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Ciclo XXIX

Structure and properties of pectin and pectic oligosaccharides from agricultural by-products

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A mia madre

A mia sorella

Ai miei angeli Olga e Giuseppe

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Stefania Baldassarre

"And those who were seen dancing were thought to be insane by those who could not hear the music..." Friedrich Nietzsche

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Abstract

The development of sustainable technologies and strategies for reusing the huge amount (about 90 million tonnes) of food wastes and by-products produced every year in EU has recently become the subject of many research projects. Indeed, food waste contains proteins, fats, sugars, antioxidants, vitamins, polyphenols or fibers with prebiotic properties, making it a suitable raw material for further re-use, such in animal feed or for the production of high-added value compounds. Anyway, its diversity makes its exploitation quite challenging.

The present thesis deals with the use of pectin derived from food waste, studying their characterization, extraction, transformation and re-use. This thesis studied, on one hand, the extraction and characterization of pectin in animal feed, produced starting from stabilized wastes, and on the other hand, the production and the characterization of potentially prebiotic pectic oligosaccharides (POS) from selected food wastes.

Pectin in animal feed produced from food waste was characterized in order to assess the effect of technological treatments on their content and structure. Furthermore, selected wastes, especially sugar beet pulp and onion skins, were exploited and investigated as source of POS.

POS mixtures extracted from sugar beet pulp and separated by using a membrane enzymatic reactor were characterized for their structure; in particular, the main detected oligomers were arabinans (DP2-10), rhamnogalacturonans (DP3-6) in both free and acetylated forms and polygalacturonans (DP3-7), in both free and methylated/acetylated forms. POS mixtures were also tested for their stimulating activity for lactic acid bacteria, monitoring the growth of *Lactobacillus* strains in presence of POS in the growth medium. The pectic oligosaccharides showed a

stimulation effect on the strains growth in a strain-specific and concentration-specific way.

The use of onion skins as new source of POS was also investigated. Pectin was extracted by using the chelator sodium hexamethaphosphate (SHMP) and hydrolyzed in a membrane enzymatic reactor. The continuous cross-flow membrane process was optimized, allowing to achieve high POS yields. The produced POS fractions were again characterized in terms of structure and stimulating activity on lactic acid bacteria. The analysis indicated the presence of polygalacturonans with DP from 2 to 8 in the free form but also in the acetylated /methylated form. Preliminary experiments indicated that POS mixtures, due to the presence of SHMP, had an inhibition effect on the growth of *Lactobacillus* strains, even if the growth, compared with the presence of SHMP alone, was enhanced by the POS. Therefore, more experiments are needed in order to remove SHMP from the extracts and elucidate the true prebiotic activity of POS from onion skins.

The analytical tools used in the present study provided a deep knowledge about the functional and compositional properties of animal feed derived from food wastes in terms of pectin content, and allowed to get deep insights in the fine chemical structure and composition of pectic oligosaccharides, obtained from selected food wastes, outlining the links with the techonology of production, from one side, and with the stimulation effect they can exert on lactic acid bacteria, on the other side. The results of this thesis clearly demonstrated that an economically viable, efficient and controllable production of POS is possible starting from food wastes and food by-products, allowing to obtain functional compounds which can potentially be used as additive and integrators for food and feed.

OUTLINE

The present PhD thesis starts with an overview on the production of food wastes in European Union, then it deals with the effect of technological treatments on food wastes in order to obtain suitable animal feed in terms of pectin content; then, the production and the characterization of pectic oligosaccharides obtained from selected food wastes is studied in deep details.

The Chapter 1 gives a general overview on the production and reuse of food waste in EU, particularly in the framework of the European project NOSHAN; moreover, an overview on the structure, properties, application of pectin is also presented with special regards to pectic oligosaccharides and their production, purification and prebiotic potential. Differently from pectin that can exert an antinutritional value, due to the gelling capability in the gastrointestinal tract, pectic oligosaccharides with low degree of polymerization (DP) (2-10) are able to stimulate the growth of probiotic strains such as *Lactobacillus,* exerting beneficial effects.

The first step of the PhD project consisted in extracting and characterizing the pectin from animal feed. The effect of the technological treatments on wastes was assessed and it was found that freeze-dried and extruded feed have a reduced pectin content, even when starting from pectin-rich food waste. (Chapter 2)

Then, sugar beet pulp (SBP) was selected as source of POS due to the high pectin content. POS obtained after extraction and hydrolysis performed using different methodologies, such as batch system or enzyme membrane reactor, were characterized by hydrophilic interaction chromatography coupled with electrospray mass spectrometry detection (HILIC/ESI-MS). The analysis highlighted the differences among POS fractions produced in different ways (Chapter 3).

POS produced starting from SBP in an enzyme membrane reactor in optimized conditions were characterized on the molecular level by HILIC/ESI-MS analysis;

moreover, POS were assessed on their ability to stimulate the growth of probiotic strains such as *Lactobacillus* strains. (Chapter 4)

In Chapter 5, the production of POS from onion skins was evaluated. POS production was initiated using different enzymes in shake flask experiments. Further the characterization of POS using HILIC/ESI-MS analysis indicated the presence of free as well substituted form of oligomers. The continuous production of POS from onion skins using the enzyme membrane reactor was optimized, achieving the highest POS yield at two different substrate concentrations. (Chapter 6)

The composition of POS fractions produced along the cross-flow membrane process was determined by HILIC/ESI-MS analysis. Then, the impedometric microbiology was used in order to assess the effect of the POS fractions on the growth of *Lactobacillus* strains. (Chapter 7)

Finally, general conclusions and future perspectives are presented in Chapter 8.

1. General Introduction

1.1 Food waste and its valorization

Food wastes are globally generated by food processes on multi tonne scale every year. Given the great quantities of waste produced and the need to find new renewable resources, food waste is more and more used as a renewable biorefinery feedstock. (Lin *et al*, 2013).

The European Union produces each year about 90 million tonnes of food waste, 38% of which is directly produced by the food manufacturing sector. (Gustavsson *et al*, 2012)

As depicted in Fig.1.1, the food waste is characterized by a diverse chemical content making the waste a suitable raw material for the production of high-added value compounds; thus, chemical and food industry could potentially benefit from the use of such a renewable resource and a circular economy could be built up. (Moran *et al*, 1999; Budarin *et al*, 2011)

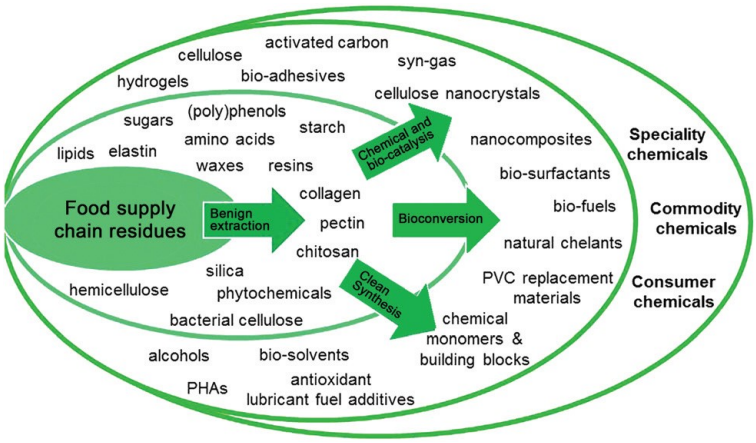

Figure 1.1: Components present in food waste and their use in common consumer applications, highlighting sectors of the chemical industry that could benefit from the use of such a renewable resource. (Pfaltzgraff *et al,* 2013)

In general, food residues contain many valuable functionalised molecules, such as flavonoids, biopolymers, fatty acids, etc. However, most of these sources are lost or underutilised when food wastes are used for electricity generation (i.e. through anaerobic digestion), fuel generation (i.e. conversion of cellulosic biomass to bioethanol) or the production of animal feed.
Therefore, the research is moving towards the development and the exploitation of technologies and strategies suitable for the recovery of valuable compounds, rather than their use as bulk material.

Many aspects should be evaluated in choosing and recycling wastes. One of them is for sure the availability: high volumes are needed in order to achieve an economically viable scale. Moreover, the variety of extractable molecules and polymers is also very important enhancing the chance to produce several components from a single waste stream. (Pfaltzgraff *et al*, 2013)
In terms of logistics, agricultural residues or food processing residues should be preferred compared to the post-consumer waste, because they are generated in a more concentrated manner (Hughes *et al*, 2011) and are more homogeneous.

Furthermore, among the food wastes, it is important to distinguish between animal-derived wastes and plant-derived wastes. Actually, the valorization of the first one is less feasible because of the sanitary risk related to transmissible encephalopaties and the strict legislation in waste managment. On the other hand, vegetable wastes show a lower risk of prions infections, even if some infections of *E.coli* or *Salmonella* species could occur in case of not proper storage of wastes. (Pfaltzgraff *et al*, 2013)

The European project NOSHAN ("Production of functional and safe feed from food waste", FP7, Grant agreement n°312140, www.noshan.eu) aimed to investigate the process and the technologies needed to use food waste of vegetal and dairy origin for feed production at low cost, low energy consumption and with the maximal valorization of starting waste material. Plant and dairy waste and by-

product could be an excellent source of, for example i) protein, fat and sugars as nutrient source; ii) functional protein hydrolysates enriched in active peptides for promoting gut health, enhance immune responce and increase bioavailability; iii) antioxidants, vitamins or polyphenols which can improve the oxidative status and enhance animal performance; iv) fibers with prebiotic properties which can improve the health of the gut.

Despite the obvious potential, the utilization of feed from waste in diet formulation has, until now, been negligeable, because of constraints imposed by several nutritional and technical considerations. (El Boushy and Var Der Poel, 2001)

Basing on this evidence, NOSHAN developed new technological/biotechnological processing tools for the production of functional and safe food starting from wastes. One of the aims of the project was the creation of a broad portfolio of characterized valorisable wastes (fruits, vegetables, root and tubers, cereals and dairy wastes products) for feed production. With this aim, a selection of wastes according to their potential nutritional properties, quantities produced, seasonality, possibility of stabilisation, safety and regulatory issues, cost and logistics was performed during the first phase of the project. The selection was done also considering the importance of the wastes in the European agricultural production.

After characterizing the wastes for their nutritional potential, suitable technologies were studied in order to stabilize and convert wastes into bulk feed; in particular waste was treated alone or mixed with other wastes looking for complementation and synergistic effects. The characterization at the molecular level of bulk feed allowed to provide the best technology and the best raw material in terms of nutritonal/functional properties.

The way of obtaining functional feed ingredients (additives) from waste was then studied, since feed additives constitute an important factor determining the final feed cost and animal functionality, and they have a larger added value than bulk feed. The production of safe and functional feeds from stabilized wastes and functional feed

additives also obtained from waste was then investigated. NOSHAN aimed also at the demonstration of the feasibility of the production at pre-industrial level by scaling-up the most efficient technologies. Moreover, it aimed at the validation of the efficacy of the functional compounds on the performance, health and welfare of weaning pigs and poultry in designed animal trials.

NOSHAN targeted to have a direct impact on the environmental field by reducing the quantity of organic waste through reuse, and reducing the GHG emissons by applying novel low energetic technologies and limiting the impact of food wastes.

1.2 Pectin

1.2.1 Origin

Pectin can be considered one of the major plant cell wall components and one of the most complex macromolecule in nature. It is found in the primary cell walls of all higher plants where it accounts for approximately the 35%, together with the 30% of cellulose, the 30% of hemicellulose and the 5% of protein (Fry *et al*, 1988). The primary cell wall between the plasma membrane and the middle lamella is represented in Fig.1.2. The pectic polysaccharide fibers are shown in blue and they are part of a complex network interacting with cellulose and hemicellulose (Alberts *et al*, 1994).

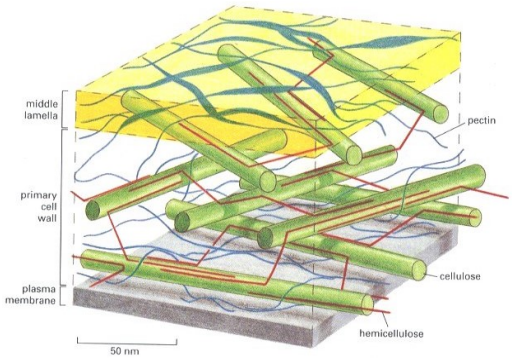

Figure 1.2: Polysaccharides in primary cell wall of higher plants (Alberts *et al*, 1994).

Pectin plays an important role in the cell wall exerting many functions that are essential for the plant as well. (McCann *et al*, 2001). The pectic matrix resists the compressive forces acting upon cell walls, provides porosity, contributes to the ionic status, and has important roles in the plant's defense mechanisms (Basic *et al*, 1988). In fact, pectin oligosaccharides are known to activate plant defense responses: they elicit the accumulation of phytoalexin which has a wide spectrum of anti-microbial activity (Jin & West, 1984; Nothnagel *et al.*, 1983). Moreover, pectin polyaccharides induce lignification and accumulation of protease inhibitors in plant tissues (Bishop *et al.*, 1984).

1.2.2 Chemical structure

Pectin is a heterogeneous carbohydrate mainly constitued by galacturonic acid (GalA) residues and rhamonose residues (Rha) linked to neutral sugars side chains. Pectin structures can be distinguished in "smooth" regions, which are homogalacturonic regions, and ramified "hairy" regions, in which most of the neutral sugars are located (De Vries, 1982).

A schematic representation of pectin structural elements is given in Fig.1.3.

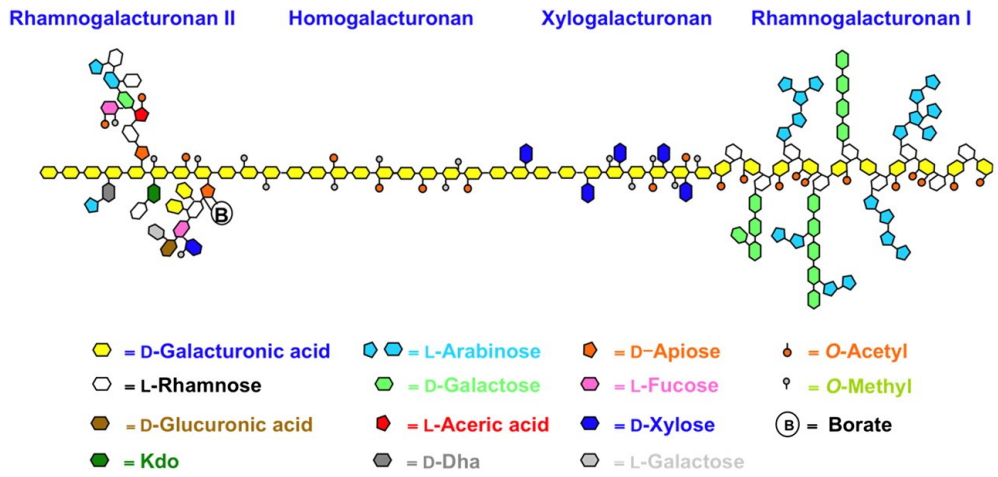

Figure 1.3: Schematic structure of pectin. (Harholt *et al,* 2010).

The major components of pectin are:

- Homogalacturonan (HG): it is a linear chain of 1,4- linked α-D-galactopyranosyluronic acid (GalpA) residues. GalA moieties within the pectin backbone may be methyl esterified at C-6 and/or *O*-acetylated at *O*-2 and/or *O*-3. The degree of methylation in native pectins is generally in the order of DM ~70-80; whereas degree of acetylation is generally much lower *e.g.* DAc ~35 for sugar beet pectin. Blocks of more than 10 non-esterified GalA residues are involved in Ca^{2+}crosslinking mediated by carboxyl groups of galacturoic acid ring (Rombouts & Thibault, 1986; Daas *et al.*, 2001).

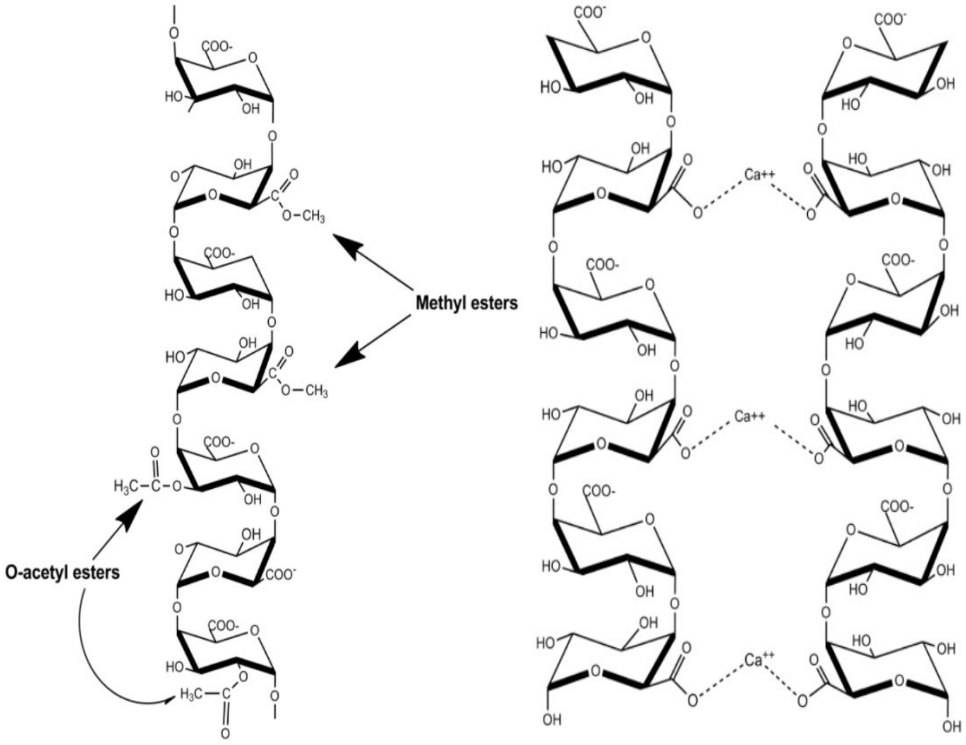

Figure 1.4: a) Homogalacturonan structure and modification. Representative sites of methyl-esterification at the C-6 and O-acetylation at the O-2 or O-3 of the carbohydrate ring are shown. (Ridley, B. L, 2001); b) The egg-box model of calcium crosslinking in HG polysaccharides (Caffal *et al*, 2009)

- Rhamnogalacturonan-I: it consists of the repetition of the disaccharide [-4)-α-D-Gal*p*A-(1-2)-α-L-Rha*p*-(1-]^n, where the galacturonic acid units may be

O-acetylated on O-2 and /or O3, but not methylated. On the other hand, the rhamnose residues can be substitued at O-4 with neutral sugar side chains such as arabinogalactan I and arabinan. (Leuroge *et al*, 1993).

- Rhamnogalacturonan-II: it is made by HG backbone of at least eight 1,4-linked α-D-GalA residues; GalA units are linked to side branches consisting of 12 different types of sugars, such as apiose, aceric acid, 3-deoxy-lyxo-2-heptulosaric acid (DHA) and 3-deoxy-manno-2-octulosonic acid (KDO), in over 20 different linkages. The structure of RGII seems to be highly conserved in the plants. (O'Neill MA *et al*, 2004)

- Xylogalacturonan: it is defined as homogalacturonan substitued at O-3 with xylose, that can be further substitued at O-4 with an additional xylose. (Zandleven *et al*, 2006).

- Arabinan: It has a backbone made by 1,5-linked α-L-arabinose residues which usually is substitued with α-L-Ara*f*-(1-2)-, α-L-Ara*f*-(1-3)-, and/or α-L-Araf-(1-3)-α-L-Araf-(1-3)-side chains. It can be present in the form of Arabinogalactan I, composed of 1,4 linked β-D- Gal*p* backbone with α-L-Ara*f* residues attached to O-3 of the galactosyl residues. Furtheremore, it can be found as Arabigalactan II made by 1,3 linked β-D-Gal*p* backbone, containing short side chains of α-L Ara*f* –(1-6)-β-D-Gal*p*-(1-6). (Ridley *et al*, 2001). Arabinogalactan II is mainly associated with proteins (3-8%), so called arabinogalactan proteins. (O'Neill MA *et al*, 2003)

The complexity of pectin can vary depending on the source.

1.2.3 Extraction and characterization

Many analytical tools are available in order to extract and characterize pectin from agricultural by-products, and in general from vegetable sources.

1.2.3.1 Extraction

In the isolation and recovery of pectin, the extraction process is a very important step since it influences the yield and the physicochemical characteristics and functional properties (Babbar *et al,* 2016; Koubala *et al*., 2008; Yapo et al., 2007; Levigne *et al,* 2002). Pectin can be extracted under acidic condition using oxalic (Koubala et al., 2008), hydrochloric (Hwang *et al*, 1998), and sulphuric acid (Garna *et al*., 2007); the use of strong acids provides high extraction yield and time-saving advantages. However, it can cause environmental problems such as the disposal of acidic wastewater, and also plays a negative role in consumer preference. Therefore, alternative methods have been developed. It is reported that chelating agents such as EDTA solution have a greater (double) extraction capacity than water. Indeed, the EDTA molecules act as chelating agents which are able to prevent the interaction of calcium ions with the pectin molecules. Therefore, pectin molecules are removed more easily from the cell wall, thus increasing the extraction performance. (Yeoh *et al,* 2008)

Alkaline extraction is also commonly investigated, but can result in a decrease of the degree of methylation (DM), acetylation (DA), and the length of the galacturonic acid main chain by β-elimination (Renard *et al*, 1990).

Overall, the highest amount of pectin is generally obtained by hot acid extraction that is also the most convenient approach for industrial purposes (May, 1990).

1.2.3.2 Purification

Various techniques are available in order to purify pectin after extraction. One of the most commonly used, especially on industrial scale, is the alcohol

precipitation (Levigne *et al*, 2002), because it is suitable for large-scale production of these polysaccharides. In this case, pectin samples must be enriched in uronide content and the other compounds (free sugars, salts, and so on) are removed by washing the precipitate with different concentrations of alcohol in order to obtain pectin with high purity.

Other precipitation techniques include dialysis (Ralet *et al,* 1994), metal precipitation (Chou, 1989) or ion exchange chromatography (Romboutsand & Thibault, 1986).

1.2.3.3 Molecular characterization

A complete characterization of pectin can be achieved thanks to different analytical methods reported in literature.

In particular, pectin can be analyzed mainly in terms of:

- Galacturonic acid and neutral sugars content, determined after pectin hydrolysis: colorimetric methods such as phenol-sulfuric acid method can be used with the disadvantage that each pectin sugar can give different degree of color intensity at the same concentration. (Melthon and Smith, 2001)

  Gas/liquid chromatography (GLC, HPLC) can also be used; they require the derivatization of monosaccharides to alditol acetates or to trimethylsilyl ethers. (Churms *et al,* 1990)

  However, a more sensitive analysis can be achieved using the high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD); in this case, the analysis can be performed without further treatment to assess the monosaccharide content and after complete hydrolysis to determine the total saccharide content. (Corradini *et al*, 2012).
- Degree of methylation and acetylation: it can be estimated by determining the proportion of methyl esters in the pectin sample in conjunction with the

total uronic acid content; a HPLC system fitted with a refractive index detector is necessary. (Voragen *et al*,1986)

H-NMR spectroscopy can also be used; the percentage of methanol and acetic acid is determined on the total amount of galacturonic acid. (Müller-Maatsch *et al*, 2014).

- Molecular distribution: the high performance size-exclusion chromatography (HPSEC) is a powerful technique for determinig the polymer distribution; it can be coupled with a differential refractive index (RI) detector. Carbohydrates are separated according to their molecular size and the separation also depends on the ratio of their molecular dimensions to the average diameter of the pores of the stationary phase. (M. Marry *et al*, 2003)

  The molecular mass distribution of the pectin can also be determined using the gel permeation chromatography (GPC). This method requires assumptions of molecular conformation or calibration using standards of known molecular weight. (Hourdet and Muller, 1991)

1.2.4 Applications

Pectin has found many application in food, pharmaceutical and other industries thanks to its structural and physical characteristics. For instance, pectin from apple or citrus is mostly made by homogalacturonan (Voragen *et al*, 2009) and it is able to form gels under defined conditions. This property is widely exploited within the food industry for the preparation of jams and jellies (Laurent *et al*, 2003)

Then, pectin can be used as stabilizer, emulsifier, film former, lubricant, coagulant and caption binding agent. (Ptchkina *et al*, 2008) Moreover, pectin is investigated as matrix for drug delivery system. Indeed, pectin is able to resist against protease and amylase which are active in the upper gastrointestinal tract. (Yu *et al*, 2009)

1.2.5 Health effects

As fibers naturally present in vegetables and agricultural by-products, pectic substances fulfill a nutritional function. Many studies are conducted in order to understand which is the effect of this polymer on health and the potential beneficial effects. For instance, Behall and Reiser suggest that pectin, as a kind of dietary fiber, lowers blood cholesterol levels and low density lipoprotein cholesterol fractions without changing high density lipoprotein cholesterol or triglycerides, which are good for health. It is also shown how pectin is able to remove metals like mercury from the gastrointestinal tract and respiratory organs (Kohn, 1982). Pectin are also found to slow down the absorption of glucose in the serum of dibetic and obese patients (Trumbo *et al*, 2002).

Furthermore, pectins are shown to have immuno-regulatory effects in the intestine (Langhout *et al*, 1999).

Despite all these positive aspects, pectin can also have some negative effects. Actually, pectin can reduce the absorption of food and rate of digestion immobilizing food components in the intestine. The absorption is influenced by the thickness of pectin layer that prohibits contact between intesinal enzyme and food, reducing the latter's avalability. Moreover, pectin can slow down the gastric empting e reduce the transit time; it can interfere with the metabolism of ions and of medicine, reducing the latter's effect. In regards to animal feeding, such effects would decrease the animal performance and wellbeing. (Dunaif & Schneeman, 1981; Flourie *et al*., 1984). Furtheremore, pectin gives a feeling of satiety due to its waterbinding capacity, thus reducing food consumption. Such effects can be negative on normal patients, but they can be used in the treatment of disorders for instance related to overeating (Di Lorenzo *et al.,* 1988).

On the other side, there are some evidences that low molecular weight pectin, thus in the form of pectic oligosaccharides (POS), have a prebiotic potential, better

than high molecular wheigth polymer. (Al-Tamimi *et al*., 2006). Some *in vitro* studies give a clear indication that POS can be successfully used to promote bifidogenic intestinal flora (Gullon *at al*, 2011). Therefore, there is an increasing interest in studying the bio-functionality of pectic oligosaccharides, thus in developing methods and procedures in order to convert pectin in short and low molecular weight saccharides.

1.2.6 Pectin depolymerization

Pectin represents the starting material to obtain pectic oligosaccharides (POS) with different degree of polymerization (DP). (Babbar et al, 2015; Combo *et al*, 2012; Mandalari *et al.,* 2006) In general, three strategies can be used for producing POS, i.e. i) extraction from plants (Ducasse *et al*, 2008); ii) synthesis (Nemati *et al*, 2008); iii) depolymerization of the polysaccharides. This latter is considered the most competitive method because it allows obtaining different kind of oligomers from one polymer. (Courtois, 2009).

Among the methods known for depolymerizing pectin, which are acid hydrolysis (Hu *et al*, 2009), physical degradation (Byun *et al*, 2006), and enzymatic hydrolysis (Zheng & Mort, 2008), the enzymatic treatment is considered as safe due to minimum adverse chemical modifications of products (Kim & Rajapakse, 2005). Various enzymes are widely used for the production of POS because of their specificity and selectivity. Moreover, given the substitutions and the complex structure of pectin, several enzymes can be required simultaneously or in sequence for degradation. (Searle-van Leeuwen *et al*, 1995)

Among the depolymerizing enzymes, the endo-polygalacturonase (Endo-PG) is widely used for pectin hydrolysis (Jayani, 2005) It is able to cleave the glycosidic bond of the α-(1-4)-polygalacturonan in a random way (Cameron *et al*., 2009) and it generally prefers a nonesterified substrate showing decreasing activity with an increasing degree of methyl esterification (Parenicova *et al*., 2000).

The “hairy” region made by rhamnogalacturonan (RG) can be degraded sequentially by rhamnogalacturonan hydrolase and rhamnogalacturonanylase both acting on α-D-1,4-GalpA-α-L-1,2-Rhap and α-L-1,2-Rhap-α-D-1,4-GalpA linkage of the RG backbone, respectively. (Mutter *et al*., 1994)

Moreover, the degradation of side chains from RGI can be achieved by using a cocktail of various enzymes such as arabinase, that can remove terminal (exo-activity) or linear regions (endo-activity) of the arabinan (Westphal *et al*., 2010), endo-galactanase, which are able to degrade relatively long (1-4)-linked galactan side-chain, or β-galactosidase which is able to remove terminal galactose residues from galactans or arabinogalactans (Pedrolli *et al*., 2012).

Depending on the desired product, specific enzymes, using different action mechanisms, can be choosen. A synergistic action or the degradation of different pectin regions can be achieved using mixtures of pectinase, especially when intense pectin breakdown is required, as for example in improving fruit/vegetable juice yield extraction and in the treatment of pectin-rich waste materials. (Van Buggenhout *et al*, 2009).

The synergistic effect of cellulolytic and pectinolytic enzymes in the hydrolysis of sugar beet pulp was studied by Spagnuolo *et al* (1997) and the combination of cellulase, hemicellulase, and pectinase was found to be very effective as compared to acid hydrolysis. They also observed that pectinase is the most important enzyme, because it favors the degradation of cellulose and hemicellulose by the other enzymes.

Babbar N. (2016) reported the conversion of sugar beet pulp pectin to POS using one step approach in central composite design (CCD); in particular, two enzymes, Celluclast and Viscozyme, were studied and the operational conditions are optimized.

The catalytic action of two arabinases (a-L-arabinofuranosidase and endo-arabinase) on sugar beet pulp was also studied (Spagnuolo *et al*, 1999): it was demonstrated that, applying commercial enzymes, the sugar beet pulp fractionation into arabinose,

pectin and cellulose result in fractions quantitatively and qualitatively similar to those obtained by traditional chemical methods, but with the advantage of avoiding the use of acids.

Combo *et al* (2012) compared the yield of pectic oligomers obtained using the endopolygalacturonase EPG-M2 with other commercial pectinases and enzyme preparations, such as pectinase 62L and Viscozyme L. As expected, EPG-M2 resulted in the most efficient enzyme in terms of POS yield, but comparable values were obtained using the other preparations, showing how they can be used for the purpose.

In general, oligosaccharides production by enzymatic hydrolysis is influenced by process parameters, such as time, temperature, enzyme concentration, absence and presence of particular enzyme (Martinez *et al*., 2009); and a specific enzyme or enzyme preparation can be chosen in order to improve POS production.

1.3 Pectic oligosaccharides (POS)

Various types of pectic oligosaccharides (POS) (substituted or non-substituted) can be obtained by pectin depolymerization. The most common and well known POS are oligogalacturonides (OGalA), galactooligosaccharides (GalOS), arabinooligosaccharides (AraOS), rhamnogalacturonoligosaccharides (RhaGalAOS), xylooligogalacturonides (XylOGalA) and arabinogalactooligosaccharides (Ar GalOS). (Concha-Olmos & Zuniga-Hansen, 2012; Martinez *et al,* 2009).

Pectic oligosaccharides are proposed as a new class of prebiotics capable of exerting a number of health-promoting effects. They are representatives of the new products with improved functional properties that global market is looking for. (Hotchkiss *et al*, 2004). In this context, the extraction of neutral and acidic polymers in the form of pectic oligosaccharide from agricultural by-products is a promising step towards the manufacture of prebiotics (Munoz *et al,* 2012; Westphal *et al*, 2010).

1.3.1 Purification and fractionation

After extraction and depolymerization of pectin, the liquid media may contain contaminants and co-extracted molecules that should be removed before utilizing the oligosaccharides. Moreover, in the enzymatic hydrolysis of pectin, the molecular weight of the final product is very important because the functional properties of pectin and its oligosaccharides depend on the molecular size. Nevertheless, the studies assessing the potential bioactivity of pectin are performed using mixtures of pectin fractions with a broad size distribution. However, it should be taken into account that using pectic oligosaccharides mixtures can blur the results and prevent the deep understanding of the structure-function relationship in relation to health promoting effect (Holck *et al*, 2011).

Hence, POS purification and/or fractionation is a necessary step, particularly starting from complex plant material or mixed hydrolysates. The most usual purification technologies are Ion Exchange Chromatography and Gel Filtration (GF) Chromatography which are useful to separate products with different properties (for example charge, molecular weight and substitution pattern), even if they do not allow the separation of tailored compounds (Guillaumie *et al,* 2006; Yu *et al*., 2010).

More recently, the use of membranes has received increasing interest for the fractionation/purification of POS. Membranes are used not only to fractionate the POS product after hydrolysis, so as downstream processing technology, but also integrated with the hydrolysis to achieve more tailored saccharide products. Therefore, the development of membrane reactors represents the result of this integration. This technology provides many advantages over the traditional batch reactor: i) a more efficient continuous process can be achieved, ii) the enzyme can be recycled and reused providing higher productivity per unit enzyme, iii) the molecular weight distribution of fractions in the hydrolysate can be controlled choosing a specific molecular weight cut off. (Giorno & Dioli, 2000)

For these reasons, the membrane reactor system is investigated as new technology to hydrolyse polysaccharides and separate the obtained oligosaccharides from the high molecular weight enzymes and remaining polysaccharides. Moreover, integrating the setup with continuous or semicontinuous product removal, the product inhibition of the enzymatic reaction can be avoided. (Guadix *et al*, 2006; P. Czermak *et al*, 2004) Some examples of the use of membrane technology are reported. A regenerated cellulose membrane of 3 kDa Molecular Weight Cut Off (MWCO) was employed by Holck, *et al* (2011). Oligomers obtained from citrus pulp pectin were purified using a 50 kDa MWCO membrane to retain the high MW compounds, and processed the resulting filtrate through a 15 kDa MWCO membrane to wash out monosaccharides and saccharose (Iwasaki and Matsubara, 2000). Moreover, Olano-Martin *et al* (2001) investigated the continuous production of pectic-oligosaccharides in an ultrafiltration dead-end membrane reactor via controlled enzymatic hydrolysis of pectin. Babbar N. (2016) investigated the continuous production of POS from sugar beet pulp in an enzymatic membrane reactor (EMR); the operational conditions of the cross-flow membrane process were optimized in order to get the highest POS yield.

1.3.2 Molecular characterization

The fractions resulting from the pectin depolymerization can be analyzed using analytical tools such as high performance anion-exchange chromatography (HPAEC), capillary electrophoresis (CE) and mass spectrometry (MS). The characterization of POS is quite difficult because of the complex chemical composition of pectins and the possible chemical alteration during POS manufacture.

HPAEC can be used thanks to the fact that many carbohydrates are weak acids with p*K*a values in the range 12-14 and, consequently, at high pH values their hydroxyl groups are partially or totally transformed into oxyanions, enabling this class of compounds to be selectively eluted as anions by high-performance anion

exchange. Quaternary-ammonium-bonded pellicular anion exchange columns are used to separate carbohydrates under alkaline conditions where the order of increasing retention is correlated with decreasing p*K*a value. In order to detect the sugars, a pulsed amperometric detector is coupled with the chromatography. (Lee, 1996). However, this technique can be limiting because the high pH eluent used for separation and detection results in saponification of methyl esters and acetyl groups from the analytes, without allowing their detection. (Ralet *et al*, 2005)

The capillary electrophoresis (CE) with laser-induced fluorescence (LIF) and mass spectrometry detection can also be used for the analysis of cell wall derived oligosaccharides (Coenen *et al,* 2008; Albrecht *et al,* 2009), even if this technique shows also some disadvantages. In fact, a fluorescent chromophore is required in order to label the oligosaccharides at the reducing end, this means that non-reducing sugars are excluded from the analysis. Moreover, the additional negative charges make the acidic oligomers too fast in migrating and subsequently difficult to separate. (Coenen *et al*, 2008)

Among the techniques used to separate oligosaccharides, there is also the porous-graphitic carbon (PGC) high performance liquid chromatography coupled with evaporative light scattering and mass spectrometry detection (HPLC–ELSD–MS). (Pabst *et al*, 2008; Ruhaak *et al,* 2009) In this case, neutral oligosaccharides are separated based on their size as well as on their type of linkage. The charged oligomers are strongly retained, and they are eluted only after adding an acid modifier. The main disadvantage of this technique is that is not selective for the size of the charged oligomers, since they are co-eluted all at the same time. (Westphal *et al*, 2010)

The development of soft ionization techniques, in the recent years, has lead to the use of mass spectrometry in the field of the oligosaccharides characterization. (Hemström *et al*, 2006) In particular, the Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) technique allows the determination of the molecular masses of neutral and acidic oligosaccharides (Papac

et al, 1996). It is characterized by the tolerance to residual salts, the relative simple sample preparation and high speed of analysis. However, the limit of this technique is that it can not be combined a with separation system since the samples have to be crystallized with a matrix that absorbs the energy of the laser and ionizes the samples. (Brüll *et al*, 1998)

In the present field, the importance of mass spectrometry has increased significantly, especially to gain more detailed structural information of a specific compound through multiple MS analysis stages, and thanks to the possibility to combine separation methodologies with more sensitive techniques.

For instance, the hydrophilic interaction chromatography (HILIC) coupled with electrospray mass spectrometry detection is an alternative method of separation of highly polar compounds. (Leijdekkers *et al*, 2011) This technique combines a hydrophilic stationary phase with an organic mobile phase and the elution is carried out by increasing the water concentration. Mono-, di-, trisaccharides, maltooligosaccharides and xylan-derived oligosaccharides are separated efficiently. (Ridlova et al, 2008; Alpert *et al*, 1994) The sensitivity of this technique is due to the high fraction of organic solvent that provides an efficient droplet formation and desolvatation within the mass spectrometry source and a faster separation due to the lower viscosity as compared to the aqueous RP-mobile phases (RP, reverse phase) (Nguyen *et al*, 2008) Moreover, the value of electrospray ionization ion trap mass spectrometry (ESI-IT-MS) is assessed also for determining the exact location of acetyl ester groups on O-2 and/or O-3 of purified oligogalacturonides, providing information on the esterification pattern of pectic oligosaccharides. Actually, this knowledge can be useful for studying the fine chemical structure of pectins as well as the action mechanisms of various pectinolytic enzymes (Que´me´ner, *et al*, 2003)

Overall, the structure of pectic oligosaccharides can not be defined using only a single analytical tool, especially dealing with complex mixtures. Therefore, combining different preparation procedures with different analytical methods is needed

to allow a deep study of the structure and composition of so complex carbohydrates such as pectin.

1.3.3 Prebiotic potential

"*A dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health*". This is the the definition of prebiotics given by the International Scientific Association for Probiotics and Prebiotics.
Pectic oligosaccharides, obtained from waste biomass or low-cost by-products (such as apple pomace, orange peels or sugar beet pulp) are considered as novel prebiotics, since they are potentially able to enhance the growth of beneficial health promoting bacteria.
It is important to highlight that the prebiotic potential of pectin-derived oligosaccharides depends on their physicochemical characteristics, especially in terms of molecular weight and degree of esterification.

Several studies prove how they are able to stimulate the growth of beneficial microrganisms. In particular, Rastall *et al* (2005) reported that oligosaccharides derived from pectins by either enzymic hydrolysis or by flash extraction show potential prebiotic properties, as they selectively increase the populations of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* and decrease undesirable bacteria such as *Clostridia.* Hotchkiss *et al* (2004) reported that oligosaccharides derived from high-methoxy citrus pectin, low-methoxy apple pectin and orange peel enhance the growth of *Bifidobacteria* and *Lactobacilli* while limiting the growth of pathogens in mixed fecal batch cultures. *In vitro* fermentation of pectic oligosaccharides derived from Valencia oranges, mainly consisting of arabinogalactan pectic side chains and xyloglucan, were fermented in vitro and showed bifidogenic effects, as revealed by the increases in acetate, butyrate and propionate concentrations upon fermentation.

(Hotchkiss *et al*., 2007) In general, these effects are based on the more selective fermentation of oligomeric compounds in comparison with the parent polysaccharides. Moreover, higher fermentation selectivity is obtained with substrates of lower degrees of methylation.

Concerning the fermentation of POS, it can be performed:

- By individual strains; Olano-Martin *et al*. (2002) studied the *in vitro* fermentation properties of pectins and POS by human gut bacteria using fecal cultures, and reported a negative influence of the degree of esterification (DE) on cell growth, and increased digestibility of POS respect to pectins.
- By fecal inocula; The fermentation of POS from orange peel by mixed fecal bacterial cultures was reported to result mainly in the production of acetic and butyric acids, and in increased numbers of *Bifidobacteria* and *Eubacterium rectale* (Manderson *et al*., 2005). Fermentation of a POS-rich extract from bergamot peel with fecal inocula resulted in increased counts of *Bifidobacteria, Lactobacilli* and *Eubacteria,* together with decreased *Clostridial* population.

  Recently, arabinooligosaccharides from sugar beet pulp (with or without feruloyl subtituents, DP 2-14) were assayed *in vitro* for fermentability using human fecal inocula (Holck *et al*., 2011).
- In humans; administration of mixtures of pectic and neutral oligosaccharides to infants resulted in increased concentrations of *Bifidobacteria* and *Lactobacilli* in stools, softer stool consistency, and decreased fecal pH, suggesting that the proposed combination is clinically safe and effective on infant microbiota (Magne *et al*., 2008).

Stimulating the growth of probiotic strains such as *Lactobacillus* strains or *Bifidobacteria* strains resulted in the production of Short Chain Fatty Acids (SCFA), which exert several healthy effects. In particular, POS were found to be capable of exerting a number of health-promoting effects, including protection of colonic cells

against Shiga toxins (Olano-Martin *et al*, 2003); prevention of the adhesion of uropathogenic microorganisms (Guggenbichler *et al*, 1997), stimulation of apoptosis of human colonic adenocarcinoma cells (Olano-Martin *et al*, 2003), cardiovascular protection *in vivo* (Li *et al*., 2010), reduction of damage by heavy metals, antiobesity effects, dermatological applications and antitoxic, antiinfection, antibacterial and antioxidan effects. Additionally, *in vivo* and *in vitro* studies confirmed that acidic POS are not cytotoxic or mutagenic, being suitable for use in food and feed (Garthoff *et al*., 2010).

Also, in feed industry there is an increasing interest in the research of new functional products that can replace the traditional additives, in order to increase animal health and decrease the use of veterinary drugs (Gaggia *et al*, 2010), and POS can contribute to the market of feed additives since they show promising potential to be used as prebiotic compounds.

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2. Characterization of pectin in animal feed derived from food waste

Abstract

The European food sector produces each year about 90 million tonnes of wastes. (Laufenberg *et al*, 2003). Vegetal wastes represent a source of valuable molecules such as sugars, proteins, fibers, antioxidants, vitamins and other bioactive agents. Among such wastes, specific agro-industrial side streams are already processed in refineries into primary substrates for other food products, but also for feed, industrial fermentation and some chemical processes. However, underutilized by-products could be exploited for the extraction and valorization of added-value compounds that can be reused by chemical, pharmaceutical, food and feed companies. Hence, the research is focusing on the development of suitable strategies and technologies in order to valorize the huge amount of available food wastes.

However, in order to ensure the quality of products derived from wastes, the latter should be stabilized for amount and composition and improved physical, chemical and biotechnological treatments should be developed.

In the frame of European project NOSHAN, food wastes, especially of vegetal origin, were selected and investigated for their potential to be converted in animal feed. One of the most important compounds present in such agricultural by-products is pectin. Pectin is classified as soluble fiber but, differently from other fibers present in vegetables and fruits, it does not exert only positive effects on health. Indeed, as a high molecular weight polymer, it might interfere with the normal metabolism and nutrients absorption in the gastrointestinal tract, due to its gelling properties. Given the negative impact it can exert on overall animal wellness, it is important to assess

the amount and the characteristics of pectin present in food wastes in order to ensure the suitability of the sources to be used as animal feed.

The present study aimed at analyzing pectin from bulk feed, feed ingredients and final feed produced starting from the selected wastes. Pectin was characterized in terms of content and structure, especially degree of methylation and acetylation, and neutral sugars content.

After technological treatments and stabilization, the matrices constitued by fresh pumpkin showed promising properties in the perspective of further feed production. Freeze dried pumpkin and extrudate pumpkin could be considered favorable in terms of nutritional properties, since they showed to have high fiber content and low pectin content.

2.1 Introduction

Food wastes represent a potential source of exploitable valuable compounds and the interest for its valorization is increasing in recent years. Actually, the amount of food waste generated every year in Europe accounts for 90 million tons, of which 38% originates from the food industry. (Laufenberg *et al,* 2003)

In order to upgrade and valorize agro-industrial side streams, improved physical, chemical and biotechnological treatments are needed and should be developed. Indeed, before being treated and reused, the wastes should be stabilized for composition to ensure the quality of the products derived from them. Thus, it is easier and more convenient to focus the attention on the wastes derived from food streams than on wastes derived from consuption. (Pfaltzgraff *et al,* 2013)

Food wastes are only partially valorized at different value-added levels (spread on land, animal feed, composting), whereas large fractions are disposed as wastes, with negative impacts on the sustainability of the food processing industry. (Federici *et al,* 2009)

Agricultural crops such as corn, wheat, rice and other cereals, sugar cane and beet, potato, tapioca, etc. are already processed in the starch and sugar refineries into relatively pure carbohydrate feedstocks (starch, sugars, *etc*), which are the primary substrates for the food industry, but also for most industrial fermentation processes and for some chemical processes. (Kamm and Kamm, 2004)

Moreover, valuable molecules such as proteins, fibers, antioxidants, vitamins and other bioactive agents, could be extracted from vegetal wastes and reused by chemical, pharmaceutical, food and feed companies. (Federici *et al*, 2009)

In this context, the European project NOSHAN was developed and during the first phase, a selection of wastes was performed, according to their potential nutritional properties, quantities produced, seasonability, possibility of stabilization, safety and regulatory issues, cost and logistics. Also, the importance of the wastes

for the European agriculture and the wastes potential, in terms of feed and feed additives, were part of the selection criteria.

Therefore, the production of bulk feed and feed ingredients, starting from the huge amount of organic waste derived from food, represented the aim of NOSHAN project. Moreover, since pigs and poultry were chosen as target group, wastes were mixted in order to win a synergistic effect in terms of nutritional values for these animals.

One of the most important component of vegetal sources is surely the fiber. Indeed, the cell wall of vegetative plants are characterized by a structure in which randomly oriented cellulose microfibrils are associated with other polymers, such as pectin, forming a complex nework. The term "dietary fiber" includes two categories, soluble and insoluble dietary fiber. Soluble dietary fiber are those components that are soluble in water and includes pectic substances and hydrocolloids. Good sources of soluble fibers include fruits, vegetables, legumes. Insoluble dietary fiber are those components that are insoluble in water and includes cellulose, hemicellulose and lignin. Whole grains are good sources of insoluble fiber. (Wardlaw *et al,* 1997)

Many studies have proved the importance of the fiber intake, given the beneficial effects of fiber consuption, especially in protection against heart disease and cancer. (Aldoori *et al,* 1998; Jenkins *et al,* 1998; Salmeron *et al,* 1997; Kritchevsky and Bonfield, 1997). However, the gel-forming polysaccharides, known to inhibit the nutrients and minerals absorption in the gastrointestinal tract, are also included in the classification as fiber. Therefore, talking about fiber, it is necessary to specify which is the polymer under study not to generate confusion, given the different effect that the same polysaccharide category can exert. (Livesey *et al*, 2004; Topping *et al,* 1991; Mathers *et al*, 1992)

In the present study, the attention will be focused on pectin that is classified as a soluble (dietary) fiber. The interest toward this complex polysaccharide is increasing. Indeed, the polymer shows interesting properties for many industrial

applications, for instance as emulsifier, thickner, gelling agent, *etc.* However, the polymer can also have a negative influence. In fact, in the form of a high molecular weight polymer, it can exert an antinutritional value, reducing the absorption of food and the rate of digestion, immobilizing food components in the intestine and reducing the latter's avalability (Dunaif & Schneeman, 1981; Flourie *et al,* 1984). Therefore, if any waste material containing pectin is intended to be used for applications such as the conversion into animal feed, a careful assessment of pectin content should be done on raw material as well as on the final product, in order to ensure the the suitability in terms of nutritional properties.

The main objective of this chapter is the characterization of pectin from feed ingredients, obtained after performing treatments on food waste, and feed produced using the same ingredients. The characterization has been performed in terms of fiber content, galacturonic acid content, degree of methylation and acetylation and neutral sugar content. Pectin from the original food wastes was characterized in a previous work by Müller-Maatsch *et al* (2016).

2.2 Materials and Methods

2.2.1 Samples

Waste-derived animal feed were provided by NOSHAN partners and they are listed in Table 2.1.

Table 2.1: List of wastes, bulk feed, ingredients and final feed analyzed for fiber and pectin content.

	Matrix Description	**Origin**
Waste	Rapeseed presscake (waste)	IGV (germany)
Bulk feed	Rapeseed presscake-milk whey mix (90:10)	IGV (germany)
Bulk feed	Rapeseed presscake-yogurt mix (75:25)	IGV (germany)
Bulk feed	Rapeseed presscake-hard cheese mix (50:50)	IGV (germany)
Waste	Malted barley germ (waste)	IGV (Germany)

	Matrix Description	Origin
Bulk feed	Malted barley germ-cheese residue-whey milk (14:25:61)	VERTECH GROUP (France)
Bulk feed	Malted barley germs-rapeseed presscake-yoghurt mix (40:40:20)	IGV (germany)
Bulk feed	Malted barley germ-yogurt mix (75:25)	IGV (germany)
Bulk feed	Malted barley germ-milk whey mix (90:10)	IGV (germany)
Bulk feed	Malted barley germ-yogurt mix (90:10)	IGV (germany)
Waste	Pumpkin kernel cake (waste)	IGV (germany)
Waste	Barley spent grain (waste)	IGV (Germany)
Bulk feed	Pumpkin kernel cake-barley spent grains-yogurt mix (40:40:20)	IGV (germany)
Waste	Fresh pumpkin (waste)	IGV (Germany)
Bulk feed	Fresh Pumpkin + acid	NS (Belgium)
Bulk feed	Fresh pumpkin	VERTECH GROUP+PROVALOR+ILVO
Bulk feed	Fresh pumpkin+pumpkin kernel cake (66:34)	VERTECH GROUP (France)
Waste	Barley spent grain (waste)	IGV (Germany)
Bulk feed	Barley spent grain-hard cheese mix (50:50)	IGV (germany)
Waste	Leek leaves (waste)	ILVO (Belgium)
Bulk feed	Leek leaves fermented	VERTECH GROUP (France)
Feed ingredient	Fresh pumpkin ingredient (ensiling and acid treatment of the waste), “FP e”	NS (Belgium)
Feed Ingredient	Pumpkin ingredient (drying the waste), “PFD”	ILVO (Belgium)
Complete feed	Extrudate pumpkin (extrusion of freeze-dried pumpkin and feed additives), “EP”	IGV (Germany)

2.2.2 Materials

Ethanol, Sodium hydroxyde 98%, Sodium tetraborate 99%, Sulfuric acid 96%, Sulfamic acid, Potassium hydroxide, 3-Phenylphenol 85%, Sodium trimethylsilil propionate (TSP), Trifluoracetic acid, N,N-dimetilformammide and BSTFA were purchesed by Sigma-Aldrich, USA.

Sugars, in specific case D-Glucose, L-Rhamnose, L-Arabinose, D-Mannose, D-Xylose, L-Fucose, D-Galactose, D-Ribose and Fenil-β-D-glucopiranoside were

purchesed by Sigma-Aldrich, USA. α- galacturonic acid 97% was provided by FLUKA St. Louis, USA.

2.2.3 Determination of alcohol insoluble residue, AIR

The AIR was determined using the method reported by Laurence D. Melton and Bronwen G. Smith (2001). In particular, 4 g of sample were placed in a graduate 50-ml centrifuge tube with conical end, then 40 ml of hot ethanol (ω= 95 %, 75 °C) were added and the mixture was heated for 10 minutes in a water bath at 85° C while stirring. Tube and contents were then centrifuged (Hettich, Zentrifugen, Universal 320R, Germany) for 15 minutes at 3000 x g and the supernatant was discarded. The precipitate was washed again with 35 ml of ethanol (ω= 63%, 75 °C) while stirring for 10 minutes in the water bath at 85°C. Thereafter it was centrifuged and the supernatant was discarded. For the determination of the total pectin, the residue left after precipitation, centrifuging and washing, was transferred into a 100-ml graduated flask. 5ml NaOH (c=1 mol/l) was added, and the solution was further diluted to 100 ml with distilled water, mixed for 30 minutes with stirring bars and allowed to stand for at least 15 min with occasional shaking. The solution was filtered, and the filtrate was frozen with liquid nitrogen to support the formation of small ice crystals and then freeze-dried (LIO 5P, VWR International PBI Milano, Italy). The procedure was performed in duplicate.

2.2.4 Determination of galacturonic acid content in pectins

The galacturonic acid was quantified following the method reported by Laurence D. Melton and Bronwen G. Smith (2001).

Sample preparation: 10 mg of pectin sample was hydrolyzed using twice 1 ml of sulfuric acid, mixed and cooled in an ice bath. Then 0.5 ml of bidistilled water was added, the mixture was stirred and further diluted with 5 ml of bidistilled water. The samples were centrifuged (eppendorf 5810R, EPPENDORF Augsburg,

Germany) and the supernatant was used for spectrophotometric measurements (Spectrophotometer, UV/VIS LAMBDA BIO 20, PERKIN ELMER Waltham (MA), USA).

Samples measurement: to 400 µl of hydrolyzed pectin sample or standard, 40 µl of 4 M sulfamic acid/potassium sulfumate solution (pH 1.6) and 2.4 ml 75 mM sodium tetraborate/sulfuric acid solution were added and vortexed. The mixture was heated (100°C) for 20 min and then cooled in ice. To the control (sample analyzed without adding the m-hydroxydiphenyl) 80 µl 0.5% NaOH was added to measure the sugar coloring. To the sample and standard 80 µl 255 M m-hydroxydiphenyl in 0.5% NaOH was added. The absorbance was measured at 525 nm against water blank. Some samples needed to be diluted with sulfuric acid.

2.2.5 Pectin extraction and fractionation

Pectin extraction and fractionation was perfromed using the method reported by Laurence D. Melton and Bronwen G. Smith (2001).

25 g of sample (reduced to small pieces) was ground to a powder under liquid nitrogen using a mortar and pestle. To stabilize the samples against enzymatic degradation, the powder was mixed with 100 ml of an 80% Phenol- 0.5 M HEPES buffer (w/v) and homogenized with Ultraturrax T-50 basic (IKA-Werke, StaufenimBreisgau, Germany, 2-3 min, 4,000 rpm). The mixture was separated in a Centrifuge 5804 (Eppendorf AG, Hamburg, Germany, 20 min, 3220 x g, RT, HDPE centrifuge bottles), and the supernatant was removed. To separate the soluble cell matrix contents, the pellet was washed three times with 50 mM HEPES buffer (pH 6.7) and the supernatant was removed after centrifugation. The pellet was treated (incubation 6 and 24 hr) with a DMSO 90% (v/v) solution to remove starch from the samples. To remove all starch residues, the pellet was furthermore incubated 1 h at 40°C with 200 U porcine pancreatic α-amylase stabilized by 20 mM HEPES buffer with 20 mM $CaCl_2$ (pH 6.9). The pellet was washed with 20 mM HEPES buffer with

20 mM $CaCl_2$ (pH 6.9) and the supernatant was removed. At this point, the wet pellet consisted only of cell walls.

Cell walls were treated twice (6 and 12 hr) with 50 mM trans-1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid (CDTA) in 50 mM potassium acetate buffer (pH 6.5), to extract the soluble pectins. The supernatant was separated by centrifuge respectively and combined. The soluble pectin was dialyzed with 0.1 M ammonium acetate buffer (pH 6.5, 1 d, 3 changes), to remove CDTA from the sample, and dialyzed with H_2O (3 d, 3 changes), to remove residues from buffers. To isolate the insoluble pectin fraction the pellet was treated with 50 mM Na_2CO_3/ 20 mM $NaBH_4$ and incubated with this extractant twice (16 and 2 hr) at 4°C. The supernatant was separated from the residues by centrifugation, and combined and neutralized. To remove the buffer from the insoluble pectin, it was dialyzed with H_2O (3 days, 3 changes). The solution was freeze-dried (LIO 5P, VWR International PBI Milano, Italy) and the residue was weighted to determine the yield of insoluble fraction related to the dry matter content of the food waste streams, reported by Muller-Maatsch J et al (2016).

The total pectin yield was taken as the combined weights of the freeze-dried CTDA and Na_2CO_3 fractions related to the dry matter content of the food waste streams.

2.2.6 Determination of the degree of methylation and acetylation of pectins by H-NMR

The degree of methylation and of acetylation of pectin was performed using the method reported by Müller-Maatsch J. *et al* (2014).

30 mg of freeze-dried pectin samples were combined with 100 µl of internal standard, 3-(trimethylsilyl)-propionate-d4 (TSP) (10 mg in 5 ml D_2O), 1 ml 0.4 M NaOH in D_2O and incubated for 2 hours at room temperature while stirring. Afterwards the supernatant was centrifuged (Centrifuge eppendorf 5810R,

EPPENDORF Augsburg, Germany), filtered using a nylon syringe filter system (0.4 μm) and transferred in NMR-tubes. 1H NMR spectra were acquired on a VARIAN-INOVA 600 MHz spectrometer, equipped with a triple resonance inverse probe (HCN), operating at 599.736 MHz for proton. Spectra were acquired at 298 K, with 32K complex points, using a 90◦ pulse length. 32 scans were acquired with a spectral width of 7196.8 Hz, an acquisition time of 2.53 and a relaxation delay (d1) of 5s. The experiments were carried out with water suppression by low power selective water signal presaturation during 5 s of the relaxation delay. The NMR spectra were processed by MestreC software. The spectra were Fourier transformed with FT size of 64K and 0.2 Hz line-broadening factor, phased and baseline corrected, and referenced to 3-(trimethylsilyl)-propionate-d4 (TSP) peak (0 ppm). The quantitative determination of acetic acid and methanol was obtained by manual integration of the corresponding signals (1.92 ppm for acetic acid, 3.358 for methanol) and the comparison with TSP area. Integrals were converted in mass value (mg) according to the following formula: (Ax*EWx/mgx) = (ATSP*EWTSP/mgTSP), where Ax = spectral area of analyte; ATSP = spectral area of internal standard; EWx = equivalent weight of analyte; EWTSP = equivalent weight of internal standard; EW = (MW/ n° of hydrogens in the signal), where MW was the molecular weight. The accuracy of the quantitative data was assured by the relaxation delay, determined by T1 measurements, which was set in order to allow the complete relaxation of the nuclei.

2.2.7 Determination of neutral sugars in pectins by GC/MS

Neutral sugars in pectin were determined following the method reported by L. D. Melton and B. G. Smith (2001). 10 mg of pectin sample were hydrolyzed with 3 ml 2 M triflouracetic acid; the sample was flashed under nitrogen and incubated for 60 min at 121°C. 1 ml phenyl-β-D-glucopyranoside (500 ppm) was added, filtrated with nylon syringe filters (40 μm) and the filtrate evaporated to dryness with nitrogen. With 800 μl N-N-dimethylformamide and 200 μl N,O-

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) the sugars were silylated for 60 min at 60°C. The GC/MS analysis was carried out with a Agilent Technology 6890N Network GC System equipped with a 5973 MS Detector, using a capillary column HP-5MS 0,25 mm x 30 m x 0,25 μm, injection volume of 1 μl, Initial Temperature 60°C, Runtime 40.50 min, Split injection (ratio 20:1), Carrier gas helium, following this temperature ramp: 10°C/min until 160°C, then 10°C/min until 220°C, then 20°C/min until 270°C.

2.2.8 Statistical analysis

All measurements were done in duplicate and the average values with the corresponding standard deviations were calculated.

2.3 Results and discussion

2.3.1 Alcohol insoluble residue AIR and galacturonic acid content in wastes bulk feed

Wastes and bulk feed were characterized for their fiber and galacturonic acid content. Pectic substances were precipitated with ethanol and the total alcohol insoluble residue, AIR, was obtained; this residue can be taken as a very rough measurement of fiber content, being made not only by pectin, but also by other cell wall compenents such as cellulose, hemicellulose, and also proteins, that could be co-extracted. Therefore, in order to determine the content of pectin in AIR, quantitative measurement of total uronic acid (since these acids are basically contained only in pectin) was performed, using a colorimetric assay aimed at quantifying this acid, released by pectin after acid hydrolysis. The bulk feed, which are listed in Table 1, were analyzed for AIR and pectin content; AIR content in the starting waste is also reported. Bulk feed are made by vegetable matrices, such as

fresh pumpkin, or vegetal matrices mixed with dairy by-products for improving the feed nutritional value.

The percentage of AIR and galacturonic acid are expressed on dry matter of waste/bulk feed.

Table 2: AIR content (% on dry matter) and Galacturonic acid content (% on dry matter) in bulk feed.

Waste/bulk feed matrix	**% AIR (d.m.)**	**% Uronic Acid (d.m.)**
Rapeseed presscake (waste)	53.11	0.85
Rapeseed presscake-milk whey mix (90:10)	38.11	1.17
Rapeseed presscake-hard cheese mix (50:50)	13.39	0.08
Rapeseed presscake-yogurt mix (75:25)	8.38	0.08
Malted barley germ (waste)	10.66	0.39
Malted barley germ-cheese residue-whey milk (14:25:61)	25.66	0.58
Malted barley germs-rapeseed presscake-yoghurt mix (40:40:20)	13.63	0.28
Malted barley germ-yogurt mix (75:25)	10.36	0.44
Malted barley germ-milk whey mix (90:10)	9.49	0.26
Malted barley germ-yogurt mix (90:10)	8.2	0.06
Pumpkin kernel cake (waste)	18.30	1.2
Barley spent grain (waste)	8.64	0.06
Pumpkin kernel cake-barley spent grains-yogurt mix (40:40:20)	13.04	0.15
Fresh pumpkin (waste)	16.38	0.06
Fresh Pumpkin + acid	**9.27***	6.83
Fresh pumpkin	38.7	2.15
Fresh pumpkin+pumpkin kernel cake (66:34)	10.46	0.69
Barley spent grain (waste)	8.64	0.06
Barley spent grain-hard cheese mix (50:50)	17.73	0.07
Leek leaves (waste)	4.42	0.27
Leek leaves fermented	21.07	0.54

*Data given on wet basis

In general, a certain variability can be observed between wastes and bulk feed derived from them.

In particular, looking at the AIR %, it decreased, compared to the waste, in bulk feed based on rapeseed presscake and pumpkin kernel cake, probably because of mixing with other compounds.

In other cases, the treatment performed on the waste resulted in an increase of the AIR %., which can be due to technological treatments which concentrate the fiber fraction or to the coextraction of other substances than fibers in the AIR fraction (Wanasundara *et al,* 2011; Pustjens *et al*, 2013, Rommi *et al*,2014)

Concerning the galacturonic acid content, a variability was also observed, certainly related to the amount of ingredients used and to the technology applied for the production of feed. As expected, the higher the vegetal fraction, the higher the fiber and pectin content, and their percentage decreased as the diary portion increased.

In terms of nutritional value, the bulk feed should provide a good fiber content, which is favorable for animal health, but of course pectin can be present when matrices of vegetal origin are used, which might have an undesired antinutritional value. Therefore, a compromise between fiber and pectin contents should be found.

Thus, in order to deeper investigate pectin composition both in food waste and in feed, an investigation was also undertaken on its molecular structure.

2.3.2 Characterization of the pectin fraction in pumpkin after technological process on food waste

Pumpkin waste, provided by the food industry, was treated by using different technological process, especially freeze-drying and ensiling combined with other industrial treatments needed for waste stabilization. Freeze-drying is a technology used for the physico-chemical stabilization of wastes. (Pinach *et al,* 2006) Ensiling

is a controlled fermentation which can be applied for the stabilization of low pH products with an adequate content of soluble hydrocarbons (8-10% of dry metter). (Jianxin, 2002)

By using such technologies, wastes were converted into feed ingredients and later into feed. In the present study, pectin was extracted from both ingredients and feed for further characterization

2.3.2.1 Pectin content in pumpkin waste and in pumpkin after ensiling

Pectin was extracted and isolated from the fresh pumpkin waste (FPw) and the fresh pumpkin waste after ensiling (FPe) following the method reported in section 2.2.5. The procedure is long and complex but it provides intact and pure pectin as it is in the matrix, without degradation due to the extraction procedure and allowing a detailed characterization of the polymer.

The procedure includes two phases: isolation and fractionation. The purpose of the cell wall isolation procedure is to obtain a preparation of cell walls that is virtually free from contamination by cytoplasmic components of the cells (e.g. membranes, nuclear material, enzymes and starch). This is achieved through a series of steps that involve methods of breaking the cells open and washing away the cell contents.

The polysaccharides in cell walls were investigated by successively treating the walls with solutions of chemicals to extract the constituent polysaccharides. These fractions were then analyzed. The walls were firstly treated with solutions of chelating agents, such as *trans*-1,2-diaminocyclohexane-*N,N,N,′N′*-tetraacetic acid (CDTA) to extract the calcium-bound pectic polysaccharides, followed by an extraction with an alkaline solution such as sodium carbonate (Na_2CO_3). The alkaline extraction with Na_2CO_3 and $NaBH_4$ was performed in order to hydrolyze and obtain the ester-bound pectin; $NaBH_4$ was added to alkaline solutions to reduce

the reducing end of the polysaccharides and therefore prevent the base peeling of polysaccharides (Melton & Smith, 2001).

This method was chosen because the chelating agents allow the extractability of pectic polysaccharides in mild conditions, obtaining pectin as intact as possible, even if co-extraction of proteins or other polysaccharides could occur. (Pustjens *et al,* 2013; Mateos- Aparicio *et al,* 2010).

Two fractions were obtained, denoted respectively as "CDTA" fraction and "Na_2CO_3" fraction; their yield was calculated on the dry matter and it is reported as % w/w.

The two fractions were then analyzed for their galacturonic acid content. After initial acid hydrolysis, 3-phenylphenol was added to sample and the absorbance was measured spectrophotometrically at a wavelenght of 525 nm. The galacturonic acid was quantified against a standard solution and it is expressed as % w/w on the extracted pectin fraction. It should be taken into account that uronic acid could be overestimated by 10-20% because of the interference of extracted cell wall components.

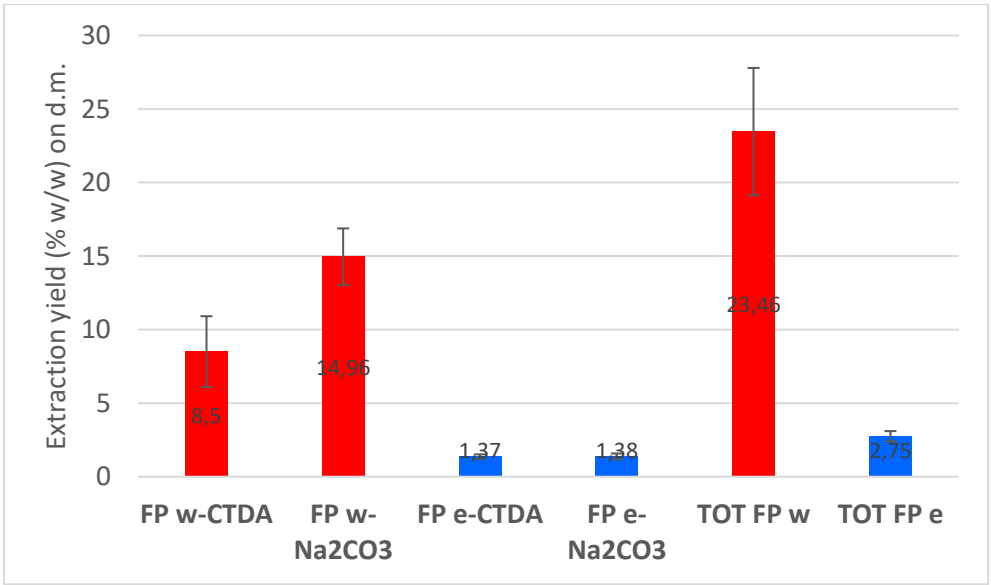

Figure 2.1. Extraction yield of soluble (CTDA) and insoluble (Na_2CO_3) pectin from the original pumpkin waste (FP w), and after fermention (FP e).

As depicted in Fig.2.1, it is evident that the pectin content is much higher in the fresh pumpkin waste (FP w) than in the sample treated by fermentation in order to obtain the feed ingredient, demonstrating that technology (ingredient mixing and technologies which reduce pectin) have a deep impact on the pectin present in the original waste material.

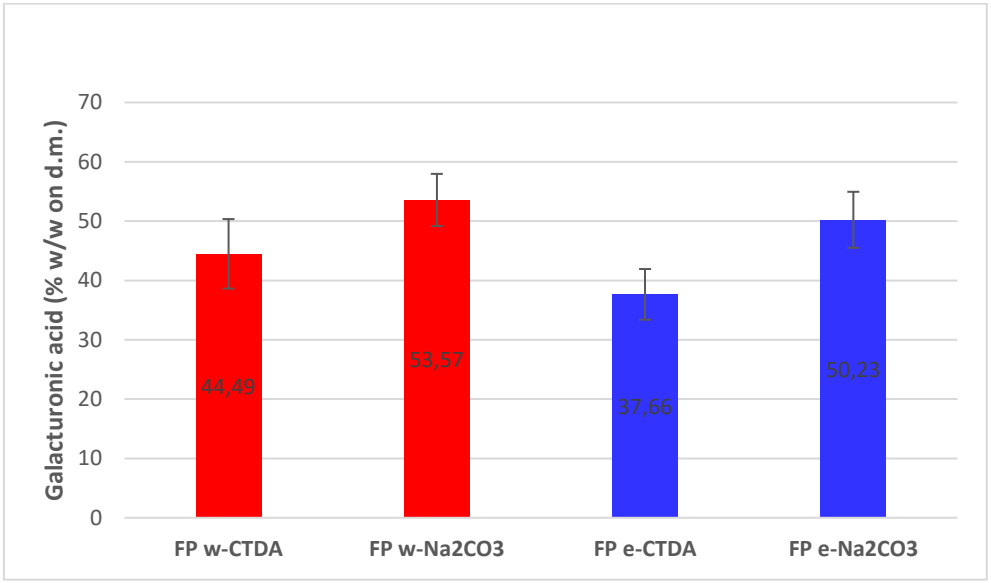

Figure 2.2: Galacturonic acid content (%) in the soluble (CTDA) and insoluble (Na_2CO_3) pectin extracted from the original pumpkin waste (FP w), and after fermentation (FP e).

Looking at the graph in Fig.2.2, it can be seen that the percentage of galacturonic acid in the pectin remained almost constant in the two pectin fractions, soluble (CTDA) and insoluble (Na_2CO_3), for both samples, indicating that, despire the very little amount present, the pectin still has a structure which resembles the one found in the orginal food waste.

2.3.2.2 Pectin in the pumpkin waste before and after freeze-drying

2.3.2.2.1 Extraction yield and pectin content

A similar characterization as for the fermented fresh pumpkin was performed also for the ingredient pumkin freeze-dried.

Pectin was extracted from the original pumpkin waste (FPw) and from pumkin freeze-dried (PFD) following the method reported by Melton and Smith (2001). Pectin was then subjected to acid hydrolysis in order to measure the galacturonic acid content using the colorimetric assay described in section 2.2.4.

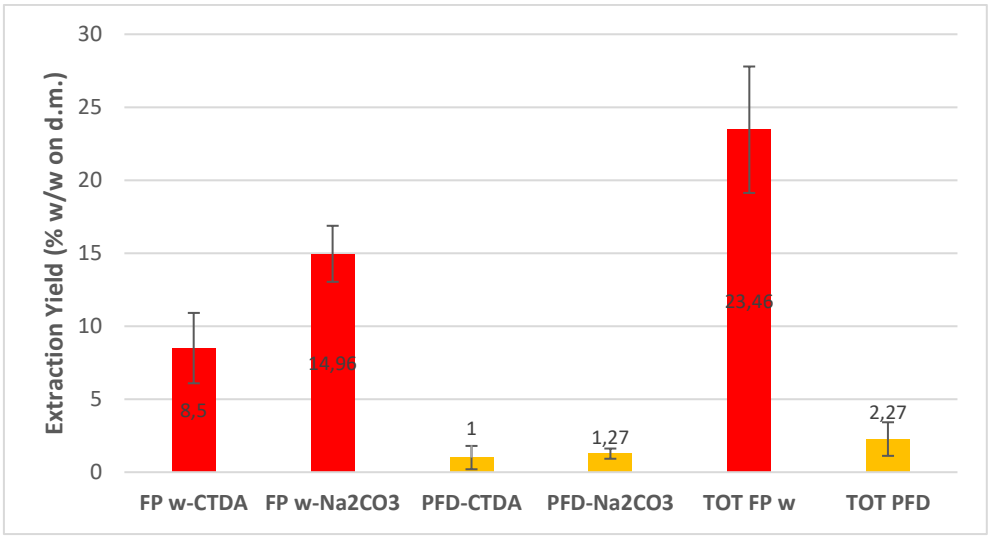

Figure 2.3: Extraction yield of soluble (CTDA) and insoluble (Na_2CO_3) pectin from the original pumpkin waste (FP w) and after freeze-drying (PFD).

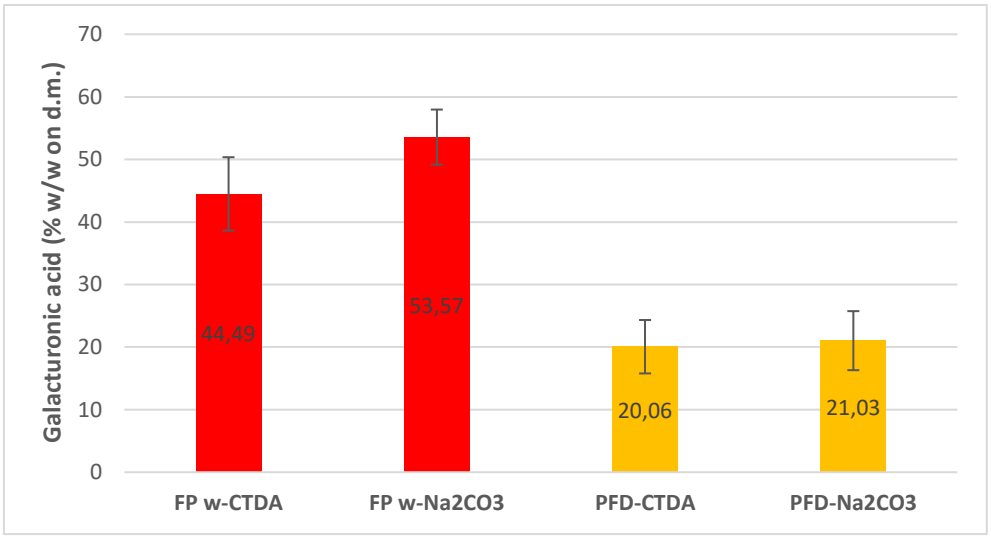

Figure 2.4: Galacturonic acid content (%) in the soluble and insoluble pectin extracted from the pumpkin waste (FP w) and after freeze-drying (PFD).

As for fermented pumpkin, also in this case a low pectin content, if compared with the original material, was noted; in particular, an extraction yield of 2.27% was observed for pumpkin freeze-dried against a yield of 23.46% for pumpkin waste.

The figure 2.4 shows the percentage of galacturonic acid in pectin extracted from the two samples. The two pectin fractions from pumpkin freeze-dried showed a lower galacturonic acid content respect to pumpkin waste indicating a lower pectin purity. This might be related to an increased difficulty in extracting pectin from the treated sample.

2.3.2.2.2 Degree of methylation and acetylation of pectin

The pectin from pumpkin freeze dried obtained from the extraction was subjected to alkaline saponification (Levigne *et al,* 2002) directly in alkaline deuterium oxide (Müller-Maatsch *et al,* 2014). The treated pectin was then analyzed by using H-NMR spectroscopy in order to quantify methanol and acetic acid. The degree of methylation and acetylation of pectins can be estimated by determining the

proportion of methyl and acetyl esters in the pectin sample in conjunction with the total uronic acid content.

The results related to pumpkin freeze-dried were compared with those concerning the pumpkin waste for both degrees of methylation and acetylation and they are reported in Fig.2.5.

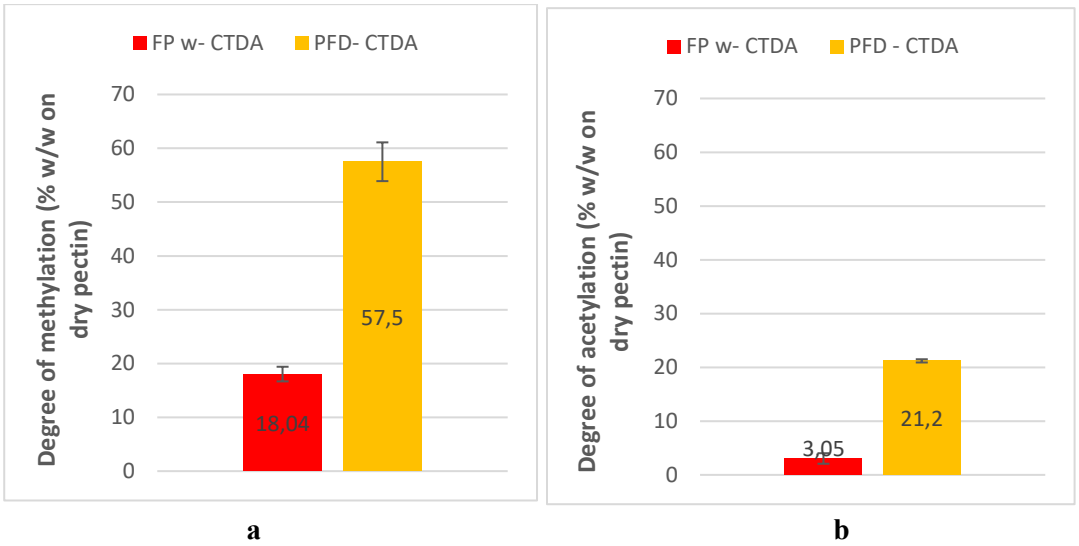

Figure 2.5: Percentage of methylation (a) and acetylation (b) on the galacturonic acid content in the original pumpkin waste (FP w), and after freeze-drying (PFD).

The data showed in the graphs indicated an increase in both degree of methylation and acetylation of extracted soluble pectin.

Combined with the reduction of the total pectin content, and the lower percentage of galacturonic acid, this data seems to indicate again that the technological treatments affect pectin content, and that methylated and acetylated pectin was more resistant to this technological treatment.

2.3.2.2.3 Neutral sugars content

The pectin extracted from pumpkin freeze-dried ingredient was characterized also for the neutral sugars content, in order to detail the structure and the composition of the extracted polymer.

In order to release the neutral sugars, pectic polysaccharides were subjected to hydrolysis using TFA. Moreover, the solution of BSTFA was used for the derivatization of sugars as silyl ethers, suitable for GC/MS analysis. (Caligiani *et al*, 2013)

In this way, it was possible to determine the neutral sugars content in pectin.

For each sugar, the responce factor was calculated using the following formula:

$$Responce\ Factor = \frac{Sugar\ Area_{sample}}{Sugar\ Area_{Standard}}.$$

Subsequently, the sugar content in samples was calculated using the following formula:

$$Sugar\ content\ (mg) = \frac{Area_{Sample*}\ Standard\ Concentration\ (mg)}{Responce\ factor*\ Area_{Standard}};$$

the sugar content can be also expressed in percentage and in mg/g using the following formula:

$$Sugar\ content\ (\%) = \frac{mg\ of\ sugar_{sample} * 100}{mg\ of\ sample}$$

$$Sugar\ content\ \left(\frac{mg}{g}\right) = sugar\ content\ (\%) * 10.$$

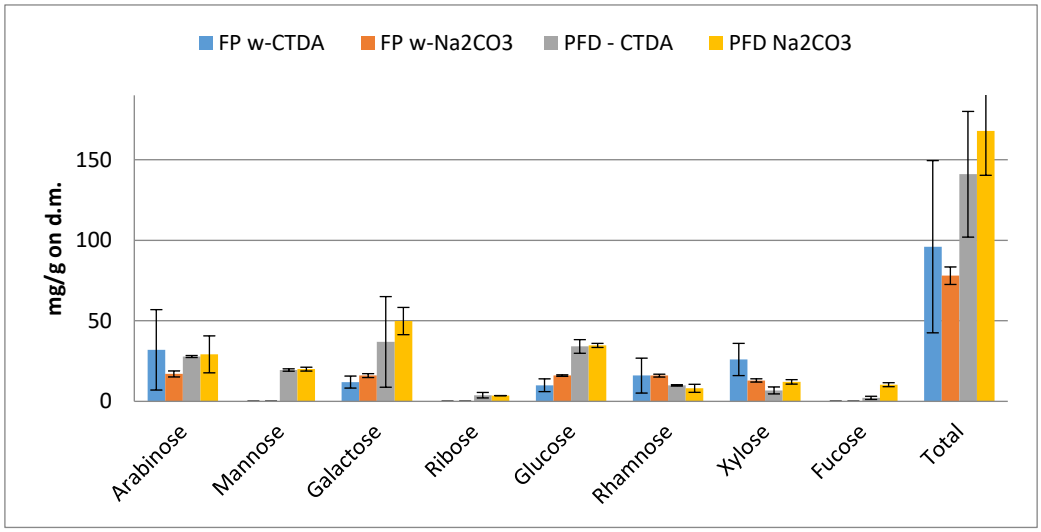

Figure 2.6: Neutral sugars content (mg/g) in the soluble and insoluble pectin from the original pumpkin waste (FP w), and after freeze-drying (PFD).

The sugars content in mg/g related to the waste matched the values reported by Muller-Maatsch *et al* (2016).

Given the higher content of neutral sugars in the feed ingredient than in the waste, the hypothesis of an effect of technological treatment on pectin seems to be confirmed: probably, ramified pectin (the one most containing neutral sugars and less uronic acid) were less affected by the technological process and were more extractable than the smooth pectin region. Thus, the drying process could have induced the gelation and concentration of "hairy" pectin.

The most abundant sugars in pumpkin freeze-dried were galactose, glucose, arabinose and mannose. This latter sugar was absent in waste probably because it was derived from the isomerization reaction of the glucose during the technological process.

2.3.2.3 Pectin content in pumpkin freeze-dried and in extrudate pumpkin (feed)

The pumpkin freeze-dried was then selected for the production of the final feed; basically, it represents the main material used for the formulation of the complete feed and, feed addtives could be added in order to improve its nutritional value. Therefore, starting from the ingredient and using the extrusion process, the extrudate pumpkin was obtained. It is intended to be used as such for animal feeding.

In this case, the rapid extraction method was used since a rough chararcterization in terms of fiber content and galacturonic acid content was needed.

The alcohol insoluble residue (AIR) was extracted from the freeze-dried pumpkin and from the extrudate pumpkin that was produced from it. In particular, samples were treated with ethanol and the soluble fraction, containing the cell walls components and enzymes, was discarded. The residue was filtered and lyophilized. The AIR, obtained following this procedure and reported in Fig.2.7, is not pure in pectins but it contains also cellulose and hemicellulose, hence the fiber fraction. The procedure is advantageous because it is rapid but it does not provide pure pectin.

In order to determine the purity in pectins, the AIR was subjected to acid hydrolysis and the galacturonic acid was quantified by using the colorimetric assay described in section 2.2.4. The analysis was performed in double. The galacturonic acid percentage was calculated on the initial sample following the formula:

$$Galacturonic\ acid_{sample}\ (\%) = \frac{Galacturonic\ acid\ (\%)_{AIR} * AIR}{g\ of\ sample}.$$

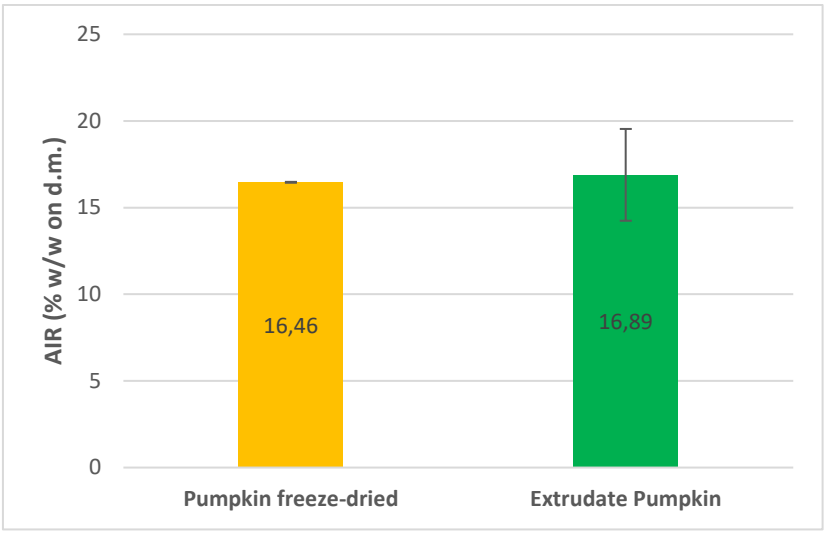

Figure 2.7: AIR (%) from pumpkin freeze-dried and from extrudate pumpkin

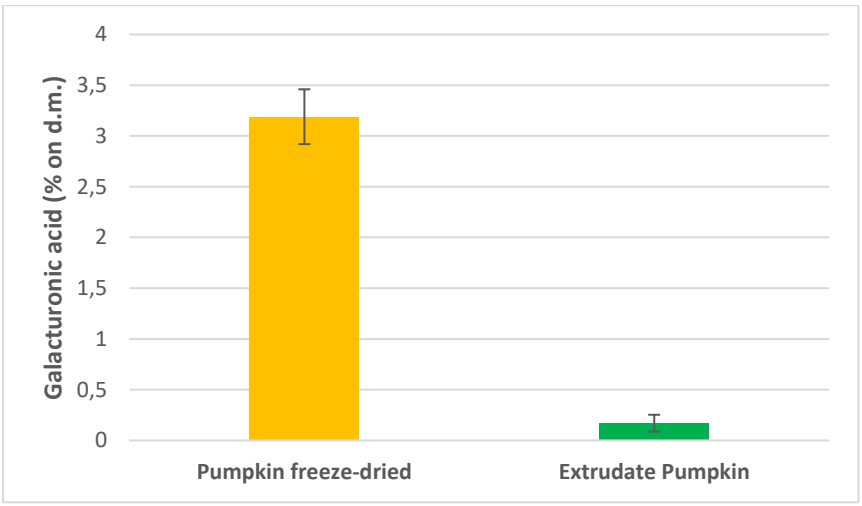

Figure 2.8: Galacturonic acid content (%) in Pumpkin freeze-dried and Extrudate Pumpkin

At first instance it can be observed that the fiber content was almost constant in ingredient and in feed; of course, in the extrudate also other extra-materials could contribute to this amount. At the same time, a strong reduction in galacturonic acid content, hence in pectin, was observed in the final feed, in fact only 0.17% was

measured in feed against 3.20% in ingredient. This effect is likely due to the extrusion treatment, where feed additives are also added, thus lowering the percentage composition in pectin.

The low pectin content makes the extrudate a suitable material to feed animals.

2.4 Conclusions

The analyses performed have allowed the assesment of fiber and pectin content in starting material, bulk feed and feed ingredients after several technological treatments on the food wastes. Fresh pumpkin was chosen for further investigation on pectin structure.

Regarding the ingredient formulated starting from pumpkin waste, it showed a reduction in pectin content as compared to the waste, probably due to the technological treatment; the process probably made the pectin, and especially the less methylated ones, less extractable, as also shown by the higher degree of esterification and neutral sugar content.

Also, the final feed formulated using the pumpkin ingredient showed a reduction in pectin content and a modification of the orginal pectin composition, indicating that the process selectively degrades or makes less extractable, some pectin structures, but not all of them, leaving untouched mostly the smooth region.

It can be conluded that, when formulating feed starting from food waste, low pectin feed ingredient and low pectin feed additives can be obtained even in the case the starting material is rich in pectin, by an appropriate choice of the technology of production and of final product formulation.

Acknowledgments

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3. Molecular characterization of pectic oligosaccharides (POS) obtained by enzymatic digestion of pectin

Abstract

In the field of prebiotics, oligosaccharides have recently been introduced as novel dietary compounds for food and feed applications. Indeed, there is an increasing interest in the use of oligosaccharidic natural substances and vegetable food waste and by-products represent one of the most suitable resources for their production. Agro-industrial by-products rich in pectin are exploitable for the production of pectic oligosaccharides (POS) possibly showing prebiotic activities.

The European project NOSHAN aimed at the production of fibers with prebiotic properties which are able to control the microbial ecology within the animal gastrointestinal tract leading to better performances. In the framework of the project, sugar beet pulp (SBP) was investigated as source of pectic oligosaccharides. POS were produced starting from raw material and using a tailor made process combining enzymatic hydrolysis and filtration cascade. Different conditions were tested and the produced POS were subsequently characterized in order to study the relation between the technology used and the POS properties, allowing the identification of the best condition to obtain the desired final ingredients.

In particular, in the present study, pectic oligosaccharides produced by enzymatic digestion of pectin were characterized at a molecular level. A colorimetric assay was used in order to quantify the amount of galacturonic acid in the POS fractions. Moreover, a HILIC-ESI/MS methodology was used in order to analyze the POS composition, the degree of polymerization and the acetylation and methylation pattern. The composition of POS fractions was studied as function of the technology

used. It was observed that, when SBP was chemically pretreated and hydrolyzed in enzyme membrane reactor (having 5kDa molecular cut off membranes), fractionation allowed to obtain following POS-fractions: (1) 0.7-1kDa, rich in arabinans, and (2) 1-5 kDa, rich in rhamnogalacturonans. With 10KDa membranes, POS in the range of 1-5KDa, rich in arabinans, and 5-10KDa, rich in rhamnogalacturonans, were produced. On the other side, batch production turned out to be rich in free galacturonic acid.

The molecular characterization provided a deep knowledge about the POS composition allowing the tailoring of the production process in order to obtain POS with specific molecular weight and specific composition.

3.1 Introduction

Carbohydrates are added to food and feed as ingredients or additives because of their many functions, i.e. emulsifying, gelling, stabilizer, sweetener, thickener, and also various dietetic functions and as source of fermentation substrate in the large intestine. (Bellisle *et al,* 1998; Voragen *et al,* 1998)

In the field of prebiotics, oligosaccharides have recently been introduced as novel dietary compounds for food and feed applications. Indeed, there is an increasing interest in the use of saccharidic natural substances and the vegetable food waste and by-products represent for sure one of the most suitable sources to produce value-added products such as potential prebiotic oligosaccharides. (Laufenberg *et al*, 2003)

Oligosaccharides can derive from chemical, physical or biological degradation of polysaccharides. According to Gibson *et al* (2004), a prebiotic oligosaccharide needs to be resistant to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption. Moreover, this oligomer has to be fermented by the intestinal microflora and to stimulate selectively the growth and/or activity of intestinal bacteria associated with health such as *Bifidobacteria* and *Lactobacilli*.

Non digestable oligosaccharides such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are the most used and commercial as prebiotics. However, novel prebiotics are being developed thanks to advancement of knowledge about polysaccharides from plant cell wall and the polysaccharides cleavage enzymes.

Pectin is one of the polymers constituting the cell wall of higher plants and it represents a source of oligomers showing prebiotic activities. (Babbar *et al,* 2015)

Agro-industrial by-products are rich in this kind of polymers, being exploitable for the production of pectic oligosaccharides (POS) and becoming very valuable in the future. (Babbar *et al*, 2015; Berrateau *et al,* 2006)

Therefore, arabinogalactooligosaccharides, arabinoxylooligosaccharides, arabinooligosaccharides, galacturonan oligosaccharides, rhamnogalacturonan oligosaccharides (which can all be defined pectic oligosaccharides), obtained after hydrolysis with specific enzymes, have been successfully experimented. These oligomers, as discussed more in detail in Chapter 1, have been fermented in pure cultures by intestinal bacteria such as *Bifidobacteria*, *Lactobacilli*, *Bacteroides* sp., *Clostridium* sp., *Escherchia coli* and *Klebsiella sp,* and they resulted in a higher stimulation of gut-beneficial bacteria than higher molecular weight molecules. (Van Laere *et al*, 2000; Olano-Martin et al, *2001*; Olano-Martin *et al,* 2002; Oosterveld *et al*, 2002)

NOSHAN contributed to a significant progress beyond the state of the art by developing new nutraceutical and feed additives; specifically, the project aimed at the production of fibers with prebiotic properties which are able to control the microbial ecology within the animal's gastrointestinal tract leading to better performances.

Fruit and vegetable waste pulps and peels rich in pectin were selected as new source of potential prebiotic compounds. In particular, NOSHAN aimed at the production of POS by developing selective technological process based on dedicated enzymes and dense ultrafiltration. The membrane technology allowed the tailoring of pectic oligosaccharides thanks to selected molecular weight cut-off (MWCO). The purpose was to produce high quality POS from a variety of fruit and vegetable waste; furthermore, by addressing the molecular structure and the functionality of the POS produced in relation to the process applied as well as the waste used, also the feasibility of waste sources was addressed.

Developing new technological process could also decrease functional ingredients cost by obtaining them from inexpensive materials, as well decreasing the total feed cost.

One of the sources investigated in the framework of the project was sugar beet pulp (SBP), due to the significant availability in Europe and the fact that this raw material has been investigated to some extent in literature.

Sugar beet (Beta vulgaris) pulp is a lignocellulosic by-product of the sugar refining industry and it is mostly used as animal feed. It was found to contain polysaccharides consisting of approximately (dry basis) 22–24 wt.% cellulose, 30 wt.% hemicelluloses, 15–25 wt.% pectin. The remaining part is composed of small amounts of fat, protein, ash and lignin at 1.4 wt.%, 10.3 wt.%, 3.7 wt.% and 5.9 wt.%, respectively. (Sun *et al*,1998)

Some studies demonstrated that SBP is a suitable starting raw material for enzymatic saccharification and conversion of sugars to value-added products, such as pectic oligosaccharides. (Levigne *et al,* 2002; Oosterveld *et al*, 2000; Oosterveld *et al,* 2002; Concha Olmos & M.E. Zúñiga Hansen, 2012)

In the framework of the project, sugar beet pulp obtained from the company IGV gmbh (Germany) was used and characterized for its pectin content in previous work by Muller- Maatsch *et al* (2016). POS were produced starting from the raw material while using a tailor made process, combining enzymatic treatment and filtration cascade. Different conditions were tested and the POS produced were subsequently characterized in order to study the relation between the technology and the POS properties, allowing the identification of the best condition to obtain the desired final ingredients, i.e. low molecular weight POS.

In particular, in the present study, POS were characterized at a molecular level. A colorimetric assay was used in order to quantify the galacturonic acid in the POS fractions. Moreover, a HILIC-ESI/MS methodology was used in order to analyze the POS composition, the degree of polymerization and the acetylation and methylation pattern. The composition of POS fractions was studied as function of the technology used, indicating which is the most suitable process in order to produce low molecular weight POS and to achieve the lowest monosaccharides formation.

3.2 Materials and Methods

3.2.1 Chemicals

The ammonium formate and the acetonitrile were provided by Sigma-Aldrich (Germany). The formic acid and the nitric acid were provided by Fisher Chemical (UK).

3.2.2 Samples

The POS fractions analyzed were obtained from sugar beet pulp. They were provided by the Flemish Institute for Technological research,VITO (Belgium) and they are listed in Table 3.1.

Table 3.1: POS fractions analyzed for pectin content and oligomers composition.

Waste	POS fraction preparation	Sample
Sugar Beet (fractionated)	Enzymatic pretreated feed- 10KDa membrane	EP-EMR10KDa-0.6 FPU/ml-0.4KDa
Sugar Beet (fractionated)	Enzymatic pretreated feed- 10KDa membrane	EP-EMR10KDa-0.6FPU/ml-0.4_0.7KDa
Sugar Beet (fractionated)	Enzymatic pretreated feed- 10KDa membrane	EP-EMR10KDa-0.6FPU/ml-0.7_1KDa
Sugar Beet (fractionated)	Enzymatic pretreated feed- 10KDa membrane	EP-EMR10KDa-0.6FPU/ml-1_5KDa
Sugar Beet (fractionated)	Enzymatic pretreated feed- 10 KDa membrane	EP-EMR10KDa-0.6FPU/ml-5_10KDa
Sugar Beet (fractionated)	HNO_3 pretreated feed – 5KDa membrane - 3FPU/ml	HNO3-EMR5KDa-3FPU/ml-0.4KDa
Sugar Beet (fractionated)	HNO_3 pretreated feed- 5KDa membrane - 3FPU/ml	HNO3-EMR5KDa-3FPU/ml-0.4_0.7KDa

Sugar Beet (fractionated)	HNO_3 pretreated feed – 5KDa membrane-3FPU/ml	HNO3-EMR5KDa-3FPU/ml-0.7_1KDa
Sugar Beet (fractionated)	HNO_3 pretreated feed – 5KDa membrane-3FPU/ml	HNO3-EMR5KDa-3FPU/ml-1_5KDa
Sugar beet pulp: not fractionated	HNO_3 pretreated feed-10KDa membrane	HNO3-EMR10KDA-1FPU/ml-NF
Sugar beet pulp: not fractionated	HNO_3 pretreated feed - 10KDa membrane	HNO3-EMR10KDA-0.6FPU/ml-NF
Sugar beet (fractionated)	HNO_3 pretrated feed – 0.6 FPU/ml - 10KDa	HNO3-EMR10KDa-0.6FPU/ml-0.4KDa
Sugar Beet (fractionated)	HNO_3 pretrated feed-0.6FPU/ml - 10KDa	HNO3-EMR10KDa-0.6FPU/ml-0.4_0.7Da
Sugar Beet (fractionated)	HNO_3 pretrated feed-0.6FPU/ml - 10KDa	HNO3-EMR10KDa-0.6FPU/ml-0.7_1KDa
Sugar Beet (fractionated)	HNO_3 pretrated feed-0.6FPU/ml- 10KDa	HNO3-EMR10KDa-0.6FPU/ml-1_5KDa
Sugar Beet (fractionated)	HNO_3 pretrated feed-0.6FPU/ml- 10KDa	HNO3-EMR10KDa-0.6FPU/ml-5_10KDa
Sugar beet (filtered)	Batch experiment 10KDa – 0.6FPU/ml	Batch-10KDa - 0.6FPU/ml

EP: Enzymatic pretreatment of substrate;
HNO3: Chemical pretreatments of substrate with HNO3;
EMR10KDa: hydrolysis in enzymatic membrane reactor with 10KDa MWCO membrane;
EMR5KDa: hydrolysis in enzymatic membrane reactor with 5KDa MWCO membrane;
Batch: hydrolysis performed in batch system;
FPU/ml: enzyme concentration;
xxKDa/xxKDa_xxKDa: MWCO membrane used in the fractionation cascade;
NF: not fractionated.

3.2.3 Production of bioactive fibres from sugar beet pulp

A downstream process was developed in order to extract and hydrolyze pectin to obtain the pectic oligosaccharides.

The process starts with the pretreatment of the feed, which can be enzymatic or chemical, then it continues with pectin hydrolysis which can be performed using a batch system or an enzyme membrane reactor (EMR). At the end, the produced POS can also be fractionated. The various methodologies which can be used are detailed as follows.

1. Extractive pretreatment step: The pectic fraction of SBP was extracted according to two different and alternative methods.
   a) *Enzyme assisted extraction (EAE*). The SBP was enzymatically treated with a mixture of a cellulase (Celluclast 1.5L, Novozymes) and an endo-polygalacturonase (Endo-pg M2, Megazyme). The SBP was suspended at a 12% (w/v) substrate loading in a citric acid buffer (pH=4.8), and Celluclast and Endo-pg were added at 20 FPU/g d.m. and 20 U/g d.m respectively (For the method of filter paper activity, see Sandhu et al (2013)). The pretreatment was done during 48 h at 48 ºC and at 160 RPM in an incubator shaker. After pretreatment, the solution was inactivated at 95 ºC for 5 minutes and centrifuged for 10 minutes at 5000 RPM. The supernatant was recovered and filtered over a 100 µm sieve. This was used as feed for the hydrolysis.
   b) *Nitric acid assisted extraction (NAE):* The SBP was pretreated with nitric acid during 4h at 5% (w/v) substrate loading, pH=1.3 and 80ºC in an incubator shaker at 125 rpm. (Kliemann *et al.,* 2009). The solution was neutralized to pH=4.5 with sodium carbonate or NaOH, and centrifuged at 5000 RPM for 10 minutes. The supernatant was separated and used for the further hydrolysis.

2. Hydrolysis: also the hydrolysis was performed in two different and alternative ways.
    a) *Batch hydrolysis*: in order to hydrolyze the SBP, Viscozyme (Viscozyme® L, Novozymes) was selected, since this enzyme has side activities which are able to hydrolyze the complex side chains of the SBP-pectin. The supernatant obtained from the pretreatment was divided in equal aliquots and added to a flask. The flasks were heated to 45°C in an incubator shaker, after which a diluted solution of Viscozyme was added in an amount of 10% (v/v) as compared to the feed. The dilution was 50x (0.6 FPU/ml).
    b) *Hydrolysis in a enzymatic membrane reactor (EMR):* In order to obtain tailored POS, the hydrolysis was also performed in a membrane enzymatic reactor. It consisted of a reactor with a coupled membrane module. As membrane, Romicon 1" hollow fiber membrane cartridges (Koch Membrane systems, Type HF 1018-1.0-43-PM10 and PM 5) containing polysulfone ultrafiltration membranes with a molecular weight cut-off of respectively 10 kDa and 5 kDa were used. The filtration surface of these membranes was 0.093 m^2. The 3 L reactor vessel was then filled with 600 mL pretreated sugar beet pulp solution at the specified substrate loading (± 50 g/L, pH 4.5). The system was thermostated at 45°C and stirred at 200 rpm. After the system was at temperature, 60 mL of a diluted solution of Viscozyme was added to the reactor vessel, being 10x (3.0FPU/ml) 30x (1.0FPU/ml) and 50x (0.6 FPU/ml). The liquid was pumped over the membrane surface, with a crossflow velocity of 1.35 m/s using a peristaltic pump (Watson-Marlow 620U, Cornwall, UK). A residence time of 30 minutes was set. For that, a permeate flow of 500 ml/h was controlled by a peristaltic pump (Watson-Marlow 520U, Cornwall,

UK). The level in the recirculation tank was kept constant by means of a level sensor and a peristaltic pump (Watson-Marlow 520U, Cornwall, UK). Each 10 minutes permeate samples were taken. Since initial tests indicated that the enzyme was completely retained in the reactor, no deactivation step was performed for the permeates. At the end of the experiment, the residue was recovered and the enzyme was deactivated by heating at 95°C for 10 minutes.

3. *Fractionation post hydrolysis*: Some of the hydrolyzed liquids, either obtained after hydrolysis in the batch or EMR reactor, were fractionated by filtration. The main goal of the fractionation was to produce pectic fractions with various molecular weights in order to be able to assess which molecular weights show the highest bioactivity. This information can then be used to adjust the membrane characteristics used in the EMR set-up. The hydrolyzates obtained from the EMR set-up were subjected to the fractionation step as such. The hydrolyzates obtained from the batch reactor, however, were first filtered over a 10 kDa membrane to remove the enzyme and other non-hydrolyzed substances. The fractionation was performed in 4 subsequent steps as schematically shown in Figure 3.1.

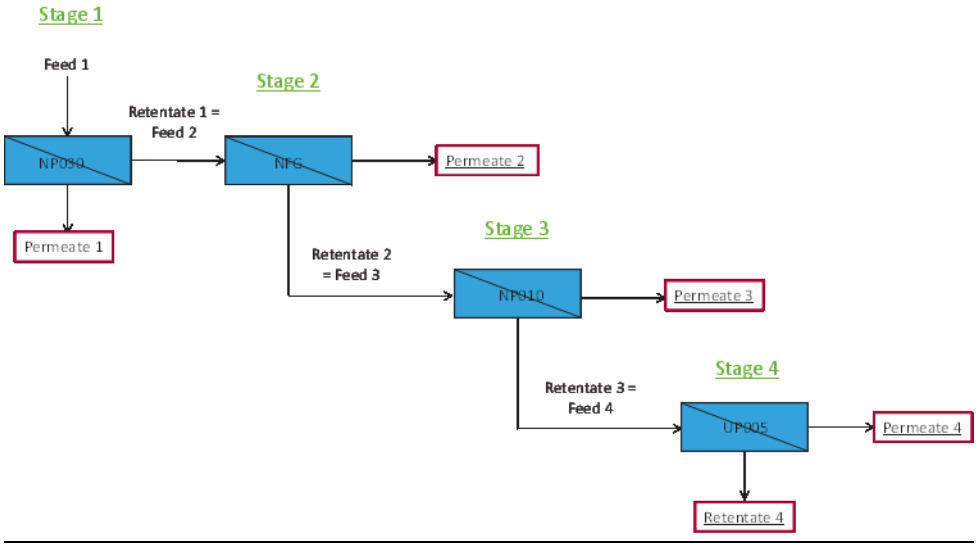

Figure 3.1: Scheme of the cascade filtration performed for the fractionation of the hydrolysate.

NP030: 0.4 KDa MWCO membrane;
NFG: 0.6-0.8 KDa MWCO membrane;
NP010: 1 kDa MWCO membrane;
UP005: 5 kDa, MWCO membrane.

Before fractionation, the hydrolysate (typically 3.5L) was immediately frozen with liquid nitrogen, and concentrated to 1-1.5 L by lyophilisation. After concentration, the hydrolysate was fractionated using four different flat sheet UF/NF (ultrafiltration/nanofiltration) membranes in a cascade filtration system i.e., 3 polyethersulfone membranes (UP005~ 5 kDa, NP010~1 kDa and NP030~400 Da from Microdyn Nadir) and 1 polyamide membrane (NFG~ 600-800 Da from Snyder). The membrane cascade was carried out one by one using the selected membranes with increasing cut-off values, whereby retentate after each filtration was used as feed in the next filtration step (see Fig. 1).

In this work, pectic oligosaccharides were produced mainly following three procedures:

A. Enzymatic pretreatment and hydrolysis with diluted feed in EMR.

The continuous crossflow filtration test was performed by using an ultrafiltration membrane of 10 kDa and Viscozyme to make tailor-made POS. The liquid as obtained from the enzymatically pretreated SBP was diluted by a factor of 2 (±25 g/L). This diluted liquid was then used as feed for the reactor test. The filtration was performed by continuous addition of buffer to the feed tank.
The different permeate fractions obtained were mixed, concentrated and fractionated by using a membrane cascade filtration system as described earlier. This resulted in 5 POS fractions described in Table1 ("EP-EMR10KDa-0.6 FPU/ml-0.4KDa", "EP-EMR10KDa-0.6FPU/ml-0.4_0.7KDa", "EP-EMR10KDa-0.6FPU/ml-0.7_1KDa", "EP-EMR10KDa-0.6FPU/ml-1_5KDa", "EP-EMR10KDa-0.6FPU/ml-5_10KDa").

B. Nitric acid pretreatment and hydrolysis in EMR with 5 & 10 kDa membrane

Another procedure was performed on chemically pretreated sugar beet pulp; in this case, water was continuously added to the feed tank of EMR. The nitric acid pretreatment was preferred above the enzymatically one, because it was expected to be more cost efficient and an additional step to remove citric acid was avoided. The optimizations of membrane and enzyme concentrations were performed to minimize monosaccharide formation (especially galacturonic acid and arabinose) and to recover maximum POS fractions.

The EMR used for pectin hydrolysis was equipped with 5KDa MWCO ultrafiltration membranes; an enzyme concentration of 3FPU/ml was used.
The different permeate fractions of reactors test were clubbed, concentrated and fractionated using the membrane cascade filtration system as described earlier. The last filtration step over UP005 was not performed, since already a 5kDa membrane was used in the reactor set-up. Therefore, in this case only 4 fractions were obtained and they are listed in Table 1 ("HNO3-EMR5KDa-3FPU/ml-0.4KDa", "HNO3-EMR5KDa-3FPU/ml-0.4_0.7KDa", "HNO3-EMR5KDa-3FPU/ml-0.7_1KDa", "HNO3-EMR5KDa-3FPU/ml-1_5KDa")

Moreover, the same methodology was used to perform a new reactor tests but changing the conditions: the 10KDa MWCO membrane and 0.6 FPU/ml enzyme preparation were used. The collected POS mixtures were fractionated by using a membrane cascade filtration system as described earlier, but adding the fractionation step on UP005 membrane, since the higher MWCO was used in EMR. This resulted in 5 fractions which are described in Table 1 (”HNO3-EMR10KDa-0.6FPU/ml-0.4KDa”, “HNO3-EMR10KDa-0.6FPU/ml-0.4_0.7Da”, “HNO3-EMR10KDa-0.6FPU/ml-0.7_1KDa”, “HNO3-EMR10KDa-0.6FPU/ml-1_5KDa”, “HNO3-EMR10KDa-0.6FPU/ml-5_10KDa”).

Furthermore, two experiments were conducted using 10KDa MWCO membrane and two diluted enzyme preparations, 1 FPU/ml and 0.6 FPU/ml. The resulting permeates were not subjected to fractionation step and they are listed in Table 1 as well (“HNO3-EMR10KDA- 1FPU/ml-NF”, “HNO3-EMR10KDA-0.6FPU/ml-NF”).

C. Hydrolysis in Batch system

In addition, an experiment was performed on a larger scale under the conditions that were found to be the best for POS production, i.e. with the 0.6 FPU/ml enzyme preparation. POS-hydrolyzates were produced in a batch system and the liquid was filtered over a 10 kDa membrane.

3.2.4 Analysis of crude pectin extract

HPAEC-PAD analysis was used in order to quantify the galacturonic acid and neutral sugars such as rhamnose, arabinose, galactose, xylose and fructose. The free monosaccharides were detected as such. The amount of dissolved pectic oligo- and polysaccharides was determined by correcting the total amount of saccharides detected after hydrolysis by the amount of monosaccharides already present in the extract. To achieve a complete hydrolysis and a full recovery of the monomers, the

extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h [29,30]. After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 2040 xg (Eppendorf centrifuge 5415 C) for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into HPAEC-PAD. The HPAEC-PAD used for analytical purpose was a Dionex ICS-5000 model (Thermo Scientific, Inc., USA) equipped with an ED-5000 electrochemical detector. The separation of monosaccharides was carried out (Lee Y.C. 1996) with a Carbopac PA -1 (4mm X 250mm X 4 mm) column coupled to a guard column Carbopac PA- 1 (4mm X 50 mm X 4mm) column. The analyses were performed using a gradient of deionized water (eluent A and D), 250 mM sodium hydroxide (eluent B) and 1 M sodium acetate (eluent C). The elution conditions were: at time zero, A: B at 25:75 (start cleanup); at 10 min, B:C:D at 6:0:47 (re-equilibration); at 30 min, B:C:D at 6:15:39.50 (stop re-equilibration and start acquisition); at 35 min, B:C:D at 50:50:0; at 36 min, B:C:D at 6:0:47; and at 46 min B:C:D at 6:0:47 (stop acquisition). The mobile phase was used at a flow rate of 1 mL/min for 46 min and the injection volume was 5 microliters. The monosaccharides were quantified by comparing them with the known concentration of standard solution (ranging from 10 mg/L to 1000 mg/L).

3.2.5 Determination of the galacturonic acid content in pectin

The galacturonic acid was quantified following the method reported by Laurence D. Melton e Bronwen G. Smith (2001).

Sample preparation: 10 mg of pectin sample were hydrolyzed using twice 1 ml sulfuric acid, mixed and cooled in an ice bath. Then 0.5 ml of bidistilled water was added, the mixture was stirred and further diluted with 5 ml of bidistilled water. The samples were centrifuged (eppendorf 5810R, EPPENDORF Augsburg, Germany)

and the supernatant used for spectrophotometric measurements (Spectrophotometer, UV/VIS LAMBDA BIO 20, PERKIN ELMER Waltham (MA), USA).
Samples measurement: to 400 µl of hydrolyzed pectin sample or standard, 40 µl of 4 M sulfamic acid/potassium sulfate solution (pH 1.6) and 2.4 ml 75 mM sodium tetraborate/sulfuric acid solution were added and vortexed. The mixture was heated (100°C) for 20 min and then cooled in ice. To the sample control 80 µl 0.5% NaOH was added to measure the sugar coloring. To the sample and standard 80 µl 255 M m-hydroxydiphenyl in 0.5% NaOH was added. The absorbance was measured at 525 nm against water blank. Some samples needed to be diluted with sulfuric acid.

3.2.6 Identification of pectic oligosaccharides by HILIC-ESI/MS analysis

Pectic oligosaccharides were separated and identified using the method reported by Leijdekkers, A.G.M *et al*, but with a slight modification. In particular, 100 mg of sample were dissolved in 1 ml of eluent B (H_2O:CH_3CN 80:20, 10 mM $HCOONH_4$ + 0.2% HCOOH). The samples were centrifuged at 2081 xg (model 5810R EPPENDORF, Hamburg, Germany), at a temperature of 4°C, for 10 minutes and the supernatant was recovered for analysis. The oligosaccharides present in the POS mixture were separated by a HILIC column (ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 3 mm X 150 mm) in an UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters ACQUITY SQD) using a gradient elution. Eluent A was H_2O:CH_3CN 20:80 (v/v), 10 mM $HCOONH_4$ + 0.2% HCOOH, eluent B was H_2O:CH_3CN 80:20 (v/v), 10 mM $HCOONH_4$ + 0.2% HCOOH; gradient: 0-60 min linear from 100% A to 60%A, 60-65 min isocratic at 60% A, 65-66 min linear from 60%A to 100%A, 66-75 min isocratic at 100%A. Flow was 0.3 ml/min; optimal run time for separation of various oligosaccharides was 75 min; column temperature 35°C; sample temperature 18°C. The injection volume was set to 10 µl; acquisition time 0-75 min; ionization ion mode: negative; capillary voltage 2.6 kV; cone voltage 60 V; source temperature

150°C; desolvation temperature 350°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. The samples were analyzed in Full Scan mode with a scan range of 200-2000 m/z. The analysis was performed in duplicate.

3.3 Results and Discussion

3.3.1 Galacturonic and neutral sugar content of the starting material

In order to determine the composition of the biomass, the sugar beet pulp was fully hydrolyzed and analyzed for the monosaccharides representative for the pectin. These are galacturonic acid as acidic sugar, and arabinose, galactose, rhamnose and xylose as neutral sugars. The galacturonic acid content of sugar beet pulp was 13 % (w/w) on d.m. basis (after the hydrolysis of polysaccharides). The arabinose content was the highest accounting for 14 % (w/w); the galactose, rhamnose and xylose content was found to be 4.5 %, 1.5 % and 1.2 % (w/w) d.m. respectively. (Babbar *et al,* 2016) The ratios between the sugars are somehow different from the pure sugar beet pectin composition found by Muller-Maatsch J *et al* (2016). In this case, on pure pectins isolated from sugar beet pulp, a galacturonic acid content of about 65% was found together with about 25% of arabinose, 3-5% of galactose and rhamnose, plus minor percentages of other neutral sugars. These differences strongly suggest that other fibers, beside pectin, are also present in the sugar beet pulp.

3.3.2 Galacturonic acid content in POS fractions

The POS fractions obtained as described in the material and method section were analyzed for their galacturonic acid content. After an initial acid hydrolysis, 3-phenylphenol was added to the sample and the absorbance was measured spectrophotometrically at a wavelenght of 525 nm. The galacturonic acid was quantified against a standard solution and expressed as % w/w on d.m.

The percentage of galacturonic acid is reported for each sample in Fig. 3.2. Different colors correspond to different batch productions which are clarified in the legend of

the figure. The same colors relate to different fractions of the same batch production, as also explained in the legend.

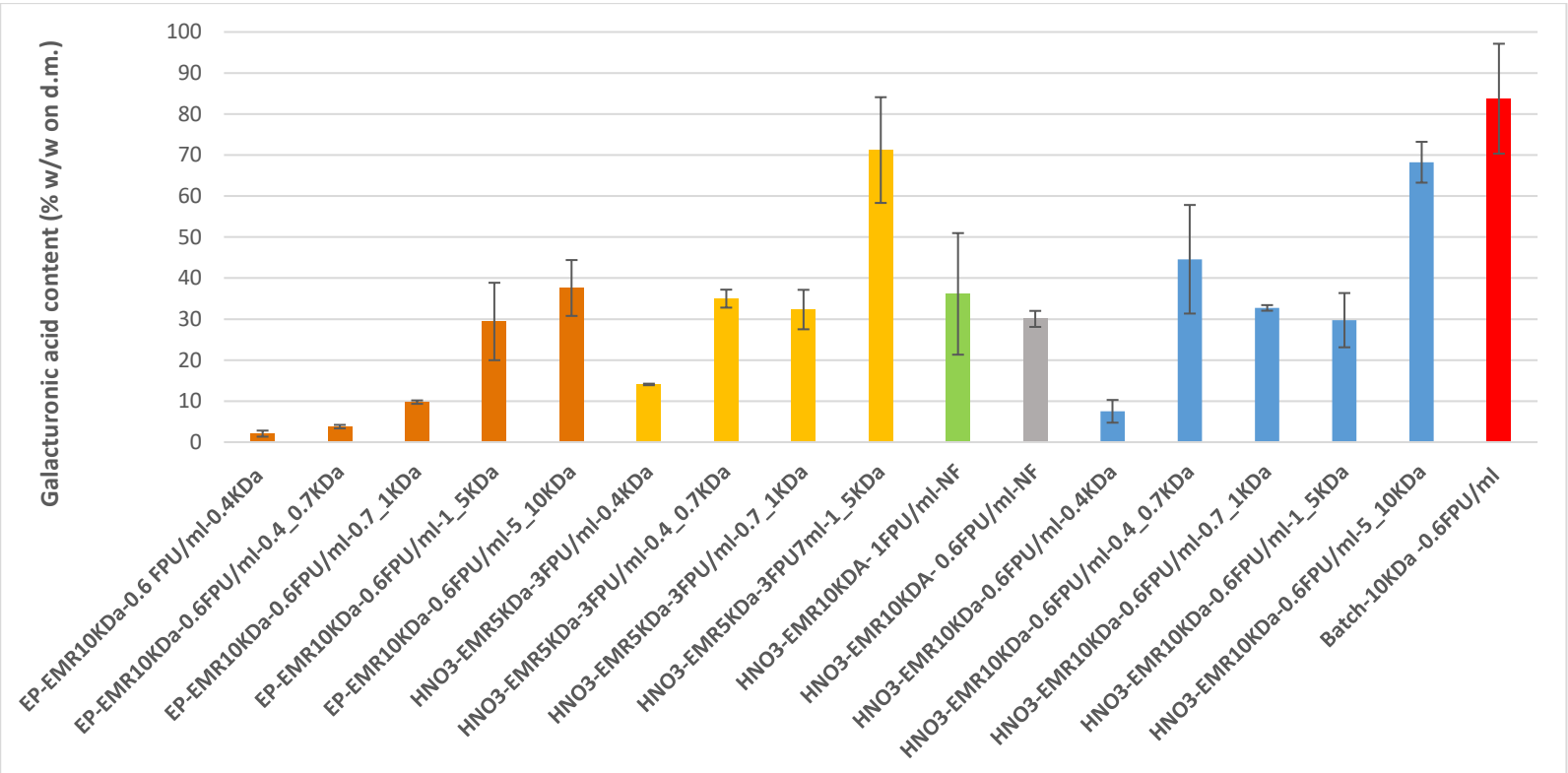

Figure 3.2: Galacturonic acid content (% w/w on d.m.) in POS fractions. EP: Enzymatic pretreatment of substrate; **HNO3**: Chemical pretreatments of substrate with HNO3; **EMR10KDa:** hydrolysis in enzymatic membrane reactor with 10KDa MWCO membrane; **EMR5KDa:** hydrolysis in enzymatic membrane reactor with 5KDa MWCO membrane; **Batch:** hydrolysis performed in batch system; **FPU/ml:** enzyme concentration; **xxKDa/xxKDa_xxKDa:** MWCO membrane used in the fractionation cascade; **NF**: not fractionated. Each color used in the graph refers to each process, as specified in the legend.

The content of galacturonic acid provided a preliminary indication on the amount of pectic oligomers contained in the different preparations. Anyway, it should be taken into account that POS could also be made by arabinans, which does not contain galacturonic acid and thus are not included in the quantification. Therefore, the amount of total pectic oligomers could be underestimated. On the other hand, the quantification performed in this way also includes free galacturonic acid, which is not a POS, and could lead to an overestimation of the pectic oligomers.

With these limitations of the method in mind, we can make some considerations on the POS content of the various batches and fractions.

The fractionation, besides having the advantage to remove the free galacturonic acid, is expected to allow to separate POS of different molecular size. Looking at the amount of galacturonic acid in each fraction (Fig.3.2), it can be observed that using the chemical pretreatment (process indicated in yellow and blue) and the batch system (process indicated in red), the highest concentration of galacturonic acid (hence possibly of POS) were found, especially in some of the fractions.

In particular, the POS seemed to be highly concentrated in the fraction of 1_5KDa, when the 5KDa MWCO membrane was used in EMR (71.21%; process indicated in yellow), as well as in the fraction of 5_10KDa for the 10KDa MWCO membrane in EMR (68.24%; process indicated in blue). The highest concentration was found using the batch system (83.73%; process in red), but this result could be strongly affected by the presence of free galacturonic acid.

Furthermore, it seemed that the POS production was not really influenced by the enzymatic dilution.

Thus, overall, the chemical pretreatments and the hydrolysis in EMR seemed to be most promising technologies in order to obtain fractions rich in POS. Anyway, in order to study more in detail the composition, the POS fraction were also analyzed by mass spectrometry technology.

3.3.3 HILIC/ESI-MS analysis of POS fractions

POS fractions were also analyzed for providing the molecular composition of pectic oligosaccharides. Samples were analyzed with a HILIC column in ESI- mode. An example of the full scan chromatogram obtained is reported in Fig.3.3. The pectic oligosaccharides were identified on the basis of retention time features, molecular weight and in source fragmentation with negative ionization mode. (Leijdekkers, *et al,* 2011) and an example of identification is reported in Fig.3.4**.** The molecular mass of the most abundant ions in the mass spectra were compared with the compounds already identified and reported in literature (Korner *et al*, 1999; Quemener *et al*, 2004; Ralet *et al,* 2009). Moreover, further masses possibly present in the mixtures were calculated considering the possible combinations of the uronic acids, the rhamnogalacturonan residues and the presence of methyl groups, acetyl groups and neutral sugar side chains.

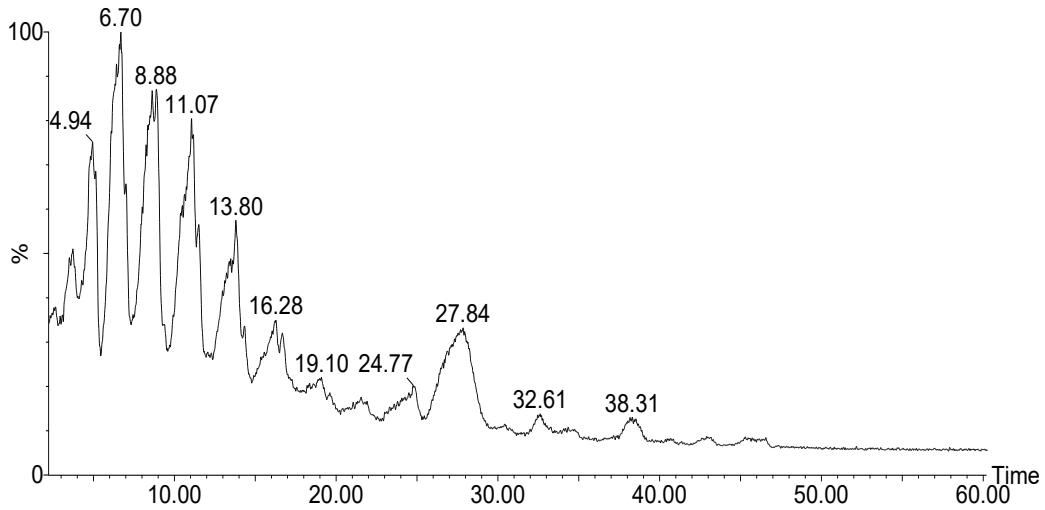

Figure 3.3: Total Ion Chromatogram (ESI-) of a pectin oligomer sample.

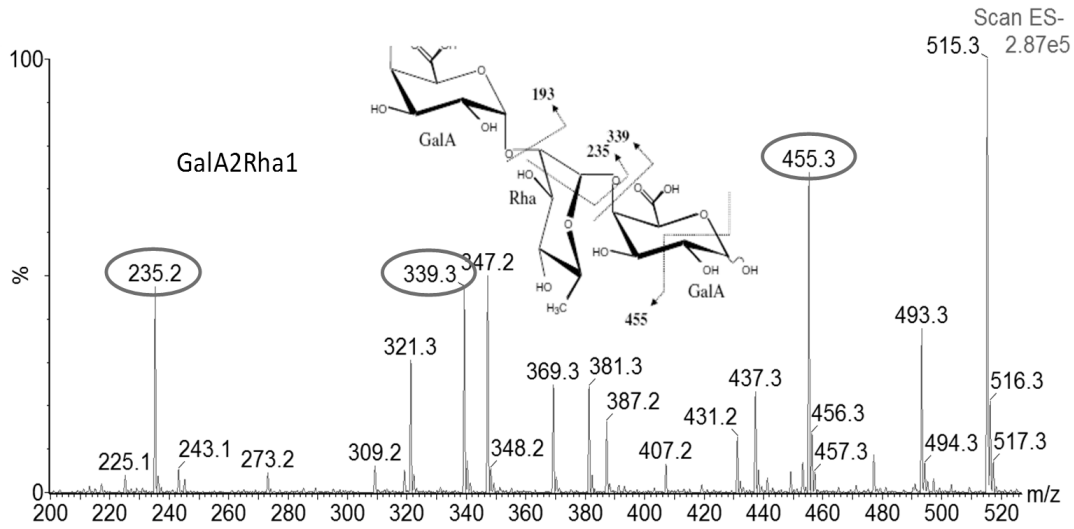

Figure 3.4: ESI- mass spectra of the rhamnogalacturonan GalA2Rha1.

Regarding the sugar composition, the occurrence of homogalacturonan, rhamnogalacturonan and arabinan was expected in the produced POS fractions. Indeed, different types of oligomers were detected: arabinans with polymerization degree (DP) from 2 to 10, rhamnogalacturonans (DP 3-6) also in acetylated form, unsaturated polygalacturonans (DP 4-5) always found in methylated or acetylated form, and polygalacturonans (DP 3-7), both free and in methylated/acetylated forms. Pectic oligomers identified are reported in Table 3.2 with the corresponding ratio m/z and the retention time.

Table 3.2: Pectic oligosaccharides identified by HILIC/ESI-MS analysis.

ID		MW (Da)	Trace (m/z)	RT (min)
Ara2	⌂⌂	282	281.3	3.59
Ara3	⌂⌂⌂	414	413.4	6.51
GalA2Rha1	●Δ●	516	515.4	26.22
Ara4	⌂⌂⌂⌂	546	545.5	6.58
GalA3	●●●	546	545.4	34.66
GalA2Rha2	Δ●Δ●	662	661.5	27.41
GalA2Rha2OAc	Δ●Δ●OAc	704	703.5	22.8

ID		MW (Da)	Trace (m/z)	RT (min)
UGalA4OMe	U●●●●OMe	718	717.5	32.56
GalA4	●●●●	722	721.5	44.92
GalA4OMe	●●●●OMe	736	735.4	38.88
GalA4Oac	●●●●OAc	764	763.6	41.18
UGalA4OMe2OAc	U●●●●OMe2OAc	774	773.5	14.33
GalA4OMeOAc	●●●●OMeOAc	778	777.5	32.44
Ara6	⌂⌂⌂⌂⌂⌂	810	809.6	13.39
GalA3Rha2	●Δ●Δ●	838	837.5	38.28
UGalA5OMe2	U●●●●●OMe2	908	907.6	35.41
GalA5OMe	●●●●●OMe	912	911.5	46.72
GalA5OMe2	●●●●●OMe2	926	925.5	39.71
Ara7	⌂⌂⌂⌂⌂⌂⌂	942	941.7	13.41
UGalA5OMe2OAc	U●●●●●OMe2OAc	950	949.6	29.41
GalA5OMeOAc	●●●●●OMeOAc	954	953.6	43.54
UGalA5OMe3OAc	U●●●●●OMe3OAc	964	963.5	20.77
GalA5OMe2OAc	●●●●●OMe2OAc	968	967.6	36.53
GalA3Rha3	Δ●Δ●Δ●	984	983.7	39.63
GalA4Rha2	●●Δ●Δ●	1014	1013.7	42.89
GalA3Rha3OAc	Δ●Δ●Δ●OAc	1026	1025.7	31.82
Ara8	⌂⌂⌂⌂⌂⌂⌂⌂	1074	1073.8	16.24
GalA6OMe2OAc	●●●●●●OMe2OAc	1144	1143.6	45.93
Ara9	⌂⌂⌂⌂⌂⌂⌂⌂⌂	1206	1205.8	18.48
GalA7OMe2	●●●●●●●OMe2	1278	1277.8+638.5	44.1
Ara10	⌂⌂⌂⌂⌂⌂⌂⌂⌂⌂	1338	1337.9	21.21

⌂ = arabinose (Ara); ● = galacturonic acid (GalA); U = unsaturated; Δ = rhamnose (Rha); OMe/OAc = methylation/acetylation.

By using HILIC chromatography, the first compounds that elute are the smaller and apolar ones, while big and charged compounds elute later. Therefore, the retention time increases with the chain length, thus with the polymerization degree. Given the unavailability of standards due to the vast variety of possible oligosaccharides structures, a semi-quantification was performed by performing an extract ion chromatogram (XIC) and integrating the corresponding peak area. In this way, the relative amount of each compound can be compared among all the samples.

The absolute chromatographic areas of all oligomers identified are reported in Fig. 3.5, which gives a rough ideas of the amount of each oligomer present. Fig. 3.6 shows the chromatographic peak area of the three main classes of identified POS, i.e. arabinans, rhamnogalacturonans and polygalacturonans.

Even if the absolute area depends on the ionization ability of every single compounds, since the structure of pectic oligosaccharides are very similar among them, it is likely to assume that the ionization is similar for every compound, hence the chromatographic area might be considered proportional to the amount. Of course, this approximation is also affected by the fact that ionization efficiency decrease by increasing the molecular mass, leading to an underestimation of the structures over 2 kDa.

Anyway, even despite these approximations, overall, based on the signal intensity in mass spectrometry, the POS abundance was found to be in agreement with concentration measured by colorimetric assay, but with some exceptions.

In fact, the POS fraction collected from the batch system, which showed the highest galacturonic acid content, did not show a very high POS amount if compared to other fractions. This difference is likely due, as suggested before, to strong the presence of free galacturonic acid in the sample. In fact, in batch system, the POS-products are not separated from the hydrolysis environment as in EMR, increasing the risk for further hydrolysis, and possibly leading to a higher contribution of free galacturonic acid to the total galacturonic acid, as measured after hydrolysis.

Furthermore, it was also remarkable that fractions in the range 0.4_0.7KDa, obtained by chemically pretreated SBP (process indicated in yellow and blue), which showed a significant galacturonic acid content (34.99 % and 44.59% respectively), were actually very poor in POS if compared to other fractions. Also in this case, the presence of free galacturonic acid, very likely not efficiently removed by the filtration, could contribute to the high acid sugar content.

On the contrary, the fraction 0.7_1 kDa, which seemed poor in POS due to the lower galacturonic acid content, was indeed found to be quite rich in it, and the reason of the discrepancy is that this fraction was mostly made by arabinans, which indeed do not contain galacturonic acid, in particular Ara2, Ara3 and Ara6.

As depicted in the figure, it can be also noted that the POS fraction collected at the end of the fractionation cascade, in particular the one at 1_5KDa (process in yellow) and the one at 5_10KDa (process in blue), showed a very similar composition in terms of oligomers abundance and in particular they were rich in rhamnogalacturonans; probably, these structure, despite the low molecular weight, could be more retained at the membrane surface along the cascade, accumulating in the last fraction. In particular, these fractions were very rich in GalA2Rha1 and GalA3Rha2.

In general, the polygalacturonans were less generated than the other two classes; this could be due, as said before, to the fact that the extracted sugar beet pulp pectin was rich in “hairy” regions (rhamnogalacturonans and side chains) and less rich in homogalacturonan, and, also, these “smooth” regions were probably less hydrolyzed.

Based on the signal intensity in mass spectrometry, it was possible to study the molecular composition and the abundance of the identified structures, considering of course the limitations mentioned above.

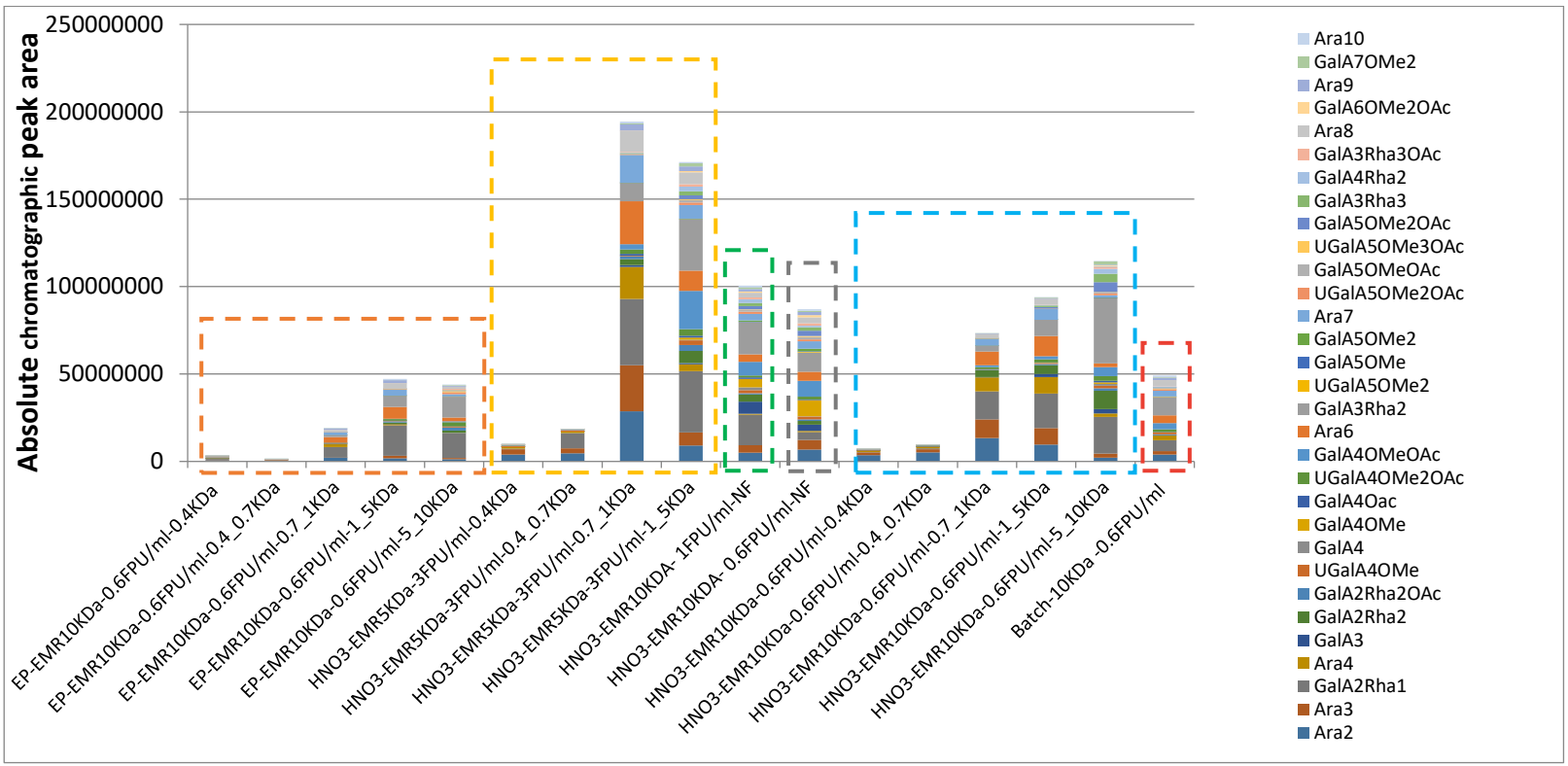

Figure 3.5. Absolute chromatographic peak areas of the identified pectic oligomers. EP: Enzymatic pretreatment of substrate; **HNO3:** Chemical pretreatments of substrate with HNO3; **EMR10KDa:** hydrolysis in enzymatic membrane reactor with 10KDa MWCO membrane; **EMR5KDa:** hydrolysis in enzymatic membrane reactor with 5KDa MWCO membrane; **Batch:** hydrolysis performed in batch system; **FPU/ml:** enzyme concentration; **xxKDa/xxKDa_xxKDa:** MWCO membrane used in the fractionation cascade; **NF:** not fractionated. Each color used in the graph refers to each process, as specified in the legend.

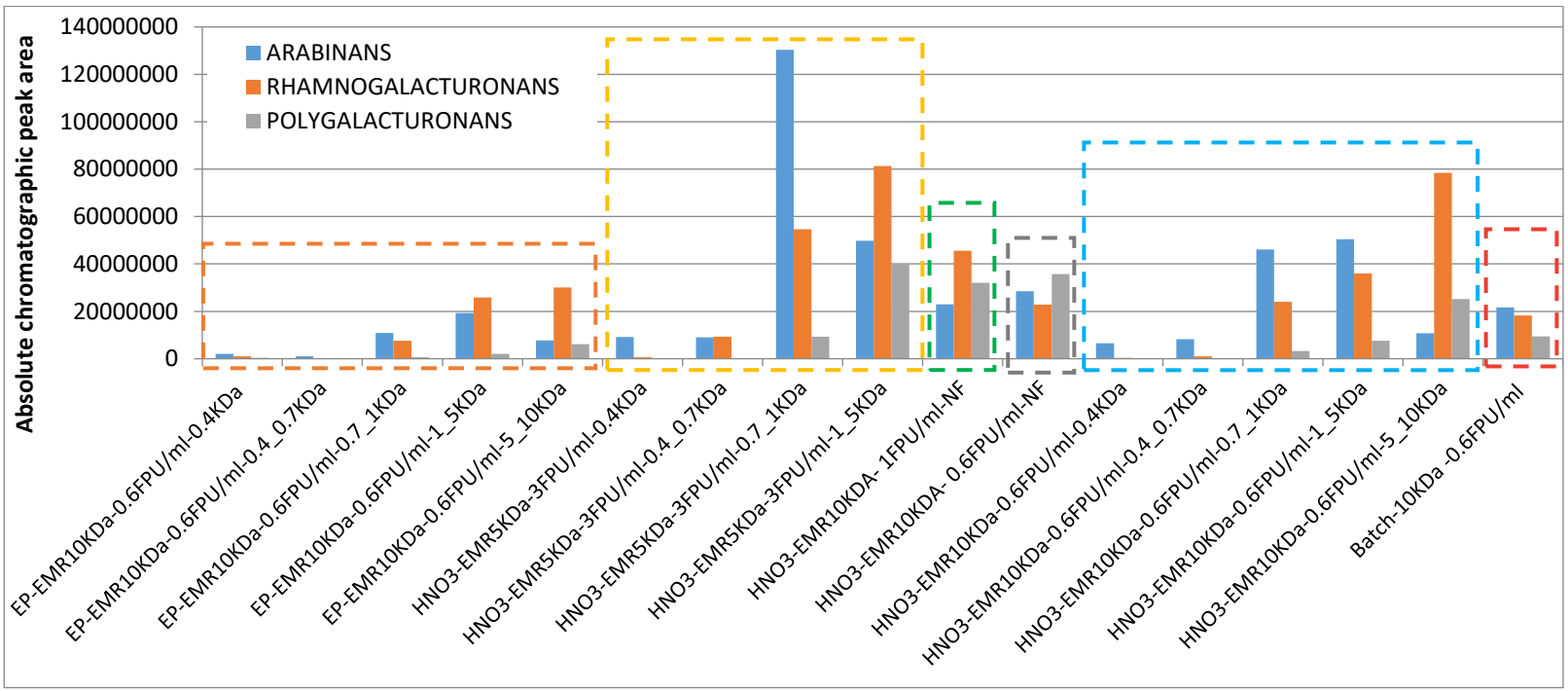

Figure 3.6: Total chromatographic peak area of arabinans, rhamnogalacturonans and polygalacturonan. **EP:** Enzymatic pretreatment of substrate; **HNO3:** Chemical pretreatments of substrate with HNO3**; EMR10KDa:** hydrolysis in enzymatic membrane reactor with 10KDa MWCO membrane; **EMR5KDa:** hydrolysis in enzymatic membrane reactor with 5KDa MWCO membrane; **Batch:** hydrolysis performed in batch system; **FPU/ml:** enzyme concentration; **xxKDa/xxKDa_xxKDa:** MWCO membrane used in the fractionation cascade; **NF**: not fractionated. Each color used in the graph refers to each process, as specified in the legend.

3.4 Conclusions

The characterization of the POS fractions on molecular level showed that using different pretreatments on the waste material and different POS production conditions, POS mixtures with different molecular properties were obtained.

The HILIC/ESI-MS analysis provided details on the composition of the POS fractions. When SBP was chemically pretreated and hydrolyzed in enzyme membrane reactor (having 5kDa molecular cut off membranes), fractionation allowed to obtain the following POS-fractions: (1) 0.7-1kDa, rich in arabinans, and (2) 1-5 kDa, rich in rhamnogalacturonans. When 10KDa membranes were used, POS in the range of 1-5KDa, rich in arabinans, and 5-10KDa, rich in rhamnogalacturonans, were produced. Therefore, the process could be tailored in order to produce POS mixtures richer in one class of oligomers than others.

The characterization performed in the present study gave the chance to investigate the production process of pectic oligosaccharides. Indeed, the process conditions could be further adjusted in order to produce POS with specific molecular weight and specific composition starting from a rich-pectin source such as sugar beet pulp.

Moreover, further experiments can be performed in order to optimize the process also in terms of residence time (contact time between substrate and enzyme in EMR) or substrate concentration.

Acknowledgments

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4. Pectic oligosaccharides (POS) from sugar beet pulp: optimization of process conditions, molecular characterization and stimulation of lactic acid bacteria

Abstract

Pectic oligosaccharide (POS) fractions derived from sugar beet pulp by enzymatic hydrolysis performed in a membrane enzymatic reactor after optimizing the process, have been characterized. In particular, the characterization was performed at the molecular level by hydrophilic interaction chromatography (HILIC) coupled with electrospray mass spectrometry detection (ESI-MS). The POS structures were identified on the basis of retention time, molecular weight and in source fragmentation. The analysis provided structural information on the composition of such mixtures, including the degree of polymerization, sugar composition and the presence of methyl esters and acetyl groups. This information also indicated the effect of the different technologies for POS production on the molecular composition of the obtained mixtures. The POS fractions were then tested for their ability to stimulate the bacterial growth of seven *Lactobacilli* strains, monitored *in vitro* using impedance microbiology. The ability to stimulate two *E. coli* strains, chosen as being common pathogens for poultry and piglet, was also evaluated. The *in vitro* studies showed that some POS preparations have the ability to stimulate the growth of *Lactobacilli* strains in a composition-specific and strain-specific way, therefore they have the potential to behave as prebiotic in feed, whereas none stimulated the *coli* strains.

4.1 Introduction

Pectin is a heterogeneous carbohydrate formed by “smooth” and “hairy” regions. The first one are called homogalacturonan and they are made by a backbone of α-(1-4)-D-galacturonic acid units (GalA). The hairy regions are represented by rhamnogalacturonan, in which the galacturonic acid units are interspersed with rhamnose residues (Rha), [→2)- α-L-Rha*p*-(1-4)- α-D-Gal*p*A-(→], linked to neutral sugar side chains, which are arabinan, galactan and/or arabinogalactan. The galacturonic acid residues can also be methyl-esterified at the C-6 or acetyl-esterified at O-2 and/or at O-3. (Caffal *et al*, 2009)

Pectic oligosaccharides (POS) are oligomers including oligogalacturonides (GalOS), arabinooligosaccharides (AraOS), rhamnooligosaccharides (RhaGalAOS), xylooligosaccharides (XylOGalA) and arabinogalactooligosaccharides (AraGalOS). They are produced from pectin by enzymatic, chemical or combined methods, starting from several vegetal sources (Gullon *et al,* 2013). Since food industry generates large amount of wastes after vegetables processing, recently the European bioeconomy is focusing on the reuse of waste as well as the recovery of functional and bioactive molecules. Pectic oligosaccharides recovery represents a relatively new field on which the research is focusing the attention (Apostolis *et al,* 2014; Howard *et al,* 2003; Lavigne *et al*, 2002; Oosterveld *et al,* 2002; Nawirska *et al,* 2004; Kush et al, 2016).

A potential source of POS is sugar beet pulp (SBP), a major by-product of the sugar production industry. The EU produces around 16.5 - 17.5 million tonnes of sugar equivalent and is the world's leading producer of sugar beet (Caffal *et al*, 2009; CIBE, 2016), containing 15-25% of pectin. (Muller-Maatsch *et al,* 2016; Oostervald *et al,* 2002)

The production and the characterization of POS from sugar beet pectin has been reported in literature (Concha Olmos *et al*, 2012; Holck *et al,* 2011). In

particular, Buchholt *et al* (2004), Funami *et al* (2011) and Martinez M. *et al* (2009) demonstrated the enzymatic production of POS starting from already available commercial sugar beet pectin by using batch system or dead-end ultrafiltration system. Recently, Babbar *et al* demonstrated that POS production can be achieved directly starting from SBP and using a continuous cross-flow enzymatic membrane reactor (EMR) with a specific cut off. This allowed the tailoring POS production also maximizing their recovery.

Recent studies indicated the prebiotic potential of pectic oligosaccharides obtained after hydrolysis with specific enzymes. They were shown to stimulate gut-beneficial microflora, such as *Lactobacillus* and *Bifidobacterium* strains (Babbar *et al*, 2015; Olano-Martin *et al*, 2002; Jun *et al*, 2006; Hotchkiss *et al,* 2003; Licht *et al,* 2010). They were also able to control the microbial ecology within animal's gastrointestinal tract, allowing better health performances. (Hotchkiss *et al,* 2003; Garthoff *et al*, 2010; Gullon *et al,* 2011)

In particular, Manderson *et al* (2005) and Mandalari *et al* (2007) demonstrated that oligomers with a degree of polymerization (DP) lower than 10 had optimal bifidogenic effects compared to higher molecular weight saccharides. Holck *et al* (2011) reported that pectic oligosaccharides with only slightly different structures have significantly different biological effects, for example, with activity on gut bacterial populations.

Moreover, the acidic POS have been proved not to be cytotoxic or mutagenic, being suitable for use in food and in feed. (Moure *et al,* 2006) In feed production, the functional ingredients represent the major cost, therefore many current studies are focusing on the development of sustainable methods to transform renewable sources, such as food wastes, into added-value products.

However, a deep study on the relationship about POS structure and MW, and their prebiotic properties, is still lacking. In the present work, in order to link the molecular composition and the prebiotic functionality of the POS produced, we

focused on the characterization of POS obtained from sugar beet pulp recovered as by-product from sugar industry, using the process described by Babbar *et al.*

The POS produced were characterized by performing HILIC-ESI/MS analysis, allowing the structural analysis in terms of degree of polymerization, sugar composition and acetylation and methylation pattern. In order to better define the structure-function relationship of POS, some POS preparations were then assessed for their prebiotic activity by monitoring their ability to stimulate the growth of seven *Lactobacilli* strains (and two *E. coli* strains taken as controls) by using impedance microbiology (Bancalari *et al*, 2016).

4.2 Materials and Methods

4.2.1 Chemicals

The ammonium formate and the acetonitrile were provided by Sigma-Aldrich (Germany). The formic acid and the nitric acid were provided by Fisher Chemical (UK).

4.2.2 Extraction of pectin

Pectin was extracted from sugar beet pulp using nitric acid, essentially following the procedure described by Babbar *et al*. Briefly, the sugar beet pulp residue at 5% (w/v) substrate loading was subjected to pretreatment with dilute nitric acid at pH 1.4. (Kliemen *et al,* 2009) The flasks were incubated in an incubator shaker at 80 °C, 125 rpm for 4h and subsequently neutralized to pH 4.8 with 10% sodium hydroxide solution. After extraction, the biomass was centrifuged at 5000 xg for 10 min and the supernatant was recovered and characterized for sugar composition.

4.2.3 Analysis of crude pectin extract

HPAEC-PAD analysis was used in order to quantify the galacturonic acid and neutral sugars such as rhamnose, arabinose, galactose, xylose and fructose. The free monosaccharides were detected as such. The amount of dissolved pectic oligo- and polysaccharides was determined by correcting the total amount of saccharides detected after hydrolysis by the amount of monosaccharides already present in the extract. To achieve a complete hydrolysis and full recovery of the monomers, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h (Martinez *et al,* 2009; Martinez *et al,* 2010). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 2040 xg (Eppendorf centrifuge 5415 C) for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into HPAEC-PAD.

4.2.4 Enzymatic hydrolysis of pectin

The crude pectin extract was subjected to an enzymatic hydrolysis in an enzymatic membrane reactor (EMR) by using Viscozyme L. The 3 L reactor was filled with 600 mL (±5 ml) pretreated sugar beet pulp solution at a substrate concentration of 25 g/L. The system was thermostated at 45°C (optimum temperature for the enzyme activity). After reaching the temperature, 60 mL of a diluted solution of Viscozyme L at pH 4.5 was added to the reactor vessel. In order to activate the enzyme, the vessel was first shaken at 200 rpm for the time chosen as residence time (time in which the substrate stays in contact with the enzyme). After this initial activation step, continuous dosing of the substrate was started while separating the POS at the same time by filtration with a 10 kDa membrane. By regulating the filtration flux, the average residence time of the substrate was set at the requested residence time. The permeates enriched in low molecular weight oligosaccharides (<10KDa) were collected at regular times (10, 20, 30, 40 and 50 minutes using the

residence time RT = 10 minutes; 20, 40, 60, 80 and 100 minutes using the residence time RT= 20 minutes; 30, 60, 90, 120 and 150 minutes using the residence time RT=30 minutes). The residue, enriched in high molecular weight saccharides (>10KDa) and containing the enzyme, was collected at the end of the process. The enzyme was inactivated at 100°C for 5 minutes.

The POS mixtures used for the assessment of the prebiotic activity were obtained after mixing three or four fractions obtained at each condition. Care was taken to select only fractions that were representative for the process condition, i.e., after stabilization of the composition. The mixtureswere freeze-dried prior to supplementation and tested for the prebiotic activity. (Liophilizer LIO 5P, VWR International PBI Milan, Italy).

4.2.5 Hydrophilic interaction liquid chromatography coupled with electrospray mass spectrometry detection

Pectic oligosaccharides were separated and identified using the method reported by Leijdekkers *et al* (2011), but with a slight modification. In particular, 100 mg of sample was dissolved in 1 ml of eluent B (H_2O:CH_3CN 80:20, 10 mM $HCOONH_4$ + 0.2% HCOOH). The samples were centrifuged at 2081 xg (model 5810R EPPENDORF, Hamburg, Germany), at a temperature of 4°C, for 10 minutes and the supernatant recovered for the analysis. The oligosaccharides present in the POS mixture were separated by a HILIC column (ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 3 mm X 150 mm) in an UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters ACQUITY SQD) using a gradient elution. Eluent A was H_2O:CH_3CN 20:80 (v/v), 10 mM $HCOONH_4$ + 0.2% HCOOH, eluent B was H_2O:CH_3CN 80:20 (v/v), 10 mM $HCOONH_4$ + 0.2% HCOOH; gradient: 0-60 min linear from 100% A to 60%A, 60-65 min isocratic at 60% A, 65-66 min linear from 60%A to 100%A, 66-75 min isocratic at 100%A. Flow was 0.3 ml/min; optimal run time for separation of various

oligosaccharides was 75 min; column temperature 35°C; sample temperature 18°C. The injection volume was set to 10 µl; acquisition time 0-75 min; ionization ion mode: negative; capillary voltage 2.6 kV; cone voltage 60 V; source temperature 150°C; desolvation temperature 350°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h. The samples were analyzed in Full Scan mode with a scan range of 200-2000 m/z. The analysis was performed in duplicate.

4.2.6 Prebiotic tests with impedance microbiology

Seven *Lactobacillus* strains were purchased from the "Belgian coordinated collection of microorganism'' (LMG, Gent, Belgium, http://bccm.belspo.be/)". The strains were selected from different species, different origins and potential probiotic nature and they are described in Table 4.1.

Table 4.1: Bacterial strains used for the impedance microbiology measurement

Strains	***Lactobacillius* species**	**Biological origin**
LMG 6400	*L. rhamnosus*	Not know
LMG 18399	*L. plantarum*	Not known
LMG 9198	*L. curvatus*	Milk
LMG 23516	*L. casei*	Human, faeces
LMG 8900	*L. fermentum*	Eight day old breast fed infant
LMG 8151	*L. acidophilus*	intestine
LMG 18223	*L. delbrueckii* subsp. *Lactis*	Acidophilus milk

Lactobacillus strains were routinely grown in Man-Rogosa-Sharpe (MRS) medium (Oxoid, Basingstoke, UK) under aerobic conditions and incubated at 30°C for mesophilic strains (*L. rhamnosus, L. plantarum, L. curvatus, L. casei)* and 37°C for thermophilic strains (*L. fermentum, L. acidophilus and L. delbrueckii* subsp. *lactis*).

Moreover, two strains of *Escherichia coli* (*E.coli* k88 and *E.coli* k99), pathogens for piglet and poultry, were gently provided by Geert Bruggeman, Nutrition Sciences, Belgium. Strains were grown in MacConkey agar medium N°2 (Oxoid, Basingstoke, UK) and incubated at 37°C under aerobic conditions. Tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) was used for the impedometric experiments.

Before the experiments, the mesophilic and thermophilic lactobacilli strains were revitalized three times in MRS broth (Oxoid, Basingstoke, UK) at 30°C and 37°C, respectively under aerobic conditions. For the *E.coli* strains, revitalization was made in TSB broth (Oxoid, Basingstoke, UK) at 37°C.

For the growth experiments, sterile broth media (MRS for lactobacilli and TSB for *E. coli*) were supplemented with two concentrations of POS, in this context 1% (w/v) and 2% (w/v) for solid POS, chosen because compatible with the concentrations actually administrable to the animals.

For lactobacilli, 50 ml of sterile MRS broth was supplemented with 0.5 g of solid POS and 50 ml of MRS sterile broth was supplemented with 1 g of solid POS, for 1% and 2%, respectively. Successively, supplemented media were filtered in 0.22 µm membrane (Millipore, Billerica, MA, U.S.A.) and the filtered fraction was used for the impedance experiment.

For *E. coli*, 20 ml of TSB broth was supplemented with either 0.2 g or 0.4 g of solid POS. Successively both broth media were filtered with a 0.22 µm membrane (Millipore, Billerica, MA, U.S.A.) and the filtered fraction was used for the impedance experiment.

The prebiotic test was carried out to assess the effect of POS mixtures on the bacterial growth of *Lactobacillus* strains, while parallel incubations with the bifidogenic substrate fructo-oligosaccharides, FOS, and no carbohydrates, were respectively used as positive and negative controls. Media supplemented with POS (1% and 2%) were inoculated with approximately 10^3 cfu/ml of each bacterial strain.

Successively, 6 ml of the media were transferred to glass cells, and incubated in the microbiological impedance analyser (BacTrac 4300® Sy-Lab, Neupurkersdorf, Austria) for 64 h at 30°C or 37°C according to the bacterial strains. For the experiments carried out in the present study, the impedance change (E-value), which is referred to as the impedance change at the electrode surface of the MRS and TSB, was used (Bancalari et al 2016). This value, recorded every 10 minutes, is revealed as a relative change in the measurement signal and shown as E% percentage in function of time (48 hours) in an impedance curve. The threshold was set at 5% E value that allows determining the time to detection (TTD), the time when the impedance curve meets the choose threshold level, by guarantying consistence results. This was previously verified by measuring serial dilutions of pure cultures (data not shown).

Both MRS and TSB supplemented with 1% (w/v) FOS (Sigma-Aldrich, St. Louis, MO, U.S.A.) and inoculated with bacterial cultures were used as positive controls. MRS and TSB, without the supplemented pectin oligosaccharides, and inoculated with each bacterial strain were used as negative controls. All experiments were made in single copy.

Blanks were made for each supplemented media without addition of pure cultures. E % values were registered and resulted to be under the threshold of 5%.

4.2.7 Statistical analysis

The statistical analysis of ESI/MS data was performed using SPSS software (IBM SPSS statistics 21). One-way analysis of variance and Tukey's multiple comparison tests were used to determine significant differences in the peak area of the identified compounds in the samples from different production conditions. Tests were considered statistically significant when *P*-values lower than 0.05 were obtained.

4.3 Results and discussion

4.3.1 Analysis of pectic oligosaccharides by HILIC/ESI-MS

The substrate of enzymatic hydrolysis in EMR was represented by the same material used in the previous chapter, i.e. the crude pectic extract obtained from the pretreated sugar beet pulp. The composition in sugars of the crude pectic extract has been reported in section 3.3.1.

In order to further fine tuning the POS production, one of the conditions used in chapter 3 was selected for further optimization. In particular, the crude pectic extract was used for the production of pectic oligosaccharides in an enzymatic membrane reactor (EMR) by using the Viscozyme L, which was found to be the most promising conditions for the production of tailored POS (see chapter 3). More specifically, the 10KDa MWCO membrane and a 0.6 FPU/ml enzyme concentration were chosen as suitable conditions to obtain the tailored POS. Then, the process was further optimized in terms of residence time (RT), hence the residence time 15, 20 and 30 minutes were tested.

The POS mixtures collected along the production process in EMR (see section 4.2.4 for process details), were characterized for their molecular composition by LC/MS. In particular, the oligosaccharides were identified using the same method as in the previous work (see chapter 3).

The POS were identified on the basis of retention time features, molecular mass and in source fragmentation, using a HILIC column coupled to an electrospray (ESI) mass spectrometer, with negative ionization mode. (Leijdekkers *et al*, 2011) An example of the full scan chromatogram obtained is reported in figure 4.1.

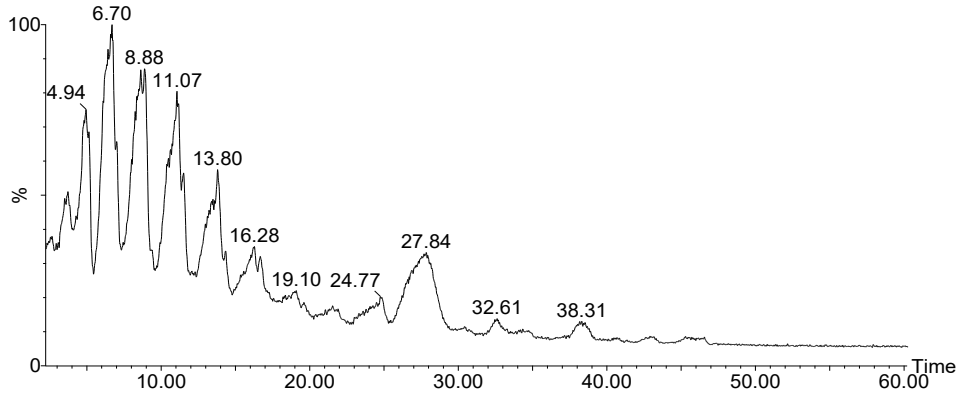


Figure 4.1: Total Ion Chromatogram (ESI-) of a pectin oligomer sample.

The molecular mass of the most abundant ions in the mass spectra were compared with the compounds already identified and reported in literature (Korner *et al*, 1999; Quemener *et al*, 2004; Ralet *et al,* 2009), and also with the structures identified in the previous study (see chapter 3). Moreover, further masses possibly present in the mixtures were calculated considering the possible combinations of the uronic acids, the rhamnogalacturonan residues and the presence of methyl groups, acetyl groups and neutral sugar side chains. The pectic oligomers identified are reported in table 4.2 with the corresponding retention time.

Table 4.2. Pectin oligosaccharides identified by HILIC/ESI-MS analysis.

ID		MW (Da)	Trace (m/z)	RT (min)
Ara2	⌂⌂	282	281.3	3.59
Ara3	⌂⌂⌂	414	413.4	6.51
GalA2Rha1	●Δ●	516	515.4	26.22
Ara4	⌂⌂⌂⌂	546	545.5	6.58
GalA3	●●●	546	545.4	34.66
GalA2Rha2	Δ●Δ●	662	661.5	27.41
GalA2Rha2OAc	Δ●Δ●OAc	704	703.5	22.8
UGalA4OMe	U●●●●OMe	718	717.5	32.56

ID		MW (Da)	Trace (m/z)	RT (min)
GalA4	●●●●	722	721.5	44.92
GalA4OMe	●●●●OMe	736	735.4	38.88
GalA4Oac	●●●●OAc	764	763.6	41.18
UGalA4OMe2OAc	U●●●●OMe2OAc	774	773.5	14.33
GalA4OMeOAc	●●●●OMeOAc	778	777.5	32.44
Ara 5	⌂⌂⌂⌂⌂	678	677.5	10.60
Ara6	⌂⌂⌂⌂⌂⌂	810	809.6	13.39
GalA3Rha2	●Δ●Δ●	838	837.5	38.28
UGalA5OMe2	U●●●●●OMe2	908	907.6	35.41
GalA5OMe	●●●●●OMe	912	911.5	46.72
GalA5OMe2	●●●●●OMe2	926	925.5	39.71
Ara7	⌂⌂⌂⌂⌂⌂⌂	942	941.7	13.41
UGalA5OMe2OAc	U●●●●●OMe2OAc	950	949.6	29.41
GalA5OMeOAc	●●●●●OMeOAc	954	953.6	43.54
UGalA5OMe3OAc	U●●●●●OMe3OAc	964	963.5	20.77
GalA5OMe2OAc	●●●●●OMe2OAc	968	967.6	36.53
GalA3Rha3	Δ●Δ●Δ●	984	983.7	39.63
GalA4Rha2	●●Δ●Δ●	1014	1013.7	42.89
GalA3Rha3OAc	Δ●Δ●Δ●OAc	1026	1025.7	31.82
Ara8	⌂⌂⌂⌂⌂⌂⌂⌂	1074	1073.8	16.24
GalA6OMe2OAc	●●●●●●OMe2OAc	1144	1143.6	45.93
Ara9	⌂⌂⌂⌂⌂⌂⌂⌂⌂	1206	1205.8	18.48
GalA7OMe2	●●●●●●●OMe2	1278	1277.8+638.5	44.1
Ara10	⌂⌂⌂⌂⌂⌂⌂⌂⌂⌂	1338	1337.9	21.21

⌂ = arabinose (Ara), ● = galacturonic acid (GalA), U = unsaturated, Δ = rhamnose (Rha), OMe/OAc = methylation/acetylation

Regarding sugar composition, the occurrence of homogalacturonans, rhamnogalacturonans and arabinans was expected in the produced POS fractions, and indeed, the main detected oligomers were arabinans (DP2-10), rhamnogalacturonans (DP3-6) in both free and acetylated forms, and polygalacturonans (DP3-7) in both free and methylated/acetylated forms. The same

compounds were identified by Holck, *et al* (2011) and Martinez *et al (*2009)*,* after hydrolysing pure commercial pectin. Also, a perfect consistency with the molecular composition of POS mixtures analysed in the previous study was observed (see chapter 3).

Given the unavailability of standards due to the vast variety of possible oligosaccharides structures, a semi-quantification was performed by performing an extract ion chromatogram (XIC) and integrating the corresponding peak area. The semi-quantification was performed at two levels, First, the abundance of each class (arabinans, rhamnogalacturonans, polygalacturonans, and unsaturated polygalacturonans) was determined by semi-quantifying the total peak area of each class against the total of the POS observed. Secondly, the relative abundance of a specific POS-molecule within its class was calculated against the total peak area of this specific class. Even if these values give an indication of the relative abundance of the various POS, it has however to be kept in mind that the peak area is not only influenced by the amount but also may be influenced by structure dissimilarity.

Differently as done in the work presented in chapter 3, in this study the POS amount in function of the process time was studied, with the main aim to investigate the process stability.

4.3.2 POS fraction composition related to the production conditions

HILIC-ESI/MS analysis of the POS fractions allowed the analysis of the fine chemical structure and composition of POS mixtures obtained from sugar beet pulp in EMR in function of the process time. The POS-fraction were collected as a function of the subsequent reactor replenishments. More specifically following samples were taken along the process at RT10 (table 4.3; fractions: 10 min, 20 min, 30 min, 40 min, 50 min and residue), at RT20 (table 4.4; fractions: 20 min, 40 min, 60 min, 80 min, 100 min and residue) and at RT30 (table 4.5; fractions: 30 min, 60

min, 90 min, 120 min, 150 min and residue). These samples were analyzed on the presence of the compounds identified in Table 4.2 and semi-quantified on two levels as explained in section 4.3.1.

Figure 4.2 shows the relative abundance of each class as a function of the production time (i.e. reactor replenishment) for the three residence times RT10, RT20 and RT30. Looking at the results obtained at the shortest residence time of RT10 (Fig.4.2a) and RT20 (Fig.4.2b), the arabinans seemed to accumulate in time, mostly at the expense of the rhamnogalacturonans that decreased in abundance at approximately the same rate. Both the polygalacturonans as well as the unsaturated polygalacturonans remained overall stably produced. The change in composition during production, indicated that the combined process of hydrolysis and filtration was not stable at these short residence times. This was most likely due to the fact that the formation of POS from rhamnogalacturonans was too slow for the feeding and filtration rate applied, resulting in a continuous decrease in abundance against other types of POS-products that were more easily formed. Also, the differences in composition between the last permeate and the residue obtained at the end of the process, suggested that the process was not stable.

At the longest residence time RT30, the trend was different. The abundance of each class remained more stable during the production, indicating a more stable process operation and, also implying that a residence time of 30 minutes was more appropriate from a production point of view.

When comparing the composition at the end of the test among the three residence times, also some differences can be noted. The smaller the residence time, the (slightly) more abundant the arabinans and rhamnogalacturonans. The polygalacturonans were however slightly more present at longer residence times.

In order to have more information at the molecular level, each class was also analysed in more detail on the various POS-compounds listed in table 4.2. The relative contribution of each compound to its class is indicated in table 4.3 for RT10,

table 4.4 for RT20 and table 4.5 for RT30. From the Tables it can be derived, that the arabinan class was composed of a variety of compounds mostly between DP3 and DP9, whereas the other classes had more dominant products, such as GalA2Rha1 and GalA3Rha2 within the rhamnogalacturonans, GalA4OMe and GalA4MeOAc within polygalacturonans and UGalA4OMe and UGalA4OMe2OAc within the unsaturated polygalacturonans.

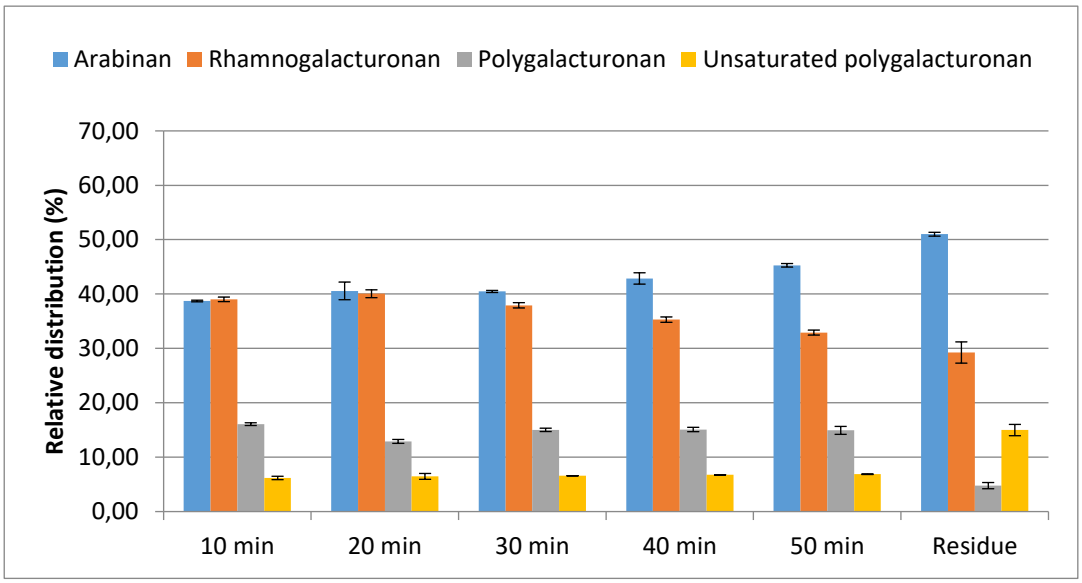
As mentioned before, the abundance of the arabinans and the rhamnogalacturonans changeed in function of production time, especially at the shortest RT10 and RT20. However, when looking into more detail at the individual POS-products in tables 4.3-4.5, it can be seen that within a class the changes were not systematic. For instance, within the arabinan class, the Ara5 and Ara8 had a general tendency to become more abundant in time at the expense of Ara9. Very similarly, even if the rhamnogalacturonan class as a whole decreased in importance in time, GalA2Rha2 seemed to be less affected than GalA3Rha3. Within the polygalacturonan class, on the other hand, GalA4OMe typically increased (at least partially) at the expense of GalA4OMeOAc. Even if the impact of filtration cannot fully be excluded, it is expected that these individual changes are related to the different formation kinetics of the various compounds, whereby the increasing ones are more easily formed than the decreasing ones. As an example, the more dominant presence of GalA4OMe could be due to the fact that the hydrolysis of acetylated galacturonans is more hampered due to steric hindrance.

At the molecular level, also a comparison was made between the compositions of the permeates obtained at the end of the process at the three investigated RTs. Even if the global composition among the various classes did not alter strongly, some remarkable differences are noted at the molecular level especially within the polygalacturonan class. From the tables 4.3-4.5, it can be observed that at short residence times GalA4OMe was very abundant (~59%), whereas at the longer residence time of 30 minutes GalA4OMeAc become the

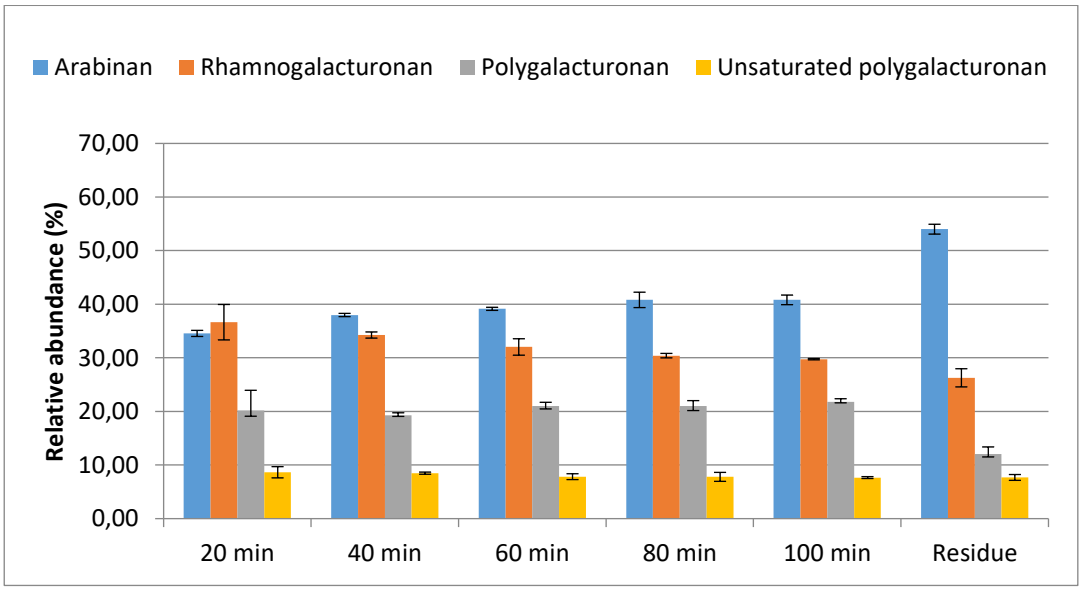
dominant POS product within the polygalacturonan class (~57%). Very striking are also the differences in composition between the last permeates and their corresponding retentates. At longer residence times, it is seen that the abundance of GalA4OMeAc in the retentate increased significantly matching much better the composition of its corresponding permeate. This observation confirms the more stable operation at a residence time of 30 minutes. Moreover, it indicates that GalA4OMeOAc probably needs more time to be formed as GalA4OMe, as can be expected from the more difficult hydrolysis of acetylated polysaccharides.

Finally, the total POS products were also evaluated in absolute terms, so based on the total intensity. As general observation, the total intensity of the residue was always lower ($p<0.05$) than the total intensity of the permeates. This could be partially due to the fact that besides POS, also other products were likely present in the residue (including polysaccharides), which might give ion suppression phenomena in mass spectrometry. Basing on this hypothesis, the low abundance of POS in the residue does not indicate the minor content of pectin but rather the presence of more coeluting compounds.

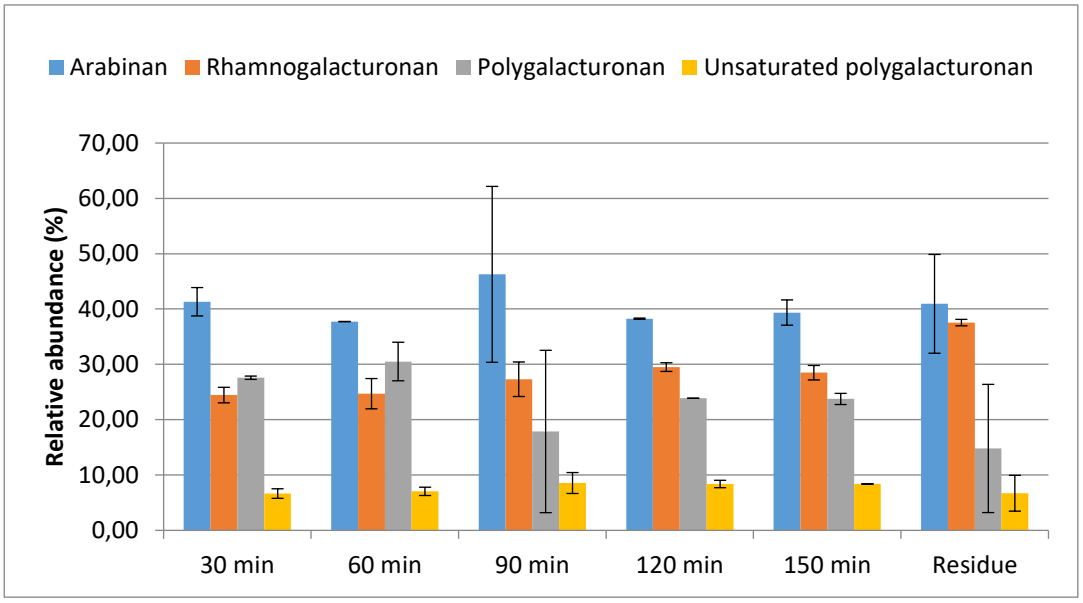
In general, the data showed that using different hydrolysis time it is possible to tailor POS amount and composition.


Arabinan
Rhamnogalacturonan
Polygalacturonan
Unsaturated polygalacturonan
Relative distribution (%)
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
10 min
20 min
30 min
40 min
50 min
Residue

(a)


Arabinan
Rhamnogalacturonan
Polygalacturonan
Unsaturated polygalacturonan
Relative abundance (%)
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
20 min
40 min
60 min
80 min
100 min
Residue

(b)



(c)

Figure 4.2: Relative abundance of each identified oligomers class in each POS fraction at RT10 (a), RT20 (b) and RT30 (c).

Table 4.3: Relative abundance of oligomers identified by performing a HILIC/ESI-MS analysis on POS fractions collected at RT10.

		10 min	Dev Std	20 min	Dev Std	30 min	Dev Std	40 min	Dev Std	50 min	Dev Std	Residue	Dev Std
Arabinans	Ara2	6.10	0.27	7.26	0.07	6.91	0.45	5.74	0.19	5.26	1.06	4.32	1.55
	Ara3	5.04	0.44	5.99	0.01	5.24	1.03	4.67	0.28	4.57	0.40	5.39	1.05
	Ara4	5.81	0.01	6.41	0.04	5.27	0.45	5.12	0.45	4.43	0.25	1.71	0.08
	Ara5	11.24	0.17	13.78	0.42	15.35	0.54	15.52	1.97	17.73	0.23	17.65	1.19
	Ara6	23.60	0.44	22.58	0.50	22.99	1.18	23.59	1.37	22.60	0.73	27.82	0.64
	Ara7	9.93	0.15	9.51	0.54	9.73	0.08	10.03	0.14	10.56	0.09	10.49	0.76
	Ara8	14.16	0.38	14.40	0.14	15.48	0.72	18.33	0.87	17.13	0.12	16.20	0.23
	Ara9	18.24	0.92	16.28	0.63	14.55	0.82	14.20	0.48	12.25	0.76	10.55	0.07
	Ara10	5.87	0.65	3.78	1.14	4.48	0.21	2.80	0.32	5.49	0.23	5.87	0.05
	Tot	38.72	0.15	40.58	1.63	40.47	0.19	42.87	1.04	45.29	0.32	51.00	0.35

		10 min	Dev Std	20 min	Dev Std	30 min	Dev Std	40 min	Dev Std	50 min	Dev Std	Residue	Dev Std
Rhamnogalacturonans	GalA2Rha1	26.34	1.60	22.76	0.65	24.09	0.19	24.54	0.19	25.24	1.33	60.07	1.66
	GalA2Rha2	9.55	0.28	11.48	1.12	11.62	0.87	12.66	0.87	12.34	1.14	0.95	0.32
	GalA2Rha2OAc	0.17	0.10	0.20	0.01	0.42	0.04	0.39	0.04	0.28	0.02	0.37	0.14
	GalA3Rha2	58.60	2.01	57.89	2.05	56.69	0.51	53.61	0.51	53.27	3.27	33.58	1.66
	GalA3Rha3	3.65	0.49	6.09	0.40	5.49	0.08	6.98	0.08	6.71	0.67	0.14	0.03
	GalA3Rha3OAc	0.70	0.23	0.81	0.24	0.54	0.12	0.54	0.12	0.77	0.04	3.00	0.07
	GalA4Rha2	0.99	0.13	0.77	0.14	1.16	0.18	1.28	0.18	1.41	0.15	1.89	0.21
	Tot	39.03	0.42	40.05	0.72	37.92	0.49	35.29	0.49	32.92	0.46	29.25	1.95

		10 min	Dev Std	20min	Dev Std	30 min	Dev Std	40 min	Dev Std	50 min	Dev Std	Residue	Dev Std
Polygalacturonans	GalA3	0.29	0.09	0.31	0.07	0.46	0.17	0.42	0.19	0.29	0.05	1.40	0.24
	GalA4	3.00	0.07	5.24	0.66	5.32	0.79	7.58	0.86	7.89	0.11	4.70	1.63
	GalA4Oac	3.92	0.02	4.58	0.09	5.00	0.19	3.34	0.45	3.99	0.15	3.80	0.38
	GalA4OMe	59.77	0.83	58.40	2.52	59.11	0.60	57.24	1.39	59.16	2.02	30.36	2.74
	GalA4OMeOAc	13.34	1.32	8.72	0.86	8.30	0.22	9.18	0.34	8.39	1.87	20.12	1.07
	GalA5OMe	4.42	0.47	5.84	1.03	5.57	0.31	5.71	0.40	5.67	0.22	8.06	1.30
	GalA5OMe2	1.98	0.19	1.38	0.19	1.67	0.04	2.40	1.07	1.65	0.07	1.05	0.76
	GalA5OMe2OAc	1.25	0.11	1.12	0.23	1.06	0.18	1.24	0.20	1.11	0.70	2.28	0.55
	GalA5OMeOAc	2.32	0.06	2.64	0.52	2.22	0.36	1.47	0.28	1.43	0.28	3.25	0.09
	GalA6OMe2OAc	1.37	0.15	1.03	0.36	2.00	0.20	1.71	0.86	0.90	0.19	3.04	0.82
	GalA7OMe2	8.35	0.23	10.73	0.53	9.29	0.12	9.72	0.92	9.51	0.52	21.94	0.34
	Tot	16.07	0.25	12.90	0.36	15.03	0.31	15.09	0.40	14.93	0.73	4.77	0.57

		10 min	Dev Std	20 min	Dev Std	30 min	Dev Std	40 min	Dev Std	50 min	Dev Std	Residue	Dev Std
Unsaturated polygalacturonans	UGalA4OMe	61.58	0.03	63.55	3.59	62.96	2.10	58.11	1.46	55.20	0.66	52.61	1.28
	UGalA4OMe2OAc	25.89	0.42	25.36	2.96	24.61	3.95	32.37	4.28	34.53	0.58	36.13	0.15
	UGalA5OMe2	8.95	0.25	7.62	0.42	7.15	0.59	6.33	1.02	5.86	0.62	8.32	1.57
	UGalA5OMe2OAc	0.33	0.06	0.31	0.15	0.25	0.04	0.40	0.07	0.41	0.12	0.23	0.05
	UGalA5OMe3OAc	3.25	0.57	3.17	0.36	5.03	1.30	2.79	1.73	4.00	0.57	2.70	0.20
	Tot	6.18	0.31	6.47	0.54	6.58	0.00	6.75	0.02	6.87	0.05	14.98	1.04

Table 4.4: Relative abundance of oligomers identified by performing a HILIC/ESI-MS analysis on POS fractions collected at RT20.

		20 min	Dev Std	40 min	Dev Std	60 min	Dev Std	80 min	Dev Std	100 min	Dev Std	Residue	Dev Std
Arabinans	Ara2	4.55	0.37	3.60	0.37	3.39	1.43	3.39	0.26	3.24	1.31	4.02	0.25
	Ara3	4.57	0.82	5.06	0.25	5.35	0.31	5.09	0.43	5.58	0.04	4.71	0.99
	Ara4	2.77	0.34	3.11	0.34	3.44	0.22	2.51	0.59	2.14	0.13	2.83	1.28
	Ara5	10.46	0.10	13.46	0.05	14.17	0.62	15.45	1.39	17.76	0.42	17.24	0.25
	Ara6	22.61	2.67	24.21	0.87	24.84	1.44	25.11	1.32	22.95	0.15	20.91	1.88
	Ara7	11.24	0.65	12.55	0.41	11.33	1.23	11.62	0.82	11.88	0.83	10.93	0.07
	Ara8	18.02	0.15	18.47	1.33	19.24	2.27	19.35	0.05	18.63	0.31	15.72	0.70
	Ara9	19.50	0.17	15.86	0.05	13.94	0.09	14.60	0.39	12.31	0.19	15.19	0.20
	Ara10	6.28	1.38	3.68	0.48	4.30	0.61	2.88	1.19	5.52	0.65	8.45	1.36
	Tot	34.55	0.57	37.99	0.30	39.14	0.29	40.81	1.44	40.82	0.90	54.00	0.92

		20 min	Dev Std	40 min	Dev Std	60 min	Dev Std	80 min	Dev Std	100 min	Dev Std	Residue	Dev Std
Rhamnogalacturonans	GalA2Rha1	31.25	0.89	28.71	0.32	28.20	0.63	29.05	0.24	29.64	0.00	44.61	3.44
	GalA2Rha2	9.13	0.71	11.93	0.09	13.13	0.18	12.09	0.17	12.66	1.07	0.47	0.17
	GalA2Rha2OAc	0.33	0.20	0.40	0.10	0.23	0.11	0.38	0.09	0.41	0.13	3.30	2.00
	GalA3Rha2	54.95	2.60	52.00	0.44	50.30	0.22	50.31	0.14	48.45	0.84	47.41	3.57
	GalA3Rha3	2.24	1.54	5.32	0.15	5.97	0.16	5.75	0.37	6.19	0.16	0.31	0.02
	GalA3Rha3OAc	1.12	0.50	0.68	0.13	0.56	0.07	0.89	0.17	0.91	0.09	2.47	1.66
	GalA4Rha2	0.99	0.18	0.95	0.05	1.61	0.48	1.53	0.33	1.75	0.15	1.42	0.01
	Tot	36.65	3.31	34.25	0.57	32.03	1.53	30.41	0.42	29.75	0.13	26.27	1.70

		20 min	Dev Std	40min	Dev Std	60 min	Dev Std	80 min	Dev Std	100 min	Dev Std	Residue	Dev Std
Polygalacturonans	GalA3	0.26	0.03	0.50	0.08	0.19	0.06	0.20	0.05	0.35	0.15	0.47	0.18
	GalA4	1.62	1.20	1.15	0.06	0.79	0.40	1.23	0.20	0.68	0.37	3.51	0.31
	GalA4Oac	1.86	0.45	2.75	0.14	3.11	0.95	3.00	0.59	2.69	0.48	4.95	0.45
	GalA4OMe	55.35	7.97	57.58	3.58	60.34	0.82	60.14	0.73	60.90	0.87	33.36	5.18
	GalA4OMeOAc	24.78	3.80	21.43	2.55	19.51	0.22	18.90	0.04	18.18	0.98	30.38	5.23
	GalA5OMe	2.05	0.14	2.33	0.17	1.64	0.21	1.54	0.27	1.89	0.04	2.43	1.11
	GalA5OMe2	0.58	0.31	0.66	0.27	1.35	0.79	1.45	0.23	1.36	0.00	3.09	0.29
	GalA5OMe2OAc	3.52	0.66	2.51	0.44	2.49	0.44	2.47	0.11	2.17	0.05	3.40	0.05
	GalA5OMeOAc	2.86	1.92	2.88	0.88	3.24	0.57	3.21	0.50	4.50	0.53	4.30	0.12
	GalA6OMe2OAc	1.19	0.40	1.25	0.38	1.04	0.56	1.09	0.17	1.29	0.58	1.60	0.99
	GalA7OMe2	5.93	0.60	6.94	0.22	6.29	0.43	6.78	0.95	6.00	0.71	12.52	0.50
	Tot	20.16	3.79	19.27	0.47	21.01	0.70	20.99	1.02	21.77	0.59	12.05	1.32

		20 min	Dev Std	40 min	Dev Std	60 min	Dev Std	80 min	Dev Std	100 min	Dev Std	Residue	Dev Std
Unsaturated polygalacturonans	UGalA4OMe	67.25	3.12	65.73	2.16	60.62	0.31	64.59	2.10	56.33	0.63	49.29	6.39
	UGalA4OMe2OAc	19.54	2.65	22.68	1.09	23.77	0.20	19.57	4.93	26.39	4.50	32.51	2.11
	UGalA5OMe2	10.63	0.33	9.15	0.54	10.57	0.37	12.15	2.79	11.70	0.42	10.36	2.64
	UGalA5OMe2OAc	0.26	0.03	0.27	0.16	1.67	2.30	0.47	0.14	2.60	2.46	0.74	0.57
	UGalA5OMe3OAc	2.31	0.11	2.17	0.37	3.37	2.43	3.22	0.10	2.98	1.22	7.10	1.06
	Tot	8.65	1.05	8.48	0.20	7.83	0.55	7.79	0.83	7.66	0.18	7.68	0.54

Table 4.5: Relative abundance of oligomers identified by performing a HILIC/ESI-MS analysis on POS fractions collected at RT30.

		30 min	Dev Std	60 min	Dev Std	90 min	Dev Std	120 min	Dev Std	150 min	Dev Std	Residue	Dev Std
Arabinans	Ara2	1.59	0.75	1.88	0.03	2.01	0.16	3.53	1.43	3.26	1.51	6.55	1.79
	Ara3	5.18	1.00	6.03	0.09	5.98	0.75	6.39	0.51	5.62	0.15	4.13	0.21
	Ara4	1.27	0.48	2.26	0.85	1.65	0.62	2.00	1.01	1.93	0.13	1.58	0.06
	Ara5	11.83	2.15	15.29	1.35	14.80	0.94	15.14	0.35	15.02	2.15	12.87	6.28
	Ara6	20.77	1.70	21.40	0.65	22.52	2.65	23.68	1.30	24.38	2.31	23.02	8.10
	Ara7	13.70	3.37	11.54	0.10	9.84	1.43	9.99	0.29	11.68	0.27	11.41	5.06
	Ara8	15.21	0.29	14.85	0.11	17.23	0.18	17.32	2.07	19.27	1.69	20.37	6.32
	Ara9	23.03	0.26	21.71	0.09	19.85	1.94	18.34	0.05	13.02	0.68	12.90	1.22
	Ara10	7.42	0.71	5.04	1.18	6.11	1.49	3.62	0.17	5.83	0.21	7.17	0.54
	Tot	41.31	2.57	37.74	0.00	46.27	15.90	38.25	0.11	39.36	2.28	40.94	8.93

		30 min	Dev Std	60 min	Dev Std	90 min	Dev Std	120 min	Dev Std	150 min	Dev Std	Residue	Dev Std
Rhamnogalacturonans	GalA2Rha1	29.71	1.60	30.73	0.62	36.32	18.05	27.61	0.00	28.22	0.55	42.72	20.18
	GalA2Rha2	9.81	0.59	8.53	0.13	9.65	3.28	10.31	0.25	11.25	1.26	7.72	0.07
	GalA2Rha2OAc	2.27	0.28	1.70	0.51	3.64	1.91	3.13	2.42	2.30	0.13	0.83	0.79
	GalA3Rha2	54.58	1.42	51.17	0.77	42.67	14.26	50.38	2.63	50.24	1.71	43.77	19.92
	GalA3Rha3	1.20	0.28	4.64	1.05	3.62	3.43	5.14	0.33	5.05	0.37	2.51	1.96
	GalA3Rha3OAc	1.26	0.20	1.57	0.12	2.27	1.08	1.39	0.23	1.39	0.88	2.03	1.04
	GalA4Rha2	1.17	0.34	1.67	0.16	1.83	0.08	2.04	0.06	1.55	0.74	0.41	0.19
	Tot	24.45	1.40	24.70	2.74	27.31	3.12	29.51	0.78	28.49	1.31	37.55	0.58

		30 min	Dev Std	60 min	Dev Std	90 min	Dev Std	120 min	Dev Std	150 min	Dev Std	Residue	Dev Std
Polygalacturonans	GalA3	0.20	0.05	0.14	0.01	0.49	2.45	0.30	0.09	0.18	0.10	3.19	13.80
	GalA4	2.71	0.01	1.66	0.86	5.14	14.64	3.58	0.35	2.80	0.06	5.35	11.93
	GalA4Oac	1.17	0.13	1.67	0.13	1.41	1.59	1.17	0.13	1.35	0.36	2.39	1.41
	GalA4OMe	30.67	2.82	36.59	7.88	19.93	11.95	19.59	0.49	19.40	0.81	23.85	11.31
	GalA4OMeOAc	50.85	0.47	46.20	6.07	52.09	29.34	57.24	0.75	57.17	1.99	50.89	26.35
	GalA5OMe	1.47	0.28	1.20	0.39	2.28	6.37	2.74	0.10	2.20	0.20	1.90	3.30
	GalA5OMe2	0.38	0.02	0.75	0.62	1.99	0.57	0.59	0.12	0.93	0.24	1.03	2.69
	GalA5OMe2OAc	3.36	0.51	3.16	0.34	3.85	1.95	4.58	0.50	4.51	1.18	4.78	1.75
	GalA5OMeOAc	4.14	1.26	3.84	0.20	4.52	2.92	4.21	0.07	5.09	0.06	4.27	2.40
	GalA6OMe2OAc	0.33	0.01	0.38	0.08	0.59	1.33	0.57	0.01	0.57	0.16	1.08	2.68
	GalA7OMe2	4.73	0.66	4.42	1.77	7.71	19.21	5.43	0.34	5.78	0.15	1.27	0.06
	Tot	27.58	0.30	30.51	3.49	17.87	14.67	23.86	0.00	23.75	1.01	14.80	11.58

		30 min	Dev Std	60 min	Dev Std	90 min	Dev Std	120 min	Dev Std	150 min	Dev Std	Residue	Dev Std
Unsaturated polygalacturonans	UGalA4OMe	44.99	1.05	37.46	3.24	41.28	8.04	37.59	1.20	41.58	2.11	61.55	1.30
	UGalA4OMe2OAc	26.92	1.95	33.38	0.02	34.44	7.83	24.76	6.35	33.53	6.63	17.08	3.23
	UGalA5OMe2	5.86	0.21	7.91	1.68	6.58	0.36	7.81	2.28	8.54	2.13	11.68	3.33
	UGalA5OMe2OAc	17.43	0.25	16.64	0.35	12.21	4.26	20.05	0.31	12.65	2.34	4.84	0.40
	UGalA5OMe3OAc	4.80	2.55	4.61	1.89	5.49	4.40	9.79	7.75	3.70	0.05	4.85	0.99
	Tot	6.65	0.86	7.05	0.75	8.56	1.89	8.38	0.67	8.40	0.03	6.71	3.24

4.3.3 Ability of POS to stimulate lactic acid bacteria

In order to assess the POS potential prebiotic effect, selected fractions were mixed and supplemented to the growth media suitable for efficient growth of each strains of lactic acid bacteria.

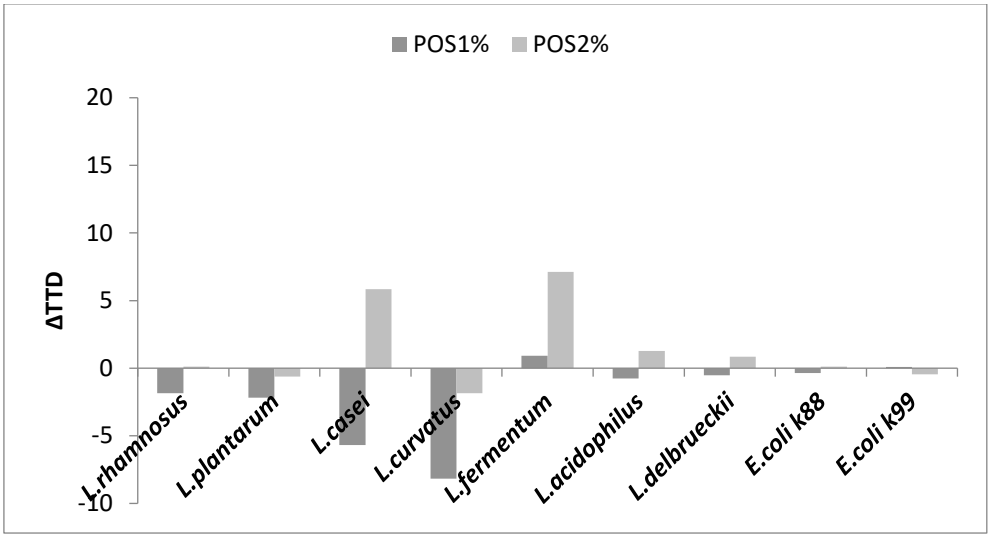
After supplementation, the growth medium was inoculated with each bacterial strain and transferred to glass cells equipped with four electrodes to which an electrical current was applied in the BacTrac 4300®. At regular intervals, the impedance was measured and plotted as function of time. As the microorganisms grow, the instrument measures the impedance changes as compared to its initial value expressed as E% (impedance of the electrodes) (Bancalari et al 2016). The time required to reach the chosen threshold level, is referred as the time to detection (TTD). Changes in the time to detection between value obtained with the strain grown in supplemented media and value obtained with the same strain grown without supplement (control), were expressed as follows:

$$\Delta TTD = TTD^{NC} - TTD^{POSX\%},$$

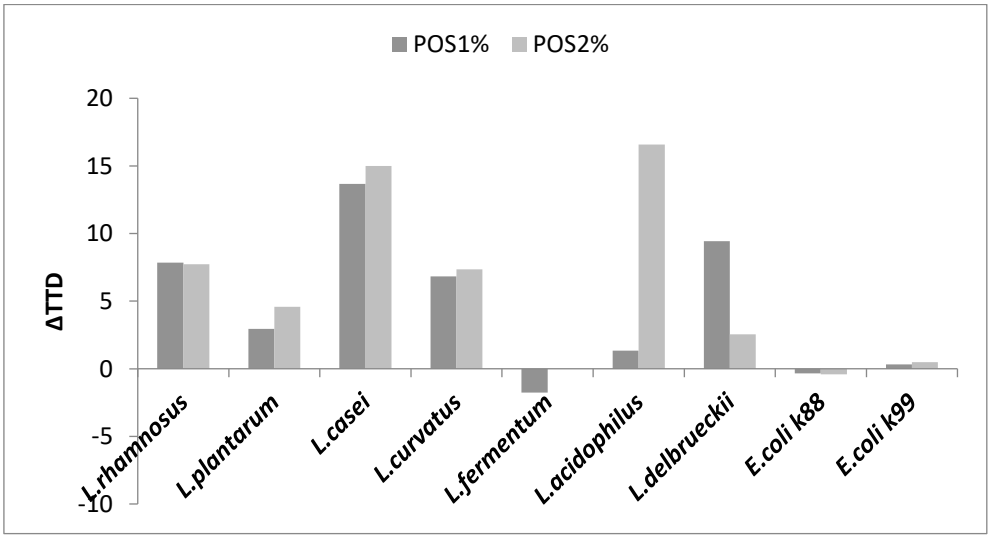
where TTD is the time to detection; NC is the negative control prepared by inoculating bacterial culture in the not supplemented media; POS X% represents the supplemented media with 1% and 2% of pectin derived-oligosaccharides.

Positive results represent a growth promotion, negative an inhibition and zero no effect.

The figure 4.3 shows the ΔTTD for each solid sample evaluated for all considered strains at the two POS concentration 1% and 2%.


POS1%
POS2%
ΔTTD
20
15
10
5
0
-5
-10
L.rhamnosus
L.plantarum
L.casei
L.curvatus
L.fermentum
L.acidophilus
L.delbrueckii
E.coli k88
E.coli k99

(a)


POS1%
POS2%
ΔTTD
20
15
10
5
0
-5
-10
L.rhamnosus
L.plantarum
L.casei
L.curvatus
L.fermentum
L.acidophilus
L.delbrueckii
E.coli k88
E.coli k99

(b)

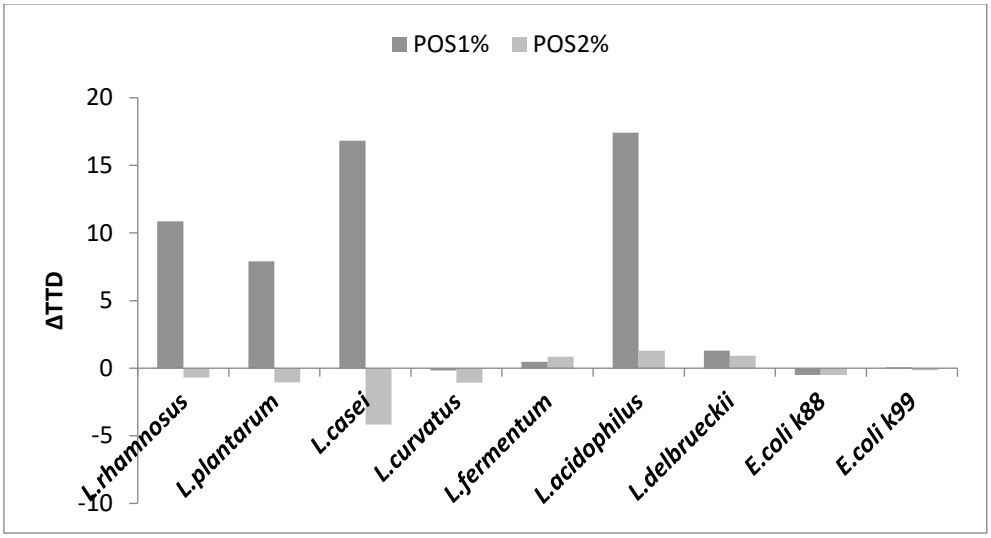


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Figure 4.3: Detection time (delta TTD vs time) of *Lactobacillus* and *E.coli* strains grown on media supplemented with 1% and 2% of mixture RT10 (a), Rt20 (b) and RT30 (c).

Concerning the process conducted at residence time 10 minutes (RT10), the fractions collected at 20, 30, 40 and 50 minutes were mixed since they had very similar composition. The mixture RT 10 (figure 4.3a) slightly promoted, only at the 2%, the growth of *L.casei* and *L. fermentum,* while seem to have none effect for the other species. Differently, the lower concentration did not affect, or even inhibited, the strains (Figure 4.3a).

Excepted for L. *fermentum*, which was not affected by its presence, the mixture RT20 (obtained mixing the fractions collected at 40, 60 and 100 minutes) showed a stimulation activity depending on the concentration and strains (Figure 4.3b).

Testing the mixture RT30, obtained mixing the fractions collected at 60, 120 and 150 minutes (figure 4.3c), it was possible to observe that *L. fermentum, L.*

delbrueckii and *L. curvatus* were not affected by its presence, while the other species were stimulated only by the highest concentration.

Almost all POS tested did not show growth stimulation or inhibition of *E. coli* strains. According to the above data, POS were able to stimulate *in vitro* the growth of lactic acid bacteria- considered the classical "microbial" signature of probiotics-, even if with strain- and concentration-specificity.

Looking at the results obtained in the present research, the stimulation was more marked when the growth medium is supplemented with mixture RT20, especially because the effect occurs at both POS concentrations. The reason for this prebiotic effect could be due both to the highest POS yield (80%) and the lowest monosaccharides yield (20%) in this fraction, as also showed by Babbar et al, as well as a specific composition. Indeed, this fraction is richer in arabinans and polygalacturonans (such as GalA4OMe), which are less abundant in the other fractions.

This suggests that not every POS is good for lactic acid bacteria stimulation, but that a specific POs composition is required, and also that not all the strains react in the same way.

4.4 Conclusions

The analyses performed allowed to get a very detailed insight on the molecular composition and on the prebiotic activity of the POS fractions from sugar beet pulp.

Clear indications on the preparations most rich in POS and on their composition in oligomeric sugars was obtained, also allowing to link the molecular composition to the technology used to obtain the POS preparation.

This study also showed evidence that POS supplementation modulates the growth of bacterial strains *in vitro*, although with a composition- and strain-

specificity. Indeed, one of the POS preparations, obtained with a residence time of 20 min, showed a marked stimulating activity, probably due to highest POS abundance and specific POS composition rich in arabinans and polygalacturonans.

According to the above results, POS can then be considered a promising product with potential prebiotic properties, also because POS fractions did not show stimulation activity on the pathogen strains. Of course, specific studies are needed in order to elucidate the effect of the oligosaccharide according to the concentration and to the specific bacterial strains. Anyway, the above studies demonstrate that pectin oligomers with a high level of functionality can be produced by enzymatic membrane reactor.

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5. Enzymatic production of pectic oligosaccharides (POS) from onion skins

Abstract

Onion skins are evaluated as a new raw material for the enzymatic production of pectic oligosaccharides (POS) with a targeted degree of polymerization (DP). The process is based on a two-stage process consisting of a chelator-based crude pectin extraction followed by a controlled enzymatic hydrolysis. Treatment of the extracted crude onion skins' pectin with various enzymes (EPG-M2, Viscozyme and Pectinase) showed that EPG-M2 was the most appropriate enzyme for tailored POS production. The experiments indicated that the highest amount of DP2 and DP3 is obtained at a time scale of 75–90 min withan EPG-M2 concentration of 26 IU/mL. At these conditions the production amounts 2.5–3.0% (w/w) d.m. for DP2 and 5.5–5.6% (w/w) d.m for DP3 respectively. In contrast, maximum DP4 production of 5.2–5.5%(w/w) d.m. is obtained with 5.2 IU/mL at a time scale of 15–30 min. Detailed LC–MS analysis allowed to identify the structure of the most abundant POS.

5.1 Introduction

Oligosaccharides are carbohydrates having sugars linked together with different degree of polymerization. In recent years, non-digestible oligosaccharides have found application in various fields, notably because of their specific prebiotic activities. (Swennen *at al,* 2006) It has been reported that prebiotic oligosaccharides benefit the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*Bifidobacteria* and *Lactobacilli*) and suppressing the activity of pathogenic organisms. (Garthoff *et al.,* 2010) The fermentation of oligosaccharides in the colon results in the generation of short chain fatty acids, which exert a number of health effects *viz.* inhibition of pathogenic bacteria, relief of constipation, reduction in blood glucose level, improvement in mineral absorption, decreased incidence of colonic cancer and modulation of the immune system. (Gullon *et al,* 2013) The studies on understanding the real mode of action of prebiotics is still going on. To date, only a few types of oligosaccharides like galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are commercially available, but there is an increasing interest in more performant and/or low cost prebiotic ingredients. In this respect, pectin derived oligosaccharides, also called pectic oligosaccharides, have been identified as emerging prebiotics. (Olano-Martin et al, 2002)

The major advantage of pectic oligosaccharides (POS) is that they are derived from the parent compound “pectin” which is a polysaccharide widely present within the primary cell wall and intercellular regions of higher plants. (Chen *et al,* 2013) More specifically, the POS is produced by tailoring the long chain pectin polysaccharides into smaller units of varying degree of polymerization. Another attractive property concerns their chemical identity which is versatile and very different from FOS and GOS due to the different chemical nature of the starting material. Pectin can be constituted of different

structural elements, such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan- I (RG-I) and rhamnogalacturoanan -II (RG-II), arabinan and arabinogalactan. As a consequence, various types of POS, *i.e.* rhamnogalacturonan-oligosaccharides, galacturonan-oligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, arabino-galactan oligosaccharides, can be produced depending upon the diverse structural elements present in pectin. (Babbar *et al,* 2014) However, only limited information is available on this new class of molecules, especially in relation to their composition and prebiotic properties, requiring further research to assess their potential.

Waste valorization of pectin rich agro-industrial residues into POS is an interesting way to use waste and by- product streams. Until now, a lot of work has been reported on the POS production from sugar beet pulp pectin, orange pectin and pure pectin. (Bako *et al,* 2007; Combo *et al,* 2012; Iwasaki *et al,* 1998; Leijdekkers *et al,* 2013; Olano *et al,* 2001; Yapo *et al,* 2007) Some scanty studies are available for POS production from potato pulp (Thomassen *et al,* 2011) and tomato processing waste (Suzuki *et al,* 2002). Nevertheless, the search for new resources and alternatives continues.

With a total production of 6.6×10^6t (Faostat, 2011), onions are an important vegetable in the EU. Part of these onions are processed in the form of dried, whole, cut, sliced and broken pieces with a totalsold volume of 3.5×10^4t in the EU (Eurostat, 2011), thereby gener-ating significant wastes including the skins. Onion skins are known to be very rich in pectin. (Alexander & Salubele, 1973) In our previous work, onion skins were found to contain around 20% (w/w) d.m of galacturonic acid. (Babbar *et al,* 2015) Historically, onion skins had various applications. Being a thin, light-weight, strong and often translucent paper. It was applied with carbon paper for typing duplicates in a typewriter. In addition, it was widely used for extracting pigment

for dyeing cotton carpet and dyeing cloth. (Bae, 2009) Nevertheless, the overall applicability has decreased enforcing the need of new valorization routes for this types of waste. Onion skins are for example currently tested for their antioxidant effect. (Albishi et al, 203; Urszula *et al,* 2013) However, given their high pectin content, they are expected to be very suitable raw material for POS production allowing a more versatile application of the waste.

The main goal of this work is to explore the use of onion skins for the production of pectic oligosaccharides. To the authors knowledge, this is the first study reporting the production and characterization of pectic oligosaccharides (POS) from this new raw material. The study is therefore taken with an objective to optimize process parameters to tailor and maximize the POS production. The research follows a two stage process *i.e.* (i) extraction of crude pectin followed by (ii) enzymatic tailoring of extracted pectin to POS.

5.2 Materials and methods

5.2.1 Raw material and chemicals

Onion skins were provided by the Institut für Getreideverarbeitung (IGV, GmbH), Germany. The skins were milled with a laboratory blender and screened on its particle size (< 1 mm) and stored in ziplock bags at room temperature until use. Celluclast 1.5 L (C-2730), predominantly (containing cellulase), Viscozyme L (V-2010) (a multienzyme complex) and Pectinase were obtained from Sigma-Aldrich (St. Louis, MO, USA). Endo-polygalacturonase M2 (EPG-M2) and polygalacturonic acid was purchased from Megazyme, Ireland. Standards for rhamnose, arabinose, galactose, xylose, glucose, fructose and galacturonic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Germany). The standards of di-galacturonic acid and tri-galacturonic acid were obtained from Sigma-Aldrich and the standard of tetra-galacturonic acid from Elicityl Oligotech (France).

The galacturonan oligosaccharide mixture DP1-DP10 was kindly provided by B. Whatelet and M. Paquot from Gembloux, Agro-Bio Tech (Belgium).

5.2.2 Total sugar composition and extraction of crude pectin from the onion skins

The total sugars present in onion hulls were estimated by following the protocol optimized in our previous study (Babbar *et al,* 2015). Based on our preliminary tests, sodium hexametaphosphate was selected as an extractant for onion skins (Babbar et al, 2015). Sodium hexametophosphate is already commonly used in food industry, though for other applications (Shirashoji *et al,* 2010)

Onion skins (1 g) were pretreated with 2% sodium hexametaphosphate at 95 °C for 0.5h in a hot water bath. The biomass was then centrifuged at 5000 x g for 10 min. The supernatant containing the crude pectin was collected and analyzed for its free monosaccharide as well as total saccharide composition. The latter was then taken as a measure for the polysaccharide content. The analysis was performed on HPAEC-PAD, as described elsewhere in the article.

5.2.3 Enzyme activity measurements

The endo-polygalacturonase activity of three enzymes was assessed on the substrate polygalacturonic acid following the protocol provided by Megazyme International, Ireland. Briefly, the method consisted of mixing 0.2 mL of a preincubated enzyme solution (suitably diluted) and 0.5 mL of preincubated substrate solution (1% w/v) in glass test tubes while vigorously mixing. The mixtures were incubated for 3, 6, 9 and 12 min at 45 ºC and measured photospectrometrically at 520 nm using the Nelson-Samogvi method. (McCleary & McGeough, 2015) The analyses of samples and standard solutions containing galacturonic acid (50µgrams *i.e.* 0.2 mL of 250

µgrams/mL in 0.2 % benzoic acid) were performed on a spectrophotometer (UV-1650 PC, Shimadzu, Koyto, Japan) against a reaction blank). One unit of endo-polygalacturonase activity is defined as the amount of enzyme required to release 1 µmole of galacturonic acid per minute from the polygalacturonic acid.

The activity of Viscozyme, Pectinase and EPG M2 expressed as EPG units was determined to be 4135, 2612 and 2600 U/mL.

5.2.4 Enzymatic pectic oligosaccharides (POS) production from the crude pectic extract from onion skins

Hydrolysis of pectin obtained from onion skins was done with three different enzymes so as to study the distribution of oligomers and monomers. Three different enzymes *i.e.* Viscozyme, Pectinase and EPG-M2 were used. All the enzymes used here are known for their diverse pectinase activity. (Combo *et al,* 2012) All the commercial enzyme solutions were diluted 50 times (accounting to 82.7, 52.2 and 5.2 U/mL). The hydrolysis was then conducted at 10% (v/v) of the diluted enzyme/ pectic solution. The hydrolysis was done for a period for 2 h at 45 °C, 150 rpm and the samples were collected at a regular interval of 15 min until 120 min. The enzymes were inactivated by thermal treatment at 100 °C for 5 min and further analyzed on HPAEC –PAD as stated elsewhere in the paper.

Based on the results obtained a further optimization of the selected enzyme (EPG-M2) was done. In the initial study, the EPG-M2 was added at 52 IU/mL, whereas in the optimization study the concentration was extended to 26 IU/mL, 5.2 IU/mL and 2.6 IU/mL. The hydrolysis was conducted at 10% (v/v) of the enzyme/ pectic solution. The hydrolysis was done at 45 °C and samples were withdrawn every 5 min unptil 90 min. The enzyme was

inactivated by thermal treatment at 100 °C for 5 min further analyzed on HPAEC – PAD.

5.2.5 Analysis of the free monosaccharides by HPAEC-PAD

The samples were adequately diluted and injected into high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for the analysis of galacturonic acid and other neutral sugars. The HPAEC-PAD used for analytical purpose is a Dionex ICS-5000 model (Thermo Scientific, Inc., USA) equipped with ED-5000 electrochemical detector. Separation of monosaccharides was carried out with Carbopac PA-1 (4 mm X 50 mm) column coupled to a Carbopac PA-1 (4mm X 250 mm) column. The analyses were performed using a gradient of deionized water (eluent A and D), 250 mM sodium hydroxide (eluent B) and 1 M sodium acetate (eluent C). The elution conditions were: at time zero to 10 min, B:C:D at 6:0:47 (start acquisition); at 30 min, B:C:D at 6:15:39.50; at 35 to 45 min, B:C:D at 0:100:0 (clean up); and at 46 min and 60 min B:C:D at 6:0:47 (re-equilibration). Mobile phase was used at a flow rate of 1 mL/min and the injection volume was 10 microliter. Analyses of monosaccharides was done by comparing them with the concentration of known standard solutions of rhamnose, arabinose, galactose, glucose, xylose, fructose and galacturonic acid.

5.2.6 Analysis of the total saccharides by HPAEC-PAD

The total saccharide composition of the crude pectin samples was determined by hydrolyzing the polysaccharides to monosaccharides with 5% (v/v) of Viscozyme at 45 °C and for 24h. Enzymatic saccharification was found to be more suitable than acid hydrolysis due to degradation of galacturonic acid to furfurals with acid hydrolysis (data not given). The enzyme was inactivated

by thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 4500 x g for 10 min to get a clear supernatant. The supernatant was collected and assayed for its free monosaccharide composition on high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as described in section 2.5.

5.2.7 Analysis of the DP- distribution of the oligosaccharides by HPAEC – PAD

Since the pectin of onion skin was found to be mostly composed of galacturonan, the analysis of the oligosaccharides was fully concentrated on the measurement of the galacturonic acid oligomers. They were characterized by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) following the method of Combo, Aguedo, Goffin, Wathelet & Paquot. (2012) The column was a Dionex CarboPac PA-100 (4 mm × 40 mm) coupled to a CarboPac guard column (4 mm × 250 mm). The mobile phase consisted of 100 mM sodium hydroxide (eluent A), 600 mM sodium acetate in 100 mM sodium hydroxide (eluent B) and 500 mM sodium hydroxide (eluent C). Elution conditions were as follows: A: B as 95:5 over 0–5 min, A:B 50: 50 at 10 min, A:B 20:80 over 15–35 min, B:C 50:50 over 36–43 min and A:B 95:5 over 44–50 min. The flow-rate was 1 mL/min and the injection volume was 25 microliter. The identification of retention time of the different oligomers was performed based on a galacturonan oligosaccharide mixture DP1-DP10. (Combo *et al,* 2012) The quantification of DP2-DP4 was done against standard solutions of DP2, DP3 and DP4 in the range of 5 to 1000 ppm. Due to a lack of standards, the quantification of DP5 to DP9 was performed by using an estimated molar response factor R_M. In the HPAEC/PAD analysis of oligomers, it is known that the molar response factor depends strongly on the DP analyzed. At low DP

typically a strong decrease in molar response factor is observed, whereas starting from DP4 and higher the decrease is much less pronounced. Since the molar response factor of a particular oligomer with a specific DPx and molecular weight MW is expected to be affected by its ability to diffuse, and the diffusion rate is inversely proportional of the square root of the molecular weight, this relationship was taken for the fitting. The general behavior matches well the expected trend of an initial fast decrease and a further stabilization at higher DP. The fitting of the molar response factors obtained for DP1-DP4 resulted in equation 1 with a goodness of fit of R^2= 0.98. The equation was used to extrapolate the molar response factor R_M for DP5-DP9.

$$RM\ (DPx) = 2.23 \times 10^4 \times \sqrt{\frac{1}{MW}} \qquad \text{(equation 1).}$$

5.2.8 Analysis of the chemical nature of the oligosaccharides by UPLC/ESI-MS

The UPLC/ESI-MS analysis was done to identify the degree of methylated and acetylated forms of the oligomers formed during hydrolysis with a slight modification of the method of Leijdekkers, Sanders, Schols & Gruppen. (2011) The distribution of the different oligomers was determined on the basis of retention time, molecular weight and in source fragmentation. (Leijdekkers *et al,* 2011) Given the unavailability of standards, a semi quantification was performed, integrating the area of each identified compound. 100 mg of freeze dried sample of the POS skins was dissolved in 1 mL of eluent B (H_2O:CH_3CN 80:20, 10 mM $HCOONH_4$ + 53 mM HCOOH). Samples were centrifuged at 15093 x g, 4°C, for 10 min and the supernatant was recovered for the analysis. Sample were separated by a HILIC column (ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 3 mm X 150 mm) in an UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole

mass spectrometer Waters ACQUITY SQD) using a gradient elution. Eluent A was H_2O:CH_3CN 20:80, 10 mM $HCOONH_4$ + 0.2% HCOOH, eluent B was H_2O:CH_3CN 80:20, 10 mM $HCOONH_4$ + 0.2% HCOOH; gradient: 0-60 min linear from 100% A to 60%A, 60-65 min isocratic at 60% A, 65-66 min linear from 60%A to 100%A, 66-75 min isocratic at 100%A. Flow 0.3 mL/min; analysis time 75 min; column temperature 35°C; sample temperature 18°C; injection volume 10 µl; acquisition time 0-75 min; ionization type negative ions; capillary voltage 2.6 kV; cone voltage 60 V; source temperature 150°C; desolvation temperature 350°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h; samples were analyzed in the full Scan mode with a scan range of 200-2000 m/z.

5.2.9 Statistical Analysis

All the experiments were conducted in duplicate and the mean and standard deviation were calculated using MS Excel software. Wherever necessary, the data were analyzed with one way ANOVA and LSD ($P < 0.05$) for tests of significance with JMP software (SAS Inc, Cary, NC, USA).

5.2.10 Calculation of the yields

As the pectin of onion skins was found to be mostly composed of homogalacturonan, the calculation of the yields was limited to galacturonic acid and its corresponding oligomers. All yields (Y) were expressed as mass of dry matter recovered of a specific entity per mass of dry matter of onion skins initially used for the extraction.

The crude pectic extract was characterized by its monosaccharide and polysaccharide composition. The monosaccharide yield was approximated by the amount of free galacturonic acid [$Y_{monoGalAc}$(pectin)] found after direct analysis of the crude pectin extract and expressed per mass dry matter of the onion skins used for extraction. Similarly, the total saccharide yield was

approximated by the total amount of galacturonic acid [$Y_{totalGalAc}$(pectin)] detected after hydrolysis and analysis of the crude pectin extract per mass of treated onion skins. The galacturonan polysaccharides present in the crude pectin extract per mass of treated onion skins [$Y_{polyGalAc}$(pectin)] was calculated by correcting the total saccharide yield by the monosaccharide yield, i.e.,

$$Y_{Poly\ GalAc}\ \ (pectin) = Y_{Total\ GalAc^{(pectin)}} - Y_{mono\ GalAc^{(pectin)}}.$$

The POS were characterized according to their DP-distribution as determined by the galacturonan oligomer analysis on HPAEC-PAD. The yields of DP1 to DP9 were calculated by using the quantified results from the oligomer analysis which were expressed per mass of treated onion skins

($Y_{DP1-9\ GalAc}$ (POS)).

The yield of the higher oligomers [> 9DP, $Y_{DP9+GalAc}$ (POS)] was estimated from the polysaccharide yield from which the yields of the smaller oligomers present in the extract were subtracted, i.e.,

$$Y_{DP9+GalAc}\ \ (POS) = Y_{Total\ GalAc^{(pectin)}} - \sum_{i=1}^{i=9}\left(Y_{DPi\ GalAc^{(POS)}}\right).$$

The percent conversion yield of pectic oligosaccharides produced from onion skins pectin was determined by the following formula:

$$\%\ conversion = \frac{Y_{DP\ GalAc^{(POS)}}}{Y_{Poly\ GalAc^{(pectin)}}} x100,$$

where $Y_{Poly\ GalAc^{(pectin)}}$ and $Y_{DP\ GalAc^{(POS)}}$ are respectively the intact crude pectin present in onion skins and the oligosaccharides with a specific DP formed during hydrolysis of pectin respectively.

5.3 Results and discussion

5.3.1 Onion skins: Composition and pectin extraction

The total sugars analyzed in onion skins account for 42.7% (w/w) on d.m. basis. The rest of the biomass can be proteins, ash, cellulose etc. Galacturonic acid represent 21.1 ± 0.2% (w/w) on d.m. forming a significant part of the total sugars. The other sugars are galactose, glucose, rhamnose and arabinose accounting for 6.9 ± 0.1 %, 14.7 ± 0.2%, 0.4 ± 0.1, 0.4 ± 0.1, respectively. The arabinose and rhamnose content of onion skins is low when compared to other pectin rich agro waste *i.e.* sugar beet pulp and olive pomace. (Coimbra *et al,* 2010; Leijdekkers *et al.,* 2013; Rodríguez, *et al,* 2007). This indicates that the pectin present in onion skins mainly comprises of homogalacturonan and nearly no contain rhamnogalacturonan regions. These results are in line with that of Alexander & Sulebele (1973) who found 11-12% of pectin in onion skins of which galacturonic acid accounted for 80% and with most recent results obtained by Mulleer-Maatsch et al. (2016).

The aim of the first part of the process, i.e., the extraction, is to release the homogalacturonan part as intact as possible. In our previous study, we found that the pectin present in onion skin is calcium bound, so ordinary extractants like nitric acid, HCl and even enzymes were not found to be effective. (Babbar *et al,* 2015) A new extraction method was developed based on sodium hexametaphosphate (SHMP). This chelator based extraction frees calcium bound pectin by loosening the egg box structures formed by the homogalacturonan and calcium complex (Ravn & Meyer, 2014).

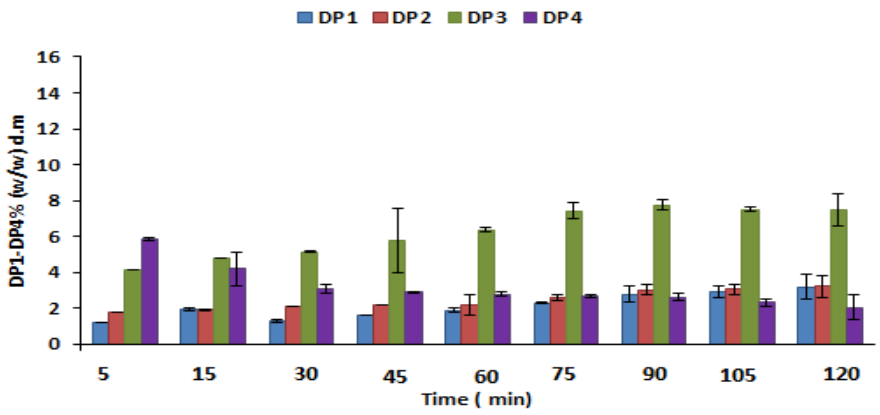
In the present study, a total polysaccharide yield of 21.3 ± 0.2% galacturonic acid was obtained without the formation of monosaccharides.

5.3.2 Pectic oligosaccharide production using EPG-M2, Pectinase and Viscozyme

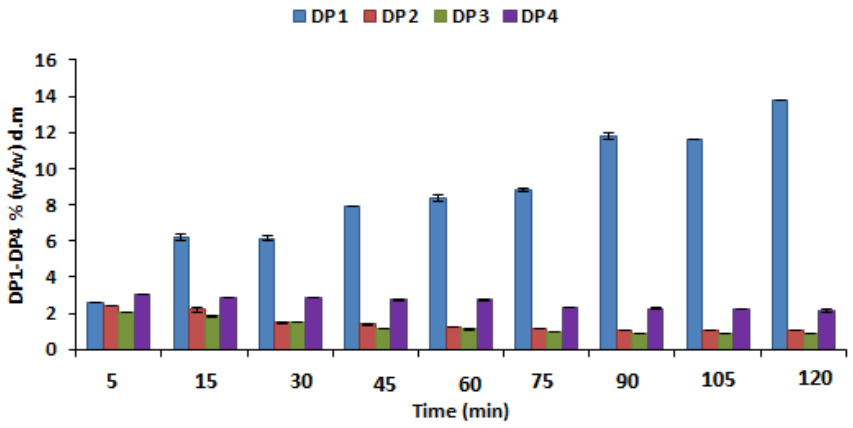
The effect of different enzymes *viz.* Pectinase, Viscozyme and EPG-M2 on POS production from onion skins' pectin in a batch system is shown in Fig 5.1. Oligomers up to DP 10 were identified. The results of POS are expressed as % (w/w) per mass of dry matter of initial biomass used.

Already after 5 min of reaction, all enzyme preparations started converting pectin to monosaccharides and oligosaccharides in varying amounts indicating the presence of both exo and endo activity of the enzymes. Fig 5.1 illustrates the conversion of pectin to free galacturonic acid (DP1). It reveals an increase in DP1 (free galacturonic acid) as the hydrolysis time progresses with Pectinase causing the largest, Viscozyme the intermediate and EPG-M2 the lowest DP1 formation. The results for EPG-M2, as shown in Fig 5.1a, indicates that only around 1.2% of DP1 is formed after 5 min. The generation is slow and 3.1% of monosaccharides are formed by the end of hydrolysis at 2h. As expected, this enzyme mostly produces DP2, DP3 and DP4. Around 2.1% of DP2 and 4.1% of DP3 are observed after 5 min, both of them almost doubling when the hydrolysis is stopped at 120 min (Fig 5.1a). Accumulation of DP4 is quite high in the beginning accounting for 6.5% but decreases to 2.28% at 120 min. This indicates the hydrolysis of both DP4 and higher oligomers to lower DPs with increasing hydrolysis time. As shown in Fig 5.2a, EPG-M2 converts around 55.8% of the crude pectin to 18.7% DP2 and 37.1 % DP3 at 120 min with a concomitant reduction in the higher oligomers (> DP4). Therefore, longer incubation favors the recovery of DP2 and especially DP3 whereas at lower times, mostly DP4 and higher oligomers (>DP4) are produced. The simultaneous formation of different oligomers suggests the random cutting behavior of endo-pg (Benen, *et al*, 1999).

On the other hand, both Pectinase (Fig 5.1b) and Viscozyme (Fig 5.1c) result in a significant DP1 yield of 2.6% and 3.1%, respectively, already after the first 5 min of hydrolysis. The monosaccharide content further increases to 11% and 6.4% respectively, by the end of 120 min. At the same time, a significant decrease in DP2, DP3 and DP4 is observed indicating predominantly exo activity of the latter enzymes. (Combo *et al,* 1012) Fig 5.2, shows the overall conversion of the onion skins crude pectin to oligomers and monomers by the three enzymes. In the case of Pectinase, a consistent decrease in DP2 and DP3 in comparison to DP4 is noticed (Fig 5.2b). On the other hand, in the Viscozyme catalyzed digestion, DP2, DP3 and DP4 decreases at almost the same rate with incubation time (Fig 5.2c). In this latter case, the accumulation of DP1 seems not only to be related to the concomitant decrease in DP2+DP3+DP4 but also due by some higher oligomers degradation. Our results are in line with those of Combo, Aguedo, Goffin, Wathelet & Paquot (2012), who also obtained similar trends of oligomer formation from pure polygalacturonic acid using different enzymes. This means that tailoring of pure polygalacturonic acid and that of crude pectic extract from onion skin progresses the same way.



(a)



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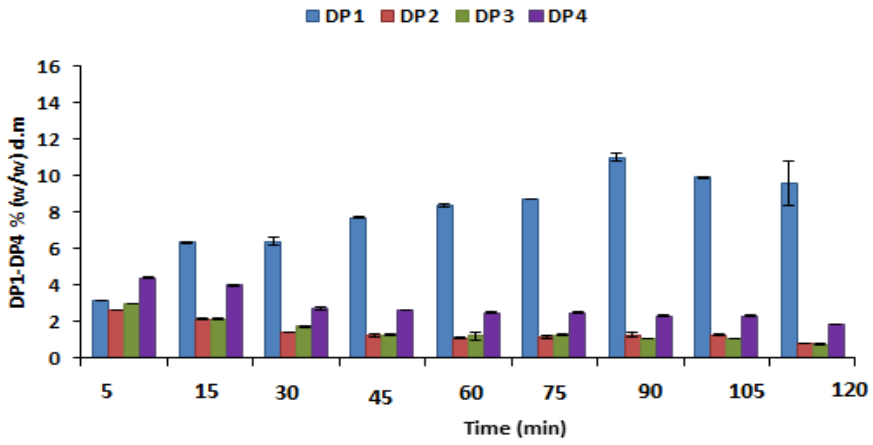
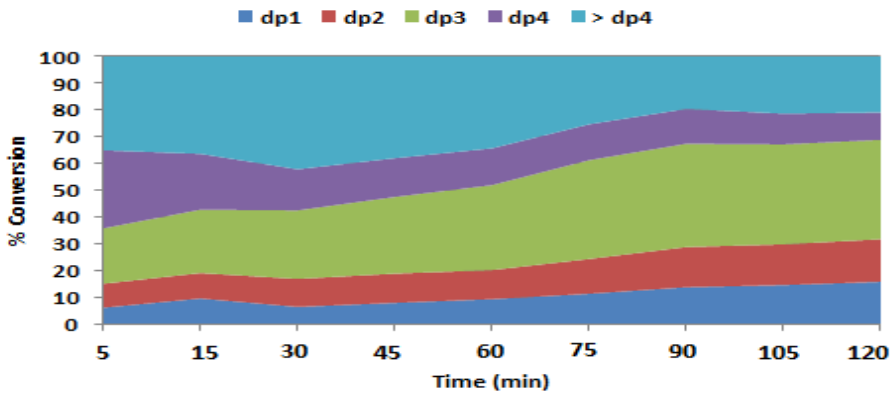
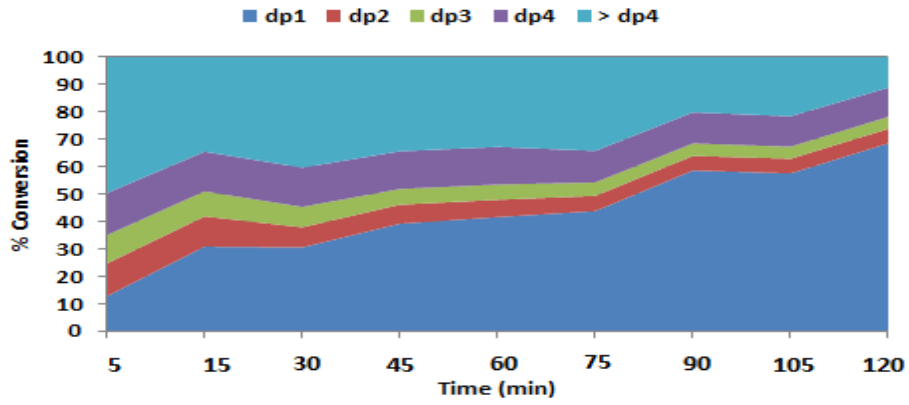


(c)

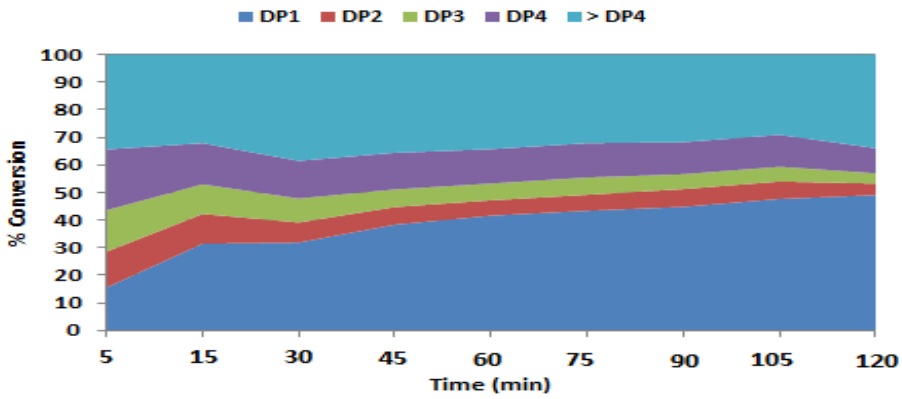
Fig 5.1: Production yield (% w/w d.m) of POS (DP1-DP4) from onion skins pectin by (a) EPG-M2 (b) Pectinase and (c) Viscozyme. LSD ($p< 0.05$) values for DP1, DP2, DP3 and DP4 for EPG M2 are 0.680, 0.710, 1.58, 0.941, for pectinase are 0.3, 0.1, 0.1, 0.1 and for viscozyme 3.1, 0.1, 0.2, 0.1 . LSD: Least significant difference; EPG M2: Endo-polygalacturonase M2; DP: Degree of polymerization; POS: Pectic oligosaccharides.


dp1
dp2
dp3
dp4
> dp4
% Conversion
100
90
80
70
60
50
40
30
20
10
0
5
15
30
45
60
75
90
105
120
Time (min)

(a)


dp1
dp2
dp3
dp4
> dp4
% Conversion
100
90
80
70
60
50
40
30
20
10
0
5
15
30
45
60
75
90
105
120
Time (min)

(b)



(c)

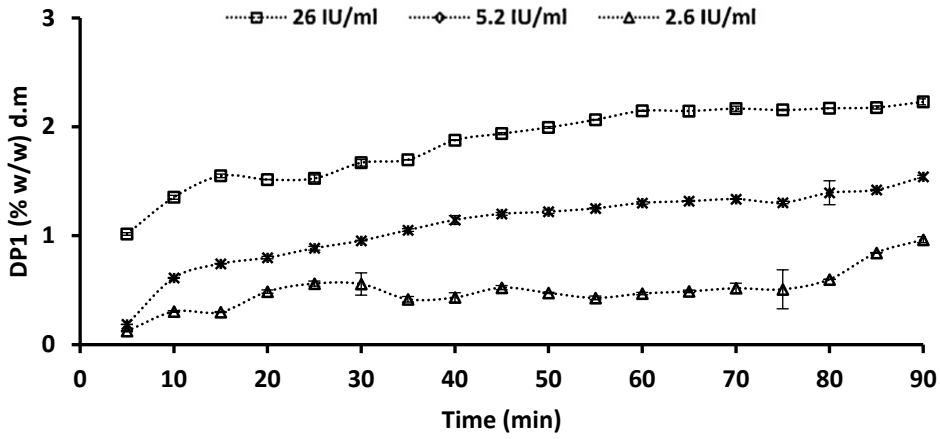
Fig 5.2: Conversion (%) of onion skins pectin to various POS fractions by (a) EPG-M2 (b) Pectinase and (c) Viscozyme. EPG M2: Endo-polygalacturonase M2; DP: Degree of polymerization>DP4 was calculated by substracting sum of low DPs (DP1-DP4) from total galacturonic acid in intact pectin.

5.3.3 POS production using different concentration of EPG-M2

Since the enzyme EPG-M2 gave the most promising results, the POS production with this enzyme was further optimized. Three different concentrations of EPG-M2 *i.e.* 26.5, 5.2 and 2.6 IU/mL were used and the products of hydrolysis were characterized on HPAEC-PAD. The results are shown in Fig 5.3, whereby the concentration of the monomer and oligomers are reported as % (w/w) per dry mass of treated biomass. A significant difference in DP1 formation is observed at the various EPG-M2 concentrations (Fig 5.3a). A continuous increase in DP1 yield is obtained as the hydrolysis time progresses following the order of EPG-M2 26.5 IU/mL > 5.2 IU/mL > 2.6 IU/mL. In the beginning of the hydrolysis (5 min), a DP1 yield of 1.0%, 0.2% and 0.1% is obtained with an EPG-M2 concentration of 26.5, 5.2 and 2.6 IU/mL. At the end of 90 min, it further accumulates to 2.2%, 1.3% and 0.5% of DP1 respectively.

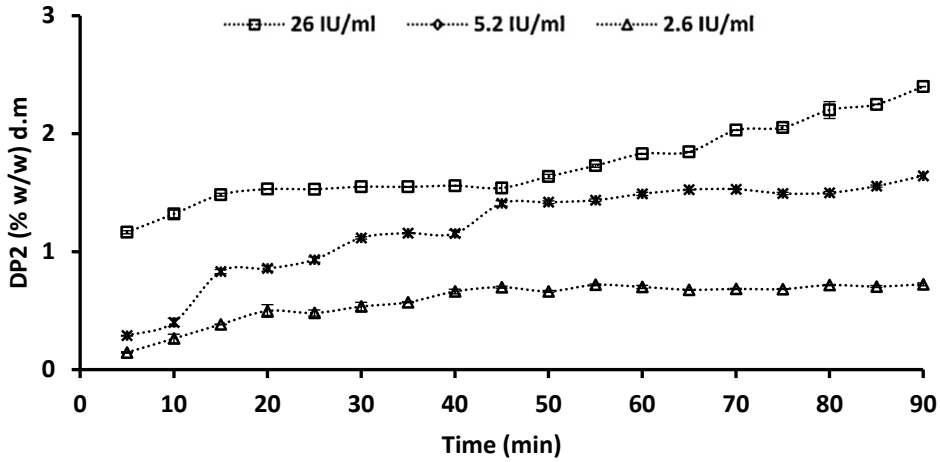
The evolution of DP2 and DP3 follows the same kinetics in function of EPG-M2 concentration (Fig 5.3 b and Fig 5.3c), whereas in case of DP4 an opposite trend is observed. The highest concentration of EPG-M2 (26.5 IU/mL) favors digestion of DP4 while lower concentration of EPG-M2 *i.e.* 5.2 IU/mL and 2.6 IU/mL results in a further increase in DP4 concentration (Fig 5.3d). This tendency indicates that less DP4 degradation occurs at lower enzyme concentration. To obtain tailored galacturonic acid oligomers with specific DP, the concentration of enzyme and contact time plays an important role as mentioned above. Fig 5.4 shows the detailed conversion efficiencies of pectin to various POS fractions. It can be clearly seen that conversion of pectin to both DP3 and DP4 remains high in comparison to other POS fractions. As depicted in Fig 5.4a, the highest conversion of pectin to DP3 accounted for 26% at a time scale of 75- 90 min with EPG M2 concentration of 26.5 IU/mL. The time scale, as defined by Iwasaki, Inove, Matsubara (1998), is the approximate time required for sufficient conversion. This can be due a continuous conversion of DP4 and other higher oligomers to DP3 with higher enzyme concentration (Fig 5.4a). Our results are in line with the results obtained by Combo, Aguedo, Goffin, Wathelet & Paquot (2012), who also obtained high DP3 from pure polygalacturonic acid catalyzed with EPG-M2. As mentioned before, the hydrolysis on crude pectic extracts of onion skins progresses in a similar way as on the pure products. The highest conversion of pectin to DP4 is catalyzed by EPG-M2 (5.2 IU/mL). Around, 26% of pectin is converted to DP4 within a time scale of 15-30 min of hydrolysis (Fig 5.4b). The formation of DP2 remains low with all three enzyme concentrations. Interestingly, the pectin conversion to higher POS fractions (> 4), is comparatively higher for lowest enzyme concentration (2.6 IU/mL) [Fig 5.4c]. This also suggests that using lower enzyme concentration (2.6 IU/mL), the tailoring of longer oligomers to fractions like DP4 and DP3 remains low and

hence higher amounts of longer oligomers. On the contrary, for higher enzyme concentration the longer oligomers are already converted to fractions like DP4 and DP3 which in turn results in lesser amounts of higher oligomers (Fig 5.4a and Fig 5.4b).



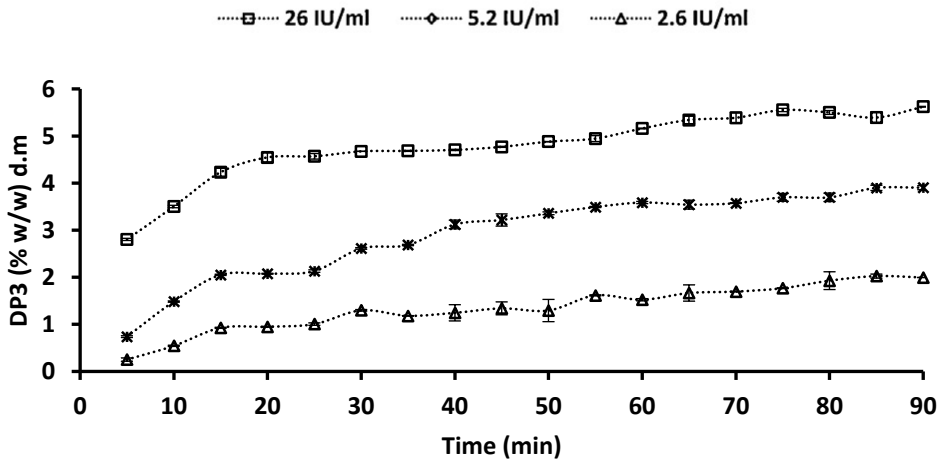
(a)

LSD ($p< 0.05$) values for 26 IU/mL, 5.2 IU/mL and 2.6 IU/mL are 0.02, 0.1 and 0.1, respectively.



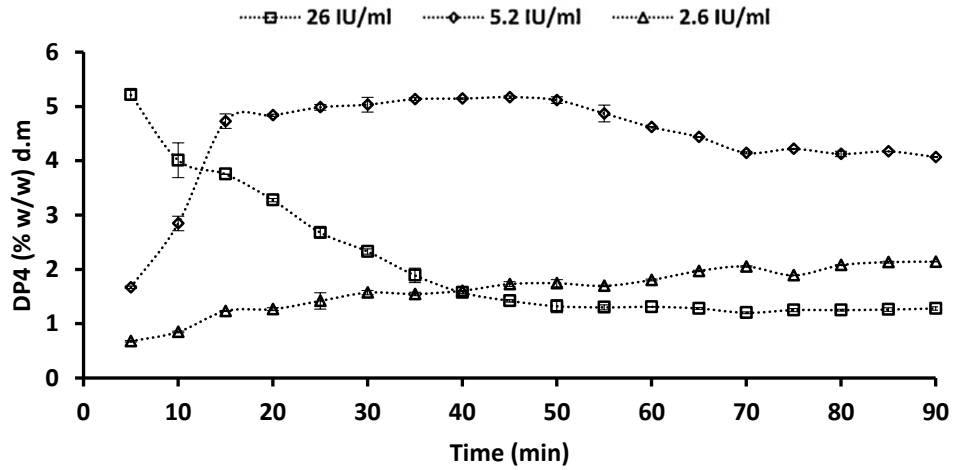
(b)

LSD ($p< 0.05$) values for 26 IU/mL, 5.2 IU/mL and 2.6 IU/mL are 0.04, 0.04, 0.1, respectively.



(c)

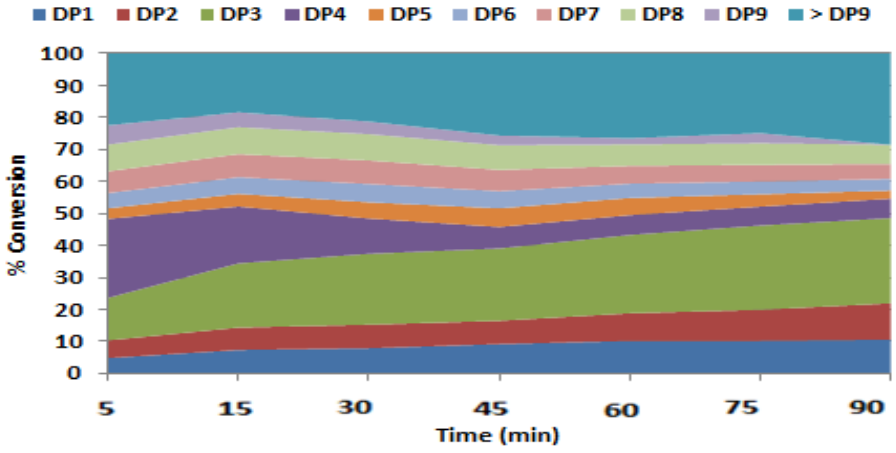
LSD ($p< 0.05$) values for 26 IU/mL, 5.2 IU/mL and 2.6 IU/mL are 0.1, 0.1, 0.2 , respectively.



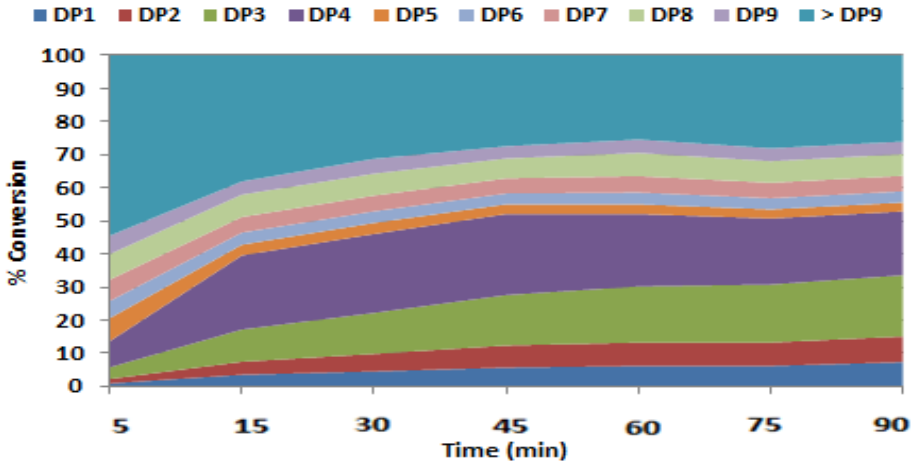
(d)

LSD ($p< 0.05$) values for 26 IU/mL, 5.2 IU/mL and 2.6 IU/mL are 0.4, 0.2, 0.2, respectively.

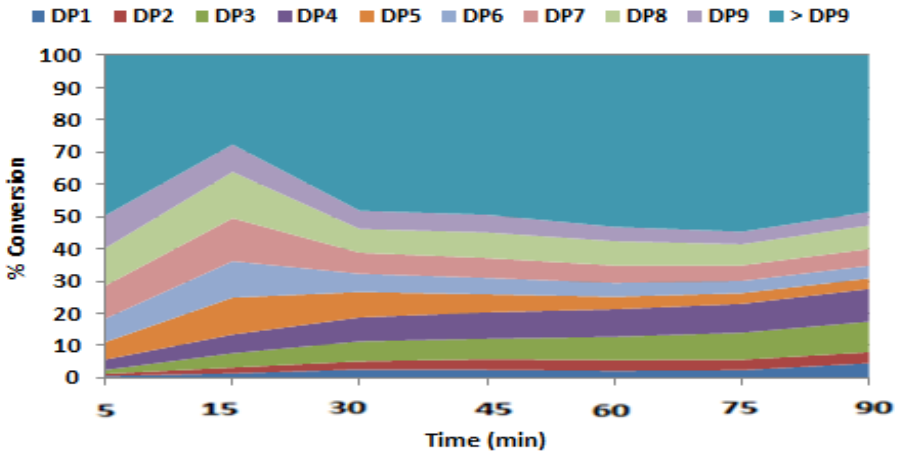
Fig 5.3: Effect of different concentrations of EPG-M2 on the production yield (% w/w d.m) of (a) DP1 (b) DP2 (C) DP3 and (d) DP4 oligosaccharides from onion skins pectin. LSD: Least significant difference


DP1
DP2
DP3
DP4
DP5
DP6
DP7
DP8
DP9
> DP9
100
90
80
70
60
50
40
30
20
10
0
% Conversion
5
15
30
45
60
75
90
Time (min)

(a)


DP1
DP2
DP3
DP4
DP5
DP6
DP7
DP8
DP9
> DP9
100
90
80
70
60
50
40
30
20
10
0
% Conversion
5
15
30
45
60
75
90
Time (min)

(b)



(c)

Fig 5.4: Conversion (%) of onion skins pectin to various POS fractions by different EPG-M2 concentration (a) 26 IU/mL (b) 5.2 IU/mL (c) 2.6 IU/mL EPG M2: Endo-polygalacturonase M2; DP: Degree of polymerization. -> DP9 was calculated by substracting sum of DP1-DP9 from total galacturonic acid in intact pectin.

5.3.4 Molecular characterization of substituted and non-substituted oligomers

All the samples were also further characterized, for understanding the distribution of substituted (methyl and acetyl esterified) and free POS fractions by using UPLC/ESI-MS. Polygalacturonans (DP2-DP8), both as free and in methylated/ acetylated forms were detected (Table 5.1).

Fig 5.5 shows the presence of different form of oligomers in the pectic digests of onion skins. As a general rule, the higher the DP, the higher the possibility to have a methylated form, due to the fact that the presence of at least one methyl group becomes statistically more likely with more galacturonic residues present. The unsubstituted form of GalA3 and GalA4 is accompanied with small amounts of substituted forms *i.e.* GalA3OMe and GalA4OMe, while GalA5 is mainly present in the form of GalA5OMe and GalA5OMe2. As a general observation, the substituted form of oligomers are

less cleaved in comparison to free forms. This can possibly be due to steric hindrance posed by additional groups attached to oligomers. Quite interestingly the trend of DP4 showed an increasing and then a decreasing trend, clearly indicating how this compound is at the same time a product of the enzymatic digestion and a further substrate for it. At the beginning of the digestion, when lot of intact pectins are present, DP4 accumulate, whereas at the more advanced stages, when pectin is mostly degraded, also DP4 gets cleaved. In the lowest concentration of EPG-M2 (2.6 IU/mL), the substituted forms are present in negligible amount (Fig 5.5c), owing to low enzyme concentration except for the substitution of DP5.

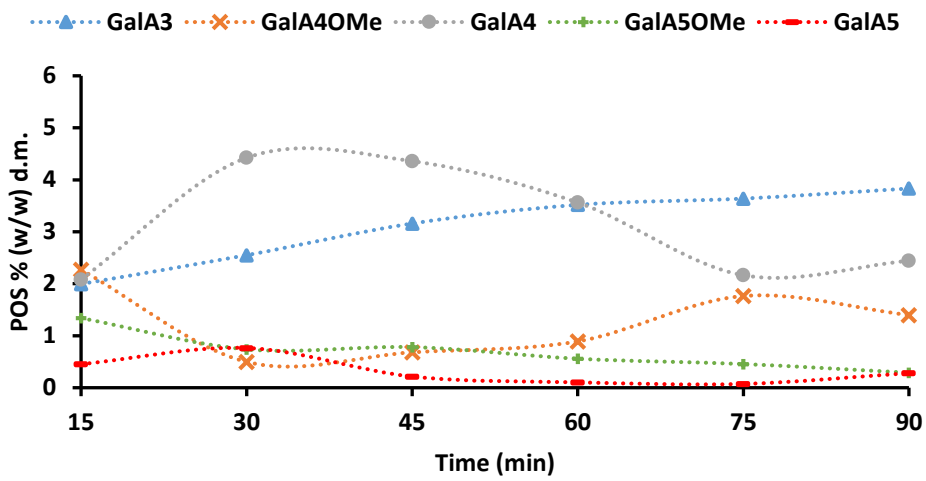
The overall information provided by HILIC-MS indicates that free form of oligomers are preferred by enzyme in cleaving in comparison to substituted forms. Also, the negligible presence of acetylated forms is due to low degree of acetylation of onion skin pectin. (Babbar *et al,* 2015). Fig 5.6 shows the relative distribution of various oligomers produced during the course of hydrolysis by different concentration of EPG-M2 at 45 min. It is quite clear that increasing concentration of the enzyme induced the cleavage of more substituted forms, whereas the diluted enzyme mostly produces less substituted POS.

Based on the information obtained from the present study, next step was facoused on the continuous production of POS from onion skin pectin using a membrane enzyme reactor.

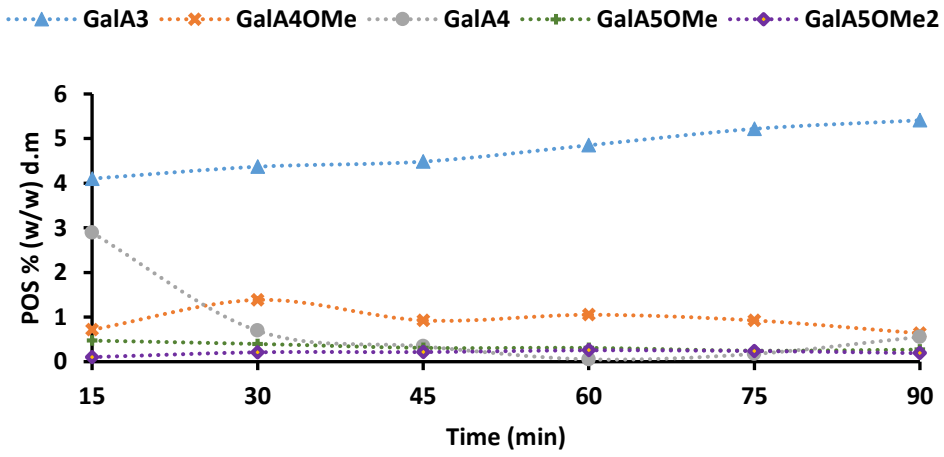
Table 5.1: Pectin oligosaccharides (POS) identified by UPLC/ESI-MS analysis.

ID		MW (Da)	Trace (m/z)
GalA2	●●	370	369.3
GalA3OMe	●●●OMe	560	559.4
GalA3OAc	●●●OAc	588	587.4
GalA3	●●●	546	545.4
GalA4OMe	●●●OMe	736	735.5
GalA4OAc	●●●●OAc	764	763.6
GalA4	●●●●	722	721.5
GalA5OMe2	●●●●●●OMe2	926	925.5
GalA5OMe	●●●●●OMe	912	911.6
GalA5OAc	●●●●●OAc	940	939.5
GalA5	●●●●●	898	897.6
GalA6OMe2	●●●●●●OMe2	1102	1101.5
GalA6OMe	●●●●●●OMe	1088	1087.5
GalA6	●●●●●●	1074	1073.5
GalA7OMe3	●●●●●●●OMe3	1078	1277.5
GalA7OMe2	●●●●●●●OMe2	1292	1291.5
GalA8OMe3	●●●●●●●●OMe3	1468	1467.5
GalA8OMe2	●●●●●●●●OMe2	1454	1453.5

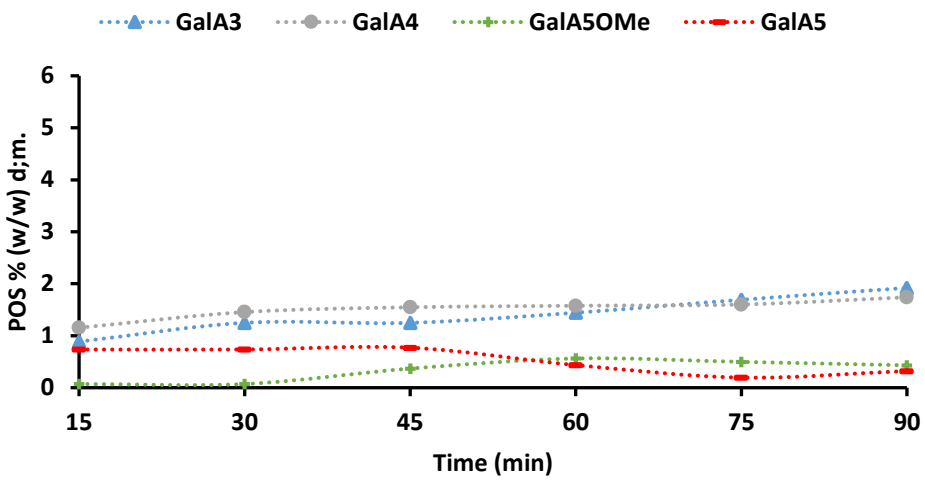
RT: Retention time; MW: Molecular weight; ●: Galacturonic acid; OMe/OAc: methylation/acetylation.


GalA3
GalA4OMe
GalA4
GalA5OMe
GalA5
POS % (w/w) d.m.
0
1
2
3
4
5
6
15
30
45
60
75
90
Time (min)

(a)


GalA3
GalA4OMe
GalA4
GalA5OMe
GalA5OMe2
POS % (w/w) d.m
0
1
2
3
4
5
6
15
30
45
60
75
90
Time (min)

(b)



(c)

Fig 5.5: Digestion pattern of unsubstituted and substituted galacturonic acid oligomers by different concentration of EPG-M2 (a) 26 IU/mL (b) 5.2 IU/mL and (c) 2.6 IU/mL.

EPG-M2: Endo-polygalacturonase M2; GalA3OMe: Galacturonic acid-3-O-methylated, GalA3OAc: Galacturonic acid-3-O-Acetylated; GalA3: Trigalacturonic acid; GalA4OMe: Galacturonic acid-4-O-Methylated; GalA4OAc: Galacturonic acid-4-O-Acetylated; GalA4:Tetragalacturonic acid; GalA5OMe: Galacturonic acid-5-O-Methylated; GalA5OAc: Galacturonic acid-5-O-Acetylated; GalA5: pentagalacturonic acid; GalA5OMe: Galacturonic acid-5-O-di-Methylated.

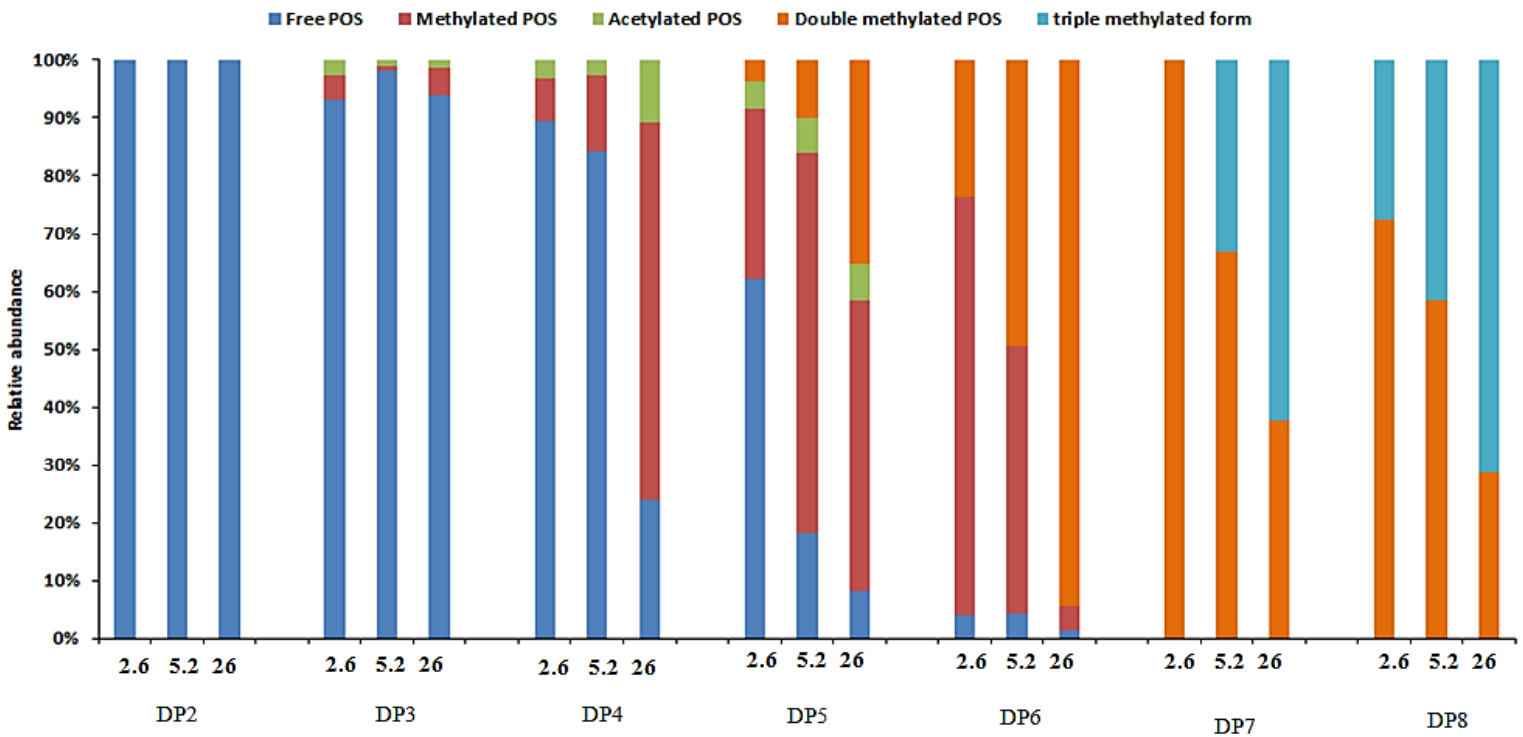
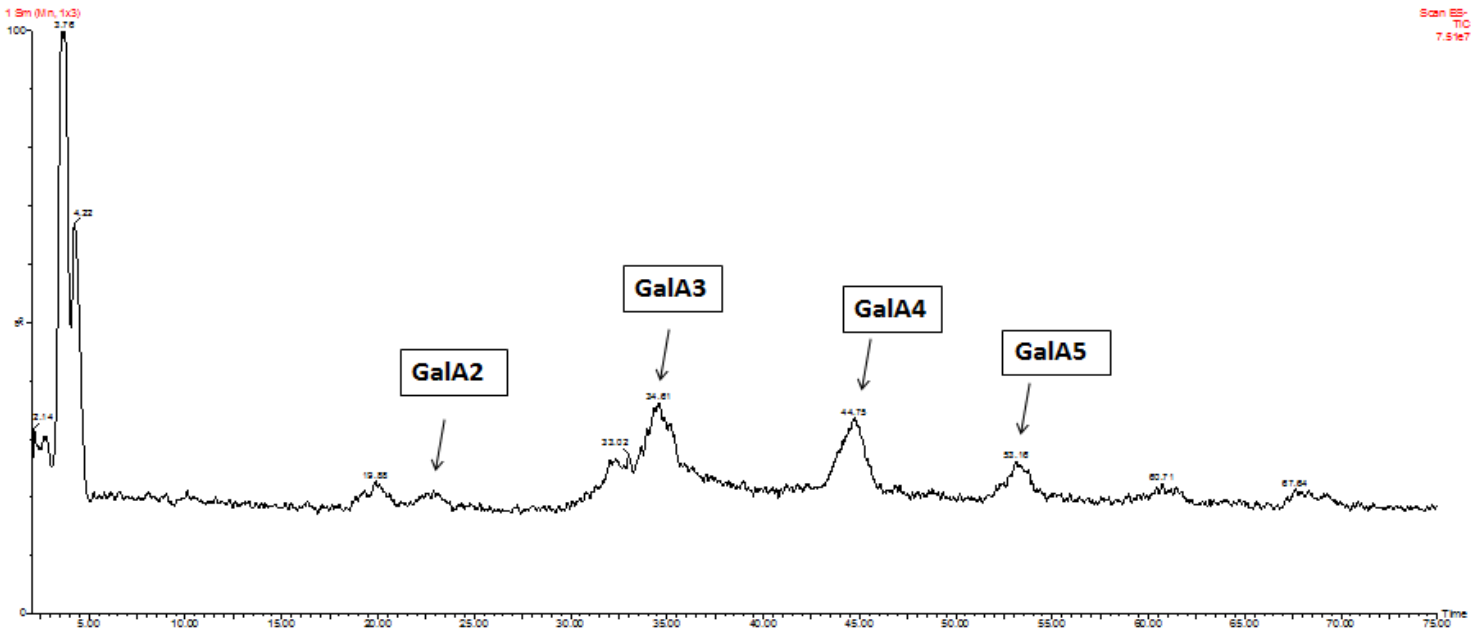
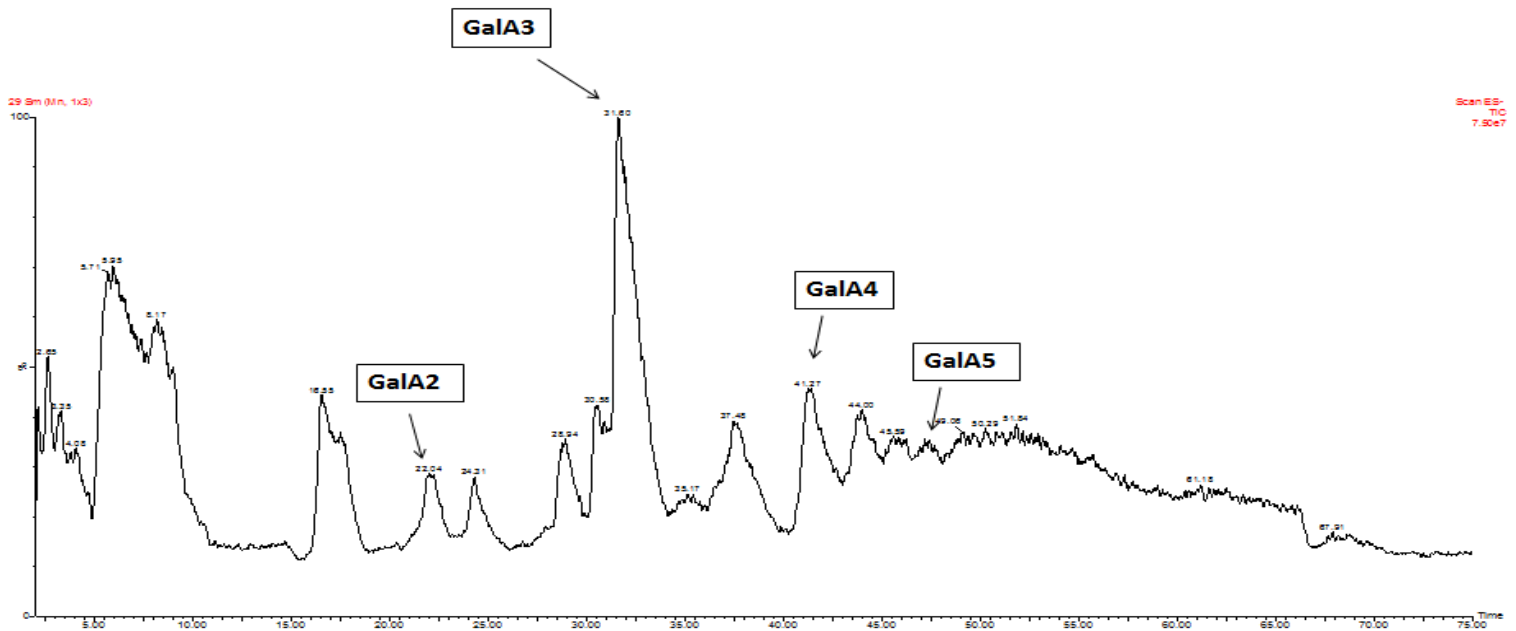


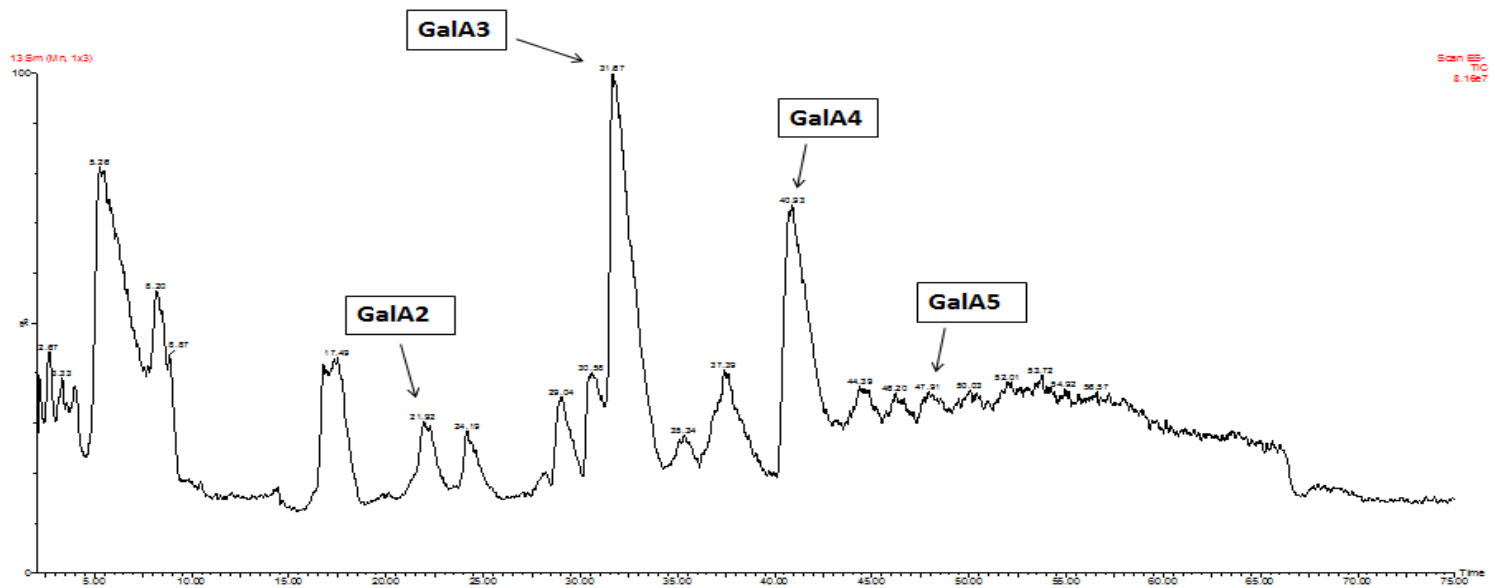
Fig 5.6: Relative abundance of different POS fractions obtained at 45 min. 2.6, 5.2 and 26 indicates enzyme concentration in IU/mL. DP: Degree of polymerization; POS: Pectic oligosaccharides.



(a)

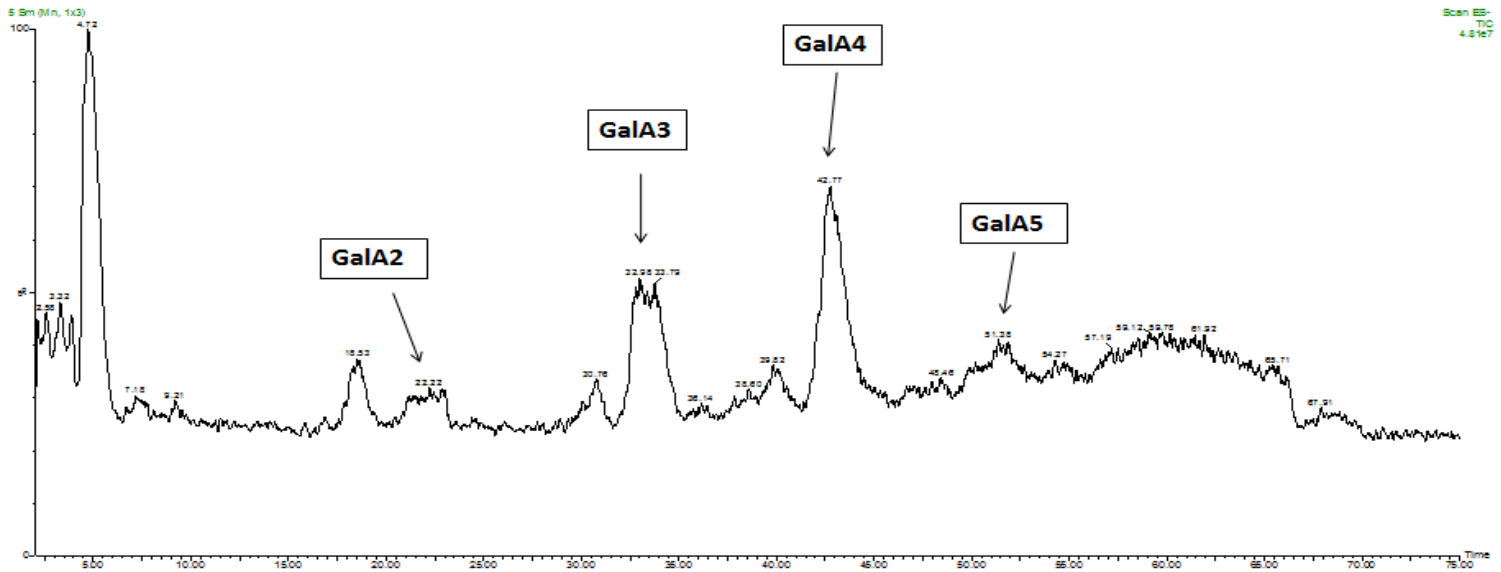

GalA3
GalA2
GalA4
GalA5
29 Sm (Mn, 1x3)
Scan ES-
TIC
7.80e7
100
%
0
5.71
5.95
6.17
2.65
5.25
4.05
16.85
22.04
24.21
25.94
30.56
31.60
35.17
37.46
41.27
44.00
45.58
49.06
50.29
51.84
61.15
67.91
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
Time

(b)

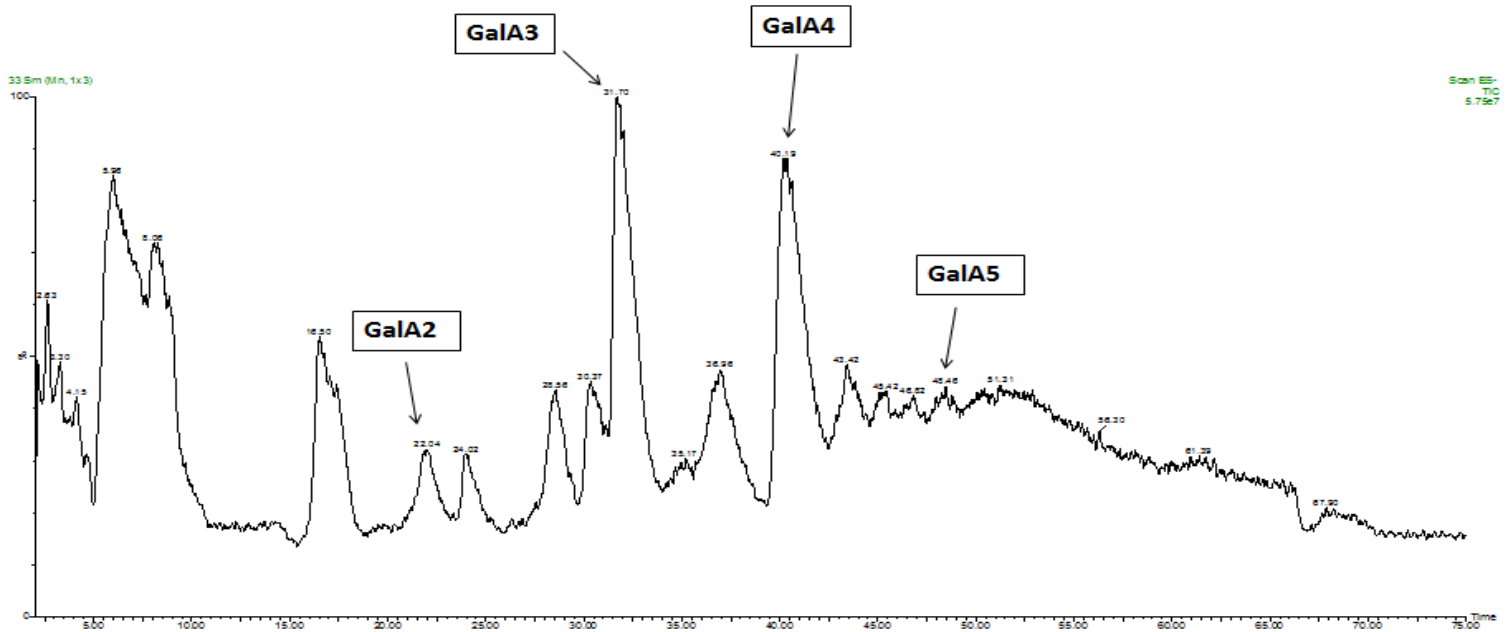


(c)

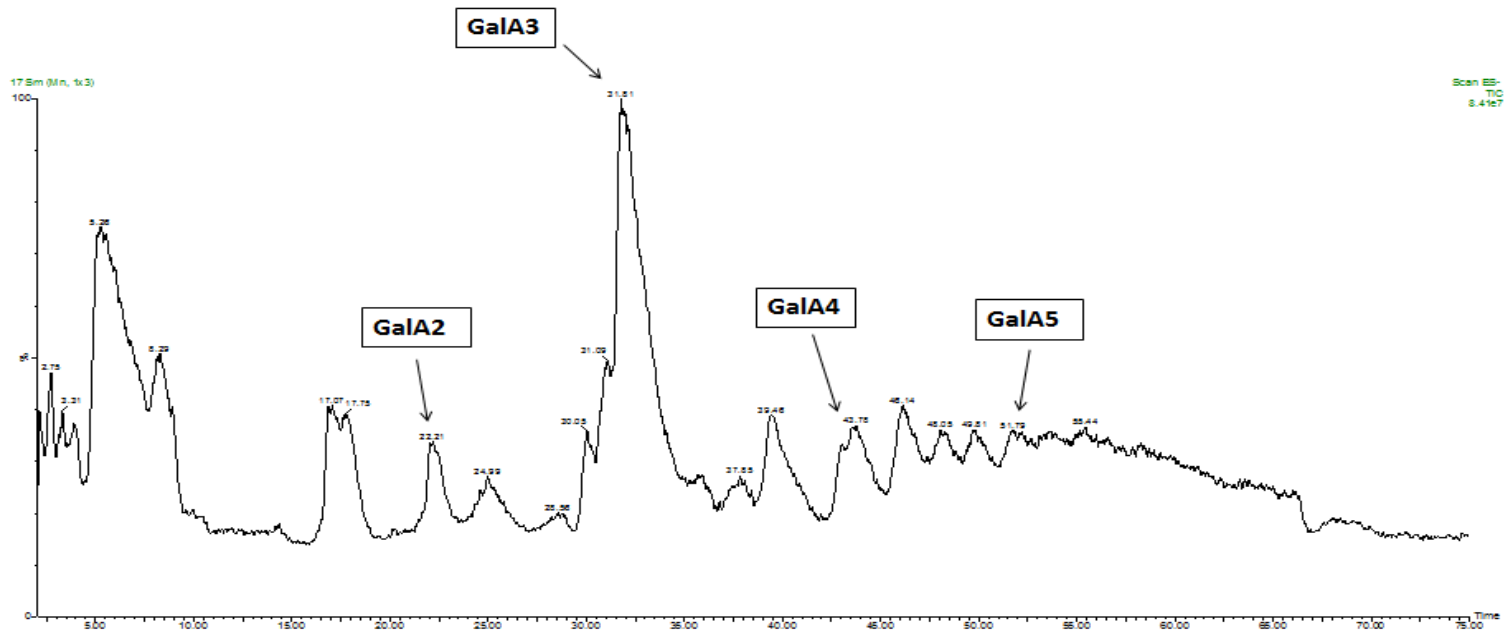
Fig 5.7: ESI-Scan of onion pectin oligomers at 15 min with EPG-M2 concentration of (a) 2.6 IU/ mL (b) 5.2 IU/ mL and (c) 26 IU/ Ml. GalA: galacturonic acid.



(a)


GalA3
GalA4
GalA5
GalA2
33 Sm (Mn, 1x3)
Scan ES-
TIC
5.75e7
100
%
0
5.98
6.08
16.50
22.04
24.02
25.56
30.37
31.70
35.96
40.19
42.42
45.42
45.82
45.46
51.31
56.30
61.39
67.90
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
Time

(b)



(c)

Figure 5.8: ESI-Scan of onion pectin oligomers at 45 min with EPG-M2 concentration of (a) 2.6 IU/ mL (b) 5.2 IU/ mL and (c) 26 IU/ mL. GalA: galacturonic acid.

5.4 Conclusions

Onions skins are evaluated as a new raw material for the enzymatic production of pectic oligosaccharides (POS) with a targeted degree of polymerization (DP). The process is based on a two stage process consisting of a chelator-based crude pectin extraction followed by a controlled enzymatic hydrolysis. The three enzymes studied in this research show the ability to produce pectic oligosaccharides (POS). However, the use of EPG-M2 results in a larger fraction of the oligomers (especially DP3) as compared to free galacturonic acid. Different concentration of EPG-M2 produce different fractions of POS with 26 IU/mL favoring DP3 formation at longer incubation times. On the other hand, the longer oligomers DP4 are favored by lower concentration of 5.2 IU/mL at reaction times between 5-15 min. Also, the production of DP2 remains high with 26 IU/mL at longer incubation times (70-90 min). The ESI scan of the pectic oligomers obtained from onion skins has shown the presence of methylated, double methylated, triple methylated and acetylated forms which are comparatively more present when the hydrolysis is done at higher enzyme concentration. The present work brings information on the POS production from onion waste which is an unexploited source of pectin, and also on the hydrolysis conditions needed to obtain extracts enriched in oligomers with targeted degree of polymerization.

Analogously to what has been done with sugar beet pulp, the hydrolysis of onion skins pectin can be moved from batch to continuous system using a cross flow membrane reactor process. The enzymatic production of POS from onion pectin by enzyme membrane reactor will be assessed in the following chapter.

Acknowledgments

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6. Pectic oligosaccharides from onion skins wastes: continuous production and separation by using a membrane enzymatic bioreactor

Abstract

The main aim of this work is to valorize a non-utilized agricultural by-product, i.e., onion peels, towards pectic oligosaccharides with potential health benefits, and to increase the hydrolysis performance and productivity by using a cross-flow continuous membrane bioreactor process on a lab-scale. Indeed, the integrated use of a biocatalytic reactor with a membrane separation provides the opportunity to produce tailored products and suppress monosaccharide formation.

In this study, the influence of various production conditions (residence time, enzyme concentration, substrate concentration) is discussed, both in terms of productivity and yield, as well as on a molecular level by assessing the molecular distribution of the obtained POS. At all conditions tested, the process reached steady state: no fouling of the membranes or loss in the enzyme activity was observed. Two conditions in particular gave the best performance. At a residence time of 15 minutes and an enzyme concentration 0.294 FPU/ml, the highest POS yield and the lowest monosaccharides yield were obtained at both substrate concentrations. Moreover, a specific productivity of 13 g/gE/h and 20 g/gE/h at substrate concentrations 25 g/L and 50 g/L respectively was obtained. According to the results shown in the present study, the membrane enzymatic reactor can be considered a promising technology towards the production of tailor-made POS from onion hulls.

6.1 Introduction

The conversion of food wastes into useful and functional compounds of higher value represents a research field of great interest. Several agricultural and agro industrial by-products, such as sugar beet pulp, citrus peels etc., rich in pectins, have been investigated as a source of useful pectin-derived products such as the pectic oligosaccharides (POS).

Structurally, the most common and well known POS are arabinogalacto-oligosaccharides, arabinoxylo-oligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, oligo-galactouronides and rhamnogalacturonan-oligosaccharides. (Olmos *et al*, 2012)

In the recent years, the research on new sources of POS has increased since these oligosaccharides have showed interesting biological properties. Some *in vitro* studies demonstrated that they are not digestible by animal digestive enzymes, they can induce changes in the activity and composition of the gastrointestinal microbiota, stimulating the growth of probiotic bacteria as *Lactobacilli* or *Bifidobacteria* and preventing the growth of pathogenic streams such as *E.coli* (Gullòn *et al*, 2011). Thanks to their functionality, POS are expected to give a significative contribution to the prebiotics market in the future.

However, POS mixtures should contain a low monosaccharides yield, which derive from pectin hydrolysis as well. In fact, monosaccharides could be absorbed in the animal gastrointestinal tract with facilitated mechanisms (Wenk *et al*, 2001) and they could contribute to diseases such as obesity.

In the present study the attention is focused on the production of pectic oligosaccharides (POS) starting from a relatively unexpoited source of pectin, i.e. onion hulls. In Europe, the waste of onion in the form of dried, whole, cut, sliced and broken onion accounts for 6.6×10^6 tons and the onion skin has

already been investigated for extracting pigments (Bae Soon Ei, 2009) or their antioxidant effect (Albishi *et al*, 2013; Urszula *et al,* 2013).

In previous work, the onion skins have been characterized for their pectin content which accounted for 28% w/w on d.m.. The pectin substances extracted were found to be mainly composed of uronic acid with a methylation degree of 19% and acetylation degree of 2%, indicative for highly polymerized polygalacturonates (Maatsh-Muller *et al,* 2016). Accordingly, the onion hulls were identified as a promising source of POS.

Batch-wise, enzymatic depolymerisation is the main approach used to prepare large amount of oligomers. (Munoz *et al,* 2012) Thereby several types of enzymes, such as pectinases, pectate lyases and other polygalacturonanses are applied to generate oligogalacturonides from pectin cleavage (Polygalacturonase catalyse the hydrolytic cleavage of the α-(1→4)-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances). (Jayani *et al*, 2005) Given the high cost of such enzymes, industrial biotechnology is looking for cheaper hydrolysing enzymes in order to develop a more suistainable production process.

In a previous research, we investigated the depolymerization of pectin extracted from onion hulls and we compared the POS and monosaccharides yields obtained using three different enzymes, being Endopolygalacturonase (Endo-PG) M2, Pectinase and Viscozyme L. The highest POS yield (especially with a degree of polymerization of 2 (DP2) and 3 (DP3)) was obtained using the Endo-PG M2, thanks to the endo-activity; on the other hand, using the Viscozyme L a certain POS yield was also observed but a high monosaccharides yield was found as well, due to the exo- and endo-activity of enzyme. (Babbar *et al*, 2016)

Therefore, in order to optimize the hydrolysis reaction, the Viscozyme L could be investigated since it is was found to be suitable for depolymerizing

pectin but, of course, the monosaccharides formation should be minimized as much as possible.

Recently, new developments have been made to increase the hydrolysis productivity and performance by using an enzyme membrane reactor (EMR). The integrated use of a biocatalytic reactor and a membrane process such as ultrafiltration is particularly important since it has shown to be able to avoid inhibition and to achieve higher productivity. (Kiss *et al*, 2018) More advantages of such technology include (i) the targeted production of tailored products by choosing an appropriate membrane cut-off, and (ii) a reduction of costs owing to a more continuous way of operating. (Giorno &Drioli, 2000; Bèfali-Bako *et al,* 2002; Bèfali-Bako, 2006)

The use of EMR for the hydrolysis of pectin has already been studied by Kulbe *et al* (1987) and Olano-Martin *et al* (2001). They applied a dead-end filtration system in a batch-wise approach suitable for the instantaneous filtration of the products formed. Nevertheless, this type of set-up does not allow a continuous production with simultaneous feeding and product removal. Moreover, dead end filtration is typically prone to clogging in case large amounts of insoluble materials are present in the substrate. The accumulation of particles onto the membrane, may result in an increased resistance to filtration and a decline in permeate flux, necessitating frequent cleaning steps and ultimately the replacement of the membrane (Pinelo *et al,* 2009). Also in these studies pure commercial pectin was used as starting material resulting in an additional cost to the process.

The aim of this work is to valorize a non-utilized agricultural by-product, i.e., onion skins, towards pectic oligosaccharides with potential health benefits, and to improve the hydrolysis performance by using a cross-flow continuous membrane bioreactor process on a lab-scale. To our knowledge,

there is no literature dealing with the continuous production of POS from onion skins pectin.

The ambition is to start from an agricultural waste, only recently mentioned for the first time as a potential source of POS (Babbar *et al*, 2016), and, passing from the extraction to the hydrolysis of the crude pectin into POS. Key to this research is that the hydrolysis is combined with simultaneous *in-situ* POS-removal through cross-flow membrane filtration, allowing the continuous production of POS and providing options for significantly increasing productivity. Moreover, it is chosen to work with an enzyme complex, Viscozyme L, because of its lower cost as compared to more specific enzymes. The presence of a cocktail of endo- and exo-activities poses, however, significant challenges to the process to produce a POS-product low in monosaccharides.

More specifically, in this study the influence of various production conditions (residence time, enzyme concentration, substrate concentration) is studied, both in terms of productivity and yield. Moreover, a detailed characterization of the POS products is performed at a molecular level.

6.2 Material and methods

6.2.1 Materials

Onion hulls were provided by the Institut fur Getreideverarbeitung (IGV, GmbH), Germany. The hulls were milled with a laboratory blender, screened to a particle size (< 1 mm) and stored in ziplock bags at room temperature until use. Viscozyme L (V-2010) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Viscozyme L is a multienzyme complex composed of pectinases, hemicellulases and arabinases (Foulk *et al,* 2008). The

endo-polygalacturonase activity activity of Viscozyme was determined by Babbar *et al* and it accounts for 4135 U/mL. Babbar N., 2016)

Romicon 1- hollow fiber membrane cartridges (Type HF 1018-1.0-43-PM10 and PM 5) containing polysulfone ultrafiltration membranes with a molecular weight cut-off of 10,000 Da were obtained from Koch membrane systems, Inc. (Stafford, GB). The total filtration surface of these membranes was 2 X 0.093 m^2. P3-Oxonia active and Ultrasil 115, the liquid disinfectants used for cleaning the reactor and the membrane modules, were purchased from ECOLAB bvba (Zellik, Belgium). Polygalacturonic acid was obtained from Megazyme International.

Standards of galacturonic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Germany). The standards of di-galacturonic acid and tri-galacturonic acid were purchased from Sigma-Aldrich and standard of tetra-galacturonic acid from Elicityl Oligotech, France. The galacturonan oligosaccharide mixture with a degree of polymerization of DP1-DP10 was kindly provided by B. Whatelet and M. Paquot from Gembloux, Agro-Bio Tech (Belgium).

6.2.2 Extraction of pectin from onion skins

Based on our preliminary study (Babbar *et al,* 2016), sodium hexametaphosphate (SHMP) was selected as pectin extractant for onion skins. Onion skins were pretreated with 2% sodium hexametaphosphate solution at 95 °C for 0.5 h in a hot water bath. The biomass was then centrifuged at 4500 x g for 10 min. The supernatant containing the crude pectin was collected and analyzed for its free monosaccharide as well as total saccharide composition. The latter was then taken as a measure for the polysaccharide content. (See section 2.4).

6.2.3 Enzymatic hydrolysis / tailoring of onion pectic polysaccharides to oligosaccharides in an EMR

The crude pectin, as obtained from the extraction process, was used as a substrate to produce the pectic oligosaccharides. The substrate was first filtered by a filter of 200 μm in order to remove the remaining solid residues still present from the extraction. Thereafter, it was hydrolyzed in a continuous way in an enzymatic membrane reactor (EMR) according the procedure described below.

6.2.3.1 General reactor operation

The EMR consists of a reactor with a coupled membrane module. The experimental set-up as used in the experiments is shown in the figure 6.1.

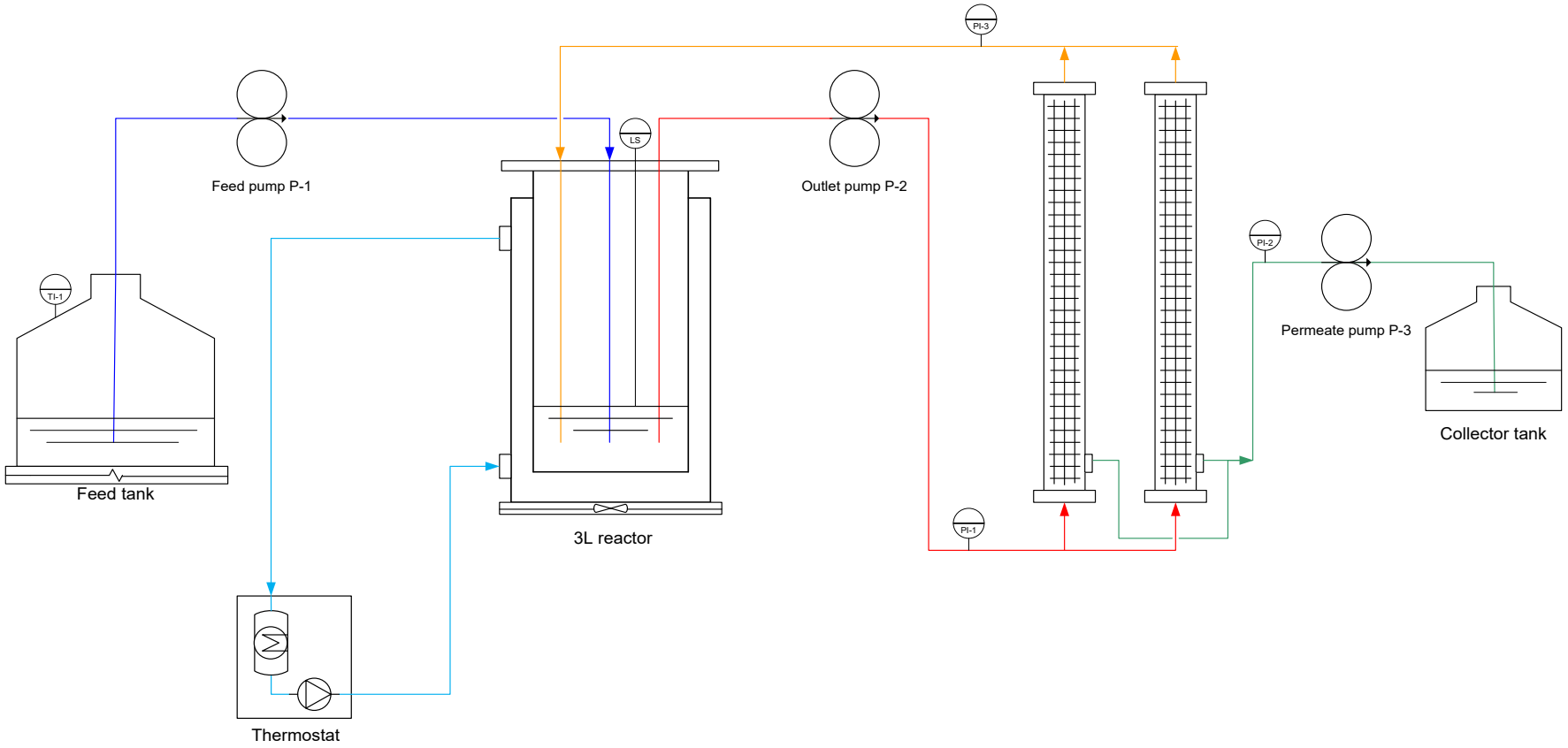


Figure 6.1: Experimental set-up of the membrane enzymatic reactor. Components: Feed tank; TI-1: Feed temperature meter; P-1: feed pump (peristaltic pump Watson – Marlow 520U); 3L Reactor; P-2: outlet pump (peristaltic pump Watson-Marlow 620 U); PI-1: feed pressure meter; KOCH Membranes; PI-2: permeate pressure meter; P-3: permeate pump (peristaltic pump Watson –Marlow 520U); Collector tank; PI-3: retentate pressure meter; LS: Level Switch: Thermostat.

The system basically consisted of a feed tank with fresh substrate, a reactor containing the reaction mixture and a cross flow membrane module coupled to the reactor. The reaction mixture, composed of the enzyme and the hydrolyzing substrate, was continuously circulated with pump P-2 over a cross flow membrane unit of 10 kDa. The POS (and other solutes) with a MW weight lower than 10 kDa were (partially) removed from the reaction mixture by permeation with pump P-3, while the remaining residue was recycled into the reactor. The permeation flux was fixed throughout the experiment and pre-set to garantee the requested average residence time of the reaction mixture in the reactor. The product removal was compensated by the continuous addition of fresh substrate from the feed tank with pump P-1 providing in this way a continuous operation.

The feed tank and reactor were thermostated to allow operation at the requested temperature of the process.

After each experiment, the whole system was thoroughly cleaned and desinfected by using using P3-Oxonia active and Ultrasil 115.

6.2.3.2 POS production process

The 3 L reactor was filled with 600 mL (±5 ml) crude pectin obtained from the extraction process. The system was thermostated at 45°C (optimum temperature for the enzyme activity). After reaching the temperature, 60 mL of a diluted solution of Viscozyme L at pH 4.5 was added to the reactor vessel. In order to activate the enzyme, the vessel was stirred at 200 rpm for the time chosen as residence time (time in which the substrate stays in contact with the enzyme).

After activating the enzyme, the continuous feeding of the substrate and the filtration of the products were started. The permeate pressure was monitored along the experiment and was found to stabilize at 0.5 ±0.1 bar.

The permeates were collected every 15 min (in case of RT15) or every 30 min (in case of RT30) until the end of the experiment. These time intervals

correspond to the time needed to obtain a new replenishment of the reactor content. The volume and the pH of the permeates collected were monitored.

Seven experiments were conducted testing three enzyme concentrations, i.e. 0.588 FPU/ml, 0.294 FPU/ml and 0.147 FPU/ml, two substrate concentrations, i.e. 25 g/l and 50 g/L, and two residence times of 15 minutes (RT15) and 30 minutes (RT30). The range in residence time and enzyme concentration was selected based on the kinetic information obtained in previous research on the hydrolysis of onion skins in a batch reactor. (Babbar *et al*, 2016) The substrate concentrations were based on earlier research that was performed on the hydrolysis of sugar beet pectin in a similar EMR set-up.

As explained before, the permeate samples were taken every 15 (RT15) or 30 (RT30) minutes. Since preliminary tests indicated that the enzyme was completely retained in the reactor, no deactivation step was performed for the permeates. At the end of the experiment, the residue was recovered as well. The residue was immediately heated at 95ºC for 10 minutes to inactivate the enzyme present. At the end the whole system was rinsed with MilliQ water and thoroughly cleaned as described above.

6.2.4 Analysis of total free monosaccharides and total pectic saccharides by HPAEC-PAD

To estimate the amount of dissolved pectic oligo- and polysaccharides in the permeates and retentates, all samples were measured in two ways: as such to determine the free monosaccharides and after additional (full) hydrolysis to determine the total amount of saccharides. The analysis was fully focused on galacturonic acid, since this was found to be the prime constituent of the pectin obtained. The neutral sugars (such as arabinose, rhamnose, xylose, ect.) were not quantified, as they were found to be very small in the permeates and below the range of calibration. The Galacturonic acid was quantified in each permeate before and

after post hydrolysis against known concentration of the standard. Based on these results the amount of oligo- and polygalacturonan was then calculated by correcting for each sample the total amount of galacturonic acid detected after post hydrolysis by the amount of the free galacturonic acid already present.

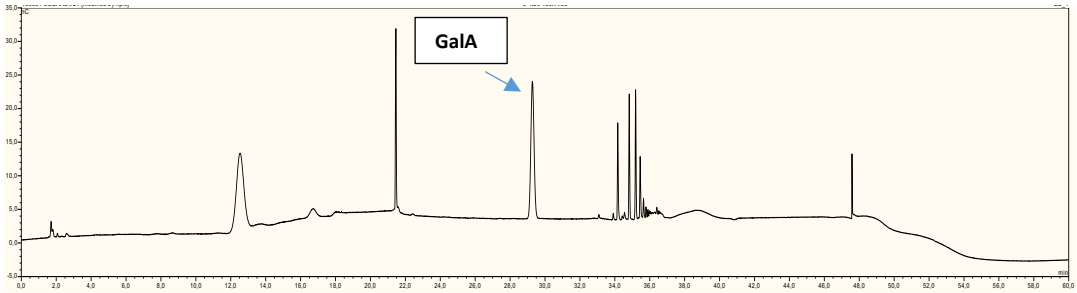
More specifically following formula was applied:

Total galacturonan oligo- and polysaccharides = Total galacturonic acid following post hydrolysis – Total monomeric galacturonic acid before post hydrolysis.

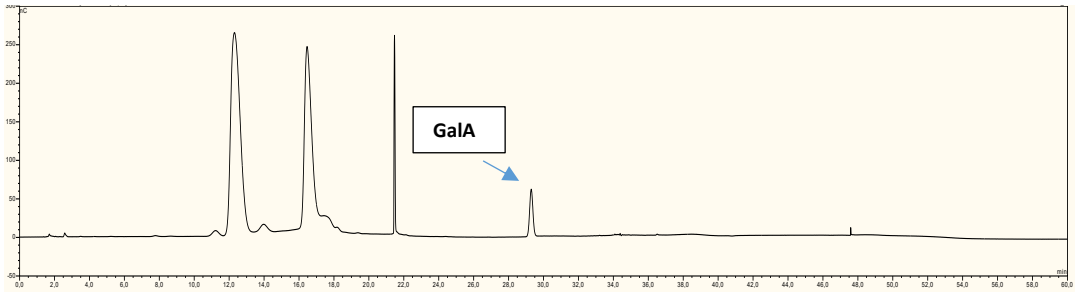
To achieve a complete hydrolysis and full recovery in the form of the galacturonic acid monomers, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h (Martinez *et al*, 2009; Babbar *et al,* 2016, a). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min, and the liquid was centrifuged at 2040 x g (Eppendorf centrifuge 5415 C) for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into HPAEC-PAD.

The HPAEC-PAD used for analytical purpose was a Dionex ICS-5000 model (Thermo Scientific, Inc., USA) equipped with an ED-5000 electrochemical detector. The separation of monosaccharides was carried out (Lee Y.C. 1996) with a Carbopac PA -1 (4mm X 250mm) column coupled to a guard column Carbopac PA- 1 (4mm X 50 m) column. The analyses were performed using a gradient of deionized water (eluent A and D), 250 mM sodium hydroxide (eluent B) and 1 M sodium acetate (eluent C). The elution conditions were: at time zero, A: B at 25:75 (start cleanup); at 10 min, B:C:D at 6:0:47 (re-equilibration); at 30 min, B:C:D at 6:15:39.50 (stop re-equilibration and start acquisition); at 35 min, B:C:D at 50:50:0; at 36 min, B:C:D at 6:0:47; and at 46 min B:C:D at 6:0:47 (stop acquisition). The mobile phase was used at a flow rate of 1 mL/min for 46 min and the injection volume was 5 microliters.

An example of chromatograms of the monomeric sugars detected before post hydrolysis and of total sugars detected after post hydrolysis is reported in figure 6.2a and 6.2b respectively.



(a)



(b)

Figure 6.2: HPAEC-PAD Chromatograms of POS mixtures as example. a) sugars detected before post hydrolysis; b) sugars detected after post hydrolysis. GalA= galacturonic acid.

The monosaccharides were quantified by comparing them with the known concentration of a galacturonic acid standard solution (ranging from 10 mg/L to 1000 mg/L). The concentrations were corrected for the volume of the permeate/retentate to obtain the total mass dissolved. against the .

6.2.5 Analysis of the oligosaccharides by HPAEC –PAD

Since the pectin of onion skin was found to be mostly composed of galacturonan, the analysis of the oligosaccharides was fully concentrated on the measurement of the galacturonan oligomers. The oligomers obtained after enzymatic hydrolysis in the enzyme membrane reactor were characterized by HPAEC-PAD by following the method of Combo, Aguedo, Goffin, Wathelet & Paquot, (2012). The column was a Dionex CarboPac PA-100 (4 mm × 250 mm) coupled to a CarboPac guard column (4 mm × 40 mm). The mobile phase consisted of 100 mM sodium hydroxide (eluent A), 600 mM sodium acetate in 100 mM sodium hydroxide (eluent B) and 500 mM sodium hydroxide (eluent C). Elution conditions were as follows: A: B as 95:5 over 0–5 min, A:B 50: 50 at 10 min, A:B 20:80 over 15–35 min, B:C 50:50 over 36–43 min and A:B 95:5 over 44–50 min. The flow-rate was 1 mL/min and the injection volume was 25 microliters. The identification of retention time of the different oligomers was performed based on a galacturonan oligosaccharide mixture DP1-DP10 (DP = degree of polymerization). The quantification of DP2-DP4 was done against standard solutions of DP2, DP3 and DP4 oligomers prepared in the range of 5 to 150 ppm.

The higher oligomers (>4 DP) were calculated using the following formula:

Total higher DP oligomers = Total galacturonan oligo- and polysaccharides – Total sum of DP2-DP4-oligomers.

6.2.6 Assessment of the monosaccharide ratio, yield and productivity

After the quantification of each permeate, the ratio of the galacturonan oligomers over the galacturonic acid monosaccharides was calculated using the formula (1):

$$Ratio = \frac{\text{GalA (POS)}(g)}{\text{GalA (monos)}(g)}, (1)$$

where GalA (POS) (g)= grams of galacturonan oligomers; GalA (monos) (g) = grams of galacturonic acid as monosaccharide.

The volumetric productivity of the POS in each permeate was calculated as a function of the collection time of each permeate using the following formula:

$$Productivity\ (g/l/h) = \frac{GalA(POS)\ (g)}{t\ (\text{min})} \times \frac{60(\text{min/h})}{0{,}6(l)}, \quad (2)$$

where g/l/h= grams per liter per hour; GalA (POS) (g)= grams of galacturonan oligomers; t (min)= collection time in minutes. The calculation was done in a cumulative way, in the sense that the volumetric productivity at a time t is calculated based on the total grams of galacturonan oligomers produced between the start and the time t under consideration.

The yield of the POS was calculated in permeate on the total sugar present in the raw material by using the following formula:

$$Yield\ (\%\ w/w) = \frac{\text{GalA(POS) (g)}}{\frac{(Vol\ (l) permeate\ x\ total\ GalA\ (monos)\ (g\ in feed)}{Tot\ vol\ feed(l)}} x100, \quad (3)$$

where % w/w= percentage weight by weight; GalA (POS) (g)= grams of galacturonan oligomers; Vol (l) permeate= total volume of collected permeate; total GalA (monos)(g in feed)= total galacturonic acid as monosaccharides present in the initial feed; Tot vol feed (l)= total volume of feed used in the experiment, where the cumulative values were used.

The yield of POS with different DP was calculated in percentage on the total sugar in the permeates by using the following formula, where the cumulative values were used:

$$Yield\ \left(\%\frac{w}{w}\right) = \frac{\text{DPx (g)}}{\frac{(Vol\ (l) permeate\ x\ total\ GalA\ (monos)\ (g\ in\ feed)}{Tot\ vol\ feed(l)}} x100, \quad (4)$$

where % w/w= percentage weight by weight; DPx (g)= grams of POS with specific (x) DP; Vol (l) permeate= volume of collected permeate; total GalA (monos) (g in feed) = total galacturonic acid as monosaccharides present in the initial feed; Tot vol feed (l)= total volume of feed used in the experiment.

The specific productivity was calculated by using the following formula:

$$Specific\ Productivity\ g\ (POS)/g\ (enzyme)/h = \frac{GalA\ (POS)\ (g)}{Time\ (\text{min})} \times \frac{60}{0.6(l)} \times \frac{g\ (enzyme)}{l(\ enzyme)}\ , \quad (5)$$

where g (POS)/ g (enzyme)/ h= grams of POS produced per gram of enzyme per hour; GalA (POS) (g)= grams of galacturonan oligomers; Time (min)= collection time in minutes; g (enzyme)= grams of enzyme used; l (enzyme)= volume in liters of enzyme used.

6.3 Results and Discussion

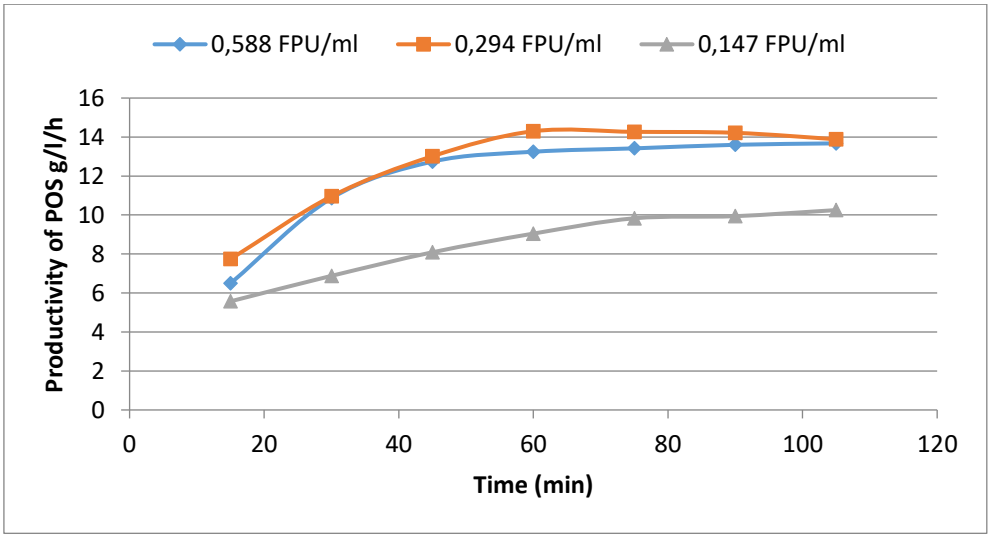
The crude pectic extract which was used as a substrate for the process was analyzed on its total saccharide composition by using the post-hydrolysis procedure described in section 2.4 and analyzing it by HPAEC-PAD. The saccharide content accounted for 28% (w/w) d.m. The rest of the biomass was likely formed by proteins, ash, unhydrolyzed cellulose and hemicellulose, etc.

The galacturonic acid content accounted for 19.3 % (w/w) on d.m., representing a significant part of the total sugar. Galactose, rhamnose and arabinose were also found and accounted for 7 %, 0.4%, and 0.3%, respectively. Given the low content of arabinose and rhamnose, it can be supposed that onion skins pectin was mostly made by homogalacturonan and very poor in rhamnogalacturonan regions. Moreover, the glucose content in the hydrolyzed samples accounted for 26.1 % w/w d.m, indicating the partial hydrolysis of cellulose. (Babbar *et al*, 2016)

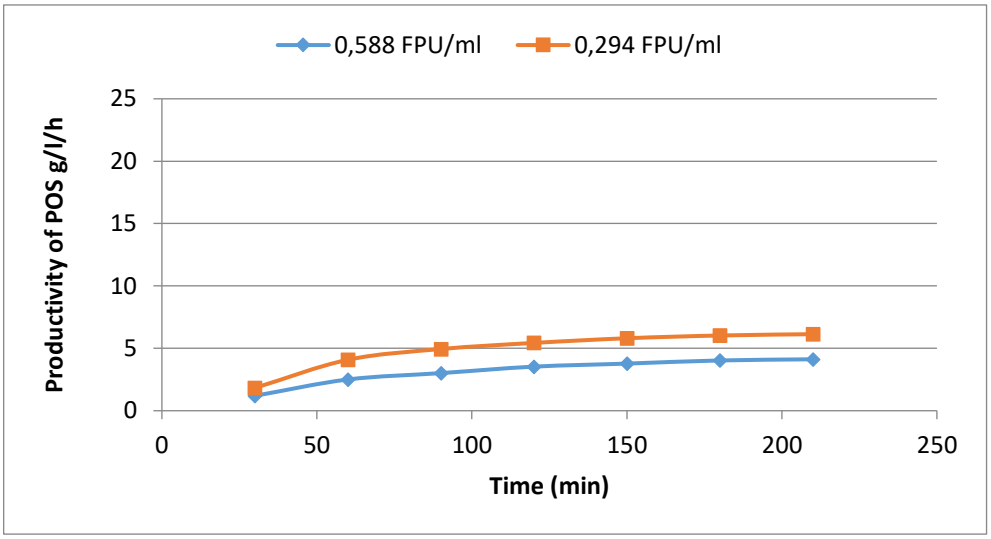
6.3.1 POS production: effect of process conditions

Six tests were carried out at a fixed substrate concentration of 25 g/L, but varying the residence time, 15 and 30 minutes, and the enzyme concentration, i.e. 0.588 FPU/ml, 0.294 FPU/ml and 0.147 FPU/ml.

In first instance, the process was evaluated on a more global level, whereby the total POS production was assessed on its yield and productivity and on its ratio against monosaccharides (monos). The results are displayed in Fig.6.3 (POS-productivity, eq 2), Fig.6.4 (ratio POS/monosaccharides, eq.1) and Fig.6.5 (POS-yield, eq.3).



(a)



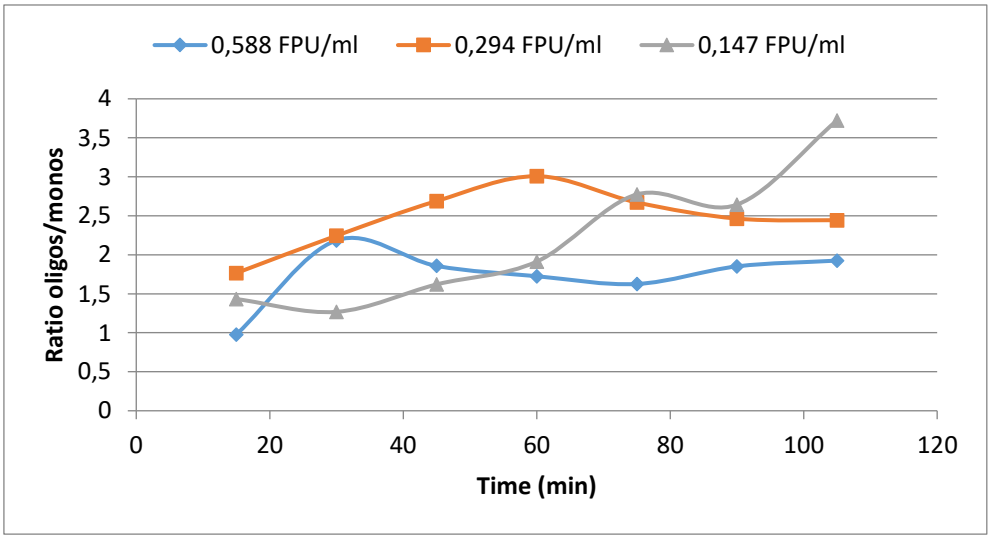
(b)

Figure 6.3: Volumetric productivity (g/l/h) of POS for the residence times 15 minutes (a) and 30 minutes (b) and at enzyme concentrations 0.147 FPU7ml, 0.294 FPU/ml and 0.588 FP/ml, using the the substrate concentration 25 g/L.

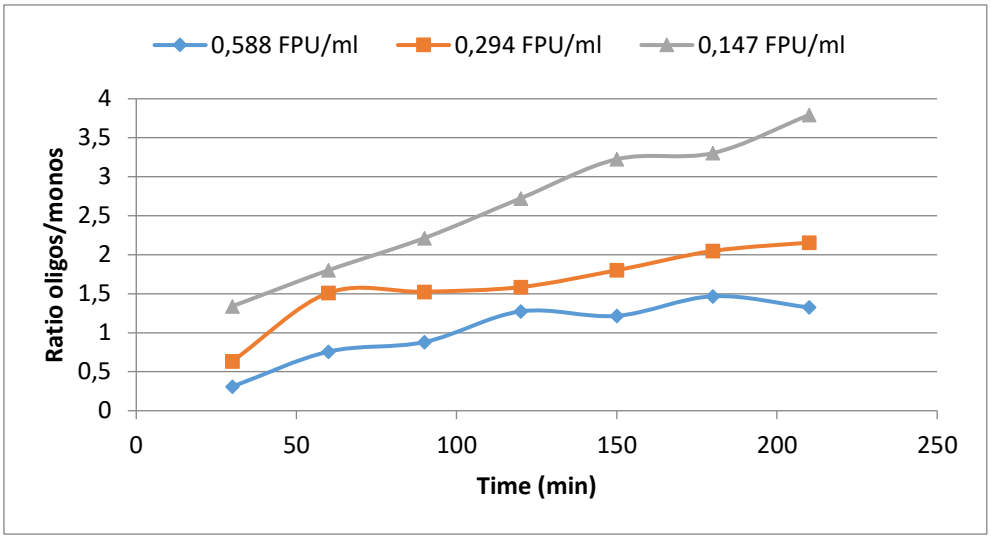
Fig.6.3 shows the volumetric productivity of POS in function of the residence time and enzyme concentration applied. At the lowest enzyme concentration, i.e. 0.147 FPU/ml, in steady state 10 g/l/h of POS were produced at RT15 (Fig.3a) and 5 g/l/h at RT30 (Fig.3b). The results are in full agreement with the expectations at a process level for the change in residence time, i.e., a doubling of productivity when halving the residence time. This indicates that the POS production itself is not altered by the shorter contact times applied. When the enzyme concentration was increased to 0.294 FPU/ml, the volumetric productivity overall increased to 14 g/l/h and 6 g/l/h at RT15 and RT30 respectively. This is attributed to a more efficient POS-formation. The change in productivity with residence time is higher than expected, indicating that also the enzymatic hydrolysis is influenced. In the presence of even more enzyme in the reactor, i.e., 0.588 FPU/ml, the productivity was 14 g/l/h at RT15 and 4 g/l/h at RT30. In this case, the POS productivity at RT30 is approximately 60% of what could be expected on a process level for the change in residence time. This

indicates that also on a reaction level the process is influenced, with relatively higher monosaccharide formation related to the high concentration of enzyme and the long residence time applied.

Besides the productivity, also a good product quality should be ensured in terms of the POS yield and the POS/mono ratio. Figure 6.4 shows the ratio between the total POS and the total monosaccharides (not cumulative) collected in the permeates in function of reaction time as determined by equation 1. The graphs in the Fig.6.5 show the yield of POS and monosaccharides in function of the hydrolysis time on percentage weight by weight-basis.

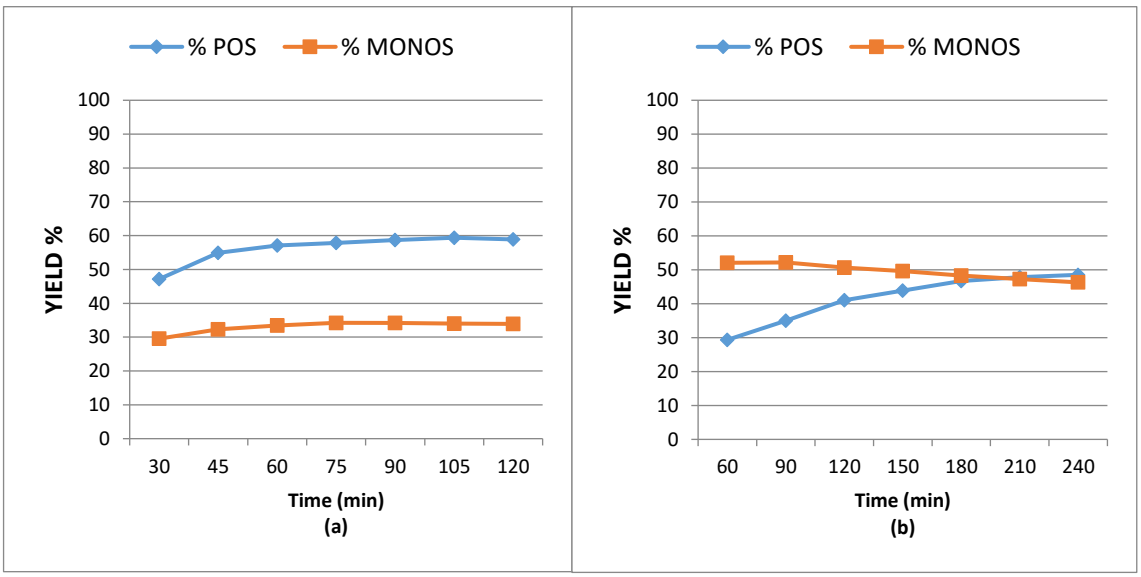


(a)



(b)

Figure 6.4: Ratio of oligosaccharides to monosaccharides calculated for the residence time 15 minutes (a) and 30 minutes (b) for the enzyme concentrations 0.147 FPU7ml, 0.294 FPU/ml and 0.588 FP/ml, using the substrate concentration 25 g/L.



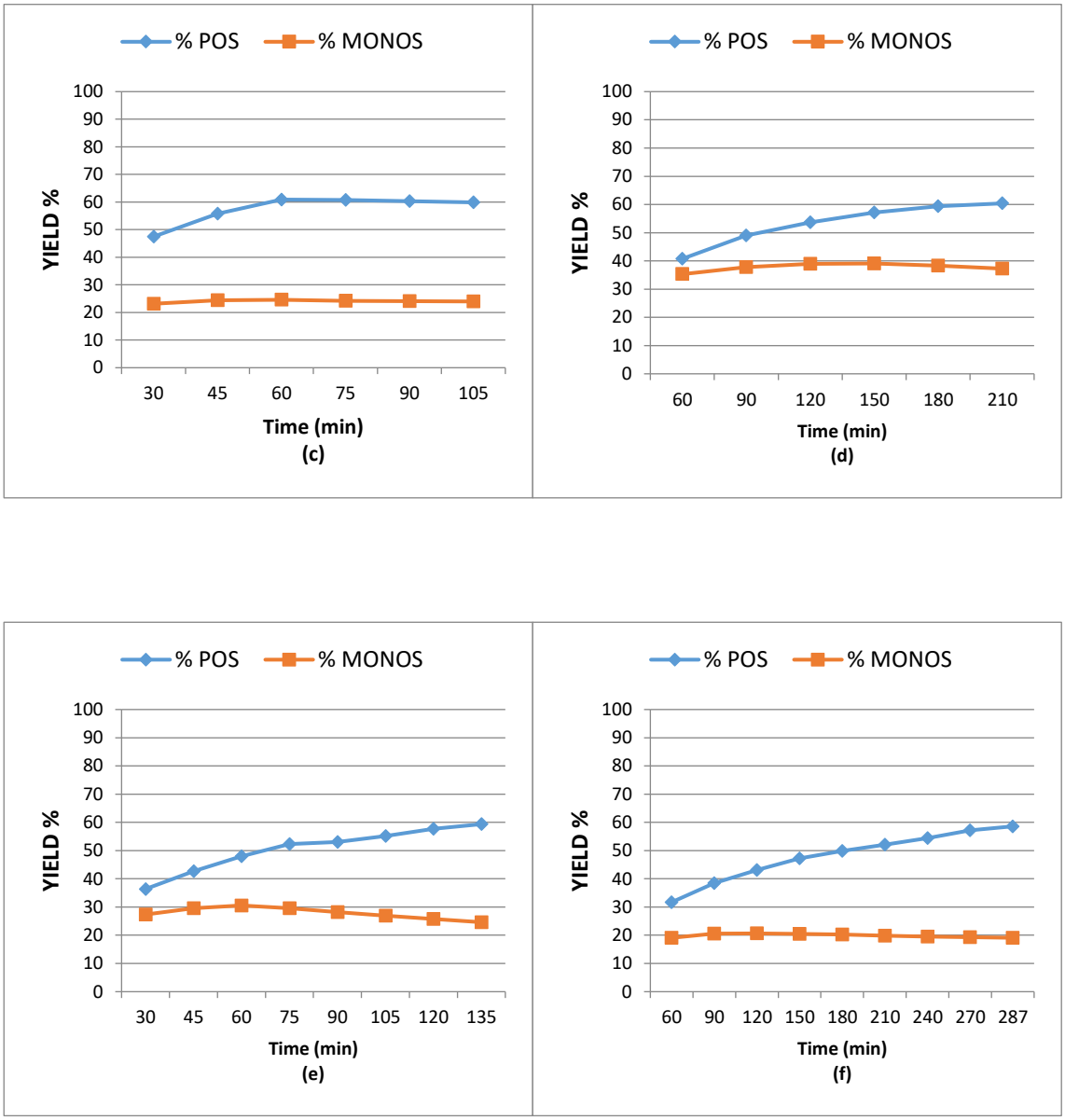


Figure 6.5: Yield (% w/w) of the POS and monosaccharides in the following conditions: substrate concentration of 25 g/L, 0.588 FPU/ml and RT 15 (a) and RT 30 (b); 0.294 FPU/ml and RT15 (c) and RT 30 (d); 0.147 FPu/ml and RT 15 (e) and RT 30 (f).

Clear differences are seen between the various production conditions. At low enzyme concentration, the permeates are typically very rich in POS with little monosaccharide formation. However, at the same time, the ratio between POS and

monos keeps on increasing in time, indicating an instable process and possibly an accumulation of saccharides in the residue. As can be expected, the effect is more pronounced at RT15 than at RT30. The two highest enzyme concentrations, on the other hand, lead to more stable POS/mono ratios indicating a more stable process window. Within this operational area, the shortest RT15 as well as the middle concentration gave the best results, whereby longer residence times as well as higher enzyme concentrations led to more extensive monosaccharide formation. In all the cases the POS/mono ratio was higher than 1, hence the process allowed the production of mixtures rich in POS.

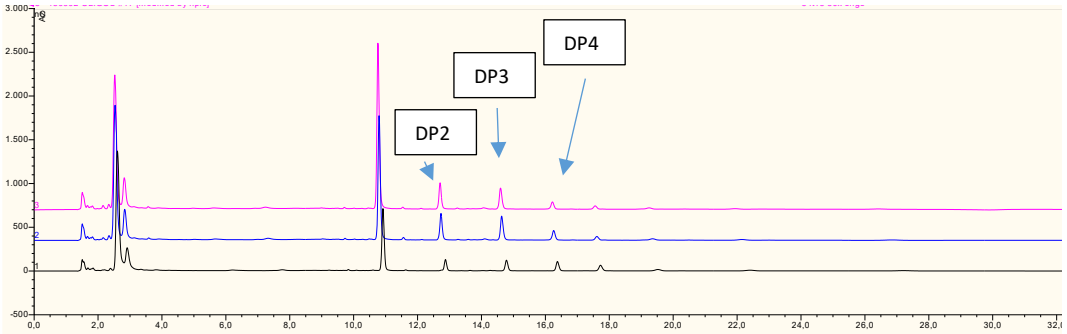
The present observations were confirmed by the behavior of the yield of POS and the monomeric galacturonic acid. Indeed, as depicted in the graphs (Fig.6.5), at the lowest enzyme concentration, 0.147 FPU/ml, the substrate was not completely hydrolyzed in comparison with the higher enzyme concentrations. In fact, until 70 and 120 minutes of reaction at respectively RT 15 (Fig. 6.5e) and RT 30 (Fig. 6.5f), the total percentage of permeation was lower than the 70%, suggesting an accumulation of the long pectic chains in the reactor. Moreover, the low monosaccharides formation (about 20-30%) was confirmed and a concomitant increase of POS yield was observed indicating a low stability of the process.

When the enzyme was more concentrated, most of the substrate fed into the reactor was hydrolyzed into POS fractions lesser than 10KDa and filtered, especially at 0.588 FPU/ml and at RT30 (100% permeation) (Fig.6.5b). However, according to the aim of the work, this latter result was not acceptable since the amount of the monosaccharides produced was too high (50%).

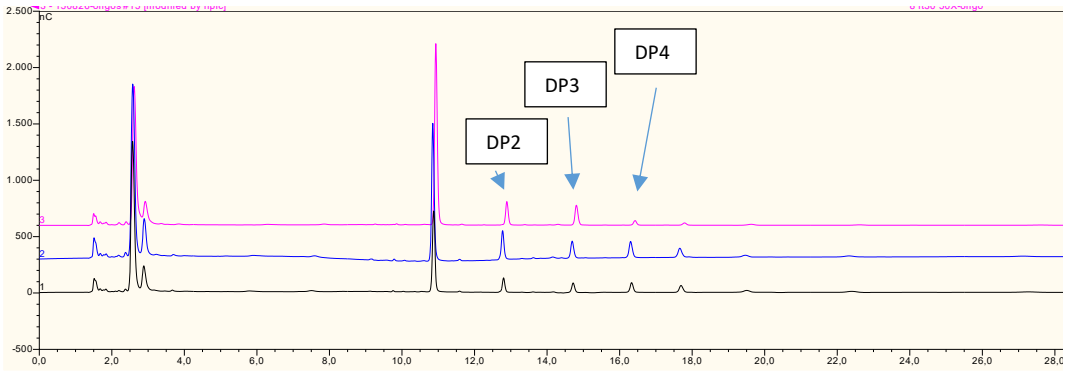
A very good compromise between POS yield and monosaccharides formation could be observed using the middle enzyme concentration and the short residence time (Fig.6.5c). Indeed, the hydrolysis resulted a POS yield of 60% and a monosaccharides yield 25%. Since the total conversion of the pectin did not reach the 100%, there still could be a bit of accumulation inside the reactor. Nevertheless,

this condition led to limited monosaccharides formation as compared to other conditions. Moreover, the process seemed to reach a stable production after 45 minutes process time, suggesting that the buildup stopped.

In order to evaluate the percentage of conversion of the pectic polysaccharides into POS, the oligosaccharides with degree of polymerization 2, 3 and 4 were quantified against a standard solution of known concentrations. The POS with DP>4 were quantified as described in the paragraph 2.6. Fig.6.6 shows the HPAEC-PAD chromatograms of POS mixtures collected in the steady state of the six processes. They give an idea on the degree of polymerization (DP) of the various POS-products obtained. Each peak corresponds to a specific oligo-galacturonan, whereby the various peaks are in order of increasing DP (the peaks before the 4 minutes analysis time correspond to salts adducts).



(a)

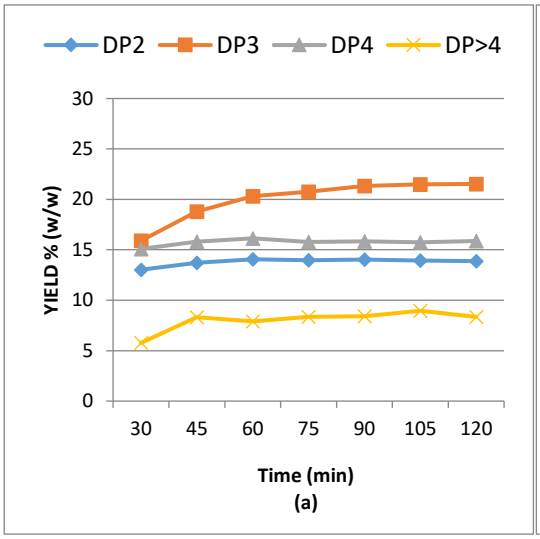


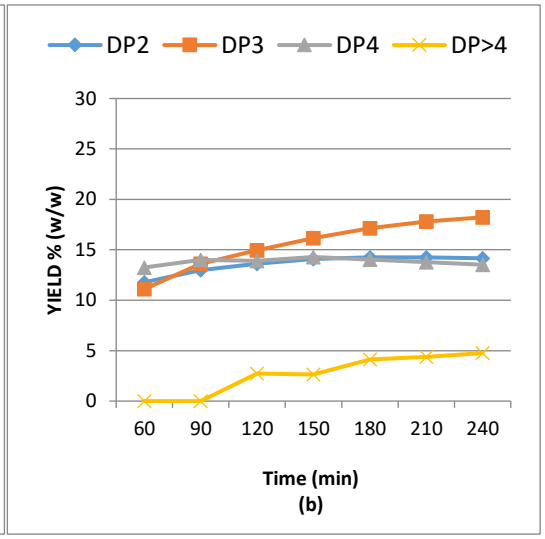
(b)

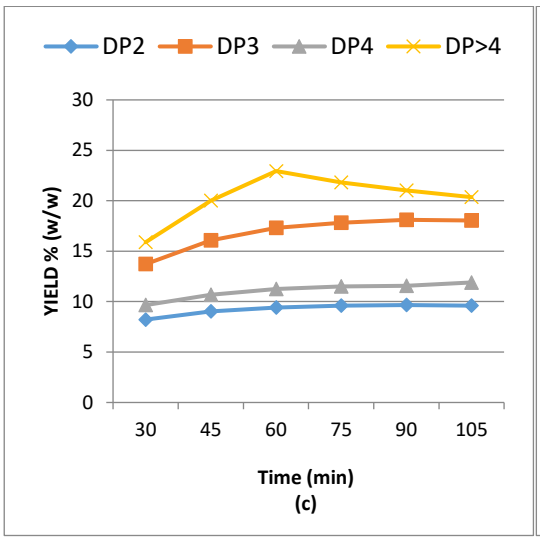
Figure 6.6: HPAEC-PAD Chromatograms of oligomers obtained after enzymatic hydrolysis in the enzyme membrane reactor. They refer to the steady state of each process. Different colors of the chromatograms correspond to different conditions: a) **black**: RT15 and 0.147 FPU/ml; **blue:** RT15 and 0.294 FPU/ml; **pink**: RT15 and 0.588 FPU/ml. b) **black**: RT30 and 0.147 FPU/ml; **blue:** RT30 and 0.294 FPU/ml; **pink**: RT30 and 0.588 FPU/ml.

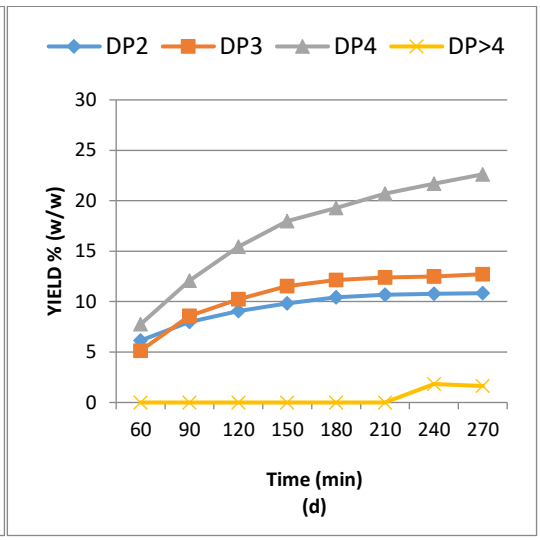
Looking at the chromatographic peaks areas detected at each condition, the highest abundance of POS, especially DP2, DP3 and DP4, can be seen at the middle enzyme concentration (chromatograms in blue).

The peak areas obtained at DP2 to DP4 were quantified as explained in the experimental section. Based on these results, the DP-yields were calculated on procentual mass/mass for each condition in function of time by using equation (4) (see Fig.6.7, a-f).


DP2
DP3
DP4
DP>4
YIELD % (w/w)
30
25
20
15
10
5
0
30
45
60
75
90
105
120
Time (min)
(a)


DP2
DP3
DP4
DP>4
YIELD % (w/w)
30
25
20
15
10
5
0
60
90
120
150
180
210
240
Time (min)
(b)


DP2
DP3
DP4
DP>4
YIELD % (w/w)
30
25
20
15
10
5
0
30
45
60
75
90
105
Time (min)
(c)


DP2
DP3
DP4
DP>4
YIELD % (w/w)
30
25
20
15
10
5
0
60
90
120
150
180
210
240
270
Time (min)
(d)

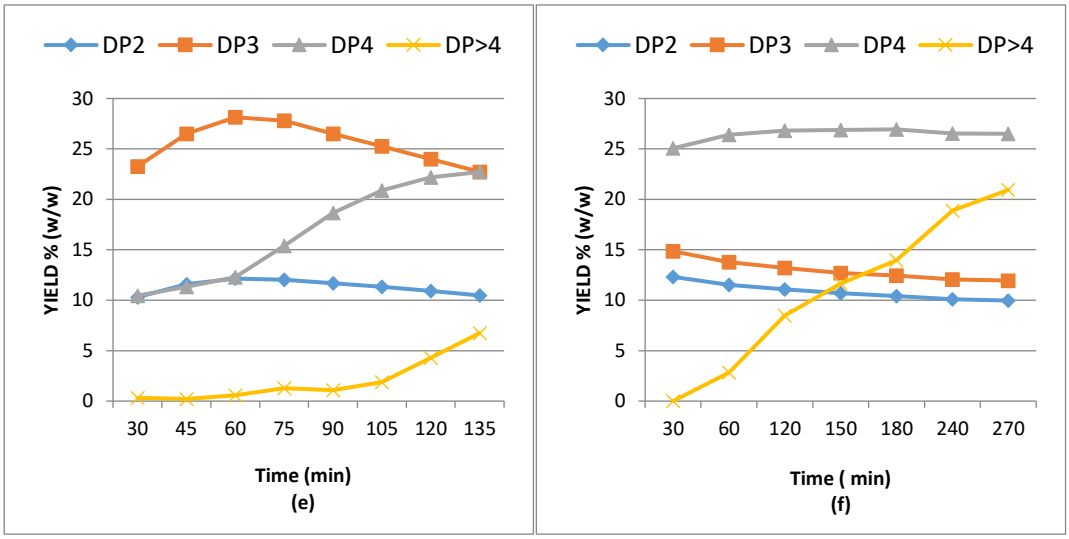


Figure 6.7: Yield (% w/w) of POS with DP2, DP3, DP4 and DP>4 at the following conditions: substrate concentration of 25 g/L, 0.588 FPU/ml and RT 15 (a) and RT 30 (b); 0.294 FPU/ml and RT15 (c) and RT 30 (d); 0.147 FPU/ml and RT 15 (e) and RT 30 (f).

The DP-yield in function of the process time immediately shows an instability of the hydrolysis at lower enzyme concentration. This confirms the earlier observations made based on the POS/monos ratio (Fig.6.4) and the POS yield (Fig.6.5). In fact, the production of small POS seems to decrease, in favor of the longer POS (DP>4) that strongly increase at the same time indicating an accumulation of long pectic chains inside the reactor.

A more stable production of short POS (DP2, DP3 and DP4) in function of time is observed at middle enzyme concentration and short residence time. As already observed in Fig.6.5, at this condition the POS production seems to stabilize after 45 process time in a POS product rich in DP3 as well as larger DP>4-oligosaccharides. It is supposed that the slight decrease of the yield of longer POS (DP>4) could continue on a longer term, potentially reducing stability problems. Higher enzyme concentrations provide, on the other hand, POS-products that are equally rich in the smaller DP2-oligomers.

Based on the fact that the membranes were not subjected to fouling or a decrease in permeability in function of time, it is strongly expected that the process could be extended for a longer time increasing further the performance. Nevertheless, dedicated experiments still need to be performed to assess the long term stability of the process. Moreover, within all reactor tests the enzyme did not lose its activity as indicated by the steady state (shown in the graphs).

The results from tests performed starting from the substrate at 25g/L demonstrated that it is possible to achieve a good yield of pectic oligosaccharides (60% w/w), especially using the middle enzyme concentration (0.294 FPU/ml) and the short residence time (15 minutes). The process leads to a POS-product composed mostly of DP3 and as well as larger DP>4-oligosaccharide. The enzyme was found to be stable during the production times applied. Nevertheless, enzyme inactivation as well as fouling on a longer term still needs to be addressed in dedicated experiments.

6.3.2 POS production: effect of substrate concentration

In order to optimize the continuous production of POS from onion hulls, also the possibility to work at higher substrate concentration was studied, i.e. 50 g/L. The hydrolysis was performed using the residence time of 15 minutes and the enzyme concentration of 0.294 FPU/ml, since the latter conditions gave the most promising results in the previous sections.

The ratio POS/monos and the volumetric productivity (g/l/h) were calculated and they accounted for about 4.5 and 21 g/l/h in the steady state respectively (data not shown).

Fig.6.8 shows the yield (% w/w) of POS and monosaccharides, as well as the yield (% w/w) of POS with DP2, DP3, DP4 and DP>4 in function of the process time.

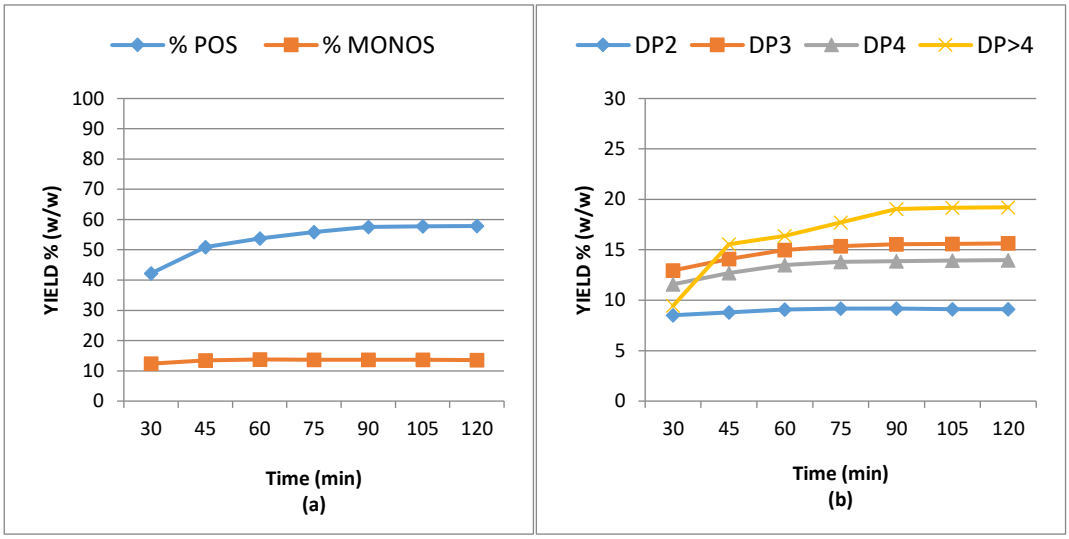


Figure 6.8: Yield (% w/w) of the POS and monosaccharides in the following conditions: substrate concentration of 50 g/L, 0.294 FPU/ml and RT 15 (a); Yield (% w/w) of POS with DP2, DP3, DP4 and DP>4 for substrate concentration 50 g/L, RT 15 and enzyme concentration 0.294 FPU/ml (b).

As depicted in Fig. 6.8a, the hydrolysis of crude pectin extract provided a good compromise between the POS yield, which accounted for 60%, and the monos yield, which was found to be around 12% in the steady state. The process seemed to reach the steady state already after 45 minutes, very similarly to the use of a lower substrate ratio (25 g/L) at the same conditions.

A stable conversion of pectin into low DP POS (DP2, DP3, DP4) was observed, even if a small increase of longer (DP>4) POS seemed to occur as well. Probably the substrate concentration of 50 g/L is at the boundaries for the enzyme concentration applied for being able to convert all pectic polysaccharides in a sufficient time.

In order to study the effect of the substrate concentration on the POS production, the specific productivity was calculated for the tests performed using the residence time RT 15, the enzyme concentration 0.294 FPU/ml and the two substrate concentrations 25 g/L and 50 g/L and in it is reported in Fig.6.9.

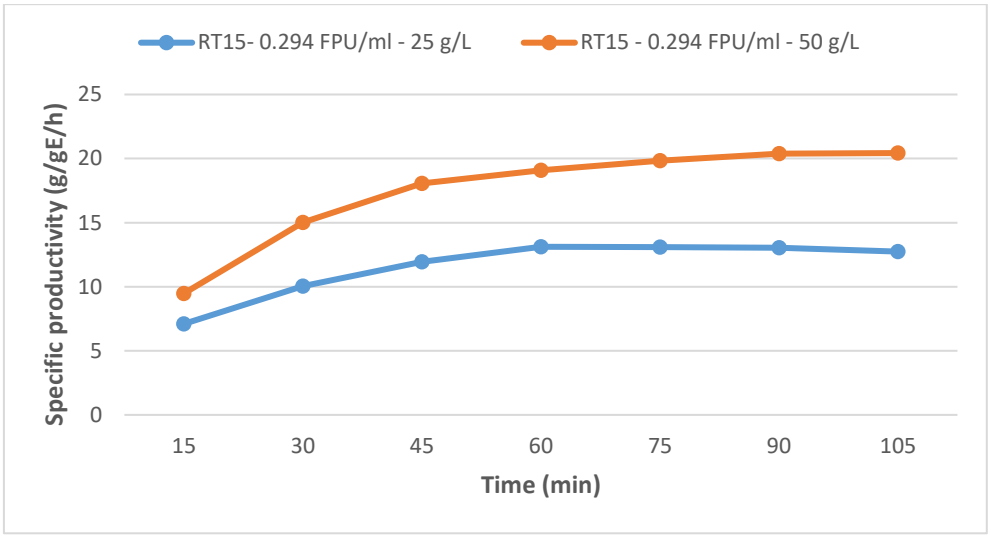


Figure 6.9: Specific productivity (g/gE/h) of POS for the residence time 15 minutes and enzyme concentration 0.294 FPU/ml, using the substrate concentrations 25 g/L and 50 g/L.

As expected, the higher the substrate concentration the higher the specific productivity. In particular, it reached 13 g/gE/h and 20 g/gE/h at the two conditions selected. This result suggests that working on undiluted substrate, results in a higher efficiency of the process. If compared to the specific productivity reported in literature for continuous citrus pectin hydrolysis, i.e. 9.7 g/gE/h (Bèfali-Bako et al, 2007) or for continuous sugar beet pulp pectin hydrolysis, i.e. 19-24 g/gE/h (Babbar N, 2016) the values reached in the present study starting from onion skins waste can be considered as very promising results.

Overall, when the substrate concentration 50 g/L was used, the best hydrolysis performance was obtained in terms of productivity and of yield. Moreover, a suppression of the monosaccharides formation could be achieved at these process conditions.

6.3.3 Continuous process vs batch production

The continuous cross-flow enzyme membrane reactor represents an innovative technology for the production of functional compounds such as pectic oligosaccharides respect to the batch production. Babbar *et al* (2016) performed the batch production of POS from onion hulls. Comparing the data collected using the two systems, the volumetric productivity of POS with DP2, DP3 and DP4 was respectively 0.01, 1.7 and 2.4 g/l/h for the batch production and 3.5, 5.8 and 5.2 g/l/h for the continuous production (RT15, 0,294 FPU/ml, 50 g/L). The productivity of the monosaccharides was of 7.7 g/l/h using the batch system and of 5.3 g/l/h using the continuous production. Accordingly, the use of the EMR provided advantages such as the suppression of the monosaccharides formation and a higher POS productivity.

6.4 Conclusions

The two-step continuous biocatalytic membrane reactor for the pectin-oligosaccharide (POS) production, in which conversion is combined with separation, was found to be very promising for the continuous production of tailor made pectin.

The present study showed that working at a residence time of 15 minutes, enzyme concentration 0,294 FPU/ml and at substrate concentrations 25 g/L or 50 g/L, the highest and stable POS productivity, the highest POS yield and the lowest monosaccharide formation could be achieved. At the mentioned conditions, a more stable production of short POS (DP2, DP3 and DP4) in function of time was also observed.

Of course, in order to gain higher POS yield and productivity, further optimization (residence time, enzyme dilution, ect.) of the hydrolysis performance is still possible.

Furthermore, the POS produced will be assessed on the molecular structure and composition by HILIC/ESI-MS analysis and on their prebiotic activity.

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7. Pectic oligosaccharides (POS) from onion skins: molecular composition and biofunctional properties investigation

Abstract

The brown skin represents one of the major by-products resulting from the industrial production of onions. Given their high pectin content (around 20 % w/w on d.m. basis), such wastes could be exploited for the production of added-value compounds such as pectic oligosaccharide (POS).

The aim of the present work was the characterization of pectic oligosaccharides obtained from the onion skins after a chelator-based extraction (using sodium hexamethaphosphate, SHMP) and enzymatic hydrolysis in an enzymatic membrane reactor (EMR). In order to evaluate the effect of the production conditions, POS fractions were analyzed in terms of molecular composition, degree of polymerization (DP) and acetylation and methylation pattern, by hydrophilic interaction chromatography (HILIC) coupled with electrospray mass spectrometry detection (ESI-MS). The analysis allowed to determine the POS fraction composition, consisting of polygalacturonans with DP from 2 to 8 in the free form as well as in acetylated/methylated form.

Moreover, an *in vitro* microbiological study was done in order to assess the POS influence on the growth of *Lactobacillus* probiotic strains. The influence on the growth of two *E.coli* strains, known to be pathogen for poultry and pigs, was also studied. However, POS mixtures showed an inhibition effect on the bacterial growth as compared to the absence of any extract, which was determined to be due to the presence of the chelator SHMP in POS fractions. However, the growth as compared

to the presence of SHMP alone was enhanced by the POS, indicating that POS might actually stimulate bacterial growth, even if more experiments will be needed in order to definitely elucidate the issue.

7.1 Introduction

Onions (*Allium cepa,* Liliaceae) represent one of the most cultivated vegetable crops not only in Europe but also in Asia, N. America and Africa, thanks to the adapting capability to a wide range of environments. (Brewster, 1994). Onions have white/yellow flesh and yellow to brown skins although red/purple onions are preferred in many parts of the world.

These vegetables are widely used in nutrition and many studies have shown their health benefits. (Griffiths *et al*, 2002) In fact, onions are rich in flavonoids which are potential antioxidants (Takahama and Hirota, 2000). Moreover, *Allium* consumption can exert a protective effect against oesophageal, stomach and brain cancer (Gao *et al.*, 1999; Hu *et al.*, 1999). Onion bulbs promote bile production and reduce sugar and lipid levels (Augusti, 1990) An anti-asthmatic activity was shown by compounds such as sulphinyl-disulphides (Dorsch *et al.*, 1989; Dorsch, 1996). Furthermore, crude extracts of onion exert potent antifungal and antibacterial properties (Kim, 1997; Yin and Tsao, 1999). Onions are also rich in inulin which improves blood lipid profiles and exerts beneficial effects on the colonic environment, being a promising functional ingredient in many processed foods (Causey *et al.*, 2000).

In 2014/2015 the EU bulb onion crop was estimated at 5.9 million tonnes which was 13 % more than in the year before. (EURONION, 2016) The huge production of onions implies as well a significant production of wastes. Indeed, approximately 450 000 tons of onion waste are produced annually, mainly from the UK, The Netherlands and Spain. The major by-products resulting from industrial peeling of onion bulbs are the brown skins, the outer two fleshy leaves and the top and bottom bulbs. Such wastes cannot be reused as animal feed due to the rapid growth of phytopathogens. However, the waste could be exploited for the production of value-added products. (Hertog *et al*, 1992; Waldron, 2001). For instance, fructans and fructooligosaccharides have been recovered from the outer two fleshy leaves

(Jaime *et al*, 2000). Actually, onions can be considered also a potentially valuable source of cell wall polysaccharides, such as pectin. (Alexander and Sulebele, 1973)

Cell walls polysaccharides derived from onion have been investigated. (Ishii, 1982; Mankarios *et al*, 1980; Redgwell & Selvendran, 1986) In particular, various preliminary treatments of the raw material have been tested. (Redgwell and Selvendran, 1986). Pectic polysaccharides, xyloglucan and a small amount of hemicellulose–pectic complexes were found in onion bulbs (Ha *et al*, 1996; Mankarios *et al*., 1980; Redgwell & Selvendran, 1986; Victoria V. Golovchenko *et al*, 2012). The pectin content of the onion bulbs was determined to be ca. 0.5 g/100 g fresh weight (O'Donoghue *et al*., 2004). The structure of water-soluble pectic polysaccharide isolated from immature onion stick was investigated: pectic polysaccharide contained d-galactose, 6-O-Me-D-galactose, 3-O-acetyl-d-methyl galacturonate and d-methyl galacturonate in a molar ratio of nearly 1:1:1:1. (Patra *et al*, 2012). Also Ha *et al* (1996) and O'Donoghue *et al* (2004) reported that onion pectin was rich in galactose residues.

Matsuura *et al* (2000) characterized the onion pectin after extraction and purification without autolysis and they found a polymer made by 93.7% uronic acid and 6.3% neutral sugars. It was also demonstrated that substantial variation exists in cell wall composition among onion tissues. (Ng *et al,* 1998). After extracting the two fractions of pectin, the one soluble in chelating agents (calcium-bound pectin) and the other one soluble in alkaline solution (ester linkage-bound pectin), a pectin content of 121 mg/g on d.m. was found in onion hulls by J. Müller-Maatsch et al (2016).

However, a limited knowledge regarding the cell wall chemistry of onion and especially the molecular composition and properties of onion pectic substances has been reported. This lack of information should be filled given the importance of onion production, consumption and processing.

Pectin represent the starting material to obtain pectic oligosccharides (POS), such as rhamnogalacturonan-oligosaccharides, galacturonan-oligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, arabino-galactan oligosaccharides, which are able to exert several health promoting effects. In fact, POS can resist digestion and reach the colon intact; at that level of the gastrointestinal tract, POS are able to enhance the growth of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli.* Moreover, they inhibit the pathogenic bacteria, for example *Clostridia* and *E.coli* population, having a "barrier effect" that limits the number of available adhesion sites. (Gullon *et al,* 2013; Mandalari *et al*, 2007)

The present study aimed at the characterization of POS obtained from onion skins after chelator based extraction and enzymatic hydrolysis in an enzymatic membrane reactor (EMR). POS were analyzed on the molecular level by HILIC/ESI-MS; in particular, the molecular composition, the degree of polymerization and acetylation and methylation pattern were studied. Moreover, POS were assessed for their prebiotic activity monitoring *in vitro* the growth of seven *Lactobacilli* strains in presence of POS extract in the growth medium.

7.2 Materials and methods

7.2.1 Chemicals

The ammonium formate and the acetonitrile were provided by Sigma-Aldrich (Germany). The formic acid and the nitric acid were provided by Fisher Chemical (UK).

7.2.2 Materials and samples

Onion hulls were provided by Institut fur Getreideverarbeitung (IGV, GmbH), Germany. The hulls were milled with a laboratory blender, screened to a particle size (< 1 mm) and stored in ziplock bags at room temperature until use. Viscozyme L (V-2010) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

POS fractions were provided by the Flemish Institute for Technological Research, VITO, Belgium.

7.2.3 Extraction of pectin

Pectin was extracted from onion hulls using the chelator sodium hexamethaphosphate, essentially following the procedure described by Babbar *et al* (2016). Briefly, onion skins (1 g) were pretreated with 2% sodium hexametaphosphate solution at 95 °C for 0.5 h in a hot water bath. The biomass was then centrifuged at 4500 x g for 10 min. The supernatant containing the crude pectin was collected and analyzed for its free monosaccharide as well as total saccharide composition. The latter was then taken as a measure for the polysaccharide content.

7.2.4 Analysis of crude pectin extract

The saccharide composition of crude pectin extract was analysed by HPAEC-PAD. The free monosaccharides were detected as such. The amount of dissolved pectic oligo- and polysaccharides was determined by correcting the total amount of saccharides detected after hydrolysis by the amount of monosaccharides already present in the extract. To achieve a complete hydrolysis and full recovery of the monomers, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h (Martinez *et al*, 2009; Martinez *et al,* 2010). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 2040 x g (Eppendorf centrifuge 5415 C) for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into HPAEC-PAD.

The HPAEC-PAD used for analytical purpose is a Dionex ICS-5000 model (Thermo Scientific, Inc., USA) equipped with an ED-5000 electrochemical detector. The separation of monosaccharides was carried out (Lee Y.C. 1996) with a Carbopac PA -1 (4mm X 250mm X 4 mm) column coupled to a guard column Carbopac PA-

1 (4mm X 50 mm X 4mm) column. The analyses were performed using a gradient of deionized water (eluent A and D), 250 mM sodium hydroxide (eluent B) and 1 M sodium acetate (eluent C). The elution conditions were: at time zero, A: B at 25:75 (start cleanup); at 10 min, B:C:D at 6:0:47 (re-equilibration); at 30 min, B:C:D at 6:15:39.50 (stop re-equilibration and start acquisition); at 35 min, B:C:D at 50:50:0; at 36 min, B:C:D at 6:0:47; and at 46 min B:C:D at 6:0:47 (stop acquisition). The mobile phase was used at a flow rate of 1 mL/min for 46 min and the injection volume was 5 microliters. The monosaccharides were quantified by comparing them with the known concentration of galacturonic acid standard solution (ranging from 10 mg/L to 1000 mg/L).

7.2.5 Enzymatic hydrolysis of pectin

The crude pectin extract was subjected to an enzymatic hydrolysis in an enzymatic membrane reactor (EMR) by using Viscozyme L. The 3 L reactor was filled with 600 mL (±5 ml) pretreated onion hulls solution. The system was thermostated at 45°C (optimum temperature for the enzyme activity). After reaching the temperature, 60 mL of a diluted solution of Viscozyme L at pH 4.5 was added to the reactor vessel. In order to activate the enzyme, the vessel was first shaken at 200 rpm for the time chosen as residence time (time in which the substrate stays in contact with the enzyme). After this initial activation step, continuous dosing of the substrate was started while separating the POS at the same time by filtration along a 10 kDa membrane. By regulating the filtration flux, the average residence time of the substrate was set at the requested residence time. The permeates enriched in low molecular weight oligosaccharides (<10KDa) were collected at regular times. The residue, enriched in high molecular weight saccharides (>10KDa) and containing the enzyme, was collected at the end of the process and the enzyme was inactivated at 100°C for 5 minutes. Two substrate concentrations were tested, i.e. 25 g/L (diluted feed) and 50 g/L (undiluted feed), together with two residence times, 15 and 30

minutes, and three enzyme concentrations, i.e. 0.588 FPU/ml, 0.294 FPU/ml 0.147 FPU/ml.

The collected POS fractions were freeze-dried prior to be analyzed (Freeze-dryer Christ, Model: Gamma 1-16 LSC, Germany).

7.2.6 Hydrophilic interaction liquid chromatography coupled with electrospray mass spectrometry detection

Pectic oligosaccharides were separated and identified using the method reported by Leijdekkers, A.G.M *et al* (2011), but with a slight modification. In particular, 100 mg of sample were dissolved in 1 ml of eluent B (H_2O:CH_3CN 80:20, 10 mM $HCOONH_4$ + 0.2% HCOOH). The samples were centrifuged at 20.817x g (model 5810R EPPENDORF, Hamburg, Germany), at a temperature of 4°C, for 10 minutes and the supernatant recovered for the analysis. The oligosaccharides present in the POS mixture were separated by a HILIC column (ACQUITY UPLC BEH Amide Column, 130Å, 1.7 µm, 3 mm X 150 mm) in an UPLC/ESI-MS system (UPLC Acquity Waters with a single quadrupole mass spectrometer Waters ACQUITY SQD) using a gradient elution. Eluent A was H_2O:CH_3CN 20:80 (v/v), 10 mM $HCOONH_4$ + 0.2% HCOOH, eluent B was H_2O:CH_3CN 80:20 (v/v), 10 mM $HCOONH_4$ + 0.2% HCOOH; gradient: 0-60 min linear from 100% A to 60%A, 60-65 min isocratic at 60% A, 65-66 min linear from 60%A to 100%A, 66-75 min isocratic at 100%A. Flow was 0.3 ml/min; optimal run time for separation of various oligosaccharides was 75 min; column temperature 35°C; sample temperature 18°C. The injection volume was set to 10 µl; acquisition time 0-75 min; ionization ion mode: negative; capillary voltage 2.6 kV; cone voltage 60 V; source temperature 150°C; desolvation temperature 350°C; cone gas flow 100 l/h; desolvation gas flow 650 l/h; samples were analyzed in the Full Scan mode with a scan range of 200-2000 m/z. The analysis was performed in duplicate.

7.2.7 Prebiotic tests with impedimetric technique

Seven *Lactobacillus* strains were purchased from the "Belgian coordinated collection of microorganism'' (LMG, Gent, Belgium, http://bccm.belspo.be/). The strains were selected from different species, different origins and potential probiotic nature and they are listed in Table 7.1.

Table 7.1: Bacterial strains used for the BacTrac analysis.

Strains	Lactobacilli species	Biological origin
LMG 6400	*L. rhamnosus*	Not know
LMG 18399	*L. plantarum*	Not known
LMG 9198	*L. curvatus*	Milk
LMG 23516	*L. casei*	Human, faeces
LMG 8900	*L. fermentum*	Eight days old breast fed infant
LMG 8151	*L. acidophilus*	intestine
LMG 18223	*L. delbrueckii* subsp. *Lactis*	Acidophilus milk

Lactobacilli strains were routinely grown in Man-Rogosa-Sharpe (MRS) medium (Oxoid, Basingstoke, UK) under aerobic conditions and incubated at 30°C for mesophilic strains (*L. rhamnosus, L. plantarum, L. curvatus, L. casei)* and 37°C for thermophilic strains (*L. fermentum, L. acidophilus and L. delbrueckii* subsp. *lactis*). Moreover, two strains of *Escherichia coli* (*E.coli* k88 and *E.coli* k99), pathogens for piglet and poultry, were gently provided by Geert Bruggeman, Nutrition Sciences, Belgium. The strains were grown in MacConkey agar medium N°2 (Oxoid, Basingstoke, UK) and incubated at 37°C under aerobic conditions. Tryptone soya broth (TSB) (Oxoid, Basingstoke, UK) was used for impedometric experiments.

Before the experiments, mesophilic and thermophilic lactobacilli strains were revitalized three times in MRS broth (Oxoid, Basingstoke, UK) at 30°C and 37°C, respectively under aerobic conditions. For the *E.coli* strains, revitalization was made in TSB broth (Oxoid, Basingstoke, UK) at 37°C.

For the growth experiments, the sterile liquid media (MRS for lactobacilli and TSB for *E. coli*) were supplemented with POS at a concentration of 1% (w/v). The latter was chosen in accordance with the concentrations actually administrable to the animals.

For lactobacilli, 50 ml of sterile MRS broth was supplemented with 0.5 g of solid POS and 50 ml of MRS sterile broth was supplemented with 1 g of solid POS, for 1% and 2%, respectively. Successively, supplemented media were filtered in 0.22 µm membrane (Millipore, Billerica, MA, U.S.A.) and the filtered fraction was used for the impedance experiment.

For *E. coli*, 20 ml of TSB broth was supplemented with 0.2 g of solid POS and 20 ml of TSB was inoculated with 0.4 g of solid POS. Afterwards, the media were filtered in 0.22 µm membrane (Millipore, Billerica, MA, U.S.A.) and the filtered fraction was used for the impedance experiment.

The prebiotic test was carried out to assess the effect of POS mixtures on the bacterial growth of Lactobacilli strains, while parallel incubations with the bifidogenic substrate fructo-oligosaccharides, FOS, and no carbohydrates, respectively, were used as controls. Media supplemented with SHMP was used as additional control, given the presence of the chelating agent in POS mixtures. Media supplemented with POS (1% w/v) were inoculated with approximately 10^3 cfu/ml of each bacterial strain. Successively, 6 ml of the media were transferred to glass cells, and incubated in the microbiological impedance analyser (Bactrac 4300® Sy-Lab, Neupurkersdorf, Austria) for 48 h at 30°C or 37 °C according to the bacterial strains. For the experiments carried out in the present study, the impedance change (E-value), which is referred to as the impedance change at the electrode surface of the MRS and TSB, was used (Bancalari *et al* 2016). This value, recorded every 10 minutes, is revealed as a relative change in the measurement signal and shown as E% percentage in function of time (48 hours) in an impedance curve. The threshold was set at 5% E value that allows determining the time to detection (TTD), the time when the

impedance curve meets the choose threshold level, by guarantying consistence results. This was previously verified by measuring serial dilutions of pure cultures (data not shown).

MRS supplemented with 1% (w/v) FOS (Sigma-Aldrich, St. Louis, MO, U.S.A.) and inoculated with bacterial cultures was used as positive controls. MRS and TSB, without the supplemented pectin oligosaccharides, and inoculated with each bacterial strain were used as negative controls. All experiments were made in double copy.

Blanks were made for each supplemented media without addition of pure cultures. E % values were registered and resulted to be under the threshold of 5%.

7.2.8 Statistical analysis

Statistical analysis of ESI/MS data was performed using SPSS software (IBM SPSS statistics 21). One-way analysis of variance and Duncan's multiple comparison tests were used to determine significant differences in the peak area of the identified compounds in the samples from different production conditions. Tests were considered statistically significant when *P*-values lower than 0.05 were obtained.

7.3 Results and discussion

A chelator-based extraction was performed in order to release the calcium-bound pectin from onion skins by loosening the egg box structures formed by the homogalacturonan and calcium complex (Ravn & Meyer, 2014). The extraction was performed using the chelator sodium hexametaphosphate (SHMP) which was found to be the most effective if compared to other ordinary extractants (Babbar *et al*, 2016)

The composition in pectic sugars of the crude pectic extract was analyzed by HPAEC-PAD and is reported in detail in the previous chapter 6 (section 6.3). Overall, it was observed that onion skins pectin was mostly made by "smooth" regions, i.e. homogalacturonan, and was very poor in "hairy" regions, i.e. rhamnogalacturonan regions.

The crude pectic extract was hydrolyzed in an enzymatic membrane reactor (EMR) as described in section 7.2.5. In particular, different process conditions were tested, i.e. two residence times (RT15 and RT30), three enzyme concentrations (0.588 FPU/ml, 0.294 FPU/ml, 0.147 FPU/ml) and two substrate concentrations (25 g/L and 50 g/L). It was observed that at a residence time of 15 min, an enzyme concentration of 0.294 FPU/ml and both substrate concentrations of 25 g/L and 50 g/L, the highest POS yield and productivity were achieved (59.89 % w/w and 14 g/l/h at 25 g/L; 57.76 % w/w and 21 g/l/h at 50 g/L) (See Chapter 6).

The POS fractions collected from the seven performed reactor tests were analyzed by HILIC/ESI-MS for their structure and composition. Further attention was then focused on the composition of POS mixtures produced at the two best hydrolysis conditions (above mentioned).

7.3.1 Analysis of pectic oligosaccharides by HILIC/ESI-MS

A HILIC column coupled to an electrospray (ESI) mass spectrometer, with negative ionization mode, was used in order to identify pectic oligosaccharides on the basis of retention time features, molecular mass and in source fragmentation (the same method as used in chapter 3 and 4 was used to analyse POS).

Fig.7.1 shows an example of the full scan chromatogram obtained. The molecular mass of the most abundant ions in the mass spectra were compared with the compounds already identified and reported in literature (Korner *et al,* 1999; Quemener *et al*, 2004). Moreover, basing on the pectin composition, the occurrence of homogalacturonan was expected in the produced POS fractions; therefore, possible combinations of the uronic acids with methyl groups or acetyl groups were considered as possibly present. Fig.7.2 shows an example of the identification.

Polygalacturonans with a degree of polymerization from 2 to 8 in both free and/or methylated/acetylated forms were detected, consistently with oligomers identified in pectic hydrolysates produced by batch system in our previous work (Babbar *et al,* 2016). The methylated forms were found to be more abundant than the acetylated

ones in agreement with Müller-Maatsch *at al* (2016), who measured a methylation degree of 19% and acetylation degree of 2% for the galacturonic acid extracted from onion hulls.

The identified oligomers are reported in Table 7.2 with the corresponding ratio m/z and retention time.

Given the unavailability of standards due to the vast variety of possible oligosaccharides structures, a semi-quantification was performed by performing an extract ion chromatogram (XIC) and integrating the corresponding peak area. Thus, a comparison of the relative amount of each compound can be done among all the samples, but not among different process due to possible different instrument response at different analysis time.

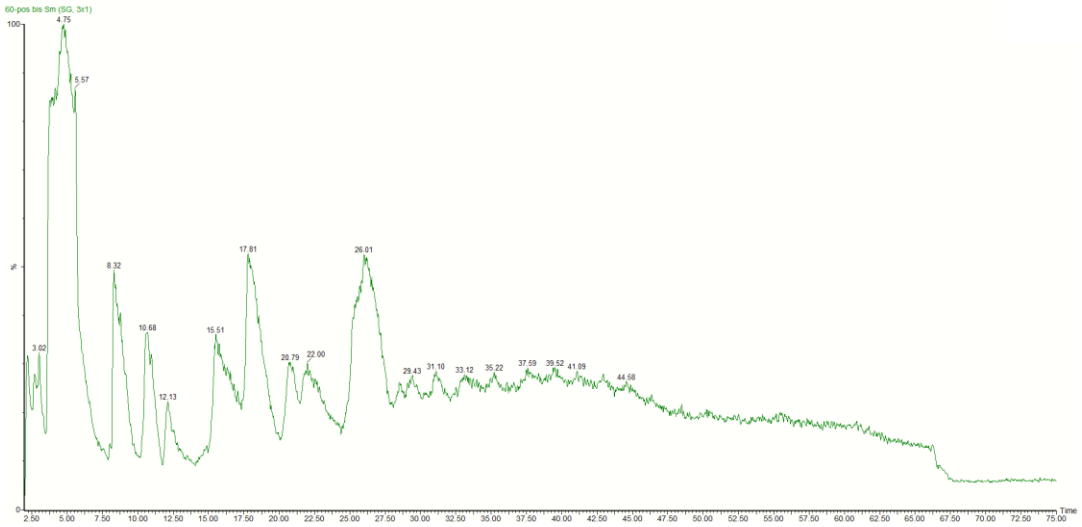


Figure 7.1: Total Ion Chromatogram (ESI-) of a pectin oligomer sample.

Figure 7.2: ESI-Mass spectrum of the polygalacturonan GalA3.

Table 7.2. Pectin oligosaccharides identified by HILIC/ESI-MS analysis.

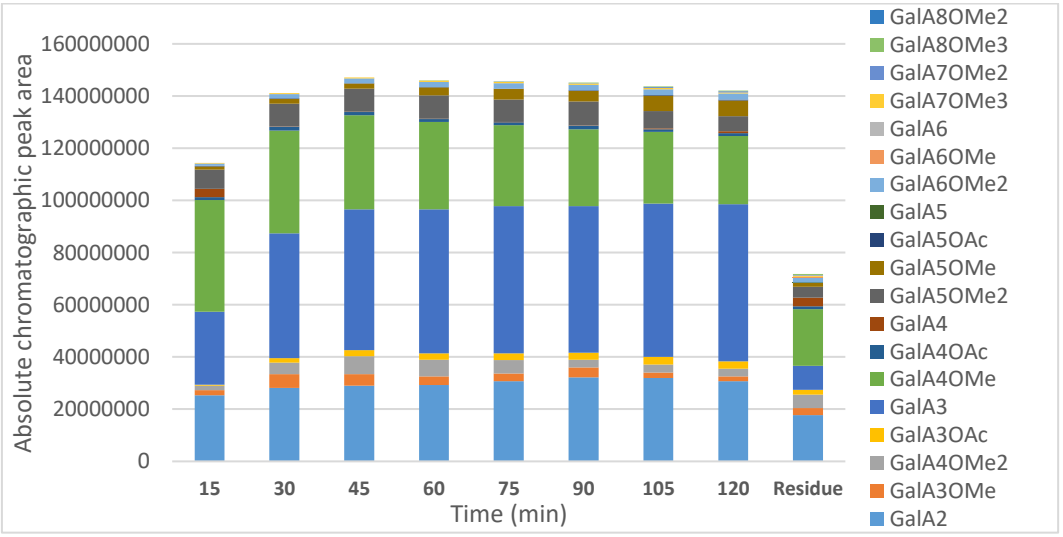
Compound		ID	MW (Da)	Trace (m/z)	RT (min)
DP2	**GalA2**	●●	370	369.3	27.92
DP3	**GalA3OMe**	●●●OMe	560	559.4	29.89
	GalA3OAc	●●●●OAc	588	587.4	33.64
	GalA3	●●●	546	545.4	37.88
DP4	**GalA4OMe2**	●●●OMe2	750	749.4	33.35
	GalA4OMe	●●●OMe	736	735.5	41.19
	GalA4OAc	●●●●OAc	764	763.6	42.99
	GalA4	●●●●	722	721.5	45.99
DP5	**GalA5OMe2**	●●●●●OMe2	926	925.5	42.22
	GalA5OMe	●●●●●OMe	912	911.6	47.98
	GalA5OAc	●●●●●OAc	940	939.5	48.97
	GalA5	●●●●●	898	897.6	53.36
DP6	**GalA6OMe2**	●●●●●OMe2	1102	1101.5	49.82
	GalA6OMe	●●●●●OMe	1088	1087.5	54.59

	Compound	ID	MW (Da)	Trace (m/z)	RT (min)
	GalA6	●●●●●●	1074	1073.5	55
DP7	**GalA7OMe3**	●●●●●●●OMe3	1292	1291.5	51.59
	GalA7OMe2	●●●●●●●OMe2	1278	1277.5	56.58
DP8	**GalA8OMe3**	●●●●●●●●OMe3	1468	1467.5	57.57
	GalA8OMe2	●●●●●●●●OMe2	1454	1453.5	63

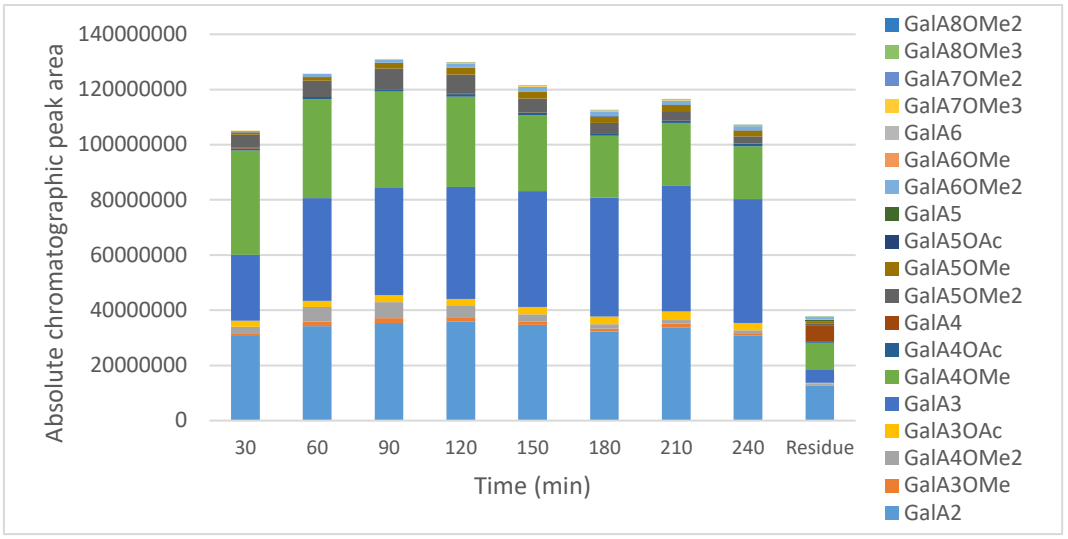
● = galacturonic acid, OMe/OAc = methylation/acetylation, DP=degree of polymerization.

7.3.2 POS fractions composition related to the production conditions

The chemical structure and composition of the analysed POS fractions were studied at the different production conditions. Fig. 7.3 reports the absolute chromatographic peak area of the identified compounds in each fraction, which can be permeate or residue, collected at each process condition.



(a)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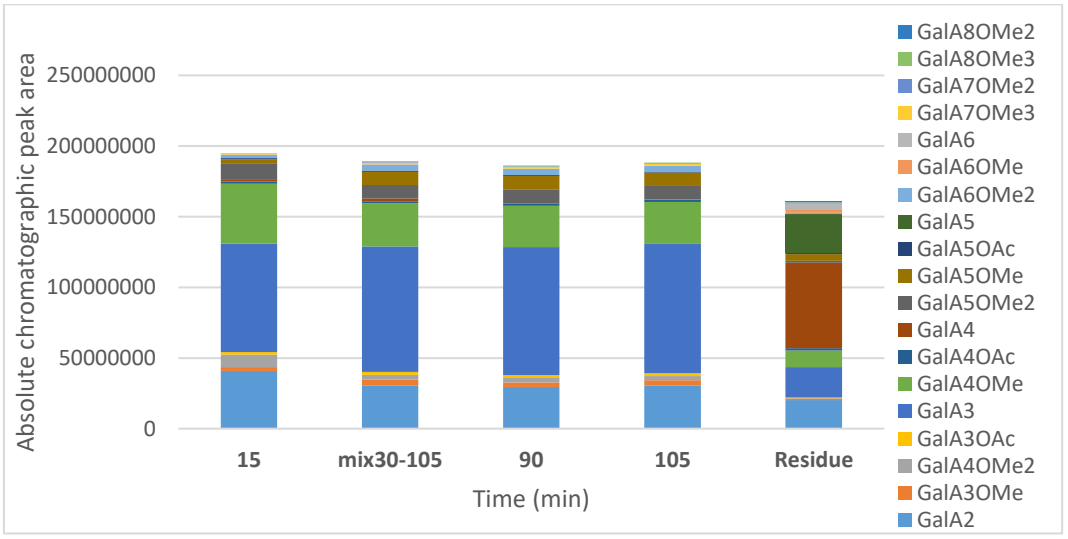
120000000
100000000
80000000
60000000
40000000
20000000
0
30
60
90
120
150
180
210
240
Residue
Time (min)
GalA8OMe2
GalA8OMe3
GalA7OMe2
GalA7OMe3
GalA6
GalA6OMe
GalA6OMe2
GalA5
GalA5OAc
GalA5OMe
GalA5OMe2
GalA4
GalA4OAc
GalA4OMe
GalA3
GalA3OAc
GalA4OMe2
GalA3OMe
GalA2

(b)


Absolute chromatographic peak area
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200000000
150000000
100000000
50000000
0
15
mix30-105
90
105
Residue
Time (min)
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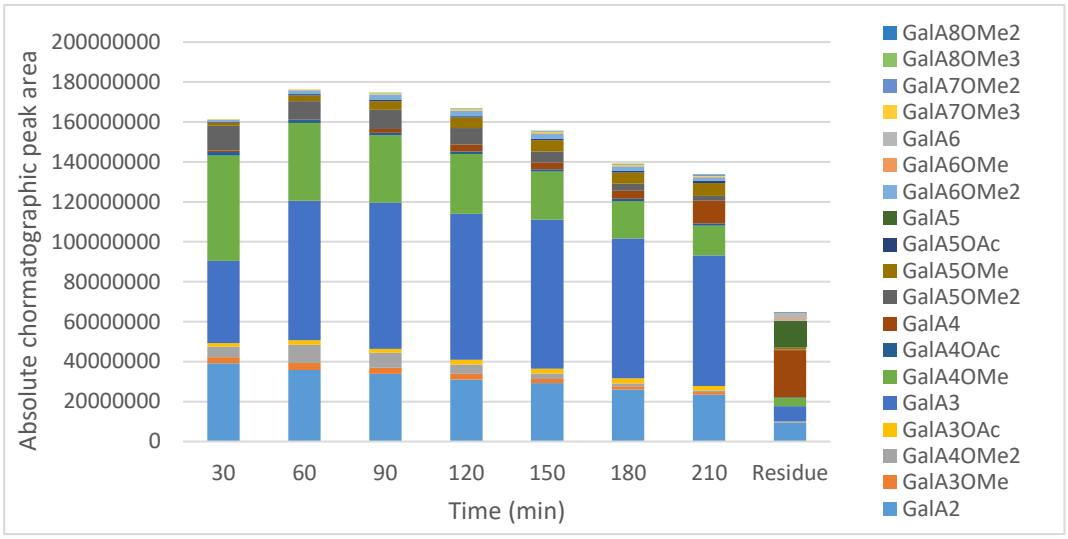
GalA8OMe3
GalA7OMe2
GalA7OMe3
GalA6
GalA6OMe
GalA6OMe2
GalA5
GalA5OAc
GalA5OMe
GalA5OMe2
GalA4
GalA4OAc
GalA4OMe
GalA3
GalA3OAc
GalA4OMe2
GalA3OMe
GalA2

(c)


Absolute chormatographic peak area
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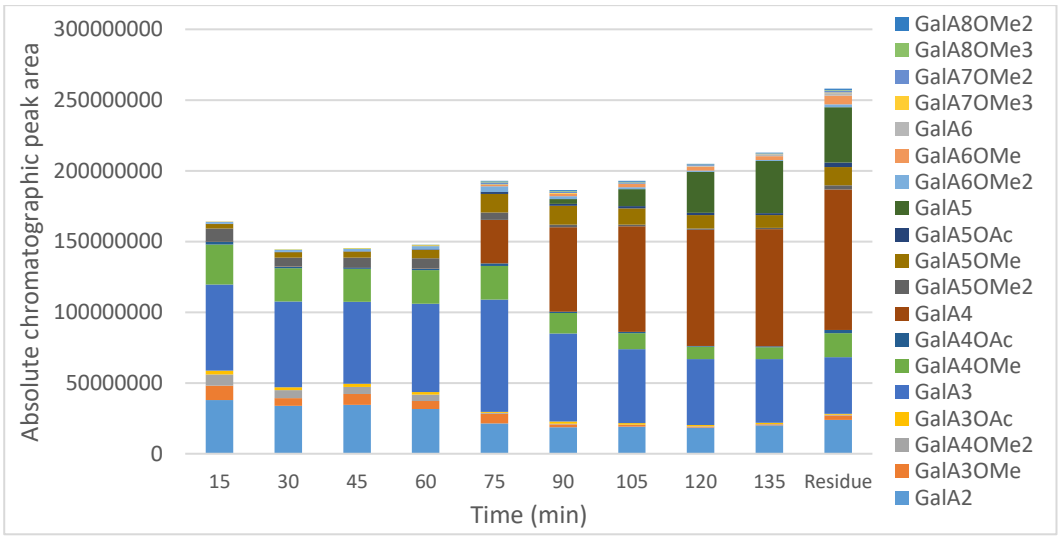
160000000
140000000
120000000
100000000
80000000
60000000
40000000
20000000
0
30
60
90
120
150
180
210
Residue
Time (min)
GalA8OMe2
GalA8OMe3
GalA7OMe2
GalA7OMe3
GalA6
GalA6OMe
GalA6OMe2
GalA5
GalA5OAc
GalA5OMe
GalA5OMe2
GalA4
GalA4OAc
GalA4OMe
GalA3
GalA3OAc
GalA4OMe2
GalA3OMe
GalA2

(d)


Absolute chromatographic peak area
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200000000
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100000000
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0
15
30
45
60
75
90
105
120
135
Residue
Time (min)
GalA8OMe2
GalA8OMe3
GalA7OMe2
GalA7OMe3
GalA6
GalA6OMe
GalA6OMe2
GalA5
GalA5OAc
GalA5OMe
GalA5OMe2
GalA4
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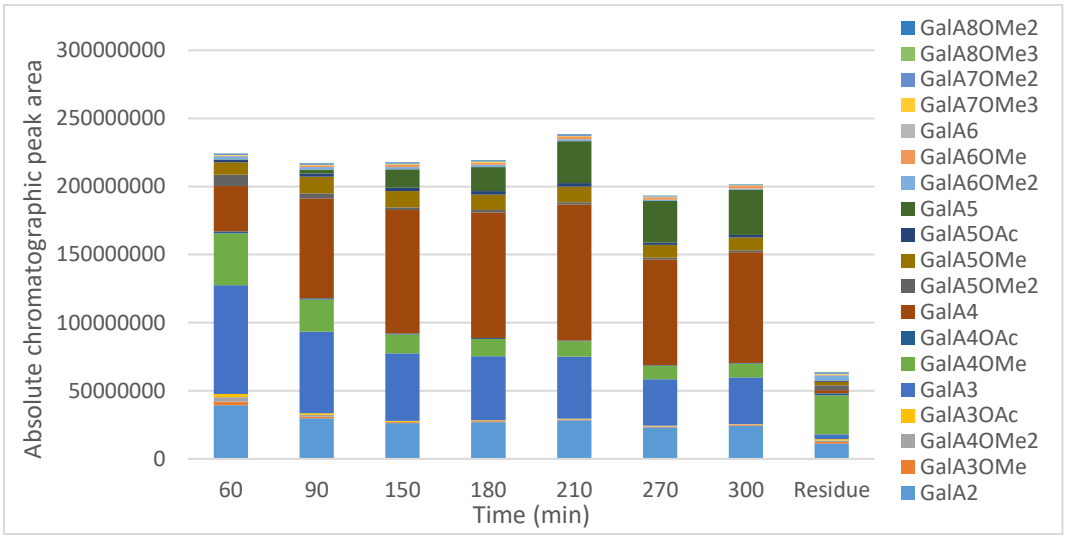
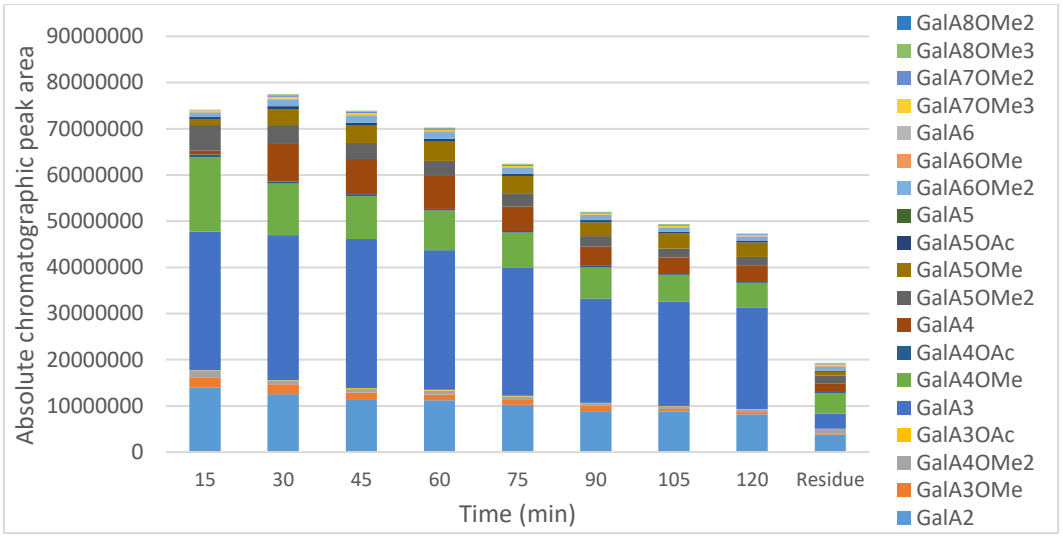
GalA4OMe
GalA3
GalA3OAc
GalA4OMe2
GalA3OMe
GalA2

(e)



(f)



(g)

Figure 7.3: Absolute chromatographic peak area of identified compounds in each POS fraction collected at the following conditions: 25 g/L, 0,588 FPU/ml and RT 15 (a) or RT 30 (b); 25 g/l, 0,294 FPU/ml and RT15 (c) or RT 30 (d); 25 g/L, 0,147 FPU/ml and RT 15 (e) or RT 30 (f); 50 g/L, 0.294 FPU/ml and RT15 (g). Residue: retentate collected from the EMR at the end of the process and subjected to enzyme deactivation.

By looking at the stability of POS production in function of time, it is evident that a high to average enzyme concentration for a short residence time was the best combination (Fig 7.3a and 7.3c), which is in agreement with what observed in terms of POS yield. At the same enzyme concentration, extending the residence time to 30 min resulted in a somehow less stable POS production (Fig. 7.3b and 7.3d). At the low enzyme concentration (Fig. 7.3e and 7.3f) or at a very high substrate concentration Fig 7.3g (which lead to low enzyme/substrate ratio), the production became even more unstable.

In the first processes the composition of the POS mixtures was also quite stable, with permeates rich in short POS such as GalA2, GalA3 and GalA4OMe, while the residue showed an abundance of the longer oligomers GalA4 and GalA5. In the lower enzyme concentration, (Fig 7.3e and 7.3f), also the composition of the permeates was markedly shifted towards a higher percentage of this longer oligomers, likely due to a lesser efficiency of the enzyme in cutting them.

As said above, the substrate concentration seemed not to influence particularly the POS mixtures composition. Indeed, the composition of permeates collected at the two substrate concentrations 25 g/L and 50 g/L (Fig.7.3 c and g respectively) was almost the same. However, a slight difference of trend in function of hydrolysis time was noted, since using the lower substrate concentration the process seemed to be more stable in function of time, indicating that in order to have a stable production in terms of yield and composition a higher enzyme/substrate ratio is more favourable.

7.3.3 POS fraction composition: influence of substrate concentration

The two conditions that provided a more stable production of POS in function of process time (basing on the results from the previous section and on the observations made in Chapter 6 as well), i.e. 15 min residence time, enzyme

concentration of 0.294 FPU/ml and both substrate concentrations 25 g/L and 50 g/L, were studied in more detail in their molecular composition.

The tables 7.3 and 7.4 report the relative abundance of all the oligomers identified at both substrates concentrations during the process, in function of the time (at a time interval of 15 min). The percentage was calculated on the total amount of all identified oligosaccharides in each fraction.

A certain variability in the distribution of each component at each stage of the process was noted. In general, the most abundant identified compounds were GalA3, GalA2, GalA4OMe and GalA4, confirming the results obtained in the preliminary experiments and reported in the previous section.

Indeed, it can be noted that the differences in composition were quite small between the two conditions. GalA3 showed the highest abundance among the POS products analyzed and accounted for about 40-48% at both conditions. It increased ($p<0.05$) at the beginning of the process, especially at 25 g/L, but it was stably produced at longer production times. As for GalA2, the behavior was different, in the sense that it first decreased ($p<0.05$) before reaching a stable production after 30 min. The effect was more evident at a low substrate loading of 25 g/L.

Among the most abundant oligomers observed, the largest differences between the two substrate loadings were noticed for GalA4OMe and GalA4. In fact, the first one showed a significant decrease ($p<0.05$) after 30 min for both processes. Moreover, around 10% of GalA4 was produced using a concentration of 50 g/L, whereas it accumulated in the residue (37.60%) at 25 g/L, being most probably further hydrolyzed into GalA3 and GalA2 in the permeates.

Depending on the condition tested, POS production was stable in terms of composition and amount among the collected permeates. However, basing on the statistical analysis of POS amount, it was noted that using the high substrate concentration 50 g/L, the production was slightly more stable than at 25 g/L. Probably, the enzyme can reach the steady state more easily using the high

concentrated substrate; this can be observed also comparing the permeate at 15 min and the permeate at 30 min: at 25 g/L there was a significant difference ($p<0.05$) for most of the oligomers, especially for GaA2, GalA3 and GalA4OMe, indicating that the hydrolysis process took time to stabilize, whereas at 50 g/L, except for GalA4OMe and GalA4, only slight differences were noted between the two permeates.

In general, the abundance in the residues of long POS could be due to the fact that the longer is the oligosaccharide, the less it is its chance to end up in the permeate, particularly if also shorter oligosaccharides are simultaneously present.

In particular, the residue collected at 25 g/l was rich in GalA4 (37.60%) and GalA5 (16.64%), which were almost not present in the permeates. Probably, these two oligosaccharides represent a product of the enzymatic hydrolysis and a further substrate for it, being converted in shorter oligos such as GalA2 and GalA3, which, on the contrary, were abundant in POS fractions, being more easily filtrated.

In case of residue collected at 50 g/L, an accumulation of GalA4OMe (23.27%) was also noted, as compared to the amount found in the permeate (about 12%); moreover, oligomers with DP5-6 were also more abundant than in residue collected at 25 g/L. This composition could be due to the higher substrate concentration, which lead to a lesser digestion of the pectic chain by the enzyme.

These trends can be compared with those one observed producing POS in batch system (Chapter 5). The batch production does not provide the possibility to separate the produced POS from the hydrolysis environment, hence they can be further hydrolyzed to monosaccharides (Chapter 5). In fact, the free galacturonic acid increased from 3,1 % after 5 min to 6,4 % after 120 min, due to the exo-activity of the enzyme. On the other hand, a decrease in function of time was noted for the DP2-DP4 forms.

On the contrary, the increase and the stable production of POS in function of time observed in the present study is due to the use of the enzyme membrane reactor,

which allowed to remove the POS as soon as they are formed, thus preventing their further degradation.

In regards to the composition of POS produced in the steady state of the two process, the Fig.7.4 and Fig.7.5 show the distribution of the free forms and of the substituted forms with different DP. As expected, the higher is the degree of polymerization, the higher is the chance to find the methylated form, because of the increased statistical chance to find a methyl group if more sugar units are present; indeed, POS with DP from 5 to 8 were mainly produced in the methylated form.

Table 7.3: Relative abundance (%) of the oligomers identified in POS fractions and residue collected at the process condition RT15, 0.294 FPU/ml and 25 g/L.

Compound		15 min	Dev Std	mix 30-105 min	Dev Std	90 min	Dev Std	105 min	Dev Std	Residue	Dev std
DP2	GalA2	20.93	0.52	16.17	0.51	15.83	0.25	16.26	0.64	12.52	1.20
DP3	GalA3OMe	1.45	0.18	2.11	0.33	1.71	0.01	1.99	0.42	0.69	0.40
	GalA3OAc	1.01	0.08	1.04	0.05	1.06	0.04	1.09	0.02	0.30	0.15
	GalA3	39.44	0.91	46.93	0.06	48.64	0.78	48.55	0.98	13.40	0.15
DP4	GalA4OMe2	4.47	0.07	1.84	0.30	1.77	0.00	1.48	0.02	0.23	3.01
	GalA4OMe	21.65	0.21	16.14	0.13	15.70	0.84	15.83	0.23	7.31	0.05
	GalA4OAc	0.82	0.06	0.78	0.07	0.73	0.00	0.79	0.11	0.93	0.28
	GalA4	0.58	0.04	1.02	0.30	0.19	0.04	0.15	0.04	37.60	0.32
DP5	GalA5OMe2	5.83	0.27	5.03	0.20	5.20	0.03	5.06	0.01	0.77	1.29
	GalA5OMe	1.69	0.08	4.95	0.28	5.22	0.04	5.09	0.13	3.05	0.26
	GalA5OAc	0.42	0.02	0.42	0.04	0.36	0.01	0.33	0.01	0.88	0.01
	GalA5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.64	0.16
DP6	GalA6OMe2	1.29	0.08	2.33	0.13	2.41	0.08	2.22	0.19	0.60	0.36
	GalA6OMe	0.00	0.00	0.07	0.01	0.00	0.00	0.00	0.00	1.61	0.04
	GalA6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.55	0.13
DP7	GalA7OMe3	0.39	0.01	0.59	0.03	0.58	0.02	0.61	0.05	0.12	0.13
	GalA7OMe2	0.04	0.00	0.34	0.01	0.32	0.00	0.31	0.01	0.38	0.00
DP8	GalA8OMe3	0.00	0.00	0.27	0.03	0.30	0.01	0.29	0.05	0.15	0.03
	GalA8OMe2	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.32	0.02
Sum of POS		100.00	-	100.00	-	100.00	-	100.00	-	100.00	-

Table 7.4: Relative abundance (%) of the oligomers identified in POS fractions and residue collected at the process conditions RT15, 0.294 FPU/ml and 50 g/L.

	Compound	15 min	Dev Std	30 min	Dev Std	45 min	Dev Std	60 min	Dev Std	75 min	Dev std	90 min	Dev Std	105 min	Dev Std	120 min	Dev Std	Residue	Dev Std
DP2	GalA2	18.91	0.31	16.19	0.57	15.30	0.14	15.99	0.08	16.41	0.61	16.96	0.76	17.76	0.23	17.30	0.21	19.55	1.14
DP3	GalA3OMe	2.79	0.98	2.77	0.38	2.16	0.24	1.77	0.03	1.66	0.12	2.30	0.86	1.14	0.10	1.01	0.09	1.68	0.58
	GalA3OAc	0.14	0.04	0.14	0.00	0.23	0.01	0.23	0.03	0.23	0.04	0.20	0.14	0.17	0.00	0.20	0.07	0.31	0.08
	GalA3	40.51	0.48	40.55	2.12	43.69	0.12	43.03	0.13	44.56	0.24	43.38	0.39	45.87	2.33	46.56	1.01	17.03	1.23
DP4	GalA4OMe2	2.11	0.26	1.03	0.00	1.01	0.12	1.20	0.14	1.20	0.02	1.04	0.51	1.14	0.02	1.06	0.04	4.60	0.59
	GalA4OMe	21.70	2.69	14.40	0.04	12.59	0.14	12.18	0.40	12.11	0.16	13.00	0.28	11.30	0.11	11.02	0.55	23.27	1.33
	GalA4OAc	0.75	0.03	0.59	0.11	0.53	0.03	0.51	0.05	0.55	0.07	0.63	0.04	0.59	0.01	0.58	0.07	0.93	0.15
	GalA4	1.14	0.04	10.47	0.78	10.34	0.41	10.14	0.41	8.40	0.52	8.02	0.41	7.33	1.17	7.81	0.67	9.74	0.41
DP5	GalA5OMe2	7.51	1.20	5.28	0.88	4.67	0.19	4.68	0.11	4.72	0.05	4.31	0.20	3.90	0.13	3.81	0.04	8.52	0.11
	GalA5OMe	1.77	0.12	4.48	0.56	5.02	0.12	5.89	0.10	5.95	0.05	5.82	0.06	6.62	0.31	6.61	0.28	4.44	0.07
	GalA5OAc	0.52	0.07	0.79	0.08	0.81	0.05	0.85	0.00	0.76	0.02	0.86	0.15	0.77	0.10	0.75	0.01	1.05	0.17
	GalA5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DP6	GalA6OMe2	1.42	0.05	1.87	0.06	2.05	0.17	2.10	0.03	2.02	0.13	2.16	0.30	1.96	0.20	1.93	0.10	4.73	0.46
	GalA6OMe	0.05	0.01	0.26	0.05	0.27	0.04	0.24	0.03	0.23	0.01	0.17	0.02	0.21	0.05	0.20	0.03	0.68	0.06
	GalA6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
DP7	GalA7OMe3	0.50	0.05	0.50	0.06	0.53	0.04	0.42	0.04	0.39	0.01	0.41	0.05	0.47	0.06	0.40	0.04	1.64	0.31
	GalA7OMe2	0.09	0.01	0.43	0.01	0.51	0.10	0.49	0.02	0.54	0.01	0.49	0.01	0.55	0.09	0.50	0.04	1.00	0.09
DP8	GalA8OMe3	0.09	0.02	0.26	0.03	0.29	0.02	0.26	0.01	0.27	0.03	0.26	0.01	0.24	0.03	0.26	0.02	0.82	0.05
	GalA8OMe2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sum of POS		100.00	-	100.00	-	100.00	-	100.00	-	100.00	-	100.00	-	100.00	-	100.00	-	100.00	-

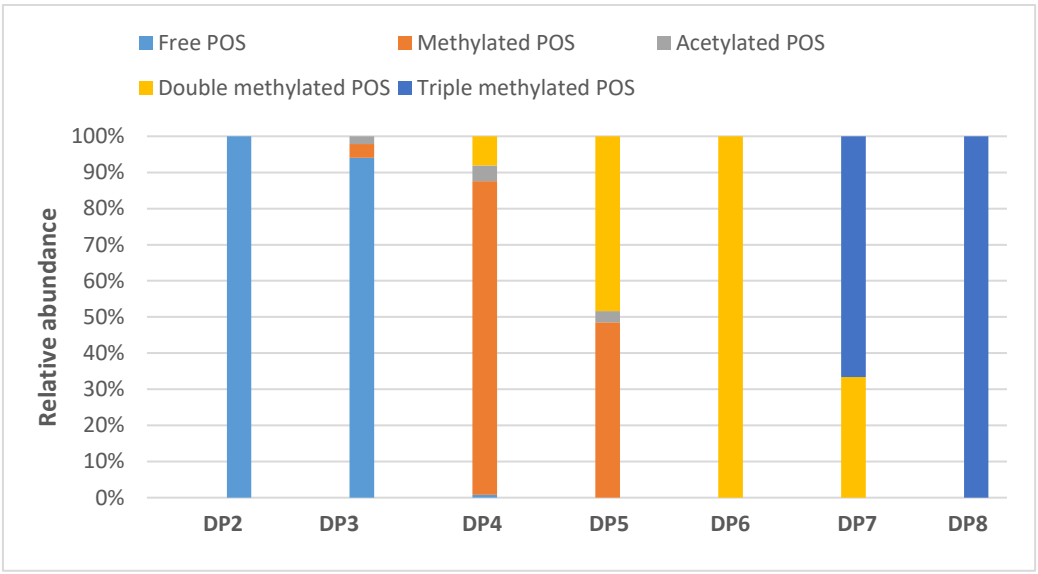


Figure 7.4: Relative abundance of free, methylated, acetylated, double methylated and triple methylated POS in the steady state at the condition RT15, 0.294 FPU/ml and 25 g/L.

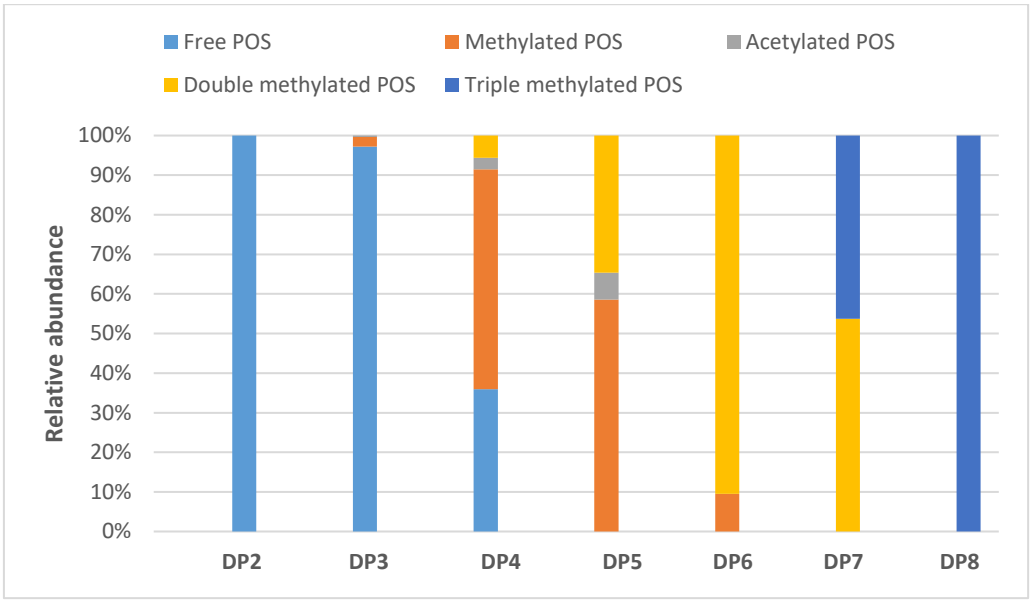


Figure 7.5: Relative abundance of free, methylated, acetylated, double methylated and triple methylated POS in the steady state at the condition RT15, 0.294 FPU/ml and 50 g/L.

7.3.4 Study of prebiotic activity on POS mixtures

The POS fraction permeates collected at the two most promising process conditions (i.e. RT15, 0.294 FPU/ml and 25 g/L or 50 g/L), were mixed all together, given their similar composition, and the resulting mixtures were assessed on their ability to simulate the growth of lactic acid bacteria. They were supplemented (or not, in the control experiment) to growth media suitable for efficient growth of each bacterial strain investigated. After supplementation, the growth medium was inoculated with the bacterial strain and transferred to glass cells equipped with four electrodes to which an electrical current was applied in the BacTrac 4300®. At regular intervals, the impedance was measured and plotted as function of time. As the microorganisms grew, the instrument measured the impedance changes as compared to its initial value expressed as E% (impedance of the electrodes) (Bancalari *et al*, 2016). The time required to reach the chosen threshold level, is referred as the time to detection (TTD). Changes in the time to detection between value obtained with the strain grown in supplemented media and value obtained with the same strain grown without supplement (control), were expressed as follows: $\Delta TTD = TTD^{NC} - TTD^{POSX\%}$,where TTD is the time to detection; NC is the negative control prepared by inoculating bacterial culture in the not supplemented media; POS X% represents the supplemented media with 1% of pectin derived-oligosaccharides. Positive results represent a growth promotion, negative an inhibition and zero no effect.

Fig.7.6 shows the impedance curves registered for the bacterial strains investigated. In particular, the curves registered for negative control (NC), for positive control (PC) and for the two POS mixtures (RT15,0.294 FPU/ml, 25 g/L, denoted as “Sample 1”, and RT15,0.294 FPU/ml, 50 g/L, denoted as “Sample2”) are shown in each graph for each strain (a-i).

The ΔTTD calculated for all bacterial strain and for both POS mixtures had negative values (data not shown). In fact, looking at the impedance curves, it can be seen that

the growth of *Lactobacilli* strains was slowed in presence of POS mixtures in growth medium compared to NC and PC, especially for mixture from RT15, 0.294 FPU/ml, 25 g/L and especially for *L.casei, L.acidophilus* and *L. delbrueckii* subsp *lactis,* whose growth was slower than other strains. The *E.coli* pathogen strains were also inhibited.

The inhibition effect of the two samples on *Lactobacillus* strains can depend on the POS mixtures composition. In fact, the SHMP, used for extracting pectin from onion skins, is present together with POS in the mixtures obtained by EMR (having a molecular mass of 611.77 Da) and it can have had such effect on the bacterial growth. Since it is used in the extraction process at a concentration of 2% w/v and it is then further concentrated after the freeze-drying, it ends up in the POS fractions, and giving his chelating effect it might have a detrimental impact on bacterila growth.

In order to test this hypothesis, the SHMP effect on the bacterial growth was assessed with pure SHMP supplemented to the growth medium at the concentration that it was supposed to be present in POS mixtures.

The growth of all *Lactobacillus* strains and of *E.coli* strains was again monitored in function of time in Bactrac. It was observed that bacteria were not able to grow in presence of SHMP, since it was not possible to register any impedance curve in any case (data not shown).

This result clearly indicated that SHMP can have an inhibition effect . Indeed, being a chelator, the SHMP could disturb the essential metal metabolism of the microorganism by interfering with metal acquisition and bioavailability for crucial reactions. (Santos *et al*, 2012)

In order to assess which is the real effect of POS mixtures on the bacterial growth, then the SHMP should be removed from the samples collected along the production process. In this way, it could be possible to investigate more in depth the potential prebiotic effect of POS from onion skins.

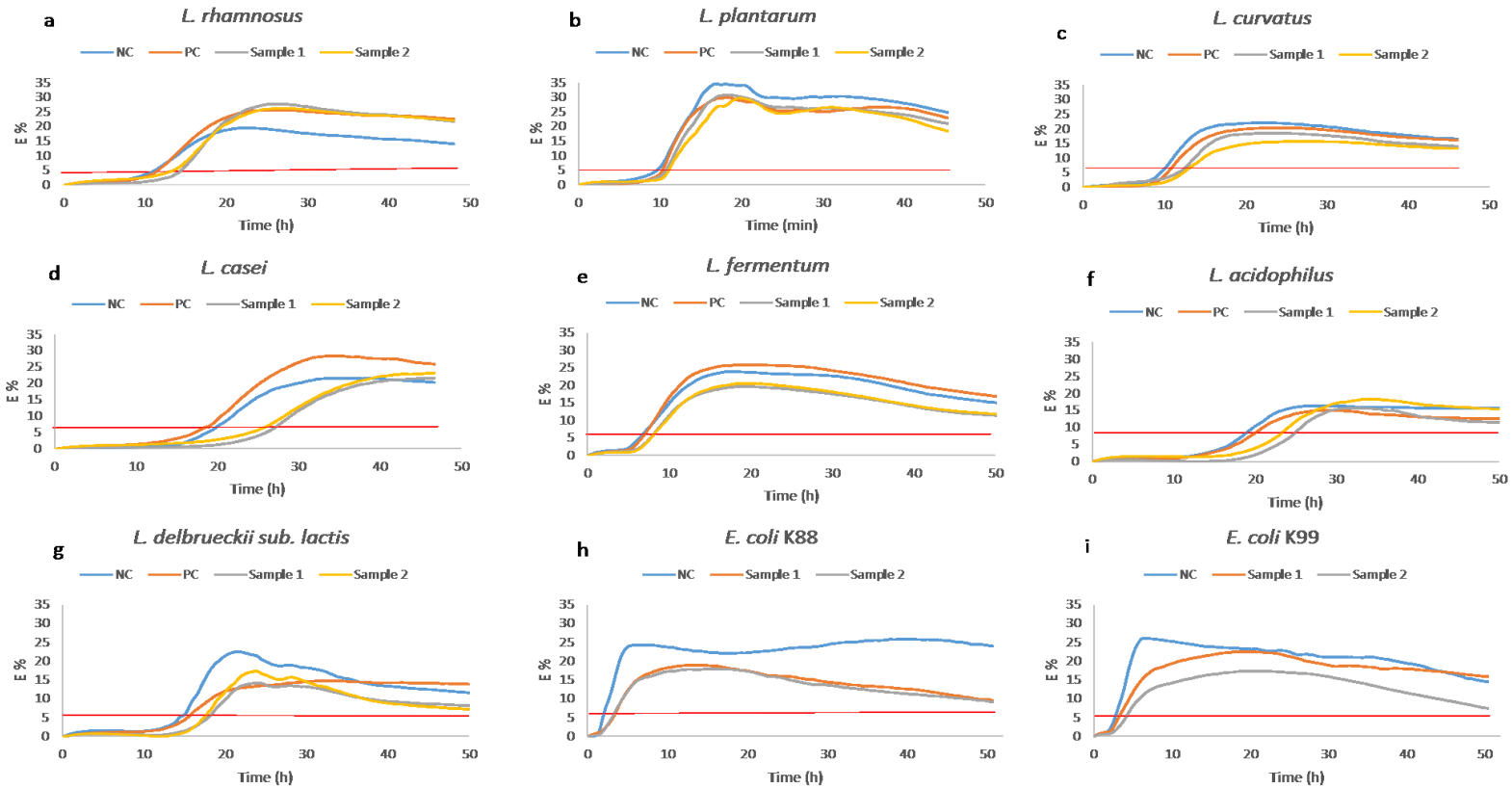


Figure 7.6: Impedance curves registered in the BacTrac 4300 ® for seven *Lactobacillus* strains and two *E.coli* strains on medium not supplemented (negative control, NC), on medium supplemented with FOS (positive control, PC) and on medium supplemented with the two samples under investigation (sample 1=POS mixture from RT15-0.294FPU/ml-25g/L; sample 2= POS mixture from RT15-0.294FPU/ml-50g/L). Red line indicates the threshold level, fixed at E=5%.

7.4 Conclusions

POS fractions obtained from onion skins by hydrolysis in an enzymatic membrane reactor were characterized in detail as far as the molecular composition is concerned. HILIC/ESI-MS analysis provided a clear indication on the POS composition, especially in terms of degree of polymerization and acetylation/methylation profile, also indicating which were the most abundant oligomers among the samples. Therefore, it was possible to find a relation between the molecular composition of the POS preparations and the technology used to obtain them. Changing the hydrolysis conditions allowed to modify the composition of the final products. The data also allowed to get indications on the best conditions, in terms of enzyme and substrate concentration, and residence time, in order to achieve a efficient and stable POS production.

POS production were also tested for their ability to stimulate that the growth of *Lactobacillus* strains, but the presence of SHMP, used for the extraction, in the POS mitxures, did not allow to achieve clear indications on this ability, since it was found to inhibit bacterial growth. Thus, SHMP is to be removed from the preparations before testing them for their prebiotic potential.

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8. General Conclusions and future perspectives

The huge amount of food wastes and by-products produced every year by the European food sector represents an underutilized resource of many valuable compounds. Indeed, the food waste could be re-used for many applications, including feed and feed additives, of course complying with the existing legislation and the economical viability of the process.

The present thesis dealt with the use of pectin obtained from food waste, studying first the amount and the composition of pectin in animal feed derived from food waste, and, then dealing with the production and the characterization of potential prebiotic pectic oligosaccharides (POS) from selected by-products.

Pectin represent a heterogeneous carbohydrate found in the cell wall of higher plants; it is characterized by a very complex structure and a very high molecular weight (20-400KDa). Pectin is mainly made by “smooth” regions, made by galacturonic acid units and known as homogalacturonan, and “hairy” regions, known as rhamnogalacturonans, in which the galacturonic acid is interspersed with rhamnose residues linked to neutral sugars side chains, such arabinose, galactose, xylose, ect. Also, this acidic polymer is classified as soluble fiber, but differently from other carbohydrates such as cellulose, hemicellulose, ect, it can exert a negative effect on health. In fact, due to its gelling properties, it can reduce the absorption of nutrients, reduce the transit time and interfere with the metabolism of ions and drugs.

Vegetables and wastes studied in the present thesis were intended to be treated and converted into animal feed; given the antinutritional value that the high molecular weight pectin could exert on animal health, their content was assessed in the matrices (bulk feed, feed ingredients and complete feed) produced starting from food waste. It was shown that it is possible to produce feed with low pectin content also starting from food waste having high pectin content, throught

the application of suitable technologies or formulations, at the same time also preserving a high amount of total fiber content

Although pectin as intact polymer has an antinutritional value, the oligosaccharides derived from it have the potential to be prebiotics. In fact, the pectic oligosaccharides are reported to be able to enhance the growth of bifidogenic bacteria, such as *Bifidobacteria* or *Lactobacillus*, promoting several health benefits. For this reason, the research of new source of POS is increasing, given the contribution that such compounds could give to the prebiotics market, also and mostly including prebiotic to be used in animal feeding. Indeed, natural compounds able to enhance the well being of animal gut microflora are of great interest, given the economic advantage coming from more healthy and productive animals and the less use of veterinary drugs. Anyway, many unknown factors are still to be investigated in the field of POS production: the economical viability of the process production, its productivity and its efficiency, the way to tailor POS composition, the structure of the POSs obtained and the link between these structures and their potential prebiotic properties.

The present thesis dealt with all these subjects, deeply investigating the production of POS from sugar beet pulp and onion skins, and the properties and the composition of the POS mixtures obtained.

Among the strategies available to hydrolyze pectin to obtain pectic oligosaccharides, the enzymatic digestion was preferred due to the specific activity and the minimum degrading effect on the polymer if compared, for instance, to acids. Batch systems are commonly used to perform this kind of enzymatic hydrolysis; however, batch systems have productivity and efficiency problems, as well as a limited control on the reaction outcome. Thus, in this thesis, an innovative technology was used for the production of POS, i.e. the enzyme membrane reactor (EMR). Scanty information on the production and almost no literature on the continuous production of pectic oligosaccharides was available,

but indeed this technology provided a very efficient way to achieve an enzymatic tailoring of long pectic chain to smaller POS.

The main disadvantage of using commercial enzymes having specific endo-activity (Endo-polygalacturonase, Pectinase, ect) is the high cost of the enzyme. Therefore, as an alternative, the low cost Viscozyme was chosen, which is mixture of enzymes having diverse activities. However, the fact that the exo-activity of such mixture leads to the generation of (undesirable) monosaccharides represent a drawback for its use. Therefore, the ambition of the study was also to use the EMR for the continuous hydrolysis of pectin allowing at the same time the separation of the products in the moment they are produced; in this way, the conversion into monosaccharides could be avoided and Viscozyme could be a suitable choice.

Sugar beet pulp (SBP) is a commonly used as pectin-rich source and the crude pectic extract obtained from it has been already used as substrate of the enzymatic hydrolysis. In this thesis, the POS fractions obtained from the optimization of the hydrolysis of SBP pectin, were charaterized for their POS content at a very detailed level, also allowing to link the composition of the mixture with the technology used. The HILIC/ESI-MS analysis of the POS fractions showed the presence of POS with degree of polymerization (DP) from 2 to 7, especially arabinans, rhamnogalacturonans and polygalacturonans. The molecular characterization gave the chance to investigate the production process of pectic oligosaccharides in terms of chemical pretreatment of the waste, the molecular weight cut-off (MWCO) of the EMR membranes, the enzyme concentration, residence time, (contact time between substrate and enzyme in EMR) and the substrate concentration. All these parameters were optimized according to the composition of the mixtures obtained, allowing to link the molecular composition to the technology used. Furthermore, the potential prebiotic activity of the produced POS mixtures were investigated by studying

their ability to stimulate the growth of lactic acid bacteria (the most important probiotic bacteria in pigs, and also very important in humans). The study showed evidence that POS can stimulate the growth of bacterial strains *in vitro*, although with a composition- and strain-specificity. Indeed, one of the POS preparations, showed a marked stimulating activity, probably due to highest POS abundance and specific POS composition rich in arabinans and polygalacturonans. Therefore, POS can then be considered a promising product which might have potential prebiotic properties, even if the real prebiotic potential will have to be studied in future studies in vivo. Quite interestingly, no one of the POS fractions did show stimulation activity on pathogen strains. Unlike sugar beet pulp, which is a relatively commonly used source of pectin, a yet unexploited source of pectin, i.e. onion skins waste, was also investigated for the production of pectic oligosaccharides. In a previous work, the treatment with the chelating agent sodium hexamethaphosphate (SHMP) was found to be the best suitable extraction method for producing a crude pectic extract from onion skins causing minimal damage to pectic polysaccharides.

In this thesis, a preliminary study on the production of POS from onion skins was conducted in a lab scale batch system, using Endo-polygalacturonase M2, since onion skins pectin is mostly made by homogalacturonan. The HPAEC-PAD analysis and the HILIC/ESI-MS analysis of onion hulls digests revealed the production of oligosaccharides with DP from 1-10 at various degree of acetylation and methylation.

The continuous production of POS from onion skins was also tested using cross flow membrane reactor process. In the research it was opted to use the cheaper enzyme complex Viscozyme with multiple activities, instead of the more expensive but also more specific Endo-polygalacturonase M2. It was aimed to tailor the products by the integrated use of a membrane.

The process was again optimized in terms of residence time, enzyme concentration and substrate concentration, allowing to achieve high and stable POS productivity, the high POS yield and the low monosaccharide formation.

Again, HILIC/ESI-MS analysis allowed to gain information on the detailed molecular composition of the obtained POS fractions, which were mostly made by polygalacturonans, with DP from 2 to 8. Looking at the relative abundance of the various POS in the mixtures, it was also possible to investigate the stability of the process in function of time. Therefore, it was possible to find a relation between the molecular composition of the POS preparations and the technology used to obtain them, allowing to tailor the hydrolysis conditions in order to modify the composition of the final products.

Even if further studies would be needed in order to elucidate the effect of the oligosaccharide according to the concentration and to the specific bacterial strains, this thesis showed that oligomers with a high level of functionality can be produced by enzymatic membrane reactor starting from food by-products, a finding that has deeper implications also for their potential use in human supplementation. The results of this thesis clearly demonstrated that an economically viable, efficient and controllable production of POS is possible starting from food wastes and food by-products, allowing to obtain functional compounds which can potentially be used as additive and integrators for food and feed.

Curriculum Vitae

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Studies

Stefania Baldassarre got the Master degree in Industrial Biotechnology (University of Parma, Italy) in November 2013 with a thesis titled "*The use of the high resolution mass spectrometry technique, DESI-MS, for a pharmaceutical application: the study of the drug-polymer interaction in controlled release systems"*. Her master degree's thesis focused on the use of the mass spectrometry to study the possible interactions between polymer and a model drug in controlled released system. The work was conducted at the Department of Pharmacy, University of Parma for a period of 6 months (2013).

In January 2014, Stefania Baldassarre started the PhD in Food Science (University of Parma, Italy), under the supervision of the Prof. Stefano Sforza and Dr. Kathy Elst. The PhD work has been characterized by a strict collaboration between Department of Food Science (University of Parma, Italy) and the Flemish Institute for Technological Research, VITO (Belgium) were she spent seven months optimizing a cross flow enzyme membrane reactor process for the

continuous production of pectic oligosaccharides from onion skins waste. Her doctoral research aimed at the characterization of pectin extracted from food wastes for feed applications; moreover, it aimed at the production and characterization of potential prebiotic pectic oligosaccharides. She used analytical methods such as mass spectrometry, anion exchange chromatography, gas-chromatography and impedometric technique. She has been also involved in the European project NOSHAN dealing with the characterization of food waste.

Scientific activity

Original papers

Pectic oligosaccharides from onion skins wastes: continuous production and separation by using a membrane enzymatic bioreactor.
Baldassarre S., Babbar N., Van Roy S, Dejonghe W., Maesen M., Sforza S.and Elst K.
Bioresource Technology (In preparation)

Pectic oligosaccharides (POS) from sugar beet pulp as functional compounds: molecular characterization and prebiotic activity.
Baldassarre S., Prandi B., Babbar N., Gatti M., Elst K. and Sforza S.
Food & Function (In preparation)

Enzymatic production of pectic oligosaccharides from onion skins.
Babbar, N., Baldassarre, S., Maesen, M., Prandi, B., Dejonghe, W., Sforza, S., Elst, K.
Carbohydrate Polymers, 2016, 146, 245-252.

Oral Communications at National and International congresses containing results achieved during the PhD Course

Production, characterization and prebiotic potential of pectic oligosaccharides (POS) from onion hulls waste stream.

Stefania Baldassarre, Neha Babbar, Sandra Van Roy, Barbara Prandi, Monica Gatti, Kathy Elst, Stefano Sforza.

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Molecular characterization of pectic oligosaccharides (POS) derived from agro-industrial by-products.

Stefania Baldassarre, Barbara Prandi, Neha Babbar, Sandra Van Roy, Monica Gatti, Kathy Elst, Stefano Sforza

7th International Symposium on Recent Advances in Food Analysis, RAFA, November, 3-6th, 2015. Prague, Czech Republic. ISBN 978-80-7080-934-1, pag.149.

Poster Communications at National and International congresses containing results achieved during the PhD Course

Production, characterization and prebiotic potential of pectic oligosaccharides (POS) from onion hulls waste stream.
Stefania Baldassarre, Neha Babbar, Sandra Van Roy, Barbara Prandi, Monica Gatti, Kathy Elst, Stefano Sforza
IFIB2016, Italian Forum on Industrial Biotechnology and Bioeconomy. September, 22-23th, 2016. Vicenza, Italy.

Composition of pectins from food waste to be used in bulk feed and as feed additives.
Stefania Baldassarre, Barbara Prandi, Marcela Santarelli, Neha Babbar, Kathy Elst, Monica Gatti, Stefano Sforza
16th European Congress on Biotechnology. July, 13-16th, 2014. EICC, Edinburgh, Scotland. Journal of New Biotechnology, New Biotechnology, · Volume 31S, pag. S212, http://dx.doi.org/10.1016/j.nbt.2014.05.995.

Participation to PhD schools, workshop, courses and project meetings

Workshop in "*Risk assessment and Communication in Food Safety and Nutrition*". University of Parma, Italy. October, 6th, 2016.

21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, Portici, Italy, September, 14-16th, 2016.

Various project meetings attended in Belgium:

- *Noshan Annual Meeting, Bruxelles, Belgium, January, 14-15th,2016.*
- *Noshan Annual Meeting, ILVO-Gent, Belgium: July, 08th,2015.*

Workshop on *Leftover, Byproducts and Food waste: New business and development opportunities for the agricultural productions*. Fiere Parma CIBUSTEC, October, 29th, 2014.

Scientific Writing Training Course, Parma Italy, October, 6-10th, 2014.

XIX Workshop on the developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Bari, Italy, Semptember, 24-26th, 2014.

Workshop: Core Shell columns: SunShell Technology Food and Pharma Applications, Unoversity of Parma, Italy, September, 8th, 2014.

Advanced course on the coupling of mass spectrometry with separation techniques in liquid phase (LC/MS analysis). Lucca, Complesso San Micheletto, June 2014.

Plenary meeting of the Panel on Genetically Modified Organisms (GMO), EFSA, April, 9-10th, 2014.