Enzymatic modification of the functional properties of xylan from lignocellulose feedstocks

by

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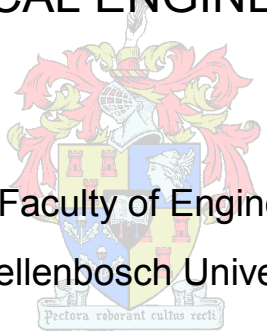

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Abstract

In the past decades, sustainable alternatives to petroleum-derived products have been explored. While fossil derived products are still the main source of energy and chemicals worldwide, they are the major contributors to the increased emission of green house gasses (GHG's), responsible for the climate change. Lignocellulose represents a more sustainable alternative since it is biodegradable, renewable and does not contribute to GHG emissions to the same extent as fossil-based resources. Hemicelluloses are the second most abundant class of polysaccharide biopolymers on earth after cellulose, based on widespread availability in nature of the lignocellulosic plant biomass in which they occur. Xylan represents between 15 and 30% of lignocellulose in hardwood plant species, between 25-35% in grasses and in lower proportions in softwood (between 7 and 12%). In pulp and paper industries, xylan is dissolved and separated from cellulose along with the lignin under harsh pulping conditions, and subsequently burned as black liquor for energy generation. This process represents an under-utilisation of hemicellulose feedstocks, due to its low specific heating value (13.6 MJ/kg) compared with lignin (27 MJ/kg). However, the xylans that can be extracted from lignocellulose, either during pulping or in dedicated processes, have limited applications due to low functionality as a biopolymer, mainly high solubility in water.

The objective of this study was to investigate the enzymatic hydrolysis of xylans, extracted from different lignocellulosic feedstocks available in South Africa and to find optimum conditions for modifying chemical and functional properties of the xylans for industrial applications.

The glucuronoxylan from *Eucalyptus grandis* was extracted using protocols adopted from Höije et al. (Höije *et al.,* 2005) and Pinto et al. (Pinto *et al.,* 2005). The arabinoglucuronoxylan from sugarcane bagasse was extracted following the protocol adopted from Höije et al. (Höije *et al.,* 2005). Beechwood xylan (Sigma) was used as the model xylan. The xylan was extracted from *E. grandis* and sugarcane bagasse using the Höije protocol with yields of 20 and 71% and uronic acid contents of 21 and 7.05%, respectively. The molecular weight distribution showed that the major fraction had a degree of polymerization of 287 in *E. grandis* xylan (Höije). The xylan was extracted from *E. grandis* by the Pinto protocol with a yield of 89%. However, the degree of polymerization was 133 due to polysaccharide degradation. The model xylan from beechwood had the highest purity levels in terms of xylose content but the lowest degree of polymerization, corresponding to 77.

Selective removal of arabinose and 4-*O*-methyl glucuronic acid by the enzymes α-L-arabinofuranosidase and α-D-glucuronidase, respectively, caused formation of water-insoluble xylan particles.

Partially purified α-D-glucuronidase at concentrations between 416 and 462 mg/L were used in the selective enzymatic hydrolysis of 4-*O*-methyl glucuronic acid side-groups in xylans extracted from different lignocellulosic feedstocks. The minimum time required for selective hydrolysis of the glucuronoxylans at concentrations ranging from 1.1 to 5.0% (w/v) in study was 24 hours. Consequently, the minimum degree of substitution required for the precipitation of beechwood xylan, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto) was 1:40, 1:6 and 1:25, respectively. The highest release of 4-*O*-methyl glucuronic acid was found at α-D-glucuronidase dosage of 6.4 mg/g combined with 1.87% (w/v) substrate in beechwood xylan, 3.08% (w/v) in *E. grandis* xylan (Hoije) and 5.03% (w/v) in E. grandis xylan (Pinto). Optimum conditions for increase in viscosity were found at an enzyme dosage of 6.4 mg/g combined with 4.98% (w/v) of substrate in beechwood xylan, 4.3% (w/v) in *E. grandis* xylan (Höije) and 4.5% (w/v) in *E. grandis* xylan (Pinto). The most significant factor for the release of 4-*O*-MeGlcA was the α-D-glucuronidase dosage (p-value < 0.03), whereas the substrate concentration ($p < 0.006$) was the most significant factor to maximise viscosity.

Enzymatic hydrolysis with both α-D-glucuronidase and α-L-arabinofuranosidase for release of 4-*O*-methyl glucuronic acid and arabinose from sugarcane bagasse xylan resulted in precipitation. A positive synergy was verified in the release of both side-chains, which was dependent on the dosage ratio between α-L-arabinofuranosidase and α-D-glucuronidase. Optimum conditions for precipitation were found at a dosage of 6.4 mg/g of α-D-glucuronidase and 150 nkat/g of α-L-arabinofuranosidase, with 25% precipitation. On the other hand, the highest release of side-chains was verified at a dosage of 6.4 mg/g of α-D-glucuronidase and 350 nkat/g of α-L-arabinofuranosidase, with 69.5% release of arabinose and 24% release of 4-*O*-MeGlcA.

Morphological analysis of the modified xylans indicated that enzymatic treatment improved both the gelling and plasticising properties. Removal of 4-*O*-methyl glucuronic acid from glucuronoxylans resulted in increased viscosity, and formation of hydrogels. Arabinose and 4-*O*-methyl glucuronic acid removal from arabinoglucuronoxylan also resulted in formation of insoluble xylan particles that settled with gravity. The highest viscosity rheological properties was observed with *E. grandis* xylan (Höije), followed by the model xylan from beechwood.

Particle agglomeration was more evident on less substituted glucuronoxylans with a minimum degree of substitution of 4-*O*-MeGlcA of 3%. The micro particles were found to increase in size with an increase in the xylan concentration, from a minimum concentration of 4.45% (w/v), during enzymatic hydrolysis. The glucuronoxylan hydrogels and arabinoglucuronoxylan insoluble particles formed at optimum conditions had particles sizes ranging from 0.4 to 1.97 µm. It was concluded that the enzymatic modification is a mild and selective chemical process that can add functionality to beechwood, *E. grandis* and sugarcane bagasse xylans, by reducing their solubility in water and can be further applied as pulp additives, paper coatings, packaging films and gel encapsulation matrices.

Opsomming

Gedurende die laaste paar dekades is ondersoek ingestel na die gebruik van volhoubare alternatiewe vir petroleum gebasseerde produkte. Alhoewel produkte afkomstig van fossielbrandstof steeds die hoof bron van energie en chemikalieë wêreldwyd is, lewer hierdie produkte ook die grootste bydrae tot die verhoogde vrystelling van kweekhuisgasse (KHG'e) wat verantwoordelik is vir klimaatsverandering. Lignosellulose is 'n meer volhoubare alternatief omdat dit biodegradeerbaar en hernubaar is en ook minder bydra tot KHG emissies as fossielbrandstof gebasseerde hulpbronne. Hemisellulose is die tweede volopste klas van polisakkaried biopolimere op aarde na sellulose, weens die wydverspreide voorkoms van lignosellulose plantaardige biomassa waarin dit in die natuur voorkom. Xilaan verteenwoordig tussen 15 en 30% van lignosellulose in hardehout plantspesies, tussen 25 en 35% in grasse en laer proporsies in sagtehout (tussen 7 en 12%). In die pulp en papier industrieë word xilaan opgelos en geskei van sellulose en lignien deur strawwe verpulpingskondisies, en word daarna verbrand as "swart vloeistof" vir energieproduksie. Hierdie proses verteenwoordig 'n nie-optimale gebruik van hemisellulose toevoermateriale weens die lae spesifieke verhittingswaarde (13.6 MJ/kg) daarvan in vergelyking met dié van lignien (27 MJ/kg). Die xilaan wat wel geëkstraheer kan word uit lignosellulose deur verpulping of toegewyde xilaan ekstraksie prosesse, het beperkte toepassings. Die rede hiervoor is die lae funksionaliteit daarvan as biopolimeer weens die hoë oplosbaarheid daarvan in water.

Die doelwit van hierdie studie was om die ensiematiese hidrolise van xilane (geëkstraheer uit verskillende lignosellulose toevoermateriale beskikbaar in Suid-Afrika) te ondersoek. Verder ook om die optimale kondisies te bepaal vir die ensiematiese wysiging van die chemiese en funksionele eienskappe van xilane vir gebruik in industriële toepassings.

Die glukuronoxilaan van *Eucalyptus grandis* is geëkstraheer deur gebruik te maak van metodes aangepas uit Höije *et al*. (2005) en Pinto *et al*. (2005). Die arabinoglukuronoxilaan afkomstig van suikerriet bagasse is geëkstraheer volgens die protokol aangepas uit Höije et al. (2005). Boekenhout xilaan (Sigma) is gebruik as 'n xilaan model. Die xilaan is geëkstraheer uit *E. grandis* en suikerriet bagasse volgens die Höije protokol en het 'n geëkstraheerde xilaan opbrengs van 20 en 71% en 'n uroonsuurinhoud van 21 en 7.05% onderskeidelik gelewer. Die molekulêre gewigsverspreiding het getoon dat die hooffraksie in *E. grandis* xilaan 'n graad van polimerisasie van 287 het (volgens die Höije protokol). Die xilaan is geëkstraheer uit *E. grandis* volgens die Pinto protokol met 'n opbrengs van 89%, maar die graad van polimerisasie was 133 weens

polisakkaried degradasie. Die model xilaan uit boekenhout het die hoogste suiwerheidsvlak gehad in terme van xilose inhoud maar ook die laagste graad van polimerisasie, naamlik 77.

Die selektiewe verwydering van syketting arabinose en 4-*O*-metielglukuroonsuur groepe deur die ensieme α-L-arabinofuranosidase en α-D-glukuronidase respektiewelik, het gelei tot die vorming van water-onoplosbare xilaan partikels.

Gedeeltelik gesuiwerde α-glukuronidase in konsentrasies tussen 416 en 462 mg/L is gebruik vir die selektiewe hidrolise van 4-*O*-metielglukuroonsuur sykettings in xilane geëkstraheer uit verskillende lignosellulose toevoermateriale. Hierdie studie het vasgestel dat 24 uur die minimum tyd is wat benodig word vir die selektiewe hidrolise van die glukuronoxilane by substraatkonsentrasies wat varieer van 1.1 tot 5.0%. Gevolglik was die minimum graad van substitusie vereis vir die presipitasie van boekenhout xilaan, *E. grandis* xilaan (volgens die Höije protokol) en E. grandis xilaan (volgens die Pinto protokol) 1:40, 1:6 en 1:25 onderskeidelik. Die hoogste vrystelling van 4-O-metielglukuroonsuur sykettinggroepe het voorgekom by ‘n ensiemdosering van 6.4 mg/g in kombinasie met 1.87% (w/v) substraat in boekenhout xilaan, 3.08% (w/v) in *E. grandis* xilaan (volgens die Höije protokol) en 5.03% (w/v) in *E. grandis* xilaan (volgens die Pinto protokol). Optimale kondisies vir ‘n toename in viskositeit is verkry by ‘n ensiemdosering van 6.4 mg/g in kombinasie met 4.98% (w/v) substraat in boekenhout xilaan, 4.3% (w/v) in *E. grandis* xilaan (volgens die Höije protokol) en 4.5% (w/v) in *E. grandis* xilaan (volgens die Pinto protokol). Die mees noemenswaardige faktor vir die vrystelling van 4-*O*-metielglukuroonsuur was die α-D-glukuronidase dosering (p-waarde < 0.03), en vir die maksimalisering van die reologiese viskositeitseienskappe was dit die substraatkonsentrasie (p < 0.0006).

Ensiematiese hidrolise met beide α-D-glukuronidase en α-L-arabinofuranosidase vir die vrystelling van 4-*O*-metielglukuroonsuur en arabinose uit suikerriet bagasse xilaan het gelei tot presipitasie. ‘n Positiewe sinergie in die vrystelling van beide sykettings is aangetoon. Hierdie sinergie was afhanklik van die doseringsverhouding van α-L-arabinofuranosidase tot α-D-glukuronidase. Optimale kondisies vir ‘n toename in viskositeit is gevind by ‘n dosering van 6.4 mg/g α-glukuronidase en 150 nkat/g α-arabinofuranosidase met 25% presipitasie. Die hoogste vrystelling van sykettings is waargeneem by ‘n dosering van 6.4 mg/g α-glukuronidase en 350 nkat/g α-arabinofuranosidase , met 69.5% vrystelling van arabinose en 24% vrystelling van 4-*O*-metielglukuroonsuur.

Morfologiese analise van die gewysigde xilane het aangetoon dat ensiematiese behandeling beide die gelvormings- en plastisiseringseienskappe daarvan verbeter het. Die verwydering van 4-*O*-metielglukuroonsuur sykettings vanaf die glukuronoxilane het tot ‘n verhoogde viskositeit

gelei asook die vorming van hidrojels. Arabinose en 4-O-metielglukuroonsuur verwydering uit arabinoglukuronoxilaan het gelei tot die vorming van onoplosbare xilaan partikels wat onder swaartekrag uitgesak het. Die hoogste reologiese viskositeitseienskappe is waargeneem by *E. grandis* xilaan (geëkstraheer volgens Höije), gevolg deur die model xilaan uit boekenhout.

Partikel agglomerasie was meer duidelik by die minder gesubstitueerde glukuronoxilane met ‘n minimum graad van 4-*O*-metielglukuroonsuur substitusie van 3%. Daar is gevind dat die mikro-partikels toegeneem het in grootte met ‘n toename in xilaan konsentrasie, vanaf ‘n minimum konsentrasie van 4.45% (w/v) gedurende ensiematiese hidrolise. Die glukuronoxilaan hidrojels en arabinoglukuronoxilaan onoplosbare partikels gevorm by die optimale kondisies het partikelgroottes gehad wat varieer van 0.4 tot 1.97 µm. Die ensiematiese modifikasie van xilaan is dus ‘n matige en selektiewe proses wat die funksionaliteit van boekenhout, *E. grandis* en suikerriet bagasse xilane kan verbeter deur hul oplosbaarheid in water te verlaag. Sodoende kan hierdie gemodifiseerde xilane verder toegepas word as verpulpingstoevoegings, papierbedekkings, verpakkingsfilms en jel inkapselingsmatrikse.

Dedication

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Table of Contents

List of Tables

List of Figures

Abbreviations and symbols

Agu: α-D-glucuronidase
Ara: α-L-arabinofuranosidase
ATR: attenuated transmittance reflectance
BSA: bovine serum albumine
DMSO: dimethyl sulfoxide
DP: degree of polymerization
DS: degree of substitution
DO: dissolved oxygen
DW: dry weight
ELS: evaporative light scattering
FTIR: Fourier transmission infra red
GHG: green house gasses
GH: glycoside hydrolyse
GRAS: generally regarded as safe
HPAEC-PAD: high performance anion exchange chromatography coupled with pulse amperometric detector
kDa: kilo Dalton
K_M: Michaelis-Menten constant
LCC: lignin carbohydrate complex
MA: maleic acid
4-*O*-MeGlcA: 4-*O*-methyl glucuronic acid
Mw: molecular weight
nkat: nano katal
p-NP: *para*-nitrophenol
p-NPA: *para*-nitrophenyl α-L-arabinofuranoside
PVA: polyvinyl alcohol
SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC: size exclusion chromatography
TAPPI: technical association of the pulp and paper industry
V_{max}: maximum reaction rate
YNB: yeast nitrogen base

CHAPTER 1: Introduction

Fossil derived products have been the most important source of fuels, chemicals, plastics and other valuable products. However, these are subjected to oil price fluctuations and are associated with the increase in the green house gasses (GHG) emissions (Cherubini, 2010). Due to their inherent thermochemical stability, hydrocarbons, aromatic compounds and plastic derived products manufactured using fossil-based raw materials are not biodegradable. They are converted to CO_2 from their waste incineration (Patel *et al.,* 2005). As a result, research towards finding more environmentally friendly raw materials for the production of fuels, chemicals and materials is in progress to minimize the global effects of the climate change, by curbing excessive reliance on fossil-based resources. Plant biomass represents the only renewable source of carbon available on earth, of which lignocellulosic biomass is the most abundant component. Lignocellulose can be a sustainable alternative for production of biofuels, biopolymers, carbon based nano- and micromaterials and other derivatives. In the process of lignocellulosic biomass conversion, care must be taken for the feedstock selection to ensure that sustainability criteria are met and this has been a topic of debate (Melamu and Von Blottnitz, 2011).

Lignocellulosic plant biomass is made up of cellulose, hemicellulose and lignin, tightly interconnected by covalent hydrogen bonds within the secondary wall of annual plants (Menon and Rao, 2012). Industrial examples of lignocellulose fractionation are primarily pulping processes, where lignocellulose is utilised inefficiently for the production of one product (pulp), while the remaining hemicellulose and lignin components are degraded and burnt (Vena *et al.,* 2009). Cellulose is a linear polymer composed of repeated α-1,4-linked glucose units forming a crystalline structure with wide applications in the pulp and paper industry and in pharmacy. Hemicellulose is a branched heterogenous class of polymers that serve as connectors between cellulose and lignin in the plant structures. Lignin is the most complex aromatic polymer composed of different cross-linked benzyl units (Timell, 1967).

Xylan is the most abundant hemicellulose, representing between 15 and 30% (dry weight basis) in hardwoods and in softwood species and cereals in lower proportions, between 7 and 12% (Timell, 1967b). Despite of its large abundance, the suitability of xylan as a speciality (bio) polymer is limited, in comparison with cellulose, due to a higher degree of substitution (DS) and a lower degree of polymerization (DP), causing it to be soluble upon dispersion in water. Commonly, the xylopyranosyl backbone is substituted by α-L-arabinofuranosyl, acetyl, feruloyl

and 4-O-methyl-α-D-glucuronic units (Ebringerová, 2005; Girio *et al.,* 2010). The solubility of xylan depends on the degree of polymerization (DP), degree of substitution (DS) and frequency (pattern) of substitution (LeBel and Goring, 1963). Selective removal of the side-chains present in the xylan backbone structure has been shown to cause visual precipitation of polymeric xylan in water (Kačuráková *et al.,* 1994; Chimphango, 2010), which will significantly improve its suitability for biopolymer applications. Such applications include as xylan packaging films, pulp additives and hydrogels (Deutschmann and Dekker, 2012). In contrast with physical and chemical methods for such side-chain removal, the enzymatic hydrolysis generates less toxic waste and is able to selectively remove the side chains without degrading the polysaccharide backbone. Consequently, there is potential to produce biodegradable polymers with small particle size, that are self-assembled and can be utilized as coatings, nanofibres and drug delivery hydrogels (Gupta *et al.,* 2002).

α-D-glucuronidase and α-L-arabinofuranosidase are accessory enzymes responsible for the hydrolysis of 4-O-methyl-α-D-glucuronic/glucuronic acid and arabinofuranosyl units, present as side-chains in the xylan backbone, respectively (Shallom and Shoham, 2003). The α-L-arabinofuranosidase has been previously produced from *Aspergillus niger* strains by expressing the *abfB* gene in *A. niger* D15 strain with an enzyme yield of 366.30 nkat g^{-1} (DW) (Chimphango, 2010). The α-L-arabinofuranosidase catalysed the removal of arabinose side-chains and decreased the solubility of the xylans extracted from softwood and grasses, with a maximal removal of 43% of the side chains present in commercial oat spelt xylan (Chimphango, 2010).

The application of α-D-glucuronidase for selective removal of 4-*O*-methyl glucuronic acid in xylans from hardwood or softwood sources has not been fully investigated. To date, the only α-D-glucuronidase capable of liberating 4-*O*-MeGlcA units from hardwood xylan was reported from the fungus *Schizophyllum commune* (Tenkanen & Siika-aho 2000; Ryabova *et al.,* 2009). In addition, it is commonly secreted at low concentrations with poor catalytic activity in glucuronoxylans. Therefore, it is necessary to develop a recombinant microbial system to selectively produce α-D-glucuronidase at higher levels of concentration and optimise the enzymatic hydrolysis process. The yeast *Saccharomyces cerevisiae* has been commonly utilized as a host organism for expression of recombinant enzymes (Mendoza-Vega *et al.,* 1994; Chambers *et al.,* 1995).

Eucalyptus grandis is the most used hardwood type of lignocellulose used in the South African paper and pulp industries. About 73.8% of the total commercial forestry area is comprised of *E. grandis* and its hybrids as the major source of cellulose in the papermaking industry (Snedden, 2001).

Sugarcane bagasse is the agricultural crop residue from sugarcane refineries; about 1 ton of sugarcane generates 280 kg of bagasse with approximately half of this amount used as source of energy, while the rest is stockpiled (Baudel *et al.,* 2005; Cardona *et al.,* 2010). However, sugarcane bagasse is presently used for pulp production, mainly using alkaline sulphite-antraquinone processes (Khristova *et al.,* 2006). Both are rich sources of hemicelluloses varying between 15 and 30% depending on the region and climate conditions. Despite the numerous efforts, existing pulping processes with *E. grandis* and sugarcane bagasse do not utilise the hemicellulose (xylan) in the feedstock for the production of valuable products, but instead degrade the hemicellulose during solubilisation/pulping, followed by burning of the hemicellulose-rich black liquor (Gabrielii *et al.,* 2000).

Many fractionation methods have been investigated for the hemicellulose extraction and recovery from lignocellulosic feedstocks, as a means to add value to this under-utilised biomaterial. However few studies are related to the modification of xylan functional properties, adding significant value as a bio-polymer, compared to the water-soluble xylans obtained from the extraction methods typically employed (Ebringerová *et al.,* 1994, 1997; Sun *et al.,* 2002; Gadhe *et al.,* 2006; Petzold *et al.,* 2006; Haimer *et al.,* 2008; Chimphango *et al.,* 2012; Postma, 2012).

1.1. Study motivation

An efficient and mild method for reduction of xylan solubility would open doors for further industrial applications of xylans from lignocellulosic biomass. Green materials obtained from cheap sources such as lignocellulose would benefit the economy of many industrial sectors especially in food packaging and pharmacy. Functionalized xylan can be incorporated as additives in pulp fibres and hydrogels for slow release of drugs (Lindblad *et al.,* 2001). Potential uses of insoluble xylan include: as viscosity modifiers, gelling agents, tablet binders for production of encapsulation hydrogels, packaging films and porous foams (cross-linking) (Ren *et al.,* 2007). The incorporation of xylan as a material for developing slow drug release matrices has been fairly well investigated (Pal *et al.,* 2009; Chimphango *et al.,* 2010). It has been found that xylan isolated from Japanese beechwood inhibits the growth rate of tumours (Ebringerová and Heinze, 2000) and has anti-cancer properties (Ando *et al.,* 2004). Other applications include as a precursor for the production of ethanol, butanol, acetone and artificial sweeteners (xylitol) via fermentation. Currently, xylan residues only contribute to between 5 and 20% in the bio-ethanol production (Polizeli et al., 2005), with little use in the pharmaceutical and paper industry.

1.2. Aim and objectives

The aim of this study is to modify the functional properties of xylans extracted from different lignocellulose feedstocks by enzymatic hydrolysis treatment. This is performed by firstly extracting the xylans from *Eucalyptus grandis* and sugarcane bagasse feedstocks and modifying their solubility properties in water, by enzymatic hydrolysis by α-D-glucuronidase and α-L-arabinofuranosidase. As a result, the objectives of this research are as follows:

- To evaluate two different extraction protocols from literature and how they affect the physical and chemical structure of extracted xylans; evaluate their suitability to be applied prior to xylan enzymatic hydrolysis.
- To characterize α-D-glucuronidase and α-L-arabinofuranosidase, produced by recombinant strains of micro-organisms and evaluate catalytic activity against different substrates.
- To study reaction and precipitation kinetics of α-D-glucuronidase hydrolysis on selected xylans from local feedstocks as well as the effect of xylan structural and chemical characteristics on the release of 4-O-MeGlcA and precipitation behaviour.
- To identify optimum conditions of xylan concentrations, α-D-glucuronidase and α-L-arabinofuranosidase dosages required for maximum side-chain release and precipitation of xylans in water solutions.

CHAPTER 2: Literature Review

2.1. Hemicelluloses

Hemicelluloses are a large group of homo and heterogenous polymers that consist mainly of β-1,4-xylopyranose, glucopyranose, mannopyranose and galactopyranose as main (backbone) units attached with different side chains (Timmel, 1967; Ren and Sun, 2010). Hemicelluloses occur along with cellulose and lignin in lignocellulose, intimately associated by covalent bonds and van der Waals interactions. However, the composition of hemicellulose may vary from one species to the other, and within a single plant depending on the plant age, growth stage or conditions at which it grows (Jeffries, 1994). The different types of hemicelluloses and distribution among plant species are illustrated as follows:

Table 2. 1: Types of hemicellulose and distribution in plant species (Girio *et al.,* 2010)

Polysaccharide type	Main occurrence	Percentage (%) of total dry weight of biomass	Backbone	Side chains	Degree of polymerization
Glucuronoxylan	Hardwoods	15-30	β-D-xylp	4-O-methyl-α-D-Glcp; Acetyl	200
Arabinoglucuronoxylan	Agricultural crops; softwoods	7-12	β-D-xylp	4-O-methyl-α-D-Glcp; β-L-Araf	100
Arabinogalactans	Softwoods	1-3; 35[1]	β-D-Galp	β-D-Galp; β-L-Araf; β-D-Glcp; β-L-Arap	200
Glucomannans	Hardwoods	2-5	B-D-Glcp; β-D-Manp	-	200
Galactoglucomannans	Softwoods Hardwoods	20-25 Less than 5	β-D-Manp; β-D-Glcp	β-D-Galp; acetyl	100

Note: xylp-xylopyranose, Galp-galactopyranose, Glcp-glucopyranose, Manp-mannopyranose, Araf-arabinofuranose, Arap-arabinopyranose

[1] Larchwood

In angiosperms (hardwood), glucuronoxylan is the major hemicellulose, comprising between 15 and 30% (dry weight). It is partially acetylated and substituted with 4-*O*-methyl-α-D-glucuronic acid residues. Gymnosperms (softwood) are mainly composed of acetylated galactoglucomannan units in up to 25% (Timell, 1967b), while the largest portion (60-70%) of the hemicellulose in grasses (graminaceaus plants) is comprised of arabinoxylans (Izydorczyk and Biliaderis, 1995). Galactoglucomannans consist of a backbone of β-1,4-glucopyranose and β-1,4-mannopyranose units, substituted with α-D-galactopyranosyl units by α-(1,6) linkages. In general, hemicelluloses have a much lower molecular weight than cellulose. The degree of polymerization (DP) of hemicelluloses ranges between 100 and 200, while for cellulose between 9,000-10,000 or even 15,000 (Goring & Timell, 1962). In plants, the secondary cell wall is composed of hemicelluloses and lignin, which cover the cellulose microfibrils, thus providing additional strength to the fibres (Guerra *et al.,* 2007). The acetyl ester groups are bound to the backbone in up to 2%. Also, ferulic and *p*-coumaric acids are found in several plant species (Lu & Ralph, 2010). In contrast with cellulose, hemicelluloses are soluble in alkali and can be easily hydrolysed by acids (Pérez *et al.,* 2002).

2.1.1. Xylan structure and supply

Xylans have a linear backbone that consists of α-1,4-xylopyranosyl units, substituted with side-chains such as acetyl, arabinofuranosyl and 4-*O*-methyl glucuronic/glucuronic acid residues, depending on the type of lignocellulose and method of extraction (Spiridon and Popa, 2008). Glucuronoxylan represents more than 90% of the hemicelluloses fraction in hardwood species and it is substituted at irregular intervals by α-1,2 linked 4-*O*-methyl-α-D-glucuronic and acetyl groups, with 4-*O*-MeGlcA and acetyl to xylose average ratios of 1:7:10 (Teleman *et al.,* 2002). Acetyl groups are substituted at positions 2, 3 or both in a ratio of approximately 24:22:10, respectively (Figure 2. 1). Glucuronoxylan has also been isolated from non wood species such as the seed pericarp of *Opuntia ficus* pear, date seeds, grape skin and hulls of Jojoba beans (Ebringerová *et al.,* 2005). After isolation, the degree of acetylation varies considerably depending on the extraction methods employed.

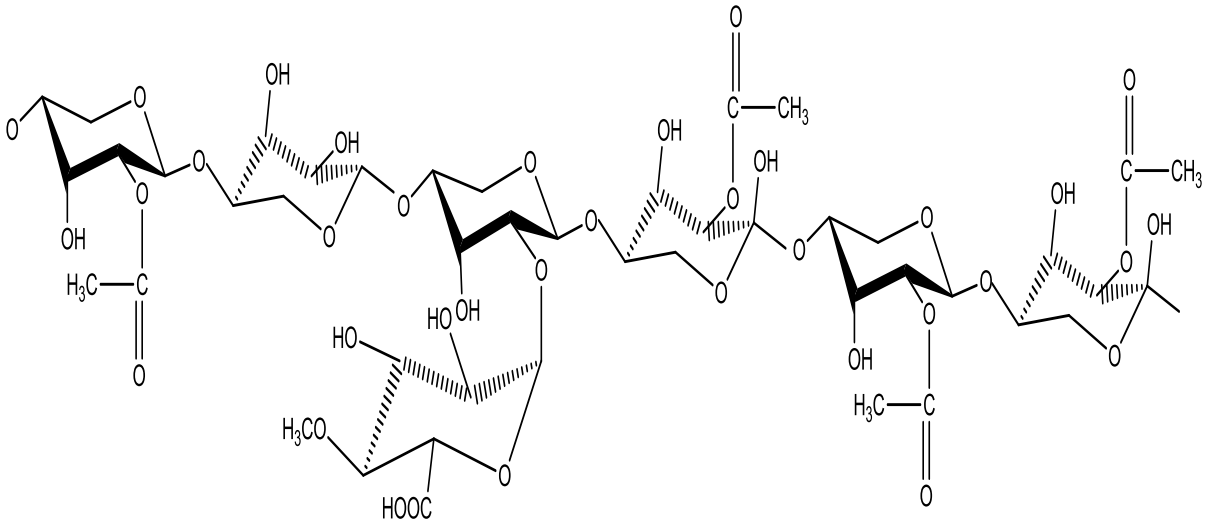


Figure 2. 1: Structure of glucuronoxylan: 4-*O*-methyl-α-D-glucuronic acid attached to β-1,4-xylopyranosyl units by α-1,2-linkages (re-drawn from Spiridon and Popa, 2008)

Arabinoxylans form part of the endosperm and outer layers of lignocellulose in cereals and are present in grasses such as bamboo, rye grass and pangola grass (Izydorczyk and Biliaderis, 1995). Arabinoxylans are substituted by α-L-arabinofuranosyl units at positions 2, 3 or both of the same monomer unit. In addition, they also contain small amounts of D-glucuronic acid or 4-*O*-methyl glucuronic acid residues (Izydorczyk and Dexter, 2008). The distribution of arabinose side-chains defines the solubility in water or alkali solvents (Sedlmeyer, 2011). For example, water insoluble xylans have arabinose to xylose ratios in the range of 1:3-5, while water-soluble arabinoxylans isolated from rye and wheat flour between 1:1.1-2 (Izydorczyk and Biliaderis, 1995).

Arabinoglucuronoxylans have 4-*O*-methyl glucuronic acids side chains in addition to the 1,3-linked α-L-arabinofuranosyl units (Figure 2. 2). They were found to have arabinose and 4-*O*-MeGlcA to xylose molar ratios of approximately 1.3:2:10. Therefore, arabinoglucuronoxylans contain higher quantities of 4-*O*-methyl glucuronic acid than arabinoxylans, but less arabinose residues. The arabinofuranosyl residues can be partially esterified at position 5 by hydroxy cinnamic acids (ferulic and *p*-coumaric acids) (Wende and Fry, 1997; Spiridon and Popa, 2008). The presence of arabinofuranosyl residues linked to C (*O*)-2 was verified in beeswing bran of wheat kernel, wheat endosperm, barley endosperm, corn cob heteroxylan and rice endosperm and bran (Fincher & Stone, 1981). They are prevalent in coniferous species and grasses (Ren and Sun, 2010).

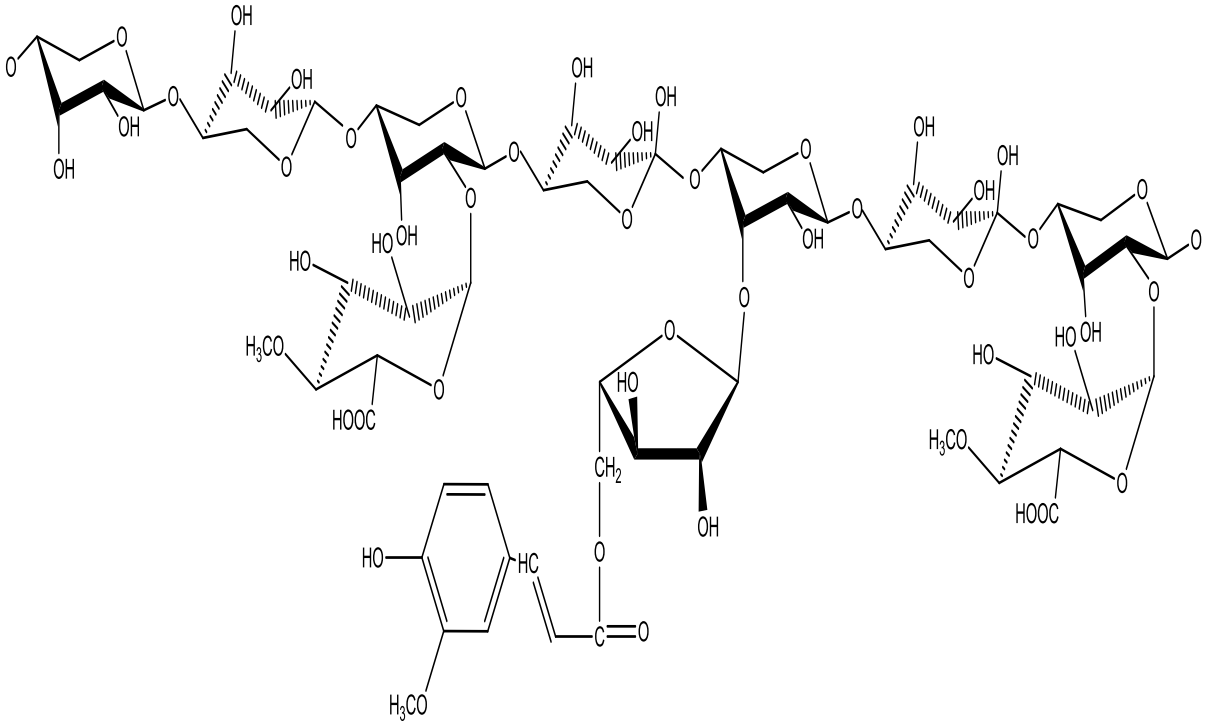


Figure 2. 2: Structure of arabinoglucuronoxylan: more heavily substituted by 4-*O*-MeGluA/glucuronic side chains with α-(1,3)-linked α-L-arabinofuranosyl units (re-drawn from Spiridon and Popa, 2008)

The hydroxyl groups and arabinofuranosyl residues attached to the xylan backbone form ester and ether linkages respectively with lignin and other wood components (Gellerstedt and Henriksson, 2008), conferring resistance to microbial attack (Jefrries, 1994). Therefore, adequate isolation of hemicelluloses is only possible when these intermolecular interactions are overcome by the ones with the extraction solvents. *Eucalyptus grandis* is a hardwood type of lignocellulosic feedstock, having glucuronoxylan as the main type of hemicellulose (Magaton et al., 2011). Sugarcane bagasse (*Saccharum officinarum L*) is the crop residue left after extracting the juice from the sugarcane for sugar production (Cardona *et al.,* 2010). Arabinoglucuronoxylan represents the major hemicellulose found in sugarcane bagasse, with residual amounts of other types of hemicelluloses (Table 2.1). For the present study, the glucuronoxylan from beechwood (*Fagus sylvatica*) was used as the model xylan, due to superior purity levels achieved after extraction (Sigma Aldrich).

2.1.2. Degree of polymerization (DP) and degree of substitution (DS)

Xylans have a degree of polymerization ranging from 100 to 300, while in cellulose the DP ranges from 10,000 in native woods to 1,000 in bleached kraft pulps (Sjöström, 1993). Previous studies showed that the solution properties of glucuronoxylans were directly associated to the degree and pattern of substitution, though molecular weight also plays an important role (Linder *et al.,* 2003). The interaction between xylan polymeric chains leads to precipitation when there are large regions of linear polymeric chains (in the absence of side-group substitutions) by a mechanism resulting in interactions through hydrogen bonds and hydrophobic constituents, such as lignin (Westbye *et al.,* 2007). Arabinoxylan can precipitate with formation of cohesive semi-crystalline films upon drying with a degree of substitution ranging from 0.30 to 0.50. Glucuronoxylans require at least 15 consecutive units of xylose per unit of 4-*O*-methyl glucuronic acid to cause precipitation. In glucuronoxylans from *E. grandis* the glucuronic acid side-chains are substituted in a blockwise mode causing them to precipitate even with a higher degree of substitution (Kabel *et al.,* 2007; Chimphango, 2010). In non-acetylated glucuronoxylan water solutions, the backbone parts without 4-*O*-methyl glucuronic acid substituents interact with each other forming insoluble aggregates, resulting in precipitation in solution (Kabel *et al.,* 2007). In addition, previous studies have shown that xylans with a lower degree of acetylation and 4-*O*-methyl glucuronic acid substituents tend to adsorb better in cellulosic materials, as compared with the highly substituted ones.

2.1.3. Interactions between lignocellulose components

The structural features of xylan may differ considerably when it is isolated or associated with other wood components such as lignin in lignocellulose. Carbohydrates and lignin are bound together forming lignin-carbohydrate complexes (LCC's) (Wallace *et al.,* 1991; Jeffries, 1994; Lu & Ralph, 2010). The nature of these covalent bonds depends of the type and source of the hemicelluloses. It has been proposed that in hardwood and grass hemicelluloses, the 4-*O*-methyl-α-D-glucuronoxylan and the arabinoglucuronoxylan form ester bonds of 4-*O*-methyl glucuronic acid with lignin from the substituents trans-*p*-coumaric and *p*-hydroxybenzoic acids. Also, radical cross-coupling reactions between the neutral sugars in carbohydrates and lignin produce ether bonds at the α-carbon of the guaiacyl or syringyl-glycerol units of lignin (Wallace *et al.,* 1991; Koshijima & Takashi, 2003).

Softwoods are differently linked to lignin since they consist of mixed portions of galactoglucomannan, arabinoglucuronoxylan and arabinogalactan. Consequently, the binding site occurs at lignin benzyl positions (Azuma and Tetsuo, 1988). In addition, hemicellulose chains form cross-linkages between the phenolic substituents (hydroxy-cinnamic acids) forming di-ferulic acids.

Other substances such as pectin also contribute to the binding of lignin to the hemicellulose. The covalent bonds from LCCs may cause resistance in delignification processes, leading to the presence of residual lignin in the isolated hemicellulose fraction (Azuma *et al.,* 1981; Höije *et al.,* 2005). In addition, they have an inhibitory effect for enzymatic attack, due to steric hindrances or multiple cross-links (Jeffries, 1990). It was found that approximately 40% of the uronic acid content in birchwood is esterified and in beechwood, up to one third is directly involved in lignin-carbohydrate linkages, that is, one glucuronic acid per 23 xylose units forms an ester linkage with lignin (Takahishi & Koshijima, 1988b). However, in grasses and cereals, LCC's occur in lower frequency and can be more easily disrupted by the acid or alkali treatment submitted during the hemicellulose isolation process (Jeffries, 1994; Peng *et al.,* 2012).

2.1.4. Xylan isolation

The degree of polymerization and the type and frequency of substitution of extracted xylans depend on the source and the isolation method (Timell, 1967; Fincher & Stone, 1981). In general, hemicelluloses are associated with cellulose in the secondary wall of plants, but they can also occur in the primary wall (Jeffries, 1994). During lignocellulose fractionation processes, the hemicelluloses are separated from other wood components based on chemical solubility properties in alkali, alcohol and acids. In conventional kraft pulping, xylan can be partially or completely degraded (Gabrielii *et al.,* 2000), which affects the quality of the cellulose fibres since the hemicelluloses fraction is greatly reduced, forcing the use of other reinforcement additives, with significant financial losses to the pulp and paper industry.

The isolation of xylan from the lignocellulose matrix is difficult since it is influenced by the side chains substitution pattern, physical entanglement, covalent bonding between the carboxyl groups of uronic acids and hydroxyl groups of the xylan backbone (Mares & Stone, 1973; Geissmann & Neikom, 1973; Fincher & Stone, 1986, Gruppen *et al.,* 1992). Previous studies have shown that a small fraction of hemicellulose is soluble in water and the remaining in alkali (Höije *et al.,* 2005). The challenge remains in developing an isolation method able to extract xylan at high yields and purity levels. The methods for xylan extraction from lignocellulose biomass differ depending on the pH range and reagents such as ammonia, sulphur, alkali, acids or oxygen. Different protocols are described elsewhere in literature (Sun and Cheng, 2002; Höije

et al., 2005; Pinto *et al.,* 2005; Kenealy *et al.,* 2007; Al-Dajani and Tschirner, 2010; Liu et al. 2011). Various multi-step extraction procedures have been studied for xylan extraction. Generally three steps precede most of the isolation methods of xylan in hardwoods and annual plants, which are as depicted in Figure 2. 3.

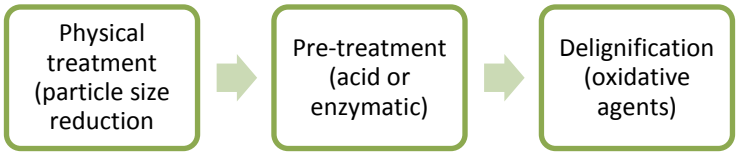


Figure 2. 3: Steps involved before performing the actual extraction of hemicelluloses from lignocellulose

Intensive milling (particle size reduction) is crucial for elimination of the structural integrity of lignocellulose and to obtain an average feedstock particle size structure. The pre-treatment prepares the biomass by removing water soluble proteins, starch and fats. It is necessary to first separate xylan from other components to avoid interferences and maximize the yield of extraction. For example, the treatment of wheat flour with 80% ethanol, before extraction with water, resulted in isolating water soluble pentosans with 2% (w/w) protein (Fincher & Stone, 1974). Other pre-treatment methods involve mild acid hydrolysis and enzymatic treatment with amylase or proteases. In Höije et al. (2005), barley husks were submitted to HCl, HCl with NH_4OH, HCl with acetic acid and enzymatic pre-treatments. The pre-treatment using HCl with acetic acid resulted in a hemicellulose extract with a final protein content of 3%, compared to 13% when only HCl is used. The enzyme treatment was unable to remove the proteins, having the highest protein content in the extracted samples (Höije *et al.,* 2005).

Most delignification methods are unable to remove all lignin; the presence of residual lignin in the extracted xylans is therefore expected. The lignin content of hardwood species is lower than in softwood species, thereby enhancing the extractability of xylan. Nevertheless, the lignin content is lower in most grass species, compared to woody biomass (Azuma and Tetsuo, 1988). The most common industrial methods for pulp delignification are kraft pulping, sulphite pulping and steam explosion. The kraft lignin is obtained with $NaOH/Na_2S$ treatment at high alkaline charge (16-20%) and temperatures between 130 and 175°C, removing between 90-95% of the lignin (Pinto *et al.,* 2005). The sulphite lignin is obtained by introducing sulphonate groups in lignin C-α position, cleaving the LCC linkages via intermediate formation of carbocations. The steam explosion as the name indicates, uses steam at high temperature followed by decreased

pressure, causing the covalent bonds in lignin to be disrupted (Gellerstedt and Henriksson, 2008). The delignification methods described, are not selective and use harsh conditions causing end-wise peeling reactions (Sjöström, 1993). In addition, the structural characteristics and the purity of the xylan recovered from kraft pulping may not suit enzymatic hydrolysis processes (Chimphango, 2010).

In hardwoods, chlorite delignification has been used extensively as a pre-treatment before alkaline extraction. A less hazardous method using H_2O_2/NaOH has been reported, to yield xylan rich polysaccharide fractions contaminated to various extents with lignin and degradation components of the cell walls (Ebringerová and Heinze, 2000). Delignification procedures were investigated for biomass samples pre-treated with HCl/NH_4OH and HCl/acetic acid with ethanol and sodium chlorite, respectively. The products of the isolation were obtained at yields above 50%, with the highest at 57% using chlorite in the delignification, since it was the most selective delignification method (Höije *et al.,* 2005). The arabinoxylan content was of 83% in the product composition. Similarly, Brienzo *et al.,* applied 6% H_2O_2 obtaining a xylan yield of 86% from sugarcane bagasse and lignin content of 5.9% (Brienzo *et al.,* 2009). Different xylan isolation methods have been employed and include:

- Auto-hydrolysis (hot water extraction): a self-catalytic process, resulting in the hydrolysis of the polymeric side-chains and the backbone, producing xylan-derived oligosaccharides and monomers. The advantages are that no chemicals are involved, with less equipment corrosion and reagents recycling (Mosier *et al.,* 2005). However it is not a selective method for isolation of hemicelluloses due to unwanted side reactions at elevated temperatures and only a small fraction of the hemicelluloses are soluble in water (Al-Dajani and Tschirner, 2010).
- Mild acid extraction is preferred to more severe conditions since these lead to unwanted side reactions, causing random chain cleaving in the hemicelluloses and celluloses which yields a product with a lower DP and DS (Al-Dajani and Tschirner, 2010). Liu *et al.* performed an acid pre-treatment for extraction of hardwood hemicelluloses with 20% H_2SO_4 at a pH of 2 at room temperature, followed by ethanol precipitation. This process had the advantage of precipitating most of the lignin during the acid treatment. The resultant hemicelluloses were present in both monomeric and oligomeric forms (1.13%), lignin (0.76%), furfural (0.17%), acetic acid (0.80%) and ash (0.43%). The presence of furfural and other contaminants can cause enzyme deactivation. Significant losses of hemicelluloses due to LCC interactions were also found in this method (Liu *et al.,* 2011). It has been found that the pre-extraction of wood chips with alkaline media gave lower yields of hemicelluloses, but improved the subsequent processes with a

reduced reaction time (10 min) and lower lignin content (Al-Dajani and Tschirner, 2010).

- Mild alkali extraction is preferred to strong alkali conditions (alkali charge > 16%) and high temperatures (150-170°C), since the later conditions will increase hemicellulose degradation due to elimination of terminal xylose reducing groups. The hemicellulose degradation increases the consumption of pulping chemicals and decreases the quality of pulp properties (Sjöström, 1993).

High yields of xylan can be obtained for low hydrolysis temperatures and short times when using mild alkaline conditions (Ebringerová, 2005). Mild alkali extraction of hardwood glucuronoxylan was investigated in Eucalyptus species and birchwood (Pinto *et al.,* 2005). These involved delignification with peracetic acid at 10% and 16% and the extraction using DMSO (30%-60% yield) or 10% KOH aqueous solution with yields above 50%. The extraction with DMSO preserves the original structure of the polysaccharides (Timell, 1967; Sjöström, 1993), since acetyl groups remain attached to the xylan backbone. Nevertheless, deacetylation reduces xylan solubility in water and increases the susceptibility of the polymer to be attacked by enzymes such as α-D-glucuronidase or α-L-arabinofuranosidase (Jeffries, 1994). Moreover, the Höije method was able to extract arabinoxylans when barley husks were treated with 1 M NaOH solution containing 0.5% of sodium borohydride. In addition, it was also applied for other types of xylans extracted from *Eucalyptus grandis*, sugarcane bagasse, Bamboo and *Pinus patula* with extraction efficiencies of 35%, 65%, 20% and 70% respectively (Chimphango, 2010). The monomeric sugar profiles, molecular weight and structural features suited enzymatic applications involving α-L-arabinofuranosidase and α-D-glucuronidase. Therefore, the mild alkali protocols by Höije *et al.* (2005) and Pinto *et al.* (2005) could be employed in this study for xylan extraction from *E. grandis* and sugarcane bagasse.

2.1.5.1. Isolated xylan properties

In practice, extracted xylans are not pure but a mixture of different wood biomass sugars where the major component is xylose in the polymeric form. The chemical composition of xylans extracted from *E. grandis* and sugarcane bagasse using mild alkali with ultrapurification and ethanol fractionation was determined by many authors (Peng *et al.,* 2009; Peng *et al.,* 2009b; Alves *et al.,* 2010; Magaton *et al.,* 2011; Postma, 2011; Chimphango *et al.,* 2012). Xylose contents have been reported between 40 and 85% for xylan extracted from *E. grandis* and from 60 to 97% for sugarcane bagasse, depending on the extraction protocol. Despite of the high xylose content, the degree of polymerization (DP) is an important factor to consider in xylan modification for biopolymer applications. The minimum DP required for formation of enzymatic

modification of soluble xylan was found to be 10 (Chimphango, 2010). The mild alkali extraction of hemicelluloses described by Höije et al. (2005) produced xylans with a major polymeric fraction with molecular weights between 35,000 to 45,000 Da (Höije *et al.,* 2005). Consequently, the degrees of polymerization obtained were above the minimum value required for enzymatic hydrolysis of xylans (Chimphango, 2010).

The degree of substitution has a remarkable influence on xylan solubility properties and adsorption mechanism in celullosic and non-cellulosic materials. Magaton *et al.* (2011) reported on extracted xylan from different *Eucalyptus* species and their pulp retention behaviour depending on the degree of 4-*O*-MeGlcA substitution. A lower uronic acid content is more favourable to the xylan adsorption process into the pulp fibres improving tensile strength and yields. Isolated xylan has been studied for drug delivery and encapsulation systems due to its ability to resist the enzymatic digestion in the upper gastric tract (Deutschmann and Dekker, 2012.). A study in xylan coated magnetic microparticles for magnetic resonance imaging (MRI) has shown that xylan-magnetite composites were far more resistant to upper digestive tract digestion than the original magnetite particles (Silva *et al.,* 2007). Therefore, the selective removal of glucuronic acid and arabinose side-chains in xylan as means for hydrogel formation is a promising tool to functionalise xylan biopolymers for various applications in the biomedical field and pulping processes.

2.2. Modification of the functional properties of extracted xylans

In general, the three-dimensional structure and the functional groups of biopolymers determine their interactions with neighbouring molecules (Matveev *et al.,* 2000). The modification of the functional properties of xylan may therefore be achieved by structural changes at molecular level. Xylan is substituted with acidic (4-*O*-methyl glucuronic acid) and neutral side-chains (arabinose) which limit intermolecular interactions between xylan polymeric chains, increasing its solubility in water due to hydrogen bonds (-NH_2, -OH, -COOH). As the hemicelluloses are the second most abundant class of bio-polymers in nature with xylan as the major component in hardwood species, it becomes important to optimise the associated fractionation process and improve its functional properties, which can in turn be converted to high valuable products. A number of physical and chemical methods have been studied to alter the nature and the degree of substitution of xylans which consequently affect solubility properties.

2.2.1. Physical methods

Ultrasonic treatment has been applied in both xylan extraction and modification of its functional and structural properties. By applying ultrasound, unstable radicals are formed by homolytical cleavage. One of the major applications consisted in reducing the polysaccharides molecular weight without loss of biological activity, by de-polymerization of UV-absorbing molecules (Ebringerová *et al.,* 1997). High-frequency ultrasound has also been applied for surface modification of lignocellulose which in turn is able to exhibit different surface properties, such as the surface free energy (Gadhe *et al.,* 2006). In addition, the influence of ultrasound radiation was investigated in pulp fibres at 23 kHz, reporting an increase in carbonyl groups and porosity due to partial oxidation. Hemicellulose extraction from wheat straw with ultra-sound, showed that applying sonication at increased periods of time resulted in higher yields of extraction, although with a reduced molecular weight (Sun *et al.,* 2002). Therefore, sonication treatments do not decrease xylan solubility in water.

2.2.2. Chemical methods

Different chemical methods have been applied for either removal of the side-chains or introduction of new hydrophobic functional groups by etherification, esterification, cross-linking reactions, among others (Ren and Sun, 2010).

Precipitation of hemicelluloses was achieved with DMSO/water mixtures using carbon dioxide as anti-solvent under super-critical conditions (Haimer *et al.,* 2010). The resulting particle size varied from less than 0.1 to more than 5 μm (spherical microgels) depending on the pressure and temperature conditions. However, agglomeration of solute molecules with subsequent precipitation of polymer particles occurred without altering the substitution pattern of xylan (Haimer *et al.,* 2010). It is believed that changing the substitution pattern in the xylan backbone can induce self-precipitation of the polymers (Haimer *et al.,* 2010).

The modification of xylan to generate hydrogels has been performed by derivatisation with maleic acid (MA) followed by polyvinyl alcohol (PVA) blending (Tanodekaew *et al.,* 2006). The blends of xylan-MA with PVA were non-toxic and showed potential to be studied for biomedical applications (Tanodekaew *et al.,* 2006).

The carboxymethylation of hemicelluloses is another alternative to reduce solubility of xylan in water by introducing non-polar side chains to the polymer backbone (Ren *et al.,* 2009). Though it proves to be a useful way of introducing anionic functions, it requires a two-step reaction of alkaline activation to obtain a high degree of substitution. This in turn causes considerable degradation and reduction in the molecular weight up to 45.9% (Ren *et al.,* 2009).

The solubilization of xylan was previously performed by quaternization of heteroxylan polysaccharides with 3-chloro-2-hydroxypropyltrimethylammonium chloride (CHMAC) in mild alkali (Ebringerová *et al.,* 1994). Other studies involved the quaternization of polysaccharides in red micro-algae cells in order to broaden their uses (Geresh *et al.,* 2000). The synthesis of cationic xylans was investigated using 2,3-epoxypropyltrimethylammonium chloride (EPTA) in aqueous NaOH, obtaining different degrees of substitution depending on the NaOH/xylan ratio. The turbidity of 1% cationic xylan in water was determined (Petzold *et al.,* 2006) and was found to have a nephelometric turbidity unit of a 100 (absence of precipitation).

Chemical treatments represent an alternative to modify the functional properties of native xylans; for example, cationic and carboxymethyl xylans can significantly improve the physical properties of handsheets (Ren *et al.,* 2009; Postma, 2012). However esterified xylan loses its biodegradability at a ratio of about 45% per degree of substitution unit (Sun *et al.,* 2011). In addition, chemical precipitation methods and thermal treatment strategies often involve harsh conditions which can result in hemicellulose degradation, due to their low thermal stability (Haimer *et al.,* 2008; Haimer *et al.,* 2010). Microscopy analysis showed that the xylan can assemble into cellulose as small particles ranging from 50 nm to a few micrometres (Linder *et al.,* 2003).

2.2.3. Enzymatic treatment (bio-catalysis)

In native wood, the binding of the polymers inhibits the access to the enzymatic hydrolysis process; hence the need of pre-treatment and fractionation steps to separate xylan from the other lignocellulosic components. Compared to physical and chemical modification methods, enzymatic hydrolysis is milder and specific towards the substrate (Peng *et al.,* 2012).

The biodegradation of xylan results from the synergistic action of a battery of enzymes, usually classified according to their mode of action or substrate specificity, depending on the chain length and substituents attached (Polizeli *et al.,* 2005).

The enzymes responsible for the degradation of xylan can be classified according to their catalytic modules: the glycoside hydrolyses (GH) cleave the glycosidic bonds and the carbohydrate esterases (CE) hydrolyse the ester bonds present in hardwood xylan species. The enzymes that hydrolyse xylan can be divided in two types: main-chain degrading enzymes, such as β-1,4-xylanases (EC 3.2.1.8) and β-1,4-xylosidases (EC 3.2.1.37); accessory enzymes which cleave the side-chains such as α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), acetyl xylan esterase (EC 3.1.1.72) and feruloyl esterase (EC3.1.1.73) (Biely, 1985; Sorensen *et al.,* 2007; Ryabová *et al.,* 2009).

Mesophilic xylan main-chain degrading enzymes have been employed in bioconversion of lignocellulose to biofuels, animal feed industry, biobleaching in pulping, clarification of juices and xylitol production (Polizeli et al., 2005). As a biopolymer, xylan could have a broader range of applications but the presence of side chains and additional acetyl groups makes xylan more soluble in water than other bio-polymers, irrespective of their lower degree of polymerization (Coughlan and Hazlewood, 1993). Accessory enzymes have been mainly employed to improve the hydrolysis efficiency of main-chain degrading enzymes, production of xylo-oligosaccharides and more recently debranching of xylan to form insoluble micro-particles (Polizeli et al., 2005).

2.2.3.1. Main-chain degrading enzymes

Endo-1,4-β-xylanases cleave the glycosidic bonds between the xylopyranosyl residues, converting the polysaccharide in shorter oligomeric units. As the hydrolysis progresses, smaller molecules might form such as mono, di and trisaccharides of β-D-xylopyranosyl. The optimum temperature for production of mesophilic β-xylanases is between 40 and 80°C, and the pH in the range between 4.0 and 6.5 (Polizeli *et al.,* 2005).

β-D-xylosidases are responsible for the hydrolysis of the oligosaccharides to its respective monomers, derived from several successive hydrolysis steps by xylanases, increasing the efficiency of hydrolysis. The affinity for oligosaccharides is inversely proportional to its degree of polymerization. The molecular weight for xylosidases ranges between 60 and 360 kDa. The temperature for maximum activity is between 40 and 80°C and the pH between 4 and 5 (Polizeli *et al.,* 2005).

β-xylanase production has also been performed from moderate to extremely thermophilic organisms, mainly bacteria (*Bacillus licheniformis, Thermotoga maritima, Thermotoga neapolitana, Thermotoga thermarum*), yeast (*Kluyveromyces lactis*) and fungus (*Trichoderma reesei, Aspergillus niger*) with optimum function temperatures ranging from 45-105°C (Bergquist et al., 2002; Niehaus et al., 1999). Fungal β-xylanases are less thermo stable than bacterial β-xylanases. In addition, β-xylanases expressed in several organisms are stable within a wide range of pH, between 2 and 10 (Kulkarni et al., 1999). In contrast, fewer studies have been done on thermo stable β-xylosidases. A thermo stable β-xylosidase has been purified and characterized from the thermophilic bacteria *Bacillus thermantacticus*, with optimum temperature of 70°C and pH 6.0 (Lama et al., 2004).

Many microbial systems are able to secrete a wide range of necessary enzymes for the complete degradation of xylan, derived from the fact that they utilize xylan as a carbon source (Den Haan and Van Zyl, 2003). However, to decrease the solubility of xylan biopolymers it is necessary to

avoid degradation the polysaccharide backbone. This is achieved if the accessory enzymes are not produced simultaneously with xylanases, since it would increase the removal of the side-chains, but decrease the polysaccharide chain length.

2.2.3.2. Side-chain removing enzymes

Acetyl xylan esterases attack the *O*-acetyl group, which is predominant in hardwood sources. *O*-Acetyl groups are present at positions 2 and 3 of the glucuronoxylan backbone (Christov and Prior, 1993). However, most alkali xylan extraction methods remove the acetyl groups, thus making the use of acetyl esterases fairly unnecessary. It was found that the presence of acetyl groups causes steric hindrances with other accessory enzymes (Polizeli *et al.,* 2005). Ferulic acid esterases and *p*-coumaric acid esterases are responsible for the cleavage of the bonds between arabinose and ferulic acid and *p*-coumaric acid respectively (Faulds *et al.,* 2003; Crepin *et al.,* 2004).

α-L-arabinofuranosidases (EC 3.2.1.55) are an important group of accessory enzymes that specifically hydrolyse non-reducing α-L-arabinofuranosyl groups present in arabinoxylans, arabinoglucuronoxylans and terminal residues in other substrates. α-L-arabinofuranosidases are assigned in five GH families: GH3, 10, 43, 51, 54 and 62, with different substrate specificities. These depend on the type of arabinofuranosyl linkage (α-1,2, α-1,3 or α-1,5) or position (terminal or reducing units) (Biely, 1985; Biely, 2003). α-L-arabinofuranosidase has synergy with other xylanolytic enzymes such as α-D-glucuronidase, having an enhancing or inhibitory effect. Most of the GH43 arabinofuranosidases hydrolyse α-1,5-arabinans and the GH62 is only active on arabinoxylans (Rémond *et al.,* 2008). GH51 and GH54 α-L-arabinofuranosidases are able to hydrolyse arabinosyl side chains that are bond through α-1,2 and α-1,3 linkages from both oligomeric and polymeric substrates. In addition, they are also able to hydrolyse the *p*-nitrophenyl arabinofuranoside (*p*-NPA), which is the substrate used for enzymatic assays (Morales *et al.,* 1995). However, it was found that GH51 α-L-arabinofuranosidase is mostly active in arabinan rather than in arabinoxylans (Biely, 2003). Among other accessory enzymes, α-L-arabinofuranosidases have been the most extensively studied for the enzymatic hydrolysis of arabinoglucuronoxylans (Siika-aho *et al.,* 1994).

The biochemical properties of α-L-arabinofuranosidases vary depending on the organism from which is expressed and from which they are sourced. For example, α-L-arabinofuranosidase expressed in *A. pullulans* is active in both α-1,3 and α-1,5 non-reducing, terminal residues, whereas the one expressed from *A. niger* hydrolyses either α-1,5 or α-1,3 arabinosyl linkages from both oligomeric and polymeric substrates (Saha, 2000). Optimum temperature and pH

hydrolysis conditions of GH54 arabinofuranosidase expressed in *Aspergillus niger* (*AbfB*) are 40°C and 5.0, respectively (Chimphango, 2010).

α-D-glucuronidases are a group of accessory enzymes that cleave 4-*O*-methyl glucuronic acid side chains attached to the xylose units by α-1,2-linkages (de Vries et al. 1998; Tenkanen & Siika-aho 2000; Shallom and Shoham, 2003). A wide range of microbial systems were investigated for the intracellular and extracellular production of α-D-glucuronidase (Table 2. 2), such as in fungae, including *Thermoascus aurantiacus, Aspergillus tubingensis, Phlebia radiata, Schizophyllum commune* (Khandke *et al.,* 1989; Smith and Forsberg, 1991; Shao *et al.,* 1995; de Vries et al. 1998; Mierzwa *et al.,* 2005; Kolenová *et al.,* 2010) and bacteria such as *Clostridium stercorarium* and *Thermoanaerobacterium saccharolyticum* (Bronnenmeier *et al.,* 1995).

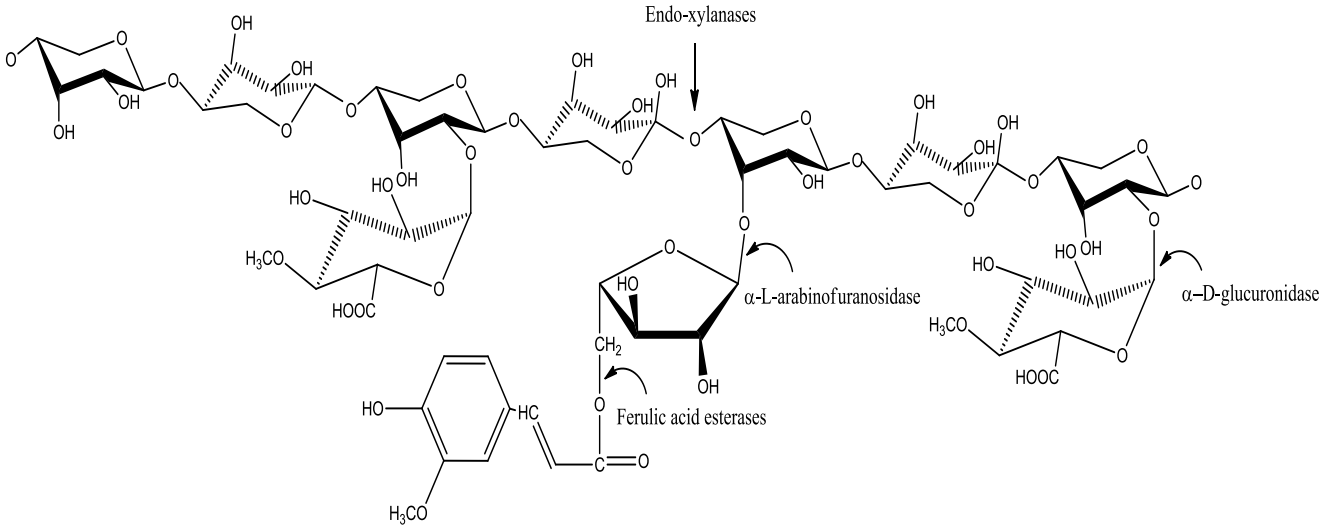


Figure 2. 4: Xylan enzymatic hydrolysis schematic view: each enzyme recognizes the site of action in a very specific way in analogy with a key-and lock system

2.3. Microbial systems for α-L-arabinofuranosidase and α-D-glucuronidase production

About 90% of industrial enzymes are recombinant and expressed in different strains of yeasts, bacteria and fungi (Cherry and Fidantsef, 2003). Many enzymes have been produced in homologous and heterologous organisms. In addition, research has been done to optimize the production, performance and prices of industrial enzymes. With the development of modern bio-technologies, foreign protein expression became possible, at the same time inhibiting unwanted proteins and activities that are commonly co-expressed in host organisms (Pérez *et al.,* 2002).

Aspergillus niger is a filamentous-type growth fungus, extensively used for the expression of homologous and heterologous enzymes (MacKenzie *et al.,* 1996). Extracellular secretion of recombinant enzymes has been possible with *A. niger* strains at relatively high yields of up to 1 g/L (Record *et al.,* 2003). GH54 α-L-arabinofuranosidases are the most active in both oligomeric and polymeric substrates. Therefore, they became the viable alternative for the selective precipitation of arabinoxylans and arabinoglucuronoxylans from different feedstocks (Chimphango, 2010). Heterologous expression of GH54 α-L-arabinofuranosidase (*AbfB*) in strains of *A. niger* resulted in production of an enzyme that was able to hydrolyse polymeric arabinoxylans and arabinoglucuronoxylans (Chimphango, 2010). The expression of α-L-arabinofuranosidase was performed under a strong constitutive promoter, the glyceralaldehyde-3-phosphate dehydrogenase. The volumetric activity of the recombinant α-L-arabinofuranosidase produced in shake-flasks and in a bio-reactor was approximately 10.0 and 8.0 nkat ml^{-1}, respectively. In addition, the protease-deficient and non acidifying pH *A. niger* mutant D15 pGTP used as expression host for *AbfB,* enabled extracellular production of the arabinofuranosidase from *A. niger* (Chimphango, 2010), therefore suitable to be applied in further research experiments.

Table 2. 2 shows a summary of previous research involving α-D-glucuronidase expression systems. As it can be verified, few of the isolated α-D-glucuronidases were able to hydrolyse polymeric xylan. The intracellular α-D-glucuronidase from *Aspergillus tubingensis* was mainly active in small substituted xylo-oligosaccharides. The thermophile strains *T. aurantiacus, C. stercorarium and T. saccharolyticum*, produced thermostable α-D-glucuronidases with optimum function temperatures of 60 and 50°C, respectively. However, α-D-glucuronidases produced from thermophilic organisms have only demonstrated activity in xylo-oligosaccharides (Bronnenmeier *et al.,* 1995).

The wood-rooting fungi *S. commune* (Tenkanen & Siika-aho 2000a; Chimphango, 2010; Chong *et al.,* 2011), was the first microbial system from which an α-D-glucuronidase that was active in polymeric xylans was isolated. It was shown to remove half of the existing 4-O-methyl glucuronic acid groups present in water-soluble arabinoglucuronoxylans (Tenkanen and Siika-aho, 2000a). *S. commune* α-D-glucuronidase was also able to cause visual precipitation in birchwood xylan (Chimphango, 2010). In addition, *S. commune* naturally produces three types of xylanases (XynA, XynB and XynC) together with glucuronidase (Kolenová et al. 2005), thus contributing to simultaneous hydrolysis of the xylan backbone due to synergy between the enzymes. In contrast with the GH67 family, the *Pichia stipitis* GH115 α-D-glucuronidase recently expressed in *P. stipitis* is based on a different amino acid sequence, with the ability to hydrolyse polymeric xylans (Ryabová *et al.,* 2009).

Table 2. 2: Different microorganisms found in literature for production of α-D-glucuronidase

Organism	**Reference**	**GH family**	**Molecular weight (kDa)**	**Activity of 1,4-xylanases/ xylosidases**	**Activity on polymeric xylan**
Agaricus bisporus; Pleurotus ostreatus (fungae)	Puls *et al.,* 1986	67	180-450	-	-
Clostridium stercorarium	Bronnenmeier *et al.,* 1995	67	72-76	+	-
Thermoanaerobacterium saccharolyticum	Bronnenmeier *et al.,* 1995		71	+	-
Thricoderma reesei	Siika-aho *et al.,* 1994	67	91	+	low
Schizophillum commune	(Tenkanen & Siika-aho 2000)	67	125	-	low
Aspergillus tubigensis	(Biely *et al.,* 2000); de Vries *et al.,* 1997	67	107	-	-
Thermoascus aurantiacus	Khandke *et al.,* 1989	67	118	β-glucosidase	low
Aspergillus niger	Kiryu *et al.,* 2005	67	58	-	-
Streptomyces olivochromogenes	Fontana *et al.,* 1988	67	Not determined	+	-
Fibrobacter succinogenes	Smith & Forsberg, 1991	67	Not determined	+	Low-released 100% of α-D-MeGluA after 240h incubation
Pichia stipitis	Ryabová *et al.,* 2009; Kolenová *et al.,* 2010	115	120	-	+
S. cerevisiae (Aerobasidium pullulans gene*)*	de Wet *et al.,* 2005	67	157	-	-

Saccharomyces cerevisiae has been widely utilized by mankind in brewing and baking, it is low in proteases and a GRAS (generally regarded as safe) organism (Gellissen and Hollenberg, 1997). Large scale cultivation of *S. cerevisiae* has been widely explored for the production of several products, particularly in the recombinant expression of enzymes (Mendoza-Vega *et al.,* 1994). It was previously used as a host organism for recombinant expression of a GH67 α-D-glucuronidase from *A. pullulans* (De Wet et al., 2006). The maximum volumetric activity obtained was 5 U/L, corresponding to 0.1 mg/L of protein. Biomass and product yields from *S. cerevisiae* can be improved by minimizing metabolic by-products such as ethanol and glycerol. The expression of foreign enzymes in *S. cerevisiae* has been a subject of study by many authors (Martin and Scheinbach, 1989; Hardjito *et al.,* 1993; Mendoza-Vega *et al.,* 1994; Birol *et al.,* 1998). Extracellular secretion of enzymes is advantageous to facilitate product recovery; *S. cerevisiae* secretes proteins in small amounts due to cell wall barrier transport resistance, influences from the growing environment, incorrect protein folding and this is especially valid when the protein exhibits a high molecular weight (Hadfield *et al.,* 1993). In addition, glycosylation can play a role in enzyme catalysed processes. It is a post-translational process in eukaryotic cells that results from binding of carbohydrate units to either N-asparagine or *O*-serine and threonine residues (Han *et al.,* 2005). It has been found that over half of all the proteins available in nature are glycosylated and this can considerably affect its catalytic properties, pH optimum and thermal stability to different levels (Qin *et al.,* 2008; Zou *et al.,* 2012). In some enzymes such as α-L-arabinofuranosidase, N-glycosylation is a necessary factor for enzymatic activity and increased storage stability. However, there is no information available on glycosylation effects and α-D-glucuronidase activity.

2.4. Microbial cultivation processes

The interaction between different cellular kinetic aspects, such as the organism morphology, type of growth, medium composition, mode of cultivation and biomass concentration, plays an important role in the expression of recombinant enzymes and its extracellular secretion. Cultivation processes can be operated in: batch, fed-batch and chemostat modes. Based on theoretical studies, fed-batch processes are more suitable to achieve high biomass yields and avoid typical problems encountered in batch cultivations of *S. cerevisiae* such as: catabolite repression, low product yields and formation of ethanol (Pham *et al.,* 1998). Most recombinant enzymes are expressed in concentrations below the levels required for sufficient catalytic activity, depending on the amount of activity required.

In filamentous-type organisms such as *A. niger*, the macro morphology varies depending on the cultivation conditions, for example if the growth occurs in shake-flasks, it assumes a pellet form

while in a bio-reactor adopts the mycelium growth (Metz and Kossen, 1977). It has been found that the growth morphology of *A. niger* recombinant strains greatly affects the production of foreign proteins.

2.5. Enzymatic hydrolysis of xylan

Growing interest in utilising renewable and biodegradable polymers has led to continuous research in the improvement of their functional properties. Xylans from different sources can greatly differ in their structural complexity and intermolecular arrangement, as referred in sections 2.1.1 and 2.1.2 (Kabel *et al.,* 2007). In section 2.3, it was mentioned that the degree and pattern of substitution are the main factors that determine the solubility of xylan in water. From literature, it was proposed that the degree of substitution is directly proportional to solubility (Wong et al. 1988; He et al. 1994). In addition, water molecules might act as bridging blocks to allow intermolecular bonding of xylan in solution (Sedlmeyer, 2011). Nevertheless, in order for precipitation to occur, the degree of polymerization should be above 10 for both hardwood and grass-type xylans (Chimphango, 2010). Few studies have been done on the optimized hydrolysis of soluble and insoluble xylans for selective removal of the 4-*O*-methyl glucuronic acid and arabinofuranosyl side-chains. The recombinant α-L-arabinofuranosidase characterized by Chimphango (Chimphango, 2010) was able to release arabinose from different arabinoxylans and arabinoglucuronoxylans. The optimum hydrolysis conditions were found to be between 14-16 hours, temperature of 38-45°C and enzyme dosage of 607 nkat g^{-1} of substrate. At these conditions, the enzyme was able to remove a maximum of 43% of the available arabinose. This caused an increase of the solution viscosity (15 mPa.s), which increased proportionally with the applied enzyme dosage. However, a lower viscosity of 2.03 mPa.s was verified at an enzyme dosage of 400 nkat g^{-1} for 9.0 hours at 45.8°C. In addition, the reaction followed the Michaelis-Menten kinetics, using *p*-nitrophenol arabinofuranosyl as a substrate.

α-glucuronidases were mainly active in aldouronic acids (De Vries et al., 1998) or co-expressed with endo-xylanases (Tenkanen and Siika-aho, 2000a). The purified α-D-glucuronidase from *S. commune* could only precipitate glucuronoxylan from birchwood and not the mild-alkali extracted xylan from *E. grandis* (Chimphango, 2010). The enzyme was able to release a maximum of 2 and 1.5% of the available 4-*O*-methyl glucuronic acid from birchwood and *E. grandis* xylans respectively, at a specific dosage of 8000 nkat g^{-1}, temperature of 40°C, pH 4.8 for a total hydrolysis time of 16 h. Only 0.5% of the total available 4-*O*-methyl glucuronic acid was released at an enzyme dosage of 18,000 nkat g^{-1}, 38°C and 11h (Chimphango, 2010). α-D-glucuronidase expressed by *S. commune* has also been able to remove up to 49% of the

available side chains from birch wood xylan at pH 5.7 and 24 hours of hydrolysis (Tenkanen & Siika-aho, 2000).

On the other hand, *Pichia stipitis* α-D-glucuronidase belonging to the GH115 family was able to remove up to 75% of the available 4-*O*-MeGlcA increasing the viscosity of beechwood glucuronoxylan (Ryabova *et al*., 2009). The specific activities for different aldouronic acids and glucuronoxylan showed that the activity decreased with increased chain length (Kolenová *et al.,* 2010). Optimum activity conditions corresponded to pH between 4.4 and 5.0 and temperature of 40°C (Ryabova *et al.,* 2009; Kolenova *et al.*, 2010). However, identifying optimum levels of enzyme dosage and xylan concentration would make the enzymatic hydrolysis of soluble xylans more efficient and economical. Other factors known to affect the yield of 4-*O*-MeGlcA release and precipitation of xylans are the lignin content and the degree of polymerization (Saake et al., 2001; Westbye et al., 2007). Therefore, it becomes important to study the kinetics of the α-D-glucuronidase and required substrate and enzyme dosages to maximise release of 4-*O*-methyl glucuronic acid side-groups and viscosity properties in polymeric glucuronoxylans.

The synergy between xylan degrading enzymes becomes evident when accessory enzymes are used in conjunction with the main-chain degrading enzymes. However, α-L-arabinofuranosidase and *S. commune* purified α-D-glucuronidase have shown a positive synergy, which released up to 21% of arabinofuranosyl units from sugarcane bagasse xylan. From the literature, it was clear that both substrate concentration and enzyme dosage were determining factors for selective removal of both arabinofuranosyl and 4-*O*-methyl glucuronic acid side chains.

2.6. Problem statement

In the wood matrix, xylan is mostly an insoluble polymer, since it is intimately bonded within the wood lignocellulosic components. Fractionation methods aim to separate (solubilise/isolate) xylan from other wood components, such as cellulose and lignin. However, in contrast with cellulose, isolated xylan has an amorphous, non-defined structural arrangement which offers little resistance in the presence of diluted acids or alkali. In the pulp industry, the hemicellulose is degraded to isosaccharinic acids, which are combusted together with the black liquor (Al-Dajani and Tschirner, 2008). Such practice is considered as a non-efficient utilisation of hemicelluloses since it only has half of heating value of lignin. In addition, the presence of hydrophilic side-groups attached to the xylan backbone and its low degree of polymerization further increase its solubility upon dispersion in water, imposing limitations on its use as a bio-polymer.The extraction of hemicelluloses prior to traditional kraft is environmentally and economically a better alternative, allowing further modification processes to be applied and adding value to a large fraction that is most likely to be continuously wasted. The enzymatic

hydrolysis of polymeric xylans is a viable method to reduce the solubility properties of different sources of xylan isolated from local feedstocks.

2.7. Research hypothesis

- α-D-glucuronidase and α-L-arabinofuranosidase optimized production from recombinant strains in the present study are able to selectively hydrolyse 4-*O*-methyl glucuronic acid and arabinofuranosyl side-groups, thus increasing intermolecular interactions, causing precipitation of soluble xylans.
- There is a synergistic effect when the two enzymes are applied simultaneously.
- Mild alkali methods for extraction of hemicelluloses from South African feedstocks represent a technically viable option to obtain high value bio-polymers suitable to be used in enzymatic modification processes.
- Different hemicellulose extraction methods may influence the catalytic action of α-D-glucuronidase.

The experimental framework of the present study involved simultaneous application of knowledge from two fields of study. The preparation of the xylan substrates and characterization required understanding in wood chemistry and the recombinant enzyme production in bio-process engineering. The application of these provided with the necessary tools to perform xylan enzymatic hydrolysis experiments. The next chapters will outline four major sections with regards to:

- The isolation and characterization of xylan from South African feedstocks.
- Production and characterization of recombinant α-L-arabinofuranosidase and α-D-glucuronidase.
- Selective enzymatic hydrolysis for optimized removal of xylan side-chains and change in functional properties.
- Morphological properties of modified xylans and potential industrial applications.

CHAPTER 3: Extraction and characterization of xylans from lignocellulosic feedstocks

3.1. Introduction

Hemicellulose is the most abundant carbohydrate biopolymer after cellulose in plant biomass, accounting for one third of all renewable carbon available on earth (Gabrielii *et al.,* 2000; Habibi *et al.,* 2003). Xylan is a type of hemicellulose composed with a linear backbone of β-1,4-linked xylose units that can be substituted with arabinose, 4-*O*-methyl glucuronic acid, galactouronic acid and D-glucuronic acid in proportions depending on the sources and methods of extraction (Pérez *et al.,* 2002; Girio *et al.,* 2010). Interest has arisen concerning effective recovery of lignocellulose constituents, mainly xylan from the lignin matrix during pulping processes. This is due to the low heating value of hemicelluloses compared to lignin, indicating inefficient utilisation of the hemicellulose content in lignocellulose by solubilisation during pulping followed by combustion. Extraction of xylans prior to pulping will isolate xylan biopolymers with potential industrial applications, as pulp and paper additives, bio-films, hydrogels for encapsulation matrices and in pharmacy (Oliveira *et al.,* 2010).

The recovery of xylan from the black liquor waste stream from pulping, used as energy source in boilers, is not feasible due to hemicellulose degradation products derived from kraft pulping processes. Hemicelluloses can be extracted from biomass prior to pulping using mild alkali, mild acid, hot water and alkaline peroxide. In the wood matrix, xylan is intimately associated with lignin through physical and covalent bonds forming the so-called LCC's, conferring resistance to the extraction of xylan. Among different extraction procedures, mild alkali extraction displayed the highest efficiencies and the lowest levels of hemicellulose degradation.

In the present study, two different mild alkali procedures were carried out to extract glucuronoxylan from *E. grandis* and arabinoglucuronoxylan from sugarcane bagasse feedstocks. A commercial source of glucuronoxylan from beechwood was obtained from Sigma Aldrich with superior purity standards. Consequently, the commercial and extracted xylans were submitted to structural and compositional analysis to evaluate their suitability for subsequent modification by hydrolysis with the enzymes α-L-arabinofuranosidase and α-D-glucuronidase (Chapter 5).

3.2. Materials and methods

Xylans were obtained from commercial sources and extracted from different lignocellulosic feedstocks. Commercial beechwood (*Fagus sylvatica*) xylan was available from Sigma-Aldrich, while *E. grandis* and sugarcane bagasse (*Saccharum officinarum L*) were used as feedstocks for xylan extraction. *E. grandis* chips were supplied from Sappi Ngodwana pulp and paper mill and sugarcane bagasse from TSB Sugar, Mpumalanga Province. The analytical grade sugars selected as standards for the feedstock and xylan characterization were: glucose, cellobiose, arabinose, xylose, xylobiose, rhamnose, galactose, mannose and glucuronic acid, at purity levels above 99%. Acetic acid (glacial), sulphuric acid (72%), sulphuric acid (95%) and calcium carbonate (>99%) were used in xylan extraction pre-treatment and total sugar characterization, all purchased from Sigma-Aldrich. The extraction protocols were performed using the following reagents: organosolvents, mainly acetone (chromasolv), ciclohexane (chromasolv), formic acid (98-100%) and methanol (chromasolv); hydrochloric acid (37%), peracetic acid (36-40%); potassium hydroxide (high purity pellets), sodium borohydride (99%) and sodium hydroxide (high purity pellets), all purchased from Sigma Aldrich. Ethanol (99%) was obtained from United Scientific (Pty), Ltd. and liquid nitrogen for freeze drying of the extracted xylans was obtained from Afrox, Ltd.

3.2.1. Feedstock preparation

E. grandis chips were pre-milled using a Condux-Werk Wolfgang bei Hanau mill, to particle size of ± 10 mm. Both *E. grandis* and sugarcane bagasse pre-milling samples were milled further in a Retsch ZM 200 mill to reduce particle size below 425 µm. The samples were further screened in a Vibratory Shaker Retsch AS200 equipped with sieves to remove particles below 250 µm and the remaining particles were used for xylan extraction and compositional analysis.

3.2.2. Characterization of feedstocks and extracted xylans

3.2.2.1. Moisture content

The moisture content was determined following the National Renewable Energy Laboratory Analytical Procedure 510-42620 (NREL-TP). The ground biomass material was placed in a pre-weighted container and dried at 105 °C until constant weight (Hames *et al.,* 2008). The percentage of total solids was obtained from the following equation:

$$Total\ solids\ (\%) = \frac{W_{sample\ (f)}}{W_{sample\ (i)}} \times 100 \qquad \textbf{Equation 3.1}$$

Where: $\mathbf{W_{sample\ (f)}}$ is the weight of the sample after oven-drying and $\mathbf{W_{sample\ (i)}}$ is the weight of the sample before oven-drying.

3.2.2.2. Extractives and ash content

Extractives and ash content were determined with the NREL-TP 510-42619 and NREL-TP 510-42622 methods, respectively (Sluiter *et al.,* 2008). The extractives present in biomass can be soluble in hot water and in ethanol; therefore both solvents were used in the summative analysis. The extractives removal was performed by adding 5±0.1 mg of biomass into a tared extraction thimble. Furthermore, 190±5 ml of distilled water or a binary system of ethanol:cyclohexane (1:2 v/v) was added into pre-weighed round flasks assembled in a Soxhlet apparatus and refluxed for 24 hours. The ash content refers to the amount of inorganic material in biomass, gravimetrically determined. A specified amount of biomass was placed in pre-weighed ashing crucibles and placed in a muffle furnace at 575°C until constant sample weight.

3.2.2.3. Carbohydrates and lignin determination

The monomeric sugar profile and lignin analysis of feedstocks and extracted xylans were performed according to the NREL-TP-510-42618 method. After removal of extractives material, 0.3 g of biomass was hydrolysed with 3 ml of concentrated sulphuric acid at 72% and 30 °C, stirring in a water bath for 60 minutes. Furthermore, diluted acid hydrolysis was performed at a concentration of 4% by addition of 84 ml of deionized water and autoclaved at 121 °C for 60 minutes. For extracted xylans, the hydrolysis time was reduced to 20 minutes. Insufficient hydrolysis time can cause incomplete hydrolysis and low carbohydrate content bias, while over-hydrolysis causes dehydration of monomers to other degradation products such as furfural and methyl furfural. The insoluble lignin was determined gravimetrically, while soluble lignin content was determined with a UV spectrophotometer at a wavelength of 525 nm within six hours after hydrolysis time. The hydrolysates were neutralized with calcium carbonate ($CaCO_3$) and filtered through a 0.22 µm filter into an auto-sampler for analysis. The HPLC system consisted of an Aminex HPX-87 P column, equipped with an evaporative light scattering detector (ELS), at a flow rate of 0.4 ml/min and distilled water as the eluent (18 MΩ). Standard deviation and external standards and controls for sugar losses during hydrolysis were included.

3.2.2.4. Degrees of substitution and polymerization of commercial and extracted xylans

The degree of substitution for beechwood, *E. grandis* and sugarcane bagasse xylans was determined with two methods: high-performance liquid chromatography (HPLC) with analytical grade standards (arabinose, D-glucuronic acid) and the carbazole-sulphuric acid method (Li *et al.,* 2007; Brienzo *et al.,* 2009) which quantifies the uronic acid content in extracted hemicelluloses. The carbazole-sulphuric acid method was performed by dissolving

0.001 g of xylan in 0.4 ml of distilled water in a test tube. Furthermore, 40 µl of 4 mol/L of sulfamic acid-potassium sulfamate was added, followed by 2.4 ml of concentrated sulphuric acid. After cooling down to room temperature, 100 µl of carbazole reagent (0.1% w/v in ethanol) was added, the mixture boiled in a water bath for 20 minutes followed by an ice water bath. Before measuring the light absorbance of the solution at 525 nm, 2.814 ml of distilled water were added. A galactouronic acid standard curve was used to determine the uronic acid content of the xylans (Appendix A). The HPLC analysis for uronic acid and arabinose contents was performed from the acid hydrolysis procedure, previously described in paragraph 3.2.2.3. The degree of polymerization of commercial and extracted xylans was determined with size exclusion chromatography, using HPLC. The degree of polymerization was obtained from the following equation:

$$DP = \frac{Total\ Mw\ of\ the\ polymer}{Mw\ of\ the\ monomer\ unit} \qquad \textbf{Equation 3.2}$$

The xylans were dissolved in water in a concentration of 1 g/L with stirring at 30 ° C. Furthermore, the xylan solutions were filtrated in a 0.22 µm filter and analysed in a Dionex Ultimate 3000 HPLC equipped with an ELS detector. A standard curve with 10 points was obtained using Pullulan (type of glucan) and the eluent utilized was deionized water with 0.05% of sodium azide at 1 ml/min.

3.2.2.5. Structural characterization with Fourier-Transformer Infrared (FT-IR) spectroscopy

The structural characterization of unmodified xylans was done on a Thermo Nicolet Nexus 870 FT-IR device with the Golden Gate ATR measuring system. The spectrum was obtained at the typical frequency (cm^{-1}) range of the FT-IR (4000-400). The spectrum was recorded in the Omnic ® 7 software. For ATR spectroscopy, no previous preparation was needed, the solid samples were placed in the measuring system and the absorption at different wavelengths was recorded in a spectrum.

3.2.2.6. Solubility properties of extracted xylans

The solubility of extracted xylans was assessed by preparing solutions at different concentrations with a maximum at 10% (w/v). The xylan samples were dissolved in water with agitation at 60 °C for approximately 6 hours. The phenol-sulphuric acid assay (Dubois *et al.,*

1956) was used to estimate the total xylan content in the soluble fraction. Xylan solutions at 10% (w/v) were centrifuged for 20 minutes at 13,200 rpm. The soluble fraction (200 µL) was mixed with 200 µL of a 5% phenol solution in a small test tube with vortex. Furthermore, 800 µL of concentrated sulphuric acid was added with vortex and the optical density measured at 490 nm. The samples were diluted to fall within the calibration curve range (0.01-0.2 g/L).

3.2.3. Mild alkali extraction of xylans

Two feedstocks were selected for xylan extraction: *E. grandis* which is a hardwood and sugarcane bagasse which is a by-product from the sugarcane refinery. The xylans were extracted using two mild alkali methods, described in Höije et al. 2005 and Pinto *et al.,* 2005. The Höije method was originally performed in barley husks, and further applied in hardwood species such as *E. grandis*. However, the Pinto method was only originally performed in hardwood species and therefore only applied to extract xylan from *E. grandis*, in the present study. The structural and chemical characterization was important to evaluate the suitability of xylans to be hydrolysed by α-L-arabinofuranosidase and α-glucuronidase.

The first step in the Höije protocol (Figure 3.1) was removing the extractives with a 0.05 M hydrochloric acid (HCl) solution at room temperature for 16 hours. The de-waxed biomass was delignified with sodium chlorite ($NaClO_2$) solution acidified to pH 4 with acetic acid (CH_3COOH). The delignification was performed at 75 °C, for 2 hours, after which the mixture was cooled down, decanted and washed several times to remove soluble lignin from the holocellulose fraction.

The hemicellulose extraction was carried out with 1 M sodium hydroxide solution (NaOH) with 0.5% sodium borohydride ($NaBH_4$) with stirring at 800 rpm at room temperature and 16 hours. The soluble xylan was separated from the cellulose residue and neutralized with 12.5 M of hydrochloric acid (HCl). Salts and other low-molecular weight impurities were dialysed in distilled water with a 12-14,000 kDa membrane for two days at 20 °C and then concentrated to 2/3 of the initial volume at 35°C using a Stereoglass Strike 300 rotary evaporator equipped with a cooling system (Julabo F-12) and a vacuum pump. Furthermore, the concentrated solution was frozen with liquid nitrogen and dried in a VirTis Bench Top 6K freeze drier, at 80 mTorr and -55 °C for 5 days. The resultant xylan was weighed to calculate the extraction efficiency.

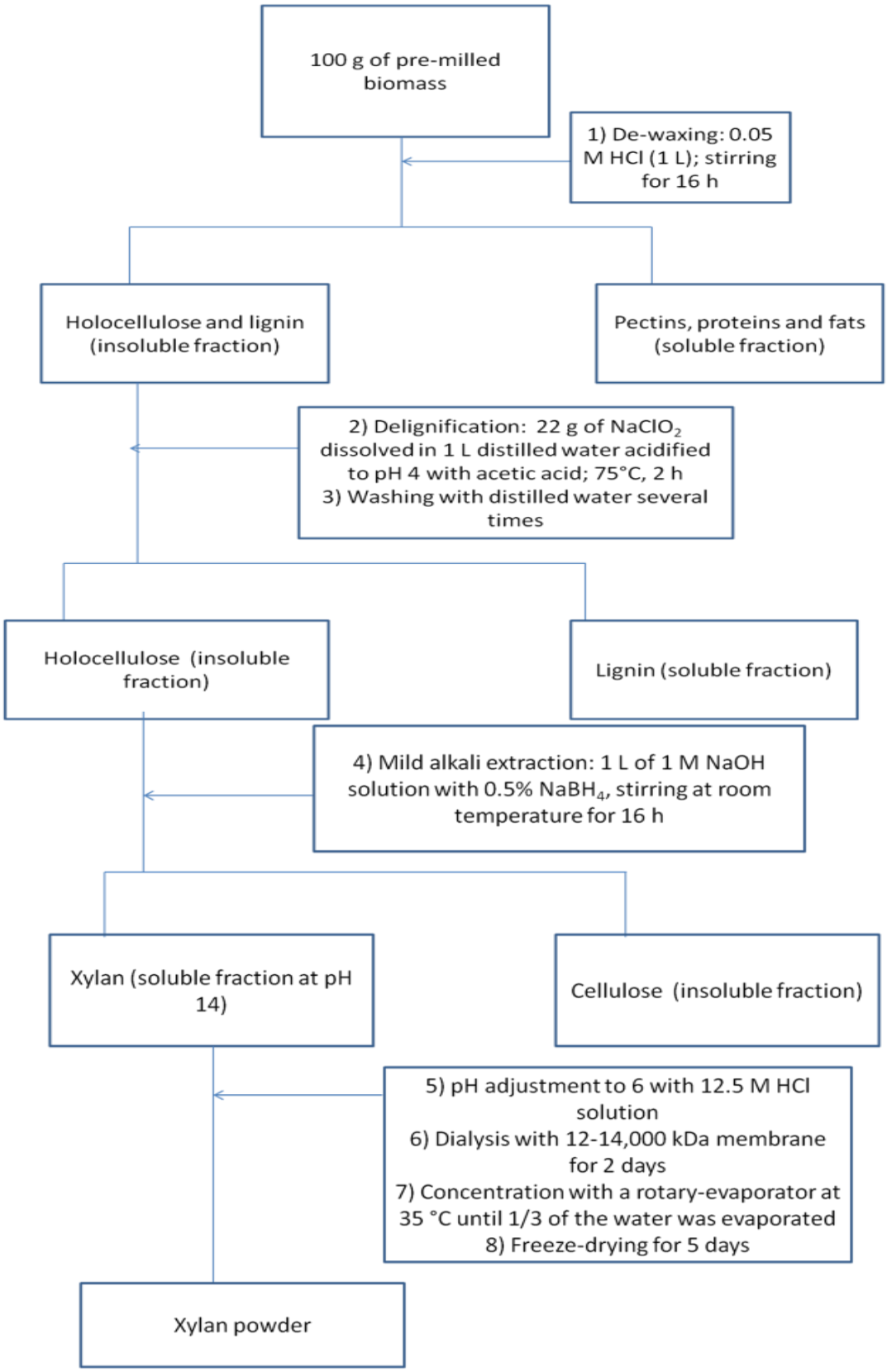


Figure 3.1: Process flow diagram for the mild alkali xylan extraction from the Höije protocol (Höije et al. 2005)

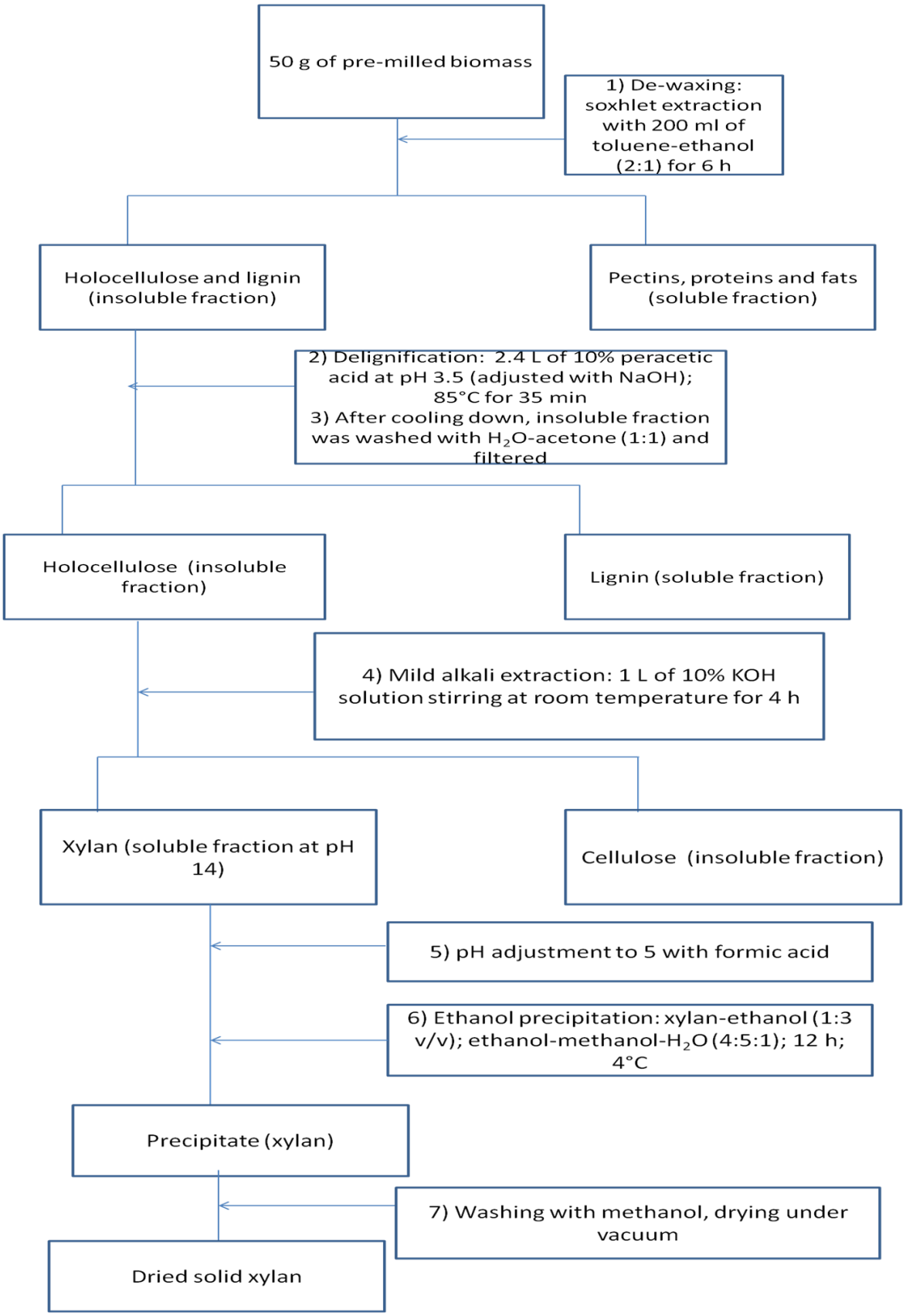


Figure 3.2: Process flow diagram for the mild alkali xylan extraction from the Pinto protocol (Pinto *et al.,* 2005)

The method described by Pinto et al. (Figure 3.2) used ethanol fractionation to extract the hemicelluloses. The de-waxing step was performed in a Soxhlet apparatus with toluene-ethanol (2:1) as a solvent refluxed for 6 hours. The ratio between the biomass and the solvent mixture was 5 g biomass per 200 ml solvent. The extractives-free biomass sample was delignified with 10% peracetic acid at pH 3.5 (adjusted with NaOH) at 85 °C for 35 minutes (2 g biomass for 0.1 L of peracetic acid). The mixture was cooled down, washed with ethanol-acetone (1:1) and filtered with a porous glass filter (0.45 µm).

The xylan solubilization was performed with potassium hydroxide at 10%, stirring at room temperature at 600 rpm for 4 hours. The residue was decanted of the xylan alkali solution and neutralized with formic acid to pH 5. Precipitation of xylan was performed with excess ethanol (ethanol-methanol-water in 4:5:1), for 12 hours at 4°C. The resultant precipitate was filtered under vacuum, washed with methanol and allowed to dry at room temperature.

3.3. Results

3.3.1. Compositional analysis of the feedstocks for xylan extraction

The compositional analysis of *E. grandis* and sugarcane bagasse is summarized in Table 3. 1. The monomeric sugar analysis of *E. grandis* indicated that cellulose was the major component with 39.91%, followed by insoluble lignin (25.57%) and hemicellulose in the form of glucuronoxylan at 21.85%. There were also minor amounts of arabinose (0.11%) and ash (0.34%).

Sugarcane bagasse had a lower cellulose content of 35.91% and a lower lignin content (20.35%) which translated into a slightly higher hemicellulose content of 22.00% (Table 3. 1). Sugarcane bagasse had an arabinose content of 1.54%, due to the presence of arabinose side-chains attached to the xylan backbone.

Table 3. 1: Average compositional analysis of *Eucalyptus grandis* and sugarcane bagasse on a wet weight basis [2]

Component	*E. grandis*	Sugarcane bagasse
Glucose (wt%)	39.91 ± 3.3	35.91 ± 2.5
Xylose (wt%)	21.58 ± 0.16	22.00 ± 1.1
Arabinose (wt%)	0.11 ± 0.027	1.55 ± 0.17
Insoluble lignin (wt%)	25.57 ± 0.005	20.35 ± 0.004
Ash (wt%)	0.34 ± 0.0003	4.79 ± 0.007
Total extractives (wt%)	3.36 ± 0.0004	5.33 ± 0.004
Moisture (wt%)	7.53 ± 0.01	6.63 ± 0.007
Summative analysis (wt%)	98.40 ± 4.2	96.55 ± 2.3

E. grandis and sugarcane bagasse had moisture levels of 7.52 and 6.63%, respectively. Sugarcane bagasse had higher extractives content corresponding to 5.3%, compared with E. grandis with 3.36%. Standard deviation values were obtained from the analysis performed in triplicates. The standard deviation and summative analysis did not exceed the 5% level confidence.

[2] Average values including standard deviation

3.3.2. Chemical and structural analysis of extracted xylans

The chemical composition of beechwood xylan (Sigma) and the xylans extracted from *E. grandis* and sugarcane bagasse is shown in Figure 3.3. The analysis of xylans showed significant differences related to the type of feedstock and extraction protocol utilised. Beechwood xylan contained minor quantities of glucose (1.60%) and lignin (3.57%), with xylose as the major component at 89.00% and uronic acid at 7.72%. The xylose content in the xylan extracted from *E. grandis* using the Höije protocol was 40.77%, accompanied with high lignin and uronic acid contents of 34.22% and 20.68%. Residual amounts of glucose were found at 1.95%. In contrast, the xylose content for the xylan extracted from sugarcane bagasse was 72.88%, accompanied by lignin, uronic acid and arabinose contents of 14.85%, 7.5% and 4.03%, respectively. Similarly, residual glucose was present at 2.22%. The extraction of glucuronoxylan from *E. grandis* using the Pinto protocol did not result in any residual sugar besides xylose, with a corresponding content of 84.90%. In addition, the delignification was more efficient giving a lignin content of 5.38%. However, the uronic acid content was lower as compared to the glucuronoxylan extracted using the Höije protocol.

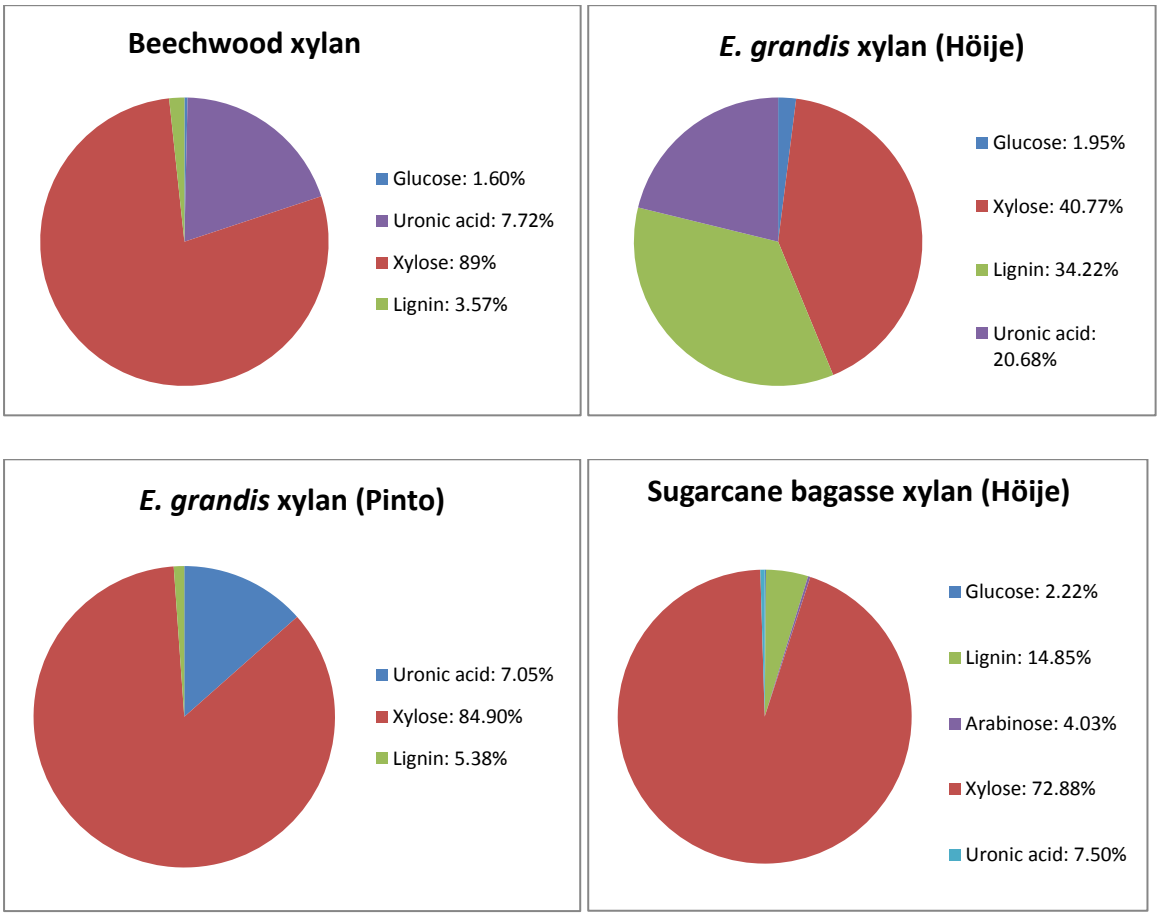


Figure 3.3: Chemical composition of: commercial beechwood xylan, *E. grandis* xylan (Höije), *E. grandis* xylan (Pinto) and sugarcane bagasse (Höije)

From compositional analysis results, the correspondent xylose to 4-*O*-methyl glucuronic acid ratios in beechwood, *E. grandis* (Höije), *E. grandis* (Pinto) and sugarcane bagasse xylans were 1:12, 1:2, 1:12 and 1:10, respectively. Sugarcane bagasse xylan had approximately 1 arabinose residue for every 18th xylose unit.

Extraction yields were calculated based on the content of xylose in the biomass feedstock and extracted fraction. Xylans were extracted from *E. grandis* and sugarcane bagasse using the Höije et al. protocol with yields of 20 and 71%, respectively. The extraction of xylan from *E. grandis* using the Pinto et al. protocol resulted in a yield of 89%.

3.3.3. Physical and water solubility properties of extracted xylans

The physical appearance of the extracted xylans is shown in Figure 3. 4. Beechwood xylan was purchased from Sigma and presented as a semi-amorphous yellow powder. *E. grandis* and sugarcane bagasse xylans (from the Höije protocol) were isolated from the extraction mixture by ultrafiltration, and were light-brown and amorphous solids after freeze-drying. On the other hand, *E. grandis* glucuronoxylan isolated from the extraction mixtures by ethanol precipitation (Pinto protocol), displayed a dark-brown colour, forming a film with a brittle structure after air-drying.

The enzymatic hydrolysis of the xylans was performed in a homogeneous system since some enzymes have restricted accessibility to the insoluble form of the substrates (Bailey and Ollis, 1986). To assess the formation of insoluble particles caused by selective removal of arabinose and/or 4-*O*-methyl glucuronic acid, xylan solutions were centrifuged and the resulting soluble fractions separated for enzymatic modification experiments. The phenol-sulphuric acid assay performed on soluble fractions of the xylans extracted using the Höije protocol and purified with ultrafiltration gave approximate concentrations of 94.3 and 25.5 g L^{-1} for *E. grandis* and sugarcane bagasse xylans, respectively. The soluble fraction of beechwood xylan from Sigma had an approximate concentration of 98.75 g L^{-1}. In contrast, *E. grandis* xylan obtained using the Pinto protocol with ethanol purification was completely soluble in water at the conditions described earlier (section 3.2.2.6). The calibration curve of the phenol-sulphuric acid assay (Appendix A) using beechwood xylan (Sigma) as a standard showed a linear relationship between concentration and light absorption at 480 nm (R^2=0.9876).

Figure 3. 4: Model and extracted xylans utilized in this study. From left to right: Beechwood xylan (Sigma), *E. grandis* xylan extracted using the Höije protocol and purified with ultrafiltration, *E. grandis* xylan extracted using the Pinto protocol and purified with ethanol precipitation, sugarcane bagasse xylan extracted using the Höije protocol and purified with ultrafiltration.

3.3.4. Distribution of molecular weight

The average molecular weight, degree of polymerization and polydispersity values obtained for the xylans in study are shown in Table 3. 2. Beechwood xylan (Sigma) had an average molecular weight (Mw) of 17,053 Da and a number average molecular weight (Mn) of 11,638 Da giving a degree of polymerization (DP) of 77. The average Mw and Mn for *E. grandis* xylan extracted using the Höije protocol with ultrafiltration were 63,809 and 42,891 Da respectively, giving a DP of 287. The glucuronoxylan extracted from *E. grandis* using the Pinto protocol with ethanol precipitation gave an average Mw of 40,021 Da with a Mn of 19,946 Da, corresponding to a DP of 133. The xylan extracted from sugarcane bagasse (Höije) with ultrafiltration showed an average Mw and Mn of 35,620 and 22,658 Da, respectively. The degree of polymerization of this type of arabinoglucuronoxylan was 151.

Table 3. 2: Molecular weight and degrees of polymerization of commercial and extracted xylans by size exclusion chromatography (SEC)

Substrate	Average molecular weight (Da)	Degree of polymerization	Polydispersity
Beechwood xylan	17,053	77	1.47
E. grandis xylan (Höije)	63,809	287	1.48
E. grandis xylan (Pinto)	40,021	133	2.01
Sugarcane bagasse xylan (Höije)	35,620	151	1.41

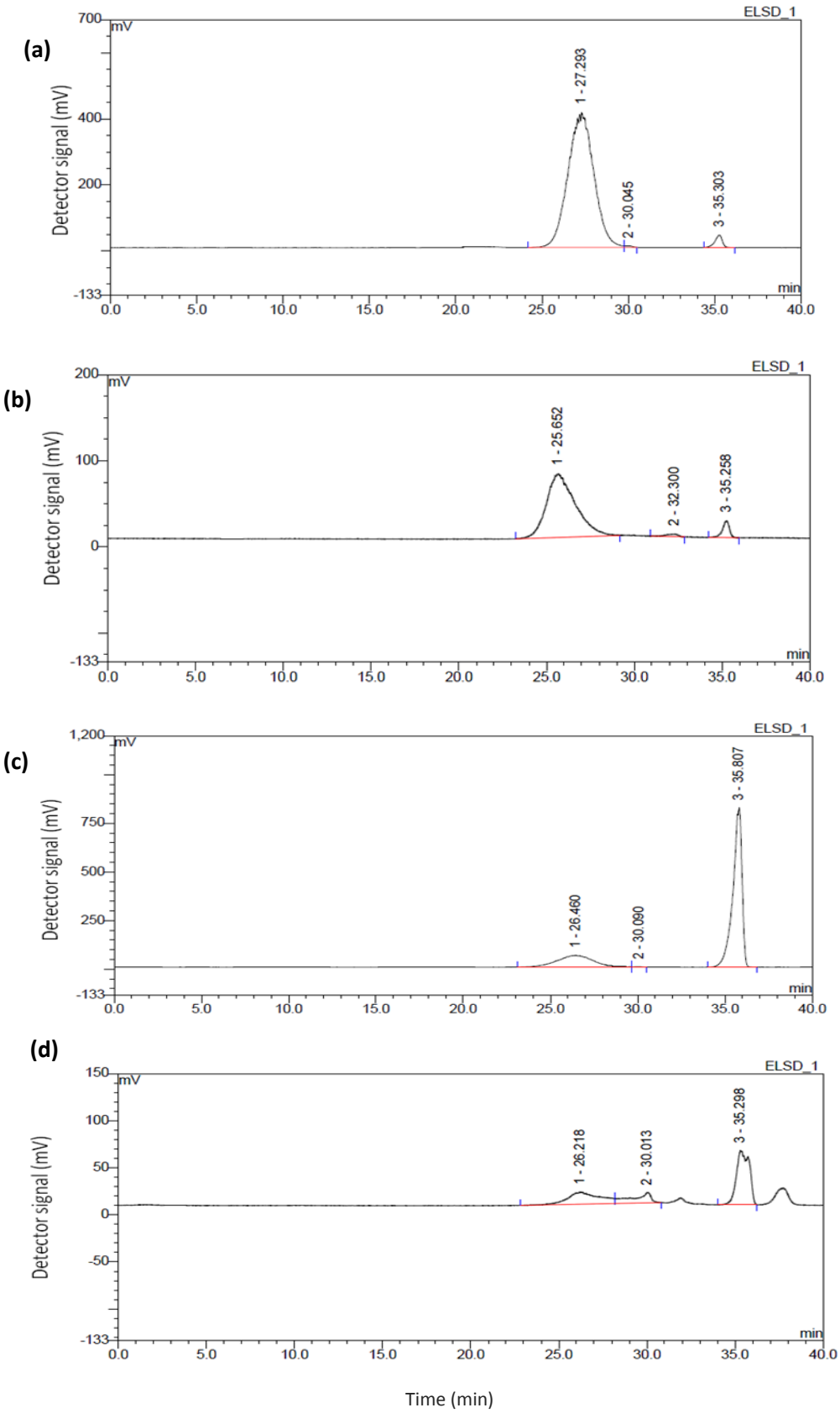


Figure 3. 5: Size exclusion chromatography elution profiles of the xylans in study, where (a): beechwood xylan (Sigma), (b) *E. grandis* xylan (Höije), (c) *E. grandis* xylan (Pinto), (d) sugarcane bagasse xylan (Höije)

The size exclusion elution chromatograms are shown in Figure 3. 5. The earliest peak corresponded to high molecular weight components (polymeric fraction), the second peak to the lower molecular weight fraction (oligomeric fraction) and the last peak corresponding to monomers. The peak correspondent to the polymeric fraction represented the major fraction in beechwood and *E. grandis* xylan (Höije). This indicates that the extraction protocol employed was able to extract hemicelluloses with minimum peeling-effects during the alkali extraction that can degrade biopolymers into oligomers and monomers. The ethanol fractionation protocol employed for *E. grandis* (Pinto protocol) was able to extract hemicelluloses with a high molecular weight; however the elution profile showed that the polymeric fraction did not represent the majority of the extracted material.

3.3.5. FT-IR analysis

The FT-IR spectra of the extracted xylans are displayed in Figure 3.6. The wide-broad absorptions at 3407, 3240 and 3368 cm^{-1} correspond to stretching of the –OH groups in the hemicelluloses (Ren and Sun, 2010). The strong absorptions at 2891 and 2921 cm^{-1} are attributed to the C-H stretching vibrations (Ren and Sun, 2010). However this shoulder had a lower intensity in the *E. grandis* xylan (Pinto). The bands at 1600, 1593, 1581 cm^{-1} correspond to the bending mode of absorbed water (Peng *et al.,* 2009a). Notably, this band was stronger in the *E. grandis* xylan (Pinto) showing its high affinity for water as a solvent and weaker in sugarcane bagasse xylan confirming water solubility results given in section 3.3.3. The shoulder peaks in the range of 1700 to 1730 cm^{-1} are attributed to the C=O double bonds stretching or the ester binds of carboxylic groups of ferulic and *p*-coumaric acids (Peng *et al.,* 2009a). The fingerprint region between 1500 and 1347 corresponds to the aromatic vibrations in lignin, particularly to the syringyl ring breathing with C_{Ar}-OCH_3 and methoxyl groups (Chimphango, 2010). The aromatic band in *E. grandis* xylan by Höije and Pinto was more intense giving indication of higher amounts of ester linkages between the 4-*O*-methyl glucuronic acid and the aromatic ring in lignin. The broad bands at 1036 and 1039 cm^{-1} are caused by C-O-C glycosydic vibrations (Ren and Sun, 2010) and they were present in all hemicelluloses, indicating that the xylan backbone was the major component in the extracted samples. The third band (987, 989 cm^{-1}) at the anomeric region correspond to the C1 carbon in the xylose ring and the first peaks at 700 and 650 cm^{-1} are caused by the α-anomers in the side-chains (Peng *et al.,* 2009b).

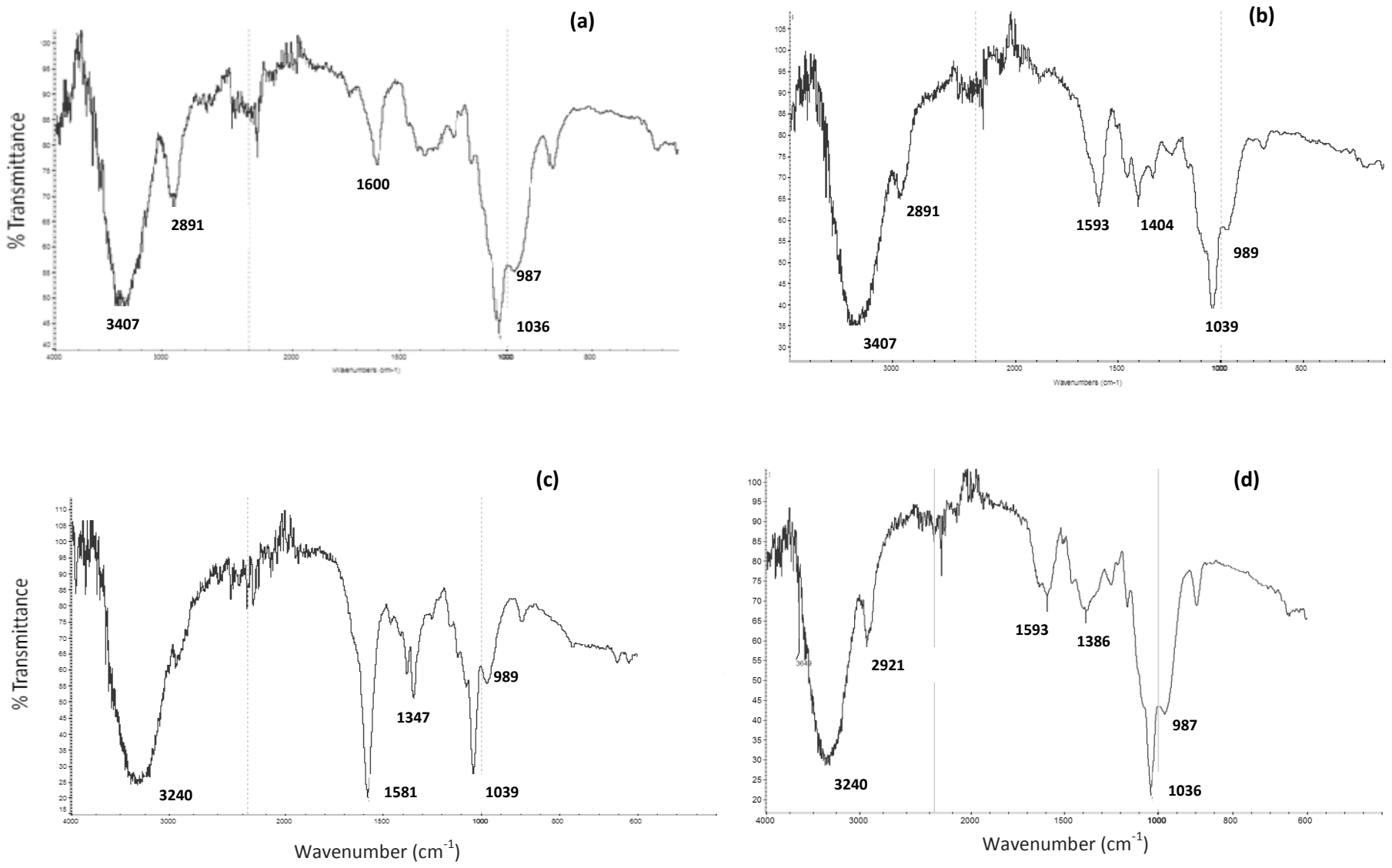


Figure 3.6: FT-IR spectra for:(a) beechwood xylan, (b) *E. grandis* xylan (Höije), (c) *E. grandis* xylan (Pinto) and sugarcane bagasse xylan (Höije)

3.3. Discussion

3.3.1. Xylan extraction efficiency

The properties of the xylans, such as purity, molecular weight, degree of substitution and extraction protocols employed are important to understand possible differences in the enzymatic hydrolysis performance.

The chemical composition of *E. grandis* and sugarcane bagasse was investigated for the extraction of xylan. Sugarcane bagasse had a slightly higher average xylan content of 22%, as compared to E. grandis with 21.58%. The xylan content for the South African sugarcane bagasse is close to values obtained previously for South American samples of sugarcane bagasse with 24.9%, respectively (Alves *et al.,* 2010). However, lower values of xylan content were reported for *Eucalyptus* at 11.5% (Alves *et al.,* 2010), as opposed to the *E. grandis* used in this study with 21.58%. The xylan content for *E. grandis* and sugarcane bagasse was in the range of the values reported in the literature, between 20-40% (Bian *et al.,* 2010). This indicates that the feedstocks selected in this study represent valid sources of xylans for extraction and fractionation. The lower xylan content in *E. grandis*, can be attributed to insufficient hydrolysis since the 1,2-xylose-glucuronic acid bond is acid resistant and it might yield a lower xylose content than expected. This was less likely to occur in sugarcane bagasse due to a lower uronic acid content as compared with *E. grandis*. Sugarcane bagasse had an arabinose content of 1.54%, derived from the arabinose side-chains in the xylan backbone. This value is similar to the ones reported in the literature (Alves *et al.,* 2010). The moisture levels were within the reported range for wood and grass feedstocks which is from 3-63% (Vassilev *et al.,* 2010). However a lower moisture level is desired to prevent microbial degradation due to excess moisture. Sugarcane bagasse had higher values of ash and extractives as compared with *E. grandis* apparently due to larger amounts of sand particles that get trapped within the sugarcane bagasse structure before chemical analysis and extraction. Therefore it is more important to do a de-waxing step before the extraction of hemicelluloses for sugarcane bagasse than in *E. grandis*.

The xylan from beechwood had the highest purity levels in terms of xylose content, with minor amounts of lignin and glucose (Figure 3.3). The mild alkali extraction protocols by Höije and Pinto were able to extract hemicelluloses in the form of xylan from *E. grandis* and sugarcane bagasse (Figure 3.3). The Höije protocol extracted xylan from *E. grandis* with the highest lignin content and lowest yield, as compared with the one extracted from sugarcane bagasse. The Hoije protocol also extracted minor amounts of glucose from *E. grandis* and sugarcane bagasse

at 1.95% and 2.22%, respectively. This has been previously verified for the extraction of arabinoxylan from barley husks using the Höije protocol (Höije *et al.,* 2005). The xylose, lignin, uronic acid and arabinose contents of the xylans extracted using the Höije protocol are in close agreement to previously reported values (Chimphango, 2010; Postma, 2012). In general, arabinoglucuronoxylans have arabinose to xylose ratios of 1:3-5 (section 2.1.1); however seasonality and type of extraction might affect the lignocellulosic composition. For example, Peng et al. performed extraction of hemicelluloses from sugarcane bagasse obtaining arabinose contents between 2.75% and 12.13% (Peng *et al.,* 2009b) at different extraction conditions.

As expected, the glucuronoxylan extraction yield for *E. grandis* was lower than for sugarcane bagasse, since the Höije method was developed for extraction of arabinoglucuronoxylans from barley husks which present structural and compositional properties more similar to sugarcane bagasse. In this case the lower yield can be attributed to the higher lignin content originally present in *E. grandis* which forms lignin-carbohydrate complexes in the form of α-benzyl ether linkages. Previous studies have indicated that grasses only have small amounts of *p*-coumaric acids which are ester linked to the lignin, whereas about 40% of the glucuronic acid residues in hardwoods are esterified to lignin (Lu and Ralph, 2010b). This makes the glucuronoxylan in *E. grandis* more resistant to the alkali extraction as opposed to sugarcane bagasse.

The extraction of glucuronoxylans using the Höije and the Pinto protocols resulted in notable differences in the chemical composition and structural characteristics (Figure 3.3). *E. grandis* xylan extracted using the Höije protocol had an exceptionally higher degree of substitution with 4-*O*-methyl glucuronic acid residues, as compared with the other xylans in this study. The lower uronic acid content in *E. grandis* xylan by the Pinto method is attributed to extensive degradation of hexeuronic acid groups by peracetic acid during the delignification step (Pinto *et al.,* 2005). It was found that about 40% of the glucuronic acid residues in hardwoods are esterified to lignin (Lu and Ralph, 2010b). As seen in Figure 3.3, the decrease in uronic acid content in the extracted glucuronoxylan using the Pinto protocol also resulted in a decrease of the lignin content, due to extensive uronic acid degradation by peracetic acid. The yield of glucuronoxylan extracted from *E. grandis* using the Pinto protocol was significantly higher, since this method was developed using different species of hardwood xylans. Consequently, the yields obtained by Pinto et al. (Pinto *et al.,* 2005) were always higher than 50%, therefore in close agreement with the glucuronoxylan extraction yield obtained in the present study.

The structural analysis confirmed the presence of the typical functional groups found in hemicelluloses (Figure 3.6). The difference in the structural properties was related to the molecular weight and degrees of polymerization (DP) (Table 3. 2). The DP for *E. grandis* xylan

(Höije) and *E. grandis* xylan (Pinto) are within the expected range for hardwood xylan types, between 100 and 300, while sugarcane bagasse showed a lower DP of 151, typical of arabinoglucuronoxylans (Girio *et al.,* 2010). In contrast, beechwood xylan (Sigma) had a lower molecular weight and consequently a lower degree of polymerization as compared with the other glucuronoxylans extracted in this study. Nevertheless, it is above the minimum DP required for precipitation of xylans to occur due to interactions between the linear regions of the polymers (Chimphango, 2010).

The conditions employed in Pinto protocol, drastically reduced the molecular weight of the extracted glucuronoxylan from *E. grandis*, resulting in major fractions in the oligomeric and monomeric forms (Figure 3.4). This indicates that this method had more severe peeling effects in the polymer backbone as compared to the one extracted using the Höije protocol. This might be due to a different delignification agent (peracetic acid) used in the Pinto protocol and because the sodium borohydride was not used as in the Höije protocol. The sodium borohydride inhibits the polymer degradation by the alkali, even at mild alkali conditions (Browning, 1967). Moreover, the delignification using peracetic acid was performed at a temperature slightly higher (85°C) decreasing the lignin content and the degree of branching. This makes the hemicellulose more susceptible to alkali degradation, due to reduced amounts of lignin and uronic acid that help preserving the structure of the hemicellulose (Pinto *et al.,* 2005). Therefore, higher amounts of lignin in the Höije protocol were accompanied with a higher molecular weight hemicellulose, whereas efficient delignification led to substantial peeling-effects for the Pinto protocol.

The degradation verified for the glucuronoxylan extracted with the Pinto protocol agrees with the highest solubility in water (10% w/v) with no insoluble residue left after centrifugation at 14,000 rpm. The low solubility of sugarcane bagasse xylan in water agrees with the low arabinose and 4-*O*-methyl glucuronic acid substitution rate as previously described in section 3.3.2. According to Ebringerová and Heinze, interchain aggregation can only occur when there are large unsubstituted areas in the xylan backbone (Ebringerová and Heinze, 2000). However, the polymeric peak verified for sugarcane bagasse xylan did not correspond to the major peak in the SEC elution profile (Figure 3.4). It has been verified that solutions of xylan in water can form aggregates (Höije *et al.,* 2005), and some can remain in solution during HPLC sample filtration causing the peak with the higher molecular weight to be less abundant than other low molecular weight components. The high molecular weight of sugarcane bagasse xylan also explains its lower solubility upon dispersion in water. Despite of this low solubility, the insoluble particles of native arabinoglucuronoxylans were found to be hygroscopic and amourphous due to random intermolecular interactions between the polymers (Escalante *et al.,*

2012). In addition, it has been reported that glucuronoxylans exhibit poor plasticising properties, as compared with arabinoglucuronoxylans (Escalante *et al.,* 2012). Thus, further removal of both arabinose and glucuronic acid side-chains might be required to form hydrophobic xylan particles to be applied as coatings, films or hydrogels.

The Höije and Pinto protocols could be optimized to extract xylans maximising the xylose content while minimising the lignin content and backbone degradation due to peeling reactions. However, it is more cost-effective to perform the extraction of a high-molecular weight xylan at a lower yield than to obtain a high yield of extraction with a low degree of polymerization. Consequently, extensive degradation to form xylo-oligomers and monomers is not desirable for applications that require xylan to perform as a polymer such as hydrogels and films with plasticising properties (Deutschmann and Dekker, 2012). In contrast, higher lignin contents can increase the aggregation behaviour of the extracted xylans due to increased intermolecular bonding between the xylan and the lignin residues (Westbye *et al.,* 2007).

3.4. Conclusions

Two mild alkali extraction protocols were used to isolate xylan from *E. grandis* and sugarcane bagasse. *E. grandis* and sugarcane bagasse represented suitable feedstocks for the extraction of glucuronoxylans and arabinoglucuronoxylans, respectively. The chemical and structural properties of the extracted xylans differed according to the type of feedstock and the extraction protocol employed. The Höije protocol was better suited for sugarcane bagasse xylan, since it was originally developed for arabinoxylans. The extraction of xylan from *E. grandis* and sugarcane bagasse using the Höije protocol resulted in higher impurity levels in terms of lignin and higher degrees of polymerization, as compared to the glucuronoxylan extracted using the Pinto protocol. The extraction of xylan from *E. grandis* using the Pinto protocol resulted in the highest yield at 89%, however the extract was mainly in the oligomeric and polymeric forms.

Results indicated that extracted hemicelluloses had xylose as the main sugar, considerable amounts of uronic acid and lower amounts of neutral sugars while the size exclusion chromatography showed that all xylans have degrees of polymerization above the minimum value of 10 required for precipitation to occur (Chimphango, 2010). Such properties are compatible to enzymatic modification by α-L-arabinofuranosidase and α-D-glucuronidase hydrolysis.

CHAPTER 4: Production and characterization of recombinant α-D-glucuronidase and α-L-arabinofuranosidase

4.1. Introduction

In nature, complete degradation/hydrolysis of xylan requires concerted action of different types of enzymes produced by organisms after the induction from the target substrates (Shallom and Shoham, 2003). α-L-arabinofuranosidases and α-D-glucuronidases are accessory enzymes that hydrolyse the arabinofuranosyl and the 4-*O*-methyl glucuronic acid side-chains attached to the xylan backbone (Kolenová *et al.,* 2010). In the past decades, the main focus has been driven to recombinant expression of enzymes to promote higher product yields and selectivity and reduce the production of inhibitory proteins.

The GH54 α-L-arabinofuranosidase has been found to cleave the arabinofuranosyl side-chains in both arabinoglucuronoxylans and arabinoxylans. *Aspergillus niger* recombinant strain D15 pGTP was successfully utilized for the production of α-L-arabinofuranosidase at volumetric activities of 10 and 8 nkat ml^{-1} (p-NPA as a substrate) in shake-flasks and bio-reactor respectively (Chimphango, 2010). Therefore it was selected in the present study for recombinant expression of α-L-arabinofuranosidase.

The first group of α-D-glucuronidases identified (GH67 family) was only able to release 4-*O*-methyl glucuronic acid residues from aldouronic acids; produced from different fungi and bacteria described earlier in literature (Chapter 2). The only α-D-glucuronidases active on polymeric xylans (GH115 family) were obtained from *S. commune* and *P. stipitis*, the first with co-expression of xylanases and the second with uncertain gene sequences (Tenkanen and Siika-aho, 2000a). It is of interest to develop a microbial system with the capability of expressing in sufficient quantities α-D-glucuronidase with activity in polymeric xylan and xylo-oligosaccharides. For this purpose the yeast *Saccharomyces cerevisiae* (baker's yeast) was selected. It has been widely used for foreign expression of proteins with minimum production of proteases. In addition, glycosylation of foreign proteins is a common characteristic and it might be a key factor for the stability or activity of certain proteins (Bitter and Egan, 1984).

The recombinant expression of *A. pululans* α-D-glucuronidase in *S. cerevisiae* has been previously performed with maximum volumetric activity of 5 U/ml which is equivalent to 0.1

mg/L. The extracellular production was performed at low concentrations and the enzyme was only active on small xylo-oligosaccharides (De Wet et al., 2006).

The aim of this chapter is to characterize α-L-arabinofuranosidase and α-D-glucuronidase produced from recombinant strains of *A. niger* and *S. cerevisiae* for selective removal of arabinose and 4-*O*-methyl glucuronic acid groups present in different types of xylan.

4.2. Materials and methods

4.2.1. Strains and medium preparation

The recombinant fungal strain of *Aspergillus niger Anabf* D15 pGTP utilized in this study was obtained at the University of Stellenbosch, Microbiology Department. The plasmid construction has been described earlier in literature (Chimphango, 2010). The spore cultivation and media preparation were performed according to Rose and Van Zyl (2002). The spore plate's cultivation was done in minimal medium containing: 1.8 % (w/v) agar, 1% (w/v) glucose, 0.2% (w/v) trypticase, 0.1% (w/v) yeast extract (Difco), 0.04 % (w/v) $MgSO_4.7H_2O$ and 0.1 % (w/v) casamino acids. This medium was supplemented with AspA salts with nitrate (1:50 v/v) and trace elements[3] (1:1000 v/v). The plates were incubated for 120 hours at 30 °C or until spore growth covered 80% of the surface. The spores were loosed with physiological saline solution of NaCl (0.9% w/v) and transferred to a 15 ml Falcon tube. The number of spores per unit volume was determined by counting in a microscope to inoculate 1.10^6 cells/ml into 125 ml shake-flasks containing 25 ml of traditional minimal medium (Table 4.1).

Table 4. 1: Medium composition for the recombinant *Aspergillus niger* shake-flask cultivation

Component	For 750 ml
Yeast extract (Difco)	7.5 g
$MgSO_4.7H_20$	0.6 g
Glucose	75 g
Casamino acids	3 g
AspA salts with nitrate	30 ml
Trace elements	1.5 ml

[3] Composition of AspA salts and trace elements is given in Appendix B.

4.2.1.1. Strain construction for α-D-glucuronidase production

The production of α-D-glucuronidase was performed from two recombinant strains of *Saccharomyces cerevisiae*, the pBKD-PsGluMH1000 and the MH1000 pK10D-Glu provided by the University of Stellenbosch, Department of Microbiology. The methodology for development of this yeast strain by DC La Grange is provided below.

A genomic copy of the *Pichia stipitis* CBS 6054 α-D-glucuronidase was isolated from *P. stipitis* 5773 (a wild type strain of *P. stipitis*). The gene was isolated using primers PSglu-L (tgcaGGATCCaaatgttgttttcatacttcccagc) and PSglu-R (gtacGGATCCctacttttttgatgtaagtttctggtgg) with genomic DNA as PCR template (Jeffries *et al.,* 2007). The 2994 bp open reading frame was cloned into an episomal *Saccharomyces cerevisiae* expression vector under the transcriptional control of the *ENO1* promoter and terminator. This gene showed 95% homology on protein level with the published sequence (GenBank CP000500). In order to prevent the loss of the episomal plasmid during propagation under non-selective conditions, the glucuronidase gene was integrated into the genome of *S. cerevisiae,* denominated as *agu1A*. The gene was cloned into the δ-integrating vector pBKD1 (McBride *et al.,* 2007) as a *Pac*I-*Asc*I fragment and integrated at the δ-locus on the chromosome of *S. cerevisiae agu1A*. Eight transformants were tested for α-glucuronidase activity and the best clones were selected for further work (data not shown) (La Grange, 2011). Stock cultures were maintained in 20% glycerol at -80° C until inoculation.

The other genomic copy of the *P. stipitis* glucuronidase (pBKD-PsGluMH1000) had 100% homology on protein level, denominated as *agu1B*. This gene was also expressed under the transcriptional control of the *S. cerevisiae ENO1* promoter and terminator. The recombinant *S. cerevisiae* strains were tested for glucuronidase activity using the method described by Kolenová *et al.* (2010).

The cultivation of *S. cerevisiae* strains was firstly done in the solid state, using double strength synthetic medium lacking uracil in order to maintain selective pressure. The medium composition for agar plate's cultivation is given in Table 4.2. The plates were incubated at 30° C for 72 hours, inoculated into test tubes containing 5 ml of the medium described in Table 4.2 to be incubated for 24 hours in a rotatory shaker at 120 rpm. The pH was adjusted to 6.0 with NaOH and glucose was autoclaved separately and added from a stock solution of 50% (w/v) to a final concentration of 2% (w/v).

Table 4. 2: Medium composition for *Saccharomyces cerevisiae* strains plate cultivations and first pre-inoculum

Component	Concentration (g/L)
Yeast nitrogen base without amino acids	3.4
$(NH_4)_2SO_4$	10
Amino acids pool without uracil	3
Succinic acid	20
NaOH	12
Glucose	20

The second pre-inoculum was done in 500 ml shake-flasks containing 200 ml working volume of synthetic medium with the following composition:

Table 4. 3: Medium composition for the cultivation of *Saccharomyces cerevisiae* strains for α-D-glucuronidase production

Component	Concentration (g/L)
Yeast nitrogen base without amino acids	6.7
$(NH_4)_2SO_4$	10
KH_2PO_4	2.4
Casamino acids	10

The pre-inoculum was incubated for 24 hours in a rotatory shaker (New Brunswick Scientific, Edison, W. J., U.S.A) at 200 rpm. To avoid an unnecessary long lag phase, all inoculum sizes were 10% of the cultivations working volume. In addition, the medium composition was the same for the pre-inoculum and the bio-reactor. The shake-flasks pre-inoculum contained glucose at 2% (w/v).

4.2.2. Mode of cultivation for production of α-L-arabinofuranosidase and α-D-glucuronidase

The production of α-L-arabinofuranosidase was performed in 125 ml shake-flasks cultures. Biomass formation was achieved in the pellet form and harvested after 88 hours of cultivation. The biomass was filtered and washed with distilled water through *Mira* cloth, transferred to pre-weighted aluminium foils, dried firstly at 50 °C in an oven and then in a desiccator until constant weight was verified. The supernatant containing the extracellular produced enzyme was filtered in a 0.22 µm paper and kept at 4°C.

The production of α-D-glucuronidase was done in a 20 L bioreactor (New Brunswick Scientific BioFlo IV, working volume 16 L) (Anane, 2013). Fed-batch cultivations are best suited to achieve high biomass formation and product yields in *Saccharomyces cerevisiae*; at the same time minimizing the formation of inhibitory metabolites such as ethanol and glycerol (Hardjito *et al.,* 1993).

4.2.3. Characterization of α-L-arabinofuranosidase

The production of recombinant α-L-arabinofuranosidase in shake-flasks was monitored by testing the activity against *para*-nitrophenyl-α-arabinofuranoside (*p*-NPA from Sigma Aldrich). The reaction mixture (100 µl) consisted of 25 µl of 5 mM *p*-NPA, 25 µl distilled water, 25 µl of enzyme supernatant (appropriately diluted 2x) and 25 µl of citrate buffer pH (5.0). The reaction was carried out at 40 °C for 10 minutes and terminated by adding 100 µl of a 1M Na_2CO_3 solution. The reaction mixture was diluted 5x and the absorbance was measured in a spectrophotometer at 405 nm. The arabinofuranosidase activity was calculated as the amount of *p*-NP released in nmol per ml per second (nkat/ml).

4.2.4. Partial purification and characterization of α-D-glucuronidase

The downstream processing (Figure 4.1) for product recovery started with biomass separation of the fermentation broth from the enzyme supernatant using a continuous centrifuge system (flow rate of 2 L/min). The resultant stream was filtered using a peristaltic pump through a 0.22 µm filter cassette and then 35-fold concentrated on a Pellicon® 5 kDa cassette membrane. This step was crucial to increase enzyme concentration and remove cultivation waste metabolites and unreacted medium components in the enzyme mixture. This fraction was 10-fold concentrated on the Amicon system (Amicon Division USA) 30 kDa cut-off membranes. The supernatant was kept at 4° C and sodium azide was added to a concentration of 0.02% (w/v) to prevent microbial growth.

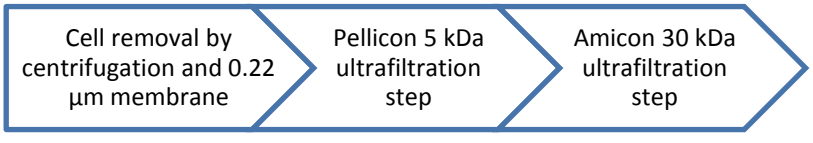


Figure 4.1: Downstream processing steps for α-D-glucuronidase partial purification

The crude supernatant, Pellicon and Amicon concentrates were assessed for enzyme quantification using 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) protocol described by Laemmli (Laemmli, 1970), and the protein bands were visualized in the gel using silver staining. The concentration of α-D-glucuronidase was estimated using image analysis (Image J® software) and densitometry, using bovine serum albumine (BSA) for the standard curve. The molecular weight was estimated using Page Ruler Prestained Protein Ladder from Fermentas with a molecular weight range from 10 to 170 kDa. The extent of glycosylation of the recombinant α-D-glucuronidases was visualized from band shifts in the SDS-PAGE.

4.2.5. α-D-glucuronidase substrate specificity

This subtopic involved assessing the substrates with which the α-D-glucuronidase reacts, translated by the release of 4-*O*-methyl glucuronic acid side-chains. The Agu1B and Agu1A α-D-glucuronidases activities were assessed in aldouronic acids and polymeric xylans extracted using the Höije protocol. The mixture of aldo-tri,-tetra and penta uronic acids in a molar ratio of 2:2:1 was obtained from Megazyme, Ireland. The aldouronic acids mixture at 120 g/L, was diluted to 20 g/L and the activity tested with partially purified Agu1B and Agu1A α-D-glucuronidases at an enzyme dosage of 5 mg/g substrate. Beechwood, *E. grandis* (Höije), sugarcane bagasse (Höije) soluble xylans were prepared at concentrations of 35 g/L and the reactions carried out at the same enzyme dosage of 5 mg/g substrate. The reactions were incubated for 24 hours at 40°C.

4.3. Results

4.3.1. Expression of α-L-arabinofuranosidase in D15 (*abfB*) *Aspergillus niger*

α-L-arabinofuranosidase was extracellularly secreted in 2x traditional minimal medium. The enzyme activity was measured over time for a period of 88 hours. The fungal growth using the medium described in Table 4.1 led to an increase of enzyme activity concentration with a maximum value at 64 hours of 11.10 nkat ml^{-1}. After 64 hours, the enzymatic activity was not increased. The average biomass concentration at the harvest point was 37.41 g L^{-1} which corresponded to an enzyme activity of 10.18 nkat ml^{-1} (against p-NPA). The standard curve was obtained using p-NP as a standard at concentrations ranging from 1.5 to 25 mM giving a linear response with a R^2 of 0.9938 (Appendix B). The specific enzyme activity achieved at harvest (88 hours incubation) was equivalent to 296.71 nkat g^{-1} on a dry weight basis.

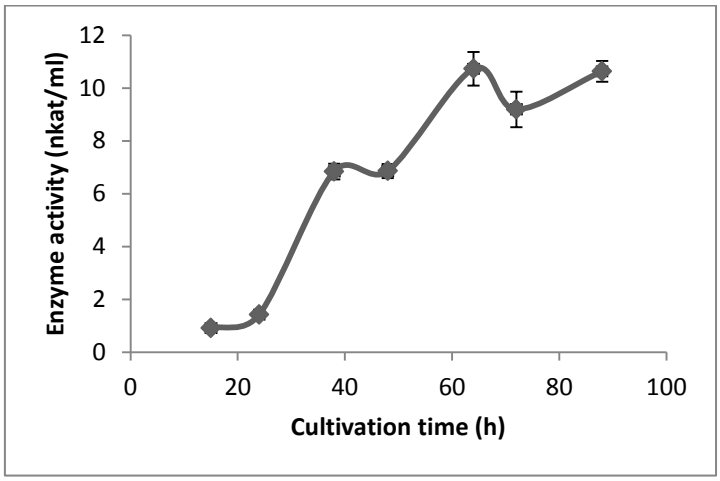


Figure 4.2: α-L-arabinofuranosidase production in shake-flasks: volumetric activity against p-NPA

4.3.3. Partial purification of α-D-glucuronidase

The protein recovery and partial purification steps for Agu1B and Agu1A α-glucuronidases are illustrated in Tables 4.4 and 4.5, respectively. The concentrations of the crude and partially purified fractions of both enzymes determined by densitometry analysis are shown in Figure 4.3. The 5 kDa ultrafiltration step aimed to decrease the amount of low-molecular weight components in the crude supernatant, such as residual glucose, ethanol and organic acids. It can be noted that about half of the total protein initially present was lost, leading to 59% and 51% recovery for Agu1B and Agu1A α-D-glucuronidases, respectively.

Table 4.4: Partial purification of Agu1B α-D-glucuronidase

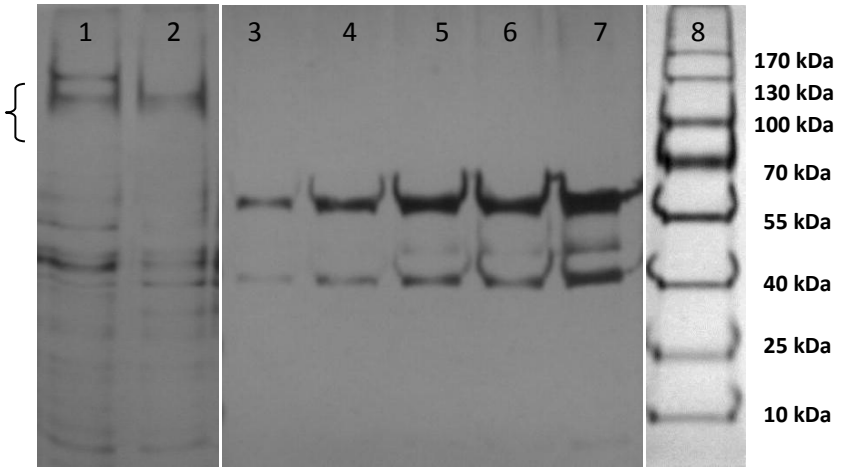
Step	Volume (L)	Concentration (mg/L)	Total amount of protein (mg)	Purification (fold)	% Recovery
Crude supernatant	10	8.3	83.6	1	100
Pellicon® 5 kDa cassete	0.5	98.6	49.2	20	59
Amicon® 30 kDa cut-off membrane	0.05	462.4	23.1	200	28

The 30 kDa membrane in the purification system was required since it separated into a much greater extent low-medium molecular weight compounds. In addition, it increased the concentration of the proteins of interest according to the molecular weight size. From step 2 to 3, the recovery factor is also higher leading to an increased concentration compared with the 5 kDa Pellicon® system. Nevertheless, the Pellicon® system was adequate as the first separation step due to large broth volumes during downstream processing.

Table 4.5: Partial purification of Agu1A α-D-glucuronidase

Step	Volume (L)	Concentration (mg/L)	Total amount of protein (mg)	Purification (fold)	% Recovery
Crude supernatant	10	11.6	116.2	1	1
Pellicon® 5 kDa cassete	0.5	117.7	58.9	20	51
Amicon® 30 kDa cut-off membrane	0.08	415.6	33.2	200	29

The final concentration of Agu1B and Agu1A α-glucuronidases was 462.4 and 415.6 mg L^{-1}, respectively. Enzymes are often quantified in terms of units of activity to differentiate the activity of a particular one from the other proteins present. In this study, the fraction purified with the 30 kDa membrane was quantified using densitometry and gel electrophoresis, performed in a single gel using a BSA standard curve (Figure 4.3, Appendix B).



Note: Lane 1-Agu1B glucuronidase at 30x dilution; 2-Agu1A glucuronidase at 30 x dilution; 3,4,5,6,7 BSA at 10, 20, 30, 40, 50 mg/L respectively; 8 protein marker

Figure 4.3: Gel electrophoresis for quantification of partially purified Agu1B and Agu1A α-glucuronidases. Quantification was performed in a single gel using bovine serum albumin as the standard protein.

The two α-D-glucuronidases had an electrophoretic mobility of 125 kDa on 10% SDS-PAGE. However, Agu1B α-D-glucuronidase displayed a double band corresponding to 125 and 130 kDa for the lower and the upper band, respectively and it indicates that it was possibly expressed with alternative glycosylation patterns. Agu1A α-D-glucuronidase displayed a single band, apparently with only one glycosylation pattern. The ultrapurification removed low molecular weight compounds but retained most of the proteins above 40 kDa as seen in Figure 4.3. The standard curve used for quantification of the partially purified enzymes is shown in Appendix B.

4.3.4. Quantitative analysis of 4-*O*-methyl glucuronic acid and substrate specificity of produced α-glucuronidases

The 4-*O*-methyl glucuronic acid was analysed by HPLC on an Xbridge™ Amide column (4.6 x 250 mm, 3.5 µm particle size) equipped with an Xbridge™ Amide precolumn (Waters). Column temperature was set to 30 °C and flow rate to 0.7 ml/min. The mobile phases used for elution were 10 mM ammonium acetate in 40% acetonitrile (A) and 10 mM ammonium acetate in 75% acetonitrile (B). Separation was carried out by gradient elution from 100% mobile phase B, decreasing to 50% mobile phase B over 39.5 minutes. This was followed by an increase back to 100% mobile phase B during the next 5 minutes. The mobile phase composition was then kept constant at 100% B for 5.5 minutes in order to equilibrate. Peaks were detected by a Varian 380-LC evaporative light-scattering detector. The HPLC-ELS chromatogram was obtained for the enzymatic hydrolysis of α-D-glucuronidase on a mixture of aldouronic acids containing aldo tri-, tetra and penta uronic acids (2:2:1) at 20 g L^{-1}, shown in Figure 4.4. The 4-*O*-methyl

glucuronic acid concentration was determined by relative quantitation using a D-glucuronic acid standard curve (shown in Appendix B). The retention time for the 4-*O*-MeGlcA was 32.84 minutes (Figure 4.5). The peak identification was based on elution profile comparison with the negative controls and the D-glucuronic acid. It can be seen that the elution profile of the aldouronic acids mixture (substrate control) differs from the one showing the enzymatic hydrolysis, with decrease and disappearance of the aldouronic acid mixture peaks at 13.85 min, 35.1 min, 41.30 min and 45.9 min, indicating the consumption of the substrates hydrolysed by α-D-glucuronidase (Figure 4.4).

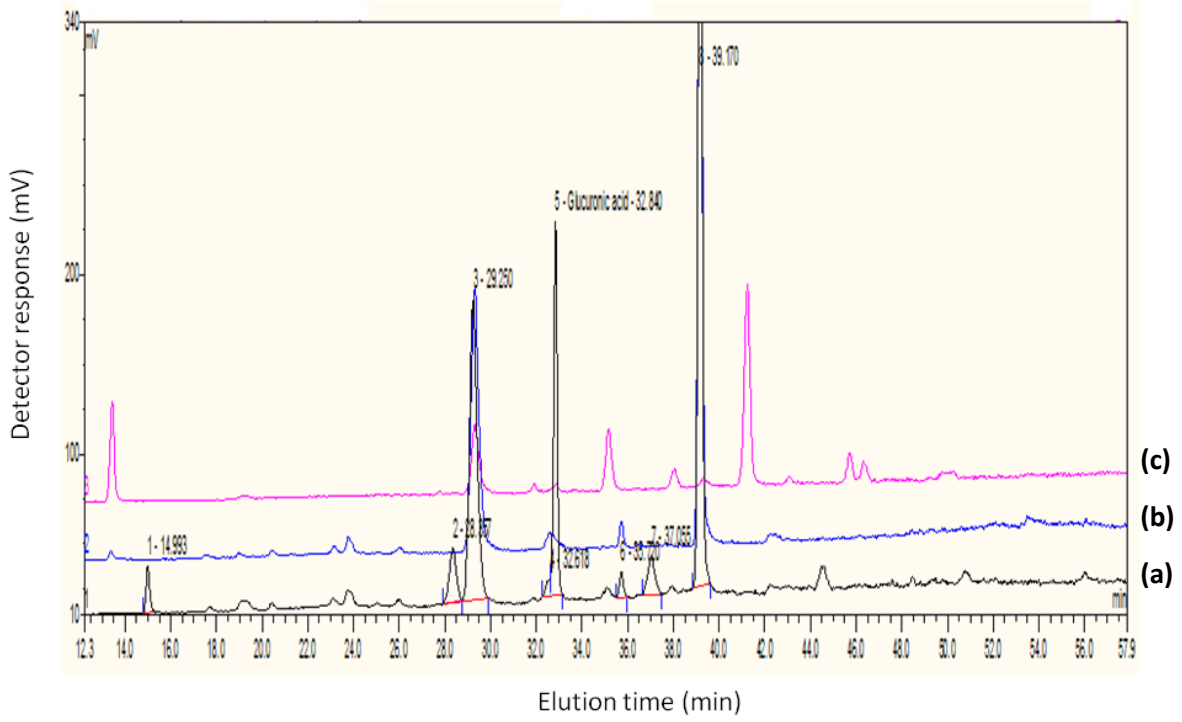


Figure 4.4: Quantitative analysis of 4-*O*-MeGlcA from the enzymatic hydrolysis of aldouronic acids at 20 g/L by Agu1B α-D-glucuronidase at 40 °C, 24 hours. Elution profiles: (a) Enzymatic hydrolysis of aldouronic acids by α-D-glucuronidase (black), (b) Enzyme control (blue), (c) substrate control (red)

The substrate preference of the two α-glucuronidases was assessed in a mixture of aldouronic acids containing tri-, tetra and penta uronic acids, in beechwood xylan (Sigma Aldrich), *E. grandis* xylan (Höije) and sugarcane bagasse xylan (Höije), using the previously described HPLC quantification method (Figure 4.4). The volumetric activities of Agu1B α-D-glucuronidase were 0.11, 0.10, 0.075 and 0.014 nkat ml^{-1} for aldouronic acids, beechwood, *E. grandis* (Höije) and sugarcane bagasse xylans respectively (data not shown). In contrast, Agu1A α- D-glucuronidase volumetric activity values were 0.022, 0.013 and 0.016 nkat ml^{-1} for aldouronic acids, beechwood and *E. grandis* xylans. No activity was verified for Agu1A α- D-glucuronidase on sugarcane bagasse xylan at the dosage of 5 mg g^{-1} substrate. The two enzymes displayed

different substrate specificities molecular properties as verified in Figure 4. 5. The Agu1B α-D-glucuronidase was able to release 70.3% of 4-*O*-MeGlcA residues in beechwood xylan, 18.6% in *E. grandis* xylan and 10.12% in sugarcane bagasse xylan (Figure 4.5). The substrate preference of Agu1A α-D-glucuronidase was not clear, even though it showed a maximum 4-*O*-MeGlcA release of 0.0014 mol L^{-1} in *E. grandis* xylan, which corresponded to 4.14% release of the total available 4-*O*-MeGlcA content (Figure 4. 5). However, the release of 4-*O*-MeGlcA from aldouronic acids was higher than in xylans, corresponding to 0.0019 mol L^{-1}.

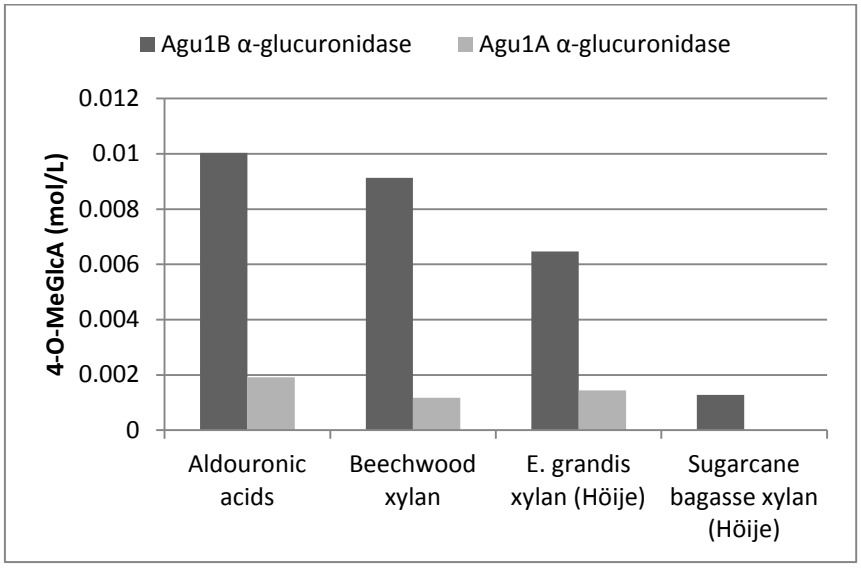


Figure 4. 5: Amount of 4-*O*-methyl glucuronic acid released by Agu1B and Agu1A α-D-glucuronidases at a dosage of 5 mg g^{-1} on aldouronic acids (20 g/L), beechwood, *E. grandis* and sugarcane bagasse xylans at 35 g L^{-1}

4.3. Discussion

4.3.1. Catalytic properties of α-L-arabinofuranosidase expressed from *Aspergillus niger* D15 *abfB*

The *Aspergillus niger* strain D15 *abfB* was able to selectively secrete α-L-arabinofuranosidase under the transcriptional control of glyceraldehyde-3-phosphate dehydrogenase (gpd_P). The crude α-L-arabinofuranosidase volumetric (10.18 nkat ml^{-1}) and specific (296.7 nkat g^{-1}) activities were close to the values obtained by Chimphango (Chimphango, 2010). Other studies have shown higher levels of α-L-arabinofuranosidase volumetric activity of 108 nkat ml^{-1} (Gomes *et al.,* 2000). However, these were accompanied by high xylanases and β-xylosidase activities (Gomes *et al.,* 2000). *A. niger* is known to be particularly effective in expressing several heterologous enzymes at high concentrations (Gokhale *et al.,* 1988; MacKenzie *et al.,* 1996; Chimphango, 2010).The protease deficient and non acidifying strain expressed α-L-arabinofuranosidase without any xylanases or xylosidase production. Therefore it was suitable to be applied in the enzymatic hydrolysis of polymeric xylans without compromising their structural integrity. The molecular weight characteristics, optimum pH and temperature were studied in depth elsewhere (Chimphango, 2010) and applied in this study.

4.3.2. Partial purification and substrate specificity of α-D-glucuronidases

Partial purification of crude Agu1B and Agu1A α-D-glucuronidases was performed in a two step process. The final concentrations for Agu1B and Agu1A α-D-glucuronidases were 462.4 and 415.6 mg L^{-1}, respectively. As compared with other production systems (Siika-aho *et al.,* 1994; Tenkanen & Siika-aho, 2000; Ryabová *et al.,* 2009), the total amount of protein was considerably higher due to concentration steps performed on the fermentation supernatant. For instance, Tenkanen and Siika-aho reported a purified fraction of enzyme of 118 mg/L using sequencial chromatography purification steps (Tenkanen and Siika-aho, 2000b).The ultrafitration using the 5 kDa and the 30 kDa cut-off membranes increased concentration levels, improving the activity levels required to hydrolyse the 4-*O*-MeGlcA side-chains in both oligomeric and polymeric xylan substrates. A lower recovery factor was verified in the first purification step using the 5 kDa cut-off membrane, possibly due to small volumetric losses during the filtration process and protein degradation as some stabilizing components are “washed out” from the enzyme supernatant.

The difference in the electrophoretic mobility of Agu1B and Agu1A α-D-glucuronidases (Figure 4.3) indicated that the two enzymes have different glycosylation patterns. Glycosylation of proteins expressed by *S. cerevisiae* strains has been reported earlier in literature. In particular, glycosylation of α-D-glucuronidase has been previously verified in *S. cerevisiae* with a molecular weight of 157 kDa (De Wet et al., 2006). In this study, the molecular weight of the α-glucuronidases is within the reported values of other expression systems (71-160 kDa) (Table 2. 2). The difference in the glycosylation results from a series of events that occur in the endoplasmic reticulum and the Golgi apparatus in eukaryotic organisms (Bretthauer and Castellino, 1999; Han *et al.,* 2005). Furthermore, the glycosylation differences could be attributed to the 5% difference present in the gene sequence homology between Agu1B and Agu1A α-glucuronidases.

GH115 Agu1B and Agu1A α-glucuronidases displayed different catalytic properties in both oligomeric and polymeric xylan substrates (Figure 4. 5). As opposed to GH67 α-glucuronidases, GH115 α-glucuronidases are able to recognise 4-*O*-MeGlcA units linked to reducing xylopyranosyl units (De Wet et al., 2006; Kolenová et al., 2010). At constant α-D-glucuronidase dosages and substrate concentrations, the Agu1B α-D-glucuronidase was able to release substantially higher amounts of 4-*O*-methyl glucuronic acid from aldouronic acids and polymeric xylans, as opposed to the Agu1A α-D-glucuronidase (Figure 4. 5). Agu1B α-D-glucuronidase showed a clear trend in substrate preference, showing the highest release of 4-*O*-MeGlcA in short aldouronic acids. Agu1A α-D-glucuronidase was equally more active in short aldouronic acids than in polymeric xylan substrates.

The different activities reported for α-D-glucuronidase are difficult to be compared because the 4-*O*-methyl glucuronic acid is not commercially available and its synthesis is labour intensive. The colorimetric assay described in the literature lacks selectivity against the 4-*O*-MeGlcA released by the enzymatic reaction, which would turn the quantitative process easier. However, the activity values for Agu1B α-D-glucuronidase are lower than the ones reported previously in literature against oligomeric and polymeric substrates (Siika-aho *et al.,* 1994; Tenkanen and Siika-aho, 2000b; Kolenová *et al.,* 2010).

The release of 4-*O*-MeGlcA from the *E. grandis* xylan (Höije) was lower than for the model xylan from beechwood (Figure 4. 5). The two glucuronoxylans had the same type of substituents, but differed in purity levels and degrees of substitution. *E. grandis* xylan (Höije) had higher lignin and 4-*O*-MeGlcA contents as compared to the beechwood xylan. Lignin is an aromatic low molecular weight polymer that can cause steric hindrances during enzymatic attack (Wallace *et al.,* 1991). However, Agu1B α-D-glucuronidase had the lowest activity against the low-lignin

sugarcane bagasse xylan. This indicated that there are other factors that might inhibit the release of 4-*O*-MeGlcA from extracted xylans. It has been suggested that the degree and pattern of substitution and the type of the side-chains attached to the xylan backbone may play a significant role in how efficiently the enzymatic attack of α-D-glucuronidase takes place (Saake *et al.,* 2001; Linder *et al.,* 2003; Westbye *et al.,* 2007). Furthermore, the presence of arabinose side groups, in addition to 4-*O*-MeGlcA, in sugarcane bagasse xylan propose the combined use of α-L-arabinofuranosidase with α-D-glucuronidase to enhance the 4-*O*-methyl glucuronic acid release and cause xylan precipitation in water.

The release of 4-*O*-methyl glucuronic acid is the key factor to improve the functional properties of xylans, mainly morphological, solubility and rheological properties. Agu1B and Agu1A α-D-glucuronidases are suitable enzymes to be studied and employed to produce functional xylans using enzymatic hydrolysis methods (investigated further in Chapter 5).

4.4. Conclusions

A. niger recombinant strain *abfB* was able to produce α-L-arabinofuranosidase with similar volumetric and specific activities as reported by Chimphango (Chimphango, 2010), thus suitable to be utilised for selective hydrolysis of the arabinoglucuronoxylan extracted from sugarcane bagasse by the Höije protocol.

Partially purified Agu1B and Agu1A α- D-glucuronidases were 58 times more concentrated than the crude supernatant and were able to release 4-*O*-MeGlcA from both polymeric and oligomeric xylan substrates. In addition, Agu1B α-D-glucuronidase had a higher preference for aldouronic acids, showing a clear trend in the substrate specificity for beechwood xylan, *E. grandis* and sugarcane bagasse xylans (Höije). However, Agu1A α-D-glucuronidase activity was much lower in all substrates, as compared with Agu1B α-glucuronidase. The two α-D-glucuronidases showed significant differences in product release, fact that might be due to glycosylation differences, which will be investigated further (Chapter 5).

Agu1B and Agu1A α-D-glucuronidase are innovative and potential accessory enzymes that can be employed to improve the functional and chemical properties of hardwood glucuronoxylans and grass-type arabinoglucuronoxylans at mild and selective conditions, without degradation of the xylan polysaccharide backbone.

CHAPTER 5: Selective enzymatic hydrolysis of xylans: reaction kinetics and optimisation

5.1. Introduction

In recent years much awareness has evolved with respect to the increase of CO_2 atmospheric emissions and oil price fluctuations. Plant biomass is the only renewable source of carbon available on earth and as such, considerable research has been implemented to improve the economics of biomass fractionation into valuable product streams. After cellulose, hemicellulose is the most abundant class of polysaccharide biopolymers. Xylan is the main hemicellulose in hardwoods and grasses, occurring between 15-30% (DW) in hardwood species and 20-35% (DW) in grasses and cereals (Timmell, 1967). Xylans also display structural differences depending of the feedstock source (Kabel *et al.,* 2007). In glucuronoxylans approximately every tenth xylose unit is substituted by 4-*O*-methyl glucuronic acid side groups whereas arabinoglucuronoxylans are more branched, containing arabinofuranosyl and 4-*O*-methyl glucuronic acid groups in an average ratio to xylose units of approximately 1.3:2:10 (Timell, 1967b; Izydorczyk and Biliaderis, 1995; Evtuguin *et al.,* 2003; Ebringerová, 2005). The xylans utilized in this study were obtained from feedstocks of great economic importance. From Chapter 3, they were found to have 4-*O*-MeGlcA to xylose ratios ranging from 1:12 to 1:2. The ratio of arabinose to xylose in the arabinoglucuronoxylan from sugarcane bagasse was 1:18, lower than the ratios reported in the literature (Chapter 2; section 2.1.1. Xylan structure and supply.

The presence of 4-*O*-methyl glucuronic acid and arabinofuranosyl side chains is the main factor that causes the high solubility of xylan in water (He *et al.,* 1994). The degree and the pattern of substitution of xylan have a remarkable influence on the conformational arrangement and intermolecular interactions, affecting their functional and chemical properties, notably the solubility in different liquids (Sedlmeyer, 2011). About 90-95% of the hemicelluloses and lignin are degraded and dissolved during the kraft cooking and usually re-precipitate due to random removal of the side chains that are hydrolysed under the strongly alkaline conditions (Polizeli *et al.,* 2005). Xylan can be more efficiently recovered from lignocellulose pulping processes without compromising the pulp quality by using pre-extraction mild alkali protocols, thereby preserving its structural properties. In addition, recovered xylan is amorphous and soluble in

water making its use substantially limited. The isolation of glucuronoxylans using alkali removes most of the acetyl groups.

Xylans with a DP that is higher than 10 (Chimphango, 2010) can be modified to improve their functional properties and incorporated in different end-uses. Insoluble particles of xylan and hydrogel aggregates are particularly valuable. Xylan nano and microparticles can be used as blending composite films (Saxena *et al.,* 2011), pulp and pharmaceutical additives and hydrogels (Gupta *et al.,* 2002). Xylan hydrogels are attractive for slow release of substances because xylan possesses anti-tumoral, anti-cancer and biocompatibility properties (Polizeli *et al.,* 2005). It has been reported that xylans can act as immunostimulators, activating human macrophages (Patel *et al.,* 2007). For example, studies have shown interesting applications of the insoluble arabinoglucuronoxylan from corn cobs as a colon-specific drug carrier, due to its ability to remain undigested in the stomach and small intestine (Oliveira *et al.,* 2010). They can replace cellulose and starch additives; since starch is mostly limited to food resources. The preparation of hydrogels from aspen wood glucuronoxylan in combination with chitosan has been reported (Hromádková *et al.,* 2005).

Physical treatments like sonication have been previously applied in different hemicelluloses including xylan but these involve harsh conditions (Ebringerová *et al.,* 1997; Sun *et al.,* 2002; Hromadkova and Ebringerová, 2003). Chemical modification has been widely explored as a way of forming insoluble particles of xylan and these include: cationization, esterification, etherification, and treatment with polyvinyl alcohol (PVA) (Ebringerová *et al.,* 1994; Tanodekaew *et al.,* 2006; Ren *et al.,* 2009). Chemical treatments have been found to reduce the biodegradability of xylan by 45% and in some cases harsh acidic conditions caused degradation of the xylan insoluble particles formed (Sun *et al.,* 2011). In this chapter, the enzymatic treatment of glucuronoxylans and arabinoglucuronoxylan by the α-L-arabinofuranosidase and α-D-glucuronidase is assessed.

In enzyme catalysis, enzymes are thought to bind to substrates in a specifically favourable position, reducing the activation energy required for a reaction to occur (Bailey & Ollis, 1986). The enzymatic hydrolysis of arabinoglucuronoxylans using α-L-arabinofuranosidase releases arabinofuranosyl sugar units, creating large non-substituted areas that favour intermolecular bonding and precipitation. Optimum conditions for formation of insoluble particles of arabinoglucuronoxylan have been found with respect to reaction time, the enzyme dosage required and the substrate concentration to allow sufficient intermolecular aggregation (Chimphango, 2010).

The kinetics of the enzymatic hydrolysis of glucuronoxylans by recombinant α-D-glucuronidase has not been fully understood neither has the enzyme-substrate reaction conditions been optimised. In addition, the application of both α-L-arabinofuranosidase and α-D-glucuronidase can reduce enzyme production costs if there is a positive synergy with respect to the two. Previous studies on the synergy of both enzymes have been performed under limited α-D-glucuronidase dosages (Chimphango, 2010).

The most important factors in enzyme hydrolysis are the time, the substrate concentration and the enzyme dosage. Optimum pH and temperature for α-D-glucuronidase activity were consistently found to be in the range of 4.8-5.0 and 38-40°C, respectively (Ryabová *et al.,* 2009; Kolenová *et al.,* 2010). However, other enzyme-related factors can play a significant role in the whole process optimization. From Chapter 4, molecular weight differences between the two α-D-glucuronidases were confirmed by band shifts displayed in the SDS-PAGE. This is caused by distinct glycosylation patterns between the two enzymes and this can be a possible reason for different catalytic properties between Agu1B and Agu1A α-glucuronidases.

In this chapter, the focus will be on studying the rate of 4-*O*-methyl glucuronic acid release and the effect of substrate concentration, enzyme dosages and xylan composition factors on the 4-O-MeGlcA release and precipitation rates for beechwood, *E. grandis* and sugarcane bagasse xylans. The preparation and characterization of these xylans were described in Chapter 3. Optimum conditions for side-chain removal by α-D-glucuronidase and α-L-arabinofuranosidase were determined using response surface methodology (RSM). The amount of 4-*O*-methyl glucuronic acid released was quantified using HPLC to determine the extent of enzymatic modification. Functional properties of modifed xylans assessed included the viscosity/precipitation and particle morphology.

5.2. Materials and methods

5.2.1. Materials

The enzymatic hydrolysis was performed with four different types of xylan: beechwood xylan as a benchmark material, containing 89% of xylose content was obtained from Sigma Aldrich. The glucuronoxylan from *E. grandis* was extracted using two mild alkali protocols (Chapter 3), and the arabinoglucuronoxylan was extracted from sugarcane bagasse. The xylans were also characterized with respect to the monomeric neutral sugar content, the insoluble lignin, the molecular weight and the uronic acid content (Chapter 3). The xylans were in the solid state and stored in sealed containers at room temperature.

The speciality enzymes for selective removal of xylan side-chains were produced in the present study (Chapter 4). α-L-arabinofuranosidase was produced and characterized with a final volumetric activity of 10 nkat ml^{-1}. α-D-glucuronidase was produced and partially purified from two recombinant strains of *S. cerevisiae* with final concentrations of 462 mg/L and 449 mg/L for Agu1B and Agu1A α-D-glucuronidases, respectively. Enzymes were stored at 4°C with 0.02% of sodium azide as a preservative. The standards utilized in this study were purchased from Sigma Aldrich (D-glucuronic acid, L-arabinose; D-glucose, galactose, cellobiose).

5.2.2. Enzymatic modification of xylans: screening tests

The screening process was performed to locate the range of enzyme dosages at which precipitation of soluble xylan occurs, using Agu1B α-glucuronidase. The dosages tried for soluble beechwood xylan (Sigma) at 1% (w/v) final reaction concentration were 6.17, 4.11, 3.08, 2.16 and 0.92 mg/g substrate. The reactions were equilibrated with 0.05 M acetate buffer pH 5.0 to a volume of 5.0 ml. The reaction conditions are presented in Table 5.1.

Table 5.1: Enzyme dosages for modification of soluble beechwood xylan at constant substrate concentration

Dosage (mg/g)	Enzyme volume (ml) at 123.50 mg/L	Substrate volume (ml)
6.17	2.500	2.50
4.11	1.664	2.50
3.08	1.250	2.50
2.16	0.875	2.50
0.92	0.375	2.50

5.2.3. Deglycosylation of Agu1B α-D-glucuronidase

The deglycosylation of native Agu1B α-D-glucuronidase was done using the Endo H (Endoglycosydase H) kit purchased from New England BioLabs®. The reaction was carried out under denaturing and non-denaturing conditions to evaluate loss of activity due to enzyme denaturation effects. The deglycosylation reaction conditions involved the preparation of 4.58 ml of enzyme diluted with 3.82 ml of distilled water, 495 µL of reaction buffer and 46 µL of Endo H, making a total reaction volume of 8.94 ml. The reaction was incubated at 37°C for 24 hours. After that, a 5 µL aliquot was taken for SDS-PAGE for mobility shift analysis before enzymatic hydrolysis experiments.

5.2.4. Optimisation of the modification of the functional properties of glucuronoxylans using α-D-glucuronidase

The effect of the α-D-glucuronidase dosage, α-D-glucuronidase type and the xylan concentration were assessed using response surface methodology (RSM), with 3 central points and 22 runs for each type of glucuronoxylan, mainly from beechwood, *E. grandis* (Höije protocol) and *E. grandis* (Pinto protocol). The experimental runs were obtained using Design Expert® version 8.0.6, shown in Table 5. 2, Table 5. 3

Table 5. 4. Each xylan was treated with the two types of α-glucuronidases, at 40°C for 24 hours and the volume equilibrated to 5 ml with 0.05 M acetate buffer (pH 5.0). Statistical analysis included analysis of variance (ANOVA) and desirability function profiles, to maximise the release of 4-*O*-MeGlcA and viscosity increases with enzymatic modification.

Table 5. 2: Experimental design (CCD) obtained for hydrolysis of 9.8% (w/v) soluble beechwood xylan

Std	Run	Coded values Dosage	Glucuronidase dosage (mg/g)	Coded values Conc	Xylan conc %	Glucuronidase type
6	1	1.41	7.5	0	3.16	Agu1B
8	2	1	6.4	1.41	4.98	Agu1B
1	3	-1	1.14	-1	1.87	Agu1B
3	6	-1	1.14	1	4.45	Agu1B
9	8	0	3.79	0	3.16	Agu1B
4	11	1	6.4	1	4.45	Agu1B
5	13	-1.41	0.042	0	3.16	Agu1B
10	14	0	3.79	0	3.16	Agu1B
7	18	0	3.79	-1.41	1.33	Agu1B
11	20	0	3.79	0	3.16	Agu1B
2	22	1	6.4	-1	1.87	Agu1B
22	4	0	3.79	0	3.16	Agu1A
18	5	0	3.79	-1.41	1.3	Agu1A
21	7	0	3.79	0	3.16	Agu1A
15	9	1	6.4	1	4.45	Agu1A
13	10	1	6.44	-1	1.87	Agu1A
16	12	-1.41	0.042	0	3.16	Agu1A
20	15	0	3.79	0	3.16	Agu1A
12	16	-1	1.14	-1	1.87	Agu1A
19	17	0	3.79	1.41	4.98	Agu1A
17	19	1.41	7.53	0	3.16	Agu1A
14	21	-1	1.14	1	4.45	Agu1A

Table 5. 3: Experimental design (CCD) obtained for hydrolysis of 9.5% (w/v) soluble *Eucalyptus grandis* xylan (Höije)

Std	Run	Coded values Dosage	Glucuronidase dosage (mg/g)	Coded values Conc	Xylan conc %	Glucuronidase type
2	1	1	6.4	-1	1.9	Agu1B
5	2	-1.41	0.042	0	3.08	Agu1B
4	3	1	6.4	1	4.27	Agu1B
10	4	0	3.79	0	3.08	Agu1B
6	5	1.41	7.5	0	3.08	Agu1B
11	11	0	3.79	0	3.08	Agu1B
9	12	0	3.79	0	3.08	Agu1B
3	13	-1	1.14	1	4.27	Agu1B
8	15	0	3.79	1.41	4.76	Agu1B
7	18	0	3.79	-1.41	1.40	Agu1B
1	21	-1	1.14	-1	1.9	Agu1B
20	6	0	3.79	0	3.08	Agu1A
17	7	1.41	7.53	0	3.08	Agu1A
13	8	1	6.4	-1	1.9	Agu1A
21	9	0	3.79	0	3.08	Agu1A
14	10	-1	1.14	1	4.27	Agu1A
22	14	0	3.79	0	3.08	Agu1A
18	16	0	3.79	-1.41	1.40	Agu1A
19	17	0	3.79	1.41	4.76	Agu1A
16	19	-1.41	0.042	0	3.08	Agu1A
15	20	1	6.4	1	4.27	Agu1A
12	22	-1	1.14	-1	1.9	Agu1A

Table 5. 4: Experimental design (CCD) obtained for hydrolysis of 10% (w/v) Eucalyptus grandis xylan (Pinto)

Std	Run	Coded values Dosage	Glucuronidase dosage (mg/g)	Coded values conc	Xylan conc %	Glucuronidase type
6	1	1.41	7.5	0	3.2	Agu1B
9	2	0	3.79	0	3.2	Agu1B
21	3	0	3.79	0	3.2	Agu1A
19	4	0	3.79	1.41	5.03	Agu1A
1	5	-1	1.14	-1	1.9	Agu1B
13	6	1	6.4	-1	1.9	Agu1A
5	7	-1.41	0.042	0	3.2	Agu1B
14	8	-1	1.14	1	4.5	Agu1A
20	9	0	3.79	0	3.2	Agu1A
15	10	1	6.4	1	4.5	Agu1A
22	11	0	3.79	0	3.2	Agu1A
8	12	1	6.4	1.41	5.03	Agu1B
4	13	1	6.4	1	4.5	Agu1B
7	14	0	3.79	-1.41	1.36	Agu1B
3	15	-1	1.14	1	4.5	Agu1B
11	16	0	3.79	0	3.2	Agu1B
17	17	1.41	7.5	0	3.2	Agu1A
16	18	-1.41	0.042	0	3.2	Agu1A
2	19	1	6.4	-1	1.9	Agu1B
12	20	-1	1.14	-1	1.9	Agu1A
10	21	0	3.79	0	3.2	Agu1B
18	22	0	3.79	-1.41	1.36	Agu1A

5.2.5. Kinetics of the enzymatic hydrolysis of extracted xylans using α-D-glucuronidase

The enzymatic hydrolysis of xylans using Agu1B, Agu1A and deglycosylated Agu1B α-D-glucuronidases was monitored over a period of 72 hours at 40°C. The xylan concentrations and α-D-glucuronidase dosages were varied at a low and a high level, as described in Table 5. 5. The final reaction volume was 15 ml in 0.05 M acetate buffer (pH 5.0). Samplings were performed at time 0, followed by 1.5, 3 and 24 hours intervals. The 4-*O*-methyl glucuronic acid released with time was measured using HPLC and the change in viscosity with time using a rheometer (Physica MCR501) at a shear rate of 10.3 s^{-1} and 25°C.

Table 5. 5: Enzymatic hydrolysis on xylans at different enzyme dosages and substrate concentrations at 40°C for 72 hours.

Substrate	Reaction 1: Low level	Reaction 2: High level
Beechwood xylan	Glucuronidase dosage: 1.14 mg/g	Glucuronidase dosage: 3.79 mg/g
	Xylan concentration: 1.87 %	Xylan concentration: 3.16 %
E. grandis xylan (Höije)	Glucuronidase dosage: 1.14 mg/g	Glucuronidase dosage: 3.79 mg/g
	Substrate concentration: 1.9 %	Xylan concentration: 3.08 %
E. grandis xylan (Pinto)	Glucuronidase dosage: 1.14 mg/g	Glucuronidase dosage: 3.79 mg/g
	Substrate concentration: 1.9 %	Xylan concentration: 3.08 %
Sugarcane bagasse xylan (Höije)	Glucuronidase dosage: 1.14 mg/g	Glucuronidase dosage: 3.79 mg/g
	Substrate concentration: 0.64%	Xylan concentration: 1.23 %

5.2.5. Enzymatic hydrolysis of sugarcane bagasse arabinoglucuronoxylan using α-L-arabinofuranosidase and α-D-glucuronidase

Optimal conditions using both enzymes were estimated using a central composite design (Design Expert® version 8.0.6) with a total of 10 runs. The experimental runs are described in Table 5.6. α-D-glucuronidase and α-L-arabinofuranosidase dosages were varied at a low level, a centre point and a high level, coded as -1, 0 and +1 respectively. The high and low levels for α-arabinofuranosidase and α-glucuronidase corresponded to 350 nkat/g combined with 6.4 mg/g and the low level to 150 nkat/g and 1.14 mg/g, respectively. The xylan concentration was kept constant at 1.23% and the reaction carried out in a 5 ml reaction mixture at 40°C for 24 hours. The action of each enzyme was performed separately to evaluate synergistic effects. The amount of arabinose and 4-*O*-MeGlcA released were measured using HPLC, whereas the precipitation of soluble xylan was measured using gravimetry.

Table 5.6: Experimental design (CCD) obtained for different levels of α-D-glucuronidase and α-L-arabinofuranosidase dosages in sugarcane bagasse xylan

Std	Run	Glucuronidase dosage (mg/g)	Arabinofuranosidase dosage (nkat/g)
9	1	3.79	250
5	2	0.042	250
10	3	3.79	250
6	4	7.53	250
8	5	3.79	391.42
3	6	1.14	350
1	7	1.14	150
2	8	6.44	150
4	9	6.44	350
7	10	3.79	108.57

5.2.6. Particle size analysis

The particle size analysis of modified xylans was conducted by a microscope system Olympus Cell^R system equipped with a F-view-II cooled CCD camera (Soft Imaging systems) and Xenon-Arc burner (Olympus Biosystems GMBH) as the light source. The Olympus UPlanApo N 100x/1.40 Oil ∞/0.17/FN26.5 were used as the objectives. The images were processed to remove unspecific background and the mean particle diameter calculated by the Soft Imaging systems software in the range between 0.2 and 20 µm. The samples were prepared by placing about 20 µL of modified xylan from the reaction mixture in a microscope slide for analysis.

5.3. Results

5.3.1. Effect of different α-D-glucuronidase dosages on precipitation of beechwood xylan

The screening process was performed to identify the range of enzyme dosages at which precipitation of soluble xylan occurs, using Agu1B α-D-glucuronidase. The change in viscosity of beechwood xylan as a function of time is shown in Figure 5.1. Agu1B α-D-glucuronidase caused precipitation of beechwood xylan at 1% (w/v) at 6.17, 4.11 and 3.08 mg/g. The change in viscosity is an indirect measurement of the extent of formation of insoluble particles in the reaction mixture. The viscosity of soluble beechwood xylan increased within 24 hours from 2.92 mPa.s to 7.6 mPa.s at 6.17 and from 2.2 mPa.s to 6.3 mPa.s at 4.11 mg/g substrate. The maximum viscosity reached was 24.7 mPa.s at 72 hours for 6.17 mg/g and 16.6 mPa.s for 4.11 mg/g substrate. The dosage of 3.08 mg/g only showed a slight increase in the viscosity at 48 hours from 2.8 mPa.s to 3.7 mPa.s. Dosages below 3.08 mg/g substrate did not show significant changes in solubility properties.

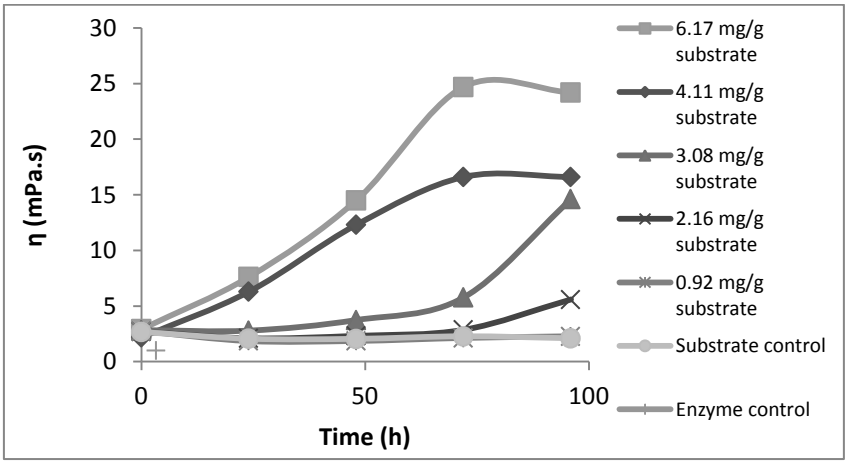


Figure 5.1: Time profile for the change in viscosity of soluble beechwood xylan (1% w/v) at enzyme dosages of 6.17, 4.11, 3.08, 2.16 and 0.92 mg/g substrate. Substrate and enzyme controls represent the conditions in the absence of enzymatic hydrolysis

5.3.2. De-glycosylation of Agu1B α-D-glucuronidase

The deglycosylation of Agu1B α-D-glucuronidase was performed as previously described in section 5.2.3. As seen from Figure 5. 2, the Endoglycosydase H was able to remove the N-linked oligosaccharides from the Agu1B α-glucuronidase. After the deglycosylation was performed (lane 4) both Agu1A and deglycosylated Agu1B α-D-glucuronidase showed an electrophoretic mobility of 125 kDa with a single band (lanes 1 and 4).

Moreover, the extent of glycosylation as a reason for the different catalytic properties between the native Agu1B and Agu1A α-D-glucuronidases can be investigated by carrying out kinetic experiments for both the native and deglycosylated enzymes.

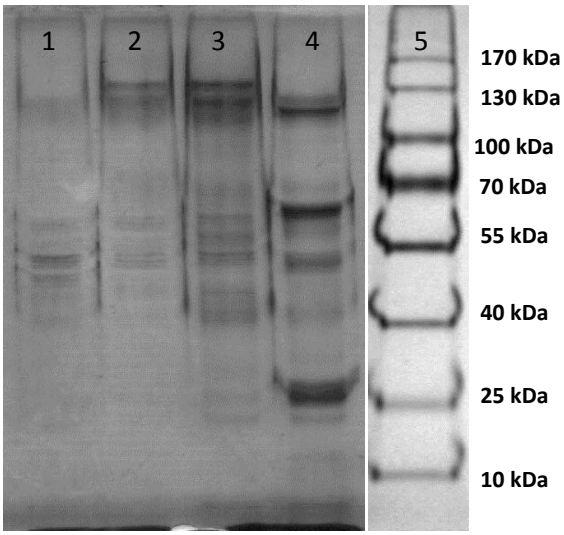


Figure 5. 2: Lanes 1-4 from left to right top bands: Agu1A glucuronidase, non deglycosylated Agu1B glucuronidase, Agu1B deglycosylation negative control and deglycosylated Agu1B glucuronidase; lane 5: protein marker

5.3.3. Time-course profile of 4-*O*-methyl glucuronic acid release and change in viscosity of polymeric xylans

The rate at which the product formation and change in functional properties occurred was assessed for beechwood xylan, *E. grandis* xylan (Höije), *E. grandis* xylan (Pinto) and sugarcane bagasse xylan. In fact, time is one of the most important factors in enzyme catalysis and therefore should be studied in most optimization processes. In this study, the enzyme kinetics was evaluated by finding the time at which no more release of product occurs, or when the functional properties of the xylans reach a plateau at the given conditions.

The release of 4-*O*-methyl glucuronic acid and the change in viscosity as a function of time were obtained for beechwood, *E. grandis* (Höije), *E. grandis* (Pinto) and sugarcane bagasse (Höije) xylans at low and high enzyme-substrate conditions. Figures 5.3a and 5.3b show the time course of the reaction at low conditions, with α-D-glucuronidase dosage of 1.14 mg/g and substrate concentrations of 1.87% for beechwood xylan and 1.9% for *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto). At these conditions, the rate at which the Agu1B and Agu1A α-D-glucuronidases released 4-*O*-methyl glucuronic acid among the three types of glucuronoxylans was remarkably different.

The differences in the reaction rate between these two α-glucuronidases can be verified at any given time; at 6 hours of hydrolysis Agu1B α-D-glucuronidase was able to release 85%[4] (1.23 g/L) of the 4-*O*-MeGlcA attached to the backbone of beechwood xylan, whereas Agu1A α-D-glucuronidase only had liberated 12% (0.17 g/L) of the available 4-*O*-MeGlcA. Agu1A α-D-glucuronidase did not liberate any 4-*O*-MeGlcA in *E. grandis* (Höije) and *E. grandis* (Pinto) xylans after 6 hours of hydrolysis, whereas Agu1B α-D-glucuronidase released 14% (0.56 g/L) and 34% (0.45 g/L) of 4-*O*-MeGlcA in *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto), respectively. The difference in catalytic properties between these two enzymes was verified earlier in Chapter 4.

The 4-*O*-MeGlcA yield over 72 hours of hydrolysis using Agu1B α-D-glucuronidase was 96% (1.39 g/L) for beechwood xylan, 19% (0.76 g/L) for *E. grandis* xylan (Höije), 49% (0.66 g/L) for *E. grandis* xylan (Pinto) and 25% (0.12 g/L) for sugarcane bagasse xylan (Höije) (Figures 5.3a and 5.3b).

In all cases, there was no increase in the viscosity due to liberation of 4-*O*-MeGlcA at 1.14 mg/g of α-D-glucuronidase dosage, combined with 1.8% (w/v) beechwood xylan, 1.9% (w/v) for *E. grandis* xylan (Höije and Pinto) and 0.64% (w/v) for sugarcane bagasse xylan (Höije). The viscosity profiles even showed a slight decrease with time (Figures 5.3a, 5.3b), when the release of 4-*O*-MeGlcA caused by the enzyme was not sufficient to promote aggregation behaviour from the linear regions in the polymer.

[4] Yield of the reaction was calculated based on the xylan concentration values in the reaction and the 4-*O*-methyl glucuronic acid content (Chapter 3).

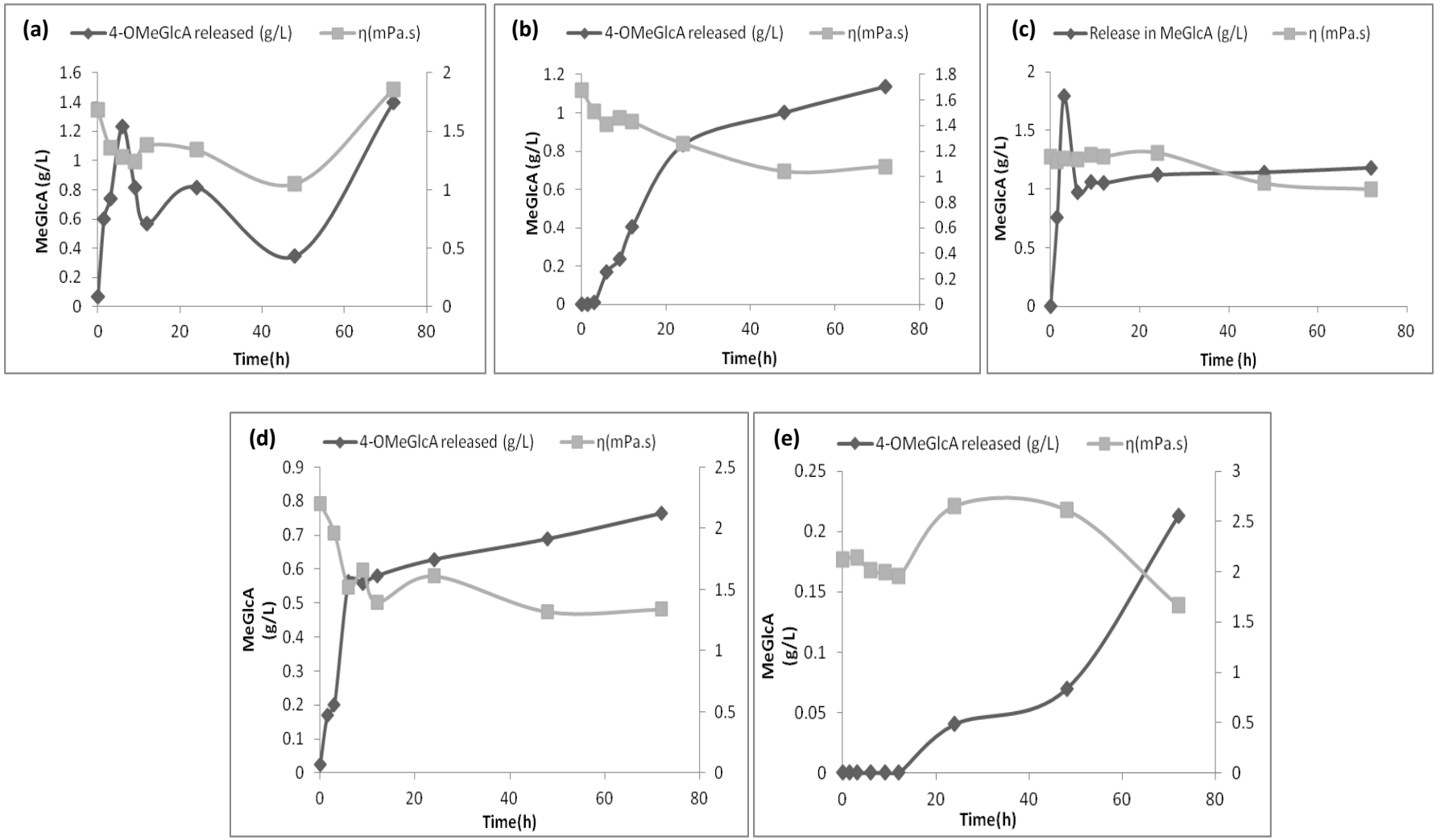


Figure 5. 3a: Time-course hydrolysis at α-D-glucuronidase dosages of 1.14 mg/g for: (a) beechwood xylan (1.8% w/v) and Agu1B α-glucuronidase, (b) beechwood xylan (1.8% w/v) and Agu1A α-glucuronidase, (c) beechwood xylan (1.8% w/v) and deglycosylated Agu1B α-glucuronidase, (d) *E. grandis* xylan (Höije) (1.9% w/v) and Agu1B α-glucuronidase, (e*) E. grandis* xylan (Höije) (1.9% w/v) and Agu1A α-glucuronidase. 4-*O*-MeGlcA: 4-*O*-methyl glucuronic acid; η: viscosity

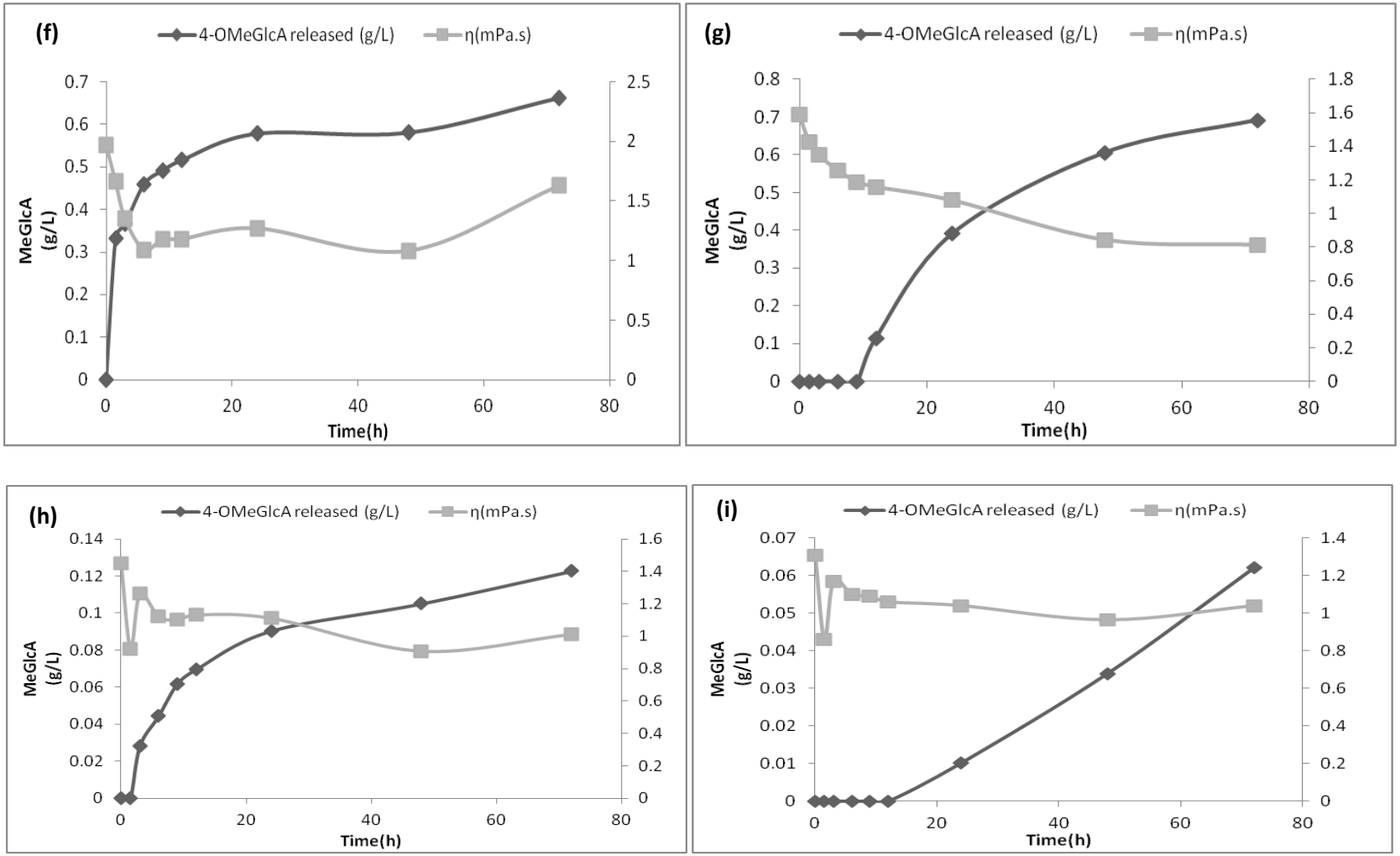


Figure 5.3 b: Time-course hydrolysis at α-D-glucuronidase dosages of 1.14 mg/g for: (f) *E. grandis* xylan (Pinto) (1.9% w/v) and Agu1B α-glucuronidase, (g) *E. grandis* xylan (Pinto) (1.9% w/v) and Agu1A α-glucuronidase, (h) Sugarcane bagasse xylan (Höije) (0.64% w/v) and Agu1B α-glucuronidase, (i) Sugarcane bagasse xylan (Höije) (0.64% w/v) and Agu1A α-glucuronidase. 4-*O*-MeGlcA: 4-*O*-methyl glucuronic acid; η: viscosity

In addition, at low enzyme-substrate hydrolysis conditions, the hydrolysis of beechwood xylan showed an unexpected drop in the 4-*O*-MeGlcA concentration when using both the native and deglycosylated Agu1B α-glucuronidases (Figure 5. 3). The decrease in the product concentration using Agu1B α-D-glucuronidase was verified at 6 hours when 85% (1.23 g/L) of the available side-chains were removed from the beechwood xylan backbone and 3 hours for the deglycosylated Agu1B α-D-glucuronidase corresponding to 100% (1.79 g/L) liberation of 4-*O*-MeGlcA side-groups (Figure 5.3a). After 6 hours of hydrolysis of beechwood xylan with the native Agu1B α-D-glucuronidase, the concentration of 4-*O*-MeGlcA was oscilating at with a final increase at 72 hours to 1.39 g/L, corresponding to 97% of 4-*O*-MeGlcA released. This also coincided with an increase in the viscosity at 72 hours. With the deglycosylated Agu1B α-D-glucuronidase, after the drop in the concentration of 4-*O*-MeGlcA, the viscosity was not further increased (Figure 5. 3a). The decrease in product concentration can be attributed to product inhibition effects, associated with low substrate concentrations in the enzymatic reaction.

Figures 5.4a and 5.4b show the time-course reaction of each substrate at high enzyme-substrate conditions, corresponding to 3.79 mg/g α-D-glucuronidase dosage, 3.16% (w/v) beechwood xylan, 3.08% (w/v) *E. grandis* xylan (Höije), 3.2% (w/v) *E. grandis* xylan (Pinto) and 1.23% (w/v) sugarcane bagasse xylan (Höije). The action of Agu1B α-D-glucuronidase on beechwood xylan was monitored over 72 hours.

The release of 4-*O*-MeGlcA reached a plateau at 9 hours, after which no significant release of product was verified. This indicated that the maximum specific reaction rate increased proportionally with the increase in the xylan concentration, thus reducing the likelihood of occurrence of product inhibition effects. Agu1B α-D-glucuronidase released 90% (2.18 g/L) of the available 4-*O*-MeGlcA in beechwood xylan, leading to an increase in viscosity after 24 hours from 2.3 to 230 mPa.s. The deglycosylated Agu1B α-D-glucuronidase showed a similar behaviour, with 75% of 4-*O*-MeGlcA side-chains released after 24 hours of hydrolysis. However, such release was equally able to increase the viscosity of soluble beechwood xylan from 1.85 to 208 mPa.s, after 24 hours of hydrolysis (Figure 5. 4a).

The reaction of Agu1B α-D-glucuronidase with *E. grandis* xylan (Höije) released a maximum of 19% of the available 4-*O*-MeGlcA at 24 hours of hydrolysis, leading to an increase in viscosity from 4.97 mPa.s to 133 mPa.s (Figure 5. 4a).

The reaction of Agu1B α-D-glucuronidase with *E. grandis* xylan (Pinto) released 43% of the 4-*O*-MeGlcA side-chains at 24 hours with a maximum release of 48% at 72 hours. This yield led to an increase in the viscosity from 4.4 mPa.s to 18.7 mPa.s at 24 hours and 38.1 mPa.s at 72 hours. In

addition, the viscosity of *E. grandis* xylan (Pinto) did not increase in 24 hours of reaction (Figure 5.4b), despite of the higher yield of side-chain release, compared with *E. grandis* xylan (Höije).

The hydrolysis of Agu1B α-D-glucuronidase with sugarcane bagasse released 16% and 21% of the available 4-*O*-MeGlcA after 24 and 72 hours of hydrolysis respectively, without xylan precipitation or viscosity increases (Figure 5. 4b).

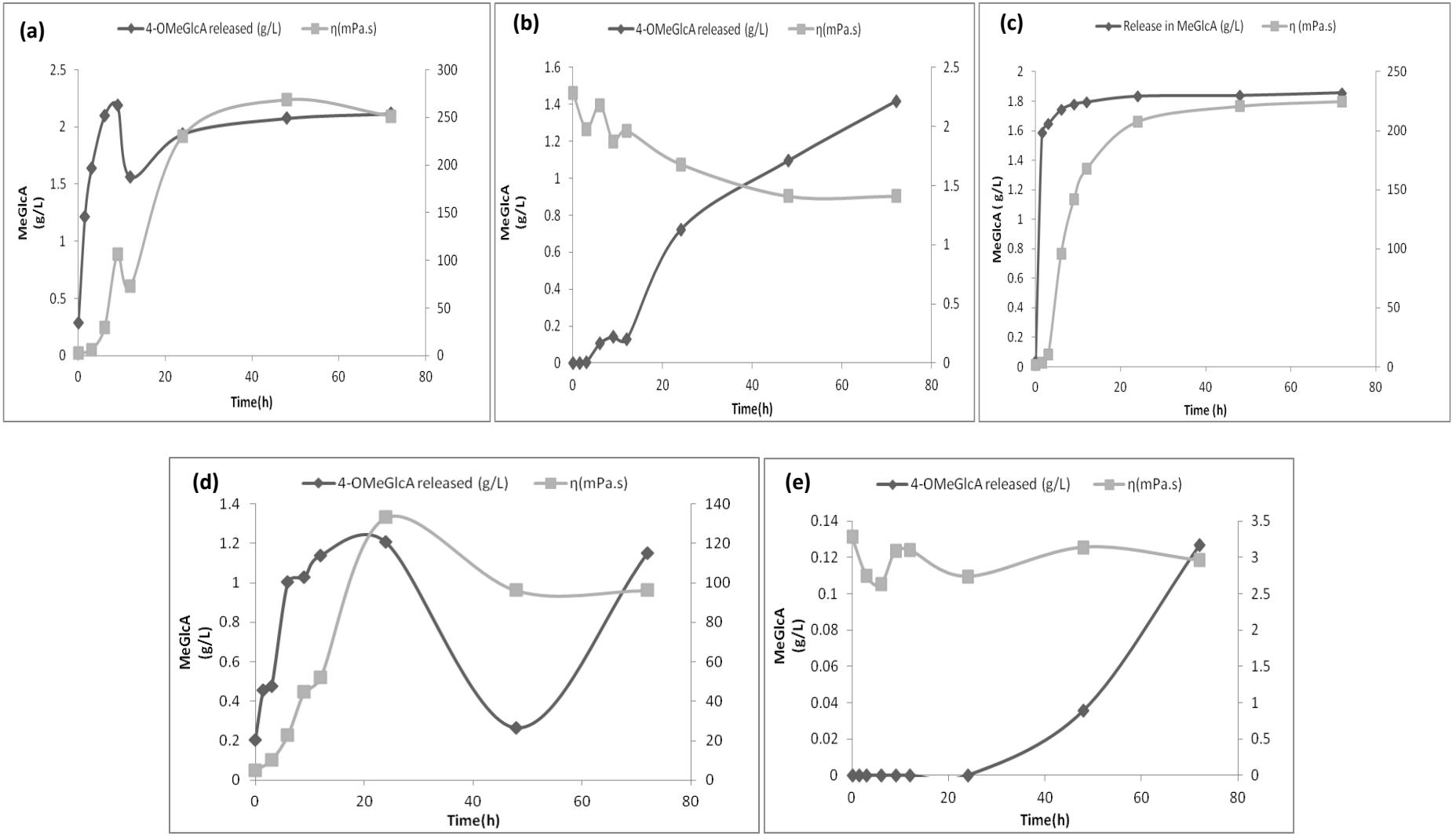


Figure 5. 4a: Time-course hydrolysis at α-D-glucuronidase dosages of 3.79 mg/g for: (a) beechwood xylan (3.16% w/v) and Agu1B α-glucuronidase, (b) beechwood xylan (3.16% w/v) and Agu1A α-glucuronidase, (c) beechwood xylan (3.16% w/v) and deglycosylated Agu1B α-glucuronidase, (d) *Eucalyptus grandis* xylan (Höije) (3.08% w/v) and Agu1B α-glucuronidase, (e) *Eucalyptus grandis* (Höije) (3.08% w/v)and Agu1A α-glucuronidase. 4-*O*-MeGlcA: 4-*O*-methyl glucuronic acid; η: viscosity

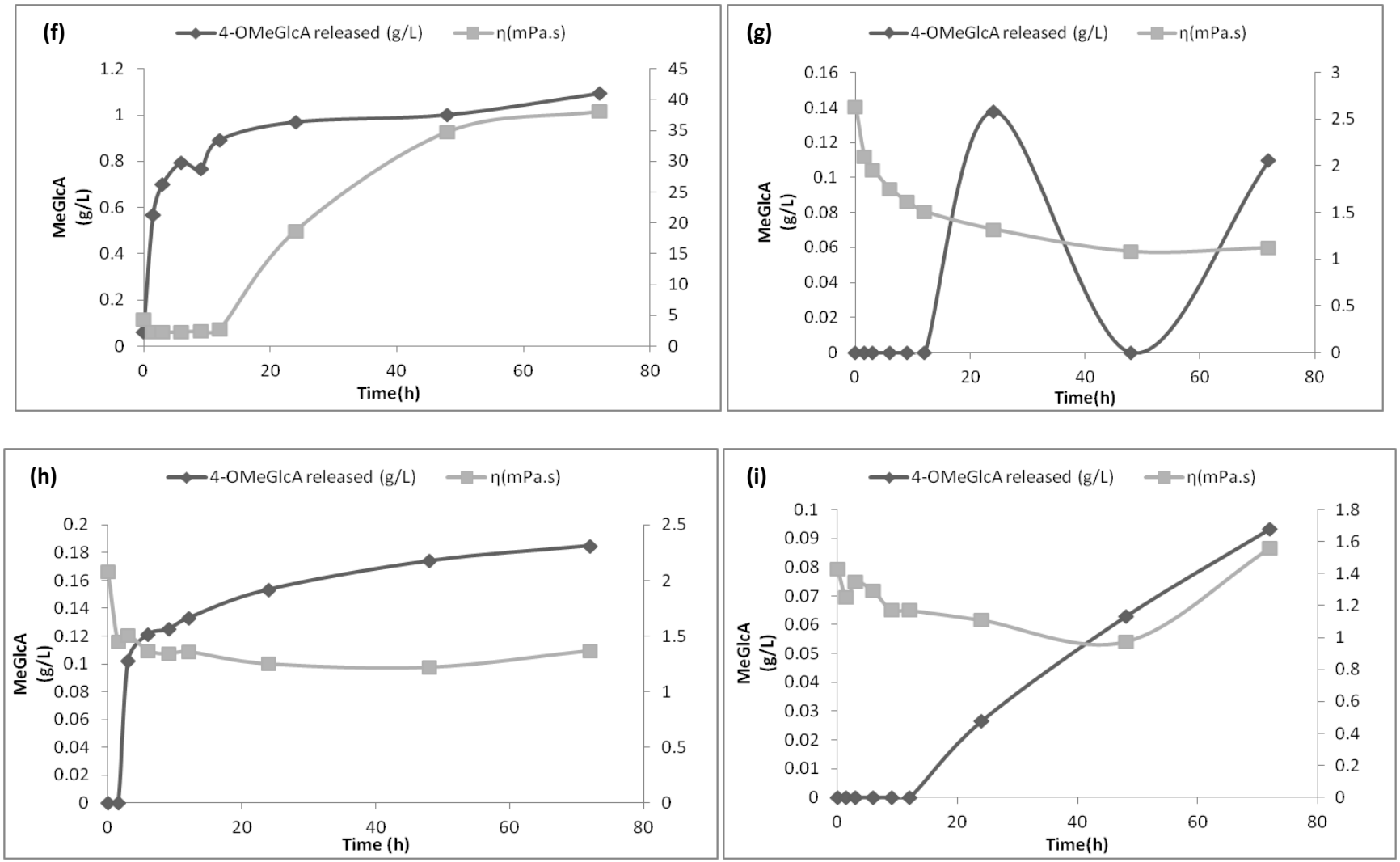


Figure 5.4b: Time-course hydrolysis at α-D-glucuronidase dosages of 3.79 mg/g for: (f) *Eucalyptus grandis* xylan (Pinto) (3.2% w/v) and Agu1B α-glucuronidase, (g) *Eucalyptus grandis* xylan (Pinto) (3.2% w/v) and Agu1A α-glucuronidase, (h) Sugarcane bagasse xylan (Höije) (1.23% w/v) and Agu1B α-glucuronidase, (i) Sugarcane bagasse xylan (Höije) (1.23% w/v) and Agu1A α-glucuronidase: 4-*O*-MeGlcA: 4-*O*-methyl glucuronic acid; η: viscosity

5.3.4. Effect of deglycosylation of Agu1B α-D-glucuronidase on the rates of 4-O-methyl glucuronic acid release and change in viscosity in polymeric xylans

To investigate the effect of glycosylation on the different catalytic properties of Agu1B and Agu1A α-D-glucuronidases, Agu1B α-D-glucuronidase was deglycosylated and the reaction kinetics studied under the same conditions employed for the native Agu1B α-D-glucuronidase, using beechwood xylan as a substrate (Figure 5.3a). The time course hydrolysis in beechwood xylan (1.8% w/v) of the native and deglycosylated Agu1B α-D-glucuronidases at 1.14 mg/g showed a very close kinetic behaviour in the rate at which the 4-*O*-MeGlcA was released, as opposed to the Agu1A α-D-glucuronidase at the same dosage (Figure 5.3a).

The initial rate at which the product was released was higher for the deglycosylated Agu1B α-glucuronidase, releasing 52% (0.75 g/L) of the available 4-*O*-MeGlcA side-chains as compared to 42% (0.6 g/L) in the native one. Furthermore, both native and deglycosylated Agu1B α-glucuronidases were able to completely remove the 4-*O*-MeGlcA side-groups present in beechwood xylan after 72 hours of hydrolysis.

When the enzyme reaction is initiated, there is an initial period of time during which the intermediates lead to the formation of product, which starts to accumulate in the reaction mixture. After this initial state, the reaction rates change relatively slowly with time, so called steady-state kinetics (Cleland, 1970). Maximum specific reaction rates at steady-state were determined by finding the points were the reaction rate was constant before approaching zero at high enzyme-substrate conditions (Figure 5. 4a).

Maximum specific reaction rates found for beechwood and *E. grandis* (Höije) xylans treated with Agu1B α-D-glucuronidase (3.79 mg/g) were 0.18 and 0.24 h^{-1} (Appendix C). Beechwood xylan treated with deglycosylated Agu1B α-D-glucuronidase (3.79 mg/g) resulted in a specific reaction rate of 0.0154 h^{-1} (Appendix C). Higher specific rates indicated that the reaction released higher amounts of 4-*O*-MeGlcA per unit time, indicating a first order reaction type relationship with the substrate. A low specific rate value of 0.0154 h^{-1} indicates a lower amount of product released per unit time approaching a zero order relationship type with regards to the substrate. The lower specific reaction rate using the deglycosylated Agu1B α-D-glucuronidase was due to the fact that from 0 to 1.5 hours (pre-steady state) there was a higher increment in product concentration, resulting in a higher initial rate, as compared with the native Agu1B α-D-glucuronidase (Figure 5.4a). Furthermore, after 1.5 hours the increments were lower as compared with beechwood and *E. grandis* (Höije) xylans treated with native Agu1B α-glucuronidase.

The maximum rates of change in viscosity were obtained in a similar manner, corresponding to 0.41 h^{-1} and 0.24 h^{-1} for beechwood xylan and *E. grandis* xylan (Höije), using Agu1B α-D-glucuronidase (3.79 mg/g). The maximum rate of change in viscosity for beechwood xylan using the deglycosylated Agu1B α-D-glucuronidase was 0.67 h^{-1} (Appendix C).

The maximum specific rates obtained were validated by comparing the predicted and the actual values at the region where both release of product and increase in viscosity were at the maximum (Figure 5. 5). The maximum specific rates were used to estimate the dependent variables using the following equations:

$$C_i = C_o e^{rt} \qquad \textbf{Equation 5. 1}$$

$$\eta_i = \eta_o e^{vt} \qquad \textbf{Equation 5. 2}$$

Where: $\mathbf{C_i}$ and $\mathbf{C_o}$ are the 4-*O*-MeGlcA concentrations at time t and time 0; $\boldsymbol{\eta}_\mathbf{i}$ and $\boldsymbol{\eta}_\mathbf{o}$ the viscosities at time t and 0; **r** and **v** represent the maximum specific rates of sugar release and increase in viscosity, respectively and **t** the correspondent hydrolysis time.

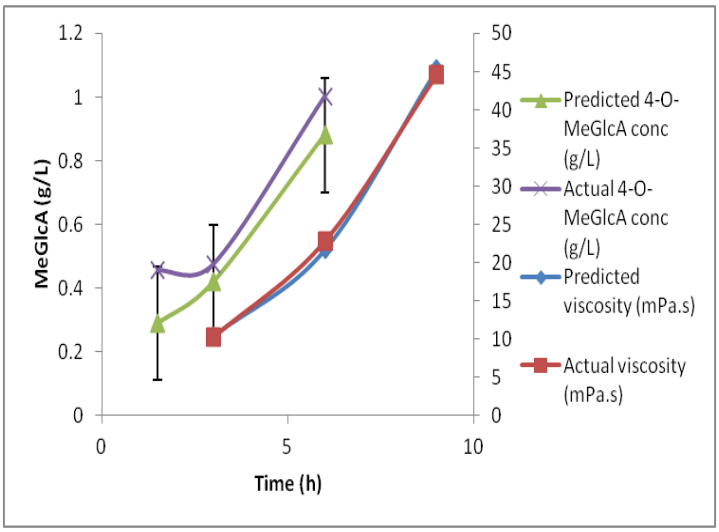


Figure 5. 5: Predicted versus actual values of 4-*O*-methyl glucuronic acid released and viscosity with *E. grandis* xylan (Höije) and Agu1B α-D-glucuronidase. Predicted values were determined using the specific rates of product release and viscosity

The increase in 4-*O*-MeGlcA and viscosity was exponential and the maximum specific rates were substituted in Equations 5.2 and 5.3 to predict the concentration of 4-*O*-MeGlcA and the viscosity before the system reaches the plateau (steady-state). The maximum specific rate of 4-*O*-MeGlcA release for *E. grandis* xylan (Höije) reproduced acceptable estimations with a negative deviation from the actual value from 11 to 36% (Figure 5. 5).

Maximum specific rate of viscosity increases led to positive errors of 1.7% and a negative deviation of 5%. This indicated that from the product release profile, the calculated specific rates can be used to predict the response variables within a range of approximately 64-98% level of confidence. The specific rates for beechwood xylan were validated in a similar manner, showing deviations within the previously mentioned range. However, Equations 5.2 and 5.3 can not be used to predict the value of the response variables where the reaction rate is not constant (non-steady state kinetics) or the reaction conditions are not the same as described before.

5.3.5. Effect of the 4-O-methyl glucuronic acid release on change in viscosity of polymeric xylans

The minimum degree of substitution required to change the functional properties of the different glucuronoxylans could be estimated by analysing the relationship between yield of 4-*O*-MeGlcA released and viscosity increases (Figure 5. 6). The minimum yield of 4-*O*-MeGlcA release required to increase the viscosity was different for each glucuronoxylan in the study. When beechwood xylan (3.16%) was treated with the Agu1B α-D-glucuronidase (3.79 mg/g), the viscosity was only increased when 67% of the 4-*O*-MeGlcA side-groups was removed (Figure 5.6a). This value corresponded to a decrease of the initial 4-*O*-MeGlcA content from 7.72% to 2.54%. The hydrolysis of beechwood at the same conditions but using the deglycosylated Agu1B α-D-glucuronidase was able to cause an increase in the viscosity when 67% of the 4-*O*-MeGlcA was released (Figure 5.6b), which agrees with the minimum yield of 4-*O*-MeGlcA release when using the native Agu1B α-D-glucuronidase.

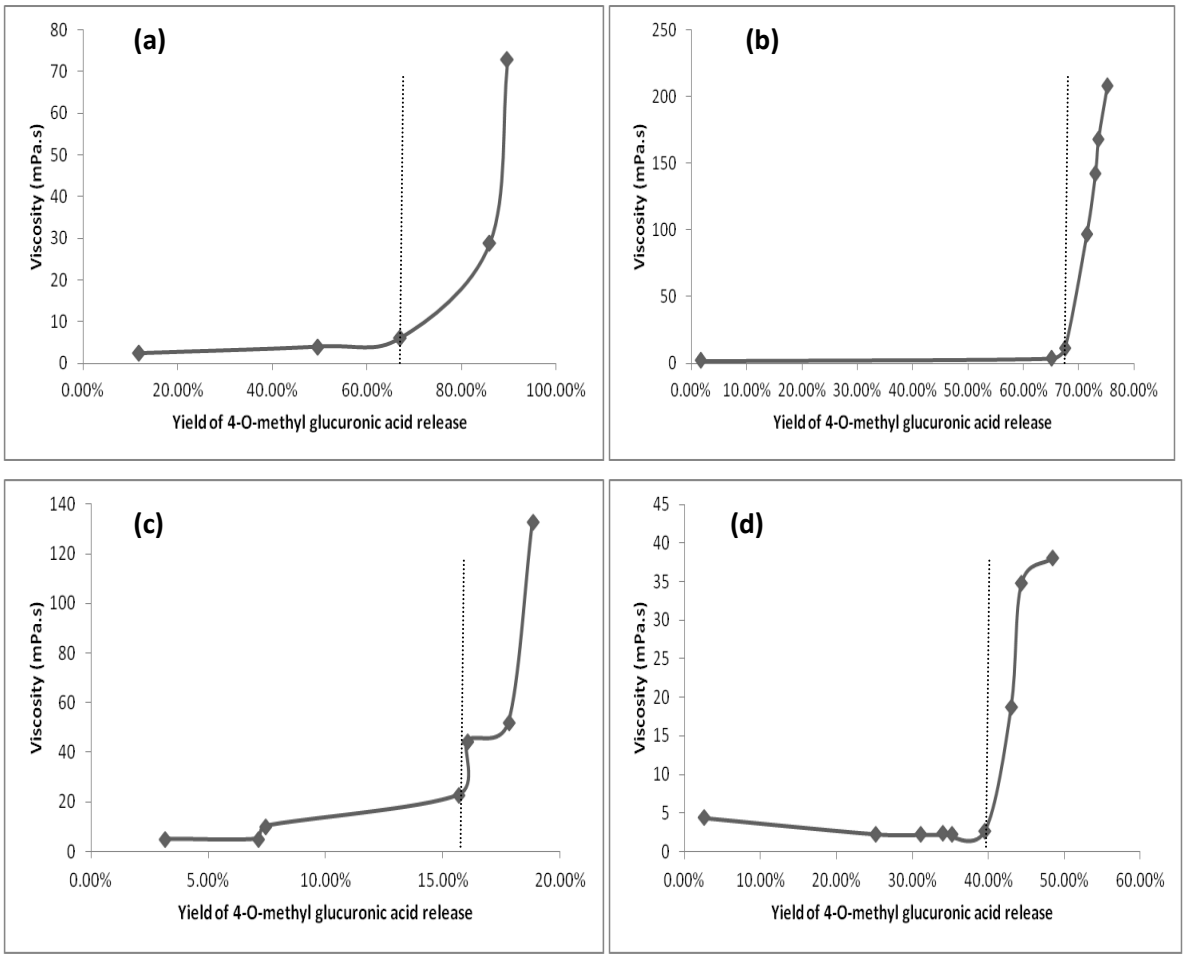


Figure 5. 6: Relationship between the yield of 4-*O*-MeGlcA release and the increase in the viscosity at α-D-glucuronidase dosage of 3.79 mg/g for: (a) beechwood xylan (3.16% w/v) and Agu1B α-glucuronidase, (b) beechwood xylan (3.16% w/v) and deglycosylated Agu1B α-glucuronidase, (c) *E. grandis* xylan (Höije) (3.08% w/v) and Agu1B α-glucuronidase; (d) *E. grandis* xylan (Pinto) (3.2% w/v) and Agu1B α-glucuronidase

In *E. grandis* xylan (Höije) (3.08%), the hydrolysis using Agu1B α-D-glucuronidase (3.79 mg/g) increased the viscosity when only 16% of the 4-*O*-MeGlcA was removed (Figure 5.6c), corresponding to a decrease of the initial 4-*O*-MeGlcA content from 20.68% to 17.44%. *E. grandis* xylan (Pinto) (3.2%) treated with the Agu1B α-D-glucuronidase showed an increase in the viscosity when 40% of the 4-*O*-MeGlcA was removed (Figure 5.6d), corresponding to a decrease of the initial 4-*O*-MeGlcA from 7.05% to 4.26%.

The relationship between 4-*O*-MeGlcA release and the change in viscosity was confirmed by plotting scatter diagrams showing the release of 4-*O*-MeGlcA versus the increase in viscosity (Figure 5.7). As it can be seen, the correlation coefficients (R^2) are all above 0.90, indicating a strong relationship between the release of 4-*O*-MeGlcA and increase in viscosity.

The equations obtained, represent the relationship between the release of 4-*O*-MeGlcA and the resulting viscosity at α-D-glucuronidase dosage of 3.79 mg/g, beechwood xylan at 3.16% and *E. grandis* xylan (Höije) at 3.08%.

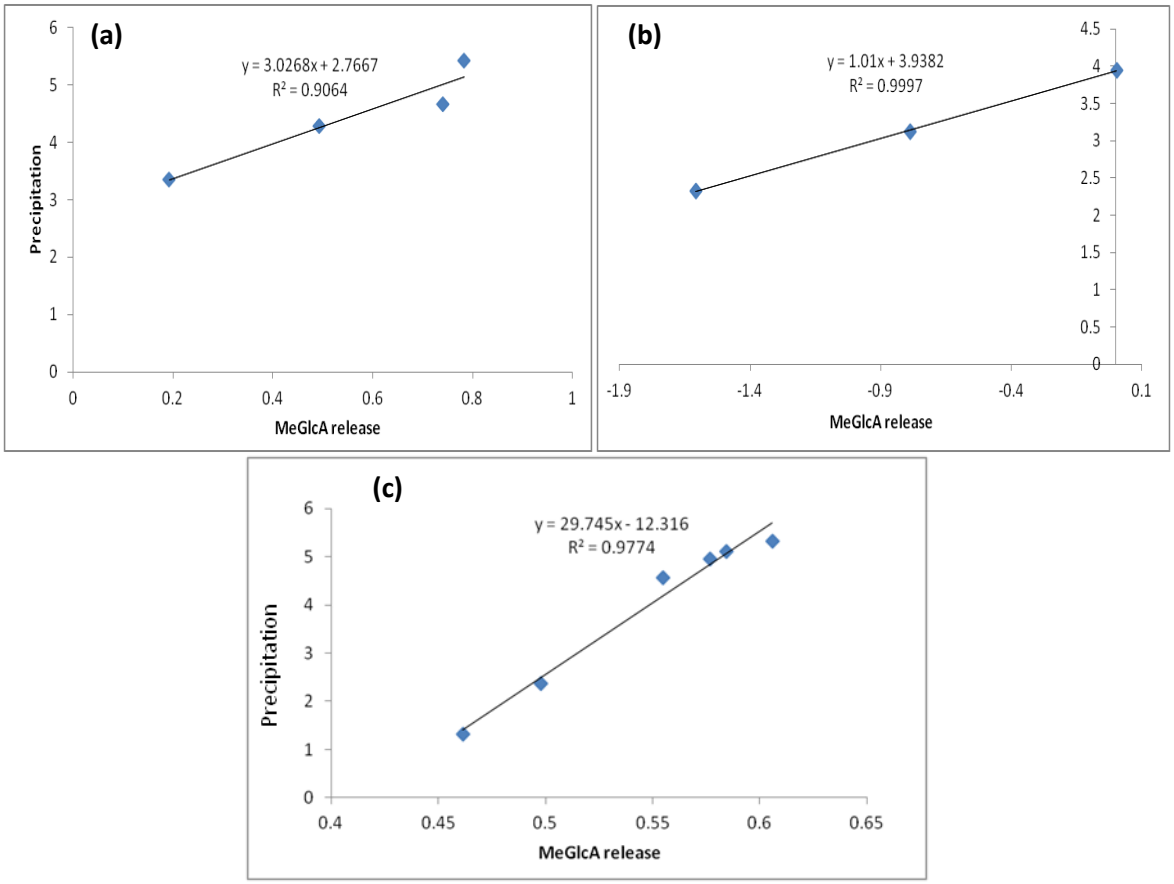


Figure 5. 7: Correlation obtained at α-D-glucuronidase dosage of 3.79 mg/g, between 4-*O*-methyl glucuronic acid release and increase in viscosity for: (a) Beechwood xylan (3.16% w/v) and Agu1B α-glucuronidase, (b) *E. grandis* xylan (Höije) (3.08% w/v) and Agu1B α-D-glucuronidase and (c) Beechwood xylan (3.16% w/v) and deglycosylated Agu1B α-glucuronidase

5.3.6. Determination of optimum conditions for enzymatic modification of glucuronoxylans by α-D-glucuronidase

Optimum conditions to modify the functional properties of the soluble glucuronoxylans obtained in Chapter 3 were investigated using a central composite design varying the α-D-glucuronidase dosage and the xylans concentration. The optimum region maximises the release of 4-*O*-MeGlcA side chains and the viscosity/precipitation, thereby improving xylan functional properties. A typical experimental design for k=2 is shown in Figure 5. 8.

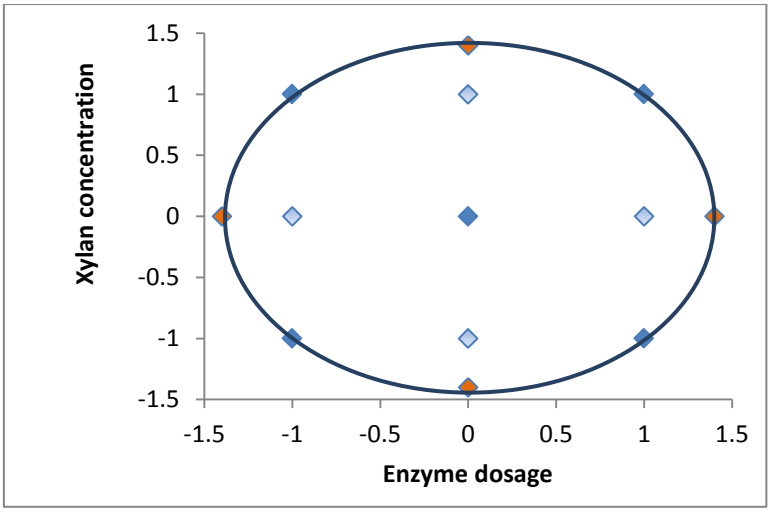


Figure 5. 8: Rotatable Central Composite Design showing the combinations between two factors and three levels-2^3

The percentage release of 4-O-MeGlcA was remarkably different between the Agu1A and Agu1B α-D-glucuronidases for all glucuronoxylans. The yields of 4-*O*-MeGlcA release obtained using the Agu1A α-D-glucuronidase did not exceed 30% for beechwood xylan, 2.98% for *E. grandis* xylan (Höije) and 8.57% for *E. grandis* xylan (Pinto), relative to the total amount present in the extracted xylans (Table 5. 7). The yields of 4-*O*-MeGlcA obtained with Agu1B α-D-glucuronidase varied within a range of 0-100% for beechwood xylan, 10.9-23% for *E. grandis* xylan (Höije) and 0-57% for *E. grandis* xylan (Pinto) (Table 5. 7). For the three glucuronoxylans, there was an increasing trend in the amount of 4-*O*-MeGlcA released from 3.79-7.53 mg/g α-D-glucuronidase dosage.

The increase in the viscosity of soluble glucuronoxylans occurred as result of 4-O-MeGlcA release from the xylan backbone. The effect of different α-D-glucuronidase dosages and xylan concentrations on the viscosity of the three glucuronoxylans can be visualised in Figure 5. 9 Figure 5. 10. It can be seen that a decrease in the xylan concentration from 4.45% to 3.16% caused a drastic decrease in the viscosity of modified materials of approximately 10-fold difference (Table 5. 7, Runs 2 and 10). Maximum viscosity for beechwood xylan was obtained by combining 6.4 mg/g α-D-glucuronidase dosage and 4.98% (w/v) beechwood xylan. At these conditions, about 90% of the available 4-*O*-MeGlcA side-chains were removed, resulting in a degree of substitution of 1:135 (0.74% 4-*O*-MeGlcA content). This led to an increase in viscosity from 3.06 to 1510 mPa.s (Figure 5. 9). The decrease in the xylan concentration to 4.45% at the same α-D-glucuronidase dosage decreased the viscosity to 1210 mPa.s while releasing 92% of the available 4-*O*-MeGlcA side-chains (Table 5.7, Run 6). The higher release of the side-chains might be caused by a higher availability of enzyme binding sites for substrate hydrolysis.

Further decrease in beechwood xylan concentration to 3.16% at α-D-glucuronidase dosage of 7.53 mg/g released 100% of the 4-*O*-MeGlcA side-chains but the viscosity was drastically decreased to 349 mPa.s.

E. grandis xylan (Höije) showed maximum viscosity (3130 mPa.s) at 4.27% (w/v) xylan concentration and 6.4 mg/g of α-glucuronidase, releasing 22% of the available 4-*O*-MeGlcA. The resultant degree of substitution was 1:6 (16% of 4-*O*-MeGlcA). The combination of 4.76% (w/v) of xylan at the same α-D-glucuronidase released 21.5% of the availabe 4-*O*-MeGlcA resulting in a viscosity of 2850 mPa.s. In this case, the increase in the xylan concentration beyond 4.27% (w/v) did not increase the 4-*O*-MeGlcA yield or the viscosity, probably due to the presence of inhibiting impurities such as lignin. Consequently, the decrease in the xylan concentration to 3.08% (w/v) at 7.53 mg/g of α-D-glucuronidase dosage released 23% of the 4-*O*-MeGlcA, but resulted in a lower viscosity of 255 mPa.s (Table 5. 7, Figure 5. 9).

E. grandis xylan (Pinto) showed maximum viscosity combining 5.03% xylan with 6.4 mg/g of α-D-glucuronidase dosage which released 57% of the available 4-*O*-MeGlcA (Table 5. 7, Figure 5. 10). This led to a degree of substitution of 1:33 (3% of 4-*O*-MeGlcA content) and a viscosity of 115 mPa.s. The combination of 4.5% (w/v) of xylan at the same enzyme dosage led to a decrease of the 4-*O*-MeGlcA yield and the viscosity to 46% and 81.4 mPa.s, respectively. Further decrease in the xylan concentration to 3.2% (w/v) at α-D-glucuronidase dosage of 7.53 mg/g removed 46% of the 4-*O*-MeGlcA but the viscosity decreased to 39.5 mPa.s (Table 5. 7).

Table 5. 7: Yield and viscosity values obtained for soluble beechwood xylan, *E. grandis* xylan (Höije), *E. grandis* xylan (Pinto) hydrolysed by α-D-glucuronidase for 24h, 40°C and pH 5.0, according to a 2^3 central composite design

			Beechwood xylan			***E. grandis* xylan (Höije)**			***E. grandis* xylan (Pinto)**		
Run	Glu type	Glu dosage (mg/g)	Xylan (%w/v)	Yield[5] (%)	Viscosity[6] (mPa.s)	Xylan (%w/v)	Yield (%)	Viscosity (mPa.s)	Xylan (%w/v)	Yield (%)	Viscosity (mPa.s)
1		7.53	3.16	100	349	3.08	23	255	3.2	46	39.5
2		6.44	4.98	90	1510	4.7	22	2850	5.03	57	115
3		1.14	1.87	57	1.34	1.9	16	1.61	1.9	43	1.27
4		1.14	4.45	89	117	4.2	19	98.3	4.5	27	2.23
5		3.79	3.16	79	230	3.08	21	108	3.2	43	18.7
6	**Agu1B**	6.44	4.45	92	1210	4.2	22	3130	4.5	46	81.4
7		0.042	3.16	0.00	1.48	3.08	10.9	2	3.2	0.00	1.57
8		3.79	3.16	100	234	3.08	19	133	3.2	30.7	16.65
9		3.79	1.33	100	1.43	1.40	19	1.56	1.36	23.2	1.16
10		3.79	3.16	100	240	3.08	22	103	3.2	35	17.92
11		6.44	1.87	100	47.9	1.9	21	10.6	1.9	28	1.38
12		3.79	3.16	30	1.68	3.08	1.62	2.57	3.2	3.07	2.01
13		3.79	1.33	7.4	1.37	3.08	0.75	1.47	3.2	2.71	1.39
14		3.79	3.16	7.2	1.91	3.08	1.00	3.01	3.2	0.00	2.74
15		6.44	4.45	6.5	2.69	4.27	2.55	4.39	4.5	8.57	2.65
16		6.44	1.87	13.4	1.58	1.9	2.28	1.95	1.9	6.98	1.49
17	**Agu1A**	0.042	3.16	1.20	1.86	3.08	0.00	2.5	3.2	0.00	2.11
18		3.79	3.16	4.85	1.99	3.08	0.00	2.74	3.2	2.96	2.21
19		1.14	1.87	0.00	1.26	1.9	0.00	2.65	1.9	0.00	2.65
20		6.44	4.98	5.24	2.94	4.76	1.13	4.53	5.03	8.49	3.00
21		7.53	3.16	6.73	2.19	3.08	2.94	2.99	3.2	6.27	2.19
22		1.14	4.45	5.13	2.38	4.27	0.48	3.87	4.5	2.29	2.58

[5] Yield of 4-O-MeGlcA calculated based on the xylan concentration and correspondent 4-*O*-MeGlcA content

[6] Viscosity measured at 10.3 s^{-1} after 24 hours of hydrolysis

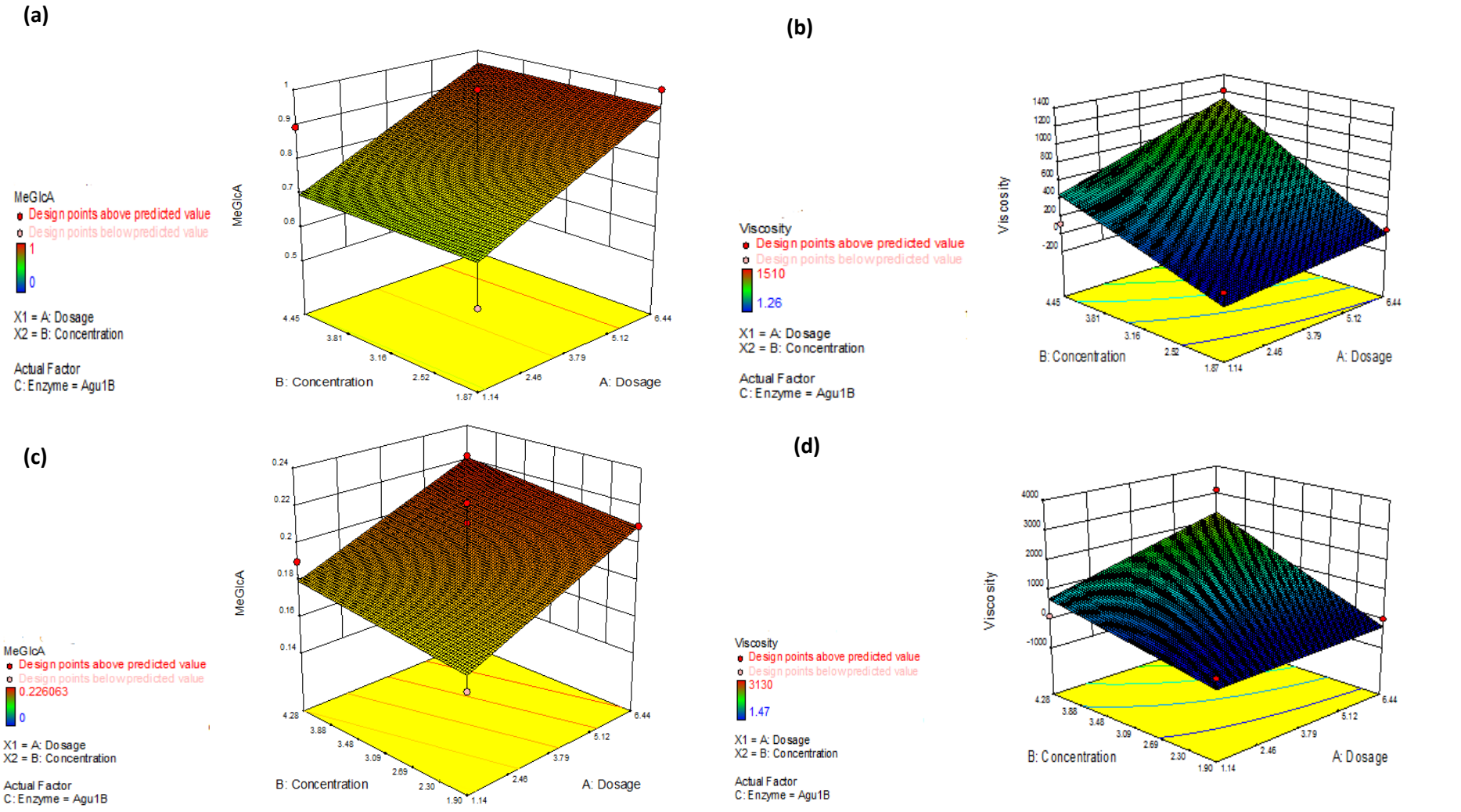


Figure 5. 9: Response surface plots for release of 4-*O*-MeGlcA (fractional yield) and change in viscosity (mPa.s) as a function of xylan concentration (%) and enzyme dosage (mg/g) in (a,b) beechwood xylan; (c,d) *E. grandis* xylan (Höije)

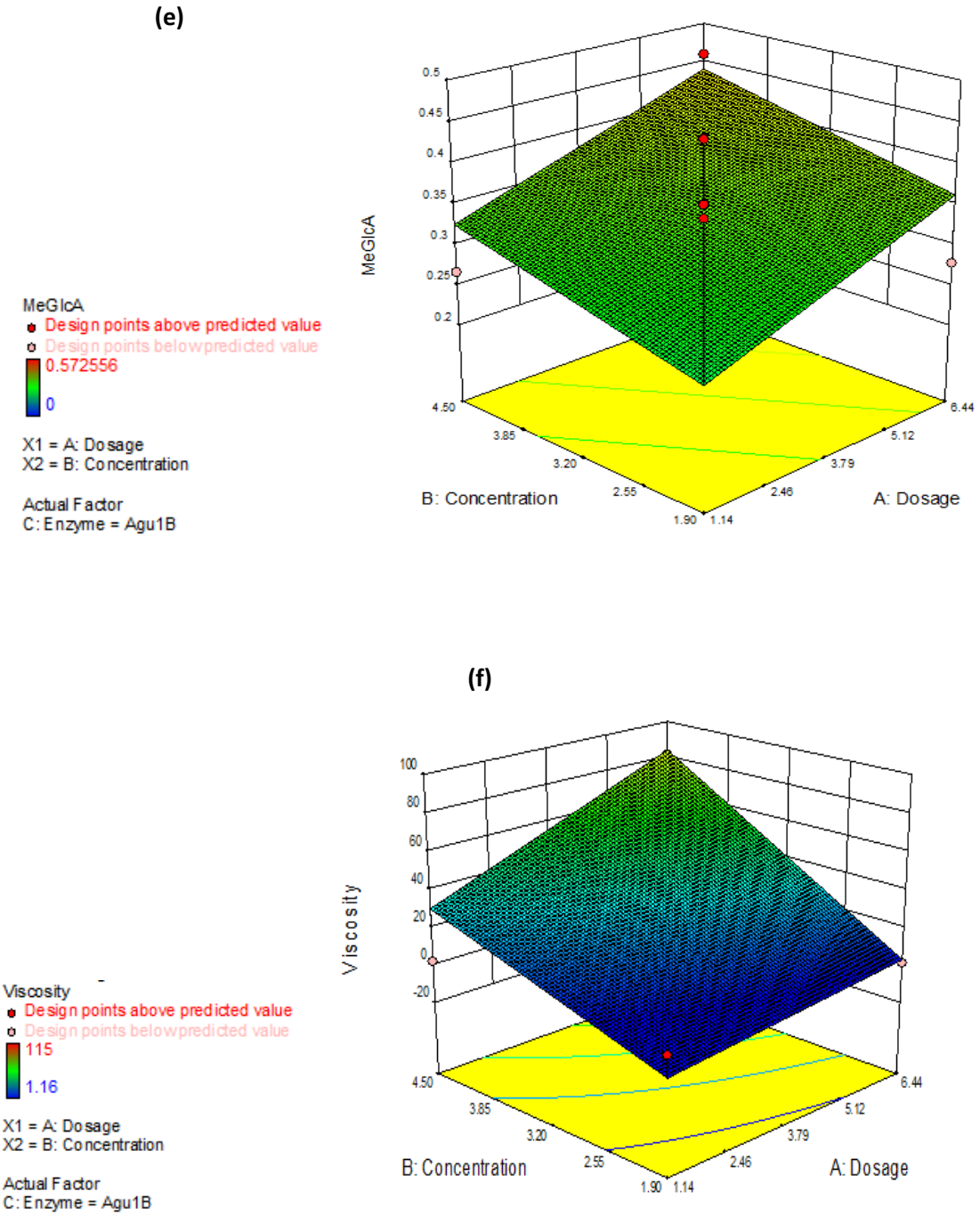


Figure 5. 10: Response surface plots for: (e) release of 4-*O*-MeGlcA (fractional yield) and (f) change in viscosity (mPa.s) as a function of xylan concentration (%) and enzyme dosage (mg/g) in *E. grandis* xylan (Pinto)

A linear model was suggested for the hydrolysis of the three glucuronoxylans for the yield of 4-*O*-MeGlcA, whereas a two factor interaction model was suggested for the change in viscosity. The analysis of variance for 4-*O*-MeGlcA release in beechwood xylan (Table 5. 8) showed that the α-D-glucuronidase dosage and α-D-glucuronidase type had a significant effect ($p < 0.0164$). However, the xylan concentration was not statistically significant (p-value of 0.93). The F-value of 28.21 indicated that the model was significant with only 0.01% chance that could be resulted from noise. The linear model fitted the response surface with an R-squared of 0.7261 (Adj R-squared 0.7954). The regression analysis on the fitted data for prediction of the release of 4-*O*-MeGlcA based on the coded values was obtained as follows:

$$\boldsymbol{Y = 0.45 + 0.13A + 0.004B - 0.37C} \qquad \textbf{Equation 5. 3}$$

Where: Y = Predicted response (release of 4-*O*-methyl glucuronic acid)

A, B and C = coded linear values for α-D-glucuronidase dosage (mg/g), xylan concentration (%) and α-D-glucuronidase type (Agu1B or Agu1A)

Table 5. 8: Analysis of variance for response surface-linear model for 4-*O*-methyl glucuronic acid release in beechwood xylan. 4-*O*-MeGlcA release = f (xylan concentration; enzyme dosage; enzyme type). DF-degrees of freedom

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	3.34	3	1.11	28.21	< 0.0001
A-Dosage	*0.28*	*1*	*0.28*	*7*	*0.0164*
B-Xylan concentration	*2.58E-04*	*1*	*2.58E-04*	*6.55E-03*	*0.9364*
C-Enzyme type	*3.06*	*1*	*3.06*	*77.63*	*< 0.0001*
AB	0.71	18	0.039		
AC	*0.64*	*14*	*0.046*	*2.81*	*0.1647*
BC	*0.066*	4	*0.016*		
Residual	4.04	21			
Lack of fit	3.34	3	1.11	28.21	0.1647
Pure error	*0.28*	*1*	*0.28*	*7*	*0.0164*
Cor Total	*2.58E-04*	*1*	*2.58E-04*	*6.55E-03*	*0.9364*

Note 1: DF- degrees of freedom

The linear model obtained to predict the release of 4-*O*-MeGlcA in *E. grandis* xylan (Höije) was significant ($p < 0.0001$). Similarly as for beechwood xylan, the significant effects were the enzyme dosage and the enzyme type ($p < 0.0002$), whereas the xylan concentration was not statistically significant (p-value 0.1928) (Table 5. 9). The linear model fitted the response surface with R-squared of 0.9561 (Adj R-squared 0.9667). The regression analysis on the fitted data to predict the release of 4-*O*-MeGlcA, resulted in the following equation based on coded values:

$$\boldsymbol{Y = 0.10 + 0.021A + 0.0059B - 0.092C} \qquad \textbf{Equation 5. 4}$$

Where: Y = Predicted response (release of 4-*O*-methyl glucuronic acid)

A, B and C = coded linear values for α-D-glucuronidase dosage (mg/g), xylan concentration (%) and α-D-glucuronidase type (Agu1B or Agu1A)

Table 5. 9: Analysis of variance for response surface-linear model for 4-*O*-methyl glucuronic acid release in *E. grandis* xylan (Höije). 4-*O*-MeGlcA release = f (xylan concentration; enzyme dosage; enzyme type)

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	0.19	3	0.064	204.34	< 0.0001
A-Dosage	6.86E-03	1	6.86E-03	21.91	0.0002
B-Xylan concentration	5.73E-04	1	5.73E-04	1.83	0.1928
C-Enzyme type	0.18	1	0.18	589.27	< 0.0001
AB	5.63E-03	18	3.13E-04		
AC	4.93E-03	14	3.52E-04	1.99	0.2649
BC	7.06E-04	4	1.77E-04		
Residual	0.2	21			
Lack of fit	0.19	3	0.064	204.34	0.2649
Pure error	6.86E-03	1	6.86E-03	21.91	0.0002
Cor Total	5.73E-04	1	5.73E-04	1.83	0.1928

The linear model for release of 4-*O*-MeGlcA in *E. grandis* xylan (Pinto) was significant (p-value < 0.0001). The significant factors were the enzyme dosage and the enzyme type (p-value < 0.03), whereas the xylan concentration was not statistically significant (p-value 0.1314). The predicted R^2 of 0.6404 is in reasonable agreement with the adjusted R^2 of 0.7381. The empirical

model on the predicted response of 4-*O*-MeGlcA release based on the coded values gave the following equation:

$$\boldsymbol{Y = 0.19 + 0.057A + 0.039B - 0.15C} \qquad \textbf{Equation 5. 5}$$

Where: Y = Predicted response (release of 4-*O*-methyl glucuronic acid)

A, B and C = coded linear values for α-D-glucuronidase dosage (mg/g), xylan concentration (%) and α-D-glucuronidase type (Agu1B or Agu1A)

Table 5. 10: Analysis of variance for response surface-linear model for 4-*O*-methyl glucuronic acid release in *E. grandis* xylan (Pinto). 4-*O*-MeGlcA release = f (xylan concentration; enzyme dosage; enzyme type)

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	0.59	3	0.2	20.73	< 0.0001
A-Dosage	0.052	1	0.052	5.42	0.0317
B-Concentration	0.024	1	0.024	2.5	0.1314
C-Enzyme	0.52	1	0.52	54.26	< 0.0001
AB	0.17	18	9.54E-03		
AC	0.16	14	0.012	5.62	0.054
BC	8.31E-03	4	2.08E-03		
Residual	0.77	21			
Lack of Fit	0.59	3	0.2	20.73	0.0540
Pure Error	0.052	1	0.052	5.42	0.0317
Cor Total	0.024	1	0.024	2.5	0.1314

Furthermore, the analysis of variance for the viscosity response indicated a two-factor interaction model for the three glucuronoxylans. The viscosity models obtained for beechwood, *E. grandis* (Höije) and *E. grandis* (Pinto) were significant ($p < 0.0001$), but showed a significant lack-of-fit (Appendix C).

The desirability plots showed the conditions that maximise the yield of 4-*O*-MeGlcA release and the viscosity by keeping the α-D-glucuronidase dosage in the range between 1.14 and 6.4 mg/g and maximising the xylan concentration (Appendix C). The beechwood xylan desirability

function located the optimum conditions at 6.4 mg/g enzyme dosage (Agu1B α-glucuronidase) and 4.45% (w/v) substrate concentration. The predicted viscosity was 1348.39 mPa.s and the yield corresponded to 96% removal of the side-chains. The desirability function for the *E. grandis* xylan (Höije) located the optimum at 6.4 mg/g enzyme dosage and 4.28% (w/v) substrate concentration. The predicted viscosity was 2316.81 mPa.s and 23% removal of the side-groups. Lastly, the desirability function for *E. grandis* xylan (Pinto) located the optimal conditions at 6.4 mg/g enzyme dosage and 4.50% (w/v) resulting in a viscosity of 83.87 mPa.s and 43% removal of side groups.

Modified beechwood xylan and the *E. grandis* xylan (Höije) displayed similar rheological behaviour when shear rate was applied. The increase in viscosity led to subsequent formation of solid hydrogels that displayed thixotropic shear-thinning behaviour when shear stress was applied (Figure 5.11). At a shear rate of 10.3 s^{-1}, *E. grandis* xylan (Höije) showed the highest viscosity (3130 mPa.s), followed by beechwood xyan (1510 mPa.s) and then *E. grandis* xylan (Pinto) with 115 mPa.s.

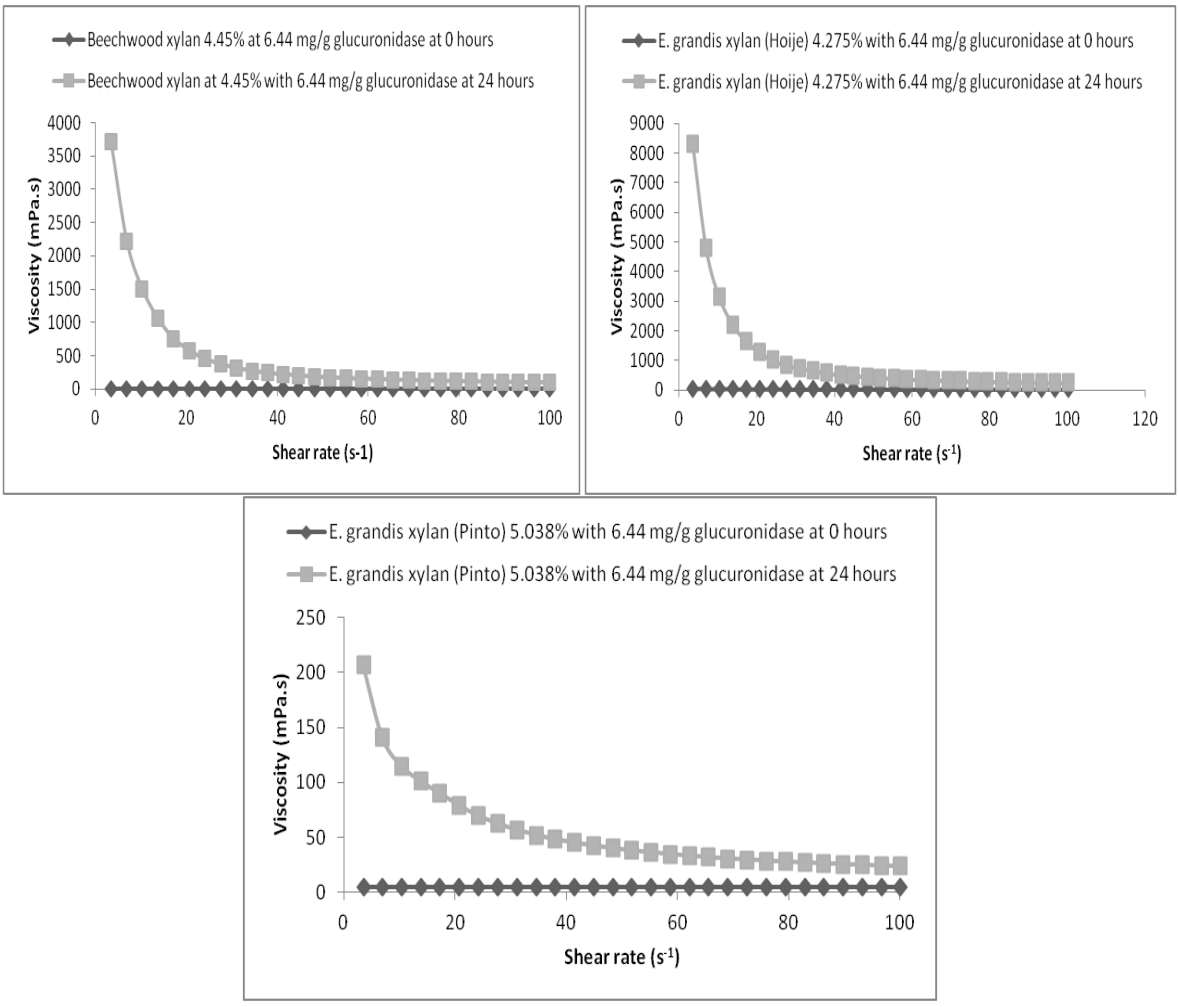


Figure 5. 11: Change of viscosity of xylans at 0 hours and 24 hours at increasing shear rate (max. shear rate 100 s^{-1})

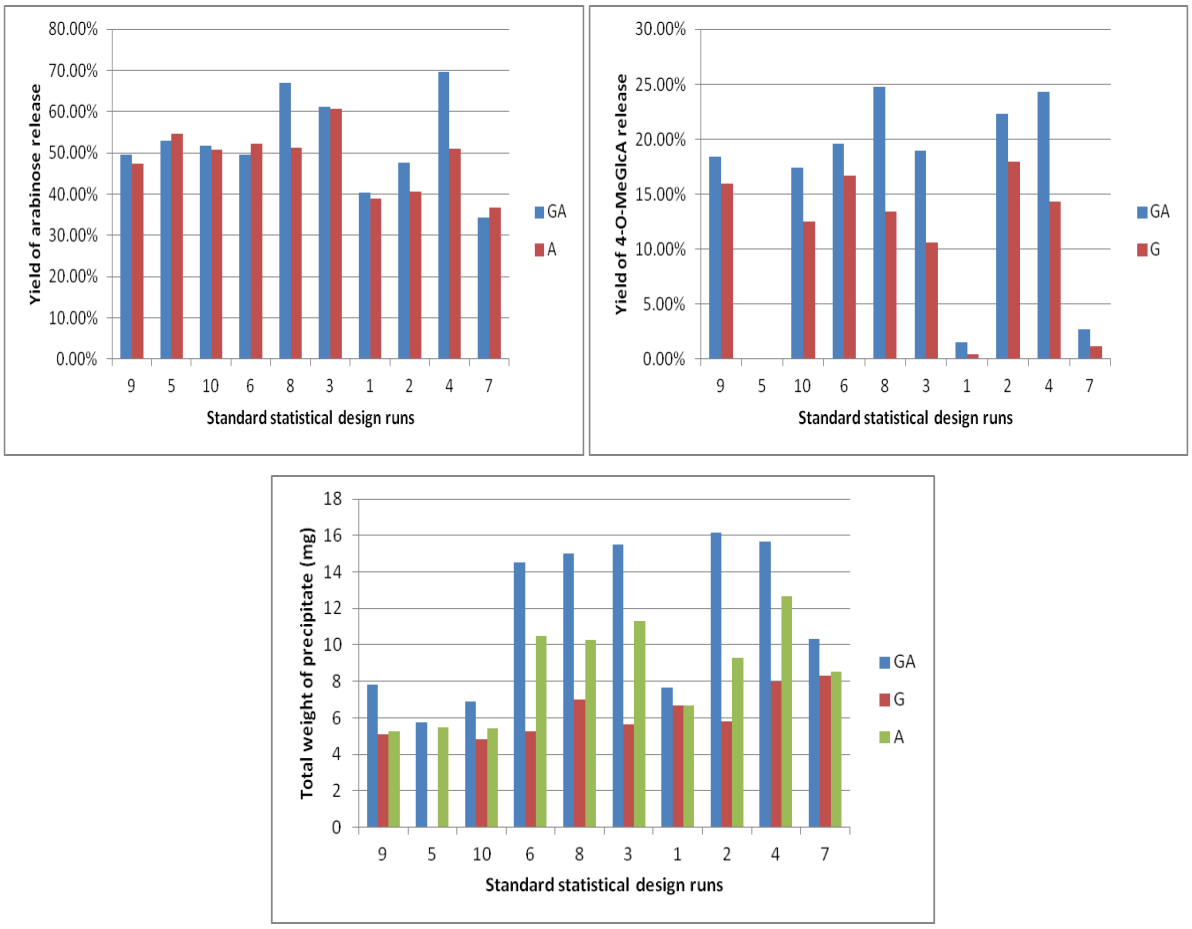
5.3.7. Effect of α-L-arabinofuranosidase and α-D-glucuronidase dosage ratios on side-chain release and change in solubility properties of sugarcane bagasse xylan

Optimum dosages of α -D-glucuronidase and α-L-arabinofuranosidase for precipitation of soluble sugarcane bagasse xylan at 1.23% (w/v) were identified as 6.4 mg/g and 150 nkat/g, respectively (Figure 5. 13). The yields of arabinose and 4-*O*-MeGlcA release varied as a response of different dosage ratios between α-D-glucuronidase and α-L-arabinofuranosidase. Positive, negative and non synergistic effects were verified at different statistical combinations of the two enzyme dosages for the release of arabinose and 4-*O*-MeGlcA (Figure 5.12). Enzymatic hydrolysis with both α-L-arabinofuranosidase and α-D-glucuronidase in treatments 4 (6.4 mg/g Agu; 350 nkat/g Ara), 8 (3.79 mg/g Agu; 391.42 nkat/g Ara), 3 (1.14 mg/g Agu; 350 nkat/g Ara), 6 (7.5 mg/g Agu; 250 nkat/g Ara) and 2 (6.4 mg/g Agu; 150 nkat/g) showed a positive synergy, releasing arabinose at 69.5%, 67%, 61%, 49.5% and 47.6%, respectively. The highest yields of 4-*O*-MeGlcA were also verified in treatments 4 (6.4 mg/g Agu; 350 nkat/g Ara), 8 (3.79 mg/g Agu; 391.42 nkat/g Ara), 3 (1.14 mg/g Agu; 350 nkat/g), 6 (7.5 mg/g Agu; 250 nkat/g Ara) and 2 (6.4 mg/g Agu; 150 nkat/g Ara), corresponding to 24%, 25%, 19%, 19.5% and 22% of 4-*O*-MeGlcA release, respectively (Figure 5.12).

The combination of 6.4 mg/g of glucuronidase with 350 nkat/g of arabinofuranosidase resulted in maximum release of arabinose at 69.5% and 4-*O*-MeGlcA at 24%. The concerted action of both enzymes at a lower glucuronidase dosage of 1.14 mg/g released less arabinose and 4-*O*-MeGlcA corresponding to 61% and 19%, respectively (Figure 5.12, treatment 3). At α-D-glucuronidase dosage of 6.44 mg/g with 150 nkat/g of α-L-arabinofuranosidase the degree of arabinose removal decreased to 47.6%, while the 4-*O*-MeGlcA was released at 22%. The 4-*O*-MeGlcA released decreased to 18% and the arabinose to 41%, when the enzymes were used individually (Figure 5.12, treatment 2).

The lowest release of 4-*O*-MeGlcA occurred with treatments 7 (3.79 mg/g Agu; 108.57 nkat/g Ara), 1 (1.14 mg/g Agu; 150 nkat/g Ara) and 5 (0.042 mg/g Agu; 250 nkat/g Ara), corresponding to 2.67%, 1.49% and 0%, respectively. At such conditions, treatments 7, 1 and 5 resulted in the release of arabinose at 34%, 40% and 53%. A small negative synergy on the arabinose removal by the action of α-L-arabinofuranosidase was verified in treatment runs 5 and 6, resulting from using either very low (0.042 mg/g) or high (7.53 mg/g) dosages of α-glucuronidase.

The precipitation of arabinoglucuronoxylan is a result from the release of arabinose and 4-*O*-MeGlcA, creating unsubstituted regions in the polymer backbone (Chimphango, 2010). The amount of insoluble arabinoglucuronoxylan obtained was in agreement with the side-chain release by the action of both enzymes.



Note: GA represents the concerted action of α-D-glucuronidase and arabinofuranosidase; G the α-D-glucuronidase single action and A the α-L-arabinofuranosidase single action

Figure 5.12: Hydrolysis of arabinoglucuronoxylan from sugarcane bagasse using α-L-arabinofuranosidase and α-D-glucuronidase with the arabinose and 4-*O*-MeGlcA released and the amount of precipitation.

In Figure 5.12 it is verified that hydrolysis conditions (treatments 7, 1, 5, 10 and 9) that gave less removal of arabinose and 4-*O*-MeGlcA side-chains also resulted in lower amounts of insoluble arabinoglucuronoxylan. However, the precipitation was increased at conditions where the removal of both arabinose and 4-*O*-MeGlcA was maximized (treatments 2, 8, 3, 4 and 6).

The combination of 6.4 mg/g α-D-glucuronidase with 350 nkat/g α-L-arabinofuranosidase (treatment 4) gave 15.65 mg of insoluble xylan. On the other hand, the one with only α-L-arabinofuranosidase at the same dosage produced 12.65 mg and the one using only α-D-

glucuronidase gave 8.0 mg. The combination of 150 nkat/g arabinofuranosidase and 6.44 mg/g glucuronidase (treatment 2) gave 16.15 mg of precipitation. The hydrolysis using α-L-arabinofuranosidase and α-D-glucuronidase indiviually gave 9.3 mg and 5.8 mg, respectively. Thus, the combined action of the two enzymes at these conditions increased the precipitation up to three times more than when using only α-L-arabinofuranosidase or α-D-glucuronidase (Figure 5.12) due to an additive effect of the action of the individual enzymes.

Optimum conditions for precipitation of sugarcane bagasse arabinoglucuronoxylan and factors significance were evaluated using response surface methodology (Figure 5.13, Table 5.11). The analysis of variance indicated that the model was significant at 97.23% confidence level. The most significant factor was found to be the α-D-glucuronidase dosage. A significant quadratic interaction was found for the α-L-arabinofuranosidase dosage (p-value 0.019). The quadratic model showed a non-significant lack of fit (p-value 0.22) relative to the pure error. However, the predicted R^2 of 0.4177 was not as close to the adjusted R^2 of 0.8133.

Table 5. 11: Analysis of variance for response quadratic model for degree of precipitation in sugarcane bagasse xylan (Höije). Degree of precipitation = f (glucuronidase dosage; arabinofuranosidase dosage)

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	146.86	5	29.37	8.84	0.0277-significant
A-Glucuronidase dosage	55.17	1	55.17	16.6	0.0152
B-Arabinofuranosidase dosage	24.44	1	24.44	7.35	0.0534
AB	17.37	1	17.37	5.23	0.0842
A2	17.85	1	17.85	5.37	0.0814
B2	47.94	1	47.94	14.43	0.0191
Residual	13.29	4	3.32		
Lack of Fit	12.89	3	4.3	10.61	0.2211-not significant
Pure Error	0.4	1	0.4		
Cor Total	160.15	9			

The regression analysis was performed on the model to predict the degree of precipitation at different combinations of α-L-arabinofuranosidase and α-D-glucuronidase dosages, giving the following equation in terms of coded values:

$$Z = 7.35 + 2.63A + 1.75B - 2.08AB + 1.98A^2 + 3.24B^2 \qquad \textbf{Equation 5. 6}$$

Where: Z = Predicted response (amount of precipitation)

A and B = coded linear values for α-D-glucuronidase enzyme dosage (mg/g) and α-L-arabinofuranosidase dosage (nkat/g)

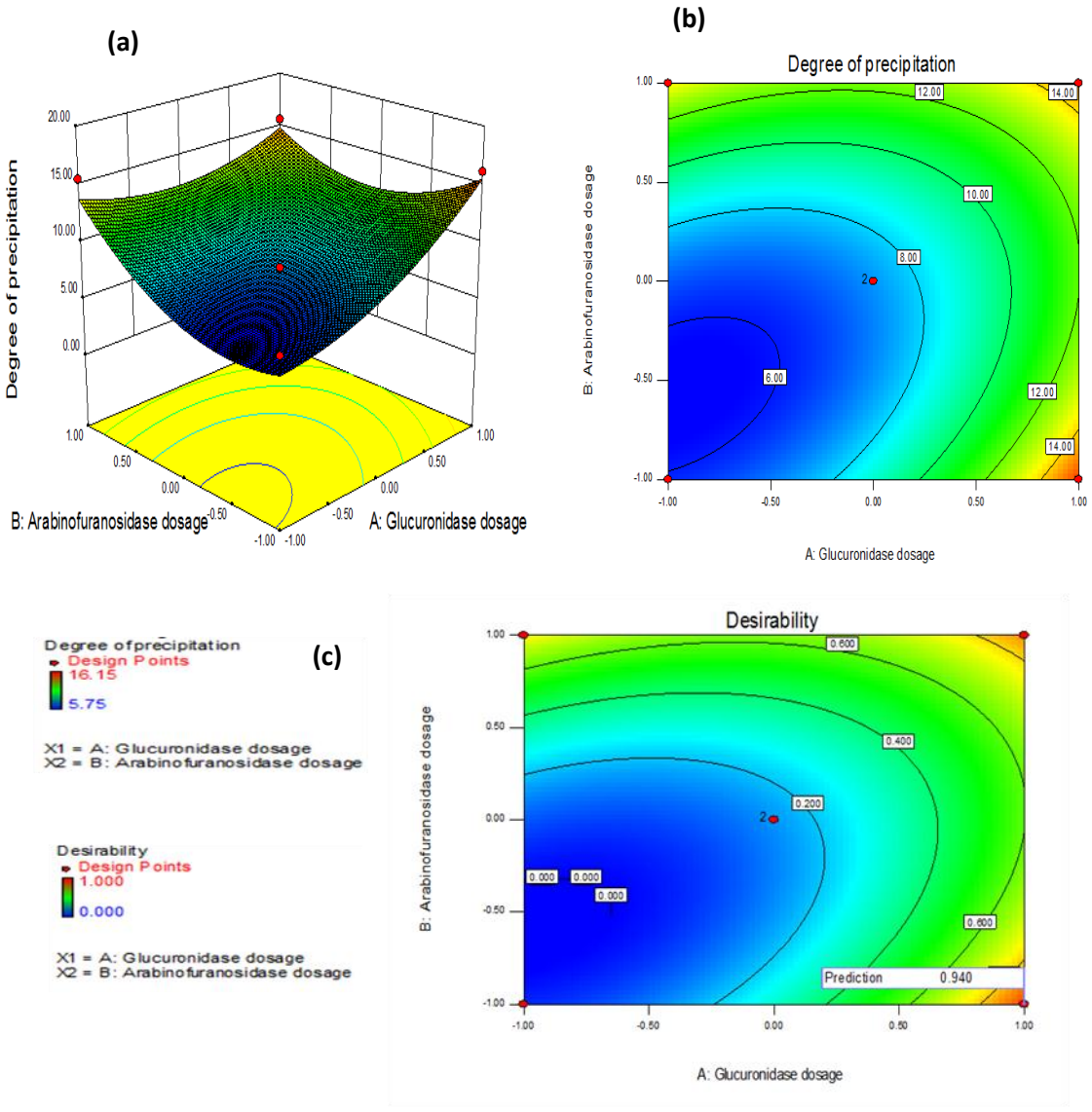


Figure 5. 13: Response surface plots (a) amount of precipitation (mg) as a function of the α-L-arabinofuranosidase (nkat/g) and α-D-glucuronidase (mg/g) dosages. (b) contour plot for the amount of precipitation. (c) desirability plot to locate optimal values for precipitation

Optimum dosages of α-D-glucuronidase (mg/g) and α-L-arabinofuranosidase (nkat/g) for soluble xylan precipitation were found at combinations of 6.4 mg/g with 350 nkat/g, 6.44 mg/g with 150 nkat/g and 1.14 mg/g with 350 nkat/g (Figure 5. 13). At enzyme combinations of 6.4 mg/g Agu-150 nkat/g Ara and 1.14 mg/g Agu-350 nkat/g Ara, 26% and 25% of the sugarcane bagasse xylan precipitated in solution, respectively.

The contour plot indicated how the degree of precipitation varied along the range of enzyme dosages applied. Looking at the path of improvement, it can be seen that the precipitation was more sensitive to α-glucuronidase changes than to α-arabinofuranosidase changes. The desirability plot located the optimal conditions at 6.4 mg/g of glucuronidase combined with 150 nkat/g of arabinofuranosidase with 16.15 mg of insoluble xylan. At these conditions, the degree of precipitation was 25%, which is the same as the actual degree of precipitation (25%). The model was able to predict the response at any given conditions within the experimental design range.

5.3.8. Particle size and morphology analysis of modified xylans

The glucuronoxylans from beechwood (Sigma) and *E. grandis* (Höije) formed solid hydrogels after enzymatic hydrolysis at optimum conditions. The glucuronoxylan extracted from *E. grandis* using the Pinto protocol formed a hydrogel with a significantly lower intrinsic viscosity. However, the arabinoglucuronoxylan formed a discontinuous association with formation of insoluble particles that settled through gravity (Figure 5.14).

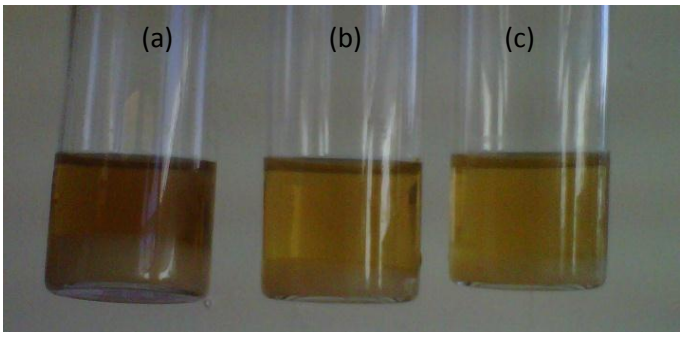


Figure 5. 14: Precipitation of sugarcane bagasse arabinoglucuronoxylan (1.23% w/v) from the concerted action of α-L-arabinofuranosidase and α-D-glucuronidase at dosages of: (a) 6.4 mg/g Agu with 150 nkat/g Ara; (b) 6.4 mg/g Agu with 350 nkat/g Ara; (c) 1.14 mg/g Agu with 350 nkat/g Ara.

The xylan concentration and enzyme dosage had a significant effect in the agglomeration properties and number of insoluble particles per unit area, as it is verified from Figure 5.15Figure 5. 16. Modified beechwood glucuronoxylan at 4.98% (w/v) showed agglomeration behaviour of the insoluble particles, with few visible isolated particles at α-D-glucuronidase dosage of 6.4 mg/g (Figure 5.15a). When a lower dosage of α-D-glucuronidase (0.042 mg/g) and xylan concentration (3.16% w/v) were employed, there were much less particles formed with no visible aggregation (Figure 5.15b). Insoluble micro-particles of the modified *E. grandis* xylan (Höije) are shown in Figures 5.15c and 5.15d. The mixture of aggregated and non-aggregated particles verified in beechwood xylan was not verified in *E. grandis* xylan (Höije). In contrast, the particles formed at 4.27% (w/v) xylan and α-D-glucuronidase dosage of 6.4 mg/g were uniformly distributed, with a regular circular shape. Fewer particles were visualised when the xylan concentration was decreased to 3.08% (w/v) and the α-D-glucuronidase dosage 0.042 mg/g (Figure 5.15d). *E. grandis* xylan (Höije) hydrogel did not show agglomeration between the insoluble particles from the release of 4-*O*-MeGlcA groups by the α-glucuronidase.

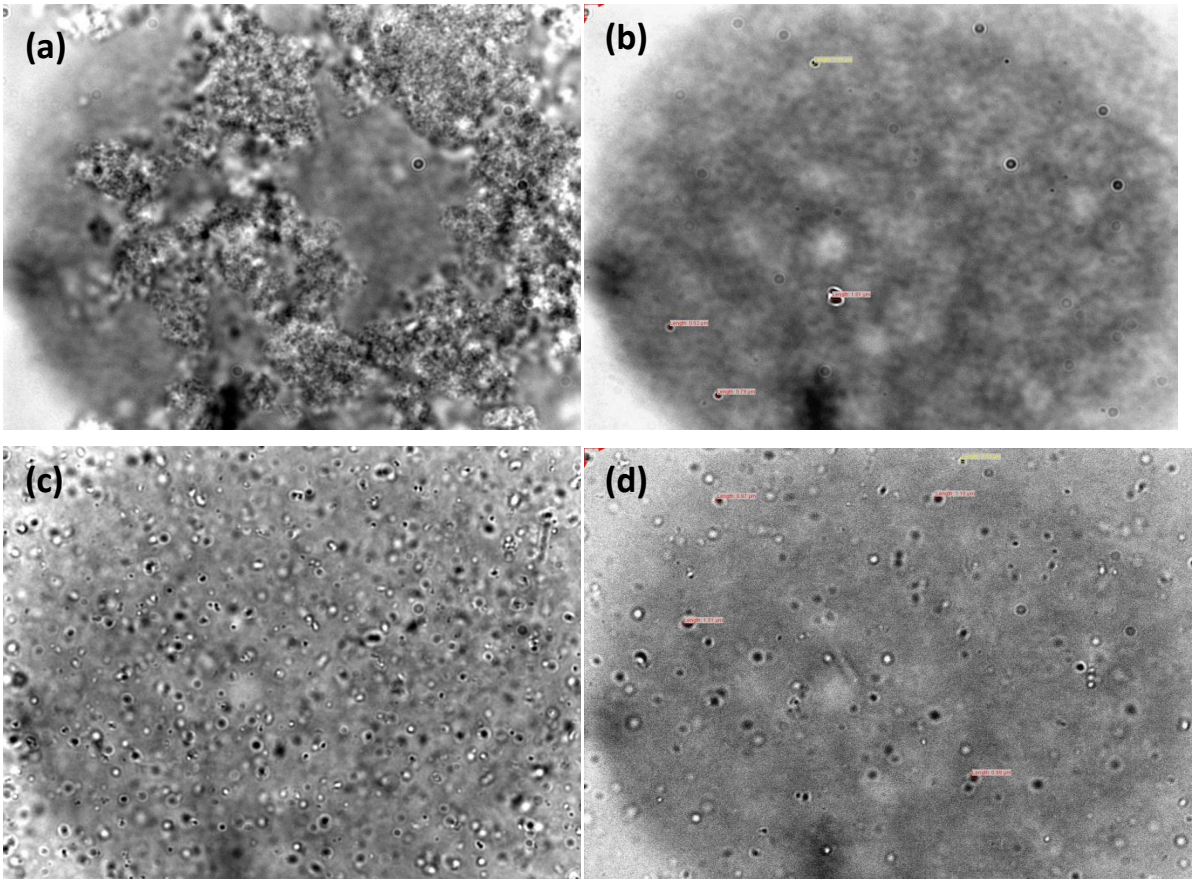


Figure 5.15: Microscope photographs (20 µm scale) of (a): beechwood xylan (6.44 mg/g glucuronidase dosage; 4.98% substrate concentration); (b): beechwood xylan (0.042 mg/g glucuronidase dosage; 3.16% substrate concentration); (c): *E. grandis* xylan (Höije) (6.44 mg/g glucuronidase dosage; 4.275% substrate concentration; (d): *E. grandis* xylan (Höije) (0.042 mg/g glucuronidase dosage; 3.0875% substrate concentration)

Fewer insoluble particles were verified in Figure 5.16e, employing a dosage of 3.79 mg/g of α-gluuronidase and *E. grandis* xylan (Höije) at 1.408% (w/v). Therefore, the substrate concentration was the most significant factor in the number of insoluble particles of xylans, particularly in the enzymatic hydrolysis of glucuronoxylans.

E. grandis xylan (Pinto) at 5.04% (w/v) modified at the optimum conditions using α-D-glucuronidase at 6.4 mg/g also showed agglomeration of the insoluble particles formed (Figure 5.16g). The higher the agglomeration, the fewer isolated particles for diameter size measurements. Sugarcane bagasse xylan at 1.23% (w/v) synergistically modified with α-L-arabinofuranosidase at 350 nkat/g and α-D-glucuronidase at 6.4 mg/g also showed particle agglomeration (Figure 5.16f).

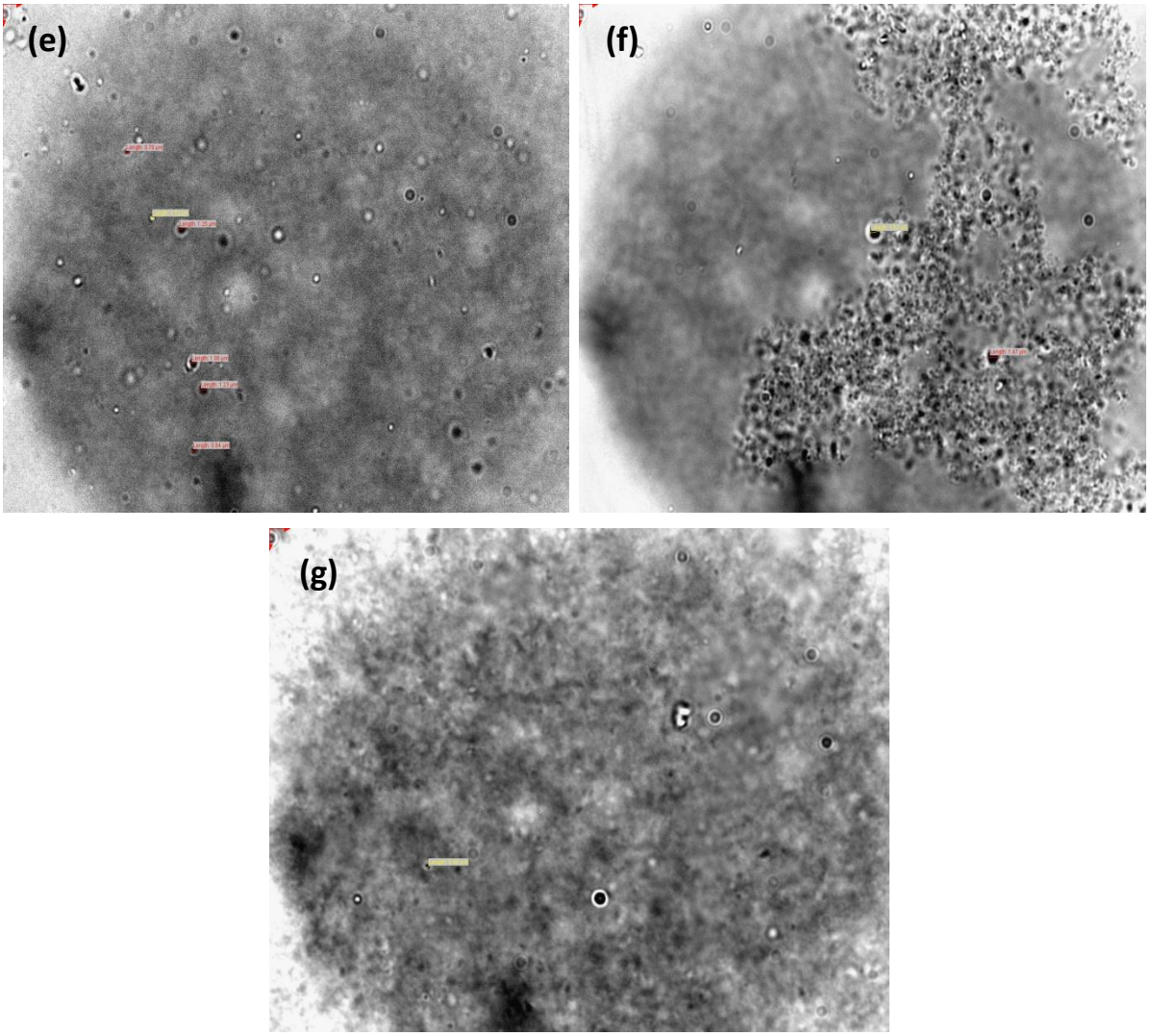


Figure 5. 16: Microscope photographs (20 µm scale) of (e): *E. grandis* xylan (Höije) (3.79 mg/g glucuronidase dosage; 1.408% substrate concentration); (f): sugarcane bagasse xylan (Höije) (6.4 mg/g glucuronidase; 350 nkat/ml arabinofuranosidase); (g): *E. grandis* xylan (Pinto) (6.4 mg/g glucuronidase dosage; 5.04% substrate concentration)

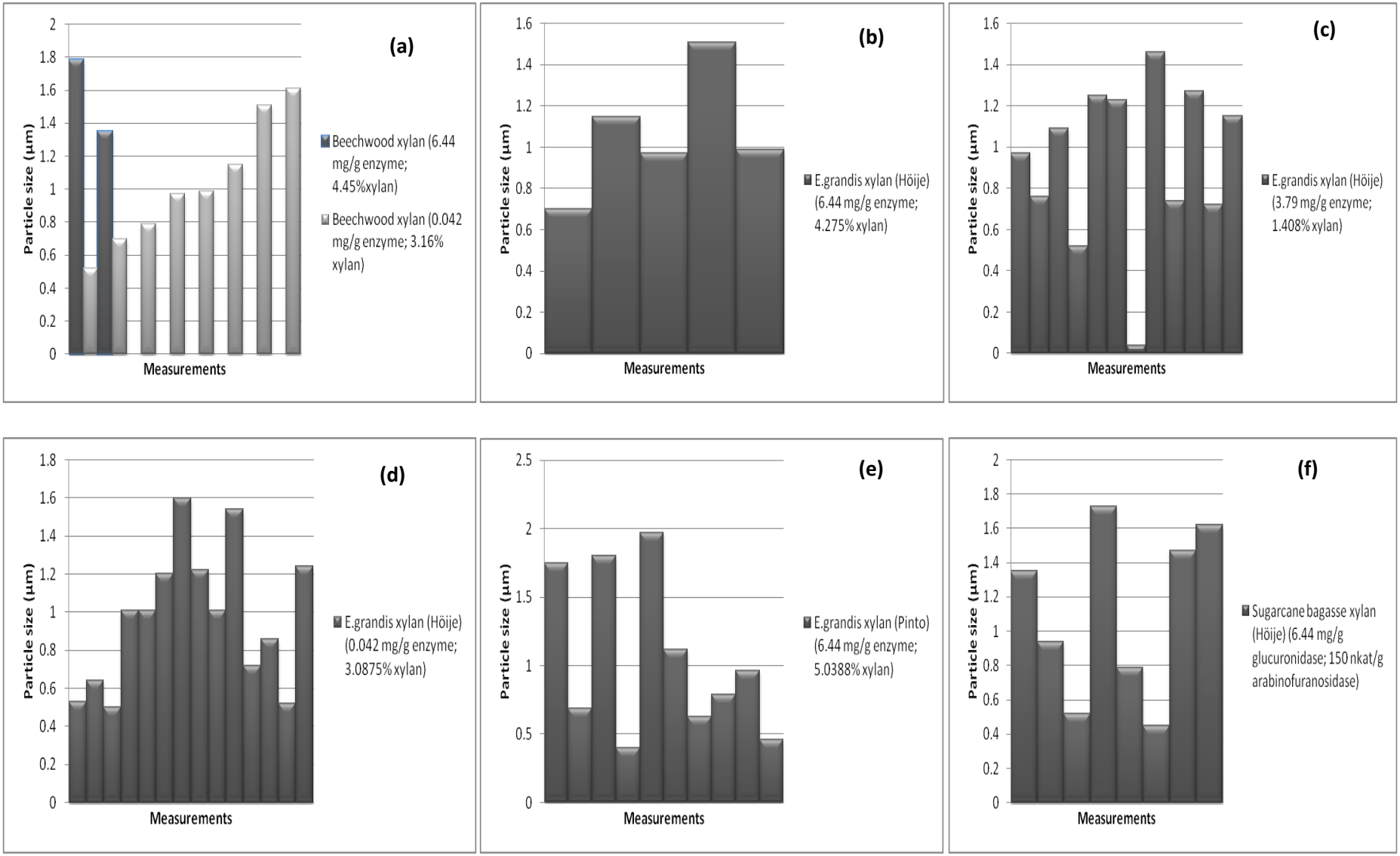


Figure 5. 17: Particle size distribution of the different modified insoluble xylans from selective hydrolysis with α-D-glucuronidase and α-L-arabinofuranosidase

The enzymatic hydrolysis of 4.45% beechwood xylan at 6.44 mg/g resulted in mean particle sizes between 1.35-1.78 µm. However, the lower concentration of 3.16% treated with 0.042 mg/g of α-D-glucuronidase resulted in smaller particles, in the range from 0.52-1.6 µm (Figure 5.17a).

E. grandis xylan (Höije) at 4.275%, treated with 6.44 mg/g α-D-glucuronidase formed hydrogels with non-agglomerated particles in the range of 0.7-1.51 µm. The decrease of the dosage to 0.042 mg/g and the xylan concentration to 3.0875%, formed fewer particles ranging from 0.5 to 1.6 µm. Further decrease in the xylan concentration to 1.408% at an enzyme dosage of 3.79% formed particles ranging from 40 nm to 1.27 µm (Figures 5.17b, 5.17c, 5.17d).

E. grandis xylan (Pinto) at 5.03% treated with a dosage of α-D-glucuronidase of 6.4 mg/g produced particle sizes ranging from 0.4 to 1.97 µm (Figure 5.17e). The modified arabinoglucuronoxylan from sugarcane bagasse had a particle size distribution between 0.45 and 1.73 µm (Figure 5.17f).

5.4. Discussion

5.4.1. Selection of α-glucuronidase dosages for precipitation of beechwood xylan

The α-D-glucuronidase dosages that were able to increase the viscosity of beechwood xylan at 1% (w/v) were investigated (Figure 5.1). The dosages of 6.17 mg/g, 4.1 mg/g and 3.08 mg/g increased the viscosity in 72 hours of reaction as opposed to when applying 2.16 mg/g and 0.92 mg/g. This can be attributed to the number of available binding sites for the enzymatic hydrolysis reaction; lower enzyme dosages imply a lower number of available catalytic binding sites, slowing down the release of 4-*O*-MeGlcA (Bailey and Ollis, 1986). As result, the viscosity of beechwood xylan at a dosage of 6.17 mg/g of Agu1B α-D-glucuronidase was the highest. A higher release of 4-*O*-methyl glucuronic acid per unit time causes a faster aggregation mechanism due to new unsubstituted regions in the polymer backbone and therefore formation of particles with higher viscosity properties (Kabel *et al.,* 2007). However, the reaction rate is directly proportional to the substrate concentration, according to the Michaelis Menten principle (Bailey and Ollis, 1986). The screening results indicated the range at which precipitation occurs but did not show the dosage required to yield maximum precipitation within the xylans solubility intervals. In addition, the rate of side-chains release and precipitation can be improved by identifying optimum time and xylan concentration.

5.4.2 Effect of the substrate concentration and enzyme dosage on the kinetics of release of 4-O-methyl glucuronic acid and change in viscosity of polymeric xylans

Glucuronoxylan concentrations between 3.08% and 3.2% combined with Agu1B α-D-glucuronidase dosages of 3.79 mg/g led to an increase in the viscosity. However, the conditions employing Agu1B α-D-glucuronidase at dosages of 1.14 mg/g and beechwood xylan at 1.87%, *E. grandis* xylan (Höije) at 1.9%, *E. grandis* xylan (Pinto) at 1.9%, released 4-*O*-MeGlcA without causing an increase in the viscosity. In this case, the polymer solution was too dilute, despite the high release of 4-*O*-MeGlcA side-chains, indicating that there is a minimum xylan concentration that enables precipitation to proceed.

The time-course profile for both native and deglycosylated Agu1B α-glucuronidases at 1.14 mg/g and beechwood xylan at 1.87% (w/v) displayed a decrease in the rate at which the 4-*O*-MeGlcA was released when 85% (1.23 g/L) of the available side-chains were removed from beechwood xylan backbone (Figure 5. 3a).

This behaviour has been verified in many enzyme-catalysed reactions, so called enzyme product inhibition (Duggleby, 1994). Substrate inhibition of the *A. pullulans* α-D-glucuronidase has been observed with short aldouronic acids (De Wet et al., 2006). In typical enzyme kinetics (Equation 5.7), the enzyme (E) binds with the substrate (S) to form an enzyme-substrate complex (ES), such step considered to be reversible. Finally, the complex dissociates releasing free enzyme (E) and the product (P), this way continuously regenerating available active sites for the substrate hydrolysis.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} P + E \qquad \textbf{Equation 5. 7}$$

However, in this case the product 4-*O*-MeGlcA might have acted as inhibitor by altering, slowing or stoping the catalytic action of the α-glucuronidase. The "lock and key" theory on competitive inhibition explains the occurrence of this phenomena (Morrison, 1969) (Figure 5. 18). The 4-*O*-MeGlcA bears structural resemblance to the substrate, causing competition on the binding site at which the substrate (xylan) is hydrolysed:

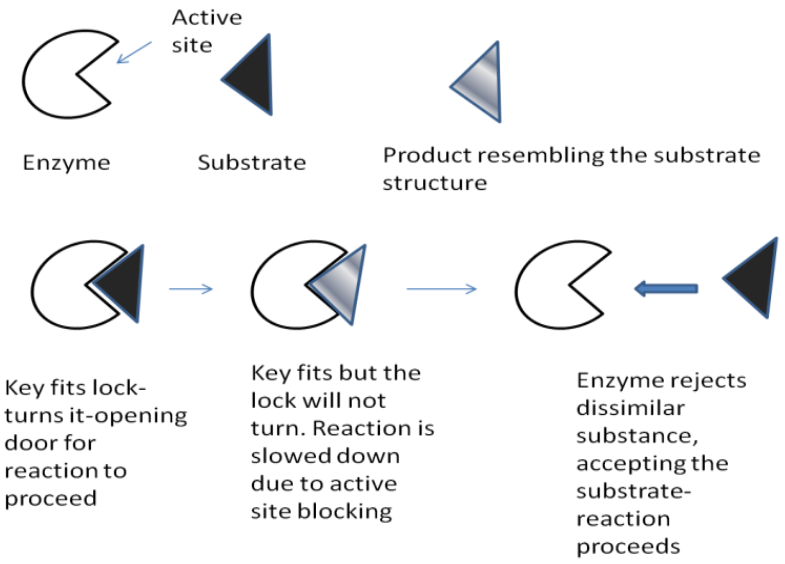


Figure 5. 18: The lock and key theory explaining the mechanism of reversible competitive product inhibition, redrawn from a publication from http://www.worthington-biochem.com/introbiochem/default.html

In this case, the product 4-*O*-MeGlcA reached a specific concentration range (1.23-1.79 g/L) before this occurred, sufficient to slow down the net reaction rate for the enzyme-substrate dissociation step. The 4-*O*-MeGlcA acted as a competitive reversible inhibitor, since the

concentration increased after a certain period of time (Duggleby, 1994). Product inhibition can significantly decrease the net reaction rate in the removal of xylan side-chains affecting negatively the rate at which the polymers aggregate due to debranching. This behaviour was not verified using the Agu1A α-D-glucuronidase possibly due to the lower yields verified on the release of 4-*O*-MeGlcA. Product inhibition can be avoided by increasing the substrate concentration and out-competing the inhibitor (Morrison, 1969). The investigation of the inhibition mechanism and the correspondent reaction constants are beyond the scope of this study, however it has been investigated in depth by many authors elsewhere (Morrison, 1969; Weiss and Cleland, 1987; Duggleby, 1994; Navarro-Lozano *et al.,* 1995; Garrido-del Solo *et al.,* 1999).

The viscosity of glucuronoxylans did not increase at the conditions previously described, therefore, it became important to find the threshold concentration at which xylan forms hydrogel aggregates. When α-D-glucuronidase dosage was increased to 3.79 mg/g and the glucuronoxylan concentrations between 3.1 and 3.2% (w/v), the viscosity increased immediately after 1.5 hours. This can be attributed to three factors, mainly a higher concentration of xylan, a higher dosage of enzyme, and as a consequence a higher release of 4-*O*-MeGlcA side-chains per unit time (Figures 5.4a and 5.4b). Agu1B and Agu1A α-D-glucuronidases also had different specific rates at which the 4-*O*-MeGlcA was released. The removal of 4-*O*-MeGlcA using native and deglycosylated Agu1B α-glucuronidases reached equilibrium at 24 hours. In contrast, the reaction using Agu1A α-glucuronidase only showed an increase of 4-*O*-MeGlcA after 24 hours of hydrolysis in all substrates (Figure 5.4a and 5.4b). Such slow reaction rate might not suit certain industrial demands, unless other process factors are optimized, such as the enzyme dosage and the xylan concentration. At the mentioned xylan concentrations and α-D-glucuronidase dosages, the viscosity increased with no significant changes after 24 hours. This indicated that 24 hours was the optimum time required for the removal of 4-*O*-MeGlcA side-chains from soluble xylans leading to an increase in the viscosity, depending on the extent of release of 4-*O*-MeGlcA (Figures 5.3a,5.3b 5.4a, 5.4b).

5.4.3. Effect of deglycosylation of Agu1B α-D-glucuronidase on the enzymatic hydrolysis of soluble beechwood xylan

The effect of deglycosylation on the catalytic activity of Agu1B α-D-glucuronidase was assessed by comparing the time-course profiles for the 4-*O*-MeGlcA release and change in viscosity before and after deglycosylation. At α-D-glucuronidase dosages of 1.14 mg/g and beechwood xylan concentration of 1.8% (w/v) both enzymes were able to remove 100% of the 4-*O*-MeGlcA side-

chains during 72 hours of hydrolysis without an increase in the viscosity. At α-D-glucuronidase dosages of 3.79 mg/g and beechwood xylan concentrations of 3.16%, 4-*O*-MeGlcA was released at 90% and 75% for the native and deglycosylated α-glucuronidases, respectively. However, the hydrolysis of beechwood xylan by Agu1A α-D-glucuronidase under the same conditions only released 30% (0.72 g/L) of the available 4-*O*-MeGlcA side-chains (Figure 5. 4a). This indicated that the extent of glycosylation was not a determining factor in the catalytic properties of α-D-glucuronidases expressed by recombinant *S. cerevisiae* strains.

5.4.4. Effect of glucuronoxylans chemical and structural properties on the rate of release of 4-O-methyl glucuronic side-chains and the viscosity

The three glucuronoxylans had different initial chemical compositions, molecular weights, degrees of polymerization and substitution that led to different yields of 4-*O*-MeGlcA released (section 5.3.3). Consequently, the release of 4-*O*-MeGlcA caused an increase in the viscosity, resulting in the formation of hydrogels with also different rheological (viscosity) properties.

The increase in the viscosity of the xylan polymers occurs due to creation of new unsubstituted regions that attract neighbouring unsubstituted polymers, resulting in the aggregation phenomena and consequently, precipitation/increase in the viscosity (Kabel *et al.,* 2007). Also, the precipitation occurs due to the new pattern of distribution of the 4-*O*-MeGlcA side-chains, rather than just the reduced degree of substitution alone (Linder *et al.,* 2003; Kabel *et al.,* 2007; Chimphango, 2010). Beechwood xylan required the highest amount of 4-*O*-MeGlcA removal (67%) as compared to the *E. grandis* xylan (Höije) (16%) and *E. grandis* xylan (Pinto) (40%). This is because *Eucalyptus* xylans have a blockwise distribution of the 4-*O*-MeGlcA substituents over the xylan backbone, leaving major parts of the xylan backbone unsubstituted (Kabel *et al.,* 2007). Thus, a lower release of 4-*O*-MeGlcA is required to increase the viscosity of *E. grandis* xylan (Höije). However, *E. grandis* xylan extracted using the Pinto protocol required a higher release of 4-*O*-MeGlcA that resulted in a lower intrinsic viscosity, as compared to the one extracted using the Höije protocol. *E. grandis* (Höije) had a higher Mw (63,809 Da) and DP (287), as compared to *E. grandis* xylan (Pinto), having a Mw of 40,021 Da and a DP of 133 (Chapter 3; Table 3. 2). The size exclusion elution profile for *E. grandis* xylan (Pinto) also showed that the xylan was mainly in oligomeric and monomeric forms, thereby reducing inter- and intramolecular polymer interactions required for precipitation to occur. In beechwood xylan, the Mw (17,053 Da) and the DP (77) were the lowest but still above the minimum required for precipitation to occur (Chimphango, 2010).

E. grandis xylan (Pinto) had a much lower initial degree of substitution (1 glucuronic acid for every 12th xylose unit), as compared to *E. grandis* xylan (Höije) (1 glucuronic acid for every 2nd xylose unit). The initial degree of substitution of beechwood xylan was the same as of *E. grandis* xylan (Pinto). In this study, the minimum degree of substitution required for formation of xylan hydrogels was 1:40, 1:6 and 1:25 for beechwood xylan, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto), respectively. It has been found that the minimum degree of substitution required for the precipitation of glucuronoxylans to occur is 1:15 (Kabel *et al.,* 2007). However, it can be seen that the degree of polymerization and the presence of lignin played an important role in this criteria, a higher DP and Mw favoured precipitation in glucuronoxylans with a higher degree of substitution. Previous studies have shown that xylans with higher molecular weights have better surface sizing properties and water resistance in papermaking processes (Bai *et al.,* 2012).

E. grandis xylan (Höije) had the highest lignin content (34.2%), compared with beechwood xylan (3.5%) and *E. grandis* xylan (Pinto) (5.3%). It has been found that lignin participates in the agglomeration phenomena of xylan in aqueous solutions (Westbye *et al.,* 2007). However, from batch kinetic results one can infer that the xylan concentration had a significant influence on the aggregation mechanism and the change of rheological properties of enzymatically modified glucuronoxylans. The presence of lignin clearly decreased the release of 4-*O*-MeGlcA in *E. grandis* xylan (Höije) as compared to beechwood xylan and *E. grandis* xylan (Pinto), as previously found from the maximum 4-*O*-MeGlcA released from each xylan (Figure 5. 4a, 5.4b). However the lignin itself played a role in the aggregation phenomena, increasing the viscosity of modified *E. grandis* xylan (Höije).

The time-course profile for Agu1A α-D-glucuronidase hydrolysis on the different glucuronoxylans displayed an initial phase without release of 4-*O*-MeGlcA (Figure 5.4a, 5.4b). This "lag-phase" effect was more prolonged in the extracted xylans than in the beechwood xylan (Sigma). This indicates that in extracted xylans, other factors affected the α-glucuronidase activity, such as the initial degree of substitution, type of side-chains, and presence of lignin (Wallace *et al.,* 1991). The release of 21% of 4-*O*-MeGlcA for sugarcane bagasse xylan (1.23%) using α-D-glucuronidase dosage of 3.79 mg/g was not able to create insoluble particles of hydrogels. In this case, it might be required the simultaneous hydrolysis using both α-L-arabinofuranosidase and the α-glucuronidase.

The kinetic experiments indicated the time at which the reaction can be terminated depending on the reaction and viscosity rates. The minimum time at which product release and viscosity reached the equilibrium was at 24 hours, using a dosage of α-D-glucuronidase of 3.79 mg/g and

xylan concentrations ranging from 3.16-3.2% (w/v). This required time agrees with the recommended time for enzymatic hydrolysis of xylans as described earlier in literature (Chimphango, 2010). This minimum reaction time can be applied to maximize the viscosity of xylan hydrogels, by finding the optimum combination of α-D-glucuronidase types, dosages and xylan concentrations.

5.4.5. Optimum conditions for the release of 4-*O*-methyl glucuronic acid from glucuronoxylans by α-glucuronidase

The soluble glucuronoxylans in this study showed an increase in the viscosity for different combinations of xylan concentrations and enzyme dosages. Optimum conditions were found when using the Agu1B α-D-glucuronidase (Figure 5. 9Figure 5. 10). Subsequently, the different combinations of Agu1B α-D-glucuronidase dosages and glucuronoxylans concentrations enhanced the rheological properties (viscosity) of glucuronoxylans.

Results in Figure 5.10 and 5.11 showed that the conditions for maximum release of 4-*O*-MeGlcA did not coincide with the ones that gave the highest viscosities for the glucuronoxylans in this study. Optimum conditions for precipitation were located using desirability functions, at a dosage of α-D-glucuronidase of 6.4 mg/g and xylan concentrations of 4.45%, 4.28% and 4.50% (w/v) for beechwood xylan, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto), respectively.

In beechwood xylan, the maximum viscosity (1510 mPa.s) corresponded to 90% removal of the 4-*O*-MeGlcA side-chains, at a dosage of 6.4 mg/g and xylan concentration of 4.45% (w/v). On the other hand, maximum release of the available 4-*O*-MeGlcA side chains occurred when the xylan concentration was 1.87% and the enzyme dosage was 6.4 mg/g (Table 5. 7). The yield of 4-*O*-MeGlcA release was mainly dependent on the type and dosage of α-D-glucuronidase (Table 5. 8). When the xylan concentration was decreased from 4.45% to 3.16% the yield of 4-*O*-MeGlcA increased from 90% to 100%, indicating that at a dosage of 6.44 mg/g, the enzyme reached the saturation point, according to the Michaelis-Menten principle (Bailey and Ollis, 1986). The factors significance levels agree with the higher release of product response obtained when the enzyme dosage is increased rather than the xylan concentration, as it can be visualised from the response surface plots (Figure 5.10, 5.11).

The analysis of variance showed that the enzyme dosage was equally significant for the removal of 4-*O*-MeGlcA in *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto), as opposed to the xylan concentration. Maximum removal of 4-*O*-MeGlcA occurred at a dosage of 6.4 mg/g and xylan concentration of 4.27%, coinciding with the conditions that resulted in the highest viscosity (3130 mPa.s). This is attributed to the lower degree of removal of the 4-*O*-MeGlcA therefore the

enzyme did not reach the saturation point in the hydrolysis of *E. grandis* xylan (Höije), as opposed to the hydrolysis of beechwood xylan. Similarly with *E. grandis* xylan (Pinto), the maximum release of 4-*O*-MeGlcA at 57% resulted in the highest viscosity, corresponding to 115 mPa.s (Table 5. 7).

On the other hand, the change in the viscosity was more affected by the xylan concentration than the enzyme dosage (Figure 5.10, 5.11). The analysis of variance (Appendix C) showed that the xylan concentration was the most significant factor to maximise the viscosity, with p-values below 0.006. In beechwood xylan, when the xylan concentration was decreased from 4.98% to 1.87% at a dosage of 6.4 mg/g, the viscosity dropped from 1510 mPa.s to 47.9 mPa.s. Similarly for *E. grandis* xylan (Höije) a decrease of the xylan concentration from 4.5% to 3.08% led to a significant decrease in the viscosity from 3130 mPa.s to 255 mPa.s (Table 5. 7). It has been found that the xylan concentration plays an active role in the aggregation phenomena (Westbye *et al.,* 2007). Consequently, this indicated that the viscosity increases due to aggregation of insoluble xylan particles is more dependent on the xylan concentration that on the enzyme dosage employed, while for the release of 4-*O*-MeGlcA, the enzyme dosage was the most significant factor.

The empirical models for the viscosity showed a significant lack of fit due to the large difference in viscosity levels achieved when the xylan concentration was increased from 3.08-3.2% (w/v) to 4.2-4.98% (w/v). Such difference corresponded to a 10-fold increase in the viscosity, forming hydrogel structures. The viscosity could not be accurately predicted since it is also dependent on the yield and on the rate of release of 4-*O*-MeGlcA, as it was verified earlier in section 5.3.3.

By increasing the xylan concentration (4.2-4.98% w/v) the rate at which the side-chains were removed was increased, leading to a higher viscosity after 24 hours of hydrolysis (Figure 5. 9). The aggregation mechanism of xylans results from intermolecular attraction forces that can only occur once the 4-*O*-MeGlcA removal overcomes the minimum DS required for precipitation to occur in the different glucuronoxylans. On the other hand, the presence of impurities such as lignin can cause deviations in the prediction of the viscosity properties that result from different combinations of xylan concentrations and α-D-glucuronidase dosages.

Maximising the xylan concentration is more economical since it reduces costs on the enzyme production. The cost of enzyme production can also be minimized if the reaction is carried out in a reactor system where the enzyme is recycled (bypass stream). In this study, the optimisation of the enzymatic hydrolysis of the glucuronoxylans led to higher yields of 4-*O*-MeGlcA release and intrinsic viscosities, as compared to other enzymatic modifications. The *S. commune* α-D-glucuronidase released 49% of the available 4-*O*-MeGlcA in soluble birchwood

xylan, causing precipitation (Tenkanen and Siika-aho, 2000b). On the other hand, the only reported α-D-glucuronidase that was able to remove 100% of the available 4-*O*-MeGlcA in polymeric xylan was the one from *Fibrobacter succinogenes,* but only after 240 hours of hydrolysis (Smith & Forsberg, 1991).

Agu1B α-D-glucuronidase was able to release more 4-*O*-MeGlcA side-chains in beechwood xylan than in the extracted glucuronoxylans from *E. grandis*. From the compositional analysis (Chapter 3; Figure 3.2), the commercial xylan had the highest xylan content and the lowest lignin content. *E. grandis* (Höije) had the highest lignin content, causing inhibition effects on the enzymatic attack by α-D-glucuronidase (steric hindrances), as it can be verified from the lower percentage of side-chain removal. However, the presence of lignin influenced the agglomeration behaviour of xylans, as investigated earlier (Ebringerová and Heinze, 2000; Westbye *et al.,* 2007). Consequently, higher α-D-glucuronidase dosages would be required to fully release the 4-O-MeGlcA present in xylans with lignin contents above 3.57%.

At optimum hydrolysis conditions the viscosity of modified *E. grandis* xylan (Höije) was higher than the modified beechwood xylan and *E. grandis* xylan (Pinto). Such difference was also due to the higher DP (287) verified for the *E. grandis* xylan (Höije), compared with the beechwood xylan (77) and the low molecular weight properties verified for *E. grandis* xylan (Pinto), which was mainly in the oligomeric and monomeric form. Also, the higher lignin content in *E. grandis* xylan (Höije) increased the aggregation of the xylan in the solution thereby leading to a higher viscosity. Insoluble xylans can be employed as coating materials, pulp strength enhancement additives and as hydrogels (biomedical field). The presence of lignin would be undesirable in the pulp industry because it causes yellowing of the paper, however it would not necessarily be the case for other applications. Beechwood xylan and *E. grandis* xylan (Höije) formed hydrogels that can suit many applications such as emulsifiers, sizing agents (Bai *et al.,* 2012) and hydrogels for drugs encapsulation (Deutschmann and Dekker, 2012.).

Results indicated that the xylan concentration was the determining factor in the formation of xylan hydrogels with high intrinsic viscosities. At optimum conditions, the threshold for the minimum 4-O-MeGlcA release required for xylan precipitation was overcome and the α-D-glucuronidase dosage was no longer a limiting factor in the aggregation mechanism. Therefore, complete removal of 4-O-MeGlcA from the xylan backbone is not a pre-requisite for precipitation.

5.4.6. Effect of α-L-arabinofuranosidase and α-D-glucuronidase dosages on side-chain release and precipitation of soluble sugarcane bagasse xylan

The removal of 4-*O*-methyl glucuronic acid and arabinose from arabinoglucuronoxylans was catalysed by α-D-glucuronidase and α-L-arabinofuranosidase, respectively. α-D-glucuronidase dosage was the most significant factor (p-value 0.015) for the precipitation of sugarcane bagasse xylan.

For example, treatment 5 (0.042 mg/g Agu; 250 nkat/g Ara) resulted in 5.75 mg of xylan precipitate, equivalent to 9.34% precipitation, whereas treatment 7 (3.79 mg/g Agu; 108.57 nkat/g Ara) resulted in 10.3 mg of xylan precipitate which is equivalent to 17% precipitation. A lower α-D-glucuronidase dosage employed caused much less precipitation, compared to when the α-L-arabinofuranosidase was minimised (Figure 5.12). Furthermore, at 6.4 mg/g α-D-glucuronidase there was a positive synergy for the release of both arabinose and 4-O-MeGlcA side-chains from soluble arabinoglucuronoxylan, even when the α-L-arabinofuranosidase dosage was decreased to 150 nkat/g (Figure 5.12).

The literature reports on different lignocellulose enzymes can act synergistically in complex substrates when employed together (Chimphango *et al.,* 2012; Van Dyk & Pletschke, 2012) . Low removal of 4-*O*-MeGlcA was verified earlier for sugarcane bagasse xylan with a maximum removal of 2%, and arabinose removal of 18% (Chimphango, 2010). In this study, the maximum release of arabinose and 4-*O*-MeGlcA was 69.5% and 25%, respectively. Some factors were found to have an influence on the action of α-glucuronidase, these include the presence of other side-chains (arabinose) that can cause steric hindrances and the conformational changes in the structure of the substrate resulting from the hydrolysis by other enzymes (Chimphango *et al.,* 2010; Tenkanen and Siika-aho, 2000b).

The initial degree of substitution also influenced the extent at which the side-chains were removed. The arabinoglucuronoxylan extracted from sugarcane bagasse (Chapter 3) was less substituted with arabinose (4.03%) and 4-*O*-MeGlcA (7.50%). The α-D-glucuronidase has a high molecular weight (125-130 kDa) with a more restrictive substrate specificity as compared to the α-L-arabinofuranosidase (de Wet *et al.,* 2006; Chimphango, 2010). However, the precipitation of arabinoglucuronoxylan was due to the removal of both arabinose and 4-*O*-MeGlcA from the synergistic action of α-L-arabinofuranosidase and α-glucuronidase.

The highest amount of precipitation was 16.15 mg, which corresponded to a degree of precipitation of 26%, releasing 48% and 22% of the arabinose and 4-*O*-MeGlcA side-chains, respectively (treatment 2) (Figure 5.12). At these conditions the arabinose content decreased

from 4.03% to 2.1% and the 4-*O*-MeGlcA content from 7.5% to 5.8%. The conditions for maximum removal of arabinose and glucuronic acid side-chains were different from the ones to obtain maximum degree of precipitation, since the additional release of the arabinose and 4-*O*-MeGlcA at 69.5% and 24%, respectively, did not result in higher precipitation levels (treatment 4, Figure 5.12). It has been found that the removal of arabinose or 4-*O*-MeGlcA in different types of xylans, not always results in immediate precipitation or increase in the viscosity (Chimphango, 2010). The stereochemistry of the hydrolysis (Kolenová *et al.,* 2010) and insufficient enzymes dosages can restrict the action of α-glucuronidase, slowing down the aggregation phenomena that leads to precipitation. The lack of increase in the release of 4-*O*-MeGlcA, as more arabinose side-chains are released, is also associated with possible changes in the xylan conformation upon hydrolysis by α-arabinofuranosidase, as earlier verified (Tenkanen and Siika-aho, 2000a). Therefore, a higher degree of 4-*O*-MeGlcA release would be required to improve the degree of precipitation, since the arabinose was significantly released from the backbone structure of sugarcane bagasse xylan.

The results showed that simultaneous hydrolysis with α-L-arabinofuranosidase and α-D-glucuronidase had a positive synergistic effect on the release of arabinose and 4-*O*-MeGlcA that produced higher degrees of xylan precipitation as compared to the hydrolysis with either α-L-arabinofuranosidase or α-D-glucuronidase (Figure 5.12). An additive effect on the precipitation from the concerted action of the two enzymes was also verified. In addition, a high dosage of α-L-arabinofuranosidase can be combined with a low dosage of α-D-glucuronidase without decreasing the degree of precipitation (treatments 2 and 3), thereby reducing costs on the enzyme production (Figure 5.12). This is economical since the media composition required for α-L-arabinofuranosidase production from recombinant *A. niger* is more costly as compared to the one for α-D-glucuronidase from recombinant *S. cerevisiae*.

5.4.7. Effect of the enzymatic hydrolysis parameters and xylan characteristics on the particle morphology

The morphologies of modified glucuronoxylans from beechwood and *E. grandis* were different from the arabinoglucuronoxylan from sugarcane bagasse. Glucuronoxylans formed solid hydrogels at optimum enzymatic hydrolysis conditions, whereas modified arabinoglucuronoxylans formed particles that settled with gravity (Figure 5. 14). The difference in the morphology of arabinoglucuronoxylan from sugarcane bagasse and glucuronoxylans from beechwood and *E. grandis* may be attributed to their different types and patterns of substitution, since the arabinoglucuronoxylans contain additional arabinose side-chains

distributed at irregular intervals along the backbone, consequently affecting the mechanism of aggregation in the solution. Earlier investigations have shown that the aggregation of glucuronoxylans was not dependent on the molecular weight but on the chemical structure (Saake *et al.,* 2001; Westbye *et al.,* 2007). In addition, the difference between the pattern of precipitation of the glucuronoxylans and the arabinoglucuronoxylans in this study can not be attributed to the degree of substitution after enzymatic hydrolysis since both modified beechwood xylan (DS of 1:135) and *E. grandis* xylan (Höije) (DS of 1:6) formed solid hydrogels.

Microscopy results indicated different agglomeration behaviours for both glucuronoxylan hydrogels and the sugarcane bagasse arabinoglucuronoxylan insoluble particles. The agglomeration was verified for beechwood xylan, *E. grandis* xylan (Pinto) and sugarcane bagasse xylan (Höije). These xylans had a lower initial degree of substitution (Chapter 3) as compared to the *E. grandis* xylan (Höije). It can be presumed that the degree of substitution by 4-*O*-MeGlcA side-groups played a major role in the aggregation behaviour, since the enzymatic treatment removed up to 91% of the 4-*O*-MeGlcA side-chains present in beechwood and 57% in *E. grandis* xylan (Pinto). In sugarcane bagasse xylan (Höije), 24% and 69.5% of the 4-*O*-MeGlcA and arabinose were removed from the backbone respectively. However, the 4-*O*-MeGlcA side chains in *E. grandis* xylan (Höije) were only removed at 22%. This indicated that there should be a balance for the extent at which the side-chains are removed to control aggregation of xylan micro-particles. Thus, E. grandis xylan (Höije) with a degree of substitution of 1:6 (16% 4-*O*-MeGlcA) did not have particle agglomeration, as opposed to modified beechwood xylan and *E. grandis* xylan (Pinto) with lower degrees of substitution of 1:135 and 1:33, respectively. Consequently, particle agglomeration occurred for the glucuronoxylans in study when the 4-*O*-MeGlcA content was below 3%. In addition, the removal of arabinose and 4-*O*-MeGlcA side-chains in sugarcane bagasse xylan (Höije) at optimal conditions decreased the arabinose content from 4.03% to 2.1% and the 4-*O*-MeGlcA from 7.5% to 5.8%. The self-aggregation of the xylans might decrease the affinity of xylan for other hydrophilic materials that could be used for gel encapsulation or implanted onto cellulosic materials (Chimphango, 2010).

The molecular weight distribution did not directly determine the agglomeration behaviour of xylans or the particle sizes formed. Therefore, it is important to control the extent at which side-chains are removed and thereby the degree of substitution after the enzymatic modification to avoid agglomeration, without compromising viscosity/gel properties.

While the aggregation behaviour was more related to the degree of substitution, the particle diameter sizes increased with the xylan concentration in the solution (Figure 5. 17). Micro particles were found to increase in size with an increase in the substrate concentration, from a

minimum of 4.45% (w/v) during enzymatic hydrolysis. Glucuronoxylan hydrogels and arabinoglucuronoxylan insoluble particles formed at optimum conditions had particles sizes ranging from 0.4 to 1.97 µm. Therefore, the particle diameter of modified xylans can be manipulated to be larger or smaller, depending on the xylan concentration, whereas the intrinsic viscosity and number of particles were associated with both xylan concentration and enzyme dosages. As mentioned before, the slight increase in particle size range can be attributed to the higher concentration of the xylan. Particle sizes for the modified glucuronoxylans in this study are within the range of previously modified xylans using chemical modification methods. Chemical modification of hemicelluloses using DMSO/water mixtures resulted in a wide particle size range, from less than 0.1 µm to more than 5 µm (Haimer *et al.,* 2010). Xylan from corn cobs extracted by Oliveira *et al.* (Oliveira et al. 2010) had a bigger particle size, ranging from 65.39 to 7.68 µm. Particles of the modified sugarcane bagasse arabinoglucuronoxylan in this study were larger in diameter than the ones reported by Chimphango et al. (Chimphango *et al.,* 2012). This is because of the lower degree of substitution which led to aggregation and formation of particles with a larger diameter. According to Kabel *et al.* xylans with fewer substituents tend to self-aggregate, which has been observed after enzymatic hydrolysis treatment (Kabel *et al.,* 2007). In addition the reason of aggregation is not attributed to the molecular weight but to the chemical structure of the xylans (Saake *et al.,* 2001).

The high lignin content of *E. grandis* xylan (Höije) did not increase the aggregation between the insoluble micro-particles, as compared with all the other xylans with lower lignin contents. The particle size and agglomeration are important characteristics to consider in the design of speciality materials for various applications, in particular for encapsulation matrices (Oliveira *et al.,* 2010). The lignin is known to create hydrophobic bonding sites in the xylan structure, that can be useful in the production of bio-based surfactants (Hromádková *et al.,* 2005). In addition, the presense of phenolic substances such as lignin can be beneficial due to its anti-oxidant properties (Hromádková *et al.,* 2005). Modified xylans had acceptable particle size diameters that could suit many end-uses such as coating materials and hydrogels in biomedical applications. The absence of agglomeration has additional advantages due to structural uniformity for slow release of drugs in hydrogels (Chimphango, 2010).

5.4. Conclusions

The release of 4-*O*-MeGlcA side-chains by α-D-glucuronidase decreased the solubility properties of glucuronoxylans from beechwood and *E. grandis*. Simultaneous release of 4-*O*-MeGlcA and arabinose by α-D-glucuronidase and α-L-arabinofuranosidase enabled precipitation of soluble sugarcane bagasse xylan. Consequently, the change in solubility properties was affected by the hydrolysis time, the enzyme dosage and the xylan concentration. Xylan chemical and structural properties influenced the rate and the degree of removal of the side-chains.

The degree of polymerization and the presence of lignin were important aspects that influenced the viscosities of the modified xylans, a higher degree of polymerization and lignin content favoured better rheological properties, in terms of higher viscosities. On the other hand, the aggregation of micro-particles verified in the modified xylans was related to the degree of substitution that resulted from the removal of the side-chains removed from the xylan backbone, rather than to the degree of polymerization. The increase in particle diameter was associated with an increase in the xylan concentration, therefore micro-particle aggregation and particle sizes observed in the hydrogels formed can be manipulated by controlling the degree of removal of the side-chains and the xylan concentration, opening doors to expand xylan end-uses as a bio-polymer. Precipitated arabinoglucuronoxylan was in the form of insoluble particles that settled with gravity. Such properties have limited applications, as compared to modified glucuronoxylans which formed insoluble hydrogels.

A linear correlation was verified between the release in 4-*O*-methyl glucuronic acid and the increase of viscosity with R-square values above 0.90. At 24 hours, the minimum percentage of removal of 4-*O*-MeGlcA for precipitation to occur was 67%, 16% and 40% for beechwood, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto), respectively. Consequently, the minimum 4-*O*-MeGlcA content for increase in the viscosity of soluble beechwood xylan, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto) was 2.54%, 17.4% and 4.26%, respectively. Product inhibition kinetic behaviour was verified at low dosages of enzyme and low substrate hydrolysis conditions, which was overcome by increasing the substrate concentration conditions to values above 1.8% (w/v).

Agu1A α-D-glucuronidase mode of action and the rate of product release were not able to alter the solubility properties of all the xylans in study, within 72 hours of hydrolysis time. In addition, the extent of glycosylation was not the key factor for the catalytic properties of the α-glucuronidases studied.

The results also showed that the enzymatic hydrolysis at xylan concentrations above 4% (w/v) and GH115 α-D-glucuronidase dosages of 6.4 mg/g maximised the yield of 4-*O*-MeGlcA released and precipitation of soluble glucuronoxylans. At these conditions, precipitated glucuronoxylans formed solid hydrogels with intrinsic viscosities ranging from 115 mPa.s to 3130 mPa.s. Degrees of substitution and polymerization of xylan, lignin content and the xylan concentration are important factors to consider for designing novel xylan-based hydrogel delivery systems, additives in the paper-making process and in cosmetics.

The enzymatic hydrolysis of arabinoglucuronoxylan from sugarcane bagasse with α-L-arabinofuranosidase and α-D-glucuronidase showed a significant increase of side-chains release and degree of precipitation when the two enzymes are employed together at 6.4 mg/g α-D-glucuronidase and 350 nkat/g α-L-arabinofuranosidase. However, maximum degree of precipitation was obtained by combining a low dosage of α-L-arabinofuranosidase (150 nkat/g) with a high dosage of α-D-glucuronidase (6.4 mg/g). This can significantly reduce costs related to the enzyme production, mainly on the α-L-arabinofuranosidase production. The quadratic model evaluated for the release of both side-groups showed that the most significant factor to maximize the degree of precipitation was the α-D-glucuronidase dosage.

Chapter 6: Conclusions and recommendations

6.1. Conclusions

The aim of this study was to investigate the selective modification of xylans from different lignocellulose feedstocks using side-chain hydrolysing enzymes, mainly α-D-glucuronidase and α-L-arabinofuranosidase and evaluate suitability of functionalised xylans to be incorporated at different levels.

The extraction protocols adopted by Höije et al. and Pinto et al. were suitable to be employed in the extraction of hemicelluloses from *E. grandis* and sugarcane bagasse. However, the Höije protocol was able to preserve the polysaccharides molecular weight original properties with lower yields of extraction and higher lignin content as opposed to the Pinto protocol. Solubility and chemical properties of purchased and extracted xylans were also suitable for the enzymatic modification. However, the challenge remains in obtaining xylans with low lignin content and little polysaccharide degradation, which are more preferred for efficient debranching by enzymatic hydrolysis.

The genomic copy of the *P. stipitis* Agu1A α-D-glucuronidase with 95% homology on the protein level showed a significantly lower catalytic activity in oligomeric and polymeric xylan substrates, as compared with the *P. stipitis* Agu1B α-D-glucuronidase with 100% homology. In addition, the extent of glycosylation was not an important factor in the catalytic properties of recombinant α-D-glucuronidase expressed in *S. cerevisiae*. The substrate specificity of the partially purified α-D-glucuronidases showed that the catalytic activity on short aldouronic acids was only slightly higher as compared to the activity in glucuronoxylans, but significantly lower in arabinoglucuronoxylan.

Selective removal of 4-*O*-methyl glucuronic acid from beechwood, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto) led to increased viscosity, forming insoluble hydrogels. The kinetics of enzymatic hydrolysis showed different profiles for 4-*O*-MeGlcA released, depending on the xylan and α-D-glucuronidase dosage levels. Low xylan concentrations of 1.8% (w/v) and α-D-glucuronidase dosages of 1.87 mg/g favoured product inhibition behaviour.

A linear relationship (R-squared values above 0.90) was found between the release of 4-*O*-methyl glucuronic acid and the increase in viscosity. Optimum conditions to maximize side-chain removal and viscosity were located at an enzyme dosage of 6.4 mg/g and xylan

concentrations of 4.45% (w/v), 4.28% (w/v) and 4.50% (w/v) for beechwood xylan, *E. grandis* xylan (Höije) and *E. grandis* xylan (Pinto), respectively.

A positive synergy was verified from the removal of both arabinose and 4-*O*-methyl glucuronic acid in sugarcane arabinoglucuronoxylan. A high dosage of α-D-glucuronidase (6.4 mg/g) and a low dosage of α-L-arabinofuranosidase (150 nkat/g) resulted in the same degree of precipitation as when employing high dosages for both enzymes. Therefore, enzyme production costs can be reduced by employing hydrolysis of sugarcane bagasse xylans with both enzymes.

The degree of polymerization of the extracted xylans is an important parameter to consider in producing insoluble particles of xylans. However the xylan concentration levels determined both the viscosity properties and the particle size distribution of the modified xylans. The aggregation behaviour of the modified xylans was related to the degree of substitution, less substituted xylans aggregated more easily due to increased intra and inter-molecular forces of the polymers. Consequently, the extent at which side-chains are removed can be controlled to avoid aggregation without compromising the viscosity properties of the hydrogels formed. Such conditions were fulfilled in *E. grandis* xylan (Höije) but not in other xylans due to their initial lower degree of substitution.

The insoluble microparticles formed represent green and new alternatives to be used along with other fossil derived additives at different levels. Modified beechwood xylan and *E. grandis* xylan (Pinto) with low degrees of substitution and solubility could be employed in pulp and paper industries as wet-strength additives to improve strength properties of cellulose. The modified *E. grandis* xylan (Höije) had higher lignin content but showed better morphological properties without inter-particle aggregation in the hydrogel structure. Therefore, it represents a promising option in pharmacy for slow release of drugs and encapsulation matrices. The enzymatic modification of xylans is a viable method to add value to a class of polymers largely wasted due their inherent high solubility in water in contrast with cellulose and starch composites.

6.2. Recommendations

The enzymatic modification of xylans can only be successful with adequate choice of hemicellulose extraction protocols that include the preparation of the feedstocks, delignification and isolation steps. In analogy with crude oil refineries, it is desirable to efficiently manage the products and waste derived from the various fermentation and thermo-chemical processes to yield high valuable end products such as fuel, chemicals and energy.

Further research is required to reduce the lignin content while preserving the original molecular weight properties of extracted glucuronoxylans using mild-alkali-low temperature extraction protocols. Consequently, this would greatly enhance final functional properties of xylans to be applicable both in pulp making processes and as encapsulation matrices. This would imply the study of the adsorption mechanism of the modified xylans on different cellulosic materials and the kinetics of drug release in xylan-based encapsulation matrices.

Optimum conditions found for the enzymatic hydrolysis of glucuronoxylans and arabinoglucuronoxylans would need to be investigated on a scale-up basis to integrate functionalised xylans along with cellulose and starch materials, which would include a life-cycle assessment on the enzyme production and xylan modification subunits. In addition, recovery and recycle of the enzymes and efficient separation of modified xylan insoluble particles from the unmodified starting material would be required to improve the process economics and efficiency.

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Appendix A

A.1. Calibration curve for phenol sulphuric acid assay

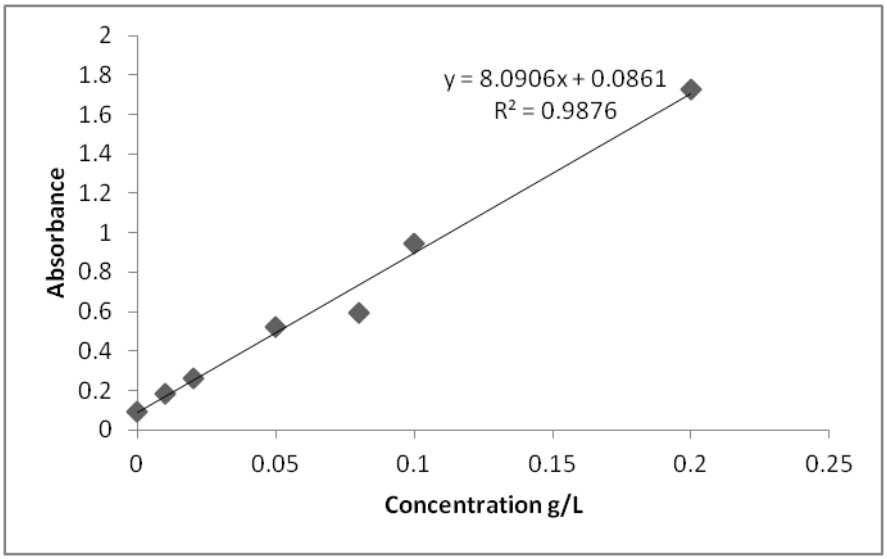

Figure A. 1: Standard calibration curve for determination of the xylan concentration in soluble xylans using the phenol sulphuric acid assay and beechwood xylan

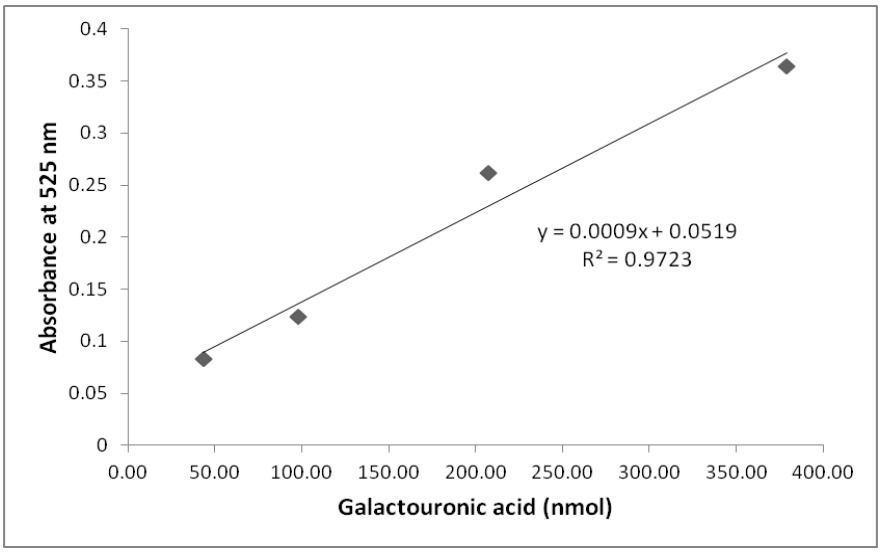

Figure A. 2: Standard calibration curve for determination of the uronic acid content of the xylans using galactouronic acid, based on the method described by (Li et al., 2007)

Appendix B

B1. Production and characterization properties of α-L-arabinofuranosidase and α-D-glucuronidase

The medium composition of AspA and trace elements was prepared in the order given below and autoclaved separately as follows:

Table B. 1: Chemical composition of AspA and trace elements used for the spore plates and shake-flask cultivations

AspA component	**For 500 ml**
$NaNO_3$	150 g
KCl	13 g
KH_2PO_4	38 g
pH adjusted to 5.5 with 10 N KOH	

Trace elements	**For 100 ml**
Distilled water	80 ml
$ZnSO_4.7H_2O$	2.2 g
H_3BO_3	1.1 g
$Mn.Cl_2.4H_2O$	0.5 g
$FeSO_4.7H_2O$	0.5 g
$CoCl_2.6H_2O$	0.17 g
$CuSO_4.5H_2O$	0.16 g
$Na_2MoO_4.2H_2O$	0.15 g
EDTA (without sodium)	5.0 g
Components were boiled and cooled down to 60 °C, pH adjusted to 6.5 with 10 N KOH	

B2. Standard curve for quantitation of partially purified α-glucuronidases

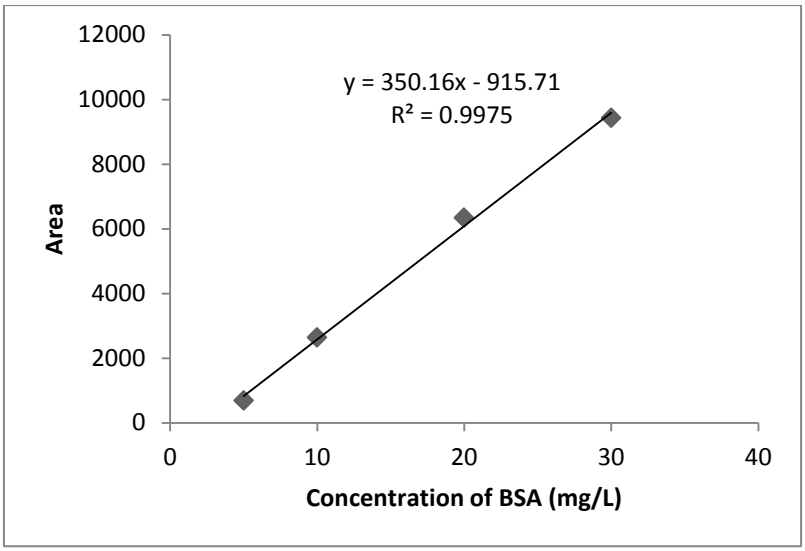

Figure B. 1: Standard calibration curve obtained using image densitometry analysis using Image J® software

B3. Standard curve for quantitative analysis of 4-O-methyl glucuronic acid

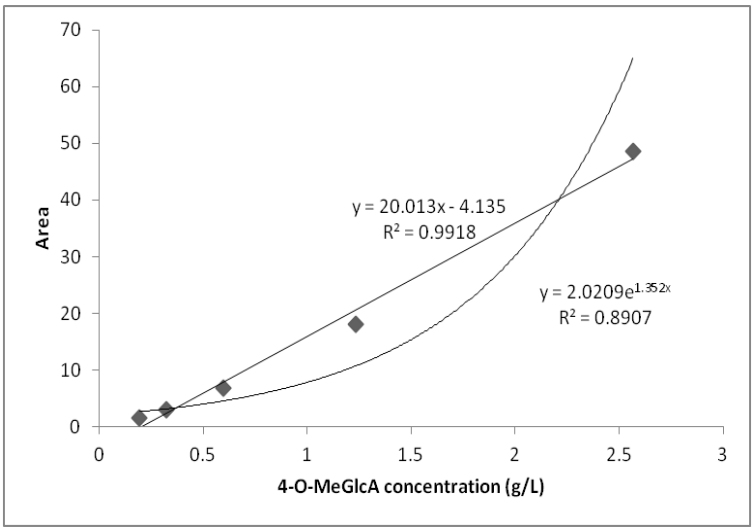

Figure B. 2: Standard calibration curve for quantitative analysis of 4-*O*-MeGlcA using D-glucuronic acid.

Appendix C

Raw data obtained from statistical Central Composite Design of glucuronoxylans in study

Table C. 1: Beechwood xylan treatment for 24 hours, viscosity measured at 10.3 s^{-1}

Std	Run	Coded values Dosage	Enzyme dosage mg/g	Coded values Conc	Substrate conc %	Glu type	Response 1 MeGlcA g/L	Response 2 Viscosity mPa.s
6	1	1.41	7.53	0	3.16	Agu1B	2.6295	349
8	2	0	3.79	1.41	4.98	Agu1B	3.4773	1510
1	3	-1	1.14	-1	1.87	Agu1B	0.8171	1.34
3	6	-1	1.14	1	4.45	Agu1B	3.0712	117
9	8	0	3.79	0	3.16	Agu1B	1.9368	230
4	11	1	6.44	1	4.45	Agu1B	3.1585	1210
5	13	-1.41	0.042	0	3.16	Agu1B	0	1.48
10	14	0	3.79	0	3.16	Agu1B	2.4966	234
7	18	0	3.79	-1.41	1.33	Agu1B	1.0367	1.43
11	20	0	3.79	0	3.16	Agu1B	2.4654	240
2	22	1	6.44	-1	1.87	Agu1B	1.4958	47.9
22	4	0	3.79	0	3.16	Agu1A	0.7217	1.68
18	5	0	3.79	-1.41	1.33	Agu1A	0.0761	1.37
21	7	0	3.79	0	3.16	Agu1A	0.1762	1.91
15	9	1	6.44	1	4.45	Agu1A	0.2262	2.69
13	10	1	6.44	-1	1.87	Agu1A	0.1942	1.58
16	12	-1.41	0.042	0	3.16	Agu1A	0.0293	1.86
20	15	0	3.79	0	3.16	Agu1A	0.1183	1.99
12	16	-1	1.14	-1	1.87	Agu1A	0	1.26
19	17	0	3.79	1.41	4.98	Agu1A	0.2016	2.94
17	19	1.41	7.53	0	3.16	Agu1A	0.1642	2.19
14	21	-1	1.14	1	4.45	Agu1A	0.1762	2.38

Table C. 2: Eucalyptus grandis xylan (Höije) treatment for 24 hours, viscosity measured at 10.3 s^{-1}

Std	Run	Coded values Dosage	Enzyme dosage mg/g	Coded values Conc	Substrate conc %	Glu type	Response 1 MeGlcA g/L	Response 2 Viscosity mPa.s
2	1	1	6.44	-1	1.9	Agu1B	0.823	10.6
5	2	-1.41	0.042	0	3.0875	Agu1B	0.7005	2
4	3	1	6.44	1	4.275	Agu1B	1.9644	3130
10	4	0	3.79	0	3.0875	Agu1B	1.2039	133
6	5	1.41	7.53	0	3.0875	Agu1B	1.4434	255
11	11	0	3.79	0	3.0875	Agu1B	1.4154	103
9	12	0	3.79	0	3.0875	Agu1B	1.3483	108
3	13	-1	1.14	1	4.275	Agu1B	1.6832	98.3
8	15	0	3.79	1.41	4.766	Agu1B	2.1219	2850
7	18	0	3.79	-1.41	1.408	Agu1B	0.5458	1.56
1	21	-1	1.14	-1	1.9	Agu1B	0.6276	1.61
20	6	0	3.79	0	3.0875	Agu1A	0	2.74
17	7	1.41	7.53	0	3.0875	Agu1A	0.1879	2.99
13	8	1	6.44	-1	1.9	Agu1A	0.0895	1.95
21	9	0	3.79	0	3.0875	Agu1A	0.0638	3.01
14	10	-1	1.14	1	4.275	Agu1A	0.0422	3.87
22	14	0	3.79	0	3.0875	Agu1A	0.1033	2.57
18	16	0	3.79	-1.41	1.408	Agu1A	0.0217	1.47
19	17	0	3.79	1.41	4.766	Agu1A	0.1114	4.53
16	19	-1.41	0.042	0	3.0875	Agu1A	0	2.5
15	20	1	6.44	1	4.275	Agu1A	0.2257	4.39
12	22	-1	1.14	-1	1.9	Agu1A	0	2.65

Table C. 3: Eucalyptus grandis xylan (Pinto) treatment for 24 hours, viscosity measured at 10.3 s^{-1}

Std	Run	Coded values Dosage	Enzyme dosage mg/g	Coded values conc	Substrate conc %	Glu type	Response 1 MeGlcA g/L	Response 2 Viscosity mPa.s
6	1	1.41	7.53	0	3.2	Agu1B	1.0363	39.5
9	2	0	3.79	0	3.2	Agu1B	0.9701	18.7
21	3	0	3.79	0	3.2	Agu1A	0	2.74
19	4	0	3.79	1.41	5.038	Agu1A	0.3017	3
1	5	-1	1.14	-1	1.9	Agu1B	0.5785	1.27
13	6	1	6.44	-1	1.9	Agu1A	0.0935	1.49
5	7	-1.41	0.042	0	3.2	Agu1B	0	1.57
14	8	-1	1.14	1	4.5	Agu1A	0.0728	2.58
20	9	0	3.79	0	3.2	Agu1A	0.0668	2.21
15	10	1	6.44	1	4.5	Agu1A	0.2719	2.65
22	11	0	3.79	0	3.2	Agu1A	0.0693	2.01
8	12	0	3.79	1.41	5.038	Agu1B	2.0336	115
4	13	1	6.44	1	4.5	Agu1B	1.4571	81.4
7	14	0	3.79	-1.41	1.36	Agu1B	0.2228	1.16
3	15	-1	1.14	1	4.5	Agu1B	0.8459	2.23
11	16	0	3.79	0	3.2	Agu1B	0.7906	17.92
17	17	1.41	7.53	0	3.2	Agu1A	0.1414	2.19
16	18	-1.41	0.042	0	3.2	Agu1A	0.0	2.11
2	19	1	6.44	-1	1.9	Agu1B	0.3726	1.38
12	20	-1	1.14	-1	1.9	Agu1A	0.0	2.65
10	21	0	3.79	0	3.2	Agu1B	0.6942	16.65
18	22	0	3.79	-1.41	1.36	Agu1A	0.026	1.39

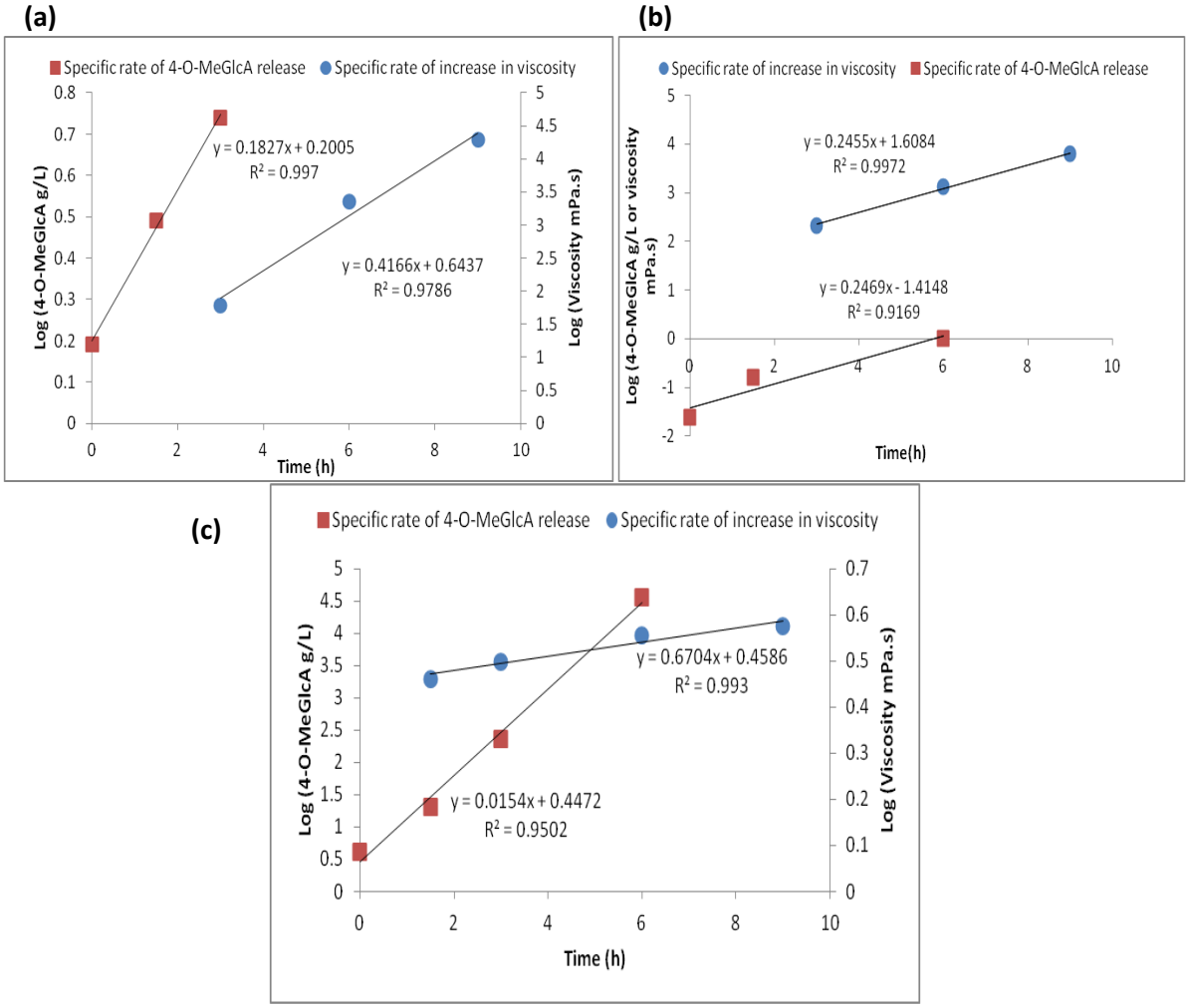

Figure C. 1: Maximum specific rates for 4-*O*-MeGlcA release and viscosity increase at α-D-glucuronidase dosage of 3.79 mg/g for: (a) Beechwood xylan (3.16% w/v) and Agu1B α-glucuronidase, (b) *E. grandis* xylan (Höije) (3.08% w/v) and Agu1B α-D-glucuronidase and (c) Beechwood xylan (3.16% w/v) and deglycosylated Agu1B α-glucuronidase

The lower rate of viscosity increase verified for *E. grandis* (Höije) using the Agu1B α-glucuronidase is in agreement with the lower viscosity obtained (133 mPa.s) after 24 hours of hydrolysis, as compared with beechwood xylan (208 mPa.s). Consequently, the high initial release of 4-*O*-MeGlcA verified in beechwood xylan (3.16%) using the deglycosylated Agu1B α-D-glucuronidase caused the rate of increase in viscosity to be higher, as compared to when using the native Agu1B α-glucuronidase.

Table C. 4: Analysis of variance for response surface-two factor interaction model for viscosity in beechwood xylan. Viscosity = f (xylan concentration; enzyme dosage; enzyme type)

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	6	4.371.E05	9.23	0.0002	significant
A-Dosage	1.665.E05	1	1.665.E05	3.51	0.0043
B-Xylan concentration	7.292.E05	1	7.292.E05	15.39	< 0.0001
C-Enzyme type	6.986.E05	1	6.986.E05	14.75	< 0.0001
AB	1	1.369.E05	2.89	0.1098	
AC	1	1.660.E05	3.51	0.0808	
BC	1	7.254.E05	15.31	0.0014	
Residual	7.105.E05	15	47368.37		
Lack of fit	7.105.E05	11	64588.62	5093.89	<0.0001-significant
Pure error	50.72	4	12.68		
Cor Total	3.333.E06	21			

Table C. 5: Analysis of variance for response surface-two factor interaction model for viscosity in Eucalyptus grandis xylan (Höije). Viscosity = f (xylan concentration; enzyme dosage; enzyme type)

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	1.12E+07	6	1.86E+06	5.8	0.0027-significant
A-Dosage	7.22E+05	1	7.22E+05	2.25	0.1544
B-Xylan concentration	3.29E+06	1	3.29E+06	10.24	0.006
C-Enzyme type	2.02E+06	1	2.02E+06	6.28	0.0242
AB	1.14E+06	1	1.14E+06	3.56	0.0787
AC	7.22E+05	1	7.22E+05	2.25	0.1545
BC	3.27E+06	1	3.27E+06	10.2	0.006
Residual	4.82E+06	15	3.21E+05		
Lack of fit	4.81E+06	11	4.38E+05	3387.72	< 0.0001-significant
Pure error	516.77	4	129.19		
Cor Total	1.60E+07	21			

Table C. 6: Analysis of variance for response surface-two factor interaction model for viscosity in Eucalyptus grandis xylan (Pinto). Viscosity = f (xylan concentration; enzyme dosage; enzyme type)

Parameter	Sum of squares	DF	Mean square	F value	p-value (Prob>F)
Model	13691.76	6	2281.96	8.49	0.0004-significant
A-Dosage	1088.08	1	1088.08	4.05	0.0626
B-Xylan concentration	3762.01	1	3762.01	13.99	0.002
C-Enzyme type	3356.98	1	3356.98	12.49	0.003
AB	805.81	1	805.81	3	0.1039
AC	1120.54	1	1120.54	4.17	0.0592
BC	3558.34	1	3558.34	13.24	0.0024
Residual	4032.27	15	268.82		
Lack of fit	4029.84	11	366.35	604.07	<0.0001-significant
Pure error	2.43	4	0.61		
Cor Total	17724.03	21			

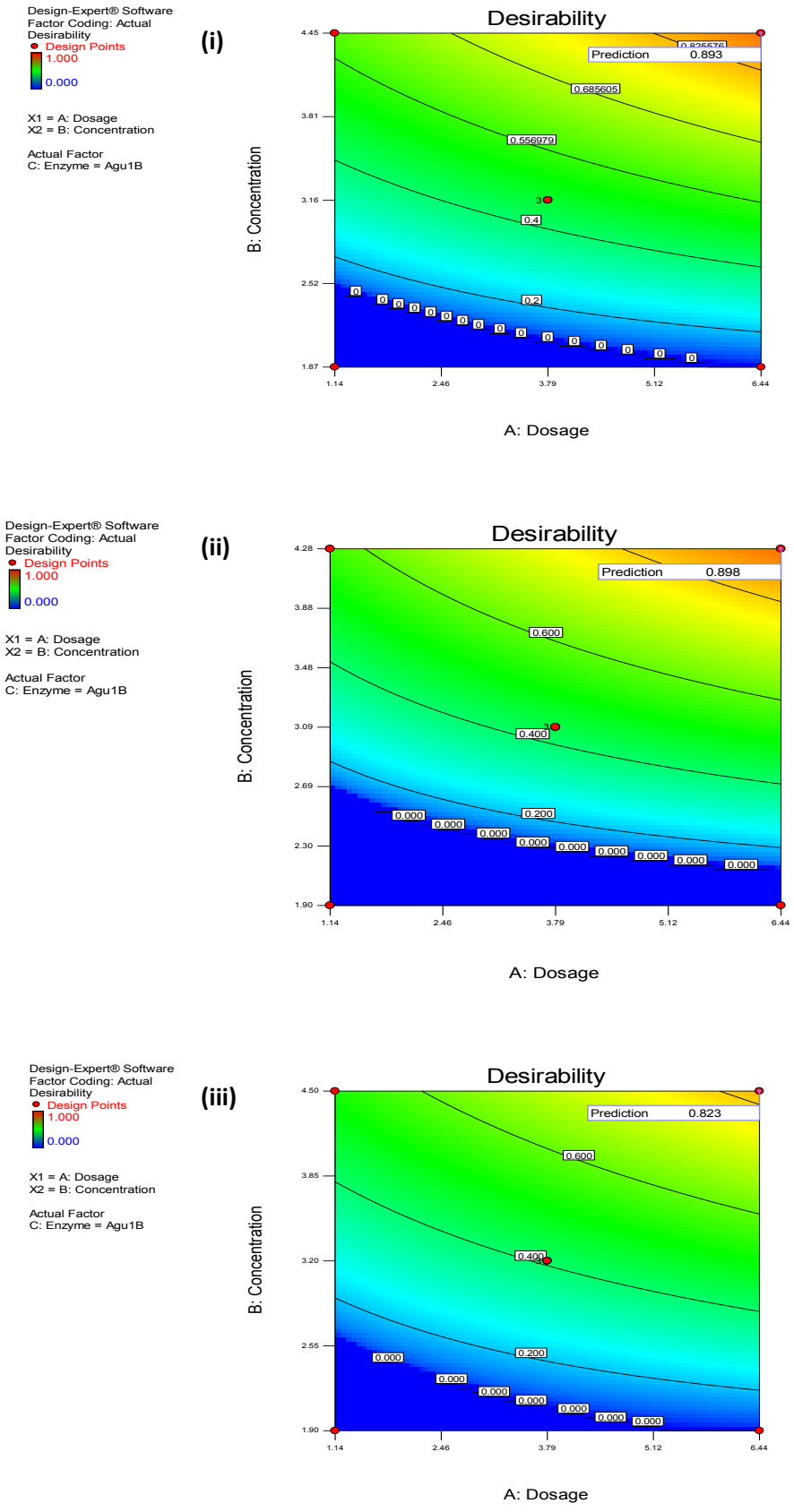

Figure C. 2: Desirability plots to determine the optimal combination of A: α-D-glucuronidase dosage, B: xylan concentration and C: Enzyme Agu1B α-glucuronidase. Desirability plots for (i): beechwood xylan; (ii) *E. grandis* xylan (Höije) and (iii) *E. grandis* xylan (Pinto). Targets: enzyme dosage in the range, substrate concentration at maximum.

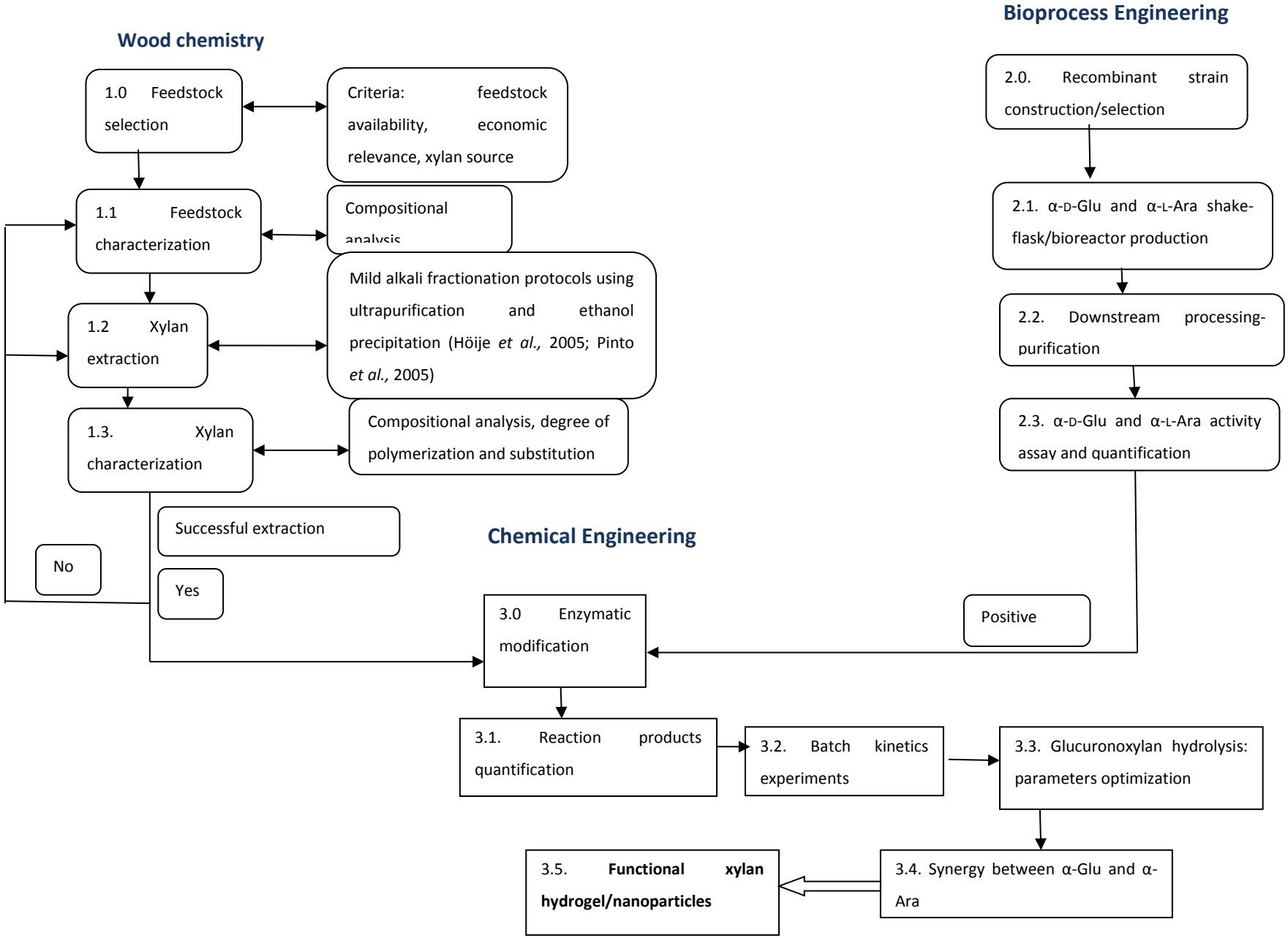

Figure C. 3: Mind map of the project