ARABINOXYLAN PREBIOTICS CO-PRODUCTION WITHIN INTEGRATED BIOREFINERIES

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Arabinoxylan prebiotics co-production within integrated biorefineries

Abstract

In cereal-based biorefineries, Distillers Dried Grains with Solubles (DDGS) is the main by-product, which is used in relatively low value animal feed formulations. A major component of DDGS is arabinoxylan (AX), which is a potential food ingredient and source of novel prebiotics. The production of AX uses large amounts of ethanol, which gives scope for feasible integration of AX extraction within a biorefinery. Meanwhile, enzymatic production of prebiotic arabinoxylan oligosaccharides (AXOS) and xylo-oligosaccharides (XOS) is of interest to animal feed formulators, as these prebiotics enhance feed conversion significantly.

The integration scenario would be implemented on the in-process streams, the Distillers Wet Grain (DWG) and the Solubles before they are combined. However, these are not readily available to study, as biorefineries are closed processes that do not allow in-process sampling of these streams. Therefore, this project used the GUNT CE-640 bioethanol unit to produce wet by-products representative of commercial DWG. Fermentation of ten batches of 6 kg of wheat yielded on average 1275 g (db) of DWG with 16% AX content and 800 g of dry Solubles with 11.4% AX content.

Enzymatic treatment of the DWG with commercial endoxylanase yielded less than 3%w/w of the prebiotic oligosaccharides. AX polysaccharides were extracted by alkaline oxidation, with and without further enzymatic purification, to give AX contents of 44% and 19%, respectively. Enzymatic treatment converted only 6% of the AX into AXOS/XOS, with significant production of undesired monosaccharides.

Limitations in the analytical methods used prompted the development of a new HPAEC-PAD method for the simultaneous measurement of mono- and oligosaccharides and uronic acids. The new method was used to quantify the profiles of XOS in commercial materials and following enzyme treatment of biomass materials and oligosaccharide standards up to DP6, the latter to reveal the xylanase mode of action. The commercial xylanase investigated showed a preference for larger XOS molecules and was incapable of acting on branched (AXOS) molecules.

Two types of AX with different branching patterns were produced; highly branched AX was extracted chemically from DWG with further purification to give 51% w/w purity, and less branched AX was produced from the Solubles fraction by ultrafiltration to give 74% purity. Enzyme treatment converted 46% of the Solubles AX into oligosaccharides, showing this to be a promising feedstock for XOS production.

Table of Contents

List of Figures

List of Tables

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List of abbreviations

AAFCO	Association of American Feed Control Officials
A/X	Arabinose to xylose ratio
A2A3XXX	23,33-di-α-L-arabinofuranosyl-xylotriose
A2XXX	23-α-L-arabinofuranosyl-xylotriose
AACC	American Association for Clinical Chemistry
ABF	Associated British Foods
anova	analysis of variance
AOAC	Association of Official Analytical Chemists
Ara	Arabinose
As	Asymmetry
AX	Arabinoxylan
AXa	First batch of AX extraction
Axb	Second batch of AX extraction
AXOS	Arabinoxylan Oligosaccharides
CAZy	Carbohydrate-Active enZYmes
CV	Coefficient of variation
Da	Dalton
db	Dry base
DDGS	Distillers Dried Grains with Solubles
DP	Degree of polymerisation
DS	Distillers Solubles
DWG	Distillers Wet Grain
EU	Europian union
FAO	Food and Agriculture Organization
FFC	Functional Food Centre
fuc	Fucose
FuFoSE	The European Commission's Concerted Action on Functional Food Science in Europe
Gal	Galactose
GalA	Galactorunic Acid
GC	Gas chromatography
Glu	Glucose
GluA	Glucorunic Acid
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High performance liquid chromatography
HPTLC	High performance thin-layer chromatography
IPOS	Innovative Physical Organic Solutions
IR	Infrared
IUBMB	International Union of Biochemistry and Molecular Biology
k'	Capacity factor
LOD	Limits of Detection

LOQ	Limits of Quantitation
Mann	Mannose
MS	Mass spectroscopy
Mw	Molecular weight
Mw	Molecular weight
NSP	Non starch polysaccharides
NSPase	Non starch polysaccharides hydrolysing enzymes
PAD	Pulsed amperometric detector
PV	Solar photovoltaics
REN21	Renewable Energy Policy Network for the 21st Century
RFA	Renewable Fuel Association
Rha	Rhamnose
RI	Refractive index
Rs	Resolution factor
RTFO	Renewable Transport Fuel Obligation
RTFO	Renewable Transport Fuel Obligation
S/N	Signal-to-noise
SCF	Short chain fatty acids
SCFA	Short chain fatty acids
SEC-MALLS	Size exclusion chromatography with multi-angle laser light scattering
TLC	Thin-layer chromatography
Uvs	Ultraviolet-Visible spectroscopy
WEAX	Water-extractable arabinoxylan
WUAX	Water-unextractable arabinoxylan
X1	Xylose
X2	Xylobiose
X3	Xylotriose
X4	Xylotetraose
X5	Xylopentaose
X6	Xylohexaose
XA2XXX	23-α-L-arabinofuranosyl-xylotetraose
XA3XXX	33-α-L-arabinofuranosyl-xylotetraose
XOS	Xylooligosaccharides
Xyl	Xylose

1 Arabinoxylan, a potential co-product of integrated biorefineries

1.1 Biorefineries in the recent UK and global context

The world is facing unprecedented challenges of providing sufficient food and fuel to cover the continuously expanding needs of the increasing population, expected to rise to 9.8 billion by 2050 (UN DESA, 2017). Today's energy production and consumption patterns indicate that the finite coal, oil and gas resources might serve humankind needs for only a few more decades (Agarwal *et al.,* 2017). Around 80% of the energy and 90% of the world's chemicals are produced from fossil resource (Maity, 2015). Such dependence on finite resources is an alarming indicator of catastrophic consequences. Escalating energy consumption is also accompanied by greater emissions of greenhouse gases, mainly CO_2, being emitted into the lower atmosphere and changing the climate at a rapid pace (Von Blottnitz and Curran, 2007; Hamit-Haggar, 2012; Saini *et al.,* 2018). The twin problems of fossil fuel depletion and climate change, in the face of an increasing, and increasingly resource demanding, global population, are the defining features of the early 21st century and exercise the resources and resolve of scientists, engineers, businesses, politicians and society globally.

In the context of increased urgency for a transition to sustainable energy resources with lower carbon footprints, and new paths that reduce the enslavement to the limited fossil fuels, renewable energy sources are continually being considered, including wind, tidal waves, solar energy and biofuels (IRENA, 2017). Among these, biofuels are the only supplier of energy in a liquid transport fuel form, in contrast to the other renewable energy sources (Bom *et al.*, 2007; Huber *et al.*, 2006; Nigam and Singh, 2011). Furthermore, unlike wind, wave and solar energy, biofuels are not intermittent and do not require energy storage systems or the employment of other fuel technology as a backup (Grahn *et al.,* 2009).

Driven by this awareness, the European Commission issued the first EU Biofuels directive in 2003 which targeted increasing the biofuel mixing rate in diesel and petrol

(Berti and Levidow, 2014). In 2005, the UK government announced the Renewable Transport Fuel Obligation (RTFO) which encouraged biofuel production from 2008 onwards. The RTFO motivated the biofuel industry to grow in the UK. For example, Vivergo Fuels in Kingston upon Hull, East Yorkshire, the UK's biggest producer of bioethanol, was started in 2007 as a joint venture between AB Sugar, BP and Du Pont to create a biorefinery for the future. In 2011 the production of bioethanol commenced, and by 2014 full production capacity of the plant was reached. However, in a setback for the emerging biorefinery industry in the UK, in September 2018 Vivergo ceased operating, citing "the Government's lack of pace over the past decade to introduce E10 [petrol blended with 10% bioethanol]." (https://vivergofuels.com/news/vivergo-fuels-bioethanol-plant-proposes-ceasing-production/). This unfortunate conclusion to the Vivergo initiative illustrates the economic fragility of biorefining in the face of unsupportive market and policy contexts, and the difficulty of bringing to fruition their potential contributions to the issues of energy security and climate change.

Prior to this latest development, in 2016 the Renewable Energy Policy Network for the 21st Century (REN21) released the Renewables Global Status report (Anon, 2016 a), which reported that in that year the world's renewable energy production had never been higher, suggesting efforts towards the transition to a more eco-friendly energy scenario were on course. However, the British Petroleum Statistical Review, also released in 2016, reported that the world's fossil fuel consumption had also never been greater (BPstats, 2016). The Renewables Global Status report showed that renewable energy production reached record levels in the year 2015, particularly from solar photovoltaics (PV), which had the most significant increase in renewable energy sector, with only a small growth rate of 3.9% in bioethanol production (Figure 1-1).

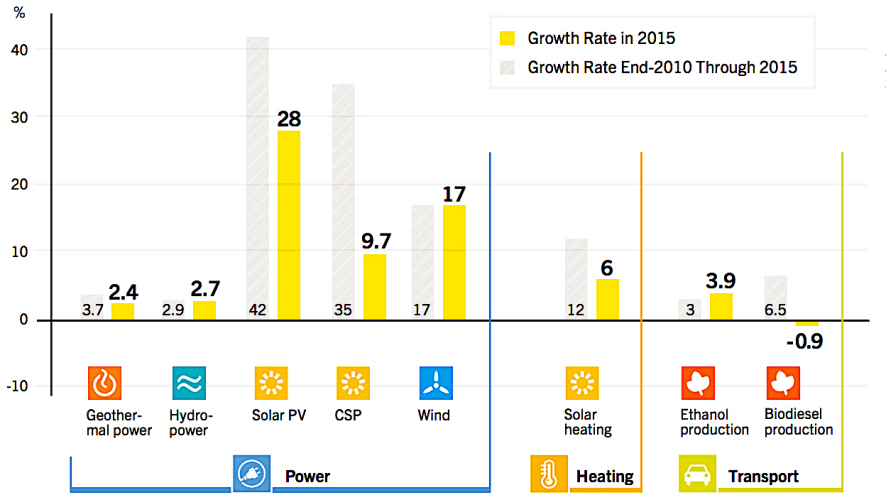

Figure 1-1. Average annual growth rates of renewable energy capacity and biofuels production, End-2010 to End-2015 (Anon, 2016 a).

The increase in renewable energy without decreasing fossil fuel consumption in part reflects the inability to directly replace fossil fuels. Despite the fact that electricity, provided by solar PV and wind, could be an attractive fuel for a small number of vehicles, unfortunately, it is not the case for the heavy duty and conventional transport applications which require a liquid type of fuel that biofuels, on the other hand, are able to deliver.

Henry Ford, as early as 1925, predicted the importance of biofuels (Agarwal *et al.,* 2017):

"The fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust—almost anything. There is fuel in every bit of vegetable matter that can be fermented."

The United Kingdom has a total biofuel production capacity, bioethanol and biodiesel, of over 1,500 million litres per year (Alberici and Toop, 2013). Figure 1-2 shows the increase in cumulative production capacity of commercial scale bioethanol plants in the UK (operational and planned), from which it is seen that the bioethanol production capacity almost doubled in the year 2013 then decreased slightly in the subsequent years.

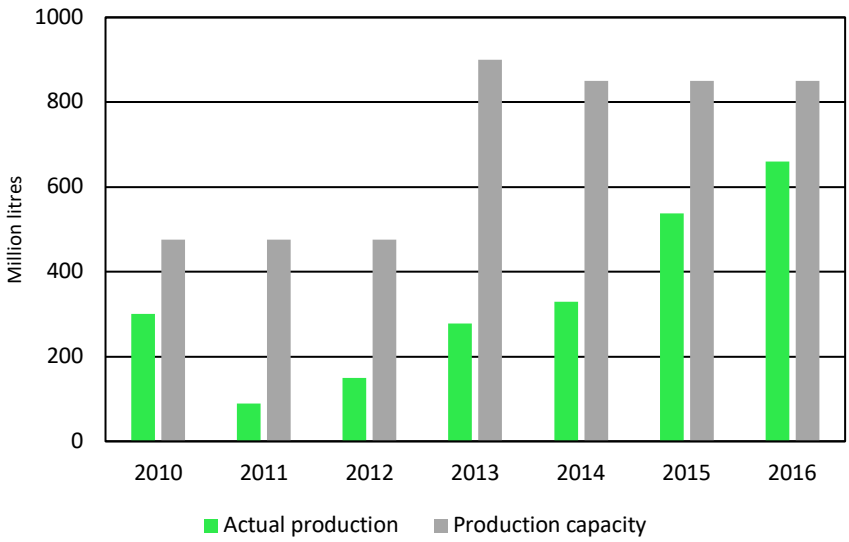

Figure 1-2. Actual bioethanol production in UK. Adapted from (Alberici and Toop, 2013; Phillips, 2017).

Despite the growth in production capability, UK bioethanol output itself has remained remarkably under capacity since 2010, particularly in 2011 when utilisation was only 17%, and the maximum utilisation percentage attained in this time period being just 65% (Alberici and Toop, 2013). However, the apparently increased production in 2016 allowed only a maximum blending of bioethanol in transport fuel of 3.3%, significantly below the aimed 10% target for 2020 (Phillips, 2017).

Moreover, Figure 1-2 shows that production capacity reached its maximum in 2013 at 900 million litres, then remained constant at 850 million litres in the years thereafter. The unchanging production capacity rate indicates the challenges facing the expansion of the bioethanol industry. Figure 1-3 presents the investments in clean energy globally. Clearly, investments in the solar and wind energy have dominated in recent years, whilst investments in biofuels have never been lower, reaching only $7bn in 2017 compared to $268bn investments in wind and solar energy.

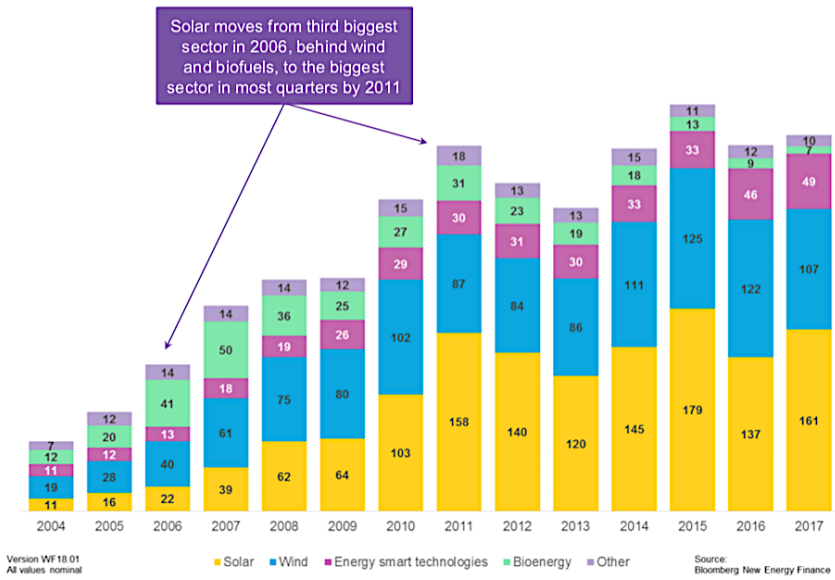

Figure 1-3. Global new investment in clean energy by sector (Louw, 2018).($bn/year)

In the UK, the investment scene in regard to clean energy sources is not dissimilar. Figure 1-4 shows the same global trend happening in the UK regarding growing investments in electrical power from wind and solar PV, with much less interest in biofuels.

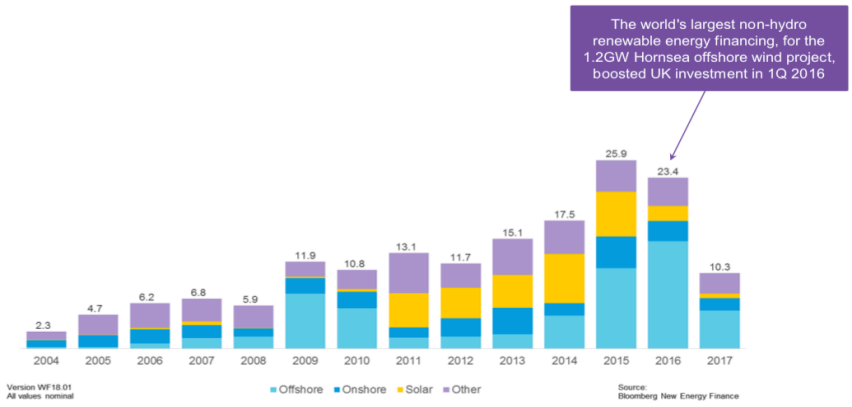

Figure 1-4. New investment in clean energy in United Kingdom, by sector (Louw, 2018).

($bn/year)

The decreasing investments in the biofuel industry are a result of the changing nature of the problems and obstacles hindering the growth of this industry. For decades, the biofuel business has been limited by multiple drivers, such as the fluctuations and rise in food commodity prices from which the "Food *vs* Fuel dilemma" ensued (Alberici and

Toop, 2013). In relation to demand, there are also several barriers regarding policies and markets; most importantly, the lack of clear, stable, long-term and profitable policy frameworks strongly stands in the way of the development of the biofuels industry (Anon, 2017). Moreover, the continued decrease in oil prices since 2014 has significantly raised the bar for biofuel production and led to the abandonment or interruption of some biofuel projects (Berti and Levidow, 2014); the focus shifted toward developing higher value applications rather than biofuels, as it has not been possible to compete with fossil fuels economically. The inability of biofuels to stand in the market as a financially viable business in the face of competition from fossil fuels and difficulties in overcoming the policy obstacles is the primary factor hindering the development of the biofuel industry, as illustrated by the closure of Vivergo.

Looking at a crude oil refinery, it is apparent that the number of commodities produced in such refineries contributes to their economic performance (Asche, Gjølberg, and Völker, 2003). Similarly in a biorefinery, a range of marketable products should be produced, alongside biofuel, to facilitate an economically-viable business that can compete against oil refineries. Also, oil refineries benefit from advanced and sophisticated process integration technologies which significantly enhance the overall efficiency and economics of oil refinery businesses (Lynd *et al.,* 2005; Lynd *et al.,* 2009; Martinez-Hernandez *et al.,* 2018). Production of a portfolio of products, such that "not a drop of oil is wasted", not only confers as much added value as possible to the whole feedstock, but also gives scope for highly integrated hence efficient and economic processes.

Although a portfolio of co-produced products is vital for biorefinery competitiveness, for generating a range of revenue streams and for providing integration opportunities as exploited within oil refineries. Not everyone working in biorefineries is as alert to the integration aspect as they are to that of revenue streams (Campbell *et al.,* 2018). Thus, for example, the definition developed by the International Energy Agency Bioenergy Task 42 Biorefinery (Anon, 2009) reads:

"Biorefinery is the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, chemicals) and energy (fuels, power, heat)."

This definition emphasises the "spectrum of marketable products" but makes no reference to integrated processes that allow economic co-production of these products. In this respect, a better definition is that given by the USA's National Renewable Energy Laboratory (NREL):

"*A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power and chemicals from biomass. The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum.*"

As Campbell *et al.* (2018) note,

"*If a biological processing facility produces multiple products, this implies a complexity of operation that gives scope and opportunity for effective integration – but it does not imply that such integration is automatically and necessarily implemented. Unless this complex facility – with its multiple products – is deliberately integrated, it remains just a biological processing facility. It is not a biorefinery.*"

The NREL definition embodies the conceptual model for tomorrow's biofuel production where high-value commodities are produced, one of them being biofuel. This could be accomplished via biomass conversion and fractionation; Integrated production of bio-based chemicals, heat, power and food within a biorefinery would present the ultimate economical option.

1.2 Arabinoxylan as a co-product of bioethanol production

In today's cereals biorefineries, the major co-product of bioethanol production is the Distillers Dried Grain with Solubles (DDGS), a relatively low-value product that goes to animal feed. DDGS come from the non-starch fraction of the cereals which mainly is the bran along with leftover protein and yeast biomass (Chatzifragkou *et al.,* 2015). Cereal

bran is the problematic fraction of cereals for biorefineries, as it comprises a large fraction of the raw material and is underexploited economically. Thus, extracting higher-value components of the bran has been a focus of attention towards producing added-value products.

Recently, studies have focused on the production of arabinoxylan (AX), a component of lignocellulosic biomasses, and a potential co-product of biorefineries that could provide a novel class of food ingredients and non-food products. DDGS composition makes it a potential source for extracting AX and arabinoxylan oligosaccharides (AXOS). Also, one process for producing arabinoxylan involves precipitating with ethanol, giving opportunities for integration with bioethanol production within biorefineries (Du *et al*., 2009; Hollmann and Lindhauer, 2005; Misailidis *et al*., 2009). Thus AX appears to be a particularly promising co-product for integration into a biorefinery to give both additional high-value revenue streams as well as scope for integration and the associated economic savings that define a true biorefinery.

Previous work has established that co-production of AX integrated with bioethanol could be economically feasible (Misailidis *et al*., 2009) and has established extraction protocols to produce AX from maize, wheat, and sugarcane bagasse (Bell 2015; Campbell *et al.,* 2019; Peng *et al.,* 2010; Rose *et al.,* 2010). Further work has introduced bioethanol pinch analysis as a formal process integration tool to minimise the use of bioethanol in the AX extraction process and hence to reduce the cost (Martinez-Hernandez *et al.,* 2013; 2018). However, the properties of extracts as functional food ingredients, as affected by source and extraction conditions, have not been extensively studied. Greater understanding of arabinoxylan functional properties is needed, as these properties vary according to feedstock and extraction conditions. Early studies on arabinoxylan extracts sometimes gave conflicting results due to differences in composition and purity of extracts (Biliaderis *et al.,* 1995; Courtin and Delcour, 2002). For the purpose of undertaking extensive functional property investigations in food systems, AX extracts need to be available in large (kg) quantities. There is currently no commercial source of AX at this scale. Hence, to progress this line of research, there is a need to establish facilities for routine production of pure AX in kg quantities.

In addition, arabinoxylan oligosaccharides (AXOS) and xylo-oligosaccharides (XOS), the small degradation products of larger AX molecules, have become a further focus of interest, as they have prebiotic properties that could benefit both human and animal health. The production of a range of AX and AXOS/XOS products, precipitated using ethanol at different concentrations, also increases the scope for bioethanol integration to reduce the costs (Martinez-Hernandez *et al.,* 2018). Thus a scenario is envisaged in which a range of AX and AXOS/XOS products are produced, each adding revenue streams to enhance the economics of the biorefinery, while reducing processing costs through the efficiencies of integrated co-production.

A major hindrance to research on DDGS has been the inability to source the in-process material that eventually becomes the dry animal feed product, DDGS. Several studies have examined DDGS as a feedstock for further processing (Chatzifragkou *et al.*, 2015; Kosik *et al*., 2017). However, this is not the material that would actually be used within a commercial process; materials for extraction would be accessed earlier in the process, prior to drying. Drying might alters the properties of the wet materials, these materials are called the Distillers Wet Grain (DWG) and the Solubles. However, existing bioethanol plants are closed systems that do not allow the in-process material to be sampled; this is why studies so far have utilised the more readily available DDGS.

A further hindrance has been the difficulty of quantifying the small oligosaccharides, due to a lack of standards and the inherent challenges of separating and detecting sugars.

Therefore, the current work undertook a wide-ranging investigation of pilot-scale production of AX materials and enzymatic studies of AXOS and XOS production from these materials. The work required development of a new method for separating and quantifying mixtures of mono- and oligo-saccharides, and applied the method to investigate the kinetics of enzymatic production of XOS using xylanases. The work thus addressed practical issues of AX and AXOS/XOS production as well as scientific issues of characterisation of the produced materials and understanding of xylanase kinetics.

1.3 Scope of the thesis

In conventional cereal-based biorefineries, the main by-product, DDGS, is a low-value co-product that goes to animal feed. The production of a portfolio of co-products is vital for the economic competitiveness of biorefineries. Recent studies have focused on the potential for production of arabinoxylan (AX), arabinoxylan oligosaccharides (AXOS) and xylo-oligosaccharides (XOS) within integrated biorefineries. The current work addresses issues of producing these materials at kg-scale and of characterising the products and the enzymatic degradation processes by which they are produced.

Arabinoxylan is the major hemicellulosic part of the cereal bran and a major component of DDGS. One of the processes for producing arabinoxylan uses ethanol for precipitation; co-production of AX with bioethanol production therefore gives scope for integrated processing and economic production of a portfolio of AX and AXOS/XOS products.

The evolving arabinoxylan story in the context of UK cereal biorefineries is presented in Chapter 2 along with the description of arabinoxylan structure, classification, physicochemical properties and the relation of these properties to the structure of the polysaccharide.

Chapter 3 describes industrial bioethanol production processes and illustrates the characteristics of the intermediate by-products of the fermentation and the effect of the drying on these intermediate products to give the final DDGS. The in-house bioethanol production procedure used in this project is also described, along with the analysis and characterisation of the products.

Chapter 4 introduces the concept of functional food ingredients and the biological importance of arabinoxylan prebiotics for human and animal nutrition. It also describes the role of xylan-hydrolysing enzymes in the in-situ production of XOS in animal feed, and presents trials performed in this project to produce XOS products from the bioethanol by-products.

Chapter 5 details the methods used for AX extraction from cereal biomass on small and large scales, and reports the large-scale extraction of AX soluble fibres from bioethanol by-products.

Having shown that characterisation of the products is a challenge and a limitation to research in this area, Chapter 6 reviews analytical methods used for the identification and quantification of arabinoxylan oligosaccharides, then details the development and validation of a powerful new analytical method for the simultaneous characterisation of mono- and oligosaccharides and uronic acids.

Chapter 7 describes arabinoxylan hydrolysing enzymes (xylanases), their classification, industrial applications and potential role in the production of AX prebiotics. Econase is a commercial endoxylanase widely used in animal feed formulations, which is believed to exert some of its benefits through production of prebiotic oligosaccharides, and for which there is a need to understand the kinetics of oligosaccharide production. The chapter presents experiments to evaluate the effects of this xylanase on XOS production and degradation.

Chapter 8 discusses the importance of the integration of AX production within biorefineries and reports the integration carried out in this project to produce two types of AX soluble fibres and the potential conversion of the AX into XOS using xylanases.

Chapter 9 concludes the thesis by summarising the main findings from the current work and presenting recommendations for progressing research in this area, in order to lead to a sufficient basis of knowledge to allow commercial production and exploitation of AX-based products within integrated biorefineries.

2 Arabinoxylan structure and physicochemical properties

2.1 Introduction

Hemicellulose is the second most abundant polysaccharide in nature after cellulose and, together with cellulose and lignin, forms the structural component of plant cell walls (Bacic *et al.,* 1988; Izydorczyk and Biliaderis, 1995). Hemicelluloses are polysaccharides of mainly 5-carbon sugars, the composition of which varies according to the plant species. In cereals, arabinoxylans (AX) are the major hemicelluloses, comprising, for example, 20-25% of wheat bran (Bacic and Stone, 1981). AX molecules consist of a linear chain of xylose with arabinose side chains and some phenolic acids attached to the arabinose branches. AX are novel polymers that possess unique functional properties that could be useful as a food ingredients or for non-food uses, offering the possibility of producing a portfolio of AX-based co-products within cereals biorefineries. This chapter reviews AX structure, classification and physicochemical properties, in order to provide the context for the objectives of the current work.

2.2 Brief background of arabinoxylan

At the beginning of the twentieth century, Hoffmann and Gortner (1927) reported a gummy non-starch polysaccharide from wheat flour which was comprised mainly of xylose and arabinose, both 5-carbon sugars; hence the new gum was named ‘pentosan’. Later, the novel pentosan gum was described as consisting of a simple type of molecule made of the association of arabinose and xylose in cereals (Preece and Hobkirk, 1953). Subsequent research reported the presence of AX in the outer layers of cereal grains such as wheat, corn, rice, barley, oats, rye and sorghum (Vinkx and Delcour, 1996). AX have received increasing attention in the field of cereal science and bio-materials in the last few decades. A bibliographic search of ScienceDirect conducted in August 2018 for “Arabinoxylan” in the title, abstract or keywords shows that publications on this topic have increased nearly ten-fold in the last twenty years (Figure 2-1).

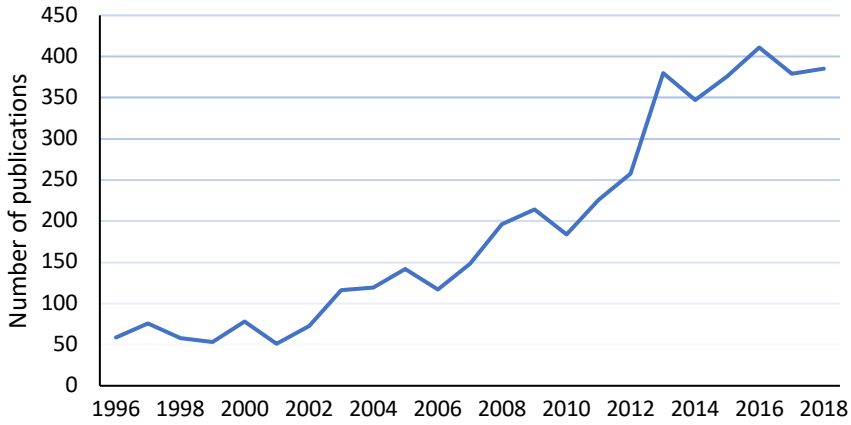

Figure 2-1. Number of publications about arabinoxylan. Source: ScienceDirect (August 2018).

The available literature describes mostly the characteristic properties of AX, the extractability on a bench-scale and the potential applications. Only a limited number of papers report larger-scale extraction, as extraction at commercial scale, or even pilot scale, is hampered by two factors: (i) the extraction requires large quantities of ethanol, which is costly and has handling implications in relation to safety and security. (ii) The absence of a clear market to commercialise AX-based products. These facts have been the reason for AX to remain only a theoretical material that might have a promising future. Thus, for example, Courtin and Delcour (2002) observed:

"In spite of early reports of their beneficial impact in breadmaking, AX have remained only a theoretical candidate for addition to the bread-recipe. This is related to the fact that studies of their functionality have sometimes yielded contradictory results and to their unavailability on a commercial scale."

In order to stimulate markets for AX, a substantial number of studies have focused on the benefits of AX in the fields of human health and prebiotic properties, breadmaking and the food industry, excipients in the pharmaceutical industry and food packaging. Table 2-1 summarises the small scale experiments for potential uses of arabinoxylan. However, the lack of commercial-scale production of AX is the main hindrance, as sufficient amounts are not available to demonstrate the technological uses of AX and promote their uptake in industry.

Table 2-1 Potential uses of AX.

Field of study	Effect of AX	Reference
Breadmaking	-Increased the loaf volume -Higher moisture content	(Biliaderis *et al.,* 1995; Li *et al.,* 2013)
Health	-Metabolic improvement during diabetes -Lower postprandial responses in serum glucose, insulin and triglycerides -Lower blood cholesterol levels -Obesity preventing -Prebiotic	(Akpinar *et al.,* 2010; Garcia *et al.,* 2007; Geraylou *et al.,* 2013; Hughes e*t al.,* 2007; Lopez *et al.,* 1999; Lu *et al.,* 2004; Staneva *et al.,* 2014; Tong *et al.,* 2014; Van Craeyveld *et al.*, 2008; D. Zhang *et al.,* 2015)
Food packaging	-Barrier for oil and fat	(Mikkonen *et al.,* 2009; Stepan *et al.,* 2014; Stevanic *et al.*, 2011)
Pharmaceuticals	-Stable emulsions -Foam stabilising -Sustained release matrix -Suspensions stabilising -Disintegrating agent	(Bashir *et al.,* 2014; Erum *et al.,* 2015; Iqbal *et al.,* 2011; Izydorczyk *et al.,* 1991; Raja *et al.,* 2014; Yadav *et al.,* 2007)

2.3 Structure of arabinoxylans

Arabinoxylan is the main hemicellulose polysaccharide in cereals; it exists mainly in the outer layer and endosperm cell walls and is associated with cellulose and lignin (Izydorczyk and Biliaderis, 1995). AX comprise a linear backbone of β-D-xylopyranosyl linked via (1-4) glycosidic linkages. Izydorczyk (2008) reported that monomers of *α*-L-arabinofuranoside are linked to some of the xylopyranosyl chain at O-2, O-3 and/or both O-2, 3 positions. According to this conformation four main structural units of AX can be observed as illustrated in Figure 2-2: mono-substituted xylopyranosyl at O-2 or O-3, di-substituted xylopyranosyl at both O-2,3 and unsubstituted xylopyranosyl (Izydorczyk and Dexter, 2008; Mendis *et al.,* 2016).

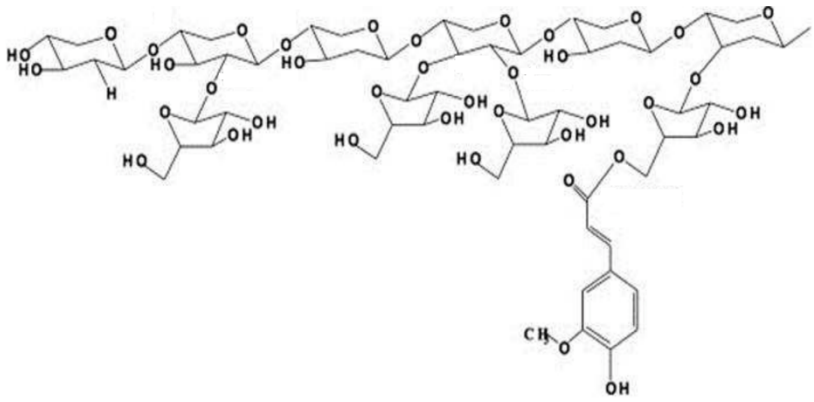

Figure 2-2. Structure of AX, showing mono- and di-substituted xylose units and a ferulic acid link (Mendis et al., 2016).

The sequence and distribution of these four conformational structural units depends on the source of arabinoxylan (Gruppen *et al*., 1993). Izydorczyk (1995) reported that the majority of arabinose side chains consist of one monomeric substitute, and just a minor proportion could contain two or more arabinose units linked via 1-2, 1-3, or 1-5 linkages (Izydorczyk and Biliaderis, 1995). The amount of arabinose in AX indicates the "embranchment" of the chains and is usually estimated by the Arabinose/Xylose (A/X) ratio, which varies between different plants and also between the different parts of a plant.

The terms "branching" and "embranchment" are widely used in the AX literature to refer to the arabinose substitutions (*e.g.* (Hoffmann *et al.,* 1992; Rattan *et al.,* 1994; Cyran and Saulnier, 2012; Coelho *et al.,* 2016)). These are not actually branches, suggesting a side-chain – the phrase "arabinose side chain" is also used, inaccurately suggesting a chain of arabinose units, so these terms are perhaps not the most helpfully precise words, but to be consistent with other literature, these terms are used in this thesis.

For example, the A/X ratio of the wheat endosperm AX is reported around 0.7 (Rattan *et al*., 1994), whereas the ratio is greater in wheat bran and reaches a value of 1.07 (Shiiba and Nagao, 1993), and a ratio of 0.21 in wheat straw (Xu *et al.,* 2006). AX was reported to have A/X ratio of 2.6-4.1 in rye grain (Hansen *et al.,* 2003) and a ratio of 1.7 in rye bran (Figueroa-Espinoza *et al.,* 2004).

Phenolic acids such as hydroxycinnamic, ferulic and *p*-coumaric acid might appear esterified to O-5 of arabinofuranoside units in AX (Smith and Hartley, 1983). Ferulic acid, the most abundant phenolic acid in cereals, can form dimers of dehydrodiferulate esters, as shown in Figure 2-3, forming a covalent cross-linkage between AX chains and between AX and other cell wall components (lignin, proteins, etc.) (Santiago *et al.,* 2006; Santiago and Malvar, 2010).

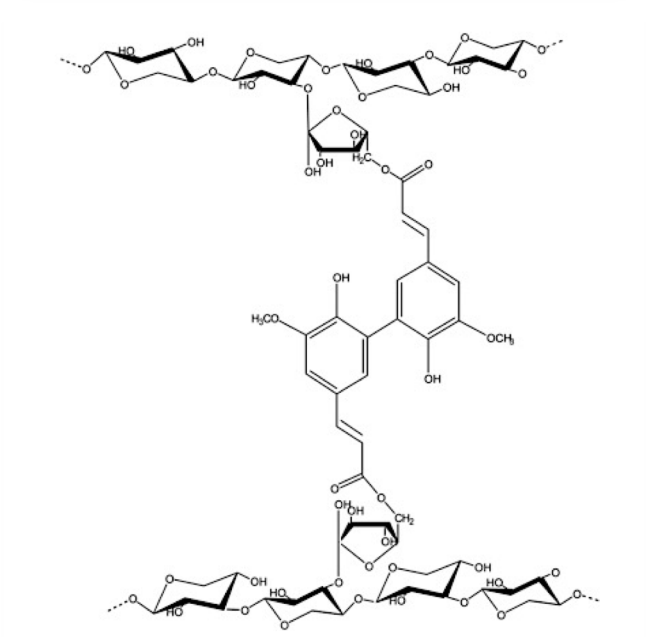

Figure 2-3. Dehydrodiferulate esters (Ferulic acid dimer) (Santiago and Malvar, 2010).

Due to the hydrogen bonds between adjacent xylose units in the unsubstituted xylan polymers, a conformation of a three-folded and left-handed helix is observed where each group of three xylose sugars forms a turn in the helix and the final conformation may look like an extended twisted ribbon (Courtin and Delcour, 2002; Fincher and Stone, 1986; Izydorczyk and Biliaderis, 1995). Arabinose substitutions can distort this conformation, as the side units can sterically hinder this formation and a semi-flexible random coil conformation is proposed (Courtin and Delcour, 2002; Dervilly-Pinel *et al.,* 2001). Moreover, the ferulic acid content in the AX chains may form linking bridges between adjacent AX chains, resulting in higher molecular weight molecules, and may change the conformation and hence the properties.

2.4 Classification of arabinoxylans

Variations in the structural configuration of AX have been the main driver for dividing the polysaccharide into two main categories: water-extractable arabinoxylan (WEAX) and water-unextractable arabinoxylan (WUAX) (Vinkx and Delcour, 1996). WEAX are loosely attached to the cell wall and can easily migrate into aqueous solutions; they are the shorter chains with less cross-linking and less linkage to the other cell wall components (Izydorczyk and Biliaderis, 1995). WUAX are the harder to extract AX, they have much larger molecular weights and greater branching (*i.e.* arabinose substitution), and are more strongly attached to the cell wall matrix by covalent (*i.e.* ester and ether bonds, diferulic acid bridges) and non-covalent (*i.e.* hydrogen bonds) linkages to adjacent AX and other cell wall constituents (Fengler and Marquardt, 1988; Izydorczyk and Biliaderis, 1995).

2.5 Occurrence of arabinoxylans

AX are one of the major hemicelluloses in the plant kingdom, occurring in a wide variety of plants. They play an essential role in strengthening the structural support system by cross-linking the cellulose microfibrils and thereby regulating the cell expansion and strengthening the wall (Gibeaut *et al.,* 1991; Scheller and Ulvskov, 2010). Hemicelluloses are synthesised in the Golgi apparatus (Carpita and Gibeaut, 1993). Briefly, the synthesis of AX goes through four main steps: xylan chain initiation, xylan chain elongation, arabinose side chain addition and extracellular deposition (Jung *et al.,* 1993). Glycosyltransferases are the enzymes responsible for the biosynthesis of hetero-polysaccharides such as AX; β-1,4-xylosyltransferase was found to be the enzyme catalysing the biosynthesis of the xylan backbone whereas AX-arabinosyltransferase adds arabinose side chains (Gibeaut and Carpita, 1991; Porchia *et al.,* 2002).

2.6 Physicochemical properties of Arabinoxylans

The reported physicochemical attributes of AX are primarily credited to its structural characteristics, *i.e.,* the length of xylan backbone, the degree of embranchment (the A/X

ratio) and the oxidative cross-linking by ferulic acid (Courtin and Delcour, 2002; Izydorczyk and Biliaderis, 1995). The main physicochemical properties of interest are water solubility, viscosity, water holding capacity and oxidative gelation.

2.6.1 Water solubility

In general, the solubility of AX in water is subject to the structure of the molecule, *i.e.* chain length, the degree of substitution (DS, equivalent to the A/X ratio), and the substitution pattern (Courtin and Delcour, 2002; Saulnier *et al*., 2007). The degree of substitution seems to be the key factor affecting AX solubility; higher DS gives greater solubility in water, as arabinose "side chains" (more accurately, arabinose substitutions) prevent the intermolecular aggregation of unsubstituted xylose chains, while removing the side chains enzymatically usually results in lower solubility (Andrewartha *et al*., 1979; Courtin and Delcour, 2002). Molecular weight (Mw) also plays an essential role in the solubility, with lower Mw AX exhibiting greater solubility (Mares and Stone, 1973; Courtin and Delcour, 2002; Saulnier *et al.,* 2007). Bian *et al*. (2010) applied a gradual precipitation on AX extracts using a range of ethanol concentrations of 10, 20, 30, 45, 60 and 80%. The high Mw AX with fewer branches precipitated first, whereas the lower Mw with more branching needed higher concentrations of ethanol to precipitate.

2.6.2 Viscosity

AX solutions are viscous and tend to exhibit pseudo-plastic behaviour due to the relatively high molecular weight and the stiff semi-flexible random coil conformation. Several factors affect the viscosity of AX solutions including Mw, A/X ratio, pH, AX concentration and ferulic acid content (Girhammar and Nair, 1992; Courtin and Delcour, 2002). Girhammar and Nair (1992) reported that higher Mw AX gave more viscous solutions, and that more viscous solutions were obtained in neutral pH; they noted it is possible that acidic and alkaline pH values break down the ester cross-linkages or even hydrolyse the polysaccharide chains in extreme pH conditions. Ferulic acid content significantly affects the solution viscosity as it may create cross-links between AX chains,

forming higher Mw molecules (Courtin and Delcour, 2002; Dervilly-Pinel *et al*., 2001; Saulnier *et al*., 2007).

2.6.3 Water Holding Capacity

Water holding capacity is one of the most attractive attributes of AX, particularly for bakers, for example. Courtin and Delcour (1998) added AX extracts to bread dough and quantified the dough behaviour by farinograph; they concluded that WUAX are capable of holding 7-10 times their weight in water, whereas WEAX presented lower water-binding capacities at 4-6 times their weight.

2.6.4 Oxidative Gelation and Gel-Forming Capacity

Izydorczyk *et al*. (1990) demonstrated that oxidising agents (*e.g.* hydrogen peroxide or peroxidase) could induce oxidative gelation in AX solutions, by catalysing the formation of covalent cross-linkages between AX molecules by the dimerisation of ferulic acid residues. Figure 2-4 describes the oxidative coupling of ferulic acid.

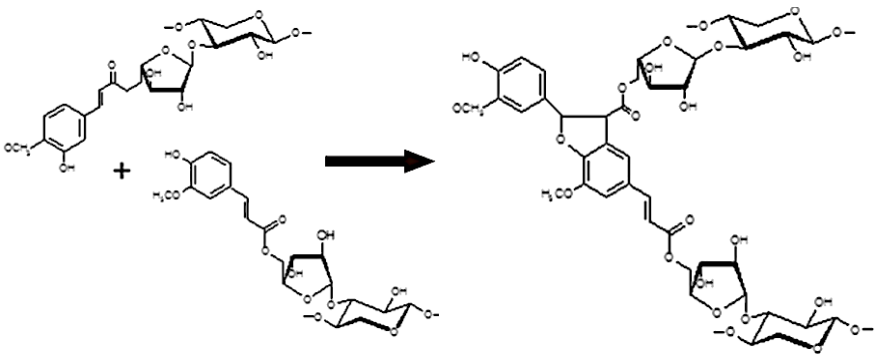

Figure 2-4. Oxidative coupling of ferulic acid (Döring et al., 2015).

These cross-linkages augment the viscosity and lead eventually to the formation of a gel (Dervilly-Pinel *et al*., 2001a; Vinkx *et al*., 1991). The high Mw, high ferulic acid and the less substituted xylan chain (low A/X) are the primary precursors of substantial cross-linking (Izydorczyk and Biliaderis, 1995). However, the rigidity of the gel is subject to the AX intrinsic viscosity, rather than the content of ferulic acid residues (Dervilly-Pinel

et al., 2001a). Other non-covalent linkages, such as hydrogen bonding and van der Waals, also contribute to the gel strength (Saulnier *et al*., 2007).

2.6.5 Foam stabilising

Arabinoxylans possess emulsifying properties and the ability to stabilise protein films against thermal disruption as they increase the viscosity of the interlamellar liquid, alter the draining properties of the foam films and mediate interactions between proteins in the absorbed layer (Izydorczyk *et al.,* 1991; Sarker *et al.,* 1998; Xiang and Runge, 2016).

2.7 Research objectives

In the context of exploring strategies for enhancing the economic income from biorefineries, the integrated biorefinery approach is an effective basis for exploiting interaction opportunities arising from the co-production of several products. Biorefineries are currently derived principally from ethanol production, and ethanol can serve as a precipitating agent for arabinoxylan, which makes arabinoxylan a natural co-product of bioethanol that offer the opportunity to bring a new class of functional food ingredients to the market.

The primary motivation of the current work was identified earlier as the pilot-scale production of arabinoxylan and arabinoxylan oligosaccharides within integrated biorefineries. In order to investigate the opportunity, several objectives were identified (some of these coming to prominence as the project progressed):

(i) to perform the bioethanol fermentation process at pilot-scale to produce and characterise the wet intermediate by-products, *i.e.* DWG and Solubles.

(ii) to investigate the direct enzymatic production of AX prebiotics from the wet by-product, DWG.

(iii) to extract AX chemically by means of the alkaline oxidative method on a pilot-scale from the DWG, and to characterise the extracts.

(iv) to develop a robust analytical method for the simultaneous determination of monosaccharides, xylo-oligosaccharides, arabinoxylan oligosaccharides and uronic acids.

(v) to study the enzymatic production and simultaneous degradation of XOS.

(vi) to produce and characterise water-extractable and water-unextractable AX within the biorefinery, and to evaluate the potential for production of XOS from these extracts.

2.8 Summary

Arabinoxylan classification and functional properties are determined by the average molecular properties such as the molecular weight, the degree of substitution and cross-linkage. Smaller AX molecules are more soluble in water and contain fewer ferulic acid cross-linkages and are usually classified as the water-extractable AX, whereas the larger molecules with high branching and more cross-linking are less soluble in water and are usually referred to as water-unextractable AX. Broadly speaking, the highly substituted WUAX shows greater water holding capacity and has the ability to form solutions of higher viscosity. Reducing the arabinose substitutions reduces both solubility and solution viscosity.

As noted above, while the scientific understanding of AX is extensive and detailed, its technological understanding has been hampered by lack of availability of larger than bench-scale quantities. Thus, one of the objectives of the current work was to produce significant quantities of AX using the School of Applied Sciences' pilot scale bioethanol plant. The production of the equivalent of industrial-scale Distillers Wet Grains (DWG) and Distillers Solubles (DS), from which to extract AX, is described in the next chapter.

3 Pilot-scale production of biorefinery by-products

3.1 Introduction

Bioethanol has become in recent decades the most important biofuel for transportation and the basis for the emergence of biorefineries. As noted in Chapter 1, the characteristics of biorefineries include a portfolio of co-products, and deployment of process integration to achieve efficiencies that will help biorefineries to become competitive with oil refineries. AX is a particularly promising candidate as a co-product with bioethanol, as ethanol is used to precipitate the AX, giving scope for integration and making production of AX economically feasible. The rise of biorefineries, giving the opportunity to create commercial sources of AX-based products, also requires the co-creation of markets for these products in the food, nutraceutical and pharmaceutical markets.

In order to successfully integrate AX production within biorefineries, the relevant streams of the bioethanol process, Distillers Wet Grain (DWG) and Distillers Solubles (DS), need to be characterised for their AX content and properties, and for the ease of recovering AX from these streams. However, the design of current bioethanol plants in the UK does not allow ready access to these in-process streams to take samples. Therefore, this part of the project aimed to operate the GUNT CE-640 bioethanol plant to produce material similar to the DWG and Solubles streams in industrial plants. This chapter reviews industrial bioethanol production and describes the operation of the GUNT pilot-scale process to produce and characterise DWG and DS for further investigations.

3.2 Bioethanol biorefineries – past, present and future

Alcoholic fermentation is one of the first bio-processes that early humankind exploited. Archaeologists report that fermented beverages existed in the Neolithic period (7000

BC) in China, Mesopotamia and ancient Egypt (McGovern *et al.,* 1996; Samuel, 1996; Steinkraus, 1997), with fermentation initiated by naturally occurring yeasts (Sicard and Legras, 2011). In China, the beverages were primary made from rice, whereas Mesopotamia and Egypt fermented fruits, honey and malt, while the utilisation of grapes was reported in Mesopotamia about 5400 BC (McGovern *et al.,* 1996). It is not documented exactly when and where fermented foods were initiated, but it is evident that fermentation has given humanity foods and drinks with pleasant flavours, aromas and textures and, in the case of drinks, with intoxicating properties.

Nowadays, fermentation continues to provide humankind not only with food and drinks but with fuel as well. The process of bioethanol production is not much different from the fermented beverage production process, except on a larger scale and without the aromatic and flavour concerns of beers and wines, for example.

In 2017, global bioethanol production reached about 100 billion litres, half of which was in the United States (Chatzifragkou *et al.,* 2015). About 95% of the world's bioethanol production is in the USA, China and Europe, utilising starch as the starting material (OECD/FAO, 2015). The production is chiefly from corn and soybeans in USA, sugar cane in Brazil, corn in China, and sugar beet, wheat and barley in Europe (Stein, 2007), with the production process varying depending on the feed material. For bioethanol production from cereals, there are two main processes: the wet milling process and the dry milling process.

3.2.1 Wet milling

Figure 3-1 illustrates the corn wet milling process. In this process, the corn is fractionated into relatively pure components (starch, gluten, oil) and germ meal and bran fractions that go to animal feed, before the starch is then hydrolysed to glucose and fermented to ethanol. Briefly, a first screening is applied to remove the foreign materials (crop residues, broken kernels, etc.), then the grains are soaked in large steeping tanks with a diluted solution of sulphur dioxide for 48 hours. The germ is separated from the soaked kernel and goes to oil production, and the leftovers are used

for feed and called the corn germ meal. The bran is separated by passing the slurry through sieves, leaving the starch and the gluten in the solution. The gluten is separated and dried to form the corn gluten meal (Blanchard, 1992; Elmekawy *et al.,* 2013; Stock, Lewis, Klopfenstein, and Milton, 2000). The process produces a range of relatively pure and high value products; however, this requires good quality feedstocks (first or second grade corn) and a sophisticated and expensive process requiring high capital investment (Belyea *et al.,* 2010).

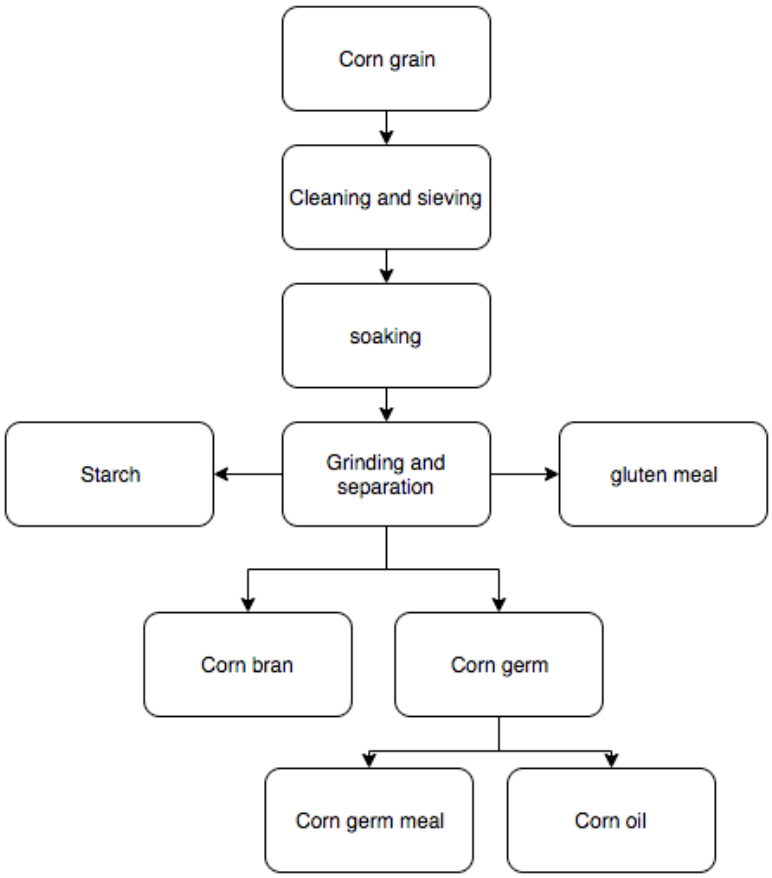

Figure 3-1. Corn grain wet milling process.

3.2.2 Dry milling

In this process, the grain or a mixture of grains is milled without applying any fractionation prior to the fermentation; the entire milled grain then undergoes the fermentation process, and the starch portion is converted into ethanol and CO_2, Figure 3-2 shows a simplified process. The fermented mash goes through various separation

processes to separate the ethanol from the bulk of the fermentation media; the non-fermented residues are dried to give the main by-product, Distillers Dried Grain with Solubles (DDGS), which goes to animal feed and contributes a significant proportion of the biorefinery's revenues. In some processes the CO_2 might be captured and sold as an additional revenue stream.

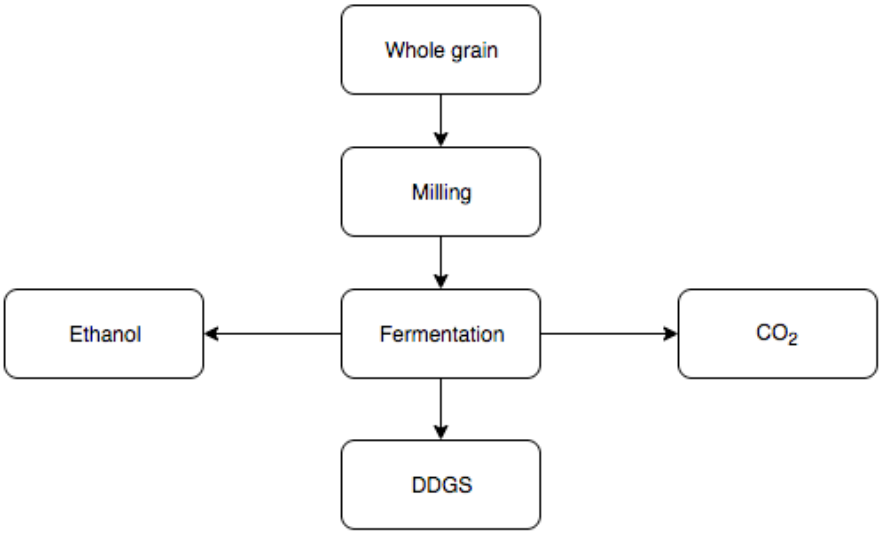

Figure 3-2. Dry milling process for bioethanol production.

The main advantage of this process, compared with the wet milling process, is the ability to process different types of grains with limited restriction on the quality of the grain (Stock *et al.,* 1999). About 82% of bioethanol production worldwide uses the dry milling method (Wood *et al.,* 2012). The prevalence of this process is resulting in increasing amounts of DDGS coming into the animal feed market, which gives issues over the nutritional value and performance of DDGS for different types of livestock (cattle, pigs, poultry and fish).

3.3 Distillers dried grains with solubles

DDGS has historically been a by-product of the alcoholic beverage industry and a rich source of energy, protein, minerals and vitamins for animal feed (Cromwell *et al.,* 1993; Klopfenstein *et al.,* 2008). Recently, the surge in dry milling bioethanol production for fuel has resulted in much greater quantities of this by-product entering the animal feed

market. The availability of DDGS is expected to increase even more in the future. Exploiting DDGS in the animal feed market contributes significantly to the profitability of the biorefinery (Klopfenstein *et al.,* 2008). However, DDGS is still a relatively low-value product, and better economical exploitation of this material would significantly assist the development of the bioethanol industry.

Extraction of the AX offers a higher value product, but also offers a further benefit; AX is dietary fibre, which is detrimental to conversion of animal feeds into weight gain (humans should eat more fibre as, in general, we need to avoid weight gain, but animal nutrition is the opposite, where rapid weight gain is desired by livestock producers). Therefore reducing the fibre content of DDGS by extracting the AX would enhance the value of the remaining reduced-fibre DDGS. Cattle can cope with high fibre in their diet, but pigs and poultry require less fibre in the diet – removing AX from DDGS would potentially allow greater use of a DDGS in animal feed formulations for pigs and poultry.

DDGS is defined by the Association of American Feed Control Officials (Świątkiewicz and Koreleski, 2008) as:

"Distillers Dried Grains with Solubles is the product obtained after the removal of ethyl alcohol by distillation from yeast fermentation of a grain or a grain mixture by condensing and drying at least ¾ of the solids of the resultant whole stillage by methods employed in the grain distilling industry."

The definition reflects the production process for DDGS, which is illustrated in Figure 3-3. Briefly, the milling, liquefaction and saccharification of the whole grain are achieved by thermal and enzymatic hydrolysis to facilitate the breakdown of starch into fermentable glucose. Then, the yeast ferments the available sugars into ethanol and carbon dioxide. Following fermentation, the ethanol is separated by distillation and dehydrated using molecular sieves. The non-fermentable and non-volatile components are separated from the slurry by filtration and centrifuging to yield a liquid fraction called thin stillage (with a solids fraction of around 5%) and a solid fraction called Distillers Wet Grains (DWG). The thin stillage is evaporated into Distillers Solubles

(typically with a solids content of about 12% (Morey *et al.,* 2009)), which is added to the DWG and dried at high temperatures to produce the final DDGS (Jensen *et al.,* 2012; Kingsly *et al.*, 2010).

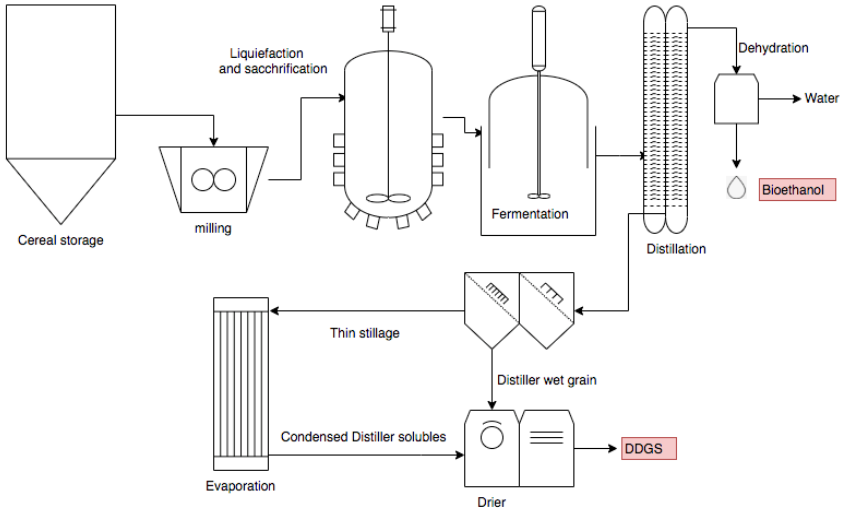

Figure 3-3. Dry-grind ethanol production processes and by-products *(simplified, adapted from Kingsly et al., 2010).*

The chemical composition of DDGS is similar to the original grain's non-starch components (it also contains yeast material from the fermentation). Variations in the raw material give variations of the composition of the final DDGS, and variations are reported among bioethanol plants and between different batches from the same production plant (Liu, 2011). The effect of the variation in the raw material (wheat, barley, maize or mixtures) has the clearest impact, but variations in grain growing, harvesting and handling also have notable impacts (Cromwell *et al.,* 1993; Kim *et al.,* 2007; Liu, 2008). Processing conditions such as the drying method and temperature also affect the final DDGS (Chatzifragkou *et al.,* 2015). Table 3-1 shows the different chemical compositions of wheat DDGS (Jarret *et al.,* 2011), maize DDGS (Spiehs *et al.,* 2002) and a blend of wheat, triticale, barley and rye DDGS (Pedersen *et al.,* 2014). Clearly, the difference in the nutrient composition of the parent grain is reflected in the DDGS; the oil in maize DDGS is greater than in wheat DDGS, whereas the latter contains a slightly greater amount of crude protein.

Table 3-1. Composition of DDGS from different plants and sources (expressed on a %, dry matter basis) (Jarret *et al.,* 2011; Pedersen *et al.,* 2014; Spiehs *et al.,* 2002).

	Maize DDGS	Wheat DDGS	blend DDGS
Dry matter	87.2–90.2	89.3–94.4	90.5–92.7
Oil	10.2–11.4	3.6–5.6	8.1–12.8
Protein	28.7–31.6	32.6–38.9	23.4–27.9
Crude fibre	8.3–9.7	6.2–10.9	9.6–10.6
Ash	5.2–6.7	4.3–6.7	3.4–7.3

Generally, after the removal of the starch by fermentation, the remaining components of the grain are concentrated by a factor of around three (Rasco *et al*., 1987). The rich protein and fibre content in the DDGS opens several exploitable possibilities other than animal feed, including the degradation of the polysaccharides for more ethanol production, production of value-added products, a source for food ingredients or use in bio-composites (Thacker and Widyaratne, 2007). However, the successful integration of any valorisation process of DDGS within biorefineries would need to be performed on the actual wet material, *i.e.* DWG and or DS, as the final dry material is not amenable to further processing (without redispersing it in water), and drying might change the characteristics of the material. The dry DDGS is easy to obtain, and a number of studies have investigated extraction from DDGS (Chatzifragkou *et al.,* 2015; Kosik *et al.*, 2017), but studies on the wet DWG would be more realistic both in terms of the characteristics of the material and the practicalities of extracting from it in the industrial context.

3.3.1 DDGS and DWG

Historically, there were two by-products of the bioethanol production process, the Distillers Dried Grain (DDG), and the Distillers Solubles (DS) (Thacker and Widyaratne, 2007). Current bioethanol plants tend to mix the spent grain and solubles during drying to produce the combined product, DDGS. In some cases, producers have arrangements with local livestock businesses that allow a moist product to be produced, as it is transported and consumed by animals before it can deteriorate, but in general a dry product is needed to give longer stability.

Figure 3-3 shows the flow of the ethanol production process in a dry-grind wheat biorefinery, where the mash is separated after distillation into the DWG (~60-70% moisture) and the thin stillage (~95% moisture) which contains all the solubles (Morey *et al*., 2009). The DWG and thin stillage undergo severe thermal treatment during the drying process, which alters the components of the DDGS. As noted section 3.3, extraction from DDGS, as well as being inappropriate in the industrial context, would yield products of different (and probably inferior) functionality compared with extraction from DWG. The severe thermal treatment has a major effect on the nutritional value of DDGS proteins (De Vries *et al*., 2013). Schroeder (2012) reported that the proportion of undegradable protein increased from 47% in the DWG to 54% in the DDG. The effect of the thermal treatment is likely to affect other components such as the cell wall polysaccharides. For instance, AX in the DDGS was reported to have a significantly lower A/X ratio as compared to the original grain AX as well as a higher proportion of water-extractable AX (Pedersen *et al.,* 2014). These structural changes and changes in AX solubility are possibly a result of alteration in the molecular structure and/or the molecular cross-linking with other components in the cell wall (Kosik *et al.*, 2017).

Hence, although some research in this area has investigated DDGS as a source of higher value materials, the current work started by producing a pilot-scale equivalent of DWG, to give a more representative material for subsequent studies of AX extraction and enzyme treatment.

3.4 Materials and equipment used for pilot-scale bioethanol and DWG production

Bioethanol and DWG were produced from milled wheat using the GUNT CE-640 bioethanol plant.

3.4.1 Chemicals and enzymes

Wheat was sourced from Target Feeds Ltd (Shropshire, UK), who provided the wheat double-milled through a 2 mm mesh. The enzymes used for starch hydrolysis, α-

amylase (SCHLIESSMANN-VF-) and gluco-amylase (SCHLIESSMANN-VZ-), the anti-foaming agent (SCHLIESSMANN-EX-PROTIN) and dry baker's yeast from the strain *Saccharomyces cerevisiae* (KORNBRAND PREMIUM), were purchased from ReKru GmbH, Germany. Sodium hydroxide pellets and sulphuric acid (>95%) laboratory reagent grade were obtained from Fisher Scientific Ltd.

3.4.2 The CE-640 bioethanol plant

The CE-640 unit supplied by GUNT Technology Ltd. (Hamburg, Germany) is designed to imitate the industrial processes of ethanol production and facilitates the in-process sampling of the processed material through all the stages of the process. (https://www.gunt.de/en/products/process-engineering/biological-process-engineering/anaerobic-processes/biotechnical-production-of-ethanol/083.64000/ce640/glct-1:pa-148:ca-251:pr-69). It comprises three main units: the mash tank, the fermentation tank and the distillation unit. Figure 3-4 shows an overall view of the unit components. The plant is controlled and monitored via the integrated programmable logic controller displayed on the right of Figure 3-4. The plant delivered to the University of Huddersfield features several modifications from the standard unit supplied by GUNT, to make it specifically suited to the AX extraction work: pumps capable of handling larger particulate solids, pH and full temperature control to both the mash and fermentation tanks, and corresponding control software modifications.

The mash tank is used for the liquefaction and saccharification of the starting materials, with a capacity of 40 litres. It is a double-jacketed stainless-steel vessel equipped with speed-regulated stirrer comprising a geared motor and a pitched blade on the shaft. The material in the mash tank is heated by direct steam injection through a jet, or via the double-jacket heating. The pH is monitored by an integrated pH probe and controlled by acid/base supply with diaphragm metering pumps.

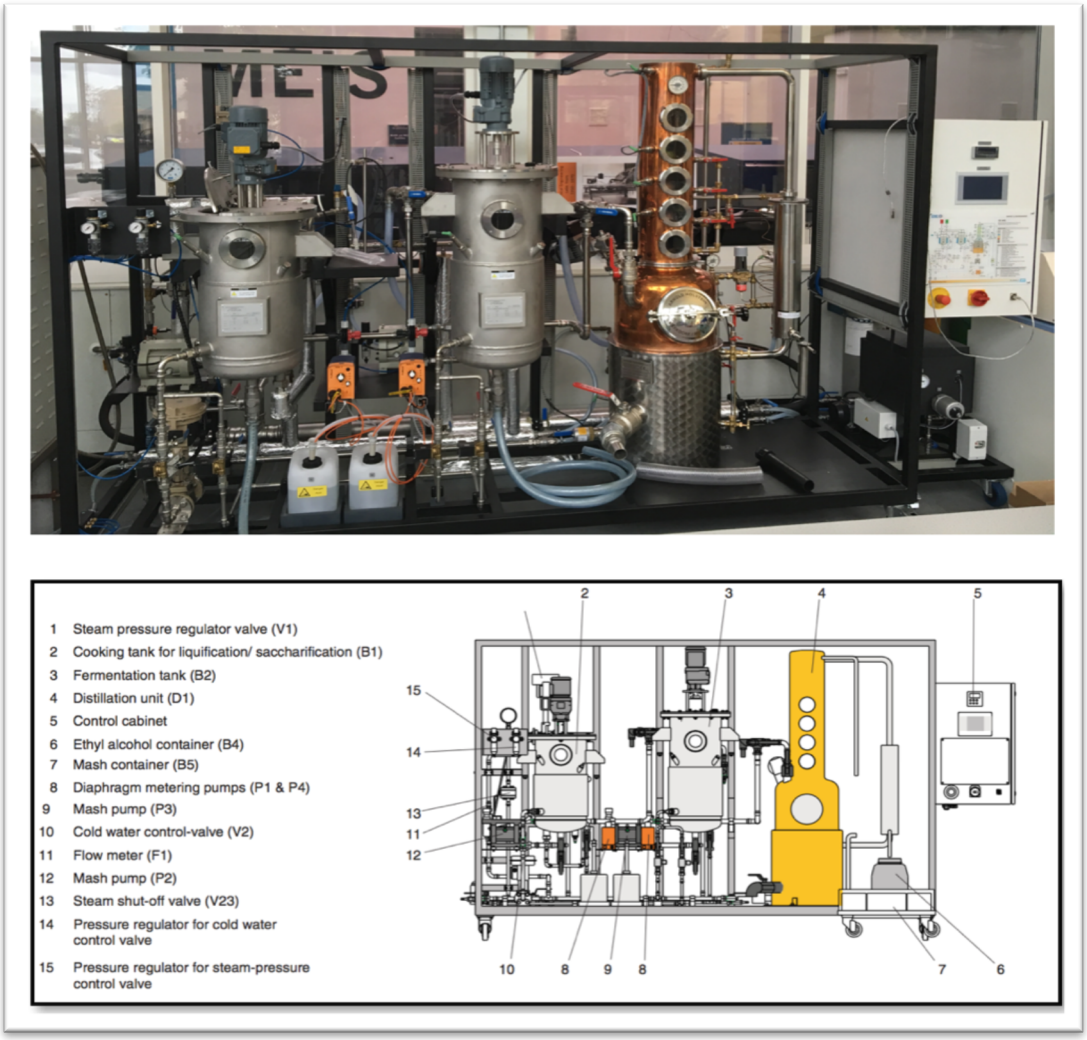

Figure 3-4. GUNT CE-640 Bioethanol plant, general view.

The fermentation unit has a capacity of 60 litres, with all the control specifications of the mashing tank. In addition, the tank is sealed airtight, and the stirrer shaft runs into the container through a fermentation lock, which employs water as a sealing liquid. The generated CO_2 during the fermentation pearls up as gas bubbles through the sealing liquid of the fermentation lock.

The distillation unit is a modified boiler heater with a water bath. It contains a bubble cap tray column, dephlegmator (a device for giving partial condensation of a multicomponent vapour stream) and condenser.

The process diagram, Figure 3-5, presents all the components and measurements on the unit, as well as the pipe connections, supply lines and the manually operated valves.

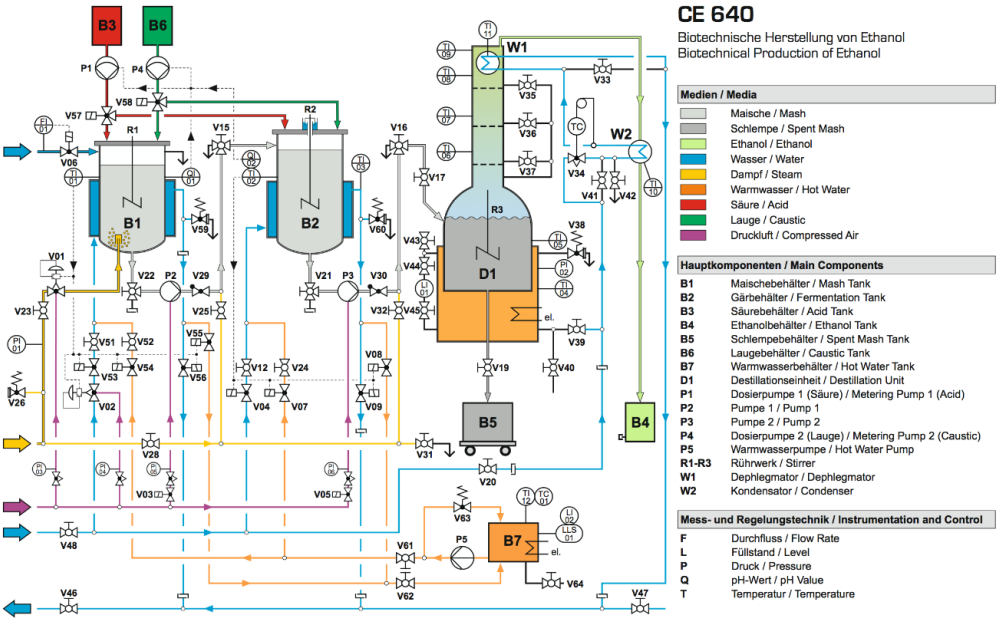

Figure 3-5. Process diagram of GUNT CE-640 Bioethanol plant.

3.5 Methods for bioethanol and DWG production

Producing the DWG product requires a fermentation process that mimics the relevant industrial conditions. The process employed for producing ethanol and DWG in the GUNT plant is described below. The fermentation was followed by the characterisation of the starting and end materials; the analytical methods used are also described.

3.5.1 Fermentation method

In order to produce a fresh fermentation by-product, DWG, that is representative of the industrial by-product, industrial production conditions should be followed. However, it was difficult to ascertain exact industrial fermentation conditions as this information is commercially sensitive and also varies between production plants. Therefore, aiming to produce a representative DWG that has the characteristics of the material produced in the large-scale biorefineries, the production conditions were set according to the recommendations of the provider of the pilot scale fermentation unit, which were similar to the conditions reported in the literature.

Figure 3-6 details the flow of the DWG production process carried out in this project. Wheat was double-milled using a 2 mm mesh by Target Feeds Ltd.; however, this left a substantial proportion of large particles and intact grains which would have been incompletely fermented. The milled wheat was therefore sieved on a 1.4 mm mesh, and the large material milled using a Retsch ZM1000 mill (Retsch GmbH, Germany) without a screen, re-sieved (again on a 1.4 mm mesh) and the material passing through then thoroughly remixed with the original smaller material, while the oversized material was re-milled.

A batch of 6 kg of milled wheat was dispensed into the mash tank with 15 L water; direct steam injection heated the mash to 85°C. The total water addition was 25 L, corresponding to a solid:liquid ratio of 1:4. Starch hydrolysis was initiated by adding 1.2 mL of α-amylase (20 mL per 100 kg) with vigorous stirring at 200 rpm for one hour. The temperature of the mash was reduced to 55°C, and pH adjusted to 4.5-5 with sulphuric acid. Further hydrolysis was accomplished by adding 3.6 mL of gluco-amylase (60 mL per 100 kg) with continuous stirring for 2 hours. The mixture's temperature was reduced to 28°C, and the mash was pumped to the fermentation vessel. Yeast (6 g, equivalent to 100 g per100 kg) was added to the mash to start the fermentation, along with 0.9 mL anti-foaming agent (15 mL per 100 kg). The mash was fermented over 72 hours with continuous control of temperature in the range 25-28°C. The end of the fermentation is recognised by the ceasing of CO_2 release from the water seal, as illustrated in Figure 3-7.

After 72 hours, the mixture was pumped to the distillation column and distilled for 4 hours to separate the ethanol, then allowed to cool to room temperature overnight. The remaining slurry was filtered through a cloth to obtain the DWG, and the filtrate — the Solubles — was dried in an oven to recover the dry solubles. Both DWG and Solubles were stored in a freezer for further experiments.

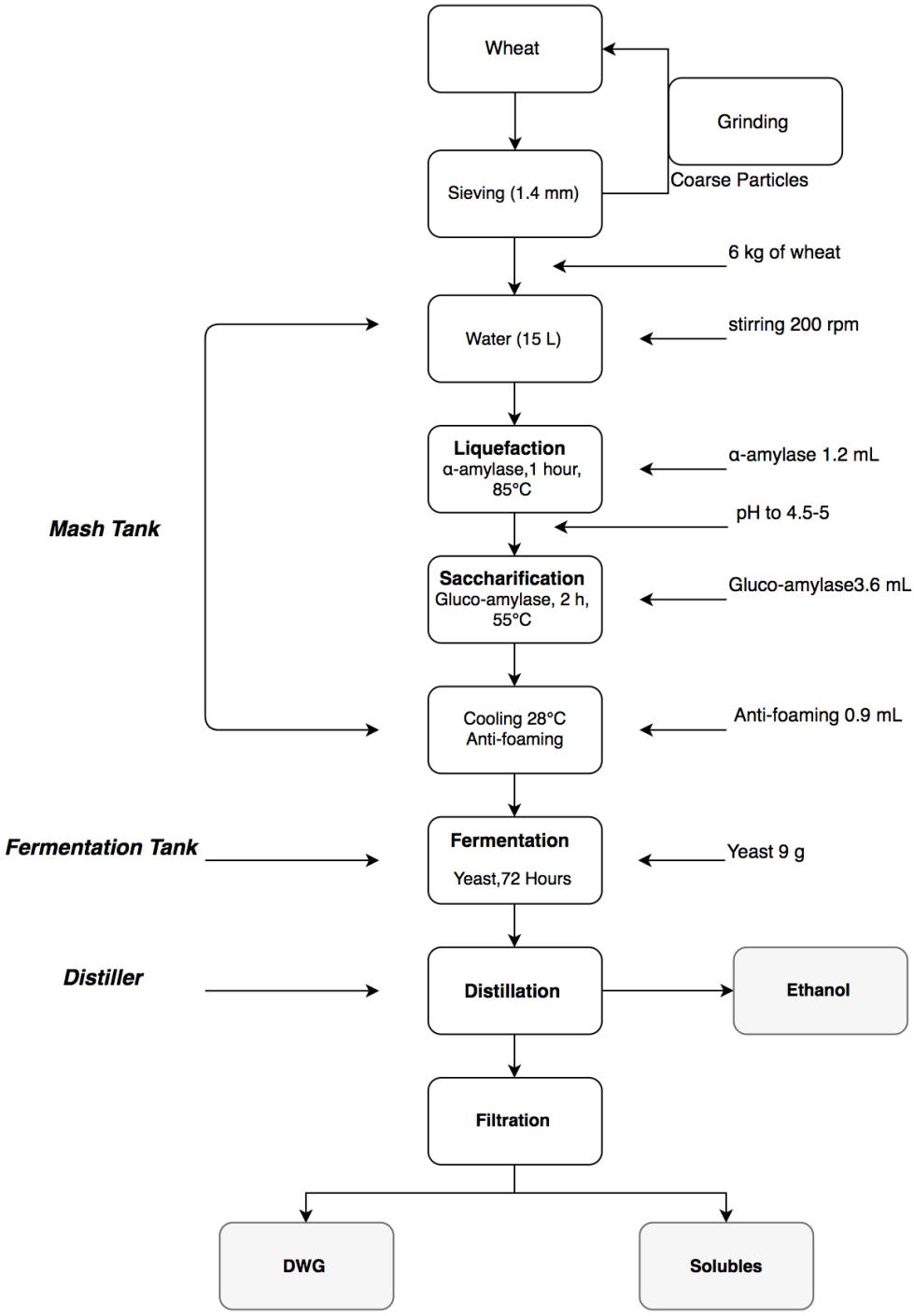

Figure 3-6. Process of DWG production using the GUNT CE-640 bioethanol plant.

Figure 3-7. CO_2 release from the water seal during fermentation.

3.6 Characterisation of the by-products

A series of tests and analyses were carried out to quantify the yields and characterise the materials produced and the changes in the distribution of sugars after fermentation.

3.6.1 Ethanol purity

Since the produced ethanol is not the primary interest of this study, the tests carried out on the ethanol were limited to the estimation of the amount and the concentration using an alcohol hydrometer. The School of Applied Sciences was required to obtain a distillery licence for the GUNT bioethanol plant from HM Revenue and Customs; in accordance with the terms of the licence, all produced ethanol was recorded and stored in our “warehouse” (a locked cupboard).

3.6.2 Constituent sugars analysis

Samples of the raw material (wheat) and the produced materials (DWG and Solubles) were freeze-dried and sent to Englyst Carbohydrate Ltd. (Southampton, UK) for constituent sugar analysis, from which arabinoxylan contents could be calculated. (Later we were able to do these analyses ourselves, but in the early stages of the project we did not have access to the necessary facilities, so made use of this external lab.)

Measurement of the non-starch polysaccharides (NSP) was carried out after enzymatic removal of starch and acid hydrolysis of the NSP to their constituent sugars. For hydrolysis, samples were subjected to treatment with 12M sulphuric acid for 30 min at 35˚C followed by 2M sulphuric acid treatment for 1 hour at 100˚C. Thereafter, sugars were separated and measured by High Performance Anion Exchange Chromatography (HPAEC) system coupled with a Dionex® CarboPac® PA-100 column (250 x 4 mm) with pulsed amperometric detection (PAD) (Englyst *et al.*, 1994).

3.6.3 Protein content analysis

Kjeldahl analysis was used for the determination of the total protein content in the wheat, Solubles and DWG. Sulphuric acid, hydrochloric acid, methyl red (pH indicator), sodium hydroxide pellets, boric acid and Kjeltabs® (digestion catalyser) were obtained from Fisher Scientific Ltd., UK. Turbotherm Rapid Digestion Systems, Gerhardt® (Model TT125) was used for sample digestion, Gerhardt Kjeldahl VAPODEST®, (Vapodest-10) was used for the steam distillation of released ammonia gas, and Schott Titronic® basic Titration Burette (auto-titrator) was used for the titration of ammonium salt.

The Kjeldahl total nitrogen determination method consisted of three stages: digestion, distillation and titration. During the digestion stage, 0.50 g of the tested material was placed into digestion tubes along with 2 Kjeltab® tablets (anhydrous sodium sulphate and a catalyst), 20 mL concentrated sulphuric acid and a few anti-bumping agents (for prevention of bubbling). The digestion process converts the nitrogen into ammonia salt ($(NH_4)_2SO_4$), and other organic matter to CO_2 and H_2O.

A control sample was also prepared and digested accordingly. Samples were digested for 1 hour and 20 minutes. At the end of the digestion stage a greenish-blue colour was developed, indicating the completion of the digestion process (increasing the digestion time was needed in order to obtain the green colour). Once digestion process was completed, digestion tubes were left to equilibrate to room temperature.

The Gerhardt Kjeldahl Vapodest® distillation unit was used to convert the ammonium sulphate into ammonia gas by the addition of sodium hydroxide. The formed ammonia was distilled to a receiving flask containing 25 mL of 3% boric acid and two drops of methyl red (pH indicator). Boric acid was used to convert the ammonia gas into ammonium borate salt.

Determination of the nitrogen content was achieved through titration of the formed ammonium borate against 0.1 M hydrochloric acid; average titration volumes from three replicates were obtained, and the blank titre value was used for calculating the nitrogen percentage presented in the samples:

$$\%N = \frac{[Sample\ titration\ volume\ (mL) - Blank\ titration\ volume(mL)] \times Molarity\ of\ HCl \times 14 \times 100}{Weight\ of\ sample\ (mg)}$$

After the determination of the total nitrogen content, the resultant nitrogen was converted to a protein percentage context by the protein conversion factor multiplication (F = 5.7) for wheat-related products (AOAC, 979.09).

3.6.4 Ash content determination

The term “ash” refers to the inorganic matter in the studied material. The origin of the ash content could be from the plant itself, such as salts absorbed from the soil during growth, or from the supply chain, for example, soil particles carried on the plant, or from the processing of the material, *e.g.* addition of acids and bases may increase the ash content. Ash content is usually determined by combusting the studied subject in a laboratory furnace under controlled conditions.

In this work, the ash content was determined according to the standard biomass analytical method published by the National Renewable Energy Laboratory (NREL), which recommends the expression of the ash as the percentage of residues remaining after dry oxidation at 550° to 600°C (Sluiter *et al.*, 2008).

Samples of the wheat, Solubles and DWG were put into porcelain crucibles and placed in a temperature-controlled furnace at 575°C ± 25°C for four hours. Crucibles were removed from the furnace directly into a desiccator and left to cool to room temperature and weighed. Crucibles were put in the furnace again and heated for one hour, transferred to the desiccator and weighed again. This was repeated until a constant weight was recorded.

3.7 Results

About 60 kg of wheat (10 batches, details are listed in appendix 1) were processed according to the procedure detailed above. On average, each batch of 6 kg yielded around 2.2 L of ethanol, 1.275 kg on dry base (db) or 5.100 kg of 75% moisture content DWG, and 0.800 kg (db) of Solubles. Thus the total yield of DDG plus S was around 2 kg or one-third of the starting wheat, in line with industrial yields.

3.7.1 Ethanol purity

The concentration of the distilled ethanol was measured by an alcohol hydrometer. Figure 3-8 shows the resulting concentration, which was typically around 89% v/v.

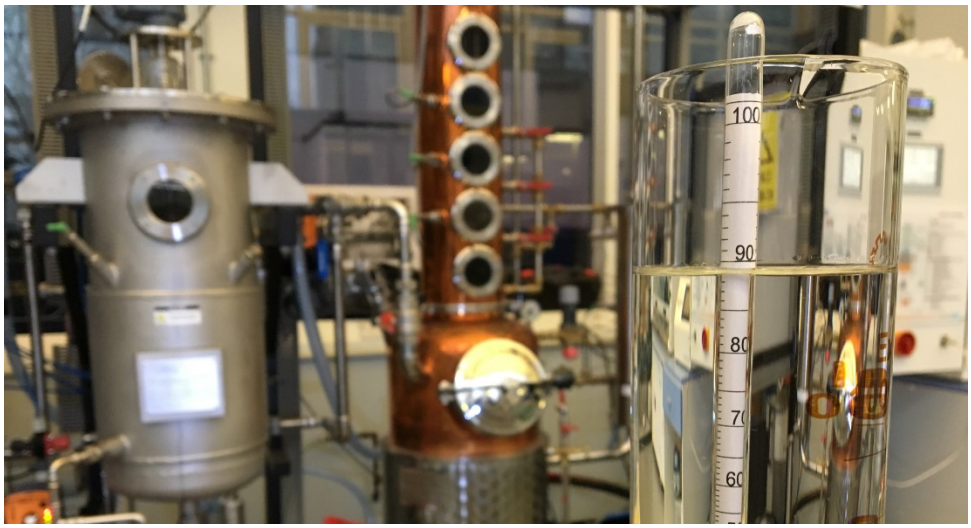

Figure 3-8. Purity of the ethanol produced in the GUNT CE-640 bioethanol plant.

3.7.2 Constituent sugar analysis results

Table 3-2 presents the results from Englyst for the distribution of the constituent sugars and starch content in the wheat, Solubles and DWG. The amount of AX in the wheat was around 5.3%, which becomes concentrated following processing to about 16.7% in the DWG and 11.4% in the Solubles. Total AX was calculated as [0.88 × (ara + xyl)]. (The factor of 0.88 accounts for the addition of a water molecule (molecular weight 18) during the hydrolysis of each monomer of molecular weight 150: (150–18)/150 = 0.88.) The starch content was about 68% in the raw material (4.08 kg starch in 6 kg of wheat), the unfermented starch by the end of the process is about 7% of DWG (around 89 g starch in 1.275 kg of DWG). Therefore, about 98.5% of the total starch was successfully fermented and converted into ethanol and carbon dioxide, while the remaining material was concentrated by a factor of around three.

Table 3-2. Constituent sugars analysis of the original wheat and the resulting DWG and Solubles following ethanol production (analyses performed by Englyst Carbohydrate Ltd.).

Sample	Constituent sugars (g/100 g)								Total NSP	Starch
	rha	fuc	ara	xyl	man	gal	glu	gal-A	g/100g	g/100 g
Wheat	0.0	0.0	2.1	3.9	0.1	0.3	2.4	0.1	8.9 ± 0.0	68.4 ± 0.7
DWG	0.1	0.0	5.4	11.3	1.2	0.9	10.2	0.4	29.6 ± 0.8	7.0 ± 0.3
Solubles	0.1	0.0	3.5	7.9	1.0	1.1	1.4	0.1	15.0 ± 0.4	0.0

3.7.3 Protein and ash content results

Table 3-3 presents the titres obtained for each sample, the calculated average titre, the % total nitrogen, and the corresponding percentage protein content. The protein contents of the two end-products are similar at around 37-39%, similar to literature values (Jarret *et al.,* 2011; Liu, 2011), and both around threefold greater than the 12.77% initial protein content of the wheat. Table 3-3 also reports the ash contents of the wheat, DWG and Solubles. It is notable that the ash content of the Solubles was much higher than of the DWG. Given their relative proportions (about 50% more DWG than Solubles was produced, on a dry basis), overall the ash content if the two were combined would be around 4.72%, again nearly three times the initial ash content of

the wheat. The ash contents of the DWG and Solubles are lower than reported in literature (Jarret *et al.,* 2011; Liu, 2011), possibly because the purity of the chemicals used.

Table 3-3. Protein and ash analysis of the original wheat and the resulting DWG and Solubles following ethanol production (db).

Sample	Titre 1	Titre 2	Titre 3	Average Titre	Average Blank	N %	Protein %	Ash %
Wheat	9.4	9.5	9.5	9.50	1.5	2.24	12.77 ± 0.09	1.8 ± 0.08
DWG	25.9	26.1	26.1	26.05	1.5	6.87	39.18 ± 0.18	2.2 ± 0.00
Solubles	25.0	25.0	24.9	24.95	1.5	6.57	37.42 ± 0.09	8.5 ± 0.40

3.8 Discussion

Ten batches, each of 6 kg of wheat, were subjected to liquefaction, saccharification and fermentation in the GUNT CE-640 bioethanol plant, each yielding about 2.2 L of ethanol at 89% v/v purity. The remaining mash was put through a solid/liquid separation process, which resulted in two end products, the Solubles (800 g db) and the DWG (1275 g db), corresponding to a combined yield of around 33%, in line with the typical yield of DDGS following ethanol production from wheat (Jensen and Björnsson, 2012).

Figure 3-9 shows the mass balance of the protein, AX and ash in the starting wheat and the two end products. In 6000 g of wheat at 12.77% protein, there was initially 766 g protein. The resulting DWG and Solubles had between them 798 g, a difference of 4% which reflects accumulated errors in the analyses. The protein was concentrated by a factor of three during the process, consistent with the literature and industrial performance.

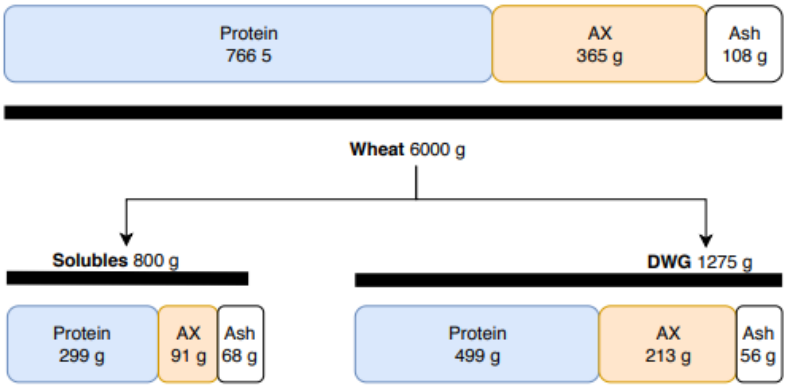

Figure 3-9. Mass balance of protein, AX and ash content before and after fermentation process.

In like manner, the 108 g of ash initially increased to 124 g ash in the two end products, an increase of 15% as a result of the enzyme solutions and the chemicals (acid and base) added during the process to adjust the pH. It is notable that the 15% increase is less than the typical increase reported in the literature (20-30%) (Jarret *et al.,* 2011). This difference is probably due to the quality of the chemicals and enzymes used during the process, the most important being the mineral content of the water. The Solubles had a much greater ash content than the wheat or DWG, indicating that the additional mineral material added during the process mostly ended up in the Solubles fraction, as expected if it is mostly coming from water and aqueous solutions.

The original wheat arabinoxylan (356 g) was divided between the DWG and the Solubles; the majority ended up in the DWG, but in total around 12% of the original AX appeared to be lost during the process. This loss could be due either to degradation during the hydrolysis of starch or during distillation. The concentration of AX in the material increased by a factor of about 2.5, from ~6% in the wheat to ~15% in the DWG and Solubles, corresponding with literature descriptions of commercial DDGS (Kosik *et al.,* 2017).

Considering the arabinose to xylose ratio (A/X), which is one of the most critical characteristics of AX besides the molecular weight, the constituent sugars analysis revealed an A/X ratio of 0.54 in the original wheat, while the ratio was lower in the DWG

(0.48) and the Solubles (0.44). Similar decreases are reported in the literature, these differences may relate to differences in their molecular structure and in their cross-linking with other components in the cell wall (Kosik *et al.,* 2017).

It can be concluded that the DWG obtained by the fermentation procedure followed in the current work has a composition similar to the intermediate by-products produced in industrial plants. Therefore, it is a suitable material for subsequent studies on enzyme treatment, AX and AXOS extraction and performance in animal feeding trials.

3.9 Summary

Distillers Dried Grains with Solubles is the second most important product from cereal biorefineries, after ethanol. DDGS is marketed for use in animal feed formulations. The protein component of DDGS is valuable for animal feed, but the fibre component, which is dominated by arabinoxylans, is deleterious in animal feed and would be better exploited for other end uses, including for increasing fibre consumption in the human food chain, while also exploiting other functional properties of AX. Meanwhile, understanding the efficacy of xylanases in animal feed formulations, in terms of their production of prebiotically beneficial arabinoxylan oligosaccharides, is a further important area of investigation.

Commercial production of AX-based products within an integrated biorefinery would access the wet materials following fermentation before it is dried, termed in the current work Distillers Wet Grains (DWG). Accessing DWG from commercial facilities in order to undertake AX extraction and other studies is problematic, as commercial bioethanol plants are closed systems in which this in-process material is inaccessible. Hence, the current work aimed to use a pilot-scale bioethanol production facility to produce equivalent and representative DWG material for further studies.

The GUNT CE-640 bioethanol plant was used to process 10 × 6 kg batches of wheat, yielding on average 2.2 L of ethanol at 89% v/v, Solubles (0.8 kg db) and DWG (1.275 kg db). The DWG produced in this work appears to have similar yields and compositions

to the material produced in large-scale biorefineries. This material was therefore used for subsequent studies on AX extraction and the production of arabinoxylan oligosaccharides, as described in the following chapters.

4 Arabinoxylan oligosaccharides production

4.1 Introduction

The identification of prebiotics, which stimulate the proliferation of healthy bacteria in human and animal digestive systems, is one of the most interesting developments in the food ingredient sector and of increasing interest in the animal feed sector as well. Arabino- and xylo-oligosaccharides exhibit promising prebiotic activity that promotes their use either by direct addition or through *in-situ* production by xylanases. This chapter reviews the importance of XOS prebiotics and presents experimental work to study the ability of xylanases to produce XOS from the freshly produced DWG, utilising different enzymes and pre-treatments.

4.2 The concept of functional food

In the 1980s, the Japanese food market witnessed the first usage of the term "functional food" to describe food commodities that contained special fortifying ingredients that offer healthy physiological advantages to the consumer (Aachary and Prapulla, 2011). Functional foods were a legislative food category called "FOFHU" and were required to fulfil three nutritional specifications: 1) to show effective results in clinical trials; 2) to be safe for clinical and non-clinical examinations; 3) to have quantifiable active components (Martirosyan and Singh, 2015). In Europe, the functional food concept was defined by the European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE) as (Diplock and Fern, 1999):

"A food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule just as normal food form."

However, European legislation considers functional food as a concept rather than a food category (Stanton *et al.,* 2005). Currently, the European food categories are: conventional foods, modified foods, foods for special dietary use and medical foods (Martirosyan and Singh, 2015). But food developers are allowed to make two claims: nutrition claims and health claims. Nutrition claims indicate the basic nutritional value and the energy provided, whereas health claims cite the food's ability to prevent, manage or heal illness.

The Functional Food Centre (http://functionalfoodscenter.net/) in the United States defines functional foods as:

"*Natural or processed foods that contains known or unknown biologically-active compounds, which in defined amounts provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease.*"

The definition underlines the necessity of specific "bioactive compounds" in functional foods that have health effects, such as probiotics and prebiotics.

The word "probiotic" is the exact opposite of "antibiotic" in the Greek language, and it means "for life". The term probiotic was first used to describe substances produced by one microorganism that stimulate the growth of another (Lilly and Stillwell, 1965). Parker (1974) defined the probiotics as:

"*Organisms and substances which contribute to intestinal microbial balance.*"

This definition connected probiotics to intestinal health, however, it does not clarify the nature of probiotics as it included both "organisms and substances". Fuller (1989) refined the definition to refer to the microorganism:

"A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance."

The recent definition declares the nature of probiotics to be viable live organisms. Lactic acid bacteria and bifidobacteria, as natural components of the intestinal microbiota, are the most studied and used probiotics in dairy products (Siró *et al.,* 2008).

Prebiotics, by contrast, are nutrients that possess the ability to modify the intestinal microbiota. Early reports described the so-called "Bifidus factor" as a component of human milk which is beneficial for the bifidobacteria in infants (Hutkins *et al.,* 2016). Thereafter, the Bifidus factor was found to be a complex of oligosaccharides and glycans. The term and definition of prebiotics were first introduced by Gibson and Roberfroid (1995) as a functional food ingredient:

"A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health."

From the latter broad definition, it is concluded that prebiotics include the oligo- and polysaccharides that resist acid and enzymatic hydrolysis in the human intestinal tract, can be fermented by microflora and stimulate the growth of gut bacteria. The most proposed and available prebiotics in the market are oligosaccharides such as lactulose, fructo-oligosaccharides, galacto-oligosaccharides, soybean oligosaccharides, lacto-sucrose, isomalto-oligosaccharides, gluco-oligosaccharides, xylan oligosaccharides (XOS) or palatinose (Macfarlane *et al.,*2006; Manning and Gibson, 2004; Okazaki *et al.,* 1990).

4.3 AXOS and XOS prebiotic properties

The prefix "oligo-" comes from the Greek *ὀλίγοι* which means "a few or containing a small number of units" (Oxford Dictionary). Oligosaccharides are small chains of a limited number of sugars connected to form one molecule; the IUB-IUPAC nomenclature defines oligosaccharides as saccharides containing three to ten monomers (Voragen, 1998), while some other authorities consider saccharides containing between three and nineteen units as oligosaccharides. However, from

a nutritional point of view, most disaccharides have similar properties to tri- and tetrasaccharides and are also considered oligosaccharides (Crittenden and Playne, 1996).

Xylan oligosaccharides are a novel class of prebiotics that occur naturally in a variety of fruits, vegetable bamboo, honey and milk (Poesen *et al.,* 2016; Rastall and Gibson, 2015; Singh *et al.,* 2015) and that could be produced from lignocellulosic biomass. The structure of XOS is similar to the parent AX: a linear β-(1-4)-D-xylopyranose with or without substitutions of arabinose, uronic acid derivatives, ferulic and coumaric acid and other phenolic acids (Gullón *et al.,* 2014). The variation in the XOS structure depends mainly on the parent plant type, the part of the plant and the method of production (Suzuki *et al.,* 2000).

In general, the prebiotic effectiveness of oligosaccharides is subject to their fermentability by the gut bacteria, which is related to their structure and degree of polymerisation (DP). Van Craeyveld *et al.* (2008) reported that AX oligosaccharides with average DP of 3 (and a maximum no greater than DP5) showed greater ability to elevate the concentration of bifidobacteria when compared with the larger DP. Furthermore, DP2 and DP3 XOS exhibited faster utilisation kinetics than DP4 and DP5 XOS (Gullón et al., 2008a). It is believed that the xylan polysaccharides are not susceptible to the gut bacteria and possess no prebiotic properties at all (Falck *et al.,* 2013; Neyrinck *et al.,* 2012). Predominantly, XOS of a degree of polymerisation of DP2-DP4 are desired for food applications (Vázquez *et al.,* 2000). The influence of the XOS substitutions on the fermentation kinetics showed that both the linear XOS and the branched AXOS are more susceptible to the intestinal microbiota than the more complex XOS containing glucuronic acid, while GluA-XOS fermentation yielded higher concentrations of short-chain fatty acids (Kabel *et al.,* 2002). Similarly, XOS extracted from wheat bran showed better prebiotic activity than the XOS extracted from Bengal Gram husk; this could be attributed to the higher arabinose substitutions in the wheat bran (A)XOS (Madhukumar and Muralikrishna, 2012). Therefore, it can be concluded that the prebiotic activity of XOS and AXOS is subject to the degree of polymerisation, substituent types and the arabinose-to-xylose ratio (degree of substitution).

Arabinoxylan oligosaccharides are a white powder, pH stable over the range 2.5 – 8.0 and thermally stable up to 100°C (Courtin *et al.,* 2009). Wang *et al.* (2009) reported that XOS could tolerate pasteurisation and high temperature sterilisation in a low pH environment. The pH and thermal stability favour XOS over inulin for a variety of food applications, such as in juice, as inulin is more susceptible to decomposition at low pH and high temperatures (Courtin *et al.,* 2009).

As prebiotics, XOS specifically invigorate the growth or the activity of a group of bacteria (Immerzeel *et al.,* 2014; Mendis and Simsek, 2014) and thereby improve health, reduce gut infections and suppress colon cancer (Macfarlane *et al.,* 2006). They are preferentially used by *Bifidobacterium* spp (Okazaki *et al.,* 1990) and utilised as a carbon source by *Weissella* strains (Patel *et al.,* 2013) and *Bifidobacterium* spp (Fujikawa *et al.*, 1991). *In vitro* tests showed that XOS are the preferred substrate for the *Bifidobacterium* spp over the other non-digestible oligo-saccharides of hexoses; their activity was comparable to raffinose oligosaccharides and higher than fructo-oligosaccharides (Jaskari *et al.,* 1998; Vázquez *et al.,* 2000). XOS seemed to improve the overall gut health and regulate the bowel movement in cases of severe constipation (Chung *et al.,* 2007; Tateyama et al., 2005) with no reports of any side effects such as increased flatulence, intestinal bloating or intestinal irritation, which have been reported to accompany the consumption of fructo-oligosaccharides (Bruggencate *et al.,* 2006).

Prebiotics can mediate the protective immune response in the intestinal tract, caused by foreign antigens from food or microbes, and help to avoid hypersensitivity reaction against antigens (Schley and Field, 2002). The mediation is mainly a result of the production of short chain fatty acids (SCFA) (acetate, propionate, butyrate and lactate). XOS demonstrated an anti-inflammatory, immunomodulatory and free radical scavenging activity (Chen *et al.,* 2012; Hromádková *et al.,* 2013). Also, the XOS fermentation products, SCFA, have an important role in adjusting the pH in the intestine to prevent the growth of unwanted pathogenic bacteria (Hughes *et al.,* 2007; Knudsen and Lærke, 2010; Wang *et al.,* 2010; Boll *et al.,* 2015; Rumpagaporn *et al.*, 2015).

XOS have been reported to deliver several health-related advantages, such as improving the clinical conditions associated with diabetes and reducing mortality rates in rodents (Gobinath *et al.,* 2010). Also, XOS demonstrated an effective role in the regulation of obesity and associated metabolic disorders in mice (Neyrinck et al., 2012). XOS were reported effectual against the induced oxidative stress associated with a high-fat diet in rats (Wang *et al.,* 2011); similarly, they played a protective role against high-protein diet-induced genotoxicity (colonic DNA damage). However, such results were not obtained for inulin (Christophersen *et al.,* 2013). This protective activity was ascribed to the production of SCFA as they seem to activate the genes responsible for cellular energy metabolism and anti-inflammatory response (Fukuda *et al.,* 2011). Moreover, XOS confer advantageous effects in regulating glucose and lipid profile and thereby on human energetic metabolism (Staneva *et al.,* 2014).

In parallel, significant efforts have been paid to increase the amount of AXOS in animal feed. Several pieces of research suggest that AXOS influence the growth performance, mortality rates and general gut health of broilers (Austin *et al.,* 1999; Courtin *et al.,* 2008; Lei *et al*., 2016). The production of AXOS in animal feed is achieved by the addition of xylanases to the feed (Lei *et al.,* 2016).

4.4 Xylanases and AXOS in animal feed

Xylanases are naturally occurring enzymes that bio-catalyse the degradation of xylan chains (Bonner, 2007). They have been exploited in a variety of industrial applications including bleaching in the paper industry, breadmaking, fruit juice and beer clarification, and animal feed (Sharma, 2013), with a global annual market share of 1 billion USD with approx. 40% of the value being for the non-starch polysaccharides hydrolysing enzymes (NSPases) in 2018 (https://www.marketsandmarkets.com/Market-Reports/feed-enzyme-market-1157.html).

Xylanases are one of the most consumed enzymes in the pig and poultry feed formulation industry, used traditionally by feed manufacturers to achieve performance consistency (Bedford and Morgan, 1996; Paloheimo *et al.,* 2010; Sitanaka *et al.,* 2018).

Despite the wide usage of xylanases in animal feed, the function of these enzymes is not completely understood. It is established that AX behave as an antinutrient in the birds' feed, which was explained by two mechanisms. The first mechanism suggests that AX are resistant to enzymatic degradation in the small intestine and thereby hinder endogenous enzyme (*e.g.* protease and amylase) gaining access to the intact endosperm cells due to the lack of functional NSPase enzyme suite in the animal (Bedford and Morgan, 1996; Pettersson and Åman, 1989). Selle *et al.* (2009) reported that utilisation of xylanases in the feed formulation improved overall feed intake and digestibility and thereby growth performance.

The second mechanism is based on the ability of AX to increase the viscosity of intestinal fluids, which is known to reduce the diffusion rate of nutrients, decrease feed passage rates and increase the population of gut flora (Feighner and Dashkevicz, 1988; Fengler and Marquardt, 1988; Salih *et al.*, 1991). Adding xylanases to the feed significantly reduces the viscosity in the intestines and improves the weight gain (Wu *et al.,* 2004; Zyłe *et al.,* 1999). The two mechanisms are connected and indicate the importance of degrading the large molecules of AX in animal feed to reach an optimum feed utilisation.

More recently, attention has turned to the beneficial role of the (A)XOS produced by xylanases (Broekaert *et al.,* 2011; Craeyveld *et al.,* 2008). This role was linked to the fermentation of AXOS in the animal's digestive system and the production of SCFA (Choct *et al.,* 1999). It was found that SCFA promotes the release of the neuropeptide peptide YY (PYY) which increases the efficiency of digestion and nutrient absorption after a meal by delaying gastric emptying (le Roux and Bloom, 2005; May *et al.,* 2015). Alijosius *et al.* (2018) reported that the xylanases affected the tenderness and colour of chicken breast meat. This effect could be attributed to the produced XOS prebiotic or the phenolic compound in the feed, although more studies are needed to reveal the impact of XOS and other prebiotics on animal feed. These studies re-emphasise the necessity to understand the susceptibility of feed substrates to release XOS when treated with enzymes. Also, it underlines the importance of producing considerable amounts of XOS and AXOS to be incorporated in animal feed trials in order to gain greater knowledge about the effects of including these prebiotics in feed formulations.

DDGS as a bioethanol by-product is marketed as a nutritional ingredient for animal feed. It is evident that the severe drying conditions reduce the nutritional value of DDGS for animal feed and change the characteristics of AX in the process (De Vries *et al*., 2013; Kosik *et al.,* 2017; Pedersen *et al.,* 2014; Schroeder, 2012). Therefore, there is a great opportunity to increase the value of DDGS via xylanase treatment prior to drying. The wet DWG produced as described in the previous chapter offers a suitable substrate to examine the enzymatic production of (A)XOS or (A)XOS-rich materials within biorefineries.

This chapter presents experiments performed to study the ability of xylanases to produce (A)XOS from freshly produced DWG, utilising different enzymes and pretreatment methods, in order to evaluate the possibility of valorising this biorefinery by-product by increasing the nutritional value of the DDGS.

4.5 Materials and methods for DWG-xylanase treatment

Aiming to produce meaningful levels of oligosaccharides from the freshly produced DWG for animal feeding, three enzymes were tested at two dosages under two pH conditions. Pretreatment with protease enzyme was also investigated.

4.5.1 Enzymes and chemicals

Xylanases: Econase XT 25L**,** Xylanase 1 EL-2016/002288 and Xylanase 2 EL-2016/002287 were kindly provided by AB Agri Ltd. The first of these is a commercial endoxylanase marketed to the animal feed industry; the other two are development enzymes. The dose of Econase when added to animal feed is usually 100 mL per tonne, preliminary results did not show any XOS production at this dosage level, hence a 500× higher dose of 50 mL per kg was used throughout this project. Citric acid monohydrate, Sodium dihydrogen phosphate dodecahydrate, sodium hydroxide and trifluoroacetic acid were obtained from Fisher Scientific Ltd, UK. Samples were treated with enzymes within a shaking water bath Thermo-Haake®, model (SWB20), and centrifuged using Eppendrof® centrifuge, model 5702.

4.5.2 Enzyme treatment

A sample of 2 g (db) of DWG was transferred into a 200 mL beaker, citrate-phosphate buffer added to give a solid-to-liquid ratio around 1:10, and the beakers moved into a shaking water bath of 42°C. Two buffer solutions were prepared at pH 5.5 (the enzyme optimum pH range) and a lower pH 2.5 in order to investigate the enzymes' performance in acidic conditions during digestion in the animal's gastric system. The studied enzyme was added according to the dosages listed in Table 4-1. The enzymes were added at 50 mL per kg dose and at 10x that dose; two blank samples were also prepared, to observe the pH effect. Samples were incubated for 1 hour, after which they were removed from the water bath and allowed to cool to room temperature. The pH was adjusted to 7±0.1 with 1M sodium hydroxide. The resultant slurry was centrifuged at 4000 rpm for 15 minutes. Supernatants were collected, filtered through a syringe filter (0.45 µm pore) and freeze-dried at 0.04 mbar and –55°C for 72 hours.

Table 4-1. Dosage and pH of the enzyme incubation test.

Sample ID		Enzyme	Enzyme Dose	pH
sample 1	Econase ×1- 2.5	Econase XT 25L	0.1 mL	2.5
sample 2	Econase ×1- 5.5	Econase XT 25L	0.1 mL	5.5
sample 3	Econase ×10- 2.5	Econase XT 25L	1 mL	2.5
sample 4	Econase ×10- 5.5	Econase XT 25L	1 mL	5.5
sample 5	XYL1 ×1- 2.5	Xylanase 1 EL-2016/002288	0.1 mL	2.5
sample 6	XYL1 ×1- 5.5	Xylanase 1 EL-2016/002288	0.1 mL	5.5
sample 7	XYL1 ×10- 2.5	Xylanase 1 EL-2016/002288	1 mL	2.5
sample 8	XYL1 ×10- 5.5	Xylanase 1 EL-2016/002288	1 mL	5.5
sample 9	XYL2 ×1- 2.5	Xylanase 2 EL-2016/002287	0.03 mL*	2.5
sample 10	XYL2 ×1- 5.5	Xylanase 2 EL-2016/002287	0.03 mL	5.5
sample 11	XYL2 ×10- 2.5	Xylanase 2 EL-2016/002287	0.3 mL	2.5
sample 12	XYL2 ×10- 5.5	Xylanase 2 EL-2016/002287	0.3 mL	5.5
sample 13	Buffer 2.5	-	x0	2.5
sample 14	Buffer 5.5	-	x0	5.5

*mL of 1 mg/mL solution

4.5.3 Protease pre-treatment

It was hypothesised that wheat protein denaturation during the fermentation and distillation process may create a protective protein matrix, preventing the xylanases

from accessing the structure of the bran particles and hence reducing the activity of the enzyme and decreasing the production of AXOS. Therefore, the DWG was treated with protease to degrade the proteins, then followed by Econase treatment as described in Table 4-2. The supernatant was collected, freeze-dried and sent for analysis at IPOS labs for sugar analysis (see below).

Table 4-2. Protease treatment prior to Econase.

Sample ID	1 (DWG + Econase)	2 (DWG + Protease + Econase)
DWG (g) db	2	2
Buffer (pH = 8) (mL)	20	20
Protease (mL)	-	0.1
Incubate for 1 hour at 50 °C		
Boil the mix for 10 min to deactivate the protease		
Adjust the pH to 2.5 by citric acid		
Econase (mL)	0.1	0.1
Incubate for 1 hour at 42 °C		
Cool, neutralise with NaOH 1M and centrifuge		

4.5.4 Analytical Methods

The analytical methods applied to the dried samples were mainly the analysis of constituent sugars after hydrolysis and screening the profile of oligosaccharides.

4.5.4.1 Constituent sugars analysis

The AX content of the released material collected in the supernatant was quantified by hydrolysing the material to its constituent arabinose and xylose, which were then measured using high performance anion exchange chromatography with a pulsed amperometric detector (HPAEC-PAD). The HPAEC Instrument used was a Dionex ICS–3000 Ion Chromatography System (Dionex Corporation, CA, USA), which comprises a Dionex Auto Sampler, Dionex ICS 3000 Eluent Organiser, Dionex ICS 3000 Detector Chromatography, Dionex ICS 3000 Dual Pump System and Dionex ICS 3000 Eluent Generator Reagent-Free Ion Chromatography. The column used was a CarboPac® PA20 analytical column, 3×150 mm 6.0 µm particle size. The chosen mobile phase was 10mM

NaOH with an adapted flow rate of 0.3 mL min^{-1} and an injection volume of 20 µL. Chromeleon® Xpress software was utilised for data processing (Englyst *et al.,* 1994).

A hydrolysis step was performed to induce chemical bond cleavage through water addition, which facilitates the conversion of AX and AXOS into their sugar moieties sub-components. 4.0 mg of the dried supernatant was transferred carefully into a pressure tube, 2 mL of 2M trifluoroacetic acid was added, the tubes were capped and heated at 120°C for 2 hours, then left to equilibrate to the ambient room temperature. A constant stream of nitrogen gas at 60°C was maintained in order to evaporate the trifluoroacetic acid. Subsequently, the dried residues were dissolved in 4 mL of ultra-purified water for HPAEC-PAD analysis.

4.5.4.2 Oligosaccharide profile analysis

Monosaccharide analysis of hydrolysed samples is relatively straightforward and readily achieved by HPAEC. Oligosaccharide analysis, by contrast, is more challenging. At this stage of the project, we had not developed the new method that is described later in Chapter 6. We therefore collaborated with Innovative Physical Organic Solutions (IPOS), Huddersfield, who were developing a method for screening of oligosaccharide distribution utilising a newly developed size exclusion chromatography method (Powles, N., unpublished work). Samples were sent to IPOS and the analysis performed using an HPLC coupled to a refractive index detector (RID). The instrument was run isocratically using ultra-pure water, at a flow rate of 0.2 mL min^{-1}.

4.6 Enzyme treatment results

The monosaccharide and oligosaccharide analyses were performed in parallel on the treated samples, aiming to construct a full qualitative and quantitative picture of the hydrolysate distribution and thereby develop a detailed understanding of the xylanases' activities at different pH values and dosages.

4.6.1 Monosaccharide analysis results

Both samples and standards were analysed via HPAEC-PAD as described in section 4.5.4.1, with calibration curves constructed for the standards of the three sugars of interest, arabinose, xylose and glucose. Figure 4-1 illustrates the standards mixture chromatogram.

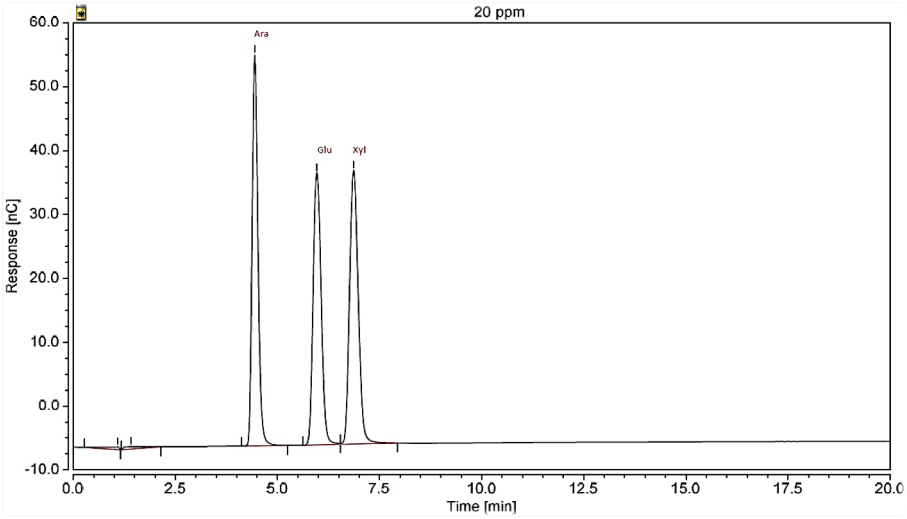

Figure 4-1. Arabinose, xylose and glucose standards separation chromatogram.

Retention times were recorded to provide a valid comparative background between the analysed samples. To construct the calibration curves, area under the curve (AUC) values were integrated via the chromatographic software Chromeleon® and plotted against concentrations for each standard.

After hydrolysing all the samples, the solutions were introduced to the HPEAC-PAD system. Comparisons were implemented between the resultant peaks and the standards for identification and quantification purposes. The amounts of the released monosaccharides in the samples were calculated and are shown in Table 4-3 and Figure 4-2. Clearly, the amounts of monosaccharides in the Econase-treated samples are higher than the samples treated with the other xylanases. In the case of Econase, the low pH proved to be a better environment, releasing more AX material into the supernatant, but this was not apparent for the other two enzymes. Increasing the dose by a factor of 10 appeared to reduce the release of AX.

Table 4-3. Total released monosaccharides from DWG after xylanase treatment (Measured by HPAEC-PAD after hydrolysis)

Sample ID		Arabinose mg	Glucose mg	Xylose mg
Sample 1	Econase ×1- 2.5	4.79 ± 0.24	5.95 ± 0.30	8.09 ± 0.41
sample 2	Econase ×1- 5.5	3.73 ± 0.19	5.34 ± 0.27	6.18 ± 0.31
Sample 3	Econase ×10- 2.5	2.96 ± 0.15	5.46 ± 0.27	5.26 ± 0.26
Sample 4	Econase ×10- 5.5	2.33 ± 0.12	6.09 ± 0.31	4.39 ± 0.22
Sample 5	XYL1 ×1- 2.5	2.84 ± 0.14	2.84 ± 0.14	4.08 ± 0.21
Sample 6	XYL1 ×1- 5.5	2.76 ± 0.14	2.89 ± 0.15	2.98 ± 0.15
Sample 7	XYL1 ×10- 2.5	2.21 ± 0.11	2.66 ± 0.13	3.35 ± 0.17
Sample 8	XYL1 ×10- 5.5	1.98 ± 0.16	2.74 ± 0.22	3.00 ± 0.24
Sample 9	XYL2 ×1- 2.5	1.68 ± 0.14	1.78 ± 0.14	1.42 ± 0.12
Sample 10	XYL2 ×1- 5.5	2.19 ± 0.18	2.01 ± 0.16	2.53 ± 0.20
Sample 11	XYL2 ×10- 2.5	2.08 ± 0.17	1.90 ± 0.15	2.38 ± 0.19
Sample 12	XYL2 ×10- 5.5	2.21 ± 0.18	2.20 ± 0.18	2.82 ± 0.23
Sample 13	Buffer 2.5	1.56 ± 0.13	1.73 ± 0.14	1.35 ± 0.11
Sample 14	Buffer 5.5	1.57 ± 0.13	1.74 ± 0.14	1.47 ± 0.12

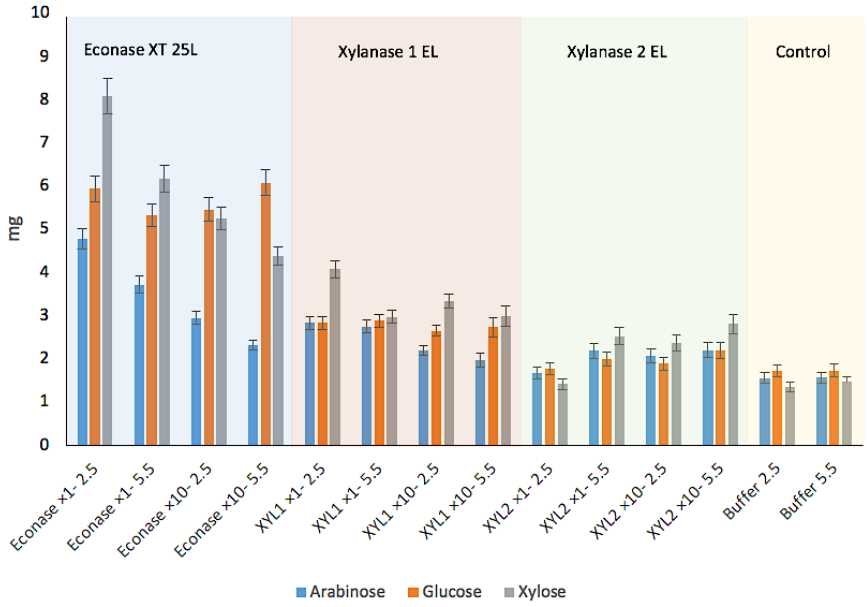

Figure 4-2. Total released monosaccharides from DWG after xylanase treatment (measured by HPAEC-PAD after hydrolysis).

The amount of AXOS released from each sample was calculated as [(Ara + Xyl) × 0.88]. Table 4-4 and Figure 4-3 report the AXOS amounts in the samples following treatment with the three enzymes at two dosages and two pH levels.

Table 4-4. Amount of AXOS released from 2 g of DWG by enzymes at different dosages and pH.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
AXOS (mg)	11.3	8.7	7.3	5.91	6.1	5.1	4.9	4.4	2.7	4.2	3.9	4.4	2.6	2.7

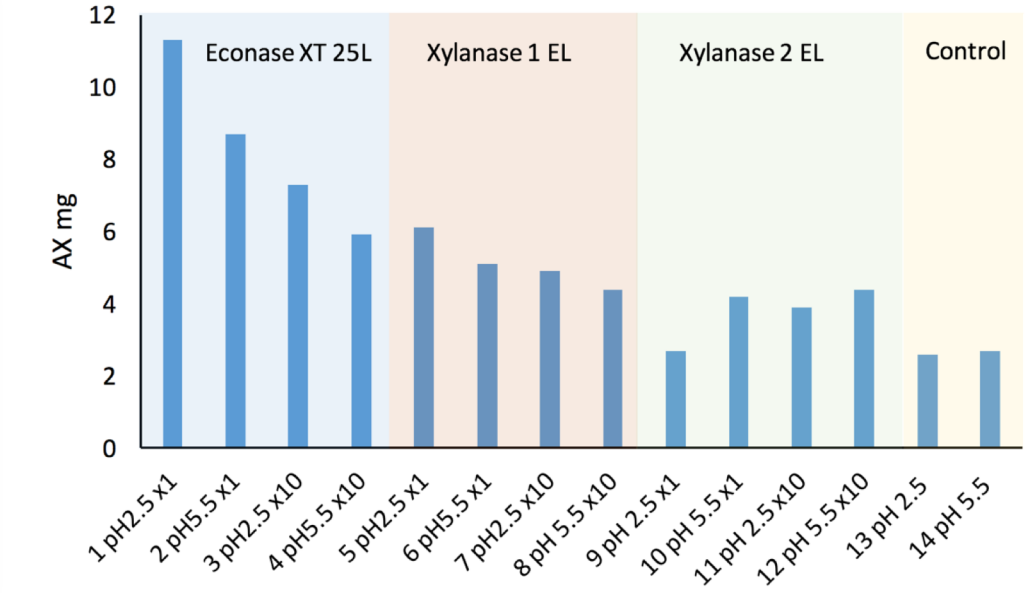

Figure 4-3. Amount of AXOS released from 2 g of DWG by enzymes at different dosages and pH.

The results suggest that lower pH can increase the effectiveness of Econase XT 25L; incubating the DWG at pH 2.5 instead of 5.5 gave 25% more AXOS. Moreover, Econase XT 25L had the strongest efficacy among the tested enzymes. For this reason, and because it is currently a commercial product, this enzyme was used for subsequent studies.

The sample treated with the lower dose of Econase XT 25L seemed to release more AXOS than the sample treated with 10 times the dosage. It is conceivable that the enzymes have limited ability to penetrate the cell wall matrix, therefore only a limited amount of AX might be released, after which the hydrolysing ability of the enzymes is directed towards the already released AX molecules, such that the released AXOS were further hydrolysed into smaller moieties. Considering that the followed analysis procedure was designed to accommodate the analysis of polysaccharides by hydrolysing

them into the monosaccharides, it is possible that the hydrolysing process degraded a considerable amount of the oligosaccharides produced by the high enzyme dosage. It is well known that monosaccharide analysis is sensitive to the hydrolysis conditions used and that the chosen conditions need to strike a balance between adequate hydrolysis and minimal degradation of the hydrolysis products, with appropriate conditions needing to be established for different analytes (Ranganathan *et al.,* 1985). The current findings probably indicate the unsuitability of the applied hydrolysis procedure for the measurement of the oligosaccharides in these samples. However, despite uncertainly over the accuracy of the analysis, it remains clear that the amount of AX/AXOS released by enzymes was very small, no more than 0.57% of the treated raw material.

4.6.2 Oligosaccharide profile analysis results

The applied chromatographic method developed by IPOS, the so-called the DPn method, offered the opportunity to examine the oligosaccharides profile by separating the oligosaccharides according to their degree of polymerisation. The technique is limited to polysaccharides with a degree of polymerisation range 1-6. Figure 4-4 shows the separation of XOS standards of DP2-6.

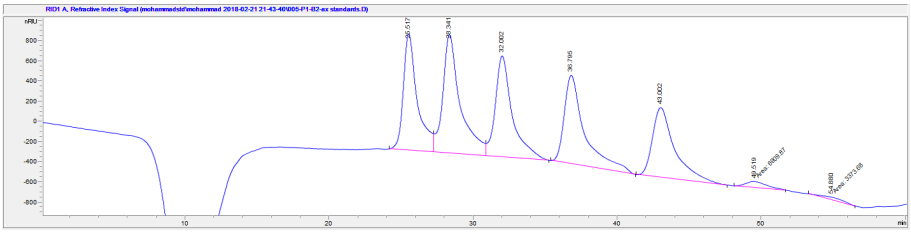

Figure 4-4. XOS standards separation by IPOS.

The analysis results of the enzymatic treatment are summarised in Table 4-5 and Figure 4-5.

Table 4-5. Oligosaccharides in the samples(%w/w of DWG) released by enzymes at different dosages and pH.

Sample ID		DP6	DP5	DP4	DP3	DP2	DP1
Sample 1	Econase ×1- 2.5	0.053	0.032	0.003	0.132	0.203	0.893
sample 2	Econase ×1- 5.5	0.074	0.081	0.049	0.347	0.683	2.683
Sample 3	Econase ×10- 2.5	0.015	0.055	0.027	0.097	0.247	1.176
Sample 4	Econase ×10- 5.5	0.056	0.058	0.006	0	0.519	2.410
Sample 5	XYL1 ×1- 2.5	0.077	0.098	0	0	0.335	1.261
Sample 6	XYL1 ×1- 5.5	0.058	0.087	0	0	0.472	1.554
Sample 7	XYL1 ×10- 2.5	0.009	0.101	0	0	0.135	0.971
Sample 8	XYL1 ×10- 5.5	0.060	0.036	0.038	0	0.151	1.149
Sample 9	XYL2 ×1- 2.5	0.007	0.085	0.114	0	0.495	1.750
Sample 10	XYL2 ×1- 5.5	0.006	0.030	0.027	0	0.142	0.392
Sample 11	XYL2 ×10- 2.5	0	0	0	0	0.278	0.745
Sample 12	XYL2 ×10- 5.5	0.060	0.063	0	0	0.407	0.993
Sample 13	Buffer 2.5	0.096	0.060	0	0	0.461	2.043
Sample 14	Buffer 5.5	0.224	0.049	0	0	0.512	2.421

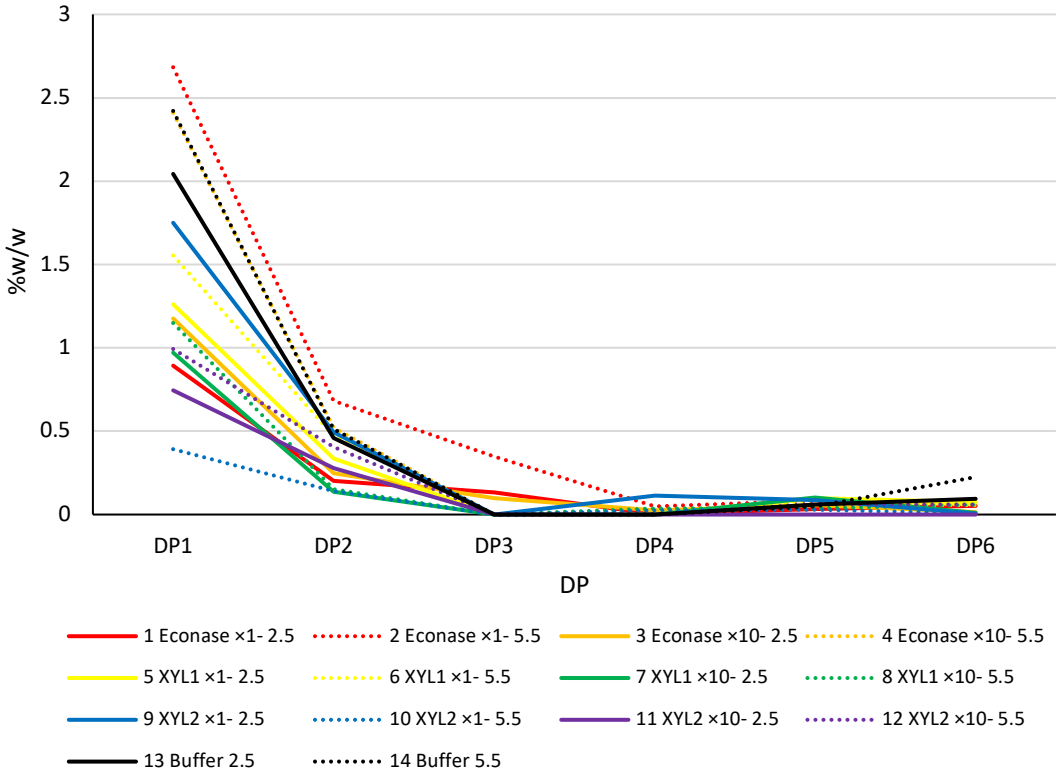

Figure 4-5. Oligosaccharides in the samples (%w/w of DWG) by enzymes at different dosages and pH.

Clearly, the amount of released AXOS was much less than 1% in all samples. In fact, the analysis shows that the monosaccharides are the overriding hydrolysate. Also, most of the liberated AXOS are DP2 and DP3 with traces, or absence, of DP4, DP5 and DP6. Contrary to the monosaccharides analysis results, higher pH (5.5) yielded higher amounts of DP2 in the Econase treated samples.

The results show a similar activity of all three enzymes in terms of the released oligosaccharides with degree of polymerisation 1 to 6. Moreover, the results suggest that the enzymes' performance in generating the AXOS is comparable to the buffer effect by itself as seen in Figure 4-5.

The high content of monosaccharides varied between the studied samples, the highest in the Econase-treated samples and the control samples at low pH values. The monosaccharide could be glucose resulting from the presence of unfermented residues of starch that had been hydrolysed during the treatment, or could be xylose resulting from the enzyme activity. The IPOS method does not distinguish the detected monosaccharides, glucose, arabinose and xylose, hindering interpretation of the results beyond the overall observation that the enzymes were not very active on this substrate.

In the case of Econase, contrary to the results from the monomers analysis, the results here suggest that the enzyme has a better performance in higher pH environment and it also shows no difference between high and low doses. This variation of results could be attributed to the fact that the DPn method is limited to measure and detect only the small oligomers (DP1-DP6) where the constituent sugar analysis was able to detect the larger polysaccharides.

Both analytical procedures indicated that only minor amounts of AXOS were released. Hence, it was investigated whether chemical extraction of AX followed by enzymatic hydrolysis could give higher yields of the desired AXOS; this investigation is reported in the next chapter.

4.6.3 Protease treatment results

The dried supernatant following protease pretreatment was also analysed for the oligosaccharides in IPOS labs; the content and the w/w% of the oligos were calculated as shown in Table 4-6 and Figure 4-6. Degradation of the protein increased the total amount of XOS released (DP2-DP6) from 3.54% to 4.0%, an increase that is probably insignificant and certainly not substantial.

Table 4-6. Protease Treatment results %w/w.

	%w/w					
	DP6	DP5	DP4	DP3	DP2	DP1
DDG + XT	0.10	0.69	0.35	0.40	2.00	5.10
DDG + Protease + XT	0.20	1.00	0.50	0.50	1.80	5.10

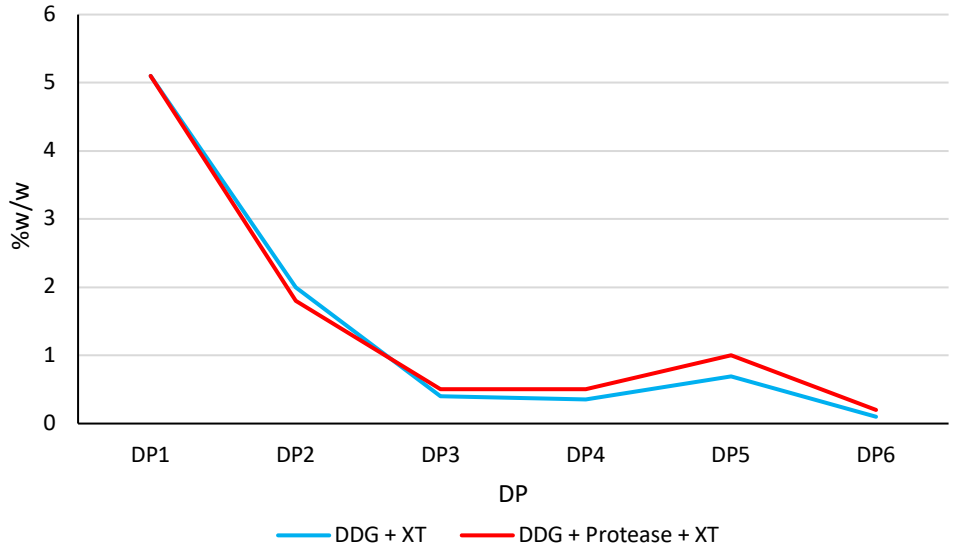

Figure 4-6. Protease treatment effect on the released AXOS.

Both Table 4-6 and Figure 4-6 present no significant outcome of the protease treatment of the substrate especially with the mono and di-saccharides. The graph shows a slightly higher content of DP5, but this could be a systemic error as there was no difference between the two samples at the other DPs.

4.7 Summary

Distillers Wet Grain was subject to a series of investigations to evaluate its potential as an XOS production source. The work focused on the enzymatic hydrolysis of DWG using a variety of xylanases and conditions, as well as pretreatment with protease to reduce any protective effect of denatured protein.

Three xylanases were tested at two dosages; 50 mL per kg and 10× that dose, and at pH values of 2.5 and 5.5. Econase outperformed the other two enzymes and was more effective at the lower pH, but increasing the dosage did not release more AX material, and in any case the amounts released were small.

The analysis method developed in IPOS labs, the DPn method, gave a qualitative picture of the distribution of the oligosaccharides that was helpful at this stage of the study; however, later in the work a better method was developed, as described below in Chapter 6.

The poor overall performance of the studied xylanases argued against further studies of AXOS production from the DWG by the direct enzymatic method. Animal feed trials would require typically 4 kg of DWG with a content of 10% AXOS in order to show measurable effect on weight gain; the results presented here showed that this was not achievable by enzymatic treatments. It might be more efficient to extract AX from DWG by chemical means, hydrolyse this more concentrated AX using Econase, and then deliver this product to the animal feed trials. Therefore, the next chapter describes studies of AX extraction from DWG.

5 Chemical extraction of AX from DWG

5.1 Introduction

The fractionation of biomass residues in order to produce valuable materials has attracted a great deal of attention recently. Globally, the amount of agricultural residues produced annually is estimated at around 5 billion tons (Naidu, Hlangothi, and John, 2018). (These estimations are based on the amounts of harvested crops, due to lack of systematic tracking of under-exploited agricultural residues (Smil, 1999)). DDGS is an increasingly available biorefinery by-product that goes to animal feed. The composition of the DDGS and the parent DWG suggests great potential for the isolation of arabinoxylan. Therefore, the extraction of AX within biorefineries where the starting material, DWG, is abundantly available seems to be a plausible and promising scenario. Isolating AX has been reported in the literature via several methods and pathways. This chapter reviews previous work on extracting AX, discusses the effects of the extraction method on the final product, and demonstrates the extraction procedure followed in the current project to isolate AX soluble fibres to be used as a substrate for the XOS production.

5.2 Arabinoxylans extraction methods

The liberation of arabinoxylan molecules from the cereal cell wall matrix is hindered by the complicated and massive structure tethering cell wall components together (Courtin and Delcour, 2001). AX naturally form ester- and/or ether-linked lignin-polysaccharide complexes, with hydrogen bonds between polysaccharides chains (Ebringerová and Heinze, 2000). Severe treatments are therefore required to separate AX from other cell wall constituents (Courtin and Delcour, 2001). Typically, the extraction process of AX follows a chemical solubilising process or mechanical-assisted hydrolysing method followed by a final purification procedure.

5.2.1 Hydrolysing and solubilising of AX

5.2.1.1 Water extraction

AX are classified into water-extractable and water-unextractable categories. The water-extracted AX was reported to preserve the native structure of the molecule, but in low yields (Ebringerová and Heinze, 2000). The low yield of this method is a result of the robust attachment to lignin through ferulate links and the hydrogen bonds between AX and cellulose (Maes and Delcour, 2002; Vinkx and Delcour, 1996). Non-covalent bonds are weak, however, the abundance of these bonds prevents AX solubilisation under moderate extraction conditions (Slavin, 2006). Applying higher temperatures and pressures during the procedure can significantly increase AX solubilisation into the extraction medium; however, the harsher conditions solubilise other cell wall components as well and consequently reduce the purity of the extracts, which therefore require further purification treatments (Schooneveld-Bergmans *et al*., 1999). Moreover, as noted above, one of the advantages of water extraction is that it preserves the molecules' original conformation; the application of high temperature and pressure was reported to modify the structure of AX and hydrolyse the polymer into smaller molecules with a lower degree of polymerisation (Josefsson *et al.,* 2002).

5.2.1.2 Alkaline extractions

The presence of the hydroxyl ions in alkaline media has the ability to induce modifications of chemical bonds and to disrupt hydrogen and covalent bonds. The alkaline breaking of hydrogen bonds between AX and cellulose and of ester links loosens the linkage between AX and the other cell wall components and eases the liberation of AX from the matrix (Cyran *et al*., 2004; Fincher and Stone, 1986). The temperature of the alkali greatly affects the rate of the hydrolysing reaction and the extraction yields. Bergmans *et al*. (1996) reported an increase of the extraction yield of AX from wheat bran from 29% at 20°C up to 50% at 95°C using barium hydroxide. However, the elevated temperature seemed to decrease the extraction yields with other alkalis, due

to the hydrolysis of the linkages between xylose units in AX and the resulting degradation of the AX chains. By contrast, the concentration of the alkali used did not show a great effect on the extraction yields; elevation of the sodium hydroxide concentration from 0.25 to 0.5M was reported to have no effect on yields (Ayala-Soto *et al*., 2016).

The nature of the alkali used for the extraction affects the extraction yield (Chanliaud *et al*., 1995). Bergmans *et al*. (1996) demonstrated that substitution of barium hydroxide with calcium or sodium hydroxide slightly reduced the yield of the extraction. Among various alkalis examined, barium hydroxide conferred relatively pure AX extracts with relatively high yields (Bergmans *et al*., 1996; Gruppen *et al*., 1991). The mechanism causing this selective action is not well explained, it was assumed that the barium ions prevent the degradation of β-glucans and they selectively interact with pentoses (Fincher and Stone, 1986; Gruppen *et al.,* 1991)

Alkaline extraction is usually associated with lignin removal or degradation by oxidation using dilute concentrations of hydrogen peroxide (Maes and Delcour, 2001). Vinkx and Delcour (1996) reported that the oxidation activity might also help solubilising recalcitrant materials. Furthermore, under the oxidation conditions, AX chains may undergo non-enzymatic oxidative gelation; inducing the oxidative coupling of ferulic acid residues may result in the formation of ferulate dimers cross-linking the AX molecules (Vinkx and Delcour, 1996). Oxidative conditions significantly elevate the yields of alkaline extraction. It was reported that a hydrogen peroxide concentration of 2% at a pH of 11.5 for four hours at 60°C was sufficient to recover 77% of the total arabinose and 65% of the total xylose of the starting wheat bran (Maes and Delcour, 2001). Increasing the hydrogen peroxide concentration showed no effect on the yield.

5.2.1.3 Acid extractions

The conversion of carbohydrates in acidic conditions into unpleasant compounds – from a food chemist's point of view – such as furfural aldehyde products has hindered to some extent the expansion of this route of extraction (Almeida *et al*., 2007; Pedersen *et*

al., 2010). Particularly under strong acidic conditions (*e.g*. high temperature and acid concentration) the furfural products might undergo a conversion reaction to produce unpleasant compounds such as formic and levulinic acids (Larsson *et al*., 1999). However, a formulation of formic acid, acetic acid and H_2O in a ratio of 30:60:10 effectively yielded 76.5% of wheat straw hemicellulose (Xu *et al*., 2006), which is comparable to the results of alkaline oxidative extraction (Maes and Delcour, 2001).

5.2.1.4 Enzyme extraction

Naturally, a variety of enzymes are capable of hydrolysing polysaccharides into lower molecular weights or shorter chains or even all the way to their constituent monosaccharides (Dekker, 1985). Other enzymes are capable of mediating the breakdown of the connections between polysaccharides and other components of plant cell wall, or hydrolysing the side chains of polysaccharides and weakening the connection of AX to other components.

Xylanases degrade the xylan backbone in a random manner resulting in a general decrease in the degree of polymerisation (Araki and Kitamikado, 1982; McCleary *et al*., 1982). Xylanases are one group of endogenous enzymes in cereals (Bergmans *et al.,* 1996; Cleemput *et al.,*1997; Kulp, 1968; Moore *et al.,* 1990; Preece and MacDougall, 1958; Slade *et al.,*1989; Taiz and Honigman, 1976). Endo-β-(1,4)-xylanases (EC 3.2.1.8) are a major group of xylanases and the most studied xylanase (Escarnot *et al*., 2012); belonging to glycan-hydrolysing families GH10 and GH11 (Biely *et al.,* 1997; Henrissat, 1991). Xylanases have been a successful tool for the extraction of AX as they can attack the polysaccharide main backbone and release (solubilise) portions of WUAX (Courtin and Delcour, 2001). GH11 xylanases appeared to be more effective than GH10 for the extraction of AX from wheat bran, with yields of 41% and 18% respectively (Maes *et al*., 2004). This difference in activity was explained by two theories: GH11 xylanases have the ability to reach the water-insoluble AX more than GH10 (Maes *et al*., 2004), or they might have greater penetration ability inside the cell wall matrix (Beaugrand *et al*., 2004).

The association of other enzymes, such as feruloyl esterase, with xylanases for the extraction of AX has been reported as a successful strategy (Faulds *et al*., 2003; Kroon *et al*., 1999). Feruloyl esterases break down the ester linkage between AX and ferulic acid, which reduces the attachment of AX to other cell wall components such as lignin, and helps degrade the di-ferulate cross-linkages between AX chains (Faulds *et al*., 2002; Faulds and Williamson, 1995).

In the same context, the cellulose hydrolysing enzyme, cellulase, has been reported to increase the yield of enzymatically extracted AX when used in combination with endoxylanases, the synergistic effect of this combination possibly a result of the simultaneous release of both cellulose and AX from the cell wall matrix (Escarnot *et al.,* 2011; Murashima *et al.,* 2003; Petit-Benvegnen *et al.,* 1998). Furthermore, α-L-arabinofuranosidases are responsible for "shaving" or removing the arabinose side branches attached to the main backbone, thereby exposing greater unsubstituted xylose regions (Beldman *et al.,* 1996). Due to this effect, the xylan chain will be more vulnerable to xylanase attack and solubilising activity (Figueroa-Espinoza and Rouau, 1998). Surprisingly, the combination of different xylanases together did not enhance the yields of released AX (Beaugrand *et al.,* 2004; Escarnot *et al.,* 2012; Morgan *et al.,* 2017).

In general, enzymatically extracted AX has a lower A/X ratio, which can be explained by the xylanase preference to attack and release the unsubstituted, or less substituted, xylan chain regions, whereas the highly branched regions remain attached to the matrix of the cell wall (Beaugrand *et al.,* 2004). Also, the overall liberation yields are much lower compared to the alkaline hydrogen peroxide method (Zhou *et al*., 2010).

5.2.2 Mechanically assisted extraction

Fragmentation of the plant cell wall, to enhance AX extraction process, has been the aim of many attempts to apply physical and mechanical technologies. The applied methods aim to force open the rigid texture of the cell wall to expose and/or release its constituents.

5.2.2.1 Ultrasound treatment

The ultrasound technique is based on the application of acoustic energy and solvents to facilitate the release of cell wall components into the solution. The mechanism is based on the creation of fluctuating pressure which propagates throughout the substrate (Reis *et al.,* 2015); the pressure fluctuation generates microscopic and unstable bubbles, which collapse immediately, creating high shear forces against any adjacent material and, as a result, cavitate the material. The temperature in the vicinity of the cavitating bubbles also increases (Ebringerová and Hromádková, 2010). The cavitation phenomenon is capable of enhancing the process of AX extraction when combined with enzymatic or alkaline pathways (Ebringerova *et al*., 2002; Minjares-Fuentes *et al*., 2016; Wang *et al*., 2014). The ultrasonic-assisted extracted AX was reported to show modified functional properties such as weak gel properties and lower intrinsic viscosity (Ebringerova and Hromadkova, 1997; Sun *et al*., 2002). These functional modifications are primarily explained by the smaller Mw of the extracted AX; it appears that ultrasound not only releases AX from the matrix but also degrades the AX polymers into shorter polymer units (Ebringerova and Hromadkova, 1997).

5.2.2.2 Steam explosion

At the beginning of the twentieth century, Mason (1928) was granted a patent for an apparatus for the disintegration of woody fibrous materials. Simply, the substrate is exposed to highly pressurised steam, then after a certain period of time, the pressure is expeditiously decreased, leading to a substantial breakdown of the structure of the lignocellulosic material (Avellar and Glasser, 1998; Cara *et al.,* 2006; Josefsson *et al.,* 2002). Steam explosion is considered an environmental-friendly and effective pretreatment to degrade biomasses. Steam explosion effectively degrades the interpolymeric phenolic cross-linkages, which leads to the general loosening of the cell wall structure (Merali *et al*., 2015). However, the powerful steam explosion impact largely depolymerises AX into the constituent oligosaccharides or even monosaccharides (Sun *et al*., 2005). This degradation is the main drawback of steam

explosion for AX fibre extraction, as it significantly affects the functional properties of the extracted AX.

5.2.2.3 Extrusion

The thermo-mechanical treatment of extrusion is carried out by applying high pressure, temperature and mechanical shear (Honců *et al.,* 2016). The implication of extrusion in AX extraction was investigated. The chemical reactions that occur during extrusion change the properties of the constituent polymers of the material; the breaking down of the side chains and the general depolymerisation notably increase the solubility of the AX chains. Extrusion exhibits many advantages such as short treatment time, low water consumption, high adaptability, productivity and environmental and cost-effective process (Jacquemin *et al*., 2012; Zeitoun *et al*., 2010). However, reported yields of AX from extrusion-based processes are relatively low with low purity (Zeitoun *et al*., 2010).

5.2.3 Purification of AX

The extraction of arabinoxylan from the cell wall structure is based mainly on the liberation and solubilisation of AX polysaccharides into the extraction medium. Unfortunately, this non-selective extraction process acts on other components of the cell wall in the same manner. Substantial amounts of protein, cellulose, lignin and other constituents are extracted as well. Hence, purification of the crude AX extracts is usually required. Generally, extract purification involves the de-activation of the enzymes applied during the extraction procedure and the employment of hydrolysis enzymes to break down the residual starch and protein in the crude extracts, after which the extracts are precipitated and dialysed to obtain the final AX-rich extracts (Saeed *et al.,* 2011; Zhang *et al.,* 2014).

5.2.3.1 Amylase and protease purification

Dervilly *et al.* (2000) successfully employed α-amylase and β-glucosidase for the degradation of residual starch in the AX extracts, leading to the partial removal of the starch from the extracts. Likewise, protease was employed to decrease the protein content in the extracts (Bell, 2015; Dervilly *et al.,* 2000; Izydorczyk and Dexter, 2008; Linares-Pastén *et al.,* 2017).

5.2.3.2 Concentration

Membrane dialysis is one of the most common purification procedures used in a laboratory (Preece and Hobkirk, 1953; Peng *et al.,* 2012). Ultrafiltration is an effective concentration method as it can be used at bench, pilot and commercial scales (Krawczyk *et al*., 2008). In previous (unpublished) work in the University of Huddersfield, following guidance from Hollmann and Lindhauer (2005) and Bell (2015), ultrafiltration was employed and successfully reduced the volume of the extracts (prior to the ethanol precipitation) to one-fifth the original volume, thereby significantly reducing the amount of ethanol used for precipitating AX and hence the cost and safety hazards. Jacquemin *et al.* (2015a) note that "the problems [of] alcohol recycling (costs and security) hinder the industrial development of xylan production". Therefore, minimising the cost of ethanol usage for AX production is key to successfully bringing new AX-based products to market (Martinez-Hernandez *et al.,* 2018), and ultrafiltration is therefore a key technology for economically viable production of AX.

Meanwhile, diafiltration – dialysis by filtration – is one of the most attractive procedures for the purification of the extracts; the injection of pure deionised water to replace the permeate effectively reduces the non-organic content of the extracts and eliminates the content of the small organic molecules that can pass the ultrafiltration membrane pores. This increases the concentration of the larger molecules (Jacquemin *et al.,* 2012, 2015; Laine *et al.,* 2015).

5.2.3.3 Precipitation

After extraction and purification, AX is recovered from the liquid extract by precipitation. Ethanol had been successfully employed to precipitate AX from the liquefied extracts; as noted throughout this thesis, the ready availability of ethanol is the basis for the economic feasibility of AX production in an integrated biorefinery. The concentration of ethanol used for the precipitation significantly affects the properties of the final product. For example, it was reported that ethanol concentration affects the A/X ratio of the extracted AX; increasing the ethanol concentration usually yields a higher ratio of A/X (Gruppen *et al*., 1992; Viëtor *et al.*, 1992). Bian *et al*. (2010) investigated in detail the effect of ethanol concentration on AX precipitation by performing a series of gradual concentrations of ethanol (10, 20, 30, 45, 60 and 80%) and observing the properties of the precipitated fractions. Variations in the branching distribution patterns were reported according to the ethanol concentration. Large molecules of AX with low branching were obtained first at low concentrations of ethanol. Whereas, increasing the ethanol strength caused the low Mw AX with higher branches to start to precipitate (Bian *et al*., 2010). Martinez-Hernandez *et al.* (2018) showed how a range of products precipitated at different ethanol concentrations increased the scope for bioethanol pinch analysis to minimise ethanol usage. Underlining how producing a portfolio of AX-based products, with different properties and end-use applications, is the way forward to creating economic processes and bringing affordable products to market.

Another precipitation technique, salting-out, has also been employed for AX recovery; it is believed that adding salt in high concentration to the solution might result in attracting the water molecules to the salt ions, hence decreasing the amount of water available to interact with the polymer. Izydorczyk *et al*. (1992) demonstrated the usage and influence of ammonium sulphate concentration on AX by adding gradually higher concentrations of the salt in the precipitation process (60, 70, 80 and 95%). The results were similar to the ethanol concentration effect, where increasing the concentration of the salt increased the A/X ratio of the resultant AX and precipitated smaller molecules

of AX (Izydorczyk and Biliaderis 1992). However, salting out does not lend itself as naturally as ethanol precipitation for integration into a bioethanol plant.

5.3 Pilot-scale extraction of AX

The lack of commercial sources of arabinoxylans has been the major barrier to establishing their potential role as food ingredients or for other applications (Courtin and Delcour, 2002; Hollmann and Lindhauer, 2005; Du *et al.,* 2009). The majority of investigations reported in the literature describing the structure, properties and potential uses of AX have been performed on bench-scale extracts. These small-scale experiments have allowed a profound understanding of AX properties and potentials. However, the utilisation of AX in food, feed or pharmaceutical investigations requires larger amounts (kg-scale quantities). Also, the characteristics of small-scale extracts might vary from those from scaled-up processes (Annison, Choct, and Cheetham, 1992). Therefore, several attempts have been made to extract AX at larger scales.

Annison *et al.* (1992) reported one of the earliest trials for the extraction of wheat bran pentosans on a large scale. The procedure employed an amylase and protease treatment to remove the starch and protein content of the bran, followed by an alkaline extraction using 0.2 M sodium hydroxide at 80°C for two hours. Thereafter the liquefied AX were separated from the solids by centrifuging. Finally, AX was precipitated by ethanol at 80%. The extraction resulted in 1200 g of AX-rich extract from 15 kg of wheat bran, a yield of 8%, with a purity of about 86%. The apparent Mw of the extract was around 500 kDa with a high polydispersity profile ranging between 5.3 – 758 kDa.

Hollmann and Lindhauer (2005) extracted AX from wheat bran in a study designed to demonstrate commercial feasibility in terms of the safety and efficacy of the materials and processes used. Five kg batches of milled (0.5 mm) wheat bran were boiled in 70% ethanol solution at 80°C for 4 hours. The washed bran was recovered using a 0.4 mm sieve and air dried. WEAX was removed by suspending 3.5 kg of the washed bran in 40 L water and heated to 40°C for 2 hours to remove coloured contaminants, fat and low Mw carbohydrates, and also to deactivate endogenous enzymes. For the extraction

of AX, 2 kg of the washed and WEAX-free bran was suspended in 40 L of 2% hydrogen peroxide solution, adjusting the pH to 11 with sodium hydroxide, and holding for 4 hours at 40°C. Thereafter, the extract was filtered, treated with protease then concentrated by ultrafiltration using a membrane with a molecular weight cut-off of 10 kDa. AX was precipitated by 65% ethanol; the collected extract was treated with β-glucosidase then exhaustively dialysed and finally freeze-dried. By the end of the procedure, 350 g (a 7% yield) of 80% purity AX was obtained, with Mw around 100-110 kDa.

Jacquemin *et al.* (2012) extracted AX from a mixture of wheat straw and wheat bran in a ratio of 6.2:1. A slurry of 86 kg material was mixed with 5.8 kg sodium hydroxide, and water (at liquid/solid ratio of 10) and injected into a co-penetrating and co-rotating twin-screw extruder. The liquids were separated by centrifugal filtration, the filtrate was concentrated by ultrafiltration using a membrane with a molecular weight cut-off of 30 kDa. The concentrated supernatant was passed over strong anion-exchange resin, then AX was precipitated using 65% ethanol. The reported yield was 33.7% with a total sugar content of 50%.

Arabinoxylan extraction requires a lengthy procedure of numerous steps, with various reports of procedures for the extraction and for the number and range of subsequent purification steps. The obvious common obstacle in all the proposed procedures is the extensive consumption of ethanol, which makes the extraction of AX expensive and hazardous. This has hindered the development of routine AX extraction to produce reasonable amounts for technological studies or for implementation at commercial scale.

Therefore, this part of this project aimed to extract AX on a larger (pilot) scale, to be used for the oligosaccharide production for animal feed trials within bioethanol biorefinery where ethanol can be used for the extraction, recovered by distillation and re-used again.

5.4 Materials and methods

5.4.1 Chemicals and enzymes

Sodium hydroxide pellets, hydrogen peroxide (>30%w/v), sulphuric acid 98% and trifluoroacetic acid 99.5% were purchased from Fisher Scientific UK Limited. The antifoaming agent used was Dimeticon SILFAR® SE 4 made by Wacker Chemie AG, Germany, and provided kindly by IMCD Group, UK. High viscosity wheat arabinoxylan was purchased from Megazyme (Bray, Ireland).

The enzymes used for the purification, α-amylase and gluco-amylase, were purchased from ReKru GmbH, Germany. Cellulase from *Aspergillus sp.* (C2605) and protease from *Bacillus licheniformis* (P4860) were purchased from Sigma Aldrich, UK.

5.4.2 Equipment

The extraction was carried out using the double jacketed mash tank of the GUNT CE-640 bioethanol plant shown earlier in Figure 3-4. Extracts were centrifuged using a Beckman centrifuge GS-6S with swing bucket rotor and capacity of 4 × 750 ml (Beckman Coulter Ltd., UK), shown in Figure 5-1.

Figure 5-1. Beckman centrifuge GS-6S.

For the concentration of the samples, a hybrid in-house assembled ultrafiltration system was employed as detailed below. Extracts were dried in a Christ Freeze Dryer Alpha 1-

4 ld plus (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Samples were prepared for the analysis using Zymark Turbovap® LV sample concentrating unit, MA, USA.

For the molecular weight evaluation, size exclusion chromatography (SEC-MALLS) was used, comprising a Shimadzu HPLC system (Shimadzu U.K. Ltd, Milton Keynes, UK) connected to three columns in series: Aquagel-OH 40, 50 and 60 (15 µm particle size, 25 cm × 4 mm, Agilent, Oxford, UK). The detection system comprised three detectors: UV/VIS detector SPD-20 A, differential index detector (Optilab rEX) and multiangle laser light scattering (MALLS) detector (mini-DAWN, Wyatt Technology, Santa Barbara, CA, USA). The eluted mobile phase was 50 mM NaNO3 (containing 0.02% NaN3 as a preservative) at a flow rate of 1 mL min^{-1}. LC Real Time® software was used for the integration and analysis of the chromatographs.

For sugar analysis, a Dionex ICS–3000 high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex Corporation, CA, USA) was used.

For concentrating the samples, two ultrafiltration systems were available: a small-scale Sartorius® Vivaflow®200 system, and a larger Alfa Laval system, both shown in Figure 5-2. The Sartorius® Stedim Vivaflow®200 Ultrafiltration system (Sartorius Stedim Biotech, Göttingen, Germany) uses cross-flow membrane cassettes each with a membrane surface area of 200 cm^2 and 10 kDa molecular weight cut-off. The system is equipped with a Masterflex® Easy-Load® peristaltic pump. The second system is an Alfa Laval LabStak® M10 ultrafiltration system (Alfa Laval Ltd, UK). The LabStak® M10 has a cross-flow flat sheet membrane with a surface area of 336 cm^2 and 10 kDa molecular weight cut-off, and an Eco® GC4-KDTTTUG gear pump.

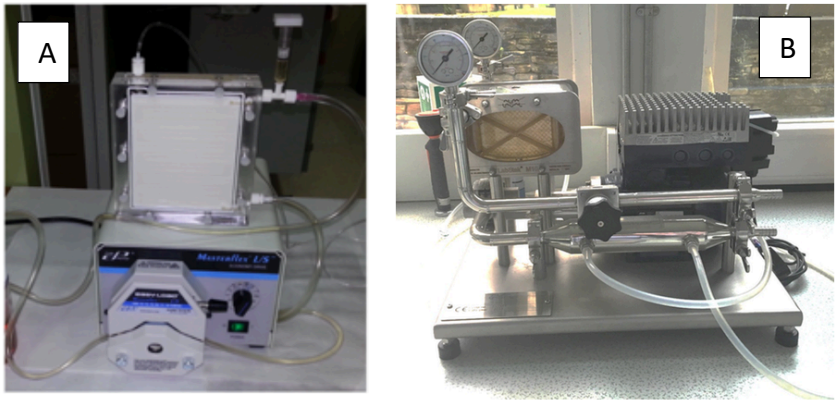

Figure 5-2. Ultrafiltration systems: (A) Vivaflow, (B) LabStak.

Both ultrafiltration systems were designed to process small samples on bench scale with maximum filtration rate around 200 mL h^{-1}. For pilot-scale extraction, the average sample volume is around 30 litres, therefore, concentrating the sample to one third would take at least two weeks of continuous operation. Looking at both systems, the Vivaflow system has high quality membrane cassettes coupled with a peristaltic pump of limited capability, whereas the Alfa Laval system is coupled with a powerful gear pump and a membrane cassette of a small surface area which limits the performance of the system.

Therefore, utilising the Eco® gear pump to feed four Vivaflow cassettes at the same time, as seen in Figure 5-3 and Figure 5-4, was implemented to scale up the ultrafiltration system and increase the overall performance. A pressure gauge and a safety pressure relief valve were attached to the new system in order to control the pressure and protect the membranes from high pressure load. All the connectors, manifold, valves and tubes needed to assemble the hybrid system were purchased from Amazon, UK.

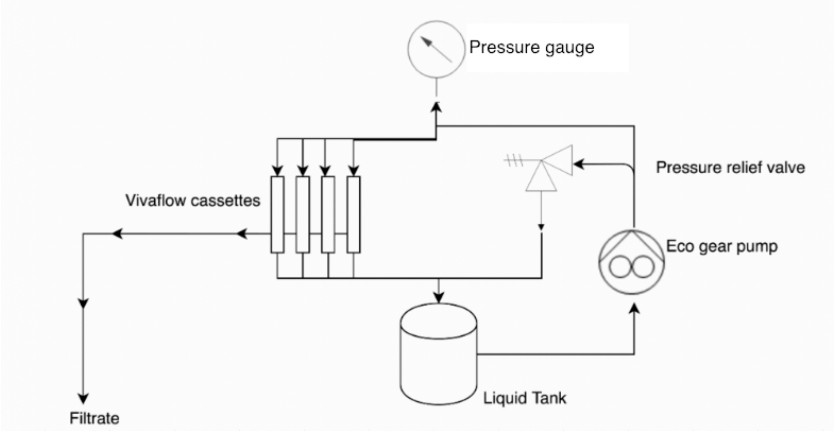

Figure 5-3. Design of the hybrid ultrafiltration system.

Figure 5-4. Assembled ultrafiltration system.

In this part of the project, two extraction batches are presented; for the first batch, two Vivaflow systems were used simultaneously, while for the second batch the hybrid system with four Vivaflow cassettes was employed.

5.4.3 Arabinoxylan extraction method

Arabinoxylan was extracted from the fresh DWG by the alkaline oxidative method; two batches of AX extracts are presented in this work (AXa and AXb), varying in the ultrafiltration system used and the subsequent purification employed. Both extraction batches were performed according to an extraction procedure adapted from Hollmann

and Lindhauer (2005) with modifications to accommodate the starting material and the available laboratory facilities.

The extraction procedure for both AXa and AXb was initiated by suspending 10.1 kg (2.55 kg db) DWG in 25 L of water and heating to 50°C in the double-jacketed mash tank of the CE-640 plant. Alpha-amylase, gluco-amylase and cellulase were added at dosages of 2 mL, 4 mL and 5 mL, respectively, and the mixture incubated at 50°C for 6 hours. The pH was adjusted to 6.5 during this incubation. Protease was added at a dose of 8 mL after adjusting the pH to 8; the incubation continued at 50°C for another 10 hours. Sodium hydroxide solution (40%) was added to raise the pH to 11, the pH was monitored during the extraction process, and alkaline solution was added when needed to maintain the alkali conditions. The temperature was increased to 60°C using direct steam injection, and 10 mL of the anti-foaming agent was added. Hydrogen peroxide was added slowly at a rate of 100 mL every 5 min, to avoid excessive foaming, while maintaining vigorous stirring. A total of two litres of 30% hydrogen peroxide was added over a period of about 3 hours; the extraction was continued for a total of 4 hours.

At the end of the extraction, the mixture was cooled to 25°C and neutralised with concentrated sulphuric acid. The whole slurry was centrifuged, supernatants were collected, the solids were washed with 5 L deionised water, the mixture was centrifuged, and the supernatant was poured into the previously collected supernatant.

For the first batch, AXa, the liquids were ultrafiltered using two Vivaflow systems over a 10 kDa membrane to reduce the total volume to one third, *i.e.* 27 L of filtrate was concentrated to 9 L. Thereafter, a sufficient amount of industrial methylated spirit (18 L) was added to give a final concentration of ethanol of 65% and left overnight at 4°C to precipitate the AX. The precipitated AX was recovered by centrifugation, freeze-dried and stored in a plastic bag. Figure 5-5 summarises the extraction procedure.

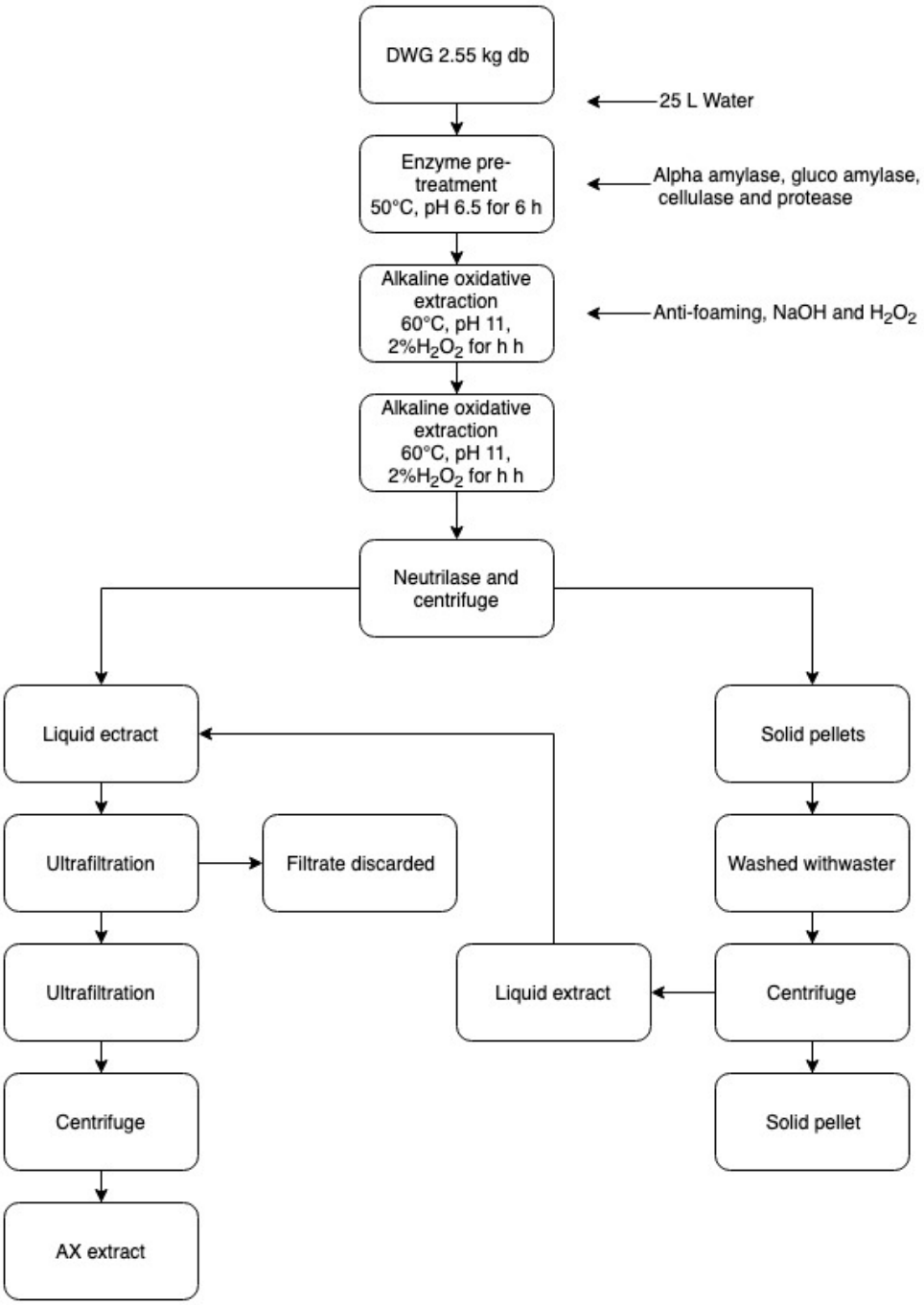

Figure 5-5. AXa extraction process.

For the second batch, AXb, the extract solution was concentrated using the hybrid ultrafiltration system, which was slightly faster than the original Vivaflow system. After concentrating the extract, AX was precipitated by ethanol, centrifuged and dried in an oven at 40°C overnight. Thereafter, the extract was dissolved in 10 L of deionised water and enzymatic treatments utilising amylase, protease and laccase were applied and

followed by ultrafiltration, ethanol precipitation, centrifuging and oven drying. Figure 5-6 shows the purification steps applied.

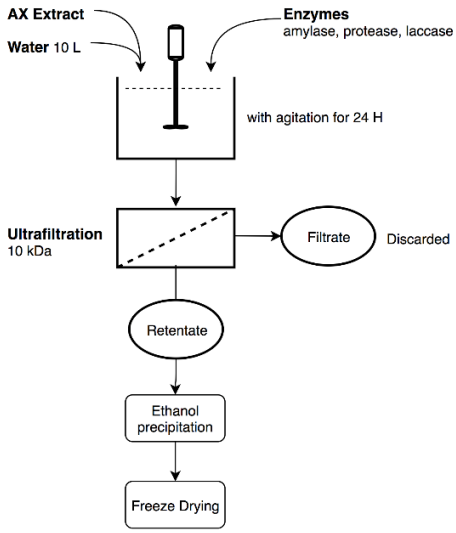

Figure 5-6. AXb extract purification method.

5.4.4 Characterisation of the extracts

Due to the strict time scale of the animal feed trials carried out by the academic partner in Nottingham Trent University, batches were characterised differently as follows:

For the first extract, AXa, the constituent sugar analysis was performed by Englyst Carbohydrates Ltd. The Kjeldahl analysis method was used for protein content (Section 3.6.3), and the ash content was determined as described in Section 3.6.4.

For the second extract, AXb, protein, ash and total carbohydrate content were analysed using the same methods used for AXa. The average molar masses (Mw, weight average molar mass and Mn, number average molar mass) were evaluated by size exclusion chromatography (SEC). The extract was solubilised in 50 mM NaNO3 solution (the mobile phase) at a concentration of 3 mg mL^{-1} under continuous stirring overnight at room temperature. The sample was injected into the system after calibration with dextran of known molecular weights, and weight average molar mass and polydispersity index calculated.

The constituent sugars analysis was performed utilising the HPAEC-PAD system. The sample was solubilised in ultrapure water (at a concentration of 2 mg mL^{-1}) under continuous stirring overnight at room temperature. Precisely 1 mL of the solution was pipetted into a pressure tube (Ace glass), trifluoroacetic acid was added to make the acid concentration equal to 4 M, the tube was closed tight and moved to a heat block at 120°C for two hours. The tubes were left to equilibrate to room temperature, then the contents were quantitatively moved to 15 mL centrifuge tubes and dried under a continuous compressed air flow at 60°C using a Turbovap® evaporator. After complete drying, the sample was re-dissolved in MilliQ water, filtered over a 0.45 µm syringe filter and injected into the chromatography system.

In order to calculate the recovery of the hydrolysis method, arabinoxylan high viscosity standard (Megazyme) was used to spike the sample at three levels of concentration (25, 50 and 75 ppm), hydrolysed according to the above method and analysed by the HPAEC system.

5.4.5 Enzyme treatment

Extract AXb was subject to enzymatic treatment with Econase to investigate the potential of XOS production from the chemically extracted AX from the Distillers Wet Grain. The extract was solubilised, Econase was added at four dosages 0X, 1X, 10X and 100X where X is 50 mL per kg, pH was adjusted to 5.5, and the incubation lasted for one hour in a shaking water bath at 55°C. At the end of the incubation period, samples were submerged in an ice bath to stop the enzyme activity, freeze-dried and submitted to IPOS for XOS analysis.

In order to investigate the branching (substitution) effect on the enzyme activity, a solubilised sample of the extract was subject to arabinofuranosidase activity for 24 hours. Then four samples were prepared following the same steps described above.

5.5 Results and discussion

5.5.1 Extracts characterisation

Extracts AXa and AXb were 900 g and 420 g, respectively, and almost identical in appearance (odourless with a non-crystalline starchy appearance after milling, and white in colour), shown in Figure 5-7.

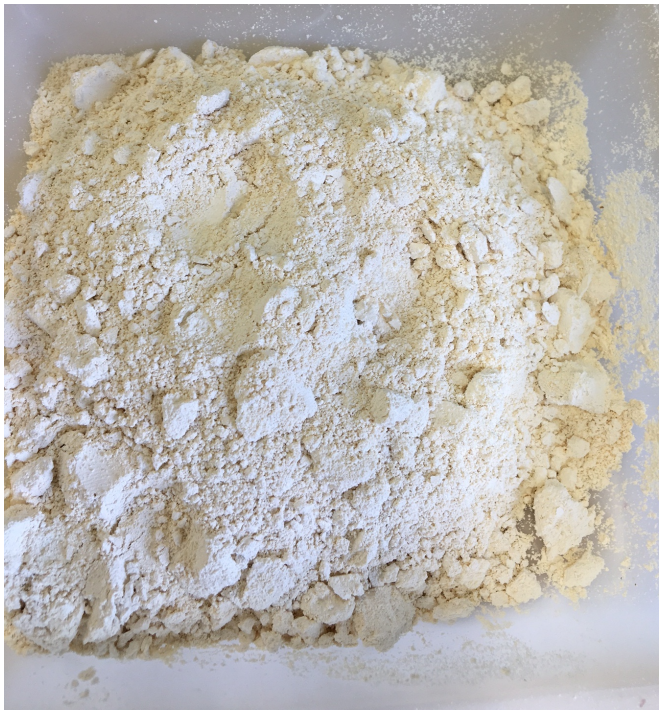

Figure 5-7. AXa extract after milling.

Protein contents were 14.00 ± 0.15 and 10.00 ± 0.20% for AXa and AXb, respectively, giving a total amount of protein of 126 g in AXa and 42 g in AXb. Evidently the purification treatment successfully decreased the amount of the protein in AXb to one third of the total protein in the un-purified extract (AXa), although other components were also removed, such that the protein concentration only decreased from 14% to 10%. The prolonged protease treatment had been expected to eliminate the protein content in the extract by degrading the total protein to smaller molecules that can pass the ultrafiltration membrane's pores; evidently the degradation was incomplete even after 24 hours. Apparently, the protease was incapable of degrading the proteins; this could be a result of the wheat protein denaturation during the bioethanol process (*i.e.*

the distillation). Both De Vries *et al*. (2013) and Schroeder (2012) reported major changes in the nutritional value of DDGS proteins and the increased amount of undegradable proteins in the DDGS. These changes were attributed to the drying process of the DWG to the DDGS, but the current results suggest these changes in protein digestibility occur partially during the ethanol distillation prior to the DDGS drying.

The ash content in the extracts was 40.08 ± 0.11% for AXa and 13.75 ± 0.38% for AXb. The huge ash content is from sodium sulphate resulting from the NaOH used in the extraction and the sulphuric acid used for the neutralisation. The purification steps applied in the second extraction, ethanol precipitation and diafiltration, significantly reduced the amounts of salt in the extracts from 360 g in AXa to 57.75 g in AXb. The ethanol precipitation step had a minor effect of the salt content as sodium sulphate is ethanol insoluble and precipitates in the 65% ethanol solution along with the AX. Hydrochloric acid should be used for the neutralisation in future subsequent extraction processes as it is more soluble in alcoholic solutions (Farelo *et al.,* 2004).

The constituent sugars analysis of the AXa extract by Englyst Labs showed an arabinose content of 8.7 ± 0.1% and xylose 11.1 ± 0.1%, the total amount of AX is calculated as 19.5 ± 0.2% of the extract. This represents about 175 g of the total 900 g extract. Considering that the amount of AX in the starting 2.55 kg DWG was 16.6% (db), corresponding to 423 g, the yield of the extraction can be estimated at 41% of the available AX in the DWG. Moreover, the AX content in the original wheat is 5.9% (713 g), this means that the extraction yielded 25% of the total AX in original wheat.

The second extract, AXb, was analysed using the HPAEC-PAD system. The analysis showed an arabinose content of 21.4 ± 0.2 % and xylose content of 24.3 ± 0.11%. The total amount of AX was 44.4 ± 0.3 % of the extract, which represents about 186 g of the total 420 g extract. This is slightly higher than the amount of AX in the first extract and represent 43.6% of the available DWG AX and 26% of the original wheat AX.

Clearly, the purification process did not reduce the total amount of AX recovered, which indicates the stability of the extracted AX and suggests further treatments could be applied to increase the purity further without compromising the absolute yield.

Traditionally, the first step in the analysis of polysaccharides is the breakdown of polysaccharides into monosaccharides, for which acidic hydrolysis had been the method of choice. Countless reports in the literature have used acidic hydrolysis for polysaccharide analysis, using an unlimited variation of conditions: hydrolysis temperature, acid concentration, hydrolysis time and, most importantly, different substrates (biomass and extracts), mostly with minimal justification for the conditions used. In general, the acid hydrolysis process can be summarised in three major steps: the breakdown of the biomass structure to release the major components (the intermolecular bonds); hydrolysing the biomass component into its basic units (the intramolecular bonds); and the degradation of the resultant units (monosaccharides into furfural products). Acid hydrolysis of polysaccharides is therefore a balance between the first two steps, the producing steps, and the third degrading step. This balance has been for a long time the "elephant in the room" due to the impact of the variation in the hydrolysed material structure, composition and degree of polymerisation on the kinetics of the hydrolysis (Ranganathan *et al.,* 1985; Puls, 1992; Willför *et al.,* 2009; Banerjee, 2014). As a wider range of polysaccharides such as AX become of increasing scientific and commercial interest, the issue of hydrolysis for reliable sugar analysis is going to become increasingly important.

Moreover, preliminary results of analysing the same AX extract sample before and after the xylanase treatment following the same hydrolysis procedure showed a huge difference in the detected amount of AX, which also emphasises the impact of the degree of polymerisation of the polysaccharide on the degradation of monosaccharides during the hydrolysis. Englyst *et al.* (1994) and Sluiter *et al.* (2008) studied the recovery of the acidic hydrolysis using standards of monosaccharides. This ignores the matrix structure of the carbohydrate as the rate of degradation of the added monosaccharides will be different from the original constituent monosaccharides which first have to be

released. According to Willför *et al.* (2009) "there is only little sense in calibrating with monosaccharides".

Fortunately, the recent availability of commercial AX pure standards with a variety of molecular weights has enabled the relatively accurate estimation of hydrolysis recovery by spiking the sample with a similar pure AX polymer. Using this approach, the calculated recovery in the current work was estimated at 90.8% of the hydrolysed arabinoxylan. Table 5-1 shows the calculated recovery of the hydrolysis method from triplicate samples. Clearly, there is a minor effect related to the amount of the AX in the sample. At all levels of added AX, the recovery was consistently 90.8%; evidently, there was a negligible effect of the amount of the AX in the sample, which reflects the effect of polysaccharide quantity on the kinetics of the hydrolysis.

Table 5-1. AX recovery following hydrolysis (IPOS).

Original sample concentration ppm	AX standard added ppm	Theoretical concentration ppm	Detected concentration ppm	% Recovery
20.14	0.00	20.14	20.14	—
20.14	25.00	45.14	42.84	90.81 ± 0.15
20.14	50.00	70.14	65.54	90.80 ± 0.01
20.14	75.00	95.14	88.22	90.78 ± 0.01

The molecular weight of AXb, measured using SEC-MALLS, showed an average Mw of 234.7 ± 0.009% kDa. The calculated polydispersity index (Mw/Mn) was 1.6 ± 0.013% kDa, which indicates a wide distribution of the molecular sizes in the extract. The average Mw was nearly double that reported by Hollmann and Lindhauer (2005), who extracted from washed wheat bran after the gentle removal of WEAX at 40°C, whereas the extraction was from DWG in the current project. Clearly there are numerous possible reasons for the difference, but it is reasonable to suppose that the distillation process (continuous boiling for 4 hours) was able to release a greater amount of small molecules of AX into the Solubles and washing them out than the less severe washing procedure reported by Hollmann and Lindhauer (2005).

5.5.2 Enzymatic treatment results

In order to explore the suitability of the DWG AX for the production of arabinoxylan oligosaccharides, samples of AXb were incubated with Econase then sent to IPOS to run their DPn method and estimate the content of the produced XOS. Theoretically, the produced extract should contain more available AX for the enzyme activity than the content of the raw material. Therefore, Econase was applied in three levels of dosages: 1× (the base level of 50 mL per kg), 10× and 100×, plus a 0× blank sample.

Table 5-2 presents the XOS percentage released from the AX extracts after the Econase treatment. Surprisingly, the enzyme showed only very small effects on the extract; at the 1× dose there was some evidence of production of the xylose monomer and xylobiose (DP2) and xylotriose (DP3), but no evidence of the higher oligosaccharides. The higher dosages increased production of DP2 and DP1, the latter unexpected as monosaccharide production should not be seen from an endoxylanase.

Table 5-2. XOS released from AXb after Econase treatment (IPOS).

%	DP1	DP2	DP3	DP4	DP5	DP6
0×	0.0	0.0	0.0	0.0	0.1	0.0
1×	2.1	0.4	0.1	0.0	0.0	0.0
10×	4.6	1.0	0.1	0.0	0.0	0.4
100×	24.8	5.9	0.2	0.0	0.4	0.0

This limited xylanase activity could be a result of the branching pattern of the extracted AX; the A/X ratio of 0.88 indicates highly branched molecules which would be less susceptible to enzyme attack, the arabinose side chains hindering access to the xylan backbone. Therefore, the extract was subject to treatment with arabinofuranosidase, an enzyme that acts to remove arabinose side chains. This “shaving” activity should expose the xylan chains and grant more access for Econase. Hence, the extract was treated for 24 hours with arabinofuranosidase at 40°C and pH 5, then treated with Econase under similar conditions to the previous treatment. The samples were coded as A0×, A1×, A10× and A100×, as shown in Table 5-3.

Table 5-3. XOS released from AXb after arabinofuranosidase and Econase treatment.

%	DP1	DP2	DP3	DP4	DP5	DP6
A0×	0.6	0.0	0.0	0.0	0.0	0.0
A1×	2.1	0.4	0.0	0.0	0.0	0.0
A10×	4.6	1.0	0.1	0.0	0.0	0.4
A100×	23.0	5.4	0.2	0.0	0.4	0.1

Clearly, pretreatment with the "shaving" enzyme had no effect on the Econase performance. The arabinofuranosidase treatment shows only a minor difference in the monosaccharides content in the first sample (A0×), compared to Econase treatment in Table 5-2, while the sample only treated with the arabinofuranosidase had almost the same monosaccharide content in the samples 100× and A100×. Since the analytical method used lacked the ability to distinguish the type of monosaccharide, it is not possible to determine whether the monosaccharides resulting from the treatment are arabinose from the arabinofuranosidase activity or xylose from the Econase activity.

Since only a minor xylanase effect was observed using the DPn method on the concentration of oligosaccharides released, samples 0X and 10X were analysed by SEC-MALLS in order to investigate the effect on the large molecules. Table 5-4 presents the molecular weight and polydispersity index for the AXb sample before and after the Econase treatment. Clearly, Econase acted to break down the large molecules; the decrease in the average Mw to about half, along with the increase of the polydispersity index, indicates the breakdown of the large AX polysaccharides into a wide range of smaller polysaccharides. So, it is evident that the Econase acted on AX molecules in the AXb extract, reducing molecular weight but with only a minor production of oligo-level saccharides. It is of course possible that the xylanase is very active towards these oligosaccharides, such that as soon as they are produced they are degraded.

Table 5-4. Molecular weight and polydispersity index for AXb before and after Econase treatment.

Samples	Mw (kDa)		Mw/Mn	
0X	234.7	± 0.009%	1.6	± 0.013%
10X	106.0	± 0.014%	2.1	± 0.020%

The change in the molecular weight distribution induced by Econase activity is clearly present in the cumulative molar mass elution profile of the extracts present in Figure 5-8. The graph shows a general reduction in the molecular weight of the sample components, as well as reduced homogeneity of the Mw distribution.

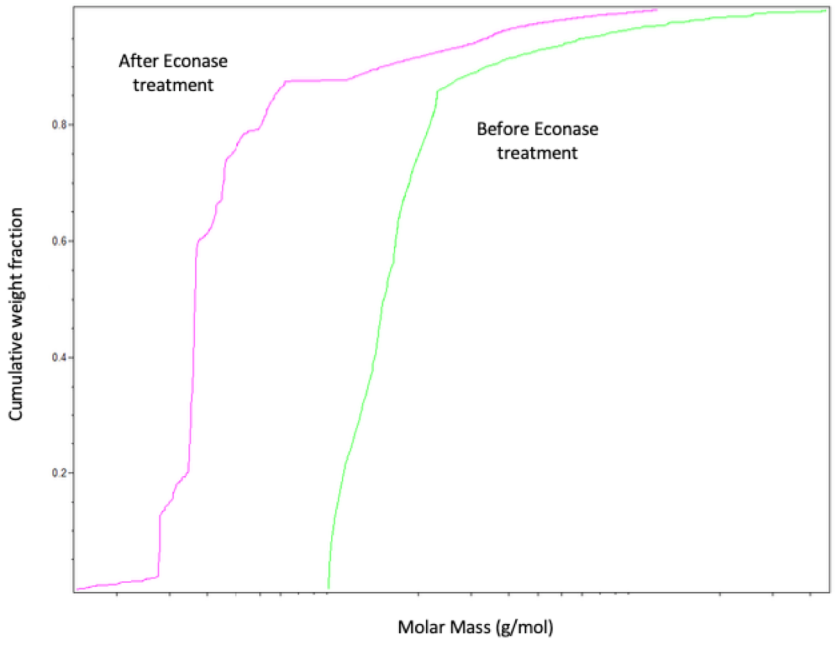

Figure 5-8. Cumulative molar mass of AXb before and after Econase treatment.

It is evident that Econase is performing on the extract, and it is plausible that more extended treatment could facilitate the degradation of AX to the desired oligosaccharides. However, the amounts of the monosaccharides produced along with the oligosaccharides would not provide the ideal oligosaccharide substrate for animal feed trials or it would require further purification steps. The inability to identify the monosaccharides produced limits the ability of the IPOS method to determine the enzyme activity pattern with any precision. These results also raise questions about the enzyme behaviour towards oligosaccharides; it is important to clarify whether the enzyme is producing and degrading the oligosaccharides to smaller oligos and all the way to monosaccharides. Such a question has not been addressed in the literature, as

the general understanding of endoxylanases is that they do not produce monosaccharides.

5.6 Summary

The extraction of AX from the bioethanol in-process material, DWG, was performed using the CE-640 plant to produce the DWG and to undertake the extractions. Two batches of AX were extracted to study the potential for XOS production using the commercial xylanase Econase. The first batch was sent to Nottingham Trent University to be tested in animal feed trials aimed at establishing the benefits of adding the AX into the diets of chickens. The second batch was subjected to several enzyme treatments aiming to produce significant amounts of XOS material. The preliminary experiments showed that Econase acted on the AX in the extract, as evidenced by the reduction in average molecular weight, but its activity appeared to be hindered by the high arabinose content. Econase could not produce XOS from the extracts as it had a high branching pattern.

The DPn chromatographic method has only been recently developed by IPOS, and the performance of this technique is not well established yet. Also, the developed method lacks the ability to distinguish the released mono- and oligosaccharides upon treatment with the enzymes. The identification of these saccharides is crucial to understand the effect of the enzymes on the extracted AX. Therefore, while the IPOS analyses allowed the project to move forward usefully at the time, the next step of this project was dedicated to developing and validating a new analytical method for the accurate screening of the enzyme degradation products.

6 Oligosaccharides analysis method development

6.1 Introduction

Arabinoxylan oligosaccharides (AXOS) and xylan oligosaccharides (XOS) are an evolving subject of interest due to their impact on human and animal health via prebiotic activity (Ou and Sun, 2014; Suwa *et al.*, 1999). The increasing interest in (A)XOS highlights the importance of establishing robust analytical procedures that can routinely measure and characterise these prebiotics in a variety of matrices; accurate measurement of the amount of prebiotics in food and feed is a prerequisite for their production and exploitation. A significant amount of research has been conducted using highly sophisticated instrumentation to characterise the conformation and structure of (A)XOS. Meanwhile, the recent availability of commercial high purity standards of these prebiotics has facilitated the development of several techniques for the measurement of (A)XOS. Chromatographic separation methods, including liquid and gas chromatography, have been applied for the separation and measurement of these oligomers. However, the currently available methods either require lengthy sample preparation steps, in the case of GC, or lack the resolution and the ability to measure both mono- and oligosaccharides simultaneously, in the case of HPLC. This chapter reviews the available analytical methods for the determination of (A)XOS in literature, then describes the development and validation of a new method for the simultaneous determination of 16 analytes including mono- and oligosaccharides and uronic acids.

6.2 Oligosaccharides analysis methods

Historically, the main challenges facing the development of methods for measuring carbohydrates were the lack of a high resolution separation method and the difficulty of detecting low concentrations of carbohydrates (Rocklin and Pohl, 1983). Classic analytical techniques used for oligosaccharide separation included paper, thin-layer, high performance thin layer, ion exchange and gel filtration chromatography (Gauch *et al.,* 1979; Voragen *et al.,* 1986). Now the fast growth of enzyme markets and the

increased demand for oligosaccharides is demanding more simplified, fast and accurate analyses that can be exploited in routine standard procedures. Table 6-1 summarises the methods reported in the literature for the analysis of oligosaccharides. Ion exchange methods with refractive index detection have been widely used, while High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) has become increasingly the method of choice.

Table 6-1. Analytical methods for the separation of oligosaccharides.

Method	Analytes	Mobile Phase	Stationary phase	Detection	Reference
Ion exchange	DP 2-4	Water	Aminex HPX-87P	Refractive index	Voragen *et al.* 1986
	DP 2-5	Water	Aminex HPX42A	Refractive index	Brienzo, Carvalho, and Milagres 2010
	DP 2-5	Water	RSO-oligosaccharides column Phenomenex	Refractive index	Sabiha-Hanim, Noor, and Rosma 2011
	DP 2-6	Water	Aminex HPX-42A	Refractive index	Uçkun Kiran *et al.* 2013
	DP 2-3	Acetonitrile and water	ZORBAX carbohydrate	Refractive index	Jayapal *et al.* 2013
	DP 2-4	Water	Aminex HPX-87C	Refractive index	De-Figueiredo *et al.* 2017
	DP 2-6	Water	Aminex HPX 42A	Refractive index	Akpinar *et al.* 2010
	DP 2-5	Water	RSO-Oligosaccharide Ag + 4 % Phenomenex	Refractive index	Wan Azelee *et al.* 2016
	DP 2-6	5 mM H_2SO_4	Aminex HPX-87H	Refractive index	Lin *et al.* 2017
	DP 2-6	Water	Aminex HPX-42A	Refractive index	Surek and Buyukkileci, 2017
RP-HPLC	DP 2-4	Sodium phosphate and acetonitrile	C18 column	UV at 245 nm	Morgan *et al.* 2017
GC-MS*	DP 2-4	Helium	HT5 Tagged "polycarborane siloxane"	Mass-spec	Hernández *et al.* 2009
HPTLC	DP 2-5	Acetonitrile and water (85:15)	TLC plates of silica Gel 60 F254	0.2% Orcinol	Chapla, Pandit, and Shah 2012

Method	Analytes	Mobile Phase	Stationary phase	Detection	Reference
	-	Butanol:Pyridine:Water (6:4.5:2.5)	TLC plates of silica Gel 60 F254	UV light at 366 nm	Gowdhaman and Ponnusami 2015
TLC	DP 2-5	n-butanol/acetic acid/water (2:1:1, v/v/v)	TLC plates of silica Gel	0·02% Orcinol	Benamrouche *et al.* 2002
HPAEC-PAD	DP 2-6	2000mM sodium acetate and 200mM sodium hydroxide	CarboPac PA-1	Pulsed amperometric detector	Gullón *et al.* 2008
	DP 3-6	400mM sodium acetate and 100mM sodium hydroxide	CarboPac PA-100		Courtin *et al.* 2009
	DP 2-6	500mM sodium acetate and 100mM sodium hydroxide	CarboPac PA200		Zhang, Xu, and Yu 2017
	DP 2-6	500mM sodium acetate and 100mM sodium hydroxide	CarboPac PA100		Álvarez *et al*. 2017
	DP 2-6	450mM sodium acetate and 150mM sodium hydroxide	CarboPac PA100		Li *et al.* 2013
	DP 2-6	500mM sodium acetate and 80mM sodium hydroxide	CarboPac PA100		Lafond *et al.* 2011
	DP 2-6	1000mM sodium acetate and 200mM sodium hydroxide	CarboPac PA100		Arruda, Pereira, and Pastore 2017
	DP 2-6	300mM sodium acetate and 100mM sodium hydroxide	CarboPac PA100		Bian *et al.* 2013, 2014

*Two-steps derivatisation was performed (oximation and trimethylsilylation)

High performance liquid chromatography (HPLC) was one of the earliest techniques used for the separation of oligomers from enzymatic degradation of plant cell wall polysaccharides after the development of fixed-ion resin columns (Schnaak, 1984). The addition of metal ions to the resin of ion exchange columns enhanced the separation of the oligosaccharides. Calcium resins proved efficient in the separation of monosaccharides, while silver and lead columns were more effective for the separation of oligosaccharides. Voragen *et al.* (1986) used an HPLC system with lead-resin column and a refractive index detector for the separation of XOS. The applied method lacked resolution as shown in Figure 6-1, while the precipitation of proteins and uronic acids

on the column required extensive cleaning and preparation of the sample prior to the analysis, as well as frequent change of the guard column. Moreover, the method was limited to the detection of DP1-DP4

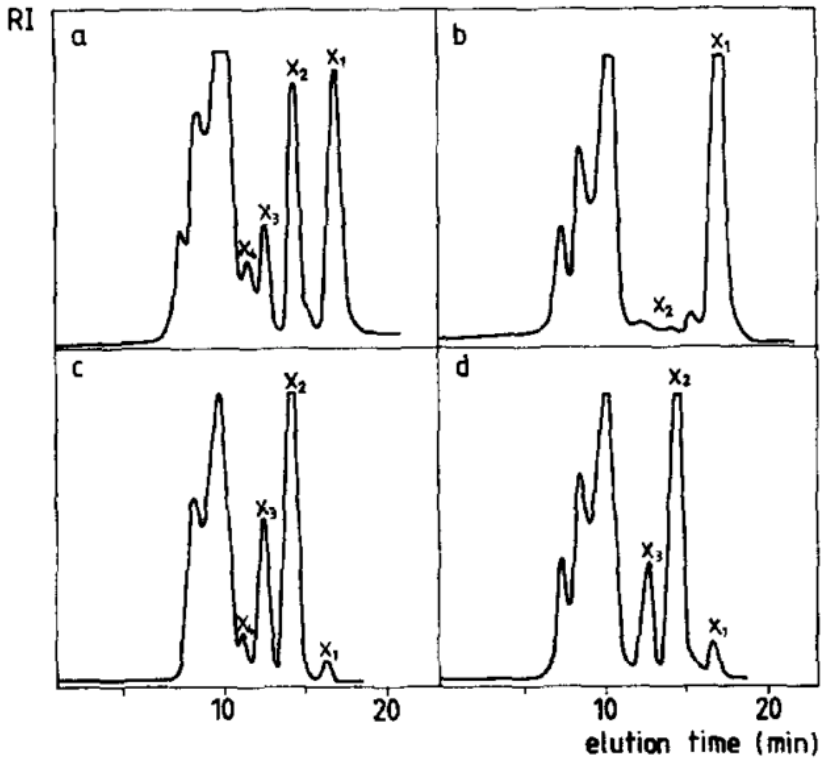

Figure 6-1. Separation of Xylooligosaccharides by HPLC-RI (Voragen et al., 1986).

Applying an ion-exchange column increases the ability to separate the oligosaccharides, and better detection can be achieved with a refractive index (RI) detector (Brienzo *et al.,* 2010; Sabiha-Hanim *et al.*, 2011; Jayapal *et al.,* 2013; Uçkun Kiran *et al.,* 2013; De Figueiredo *et al.,* 2017). The main drawback with this method is the low sensitivity of the RI detector for carbohydrates as well as the high sensitivity of the detector to any factor that affects the refractive index such as temperature, pressure and mobile-phase composition. Furthermore, the incompatibility of the RI detector with gradient elution (as the gradient elution changes the refractive index of the mobile phase) significantly hampers the efficiency of the separation (Swartz, 2010).

A degree of in-depth understanding of the formation and the structure of XOS and AXOS has been obtained via more sophisticated methods such as mass spectroscopy (MS) and

Nuclear magnetic resonance (NMR) (Teleman *et al.,* 1995). Gruppen *et al.* (1992) used NMR to characterise the products of enzymatic hydrolysis of alkali-extracted AX from wheat flour after ion exchange separation, revealing the type and location of the arabinofuranosyl groups. Hoffmann *et al.* (1992), using the same technique, described the sequence of the branching of XOS by studying the structure of hepta- to tetradeca-saccharides obtained by digestion of wheat endosperm arabinoxylan with endoxylanase, concluding that AXOS with two 2, 3-branches are separated by one or two unbranched xylose units. Despite the insights revealed by these techniques (achieved despite the lack of commercial standards at the time), the cost of the equipment used for the analysis limits the availability of these methods for routine analysis.

High performance liquid chromatography (HPLC) has become one of the most reliable techniques used in food and pharmaceutical research. Morgan *et al.* (2017) used a reversed phase HPLC system with C18 column and UV detection at 245 nm with a mixture of sodium phosphate buffer and acetonitrile as a mobile phase for the separation of XOS. The method was limited to measuring XOS of degree of polymerisation up to four monomers (DP4), as shown in the chromatogram in Figure 6-2. Moreover, the lack of chromophore groups in the carbohydrates limits the UV detection ability to detect low concentration.

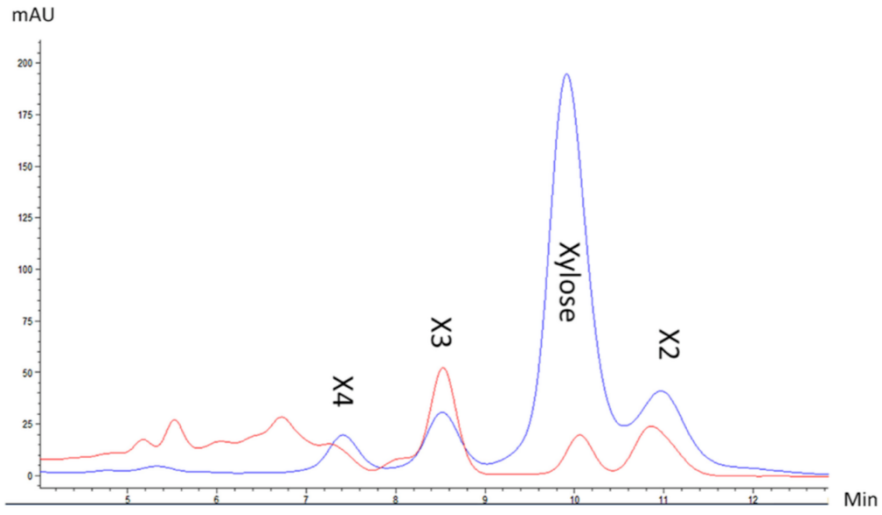

Figure 6-2. Chromatogram of XOS standards DP 2-4 (1000 ppm) analysed by RP-HPLC (Morgan et al., 2017).

Apparently, the utilisation of RP-HPLC does not give the optimum picture of the xylanase activity or the oligosaccharide profile in the samples, especially as very high concentrations of the analytes are needed in order to be detected.

Gas chromatography (GC) is widely used for carbohydrate determination; however, due to the non-volatile nature of monosaccharides and oligosaccharides, a lengthy derivatisation procedure is required prior to the analysis. Methylation of sugars for GC analysis is used to determine linkage types between component monosaccharide units in the oligo- and polysaccharides. This is usually accomplished by treating the polysaccharide with methylsulphinyl carbanion to create polyalkoxide ions, followed by treating the mixture with methyl iodide to add a methyl group (Harris *et al.,* 1984; Sandford and Conrad, 1966). The methylated polysaccharides then undergo a hydrolysis step to break down the polysaccharide into the constituent monosaccharides, which undergo a reduction step followed by acetylation. The resultant alditol acetates of methylated sugars are separated by GC-MS. Hernández *et al.* (2009) reported a successful quantification method that separates mono-, di- , tri- and tetra-saccharides using gas chromatography–mass spectrometry (GC–MS) with a two-step derivatisation involving oximation and trimethylsilylation.

Despite the importance of methylation analysis in evaluating the qualitative linkage pattern in polysaccharides, the quantitative utilisation of this procedure is hindered by several factors. The alditol acetate derivatives of the methylated sugars might result in under-methylation, and the kinetics of the reaction are not well studied. Also, the recovery of the acidic hydrolysis, the monosaccharides' degradation and demethylation during the hydrolysis, are not well established. Another factor is the unstudied recovery of both reduction and acetylation reactions, and the losses of volatile products during evaporation of solvents while performing the various steps. All these factors hinder the utilisation of the methylation method for quantitative analysis.

Among all the chromatographic techniques, high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been the method of choice for carbohydrates analysis (Rocklin and Pohl, 1983). The utilisation of

pulsed amperometry has enabled sensitive detection, which is indispensable in the case of complex samples. Recently, the commercial availability of XOS standards up to DP6 and a smaller number of AXOS standards has facilitated the separation of these analytes in a simple and direct analysis using an HPAEC-PAD system. Falck *et al.* (2014) determined the XOS and AXOS produced by different xylanases using a CarboPac PA200 column and a fixed 100 mM sodium hydroxide with a linear increase of sodium acetate from 0 to 120 mM within a 30 minutes run, as illustrated in Figure 6-3. The accurate determination of xylanase activity by directly measuring the produced oligosaccharides has thus become a routine analysis.

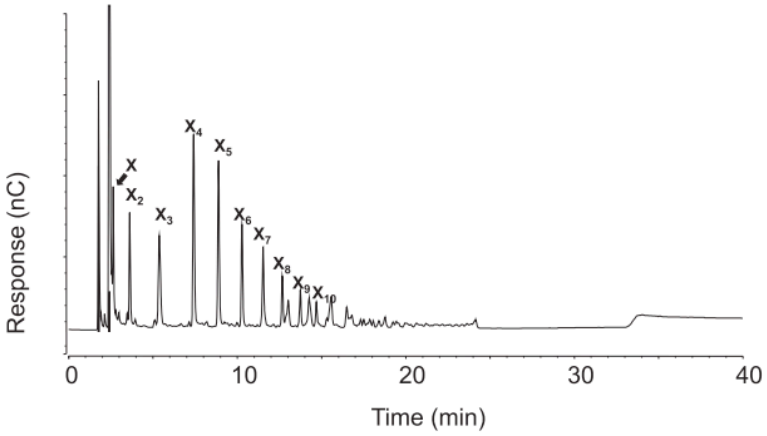

Figure 6-3. Analysis of XOS with HPAEC-PAD (Falck et al., 2014).

Cürten *et al.* (2018) reported a faster method for the analysis of XOS as an automated method for the assay of xylanases activity using a CarboPac PA100 column with a more concentrated mobile phase composition. They also studied the effect of sodium hydroxide concentration, sodium acetate concentration, column temperature and flow rate, as illustrated in Figure 6-4.

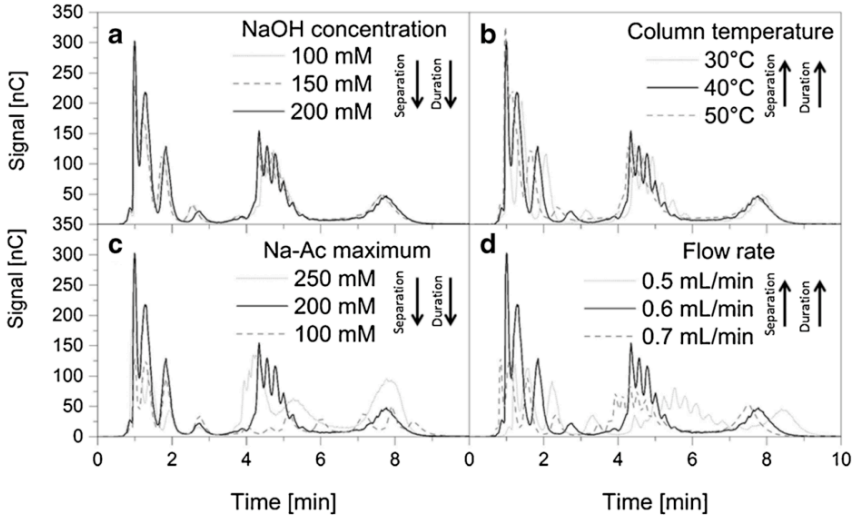

Figure 6-4. Effect of mobile phase strength, column temperature and flow rate on the separation of XOS (Cürten et al., 2018).

Other reports have showed the effective usage of HPAEC-PAD for the quantification of XOS and/or AXOS, variously using CarboPac columns PA1, PA100 and PA200 to measure XOS in the range DP2-6 (Gullón *et al.,* 2008; Courtin *et al.,* 2009; Lafond *et al.,* 2011; Li *et al.*, 2013; Bian *et al.*, 2013, 2014; Arruda *et al.,* 2017; Zhang *et al*., 2017). All of these used a fixed (isocratic) concentration of the main solvent, sodium hydroxide, with gradient elution of sodium acetate, in varying proportions and molarities.

The fast and reliable analysis has made the HPAEC-PAD technique the favoured choice for oligosaccharides analysis. However, the isocratic elution of strong sodium hydroxide solution is recommended by the manufacturers, as the manual of CarboPac PA 200 column reads (Dionex Corporation, 2004, p. 11):

"To maintain baseline stability, it is important to keep the sodium hydroxide concentration constant during the sodium acetate gradient, because acetate has no buffering capacity at high pH."

The isocratic elution of strong eluents severely reduces the resolution of the separation of the monosaccharides and to some extent the oligosaccharides; these recommendations are probably inherited from the first generations of ion exchange techniques when RI detectors were the only option.

Practically, the low retention of the small oligosaccharides (DP2-3) makes the analysis of these XOS almost impossible in complex samples such as food and feed products, which can be rich with proteins and amino acids. Figure 6-5 shows a chromatogram of an isocratic elution method for the analysis of XOS content in a real animal feed sample performed in AB Enzymes labs (unpublished work). Clearly, the amount of interfering materials has completely covered the area of the small XOS elution, and it was impossible to determine the content of the oligosaccharides without developing a higher resolution method.

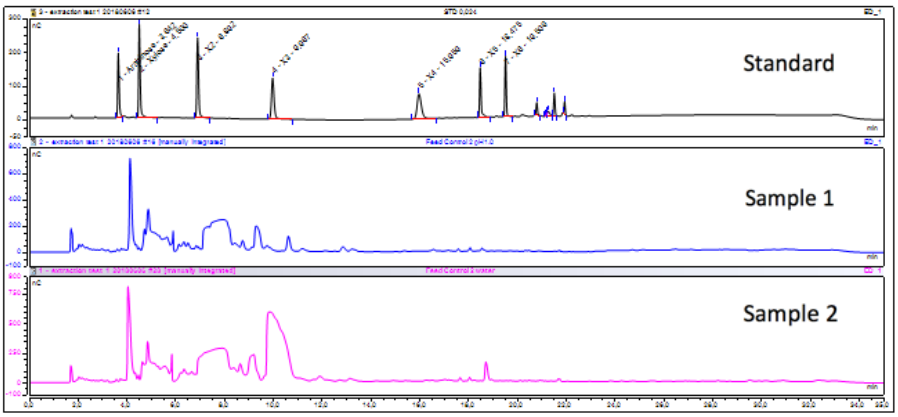

Figure 6-5. XOS analysis in animal feed sample (Unpublished work, AB Enzymes labs).

Moreover, the analysis of monosaccharides required different conditions from those for oligosaccharides, as the strong solvent elution used for the oligosaccharides elution is not suitable for the monosaccharides; hence, to get the full picture of the enzyme hydrolysis activity, each sample needs to be analysed twice under two different conditions with two different columns, to quantify the monosaccharides and the oligosaccharides separately (Brienzo et al., 2010; Mathew, Karlsson *et al.*, 2017; Sabiha-Hanim *et al*., 2011; Zhang *et al*., 2017). Dionex®, the leading manufacturer of HPAEC-PAD, is marketing two major versions of columns for the carbohydrates analysis: CarboPac PA10 and PA20 for the analysis of monosaccharides and CarboPac PA100 and PA200 for the analysis of oligosaccharides, requiring two sets of conditions.

However, previous attempts to perform simultaneous separation of mono- and oligosaccharides using HPAEC technique following a multistep gradient elution approach, where a low concentration of NaOH is used to elute monosaccharides and

followed by sudden increase of the concentration of the mobile phase to elute the oligosaccharides and uronic acid (Anders *et al.,* 2015; Wang *et al.,* 2012). The multistep gradient elution successfully separated the analytes in the presented reports, but it required a previous knowledge of the peaks and their retention time. Also the sudden change in the mobile phase causes the disruption and increased noise of the base line as illustrated in Figure 6-6 which affects the overall peak integration and the method repeatability (Snyder *et al.,* 2012).

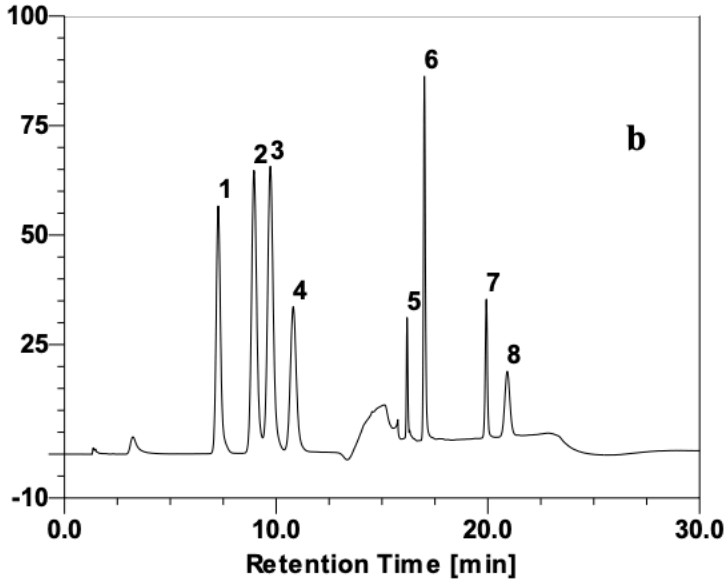

Figure 6-6. Separation of 1. arabinose; 2. galactose; 3. glucose; 4. xylose; 5. xylonic acid; 6. gluconic acid; 7. galacturonic acid; 8. glucuronic acid with step gradient method (Wang et al., 2012).

Due to these various technical limitations, the precise activity of xylanases in terms of their activity on oligosaccharides, particularly those from wheat, has not been well described in the literature.

Therefore, this chapter introduces a novel high-resolution HPAEC-PAD method based on the linear gradient elution of both sodium hydroxide and sodium acetate to give a high-resolution method for the simultaneous determination of cereal monosaccharides, xylooligosaccharides, arabinoxylooligosaccharides and uronic acids (glucuronic and galacturonic acid). The new method allows a comprehensive description, quantitative and qualitative, of the modes of action of xylan-degrading enzymes as well as the

substrate specificity of the enzymes. The chapter reports the materials and methods used, followed by the description of the method performance by reporting relevant validation parameters. The new method was demonstrated to evaluate the performance of Econase to release XOS from a relatively clean substrate (wheat bran) and to measure the XOS content in some external samples submitted to the University of Huddersfield from industrial and academic partners.

6.3 Materials and methods

6.3.1 Materials

Standards of monosaccharides fucose (as internal standard), arabinose, galactose, glucose and xylose were obtained from Sigma UK. Xylo-oligosaccharides (xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6)) and arabinoxylan oligosaccharides (2^3-α-L-arabinofuranosyl-xylotriose (A2XXX), 3^3-α-L-arabinofuranosyl-xylotetraose (XA3XXX), 2^3-α-L-arabinofuranosyl-xylotetraose (XA2XXX) and 2^3,3^3-di-α-L-arabinofuranosyl-xylotriose (A2A3XXX)) were purchased from Megazyme (Bray, Ireland). Glucuronic acid, galacturonic acid, sodium acetate, sodium hydroxide (52%), citric acid and sodium phosphate mono-basic were obtained from Fisher Scientific UK.

Commercial xylanase Econase XT 25L was supplied by AB Vista (Marlborough, UK). Wheat bran was kindly provided by the Biorenewables Development Centre, York, UK.

The chromatography system used was the Dionex ICS-3000 described earlier in Section 4.5.4.

6.4 Chromatographic conditions

Gradient elution of two mobile phases was performed: Solution C comprising 10 mM sodium hydroxide; and Solution D comprising a mixture of 200 mM sodium hydroxide and 125 mM sodium acetate. Figure 6-7 presents the elution conditions applied;

starting with 100% Solution C at time 0, a linear increase of Solution D was applied to reach 100% after thirty minutes, then held at 100% D for five minutes. Column equilibration was performed by maintaining starting conditions (100% C) for at least 15 min before sample injection. The mobile phase was sparged with helium and kept under helium during the analysis. The elution flow rate was maintained at 0.3 mL min^{-1} and the injection volume was 20 µL. Pulsed wave form "carbohydrates (standard quad)", wave form description "carbohydrates (std. quad. Potential)" was used for the detection. Fucose was used as an internal standard. Solutions A and B are water and 200 mM sodium hydroxide respectively, and are used for system washing.

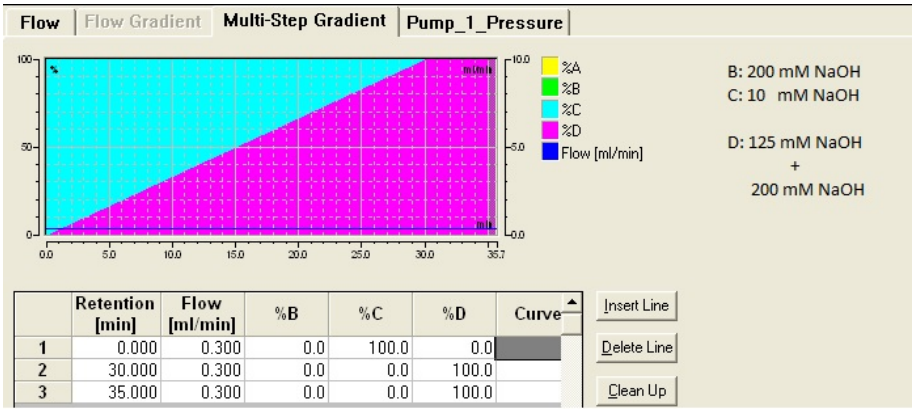

Figure 6-7. Developed HPAEC-PAD method condition for the new method.

6.4.1 Preparation of standard solutions

A primary stock solution of each standard was prepared in MilliQ (18.2 MΩ·cm) water to obtain a concentration of 2 mg mL^{-1}. Then a secondary stock solution of a mixture of all the standards was prepared at concentrations of 100 ppm each. Serial dilutions with water were performed to prepare the calibration curve for each standard. The concentration range for working standard solutions was 1-20 ppm. The standards solution was found to be stable when kept refrigerated at 4°C.

6.4.2 Sample preparation

6.4.2.1 Aberystwyth University samples

Two samples of defatted steam exploded brewers spent grain were received to measure the oligosaccharide yield of the steam explosion pretreatment. The samples were dry, so were solubilised in ultrapure water to yield a concentration of 10 mg mL^{-1}, centrifuged and filtered over 0.45µm syringe filters.

6.4.2.2 AB Vista samples

AB Vista is part of AB Agri, the agricultural part of Associated British Foods (ABF), and is one of the leading international companies in the field of animal nutrition technologies. Four samples of XOS condensed syrup were submitted by AB Vista to measure the content of oligosaccharides following dilution with water and treatment with xylanases. Samples were diluted, filtered and analysed via HPAEC-PAD using the new method. The samples were labelled as:

- 108-656 (XOS syrup)
- 109-580 (Water + XOS syrup (2:1))
- 109-581 (Xylanase 1 + XOS syrup (2:1))
- 109-582 (Xylanase 2 + XOS syrup (2:1))

6.4.2.3 Wheat bran—Econase treatment

Wheat bran was washed with water at 70°C with continuous stirring for 60 minutes, then strained over a 250 µm sieve three times to ensure the removal of any residual starch. The washed bran was oven-dried overnight at 75°C.

A sample of 20.0 g of the washed dried bran was weighed into a flask with 200 mL of citrate/ phosphate buffer at pH 4.5 and placed in a shaking water bath at 50°C. One mL of the enzyme was added (equivalent to 50 mL per kg of dry sample), after which 10 mL

samples of the solution were taken after 0, 30, 60 and 120 minutes and submerged in an ice bath to halt the enzyme activity. The samples were diluted and filtered over 0.45 μm syringe filters and analysed in the HPAEC system. Samples were injected manually directly after removal from the ice bath (thus samples remained cold such that enzyme activity did not resume prior to analysis).

6.5 Results and discussion

6.5.1 Chromatography and specificity

The general concept of chromatographic separation is based on the variation in the distribution of the analysed substances between two dissimilar phases, the stationary and mobile phases. In liquid chromatography, the mixture of analytes is dissolved in the mobile phase and separated by passing through the column which contains the particles of the stationary phase. The separation occurs as each molecule in the sample act in a different way with the stationary phase. Molecules of strong solubility in the mobile phase and less affinity to the stationary phase move faster throughout the column and have lower retention times. Considering the analysis of monosaccharides and oligosaccharides with HPAEC, it is clear that the strength of the mobile phase is the key driver behind the solubility of the sugars in the mobile phase and thereby the different retention times of monosaccharides of similar Mw. The molecular weight and shape also plays an important role as well. The applied gradient elution allows varying interaction with the mobile phase to take place and yields in a gradual separation of monosaccharides followed by the migration of linear XOS according to their molecular weight, then the elution of the branched AXOS and the uronic acids.

Figure 6-8 shows a chromatogram of the standards mixture, each at a concentration of 20 ppm, obtained using the CarboPac PA200 column. The chromatographic run time of 35 minutes was sufficient for the elution of all the standards. The baseline was smooth and stable in all the analysed samples, and the gradient elution gave a high resolution separation and did not affect the baseline.

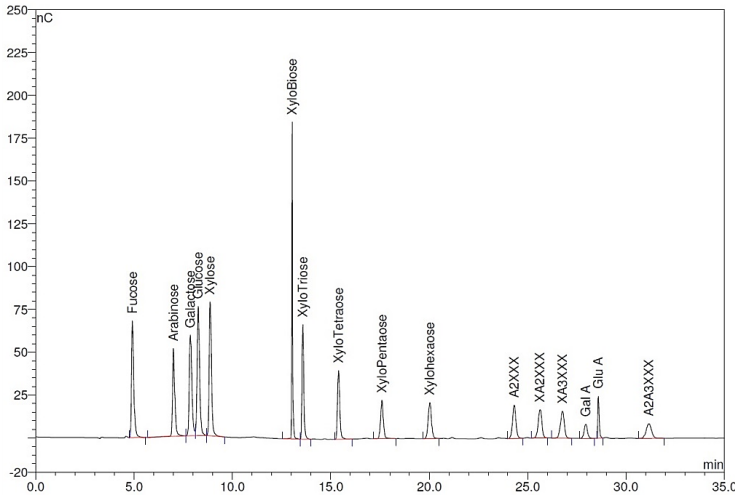

Figure 6-8. Chromatogram of mono and oligosaccharides mixture using CarboPac PA200.

In fact, the linear gradient elution of the alkaline solvent not only enhanced the separation of oligosaccharides, it also increased the capability of the columns used for the separation. The CarboPac PA20 is commercialised as the column of choice for mono- and disaccharides, but with the applied conditions the PA20 was also able to separate the oligosaccharides efficiently although over a longer time, as illustrated in Figure 6-9. The PA200 was used for the remainder of the current work as it allowed faster run times.

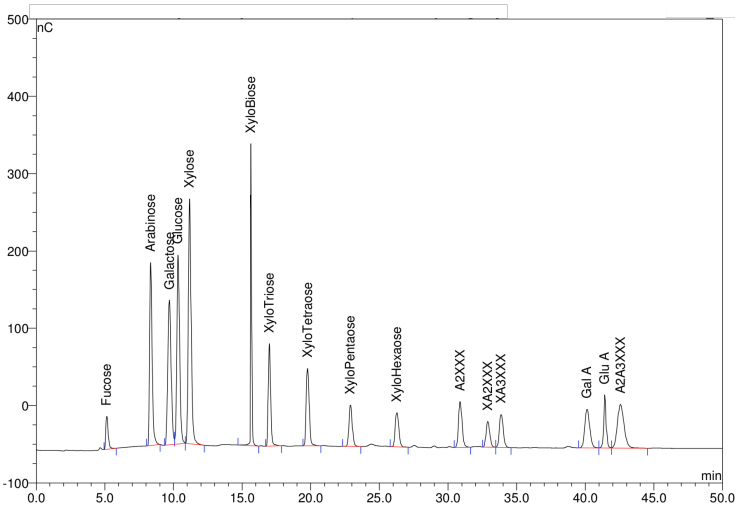

Figure 6-9. Chromatogram of mono and oligosaccharides mixture using CarboPac PA20.

The validation of analytical methods addresses the objective of obtaining consistent, reliable and accurate measurements. The validation procedure followed in this

research complies with the International Conference of Harmonisation (ICH) guidelines on method validation (ICH Q2(R1), 1994).

6.5.2 Linearity

To assess linearity, calibration curves were plotted using the ratio of the analyte peak area to the internal standard peak area against concentrations of different dilutions (n = 9) covering the range 1-100 ppm for monosaccharides and 1-20 ppm for XOS, AXOS and uronic acids. The correlation coefficient values were acceptable for all the standards and ranged between 0.985 and 0.999. The correlation coefficients (R^2), slopes and intercepts are reported in Table 6-2, along with Limits of Detection (LOD) and Quantitation (LOQ).

Table 6-2. Method linearity, LOD and LOQ for each analyte.

	R2	Intercept	Slope	LOD (ppm)	LOQ (ppm)
Arabinose	0.999	0.039	0.154	0.03	0.08
Galactose	0.996	-0.176	0.132	0.08	0.1
Glucose	0.996	-0.231	0.149	0.08	0.1
Xylose	0.997	0.087	0.213	0.04	0.1
Xylobiose	0.984	0.226	0.079	0.001	0.008
Xylotriose	0.983	0.221	0.075	0.005	0.01
Xylotetraose	0.981	0.202	0.056	0.2	0.4
Xylopentaose	0.985	0.112	0.041	0.3	0.5
Xylohexaose	0.986	0.12	0.039	0.03	0.1
A3XXX	0.994	0.073	0.049	0.04	0.08
XA2XXX	0.993	0.057	0.045	0.01	0.03
XA3XXX	0.990	0.081	0.043	0.01	0.03
Gal A	0.984	-0.031	0.033	0.06	0.2
Glu A	0.997	-0.021	0.056	0.06	0.1
A2A3XXX	0.995	0.047	0.035	0.08	0.2

6.5.3 Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ measure analytical method sensitivity to low concentrations of analyte. LOD is the lowest concentration detected and LOQ is the minimum quantifiable concentration measured by the method. Signal-to-noise (S/N) ratios of 3:1 and 10:1 were used to define LOD and LOQ, respectively, and were calculated by the Chromeleon® software. The peaks were clear to identify after the dilutions and had acceptable precision. LOD and LOQ values for all the analytes are listed in Table 6-2. The table shows variation in the detection and quantification limits of the standards, which is a reflection of the variation in the peak heights and areas for the same concentrations, as evident in Figure 6-8. In general, the monosaccharides showed lower detectable concentrations than the oligosaccharides, except for xylobiose which is more readily detected than xylose and gave the lowest values of LOD and LOQ.

6.5.4 Accuracy and precision

The accuracy of the method was studied by analysing triplicates of control samples covering three concentration levels (5, 10, 15 ppm); the results are listed in Table 6-3 as percentage recovery. The calculated recoveries for all the studied standards ranged acceptably between 97-103%.

The precision of the method reflects the agreement among individual test results. Intra-day precision (repeatability) was estimated by analysing seven replicates of a control sample in the same HPAEC run. Inter-day precision (intermediate precision) was assessed from nine triplicates over three consecutive days (three runs per day) using the same instrument with three different batches of the mobile phase. Both repeatability and intermediate precision results are listed in Table 6-3 as the percentage coefficient of variation (CV%). Both the precision and the intermediate precision are in acceptable ranges for all the analytes.

Table 6-3. Method accuracy (reported as percentage recovery) and precision (reported as CV%).

		Arabinose	Galactose	Glucose	Xylose	Xylobiose	Xylotriose	Xylotetraose	Xylopentaose	Xylohexaose	A3XXX	XA2XXX	XA3XXX	A2A3XXX	Gal A	Glu A
Recovery %	15 ppm	101.02 ±0.64	100.46 ±1.46	100.28 ±0.82	100.42 ±1.2	100.81 ±0.78	99.99 ±1.23	100.34 ±0.31	100.4 ±0.86	102.67 ±3.2	102.13 ±3.33	102.07 ±2.42	100.01 ±1.12	100.12 ±0.6	100.4 ±1.02	100.26 ±0.41
	10 ppm	101.21 ±2.52	100.91 ±1.22	102.38 ±3.42	99.89 ±2.73	101.23 ±1.93	103.29 ±4.63	99.68 ±1.08	101.14 ±2.4	100.59 ±0.64	99.41 ±1.1	101.55 ±1.41	99.97 ±0.9	100.26 ±2.25	99.81 ±2.1	101.2 ±1.15
	5 ppm	100.19 ±3.03	100.5 ±3.07	101.95 ±2.87	100.09 ±0.87	97.70 ±2.83	103.91 ±4.61	100.20 ±1.88	99.60 ±1.76	100.08 ±0.33	99.57 ±0.51	101.33 ±3.17	100.79 ±2.97	100.07 ±1.83	99.55 ±0.92	100.74 ±2.18
Precision CV%		0.006	0.015	0.008	0.012	0.008	0.012	0.003	0.009	0.031	0.033	0.024	0.011	0.006	0.01	0.004
Intermediate precision CV%	Day 1	0.03	0.017	0.047	0.005	0.014	0.026	0.011	0.002	0.05	0.049	0.018	0.018	0.017	0.019	0.017
	Day 2	0.015	0.014	0.012	0.025	0.01	0.024	0.017	0.015	0.041	0.022	0.004	0.02	0.014	0.016	0.003
	Day 3	0.006	0.015	0.008	0.012	0.008	0.012	0.003	0.009	0.031	0.033	0.024	0.011	0.006	0.01	0.004

6.5.5 System suitability tests

The fitness of the chromatography system was estimated by a range of system suitability test parameters: Capacity factor (or retention factor, *k'*), Resolution factor (R_s), number of theoretical plates (Column efficiency) and Asymmetry (A_s), as calculated by the Chromeleon® software. The results of these parameters for each analyte are listed in Table 6-4. The values of the resolution factor reflect high-resolution separation of the analytes except for galactose where the R_s was only 1.71. In general, all the calculated parameters gave acceptable values; the asymmetry factor was between 0.95 and 1.37, the capacity factor was more than 2 and the number of theoretical plates was greater than 15000 for all the analysed standards.

Table 6-4. Method resolution, asymmetry factor, capacity factor and number of theoretical plates.

	Resolution factor R_s	Asymmetry A_s	Capacity factor *k'*	theoretical plates
Arabinose	3.63	1.35	2.676	17620
Galactose	1.71	1.10	3.114	15701
Glucose	2.64	1.08	3.334	18981
Xylose	10.23	1.26	3.649	26455
Xylobiose	26.6	1.10	5.851	1081600
Xylotriose	9.46	1.23	6.132	112710
Xylotetraose	9.15	1.33	7.071	80127
Xylopentaose	8.25	1.09	8.228	69836
Xylohexaose	13.27	1.04	9.500	61511
A3XXX	3.91	1.10	11.737	91312
XA2XX	3.13	0.95	12.430	82712
XA3XX	3.69	0.95	13.026	82668
Gal A	3.06	1.07	13.684	130671
Glu A	7.47	1.37	14.035	793131
A2A3XXX	23.52	1.05	15.298	57549

6.5.6 Sample analysis results

This subject area is of active interest to other industrial and academic parties who, knowing of our development of this new analytical method, were keen to send us samples for analysis. This section illustrates the new method and the sorts of samples

for which it is relevant, by reporting results for samples sent from Aberystwyth University and AB Vista. The new method was also used to detect the results of Econase treatment of wheat bran.

6.5.6.1 Aberystwyth University samples results

The chromatograms from the two spent grain samples from Aberystwyth University are shown in Figure 6-10. The chromatograms present clearly the complex nature of the samples with the presence of unidentified peaks. However, they also show that the targeted XOS were separated efficiently and could be identified. The samples were also spiked with the standards solution and reanalysed, to confirm that the peaks were identified correctly.

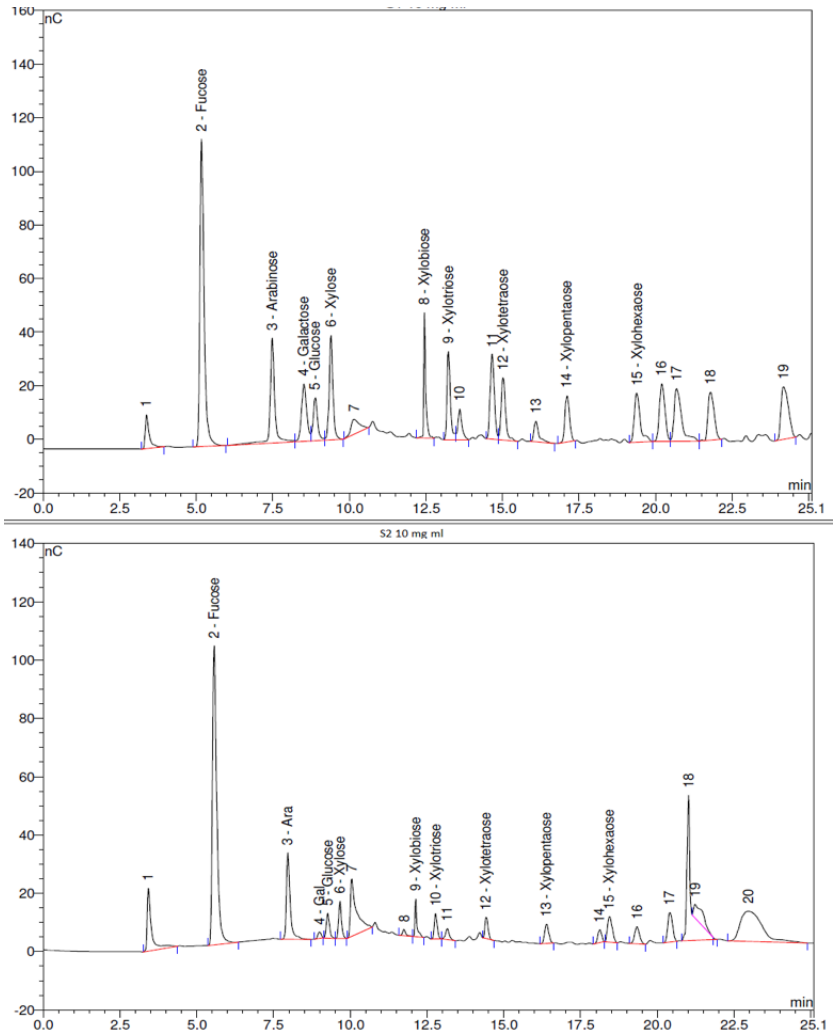

Figure 6-10. Chromatographs of Aberystwyth University spent grain samples.

Table 6-5 reports the percentage content of the analysed mono- and oligosaccharides in the analysed samples. Clearly, the content of XOS in the samples was almost negligible and the pretreatment with steam explosion was not sufficient to produce significant amounts of the desired XOS.

Table 6-5. Concentration of XOS in Aberystwyth University spent grain samples.

	Concentration of the sample (%)							
	Ara	Glu	Xylose	X2	X3	X4	X5	X6
S1	0.04	0.01	0.02	0.05	0.05	0.05	0.05	0.08
S2	0.03	0.01	0.01	0.01	0.01	0.01	0.05	0.03

6.5.6.2 AB Vista samples

Figure 6-11 shows the chromatogram of the first of the four samples. Clearly, there was a significant amount of XOS in the syrup, particularly X2 and X3.

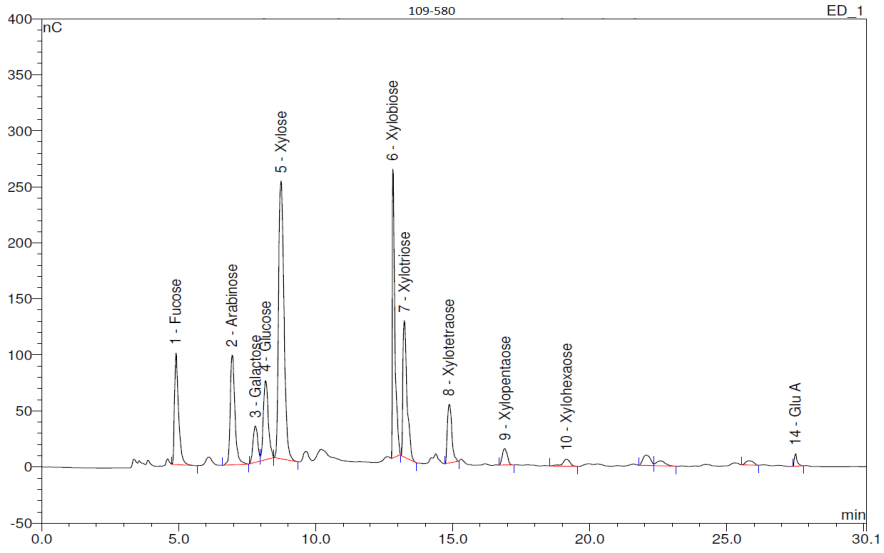

Figure 6-11. AB Vista (108-656 XOS syrup) sample chromatogram.

Figure 6-12 reports the amounts of each mono- and oligosaccharide in the four samples. In the original XOS syrup (Sample 108-656), xylose (X1) and xylobiose (X2) dominate, followed by X3, with significant amounts of X4-X6 as well as arabinose, galactose and glucose. Dilution reduced the concentration of all components by a factor of three (Sample 109-580). Both enzymes then increased dramatically the concentrations of X1

and X2, apparently by acting on X3, X5, X6 and presumably larger oligos not measured (the increase in X1 and X2 is more than can be accounted for by the decreases in X3, X5 and X6) but, interestingly, appearing to leave X4 largely untouched. The apparent lack of change in X4 is probably an illusion arising from the dynamic balance between the production of X4 from larger oligos (X5, X6 and beyond) and its degradation; probably largely by coincidence, under the conditions used, X4 happened to be produced as fast as it was degraded, while for the other oligos these were not balanced. This result demonstrates how fully understanding the enzymatic production of oligosaccharides requires population balance modelling of the evolution of the oligosaccharide profile, a theme picked up in the next chapter. For now, the results demonstrate that the new method can be applied to characterise commercial materials containing XOS and to determine the effects of enzymes on these materials, in order to maximise XOS production. It is also noteworthy that the enzymes produced significant amounts of the xylose monosaccharide, which is not expected from an endo-xylanase.

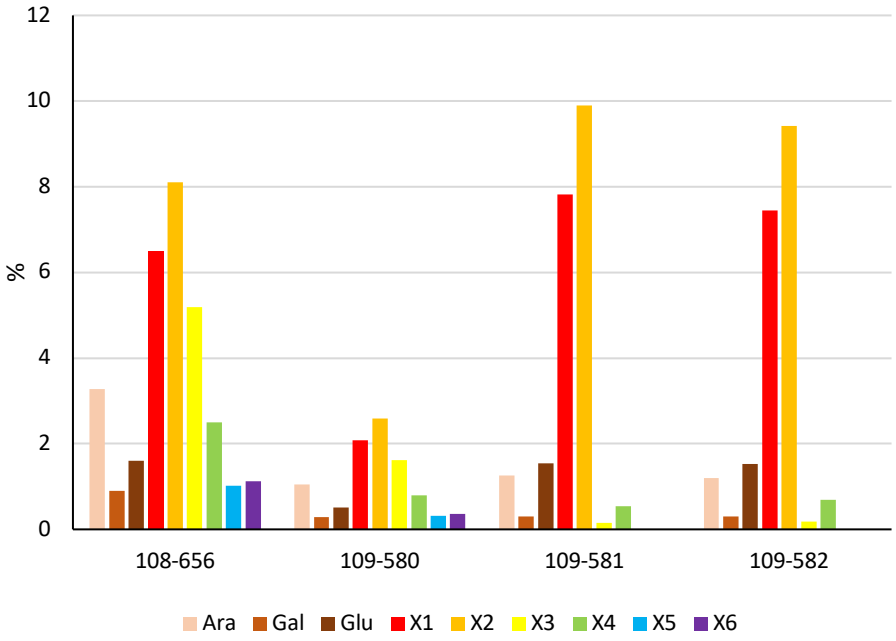

Figure 6-12. XOS syrup enzyme treatment samples.

6.5.6.3 Wheat bran—Econase treatment

The oligosaccharides released from wheat bran by xylanase treatment were measured and estimated as a percentage of the original wheat bran on a weight basis. Figure 6-13 shows the release of each analyte over time for up to 180 minutes. (For comparison, the release in buffer solution without enzyme after 180 minutes is also shown; significant extraction of endogenous glucose and xylose into buffer occurred without enzyme, but this was not the case for the oligosaccharides or GluA and GalA, indicating these were not present endogenously in the original wheat bran in detectable amounts.) Clearly both glucose and xylose increased over time, the former suggesting some amylolytic side activity or contamination in the enzyme. Xylobiose (X2) also increased monotonically, but by contrast, X3 and X4, after an initial burst of production, decreased over time as they were further degraded by the xylanase. Small amounts of X5 and X6 also appeared briefly but were degraded even more quickly. Significant amounts of GalA and GluA were also released, to levels that appeared to become stable over time.

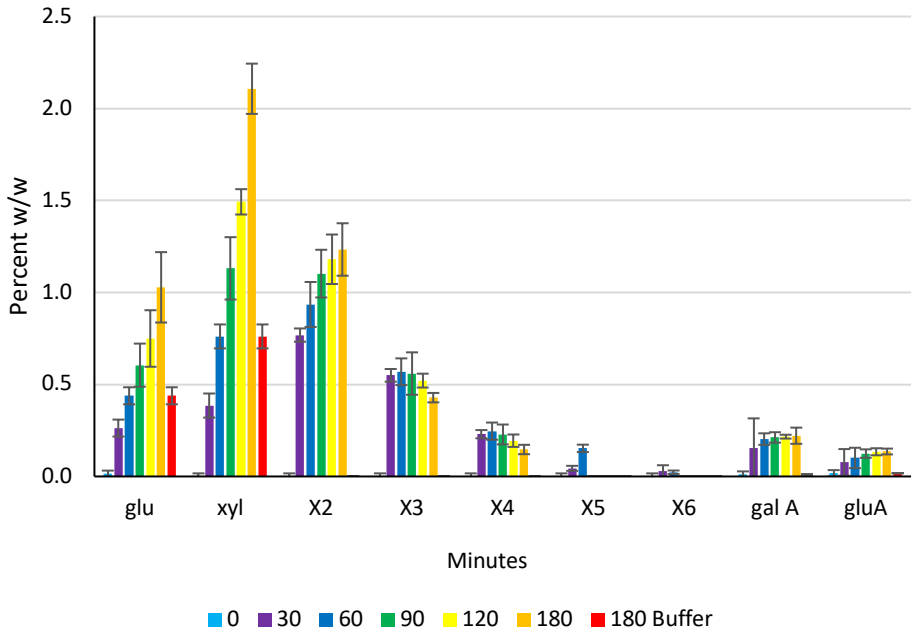

Figure 6-13. Mono- and oligosaccharides released from wheat bran by xylanase treatment for 180 minute at 50°C.

Figure 6-14 illustrates more clearly the dynamics by showing the evolution of X1-6 over time. The results indicate a pattern of the creation and degradation of the xylan oligosaccharides as initially fragments are released by enzyme action on larger AX polysaccharides, then the enzymes turn their action onto the released oligos. Thus the majority of the oligos were produced in the first 30-60 minutes, including the brief appearance of X6 and X5 during this time. Thereafter, a balance of producing new small oligos (X2) and degrading the larger oligos (X3, X4, X5 and X6) becomes evident. The steady increase of X2 during the treatment suggests that this endoxylanase cannot act on the disaccharide. On the other hand, the increasing amounts of xylose, which is not expected for endoxylanases (Araki and Kitamikado, 1982; McCleary *et al.*, 1982) (as they attack the middle of xylan chains), could be explained by three hypotheses: endoxylanases could have a side activity as exoxylanases; there could be some contaminating exoxylanase in the enzyme preparation; or (least likely for steric hindrance reasons), the xylose production could be the result of two enzyme molecules attacking the same xylan chain one unit apart at the same time.

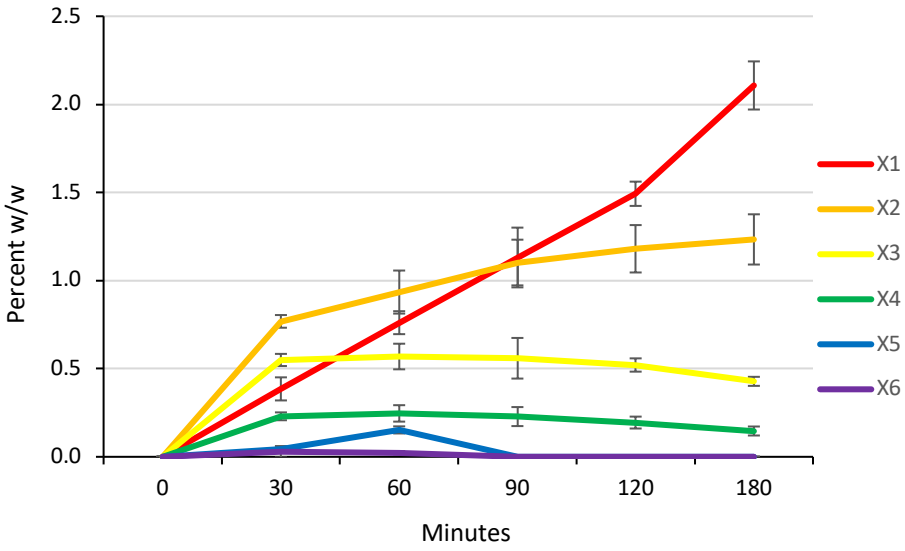

Figure 6-14. Evolution of XOS from wheat bran over time following xylanase treatment.

These results confirm the efficacy and usefulness of this new method for analysing samples containing XOS, and for understanding the evolving profile as enzymes create and then destroy oligosaccharides. These preliminary trials were on an arbitrary selection of industrially relevant samples, to demonstrate the method and to begin to

gain insights into the patterns of enzyme behaviour. Having done so, it becomes clear that there is a need for more fundamental and controlled studies to understand the behaviour of xylanases on individual XOS molecules.

6.6 Summary

The analysis of xylan and arabinoxylan oligosaccharides has been a major obstacle in the path to develop and study these products. High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has proved to be well suited for the separation and analysis of XOS. Previously reported methods showed good performance in characterising the XOS with DP2-6. However, the resolution of the separation has made it difficult to apply these methods to real samples such as food and animal feed formulations. Also, the need to perform a separate analysis for monosaccharides increases the time and effort to identify the full carbohydrate profile of a sample. Moreover, the measurement of uronic acids in the samples, which are a major component of wheat AX, required a third run with different conditions as well.

Therefore, this part of the work has presented a novel and reproducible HPAEC-PAD method for the simultaneous separation of cereal monosaccharides, xylan oligosaccharides, arabinoxylan oligosaccharides and uronic acids. The method is based on gradient elution of the solvents, which was shown to give excellent separation of xylose, arabinose and glucose as well as the xylo-oligosaccharides in the range X2-X6 plus galacturonic and glucuronic acids. The new method is applicable for testing and developing xylan-degrading enzymes and for measuring their production of mono- and oligosaccharides in food and feedstuffs.

The developed method was applied to measure the XOS content of some commercial and research samples from collaborators, and also allowed the precise observation of Econase action on wheat bran. The preliminary investigation raised vital questions about the mode of action of this endoxylanase towards the production and degradation

of XOS. As the new method can give a clear picture of the mono- and oligosaccharide profile, the next step of this project aimed to observe the action of Econase on XOS pure standards, to clarify the precise behaviour of this enzyme on these small oligosaccharides.

7 Endoxylanases degradation of XOS standards

7.1 Introduction

The possibility of enzymatic production of xylan and arabinoxylan oligosaccharides has been reported extensively in the literature; this has mostly been addressed by the employment of xylanases. The common understanding of the activity of xylanases divides them into two main categories: exo-xylanases (β-xylanases), which possess the ability to attack the terminal units in the xylan chain and produce xylose monomers; and endo-xylanases, which attack the middle of the xylan polysaccharide to produce smaller polymer molecules such as oligomers.

In the experiments reported in Chapter 6, it was observed that significant amounts of xylose monomers were present after the utilisation of the commercial endoxylanase Econase. Such presence of monosaccharides was not expected to accompany the production of XOS/AXOS. This unexpected result underlines that the role of endoxylanases in degrading oligosaccharides is not well established. Having developed the new method for the simultaneous determination of mono- and oligosaccharides, and with the recent availability of high purity AXOS standards, it became possible to investigate and explore the precise effect of xylanases on the evolution of the oligosaccharide profile. This chapter presents a brief review of the origin, classification and usages of xylanases. It then describes experimental work to treat XOS standards with Econase and follow the evolving production and degradation of the profiles of xylo-oligosaccharides alongside the xylose monomer.

7.2 Xylanases – a review of their features and applications

In the nineteenth century, Schulze (1891) isolated a fraction of plant material using dilute alkaline solution, which was made of sugars but not cellulose, which he named "hemicellulose". Hemicelluloses comprise a complex family of structures that can be formed from a variety of polysaccharides such as glucuronoxylan, arabinoxylan, glucomannans, arabinogalactan, and galactoglucomannans (Kulkarni *et al.*, 1999; Subramaniyan and Prema, 2002). They contribute to the structural integrity of plant cell walls and are thus closely associated with the other structural components, cellulose and lignin. The distribution of these polysaccharides varies according to the plant species; unlike cellulose, hemicellulose is not a defined chemical compound or material, but more like a class of plant polysaccharides with a widely variable set of properties peculiar to the constituent polysaccharides. Courtin and Delcour (2002) described cereal hemicelluloses, *i.e*. arabinoxylans, as a "peculiar group of molecules", reflecting their strange diversity of features and behaviours.) The most common polysaccharide in hemicelluloses is Xylan, which can be degraded by xylanolytic enzymes (Bajpai, 2014b). Hemicellulose degradation was first reported by Hoppe-Seyler (1889, cited in Sharma, 2013) who described the degradation of xylan wood slurry by microbes from river mud. As hemicelluloses are made up of 5-carbon sugars, they have been known as "pentosans", and in 1955, xylan-degrading enzymes were reported as pentosanases (Whistler and Masak, 1955; Collins *et al.,* 2005). Thereafter, they were recognised by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 and were given the enzyme code (EC 3.2.1.8.) (Collins *et al.,* 2005).

Naturally, xylanases are as diverse as the composition and structure of xylan polysaccharides; they can be produced by both plants and microorganisms to serve various purposes. In the plant kingdom, xylanases have been found in cereal grains where they help in the remodelling and the expansion of cell wall during seed growth and also for the cell wall degradation during seed germination (Dornez *et al.,* 2009). Xylanases have been found in wheat grain (Preece and MacDougall, 1958; Kulp, 1968), wheat bran (Bergmans *et al*., 1996), wheat flour (Cleemput *et al*., 1997), barley (Taiz and Honigman, 1976) and in germinated barley seeds (Slade *et al*., 1989). Xylanases

expressed by microorganisms (bacteria, fungi, actinomycetes and yeast) serve a wide range of purposes: some organisms excrete a number of hydrolysing enzymes including xylanases in order to provide a survival carbon source (Prade, 1995), whereas other organisms produce xylanases as a part of infecting and colonising plant cells (Hrmová *et al.,* 1984; Sunna and Antranikian, 1997; Bajpai, 2014a). In a broader context, xylanases have also been reported in marine algae, protozoans, crustaceans, insects and snails (Sunna and Antranikian, 1997).

7.3 Classification of xylanases

The number of the identified xylanases had been increasing due to the heterogeneity of xylan polysaccharides in nature and the tremendous number of xylanase producing microorganisms, considering that many organisms can produce several xylanases (Gilbert and Hazlewood, 1993; Collins *et al.*, 2005). Xylanases are thus known for their wide diversity, with overlapping and complex physicochemical properties, structures, specific activities and yields. Several classifications have been attempted for these enzymes. Wong *et al.* (1988) classified xylanases according to their physicochemical properties and categorised them into two groups: small molecular weight (<30 kDa) and basic isoelectric point, and large molecular weight (>30 kDa) and acidic isoelectric point. Nearly 30% of the characterised xylanases do not fit into this classification, particularly the fungal xylanases (Matte and Forsberg, 1992; Sunna and Antranikian, 1997).

A broader and more comprehensive classification was constructed for all the glycosidase enzymes (EC 3.2.1.x.) by the International Union of Biochemistry and Molecular Biology (IUBMB) (Henrissat *et al.*, 1989). This new classification has become the standard classifying system for all the hydrolysing enzymes; its basis, according to the carbohydrate active enzyme (CAZy) website (http://www.cazy.org/Glycoside-Hydrolases.html), is:

"Glycoside hydrolases (EC 3.2.1.x) are a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The IUBMB Enzyme nomenclature of glycoside hydrolases is based on their substrate specificity and occasionally on their molecular mechanism."

The IUBMB classification relies mainly on the substrate specificity, indicated in the numbering system (EC 3.2.1.x) in which x defines the substrate; for example, β-glucosidase is referred to as (EC 3.2.1.21), and β-galactosidases are (EC 3.2.1.23).

It was recognised that a varied group of enzymes collectively degrade xylan in nature; they were therefore classified under several Enzyme Commission numbers (EC): endo-xylanase (endo-1, 4-β-xylanase E.C.3.2.1.8), β-xylosidase (1, 4-β-xylosidase E.C.3.2.1.37), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), acetyl-xylan esterase (E.C.3.1.1.72) and feruloyl esterase (EC 3.1.1.73).

Exo-xylanases or β-xylosidase (1,4-β-xylosidase E.C.3.2.1.37) have the ability to hydrolyse xylan and xylo-oligosaccharides, releasing xylose residues by attacking the terminal units of the chain (Araki and Kitamikado, 1982; McCleary *et al*., 1982), as Figure 7-1 illustrates. By contrast, endoxylanases or endo-β-(1,4)-D-xylanohydrolase, (E.C. 3.2.1.8) hydrolyse xylan to β-D-xylopyranosyl oligomers by attacking the glycosidic bonds between xylose units in the xylan backbone resulting in a general reduction in the degree of polymerisation (Mandal, 2015).

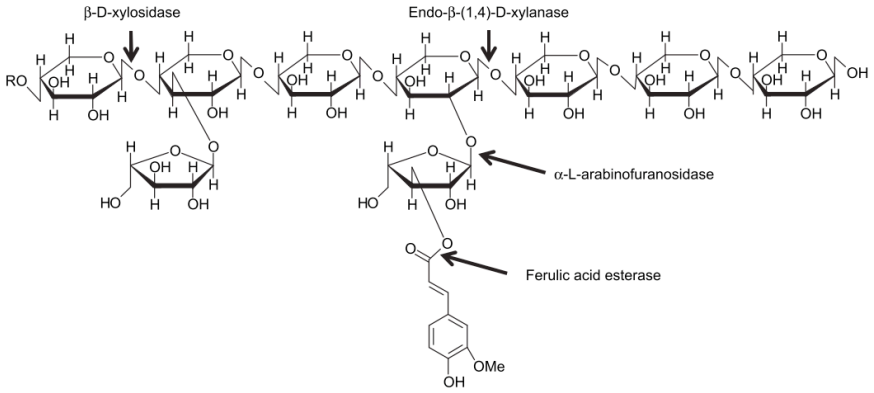

Figure 7-1. AX hydrolysing enzymes site of attack (Dornez et al., 2009).

Under this classification, endoxylanases from different origins are divided into several glycoside hydrolase families (GH) (5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62) (Moreira and Filho, 2016; Uday *et al.,* 2016). Among these families, GH10 and GH11 are well-studied in the literature, and present differences in the substrate susceptibility. It is believed that the GH10 family has the ability to hydrolyse substituted xylan chains and thereby produces substituted or branched oligosaccharides. By contrast, xylanases from the GH11 family, customarily known as "the true xylanases", are strictly able to attack only the xylan backbone and perform only on the unsubstituted regions of the polysaccharide (Collins *et al.,* 2002; Imjongjairak *et al.,* 2015). Studies on the modes of action of β-xylosidases and endoxylanases revealed that the endoxylanases have no effect on small XOS of DP2-3, whereas β-xylosidases can hydrolyse them (Deshpande *et al.*, 1986).

7.4 Industrial Applications of Xylanases

In the last few decades, enzymes have witnessed dramatic growth, particularly in the field of biotechnology. The enzyme global market share reached one billion dollars in 1990 and two billion in 2005, the market share climbed to nearly $4.6 billion and $4.9 billion in 2014 and 2015 respectively and is expected to soar upwards to $6.3 billion in 2021 according to bccResearch (Anon, 2017b). Polizeli *et al.* (2005) reported that xylanases, cellulases and pectinases collectively account for 20% of the world enzymes market.

Xylanases have been exploited in several industrial fields, of which paper and pulp bleaching is the largest market so far. In this context, cellulose is the main component of paper, coming primarily from the wood. In order to remove the lignin component from the woody starting material, several chemical approaches are used incorporating chlorine, hydrogen peroxide and oxygen. However, the environmental effect of this industry was the primary precursor of searching for other cleaning methods; since xylan plays the role of the connector between the undesirable lignin and the desirable cellulose, the enzyme-mediated degradation of this linkage has become of interest as it is low cost and eco-friendly (Amin, 2006; Bajpai, 1999; Paice *et al.,* 1992; Viikari *et al.,* 1994). Xylanase treatment in paper-making is usually called pre-bleaching or bleach boost, as the enzyme does not affect the chromophores of the lignin, but it degrades the xylan network surrounding and protecting the lignin. This effect makes lignin more vulnerable to chemical bleaching and reduces the amounts of chemical bleachers used in the process (Saleem *et al.,* 2009; Ko *et al*., 2011; Walia *et al.,* 2017).

Xylanases are also widely used in the food industry, particularly bread making, along with other enzymes such as α-amylase, malting amylase, glucose oxidase and proteases (Butt *et al.,* 2008; Harris and Ramalingam, 2010). Bread making is the most established xylanase application in the food world; the hydrolysis of non-starch polysaccharides seems to improve the rheological behaviour of bread dough and yield a general improvement in bread specific volume, crumb firmness, loaf volume and crumb structure (Baillet *et al.,* 2003; Guy and Sarabjit, 2003; Shah *et al.,* 2006). Xylanases also decrease starch retrogradation, reducing the initial crumb firmness during storage and having as anti-staling effect (Haros *et al.,* 2002; Jiang and Tan *et al.*, 2005; Jiang and Li *et al.*, 2005). In biscuit-making, xylanases help to improve wafer palatability, texture and uniformity (Polizeli *et al.,* 2005). Xylanases, with other glycosidic hydrolases, are applied in fruit and vegetable juice making as they increase both the yield and quality of the juice by facilitating the liquefaction process and increasing the recovery of essential oils, vitamins, mineral salts and edible dyes (Bajpai, 2014a; Polizeli *et al.,* 2005). Xylanases in the brewing industry improve the filterability of the mash by reducing the viscosity (Sadosky *et al.,* 2002).

The recent utilisation of xylanases for the production of xylan prebiotics is a promising and exciting field. The increasing amounts of lignocellulosic biomasses produced in several industrial domains, along with the insatiable need of the food market for novel and healthy products, have developed a great opportunity for the production of xylooligosaccharides.

The most convenient method for XOS production is the partial breakdown of hemicellulosic xylan chains into smaller chains. Interestingly, the conversion of hemicellulose into valuable XOS material has received a great deal of attention in the last decade. Several reports suggested hydrothermal (Vegas *et al.,* 2005; Ho *et al.,* 2014; Rose and Inglett, 2010; Buruiana *et al.,* 2017; Surek and Buyukkileci, 2017) and enzymatic treatments for the xylan degradation in different biomasses (Rydlund and Dahlman, 1997; Swennen *et al.,* 2006; Escarnot *et al.,* 2012; Falck *et al.,* 2013; Falck *et al.,* 2018). Table 7-1 illustrates the most relevant papers describing XOS production by enzymatic means, utilising xylanases from different origins to produce XOS from a variety of substrates including cereals, sugarcane bagasse, maize and tobacco stalks. The yields varied according to the substrate and the enzymes applied and the pretreatment method.

The production of XOS and AXOS could be achieved in several ways; the most direct method is utilising xylanases to hydrolyse the xylan chain in biomass, but although this method is cost effective, the produced yields are very low. Alternatively, chemical extraction of AX followed by enzyme treatments can give a higher yield. Utilising harsher treatments such as steam and hydrothermal processes could significantly increase the yield. However, the cost of the process and the simultaneous production of undesired monosaccharides and other chemicals, such as furfural, promotes the use of xylanases as the optimum route (Bajpai, 2014a; Rose and Inglett, 2010).

Table 7-1. Methods reported for XOS production.

Biomass	Pre-treatment	Enzymes	Yields of XOS from original material*	Reference
Sugarcane bagasse	Aqueous ammonia	Bacterial arabinofuranosidase + β-xylosidase	*	Reddy and Krishnan, 2016
Sugarcane bagasse	Alkaline extraction	Bacterial cellulase + endoxylanase	6%	Xue *et al*., 2016
Wheat bran	Destarching	Fungal endoxylanase + Feruloyl esterase	**	Wu *et al*., 2017
Wheat chaff	Ultrasound	Endoxylanase	8% (+2-3% xylose)	Antov and Đorđević, 2017
Wheat straw	Alkaline extraction	Bacterial endoxylanase	11%	Faryar *et al.*, 2015
Wheat straw	Steam explosion	Commercial endoxylanase + glucosidase	8.9%	Álvarez *et al.,* 2017
Birchwood	-	Fungal endoxylanase GH11	28.8%	Nieto-Domínguez *et al.*, 2017
Birchwood, Rye	-	Bacterial xylanase	20% birchwood 3.3% rye	Falck *et al.*, 2013
Kenaf stems (*Hibiscus cannabinus*)	$Ca(OH)_2$ followed by peracetic acid	Endoxylanase and arabinofuranosidase	35.2%	Wan Azelee *et al.*, 2016
Corncob	Alkaline extraction	Bacterial endoxylanase	**	Gowdhaman and Ponnusami, 2015
Corncob	Alkaline extraction	Fungal β-xylosidase	10.7%	Chapla *et al.,* 2012
Corncob	Acidic hydrolysis	Cellulase	14% (+ 32.8% glucose, 14.8% xylose)	Zhang *et al.,* 2017
Corncob	Hydrothermal + 16% alkali	Commercial endoxylanase	35% AX extract containing 9% XOS	Samanta *et al*., 2015
Corncob	Alkali/acid pressure cooking	Fungal endoxylanase	**	Aachary and Prapulla, 2009
Tobacco stalk	Alkaline extraction	Commercial endoxylanase	11%	Akpinar *et al.,* 2010

* Yields are measured and reported in a variety of ways and are indicative rather than directly comparable.

** Insufficient data

Studies of enzymatic XOS production have only occasionally reported the accompanying release of monosaccharides. Uçkun Kiran *et al.* (2013) reported that two commercial endoxylanases released different amounts of undesired xylose monosaccharide in the extracts. Antov and Đorđević (2017) advised that the endoxylanase treatment time is crucial to minimise the production of undesired monosaccharides.

Generally, the activity of endoxylanases on oligosaccharides is not well understood. For example, only a limited number of contradictory reports in the literature discuss whether endoxylanases produce monosaccharides or not (Antov and Đorđević, 2017; Cürten *et al.,* 2018; Uçkun Kiran *et al.,* 2013). Also, the action or preference of endoxylanases for larger or smaller oligosaccharides chains has not previously been reported, as the interest in the hydrolysing enzymes has been primarily their efficacy in breaking down the polysaccharides either all the way to the constituent monosaccharides (as a fermentation feedstock, for example) or just to reduce viscosity. Now, with the increasing interest in oligosaccharides arising from their prebiotic functionality, it has become essential to investigate in detail the mode of action of xylanases on the production and degradation of small xylo- and arabinoxylo-oligosaccharides. Such investigations have faced the difficult technical challenges of measuring these oligosaccharides; however the new HPAEC-PAD method developed in the last chapter now makes such studies feasible.

Therefore, the aim of this part of the project was to study and observe Econase activity on the small oligosaccharides, using XOS standards, to identify the substrate preference and the end products of the enzyme, aiming to increase our understanding of xylanase behaviour which will allow better deployment of this and other endoxylanases to produce XOS.

7.5 Materials and methods

Arabinoxylan oligosaccharide standards were obtained from Megazyme as described in Section 6.3.1. For the analysis of oligosaccharides following treatment with Econase, the newly developed method described in Chapter 6 was used.

Solutions containing the XOS standards were incubated with Econase at a dose of 50 mL per kg of dry sample, at pH 5.5 phosphate/citrate buffer in a water bath at 50°C. (Note that this dosage is 500× the recommended commercial dose, as the purpose of current commercial usage is based on viscosity reduction, which can be achieved by low dosages, whereas the current study aimed to accelerate XOS production which was expected to require higher enzyme dosages; earlier studies with the IPOS DPn method had shown undetectable effects at lower dosages when applied to wheat bran.) Samples were taken during the hydrolysis to evaluate the XOS production and degradation over time. The samples were diluted using phosphate-citrate buffer (at pH 7) and boiled for 20 minutes to deactivate the enzyme permanently (to allow queuing in the autosampler without further enzyme activity), then kept at room temperature until analysis.

7.6 Results and discussion

Figure 7-2 shows the breakdown of xylohexaose by Econase and the evolution of smaller oligos over time. Clearly, the X6 is rapidly degraded by the Econase at this high dosage. The predominant product is the X3, as expected as cleavage in the middle of the X6 chain would result in two X3 molecules, while cleavage elsewhere would give X2+X4 (a single molecule of each) or X1+X5 (although terminal cleavage to produce a monomer is not expected from an endoxylanases). Thus less X4 than X3 was produced at the beginning of the treatment, which soon were subject to further breakdown into xylobiose units. The presence of the X5, in minor amounts in the first 10 minutes, suggests that the enzyme could attack the terminal units, while the increasing amounts of the monosaccharide, xylose, indicate the unexpected breakdown of the small oligos (X3 and X2) into monosaccharides.

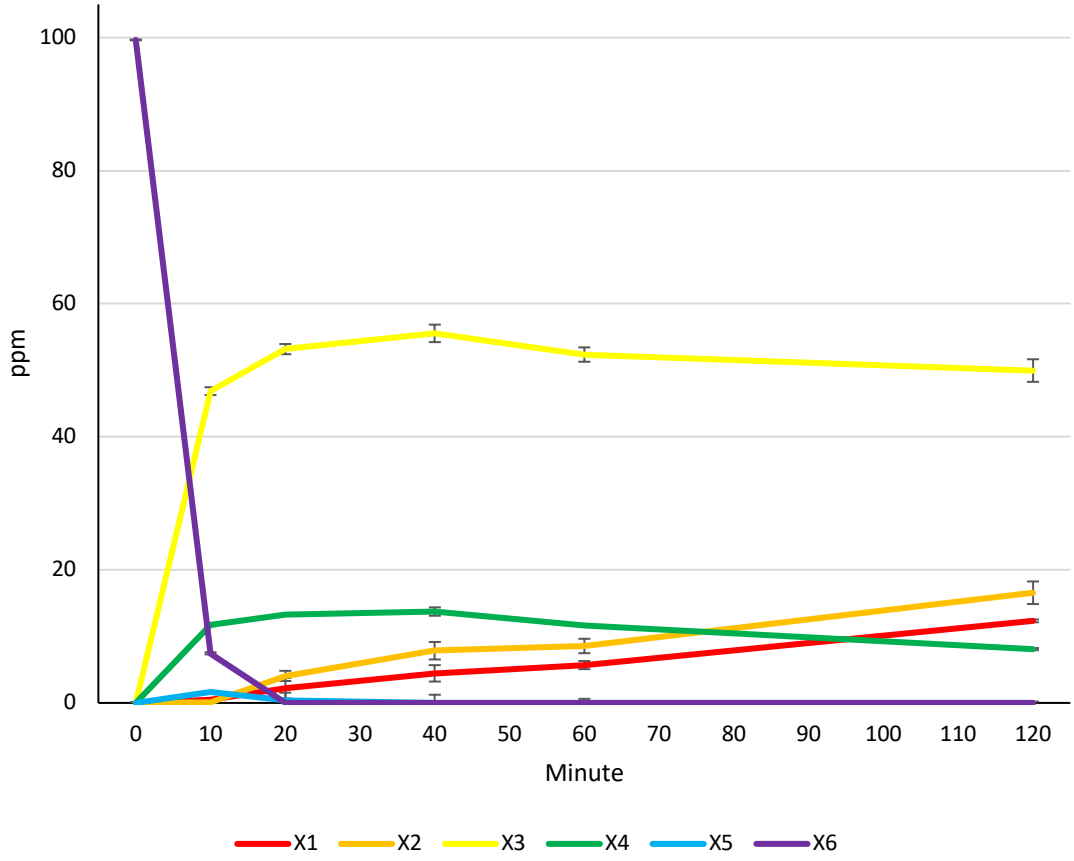

Figure 7-2. Breakdown of xylohexaose upon Econase treatment.

The breakdown of xylopentaose by Econase shows a similar behaviour of attacking the middle of the chain, resulting in production of X2 and X3, as shown in Figure 7-3. The presence of increasing – but small – amounts of monosaccharides again suggests breakdown of the small oligos into xylose. Terminal unit attack appeared to be small as only small amounts of X4 appear along the treatment. The rate of the breakdown of X5 was slower than the breakdown of X6, as 90% of X5 was degraded in the first hour, whilst the whole amount of X6 was degraded after only 20 minutes of treatment.

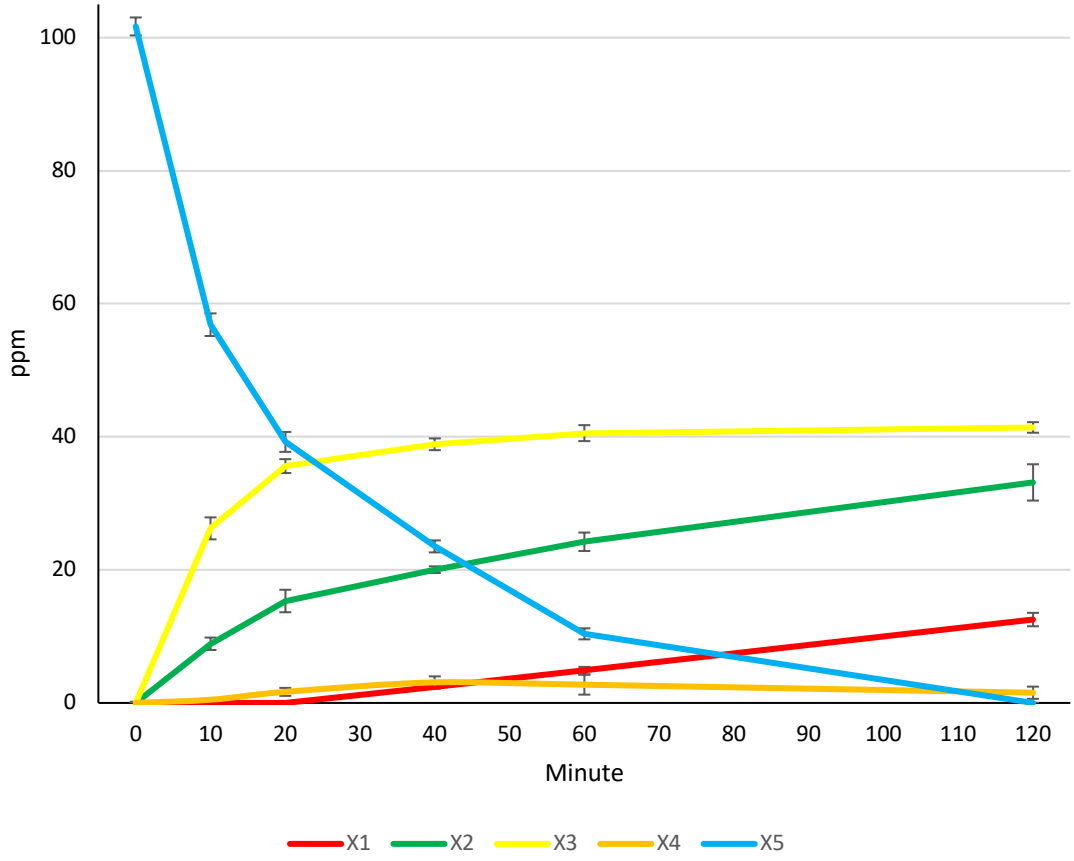

Figure 7-3. Breakdown of xylopentaose upon Econase treatment.

The difference in the degradation speeds of X6 and X5 indicates a preference of the enzyme for the longer chain oligos. This preference was shown more clearly when a mixture of X6 and X5, each at 100 ppm, was treated with Econase at double the dosage, as presented in Figure 7-4. Again, degradation of X6 was complete within 20 minutes, whereas the X5 again took 120 minutes with little change in the curve shape, which suggests that the presence of the longer X6 chain did not slow the degradation of the X5 (the overall enzyme:substrate ratio staying constant). It can be concluded that possibly the rate of the reaction is the main driver behind the enzyme preferences.

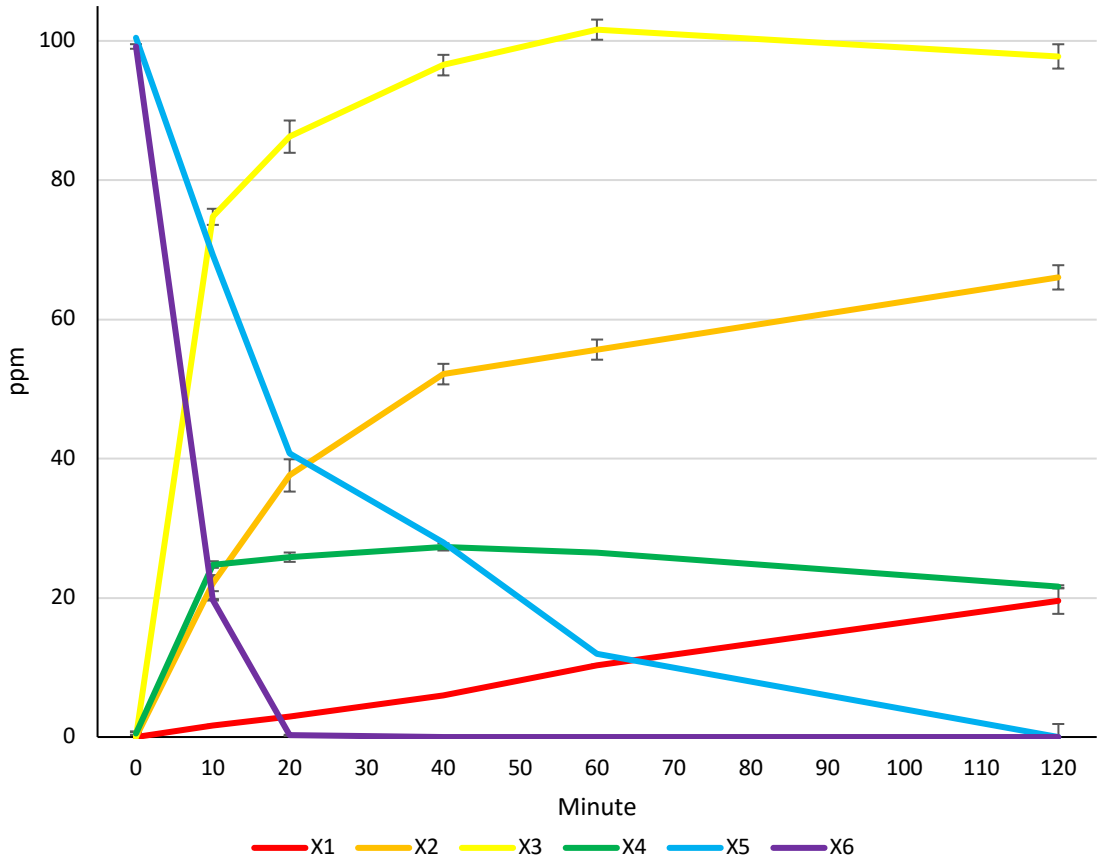

Figure 7-4. Econase degradation of a mixture of xylohexaose and xylopentaose.

Figure 7-5 reports the degradation of X4 upon treating with Econase. In this case, the main degradation product is X2 with the presence of very small amounts of X3 and corresponding X1 monosaccharides. However, the relative quantities of the monosaccharides and xylotriose does not support that the presence of xylose is a result of breaking X4 into X3 and X1 only, which ought to result in almost three times as much X3 as X1 on a mass basis; the greater mass of X1 implies some degradation of X2. Again, this is unexpected for an endo-xylanase; as noted in the previous chapter, this may indicate some side activity or, more likely, indicates the presence of a contaminating exo-xylanase in the Econase preparation.

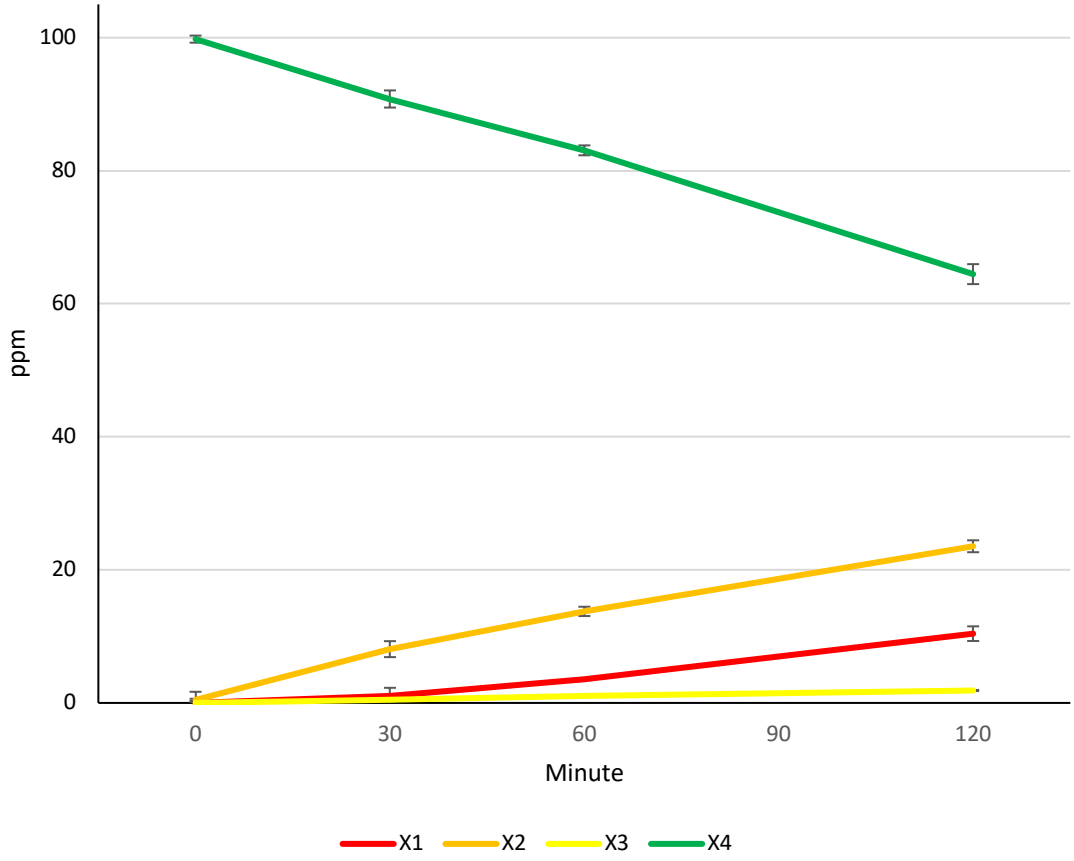

Figure 7-5. Breakdown of xylotetraose upon Econase treatment.

Figure 7-6 and Figure 7-7 show the effect of Econase treatment on the smaller oligosaccharides X3 and X2, showing that Econase can perform very slowly on the small oligos, and again confirming the production of the monosaccharide.

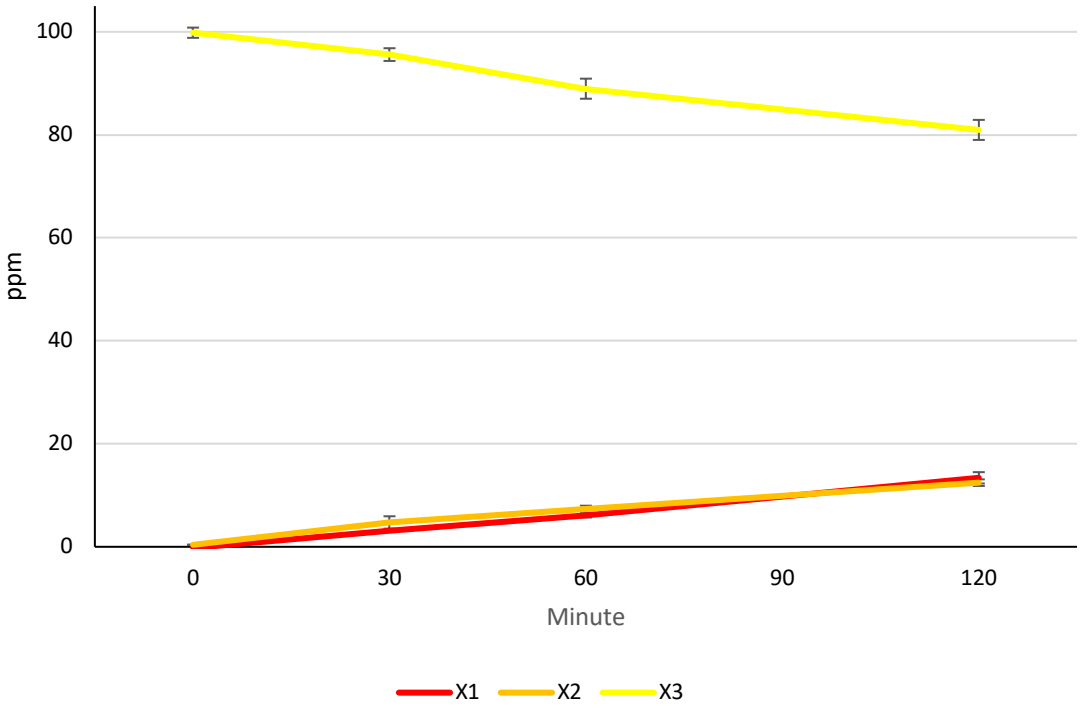

Figure 7-6. Breakdown of xylotriose upon Econase treatment.

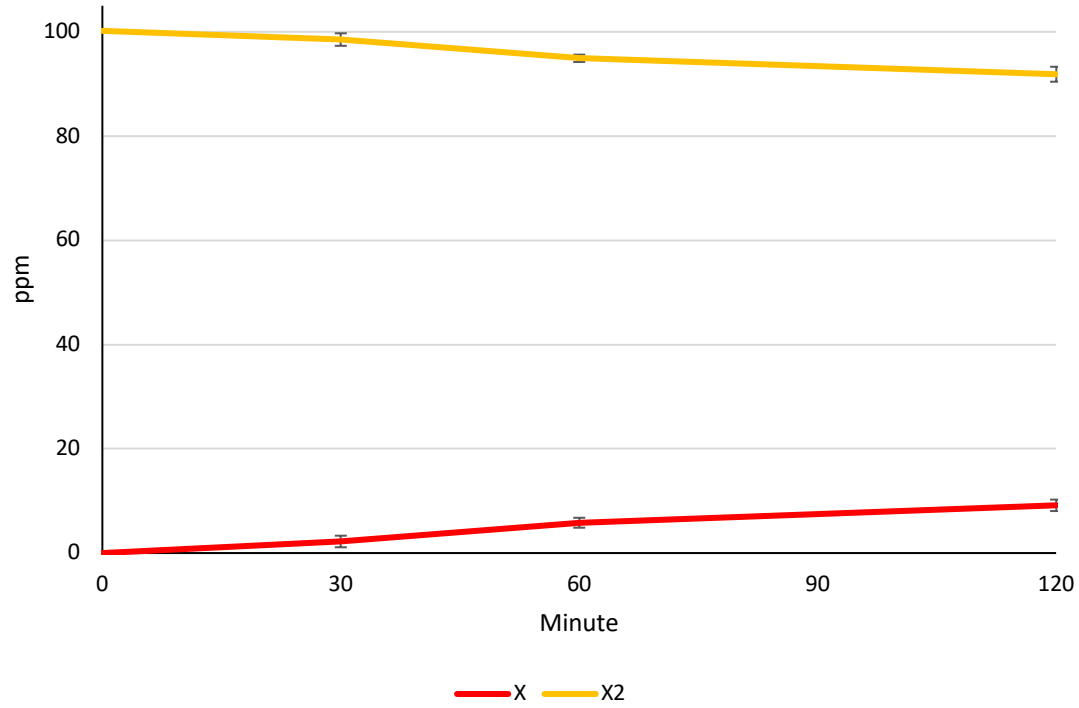

Figure 7-7. Breakdown of xylobiose upon Econase treatment.

These results are in accordance with the findings of Biely *et al.* (1981) who found the Bond-cleavage frequency of xylotriose, xylotetraose and xylopentaose to be

concentration dependent. However, these results differ from those of Mitsuishi *et al.* (1988) who studied three endoxylanases and reported that the enzymes were able to hydrolyse the oligos of X8-X3, but none of the enzymes was able to hydrolyse xylobiose. However, both papers report the dependency of the endoxylanase hydrolysing activity upon the concentration of the substrate. In other words, it might be more plausible to correlate the activity of the enzyme to the substrate-enzyme ratio, or to the enzyme dosage. Figure 7-8 shows the effect of Econase on xylobiose with 20 times the dose used earlier. Surprisingly, the enzyme was capable of degrading almost 99% of the xylobiose substrate, which agrees with Biely *et al.*'s (1981) observation in relation to dose effects, and confirms that, at high dose and in the absence of a preferable substrate, the commercial Econase preparation is able to cleave xylobiose to the monomer.

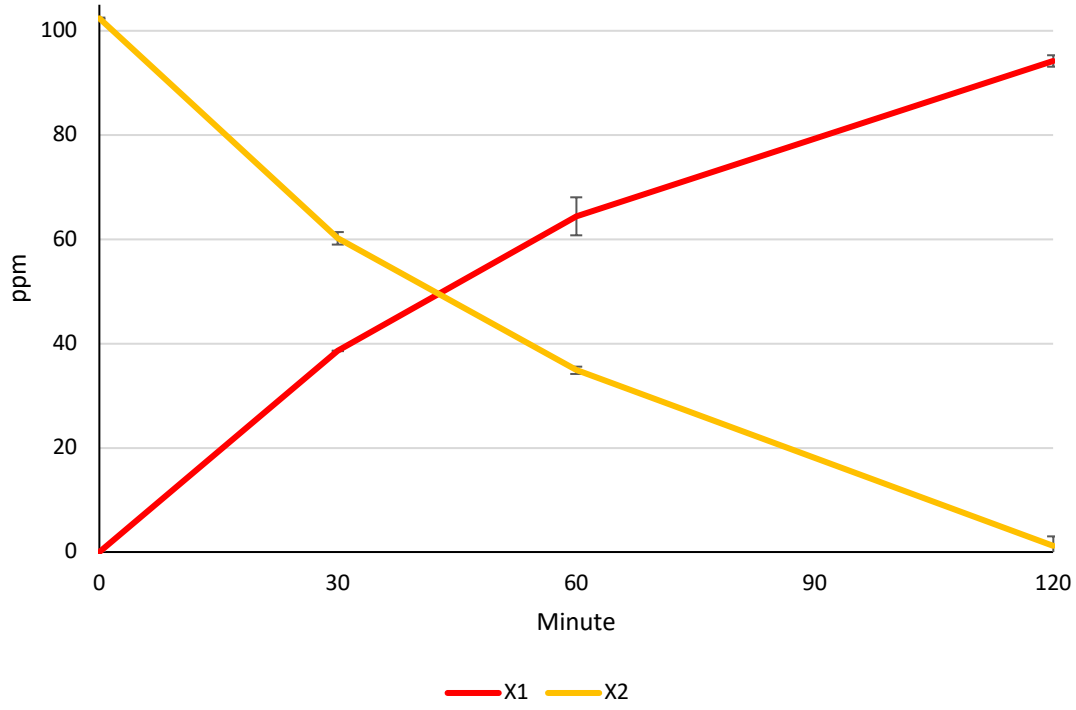

Figure 7-8. Breakdown of xylobiose upon severe Econase treatment.

The conflicting findings and reports in the literature are a reflection of the immensity of the xylanases world and the limited knowledge of their behaviour on small oligosaccharides. For the studied enzyme, Econase, it can be concluded that it is able to hydrolyse linear oligosaccharides of degrees of polymerisation in the range DP6-DP2,

and that the rate of the hydrolysis is greater for the larger molecules. Figure 7-9 compares the rates of degradation of the studied oligos, showing the much faster degradation of the larger chains.

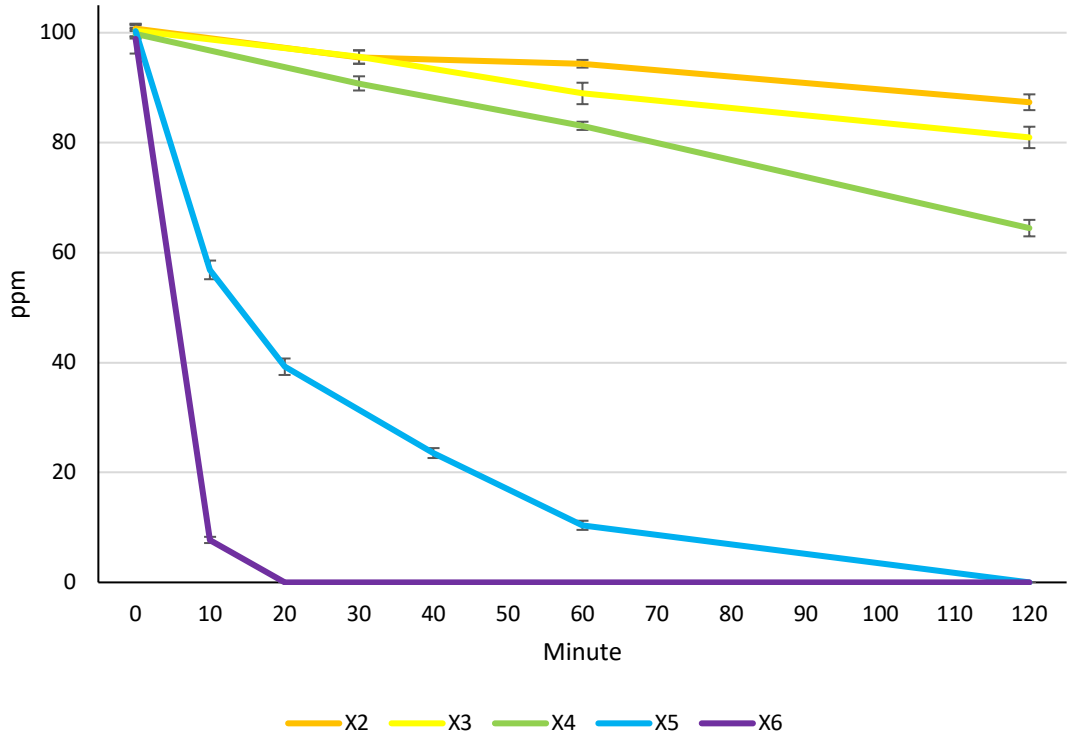

Figure 7-9. Degradation of XOS (DP6-DP2) upon Econase treatment.

Figure 7-12 shows the treatment of 3^3-α-L-arabinofuranosyl-xylotetraose and 2^3-α-L-arabinofuranosyl-xylotetraose with Econase, both are illustrated in Figure 7-10. The experiment showed that Econase activity was different in the presence of the arabinose side unit. Apparently, the 3^3-α-L-arabinofuranosyl-xylotetraose molecule was not susceptible to Econase activity during the treatment, whereas Econase slightly attacked the 2^3-α-L-arabinofuranosyl-xylotetraose molecule and chopped off the terminal xylose unit producing 2^3-α-L-Arabinofuranosyl-xylotriose, shown in Figure 7-11.

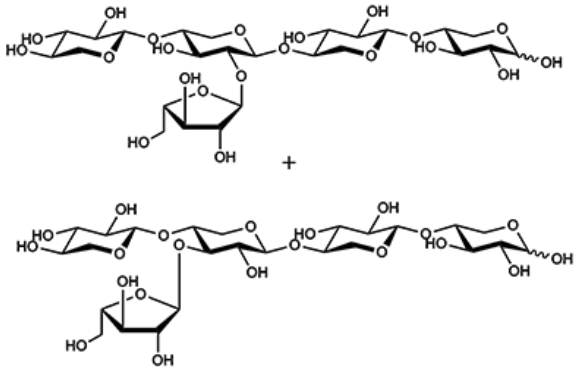

Figure 7-10. Structure of 3^3-α-L-arabinofuranosyl-xylotetraose and 2^3-α-L-arabinofuranosyl-xylotetraose.

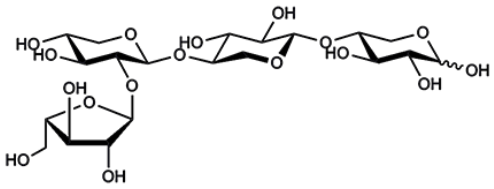

Figure 7-11. Structure of 2^3-α-L-Arabinofuranosyl-xylotriose.

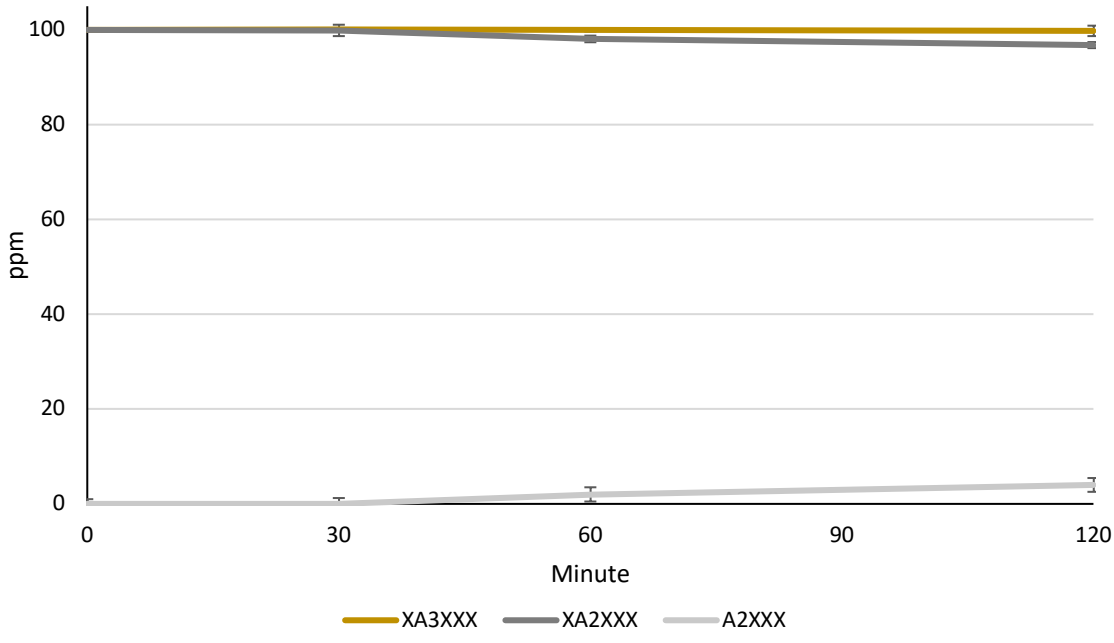

Figure 7-12. Treatment of 3^3-α-L-arabinofuranosyl-xylotetraose and 2^3-α-L-arabinofuranosyl-xylotetraose with Econase.

Clearly, Econase affinity towards the substituted oligosaccharide varied according to the O-3 and O-2 α-L-arabinofuranosyl groups. In either case, the middle of the tetra-xylan chain was protected by the arabinose side unit, and the enzyme was unable to break it down into xylobiose. However, in the case of O-2 α-L-arabinofuranosyl group, it seems that the steric hindrance of the side chain could not protect the terminal xylose unit and a "slow" or weak hydrolysis occurred, yielding tiny amounts of xylose and a smaller oligosaccharide.

7.7 Summary

Xylanases are a widely varying group of enzymes that hydrolyse the xylan chains in hemicelluloses. There is an unlimited number of sources of xylanases including plants, insects, fungi and bacteria and thereby a huge variation in their nature, characteristics and mode of action. It can be said that the only thing in common among xylanases is the fact that they hydrolyse xylan polysaccharides. Xylanases are widely used in industry, with paper and pulp bleaching the largest market, and they are also widely used in the food industry, particularly as an ingredient in breadmaking. Xylanases now offer a promising new market for the production of oligosaccharides with prebiotic functionality.

In earlier trials carried out in this project aiming to produce AXOS, it was observed that Econase, the endoxylanase used, was yielding only small amounts of oligosaccharides and monosaccharides with an evolving profile as treatment progressed. Knowledge about the precise effects of xylanases on production and degradation of oligosaccharides is not well established; as this has only become of interest relatively recently, there has not been a good method to measure these oligosaccharides, and there has not been the availability of standards. Therefore, it was essential to observe the Econase mode of action on oligosaccharide production and degradation with time during treatment.

The general understanding of endoxylanases is that they perform the hydrolysis in the middle of the polysaccharide chain to create two smaller polysaccharides, in contrast to

β-xylanases which hydrolyse the terminal sugar unit, reducing the polysaccharide chain into monosaccharides. The studied commercial enzyme preparation, Econase, broke down larger oligosaccharides more rapidly than smaller ones, while showing unexpected production of xylose monomer. Econase treatment of larger molecules, X6 and X5, gave a complex evolving profile of smaller oligos as these were created and then destroyed. Even X3 and X2 were susceptible to Econase degradation, especially at high dosages of the enzyme, possibly due to side activity or, more likely, contamination with an exo-xylanase in the enzyme preparation. Minimising or eliminating the production of the monomer is crucial for maximising the creation of the larger molecules that have the prebiotic functionality.

The substitution pattern of the substrate is a major key in the xylanase mode of action. It was clear that Econase has limited performance in the presence of arabinose side units, as the side chains protect the xylan backbone by steric hindrance. This hindrance is also subject to the location of the arabinose linkage to the xylose unit in the chain; the O-3 linkage showed complete protection of the tetra-xylose chain in the studied AXOS, whereas the O-2 linkage allowed some hydrolysis of the terminal xylose unit, releasing small amounts of xylose. This chain protection may explain the low XOS yields obtained from the treatment of DWG AX in Section 5.5.2, as the extracted AX had a relatively high A/X ratio which represent a high branching pattern that would tend to protect the xylan chain from enzyme attack.

Thus, producing AX extracts of lower A/X ratio may be key for the conversion of AX into (A)XOS. The water-extractable arabinoxylan with its lower A/X ratio might provide a more suitable substrate for the XOS production. Therefore, the next step in this project aimed to integrate the extraction of WEAX from the Solubles fraction within the bioethanol production process and to evaluate the conversion of the produced AX into (A)XOS, in comparison with the water-unextractable AX from the DWG.

8 Extraction of water-extractable and water-unextractable arabinoxylan within biorefineries

8.1 Introduction

The need to reduce the dependence on fossil fuel along with the ambition to reduce greenhouse gas emissions and climate change have led to the emergence of biorefineries. However, the challenges facing the development and expansion of biorefineries industry were greater than their economic performance could sustain, as demonstrated by the recent Vivergo closure. The transformation into the integrated biorefinery where multiple products are produced and achieve economic viability through successful integration is key for the biorefinery sector to survive and compete with the oil refinery industry which already benefits from extensive process integration. Arabinoxylans are promising candidates to be co-produced within a biorefinery via integration with ethanol production to give a range of AX products including high viscosity AX (high branching WUAX), medium viscosity AX (medium branching, WEAX) and (A)XOS prebiotics. This chapter reviews possible integration scenarios for the development of biorefineries, and presents trials performed to integrate the production of a range of AX products within the biofuel production process using the GUNT bioethanol plant.

8.2 Integrated biorefineries status

Biorefineries are one of the evolving bio-economies that possess great potential to deliver economically competitive and environmentally favourable models of the chemical and energy industries in the 21st century. However, the emergence of biorefineries has been a fragile undertaking thus far, as illustrated by the closure in late 2018 of the Vivergo plant in the UK. The development of the biorefinery industry, as an immature industry, is still struggling against political, technological and economic barriers. Integrated processing, already a key feature of the oil refineries with which

biorefineries must compete, will be key to the technological and economic success of biorefineries. AX-based materials offer a natural co-product for integration with bioethanol, and therefore represent a strategically important opportunity to enhance the viability of biorefineries, while bringing new food, feed and non-food products to these markets.

On the technological and economic side, the integration of biorefineries within an industrial cluster can significantly increase energy efficiency and reduce production costs (Ng *et al.,* 2015). In such a scenario, the industrial cluster will provide both sources and sinks of heat and energy that can be optimised through integration, thereby minimising the process demand for energy. Also, the co-location of several production processes in the same cluster will reduce transportation costs if the feedstock of one process is produced by a co-located process. Another important advantage of this scenario is the reduction in the cost of the infrastructure as facilities of water treatment, steam, safety, electricity, *etc.* can be shared among several processes (Kimm, 2008). Figure 8-1 represents an overview of the biorefinery cluster concept and the potential products of fuel, energy and biochemicals. However, this scenario lacks the production of food and nutraceuticals; in general biorefineries and food have been seen as incompatible and have been kept separate in government policy (*e.g*. in the emphases of research initiatives), when in fact there are opportunities for synergies that could benefit both biorefineries and the food industry; AX-based products are a notably promising example (Martinez-Hernandez *et al*., 2018). Nevertheless, the concept of multiple co-products made economically viable through co-located processes that benefit from integration remains at the heart of scenarios for successful biorefineries.

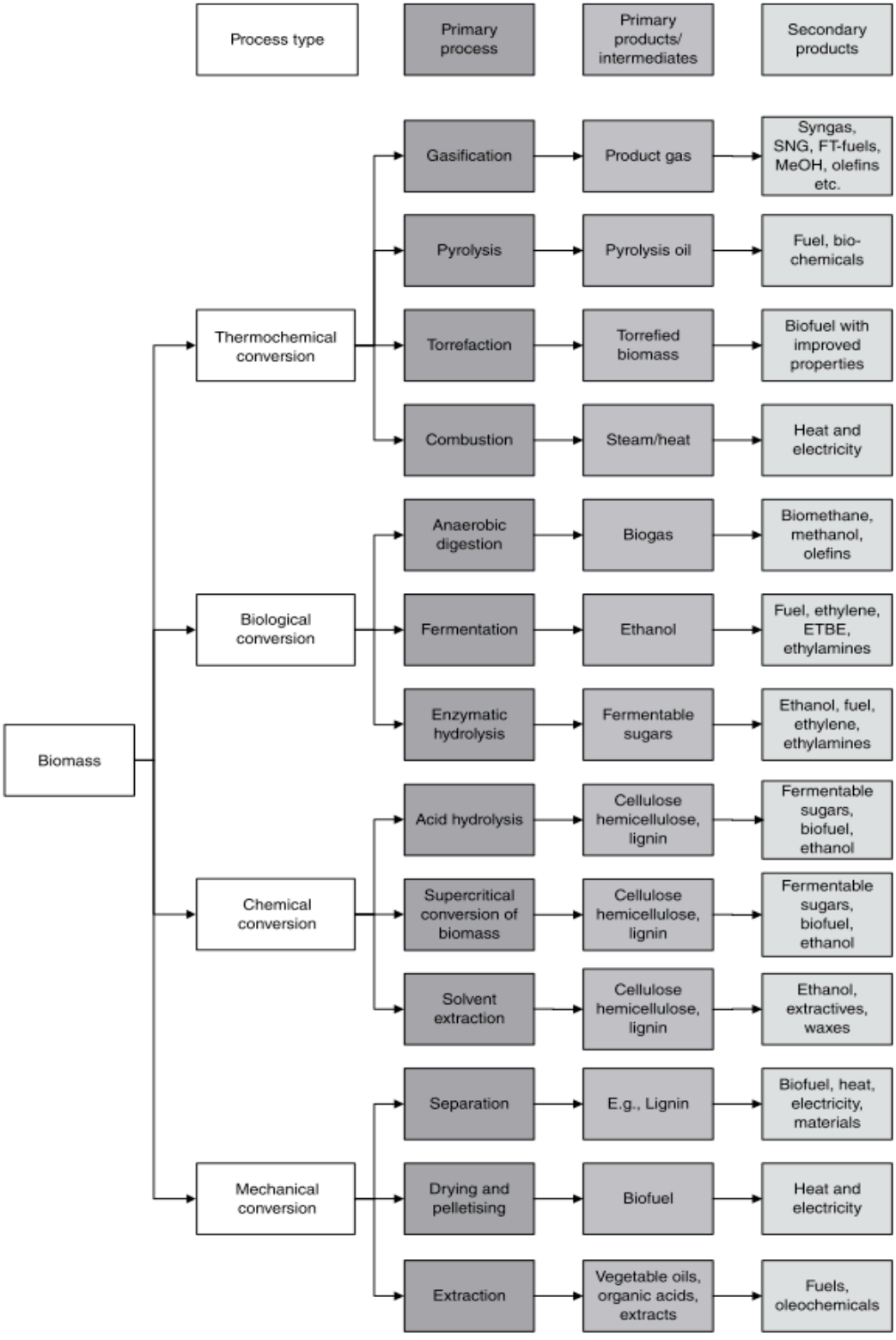

Figure 8-1. Overview of biomass conversion processes and potential products (Ng et al.,2015).

Misailidis *et al.* (2009) presented a process simulation and economic analysis of the co-production of arabinoxylan alongside ethanol within biorefineries, showing that in this

context production of AX, while not cheap, could be economically viable. The integration of the extraction of relatively pure AX from wheat bran, recovered by pearling technology prior to the bioethanol fermentation, with the utilisation of the biorefinery's facilities to recover the used ethanol, seems to offer a feasible pathway to commercialise the production of AX. Recently, Martinez-Hernandez *et al.* (2018) presented a conceptual design for the integration of AX and AXOS production within a bioethanol production facility using mass pinch analysis to minimise bioethanol usage; a range of AX and AXOS products, precipitated at varying ethanol concentrations, increased the scope for integration and for minimising the costs. The work illustrated that retrofitting existing bioethanol processes for integrated AX and AXOS production might offer significant new revenue streams for biorefineries.

However, the production of AX from bioethanol by-products and evaluation studies of integrated AX production in the literature have focused on the solid intermediate wet by-product (DWG), the final dry by-product (DDGS) or the wheat bran fraction before fermentation as the source of AX. The earlier analysis carried out in the current project of the intermediate by-products of bioethanol process, DWG and Solubles, illustrated that around 25% of the total AX of the starting wheat is located in the Solubles fraction (Figure 3-9); evidently water-extractable AX was released from the cell wall structure and solubilised during the hydrolysis and the distillation thermal treatment such that it ended up in the Solubles fraction.

This fraction of AX has been perhaps neglected in the literature due to the assumption that other components of unfermented materials will also be solubilised, such as the plant pigments, proteins and oligopeptides, cellulose oligosaccharides and fractions from the yeast cell wall. Also, it is perhaps assumed that these soluble AX molecules have a small Mw and the separation and purification of the WEAX fraction will not be effective. However, now that it is clear that a range of AX products, including the small XOS and AXOS molecules, are of interest, it is appropriate to examine the AX material in the Solubles stream of a bioethanol plant.

Therefore, this part of the work presents trials to recover the WEAX from the Solubles while also extracting the WUAX from the DWG. Both extracts were characterised and then tested as substrates for enzymatic conversion to oligosaccharides.

8.3 Materials and methods

The materials used in this study (the starting raw material, chemicals and enzymes) are as listed in Sections 3.4.1 and 5.4.1. The CE-640 bioethanol plant, described in Section 3.4.2, was used for both the ethanolic fermentation to produce the DWG and Solubles stream and the AX extraction from both DWG and Solubles.

For the liquid-solid separation, a Carlson® filter press was used, shown in Figure 8-2 (Carlson Filtration Limited, UK), with an EE4.6HF depth filter sheet (20 × 20 cm).

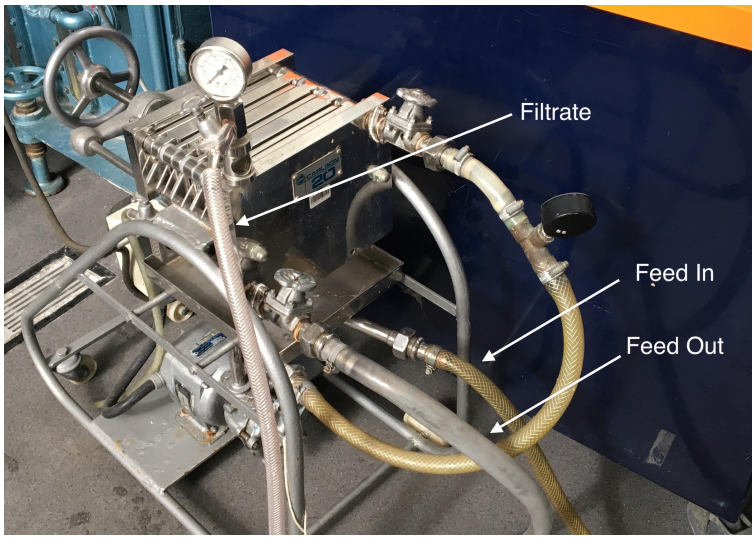

Figure 8-2. Carlson filter press.

For the ultrafiltration concentration and purification, the hybrid version of the ultrafiltration system (described in Section 5.4.2) was further upgraded by combining the Eco® gear pump with a high performance AMI® spiral membrane polyethersulfone with 10,000 MWCO cut-off, 10 m^2 surface area and 4" diameter × 40" length dimensions, presented in Figure 8-3, obtained from Wateranywhere®, USA.

Figure 8-3. AMI® spiral ultrafiltration membrane and housing.

The combination of the powerful gear pump with the large surface area spiral membrane enabled fast and effective concentration and diafiltration of the extract with a filtration rate of 1.5-2 litre per minute. The system is described diagrammatically in Figure 8-4 and the final assembled kit is shown in Figure 8-5.

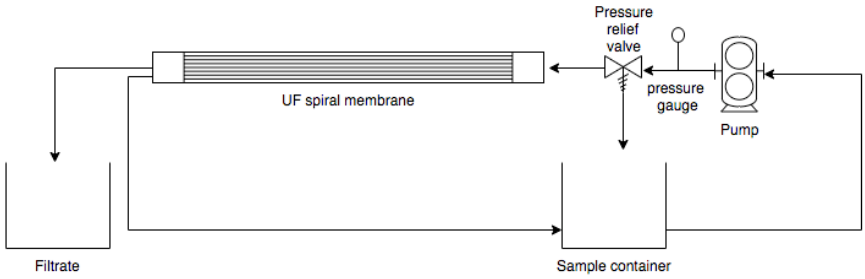

Figure 8-4. UF system diagram.

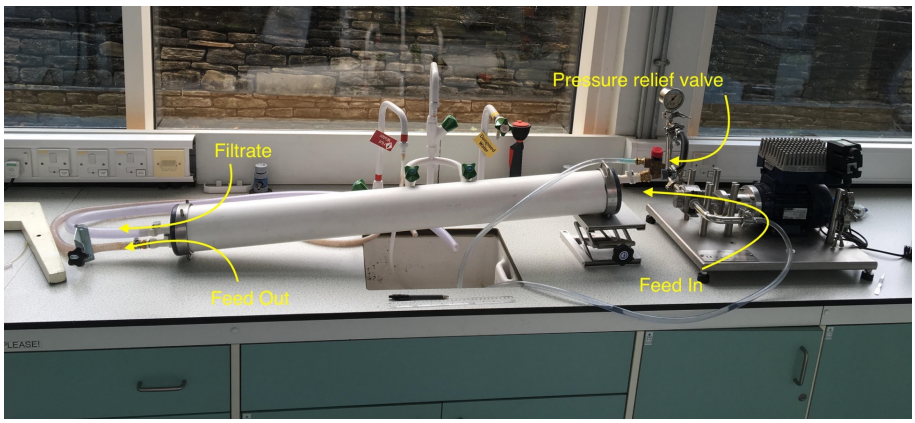

Figure 8-5. Assembled large-scale ultrafiltration kit.

8.3.1 Methods

Aiming to extract both WEAX and WUAX from DWG and Solubles, the whole process was optimised. The liquefaction was performed in the distiller of the fermentation plant as it offers the facility boil the material. The saccharification, fermentation and the chemical extraction of WUAX were similar to the process carried out in earlier parts of this project. Figure 8-6 illustrates the complete process of WEAX and WUAX production.

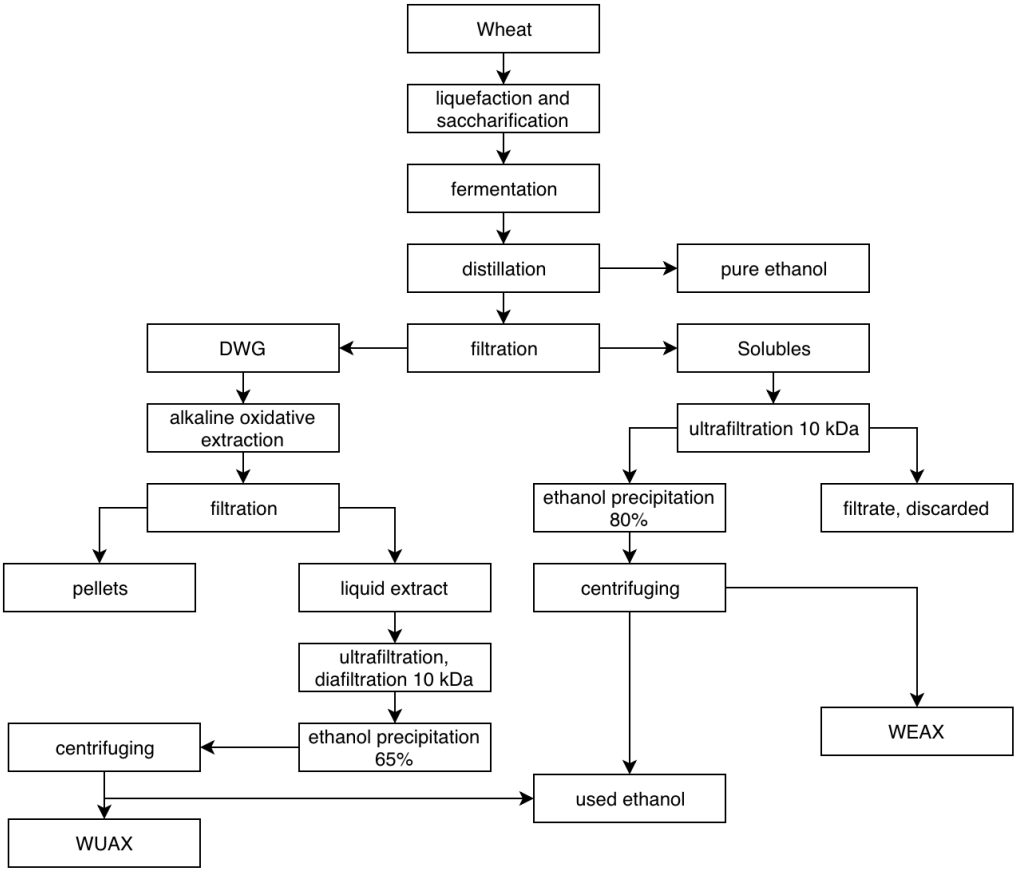

Figure 8-6. The process of WEAX and WUAX production.

A batch of 10 kg of ground wheat grain was mixed with 25 litres of water and boiled at 100°C inside the CE-640 distiller for 3 hours. Thereafter, the mixture's temperature was reduced to 90°C, and the whole mixture was transferred manually to the mash tank. The distiller tank was rinsed with 5 litres of water, and the wash was added to the mixture in the mash tank. The enzymatic hydrolysis of the grain starch was commenced by adding α-amylase with continuous stirring at 90°C for one hour. The hydrolysis was

continued for 3 hours with gluco-amylase after reducing the mash temperature to 55°C and adjusting the pH to 4.5.

By the end of the hydrolysis, the mash was transferred to the fermentation tank where the temperature was adjusted to 28±2°C, and the yeast was added. The fermentation was allowed to take place over 72 hours with intermittent stirring. Thereafter, the fermented mash was transferred to the distillation unit where the produced ethanol was separated over 4 hours. After the completion of the distillation procedure, the ethanol was placed into secure storage and the power was switched off to allow the material inside the distiller to cool to the ambient temperature overnight.

The leftover material from the solid fermentation was separated from the mash by filtration using the Carlson filter press and washed with 20 litres of water to remove the adsorbed Solubles from the Distillers Wet Grain.

The DWG was suspended in 20 litres of water in the mash tank, and the pH was adjusted to 8 to be treated with protease at 60°C for four hours. The protein-reduced DWG was filtered and recovered, and the filtrate was discarded. The filtration cake was suspended again in 20 litres of water in order to extract the Water-unextractable arabinoxylan, the pH was adjusted to 11+, the temperature was elevated to 60°C, antifoaming agent was added, and hydrogen peroxide was added gradually with vigorous stirring to reach the concentration of 2%, with the total extraction time being 4 hours. The mixture was cooled to 25°C, the pH was neutralised with hydrochloric acid, the solubilised AX extract was separated from the solids by filtration, the cake was washed with 10 litres of fresh water and filtered, and the filtrate was added to the original filtrate. The total extract was concentrated by ultrafiltration, then diafiltered by adding 20 litres of water to the solution and concentrated again. WUAX was precipitated with 65% ethanol overnight, and precipitated WUAX was collected by centrifugation, dried in a 40°C oven overnight, and stored for analysis and characterisation. The ethanol was recovered by distillation.

The Solubles portion from the fermentation, about 50 litres, was subject to ultrafiltration over 10 kDa to remove the small molecules and recover the water soluble arabinoxylan. The concentrated Solubles were precipitated with 80% ethanol overnight, and precipitated WEAX was collected by centrifuging, dried and stored for further analysis. The ethanol was again recovered again by distillation.

8.3.2 Characterisation of the extracts

Both extracts of WEAX and WUAX were characterised for AX content, average molecular weight, protein, ash, moisture and fat contents. The constituent sugars analysis was carried out using the HPAEC-PAD, and SEC-MALLS was used for the Mw as described in Section 5.5.1. The protein, ash and moisture contents were evaluated as described in Sections 3.6.3 and 3.6.4.

8.3.3 Enzymatic treatment of WEAX and WUAX

For the enzymatic oligosaccharide production, 1 g from each extract was suspended in 100 mL of phosphate-citrate buffer at pH = 5, the samples were stirred overnight to ensure the complete solubilisation of AX in the solution. Samples were placed in a water bath at 50°C and Econase was added at a dose of 50 mL per kg. Sub-samples were taken after 1 and 2 hours and diluted with phosphate-citrate buffer of pH = 7, then boiled for 20 minutes to deactivate the enzyme permanently.

During this research, Econase has been used at a dose of 50 mL per kg which is 500× the commercially recommended dose. This dose was chosen according to preliminary results where the normal dose did not show any effect (Chapter 4). However, the findings presented in Chapter 7 clarified that Econase has the ability to degrade XOS. Thus it became clear that the high dosages employed to accelerate AX breakdown may have also degraded any XOS produced. The development of the new HPAEC-PAD method for the accurate measurement of XOS, and the extraction of more suitable substrate, the WEAX, offered the opportunity to investigate the effect of the enzyme dosage on the production of XOS. Therefore, samples of WEAX was solubilised in buffer

at pH 5, placed in a water bath at 50°C and Econase was added at a doses of 50 mL per kg, 50 mL per 10 kg, 50 mL per 100 kg and 50 mL per 1000 kg for 2 hours. The samples were again diluted with phosphate-citrate buffer of pH = 7 and boiled for 20 minutes to deactivate the enzyme permanently.

The ability of Econase to produce xylose monosaccharide as shown in Chapter 7, and the unusual behaviour of the enzyme towards the branched AX oligosaccharides discussed in Section 7.6, suggest the possibility of Econase being contaminated with traces of an exo-xylanase which might be responsible for the monosaccharides production. As Econase contains a thermal stable endo-xylanase, thermal deactivation is a strategy for eliminating contaminating enzymes. To test this strategy and clarify the reason for the appearance of xylose monomer, Econase solution was heated to 70°C for 30 minutes then applied to the WEAX extract at dose of 50 mL per kg as described above.

8.4 Results and discussion

The fermentation procedure yielded from 10 kg wheat a total of 3.2 litres of 90% ethanol, 2.1 kg (db) of solids in the form of DWG and around 50 litres of Solubles solution. The chemical extraction of WUAX yielded 85 g of crude extract (a 0.85% yield) while the extraction of WEAX resulted in 92 g of crude extract (a 0.92% yield).

The characterisation of the extracts included the analysis of the AX purity, moisture, protein and ash, as shown in Table 8-1. The determination of AX purity was performed by measuring the arabinose and xylose sugars in the sample using HPAEC-PAD after hydrolysing the sample with 2M trifluoroacetic acid for one hour. The recovery of the hydrolysis was calculated for each extract using a suitable mixture of low viscosity AX (50 kDa) and medium viscosity AX (300 kDa) Megazyme standards. To ensure these standards were sufficiently similar to the extracted material to allow recoveries to be calculated, the molecular weight distribution of the samples was evaluated.

Table 8-1. Chemical composition of WEAX and WUAX

	WEAX	WUAX
AX purity %	74.24 ± 0.13	51.62 ± 0.09
A/X ratio	0.49	1.18
Average Mw (kDa)	70	110
Total crude protein %	5.8 ± 0.21	9.8 ± 0.31
Ash %	0.72 ± 0.1	4.54 ± 0.1
Moisture %	8.90 ± 0.49	14.15 ± 0.47

The original 10 kg wheat contained 590 g AX which was divided between ~350 g in DWG and ~150 g in the Solubles. The recovery of 92 g of WEAX of 74% purity implies that 70 g of the Solubles AX was recovered (around 47% of the available 150 g). The purity of the 85 g of WUAX was 51%; this means only 42 g of DWG AX was recovered (around 12% of the available 350 g) which is less than the AX obtained by the extraction performed in Section 5.5.1 where 43% of the DWG AX was recovered. However, the purity of AX extraction had been a challenge (Bell 2015; Jacquemin *et al.,* 2015b) and the purification applied in this project significantly increased the purity of the extract.

The molecular weight distribution of both WEAX and WUAX was measured using SEC-MALLS, giving average weights of 70 and 110 kDa, respectively. The Mw of the water-extractable AX was lower than the water-unextractable AX as expected, because the WEAX naturally comprises AX units with fewer linkages to the cell wall matrix, hence fewer arabinose side branches, whereas WUAX consists of larger molecules with more linkages to the other cell wall components. However, the extracted WUAX presented a smaller Mw than the previously extracted AX, described in Section 5.5.1, which is a result of changing the extraction conditions, particularly the solids–liquid ratio of the extraction. This change in the Mw as a result of the different extraction conditions explains the low yields obtained, as the WUAX has a lower average molecular weight than the earlier material, more AX was lost during the diafiltration procedure.

The total crude protein content in the Solubles WEAX is lower than the DWG WUAX, (5.8% compared with 9.8), even though the dried Solubles had a total protein content of 37% (Section 3.7.3). It is convenient that the majority of the proteins in the Solubles appear to have been small molecules that were able to pass the 10 kDa ultrafiltration

membrane. However, the extraction of WEAX in this project was performed according to a minimum cost; a purification step of protease treatment might be useful in reducing the 5% protein content to an even lower level.

The water-unextractable AX protein content was about 10%, which is lower than the 14% protein content of the previous extracts (Section 5.5.1). This indicates that protease treatment could be more effective when it precedes the AX extraction, as performed in this current experiment.

The low ash content in the WEAX compared to the WUAX is due to the chemical-free recovery method used in this project. However, the ~5% ash content in the WUAX is significantly less than the ash content in the previous extracts, around 13%; this is a reflection of the performance of the upgraded ultrafiltration system and the use of diafiltration.

The low arabinose substitution of the WEAX suggested this might be a suitable substrate for oligosaccharide production via enzymatic treatment. 1 g of this extract was therefore resolubilised in 100 mL of buffer at pH 5 and treated with 50 µL Econase at 50°C for 1 and 2 hours. The amounts of xylose plus oligosaccharides up to X6 produced were measured.

Figure 8-7 shows the percentage conversion of the extracted AX after Econase treatment for one and two hours. Clearly, Econase treatment of the WEAX for 1 hour produced significant amounts of X2 and X3 as well as some xylose monomer, but there was no evidence of the larger oligos (X4-X6). Longer treatment degraded the X3 into X2 and X1. In general, the treatment yielded 46% conversion of the material in the form of small XOS (xylobiose 27% and xylotriose 19%). A small portion (11%) of the WEAX was converted into monosaccharide after one-hour treatment. The longer treatment reduced the amount of xylotriose to 40% and doubled the amount of xylose.

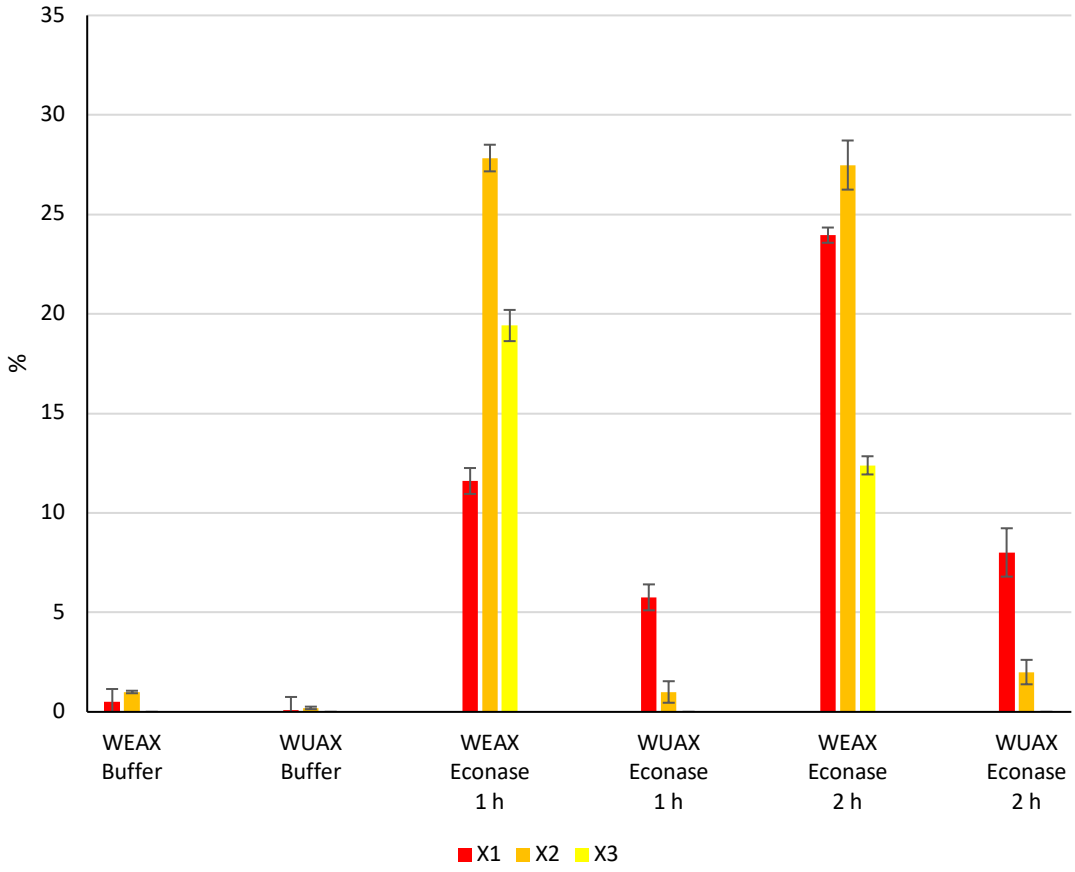

Figure 8-7. XOS production from WEAX and WUAX via Econase treatment for 1 hour and 2 hours.

For the WUAX, the treatment was much less effective, yielding mostly xylose and a smaller amount of xylobiose after 1 hour, and a little more after two hours. Evidently the high branching nature of WUAX protects the molecule against the enzymatic hydrolysis apart from the release of some xylose and traces of xylobiose, whereas the low substituted WEAX seems to be a more susceptible substrate for the enzyme activity.

Although the WUAX might not be the optimal substrate for prebiotic production, this does not compromise its importance for utilisation in other application such as food and pharmaceutical industry as a novel biopolymer that can serve several purposes (such as a thickener, gel making, film making) (Chen *et al.,* 2016; Erum *et al.,* 2015; Khathuriya *et al.,* 2015; Lazaridou and Biliaderis, 2007; Phan *et al.,* 2002).

Figure 8-8 presents the Econase treatment of WEAX when used at a doses of 50 mL per kg, 50 mL per 10 kg, 50 mL per 100 kg and 50 mL per 1000 kg (500×, 50×, 5× and 0.5× the commercial dose) for 2 hours. Clearly, the monosaccharide concentration was greater with the high dose (about 23%), whilst with lower doses the xylose concentration did not exceed 3%. The total released XOS was about 53% with the high (500×) dose comprising mainly X2 (32%), X3 (17%) and X4 (3%); whilst the total XOS was smaller with the lower doses (3% for 0.5×, 34% for 5× and 45% for 50×). The large XOS (X5 and X6) appears only with the lower dosages.

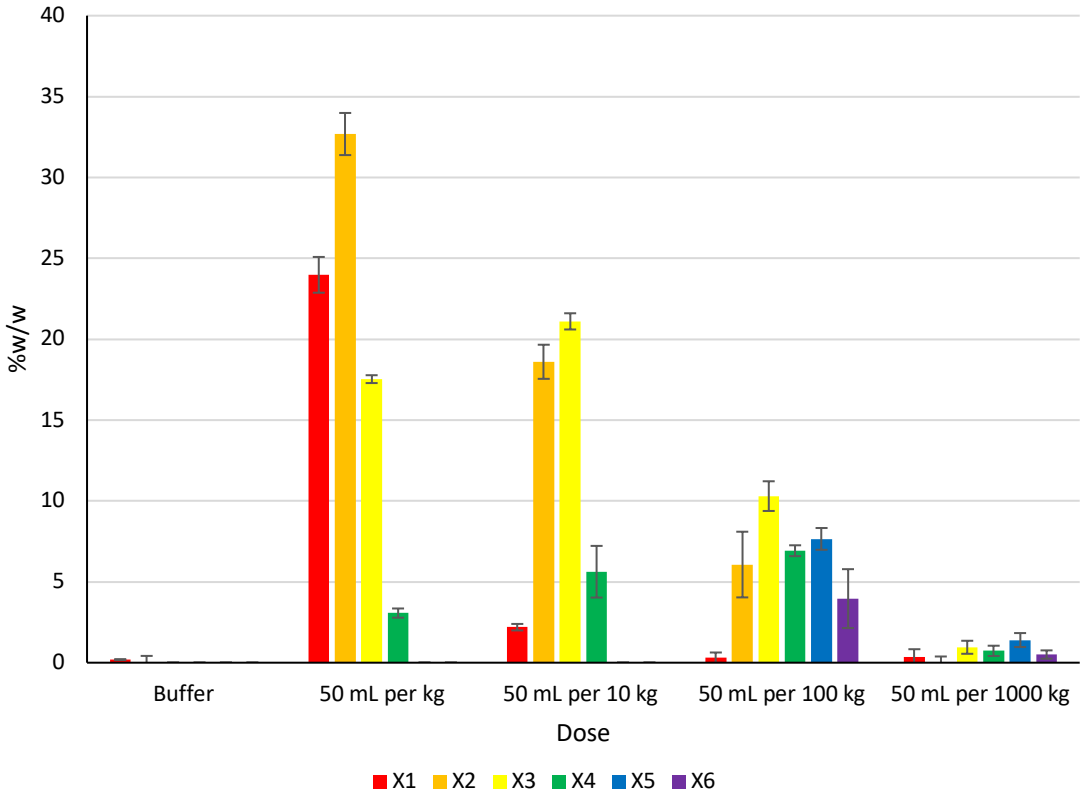

Figure 8-8. WEAX treatment with Econase at 500×, 50×, 5× and 0.5× the commercial dose.

Evidently, the high dose of the Enzyme produced greater amounts of the total XOS with notable elimination of the larger XOS (X5 and X6). However, the high yield of X2 and X3 is desired as these small oligos have the highest prebiotic activity (Gullón *et al.*, 2008a; Van Craeyveld *et al.,* 2008).

The ability of Econase to produce undesired monosaccharides was significantly reduced after thermal treatment. Figure 8-9 shows the difference in Econase effect on WEAX (at a dose of 50 mL per kg) before and after heating the enzyme solution to 70°C for 30 minutes. The amount of released monosaccharide was significantly decreased from 20% to 1.5% after the thermal treatment. Also, the total XOS produced (X2, X3 and X4) was increased from 51% to 59%, also the XOS profile shows a higher X3 after thermal treatment, whilst X2 was predominant in the direct treatment.

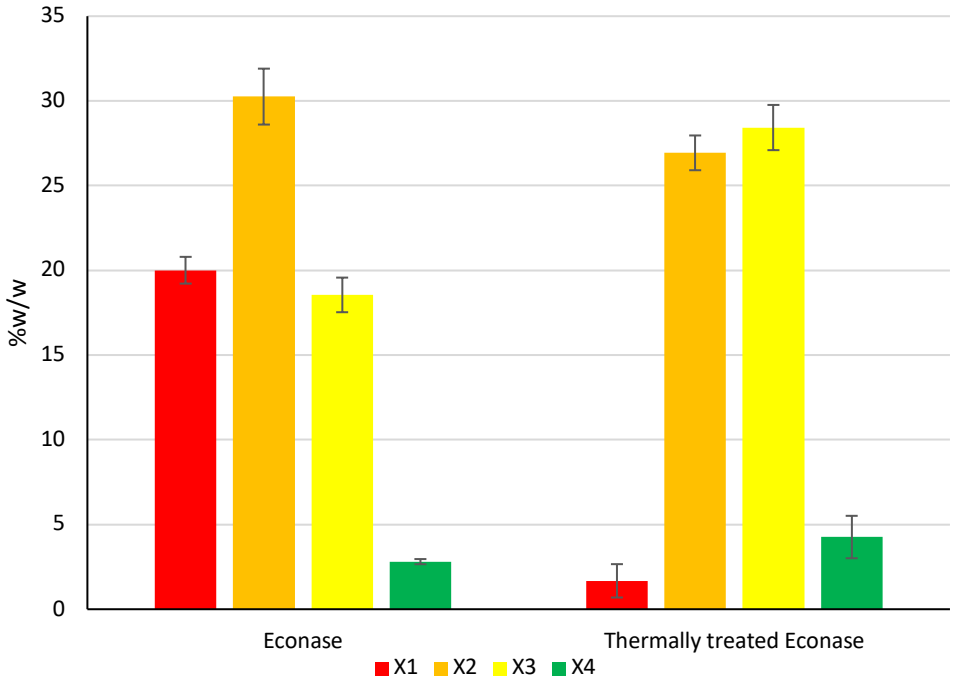

Figure 8-9. Econase effect before and after thermal treatment at 70°C for 30 minutes.

Clearly, the results suggest that the commercial Econase preparation does not contain a pure endoxylanase, and a contaminant exo-xylanase is affecting the overall behaviour of the Econase. The increased amount of X3 implies that the contaminant enzyme has an exo-xylanase activity that converts the X3 into X2 and monomeric xylose. It is not evident but possible that this contamination is affecting all the produced XOS. Also, this contamination explains the Econase behaviour towards the branched oligos discussed in Section 7.6 where Econase performed on the terminal xylose in the branched AXOS.

Building on the emerging picture from the earlier chapters, these trials have drawn together several threads of evidence to demonstrate that the Solubles stream from a bioethanol plant is a promising source of AX for enzymatic production of (A)XOS; the low A/X ratio of the WEAX extracted from Solubles makes them susceptible to enzyme degradation to produce the oligosaccharides. This work has also confirmed that Econase contains some contaminating exo-xylanase, the activity of which could be removed through a simple thermal treatment. Eliminating the production of xylose monomer increased the yield of the desired xylobiose and xylotriose.

The discovery of this contamination compromises the interpretation of the enzyme dynamics from the study presented in Chapter 7 on Econase treatment of standards. That study also required a greater range of enzyme/substrate ratios to obtain a full picture of Econase kinetics. Nevertheless, the results from the last few chapters have increased our understanding of Econase behaviour and have demonstrated how this endoxylanase, or other xylanases, could be deployed to optimise production of (A)XOS prebiotics from bioethanol by-products.

8.5 Summary

The bioethanol production process yields two intermediates by-products, Distillers Wet Grain and Solubles, to which thermal evaporation and drying procedures are applied to combine the intermediate by-products into the final and major by-product in biorefineries, Distillers Dried Grain with Solubles (DDGS).

The isolation of arabinoxylans from the DWG and the Solubles, prior to the drying procedure, yields different AX materials with different properties, including differing susceptibilities to xylanase treatment to produce oligosaccharides. Production of these two AX products and of XOS/AXOS products from them could significantly enhance the economic performance of cereal-based biorefineries, by creating new products that could be commercialised for the food market.

In this project, two arabinoxylan products, the water-extractable AX from the Solubles and the water-unextractable AX from the DWG, were successfully produced. The water-extractable AX was extracted from the aqueous phase of the fermentation mash, the Solubles. The thermal treatment of the wheat during the process of ethanol production, *i.e.* the starch hydrolysis prior to fermentation and the ethanol distillation after fermentation, was sufficient to release a substantial portion of AX into the Solubles. The isolation of WEAX from the Solubles was performed using an in-house assembled ultrafiltration system with a membrane of 10 kDa cut-off.

The WUAX was chemically extracted from the DWG; the oxidative alkaline extraction was able to break the material open and release the tightly connected AX from other cell wall components. After extraction, the liquid extracts were concentrated, diafiltered and precipitated with 65% ethanol. The purity of the extracted WUAX was higher than the purity of the extract in Chapter 6 due to the utilisation of a more effective ultrafiltration system. However, the yield was lower and the average molecular weight was smaller due to changing the extraction conditions, particularly the liquid-solid ratio.

WEAX was recovered from the Solubles by mean of ultrafiltration followed by ethanol precipitation. The yield was about 1% of the original wheat, and about 47% of the available AX in the Solubles. Despite the relatively low yield, the low cost of the process where no chemical extraction is required, and the high purity of the recovered WEAX (74%) make this route of AX production viable in the context of biorefineries.

The enzymatic conversion of WEAX into XOS prebiotics yielded a 46% conversion of the WEAX into XOS mainly xylobiose and xylotriose.

The enzyme dosage trial showed that higher doses of the enzyme produce greater amounts of XOS (X2, X3 and X4) with higher concentrations of xylose monosaccharide, whilst the lower doses produced a wide range of XOS molecules (X2 – X6) but the total XOS was less. The production of monosaccharides was confirmed to be the result of the

contamination of the enzyme with traces of an exo-xylanase, which could be deactivated via gentle thermal treatment.

The results demonstrate a previously unconsidered source of AX, the Solubles fraction, and show that its low A/X ratio makes it potentially well suited to enzymatic production of XOS. The ability to measure the XOS profile accurately and to understand its dynamic evolution opens the way to optimise production of the desired prebiotic oligosaccharides.

9 Conclusions and Recommendations

9.1 Progress made in the current work

In the context of exploring strategies for enhancing the economic income from biorefineries, the literature advocates the integrated biorefinery approach as an effective basis for exploiting interaction opportunities arising from the co-production of several products. Biorefineries are currently derived principally from ethanol production, and ethanol can serve as a precipitating agent for arabinoxylans, which makes arabinoxylans potentially a natural co-product of bioethanol, and raising the opportunity for bringing a new class of functional food ingredients to the market.

The primary motivation of the current work was identified earlier as the pilot-scale production of arabinoxylan and arabinoxylan oligosaccharides within integrated biorefineries. In order to investigate this opportunity, several objectives were identified.

The first objective was to perform the bioethanol fermentation process on a pilot-scale to produce the wet intermediate by-products. The wet materials produced during the project, DWG and Solubles, had similar characteristics to the materials reported in the literature, and are representative of the intermediate by-products produced in commercial bioethanol plants.

The second objective was to investigate the direct enzymatic production of AX prebiotics from the DWG. The investigations revealed that the enzymes were incapable of releasing the xylooligosaccharides from the cell wall matrix, which emphasised the importance of the third objective.

The third objective was to extract AX chemically by mean of the alkaline oxidative method. The extraction process yielded two extracts with different characteristics according to the purification methods used. Use of enzymes and ultrafiltration increased the purity of the extract from 19% (the first un-purified extract) to 44% after

purification. The first extract was sent to Nottingham Trent University, where collaborating academics introduced the extract to chicken feed formulation trials to assess the effect of the AX polysaccharide on poultry nutrition. The second extract was subjected to several enzymatic treatments aiming to produce significant quantities of XOS. The trials produced only small amounts of XOS, and revealed the limitations of the analytical techniques used for XOS measurement. The trials also stressed the importance of understanding the studied enzyme's mode of action.

The fourth objective addressed in this project was the development of a robust separation and measurement technique for the simultaneous determination of monosaccharides, xylo-oligosaccharides, arabinoxylan oligosaccharides and uronic acids. The developed HPAEC-PAD method is a novel and effective tool for XOS analysis and enzymes activity studies. The simultaneous screening of all the potential products of enzyme activity significantly enhances the basis for enzyme development in relation to XOS production, and gives the potential for precise and conclusive descriptions of enzyme modes of action. The method was validated, and several samples were sent to our lab from academic and industrial partners to be analysed, indicating the timeliness and value of this new method.

The fifth objective was to study the enzymatic production and simultaneous degradation of XOS and to evaluate the ability of Econase, an endoxylanases, to produce monosaccharides. The study showed that Econase was active on all the XOS standards up to DP6, acting more rapidly on the larger oligosaccharides, and with significant and unexpected monosaccharide production. The enzyme had only minimal ability to hydrolyse the branched substrates. Therefore, an AX substrate with less branching (a lower A/X ratio) is likely to be a more suitable feedstock for XOS production.

The final objective of the project was to produce both the Water-Extractable and the Water-Unextractable AX within the biorefinery. The WUAX was extracted chemically following the same methods used earlier, but with more extensive diafiltration purification. The WEAX was recovered from the Solubles fraction by ultrafiltration over a 10 kDa membrane. The processing of 10 kg of wheat yielded only 85 g of 51% WUAX

and 92 g of 74% WEAX. The enzymatic conversion of WEAX into XOS was capable of converting 46% of the material into oligosaccharides and 11% into monomers. Thermal treatment of the Econase largely eliminated the production of xylose, indicating this had arisen from a heat-labile contaminant exo-xylanase.

Overall the work has moved forward our understanding of the practicalities of producing AX materials at scale from the DWG and Solubles streams in a bioethanol plant, has introduced a powerful new method for analysing mono- and oligosaccharides, has clarified the kinetics of Econase activity in relation to production and degradation of oligosaccharides, and has identified the Solubles stream as a promising source of AX with a low A/X ratio, suitable for enzymatic production of XOS.

9.2 Recommendations for future work and industrial uptake

The AX extract produced suffered from low yields and purities, in common with results from other researchers (Bell 2015; Jacquemin *et al.,* 2015b). Steps to improve purity naturally reduced yields, but were able to get up to 74% AX content. Purification of the extracts might be enhanced by the utilisation of large-scale ion exchange columns. Also, a high-speed centrifugation might significantly enhance the extraction. In general, an objective of future work ought to be to continue to explore ways of enhancing AX yields and purities. It is also recommended to satudy the effect of the drying method on the characteristics of the final AX extracts.

The enzymatic production of arabinoxylan oligosaccharides was accompanied with the creation of undesired xylose monomers. In part this was because, having created oligosaccharides from the larger AX, the xylanase then turns its attention to these molecules and further degrades them. Therefore, it may be plausible to perform the enzymatic hydrolysis simultaneously with ultrafiltration, where the small produced oligos would be removed from the solution, and the enzyme activity would be directed towards the large molecule. In general, there is a need for greater understanding of the dynamics of the evolution of the profile of oligosaccharides, in order to implement and

optimise enzyme treatment to maximise production of the desired XOS with prebiotic functionality.

The current work successfully produced two types of soluble AX, WEAX from the Solubles and WUAX from the DWG. The first had a lower A/X ratio indicating less substitution, hence was more readily hydrolysed by xylanase and more suitable for enzymatic conversion into XOS, while the WUAX was resistant to enzyme activity but may have food ingredient uses as an emulsifier, thickener or just a source of fibres for fibre-rich diets, or may find uses in non-food applications.

The techno-economic study of Misailidis *et al*. in 2009 formed the original justification for ongoing work into AX production in the context of integrated biorefineries. Since then it has become clear that a portfolio of products, including prebiotic AXOS, would be more economically attractive than a single AX product. Ongoing techno-economic studies will be vital to determine the cost and the feasibility of the AX and AXOS production procedures presented in this project prior to introducing them to industrial scale production.

The progress achieved in this work started with the pilot-scale production of representative bioethanol co-products to allow the investigation of functional AX and prebiotic AXOS production within biorefineries. The new method for mono- and oligo-saccharide analysis has opened the way to understanding the mode of action of arabinoxylan hydrolysing enzymes and refining the targeted production of desired products. The new method is also applicable for other fields of study regarding production of oligosaccharides, acidic and amino sugars. The identification of the AX from the Solubles fraction as a promising source of prebiotic AXOS is a significant milestone in creating marketable products. More generally this work, by clarifying the scope for multiple AX-based co-products and establishing the basis for their effective production and characterisation, has helped moved the entire sector forwards toward multiple product and economically successful biorefineries that can deliver their unique role in providing food, biomaterials and fuel sustainably for future generations.

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Appendix

DWG production batches

Batch No.	Starting wheat kg	DWG kg	Moisture %
1	6.0	5.1	75
2	6.0	5.2	77
3	6.0	5.2	75
4	6.0	5.5	77
5	6.0	6.5	80
6	6.0	4.5	71
7	6.0	4.9	75
8	6.0	5.2	75
9	6.0	4.7	72
10	6.0	4.9	74

Arabinoxylan production batches

Batch	Starting wheat kg	DWG kg db	Crude extract g	AX purity %
AXa	12	2.55	900	19.5
AXb	12	2.55	420	44.4
WEAX	10	2.1	92	74.2
WUAX			85	51.6

Conferences

Oral presentations

- American Association of Cereals Chemists International (AACCI) Conference 2018, London.
- P2P Plants to Products Final Showcase 2018, Birmingham.

Poster presentation

LBnet International conference 2018, Manchester.