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Conversion of xylan into value-added products

REZA FARYAR | BIOTECHNOLOGY | LUND UNIVERSITY

Conversion of xylan into value-added products

Conversion of xylan into value-added products

Reza Faryar

LUND
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DOCTORAL DISSERTATION

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Abstract

Today's high demand on finding sustainable energy sources, replacing petroleum-based products and the need for healthier food alternatives have forced us to search for natural recoverable resources and explore their many hidden applications. One such resource is xylan, which has gained much attention as possible raw material for various exploitations. However, its wider industrial application puts demand on research achievements to reach a marketable product. The work throughout this thesis was focused on studying some of these applications with the aim to improve the final product or its production process.

Production of alkyl xylobioside and alkyl xylotrioside was achieved using a highly thermostable recombinant endoxylanase from *Thermotoga neapolitana*. This enzyme made it possible to directly react xylan with an alcohol to produce surfactant without the need for its hydrolysis to xylooligosaccharides (XOS). Furthermore, the effect of xylan concentration, enzyme dose, reaction water content, reaction temperature and initial pH on the yield of these surfactants was studied (**Paper I**).

XOS as prebiotic, another remarkable product from xylan, was produced from wheat straw. Xylan was first extracted with alkali and later hydrolysed to XOS using a variant of an alkali-tolerant endoxylanase from *Bacillus halodurans S7*. Its prebiotic properties were confirmed by growth of a putative probiotic strain; *Lactobacillus brevis* DSM 1269 (**Paper II**). To further study this strain and understand how it utilizes the XOS we studied one of its enzymes, a β-xylosidase from glycoside hydrolase family 43 (GH43). The kinetic studies using XOS of different chain length revealed that the enzyme displayed a higher catalytic efficiency for shorter chain length substrate, in line with data showing that XOS of short chain length can be taken up by *L. brevis*. Moreover, the molecular structure of this enzyme was solved using X ray crystallography showing a tetrameric structure, which further assisted to better understand its catalytic activity in relation to its structure (**Paper III**).

The potential application of xylan as film and coating was the driving force for our final study. For this purpose, arabinoxylan was mixed with tragacanth as a second polymer and cross-linked with malic acid. Based on the initial type of arabinoxylan used, two types of films were produced with similar tensile strength and thermal decomposition curves but significantly different elongation at break and swelling ratios. With further studies these films have the potential to be used as edible films, wound dressing or drug coating (**Paper IV**).

Key words: xylooligosaccharides, biosurfactant, prebiotic, xylan-based film, *Lactobacillus brevis*

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MADE IN SWEDEN

To my family

Table of Contents

Abstract

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Popular Science

Xylan is the second most abundant biomass in nature. It is present in plant cell walls and exists in massive amounts as an agricultural and forestry waste. Up to present it has mainly been used as starting material for xylitol production. However, its many new applications are being discovered everyday, but have not yet been exploited sufficiently on an industrial scale.

One of the products from xylan that has gained a lot of interest is surfactants. Today there is a great demand for bio-based surfactants due to both health and environmental concerns. These xylan-based surfactants, also known as alkyl xylosides, are a great option in that respect. They have good surface activity and excellent biodegradation properties and can be used in various products such as in personal care and cosmetics. Producing alkyl xylosides with better surfactant properties and optimizing its production towards greener processes and higher yields is crucial for its market growth. One of out goals in current thesis work was to use enzymatic methods to produce these types of surfactants and to improve its production process by careful analysis of main factors affecting the enzymatic process.

Partial degradation of the xylan polymer results in production of xylooligosaccharides (XOS). Studies have shown that XOS have prebiotic potential meaning that they can selectively stimulate the growth of health promoting bacteria (probiotic) in a host. Production of XOS as an emerging prebiotic is in early stages. Thus finding new sources for XOS production and optimizing this production process can be of great importance, which was also a focus in our studies. Furthermore, understanding how XOS are utilized by probiotics can further assist in better formulation of prebiotic XOS. Structural and functional characterization of enzymes from probiotic bacteria can shed some light on how these organisms utilize XOS that has been taken up by the microbe.

Another foreseeable product from xylan is film and coating with biomedical and food packaging applications. Xylan is already being used as an additive, as films in packaging applications but their use as the main component in packaging films has not yet been fully established. Most often xylan-based films are too brittle with low elasticity and have high solubility which limits their application. These hurdles need to be tackled to be able to compete with existing products. Testing xylan from various sources, addition of a second polymer and cross-linking these

polymeric chains can improve the properties, which were evaluated in our final study. The work throughout this thesis was to further elucidate the potential of xylan as a natural and recoverable starting material for production of a vast array of commodities.

List of papers

This thesis is based on the following collection of papers, referred in the thesis with Roman numbers I-IV. The papers are attached as appendices at the end of the thesis with permission from the publishers.

Paper I. Surfactants from xylan: Production of n-octyl xylosides using a highly thermostable xylanase from *Thermotoga neapolitana*, Mamo G., Kasture S., **Faryar R.**, Hashim S., Hatti-Kaul R., (2010) *Process Biochemistry* 45: 700–705

Paper II. Production of prebiotic xylooligosaccharides from alkaline extracted wheat straw using the K80R-variant of a thermostable alkali-tolerant Xylanase, **Faryar R.**, Linares-Pastén J., Immerzeel P., Mamo G., Andersson M., Stålbrand H., Mattiasson B., Nordberg Karlsson E., (2015) *Food and Bioproducts Processing* 93: 1–10

Paper III. Structural and functional studies of a β-1,4-D-xylosidase (family GH43) from the putative probiotic *Lactobacillus brevis* DSM1269, Linares-Pastén J, **Faryar R.**, Shouker B., Abou Hachem M., Logan D. T., Nordberg Karlsson E., (*Manuscript*)

Paper IV. Synthesis and characterization of arabinoxylan-tragacanth based films, **Faryar R.**, Engqvist J., Olsson J., Jannasch P., Nordberg Karlsson E., (*Manuscript*)

My contribution to the papers

Paper I. I performed part of the experiments related to production of surfactant in batch process. I helped with writing of the first manuscript.

Paper II. I designed the study and performed the experiments including extraction of xylan, its conversion to XOS and prebiotic potentials. I wrote the first draft of the manuscript and took active part in the revision.

Paper III. I took active part in the enzymological study of the work and wrote the parts of the manuscript that corresponded to this study.

Paper IV. I designed the study, did the major part of the experiments and wrote the first version of the manuscript.

1. Introduction

A large portion of chemicals used today in major industries around the world are based on crude oil. However, with the depletion of crude oil, more attention has gone towards the use of natural recoverable resources for production of bio-based chemicals. Lignocellulosic biomass holds high potentials to be used as a raw material for production of many kinds of chemicals that are at the moment produced mainly from crude oil. It is found in almost all plant-derived materials, such as wood and grass, agricultural residues and municipal solid wastes. Lignocelluloses consist of mainly cellulose, hemicellulose, and lignin. Xylan is the most common form of hemicellulose with various structural diversity, depending on its source.

Since the 20th century up to now, the major product from xylan with high marketability has been xylitol. Later, with the emergence of second-generation biofuels, the research activities on xylan were mainly aimed at bio-converting xylan together with cellulose to biofuel. In the last two decades, studies have shown a greater application potential of this biopolymer in various areas including food and non-food applications.

Alkyl xylosides as xylan-based surfactants, which are less studied compared to hexose-based surfactants have shown excellent properties. Studies on this group of surfactants are still in its early stages. Evaluating different sources of xylan and improving its production and purification techniques are of great importance to reach a marketable product.

Another interesting application of xylan is its potential to be utilized as a prebiotic in form of xylooligosaccharides (XOS). Meaning that they can actively enhance the growth of health promoting bacteria in human gastrointestinal tract (GIT). Thus, supplementation of the diet with XOS can manipulate the GIT microbiota, to prevent or alleviate various disease conditions associated with the lower gastrointestinal system. Currently different aspects of utilizing XOS as prebiotic are being studied with more focus on studying XOS from various sources and its effect on different probiotic strains in GIT.

In packaging industry, xylan is already being used as an additive to plastics to increase strength and biodegradability. Recent studies on its modification and

functionalization have shown their potential wider application in film and coating for food as well as biomedical products.

The aim of this thesis is to present an overview about the structural diversity of xylans, its extraction methods, enzymes involved in its catalysis and some of its application possibilities.

1.1. Scope of the thesis

The main goal of this work was to utilize hemicellulose as a valuable resource to produce different compounds. Xylan from various sources i.e. wheat straw; wheat bran and birchwood were used throughout this study. Also, whenever possible, enzymatic processes were applied instead of chemical processes, using xylanases, in order to prevent the use of harsh or harmful chemicals.

In **Paper I** xylan was used to produce the surfactants n-octyl xylobioside and n-octyl xylotrioside using an enzymatic process. In **Paper II**, wheat straw, an abundant agricultural byproduct was converted to XOS, which was further evaluated for its prebiotic potentials using *Lactobacillus brevis*. Characterization of novel enzymes from probiotics such as *Lactobacillus brevis* has also been one of the goals of this study in order to better understand the XOS degradation mechanism (**Paper III**). In **Paper IV** the effort was to produce xylan-based films with various characteristics based on its source.

2. Xylan - the major type of hemicellulose and its applications

2.1. Hemicelluloses

Hemicelluloses are heterogeneous polysaccharides, which together with cellulose and lignin are the main cell wall components. They are located in the primary and secondary layers of the plant cell wall as well as in the middle lamella (the layer between two adjoining plant cells). (Figure 1) (Ramos 2003). The distribution of cellulose, hemicelluloses and lignin varies among different layers of cell wall. Cellulose is the major fraction in the secondary wall. The primary wall and middle lamella, on the other hand holds the major part of the lignin. The hemicellulose fraction is relatively constant in the cell wall but the secondary cell wall holds the major portion of hemicellulose due to its thickness compared to other layers (Liu 2015).

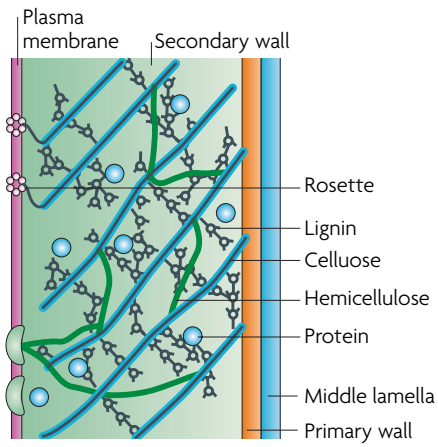


Figure 1. Plant cell-wall structure, to the right is the cell boundary and to the left is the interface with the cell. The secondary cell wall is shown containing cellulose fibers, lignin and hemicellulose, (Sticklen 2008)

In the plant cell wall hemicelluloses are closely linked to both cellulose and lignin mainly by hydrogen bonds and ionic interactions, respectively (Beg et al. 2001). Cellulose fibrils give rigidity to the plant tissue, whereas lignin acts as a water channel, and water and microbial repellent (Deutschmann and Dekker 2012). In this matrix, hemicelluloses play a significant role as a flexible bridge and a coating between and around cellulose fibrils, which maintains structural integrity of cell wall (Deutschmann and Dekker 2012, Scheller and Ulvskov 2010, Thomson 1993). The percentage of the cell wall components can vary significantly between different biomass and different components of an individual organism.

Hemicelluloses are mainly heteropolymers in both linear and branched form. They are composed of monosugars such as D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, L-rhamnose, L-fucose and D-glucuronic acid, which might be acetylated or methylated. Most hemicelluloses contain two to six of these sugars. Hemicelluloses are usually classified according to the main sugar residues in the backbone, such as xylans (xylose backbone), mannans (mannose backbone), β-glucans with mixed linkages (homoplymer of glucose), and xyloglucans (glucose backbone with xylose decoration) (Deutschmann and Dekker 2012, Saha 2003). The dominant hemicellulose in hardwood is xylan (glucoronoxylan), whereas in softwood it is mainly mannan (glucomannans and galactoglucomannans) (Ramos 2003). Cell wall components and hemicellulose composition of wheat straw (**Paper II**), wheat bran (**Paper IV**), birch wood (**Paper I**) as an example of a hard wood and spruce wood as an example of soft wood can be compared in Figure 2.

Figure 2. Cell-wall components (cellulose, hemicellulose and lignin) and hemicellulose composition for a variety of biomass, i.e. wheat straw (Lynd et al. 1999, Wiselogel et al. 1996), wheat bran (Immerzeel et al. 2014, Merali et al. 2015), hardwood (birch; Betula pendula) (Garrote et al. 1999, Lehto and Alen 2013), softwood (spruce; Picia abies) (Fengel et al. 1989, Willfor et al. 2005).

2.2. Xylans (d-Xyloglycans)

Xylans are polysaccharides possessing D-xylopyranoside monomer units as their backbone with various substituents such as arabinofuranosyl, glucopyranosyl, uronic acid derivatives, acetyl and phenolic acids such as ferulic and coumaric acid, which are either ether or ester substituted to the hydroxyl group of xylose units (Singh et al. 2015). They are available worldwide in large amounts as by-products from agriculture, forestry, and pulp and paper industries (Ebringerova et al. 2005).

In nature xylans can be found with various structures based on their botanical source or tissue type. Based on their structure they can be categorized as homoxylans and heteroxylans. Heteroxylans can be subdivided into glucuronoxylans (MGX), (arabino)glucuronoxylans (AGX), (glucurono)arabinoxylans (GAX), arabinoxylans (AX), and complex heteroxylans. Table 1 shows structure and occurrence of various xylans, which will be discussed further in the following section.

Table 1. The main types of xylans and their characteristics (Ebringerova et al. 2005, Peng et al. 2012, Saha 2003)

Xylan	Main chain	Side chain	Occurrence
Homoxylan	β-(1→3))-d-xylopyranose or β-(1→3, 1→4))-d-xylopyranose	-	Seaweed
Glucoronoxylan (MGX)	β-(1→4)-d-xylopyranose	MeGlA or GlA at position 2 Xyl: GlA 10:1 Acetylated at 2 and 3, one acetyl groups per 10 xylose unit May contain small amounts of L-rhamnose and galacturonic acid	Main hemicellulose in hardwood
(Arabino)glucoronoxylan (AGX)	β-(1→4)-d-xylopyranose	MeGlA at position 2 Xyl: GlA 5-6:1 α-L Araf position 2 or 3 Disaccharides can be seen arabinose: glucuronic acid:xylose is 1:2:8 Ester groups such as ferulic and p-coumaric acid are attached at position 5 of Araf Less acetylated than hardwood May contain low amounts of galacturonic acid and rhamnose	Can be found in softwood Main hemicellulose in lignified tissues of grass and cereals (e.g. straw)
(glucorono)arabinoxylan (GAX)	β-(1→4)-d-xylopyranose	Compared to AGX has 10 times less MeGlA than Arabinose α-L Araf position 2 and/or 3. Disaccharides can be seen xylose can be double substituted by uronic acid and Araf. Ester groups such as ferulic and p-coumaric acid are attached at position 5 of Araf	Non-endospermic tissues of cereals (e.g. bran)
Arabinoxylan (AX)	β-(1→4)-d-xylopyranose	α-L Araf position 2 and/or 3 May also be esterified with ferulic or p-coumaric acid attached at position 5 of Araf	Main hemicellulose in cell wall of starchy endospermic tissues of cereals e.g. wheat, rice, oat,.. (flour) and outer layer (bran)
Complex heteroxylan (CHX)	β-(1→4)-d-xylopyranose	Various mono and oligosaccharides attached at position 2 and 3 Heavily substituted by Xyl, Ara, Glu	Cereals, seeds, gum exudates, and mucilages

Homoxylans

Homoxylans have backbone consisting of Xylp residues with β-(1→3) (Figure 3 Aa) or mixed β-(1→3, 1→4)-glycosidic linkages (Figure 3 Ab) which can be found in seaweeds (Ebringerova et al. 2005).

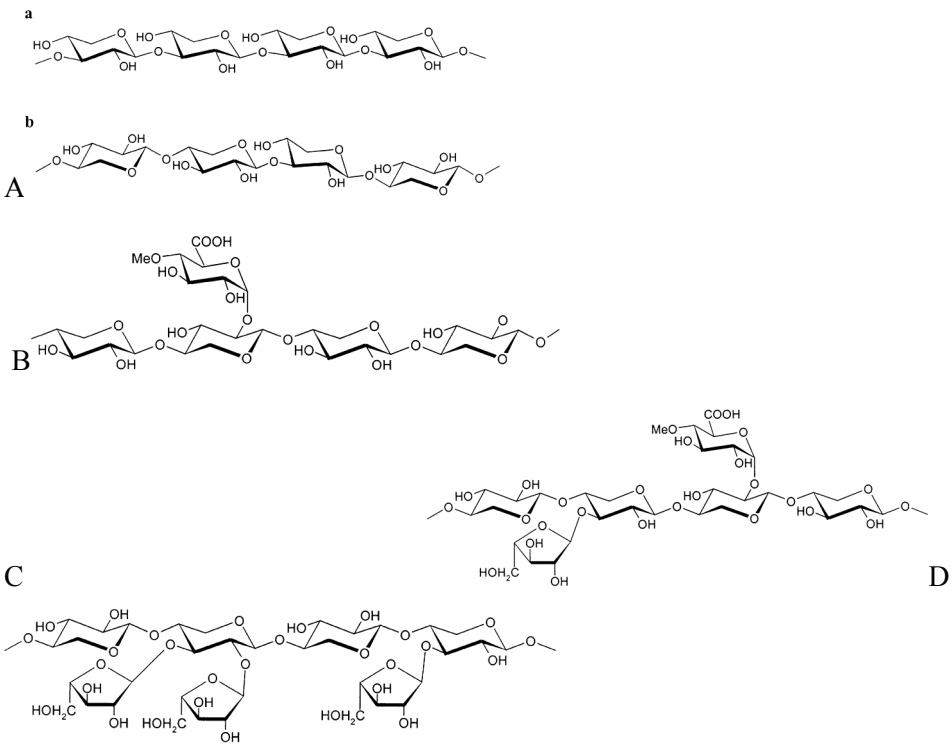


Figure 3. Primary structure of A)Homoxylan as a) β-(1→3)-d-xylan and b) β-(1→3, 1→4)-d-xylan, B) 4-O-methyl-d-glucurono-d-xylan (MGX), C) (l-arabino)-4-O-methyl-d-glucurono-d-xylan (AGX), D) water-soluble l-arabino-d-xylan (AX). Figures show only a typical structure of the respective polysaccharide and are not a repeating unit in the polymer chains.

Glucuronoxylans

Glucuronoxylans have a Xylp backbone with glucuronic acid side chain attached at position 2, which can be present both as methylated (MeGlcA) and non-methylated forms (GlcA) (Figure 3 B).

Glucuronoxylans are the major hemicellulose component of hardwoods and depending on the extraction conditions they have a Xyl : MeGlcA ratio of about 4 : 1 to 16 : 1. They have also been isolated from various fruits and dicotyls.

Glucuronoxylans are acetylated in native form. In hardwood 60-70% of the xylose backbones are acetylated (Saha 2003). However during the alkaline extraction process these acetyl groups are split which results in partial or full water-insolubility of the xylan preparations (Ebringerova et al. 2005)

(Arabino)glucuronoxylan and (glucurono)arabinoxylan

The β-(1→4)-d-xylopyranose backbone of both (arabino)glucuronoxylan (AGX) and (glucurono)arabinoxylan (GAX) have single MeGlcA at position 2 and α-l-Araf residues attached at position 2 and/or 3 (Figure 3 C). Xylp backbone is usually slightly acetylated. Disaccharide side chains might be found both in AGX and GAX. Their main difference is in the content of α-l-Araf and uronic acid side chains. In GAX the α-l-Araf content is higher than in AGX. In AGX the Araf unit might be esterified by ferulic acid (FA) at position 5 but similar to acetyl groups, they are mostly lost during alkaline extraction of AGX.

AGX is the dominant hemicellulose in the cell walls of lignified supporting tissues of grasses and cereals and have been isolated from corncob and wheat straw. They also occur in significant amounts in coniferous species. GAX can be found in the non-endospermic tissues of cereal grains such as in wheat, corn, and rice bran (Ebringerova et al. 2005).

Arabinoxylan

In Arabinoxylan (AX) the linear Xylp backbone is partially substituted by α-l-Araf residues at position 2 or 3 or on both 2 and 3 of the Xylp monomer units (Figure 3 D). Some of the Araf may be esterified by phenolic acids such as ferulic and coumaric acid at position 5. In addition, acetylation of the Xylp backbone can also occur. AXs can be seen as either neutral or slightly acidic. In the latter case they are usually included in GAX group (Ebringerova et al. 2005).

AXs are the dominant hemicellulose in the starchy endosperm (flour) and outer layers (bran) of the cereal grain such as wheat, rice, oat, barley, rye and corn. But their content can vary significantly depending on the source.

In wheat endosperm cell wall AX take up 85% of the total non-starch polysaccharide content (Courtin and Delcour 2002). Based on the ability to extract AX they can be either water soluble (WS-AX) or water insoluble (alkaline extractable) (WI-AX). WS-AX are assumed to be loosely bound to the outside of the cell wall whereas the WI-AX are covalently bound with cell wall constituents such as proteins, lignin or cellulose, within the cell wall (Courtin and Delcour 2002, Finnie et al. 2006).

Solubility of AX can depend on various factors such as degree of substitution. Accordingly, water insoluble AX which makes up the major portion of AX has a lower Ara to Xyl ratio of up to ~0.2–0.3 compared to water soluble portion with Ara to Xyl ratio of 0.3-1.2 (Ebringerova et al. 2005, Finnie et al. 2006). Water insoluble AX also have a slightly higher molecular weight which decrease their solubility (Kiszonas et al. 2013).

Another factor that affects solubility is the cross-linking between ferulic acids to form di-ferulic acids or cross-linking between ferulic acids and lignin or ferulic acids and protein (via tyrosine) (Finnie et al. 2006). Even though the amount of ferulic acid is very low and represents 0.2–0.4% of WS-AX (w/w) and 0.6–0.9% of WI-AX in wheat it has an important role in structural characteristics of AX. In WS-AX from wheat, the amount of dehydrodiferulic acids detected is 10–15 times less than ferulic acid while for WI-AX it is 4 times lower than ferulic acid (Dervilly-Pinel et al. 2001, Saulnier et al. 2007).

Complex Heteroxylans

The complex heteroxylans (CHX) have a Xylp backbone heavily substituted by various monosaccharaides (such as β-d-Xylp, β-l-Araf , α-d-GlcpA at position 2 and/or 3) and oligosaccharide side chains. They have been identified in cereals, seeds, gum exudates, and mucilages (Ebringerova et al. 2005).

2.3. Current and potential applications of Xylan

The depletion of non-recoverable resources and impact on the environment has forced us to look for better alternatives for production of energy and materials. There is an ever increasing research on xylan-based products and their use in wide range of industries. To date, several of these products have found commercial applications such as ethanol and xylitol.

First generation biofuels are made by biological conversion of sugar, starch and vegetable oils. Due to the demand on food commodities and concern over land use the goal of second-generation biofuels was to use lignocellulosic feedstock to produce fuels. However due to the more complex nature of lignocellulosic biomass its fermentation process is much more challenging (Mamo et al. 2013).

Xylitol is the most widely produced xylan derived product that was discovered more than a hundred years ago (Makinen 2000). It is used in diabetic products as a low-caloric sweetener and in chewing gums, toothpastes as a preventative agent against dental caries. It is produced by either chemical or biotechnological

methods (Jain and Mulay 2014). In the chemical method, xylan is hydrolysed by acid hydrolysis. After purification of xylose it is hydrogenated at 80-140°C and at the pressure up to 50 atm (Parajo et al. 1998). Chemical processes are labor and energy intensive, require extreme conditions and costly purification steps, and has low final product yield (Deutschmann and Dekker 2012, Parajo et al. 1998). Biotechnological methods offer a more environmentally friendly approach that are based on microbial fermentation or enzymatic conversion of xylose to xylitol (Parajo et al. 1998).

Development of bio-based value-added chemicals from xylan holds great potentials as an alternative to the petrochemically based chemicals. In the following chapters other emerging products from xylan such as surfactant, film and coating and prebiotics will be discussed. In Figure 4 a schematic representation of various xylan-based products is illustrated.

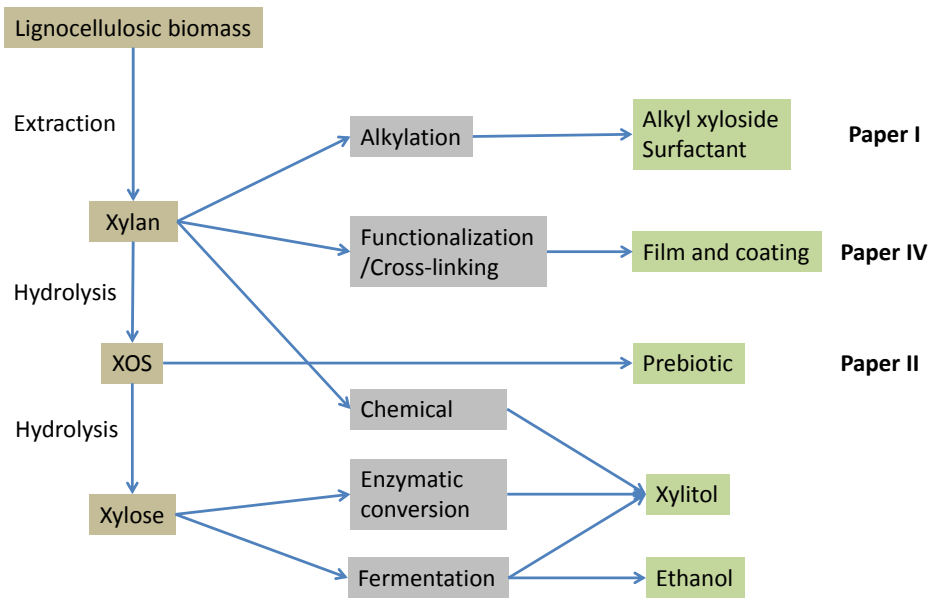


Figure 4. Current and potential products from xylan in biorefinery concept of lignocellulosic biomass.

3. Xylan extraction

Xylan from biomass requires mechanical, chemical, biological or a combination of treatments to produce a material suitable for conversion into various value added products. The choice of extraction method depends primarily on the type of target product and the purpose of extraction. Based on the extraction method used and its severity, the recovered hemicelluloses could be of different lengths, polymeric, oligomeric or monomeric sugars (Al-Dajani and Tschirner 2008).

The extractability of hemicelluloses is greatly affected by their chemical or physical interactions with other cell wall components. Xylan in particular can have different types of bonds to lignin such as ether and ester linkages (Peng et al. 2012). Xylan can also form cross-links by di-ferulic bridges, which can form a network surrounding cellulose microfibrils. Cell wall proteins might be cross-linked by isodityrosine bridges and can also link to feruloylated xylan (Saha 2003). Xylan-protein interactions have been observed in maize bran, and have been suggested to be the main cause of its xylan insolubility (Saulnier et al. 1995). Due to the linear structure of the xylan backbone, there is a great likelihood of its partial alignment to cellulose microfibrils and their interactions through hydrogen bonds (Ebringerova et al. 2005). Xylans can also be partially trapped in the cellulose microfibrils during crystallization just after synthesis (Scheller and Ulvskov 2010).

In general, for an effective extraction method, several criteria must be considered such as; having high selectivity towards hemicellulose, high extraction yields with less production of unwanted by-products, low capital and operational costs and low environmental impact (Bozell et al. 1997). In this thesis, methods for extraction that are more commonly used and studied in literature for hemicelluloses and in particular for xylan, are presented. In Table 2 advantages and disadvantages of each method are mentioned.

Table 2. Some of the advantages and disadvantages of different hemicellulose extraction methods.

Methods	Advantage	Disadvantage	References
Dilute acid pretreatments	Low cost High hemicellulose yield	Corrosion of equipments Degradation of hemicellulose into monomers Formation of furfural Production of harmful fumes Chemical recovery problems	(Buranov and Mazza 2010, Huang et al. 2008, Khan 2010)
Liquid hot water extraction	Low corrosion of equipments Low xylose degradation Less by-products including inhibitory compounds	Under more drastic conditions formation of by-products such as furfural and hydroxymethyl furfural	(Huang et al. 2008, Sun et al. 2005b)
Steam explosion-based extraction	Environmentally friendly Cost effective	Under more drastic conditions formation of by-products such as furfural and hydroxymethyl furfural are inhibitory to microbial fermentation Fragmentation of fibers	(Huang et al. 2008, Sun et al. 2005b)
Alkaline extraction	Compared to autohydrolysis (water or steam treatment) and dilute acid, xylans extracted have longer chain polymers due to stability in alkali with slow endwise peeling.	Environmental concerns High cost of product recovery	(Buranov and Mazza 2010)
Organic solvent extraction	Used for studying hemicellulose Easy recovery of solvent	High price of organic solvent Safety issues regarding handling of solvent	
Ultrasonic irradiation extraction	Simple and more rapid		(Sun et al. 2004a)
Microwave-assisted extraction	Rapid Constant condition	It is not suitable for large scale because microwaves penetrate only a few centimeters into the material. Long heating and cooling period	(Palm and Zacchi 2003)

3.1. Dilute acid pretreatments

Previously acid pretreatment was explicitly used to facilitate conversion of the biomass to ethanol by removing hemicelluloses either in combination or prior to acid hydrolysis of cellulose to glucose. It is now being applied as an extraction method of hemicellulose from varieties of lignocellulosic materials (Liu 2015).

Dilute acid pretreatment typically employs sulfuric acid but other acids such as nitric, hydrochloric, phosphoric sulfur dioxide have also been evaluated (Jeong et al. 2010, Mosier et al. 2005). In acid treatment, hydrogen ions induce depolymerization of polysaccharides by hydrolyzing glycosidic bonds (Liu 2015). Depending on the severity of the hydrolysis condition (such as acid content, temperature) different contents of oligomeric and monomeric products

are produced from the hemicellulose polymer (Hendriks and Zeeman 2009). Hardwood xylans are more labile towards acid hydrolysis compared to softwood. This is due to the fact that they have higher content of pentosans (such as 4-O-methyl glucuronoxylans) compared to softwood which is mostly hexosans (such as glucomannans and galactoglucomannans). Also the high content of acetyl groups in hardwood xylan forms an acidic condition, which subsequently can cause auto-hydrolysis (Ramos 2003).

Jeong et al. (2010) used rapeseed straw as the raw material and studied the effect of acid concentration, temperature and retention time on hemicellulose yield, production of monomeric sugars and by-products (furfural, 5-hydroxymethyl furfural, and acetic acid). Optimum conditions for maximum xylan extraction were 1.76% H_2SO_4, temperature 152.6 ˚C and retention time of 21 min with 78.9% recovery of xylan, mannan and galactan.

There are several drawbacks in using this method of extraction such as corrosion of equipment, need for an extra step for acid neutralization before downstream enzymatic or fermentation processes and formation of inhibitory degradation products (Mosier et al. 2005, Sun and Cheng 2002)

3.2. Liquid hot water extraction

In liquid hot water extraction, hot water is used to extract mainly hemicelluloses (Hendriks and Zeeman 2009). Due to the high dielectric constant of water, ionic substances in lignocellulosic biomass such as lignin and hemicellulose can dissociate and dissolve in liquid hot water (Liu et al. 2012). Acetyl and uronic acid substitutions of hemicellulose are cleaved, resulting in production of organic acids. These acids can further degrade hemicellulose to oligosaccharides. However under more severe conditions they can be hydrolysed to monosugars and aldehydes (e.g. furfural and 5-hydroxymethyl furfural) (Liu et al. 2012, Tunc et al. 2014). However the formation of degradation products can be controlled by keeping the pH between 4 and 7 (Hendriks and Zeeman 2009).

Mok and Antal (1992) investigated the extraction of lignocellulosic components from wood and herbaceous biomass. They used compressed liquid water for 0-15 minutes and a temperature between 200 and 230˚C which resulted in 40-60% solubilization of sample mass and 100% solubilization of hemicellulose, of which 90% was recoverable monomeric sugar when acid was used to hydrolyze the resulting liquid. Their results showed that high lignin solubilization can hinder recovery of hemicellulose sugars.

3.3. Steam explosion extraction

In steam explosion extraction, pressurized steam is used which is followed by rapid release of pressure causing fractionation, depolymerization and easier hydrolyzation of hemicelluloses. It is well known as a method to separate lignocellulosic materials into its main components; cellulose, hemicellulose and lignin (Mcmillan 1994). Both steam explosion and hot water extraction are considered to be "autohydrolysis" due to the natural acidic conditions and the influence of hot water and steam. Autohydrolysis is due to the deacetylation of hemicellulose, which releases acetic acid and this acidic condition causes the hydrolysis of hemicelluloses to soluble sugars (Liu et al. 2012).

This method has been used for variety of lignocellulosic biomass such as cereal straw and wood (Agudelo et al. 2016, Cotana et al. 2014, Horn and Eijsink 2010, Lopez-Linares et al. 2015, Monschein and Nidetzky 2016).

The advantage of this method is that it is environmentally friendly and more cost effective compared to other extraction methods (Cara et al. 2006). However, the drawback of this process is that under severe conditions hemicellulose can undergo secondary reactions forming furfural and hydroxymethylfurfural (Liu et al. 2012). Thus it is crucial to control process conditions such as time and temperature in order to decrease material loss due to side reactions (Josefsson et al. 2002).

Ibrahim and Glasser (1999) utilized steam explosion at low severities of 23 bar, 222˚C and 2.5 minutes retention time, reaching maximum hemicellulose yield of up to 72% from red oak wood chips.

It is possible to utilize steam explosion as a pretreatment step before chemical extraction of hemicelluloses. Sun et al. (2005b) used a two stage extraction by steam explosion and alkaline peroxide to isolate hemicellulose and lignin from wheat straw. The two stage treatment yielded 77-87.6% of the total original hemicellulose and 92.3-99.4% of total original lignin.

Steam explosion can also be used in combination with chemical extraction methods. Tucker et al. (2003) utilized a combined dilute acid- steam explosion for recovery of xylose from corn stover. The substrate was treated with 1% H_2SO_4 for 70-840 seconds in a steam explosion reactor. Using this process xylose recovery of 63-77% at 160-180˚C and 90% at 190˚C was reached.

Chen and Liu (2007), coupled steam explosion with ethanol extraction for fractionation of wheat straw and reached lignin, hemicellulose and cellulose recovery of 75, 80 and 94%, respectively.

3.4. Alkaline extraction

One of the most common and effective methods of hemicellulose extraction is by alkaline solutions (Gabrielii et al. 2000, Sun et al. 2005a, Zhang et al. 2011). Alkaline conditions can disrupt cell wall of lignocellulosic materials by dissolving hemicelluloses and lignin and decreasing cellulose crystallinity. Alkaline treatment deacetylates hemicellulose and hydrolyzes ester bonds between lignin and hemicellulose (Buranov and Mazza 2010). However the ether bonds between lignin and hemicellulose are much more stable under alkaline conditions (Sjöström 1993).

Depending on the substrate and the target product alkaline extraction can be performed on raw substrate or pretreated substrate which is dewaxed and/or delignified. In the latter a higher purity of hemicellulose with a lighter color is obtained. When the lignin content is higher, the final hemicellulose obtained becomes more brownish (Peng et al. 2012).

The yield of extracted hemicellulose depends on several factors such as alkali type, alkaline concentration, time, and temperature. Different alkaline solutions namely sodium, potassium, lithium, barium, calcium, and ammonium hydroxide have been evaluated so far. However sodium and potassium hydroxide are typically preferred since higher yields can be reached (Lawther et al. 1996).

Alkaline peroxide extraction has been found to be an effective agent for solubilization of hemicelluloses from straw (Fang et al. 2000, Saha and Cotta 2006). In alkaline solution hydrogen peroxide readily decomposes into hydroxyl radicals (HO.) and superoxide anion radicals (O_2^-.). It is believed that these radicals oxidize lignin, thus introducing hydrophilic (carboxyl) groups into its structure and cleaving some inter-unit bonds which results in dissolution of lignin and hemicelluloses (Sun et al. 2004b).

To date, alkaline extraction has been used to extract xylan from many different agricultural residues and by-products. Hespell (1998)worked on corn fiber (containing more than 30% xylan) for extraction of hemicelluloses using different alkalines: 2% calcium hydroxide, 15% ammonium hydroxide and potassium hydroxide and achieved xylan yields of upto 15%. Al-dajani and Tschirner (2008) utilized aspen chips with treatment conditions of 1.04, 1.07 and 2.08 M NaOH at 50-90˚C which resulted in a recovery of 40-50 kg of hemicellulose per ton of chips (with 63% xylan content in the extracted hemicellulose). Sun et al. (2004b) used sugarcane bagasse for extraction of hemicelluloses. First they dewaxed the substrate by toluene and ethanol for 6hrs then pretreated it with water at 55˚C for 2hr and then treated with alkaline peroxide (0.5 M NaOH, 0.5 % H2O2) at pH of 11.5, 55˚C for 2hr. In **Paper II** alkaline extraction using 2% NaOH at 80˚C for 90 min was used to extract xylan from wheat straw, which resulted in 56.5 g

xylose equivalent per kg dried wheat straw. In a similar study Ruzene et al. (2008) extracted 49.3% hemicellulose of the original hemicellulose in wheat straw by using 2% NaOH at 55˚C for 2 h. In order to reach higher yields of xylan extracted from wheat straw, alkaline extraction in combination with autohydrolysis (hot water) has been suggested (Wu et al. 2018).

3.5. Organic solvent treatment

Organic solvents such as dimethyl sulfoxide (DMSO), ethanol and methanol can be applied to extract hemicellulose. Dimethyl sulfoxide (DMSO) is the most common neutral (non-destructive) solvent that has been utilized. It is an efficient solvents for low branched heteroxylans (Ebringerova and Heinze 2000). The main drawback of using DMSO is its high cost and potential hazards of handling its large volumes (Peng et al. 2012).

During organic solvent extraction of hemicellulose, acetyl ester compounds and the glycosidic linkages remains intact which can be used to study hemicellulose structure (Peng et al. 2012). Xu et al. (2006) compared various organic solvents such as acetic acid, formic acid, methanol and ethanol for extraction of hemicellulose from wheat straw. Extraction yield of up to 76.5% was reached using a mixture of formic acid, acetic acid and water as solvents.

They also have the advantage of easy recovery of solvent by distillation, efficient pollution control, and low energy consumption (Pan et al. 2006, Vila et al. 2003).

3.6. Ultrasonication

It has been well known that ultrasound can be used as an alternative method for extraction (Pico 2013, Vilkhu et al. 2008). The potential of ultrasonication to enhance extraction of hemicellulose in different plants and plant materials have been summarized by Ebringerova and Hromadkova (Ebringerova and Hromadkova 2010). During ultrasonication, ultrasound waves are irradiated into lignocellulosic materials, creating cavitation bubbles. These bubbles collapse; causing microfractures in cell wall biomass and consequently increasing the diffusion and mass transfer during chemical extraction process (Ebringerova and Hromadkova 2010).

This method has been used to improve extraction of hemicellulose from grape pomace, buckwheat hulls, wheat straw, corn bran, sugarcane bagasse, (Ebringerova and Hromadkova 2002, Hromadkova and Ebringerova 2003,

Minjares-Fuentes et al. 2016, Sun et al. 2004a, Sun et al. 2002, Sun and Tomkinson 2002).

Sun et al. (2004a) utilized sugarcane bagasse. First they dewaxed the substrate by toluene and ethanol for 6hrs then used ultrasonic irradiation at 55˚C for 40 minutes and then treated with alkaline or alkaline peroxide leading to release of over 90% of hemicellulose and lignin. They suggested that ultrasonication cleaves the ether linkage between lignin and hemicellulose thus increasing the accessibility and extractability of hemicellulose.

3.7. Microwave irradiation

In microwave assisted extraction, the electromagnetic energy which is absorbed by lignocellulosic matter and aqueous solution is transformed into heat. This method has the advantage of having shorter process time and constant conditions can be maintained. It can also be an eco-friendly extraction alternative (Buranov and Mazza 2010, Peng et al. 2012). Palm and Zacchi (2003) employed microwave treatment for extraction of hemicellulosic oligosaccharides from spruce. Under microwave treatment conditions of 200 °C and 5 min, highest extraction yield of mannan (70%) was achieved. In order to reach higher recovery Lundqvist et al., (2002) combined microwave irradiation and NaOH-impregnation for fractionation of spruce hemicelluloses. One of the disadvantages of using microwave-assisted extraction is the difficulty to prevent extensive degradation of the hemicelluloses and at the same time achieving high yields (Peng et al. 2012).

4. Xylanases and xylosidases - catalytic tools acting on xylans

The industrial use of lignocellulosic biomass including hemicellulose often requires the use of glycoside hydrolases (EC 3.2.1.-) in order to degrade plant cell wall polysaccharides. Thus knowledge of the biochemical and molecular aspects of various glycosyl hydrolases seems crucial for their further application. In this regard xylan degrading enzymes have been the focus of many studies and some are being commercially produced. Xylanases have found applications in industries as a processing aid in saccharification prior to fermentation (for production of e.g. lactic acid or ethanol, or for xylitol) or as additives in feed (improve digestibility) and food (to improve bread quality) (Linares-Pasten et al. 2018). In recent years the use of xylanases for production of XOS as prebiotic have received increased interest (Mano et al. 2018).

In nature a large variety of enzymes are required to breakdown the complex and heterogenic structure of xylans. These enzymes include endo-xylanases (EC 3.2.1.8) which hydrolyze the xylan backbone in an endo- acting manner, exo-xylanases (EC 3.2.1.157) which releases xylose and short chain oligosaccharides from the reducing end of long xylooligomers, β-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylooligosaccharides and while removal of the side groups is catalysed by α -L -arabinofuranosidases (EC3.2.1.55), α -D -glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC3.1.1.73) and p -coumaric acid esterases (EC 3.1.1.-) (Figure 5). Theses enzymes have been found to be widespread among fungi, actinomycetes and bacteria and are most commonly located in environments with accumulated plant material, as well as in the rumen of ruminants (Collins et al. 2005). Here in this chapter the focus will be on the endo-acting xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37).

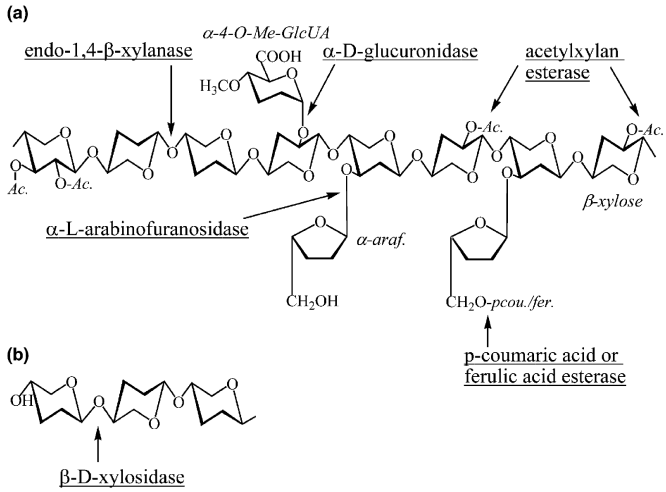


Figure 5. a) The structure of xylan and the sites of attack by endoxylanase and debranching enzymes b) xylotriose and site of attack by β-xylosidases (Collins et al. 2005).

4.1. Classification

Glycoside hydrolases (GH) can be classified into families based on the amino acid sequence similarities of the catalytic modules. The primary structural comparison reflects both structural features and molecular mechanism, which can also help to find evolutionary patterns. Based on this classification endoxylanases (EC 3.2.1.8) have predominantly been found in families 10 and 11. They have also been found in families such as 5, 8, 30 and 43 (Linares-Pasten et al. 2018). The β-xylosidases have been found in families such as 3, 39, 43, 52 and 120 (Lagaert et al. 2014).

The catalytic mechanism of GH can be either a double displacement mechanism or single displacement mechanism. In the double displacement mechanism, which is a two step process, a covalent glycosyl-enzyme intermediate is formed by two carboxylic acid residues located in the active site, one acting as a general acid or base catalyst and the other as a nucleophile performing a nucleophilic attack. In this mechanism the formed anomeric carbon configuration is retained. In the single displacement mechanism, which is a one step process, one carboxylate in the active site provides for a general acid and the second functions as a general base, activating a nucleophilic water molecule to attack the anomeric carbon, and leading to an inversion of the configuration at the anomeric carbon (Rye and Withers 2000).

Endoxylanases in GH families 5, 10, 11 and 30 perform hydrolysis with retention of the anomeric configuration, while members of GH 8 and 43 catalyse hydrolysis with inversion of anomeric configuration. All β-xylosidases families mentioned above are retaining GH except for GH 43, which as said is an inverting GH (Carbohydrate-active enzyme server (CAZY) at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/.).

4.2. Endo-xylanases in GH 10

The structure of this family is an (α/β)8 TIM-barrel fold, consisting of eight major parallel β-strands in the center forming a cylinder which are surrounded by eight major α helices. From the side the structure resembles a salad bowl (Figure 6). Top face of the molecule has a large radius (approximately 45 Å) composed of an elaborate β-α loop architecture. This elaborate architecture forms the large substrate binding cleft. The bottom face, which mainly consists of α-β turns, has a smaller radius of approximately 30 Å (Collins et al. 2005). The active site is located at the wider face where the acid/base and nucleophile catalytic residues are at the C-terminal end of β-strand 4 and 7, respectively (Linares-Pasten et al. 2018).

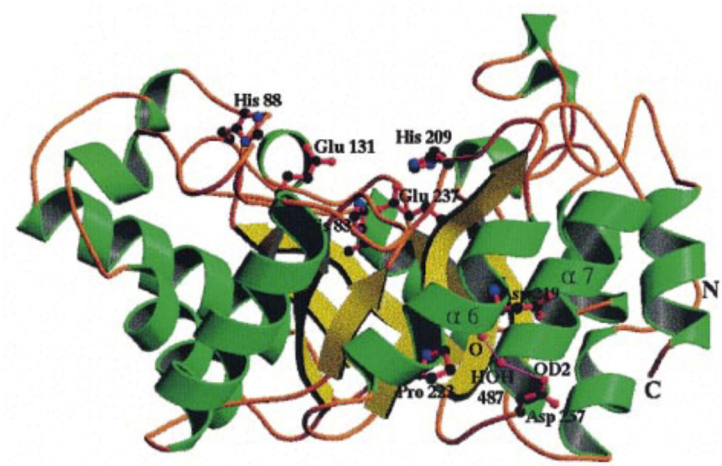


Figure 6. The crystal structure of family 10 endoxylanase from *Thermoascus aurantiacus* depicting the (α/β)8 TIM-barrel fold at 1.8 Å resolution (Natesh et al. 1999).

Enzymes of this family have shown endo-1,4-β-xylanases (EC 3.2.1.8), endo-1,3-β-xylanases (EC 3.2.1.32) and cellobiohydrolase (EC 3.2.1.91) activities (Collins et al. 2005). Family 10 endo-1,4-β-xylanases have the possibility to hydrolyse

substituted regions of xylans. Crystal structure analyses revealed that arabinose and methylglucuronic acid substitutions could be accepted mainly in -2 and -3 subsites, respectively (Pell et al. 2004, Pollet et al. 2010). But no substitution is accepted at -1 subside (Pollet et al. 2010). Crystal structural studies of the enzyme and substrate reveals stronger binding in the glycone subsites with several hydrogen bonds at -1 and -2 subsites, compared to the aglycone sites in which hydrophobic interaction are favoured (Fujimoto et al. 2004, Schmidt et al. 1999).

Family 10 xylanases typically have four to five substrate binding sites which is fewer than family 11 (with usually five to seven subsites) (Linares-Pasten et al. 2018). Thus in contrast to family 11, they show preference for shorter xylooligosaccharides (Falck et al. 2014). The shortest substrate that can be hydrolyzed is X3 due to its low affinity to xylobiose at -1 and +1 subsites (Pollet et al. 2010). This is in accordance with our findings that X2 and X3 were the main products from wheat straw hydrolysis by endoxylanase GH 10 of *B. halodurans* (**Paper II**). Also GH10 compared to GH11 showed less affinity towards insoluble xylan.

The choice of xylanase used for any industrial application depends primarily on the final products resulting from enzymatic hydrolysis. For production of XOS as prebiotics, GH 10 and GH11 endoxylanase are good candidates since their main hydrolysis products are XOS and they produce less xylose (Mano et al. 2018). Studies have shown that endo-xylanases from family 10 have high affinity for short chain XOS producing preferably XOS with lower DP (X2-X5), and family 11 enzymes are most active on long chain XOS producing XOS with higher DP (X2 -X6) (Chen et al. 2009). The higher activity of GH11 on larger substrate molecules could relate to its larger substrate binding clefts and smaller size (commonly as single domain) which can increase its accessibility to complex xylan structure (Abou-Hachem et al. 2003, Collins et al. 2005).

4.3. Extremophilic GH10 xylanases

Xylanases from extremophilic origins are in high demand for biotechnological applications. Their stability under extreme temperature and pH conditions makes them industrially suitable.

For example, the use of themostable xylanases in transglycosylation reactions allows the use of high temperature to facilitate mixing of the two phases (water and alcohol) and to improve xylan solubility. In **Paper I** GH10 endoxylanse from hyperthermophilic *Thermotoga neapolitana* was utilized for production of alkyl xylosides as surfactant (Mamo et al. 2010). *T. neapolitana* is a strictly anaerobic Gram negative marine bacterium with optimum growth temperature of 80 °C

(Belkin et al. 1986). Three different domains have been identified in this endo 1,4-β-xylanases enzyme as; the core domain which is the catalytic domain belonging to family 10, the N- and C-terminal domains (Zverlov et al. 1996). The N-terminal and C-terminal domains are believed to contain carbohydrate-binding modules (CBM). Two carbohydrate-binding modules (CBM) have been identified in the N-terminal domains which specifically bind to xylan and not to cellulose (Mamo et al. 2007).

Another example of extremophilic xylanases that have gained importance is alkaline xylanases. Their ability to hydrolyse xylan in alkaline solutions is important especially for paper and pulp industries (Kulkarni et al. 1999).

Bacillus halodurans S7 for example is an alkaliphilic bacteria isolated from a soda lake (Mamo et al. 2006b). The extracellular endo-1,4-β-xylanase belong to family GH10 and is optimally active at pH 10 and high temperature of 70 °C. The crystal structure of the catalytic domain shows a common eight-fold TIM-barrel structure of family 10 xylanases. However, compared to a number of non-alkaline active GH10 endo-xylanases they have some striking features such as higher percent composition of acidic amino acids, higher negatively charged surface and a deeper active site cleft (Mamo et al. 2009). It was proposed that the ability of this strain to withstand high salt concentration and high pH could relate to the excess negative charge on the protein surface forming a protective water shield around the catalytic domain. Like most other xylanases from alkaliphiles, the *B. halodurans* xylanase contains a single domain and no xylan binding domains (Mamo et al. 2006a).

In **Paper II** a variant of this endo-xylanse was used to further degrade alkaline extracted hemicellulose to XOS as a potential prebiotic. The use of alkaliphilic xylanase to hydrolyse alkaline extracted hemicellulose is preferred due to its stability in high pH conditions present in the extraction process and thus can reduce the need for several washing of the hemicellulose before enzymatic degradation. To increase thermostability, Lys 80, a surface exposed residue at the edge of the glycone binding region of the catalytic cleft (approximately 21 Å from the catalytic nucleophile, Glu 265) in the wild type enzyme was mutated to Arg. The mutant was preferred over the wild type due to its higher specific activity on birchwood xylan at pH 8, 9 and 10. The increase in activity could relate to slight increase in the affinity for long substrates forming additional hydrogen bonds. However, the mutation did not increase the thermostability due to less probability of salt-bridge formation with Glu41.

4.4. β-xylosidases in GH 43

Glycoside hydrolase family 43 have exo-acting β-xylosidases and α-arabinofuranosidase activity. This family of enzymes are especially important for degradation of XOS and AXOS in lactic acid bacteria (LAB) and bifidobacteria. Thus in order to better control the gut microbiota it is essential to know how these enzymes work to degrade prebiotics. With their ability to degrade XOS to xylose this family of enzymes could also potentially be useful for industries converting biomass to fermentable sugars such as bioethanol production.

Few studies on LAB have determined the specific activity towards XOS with different lengths. One of few studies made comes from two other strains of *Lactobacillus brevis* and includes the enzymes termed LbX (from *L. brevis* strain ATCC367) and XynB2 from *L. brevis* strain DSM20054, both being homologues to the currently studied GH43 enzyme XynB from *L. brevis* DSM1269 (**Paper III**). The specific activity LbX from *L. brevis* ATCC367 was higher for xylobiose than for xylotriose (Jordan et al. 2013). XynB2 from *L. brevis* DSM20054 has also shown high catalytic efficiency with respect to xylobiose indicating that these enzymes have two subsites in the active site (Michlmayr et al. 2013). In **Paper III** XOS with a chain length ranging from xylobiose to xylotetraose were studied, using LbXyn43B. The enzyme displayed a decrease in the catalytic efficiency (kcat/Km) and the turnover number (kcat) with increasing chain length, despite the lower affinity of the enzyme for shorter chain XOS, as indicated by a decrease in the *Km* values. In a recent study, a homologous enzyme WXyn43 from *Weissella* strain 92 demonstrated higher catalytic efficiency on xylobiose and xylotriose but had similar efficiency towards xylotetraose conversion (Falck et al. 2016).

These studies show that XOS interact with all GH 43 xylosidases at two subsites (−1 and +1). In **Paper III** docking of xylobiose substrate in the active site of the crystallographic structure of xylobiosidase in *L. brevis* DSM1269 clearly revealed these two sub-sites. The sub-site -1 had Asp128, Asp15, Arg290 and Phe32 as the amino acids surrounding this sub-site, forming hydrogen bond interactions with the ligand. In the subsite +1 Phe509 and Phe510 were in direct contact with the ligand through hydrophobic interactions. The catalytic donor proton Glu188, as well as the catalytic nucleophile/base Asp15 and the auxiliary residue Asp120, which are highly conserved in this family, were also identified (Linares-Pasten et al. 2017).

The structures of GH43 enzymes exist as tetramers, dimers or monomers. XynB2 from strain DSM20054 was however reported as an apparent trimer (Michlmayr et al. 2013), which is unexpected.

Family 43 catalytic domain is a 5-bladed β-propeller structure which is located in the N-terminal. The C-terminal domain is a β-sandwich fold which function is still unclear (Lagaert et al. 2014). The crystallographic structure of β-xylosidase in *L. brevis* DSM1269 demonstrated a tetrameric structure (Figure 7). The homo-tetramer is a dimer of dimers where each dimer is stabilized in antiparallel way meaning that the catalytic domains interact with the β-sandwich domains of the other subunit. The interaction is between two loops from the catalytic domain, Lys92-Asp100 and Thr144-GLy156 and the β-sandwich domain. This strong interaction creates a big cavity between the two dimers. The active site of each subunit, which is located in the five bladed propeller that builds up the catalytic domain is in the oligomeric complex, located inside this central cavity, making it necessary for the substrate to pass to reach the active site.

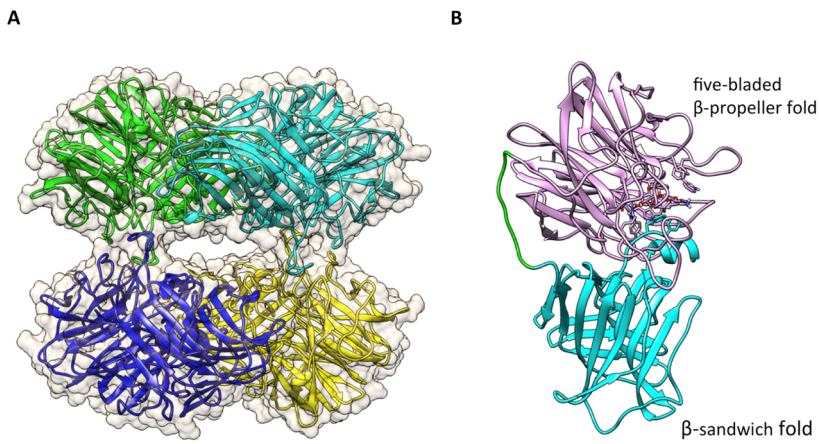


Figure 7. Crystallographic structure of the β-xylosidase from *L. brevis* DSM 1269 solved at 1.9Å of resolution. (A) Tetramer where each subunit is represented in different colors. (B) Detail of each subunit showing the catalytic domain in purple in the N-terminal, the β-sandwich domain in cyan in the C-terminal and the loop which link both domains in green.

To understand why some GH43 β-xylosidases have monomer or dimer or potentially a trimer structure, in **Paper III** threonine in the position 274 of *L. brevis* DSM 1269 was mutated to alanine to remove the potential interaction between the monomers, mimicking the sequence conservation in XynB2. The apparent molecular mass of the mutant enzyme was slightly decreased, indicating a change in the interactions. In addition, the results showed that unlike the wild type enzyme the catalytic efficiency was higher for longer chain substrate which might relate to change in binding affinity in the active site due to disturbance in oligomerization or a change in tetrameric structure which helps in better accessibility for longer chain to reach the active site.

5. Enzymatic synthesis of xylan-based surfactants

There is an ever-increasing interest for bio-based surfactants because of their great demand in production of biodegradable products. As an example of such surfactants are sugar-based amphiphiles which are produced by conjunction of carbohydrates with fatty acids, long chain alkanols or other natural hydrophobic species. In this category alkylglycosides have shown good surface activity and excellent biodegradation properties and thus have found applications in various fields such as for personal care products and cosmetics (Nowicki et al. 2018).

These surfactants contain a hydrophilic and a hydrophobic region thus enabling them to accumulate between fluid phases and thus reducing the interfacial tensions between the two phases (Sharma et al. 2013).

The most studied compound of this group has been alkylglucosides (Greffe et al. 2005, Jiang et al. 2004, Matsumura et al. 1997, Tramice et al. 2007). However, less studies has been done on the production of alkylpentosides such as alkylxylosides and alkylarabionosides which hold a great potential due to the utilization of hemicellulose as a cheap starting material. Studies of long-chain alkyl xylosides namely octyl D -xyloside (C8 X), nonyl D -xyloside (C9 X), decyl D -xyloside (C10 X) and dodecyl D -xyloside (C12 X) have shown their interesting physico-chemical properties and eco-toxicological profiles making them good candidates for environmentally friendly surfactants. They have shown considerable surface tension reduction, even at relatively low concentrations. They also have good emulsifying and foaming properties with no toxic influence on two *Pseudomonas* strains at concentrations below 25 mg/L (Smulek et al. 2017).

5.1. Synthesis

Chemical synthesis is the most common method for the synthesis of alkyl glycosides. The synthesis is carried out by Fischer's glycosylation mechanism. In Fischer glycosylation the aldose or ketose group of a glycoside reacts with an alcohol in the presence of an acid as catalyst (Nowicki et al. 2018).

Chemical synthesis of alkyl glycosides generally requires harsh conditions, generate non-recyclable wastes and are not stereospecific. Enzymatic synthesis of alkyl glycosides compounds is preferred over chemical synthesis as the enzymes are stereoselective and the synthesis is carried out in mild conditions and does not generate non-recyclable waste (Beg et al. 2001, Ochs et al. 2011, van Rantwijk et al. 1999).

The most efficient enzyme to be used for production of alkylglycosides are glycosidases GH. In vivo they catalyse the hydrolysis of glycosides, but in vitro many of them can be used for synthesis of alkyl glycosides in a low water activity and high concentration of alcohol (van Rantwijk et al. 1999). Main advantages of enzymatic methods are high selectivity of enzymes, mild reaction conditions, no use of toxic reagents and fewer steps (**Paper I**). Two mechanisms used for enzymatic glycosylation are reversed hydrolysis which is thermodynamically controlled and transglycosylation which is kinetically controlled.

In reverse hydrolysis monosaccharides are used as glycosyl donors which react with a nucleophile such as an alcohol producing an alkyl-glycoside and water. Since the reaction is thermodynamically controlled it continues until an equilibrium is reached (Turner et al. 2007).

Glycosyl-OH + $R^1OH \leftrightarrow$ Glycosyl-OR^1+ H_2O

(General scheme of the reverse hydrolysis reaction where R^1 is an alkyl group)

In transglycosylation an activated substrate such as disaccharides or *p*-nitrophenyl glycosides are used as glycosyl donors. Since this mechanism is kinetically controlled it can exceed the equilibrium conversion and thus most often it has higher reaction rates and higher yields compared to reverse hydrolysis. Enzyme properties can have large influence in this case since it is kinetically controlled (Turner et al. 2007). GH enzymes can either have retaining or inverting mechanism but only GH acting with retention of the anomeric configuration, can catalyse transglycosylation reaction (Brusa et al. 2018).

Glycosyl-OR^1+ $R^2OH \rightarrow$Glycosyl-OR^2+R^1OH

(General scheme of the transglycosylation reaction where R^1 is a glycosyl group and R^2 is an alkyl group)

5.2. The choice of enzyme

In both reverse hydrolysis and transglycosylation the water activity should be kept as low as possible to prevent hydrolysis. But on the other hand glycosidases need at least some water in order to stay active. Thermostable enzymes are most often stable at lower water activity at the same time by working at higher temperatures reactants (glycosides) can become more solubilized in organic phase.

In **Paper I** a thermostable endoxylanase from GH10 from *Thermotoga neapolitana* was used for the production of n-octyl xylobioside and n-octyl xylotrioside at 70 ^{o}C from birchwood xylan. *Thermotoga neapolitana* is an extremophilic marine bacterium with an optimum growth temperature of 80 ^{o}C belonging to the Thermotogales order. Members of this order possess an array of important hyperthermophilic glycosyl hydrolases (Henrissat and Coutinho 2001). The main detected products from transglycosylation reaction in this study were n-octyl xylobioside and n-octyl xylotrioside followed by n-octyl xyloside. There was no evidence of n-octyl xylosides formation by reacting n-octanol with xylobiose. This is in accordance with family 10 endoxylanase activity since the smallest substrate they can hydrolyze is X3.

The concentration of the enzyme is also an important factor. Ochs et al. (2011) compared two enzymes, a recombinant thermophilic family 10 endo-xylanase (Tx xylanase) from *Thermobacillus xylanilyticus* and a commercial thermophilic endo-xylanase from Novozymes (NS-50030). For the two enzymes the main product was pentyl β-D-xylobioside followed by pentyl β-D-xylotrioside, but in higher enzyme concentrations their product profile differed; in case of Tx-xylanase, pentyl β-D-xylotrioside decreased due to secondary hydrolysis to pentyl β-D-xyloside. For the commercial xylanase however the production pentyl β-D-xyloside was only detected as traces for high xylanase loading.

Brusa et al. (2018) compared family 10 and 11 GH for their transxylosylation abilities in the presence of various acceptors using two model xylanases; Tx-xyn10 and Tx-xyn11 from *Thermobacillus xylanilyticus.* Their results showed that Tx-xyn10 has a broader specificity for various acceptors compared to Tx-xyn11. The difference could be a consequence of their different three-dimensional structures since GH10 xylanases with an (β/α)8 conformation forms a wide, shallow groove, while that of GH11 display a β-jelly roll structure and forms a deep, narrow cleft making it harder for different substrates to reach the active site. Also xylanases GH10 have the ability to catalyse hydrolysis between substituted xylosyl residues whereas xylanases GH11 can only catalyze hydrolysis between two unsubstituted xylose residues. On the other hand, GH11 are more capable of hydrolyzing insoluble xylans than GH10.

5.3. Process optimization

The choice of the starting material and its pretreatment can be an important factor to reach higher conversion rates. In **Paper I** birchwood xylan was used for transglycosylation reaction without any pretreatment. Ochs et al. (2011) use XOS recovered after hydrothermally pretreatment wheat bran to produce alkyl xylosides and showed that use of pretreated substrate increased the yield of conversion compared to non-pretreated wheat bran. The same study also showed that the yields could be improved by using a co-solvent such as tert-butanol in the reaction medium.

As discussed, the type of enzyme used can have a significant influence on the type of products and the obtained yields. Apart from the use of thermostable enzymes, to keep enzymes active in organic phase, methods such as adsorption of enzyme on an inert material, entrapment of enzyme, solubilisation of enzyme in organic media by attachment of non polar substituents and solubilisation of enzyme in organic media by physical binding of enzyme to a modifier can be used (van Rantwijk et al. 1999).

Despite the advantages of enzymatic method for production of alkyl pentosides (such as alkylxylosides and alkylxylobiosides) compared to chemical methods, this method is still very limited due to low conversion mainly due to presence of water. Thus methods such as protein engineering to produce more stable enzymes and finding new extremophilic enzymes that can withstand low water activity can be good alternatives to increase the production yields.

6. Prebiotic potential of XOS

6.1. Intestinal microbiota

Humans just like other vertebrates harbor a densely populated microbial community called microbiota, which consists of bacteria, viruses and fungi. They are particularly found in mucosal organs, such as the oral cavity and the intestine (Kamada et al. 2013).

The number and composition of microbiota varies in different gut regions, depending on physicochemical conditions such as pH, transit time and nutrient availability of each region. The large intestine hosts the major bacterial community (approximately 10^{11} –10^{12} cfu per gram of contents) due to its pH, slow transit time, availability of nutrients and anaerobic conditions that favors microbial growth (Gibson et al. 2010, Lambert and Hull 1996).

The main substrates for colonic microbiota are starches, dietary fibers (usually polysaccharides, DP > 10), oligosaccharides, some non-absorbable sugars and sugar alcohols, proteins and amino acids, and other materials, including mucins, bacterial metabolites and products from cell lysis (Gibson et al. 2010).

Microorganisms in colonic microbiota can be categorized as either pathogenic or health promoting, based on their effect on their host. Bacteria exclusively with saccharolytic metabolism (e.g. no proteolytic activity) such as lactobacilli and bifidobacteria are generally considered as potentially beneficial. On the other hand microorganisms having a peptolytic or mixed saccharolytic/peptolytic metabolism are either less beneficial or even harmful by producing toxins or by being a pathogen or opportunistic pathogen (Gibson et al. 2010).

A “balanced” microbiota plays an important role in many host physiological processes such as enhancement of the intestinal epithelial barrier, reinforcement of natural immune system and increasing the bioavailability of nutrients. Their major function however, is to protect the host against colonization by pathogens (Kamada et al. 2013). Thus, increasing the number of preferred microorganisms in microbiota and suppressing the growth of potentially harmful bacteria is of great importance for human well-being.

The concept of modifying the intestinal microbiota was introduced as early as 1907 by Metchnikoff, who suggested that since the intestinal microbes are dependent on food then it is possible to modify the flora and replace harmful microorganisms with beneficial ones (Metchnikoff 2004). Since then the great deal of research effort has enabled us to actualize this concept. Today, modulation of intestinal microbiota can be achieved by addition of beneficial bacteria (probiotics), ingredients that stimulate the growth of these bacteria (prebiotic), or a combination of both (synbiotics) into our diet.

6.2. Probiotics

Probiotics or live dietary microbes are now commonly used to modulate the gut microbiota composition. The term probiotic refers to "live microorganisms which, when administered in adequate amount, confer a health benefit to the host" (WHO 2002). To date, many types of microorganisms have been evaluated both *in-vitro* and *in-vivo* and some have been commercialized. Some of the most commonly used probiotics are *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Bifidobacteria* and certain strains of *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus coagulans*, *Escherichia coli* strain Nissle 1917, certain *Enterococci*, especially *Enterococcus faecium* SF68, and the yeast *Saccharomyces boulardii* (Gibson et al. 2010, Pandey et al. 2015). Nevertheless, new strains of probiotics are continuously being discovered with the help of more advanced and extensive research.

The probiotic mechanism of action to restrain pathogen growth is applied through production of metabolites such as bacteriocins and short chain fatty acids (SCFA) (decrease in pH), making an unfavorable condition for pathogenic growth (Oelschlaeger 2010). Other mechanisms are competition for nutrients and localization with pathogens and induction of host immune responses (Wohlgemuth et al. 2010).

The beneficial effects of probiotics have been studied extensively, which include reduced cholesterol and/or triglyceride levels, better absorption of minerals, increased lactose tolerance, increased insulin sensitivity and stimulation of the immune system (Gibson et al. 2010). The positive effects of probiotics against disorders and diseases connected to gut microbiota such as diarrhea/constipation, obesity, type 2 diabetes, inflammatory bowel diseases, cardiovascular disease and colon cancer have been observed (Pandey et al. 2015, Saarela et al. 2002, Yoo and Kim 2016). The effects of probiotics as health promoting microorganisms have been summarized in Figure 8.

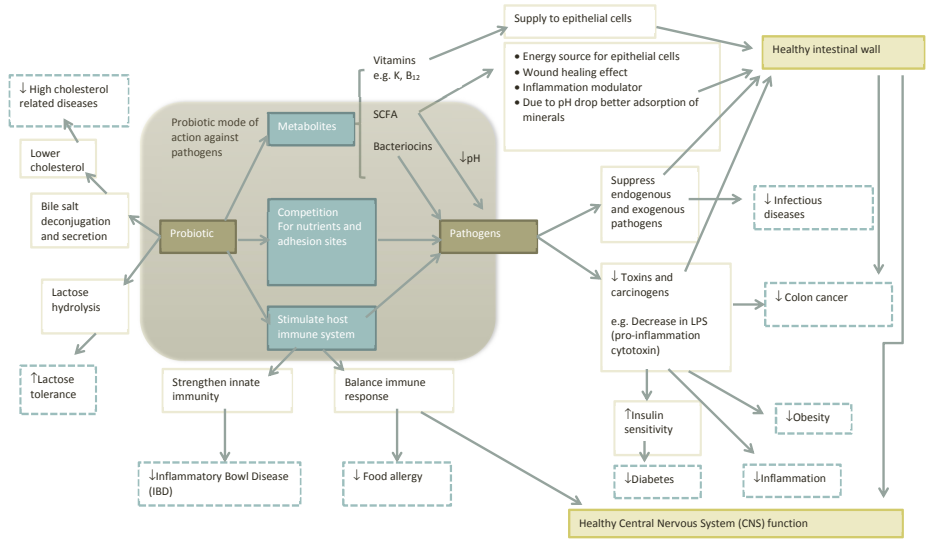


Figure 8. Different mechanisms underlying the probiotic activity (Cani and Delzenne 2009, Saarela et al. 2002)

6.3. Prebiotics

An alternative approach to modulate gut microflora is through the use of prebiotics. Dietary prebiotics are defined as "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al. 2010). Most prebiotics, are digested by bifidobacteria and lactobacilli and stimulate their growth (Gibson et al. 2010). Gibson et al. (2004) suggested that for an ingredient to be considered as prebiotic it should have these three criteria: 1) resistance towards gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; 2) fermentable by the intestinal microflora and 3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being.

Examples of prebiotics that fulfill these three criteria are lactulose, fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO) and XOS (Singh et al. 2015, Yoo and Kim 2016). They are present in fruits, vegetables, bamboo, honey and milk. Some examples of prebiotics, their natural source and chemical composition are summarized in Table 3.

Table 3. Some examples of prebiotics

Type of oligosaccharide	Natural occurrence	Chemical structure *	Production	Reference
Lactulose	Cow milk	Gal–Fru	Isomeration of lactose	(Villamiel et al. 2002)
FOS	asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye	(Fr)n–Glu	Two common methods 1. transfructosylation of sucrose by β-fructofuranosidase 2. controlled enzymatic hydrolysis of the polysaccharide inulin which can be extracted from chicory roots, for example	(Crittenden and Playne 1996, Park and Almeida 1991)
GOS	Human milk, cow milk	(Ga)n–Glu	Transglycosylation of lactose by β-galactosidases	(Sako et al. 1999)
IMO	Honey, sugarcane juice	(Glu)n	Two stage process: First starch is liquefied using α-amylase. In the second-stage that requires both β-amylase and α-glucosidase, β-amylase first hydrolyses the liquefied starch to maltose and then by transglucosidase activity of α-glucosidase IMO is produced	(Kaneko et al. 1994, Mussatto and Mancilha 2007)
XOS	Hardwood, corncob, wheat straw, rice hull, barley straw	(Xyl)n	Extraction of xylan from lignocellulosic material followed by hydrolysis of xylan by chemical or enzymatic method using endo xylanases	(Moure et al. 2006, Singh et al. 2015)

* Ga: galactose, Glu: glucose, Fr: fructose, Xyl: xylose.

Even though prebiotics can naturally occur in many foods, their intakes are commonly lower than the recommended daily fiber requirement of 25 g for a 2000 kcal diet (FDA). This makes it necessary to supplement our diet with prebiotics.

6.4. XOS as prebiotics

XOS have been found to have prebiotic properties and are now recognized as a new class of prebiotics. XOS have a degree of polymerization (DP) ≤20 and are naturally present in lignocellulosic materials such as wheat bran and straw, spent wood, barley hulls, brewery spent grains, almond shells, bamboo and corn cob (Belorkar and Gupta 2016).

XOS properties such as its resistance to heat (up to 100 °C) and stability over a wide range of pH (2.5-8.0) make them superior compared with other non-digestible oligosaccharides such as FOS and inulin (Vazquez et al. 2000). XOS owe much of their stability to their β-linkages, which are stronger than α-linkages (Carvalho et al. 2013). They are commercialized as a white powder with moderately sweet taste with no carcinogenic effects (Singh et al. 2015).

In recent years, many studies have described the beneficial effects of XOS consumption due to selective growth stimulation of health promoting bacteria by reducing the risk of various diseases related to gut microbiota, such as colon infection, diabetes, cardiovascular diseases and colon cancer (Carvalho et al. 2013, Deutschmann and Dekker 2012). Moreover, studies have shown that XOS consumption increases the level of antioxidant enzymes such as peroxide dismutase and glutathione peroxidase (Sheu et al. 2008, Wang et al. 2011)

In vitro studies have shown that XOS can selectively stimulate the growth of *Bifidobacterium* such as *Bifidobacterium adolescentis*, *B. infantis*, *B. longum*, *B. bifidum* and some strains of Lactobacillus bacteria (Li et al. 2015, Mathew et al. 2018, Riviere et al. 2014). Many strains of *Bifidobacteria* are able to ferment XOS and other structurally complex oligosaccharides due to their wide range of glycosidases. On the other hand the *Lactobacilli* that have been studied so far are not able to utilize XOS as a sole carbon source with the exception of *Lactobacillus brevis* (Crittenden et al. 2002, Moura et al. 2007). In **Paper II** *L. brevis* could efficiently utilize XOS (X2, X3) extracted from wheat straw as the sole carbon source.

The prebiotic potentials of XOS depend on the degree of polymerization and its substituents. It has been found that AXOS and XOS with low DP (2-5) have stronger bifidogenic properties compared to higher DP of above 5 (Moura et al. 2007). Moreover, substitution pattern can affect the digestibility of XOS. It has been observed that *B. adolescentis* could ferment both unsubstituted and arabinose substituted XOS as carbon source, in contrast to *L. brevis* which could only utilize unsubstituted XOS (Falck et al. 2013). Moreover, linear XOS and AXOS were fermented faster than highly substituted XOS with acetyl and uronic acid side groups by fecal inocula (Kabel et al. 2002).

6.5. XOS production

In order to produce XOS, a two stage process is commonly used; where in the first stage a xylan rich substrate is extracted from lignocellulosic material (pretreatment) followed by further degradation of xylan to XOS in the second stage. The first stage is usually a mechanical/chemical process followed by chemical or enzymatic degradation. In Table 4 a few examples of XOS production from low-cost agricultural residues and their bioprocess characteristics are mentioned.

Xylan extraction is often performed by alkaline extraction, dilute acid extraction, hot water (steam) extraction or a combination of these methods as described previously. The choice of extraction method and the need for additional steps

depends mainly on the type of raw material. For example, in cereal bran addition of amylases for starch removal could be necessary prior to hot water extraction to improve purity (Immerzeel et al. 2014). Also, addition of proteases to remove excess of gluten has been added to the treatment process (Falck et al. 2014). It was observed that the addition of amylase and protease in rye bran increased the purity of the product but it also increased the formation of Maillard products after heat treatment (Falck et al. 2014). Also depending on the target product, the sensitivity of xylan substituents towards processing conditions should be considered. For example, substituents such as feruloyl and acetyl groups are removed in high temperature and alkaline conditions.

The advantage of using alkaline extraction is that compared to hot water and dilute acid extraction it uses a lower temperature thus producing less (undesirable) side products such as furfural and 5 hydroxymethylfurfural that could potentially inhibit the enzymatic activity to produce XOS. In **Paper II** alkaline extraction with NaOH (2%) followed by ethanol precipitation and neutralization was used. Also, production of xylose is lower in this method. Production of xylose is undesirable because it first of all does not have prebiotic properties and secondly it inhibits the xylanase activity for further production of XOS.

After extraction, the xylan rich substrate is commonly subjected to enzymatic degradation to XOS. As previously discussed, endoxylanases from GH10 and GH11 are used mainly for production of XOS. Examples of endoxylanases studied are mentioned in Table 4. Bacterial endo-β-xylanases from Gram-positive bacteria from classes *Bacilli* (*Bacillus aerophilus*, *B. halodurans*, *B. mojavensis*, *B. licheniformis*, *B. subtilis*, *Geobacillus thermodenitrificans* and *G. thermoleovorans*), *Clostridia* (*Clostridium thermocellum*) and *Streptomyces* (*Streptomyces rameus*, *S. halstedii*, *S. matensis*, *S. olivaceovirides*) have mainly been used. Also, thermostable GH10 xylanase from the Gram negative thermophile *Rhodothermus marinus* have been used in AXOS and XOS production from hardwood and cereal xylans (Falck et al. 2014). Most common fungal endoxylanases used have been from different strains of *Aspergillus* (*Aspergillus foetidus*, *A. niger*, *A. oryzae*, *A. versicolor*) also from *Penicillum*, *Trichoderma*, *Thermomyces*, etc. (Linares-Pasten et al. 2018).

These enzymes are obtained using recombinant or native organism production system. Production of XOS as prebiotic as food ingredient requires the use of GRAS (generally recognized as safe) organism or a recombinant GRAS host. Otherwise the produced enzyme has to be further purified to assure a safe product for human consumption (Linares-Pasten et al. 2018).

Table 4. Examples of research studies on XOS production.

Xylan source	Pretreatment	Xylan extraction yield	Xylanase characteristics	XOS production	Reference
Rice husk	Alkaline extraction (NaOH)	Maximum yield of 54.49 ± 0.61% with alkaline concentration, steaming time, and temperature of 12%, 30 min, and 133.64 °C, respectively	Endoxylanase from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae* (Pentopan™ MonoBG)	17.35 ± 0.31 mg XOS per mL xylan in the run conditions of 6.25 mg enzyme per g xylan, 9 h of incubation time, and 5% of xylan	(Khat-Udomkiri et al. 2018)
Sweet sorghum bagasse	Alkaline extraction (NaOH, KOH)	Maxium yield 33.3 ± 0.7% at 3.91 h, extraction temperature of 86.1°C, and NaOH concentration (w/w) of 12.33%	*Bacillus subtilis* MR44, an engineered biocatalyst to secrete only the XynC xylanase and Axh43 arabinoxylan hydrolase is capable of processing $MeGAX_n$ to exclusively U-XOS	average degrees of polymerization of 11–12 acidic (uronic acid) xylooligosaccharides (U-XOS)	(Wei et al. 2018)
Corn cob	ultra-high pressure (UHP) pretreatment	100% recovery of hemicellulose	endo-xylanase produced from *Streptomyces thermovulgaris*TISTR1948	maximum XOS yield of 35.6 mg/g substrate	(Seesuriyachan et al. 2017)
Rye bran	Enzymatic removal of starch and proteins followed by heat treatment	62% (w/w) AX	*Rhodothermus marinus* *Rm*Xyn10A from GH10 and Pentopan Mono BG from GH11	GH10 gave a higher yield of short oligosaccharides (60% w/w) with xylobiose as the main product; xylobiose and xylotriose were the main products with GH11 (40% w/w).	(Falck et al. 2014)
Wheat straw	Alkaline extraction (NaOH)	56.5g xylose /kg wheat straw	Mutated endoxylanses *from Bacillus halodurans*	36% conversion of the xylan to predominantly xylobiose 0.38g XOS/g Xylan pH 7 0.36g pH 8	**Paper II**
Wheat straw (also tobacco stalk, cotton stalk, sunflower stalk and)	Alkaline extraction (KOH)	Sugar content of extracted xylan:79.9% xylose 10.8% arabinose	*Aspergillus niger and Trichoderma longibrachiatum*	A. niger 0.079gX2-X5/g Xylan T. *longibrachiatum* produced significant amounts of xylose	(Akpinar et al. 2009)
Corncob	Alkaline extraction NaOH	Not indicated	*Bacillus halodurans* (free and immobilized)	0.34g X2- X3/g Xylan (Free enzyme)	(Lin et al. 2011)
Sugarcane bagasse	Alkaline extraction (alkaline hydrogen peroxide)	80.9%	Crude xylanase extracts from Thermoascus aurantiacus	37.1%	(Brienzo et al. 2010)
Natural grass	Alkaline (NaOH) and steam	98%	endoxylanase from *Trichoderma viridae*	11 % xylobiose based on Response surface model (RSM)	(Samanta et al. 2012)

7. Films and hydrogels from xylan

Xylans have varying chemical structure depending mainly on their source and extraction method. They thus have varying physical and chemical properties making them a good starting material for many applications. The main xylan polymer-based applications have focused on the formation of films and coatings, foams and gels, for food and medicinal industry including active food packaging, wound dressings, and drug capsules. For most applications their chemical modifications are necessary to enhance their properties, or to create materials with new applications. The hydroxyl groups in the backbone of xylan chain makes it possible for modification using different chemical methods.

7.1. Films and coatings

The great majority of film materials in use today are based on fossil raw materials. The increasing environmental and health awareness on their utilization has forced us to look for bio-based films and coatings. However the share of bioplastics in the global plastic market still remains low (only represent about 1% of around 320 million tonnes of plastic produced annually) (Bioplastics 2017). In this context, xylans as an abundant biodegradable resource and with great oxygen barrier properties have received increasing interest (Escalante et al. 2012). Agricultural and wood sources of xylan have been studied for packaging films, edible food and drug coating.

The film properties of different xylans depend mainly on its characteristics such as crystallinity, purity, degree of polymerization, heterogeneity, frequency and regularity of substituents. For example in **Paper IV** using wheat bran soluble AX and insoluble AX, two xylan-based films were produced which had significantly different properties. The elongation at break and the water uptake was 5.3 and 7.6 times higher for the film containing soluble AX than the film containing insoluble AX. As mentioned previously (Chapter 2) soluble AX has more arabinose side chain with slightly lower molecular weight compared to insoluble AX. The more irregular and substituted the xylan chain is, the less crystalline the material will become and there will be higher probability of film formation. As an example the loss of acetyl and methylglucuronic acid groups during extraction or the pulp

bleaching process will diminish film forming properties (Sousa et al. 2016). The presence of 1% lignin (w/w lignin/xylan) after xylan purification for both cotton stalk and birchwood xylan can also induce self-supporting film formation (Goksu et al. 2007). Höije et al. (2005) showed the possibility of producing flexible film from pure barley husk AX with high tensile strength (stress at break 50 MPa) however it was highly hygroscopic with low flexibility (2.5 % elongation at break).

The desired specific properties of the film depend on the final application of the produced film which could defer greatly depending on the application. As an example while low water solubility is desired in food packaging in order to maintain product integrity over time, in edible films high water solubility is an advantage (Goksu et al. 2007). In general, for food packaging material; films should have properties such as low oxygen permeability, water resistance, mechanical strength and flexibility.

Most pure xylans cannot form a self-supporting film with acceptable mechanical properties and must be modified to form a film with necessary physical properties. These modification methods that could be used in combination or alone include addition of: plasticizer, second polymer and cross-linker, which will be discussed in the following section.

7.1.1. Addition of plasticizers

Plasticizers have been used to increase flexibility of xylan films and prevent cracking. Low molecular plasticizers such as glycerol, sorbitol and xylitol can enter the polymer chain network increasing the free volume of the material and thus increasing macromolecular mobility of the polymer (Aydinli and Tutas 2000, Wang et al. 2014a). They can also decrease intermolecular forces, which can negatively affect the strength and gas permeability (Goksu et al. 2007, Gontard et al. 1993).

The use of glycerol and sorbitol for xylan film production has shown to be successful. The effects of different concentrations of glycerol, propylene glycerol or sorbitol on the properties of corn hull AX films as edible coating have been evaluated (Zhang and Whistler 2004). It was observed that glycerol had higher plasticization effect than sorbitol and propylene glycerol. However, glycerol showed higher water vapor permeability than sorbitol. The authors concluded that since glycerol compared to sorbitol has lower molecular weight and is hygroscopic it is more effective in breaking chain-to-chain hydrogen bond interactions between polysaccharide chains, and thus introduce more free volume in the AX film matrix. Consequently, glycerol-AX films showed higher

elongation at break but lower tensile strength and were more permeable towards water compared to sorbitol-AX films. In **Paper IV** glycerol was used as a plastisizer to produce arabinoxylan-tragacanth film. Our preliminary studies showed that its addition was necessary to produce a flexible film.

Gröndahl et al. (2004) also showed that addition of sorbitol and xylitol facilitated film formation from aspen wood glucoronoxylan. With increasing the amount of plasticizers the elongation at break increased, but the tensile strength and Young's modulus of the films decreased. At high amount of plasticizer, it was found that films with xylitol gave lower extensibility and more brittleness due to crystallization. Addition of plasticizer greatly decreased the glass transition temperatures. The films with sorbitol also showed low oxygen permeability comparable to poly(vinyl alcohol) (PVOH) films, which are excellent oxygen barrier materials.

Goksu et al. (2007) showed that addition of glycerol to cotton stalk xylan/lignin films significantly affected its mechanical properties by decreasing the tensile strength from 1.3 to 0.8 MPa but increasing the strain at break values from 49% to 90%.

Mikkonen et al. (2009) showed that addition of plasticizer, either glycerol or sorbitol, was necessary for film formation from oat spelt AX. The films containing glycerol had higher elongation at break at two different concentrations tested. They also had higher tensile strength at 10% w/w concentration than the films containing sorbitol, but with a 40% plasticizer content, the result was the opposite. However, the films with sorbitol had lower water vapor permeability than films with glycerol. Addition of lipids, such as fatty acids have also proven to be efficient in improving water vapor barrier properties of xylan-based films (Queiros et al. 2017).

The characteristics of xylan-based films can be further diversified with addition of a second polymer and/or a cross-linker to match the requirements needed for various application.

7.1.2. Addition of second polymer

Another method to enhance film properties of xylan is to add another polymeric material.

The et al. (2009) studied the effect of blending agar, cassava starch and AX to produce edible film. Their results showed that the mechanical properties of agar based films were degraded when cassava starch or AX was added. The positive effect of AX was to increase moisture barrier efficiency of agar based films. Peng

et al. (2011b) could significantly improve mechanical properties and thermal stability of xylan rich hemicellulose films by adding cellulose nanofibers. A combination of xylan and chitosan has also been previously tested (Costa et al. 2015, Schnell et al. 2017). Schnell et al. (2017) combined beechwood xylan and chitosan in different ratios with 10% glycerol. They concluded that xylan-chitosan films with mass ratio of 70/30 (xylan/chitosan) were the most promising films with more transparency, uniformity and flexibility with tensile strength of 10 MPa and elongation at break of 12.2%. Increasing the amount of xylan resulted in increase in tensile strength to 23.1 MPa, however it negatively affected its flexibility. This is due to the fact that xylan has a lower molecular weight than chitosan and thus can crystallize easier than chitosan. This increase in intermolecular hydrogen bonds due to higher crystallization results in a film with higher stress at break but with lower strain at break (lower ductility).

The addition of tragacanth as a second polymer, which had not previously been examined, was evaluated in **Paper IV**. Gum tragacanth is exudates from stem and branches of the plant Astragalus genus mostly found in semi desert and mountainous regions of south west Asia, from Pakistan to Greece and in particular in Iran and Turkey (Whistler 1993). Tragacanth is an anionic polysaccharide gum with monosaccharides composition of D-galacturonic acid, D-galactose, L-fucose (6-deoxy-Lgalactose), D-xylose, L-arabinose, L-rhamnose. The proportions of each sugar varied significantly among the gums from the different species of *Astragalus* (Balaghi et al. 2011). Tragacanth is composed of two main fractions. One fraction which is often called Bassorin is insoluble in water but has the capacity to swell and form a gel. The other fraction termed Tragacanthin is soluble in water and gives a colloidal, hydrosol solution (Balaghi et al. 2011, Eastwood et al. 1984, Gavlighi 2012, Tischer et al. 2002). The main structural difference between Bassorin and tragacanthin is their uronic acid and methoxyl content and it has been suggested that bassorin is a complex structure of polymethoxylated acids and on demethoxylation, probably yields tragacanthin (Balaghi et al. 2010). Tragacanthin itself has an ethanol soluble (arabinogalactan) fraction and ethanol insoluble (tragacanthic acid) fraction which depending on the sugar composition in different *Astragalus* species can be arabinogalacturonan, xylogalactorunan or fucogalactorunan (Balaghi et al. 2011).

Tragacanth has been in use for thousands of years and since 1961 has been accepted as GRAS and its positive health effects have long been proven (Eastwood et al. 1984). Due to its high viscosity at low concentration, unusually high stability to heat and acidity and emulsifying properties it is commonly used as stabilizer, thickener, emulsifier, adhesive agent, anti-freezing agent in food, pharmaceutical, cosmetic, textile and leather industries (Balaghi et al. 2011, Mohammadifar et al. 2006, Saha et al. 2017).

Recent studies on its resistance to microbial attack and prebiotic and mucoadhesive (Nur et al. 2016) properties have opened up a whole new range of applications of tragacanth especially in food and medical industries. Different types of materials based on tragacanth gum have been studied such as hydrogels, films, nanofibers and nanoparticles, mentioned in Table 5. Tragacanth hydrogels have been produced for applications such as edible films, wound dressing, drug delivery systems, tissue engineering and separating membranes. Having excellent mucoadhesive properties and a hydrogel network makes it a good candidate for slow release of drugs. Moreover, studies have shown prebiotic potentials of oligosaccharides from tragacanth (Gavlighi 2012) which could be valuble for colon targeted drug delivery system, as they can only be degraded in colon where the drug needs to be released. Thus the arabinoxylan-tragacanth based films produced in our study (**Paper IV**), with potential prebiotic properties observed for oligosaccharides from the respective materials, can possibly be used as a coating for food or colon targeted drug delivery systems.

Table 5. Examples of different materials produced from tragacanth and their applications

Type of material	Application	Additives	Reference
Hydrogel	Wound dressing	Using radiation to cross-linking PVA with tragacanth and alginate Or tragacanth and polyvinyl pyrrolidone (PVP)	(Singh et al. 2016) (Singh et al. 2017)
	Drug delivery system Tissue engineering Separating membrane	Using heat and Cross-linker (glycerin, ethylene glycol, triethylene glycol or glutaraldehyde)	(Kiani and Asempour 2012, Kiani et al. 2012)
Film	Edible film	Blend with whey protein Blend with locust bean gum	(Tonyali et al. 2018) (Mostafavi et al. 2016)
Nanofiber	Wound dressing	Electrospinning of tragacanth and PVA solution	(Ranjbar-Mohammadi et al. 2013)
Nanoparticle	Drug delivery system	Acid induced gelation of tragacanth to entrap insulin	(Nur and Vasiljevic 2018)

7.1.3. Addition of cross-linker

Xylans having ferulic acid side chains, such as AX, can naturally form covalent cross-linking via diferulate bridges. Cross-linking between these ferulic acid residues or ferulic acid and tyrosine/protein and ferulic acid and lignin determines the solubility of AX (chapter 2). In order to further induce cross-linking it is possible to add a cross-linker. Natural dicarboxylic and polycarboxylic acids have

been used as cross-linking agents for polysaccharides. They have the advantage of being biocompatible and unlike most synthetic cross-linkers - which are toxic if are not reacted - any unreacted acid will be nutritionally acceptable and can also act as plasticizer (Azeredo et al. 2015). During cross-linking the carboxyl groups of the dicarboxylic and polycarboxylic acids form covalent ester linkage with the hydroxyl groups of the polysaccharide forming a bridge between the polysaccharide chains. In **Paper IV** FTIR results indicated a high degree of esterification when malic acid was added which indicates cross-linking of the polymers. Formation of these inter and intramolecular bonds within the polysaccharide chain thus increase its mechanical strength, and barrier properties which can also reduce solubility, water uptake and the mobility of the polymeric chains (Olsson et al. 2013).

Citric acid has commonly been used as cross-linker in biofilm production from polysaccharides such as agarose (Awadhiya et al. 2016) starch (Olsson et al. 2013) and xylan (Azeredo et al. 2015, Wang et al. 2014b). Previously citric acid with varying concentrations together with glycerol has been added to wheat straw hemicellulose for food packaging applications (Azeredo et al. 2015). The results showed improved water resistance and water vapor barrier properties of the films. The tensile properties showed plasticizing effect of citric acid especially at higher concentrations.

The addition of citric acid and dicarboxylic acid to polysaccharides for production of hydrogels and foams for medical applications such as wound dressing have also been evaluated (Chen et al. 2008, Salam et al. 2011, Tsao et al. 2011). As an example Salam et al. (2011) used citric acid to cross-link hemicellulose to chitosan and produced an elastic and highly porous film. Their analysis showed that the addition of citric acid greatly improved mechanical properties (higher storage modulus) and water absorption capacity relative to hemicellulose-chitosan without the addition of acid. Tsao et al. (2011) compared two dicaroxylic acids (glutamic acid and succinic acid) as cross-linker for producing chitosan hydrogels. The in vitro cell culture and histological examinations showed the potential use of the produced hydrogel as wound dressing. Other dicarboxylic acids such as oxalic acid, succinic acid, malic acid, and adipic acid have also been used to produce porous chitosan membranes (Chen et al. 2008). The results showed that the material properties like the water uptake and tensile strength were improved when using dicarboxylic acid solutions compared to commonly used acetic acid solvent for chitosan dissolution.

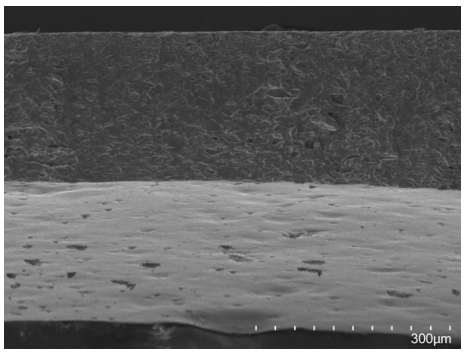
7.2. Foams and hydrogels

There has been a great interest in the production of biocompatible and biodegradable hydrogels for the manufacturing of biomedical products such as contact lenses, hygiene products, tissue engineering scaffolds, drug delivery systems and wound dressings (Calo and Khutoryanskiy 2015, Hoare and Kohane 2008). Natural based hydrogels are preferred over most synthetic hydrogels due to their biocompatible and biodegradable nature and greener manufacturing process.

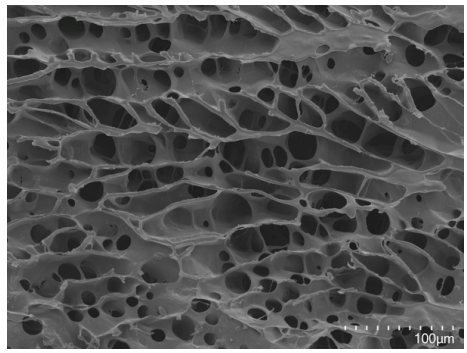
Xylans have been used as starting material for production of hydrogels. To form three-dimensional structures needed for hydrogel formation the polymer chain must be cross-linked or there should be a network of secondary forces (H-bonds) strong enough to support the structure. For example, xylan-based hydrogels have been prepared by copolymerization of xylan with acrylic acid, acrylamide or N-propylacrylate (Gao et al. 2015, Peng et al. 2011a, Zhang et al. 2015). Cross-linking of xylan with poly (ethylene glycol)-β-poly (propylene glycol)-β-poly (ethylene glycol) (PEG-PPG-PEG) for hydrogel preparation has also been carried out previously (Pahimanolis et al. 2014).

Other milder processes include addition of another polymer either physically mixed or cross-linked to xylan. Gabrielii et al. (2000) produced a xylan-chitosan film with hydrogel properties when placed in aqueous solution. Salam et al. (2011) cross-linked birchwood xylan and chitosan with citric acid and produced an elastic foam with good water absorption and the capability of absorbing salts from aqueous solutions.

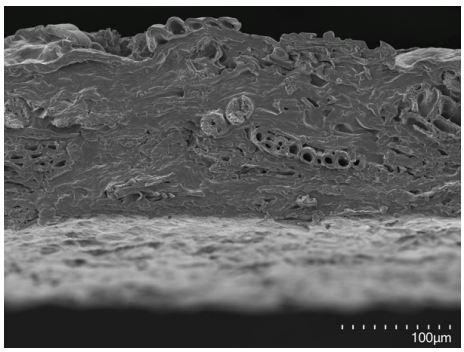
In **Paper IV**, the addition of tragacanth (as a second polymer) and malic acid (as a cross-linker) to soluble arabinoxylan were effective in producing a network strong enough to form a hydrogel. However, they did not have the same effect when added to insoluble arabinoxylan. The arabinoxylan-tragacanth based films containing soluble arabinoxylan had the ability to uptake water and produce a hydrogel with homogeneous macroporous structure as shown in SEM images (Figure 9).



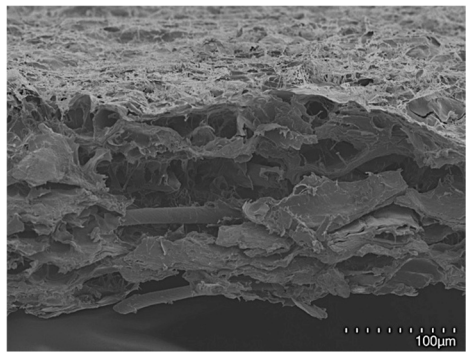
A)



B)



C)



D)

Figure 9. SEM images of arabinoxylan-tragacanth based films. A) dried films, containing soluble AX, B) freeze-dried sample after 30 min swelling, containing soluble AX, C) dried films, containing insoluble AX, D) freeze-dried sample after 30 min swelling, containing insoluble AX.

8. Conclusion and future perspective

The future of chemical production relies on renewable, recoverable resources. For this, lignocellulosic biomass is an excellent source. However, until now xylan as the second most abundant biomass in nature, has been under-utilized and most often treated as a source of biofuel, or as agricultural waste.

In our studies, we focused on xylan-based products which we believe have higher future prospects. Enzymatic production of alkylxylosides, as an emerging pentose based bio-surfactant, was studied. The process was optimized and scaled up in a semi-continuous process, which resulted in significantly higher yields. However, there is much room for improving such process to decrease limitations with enzymatic synthesis such as product inhibition and degradation.

Another emerging industry in this area is the production of prebiotics from xylan. Finding new starting materials and optimizing the processing technologies is indeed essential. For this reason, we chose wheat straw as a cheap and abundant raw material to produce XOS. By choosing a thermostable alkali-tolerant family 10 endoxylanase, it was possible to produce XOS mainly X2 followed by X3 with significantly low amounts of xylose which is not selective for stimulation of prebiotic organisms. The prebiotic potential of the resultant XOS product was confirmed by *L. brevis*, a putative prebiotic strain. However, to further continue with this study it is necessary to evaluate the utilization of our produced XOS with other prebiotic strains to have a better picture of its effect on GIT microorganisms. Xylan extraction process and following selective hydrolysis can also be improved to increase the yield of XOS.

To produce XOS prebiotics with more efficiency in stimulating probiotic microorganism, it is important to know how they are metabolized and degraded by GIT microorganisms. *L. brevis*, which is known to be one of the best XOS-fermenters among LAB, was chosen for further studies. The 3D-structures of *β*-xylosidases in *L. brevis* studies have shed some light into their mechanism. However, the importance of the oligomeric structure for function is still not clear. Other sites of mutations than what we studied here could assist in this regard and also help us to understand the function of different regions of the enzyme.

The vast array of chemical structures in xylan makes it a perfect biopolymeric starting material for producing various materials. However some modifications

and functionalizations are needed to reach the target material with its specific properties. Cross-linking of xylan with other biopolymers can assist in tuning in to desired characteristics. Two produced xylan-tragacanth based films in our studies with significantly different characteristics are examples of how versatile xylan films can be, based on their source.

Research on xylan and its many applications are ever increasing. Today, both technical and economical aspects have to be addressed to be able to compete with current petroleum based industries. We can foresee, in the future, a tremendous increase in the number of industries utilizing xylan as starting material for production of various chemicals and products.

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