Biotechnology), ligated to the vector pFD666 and packaged using the MaxPlax Lambda Packaging Extracts kit (Epicentre Biotechnology). Packaged DNA was transfected into *E.coli* Genehog ($recA^-$), and incubated overnight at 37˚C on Luria-Bertani agar (LBA) containing 25µg/ml Kanamycin. Glycerol stocks were prepared as follows: a single colony was transferred to a microtitre plate well that contained 200 µl LB and 25µg/ml Kanamycin, and incubated at 37 ˚C overnight. Cultures were then divided between two microtitre plates, 20 % glycerol added, and stored at -80˚C for future screening.

3.1.2.2 Library screening for α-L-arabinofuranosidase activity

The cosmid library was screened using three different methods as described below. These methods were first tested on the positive control, *Bacillus subtilis* 168, which has known AFase activity (Inacio *et al*., 2008). *E.coli* Genehog containing the vector pDF666 lacking a DNA insert was used as a negative control.

Solid-phase screening with pnp-arabinofuranosidase. This screen required the solubilisation of 50 mg of *p*np- arabinofuranoside per 100 ml LB agar. Following inoculation of the clones onto the plates, the plates were sealed with foil and incubated overnight at 37˚C.

Liquid-phase screening. AFase activity was induced in LB media containing 1 mM *p*np-α-L-arabinofuranoside as described by Margolles-Clark *et al.* (1996) and incubated at 37˚C. Amendments made to the protocol included an increase in sampling on days one, three, four and six.

Solid-phase screening that incorporates cell lysis. The protocol devised by Lee *et al* (2009) was amended as follows. The microtitre plates containing the library were pooled, and incubated overnight at 37 ˚C in LB/kanamycin. The overnight culture was inoculated onto a LBA/kanamycin plate and grown overnight at 37˚C. The colonies formed were transferred via a membrane from the LBA plate to a 1% agarose plate (50 mg Lysozyme, 1 mM *p*np-arabinofuranoside, 50 mM potassium phosphate buffer pH 7) and incubated at 30˚C overnight.

3.1.3 Gene amplification and sequencing

3.1.3.1 Primers and PCR parameters

The oligonucleotide primers utilized in this study are represented in Table 3.1. The cycling parameters for the individual polymerase chain reactions are defined in Table 3.2.

Table 3.1: Oligonucleotide sequences and functions

Primer name	Sequence (5'- 3')	Target
E9F	GAGTTTGATCCTGGCTCAG	Amplification of the 16S rRNA gene
U1510R	GGTTACCTTGTTACGACTT	
F1	AGAGTTTGATCITGGCTCAG	Universal 16S rRNA gene sequencing primer
R5	ACGGITACCTTGTTACGACTT	
AF2F1	CSMCGGCGGCRACKTCGTC	Amplification of the AFase gene
AF2R1	CTCGACGMCGGCGAGGCGG	
M13 Forward	CGCCAGGGTTTTCCCAGTCACGAC	Amplification and sequencing of the gene inserted into the multiple cloning site of pGEM T-easy vector
M13 Reverse	GAGCGGATAACAATTTCACACAGG	
AFO1C9	AGTACGAAGGACGAGCGCGGCACGTA	Sequencing of the AFase gene

Table 3.2: PCR parameters

Primer set	Main cycling conditions : temperature (˚C) and time(mins)					Final extension
	Initial denaturation	**No. of cycles**	**Denaturing**	**Annealing**	**Extension**	
E9F/U1510R	94˚C, 4 min	30	94˚C, 0.5 min	52˚C, 0.5 min	72˚C, 1.5 min	72˚C, 10 min
AF2F1/ AF2R1 Touchdown	96˚C, 5 min	2 X 9	96˚C, 0.5 min	(60˚C , 0.5 min) every alternate cycle - 1˚C until 51˚C is reached	72˚C , 1 min	72˚C, 10 min
		20	96˚C, 0.5 min	50˚C , 0.5 min	72˚C , 1 min	
M13 forward and reverse	95˚C, 10 min	30	95˚C, 0.5 min	55˚C, 0.5 min	72˚C, 1.5 min	72˚C, 5 min

3.1.3.2 16S rRNA characterisation

DNA was extracted from strains ORS #1, NDS #4 and WBDS #9 via the Wang method (Wang *et al*., 1996)**.** The 16S rRNA gene was amplified using primers E9F and U1510R (Table 3.1) with PCR parameters as described in Table 3.2. The 16S rRNA amplicons were purified using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). Sequencing reactions were carried out by the University of Stellenbosch sequencing facility with primers F1 and R5 (Table 3.1).

3.1.3.3 Amplification of the α-L-arabinofuranosidase gene

Six *Streptomyces* α-L-arabinofuranosidase protein sequences that represent the GH families 62, 43 and 51 were obtained from the NCBI (National Center for Biotechnology Information) database and aligned using DNAman. Although the *Streptomyces* AFases were similar on the protein level, there was a high degree of degeneracy at the nucleotide level. For this reason, the degenerate primers AF2F1 and AF2R1 were designed to flank an 825 base region that represented 55% of the AFase gene.

Touchdown PCR (Table 3.2) was performed in a 50 µl reaction with 200 ng of genomic DNA from strain ORS #1, NDS #4 and WBDS #9. Each reaction contained 200 µM of each DNTP, 0.5 µM of the degenerate primers, AF2F1 and AF2R1 (Table 3.1), 0.5 U of Robust Taq polymerase and 1 X GC buffer (Kappa Biosystems) as directed for high GC template DNA. A magnesium chloride (Fermentas) gradient of 1.5 mM to 3.5 mM was included.

Troubleshooting: Additional PCR's were conducted with 200 ng of WBDS #9 and NDS #4 DNA. These PCR's included all current optimisation available for the Robust PCR kit i.e. the use of 1 X GC buffer with 4% DMSO **(**Sigma) and also 1 X buffer A instead of the GC buffer combined with enhancer A and 5% DMSO. For high fidelity, 0.5 U PrimeSTAR^{TM}HS DNA polymerase (Takara) was employed along with 1X PrimeSTAR buffer, 200 µM dNTP mixture containing 1 mM $MgCl^{2+}$ and 0.3 µM of primers AF2F1 and AF2R1. All amplicons where visualised on a 1% agarose gel.

3.1.3.4 Cloning and sequencing of the α-L-arabinofuranosidase gene

With reference to the molecular weight marker, a band approximately 825 base pairs in size was excised from a 2% agarose gel and the PCR product purified using the Nucleospin kit (Macherey-Nagel). The gene was ligated using T4 ligase (NEB) to pGEM T-Easy (Promega), transformed into *E.coli* Genehog by electroporation and screened using blue/white colony selection.

DNA for colony PCR was prepared by immersing a single colony in sdH_2O followed by a ten minute incubation step at 96˚C. Cellular debris was collected via centrifugation at 8000 rpm and the supernatant retained. The insert was confirmed through M13 PCR using the M13 forward and reverse primer set (Table 3.1). Plasmid DNA (pDNA) was isolated from the selected clones using the alkaline lysis mini prep method for pDNA isolation (Ish-Horowicz and Burke, 1981) and sequenced with the M13 and AFO1C9 oligonucleotides (Table 2.1).

3.1.4 Protein purification and characterisation

3.1.4.1 Growth curves and protein production

Strain ORS #1 was inoculated into 1L ISP 9 media supplemented with 1% (w/v) Birchwood xylan and incubated at 45˚C for 30 days. Aliquots (100 ml) were removed daily and cell free extracts assayed for intracellular and extracellular AFase activity. A growth curve was generated and used to determine the optimal growth stage for highest AFase production.

3.1.4.2 Ammonium sulphate protein precipitation and desalting

Cell free extract (Section 3.1.1.3) was suspended in 50 mM potassium phosphate buffer (pH 7) and the proteins precipitated to 80% ammonium sulphate saturation at 4˚C. The proteins were pelleted at 10 000 rpm for 40 minutes and resuspened in 50 mM phosphate buffer. The precipitated protein was desalted by dialysis against 100 mM potassium phosphate buffer pH 7 at 4˚C overnight.

3.1.4.3 Ion-exchange chromatography (IEC)

Dialysed protein was applied to a Q-sepharose column (Amersham Bioscience) equilibrated with potassium phosphate buffer (pH7). The bound protein was eluted against a 0-1 M NaCl linear gradient with a flow rate of 1.5 ml/minute and peak fraction volume of 1 ml. The fractions were screened for β-D-xylosidase activity on a 1% agarose plate containing 0.5 mM 4-methylumberferyl-xyloside [MUX (Sigma)] and α-L-arabinofuranosidase activity using the standard assay (section 3.1.1.4). Active peaks were pooled, diluted in 0.5 volumes of sdH_2O and reapplied to the Q-sepharose column. The flow rate was further decreased to 1.25 ml/min and the gradient set at 70% 1 M NaCl. The separation efficiency was determined through SDS-PAGE analysis.

3.1.4.4 Hydrophobic interaction chromatography

The peak containing the AFase protein was precipitated to 30% ammonium sulphate saturation and applied to a Phenyl-sepharose column (Amersham Pharmacia Biotech) equilibrated with 50 mM potassium phosphate buffer (pH 7) containing 1M ammonium sulphate. The bound proteins were eluted with 50 mM potassium phosphate buffer (pH 7) at a flow rate of 2 ml/min.

3.1.4.5 Gel electrophoresis

Purified proteins were prepared by boiling the sample with equal volumes of sample buffer [1M (v/v) Tris pH 6.8, 20% (v/v) glycerol, 10% (v/v) SDS, 10 mM DTT, 0.01% bromophenol blue (BPB)] prior to protein separation in the first dimension (Laemmli, 1970). Bands were visualised by staining with Coomassie Brilliant Blue R-250 [CBB (Sigma)]. The molecular weight was estimated according to the PageRuler (Fermentas).

3.1.4.5 Activity staining

SDS gels were washed in 250 ml sdH_2O with gentle shaking for 30 minutes to remove all traces of the SDS buffer. The proteins were refolded in R-buffer (25 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100) for 14- 18 hours at 37˚C whilst gently shaking (Ostile *et al*., 2002). The gel was washed with 400 ml of 400 mM Tris-HCl (pH 6.5) and sandwiched against a 1%

(w/v) agarose gel containing 10 mM *p*np-arabinofuranoside. The gels were bound in cling wrap and incubated at 50˚C for one hour (Buchmann and McCarthy, 1991).

The in-gel assay for β-D-xylosidase activity was performed according to the protocol established by Watson *et al.* (2009) with minor amendments. The SDS-PAGE was washed twice with sdH_2O and incubated in 30 ml refolding buffer (20 mM PIPES [Pipreazine-N,N'-bis [2-ethanesulphonic acid] buffer (pH 6.8), 2.5% (v/v) Triton X-100, 2 mM DTT [Dithiothreitol], 2.5 mM $CaCl_2$) at 20ºC for one hour. Fresh refolding buffer was applied and the gel incubated overnight at 4ºC. The gel was rinsed twice with 20 mM PIPES (pH 6.8) and incubated at 37ºC for 12 hours in fresh buffer. β-D-xylosidase activity was determined by incubating the renatured protein in 50 mM potassium phosphate (pH7) containing 0.5 mM MUX at 45 ºC for one hour.

3.1.4.6 Protein sequencing

Purified protein was sent for sequencing at the Proteomics Research Group (UWC) where it was subjected to trypsin in-gel digestion and peptide separation via MALDI- TOF mass-spectrometry. The peptide mass fingerprints produced were queried in the MASCOT database (Matrix Science). Proteins with a MOWSE score greater than 83 were considered to be significant.

3.1.5 Characterisation of the precipitated protein

3.1.5.1 pH optimum and stability

All characterisation was performed on the desalted ammonium sulphate precipitate (section 3.1.4.2). The pH optimum was determined by incubating 10 µl of protein with 10 mM *p*np-α-L-arabinofuranoside (*p*np) and 50 mM buffer at 49˚C for 10 minutes. The reaction was quenched by the addition of 550 mM Na_2CO_3, and an absorbance reading taken at a wavelength of 410 nm. The buffers utilized were as follows: citrate phosphate (pH 3-6), potassium phosphate (pH 6-8) and Tris-HCl (pH 8-9). The pH stability of the enzyme was determined by incubating the enzyme in each of these buffers at 4˚C for 24 hours and subsequently assaying for residual AFase activity.

3.1.5.2 Temperature optimum and stability

The temperature optimum of the enzyme was established by assaying the effect that temperature has on the catalytic ability of the AFase. The enzyme was assayed for 10 minutes in 50 mM potassium phosphate (pH7) buffer over the temperature range of 30˚C- 80˚C. The temperature stability of the AFase was examined by incubating the AFase in 50 mM potassium phosphate (pH7) buffer over the predetermined temperature range for 19 hours. Aliquots were removed at 30 minute intervals over a period of two hours and at 19 hours, and assayed for residual AFase activity at 49˚C.

3.1.5.3 Enzyme Kinetics

The kinetic parameters of the α-L-arabinofuranosidase were determined by assaying its activity over a range of substrate concentrations (0.5 mM- 25 mM) monitored for ten minutes at a wavelength of 410 nm by the Cary 300 Win/ UV spectrophotometer. V_{max} and Km were deduced through non-linear regression fitting the data to equation 1, in GraphPad Prism 4 (GraphPad software).

Equation 1: Y= Vmax*X/ (Km+X)

3.2 RESULTS

3.2.1 Substrate depolymerisation

Twenty *Actinomycete* isolates were grown on carbon utilization media (ISP9) in the presence of Birchwood xylan. All isolates listed in Table 3.3, with the exception of WBDS #11, BSII #7 and BSII #1, were able to depolymerise xylan as indicated by sporadic growth. Screening with *p*npAraf at 49°C rendered five isolates (ORS #1, ORS #2, NDS #4, NDS #9 and WBDS #9) with considerable intracellular and extracellular AFase activity.

Table 3.3: Screening for α-L-arabinofuranosidase (AFase) activity: xylan degradation and *p*np-α-L-arabinofuranoside hydrolysis by *Actinobacteria.*

Isolate	1% (w/v) Birchwood Xylan	Intracellular AFase activity (*p*npAraf, U.mg)	Extracellular AFase activity (*p*npAraf, U.mg)
BSII #1	-	-	-
BSII #7	-	-	-
GSIV #1	+	-	-
NDS #4	+	1.4	14.6
NDS #5	+	*	*
NDS #9	+	0.343	-
NDS #10	+	*	*
NDS #11	+	-	-
NDS'#14	+	-	-
NDS #15	+	-	-
ORS #1	+	1.4	0.2
ORS #2	+	0.4	-
ORS #3	+	-	-
ORS #10	+	*	*
ORS #13	+	-	-
WBDS #6	+	-	-
WBDS #8	+	-	-
WBDS #9	+	13.3	4.6
WBDS #10	+	-	-
WBDS #11	-	*	*

*p*npAraf *p*np-α-L-arabinofuranoside, + positive, - negative, * trace levels,

Screening for AFase activity in intracellular cell free extract at 60˚C indicated that an increase in assay temperature by 11ºC decreased the specific activity of the protein (Figure 3.1). Isolate WBDS #9 displayed highest specific activity (30.2 U.mg).

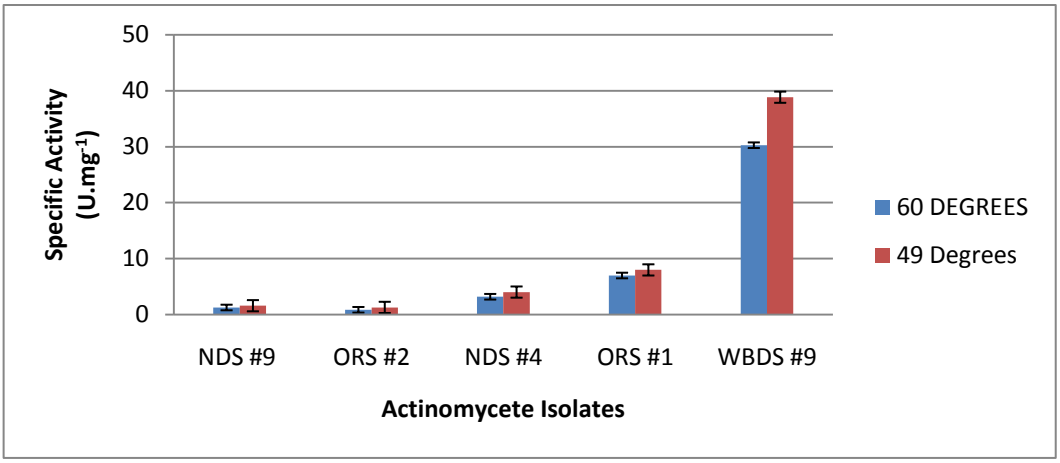

Figure 3.1: **Intracellular AFase specific activity at different temperatures**.

3.2.2 Library screening for α-L-arabinofuranosidase activity

The cosmid library was constructed from genomic DNA extracted from ORS #1, NDS #4 and WBDS #9. All methods utilised in cosmid library screening proved to be highly effective in the detection of AFase activity displayed by the positive control, *B. subtilis* 168. Hydrolysis of *p*np-Araf was confirmed by the production of a yellow product (Figure 3.2). These methods were, however, unable to detect AFase activity within the cosmid library.

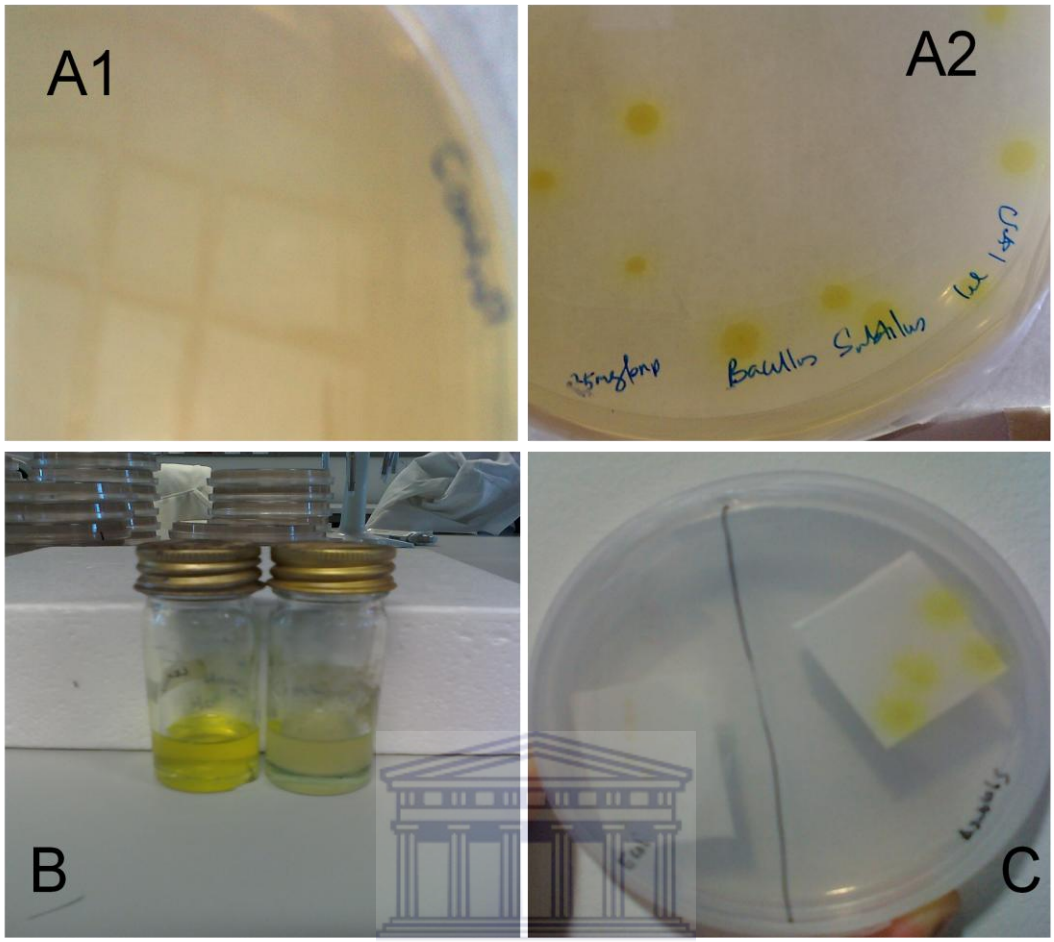

Figure 3.2: Methods and controls used to screen a Cosmid library for α-L-arabinofuranosidase (AFase) activity. *E.coli* (negative control) displayed no activity toward the substrate. *B.subtilis* 168 (positive control) displayed AFase activity as indicated by the formation of a bright yellow colour in the media. **A,** *p*np-Araf/LBA method: (A1) *E.coli* and (A2) *B.subtilis* 168. **B,** *p*np/LB method: *B.subtilis* on the left and *E.coli* on the right. **C,** Colony transfer method visualised on an agarose plate containing 15mg/ml of lysozyme and 15mg/ml *p*np-Araf: *E.coli* on the left and *B.subtilus* on the right.

3.2.3 Gene sequencing

3.2.3.1 16S rRNA gene analysis of ORS #1, NDS #4 and WBDS #9

The 16S rRNA gene was amplified from isolates ORS #1, NDS #4 and WBDS #9 (Figure 3.3).

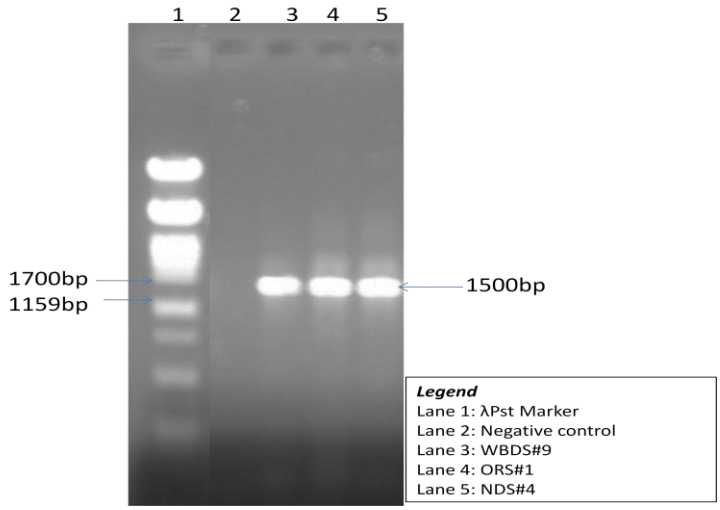

Figure 3.3: Gel electrophoresis of 16SrRNA amplicons on a 0.8% agarose gel.

Sequencing analysis indicated that all isolates belonged to the genus *Streptomyces* (Table 3.4). The 1414 base pair amplicon obtained for ORS#1 shares 97% sequence similarity with its closest documented relative, *Streptomyces albogriseolus* (Figure 3.4).

Table 3.4: BLAST results for the 16S rRNA gene.

Isolate	Closest known relative	Identity (%)
NDS#4	*Streptomyces collinus*	99
ORS#1	*Streptomyces alborgriseolus*	97
WBDS#9	*Streptomyces radiopugnans*	99

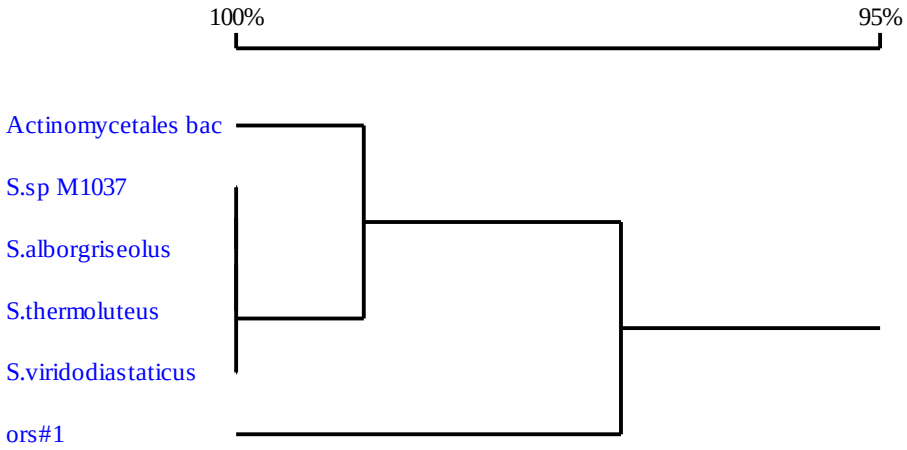

Figure 3.4: Homology tree of strain ORS #1. The diagram indicates the degree of relatedness (%) between ORS #1 and other *Streptomyces species* (constructed in DNAman).

3.2.3.2 Amplification of the putative AFase gene

Amplification of the AFase gene from ORS #1 with degenerate primers resulted in the visualisation of four distinct bands (Figure 3.5). The most prominent band was of the desired size (825bp).

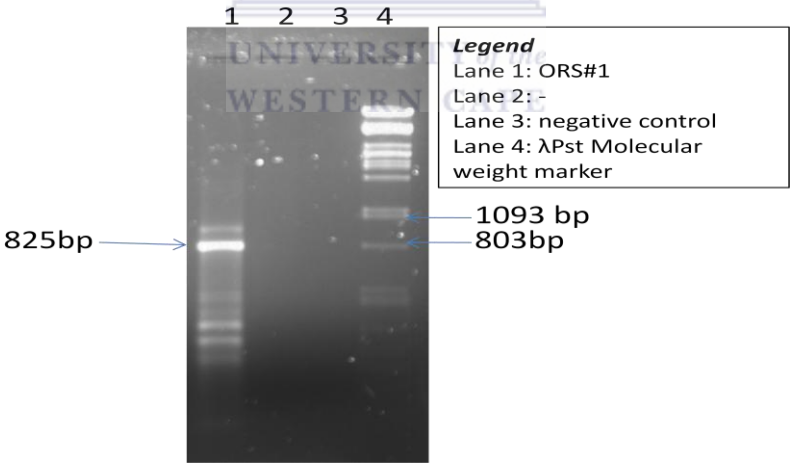

Figure 3.5: PCR amplicons of the AFase gene obtained with primers AF2F1 and AF2R1, visualised on a 1% agarose gel.

Cloning of the 825 bp amplicon into *E.coli* Genehog was successful as indicated by the presence of white colonies on the LB/ampicillin/IPTG/Xgal agar plates. Colony PCR rendered the appropriately sized band (Figure 3.6).

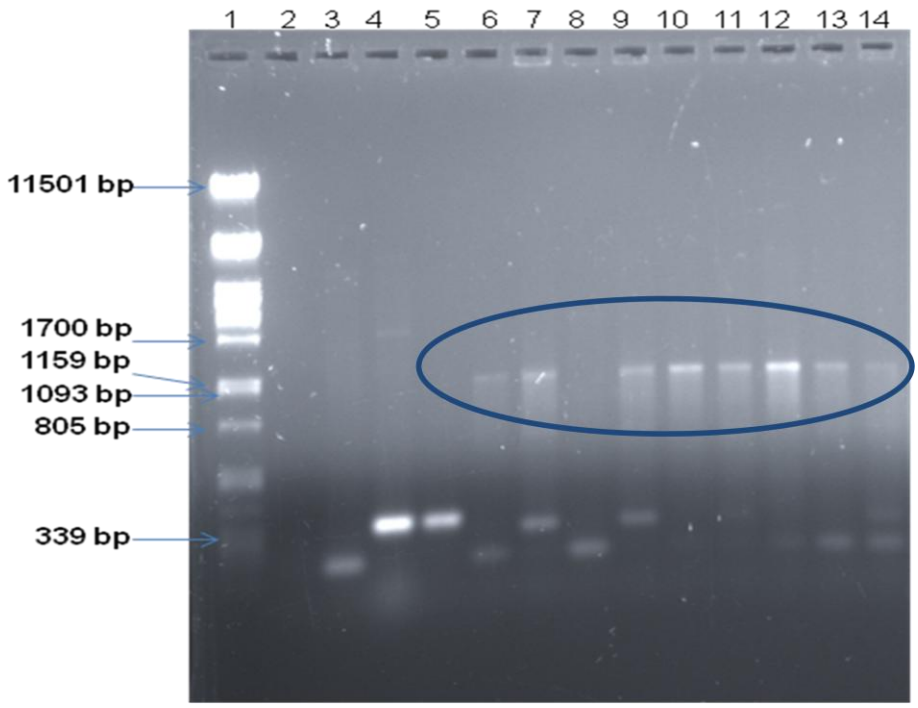

Figure 3.6: M13 colony PCR. The amplicons were resolved on a 0.8% agarose gel. The AFase insert was identified in the range of 1093 base pairs. **1**: Lambda Pst molecular weight marker. **2**: negative control. **3**: pUC 19 M13 positive. **4**: pGEMT with insert. **5**: pGEM lacking insert. **6**: O1C1. **7**: O1C2. **8**: O1C3. **9**: O1C4. **10**: O1C5. **11**: O1C10. **12**: O1C9. **13**: O1C11. **14**: O1C15 (within the blue oval).

Sequencing of clone O1C9 resulted in an 843 base product. BLASTx (http://www.ncbi.nlm.gov/BLAST/) analysis showed 41% DNA sequence homology with a recombinase gene from *Streptomyces sp*. SPB74. Alignment of the sequencing product with the *Streptomyces* AFase's used to design the degenerate primers revealed only 6.1% homology.

3.2.4 ORS #1: α-L-arabinofuranosidase purification

3.2.4.1 Cellular growth and α-L-arabinofuranosidase production

The rate at which ORS #1 produces α-L-arabinofuranosidase was monitored daily by examining the correlation of the number of enzyme units produced with total cellular protein. Highest specific AFase activity was displayed on day three (Figure 3.7). Total protein content was also highest by day three (Figure 3.8). Although low levels of activity were

detected in extracellular samples (Figure 3.7), no proteins were visualised by SDS-PAGE in these fractions (data not shown).

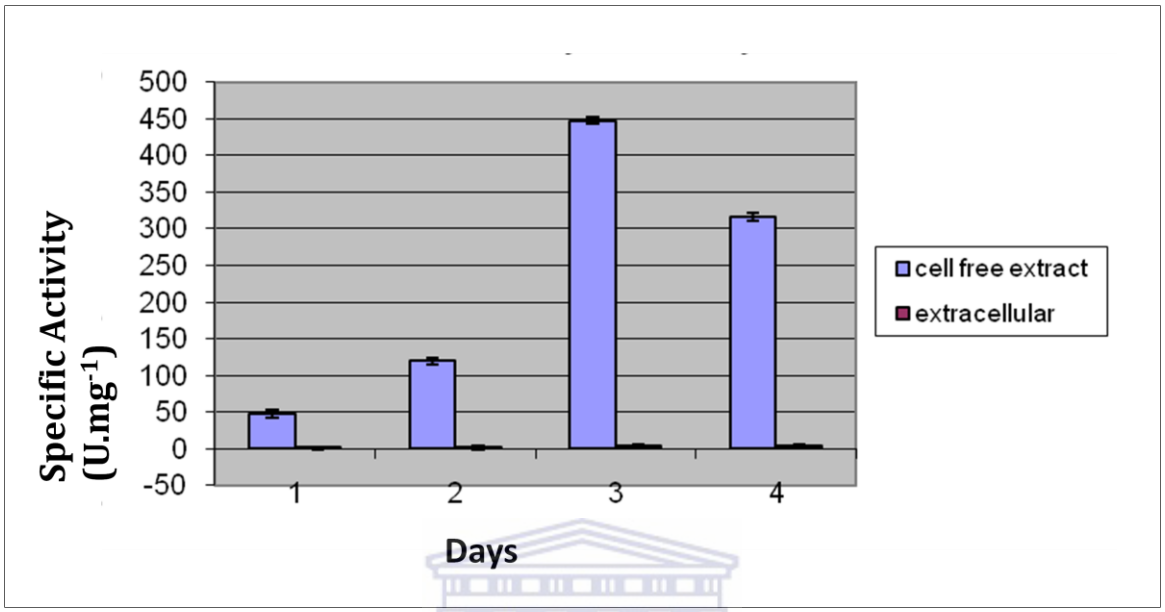

Figure 3.7: Localization of AFase activity in ORS #1.

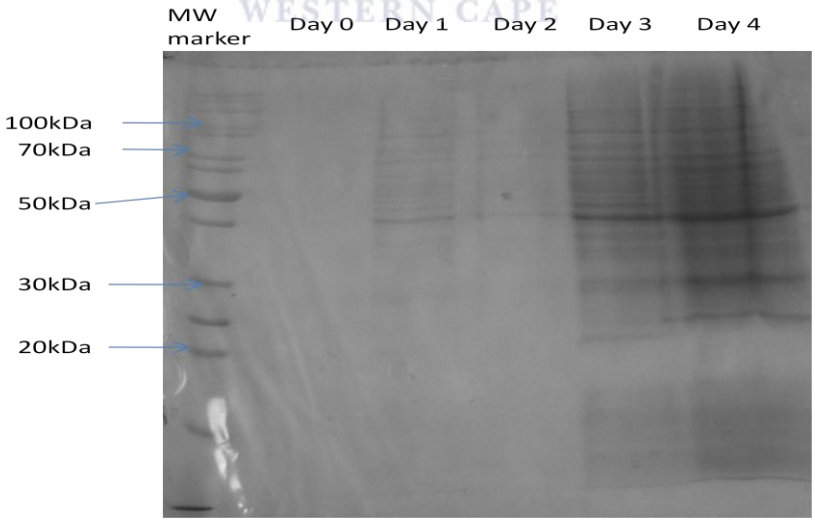

Figure 3.8: Changes in intracellular protein profile of ORS #1 over four days. Subtle changes in the proteins expressed were observed for days one and two. A notable change in the banding pattern was visualised for day three and four with a clear up-regulation of proteins in the range of 40 kDa- 100 kDa.

3.2.4.2 Protein separation and activity assays

Intracellular proteins were separated by IEC. The initial run was conducted at a flow rate of 1.5 ml per minute and resulted in the spectrophotometric detection of nine peak fractions (Figure 3.9.A). The sixth peak tested positive for AFase activity. Further separation (1.25 ml/min) of proteins in this peak resulted in the resolution of ten peaks (Figure 3.9.B) of which one was positive for both AFase and Xylosidase activity (Figure 3.10). Active fractions were pooled and subjected to hydrophobic interaction chromatography but could not be recovered.

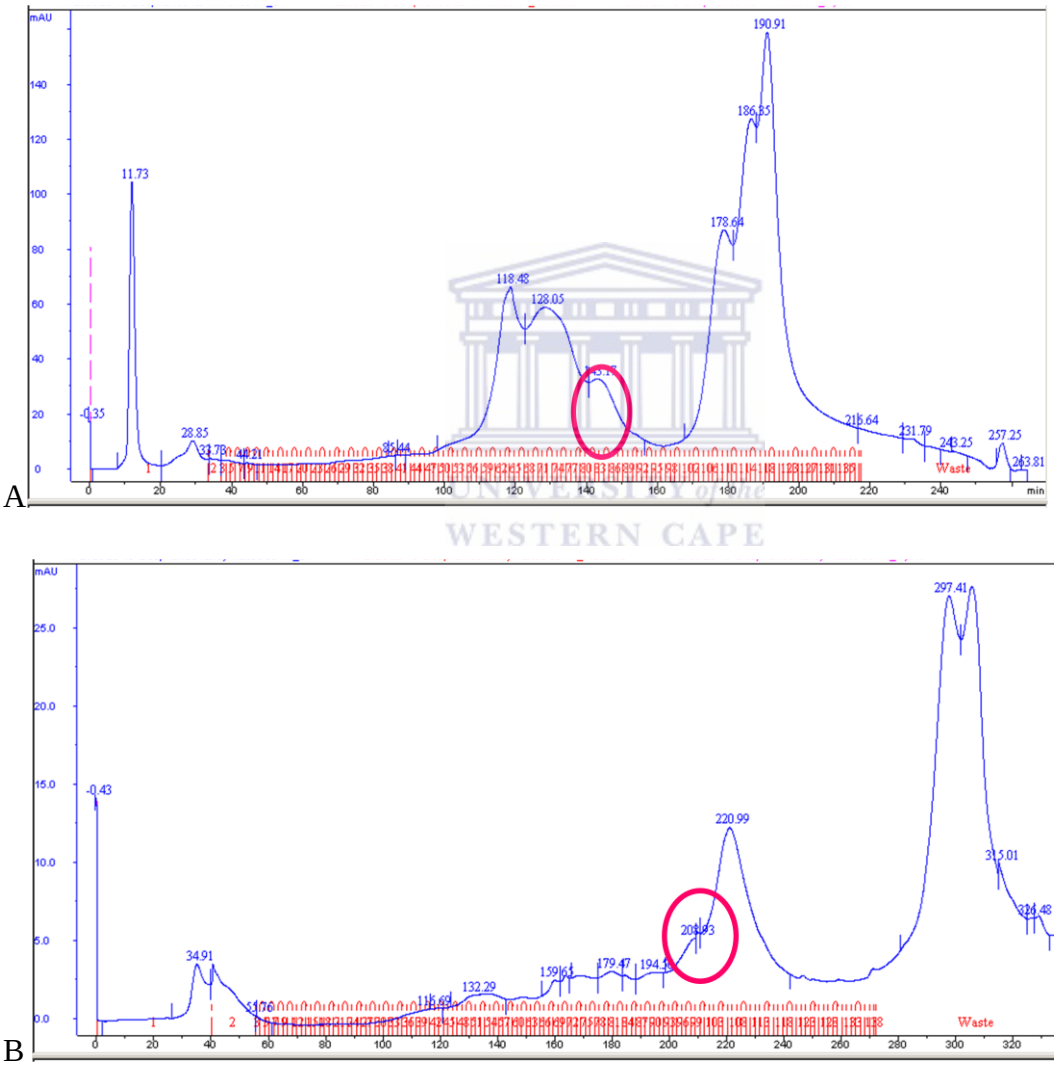

Figure 3.9: The effect of the flow rate on protein separation. A, Protein separation at 1.5 ml/min resulted in the resolution of nine peak fractions. B, Separation of the active peak at 1.25 ml/min resulted in major separation with ten visible peaks. AFase active peaks are denoted by the pink circles.

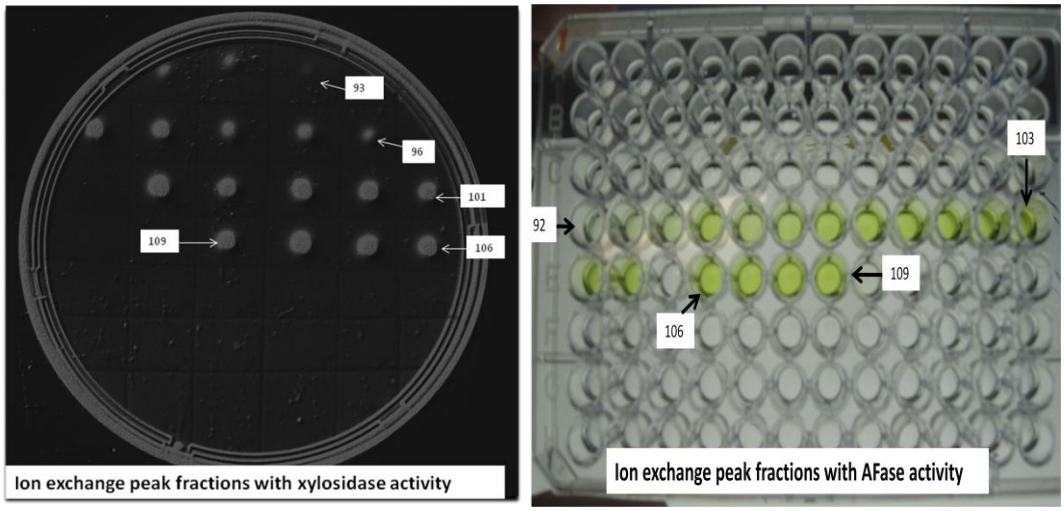

Figure 3.10: Enzyme activity determination of the ion exchange peak fractions. β-D-xylosidase activity (**left**) was detected on MUX-agarose plates while AFase activity (**right**) was identified with *p*np-Araf in fractions 93- 109**.**

The efficacy of IEC separation was determined through the construction of a purification table (Table 3.5) and SDS- PAGE analysis (Figure 3.11. A). Overall AFase yield achieved was 1.2 % with a purification factor of 15.3. Purification of the AFase to homogeneity for sequencing purposes was however not obtained. SDS-PAGE analysis revealed numerous faint bands. Zymogram development for AFase and Xylosidase activity was unsuccessful. The sizes of the proteins obtained in the active fraction was compared to the documented molecular weights of AFases and appropriately sized bands excised from the SDS- PAGE and sequenced (Figure 3.11. B).

Table 3.5: Purification table for the sub-cellular AFase produced by ORS #1.

Fraction	**Volume (ml)**	**Total protein (mg)**	**Activity (U)**	**Total activity (U)**	**Specific activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude	70	12.7	1700	119000	133.9	1	100
Ammonium sulphate precipitation	6	0.74	3559	2135.4	480.9	3.6	1.8
Q-sepharose	2.5	0.28	572.6	1431.5	2045	15.3	1.2

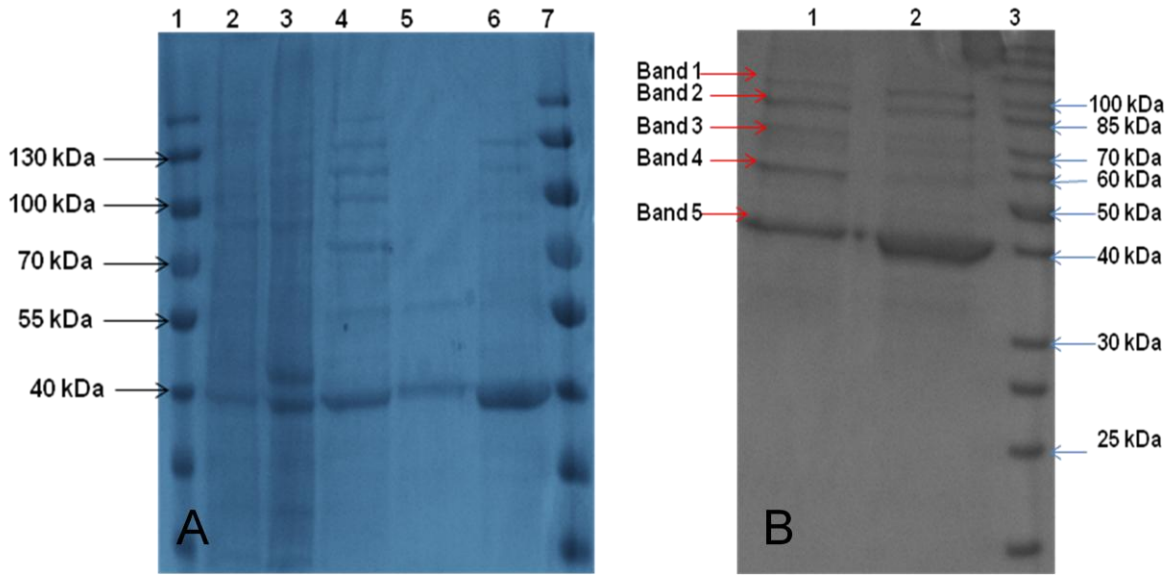

Figure 3.11: SDS-polyacrylamide gel electrophoresis of protein from ORS#1. A: Purification of the AFase**. 1** and **7**: Prestained PageRuler**. 2**: cell lysate. **3**: 80% ammonium sulphate saturation. **4: Ion** exchange chromatography resulted in the visualisation of 7 distinct bands. **5:** Nested AFase fraction separation. **6**: IEC fraction with dominant xylosidase activity. **B:** diagrammatic representation of the potential AFase bands excised and sent for sequencing. **1**: 10 µl protein**. 2**: 5µl protein. **3**: Unstained PageRuler.

3.2.4.3 Protein Sequencing

MALDI- TOF MS analysis of the fifth protein band gave peptide mass fingerprints which led to the positive identification of a xylose isomerase (Table 3.6). Sequence coverage, depicting the percentage homology between the protein query and the protein identified is highlighted in Figure 3.12.

Table 3.6: Mascot results obtained for protein band 5 confirming their identity and essential properties.

Band No.	Protein identified	Organism	MOWSE Score	Mass in Da
5	Xylose Isomerase	*Streptomyces avermitilis*	150	42 807

```
  1 MNYQPTPEDR FTFGLWTVGW QGRDPFGDAT RRALDPVETV QRLAGLGAHG
 51 VTFHDDDLIP FGSSDTERES HIKRFRQALD ATGMAVPMAT TNLFTHPVFK
101 DGAFTANDRD VRRYALRKTI RNIDLAAELG AKTYVAWGGR EGAESGAAKD
151 VRVALDRMKE AFDLLGEYVT AQGYDLRFAI EPKPNEPRGD ILLPTVGHAL
201 AFIERLERPE LYGVNPEVGH EQMAGLNFPH GIAQALWAGK LFHIDLNGQS
251 GIKYDQDLRF GAGDLRAAFW LVDLLESAGY EGPKHFDFKP PRTEDLDGVW
301 ASAAGCMRNY LILKERTAAF RADPEVQEAL RAARLDELAQ PTAGDGLTAL
351 LADRTAFEDF DVEAAAARGM AFEQLDQLAM DHLLGARG
```

Figure 3.12: Mascot results obtained for protein band 5 confirming its identity and essential properties. Alignment of polypeptide composition in the Mascot database illustrates the 33 % sequence coverage of band 5 where the highlighted red text depict the sequence similarities it shares with the xylose isomerase protein.

3.2.5 Characterisation of the crude α-L-arabinofuranosidase

Partially purified AFase active fractions obtained with IEC lost all activity within 24 hrs. Characterisation studies were therefore performed with crude protein extract.

3.2.5.1 pH characterisation

AFase activity was optimum at pH 6 to 7 (Figure 3.13). No significant activity was observed below pH 5 or above pH 9. At 4°C AFase activity was stable at pH 6 and pH 7 and retained more than 98% of its activity for at least 24 hours (Figure 3.14).

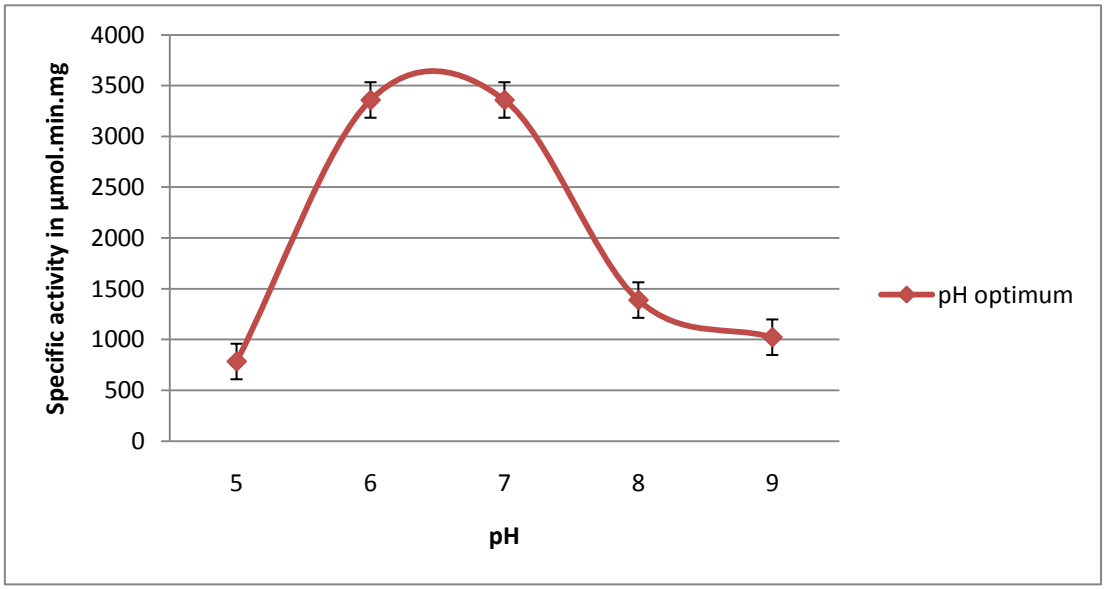

Figure 3.13: pH optimum of the AFase. The α-L-arabinofuranosidase produced by ORS #1 function optimally at pH7.

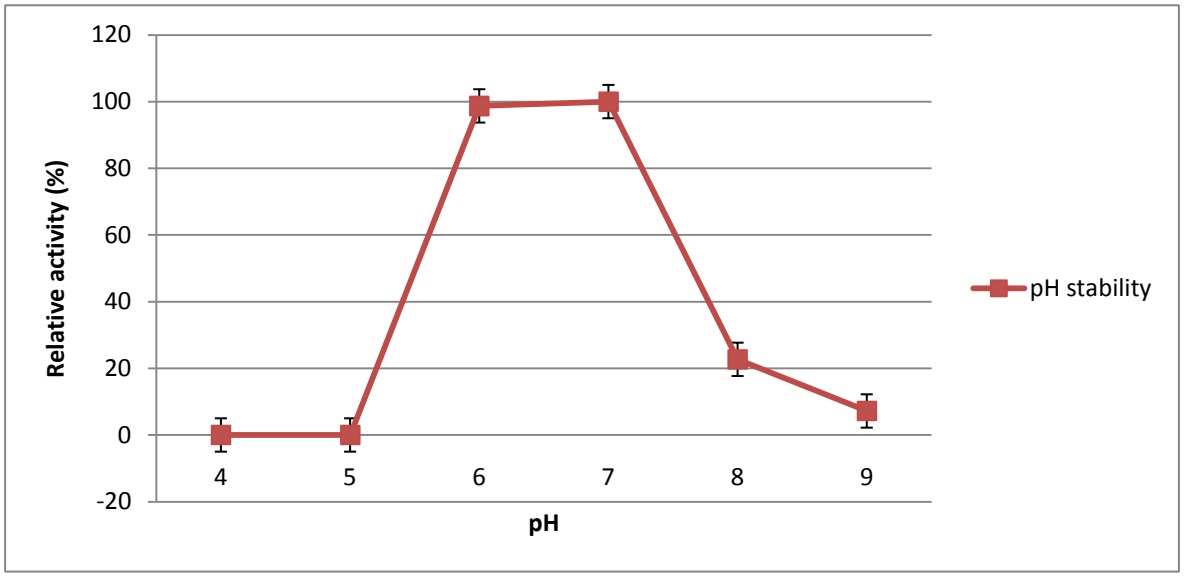

Figure 3.14: pH stability at 4˚C for 24 hours. The enzyme is highly stabile at pH six and seven for 24 hours.

3.2.5.2 Temperature constraints

The AFase displayed a temperature optimum of 50˚C (Figure 3.15) and was stable at 40˚C for up to 1.5 hours. AFase activity was rapidly lost with an increase in temperature, with no residual activity detected after 30 minutes at 70˚C (Figure 3.16).

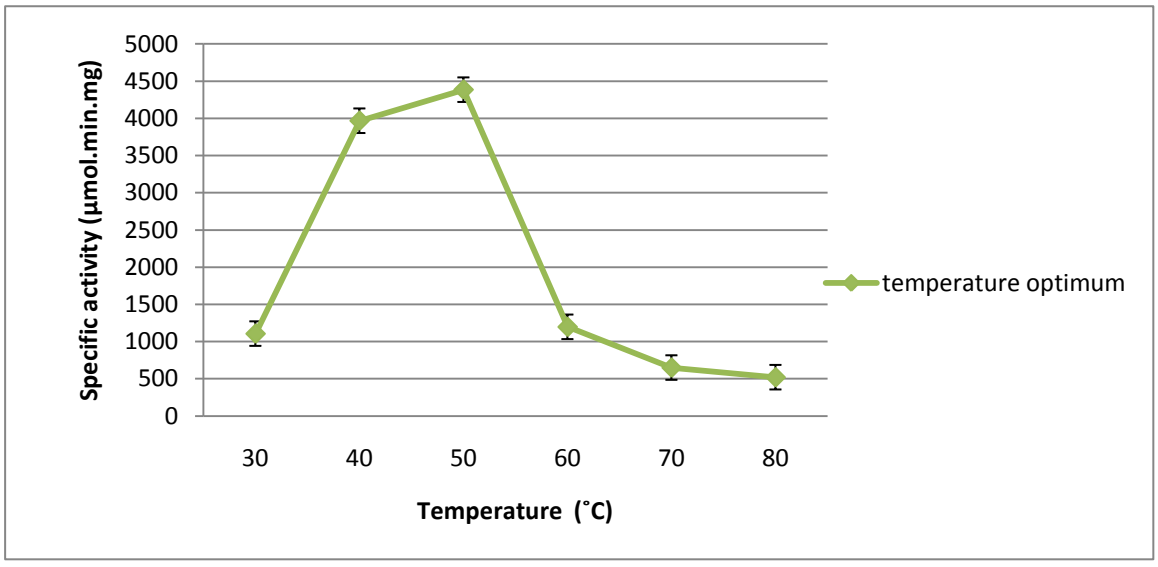

Figure 3.15: AFase activity over a range of temperatures. AFase activity was found to be optimal at 50ºC

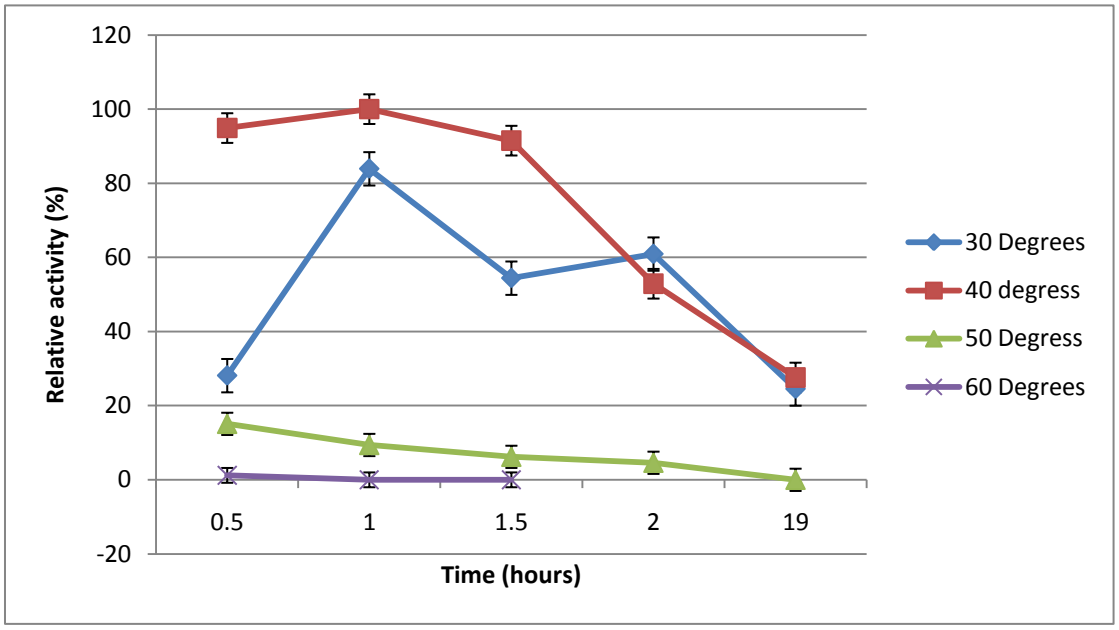

Figure 3.16: Stability of the AFase over a temperature range of 30˚C- 70˚C. The AFase is stable at 40ºC for up to one hour and thereafter gradually starts to lose activity.

3.2.5.3 Kinetic parameters

The kinetic parameters were determined through non linear regression. Data was applied to the Michaelis-Menten equation to generate the Michaelis-Menten curve (Figure 3.17). The α-L-arabinofuranosidase displayed a V_{max} of 3958 U.mg and a Km of 8.2 mM.

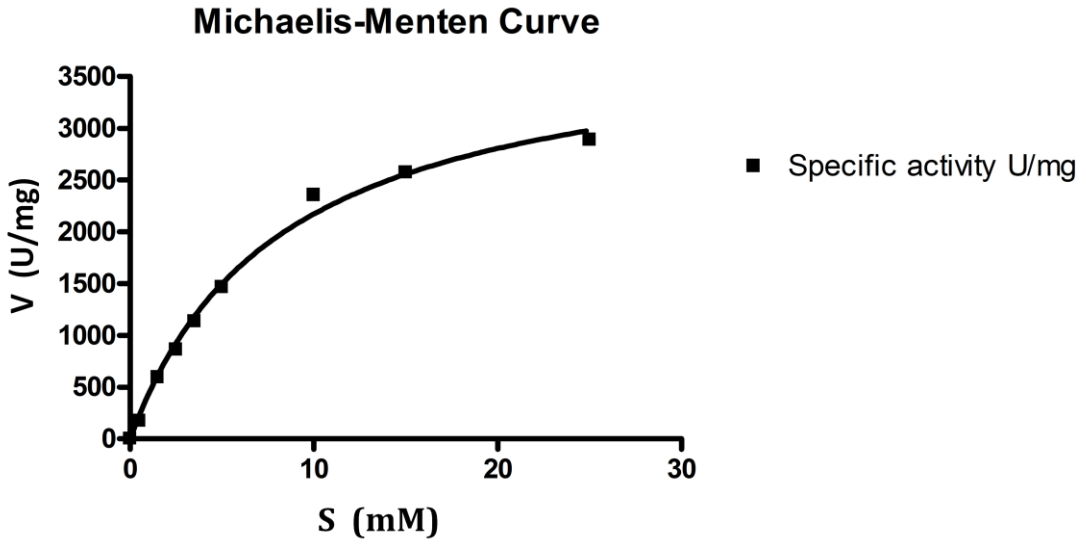

Figure 3.17: The Michaelis-Menten Curve for the kinetic activity of α-L-arabinofuranosidase on *p*np-α-L-arabinofuranoside.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

CHAPTER 4

4.1 General Discussion

4.1.1 Identification of isolates with α-L-arabinofuranosidase activity

α-L-arabinofuranosidases (AFases) are hemicellulases which cleave arabinofuranosyl residues from arabinose-rich polysaccharides and are industrially significant for the synergistic effect they have on other hemicellulases and are therefore frequently isolated and purified (Saha, 2000; Numan and Bhosle, 2006). Considering that *Actinobacteria* are ubiquitous in terrestrial environments these organisms were expected to dominate Namibian and Zambian soil isolates. *Actinobacteria* are well characterised for their biodegradative capabilities (McCarthy and Williams, 1992) and are therefore a perfect reservoir for lignocellulytic enzymes. Hemicellulose is comprised of many different forms of xylan to which arabinofuranosyl residues are covalently linked. Screening for xylanolytic activity with xylan as the inducer is thus instrumental in the isolation of AFases (Khanderparker *et al*., 2008; Pei and Shao, 2008). Screening of the soil isolates for xylan degradation on the carbon utilization media, ISP9, rendered seventeen positives. AFase activity is identified by the cleavage of arabinofuranoside from the chromogenic substrate *p*np-arabinofuranoside (*p*np-Araf) and is detected as a yellow product. Four isolates were able to hydrolyse *p*np-Araf of which three (ORS#1, NDS #4 and WBDS #9) with highest AFase activity were selected as potential candidates for genebank construction pending phylogenetic analysis.

Because of its ubiquity and conservation of function, the 16S rRNA gene is the most common housekeeping gene employed in determining bacterial phylogeny (Janda and Abbott, 2007). Approximately 94% of the 1.5kb 16S rRNA gene was sequenced for all isolates. Sequence analysis of WBDS #9 and NDS #4 revealed a 99 % homology with *Streptomyces radiopuqnans* and *Streptomyces collinus* respectively. The 16S rRNA sequence of ORS #1 shared 97 % homology with *Streptomyces alborgriseolus*, suggesting that ORS #1 may be a novel *Streptomyces* species. The strain's 16S rRNA gene falls just short of the 97.5 % threshold value that delineates a bacterial species (Wayne *et al*., 1987, Bauer *et al*., 2009). Novel organisms, especially those isolated from thermophilic environments, are exciting as they are more likely to harbour unknown gene products and enzymes with new catalytic mechanisms which are particularly interesting for

industrialization (Ferrer *et al*., 2007). In order to confirm that ORS#1 is truly a novel organism, DNA-DNA hybridization studies would need to be conducted.

4.1.2 α-L-arabinofuranosidase gene discovery

Streptomyces species are well-documented for their ability to produce AFases and have previously been isolated from genomic libraries through functional screening (Margolles-Clark *et al*., 1996; Matsumura *et al*., 2004). In this project, functional screening of the cosmid library containing the AFase-producing strains required an array of techniques, all of which proved to be unsuccessful in the identification of an AFase. The liquid-phase screening method that incorporated the substrate *p*np-Araf was shown to be effective in the identification of AFases from a yeast (*Trichoderma reesei)* expression library (Margolles-Clarke *et al*., 1996). The adaptable solid-phase screen method devised by Lee *et al* (2009) proved to be a robust method for detecting AFase activity under the amended conditions as displayed by *Bacillus subtilus* 168 (Figure 1.4 C). This method also ensures the detection of any AFase that may have been internalized by the host organism. Ineffective functional screening experienced with the cosmid library therefore implies that no functional AFase was translated by the *E.coli* host. Problems associated with heterologous expression of *Streptomyces* genes may be the contributing factor. Differences in codon usage, regulatory signals for transcription and post translational modification (PTM) between expression host and the organism from which the gene originates is a major hurdle in the expression of *Actinobacteria* genes (Connell, 2001). Transcription efficiency is determined by the specificity of the interaction between RNA polymerase's sigma (σ) factor with the promoter region of a gene and is dependent on G+C content. The high G+C content of *Actinobacteria* genes adversely affects the promoter recognition by prokaryotic bacterial σ factors resulting in a limited degree of transcription (Helmann and Chamberlin, 1988; Patek *et al*., 1996). Furthermore, α- L-arabinofuranosidase requires PTM in the form of glycosylation in order to be active (Axamawaty *et al*., 1990; Filho *et al*., 1996). In most instances, heterologous host systems lack the cellular machinery to perform these modifications (Nakashima *et al*., 2005). Although the library was constructed in a *Streptomyces-E.coli* shuttle vector pDF666 in anticipation of the problems associated with high G+C % genes, a great deal of fundamental research is still necessary to render *Streptomyces* systems competitive for library screening.

A PCR screen based on consensus primers designed to specific AFase genes may be used to confirm the presence of the AFase gene within the construct. This approach has had previous success with an AFase from a metagenomic library (Wagschal *et al.*, 2007). Considering this approach, we set out to design consensus primers and subsequently used the gene product as a probe during library screening. High levels of degeneracy were encountered at the DNA level, and degenerate primers were designed based on DNA sequence of a few *Streptomyces* strains. PCR amplification of the putative AFase gene was complicated due to the high G+C content inherent to *Actinomycete* DNA. Initial amplification reactions were conducted under conventional and gradient PCR conditions but with no success. Touchdown PCR, wherein the template DNA anneals with primer over a gradient of ten degrees Celsius for eighteen cycles, was employed and resulted in the amplification of a DNA fragment that correlated with the estimated product size of 825 base pairs from *Streptomyces* isolate ORS #1 using the KappaRobust system. The same sized band could however not be amplified from isolates NDS #4 and WBDS #9 with any of the optimization parameters suggested by the manufacturer. The PrimeStar high fidelity system produced faint bands with the expected size for both these isolates but was irreproducible. The PrimeStar dNTP's frequently hybridized with the primer to produce DNA fragments of up to 803 base pairs evident in the negative control, suggesting a problem with the PCR reagents. New reagents also produced no amplification. PrimeStar has however been shown to effectively amplify an AFase from *Fibrobacter succinogenes* (Yoshida *et al*., 2010) and a xylanase from *Penicullium griseofulvum* (Tison *et al*., 2009).

Cloning of the approximate 825 base pair fragment from ORS #1 into *E. coli* produced an approximate 1000 base pair fragment upon colony PCR. This indicates that the gene was successfully integrated into the pGEMT backbone. Sequencing of the gene rendered an 843 base pair product which displayed 41% homology with a recombinase gene from *Streptomyces sp*. SPB74 and 6.10% similarity with the *Streptomyces* AFases used to design the degenerate primers. This suggests that the partial gene obtained is unlikely to be an AFase. Successful amplification of the AFase gene from *Streptomyces* has been achieved by Vincent *et al.* (1997), Tsujibo *et al.* (2002), Wagschal *et al.* (2007), and Ichinose *et al.* (2008). Consensus primers were based on the sequence of specific GH families. This approach was not considered as there was no way of determining which GH families our isolates housed. Moreover, a literature survey revealed that *Streptomyces chartreusis* GS901

and *Aspergillus awamori* IFO 4033 are capable of producing multiple, functional AFases that belong to different GH families (Kaneko *et al*., 1998; Matsuo *et al*., 2000).

4.1.3 Purification of the α-L-arabinofuranosidase

AFases have routinely been purified from both the culture filtrate and the cell free extract of culturable microorganisms (Tajana *et al*., 1992; Sakamoto and Kawasaki, 2003; Pei and Shao, 2008). In the current study, the growth rate of ORS #1 could not be reliably determined through the conventional OD measurement method, as *Streptomyces* growth is filamentous and the strain grew in the form of balls. A growth curve was therefore derived though examination of the relationship between AFase activity and cellular proteins. In the current study, intracellular protein concentration gradually increased over days one and two then greatly increased on days three and four. This protein profile suggests that the organism progresses from a lag phase (days 1 and two) into the exponential phase by day three, while day four possibly denotes the stationary growth phase as suggested thereafter by the constant decrease in AFase activity and the unaltered total protein concentration (data not shown). Growth of ORS #1 in media containing xylan rendered optimal AFase synthesis on day three and is consistent with the rate of AFase production displayed by *Streptomyces lividans* 66 (Manin *et al*., 1994). Other *Streptomyces* species, which include *S. chartreusis* GS901, *S. flavogriseus* ATCC 33331 and *S. olivochromogenes* NBRC 2258, attained optimal AFase levels on day four and in the case of *Streptomyces sp*. PC22, in five days (MacKenzie *et al*., 1987; Matsuo *et al*., 2000; Raweesri *et al*., 2008). This suggests that ORS #1 is an efficient AFase producer. Low levels of AFase activity was detected in the culture supernatant although no protein was detected through CBB staining. The presence of the AFase in the culture media may be a result of cell lysis as postulated by Manin *et al.* (1994).

Ion exchange chromatography separates proteins on the basis of ionic interactions and has been described as the most frequently utilized technique for protein separation. This method is routinely employed for its versatility, high protein–binding capacity, high resolving power and efficacy (Karlsson and Ryden, 1998). Many AFases have been isolated using this method as the first step in protein fractionation (Kaji *et al*., 1981; Gilead and Shoham, 1995; Kaneko *et al*., 1998; Saha and Bothast, 1998; Matsuo *et al*., 2000; Sakamoto and Kawasaki, 2003; Shin *et al*., 2003; Matsumura *et al*., 2004). The use of the Q-Sepharose ion exchange

column in this work required some optimization to ensure thorough protein separation. By decreasing the flow rate protein bands present in the active peak were reduced. AFase was however not purified to homogeneity by ion exchange alone. Active fraction was applied to a hydrophobic interaction column, from which the enzyme could not be recovered. This phenomenon was unexpected as hydrophobic interaction is routinely used as either a primary or secondary step in AFase purification (Nogawa *et al*., 1999; Birgisson *et al*., 2004). The prominent protein bands revealed by SDS-PAGE were all treated as potential AFases as the enzyme has been shown to have molecular weights of 108 kDa, 85 kDa, 70 kDa, 55 kDa, and 40 kDa (Hespell and O'Bryan, 1992; Bezalel *et al*., 1993; Beylot *et al*., 2001; Numan and Bhosle, 2006; Fritz *et al*., 2008). Sequencing of the most prominent protein band in the AFase active fraction revealed a protein with similarity to a 42 kDa xylose isomerase in the MASCOT database. Xylose isomerase is an enzyme that plays a pivotal role in the pentose phosphate cycle by converting *D*-xylose into *D*-xylulose, the initial step in xylose assimilation (Sanchez and Smiley, 1975; Liu *et al*., 1996). This enzyme has not been well documented though it is significant to note that a few xylose isomerases are bifunctional enzymes that also display β-D-xylosidase activity (Belfaquih and Penninchx, 2000). Coincidentally, many β-D-xylosidases have also been shown to display AFase activity (Poutanen and Puls, 1988; Utt *et al*., 1991; Shao and Wiegel, 1992; Wagschal *et al*., 2007). Xylose isomerases are not routinely tested for AFase activity so it is possible that this single protein is responsible for all three activities. Fractions active for AFase activity were shown to also display xylosidase activity. Xylose isomerase activity was however not confirmed. All attempts at activity staining of the SDS-PAGE for AFase and Xylosidase functionality through zymogram development were unsuccessful. The four remaining prominent bands were also subjected to MALDI-TOF (results pending).

4.1.4 Preliminary characterisation

Determining the temperature and pH parameters of an enzyme is vital as these parameters dictate the industrial viability of the enzyme**.** At temperatures higher than the optimal defined for a given enzyme, the protein will become inactive thereby rendering the fermentation process inefficient (Peterson *et al*., 2007). Fermentations conducted under thermophilic conditions (45ºC- 80ºC) are highly desirable as the risk of contamination by omnipresent

mesophilic organisms is reduced, thereby ensuring maximal product yield (Haki and Rakshit, 2003). For their robust nature, thermostable enzymes are highly sought after to drive these industrial processes. The thermoactivity of the AFase isolated in this study was optimal at 50ºC which corresponds to the thermoactivity reported for various AFase from *Aspergillus* species and *Penicillium chrysogenum* (Van der Veen *et al*., 1991; Le Clinche *et al*, 1997; Sakamoto and Kawasaki, 2003). This is surprisingly low considering that most known thermophilic AFases display optimal activity between 60- 75ºC (Saha and Bothast, 1998; Gilead and Shoham, 1995; Tsujibo *et al*., 2002; Tuncer and Ball, 2003; Canakci *et al.*, 2008; Pei and Shao, 2008). The AFase was most stable at 40ºC and retained 30% of its activity after 19 hours. The enzymes produced by *Trichoderma reesei* and *Aspergillus niger* are the only other documented AFases that display optimal thermostability at 40ºC (Nogawa *et al*., 1999; Yang *et al*., 2006). Other microbial AFases display thermostability ranging from 45ºC- 90ºC (Tajana *et al*., 1992; Debeche *et al*., 2000; Margolles and de los Reyes-Gavilan., 2003; Canacki *et al*., 2007; Morana *et al*., 2007; Canacki *et al*., 2008). The temperature profile of AFase produced by S*treptomyces* isolate ORS #1 suggests that the enzyme is not an ideal candidate for industrial thermophilic fermentations above 40°C. This AFase may be an ideal candidate for use in mesophilic fermentation processes at 40°C (Numan and Bhosle, 2006).

It is imperative that the pH parameters of an enzyme be defined prior to its industrialization. The pH of the environment affects the affinity of the enzyme for the substrate by altering the charge on both the substrate and the enzymes active site, thus hindering catalytic activity (Dixon, 1953). The partially purified AFase displayed an optimum range of pH 6-7 and retained 100% of its activity at pH 7, whilst at 4ºC for at least 24 hours. This coincides with the AFases purified from *Streptomyces sp*. PC22, (Raweesri *et al*., 2008), *Streptomyces lividans* 66 (Manin *et al*., 1994) and *Streptomyces avermitilis* NBRC 14893 (Ichinose *et al*., 2008). Some microbial AFases function optimally over a pH range of 2-8 (Uesaka *et al*., 1978; Khanderparker *et al*., 2008). Many industrially utilized microorganisms function optimally at neutral pH. The pH stability of this AFase ensures that it can be utilised in mesophilic bioethanol fermentation wherein simultaneous saccharification is employed.

The catalytic activity of the AFase was determined by measuring the rate of hydrolysis of *p*np-arabinofuranoside under optimal conditions (40ºC, pH 7), over a substrate concentration range of 5-25 mM. The V_{max} and K_m of the AFase was estimated to be 3958 U.mg and 8.223 mM, respectively. This is the second highest V_{max} documented for an AFase and the highest

for a partially purified enzyme. The highest recorded V_{max} for a purified AFase is 6610 U.mg, isolated from *Hordeum vulgare* (Lee *et al*., 2003).

4.2 Conclusion/future work

α-L-arabinofuranosidase is a key enzyme in hemicellulose deconstruction for its cleavage of arabinofuranosyl residues and the catalytic effect it has on other hemicellulases. This study reports on an AFase isolated from a *Streptomyces* strain. The enzyme was partially purified and characterized. Future work includes gene amplification and cloning into a protein expression system.

CHAPTER 5

REFERENCES

CHAPTER 5

Adams, E.L., Kroon, P.A., Williamson, G., Gilbert, H.J. and Morrisa, V.J. 2004. Inactivated enzymes as probes of the structure of arabinoxylans as observed by atomic force microscopy. *Carbohydrate Research*. **339**: 579- 590.

Ahmed, Z., Banu, H., Rahman, M.M., Akhter, F. and Haque, M.S. 2001. Microbial activity on the degradation of lignocellulosic polysaccharides. *Journal of Biological Sciences*. **1**: 993-997.

Antoni. D., Zverlov, V.V. and Schwarz, W.H. 2007. Biofuels from microbes. *Applied Microbiology and Biotechnology*. **77**: 23- 35.

Aristidou, A. and Penttila, M. 2000. Metabolic engineering applications to renewable resource utilization. *Current Opinions in Biotechnology*. **11**: 187- 198.

Axamawaty, M.T., Fleet, G.W., Hannah, K.A., Namgoong, S.K. and Sinnott, M.L. 1990. Inhibition of alpha-L-arabinofuranosidase III of *Monilinia fructigena* by 1,4-dideoxy-1,4-imino-L-threitol and 1,4-dideoxy-1,4-imino-L-arabitol. *Biochemistry*, **266**: 245-249.

Balat, M., Balat, H. And Oz, C. 2008. Progress in bioethanol processing. *Progress in Energy and Combustion Science*. **34**: 551- 573.

Buchmann, S.L. and McCarthy, A.J. 1991. Purification and co-operative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca. Applied Microbiology and Biotechnology*. **57**: 2121- 2130.

Bauer, R., Bekker, J.P., van Wyk, N., du Toit, C., Dicks, L.M.T. and Kossmann, J. Exopolysaccharide production by lactose-hydrolyzing bacteria isolated from traditionally fermented milk. *International Journal of Food Microbiology*. **131**: 260- 264.

Beg, Q.K., Kapoor, M., Mahajan, L. and Hoondal, G.S. 2001. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology*. **56**: 326- 338.

Belfaquih, N. And Penninckx, M.J. 2000. A bifunctional β-D-xylosidase–xylose isomerase from *Streptomyces sp*. EC 10. *Enzyme and Microbial Technology*. **27**: 114-121.

Beldman, G., Schols, H.A., Pitson, S.M., Searle-van Leeuwen, M.J.F. and Voragen, A.G.J. 1997. Arabinans and arabinose degrading enzymes. *Advances in Macromolecules Carbohydrate Research*. **1**: 1- 64.

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

Beylot, M.H., Emani, K., McKie, V.A., Gilbert, H.J. and Pell, G. 2001. *Pseudomonas cellulosa* expresses a single membrane-bound glycoside hydrolase family 51 arabinofuranosidase. *Biochemistry Journal*. **385**: 599-605.

Bezalel, L., Shoham, Y. and Rosenberg, E. 1993. Characterization and delignification activity of a thermostable alpha-L-arabinofuranosidase from *Bacillus stearothermophilus. Applied Microbiology and Biotechnology*. **40**: 57-62.

Birgisson, H., Fridjonsson, O., Bahrani-Mougeot, F.K., Hreggvidsson, G.O., Krisjansson, J.K. and Mattiasson, B.A. 2004. New thermostable α-L-arabinofuranosidase from a novel thermophilic bacterium. *Biotechnology Letters*. **26**: 1347- 1351.

Blumer-Schutte, S.E., Kataeva, I., Westphiling, J., Adams, M.W.W. and Kelly, R.M. 2008. Extreme thermophilic micro-organisms for biomass conversion: status and prospects. *Current Opinion in Biotechnology*. **19**: 210- 217.

Bohlmann, G.M. 2006. Process economic considerations for production of ethanol from biomass feedstocks. *Industrial Biotechnology*. **2**: 14- 20.

Bradford M.M. 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248- 254.

Brown, M.A., Levine, M.D., Romm, J.P.R.A.H. and Koomey, J.G. 1998. Engineering economic studies of energy technologies to reduces green house gas emissions: opportunities and challenges. *Annual Reviews in Energy and Environment*. **23**: 31- 39.

Canakci, S., Belduz, A.O., Saha, B.C., Yasar, A., Ayaz, F.A. and Yayli, N. 2007. Purification and characterization of a highly thermostable alpha-L-arabinofuranosidase from *Geobacillus caldoxylolyticus* TK4. *Applied Microbiology and Biotechnology*. **75**: 813- 820.

Canakci, S., Kacagan, M., Inan, K., Osman Belduz, A. and Saha, B.C. 2008. Cloning, purification and characterisation of a thermostable α-L-arabinofuranosidase from

Anoxybacillus kestanbolensis AC26Sari. *Applied Microbiology and Biotechnology*. **81**: 61-68.

Chum, H.L. and Overend, R.P. 2001. Biomass and renewable fuels. *Fuel Processing and Technology*. **71**: 187- 195.

Connell, N. D. 2001. Expression systems for use in *Actinomycetes* and related organisms. *Current Opinion in Biotechnology*. **12**: 446- 449.

Debeche, T., Cummings, N., Connerton, I., Debeire, P. and O'Donohue, M.J. 2000. Genetic and biochemical characterization of a highly thermostable alpha-L-arabinofuranosidase from *Thermobacillus xylanilyticus. Applied Environmental Microbiology*. **66**: 1734- 1736.

De Vries, R.P., Kester, H.C.M., Poulsen, C.H., Benen, J.A.E. and Visser, J. 2000. Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. *Carbohydrate Research*. **327**: 401- 410.

Deobald, L.A. and Crawford, D.L. 1997. Lignocellulose biodegradation. Washington DC. ASM Press

De Vries, R.P. and Visser, J. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews*. **65**: 497- 522.

Dixon, M. 1953. The Effect of pH on the Affinities of Enzymes for Substrates and Inhibitors. *Biochemistry Journal*. **55**: 61- 70.

Ferre, H., Broberg, A., Duus, J.O. and Thomsen, K.K. 2000. A novel type of arabinoxylan arabinofuranohydrolase isolated from germinated barley. Analysis of substrate preference and specificity by nano-probe NMR. *European Journal of Biochemistry*.**267**: 6633- 6641.

Ferrer, M., Golyshina, O., Beloqui, A. and Golyshina, P.N. 2007. Mining enzymes from extreme environments. *Current Opinion in Microbiology*. **10**: 207- 214.

Filho, E. X., Puls, J. and Coughlan, M.P. 1996. Purification and characterisation of arabinofuranosidases from solid-substrate cultures of the fungus *Penicillium capsulatum*. *Applied Environmental Microbiology*. **62**: 168- 178.

Fritz, M., Ravanal, M.C., Braet, C. and Eyzaguirre, J. 2008. A family 51 alpha-l-arabinofuranosidase from *Penicillium purpurogenum*: purification, properties and amino acid sequence. *Mycology Research*. **11**: 933- 942.

Galbe, M. and Zacchi, G. 2002. A review of the ethanol production from softwood. *Applied Microbiology and Biotechnology*. **59**: 618- 628.

Gallagher, S.R. 1999. One-Dimensional-Electrophoresis using non-denaturing conditions. *Current Protocols in Molecular Biology Supplement.* **47**: 2B1- 11.

Gilead, S. and Shoham, Y. 1995. Purification and characterisation of a α-L-arabinofuranosidase from *Bacillus stearothermophilus* T-6. *Applied Environmental Microbiology*. **61**: 170- 174.

Goodfellow, M. and Williams, S.T. 1983. Ecology of *Actinomycetes*. *Annual Reviews in Microbiology*. 37: 189-216.

Grassi, G. 1999. Modern Bioenergy in the European Union. *Renewable Energy*. **16**: 985- 990.

Gray, K.A., Zhao, L. and Emptage, M. 2006. Bioethanol. *Current Opinion in Chemistry and Biology*. **10**: 141- 146.

Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Liden, G. and Zacchi, G. 2006. Bioethanol- the fuel of tomorrow from the residues of today. *Trends in Biotechnology*. **24**: 549- 556.

Haki, G.D. and Rakshit, S.K. 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource Technology*. **83**: 17- 34.

Helmann, J.D. and Chamberlin, M.J. 1988. Structure and function of bacterial σ factors**.** *Annual Reviews in Biochemistry.* **57**: 839- 872.

Heredia, A., Jimenez, A. and Guillen, R. 1995. Composition of plant cell walls. *Z Lebensm Unters Forsch*. **200**: 24- 31.

Inacio, J.M., Correia, I.L. and de Sa-Nogueira, I. 2008. Two distinct arabinofuranosidases contribute to arabino-oligosaccharide degradation in *Bacillus subtilis*. *Microbiology*. **154**: 2719- 2729.

Inchinose, H., Yoshida, M., Fujimoto, Z. and Kaneko, S. 2008. Characterisation of a modular enzyme of exo-1,5-α-L-arabinofuranosidase and an arabinan binding module from *Streptomyces avermitilis* NBRC 14893. *Applied Microbial Biotechnology*. **80**: 399- 408.

Ish-Horowicz, D. and Burke, J.F. 1981. Rapid and efficient cosmid cloning. *Nucleic Acid Research*. **9**: 2989- 2998.

Izydroczyk, M.S. and Biliaderis, C.G. 1995. Cereal arabinoxylans: advances in structure and physiochemical properties. *Carbohydrate Polymers*. **28**: 33- 48.

Janda, M.J. and Abbott, S.L. 2007. 16S rRNA gene sequence for bacterial identification in the diagnostic laboratory. *Journal of Clinical Microbiology*. **45**: 2761- 2764.

Janson, J-C. and Ryden, L. 1998. Ion Exchange chromatography. *In Protein purification: Principals, high resolution methods and applications. Second edition*. New York: Wiley-VCH, 145- 206.

Kaji, A. 1984. L-arabinosidases. *Advances in Carbohydrate Chemistry and Biochemistry*. **42**: 382- 394

Kaji, A. and Tagawa, K. 1964. Studies on the enzymes acting on arabinan.V. Action of arabinase producing *Aspergillus niger*. *Nippon Nogei Kagaku Kaishi*. **38**: 580- 584.

Kaji, A., Tagawa, K. and Matsubara, K. 1967. Studies on enzymes acting on arabinan. VIII. Purification and properties of arabinases produced by *Aspergillus niger*. *Agriculture, Biology and Chemistry*. **31**: 1023- 1028.

Kaji, A., Tagawa, K. and Ichimi, T. 1969. Properties of purified α-L-arabinofuranosidase from *Aspergillus niger*. *Biochimica et Biophysica Acta*. **171**: 186- 188.

Kaji, A. and Tagawa, K. 1970. Purification, crystallisation and amino acid composition of α-L-arabinofuranosidase from *Aspergillus niger*. *Biochimica et Biophysica Acta.* **207**: 456-464.

Kaji, A. and Yoshihara, O. 1971. Properties of purified α-L-arabinofuranosidase from *Corticium rolfsii*. *Biochimica et Biophysica Acta* **250**: 367– 71.

Kaji, A., Sato, M. and Tsutsui, Y. 1981. An α-L-arabinofuranosidase produced by wild type *Streptomyces sp.* No. 17-1. *Agriculture, Biology and Chemistry*. **45**: 925- 931.

Kaneko, S., Arimoto, M., Ohba, M., Kobayashi, H., Ishii, T. and Kusakabe, I. 1998. Purification and substrate specificities of two α-L-arabinofuranosidases from *Aspergillus awamori* IFO4033. *Applied and Environmental Microbiology*. **64**: 4021- 4027.

Kellett, L.E., Poole, D.M., Ferreira, L.M.A, Durrant, A.J., Hazelwood, G.P. and Gilbert, H.J. 1990. *Biochemistry Journal*. **272**: 369- 376.

Khanderparker, R., Numan, M.T.H., Mukherjee, B., Satwekar, A. and Bholse, N.B. 2008. Purification and characterisation of α-L-arabinofuranosidase from *Arthrobacter sp*. MTCC 5214 in solid state fermentation. *Process Biochemistry*. **43**: 707- 712.

Kumar, R., Singh, S. and Singh, O.V. 2008. Bioconversion of lignocellulose biomass: biochemical and molecular prospective. *Journal of Industrial Microbiology and Biotechnology*. **35**: 377- 391

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680- 685.

Le Clinche, F., Pinaga, F., Ramon, D. and Valles, S. 1997. alpha-L-arabinofuranosidases from *Aspergillus terreus* with potential application in enology: Induction, purification, and characterization. *Journal of Agriculture and Food Chemistry*. **45**: 2379- 2383.

Lee, R.C., Hrmova, M., Burton, R.A., Lahnstein, J. and Fincher, G.B. 2003. Bifunctional family of 3 glycoside hydrolases from barley with alpha-L-arabinofuranosidas and beta-D-xylosidase activity. *Journal of Biology and Chemistry*. **278**: 5377- 5387.

Lee, C.C., Wagschal, K., Kibblewhite-Accinelli, R.E., Orts, W.J., Robertson, G.H. and Wong, D.W.S. 2009. An α-Glucuronidase enzyme activity assay adaptable for solid phase screening. *Applied Biochemistry and Biotechnology*. **155**: 314- 320.

Lin, Y. And Tanaka, S. 2006. Ethanol fermentation from biomass resources: current state and prospect. *Applied Microbiology and Biotechnology*. **69**: 627- 642.

Liu, S-Y., Wiegel, J. and Gherardini, FC. 1996. Purification and cloning of a thermostable xylose (glucose) isomerase with an acidic pH optimum from *Thermoanaerobacterium* strain JW/SL-YS 489. *Journal of Bacteriology*. **178**: 5938–5945.

Lynd, L.R. 1996. Overview and evaluation of fuel ethanol production from cellulosic biomass: technology, economics and the environment, a policy. *Annual Reviews in Energy and Environment*. 403- 465.

MacKenzie, R.C., Bilous, D., Schineider, H. and Johnson, K.G. 1987. Induction of cellulolytic and xylanolytic enzyme systems in *Streptomyces spp*. *Applied Environmental Microbiology*. **53**: 2853- 2839.

Malherbe, S. and Cloete, T.E. 2002. Lignocellulosic Biodegradation: fundementals and applications. *Reviews in Environmental Science and Biotechnology*. **1**: 105: 114.

Manin, C., Shareek, F., Morosoli, R. and Kleupfel, D. 1994. Purification and characterisation of an α-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (*abf*A). *Biochemistry Journal*. **302**: 443- 449.

Margolles-Clark, E., Tenkanen, M., Nakari-Setala, T. and Penttila, M. 1996. Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*. **62**: 3840- 3846.

Margolles, A. and de los Reyes-Gavilan, C.G. 2003. Purification and functional characterisation of a novel α-L-arabinofuranosidase from *Bifidobacterium longum* B667. *Applied and Environmental Microbiology*. **69**: 5096- 5103.

Matsumura, K., Obata, H., Hata, Y., Kawato, A., Abe, Y. and Akita, O. 2004. Isolation and characterisation of a novel gene encoding α-L-arabinofuranosidase from *Aspergillus oryzae*. *Journal of Bioscience and Bioengineering*. **98**: 77- 84.

Matuso, N., Kaneko, Kuno, A., Kobayashi, H. and Kusakabes, I. 2000. Purification, characterisation and gene cloning of two α-L-arabinofuranosidase from *Streptomyces chartreusis* GS901. *Biochemical Journal*. **34**: 9- 15.

McCarthy, A.J. and William S.T. 1992. *Actinomycetes* as agents of biodegradation in the environments- a review. *Gene.* **115:** 189- 192.

McKendry, P. 2002. Energy production from biomass: overview of biomass. *Bioresources Technology*. **83**: 37- 43.

Miyazaki, K. 2005. Hyperthermophilic α-L-arabinofuranosidase from *Thermotoga maritima* MSB8; molecular cloning, gene expression and characterisation of the recombinant protein. *Extremophiles*. **9**: 399- 406.

Monique, H., Fajj, A., Van den Broek, R., Berndes, G., Gielen, D. and Turkenburg, W. 2003. Exploration of the ranges of global potential for biomass energy. *Biomass Energy*. **25**: 119- 133.

Morana, A., Paris, O., Maurelli, L., Rossi, M. and Cannio, R. 2007. Gene cloning and expression in *Escherichia coli* of a bi-functional beta-D-xylosidase/alpha-L-arabinosidase from *Sulfolobus solfataricus* involved in xylan degradation. *Extremophiles*. **11**: 123- 132.

Moreira, L.R.S. and Filho, E.X.F. 2008. An overview of mannan structure and mannan degrading enzyme systems. *Applied Microbiology and Biotechnology*. **79**: 165- 178.

Morrison, L. 2008. Second-generation biofuels may offer a way forward. Taylor.L. (ed). www.engineeringnews.co.za. Accessed: 7 May 2009.

Mosier, N., Wyman,C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*. 96: 673- 686.

Nakashima, N. Mitani, Y. and Tamura, T. 2005. *Actinomycetes* as host cells for production of recombinant proteins. *Microbial Cell Factories*. **4**: 1- 5.

Nogawa, M., Yatsui, K., Tomioka, A., Okada, H. and Morikawa, Y. 1999. An α-L-arabinofuranodidase from Trichoderma reesi containing a non catalytic xylan-binding domain. *Applied and Environmental Microbiology*. **65**: 3964- 3968.

Numan, M.T.H. and Bholse, N.B. 2006. α-L-arabinofuranosidases: the potential applications in biotechnology. *Journal of Industrial Microbiology and Biotechnology*. **33**: 247- 260.

Ostile, H.M., Vergarud, G. and Langsrud, T. 2002. Autolysis of *Propionibacteria*: detection of autolytic enzymes by renaturing SDS-PAGE and additional buffer studies. *International Journal of Food Microbiology*. **117**: 167- 174.

Patek, M., Eikmanns, B.J., Patek, J. and Sahm, H. 1996. Promoters from *Corynebacterium glutamicum*: cloning, molecular analysis and search for a consensus motif**.** *Microbiology*. **142**: 1297- 1309.

Pei, J. and Shao, W. 2008. Purification and characterisation of an extra-cellular α-L-arabinosidase from novel *Bacillus pumilus* ARA and its over expression in *Eschericia coli*. *Applied Microbiology and Biotechnology*. **78**: 115- 121.

Peterson, M.E., Daniel, R.M., Danson, M.J. and Eisenthal, R. 2007. The dependence of enzyme activity on temperature: determination and validation of parameters. *Biochemistry Journal*. **402**: 331-337.

Perez, J., Munoz-Dorado, J., De La Rubia, T. and Martinez, J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology*. **5**: 53- 63.

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

Poutanen, K. and Puls, J. 1988. Characteristics of *Trichoderma reesei* β-xylosidase and its use in the hydrolysis of solubilised xylans. *Applied Microbiology and Biotechnology*. **28**: 425- 432.

Raweesri, P., Riangrungrojana, P. And Pinphanichakarn, P. 2008. α-L-arabinofuranosidase from *Streptomyces sp*. PC22: Purification, characterisation and its synergistic action with xylanolytic enzymes in the degradation of xylan and agricultural residues. *Bioresource Technology*. **99**: 8981- 8986.

Rosegrant, M.W. 2008. Biofuels and grain prices: impacts and policy response. International Food Policy Research Institute. Washington DC, USA.

Rye, C.S. and Withers, S.G. 2000. Glycosidase mechanisms. *Current Opinion in Chemical Biology*. **4**: 573- 580.

Saha, B.C. and Bothast, R.J. 1998. Purification and characterisation of a novel thermostable α-L-arabinofuranosidase from a color-variant strain of *Aureobasidium pullulans*. *Applied and Environmental Microbiology*. **64**: 216- 220.

Saha, B.C. 2000. α-L-arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. *Biotechnology Advances*. **18**: 403- 423.

Saha, B.C. 2003. Hemicellulose Bioconversion. *Journal of Industrial Microbiology and Biotechnology*. **30**: 279- 291.

Saha, B.C. and Bothast, R. 1998. Purification and characterisation of a novel thermostable α-L-arabinofuranosidase from a colour-variant strain of *Aureobasidium pullulans. Applied and Environmental Microbiology*. **60**: 216- 220.

Sakamoto, T. and Kawasaki, H. 2003. Purification and properties of two type-B α-L-arabinofuranosidase produced by *Penicillium chrysogenum*. *Biochimica et Biophysica Acta*. **1621**: 204- 210.

Sanchez, C. 2009. Lignocellulose residues: biodegradation and bioconversion by fungi. *Biotechnology Advances*. **27**: 185- 194.

Sanchez, S and Smiley, K.L. 1975. Properties of D-Xylose Isomerase from *Streptomyces albus*. Applied Microbiolgy. **29**: 745-750.

Sanchez, O.J. and Cardona, C.A. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*. **99**: 5270- 5295.

Shallom, D. and Shoham, Y. 2003. Microbial hemicellulases. *Current Opinion in Microbiology*. **6**: 219- 228.

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

Shao, W. and Wiegel, J. 1992. Purification and characterisation of a thermostable β-xylosidase from *Thermoanaerobacter ethanolicus*. *Journal of Bacteriology*. **174**: 5848- 5853.

Shin, H-Y., Park, S-Y., Sung, J.H. and Kim, D-H. 2003. Purification and characterisation of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing Ginsenoside Rb2 and Rc. *Applied and Environmental Microbiology*. **69**: 7116- 7123.

Shirling, E.B. and Gottlieb, D. 1966. Methods for characterisation of *Streptomyces* species. *International Journal of Systematic Bacteriology*. **16**: 313- 340.

Smith, M.M. and Hartley, R.D. 1983. Occurrence and nature of ferulic acid substitution of cell wall polysaccharides in graminaceous plants. *Carbohydrate Research*. **118**: 65- 80.

Sommer, P., Georgieva, T. and Ahring, B.K. 2004. Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochemical Society Transactions*. **32**: 282- 288.

Steward, D., Azzini, A. and Morrison, I. 1997. Sisal fibres and their constituent non-cellulosic polymers. *Industrial Crop Production*. **6**: 17- 26.

Sun, Y. And Chen, J. 2002. Hydrolysis of lignocellulosic material for ethanol production: A review. *Bioeresource Technology*. **83**: 1- 11.

Tajana, E., Feichter, A. and Zimmermann, W. 1992. Purification and characterisation of two α-L-arabinofuranosidases from *Streptomyces diastaticus*. *Applied and Environmental Microbiology*. **58**: 1447- 1450.

Takao, M., Akiyama, K. and Sakai, T. 2003. Purification and characterisation of the thermostable endo-1,5- α-L-arabinase from strains of *Bacillus thermodentrificans*. *Applied Environmental Microbiology*. **68**: 1639- 1646.

Thomas, J.A. 1993. Molecular biology of xylan degradation. *FEMS Microbiology Reviews*. **10**: 65- 82.

Tison, M.C., Andre-Leroux, G., Lafond, M. Georis, J., Juge, N., and Berrin J-G. 2009. Molecular determinants of substrate and inhibitor specificities of *Penicillium griseofulvum* family 11 xylanases. *Biochemica et Biophysica Acta- Proteins and Proteomics*. **1804**: 438-445.

Tsujibo, H., Takada, C., Wakamatsu, Y., Kosaka, M., Tsuji, A., Miyamoto, K. and Inamori, Y. 2002. Cloning and expression of an α-L-arabinofuranosidase gene (stx*IV*) from *Streptomyces thermoviolaceus* OPC- 520. *Bioscience, Biotechnology and Biochemistry*. **66**: 434- 438.

Tuncer, M. 2000. Characterisation of β-xylosidase and α-L-arabinofuranosidase activities from *Thermomonospora fusca* BD25. *Turkish Journal of Biology*, **24**: 753-767.

Tuncer, M., Ball, A.S., Rob, A. and Wilson, M.T. 1999. Optimization of extracellular lignocellulolytic enzyme production by the thermophilic *Actinomycete Thermomonospora fusca* BD25. *Enzyme Microbiology and Technology*. **25**: 38- 47.

Tuncer, M. and Ball, A.S. 2003. Co-operative actions and degradation analysis of purified xylan-degrading enzymes from *Thermomonospora Fusca* BD25 on oat-spelt xylan. *Journal of Applied Microbiology*. **94**: 1030- 1035.

Uesaka, E., Sato, M., Raiju, M. and Kaji. 1978. α-L-arabinofuranosidase from *Rhodotorula flava*. *Journal of Bacteriology*. **133**: 1073- 1077.

Uffen, R.L. 1997. Xylan degradation: a glimpse of microbial diversity. *Journal of Industrial Microbiology and Biotechnology*. **19**: 1- 6.

Utt, E.A, Eddy, C.K., Keshav, K.F. and Ingram, L.O. 1991. Sequencing and expression of *Butyrivibrio firisolvens* XylB gene encoding a novel bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities. *Applied Environmental Microbiology*, **57**: 1227- 12234.

Van der Veen, P., Flipphi, M.J.A., Voragen, A.G.J. and Visser, J. 1991. Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. *Archives of Microbiology*. **15**: 23- 28.

Van Laere, K.M.J., Beldman, G. and Voragen, A.G.J. 1997. A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove the arabinosyl residue from double substituted xylose units in arabinoxylan. *Applied Microbiology and Biotechnology*. **47**: 231- 235.

Van Laere, K.M.J., Voragen, C.H.L., Kroef, T., Van den Broek, L.A.M., Beldman, G. and Voragen, A.G.J. 1999. Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescentis* DSM 20083. *Applied Microbiology and Biotechnology*. **51**: 606- 613.

Vincent, P., Shareck, F., Dupont, C., Morosoli, R. and Kluepfel, D. 1997. New α-L-arabinofuranosidase produced by *Streptomyces lividans*: cloning and DNA sequencing of the afB gene and characterisation of the enzyme. *Biochemistry Journal*. **322**: 845- 852.

von Sivers, M. and Zacchi, G. 1996. Ethanol from Lignocellulosics; a review of the economy. *Bioresource Technology*. **56**: 131- 140.

Wagschal, K., Heng, C., Lee, C.C. and Wong, D.W.S. 2009. Biochemical characterisation of a novel dual function arabinofuranosidase/ xylosidase isolated from a compost starter mixture. *Applied Microbiology and Biotechnology*. **81**: 855- 863.

Wang, Y., Zhang, Z. And Ruan, J. 1996. A proposal to transfer *Microbispora bispora* (Lechevalier 1965) to a new genus, *Thermobispora* gen.nov., as *Thermobispora bispora* comb nov. *International Journal of Systematic Bacteriology*. **46**: 933- 938.

Watson, B. J., Zhang, H., Longmire, A. G., Moon, Y. H. and Hutcheson, S.W. 2009. Processive endoglucanases mediate degradation of cellulose by *Saccharophagus degradans. Journal of Bacteriology*. **191**: 5697– 5705.

Wayne, L.G., Brenner, D.J., Cowell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, H.L., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., star, M.P. and Trupper, H. G. 1987. Report of the ad hoc committee on reconciliation of of approaches to bacterial systematic. *International Journal of systematic Bacteriology*. **29**: 463- 464.

Wheals, A.E., Basso, L.C., Alves, D.M.G. and Amorim, H.V. 1999. Fuel ethanol after 25 years. *Trends in Biotechnology*. **17**: 482- 487.

Wiselogel, A., Tyson, J. and Johnsson, D. 1996. Biomass feedstock resources and composition. In *Handbook on bioethanol: production and utilization* Wyman, C.E. (ed). Washington DC: Taylor and Frances, 19- 30.

Wyman, C.E. 2003. Potential synergies and challenges in refining cellulosic biomass to biofuels, chemicals and power. *Biotechnology Progress*. **19**: 254-262.

Yang, H., Ichinose, H., Nakajima, M., Kobayashi, H. and Kaneko, S. 2006. Synergy between and alpha-L-arabinofuranosidase from *Aspergillus oryzae* and an endo-arabinanase from *Streptomyces coelicolor* for degradation of arabinan. *Food Science Technology Research*. **12**: 43- 49.

Yoshida, S., Hespen, C.W., Beverly, R.L., Mackie, R.I. and Cann I.K.O. 2010. Domain analysis of a modular α-L-arabinofuranosidase with a unique carbohydrate binding strategy from the fibre degrading bacterium *Fibrobacter succenogenes*. *Journal of Bacteriology*. **585**: 483- 493.

Zaldivar, J., Neilsen, J. and Olsson, L. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering. *Applied Microbiology and Biotechnology*. **56**: 17- 34.

Internet resources

http://www.biosmartpackaging.com. Accessed: 7 May 2009

http://www.khalipakistan.com/trade/html Accessed: 7 May 2009

http://www.moosecrossingardencenter.com/store Accessed: 7 May 2009

http://www.sciencedaily.com/releases/2007/07/070709103138.htm Accessed: 7 September 2009