Enzymatic hydrolysis with commercial enzymes of a xylan extracted from hardwood pulp

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by

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SYNOPSIS

In the forest products industry the opportunity exists to extract currently under-utilised compounds from the process or waste streams and thereby derive more value from the wood entering the process. A big portion of the hemicellulose content of wood does not form part of the final product. Extracting the hemicelluloses from the waste streams or other locations in the process would allow them to be used more effectively.

The predominant hardwood hemicellulose, xylan, is polymeric xylose. Xylose is an important platform sugar in bioconversion strategies and can be converted to fuels and other valuable chemicals. The xylan polymer can be hydrolysed to its xylose monomers by a number of conversion strategies; the most widely known being chemical and enzymatic digestion. Chemical conversion is usually done using acid at elevated temperatures, but high yields are often offset by degradation of the product. On the other hand, enzymatic hydrolysis can be better regulated to prevent unwanted degradation of the monomeric sugar products. Enzymatic hydrolysis has been pronounced the environmentally friendly choice of technology, although it is hampered by low conversions and high cost of enzymes. To date commercial enzymes for biomass conversion are not readily available most of which are still in development.

In understanding how to best utilise a xylan, recovered from the pulping process, the potential to convert hardwood xylan to xylose with enzymes currently available on the market was studied. A hardwood xylan extracted from fully bleached Eucalyptus pulp with a chelating agent, Nitren, was used as substrate to evaluate the ability of some commercial enzymes to degrade the extracted xylan to xylose monomers.

The enzymes used in this study were not dedicated biomass conversion enzymes, but rather chosen for their xylan degrading potential, i.e. xylanase content. By means of hydrolysis profiles on commercial Birchwood and Oat Spelts xylan as substrates and enzyme characterisation, Multifect xylanase was identified as most promising enzyme for

xylan conversion. Multifect contained high levels of xylanase and xylosidase activity in the enzyme preparation.

Commercial Birchwood xylan and the extracted Eucalyptus xylan were found to be chemically similar, both composed predominantly of xylose. The hydrolysis profiles obtained on Birchwood xylan could therefore serve as a benchmark against which the hydrolysis of Eucaluptus xylan could be compared.

Full conversion of the Eucalyptus xylan with Multifect could not be achieved, although Multifect completely degraded the Birchwood xylan. The maximum xylose yield that could be obtained on Eucalyptus xylan was 80 % and it was concluded that the remaining 20 % was unhydrolysable by the enzyme, most likely due to the limitations in the employed extraction method.

It was however noted that up to the point of 80 % conversion higher hydrolysis rates were observed on Eucalyptus xylan than Birchwood xylan with equal charges of Multifect. The differences in hydrolysis rates may have indicated that the Eucalyptus xylan is more accessible to enzyme attack than the Birchwood xylan, likely as a result of the extraction methods used to prepare the xylans.

A simple economic evaluation illustrated the weight of various costs in process profitability. The most economic operation of a continuous steady state reactor is at a low enzyme charge, 17 IU/ℓ, and a long retention period, five days, due to the high cost of the enzyme compared to other factors. For a reduced retention time, an investigation into enzyme immobilisation and the use of a packed-bed type reactor is recommended.

KEYWORDS: commercial xylanase, xylosidase, Birchwood xylan, Oat Spelts xylan, Eucalyptus xylan, enzymatic hydrolysis, xylose, nitren extraction

ACKNOWLEDGEMENTS

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NOMENCLATURE

α Parameter 1 of the logarithmic expression

β Parameter 2 of the logarithmic expression

η Stoichiometric parameter of loss of activity

τ Retention time h

C Cost index constant   R

C_{D0} Initial concentration of degradation products g/ℓ

C_D Concentration of degradation products g/ℓ

C_{H0} Initial concentration of hemicellulose substrate g/ℓ

C_H Concentration of hemicellulose substrate g/ℓ

C_{M0} Initial concentration of monomers g/ℓ

C_M Concentration of monomers g/ℓ

C_{S0} Initial substrate concentration $kmol/m^3$

C_{Si} Concentration of substrate in inlet stream $kmol/m^3$

C_{So} Concentration of substrate in outlet stream $kmol/m^3$

C_{St} Concentration of the sugar standard mg/ℓ

C_S Substrate concentration at given time $kmol/m^3$

C_U Concentration of the sugar in the sample mg/ℓ

CMC Carboxymethyl cellulose units   CMC/mℓ

$Cost_a$ Annual operational cost   R/a

$Cost_E$ Cost of the enzyme   R/d

$Cost_F$ Cost of the feed   R/d

$Cost_P$ Worth of the product   R/d

$Cost_V$ Cost of the vessel   R

D Total degradation products

D_f Dilution factor used in sample preparation

E_l Enzyme loading   IU/mℓ

F_E Enzyme flow rate   kg/d

F_F Feed flow rate   kg/d

F_i Flow rate of inlet stream   kmol/h

F_o Flow rate of outlet stream   kmol/h

F_P Product flow rate   kg/d

FPU Filter paper units   FPU/mℓ

GF Goal function

H_t Total hemicellulose

i Required return on investment

I_h Peak height of internal standard added to the sample   nC

k Rate constant of hydrolysis

k' General rate constant of enzymatic hydrolysis   h^{-1}

k_1 Rate constant of reaction 1   min^{-1}

k_2 Rate constant of reaction 2   min^{-1}

k_d Rate constant of loss of activity   h^{-1}

M Total monomeric sugars

n	Number of years	
N_S	Number of moles of substrate at give time	kmol
O	Total oligomers	
p	Power of cost index	R
Q	Volumetric flow rate	m^3/h
r_A	Rate expression of enzymatic hydrolysis	$kmol/m^3 h$
R_{EA}	Residual enzyme activity	
S_h	Peak height of sugar in the standard	nC
t	Time of incubation	h
U_h	Peak height of sugar in the sample	nC
V	Volume of the reactor	m^3
x	Conversion	

"No pessimist ever discovered the secrets of the stars, or sailed to an uncharted land, or opened a new heaven to the human spirit"

– **Helen Keller**

CHAPTER 1

Introduction

As the world's energy needs increase, the use of renewable feedstocks, i.e. biomass, is becoming a realistic alternative to petrochemicals. Although more research is based on the bioconversion of agricultural wastes, the potential contribution of forest products is receiving increasing interest.

The forest biorefinery is well suited to play a significant role in biomass conversion by adapting and modifying current process facilities to produce more than wood pulp thereby transforming the pulp mill into a biorefinery. Analogous to the modern day petroleum refinery, the biorefinery seeks to utilise all components of biomass to produce fuels, power and chemicals (Ragauskas et al., 2006). The pulp and paper mill is an ideal platform for transformation, since a large number of technologies required for biomass conversion are already in use. As a first step, there is currently the potential to extract the valuable components directly from the wood chips, from process streams or spent process liquors.

The wood entering a pulp mill is composed mainly of cellulose, hemicellulose and lignin. Depending on the source, hemicelluloses can account for up to 30 % of the hardwood mass. During chemical pulping, almost half of this is lost into the spent liquors. When dissolving pulp is the product, a cellulose content higher than 90 % is desired in which case almost all the hemicelluloses are extracted during pulping and bleaching. The spent liquors from all these processes are incinerated in furnaces. However, hemicelluloses only account for a quarter of the energy generated (Ragauskas et al., 2006). If hemicelluloses could be extracted from the process prior to combustion, it could be purified as a step towards conversion into other valuable products.

Several hemicelluloses exist in nature; the major hemicellulose present in hardwoods being glucoronoxylan, which is composed predominantly of the monomeric sugar, xylose. Xylose is an important platform chemical in biorefineries mainly as a potential raw ma-

terial for the manufacture of ethanol and other chemicals traditionally derived from fossil fuels.

The conversion of xylan to xylose can be achieved by acid or enzyme catalysed hydrolysis. Acid hydrolysis is generally carried out at high temperatures, but the corrosive conditions can lead to degradation of the product. Further processing of the xylose, especially fermentation, is then hampered by the degradation products in the hydrolysate. Enzymatic hydrolysis does not have any adverse effects on the sugar product, but the disadvantages of this technology include low yields and high enzyme costs.

As the bioconversion of hemicellulose and other biomass components has grown in importance, the prominence of enzymes, a favoured greener technology, has increased. Much of the research in this area is focused on isolating and engineering enzymes from various sources to better convert the biomass substrates. However, to date commercial enzymes for biomass conversion are not available. Most are still in the development phase.

In understanding how to utilise xylan recovered from the pulp process, the enzymatic degradation to its constituent monosugars was investigated. A hardwood xylan was extracted from fully bleached Eucalyptus pulp with a chelating agent, Nitren and the ability of several commercial enzymes to degrade the extracted xylan to xylose monomers was determined. As the commercial enzymes currently available are not specifically intended for biomass conversion, hydrolysis profiles on commercial xylans as well as enzyme characterisations were done to investigate the xylan conversion potential of the enzymes. The enzymatic hydrolysis of the commercial xylans by the enzymes used in this investigation served as a benchmark against which the conversion of the extracted xylan could be compared.

The techniques and procedures developed have direct application to laboratory scale only. The fitted curves are empirical and therefore only applicable to the data presented here.

CHAPTER 2

Literature Survey

2.1 Background of biomass conversion

Energy needs are steadily increasing as the world population grows and more countries are becoming industrialised (Sun & Cheng, 2002). The inevitable depletion of the world's crude oil sources and the phenomenon of global warming have increased the need to find alternative sources of energy and chemicals production. Biomass is an obvious choice to relieve the world's dependence on crude oil sources. Not only can biomass be converted to ethanol and other valuable chemical products, it also results in a sustainable carbon cycle. No harmful greenhouse gasses are added to the atmosphere when the biomass is converted. In South Africa, the use and reuse of lignocellulosic biomass and wastes will become a necessity in the near future. The natural pastures of the country are threatened by the expansion of the Karoo, overgrazing and other malpractices and much lignocellulosic waste is currently disposed of by biomass burning (Malherbe & Cloete, 2002).

Increasing interest in the conversion of lignocellulosic materials to various chemicals has lead to the anticipation of integrated production facilities, i.e. biorefineries where fuels, chemicals and power is derived from organic biomass (Lynd et al., 2003). In particular, hemicellulose bioconversion is receiving renewed attention. As the second most abundant polysaccharide in nature, its utilisation is essential to achieve economical conversion of lignocellulosic materials.

Many studies indicate that to economically convert lignocellulose to ethanol, utilising the xylose, the main constituent of xylan hemicelluloses, is crucial (Lee, 1997; Saha, 2003). The production costs of ethanol can be reduced from $ 1.65 per gallon to $ 1.23 per gallon by fermenting the xylose in addition to glucose, the main constituent of cellulose (Lee, 1997). Xylose can also be used to replace glucose as a feedstock for fermentation.

Glucose is usually the main feedstock for microbial fermentation, but in Europe it can only be obtained from sugar beet or sweet sorghum and starch hydrolysates (Danner & Braun, 1999), which limits production opportunities. Whatever the intended application, it is clear that hemicelluloses and specifically the carbohydrates that it comprises are important considerations in any biomass conversion strategy.

2.2   Overview of Lignocelluloses

Biomass is the collective term used to refer to all organic material originating from plants. The energy potential of biomass is stored in the chemical bonds of the carbon, hydrogen and oxygen. Biomass is a promising feedstock for conversion to chemicals and fuels as it presents the unique opportunity of a closed carbon cycle. The CO_2 and H_2 released during biomass conversion is taken up again in plant photosynthesis to produce new plant material in a cyclical process. With emphasis today on reducing carbon emissions, according to the Kyoto protocol of 1997, the benefits of using a renewable and sustainable energy source are obvious.

Biomass is simply divided into four main categories (McKendry, 2002) namely:

- woody plants

- herbaceous plants and grasses

- aquatic plants

- manures

Biomass contains various amounts of cellulose, hemicellulose and lignin hence the term lignocellulose. Lignocellulosic biomass is the most abundant renewable organic source available on earth. At an annual production of $10 - 50$ x 10^{12} kg, it accounts for 50 % of the total biomass in the world (Galbe & Zacchi, 2002). The main sources of lignocellulose are hardwood, softwood, grasses and agricultural residues (Lee, 1997). The proportion of cellulose, hemicellulose and lignin in biomass sources vary depending on the plant species.

The composition of a few biomass sources is given in Table 2.1 for illustrative purposes (McKendry, 2002; Pandey et al., 2000). Woody sources contain a greater portion of lignin than wheat straw. The unique ratio of cellulose:hemicellulose:lignin gives plants their structural characteristics.

2.3   Lignocellulose chemistry

Lignocellulose is composed of celluloses, hemicelluloses and lignin. Hemicelluloses form a coat around the underlying cellulose fibre, bound by hydrogen bonding. The overlaying

Table 2.1: The composition of lignocellulose for various biomass sources (Values expressed in wt%)

Biomass source	Lignin %	Cellulose %	Hemicellulose %
Softwood	27 − 30	35 − 40	25 − 30
Hardwood	20 − 25	45 − 50	20 − 25
Wheat straw	15 − 20	33 − 40	20 − 25

sheath of lignin is interspersed, intertwined and covalently linked to the hemicellulose (Beg et al., 2001). The lignocelluloses contained in the cell walls of plants are responsible for the specific mechanical properties. During cell formation, the middle lamella and primary wall are formed first. The subsequent cell-thickening is onset when the bulk of the cellulose and hemicellulose are deposited in the secondary wall (Timell, 1964). Celluloses are deposited to form microfibrils, which are key to the mechanical strength of the cell. The hemicelluloses are linked to the celluloses in such a manner as to orientate the microfibrils. The orientation of the fibrils gives the cell flexibility and directional strength (Lima et al., 2001). Lignin forms in the middle lamella and secondary cell wall and serves to bind the fibres in the wood. When lignification is completed, the cell dies. In the simplest form, therefore, celluloses form the skeletal structure, which is surrounded by hemicelluloses and bound by lignin (Sjöström, 1993: p. 12).

It has been found that structurally different carbohydrates can perform analogous functions in plants of different taxonomic groups, hence the difference in types of hemicelluloses occurring in nature The main polysaccharides that the cell walls of plants are composed of are shown in Table 2.2. The complexity of the polysaccharides can be gauged from the sheer number of enzymes needed for complete degradation.

2.3.1 Cellulose

Cellulose is the main constituent of wood, typically 40 − 50 % of the wood by weight is made up of it. On a molecular level cellulose is a homopolysaccharide; a linear polymer of anhydro-D-glucose connected by β–$(1 \rightarrow 4)$–linkages. The degree of polymerisation (DP) is above 10000 for unaltered wood, but may be less than 1000 in highly bleached Kraft pulp (Biermann, 1996: p.32).

Cellulose can occur in two forms. About 50 − 70 % of wood cellulose is the highly recalcitrant, crystalline form and the rest the amorphous form (Biermann, 1996: p.32). As mentioned before, cellulose polymers are completely linear. Bundles of cellulose molecules clump together and form crystalline regions with intermolecular hydrogen bonds and alternate less ordered amorphous regions. The smallest bundle is a microfibril. Microfibrils

Table 2.2: The main polysaccharides found in plant cell walls, their sugar composition and the enzymes required to degrade them (Lima et al., 2001)

Polysaccharide	Main chain residue	Branch residue	Enzymes
Mannans	Mannose	Sometimes galactose	Endo–β–mannanase
Glucomannans	Mannose Glucose	Galactose	Endo–β–mannanase Endo–β–glucanase α–galactosidase
Galactomannans	Mannose	Galactose	Endo–β–mannanase α–galactosidase Exo–β–mannanase
Xyloglucans	Glucose	Xylose Galactose Fucose Arabinose	Xyloglucan–endo–β-transglucosilase Xyloglucan–endo–β–glucanase β–galactosidase α–xylosidase β–glucosidase α–fucosidase
Galactans	Galactose	Arabinose	Exo–galactanase α–arabinosidase
Arabinoxylans	Xylose	Arabinose Glucoronic acid	β–xylosidase Endo–β–xylanase α–arabinosidase α–glucuronodase
β–Glucan	Glucose	None	Lichenase β–(1,3)–(1,4)–glucosidase
Callose	Glucose	None	β–(1,3)–glucanase
Cellulose	Glucose	None	Cellulase endo–β–(1,4)–glucanase

build to form fibrils and fibrils form cellulose fibres. Cellulose has a high tensile strength and is insoluble in most solvents because of the regular occurrence of hydrogen bonding and the fibrous structure (Sjöström, 1993: p.51).

Naturally occurring celluloses are known as cellulose I in which the cellulose molecules are arranged in a parallel manner. The second crystalline structure in which cellulose occurs in is cellulose II. Molecules are arranged in an anti-parallel manner and is much more stable than cellulose I. Conversion from cellulose I to II, also called mercerization,

has been reported although the reversal of the process have never been achieved. Cellulose II fibres are stronger than those of cellulose I (Hyatt et al., 1998).

2.3.2   Hemicelluloses

Originally hemicelluloses were believed to be the intermediates in the biological synthesis of cellulose hence the name. Today it is known that hemicelluloses are formed through entirely different biosynthetic pathways and in contrast to cellulose, are hetero–polysaccharides (Sjöström, 1993: p.63). When used in the plural form, hemicelluloses are used to describe a class of materials. These are white, solid materials that are rarely crystalline or fibrous in nature, but fill out the flesh of the wood fibre.

Hemicelluloses that are found in the supporting tissue of woody plants are primarily modified xylans, galactoglucomannans, glucomannans and arabinogalactans (McGinnis & Shafizadeh, 1980 - 1983). They are more often than not branched structures with lower molecular weights than cellulose. The degree of polymerisation is usually less than 200 (McGinnis & Shafizadeh, 1980 - 1983; Gong et al., 1981). All hemicellulose polymers consists of some or all of the following: the 6–carbon sugar residues mannose, galactose, glucose and 4–O–methyl–D–glucuronic acid and the 5–carbon sugars xylose and arabinose. The average degree of polymerization is 100 - 200 sugar units per molecule. They are essentially linear polymers with single sugar sidechains and acetyl substituents (Biermann, 1996: p.33).

Although the hemicelluloses in softwood and hardwoods are related, they differ in characteristics and composition (McGinnis & Shafizadeh, 1980 - 1983). In hardwoods the predominant hemicellulose is glucuronoxylan (Sjöström, 1993: p.67) or partially acetylated 4–O–methylglucuronoxylan (McGinnis & Shafizadeh, 1980 - 1983) with minor amounts of glucomannan. The dry weight of plant material can consist of 10 – 35 % 4–O–methylglucuronoxylan and 2 – 5 % of glucomannan (Puls & Schuseil, 1993). In grasses 20 – 40 % of the plant material is composed of arabinoxylan (Puls & Schuseil, 1993). In softwood the hemicelluloses consist of partially acetylated galactoglucomannans with smaller amounts of arabino–(4–O–methylglucurono)xylan (McGinnis & Shafizadeh, 1980 - 1983).

Xylans

The term, xylan is used to broadly refer to all hemicelluloses that have a backbone composed of β–1,4–linked xylopyranose units. After cellulose, xylans are the most abundant polysaccharides in nature (Biely, 1993: p.30). They account for up to 30 % of the cell wall material of annual plants, 15 – 30 % in hardwoods and 7 – 10 % in softwoods (Subramaniyan & Prema, 2003). Typically, hardwoods from temperate zones contain more xylan and less glucomannan and lignin than hardwoods from tropical climates (Timell,

1964).

The structure and composition of xylans differ depending on the biomass source. The biggest differences are seen in the xylans from hardwoods, softwoods and cereals. A brief description of each is presented below.

- **Acetylglucuronoxylan (glucuronoxylan)**

  The most prominent hemicellulose in hardwoods is O–acetyl–4–O–methylglucuronoxylan. Between 15 and 30 % of the dry wood mass consists of this polymer. The backbone consists of 1,4–linked β–D–xylopyranose units with an α–1,2–linked 4–O–methylglucuronic acid on about every tenth xylose unit. On average, about 70 % of the xylose units is acetylated on C-2 or C-3. The DP of the hardwood glucuronoxylan is 15 – 200 (Coughlan et al., 1993). A typical hardwood xylan is shown in Figure 2.1.
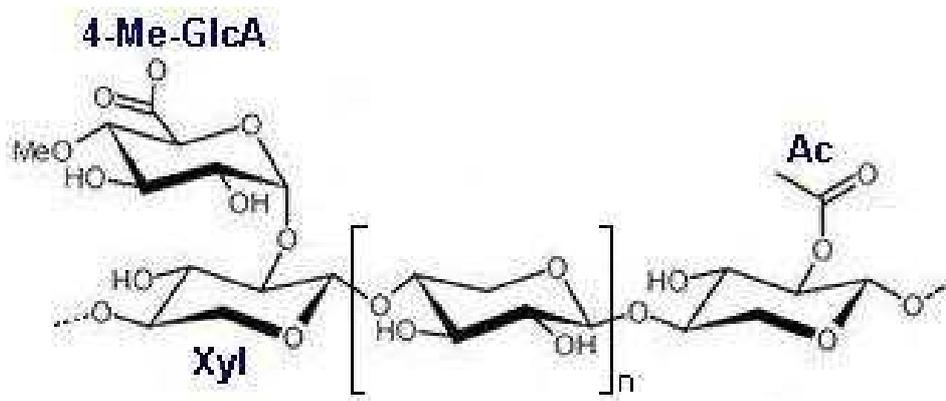
Figure 2.1: A typical hardwood xylan (adapted from Ebringerová & Heinze (2000)), Ac – acetyl, Me-GlcA – methylglucuronic acid, Xyl – xylose

  The 4–O–methylglucuronic acid link is the strongest link in the polymer structure and is believed to act as a barrier against wood degradation (Subramaniyan & Prema, 2003). D-Glucuronic acids are commonly found attached as side chains to the main backbone and are not known to be present at the terminal point of the xylan structure (Aspinall, 1959).

- **Arabino–4–O–methylglucuronoxylan**

  Softwood xylans consist of β–1,4–linked xylose units as is the case with hardwood xylans. Softwood xylans however, are not acetylated, but instead contain α–L–arabinofuranose residues, attached to about every ninth xylose unit. About 10 – 15 % of the softwood mass consists of this water soluble hemicellulose (Puls & Schuseil, 1993). Figure 2.2 shows the structure of a typical softwood xylan.
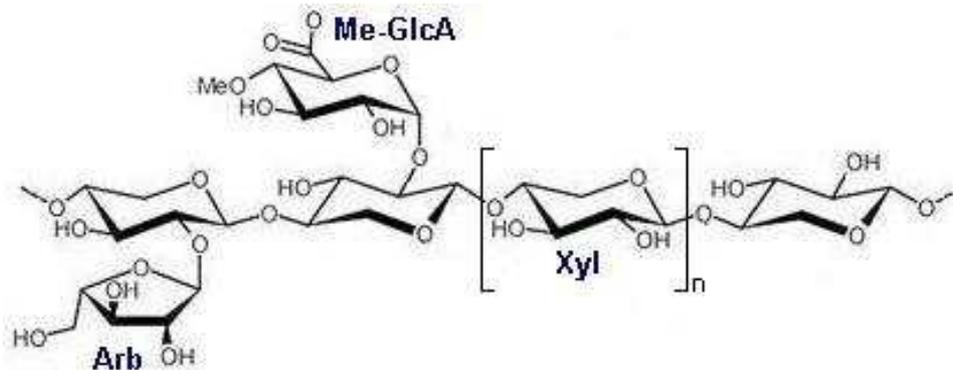
Figure 2.2: A typical softwood xylan (adapted from Ebringerová & Heinze (2000)), Arb – arabinose, Me-GlcA – methylglucuronic acid, Xyl – xylose

- **Arabinoglucuronoxylan**

  The hemicelluloses portion of cereals is reported to consist mainly of arabinoxylans (Montgomery & Smith, 1956). In softwoods, 7 – 8 % of the dry weight can consist of this polymer. The xylan backbone is made up of 1,4–linked xylose units where 20 % of the xylose residues are substituted at C-2 with $4-O-D$–glucuronic acid and 10 – 15 % with arabinose residues which are usually attached to the xylose ring at C2 or C3 (Coughlan et al., 1993).

  From various analysis methods it has been proven that the polysaccharide ends are made up of L–arabinofuranose units. These non-reducing end-groups are attached to a xylose residue, whether the xylose residue is part of the xylan backbone or attached to the backbone as a branch structure (Aspinall, 1959). Consequently a high portion of arabinose to xylose would indicate a high degree of branching (Montgomery & Smith, 1956). The DP of arabinoglucuronoxylans is usually between 70 – 130 (Coughlan et al., 1993).

  The degree of branching and substitution are different for grasses and cereal grains; in wheat about every seventh xylose residue is substituted with an arabinose side chain (Puls & Schuseil, 1993). The xylans from graminaceous plants contain up to 2 % of the dry weight of the plant material in acetyl groups and it is reported about every 30th arabinose residue is esterified with p–coumaric acid and every fifteenth with ferulic acid (Puls & Schuseil, 1993). A generic arabinoxylan structure is shown in Figure 2.3.

Galactoglucomannans

Between 5 – 10 % of softwood biomass can consist of galactoglucomannan. This water-soluble polysaccharide contains mannose, glucose and galactose in a ratio of 3:1:1 (Puls & Schuseil, 1993). The sugar residues are distributed at random in the polysaccharide
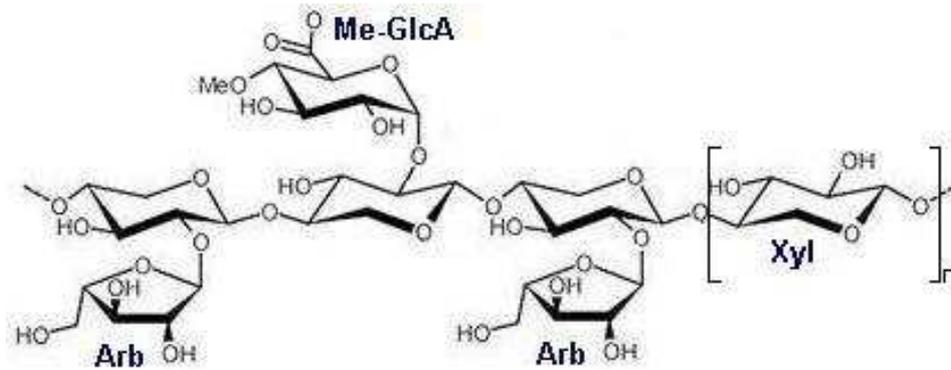
Figure 2.3: A typical cereal xylan (adapted from Ebringerová & Heinze (2000)), Arb – arabinose, Me-GlcA – methylglucuronic acid, Xyl – xylose

structure linked by β–1,4–bonds (Puls & Schuseil, 1993).

Glucomannan

Hardwoods are reported to only consist of 3 – 5 % glucomannan, the glucose:mannose ratio of the polymer ranging from 1:1 to 1:2 (Puls & Schuseil, 1993). Glucomannan is more abundant in softwoods than hardwoods and is found in quantities of 10 – 15 % of the wood biomass.

The sugar composition of the softwood glucomannan also differs from its hardwood counterpart; a larger fraction of mannose is present. A ratio of glucose to mannose of 1:3 is reported, with a small amount of galactose also being present, usually in ratio of 0.1:1 of the glucose content (Puls & Schuseil, 1993). The softwood glucumannans are sometimes also referred to as galactoglucomannans (Coughlan et al., 1993). In both the hardwood and softwood glucomannan, the sugars are distributed at random in a backbone of β–1,4–linked residues.

Arabinogalactan

This polysaccharide is known to occur to a lesser degree in softwoods and hardwoods but can be found in larchwood in quantities of 10 – 20% of the dry wood weight (Coughlan et al., 1993). It consists of a β–1,3–linked galactose backbone of which each residue is substituted with a β–1,6–linked galactose side chain. Some of the side chains may contain a terminal L-arabinofuranose or 3–O–β–L–arabinopyranosyl–L–arabinofuranose residues (Puls & Schuseil, 1993).

Arabinan (araban)

As follows from the naming convention, araban is a polymer of α–1,5–linked L–arabinofuranose residues. Single unit side chains of arabinose are sometimes attached to the

backbone, with some galactose also being present. Araban can be extracted from beet pulp which contains between 20 and 25% of this polysaccharide (Coughlan et al., 1993).

Xyloglucan

Xyloglucans consist of a 1,4–linked backbone of β–glucose residues with side chains of 1,6–α–xylose residues. Xyloglucans act as the interface between cellulose and other polysaccharides in the primary cell walls of plants (Coughlan et al., 1993).

2.3.3 Lignin

Cellulose and hemicelluloses are bound together by lignin, which is generally responsible for wood hardening. Lignin is a highly oxygenated aromatic polymer with a repeating phenylpropane skeleton (McGinnis & Shafizadeh, 1980 - 1983: p.38). The three basic monomers found in lignin are ρ–coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Grasses and straw contain all three monomers, whereas hardwoods contain coniferyl and sinapyl alcohol and softwoods only coniferyl alcohol (Biermann, 1996: p.36).

The first step of lignin polymerisation is the formation of a free radical at the phenolic hydroxyl group. This structure has five resonance forms with free radicals occurring at the various carbon atoms (Biermann, 1996: p.36). All the different linkages that are possible lead to the complex nature of lignin.

2.3.4 Extractives and Ash

Extractives are by definition the compounds that are extracted from the wood by organic solvents or water. The amount of extractives present varies with the wood species in question but is usually between 1 and 8 % of the total composition (Biermann, 1996: p.39). Terpenes are a class of compounds that are present in softwood in large quantities but not usually found in hardwoods. Terpenes can be collected in large enough quantities for resale when processing high-resin wood species. Triglycerides are saponified in the Kraft cooking process to produce free fatty acids and are recovered during black liquid evaporation.

A small quantity of ash is found in wood; less than 0.5 % of the weight composition. Wood ash is made up of metallic ions like sodium, potassium, calcium and the corresponding anions, for example carbonate, phosphate, silicate and sulphate. The composition of the ash is determined by the controlled combustion of the wood (Biermann, 1996: p.39).

2.4 Bioconversion of Lignocelluloses

A biorefinery is analogous to a petroleum refinery in that it seeks to utilise all components of biomass to produce fuels, power and chemicals (Ragauskas et al., 2006). In a classic biorefinery, converting lignocellulose into other products would involve the following steps:

1. **Pretreatment:** Delignification to liberate the cellulose and hemicellulose fibres from the lignin complex

2. **Hydrolysis:** Depolymerisation of the carbohydrate polymers to produce the free sugar residuals

3. **Fermentation:** Microbial conversion of the sugars to produce the value-added products

Lignocellulose is a highly recalcitrant substrate and its delignification is the most difficult step to accomplish (Lee, 1997). Native biomass is structurally very complex and resistant to enzymatic hydrolysis, because cellulose fibres are embedded in a lignin-polysaccharide matrix (Saha, 2003), hence the need for pretreatment. Various methods are employed to fractionate, solubilise, hydrolyse and separate the cellulose-hemicellulose-lignin matrix. During pre-treatment the biomass is reduced in size and the structure is physically opened to allow for easier access to the fibres for subsequent treatment processes.

Instead of developing a grassroots biorefinery, however, a unique opportunity exists in the forest products industry. The pulp mill is well suited for transformation into a biorefinery, in that many of the current process facilities can be adapted and modified to produce more than wood pulp. More value can be derived from the wood entering the pulp mill by utilising the byproducts more effectively. Depending on the source, up to 30 % of the wood used in the making of pulp and paper consists of hemicelluloses. A large fraction of the hemicelluloses is lost into the waste liquors during wood processing from where it is incinerated in the recovery furnace, although hemicelluloses only account of a quarter of the energy provided (Ragauskas et al., 2006). If hemicelluloses could be extracted from the process prior to combustion, it has the potential to be purified into an additional product or converted into other valuable chemicals.

There are several opportunities to recover hemicelluloses from the pulping process before incineration. The hemicelluloses can be pre-extracted from the wood chip, prior to pulping. This would involve liberating it from the cellulose and lignin intertwined matrix. The harsh conditions and high temperatures needed to extract the hemicelluloses are non-specific and usually have a derogatory effect on the cellulose as well. Another consideration is that the hemicelluloses have an important part to play in the final paper properties; they have been related to paper bond strength (Ragauskas et al., 2006). It is

therefore a fine balance to pre-extract enough hemicelluloses for bioconversion applications without negatively impacting on the final paper product.

During pulping the loss of some polysaccharides from the product is inevitable. In the pulping of birchwood it was shown that almost half of the original xylan present in the wood was lost into the waste liquors (Ragauskas et al., 2006). A cellulose content higher than 90 % is desired when dissolving pulp is the product and extensive purification is required with an additional alkali treatment step to remove and destroy hemicelluloses. The hemicellulose portion of the lignocelluloses is undesired in the final product as it adversely affects the strength, solubility and filterability properties (Gubitz et al., 1997). Removal of hemicelluloses increases the cost of dissolving grade pulp and reduces the yield as some of the cellulose is attacked as well (Heikkila et al., 2003). A classic method for the removal of hemicelluloses is hydrolytic disintegration by subjecting the pulp to superheated steam at high pressure. The decomposition of hemicelluloses is, however, not selective either and damage to the cellulose occurs. Hydrolysis under acid conditions can be employed as well, but the same problem of non-selective degradation is observed (Kettenbach & Stein, 2007).

Another source of hemicelluloses is the carbohydrates in the waste liquors. Since these lost hemicelluloses do not form part of the paper product, the extraction thereof is an ideal source of carbohydrates for bioproducts. The recovery of hemicelluloses from the liquor streams is becoming more feasible as technology advances. Membranes are being developed to separate the high molecular weight xylans from the lignin and other contaminants (Axegard, 2006).

A new approach is to upgrade the so called "paper-grade" pulp to dissolving pulp by extracting the hemicelluloses. In this context, enzymatic removal has been investigated. High retention times and close environmental control is needed to achieve hemicelluloses extraction in this method (Gubitz et al., 1997).

Alternatively, a new, more effective process of nitren extraction is proposed. In the nitren process, a metal complex of tris–(2–amino-ethyl)amine and nickel(II)hydroxide selectively extracts hemicelluloses from paper-grade pulps (Kettenbach & Stein, 2007). The increase of the cellulose content from 81 % to 96 % by a two-stage extraction with 3 % nitren on Eucalyptus Kraft pulp has been reported (Janzon et al., 2006). Xylans of high quality could be precipitated out from the extraction liquors. These xylans would be an excellent source for bioconversion, since they do not form part of the process and their extraction improves the quality of the product. The use of fully bleached pulps is ideal for this application because the high degree of delignification facilitates the extraction of pure xylans (Janzon et al., 2007).

It is important to consider that the pulping process negatively affects the structure of the xylans. The harsh processing conditions can partially degrade the xylan and therefore alter the properties from what would be observed in the native xylan. Nevertheless,

recovery of the underutilised xylans from the pulp mill would still ensure an excellent source for bioconversion feedstocks.

2.5   Enzyme technologies

The industrial applications of commercial enzymes are growing at an astonishing rate. In 1998 the world-wide sales of enzymes amounted to $1.5 billion with predicted increases of 15 % in paper production and up to 25 % in feed enzymes (Van Beilen & Li, 2002). The real value of enzyme technology, however, is in enzyme-based processes, where operating costs can be typically reduced by anything from 9 to 90 % and are accompanied by savings in energy and raw materials (Van Beilen & Li, 2002). Of the enzymes available commercially, up to 75 % are of the hydrolytic type (Godfrey & West, 1996: p.3).

Xylanases account for a large portion of the hydrolytic enzymes and are extracted from both bacterial and fungal sources. Enzymes from the bacterial sources such as *Clostridium*, *Cellulomonas*, *Bacillus* and *Thermomonospora* genera have been used widely. Fungal enzymes such as *P. chrysosporium*, *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* have been extensively studied for hemicellulase production. Because of the difficulties caused by the anaerobic nature of some bacteria species and the generally low yields extracted from bacteria, more focus has fallen on the use of fungal strains for commercial enzyme preparations (Sun & Cheng, 2002).

Currently, the most widely used application of xylanases is in the pre-bleaching of Kraft pulp. The use of xylanases in bleaching is known to reduce the number of bleaching steps necessary as well as to reduce chemical consumption (Beg et al., 2001). Other applications of commercial xylanolytic enzymes include

- the use as an additive in poultry feed to improve weight gain of chicks and feed conversion efficiency

- use in combination with amylase to improve specific volume of bread in bakery industry

- the treatment of waste waters

- improving the nutritional properties of agricultural feeds

- degumming bast fibres such as hemp, jute and ramie

- and the clarification of fruit juices.

2.6 Enzymes in the hydrolysis of hemicelluloses

The composition of hemicelluloses is more complex than that of cellulose, but it does not form the tightly packed crystalline structure normally associated with cellulose. Therefore hemicelluloses are more readily accessed by degradation enzymes (Saha, 2003). The complex nature and variable structure of hemicelluloses, however, require the action of a multitude of enzymes for their complete degradation. Table 2.3 summarises the action of the enzymes and their roles in the complete degradation of hemicelluloses (Beg et al., 2001; Shallom & Shoham, 2003; Saha, 2003). The actions of the enzymes involved in the breakdown of typical hardwood, softwood and cereal xylans are shown in Figure 2.4.

Table 2.3: Enzymes associated with hemicellulose degradation and their specific mode of action

Enzyme	Mode of action	Catalytic classification
Exo–xylanase	Hydrolyses β–1,4–xylose linkages releasing xylobiose	GH
Endo–xylanase	Hydrolyses mainly interior β–1,4–xylose linkages of the xylan backbone	GH
β–Xylosidase	Releases xylose from xylobiose and short chain xylooligosaccharides	GH
α–Arabinofuransidase	Hydrolyses terminal non-reducing α–Arabinofuranose from arabinoxylans	GH
α–Glucuronidase	Releases glucuronic acid from glucuronoxylans	GH
Acetyl xylan esterase	Hydrolyses acetylester bonds in acetyl xylans	CE
Ferulic acid esterase	Hydrolyses feruloyester bonds in xylans	GH
p–Coumaric acid esterase	Hydrolyses p–Coumaryl ester bonds in xylans	GH
Endo–β–1,4–mannanase	Hydrolyse mannan-based hemicelluloses, liberate β–1,4–mannooligomers	GH
Exo–β–1,4–mannosidase	Hydrolyse β–1,4–mannooligomers to mannose	GH
Endo–galactanase	Hydrolyses β–1,4–galactan	GH
Acetyl mannan esterase	2– or 3–O–acetyl xylan	CE

GH: glycoside hydrolysis
CE: carbohydrate esterases

2.6.1 Classification of enzymes

Hemicellulases can be grouped based on their catalytic properties. The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyse glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester bonds (Table 2.3). These enzymes

(a) The enzymes involved in the degradation of a typical hardwood xylan

(b) The enzymes involved in the degradation of a typical softwood xylan

(c) The enzymes involved in the degradation of a typical cereal xylan
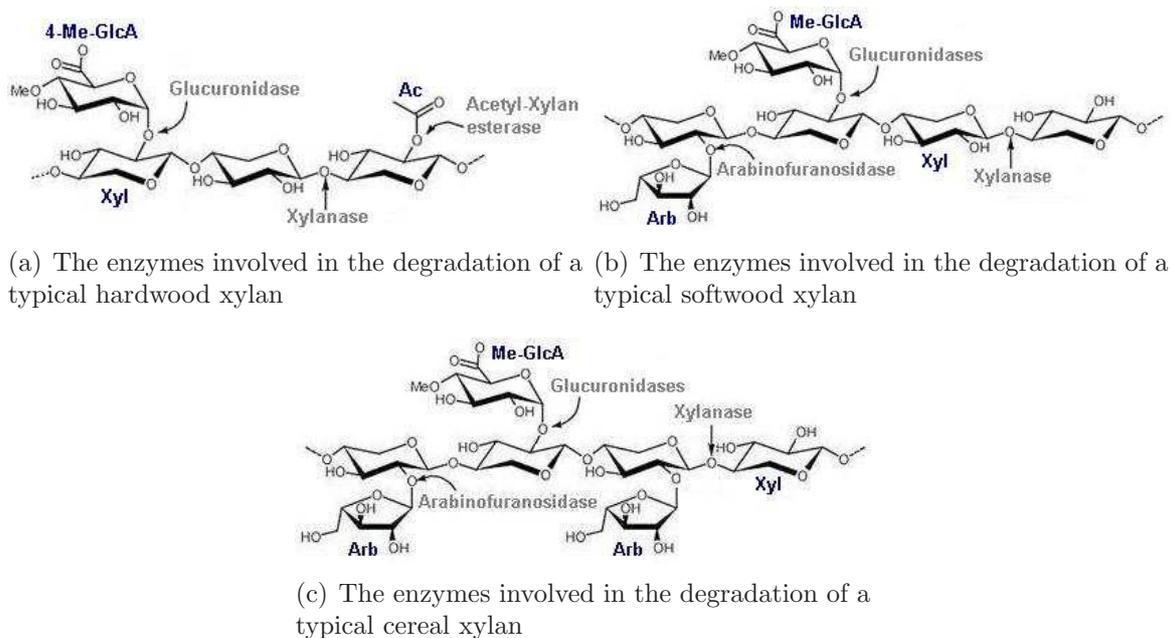
Figure 2.4: The enzymes involved in the degradation of typical xylans (Adapted from Ebringerová & Heinze (2000), Arb – arabinose, Ac – acetyl, Me-GlcA – methyl-glucuronic acid, Xyl – xylose)

are further assigned to numbered families, based on their primary catalytic activities as determined by their active site topography. Historically, enzymes were grouped into clans, marked alphabetically, based on the similarity in protein folds. Enzymes categorised into numbered families and those into alphabetical clans, have been known to exhibit similar catalytic properties. This is because the classification criteria, enzyme fold and active site topography are so closely related in determining the catalytic behaviour of the enzyme (Coughlan et al., 1993; Gebler et al., 1992).

Most of the known xylanases belong to GH families 10 and 11 (also F and G), but there are also xylanases in families 5, 8 and 43 . They are grouped into these families according to their physico-chemical properties such as molecular mass and isoelectric point. Family 10 contains high molecular mass molecules with low pI values, while low molecular mass molecules with high pI values belong to family 11. More importantly, there are differences in catalytic activity between the two families. The enzymes of family 10 show less substrate specifity; they have been known to also cleave the gluconic linkage and hydrolyse β–D–cellobiosides (Biely, 1993). The endo-xylanases of family 10 are capable of attacking the glucosidic linkages next to branch points and towards the non-reducing end, whereas those of family 11 are not (Subramaniyan & Prema, 2003). Also, the endo-xylanases of family 10 require two unsubstituted xylopyranosyl residues between branches, whereas the endo-xylanases of family 11 require three consecutively unsubstituted xylopyranosyl residues. According to Biely (1993), the endo-xylanases of family 10 possess catalytic properties which make them compatible with β-xylosidases.

β–Xylosidases are found in GH families 3, 39, 43, 52 and 54 (Shallom & Shoham, 2003).

Similar to chemical molecules, enzymes are grouped into enzyme classes, EC, with IUPAC numbering to facilitate nomenclature. The EC numbers are based on the enzyme designation as in Table 2.3.

2.6.2 Mode of metabolism and stereochemistry in hemicellulases

Reactions catalysed by enzymes follow three steps. First is the adsorption of the enzyme onto the polymeric substrate, which is followed by the subsequent cleavage of the bond as catalysed by the active sites on the enzyme. Thereafter, desorption of the enzyme from the substrate follows (Sun & Cheng, 2002). Enzymes are known to catalyse stereoselective hydrolysis, where the configuration about the anomeric centre of the monomeric sugar is either retained or inverted upon cleavage. Two mechanisms of hydrolysis are observed in the action of xylanases. The one is the single displacement mechanism, where the configuration about the anomeric centre is inversed and the other is the double displacement mechanism, where the configuration is retained.

The most common is the double displacement mechanism and all enzymes classed into families 10 and 11 exhibit this behaviour (Biely et al., 1994). Exoxylohydrolase has been shown to preferentially remove xylose from the non-reducing ends of xylans and xylooligomers with inversion of configuration according to the single displacement mechanism (Coughlan et al., 1993).

Regardless of the stereochemistry of hydrolysis, in a hydrolysate a racemic mixture will always be obtained because of mutarotation. The equilibrium concentration of xylose in water at 25 °C is 45:53 α to β (Franks, 1987), which makes the stereoselective nature of the enzyme a minor consideration.

2.6.3 Xylanolytic enzymes

Xylanolytic enzymes are a collective group of enzymes required for the degradation of plant xylan. Like all hemicelluloses, xylan is a heterogenic polymer with a complex and varied structure and therefore necessitates a proper mix of enzymes for its complete degradation to monomeric constituents.

The xylanolytic enzyme system consists of the following enzymes (Table 2.3):

- the main depolymerising enzyme; β–1,4–endoxylanase

- accessory xylanolytic enzymes; β–xylosidase, α–L–arabinofuranosidase, α–glucuronidase, acetyl xylan esterase and phenolic acid (ferulic and p–coumaric acid) esterase

The accessory enzymes are responsible for debranching of the substrate (Beg et al., 2001).

Endo–1,4–β–xylanases

Endo–1,4–β–xylanases, (1,4–β–D–xylanxylanohydrolase, EC 3.2.1.8), depolymerise the xylan hemicellulose by random hydrolysis of the xylan backbone, yielding short chain xylooligomers. The endo–xylanase enzymes require a long chain of consecutively unsubstituted xylopyranose units on which to act. The required chain length depends on the family of endo–xylanase used, but is usually more than eight. Branching and sidechains cause steric hindrance to the action of the enzyme (Coughlan et al., 1993).

β–Xylosidase

1,4–β–xylosidase (1,4–β–D–xylan xylohydrolase, EC 3.2.1.37) hydrolyse short xylo-oligomers chains into the monosugar, xylose. The action of the β–xylosidase is preferential to removal of monomers from the non-reducing end of the xylooligomer with affinity increasing with decreasing DP (Coughlan et al., 1993). Generally β–xylosidases do not show any activity on the polymeric xylans. An important consideration in the use of β–xylosidase is its susceptibility to inhibition by the xylose end-product (Poutanen et al., 1991).

α–Arabinofuranosidases

Less than a dozen arabinofuranosidase (EC 3.2.1.55) enzymes have been isolated and characterised since their activity have been identified. These enzymes are reported to liberate arabinose from arabinans, arabinoxylans and arabinogalactans. When the arabinose is a substituent on a xylan, the enzymes shows preference to the arabinose-linked oligosaccharides, rather than the xylan itself (Coughlan et al., 1993; Subramaniyan & Prema, 2003).

α–Glucuronidases

α–D–Glucuronidases (EC 3.2.1.139) are responsible for releasing D–glucuronic acid by hydrolysing the α–1,2–glucosidic linkage with the xylopyranose unit. In hardwood xylans, the D-glucuronic acid is found in the form of its 4–O–methyl ether. The α-1,2–linkage is by far the most stable and is often seen as the bottleneck in enzymatic hydrolysis of xylans. The 4–O–methyl–glucuronic acid linkage forms acts as a barrier against wood degradation (Subramaniyan & Prema, 2003).

As is the case with arabinosidases, few glucuronidases have been isolated. The preferred substrate is also short xylooligomers like 4–O–methylglucuronoxylobiose, with little to no activity towards the xylan polysaccharide (Poutanen et al., 1991).

Esterases

The glycosidic enzymes discussed above hydrolyse the glycoside linkages in the xylan polymer, but another type of enzyme is required to remove substituents bound by ester-linkages. The O–acetyl group found commonly in hardwood and cereal xylans is liberated by acetyl xylan esterases. These esterases have been found to act on both the xylan polymer and xylooligomers. Feruloyl esterases remove ferulic acid bound to the arabinoxylans of certain cereal xylans.

2.6.4   Synergism in the enzyme system

Generally the best synergism examples are found when accessory enzymes are used in conjunction with backbone hydrolysing enzymes. The side-chain cleaving enzymes improve accessibility to the backbone, improving yield of all the sugars. It has been proven over and over that endo-type enzymes show a low affinity to polysaccharides. The reason is suspected to be the complex nature of most polysaccharides and the resultant interference of the sidechains (Coughlan, 1992). However when deacetylation enzymes were used to pretreat the xylan before exposure to xylananses and xylosidases, a lower yield was observed. This suggested that the sequence of enzyme application not only affected the yield of liberated sugars but also influenced the nature of the oligomers, rendering it unsuitable for further degradation by enzymes (Poutanen et al., 1991). Deactelytion before xylanase action lowers the solubility of the xylan in water and also causes intermolecular aggregation (Tenkanen & Poutanen, 1992).

Many xylanases show no activity towards substituted xylose units. Their action is dependent on the accessory enzyme completely removing the side chains from the backbone (Saha, 2003). However, several of the accessory enzymes only show preference to the substituents on the oligomers and not the polymer (Saha, 2003). The highest yields have been observed when the enzymes are used simultaneously (Poutanen et al., 1991) and indeed xylan degrading organisms have been found to produce a whole spectrum of xylan hydrolyses. During xylan hydrolysis, synergism has been observed between the enzymes acting on the xylan backbone and the side chains. In the case of acetylated xylan, the release of acetic acid by acetyl xylan esterase increased the accessibility of the xylan backbone for endoxylanase attack. The products of endoxylanase activity are shortened acetylated polymers, which in turn is the preferred substrate of the esterases (Beg et al., 2001).

2.7   Products derived from monosugars

The most well-known method of bioconverting monosugars to other products is fermentation. Currently a small number of chemicals are produced from renewable sources via

fermentation. In Europe, the biotechnological production of lactic acid, acetic acid and ethanol are the only processes that can compete with the traditional petrochemical routes (Danner & Braun, 1999).

The fermentation of 6-carbon sugars have been studied and implemented widespread. A number of organisms have been identified that can ferment glucose with ease. The production of ethanol especially has been researched extensively. However, when considering the amount of hemicelluloses present in various biomass sources, it has been shown that the conversion of the major sugar in hemicelluloses, xylose, is paramount to ensure the feasibility of biomass conversion strategies. Various valuable products can be obtained by the fermentation of xylose.

2.7.1   2,3–Butanediol

Butanediol is produced during oxygen limited growth by the mixed-acid-butanediol fermentation pathway. It is also known as 2,3-butylene glycol and used in applications as a solvent, liquid fuel and precursor to various synthetic polymers and resins. 2,3-Butanediol has a heating value of 27,100 kJ/kg and is comparable to methanol and ethanol for used as liquid fuel and fuel additive. The solvent methyl ethyl ketone can be prepared by the dehydration of 2,3-butanediol. Further dehydration yields 1,3-butadiene, which is a precursor for synthetic rubber and various polymers (Saha, 2003). The theoretical maximum yield from xylose if 0.2 g/g (Magee & Kosaric, 1987). The organisms *Enterobacter cloacae* NRRL B-23289 (Saha, 2003) has been reported to be able to ferment glucose, arabinose and xylose to 2,3 butanediol with yields up to 0.43 g diol/g substrate.

2.7.2   Ethanol

The world demand for fuel ethanol is estimated at about 13 million tonnes per annum (Wilke, 1999). Much of this demand can be met by microbial production. Fermentation of glucose to ethanol using bacteria and fungi has been extensively developed, but xylose however, is hardly fermentable by microorganisms (Lee, 1997).

Many studies indicate that to economically convert lignocellulose to ethanol, utilising the xylose is crucial (Lee, 1997; Saha, 2003). It has been demonstrated by Hinman (quoted by Lee (1997)) that the production costs of ethanol can be reduced from $ 1.65 per gallon to $ 1.23 per gallon by fermenting the xylose to ethanol as well.

2.7.3   Lactic acid

With applications in the food, pharmaceutical and cosmetic industries, lactic acid is another value-added product that can be produced from fermentation of hemicellulose hydrolysates. The world demand for lactic acid is estimated at 70 000 tonnes per annum

(Wilke, 1999). New applications include the use of ethyl esters to replace hazardous solvents (Danner & Braun, 1999). Lactic acid may also be polymerised to poly(L-lactic acid), a biodegradable plastic (Tanaka et al., 2002).

In industrial applications sugars are converted to lactic acid under anoxic conditions. Fungal and bacterial strains of *Rhizopus* and *Lactobacillus sp.* cultured on agricultural residues such as sugarcane bagasse are commonly used to produce lactic acid (Krishna, 2005). Recombinant strains have been known to ferment xylose to lactic acid at yields of 56 - 63 g lactic acid from 100 mg/ℓ xylose (Saha, 2003).

2.7.4 Xylitol

Xylitol is used as an alternative food sweetener for diabetics. Slow adsorption of xylitol into metabolic pathways is independent of insulin and does not cause rapid fluctuations in blood sugar levels. It is also used in orthodontical applications because of its beneficial properties such as teeth hardening, remineralisation of tooth enamel and antimicrobial properties. When used in gum and toothpaste manufacture it gives a cool, refreshing taste due to its negative heat of solution (Winkelhausen & Kuzmanova, 1998; Saha, 2003; Nigam & Singh, 1995).

Several other applications of xylitol are also receiving renewed interest, such as its use as sweetener in sweets and chocolates, baked goods and food marinades and sauces. It can also be used as sweetener in liquid pharmaceutical applications as it will not cause damage to teeth (Emodi, 1978).

Currently the world xylitol market is estimated at $ 28 million/annum (Anon, 2000a), but is expected to grow fast once its potential applications are utilised fully. At the current price of $ 3/lb, the market volume can be estimated at 4'200 tonnes/annum (Anon, 2000b).

2.7.5 Furfural

Furfural is the product of the degradation of xylose sugars. In commercial processes, furfural is produced by the acid catalysed degradation of xylose (Galbe & Zacchi, 2002). Furfural is used in the manufacture of furfural-phenol plastics, varnishes and pesticides (Howard et al., 2003). Over 200'000 tonnes of furfural are produced annually at a market price of $ 1700/ton (Montané et al., 2002).

2.8 Concluding remarks from literature review

Higher energy demands and concern for the environment has renewed interest in the conversion of biomass sources to fuels and other chemicals. Particularly, interest in the

recovery and use of hemicelluloses is gaining momentum. As a source of various sugars, hemicelluloses make up a significant portion of biomass, and have been neglected regularly in bioconversion strategies.

In the pulp and paper industry a unique opportunity exists for the efficient use of the wood that enters the mill. Hemicelluloses, and xylan in particular, can potentially be recovered from a number of different locations within the pulping process. The major constituent sugar of xylan, xylose, is an important platform chemical for conversion to fuels and other chemicals.

Hemicelluloses are more complex than cellulose and often comprise several types of base sugars. Hardwood xylans are primarily O–acetyl–4-O-methyl-glucuronoxylans, whereas cereal xylans also contain significant amounts of arabinose and are called arabinoglucuronoxylans. Both are composed of a 1,4–linked β–D–xylose backbone. In hardwood xylans approximately 70 % of these xylose units are acetylated with methylglucuronic acid. In cereal xylans about 20 % of the xylose units are acetylated and 10 – 15 % are substituted with an arabinose residue. In addition to carrying more substituents, cereal xylans are generally more heavily branched than their hardwood counterparts.

Due to the complex nature of hemicelluloses, the enzymatic hydrolysis of xylans requires the synergistic workings of several enzymes. The main depolymerising enzyme is endo–xylanase, which is responsible for the hydrolysis of the xylose-backbone. The endo–xylanase randomly cleaves the xylose polymer into shorter xylose-oligomers. The action of the xylanases is supplemented by accessory enzymes that cleave substituents off the main chains and hydrolyse oligomers into monomers. In the degradation of xylan, the most important accessory enzyme is β–xylosidase, which converts xylose–oligomers into monomeric xylose.

From this literature study it is evident that the xylans extracted from hardwood pulps could be a good source for bioconversion to xylose. When studying their enzymatic hydrolysis, however, the species from which they are derived may not be the only factor contributing to their susceptibility to degradation, the method used for extraction should also be considered.

The complete hydrolysis of hardwood xylans with enzymes may require the action of endo–xylanases, xylosidases and esterases, depending on the composition of the xylan. Commercial enzymes often contain several enzyme activities depending on the source from which they were isolated and their intended application. Their suitability to degrade the wood xylans would depend on the types and quantities of enzyme activity present.

CHAPTER 3

Carbohydrate analyses

3.1 Introduction

In carbohydrate research, colorimetric techniques are often used to estimate the sugar concentrations in samples. Particularly, the Dinitrosalicylic acid (DNS) assay (Bailey et al., 1992) and the Arsenomolybdate assay by Somogyi and Nelson (S-N assay) (Somogyi, 1945; Nelson, 1944) are the preferred methods for the evaluation of enzyme activity (Ghose & Bisaria, 1987). Despite the disadvantages such as low reproducibility and low accuracy (Jeffries et al., 1998; Deschatelets & Yu, 1986), their ease of use make them the preferred method of choice in routine assays (Bailey et al., 1992).

Historically, the reducing sugar content and monosugar content of carbohydrate hydrolysates were determined with colorimetric techniques in parallel with gas chromatography for determining the various fermentation products (Schwald & Saddler, 1988). Significant advantages in chromatography now provides a simple and effective method for quantifying sugar mixtures. Column developments allows for the quantification of all the sugar and fermentation products in one run (Kaar et al., 1991; Schwald & Saddler, 1988; Buchert et al., 1993).

Since both colorimetric and chromatographic methods have distinct advantages, the Dinitrosalicylic acid (DNS) and Somogyi-Nelson (S-N) colorimetric techniques and high performance ion exchange chromatography (HPIC) were compared based on their ability to detect sugars in hydrolysates from enzymatic and acid conversions.

3.2 Materials and Methods

3.2.1 Total reducing sugars

The reducing sugar concentration was estimated as xylose equivalent by dinitrosalicylic acid (DNS) (Bailey et al., 1992) and Somogyi-Nelson (S-N) colorimetric assays (Somogyi, 1945; Nelson, 1944).

3.2.2 Monosugars

Monosugar analysis was conducted using high pressure ion chromatography (HPIC) on the Dionex ICS-2000. The detector was an ED-50 electrochemical detector (Dionex) using Pulsed Amperometric Detection with disposable gold electrodes and the Dionex recommended voltage waveform. The column was a CarboPac PA20 with no guard column or amino trap installed. The column temperature was maintained at 30 °C. Dionex sample vials (6 mℓ) were fitted with filtration caps and analysed sequentially from an AS-40 autosampler (also from Dionex). The results were analysed with Chromeleon v.8 software.

The column was eluted with ultrapure water (18 MΩ) at a flowrate of 0.40 mℓ/min and pressure of 2000 psi. Before the detector, 400 nM NaOH was added post-column at a flowrate of 0.40 mℓ/min and pressure of 1000 psi with a Dionex AXP pump. Backpressure tubing was installed to enhance the effect of the pulse dampener on the AXP. The sample volume in the sample loop was approximately 10 $\mu\ell$.

The analysis run was 20 minutes, where after the column was rinsed with 400 mM NaOH for 25 minutes. The hydroxide flow was switched from post-column addition to flowing through the column with a switching valve. After rinsing, the column was stabilised with ultrapure water for 15 minutes. The total run time per sample was therefore 60 minutes.

The method was based on recommendations in the CarboPac PA20 technical manual (Dionex, 2005) and on experience from the instrument operator.

3.3 Results and Discussion

DNS and S-N analyses yielded monosugar content as total reducing sugar concentration; the assays do not allow for differentiation between the individual sugars. Individual monosugar concentrations as well as total monosugar yield could be determined using the HPIC, which has been shown to be very accurate and highly reproducible when applied to carbohydrate analysis (Deschatelets & Yu, 1986; Schwald & Saddler, 1988). The HPIC was therefore used as the benchmark to validate results from the DNS and S-N assays.

3.3.1 Comparison of analysis techniques

Acid hydrolysis of the xylan substrates produced monosugars, as discussed in Appendix A. Analysis of the hydrolysates (Oat Spelts and Birchwood xylan hydrolysed with different concentrations of H_2SO_4 for 1 hour) by DNS, S-N and HPIC methods yielded different results for the percentage conversion of the xylans to their monomeric sugars (Figures 3.1 and 3.2). Regardless of the substrate, the same trend of hydrolysis was obtained from all three analytical methods; a higher acid concentration led to a lower sugar yield.
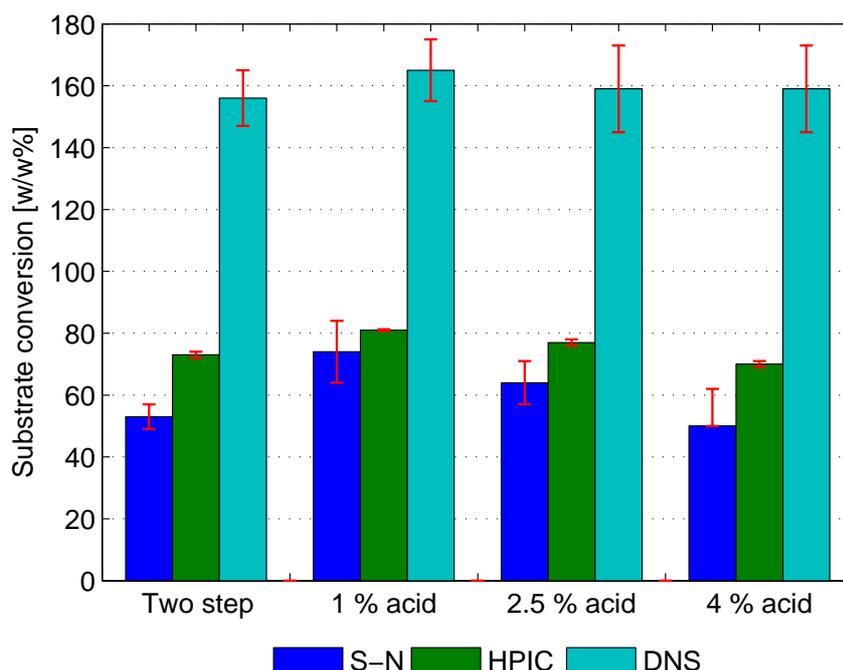
Figure 3.1: Comparison of the DNS, S-N and HPIC methods for determining conversions after acid conversion of Oat Spelts xylan.

Sugar analysis by the Somogyi-Nelson method

When compared to results from HPIC analysis, the S-N method underestimated the concentration of total sugars by up to $10 - 30\%$, the difference becoming more pronounced as the total sugar concentration decreased.

The S-N assay for reducing sugars was initially developed to determine glucose concentrations in blood (Somogyi, 1945; Nelson, 1944), which is usually present in small quantities. The optimisation of the S-N assay for this application may have led to the reagents only being sensitive to sugars in low concentrations. In this study the calibration curve was found to be linear for xylose concentrations below 0.5 g/ℓ, which resulted in the need for excessive dilutions. The experimental error introduced by dilution needed for higher concentrations of sugar may explain the progressive decline in accuracy of the S-N assay as seen in Figures 3.1 and 3.2.
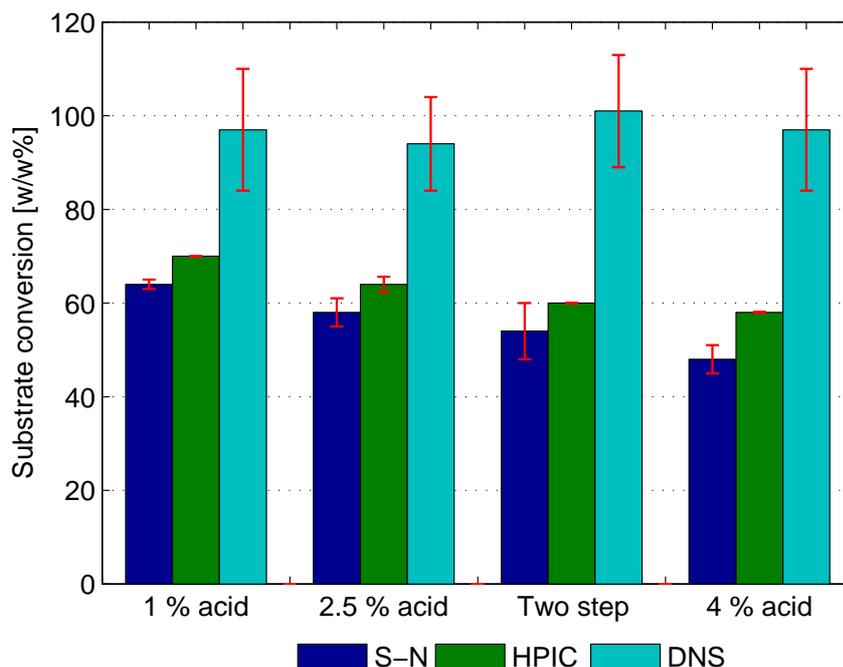
Figure 3.2: Comparison of the DNS, S-N and HPIC methods for determining conversions after acid conversion of Birchwood xylan.

Analysis by S-N underestimated sugar concentrations in hydrolysates (Bailey et al., 1992), but it was illustrated (Figures 3.1 and 3.2), that the results are closer to that of HPIC than could be obtained with DNS. Compared to DNS, S-N therefore yielded more accurate results. However, the S-N method involves more steps and reagents, and has not yet replaced DNS in routine enzyme assays. Nevertheless, the method can be applied to carbohydrate analysis with some success as shown here, and is the method of choice in some laboratories (Ghose & Bisaria, 1987).

Sugar analysis by the Dinitrosalicylic acid method

When DNS is added to the carbohydrate mixture, the 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid, while the aldehyde groups of the carbohydrate are oxidised (Miller, 1959). The DNS method overestimated sugar concentrations by at least 60 % over the possible maximum (Figure 3.1). It also reads over 100 % higher than the HPIC analysis. From the chemical stand point, the analysis reagents react with all monomeric sugar species as well as the non-reducing ends of oligomers, thereby increasing the observed yield and overestimating conversion (Deschatelets & Yu, 1986; Jeffries et al., 1998). While this explains the over-estimation obtained with acid hydrolysis, it is also a problem when enzymatic hydrolysis is used. Both acid and xylanases randomly cleave the xylan backbone, yielding oligomers of various chain lengths. The differences in analysis by DNS and HPIC can be seen when Figures 3.3 3.4 and are compared. Initially

the conversions and trends were similar, but after 24 hours the DNS analysis started to deviate from the HPIC analysis, indicating higher conversions. This difference may be explained by the increased presence of oligomers at higher hydrolysis times, which interferes with the DNS analysis but not the HPIC analysis.
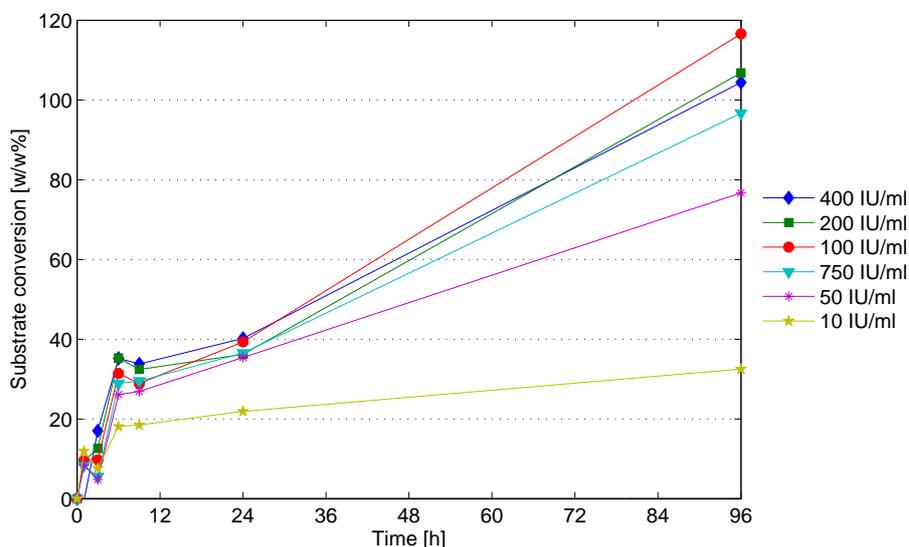
Figure 3.3: DNS analysis of the conversion of Birchwood xylan to total monosugars by Multifect Xylanase.

While the reaction with oligomers in the DNS analysis may have led to over-estimation of the monomeric sugars, it does not account for the conversions higher than 100 %. This may be explained as a result of interference from other sugars in the hydrolysate. Different sugars yield different amounts of colour (Miller, 1959) and because the calibration curve was constructed with xylose only, the presence of other sugars may have imparted a darker colour that would have been interpreted as a higher concentration.

This possibility is supported by the comparison of the sugar yields obtained for the DNS analysis of Oat Spelts xylan hydrolysates (Figure 3.1) to that of Birchwood xylan hydrolysates (Figure 3.2). Here the total sugar yield detected by HPIC analysis differed by 20 % for the two xylans, while DNS analysis indicated a difference of more than 60 %. The larger difference observed for Oat Spelts xylan may be explained by the effect of the other sugars present in the hydrolysate that are not found in the hydrolysate of Birchwood.

In order to obtain the most accurate results from the colorimetric techniques, the following recommendations are made:

- Experiments should be planned so that the minimum and maximum amount of sugar to be detected falls within the linear range of the calibration curve, thus eliminating the use of extensive dilution.

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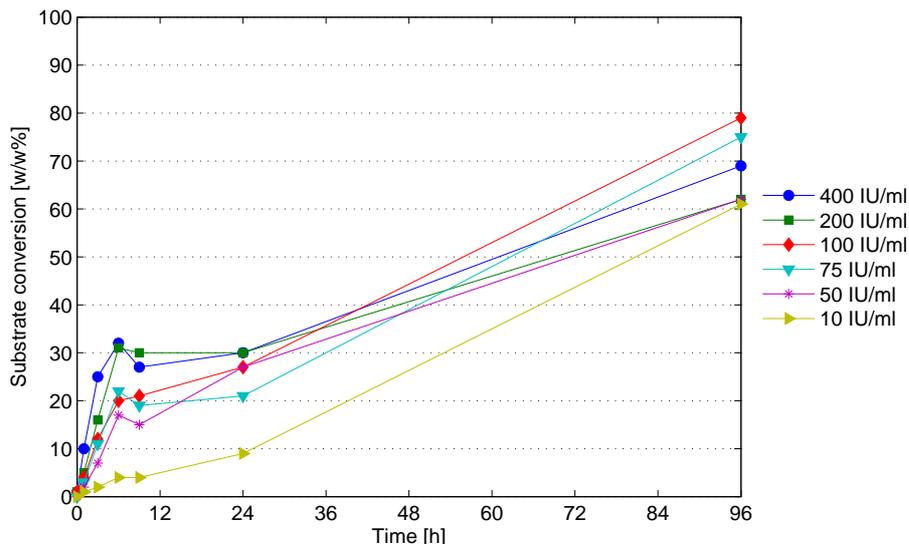
Figure 3.4: HPIC analysis of the conversion of Birchwood xylan to total monosugars by Multifect Xylanase.

- Using sugar standards in comparable compositions may improve accuracy, especially in the DNS assay.

- As is stressed in literature standards, blanks and samples should be heat treated simultaneously as the colouring agents are very sensitive to temperature and variations can lead to inaccurate results. For the same reason the analyses to be compared should be colour-developed in the same batch to increase accuracy and repeatability.

3.3.2 Sugar analysis by high performance ion exchange chromatography

In the remainder of the investigation HPIC was the preferred method of analysis, with DNS and S-N analyses used only in enzyme assays and trend determinations. Despite the increased accuracy of HPIC over the colorimetric techniques, some optimisation of the method was needed to improve repeatability and instrument stability.

Deionised water was used as eluent in the ion exchange chromatography method to enable the separation of xylose and mannose in the column. Hydroxide was added post-column to enhance detector response. Sugar standards were included after every fifth sample to improve the accuracy of the results. Arabinose, rhamnose, galactose, glucose, xylose and mannose (Sigma-Aldrich) in concentrations of 50 mg/ℓ were used as sugar standards. With the employed method, arabinose and rhamnose co-eluted (Figure 3.5), with slight peak overlaps between xylose and mannose. As the xylans used in this investigated were not expected to contain significant rhamnose or mannose, the analysis

of xylose and arabinose was not compromised. In the optimisation of the method, the guard column was removed as this caused tailing of the peaks. The removal of the amino trap improved the resolution of the analysis. The absence of these two columns did not affect the lifespan of the main analysis column negatively. Column fouling by the unhydrolysed portion of the substrate in the samples was limited by rinsing the column with hydroxide after each sample injection to remove highly retained ions from the active sites. Despite these precautions low repeatability was observed on consecutive analyses of the same samples (Table 3.1).
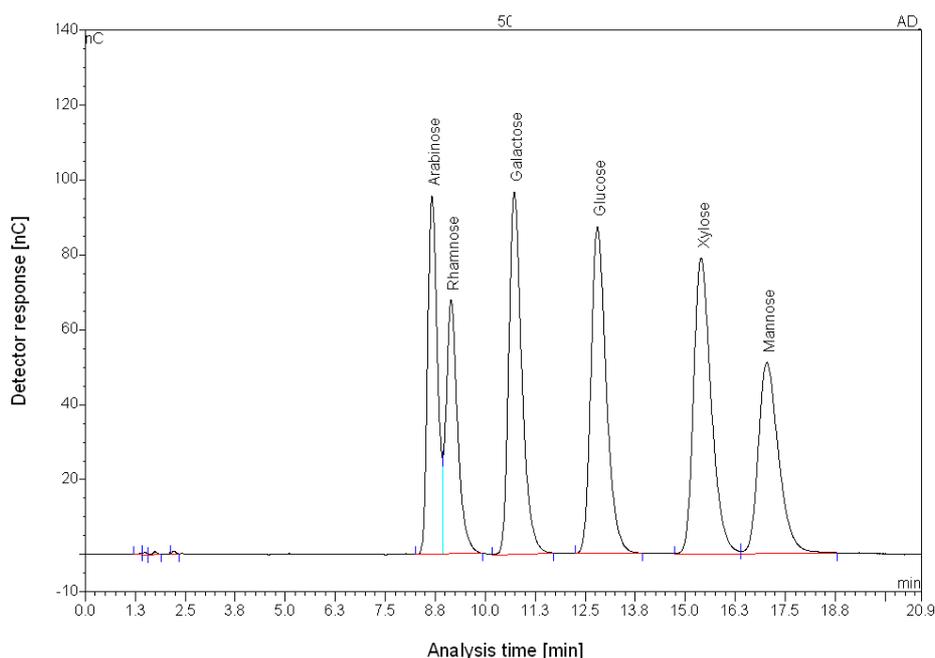
Figure 3.5: Chromatogram of a standard sugar solution analysed on the HPIC.

Large discrepancies were observed between analyses on different days, which were particularly evident for the xylose standard solution (Table 3.1). There was no change in instrument setup between the different runs and the samples were kept frozen until analysed to limit degradation of the sugars.

In order to illustrate the stability of the baseline and repeatability of analysis, sequential injections of a 50 ppm composite sugar standard were made. The sugar standard contained arabinose, glucose, galactose, xylose and mannose at concentrations of 50 mg/ℓ. The variation in concentrations measured by the instrument can be seen in Figure 3.6. It was found that the detected concentration continued to decrease with repeated analysis, with a significant decrease already apparent after six injections. Any number of factors could have contributed to this effect, including column fouling, detector fouling, changes in the hydroxide concentration or sugar degradation of the standards.

Fluctuations of the hydroxide concentration entering the detector could have man-

Table 3.1: Repeated HPIC analysis of some acid hydrolysed samples (% xylose yield)

Sample	08–06–2007	06–09–2007 *	20–09–2007 **	17–12–2007 **	18–10–2007 **
Xylose (0 % acid, 1 h)	88 %	110 %	87 %	89 %	82 %
Xylose (1 % acid, 1 h)	96 %	114 %			63 %
Oat Spelts (1 % acid, 1 h)	81 %	73.8 %			48 %
Xylose standard solution			87 %	105 %	

* new samples were prepared and analysed

** the samples of 06–09–2007 were reanalysed

ifested as changes in concentration. However this was an unlikely occurrence as the concentration of hydroxide was regulated by an online eluent generator. Fouling of the detector was another probable cause ruled out as there was good reproducibility of the peak heights. Possible fouling of the column was considered since it is a known problem in the analysis of carbohydrates from enzyme hydrolysates. This was identified by peak shifting which was observed over the analysis period. Extensive rinsing of the column had already been implemented and adding further rinsing time was not feasible as the analysis already took one hour per sample.

Degradation of the sugars while stored in the autosampler was another possible cause, as the autosampler was not refrigerated. A closer investigation of the sugar standards however revealed that freshly prepared standards immediately injected unto the column and standards treated with an anti-fungal agent showed the same decrease in measured concentration as observed with the original standard solution. It was therefore apparent that the change in measured concentration was not due to sugar degradation.

Column fouling was concluded to be the main contributing factor towards the decreased accuracy of the measured concentrations. Column fouling would have affected the analysis of all sugars equally and could therefore be compensated for by the addition of an internal standard. A number of sugars were screened for their suitability as internal standards including, inositol, sorbitol, fucose, sucrose and fructose. Inositol eluted after one minute, which was not suitable as all components not strongly retained by the column would interfere with this peak, causing inaccuracies in the analysis. Sorbitol eluted after 2.5 minutes, but a closer investigation revealed that the enzyme preparation con-
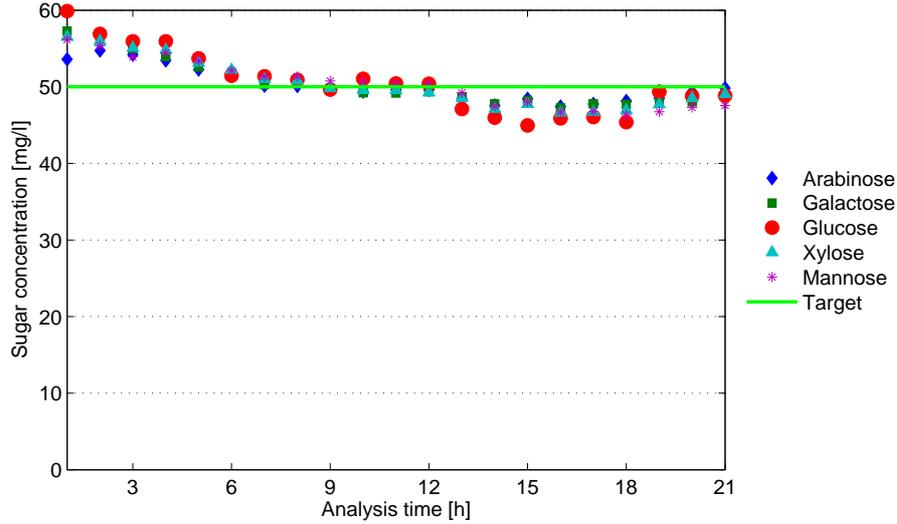
Figure 3.6: Repeated HPIC analysis of a 50 ppm composite sugar standard showing variation in measured concentrations.

tained copious amounts of sorbitol, most likely as a preservative. Fucose eluted after five minutes, but as some wood substrates may contain fucose, it was not a reliable internal standard. Using the current analysis method, sucrose had similar retention to glucose, rendering it unsuitable. The fructose peak eluted after 21 minutes, which was well clear of the other sugar peaks. Its positioning was ideal to compensate for column fouling as it eluted toward the end of the analysis period.

The concentration of all sugars in the sample was calculated by relating the measured peak height of the sugar in the sample to that of the internal standard with a concentration of 50 mg/ℓ using Equation 3.1:

$$C_U = (D_f) \left(\frac{C_{St}}{average \left(\frac{S_h}{I_h} \right)} \right) \left(\frac{U_h}{I_h} \right) \tag{3.1}$$

where C_U is the calculated concentration of the sugar in the sample, C_S the measured concentration of the sugar standard, D_f the dilution factor, S_h measured peak height of the sugar standard, I_h measured peak height of the internal standard included in the sample and U_h peak height of sugar in the sample.

To improve accuracy of results reported, samples that were to be compared were analysed in the same batch on the HPIC as far as possible. Despite this, inconsistencies were observed in some results that had made data analysis a difficult task. The internal standard was added to the analysis vials in exact amounts, which should have allowed compensation for fluctuations of the instrument between sample analyses. Figure 3.7, however shows how the peak height and width of the internal standard as measured by the instrument, varied by 27.95 and 29.10 % respectively during an analysis batch.
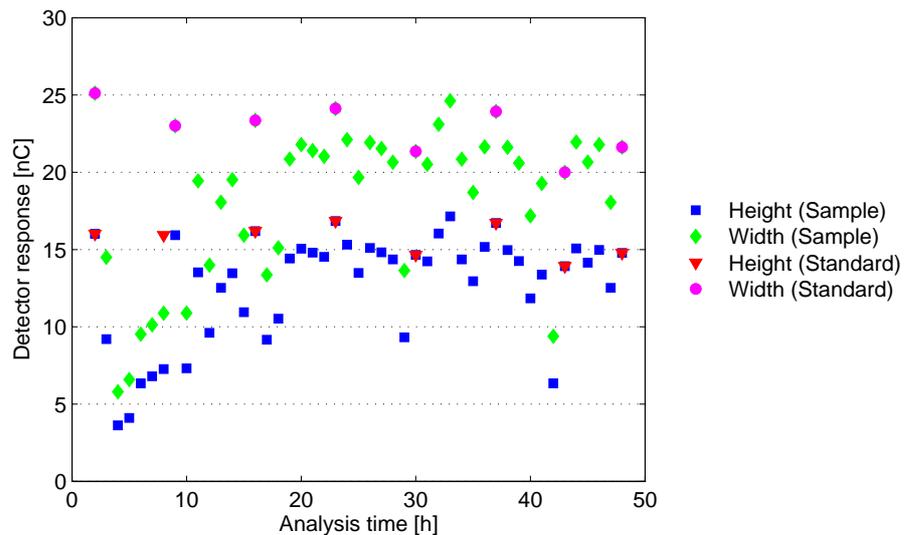
Figure 3.7: The detector response for peak height and width of the internal standard as measured on the HPIC.

For each analysis batch, the concentrations were adjusted according to the average peak height of the internal standard in the standard preparations (Equation 3.1). However, when the variations were larger than 5 %, as seen in Figure 3.7, the results were deemed unusable (see Section 4.3.7) and the analysis was repeated.

CHAPTER 4

Enzymatic hydrolysis of selected xylans

4.1 Introduction

Biomass conversion rates by specific enzymes are mainly dependent on the enzymes used, substrate source and the state in which the substrate is supplied. For example, the protective sheath that the lignin and hemicellulose form around the cellulose fibrils (see Section 2.3), make all the components of a native fibre less susceptible to enzyme action.

The ability of the commercial enzymes to convert hemicelluloses to monomeric sugars was investigated using two sources of xylan: Birchwood xylan, a typical hardwood xylan and Oat Spelts xylan, a graminaceous xylan. As was discussed in Section 2.3.2, the composition of each of these xylans necessitates the use of more than one enzyme to achieve complete conversion. The monomeric sugar composition of the xylans used in this investigation was determined by conversion with acid (See Appendix A). Birchwood xylan is composed almost entirely of xylose and the enzymes required for hydrolysis would therefore include xylanases and xylosidases. The Oat Spelts contained additional substituents such as glucose and arabinose, therefore in addition to the xylanases and xylosidases, side-chain cleaving enzymes might also be needed.

The commercial enzymes used in this investigation were chosen mainly on the basis of their xylanase activity. Typically, the exact makeup of the enzyme preparation is not disclosed to the customer as it is often proprietary information. The commercial enzymes used in this investigation have been developed by suppliers for different applications and therefore were expected to not exhibit exactly the same behaviour. Although Xylanase P and Optimase are both bleaching enzymes for use mainly on wood pulps, they are from different suppliers and are extracted from different organisms. Xylanase P is an enzyme intended for acid conditions, whereas Optimase is an alkalophilic enzyme (Genencor, 2007b) from genetically modified strains of *Bacillus lichenifermis* and *Bacillus*

alcalophilus respectively. Multifect is used for the partial digestion of animal feeds and is purified from a genetically modified strain of *Trichoderma reesei* (Genencor, 2007a). A limited amount of purified xylosidase from Sigma-Aldrich was also available. Although this was a laboratory grade enzyme that cannot be applied in industry, it could still serve as adequate indication of the hydrolysis potential of a xylosidase.

The hydrolysis potential of the commercial xylanases was investigated by looking at different enzyme properties. Enzyme characterisation was conducted to determine enzyme activities. The enzymes were assayed for xylanase, xylosidase and cellulose activities. Furthermore the enzyme preparations were also evaluated for residual sugars.

4.2 Materials and Methods

4.2.1 Enzymes

Commercial xylanase preparations were used in the investigation. The following samples for laboratory application were obtained: Xylanase P from Iogen (Canada), Multifect Xylanase and Optimase CX72L from Genencor (Finland). In addition, a purified β–xylosidase was obtained from Sigma–Aldrich (Germany).

4.2.2 Enzyme characterisation

Xylanase assay: Xylanase P

The xylanase activity of Xylanase P enzyme was determined by the DNS assay for reducing sugars as described by Bailey et al. (1992) using Birchwood and Oat Spelts xylan as substrates. The appropriately diluted enzyme, relevant blanks and standards were incubated with the xylan substrate (1 % w/w, citrate-phosphate buffer, 0.05 M, pH 6.0) and incubated at 60 °C for 5 minutes. The colour was read on a spectrophotometer (Helios–β–Unicam, England) and translated to concentration with the standard absorbance curve. The activity of the enzyme in International Units (IU)/mℓ was subsequently calculated, where 1 IU is the number of μmoles of xylose produced per minute of hydrolysis per mℓ of enzyme used (Ghose & Bisaria, 1987). Throughout the experimental work, substrate concentrations of 1 % were chosen because of the limited solubility of the polymeric substances in aqueous solutions. Also, an enzyme:substrate ratio of 1:9 ensured good substrate availability to the enzyme allowing for linearity in the assays and hydrolysis experiments (Bailey et al., 1992).

Xylanase assay: Multifect Xylanase

Xylanase activity was determined as described by Bailey et al. (1992). To determine the effect of temperature on activity, the appropriately diluted enzyme and Oat Spelts xylan

(1 % in citrate-phosphate buffer, 0.05M, pH 5.0) were incubated at 50, 55, 60, 65 and 70 °C. The enzyme activity was also determined at different pH values (Bachmann & McCarthy, 1991). The appropriately diluted enzyme and Oat Spelts xylan suspended in Britton-Robinson buffer (0.05 M, pH ranging from 2.0 to 8.0) was incubated at 60 °C for 5 minutes.

The activity of the enzyme was also determined using Birchwood xylan as substrate (1 % in citrate-phosphate buffer, 0.05 M, pH 5.0) incubated at 55 °C. Similarly, the activity of Multifect was evaluated on Eucalyptus xylan as substrate at 55 °C and pH 5.0.

Xylanase assay: Optimase Xylanase

The xylanase activity of Optimase was determined with the method of Bailey et al. (1992). To determine the effect of temperature, the Optimase and Oat Spelts xylan were suspended in buffer (citrate-phosphate, 0.05 M, pH 8.0) and incubated at temperatures ranging from 40 to 80 °C. The enzyme activity was also determined at different pH values (Bachmann & McCarthy, 1991). The enzyme and substrate suspended in buffer (Britton-Robinson, 0.05 M, pH values ranging from 2.0 to 10.0) were incubated at 65 °C.

The xylanase activity of Optimase on Birchwood xylan was determined by suspending the enzyme and substrate in buffer (citrate-phosphate, 0.05 M, pH 7.0) and incubating at 65 °C as described previously.

β–Xylosidase assay

The commercial enzymes were evaluated for β–xylosidase activity using a nitrophenyl glycoside (NPG) substrate (Ghose & Bisaria, 1987).

The effect of temperature on xylosidase activity in the xylanase enzymes was investigated with assays conducted at pH 4.0 and a temperature range of 40 to 70 °C using p-nitrophenyl-β-xylopyranoside (PNPX) as substrate and Britton-Robinson buffer as diluent (0.05 M, pH 4.0). The enzyme and substrate were incubated at the various temperatures for 10 minutes after which the reaction was stopped by the addition of 0.5 mℓ of a 1 M NaH_2CO_3 solution. The xylanases were also evaluated for xylosidase activity at varying pH values ranging from pH 4.0 to 8.0, with samples incubated at 50 °C for 10 minutes.

Sample absorbances were read at 400 nm on the spectrophotometer and the activities calculated. Enzyme activity of 1 IU is the number of μmoles of ρ-nitrophenol produced per minute of hydrolysis per mℓ of enzyme solution used (Ghose & Bisaria, 1987).

Filterpaper assay for cellulases

The cellulase side-activity of the commercial xylanases was determined using the Filter paper assay (Ghose, 1987). Whatman No. 1 filterpaper strips of 1.0 cm x 6.0 cm were

used as substrate and 0.05 M citrate-phosphate buffer (0.05 M, pH 4.8) as diluent. The assay was conducted at 50 °C with samples incubated for 1 hour. After incubation the sample absorbances were determined at 540 nm with the spectrophotometer and enzyme activity expressed as filter paper units (FPU). 1 FPU is described as the amount of enzyme that produces 2.0 mg of glucose in 60 minutes hydrolysis time. As the FPU assay is non-linear, the use of IU is inappropriate, and FPU is calculated as follows (Ghose, 1987):

$$FPU = \frac{0.37}{\text{enzyme concentration to release 2.0 mg glucose}} \qquad (4.1)$$

where 0.37 is a unit correction factor.

Carboxymethyl Cellulase assay for endo–1–β–glucanase

The endo–1–β–glucanase side activity of the commercial xylanases was determined using the carboxymethyl assay (Ghose, 1987). Carboxymethyl cellulose- dissolved in citrate-phosphate buffer (0.05 M, pH 4.8) was used as substrate. The enzyme and substrate were incubated at 50 °C for 30 minutes, where after the absorbance of the samples were determined at 540 nm and translated to activity. The endo–1–β–glucanase of the enzymes were expressed in carboxymethyl cellulose units (CMC) where 1 CMC represents the amount of enzyme needed to release 0.5 mg glucose in 30 minutes hydrolysis time. Analogous to the FPU assay, the CMC assay is non-linear and the activity is calculated as follows (Ghose, 1987):

$$CMC = \frac{0.185}{\text{enzyme concentration to release 0.5 mg glucose}} \qquad (4.2)$$

where 0.185 is a unit correction factor.

4.2.3 Determination of residual sugars in the enzyme preparations

Residual sugars in the enzyme preparation were determined by diluting the enzyme preparations in buffer and placing in a boiling water bath for 5 minutes to deactivate the enzyme. Suspended solids were removed from the samples by centrifuging at 8000 rpm for 8 minutes and the supernatant subsequently analysed for carbohydrate content with HPIC.

4.2.4 Enzymatic hydrolysis of xylan substrates

Xylanase P

Birchwood xylan (1 %) and appropriately diluted enzyme in buffer (citrate phosphate, 0.05 M, pH 6.0) in a ratio of 9:1 were incubated in an orbital shaking incubator (LM-510R SciLab, Taiwan) set at 65 rpm. The temperature in the incubator was maintained at 60 °C, the optimum of the Xylanase P enzyme. Aliquots were drawn off at various time intervals and immediately placed in a boiling waterbath for 5 minutes. Thereafter, samples were centrifuged at 8000 rpm for 8 minutes at 4 °C to remove suspended solids. The samples were frozen and stored till further analyses.

Enzymatic hydrolysis with Oat Spelts xylan as substrate was done according to the method described above.

Multifect Xylanase

Birchwood xylan (1 %) and Multifect in buffer (citrate phosphate, 0.05 M, pH 5.0) were incubated in an orbital shaking incubator set at 65 rpm and 55 °C, the optimum temperature of the Multifect enzyme. Hydrolysis was carried out as described previously. Enzymatic hydrolysis with Birchwood and Eucalyptus xylan as substrates was also done.

Optimase Xylanase

Birchwood xylan (1 %) and Optimase in buffer (citrate phosphate, 0.05 M, pH 7.0) were incubated in an orbital shaking incubator set at 65 rpm and 65 °C, the optimum temperature of the Optimase. Hydrolysis was carried out as described previously. Enzymatic hydrolysis with Birchwood xylan as substrate was done as well.

4.2.5 Hydrolysis of Oat Spelts xylan with Optimase and β–xylosidase

Oat Spelts xylan (1 %) was suspended in buffer (citrate-phosphate, 0.05 M, pH 7.0) and added to appropriately diluted Optimase in a ratio of 9:1. The substrate and enzyme were incubated in an orbital shaking incubator set at 65 rpm for six hours. The temperature was maintained at 65 °C, which is the optimum tempature of the Optimase enzyme. Thereafter the samples were adjusted to pH 5.0 with dilute H_2SO_4. β–xylosidase (Sigma-Aldrich) was added to the samples in a ratio of 1:50 and the samples were incubated in an orbital shaking incubator, 65 rpm, set to 50 °C for 72 hours. The adjusted temperature of 50 °C was the optimum tempature of the xylosidase.

Aliquots were drawn off at various time intervals and immediately placed in a boiling waterbath for 5 minutes. The samples subsequently were centrifuged at 8000 rpm for 8

minutes at 4 °C to remove suspended solids. The samples were frozen and stored until further analysis.

4.2.6 Effect of multiple dosing on xylan hydrolysis

Birchwood xylan (1 %) was suspended in buffer (citrate-phosphate, 0.05M, pH 5.0) and added to appropriately diluted enzyme. The substrate and enzyme were incubated in an orbital shaking incubator set at 65 rpm. The temperature was maintained at 55 °C, which is the optimum of the Multifect xylanase. At various time intervals enzyme was added to the samples. Aliquots were drawn off at various time intervals and immediately placed in a boiling waterbath for 5 minutes. The samples were centrifuged to remove suspended solids and frozen until further analysis.

4.3 Results and Discussion

4.3.1 Enzyme characterisation

Enzyme characterisation involves identifying the activity of the enzyme and investigating the change in activity at different pH and temperature values. Although the enzymes' pH and temperature optima as well as relative activity are given by the supplier, these properties were verified in the current study. The enzymes could be dosed in specific quantities to yield similar final activities in the substrate solution. Since some of the enzymes investigated are multi-component systems, the individual enzyme activities were determined and selectively characterised.

Endo-xylanase

The optimum hydrolysis conditions of the enzyme is not only where the activity is highest, but also where the stability of the enzyme over the hydrolysis period is best. Characterisation was carried out by first determining the optimum temperature and then the optimum pH of the xylanase. Temperature stability was performed at the optimum pH and the optimum temperature as well as 5 °C below and above the latter.

Xylanase P had been characterised before and the pH and temperature optima are reported to be 60 °C and pH 6.0 respectively (Chipeta, 2006). The activity of the enzyme was subsequently determined under the above conditions on both Oat Spelts and Birchwood xylan as 11 000 IU/mℓ. The thermostability study of this enzyme under these conditions revealed that the enzyme retained up to 70 % of its activity in the first four hours with a decrease to 40 % after 24 hours (Figure 4.1).

The activity of the Multifect was assayed over the temperature range, shown in Figure 4.2a, at a constant pH of 5.0 (the optimum pH recommended by the supplier, Genencor
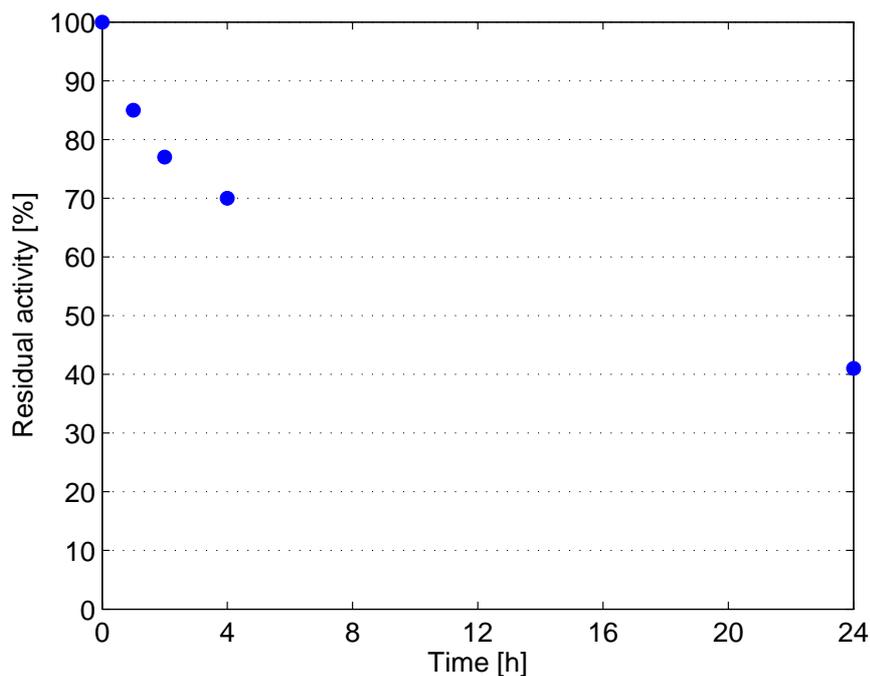
Figure 4.1: Thermostability of Xylanase P at 60 °C and pH 6.0 on Oat Spelts xylan.

(2007a)). The pH profile was subsequently constructed at the determined temperature optimum (Figure 4.2b). The maximum activity of Multifect was found to be 40 000 IU/mℓ at 60 °C and pH 5.0. This corresponded well to the product datasheet (Genencor, 2007a), which recommended use of the preparation at a temperature of 55 °C and a pH of 5.0.

(a) Temperature profile at pH 5.0.

(b) pH profile at 60 °C.
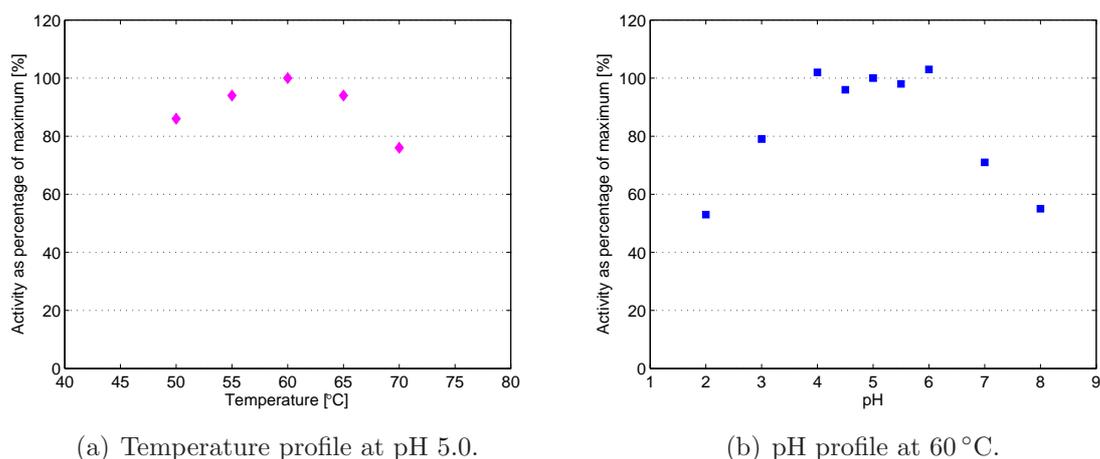
Figure 4.2: Enzyme characterisation of Multifect on Oat Spelts xylan.

Multifect is derived from a genetically modified strain of *Trichoderma reesei* (Genencor, 2007a). The results obtained in this study also corresponded to the work of Dekker (1983), who reported similar trends with *Trichoderma reesei* QM 9414. This indicated

that the Multifect behaved consistently with what is expected from the source strain.

The thermostability curves (Figure 4.3), indicated that the initial loss of activity was more severe at higher temperatures, although the residual activities after 48 hours were similar for all temperatures investigated. The most effective use of the enzyme would therefore be at the lower temperature, where high activity is still obtained, with less deactivation and less energy input.
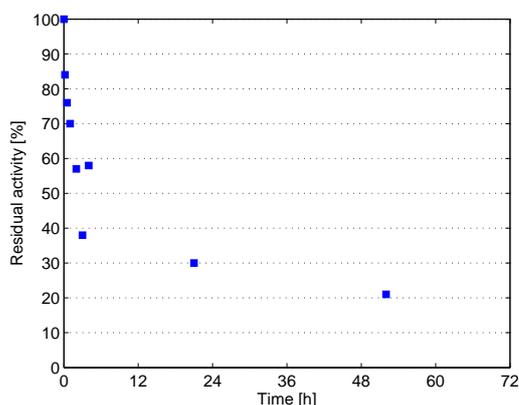
(a) Thermostability at 55 °C.
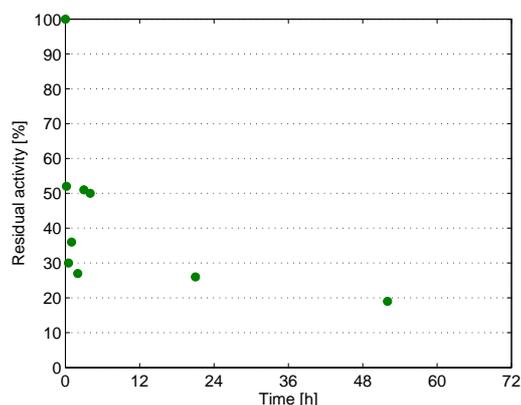
(b) Thermostability at 60 °C.
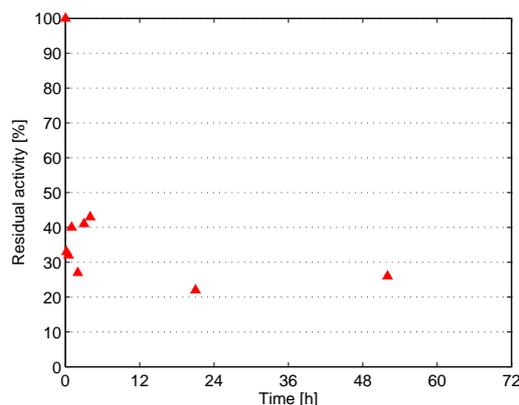
(c) Thermostability at 65 °C.

Figure 4.3: Thermostability of Multifect at different temperatures and pH 5.0 on Oat Spelts xylan.

Determination of the optimum temperature for Optimase was conducted at pH 8.0 (Genencor, 2007b). The pH optimum was subsequently determined at this optimum temperature. The temperature and pH profiles are shown in Figures 4.4a and b. Optimase exhibited maximum activity at a temperature of 65 °C (Figure 4.4a). This was much higher than the reported temperature optimum of 50 °C for this enzyme (Genencor, 2007b). This enzyme was, however, developed for use in existing processes in the pulp and paper industry, which are seldom operated at temperatures higher than 50 °C and therefore the product sheet may recommend use at a temperature which is not necessarily the optimum. Likewise, the product information sheet emphasises that this enzyme be

applied in alkaline conditions, such as Kraft processes, which may explain the discrepancy in the determined and supplied pH optima.

(a) Temperature profile at pH 8.0.

(b) pH profile at 65 °C.
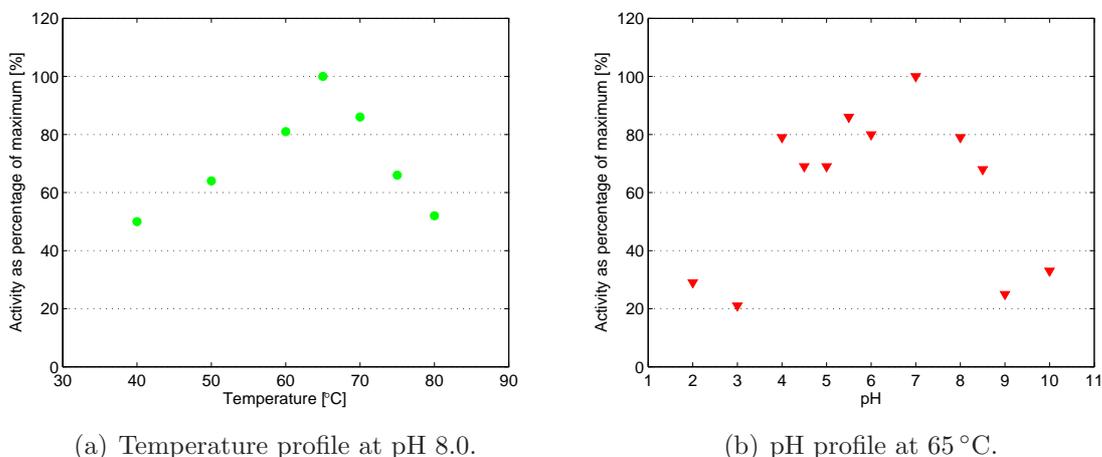
Figure 4.4: Enzyme characterisation of Optimase on Oat Spelts xylan.

The thermostability profiles of Optimase (Figure 4.5) showed that xylanase activity remained at high levels up to 72 hours at temperatures of 60 and 65 °C. However, at 65 °C significantly higher activities were obtained during the first six hours. Optimase was therefore used at 65 °C and pH 7.0 in the subsequent hydrolysis studies, which corresponded to an activity of 30 000 IU/mℓ.

Xylosidase from Sigma–Aldrich (SA) exhibited no detectable activity on the xylan substrates. This was consistent with the reported behaviour of a β–xylosidase, which only acts on oligomers with a chain length of six monomers or shorter (Coughlan et al., 1993: p.69).

The most effective use of the enzymes is at conditions corresponding to the pH and temperature optima of the different enzymes, i.e. where the highest enzyme activity is obtained. The effect of temperature on enzymes is however twofold; it influences both the rate of the catalysed reaction and the protein structure of the enzyme (Leidler & Peterman, 1983: p.149). As the rate of the enzyme catalysed reaction increases with temperature, a higher enzyme activity is observed (Cornish-Bowden, 2004: p.235). The Arrhenius temperature dependence of the rate is predominant at lower temperatures of about 30 °C. The other effect of temperature is thermal denaturing of the enzyme. The enzyme structure is maintained by hydrogen bonds and other weaker interactions that are broken during high temperature treatment, causing the structure of the enzyme to open (Cornish-Bowden, 2004: p231). The opened structure cannot exhibit the same catalytic properties and the observed enzyme activity is lower. Thermal denaturing is worsened by prolonged exposure to high temperatures (Leidler & Peterman, 1983: p.233). The thermostability curve therefore indicates the optimum temperature where the highest level of sustained activity is obtained over the investigated hydrolysis period.

41
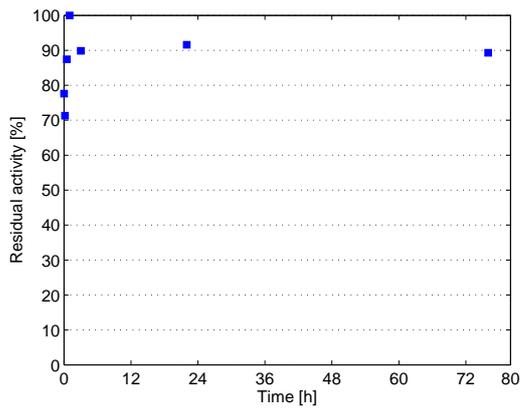
(a) Thermostability at $60\,^{\circ}\mathrm{C}$.
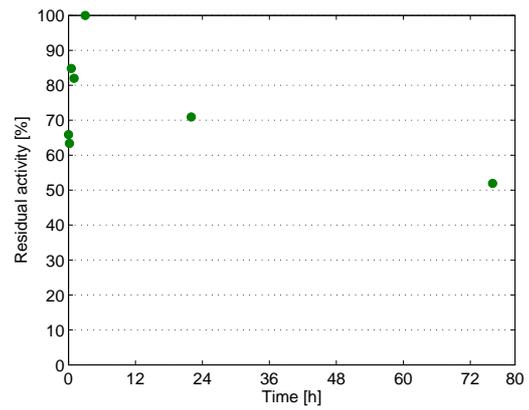
(b) Thermostability at $65\,^{\circ}\mathrm{C}$.
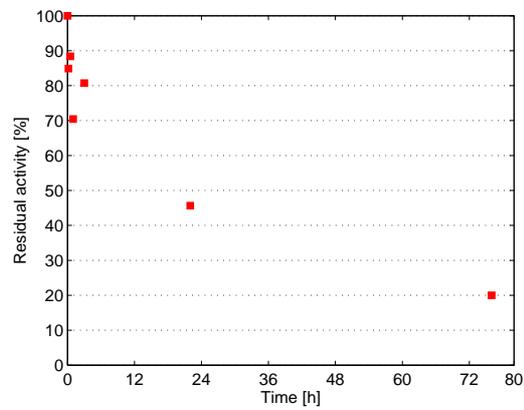
(c) Thermostability at $70\,^{\circ}\mathrm{C}$.

Figure 4.5: The thermostability of Optimase at pH 7.0 on Oat Spelts xylan.

The effect of pH follows a similar mechanism of activation-inactivation. The enzyme looses protons due to changes in pH, causing denaturing of the protein and subsequent decreases in activity. The pH where denaturing is slowest corresponds to the highest observed activity (Cornish-Bowden, 2004: p.214), which explains the bell-shaped curves observed in pH profiling.

Xylosidase activity

The xylosidase assay was carried out on nitrophenyl-xylosidase, a substrate that is unhydrolysable by xylanases, so that monomers released can be attributed to xylosidase activity alone. The presence of xylosidase in the commercial preparations is an important factor to be considered when choosing a enzyme for the hydrolysis of the xylans.

The temperature and pH profiles of the xylosidase from Sigma-Aldrich, purified from *Aspergillus niger*, are shown in Figures 4.6a and b, with residual activity expressed as a percentage of activity at the standard conditions of the assay, 50 °C and pH 4.0. While the xylosidase activity increased with higher temperatures over the range tested in this study (Figure 4.6a), it was expected that the activity would rapidly decrease at temperatures higher than 65 °C. It has been shown that xylosidases from *Aspergillus niger* are much less stable at temperatures above 50 °C (Poutanen & Puls, 1988), due to the effects of thermal denaturing. A limited amount of xylosidase was available and the experimentation was judiciously carried out so that the temperature profile not extended beyond 60 °C as based on the information gathered from literature.

As per the method of Bailey et al. (1992), the pH profile was conducted at the assay temperature of 50 °C. At this temperature the xylosidase was more active at lower pH's (Figure 4.6b). The lowest pH investigated was 4.0. The intention was to use the Xylosidase in addition to the xylanases. But all the xylanases investigated had shown a decline in activity at pH values lower than 5.0. Therefore, application of the Xylosidase under pH 4.0 was not feasible as this would have been detrimental to the activity of the xylanase. The activity of Xylosidase at pH 4.0 and 50 °C was determined as 0.018 IU/mℓ. Due to the limited amount of xylosidase enzyme available, thermostability was not investigated.

When the xylosidase activities of the commercial enzymes were assayed it was found that the Xylananse P and Optimase showed very little activity towards the substrate of the xylosidase assay. Activities of 0.0041 and 0.0003 IU/mℓ were determined for Xylanase P and Optimase respectively. In contrast, the xylosidase component of Multifect, had a higher activity than the Xylosidase. At assay conditions it was determined to be 1.208 IU/mℓ. It was apparent that a substantial amount of the activity determined in the endo-xylanase assay could have been attributed to the xylosidase component.

Characterisation of the xylosidase component in Multifect with regards to temperature and pH revealed a similar behaviour to what was observed with the purified xylosidase.
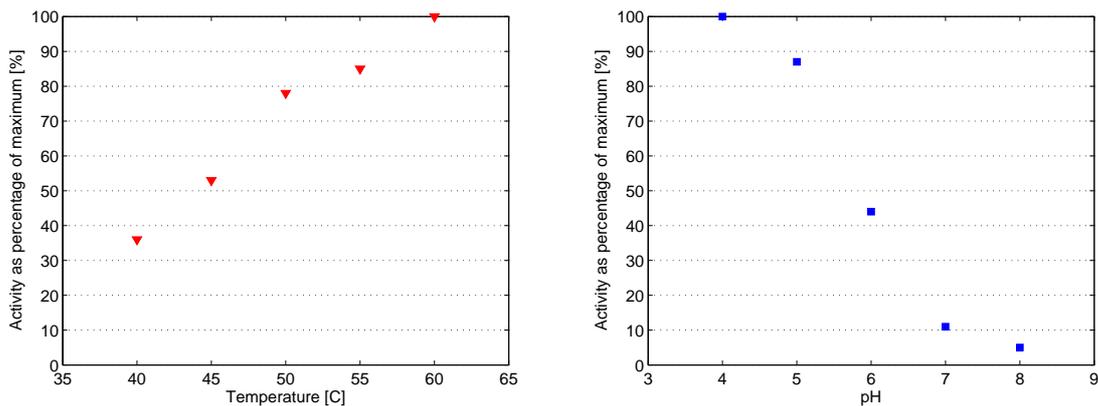
(a) Effect of temperature on the xylosidase activity at pH 4.0.

(b) The effect of pH on the xylosidase activity at 50 °C.

Figure 4.6: Enzyme characterisation of a xylosidase from Sigma-Aldrich.

At pH 4.0, the Multifect showed an increase in activity at temperatures above 50 °C, with a sharp decrease after 70 °C (Figure 4.7a). At 50 °C, the enzyme showed the highest activity at pH 4.0, with a sharp decrease at lower pH values (Figure 4.7b). The temperature stability of the xylosidase component of Multifect was investigated at optimum pH and temperature as well as 55 and 60 °C and the results are shown in Figure 4.8. A rapid decrease in activity was seen for all temperatures investigated. This illustrated the sensitivity of the enzyme to temperature and the need to take thermostability into consideration when choosing hydrolysis conditions.
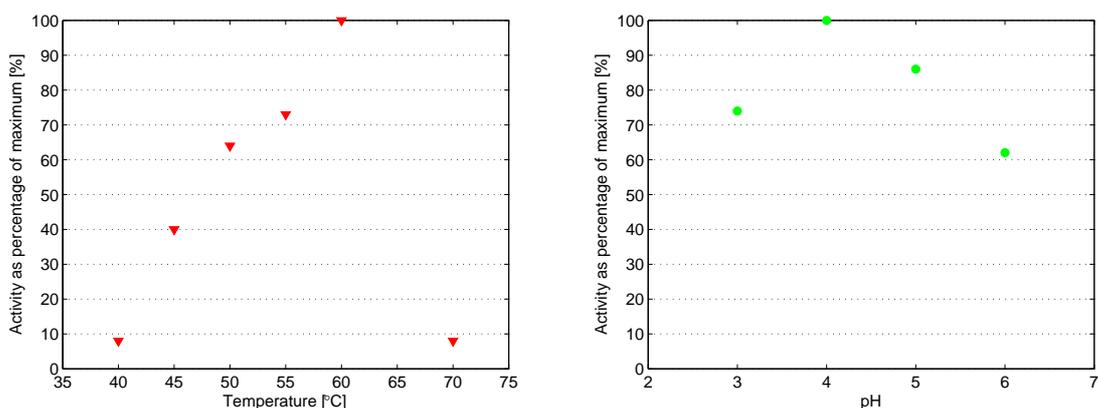
(a) The effect of temperature on Multifect at pH 4.0.

(b) The effect of pH on Multifect at 50 °C.

Figure 4.7: Enzyme characterisation of xylosidase component in Multifect.

The xylosidase in Multifect compared well to other enzymes isolated from the same organism, *Trichoderma reesei*. Dekker (1983) reported optima of 60 °C and pH 4.0, with 50 % of the activity retained in an hour when incubated at 50 °C. It was reported that the enzyme was unstable at pH's lower than 2.0 and higher than 6.0.
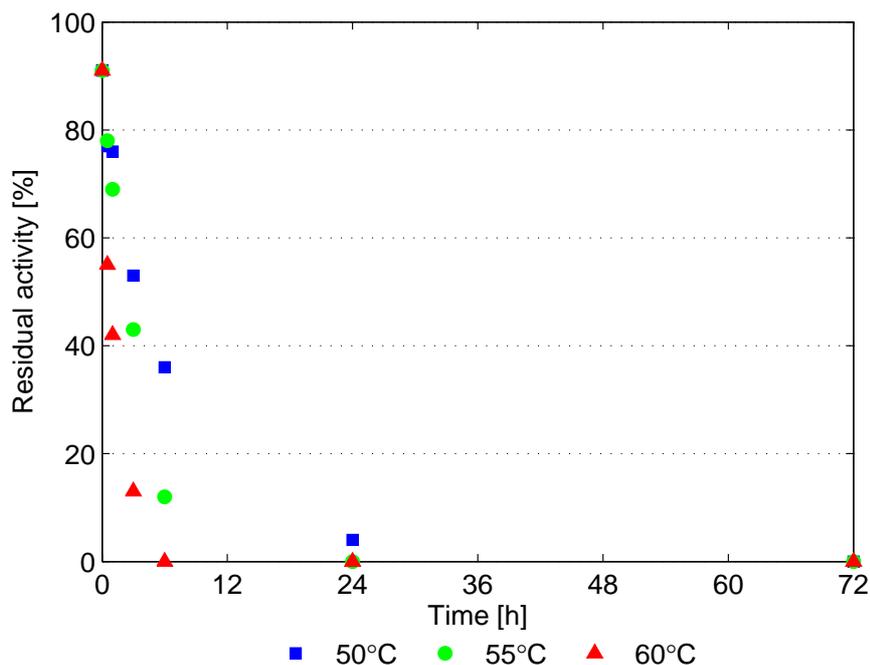
Figure 4.8: The themostability of xylosidase in Multifect at pH 4.0 and different temperatures.

Cellulase and endo-glucanase activity

The source of the extracted xylan was fully bleached hardwood pulp. In the case that small residual cellulose fragments are present, the presence of cellulose degrading enzymes would be important as glucose would be released into the hydrolysate. The cellulase activity of the commercial enzymes was determined with the Filter paper assay and the endo–glucanase degrading potential with the Carboxymethyl cellulose assay (Ghose, 1987).

The assays indicated that Multifect showed the highest cellulase and endo–glucanase activities. This is to be expected as Multifect is intended to degrade all biomass components to monomeric sugars and would therefore include a whole spectrum of enzyme activities (Genencor, 2007a). Xylanase P also showed high cellulase and endo–glucanase activities, despite it being an enzyme for pulp bleaching applications (Chipeta, 2006). Enzymes used in the bleaching of pulps target the hemicellulose fraction of the pulp, leaving the celluloses relatively unaltered as to minimise the impact on pulp yield and quality. The high glucose concentrations obtained in the assays could have been due to the high concentration of glucose present in the enzyme preparation which affected the sensitivity of the assay. It has been shown (Section 3.3.1) that the colorimetric techniques are highly susceptible to interferences such as background concentration. The determined activities of the commercial enzymes are shown in Table 4.1. The results are expressed in FPU/mℓ and CMC/mℓ as per the convention (Ghose, 1987).

Table 4.1: Cellulase and endo-glucanase activities of the commercial enzymes.

Enzyme activity	Xylanase P	Multifect	Optimase	SA Xylosidase
Cellulase (FPU/mℓ)	1.95	1.3	1.3	n.a
Endo-glucanase (CMC/mℓ)	3.25	3.56	n.a.	n.a.

Residual sugars in the enzyme preparations

Depending on the enzyme production and purification methods, various monomeric sugars may be present in the commercial enzyme preparations (Jacobs et al., 2003). Usually large quantities of preservatives are also added to prolong the shelf life of the product. It was, therefore, necessary to investigate the presence of sugars in the commercial enzyme preparations.

The enzymes were deactivated by placing in a boiling water bath and subsequently analysed for monomeric sugars. Deactivation was needed as the presence of higher order sugar oligomers could not be discounted and these would be hydrolysed by the enzymes upon reactivation, when the enzyme is taken out of cold storage. The enzyme preparations were acid hydrolysed to release all bound and higher order sugars. Hydrolysis conditions of 1 % acid and 30 minutes hydrolysis time were chosen based on the good sugar yields obtained with little degradation (see Section A.3.1). Analysis of a 10 mℓ/ℓ enzyme solution, both acid hydrolysed and unhydrolysed, yielded several species of sugar. The concentration of the sugars relevant to the current study is shown in Table 4.2. Other sugars identified included fucose, sorbitol, inositol and sucrose.

Table 4.2: Sugar concentrations (mg/ℓ of a 10 mℓ/ℓ enzyme solution) detected in the enzyme preparation: acid hydrolysed versus nonhydrolysed.

Treatment	Arabinose	Galactose	Glucose	Xylose	Mannose
Xylanase P					
Enzyme blank	0.00	2.03	1619.54	0.20	5.99
Acid hydrolysed	0.19	8.19	951.04	0.00	19.04
Multifect					
Enzyme blank	0.00	11.27	0.00	0.00	10.07
Acid hydrolysed	0.52	7.50	16.56	0.47	12.81
Optimase					
Enzyme blank	1.53	1.72	0.13	4.19	0.00
Acid hydrolysed	3.52	11.95	51.68	0.89	1.63

There were differences in the sugar concentrations obtained for the enzyme blank

and the acid hydrolysed samples. Lower concentrations after acid hydrolysis could be attributed to the degradation of monosugars in the acid conditions whereas the increase concentrations were due to the release of monosugars from bound forms. The glucose in Xylanase P was the only sugar detected in significant quantities that could possibly interfere with the results of subsequent experimental work. In light of these findings, the results of further hydrolysis experiments were adjusted to compensate for the background sugar concentrations.

Summary of enzyme activities

To facilitate further discussion and comparison of the enzymes used in this study, a summary of the enzyme activities is given in Table 4.3.

Table 4.3: A summary of the enzyme activities present in the commercial enzymes used in this study.

Enzyme activity	Xylanase P	Multifect	Optimase	SA Xylosidase
Endo-xylanase on Birchwood Xylan (IU/mℓ)	10 000	40 000	30 000	n.a.
Xylosidase (IU/mℓ)	0.004	1.2	0.0003	0.018
Endo-glucanase (CMC/mℓ)	3.25	3.56	n.a.	n.a.
Cellulase (FPU/mℓ)	1.95	1.3	1.3	n.a

4.3.2   Enzyme catalysed hydrolysis of Birchwood xylan

The hydrolysis of Birchwood xylan was used as an indication of the ability of the commercial enzymes to convert typical hardwood xylans. It was found that Birchwood xylan is composed only of xylose (see Chapter A) and therefore other sugars found in the hydrolysate would have originated from the enzyme preparation. Only the yield of xylose during hydrolysis of Birchwood xylan was used as a measure of the action of the commercial enzymes.

The hydrolyses were performed at each enzyme's optimum pH and temperature as determined previously. The enzymes were diluted to yield similar activities in the final samples as to allow for comparison of the results. At an enzyme charge of 100 IU/mℓ Multifect, a xylose yield of nearly 45 % was obtained in 72 hours (Figure 4.9), whereas

Xylanase P and Optimase only yielded 13 and 6 % respectively (see Figures 4.10 and 4.11). The results are not surprising as it was determined in the enzyme assays presented in Section 4.3.1 that neither Xylanase P nor Optimase had any significant xylosidase side-activities and were primarily endo–xylanases. When considering that the action of an endo–xylanase is to cleave the xylan backbone into shorter oligomers, with limited ability to free monomers from the non-reducing end of the polymer, low monomer yields were therefore expected.
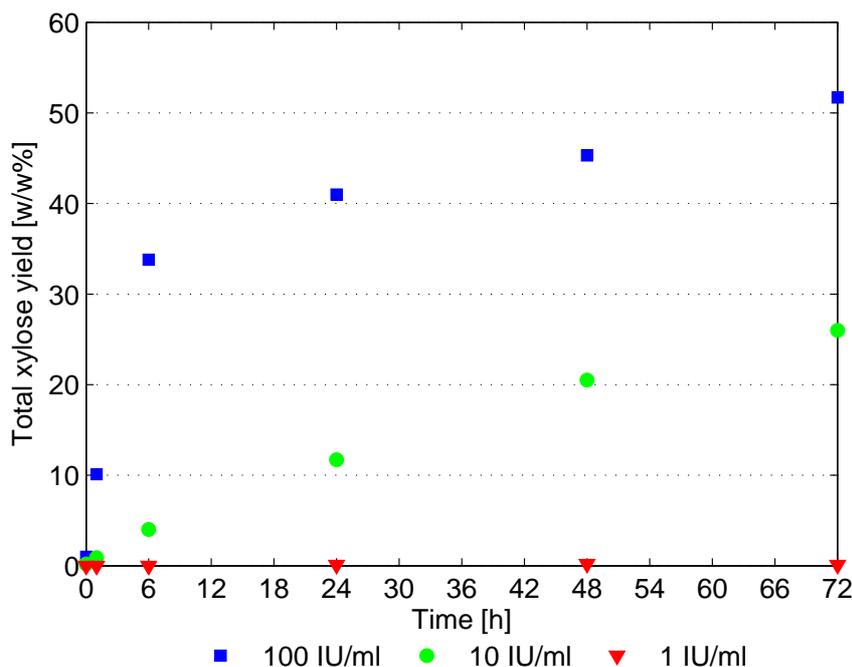
Figure 4.9: The xylose yield from the enzymatic hydrolysis of Birchwood xylan with Multifect at 55 °C and pH 5.0.

Multifect was found to contain xylosidase activity at a higher level than even that of the pure xylosidase. Since Multifect contained both xylosidase and endo–xylanase activity, the hydrolysis rate and total monosugar yield obtained were much greater than from the other commercial enzymes. Despite this, substrate conversion of more than 50 % could not be obtained even with an enzyme charge of 100 IU/mℓ and a hydrolysis period of 72 hours. The yields obtained were however comparable to results reported in literature, where conversions of less than 40 % in 24 hours was observed with a similar xylanase and xylosidase system (Kormelink & Voragen, 1993).

4.3.3 Enzyme catalysed hydrolysis of Oat Spelts xylan

Oat Spelts xylan was used a substrate representing a typical cereal xylan. The characterisation of the monomeric composition of this substrate (Section A.2.3) had shown that in addition to xylose, other sugars were present as well. From literature, it is known that
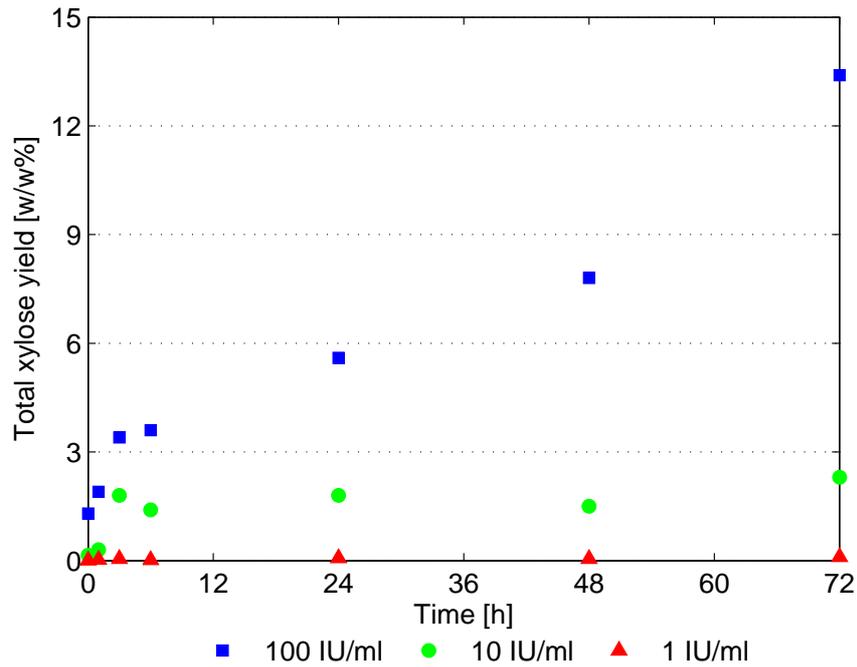
Figure 4.10: The xylose yield from the enzymatic hydrolysis of Birchwood xylan with Xylanase P at 60 °C and pH 6.0.
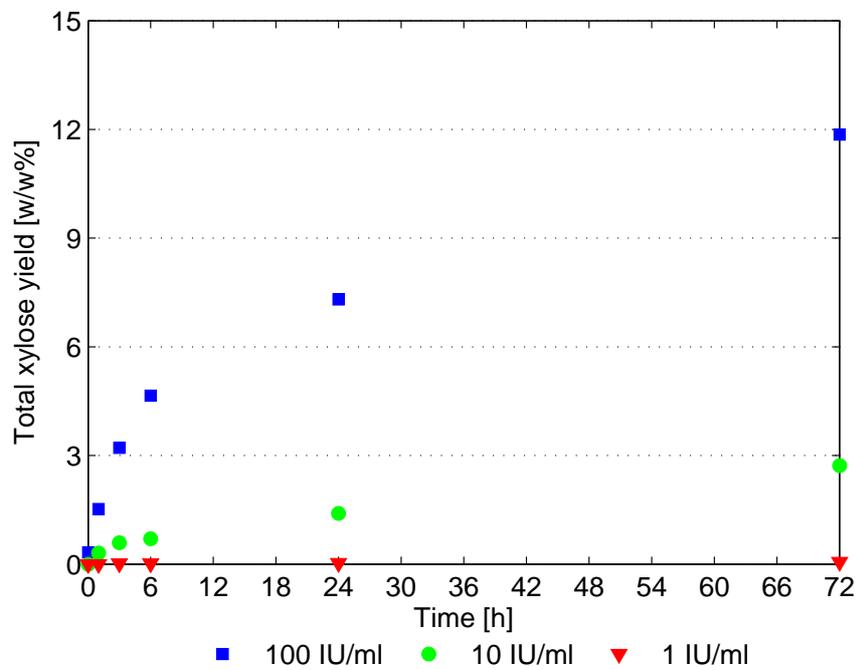
Figure 4.11: The xylose yield from the enzymatic hydrolysis of Birchwood xylan with Optimase at 65 °C and pH 7.0.

the xylose forms the xylan backbone of the polymer, with the other sugars present as substituents or short side chains (see Section 2.3.2). The hydrolysis patterns obtained from the action of the commercial enzyme on Oat Spelts xylan was, therefore, expected to be different to than of Birchwood xylan, due to the more complex structure of the substrate. It was also observed in Chapter A that Oat Spelts was harder to hydrolyse with acid than Birchwood xylan.

As was the case with Birchwood xylan as substrate, the highest conversion of Oat Spelts xylan was seen with Multifect; over a similar hydrolysis period more than 45 % conversion was obtained, with conversions from Xylanase P and Optimase less than 10 and 6 % respectively. Figures 4.12, 4.13 and 4.14 show the hydrolysis profiles of the commercial enzymes.
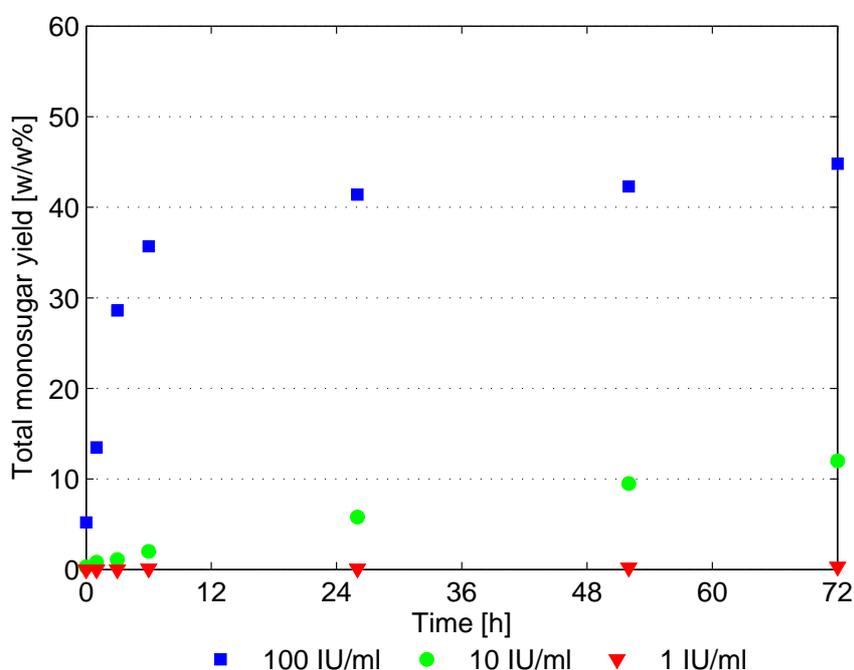
Figure 4.12: Substrate conversion of Oat Spelts xylan with Multifect Xylanase at 55 °C and pH 5.0.

The substrate conversion of Oat Spelts xylan was lower than that of Birchwood xylan for all three enzymes investigated. From the characterisation of the monomeric composition of the substrate glucose, galactose and arabinose were identified in addition to xylose, illustrating the complexity of the xylan. The presence of these sugars as substituents is typical of the branched nature of an arabinoxylan and may have caused steric hindrances, which interfered with the action of the enzymes leading to lower hydrolysis yields.

When looking at the sugar composition of the hydrolysates, it could be seen that the individual sugars were liberated to different extents by the enzymes. At an enzyme charge of 100 IU/mℓ and a hydrolysis period of 72 hours, xylose was the major sugar released by
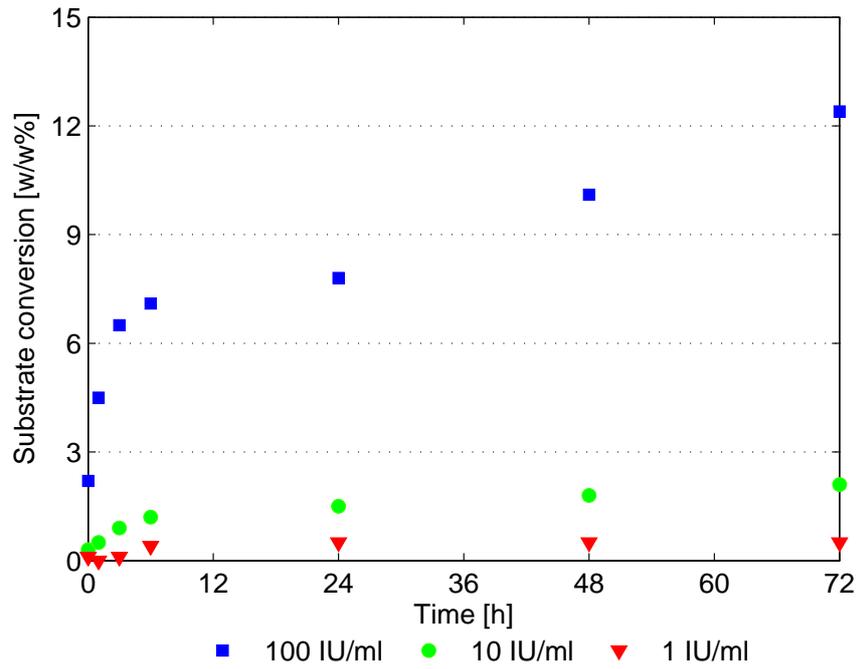
Figure 4.13: Substrate conversion of Oat Spelts xylan with Xylanase P at 60 °C and pH 6.0.
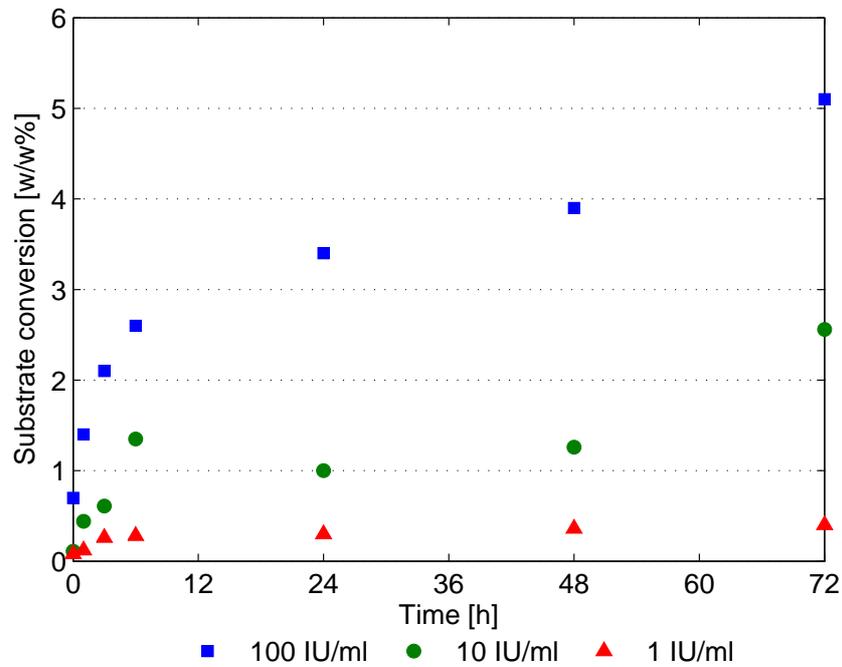
Figure 4.14: Substrate conversion of Oat Spelts xylan with Optimase at 65 °C and pH 7.0.

Multifect and Optimase and glucose was the major sugar released by Xylanase P (Table 4.4).

Table 4.4: Sugar composition of the hydrolysates from the enzyme hydrolysis of Oat Spelts xylan with various enzymes at charges of 100 IU/mℓ and hydrolysis time of 72 hours .

Enzyme	Xylose	Glucose	Arabinose	Galactose
Xylanase P	28.6 %	57.2 %	n.a	14.3 %
Multifect Xylanase	84.1 %	11.2 %	4.6 %	0.0 %
Optimase	90.0 %	5.5 %	4.6 %	0.0 %

As was discussed previously, the synergistic working of endo–xylanase and xylosidase is responsible for the release of xylose from the xylan backbone. In the absence of xylosidase, e.g. for Optimase and Xylanase P, the endo–xylanase released limited amounts of xylose from the non-reducing ends of the oligomeric chains. In the hydrolysates of Multifect and Optimase, some arabinose was detected to a limited extent. Since no arabinofuranosidase assay was performed, the presence of arabinose-sidechain liberating activities in the enzyme solutions could not be discounted. It was, however, also possible that the arabinose was liberated by the action of the xylosidase, especially in Multifect. The arabinose-xylose bond is very similar to that of xylose-xylose (Coughlan et al., 1993; Poutanen & Puls, 1988: p.69). The limited release of galactose by Xylanase P could have been due to the presence of α–galactosidase, the activity of which was not determined by an enzyme assay.

Of all the commercial enzymes used in this investigation, Multifect Xylanase was the most suitable to degrade the more complex xylan.

4.3.4 Hydrolysis of Oat Spelts xylan with Optimase and an accessory enzyme

To increase the yield of monosugars by the commercial xylanases, the effect of adding an accessory enzyme, β–xylosidase was investigated. The preferred substrate of xylosidase enzymes is xylo-oligomers; xylosidase usually shows no preference to the xylan backbone. The most pronounced effect of added xylosidase would, therefore, be seen when used in conjunction with a pure endo-xylanase. Of the commercial xylanases investigated, Optimase exhibited the least amount of auxiliary enzyme activity and is most representative of a pure endo-xylanase. The combined action of the xylosidase and Optimase was investigated using Oat Spelts xylan as a substrate.

The Oat Spelts xylan was pre-hydrolysed with Optimase at charges of 10 and 100

IU/mℓ and the optimum pH and temperature of the xylanase. The hydrolysis patterns in Figures 4.15a and b conforms to the trends in Figure 4.14; less than 3 % conversion was achieved with Optimase after six hours hydrolysis time. The action of xylosidases is dependent on the ability of the xylanase to liberate oligomers and no substrate conversion was obtained with use of the xylosidase when used on its own.

After six hours pre-hydrolysis, the hydrolysate was adjusted to the optimum conditions for hydrolysis with xylosidase. The pre-hydrolysis step allowed for more effective use of the xylosidase as it rapidly loses activity due to temperature exposure (see Section 4.3.1). After the xylosidase was added, the rate of hydrolysis increased markedly (Figure 4.15a and b). The dependency of the xylosidase activity on that of the xylanase can be seen in Figures 4.15a and b. A higher enzyme charge of Optimase led to a faster hydrolysis rate when the xylosidase was subsequently added. After 72 hours of hydrolysis with Optimase at 100 IU/mℓ and xylosidase at 0.02 IU/mℓ, the substrate conversion increased to over 40 %, with a sugar composition as shown in Table 4.5.
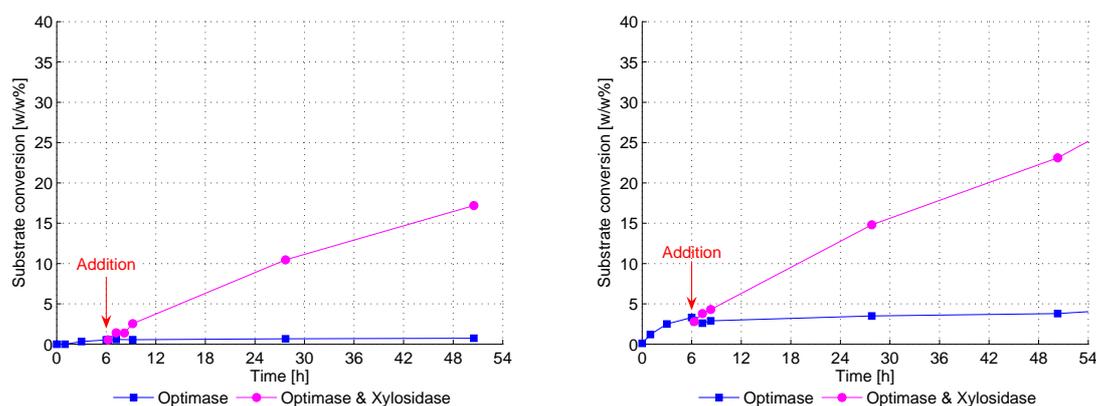
(a) Substrate conversion of Oat Spelts xylan when pre-hydrolysed with 10 IU/mℓ Optimase and subsequently added xylosidase.

(b) Substrate conversion of Oat Spelts xylan when pre-hydrolysed with 100 IU/mℓ Optimase and subsequently added xylosidase.

Figure 4.15: Enzymatic hydrolysis of Oat Spelts xylan with Optimase and xylosidase.

Table 4.5: The sugar composition of the hydrolysate of Oat Spelts xylan hydrolysed with Optimase by itself and added xylosidase

Treatment	Arabinose	Glucose	Xylose
Optimase	5.6 %	4.2 %	90.2 %
Optimase and Xylosidase	1.2 %	10.4 %	88.5 %

Even though the main sugar released by Optimase and xylosidase was xylose, the release of glucose was also increased by the addition of xylosidase (Table 4.5). As was discussed previously, the presence of glucosidase and arabinofuranosidase activities in xy-

losidase could not be excluded, but it was also possible that xylosidase catalysed the hydrolysis of other glycosidic bonds as well to cleave the xylose-glucose and xylose-arabinose bonds.

The synergism between endo–xylanase and xylosidase was seen in the hydrolysis of Oat Spelts xylan with Optimase and xylosidase. Not only was the substrate conversion improved substantially, the release of sugars other than xylose was improved as well. This indicated that the low yields obtained from the commercial enzymes may be improved by the addition of accessory enzymes, particularly β–xylosidase.

4.3.5 Selection of the most suitable enzyme for xylan degradation

With enzyme characterisation and the hydrolysis of Birchwood and Oat Spelts xylan, insight into the hydrolysis potential of the commercial enzymes was gained. It was observed that in addition to xylanase activity, the presence of high levels of xylosidase activity is pivotal in achieving high monosugar yields; the yield of the commercial enzyme, Optimase was improved when used in conjunction with added xylosidase. In understanding how to best apply the enzymes to xylan degradation and to select the enzyme most suitable for the task, it was needed to determine the extend to which yield can be improved by the addition of xylosidase.

The xylans were pre-hydrolysed with the commercial xylanases at equal charges of 100 IU/mℓ and each enzyme's optimum pH and temperature for 24 hours. After 24 hours, the conditions were adjusted to the optimum pH and temperature of the xylosidase and equal xylosidase (from Sigma-Aldrich) charges were added to the pre-hydrolysed samples. The xylose yield of the samples with added xylosidase and those without were compared to determine the effect of the xylosidase on yield (Figure 4.16). The xylose yields obtained without xylosidase addition were consistent with previous results; Multifect released up to four times more xylose than the other enzymes in the same hydrolysis period. The addition of xylosidase to Optimase and Xylanase P significantly increased the xylose yield over what had been obtained when the enzymes were used on their own. The addition of xylosidase to Multifect, however, has little impact on the xylose yields, most likely because the xylosidase side activity of Multifect was at a higher level than that of the pure xylosidase.

It is clear that Multifect was the enzyme most suitable for the degradation of the xylans and was used in the remainder of the study. High yields could be obtained with this enzyme, because of the high levels of endo–xylanase and xylosidase activities present in the preparation. Thermal denaturing, however, influenced both components at the temperatures used in this investigation.
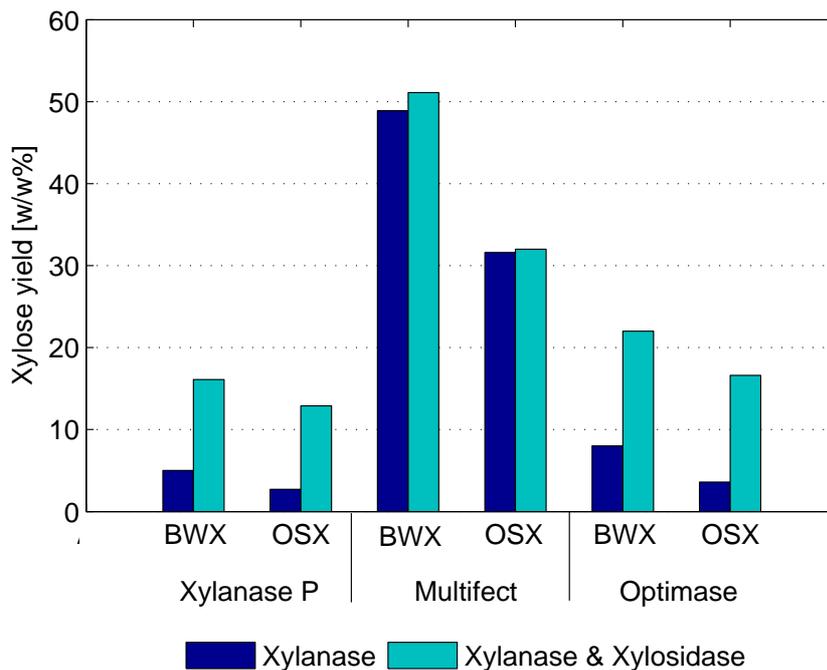
Figure 4.16: Xylose yield from the enzymatic conversion of Birchwood and Oat Spelts xylans with commercial xylanases and xylosidase after 48 hours total hydrolysis time.

4.3.6 Enzymatic hydrolysis of Birchwood xylan with Multifect

Multifect was chosen as the most suitable enzyme for xylan degradation. In order to understand how to most effectively use the enzyme in hydrolysis, a deeper investigation into enzyme dosage was needed. However, before the Multifect could be applied to the Eucalyptus xylan, a benchmark was needed to compare the results. The hydrolysis of Birchwood xylan with Multifect was selected, as Birchwood xylan is a commercially available xylan that has been studied extensively and as it is a hardwood xylan like Eucalyptus xylan, the results should be comparable.

Effect of various enzyme charges

At this point in the investigation, Multifect had been selected as most suitable commercial enzyme preparations to proceed with, but optimum dosages had not yet been determined. During preliminary investigations, yields lower than 60 % were obtained over hydrolysis periods of 72 hours and it appeared that there was scope to improve on the extent and rate of conversion.

It is generally known that increasing the enzyme dosage increases the rate of conversion only up to the point of overdosing, where after no increase in rate is obtained because the enzyme:substrate ratio is too high. Enzymes catalyse hydrolysis by binding onto sites on the substrate; a higher enzyme concentration allows binding to more sites on the substrate and therefore faster hydrolysis rates are observed. At high enzyme dosages,

a point exists were all the possible sites on the substrate are occupied by the enzymes and steric hindrance becomes a problem (Cornish-Bowden, 2004). A higher concentration of enzyme would then not increase the hydrolysis rate further. It is therefore clear that determining the dosage end-point is an economic consideration as well as a practical issue.

As the Multifect charge was increased, the hydrolysis rates on Birchwood xylan increased. The dosage end-point was reached at 600 IU/mℓ (Figure 4.17). The gradual increase in yield after 24 hours hydrolysis time could be attributed to the limited solubility of the substrate. Commercial Birchwood xylan is usually prepared with alkaline extraction, which is known to decrease the xylans solubility in aqueous solutions (Puls & Schuseil, 1993; Tenkanen & Poutanen, 1992). Since enzymatic hydrolysis only occurs in the liquid phase, this would affect hydrolysis. As the reaction proceeds, more of the xylan is solubilised, leading to the continual increase in yield observed. The hydrolysis rate is therefore not only limited by the enzyme activity, but also the concentration of substrate in the liquid phase.
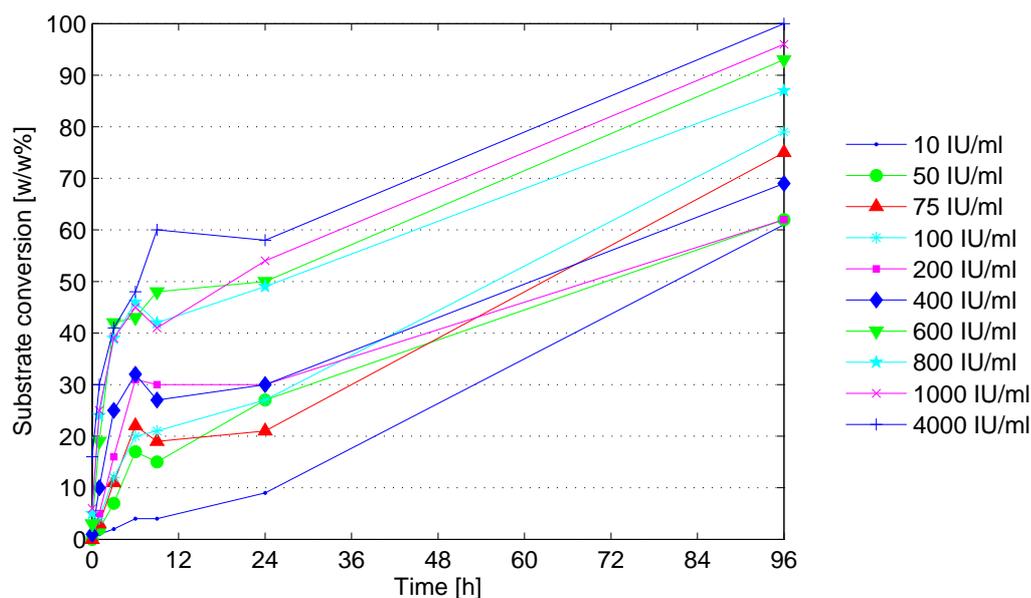
Figure 4.17: Substrate conversion of Birchwood xylan with various enzyme dosages.

Is it known that when xylans are dried, interchain aggregation occurs due to the formation of strong hydrogen bonds causing the polymeric structure to collapse (Ebringerová & Heinze, 2000; Puls & Schuseil, 1993). Not all of these bonds are broken when the xylan is rehydrated and the polymer does not fully unfold again. The partially collapsed structure is inaccessible to enzyme attack and would lead to incomplete conversions to be observed. As full conversion of Birchwood xylan was obtained with Multifect Xylanase, interchain aggregation of the xylan, if it did occur, did not influence the extent of enzyme hydrolysis. The hydrolysis rate would have been affected, which was apparent when the

results were compared to that of Eucalyptus xylan.

Effect of multiple dosages

Substrate conversion of 100 % was obtained with an enzyme charge of 600 IU/mℓ, however only after 96 hours hydrolysis time. An enzyme charge of 600 IU/mℓ corresponds to a dosage of 1.5 % (v/v), which is very high compared to dosages of 0.3 IU/mℓ (Dekker, 1983) reported in literature or the recommended charge of 0.002 % (Genencor, 2007a). Although the initial hydrolysis rate was rapid, it decreased after six hours increasing the hydrolysis time required for high conversions considerably. The decrease in rate coincided with the loss of xylosidase activity (see Section 4.3.1). To shorten the hydrolysis time, more effective use of the Multifect was required, i.e. shorter exposure to the high temperature environment that causes thermal denaturing of the enzyme. Multifect was therefore added in multiple dosages of 100 IU/mℓ to use the faster initial hydrolysis rates (Figure 4.18). A description of the treatments used is shown in Table 4.6.
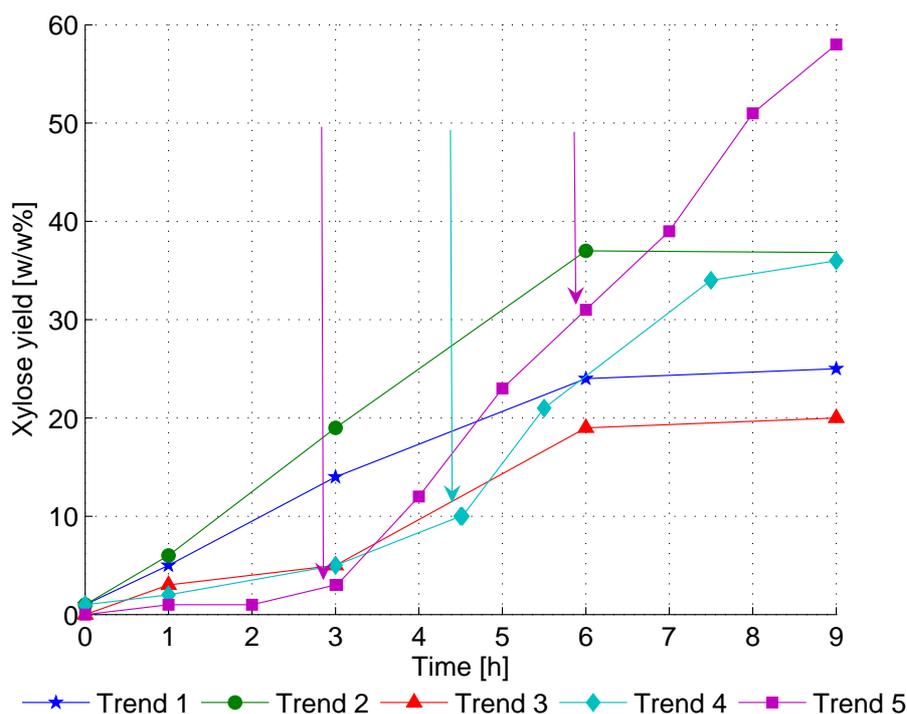
Figure 4.18: Substrate conversion of Birchwood xylan with multiple dosages of Multifect.

The curves for the enzyme charges of 100* and 200* IU/mℓ (Trends 1 and 2) were taken from the dosing curve (Figure 4.17) are repeated here to facilitate discussion. The initial rate of hydrolysis observed in this experiment was much slower than was observed in the previous results for a charge of 100 IU/mℓ (Treatment 3 in Figure 4.18). The sampling frequency in this experiment was much higher than previous ones and good temperature regulation of the incubator could not be achieved between sampling times,

Table 4.6: Description of treatments as applied for multiple enzyme additions

Treatment	Trend	Description
1	Trend 1	100* IU/mℓ, from previous results
2	Trend 2	200* IU/mℓ, from previous results
3	Trend 3	100 IU/mℓ, this experiment, dosed at 0 h
4	Trend 4	100 IU/mℓ, this experiment, dosed at 0 and 4.5 h
5	Trend 5	100 IU/mℓ, this experiment, dosed at 0, 3 and 6 h

which would have led to lower hydrolysis rates. This was considered in the interpretation of the results.

For a single treatment (Trend 3) of 100 IU/mℓ the effect of temperature denaturing on the enzyme can be seen when the rate started dropping off after six hours. Where an additional dosage of enzyme was administered after three hours, an immediate increase in rate was seen (Trend 5). Although the total enzyme charge was 200 IU/mℓ immediately after the second addition, the initial rate was still slower than the initial rate of a single enzyme dose of 200 IU/mℓ. It is assumed that this is the result of adding a fresh enzyme dose on top of the original enzyme dose where temperature denaturing resulted in reduced activity. A further addition of fresh enzyme after six hours merely caused the reaction rate to remain constant, indicating the pronounced effect of denaturing on enzyme activity.

From this experiment it was clear that the effect of thermal denaturing set in rapidly. The administration of new enzyme charges yielded rates comparable to the initial rates observed with the first enzyme dosage. The use of multiple additions of enzymes allowed for higher reaction rates throughout the hydrolysis period, thereby shortening the time required over that of single-dose hydrolysis.

Multifect is expected to exhibit similar behaviour on the Eucalyptus xylan as was observed on Birchwood xylan. The hydrolysis profiles will, however, not be an exact match as the different extraction methods used to prepare the xylan may have altered the structures and thereby affected the enzyme action. Conducting a study on Eucalyptus xylan, similar to what was presented in this section, will facilitate a comparison and thereby give insight into potential of enzymatically degrading the extracted xylan.

4.3.7 Enzymatic hydrolysis of Eucalyptus Xylan with Multifect

The extracted Eucalyptus xylan was obtained in a gelatinous form of 9.35 % consistency and had never been dried. To facilitate direct comparison between the enzymatic hydrolysis of the two xylans, the same experimental procedures were used on the Eucalyptus xylan as on the Birchwood xylan described earlier.

Characterisation of the monosugar composition of the Eucalyptus xylan was required so that yields could be correctly reported. It was also needed to determine the residual nickel content of the extractive originating from the extraction method as it could have

affected the enzyme action.

Determining the constituent monomers

The monomeric sugar content of the Eucalyptus xylan was determined by acid hydrolysis. True to the extraction method used, the gelatinous preparation was expected to contain only xylan, unbound to lignin or cellulose (Kettenbach & Stein, 2007). It was assumed that this xylan would be easily hydrolysed due to its open structure, which was expected to be uncollapsed since it had not been dried prior to use. The xylan was degraded to monomers with 1 % acid and 30 minutes hydrolysis time according to the developed protocols (Section A.3.1) where less than 5 % degradation of the unbound sugars was expected.

The total sugars yield from hydrolysis of Eucalyptus xylan with this protocol was only 60 %, but it could be determined that the hydrolysate contained only xylose as monosugar as had been reported by previous work on the substrate (Lax, 2007b). The extraction method used is expected to remove the $4-O$–methylglucuronic acid leaving the xylan with an unhindered structure that should be readily accessible to enzyme action (Puls et al., 2006).

Determining the heavy metal content

As a result of the method used to extract the xylan, nickel ions were present in the extractive (Kettenbach & Stein, 2007). Many nickel compounds are known carcinogens and nickel itself is an allergenic substance (Janzon et al., 2006). There was concern that the nickel content could have been detrimental to the functioning of the hydrolytic enzymes as it is known that heavy metals adsorb onto the active sites of the enzyme, lowering activity and eventually deactivating the enzyme (Godfrey & West, 1996).

Therefore, the heavy metal content was determined and found to be 50 ppm nickel in 1 % (w/v) solution of xylan (Lax, 2007a). Extensive washing is required to reduce the nickel levels further (Janzon et al., 2007). However, removing nickel from the xylan was not the purpose of the investigation and this level of nickel was deemed low enough to continue with the enzymatic hydrolysis.

Activity of Multifect on Eucalyptus xylan

In order to have comparable results, the enzyme had to be dosed in charges equal to those used for the hydrolysis of Birchwood xylan. Whether the Multifect would have the same activity on the Eucalyptus xylan considering the high nickel content, was a concern. The xylanase assay described in Section 4.2.2 was conducted with Eucalyptus xylan as substrate. The activity was determined as 30 000 IU/mℓ. Even though the Eucalyptus xylan structure was expected to be more accessible than the Birchwood xylan, the activity

was lower. This may have indicated that the nickel interfered with the functioning of the enzyme.

Effect of various enzyme charges

The effect of different enzyme charges on the hydrolysis of Eucalyptus xylan with Multifect was investigated to determine the extent of deactivation caused by the nickel. The dosing curve obtained for the Birchwood xylan was used as a guideline and the enzyme was dosed to yield similar enzyme charges. The dosing curve obtained on Eucalyptus xylan was similar to that on Birchwood (Figure 4.19), where an initial fast hydrolysis rate was followed by a distinct decrease after six hours hydrolysis time.
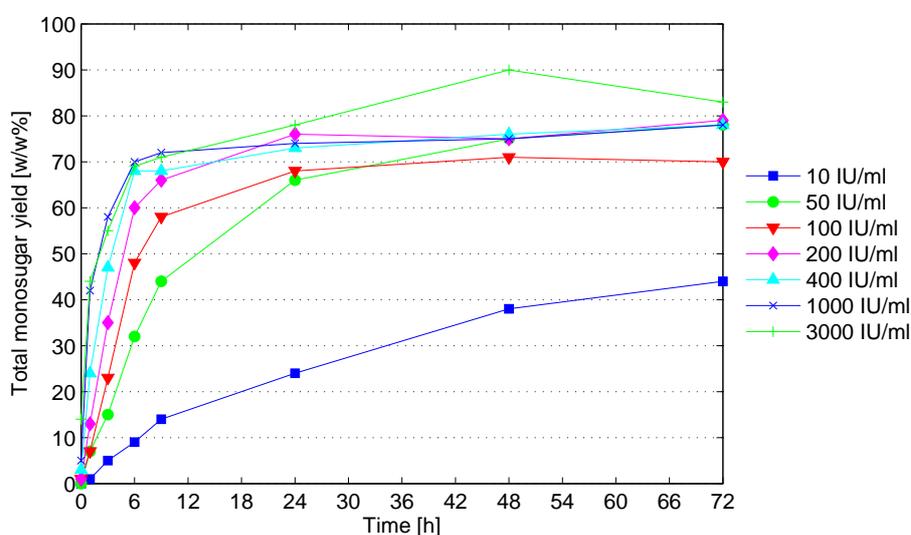
Figure 4.19: Substrate conversion of Eucalyptus xylan at various charges of Multifect.

The dosage end-point of Multifect on Eucalyptus xylan was at 400 IU/mℓ, which was lower than what was obtained on Birchwood. Also, substrate conversion of 100 % could not be obtained with the Multifect, even at enzyme charges of 10 % (v/v). When considering these two observations, it was concluded that not all of the Eucalyptus xylan structure was hydrolysable. This was most likely a results of the extraction method used, as Birchwood xylan could be degraded fully with Multifect under similar conditions.

Another cause of the incomplete conversion could have been the continued deactivation of the enzyme by nickel. The activity assay of Multifect on Eucalyptus xylan yielded lower activities presumably because of the presence of nickel in the substrate. The hydrolysis time in the assay, however, was only five minutes, whereas in the hydrolysis experiments the enzyme was exposed to the nickel for up to 72 hours. During prolonged exposure the nickel would continue to block the active sites on the enzyme, thereby decreasing the observed activity. This effect would be most pronounced at low enzyme charges where the nickel to enzyme ratio is high. However, at an enzyme dosage of 10

IU/mℓ conversion continued to increase over the hydrolysis time investigated indicating that nickel deactivation was not the limiting factor (Figure 4.19).

Although complete conversion could not be obtained on the Eucalyptus xylan with Multifect, up to 80 % conversion, the hydrolysis rates were higher than those obtained on Birchwood xylan. Both xylans were composed only of xylose and there were no other substituents present in Birchwood xylan that could interfere with hydrolysis and slow the rate. The only difference between the two xylans was structural as a result of either the wood species or extraction method used to prepare the xylans. Both extraction methods are expected to alter the structure from the in-situ xylan, however it is known that alkaline extraction leaves the xylan more branched than extraction with Nitren (Janzon et al., 2007). Nitren extraction is also known to remove the 4–O–methylglucuronic acid, leaving the xylan with an unhindered structure that should be readily accessible to enzyme action (Puls et al., 2006). Another cause for the slower hydrolysis rates observed on Birchwood xylan, could have been the decreased accessibility of the structure to enzyme attack due to interchain aggregation. The Birchwood xylan was supplied in powdered form, which means it was subjected to a drying process. Is it known that when xylans are dried, interchain aggregation occurs due to the formation of strong hydrogen bonds (Ebringerová & Heinze, 2000; Puls & Schuseil, 1993) and the polymeric structure collapses. Not all of these bonds are broken when the xylan is resuspended in liquid and the polymer does not fully unfold. The partially folded structure is less susceptible to the action of the enzyme. It follows from these reasons that the Eucalyptus xylan had a more open structure, hence the higher hydrolysis rates observed.

The preparation methods influence the solubility of the xylan in aqueous solutions. Commercial xylans are usually extracted with alkaline extraction and a low solubility is observed due to the saponified ester-linkages (Puls & Schuseil, 1993; Tenkanen & Poutanen, 1992). Birchwood xylan was observed to be less soluble than Eucalyptus xylan and needed boiling before a uniform suspension could be obtained. Since enzyme degradation only occurs in the liquid phase, transport of the substrate into the aqueous phase would have slowed the hydrolysis rate.

Effect of multiple dosages

The effect of multiple dosages of Multifect on the hydrolysis of Eucalyptus xylan was investigated. In this experiment, to reduce the effect of frequent sampling on the enzyme hydrolysis as seen with Birchwood in Section 4.3.6, sampling intervals were further apart to ensure good temperature regulation in the incubator. All samples that were compared were incubated simultaneously to minimise variation. Despite these precautions, the results in Figure 4.20 was not comparable with results in Figure 4.19. Discrepancies were apparent even at the beginning of hydrolysis where the sugar concentrations should be

close to zero. An analysis of the consistency of the concentration of the internal standard revealed large variations (Section 3.3.2).
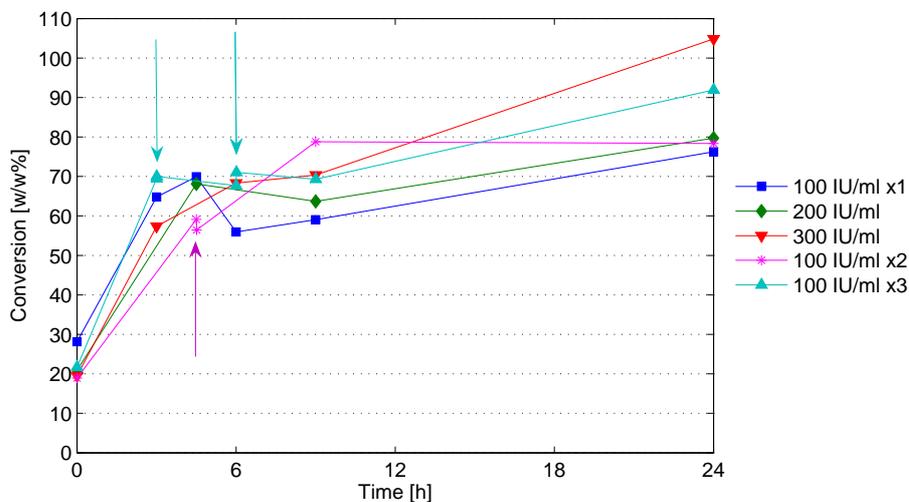
Figure 4.20: Substrate conversion of Eucalyptus xylan with multiple dosages of Multifect

As an additional comparison of the hydrolysis of Eucalyptus xylan to Birchwood xylan, multiple enzyme dosing was performed. The more accurate profiles of this experiment would also have served as a cross-verification of the profiles obtained in Figure 4.18. Multiple dosages of enzyme allows for the more effective use of the initial fast hydrolysis rates, with another dose of fresh enzyme being introduced when thermal denaturing starts to occur. The high reaction rates are therefore sustained and the reaction time needed to achieve complete conversion is shortened. A shorter reaction time translates to a smaller vessel and lower capital cost in a continuous process. From the economic model (see Section 5.2) is was however shown that retention time is not the most important consideration in a cost analysis, but enzyme cost is. The addition of more enzyme to the substrate does not seem to be a desired option. Therefore, although conclusive results could not be obtained from the data presented in this section, it was deemed unnecessary to repeat the experiment as it would not add more weight to the conclusions presented.

4.3.8 Concluding remarks

With the correct dosage of Multifect Xylanase, 100 % conversion of the Birchwood xylan was obtained. However, only 80 % of the Eucalyptus xylan could be degraded by Multifect, despite increases in enzyme dosage. The conversion levelled off after a period of 24 hours, which indicated that the remaining 20 % was unhydrolysable by enzymes.

Higher hydrolysis rates could be observed on Eucalyptus than on Birchwood xylan, even though high nickel levels were present in the Eucalyptus xylan. Since the two xylans were chemically similar, the difference in rate could only be attributed to the

structural properties of the two xylans obtained by different extraction methods. Alkaline extraction, as used to prepare commercial Birchwood xylan, hydrolyses ester-linkages and subsequently decreases the solubility of the xylan in aqueous solutions. It was also dried, which is known to partially collapse the structure and decrease enzyme accessibility. Both of these would lead to a lower rate of hydrolysis than would be seen on Eucalyptus xylan, which has never been dried and had been prepared differently.

From these results, it can be seen that Eucalyptus xylan is a promising source of xylose when hydrolysed with Multifect. High enzyme dosages or long retention times are however required to in order to reach high xylose yields.

CHAPTER 5

Modelling and scale up considerations

Decisions concerning the implementation and modification of process applications are usually based on economic considerations. The process optimum is more often than not the point of most profitable operation rather than the highest conversion. A preliminary cost evaluation was done to determine the economic implications of a simple steady state reactor based on the results from Section 4.3.7. The monetary values of the following factors were considered:

- Feed worth

- Product worth

- Enzyme cost

- Reaction vessel cost

All the above mentioned factors are functions of the variables conversion and hydrolysis time, i.e. retention time in a continuous operation. This empirical model developed was applicable to this investigation only and was only used to determine the point of most economic expression.

5.1 Steady state reactor model

5.1.1 Basic enzyme kinetics

The first step in constructing the economic model was to find an expression that described conversion with enzyme dosage and retention time. In literature, various types of expressions are used to describe enzyme kinetics, e.g. Michaelis-Menten or Briggs-Haldane kinetics (Cornish-Bowden, 2004; Shuler & Kargi, 1992). The Michaelis-Menten

expression was derived for a single substrate–single enzyme system and modification to represent more complex systems becomes a tedious task. This is by no means impossible, but the results of this investigation did not allow for it, as different substrate concentrations were not used to construct hydrolysis curves. For this investigation, the hydrolysis curves of Figure 4.19 where described by an empirical first order reaction.

Enzyme deactivation

Thermal denaturing causes the enzyme to lose activity after prolonged exposure to a high temperature environment as was discussed in Section 4.3.1. This effect has a pronounced effect on the hydrolysis profiles obtained when using the enzymes for extended incubation times. The loss of activity has a bigger effect on the rate of hydrolysis than the that of the decrease of substrate concentration (Section 4.3.7). An important factor to be included in a kinetic expression of the hydrolysis profile is therefore the loss of activity of the enzyme with time.

A mathematical expression was derived to describe the loss of activity of Multifect Xylanase during incubation as shown in Figure 4.3. Multifect Xylanase was used at a constant temperature of $55\,°C$ in experimental work and therefore no temperature dependence was included. The residual enzyme activity, R_{EA} can be described by Equation 5.1. The shape and parameters were determined by the sum of least squares as fitted to the experimental data in Figure 4.3.

$$R_{EA} = \frac{k_d}{t^\eta} \tag{5.1}$$

where R_{EA} is the residual enzyme activity, k_d, the rate constant of the loss of activity and η, the stoichiometric parameter of the expression. k_d and η were determined as 0.7 and 0.3 respectively. The fitted curve is shown in Figure 5.1.

When comparing the residual activity as a fraction of the maximum activity from Section 4.3.1 and the fitted curve of Equation 5.1, it can be seen that the hyperbolic expression adequately describes the loss of activity resulting from the duration of temperature exposure.

Product formation

The rate of any reaction is dependent on the concentration of the substrate, the hydrolysis time and in the case of a catalyst, the concentration of the catalyst. Enzymatic hydrolysis is an example of a catalysed reaction, where an increase in enzyme concentration increases the rate of hydrolysis up to the point of endpoint dosage (Section 4.3.6). The kinetics of the hydrolysis of Eucalyptus xylan by Multifect Xylanase was determined based on the results presented in Figure 4.19.
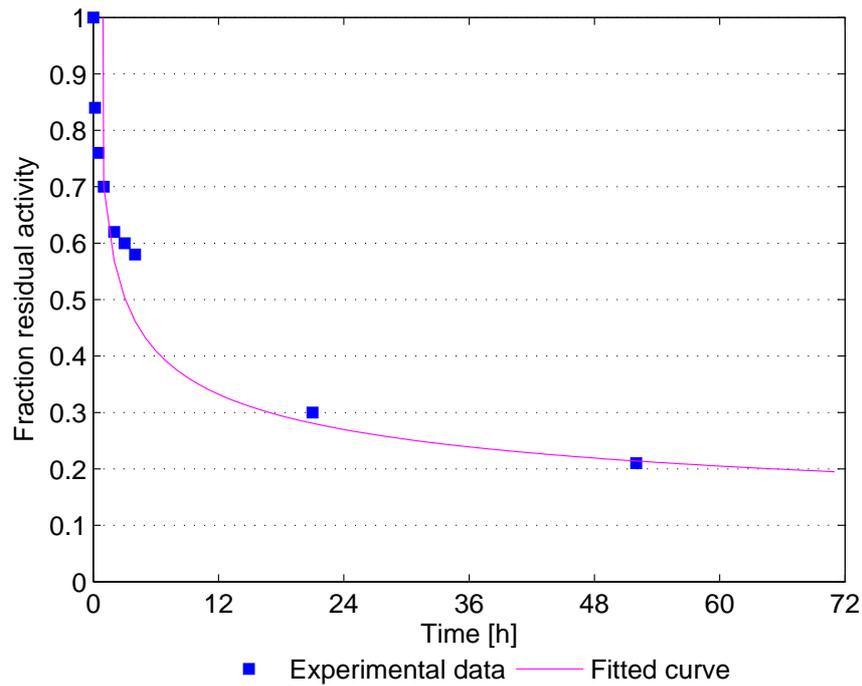
Figure 5.1: The residual activity of Multifect Xylanase over time and the expressions thereto

The hydrolysis was appropriated by first-order kinetics:

$$-r_A = k'C_S \tag{5.2}$$

where r_A is the rate expression of reaction A, C_S, the substrate concentration at any given time, C_{S0}, the initial substrate concentration and k' the general rate constant of the reaction.

The rate expression (Equation 5.2) was substituted into the differential mass balance for a batch system

$$\frac{dN_S}{dt} = r_A V = -k'C_S V \tag{5.3}$$

where N_S was the number of moles of substrate at give time, V the volume of the reactor and x conversion.

The mass balance (Equation 5.3) was rearranged and differentiated

$$\frac{V\,dC_S}{dt} = -k'C_S V \tag{5.4}$$

$$\frac{dC_S}{C_S} = -k'dt \tag{5.5}$$

$$\int \frac{dC_S}{C_S} = \int -k'dt \tag{5.6}$$

$$ln\frac{C_S}{C_{S0}} = -k't \tag{5.7}$$

Approximating the average molecular mass to be constant throughout the experiment (which is not strictly true but simplifies the calculation) C_S can be expressed as C_{S0} using conversion:

$$C_S = C_{S0}(1-x) \tag{5.8}$$

and substituted into Equation 5.7 and rearranged to yield Equation 5.9:

$$x = 1 - e^{-k't} \tag{5.9}$$

To adjust for the 20 % of Eucalyptus xylan that was hydrolysable by the enzyme (Section 4.3.7), Equation 5.9 was multiplied by 0.8 for a better fit to the experimental data. The general kinetic constant, k' consisted of the rate constant of hydrolysis, k multiplied by the residual activity, A_E (Equation 5.10).

$$x = 0.8 \left(1 - e^{-ktR_{EA}}\right) \tag{5.10}$$

Equation 5.10 could now be fitted to the experimental data in Figure 4.19 to determine the kinetic parameter, k for each enzyme loading. The experimental data and fitted equation is shown in Figure 5.2.
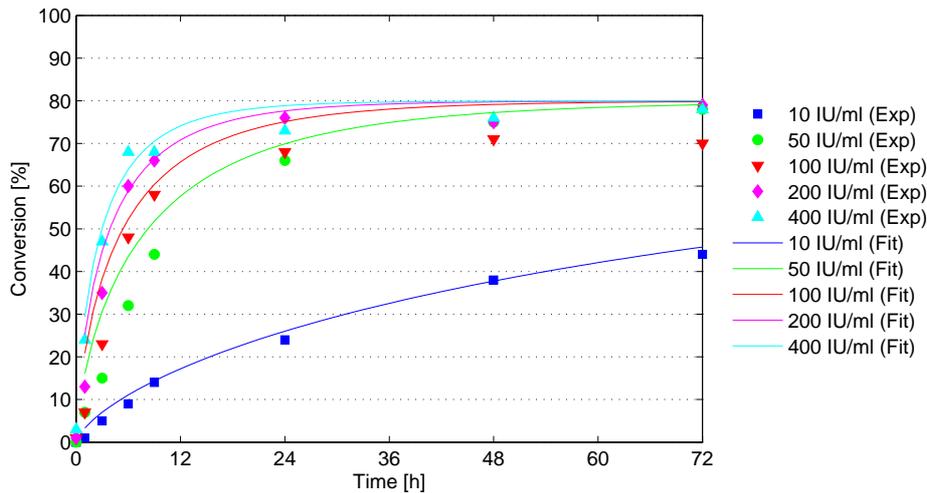
Figure 5.2: The hydrolysis curves of Multifect on Eucalyptus xylan and the fitted expression

The rate constant, k, varies with enzyme loading. The relationship between the kinetic parameter and enzyme dosage followed a logarithmic trend and could be expressed as

follows:

$$k = \alpha ln(E_l) - \beta \tag{5.11}$$

where E_l was enzyme loading and α and β were parameters of the expression.

The parameters, α and β were determined as 0.161 and 0.31 respectively. The k values determined from Equation 5.10 for different enzyme charges and the curve of Equation 5.11 fitted to it is shown in Figure 5.3.
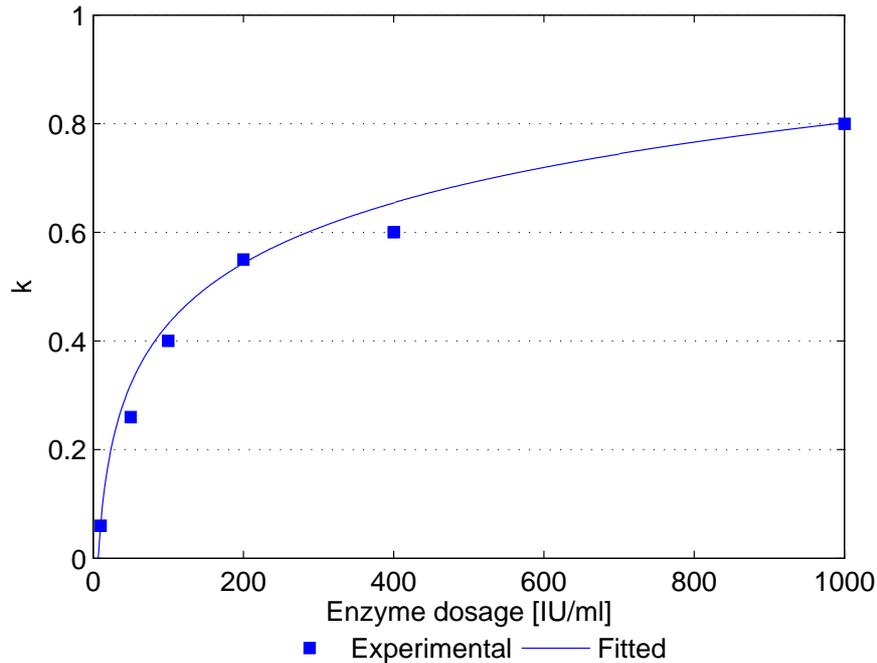
Figure 5.3: The rate constant with enzyme loading

The rate constant used in Equation 5.10 was expanded with Equation 5.11 to account for enzyme loading:

$$k = \left((0.161E_l - 0.31) \left(\frac{0.7}{t^{0.3}} \right) \right) \tag{5.12}$$

The rate constant was substituted in the first order rate equation, Equation 5.2:

$$-r_A = \left((0.161E_l - 0.31) \left(\frac{0.7}{t^{0.3}} \right) \right) C_S \tag{5.13}$$

5.1.2 Scale up considerations

The proposed process is a continuously stirred tank reactor (CSTR) with an inlet feed and enzyme stream and outlet product stream. The mass balance across the reactor is described as follows:

$$F_i - F_o = -r_A V \tag{5.14}$$

where F_i is the flow rate of inlet stream, F_o the flow rate of outlet stream, V the volume of the reactor and r_A the reaction rate.

For the modelling the continuous reactor, the assumption of steady-state was made. The use of retention time, τ was therefore more appropriate than hydrolysis time, t. τ could be related to reactor volume, V and volumetric flow rate, Q:

$$\tau = \frac{V}{Q} \tag{5.15}$$

The expression for reaction rate derived previously (Equation 5.2) was substituted into Equation 5.14:

$$F_i - F_o = k'C_{So}V \tag{5.16}$$

The mass flow rate, F in Equation 5.16, is related to concentration and volumetric flow rate:

$$F_i = (Q)(C_{Si}) \tag{5.17}$$

where C_{Si} is the concentration of substrate in inlet stream and C_{So} the concentration of substrate in outlet stream. Both inlet and outlet flow rates was converted to concentration and substituted into equation 5.16:

$$Q(C_{Si} - C_{So}) = k'C_{So}V \tag{5.18}$$

Equation 5.18 was rearranged and V was substituted by Equation 5.15:

$$\frac{C_{Si} - C_{So}}{So} = k'\tau \tag{5.19}$$

The concentration of the substrate was expressed as conversion as follows, again assuming that the average molecular mass of the substrate remains constant:

$$C_{So} = C_{Si}(1 - x) \tag{5.20}$$

Equation 5.20 was substituted into Equation 5.19 and rearranged to yield:

$$\frac{x}{1 - x} = k'\tau \tag{5.21}$$

The reaction rate defined in Equation 5.12 was substituted into Equation 5.21 to yield the final design equation for a CSTR:

$$\frac{x}{1 - x} = (0.161 ln(E_l - 0.31)) \left(\frac{0.7}{\tau^{0.3}}\right) \tau \tag{5.22}$$

5.2 Economic considerations

5.2.1 Cost equations

The only operating costs that were considered in the evaluation were the feed, product and enzyme worths with vessel cost the only capital expense included. The purpose of this model was to illustrate the regions of conversions and retention times that would lead to economic operation.

Xylan feed

The feed to the proposed process is the extracted Eucalyptus xylan from a hardwood pulp line. The flow rate of such a pulp process is approximated to be 600 000 t/a (Anon, 2007b). Up to 10 % of this pulp is xylan where about 50 % can be successfully extracted (Pearcy, 2007).

The feed flow rate was therefore constant, calculated as follows:

$$F_F = \frac{(600000)(1000)(0.1)(0.4)}{300} \tag{5.23}$$

where F_F was the feed flow rate with numerical numbers for unit correction.

The cost of the xylan was based on the current market value. Xylan sold for \$ 0.06/lb (Amidon, 2002) in 2002 and was adjusted with the Marshall and Swift (MS) index (Seider et al., 2004; Anon, 2007a) and exchange rate (Anon, 2007c) to reflex a 2007 worth in Rands. This didn't necessarily reflect the actual cost of extracting the xylan, but served as good estimation as it would indicate whether hydrolysis to monomers added more value to the xylan. The running cost of the xylan, $Cost_F$ was therefore:

$$Cost_F = (F_F)(0.93) \tag{5.24}$$

The consistency of the feed stream was chosen to be the same as used in this investigation, 10 g/ℓ. The density of the feed was therefore assumed to be close to that of water, 1000 kg/m^3.

Enzyme catalyst

The cost of enzyme was approximated as €20/kg (Wolfaardt, 2007), with density 1100 kg/m^3 (Genencor, 2007a). The enzyme flow rate is proportional to the feed flow rate according to the dosage and was calculated as follows:

$$F_E = \left(\frac{E_l}{E_a}\right)\left(\frac{F_F}{1000}\right)(1100) \tag{5.25}$$

where F_E was the enzyme flow rate, E_l enzyme loading and E_a the maximum enzyme activity. The associated cost, $Cost_E$ was:

$$Cost_E = (200)F_E \qquad (5.26)$$

Xylose product

The xylose product worth was determined by adjusting a market value of xylose, $ 45.5/kg in 1999 (Anon, 1999), with the Marshall and Swift index to a current value. The product flow rate is dependent on the conversion achieved in the reactor. The flow rate was calculated as follows:

$$F_P = (F_F)(x) \qquad (5.27)$$

with associated cost, $Cost_P$:

$$Cost_P = (56.07)F_P \qquad (5.28)$$

Net annual cost

The above cost expression for the feed, enzyme and product worths, equations 5.24, 5.26 and 5.28 respectively, was combined to yield an annual operational cost, assuming 300 operation days in a year:

$$Cost_a = (300)\left(Cost_P - (Cost_E + Cost_P)\right) \qquad (5.29)$$

A positive cost, $Cost_a$, indicated a profit according to the adopted convention.

Capital cost

The only capital cost considered in this economic model was that of the reaction vessel. The volume of the tank was calculated based on the retention time:

$$V = \tau\left(\frac{F_f}{24}\right) \qquad (5.30)$$

where V is the volume of the reactor. The cost of the vessel in 1996, $Cost_V$, was determined from cost functions (Sinnott, 1999: p.258):

$$Cost_V = C(V)^p \qquad (5.31)$$

where C = 3609.15 and n = 0.57. The cost of the vessel was therefore calculated as follows and adjusted with Marshall and Swift cost index and the exchange rate:

$$Cost_{vessel} = 3609.15(V)^{0.57}(7)\left(\frac{1306.3}{1060.1}\right) \tag{5.32}$$

5.2.2 Discounted cash flow calculation

The Discounted Cash Flow (DCF) approach was used to assign a time-value to money. In this investigation, the required rate of return on the money invested was set at 20 % with a payback of 3 years. The DCF analysis was used to determine the value gained from the capital cost investment over the payback period. A goal function was defined with all costs converted to present day values using the required return on investment (Blank & Tarquin, 2005):

$$\text{Goal function} = \text{Capital cost} + \text{Annual cash flow}(P/A,i,n) \tag{5.33}$$

where i was 20 % and n = 3 years and $(P/A, i, n)$ is the discrete cash flow formula defined as follows (Blank & Tarquin, 2005):

$$(P/A, i, n) = \left(\frac{(1 + 0.2)^3 - 1}{0.2(1 + 0.2)^3}\right) \tag{5.34}$$

All the previously determined cost formulae were substituted into Equation 5.33 to yield:

$$GF = -Cost_{vessel} + Cost_a(P/A, i, n) \tag{5.35}$$

where GF is the goal function.

The numerical value of the goal function indicated the feasibility of the scenario. When the value of the goal function was zero, the return on investment was exactly 20 % with a payback of 3 years. A positive value indicated a higher return on investment, whereas negative values indicated non-feasible scenarios where the return on investment is lower than the specified 20 %.

The minimum enzyme loading that yielded a real solution of the goal function was 7 IU/mℓ and the goal function was calculated for enzyme loadings of 7 to 100 IU/mℓ. Hydrolysis times, i.e. retention times τ, between 1 and 120 hours where chosen, as 5 days is the maximum retention time normally used in commercial enzyme hydrolysis processes (Eduardo et al., 2006). The values of the goal function with retention time and enzyme loading (Figure 5.4) showed that feasible operation is only achieved at very low enzyme charges.

A low enzyme charge, 1 mℓ per 1 ℓ substrate solution, with a 5 – 15 % solids loading is used in Iogen's cellulose–to–bioethanol plant. This corresponds to a retention time
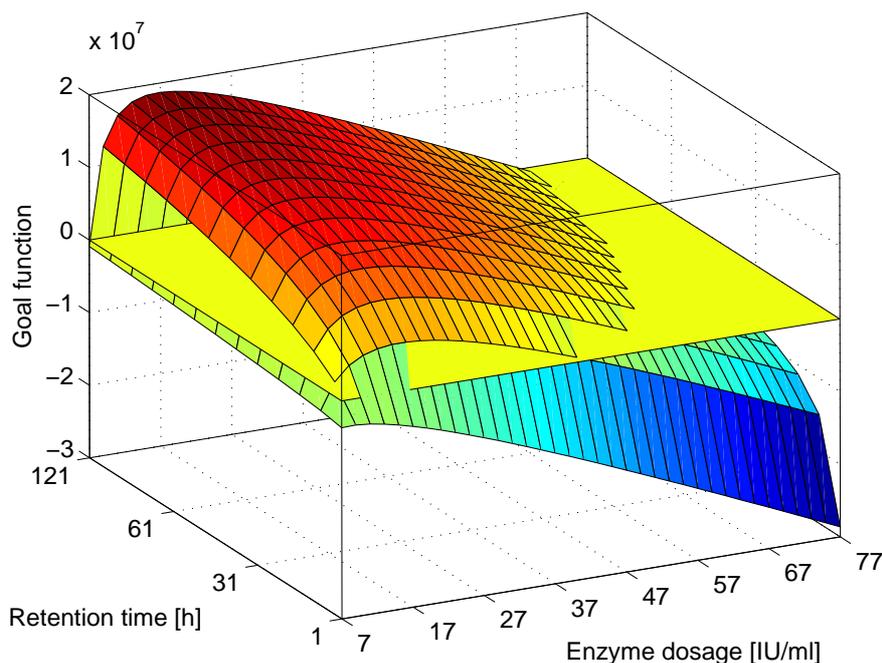
Figure 5.4: Effect of enzyme loading and retention time on the goal function

of 5 days to achieve a conversion of 80 % (Eduardo et al., 2006). When looking at the operational parameters, enzyme was by far the highest cost, which explains the need for low enzyme charges and long retention times as seen in commercial applications.

With 5 days the maximum retention time considered, the most profitable scenario corresponded to an enzyme loading of 17 IU/ℓ, retention time of 120 hours and a conversion of 76 % (Figure 5.4).

5.2.3 Multiple enzyme dose analysis

From the economic analysis it was shown that low enzyme dosages are required for feasible operation. In a continuous process this translates to a long retention time and large reactor volume. In the multiple dosage experiments (Section 4.3.6) it was shown that when an enzyme charge was split over several dosages, higher yields could be obtained in the same hydrolysis time as with a single dosage.

In a continuous process this would require the use of sequential reactors, although each would be smaller in size, the capital cost would still increase. From the experimental data available, the number of dosages for optimum economic operation cannot be determined, because the data in Section 4.3.6 is not accurate enough to permit adequate fits. However considering the fact that the hydrolysis profiles for single dosing could be successfully approximated as a first order reaction, it follows from the Levenspiel-plot that a plug flow reactor would be the best choice as the desired yield can be obtained in the smallest

volume.

In an enzyme applications, a plug flow reactor (or trickle bed type), would require immobilisation of the enzyme. This type of reactor cannot be modelled with the data presented in this investigation as it is known that immobilisation alters the characteristics of the enzyme. In order to pursue this option research on the best immobilisation and reactor configuration is required.

Process implementation was not the objective of this study, but instead the economic model of a process was used as a tool to obtain approximate enzyme charges and reactor volumes needed for feasible operation. In understanding the utilisation of the extracted xylan, these estimations are essential in guiding further work along this avenue of research.

CHAPTER 6

Conclusions and Recommendations

The forest products industry is well suited to play a significant role in biomass conversion to alleviate the use of fossil fuels for energy and chemicals production. Currently, the pulp mill presents the opportunity to recover under-utilised xylan from the pulp or waste streams in the process. Hardwood xylan is composed mainly of the sugar monomer xylose, which is a platform chemical for conversion to fuels and other valuable chemicals. In order to better understand possible uses for the recovered xylan, an investigation into the enzymatic hydrolysis thereof was conducted. A hardwood xylan extracted from fully bleached Eucalyptus pulp with a chelating agent, Nitren, was obtained and the ability of several commercial enzymes to degrade the extracted xylan to xylose monomers was determined.

Carbohydrate analysis

The carbohydrate content of the acid and enzyme hydrolysates were analysed by two colorimetric assays, Dinitrosalicylic acid (DNS) and Somogyi-Nelson (S–N), and chromatographically by high performance ion exchange chromatography (HPIC). The methods compared well as far as trend analysis was concerned, but in absolute values, the DNS method overestimated the sugar concentration, whereas S–N yielded more conservative results when compared to HPIC analysis. Ease of use, however, made DNS analysis the method of choice for enzyme assays and trend analyses. HPIC was used where more accurate results were required. The repeatability of analysis by HPIC was improved with the inclusion of an internal standard and results to be compared were analysed in the same batch to minimise the effect of instrument variation.

Determination of the sugar composition of the commercial xylans

Chemical conversion was used to quantify the monomeric composition of the xylans used in this investigation. The acid hydrolysis protocols were optimised for the equipment as well as the substrate used, by varying acid concentration and autoclave time. The maxi-

mum conversion of Birchwood xylan with acid (1 % H_2SO_4, 30 minutes autoclaving) was 76 %, with xylose the only monomer present in significant quantities in the hydrolysate. The maximum conversion of Oat Spelts xylan (1 % H_2SO_4, 60 minutes autoclaving) was 80 %, with the hydrolysate composed of 78 % xylose, 11 % arabinose, 9 % glucose and 2 % galactose. For both substrates, the compositions compared well to those reported in literature and the protocols could be applied to characterise the carbohydrate content of the extracted xylan with confidence.

Enzyme catalysed hydrolysis of the xylans

Enzyme characterisation was done to gauge the level of endo-xylanase and accessory enzyme activity in the commercial enzyme preparations and to determine the effect of pH and temperature on the enzymes' behaviour. Decreased activity due to thermal denaturing after prolonged exposure to high temperatures was observed for all the enzymes investigated.

When the commercial xylans were degraded with the enzymes at equal charges, the conversion of Birchwood xylan was higher than that of Oat Spelts xylan in the predetermined hydrolysis times for all enzymes investigated. The hydrolysis of the chemically homogeneous Birchwood xylan appeared to be faster than that of the more complex Oat Spelts xylan. Oat Spelts xylan contains glucose, arabinose and galactose, presumably as sidechains on the xylan backbone, which could slow the rate of enzyme hydrolysis by sterically interfering with the enzyme action. The highest yield of monosugars was obtained with Multifect on both Birchwood and Oat Spelts xylan as substrates. The high level of endo–xylanase and β–xylosidase activities present in the enzyme preparation made it the most suitable out of the enzymes investigated for xylan degradation.

Different dosages of Multifect were applied to Birchwood xylan to determine the optimum dosage. At an enzyme charge of 600 IU/mℓ, 100 % conversion of Birchwood xylan to xylose was obtained in 72 hours hydrolysis time. However, a distinct decrease in hydrolysis rate was observed after six hours of hydrolysis time, which was found to be a result of thermal denaturing of the enzyme. To more effectively use the higher hydrolysis rates before thermal denaturing sets in, multiple enzyme dosages were applied throughout the course of the hydrolysis experiment. This allowed for higher xylose yields to be obtained with the same overall enzyme charge and hydrolysis time.

The chemical conversion of Eucalyptus xylan yielded only xylose as monomer. As its composition was similar to that of Birchwood xylan, Birchwood xylan could serve as a benchmark to which the hydrolysis of Eucalyptus xylan could be compared. The structures of the two xylans were not determined in this study. It would not be identical though, as the xylans were sourced from different wood species and prepared using different extraction methods.

As a result of the difference in xylan structures, the hydrolysis rate obtained using Eucalyptus xylan as substrate was higher than using Birchwood xylan as substrate with

equal enzyme charges of Multifect. Nitren extraction removes all the branches and substituents, leaving the xylan backbone accessible to enzyme action. Alkaline extraction does not have this effect on the Birchwood xylan; the branches caused steric hindrance, resulting in reduced hydrolysis rates.

As a result of Nitren extraction, the Eucalyptus xylan contained high levels of nickel. When looking at the hydrolysis profiles for various charges of Multifect on Eucalyptus xylan, however, it was apparent that the nickel content did not severely affect the functioning of the enzyme.

Despite the higher hydrolysis rates, full conversion of Eucalyptus xylan to xylose could not be obtained with Multifect. The complete hydrolysis of Birchwood xylan, however, indicated all the enzymes required for xylan conversion were present in Multifect. It was, therefore, concluded that the unconverted fraction of the Eucalyptus xylan was unhydrolysable by enzymes as a result of the extraction method used.

However, up to the point of 80 % conversion, higher hydrolysis rates were obtained on Eucalyptus xylan than Birchwood xylan with equal charges of Multifect. The alkaline extraction is expected to leave the Birchwood xylan branched, which would hinder the access of enzyme to the structure thereby slowing the overall hydrolysis rate. Nitren extraction on the other hand, removes all substituents and branches leaving the xylan backbone readily accessible to the enzyme, barring the unhydrolysable 20 %.

Simple economic model of a continuous reaction process

A simple economic evaluation was done based on the results from the experimental work. Empirical equations were derived to describe the enzyme kinetics, accounting for thermal denaturing. A CSTR was modelled as an example of a continuous reaction process. The costs involved regarding the xylan feed, xylose product and enzyme catalyst as well as the capital cost of the reaction vessel was considered. A discounted cash flow (DCF) analysis was done with a 20 % return on investment required in 3 years, with enzyme charge and retention time as variables.

From the DCF analysis it was seen that the optimum economic potential of the process was with an enzyme charge of 17 IU/ℓ and a retention period of five days. This corresponded to a substrate conversion of 76 %. High enzyme costs necessitate the use of low enzyme charges and long retention times. The use of multiple enzyme dosing would allow for this yield to be achieved in a shorter hydrolysis time. However, it is postulated that the use of a plug flow-type reactor might be more economical than several sequential enzyme additions, in which case it is recommended that enzyme immobilisation be studied.

In understanding the utilisation of the extracted xylan, it is essential to establish the potential of degrading it to monomers with commercial enzymes. It was shown that a hardwood xylan, extracted from fully bleached Eucalyptus pulp with Nitren, could be successfully converted to xylose with the commercial enzyme Multifect.

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APPENDIX A

Determination of the constituent monomers

A.1 Introduction

The monosugar composition of xylans is unique to the wood species it is sourced from. In Section 2.3.2 the expected monomeric composition of xylans from some sources were given, which could serve as an estimate of the approximate sugar composition. Exact quantification of the monosugar content of the xylan is however required before any meaningful investigation can be done using it as substrate. It was important that the correct enzymes be applied in the enzymatic hydrolysis protocols and also that the monomeric sugar yields be correctly calculated. The sugar composition of the xylans under investigation was therefore determined.

In the conversion of hemicelluloses to monomers for carbohydrate quantification, chemical and enzymatic hydrolysis are the two methods most commonly employed. In enzymatic hydrolysis, a cocktail of different commercial enzymes is used to completely degrade the hemicellulose (Vaaler et al., 2001; Buchert et al., 1993; Schwald & Saddler, 1988). Chemical conversion can be achieved by methods such as mineral acid conversion (TAPPI, 2001; Jacobs et al., 2003; Kaar et al., 1991), acid methanolysis (Sundberg et al., 1996) and trifluoroacetic acid (THF) conversion (Garna et al., 2006).

Acidic hydrolysis by mineral acids is a quick and simple method, but is usually accompanied not only by uncontrollable yield losses (Vaaler et al., 2001), but also the complete loss of acidic sugars, meaning that only neutral sugars can be quantified (Sundberg et al., 1996). Enzymatic hydrolysis, acid methanolysis and hydrolysis with trifluoroacetic acid (THF) all have little to no product losses, but are more lengthy procedures involving more reagents and experimentation time. In view of the following deciding factors, conversion with mineral acid was however chosen:

- Ease of method: no complicated reagent preparation or laborious procedures were

involved. Also the results could be obtained quickly, relative to the other proce-
dures. Both enzymatic and THF hydrolysis requires long periods to obtain good
yields.

- Desired products: the sugars that were of importance are the neutral sugars xylose,
  glucose, galactose and arabinose. Since the commercial substrates used were prob-
  ably prepared by alkaline extraction, no acetylated products were expected that
  would require the use of more involved procedures.

- Limited degradation can be tolerated: Sugar degradation is expected from acid
  hydrolysis with mineral acids, but since identification of the constituent monomers
  and not complete conversion was the ultimate goal, this was acceptable.

- Repeatability: Acid hydrolysis is known to deliver repeatable results. Hydrolysis
  with acetic acid may cause less degradation, but does not give easily reproducible
  results.

Mineral acid hydrolysis can be carried out under mild conditions, typically 120 –
170 °C with sulphuric acid catalyst at less than 1 % (Lee & McCaskey, 1983) or with
strong sulphuric acid, 72 %, at room temperature (TAPPI, 2001). Both methods are
modified from the Klason method for carbohydrate analysis (Kaar et al., 1991). The
applicability of the method used depends on the source and physical state of the substrate;
a wood chip requires far harsher treatment than an extracted xylan. For the sake of
completeness both methods were used in this investigation, with the acid concentration
and hydrolysis time optimised for the specific xylan.

A.2 Materials and Methods

A.2.1 Xylan substrates

Commercial Birchwood Xylan (X0502) and Oat Spelts Xylan (X0627) from Sigma–
Aldrich (Germany) were used as representative substrates of a hardwood and cereal
xylan. As a representative sample of a xylan recovered from the pulping process, the
xylan product extracted from fully bleached Eucalyptus hardwood pulp using a method
involving a chelating complex, Nitren (Kettenbach & Stein, 2007), was obtained.

A.2.2 Two–step acid hydrolysis

A modification of the Tappi method T–223 for the determination of pentoses in wood
pulp (TAPPI, 2001) was used to develop this method. A 72 % H_2SO_4 solution (1.5
mℓ) was added to 0.15 g xylan in a 5 mℓ Hach test tube, ensuring that the xylan was

moistened. As controls, 0.15 g xylan and 0.15 g xylose each in deionised water (1.5 mℓ) and 0.15 g xylose in 72 % H$_2$SO$_4$ (1.5 mℓ) were included. The samples and controls were placed on the bench top at ambient temperature, +/- 28 °C, for 1 h.

The samples and controls were sequentially transferred into 250 mℓ Erlenmeyer flasks and diluted to 2.5 % H$_2$SO$_4$ with the addition of 41 mℓ deionised water after which they were placed in an autoclave (Hirayama HV-50 Hiclave, Tokyo), at 121 °C for 1 h.

The autoclaved samples and controls were then centrifuged at 10 000 rpm for 10 min at 4 °C (Eppendorf 5130R, Germany), filtered through 0.45 μm Milipore filters and each made up to a total volume of 100 mℓ.

A.2.3 Dilute acid hydrolysis

A modification of the methods described by Nee & Yee (1976) was done to develop this method. Different acid solutions (42.5 mℓ of 0.5, 1, 2.5 and 4 % H$_2$SO$_4$) were added to 0.15 g xylan in 250 mℓ Erlenmeyer flasks, ensuring that the xylan was moistened. As controls, 0.15 g xylan and 0.15 g xylose each in deionised water (42.5 mℓ) and 0.15 g xylose in different acid solutions (42.5 mℓ of 0.5, 1, 2.5 and 4 % H$_2$SO$_4$) were included. The samples and controls were autoclaved in time periods of 0.5, 1, 3 and 6 h. The autoclaved samples and controls were subsequently treated as described above. Hydrolysis with sulphuric and hydrchloric acid has been reported to yield similar results (Qiabi et al., 1994), therefore only sulphuric acid was used.

A.3 Results and Discussion

A.3.1 Establishing the protocols for acid catalysed hydrolysis

Effect of hydrolysis conditions on monosugars

It is a well known fact that sugars degrade in an acid environment and this is indeed the basis for assaying pentoses where the furfural production is measured as an indication of the original amount of pentoses present in the sample (TAPPI, 2001). Analysis with HPIC and colorimetric methods are however done on the monosugars and degradation is not desired. It is imperative that the method used to convert the substrate into monomeric sugars results in minimum losses to degradation.

Results showing the effects of exposing xylose to various concentrations of acid at different autoclave times are depicted in Figure A.1. The effect of temperature on monomeric xylose was established by autoclaving a known amount of xylose in water for different periods of time. The combined effect of temperature and acid concentration on monomeric xylose was then determined by autoclaving a known amount of xylose for different periods of time with acid ranging from 0.5 – 4 % in concentration. It was observed that higher

acid concentrations and longer autoclaving times led to lower sugar recoveries. The presence of acid at high temperature exacerbated sugar decomposition far above what was seen for the temperature controls alone.
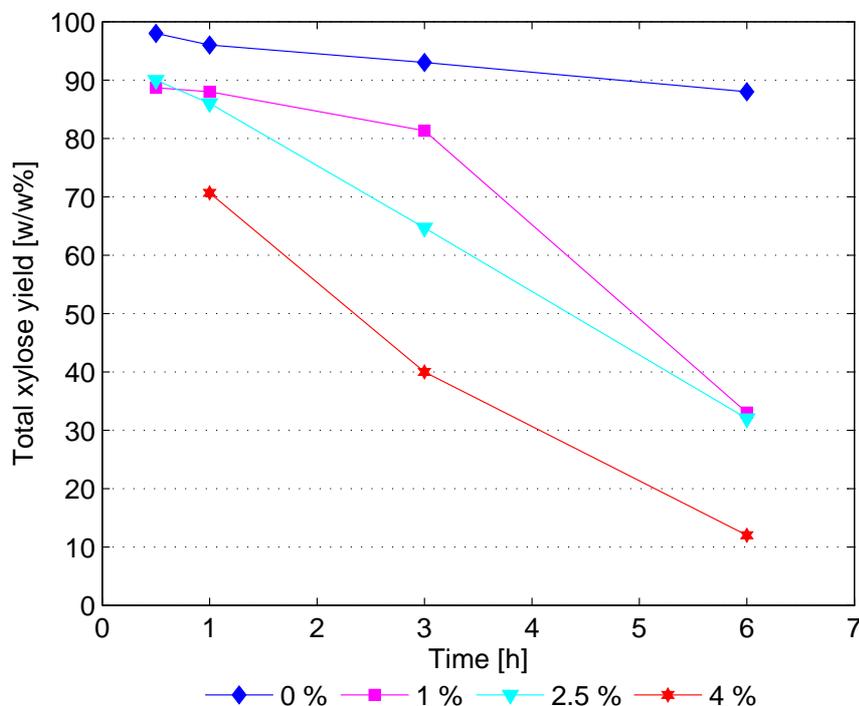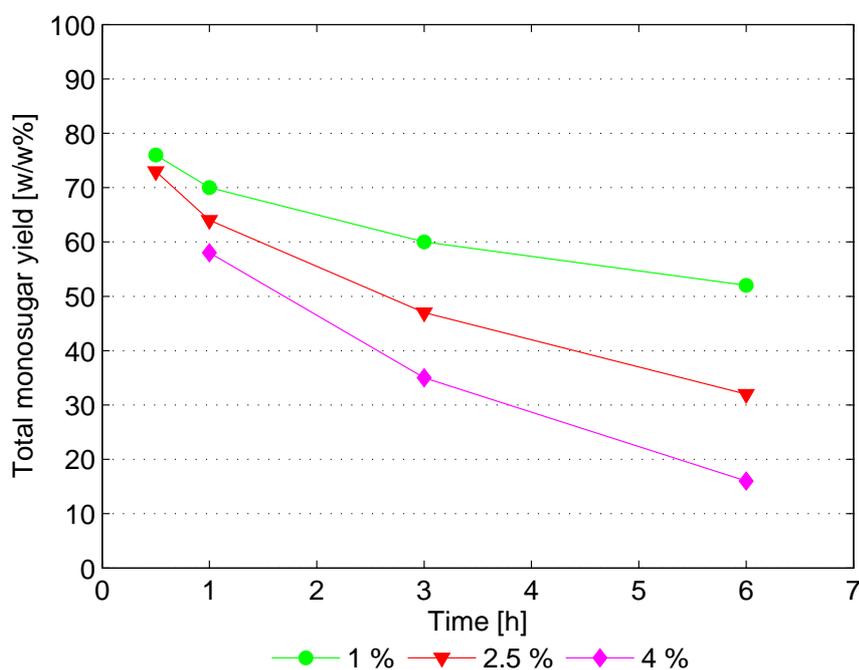

Figure A.1: Total xylose recovered after treatment with acid at various concentrations for different periods of time.

The two–step acid hydrolysis protocol is usually used in the conversion of protected fibre bundles, like bagasse and wood chip, to monomers, where the strong acid step serves to pre-treat the substrate (Lavarack et al., 2002; Singh et al., 1984). In this investigation it was interesting to observe that the two–step protocol resulted in slightly less degradation of the xylose than the 4 % dilute acid protocol despite the 72 % acid pre-treatment step. With the two step protocol, 71 % of the xylose was recovered. This can be explained by the effect of the high temperature in the dilute acid protocol, which increases the hydrolysis rate of the acid catalysed reaction. The use of 0.5 % acid in the dilute acid protocol, yielded xylose recoveries of 96 % and 89 % when autoclaved for 30 minutes and one hour respectively.

The conversion of xylan with acid hydrolysis inevitably leads to degradation of the liberated monosugars. As these reactions occur simultaneously, hydrolysis of the xylan is always balanced against degradation of the product. When the monosugar content of xylans are characterised with acid hydrolysis, the optimum conditions are sought where monosugar recovery is maximised and minimum product losses are obtained.
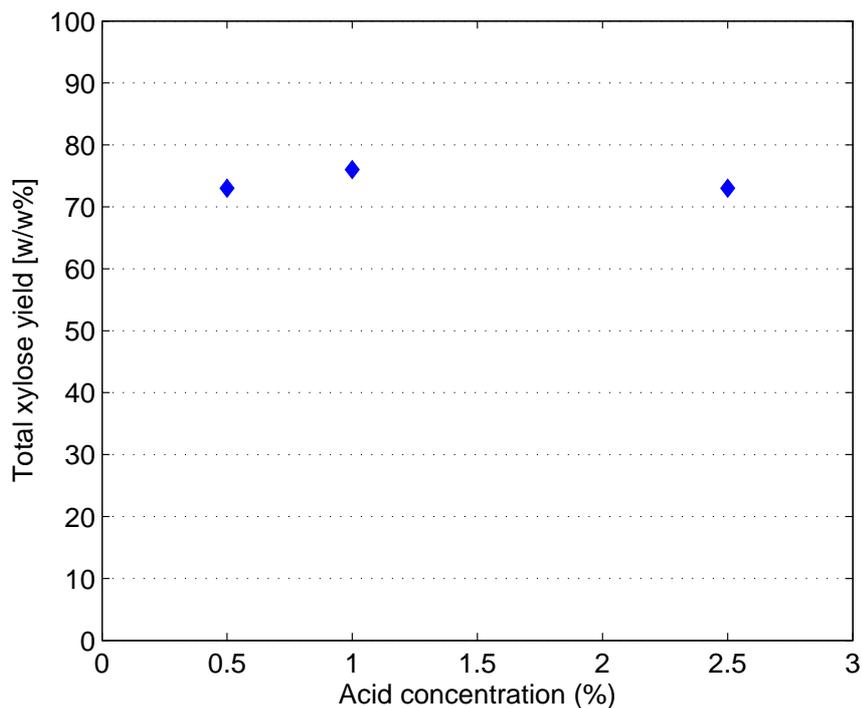
Acid catalysed hydrolysis of Birchwood xylan

Birchwood xylan was hydrolysed using both the dilute acid and two–step hydrolysis protocols. In the hydrolysate of Birchwood xylan, xylose was the only sugar detected in significant quantities. The highest conversion of Birchwood xylan to xylose was obtained with an acid concentration of 1 % and autoclaving duration of 30 minutes (Figure A.2). From the trends it appeared as if a higher monosugar yield could have been obtained at a shorter autoclaving time, but investigating a period shorter than 30 minutes was impractical due to the physical limitations of the equipment.

Figure A.2: Total xylose determined after treatment of Birchwood xylan with acid at various concentrations for different hydrolysis periods.

When using the two step protocol, 60 % of the xylose was recovered, whereas the use of 0.5 % acid in the dilute acid protocol yielded 73 % and 66 % xylose recovery when autoclaved for 30 minutes and one hour respectively.

When looking at the use of different acid concentrations for an autoclaving period of 30 minutes, 1 % was the optimal acid concentration at which the highest sugar yield was obtained (Figure A.3). In Section A.3.1 it was seen that a lower acid concentration could lead to less degradation of the xylose. However, the yield with 0.5 % acid was lower than with 1 % acid, because a lower acid concentration also means less substrate is converted to monosugars. With the protocol employed, the highest sustrate conversion that would be obtained was 78 %.

Figure A.3: Total xylose determined from the treatment of Birchwood xylan with acid at various concentrations and 30 minutes hydrolysis time.

Acid catalysed hydrolysis of Oat Spelts xylan

Acid hydrolysis of Oat Spelts xylan with the dilute acid and two–step hydrolysis protocols yielded the carbohydrate monomers xylose, glucose, arabinose and galactose. Up to 80 % of the original substrate mass could be recovered as monomers (Figure A.4), the maximum sugar yield being obtained at 1 % acid autoclaved for one hour. A shorter hydrolysis time and a lower acid concentration led to lower sugar yields, presumably due to the decreased extent of substrate hydrolysis. A longer hydrolysis time was needed to obtain a conversion similar to that with Birchwood xylan, which seemed to indicate that Oat Spelts xylan is a more recalcitrant substrate because it is less susceptible to acid attack.

The sugar composition of the Oat Spelts xylan hydrolysate varied with the hydrolysis conditions (Figure A.5); the fraction of xylose in the total monosugar hydrolysate decreased with higher acid concentrations and longer autoclave periods. When the two step protocol was employed, the total monosugar yield was 73 %, of which 76 % consisted of xylose. With the use of 0.5 % dilute acid for autoclaving durations of 30 minutes and one hour, 73 % and 71 % total sugar yield were obtained respectively. The hydrolysate from both hydrolysis durations consisted of 84 % xylose.

It is known that acid catalyses the hydrolysis of the polymer by facilitating random cleavage of the structure with no regard to sugar or bond type, but the hydrolysis of polysaccharides composed mainly of pentoses are 10 to 1000 times faster than those
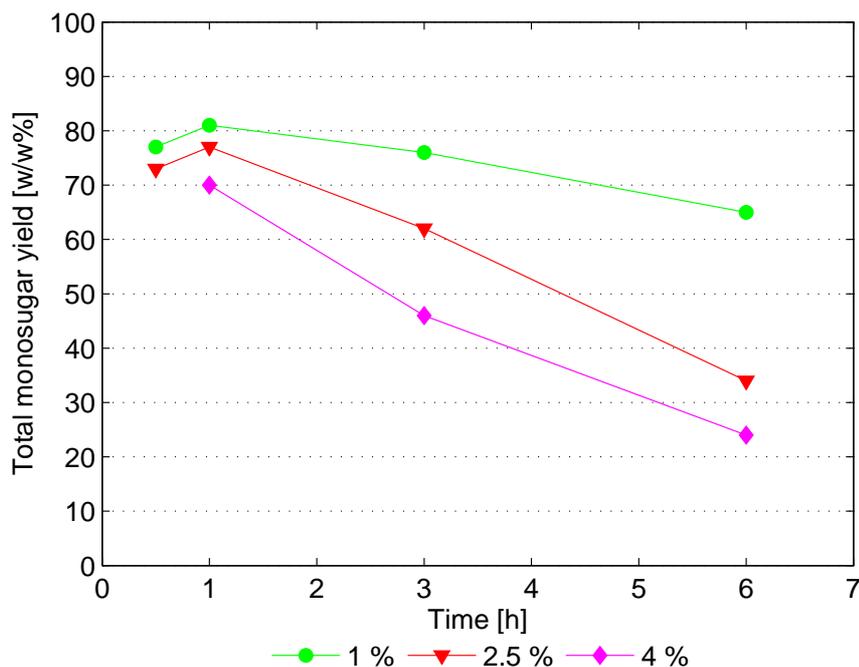
Figure A.4: The total sugar yield after treatment of Oat Spelts xylan with various acid concentrations and different hydrolysis times.

composed mainly of hexoses (Kennedy & White, 1983). If this was applicable here, xylose would be liberated at a faster rate than the other sugars and would consequently be exposed to the degrading environment for longer, explaining the trend in Figure A.5.

In Figure A.4, it was seen that the optimum conditions for maximum conversion is a balance between substrate hydrolysis and product degradation. It was assumed that at these optimum conditions the most representative sugar composition of the substrate would be obtained. The sugar composition determined with this protocol was comparable to results presented in literature for the same substrate (Table A.1). In the work of Kormelink & Voragen (1993), a THF hydrolysis protocol was used, which is considered to be more accurate than mineral acid hydrolysis, as the THF does not have a degrading effect on the sugar product. From this comparison, the acid hydrolysis protocol was deemed adequate for the purposes of this investigation.

A.3.2 Hydrolysis kinetics

In the chemical conversion of hemicelluloses, the polymer is hydrolysed to its constituent monomers by the catalytic action of the acid. The reaction rate is increased with the use of high temperature such as autoclaving (Lavarack et al., 2002; Singh et al., 1984). In the chemical digestion of natural hemicelluloses, two distinct rates of hydrolysis are often observed (Maloney et al., 1985); the first 60 – 70 % of the xylan, hemicellulose A, hydrolyses according to fast first order kinetics, with the remainder, hemicellulose B,
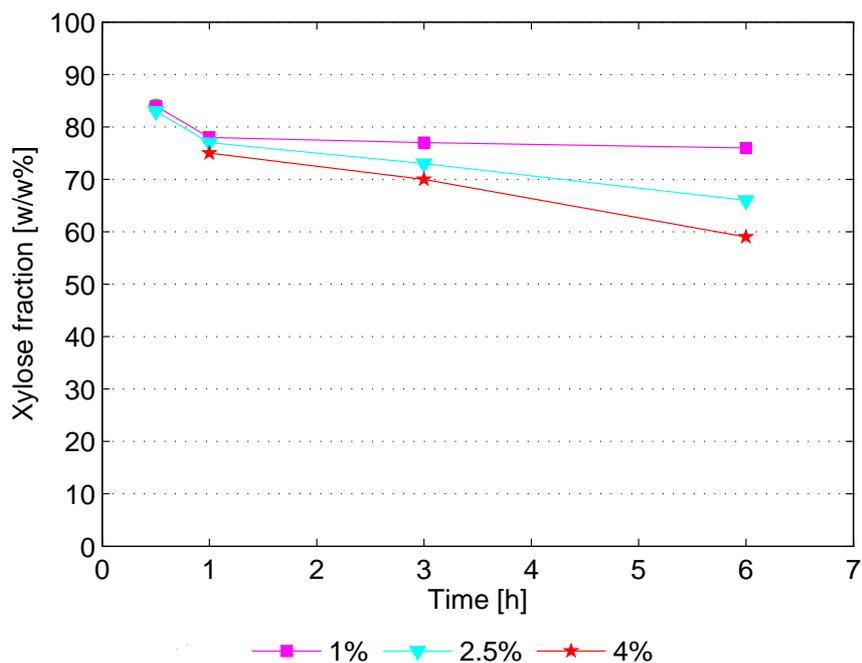
Figure A.5: The xylose fraction in the total monosugars obtained from the acid hydrolysis of Oat Spelts at different acid concentrations and hydrolysis periods.

Table A.1: The sugar composition of Oat Spelts xylan as determined in this investigation and from literature

Source	Arabinose	Galactose	Glucose	Xylose	Mannose
This study	11 %	2 %	9 %	78 %	n.a
Kormelink & Voragen (1993)	9.7 %	1.1 %	3.4 %	81.4 %	n.a.

hydrolysing at a slower rate. In native xylans, hemicellulose A is identified as the more reactive xylan whereas hemicellulose B is more resistant to degradation. The reactivity of the xylan is an inherent characteristic of its location within the cellulose-hemicellulose-lignin matrix. The more resistant hemicellulose is more intertwined and less accessible to attack.

The xylans used in this study have been pre-extracted from the source with commercial procedures. It was, therefore, assumed that the structure of the xylan from the vendor would be homogeneous in terms of reactivity and would hydrolyse at one rate. In the acidic conditions, the released monomeric sugars are degraded in subsequent decomposition reactions. The reaction scheme for a homogeneous hemicellulose can be described by Equation A.1 (Maloney et al., 1986):

$$H_t \rightarrow O \rightarrow M \rightarrow D \tag{A.1}$$

where H_t is the total hemicelluloses, O the total oligomers, M the total monomeric sugars and D the total degradation products.

In the first reaction, the acid catalyses a random breakdown of the xylan polymer to oligomers of various degrees of polymerisation (Lee & McCaskey, 1983). The rate of hydrolysis of the oligomers is dependent on the chain length and this complicates an exact reaction model of hydrolysis. The rate of hydrolysis of the soluble oligomers is however much faster than that of the xylan and the reaction mechanism can be simplified to the following with little loss in accuracy (Maloney et al., 1986):

$$H_t \xrightarrow{k_1} M \xrightarrow{k_2} D \tag{A.2}$$

The individual reactions in Equation A.2 can be appropriated by first order kinetics (Lee & McCaskey, 1983). The rate of the hydrolysis reaction, k_1, is much faster than that of the decomposition reaction, k_2, which is why high yields can be obtained with acid hydrolysis (Lee & McCaskey, 1983). Using first order kinetics and the reactions described in Equation A.2, the concentrations of the substrate and products can be expressed as follows:

$$C_H = C_{H0}e^{-k_1t} \tag{A.3}$$

where C_H is the concentration of hemicellulose substrate in the reaction mixture at any given time, C_{H0} the initial hemicellulose substrate, k_1 the rate constant of the hydrolysis reaction and t the hydrolysis time.

$$C_M = C_{H0}k_1 \left(\frac{e^{-k_1t}}{k_2 - k_1} + \frac{e^{-k_2t}}{k_1 - k_2} \right) \tag{A.4}$$

where C_M is the concentration of monomers in the reaction mixture at any given time and k_2 the rate constant of the degradation reaction.

$$C_D = C_{H0} \left(1 + e^{-k_1t} \left(\frac{k_2}{k_1 - k_2} \right) + e^{-k_2t} \left(\frac{k_1}{k_2 - k_1} \right) \right) \tag{A.5}$$

where C_D is the concentration of degradation products in the reaction mixture at any given time.

The total monomeric sugar products from the acidic hydrolysis of Oat Spelts xylan, as shown in Figure A.4, could be described with Equation A.4, with the values of k_1 and k_2 determined by a least squares estimation (Figure A.6).
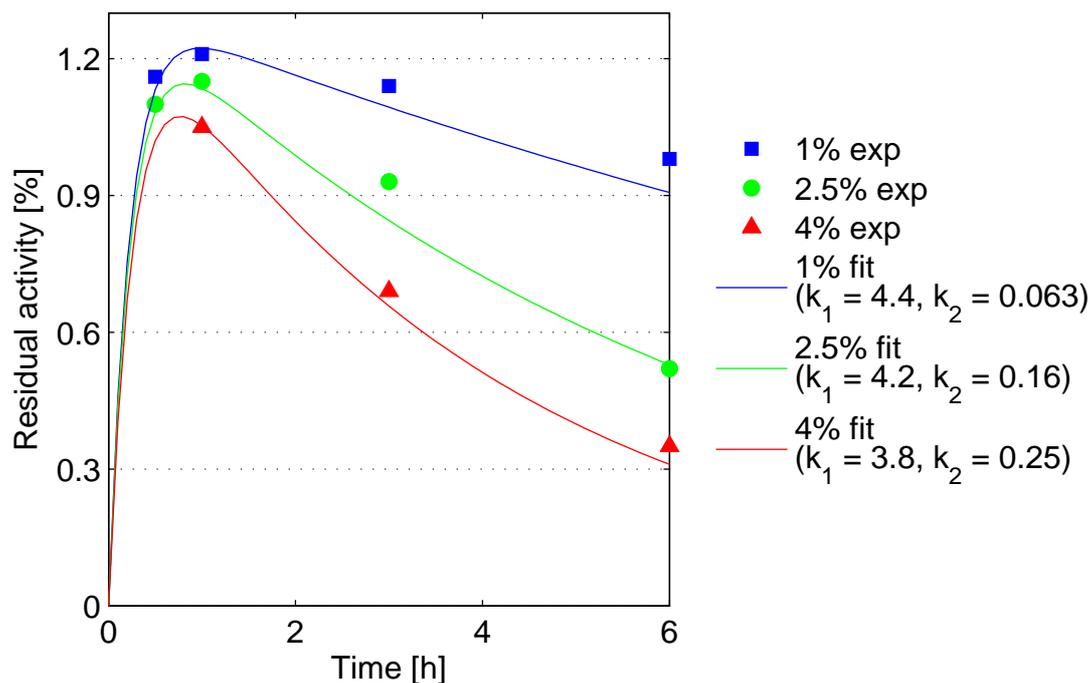
Figure A.6: Experimental and hypothetical values for total monosugar yield from the acid hydrolysis of Oat Spelts xylan

The hypothetical curves and experimental values for the total monosugar concentrations are shown (Figure A.6). The turning point observed in the experimental data can be successfully described by the sequential first order hydrolysis and degradation reactions. Maximum monosugar yield is obtained when hydrolysing the xylan with 1 % acid for 1 hour, which would correspond to the most accurate estimation of the composition of the xylan.