

The α -L-arabinofuranosidase of *Aureobasidium pullulans*

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Promoter: Prof. B. A. Prior

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted to any university for a degree.

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SUMMARY

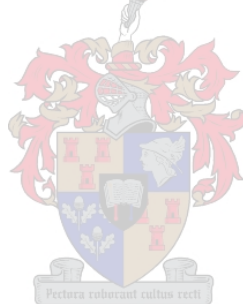
The euascomycetous fungus *Aureobasidium pullulans* produces xylanolytic accessory enzymes, including an α -L-arabinofuranosidase. The deduced amino acid sequence of the *abfA* gene encoding α -L-arabinofuranosidase was 69-76% identical to family 54 glycoside hydrolases. The *abfA* gene encoded a 498 amino acid polypeptide including a signal peptide consisting of 20 amino acids. The mature protein had a calculated molecular weight of 49.9 kDa. One putative *N*-glycosylation site was found and an iso-electric point of 4.97 was calculated. *A. pullulans* AbfA was also found to consist of an N-terminal catalytic domain (residues 1-317) and a C-terminal arabinose-binding domain (residues 318-442).

The *abfA* gene from the colour-variant strain of *A. pullulans*, NRRL Y-2311-1, was recently transferred in *Saccharomyces cerevisiae* Y294. The yeast culture was grown on synthetic defined medium and α -L-arabinofuranosidase was expressed successfully, secreted from the cells and was purified from the supernatant in a single step using gel filtration. It had an apparent mobility of 52.7 kDa on SDS-PAGE and 36 kDa estimated by gel filtration.

The heterologous enzyme was characterized according to pH and temperature dependence and stability, apparent mobility and kinetic properties. The temperature optimum of the recombinant α -L-arabinofuranosidase was 55 °C and it was stable over 3 h at 40 °C. The enzyme displayed optimum activity between pH 4 and 4.5 and was stable at pH 4 over 3 h. Kinetic analysis on *p*-nitrophenyl- α -arabinofuranoside yielded a K_m of 1.43 mM and a V_{max} of 23.7 U/mg. Product inhibition was observed and a K_i of 28 ± 3 mM was determined during assaying in the presence of arabinose. A specific activity of 3.85 ± 0.008 U/mg was determined on *p*-nitrophenyl- α -L-arabinofuranoside and no activity was found on chromogenic substrates which contained a β -linked arabinofuranosyl. The enzyme showed low activity against the 1,5- α -L-arabino-oligosaccharides and cleaved arabinose from corn fibre, oat spelt arabinoxylan and to a lesser degree wheat arabinoxylan. No release of arabinose was observed from larch wood arabinogalactan, α -1,5-debranched arabinan and lignin-arabinose substrates. Linkage preference showed less activity against α -1,5-linked than α -1,2 or α -1,3-linked arabinofuranosyl subunits. Synergism between α -L-arabinofuranosidase and endo- β -1,4-xylanase occurred when measuring the increase in arabinose during wheat arabinoxylan degradation.

A. pullulans NRRL Y-2311-1 was grown on synthetic defined medium and native α -L-arabinofuranosidase was expressed and secreted into the culture medium. The native enzyme was partially purified from the supernatant in two steps using gel filtration. The native α -L-arabinofuranosidase had an apparent mobility of 51.5 kDa on SDS-PAGE, displayed optimum activity at 50°C and pH 3. Kinetic analysis on *p*-nitrophenyl- α -arabinofuranoside gave a K_m of 8.33 mM and a V_{max} of 1.54 U/mg, and the enzyme showed slight activity against 1,5- α -L-arabinotriose. The properties of the native enzyme were similar to that of the heterologous α -L-arabinofuranosidase.

Hydrolysis of sugar cane bagasse by heterologous α -L-arabinofuranosidase and xylanase revealed that pre-treatment with liquid ammonium was more effective in releasing component sugars than a pre-treatment with water at 140° C. A three-dimensional homology model of the heterologous α -L-arabinofuranosidase was constructed using the solved crystal structure of arabinofuranosidase (AkabfB) from *Aspergillus kawachii*, which was 71 % identical.



OPSOMMING

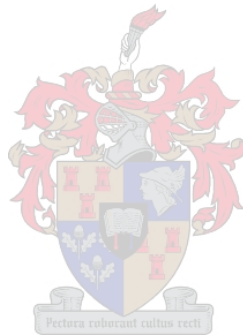
Die euaskomietiese swam *Aureobasidium pullulans* produseer xilanolitiese ensieme, insluitend 'n α -L-arabinofuranosidase. Die afgeleide aminosuurvolgorde van die *abfA* geen wat α -L-arabinofuranosidase encodeer, was 69-76% identies aan familie 54 glikosied hidrolase. Die *abfA* geen encodeer 'n 498 aminosure polipeptied, insluitend 'n sein peptied bestaande uit 20 aminosure. Die volwasse proteïen het 'n berekende molekulêre gewig van 49.9 kDa. Een moontlike N-glikosilasie plek is gevind en 'n iso-elektriese punt van 4.97 is bereken. *A. pullulans* AbfA bestaan uit 'n N-terminaal katalitiese gebied (residu 1-317) en 'n C-terminaal arabinose-bindingsgebied (residu 318-442).

Die *abfA* geen van die kleur-variante ras van *A. pullulans*, NRRL Y-2311-1, is onlangs na *Saccharomyces cerevisiae* Y294 oorgedra. Die giskultuur is op 'n sinteties gedefinieerde medium gegroei en α -L-arabinofuranosidase was suksesvol uitgedruk, uitgedra van af die selle en van uit die supernatant gesuiwer in 'n enkele stap deur gel filtrasie te gebruik. Dit het 'n berekende mobiliteit van 52.7 kDa op SDS-PAGE en 36 kDa geskat deur middel van gel filtrasie.

Die heteroloë ensiem is gekarakteriseer volgens pH en temperatuurafhanklikheid en stabiliteit, klaarblyklike mobiliteit en kinetiese eienskappe. Die temperatuur optimale van α -L-arabinofuranosidase was 55 °C en dit was stabiel oor 3 ure teen 40 °C. Die ensiem het optimale aktiwiteit tussen pH 4 en 4.5 getoon en was stabiel teen pH4 en oor 3 ure. Kinetiese analiese op *p*-nitrofeniel- α -L-arabinofuranosied het 'n K_m van 1.43 gelever en 'n V_{max} van 23.7 U/mg. Produk-inhibisie is opgelet en 'n K_i van 28 ± 3 mM is vasgestel gedurende toetsing in die teenwoordigheid van arabinose. 'n Spesifieke aktiwiteit van 3.85 ± 0.008 U/mg is vasgestel op *p*-nitrofeniel- α -L-arabinofuranosied en geen aktiwiteit was gevind op chromogeniese substrate wat 'n β -verbinding arabinofuranosiel bevat het nie. Die ensiem het 'n lae aktiwiteit getoon teen die 1,5- α -L-arabino-oligosakkaried en het die arabinose van mielievesel, hawerspelt arabinoxilaan en tot 'n mindere mate koring arabinoxilaan geskei. Geen vrystelling van arabinose is van af lorkehout arabinogalactan, α -1,5-onvertakte arabinan en lignin-arabinose substrate nie opgemerk. Verbindingsvoorkeure het minder aktiwiteit teen α -1,5-verbindinge as α -1,2 of α -1,3-verbindinge arabinofuranosiel subeenhede getoon. Sinergisme tussen α -L-arabinofuranosidase en endo- β -1,4-xilanase het plaasgevind met die bepaling van meer arabinose gedurende koring arabinoxilaan degradasie. *A. pullulans* NRRL Y-2311-1 is op sinteties gedefinieerde medium gegroei en α -L-

arabinofuranosidase was uitgedruk en uitgeskei in die kultuur medium. Die inheemse ensiem was gedeeltelik gesuiwer van die supernatant in twee stappe met die gebruik van gel filtrasie. Die inheemse α -L-arabinofuranosidase het 'n klaarblyklike mobiliteit van 51.5 kDa op SDS-PAGE, het optimum aktiwiteit vertoon by 50°C en pH 3. Kinetiese analiese op *p*-nitrofeniel- α -arabinofuranosiede het 'n K_m van 8.33 mM en 'n V_{max} van 1.54 U/mg, en die ensiem het effense aktiwiteit teen 1,5- α -L-arabinotrios getoon. Die eienskappe van die inheemse ensiem was soortgelyk aan die van die heteroloë α -L-arabinofuranosidase.

Hidroliese van suikerrietbagasse met heteroloë α -L-arabinofuranosidase en xilanase het aan die lig gebring dat vooraf behandeling met vloeistof ammonium meer effektief is in die vrystelling van komponent suikers as 'n vooraf behandeling met water teen 140° C. 'n Driedimensionele homologiese model van die heteroloë α -L-arabinofuranosidase is gekonstrueer deur die gebruik van die verklaarde kristal struktuur van arabinofuranosidase (AkabfB) van *Aspergillus kawachii*, wat 71% identies was.



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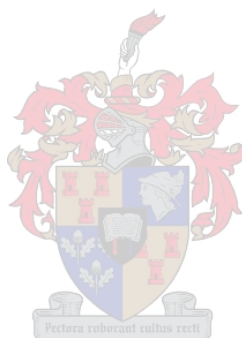
My friends and family for support and encouragement.



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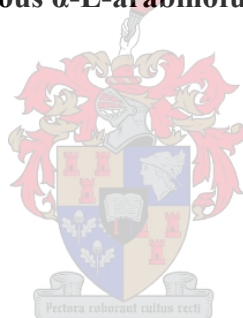
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COMMONLY USED ABBREVIATIONS

AbfA	α -L-arabinofuranosidase of <i>Aureobasidium pullulans</i>
EDTA	Ethylenediaminetetraacetic acid
His	Histidine
HIS3	Auxotrophic mutation for histidine production
HPAEC	High-pH anion-exchange chromatography
k_{cat}	Catalytic constant
kDa	kilo Dalton
K_i	Inhibition constant
K_m	Michaelis constant
leu	Leucine
LEU2	Auxotrophic mutation for Leucine production
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulphonyl fluoride
SC	Synthetic complete
SDS	Sodium dodecyl sulphate
trp	Tryptophan
TRP1	Auxotrophic mutation for tryptophan production
ura	Uracil
URA3	Auxotrophic mutation for uracil production
V_{max}	Maximum enzyme activity
YPD	Yeast peptone dextrose
PAGE	Polyacrylamide gel electrophoresis

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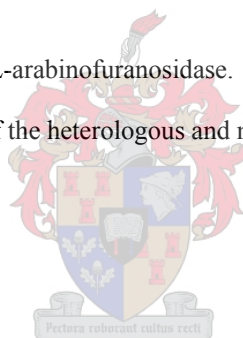
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1. INTRODUCTION

Plant cell wall material (lignocellulose) is the most abundant naturally occurring organic polymer in the biosphere and is composed of 40-50% cellulose, 20-30% hemicellulose and 20-30% lignin in dry weight. The exact composition of plant cell wall material varies between different types of species of plants and the type of tissue. Cellulose is the structural base of cell walls and is a polymer glycan with a uniform chain structure made up of D-glucose units which are bound by β -1,4-glycosidic linkages. The regular system of linkages results in a crystalline lattice, which makes the cellulose fibres insoluble in water. Lignin consists of phenylpropane units, has an amorphous structure and acts as a glue-like filler material. This polymer increases the mechanical strength and density properties of plant cell walls. Hemicelluloses are a group of alkali extractable heteropolysaccharides composed of a homopolymer backbone of various sugar units and contain branching side groups (Fengel & Wegener, 1989).

Xylans, a major hemicellulose, consist of a backbone of D-xylose units linked by β -1,4-glycosidic bonds. In hardwood the xylan backbone is linked to 4-O-methyl-D-glucuronic acid by α -1,2-glycosidic bonds at irregular intervals and a large percentage of the hydroxyl groups at C2 and C3 of the D-xylose units are substituted by O-acetyl groups. Softwood xylans contain 1,3-linked α -L-arabinofuranosyl residues, have a higher occurrence of 4-O-methyl-D-glucuronic acid side groups and do not contain acetyl groups. The average degree of polymerization of the xylan backbone from softwood is shorter (70-130) than hardwood (100-200) (Fengel & Wegener, 1989).

L-Arabinofuranose is a component of a number of plant cell wall polysaccharides, including arabinoxylan, arabinogalactan and arabinan. Arabinans contain α -L-arabinofuranose both as the 1,5-linked backbone, as well as the 1,2- and 1,3-linked side-chains, while α -L-arabinofuranose is found as 1,3- and 1,6-linked side-chains in arabinogalactans. Arabinoxylan is a major component of the cell walls of softwoods as well as grasses and cereals and consists of a backbone of 1,4-linked β -xylopyranose units that carries α -L-arabinofuranose substituents at the O-2 and O-3 positions (Bacic *et al.*, 1988).

Hemicelluloses from forestry and agricultural biomass are a significant renewable resource for bioconversion into chemicals and fuels. The polysaccharide can be hydrolysed into component

saccharides by enzymatic or chemical action. The class of enzymes, glycoside hydrolases, act by degrading the plant cell wall structure and this results in component release. The combined action of numerous glycoside hydrolases produced by plant pathogens and microbial saprophytes is required for the complete hemicellulose degradation into sugar molecules during growth (Dekker, 1989). One of the accessory enzymes involved in arabinoxylan, arabinogalactan and arabinan degradation is α -L-arabinofuranosidase. α -L-Arabinofuranosidases (E.C. 3.2.1.55) catalyse the hydrolysis of single α -L-arabinofuranose residues from the non-reducing ends of arabinose-containing polysaccharides and play an integral role in the biodegradation of hemicelluloses. These enzymes have hydrolytic activity for both α -1,3 and α -1,5-linked, non-reducing, terminal arabinosyl residues of arabinoxylan and are rate limiting during arabinoxylan degradation (Saha, 1998b).

α -L-arabinofuranosidases have been employed in numerous industrial applications, usually complementing the action of endo- β -1,4-xylanase in the enzymatic degradation of xylan. Specific applications have included the pre-treatment of paper pulp, the pre-treatment of lower grade animal feeds for increased digestibility and the enzymatic clarification of fruit juices. Potential applications in biotechnology include clarification and thinning of juices, bioconversion of lignocellulosic materials to fermentable products and the hydrolysis of grape monoterpenyl α -L-arabinofuranosyl glucosides to increase aroma during wine making (Beg *et al.*, 2001; Saha, 2000).

α -L-arabinofuranosidases have been placed into four glycoside hydrolase families (43, 51, 54 and 62) based on amino acid sequence similarity (<http://afmb.cnrs-mrs.fr/CAZY/>). Family 54 enzymes are found only in fungi, consist of monomeric enzymes that have molecular weights of about 50 kDa and acidic isoelectric points around pH 5. They have hydrolytic activity for the α -1,2 or α -1,3-linked and to a lesser degree α -1,5-linked, non-reducing, terminal arabinosyl residues of arabinoxylan, arabinan and arabinogalactan. These enzymes do not hydrolyze internal α -arabinosyl linkages and do not act on double arabinosylated xylopyranosyl residues. They have highly conserved primary sequences, substrate specificity and regulation of expression and are able to hydrolyse the synthetic aryl glycoside *p*-nitrophenyl α -L-arabinofuranoside (Saha, 2000). The arabinofuranosidase B from *Aspergillus niger* was the first family 54 α -L-arabinofuranosidase to be purified and characterized (Flipphi *et al.*, 1993). The gene *abfA* encoding an arabinofuranosidase from *Aspergillus niger* was cloned and was expressed in *Saccharomyces cerevisiae* (Crous *et al.*, 1996). The three-dimensional crystal structure of the arabinofuranosidase (AkAbfB) from *Aspergillus kawachii* IFO4308 has been solved and it consists of a β -sandwich fold and has been grouped in the clan-B glycoside hydrolases (Miyanaga *et al.*, 2004).

Aureobasidium pullulans is an ascomycetous saprophyte that produces xylan hydrolyzing enzymes and belongs to the family *Dothideales*. It is found on leaves of a wide range of crops in many countries including South Africa and is a plant pathogen causing softening of plant tissue. The imperfect stage has a diverse morphology and it shows polymorphism where it can grow as a mycelium or as a budding yeast depending on environmental conditions (Myburgh *et al.*, 1991b). Starting as cream, yellow, light brown or light pink in colour, colonies of *A. pullulans* become black due to chlamydospore production. Colour variants of *A. pullulans* produce a high level of β -xylanase when cultivated on xylan and D-xylose (Saha *et al.*, 1998). The colour variant strain NRRL Y-2311-1 secreted xylanase with the highest specific activity (Leathers *et al.*, 1986). The α -L-arabinofuranosidase of *A. pullulans* strain NRRL Y-2311-1 has only been characterized in terms of temperature and pH properties in crude extracts. Maximum activity was observed at 60 °C and the enzyme was stable over 3 h at the same temperature, but was inactivated at higher temperatures. The pH optimum was 5.0 but the enzyme was most stable at 60° C (Myburgh *et al.*, 1991b).

The *abfA* gene from *A. pullulans* strain NRRL Y-2311-1 encoding α -L-arabinofuranosidase was recently cloned and expressed in *S. cerevisiae* Y294 (de Wet, 2003). The aim of this study was the following:

- 1) Express and purify the heterologous α -L-arabinofuranosidase (EC 3.2.1.55) from *S. cerevisiae*
- 2) Establish the biochemical properties of the heterologous enzyme
- 3) Establish the specificity of the enzyme for various substrates
- 4) Investigate the synergistic action with xylanase (EC 3.2.1.136) against arabinoxylan
- 5) Establish the effect of inhibitors and calculate the inhibition constant
- 6) Establish the degree of binding of the heterologous enzyme to various substrates
- 7) Create a three-dimensional model of the heterologous α -L-arabinofuranosidase
- 8) Express and attempt to purify the native α -L-arabinofuranosidase of *A. pullulans* NRRL Y2311-1
- 9) Establish the biochemical properties of the native enzyme
- 10) Determine the specificity of the native enzyme
- 11) Evaluate enzymatic degradation of sugar cane bagasse pretreated by various methods

2. LITERATURE REVIEW

2.1. STRUCTURE OF PLANT CELL WALLS

2.1.1. Ultrastructure

Plant cells have a rigid protective cell wall structure that encapsulates plant cells, forming a skeleton that determines plant architecture. It consists of a structural matrix of cellulose and hemicellulose fibres that are covered in an unstructured matrix of lignin. Primary cell walls are associated with young, differentiating and growing tissues. Secondary cell walls are associated with a terminal developmental phase where cell shape and size are established (Fengel & Wegener, 1989).

Cells are surrounded by a polysaccharide-rich primary wall during the growth phase. This wall is part of the apoplast, which contains all that is located between the cuticle and the plasma membrane. Primary walls isolated from higher plant tissues are between 0.1 and 0.2 μm thick, and are made up of mostly polysaccharides, which consists of a network of cellulose microfibrils and several hemicelluloses. Smaller amounts of structural glycoprotein, covalently and ionically bound minerals such as boron and calcium, phenolic esters, and enzymes are also present. Proteins known as expansins regulate wall expansion. The primary wall is able to endure the turgor pressure in the plant, as it is rigid, but still allows for longitudinal growth. At the end of the growth phase in specialised load-bearing cells (xylem, phloem and the sclerenchymal fibre cells), a thick secondary wall is formed (Leea *et al.* 2004).

The secondary wall is made up of three layers. The outer layer is 0.1-0.3 μm thick and consists of a mesh network of cellulose microfibrils. The middle layer of the secondary wall is 1-5 μm thick and the cellulose microfibrils are arranged parallel to the cell's vertical axis. The inner layer is thinner (0.1 μm) and consists of a layer of microfibrils that are arranged horizontally to the cell's vertical axis.

Between the primary walls of individual cells, the middle lamella exists and contains lignins. After secondary wall formation, lignification takes place and results in any residual spaces between the fibres in the primary and secondary walls to be encased in the lignin. Most of the apoplast in

growing tissue is accounted for by the middle lamella and primary wall (Timmel, 1967; Reiter, 2002). Figure 2.1 presents a schematic diagram of the plant cell.

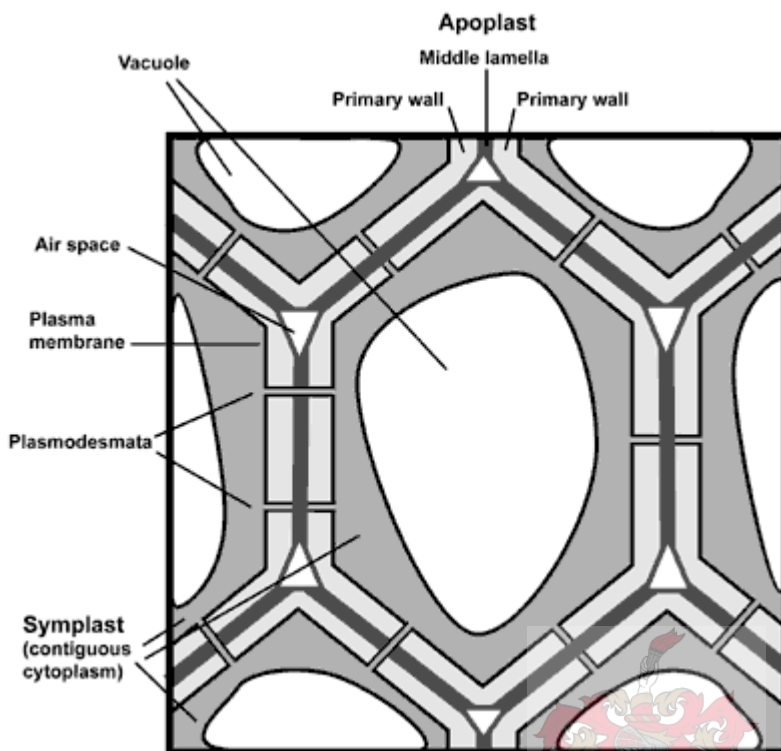


Figure 2.1. The structure of a plant cell wall. This cross-section shows the distribution of the primary wall, middle lamella and symplast. (<http://www.crc.uga.edu/~mao/intro/outline.htm>)

The plant cell wall serves a range of functions. Other than structural and mechanical support and maintenance of the cell shape, the cell wall controls the rate and direction of growth. Cell walls also regulate flow of water and nutrients through the apoplast, resist internal turgor pressure and provide a barrier against infection by pathogens and dehydration (Fengel & Wegener, 1989).

Plant cell wall material (lignocellulose) is the most abundant naturally occurring organic polymer in the biosphere and is composed of 40-50% cellulose, 20-30% hemicellulose and 20-30% lignin in dry weight. The variation in composition is dependent upon plant species, growth conditions and location in the plant. Figure 2.2 shows the distribution of lignocellulose within the plant cell wall. Cellulose is a polysaccharide composed of 1,4-linked β -D-glucose residues. Hemicellulose is a branched polysaccharides which is structurally homologous to cellulose as they both include a backbone composed of 1,4-linked β -D-hexosyl residues. The major hemicellulose in many plant cell walls is xyloglucan, arabinoxylan, glucomannan, and galactomannan.

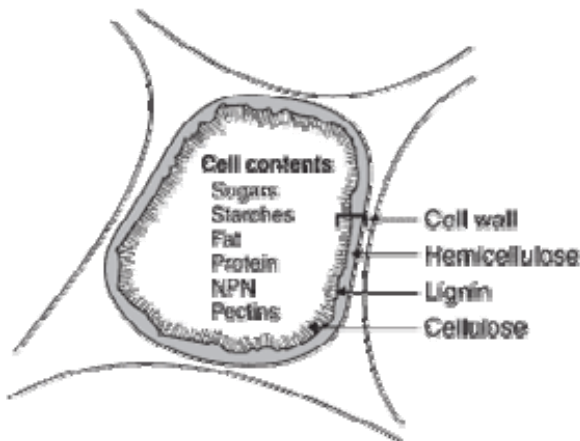


Figure 2.2. Distribution of lignocellulose in the plant cell wall (Schroeder, 2004).

The exact composition of plant cell wall material varies between different types of species of plants and the type of tissue. Its biodegradation is important in the carbon cycle in nature and in many industrial processes (Fengel & Wegener, 1989).

2.1.2. Cellulose

Cellulose is the structural base of cell walls and is a polymer of glycan with a uniform chain structure made up of glucose units bound by β -1,4-glycosidic linkages. Cellulose has a high degree of polymerisation and single polymer chains normally contain 10000-15000 glucose residues (Figure 2.3). The hydroxyl groups of cellulose form different types of hydrogen bonds, ones between hydroxyl groups of adjacent glucose units in the same cellulose molecule, called intramolecular linkages, and ones between hydroxyl-groups of adjacent cellulose molecules, called intermolecular linkages (Fengel & Wegener, 1989). The cellulose polymer is unbranched and composed of neutral carbohydrate moieties, which results in a high degree of intra- and intermolecular hydrogen bond formation. The regular system of linkages results in a crystalline lattice, which makes the cellulose fibres insoluble in water.

Hexagonal structures present in the plasma membrane of the plant cell wall are involved in cellulose synthesis and export. Each microfibril forms spontaneously during synthesis and is composed of 36 single cellulose chains. Six 1,4- β -D glucan synthases found in the hexagonal structures synthesize and export 36 cellulose chains. The cellulose synthase complex combines the single chains into a microfibril. Orientation of the microfibrils around the cell directs the movement of this complex along the cellular cytoskeleton (Williamson *et al.*, 2002; Reiter, 2002).

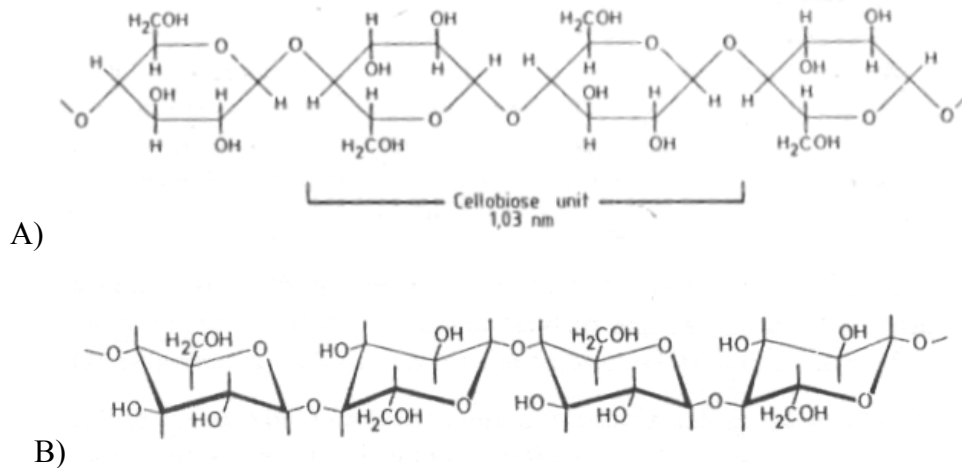


Figure 2.3. The structure of cellulose, A) central section of the molecular chain, B) stereo-chemical formula (Fengel & Wegener, 1989).

2.1.3. Hemicelluloses

Hemicelluloses are a group of alkali extractable heteropolysaccharides composed of a homopolymer backbone of various sugar units and contain branching side groups. Compared to cellulose, hemicelluloses have a lower degree of polymerization, consisting of less than 200 glycosidic units (Fengel & Wegener, 1989). Hemicelluloses are frequently soluble in alkaline solutions and occasionally in water. They consist of many carbohydrate components including D-mannose, D-xylose, 4-*O*-methyl glucuronic acid, D-glucose, L-arabinose, and D-galactose. The percentage of these carbohydrate components differ in hardwood and softwoods. Hardwoods consist of a higher amount of acetyl and xylose units while softwoods consist of a higher amount of mannose and galactose units. Hemicelluloses also contain esterified organic acids like acetic acid and cinnamic acids (ferulic and *p*-coumaric acid). Hemicelluloses are synthesised in the Golgi cisternae and secreted by exocytosis (Brett & Waldron, 1996).

Mannan, another form of hemicellulose, is a heteropolymer consisting of D-mannose and D-glucose subunits linked by β -1,4-glycosidic bonds. Softwood mannans contain acetyl groups and galactose residues attached to a glucomannan backbone. Hardwood mannans consist only of glucose and mannose units.

Xylan is one of the main hemicelluloses found in plant cell walls and consists of a homopolymer backbone of β -1,4 linked D-xylose units. In hardwood the xylan backbone is substituted with α -1,2 linked 4-*O*-methylglucuronic acid on every tenth D-xylose unit. Approximately 70% of the

hydroxyl groups at C2 and C3 of the xylose units are esterified with acetic acid. Depending on plant species, the average degree of polymerization of the xylan backbone ranges from 100 and 200. Softwood xylans contain 1,3-linked α -L-arabinofuranosyl residues on approximately every ninth xylose unit. These xylans have a higher occurrence of 4-*O*-methylglucuronic acid side groups with an average ratio of 6 xylose units to 1,4-*O*-methylglucuronic acid. Softwood xylans do not contain acetyl groups and the degree of polymerization of the backbone is between 70 and 130. Softwoods have a much higher degree of lignification and therefore need to be delignified prior to the alkaline extraction of xylan (Timmel, 1967; Fengel & Wegener, 1989; Sjöström, 1993).

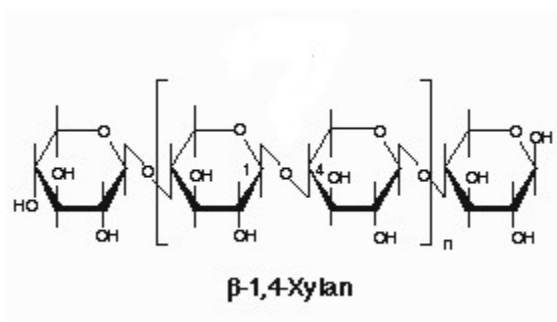


Figure 2.4. The structure of xylan (www.nakamura.bio.titech.ac.jp/xyn/xyn.html).

Xylans have been characterized after being isolated from numerous species in the commercially important Gramineae family, which includes cereals and grasses. Grasses and cereal xylans are classified as arabino-4-*O*-methylglucuronoxylan, but also contain *p*-coumaric acid or ferulic acid esterified to the C5 of arabinofuranose side-chains. In xylans from the different Gramineae species, the position of the arabinofuranose moiety varies between C2 and C3 (Wilkie, 1979; Bacic *et al.*, 1988). In barley straw 1/120 of the xylose residues have an arabinofuranose moiety esterified with ferulic acid and 1/240 of the residues have an arabinofuranose moiety esterified with *p*-coumaric acid. In wheat bran 1/150 xylose residues contain an arabinofuranose moiety which is esterified with ferulic acid (Mueller-Harvey & Hartley, 1986).

2.1.4. Lignin

Lignin is the second most abundant organic polymer, has an amorphous structure and acts as a glue-like filler material. It is a polymer of randomly cross-linked phenyl propane units, which surrounds the cellulose fibres and increases the mechanical strength and density properties of plant cell walls. It consists of phenylpropane units including coniferyl, *p*-coumaryl and sinapyl alcohol linked

carbon-carbon and ether bonds (Figure 2.5). Half of the lignin linkages in hardwood and softwood is β -O-4 and other major kinds are β -5, α -O-4 and 5-5 linkages (Brett & Waldron, 1996).

During the final phase of the differentiating development of a plant cell, lignin is incorporated into the polysaccharide cell wall. Polymerisation of the phenylpropane units happens after longitudinal cell growth and the procedure continues until cinnamyl alcohols in the cell wall are depleted. Lignin displaces water molecules by occupying the spaces between single microfibrils, which results in an anhydrous environment. It is a morphological and chemical component of tissues belonging to higher plants. It usually occurs in the vascular tissues (Fengel & Wegener, 1989).

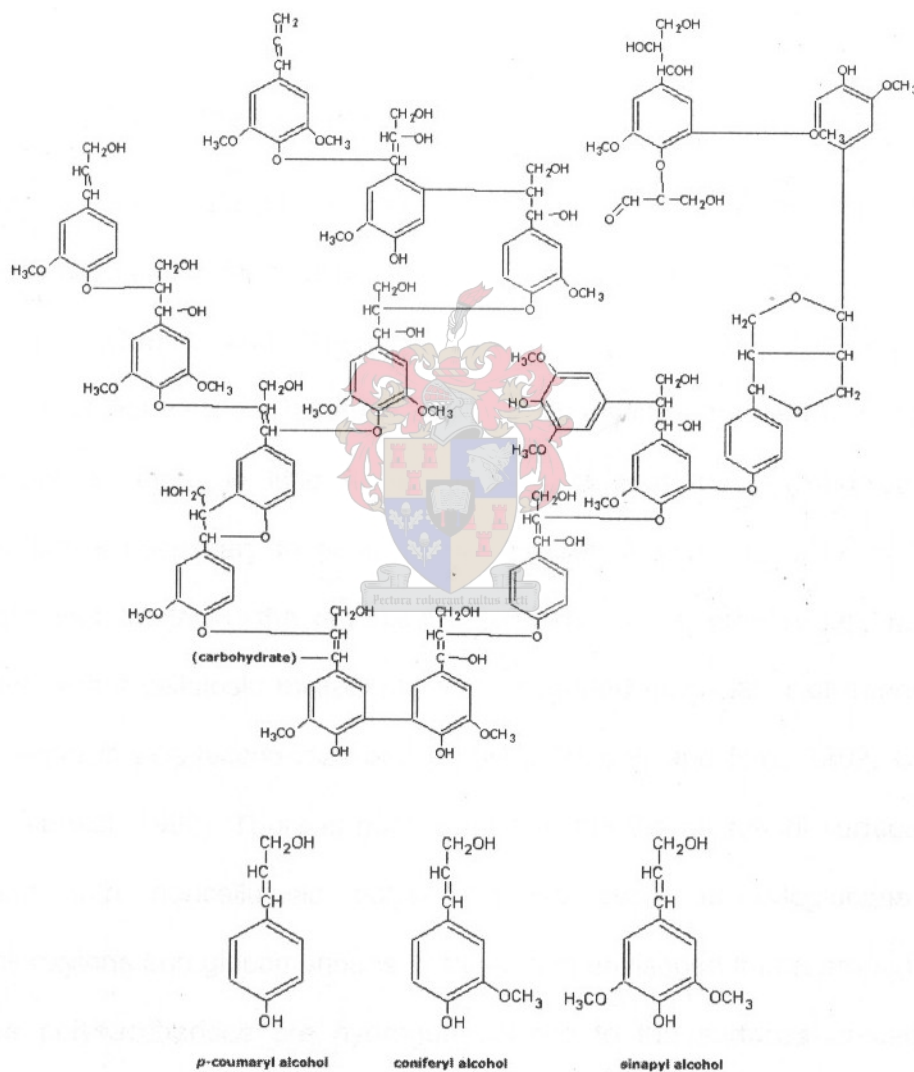


Figure 2.5. The structure of lignin including the phenylpropane units *p*-coumaryl, coniferyl and sinapyl alcohol (Iiyama *et al.*, 1990)

2.2. STRUCTURE OF LIGNOCELLULOSES CONTAINING ARABINOSE

L-Arabinose is present in many constituents of plant material. In hardwood xylans, L-arabinose makes up about 2% of the dry weight and forms non-reducing end groups. Softwood xylans contain L-arabinofuranose units which are α -1,3-linked to the xylan backbone and makes up about 6% of the dry weight. In this section, the lignocelluloses which are relevant in this study are given more attention (Fengel & Wegener, 1989).

2.2.1. Hemicelluloses which contain L-arabinose.

Xylan is one of the major hemicelluloses present in plant cell walls, consisting of 10 - 35% of the dry weight (Timmel, 1967; Dekker, 1989). Softwood xylans contain 1,3-linked α -L-arabinofuranosyl residues whereas grasses and cereals contain 1,5-linked arabinofuranose side-chains. These and other xylans containing L-arabinose side-chains are known as arabinoxylans.

Arabinoxylans

Arabinoxylan is a major component of plant hemicellulose and consists of α -L-arabinofuranose residues attached either to position O-3 or to positions O-2 and O-3 as side branches of the linear β -1,4-linked D-xylopyranose polymeric backbone chains (Figure 2.6). They generally also contain heterogenous substituents like O-acetyl, and ferulic, *p*-coumaric and 4-O-methylglucuronic acids. Arabinoxylans are mainly found in the grasses of the *Graminae* family. Phenolic compounds such as feruloyl and *p*-coumaroyl substituents are esterified to a part of the L-arabinofuranose present in arabinoxylans (Chaplin, 2004; Koseki *et al.* 2003)

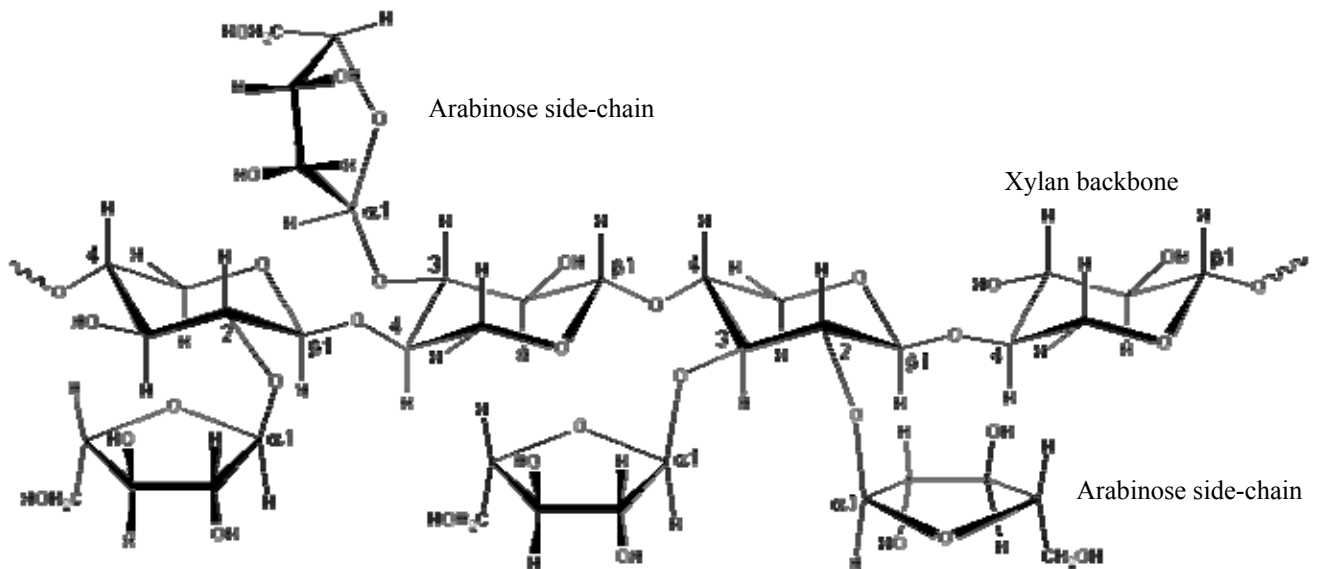


Figure 2.6. The structure of arabinoxylan showing the β -1,4-linked D-xylopyranose backbone and arabinose side chain substituents (Chaplin, 2004).

In barley straw, every 1 in 240 of the xylose backbone residues contains an arabinofuranose moiety esterified with *p*-coumaric acid and every 1 in 120 of the residues contain an arabinofuranose moiety esterified with ferulic acid. In larchwood xylan the arabinose is primarily O-2 linked to xylose, while in oat spelt xylan, it is O-3 linked (Timmel, 1967; Dekker, 1989).

The cell walls of wheat endosperm consist mainly of arabinoxylans (70% w/w) and 1,4-linked-D-glucans (20% w/w). They also have small amounts of cellulose (4% w/w), which is usually associated with glucomannans (6% w/w). Arabinoxylans are the main non-starch polysaccharides of wheat and rye grains. The L-arabinose to D-xylose ratio of wheat arabinoxylans is 0.51-0.61. In wheat bran, one in 150 D-xylose backbone residues contain an L-arabinofuranose moiety which is esterified with ferulic acid (Mathlouthi *et al.*, 2002).

Arabinoxylooligosaccharides are short arabinoxylans consisting of a xylose backbone and single side-chain arabinose moieties. These are produced after degradation, for example by the enzyme *endo*- β -1,4-xylanase (Figure 2.7).

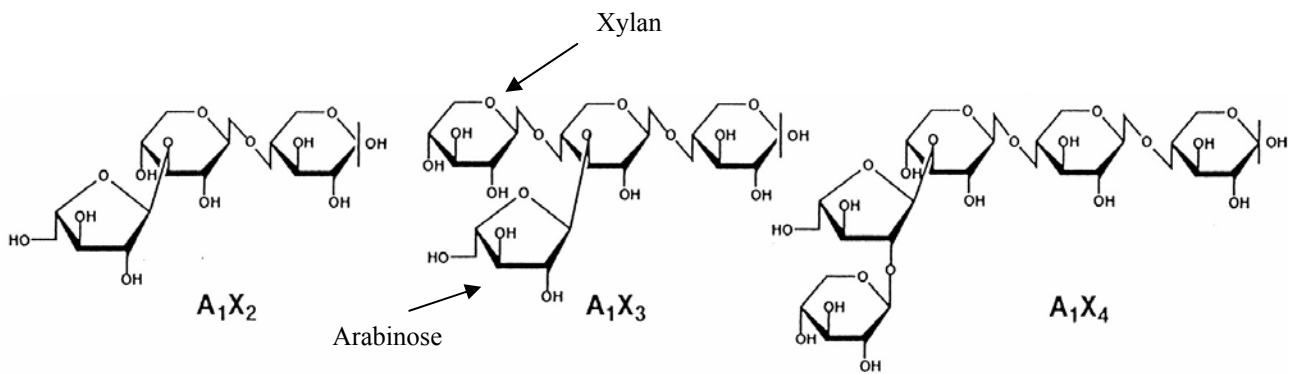


Figure 2.7. Structures of arabinoxylooligosaccharides (Kaneko *et al.* 1998).

Glucans

These occur in the cell walls as minor components. The most abundant type of hemicellulose in the primary cell walls of dicots are xyloglucans. Xyloglucan consists of a 1,4-linked β -glucopyranose backbone which is highly substituted with 1,6-linked α -xylopyranose side-chains. The xylose units also contain 1,2-linked β -galactopyranose, 1,2-linked α -arabinofuranose, or α -1,2-fucopyranosyl- β -1,2-galactopyranose as additional extensions of the side-chains (Fengel & Wegener, 1989; Brett & Waldron, 1996).

Galactans

Arabinogalactan is a principal water-extractable hemicellulose in larchwood (10-25% w/w). It consists of a backbone made from 1,3-linked β -galactopyranose and is highly branched (Figure 2.8). The backbone is substituted with 1,6-linked α -L-arabinofuranose and 1,6-linked β -D-glucuronic acid units. Longer side-chains of 1,6-linked β -D-galactopyranose containing β -arabinopyranose and 1,3-linked α -arabinofuranose are also present. Rhamnoarabinogalactans are found in hardwood and consist of a 1,3-linked β -galactopyranose backbone. These galactans are also substituted with D-rhamnose residues with the ratio of galactose to arabinose to rhamnose is 17:10:2 (Timmel, 1967; Fengel & Wegener, 1989; Sjöström, 1993).

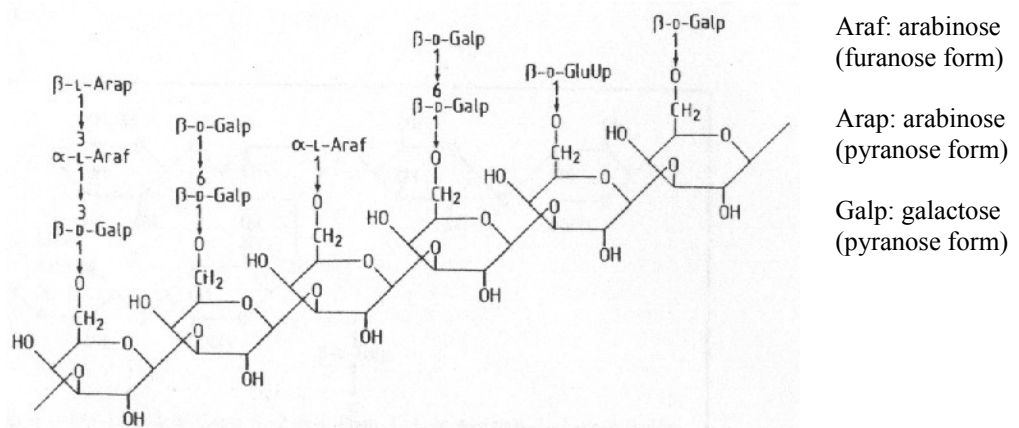


Figure 2.8. The structure of larch wood arabinogalactan. The backbone consists of β -1,3-linked galactose units and side chains of β -1,6-linked arabinose and galactose units. (Fengel & Wegener, 1989).

Arabinans

Arabinans have a backbone of 1,5-linked α -arabinofuranose units and is branched with varying numbers of α -arabinofuranose residues at the C2 and C3 positions (Figure 2.9). L-Arabinose is a major component of arabinan, which has a sweet taste, but is not easily absorbed by the human body (Bacic *et al.*, 1988; Matsuo *et al.*, 2000; Williamson *et al.*, 2002; Koseki *et al.* 2003).

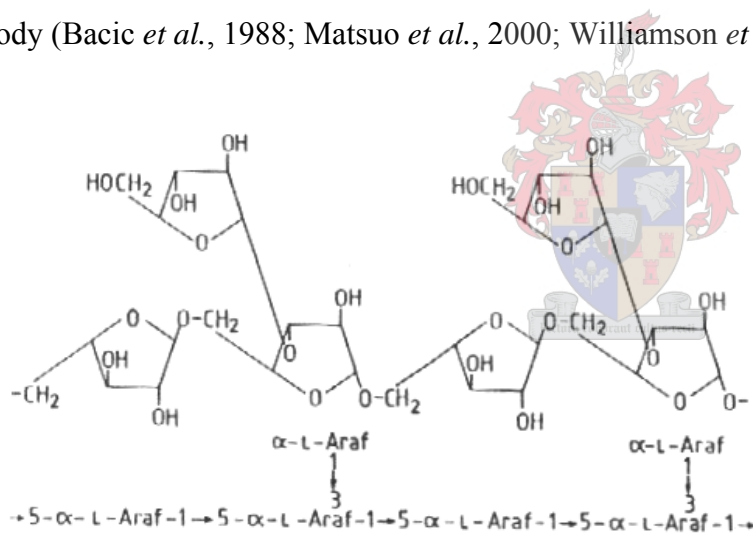


Figure 2.9. The partial structure of arabinan. The backbone consists of α -1,5-linked arabinose units and side chains of α -1,3-linked arabinose units (Fengel & Wegener, 1989).

2.2.2. Lignin linked to arabinose

Hemicelluloses containing arabinoxylans often form cross-links with lignins through arabinose and xylose by means of both ether and glycosidic bonds in the cell walls of wheat straw. Therefore samples of extracted lignins often contain trace amounts of arabinose (Sun *et al.*, 2005).

2.2.3. Artificial substrates containing arabinose

Small chromogenic substrates which consist of *p*-nitrophenyl glycosides are often used to assay glycoside hydrolases. They are used to evaluate the type of bonds these enzymes can cleave. *p*-nitrophenyl glycosides yield a soluble yellow coloured product with an absorption maximum at 450 nm which is used to calculate the enzyme activity. Figure 2.10 shows the substrate *p*-nitrophenyl- α -L-arabinofuranoside.

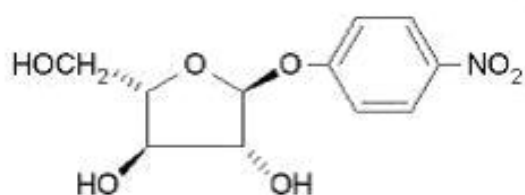


Figure 2.10. Chromogenic substrate with α -linked arabinose (Sigma).

Artificial substrates are also synthesized as an alternative to the isolation of naturally occurring substrates. For example, substrates which contained esters of ferulic acid on different positions of 4-nitrophenyl glycosides of α -L-arabinofuranose and β -xylopyranose, were designed to replace feruloylated oligosaccharides as substrates in the determination of activity of feruloyl esterases (Mastihubová *et al.*, 2003). Two of these substrates, 4-nitrophenyl 2-O-trans-feruloyl- α -L-arabinofuranoside and 4-nitrophenyl 5-O-trans-feruloyl- α -L-arabinofuranoside, were used for testing the activity of feruloyl esterases (Figure 2.11). The coupling of the action of feruloyl esterase with α -L-arabinofuranosidase converts the two 4-nitrophenyl feruloyl- α -L-arabinofuranosides into chromogenic substrates for feruloyl esterases. Another assay used the same substrates 4-nitrophenyl-2-O-trans-feruloyl- α -L-arabinofuranoside and 4-nitrophenyl-5-O-trans-feruloyl- α -L-arabinofuranoside to evaluate feruloyl esterase activity by measuring the changes in the absorbance at 340 nm of ferulic acid esters and the reaction products (Biely *et al.*, 2002).

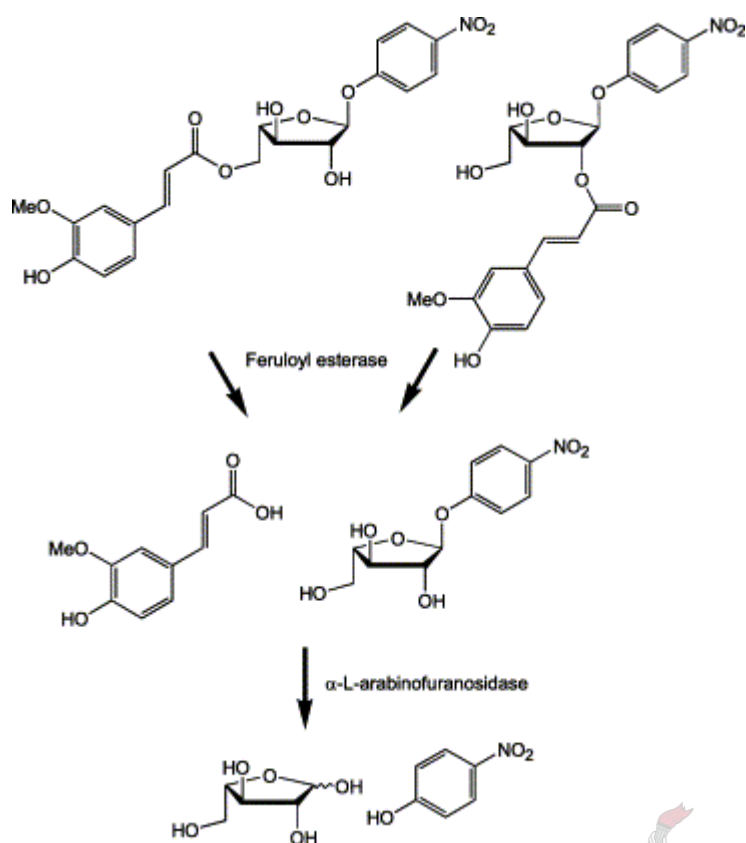


Figure 2.11. The α -L-arabinofuranosidase-coupled assay of feruloyl esterase on two differently feruloylated α -L-arabinofuranosides (Biely *et al.*, 2002).

2.3. MICROORGANISMS PRODUCING ARABINOFURANOSIDASES

2.3.1. Biodegradation of lignocelluloses by microorganisms

Hemicelluloses from forestry and agricultural biomass are a significant renewable resource for bioconversion into chemicals and fuels. The combined action of numerous glycoside hydrolases and esterases produced by plant pathogens and microbial saprophytes is required for the complete xylan degradation into sugar molecules during growth. (Dekker, 1989; Saha, 1998b).

This combined action of these enzymes has a much larger effect than the sum of each of enzyme acting individually. This synergistic effect is a result of substrate specificity of the enzymes and the complex structure of xylan. The main chain of xylan is cleaved internally by *endo*- β -1,4-xylanase into oligosaccharides such as the disaccharide xylobiose. β -D-xylosidase hydrolyses xylobiose into two D-xylose residues. L-Arabinose residues make up monomeric or oligomeric side-chains on the D-galactose or β -1,4-linked xylose backbones in xylans, arabinogalactans, and arabinoxylans. These side-chains limit the enzymatic hydrolysis of hemicelluloses by the *endo*- β -1,4-xylanases

(E.C. 3.2.1.8) which cleave the internal xylosidic bonds of the backbone. This results in oligosaccharides of different lengths. β -Xylosidase (E.C. 3.2.1.37) cleaves single xylose units from the non-reducing ends of unsubstituted xylo-oligosaccharides. Accessory enzymes like α -L-arabinofuranosidases, feruloyl esterases (EC 3.1.1.73), α -glucuronidases (EC 3.2.1.139) and acetyl xylan esterases (EC 3.1.1.72) cleave the side-chains from the backbone. Two enzymes which degrade arabinan are α -L-arabinofuranosidase (EC 3.2.1.55) and endo-1,5- α -arabinanase (EC 3.2.1.99). Acetylxylan esterase hydrolyses acetyl groups from the backbone and α -glucuronidase is an accessory enzyme which hydrolyses the 4-*O*-methyl-glucuronic acid side-chains (Figure 2.12). The majority of these side-chain cleaving accessory enzymes only remove the subunits from the non-reducing ends of the smaller oligosaccharides (Dekker, 1989; Birsan *et al.*, 1998; Koseki *et al.* 2003).

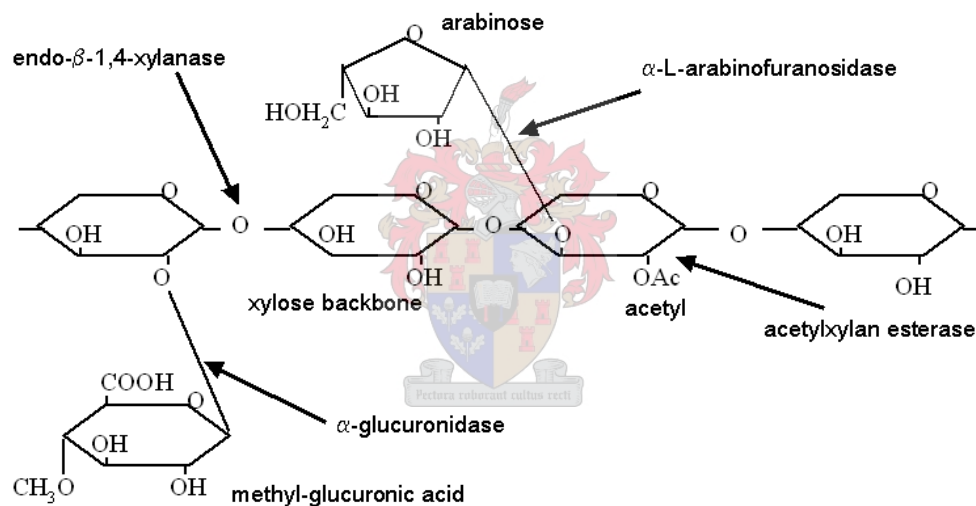


Figure 2.12. The structure of xylan including the enzymes which degrade it. The arrows indicate the cleavage sites where the enzymes act on the structure (Adapted from Saha, 2000).

The hydrolysis of single α -L-arabinofuranose residues from the non-reducing ends of polysaccharides and oligosaccharides which contain arabinose, is catalysed by α -L-arabinofuranosidases (E.C. 3.2.1.55). These are exotype enzymes that hydrolyze terminal non-reducing α -L-arabinofuranosyl residues from L-arabinose containing polysaccharides. This enzyme is part of the complex necessary for complete breakdown of xylans and is important in the biodegradation of hemicelluloses (Biely, 1993).

Growth on lignocellulosic substrates stimulates the microbial production of xylanolytic enzymes. Cellulose and xylan are not able to move through the microbial cell wall and therefore are hydrolyzed in the extracellular culture fluid. After degradation of these polymers by base-level amounts of glycoside hydrolases, the resulting small molecules act as transcriptional inducers. Permeases transport the small molecules across the cell wall and this causes the induction of enzyme systems. The induction of xylanolytic enzymes is repressed by D-glucose (Beg *et al.*, 2001).

The transcriptional control of the xylanolytic enzymes of fungi have been studied. Two examples are discussed below: When grown on cellulose or different sources of xylan, the xylanolytic genes of *Trichoderma reesei* are induced. Different xylans caused different responses in the two *endo*- β -1,4-xylanase genes, *xyn1* and *xyn2*. The small molecules sophorose, arabitol and xylobiose induced the transcription of *endo*- β -1,4-xylanase genes. The *xyn1* gene did not respond to cellobiose but the *xyn2* did. When D-glucose was present there was no expression of the xylanolytic genes, but induction by D-glucose depletion took place in all the xylanolytic genes except *xyn1*. The α -arabinofuranosidase gene *abf1* was induced by the same small molecule inducers as the *endo*- β -1,4-xylanases but was only induced by xylan which was substituted with L-arabinose (Margolles-Clark *et al.*, 1996; La Grange *et al.*, 2000).

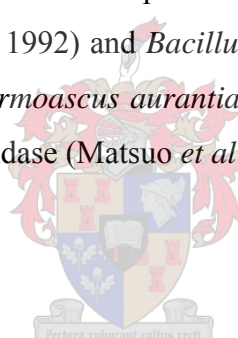
In *Aspergillus niger* the xylanolytic regulator gene *xlnR* is induced by growth on xylose or xylan and causes the induction of the *xlnB*, *xlnC*, and *xlnD* genes encoding xylanolytic enzymes endoxylanases B and C and β -xylosidase, respectively. XlnR was also found to control the transcription of the genes encoding accessory enzymes (α -glucuronidase A, acetylxyylan esterase A, arabinoxyylan arabinofuranohydrolase A, and feruloyl esterase) involved in xylan degradation (van Peij *et al.*, 1998). General inducers of arabinofuranosidase production in fungi include arabitol and arabinose and general repressors are xylose, xylitol and glucose.

The regulation of each xylanolytic gene is most likely controlled by different transcription factors and separate regulation of each xylanolytic gene will permit optimal production of each enzyme when a certain substrate is present.

2.3.2. Microorganisms which produce arabinofuranosidases

Arabinofuranosidases are produced by fungi and bacteria and are located mostly extracellular. These enzymes are secreted to assist in the degradation of plant cell wall material for the production of smaller molecules and sugars used as nutrients for these organisms. The enzymes have been purified and characterized from numerous microorganisms, with the most attention given to the genus *Aspergillus* (Saha, 2000; <http://afmb.cnrs-mrs.fr/CAZY/acc.html>), including strains of *A. kawachii* (Koseki *et al.*, 2003), *A. niger* var. *awamorii*, *A. oryzae* (Koseki *et al.*, 2003) and *A. oryzae* (Hashimoto & Nakata, 2001).

Other fungi which produce arabinofuranosidases include *Aureobasidium pullulans* (Saha *et al.*, 1998b), *Cochliobolus carbonum* (Ransom & Walton, 1997), *Emericella nidulans* (Gielkens *et al.*, 1999), *Hypocrea jecorina* (Margolles-Clark *et al.*, 1996), *Neurospora crassa* (Galagan *et al.*, 2003), *Cytophaga xylanolytica* (Renner & Breznak, 1998) and *Penicillium purpurogenum* (De Ioannes *et al.*, 2000). Arabinofuranosidases have also been purified from many bacterial species including *Streptomyces diastaticus* (Tajana *et al.*, 1992) and *Bacillus polymyxa* (Morales *et al.*, 1995). Of all the microorganisms studied so far, *Thermoascus aurantiacus* and certain *Bacillus* species produce the highest quantities of arabinofuranosidase (Matsuo *et al.*, 2000).



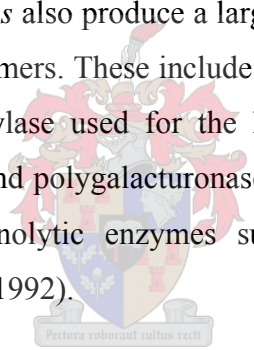
2.3.3. *Aureobasidium pullulans*

A. pullulans is a euscomycetous saprophyte that produces xylan hydrolyzing enzymes and belongs to the family *Dothideales* (see Figure 2.13). It is found on leaves of a wide range of crops in many countries including South Africa, is a plant pathogen causing softening of plant tissue and is associated with tropical fruit and certain crop species. The imperfect stage has a diverse morphology and it shows polymorphism where it can grow as a mycelium or as a budding yeast depending on environmental conditions. *A. pullulans* is most often present in temperate climates and has been isolated from a range of fresh-water and marine sediments and soils (Deshpande *et al.*, 1992).



Figure 2.13. Micrographs of *A. pullulans* growing as a mycelium.
 ([http://www.mycology.adelaide.edu.au/Fungal_Descriptions/Hyphomycetes_\(dematiaceous\)/Aureobasidium/](http://www.mycology.adelaide.edu.au/Fungal_Descriptions/Hyphomycetes_(dematiaceous)/Aureobasidium/))

Certain strains of *A. pullulans* have been used in industry. It produces an exo-polysaccharide slime known as pullulan, which is made from linear chains of α -1,4 and α -1,6-linked maltotetraose and maltotriose units. Pullulan can be used to make a unique thin film which is resistant to oils and impermeable to oxygen (Roukas, 1999). *A. pullulans* has the ability to secrete great amounts of hydrolytic enzymes and has been used in the biodegradation of agricultural lignocellulosic waste (Lee *et al.*, 1999). Strains of *A. pullulans* also produce a large range of enzymes which are involved in the degradation of carbohydrate polymers. These include sucrase, invertase used in production of invert sugar, glucoamylase and α -amylase used for the hydrolysis of starch to D-glucose and maltodextrins, pectin methyl-esterase and polygalacturonase used for fruit pulp maceration and the clarification of fruit juice, and xylanolytic enzymes such as *endo*- β -1,4-xylanase and α -L-arabinofuranosidase (Deshpande *et al.*, 1992).



The colour morphology of *A. pullulans* shows considerable variability. Starting as cream, yellow, light brown or light pink in colour, colonies of *A. pullulans* become black due to chlamydospore production. Colour variants of *A. pullulans* have been recognized as high producers of xylanase, amylases, and β -glucosidase (Saha *et al.*, 1998b). The colour variant strain NRRL Y-2311-1 secretes a hundred-fold greater activity of *endo*- β -1,4-xylanase than the normally pigmented variants of the organism (Leathers *et al.*, 1986).

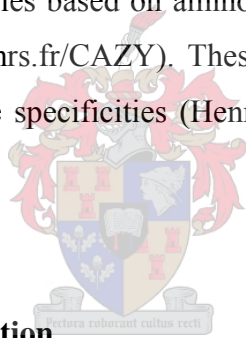
The production of xylanolytic accessory enzymes like β -xylosidase, α -arabinofuranosidase and acetyl esterase from *A. pullulans* NRRL Y2311 in response to growth on xylan and D-xylose has been studied. Another colour variant strain of *A. pullulans*, NRRL Y-12974, produced α -L-arabinofuranosidase when grown in liquid culture on L-arabinose, wheat arabinoxylan, sugar beet arabinan, L-arabitol, oat spelt xylan, xylitol, xylose, arabinogalactan or maize fibre. Lower enzyme activity was found in cultures grown on arabinogalactan and maize fibre whereas greater enzyme activity was found when xylose, wheat arabinoxylan, oat spelt xylan, sugar beet arabinan and

xylitol was used as carbon sources to support α -L-arabinofuranosidase production. L-Arabinose and L-arabitol gave the highest extracellular production of α -L-arabinofuranosidase by *A. pullulans* (Myburgh *et al.*, 1991a; Saha *et al.*, 1998b).

2.4. CLASSIFICATION OF ARABINOFURANOSIDASES

2.4.1. Classification system of glycoside hydrolases

The glycosidic bond between two sugars is cleaved by glycoside hydrolases such as hemicellulases and cellulases. The glycoside hydrolases are classified according to substrate specificity and primary amino acid sequence. Evolutionary relatedness of structural domains in glycoside hydrolases can also be compared using such a classification system. Glycoside hydrolases have been classified into more than 90 families based on amino acid sequence similarity (Carbohydrate Active enZYmes server at afmb.cnrs-mrs.fr/CAZY). These enzymes have considerable structural diversity and a wide range of substrate specificities (Henrissat & Davies, 1997; Miyanaga *et al.*, 2004b).



2.4.2. Arabinofuranosidase classification

Arabinofuranosidases (EC. 3.2.1.55) are exo-type enzymes and have been placed into four glycoside hydrolase families (43, 51, 54 and 62) based on amino acid sequence similarity (<http://afmb.cnrs-mrs.fr/CAZY/>). Arabinofuranosidases can also be classified according to substrate specificity (Pitson *et al.*, 1996).

There are two reaction mechanisms of glycoside hydrolases. The one mechanism causes inversion of the anomeric configuration from substrate to the product and the other results in retention of the anomeric configuration in the product. Catalysis for these two mechanisms is dependent on a pair of carboxylate side-chains (see Figure 2.14). Transglycosylation activity is possible in the retaining glycoside hydrolases, while the inverting glycoside hydrolases are not able to perform transglycosylation reactions (McCarter & Withers, 1994).

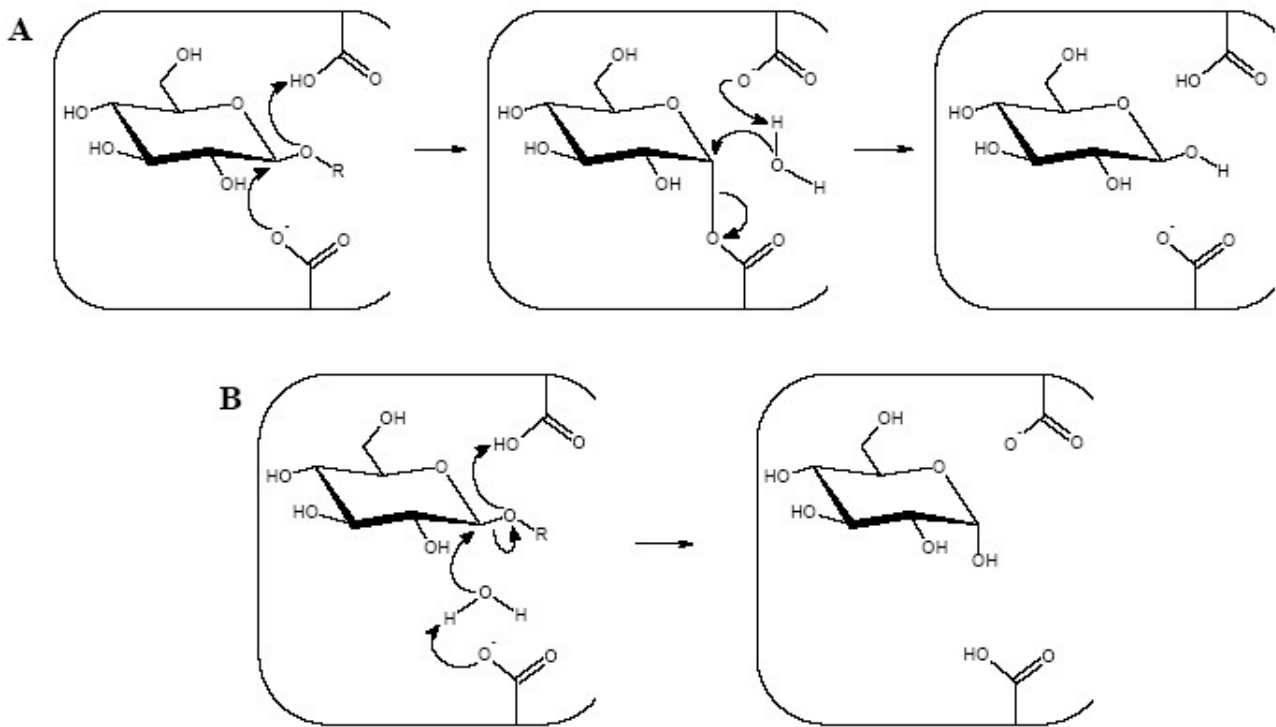


Figure 2.14. Diagram of the (A) retaining and (B) inverting reaction mechanisms of glycoside hydrolases. (A) demonstrates the action of nucleophilic carboxylate on the anomeric carbon, where the transfer of a proton from the general acid to the glycosidic oxygen occurs. Water is activated by the general base and it then acts on the anomeric carbon and the transfer of the bond electrons back to the nucleophile occurs. (B) The catalytic base activates water and acts directly on the anomeric carbon, then the transfer of the bond electrons to the glycosidic bond oxygen and the transfer of a proton from the general acid to the glycosidic oxygen occurs. (McCarter and Withers, 1994)

2.4.3. Family 43 arabinofuranosidases



Family 43 enzymes follow an inverting single-displacement mechanism with two conserved carboxylic acids, a general base and a general acid and these enzymes are found only in bacteria (Figure 2.15). They have bifunctional α -arabinofuranosidase, xylanase, arabinanase and β -xylosidase activity and have molecular weights ranging from 50 to 70 kDa (<http://afmb.cnrs-mrs.fr/CAZY>). The three-dimensional crystal structure of the α -L-arabinanase (Arb43A) from *Cellvibrio japonicus* has been solved and it consists of a 5-fold β -propeller fold and is grouped in the clan-F glycoside hydrolases (Nurizzo *et al.*, 2002).

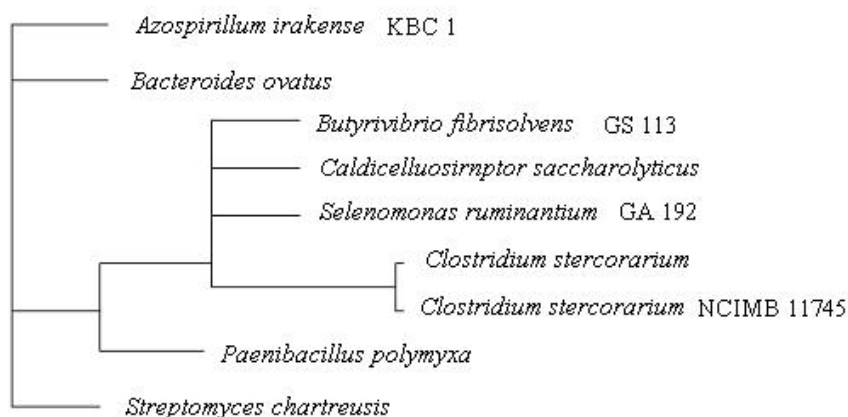


Figure 2.15. Phylogenetic tree of the arabinofuranosidases in glycoside hydrolase family 43 (adapted from ncbi.com).

2.4.4. Family 51 arabinofuranosidases

Family 51 enzymes follow a retaining mechanism and are α -arabinofuranosidases and endoglucanases and are found in bacteria, plants and fungi (Figure 2.16). Bacterial α -arabinofuranosidases in this family have a preference for α -1,2- and α -1,3-linked L-arabinose side-chains above α -1,5-linked side-chains and can hydrolyse *p*-nitrophenyl α -L-arabinofuranoside. These enzymes are tetrameric consisting of monomeric molecular weights ranging from 57 to 72 kDa (Schwarz *et al.*, 1995; Beylot *et al.*, 2001). The three-dimensional crystal structure of the arabinofuranosidase (AbfA) from *Geobacillus stearothermophilus* T-6 has been solved and it consists of a $(\beta/\alpha)_8$ inferred fold and has been grouped in the clan-A glycoside hydrolases (Hoewel *et al.*, 2003).

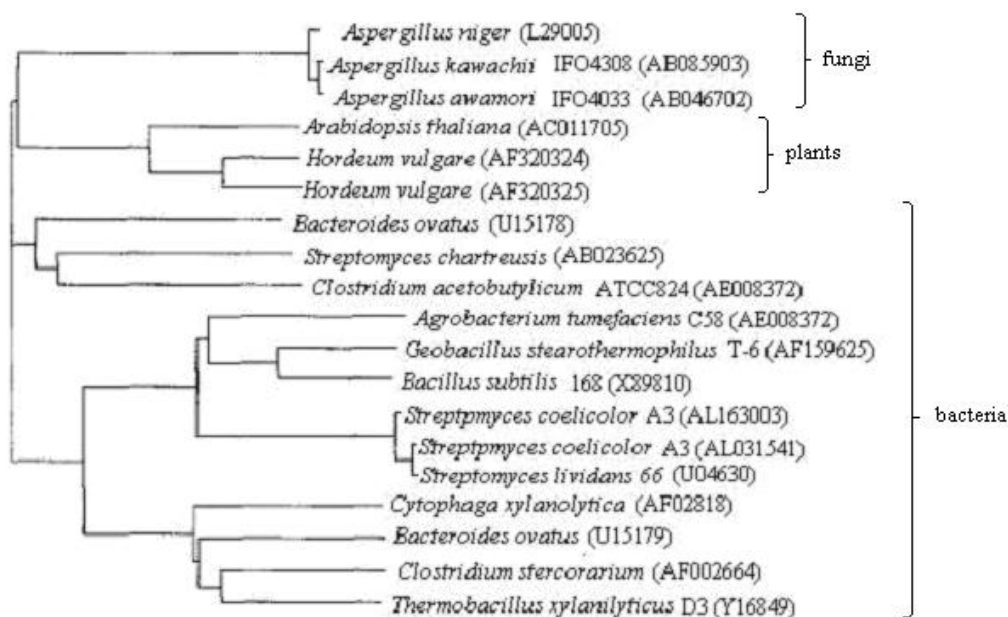


Figure 2.16. Phylogenetic tree of the arabinofuranosidases in glycoside hydrolase family 51. Nucleotide sequence data accession number is in parentheses (Koseki *et al.*, 2003).

2.4.5. Family 54 arabinofuranosidases

Family 54 enzymes are found only in fungi to date and follow a retaining mechanism (Figure 2.17). They consist of monomeric enzymes that have molecular weights of about 50 kDa and acidic isoelectric points around pH 5. They have hydrolytic activity for the α -1,2 or α -1,3-linked and to a lesser degree α -1,5-linked, non-reducing, terminal arabinosyl residues of arabinoxylan, arabinan and arabinogalactan. These enzymes do not hydrolyze internal α -arabinosyl linkages and do not act on double arabinosylated xylopyranosyl residues. They have highly conserved primary sequences, substrate specificity and regulation of expression and are able to hydrolyse the synthetic aryl glycoside *p*-nitrophenyl- α -L-arabinofuranoside (Saha, 2000). The three-dimensional crystal structure of the arabinofuranosidase (AkAbfB) from *Aspergillus kawachii* IFO4308 has been solved and it consists of a β -sandwich fold and has been grouped in the clan-B glycoside hydrolases (Miyanaga *et al.*, 2004a).

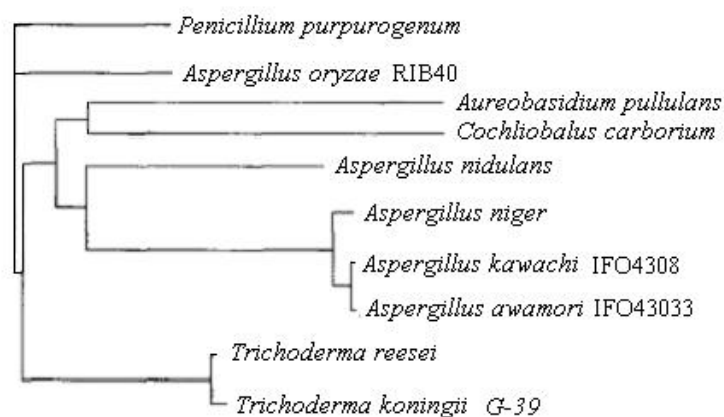
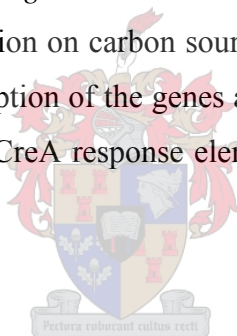


Figure 2.17. Phylogenetic tree of arabinofuranosidases in glycoside hydrolase family 54 (Adapted from Koseki *et al.*, 2003).

The arabinofuranosidase B from *Aspergillus niger* was the first family 54 α -L-arabinofuranosidase to be purified and characterized (Flippi *et al.*, 1993). The gene *abfA* encoding an arabinofuranosidase from *Aspergillus niger* was cloned and was expressed in *Saccharomyces cerevisiae* (Crous *et al.*, 1996). Cultivation on carbon sources containing L-arabinofuranose and L-arabitol caused induction of the transcription of the genes and enzyme production. The induction of the enzyme was repressed through the CreA response element by the occurrence of glucose in the growth medium (Gielkens *et al.*, 1999).



2.4.6. Family 62 arabinofuranosidases

Family 62 enzymes are found in bacteria and fungi and follow an inverting mechanism and have an exclusive preference for arabinoxylan and its breakdown products (Figure 2.18). They remove α -1,2- and α -1,3-linked L-arabinose residues and can cleave arabinose without prior degradation of arabinoxylan to oligosaccharides. These enzymes cannot act on double arabinosylated xylopyranosyl residues. They have molecular weights ranging from 30 to 70 kDa. The three-dimensional crystal structure of the arabinofuranosidase (AxA) from *Aspergillus niger* has been solved and it has been grouped in the clan-F glycoside hydrolases (Gielkens *et al.*, 1996).

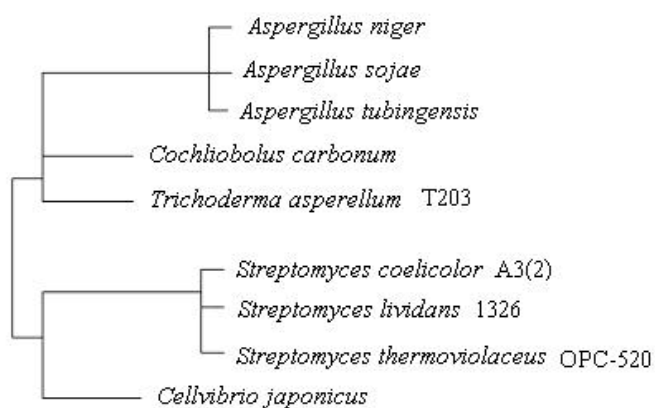


Figure 2.18. Phylogenetic tree of arabinofuranosidases in glycoside hydrolase family 62 (adapted from ncbi.com).

2.5. MOLECULAR AND BIOCHEMICAL DESCRIPTION OF ARABINOFURANOSIDASES

2.5.1. Arabinofuranosidases from bacteria

Numerous bacteria have been found to produce L-arabinofuranosidases which have been placed in families 43, 51 and 62 (Table 2.1).

Table 2.1: Examples of bacterial arabinofuranosidases which have been characterized. (<http://afmb.cnrs-mrs.fr/CAZY> ; Saha, 2000)

Organism	Family	MW (kDa)	Protein
<i>Butyrivibrio fibrisolvens</i> H17c	43	24	Arabinosidase
<i>Cellvibrio japonicus</i>	43	39	ArbA
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168	51	65	arabinofuranosidase 2
<i>Streptomyces lividans</i> 1326	62	47	arabinofuranosidase B (AbfB)

Some bacteria produce more than one arabinofuranosidase. *Streptomyces chartreusis* GS901 secreted α -L-arabinofuranosidases I and II and these had molecular weights of 80 kDa and 37 kDa respectively. Their pI values were 6.6 and 7.5 and both could partially hydrolyse arabinoxylan and arabinogalactan but could not hydrolyse arabinoxylo-oligosaccharides or gum arabic. The α -linkage types that occur between two α -L-arabinofuranosyl residues were all hydrolysed by α -L-arabinofuranosidase I in decreasing order of reactivity for α -1,2 α -1,3 and α -1,5. The 1,3-linkages were cleaved in preference to the 1,5-linkages of the arabinosyl side-chains of methyl 3,5-di-O- α -L-arabinofuranosyl- α -L-arabinofuranoside. About 30% of the arabinan was hydrolysed by α -L-arabinofuranosidase I but only a very low amount of linear arabinan was hydrolysed. α -L-

Arabinofuranosidase II hydrolysed linear 1,5-linked arabinan, but was less effective on the arabinan. The two enzymes converted arabinan and debranched arabinan into arabinose and therefore acted in synergism. After the amino acid sequence of α -L-arabinofuranosidase I was determined, it was classified as a family 51 glycoside hydrolase. α -L-arabinofuranosidase II is an *exo*-1,5- α -L-arabinofuranosidase and was classified into family 43 (Matsuo *et al.*, 2000).

In *Pseudomonas cellulosa*, a single gene (abf51A) encoding an enzyme which had 4-methylumbelliferyl- α -L-arabinofuranosidase activity was isolated. This gene encoded a glycoside hydrolase family 51 arabinofuranosidase which had a molecular weight of 57 kDa. This enzyme preferentially cleaves α -1,2, α -1,3-linked arabinofuranose side-chains from arabinan or arabinoxylan, and hydrolysed α -1,5-linked arabinofuranose side-chains from arabinooligosaccharides at a 300 fold lower rate (Beylot *et al.*, 2001).

Another family 51 enzyme from *S. chartreusis* preferentially cleaved methyl 2-*O*- α -L-arabinofuranosyl- α -L-arabinofuranoside and methyl 3-*O*- α -L-arabinofuranosyl- α -L-arabinofuranoside, compared with methyl 5-*O*- α -L-arabinofuranosyl- α -L-arabinofuranoside. A preference for α -1,2- and α -1,3-linked arabinofuranose units over α -1,5-linked units is a property of family 51 enzymes (Matsuo *et al.*, 2000).

Family 51 enzymes show different substrate specificity. The family 51 enzyme of *S. chartreusis* showed low activity on arabinoxylan compared with arabinan and it could not hydrolyse arabinoxylo-oligosaccharides. The *P. cellulosa* arabinofuranosidase showed higher activity on α -1,5-arabino-oligosaccharides which consisted of three or more sugars than on the disaccharide. The *endo*- α -1,5-arabinanase from *P. cellulosa* produces mostly arabinotriose from linear arabinan, while the arabinofuranosidase from *A. niger* produces arabino-oligosaccharides and large amounts of arabinobiose. (Beylot *et al.*, 2001)

The AbfD3 gene from *Thermobacillus xylanilyticus* D3 encoding a α -L-arabinofuranosidase was expressed in *Escherichia coli* and was found to be stable up to 90°C in the pH range 4-12. It had a molecular weight of 56 kDa and was classified in family 51 of the glycosyl hydrolases after the amino acid sequence was determined. However AbfD3 showed high activity on polysaccharide substrates, unlike most family 51 enzymes. By increasing enzyme concentrations the amount of arabinose hydrolysed from wheat arabinoxylan could not be increased, demonstrating that the residual arabinose was not available to hydrolysis. This could be due to linkage or arabino-

disubstituted xylose might be a limiting factor. This suggestion is likely because only one enzyme known to be able to cleave arabinosyl residues from disubstituted xylose has been isolated so far. The loss of arabinose units decreases the solubility of the substrate, which might also cause a decreased rate of hydrolysis (Debeche *et al.*, 2000).

2.5.2. Arabinofuranosidases from fungi

Fungal arabinofuranosidases generally have a pH optima of between 3.0 and 7.0 and temperature optima of 40-75°C. The molecular weights range between 34 kDa and 500 kDa and some examples of these are listed in Table 2.2.

Table 2.2: Examples of fungal arabinofuranosidases which have been characterized.
(<http://afmb.cnrs-mrs.fr/CAZY> ; Saha, 2000)

Organism	Family	MW (kDa)	Protein
<i>Aspergillus nidulans</i> FGSC A4	43	65	AN1-8
<i>Aspergillus niger</i>	43	60	<i>endo</i> -arabinase A
<i>Cytophaga xylanolytica</i>	51	160	arabinofuranosidase I
<i>Cytophaga xylanolytica</i>	51	240	arabinofuranosidase II
<i>Aspergillus nidulans</i> FGSC A4	54	65	AN1571.2
<i>Aspergillus niger</i> MRC11624	54	67	arabinofuranosidase 2
<i>Aspergillus niger</i>	54	53	arabinofuranosidase B
<i>Cochliobolus carbonum</i>	54	63	ARF2
<i>Penicillium purpurogenum</i>	54	58	Abf
<i>Cochliobolus carbonum</i>	62	63	arabinofuranosidase ARF1
<i>Aspergillus sojae</i>	62	34	arabinofuranosidase (Arf)

The α -L-arabinofuranosidase B encoded by the *abfB* gene in *Aspergillus niger* N400 was the first eukaryotic gene coding for α -L-arabinofuranosidase to be characterized and the protein consists of 499 amino acids (Flippin *et al.*, 1993). The mature α -L-arabinofuranosidase B, lacking the signal sequence, consists of 481 amino acids and has a molecular weight of 50.7 kDa. The arabinofuranosidase (*abfB*) genes of *A. niger* MRC11624 have been cloned and expressed in *Saccharomyces cerevisiae* using a multicopy episomal plasmid. Functional α -L-arabinofuranosidase was secreted by *S. cerevisiae* and showed a 94% similarity to the α -L-arabinofuranosidase B of *A. niger* N400. When grown in SC medium, a maximum enzymatic activity of 0.020 U/ml was

obtained after 48 h and when grown in YPD medium a maximum activity of 1.40 U/ml was measured after 48h (Crous *et al.*, 1996).

The family 54 α -L-arabinofuranosidase of *T. reesei* encoded by the *abf1* gene showed a high similarity to the α -L-arabinofuranosidase B of *A. niger*. This gene coded for a protein of 500 amino acids which included the theoretical N-terminus signal sequence. The *abf1* gene was cloned into *S. cerevisiae* and the expressed enzyme was tested on different substrates. This α -L-arabinofuranosidase released L-arabinose from the synthetic substrate *p*-nitrophenyl- α -L-arabinofuranoside and from natural arabinoxylans. The enzyme also showed trace residual β -xylosidase activity toward *p*-nitrophenyl- β -D-xylopyranoside. The enzyme was characterized and a molecular weight of 53 kDa and pH optimum of 4.0 was obtained (Margolles-Clark *et al.*, 1996; Poutanen, 1998).

The α -L-arabinofuranosidase of *Aspergillus nidulans* was also characterized and an apparent mobility of 65 kDa was observed on SDS-PAGE and the isoelectric point was 3.3. The pH and temperature optima were 4.0 and 65 °C respectively. The DNA sequence of the *A. nidulans abfB* gene showed a high degree of sequence identity with the *A. niger abfB*, *T. koningii abf-1* and *T. reesei* α -L-arabinofuranosidase genes. The expression of the *abfB* gene, the total α -L-arabinofuranosidase activity and arabinofuranosidase levels were determined in a wild-type and mutant strains of *A. nidulans*. Results indicated that the expression of the *A. nidulans abfB* gene was increased during growth in media with an acidic pH and that arabinofuranosidase is the main enzyme involved in hydrolyzing *p*-nitrophenyl α -L-arabinofuranoside (Ramon *et al.*, 1993; Gielkens *et al.*, 1999).

α -L-arabinofuranosidases from *Aspergillus kawachii* have been purified and characterized. These two enzymes, together with xylanase, degraded arabinoxylan in synergism. These two different genes encoding α -L-arabinofuranosidase were named *AkabfA* and *AkabjB*, and were also cloned and characterized, together with two α -L-arabinofuranosidases from *Aspergillus awamori*, named *AwabfA* and *AwabjZ3*. The two enzymes from *A. kawachii* were acidophilic, had a pH optimum of 4.0 and were stable between pH 3.0 and 7.0. The properties of these enzymes were similar to those of *A. awamori* and the difference between the sequences of *AwabfA* and *AkabfA* was one nucleotide. This led to an amino acid difference in the sequence, and as a result, the enzymes were assigned as family 51 glycoside hydrolases. There was a difference of two nucleotides in the sequences of *AkabjB* and *AwabjB* and this resulted in one amino acid difference. These two enzymes were

assigned as family 54 glycoside hydrolases. The genes encoding α -L-arabinofuranosidases (*abfA* and *abfI*) in *A. niger*, *A. kawachii* and *A. awamori* were compared and the two genes from *A. awamori* and *A. kawachii* were more closely related than those between *A. niger* (Koseki *et al.* 2003).

Aspergillus awamori IFO 4033 secreted α -L-arabinofuranosidases I and II and these had molecular weights of 81 kDa and 62 kDa respectively. Both enzymes had an optimum pH of 4.0, were stable at pH 3-7 values and had pIs of 3.3 and 3.6, respectively. They had an optimum temperature of 60 °C and were able hydrolyze *p*-nitrophenyl- α -L-arabinofuranoside, *O*- α -L-arabinofuranosyl-1,3-*O*- β -D-xylopyranosyl-1,4-D-xylopyranose, and arabinose-containing polysaccharides. These enzymes could not hydrolyze *O*- β -D-xylopyranosyl-1,2-*O*- α -L-arabinofuranosyl-1,3-*O*- β -D-xylopyranosyl-1,4-*O*- β -D-xylopyranosyl-1,4-D-xylopyranose. In addition, α -L-arabinofuranosidase I could also hydrolyse arabinose from *O*- β -D-xylopyranosyl-1,4-[*O*- α -L-arabinofuranosyl-1,3]-*O*- β -D-xylopyranosyl-1,4-D-xylopyranose. α -L-Arabinofuranosidase I hydrolyzed the linkages present between two α -L-arabinofuranosyl residues in decreasing order of reactivity for 1,5-, 1,3-, 1,2-linkage. The order for α -L-arabinofuranosidase II was 1,5-, 1,2-, 1,3-linkage. α -L-arabinofuranosidase II hydrolyzed the arabinosyl side-chain linkage of arabinan while α -L-arabinofuranosidase I cleaved arabinose from the non-reducing ends of arabinan (Kaneko *et al.*, 1998).

Penicillium purpurogenum secreted arabinofuranosidase and the maximum level of enzyme obtained was 1.0 U/ml when grown on L-arabitol. When grown on sugar beet pulp and oat spelts, lower yields of enzyme were obtained. One of the three arabinofuranosidases produced by *P. purpurogenum* was purified and characterised and was found to be highly specific for α -L-arabinofuranosides and cleaved arabinose from arabinoxylan. The substrate specificity was also tested on a range of nitrophenyl derivatives (*p*NP- α -L-arabinopyranoside, *p*NP- β -D-mannopyranoside, *p*NP- β -D-xylopyranoside, *p*NP- α -D-mannopyranoside, *p*NP- β -D-glucopyranoside, *p*NP- α -D-galactopyranoside, and *p*NP- β -D-galactopyranoside). All these synthetic substrates showed less than 2% of the activity of that found for *p*-nitrophenyl- α -L-arabinofuranoside, which indicated a high specificity for the α -L-arabinofuranosyl linkage. The enzyme was a monomer with molecular weight of 58 kDa and a pI of 6.5. Its optimum temperature was 50°C, the optimal pH was 4.0 and the enzyme displayed hyperbolic kinetics with a *K*_m of 1.23 mM when tested on *p*-nitrophenyl- α -L-arabinofuranoside. This enzyme was classified into the

family 54 glycosyl hydrolases due to a N-terminal sequence which showed 70% identity to that of other arabinofuranosidases belonging to family 54 (De Ioannes *et al.*, 2000).

2.5.3. Arabinofuranosidases from *Aureobasidium pullulans*

The α -L-arabinofuranosidase of a thermophilic strain of *A. pullulans* was purified to homogeneity and was found to be a homodimer with an apparent mobility of 210 kDa. The temperature optimum was 75 °C and a pH optimum of between 4 and 4.5 was determined (Saha *et al.*, 1998). The α -L-arabinofuranosidase of *A. pullulans* strain NRRL Y-2311-1 has only been characterized in terms of temperature and pH properties in crude extracts. Maximum activity was observed at 60 °C and the enzyme was stable over 3 h at the same temperature, but was inactivated at higher temperatures. The pH optimum was 5.0 but the enzyme was most stable at 60° C (Myburgh *et al.*, 1991b).

2.6. ARABINOFURANOSIDASE STRUCTURE ANALYSIS

2.6.1. Comparative homology modelling

There has been a recent increase in the number of genomes being sequenced and therefore many amino acid sequences of proteins are available (Grunenfelder & Winzeler, 2002). The functions of these proteins now can be investigated and the determination of the three-dimensional structures will provide valuable information concerning their function. Three-dimensional crystal structures are solved using experimental techniques like nuclear magnetic resonance spectroscopy, X-ray crystallography and electron microscopy. Most proteins are too large for nuclear magnetic resonance spectroscopy, are complicated to crystallize or are insoluble under the conditions required by these techniques. Alternative techniques have been developed where no experimentally determined structure can be acquired. The most popular technique is comparative homology modelling which creates a model of a protein, based on the three-dimensional crystal structures of closely related proteins (Sali & Blundell, 1993).

Proteins with closely related amino acid sequences often have similar three-dimensional structures. Using the amino acid sequence of the target protein and the experimentally determined three-dimensional structures of proteins with closely related sequences, a prediction of the target structure

can be determined. This method helps assign function to proteins using existing structures and therefore also increases the value of genome sequencing. Naturally these comparative homology models will be less accurate than those derived experimentally but they still provide important hypothetical data. There is a large variation in the accuracy of comparative homology models. Some models are comparable to three-dimensional crystal structures determined by low resolution X-ray crystallography but some models would only have a correct fold compared to the crystal structure (Baker & Sali, 2001).

2.6.2. The construction of a three-dimensional homology model

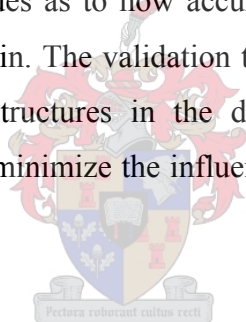
Comparative homology modelling predicts the structure of a target protein, for which an amino acid sequence is available but an experimentally determined three-dimensional structure is not available. Other than the amino acid sequence of the target protein, the three-dimensional crystal structures of proteins with closely related (approximately more than 60%) amino acid sequences (templates) are required. The accuracy of comparative homology modelling relies on the alignment of the amino acid sequences of the target and templates. Homology modelling computer programs use two methods, COMPOSER (Sutcliffe *et al.*, 1987) and SWISSMODEL (Guex & Peitsch, 1997) use fragment based steps and MODELLER (Sali & Blundell, 1993; <http://salilab.org/modeller>) uses a single step method. These programs model the amino acid sequence of the target protein in the most likely conformation based on the three-dimensional structure of a template protein (Kirton *et al.*, 2002).

2.6.3 Model accuracy and quality

Direct comparison between an experimentally derived crystal structure and a model is not possible, since homology modelling is used to predict the three-dimensional structure of a protein when the experimentally derived structure is not obtainable. Many errors can occur during homology modelling such as incorrect alignment and fold assignment, side chain packing and distortions of core segments of folds. The most damaging errors involve incorrect alignment of the amino acid sequences, since these often occur and have a large negative influence on the model's accuracy. There are no homology model building programs which can recover from input alignment errors and because of their frequency, they result in the single foremost limitation in homology modelling (Venclovas *et al.*, 2001).

Certain independent structural validation tests are used to evaluate the model's quality, testing properties such as the root mean square deviation. This test measures the root mean square deviation between main chain atoms in the model and the template with the highest homology to the target protein. There is a relationship between the root mean square deviation and the percentage sequence identity of the main chain atoms in homologous sections of any two proteins. Template proteins used to produce the model of the target protein are directly compared to the results from validation tests to check for errors. Achieving similar values between the templates and the models for these validation tests demonstrates a valid model (Venclovas *et al.*, 1997).

Modelling programs do provide a wide range of validation tests, which detect certain errors in the homology models. Programs such as COMPOSER, DeepView (Guex *et al.*, 1999 <http://www.expasy.org/spdbv>) and MODELLER compare a model's properties with many crystal structures taken from the protein data base (www.rcsb.org/pdb ; Berman *et al.*, 2000). These programs unfortunately cannot give clues as to how accurately a homology model represents the true crystal structure of the target protein. The validation tests do however indicate if a model has related properties to known crystal structures in the data base (Sippl, 1993). Programs like MODELLER use functions which can minimize the influence of an incorrect input alignment (Sali & Blundell, 1993).



2.6.4. Docking: Associations with ligands

Small molecule ligands bind to protein's active sites in numerous biological processes. This binding mode of a ligand with its receptor protein can be accurately predicted using automated molecular docking (Taylor *et al.*, 2002). Most docking programs use an estimate of the binding affinity of the ligand to the receptor protein for the energy function. Automated docking methods with energy functions are used to predict the most energetically correct orientations of ligands in the interior space of the active site of proteins. The first step in the method is to generate many possible ligand-receptor protein orientations which are then evaluated using an energy function (Graaf *et al.*, 2005). The most likely binding mode of the ligand is the one with the most energetically correct value. As a reference, the three-dimensional structure of a ligand is docked into the active site of the receptor protein with an experimentally determined three-dimensional structure. The accuracy of docking ligands into homology models can be evaluated by comparing the predicted binding modes and

affinities with these experimentally determined structures. Automated docking methods use two properties when predicting the most energetically correct ligand–receptor orientations. These are docking algorithms, which predict the binding orientation, and scoring functions, which predict the binding free energy. These two properties are used together to generate different orientations and to determine the tightness of ligand–receptor interactions. Many programs are available which specialize in automated docking, including GOLD, AutoDock, DOCK and FlexX (Bissantz *et al.*, 2002; Paul *et al.*, 2002)

2.6.5. Experimentally solved arabinofuranosidase structures

A small number of arabinofuranosidases have had their three-dimensional crystal structures solved using experimental techniques. The arabinofuranosidase structures listed in the protein database (www.rcsb.org/pdb ; Berman *et al.*, 2000) are discussed below.

The only family 43 glycoside hydrolase to have its crystal structure solved is the xylanase D / α -L-arabinofuranosidase (XynD) from *Paenibacillus polymyxa*. The three-dimensional structure of the carbohydrate binding module (CBM36) domain of this protein was determined at a resolution of 0.8 Å. Experimental techniques such as isothermal titration calorimetry, affinity electrophoresis and UV difference spectroscopy indicated that this binding domain is novel and is dependent on Ca^{2+} ions when binding xylan. Conformational changes in the free three-dimensional structure of CBM36 were compared with the xylotriose- and Ca^{2+} - complex structure which was determined at a resolution of 1.5 Å. The conformational changes provide molecular evidence of the novel Ca^{2+} -dependent binding method of xylooligosaccharides to the CBM36 (Jamal-Talabani *et al.*, 2004).

The structure of the family 51 glycoside hydrolase from *Geobacillus stearothermophilus* T-6 has been determined using X-ray diffraction. This α -L-arabinofuranosidase consists of 6 monomers, each arranged into two domains. A β sandwich consisting of 12 strands with a jelly-roll topology and a β/α 8 barrel structure. The structure of a transient covalent reaction enzyme intermediate was determined. The structure of arabinofuranosidase in association with a natural substrate (arabinose- α -1,3-xylanase) and a synthetic substrate (*p*-nitrophenyl-arabinofuranoside) was also determined. These structures can be used for testing the properties of the catalytic mechanism, because they represent two stable states in the double displacement mechanism. It was found that a complex of hydrogen bonds bound the arabinofuranose sugar to the enzyme and caused distortion of the sugar (Hövell *et al.*, 2003).

The first family 54 glycoside hydrolase crystal structure to be solved was α -L-arabinofuranosidase (AkAbfB) from *Aspergillus kawachii* IFO4308 using X-ray diffraction. The free enzyme and the arabinose-complex forms were solved by Miyanaga *et al.* (2004a). AkAbfB consists of a C-terminal arabinose-binding domain which can bind two arabinofuranose molecules and a N-terminal catalytic domain which can bind one arabinofuranose molecule. The arabinose-binding domain was found to have certain properties similar to carbohydrate-binding module family 13, such as a β -trefoil fold. The catalytic domain has properties similar to clan-B glycoside hydrolases including a β -sandwich fold (Figure 2.19). The signal peptide was 17 residues and there was two potential *N*-glycosylation sites found. This enzyme displayed many properties which are conserved in all family 54 enzymes, including three disulphide bonds in the catalytic domain, one disulphide bond in the arabinose-binding domain and certain important residues involved in catalysis and substrate binding.

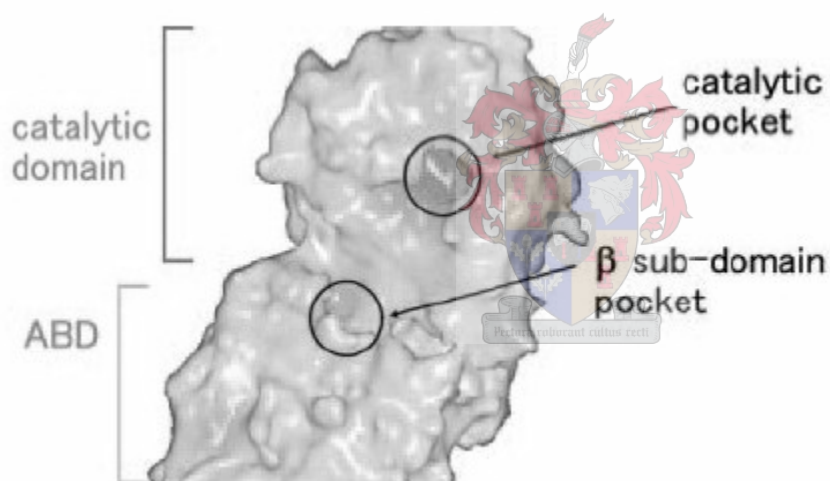


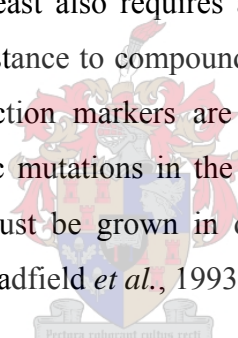
Figure 2.19. Molecular surface of AkabfB indicating the catalytic and arabinose-binding domains (Miyanaga *et al.*, 2004a).

2.7. CLONING AND EXPRESSION OF ARABINOFURANOSIDASE IN *S. CEREVISIAE*

Saccharomyces cerevisiae is a eukaryotic microorganism like *Aureobasidium pullulans* and therefore has many advantages over *E. coli* as an expression host. Eukaryotic cells have a polypeptide processing pathway involving the endoplasmic reticulum and Golgi apparatus which is not present in prokaryotes. Proteins that have a recognisable signal sequence are secreted by *S. cerevisiae* but if the native signal sequence is not recognized, it can be replaced by one of the

identified secretion signals which function in yeast. *S. cerevisiae* secretes a low amount of proteins into the culture medium, which is an advantage when producing secreted heterologous proteins because the purification of the heterologous protein is simplified. Under controlled conditions *S. cerevisiae* can be grown to a high cell density which results in improved production of heterologous proteins. It also has GRAS (generally regarded as safe) status and can be cultivated on low-cost media (Romanos *et al.*, 1992). A disadvantage is that *S. cerevisiae* tends to glycosylate proteins although glycosylation has not been found to hinder the usual functioning of heterologously produced enzymes. Shuttle vectors are usually used to transform in yeast. Promoters can be inducible, constitutive or modulated by physiological conditions, like the *ADH2* promoter which is induced by the glucose depletion and the adjustment from aerobic to fermentative metabolism (Hadfield *et al.*, 1993).

If the codon bias of the native organism is different than that of *S. cerevisiae*, the rare tRNAs are exhausted and this results in a lower production of the heterologous protein (Romanos *et al.*, 1992). Maintenance of episomal vectors in yeast also requires a selectable marker. Dominant selection markers consist of genes encoding resistance to compounds like antibiotics and permit selection in rich medium. The most popular selection markers are auxotrophic, which contain genes that complement corresponding auxotrophic mutations in the yeast, such as *URA3*, *LEU2*, *TRP1* and *HIS3*. The auxotrophic yeast strain must be grown in defined medium lacking uracil, leucine, tryptophan, or histidine, respectively (Hadfield *et al.*, 1993).



Xylanolytic glycoside hydrolases from prokaryotic and eukaryotic origin have been expressed with success in *S. cerevisiae*. The family 11 *endo*- β -1,4-xylanases have been frequently expressed in yeast. Examples include: *Aspergillus kawachii xynC*, *Aspergillus nidulans xlnA* and *xlnB*, *Trichoderma reesei xyn2* and *Bacillus pumilus xynA* (Perez-Gonzalez *et al.*, 1996; la Grange *et al.*, 2000). The single family 10 *endo*- β -1,4-xylanase that has been expressed in *S. cerevisiae* is the *XLN* gene from *Cryptococcus albidus* (Moreau *et al.*, 1992).

The α -arabinofuranosidase from *Aspergillus niger*, *abfB*, was expressed in *S. cerevisiae* commercial wine strain T73 under control of the constitutive actin promoter (Sanchez-Torres *et al.*, 1996) and in laboratory strain Y294 under control of the constitutive *PGK1* promoter (Crous *et al.*, 1996). The biochemical properties of the heterologously produced enzyme were similar to the native protein in these reports. Because many xylanolytic glycoside hydrolases have been cloned into and secreted from *S. cerevisiae* it indicates that it is a suitable host for heterologous production. The gene from *A. pullulans* encoding *endo*- β -1,4-xylanase was successfully expressed in *S. cerevisiae*, which

indicated that xylanolytic genes from this euascomycetous fungus should be able to be expressed in yeast (Li & Ljungdahl, 1996).

2.8. BIOTECHNOLOGICAL APPLICATION OF ARABINOFURANOSIDASES

2.8.1 Industrial importance of plant cell wall components as substrates

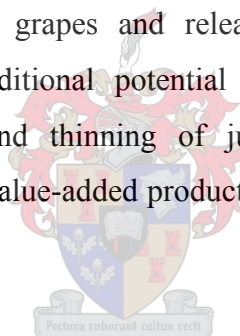
In the world the most abundant source of renewable carbon is plant cell wall material. Approximately 1000 billion tons of these polymers are produced yearly, with an energy content corresponding to 640 billion tons of oil. Plant cell wall material is an important substrate in the energy, detergent, human food, animal feed, pulp and paper and textile industry. The availability and price of petroleum based products are uncertain and the enzymatic degradation and modification of these materials can be used to generate biofuels (Dekker, 1989).

One of the major hemicelluloses found in plant cell walls is xylan, which contributes 10-35 % w/w of the biomass. The enzymatic degradation of xylan is significant in numerous industrial processes and in the carbon cycle in nature. The hydrolysis of xylan using enzymes has many advantages over using chemical procedures, such as better specificity in the hydrolysis of certain chemical bonds and a lower production of toxic waste. The enzymes which hydrolyse xylan are used in the pre-digestion of animal feeds, bleaching of paper pulp, the handling of lignocellulosic wastes from food and agricultural industry and the enhancement of the consistency of bread dough (Timmel, 1967; Beg *et al.*, 2001).

Hemicellulose, such as wheat arabinoxylan, is degraded to monosaccharides by enzymatic or acidic hydrolysis. Enzymatic hydrolysis results in a lower production of products that inhibit the subsequent microbial fermentation. The conversion of wheat endosperm starch to glucose is catalyzed by enzymes during the industrial production of bioethanol for potable spirits. Enhanced utilization of wheat hemicellulose in ethanol fermentation is dependent on the hydrolysis of arabinoxylan. Substitutions on the xylan backbone of arabinoxylan hinder the enzymatic degradation by xylanases. The enzymatic hydrolysis of water soluble wheat arabinoxylan is dependent on synergy between *endo*-1,4- β -xylanases, α -L-arabinofuranosidases and β -xylosidase (Sørensen *et al.*, 2003).

2.8.2. Industrial importance of arabinofuranosidases

Lately, α -L-arabinofuranosidases have received a great deal of attention because of their practical applications in a range of agroindustrial processes. In industry, α -L-arabinofuranosidases are used for aromatizing musts, wines and fruit juices. They interact with *endo*-xylanases to facilitate the degradation of arabinoxylans in grasses, increase the digestibility of animal feed and can be used in the delignification of pulp (Saha *et al.*, 1998b). The arabinofuranosidase from *B. stearothermophilus* was used to treat softwood kraft pulp and led to a 2.3% lignin release. In the delignification process, this enzyme acted in synergism with a thermophilic xylanase in the delignification process and resulted in a 19.2% release of lignin (Beldman *et al.*, 1993). Certain monoterpenes of grape cultivars affect the flavour of wine and they are found in must as aroma precursors such as terpenes. Enzymatic hydrolysis can release glycosidically bound terpenes. The arabinofuranosidase from *A. niger* was active against monoterpenyl α -L-arabinofuranosylglucosides present in grapes and released monoterpenyl β -D-glucosides and arabinose (Gunata *et al.*, 1990). Additional potential applications of arabinofuranosidase in biotechnology include clarification and thinning of juices, bioconversion of lignocellulosic materials to fermentable products and value-added products such as ethanol (Saha *et al.*, 2000; Beg *et al.*, 2001).



3. MATERIALS AND METHODS

3.1. STRAINS

3.1.1. *AUREOBASIDIUM PULLULANS*

Wild type strain *A. pullulans* NRRL Y2311-1 was kindly provided by the National Center for Agricultural Utilization Research, Peoria, Illinois, USA.

3.1.2. *SACCHAROMYCES CEREVISIAE*

Three strains of *S. cerevisiae* Y294 (α *leu2-3,112 ura3-52 his3 trp1-289*) were used and are described in the thesis of de Wet (2003): *S. cerevisiae* Y294 [pDLG1-APabfA] (an auxotrophic strain), *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] (an autoselective strain) and *S. cerevisiae* Y294 [pDLG1] (a strain containing the vector without an insert). The *abfA* of *A. pullulans* was cloned and expressed in *S. cerevisiae* as described by de Wet (2003). Details are provided in Appendix A.



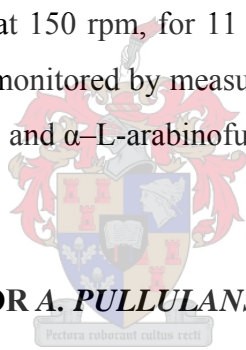
3.2. GROWTH CONDITIONS

3.2.1. GROWTH CONDITIONS FOR *S. CEREVISIAE*, HETEROLOGOUS EXPRESSION AND RECOVERY OF α -L-ARABINOFURANOSIDASE

One litre of synthetic complete (SC) medium (containing, per litre, 6.7 g yeast nitrogen base lacking amino acids and ammonium sulphate [Difco], 20 g glucose, 5 g ammonium sulphate and 1.5 g amino acid mixture lacking uracil [adenine 2.5 g, L-tryptophan 2.4 g, L-histidine 1.2 g, L-arginine-HCl 1.2 g, L-methionine 1.2 g, L-tyrosine 1.8 g, L-leucine 3.6 g, L-lysine-HCl 1.8 g, L-phenylalanine 3 g, L-threonine 12 g, proline 2.5 g, asparagine 2.5 g, isoleucine 2.5 g, aspartic acid 6 g, glutamic acid 6 g, serine 22.5 g, valine 9 g]) was inoculated with *S. cerevisiae* Y294 [pDLG1-APabfA] and incubated at 26°C for 8 days with shaking at 150 rpm. The plasmid control

strain containing the vector without insert was separately inoculated in the same way. Cultures were inoculated to an absorbance of 0.010 and growth was monitored by measuring the absorbance at 600 nm. Samples of supernatant were taken every 24 hours and α -L-arabinofuranosidase activity was assayed. Each reaction mixture contained 50 μ l culture supernatant, 25 μ l acetate buffer (50 mM, pH 4) and 25 μ l *p*-nitrophenyl- α -L-arabinofuranoside (5 mM) and was incubated at 40°C for 30 min. Cell associated protein was released by vortexing cell pellets with 2 μ m glass beads after centrifugation. The cell pellets were vortexed five times for a minute and incubated on ice for a minute inbetween. After centrifugation, the supernatant was resuspended in 50 mM acetate buffer (pH 4) equal to the volume of the original sample and this was assayed for α -L-arabinofuranosidase activity. *S. cerevisiae* Y294 [pDLG1] was grown using the same method.

The autoselective strain, *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA], was grown in YPD medium (containing per litre, 20 g glucose, 20 g peptone and 10 g yeast extract, adjusted to pH 5) and incubated at 26°C, with shaking at 150 rpm, for 11 days. This culture was inoculated at an absorbance of 0.010 and growth was monitored by measuring absorbance at 600 nm. Samples of supernatant were taken every 24 hours and α -L-arabinofuranosidase activity was assayed.



3.2.2. GROWTH CONDITIONS FOR *A. PULLULANS* ENZYME PRODUCTION

A. pullulans NRRL Y2311-1 was recovered from frozen glycerol stock cultures by growing on YPD medium (containing per litre, 10 g yeast nitrogen base (Difco), 20 g glucose, 20 g peptone (Difco) and 20 g agar) for 3 days at 30°C. A starter culture was grown in YPD medium for 2 days, 20 ml was used to inoculate defined medium. *A. pullulans* was cultured in 1 litre defined medium (containing per litre, 6.7 g yeast nitrogen base (Difco), 10 g birchwood xylan (Roth), 5 g monobasic potassium phosphate and 2 g L-asparagine) for 4 days at 30°C with shaking at 150 rpm for optimal enzyme production. Two samples of supernatant were taken every 12 hours and α -L-arabinofuranosidase activity and protein concentration was assayed. Cell associated protein was released by vortexing cell pellets with glass beads as described above after centrifugation and assayed for α -L-arabinofuranosidase activity.

3.3. PURIFICATION OF THE α -L-ARABINOFURANOSIDASE

EDTA, sodium azide and PMSF (phenyl methyl sulphonyl fluoride) were added to the supernatant to a final concentration of 5 mM, 0.02% and 1 mM respectively and the supernatant was stored at 4°C until purification.

3.3.1. PURIFICATION OF THE HETEROLOGOUS α -L-ARABINOFURANOSIDASE

The *S. cerevisiae* Y294 [pDLG1-APabfA] and *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] supernatants were harvested after 8 days growth by centrifugation (8000 rpm in a Beckman JA14 rotor for 5 min). The supernatant of strain Y294 [pDLG1-APabfA] was concentrated 2000-fold by ultrafiltration using a Minitan cross-flow ultrafiltration device (Millipore), an Amicon ultrafiltration cell (Millipore) and a Amicon Centriplus centrifugal filter device (Millipore) all fitted with 10 kDa membranes. The *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] supernatant was only concentrated 13-fold. Samples of the concentrated supernatants were filtered through a 0.22 μ m membrane filter (GE Infrastructure) before purification.

The heterologous α -L-arabinofuranosidase was purified by single step gel filtration on a Superdex 75 HR 10/30 column (300 x 100 cm) connected to an AKTA Purifier chromatography system (Amersham Pharmacia Biotech). The sample was eluted in 0.5 ml fractions with 50 mM acetate buffer pH 4 and 400 mM NaCl with a flow rate of 0.5 ml/min. The presence of the protein in each fraction was determined by assaying for α -L-arabinofuranosidase activity and by SDS-PAGE.

Hydrophobic interaction chromatography was attempted using the HiTrap 1 ml Phenyl FF (highsub) column (Amersham Pharmacia biotech) on the AKTA purifier system. A pI of 4.97 was calculated from the amino acid sequence of α -L-arabinofuranosidase (de Wet, 2003), therefore a buffer at pH 5 was used. The 300 μ l concentrated supernatant sample was eluted in 1.0 ml fractions using a salt gradient consisting of 100 mM acetate buffer at pH 5 with 1 M ammonium sulphate and 100 mM acetate buffer at pH 5 with no salt. A flow rate of 0.5 ml/min was used and the presence of α -L-arabinofuranosidase in each fraction was determined by assaying under standard conditions.

3.3.2. PURIFICATION OF THE NATIVE α -L-ARABINOFURANOSIDASE

The *A. pullulans* NRRL Y2311-1 was cultured as described above and the supernatant was harvested after 4 days by centrifugation (8000 rpm in a Beckman JA14 rotor for 5 min). The supernatant was concentrated 63-fold by ultrafiltration using an Amicon ultrafiltration cell (Millipore) fitted with a 10 kDa membrane. Samples of the concentrated supernatant were filtered through a 0.22 μ m membrane filter (GE Infrastructure) before purification.

A series of steps involving combinations of gel filtration and anion exchange chromatography were followed to purify of α -L-arabinofuranosidase:

Step 1: Purification was attempted using gel filtration following the same method as used for the heterologous enzyme as described above. The presence of the protein in each fraction was determined by assaying for α -L-arabinofuranosidase activity and by SDS-PAGE. The fraction from gel filtration which contained the highest activity, was run again on the same column and the resulting fraction which contained the highest activity showed partially pure α -L-arabinofuranosidase on SDS-PAGE.

Step 2: Anion exchange chromatography was also attempted using the slightly basic Resource Q 1 ml column (Amersham Pharmacia Biotech) on the AKTA purifier system. The α -L-arabinofuranosidase has a pI of 4.97 and at pH 8 it is negatively charged. The 300 μ l concentrated supernatant sample was eluted in 1.0 ml fractions using a buffer gradient consisting of 50 mM phosphate pH 8 and 50 mM phosphate pH 8 with 50 mM NaCl. A flow rate of 0.5 ml/min was used and the presence of α -L-arabinofuranosidase in each fraction was determined by assaying for activity and by SDS-PAGE.

Step 3: The sample containing the highest activity from the second round of gel filtration was subsequently run on the anion column to acquire a higher degree of purification.

Step 4: The sample containing the highest activity from the anion exchange column was subsequently run on the gel filtration column to acquire a higher degree of purification.

Step 5: The sample containing the highest activity from the first round of gel filtration was subsequently run on the anion column to acquire a higher degree of purification.

3.4. CHARACTERISATION OF THE NATIVE AND HETEROLOGOUS α -L-ARABINOFURANOSIDASES

3.4.1. TEMPERATURE AND pH DEPENDENCE AND STABILITY

Pure heterologous α -L-arabinofuranosidase was characterized in terms of pH and temperature dependence, and pH and temperature stability and kinetic constants using *p*-nitrophenyl- α -L-arabinofuranoside as substrate (Sigma). The temperature dependence of α -L-arabinofuranosidase activity was determined by assaying the activity at different temperatures ranging from 30 to 80°C for 5 min. The pH dependence of α -L-arabinofuranosidase activity was determined by assaying at 40°C over a pH range between 2.2 and 6. McIlvaine's buffer (0.1 M citrate acid and 0.2 M dibasic sodium phosphate buffer mixed in different ratios) was used during the 10 min assay. The temperature stability over 3 h was determined by incubating the enzyme at 50, 55 and 60°C. At regular time intervals samples were assayed. The short-term pH stability over 3 h was tested using a pH range between 2.2 and 6. Assays were performed at 55°C in McIlvaine's buffer. All experiments were repeated twice and the average values were used.

The activity of partially purified native α -L-arabinofuranosidase was characterized in terms of pH and temperature dependence. The temperature dependence was determined by assaying from 30 to 80°C for 30 min. The pH dependence was determined by assaying at 40°C over a pH range between 2.2 and 6 with McIlvaine's buffer for 30 min. Both experiments were repeated twice.

3.4.2. KINETIC ANALYSIS

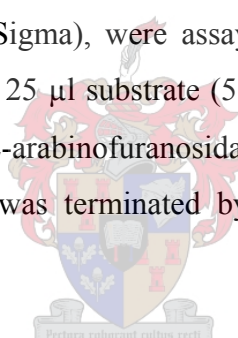
The substrate concentration dependence of the purified heterologous α -L-arabinofuranosidase was determined by assaying with a range of 0.2 to 30 mM *p*-nitrophenyl- α -L-arabinofuranoside. Two repeat assays were performed at 55°C for 5 min and the results were plotted as a hyperbolic graph using Sigma Plot (SPSS Inc.). The K_m and V_{max} were determined using the Lineweaver-Burk and Hanes plots. The k_{cat} and specificity constant were also calculated.

The substrate concentration dependence of the partially purified native α -L-arabinofuranosidase was determined by assaying with a range of 0.5 to 15 mM *p*-nitrophenyl- α -L-arabinofuranoside. Two repeat assays were performed at 55°C for 5 min and the K_m , V_{max} , k_{cat} and specificity constant were calculated.

3.4.3. SUBSTRATE SPECIFICITY

Purified heterologous α -L-arabinofuranosidase was assayed under standard conditions with chromogenic substrates, arabino-oligosaccharides and polysaccharides containing arabinose. Reaction products were assayed either by spectrophotometry or by High-pH anion-exchange chromatography (HPAEC).

Five chromogenic substrates, *p*-nitrophenyl- β -D-mannopyranoside, *p*-nitrophenyl- β -D-xylopyranoside, *o*-nitrophenyl- β -D-galactopyranoside, *p*-nitrophenyl- α -L-arabinofuranoside and *p*-nitrophenyl- β -D-glucopyranoside (Sigma), were assayed in 100 μ l reactions for four repeat experiments. Each reaction contained 25 μ l substrate (5 mM), 25 μ l acetate buffer (50 mM, pH 4), 20 μ l purified heterologous α -L-arabinofuranosidase (0.01 U/ml) and 30 μ l H₂O. Each reaction was run for 120 min and was terminated by addition of 100 μ l saturated sodium tetraborate.



The activity of the purified heterologous α -L-arabinofuranosidase on four arabino-oligosaccharides (1,5- α -L-arabinobiose, 1,5- α -L-arabinotriose, 1,5- α -L-arabinotetraose and 1,5- α -L-arabinopentaose, Megazyme) was tested for 120 min. Each reaction contained 25 μ l substrate (2 mM), 25 μ l acetate buffer (50 mM, pH 4), 20 μ l purified α -L-arabinofuranosidase (0.043 U/ml) and 30 μ l H₂O. Four repeats of these reactions were assayed at 40°C and the reaction was terminated by boiling. Additionally the 1,5- α -L-arabinotriose was assayed over 24 h at 40°C in duplicate.

All samples analysed by HPAEC (Dionex) on a PA-100 column (Dionex) were diluted to 1 ml in MilliQ H₂O. Standards were set by running arabinose (Merck), arabinobiose (Megazyme), arabinotriose (Megazyme), arabinotetraose (Megazyme) and xylose (Fluka Chemie) on the column to determine the retention time for each sugar. The peak area for each sugar was used to calculate the concentration using standard curves.

Three arabinoxylans were assayed under standard conditions for 0, 30, 60 min and 24 h. The substrates wheat arabinoxylan (low viscosity, arabinose:xylose = 41:59, 37% arabinose, Megazyme), corn fibre (30% arabinose, kindly provided by Dr Shin Li, NRRL, Peoria, IL, USA) and oat spelt xylan (10% arabinose, Sigma, X-0627) were prepared in 1% w/v solution. The arabinoxylan samples were solubilized by dissolving 0.1 g substrate in 0.8 ml ethanol (95%) followed by the addition of 9 ml distilled water and stirred at 100°C for 10 min. This was allowed to cool and the volume was finally adjusted to back to 10 ml. Each type of arabinoxylan was assayed in 100 µl samples and this was repeated at least in duplicate for the four time points. Each reaction contained 25 µl substrate (5 mM), 25 µl acetate buffer (50 mM, pH 4), 15 µl purified heterologous α -L-arabinofuranosidase (0.043 U/ml) and 35 µl distilled water. These were incubated at 40°C and the reaction was terminated by boiling. Each sample was diluted to 1 ml with distilled water and analyzed by HPAEC.

Larch wood arabinogalactan (arabinose:galactose = 15:85, Megazyme) was prepared at 1% (w/v) concentration in distilled water. This was assayed under standard conditions for 0, 30 and 60 min. Four repeats of each time point was performed with 100 µl samples containing 25 µl substrate (5 mM), 25 µl acetate buffer (50 mM, pH 4), 20 µl purified heterologous α -L-arabinofuranosidase (0.043 U/ml) and 30 µl distilled water. These were incubated at 40°C and the reaction was terminated by boiling. Each sample was diluted to 1 ml with distilled water and analyzed by HPAEC. Additionally this substrate was also assayed over 24 h at 40°C in duplicate.

Debranched arabinan (Ara:Gal:Rha:GalUA = 88:4:2:6, Megazyme) was prepared at 1% (w/v) concentration by dissolving in distilled water at 70°C. This was assayed four times under standard conditions for 0, 30, 60, 90 and 120 min. Samples contained 25 µl substrate (0.25 % w/v), 25 µl acetate buffer (50 mM, pH 4), 20 µl purified α -L-arabinofuranosidase (0.043 U/ml) and 30 µl distilled water. These were incubated at 40°C and the reaction was terminated by boiling. Each sample was diluted to 1 ml with distilled water and analyzed by HPAEC. Additionally this substrate was also assayed over 24 h at 40°C in duplicate.

Three types (R1000, HiA350, LS3000) of residual lignin samples linked to arabinose were tested for arabinose release. These substrates were obtained from kraft type pulps by enzymatic hydrolysis using cellulolytic enzymes (supplied by B. Hortling, Keskuslaboratorio-Centrallaboratorium, Finland). The three types of residual lignins (also known as lignin-carbohydrate complexes) contained similar amounts of arabinose: R1000 0.41%, HiA350 0.36%

and LS3000 0.35% (see Appendix B for more details). These substrates were prepared at 1% w/v by dissolving in 0.1 mM NaOH. The hydrolysis of the substrates were assayed four times under standard conditions for 0, 30, 60, 90 and 120 min. Samples contained 25 μ l substrate (5 mM), 25 μ l acetate buffer (50 mM, pH 4), 20 μ l purified α -L-arabinofuranosidase (0.035 U/ml) and 30 μ l distilled water. These were incubated at 40°C and the reaction was terminated by boiling. Each sample was diluted to 1 ml with distilled water and analyzed by HPAEC. Due to the uncertain composition of these substrates and the possibility of degradation caused by the preparation method, control tests were performed to investigate for arabinose release due to NaOH or incubation at 40°C over 2 h. In the first control experiment, the substrates were suspended in distilled water, vortexed and analyzed by HPAEC. In the second control experiment the substrates were suspended in 0.1 mM NaOH for 2 h at 40°C and analyzed by HPAEC. Finally a zero time reaction was performed to test for arabinose release due to acetate buffer.

Native α -L-arabinofuranosidase was assayed over 24 h at 40°C in duplicate with 1,5- α -L-arabinotriose as substrate. Each reaction contained 5 μ l substrate (1 mM), 25 μ l acetate buffer (50 mM, pH 4), 10 μ l partially purified native α -L-arabinofuranosidase (0.032 U/ml) and 60 μ l H₂O.

3.4.4. SYNERGISTIC ACTION WITH XYLANASE

Five reactions containing 1 % w/v low viscosity wheat arabinoxylan (Megazyme) as substrate with combinations of endo- β -1,4-xylanase (100 U/ml, M4 from *A. niger*, family GH11, Megazyme. EC 3.2.1.136) and heterologous α -L-arabinofuranosidase (0.081 U/ml) were prepared. Each reaction contained 25 μ l substrate (5 mM), 25 μ l acetate buffer (50 mM, pH 4) and varying activities of enzyme, as described below. Each reaction was made up to 100 μ l with distilled water and was incubated at 40°C for 0 min (zero time reaction) and 12 h. The reaction was terminated by boiling. Reaction 1 contained 20 μ l (0.0016 U) α -L-arabinofuranosidase. Reaction 2 contained 5 μ l (0.5 U) xylanase. Reaction 3 contained 5 μ l xylanase, incubated for 12 h, boiled and then 25 μ l α -L-arabinofuranosidase was added (1/5 of the volume) and incubated for a further 12 h. Reaction 4 contained 5 μ l xylanase and 20 μ l α -L-arabinofuranosidase. Reaction 5 contained no enzyme (buffer control). Each reaction was performed in duplicate.

The five zero time and five 12 h reaction samples were diluted to 1 ml in distilled water and analysed by HPAEC on a PA-100 column together with arabinose (0.5 mM) and xylose (1 mM) standards for the determination of retention times for the reaction products. Wheat arabinoxylan (1 % w/v) consists of 37 % arabinose and 61 % xylose. Therefore 0.093 mg potential arabinose was available in 25 μ l substrate for every reaction. The amount of arabinose formed after the 12 h reactions was compared to the initial amount available from the substrate.

The xylanase was also tested for α -L-arabinofuranosidase activity by assaying 5 μ l at the same standard conditions with *p*-nitrophenyl- α -L-arabinofuranoside used for α -L-arabinofuranosidase assays.

3.4.5. EFFECT OF INHIBITORS AND DETERMINATION OF INHIBITION CONSTANTS

Six carbohydrates were included in the standard assay using *p*-nitrophenyl- α -L-arabinofuranoside. Stocks of D-glucose (Kimix), D-xylose (Fluka Biochemika), D-mannose (Nutritional Biochemicals Corporation, Cleveland Ohio), L-arabinose (Merck), D-cellobiose (Sigma) and D-maltose (Saarchem) were prepared in distilled water at 200 mM. Reactions of 100 μ l were prepared containing 15 μ l purified heterologous α -L-arabinofuranosidase (0.01 U/ml), 25 μ l substrate (5 mM), 25 μ l buffer (50 mM acetate pH 4), 25 μ l carbohydrate stock and 10 μ l distilled water. For each carbohydrate reaction, four repeats were performed and a control reaction containing 35 μ l distilled water with no carbohydrate was included. The assays were assayed at 40°C for 30 min. Specific activities were determined for each reaction.

A series of reactions containing the product arabinose, was performed to calculate the inhibition constant. Each 100 μ l reaction contained 15 μ l purified heterologous α -L-arabinofuranosidase (0.01 U/ml) and 25 μ l buffer. Four different product concentrations were used (50, 25, 10, 0 mM) with five different substrate concentrations (5, 4, 3, 2, 1 mM). The volumetric activity of each reaction was calculated and given as a % relative to the control (0 mM product reactions). Primary and secondary Lineweaver-Burk plots were used to calculate the inhibition constant.

3.4.6. EVALUATION OF SUBSTRATE PRETREATMENT ON SUGAR CANE AND BAGASSE ENZYMATIC HYDROLYSIS

Four samples (CLM/140°C, BAG/140°C, Pith/AFEX, CLM/AFEX) of sugarcane and bagasse from Audubon Sugar Institute (Louisiana, USA) were tested. CLM/140°C was sugar cane leaf matter treated with water at 140°C for 1 h. The extract was then removed. BAG/140°C was bagasse treated with water at 140°C for 1 h. Pith/AFEX was sugar cane pith treated with liquid NH₃ at 100°C for 30 min. CLM/AFEX was sugar cane leaf matter treated with NH₃ (2:1) at 100°C for 30 min. Enzyme treated samples contained 0.1 g substrate, 100 µl acetate buffer (200 mM, pH 4), 30 µl purified heterologous α-L-arabinofuranosidase (0.035 U/ml), 1 µl xylanase (210 U/mg, M4 from *A. niger*, family GH11, Megazyme) and 900 µl distilled water. Controls for the four substrates contained no enzymes. Duplicates of the controls and samples were incubated at 40°C for 4 h and the reaction was terminated by boiling. Each sample was filtered using a 0.45 µm acetate filter (GE Infrastructure) and run on the HPAEC (Dionex) with a PA-100 column. Concentrations of arabinose, xylose and xylobiose release were determined from standard curves using L-arabinose (Merck), D-xylose (Fluka Chemie) and 1,4-β-D-xylobiose (Megazyme).

3.4.7. SUBSTRATE-BINDING ASSAY OF HETEROLOGOUS α-L-ARABINOFURANOSIDASE

Four insoluble substrates, wheat arabinoxylan (low viscosity, Megazyme), oat spelt xylan (Sigma X-0627), birchwood xylan (Roth 7500.7) and avicel (crystalline cellulose; Fluka BioChemica 11365) were used for the substrate-binding assay. These were prepared by washing 0.1 g substrate in 50 mM acetate buffer (pH 4) three times. The resultant pellet was suspended in 2 ml buffer to give the concentration of 50 mg/ml.

The substrate-binding assay included 50 µl substrate suspension, five purified heterologous α-L-arabinofuranosidase concentrations (0.027, 0.056, 0.088, 0.095 and 0.135 mU/ml) and distilled water to a final volume of 100 µl. These were stored at 4°C for 20 min and the supernatant was removed after centrifugation for 2 min at 13 000 rpm.

To determine remaining enzyme activity, the supernatant (50 μ l) was assayed with 25 μ l *p*-nitrophenyl- α -L-arabinofuranoside and 25 μ l distilled water for 30 min at 40°C. Initial activity was determined under the same conditions by assaying the same amount of enzyme which was added in the substrate-binding assay.

3.5. ANALYSES

3.5.1. α -L-ARABINOFURANOSIDASE ASSAY

The standard assay method is described below but different modifications of this method were used in the above sections. α -L-arabinofuranosidase activity was assayed using the synthetic substrate *p*-nitrophenyl- α -L-arabinofuranoside (Sigma). Reaction mixtures contained 5 mM *p*-nitrophenyl- α -L-arabinofuranoside, 50 mM acetate buffer pH 4, and an appropriate amount of α -L-arabinofuranosidase in a final volume of 100 μ l. Reactions took place at 40°C and were terminated by the addition of 100 μ l saturated sodium tetraborate. The reaction absorbance at 405 nm was measured in a microtitre plate reader (Anthos 2001, Austria). Enzyme activity was calculated from the slope of a standard plot of absorbance at 405 nm against *p*-nitrophenyl concentration. One unit of α -L-arabinofuranosidase activity is the amount of enzyme which can cleave 1 μ mol *p*-nitrophenol from *p*-nitrophenyl- α -L-arabinofuranoside in one min during standard assay conditions.

3.5.2. PROTEIN DETERMINATION

Protein was determined by direct measurement at 280 nm using a bovine serum albumin standard (Sigma A-8531) (Ausubel *et al.*, 1995).

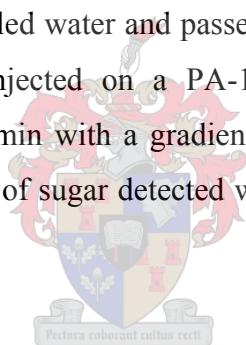
3.5.3. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed by the Tris-glycine discontinuous buffer system using 10% gels. Samples were prepared in loading buffer [250 mM Tris-Cl (pH 6.8), 2 % (m/v) SDS, 20 % (v/v) glycerol, 0.1% (m/v) bromophenol blue and 5% (v/v) β -mercapto ethanol], boiled for 4 min and cooled to 4°C. Rainbow Molecular weight marker

(Pharmacia) with an apparent mobility range of 14.3 to 220 kDa was used and silver staining was used to visualize protein (Blum *et al.*, 1987). SDS-PAGE gels were placed in fixing solution (50% methanol, 12% acetic acid, 0.0185% formaldehyde) for 1 h and washed in 50% (v/v) ethanol twice for 10 min each. Staining of the gels was achieved by treating with 0.002% sodium thiosulphate pentahydrate for 1 min, washed three times for 30 seconds with water, treated in 0.2% silver nitrate solution containing 0.028% formaldehyde for 10 min, and finally washed twice for 20 sec with water. Subsequently the gels were treated in developing solution (6% sodium carbonate, 0.4 mg/l sodium thiosulphate pentahydrate and 0.0185% formaldehyde). The gel was removed from this solution and development was terminated by the addition of a methanol (50%)-acetic acid (12%) solution.

3.5.4. HIGH-pH ANION-EXCHANGE CHROMATOGRAPHY

Samples were diluted to 1 ml in distilled water and passed through a 0.22 µm acetate filter (GE Infrastructure). Each sample was injected on a PA-100 column (Dionex) using HPAEC (Dionex) and allowed to run for 40 min with a gradient of 250 mM NaOH and 1 M sodium acetate buffers. The type and amount of sugar detected was interpreted from the HPAEC scans using standards.



3.5.5. DEGLYCOSYLATION

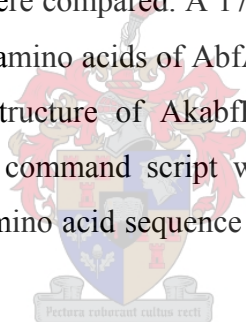
Carbohydrates were cleaved from the α -L-arabinofuranosidase by adding 1 U N-Glycosidase F (recombinant *E. coli*, 1365185, Roche Diagnostics GmbH Mannheim, Germany) in 100 µl 200 mM acetate buffer (pH 5) to 50 µl pure enzyme (50 µg/ml) and incubating for 15 h at 30°C. The reaction was terminated by boiling and run on SDS-PAGE (3.5.3) against the untreated pure enzyme sample.

Carbohydrates were also cleaved from the α -L-arabinofuranosidase using an alternative method. PNGase F, an amidase that cleaves between the asparagine and N-acetylglucosamine residues (New England Biolabs P0704S) was used. A sample of 50 µl pure enzyme (0.07 U/ml) was treated as described by the supplier. The molecular weight of the deglycosylated samples and the untreated purified sample was determined by SDS-PAGE.

3.5.6. MODELLING OF α -L-ARABINOFURANOSIDASE

The arabinofuranosidase (AbfA) from *A. pullulans* expressed in *S. cerevisiae* was sequenced by de Wet (2003). The arabinofuranosidase (AkabfB) from *Aspergillus kawachii* was sequenced by Koseki *et al.* (2003) and the crystal structure was solved by Miyanaga *et al.* (2004a). Both of these arabinofuranosidases are grouped in the family 54 glycosyl hydrolases. These sequences were used for a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) and AkabfB was found to be 71% identical to AbfA.

The protein data base creating program, MODELLER (Sali & Blundell 1993; <http://salilab.org/modeller>), was used to create a homology model of AbfA based on the crystal structure of AkabfB. An alignment text file (consisting of both amino acid sequences) was constructed and conserved residues were compared. A 17 amino acid signal peptide was present in AkabfB which corresponded to 18 amino acids of AbfA but was not included in the sequence which was modelled. The crystal structure of AkabfB was downloaded from the protein databank (www.rcsb.org/pdb) and a command script was written to direct MODELLER to create a homology model using the amino acid sequence of AbfA based on the crystal structure of AkabfB.



The protein data base file (homology model) of AbfA created by MODELLER was opened in DeepView: Swiss-pdb viewer (Guex *et al.*, 1997, GlaxoSmithKline, <http://www.expasy.org/spdbv/>). This program was used to generate images of the homology model using different structural styles and to validate the model using the energy minimisation tool. This energy value was compared to the value obtained from the crystal structure of AkabfB. Because these 2 enzymes are so similar, there was little difference in energy values and therefore no further corrections were made to the homology model. The three active sites on the image of the molecular surface structure were also identified using important residues highlighted in the paper by Miyanaga *et al.* (2004a).

4. RESULTS

4.1. CLONING AND ANALYSIS OF THE *A. PULLULANS* *ABFA* GENE

The *abfA* of *A. pullulans* was cloned by de Wet (2003) into *S. cerevisiae* Y294. This gene was sequenced (deposited at National Center for Biotechnology Information; AY495375) and was classified as belonging to the glycoside hydrolase family 54 according to the Carbohydrate Active enZYmes database found at <http://afmb.cnrs-mrs.fr/CAZY/>. The nucleotide sequence of *A. pullulans abfA* with the deduced α -L-arabinofuranosidase amino acid sequence as determined by de Wet (2003) is shown in Figure 4.1.

Initial analysis of the *abfA* gene by de Wet revealed a putative secretion signal of 20 amino acids, which gave a mature protein with a predicted molecular weight of 49.9 kDa (Figure 4.1). One possible *N*-glycosylation site was found and an iso-electric point of 4.97 was calculated.

Further analysis of the *abfA* gene revealed additional properties after comparing it to the *AkabfB* gene of *A. kawachii* which is also placed in family 54 of the glycosyl hydrolyses. From the homology modelling of *A. pullulans* AbfA based on the crystal structure of AkabfB, many family 54 glycosyl hydrolyse properties were found to be conserved in AbfA (see section 4.9). An 18 amino acid sequence in AbfA aligned with the 17 amino acid signal peptide of AkabfB. However the signal peptide sequence of AbfA was 2 amino acids shorter than the secretion sequence described by de Wet (2003). *A. pullulans* AbfA was also found to consist of an N-terminal catalytic domain (residues 1-317) and a C-terminal arabinose-binding domain (residues 318-442) (see section 4.9).

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-185                                     gatcc
-180 cgccggattacggaaaataacagagcgaggttcgtagcgatgatcttcgctggagatgtg
-120 ctacatccacagctcgaacataaatagagaagacaatgccgcctggctgtccaacatcaa
-60  ctcctctcatalccgcaagcttctctgtcaaccctcctcacagttcgctcatcaactcaaac
  1  ATGCGTTCCAGGACGAACATCGCTCTTGGCCTAGCTGCCACTGGTCCCTAGTCCGCTGCC
  1  M R S R T N I A L G L A A T G S L V A A
  61  GCGCCTTGGGATATCTATCAGAAATGGCGGTACTCCTTGGCTAGCTGCTCACGGCACAACT
  21  A P C D I Y Q N G G T P C V A A H G T T
  121 CGCGCATTGTATGATTCTACACTGGTCTCTCTACCAACTTAAGAGAGGGCTCAGATGGC
  41  R A L Y D S Y T G P L Y Q L K R G S D G
  181 ACTACGACCGATATTTCTCCTTTGCTGCTGGTGGTGTGCCAATGCTGCTGCTCAGGAC
  61  T T T D I S P L S A G G V A N A A A Q D
  241 TCTTTCTGCAAGGGTACTACCTGCTCTATCAGTATTATCTACGATCAGTCTGGGGCTGCA
  81  S F C K G T T C L I S I I Y D Q S G R A
  301 AACCATCTTTATCAGGCCAGAAAGGTGCTTTCAGCGGACCAGATGTCAACGGAACGAC
  101 N H L Y Q A Q K G A F S G P D V N G N D
  361 AACTTGGCAGGCGCTATTGGAGCACCAGTGACTTTGAATGGCAAGAAGGCATATGGCGTG
  121 N L A G A I G A P V T L N G K K A Y G V
  421 TTCATCTCGCCGACTGGGTACAGAAACGACGAAGTCAAGCGGCACGGCCACTGGAAAC
  141 F I S P G T T G Y R N D E V S G T A T G N
  481 GAACCTGAGGGCATGTATGCTGTTCTTGACGGCACTATTACAACGATGCTTGCTGCTTT
  161 E P E G M Y A V L D G T H Y N D A C C F
  541 GACTACGGAAACGCGGAAATCAGCAACACGGATACTGGTAACGGACATATGGAGGCCGTC
  181 D Y G N A E I S N T D T G N G H M E A V
  601 TACTATGGTAACAACAGATTGGGGCAGTGGCTCTGGCAGCGTCCCTGGCTCATGGCC
  201 Y Y G N N T I W G S G S G S G P W L M A
  661 GACCTTGAGAACGGTTTGTCTCTGGCCAGGGTACCAAGCAGAACACTGCAGACCCTTCA
  221 D L E N G L F S G Q G T K Q N T A D P S
  721 ATCTCCAACAGATTCTTACCAGGAAATGGTCAAGGGAGAGCCTAACCAGTGGGCGCTTCGC
  241 I S N R F F T G M V K G E P N Q W A L R
  781 GGTAGCAATGCCGCGTCCGGTCCCTGTTCGACCTACTACAGTGGCGCTCGTCCCACCGTC
  261 G S N A A S G S L S T Y Y S G A R P T V
  841 GCGGTTTACAACCCCATGAGCCTCGAGGGCGCCATTCCTTGGCATCGGTGGCGATAAC
  281 G G Y N P M S L E G A I I L G I G G D N
  901 AGCAATGGCGCTCAGGGCACTTCTATGAGGGGGTCACTGACCTCGGGCTACCCGTCTGAT
  301 S N G A Q G T F Y E G V M T S G Y P S D
  961 GCCACTGAAGCCTCGGTGCAGGCCAACATTGTGGCTGCGAAGTACGCTACCACATCTTTG
  321 A T E A S V Q A N I V A A K Y A T T S L
  1021 AACACAGCACCCTCACTGTCCGCAACAAGATTCGATCAAGGTGACCACCCCGGCTAC
  341 N T A P L T V G N K I S I K V T T P G Y
  1081 GACACCCGCTATCTGGCACACACCGGACCCGTC AACACGCAGGTTGTCTCTTTCATCT
  361 D T R Y L A H T G A T V N T Q V V S S S
  1141 AGCGCGACTAGCCTCAAGCAGCAGGCCAGCTGGACTGTTGCGACAGGCCCTCGGTAACAGC
  381 S A T S L K Q Q A S W T V R T G L G N S
  1201 GGCTGTTACTCTTTCGAGTCCGGTGTACACCTGGAAGCTTCATCAGACACTACAACCTTC
  401 G C Y S F E S V D T P G S F I R H Y N F
  1261 CAGCTCCAGCTCAACGCGAATGACAACACCAAGGCTTTCAAGGAAGACGCGACTTTCTGC
  421 Q L Q L N A N D N T K A F K E D A T F C
  1321 TCTCAGACCGGTCTTGTACCGGCAACACTTCAACTCGTGGAGCTACCCCTGCCAAGTTC
  441 S Q T G L V T G N T F N S W S Y P A K F
  1381 ATCCGCTCACTACAACAATGTTGGATACATCGCCAGCAACGGTGGTGTTCACGACTTTGAC
  461 I R H Y N N V G Y I A S N G G V H D F D
  1441 TCTGCTACAGGCTTCAACAACGATGTCTAGCTTTGTGGTTGGAAGCAGCTTTGCTTAGatg
  481 S A T G F N N D V S F V V G S S F A *
  1501 taaaaggctcaggatgaatatgatggatgtttatgacaaaagaagttatgagtttgtagtt
  1561 atggaatcttagctgtagcttttgaaagcctttgggatatcagatggtttgtctcttgttc
  1621 atgtgccgttgcaaaagaagaaaagaaggagcagcaagcagtgaggctcttatcgggacgat
  1681 agggctagatc

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Figure 4.1. *A. pullulans abfA* nucleotide sequence showing the α -L-arabinofuranosidase amino acid sequence in bold. Non-coding sequence is displayed as small letters and coding sequence in capitals. The N-linked glycosylation consensus sequence is dotted underlined and the putative signal peptide is underlined (de Wet, 2003).

4.2. EXPRESSION OF THE *A. PULLULANS* ABFA GENE IN *S. CEREVISIAE*

Growth of *S. cerevisiae* Y294 [pDLG1-APabfA] in SC medium was monitored over 72 h and compared to the time course of α -L-arabinofuranosidase production (Figure 4.2). Stationary phase took 30 h to reach. The extracellular activity of α -L-arabinofuranosidase reached a plateau after 120 h and remained relatively constant at an enzymatic activity of 0.0135 U/ml. Cell associated α -L-arabinofuranosidase activity was determined and found to be 25-fold less than the activity present in the supernatant.

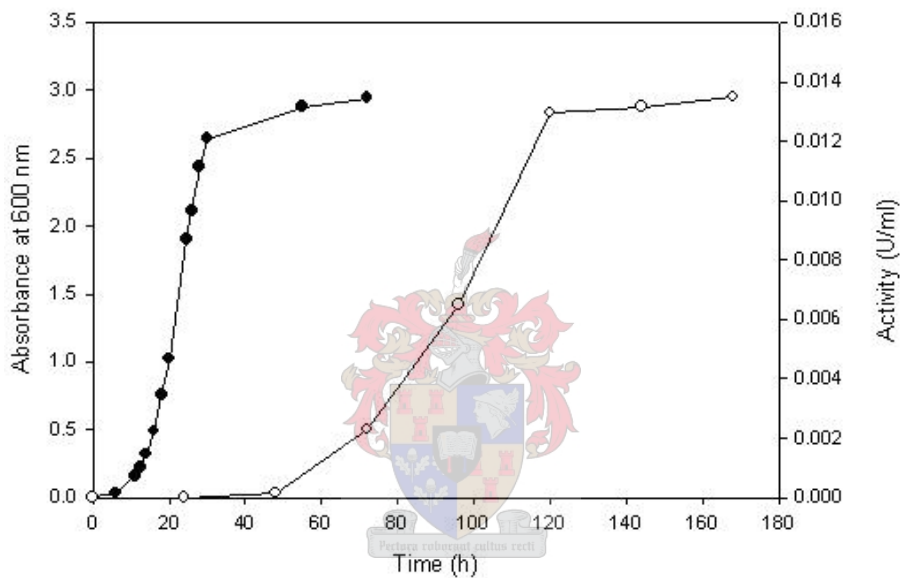


Figure 4.2. Growth curve (●) of the *S. cerevisiae* Y294 [pDLG1-APabfA] culture and volumetric activity (○) of α -L-arabinofuranosidase in the supernatant.

The growth and activity levels of *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] were monitored in YPD medium over 48 h and 260 h respectively (Figure 4.3). The cell density increased up to 9 A_{600nm} at 48 h and extracellular α -L-arabinofuranosidase activity was only first observed at 48 h. Production rate slowed down after 144 h and appeared to level off after 240 h at approximately 0.9 U/ml.

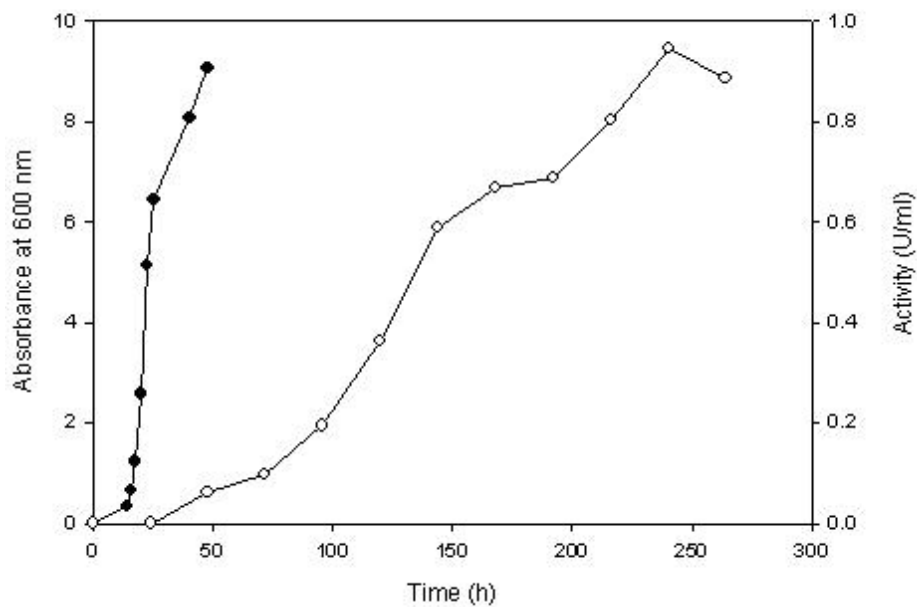


Figure 4.3. Growth curve (●) of the *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] culture and volumetric activity (○) of α -L-arabinofuranosidase in the supernatant.

The concentrated supernatants of *S. cerevisiae* Y294 [pDLG1-APabfA] and the plasmid control strain *S. cerevisiae* Y294 [pDLG1] were run on SDS-PAGE. A single additional band was visible in the lane which contained the supernatant of the α -L-arabinofuranosidase producing strain and had an approximate apparent mobility of 52 kDa.

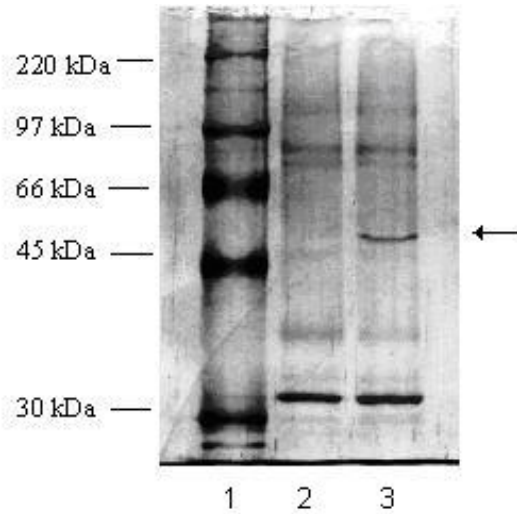


Figure 4.4. SDS-PAGE gel of the concentrated supernatant from *S. cerevisiae* Y294 [pDLG1-APabfA] and the plasmid control strain. Lane 1, apparent mobility standards; lane 2, plasmid control concentrated supernatant; lane 3, *S. cerevisiae* Y294 [pDLG1-APabfA] concentrated supernatant. The arrow points to the α -L-arabinofuranosidase. The standards (kDa) were myosin (220), phosphorylase B (97), bovine serum albumin (66), ovalbumin (45) and carbonic anhydrase (30).



4.3. PURIFICATION OF THE HETEROLOGOUS α -L-ARABINOFURANOSIDASE.

The α -L-arabinofuranosidase was purified from the concentrated supernatant of the *S. cerevisiae* Y294 [pDLG1-APabfA] by gel filtration. The protein elution during gel filtration was monitored at 280 nm and α -L-arabinofuranosidase was present in a single peak at 12 ml (Figure 4.5).

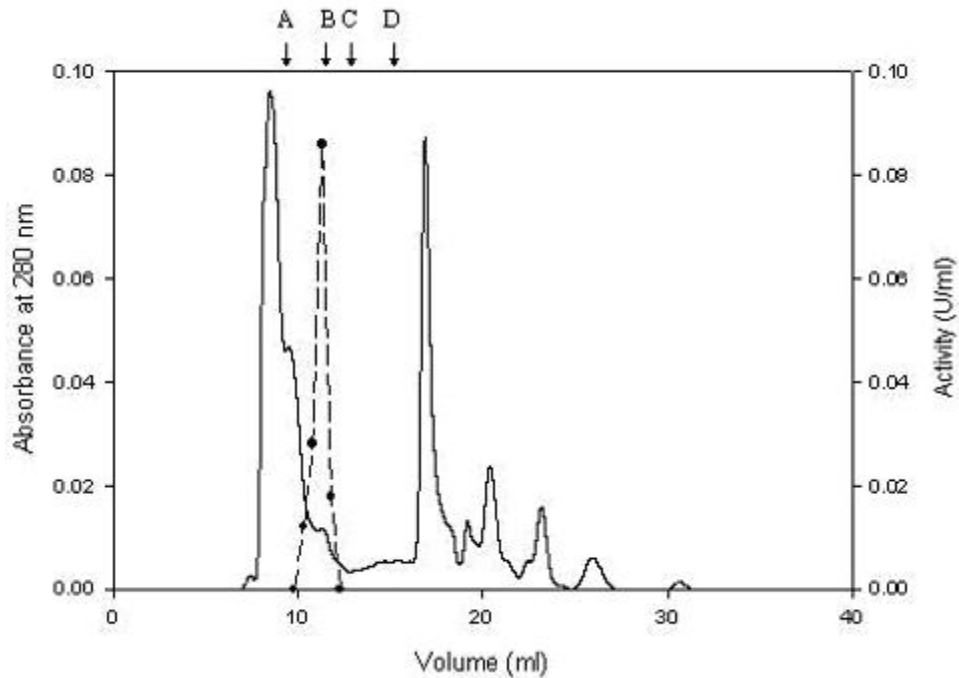


Figure 4.5. Elution profile on gel filtration of the concentrated supernatant from *S. cerevisiae* Y294 [pDLG1-APabfA]. The absorbance at 280 nm is plotted as a solid line and α -L-arabinofuranosidase activity is plotted as a dashed line. The protein standards used to calibrate the column are indicated with arrows at their respective elution volumes. The standards (kDa) were bovine serum albumin 66 (A), carbonic anhydrase 29 (B), cytochrome C 12.4 (C) and aprotin 6.5 (D).

The fraction containing highest activity was found to be electrophoretically pure and used for characterization experiments (Figure 4.6).

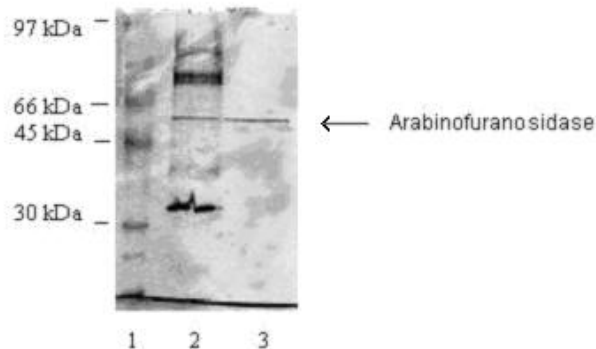


Figure 4.6. SDS-PAGE gel of the concentrated supernatant from *S. cerevisiae* Y294 [pDLG1-APabfA] and the gel filtration fraction containing the highest activity. Lane 1, MW standards; lane 2, *S. cerevisiae* Y294 [pDLG1-APabfA] concentrated supernatant; lane 3, gel filtration fraction containing highest activity. The arrow points to the α -L-arabinofuranosidase.

The heterologous enzyme was purified 7.2-fold with a yield of 30.9 % from the culture supernatant of *S. cerevisiae* Y294 [pDLG1-APabfA] (Table 4.1).

Table 4.1. Purification table comparing the concentrated supernatant to the pure sample.

Purification step	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Fold purification	Yield %
Concentrated supernatant	0.55	1.17	0.47	0	100
Gel filtration	0.17	0.05	3.4	7.2	30.9

The concentrated supernatant from the *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] culture grown on rich medium could not be fully purified by a single gel filtration step. The elution profile differed due to *S. cerevisiae* secreting more extracellular proteins (see Figure 4.7, lane 3) when grown on rich medium. The α -L-arabinofuranosidase eluted at the same fraction volume as the culture grown in defined medium (Figure 4.8).

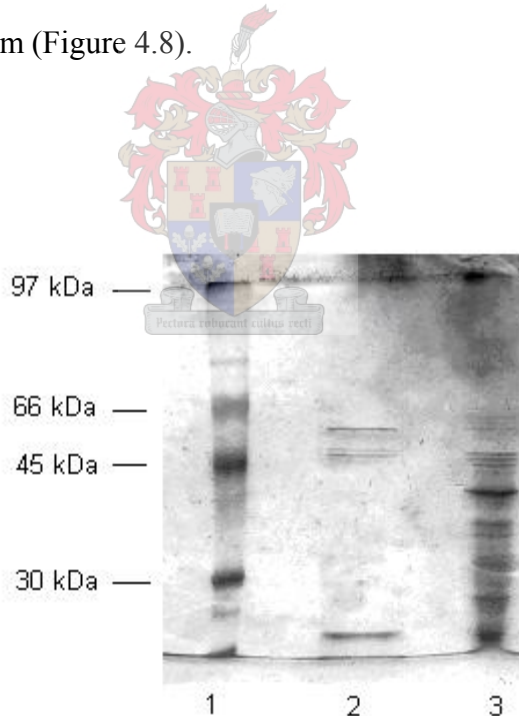


Figure 4.7. SDS-PAGE gel of the concentrated supernatant from *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] and the gel filtration fraction containing the highest activity which eluted at 11.8 ml in Figure 4.5. Lane 1, MW standards; lane 2, gel filtration fraction; lane 3, *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] concentrated supernatant.

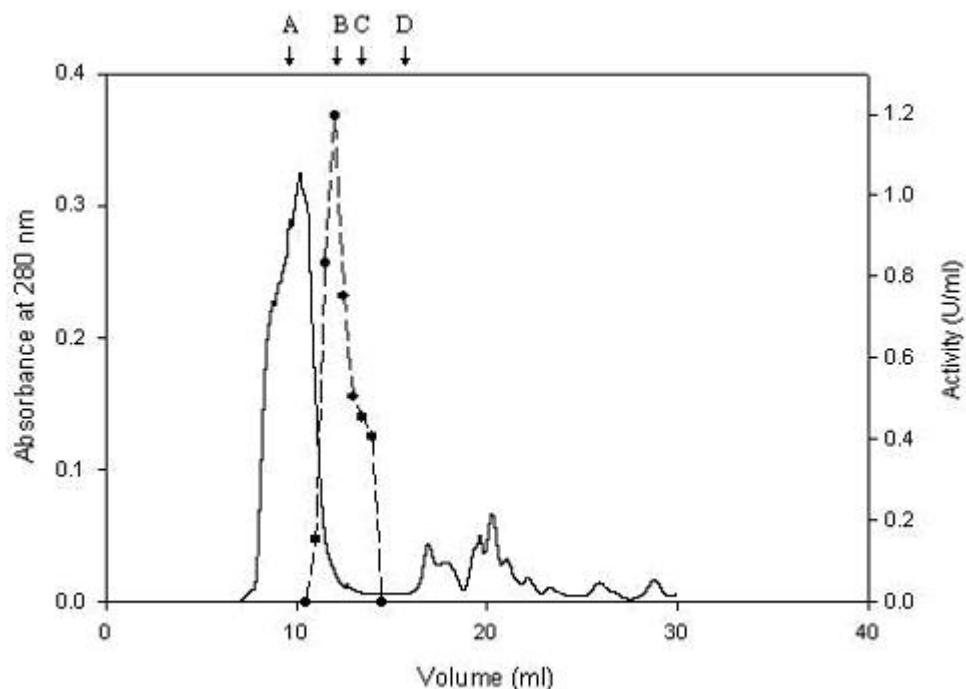


Figure 4.8. Gel filtration elution profile of the concentrated supernatant from *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA]. The elution volume where α -L-arabinofuranosidase activity was present is indicated by a dashed line representing volumetric activity. The protein standards used to calibrate the column are indicated with arrows at their respective elution volumes. The standards (kDa) were bovine serum albumin 66 (A), carbonic anhydrase 29 (B), cytochrome C 12.4 (C) and aprotin 6.5 (D).

The activity of the partially purified enzyme present in the elution fractions in the peak in Figure 4.8 were compared to the *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] concentrated supernatant. The yield was much higher than that of the *S. cerevisiae* Y294 [pDLG1-APabfA] culture, but the eluted fractions did not contain pure enzyme (see Figure 4.7, lane 2). There were at least two contaminating bands, at approximately 45 kDa and 20 kDa. This enzyme preparation was not characterized further.

The α -L-arabinofuranosidase (2.5 U/ml) from the concentrated supernatant of the *S. cerevisiae* Y294 [pDLG1-APabfA] was also run on a hydrophobic interaction column. The protein elution during gel filtration was monitored at 280 nm (Figure 4.9).

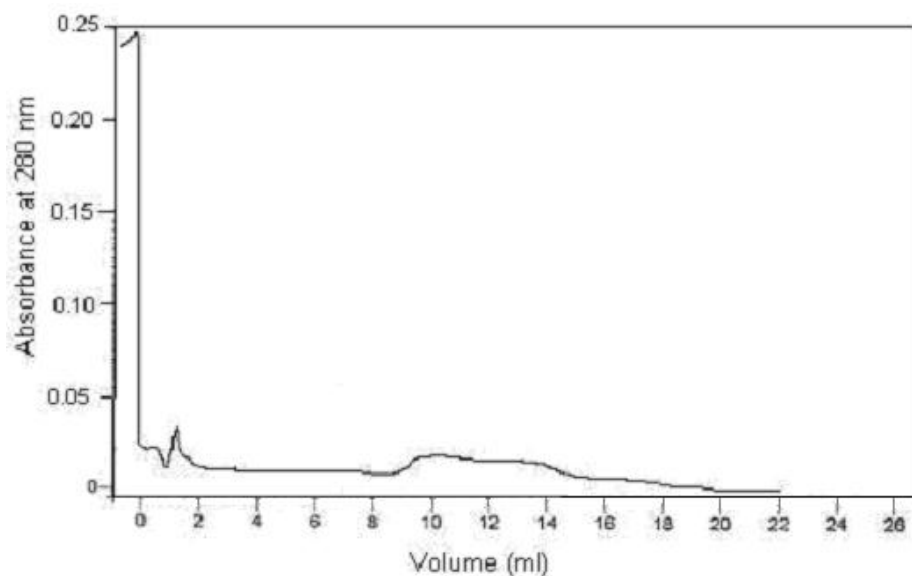


Figure 4.9. Elution profile on the hydrophobic interaction column of the concentrated supernatant of the *S. cerevisiae* Y294 [pDLG1-APabfA].

The elution profile indicated that this method was unsuccessful and therefore no fractions were assayed.



4.4. CHARACTERISATION OF THE HETEROLOGOUS α -L-ARABINOFURANOSIDASE

The fraction containing the highest activity from the *S. cerevisiae* Y294 [pDLG1-APabfA] culture (Figure 4.6, lane 3) was used to characterize α -L-arabinofuranosidase.

4.4.1. Molecular weight estimation by SDS-PAGE. The apparent mobility of the heterologous α -L-arabinofuranosidase was estimated at 52.7 kDa (Figure 4.10).

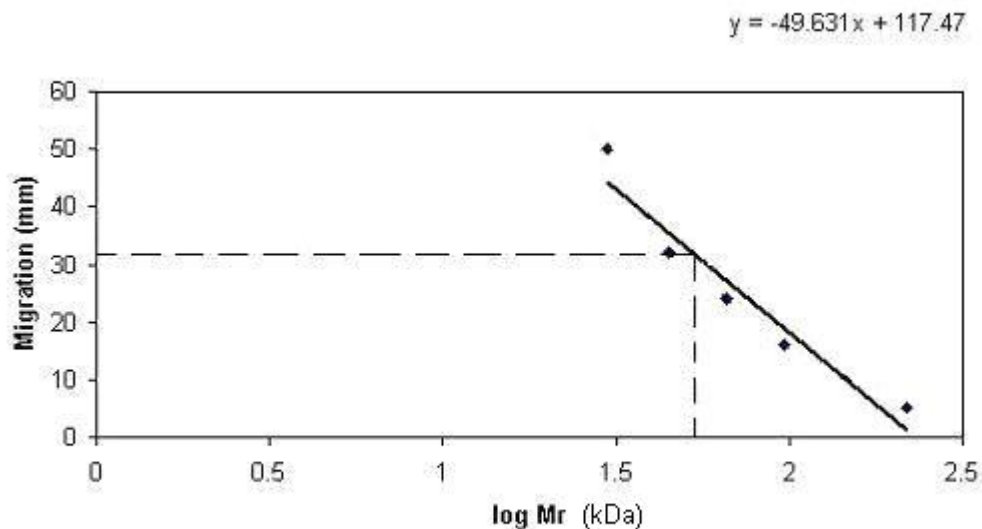


Figure 4.10. The log Mr of the protein markers (Rainbow Molecular weight markers, Pharmacia. 220, 97, 66, 45 and 30 kDa) against distance migrated on the SDS-PAGE gel. The dashed line represents the plotted α -L-arabinofuranosidase.

4.4.2. Molecular weight estimation by gel filtration. The apparent mobility was estimated at 36 kDa by gel filtration (Table 4.2 and Figure 4.11).

Table 4.2. The protein standards run on the gel filtration column. These values were used together with dextran blue (V_0) to construct the standard curve.

protein	Mr	V_e (ml)	V_e/V_0	log Mr
BSA	66000	9.78	1.228643	4.82
Carbonic Anhydrase	29000	11.97	1.503769	4.46
Cytochrome C	12400	13.73	1.724874	4.09
Aprotin	6500	15.85	1.991206	3.81

V_e of dextran blue = 8.0 ml

The V_e of α -L-arabinofuranosidase was 12 ml. Therefore a V_e/V_0 value of 1.5 was calculated and this corresponded with a Mr of 36 kDa.

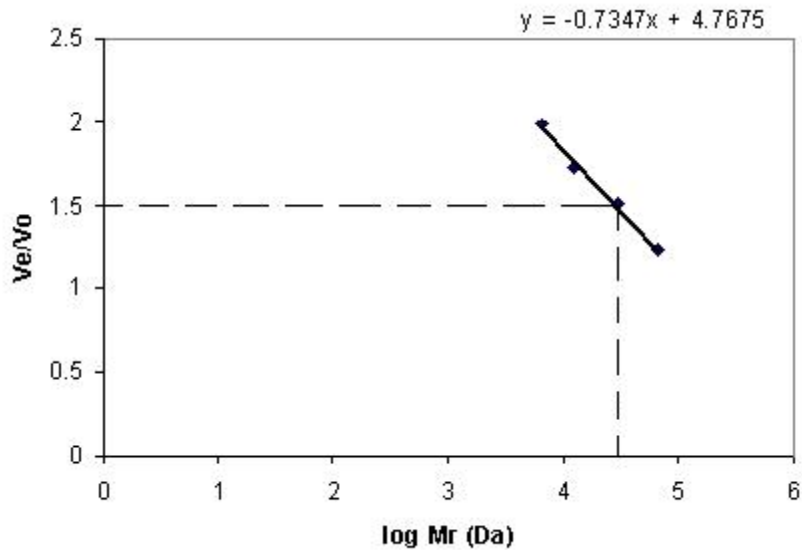


Figure 4.11. The protein standards in Table 4.2 were used to construct a calibration curve to calculate Mr from the elution volume. The dashed line represents the plotted α -L-arabinofuranosidase.

4.4.3. Molecular weight estimation from sequence

The gene encoding the AbfA mature enzyme was also sequenced (Figure 4.1) and the molecular weight of the enzyme was calculated to be 49.9 kDa.

4.4.4. Glycosylation. From the amino acid sequence, only one glycosylation site was found for *abfA* (Figure 4.1, de Wet, 2003). *S. cerevisiae* tends to glycosylate proteins and therefore purified α -L-arabinofuranosidase was treated with N-Glycosidase F which cleaves carbohydrates from proteins. Two gel filtration fractions containing pure samples of α -L-arabinofuranosidase were treated with N-Glycosidase F and run on SDS-PAGE together with the untreated samples but no difference in the α -L-arabinofuranosidase band at approximately 50 kDa could be identified using this method. If the enzyme was partially de-glycosylated there would have been a possible double band at around 50 kDa. The sugar molecules would only account for about 1-2 kDa difference, which could not be detected using this method. An alternative method using a PNGase F kit was followed. Pure samples and PNGase treated samples were also run on SDS-PAGE but no change in mass could be observed

using this method. Both untreated and de-glycosylated samples would need to be further accurately evaluated using mass spectrometry.

4.4.5. Temperature dependence and stability. The purified enzyme showed maximum activity at 55°C in 50 mM acetate at pH 4 (Figure 4.12). The enzyme was stable over 180 min at 40°C, lost half of its activity at 50°C after 60 min and lost almost 90 % activity after 60 min at 60°C at pH 4 (Figure 4.13).

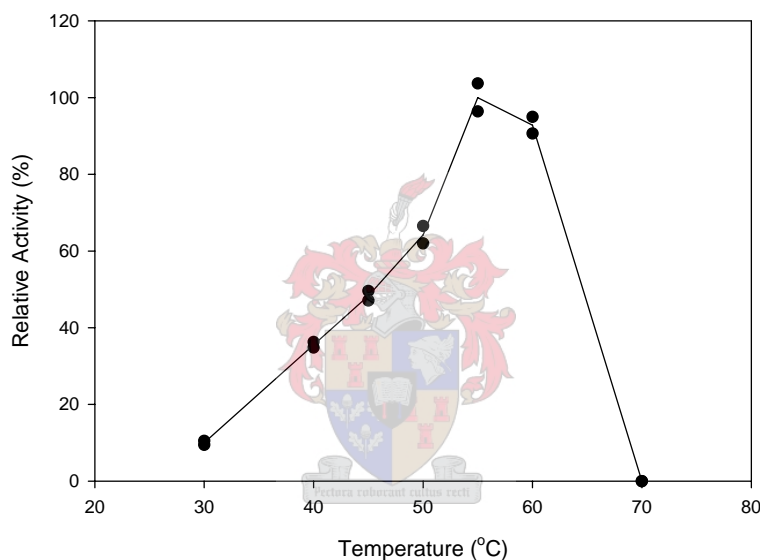


Figure 4.12. Effect of temperature on the activity of the purified α -L-arabinofuranosidase. The experiment was performed in duplicate and both activity values are indicated. 100 % activity corresponds to 0.831 U/ml.

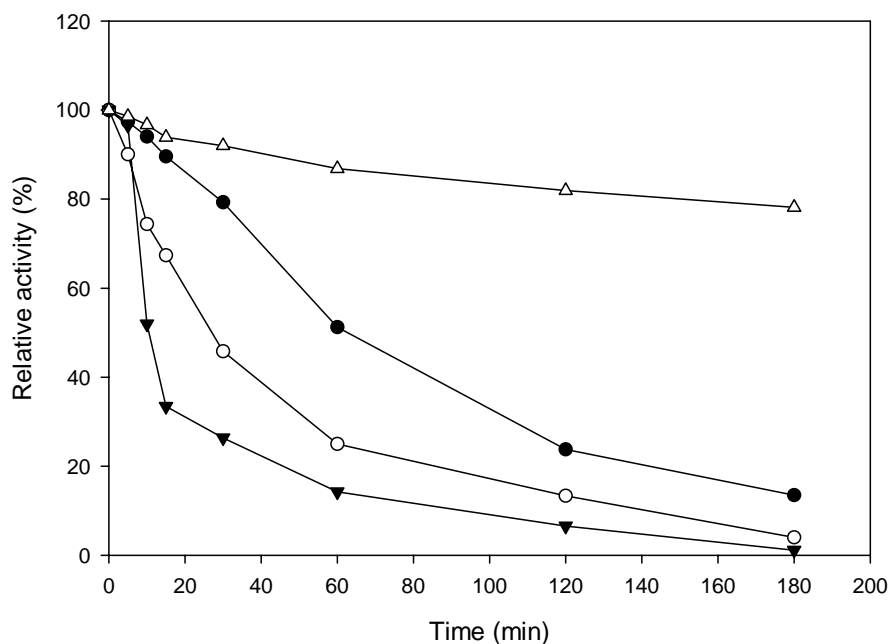


Figure 4.13. Effect of temperature at 40 °C (△), 50 °C (●), 55 °C (○) and 60 °C (▼) on the stability of α -L-arabinofuranosidase from *S. cerevisiae* Y294 [pDLG1-APabfA]. The 100 % activity was 0.353 U/ml. Each data point represents the mean of two determinations.



4.4.6. pH dependence and stability. α -L-arabinofuranosidase displayed maximum activity between pH 4 and 4.5 (Figure 4.14). The enzyme was stable at pH 4 over 3 h and at pH 3 it showed 90% activity over 3 h. At pH 6 α -L-arabinofuranosidase displayed 30 % activity at 1 h and at pH 2.2 the activity dropped to 10 % of the maximum activity within 20 min (Figure 4.15).

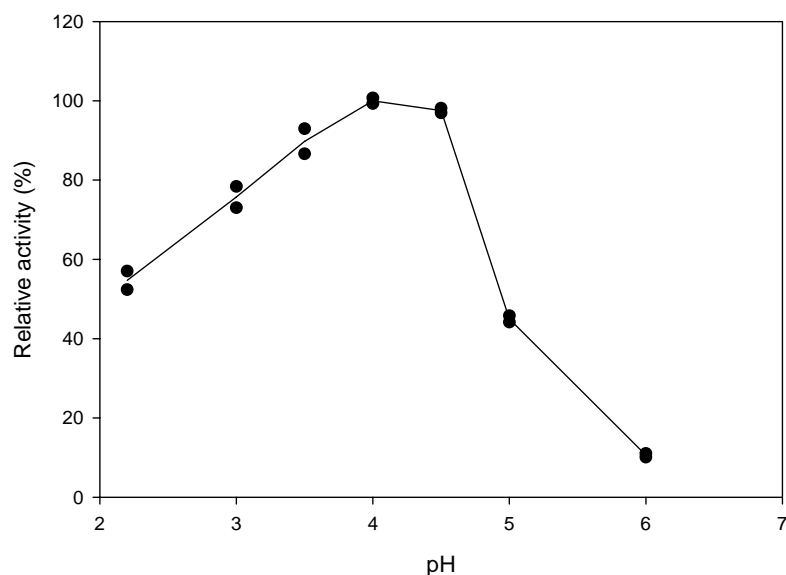


Figure 4.14. Effect of pH on the activity of the purified α -L-arabinofuranosidase from *S. cerevisiae* Y294 [pDLG1-APabfA]. The experiment was performed in duplicate. 100 % activity corresponds to 0.299 U/ml.

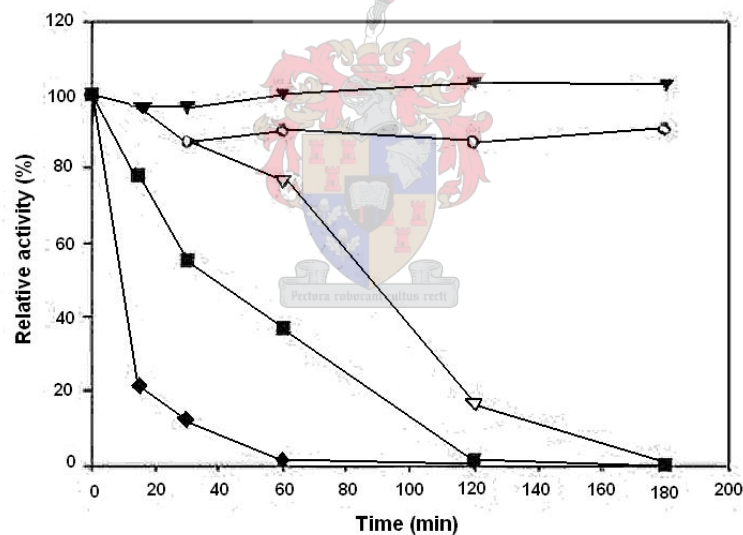


Figure 4.15. Effect of pH at 2.2 (◆), 3 (○), 4 (▼), 5 (▽) and 6 (■) on the stability of α -L-arabinofuranosidase from *S. cerevisiae* Y294 [pDLG1-APabfA]. The 100 % activity was 0.85 U/ml. Each data point represents the mean of two determinations.

4.4.7. Kinetic analysis. The rate dependence of the enzymatic degradation of *p*-nitrophenyl- α -L-arabinofuranoside was measured at different concentrations. α -L-arabinofuranosidase activity was assayed at 55°C and the direct plot of the data is shown in Figure 4.16.

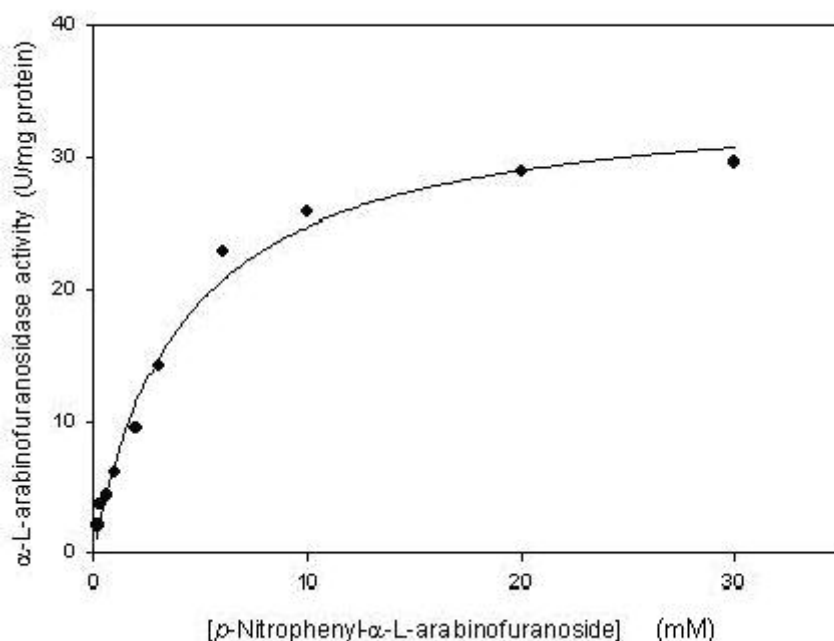


Figure 4.16. α -L-arabinofuranosidase activity as a direct function of *p*-nitrophenyl- α -L-arabinofuranoside concentration. A range from 0.2 to 30 mM substrate was assayed. Each data point represents the mean of two determinations.

From the Lineweaver-Burk plot, a K_m of 1.43 mM and a V_{max} of 23.7 U/mg was calculated whereas the Hanes plot gave a K_m value of 4.51 mM and a V_{max} of 41.5 U/mg (Figure 4.17).

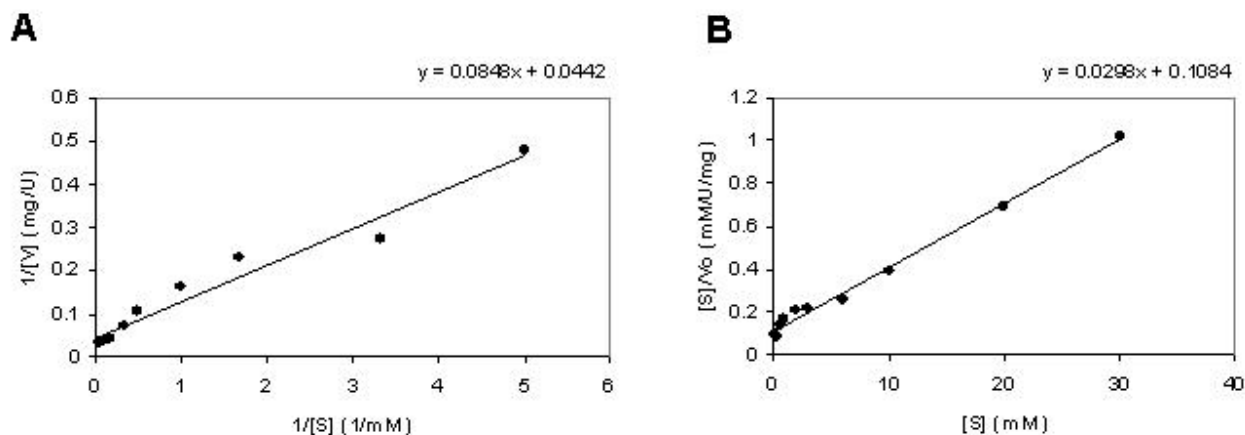


Figure 4.17. Lineweaver-Burk (A) and Hanes (B) linear plots for the effect of *p*-nitrophenyl- α -L-arabinofuranoside concentration on the initial rate of the reaction.

The turnover number (k_{cat}) was calculated from the Lineweaver-Burk V_{max} and total concentration (E_t) of the α -L-arabinofuranosidase used in the reaction $V_{max} / E_t = 18160 \text{ s}^{-1}$. This indicates the maximum number of moles of substrate that could be converted to products per mole of α -L-arabinofuranosidase every second. The specificity constant (k_{cat} / K_m) was $12699 \text{ M}^{-1}\text{s}^{-1}$. The k_{cat} calculated from the Hanes plot was 31800 s^{-1} and the specificity constant was $7051 \text{ M}^{-1}\text{s}^{-1}$.

4.4.8. Effect of inhibitors and inhibition constants.

Table 4.3. Effect of carbohydrates on the purified heterologous α -L-arabinofuranosidase activity ^a

Carbohydrate (50 mM)	Specific activity (U/ml) ^b	Enzyme activity (%)
control	0.0141 ± 0.0006	100.0 ± 4.3
D-glucose	0.0130 ± 0.0004	92.2 ± 2.8
D-xylose	0.0127 ± 0.0009	90.2 ± 6.4
D-mannose	0.0133 ± 0.0018	94.3 ± 12.3
L-arabinose	0.0062 ± 0.0022	43.9 ± 15.6
D-cellobiose	0.0157 ± 0.0013	111.3 ± 9.2
D-maltose	0.0134 ± 0.0009	95.0 ± 6.4

^a The effect of carbohydrates on enzyme activity was determined using *p*-nitrophenyl- α -L-arabinofuranoside as substrate.

^b Mean ± SD of 4 determinations.

Table 4.3 shows the effect of various carbohydrates on the activity of α -L-arabinofuranosidase. Only arabinose inhibited the activity of α -L-arabinofuranosidase. Most showed a slight decrease in activity, whereas cellobiose showed a slight increase. However there was a 56% decrease in activity in the presence of 50 mM arabinose pointing to product inhibition by this carbohydrate.

A series of reactions containing different concentrations of arabinose and substrate was performed to determine the inhibition constant (Figure 4.18).

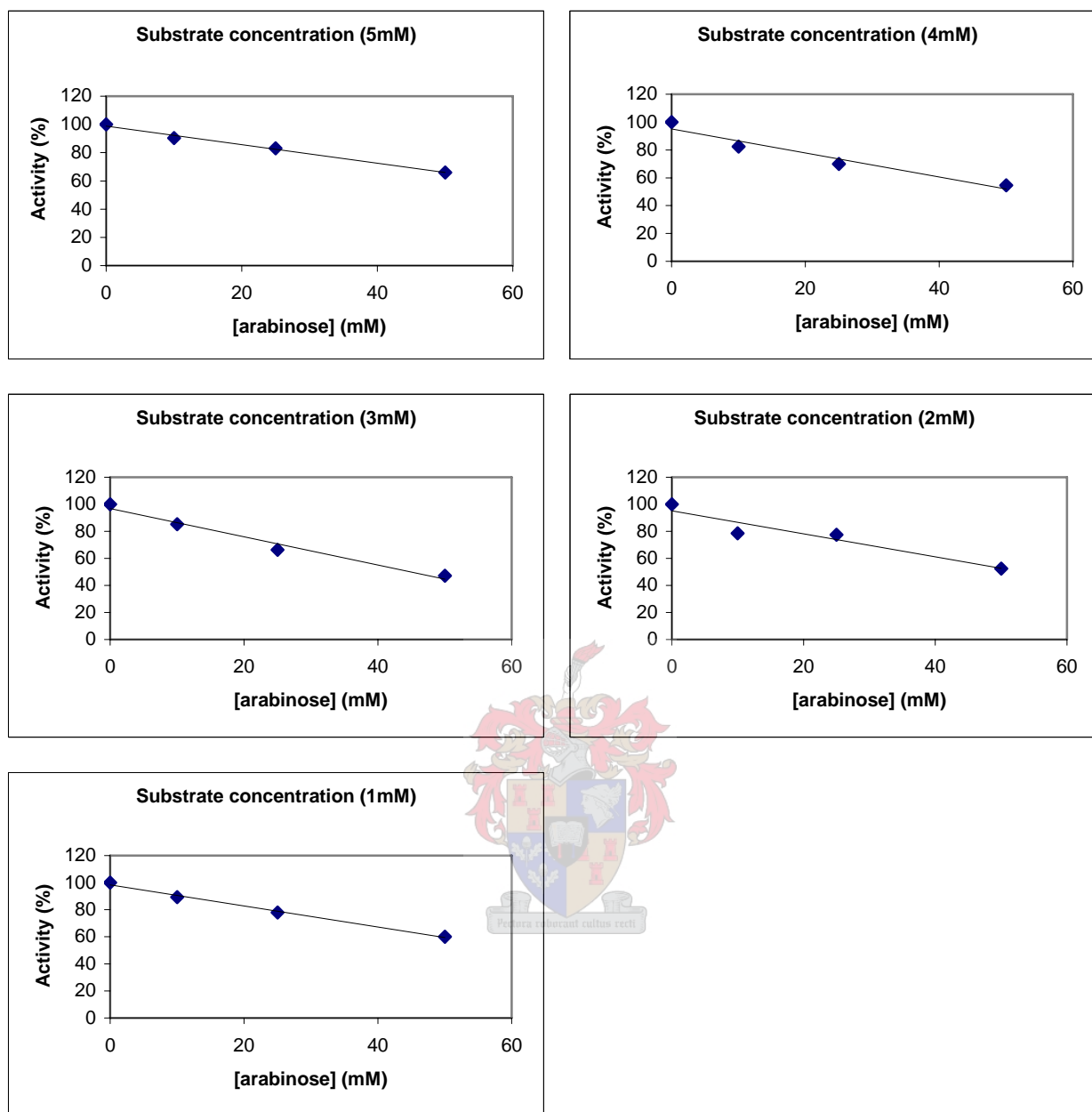


Figure 4.18. The relationship between α -L-arabinofuranosidase activity and *p*-nitrophenyl- α -L-arabinofuranoside concentration (1-5 mM) in the presence of arabinose.

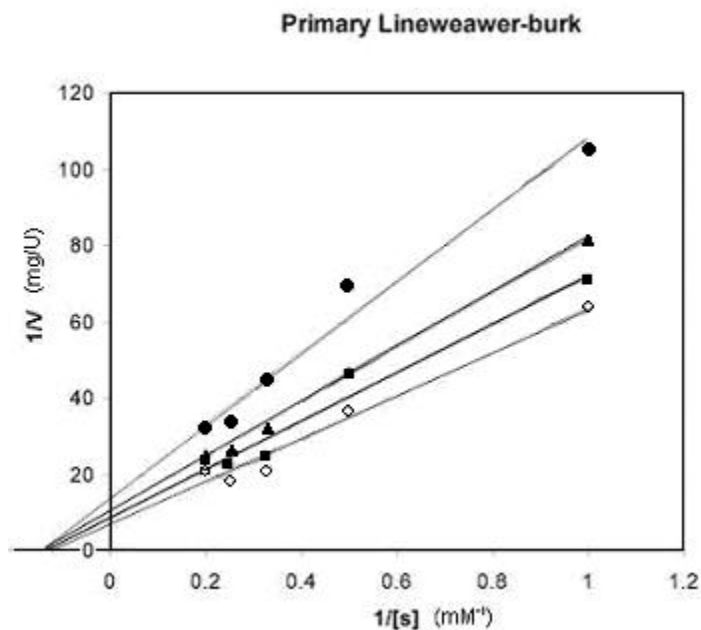


Figure 4.19. The effect of L-arabinose concentrations at 0 mM (o), 10 mM (■), 25 mM (▲) and 50 mM (●) on enzyme activity.

Plotting of data revealed that arabinose is a non competitive inhibitor as the enzyme activity decreased as the inhibitor concentration increased.

The Dixon secondary plot was used to calculate the inhibition constant by extrapolating the linear relationship of 1/V versus I to intercept the negative x-axis (Figure .4.20).

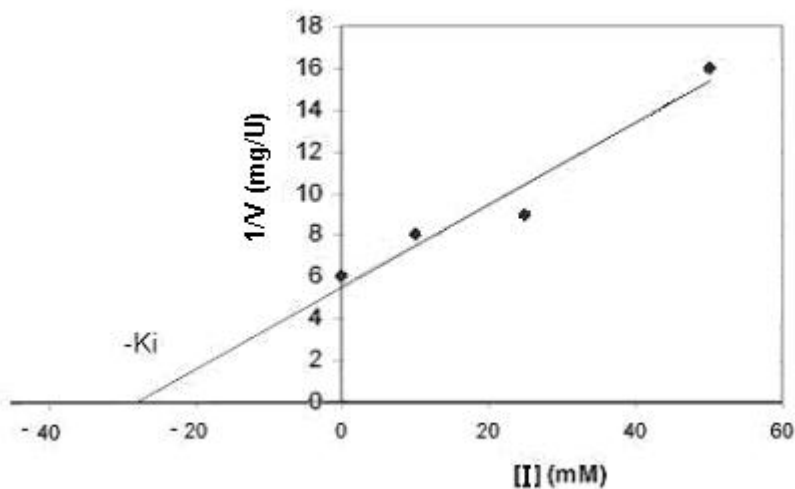


Figure 4.20. The Dixon secondary plot for non competitive inhibition by arabinose ($R^2 = 0.866$). The x-axis intercept was used to calculate the inhibition constant.

A K_i value of 28 ± 3 mM was determined.

4.4.9. Substrate specificity of α -L-arabinofuranosidase. Purified α -L-arabinofuranosidase was assayed under standard conditions with chromogenic substrates, arabino-oligosaccharides and polysaccharides.

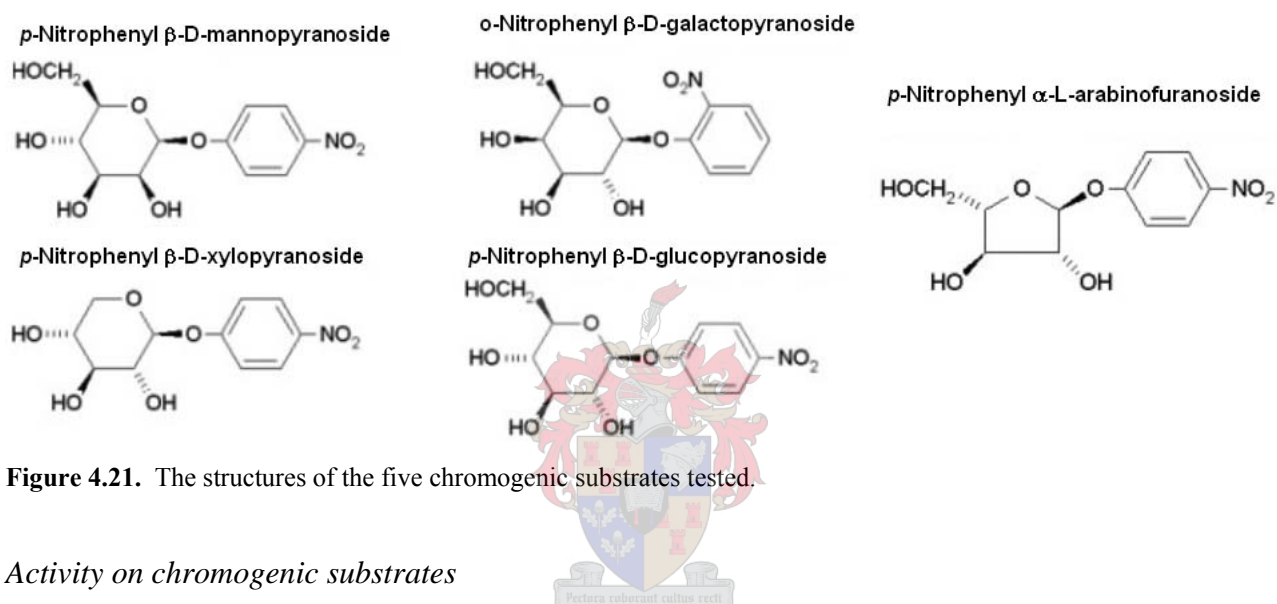


Figure 4.21. The structures of the five chromogenic substrates tested.

Activity on chromogenic substrates

No activity was found after a 2 h reaction with the chromogenic substrates which contained a β -bond. For *p*-nitrophenyl- α -L-arabinofuranoside a specific activity of 3.85 ± 0.008 U/mg was determined.

Activity on arabino-oligosaccharides

The activity of α -L-arabinofuranosidase on the arabino-oligosaccharides was determined using HPAEC to detect sugar substrates and breakdown products. Standards were required to determine the retention times of each sugar and to calculate the concentration from the peak area. In Figure 4.22 arabinose and arabinobiose were run on the HPAEC and arabinose (A) had a retention time of approximately 3.8 min and arabinobiose (B) 19.5 min.

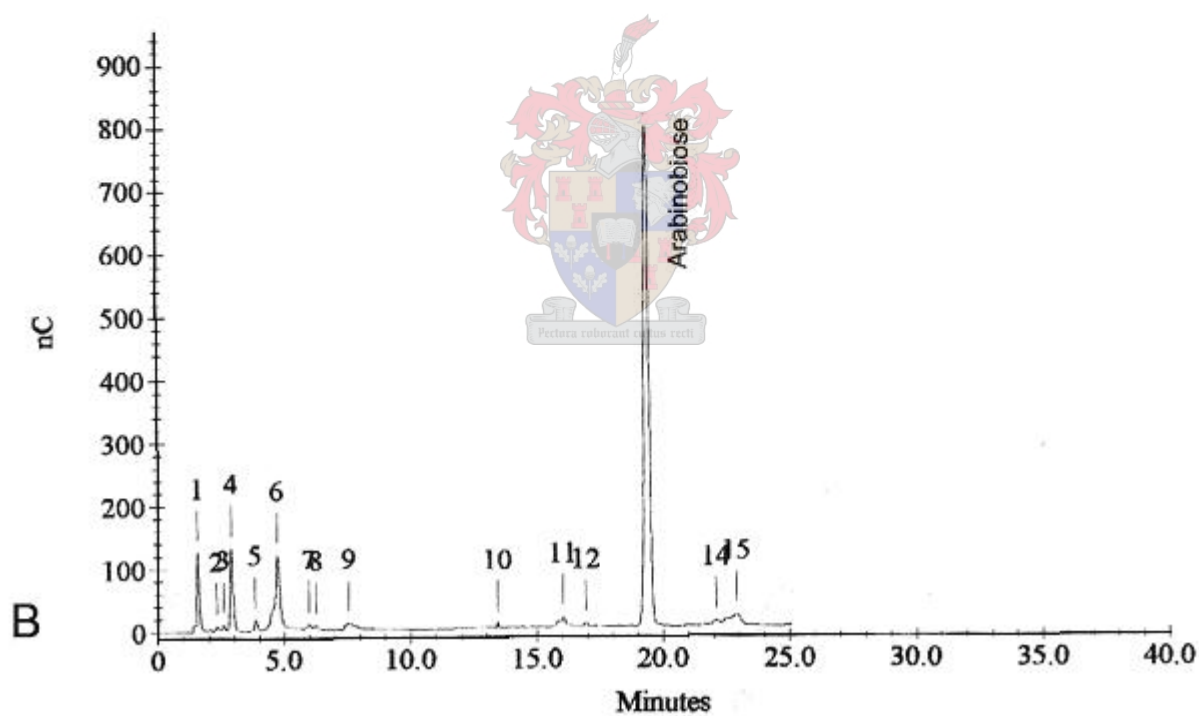
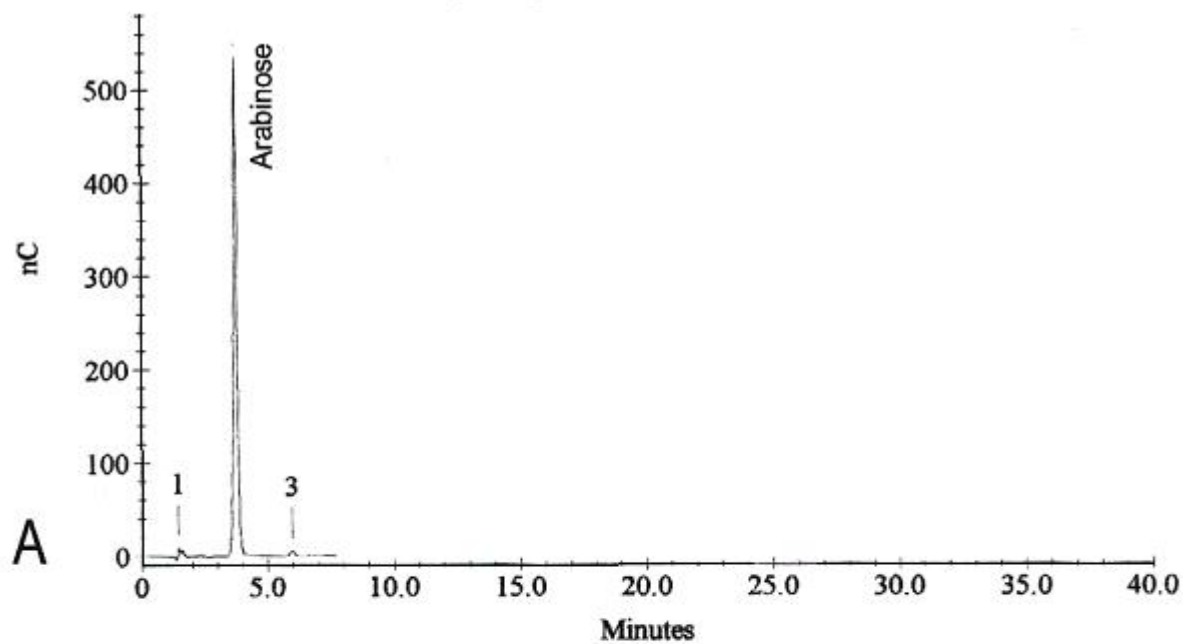


Figure 4.22. HPAEC standards for arabinose (A) and arabinobiose (B) at concentrations of 100 $\mu\text{g/ml}$ each. This was used to calculate concentration from peak area (nC) and to identify the sugar using retention time.

A series of concentrations of arabinose and arabinobiose were run and from the corresponding peak areas, standard curves were drawn (Figure 4.23).

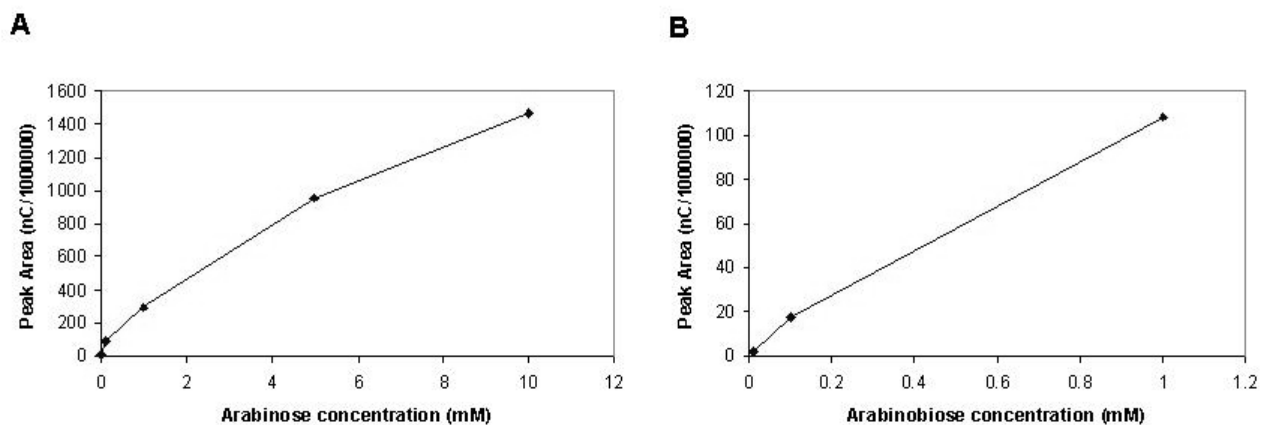
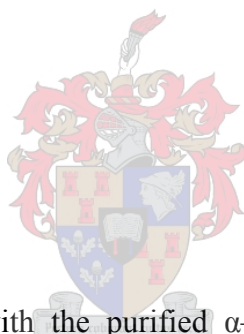


Figure 4.23. HPAEC standard curves for arabinose (A) and arabinobiose (B).

In each sample reaction the breakdown products, such as arabinose and arabinobiose, were also monitored by HPAEC together with the substrate concentration over the time points of 0, 30, 60, 90 and 120 min.



Activity on 1,5- α -L-arabinobiose

Arabinobiose was allowed to react with the purified α -L-arabinofuranosidase for 2 h but no changes in the arabinose or arabinobiose concentration were found for 0 min and 120 min reactions (Figure 4.24).

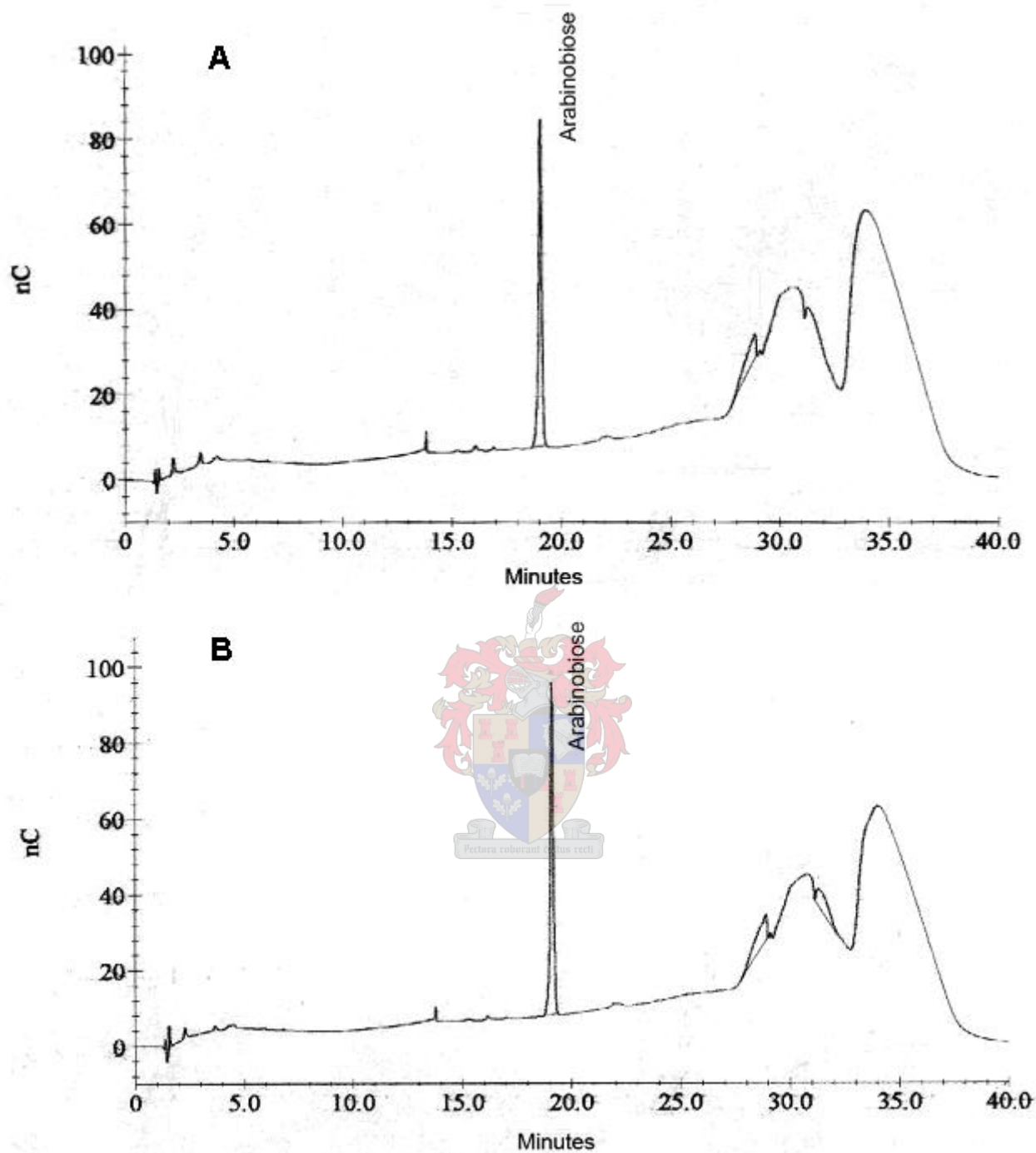


Figure 4.24. High pH anion exchange chromatograph after 0 min (A) and 120 min (B) reaction by α -L-arabinofuranosidase on 1,5- α -L-arabinobiose.

Average values of peak areas at each time point of four repeats were taken and a graph was drawn to represent changes in substrate and product concentration over time (Figure 4.25).

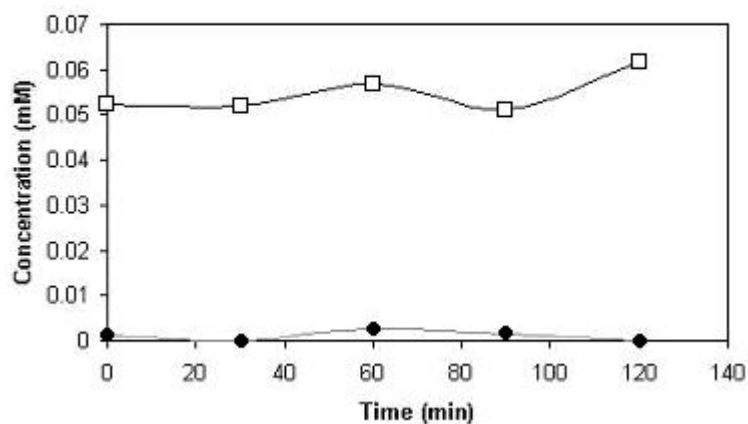


Figure 4.25. Concentration of arabinose (●) and arabinobiose (□) measured by HPAEC during the reaction of α -L-arabinofuranosidase on 1,5- α -L-arabinobiose. Each data point represents the mean of four determinations.

Therefore there was no significant hydrolytic action of α -L-arabinofuranosidase against 1,5- α -L-arabinobiose over 120 min.



Activity on 1,5- α -L-arabinotriose

The 0 min reaction for arabinotriose is shown in Figure 4.26 A. The breakdown products arabinose and arabinobiose were both present in small quantities at the start of the reaction. After 120 min little change in the concentration of the three sugars was observed (Figure 4.26 B).

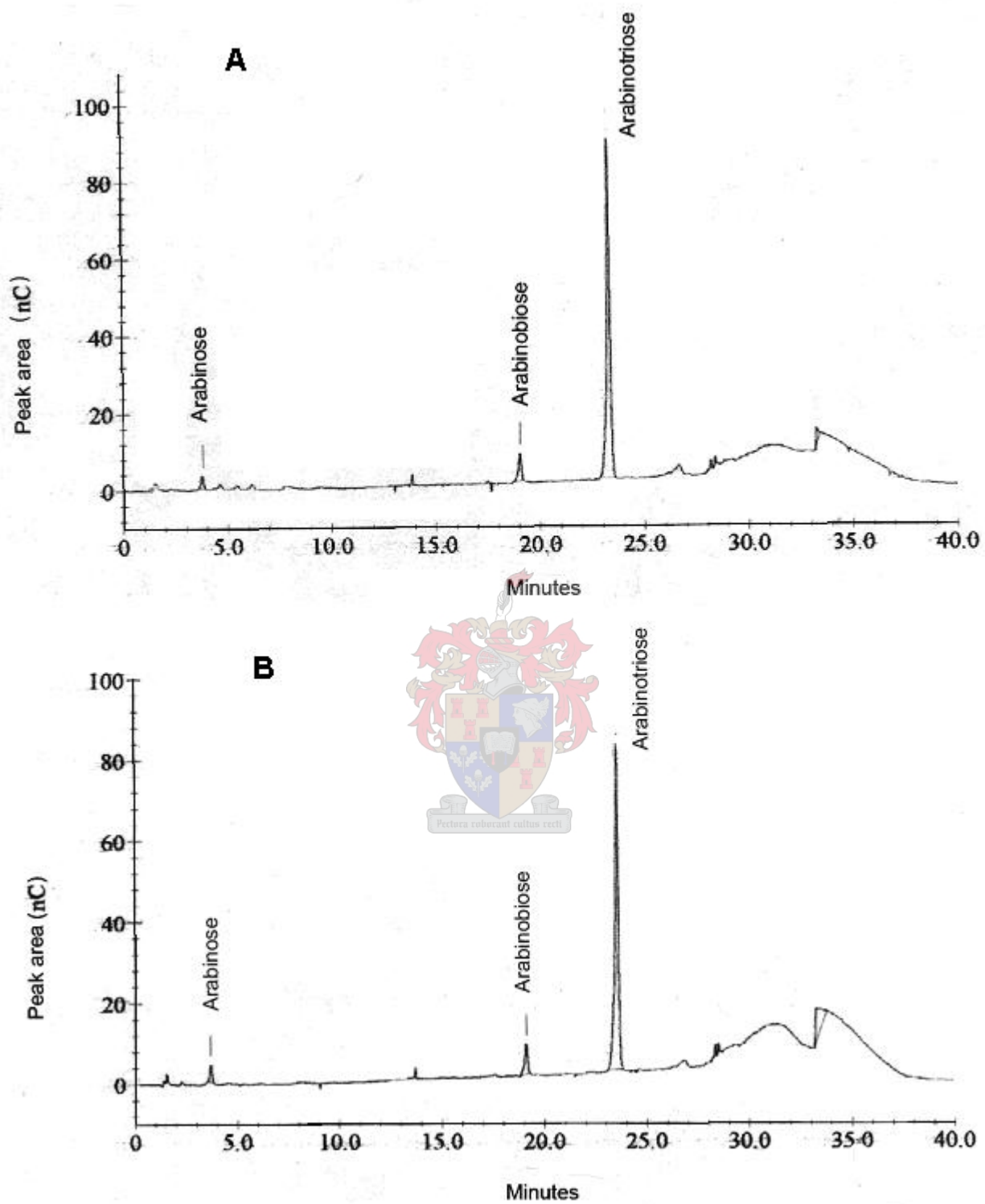


Figure 4.26. High pH anion exchange chromatograph after 0 min (A) and 120 min (B) reaction by α -L-arabinofuranosidase on 1,5- α -L-arabinotriose.

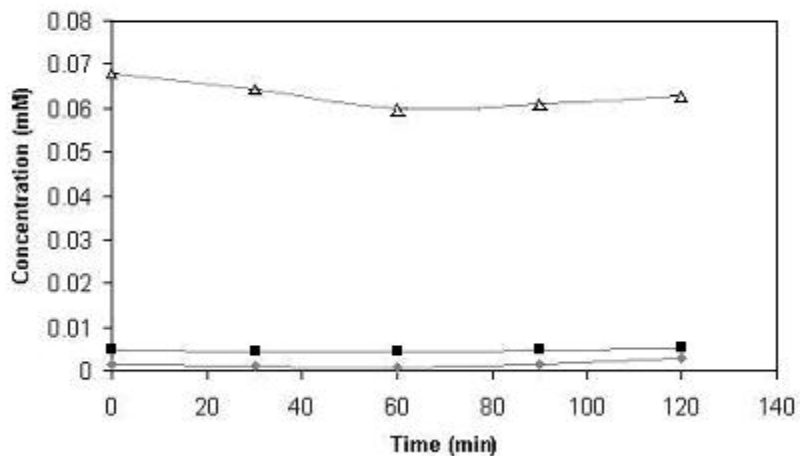


Figure 4.27. Concentrations of arabinotriose (Δ), arabinobiose (\blacksquare) and arabinose (\bullet) measured by HPAEC during the reaction of α -L-arabinofuranosidase on 1,5- α -L-arabinotriose. Each data point represents the mean of four determinations.

A slight decrease in arabinotriose was observed but no simultaneous increase in arabinobiose or arabinose was noted (Figure 4.27).

Additionally the 1,5- α -L-arabinotriose was assayed over 24 h to investigate possible breakdown over longer periods. The 0 h reaction for arabinotriose is shown in Figure 4.28 A. The breakdown products arabinose and arabinobiose were both present in small quantities at the start of the reaction but after 24 h there was a significant increase in the concentration of the products arabinose and arabinobiose (Figure 4.28 B).

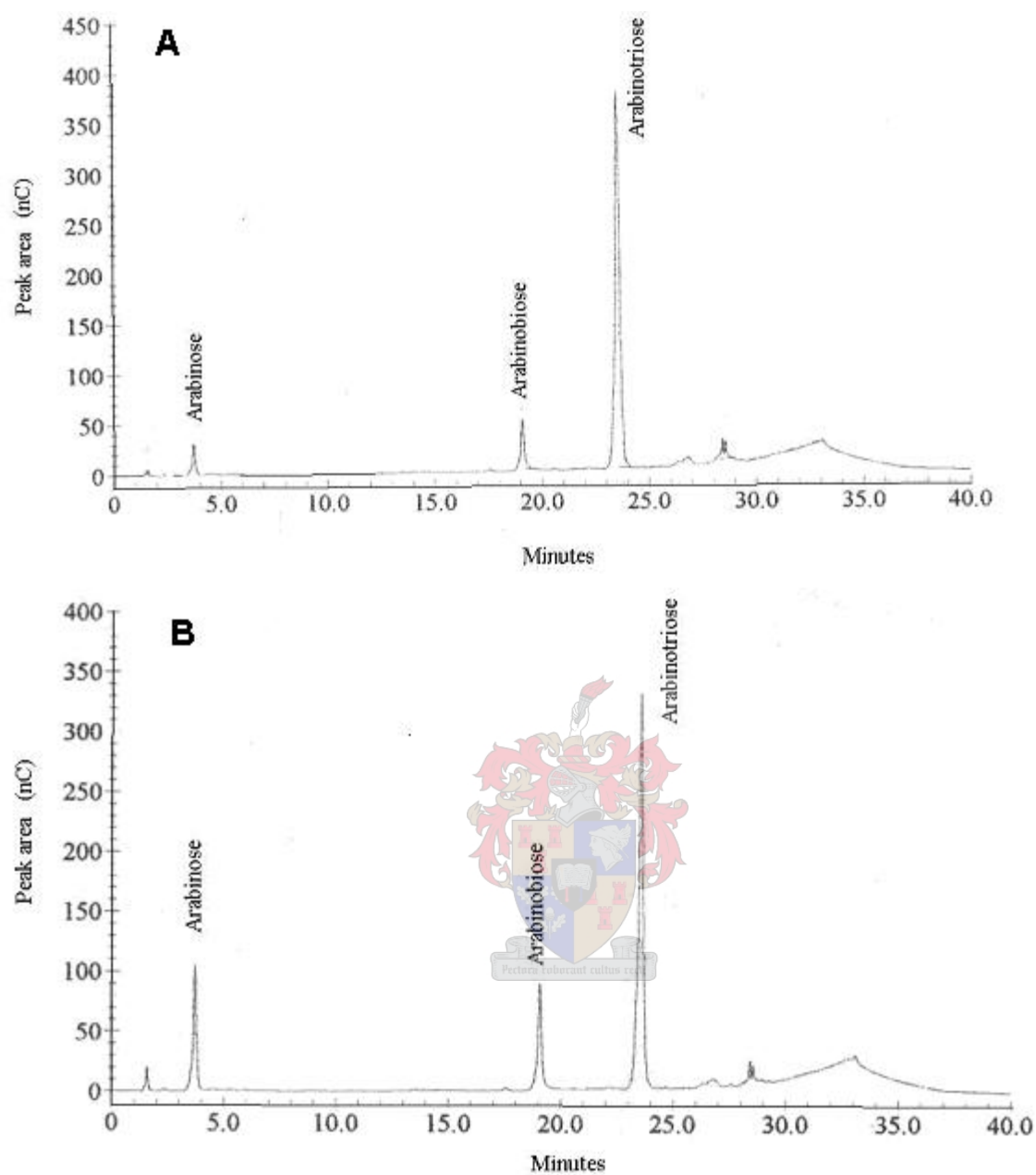


Figure 4.28. High pH anion exchange chromatograph after 0 h (A) and 24 h (B) reaction by α -L-arabinofuranosidase on 1,5- α -L-arabinotriose.

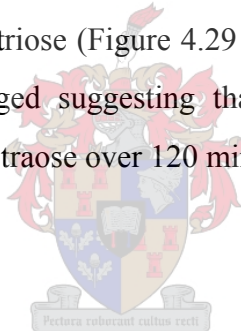
An increase in arabinose and arabinobiose was observed together with a decrease in arabinotriose concentration (Table 4.4). The concentration of the two hydrolysis products showed a small increase over time whereas the substrate showed a significant decrease although a stoichiometric relationship could not be established. This result indicated that a longer reaction time was needed to release products by the α -L-arabinofuranosidase.

Table 4.4. Analysis of 1,5- α -L-arabinotriose degradation by α -L-arabinofuranosidase over 24 h.

Sugar	Concentration (mM)		
	0 h	24 h	Change (mM)
Arabinose	0.0290 \pm 0.0032	0.0971 \pm 0.0189	+ 0.0681
Arabinobiose	0.0422 \pm 0.0055	0.0718 \pm 0.0064	+ 0.0296
Arabinotriose	3.8937 \pm 0.3548	3.5087 \pm 0.0935	- 0.3850

Activity on 1,5- α -L-arabinotetraose

The 0 min reaction for arabinotetraose is shown in Figure 4.29 A. One of the breakdown products, arabinotriose, was detected in small quantities in the initial reaction. After 120 min there was little change in the concentrations of arabinotriose (Figure 4.29 B). The concentrations of arabinose and arabinotetraose also remained unchanged suggesting that the α -L-arabinofuranosidase had no significant activity on 1,5- α -L-arabinotetraose over 120 min (Figure 4.30).



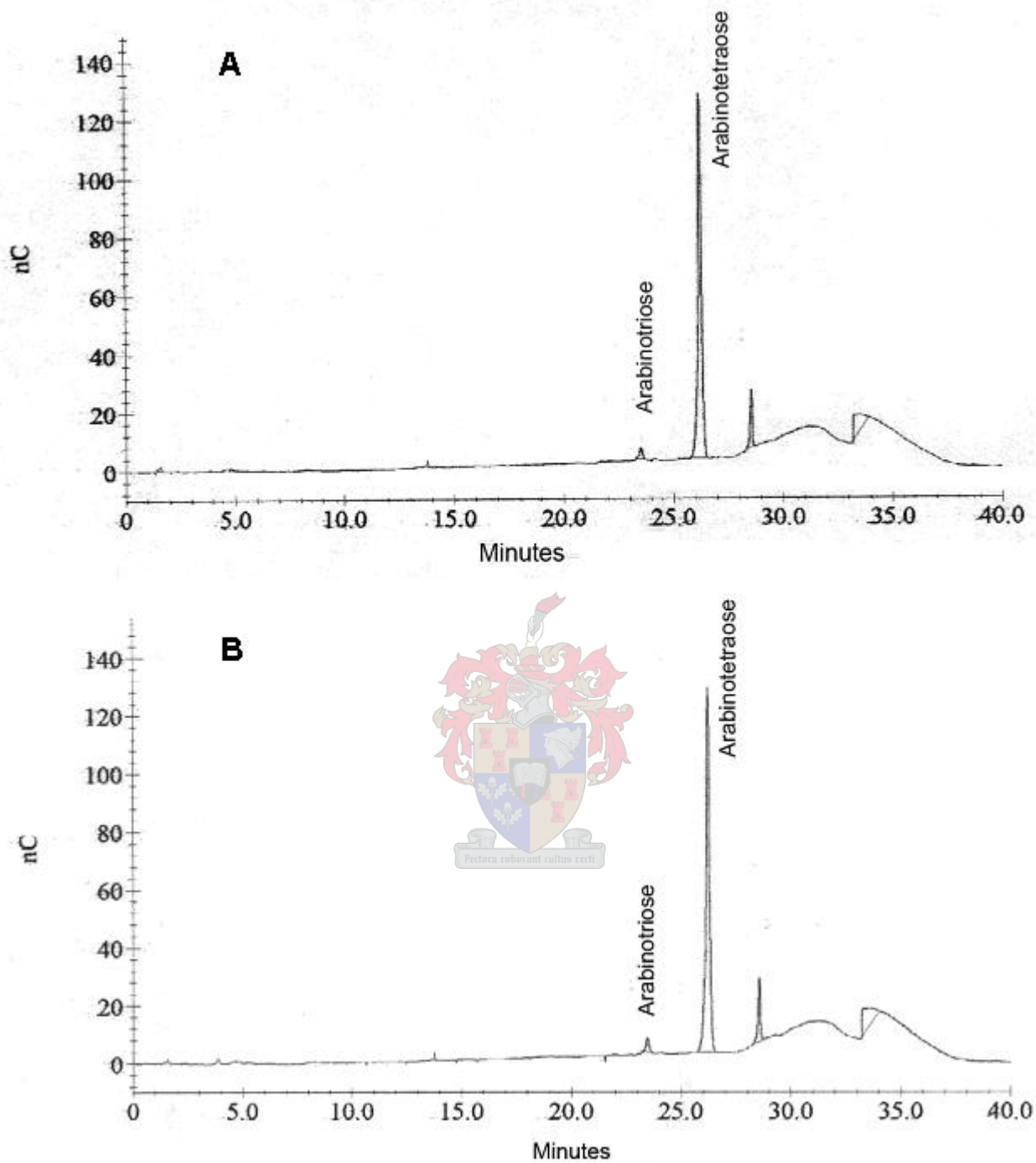


Figure 4.29. High pH anion exchange chromatograph after 0 min (A) and 120 min (B) reaction by α -L-arabinofuranosidase on arabinotetraose.

The substrate remained constant over time and average values of peak areas at each time point were taken and a graph was drawn to represent changes in substrate and product over time (Figure 4.30).

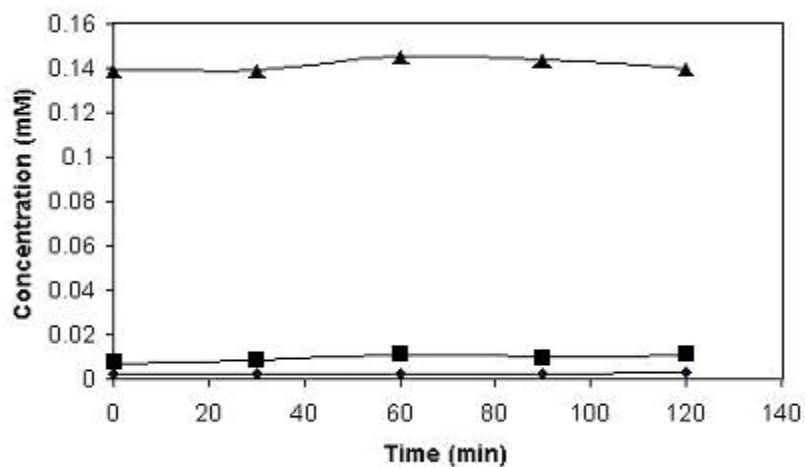
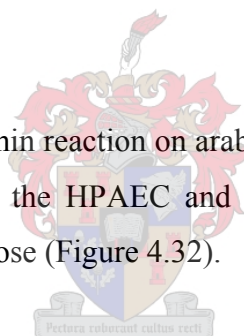


Figure 4.30. Concentration of arabinotetraose (▲), arabinotriose (■) and arabinose (◆) measured by HPAEC during the reaction of α -L-arabinofuranosidase on 1,5- α -L-arabinotetraose. Each data point represents the mean of four determinations.

Activity on 1,5- α -L-arabinopentaose

The chromatograph for 0 min and 120 min reaction on arabinopentaose is shown in Figure 4.31. No breakdown products were detected by the HPAEC and after 120 min there was no significant change in the peak area of arabinopentaose (Figure 4.32).



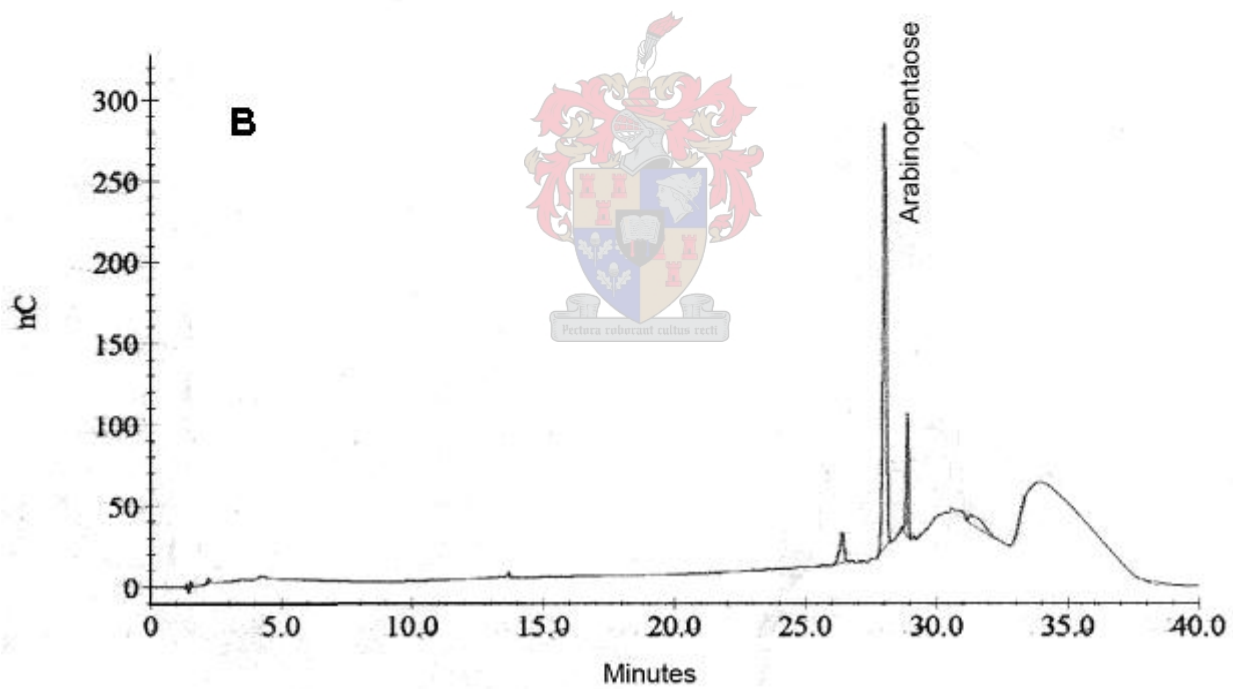
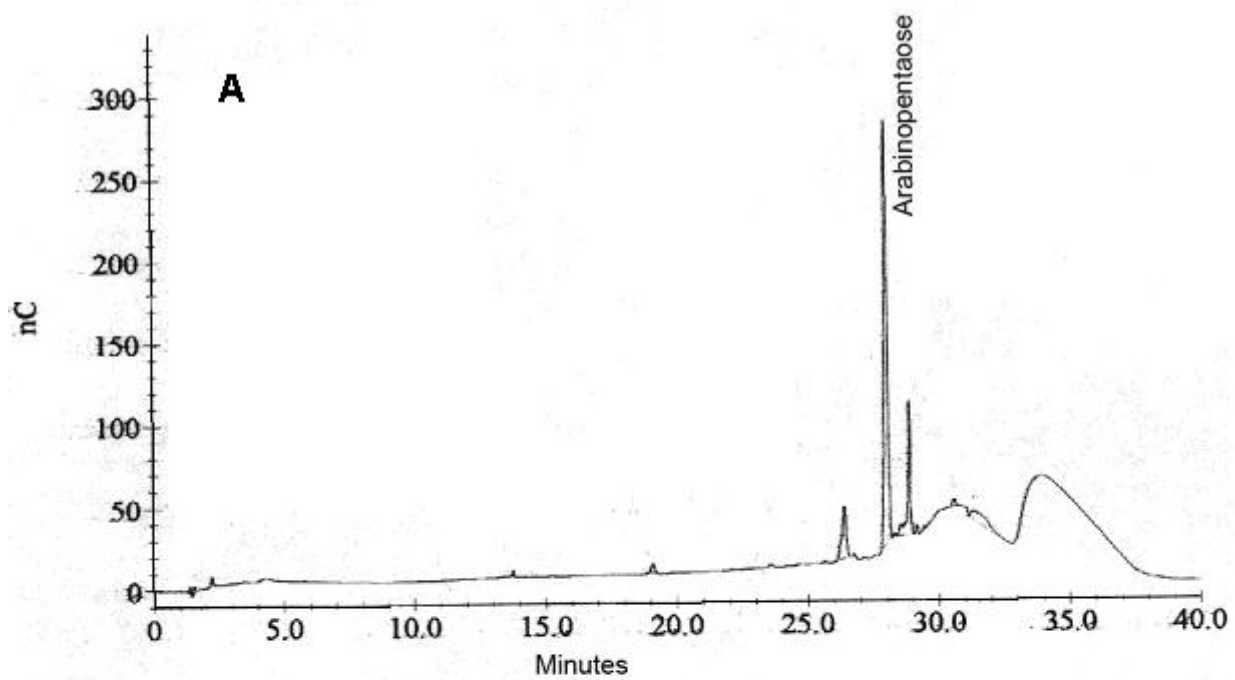


Figure 4.31. High pH anion exchange chromatograph after 0 min (A) and 120 min (B) reaction by α -L-arabinofuranosidase on arabinopentaose.

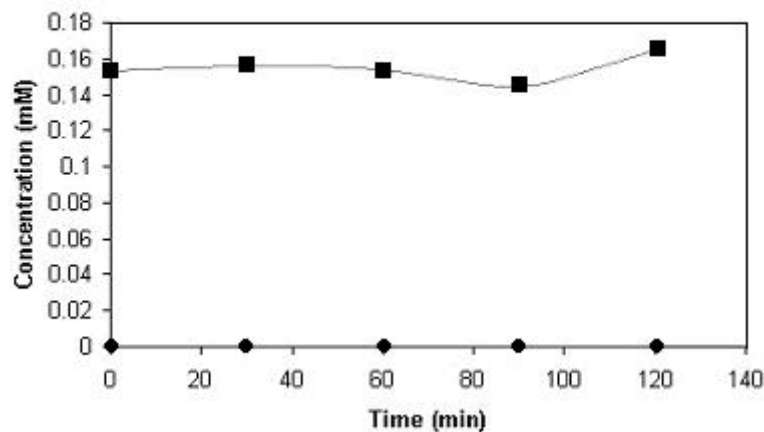
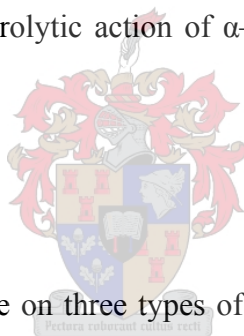


Figure 4.32. Concentration of arabinopentaose (■) and arabinose (●) measured by HPAEC during the reaction of α -L-arabinofuranosidase on 1,5- α -L-arabinopentaose. Each data point represents the mean of four determinants.

Therefore there was no significant hydrolytic action of α -L-arabinofuranosidase against 1,5- α -L-arabinopentaose over 120 min.



Activity on arabino polysaccharides

The activity of α -L-arabinofuranosidase on three types of arabinoxylans were all assayed over 60 min. Corn fibre arabinoxylan was partially hydrolysed as indicated by the increase in arabinose release over 60 min (Figure 4.33).

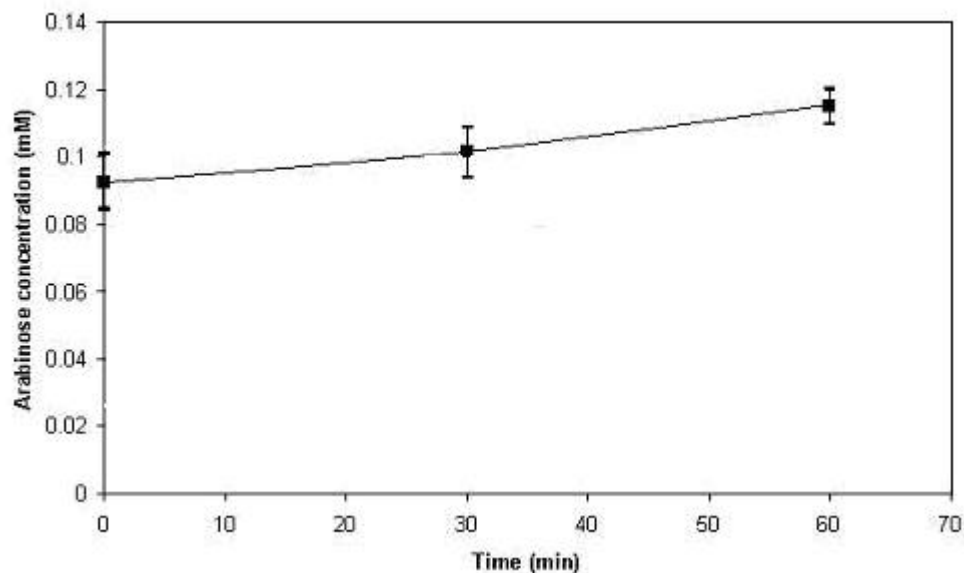
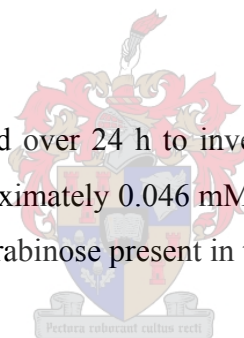


Figure 4.33. Increase in arabinose concentration (mean \pm standard deviation of four determinations) measured by HPAEC during the reaction of α -L-arabinofuranosidase on corn fibre arabinoxylan.

Additionally the corn fibre was assayed over 24 h to investigate possible breakdown over longer periods. There was an increase of approximately 0.046 mM in arabinose concentration measured by HPAEC which was about 23 % of the arabinose present in the corn fibre.



Oat spelt arabinoxylan showed partial hydrolysis since there was an increase in arabinose detected by the HPAEC (Figure 4.34).

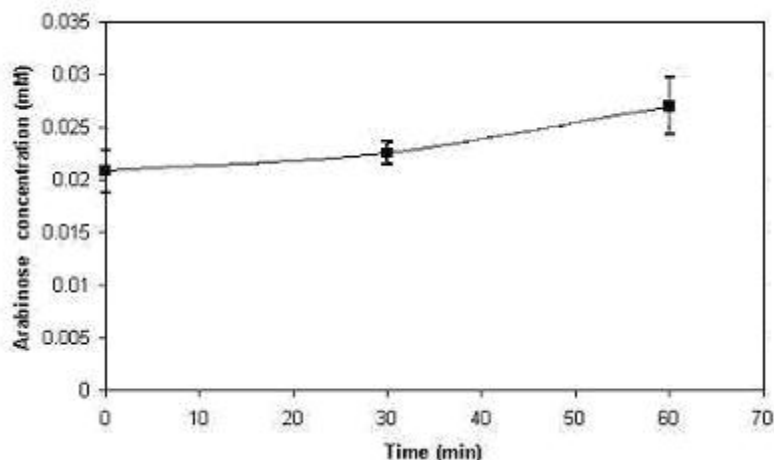


Figure 4.34. Increase in arabinose concentration (mean \pm standard deviation of four determinations) measured by HPAEC during the reaction of α -L-arabinofuranosidase on oat spelt xylan.

Additionally this substrate was assayed over 24 h to investigate possible breakdown over longer periods. There was an increase of approximately 0.04 mM in arabinose concentration which is approximately 6 % of the arabinose in the oat spelt xylan and this therefore indicated that a longer reaction time was needed to release products from oat spelt arabinoxylan.

When α -L-arabinofuranosidase was incubated with wheat arabinoxylan no release of arabinose over 60 min was observed. Over 24 h there was an increase of approximately 0.0025 mM in arabinose release which represented only 1 % of the total arabinose in the substrate. This indicated that wheat arabinoxylan was more recalcitrant to α -L-arabinofuranosidase hydrolysis than either oat spelt or corn arabinoxylan. However when xylanase was also present, much greater amounts of arabinose were released (see section 4.4.10 for details).

No release of arabinose was observed when larch wood arabinogalactan was incubated with α -L-arabinofuranosidase over 60 min and 24 h. There was no arabinose detected by the HPAEC in the 0 min reactions either, indicating there was no residual arabinose present with this substrate and no arabinose was cleaved by arabinofuranosidase (data not shown).

Debranched arabinan showed partial release of arabinose over time, although arabinose was also detected by the HPAEC in the 0 min reactions (Figure 4.35). There was a slight increase in arabinose released during a reaction of debranched arabinan with α -L-arabinofuranosidase over 120 min. However the experimental error was large enough to account for this increase and therefore it would appear that α -L-arabinofuranosidase is unable to release arabinose from 1,5- α -L-arabinan.

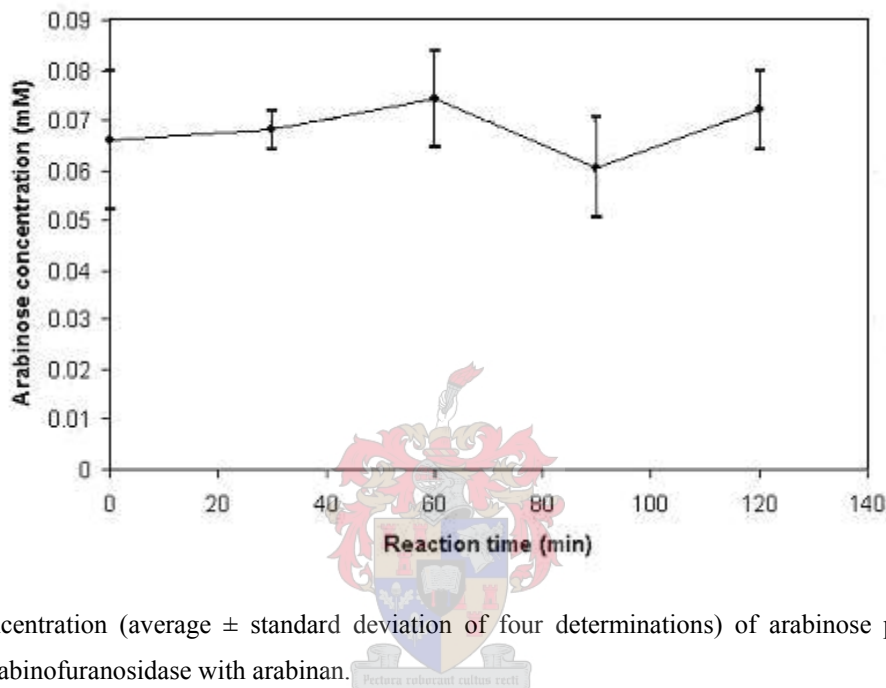


Figure 4.35. Concentration (average \pm standard deviation of four determinations) of arabinose present during the reaction of α -L-arabinofuranosidase with arabinan.

Additionally the debranched arabinan was assayed over 24 h to investigate possible breakdown over longer periods. There was approximately 0.015 mM in arabinose released and therefore indicated that the longer reaction time result suggested that α -L-arabinofuranosidase had little activity on debranched 1,5- α -L-arabinan.

Activity on lignin-arabinose substrates

Due to the uncertain structure (details of composition are given in Appendix B) and bonds of three lignin-arabinose substrates (R1000, HiA350, LS3000), control tests were performed to investigate the release of arabinose when the substrates are dissolved in water or NaOH during assay conditions. The arabinose release in all these control tests gave concentration values of between 0

and 0.01 mM arabinose. Any concentration value found in this range was not considered as arabinose release due enzyme degradation.

When α -L-arabinofuranosidase was incubated with lignin-arabinose substrate R1000 no significant release of arabinose over time was observed, although there was a small amount of arabinose detected by the HPAEC in the initial reaction (Figure 4.36). There appeared to be a slight increase of arabinose over time although the large error in the data suggests that the increase in arabinose was not statistically significant.

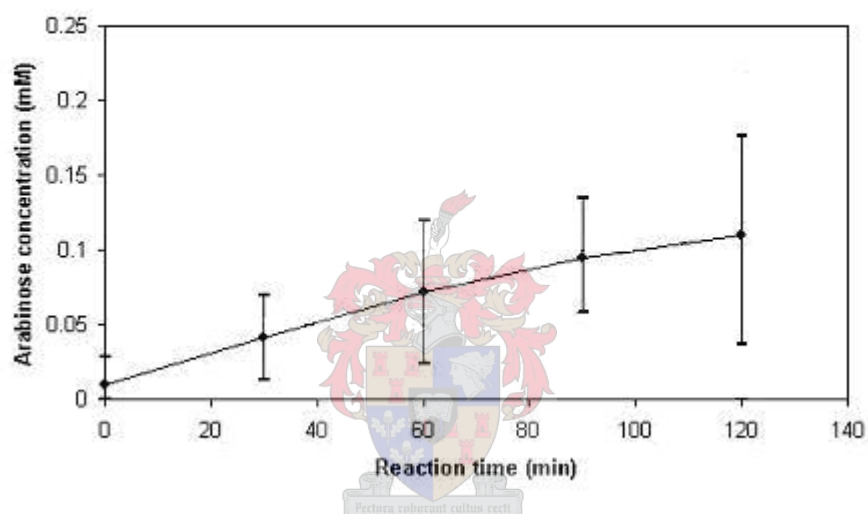


Figure 4.36. Concentration (average \pm standard deviation of four determinations) of arabinose present during the reaction of α -L-arabinofuranosidase with lignin-arabinose substrate (R1000).

α -L-Arabinofuranosidase showed no significant release of arabinose over time from the lignin-arabinose substrate HiA350 although there was a small amount of arabinose in the initial reaction (Figure 4.37).

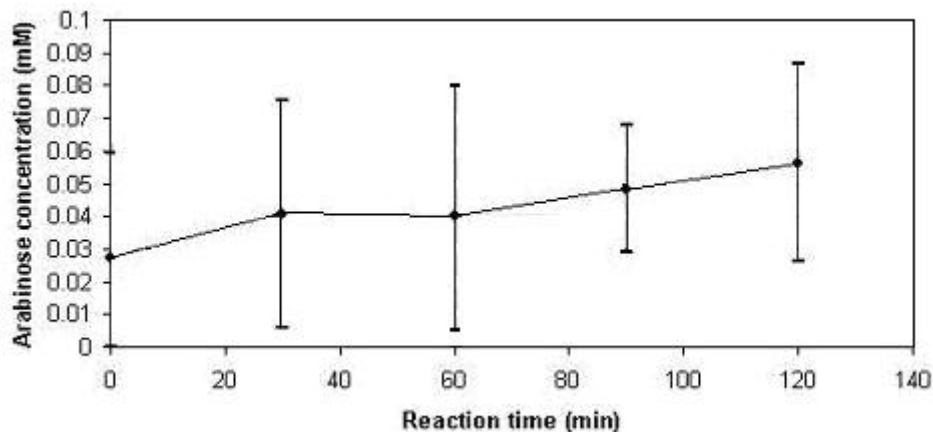


Figure 4.37. Concentration (average \pm standard deviation of four determinations) of arabinose present during the reaction of α -L-arabinofuranosidase with lignin-arabinose substrate (HiA350).

Addition of α -L-arabinofuranosidase to lignin-arabinose substrate LS3000 resulted in no significant release of arabinose over time and there was an arabinose concentration of more than 0.03 mM in the 0 min reactions (Figure 4.38). The peak area for arabinose over time remained relatively constant and there was also a high standard deviation of the average value.

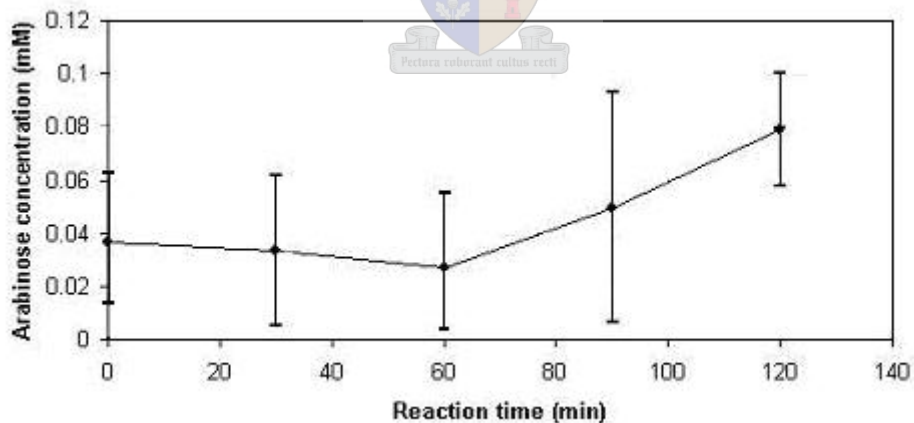


Figure 4.38. Concentration (average \pm standard deviation of four determinations) of arabinose present during the reaction of α -L-arabinofuranosidase with lignin-arabinose substrate (LS3000).

Therefore the results of these experiments suggested that the α -L-arabinofuranosidase could not cleave arabinose from these lignin-carbohydrate complexes. This result was somewhat surprising as

arabinose is often linked to lignin by ferulic acid and should be amenable to hydrolysis (Jeffries, 1990).

A summary of all the substrates tested and average specific activities is presented in Table 4.5.

Table 4.5. Substrate specificity of purified heterologous α -L-arabinofuranosidase.

Substrate	Reaction time (h)	Released μ mol arabinose / min / mg protein
<i>p</i> -Nitrophenyl α -L-arabinofuranoside	2	3.85 \pm 0.008 ^a
<i>p</i> -Nitrophenyl β -D-glucopyranoside	2	ND
<i>o</i> -Nitrophenyl β -D-galactopyranoside	2	ND
<i>p</i> -Nitrophenyl β -D-mannopyranoside	2	ND
<i>p</i> -Nitrophenyl β -D-xylopyranoside	2	ND
1,5- α -L-Arabinobiose	2	ND
1,5- α -L-Arabinotriose	2	ND
1,5- α -L-Arabinotriose	24	0.110 \pm 0.030 ^b
1,5- α -L-Arabinotetraose	2	ND
1,5- α -L-Arabinopentaose	2	ND
Arabinoxylan (wheat flour Ara 41:59 Xyl)	1	ND
Arabinoxylan (wheat flour Ara 41:59 Xyl)	24	0.019 \pm 0.020 ^b
Arabinoxylan (corn fibre 30% arabinose)	1	0.070 \pm 0.036 ^b
Arabinoxylan (corn fibre 30% arabinose)	24	0.165 \pm 0.006 ^b
Arabinoxylan (oat spelt 10% arabinose)	1	0.013 \pm 0.008 ^a
Arabinoxylan (oat spelt 10% arabinose)	24	0.054 \pm 0.002 ^b
Larch Arabinogalactan (Ara 15:35 Gal)	1	ND
Larch Arabinogalactan (Ara 15:35 Gal)	24	ND
Debranched arabinan (Ara:Gal:Rha:GalUA = 88:4:2:6)	1	ND
Debranched arabinan (Ara:Gal:Rha:GalUA = 88:4:2:6)	24	ND
Residual Lignin (HiA350 proRL)	2	ND
Residual Lignin (R1000 proRL)	2	ND
Residual Lignin (LS3000 proRL)	2	ND

^a Average \pm standard deviation of four determinations.

^b Average \pm range of two determinations.

ND – Activity not detected.

In summary, it can be concluded that none of the chromogenic substrates containing a β -bond were cleaved. The arabino-oligosaccharides were all polymers of arabinose joined by a 1,5- α -L-bond and no activity was found after 2 h, but there was slight activity over 24 h. After 1 h wheat arabinoxylan showed no arabinose release but over 24 h slight release was observed. A greater degree of arabinose release was observed when α -L-arabinofuranosidase was reacted with oat spelt and corn

arabinoxylan at 1 and 24 h. The α -1,5-debranched arabinan showed no arabinose release after 2 h or 24 h and larch arabinogalactan showed no arabinose release after 1 h or 24 h. The three lignin samples also showed no significant arabinose release after 2 h.

4.4.10. Synergistic action with xylanase. Five reactions containing wheat arabinoxylan as substrate with combinations of endo- β -1,4-xylanase (family 11) and α -L-arabinofuranosidase were performed. Reaction 1 contained α -L-arabinofuranosidase, reaction 2 contained xylanase, reaction 3 contained xylanase, incubated for 12 h, boiled and then α -L-arabinofuranosidase was added and incubated for a further 12 h. Reaction 4 contained xylanase and α -L-arabinofuranosidase and reaction 5 contained no enzyme and served as a buffer control. The reaction samples were analyzed by HPAEC and no arabinose (would be found at a retention time of \pm 4 min) was detected in the initial samples (Figure 4.39) or in the buffer control after a 12 h reaction (Figure 4.40).

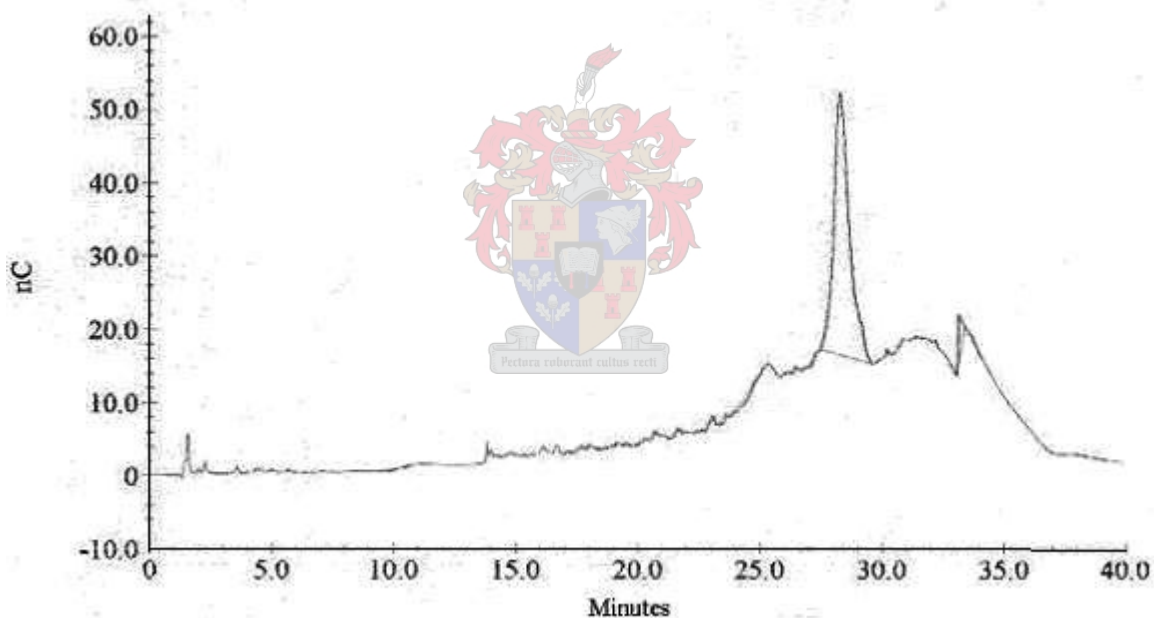


Figure 4.39. Products released after wheat arabinoxylan hydrolysis for 0 h by α -L-arabinofuranosidase (0.081 U/ml) in reaction 1. The zero time reactions for the other four reactions were similar and therefore not displayed.

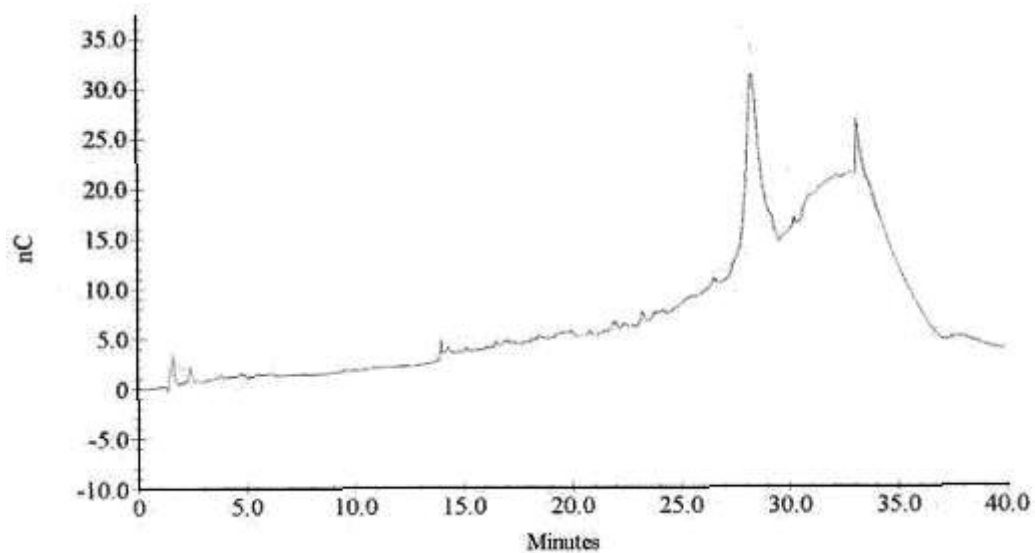


Figure 4.40. Products released in reaction 5 (buffer control) after 12 h.

When wheat arabinoxylan was reacted with α -L-arabinofuranosidase over 12 h (reaction 1) only arabinose was identified in the reaction mixture (Figure 4.41).

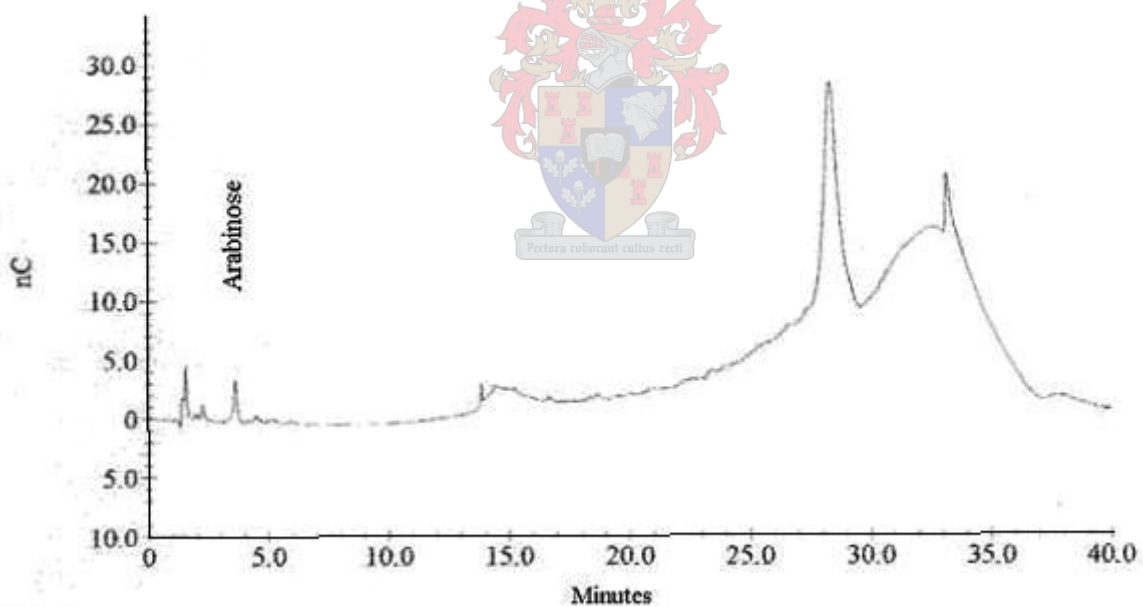


Figure 4.41. Products released after wheat arabinoxylan hydrolysis for 12 h by α -L-arabinofuranosidase (0.081 U/ml) in reaction 1.

In the HPAEC profiles of the 12 h reactions, arabinoxylo-oligosaccharides were also detected but only arabinose, xylose and xylobiose peak areas were evaluated further.

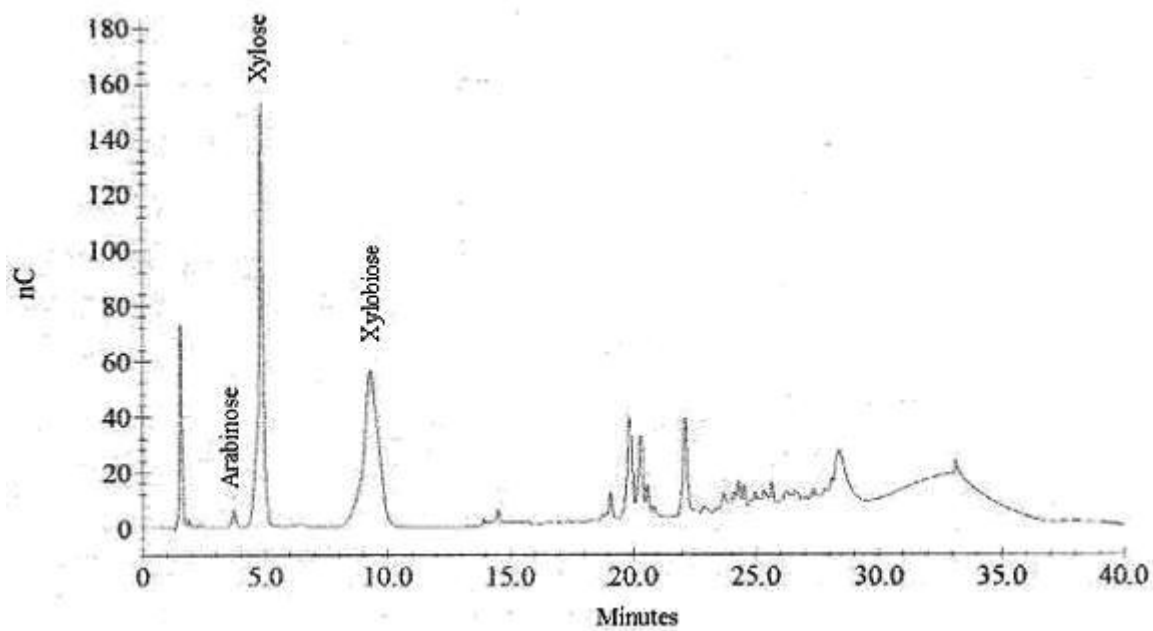


Figure 4.42. Products released after wheat arabinoxylan hydrolysis for 12 h by xylanase (100 U/ml) in reaction 2.

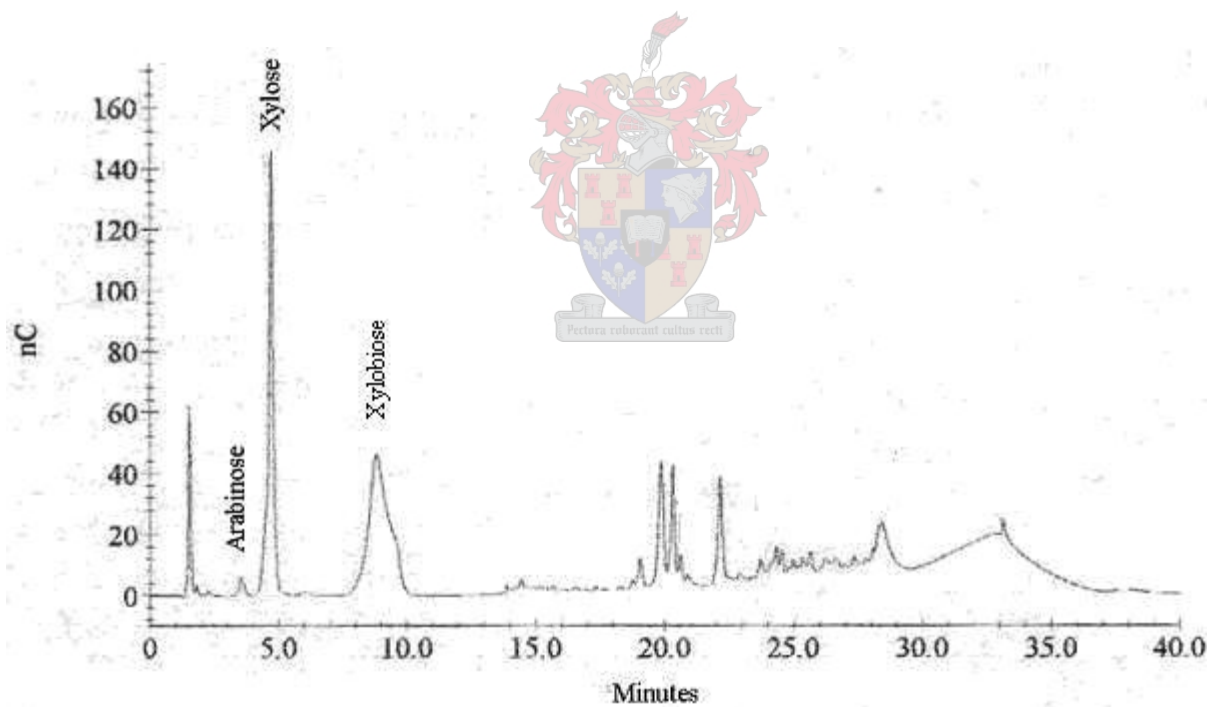


Figure 4.43. Products released after wheat arabinoxylan hydrolysis with α -L-arabinofuranosidase (0.081 U/ml) for 12 h, followed by xylanase (100 U/ml) for 12 h in reaction 3.

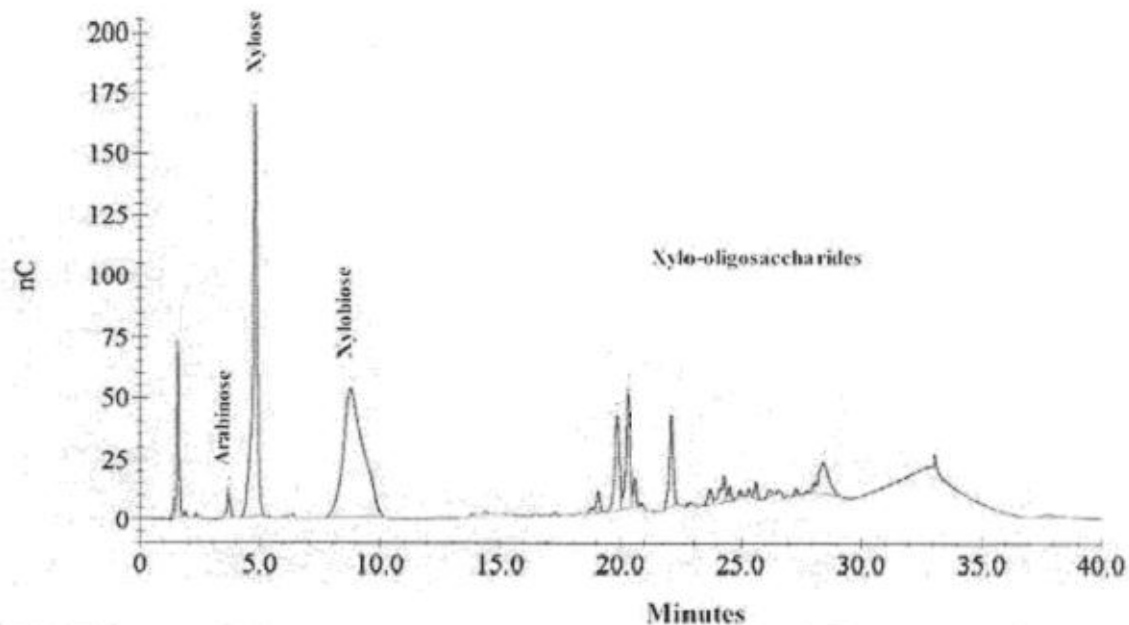


Figure 4.44. Products released after wheat arabinoxylan hydrolysis for 12 h by α -L-arabinofuranosidase (0.081 U/ml) and xylanase (100 U/ml) in reaction 4.

For the 12 h reactions, the arabinose, xylose and xylobiose were identified and concentrations determined in reactions 1 to 4. In reaction 1 no xylose was present but a concentration of 0.00195 mM was detected for arabinose, which was expected as α -L-arabinofuranosidase was present (Figure 4.41). In reaction 2 peaks for arabinose, xylose and xylobiose were detected (Figure 4.42). The presence of arabinose was not expected as only xylanase was present. This result prompted an investigation into the possibility that there was α -L-arabinofuranosidase activity in the xylanase M4 from Megazyme. A standard assay with *p*-nitrophenyl- α -L-arabinofuranoside resulted in no activity being found for this xylanase, although some family 11 xylanases have been found to have arabinose-releasing properties (Wong *et. al.*, 1988). For reaction 3 there was an increase in the amount of arabinose released. This reaction allowed xylanase to first partially hydrolyse the arabinoxylan for 12 h. Then the α -L-arabinofuranosidase may have hydrolysed the remaining arabinoxyl oligosaccharides together with the xylanase. Reaction 4 tested whether synergy took place. Both enzymes were added and allowed to act together for 12 h. The peak areas for all three compounds were the highest of all reactions (Figure 4.44). The concentrations for arabinose, xylose and xylobiose in all five reactions are compared in Figure 4.45.

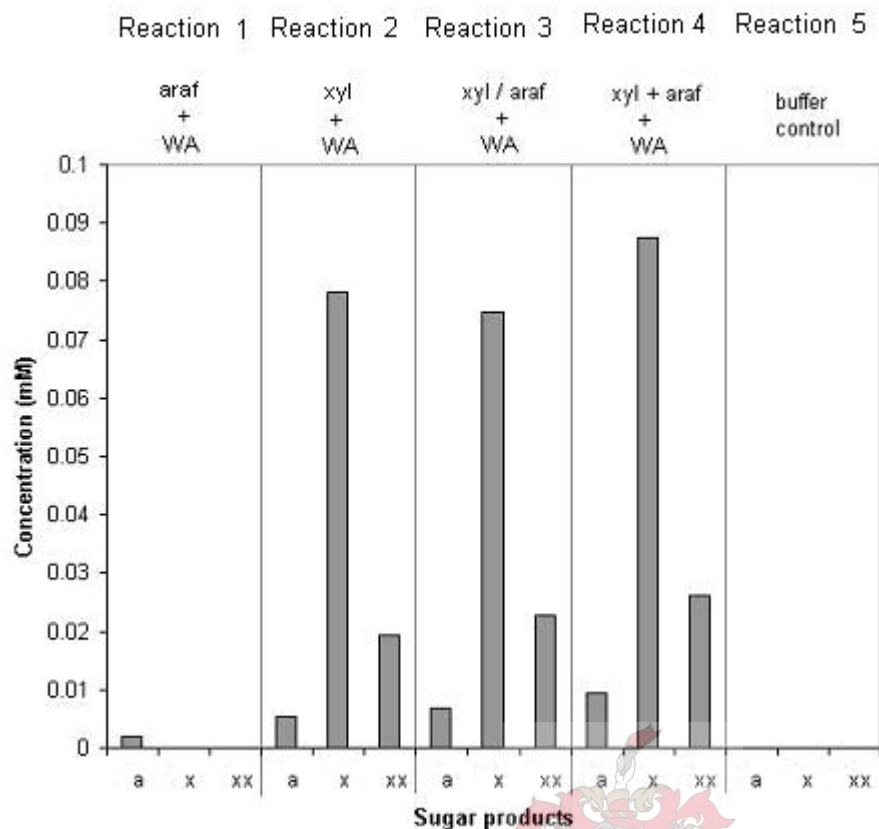


Figure 4.45. Mean concentrations of arabinose (a), xylose (x) and xylobiose (xx) after treatment of wheat arabinoxylan with α -L-arabinofuranosidase (reaction 1), xylanase (reaction 2), xylanase followed by α -L-arabinofuranosidase (reaction 3), xylanase and α -L-arabinofuranosidase (reaction 4) and no enzyme (reaction 5).

There was approximately 600 μ mol arabinose available from the substrate for each reaction. When the concentration values of the five reactions were converted to amount of arabinose (μ mol), there was a clear increase in arabinose through reactions 1 to 4 (Figure 4.46). Even though there was an unexplained amount of arabinose in reaction 2, the sum of reactions 1 and 2 (24.1 μ mol) was still lower than reaction 4 (31.6 μ mol). Therefore synergism occurred. In reaction 3 there was more arabinose released than in reaction 2. This indicated that after treated with xylanase, arabinose release from arabinoxylan was enhanced as the xylan backbone was degraded and α -L-arabinofuranosidase could leave side-chain groups more efficiently. But reaction 4 still displayed the highest arabinose release, therefore both enzymes should be present simultaneously for the best synergistic effect.

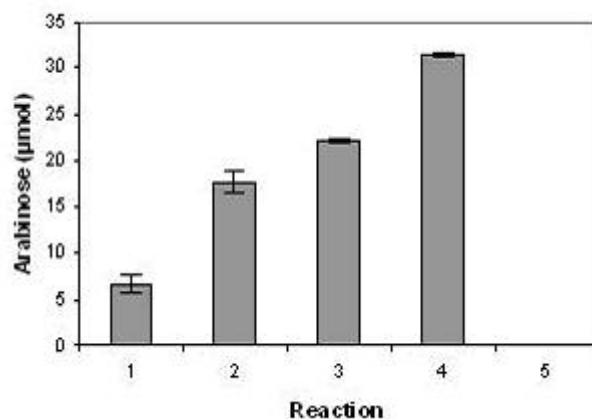


Figure 4.46. Amount (mean \pm standard deviation of four determinations) of arabinose release from wheat arabinoxylan when α -L-arabinofuranosidase (0.081 U/ml) was added to the five reactions. (see Figure 4.45 for further details of the reactions)

Table 4.6. Summary of the synergism reactions with the concentration of each sugar product given for each reaction.

Reaction number	Enzyme in reaction	Arabinose (mM) ^a	Xylose (mM) ^a	Xylobiose (mM) ^a
1	arabinofuranosidase	0.0019 \pm 0.0002	0	0
2	xylanase	0.0053 \pm 0.0007	0.0782 \pm 0.0010	0.0193 \pm 0.0057
3	arabinofuranosidase / xylanase	0.0067 \pm 0.0001	0.0746 \pm 0.0001	0.0227 \pm 0.0004
4	arabinofuranosidase + xylanase	0.0095 \pm 0.0001	0.0876 \pm 0.0021	0.0261 \pm 0.0024
5	buffer control	0	0	0

^a Mean \pm standard deviation of two determinations

4.4.11. Substrate-binding assay. The adsorption on four different insoluble substrates was tested with the purified heterologous α -L-arabinofuranosidase. In each case an amount of the added enzyme was adsorbed by these substrates. This was shown by the remaining activity after adsorption in the five enzyme concentration points which were less than initial activity values (Figure 4.47). The α -L-arabinofuranosidase appeared to bind most strongly to oat spelt arabinoxylan (33%) followed by birchwood xylan (22%) but bound poorly to wheat arabinoxylan (11%). Some binding to crystalline cellulose (Avicel) also occurred.

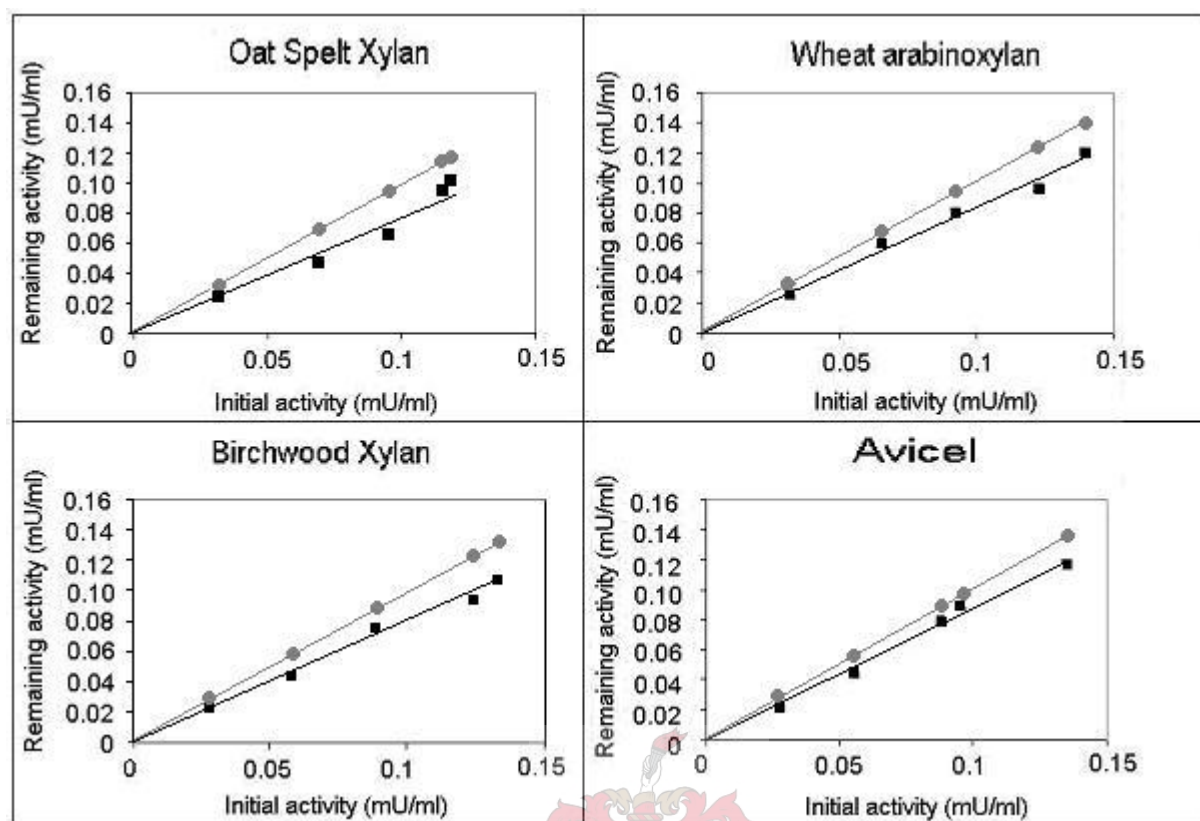


Figure 4.47. Adsorption of purified heterologous α -L-arabinofuranosidase on four insoluble substrates. Both initial (●) and remaining (■) activities are shown. Five enzyme concentration points were taken for each substrate.

4.5. PRODUCTION OF α -L-ARABINOFURANOSIDASE BY *A. PULLULANS* NRRL Y2311-1

Production of α -L-arabinofuranosidase only commenced after 35 h and attained an activity of 0.072 U/ml after 96 h (Figure 4.48). Most enzyme was extracellular as the cell associated activity was approximately 114-fold less (0.00063 U/ml) than that of the activity present in the supernatant.

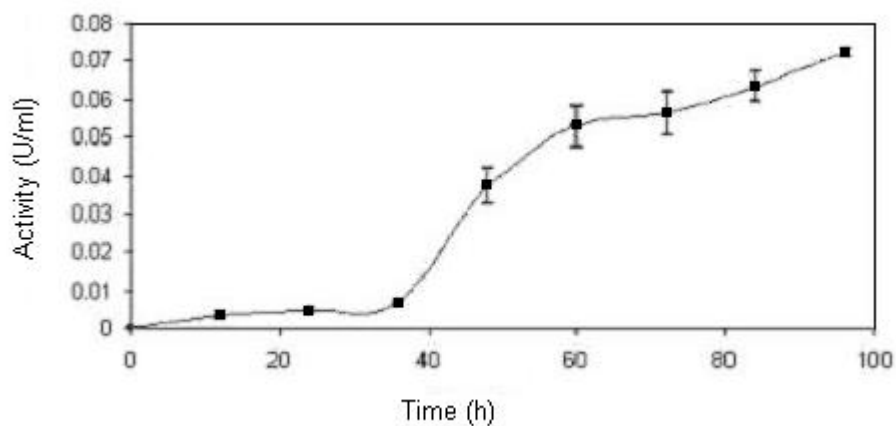
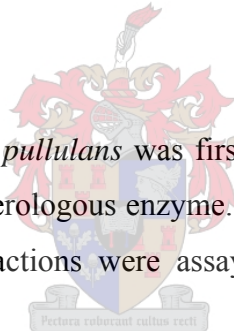


Figure 4.48. Production of extracellular α -L-arabinofuranosidase in the supernatant by *A. pullulans* NRRL Y2311-1 at 30°C.

4.6. PARTIAL PURIFICATION OF THE NATIVE α -L-ARABINOFURANOSIDASE

Step 1:

The concentrated supernatant of the *A. pullulans* was first run on the same gel filtration column using the same procedure as for the heterologous enzyme. The protein elution during gel filtration was monitored at 280 nm and the fractions were assayed for α -L-arabinofuranosidase under standard conditions (Figure 4.49).



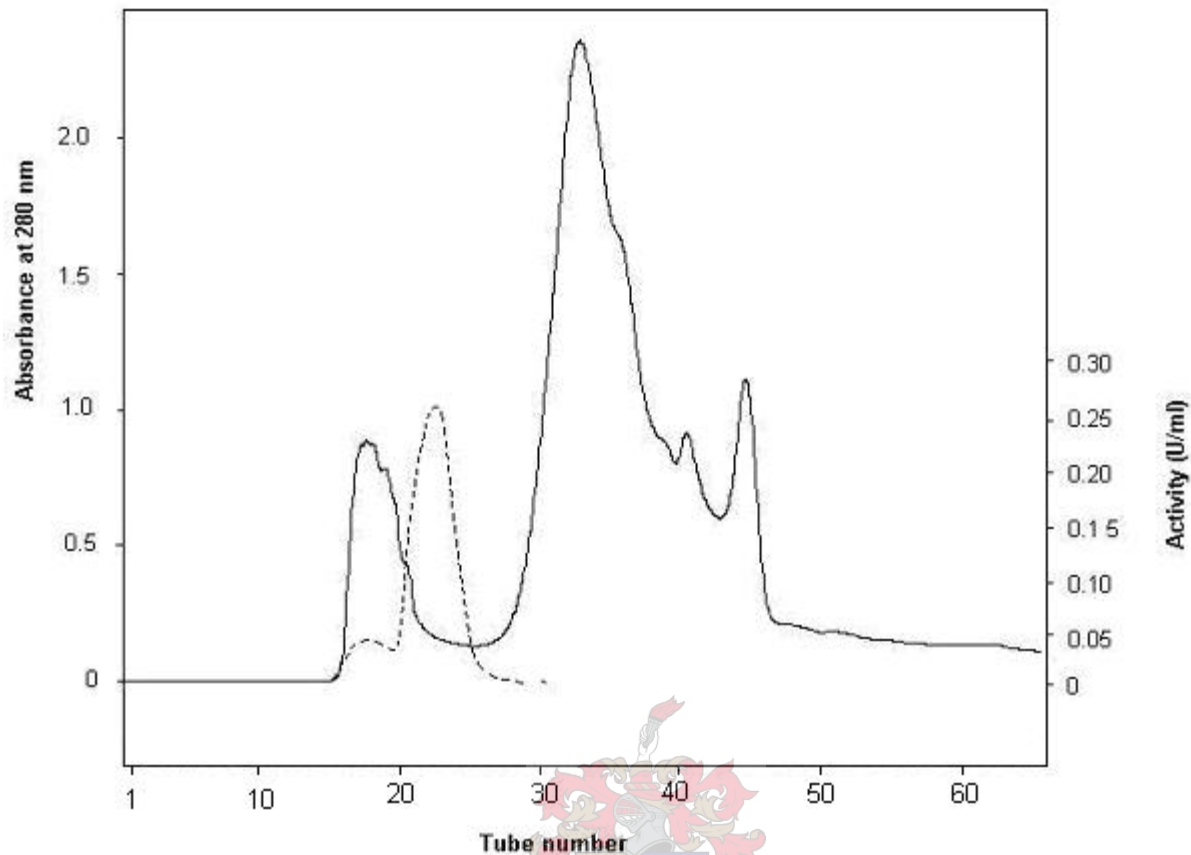


Figure 4.49. Gel filtration elution profile of the concentrated supernatant from *A. pullulans*. The absorbance at 280 nm is plotted as a solid line. The elution volume where α -L-arabinofuranosidase activity was present is indicated by a dashed line representing volumetric activity. Each tube contained 0.5 ml.

The tube containing the highest activity, 22 (0.255 U/ml), was applied again to the gel filtration column and the tubes were tested for activity (Figure 4.50).

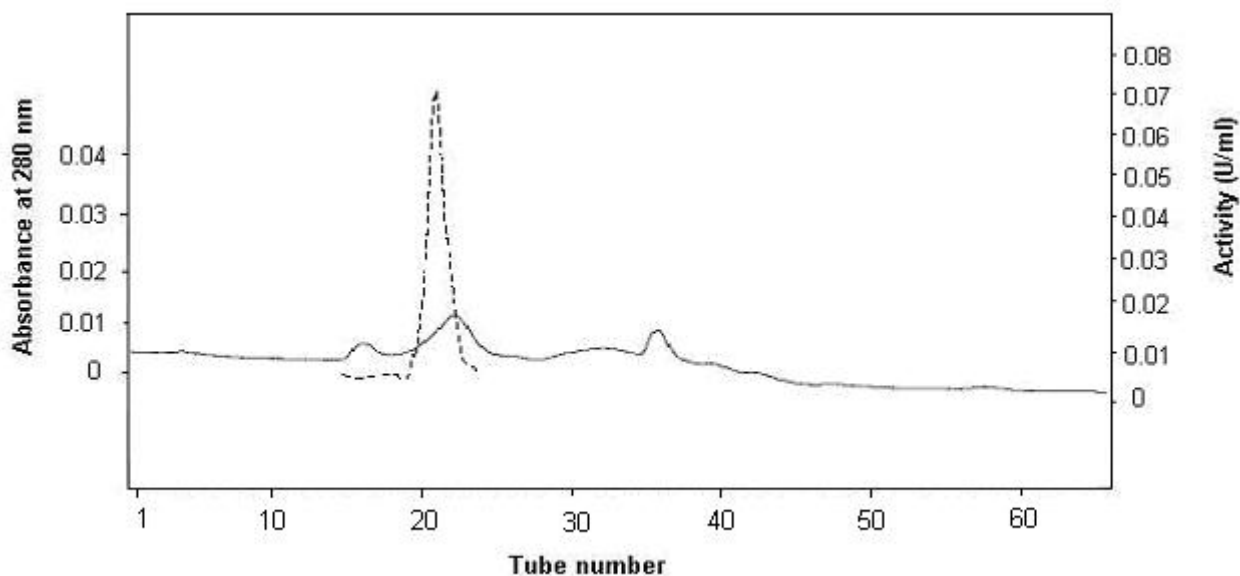


Figure 4.50. Gel filtration elution profile of the tube 22. The absorbance at 280 nm is plotted as a solid line. The elution volume where α -L-arabinofuranosidase activity was present is indicated by a dashed line representing volumetric activity. Each tube contained 0.5 ml.

The samples from the tubes with the highest activity, 21 (0.072 U/ml) and 22 (0.032 U/ml) from the second gel filtration, together with the concentrated supernatant and tubes 22 and 23 from the first gel filtration were separated on SDS-PAGE (Figure 4.51).

In the second gel filtration absorbance profile (Figure 4.50), it is clear that the peak for α -L-arabinofuranosidase is on the shoulder of another protein indicated by the absorbance at 280 nm. This protein was a contaminant in the first round of gel filtration and its presence was confirmed by SDS-PAGE. In lanes 2 and 3 of the SDS-PAGE, which contained tubes 22 and 23 from the first gel filtration, there were dark bands at 60 kDa. In lanes 4 and 5, which contained tubes 21 and 22 from the second gel filtration, these dark bands were less obvious, but the bands at 50 kDa, representing α -L-arabinofuranosidase, was still present.

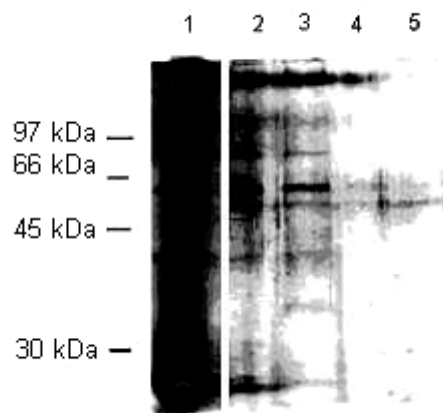


Figure 4.51. SDS-PAGE gel of the concentrated supernatant from *A. pullulans* (lane 1) and the gel filtration fractions containing the highest activity of both experiments. Lane 2: tube 22 of first gel filtration. Lane 3: tube 23 of the first gel filtration. Lane 4: tube 21 of the second gel filtration. Lane 5: tube 22 of the second gel filtration in step 1.

Step 2:

The α -L-arabinofuranosidase (2.5 U/ml) from the original concentrated supernatant of *A. pullulans* was then run on the anion exchange column. The protein elution was monitored at 280 nm and the fractions were assayed under standard conditions (Figure 4.52).

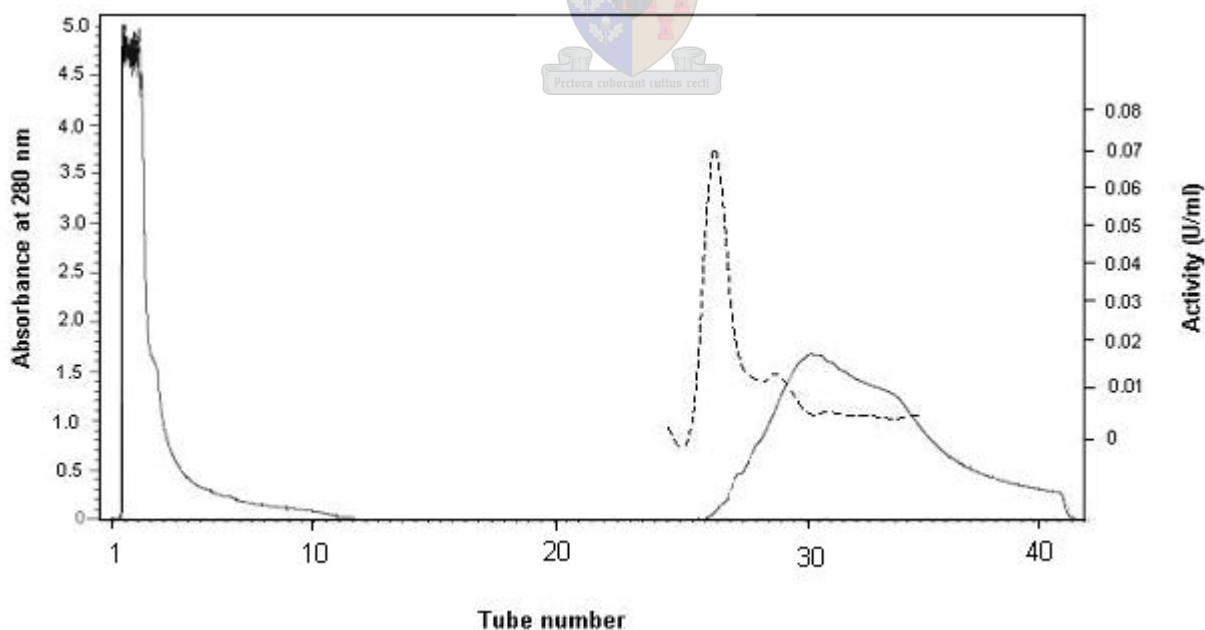


Figure 4.52. Anion exchange elution profile of the concentrated supernatant from *A. pullulans*. The absorbance at 280 nm is plotted as a solid line. The elution volume where α -L-arabinofuranosidase activity was present is indicated by a dashed line representing volumetric activity. Each tube contained 0.5 ml.

The tubes with the highest activity, 26 (0.069 U/ml), 27 (0.021 U/ml) and 28 (0.012 U/ml) were run on SDS-PAGE (see lane 4, 5 and 6 in Figure 4.53).

Step 3:

The tube 22 from the second round of gel filtration (0.032 U/ml) was run on the anion exchange column. The protein elution was monitored at 280 nm and the fractions were assayed under standard conditions but no activity was found. The tube fractions from this anion exchange column run that were expected to contain α -L-arabinofuranosidase (26, 27, 28) were also run on SDS-PAGE (see lanes 1, 2 and 3 in Figure 4.53).

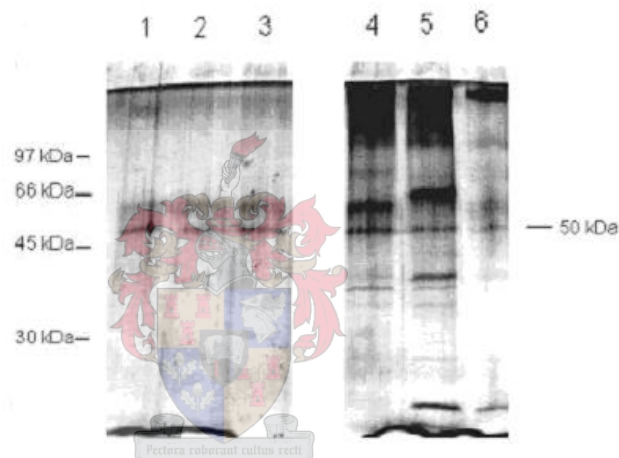


Figure 4.53. SDS-PAGE of three samples from the second gel filtration tube 22 run on the anion exchange column (step 3) and three samples from the concentrated supernatant run on the anion exchange column (step 2). Lane 1: tube 26 of gel filtration fraction 22 run on the anion exchange column. Lane 2: tube 27 of gel filtration fraction 22 run on the anion exchange column. Lane 3: tube 28 of gel filtration fraction 22 run on the anion exchange column. Lane 4: tube 26 of the concentrated supernatant run on the anion exchange column. Lane 5: tube 27 of the concentrated supernatant run on the anion exchange column. Lane 5: tube 28 of the concentrated supernatant run on the anion exchange column.

There were bands at 50 kDa indicating the presence of α -L-arabinofuranosidase in lanes 1, 2 and 3 but no activity was found in these samples. Activity was found for the samples run in lanes 4, 5 and 6 but these were not pure.

Step 4:

The tube with the highest activity, 26 (0.069 U/ml), from the anion exchange column of the concentrated supernatant (step 2 and lane 4, Figure 4.53) was run on the gel filtration column. The protein elution was monitored at 280 nm and the fractions were assayed under standard conditions (Figure 4.54).

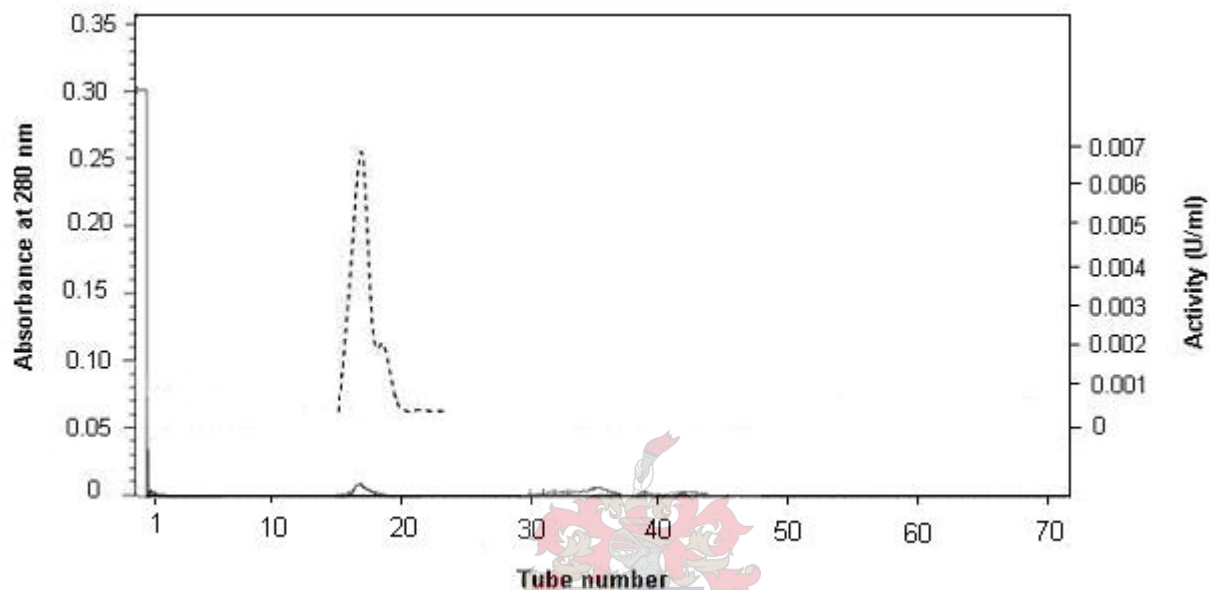


Figure 4.54. Gel filtration elution profile of tube 26 from step 2 of anion exchange chromatography. The absorbance at 280 nm is plotted as a solid line. The elution volume where α -L-arabinofuranosidase activity was present is indicated by a dashed line representing volumetric activity. Each tube contained 0.5 ml.

The tubes with the highest activity, 16 (0.004 U/ml), 17 (0.007 U/ml) and 18 (0.002 U/ml) were run on SDS-PAGE (see lane 1, 2 and 3 in Figure 4.55).

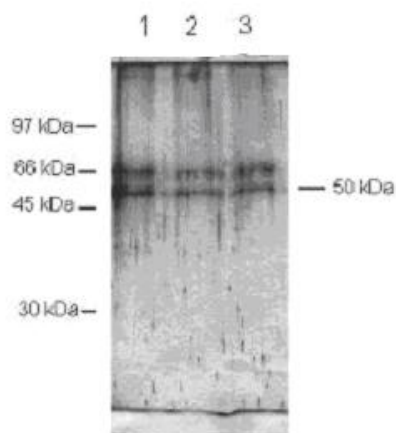


Figure 4.55. SDS-PAGE of three of the gel filtration samples of tube 26 from anion exchange (step 4). Lane 1: tube 16, Lane 2: tube 17, Lane 3: tube 18.

In all three lanes there was one contaminating band at approximately 60 kDa in addition to the band at 50 kDa that represented the α -L-arabinofuranosidase.

Step 5:

The tube 23 (Figure 4.49 and Figure 4.51 lane 3) from the first round of gel filtration in step 1 (0.225 U/ml) was run on the anion exchange column. The protein elution was monitored at 280 nm and the tubes 21-30 were assayed under standard conditions but no activity was found.

Hydrophobic interaction was attempted on the heterologous enzyme and was completely unsuccessful so therefore this was not attempted again with the native enzyme. Table 4.8 summarizes the most successful step and the yield achieved. The first step using a double round of gel filtration was the most pure as indicated by SDS-PAGE.

Table 4.8. Purification summary of the concentrated supernatant and the partially successful step 1.

	Activity (U/ml)	Protein (mg)	Specific activity (U/mg)	Yield %
Concentrated supernatant	1.128	0.75	1.5	100
Purification step 1	0.072	0.22	0.33	21.8

4.7. CHARACTERISATION OF THE NATIVE α -L-ARABINOFURANOSIDASE

Since the native enzyme was only partially purified, the most pure sample according to SDS-PAGE results was used. The tube 21 (0.072 U/ml) from the second gel filtration attempt (Figure 4.50) of step 1 (see lane 4 of Figure 4.51) was used for characterization experiments.

4.7.1. Molecular weight estimation by SDS-PAGE. From Figure 4.51 lanes 4 and 5, it is visible that tubes 21 and 22 (Figure 4.50, step 1) contain a band at approximately 51.5 kDa (Figure 4.56). These samples had activities of 0.072 U/ml and 0.032 U/ml. Similar results were found for the heterologous enzyme (see section 4.4.1.).

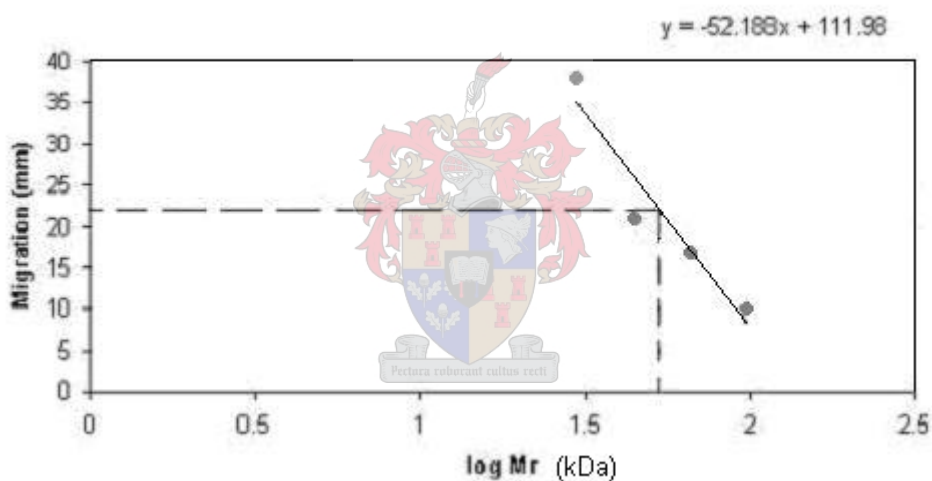


Figure 4.56. The log Mr of the protein markers (Rainbow Molecular weight markers, Pharmacia. 220, 97, 66, 45 and 30 kDa) against distance migrated on the SDS-PAGE gel. The dashed line represents the plotted α -L-arabinofuranosidase.

4.7.2. Temperature dependence. The enzyme showed maximum activity at 50°C in 50 mM acetate buffer at pH 4 (Figure 4.57).

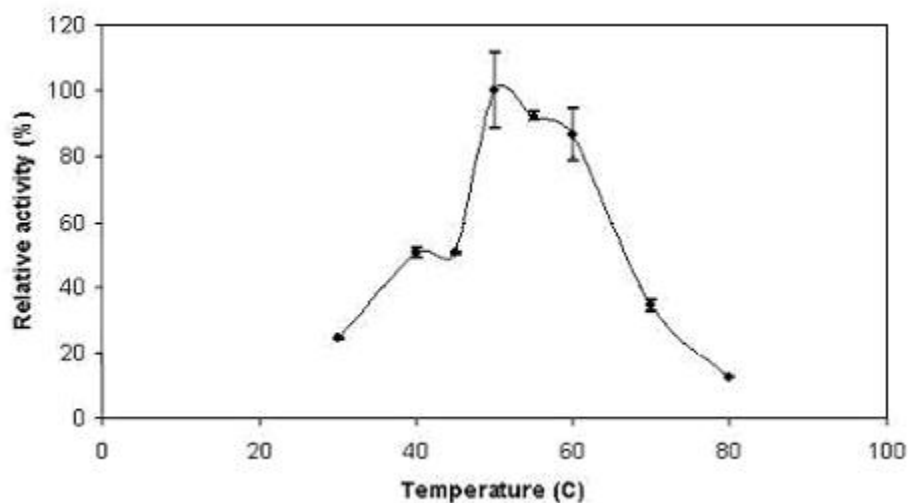


Figure 4.57. Effect of temperature on the activity (mean \pm range of 2 determinations) of the partially purified native α -L-arabinofuranosidase. 100 % activity corresponds to 0.124 U/ml.

4.7.3. pH dependence. α -L-arabinofuranosidase displayed maximum activity at pH 3 (Figure 4.58).

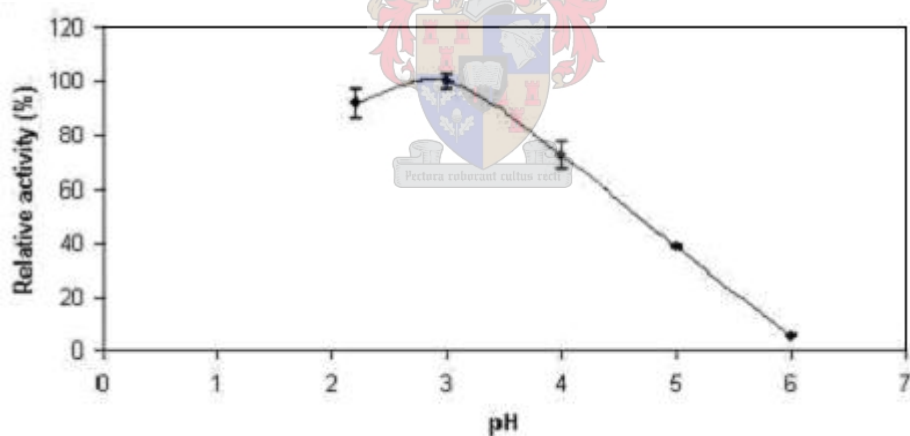


Figure 4.58. Effect of pH on the activity (mean \pm range of 2 determinations) of the partially purified native α -L-arabinofuranosidase. 100 % activity corresponds to 0.928 U/ml.

4.7.4. Kinetic analysis. The rate dependence of the enzymatic hydrolysis of *p*-nitrophenyl- α -L-arabinofuranoside was measured at different concentrations. α -L-arabinofuranosidase activity was assayed at 55°C and the direct plot of the data is shown in Figure 4.59.

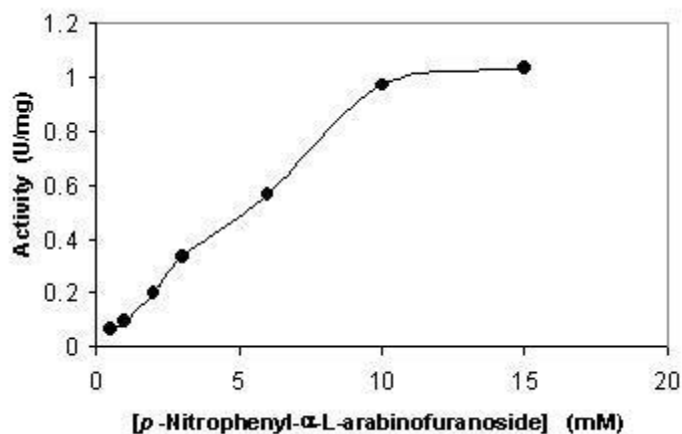


Figure 4.59. α -L-arabinofuranosidase activity as a direct function of *p*-nitrophenyl- α -L-arabinofuranoside concentration. A range from 0.5 to 15 mM substrate was assayed. Each data point represents the mean of two determinations.

From the Lineweaver-Burk plot, a K_m of 8.33 mM and a V_{max} of 1.54 U/mg was calculated whereas the Hanes plot gave a K_m value of 31 mM and a V_{max} of 3.52 U/mg (Figure 4.60).

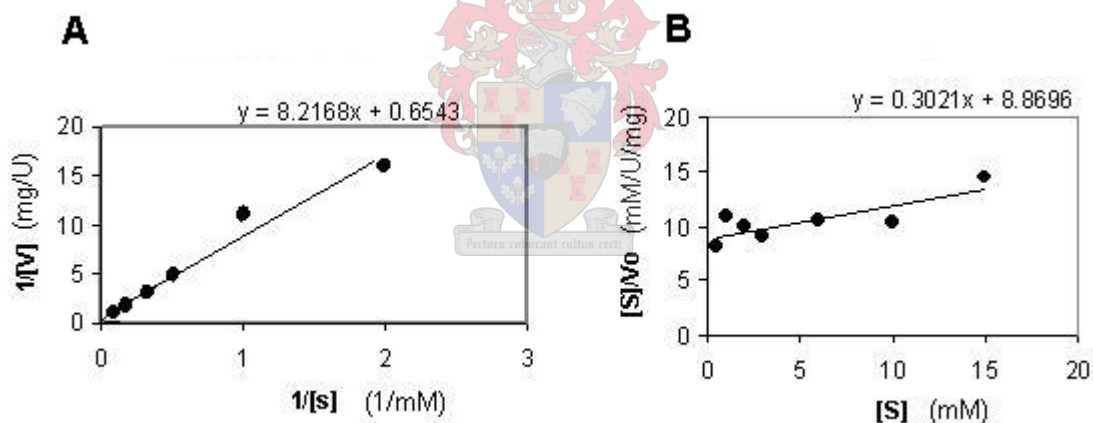


Figure 4.60. Lineweaver-Burk (A) and Hanes (B) linear plots for the effect of *p*-nitrophenyl- α -L-arabinofuranoside concentration on the initial rate of the reaction.

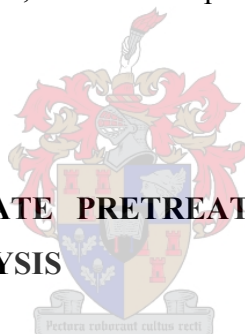
The kinetic constants obtained using Lineweaver-Burk and Hanes plots show that the reaction rate of the native partially pure α -L-arabinofuranosidase for the *p*-nitrophenyl- α -L-arabinofuranoside was lower than the pure heterologous enzyme. These differences could be ascribed to the partial purity of the native enzyme but the two enzymes appear to have similar kinetic properties.

Table 4.9. Comparison of the kinetic properties of the heterologous and native enzymes.

Arabinofuranosidase	Kinetic method	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat} / K_m ($M^{-1}s^{-1}$)
Heterologous	Lineweaver-Burk	1.43	22.7	18160	12699
	Hanes	4.51	41.5	13800	7051
Native	Lineweaver-Burk	8.33	1.54	24062	2888
	Hanes	31	3.52	58666	1892

4.7.5. Substrate specificity on α -L-arabinotriose. The 1,5- α -L-arabinotriose was assayed over 24 h to investigate possible arabinose and arabinobiose release. An increase in arabinobiose and arabinose was observed over 24 h and therefore indicated that the native enzyme had similar properties to that of the heterologous enzyme. An arabinose increase of 0.017 mM was calculated which was equivalent to 0.0015 μ mol arabinose/min/mg protein, which indicated a very low activity on the 1,5- α -L-linkage. This rate was considerably lower than that found with the pure heterologous enzyme but this can be ascribed to the native enzyme only being partially pure and it also underwent two steps of gel filtration, whereas the pure heterologous enzyme only underwent one step of gel filtration.

4.8. EVALUATION OF SUBSTRATE PRETREATMENT ON SUGAR CANE AND BAGASSE ENZYMATIC HYDROLYSIS



The four samples (CLM/140°C, BAG/140°C, Pith/AFEX, CLM/AFEX) of sugarcane and bagasse showed varying amounts of arabinose, xylose and xylobiose release due to the different methods used to prepare these substrates. All four substrates were allowed to react with α -L-arabinofuranosidase and xylanase and the release of sugars was compared to the amount present in the control, which contained no enzymes.

The average amount of arabinose, xylose and xylobiose detected by HPAEC is displayed for the four substrates (Figures 4.61).

The substrates treated with liquid ammonia (AFEX) showed greater release of arabinose and xylobiose but not xylose, than substrates treated with water at 100°C.

Further investigation of sugar peak profiles detected by HPAEC in the control and assay samples was performed using Figure 4.62. Sugar cane leaf matter treated with water at 140°C for 1 h (CLM/140°C) and with NH₃ at 100°C for 30 min (CLM/AFEX) were compared. Sugar cane bagasse treated with water at 140°C for 1 h (BAG/140°C) and sugar cane pith treated liquid ammonia at 100°C for 30 min (Pith/AFEX) were also compared (Figure 4.63).



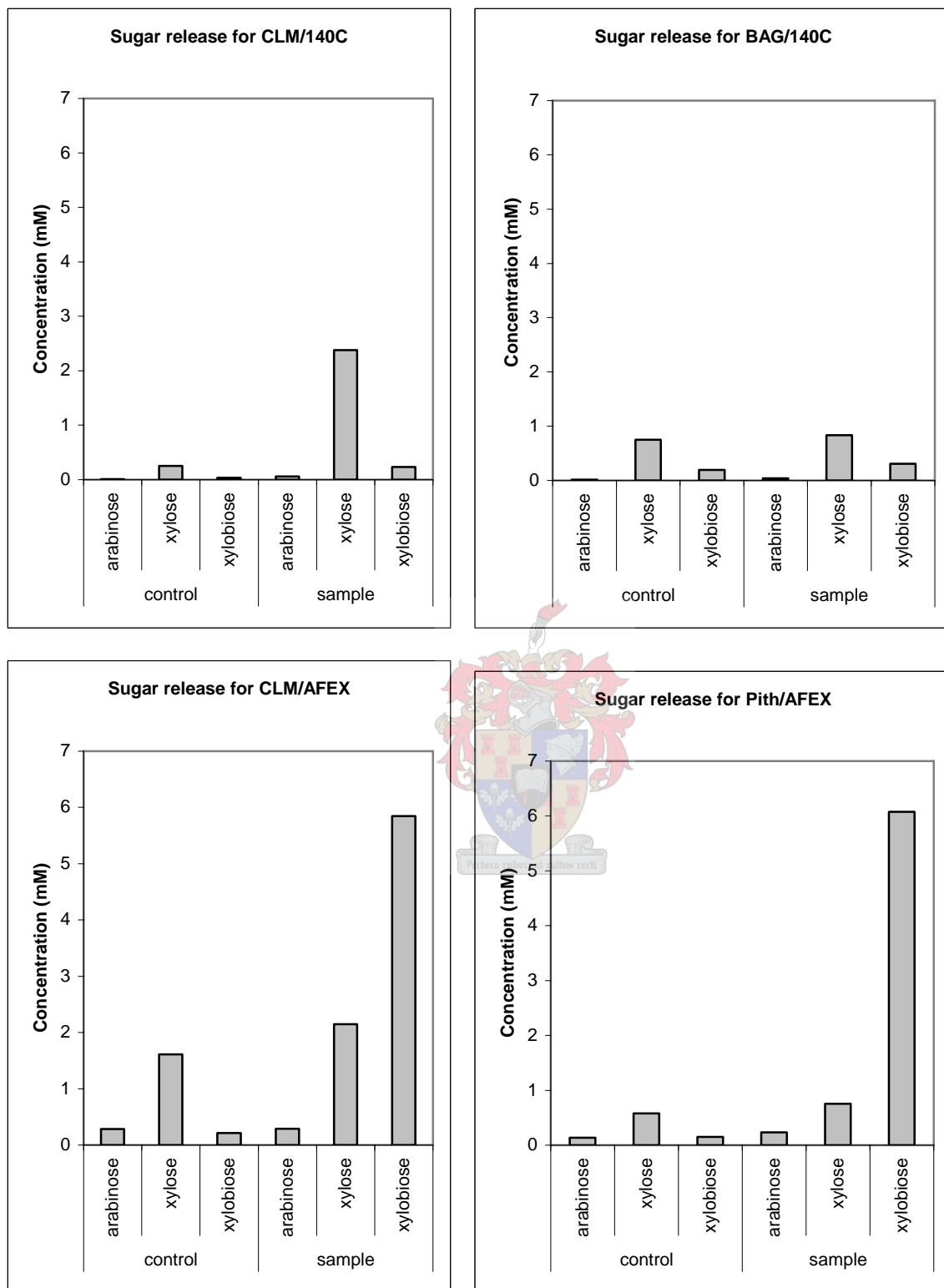


Figure 4.61. Change in concentration for the average amount of arabinose, xylose and xylobiose detected by HPAEC in the control and assay samples. CLM/140°C = sugar cane leaf matter treated with water at 140°C for 1 h. BAG/140°C = bagasse treated with water at 140°C for 1 h. CLM/AFEX was sugar cane leaf matter treated with NH₃ (2:1) at 100°C for 30 min. Pith/AFEX = sugar cane pith treated with liquid NH₃ at 100°C for 30 min.

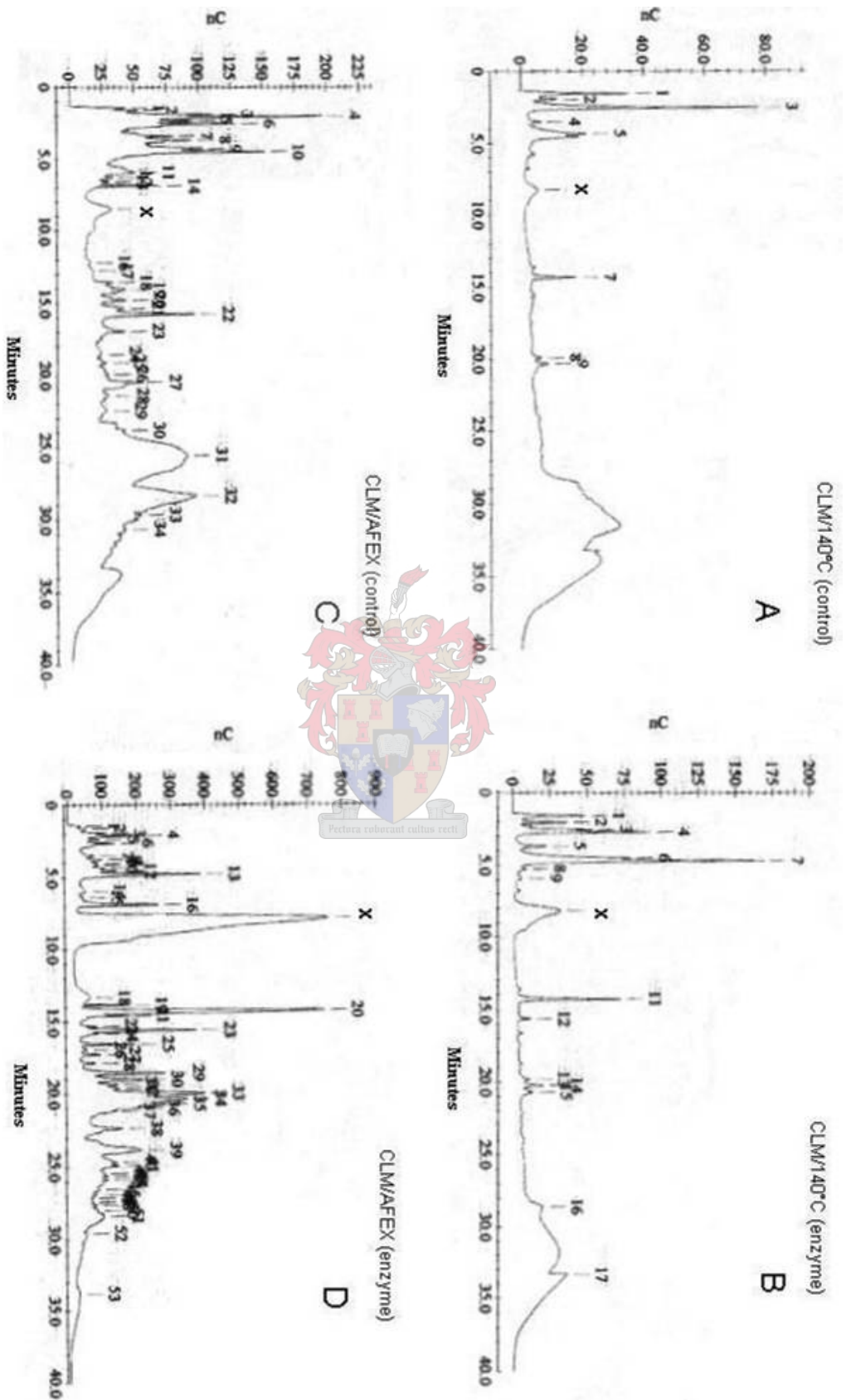


Figure 4.62. HPLC profiles of sugarcane leaf matter treated with water at 140°C for 1 h (A, B) or with liquid ammonia at 100°C for 30 min (C, D). Samples treated with xylanase and α -L-arabinofuranosidase for 4 h are shown in B and D. The peak representing xylobiose is marked with an X.

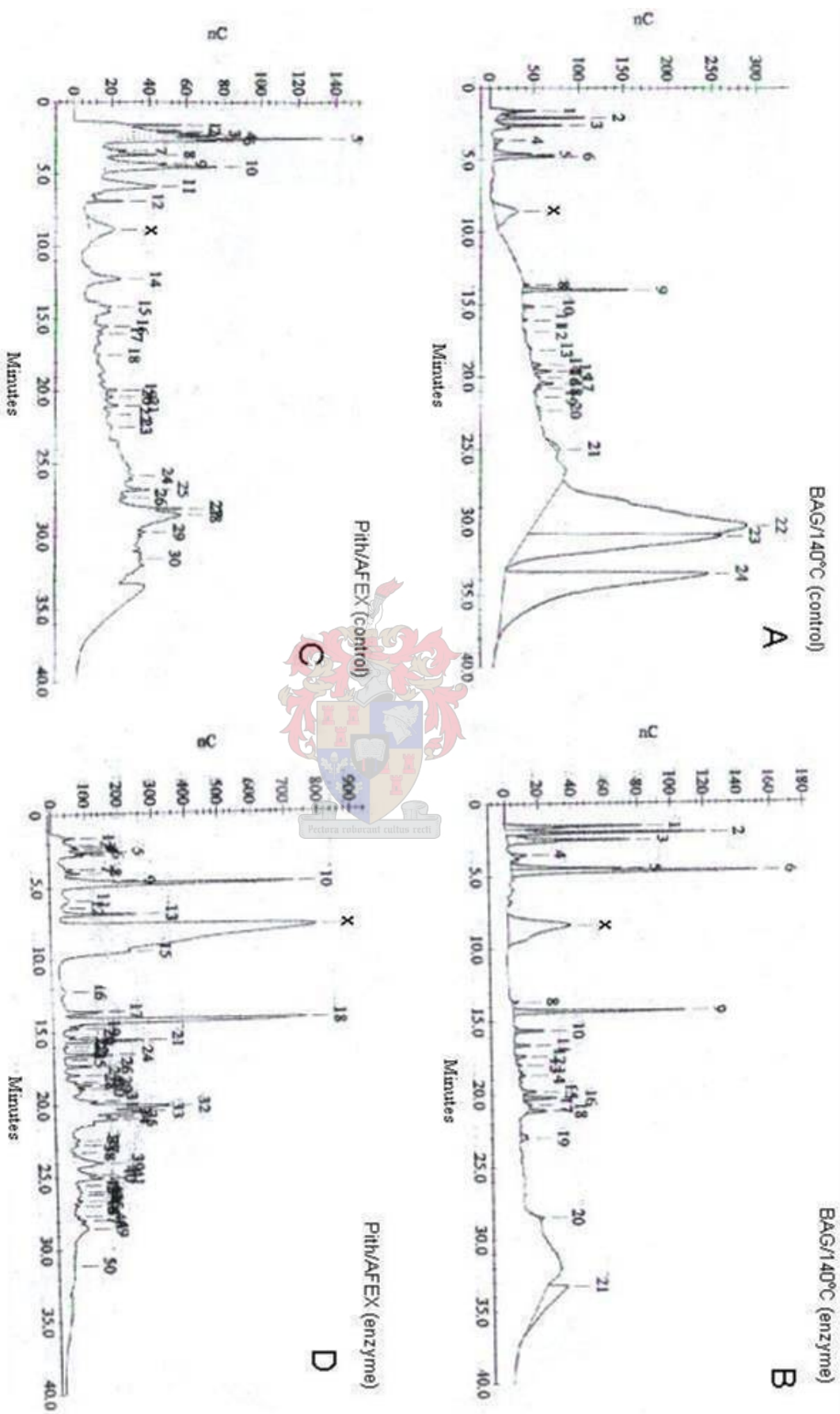


Figure 4.63. HPAEC profiles of sugar cane bagasse treated with water at 140°C for 1 h (A, B) or sugar cane pith treated liquid ammonia at 100°C for 30 min (C, D). In addition, HPAEC profiles of samples treated with xylanase and α -L-arabinofuranosidase for 4 h are shown in B and D. The peak representing xylobiose is marked with an X.

A comparison of samples treated with water at 140°C with those treated with liquid ammonia showed that there was a significant concentration of sugars present in the liquid ammonia treatment before enzyme treatment which suggested that partial sugar release had already occurred. In both Figures 4.62 and 4.63 there is a large increase in the peak area (nC) for xylobiose (X) in the assay samples, which appeared at approximately 8 minutes. Xylobiose is a breakdown product caused by the action of xylanase.

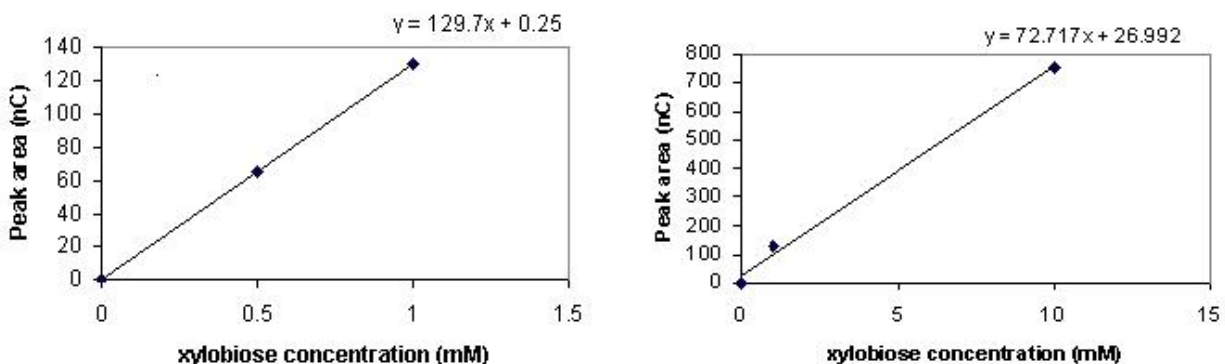


Figure 4.64. Standard curves used to determine the concentration of the sugar from the HPAEC profiles.

From the standard curve (Figure 4.64), an increase in xylobiose concentration was calculated in the CLM samples for direct comparison. In the CLM/140°C control there was 0.0366 mM xylobiose present, while in the after enzyme treated sample there was 0.229 mM present, which was 6.3 times more. The initial xylobiose concentration in the CLM/AFEX sample was 0.214 mM and was 5.846 mM in the after enzyme treated sample. This was 27.3 times more and indicated that the liquid ammonia treatment made the substrate more accessible to xylanase and possibly other hemicellulose-degrading enzymes. It was also noted that the initial CLM/140°C control sample contained 5.8 times less xylobiose than the CLM/AFEX assay sample.

Other than xylobiose showing a significant increase in samples treated liquid ammonia compared to those treated with water at 140°C, there were also many other peaks that increased in the CLM/AFEX samples.

4.9. MODELLING OF THE HETEROLOGOUS α -L-ARABINOFURANOSIDASE

The α -L-arabinofuranosidase (AkabfB) from *A. kawachii* was the first family 54 glycosyl hydrolase to have its crystal structure solved (Miyanaga *et al.*, 2004a). This structure was used as a structural template for the construction of a homology model of the α -L-arabinofuranosidase (AbfA) from *A. pullulans* expressed in *S. cerevisiae*. After a BLAST comparison of the two sequences, they were found to be 71% identical and therefore AbfA was similar enough to AkabfB for further modelling experiments. From the alignment file created with MODELLER, it was apparent that there was an 18 amino acid sequence in AbfA that corresponded with the 17 amino acid signal peptide of AkabfB. This 18 amino acid sequence was removed from the protein's sequence prior to modelling (Figure 4.65).



```

_aaln.pos      10      20      30      40      50      60
AkabfB      MGPCDIYEAGDTPCVAAHSTTRALYSSFSGALYQLQRGSDDTTTTISPLTAGGIADASAQDTFCANTT
AbfA        AAPCDIYQNGGTPCVAAHGTTRALYDYSYTGPLYQLKRGSDGTTTDISPLSAGGVANAAAQDSFCKGTT
_consrvd     ***** * ***** ***** * * ***** ***** ***** ***** * * * * * * * *
_aaln.p       70      80      90      100     110     120     130
AkabfB      CLITIIYDQSGNGNHLTQAPPGGFDGPDTDGYDNLASAIIGAPVTLNGQKAYGVFMSPGTGYRNEATG
AbfA        CLISIIYDQSGRANHLTYAQKGAFGSPDVNGNDNLAGAIGAPVTLNGKAYGVFISPGTGYRNDVSG
_consrvd     *** ***** ** * * * * * * * * * * * * * * * * * * * * * * * *
_aaln.pos     140     150     160     170     180     190     200
AkabfB      TATGDEAEGMYAVLDGTHYNDACCFDYGNAETSSTDTGAGHMEAIYLGSTTWGYGAGDGPWIMVDME
AbfA        TATGNEPEGMYAVLDGTHYNDACCFDYGNAEISNTDTGNHMEAVYGNNTIWSGSGSGPWLMADLE
_consrvd     **** * ***** ***** * * * * * * * * * * * * * * * * * *
_aaln.pos     210     220     230     240     250     260     270
AkabfB      NNLSFGADEGYNSGDPSISYRFVTAAVKGGADKWAIRGANAAAGSLSTYYSGARPDYSGYNPMSKEGA
AbfA        NGLFSGQGTQNTADPSISNRFFTGMVKGEPNQWALRGSNAASGSLSTYYSGARPTVGGYNPMSLEGA
_consrvd     * **** * ***** ** * * * * * * * * * * * * * * * * * *
_aaln.pos     280     290     300     310     320     330     340
AkabfB      IILGIGGDNENGAQGTFFYEGVMTSGYPSDDTENSVDQENIVAACYVVGSLVSGPSFTSGEVVSLRVVTP
AbfA        IILGIGGDNENGAQGTFFYEGVMTSGYPSDATEASVQANIVAACYATTSLNTAP-LTVGNKISIKVTP
_consrvd     ***** ***** * * * * * * * * * * * * * * * * * *
_aaln.pos     350     360     370     380     390     400
AkabfB      GYTTRYIAHTDTTVNTQVVDDSSSTTLKEEASWTVVTGLANSQCFSFESVDTPGSYIRHYNFELLNA
AbfA        GYDTRYLAHTGATVNTQVVSSSATSLKQASWTVRTGLGNSGCYSFESVDTPGSFIRHYNFQLQLNA
_consrvd     ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
_aaln.p       410     420     430     440     450     460     470
AkabfB      NDGKQFHEDATFCPQAALNGEGTSLRSWYPTRYFRHYENVLYAASNGGVQTFDSKTSFNNDVVSFEI
AbfA        NDNTKAFKEDATFCSQTGL-VTGNTFNSWYPAKFI RHYNVVGVIASNGGVHDFDSATGFNNDVVSFVV
_consrvd     ** * * * * * * * * * * * * * * * * * * * * * * * * * * * *
_aaln.pos     480
AkabfB      ETAFAS |
AbfA        GSSFA-
_consrvd     **

```

Figure. 4.65. Alignment file text file of the two amino acid sequences from *A. kawachii* (AkabfB) and *A. pullulans* (AbfA) built with MODELLER. (* indicate conserved residues).

The energy report from MODELLER for the homology model of AbfA was compared to the energy report of AkabfB. This energy report represents the status of all the forces, including ionic and van der Waals, involved in a structure. The more negative the energy value, the more favourable the atomic arrangement is. Any crystal structure would naturally have a low energy value. A homology model will therefore have a higher energy value than the structural template it was based on. The closer the two energy values are, the more accurate the homology model is. The energy value for AkabfB crystal structure was -24710 and for the AbfA homology model was -20608. Because these values were similar, no further correction of errors was undertaken.

Schematic diagrams of the homology model of AbfA were produced using Swiss-pdb viewer. Both AbfA and AkabfB had a similar structure (see Figure 4.66), consisting of a catalytic and an arabinose-binding domain. The catalytic domain of the *A. kawachii* AkabfB could bind one arabinose and the arabinose-binding domain could bind two arabinose molecules (Miyanaga *et al.*, 2004a).

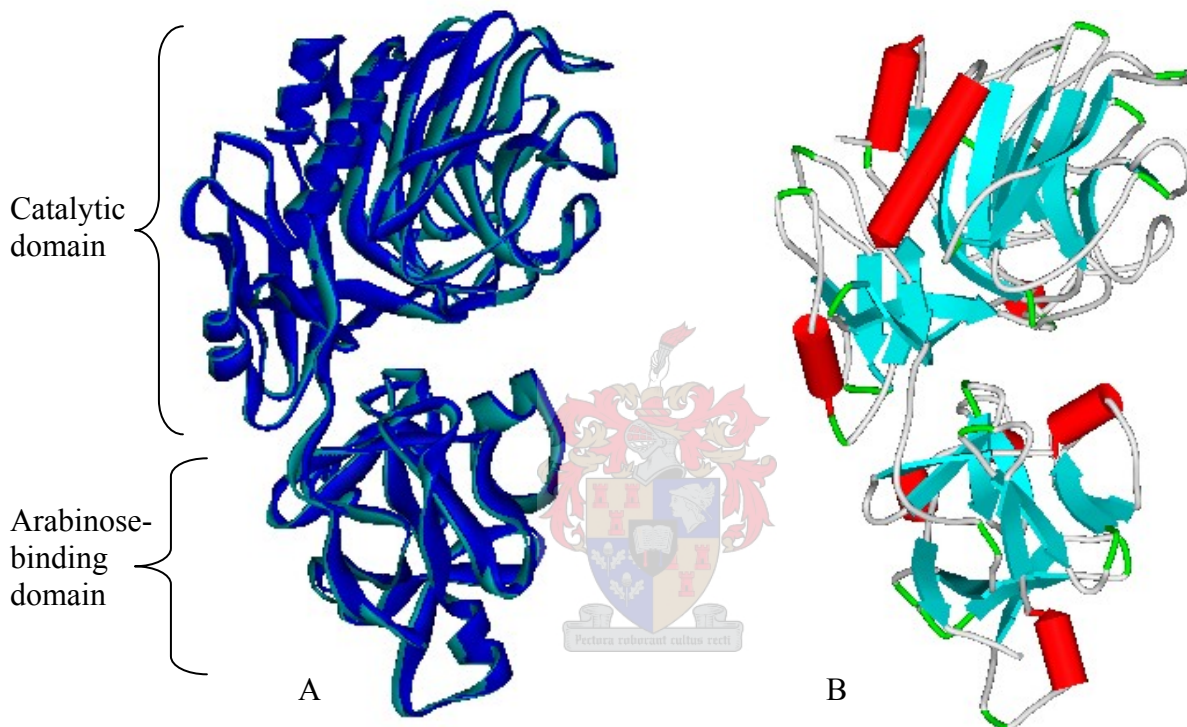


Figure 4.66. The ribbon overlay structure (A) shows the AkabfB crystal structure (green) and the AbfA homology model (blue). The schematic structure shows the β -sheets and α -helices of the homology model (B).

The differences and similarities of the *A. kawachii* AkabfB crystal structure and the *A. pullulans* AbfA homology model were then compared. The properties conserved in all Family 54 glycosyl hydrolase structures are 4 disulphide bonds and the same number of important residues involved in catalysis and substrate binding. In AkabfB there were 3 disulphide bonds in the catalytic domain, Cys¹⁷⁶-Cys¹⁷⁷, Cys⁸¹-Cys⁸⁶, Cys²¹-Cys³¹, and 1 in the arabinose-binding domain, Cys⁴⁰¹-Cys⁴³⁹ (Miyanaga *et al.*, 2004a). All these disulphide bonds were also found in AbfA at the same positions (Figure 4.67 A). Important residues involved in catalysis and substrate binding, Asp²⁹⁷, Leu²²⁴, Asn²²², Glu²²¹, Asp²¹⁹, Trp²⁰⁶, Met¹⁹⁵, Cys¹⁷⁷, Cys¹⁷⁶ were also present in the homology model

(Figure 4.67 B). On both sides of the anomeric C-1 carbon of α -L-arabinofuranosidase the carboxyl groups of Asp297 and Glu221 are found which confirms that these are catalytic residues.

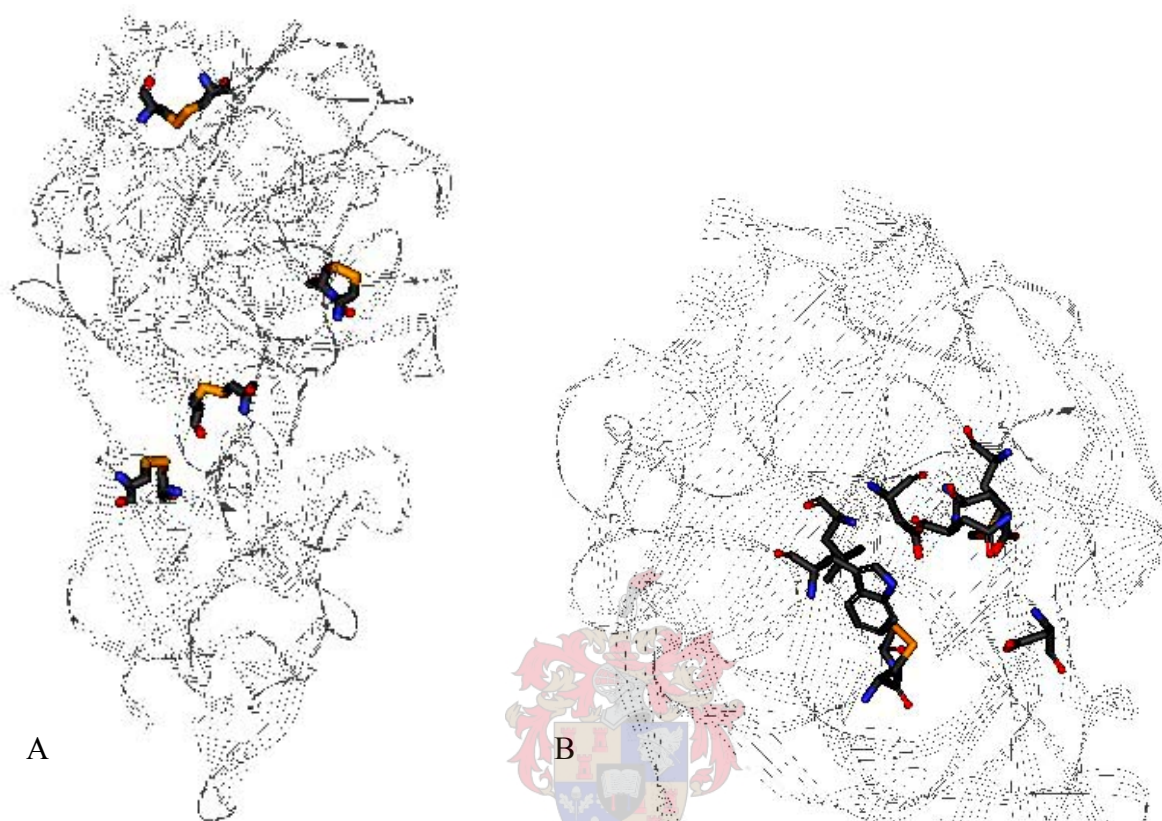


Figure 4.67. AbfA homology model highlighting the four disulphide bonds (A). A different angle of the homology model highlighting the important residues involved in catalysis and substrate binding (B).

The active site in the catalytic domain of AbfA was located using the important residues involved in catalysis. These residues (Asp²⁸⁰, Leu²⁰⁷, Asn²⁰⁵, Glu²⁰⁴, Asp²⁰², Trp¹⁸⁹, Met¹⁷⁸, Cys¹⁶⁰, Cys¹⁵⁹) were present in the region of the negatively charged pocket (Figure 4.68 A). The two active sites in the arabinose-binding domain were located in the region of the Tyr⁴⁰⁰ in the β subdomain and the Tyr⁴³⁹ in the γ subdomain (Figure 4.68 B). The residues were highlighted in the ribbon structure as yellow structures.

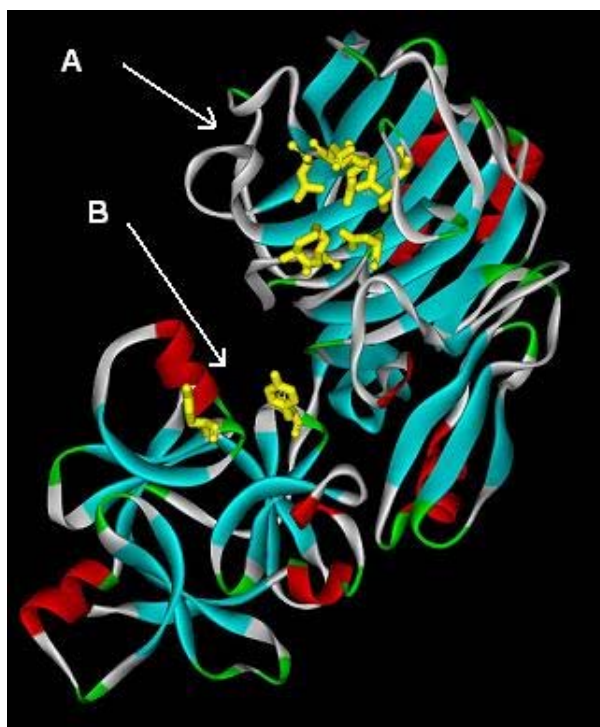


Figure 4.68. Ribbon structure of the AbfA homology model. The important residues involved in catalysis (A) in the catalytic domain and the Tyr residues (B) in the arabinose-binding domain are shown in yellow.

The molecular surface of the homology model was rendered showing the active sites (Figure 4.69).

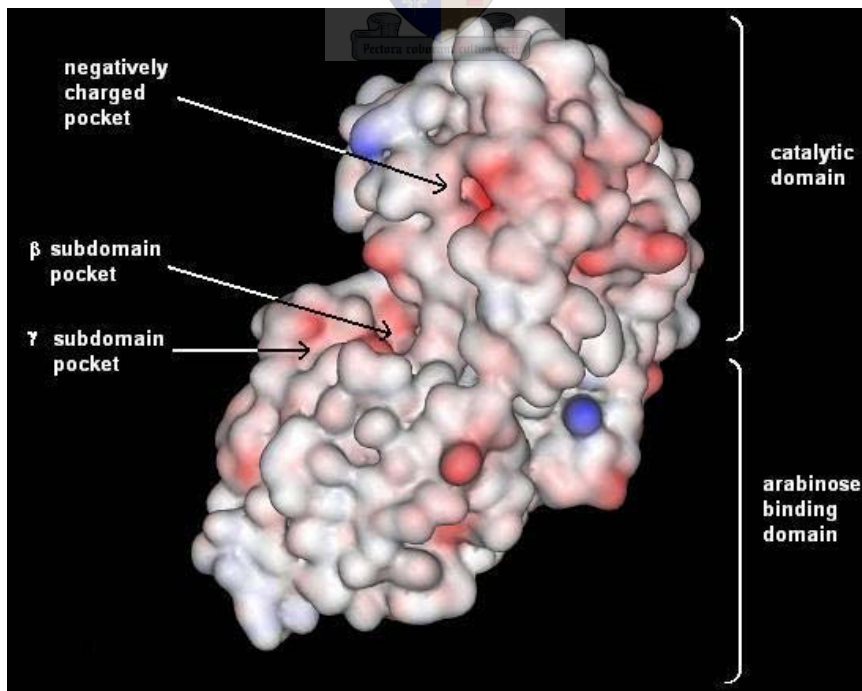
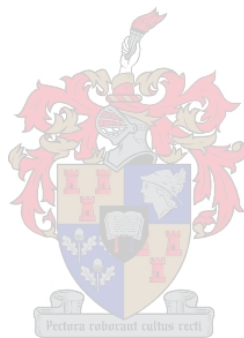


Figure 4.69. The molecular surface of AbfA.

Further analysis and accurate three-dimensional enzyme-ligand binding could be achieved by using docking software such as AutoDock (Goodsell *et al.*, 1990; Morris *et al.*, 1996; Morris *et al.*, 1998; <http://www.scripps.edu/mb/olson/doc/autodock>). This would allow correlation of experimental data from the kinetic studies of the substrates already tested. The substrates tested such as arabinoxylans and arabino-oligosaccharides could be modelled and docked with the α -L-arabinofuranosidase homology model to generate binding values. From these values an indication of three-dimensional spatial interaction could be compared to the amount of activity α -L-arabinofuranosidase had against each substrate. If, for example, the binding values were low for a given substrate and experimentally there was little activity against the substrate, both sets of data would be supported by each other.



5. DISCUSSION

Comparing heterologous α -L-arabinofuranosidase production of the two yeast strains.

(Refers to section 4.2)

The production level of α -L-arabinofuranosidase produced by *S. cerevisiae* Y294 [pDLG1-APabfA] was similar to that found for *A. pullulans* NRRL Y-2311-1 (Myburgh *et al.* 1991b).

Growth of *S. cerevisiae* Y294 [pDLG1-APabfA] on SC was restricted, resulting in lower cell density and enzyme production. The *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] culture was grown on rich medium due to its ability to retain pDLG1-APabfA and at stationary phase showed a 3-fold higher cell density.

S. cerevisiae Y294 [*fur1::LEU2* pDLG1-APabfA] produced the heterologous enzyme over a longer period (10 days) compared to the culture grown on defined medium (7 days). Due to the higher cell density and longer production period, the culture grown on rich medium produced 70-fold more α -L-arabinofuranosidase activity than what was produced by the culture grown on SC medium.

Purification of heterologous α -L-arabinofuranosidase. (Refers to section 4.3)

To acquire pure α -L-arabinofuranosidase from the culture supernatant, three methods were attempted. The first purification step attempted after 2000-fold concentration of the *S. cerevisiae* Y294 [pDLG1-APabfA] supernatant was anion exchange chromatography. A Resource Q (1 ml) weakly basic column was used together with a phosphate buffer of pH 8 but the sample did not bind to the column. Hydrophobic interaction was also attempted. A HiTrap 1 ml Phenyl; FF (highsub) column was used together with a 100 mM acetate buffer and 1 mM ammonium sulfate. No activity was present in the fractions and furthermore it is a possibility that the enzyme bound irreversibly to the column. Gel filtration did prove successful, provided that the growth of *S. cerevisiae* was under conditions which resulted in low extracellular protein production.

α -L-arabinofuranosidase had to be produced by *S. cerevisiae* Y294 [pDLG1-APabfA] on defined medium to provide the required properties required of the supernatant so that the enzyme could be purified in only one step.

The results of the two different cultures during gel filtration are compared: The elution profile in Figure 4.5 is different to the profile in Figure 4.8 because the latter was grown in rich YPD medium and more extracellular proteins were produced under these conditions. The amount of volumetric activity in the elution fraction that contained the highest activity in Figure 4.5 was 13-fold less than in the fraction that contained the highest activity in Figure 4.8. The enzyme production in *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] was higher due to growth on rich medium. Because the growth took place in rich medium, there were many more proteins secreted compared to what was present in the *S. cerevisiae* Y294 [pDLG1-APabfA] culture supernatant (see Figure 4.6, lane 2 and Figure 4.7, lane 3). During gel filtration these extracellular proteins could have possibly bound to the column and resulted in a higher amount of α -L-arabinofuranosidase being eluted. In the case of the *S. cerevisiae* Y294 [pDLG1-APabfA], it is thought that most of the α -L-arabinofuranosidase bound to the column and this gave a yield of 30.9 %. Although the yield was now much higher, the elution fractions of the *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] culture did not contain pure enzyme (see Figure 4.7, lane 2). At least two different proteins were secreted during growth on rich medium and this resulted in the fractions requiring further purification steps. The elution fractions of the *S. cerevisiae* Y294 [pDLG1-APabfA] culture were pure (Figure 4.6, lane 3) although more than 66% of the activity was lost. Since pure α -L-arabinofuranosidase could be obtained in one step after grown on SC medium, even though 66% activity was lost, this was the most convenient purification protocol.

This α -L-arabinofuranosidase cloned from *A. pullulans* strain NRRL Y-2311-1 and heterologously produced by *S. cerevisiae* Y294 [pDLG1-APabfA] had similar properties of other family 54 glycoside hydrolases.

Comparison of the molecular weight and glycosylation of heterologous α -L-arabinofuranosidase to other related arabinofuranosidases previously characterised. (Refers to section 4.4.1, 4.4.2, 4.4.3, 4.4.4.)

An apparent mobility of 52.7 kDa was estimated for the enzyme using SDS-PAGE and this was similar to the α -L-arabinofuranosidase of *A. niger* N400 (50.7 kDa) and of *T. reesei* (53 kDa) (Flippi *et al.* 1993; Poutanen, 1998). A molecular weight of 49.9 kDa was calculated after sequencing of the DNA encoding for the mature protein and there was only one potential glycosylation point discovered (de Wet, 2003). According to Miyanaga *et al.* (2004b), α -L-arabinofuranosidase B from *A. kawachii* contains two potential glycosylation sites. Because α -L-arabinofuranosidase runs at a similar apparent mobility on SDS-PAGE to the calculated molecular weight, glycosylation is unlikely. *S. cerevisiae* tends to glycosylate proteins although glycosylation has not been found to hinder the usual functioning of heterologously produced enzymes. The apparent mobility estimate of the protein on gel filtration (36 kDa) was smaller than the calculated molecular weight (49.9 kDa). This shows that α -L-arabinofuranosidase is a monomer but the considerably lower mobility found by gel filtration could be due to additional binding to the column matrix.

The α -L-arabinofuranosidase was de-glycosylated but no difference could be observed on SDS-PAGE. Since there is only one potential glycosylation site, the molecular weight difference between untreated and de-glycosylated α -L-arabinofuranosidase would be approximately 2-4 kDa. Mass spectrometry would have served as an accurate method for further evaluation of these samples and establish whether the heterologous enzyme is glycosylated.

Temperature optimum of heterologous α -L-arabinofuranosidase. (Refers to section 4.4.5.)

The temperature optimum of α -L-arabinofuranosidase was 55 °C (Figure 4.12) which is similar to that of the *A. nidulans* produced enzyme (Ramon *et al.* 1993). The temperature optima of the enzyme produced by the thermophilic strain of *A. pullulans* was 75 °C, which was substantially higher than what was found for other α -L-arabinofuranosidases produced in fungi. This suggests that Saha & Bothast (1998a) probably characterised another α -L-arabinofuranosidase than that studied here. α -L-arabinofuranosidase was stable over 3 h at 40 °C and showed 50 % activity after 1 h at 50 °C, but was not stable for more than 1 h at 60 °C (Figure 4.13). In comparison the crude native α -L-arabinofuranosidase of the same strain of *A. pullulans* was stable for 3 h at 60 °C and had a temperature optimum of 60 °C (Myburgh *et al.* 1991a).

Optimum pH of heterologous α -L-arabinofuranosidase. (Refers to section 4.4.6.)

The enzyme displayed optimum activity between pH 4 and 4.5 (Figure 4.14). These results are similar to that obtained for α -L-arabinofuranosidase produced by *T. reesei* and *A. nidulans* which both had optimal activity at pH 4.0 (Ramon *et al.* 1993; Poutanen, 1998) and for the thermophilic strain of *A. pullulans* which had a pH optimum of between 4 and 4.5 (Saha & Bothast, 1998a). The enzyme was stable at pH 4 over 3 h and at pH 3 it showed 90% activity over 3 h. At pH 6 α -L-arabinofuranosidase displayed 30 % activity at 1 h and at pH 2.2 the activity dropped to 10 % of the maximum activity within 20 min (Figure 4.15). The crude α -L-arabinofuranosidase of *A. pullulans* strain NRRL Y-2311-1 was previously characterized and it was found that the pH optimum was 5.0 and that the enzyme was most stable at this pH value (Myburgh *et al.* 1991b).

Heterologous α -L-arabinofuranosidase kinetics. (Refers to section 4.4.7.)

From kinetic analysis using *p*-nitrophenyl α -L-arabinofuranoside, the K_m (1.43 mM) and V_{max} (22.7 U/mg) values were determined using Lineweaver-Burk and a K_m (4.51 mM) and V_{max} (41.5) were determined using the Hanes plot. The Hanes plot places greater emphasis on the data at higher substrate concentrations. This data is less subject to experimental error and therefore Hanes is the statistically preferred plot. This enzyme followed Michealis-Menten kinetics and showed similarity to the results found for other family 54 glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/GH_54.html). A family 54 α -L-arabinofuranosidase from *Penicillium purpurogenum* showed hyperbolic kinetics against *p*-nitrophenyl α -L-arabinofuranoside with a K_m of 1.23 mM (De Ioannes *et al.*, 2000). A family 51 α -L-arabinofuranosidase from *Cytophaga xylanolytica* showed a K_m of 0.504 mM with *p*-nitrophenyl α -L-arabinofuranoside (Renner *et al.*, 1998). Another family 51 α -L-arabinofuranosidase from *Streptomyces lividans* exhibited a K_m of 0.6 mM (Manin *et al.*, 1994). There appeared to be a general pattern whereby family 54 enzymes give a slightly higher K_m than family 51 enzymes on *p*-nitrophenyl α -L-arabinofuranoside.

Effect of carbohydrates on heterologous α -L-arabinofuranosidase activity. (Refers to section 4.4.8.)

Glucose, xylose, mannose and maltose showed little effect on the activity of α -L-arabinofuranosidase against *p*-nitrophenyl- α -L-arabinofuranoside. Cellobiose appeared to increase the enzyme's activity, but all these results are possibly due to experimental error and were not considered significant. What was significant however, was the large negative effect of the product arabinose. Approximately 56% decrease in activity was caused by the presence of the arabinose and this suggests product inhibition. Using the Lineweaver-Burk method, an inhibition constant of 28 ± 3 mM was calculated. Other inhibitors of α -L-arabinofuranosidase include 1,4-dideoxy-1,4-imino-L-threitol and 1,4-dideoxy-1,4-imino-L-arabinitol which were competitive inhibitors of *Monilinia fructigena* α -L-arabinofuranosidase III and L-arabinono-1,4-lactone which was an inhibitor of α -L-arabinofuranosidase from *Aspergillus niger* (Schwabe *et al.*, 1978; Axamawaty *et al.*, 1990).

From the crystal structure of *A. kawachii* arabinofuranosidase B, Miyanaga *et al.* (2004a) found that this enzyme can bind three arabinose molecules. After homology modeling of the heterologous α -L-arabinofuranosidase based on the crystal structure of *A. kawachii* arabinofuranosidase B, it was found that there were also three arabinose binding regions.

Substrate specificity of heterologous α -L-arabinofuranosidase. (Refers to section 4.4.9.)

Many different substrates containing arabinose were tested and the results were compared to the known properties of family 54 glycoside hydrolases. According to literature they have hydrolytic activity for the α -1,2 or α -1,3-linked and to a lesser degree α -1,5-linked, non-reducing, terminal arabinosyl residues of arabinoxylan, arabinan and arabinogalactan. These enzymes do not hydrolyze internal α -arabinosyl linkages and do not act on double arabinosylated xylopyranosyl residues. They are also able to hydrolyse the synthetic aryl glycoside *p*-nitrophenyl α -L-arabinofuranoside (Saha, 2000).

The first five substrates tested were the synthetic chromogenic *p*-nitrophenyl group. These included *p*-nitrophenyl α -L-arabinofuranoside, which was the standard enzyme used to determine activity in most experiments. The α -L-arabinosyl bond was cleaved efficiently as expected, but the other four substrates which contained β -D-arabinosyl bonds were not cleaved as expected.

The second group of four substrates were the 1,5- α -L-arabino-oligosaccharides, arabinobiose, arabinotriose, arabinotetraose and arabinopentaose. All these showed no significant arabinose release after 120 min. But family 54 enzymes have been found to attack α -1,5-linked terminal L-arabinofuranosyl bonds and this was shown in the 24 h assay on 1,5- α -L-arabinotriose. There was an increase in arabinose and arabinobiose and therefore the α -L-arabinofuranosidase was able to cleave this bond, but at a much lower activity. Unfortunately there is a lack of appropriate substrates available to test the α -1,2 and α -1,3 cleaving ability.

The family 51 α -L-arabinofuranosidase from *Bifidobacterium longum* B667 was found to release L-arabinose from 1,5- α -L-arabinobiose, arabinotriose, arabinotetraose and arabinopentaose (Margolles *et al.*, 2003). Family 51 is most closely related to family 54, but has slightly different substrate specificities.

The three types of arabinoxylans were all tested for arabinose release after 60 min reactions with α -L-arabinofuranosidase. Corn fibre showed partial hydrolysis but no further information on the type of bonds that occur has been reported in literature. L-arabinose residues could be 2- or 3-substituted or di-substituted. Oat spelt arabinoxylan showed partial hydrolysis since there was an increase in arabinose detected by the HPAEC. Oat spelt contains only α -1,3-linked arabinose residues (Debeche *et al.*, 2000) and this was the arabinoxylan which showed the most arabinose release.

Wheat arabinoxylan showed no release of arabinose after 60 min reactions with α -L-arabinofuranosidase and showed a low activity after 24 h. This arabinoxylan contains both 2- and 3- substituted and di-substituted α -L-arabinofuranosidase residues. This substrate was expected to release some degree of arabinose after reaction with α -L-arabinofuranosidase since 3- substituted residues are present. It is possible that these α -1,3-linked arabinose residues occur at a lower ratio. However, when allowed to react together with xylanase and α -L-arabinofuranosidase over 12 h (see section 4.4.10.), this substrate did show increased arabinose release.

The results for the linkage preference of α -L-arabinofuranosidase were similar to the study on the family 51 arabinofuranosidase (Abf51A) from *Pseudomonas cellulosa* (Beylot *et al.*, 2001). Instead of cleaving the arabinose at the non-reducing end of the backbone of the sugar beet

arabinan polymer, family 51 enzymes prefer to cleave the arabinose side-chains. Their results also indicated that Abf51A showed approximately 300-fold less activity against α -1,5-linked arabinooligosaccharides than those with α -1,2 or α -1,3-linked subunits.

Since α -L-arabinofuranosidase did hydrolyze α -1,3-linkages in oat spelt xylan, it was surprising that wheat arabinoxylan needed such a long reaction time and the addition of xylanase to show sufficient arabinose release. Wheat arabinoxylan contains both α -1,2 and α -1,3-linked arabinose side-chains. Since no literature was found that could explain this issue in family 54 enzymes, results in family 51 enzymes were compared. It has been shown that some family 51 arabinofuranosidases show limited action against arabino-disubstituted xylose in wheat arabinoxylan (Beylot *et al.*, 2001). This could explain the results found for these arabinoxylan experiments.

Larch arabinogalactan is composed of β -1,3-linked D-galactopyranose residues and contains a high degree of branching. Most of the residues on the backbone are linked to a side chain which consists of two β -1,6-linked D-galactopyranose residues. The other side chain found in this substrate is 3-O- β -L-arabinopyranosyl-L-arabinofuranose. A low amount of L-arabinofuranose and D-glucuronic acid terminal residues are also present (Coughlan & Hazelwood, 1992). There was no arabinose detected by the HPAEC after repeat 60 min and 24 h reactions with larch arabinogalactan. The amount of available arabinose in this substrate was low and therefore these results were expected.

Debranched arabinan showed no significant release of arabinose after 120 min and 24 h reactions with α -L-arabinofuranosidase. Since this substrate has been treated and all side chains were removed, only the backbone consisting of α -1,5-arabinan units was present. Once again α -L-arabinofuranosidase showed no activity against this type of bond.

The three lignin substrates, which contained small amounts of arabinose of unknown linkage, showed inconsistent results. In some cases the HPAEC detected initial amounts of arabinose present but no significant increase followed after 120 min reaction with heterologous α -L-

arabinofuranosidase. Since the arabinosyl bonds are unknown, no conclusions can be made about the bond preference of α -L-arabinofuranosidase from these three lignin substrates.

Therefore α -L-arabinofuranosidase was able to cleave α -1,3-linked arabinose and the preference for α -1,5-linked arabinose was much lower. The experiments with the chromogenic substrates also showed that α -L-arabinofuranosidase was not active against β -bonds.

This α -L-arabinofuranosidase was classified as a member of family 54 because of its amino acid sequence similarity (de Wet, 2003). It has also been previously shown that all family 54 enzymes cleave bonds with a retaining mechanism, consist of a β -sandwich fold and have been grouped in the clan-B glycoside hydrolases. The experimental data presented here could not confirm these properties. However, these experiments did show that this α -L-arabinofuranosidase displays the expected substrate specificity of this family.

Heterologous α -L-arabinofuranosidase synergistic action with xylanase. (*Refers to section 4.4.10*)

Synergism between these two enzymes was shown to occur when measuring the increase in xylose and arabinose during wheat arabinoxylan degradation. Although the results and Figures 4.45 and 4.46 are discussed in section 4.4.10, a few more points are made regarding the apparent arabinofuranosidase activity of xylanase M4 in reaction 2. Although there was no activity found against *p*-nitrophenyl- α -L-arabinofuranoside, this test did not prove that xylanase M4 has no arabinofuranosidase activity against wheat arabinoxylan. Some family 11 xylanases have been shown to have arabinofuranoside activity, for example the xylanase I from *A. niger* (Wong *et al.*, 1988). They found that the hydrolysis of arabinoxylan by this enzyme caused a release in arabinosyl substituents.

Wheat arabinoxylan showed no arabinose release after 1 h in the results in section 4.4.9. where only α -L-arabinofuranosidase was present in the reaction. In this synergism experiment, arabinose was released after 12 h and even more was released when the reaction contained xylanase. Therefore the heterologous α -L-arabinofuranosidase was able to cleave 1,2- α -L-arabinosyl bonds found in wheat arabinoxylan, but to a lower extent than the 1,3- α -L-arabinosyl bonds found in oat

spelt xylan. It can also be noted that α -L-arabinofuranosidase showed no xylanolytic activity since no xylose was present in reaction 1.

Substrate-binding assay. (Refers to section 4.4.11.)

All four insoluble substrates tested showed significant adsorption of the heterologous α -L-arabinofuranosidase. Oat spelt xylan could bind the most α -L-arabinofuranosidase (33%) and avicel the least (Table 4.7). This is expected since avicel is microcrystalline cellulose and therefore theoretically does not bind α -L-arabinofuranosidase. However avicel can be heterogeneous and therefore binding results can be unclear. As shown in Figure 4.47 there is significant binding of the two arabinoxylan substrates tested previously for specificity, namely wheat and oat spelt.

Comparison of native and heterologous α -L-arabinofuranosidase production. (Refers to section 4.5.)

After 4 days *A. pullulans* secreted 0.072 U/ml of α -L-arabinofuranosidase activity into the culture, compared to *S. cerevisiae* Y294 [pDLG1-APabfA] which secreted 0.0135 U/ml activity after 5 days. Therefore the native organism was more efficient in producing the enzyme. *S. cerevisiae* Y294 [pDLG1-APabfA] was grown in defined medium lacking uracil and had to depend on the pDLG1-APabfA plasmid for survival, which would of put extra stress on the yeast. *S. cerevisiae* showed a much lower relative secretion of the enzyme as cell associated activity was 25-fold less than the activity present in the supernatant. *A. pullulans* showed approximately 114-fold less cell associated activity than that present in the supernatant. Therefore *S. cerevisiae* was less efficient in secreting the foreign α -L-arabinofuranosidase but still showed significant recognition of the secretion signal. Since most of the α -L-arabinofuranosidase is secreted by *A. pullulans*, it can be deduced that the enzyme is involved in debranching of the substrate before uptake by the cell.

Purification of the native α -L-arabinofuranosidase. (Refers to section 4.6.)

Unfortunately this was only partially successful and the most pure sample of native α -L-arabinofuranosidase still showed signs of contamination from other proteins secreted by *A. pullulans* (Figure 4.51 lane 4). Gel filtration and anion exchange were attempted but more than one step was always required. During each step a large amount of activity was lost and if continued, it therefore would have not been possible to continue with further characterization experiments. A yield of 4.4 % was achieved after two steps involving gel filtration, compared to the 30.9% yield

for the heterologous enzyme during a single purification step in *S. cerevisiae* Y294 [pDLG1-APabfA]. This was the main reason why the *abfA* gene was cloned into *S. cerevisiae* by de Wet (2003), as to avoid the problem of many proteins secreted by *A. pullulans*. This made purification from supernatant simpler and more suitable for up-scale production of α -L-arabinofuranosidase. The reason why initial characterization experiments (optimum temperature and pH) were performed on the partially purified native α -L-arabinofuranosidase was to note possible changes in basic properties caused by being secreted by a different organism.

Comparison of the optimum temperature, pH and molecular weight of native and heterologous α -L-arabinofuranosidase. (Refers to section 4.7.)

The gel filtration sample in lane 4, Figure 4.51 was used for two characterization experiments and the apparent mobility of the native enzyme in this SDS-PAGE appeared to be the same as that found for purified heterologous α -L-arabinofuranosidase in lane 3, Figure 4.6.

The optimum temperature varied slightly from the heterologous enzyme, which showed maximum activity at 55°C (Figure 4.12). The native α -L-arabinofuranosidase showed optimal activity at 50°C and appeared to be more stable at higher temperatures (Figure 4.57). The heterologous enzyme showed the most activity between 45°C - 60°C whereas the native enzyme between 40°C – 70°C. These differences were slight and not considered significant enough to suspect changes in the properties of the two enzymes due to secretion by different organisms.

The optimum pH also varied slightly from the heterologous enzyme. The native enzyme showed at optimum activity at pH 3 whereas the heterologous enzyme was most active at pH 4. But the native enzyme showed approximately 70 % relative activity at pH 4, and the heterologous enzyme showed 75 % relative activity at pH 3. Therefore the partially purified native enzyme appeared to be slightly more active at lower pH values. But again these differences were slight and not considered significant enough to suspect changes in the properties of the two enzymes. These results are both consistent with the properties of family 54 glycoside hydrolases, which have acidic isoelectric points around pH 5 (Saha, 2000).

Comparison of the kinetics and substrate specificity of native and heterologous α -L-arabinofuranosidase. (Refers to section 4.7.4. and 4.7.5.)

The K_m value calculated from the Lineweaver-Burk plot for the native enzyme was 8.33 mM, which was slightly more than the heterologous enzyme (1.43 mM). This indicated that the native enzyme was not as active against the substrate compared to the heterologous enzyme. This could have been caused by impurities, since the native enzyme was partially purified. The larger variation in V_{max} and k_{cat} values were most likely due to the presence of impurities which influenced the protein concentration in the sample. These variations were not considered significant and the kinetic properties of both heterologous and native enzymes were considered similar.

The heterologous enzyme showed a slightly higher increase in arabinose and arabinobiose after the 24 h assay with arabinotriose, compared to the native enzyme. This difference in increase in peak area of the products was not significant enough to suspect any changes in the cleaving properties of the native and heterologous enzymes. The increase in arabinobiose and arabinose over 24 h indicated that the native enzyme recognized and cleaved the same 1,5- α -L-arabinosyl bond as the heterologous enzyme.

Effect of sugar bagasse sample preparation on enzymatic degradation. (*Refers to section 4.8.*)

The four variants of bagasse samples were prepared in two different ways and the enzymatic breakdown was investigated. CLM/140°C and BAG/140°C were treated with water at 140°C and Pith/AFEX and CLM/AFEX were treated with liquid NH_3 at 100°C. A higher sugar release was expected for the AFEX substrates and since a combination of α -L-arabinofuranosidase and xylanase was used, arabinose, xylose and xylobiose were the sugars monitored. In the control samples there were initial amounts of both sugars and in the enzyme samples there was an increase in released sugar, indicating that the enzymes were active against the substrates (Figure 4.61). Arabinose release in enzyme samples were low for BAG/140°C and CLM/140°C (under a concentration of 0.2 mM) but Pith/AFEX and CLM/AFEX showed higher arabinose release (concentration of around 0.5 mM) in both initial controls and enzyme samples. Pith/AFEX and BAG/140°C showed significant release of arabinose when comparing the control and enzyme samples. The level of arabinose in the two CLM substrates stayed relatively constant and therefore no significant release was observed. Initially BAG/140°C and the two AFEX substrates showed a large amount of xylose but there was not significant release in the enzyme sample. The AFEX substrates treated with liquid ammonia did not show any real improvement in these two sugar's

release. However, treatment with liquid ammonia improved general sugar release when comparing control samples of CLM/140°C and CLM/AFEX. There was also a large increase in xylobiose, a breakdown product cause by the action of xylanase. This was found to be the case in all assay samples of the four substrates when comparing them to control samples.

Three-dimensional homology model of the heterologous α -L-arabinofuranosidase. (Refers to section 4.9)

Since AbfA from *A. pullulans* produced by *S. cerevisiae* was found to be 71% identical to AkabfB from *A. kawachii* (Miyana *et al.*, 2004a), both the three-dimensional models displayed similar properties. All the properties displayed by family 54 of the glycosyl hydrolyse were found in both the crystal structure of AkabfB and the homology model of AbfA. These include the highly conserved primary sequence, 4 disulphide bonds and the same number of important residues involved in catalysis and substrate binding. There were also three arabinose binding sites found in both, one in the catalytic domain and two in the arabinose-binding domain. The AkabfB had a β -sandwich fold present in the catalytic domain and this is a property found in the clan-B glycosyl hydrolyses, to which family 54 belongs. The AkabfB arabinose-binding domain contained a β -trefoil fold similar to those found in carbohydrate-binding module family 13. AbfA from *A. pullulans* most likely shares these domain properties.

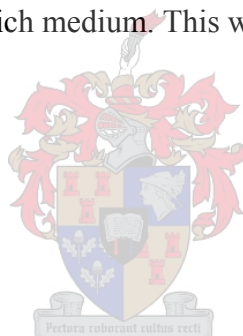
Differences between the two α -L-arabinofuranosidases were also found during the modelling process when comparing the amino acid sequences. The signal peptides differed by a single amino acid and only one potential glycosylation site was found for AbfA, whereas AkabfB contains 2 potential glycosylation sites.

AkabfB from *A. kawachii* was also crystallized with two arabinose molecules in the arabinose-binding domain and one in the catalytic domain. This lead to the active sites being identified from key amino acid residues. From the corresponding residues in the homology model of AbfA, the three active sites were located and highlighted. This data could be used for further docking arrangements using three-dimensional models of various substrates. Results from the theoretical three-dimensional docking of ligands into the active sites could be then compared to the experimental assay results.

6. APPENDIX A

Cloning α -L-arabinofuranosidase from *A. pullulans* into *S. cerevisiae*.

The *abfA* gene of *A. pullulans* strain NRRL Y-2311-1 was cloned by PCR methods including degenerate PCR, CODEHOP (Rose *et al.*, 1998) and inverse PCR and expressed in *S. cerevisiae* Y294 (α *leu2-3,112 ura3-52 his3 trp1-289*) (la Grange *et al.*, 1996) under control of the *ADH2* promoter. This promoter is upregulated under conditions of glucose depletion and allows for growth of the culture to stationary phase before the production of the heterologous protein occurs (Romanos *et al.*, 1992). Two constructs were created: *S. cerevisiae* Y294 [pDLG1-APabfA] which was auxotrophic (*ura*-) and designed to grow on defined medium lacking uracil, and *S. cerevisiae* Y294 [*fur1::LEU2* pDLG1-APabfA] (constructed by *fur* disruption) which was autoselective and could be grown on rich medium. This work was reported by de Wet (2003).



7. APPENDIX B

Three residual lignins (lignin-carbohydrate complexes) samples were obtained by isolation from kraft type pulps by enzymatic hydrolysis using cellulolytic enzymes. In Table 7.1 the content of protein impurities present in the samples is given together with sugar content.

Table 7.1. The correction for protein impurity is given together with the monosaccharide content in each sample.

Residual lignins	N (%)	Protein (%) (=6.25 N%)	Ara (%)	Gal (%)	Glu (%)	Xyl (%)	Man (%)	Total	Lignin content (%)
								monosaccharide content (%)	
R1000proRL	0.8	5	0.41	2.13	1.03	1.44	1.37	6.392	88.61
LS3000proRL	1.04	6.5	0.35	1.18	0.95	1.44	1.22	5.139	88.36
HiA350proRL	0.94	5.875	0.36	2.03	1.39	0.95	1.87	6.603	87.54

Supplied by B. Hortling, Keskuslaborio-Centrallaborium, Finland



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