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Elucidation of enzymatic consortia for structural chemotyping of dicot hemicelluloses

JURY

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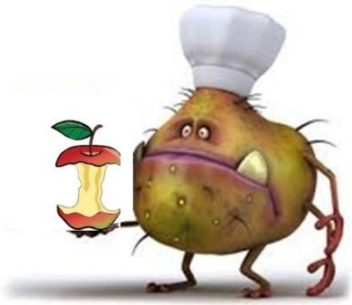
Elucidation of enzymatic consortia for structural chemotyping of dicot hemicelluloses



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Abbreviations

AIR	Alcohol Insoluble Residue
AX	ArabinoXylan
CAZy	Carbohydrate-Active enZymes
AEC	Anion Exchange Chromatography
CWR	Cell Wall Residue
DMSO	DiMethylSulphOxide
HPAEC	High Performance Anion Exchange Chromatography
HPSEC	High Performance Size Exclusion Chromatography
GalM	GalactoMannan
GAX	GlucuronoArabinoXylan
GlcM	GlucoMannan
GgM	GalactoglucoMannan
GgMOs	GalactoglucoMannanOligosaccharides
GuX	GlucuronoXylan
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry
pDAIR	partially Depectinated Alcohol Insoluble Residue
SEC	Size Exclusion Chromatography
XOs	XylanOligosaccharides
XyG	XyloGlucan
XyGOs	XyloGlucanOligosaccharides

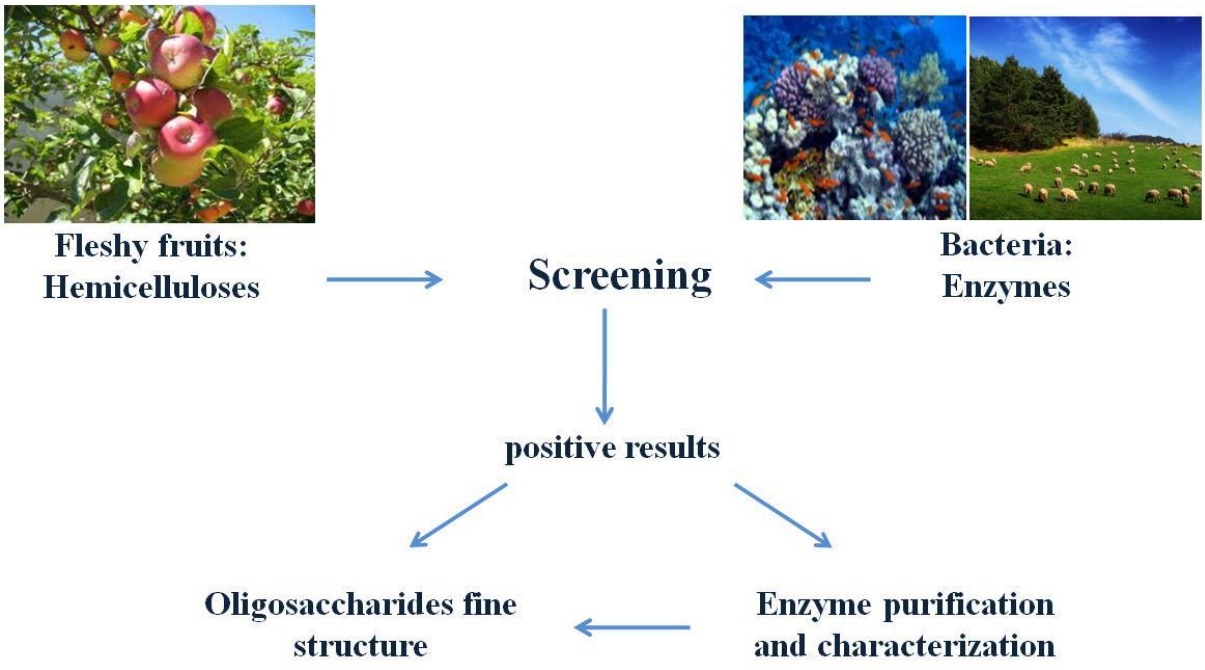
Thesis outline

Plant cell walls are the key components in controlling the mechanical properties of tissues and organs, intercellular exchanges and defense against pests and diseases. They consist mainly of polysaccharides including cellulose, hemicellulose and pectin. Cellulose constitutes the rigid framework of the wall through a crystalline organization and interaction between fibrils. These interactions are controlled by the hemicelluloses, which adsorb on and between the fibrils and allow there organization of the cellulose throughout cell life. From a functional point of view, hemicelluloses play a key role in regulating the mechanical properties of organs and have an additional direct impact on the uses of crops including the ability of fruits and vegetables processing, or the assessment of the texture by the consumer.

Hemicelluloses are divided into three structural families: xyloglucans (XyG), xylans and mannans. The amount and the structure of all of them largely depend on the type of cell wall, the tissue, the state of development and the plant considered. Upon these three families XyGs are the predominant components in the fleshy fruit walls. In these organs, the xylan population consists mainly of glucuronoxylan (GuX), whereas mannans refer to as galactoglucomannan (GgM). Very little information are known about the structure, properties and functions of these polysaccharides in the plant (Ulvskov & Scheller, 2010), as well as the enzymes involved in their establishing and remodeling. Moreover, little is known about the location of these polysaccharides *in muro* and their interaction with other polysaccharide and non-polysaccharide partners in the wall.

In order to better understand the relation between the fine structure of the hemicellulose (XyG, GgM and GuX) and their function within the walls, a detailed structural study is needed with respect to their glycosidic linkages, the distributions of ramifications and non-sugar substituents, and the location *in planta*. Due to their very narrow specificity, polysaccharide-degrading enzymes can be considered as performant analytical tools to elucidate the polysaccharide structure. This type of study needs to use highly purified enzymes and to know precisely their specificity and mode of action, in order to be able to anticipate their action on an unknown polysaccharide. Many enzymes active on hemicelluloses were reported to date and can be used for this purpose. However, they have some limitations and do not allow describing fully the more complex structures. The objective of this project is thus to expand the range of enzymatic tools that are specific of hemicelluloses in order to better understand their fine structure. In this context in addition to

commercial sources, bacterial enzymes belonging to potential glycoside hydrolase families responsible for hemicelluloses degradation will be investigated.



Scheme of the thesis project.

To achieve this goal, the project consisted of three consecutive steps:

Step 1 consisted of the preparation of different hemicelluloses from apple *var*. Gala in their native form, using the LiCl/DMSO solvent system combined with different chromatographic purifications and identification using commercial enzymes as analytical tools. This part of the project was realized in the 'Cell Wall' team in INRA Nantes.

Step 2 consisted of the data mining of bacterial genomes for identifying new hemicellulases, and their functional validation in selected bacteria using different culture conditions, and different polysaccharides as substrates, including the apple hemicelluloses previously purified. This part of the project was realized partly in the 'Station Biologique' in Roscoff (1/3rd) and in CERMAV in Grenoble (2/3rd).

Step 3 consisted of the isolation of enzymes of interest in selected bacterial culture supernatants. Further, a preliminary approach on their mode of action on apple and other

hemicelluloses was demonstrated. This last part of the study was realized in Nantes.

The first section of this manuscript presents a bibliographic review about the plant cell wall, the hemicelluloses and related enzymes, and ends with the aim of the thesis work. In the second section, the results of this project are presented in three parts corresponding to the three steps of the strategy explained above. The first part deals with the gentle extraction of apple hemicelluloses and their structural characterization, and corresponds to a paper published in **Carbohydrate Polymers**. In the third part, bacteria were selected as promising sources of new hemicellulases and the produced enzymes were functionally validated. This is the object of a paper submitted in **Applied Microbiology and Biotechnology**. In the fourth and last part of the results, some selected bacterial extracts were fractionated and a preliminary investigation on the specificity and mode of action of the present enzymes was carried out. The manuscript ends with a general discussion of the results and the perspectives that can be drawn from these results.

In addition to the papers, this thesis work has been presented in different congress listed below.

Oral presentations

1. Congrès GLUCIDOC 2013, Landeda, France.
Hemicellulose fine structure in apple as investigated by enzymatic profiling.
Sayani Ray, Jacqueline Vigouroux, Bernard Quemener, Estelle Bonnin, Marc Lahaye.

2. 27th International Carbohydrate Symposium 2014, Bangalore, India.
Elucidation of enzymatic consortia for the structural chemotyping of dicot hemicelluloses.
Sayani Ray, Maude Fer, William Helbert, Marc Lahaye, Estelle Bonnin.

Poster presentations

1. Summer Course Glycosciences, 2012, Groningen, Netherlands.
Screening of bacterial enzymes for the degradation and modification of hemicelluloses of fleshy fruit.
Sayani Ray, Maude Fer, William Helbert, Marc Lahaye, Estelle Bonnin.

2. Plant and Seaweed Polysaccharides Symposium 2012, Nantes, France.
Screening of bacterial enzymes for the degradation and modification of hemicelluloses of fleshy fruit.
Sayani Ray, Maude Fer, William Helbert, Marc Lahaye, Estelle Bonnin.

3. The XIIIth Cell Wall Meeting 2013, Nantes, France.
Hemicellulose fine structure in apple as investigated by enzymatic profiling.
Sayani Ray, Jacqueline Vigouroux, Bernard Quemener, Estelle Bonnin, Marc Lahaye.

4. The International conference on challenges in chemistry and biology of carbohydrates, 2014, Dehradun, India.
Novel fine structure in apple hemicelluloses as revealed by enzymatic profiling.
Sayani Ray, Jacqueline Vigouroux, Bernard Quemener, Estelle Bonnin, Marc Lahaye

Chapter I

Hemicelluloses and hemicelluloses modifying enzymes: structure, properties and applications

I.1. Plant cell wall

In contrast to vertebrate animals, plants do not have specialized skeletal systems to bear up the gravitational forces. This is instead provided by the cumulative strength of the walls that surround individual cells together with the cellular water turgor pressure. Plant cell wall plays also many other essential biological roles with regard to cell growth and organ development, cell-to-cell signaling, and environment-cell signaling (Keegstra, 2010). Cell walls represent the major part of plant biomass that plays an important part in human civilization providing housing, clothing, food, energy, pharmaceuticals and fiber material, such as paper.

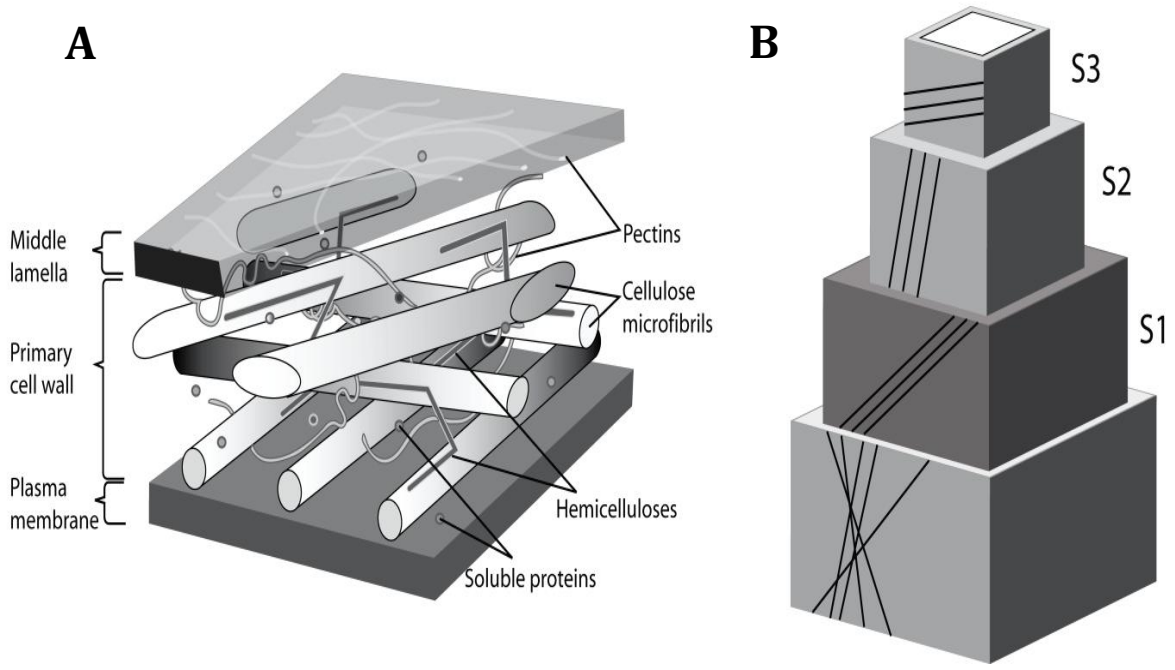


Figure I-1: Plant primary (A) and secondary (B) cell wall structure (Eklöf, 2011).

On the basis of cell wall development and source origins, the cell wall can be differentiated into two major types of cell walls, primary and secondary. The primary cell wall (Figure I-1A) has many important functions such as structural and mechanical support, protection against pathogens and dehydration, maintenance and determination of cell shape and regulation of internal turgor pressure. Moreover, primary wall provides plasticity allowing cell expansion and cell division during growth. The primary cell wall is mostly composed of cellulose and matrix polysaccharides that differ in their ratio between different

tissues and plants (Fry, 2004). Accordingly, they are classified into three classes: type I found in dicots and noncommelinoid monocots (Fry, 2004), type II found in grass species such as rice (McCann & Carpita, 2008) and type III found typically in many ferns (Silva et al., 2011).

The secondary cell wall is present in highly specialized cells such as vessel elements or fiber cells. It is formed after cell expansion and elongation between plasma membrane and primary wall (Figure I-1B).With the formation of secondary wall, cell wall becomes thicker and stronger. This wall mainly consists of three layers: a thin inner layer S3 that is surrounded by a thick and multilayered middle layer S2 and a thin outer layer S1 (Boerjan et al., 2003). The presence of these layers enables the cell wall to withstand several environmental stress situations like internal pressure or compression. Furthermore the contribution of secondary wall to plant biomass is much higher than that of primary wall.

There is a great interest in understanding primary wall structure and organization in relation to their biological role and their enormous utilization in human civilization, in particular in different agro-industries.

I.2. Cell wall polysaccharides

The cell wall is composed of polysaccharides, proteins, minerals such as calcium and boron, and in some cells, non-polysaccharide polymers such as polyphenolic compounds. The proportion of the different polymers depends on plant origin, function of the cell and stage of development (Albersheim et al., 2011). The different cell wall polysaccharides including cellulose and non-cellulosic polysaccharides such as pectins and hemicelluloses will be briefly described.

I.2.a. Cellulose

Cellulose is the main reinforcing component in the plant cell wall. Due to its high abundance in nature and its importance in the mechanical properties of cells and organs, its structure has been studied in depth. Cellulose is a linear homopolysaccharide, composed of 1,4-linked β-D-glucopyranose (Glc*p*) units where the repeating unit is the disaccharide cellobiose. This structure is built on two 1,4-linked β-D-Glc*p*. The conformation provided by the β-1,4-glucan backbone lead to a strong tendency to form intramolecular hydrogen bonds and when two glucan chains are present, intermolecular bonds (Kataoka & Kondo, 1998)

(Figure I-2). These bonds result in the lateral aggregation and crystallization of the β-1,4-glucan backbone into a microfibrille structure that is responsible for the strength and stiffness of the cell wall. The native form of the glucan chains is referred to as cellulose I where the molecules are oriented in parallel direction (Kroon-Batenburg & Kroon, 1997).

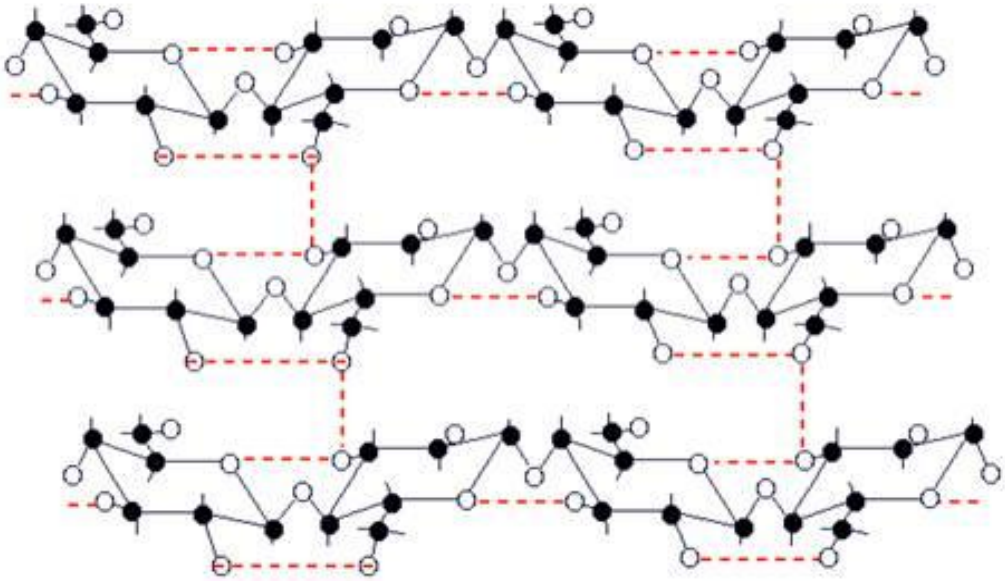


Figure I-2: Schematic representation of intra and inter molecular H-bonding (presented as dotted lines) in between cellulose microfibriles.

These crystalline regions are interrupted by amorphous regions. Strong basic treatments of cellulose I cause the conversion into cellulose II, in which glucan chains are arranged into anti parallel direction (Albersheim et al., 2011).

I.2.b. Pectin

Pectins are a highly heterogeneous group of polymers defined by the presence of galacturonic acid as the main monosaccharide moiety. They play an important role in the plant cell walls by influencing porosity, surface charge, pH and ion balance. They are therefore responsible for the ion transport in the cell wall (McNeil et al., 1984). Generally, pectin in the cell wall associates smooth regions, that comprises the homogalacturonan (HG) and xyloglacturonan (XGalA) and rhamnogalacturonan II (RG-II), as well as hairy regions, containing rhamnogalacturonan I (RG-I) carrying galactan and arabinanan side chains (Figure I-3).

The HG region consists of a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (Gal*p*A) residues (Figure I-3A) (McNeil et al., 1984) and accounts for around 60% of total pectin present in the cell wall (O'Neill et al., 1990; Mohnen et al., 2003).The carboxyl groups of the Gal*p*A residues can be methyl esterified and *O*-acetylated at C-2 and/or C-3 (Ishii, 1995). The degree of esterification and their distribution along the backbone affect the functional properties, such as surface charge, pH and ion balance of HG. When the carboxylic group is free of methanol, HG chains can interact via divalent ion bridges, such as calcium, forming junction zones and leading *in vitro* to gelation (Thibault & Rinaudo, 1986).

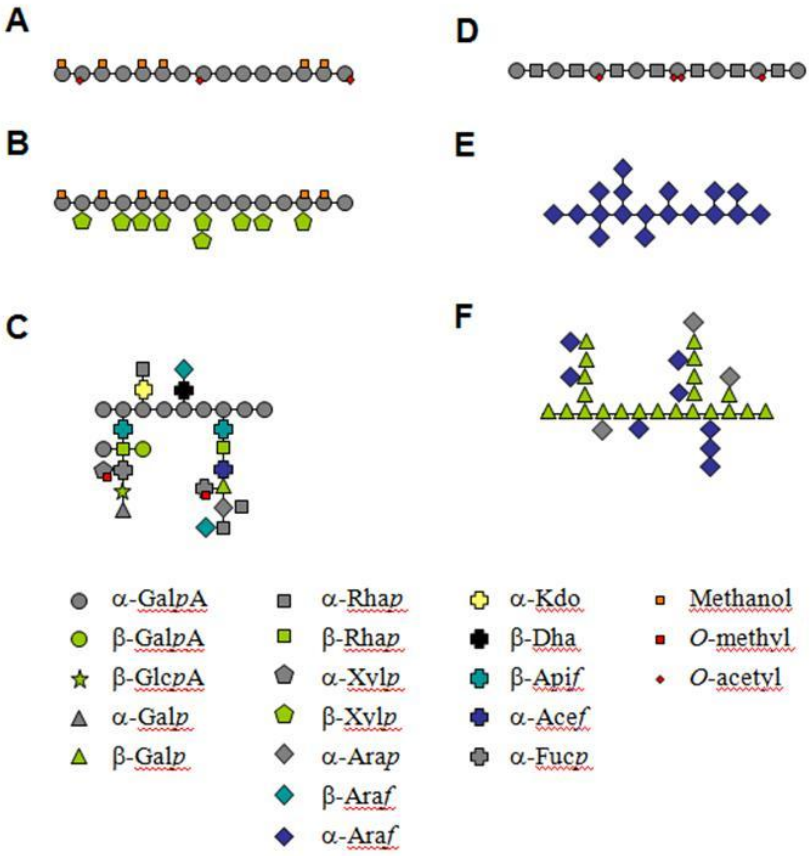


Figure I-3: Schematic representative structures of pectic structural domains: homogalacturonan (A), xylogalacturonan (B), rhamnogalacturonan II (C), rhamnogalacturonan I (D), arabinan (E) and arabinogalactan (F) (Bonnin et al., 2014). Galactopyranosyluronic acid (Gal*p*A), glucuronopyranosyluronic acid (Glc*p*A), galactopyranosyl (Gal*p*), rhamopyranosyl (Rha*p*), xylopyranosyl (Xyl*p*), arabinofuranosyl (Ara*f*), arabinopyranosyl (Ara*p*), 3-deoxy-D-manno-2-octulosonic acid (Kdo), 3-deoxy D-lyxo-2-heptulosonic acid (Dha), apiofuranosyl (Api*f*), aceric furanosyl acid (Ace*f*), fucopyranosyl (Fuc*p*).

Homogalacturonan substituted with 1,3 linked β-D-xylopyranosyl (Xyl*p*) unit side

chains is called xylogalacturonan (Figure I-3B). It is mostly found in the reproductive tissues such as fruits and seeds (Schols et al., 1995; Daas et al., 2001) and the degree of substitution by Xyl varies with the sources.

RG-II is composed of 1,4-linked α-D-Gal*p*A residues (Figure I-3C).The backbone carries four different side chains made not only of Gal*p*A and Rha*p* but also of rare characteristic sugar residues, such as apiose, 2-*O*-methyl-xylose,2-*O*-methyl-fucose, 3,4-linked fucose, 2,3,4-linked rhamnose, 2-linked glucuronic acid, aceric acid, 3-deoxy-D-manno-2-octulosonic acid (Kdo), and 3-deoxy D-lyxo-2-heptulosonic acid (Dha) (McNeil et al., 1984; O'Neill, Albersheim & Darvill, 1990; Strasser & Amadö, 2002). RGII structure is highly conserved and can be considered as a complex part of HG. RGII can dimerize with boron, forming a borate–diol ester, using only the apiofuranosyl residues of the 2-O-methyl-D-xylose-containing side chains in each subunit (Ishii et al., 1999).

The hairy regions structure is based on a rhamnogalacturonan I (RGI) (Figure I3D) backbone composed of alternating sequence of 1, 2 linked-α-L-rhamopyranosyl (Rha*p*) and 1, 4 linked-α-D-Gal*p*A acid (Schols et al., 1995; Albersheim et al., 1996). The Gal*p*A residue in RGI backbone may be highly *O* acetylated on position *O*-2 and/or *O*-3 (Carpita & Gibeaut 1993; Schols & Voragen, 1994) but presumably do not carry any methyl esterification. Rha*p* can carry side chains at C-4 consisting of arabinans (Figure I-3E), galactans or arabinogalactans (Figure I-3F). There are two types of arabinogalactan according to the linkages. Their degree of branching varies between 20-80% depending on the source (Albersheim et al., 1996).

I.3. Hemicelluloses

The hemicellulosic polysaccharides comprise xyloglucans, mannans, xylans and β-glucans, where the backbone sugars are β-1,4-D-Glc*p*, β-1,4-D-mannopyranosyl (Man*p*), β-1,4-D-Xyl*p* and β-1,3-D-Glc*p* respectively. In each case the backbone is further substituted by a variety of sugars and acetyl groups depending on the source (Albersheim et al., 2011). As this work is mainly focused on hemicelluloses, such as xyloglucan, heteromannan and heteroxylan, their structures will be described in more details.

I.3.a. Xyloglucan

Xyloglucan (XyG) is found in all terrestrial plants (Popper & Fry, 2004; Popper, 2008). It is the most abundant hemicellulosic polysaccharide in the primary cell walls of non-graminaceous plants, often encounting for 10-20% of the dry mass of the wall (Hayashi, 1989).

Table I-1: Short hand notation for xyloglucan oligosaccharides (Fry et al., 1993).

Notation	Monosaccharides and linkages
G	Glc*p*
X	α-D-Xyl*p*-(1→6)-Glc*p*
L	β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p*
F	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p*
U	β-D-Xyl*p*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p*
S	α-D-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p*
T	α-D-Ara*f*-(1→3)-α-D-Ara*f*(1→2)-α-D-Xyl*p*-(1→6)-Glc*p*
J	α-L-Gal*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p*
Q	α-L-Gal*p*-(1→4)-β-D-Gal*p*A-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p* β-D-Gal*p*-(1→4)-
P	β-D-Gal*p*A-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p* β-D-Gal*p*-(1→4)-
M	α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p* β-D-Gal*p*-(1→4)-
N	α-L-Ara*f*-(1→2)-α-D-Xyl*p*-(1→6)-Glc*p* β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-

XyG has a 'cellulosic' backbone consisting of 1, 4-linked β-D-Glc*p* residues where up to 75% of the backbone residues are branched at *O*-6, bearing α-D-Xyl*p* (Voragen et al., 1986; Fry et al., 1993). The Xyl*p* residues can further bear one β-galactopyranosyl (Gal*p*) or α-arabinofuranosyl (Ara*f*) units at *O*-2. The Gal*p* residue can be substituted at *O*-2 with α-L-fucose. The presence of side chains on XyG dramatically change the physical properties of the polymer compared to cellulose. Indeed XyG is highly water-soluble and can not form

ordered crystalline microfibrils like cellulose does (Fry, 1989). The native XyG can be acetylated and the acetyl esterification occurs usually on the *O*-6 of Gal*p* residue and/or on *O*-3, *O*-2 of the glucan backbone (Kiefer et al., 1990; Pauly et al., 1999). The backbone structure is fixed, only the side chain structure and distribution vary with biological origin. Fry *et al*. (1993) described a simple nomenclature by listing the pattern of side chain substitution for each Glc*p* residue in the backbone (Table I-1). The unbranched Glc*p* residue is designated by the uppercase letter **G**, while **X** denotes α-D-Xyl*p*-(1→6)-Glc*p*. The Glc*p* carrying a disaccharide side chain Xyl*p*-Gal*p* or Xyl*p*-Ara*f* are denoted by **L** and **S**, respectively. The Glc*p* carrying a trisaccharide side chain Xyl*p*-Gal*p*-Fuc is designated **F**. Similarly, the Ara*f* residue in the **S** side chain can further carry a β-l-Ara*f* referred to as **T** (York et al., 1996). Some unusual side chains are also reported. Hantus et al. (1997) reported an α-L-Gal*p*-(1→2)-β-D-Gal*p*-(1→2)- α-D-Xyl*p*- side-chain represented by the letter **J** in jojoba seed XyG whereas β-D-Xyl*p*-(1→2)-α-D-Xyl*p* side-chains designated by the letter **U** were also found in tissues of bilberry (Hilz et al., 2007) and argan leaves (Ray et al., 2004). More recently, Pena et al. (2008) found XyG with a 2,4-linked Xyl*p* residue, where Xyl*p* can be substituted with a β-D-Gal*p*A at *O*-2 and a β-D-Gal*p* at *O*-4 (**P** motif), with an α-L-Ara at *O*-2and a β-D-Gal*p* at *O*-4 (**M** motif), an α-L-Ara at *O*-2 and the disaccharide β-D-Gal*p*-(1→6)-β-d-Gal*p*A at *O*-4 (**N** motif), or the disaccharide β-D-Gal*p*-(1→6)-β-D-Gal*p*A at *O*-2 and a β-D-Gal*p* at *O*-4 positions (**Q** motif) in mosses and liverworts.

XyGs structure has been thoroughly characterized. Two types of backbones are distinguishable 'XXXG-type' and 'XXGG-type', based on the number of backbone glucosyl residues carrying side chains (Vincken *et al.*, 1997a). XXXG-type XyGs have three consecutive backbone residues bearing an α-D- Xyl*p* substituent and a fourth unbranched glucosyl residue resulting in a polymer composed of subunits that have an XXXG core (Figure I-4A). Fucosylated XyGs with XXXG-type structure are constituted mainly of XXXG, XXFG, XLFG and XXLG subunits and are present in different proportions depending on the plant tissue and developmental stage (Pauly et al., 2001; Obel et al., 2009). Both XLLG, XLXG galactosylated oligosaccharides are found in tamarind, black currant and *Afzelia africana* XyG (Marry et al., 2003; Ren et al., 2004; Hilz et al., 2006). Hilz et al. (2007) demonstrated the presence of XXUG, XULG, XUFG and XUUG oligosaccharides with small amounts of reduced oligomers (XXUGol and XXLGol) in bilberry. However, it was not clear if the reduced oligomers originate from the source or if they were produced artifactually during the extraction. In addition, acetyl esterification on the Gal*p* residues in XXLG, XXFG,

and XLFG was found in *Arabidopsis* (Gille et al., 2011) and sycamore XyG (Kiefer et al., 1989). In contrast, Quemener et al. (2014) reported mono acetylated Fuc residue in the XXFG oligomer from apple.

In XXGG-type XyG, two consecutive glucosyl residues bear an α-D-Xyl*p* substituent at *O*-6, and the third and fourth backbone residues are free of side chain (Figure I-4B). The less substituted XXGG- pattern is found in cell walls of commelinid monocots (such as bamboo) (Scheller & Ulvskov, 2010) and in solanaceous plants (such as tobacco and tomato)

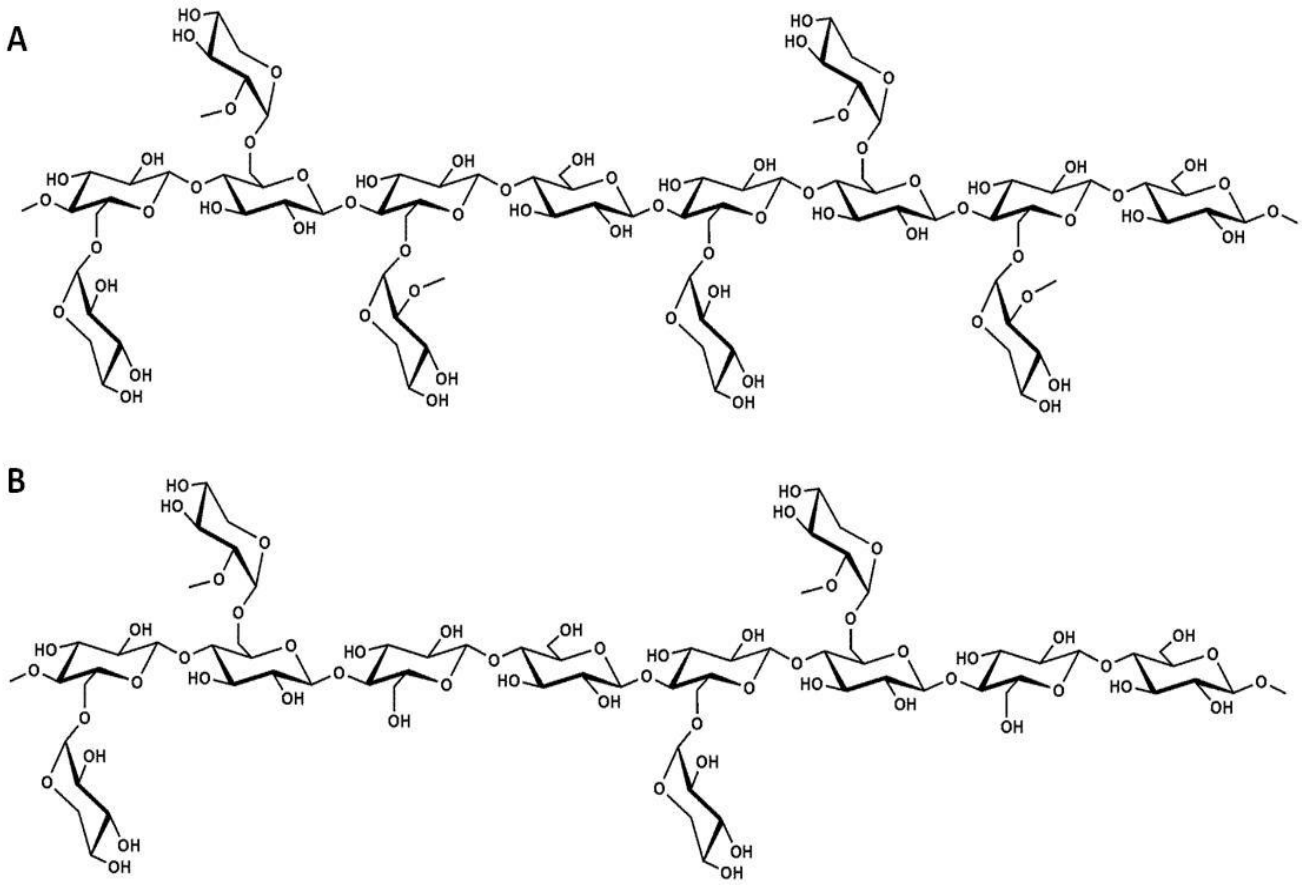


Figure I-4: Schematic representation of the backbone structure of XXXG (A) and XXGG (B) type xyloglucan.

(York et al., 1996; Jia et al., 2003). These types of XyGs do not contain any fucosyl residue and have minor amounts of Gal*p* units. In tomato and tobacco, the Xyl*p* residues are mostly substituted with arabinosyl residues while they are substituted by both Ara and Gal in equal proportion in potato (Ring & Selvendran, 1981; Hayashi 1989, York et al., 1996). Tomato and tobacco XyG have the same side-chains but, in tomato, some Xyl*p* residues may be also

substituted by β-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→2)-chain (Vincken *et al.*, 1997a; York et al., 1996; Jia et al., 2003). Presence of XXXG type oligosaccharide (GLUG) has been recently revealed by Quemener et al. (2014) for the first time in tomato XyG upon endo-glucanase digestion. The acetyl esterification of solanaceae XyG is considered to occur mostly at position *O*-6 of the glucan backbone or at *O*-5 of the terminal arabinosyl residue (York et al. 1988; Sims et al., 1996).

I.3.b. Heteromannan

Heteromannan is another class of hemicelluloses widely distributed in plants, including hardwoods and softwoods, seeds of leguminous plants, fleshy fruits and algae. This class of hemicelluloses can be divided in four subfamilies: linear mannan, glucomannan (GlcM), galactomannan (GalM), and galactoglucomanan (GgM) (Petkowicz et al., 2001). Each of them contains a backbone of β-1,4- linked D-mannopyranosyl (Man) or a mixture of β-1,4- linked D-Man and D-Glc. The backbone can further be substituted by Gal residues and acetyl groups (Figure I-5).

- **Linear mannan**

Linear mannans are homopolysaccharides composed of a linear backbone of 1,4-linked β-D-Man (Figure I-5A). This type of mannan is usually found in woods and in seed endosperms such as Palmae, ivory nuts and green coffee beans (Aspinall, 1959; Wolfrom et al., 1961; Hopf & Kandler, 1977). The mannans from ivory nuts can be separated into two domains depending on their solubility and degree of polymerization (Petkowicz et al., 2007). Mannan I, also called mannan A, is highly crystalline and has low molecular mass (Moreira & Filho, 2008). In contrast, mannan II is built up microfibriler structure similar to celluloses and has high molecular mass than mannan I. Petkowicz et al. (2001) reported microfibrillar crystalline mannan II in the endosperm of *Schizolobium amazonicum* (Leguminosae). Similar mannans were also found in various species of green and red algae (Preston, 1979; Painter 1983).

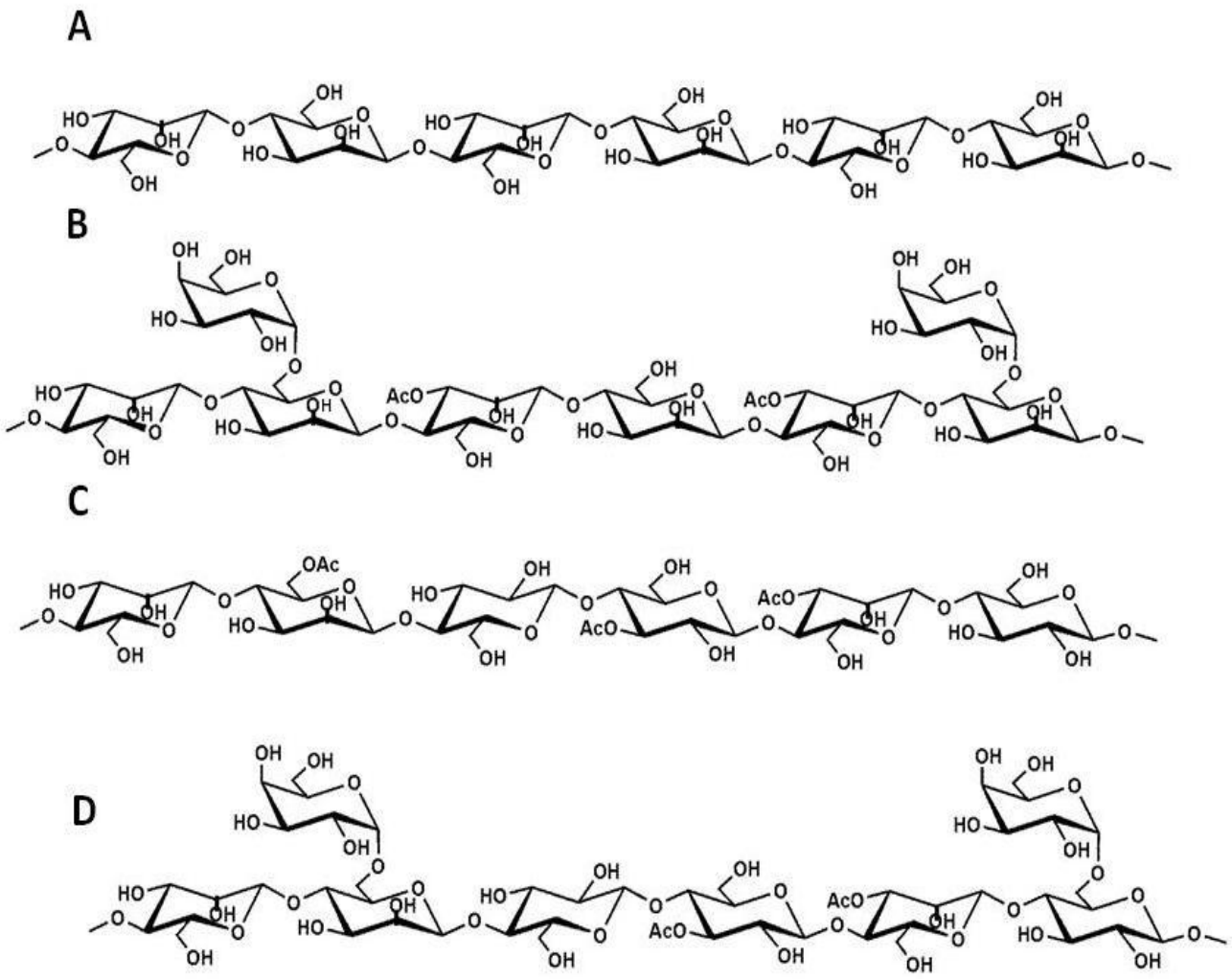


Figure I-5: Schematic representation of the structure of linear mannan (A), glucomannan (B), galactomannan (C) and galactoglucomannan (D).

- **Glucomannan (GlcM)**

GlcMs are present in large amounts in the hemicellulose fraction of softwood. It is a linear chain composed of D-Man and D-Glc residues which are connected to each other by β-1,4-linkages (Figure I-5B) (Northcote 1972; Popa & Spiridon 1998). Hardwoods contain 2–5% of GlcM with a mannose/glucose ratio between 1:1.5–2 depending on the source (Timell 1967; Hongshu et al., 2002). The konjac plant is the most widely used source of GlcM. It has been detected in various tissues such as the tuber where it represents 60-80% (Gao & Nishinari, 2007). It consists of Man and Glc in a molar ratio of 1.6:1, with a molecular weight varying from 200-2000 kDa (Maeda et al., 1980; Nishinari et al., 1992). Usually, 5–10% of

the backbone residues of konjac GlcM are esterified by acetyl groups (Maekaji, 1978). GlcMs are also present in small amounts in angiosperm (Northcote, 1972). The presence of GlcM has also been found in seeds of *Lupinus varius*, an annual herb (Ishurd et al., 2006). Methylation analysis of this structure stated that this polymer mainly consisted of 1,4 linked GlcM with only traces of branched sugar residues. The ^{1}H NMR revealed that the Man residues of this GlcM are mainly acetylated either at C-6 or at C-2 and/or C-3 positions.

- **Galactomannan (GalM)**

GalM occur in large amounts in the endosperm of the seeds of many Leguminoseae and consist of water-soluble 1,4-linked β-D-mannopyranosyl residues with side chains of single 1,6-linked α-D-galactopyranosyl residue (Figure I-5C) (Dey, 1978). Different amounts and distribution of D-galactosyl units along the mannan structure are found in GalMs from different sources. Locust bean, guar and tara gums have mannose to galactose ratios of 4:1, 2:1 and 3:1, respectively (McCleary & Matheson, 1975, Dea, 1993). In *Retama raetam*, a wild plant belonging to the Fabaceae family, another member of the Leguminoseae, Ishurd et al. (2004) reported an unusual GalM backbone containing (1,3)-linked residues together with a small proportion of (1,4)-linked β-D-mannopyranosyl residues with galactopyranosyl units attached at position 6. GalMs are also found in seeds of Coffee. The GalMs of green and roasted coffee grains have unique structural features (Nunes et al., 2005). Both fractions are acetylated: 11 mol% for the green and 8% for the roasted coffee. The electron spray ionization mass spectrometry (ESI-MS) study of β-mannanase-digested GalM revealed that the acetylation occurs preferentially at *O*-2 of Man residues though presences of doubly acetylated hexose residues were also reported. Very small amounts of β-1,4-linked Glc were reported in the mannan backbone. These Glc residues were found as backbone constituents in the green coffee, whereas they were found only at the reducing end of the roasted coffee mannan backbone. Additionally, arabinose (Ara) residues were detected as side chains at *O*-6 of the mannose residues in addition to galactose substitution. Presence of GalM in *Peltigera cannina*, a lichen species revealed (Omarsdottir et al., 2006) that this highly branched GalM was composed of (1,6)-α-D-mannopyranosyl backbone substituted at *O*-2 and *O*-4 with α-Man and β-Gal, respectively.

- **Galactoglucomanan (GgM)**

GgM have been found in mosses (Geddes & Wilkie, 1972), ferns (Bremner & Wilkie, 1971), secondary walls of gymnosperms (Wenda et al., 1990) and angiosperms (Jakimow-Barras, 1973; Kubackova et al.1992; Fischer et al. 1996). All GgMs consist of a mixed backbone of 1,4-linked β-D-mannopyranosyl and 1,4-linked β-D-glucopyranosyl residues. Both monomers can be further substituted at *O*-6 with D-galactopyranosyl residues (Figure I-5D) (Aspinall et al., 1962). The reported ratio of Gal to Glc to Man varies, depending on the plant source and developmental stage of the tissue. For example the Gal to Glc to Man ratio in kiwifruit, tomato and apple were 1:2:2, 1:1.5:2, 1:3:5.7, respectively (Schröder et al., 2001; Nara et al., 2004; Assor et al., 2013). Part of the mannosyl units are *O*-acetyl-esterified, either on C-2 or on C-3 positions (Popa & Spiridon, 1998; Willför et al. 2003). Sims et al. (1997) reported GgM from suspension cultured cells of *Nicotiana plumbaginifolia* where the backbone predominantly contains alternating 1,4-linked β-D-mannopyranosyl and 1,4-linked β-D-glucopyranosyl residues. In addition to single α-D-galactopyranosyl residues or the disaccharide β-D- galactopyranosyl-(1,2)-α-D- galactopyranosyl substitutions, small amount of arabinofuranosyl and xylopyranosyl residues were shown to be covalently linked to the *O*-6 of the mannopyranosyl backbone. More recently, presence of pentose (arabinose and/or xylose) sugar was evidenced in tomato GgM (Assor et al., 2013). GgM were also reported in alga (Lechat et al., 2000).

I.3.c. Heteroxylan

Xylans form a complex family for which the common feature is the presence of a backbone of xylopyranose residues linked by β-1,4-glycosidic bonds (Whistler & Richards, 1970). According to the source, they can be linear or branched polysaccharides and can be further subdivided into homoxylan, arabinoxylan (AX), glucuronoxylan (GuX) and glucuronoarabinoxylan (GAX) (Figure I-6).

- **Homoxylan**

Homoxylans consist exclusively of xylopyranose residues. This type of xylan is not widely spread in nature. The homopolymer occurs in seaweeds such as *Palmaria palmata*. These walls are essentially made of mix-linked β-(1→3) and β-(1→4)-D-xylans with minor β-(1→4)-D-xylans (Deniaud et al., 2003; Lahaye et al., 2003). Analysis of the sequentially extracted homoxylans demonstrated that they are partly acidic, likely due to the presence of sulfate, phosphate and proteins (Deniaud et al., 2003). Furthermore, presence of homoxylan is also reported in esparto grass (Chanda et al., 1950), tobacco stalks (Eda et al., 1976), and guar seed husk (Montgomery et al., 1956).

- **Arabinoxylan (AX)**

Arabinoxylans are mostly abundant in the cell walls that surround the cells of the starchy endosperm and the aleurone layer (about 60%-70%) of the cereal grains (Fincher & Stone, 1986). The backbone of these polysaccharides is formed by xylopyranose residues linked by β-1,4-glycosidicbonds, and the xylopyranose residues can be both mono- and di-substituted at *O*-2 and *O*-3 positions by α-L-arabinofuranosyl units (Figure I-6A). The arabinose substitution frequency is evaluated through the Ara/Xyl ratio and depends on the plant species, the cell wall type and the stage of development. In wheat endosperm, the Ara/Xyl ratio can vary from 0.3 to 1.1(Saulnier et al., 2007). For the extractable rye AX Ara/Xyl ratio of varies from 0.3 to 0.9 (Knudsen & Laerke, 2010). The Ara/Xyl ratio of maize AX varies from 0.06 to 0.6 depending on the extraction conditions and showed some different structural characteristics (Chanliaud et al., 1995). In addition to Ara substitution, the xylan backbone can be further decorated with xylopyranosyl and galactopyranosyl residues (Saulnier et al., 1995a; Saulnier et al., 1999). Even the substituted Ara can be further decorated with xylopyranosyl, galactopyranosyl or one or more arabinofuranosyl residues (Saulnier et al., 1995a; Saulnier et al., 1995b). Pastell et al. (2009) studied the substitution of the arabinoxylan chain in eight different cereal by-products by combining NMR and HPAEC-PAD (high performance anion exchange chromatography-pulsed amperometric detection) techniques.

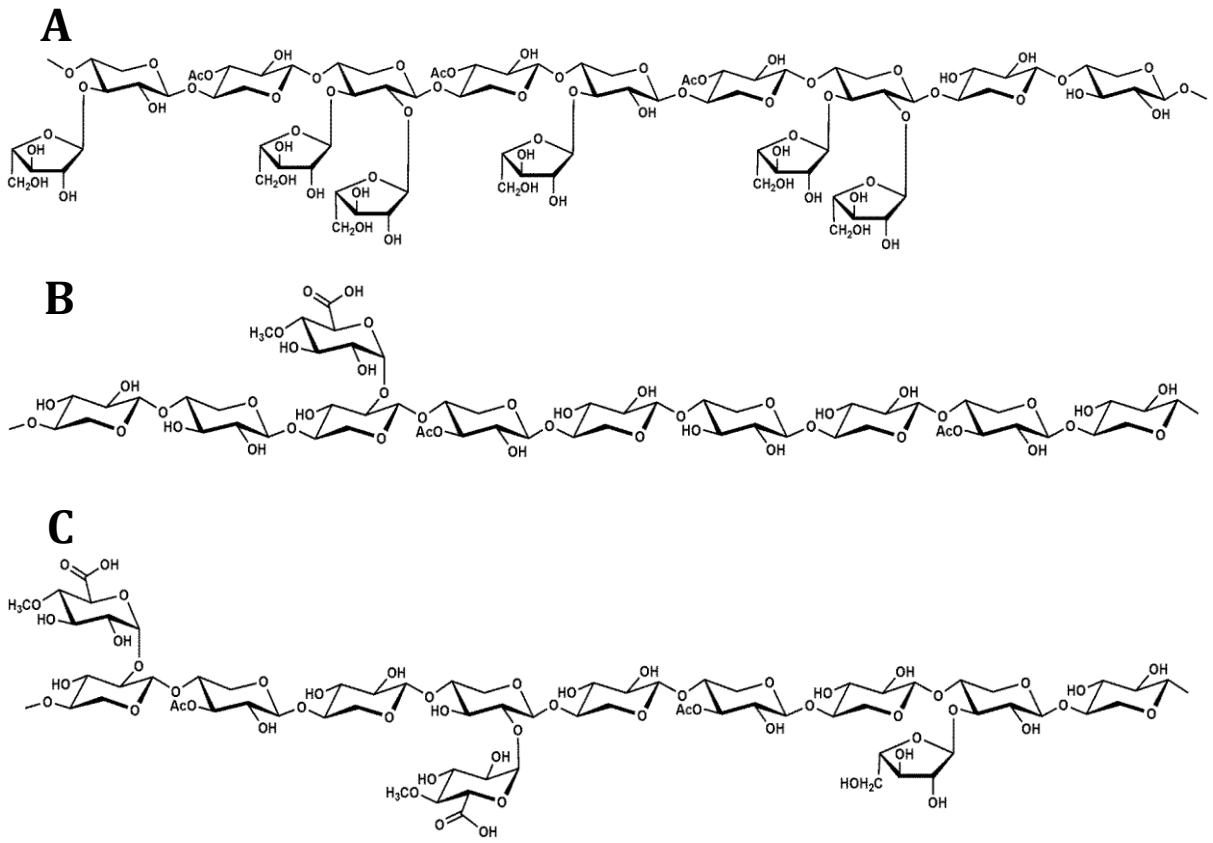


Figure I-6: Structure of arabinoxylan (A), glucuronoxylan (B) and glucuronoarabinoxylan (C).

AX extracted from wheat, rye and oat brans showed similar substitution patterns, with both mono- and di-arabinose substitution. Other AX, originating from more lignified plant tissues such as rice and barley husk, oat spelt or corn cob has been found to contain mostly mono-substituted xylan chains. In addition, phenolic acids such as ferulic and coumaric acids have been found to be esterified to *O*-5 of some Ara*f* residues in AX (Ebringerova, 2005).

- **Glucuronoxylan (GuX)**

The predominant hemicellulose in hardwood is *O*-acetyl-4-methyl-glucuronoxylan (Puls & Schuseil, 1993). It represents 10-35% of the dry wood mass of hardwoods. The polysaccharide backbone consists of β-(1→4)-linked xylopyranosyl residues that are further substituted with α-(1→2)-linked glucuronopyranosyl and 4-*O*-methyl glucuronopyranosyl residues(Figure I-6B) (Lindberg *et al*., 1973; Puls et al., 1993). About 70% of the

xylopyranosyl units are acetylated at C-2 and/or C-3. Acids easily hydrolyze the xylosidic bonds between the xylose units, but the 4-*O*-methyl glucuronic acid is strongly linked to the xylan backbone and this is believed to act as a strong barrier preventing wood from degradation (Subramaniyam & Prema, 2003). Small amounts of rhamnose and galacturonic acid may also form part of the main chain (Coughlan & Hazlewood, 1993). The structure of -*O*-β-D-Xyl*p*-(1→4)-*O*-β-D-Xyl*p*-(1→3)-*O*-α-L-Rha*p* (1→2)-*O*-α-D-GalA*p*-(1→4)-D-Xyl*p* have been identified at the reducing end of birch xylans (Johansson & Samuelson, 1977; Evtuguin et al., 2003). More recently, the presence of GuX has been observed in primary cell walls of fleshy fruits, such as tomato (Prakash et al., 2012; Assor et al., 2013).

- **Glucuronoarabinoxylan (GAX)**

The softwood secondary cell walls contain about 5% to 10% GAXs and/or *O*-methyl GAXs (Puls & Schuseil, 1993). These polysaccharides are formed by β-1,4-linked D-xylopyranosyl backbone, partly substituted at *O*-2 either by α-(1 → 2)-linked glucuronopyranosyl and/or 4-*O*-methyl glucuronopyranosyl residues (Xyl:GlcA ratio of 5:1). Single α-L-arabinofuranosyl residues (Xyl:Ara ratio 10:1.3) are additionally substituted at *O*-3(Figure I-6C) (Joseleau *et al*., 1992; Puls & Schuseil, 1993). Furthermore some of these arabinosyl units can carry feruloyl or *p*-coumaroyl acids at C-5 position. The softwood GAX is less branched and contains less α-1, 2-linked 4-*O*-methylglucuronic acid in than hardwood xylans (Joseleau *et al*., 1992; Puls & Schuseil, 1993; Sunna & Antranikian, 1997). Though the heteroxylans in grasses are often called AX, they tend to contain significant amount of glucuronic acid and 4-*O*-methyl glucuronic acid, making GAX a more appropriate name (Scheller & Ulvskov, 2010). do Nascimento et al. (2013) reported the structure of a galactoarabinoglucuronoxylan from tamarillo (*Solanum betaceum*), a tropical exotic fruit.

I.3.d. Mixed β-glucan

The mixed β-glucans are found in the monocotyledon primary cell wall, particularly in Poaceae but not in dicots (Scheller & Ulvskov, 2010). These polysaccharides are made of a

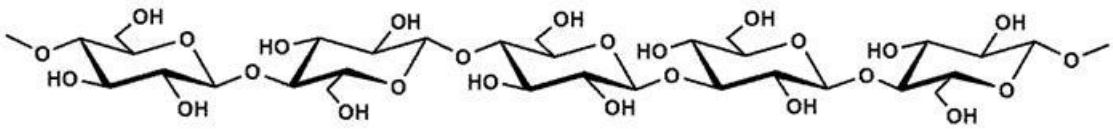


Figure I-7: Schematic structure of mixed β-glucan.

glucopyranosyl backbone and mixed (1-4) and (1-3) linkages. Cellotriosyl and cellotetrosyl oligosaccharides are linked by (1-3) bounds, but (1-4) stretches longer than 4 can occur (Figure I-7). The fine structural variation of β-glucan depends on their origin and their difference are characterized by the molar ratio of cellotriosyl to cellotetraosyl units (Wood et al., 1994; Cui et al., 2000; Roubroeks et al., 2000 a, b).This ratio is around 4.5 for wheat, 3.3 for barley, 3.0 for rye and 2.2 for oat β-glucans (Wood et al., 1994; Cui et al., 2000; Roubroeks, et al., 2000a, b). This hetero-linkage alters the polymer strength and hence increases polymer flexibility**.** Ebringerova et al. (2005) described that such polymers exhibit a tendency for interchain aggregation through strong hydrogen bonding along the cellulose-like regions and hence lower solubility. This structure has a role in the control of cell expansion and their amount is growth-stage dependent (Scheller & Ulvskov, 2010).

I.4. Hemicelluloses in wall matrix

Polysaccharides are organized in plant cell walls and form complex interactions that need to be understood in order to figure out their function in plant cell wall biology and plant uses. The next section will be focused on different linkages or interactions found in the cell wall between cellulose-hemicelluloses, hemicelluloses-pectins, hemicelluloses-hemicelluloses and pectins-pectins.

I.4.a. Cellulose-hemicelluloses interactions

The interaction between XyG and cellulose makes a major contribution to plant cell wall assembly, structure and function. Based on extractability and binding behavior of XyG (Hayashi & Maclachlan, 1984; Hayashi et al., 1987; McCann et al., 1990; Pauly et al, 1999) Pauly et al. (1999) proposed three different types of cellulose-XyG interactions in the plant cell wall. The first domain, which can be degraded by endo-glucanase, includes the

hypothetical tethering, loops and free strands XyG that create the space between cellulose microfibrils. The second domain, inaccessible to endo-glucanase in the plant cell wall and extractable with concentrated alkali, contains the major part of XyG and covers the cellulose microfibrils. The third domain, representing a small part of XyG, is entrapped within the amorphous cellulose microfibrils and is released after complete wall digestion. Bootten et al. (2004) checked mung bean XyG with different mobilities using ^{13}C-NMR spectroscopy and concluded that partly rigid XyG form cross-linking between adjacent cellulose microfibrils and/or between cellulose microfibrils. They demonstrated the absence of XyG within cellulose microfibrils. These results were partly consistent with Pauly et al. (1999) scheme.

To better understand XyG-cellulose interactions, synthetic models were developed. *In vitro* building of XyG-cellulose complexes allows studying the relationship between molecular length and decoration pattern and interacting abilities. Addition of cellulose microfibrils or microcrystalline cellulose to XyG solutions highlighted the pattern of interaction. Hayashi and co-workers have demonstrated by observing high temperature annealing (120-200°C) at pH 5.5 that XyG oligomer having eight or more Glc residues efficiently bind amorphous cellulose, microcrystalline cellulose, and filter paper (Hayashi et al. 1994 a, b). Furthermore, Vincken et al. (1995) confirmed that XyG made of 16 or more Glc backbone quantitatively binds to microcrystalline cellulose at 40°C, pH 5.8. More recently Lopez et al. (2010) highlighted that a minimum of 12 Glc backbone is required to observe significant interactions and an increase of XyG interaction capacity occurred with increasing XyG molecular weight. The XyG-cellulose interaction is thus depending on the XyG backbone length. The binding of tamarind XyG to microcrystalline cellulose is largely independent of pH between 2 and 8 (de Lima & Buckeridge, 2001; Lima et al., 2004). But XyG does not bind to cellulose in strongly alkaline solutions. Therefore, these conditions typically allow extracting hemicelluloses from cell wall material. Still the influence of XyG side chains on binding capacity remains unclear. Higher XyG binding capacity appears related to galactosyl content or specific pattern of substitution (Vincken et al., 1995, de Lima & Buckeridge, 2001). Fucosyl substitution was claimed to increase the adsorption affinity (Hayashi et al., 1994) but it has no influence on binding capacity (Lima et al., 2004). Lopez et al. (2010) reported that both the presence of trisaccharide side chains with fucosyl residues and an increase of unsubstituted Glc residues enhanced XyG-cellulose interactions.

However, little is known about interaction of cellulose with hemicelluloses other than XyG. Several reports proposed the role of xylans to tether neighbouring cellulose microfibrils in gramineous monocotyledon primary walls in a manner similar to that proposed for XyG in

dicotyledons (Brett et al., 1997; Migné et al., 1994). Reis et al. (1991) performed *in vitro* re-association of isolated cellulose/GuX components. Initially dispersed suspensions were shown to be re-associated into cholesteric-like ordered structure that was similar to the *in situ* native organization. In contrast, cellulose flocculated in the same system without GuX. Xylan-cellulose interaction was studied by exposing bacterial cellulose gels to xylan solutions under autoclave treatments and the complex formation was visualized by atomic force microscopy (Linder et al., 2003). Instead of forming multilayer on the cellulose surfaces, globular xylan particles were found on the cellulose surfaces even after short treatment times. Dynamic light scattering measurements suggested that preformed xylan aggregates were absorbed on the cellulose surface. Kabel et al. (2007) demonstrated the adsorption of various xylans to bacterial cellulose. The unsubstituted linear xylan favorably adsorbed to cellulose and the affinity could be related to xylan length. Furthermore, presence of substitution on the xylan backbone, such as arabinosyl and *O*-acetyl substituents, decreased the adsorption. Most likely, increasing the number of unsubstituted xylosyl residues induced the formation of xylan–xylan interactions, which contributed to adsorption to bacterial cellulose.

I.4.b. Pectin-hemicelluloses interactions

A covalent linkage between XyG and pectin is thought to make a major contribution to cell wall structure and metabolism, although the mechanism has not been established yet. The occurrence of covalent XyG-pectin linkages within dicot cell wall was first hypothesized by Keegstra et al. (1973). They proposed a cell wall model containing glycosidic linkages between the reducing end of XyG and what would today be termed the RG-I side-chains. The evidence was based on endo-polygalacturonase (EGP) digestion of *Acer* primary cell walls, where EPG solubilized approximately 50% of pectin with 10% of XyG. Consequently, Baur et al. (1973) treated EPG-digested cell walls with 8 M urea or 0.5 M NaOH or endo-β-glucanase and showed the presence of neutral sugars originating from XyG and pectin. At that time Xyl residues were not distinguished from 2-*O*-methylxylose, and all Xyl residues were attributed to XyG, whereas all uronic acid residues were attributed to pectin. The later discovery of single polymer containing both Xyl (and/or 2-*O*-methylxylose) and uronic acid such as GuX or RG-II (Spellman et al., 1983; Prade et al., 1999) supports XyG and RG-I as belonging to independent networks (McCann & Roberts, 1991; Carpita & Gibeaut, 1993). Further, RG-I was shown to co-elute in anion exchange chromatography with XyG extracted

by concentrated alkali from cotton cell-suspension (Fu & Mort, 1997). They suggested connections between XyG and RG-I through α-(1,5)-L-arabinan by enzymatic digestion of the complex. Similar observations were reported by other authors in suspension rose cell culture and red wine (Thompson & Fry, 2000; Vidal et al., 2003). In addition Thompson & Fry (2000) showed dual behavior of the complex, migration towards the anode during electrophoresis like pectin and binding capacity to cellulose like XyG. The enzymatic digestion of the remaining XyG of the anionic complex after treatment with 8 M urea, 6 M NaOH or proteinase suggested the linkage between XyG and an Ara/Gal-rich pectic domain, probably RG-I (Thompson & Fry, 2000). Abdel-Massih et al. (2003) showed that after ^{14}C labeling of pea shoots, cell wall polysaccharides containing [^{14}C]-galactan chains had a strong affinity for paper (cellulose), which drastically decreased after treatment by endo-β-glucanase (Brett et al., 2005). All together, these results provide further evidence for XyG-pectin complexes. XyG–pectin covalent complexes, accounting for 30-70% of the total XyG, were found in a broad variety of angiosperm cell-suspension cultures (*Arabidopsis*, sycamore, rose, tomato, spinach, maize and barley), despite wide variation in their XyG structures and in their overall primary cell wall compositions (Popper & Fry, 2005). These authors suggested that this complex is evolutionarily conserved among the angiosperms. More recently, Popper & Fry (2008) pulse-labeled *Arabidopsis* cell-cultures with [^{3}H] arabinose and monitored the radio labeling of anionic XyG. [^{3}H] XyG complexed with pectin were detected as early as 4 min after radio feeding and the authors concluded that about 50% of the XyG was synthesized within endo-membrane system on a pectic polymer primer.

Similarly evidence for other hemicelluloses-pectin cross-linking has also been found. The formation of xylan–pectic polysaccharide complexes during maturation of asparagus stems has been reported (Waldron & Selvendran, 1992). Furthermore, the maturation of cauliflower stems was accompanied by an increase in the acidic xylans (Femenia et al., 1999a). Extraction with 0.5 M KOH followed by graded ethanol precipitation by 40-60% and 60-80% released fractions rich in acidic xylans and containing large amounts of XyG and pectic polysaccharides. To investigate the possibilities for linkages between xylan, XyG and pectic components further study was carried out (Femenia et al., 1999b). Additionally, pectic-xylan-XyG complex from cauliflower stem, obtained by graded ethanol precipitation of 0.5 M KOH soluble fraction, were further purified by anion-exchange and gel-permeation chromatographic methods (Femenia et al., 1999b). Upon *endo*-β-xylanase and polygalacturonase of xylan and pectic polysaccharides caused a decrease in the molecular weight of the XyG, thus suggesting a probably covalent interaction between the different

polymer species. On the other hand, a pectic polysaccharide was purified from boiling water extract of Diospyros kaki leaves, a medicinal plant (Duan et al., 2004). It contained rhamnose, arabinose, galactose, xylose and galacturonic acid in the molar ratios 0.4:3.4:2.4:1.0:0.8. The fine structural analysis revealed that short linear chains of 4-linked Xyl residues were directly linked to *O*-4 of rhamnose of the backbone. Furthermore, fractionated arabinogalactan protein (AGP) from suspension culture medium of *Arabidopsis* showed the presence of different monomeric sugars corresponding to xylan and pectin (Tan et al., 2013). This led to the question whether the pectin and xylan-like chains were covalently attached to the AGP. The fine structural analysis revealed that one AX was directly attached to a side chain Ara residue of the AGP domain, whereas another AX was linked to the *O*-4 of Rha in the RG-I domain.

I.4.c. Hemicelluloses-hemicelluloses interactions

The presence of Xyl and a minor proportion of Ara have been reported in GgMs from cell walls of suspension-cultured cells of *N. tabacum* (Eda et al., 1985) and *N. plumbaginifolia* (Sims et al., 1997). Linkage between GgM and GuX was recently revealed (Prakash et al., 2012). Two different populations of GgM-GuX were purified from DMSO and 4 M KOH extractions from cell walls of red ripe tomato pericarp. Both fractions consisted of Man, Glc, Gal together with GlcA and Xyl, indicating the presence of GgM and GuX. They were purified as a single peak in size exclusion chromatography. Upon β-xylanase digestion, there is decline in molecular weight of the complex, suggesting that the enzyme released fragments of GuX, leaving a GgM of lower molecular weight. This indicated that GgM and GuX domains were associated and co-purified along the process. Similar phenomenon was observed using β-mannanase. In contrary, Assor et al. (2013) purified GuX and GgM as two different fractions from tomato using DMSO doped with LiCl. The authors explained that the GuX recovery as an isolated fraction could arise from different varieties of fruit and/or different developmental stages. Another hypothesis was that the interaction between the two polysaccharide families was cleaved during LiCl-DMSO extraction. Poor resolution of the size exclusion chromatography can be ignored. These observations lead to a better understanding of the fine structure of polysaccharides to better understand wall architecture.

I.4.d. Different cell wall models

The different fine structures of the different polysaccharides and the presence of their possible cross links question about the plant cell wall architecture. Based of numerous research experiments over the years, different models have been developed. These models not only describe the plant cell wall and its architecture but help in understanding of the major influence of wall architecture on its degradability, physical strength and on the function of its the individual components.

The first cell wall model was described by Keegstra et al. (1973) based on sycamore cell culture. The authors described the cell wall of dicotyledon as one macromolecular network that consisted of cellulose, hemicelluloses, pectins and proteins. Cellulose fibrils and hemicelluloses were thought to interact via hydrogen bonds. XyG were thought to be covalently linked to galactan side chains of pectins, whereas pectins were found to bind with wall proteins.

A more current model was proposed by Carpita & Gibeaut (1993) who suggested that cell walls of flowering plants can be grouped into two categories: type-I and type-II walls, according to the differences of structural architecture, chemical components, and biosynthetic process. In both cases, three structurally independent but interacting networks were proposed. The network containing cellulose microfibrils coated with branched non-cellulosic polysaccharides (XyG) were embedded in an independant pectin network. The third network contained structural proteins. In this model XyG-cellulose network is thought independent of pectin.

Furthermore, based on microscopic and spectroscopic results, Cosgrove (2000) proposed three cell wall models. The first model was called as "sticky network model" and was based on the model proposed by McCann et al. (1990). This model initially proposed that cellulose-XyG networks are embedded in an independent pectin matrix where the XyG hold the cellulose microfibrils apart. The model also suggested clear roles for a number of enzymes that appear to be associated with growth, such as xyloglucan-endotransglycosylase (XET) (Fry et al., 1992; Palmer & Davies, 1996) and endo-glucanases (Loopstra et al., 1998; Park et al., 2003) in breaking hemicellulose tethers. The non-enzymatic expansins are thought to break hydrogen bonds between cellulose microfibrils and hemicelluloses (McQueen-Mason & Cosgrove, 1994).

The second model referred to as "multi-coat model" was initially proposed by Talbott & Ray (1992). This model on polysaccharides extraction and mobility was assayed by NMR

spectroscopy (Ha et al., 1997). Here the XyG-coated cellulose microfibrils are embedded in layers of successively less tightly bound hemicellulosic polymers and finally pectins. In this model, instead of having tethers linking, microfibrils are held together only by the cohesive forces between successive layers of laterally associated matrix polymers. In this model, XyGs do not necessarily hold the key to wall expansion. Higher molecular mobility of pectin and other hemicelluloses polymers as observed by NMR (Fenwick et al., 1997; Ha et al., 1997) stated that they contributed to an easy slippage of microfibrils during wall expansion. The "stratified wall model" proposed by Cosgrove (2000) is a hybrid of the sticky network and the multi coat models. The main polymers that rivet cellulose microfibrils are XyG (sticky network model). Pectins filled the gap between each layer and thereby controlled the spacing and interaction between the different polysaccharides.

A slightly modified cell wall model was proposed by Bootten et al. (2004) based on detailed structural and molecular organization of mung bean cell wall by solid-state ^{13}C NMR. This study showed the presence of minor rigid and major partly rigid XyG domains but do not allow defining the exact location of these two areas. However, the majority of XyGs present in the walls is assumed to link the cellulose microfibrils together or form associations with other cell wall polymers such as pectins. Based on these different results, Bootten et al. (2004) proposed a new model in which most XyGs form bridges between the microfibrils or interact with other polysaccharides, whereas only a very small amount of XyGs covers the cellulose surface.

I.5. Functions of hemicelluloses *in planta*

The results discussed above about the polysaccharide-polysaccharide interactions not only describe the plant cell wall and its architecture but also envisionne the role of the individual components on the cell wall architecture, physical strength and degradability. The next section will be focused on the functions of hemicelluloses *in plantar*.

I.5.a. Xyloglucan

The XyG-cellulose network forms a load-bearing assembly in the wall which loosening is carefully controlled by several enzymes and other proteins during cell wall

expansion (Hayashi, 1989; McCann & Roberts, 1991; Carpita & Gibeaut, 1993; Somerville et al., 2004). These wall loosing enzymes/proteins include mainly expansin, endo-glucanase and xyloglucan endo-transglucosylase/hydrolase (XET/XTH). Expansins are proteins that alter hydrogen bonds between XyG and cellulose and promote wall loosening in various situations, such as fruit softening (Roseet al., 1997; Brummell et al., 1999). Both endo-glucanase and XET/XTH cleave the polysaccharide backbone, whereas XET ligates the XyG backbone making flexible network during cell expansion. XTHs carry out various functions including wall loosening as well as strengthening (Fry et al., 1992; Antosiewicz et al. 1997), integrating new XyG into the wall (Thompson et al., 2001), trimming XyG strands that are not tightly stuck to the surface of cellulose (Thompson et al., 1997). More recently Park & Cosgrove (2012), digested the primary cell wall of cucumber by three endo-glucanases hydrolysing specifically XyG, cellulose or both. The results highlighted the inaccessible XyG-cellulose structure for the mechanical importance of the cell wall.

Mutants with modified enzymatic activities play an important role in the understanding of the XyG-cellulose network and their mechanical output in the cell wall. For instance, different mutants of *Arabidopsis thaliana* were studied. The mutant *mur2* lacks the xyloglucan specific fucosyltransferase but has no visible phenotype compared to the wild-type (Hoffman et al., 2005). Peña et al. (2004) reported a slightly lower tensile strength for this mutant compared to the wild type plant. MUR3 encodes a XyG galactosyltransferase, inducing that *mur3* XyGs has modified galactose content (Madson et al., 2003). Analysis of XyG composition revealed that more than 80 % of XXXG structures were devoid of D-galactose and L-fucose side chains and at the cellular level this mutation caused abnormal swelling and bulging of cells in the cortex and epidermis and an increase of cell diameter. In contrary to *mur2* plants, *mur3* hypocotyls showed a drastic decrease in stiffness and ultimate stress (Ryden et al., 2003; Peña et al., 2004; Burgert, 2006). Among other *A. thaliana* mutants affected in the XyG biosynthesis, Cavalier et al. (2008) studied the single and double mutants affected in xylosyltransferase genes *xxt1* and *xxt2*. Driselase-susceptible crude cell wall preparations from wild type and three mutants were observed by HPAEC-PAD analysis. Driselase lacks α-xylosidase activity; thus, digestion of XyG produces isoprimeverose (IP). The *xxt1* and *xxt2* single mutants had reductions in IP content of 10.2 and 20.8%, respectively. More interestingly, analysis of the products released by Driselase digestion of cell walls from the *xxt1 xxt2* double mutant indicated that there was no detectable IP released. The single mutants showed a modest reduction in XyG content, whereas in the *xxt1 xxt2* double mutant, XyG level was appreciably lower. Though all the mutants were viable under laboratory

conditions, they exhibited significant reductions in hypocotyl stiffness and failure stress (Cavalier et al., 2008). The *xxt5* single mutant had a 50% reduction in XyG content and the mutation in XXT5 did not eliminate the xylosylation of any particular Glc in XyG (Zabotina et al., 2008). Assessment of cell wall structure in wild-type and *xxt1 xxt2 xxt5* mutant plants by solid-state NMR demonstrated extensive surface interaction between pectin and cellulose microfibrils (Dick-Pérez et al., 2011). Zabotina et al. (2012) demonstrated that the lack of detectable XyG in the cell wall did not cause significant compensation by other polysaccharides. Instead, the structural rearrangements of the polysaccharide network appeared responsible for maintaining wall integrity in the absence of XyG, thereby allowing nearly normal plant growth.

I.5.b. Heteromannan

Heteromannans are less widely characterized and no clear role has been established for them within the overall architecture of the wall despite their presence throughout the plant kingdom. Mannans main functions are as energy storage and/or structural components (Matheson, 1990). In the cell wall of legume endosperm, GalM is a storage component occupying approximately up to 30% of the seed dry weight (Buckeridge, 2010). Their water holding capacity also protects seeds from complete drying that would lead to protein denaturation, especially of enzymes essential for seed germination. The D-galactosyl side branches of the polymer determine the hydrophilic nature of the molecule, and the solubility in water increases with the galactose content (Dea & Morrison, 1975). In the primary cell wall of many ferns, mannans replace XyGs and GAXs as the cellulose cross-linking hemicellulose (Rodriguez, 2012). In tomato, mannan-endotransglycosidase/hydrolase reshuffles the GgM (Schröder et al., 2006) probably during its metabolism during fleshy fruit development (Percy et al. 1997). Mannans have been also observed to contribute to cell adhesion in tomato fruit pericarp parenchyma (Ordaz-Ortiz et al., 2009). In the secondary wall of softwoods, GgM is the principal hemicellulose interacting with cellulose to form a network embedded in lignin (Rowell et al., 2005). In some algae species, crystalline mannan replaces cellulose as the main cell wall glycan (Painter, 1983). The change of sugar configuration leads to a different hydrogen bonding pattern with lower stability than cellulose. Besides their function as storage and structural polysaccharides, mannans have also an important role as signaling molecules in plant cell differentiation (Benova, 2006). Zinnia cultures with exogenous GgM

oligosaccharides (GgMOs) caused an increase in cell population density with a moderate decrease in protoxylem to metaxylem tracheary elements ratio (Benova, 2006). Using macroarrays probed with cDNAs from cells cultured in the presence or the absence of GgM oligosaccharides indicated that significantly more genes were down-regulated rather than up-regulated. Currently, there is a great interest in mannan polysaccharides. Due to their ill-defined fine chemical structure, their properties and role *in planta* are still under discussion.

I.5.c. Heteroxylan

The heteroxylans are widely distributed in the nature mainly in hardwood, seeds, fleshy fruits, and algae. It is likely that in both dicot and commelinoid monocot cell walls, heteroxylans interact non-covalently with cellulose and form inter-microfibril cross-links, in a manner similar to that of either XyG or mannans (Carpita, 1983). Highly-substituted acidic xylans have been suggested to replace pectin in gramineous primary walls and may thus perform similar roles such as the control of porosity (Carpita, 1996). In addition, gramineous xylans carry feruloyl moieties, which probably contribute to wall tightening by oxidative phenolic coupling (Kamisaka et al., 1990; Fry et al., 2000). Furthermore, GuXs possess surface charges and flexible molecular structure that regulate the generation of fine fibrils as a selective moderator (Atalla, 1991). Several studies revealed that GuX could play a role of ‘twisting agent’, ‘helper molecules’, ‘molecular organizers’ or ‘lubricating agents’ driving the cellulose microfibrils into helicoidal rotation (Nieduzinski & Marchessault, 1972; Abeysekera & Willison, 1988; Neville, 1988). Heteroxylan, mostly GuX and GaX, undergo potential modifications by cell wall modifying enzymes during the cell wall loosening that allows plant growth as well as fruit softening. Among these enzymes, endo-(1,4)-β-xylanases are well known in germinating seeds of some monocots (notably cereals) and dicots, and in ripening fruit tissues of some dicot species (Brummell & Schroder, 2009). Paull & Chen (1983) demonstrated that large increase in endo-xylanase activity accompanies fruit ripening in papaya, suggesting heteroxylan depolymerisation during fruit softening. Furthermore, the presence of xylan endotransglycosylase activity was proposed (Schroder et al. 2009; 2010) that was analogous to XET and mannan endotransglycosylase in higher plants. More recently, Johnston et al. (2013) purified xylan endotransglycosylase from ripe papaya fruit. This enzyme showed both endoxylanase and xylan endotransglycosylase activities *in vitro*, suggesting that a single enzyme possesses dual activity *in planta*. They further proposed that

xylan endotransglycosylase could be involved in remodelling or re-arrangement of heteroxylans of the cell wall framework.

I.6. Industrial applications for hemicelluloses

Besides the biologically related functionality of hemicelluloses in plant cell wall, these polysaccharides provide a wide variety of applications. Some of their uses and potential uses are discussed in the next section.

I.6.a. Food industry

Polysaccharides able to show rheological properties are widely used as functional ingredients in the food industry. The high solubility of XyG in water and its rheological properties makes it a useful food additive as thickener, stabilizer and gelling agent (Miyazaki et al., 1998; Yamatoya & Shirakawa, 2003). XyG strongly retains water and the addition of hydrolyzed XyG dramatically improves the hardness and reduces water release of freeze-thawed carrageenan gel (Shankaracharya, 1998; Yamatoya & Shirakawa, 2003). XyG improve the physical properties of dough and bread quality. The addition of XyG and AX increases the stability of the dough and improves the softness of bread. It significantly improves various features of the final products, such as loaf volume, storage properties and appearance (Mishra & Malhotra, 2009). In addition to XyG, the mannans (GalM) have been employed in a wide variety of industrial and food applications for their rheological properties, as thickener, stabilizer and gelling agent (Chandrasekaran et al., 1998; Sittikijyothin et al., 2005).

I.6.b. Paper and pulp industry

Hemicelluloses, specially XyG and mannan (GalM) are used in paper-making (Shankaracharya, 1998). XyG has been tested as a wet end additive (Christiernin et al., 2003; Lima et al., 2003) and thus the addition of XyG to the pulp reduces the friction of the fibers and may thereby improve sheet formation (Stiernstedt et al., 2006). Paper sprayed with XyG showed improved mechanical strength (Ahrenstedt et al., 2008).

The main use of XyG, most often from tamarind seeds, lies in the manufacturing of textile sizing powder. XyG is widely used in sizing jute and cotton yarns due to its high availability and low cost (Mishra & Malhotra, 2009). Anionic derivatives of XyG have been shown to offer some advantages over regular XyG in print paste thickeners. Adducts of XyG with polyacrylic acid are useful in textile printing (Abo-Shosha et al., 2008).

I.6.c. Film and coating making

Films and coatings from renewable materials have numerous potential applications in the food and medicinal industry including active food packaging, wound dressings, and drug capsules (Tharanathan, 2003; Malafaya et al., 2007). Food packaging material derived from sustainable resources is now receiving considerable interest for their advantages in product marketing. In addition to good mechanical properties such as high strength and flexibility, low oxygen permeability can be a key requirement for food packaging materials. Hemicellulose-derived packaging materials have been produced with higher oxygen permeability than other biopolymers such as amylose, amylopectin and chitosan, and thus can be used as an alternative (Hansen & Plackett, 2008). Edible coatings for food stuffs are often necessary to prolong shelf life and maintain organoleptic properties such as texture, taste and mouth feel. The uptake of moisture is often a crucial factor in the rate of degradation of a given product. Therefore coatings acting as moisture barriers are of the utmost interest in the food industry and hemicelluloses play an important role for the edible coating formation (Hansen & Plackett, 2008).

I.6.d. Health aspects

Considerable research is done in the area of hemicellulose applications for biomedical purposes due to their hydrophilicity, biodegradability, biocompatibility, low toxicity and high stability (Ebringerova, 2006; Zhou et al., 2007). Blending hemicelluloses with chitosan (Ranucci et al., 1998), polyvinyl alcohol (Gaillard & Thompson, 1971), and co-polymerization with 2-hydroxyethyl methacrylate (Lindblad et al., 2005) were some of the methods investigated for the formation of hydrogels. Advances in the pharmaceutical industry lead to increased demands on materials for specific applications. Therefore, more specialized drugs and new methods of drug delivery will be necessary to fulfill requirements. XyG sulfate

derivative has an inhibitory effect on the initial stages of the rubella virus (RV) infection and has the highest inhibitory effect on RV antigen synthesis, by blocking a step in virus replication subsequent to virus attachment (Mishra & Malhotra, 2009). Magnetic microparticles, coated with corncob xylan and intended for oral intake, are applied as magnetic markers for monitoring the gastro intestinal motility (Silva et al., 2007). Here, the aim of the polymer coating is to reduce dissolution of the particles in the patient's stomach, ensuring passage into the colon. A number of hemicelluloses such as guar gum (GalM) and konjac GlcM are already commonly used for this application, but other less investigated hemicelluloses may become useful in the future (Hansen & Plackett, 2008).

I.6.e. Next generation fuel

In the era of depleted fossil fuel field, mankind is awaited for the tremendous energy crisis. Hence, much more attention is taken to explore the non-fossil carbon energy sources. In this context the efficient bioconversion of lignocellulosic biomass, originating from agriculture and forest residues, into biofuel becomes a challenging research area (Limayem & Ricke, 2012). Biofuel not only provides alternative energy source but can be also utilized as oxygenate gasoline elevating its oxygen content, allowing a better oxidation of hydrocarbons and thus reducing the amounts of greenhouse gas emissions into the atmosphere (Hill et al., 2006). The lignocellulosic biomasses are mainly composed of cellulose, hemicelluloses and lignin. The essential steps in biofuel production consist of physico-chemical pretreatments of bio mass followed by enzymatic degradation in the monomeric form and then fermentation of the monomers into ethanol (Limayem & Ricke, 2012). Among the lignocellulosic biomass, hemicelluloses currently represent the largest polysaccharide fraction wasted in most cellulosic ethanol pilot and demonstration plants around the world (Gírio et al., 2010). The reasons are based on the heterogeneous polymeric nature of these polysaccharides and the low fermentability of pentoses by the most common industrial yeast strains.

I.7. Isolation of hemicelluloses

In order to better understand the hemicelluloses functionality *in planta* and to improve their industrial usages, their mode of isolation from biomass has received attention in the

recent years. It has been a great challenge for the researchers to design extraction and isolation processes due to the number and diversity of linkages between hemicelluloses and other cell wall components. Much attention has been paid to developing effective isolation and purification methods to obtain hemicelluloses with both high purity and yield. In this section, the methods currently available in literature for isolating hemicelluloses from different sources will be discussed.

I.7.a. Extraction of hemicelluloses

Extraction of hemicelluloses from plant cell wall involves different techniques. Alkali solutions are classically used to extract hemicelluloses since alkali cleave hydrogen bonds between cellulose and hemicelluloses (Fry, 1988). But at such alkaline pH the ester groups are saponified and peeling as well as β-elimination reactions can also occur. Other physico-chemical methods have been introduced for extracting hemicelluloses. Microwave irradiation technique (Passos et al., 2014) was shown to be a very rapid technique to isolate different populations of deacetylated oligomannans according to treatment times. "Active oxygen cooking" used to date for hemicelluloses extraction (Shi et al., 2014). Cooking for different times and temperatures in oxidative conditions generated by MgO and hydrogen peroxide extracted deacetylated AX of different molecular weights, from 3 to 20 kDa. These two extraction methods have limited uses due to the partial depolymerisation and deacetylation of hemicelluloses. Ionic liquids were used to extract hemicelluloses from spruce wood but the yield and the fine chemical characteristics of the isolated polysaccharides were not reported (Anugwom et al., 2012). Cellulose material exhibits considerable inter-micellar swelling in DMSO thus Hagglund et al. (1956) used DMSO to extract hemicelluloses. DMSO-assisted extraction has been optimized with different concentrations of water (Hu et al., 2008; Willfor et al., 2008) or with 8.4% LiCl (Assor et al., 2013). In these conditions, the particular preservation of the acetyl esters represents an advantage to characterize hemicellulose fine structure.

I.7.b. Fractionation of hemicelluloses

The extract obtained from the above-discussed techniques is a mixture of different polysaccharides. So, purification is an on ward way for obtaining homogeneous

hemicelluloses and can be done by various techniques including precipitation, column chromatography, and membrane filtrations. Graded precipitation mainly involves adding miscible organic solvent mostly ethanol, or by adding different salts such as ammonium sulphate in various concentrations. By this way, AX having different Ara/Xyl ratio, branching pattern, hydrodynamic properties were fractionated (Dervilly et al., 2000; Rao & Muralikrishna, 2006; Bian et al. 2010). Recently, Peng at al. (2012) fractionated linear xylan with low UA/Xyl ratio from the alkali soluble fraction of delignified pea shrub by using iodine-potassium iodide precipitation. In this method, iodine complex preferentially precipitates the more linear regions of the polysaccharide (Gaillard, 1961). Barium hydroxide makes complexes with the 2, 3-vicinal hydroxide of Man thus taking part in the precipitation of the Man- containing polymers (Prakash et al., 2012). Membrane filtrations such as microfiltration, ultra filtration are the eco-friendly methods for purifying AX or acetyl-GgM having different sizes (Willfor et al., 2003; Krawczyk et al., 2008). This method can efficiently reduce alcohol utilization but does not permit separating the polysaccharides of high viscosity. Varies chromatographies are widely used techniques for the fractionation of the polysaccharides. Amongst different chromatographic technique used to date, the anion exchange, size exclusion and reversed phase chromatography were widely used for polysaccharide separation. Separation through anion exchange chromatography depends on reversible absorption of negatively charged molecule onto the positively charged matrix. In this way co-extracted pectin can be easily removed from the extract (Capek et al., 1995; Hilz et al., 2007) and charged xylan can be separated from the neutral hemicellulose fraction (Prakash et al., 2012; Assor et al., 2013).

I.8. Introduction to Carbohydrate Active enZymes (CAZymes)

The isolated purified polysaccharides need structural characterization for their better usages and/or understanding of their functionality in cell wall. However, they are macromolecules so it is nearly impossible to detail their fine in intact form. Enzymatic degradation followed by chemical characterisation of the products provides one of the leading tools to resolve the structure of the polysaccharides. The use of the very narrow specificity of enzymes will allow producing oligosaccharides with known initial links and that can be further analysed by chromatography or mass spectrometry techniques.

I.8.a. CAZymes families

All the enzymes are classified according to their catalyzed reaction through an EC number defined by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology. The enzymes mostly involved in the degradation of polysaccharides are glycoside hydrolases, polysaccharides lyases, and carbohydrate esterase. All the polysaccharide hydrolases are referred to as EC 3.2.1., whereas polysaccharide lyases are referred to as EC 4.2.2. In both cases a fourth number refers to the substrate. As early as 1991, most of the polysaccharide-specific enzymes have been grouped into the continuously updated Carbohydrate-Active enZymes (CAZy) database (http://www.cazy.org). The grouping of enzymes into different families depends on their sequence similarity together with certain features shared, such as the overall 3-D structure and the catalytic mechanism (Henrissat & Davies, 1997). Within one family, the substrate specificity can be unique or diverse. Moreover, some families are clustered into clans, each clan representing a general fold pattern. The proteins gathered in the CAZy database are organized into five main classes: glycoside hydrolases (GH) (Henrissat & Davies, 1995), glycosyl transferases (GT) (Campbell et al. 1997), polysaccharide lyases (PL) (Coutinho & Henrissat, 1999), carbohydrate esterases (CE) (Coutinho & Henrissat, 1999), and carbohydrate binding modules (CBM) (Boraston et al., 2004). Very recently, a new class was added for 'Auxiliary activities' corresponding to lytic polysaccharide mono-oxygenases (LPMO) and lignin degrading enzymes (Lombard et al., 2014).

PLs (EC 4.2.2) cleave polysaccharides containing uronic acid via a β-elimination reaction to produce an unsaturared sugar moiety and a new reducing end. These types of enzymes efficiently catalysed degradation of pectin as well as algal polysaccharides like alginate, ulvans carrying uronic acid. As this work did not use PLs, the next section will be focused on CEs and GHs families and their active participation in hemicellulose degradation.

I.8.b. Carbohydrate esterases

Carbohydrate esterases (CEs) are enzymes catalyzing the *O* or *N*-deacylation of substituted saccharides. These enzymes show a great diversity in substrate specificity. In the CAZy database they are currently classified in 16 different CE families including acetylxylan esterases, acetyl esterases, chitin deacetylases, peptidoglycan deacetylases, feruloyl esterases, pectin acetyl esterases, pectin methylesterases, glucuronoyl esterases and enzymes catalyzing *N*-deacetylation of low molecular mass amino sugar derivatives. But many enzymes, mainly feruloyl esterases, do not fit into the established CE families and have been separately classified based on sequence similarities (Gupta Udatha et al., 2011). Here we will focus on acetyl esterases.

- **Acetyl esterases**

Hemicelluloses are present in acetylated forms in plant cell wall. Acetylation not only modifies the physicochemical properties of polysaccharides but also prevents them from attack by polysaccharides modifying GHs. Acetyl esterase is an important class of enzymes to overcome the steric problems provided by acetyl substituent. Plant cell wall degrading organisms have developed acetyl esterases whose function is to deacetylate the polysaccharides prior to, or concomitant with glycoside hydrolases (Taylor et al., 2006). The acetyl esterases have been grouped into CE family 1, 2, 3, 4, 5, 6, 7, 12 and 16 in the CAZy database (http://www.cazy.org). Taylor et al. (2006) expressed two distinct de-*O*-acetylases from family CE4, the *Clostridium thermocellum* (CtCE4) and *Streptomyces lividans* (SlCE4) acetylxylan esterases. Both enzymes showed no detectable activities on *p*NP-acetate. Thus authors used a novel linked assay system using *p*NP-2-*O*-acetyl-xyloside and a β-xylosidase, from which CtCE4 and SlCE4 released acetyl esterification producing *p*NP-xyloside that was further degraded by the β-xylosidase liberating xylose. Thus these two enzymes were very sugar-specific for acetylated xylose. Topakas et al. (2010) tested three acetyl esterases (AcEs) from the saprophytic bacteria *Cellvibrio japonicus* (CjCE2B, CjCE2C) and *Clostridium thermocellum* (CtCE2), members of the CE2 against methyl-6-*O*-acetyl-glucopyranosides, 6-*O*-acetyl-mannopyranosides and methyl-diacetylated (3, 4; 2, 3 and 2, 4)-xylopyranosides. They showed preference for deacetylation of the first two sugar residues. To probe further the positional specificity for xyloside, these enzymes were tested against three different (2, 3 and 4) monoacetylated *p*NP-xyloside and revealed to preferentially degrade acetyl substitution on

positions 3 and 4. Similar positional specificity was observed by the CE16 (Aes 1) from *Hypocrea jecorina* (Li et al., 2008). Drzewiecki et al. (2010) purified an esterase which is encoded within a *Thermotoga maritima* chromosomal gene cluster for xylan degradation and utilization was characterized after heterologous expression of the corresponding gene in *Escherichia coli* and purification of the enzyme. The recombinant enzyme (AxeA) displayed activity on *p*-nitrophenyl-acetate as well as on various acetylated sugar substrates such as glucose penta-acetate, acetylated oat spelts xylan, DMSO-extracted Beachwood xylan. *Thermotoga maritima* AxeA represents the most thermo stable acetyl xylan esterase known to date.

I.8.c. Glycoside hydrolases

Glycoside hydrolases (GHs) are enzymes that catalyze the hydrolysis of glycosidic linkages between sugar moieties. GHs cleave glycosidic bonds by acid-base catalysis *via* either a single or double displacement mechanism, leading to inversion or retention of anomeric configuration, respectively (Koshland, 1953).

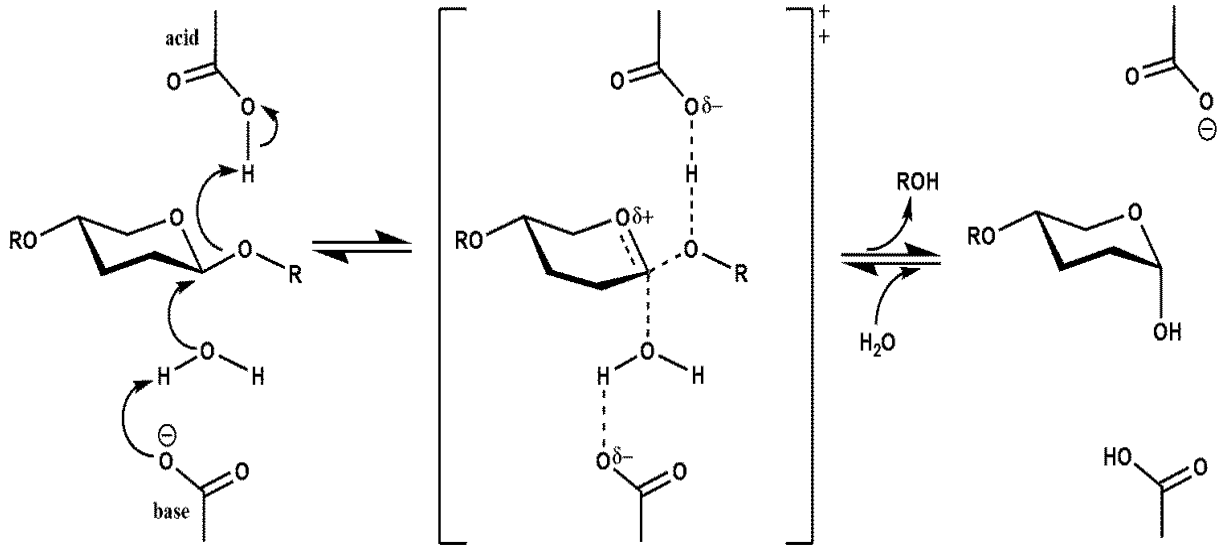


Figure I-8: The converting hydrolysis mechanism.

In the inverting mechanism a carboxylic amino acid acts as a general base. This amino acid activates a water molecule that undergoes a nucleophilic attack on the anomeric carbon at the centre of the glycosidic bond (Figure I-8). Simultaneously, a second carboxylic acid

residue, usually aspartate or glutamate, facilitates the departure of the leaving group by protonation thus permitting the breaking of the glycosidic bond (Koshland, 1953). Consequently, the overall stereochemistry of the anomeric centre is inverted.

On the opposite, the retaining mechanism consists of a two-step mechanism involving nucleophile and acid-base functionality (Figure I-9). In the first step, a carboxylic acid residue acts as a nucleophile that attacks the anomeric carbon forming glycosyl-enzyme intermediate with first inversion of the conformation in the anomeric center. A second carboxylic acid attacks the intermediate inducing a second inversion with final retention of the anomeric carbon configuration (Koshland, 1953). Again, the catalytic residues are usually aspartate or glutamate, whereas a tyrosine acts as the nucleophile (Watts et al., 2003).

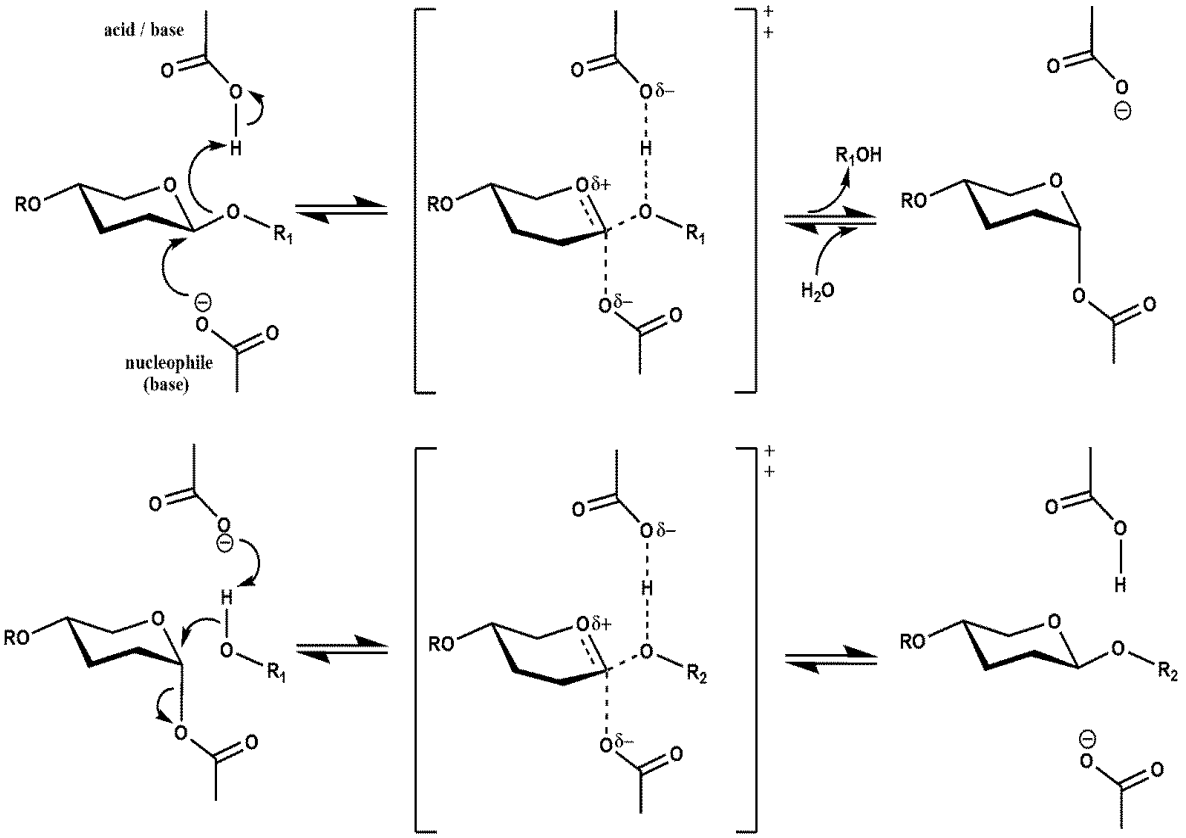


Figure I-9: The retaining hydrolysis mechanism.

More recently, completely distinct mechanisms of glycoside hydrolases involving elimination and/or hydration steps have been discovered (Jongkees & Withers, 2014). For example, in GH families 4 and 109, the reaction proceeds involving both the elimination and hydration reactions. The first step involves NAD^+-mediated transient oxidation of C3 hydroxyl group causing the acidification of the C2 hydrogen and thus allowing elimination

across the C1-C2 bond. The second step involves a nucleophilic attack by water molecule on the α,β-conjugated system consequently reducing the C3 ketone.

Biodegradation of hemicellulose structures involves the concerted action of a variety of hydrolytic enzymes. Currently, 44 of the 133 GH families contain enzymes that contribute to plant cell wall deconstruction.

- **Diversity of xyloglucanases and β-glucanases**

The complete degradation of XyG involves concerted and sequential action of *endo*-acting xyloglucanases, working on the backbone and a variety of *exo*-acting enzymes working on the side chains and including α-xylosidases (GH31), β-galactosidases (GH1, 2, 36, 42 and 59), α-arabinofuranosidases (GH2, 3, 42, 43, 51, 54 and 62), α-fucosidases (GH 29, 95), *O*-acetyl esterases when required according to the source of XyG (Figure I-10). By degrading specifically the XyG backbone, the *endo*-acting xyloglucanases (EC 3.2.1.151) produce XyG oligosaccharides (XGOs) and thus play an important role in the structural determination of the XyG fine structure.

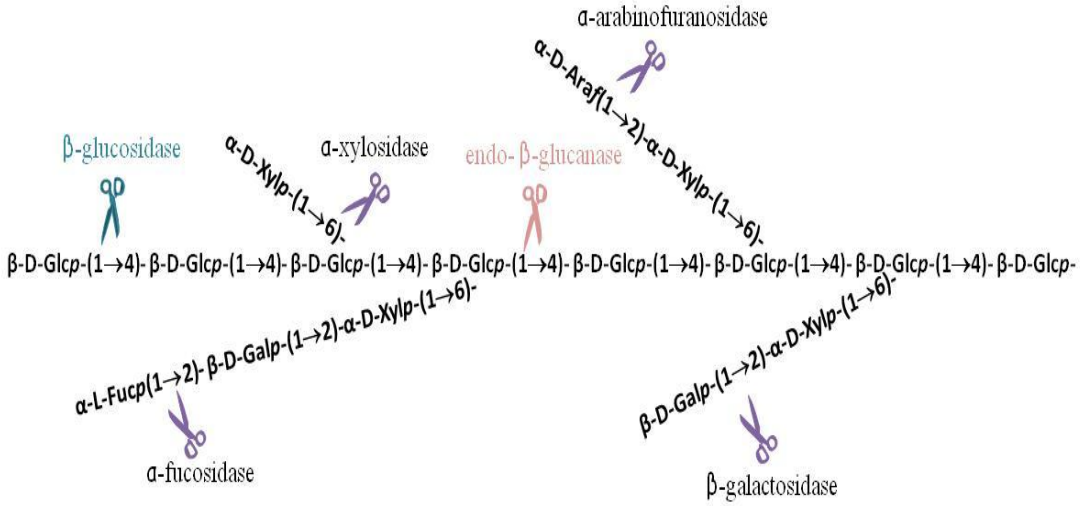


Figure I-10: Overview of the of the enzyme portfolio required for the complete digestion of xyloglucan.

These types of enzymes are classified in GH families 5, 9, 12, 16, 44 and 74 (Gilbert et al., 2008). GH5, 12, 16 and 44 act by retaining mechanism, whereas GH9 and 74 acts by inverting mechanism. The cleaving site of most of these xyloglucanases includes one or more

unsubstituted glucosyl unit at the left of the broken linkage. However Ariza et al. (2011) showed an unusual cleavage pattern for a GH44 from *Paenibacillus polymyxa*. The main products of XXXGXXXG degradation were XXX and GXXXG. This is thus the first xyloglucanase shown to preferentially cut XyG between an X motif in the -1 sub site and a G in +1 sub site of the active site. Gloster et al. (2007) compared the different degradation extent of 2-chloro-4-nitrophenol derivatives of GGGG, XXXG and XLLG using different xyloglucanases secreted either by *Pp*XG5 (GH5) from *Paenibacillus pabuli* or by *Bl*XG12 (GH12) from *B*. *licheniformis*. Substitution of the glucan backbone by both xylose and galactose caused a significant decrease in the reaction rate of *Bl*XG12. In contrast, substitution of glucan backbone either by xylose or galactose increments the reaction rate of *Pp*XG5. In similarity with *Bl*XG12, another GH44 xyloglucanase (*Pp*XG44) from *Paenibacillus polymyxa* revealed decrease in velocity for the same oligo derivatives (Ariza et al., 2011). Altogether, these results make evidence for the role of side chain decoration in the recognition process. *Pp*XG44 showed unusual pattern of degradation, upon degradation of XXXG-XXXG by *Pp*XG44 revealed to produce XXX and GXXXG type of oligosaccharides. Some GH74 members also showed another pattern of XyG degradation. The oligoxyloglucan reducing end-specific xyloglucanobiohydrolase from *Aspergillus nidulans* (Bauer et al., 2005) release XG, LG or FG from XyG. Another oligoxyloglucan specific β-glycosidase from *Geotrichum* sp. M128 revealed similar activity (Yaoi & Mitsuishi, 2002). Xyloglucanase (Cel74A) from *Hypocrea jecorina*, tropical fungus, preferably cleaves XyG and XyG oligosaccharides between two X motifs of XyG backbone (Desmet et al., 2007).

Glucanases can cleave (1-4)-β-glycosidic linkages in a variety of substrates such as cellulose (EC 3.2.1.4), or (1-3)-, (1-4)-β-D-glucan (EC 3.2.1.6). Furthermore, it has been long recognized that endo-β-(1-4)-glucanases can catalyze the hydrolysis of XyGs as a direct consequence of the backbone homology with cellulose. The digestion of XyG by endo-β-glucanases typically produces well defined oligosaccharide mixtures arising from cleavage at specific, usually unbranched, glucosyl residues. Vincken et al. (1997b) reported the mode of action of three endoglucanases, endoI, endolV, and endoV, from *Trichoderma viride* towards various XyG derived from apple fruit and potato by monitoring the released oligosaccharides and demonstrated that EndolV differed from the two other endoglucanases. All three were concluded to bind preferentially unsubstituted Glc. However, contrary to endolV, endoI and endoV released XLXG by a slower rate than XXXG suggesting that they were more affected by the substitution of the substrate. On the contrary endolV showed the slower release of

XXLG compared to XLXG suggesting that the Gal position is predominant to hamper or not the action of endolV. Similarly, due to the structural proximity of GlcM and cellulose, endo-β-glucanase from *Trichoderma longibrachiatum* was shown to degrade the GlcM backbone of tomato and apple acetylated GgMs (Galvez-Lopez et al., 2011; Assor et al., 2013). Presently β-glucanases, with various tolerances for the highly branched XyG chain, belong to 17 GH families, namely families 5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 74, 81 and 124. Recently many research groups have purified and characterized cellulases isolated from different bacteria viz. *Cellulomonas sp.* YJ5 (Yin et al., 2010), *Bacillus sp* (Acharya & Chaudhury, 2011; Ashabil et al., 2011) but their mode of action on XyG, GlcM or GgM is not well known yet.

- **Diversity of β-mannanases**

As heteromannans contain mannose or glucose-mannose backbone carrying galactose and acetyl groups (Petkowicz et al., 2001), their enzymatic hydrolysis requires the combined action of several hydrolases, endo β-1,4-mannanases, β-mannosidases, β-glucosidases as well as α-galactosidases and *O*-acetyl(mannan) esterases removing substitutions (Figure I-11).

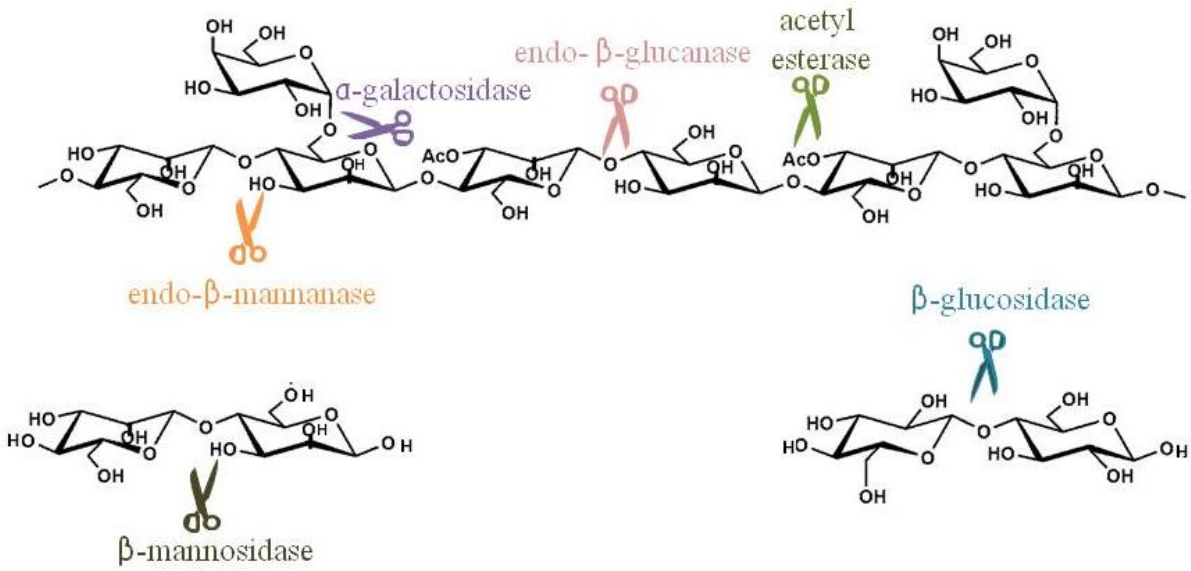


Figure I-11: Overview of the enzymatic consertia required for the complete digestion of galactoglucomannan.

The β-mannanases (EC 3.2.1.78) are responsible for catalyzing the hydrolysis of the β-1,4-linkage between two mannose units. They belong to 4 CAZy families GH5, 26 and 113. All these

families are grouped in the largest glycoside hydrolase clan GH-A, which share the TIM (triose phosphate isomerase) $(\beta/\alpha)_8$ barrel fold and a retaining reaction mechanism. The crystal structures of β-mannanases generally reveal an open active-site cleft with at least four sub sites and the strictly conserved catalytic glutamates (nucleophile and acid/base) presented on β-strands 4 and 7, respectively (Le Nours et al., 2005; Larsson et al., 2006). The β-mannanases usually recognize mannose at -1 sub site and are thus able to hydrolyze mannosidic bonds. The way of enzyme - substrate interaction varies. The mannotriose binds to the GH5 *Thermobifida fusca* mannanase (TfMan5) at sub sites -2, -3, and -4 (Hilge et al., 1998), whereas the GH26 *Cellvibrio japonicas* mannanases A (CjMan26A) uses sub sites -1, -2, and -3 for binding to the same oligosaccharide (Hogg et al., 2001). Some β-mannanases are able to hydrolyze GlcM that contain a β-1,4-linked backbone of Glc and Man, but the respective position of the two monomers in the active site is not yet known. Recognition of Man and Glc at sub site -1 is highly variable, although some general trends for the difference in specificity between GH5 and GH26 mannanases was recently revealed. Both GH5 *Bacillus agaradhaerens* mannanase (BaMan5A) and GH26 *Bacillus subtilis* mannanase (BsMan26A) can accommodate mannose at the negative binding sites. On the contrary BaMan5A also has the capacity to bind Glc at sub sites -2 and +1 (Tailford et al., 2009). Thus, the flexible substrate binding cleft of BaMan5A allows the enzyme to hydrolyze glucomannan with a variable backbone of mannose and glucose units. The presence of arginine at the -2 sub site in GH26 mannanases may explain extensive interactions with the substrate and appears to confer unusually high activity against small manno-oligosaccharides (Ducros et al., 2002; Cartmell et al., 2008).

- **Diversity of β-xylanases**

Complete degradation of xylan requires the synergistic action of a variety of enzymes, including β-xylanase, β-xylosidase (GH1, 3, 8, 30, 39, 43, 51, 52, 54, 116, 120), α-glucuronidase (GH31, 62, 67, 115), α-arabinofuranosidase (GH2, 3, 42, 43, 51, 54, 62), acetylxylan and feruloyl esterases (Shallom & Shoham, 2003) (Figure I-12). Among them, β-xylanase (EC 3.2.1.8) plays a crucial role in the hydrolysis of the xylan backbone, in which it cleaves the β-1, 4-glycosidic bond between xylose residues to release xylo-oligosaccharides. Most of the microbial β-1,4-xylanases are grouped into 7 GH families: GH5, 8, 10, 11, 30, 43 and 51 (Shallom & Shoham, 2003). Currently, the best known β-1,4-xylanases belong to

families GH10 and GH11 (Berrin & Juge, 2008; Paës et al., 2012). In general, GH10 xylanases have higher molecular weights and lower isoelectric point (pI) values than GH11 xylanases. Both GH10 and GH11 hydrolyzed the xylan backbone by configuration-retaining mechanism (Joshi et al., 2001; Pell et al., 2004).

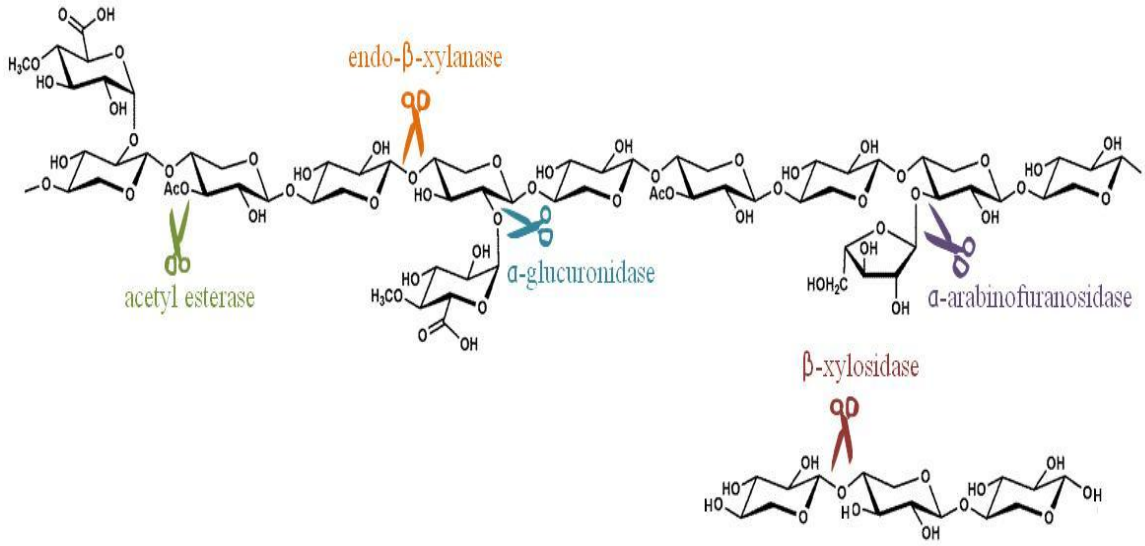


Figure I-12: Overview of the enzymatic consertia required for the complete digestion of glucuronoarabinoxylan.

The three-dimensional structures of GH10 and GH11 xylanases show a $(\alpha/\beta)_8$ barrel fold and a β-jelly roll, respectively, and glutamic acid (Glu) residues play an important role as the catalytic nucleophile/base or proton donor. Hydrolysis studies showed that the family 10 xylanases can attack the xylosidic linkage on the non-reducing end of a substituted residue and can only cleave at the third xylosidic linkage after a substituted residue (Biely et al., 1997). More recently, Kolenova et al. (2006) showed that GH10 and 11 xylanases differ by their substrate specificity, while incubated with purified xylo-oligosaccharides substituted with methylglucuronic acid (MeGlcA). This result revealed that GH10 enzymes interact with xylose substituted with MeGlcA at +1 subsite, whereas GH11 enzymes cleave xylan when MeGlcA is appended at the +2 subsite. More recently, Zhang et al. (2011) purified two xylanases from *Nonomuraea flexuosa* (NfXyn11A) and from *Thermoascus aurantiacus* (TaXyn10A). Their mode of action were observed by degrading birchwood GuX, insoluble oat spelt AX and observing the released xylo-oligosaccharides. Both of them produced xylose and xylobiose as the major end products from birchwood GuX and oat spelt AX. In addition they showed that NfXyn11A produced more xylotriose than TaXyn10A. Similarly, GH10

enzymes degrade xylan with arabinose substituted at +1 subsite, whereas GH11 enzymes cleave xylan only when the two corresponding xylose moieties in subsites − 1 and + 1 are not branched (Biely et al., 1997) and arabinose is appended at the +2 subsite (Maslen et al., 2007). For all these reasons, GH11 are often qualified as 'true xylanases' as they are less tolerant to substrate substitution than GH10 xylanases. A GH5 xylanase displays an absolute requirement for 4-*O*-methyl-D-GlcA appended to the Xyl positioned at the -2 subsite (Vrsanska et al., 2007). Thus not only the degree of substitution of xylan but also the distribution and the nature of substituents along the main chain will influence the hydrolysis products released by xylanases.

I.8.d. Hemicellulases as analytical tools

Several literature studies used enzymatic hydrolysis of the hemicelluloses followed by product analysis by various chemical and/or chromatographic and spectroscopic methods. This approach is widely used to identify different patterns of side chain substitution that are present within the ensemble of molecules and allows deducing their fine structure. Furthermore, hemicellulases have been developed as probes for *in situ* plant cell wall investigations. For example, the cell wall network and tissular organization recalcitrance of wheat bran was evidenced by immunocytochemical localization of xylanase in industrial products (Beaugrand et al., 2004b) and in developing cell walls (Beaugrand et al., 2004c). Visualizing the complex between water-soluble wheat AXs and inactivated enzymes by atomic force microscopy provided the structural heterogeneity of individual polysaccharide molecules (Adams et al., 2004). This study demonstrated the potential uses of inactivated enzymes. Furthermore, for the screening and identification of mutants with altered cell wall structure is challenging. Various biochemical, chemical, and microspectroscopic methods coupled with enzymatic fingerprinting procedures using high-performance anion-exchange-liquid chromatography, fluorophore-assisted carbohydrate electrophoresis, and matrix-assisted laser-desorption offers a powerful tool to screen and identify cell wall mutants rapidly and efficiently and, more importantly, is able to give initial insights into the structural composition and/or modification that occurs in mutants (Lerouxel et al., 2002).

I.9. Industrial applications of hemicellulases

In addition to their role in elucidating the fine chemical structure of hemicelluloses, the various substrate specificity of hemicellulase imparts a variety of applications in food- and feed industry, pulp- and paper industry, textile- and laundry industry, baking industry, waste treatment or ethanol production from biomass. The following section will discuss some of the most promising and newly explored applications of hemicellulases.

I.9.a. Food and feed industry

Hemicellulases play an important role in food processing. The production of fruit and vegetable juices needs several treatment steps such as extraction, clarification and stabilization whereas alternatively xylanase and cellulases are used along with pectinase for liquefying fruits and vegetables and their further clarification (Biely, 1985, Camacho et al., 2003). Moreover, in beer production process, enzymes are used to supplement the malt's own enzymes in order to increase the extract yield, shorten the wort separation, accelerate the fermentation process and increase the alcohol production.

A combination of xylanases from different GH together with amylases, glucose oxidases and proteases is widely used in baking industry where the wheat-flour hemicelluloses (AX) are effectively hydrolysed by xylanase leaving the dough softer by enhancing water absorption and increasing the bread volume (Maat et al., 1992; Camacho et al., 2003). In pasta processing, GH10 and GH11 xylanases can act on durum wheat semolina. They have been reported to modify significantly the rheological properties and consistency of spaghetti dough (Ingelbrecht et al., 2001).

The mannanase degradation of the mannans in the coffee extract efficiently reduced viscosity and thus allows concentrating the coffee bean extracts by a low cost procedure such as evaporation (Nunes et al., 2006). Furthermore, mannanases are efficient in oil extraction from coconut by degrading heteromannans (Chen & Diosady, 2003).

Another economically important application of hemicellulases deals with animal feeding. Numerous enzyme preparations have been marketed to improve the metabolisable energy of cereal-based poultry diets and are exhaustively studied to understand their mechanism in the complex conditions of gut (Choct, 2006, Maisonnier-Grenier et al., 2006, Lafond et al., 2011). In the last decade, xylanases have also been employed for the production of more or less tailored XOS for prebiotic action for food and feed sectors (Carvalho et al.,

2013).

I.9.b. Paper and pulp industry

The most potent application of hemicellulases, in particular, mannanases and xylanases would be in the bleaching of softwood pulps. The extraction of lignin from wood fibers is an essential step in bleaching of pulps. Pretreatment of pulps in the alkaline conditions hydrolyzes hemicelluloses covalently bound to lignin and facilitates its subsequent removal. The major drawback of alkaline treatment is the release of chlorinated compounds in the environment. In this context, mannanase and xylanase degradation of the hemicelluloses can provide an eco-friendly alternative for the pulp delignification (Ragauskus et al., 1994; Gubitz et al., 1997; Vicuna et al., 1997; Benech et al., 2007). Moreover, the process of pulping is carried out at high temperature and alkaline pH, which needs highly thermo stable and alkaline stable enzymes (He et al., 2008; Pan et al., 2011).

I.9.c. Production of biofuels

Another potential usage of the cellulases and hemicellulases is the saccharification of lignocelluloses in the process of bioconversion of lignocellulose to biofuels and biochemicals (Saha et al., 2003). Cellulase and hemicellulase enzymes play an important role in the hydrolysis of biomass to fermentable mono- and oligosaccharides (Kumar et al., 2009). The recent focus on high-solid loadings renders the conditions for enzymatic process even more unfavorable (Modenbach & Nokes, 2013).

I.10. Aims of the study

The preceding chapter stressed on the complexity of the diverse xylans, mannans and xyloglucans structures *in planta*. It also pointed to the requirement of numerous enzymes to modify and degrade them. However, hemicellulose structure is still partly unknown and new enzymes are necessary to go deeper into their structural analysis. Thus, the aim of the research presented here was to better understand the fine structure of different hemicelluloses by the help of enzymatic tools. To that end, the aim of this work was twofold: 1) further our understanding on the hemicelluloses fine chemistry, as a prerequisite of future works on their functions *in planta* and 2) identify new sources of hemicellulases from the broad diversity of unexplored bacterial system to allow investigating hemicelluloses structure.

These two objectives are described in the following chapters:

Chapter 2 describes the preparation and identification of different natively acetylated glucuronoarabinoxylan, galactoglucomannans and xyloglucans hemicelluloses from apple *var*. Gala using the LiCl/DMSO solvent system combined with different chromatographic purifications, and commercially available enzymes as analytical tools.

Chapter 3 describes the data mining of bacterial genomes for identifying new hemicellulases and their functional validation in selected bacteria using different culture conditions, and numerous polysaccharides as substrates, including previously prepared apple hemicelluloses.

Chapter 4 describes the isolation of enzymes of interest in selected bacterial culture supernatants and a first approach on their mode of action on apple and other hemicelluloses.

Chapter 5 summarizes and discusses the results of the different chapters.

I.11. References

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Chapter II

Novel and diverse fine structures in LiCl-DMSO extracted apple hemicelluloses

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This chapter aimed at the preparation of different acetylated hemicellulosic polysaccharides from apple and their preliminary structural characterisation. To that end, apple hemicelluloses were sequentially extracted by DMSO doped by LiCl followed by potassium hydroxide. The weakly bounded hemicelluloses in the LiCl-DMSO soluble extract were fractionated by ion exchange (AEC) and size exclusion (SEC) chromatographies. The structure of all the extracts and fractions was established by enzymatic fingerprinting using commercial β-glucanase, β-mannanase and β-xylanase. Molecular weight of the fraction was established by high performance size exclusion chromatography (HPSEC). Mass spectrometry (MS) as well as high performance anion exchange chromatography (HPAEC) analyses of the enzyme digests revealed the remarkable diversity of apple hemicelluloses. Different xyloglucan (XyG), galactoglucomannan (GgM) and glucuronoarabinoxylan were isolated along the extraction and fractionation process. All LiCl-DMSO soluble fractions were acetyl-esterified. Moreover, the LiCl-DMSO soluble XyG differed from the 4 M KOH extracted one essentially on the basis of its molecular weight. At least two populations differing in their content and distribution of glucose and mannose composed GgM. Moreover, galactose ramifications occurred on mannose blocks in the glucose rich fraction. These results open the way for future studies on the complex structure-function relationship of hemicelluloses in plant cell walls.

Keywords: cell wall, xyloglucan, galactoglucomannan, glucuronoarabinoxylan, LiCl-DMSO, MALDI-TOF MS

II.1. Introduction

Fruit texture is a major quality criterion that influences consumer acceptance and postharvest technical itineraries. It results from coordinated events implying different determinants at several scales involving cell walls (Harker, Redgwell, Hallett, Murray & Carter, 1997; Waldron, Park & Smith, 2003). In fleshy fruit, cell walls are assimilated to the primary cell wall of elongating cells due to their composition, hydrophilicity and their viscoelastic mechanical behaviour (Albersheim, Darvill, Roberts, Sederoff & Staehelin, 2011). The macromolecular assemblies composing them are responsible for the cell shape, provide mechanical support able to sustain large pressures arising from cell turgor and contribute to the structural integrity of the plant organs through cell-cell bonding. These cell walls consist of cellulose microfibrils embedded in and interacting with networks of an amorphous matrix of hemicelluloses, pectins and glycoproteins (Albersheim, Darvill, Roberts, Sederoff & Staehelin, 2011). On ripening and softening, fleshy fruit cell walls are reorganized and disassembled through the reshuffling and degradation of the matrix polymers that hold cellulose microfibrils in place. The complex biochemical processes involved coordinated sets of "wall-loosening" enzymes and proteins (Brummell, 2006; Goulao & Oliveira, 2008). Wall glycosidases, lyases and esterase modify and/or degrade the complex homogalacturonan and rhamnogalacturonan domains of pectins (Atmodjo, Hao & Mohnen, 2013) affecting their important role in cell-cell interactions and cell wall rigidity (Jarvis, Briggs & Knox, 2003; Peaucelle, Braybrook & Hofte, 2012). Other glycosidases, transglycosylases and expansins act on the fine chemical structure that impacts organization and interactions of the three families of polysaccharides composing the hemicelluloses: xyloglucan (XyG), galactoglucomannan (GgM) and glucurono(arabino)xylan polysaccharides (Scheller & Ulvskov, 2010). Expansins break hydrogen bonds between cellulose and XyG allowing slippage of cellulose microfibrils (Cosgrove, 2005) and making cell wall polysaccharides susceptible to further enzymatic degradation (Brummell, Harpster, Civello, Palys, Bennett & Dunsmuir, 1999; Wei, Yang, Luo, Lu, Wu & Yuan, 2010). Xyloglucanendotransglucosylase / hydrolase (XTH) cleaves and ligates XyG chains to temporarily loosen cell wall (Fry, Smith, Renwick, Martin, Hodge & Matthews, 1992). Other transglycosylases acting on tomato GgM and on papaya xylan have been reported (Johnston et al., 2013; Schröder, Atkinson & Redgwell, 2009). Glycosidases, some having transglycosylases activities, can also remodel XyG during organ development (Frankova & Fry, 2012; Pauly et al., 2013) with yet unclear functions on wall mechanical properties and fruit texture.

The function of hemicelluloses in plant development and fruit texture has been particularly studied with regard to XyG (Atkinson, Johnston, Yauk, Sharma & Schröder, 2009; Miedes, Herbers, Sonnewald & Lorences, 2010; Miedes et al., 2013; Somerville et al., 2004). In apple, an important fruit commodity with contrasted texture affecting consumption and transformation, 11 XTH (Atkinson, Johnston, Yauk, Sharma & Schröder, 2009) and 8 expansin genes (Trujillo, Mann & Tong, 2012) have been reported to date targeting potentially XyG and its interactions with cellulose. Differences in gene expression of some of them were shown to contribute to differentiate slowly from rapidly softening apples (Harb, Gapper, Giovannoni & Watkins, 2012). The number of genes and their regulated expression during development and ripening suggest that they might have different specific functions and targets in the cell wall as the fruit develops and ripens. Besides XyG, little is known on structure, function and enzyme remodelling or metabolizing of apple GgM and heteroxylan.

Apple XyG is a fucogalactoxyloglucan composed of a cellulose-like backbone of 1,4 linked β-D-glucopyranose residues, of which around 75% carry side-chains at *O*-6 (Fry et al., 1993; Voragen, Schols & Pilnik, 1986). The main side chains are α-D-Xyl*p*-(1→ noted **X** (cf (Fry et al., 1993) for nomenclature), β-D-Gal*p*-(l→2)-α-D-Xyl*p*-(1→ noted **L**, and α-L-Fuc*p*(1→2)-β-D-Gal*p*-(1→2)- α -D-Xyl*p*-(1→ noted **F**. Xyloglucanendoglucanase degrades XyG in a regular manner producing at least one unbranched Glc (**G**) at the reducing end of the oligomers produced (XyGOs). The major apple XyGOs are **XXXG, XXFG** and **XLFG** the two latter being present mainly as acetylated derivatives (Galvez-Lopez, Laurens, Quemener & Lahaye, 2011; Renard, Lomax & Boon, 1992; Vincken, van den Broek, van der Lei, Beldman & Voragen, 1997). Acetyl esterification in dicots XyGOs occurs usually on the galactose residue at position *O*-6 and on *O*-3, *O*-4 of the glucan backbone (Kiefer, York, Albersheim & Darvill, 1990; Pauly, Albersheim, Darvill & York, 1999).

Alkali-soluble apple GgM were shown to consist of a β-(1→4)–linked Glc*p* and Man*p* backbone, some of which are substituted by single Gal and di-Gal chains at *O-6* position (Nara, Ito, Kato & Kato, 2004). In the cell wall, GgM are acetylated (Galvez-Lopez, Laurens, Quémener & Lahaye, 2011). Percy, Melton & Jameson (1997) showed changes in the content of the hemicellulosic mannose (Man) during apple development, indicating fine structural changes in Man containing polymer (GgM). Usually, dicots xylan consists of linear (1,4)-linked-β-D-xylopyranosyl (Xyl) backbones substituted by (1,2)-linked (4-*O*-methyl)-α-D-glucopyranosyluronic acid and by acetyl esters. Arabinose (Ara) is a minor substituent in dicot hemicelluloses (Scheller & Ulvskov, 2010). In apple, Ara was proposed to be linked on C-2 of xylan (Voragen, Schols & Pilnik, 1986).

The full knowledge of the mechanisms of enzyme and protein consortia targeting hemicelluloses in the development of fruit texture requires a better understanding of their mode of action and functions in the wall as well as an in depth knowledge of the structural and physicochemical characteristics of their polysaccharide substrates. To that end, the aim of this study focussed on the composition and fine chemical structure of native hemicelluloses isolated from apple. The polysaccharides were extracted by 8.4% LiCl DMSO, as optimised by Assor, Quemener, Vigouroux & Lahaye, 2013, before being fractionated by anion exchange and gel permeation chromatography. After they were degraded by glucanase, xylanase and mannanase, the produced oligosaccharides were characterized by high performance anion exchange chromatography and MALDI-TOF mass spectrometry.

II.2. Material and methods

2.1. Cell wall material preparation

Cell wall material was prepared as an alcohol insoluble residue (**AIR**). Unripe apples (var. Gala; 5.6 kg) were harvested in July 2011 from INRA orchard, Angers, France and kept at 4°C for 5 months during which the fruits ripened. Apples were peeled, cut and boiled in ethanol 96% (humid/liquid ratio as 1/3 w/v) for 20 min and recovered on a nylon cloth (90 µm). The cell wall material was washed repeatedly with 70% ethanol (solid/liquid ratio as above, 5 times for 4 h) until the ethanol extract was free of sugar (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Finally 1.18 kg of humid **AIR** was obtained.

*2.2. Isolation of polysaccharides from **AIR***

The sequential extraction procedure used is shown in Figure II-1**.**

(i) **AIR** (380.9 g fresh weight) was first partially depectinated with water (2 L, 2 h) at 80 – 85 °C. Solubilized material was separated from the insoluble residue by filtration on a nylon cloth (90 µm) and centrifugation (15 min, 20 °C, 9000 g). The soluble extract (**HWE)** was concentrated, dialyzed against water, concentrated and freeze dried.

(ii) The residue after hot deionised water extraction, named partially depectinated AIR (**pDAIR**), was washed twice with DMSO at 20 °C (1 g of equivalent dry weight for 40 mL of DMSO, 30 min), centrifuged (20 min, 20 °C, 9000 g) and filtered on sintered glass (40-100

µm).

(iii) The DMSO insoluble residue was extracted with 8.4% LiCl-DMSO (1 g equivalent residue dry weight for 200 mL LiCl-DMSO) at 100 °C during 5 h under continuous agitation and under N_2 (Flores, Stortz & Cerezo, 2000). The suspension was centrifuged as above and the supernatant solution was filtered on sintered glass (40-100 µm).

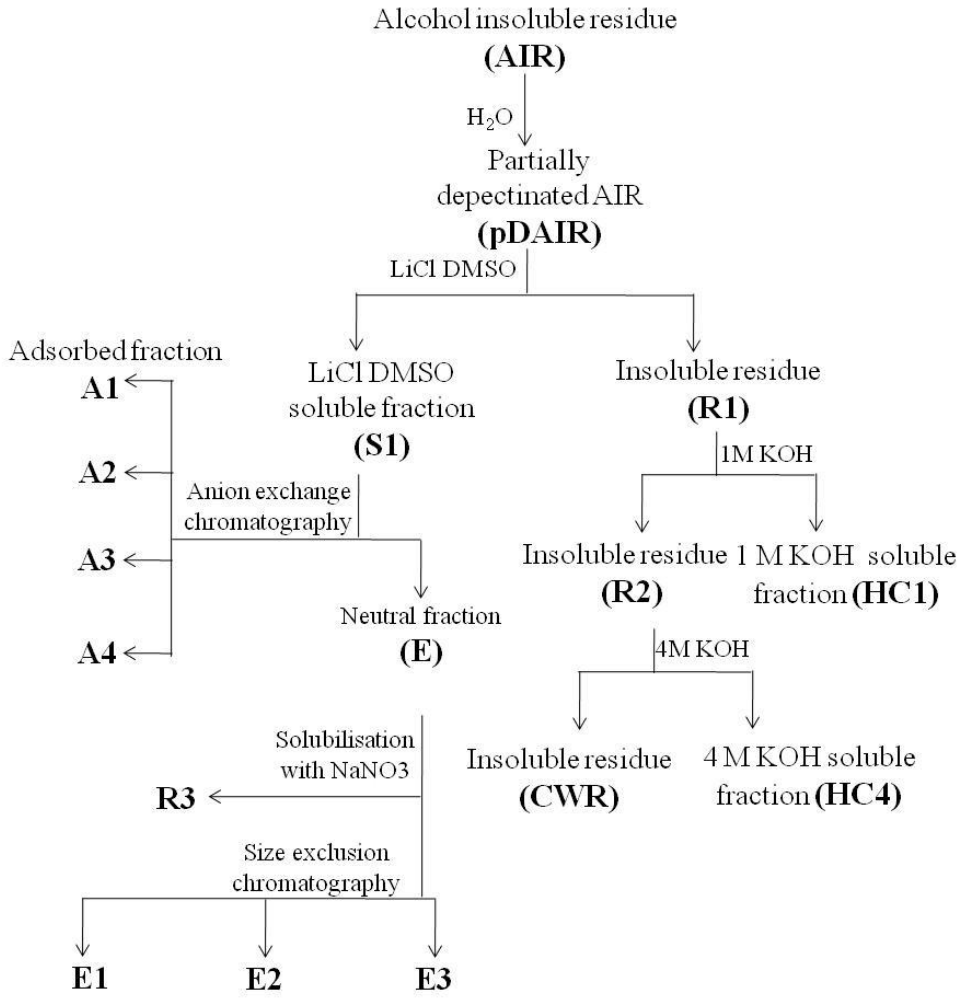

Figure II-1: Sequential extraction and fractionation of hemicelluloses using apple alcohol insoluble residue (AIR) as starting material.

The insoluble residue (**R1**) was washed twice with LiCl-DMSO and then kept at 4 °C. The LiCl-DMSO extract and washing water were pooled and concentrated by evaporation at 60 °C under vacuum. The concentrated solution was brought to 750 mL with deionised water and precipitated with 4 volumes of 96% ethanol under stirring over night at 4 °C. The crude hemicellulose precipitate (**S1**) was recovered by centrifugation (10 min, 4 °C, 12600 g) followed by filtration on sintered glass (16-40 µm), washed with ethanol 96% and dried with

acetone.

(iv) The insoluble fraction **R1** was extensively washed with deionized water. The remaining hemicelluloses in the residue (1 g residue dry weight) were then extracted with 400 mL of 1 M KOH solution containing 20 mM $NaBH_4$ for 17 h at room temperature (Carpita, 1984). The extract was acidified with cold 4 M acetic acid in an ice bath to pH 6 and dialyzed extensively against deionized water (MWCO 6000-8000). The suspension was centrifuged (20 min, 20 °C, 9000 g) and the supernatant filtered (40-100 μm). **R1** was extracted once more for 1 h in the same conditions. The two extracts were pooled and designated as **HC-1**, and freeze-dried. The residue (**R2**) was submitted to a 4 M KOH extraction following the same protocol. It was washed with 0.1 M acetic acid and with deionised water, freeze-dried and referred to as cell wall residue (**CWR**). The corresponding extracts (**HC-4**) were treated as **HC-1**.

2.3. Fractionation of hemicelluloses

2.3.1. Anion exchange chromatography (AEC)

Crude hemicelluloses fraction **S1** was solubilized in water at a final concentration of 12 g L^{-1}. It was then fractionated on a DEAE-Sepharose Fast Flow column (5 x 15 cm, GE Healthcare, Uppsala, Sweden) equilibrated with deionized water. Elution was performed at a flow rate of 2 mL min^{-1} with water (2 column volumes, 600 mL) to recover the unbound fraction (**E**) that was concentrated and freeze-dried. Bound polysaccharides were eluted using a gradient (6 column volumes) from 0 to 100% of NaCl 1 M. Elution was monitored by colorimetric measurement of total sugars (Tollier & Robin, 1979) and uronic acids (Blumenkrantz & Asboe-Hansen, 1973). The fractions eluted in the gradient (**A1**, **A2**, **A3** and **A4**) were concentrated, dialyzed against deionized water, concentrated under vacuum and freeze-dried.

2.3.2. Preparative size-exclusion chromatography (SEC)

Preparative SEC was performed on Sephacryl S-300 column (5 x 87 cm, GE Healthcare, Uppsala, Sweden) using 50 mM $NaNO_3$ as eluent at a flow rate 1.6 mL min^{-1}. A solution of **E** was prepared in 50 mM $NaNO_3$ pH 5 at a final concentration of 23.8 g L^{-1} and centrifuged prior to chromatography. The insoluble part was referred to as **R3**. Chromatographic fractions of the $NaNO_3$ soluble **E** were assayed for total neutral sugar and uronic acid contents as above. Fractions **E1**, **E2**, **E3** were pooled according to elution profile, dialysed and freeze dried.

2.4. Chemical cell wall characterization of polysaccharide fractions

2.4.1. Total neutral sugar and uronic acid content

The total neutral sugar content was determined colorimetrically with an automated orcinol/sulphuric acid assay (Tollier & Robin, 1979). Glucose was used as a standard.

Uronic acids content was measured colorimetrically using m-hydroxydiphenyl and concentrated sulphuric acid hydrolysis (Blumenkrantz & Asboe-Hansen, 1973) as automated by (Thibault, 1979). Galacturonic acid was used as a standard.

2.4.2. Neutral sugar composition

Fractions obtained by sequential extraction were hydrolysed in 1 M H_2SO_4 (2 h, 100 °C) for measurement of individual neutral sugar. Sugars were reduced, acetylated and analysed as alditol acetate by GLC (Blakeney, Harris, Henry & Stone, 1983) on a Perkin Elmer AutoSystem (Courtaboeuf, France) mounted with a DB 225 capillary column (J & W Scientific, Folsorn, CA, USA; temperature 205 °C, carrier gas H_2). Standard sugars solution and inositol as internal standard were used for calibration.

2.4.3. Degree of methylation and acetylation

Acetyl ester content was measured by HPLC as the amount of acetic acid released by saponification of 5 mg sample in 1 mL NaOH 0.50 M for 1 h at 4 °C. HPLC was carried out on C18 (4 x 250 mm, Lichrospher 100 RP-18e (5µm), Interchim, France) column thermostated to 25 °C using a refractometric detector (Waters, 2414). An isocratic elution of 4 mM H_2SO_4 was used at a flow rate 1.0 mL min^{-1}(Levigne, Thomas, Ralet, Quemener & Thibault, 2002). Standard methanol, acetic acid and isopropanol as internal standard were used for calibration.

2.4.4. Protein content

Soluble proteins were measured by the Bradford colorimetric method with the Bio-Rad reagent and using BSA as a standard (Bradford, 1976). Insoluble proteins were measured by the Kjeldahl method (N x 6.25).

2.5. Structural characterization of hemicelluloses

2.5.1. Enzymatic degradations

Cell wall material, chromatographic fractions and extraction residues were degraded by commercial enzymes from Megazyme, Bray, Ireland: endo-1,4-β-glucanase (EGII), endo-1,4-β-xylanase (M2) both from *Trichoderma longibrachiatum*, endo-1,4-β-mannanase from *Aspergillus niger*, α-L-arabinofuranosidase from *Aspergillus niger* and α-D-galactosidase from Guar. The samples (2 mg) were suspended in deionised water containing either 4 U of β-glucanase (Galvez-Lopez, Laurens, Quemener & Lahaye, 2011), 1.8 U of β-mannanase, 5.2 U of β-xylanase, 10 U of α-L-arabinofuranosidase or 10 U of α-galactosidase and incubated at 40 °C under gentle agitation during 17 h. After centrifugation (15200 g, 10 min at 20 °C) and enzyme inactivation by 10 min boiling, supernatant solutions were passed through 0.45 µm filter (Millex-Hv, PVDF, Millipore, St Quentin en Yvelines, France) prior to analysis of the oligosaccharides by HPAEC, and MALDI-TOF-MS.

2.5.2. Analysis of oligosaccharides by HPAEC

XyG and GgM oligosaccharides (XyGOs and GgMOs, respectively) were chromatographed through on a Carbo-Pac PA 200 column (3 x 250 mm, Dionex, Sunnyvale, USA) whereas xylan oligosaccharides (XOs) were analysed on a Carbo-Pac PA 1 column (2 x 250 mm, Dionex, Sunnyvale, USA) thermostated at 30 °C. Elution was realized using a linear gradient of sodium acetate from 0 to 170 mM in 100 mM NaOH (Quemener, Bertrand, Marty, Causse & Lahaye, 2007) and detection was done by amperometry using an ED electrochemical detector (Dionex, Sunnyvale, USA).

2.5.3. Analysis of oligosaccharides by MALDI-TOF MS

Enzyme hydrolyzates (1 µL) were mixed with N,N-dimethylaniline / 2,5-dihydroxybenzoic acid matrix (1 µL) (Ropartz et al., 2011) on the target plate and left to dry for 3 h at room temperature. For each hydrolyzate, three replicates were realized. MALDI-TOF MS analysis was performed in the positive mode on a MALDI-TOF/TOF (Autoflex III Smartbeam, Bruker, Germany) equipped with a Yag laser (355 nm). Analyses were carried out in the reflector mode using a laser frequency of 200 Hz and an accelerating voltage of 19 kV. Spectra were recorded in the mass range m/z 500–3000. A low mass gate value of m/z 500 was selected for analysis in order to avoid saturation of the detector. The instrument was externally calibrated using the monoisotopic masses of main oligosaccharides ($[M+Na]^+$ ion)

released from XyG (**XXG**: 791.243 Da, **XXXG**: 1085.338 Da, **XXFGa1**: 1435.459 Da, **XLFGa1**: 1597.512 Da). Nomenclature of XyG oligosaccharides followed that of (Fry et al., 1993) extended to account for acetyl, methyl and uronic acid (UA) groups noted as **a**, **m** and **U,** respectively. Hexoses containing oligosaccharides attributed to GgM were noted **H** and pentoses containing oligosaccharides recovered after xylanase degradation were noted **P**. The figure following the structure codes denoted the number of building structures and acetyl groups in the oligosaccharides ($\mathbf{H_4a_2m_1}$ corresponds to an oligomer with 4 hexoses, 2 acetyl and 1 methyl groups).

2.5.4. Analytical size-exclusion chromatography (HPSEC)

The HPSEC was performed on isolated hemicellulose fractions. The system consisted of a Shodex OHpak SB-G guard column (6 x 50 mm, Shodex, Tokyo, Japan) in front of a series of OHpak SB-805HQ and OHpak SB-804HQ columns (8 x 300 mm, Shodex, Tokyo, Japan) connected to pump (Jasco PU-1580, Tokyo, Japan) and injector (PerkinElmer, series 200 auto sampler, Courtaboeuf, France). Around 3 mg of sample were solubilized in 1mL of 50 mM $LiNO_3$, centrifuged (10 min, 4 °C, 7400 g) and filtered through 0.45 µm membrane (Millex-HV, PVDF) prior to injection. Elution was performed with 50 mM $NaNO_3$ at a flow rate of 0.7 mL min^{-1} and monitored by i) differential refractometry (Viscotek VE 3580 RI dectector, Malvern Instruments, Orsay, France), ii) light scattering (LS) detection and iii) differential pressure viscometry (both from Viscotek 270 dual detector, Malvern Instruments, Orsay, France). Weight average molecular weight and molecular weight distribution were obtained using the Omnisec 4.5 software (Software Viscotek 270 dual detector, Malvern Instruments, Orsay, France) and calibration was done with using pullulan-150K (Viscotek, Malvern Instruments, Orsay, France).

II.3. Results and discussion

3.1. Sequential extraction and fractionation of the cell wall material and their composition

Cell wall material from apple parenchyma was prepared as alcohol insoluble residue (**AIR**). It was then sequentially extracted with hot water, LiCl-DMSO, 1 M and 4 M KOH leaving an insoluble cell wall residue. The overall extraction and fractionation sequences are presented in Figure II-1. The yield and sugar composition of cell wall preparations, soluble

and insoluble fractions are given in Table II-1. The hot water extract (**HWE**) was mainly composed of uronic acid (UA, 49.2 dry weight% of the extract), arabinose (Ara, 11.7 dw%), galactose (Gal, 4.4 dw%) and contained methyl ester (9.4 dw%) in agreement with the presence of water-soluble pectic polysaccharides (Renard, 2005). The partially depectinated **AIR** (**pDAIR**) was mainly composed of glucose (Glc, 24.1 dw%), UA (17.4 dw%), arabinose (Ara, 8.9 dw%), xylose (Xyl, 7.6 dw%) and mannose (Man, 3.5 dw%) suggesting the presence of cellulosic, hemicellulosic and residual pectic polysaccharides.

Alkali solutions are classically used to extract hemicelluloses since alkali cleaves hydrogen bonds between cellulose and hemicelluloses (Fry, 1988). But at such pH the ester groups are saponified and peeling as well as β-elimination reactions can occur.

Table II-1: Yield and chemical composition of apple cell wall fractions on the percent dry weight basis. HWE, hot water extract; pDAIR, depectinated alcohol insoluble residue; fractions obtained by sequential extraction (cf Figure II-1): LiCL-DMSO (S1, R1) and KOH (HC1, HC4, CWR); DEAE anion exchange chromatography (E, A1, A2, A3, A4; cf Figure II-2) and S-300 size exclusion fractionation (E1, E2, E3, R4; cf Figure II3). The yields are calculated on a dry weight pDAIR basis.

Fraction	Yield	Sugar									Acetyl	Methyl	Protein
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	TS	ester	ester	
HWE		1.2	0.0	11.7	1.0	0.6	4.4	2.5	48.9	70.3	0.0	9.4	
pDAIR		1.1	1.5	8.9	7.6	3.5	7.8	24.1	17.4	71.9			
S1	21.1	1.3	2.1	8.9	10.0	7.9	9.3	16.9	27.9	84.3			
R1	61.2	0.9	1.1	6.2	5.7	0.8	5.7	20.9	10.7	52.0			6.7
HC1	6.7	0.8	0.4	3.4	1.5	0.8	3.9	1.3	3.6	15.7			36.3
HC4	9.2	0.4	1.9	2.3	8.1	0.8	4.9	12.9	4.4	35.7			2.2
CWR	35.9	1.1	1.1	6.6	5.8	1.5	5.8	72.9	4.1	98.9			2.1
E	18.3	0.8	2.4	7.8	10.8	9.5	9.2	18.4	5.8	64.7			
A1	0.2	2.3	0.5	3.3	4.4	29.5	19.4	27.8	1.4	88.6	2.5	0.0	1.8
A2	0.2	1.1	0.0	14.9	44.5	1.6	7.9	1.5	11.2	82.7	3.8	0.7	1.8
A3	0.6	4.4	0.2	24.7	13.6	0.0	10.4	0.5	24.3	78.1	2.4	1.3	2.7
A4	1.4	2.0	0.0	3.7	1.4	0.0	2.4	0.4	39.5	49.4	0.6	1.2	2.2
E1	2.9	1.3	4.9	6.8	19.0	1.3	11.1	34.5	12.6	91.5	3.7	0.7	2.7
E2	8.6	0.7	3.8	4.8	14.2	16.0	9.9	34.8	6.8	91.0	4.8	0.4	3.1
E3	0.8	0.6	2.7	5.4	10.6	20.5	9.1	28.9	6.4	84.2	5.8	0.3	2.1
R3	1.4	3.1	0.5	12.0	6.2	3.8	10.9	6.5	24.9	67.9	3.3	1.5	12.7

Other methods have been introduced for extracting hemicelluloses, such as microwave irradiation (Passos, Moreira, Domingues, Evtuguin & Coimbra, 2014), active oxygen cooking (Shi, Yang & Lin, 2014), or ionic liquids (Anugwom et al., 2012). Microwave irradiation technique (Passos, et al., 2014) was shown to be a very rapid technique to isolate different

populations of deacetylated oligomannans according to treatment times. Similarly, cooking for different times and temperatures in oxidative conditions generated by MgO and hydrogen peroxide extracted deacetylated arabinoxylan of different molecular weight (from 3 to 20 kDa). These two extraction methods have limited uses due to the partial degradation of hemicelluloses and particularly their deacetylation. Liquid ionic was used to extract hemicelluloses from spruce wood but the yield and the fine chemical characteristics of the isolated polysaccharides were not reported (Anugwom et al., 2012). According to Hagglund et al. (1956) DMSO extracts hemicelluloses in a non-destructive way (Peng, Peng, Xu, & Sun, 2012) where cellulose material exhibits considerable inter-micellar swelling. DMSO has been used in optimized ways with different concentrations of water (Hu et al., 2008; Willfor et al., 2008) or with 8.4% LiCl (Assor et al., 2013) to extract hemicelluloses. The particular preservation of the acetyl esters on these polysaccharides represents an advantage to characterize their fine structure. In the present work, natively acetylated hemicelluloses were extracted from **pDAIR** with the polar aprotic solvent DMSO doped with 8.4% LiCl according to Assor et al. (2013). This condition successfully extracted acetylated XyG, GgM and glucuronoxylan from tomato cell wall material (Assor, Quemener, Vigouroux & Lahaye, 2013). Applied to apple **pDAIR** this extraction yielded 21.1% dry weight (dw%) of soluble polysaccharides (fraction **S1**) that mainly contained 56, 39, 32 and 17% of **pDAIR** Man, UA, Xyl and Glc, respectively, indicating the presence of XyG, GgM and residual pectin or/and glucuronoxylan. On the other hand, **R1** corresponded to 61.2 dw% of **pDAIR** and contained 20.9 dw% Glc attributed to cellulose, and 6.2 dw%, 5.7 dw% and 5.7 dw% of Ara, Xyl and Gal together with 10.7 dw% of UA, indicating the presence of residual pectin and unextracted XyG or/and glucuronoxylan. To access at residual hemicelluloses, **R1** was sequentially treated with 1 M and 4 M KOH. The 1M KOH soluble fraction (**HC1,** yield 6.7 dw% of **pDAIR**) was composed of Gal, UA and Ara (3.9, 3.6 and 3.4 dw%, respectively) and 36.3 dw% of water-soluble protein indicating the probable presence of arabinogalactan protein. This result contrasted with a previous study (Renard, Lomax & Boon, 1992) showing that part of apple XyG was extracted with 1 M KOH. Only trace amounts of Xyl and Glc (1.5 and 1.3 dw%, respectively) were measured in **HC1**, showing the absence of XyG in this fraction. In contrast, the 4 M KOH soluble fraction **(HC4,** 9.2 dw% of **pDAIR)** contained mainly Glc, Xyl, Gal and UA (12.9, 8.1, 4.9 and 4.4 dw%, respectively), suggesting the presence of XyG with a trace amount of pectic polysaccharides. Thus, LiCl-DMSO extraction solubilized loosely bound XyG, GgM and pectin whereas part of the tightly bound XyG in the cell wall was solubilized by 4 M KOH. The residue after 4 M KOH extraction (**CWR**) still contained

Ara (6.6 dw%), Xyl (5.8 dw%), Gal (5.8 dw%), UA (4.1 dw%), Man (1.5 dw%) attesting for residual pectin and hemicelluloses. The amount of Man in the different fractions showed that about 56% of the initial **pDAIR** Man was recovered from LiCl-DMSO extraction whereas 23% were recovered in **HC1, HC4** and **CWR** and thus were more tightly associated in the wall. Conversely, 21% of Man was lost during the extraction process which was lower than that reported for tomato (Assor, Quemener, Vigouroux & Lahaye, 2013). Overall, about 15 dw% of **pDAIR** was lost with alkaline extractions, attributed to incomplete recovery of polysaccharides and degradation of protein.

Anion exchange chromatography (AEC) allowed the separation of **S1** into one neutral fraction (**E**) and 4 different populations of polysaccharides eluted with the NaCl gradient (**A1**, **A2**, **A3**, **A4**; Figure II-2, Table II-1). The neutral fraction **E** contained 62, 69 and 62% of **S1** Xyl, Man, and Glc, respectively, together with 4 dw% of acetyl esters, which were attributed

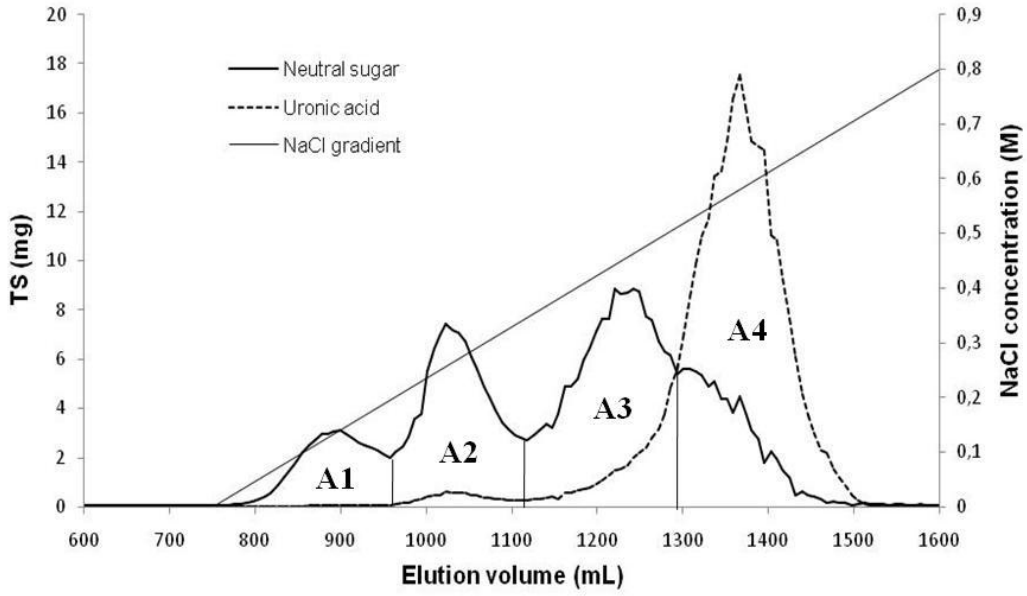

Figure II-2: Fractionation of LiCl-DMSO soluble fraction (S1) by anion-exchange chromatography on DEAE-Sepharose FF with a NaCl gradient. The elution was monitored using orcinol and MHDP assays for measuring sugar content.

to highly acetyl esterified XyG and GgM. Presence of Ara (7.8 dw%), UA (5.8 dw%), Rha (0.8 dw%) was attributed to a small amount of pectin. Fraction **A1** was slightly bound to the anion exchange matrix as its elution occurred just after the beginning of the NaCl gradient. This neutral fraction was mainly composed of Man, Glc and Gal (39.6, 27.8 and 16.4 dw%, respectively) indicating a population of GgM. The retention of this neutral fraction on the anion exchanger may be related to the characteristics of the Sepharose matrix. This gel is

based on cross-linked agarose, which is known to interact through hydrogen bonds with mannans and glucomannans (Dea, 1981). **A2** was essentially made of Xyl, Ara and UA (44.5, 14.9 and 11.2 dw%, respectively), suggesting a substituted xylan. Fraction **A3** was mainly composed of Ara (24.7 dw%), UA (24.3 dw%), Xyl (13.6 dw%), Gal (10.4 dw%) and Rha (4 dw%) indicating the presence of rhamnogalacturonan together with a small amount with glucuronoxylan or xylogalacturonan, a pectic structural domain known to be present in apple pectins (Schols, Bakx, Schipper & Voragen, 1995). The major fraction from the NaCl gradient **A4** was mainly composed of UA (39.5 dw%), which was attributed to homogalacturonan rich pectin.

Polysaccharides in fraction **E** from the AEC were fractionated on the basis of their hydrodynamic volume through size exclusion chromatography. **E** was dissolved in 50 mM $NaNO_3$ but a part of the fraction remained insoluble (**R3**, 4% of **pDAIR**) likely due to

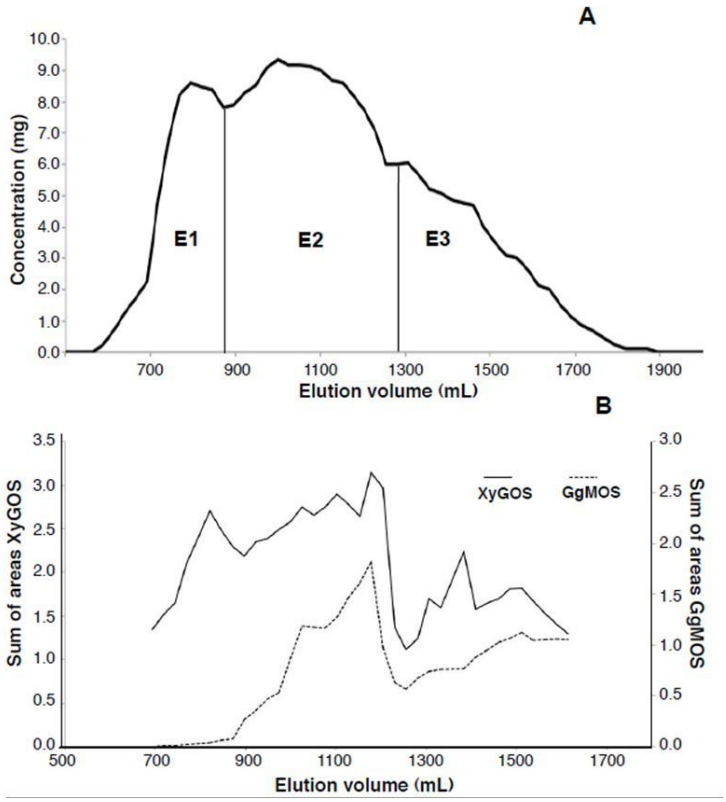

Figure II-3: (A) Size-exclusion chromatography (SEC) on S-300 Sephacryl of the neutral fraction E obtained after anion-exchange chromatography. The elution was monitored using orcinol assay for sugar content. (B) The distribution of XyG and GgM through the elution profile of the S-300 was evaluated by considering the specific XyG oligosaccharides (XXXG, XXFG and XLFG; cf Figure II-5) and GgM oligosaccharides released by endo-glucanase degradation of each collected fraction (peaks eluting between 3 and 10 min cf Supplementary Figure II-S1).

polymer aggregation during the fraction processing. The soluble fraction yielded 3 main peaks, **E1** (3 dw% of **pDAIR**), **E2** (9 dw% of **pDAIR**) and **E3** (1 dw% of **pDAIR**) (Figure II-3A). The sugar analysis (Table II-1) suggested **E1** was essentially made of XyG (Glc 34 dw%, Xyl 19 dw% and trace amount of Man), whereas **E2** (Glc 34 dw%, Man 15 dw% and Xyl 14 dw%) and **E3** (Glc 28 dw%, Man 20 dw% and Xyl 10 dw%) were a mixture of XyG and GgM. The distribution of XyG and GgM through the elution profile was evaluated by considering the release of specific oligosaccharides after endo-glucanase degradation of each collectedfraction. The result (Figure II-3B) suggested that **E1** contained almost exclusively XyG, whereas **E2** and **E3** contained various proportions of XyG and GgM**.**

3.2 Fine structural analysis of the hemicellulosic fractions

3.2.1. Xyloglucan

Two different populations almost exclusively composed of XyG (**E1** and **HC4**) were obtained from the **pDAIR** by two approaches. **E1** was obtained after LiCl-DMSO extraction followed by anion exchange and size exclusion chromatographies whereas **HC4** was extracted with 4 M KOH from the LiCl-DMSO residue (Figure II-1). The two fractions differed in their molecular weight: 156 kDa and 200 kDa for **E1** and **HC4**, respectively (Figure II-4A). The disappearance of the major HPSEC peak after treatment of **E1** with endo-β-glucanase confirmed its attribution to XyG (Figure II-4A). As the two fractions behaved differently along the fractionation process, they were believed to be differently associated with the other cell wall polysaccharides due to fine structure differences. These were sought by HPAEC and MALDI-TOF MS analyses of the oligosaccharides released by endo-β-glucanase hydrolysis. The HPAEC elution pattern confirmed the presence of XyGOs (Figure II-5A, 5B) that were identified using retention times of known oligosaccharides from apple XyG (Renard, Lomax & Boon, 1992). The two XyGOs profiles were typical of apple XyG with major **XXXG**, **XXFG**, **XLXG** and **XLFG** building blocks together with minor amounts of **XXG**-type oligomers. Beside the loss of acetyl esterification due to the alkaline conditions of **HC4** extraction, the XyGOs profile of the two XyG fractions only differed by the presence of additional small unidentified peaks eluting between 3.3 and 10.3 min on the **HC4** chromatogram. The attribution of the main oligomers was supported by the MALDI-TOF MS spectra of the β-glucanase digest (Figure II-6A, 6B).

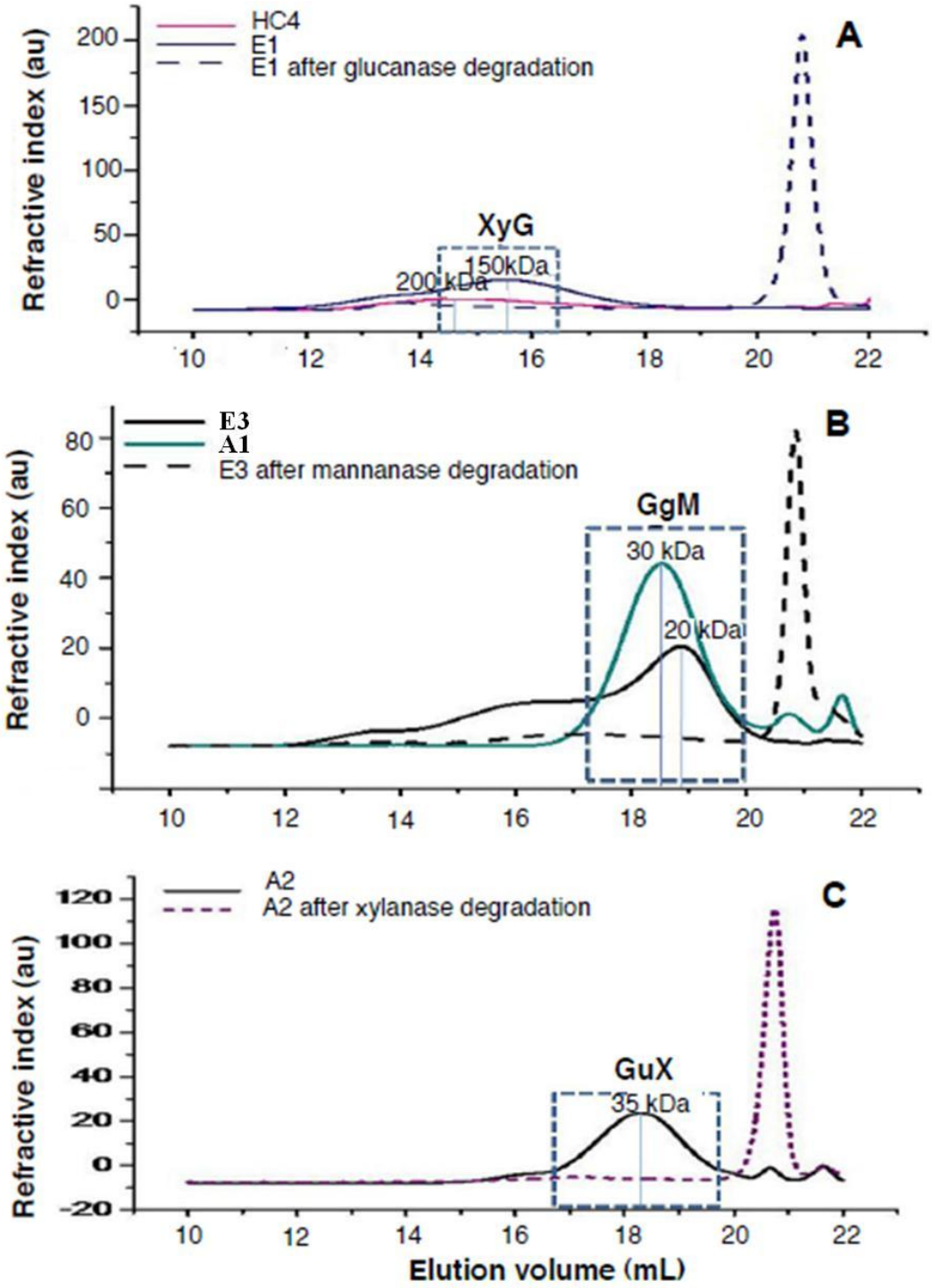

Figure II-4: Normalised high performance size-exclusion chromatogram (HPSEC) of different populations of XyG (A), GgM (B) and glucuronoxylan (C), either as native fraction or after endo-glucanase (A), endo-mannanase (B) and endo-xylanase (C) degradation.

Fraction **E1** yielded mainly acetylated XyGOs (Figure II-6A) corresponding to **XLFGa1**, **XXXG** and **XXFGa1** together with minor structures identified as **GFG**, **XFG**, **XXG**, **XLXG**, **XLXGa1**, **XXFG**, **XXFGa2**, **XLFG** and **XLFGa2** as previously reported (Galvez-Lopez,

Laurens, Quemener & Lahaye, 2011; Renard, Lomax & Boon, 1992; Vincken, van den Broek, van der Lei, Beldman & Voragen, 1997).

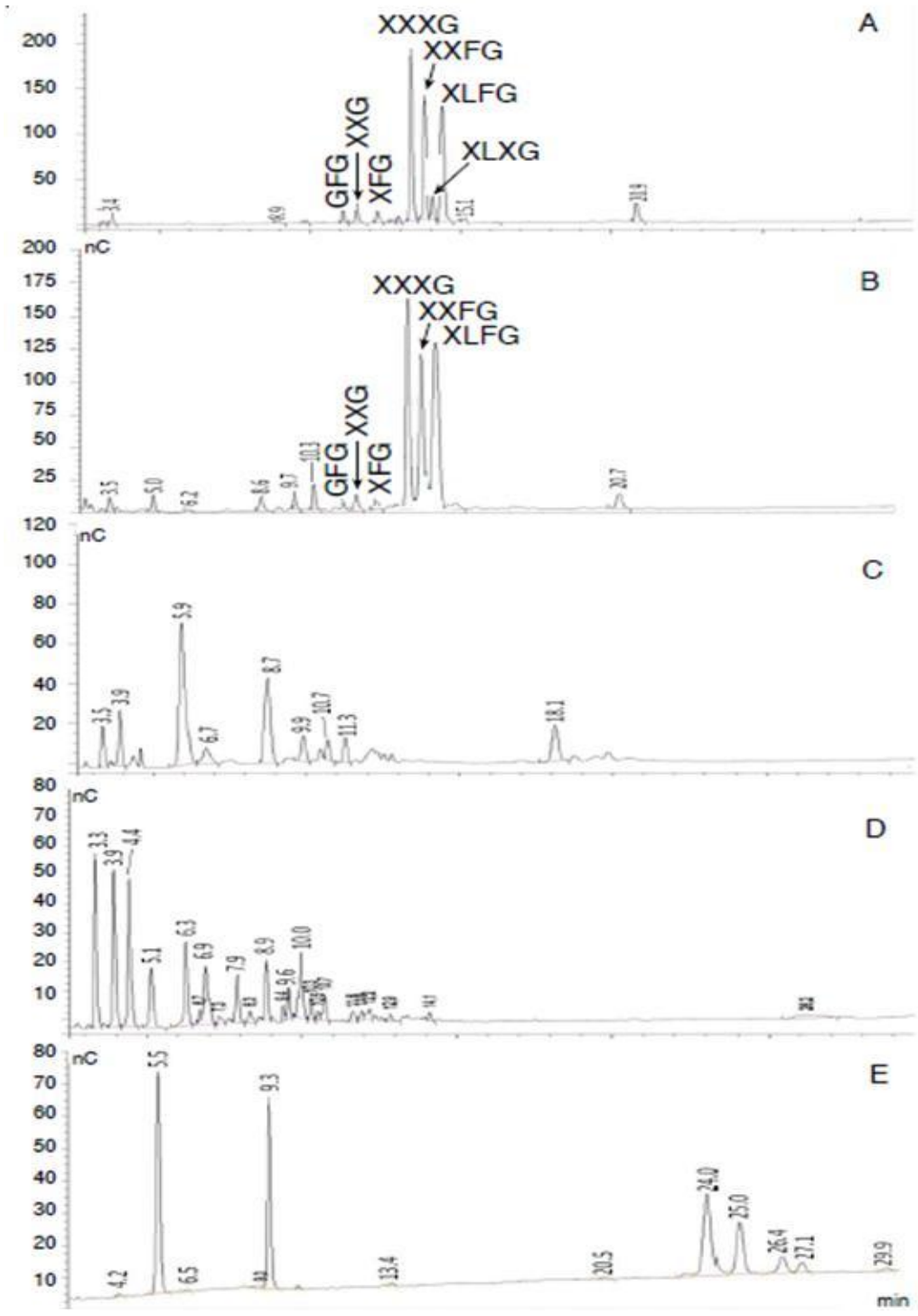

Figure II-5: HPAEC analysis of enzymatic digests of hemicelluloses fraction: endo-glucanase digest of E1 (A), endo-glucanase digest of HC4 (B); endo-mannanase digest of A1 (C), endo-mannanase digest of E3 (D) and endo-xylanase digest of A2 (E).

The XyGOs MS profile of **HC4** (Figure II-6B) yielded a close profile as that of **E1** taking into account the deacetylation and the potassium adducts of the ions resulting from the KOH extraction of the fraction. This was in agreement with previous work showing no major average structural differences between enzyme and alkali extracted XyG from pea (Pauly, Albersheim, Darvill & York, 1999; Pauly, Qin, Greene, Albersheim, Darvill & York, 2001). According to the tethered network model of primary cell walls (Albersheim, Darvill, Roberts, Sederoff & Staehelin, 2011), XyG would act as spacer molecule hydrogen bounded to cellulose and thus prevent cellulose self-aggregation and enable the formation of a strong but flexible XyG-cellulose network.

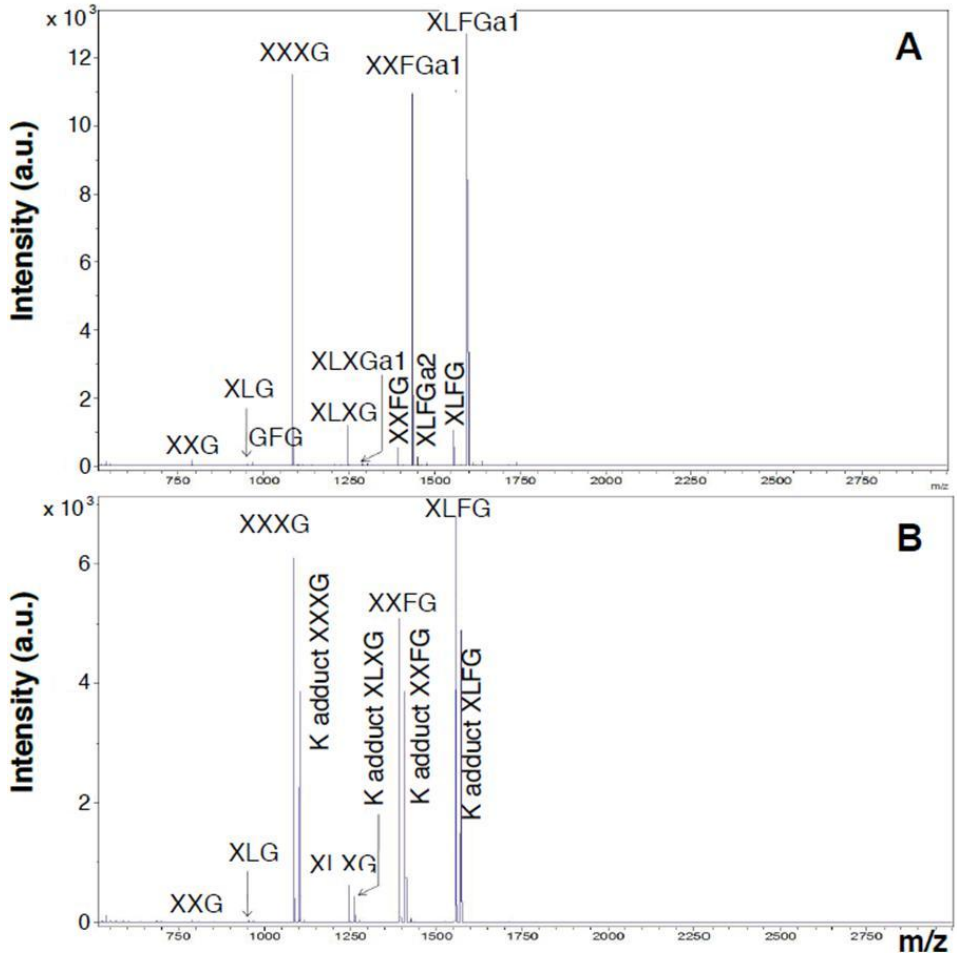

Figure II-6: MALDI-TOF MS spectrum of the endo-glucanase digest of E1 (A) and HC4 (B). The nomenclature of oligosaccharides is as described in the text.

XyG varies in its accessibility to solvents or enzymes (Pauly, Albersheim, Darvill & York, 1999). Part of it is accessible to endo-glucanase degradation and would include structures tethering cellulose microfibrils in the tethered network model of primary cell walls (Albersheim, Darvill, Roberts, Sederoff & Staehelin, 2011). A second part involves XyG resisting glucanase degradation and extracted by concentrated alkali. This constitutes the

major part of XyG that is thought to coat cellulose microfibrils. A third part, representing a very minor part of XyG (< 0.1%), corresponds to domains entrapped within cellulose microfibrils and appears determinant in cell wall mechanical properties (Bootten, Harris, Melton & Newman, 2004; Park & Cosgrove, 2012; Pauly, Albersheim, Darvill & York, 1999). Studies reporting mechanical assays after xyloglucanase, cellulase and glucanase degrading the minor cellulose-entrapped XyG, do not support the tethered cell wall model and question the function of XTH in the control of cell wall mechanical properties (Park & Cosgrove, 2012). These authors proposed a model where XyG and cellulose are entangled with a minor amount of XyG interacting with two adjacent cellulose microfibrills. From the present results, apple XyG fractions assumed to have different cell wall affinity in the light of their extraction did not differ on their average fine chemical structure but had different molecular weight. Considering that residual XyG was present in the **CWR**, LiCl-DMSO, 4 M KOH and residual XyG may reflect chains with increasing molecular weight. The longer the chains, the more potential segments interacting with cellulose microfibrils and the lower the diffusion of the entangled and interacting chains will be from the cell wall. One should also consider that enzymatic profiling of XyG yields only a mean view of the fine structure and does not inform on the distribution of the different motifs. Furthermore, the degree of acetylation of more tightly bound XyG could not be measured. In tomato, the acetyl ester content of the cell wall residues after LiCl-DMSO extraction indicated that residual hemicelluloses were still esterified (Assor, Quemener, Vigouroux & Lahaye, 2013). Block and/or random distribution of **XXXG-** and **XXG**-based motifs as well as acetyl ester substituents may also contribute to the physicochemical properties of XyG in the cell wall. The present scheme of hemicelluloses preparation offers the possibility to have access at different population of XyG that will be useful to study the interaction abilities and mechanical properties of assemblies with cellulose. To our knowledge, it is the first time that acetyl-esterified apple XyG is purified as a single fraction.

3.2.2. Galactoglucomannan

Two different populations (**A1** and **E3**) enriched in apple GgM were obtained from the LiCl-DMSO extract. **A1** was the first fraction eluted in the NaCl gradient from the anion exchanger while **E3** was obtained by gel permeation of the fraction unbounded on the anion exchanger (Figure II-1). **A1** and **E3** were first compared with respect to their molecular weight. **A1** eluted as a single peak at 30 kDa whereas **E3** eluted as a predominant peak at 20

kDa and was contaminated by polysaccharides with higher molecular weight (Figure II-4B). Disappearance of the major **E3** HPSEC peak after endo-β-mannanase treatment confirmed the presence of mannan in this population (Figure II-4B). Differences in their fine structure were assessed by HPAEC and MALDI-TOF MS of oligosaccharides released by β-mannanase hydrolysis. Elution profiles of β-mannanase digests are shown on Figure II-5C, 5D. The released GgMOs at retention time around 5.9 and 8.7 min were major in **A1** and minor in **E3** whereas peaks eluting up to 5.1 min were major in **E3** and minor or absent in **A1**. The presence of higher molecular weight GgMOs as major structures in **A1** in contrast to **E3** revealed fine structural differences between the two GgM fractions.

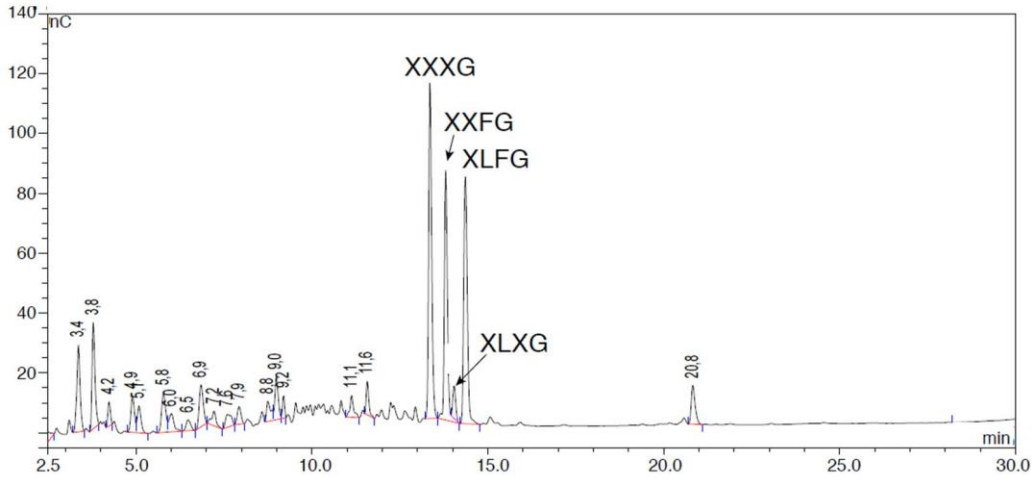

Figure II-S1: HPAEC analysis of enzymatic digest of E3 by endo-glucanase.

The MALDI-TOF spectra of β-mannanase hydrolyzates from **A1** (Figure II-7A) and **E3** (Figure II-7D) demonstrated the presence of a series of more or less acetyl-esterified hexo-oligosaccharides with degrees of polymerization from 3 to 14 attributed to GgM. Mass spectra (Figure II-7A, 7D) were in agreement with HPAEC chromatograms (Figure II-5C, 5D): the **A1** mannanase digest contained higher molecular weight oligosaccharides structures compared to **E3**. In **A1** digest, $\mathbf{H_6a_1}$ was the most intense peak followed by $\mathbf{H_6}$ and $\mathbf{H_8a_1}$. **E3** hydrolyzate contained $\mathbf{H_3a_1}$ as highly intense peak followed by $\mathbf{H_4a_1}$, $\mathbf{H_4a_2}$ and others. In order to further investigate the structural differences in these two GgM populations, a sugar composition analysis was realized on the β-mannanase released GgMOs. Results showed a Gal:Man:Glc ratio of 1:1.5:1.4 and 1:4.3:1.4 in **A1** and **E3,** respectively, indicating that **A1** was GgM whereas **E3** was rather a mannan. To confirm this statement **E3** and **A1** were hydrolysed by β-glucanase and the released GgMOs were again compared with that of the mannanase digest. The MALDI-TOF MS of the β-glucanase oligosaccharides from **A1**

(Figure II-7B) contained **H_4a_1** as major ion followed by **H_3a_1**, **H_4**, **H_4a_2**, and **H_6a_1** confirming its GgM nature. In contrast, the **E3** glucanase digest yielded major ions attributed to XyGOs and minor ions attributed to GgMOs (Figure II-7E). The presence of XyG in this fraction agreed with the HPSEC analysis (Figure II-4B), which showed a peak at 15.8 mL of close retention time as the XyG in **E1**. Among the GgMOs, **E3** released minor ions attributed to **H_4a_1**, **H_5a_1** and **H_6a_1** followed by **H_3**, **H_3a_1**, **H_4**, **H_4a_2**, **H_5a_2**, **H_6a_2**, and **H_7a_1**. These minor GgMOs and major XyGOs were also observed by HPAEC (Figure II-S1) indicating that Man in this fraction composed mannan segments.

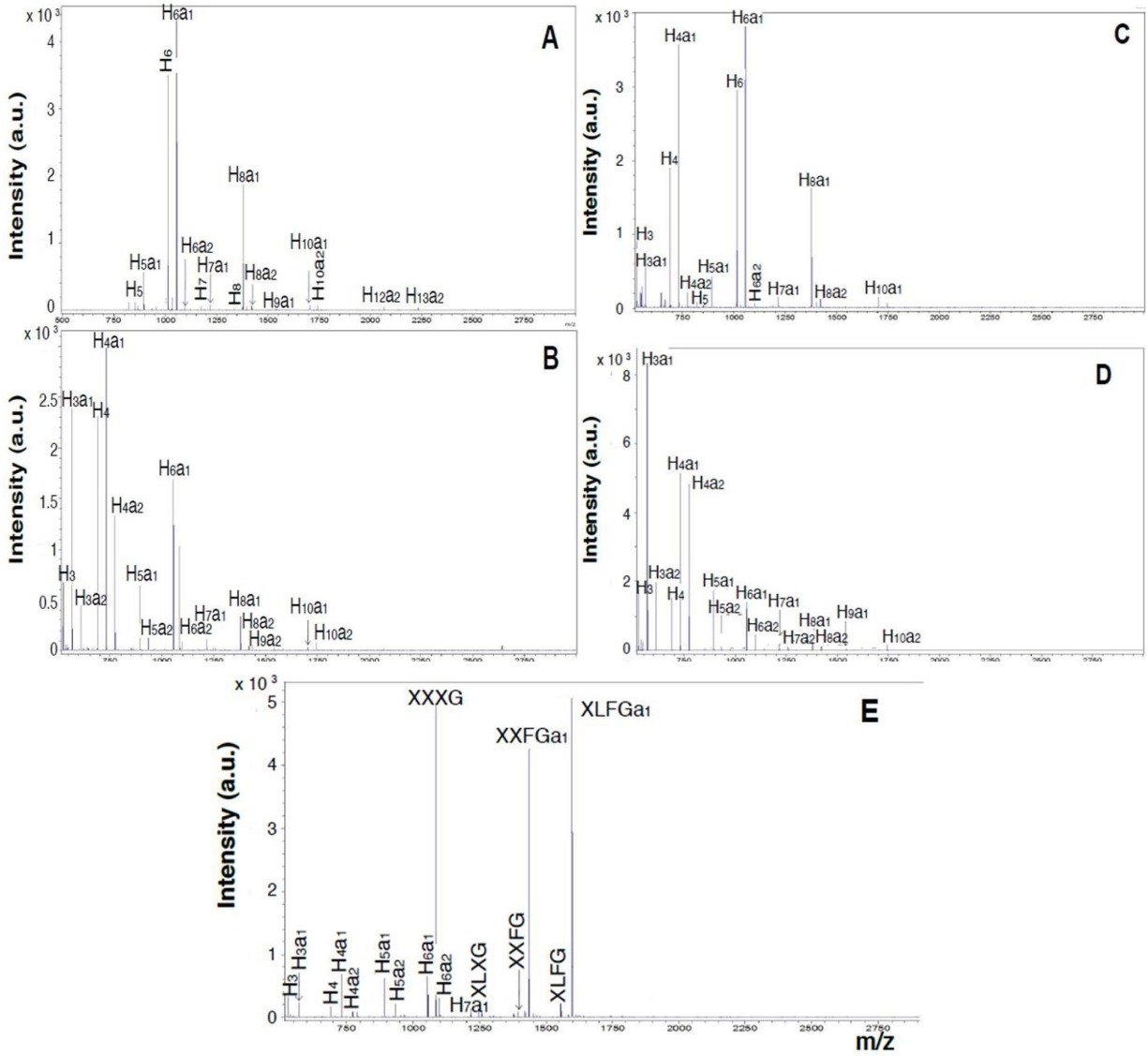

Figure II-7: MALDI-TOF MS spectra of acetylated GgM A1 (A, B, C) and GgM E3 (D, E) after degradation by endo-mannanase (A, D), endo-glucanase (B, E) and endo-mannase + α-galactosidase (C). The nomenclature of oligosaccharides is as described in the text.

To test for galactose ramifications on the **A1** backbone, this fraction was degraded by a combination of the β-mannanase and a α-galactosidase. The MS profile of the digest obtained (Figure II-7C) was compared with that of GgMOs produced by the β-mannanase alone. The high molecular mass β-mannanase GgMOs (**$H_{12}a_2$** and **$H_{13}a_2$**) disappeared after β-mannanase + α-galactosidase treatment with a concomitant appearance of ions attributed to**H_3**, **H_3a_1**, **H_4**, **H_4a_1**. Thus, Gal ramification was responsible for the presence of high molecular weight oligosaccharides in mannanase released GgMOs from **A1**. Similar treatment of fraction **E3** failed to change the MS GgMOs profile (data not shown). This shows Gal hindered β-mannanase action on **A1** and supports Gal ramification at least one Man. Furthermore, the disappearance of high molecular weight oligosaccharides after galactosidase treatment suggested that galactosylated Man occurred as small blocks in Glc-rich GgM. Nara, Ito, Kato & Kato (2004) suggested Gal ramification is possible on both Glc and Man backbone residues in apple GgM. GgM were reported in other fleshy fruits, such as kiwi and tomato (Prakash et al., 2012; Schröder, Nicolas, Vincent, Fischer, Reymond & Redgwell, 2001). In kiwi fruit, Gal ramifications occur on C-6 of mannose and include the disaccharide β-D-Gal-(1→4)-α-D-galactose. The function of this hemicellulose is unclear though it is known to be able to hydrogen bound to cellulose (Melton, Smith, Ibrahim & Schröder, 2009), to be related to cell-cell adhesion in tomato (Ordaz-Ortiz, Marcus & Knox, 2009), to be likely reshuffled by mannanendotransglycosidase/hydrolase as shown in tomato (Schröder, Wegrzyn, Sharma & Atkinson, 2006) probably during its metabolism during fruit development (Percy, Melton & Jameson, 1997). This work revealed that GgM are at least under two different forms in apple cell wall according to the content and distribution of the Man.

3.2.3. Glucuronoxylan

As previously indicated, **A2** was mainly made of Xyl, Ara and UA and also contained high amount of acetyl groups (Table II-1), suggesting an acetylated heteroxylan. Its molecular weight was around 35 kDa. Treatment of **A2** by β-xylanase induced the disappearance of the major HPSEC peak (Figure II-4C) and the appearance of a series of oligosaccharides (Figure II-5E), thus confirming its xylan backbone. The MADI-TOF MS analysis of β-xylanase degradation products (Figure II-8) showed the presence of **$P_5U_1a_1m_1$**, **$P_6U_1a_1m_1$** and **$P_7U_1a_1m_1$** as major ions and **$P_4U_1a_1m_1$**, **$P_5U_1m_1$**, **$P_6U_1m_1$** and **$P_8U_1a_1m_1$** as minor ions. The high molecular mass oligomers such as **P_6**, **P_7**, **P_8** in the end products of **A2** degradation probably originated from different ramifications of the xylan limiting further degradation. In some fleshy fruits like tomato (Assor, Quemener, Vigouroux & Lahaye, 2013; Prakash et al.,

2012), or tamarillo (do Nascimento, Hamm, Baggio, Werner, Iacomini & Cordeiro, 2013) xylans are substituted with (4-*O*-methyl-)glucuronosyl residues resulting in glucuronoxylan. Besides commelinid monocots that usually contain arabinoxylan, arabinosylated glucuronoxylans are rarely reported in the primary cell wall of dicots (Scheller & Ulvskov, 2010).

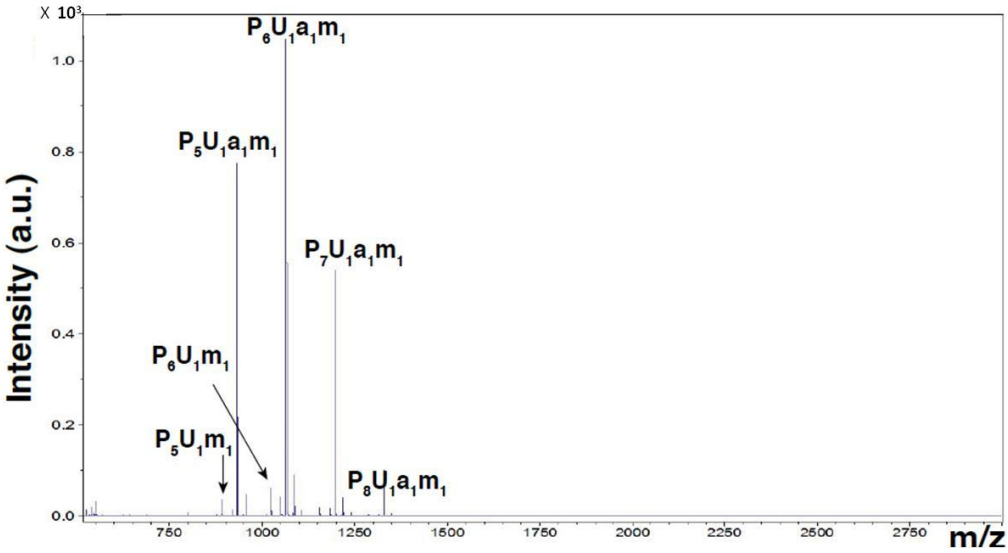

Figure II-8: MALDI-TOF MS spectrum of acetylated glucuronoxylanA2 after endo-xylanase treatment. Treatment of A2 by endo-xylanase + α-arabinofuranosidase in combination produced the same profile.

The sugar composition analysis of the xylanase derived oligosaccharides (XOs) from **A2** showed the presence of Ara and Xyl in a ratio of 1:3 (Table II-1) indicating the presence of glucuronoarabinoxylan. Ara substitutions on dicot xylans are reported at *O*-2 (Darvill, McNeil, Darvill & Albersheim, 1980; Zablackis, Huang, Muller, Darvill & Albersheim, 1995), while3-linked Ara and doubly substituted Xyl residues have been described in psyllium mucilage and flax seeds, respectively (Fischer, Yu, Gray, Ralph, Anderson & Marlett, 2004; Naran, Chen & Carpita, 2008). To investigate the position of Ara as ramification or in the backbone, **A2** fraction was treated with a combination of the xylanase and the α-arabinofuranosidase from *A. niger*. Both the HPAEC elution profiles and MALDI-TOF MS spectra were similar prior and after degradation (data not shown). The arabinofuranosidase used preferentially releases arabinosyl side chains from arabinan and cuts 1-5 linkages better than 1-2 and 1-3 (Kaneko, Arimoto, Ohba, Kobayashi, Ishii & Kusakabe, 1998). This result suggested that Ara was not linked in 1-5, but no clear evidence has been

found regarding its position in glucuronoxylan. Thus, the presence of a minor amount of xylan in apple hemicelluloses is confirmed (Voragen, Schols & Pilnik, 1986) most likely as a glucuronoarabinoxylan. Occurring as a distinct fraction from GgM, it did not appear linked to this other hemicellulose as reported under certain conditions in tomato (Prakash et al., 2012).

II.4. Conclusions

Extraction of hemicelluloses from partially depectinated apple cell walls by LiCl-DMSO revealed that most of these polysaccharides were loosely bounded to the cell wall. The solubility of the acetyl-esterified XyG, GgM, and glucuronoarabinoxylan in LiCl-DMSO and strong base as well as their entanglement in the cellulose rich cell wall residue likely resulted in part from their molecular weight. In addition, at least with regard to XyG, the results question the role of the distribution of the structural motifs and ester substitutions on the interaction and solubility of this polysaccharide. In that respect, acetylated GgM was revealed to exist at least in two forms according to Glc and Man content and distribution. Furthermore, the distribution of galactose ramifications in Glc-rich GgM includes block domains. The diversity and the structural complexity revealed for apple hemicelluloses are at the image of the complex gene families that potentially modify their structure and interaction during fruit development and ripening. The methods set up in this work for obtaining distinct native hemicellulose fractions provide a mean to investigate in more details the cell wall enzymology and the function of these polysaccharides in cell wall mechanical properties and fruit texture.

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Chapter III

Data mining of bacterial genomes for identifying new hemicellulases and functional validation

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From the previous chapter, new characteristics of apple hemicelluloses were stated about their structure and their interactions, notably with cellulose. But some questions stayed unsolved that need new means of investigation. This chapter is focused on the finding of new enzymatic tools to better resolve the apple xyloglucan, mannan and xylan structure in order to better understand their functionality *in planta*. Hemicellulolytic microorganisms secrete enzyme consortia that offer a powerful tool for enzymatic structural finger printing of these polysaccharides. Starting from gene survey, 4 aerobic, nonpathogenic culturable bacterial strains from marine (*Pseudoalteromonas altantica, Cellulophaga algicola*), oligotropic (*Caulobacter crescentus*) and terrestrial (*Paenibacillus* sp.) environment with hemicellulolytic potential were selected. Different culture conditions were tested, including different added hemicelluloses targeting the putative glycoside hydrolases (GH) annotated in each genome. Their extracellular and intracellular extracts were recovered for testing GH activities by colorimetric assay against 21 different hemicelluloses and cellulose analogues, and 21 diverse polysaccharides both from marine and terrestrial origins. Effectiveness of the colorimetric assay was confirmed by analyzing the oligosaccharides production by high performance anion exchange chromatography. Among the 4 strains, *Pseudoalteromonas atlantica* and *Caulobacter crescentus* culture supernatants were shown for the first time to contain activities towards hemicelluloses, in accordance with genome annotation. This work enables the selection of enzymes from these strains to provide novel insights of hemicellulose complexity. In the opposite, *Cellulophaga algicola* and *Paenibacillus* sp. extracts were respectively poorly active and inactive on hemicelluloses. This study points out the successful approach combining genomic data mining with experimental results for the identification of new hemicellulases and optimization of their production from bacteria.

Keywords: hemicelluloses, genome mining, bacteria, screening, hemicellulases, HPAEC

III.1. Introduction

Hemicelluloses are among the most abundant polysaccharides present in the plant cell wall after cellulose. They play an important role in the regulation of cell wall mechanical properties during plant growth, which impacts the various applications of plant crops quality and their agro-industrial processes (Saha et al. 2003; Hansen and Plackett 2008). Hemicelluloses include several polysaccharides, such as heteroxylans, heteromannans, xyloglucans (Albersheim et al. 2011). Heteroxylan consists of a linear (1→4)-linked-β-D-xylopyranosyl (Xyl*p*) backbone substituted with (1,2)-linked α-D-glucuronic acid, its 4-*O*-methyl derivative and acetyl groups (glucuronoxylans, GuX) (Aspinall 1980; Komiyama et al. 2009). In monocot endosperm, xylans carry (1,3)- and/or (1,2)- linked α-L-arabinofuranosyl units as the main substituents to form arabinoxylans (AX) (Izydorczyk and Biliaderis 1995; Sárossy 2011). Heteromannans can be classified in four subfamilies: linear mannan, glucomannan (GlcM), galactomannan (GalM), and galactoglucomanan (GgM) (Petkowicz et al. 2001). Each of these polysaccharides presents a β-1,4-linked D-mannose backbone or a combination of β-1,4-linked D-glucose and mannose residues. In GalM and GgM, the backbone is substituted with α-1,6-linked D-galactose residues. Xyloglucan (XyG) is composed of a cellulose-like backbone of 1,4 linked β-D-glucopyranose residues, on which α-D-xylose can be branched at *O*-6 (Scheller and Ulvskov 2010). The xylose residues at *O*-2 position can further carry β-L-arabinose or α-D-galactose that can be further α-L-fucosylated. A deeper knowledge on the fine structural details of hemicelluloses is required for a better understanding of their function in the cell wall and can provide better usages of these polysaccharides.

Enzymatic degradation followed by chemical, chromatographic and spectrometric analysis is one of the leading tools to solve the fine structure of polysaccharides. As early as 1991, the polysaccharide-active enzymes have been grouped into the continuously updated Carbohydrate-Active EnZymes (CAZy) database (http://www.cazy.org), where they are classified in families based on amino-acid sequence similarities. The database includes mostly glycoside hydrolases (GH, 133 families), some of them being involved in the degradation of hemicelluloses (44 GH families). Among these GH families, some are mono-specific, such as for instance, GH6 or GH45, where all the enzymes hydrolyze a β-(1-4) link between two glucose residues. Some others include a large variety of activities, such as GH5 gathering to date 20 different EC numbers.

Due to their structural complexity, hemicellulose enzymatic degradation requires a large variety of synergistic enzymes. Complete degradation of heteroxylan requires endo-β-1,4-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase (Shallom and Shoham 2003). Among them, β-xylanase (EC 3.2.1.8) play a crucial role in the hydrolysis of the xylan backbone, in which it cleaves the β-1,4-glycosidic bond between xylose residues to release xylo-oligosaccharides. Most of the microbial β-1,4-xylanases are grouped into 7 GH families: GH5, 8, 10, 11, 30, 43 and a bifunctional cellulase/xylanase belongs to GH51 (http://www.cazy.org). Currently, the best studied β-1,4-xylanases belong to families GH10 and GH11 (Kolenova et al. 2006; Berrin & Juge 2008; Zhang et al. 2011). Heteromannan degradation depends on endo-β-1,4-mannanases that catalyze the hydrolysis of the β-1,4-linked backbone. They have been classified into the GH families 5, 26 and 113. All these families are gathered in the largest clan GH-A, which shares the TIM (triosephosphateisomerase) (β/α) 8 barrel fold and has retaining reaction mechanism. With regard to xyloglucans, splitting the β-1,4-link between two glucose residues in XyG backbone requires either broadly specific endo-β-1,4-glucanases (EC 3.2.1.4) or highly specific endo-acting xyloglucanases (EC 3.2.1.151). The xyloglucanases are classified in GH5, 12, 16 and 44 acting by retaining mechanism, and GH9 and 74 acting by inverting mechanism (Gilbert et al. 2008). To date, β-glucanases belong to 13 GH families, namely families 5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 74, 124 and 131, the latter having a broad specificity towards 1-3, 1-4, and 1-6 linkages. Some of them are capable of degrading highly branched xyloglucan (Vincken et al.1997).

Finding novel enzymes is challenging to date. Function-based genomic and metagenomic screening using gene cloning technique is a rapidly progressing technology that has been widely used for searching of specific enzymatic activities (Brennan et al. 2004; Lee et al. 2004; Yun et al. 2004; Solbak et al. 2005) from not readily culturable microorganisms present in natural environments. However, although up to several hundred thousand clones can be analyzed in a single screen run, less than ten active clones are usually detected (Henne et al. 1999, 2000; Majernik et al. 2001) due to low heterologous gene expression and/or insolubility or toxicity of the expressed protein. On the other hand, in light of the multiple GH families of hemicellulolytic enzymes, the selection of microorganisms having the highest potential to degrade these polysaccharides is challenging. On this aim, the 'CAZome', defined as the collection of carbohydrate-active enzymes encoded by the genome of an organism, provides an insight into the nature and extent of the metabolism of complex carbohydrates by different species. Now a day, CAZy covers over several thousand genomes of organisms: Bacteria, Archea, Eukaryota and Viruses. However, the link between genome annotation and

enzyme activity produced by a given organism is often unknown. In this context, our study focused on selecting unexplored culturable bacterial strains, which potentially produced hemicelluloses-modifying enzymes according to the expert genome annotation available in CAZy database. Among the species evaluated, 4 aerobic and nonpathogenic bacteria were grown in different culture conditions and further screened by a colorimetric medium throughput method and HPAEC analysis of degradation products to evaluate their ability to produce some new hemicellulose-degrading enzymes.

III.2. Material and methods

III.2.1. Selection of polysaccharides and their preparation

Forty-two terrestrial or marine polysaccharides of diverse structures and origins were used as substrates (Table III-1). All the polysaccharides were dissolved in deionized water (0.4% w/v). Solutions of commercial polysaccharides were diafiltered on 10 kDa membrane (PES, Millipore, Billerica, MA, USA) using a 50 mL Amicon system 8050 (Millipore). Polysaccharides from the laboratory collection purified from apple and tomato were directly dissolved in deionized water.

III.2.2. Selection of bacteria and their culture conditions

Selected bacteria were grown in their optimum growth conditions mentioned in Table III-2. These conditions were reported either by different research groups or by the international microorganism collections from which the strains were purchased (ATCC, USA; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany; Bacillus Genetic Stock Center, USA).

A preculture was carried out by inoculating frozen cells in 10 mL of specific culture medium (Table III-2) in a 100 mL Erlenmeyer flask. After 36 h incubation at the corresponding temperature with shaking at 150 rpm in a New Brunswick incubator, 1 mL of the culture was inoculated in 50 mL of fresh medium and incubated for additional 8 h under similar conditions. Then, 50 mL were inoculated in 1 L of fresh medium in 5 L shaker flask. After 36 h, the culture was centrifuged at 5,000 × g for 30 min at 4°C.

Table III-1: List of polysaccharides used as substrates for the screening of polysaccharides degrading enzymes. The list includes 21 different hemicelluloses and 21 other polysaccharides coming from different environments.

Number	Polysaccharide	Biological origin	Source
1	**Arabinoxylan (AX)**	Maize	INRA
2	**Arabinoxylan (AX)**	Rye	Megazyme
3	**Arabinoxylan (AX)**	Wheat	Megazyme
4	**Xylan**	Beechwood	Sigma
5	**Glucuronoxylan (GuX)**	Tomato	INRA
6	**Glucuronoarabinoxylan (GAX)**	Apple	Ray et al. 2014
7	**Glucomannan (GlcM)**	*Amorphopallus konjac* (Konjac powder)	Dextra lab
8	**Galactomannan (GalM)**	*Caesalpina spinosa* (Tara gum)	Dextra lab
9	**Galactomannan (GalM)**	*Cyamopsis tetragonolobus* (Guar gum)	Dextra lab
10	**Galactomannan (GalM)**	*Ceretonia siliqua* (Locoust bean gum)	Dextra lab
11	**β-1,4-Mannan**	Carob	Megazyme
12	**Galactoglucomannan (GgM; fraction E3)**	Apple	Ray et al. 2014
13	**Galactoglucomannan (GgM; fraction A1)**	Apple	Ray et al. 2014
14	**Galactoglucomannan (GgM)**	Tomato	INRA
15	**CM-Cellulose**		Sigma
16	**Avicel**		Sigma
17	**Xyloglucan XyG**	*Taramindus indica* (Tamarind gum)	Dextra lab
18	**Acetylated xyloglucan (XyG; fraction E1)**	Apple	Ray et al. 2014
19	**Xyloglucan (XyG; fraction HC4)**	Apple	Ray et al. 2014
20	**Xyloglucan (XyG; 4MK)**	Tomato	INRA
21	**partially depectinated Alcohol insoluble residue (pDAIR)**	Apple	Ray et al. 2014
22	**Pectin (fraction HWE)**	Apple	Ray et al. 2014
23	**Pectin Me 30%**	Citrus	Cargill
24	**Rhamnogalacturonan (RG)**	Carrot	INRA
25	**Rhamnogalacturonan (RG; fraction A4)**	Apple	Ray et al. 2014
26	**Arabinan**	Sugar Beet	Megazyme
27	**Welan Gum**	*Alcaligenes* sp.	Dextra lab
28	**Arabic Gum**	*Acacia* sp.	Dextra lab
29	**Tragacanth Gum**	*Astragalus* trees	Dextra lab
30	**Amylose**	Potato	CNRS
31	**Amylopectin**	Waxy corn	TCI
32	**Pullulan**	*Scerotium rolfsii*	Dextra lab
33	**Dextran**	*Leuconostoc mesenteroides*	Dextra lab
34	**Xanthan**	*Xanthomonas campestris*	Dextra lab
35	**Alginate de sodium**	*Ascophyllum* sp.	CNRS
36	**α,α-trehalose**		Carbosynth Ltd
37	**ι-ν- carrageenan**	*Kappaphycus alvarezzi*	CP-Kelco
38	**κ-μ–carrageenan**	*Eucheuma denticulatum*	CP-Kelco
39	**κ-carrageenan**	*Kappaphycus alvarezzi*	CP-Kelco
40	**Porphyran**	*Porphyra umbicalis*	INRA
41	**Mannuronan**		CNRS
42	**Ulvan rot**	*Ulva rotundata*	CNRS

Culture media and cell pellets were collected and treated separately prior to the screening assay. The cell free culture supernatant was filtered overnight on a 300 kDa membrane (PES,

Millipore, Billerica, MA, USA) using a 400 mL Amicon system 8400 (Millipore). Afterwards, the filtrate was diafiltered and concentrated to 20 mL, with 50 mM Tris–HCl buffer pH 8.5 containing anti-protease (Roche, Indianapolis, IN, USA), on a 10 kDa membrane (PES, Millipore, Billerica, MA, USA) under compressed air (0.5 bar) to remove molecules smaller than the cut-off.

Table III2: Source, origin, culture conditions of 4 different aerobic and non-pathogenic bacteria used as source for the screening of polysaccharides-degrading enzymes. Occurrence of the hemicelluloses modifying GH families in these four different bacterial strains.

	Bacterial strain	*Pseudoalteromonas atlantica* T6c	*Caulobacter crescentus* CB15	*Cellulophaga algicola* DSM14237	*Paenibacillus* sp. JDR-2
	Source	ATCC BAA-1087	DSMZ 4727	DSMZ 14237	BGSCID 35A1
	Origin	Marine	Oligotrophic	Marine	Terrestrial
Culture conditions	Medium	Bacto marine broth (DIFCO 2216)	CAULOBACTER medium	Bacto marine broth (DIFCO 2216)	LB medium
	pH	7.4	7.0	7.4	7.0
	T (°C)	20	28	20	30
	Reference	Gauthier et al. 1995	DSMZ	Birte et al. 2011	BGSC
Sequence number in each GH families	5	1	1	4	7
	6				
	8	1			1
	9		1		1
	10	1	2	4	5
	11			1	1
	16	4	1	8	3
	26			3	1
	30		1		5
	43	3	3	3	25
	48				
	51		1		7
	74				2
	113			1	1
	Total	10	10	24	59

Cell pellets were washed twice with 500 mL of 50 mM Tris–HCl buffer pH 8.5. The cell pellets were resuspended in 10 mL of the same buffer containing anti-protease mixture. Bacterial cells were disrupted by Constant cell disruption systems (Constant systems Ltd, Northants, UK) and the membrane fragments were removed by centrifugation at 20,000 × g for 60 min at 4°C. The clarified lysate was diluted 10 times, filtered overnight on a 300 kDa membrane and finally concentrated, diafiltered with 50 mM Tris–HCl buffer pH 8.5 on a 10

kDa membrane to a final volume of 20mL. Both cell lysates and culture supernatants were stored at 4°C until incubation with polysaccharides, which was performed on the same day.

III.2.3. Screening assay

The 42 polysaccharide solutions (150 µL) and 5 controls containing water were dispensed into half of the wells of 96-well filter microplate (10 kDa, PES, Pall) with an equal volume (150 µL) of bacterial extract (Fer et al. 2012). Similarly, polysaccharide controls were done in the remaining half of the plate by incubating 150 µL of sample solutions with 50 mM buffer solution (Tris-HCl, pH 8.5 or acetate, pH 5.2) instead of bacterial extract. Microplates were sealed with a plastic film and incubated overnight with shaking at 34°C. The incubation medium was filtered on a multiscreen HTS vacuum manifold (MSVMHTS00, Millipore) connected to a high-output vacuum pressure pump (Millipore) for 3 h with the vacuum maintained at about 0.5 bar.

For the colorimetric assay, 40 µL of each incubation filtrate were transferred to a microplate (Dutscher, Brumath, France). Then, 200 µL of ferricyanide solution (1.5 $g.L^{-1}$ ferricyanide, 24 g Na_2CO_3, 1 mL 5 M NaOH, QSP 1 L, Kidby and Davidson 1973) were added to the sample and the microplate was sealed with plastic film. The plate was heated at 95°C for 15 min in a thermocycler (GenAmp PCR system 2700, Applied Biosystems France, Villebon-sur-Yvette, France) and cooled to room temperature. The occurrence of reducing ends was revealed by analyzing the absorbance at 420 nm of 200 µL of the samples in a microplate reader (Nunc, Roskilde, Denmark) using a Wallac 1420 multilabel counter (PerkinElmer Life Sciences, Boston, MA, USA). The amount of reducing ends was expressed in absorbance units. The optical density (OD) of the hydrolyzates for which absorbance was higher than that of the bacterial extract alone (control) minus 0.15, were considered as polysaccharides of negative hits. The mean (X) and the standard deviation (σ) of the OD of the incubation of these negative hit polysaccharides were calculated. When the OD of the hydrolyzate was lower than X-2σ, the polysaccharide sample was considered as degraded. The hit polysaccharides were classed according to four intervals based on the control X and its σ.

III.2.4. Analysis of oligosaccharides on High Pressure Anion Exchange Chromatography (HPAEC)

Reaction media were chromatographed through on a Carbo-Pac PA 200 column (3 x 250 mm,

Dionex, Sunnyvale, USA) thermostated at 30°C. Samples were eluted using a linear gradient of sodium acetate from 0 to 170 mM in 100 mM NaOH (Quémener et al. 2007) and monitored by amperometry using an ED electrochemical detector (Dionex).

III.2.5. Protein content of bacterial extracts

The concentration of protein was determined in the bacterial extracts by adding 200 µL of each to 50µL of the Bradford reagent (Bio-Rad, Marnes-la-Coquette, France) (Bradford 1976). Bovine serum albumin (BSA, Sigma) was used as a standard (0–25µg.mL^{-1}).

III.3. Results

III.3.1. Selection of bacteria

The first criterion for the selection of the bacteria was that their genome must contain genes coding for proteins classified in GH families encompassing backbone destructing hemicellulase activities, β-glucanase, xyloglucanase, β-mannanase and β-xylanase (GH5, 6, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 45, 48, 74, 113, 124). From the CAZy database, a total of 3692 culturable bacteria were found as putatively possessing these families in their genome. Among them, 135 were selected because their genome contained at least 10 sequences of interest and for experimental reasons pathogenic and anaerobic strains were eliminated. To reduce the number of the bacterial strains to study, *Caulobacter crescentus* CB15, *Paenibacillus* sp. JRD-2, *Cellulophaga algicola* DSM 14237 and *Pseudoalteromonas atlantica* T6c strains were chosen because they come from oligotrophic, terrestrial and marine environments. The number of potential hemicellulases and their repartition in CAZy families are indicated in Table III-2. When comparing the number of GH of interest between the four strains, *Paenibacillus* sp. JRD-2 and *Cellulophaga algicola* DSM 14237 appeared to be most promising strains. However, as already stated above, some families, such as GH5, 16, 43 are multispecific, and thus, the sequences belonging to these families are not all of hemicellulose-degrading enzymes. For example, *P. atlantica* T6c contain 4 GH16 sequences among which 3 sequences (Palt_0805, 0824 and 0843) contain a β-agarase domain.

III.3.2. Enzymatic screening

At first, the selected bacteria were grown in the absence of any added polysaccharides (Table III-2) and their enzymatic activities were screened towards 42 polysaccharides through the medium throughput protocol of Fer et al. (2012). The enzymatic hydrolysis was assessed by a colorimetric method revealing the appearance of reducing power (Table III-3).

The protein concentration of the extracellular extract of *P. atlantica* was 1265 $\mu g.mL^{-1}$ and its activity was observed to be the highest on tomato GgM, konjac GlcM, carboxymethyl cellulose (CMC), porphyran and α,α-trehalose. This bacterial extract was moderately active on apple GgM, deacetylated tomato XyG, beechwood xylan and AX from wheat and rye. It was slightly active on apple (acetylated or not) XyG and GAX, maize AX, apple and citrus pectin. The intracellular extract from the same bacterium, having a protein concentration of 1470 $\mu g.mL^{-1}$, showed a faint activity towards maize AX, apple GgM (E3) and CMC. Similarly the extracellular extract from *C. crescentus* (protein concentration of 350 $\mu g.mL^{-1}$) was highly active on tomato GgM and apple rhamnogalacturonan, moderately active on xanthan and very slightly active on apple GgM, maize AX, apple glucuronoarabinoxylan (GAX), linear arabinan, tomato and apple XyG fractions. The intracellular extract from *C. crescentus* (protein concentration of 1360 $\mu g.mL^{-1}$) showed a low activity on arabic gum, pullulan, wheat AX, and tomato GgM and XyG. The *C. algicola* extracellular extract demonstrated very little activity towards pullulan, tomato GuX and apple acetylated XyG and GgM (E3) while no intracellular activity was found against these polysaccharides. The protein concentration of both extra and intracellular extracts was very low compared to other bacteria (65 and 92 $\mu g.mL^{-1}$, respectively). This can be partly linked to the limited growth of the strain (data not shown). The extracellular extract of *Paenibacillus* sp. (protein concentration of 269 $\mu g.mL^{-1}$) showed no enzyme activity on studied hemicelluloses. However, its intracellular extract (protein concentration of 800 $\mu g.mL^{-1}$) had a low activity on tomato GuX. By comparing all the colorimetric assay results (Table III-3), it was clear that most of the hemicelluloses-degrading activities were in the extracellular extracts of the four bacteria. These were thus further investigated.

Table III-3: Colorimetric analysis of enzymatic activities after the incubation of the 42 different polysaccharides with each of 4 different bacterial cultivated in the absence of added polysaccharides. Positive hits of polysaccharide degradation were classed according to 4 intervals based on the control mean (x) and standard deviation (σ) of negative hit polysaccharides. The extent of degradation was indicated according to gray intensity, :x −2σ<absorbance < x−4σ; : x−4σ< absorbance < x−6σ; : x−6σ< absorbance < x−8σ; : x−8σ<.

	P. atlantica		*C. crescentus*		*C. algicola*		*Paenibacillus* sp.	
Activity	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular	Extracellular	Intracellular
Protein concentration (µg/mL)	1265	1470	350	1376	65	92	269	800
Polysaccharides								
Maize AX								
Rye AX								
Wheat AX								
Beechwood Xylan								
Tomato GuX								
Apple GaX								
Konjac GlcM								
Tara GalM								
Guar GalM								
Locust GalM								
1,4-β- mannan								
Apple GgM (E3)								
Apple GgM (A1)								
Tomato GgM								
Cm-cellulose								
Avicel								
Tamarind XyG								
Apple acetylXyG (E1)								
Apple XyG (HC4)								
Tomato XyG (4MK)								
pDAIR								
Arabinan linear								
Welan gum								
Tragacanth gum								
Arabic gum								
Amylopectin								
Amylose								
Pullulan								
Apple pectin								
Citrus pectin								
Carrot RG								
Apple RG (A4)								
Xanthan								
Dextran								
α,α-trehalose								
ι-ν- carrageenan								
κ-µ–carrageenan								
κ-carrageenan								
Porphyran								
Mannuronan								
Ulvan								
Na alginate								

III.3.3. Confirmation of the hemicellulose degradation

In support of the results obtained by colorimetry some hydrolyzates were selected and analysed by HPAEC.

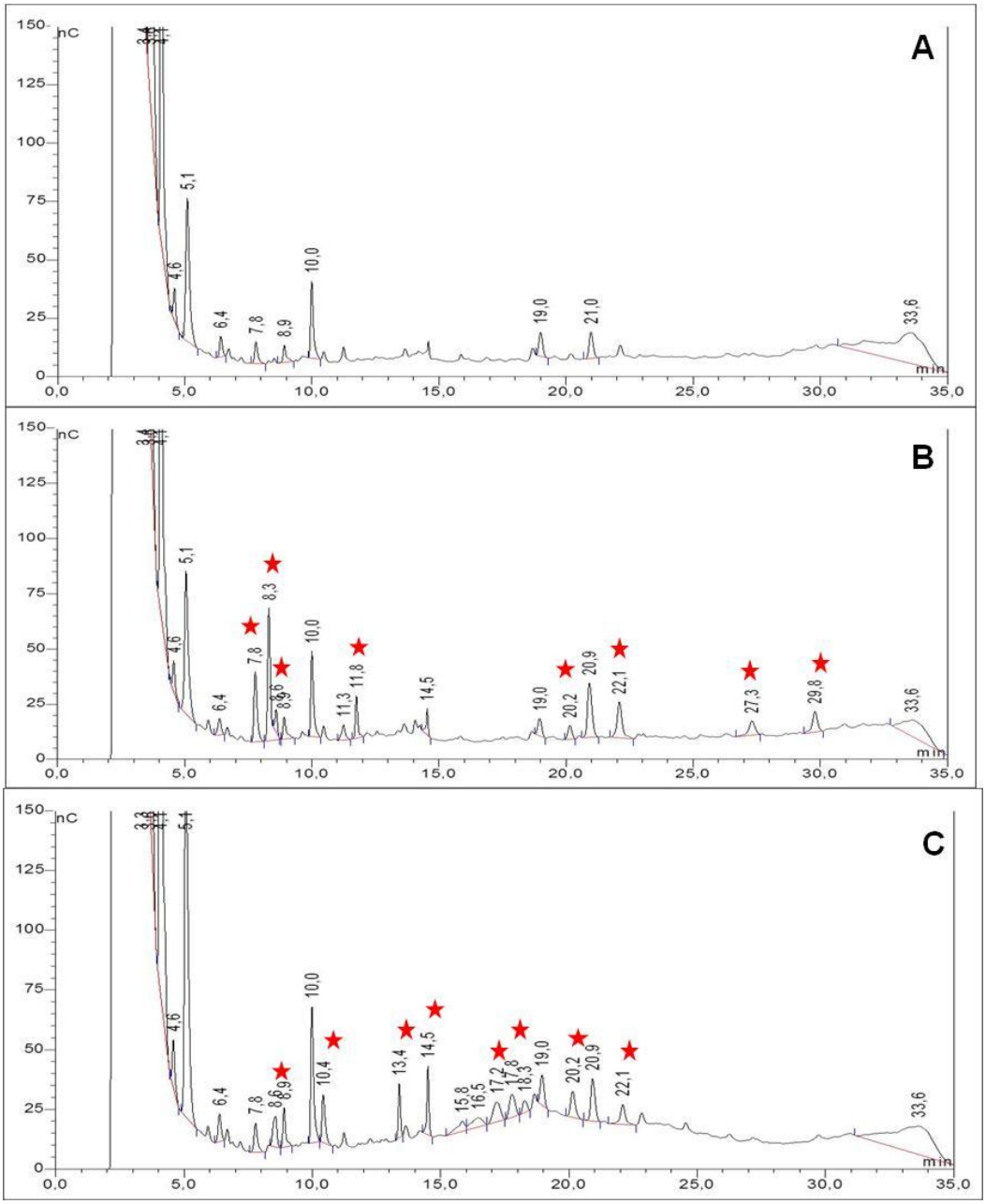


Figure III-1: HPAEC of *C. crescentus* extracellular extract: (A) control without substrate, (B) after incubation with tomato XyG, (C) after incubation with apple GgM. The red stars indicate the peaks of the newly produced oligosaccharides.

The extracellular extract of *C. crescentus* and *C. algicola* were chosen to confirm the low

hemicellulose degradation observed by the colorimetric method. The figure III-1 presents the products resulting from the incubation of tomato XyG with *C. crescentus* extract at pH 8.5 (Figure III-1B), compared with the bacterial extract alone as control (Figure III-1A). The new signals eluting at 7.8, 8.3, 8.6, 11.8, 20.2 and 22.1 min present in the Figure III-1B but absent in the Figure III-1A confirmed the enzymatic activity of *C. crescentus* towards XyG. Similarly, the activity of the *C. crescentus* extract towards apple GgM was confirmed by the appearance of new peaks eluting at 8.6, 10.4, 13.4, 14.5, 17.2, 17.8, 20.2 and 22.1 min in the chromatogram shown on Figure III-1C. The same method was applied to the analysis of incubation of tomato GuX with *C. algicola* extract at pH 8.5.

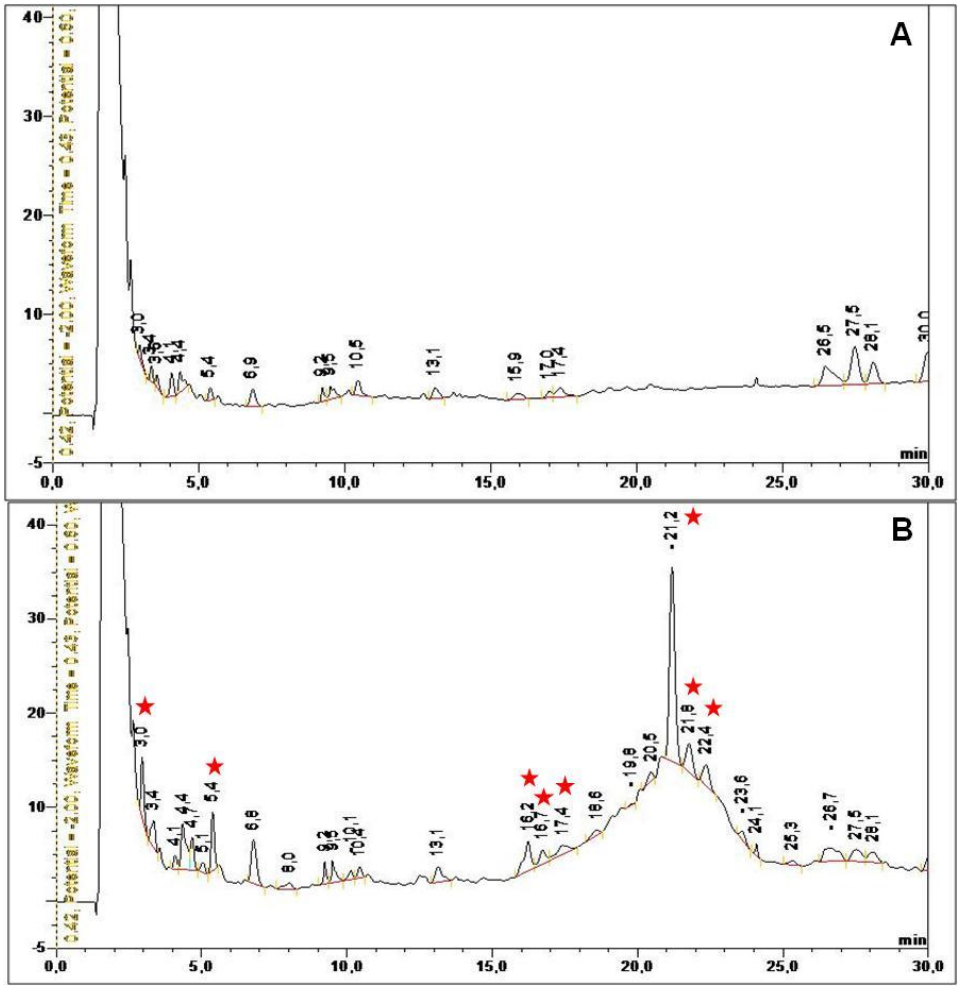


Figure III-2: HPAEC of *C. algicola* extracellular extract: (A) control without substrate, (B) after incubation with tomato GuX. The red stars indicate the peaks of the newly produced oligosaccharides.

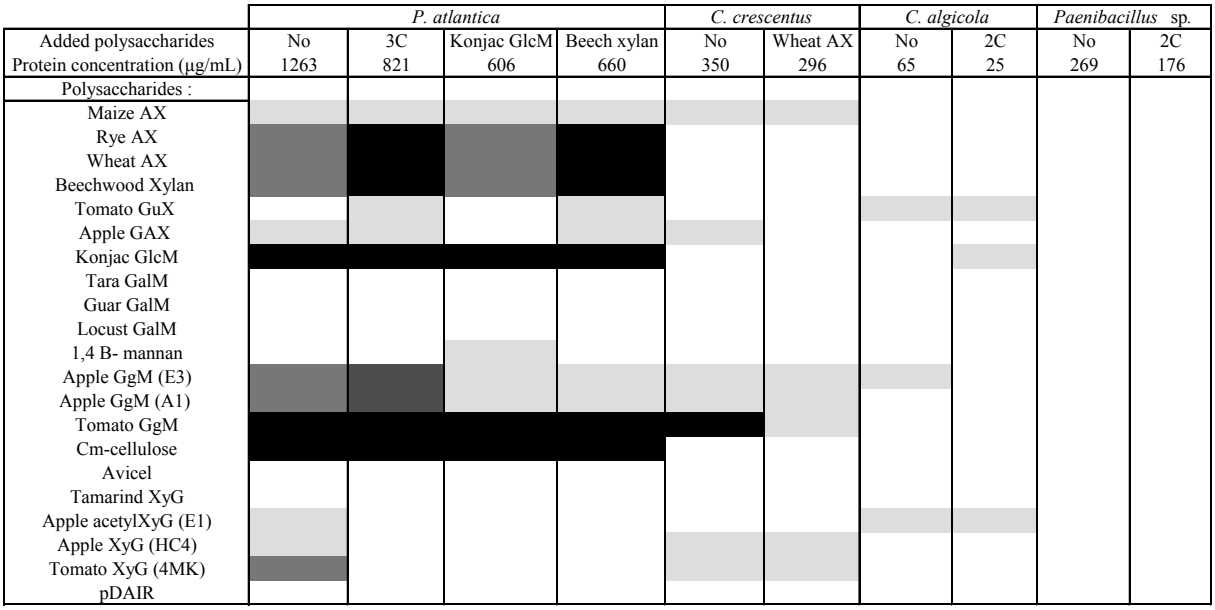
Appearance of new peaks eluting at 3.0, 5.4, 16.2, 16.7, 17.4, 21.2, 21.8 and 22.4 min (Figure III-2B), which were absent in the bacterial extact alone used as control (Figure III-2A),

suggested the production of oligo-xylans. As the colorimetric screening showed a slight response for all these incubations (in light gray in Table III-3), the chromatographic analysis confirmed the release of oligosaccharides.

III.3.4. Optimization of the culture conditions by the use of polysaccharide inducers

Addition of selected carbon sources in the culture medium of the bacteria can play an important role in the induction of enzymatic activity (El-Helow et al. 1997; Ferreira and Filho 2004; Gomes et al. 2007; Rashid et al. 2010; Kim et al. 2011ab). Hence, to enhance the enzyme production, different hemicelluloses were added to the culture media.

Table III-4: Colorimetric profiling of the hemicelluloses-degrading enzymes of extract obtained from bacteria culture without and with polysaccharides inducers. Inducer: for *P. atlantica* mixed CMC, tara GalM and beechwood xylan (abbreviated as 3C), konjac GlcM and beechwood xylan; for *C. crescentus* wheat AX and for both *C. algicola* and *Paenibacillus* sp. a mixture of CMC and tara GalM (abbreviated as 2C) were used as added polysaccharides.



These were chosen according to the hemicelluloses degraded by bacterial extract and/or taking into account the GH families potentially present in the bacteria. In CAZy database, 1 protein sequence of *P. atlantica* is classified in GH5, 1 in GH8, 1 in GH10, and 3 sequences in GH43 (Table III-2). These 6 proteins potentialy degrade β-linked-1,4-mannan, glucan and xylan.

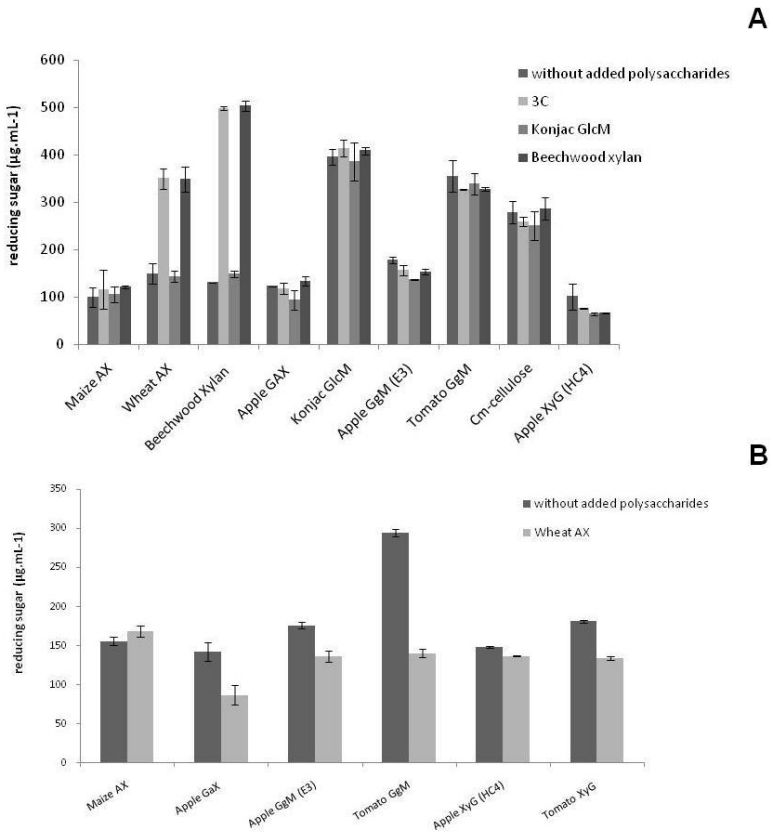


Figure III-3: Reducing sugars released by extracellular extracts incubated with various hemicelluloses (A) *P. atlantica* grown without inducer, with mixed CMC, tara GalM and beechwood xylan (abbreviated as 3C), with konjac GlcM and with beechwood xylan as inducers; (B) *C. crescentus* grown without inducer and with wheat AX as inducer.

When the culture medium of *P. atlantica* was complemented with a mixture of CMC, tara GalM and beechwood xylan (abbreviated 3C source) the extracellular bacterial extract showed highest activity towards tomato GgM, Konjac GlcM, CMC, beechwood xylan, rye and wheat AX (Table III-4). It showed moderate activities against both apple GgM fractions, and low activities towards maize AX, tomato GuX and apple GAX. Similarly, this bacterium when grown with konjac GlcM as sole added carbon source showed high extracellular activity on konjac GlcM, tomato GgM and CMC, moderate activity on beechwood xylan, rye and wheat AX and low activity on maize AX, β-mannan, and both apple GgM fractions (Table III-4). When the culture medium of *P. atlantica* was complemented with beechwood xylan, the extracellular extract showed the highest activity towards tomato GgM, konjac GlcM, CMC, beechwood xylan, rye and wheat AX, and low activities towards maize AX, tomato GuX and apple GAX (Table III-4). The released reducing ends were compared between

different bacterial extracts of *P. atlantica* incubated with maize and wheat AX, beechwood xylan, apple GAX, konjac GlcM, apple and tomato GgM, CMC and apple XyG (Figure III-3A) due to their positive degradation profile (Table III-4). The degradation of wheat AX and beechwood xylan increased a lot when xylan was added in *P. atlantica* culture medium, whereas very little effect was noticed on the degradation of maize AX and apple GAX. Moreover, the addition of hemicellulose carbon source in *P. atlantica* culture medium seemed to have no effect on the degradation of glucan and GlcM backbones.

Due to the presence of xylan-modifying enzymes of GH10, 30, 43 and 51 in its genome, *C. crescentus* was grown with wheat AX as carbon source. However, the extracellular extract contained a very low activity towards wheat AX, apple and tomato GgM and XyG. Thus, it did not show a major increase of activity on xylan and even showed a decrease of the activity on GgM compared to the not complemented culture (Table III-4). Similarly, the reducing ends from incubations of maize AX, apple GAX, apple tomato GgM and non-acetylated XyG with different *C. crescentus* bacterial extracts were compared (Figure III-3B). Presence of AX in culture medium had a low effect on the activity towards glucan and GlcM and did not affect significantly the AX and GAX backbone.

For *C. algicola* and *Paenibacillus* sp. both CMC and tara GalM were added to their respective culture broth as their genome were annotated to contain members of the GH5, 16, 26, 113 and GH5, 10, 16, 26, 74, respectively (Table III-4). However, adding these hemicelluloses as carbon sources had only little effect on the activity against these polysaccharides.

Finally these experiments aiming at improving secretion of hemicellulolytic enzymes in the culture supernatant of these 4 bacteria showed a positive effect of the induction mainly with *P. atlantica*. Thus highly active *P. atlantica* and moderately active *C. crescentus* bacterial extracts were selected for optimizing the recovery of enzymatic activities.

III.3.5. Optimization of the recovery of P. atlantica and C. crescentus hemicellulolytic activities

To optimize the recovery of activity from the culture supernatant, the effect of pH during diafiltration of the extract was tested on the hemicellulolytic activities.

Table III-5: Colorimetric enzymatic activityin the extracellular culture medium filtered at pH 5.2 and 8.5 from (A) *P. atlantica* and (B) *C. crescentus* in the absence or in the presence of added polysaccharides.

A

	P. atlantica							
Added polysaccharides	No		3C		Konjac GlcM		Beechwood xylan	
pH	8.5	5.2	8.5	5.2	8.5	5.2	8.5	5.2
Protein concentration (µg/mL)	1263	930	821	748	606	541	660	515
Polysaccharides :								
Maize AX								
Rye AX								
Wheat AX								
Beechwood Xylan								
Tomato GuX								
Apple GAX								
Konjac GlcM								
Tara GalM								
Guar GalM								
Locust GalM								
1,4 B- mannan								
Apple GgM (E3)								
Apple GgM (A1)								
Tomato GgM								
Cm-cellulose								
Avicel								
Tamarind XyG								
Apple acetylXyG (E1)								
Apple XyG (HC4)								
Tomato XyG (4MK)								
pDAIR								

B

	C. cresentus			
Added polysaccharides	No		Wheat AX	
pH	8.5	5.2	8.5	5.2
Protein concentration (µg/mL)	350	289	296	227
Polysaccharides :				
Maize AX				
Rye AX				
Wheat AX				
Beechwood Xylan				
Tomato GuX				
Apple GAX				
Konjac GlcM				
Tara GalM				
Guar GalM				
Locust GalM				
1,4 B- mannan				
Apple GgM (E3)				
Apple GgM (A1)				
Tomato GgM				
Cm-cellulose				
Avicel				
Tamarind XyG				
Apple acetylXyG (E1)				
Apple XyG (HC4)				
Tomato XyG (4MK)				
pDAIR				

The two different pH 5.2 and 8.5 affected diversely the hemicellulolytic activities (Table III-5A, 5B). In the case of *P. atlantica*, the extracts arising from the culture without or with

added polysaccharides (konjac GlcM, beechwood xylan, mixture of beechwood xylan, tara GalM, CMC) at both pH 5.2 and 8.5 exhibited glucan- and GlcM-degrading activities (Table III-5A). In the presence of konjac GlcM in the growth medium, the extract at pH 5.2 showed the highest activity on locust GalM, intermediate activities on guar GalM and very low activity on 1,4-β-mannan. At pH 8.5, this extract had no activity towards GalM.

At pH 5.2, the extract from *C. crescentus* grown without added hemicelluloses showed a low activity towards 1,4-β-mannan and maize AX while at pH 8.5 it showed the highest activity on tomato GgM and a very low activity on maize AX, apple GgM, tomato and apple XyG (Table III-5B). In the presence of wheat AX in the growth medium, the *C. crescentus* extract at pH 5.2 was moderately active towards maize AX and showed a very little activity towards guar gum and 1,4-β-mannan. At pH 8.5 it demonstrated a faint activity towards apple GgM and de-acetylated XyG, tomato GgM and de-acetylated XyG. As expected, pH is an important parameter that modulates the enzyme activities occurring in bacterial extracts and the most favourable pH depends on the bacterial extract and the targeted enzyme activity.

III.4. Discussion

Bacteria investigated in this study were selected because their genome contained several genes coding for putative hemicellulose-modifying enzymes classified in the expert CAZy database (http://www.cazy.org). The enzymatic activities occurring in bacterial extracts were screened with a medium-throughput colorimetric profiling method and validated using chromatographic analysis. The bacteria were grown in different culture conditions, notably, in the presence of hemicellulose as added source of carbon aiming at stimulating potential secretion of hemicellulases. Altogether, combining data mining with biochemical validation successfully allowed confirming *P. atlantica* and *C. crescentus* as potential sources of new hemicellulases whose most were secreted in the culture media. Conversely, *C. algicola* and *Paenibacillus* sp. whose genome contained 24 and 59 genes coding for potential hemicellulases, respectively, showed unexpectedly poor or absence of hemicellulolytic enzymes.

The annotated genomic sequence of *P. atlantica* T6c (Copeland et al. 2006) suggested 10 genes coding for putative hemicellulases. One protein of *P. atlantica* was found in the family GH5, which has been divided in sub-families to better predict substrate specificities of GH5 enzymes. The Patl_1404 gene coded for a protein classified in GH5_2 sub-family,

which encompassed only cellulases or endo-glucanases. The GH10 family encompassed enzymes having endo-xylanase activity suggesting that Patl_2657 gene also coded for a xylanase. These predicted endo-glucanase and endo-xylanase could explain the presence of glucan, GlcM- and xylan-modifying activities in *P. atlantica* extracellular extracts. The GH8, GH16 and GH43 contained several enzymes activities and, in contrast to GH5 family, no sub-classification has been reported and substrate specificity was then less straightforwardly predictable.

In contrast to *P. atlantica*, no hemicellulose degradation products were observed for *Paenibacillus* sp. JDR-2 though its genome annotation suggested the presence of many hemicellulases (Lucas et al. 2009). Six proteins were classified in family GH10 (Pjdr2_4255, 4260, 4244, 0221, 1324) and GH11 (Pjdr2_4664), which gathered only endo-xylanases suggesting that this strain was able to degrade xylan. Seven proteins were classified in GH5 including 3 proteins (Pjdr2_4071, 0674, 0680) that were not sub-classified because their sequences were distantly related and therefore no substrate specificity could be predicted. Three proteins were found in subfamilies GH5_13 (Pjdr2_1620), GH5_35 (Pjdr2_4271) and GH5_46 (Pjdr2_5185) for which no substrate specificity was biochemically demonstrated. The seventh protein (Pjdr2_6111) was the only one classified in the monospecific GH5_22 sub-family, which contains cellulases or endo-glucanases. The other potential hemicellulases were classified in families having members catalyzing a wide diversity of substrates, whose specificity was not predicted. However, because of the high number of putative hemicellulases, we were expecting to observe hemicellulose degradation.

Similar detailed investigations on putative hemicellulases of *C. crescentus* CB15 and *C. algicola* suggested also the occurrence of potential 10 and 24 hemicellulases respectively. For *C. crescentus* CB15 some substrate specific GgM, XyG and xylan degradation were obtained whereas for *C. algicola*, very poor degradation productions was observed. Altogether, our screening results highlighted a gap between genome prediction and functional validation. This could be explained by the selection of hemicellulose substrates used in the screening and culture conditions, which were not optimal for hemicellulase expression.

To succeed in evidencing enzymes, the choice of substrates is crucial. We observed that the activities of different *P. atlantica* bacterial extracts towards the xylan backbone differed likely due to variations in the fine structure of the xylan substrates (Figure III-3A). In particular, the concentration of reducing ends released from maize AX was very low in comparison to others. This AX has a complex structure that may make it more resistant to enzyme than others. In addition to mono-arabinose substitution, the xylan backbone of maize

can be further decorated with xylopyranosyl, galactopyranosyl (Saulnier et al. 1995a; Saulnier and Thibault 1999). The substituting arabinose can be further decorated with xylopyranosyl, galactopyranosyl, ferulic acid or one or more arabinofuranosyl residues (Saulnier et al 1995a; Saulnier et al. 1995b). Therefore, *P. atlantica* endo-xylanase likely accommodates in its active site less substituted xylan backbone. Similarly, *C. crescentus* bacterial extract showed high activity on tomato GgM whereas it showed very low activity on apple GgMs (Figure III-3B). These observations suggested that absence of hemicellulose degradation with *C. algicola* and *Paenibacillus* sp. could be relied on the selection of hemicellulose substrates.

Furthermore, the production of the hemicellulases by microorganisms is most often inducible (El-Helow et al. 1997; Ferreira and Filho 2004; Gomes et al. 2007; Rashid et al. 2010; Kim et al. 2011ab). Unfortunately in our experiments, addition of polysaccharides as inducers in the growth medium of the 4 bacteria was only beneficial for *P. atlantica*. The choice of the proper polysaccharide used as inducer is not easily predictable. The hemicellulase activity can be stimulated using the polysaccharides directly related with its metabolism. For example, several studies showed that xylanases were secreted in media containing xylan like birchwood xylan (Tsenga et al. 2002; Sharma et al. 2013) or xylan-rich residues like wheat bran (Ninawe et al. 2008; Sanghi et al. 2010). In agreement with these studies, the presence of xylan in the culture medium of *P. atlantica* is likely responsible for the stimulated secretion of enzymes that modify xylan backbone. Further, it can be pointed out that though maize AX has xylan backbone, using it as carbon source might have enhance the induction of the xylanase and related enzymes. It has been also shown that the hemicellulase activity could be induced by polysaccharides, which are not directly related to its metabolism. As an example, Fer et al. (2012) demonstrated that *P. carrageenovora* showed activities against konjac GlcM, wheat and rye AX when the strain was grown in a culture medium complemented with carrageenan, which has no structural or physiological link with hemicellulases. Therefore one can hypothesized that the suitable polysaccharide inducers had not been identified in our study of *C. crescentus* CB15, *Paenibacillus* sp. and *C. algicola*.

Activity and stability of microbial hemicellulases are greatly influenced by the pH. In general, optimum pH for activity of most of the bacterial hemicellulases has been reported in the neutral pH range (Gupta et al. 2000; Kim et al. 2011; Mou et al. 2011). However, alkalophilic hemicellulases exhibit pH optima around 9 (Gessesse et al. 1998; He et al. 2008), whereas acidophilic hemicellulases show an optimum around pH 4 (Rashid et al. 2010; Mohamad et al. 2011). In our study, the glucan- and GlcM-degrading activity, likely β-glucanase from *P. atlantica*, was observed at both slightly acidic and alkaline pH, whereas the

same activity from *C. crescentus* was observed only at alkaline pH. So, activity and/or stability of enzymes in pH used for *Paenibacillus* sp. and *C. algicola* have to be further investigated.

Apart induction, growth and pH of the culture media, the production of enzymes was shown to be greatly influenced by nutritional and physicochemical factors, such as nitrogen sources, inorganic salts, dissolved oxygen concentration, incubation time, temperature, and agitation (Aziz et al. 2008; Moreira and Filho 2008). *In vivo* bacteria find their nutritional elements in their environment thus, it is expected that the physiology and secretion of enzymes is regulated by the environmental conditions. For example, *Paenibacillus* sp. JDR-2 was firstly isolated from fresh cut disks of sweet gum stem wood while incubated below the soil surface in sweet gum stand (John et al. 2006). Its ability to use effectively sweet gum methylglucuronoxylan in the medium confirmed the important role of environmental condition for the expression of specific activities. On the other hand, bacteria usually live in consortia where they are facing to dynamic changes of nutrient profile due to either environmental or metabolic causes. Thus presence of the complementary partners is required for growing and producing their degrading activities (Moons et al. 2009).

Apart from these drawbacks, the approach used in this study allowed finding novel hemicellulases from unexplored bacteria. To date, this is the first report of extracellular extracts from *P. atlantica* highly active on xylan and cellulose-like backbones, in agreement with the genomic-predicted enzymes. Similarly, extracellular extracts from *C. crescentus* showed activity on GgM, XyG and AX backbones predicted from the genome exploration. This genome data mining approach can further be used for obtaining enzymatic consortia from any organism by capitalizing on the polysaccharides used as inducers. Furthermore this study paves the way to purify these enzymes and analyse their modes of action, and further use them as new analytical tools to investigate in depth the hemicellulose fine structure /function relationship in cell wall.

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Chapter IV

New progress on hemicelluloses fine structure through the use of β-glucanase and β-xylanase from *Pseudoalteromonas atlantica* T6c

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In this chapter the secretome of the marine bacterium *Pseudoalteromonas atlantica* T6c was fractionated by ion-exchange chromatography to isolate endo-β-glucanase and endo-β-xylanase activities previously detected through a screening test. The mode of action of the enzymes was assessed on hemicellulosic substrates of different origins coupled to high performance anion exchange chromatographic analysis of the oligosaccharides released. The β-glucanase degraded glucose-rich polysaccharides, such as xyloglucan, galactoglucomannan and glucomannan from apple, tomato and konjac and thus demonstrated some degree of specificity for highly branched glucans. Similarly, the β-xylanase hydrolyzed various xylans from beechwood, wheat, tomato and apple and produced xylooligosaccharides, together with more complex and substituted oligosaccharides. The presence of β-xylosidase and α-arabinofuranosidase was revealed for the first time in this strain. The isolated proteins degraded efficiently *o*-NP β-D-xylopyranoside and *p*-NP α-L-arabinofuranoside, respectively. Taken together, *P. atlantica* T6c produces a promising enzymatic consortium for the degradation of complex hemicelluloses that can be useful to reveal new insights on the complexity of these polysaccharides from different origin.

Keywords: fractionation, β-xylanase, β-glucanase, HPAEC, xylooligosaccharides, glucanoligosaccharides

IV.1. Introduction

Hemicelluloses refer to as the non-cellulosic cell-wall polysaccharides of plant cell wall and represent an immense renewable resource of biopolymers. Although they share a β-linked sugar backbone, they occur in a large variety of structural types. The hemicellulosic polysaccharides comprise xylans, mannans and xyloglucans, where the backbone sugars are D-xylose, D-mannose and D-glucose, respectively. Those main chains are further substituted by a variety of sugars and acetyl groups depending on the source (Albersheim et al., 2011). Xylan is a heterogeneous polysaccharide consisting of a backbone of β-1,4 linked D-xylopyranosyl units carrying short side-chains mostly consisting of *O*-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl and/or 4-*O*-methyl glucuronyl residues (Scheller & Ulvskov, 2010). Heteromannan contains β-1,4- linked mannopyranose or mixture of β-1,4-linked mannopyranose and glucopyranose as the main backbone which can be further substituted, most often by galactose residues and acetyl esterifications (Petkowicz et al., 2001). Xyloglucans (XyG) have a glucan backbone composed of up to 75% of the 1,4-linked β-D-glucosyl residues substituted at *O*-6 with mono-, di-, or triglycosyl side-chains through a α-D-xylosyl unit (Scheller & Ulvskov, 2010). The xylose residues at *O*-2 generally carry different substituents like β-arabinose and/or α-galactose and/or α-L-fucosylated galactose depending on the origin. They present a large diversity in the composition and in the distribution of repeating structural patterns. To date, the partial knowledge on hemicelluloses structure does not allow establishing clear relationships between their structure and their functions *in planta*.

Out of different analytical tools existing to date, the coupling of enzymatic degradation and chemical analyses of the degradation products provides an essential tool to solve the fine structure of these complex macromolecules. Degradation of hemicellulose backbone involves β-xylanases, β-glucanases, xyloglucanases and β-mannanases. Auxiliary enzymes remove side chain decorations and include mainly α-arabinofuranosidase, α-galactosidase, β-mannosidase, β-glucosidase, α-xylosidase, β-galactosidase, α-fucosidase, β-xylosidase, acetyl esterase. The β-xylanase (EC 3.2.1.8) cleaves the β-1,4-glycosidic bond between xylose residues to release xylooligosaccharides. Most of the β-1,4-xylanases are grouped into 6 glycoside hydrolases (GH) families: GHs 5, 8, 10, 11, 30 and 43 (www.cazy.org) (Shallom & Shoham, 2003). Currently, the best studied β-1,4-xylanases belong to families GH10 and GH11. Several literature studies (Kolenova et al., 2006; Berrin

& Juge, 2008; Zhang et al., 2011) showed that GH10 and GH11 enzymes showed different substrate specificity while incubated with branched xylan. The β-glucanase can cleave (1-4)-β-glycosidic linkages in a variety of substrates such as cellulose, carboxymethyl cellulose (CMC), (1-3)(1-4)-β-D-glucan, glucomannan (GlcM), galactoglucomannan (GgM). Furthermore, it has been long recognized that certain cellulases, namely endo-β-(1–4)-glucanases (EC 3.2.1.4), can catalyze the hydrolysis of XyGs because of the homology of the backbone with cellulose (Vincken et al., 1997). Presently β-glucanases belong to 17 GH families, namely families GH5, 6, 7, 8, 9, 12, 16, 17, 44, 45, 48, 51, 55, 61, 74, 81 and 124 (www.cazy.org). The use of the very narrow specificity of enzymes will allow producing oligosaccharides, which structure can be further analysed by chromatography, mass spectrometry or nuclear magnetic resonance spectroscopic techniques. In that aim, endo-acting enzymes play an important role in the structural elucidation of these complex macromolecules. However, the complexity of the structure of the hemicelluloses and the high substrate specificity of enzymes may compel us to find new enzymes in order to investigate motives not revealed to date.

Micro-organisms are a source of multiple β-xylanases (Tsenga et al., 2002; Ninawe et al., 2008; Sanghi et al., 2010; Sharma et al., 2013) as well as β-glucanases (Akiba et al., 1995; Mawadza et al., 2000; Yin et al., 2010; Vijayaraghavani & Vincent, 2012) that differ by their physicochemical and catalytic properties. Several marine bacterial genomes contains numerous uncharacterized GH sequences, some of which are hypothesized or were shown to be active on higher plant cell wall hemicelluloses presenting structural analogies to marine algal glucan, xylan or mannan polysaccharides. In numerous studies (Tamaru et al., 1995; Politz et al., 2000; Hung et al., 2011; Prasad & Sethi, 2013) new specific enzymes depolymerising hemicelluloses were observed from marine bacteria. The annotated genomic sequence (Copeland et al., 2006) of *P. atlantica* T6c strain suggested the presence of different sequences such as putative β-glucanase (Palt_1404), β-xylanase (Palt_2657), glycoside hydrolase 8 (Palt_0810) (http://www.cazy.org) and other auxiliary activities belonging to GH families 2, 3, 29, 36, 42 and 43. Our previous work (Chapter III) confirmed the presence of different hemicellulases in this bacterium by observing the degradation of various hemicelluloses by its extracellular extracts prepared with or different polysaccharides such as konjac GlcM, beechwood GuX, mixture of CMC, tara GalM and beechwood GuX. The present study aimed at isolating β-xylanase and β-glucanase activities and characterizing their mode of action on different hemicellulosic substrates. Analysis of the reaction products by high performance anion exchange chromatography (HPAEC) was realized to gain new

insights of hemicelluloses fine structures. Two glycosidases, β-xylosidase and α-arabinofuranosidase, were also revealed for the first time to our knowledge in the *P. atlantica*s secretom.

IV.2. Material and methods

IV.2.1. Selection and preparation of polysaccharide substrates

Twelve different terrestrial hemicelluloses of diverse structures and origins (Table IV-1) and 8 different nitrophenyl-sugar derivatives were used as substrates. All the substrates were dissolved in deionized water. The polysaccharides were dissolved at 0.4% w/v, and the sugar derivatives at 4 mM. The solutions were kept in -20 °C before use.

Table IV-1: Hemicelluloses and sugar derivatives used as substrates for the screening of hemicellulases present in the different bacterial extracts of *Pseudoalteromonas atlantica* T6c.

			Sugar composition (% weight)							
Substrate	**Biological origin**	**Source**	**Rha**	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**UA**
Xylan	Beechwood	Sigma								
Arabinoxylan (AX)	Wheat	INRA	0.0	0.0	28.4	53.9	0.2	0.7	0.4	0.0
Glucuronoxylan (GuX)	Tomato	Assor et al., 2012	0.0	0.0	1.5	73.2	0.0	6.7	0.0	8.3
Glucuronoarabinoxylan (GAX)	Apple	Ray et al., 2014	0.2	1.1	0.0	14.9	44.5	1.6	7.9	11.2
Glucomannan (GlcM)	Konjac powder	INRA	0.0	0.0	0.5	0.0	52.6	0.3	30.6	0.0
Galactoglucomannan (GgM; fraction E3)	Apple	Ray et al., 2014	0.6	2.7	5.4	10.6	20.5	9.1	28.9	6.4
Galactoglucomannan (GgM; fraction A1)	Apple	Ray et al., 2014	2.3	0.5	3.3	4.4	29.5	19.4	27.8	1.4
Galactoglucomannan (GgM)	Tomato	INRA	0.8	0.0	0.8	4.7	31.0	14.2	30.3	1.8
Acetylated xyloglucan (XyG; fraction E1)	Apple	Ray et al., 2014	1.3	4.9	6.8	19.0	1.3	11.1	34.5	12.6
Xyloglucan (XyG; fraction HC4)	Apple	Ray et al., 2014	0.4	1.9	2.3	8.1	0.8	4.9	12.9	4.4
Xyloglucan (XyG; 4MK)	Tomato	Assor et al., 2012	0.0	0.0	6.9	13.0	0.0	16.8	25.8	3.5
CM-Cellulose		Sigma								
***o*-NP β-D-xylopyranoside**		Sigma								
***p*-NP α-L-arabinofuranoside**		Sigma								
***p*-NP α-D-galactopyranoside**		Sigma								
***p*-NP β-D-mannopyranoside**		Sigma								
***p*-NP β-D-glucopyranoside**		Sigma								
***p*-NP α-D-xylopyranoside**		Sigma								
***p*-NP β-D-galactopyranoside**		Sigma								
***p*-NP α-L-fucopyranoside**		Sigma								
***p*-NP acetate**		Sigma								

IV.2.2. Bacterial strain stock

A glycerol stock of *Pseudoalteromonas atlantica* T6c strain was used to inoculate10 mL of sterile bactomarine broth (Difco 2216, Fisher, Illkirch, France) culture medium. Bacteria were grown at 18 °C for 36 h prior to the transfer of 0.5 mL of the culture into 1 mL vial containing 0.5 mL of 20% (v/v) glycerol in bactomarine broth and the suspension was kept in -80 °C for further use.

IV.2.3. Bacteria growth kinetics

Two pre-cultures of *P. atlantica* were carried out by inoculating frozen cells (0.5 mL) in 10 mL of sterile bactomarine broth culture medium in a 100 mL Erlenmeyer flask at 12 h interval. After 36 h incubation at 20 °C and shaking (180 rpm, New Brunswick incubator), 1 mL of culture was inoculated in 250 mL of fresh medium. Growth of the bacteria was monitored every hour for 48 h by its absorbance at 600 nm.

Growth kinetics was also followed with Konjac GlcM or Beechwood glucuronoxylan (GuX) added at 1 $g.L^{-1}$ as inducer.

IV.2.4. Preparation of the bacterial extract

A preculture of *P. atlantica* was carried out in 10 mL of sterile bactomarine broth culture medium. After 16 h incubation at the 18°C with shaking as above, 1 mL of the culture was inoculated in 50 mL of fresh medium and incubated for 8 h in similar conditions. Then, 50 mL were inoculated in 1 L of fresh medium in 5 L shaker flask in the same conditions. After 16 h, the culture was centrifuged at 5000 × g for 30 min at 4°C and culture medium (named as extract **P**) was separated from the cell pellet.

Similarly bacterial extracts were obtained by culturing the bacteria in sterile bactomarine broth culture medium containing konjac GlcM (extract **K**) or beechwood GuX (extract **B**) at 1 $g.L^{-1}$ for 18 and 16 h, respectively.

IV.2.5. Fractionation of bacterial extract

IV.2.5.1. Filtration

The cell free culture supernatants were filtered overnight on a 300 kDa membrane (PES, Millipore, Billerica, MA, USA) using a 400 mL Amicon system 8400 (Millipore). Afterwards, the filtrates were diafiltered and concentrated to 20 mL, with deionized water on a 10 kDa membrane (PES, Millipore) under compressed air (0.5 bar) to remove molecules smaller than the membrane cut off. The concentrated filtrates were separated into 2 x 10 mL and the pH of each half was adjusted at 8.5 or 5.2 by adding 100 mM Tris-HCl or 100 mM acetate buffer, respectively. By this way, 6 culture supernatants were obtained: **P** at pH 8.5 and 5.2, **K** at pH 8.5 and 5.2, and **B** at pH 8.5 and 5.2. All were stored at 4°C before use.

IV.2.5.2. Anion exchange chromatography (AEC)

Crude extracts **K** and **B** were fractionated on a Mini Q^{TM} column (4.6 x 50 mm, GE Healthcare, Uppsala, Sweden) installed on an AKTA purifier (GE Healthcare) and equilibrated with 50 mM Tris-HCl buffer at pH 8.5. Elution was performed at a flow rate of 0.8 mL min^{-1} with 50 mM Tris-HCl buffer pH 8.5 (7 column volumes) to recover the unbound fraction. Bound proteins were eluted using a gradient on 10 column volumes from 35 to 75% of NaCl 1 M in 50 mM Tris-HCl buffer pH 8.5. The fractions of interest were dialyzed through a 10 kDa cut off dialysis tube (Nanosep 10K Omega, Pall Life Science, USA) against 50 mM Tris-HCl buffer at pH 8.5, adjusted to a final volume of 3 mL and stored at 4 °C.

IV.2.6. Characterisation of protein fractions

IV.2.6.1. Protein assay

The concentration of protein was determined in a microplate by adding 200 µL of sample to 50 µL of the Bradford reagent (Bio-Rad, Marnes-la-Coquette, France) (Bradford, 1976). Bovine serum albumin (Sigma) was used as a standard (0-25 µg.mL^{-1}) and the optical density was read at 600 nm on a microplate reader (Multiskan Ex, Thermo Electron Corporation, St Herblain, France).

IV.2.6.2. Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions by treating the sample with β-mercaptoethanol in a Apelex (Massy, France) tank. The gel was prepared using 12% (w/v) polyacrylamide solution with Tris-glycine buffer (pH 8.5) containing 0.1% (w/v) SDS. The resolved protein bands were visualized after staining with 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol, acetic acid, and distillated water (5:1:5, v/v/v). The standard molecular mass markers were used in the range of 10-170 kDa (Fermentus Inc., Boston, USA).

IV.2.7. Enzymatic assays

Enzymatic activities towards polysaccharides were investigated by Somogyi-Nelson method (Nelson, 1944) adapted to microplate (Sturgeon, 1990). Two hundred µL of polysaccharide solution were incubated overnight with 200 µL of bacterial extract or protein fraction. After withdrawing 100 µL aliquote, the enzymatic reaction was stopped by adding 100 µL of the Somogyi copper reagent and boiling in a water bath for 15 min. Then it was cooled to room temperature and 100 µL Nelson arseniomolybdate reagent was added. The mixture was agitated for 10 min after adding 1 mL water. The amount of reducing ends was revealed by reading the absorbance at 600 nm of 250 µL of the mixture in a microplate reader (Multiskan Ex). The amount of reducing ends was expressed in $\mu L.mL^{-1}$ unit using standard curve prepared with appropriate sugar. Controls were prepared similarly with bacterial extracts and polysaccharide solutions.

Exo-activities were assayed by incubating 100 µL bacterial extracts with 100 µL 4 mM *para*-nitrophenyl (*p*-NP) derivatives of α-L-arabinofuranoside, α-D-galactopyranoside, β-D-mannopyranoside, β-D-glucopyranoside, α-D-xylopyranoside, β-D-galactopyranoside, α-L-fucopyranoside, acetate and *ortho*-nitrophenyl (*o*-NP) β-D-xylopyranoside for 16h at 40°C. The incubation was stopped by adding 0.6 mL of 1 M sodium bicarbonate solution. The occurrence of nitro-phenol was revealed by reading the absorbance of 250 µL of the samples in a microplate reader at 405 nm. The absorbance was compared to a *p*-nitrophenol or *o*-nitrophenol standard curve accordance to the substrate, and the liberation of nitro-phenol was reported as $nmole.mL^{-1}$. Control bacterial extracts and sugar derivatives solutions were prepared similarly.

IV.2.8. Analysis of oligosaccharides by HPAEC

XyG and GgM oligosaccharides (XyGOs and GgMOs, respectively) were chromatographed on a Carbo-Pac PA 200 column (3 x 250 mm, Dionex, Sunnyvale, USA), whereas xylan oligosaccharides (XOs) were analysed on a Carbo-Pac PA 1 column (2 x 250 mm, Dionex, Sunnyvale, USA) thermostated at 30 °C. Elution was conducted using a linear gradient of sodium acetate from 0 to 170 mM in 100 mM NaOH (Quemener et al., 2007) and monitored by amperometry using an ED electrochemical detector (Dionex, Sunnyvale, USA).

IV.3. Results and discussion

IV.3.1. Growth kinetics

In vivo enzyme production is greatly associated to growth of the micro-organism. Composition of culture medium, dissolved oxygen concentration, temperature, pH and agitation are important parameters that determine growth rates of microorganisms and hence enzyme production (Aziz et al., 2008; Moreira & Filho, 2008; Ahamed & Vermette, 2009).

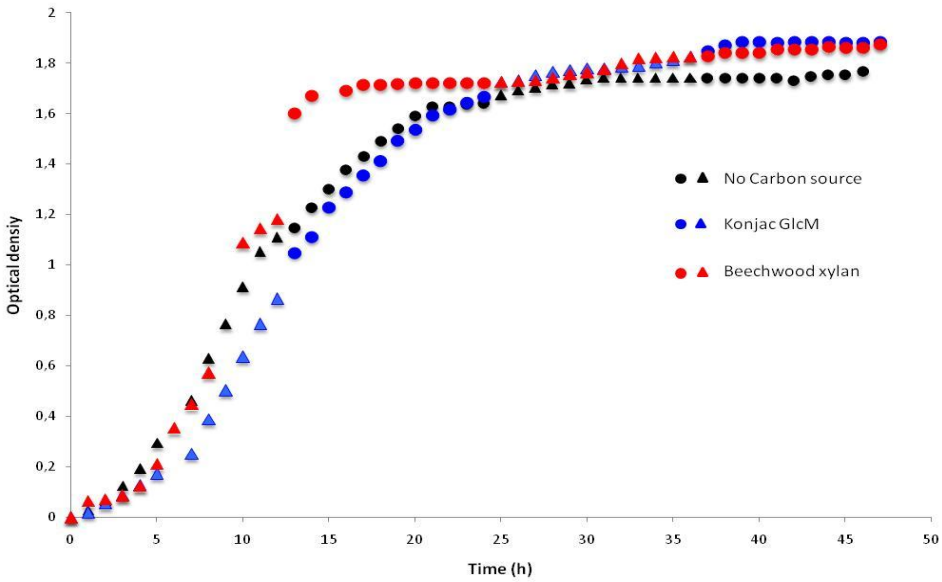

Figure IV-1: *P. atlantica* growth in the absence (black markers) and in the presence of konjac GlcM (blue markers) and beechwood GuX (red markers).

Growth of *P. atlantica* without any inducer was followed over 48 h (Figure IV-1). Growth was slow for the first 5 h and increased rapidly to reach a plateau after 20 h incubation. Addition of polysaccharide inducers in the culture medium was expected to boost of enzymatic production (El-Helow et al., 1997; Ferreira & Filho, 2004; Gomes et al., 2007; Rashid et al., 2010; Kim et al., 2011ab). Following our previous study (Chapter III), konjac GlcM and beechwood xylan were selected as added polysaccharides in the culture medium (Figure IV-1). However, konjac GlcM induced no significant change in the bacterial growth in comparison to culture in the absence of carbon source. Conversely beechwood GuX induced stiffer bacterial growth from 10 to 20 h. This study revealed that beechwood GuX positively affected the growth rate of *P. atlantica*.

IV.3.2. Preparation of bacterial extracts

Following the kinetics study, bacteria were grown either without added polysaccharides, or in the presence of konjac GlcM or beechwood GuX to produce the respective bacterial extracts **P**, **K** and **B**. During the culture without added inducer, bacteria metabolize their own sugars. On the contrary external carbohydrates, here konjac GlcM and beechwood GuX, provide the energy source when added, but also release poly- and oligosaccharides in the culture medium. For this reason filtration steps were carried out. 300 kDa filtration eliminated only 2 to 10% of the reducing ends initially present in the extract, but dramatically improved the sensitivity and the reproducibility of the colorimetric assay by decreasing the reducing compounds produced in the bacterial extracts (Fer at al., 2012). Furthermore, the 10 kDa filtration was absolutely necessary to remove the large excess of lower molecular weight reducing compounds in bacterial extracts. Bacterial extracts were tested for enzymatic activity on 12 different hemicelluloses and 8 nitrophenyl-derivatives at pH 5.2 and 8.5 to identify the secreted activities (Figure IV-2).

P extract (Figure IV-2A) and **K** extract (Figure IV-2C) exhibited roughly similar activity profiles. Both showed the highest activity on significantly substituted and/or unsubstituted glucan and glucomannan at both pH. On the contrary, pH had a more intense effect on heteroxylan degradation, whatever the polysaccharide source. In agreement with our previous study (Chapter III), these activities towards glucose-rich polysaccharides and xylan backbone could be related to the presence of genes annotated as β-glucanase (Palt_1404) and β-xylanases (Palt_2657), respectively. **P** and **K** extracts at both pH showed for the first time activity towards *p*-NP α-L-arabinofuranoside, *p*-NP acetate and *o*-NP β-D-xylopyranoside but

the exo-activities were predominant at pH 5.2 (Figure IV-2B, D). The first two activities could be explained by the presence of genes annotated as two putative α-arabinofuranosidases (Palt_0842 and Palt_0823), five putative polysaccharide acetyl esterases (Palt_2422, Palt_2718 fragment, Palt_2733, Palt_2734, Palt_4235 and Palt_2308). Although no sequence in the genome of *P. atlantica* was annotated as a β-xylosidase, an activity towards *o*-NP β-D-xylopyranoside was demonstrated.

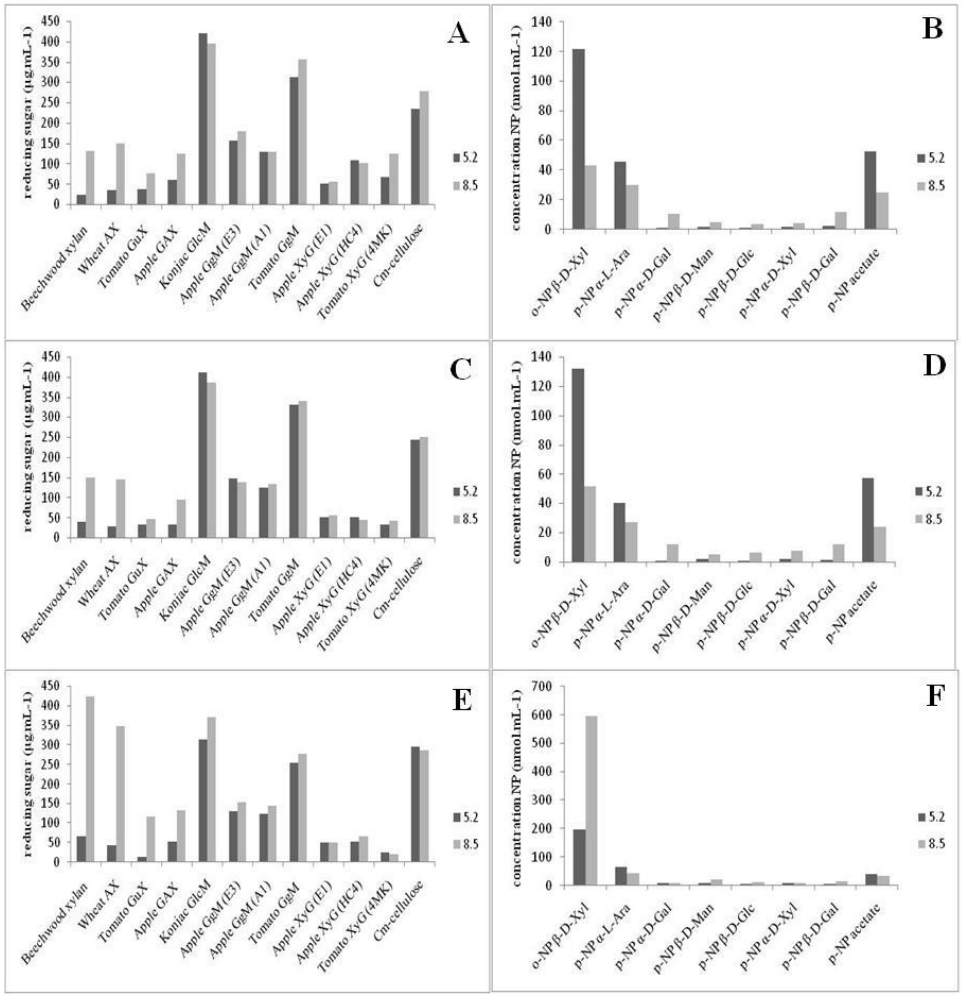

Figure IV-2: Enzymatic activities of P, K and B extracts on hemicelluloses: beechwood and tomato GuX, wheat AX, apple GAX, konjac GlcM, tomato and apple GgMs, XyGs and CMC (A, C, E, respectively) and sugar derivatives: (*p*-NP) derivatives of α-L-arabinofuranoside, α-D-galactopyranoside, β-D-mannopyranoside, β-D-glucopyranoside, α-D-xylopyranoside, β-D-galactopyranoside, α-L-fucopyranoside, acetate and (*o*-NP) β-D-xylopyranoside (B, D, F, respectively).

P. atlantica genome contained GH8-like protein (Palt_1069), and GH8 includes β-glucanase, β-xylanase, chitosanase, licheninase and reducing-end-xylose releasing exo-

oligoxylanase. The genome also contained two GH3-like protein sequences with unknown functions (Palt_3730, Palt_4162). As GH3 includes β-xylosidase, α-arabinofuranosidase and β-glucosidase, there is two possible explanations for this β-xylosidase activity, either the GH8 reducing-end-xylose releasing exo-oligoxylanase, or a GH3 β-xylosidase. In both cases, revisiting the annotation could support potential xylosidase activity. Due to spatial similarity between D-xylopyranose and L-arabinofuranose, the glycosidic bonds and hydroxyl groups can be overlaid, thus several β-xylosidases exhibit bifunctional β-D-xylosidase/α-L-arabinofuranosidase activity (E.C. 3.2.1.37 / 3.2.1.55) (Mai et al., 2000; Lee et al., 2003; Jordan & Li, 2007). Many characterised GH43 enzymes showed also dual-function by accommodating D-xylopyranoseand L-arabinofuranose residues at subsite −1 (Wagschal et al. 2009; Ravanal et al., 2010; Viborg et al., 2013; Zhou et al., 2012). Consequently, the two putative GH43 α-arabinofuranosidases of *P. atlantica* (Palt_0842 and Palt_0823), may exhibit the duel activity.

In contrast, shaving activities, such as α-galactosidase, β-mannosidase, β-glucosidase, α-xylosidase, β-galactosidase that remove decorations from XyG and GgM,were not detected in the bacterial extracts (Figure IV-2B, 2D), thus explaining the relatively low content in reducing sugars produced from the different XyG (Figure IV-2A, 2C). The β-glucanase detected in the previous work (Chapter III) was confirmed here by the degradation of CMC, XyG, GlcM and GgM. Comparison of Figure IV-2A and IV-2B and Figure IV-2C and IV-2D demonstrated that addition of konjac GlcM in the culture medium did not improve production of hemicellulases as well as glycosidases. This result agreed with the growth kinetics study, which showed that konjac GgM did not significantly enhance the bacterial growth. Altogether, this suggests that konjac GlcM was not a suitable inducer either for the degradation of glucan-based polysaccharides (CMC, XyG, GlcM or GgM), or for exo-activities required for side chain release.

Addition of beechwood GuX in the culture medium induced a large increase of heteroxylan degrading enzymes by the bacterial extract **B** at pH 8.5, but these activities were lost at pH 5.2 (Figure IV-2E). This behaviour is similar to the GH10 β-xylanase from a marine bacterium, *Thermoanerobacterium saccharolyticum* NTOU1 that showed low activity around pH 5 and an appreciable activity at pH 8 (Hung et al., 2011). Similarly, **B** showed increased activities on *o*-NP β-D-xylopyranoside at pH 8.5. Thus, beechwood GuX is an inducer of β-xylanase, β-xylosidase in *P. atlantica*. This result is in line with several studies showing that xylanases were secreted in media containing xylan or xylan-rich residues like birchwood xylan (Tsenga et al., 2002; Sharma et al., 2013) and wheat bran (Ninawe et al.,

2008; Sanghi et al., 2010).

IV.3.3. Fractionation of bacterial extracts

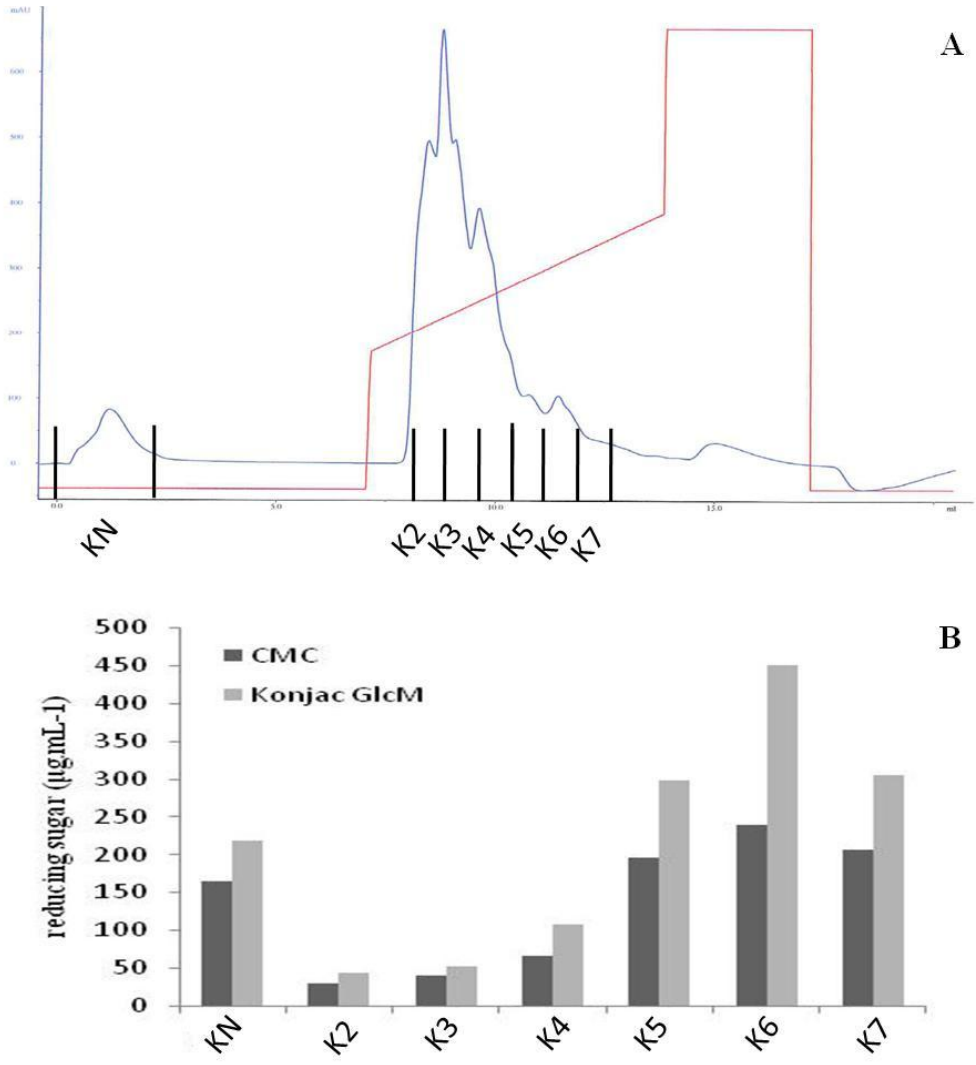

Figure IV-3: Elution profile on anion exchange chromatography of the fraction K at pH 8.5 (A). The glucanase activity on CMC and Konjac GlcM in the eluted fractions was measured by reducing power assay (B).

Bacterial extract **K** at pH 8.5 having protein concentration 1.2 $mg.mL^{-1}$ was fractionated by ion exchange chromatography into one neutral fraction (**KN**) and 6 different populations of retained proteins (**K2**, **K3**, **K4, K5, K6** and **K7**; Figure IV-3A) eluted in the 0 to 1 M NaCl gradient. The β-glucanase activity against konjac GlcM and CMC degradation was assessed in all the fractions collected (Figure IV-3B). The result suggested that **KN** contained appreciable amount of activity towards these two polysaccharides. Among the

bound fractions, the activity was present in **K4** to **K7** and the activity was higher towards GlcM than towards CMC.

The molecular weights of the proteins present in these fractions were checked by SDS-PAGE (Figure IV-4). The second major band in **K** corresponded to a molecular weight around 45 kDa and was found in the fractions **K5** to **K7**. It could correspond to the calculated molecular weight of the amino acid sequence of the annotated gene of β-glucanase.

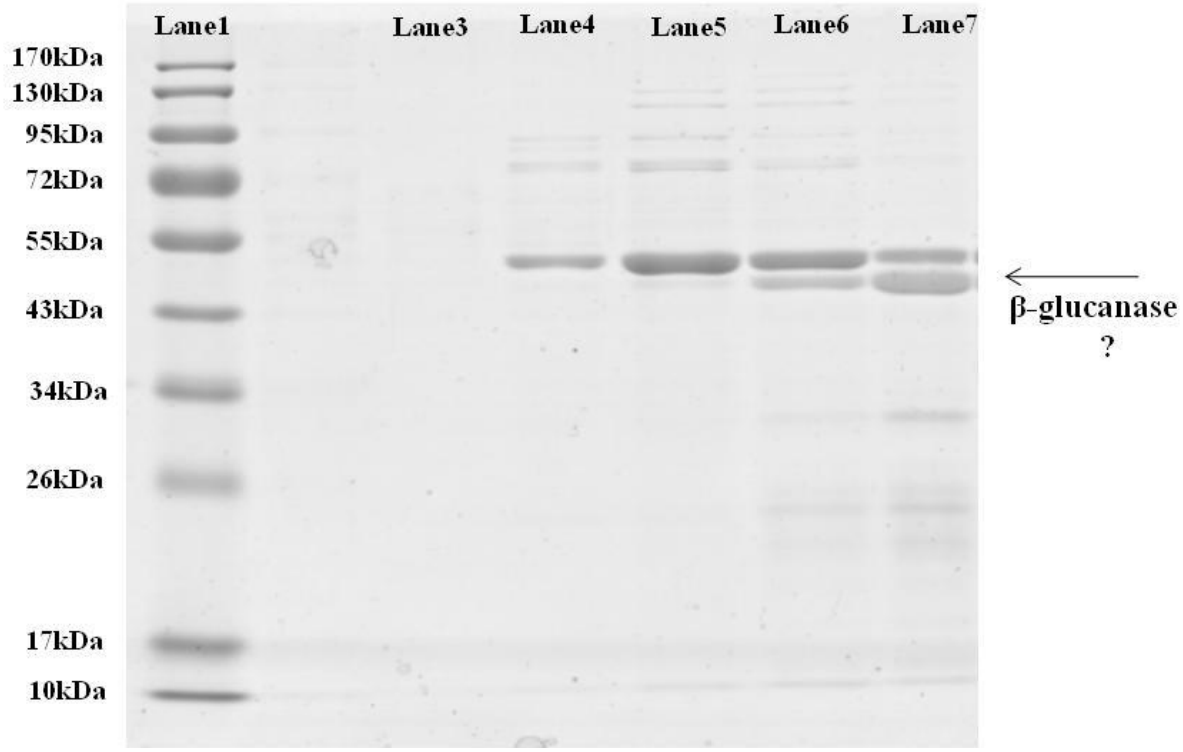

Figure IV-4: SDS-PAGE of the bacterial extract K at pH 8.5: molecular weight markers (lane 1), fraction K3 (lane 3), fraction K4 (lane 4), fraction K5 (lane 5), fraction K6 (lane 6) and fraction K7 (lane 7).

Similarly fraction **B** at pH 8.5 was chromatographed into one neutral fraction (**BN**) and 12 different fractions eluted in the NaCl gradient (**B2** to **B13**; Figure IV-5A). The hemicellulases activities in the collected fractions were evaluated on konjac GlcM, CMC, beechwood GuX and wheat AX substrates. The colorimetric results (Figure IV-5B) suggested that fractions **B4** to **B7** only degraded konjac GlcM and CMC. The gel electrophoresis of these fractions showed one band near 45 kDa (Figure IV-6) that was similar to that of **K4** to **K7** fractions obtained from konjac GlcM induction (Figure IV-4).

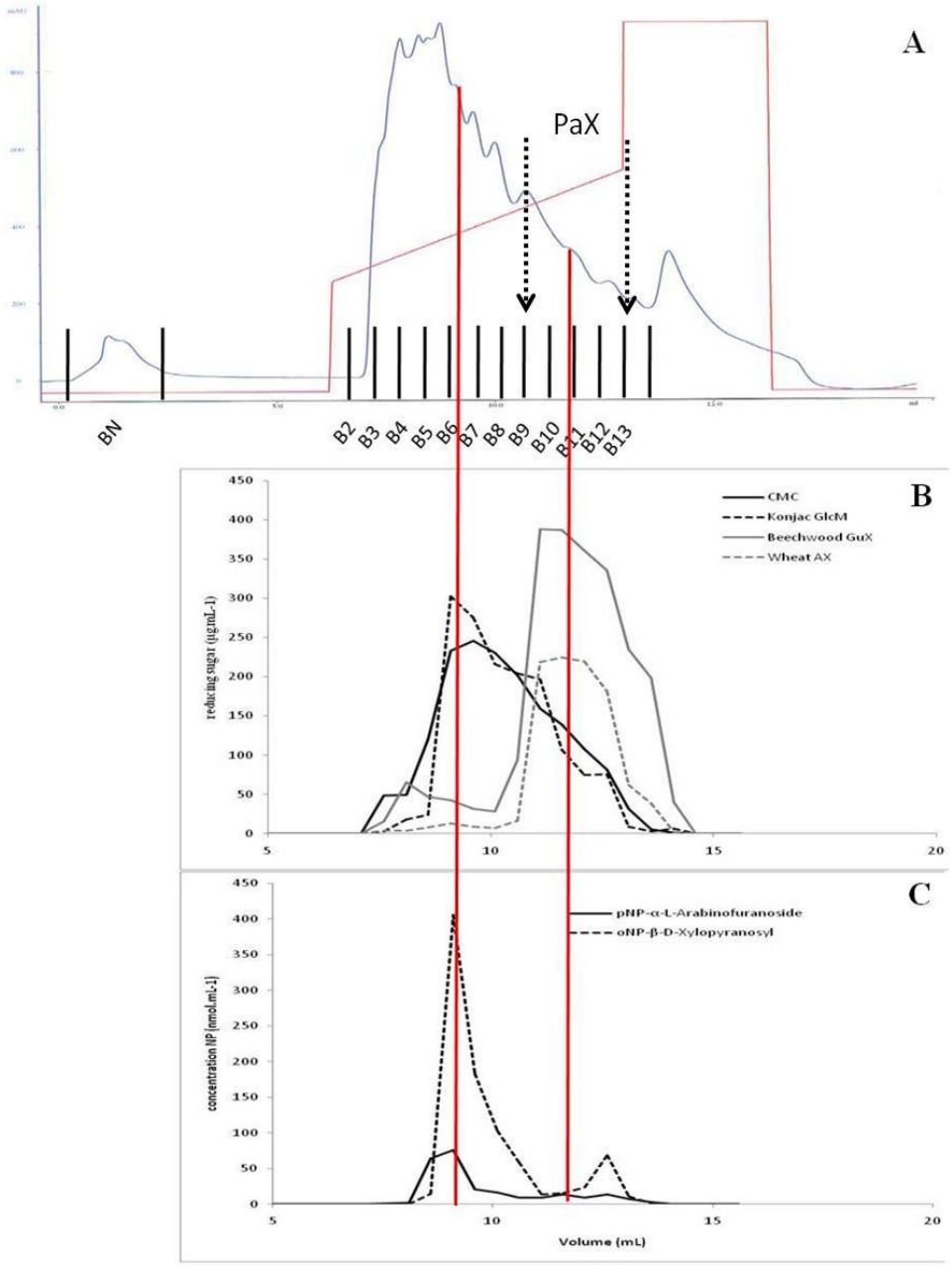

Figure IV-5: Elution profile on anion exchange chromatography of the fraction B at pH 8.5 (A). The glucanase activity on: CMC, konjac GlcM, xylanase activity on: beechwood GuX, wheat AX (B) and osidase activities on: *o*-NP β-D-xylopyranoside, *p*-NP α-L-arabinofuranoside (C) in the eluted fractions were measured by the reducing power assay.

The beechwood GuX and wheat AX degradation mainly occurred from **B8** to **B14**. The calculated molecular weight of the putative GH10 β-xylanase (Palt_2657) was 42 kDa, close to the putative β-glucanase molecular weight (45 kDa). Only one band was observed on the SDS gel around this molecular weight. However, especially in **B7** and **B8**, the band was quite large and could correspond to the co-migration of the two proteins. Taking together the

activity profile of the chromatography (Fig IV-5B, 5C) and the SDS gel, the same band could correspond to the β-glucanase in the earlier fractions (**B5**-**B6**), to a mixture of β-glucanase and β-xylanase in the intermediate fractions (**B7**-**B8**) and to the β-xylanase in the later fractions (**B9** to **B12**). Another band around 96 kDa appeared from the fraction **B9** to **B12** and could correspond to the GH8 protein (Patl_1069). It is thought to be a β-xylanase and/or a reducing-end-xylose releasing exo-oligoxylanase. Fractions **B9** to **B12** were grouped together and further called as **PaX**.

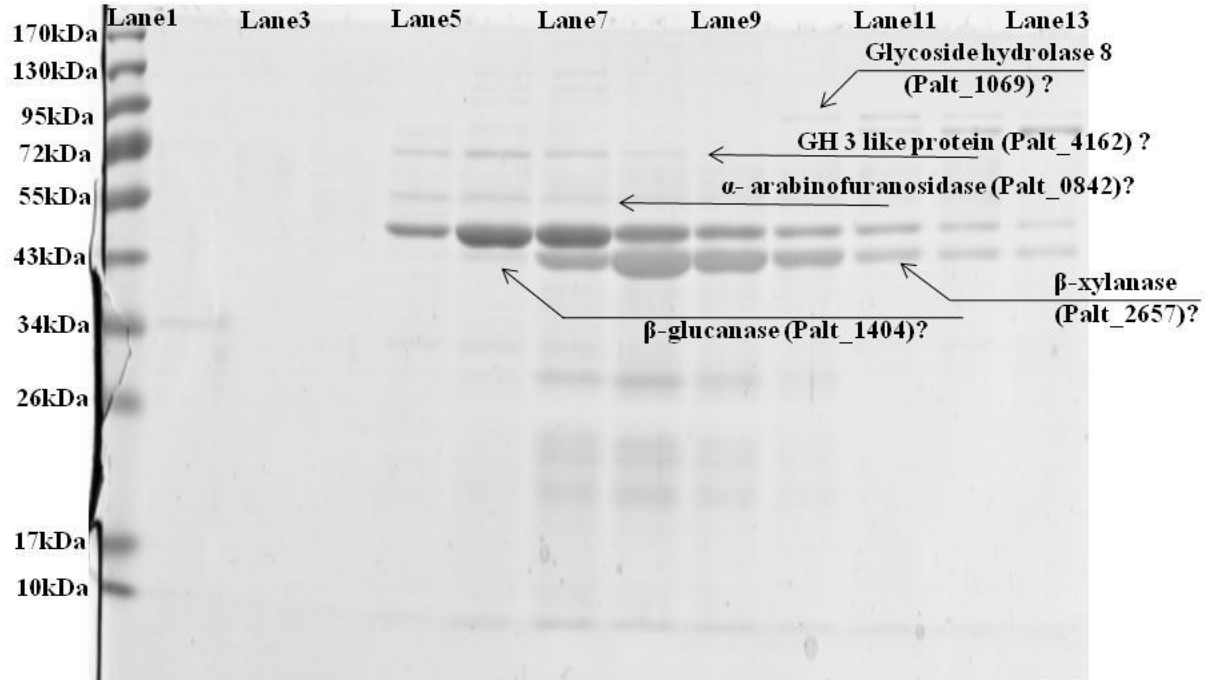

Figure IV-6: SDS-PAGE of bacterial extract B at pH 8.5: molecular weight markers (lane 1), fraction B2 (lane 3), fraction B3 (lane 4), fraction B4 (lane 5), fraction B5 (lane 6), fraction B6 (lane 7), fraction B7 (lane 8), fraction B8 (lane 9), fraction B9 (lane 10), fraction B10 (lane 11), fraction B11 (lane 12) and fraction B12 (lane 13).

Profiling β-xylosidase and α-arabinofuranosidase activities in all the collected fractions (Figure IV-5C) revealed high activity towards *o*-NP β-D-xylopyranoside on the one hand in **B6** and **B7,** and on the other hand in **B12**. α-arabinofuranosidase activity was only demonstrated in **B5** and **B6**, but at a much lower extent. Depending on the reports, β-xylosidase / α-arabinofuranosidase bifunctional enzymes may have similar or highly different activities towards the two substrates (Wagschal et al., 2009; Ravanal et al., 2010; Zhou et al., 2012; Viborg et al., 2013). Therefore, it was not possible to assess if the activities found in **B6**

were due to one or two proteins. Moreover, a band potentially corresponding to GH3-like protein Palt_4162 was observed in fractions **B4** to **B7** and could correspond to a β-xylosidase. In the same way, the SDS-PAGE of these fractions contained a band around 58 kDa that could be attributed to the molecular weight calculated for GH43 (Palt_0842) putative α-arabinofuranosidase.

IV.3.4. Characterisation of oligosaccharides

The bacterial extract **P** at pH 5.2 contained β-glucanase with other enzymes like acetylesterase, β-xylosidase and α-arabinofuranosidase (Figure IV-2A, 2B) that were not involved in the degradation of glucan backbones. The extract incubated in the presence of apple XyG and GgM released oligosaccharides that were analysed by HPAEC. Compared to the control chromatograms of bacterial extract and polysaccharide alone (not shown), area of peak at 4.6, 10.6, 11.4, 12.7 and 18.6 min increased and confirmed the enzymatic degradation of acetylated apple XyG **E1** (Figure IV-7A). The non acetylated apple XyG population (**HC4**) was similarly degraded and produced oligosaccharides with retention times 3.6, 4.3, 4.6, 12.1, 12.9, 13.6, 13.9, 14.4, 14.8 and 17.2 min. (Figure IV-7B). The HPAEC elution pattern of the two XyG fractions using commercial β-glucanase (EGII from *Trichoderma longibrachiatum*) revealed the presence of **XXXG**, **XXFG**, **XLXG** and **XLFG** building blocks with retention time 13.5, 13.8, 14.1, 14.4 min. (Chapter II). As a consequence, comparison of these two different profiles suggested that the **P** extract proceeded to a degradation of apple XyG in a different way than EGII. The colorimetric assay revealed that **P** did not contain any α-xylosidase, β-galactosidase and β-glucosidase (Figure IV-2B) but α-fucosidase activity was not measured. The genome sequence predicts the presence of a GH29 enzyme annotated as an α-fucosidase (Patl_0831).

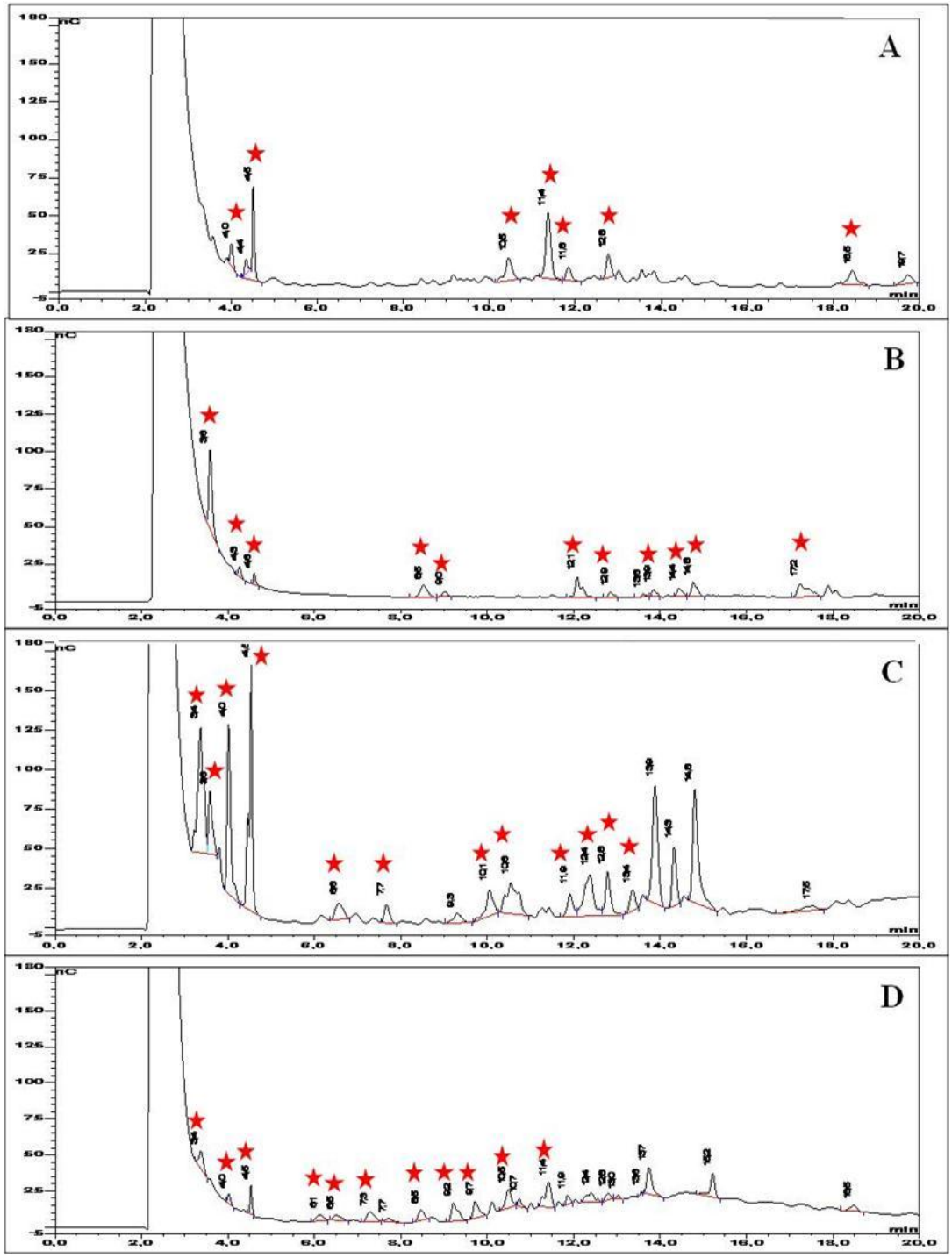

Figure IV-7: HPAEC analysis of enzymatic digests of hemicellulose fraction: apple acetylated XyG E1 (A), nonacetylated XyG HC4 (B), GgM A1 (C) and GgM E3 (D) by bacterial extract P at 5.2. The red stars indicate the peaks of the newly produced oligosaccharides.

In contrast to the β-glucanase (EGII) (Chapter II), *P. atlantica* β-glucanase released oligomers of different retention times from the two XyG fractions. Further investigations on the structure of these products might be interesting to reveal fine structural difference between

these two XyG populations. Similarly, the *P. atlantica* β-glucanase degradation profiles of the two apple GgM fractions **A1** and **E3** were compared with the controls. They revealed an increase in the area of several peaks noted by a star on Figure IV-7C, 7D, consecutive to the enzymatic hydrolysis. **A1** released preferentially lower retention time oligomers (3.4, 3.6, 4.0, 4.5 min.) in agreement with what was shown in Chapter II. Peaks at 13.9, 14.3 and 14.9 min were attributed to the auto-degradation of polysaccharides during the incubation period (visible on the control chromatogram, data not shown). The presence of small peaks at 10.5, 11.4, 12.8 and 18.5 min in **E3** hydrolyzate can be attributed to the presence of oligoXyGs in addition to others related peaks (3.4, 4.0, 4.5 min.) to oligoGgMs (Chapter II). The high molecular weight oligomannans (6.6, 7.7, 10.1, 10.6, 11.9, 12.4, 12.8, 13.4 min for **A1,** and 6.1, 7.3, 8.5, 9.2, 9.7 min for **E3**) produced by the bacterial extract **P** can be attributed to incomplete enzymatic digestions. However, even if they did not constitute final products from GgM hydrolysis, the characterization of their structures would give new insights on polysaccharide fine structure.

In order to better understand the action of the β-xylanase, beechwood and tomato GuX, wheat AX and apple GAX were incubated with fraction **PaX**, corresponding to the pool of fractions **B8** to **B12** isolated by ion exchange chromatography. The hydrolysis products of these different xylan-based polysaccharides were analysed by HPAEC and compared to the retention time of standard xylose (**X**), xylobiose (**X2**), xylotriose (**X3**), xylotretraose (**X4**) and known oligoheteroxylans (XOs) (Chazal, 2014). The hydrolysis by **PaX** produced mainly **X**, **X2**, **X3**, **X4** and more complex unknown oligosaccharides (star marked peaks) (Figure IV-8). Hydrolyzed apple GAX showed oligosaccharides eluting as **X4U1m1** (4 xylose, 1 uronic acid, 1 methyl), **X5U1m1**, **X6U1m1**, **X5U1**, **X6U1**, and other oligosaccharides eluting later (Figure IV-8A). In tomato GuX incubation medium, only 4 complex oligosaccharides **X6U1m1**, **X4U1**, **X5U1**, **X6U1** were recovered in intermediate amount (Figure IV-8B). Degraded beechwood GuX showed the same peaks, except that X4U1m1 was almost absent, while all the others were in much larger amounts (Figure IV-8C). Teleman et al. (2002) using NMR spectroscopy revealed that beechwood GuX contained one 4-*O*-methylglucuronic acid substituent for approximately 15 D-xylose residues. By revealing the presence of appreciable amount of unsubstituted **X**, **X2**, **X3** and **X4** our study was in complete in agreement with Teleman et al. (2002). In degraded wheat AX several unknown complex oligosaccharides were found to be present (Figure IV-8D).

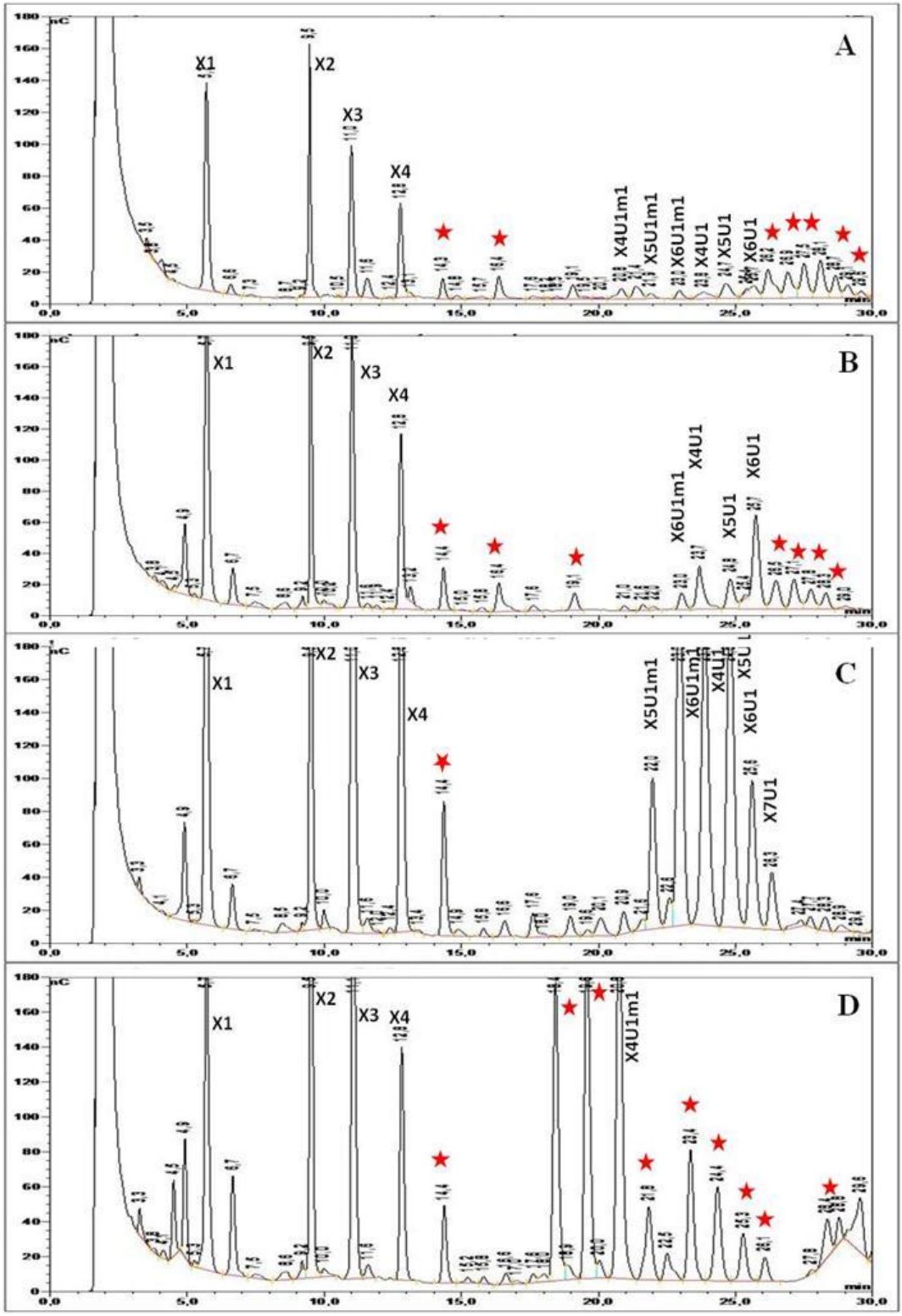

Figure IV-8: HPAEC analysis of hydrolysates on apple GAX (A), tomato and birchwood GuX (B and C), wheat AX (D) by PaX enzyme. X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotretraose; X4U1m1, 4 xylose 1 uronic acid 1 methyl; X5U1, xylose5 uronic acid1 and so on. The red stars indicate the pick unknown and newly produced peaks.

Tseng et al. (2002) purified two xylanases having molecular weights of 45 kDa and 23 kDa from *Bacillus firmus*. The mass spectrometric analysis of birchwood xylan hydrolysate mainly contained **X**, **X3** and **X6**, indicating that these enzymes preferentially degraded internal glycosidic bonds of xylo-oligosaccharides. More recently, Zhang et al. (2011) purified two xylanases from *Nonomuraea flexuosa* (Nf Xyn11A) and from *Thermoascus aurantiacus* (Ta Xyn10A). Their modes of action were investigated by degrading birchwood GuX, insoluble oat spelt AX and analysing the released XOs by HPAEC. Both of them produced **X** and **X2** as the major end products. In addition the authors showed that Nf Xyn11A produced more **X3** than Ta Xyn10A. In our study, the presence of linear xylooligosaccharides could be attributed to incomplete digestion. However they indicate that the initial polysaccharide backbones contained long unsubstitued streches.

IV.4. Conclusion

In the present study, one β-glucanase and one β-xylanases were purified from *P. atlantica* bacterial extracts induced by konjac GlcM and beechwood xylan. The presence of β-xylosidase, α- arabinofuranosidase and acetyl esterase was also observed for the first time in this secretome. To to our knowledge, it is the first β-glucanase obtained from marine bacterium that revealed high catalytic efficiency in degrading different acetylated and/or nonacetylated GgMs and XyGs. The HPAEC profiles of oligosaccharides released from apple XyG highlights the remarkable fine structural diversity between the two populations. The β-xylanase was capable of hydrolyzing various acetylated and/or nonacetylated xylan backbones such as apple GAX, tomato and beechwood GuX, wheat AX. The HPAEC profiles of the hydrolyzates revealed the release of simple sugars as xylose, xylobiose, xylotriose, xylotetraose with other complex xylo-oligosaccharides. This study successfully provided novel insights of xylan complexity. In the future, these enzymes would be powerful new tools to perform the fine structural chemotyping of hemicelluloses and thus paved the way to better understand the function of these polysaccharides in cell wall.

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Chapter V

General discussion and Perspectives

V.1. General discussion

Fleshy fruits softening during ripening involves solubilization, depolymerization and rearrangements of cell wall polysaccharides caused by the action of several cell wall-modifying enzymes and proteins acting on both pectins and hemicelluloses (Brummell, 2006; Goulao & Oliveira, 2008). The modification of pectic domains during this complex biological process has been well studied (Johnston et al., 2002; Brummell, 2006; Goulao & Oliveira, 2008). But, in comparison, little is known about the fate of hemicelluloses apart their possible hydrolysis and transglycosylation assumed to result from XET/XTH, MTH, xylan transglycosylases activities reported during this process (Rose et al., 1997; Brummell et al., 1999; Schröder et al., 2006; Johnston et al., 2013). In tomato, the molecular weight of the tightly bound xyloglucan fraction decreases while the amounts of cellulose and XyG remain constant (Maclachlan & Brady, 1994). The fine structure of their hemicelluloses is also affected during ripening (Lahaye et al., 2012). In apple, Percy et al. (1997) showed changes in the content of the Glc, Man, and Xyl during fruit development, indicating structural changes in hemicelluloses. But the establishment of clear relationship between hemicelluloses structure and fruit texture remains a challenge due to the ill-defined fine structure of these polysaccharides. To that end, the main objective of this thesis was to further our understanding on the fine structures of these polysaccharides taking apple as a model. The choice of this fruit was made on the fact that it represents a major production worldwide among fleshy fruits.

Xyloglucan (XyG), galactoglucomannan (GgM) and glucuronoxylan (GuX) represent the hemicellulosic components in the fleshy fruit primary cell wall. Our experimental set up (Chapter II) allowed the recovery of two XyG populations. The endo-β-glucanase structural profiling confirmed the presence of **XXXG**, **XXFG**, **XLXG** and **XLFG** as major building structures of these XyG fractions with minor amounts of **XXG**-type oligomers and natively acetylated derivatives, as previously reported (Renard et al., 1992; Vincken et al., 1997; Galvez-Lopez et al., 2011). The gentle way of extraction used in our study allowed the isolation of acetylated XyG. In the **XXXG** type backbone of *Arabidopsis* XyG, the acetyl esterification was found on the Gal*p* residues (Gille et al., 2011) whereas more recently, Quemener et al. (2014) reported mono acetylated Fuc residue in apple XyG. This suggested that position of acetyl esterification on XyG might be dependent on the botanical source. In agreement with Assor et al. (2013), our study revealed that LiCl-DMSO solubilised the

loosely bound XyG, whereas harsh alkaline conditions such as 4 M KOH solubilised the XyG population that likely covers the celluloses microfilbrils (Pauly et al., 1999). *In vitro* studies suggested that galactosyl content or its specific distribution on the backbone appeared to impact XyG binding on cellulose (Vincken et al., 1995, de Lima & Buckeridge, 2001). The two apple XyG populations recovered in the present study produced the same XyG oligosaccharides profile on endo-β-glucanase degradation but the distributions of the structural domains are questioned particularly with regard to the galactosylated structures. Furthermore, the extent and distribution of acetyl esterification of these XyG fractions that could not be assessed due to loss of esters in the alkaline extraction, are likely to play an important role in respect to cellulose interaction. The presence of *O*-acetyl esters may inhibit the H-bond formation with the hydroxyl group of cellulose by decreasing the polarity of the XyG oxygen. It may also introduce a hydrophobic character of XyG domains that favour interactions with cellulose hydrophobic surfaces. The LiCl-DMSO soluble XyG accounted for 3.7 weight % acetyl esterification but the ester substitution of the more tightly bound XyG could not be measured due the use of alkaline extraction conditions. In tomato, the LiCl-DMSO extraction residues were shown to contain acetyl esters suggesting that residual hemicelluloses were acetyl esterified (Assor et al., 2013). Additionally, the tightly bound XyG had a higher molecular weight than the LiCl-DMSO soluble one in agreement with several studies that indicate molecular weight as an important parameter controlling XyG-cellulose interaction (Vincken et al., 1995; Lopez et al., 2010). Besides, LiCl-DMSO soluble XyG was the quantitatively most important fraction, which is partly in agreement with Bootten et al. (2004) who presented tethering XyG as the major XyG fraction. The presence of Xyl and Gal in the cell wall residue was consistent with the presence of entrapped XyG in cellulose microfibrils, in agreement with the model of Pauly et al. (1999). Several studies established XyG-pectin RG-I covalent linkages through arabinan and/or galactan side chains that may contribute to the retention of XyG in the cell wall matrix (Fu & Mort, 1997; Thompson & Fry, 2000; Abdel-Massih et al., 2003). Such linkages were unlikely in the extracted apple XyG as HPSEC applied on the extract was able to separate pectins from hemicelluloses. Our results are in accordance to other fleshy fruits like tomato and bilberry where XyG was purified as a pectin-free fraction (Hilz et al., 2007; Assor et al., 2013), indicating pectin-XyG linkage, if present in fruit parenchyma cell wall, may depends on genetic and/or developmental factors or may be cleaved during the extraction procedure.

In contrast to XyG, the galactoglucomannan (GgM) has received less attention despite their presence throughout the fruit cell walls and in particularly in apple (Nara et al., 2004;

Galvez-Lopez et al., 2011). Alkali-soluble apple GgM were shown to consist of Man, Glc, Gal in the molar ratio of 5.7:3.0:1.1 where the glucomannan backbone was substituted by single Gal and di-Gal chains on both Glc and Man residue (Nara et al., 2004). The present study revealed for the first time the presence of two different acetylated GgM populations in apple like in tomato (Assor et al., 2013). In Solanaceae the GgM populations differ on the distribution of pentose (Ara and/or Xyl) side chains, some fractions being nearly free of these monomers whereas others contain substituted block domains. In contrast, only one GgM population was revealed in kiwi fruit. It was reported as constituted predominantly of an alternating β-(1,4)-linked D-Glc*p* and D-Man*p* residues backbone interspersed by small unsubstituted mannan region (Schroder et al., 2001). The two GgM fractions in apple contained small glucan domains but differed on their mannan domain distribution. Hydrolysis of these fractions by β-mannanase together with α-galactosidase indicated that galactosylated mannose occurred as small blocks. The Gal:Man:Glc ratio of these two fractions differed between the anion exchanger retained fraction with a ratio of 1:1.5:1.4 compared to the non-retained fraction that was richer in mannose (1:4.3:1.4). These results markedly differ from that reported by Nara et al. (2004) suggesting that GgM structure may be dependent of fruit development stage and/or genetics. The two GgM fractions had nearly the same molecular weight (15-30 kDa), close to that of kiwi fruit GgM (16-42 kDa) (Schroder et al., 2001). Recently, the linkage between GgM and GuX was revealed in red ripe tomato cell walls in both DMSO and 4 M KOH extracts (Prakash et al., 2012) in contrast to Assor et al. (2012) who used LiCl-DMSO extraction. The latter separated GgM and GuX suggesting that the extraction conditions may have cleaved the GgM-GuX linkages or other biological factors may play an important role on the linkage. In apple, the present results showed that the GgM fraction retained on anion exchange chromatographic matrix was eluted first prior to that of GAX. The retention of a neutral GgM on the anion exchange column remains unclear. It can be proposed that apple GgM and GAX were extracted by LiCl-DMSO in the form of a complex which was disaggregated by the NaCl gradient. Such hypothesis would indicate that LiCl-DMSO extraction partially destroy the linkage between the two hemicelluloses. The different degree of acetyl esterification of apple mannan fractions may also play an important role in their interaction behavior. The hydrophilic low acetylesterified GgM is likely to adsorb better on the anion exchange chromatography matrix than the more acetylesterified hydrophobic one. Indeed, the fraction retained on the anion exchanger was less acetylesterified than the excluded one, though the latter contained XyG as small impurity that prevented exact quantification of its acetyl ester content. Besides regulating polymer

interactions, acetyl esterification may also control the action of mannan-endotransglycosidase/hydrolase modification *in planta* upon fruit development and ripening (Schröder et al., 2006). Similarly to what was observed for the interaction ability of acetylated pectin (Pippen et al., 1950), it can be imagined that acetyl distribution on GgM plays an important role in controlling their behavior *in planta*. Further more precise structural studies are required to better understand of their different behavior.

The third group of fleshy fruit hemicelluloses consists in GuX. Although apple hetero-xylan was reported by Voragen et al. (1986), our experimental setup led to the first successful purification of a natively acetylated xylan. Due to its LiCl-DMSO extraction, it is like that the xylan is loosely bound in apple parenchyma cell walls. The MADI-TOF MS, HPAEC and sugar composition analyses of β-xylanase degradation products revealed high molecular mass oligomers and the presence of Ara in a molar ratio of 1:3 (Ara:Xyl). These results suggest that the presence of different ramifications, notably Ara, on the xylan backbone restricts the enzymatic degradation. The presence of GuX as a minor hemicelluloses constituent was also revealed in tomato (Quemener et al., 2007; Prakash et al., 2012; Assor et al., 2013) and in the tropical fruit tamarillo (do Nascimento et al., 2013). Like apple, tamarillo GuX carries Ara. In this fruit, it was localized at the *O*-5 position on the backbone Xyl and the extract also contained β-D-Gal*p*. Acidic xylan (GuX and/or GAX) can be thought to contribute in the control of the wall porosity and pH as proposed in the pectin-devoid gramineous primary walls (Carpita, 1996). Furthermore, with surface charges and a flexible conformation, GuX and/or GAX may regulate the assembly of cellulose as observed *in vitro* (Atalla, 1991). Similar re-association assay was done by Reis et al. (1991). These authors showed cholesteric-like ordered structure of the cellulose-GuX system whereas, cellulose flocculated in the same system without GuX. Xylan-cellulose interaction was studied by exposing bacterial cellulose gels to xylan solutions under autoclave treatments and the complex formation was visualized by atomic force microscopy (Linder et al., 2003).

Endo-xylanase and/or xylan endotransglycosylase activities are likely to reshuffle these hemicelluloses in the cell walls of the ripening fruit (Brummell & Schroder, 2009; Schroder et al. 2009; 2010), under the putative control of acetyl esterification, as for GgM and XyG.

This first part of the study revealed the great complexity of apple parenchyma cell wall hemicellulose types and fine structure. Several questions arose during this study: how side chains distribute along the glucan backbone of the two types of XyG? What are the locations of Ara residue in the GuX backbone? How acetyl esterification distributes along the

backbone and/or side chains. Further analyses are thus required for the better understanding of these structures.

There are different complementary analytical approaches that can conceptualize and confine our current understanding of the hemicellulosic polysaccharides. Sugar linkage analysis by permethylation is an important analytical method in this context (Anumula & Taytor, 1992). It involves three essential steps: methylation of the free hydroxyl groups of the polymer, acid hydrolysis of the methylated polymer in monomeric units followed by alditol acetate derivatisation of the partially methylated monomeric sugar units for their chromatographic separation their identification. This common approach has been widely used for the structural determination of hemicelluloses (Femenia et al., 1999; Laine et al., 2002; Nara et al., 2004; do Nascimento et al., 2013). Its disadvantage is that for the highly branched hemicelluloses made of several different sugars, where the fine structure of the polymer is difficult to predict. As an example, it cannot anticipate the location of a ramified sugar either in the backbone or in the long side chain. Another approach using partial acid hydrolysis, chromatographic and spectroscopic analyses of the oligosaccharides released have led to the recognition of molecular core and the sequence of the extremely complex arrays of sugar units (Srivastava & Smith, 1957; Igartuburu et al., 1998, 2001; Reis et al., 2003). This chemical strategy is powerful but tedious. Moreover, the selectivity of glycosidic bond cleavage by acids is low. Using Smith degradation it is possible to obtain information on backbone structure and other finer structural details (Revanappa at al., 2010). This procedure selectively degrades a polysaccharide to either a shorter polysaccharide or oligosaccharides, from which structural information can be deduced. It involves three steps: oxidation with periodate, reduction to a polyalcohol followed by hydrolysis with dilute acid under mild conditions and characterization of the fragments by various chromatographic and analytical means (Goldstein et al., 1965). During the hydrolysis step only acetals of oxidised sugar rings are cleaved. The product may range from a monosaccharide glycoside to a polysaccharide. The main *disadvantages* of this method are its laboriousness and the necessity for the presence of molecular fragments that are susceptible to oxidation by periodate. The use of analytical tools that would allow mapping and sequencing of larger oligomeric motifs is critical to gaining a better understanding at the structural level.

To that end, enzymes work in mild conditions and give specific fragments. The complexity of the structure of the hemicelluloses and the high substrate specificity of enzymes has compelled us to search new enzymes in order to investigate motifs not revealed to date. Hence, the next step of this project (chapter III) was to investigate unexplored sources

of hemicellulases to get new analytical tools allowing access to these structures and answer the above questions. There are different ways to find new enzymes. It can be achieved by metagenomic approach or direct growth of microorganisms sampled from the environment. A number a micro-organisms in nature cannot be grown using conventional methods. To that end, functional metagenomic screening approach enables rapid generations of clones with potential activities of interest (Brennan et al., 2004; Lee et al., 2004; Yun et al., 2004; Solbak et al., 2005). For this issue selection of good host is crucial. *Escherichia coli* is still the preferred host (Kim et al., 2011), though very recently other hosts have emerged such as *Bacillus megaterium* (Summpunn et al., 2011), *Brevibacillus brevis* (Zhou et al., 2011), *Lactobacillus plantarum* (Scheirlinck et al., 1990). Often several hundred thousand clones have been analyzed to detect less than ten active clones in a single screen (Henne et al., 1999; Henne et al., 2000; Majernik et al., 2001). According to Streit & Schmitz (2004) this limited positive output is mainly owing to a lack of efficient transcription of the metagenome-derived genes in the host strain. This effect might be worsened by a weak translation in combination with a poor secretion of the foreign protein by the employed host strain. Furthermore, in many cases, the desired protein is not folded correctly because required chaperones are absent in the host strain. Co-factors might not be synthesized by the foreign host strain and/or not inserted correctly into the recombinant metagenomic protein. Finally, a different codon usage could limit protein expression and low activities.

Due to these limitations, a different strategy was preferred in this work. Unexplored bacteria with known genome were selected on the basis of the presence of putative hemicellulase coding sequences. The occurrence of predicted enzymatic activities was validated through the screening of culture supernatants of selected bacteria grown in various conditions with a collection of polysaccharides. Finally, some culture supernatants were partially purified to investigate the enzymes produced in more details. Characterization of polysaccharide degradation is a challenging task and since many years several methods have been developed to detect and/or quantify the activities of glycosyl hydrolases (Dashtban et al., 2010; Santoro et al., 2010). Several polysaccharides can specifically or nonspecifically interact with dyes like Congo Red, Ruthenium Red, Calcofluor White, iodine, Direct Green, Remazol Brilliant Blue. A clear hole in a colored gel can be visualized when the polysaccharide substrate is embedded in a gelled matrix to observe its enzymatic degradation (Ruijssenaars & Hartmans, 2001, Zhang et al., 2006). Similarly, the insolubility of polysaccharides in numerous solvents such as ethanol, acetone or isopropanol can be exploited. When polysaccharide substrate is immersed in the solvent, opacity shows non-

degradation while a transparent halo reveals the activity of an enzyme (Ruijssenaars & Hartmans, 2001). Monitoring the changes of viscosity of the polymer solutions incubated with the enzymes is one of the well-established ways to observe enzymatic degradation (Kühn et al., 2004; Pala et al., 2007) but large amount of polysaccharide requirements limits this procedure only to the industrial scale. The above-mentioned behaviors are dependent on polysaccharides properties. Detection of reducing end produced after cleavage of the glycosidic linkage of the polysaccharide by enzyme is one of the most established ways to evidence enzymatic degradation. A colorimetric assay, that allows detecting rapidly reducing sugars at micro-plate scale (Miller, 1959; Xiao et al., 2004, 2005; King et al., 2009) is suitable for comparing the relative activities of many samples using medium- or high-throughput screening methodology (Chen et al., 2008; Hu et al., 2008). However the interference from complex bacterial extracts containing reducing ends greatly decreased the sensitivity of the colorimetric assay (Meeuwsen et al., 2000; Rigouin et al., 2009). In this work a medium throughput profiling method based on colorimetric detection technique was used to screen the polysaccharide-degrading enzymes in crude bacterial extracts. This method was chosen as it is applicable to any panel of polysaccharides having known or unknown structures (Fer at al., 2012). The strategy of the screening methodology was based on a series of filtrations to eliminate any reducing sugars present in the raw bacterial extract. Such step greatly increases the sensitivity and reproducibility of the colorimetric assay. Different culture conditions were tested, including different added hemicelluloses targeting the putative GH annotated in each genome. Both the extra- and the intracellular extracts were recovered for testing GH activities by colorimetric assay against the previously prepared apple hemicellulose fractions and many other diverse polysaccharides from both marine and terrestrial origins.

The genome analysis revealed the presence of 24 and 59 sequences of interest in *C. algicola* DSM14237 (Abt et al., 2011) and *Paenibacillus* sp. JDR-2 (Lucas et al., 2009), respectively. But unfortunately, very little or none of these activities were observed in the bacterial extracts prepared without or with polysaccharide inducers. These disappointing results could be partly due to errors on the sequencing and/or annotations of genes (Farrer et al., 2009; Poptsova et al., 2010). However, these results question the choice of inducer polysaccharides used which may not have been properly chosen for *Paenibacillus* sp. and *C. algicola* growth and production of hemicellulases. The production of enzymes is also greatly influenced by nutritional and physicochemical factors (Aziz et al., 2008; Moreira & Filho, 2008) and suggests that culture conditions of these bacteria required some more improvement. On the contrary, the enzyme activity found in the extracellular extract of *C. crescentus* CB15

cultured without added carbon source confirmed for the first time the presence of specific glucan, mannan and xylan-degrading activities in partial accordance with the gemonic prediction (Nierman et al., 2001). However wheat AX used as carbon source did not neither stimulate the production of putative enzymes annotated as a β-xylanase, xylan-1, 4-β-xylosidase and α-L-arabinofuranosidase, nor significantly enhance the glucan and/or GlcM-modifying activities. It should be mentioned that biopolymers are important substrates for heterotrophic bacteria in (ultra)oligotrophic environments, such as *C. crescentus* (Sack et al., 2014).

Several marine micro-organism's genomes contain numerous uncharacterised GH sequences whose some are hypothesized to be active on higher plant cell wall hemicelluloses presenting structural analogies to marine polysaccharides, and in fact new hemicellulases were observed in marine bacteria (Tamaru et al. 1995; Hung et al. 2011; Prasad & Sethi 2013). Similarly, *P. atlantica* T6c showed the presence of β-glucanase and β-xylanase in complete agreement with activities expected from genomic annotation. In protein extract of *P. atlantica* grown with a mixture of CMC, tara GalM and beechwood xylan or with beechwood xylan alone, the secretion of β-xylanases was stimulated in agreement with several literature studies where heteroxylan was used as β-xylanase inducer (Tsenga et al., 2002; Ninawe et al., 2008; Sanghi et al., 2010; Sharma et al., 2013). The presence of tara GalM, CMC or konjac GlcM as added carbon sources did not improve the secretion of enzymes specific for glucomannan and glucan backbone. In some cases, supplementing the culture with a polysaccharide is not sufficient, and adding a polysaccharide and a monomer better enhances the activity. This is well shown in the very systematic study of Kyu et al. (1988) who tested xylan and various monomers to induce *Bacillus circulans* B_6 xylanase. Ara was the most efficient sugar although it was not used as carbon source by the bacterium and it did not improve growth or the extracellular protein level. This can be hypothesized that Ara can participate to activate the promoter that regulate xylan-modifying activity and at the same time it suppress other extracellular activities so that the overall extracellular protein concentration remains constant. A similar situation has been found for *P. atlantica* growing in the presence of beechwood GuX. This inducer did not change the total protein concentration but its presence increased the xylan modifying activities and at the same time lowered the α,α-trehalase activity. Apart from expression levels, one explanation can also be found in the polysaccharide inducers themselves. The linkages in such complex biopolymers are not all equivalent and equally accessible to hemicellulolytic enzymes. The accessibility to linkages can change during the course of hydrolysis. The production of an enzyme consortium, each

enzyme having a specialized function, is one of the strategies that a micro-organism may use to achieve efficient polysaccharide hydrolysis.

This part of the study supported the genome data mining as a good strategy for identifying unexplored bacteria that potentially produce novel hemicellulose-degrading enzymes. Out of 4 initially identified non-pathogenic bacteria, the secretome of *P. atlantica* was revealed to be the most promising for the production of hemicellulose-degrading enzymatic consortia. Further analyses are now required for the better understanding of their mode action, to identify the degradation products that can, in particular, bring answers about the fine structure of apple hemicelluloses.

To that end, the secretome of the marine bacterium *P. atlantica* T6c was fractionated and a β-glucanase and a β-xylanase were isolated and tested at pH 5.2 and 8.5. Stability and activity of bacterial hemicellulases are greatly influenced by pH. Optimum pH for activity of most of the bacterial hemicellulases has been reported in the neutral pH range (Gupta et al., 2000; Kim et al., 2011; Mou et al., 2011). However, some alkalophilic hemicellulases exhibited pH optima around 9 (Gessesse et al., 1998; He et al., 2008), whereas some acidophilic hemicellulases showed an optimum pH around 4 (Rashid et al., 2010).The β-glucanase of *P. atlantica* showed similar activity at both pH 5.2 and 8.5, whereas the β-xylanase was active only at pH 8.5. The β-glucanase was capable of degrading different glucose-rich polysaccharides such as different XyG and GgM backbones and thus has some degree of specificity for the highly branched glucan chain. Moreover, the way of hydrolysis of these two substrates is similar at both pH. β-Xylanase is able to hydrolyze a series of heteroxylan from different botanical sources. In parallel, the presence of acetylesterase, β-xylosidase and α-arabinofuranosidase was evidenced for the first time in this strain.

These panel of new enzymes produced hemicellulose oligosaccharides that were subjected to analysis aiming at obtaining complementary structural information. The newly isolated β-glucanase treatment of two different apple XyG populations revealed the presence of different oligosaccharides that differed with those obtained by commercial β-glucanase (EG II) degradation (chapter II). The EG II β-glucanase from *Trichoderma longibrachiatum* degraded the apple XyG backbone in typical well defined oligosaccharide mixtures arising from cleavage at specific unbranched glucosyl residues (Quemener et al., 2014). However, the β-glucanases (EG II) belong to GH 7 while the putative *P. atlantica* β-glucanases is classified in GH5. This difference might explain their different behavior upon substrate degradation. The GH5 *P. atlantica* β-glucanase was shown to degrade the two apples GgM and the structural information obtained from the new tool is in agreement with β-glucanase (EG II)

degradation products (Chapter II) though the two enzymes are not from the same GH family. So, an interesting conclusion can be drawn from the overall investigation that the both β-glucanases showed similar tolerance to mannose-containing glucan. Conversely XyG degradation demonstrated that the two enzymes show different tolerance to backbone substitution, resulting in different XyG oligosaccharides. Another important aspect is that the new GH5 β-glucanase in similarity with EG II β-glucanase (GH7) was shown to tolerate the acetyl esterification of both the GgM and XyG backbones as all these substrates were initially acetylated.

The hydrolysis of apple acetylated GAX by *P. atlantica* β-xylanase fraction (**PaX**) produced predominantly xylose (**X**), xylobiose (**X2**), xylotriose (**X3**), xylotetraose (**X4**), with small amounts of various substituted XOSs. The degradation of the GAX with commercial GH11 β-xylanase from *T. longibrachiatum* revealed different HPAEC profiling as it produced mainly **X** and **X2** with other different high retention time XOSs. This difference can be explained in two ways. First, the enzymatic degradation by **PaX** was partial as there was a series of **X**, **X2**, **X3** and **X4**. Indeed, *P. atlantica* β-xylanase may be classified in GH10 family in contrast to the GH11 of *T. longibrachiatum*. In literature the substrate specificity of GH10 and 11 xylanases has been well established: GH10 xylanase was known to be more tolerant to the substitution than that of GH11 (Biely et al., 1997; Bonnin et al., 2006; Kolenova et al., 2006). Additionally from the **PaX** degradation products it is clear that apple GAX contain unsubstituted β-(1,4)-linked xylan stretches of 4 residues, in addition to the substituted area. These domains may be of importance for the cell wall as *in vitro* xylan-cellulose interaction study suggested the formation of globular xylan particles on the cellulose surfaces (Linder et al., 2003), and the unsubstituted linear regions were shown to interact more favorably with cellulose (Kabel et al., 2007). So, this unsubstituted GAX xylan region can serve as potential segment to interact with cellulose microfibrils.

Finally, this entire work allowed progressing on the one hand on the apple hemicellulose structure, and on the other hand, on the characterization of new hemicellulose-specific enzymes.

V.2. Perspectives

New hemicellulases consortia were revealed in this work by exploring bacterial genome sequences. The marine bacterium *P. atlantica* T6c was revealed as a promising source of new enzymic tools. Nevertheless, a number of questions remain unanswered.

Further works are necessary to purify the enzymes from the hemicellulases consortia (backbone hydrolyzing enzymes and auxiliary enzymes) produced by *P. atlantica*, check for their amino acid sequences with that predicted from the genomic sequences, and precisely characterize their detailed mode of action as isolated enzymes and on model substrates. This will offer a panel of new enzymes usable as tool for the elucidation of hemicellulose fine structure and for the understanding of synergistic actions of enzymes in consortia in the process of the saccharification of hemicelluloses. With these data at hands, more detailed structural studies of apple hemicelluloses using the different enzymes from *P. atlantica* are expected to further our understanding on hemicelluloses functions *in planta* and in particular on their evolution during organ development, such as fruit ripening. Fragments of hemicelluloses generated by these new tools can prove to be in valuable structures to develop new probes, such as antibodies, to locate and follow specific hemicellulose structures within the cell wall during plant development.

V.3. References

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Résumé

Elucidation des consortia enzymatiques pour le chémotypage structural des hémicelluloses de dicotylédones

Les hémicelluloses sont définies comme des polysaccharides non-cellulosiques présents dans la paroi végétale, parmi lesquels on trouve les xyloglucanes, xylanes et mannanes. Elles représentent une source importante de biopolymères renouvelables. Bien que leur chaîne principale soit toujours basée sur des liaisons β-glycosidiques, elles présentent une grande diversité structurale due à la variabilité de leurs chaînes latérales osidiques et de leurs substituants non-osidiques. A l'heure actuelle, cette diversité de composition et de distribution des substituants n'est pas suffisamment connue pour permettre d'établir une relation entre la structure des ces polysaccharides et leur fonction dans l'architecture de la paroi. Ainsi, dans les fruits charnus tels que la pomme ou la tomate, les hémicelluloses jouent un rôle clé dans la régulation des propriétés mécaniques des organes et ont une incidence directe sur les utilisations de ces produits végétaux cultivés (i.e. aptitude des fruits à la transformation, appréciation de la texture par le consommateur...).

Différents outils analytiques sont exploitables pour élucider la structure fine des polysaccharides, parmi lesquels la dégradation enzymatique et l'analyse des produits de réaction s'avère une stratégie performante. La dégradation enzymatique du squelette principal des hémicelluloses requiert des β-xylanases, β-glucanases, xyloglucanases et β-mannanases.

Ainsi, la première partie de ce travail est focalisée sur la **structure des hémicelluloses de pomme**, et fait l'objet d'un article paru dans Carbohydrate Polymers (Ray S., Vigouroux J., Quémener,B., Bonnin E., & Lahaye M. (2014). Novel and diverse fine structures in LiCl-DMSO extracted apple hemicelluloses. *Carbohydrate Polymers, 108*, 46-57).

Les hémicelluloses de pomme ont été extraites à partir de matériel pariétal isolé. Une extractionséquentiellea été réalisée par un mélange LiCl-DMSO, puis par de l'hydroxyde de potassium. Le premier extrait, contenant les hémicelluloses associées à la matrice pariétale par des liaisons de faible énergie, a été fractionné par des techniques de chromatographies d'échange d'ions ou d'exclusion stérique. Les populations isolées ont été dégradées par des enzymes commerciales, β-glucanase, β-mannanase et β-xylanase, et la structure des oligosaccharides produits a été élucidée par chromatographie haute performance d'échange d'anions (HPAEC) et spectrométrie de masse (SM). A partir de l'extrait LiCl-DMSO, une

fraction riche en xylanes a été purifiée par chromatographie d'échange d'ions. Sa masse moléculaire moyenne est d'environ 35 kDa. Elle contient majoritairement du xylose, mais également de l'arabinose et de l'acide glucuronique, ainsiqu'une forte proportion de groupements acétyles, suggérant ainsi un hétéroxylane acétylé. L'analyse en spectrométrie de masse MALDI-TOF des produits d'hydrolyse libérés par une β-xylanase montre la présence de différents oligomères formés de pentose (P), acide uronique (U), acétyle (a) et méthyle (m). Les composés majoritaires sont $P_5U_1a_1m_1$ (5 pentoses, 1 acide uronique, 1 acétyle et 1 méthyle), $P_6U_1a_1m_1$, and $P_7U_1a_1m_1$, accompagnés de composés mineurs tels que $P_4U_1a_1m_1$, $P_5U_1m_1$, $P_6U_1m_1$, and $P_8U_1a_1m_1$. La présence, parmi les produits finaux, d'oligosaccharides de degré de polymérisation (DP) élevé contenant jusqu'à 8 pentoses trouve vraisemblablement son origine dans la structure très substituée de ces hétéroxylanes, qui s'oppose à leur dégradation. L'analyse de la composition osidique des oligosaccharides issus de cette fraction indique la présence d'arabinose et de xylose dans un rapport 1:3. Les résultats de spectrométrie de masse et de composition permettentde conclureà la présence glucuronoarabinoxylanes (GAX). Pour élucider les positions respectives de l'arabinose et du xylose, l'une des fractions éluées de la chromatographie d'échange d'ions a été traitée par une combinaison xylanase-arabinofuranosidase. L'arabinofuranosidase libère les chaînes latérales d'un arabinane branchée par des liaisons 1-5. Les mêmes produits d'hydrolyse sont obtenus après dégradation par la xylanase seule ou par la combinaison des deux enzymes, ce qui suggère que l'arabinose présent dans le GAX de pomme n'est pas lié en 1-5.Par contre, sa position exacte n'a pu être précisée.

L'extraction par le LiCl-DMSO solubilise également deux populations enrichies en galactoglucomannanes. Ces deux populations ont été séparées pendant les étapes de purification par échange d'ions et exclusion stérique. Les deux fractions ont des masses moléculaires proches mais diffèrent par leur composition.La composition osidiquedela fraction retenue sur échange d'ions montre un rapport galactose : mannose : glucose de 1:1.5:1.4, alors qu'il est de 1:4.3:1.4dans la fraction isolée par exclusion stérique, traduisant une proportion beaucoup plus important de mannose. De plus, une dégradation de ces deux fractionspar la mannanase produit des oligosaccharides différents. Ainsi, la fraction retenue en chromatographie d'échange d'ions est dégradée en oligosaccharides de DP élevé : H_6a_1 (6 hexoses, 1 acétyle), H_6et H_8a_1. A l'inverse, la fraction purifiée par exclusion stérique est dégradée en trimère ou tétramère mono- ou di-acétylés. Après une dégradation par la β-glucanase, les deux fractions produisent des oligosaccharides de DP entre 3 et 6, dont la majorité est mono-acétylée. L'ensemble de ces résultats suggère que les deux fractions

contiennent de petits domaines glucane, séparés, dans un cas par de larges domaines mannane, et dans l'autre cas par de petits domaines mannanes. Un traitement de la fraction retenue sur échange d'ions par une combinaison mannanase-galactosidase induit la disparition des oligosaccharides de hautes masses moléculaires. Cela montre que des ramifications de galactose étaient présentes sur le squelette mannane et qu'elles gênaient l'action de la mannanase. Cela montre également que les mannoses substitués par du galactose formaient de courts blocs sur le squelette principal du polysaccharide.

La dernière fraction d'intérêt parmi les hémicelluloses de pomme est une fraction xyloglucane (XyG). Deux fractions de cette nature ont été isolées à partir des parois de pomme : la première est solubilisée dans l'extrait LiCl-DMSO puis isolée par chromatographies d'échange d'ions puis de gel-filtration ; la seconde est solubilisée en 4M KOH. Les conditions différentes requises pour l'extraction de ces deux fractions sont probablement liées à des structures et à des associations différentes avec les autres constituants pariétaux. La fraction solubilisée en LiCl-DMSO a une masse moléculaire d'environ 156 kDa. L'analyse de sa structure par hydrolyse par la β-glucanase et analyse des produits de dégradation montre un profil typique de XyG, contenant les principales sous-unités constitutives de ce polysaccharide. De plus, l'utilisation de la spectrométrie de masse MALDI-TOF a permis de mettre en évidence l'acétylation de ces oligosaccharides, notamment parmi ceux présents en plus grande proportion. Les conditions alcalines drastiques (4M KOH) nécessaires pour extraire la seconde fraction XyG sont à rapprocher des étroites associations qui relient XyG et microfibrilles de cellulose. Les oligosaccharides produits par la dégradation de cette fraction par la β-glucanase sont très comparables à ceux obtenus à partir de la fraction soluble en LiCl-DMSO. Par contre, sa masse moléculaire de 200 kDa est significativement plus importante. La distribution des oligosaccharides constitutifs est vraisemblablement un paramètre déterminant pour la capacité d'association des XyG sur les autres polysaccharides de la paroi, et notamment la cellulose. Malheureusement, cette distribution n'est pas révélée par les analyses menées ici par la dégradation enzymatique de ces fractions par des enzymes commerciales. De la même façon, le degré d'acétylation des XyG influencent leur aptitude aux interactions. Du fait de l'extraction en conditions alcalines fortes, ce paramètre n'a pas pu être étudié sur la fraction extraite en 4M KOH. Ainsi, ce travail d'extraction et caractérisation des hémicelluloses de pomme a permis de mettre en évidence différentes fractions jusqu'alors non montrées mais n'a pas permis de révéler totalement leur structure, ni de rapprocher ces structures des interactions mises en jeu dans la paroi entre les différentes familles d'hémicelluloses et les

autres constituants de la paroi.Ainsi, la complexité des structures à élucider et la spécificité très étroite des enzymes nous amène à rechercher de nouvelles enzymes pour résoudre la structure de motifs jusqu'alors non résolus.

De ce fait, la suite de ce projet a consisté à **explorer de nouvelles sources d'hémicellulases** pour obtenir de nouveaux outils enzymatiques permettant de mieux accéder aux subtilités structurales de ces polysaccharides. Ce travail fait l'objet du deuxième article présenté dans cette thèse et soumis à Applied Microbiology and Biotechnology (Data mining of bacterial genomes for identifying new hemicellulases and functional validation, Ray S., Fer M., Lahaye M., Bonnin E., Helbert W.) Les hémicellulases sont produites par différents organismes, dont les bactéries, les champignons, les plantes et les animaux. Les hémicellulases bactériennes sont secrétées dans le milieu de culture et permettent à la bactérie de dégrader la biomasse végétale en monomères qu'elleconsommeensuite. Nous avons choisi ici d'explorer des bactéries dont le génome est séquencé. Pour cela, le « CAZome », défini comme la collection d'enzymes actives sur les polysaccharides codée par un génome, fournit une vue des aptitudes d'un organisme à métaboliser les polysaccharides. A l'heure actuelle, la base de données CAZy (www.cazy.org) couvre plusieurs milliers de génomes, dont la majorité sont d'origine bactérienne. Mais une faible minorité des gènes ainsi définis ont une fonction reconnue. Parmi ces nombreux génomes, nous avons sélectionné ceux qui codent potentiellement pour des enzymes spécifiques des hémicelluloses, c'est à dire pour des glycosyl hydrolases (GH) des familles CAZy 5, 6, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 45, 48, 51, 74, 113, 124. 3692 génomes bactériens répondent à ce critère. 135 ont été retenus sur la base de leur nature aérobie et non-pathogène et sur la présence d'un minimum de 10 séquences d'intérêt dans chaque génome. Finalement, les souches *Caulobacter crescentus* CB15, *Paenibacillus* sp. JDR-2, *Cellulophaga algicola* DSM 14237 et *Pseudoalteromonas atlantica* T6c, provenant d'environnementsoligotrophe, terrestre et marin, ont été sélectionnées. Différentes conditions de culture ont été testées pour chaque bactérie, et notamment l'apport comme source carbonée des hémicelluloses potentiellement ciblées par les GH annotées sur chaque génome. Les activités GH ont été criblées par un test colorimétrique sur les extraits bactériens intra- et extracellulaires sur 42 polysaccharides, dont les hémicelluloses de pomme préalablement préparées. Les résultats ont été confirmés par l'analyse chromatographique des produits d'hydrolyse.

Le génome de *P. atlantica* T6c est annoté comme codant 10 GH classées dans des familles contenant des enzymes spécifiques des hémicelluloses et validées biochimiquement.

La présence de β-glucanase et β-xylanase est confirmée dans le surnageant de culture obtenu en l'absence de source carbonée, et pourrait correspondre aux annotations Palt_1404 et Palt_2657, respectivement. En effet, Palt_1404 est classée dans la sous-famille 2 de la famille GH5 qui ne contient que des cellulases, et Palt_2657 appartient à la famille GH10 qui ne contient que des xylanases. A notre connaissance, c'est la première fois que ces activités sont rapportées chez *P. atlantica*. A l'inverse, ces activités ne sont trouvées qu'à l'état de traces dans l'extrait intracellulaire. Par contre, les deux extraits présentent des activités sur porphyrane et α-α tréhalose, qui sont probablement à relier aux annotations d'une séquence GH37, famille qui comprend les α-α tréhalases, et de séquences des familles GH16, 50 et 86, qui regroupent les agarases.

Lorsqu'il est cultivé en présence d'un mélange de carboxyméthyl cellulose (CMC), galactomannane (GalM) de gomme tara, et xylane de bois de hêtre, *P. atlantica* dégrade plus efficacement les polysaccharides formés d'un squelette xylane, tels que les arabinoxylanes de blé ou de seigle ou les xylanes de hêtre. Les xylanes utilisés comme inducteur stimuleraient donc la production des xylanases Palt_2657 et Palt_0810. A l'inverse, la présence dans le milieu de culture de GalM, CMC ou glucomannane (GlcM) de konjac ne renforce pas la sécrétion des enzymes correspondantes, alors que ces enzymes sont pressenties par l'annotation du génome. Ainsi, il semble que les mannanes et GlcM n'induisent pas la GH5 Palt_1404, annotée comme cellulase putative.

L'analyse du génome de *Caulobacter crescentus* CB15 révèle 10 séquences potentiellement impliquées dans la dégradation des hémicelluloses. Le surnageant de culture obtenu sans source carbonée contient des activités β–glucanase, β–mannanase et β–xylanase, mises en évidence ici pour la première fois dans cette souche bactérienne. Par contre, lorsque des arabinoxylanes de blé sont ajoutés au milieu de culture, ils ne stimulent pas l'expression du gène de la β–xylosidase spécifique des xylanes (CC0989, GH43) ou de l'α-arabinofuranosidase (CC1422, GH51), et la dégradation des hémicelluloses testées n'est pas modifiée.

Chez *Cellulophaga algicola* DSM 14237, 24 gènes coderaient pour des enzymes spécifiques des hémicelluloses, parmi lesquels 3 cellulases et 5 xylanases. Cultivée sans source carbonée, cette bactérie exprime faiblement les enzymes nécessaires à la dégradation de GgM, XyG et GgM extraits de pomme, indiquant pour la première fois la présence d'une mannanase, d'une xyloglucanase et d'une xylanase. Par contre, les activités cellulases attendues n'ont pu être démontrées.

De même, l'analyse du génome de *Paenibacillus* sp. JDR-2 révèle la présence de 38 enzymes putatives impliquées dans la dégradation des hémicelluloses, parmi lesquelles des cellulases, xylanases, mannanases. Malheureusement, aucune de ces activités n'a pu être démontrée dans les conditions de culture qui ont été testées. En particulier, l'expression de cellulases et mannanases par *C. algicola* et *Paenibacillus* sp. n'ont pas été mises en évidence, y compris en utilisant CMC et GalM comme inducteurs.

Finalement, parmi les 4 souches initialement retenues, les activités attendues ont été mises en évidence pour *P. atlantica* et *C. crescentus* mais pas pour *C. algicola* et *Paenibacillus* sp. Ces résultats peuvent être partiellement expliqués par des erreurs d'annotations des génomes. De plus, les conditions de culture, et en particulier le choix des sources carbonées, sont cruciaux pour l'expression d'enzymes. Ainsi, l'inducteur le mieux adapté peut ne pas avoir été trouvé pour *C. algicola* et *Paenibacillus* sp. De plus, la production d'enzymes est liée à la croissance bactérienne, et on remarque ici que la teneur en protéines du surnageant de culture de *C. algicola* montre une concentration en protéines beaucoup plus faible que les autres surnageants, traduisant une faible croissance. Malgré cela, cette étude montre l'intérêt de la démarche couplant exploration des données d'annotations et résultats expérimentaux pour la découverte de nouvelles hémicellulases bactériennes et l'optimisation de leurs conditions de production. A l'issue de ce travail, *P. atlantica* est la souche la plus intéressante pour la dégradation des hémicelluloses de pomme. **L'exploration plus approfondie du sécrétome de *P. atlantica* T6c** fait donc l'objet de la troisième partie des résultats présentés ici.

Les sécrétomes obtenus en présence de GlcM de konjac ou de glucuronoxylanes de hêtre commesource carbonée ont été fractionnés par chromatographie d'échange d'ions. Une β−glucanase et une β−xylanase ont ainsi été isolées. L'influence du pH a été testée sur ces deux enzymes, et l'activité de la β−glucanase est de même niveau en conditions acides (pH 5,2) ou basiques (pH 8,5), alors que la xylanase n'est active qu'en conditions basiques. Les oligosaccharides libérés lors de la dégradation de différentes hémicelluloses par ces deux enzymes ont été analysés en HPAEC. La β−glucanase est capable d'hydrolyser différents polysaccharides riches en glucose, tels que les XyG, GgM et glucomannanes de différentes origines, telles que tomate, pomme et konjac. Cela permet de conclure à une certaine spécificité de cette enzyme pour les chaînes de glucanes portant des ramifications. De la même façon, la β−xylanase hydrolyse différents squelettes xylanes tels que ceux qui forment les xylanes de hêtre, les arabinoxylanes de blé ou les GAX de tomate ou pomme. Les

hydrolysats contiennent majoritairement des xylooligosaccharides, mais également des oligosaccharides plus complexes et plus substitués. Ainsi, cette analyse révèle la présence de régions de xylane non substituées à coté des régions fortement substituées dans les squelettes de ces hétéroxylanes. De plus, une β−xylosidase et une α−arabinofuranosidase ont été mises en évidence pour la première fois chez cette bactérie, dont seule l'arabinofuranosidase correspond à une annotation préalable du génome (Palt_0842 et Palt_0823). Après isolement, l'activité de chacune a été démontrée sur *p*NP-xyloside et *p*NP-arabinoside, respectivement. Leur implication dans la dégradation des hémicelluloses complexes a également été montrée. Ainsi, cette étude aura non seulement démontré la présence de nouvelles activités enzymatiques chez *P. atlantica*, mais aussi de nouveaux motifs structuraux dans les hémicelluloses de différentes origines.

En résumé, ce travail fournit un schéma d'isolement et purification des différentes hémicelluloses de pomme, ainsi qu'une analyse de leur structure par des enzymes commerciales utilisées comme outil. Il montre ensuite une approche d'exploration des données génomiques de bactéries couplée à un criblage moyen-débit pour mettre en évidence de nouvelles sources d'enzymes d'intérêt pour la dégradation des hémicelluloses. Ainsi, une β−glucanase, une β−xylanase, une β−xylosidase et une α−arabinofuranosidase ont été purifiées de *P. atlantica* T6c, et leur spécificité a été étudiée.

Cependant, des questions restent à élucider, dont notamment le mode d'action de ces nouvelles enzymes sur des substrats modèles de structure parfaitement connue. Une fois ce mode d'action établi, ces enzymes pourront être utilisées pour compléter l'analyse structurale des hémicelluloses de pomme, c'est à dire la distribution relative du glucose et du mannose dans les GlcM, la longueur des blocs correspondants, la nature des oses acétylés, la distribution des oligosaccharides constitutifs des XyG, le degré d'acétylation et la distribution des groupements acétyles dans la population de XyG la plus fortement associée à la cellulose…

Une détermination plus précise de ces structures permettra de mieux appréhender les propriétés de ces hémicelluloses, comme par exemple leurs capacités d'interactions, et par conséquent leur fonction dans la paroi. D'autre part, si ces nouvelles activités montrent des spécificités différentes des enzymes connues à ce jour, cela complètera l'arsenal d'enzymes utilisable pour la saccharification de la biomasse.

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PÔLE DE RECHERCHE ET D'ENSEIGNEMENT SUPÉRIEUR

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Sayani Ray

Elucidation of enzymatic consortia for structural chemotyping of dicot hemicelluloses

Résumé

Xyloglucanes (XyG), galactoglucomannanes (GgM) et glucuronoarabinoxylanes (GAX) sont des hémicelluloses clés pour la régulation des fonctions de la paroi chez les dictoylédones. Cependant, l'impact de leur composition variée et de leur distribution dans la paroi reste à élucider. Pour explorer leur structure fine, la dégradation par des enzymes commerciales des fractions natives de XyG, GgM et GAX acetylés a révélé des structures différentes entre différentes populations de chaque hémicellulose, avec en particulier la première mise en évidence de substitutions arabinose et/ou xylose sur les GAX. Des détails structuraux restent toutefois à élucider, ce qui nécessite de nouveaux outils enzymatiques dotés de nouvelles spécificités. A cette fin, nous avons utilisé une démarche d'analyse du génome couplée à un criblage moyen-débit pour sélectionner des bactéries de diverses origines produisant des hémicellulases. Ainsi, 4 souches aérobies et non-pathogènes (*Pseudoalteromonas atlantica, Cellulophaga algicola, Caulobacter crescentus*, *Paenibacillus* sp.) ont été cultivées dans différentes conditions. *P. atlantica* s'est avéré le plus efficace pour produire des hémicellulases. Son sécrétome a été fractionné par diverses méthodes chromatographiques, ce qui a permis d'isoler des β-glucanase, β-xylanase, β-xylosidase and α-arabinofuranosidase. L'analyse des hydrolysats d'hémicelluloses de pomme par ces enzymes a révélé des spécificités de substrats diverses et l'analyse structurale des oligosaccharides a fourni de nouvelles avancées sur la structure de ces polysaccharides.

Mots clés
Pomme, hémicelluloses acétylées, exploration du génome, bactéries, hémicellulases, HPAEC

Abstract

Xyloglucan (XyG), galactoglucomannan (GgM) and glucuronoarabinoxylan (GAX) are key hemicelluloses regulating plant cell wall functions in dicots. However, the impact of their diverse sugar composition and distribution *in planta* remains unclear. To detail their fine structure, degradation by commercial enzymes of purified native acetylated XyG, GgM and GAX fractions from apple revealed structural variations between different XyG and GgM populations and demonstrated first evidence for arabinose and/or xylose substitution on GAX. However, more detailed fine structural aspects of these polysaccharides require new enzymatic tools with regard to cleaving site specificities. In this aim, we used a genome mining approach coupled with a medium-through put screening procedure to identify hemicellulose-degrading enzymes from marine and land bacteria. Based on the presence of potential hemicellulases in their annotated genome, 4 culturable aerobic and nonpathogenic bacterial strains from marine (*Pseudoalteromonas atlantica, Cellulophaga algicola*), oligotropic (*Caulobacter crescentus*) and terrestrial (*Paenibacillus* sp.) environment were selected. Of the 4 strains, *P. altantica* was chosen for its culture supernatant appreciable activity towards fruit hemicelluloses. The secretomes were fractionated by ion-exchange chromatography to isolate β-glucanase, β-xylanase, β-xylosidase and α-arabinofuranosidase. The analysis of hydrolyzates revealed different substrate specificities, which together with structural analyses of isolated oligosaccharides provided novel insights of apple hemicelluloses complexity.

Key Words
Apple, acetylated hemicelluloses, genome mining, bacteria, hemicellulases, HPAEC