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DEVELOPMENT OF INTEGRATED
BIOPROCESSING TECHNOLOGIES FOR
THE PRODUCTION OF PECTIC
OLIGOSACCHARIDES (POS) FROM
AGRO-PROCESSING RESIDUES

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PREFACE

This thesis is submitted in fulfillment of the requirements for the Doctorate (PhD) degree at the University of Parma, Italy. The work presents in this thesis was conducted during my PhD study at the Department of Separation and Conversion Technology, VITO-Flemish Institute for Technological Research, Belgium and university of Parma, Italy. For completing this study I am most grateful to Dr. Kathy Elst and Prof Stefano Sforza for their supervision and continuous support throughout this study. I also acknowledge the work supported by the European commission (FP7, NOSHAN, contract no. 312140). The PhD study was financed by a scholarship grant from VITO and University of Parma, Italy. Most of the experiments were performed at Enzyme Laboratory and analytical laboratory at VITO. I acknowledge Sandra Van Roy for her support and guidance with the continuous cross flow reactor set up and Mrs. Miranda Maesen for regular guidance with HPAEC-PAD and other analytical instruments. For completing this study, I am also grateful to Dr Winnie Dejonghe, for the valuable guidance and comments.

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LIST OF ABBREVIATIONS

Abn1	Arabinohydrolase I
Abn2	Arabinohydrolase II
Abn3	Arabinohydrolase III
AOS	Arabino-oligosaccharides
AP	Apiogalacturon
<i>Araf</i>	Arabinofuranoside
BP	Berry pomace
D _{Ac}	Degree of acetylation
DM	Degree of methylation
DP	Degree of polymerization
DP1	Galacturonic acid
DP2	Di-galacturonic acid
DP3	Tri-galacturonic acid
DP4	Tetra-galacturonic acid
EPG-M2	Endo-polygalacturonase M2
EAE	Enzyme assisted extraction
EMR	Enzyme membrane reactor
FOS	Fructo-oligosaccharides
FPU	Filter paper units
<i>GalpA</i>	Galacturonic acid
GalAOS	Galacturonic acid oligosaccharides
GalA3OMe	Galacturonic acid-3-O-methylated
GalA3Oac	Galacturonic acid-3-O-Acetylated
GalA4	Tetragalacturonic acid
GalA4Ome	Galacturonic acid-4-O-Methylated
GalA4Oac	Galacturonic acid-4-O-Acetylated
GalA5Ome	Galacturonic acid-5-O-Methylated
GalA5Oac	Galacturonic acid-5-O-Acetylated
GalA5Ome	Galacturonic acid-5-O-di-Methylated.
GalA5	Pentagalacturonic acid
GC	Gas chromatography
GIT	Gastro-intestinal tract
GOS	Galacto-oligosaccharides
GPC	Gel permeation chromatography
HPAEC	High performance anion exchange chromatography-pulsed amperometric detection
HG	Homogalacturonan
HILIC	Hydrophilic Interaction Liquid Chromatography
HMF	Hydroxy methyl furfural
HPLC	High performance liquid chromatography
IU	International units
kDa	Kilo Dalton
LSD	Least significant difference
Mono	Monosaccharides
MW	Molecular weight
NAE	Nitric acid assisted
ND	Not detectable
OH	Onion hulls

Oligosaccharides	Oligos
POS	Pectic oligosaccharides
Poly	Polysaccharide
PP	Pressed pumpkin
RSM	Response surface methodology
RT	Retention time
RG	Rhamnogalacturonan
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
Rhap	Rhamnose
SBP	Sugarbeet pulp
SCFA	Short chain fatty acid
SEC	Size exclusion chromatography
SHMP	Sodium hexa meta phosphate
UPLC	Ultra performance liquid chromatography
XG	Xylogalacturonan
XOS	Xylooligosaccharides

ABSTRACT

This research deals with the production of pectic oligosaccharides (POS) from agro-industrial residues, with specific focus on development of continuous cross flow enzyme membrane reactor. Pectic oligosaccharides have recently gained attention due to their prebiotic activity. Lack of information on the continuous production of POS from agro-industrial residues formed the basis for the present study. Four residues *i.e* sugar beet pulp, onion hulls, pressed pumpkin cake and berry pomace were taken to study their pectin content. Based on the presence of higher galacturonic acid and arabinose (both homogalacturonan and rhamnogalacturonan) in sugar beet pulp and galacturonic acid (only homogalacturonan) in onion hulls, further optimization of different extraction methods of pectin (causing minimum damage to pectic chain) from these residues were done. The most suitable extractant for sugar beet pulp and onion hulls were nitric acid and sodium hexametaphosphate respectively. Further the experiments on the continuous production of POS from sugar beet pulp in an enzyme membrane reactor was initiated. Several optimization experiments indicated the optimum enzyme (Viscozyme) as well as feed concentration (25 g/L) to be used for producing POS from sugar beet pulp in an enzyme membrane reactor. The results highlighted that steady state POS production with volumetric and specific productivity of 22g/L/h and 11 g/gE/h respectively could be achieved by continuous cross flow filtration of sugar beet pulp pectic extract over 10 kDa membrane at residence time of 20 min. The POS yield of about 80% could be achieved using above conditions.

Also, in this thesis preliminary experiments on the production and characterization of POS from onion hulls were conducted. The results revealed that the most suitable enzyme for POS production from onion hulls is endo-polygalacturonase M2. The POS produced from onion hulls were present in the form of DP1 -DP10 in substituted as well as unsubstituted

forms. This study clearly demonstrates that continuous production of POS from pectin rich sources can be achieved by using cross flow continuous enzyme membrane reactor.

OBJECTIVES AND OUTLINE

The goal of this PhD was to develop a continuous cross flow enzyme membrane reactor for the production of pectic oligosaccharides (POS) from agro-industrial residues. The major objective for using enzyme membrane reactor was to separate the POS fractions by means of a membrane, so that they are not further tailored to monosaccharides and at the same time maintaining a continuous feed supply in order to replace the washed POS fractions.

In Chapter 1, a general overview on the production of POS from various agro-industrial residues is presented. This overview makes clear that how use of different enzymes can effect POS production. Also, a summary on the most common processes used for separating the POS is given.

So, the first step of the PhD project was started with optimizing the various parameters like particle size, enzyme concentration and time on the production of POS from sugar beet pulp using response surface methodology in a one step process i.e directly from the sugar beet pulp (Chapter 2). Although, interesting results were obtained from these experiments. But, since the final aim was to produce POS in an enzyme membrane reactor, it was realized that with one step POS production, the membrane could easily foul.

Then a second approach using two-step process *i.e.* (i) extraction of crude pectin and (ii) production of POS was conducted. In order to carry out these experiments, it was necessary to understand the damage caused to pectin during various extraction processes (Chapter 3). So the process causing minimum damage to pectin chain was selected. Out of four residues *i.e.* sugar beet pulp, berry pomace, pressed pumpkin cake and onion hulls, sugar beet pulp and onion hulls were selected (based on pectin content) for further production of POS using nitric acid and sodium hexametphospahte as extractants respectively (Chapter 3).

In Chapter 4, the production of POS using crude pectic extract from sugar beet pulp in an enzyme membrane reactor was optimized. Feedback from various experiments done in

batch indicated the suitable enzyme concentration of 82.7 U/mL (viscozyme -PG units) to be used for POS production from crude pectic extract of sugar beet pulp in an enzyme membrane reactor. Both semi-continuous (flushing with water) and continuous (pumping with feed) were evaluated in order to produce POS of high quality with lesser monosaccharides and higher POS productivities. Continuous feeding of diluted substrate (25 g/L) at retention time of 20 min was the most optimum for continuous POS production from sugar beet pulp. Using these parameters the POS production could be continuously operated for 36 h.

The production of POS from onion hulls was evaluated in Chapter 5. POS production from onion hulls was initiated using different enzymes in shake flask experiments. Further the characterization of POS using LC-MS analysis revealed the presence of free as well substituted form of oligomers from onion hulls.

Finally, the general conclusions and future perspectives are presented in Chapter 6.

1. GENERAL INTRODUCTION

Based on: Neha Babbar, Winnie Dejonghe, Monica Gatti, Stefano Sforza and Elst Kathy (2014). Pectic oligosaccharides from agricultural by-products: production, characterization and health benefits. Critical reviews in Biotechnology, DOI: 10.3109/07388551.2014.996732.

1.1 PECTIN AND PECTIC OLIGOSACCHARIDES (POS)

Pectin is a complex and heterogeneous polysaccharide present within the primary cell wall and intercellular regions of higher plants.¹ Pectin comprises a family of acidic polymers, known as homogalacturonan (HG) and rhamnogalacturonan (RG) with several neutral sugars/polymers such as arabinans, galactans and arabinogalactans (attached as side chains).^{2,3} The extraction of these neutral and acidic polymers in the form of pectic oligosaccharide (POS) is a promising step towards the manufacture of prebiotics from agricultural by-products.^{4,5} Pectic oligosaccharides (POS) are non-digestible oligosaccharides which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*Bifidobacteria* and *Lactobacilli*).⁶⁻¹¹ Pectic oligosaccharides have been reported to suppress the activity of entero-putrefactive and pathogenic organism.⁶⁻¹¹ The colonic fermentation of prebiotic POS results in the generation of short chain fatty acids (SCFA), which exerts a number of health effects like 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.¹² Literature also suggests that POS can act as phytoalexin elicitor, flowering inducer and antibacterial agent in plants.¹³

Agricultural by-products have been studied extensively for bioethanol production, enzyme production, protein enriched cattle feed.¹⁴⁻¹⁸ Some agricultural by-products like apple pomace, sugar beet pulp, berry pomace also contain significant amount of pectin.^{5,19} The production of POS from these agricultural residues is an interesting way to reuse waste streams for both environmental and economic benefits. The most common and well known

POS are arabinogalacto-oligosaccharides, arabinoxylo-oligosaccharides, arabinooligosaccharides, galacto-oligosaccharides, oligo-galactouronides, rhamnogalacturonan-oligosaccharides.^{20, 21}

1.2 NATURE AND CHEMISTRY

1.2.1 Pectin

Pectin is a complex macromolecule made up of several monosaccharides containing diverse linkages. The structure of pectin is hypothesized to be made up of “smooth” homogalacturonic (polygalacturonic acid) and branched “hairy” rhamnogalacturonic regions (in which most of the neutral sugars are located).²² A schematic representation of the structure of pectin and the constituent sugar in each region is presented in Fig 1.1. Four main pectic components have been identified namely, homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II) and xylogalacturonan (XG).²²⁻²⁶ All these pectic components are connected by either covalent or ionic cross links.²⁷ The most abundant pectic polysaccharide HG, is made of galacturonic acid (Galp) residues with α -1,4-linkages and comprises more than 65% of pectin.²² It can be partly methyl-esterified at C-6 and possibly partly acetyl esterified at O-2 and O-3.²⁸

The RGI backbone is composed of [\rightarrow 2)- α -L-Rhap- (1 \rightarrow 4)- α -D-GalpA-(\rightarrow 1] repeats.⁴ Rhamnose is a minor component of the pectin backbone and introduces a kink into the straight chain. The length and quantity of HG and RG-I components can vary in different plants¹². For instance, in sugar beet pectin, the length of HG polymer is shorter than that of pectin of citrus and apple. On the other hand, RG-I is abundant in sugar beet pulp than in citrus and apple.¹² Rhamnogalacturonan I has number of side chains in the form of sugars and branched oligosaccharides attached to its backbone.²⁹ The length of these side chains can vary from single neutral glycosyl to polymeric side chains of different types viz. (1 \rightarrow 5)- α -L-arabinans, (1 \rightarrow 4)- β -D-galactans, arabinogalactans-I, arabinogalactans-II².

Rhamnogalacturonan II is a structurally complex pectin and accounts for more than 10% of pectin.³⁰ The building blocks of RG-II are galacturonic acid, rhamnose, galactose and unusual neutral sugars. The structure of RG-II is characterized as a distinct region within HG that contains cluster of side chains of rare sugar residues, such as apiose, aceric acid, 3-deoxy-lyxo-2 heptuloasarinic (DHA) and 3-deoxy-manno-2-octulosonic acid.³¹ Xylo-galacturonan (XGA) is a substituted HG with a single unit of β -D-Xylp-(1 \rightarrow 3) side chain.²⁴ The presence of XGA has been mainly identified in reproductive organs or storage tissues such as in cell walls of peas, soybeans, watermelons, apples, pears, onions, potatoes, pine pollen, and cotton seed.^{31,32}

[4)- α GalA-(1,4)- α GalA-(1,4)- α GalA-(1,4)- α GalA-(1,4)- α GalA-(1,4)- α GalA-(1,4)- α GalA-(1,)]_n

Homogalacturonan (HG)

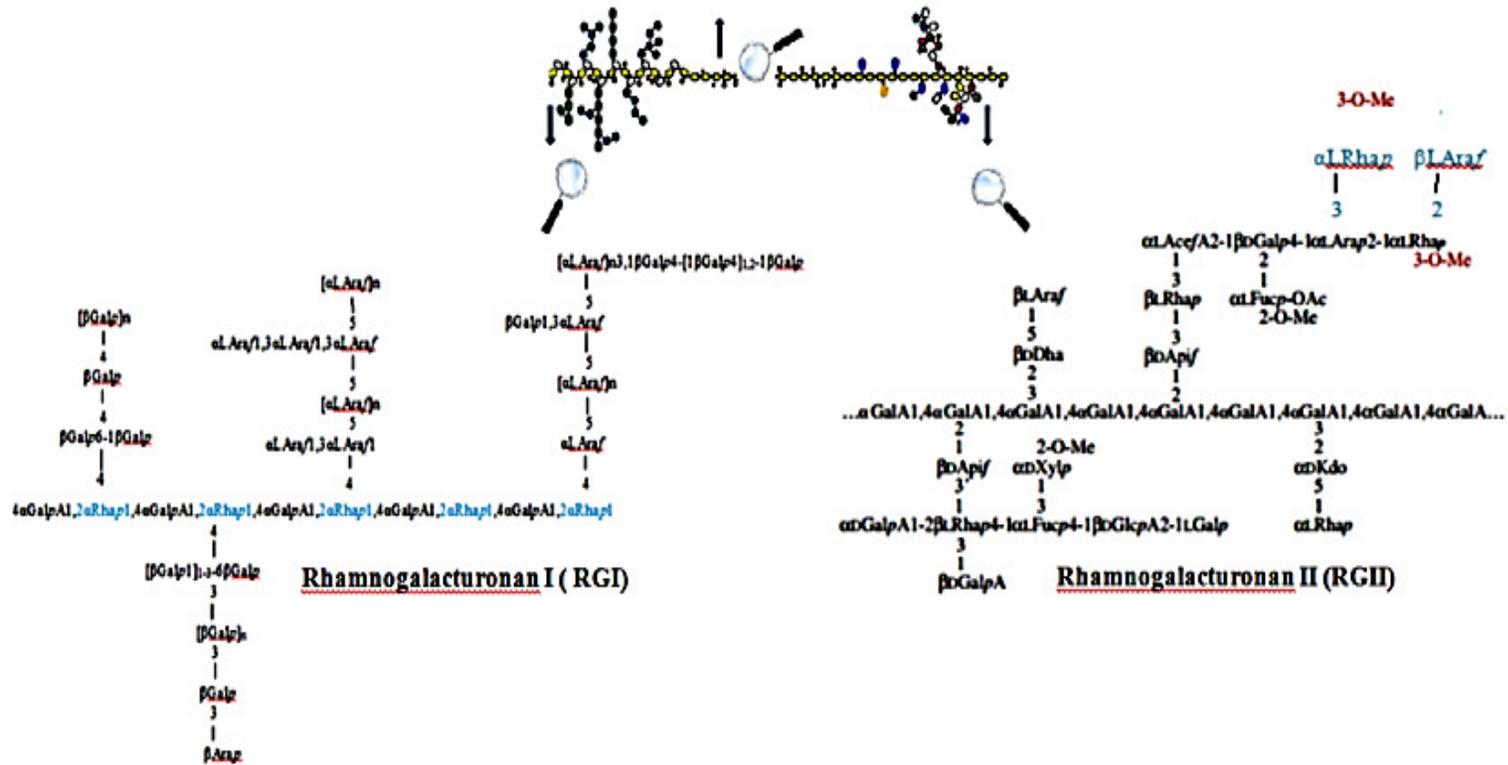


Fig 1.1: Structure of pectic polysaccharide

1.2.2 Pectin Complexes

There are three type of pectin complexes *viz.* HG calcium, RG-II borate and uronyl ester complex. The HG calcium complex is formed by two unesterified HG chain, whereby the carboxyl groups of two GalpA residues form a negatively charged pocket that binds with a Ca^{2+} cation. Minimum of ten continuous unesterified GalpA residues are needed to build a stable cross-link between the chains.³³ Calcium crosslinking of HG contributes to the cell wall strength by bringing blocks of unmethylesterified HG chains into a tightly packed conformation.³⁴ The second pectin crosslink is known as the borate diol ester (RG-II borate). This is formed by two RG-II molecules with boron. Only the apiofuranosyl residues of the 2-O-methyl-D-xylose containing side chains in each subunits of the dimer can participate in the cross-linking. Borate-diol esters can also crosslink two HG chains as RG-II is an integral part of HG chain. Cations such as Ca^{2+} Pb^{2+} , Sr^{2+} and La^{3+} promote dimer formation *in-vitro*.³⁴ Homogalacturonan can also cross-link to other components by uronyl ester. Approximately 2% of GalpA residues can be cross-linked this way. Homogalacturonan are mainly found in plant cell walls in a methyl-esterified form and it is clear that these molecules hold an enormous potential for cross-linking.³⁴

1.3. POTENTIAL SOURCES OF PECTIC OLIGOSACCHARIDES

Pectin containing by-products in addition to their conventional uses, can also be exploited for POS production. Table 1.1 illustrates the quantity of pectin rich agricultural by-products produced in Europe along with the content of HG and RG sugars. Further, Table 1.2 gives a detail insight of the pectin content as well as acidic and neutral sugar composition of important agricultural by-products.

1.3.1 Olive (*Olea europaea*) pomace

Olive pomace is the by-product of olive oil processing. Spain is the leading producer of olive oil in the world. The production of olive oil generates huge quantities of olive pomace. Cell wall material of olive pomace comprises of a number of molecular components, with considerable quantities of pectic polysaccharides (39%), cellulose (30%), hemicellulosic polymers rich in xylans and glucuronoxylans (14%), xyloglucans (15%) and mannans (2%).^{35,36} The pectic polysaccharides of olive pomace are unique due to presence of arabinan. The degree of methylesterification and acetylation in olive pomace was determined to be 48 and 11 % respectively by Cardoso et al which suggests high gelling properties of pectin.³⁷ Owing to high arabinan and galacturonic acid, olive pomace can be considered as a potential source of POS.^{5, 38, 39} Hydrothermal processing of olive pomace has been reported to produce tetra-, tri- and di-galacturonic acid and different structures of neutral and acidic xylo-oligosaccharides.⁵

1.3.2 Sugar beet (*Beta vulgaris*) pulp

Sugar beet pulp is a by-product of sugar refining industry and is used mostly as animal feed. It is combined with molasses and dried to give a high energy feed for ruminants. Sugar beet pulp polysaccharides consists approximately of 22-24 % cellulose, 30 % hemicellulose, 15-25 % pectin, 3 % ash and 5.9 % lignin.⁴⁰ Beet pulp contains low amounts of protein, lignin and fat. The combination of shorter HG chain length, high degree of acetylation and the higher concentration of side chains (containing neutral sugars) contribute to the poor gelling properties of sugar beet pectin. Production of POS from sugar beet pulp (SBP) has been successfully carried out by various researchers.^{21,41} Al Tamimi et al. isolated sugar beet arabinan (MW 5700 to 10000 D) and arabino-oligosaccharides from sugar beet pulp. Kuhnel et al. characterized branched arabino-oligosaccharides [having an α -(1,5)-linked backbone of L-arabinosyl residues] from sugar beet pulp produced by a mixture of arabinohydrolases.^{42,43}

1.3.3 Potato (*Solanum tuberosum*) pulp

Potato pulp is a by-product of the potato industry. Potato pulp consists of pectic polysaccharides (56%), starch (12%), proteins (5%), ash (4%) fat (0.3 %) and water (9%). The pectin of potato pulp contains high proportion of RG-I with long galactan side chains (75 %).^{44,45} Previous studies have reported successful extraction of HG and RG-I oligosaccharides from potato pulp.^{46,47} The RG-I in potato pulp contains complex arabinogalactan structure which can be converted to POS.²

1.3.4 Citrus Waste

Citrus fruits are the most important fruits grown and consumed all over the world.⁴⁸ The waste from orange juice processing industry ranges between 40-60% of the fruit weight and is made up of peel and segment membranes.⁴⁹ *Citrus reticulata*, an important tropical crop contains 10% cellulose, 4.28% hemicellulose, 0.56% lignin, 5.78% protein, 22.6 % pectin and 3.23% ash.⁵⁰ The presence of low lignin makes citrus by-product ideal for use in the area of bioprocessing. Some information on POS production has been reported in orange peel wastes.¹⁹ Cancalon found significant amounts of oligosaccharides of DP > 2 in naturally fermented citrus juices.⁵¹ The presence of transfructosidase activity (present in invertases) catalyzes the synthesis of various oligosaccharides during such fermentations. Kang et al., evaluated positive effects of POS produced by irradiation (10kGy/hr) from citrus pectin on levels of serum triglyceride, total cholesterol, and LDL-cholesterol in the blood of mice fed high-cholesterol diets.⁵²

1.3.5 Apple (*Malus domestica*) Pomace

Apple pomace a by-product of apple processing industry accounts for 25-35 % of the dry mass of apple.^{53,54} It contains 7 % protein, 1.4 % ash, 8.3 % pectin, 58.3 % neutral polysaccharides (cellulose and hemicellulose).²⁴ The residue of apple contains highly

branched RG and XG polysaccharide.⁵⁵ These polysaccharides can be further degraded to produce oligomers of desired chain length. Watt et al. and Renard et al. obtained fucogalactoxyloglucan oligosaccharides from apple pomace by alkaline pretreatment.^{53,56} Gullon et al., found 32-45 % of alcohol soluble compounds in apple pomace (inclusive of monosaccharides, oligosaccharides and malic acid).⁵⁷ Oligosaccharides were mainly present in the form of gluco-oligosaccharides, xylo-oligosacchrides and arabino-oligosaccharides.

1.3.6 Others

Oligo-galacturonic acid (DP 6-12) from tomato processing waste isolated by acid hydrolysis was found to be potent plant growth promoter.⁵⁸ Hydrolysis of Lucerene (*Medicago sativa*) led to the production of acidic oligosaccharides.⁵⁹ Montella et al., isolated galacto-oligosaccharides and xyloglucans from hazel nut skin by alkaline and water extraction.⁶⁰ Bilberries and black currants, an important crop in Scandinavian countries contains pectin. Due to formation of pectin gel after mashing, some pectinolytic enzymes are added to the mash to release the juice. After degradation, some polysaccharides remain in the mash in the form of RG-II which can be used for POS production.⁶¹ Zywinska et al., obtained POS of different molecular weight from chicory roots, citrus peel, cauliflower floret/ leaves and sugar beet pulp.⁶² Pectic oligomers obtained by hydrolyzing the soybean polysaccharides were of RG origin.⁶³ Cello-oligosaccharides (cellopentaose, cellotetraose, cellotriose, cellobiose) and galactooligosaccharides (galactotetraose, galactotriose) from carrot pomace were obtained after alkaline pretreatment.⁶⁴

Table 1.1: The pectin and side chain composition of various agro- industrial by-products

Source	Pectin content (%)	HG *	RG I	NSC	RG II	References
Citrus waste	30	77	5	4	0.3	19, 108
Apple pulp	20.9	36	1	47	10	24, 109
Sugarbeet pulp	16.2	29	4	48	4	22,24, 110
Olive pomace	34.4	-	-	38.8	-	35
Potato pulp	15	20	75	-	-	111

NSC- Neutral side chains
HG- Homogalacturonan
RG- Rhamnogalacturonan
*of total pectin

Table 1.2: Different pretreatment methods, quantification, identification and yield of POS from various agro-industrial residues.

Substrate	Pre-treatment		Quantification		of Yield	References
	Type	Conditions	Type	Method		
Alperujo*	Steam	15 min at 170 °C	PH (Acid) PH (Enz)	2N TFA at 121°C for 2h Olivex® at 40 °C, pH 5.0 for 24 h	23% (w/w) POS of total polysaccharides (3000-1000 Da)	5
Poly-galacturonic acid (model)	Enz (EPG-M2, Pectinase, Pectinex ultra SP-L, Pectinex 62 L, Macer 8 FJ)	35-40 °C, pH 3.8 - 5.0	Direct	Identification with Oligomer standards (di and tri PGA)	EPG-M2, 2h: 58% (w/w) DP3 18% (w/w) DP2 13% (w/w) DP1 of total polysaccharides	60
Sugarbeet pulp	Enz (Viscozyme L, Pectinase and combinations)	45 °C up to 48 h	PH (Acid)	1h, 30°C in 72% (w/w) H ₂ SO ₄ , followed by 3 h, 100 °C in 1M H ₂ SO ₄ .	-	37
Sugarbeet pulp	Hydrothermal	287 min at 160 °C; 357 min at 163 °C	PH (Enz)	Viscozyme L - 45U/g 40h, 37°C, pH 5	31.2% (w/w) POS 29.9% (w/w) POS of oven dried SBP	20
Orange peel	Hydrothermal	288 min at 160 °C	PH (Enz)	Viscozyme L - 45U/g, Cellulase - 5 FPU/g	25.1% (w/w) POS of oven dried raw material.	19
Pectin (apple,	Acid (HCl 0.1M)	72 h at 80 °C	Direct	45h, 37 °C, pH 5 MW assessment by	DP 6-20	112

sugar beet and citrus)				Oligogalacturonides and maltodextrins of 980-1630 D	
			PH (Acid)	3h, 120 °C in 2M TFA	
XG, PGA, Apple MHR	Enz (XG: XG hydrolase, 16 h at 37 °C PGA: EP, apple MHR: RG hydrolase)		Direct	- **	113
SugarbeetArabina n	Enzymatic (Araf, Abn and mixtures)	-	PH (Acid)	1h, 30 °C in 72% (w/w) H ₂ SO ₄ followed by 3h, 100 °C in 1M H ₂ SO ₄ ;	Mixtures of DP 1- 6 depending on conditions
			Direct	Quantification by arabinose oligomers	39

Araf- Arabinofuranosidase

Abn- Arabinohydrolase

AOS- Arabino-oligosaccharides

Direct- Direct quantification/ identification of the oligomers present in the mixture.

DHPM- Dynamic high pressure microfluidization.

DP- Degree of polymerization

EA- Endo-Arabinase

Enz- Enzymatic

EP- Endopolygalacturonase

EXA- Exo-Arabinase

GO- Gluco-oligosaccharides

GalO- Galacto-oligosaccharides

GalacidO- Galacturonic acid oligosaccharides

HNO₃- Nitric acid

MHR- Modified hairy regions

PH- Post hydrolysis (Quantification of the additional monomers formed by post hydrolyzing the mixture).

POS- Pectic oligosaccharide

PGA- Polygalacturonic acid

RG- Rhamnogalacturonase

SBP-Sugarbeet pulp

XG- Xylogalacturonan

*Semi-solid by-product of virgin olive oil processing

** Structural information with MS

1.4 PROCESSES FOR THE PRODUCTION OF PECTIC OLIGOSACCHARIDES

Pectic polysaccharides are covalently cross linked and therefore certain pretreatment is required to separate HG, RG-I and RG-II from each other. Pectic oligosaccharides can be obtained by depolymerization of suitable raw materials by different pretreatment methods *viz.* enzymatic, chemical and physical.^{1,20,65,66} Table 1.3 comprehensively covers different pretreatment approaches for the extraction of POS from different agricultural by-products.

Table 1.3: Effect of POS obtained from different agro-residues on health

Source/ Type of POS	Effective DP	<i>in vivo</i> / <i>in vitro</i> effects	References
*** Sugarbeet	2-10 and 7-14	* <i>Bifidiobacterium adolescentis</i> , <i>Bifidobacterium longum</i> , <i>Bacteroides vulgatus</i>	38, 72, 86
Orange peel	-	* <i>Bifidiobacterium</i> and <i>Eubacterium rectale</i>	9
Acidic (galacturonic acid) POS	-	Improved immune response	95, 115
Acidic POS	-	Reduced allergic asthma; <i>Bifidobacterium</i> and <i>Lactobacilli</i> *	95, 116
Bergamot peel	-	* <i>Bifidibacterium</i> , lactobacilli, <i>Eubacteria</i> and clostridia**	62
Potato pulp	> 100 KDa	* <i>Bifidibacterium</i> and <i>Lactobacillus</i>	42
Sugarbeet arabinan	-	* <i>Bifidibacterium</i> and <i>Lactobacillus</i>	116

DP: Degree of polymerization

POS: Pectic Oligosaccharides

* Increase in numbers

** Decrease in numbers

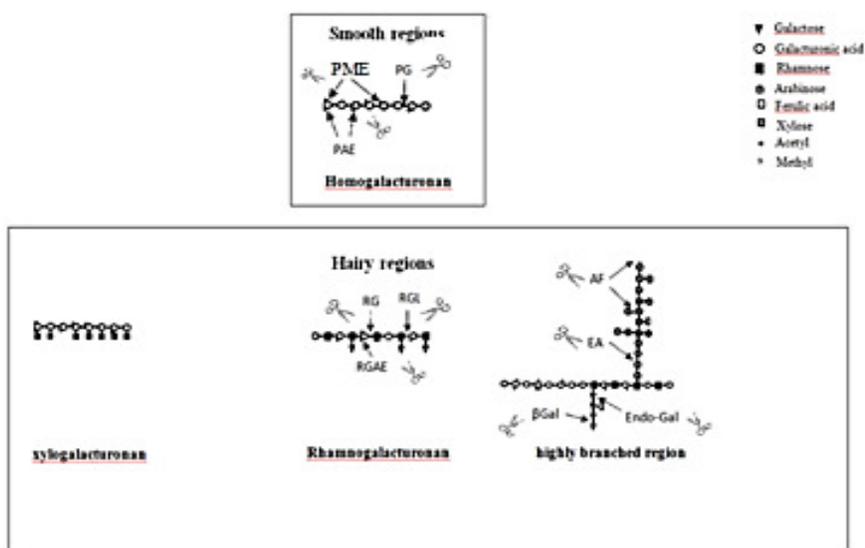
*** Feruloyated and non feruloyated arabino-oligosaccharides

1.4.1 Enzymatic processes

Various enzymes have been widely used for the production of POS because of their specificity and selectivity. Also, the use of enzymes over other pretreatment methods is regarded safe due to minimum adverse chemical modifications of products.⁶⁷ Some specific pectin degrading enzymes have been used, which acts synergistically to produce POS.^{20, 21, 24, 66, 68, 69} Pedrolli et al., reviewed the type of enzymes needed for the production of POS. Fig 1.2 illustrates various pectic enzymes active on smooth and hairy regions of pectin. The methyl esters and acetyl groups from galacturonic acid residues are removed by pectin methyl esterase (PME) and pectin acetyl esterase (PAE) respectively.^{69,70} Both the enzymes act before endo-polygalacturonase (Endo-PG). Endo-polygalacturonase (Endo-PG) is able to cleave the glycosidic bond of the α -(1 \rightarrow 4)- polygalacturonan in a random fashion.⁷¹ Endo-Polygalacturonase generally prefers non-esterified substrate and shows decreasing activity with increasing degree of methyl esterification.⁷² Exo- polygalacturonase (Exo-PG) attacks the substrate from the non-reducing end and is able to remove terminally (1-) linked Gal A residues from HG chains.⁷³

The RG subunit of the ramified 'hairy' regions can be degraded sequentially by rhamnogalacturonan hydrolase (RG), rhamnogalacturonanlyase (RGL) 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 RG backbone respectively. Rhamnogalacturonan acetyl esterase (RGAE) is an exo-acting pectinase active on the acetyl groups and also removes terminal rhamnosyl residues.⁷⁴ The removal of side chains from RGI can be achieved by cocktail of various enzymes like (i) arabinofuranosidase B (Araf) which removes terminal arabinose residues from the arabinan side-chains of pectins (ii) Endoarabinase (EA) hydrolyze the linear regions of the arabinan backbone and release a mixture of arabinose oligomers (iii) Exoarabinases (ExA) releases arabinose, arabinobiose, arabinotriose to from linear α - linked arabinan.^{4,75,76,77} These enzymes acts in a synergistic

fashion, leading to a rapid degradation of the arabinans. On the other hand, the relatively long (1→4)-linked galactan side-chains can be degraded by endogalactanase while, β -galactosidase is able to remove terminal galactose residues from galactans or arabinogalactans.⁶⁹ Eight neutral branched arabino-oligosaccharides (α -1,5 linked backbone of L-arabinofuranosyl residues) from sugar beet arabinan was obtained by mixture of arabinohydrolases, Abn 1 (endo-arabinase), Abn2 (exo-arabinase) and Abn 4 (arabinofuranosidase)⁴. Holck et al. separated sugar beet pectin into HG and RG-I by sequentially applying enzymes *viz.* pectin lyase, β -galactosidase-1, β -galactosidase-2, galactanase, arabinofuranosidase, and arabinanase.⁷⁸



PME- Pectin methylesterase; PAE- Pectinacetyl esterase; PG- Polygalacturonase; RGL- Rhamnogalacturonan lyase; RGAE- Rhamnogalacturonan acetyl esterase; RG- Rhamnogalacturonan hydrolase; AF- Arabinofuranosidase; EA- Endo-arabinase; Endo-Gal- Endo-galactanase; β Gal- β Galactosidase

Fig 1.2: Enzyme active on smooth and hairy regions of pectin

The process parameters, like time, temperature, enzyme concentration, absence and presence of particular enzyme influence oligosaccharides production.²⁰ Leijdekkers et al., and

Kuhnel et al., concluded that branched arabino-oligosaccharides can be produced if the enzyme mixture lacks arabinofuranosidase.^{41,43} Same authors observed that the higher enzyme loadings results in increased arabinan conversion to arabinose. The presence of galacturonic acid and low DP oligomers indicated the presence of Exo-PG which cleaves the polygalacturonic acid oligomers.^{41,66} The absence of RG rhamnohydrolase and RG galacturonase lead to the production of recalcitrant oligosaccharides, while presence of rhamnogalacturonase resulted in production of rhamnogalacturonan oligomers.^{41,56} Feruloylated arabinose di, tri, hexa, hepta and octa saccharides as well as feruloylated galactose disaccharides were obtained after the hydrolysis of sugar beet pulp with driselase.⁷⁹ Potato pulp was hydrolysed with pectin lyase, polygalacturonase and pectin methyl esterase for the production of HG and RG-I oligosaccharides⁴⁶.

1.4.2 Physical and Chemical processes

For POS production, physical pretreatments like hydrothermal, Dyanamic high pressure microfluidization (DHPM) and irradiation have been tried. During hydrothermal pretreatment, pectin is partially hydrolysed and oligosaccharides can be effectively released from the biomass. Arabino and galacto-oligosaccharides were successfully produced from various agro- residues by hydrothermal hydrolysis.^{5,20,80,81} Another physical pre-treatment DHPM which is based on the principal of powerful shear, turbulence, impaction and cavitation has been used for POS production from apple pectin¹. Various DP of POS were obtained and identified as oligo-galacturonides, arabino-oligosaccharides and galacto-oligosaccharides.

Chemical hydrolysis of pectin for the production of POS has not been studied extensively except for the alkaline pre-treatment which is generally used for the production of RG-I pectin. Zywinska et al., used alkaline extraction for the production of RG-I oligosaccharides from potato pulp.^{82,83} There are some disadvantages of chemical hydrolysis

processes, as these are generally not safe for environment and there is also a limitation to achieve the desired degree of polymerisation.⁶⁷

1.5 CHARACTERISATION OF POS

As mentioned previously, pectin is often pretreated to produce POS of varying DPs (Table 1.3). The effect of this degradation results in fragments which are in the range of a broad set of analytical techniques, ranging from liquid chromatography, to capillary electrophoresis (CE), gas chromatography (GC) and mass spectrometry (MS).²⁷ Liquid chromatographic analyses are the most commonly used and are often done using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Sugar oligosaccharides are separated based on their charge differences with HPAEC, with the separation is performed at pH 12. The negatively charged sugars bind to the column and elute through competitive binding with an increasing salt gradient.^{84,85} A series of galacturonic acid oligomers (DP1-DP10) formed by depolymerization of polygalacturonic acid were identified on HPAEC-PAD⁶⁶. Another possibility recently receiving more and more attention is the use of hydrophilic interaction liquid chromatography (HILIC). POS oligomers have recently been efficiently separated, identified and quantified using HILIC with online electrospray ionization ion trap mass spectrometry (ESI-IT-MSⁿ) and evaporative light scattering detection (ELSD)⁸⁶. The molecular weight of POS can also be estimated with size exclusion chromatography (SEC).⁸⁷ The presence of tetra, tri and di-galacturonic acid prepared from olive by-products were confirmed by adsorption/ SEC and identified by HPLC, GC, ESI-MS and ESI-MS/MS.⁵ Beside chromatography, matrix assisted laser desorption ionisation mass spectrometry, due to its tolerance to residual salts, ease of simple sample preparation, and high speed of analysis, is often used for offline MS analysis in order to identify the DP and the composition of the separated oligomers.⁸⁸ Arabino-oligosaccharides prepared from sugar beet pulp were identified by MALDI-TOF MS and HPAEC-PAD.⁴

1.6 ENZYME MEMBRANE REACTOR FOR POS PRODUCTION

In an enzymatic membrane reactor, the membrane governs the mass transport across itself thus also retaining the enzymes inside the reactor and achieving some level of product separation as well. Membrane bioreactors represent an alternative approach of biocatalyst immobilization while integrating catalytic conversion, product separation and catalyst recovery in single operation. The membrane has the property to allow certain components more easily than others, due to differences in physical and chemical properties of both membrane as components. Transport through the membrane takes place as a result of a driving force acting on the components in the feed. In the majority of cases is the magnitude of the driving force is directly proportional to the size of the permeate stream. This driving force may be an electric potential difference or a pressure, concentration or temperature gradient. The effect of factors most likely to affect reactor performance (used for POS production and filtration), are substrate concentration, enzyme concentration and residence time. Further there are different set ups which can be used for POS production. Most commonly dead end set up and cross flow set up are the most common each with its own advantages and disadvantages (Fig 1.3).

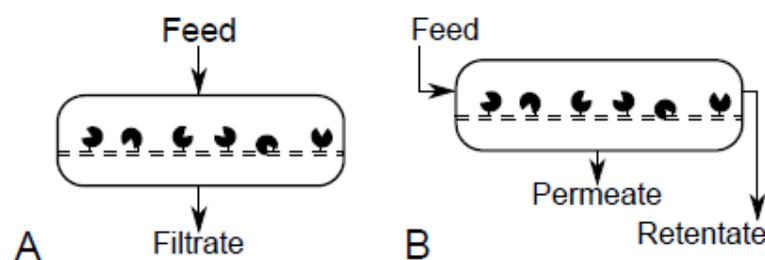


Fig 1.3: Filtration A: Dead end filtration B: Cross flow filtration

Purification of POS can be done by membrane based separation or other chromatography based purification techniques described elsewhere in this paper.^{41,89} Holck et al., employed regenerated cellulose membrane of 3 KDa molecular weight cut off for POS

purification.⁷⁸ Munoz et al., obtained POS by ultrafiltration through 1000, 3000, 5000 and 10,000 Da cellulose regenerated molecular weight cut-off membranes.⁵ Different techniques based either on the membrane based technology or those based on chromatography are employed for separation of POS of different DPs. It is important to select the membrane and its cut-off on the basis of molecular weight of a specific compound. Similarly, the chemistry of the resin/ matrix used during chromatography is important for separation of a specific POS of a desired DP. In brief, the separation and purification techniques are generally chosen according to the compound/ mixture of compounds to be separated from a mixture.

1.7 HEALTH BENEFITS OF POS

The health effects imparted by oligosaccharides make them active ingredients of ‘functional foods’ which are similar in appearance to conventional foods that are consumed as part of a normal diet and have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions.⁹⁰ As food ingredients, prebiotics have an acceptable odor, and are low-calorie, allowing their utilization in anti-obesity diets. It has been stated that the prebiotic effect of POS depends upon the molecular weight of the fractions.^{1,89} Martin et al. were the first to compare the effect of pectin and POS on the growth of pure cultures of various species indigenous to the gastrointestinal tract.⁹¹ Several authors have reported that low molecular weight POS have prebiotic potential better than high molecular weight POS.⁴² *In- Vitro* studies have given a clear indication that POS can be successfully used to promote Bifidogenic flora. Pectic oligosaccharides of DP 3-7 were produced by enzymatically from bergamot peel and successfully evaluated for prebiotic properties in fecal batch cultures⁶⁸. Chen et al., used apple pectin POS and found a decrease in number of *Bacteroides* and *Clostridia*.¹ The fermentative capability of some intestinal strains *viz.* *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Klebsiella* and *E coli* was tested on POS prepared from soy arabinogalacturonan, sugar beet arabinan, wheat flour arabinoxylan, polygalacturonan and

rhamnogalacturonan fraction from apple. Except for *Bacteroides*, all other species were able to ferment *in-vitro*.⁹² Small oligomers of galacturonic acid (with DP 2 to 7) were responsible for inhibiting the adherence of bacteria to epithelial cells, the initial and crucial stage of infection.⁹³ The *in-vitro* fermentability of apple pomace oligosaccharides showed an increase in short chain fatty acids and increased bacterial growth.⁹⁴ Holck et al., reported that the long chain arabino-oligosaccharides from sugar beet pulp has a large bifidogenic effect in comparison to short chain arabino-oligosaccharides.⁷⁸ An increase in *Eubacterium rectale* population and butyrate levels was observed with the use of orange peel POS.⁹ Stimulation of *Bifidobacteria* and *Lactobacilli* population was seen with the use of low molecular weight arabino-oligosaccharides.⁴²

Pectic oligosaccharides have also been shown to possess antioxidant activity and significant effect in lowering the serum levels of total cholesterol ($p < 0.01$) and triglycerides ($p < 0.05$) and inhibition in the accumulation of body fat.^{95, 96} Pectic oligosaccharides have been reported to protect against cardiovascular diseases *in-vivo*.⁹⁶ Treatment with haw POS at higher doses (150–300 mg/kg) significantly suppressed weight gain in mice.⁹⁶ Scanty literature is available on the evaluation of POS isolated from agro-residues on the health benefits. Table 1.4 summarizes the biological and prebiotic effects of POS from agro-processing residues. There have been reports that POS regulate lipid and glucose metabolism with decreased glycemic response and blood cholesterol levels.⁸⁹ Pectic derived acidic oligosaccharides (pAOS) have been evaluated for their genotoxic potential and the safety of pAOS for human consumption was tested by Garthoff et al., Increase in *Bifidobacteria* population and a considerable decrease in *Clostridium lituseburensel* *Clostridium histolyticum* group was observed in HIV patients after ingesting a mixture of POS.^{7,97}

Other health promoting effects of POS are protection of colonic cells against *Shigella* toxins, prevention of adhesion of uropathogenic microorganisms and stimulation of apoptosis

of human colonic adenocarcinoma cells.^{93,98-100} *In- vivo*, the synergistic empowerment of immunomodulation caused by galacto-oligosaccharides (GalOS) and fructo-oligosaccharides (FOS) was studied by Vos et al., and Makker et al., have reported inhibition of tumour growth and metastasis by galactan oligomers.^{101,102} Anti-tumour activity of galacturonide (1 KDa) oligosaccharide obtained from citrus pectin was successfully tested on mouse and human tumour cells.¹⁰² However, a precise study on the effect of individual arabino-oligomer, galacto-oligomer, arabino-oligomer is lacking, because POS are generally produced in mixture and the complex nature of POS makes them difficult to separate.

The disadvantages of *in- vitro* methods are the absence of synergistic, antagonistic, and/or competitive effects as well as the absence of an immune system. In the field of prebiotics, POS are an exciting new development as they can be manufactured from low cost agricultural by-products.

1.8 POS IN FOOD AND FEED INDUSTRY

Information on the prebiotic activity of POS stated above is mainly from *in-vitro* models representing the human colon. But the mechanisms operating *in-vivo* need to be elucidated to interpret if these studies can be extended to human needs as well. Worldwide awareness of consumers towards diet and health has opened up new opportunities for food industries in research and development of functional foods. Foods that contain pre- and probiotics are drawing special attention of consumers and are potentially exciting component of the food market. Different prebiotics can be used for the fortification of different food products for the designing of functional foods for the special target groups. Moreover prebiotics from other sources have been successfully tested for their stability at high temperature and low pH and can therefore be added in bakery product, pasteurized juices, acidic foods like yogurts.¹⁰³ The importance of prebiotic foods lies in their active stimulation of growth of beneficial bacteria, thereby adding up to potential health and nutritional

benefits.¹⁰⁴ However, to further substantiate the claim of the prebiotic efficacy and other health benefits of POS, more rigorous *in-vitro* investigations are required and *in- vivo* studies will validate the claim. Potential applications of prebiotics (both the food and the non-food) in general are given in Fig 1.4.

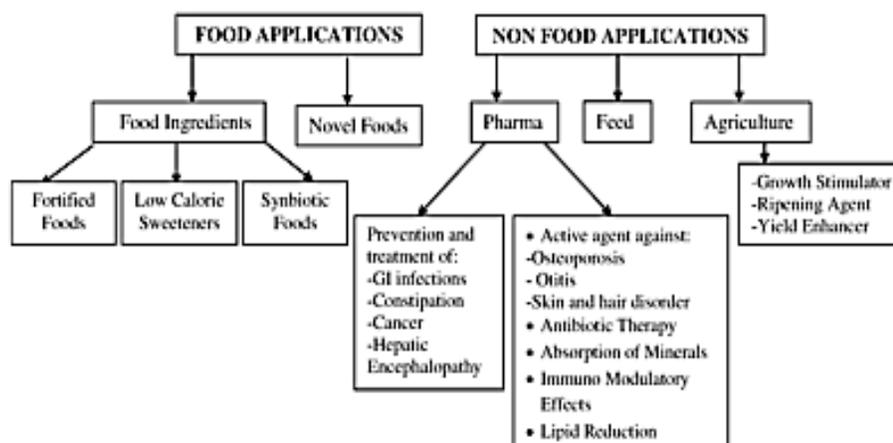


Fig 1.4: Potential applications of prebiotics (Panesar et al., 2013)

As antibiotics are banned in many countries due to transfer of the genes which resists anti-microbial/antibiotic action from animal to human microbiota and hence an alternative is needed that could enhance the natural defense mechanisms of animals.¹⁰⁵ Poultry flocks are the main sources of infection of *Camphylobacter jejuni*, Clostridia and Salmonella infections. Pigs have been found to be more prone to *E. coli* infections.¹⁰⁶ Oligosaccharides have been found to prevent this kind of invasion by binding to the microbe's carbohydrate-binding proteins and pathogens are cleared by the physiological mechanism characteristic of the specific tissue.¹⁰⁷⁻¹⁰⁹ In addition, in diets containing reasonable quantities of carbohydrates saccharolytic fermentation prevails, thus the pH of the GIT remains stable and subsequently reduces the onset of Clostridia infections, as a more alkaline pH is required by species of this

genus. Inulin added to rabbit feed was fermented in the caecum produced SCFA, and reduced the risk of clostridiosis.¹¹⁰ Pectic oligosaccharides are reported with this activity and have been tested against some pathogens or toxins.⁹⁹ Ganan et al. found that POS significantly inhibits cell invasion.¹¹¹ Gaggia et al., have reviewed in detail the application of prebiotics in animal feeding.¹¹² As mentioned previously, there are a number of reports available on application of POS in food and pharmaceutical industry, however, its potential in the feed industry is yet to be exploited. There is only a limited information available on the use of POS in animal feeds in promoting the health of the animal or acting as therapeutic agents. *In- vitro* studies show that POS have a potential to be used as feed additives. However, extensive *in- vivo* studies may be required in different animal models due to complex structure of the GIT and diverse microflora.

1.9 MARKET DEMAND OF PREBIOTICS

According to the Global Industry Analysts (GIA) report, the European and the US market for prebiotics is projected to reach \$1.17 billion and \$225.31 million respectively by the year 2015. While the European market is driven by the expansion of prebiotic ingredient manufacturers into new application areas such as meat and snack products, the US market is driven by continued demand for fructans, which includes both the inulin as well as fructo-oligosaccharides. Global market for prebiotics is projected to reach US\$4.8 billion by 2018, driven by rising awareness of health and nutrition, growing consumer acceptance of the benefits of prebiotics, expanding applications and rapid innovations in prebiotics based food products. The potential in the world market for emerging prebiotics in terms of their production and purification is yet to be completely realized and can be optimized from cellulosic and pectic biomass pretreatments. Fructans represents the largest product market worldwide. Prominence of GOS (Galacto oligosaccharides) is growing led by the inherent benefits offered by this class of oligosaccharides in their versatility for use in a wide range of

products including clear beverages, juices and bakery products. Since pectic oligosaccharides are not yet commercial, it is difficult to predict their contribution to the prebiotic industry in economic terms, but it is felt that the POS are likely to contribute significantly to the prebiotic market in the years to come. Development of POS from relatively cheaper by-products such as agro-residues for application in food, feed and pharmaceutical industry will set a new direction for future research.

1.10 CONCLUSIONS

Pectin containing agricultural by-products are potential sources of a new class of prebiotics known as pectic oligosaccharides (POS). In general, pectin is made up of homogalacturonan (HG, α -1,4-linked galacturonic acid monomers) and rhamnogalacturonan (RG, alternate galacturonic acid and rhamnose backbone with neutral side chains). Controlled hydrolysis of pectin containing agricultural by-products like sugar beet, apple, olive and citrus by chemical, enzymatic and hydrothermal can be used to produce oligo-galacturonides (GalpOS), galactooligosaccharides (GalOS), rhamnogalacturonan-oligosaccharides (RGOS), etc. Out of several production methods, continuous production and separation of POS using an enzyme membrane reactor exhibits a huge potential for the production of tailored POS. To achieve the utmost productivity of POS in an enzyme membrane reactor it is essential that several factors like enzyme concentration, substrate loading are optimized.

1.11 ACKNOWLEDGMENT

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2.ENZYMATIC PECTIC OLIGOSACCHARIDES (POS) PRODUCTION FROM SUGAR BEET PULP USING RESPONSE SURFACE METHODOLOGY

Based on Babbar N, Dejonghe W, Elst K and Sforza S. Enzymatic pectic oligosaccharides (POS) production from sugar beet pulp using response surface methodology. Submitted with LWT Food Science and Technology

2.1 INTRODUCTION

Sugar beet (*Beta vulgaris*) is a temperate crop abundantly produced in Europe. After the extraction of sugar, a by-product in the form of pulp is generated which is mainly used as animal feed. In addition, a current commercial product known as fibrex, a form of dietary fibre is also available.¹ The amount of sugar beet pulp produced in Europe is 9.1×10^7 tonnes.² The major component of sugar beet pulp is hemicellulose (30%) followed by cellulose (22-24%), pectin (15-25 %) and others (9-10%).³ Cellulosic and hemicellulosic fractions of sugar beet pulp have been extensively exploited for the production of bioethanol and biogas.⁴ Current research is fostered on the production of oligosaccharides from the pectic fraction of sugar beet pulp.⁵⁻⁷ Pectin is a complex and heterogenous group of polysaccharides which is composed of distinctive domains covalently linked to one another.⁸ The main structural domain in pectin is homogalacturonan-I (HG), rhamnogalacturonan I and II (RGI and RG II) often described as the smooth and hairy regions respectively.⁹ Homogalacturonan (HG) is composed of (1,4)- linked α -D-GalpA residues that can partly be methyl-esterified at C-6 and possibly partly acetyl esterified at O-2 and O-3.¹⁰ Rhamnogalacturonan (RG I) is made up of chains with alternate units of galacturonic acid and rhamnose having branched arabinan, galactan or even arabinogalactan chains at O-4 of Rhap residues. Arabinan side chains are composed of α (1-5) linked Araf residues which can be further branched at α -L-Araf units at O-2 and O-3, whereas galactan side chains are constituted of (1-4) linked beta-D-galp units.⁸ Rhamnogalacturonan II (RG II) is present in minor amount and consists of galacturonic acid,

rhamnose, galactose and neutral sugars.⁵ In addition xylogalacturonan (XG) is also a part of pectin.⁹ These acidic and neutral sugars are arranged in such a way that the use of specific hydrolysis processes generates an oligosaccharide mixture that can contain arabino-galactooligosaccharides, arabino-xylooligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, oligo-galactouronides, rhamno-galacturonan oligosaccharides.³

The major interest in oligosaccharides lies in their physico-chemical and physiological properties.¹¹ Such properties include selective stimulation of growth of beneficial bacteria in colon.¹² Therefore, pectin-derived oligosaccharides are considered as “novel candidate prebiotics” and “emerging prebiotics”. A prebiotic is defined as a selectively fermented ingredient that allows specific changes in the composition and/ or activity of the gastrointestinal microbiota that confers benefits upon host well-being and health by selectively providing growth factors and energy substrates to some host microflora, or alternatively *via* binding and eliminating others.¹³ In addition, pectic oligosaccharides (POS) have also been investigated for other health related effects *viz*, apoptosis of colon cancer cells, inhibition of *Camphylobacter jejuni* on human Caco -2 cells, and protection against various pathogens, repression of lipid accumulation.¹⁴⁻¹⁶

Enzymatic hydrolysis of sugar beet pulp for the production of POS has been investigated by Concha & Zuniga, Martinez, Gullon, Schols, Alonso & Parajo, Leijdekkers, Bink, Geuthes, Schols, Gruppen.^{3,5,7} The production of pectic oligosaccharides (POS) using enzymes needs an optimization of various process parameters). Using response surface methodology (RSM) to optimize process parameters saves time and also provides information about interactions between parameters.¹⁷ A very limited literature on the statistical optimization of the use of enzymes in relation to other operational parameters is available for POS production from sugar beet pulp. In the work of Martinez, Gullon, Yanez, Alonso & Parajo, lower yield of galacturonic acid oligomers and arabino oligomers were obtained.¹⁸

The present study was thus undertaken to optimize particle size, enzyme concentration and time for converting sugar beet pulp pectin to POS using one step approach in central composite design (CCD). Two enzymes *viz.* (i) Celluclast (for solubilizing of pectin) (ii) Viscozyme (to cleave the side chains) were studied. The optimized parameters were then used for validation experiment to establish the correlation between the actual yield and the yield predicted by the Design Expert software.

2.2 MATERIAL AND METHODS

2.2.1 Raw material

Sugar beet pulp with a dried matter content of 94.7 % (w/w) was provided by the Institut für Getreideverarbeitung (IGV GmbH), Germany. The dried sugar beet pulp was ground using a Robot coupe R 20.V.V mixer at room temperature and 3500 rpm for 30 min, sieved to different particle sizes (as given by the design) and stored in ziplock bags at room temperature until use. The polygalacturonic acid, 4-nitrophenyl- β -D-glucopyranoside and 4-nitrophenyl- α -L-arabinofuranoside and standards for monomeric sugars were procured from Sigma Aldrich, USA, while linear arabinan was bought from Megazyme, Ireland. All other analytical chemicals were purchased from Sigma Aldrich, USA or Merck, Germany.

2.2.2 Total sugar composition of the raw material

The total sugars present in sugar beet pulp were estimated by following the protocol optimized in our previous study.¹⁹ The supernatant (containing the monosaccharides) was assayed for its monosaccharide composition by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, see Section 2.2.6).

2.2.3 Enzyme activity assays of Viscozyme L and Celluclast 1.5 L on different substrates

The different enzyme activities present in Viscozyme L and Celluclast 1.5 L were determined using appropriate substrates. The activities tested were selected based on the

approximate composition of sugar beet pulp. The activities of polygalacturonase, endo arabinase, β -D-glucosidase and α -L-arabinofuranosidase were respectively determined by measuring the amount of reducing sugars released from the hydrolysis of poly-galacturonic acid, linear arabinan, 4-nitrophenyl- β -D- glucopyranoside and 4-nitrophenyl- α -L-arabinofuranoside.²⁰ The filter paper activity of the enzymes was analyzed using laboratory analytical procedure of National Renewable Energy Laboratory.²¹

2.2.4 Experimental design for optimization

A three-factor CCD consisting of 20 experimental runs with three replications at the central point (Table 2.1) was used to optimize the independent variables, *i.e.* particle size (X_1), Viscozyme concentration (X_2) and incubation time (X_3) on arabino-oligosaccharides (AOS), galacto-oligosaccharides (GalOS), rhamno-oligosaccharides (RhOS), xylo-oligosaccharides (XOS) and galacturonic acid-oligosaccharides (GalaOS). The selected ranges for Viscozyme concentration, time and particle size were (0.75-2.0) filter paper units per gram dried substrate FPU/g d.m, (1-3) h, and (0.3-0.8) mm respectively. To assess the POS production by enzymatic hydrolysis, a set of preliminary experiments was carried out to identify the most influential parameters and their range of practical interest (data not shown). Viscozyme is basically a cell wall degrading complex, so for the sake of simplicity the addition of Viscozyme was done on the basis of the total Celluclast activity. In addition to Viscozyme, Celluclast was also added in all the experimental runs at a fixed concentration of 10 FPU/ g d.m. based on the optimization of the enzyme units in preliminary experiment (data not shown). The aim of this study was to obtain high oligosaccharide yield, thus, the selected time range was lower than that used by previous researchers.⁵ Since hydrolysis was done for oligosaccharide production, monosaccharide concentration was not considered as a response. The experiment was performed in capped fermentation flasks, each containing 1 g of dried sugar beet pulp. Hydrolysis was conducted at a substrate concentration of 13.5% (w/v) and a

temperature of 45° C in an incubator shaker at 150 rpm. The enzyme concentrations, particle size and time of hydrolysis were selected according to data generated by the RSM software (Table 2.1). After hydrolysis the flasks were heated to 100 °C for 5 min to inactivate the enzyme. At the end of the process, the hydrolysate was separated by centrifugation (5000 xg for 10 min) and analyzed for monosaccharides and oligosaccharides. Experimental data from the CCD were analyzed with RSM algorithm Design Expert 8.1 and fitted according to Eq. (1) as a second order polynomial equation including the main effects and interaction effects of each variable

$$Y = \beta_o + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii}^2 X_i^2 + \sum_{j=i+1}^3 \beta_{ij} X_i X_j$$

With Y the predicted response, β_o the constant coefficient, β_i the linear coefficient, β_{ii} the quadratic coefficient, and β_{ij} the interaction coefficient.

Table 2.1: RSM design employed during optimization studies for three independent variables

Run no	Variables		
	Particle size (mm) X_1	Enzyme Concentration (FPU/ gds ⁻¹)* X_2	Time (h) X_3
1	1.0	1.4	2.0
2	0.5	1.4	2.0
3	0.8	0.7	3.0
4	0.5	1.4	2.0
5	0.5	1.4	2.0
6	0.3	0.7	1.0
7	0.3	0.7	3.0
8	0.5	1.4	2.0
9	0.5	1.4	3.7
10	0.8	2.0	3.0
11	0.5	1.4	2.0
12	0.8	2.0	1.0
13	0.3	2.0	1.0
14	0.1	1.4	2.0
15	0.5	0.5	0.3
16	0.8	0.7	1.0
17	0.5	0.3	2.0
18	0.5	1.4	2.0
19	0.5	2.4	2.0
20	0.3	2.0	3.0

*Concentration of Viscozyme was varied while that of Celluclast was kept constant

2.2.5 Statistical analysis

The analysis of variance (ANOVA) and surface plots were generated using Design Expert 8.0, and the optimized values of three independent variables for maximum response were determined using the numerical optimization package of the same software.

2.2.6 Analysis of total free monosaccharides and pectic polysaccharides by HPAEC-PAD

All extracts were analyzed on HPAEC-PAD in two ways, *i.e.*, without further treatment to assess its monosaccharide content, and after complete hydrolysis to determine its total saccharide content. 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 of galacturonic acid and the neutral sugars, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h based on the method of Martinez, Gullon, Schols, Alonso & Parajo.⁵ Viscozyme L is a multienzyme complex composed of pectinases, hemicellulases and arabinases.²² It is worth mentioning here that the commonly used acid hydrolysis was not suitable for these pectin rich extracts, even when applying the mild conditions of the seamans hydrolysis. In all cases low amounts of galacturonic acid were found due to degradation (data not given). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min, and the liquid was centrifuged at 5000 x g for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into a high performance anion exchange chromatography with pulsed amperometric detection (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 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 mobile phase was used at a flow rate of 1 mL/min for 46 min. The monosaccharides were quantified by comparing them with the concentration of known standard solutions (ranging from 10 mg/L to 1000 mg/L) of rhamnose, arabinose, galactose, glucose, xylose, fructose and galacturonic acid.

2.3 RESULTS AND DISCUSSION

2.3.1 Total sugar (galacturonic acid and neutral sugar) composition of sugar beet pulp

To determine the composition of the biomass, the biomass was fully hydrolyzed and analyzed for the monosaccharides representative for the pectin. These are galacturonic acid as acidic sugar, and arabinose, galactose and rhamnose as neutral sugars. The results are expressed as % (w/w) d.m. The galacturonic acid content of sugar beet pulp was $16.5 \pm 0.8\%$. The neutral sugars comprised of arabinose, rhamnose and galactose. The arabinose content was the highest accounting for $16.0 \pm 0.6\%$. The rhamnose and galactose content of sugar beet pulp was found to be $1.1 \pm 0.3\%$ and $5.6 \pm 0.2\%$, respectively. Also, some cellulosic and hemicellulosic sugars in the form of glucose and xylose were present. The glucose content of $18.4 \pm 0.2\%$ and xylose content of $2.1 \pm 0.2\%$ was found. In general, the compositional data fall within the range reported in literature.⁵

Given the high pectic content and the versatility of the pectin structure, sugar beet pulp was considered to be an interesting substrate to produce POS.

2.3.2. Enzymatic hydrolysis of sugar beet pulp for POS production

Table 1 depicts the design used in the optimization experiment involving three independent variables. The use of the two enzymes (*i.e.* Viscozyme and Celluclast) for the

hydrolysis of sugar beet pulp had a positive effect on the production of different POS. Table 2.2 shows the effect of three independent variables on the production of various oligosaccharides from sugar beet pulp. The highest concentration of GalAOS were 13.3 and 13.5 g/L obtained at 0.7 & 0.3 FPU/g d.m. of Viscozyme respectively (run 16 and 17). Use of higher enzyme concentration above 0.7 FPU/ g d.m., while keeping the other two parameters the same as that of run 16, results in lower GalAOS yield (run 2, 4, 12 & 19). This indicates that lower enzyme concentration is required to produce GalAOS oligosaccharides. Table 2.3 shows the corresponding conversion of polygalacturonic acid to GalAOS and galacturonic acid (GalA). Although the overall production of monosaccharides remains low, an increase in the concentration of monosaccharides is observed with increase in enzyme concentration. This effect is due to the exo-activity of Viscozyme.²³ Therefore, for the production of GalAOS, the optimum concentration of Viscozyme is between 0.3 and 0.7 FPU/ g d.m.. On the other hand in case of AOS, the highest production of 20.3 g/L and 20.2 g/L was obtained with 2.4 and 2 FPU/ g d.m. of Viscozyme respectively. It should be noted that AOS production remains more than 15 g/L when higher doses of Viscozyme (1.4 FPU/ g d.m.) was used (run 1 2, 4, 5, 8, 9, 11, 14 and 18). The results obtained at run 17 suggests that if other two parameters are kept constant and enzyme concentration is lowered, then AOS production is significantly affected. This observation is contrary to what Martinez, Gullon, Schols, Alonso & Parajo found.⁵ The difference could be due to differences in enzyme loading. This could be related to a lower arabinan degrading activity of Viscozyme which would result in a lesser yield of AOS when lesser enzyme concentration is used (Table 2.4). On the other hand, higher enzyme concentration leads to a rapid hydrolysis of GalAOS oligomers as described elsewhere in the article. So a balance is needed to obtain both GalAOS and AOS at the same time. Table 2.3 shows the conversion of arabinan to AOS and arabinose. The arabinose monosaccharide remains low when compared to monosaccharides of galacturonic acid.

Data in Table 2.2 shows that a higher concentration of enzyme is needed to produce XOS. The xylose is a partly from the hemicellulose fraction and a high xylanase activity is needed to produce XOS. Enzymes used in the present study possesses lower xylanase activity (Table 2.4). The highest yield of XOS 2 g/L was obtained when 2.4 FPU/ g d.m. of Viscozyme was applied (run 19). On the other hand, RhOS and GalOS were not much affected by the varyiation in enzyme concentration.

As shown in Table 2.2, time is also a critical parameter for the production of oligosaccharides. Longer time is needed for AOS, GAIOS, XOS and RhOS production but comparatively lesser time is requested for GalAOS formation (run 2 and 9) (run 3 and 16), (run 6 and 7) (run 10 and 11) (run 13 and 20). The change in particle size doesnot significantly affected POS production from sugar beet pulp.

Table 2.2 : Pectic oligosaccharides yield during the hydrolysis of sugar beet pulp with viscozyme (varied concentration) and Celluclast (10 FPU/ gds⁻¹)

Run no	GalAOS	AOS (g/l)	GalOS	ROS	XOS
1	10.4	18.0	5.5	1.0	1.7
2	9.7	17.0	5.3	0.9	1.7
3	12.1	18.0	5.3	1.0	1.6
4	9.8	18.0	5.7	1.0	1.8
5	10.0	18.3	5.7	1.0	1.6
6	12.3	15.0	4.9	0.9	1.5
7	12.0	16.0	5.2	1.0	1.5
8	9.8	18.4	5.7	1.0	1.7
9	8.7	19.9	5.7	1.0	1.8
10	7.2	20.2	5.9	1.1	1.9
11	8.2	16.7	5.8	0.8	1.7
12	9.8	17.0	5.3	0.8	1.6
13	10.0	17.7	5.5	0.8	1.6
14	12.4	18.9	5.8	0.9	1.4
15	8.3	13.1	4.7	0.7	1.3
16	13.3	17.5	4.9	0.7	1.4
17	13.5	13.5	4.3	0.8	1.3
18	11.3	18.8	5.9	0.9	1.8
19	7.9	20.3	6.0	1.0	2.0
20	7.2	19.9	6.0	0.9	1.9

GalAOS: Galacturonic acid oligosaccharide, AOS: Arabino oligosaccharides; GalOS: Galactose oligosaccharides, XOS: Xylo oligosaccharide, ROS: Rhamno-oligosaccharides.

Table 2.3: Conversion of pectin to various oligosaccharides and monosaccharides

Run no	Galacturonic acid		Arabinose		Galactose		RhOS		XOS	
	Mono	Oligos	Mono	Oligos	Mono	Oligos	Mono	Oligos	Mono	Oligos
	% conversion									
1	22.8	46.7	12.0	83.3	22.9	71.9	16.2	65.2	ND	85.0
2	24.6	43.5	13.9	79.1	22.5	69	21.0	54.9	ND	86.3
3	15.2	54.5	6.4	83.3	20	69.1	12.0	61.7	ND	79.3
4	26.5	44.3	13.4	83.4	23	72.8	18.8	61.4	ND	88.3
5	26.3	45.2	13.5	84.5	24	73.6	18.8	63.2	ND	79.2
6	9.1	55.5	3.7	69.5	12	64.2	10.6	54.9	ND	74.3
7	14.4	53.6	6.1	74.1	19	68.8	14.4	62.8	ND	76.2
8	27.0	44.1	13.7	85.1	25.1	73	20.6	59.4	ND	85.3
9	31.8	39.2	4.1	92.1	25.2	73.2	19.1	63.4	ND	88.0
10	34.8	32.4	2.2	93.7	23.1	76.6	26.2	68.2	ND	93.3
11	26.5	37.1	14.0	77.2	23	75.3	20.9	50.4	ND	84.8
12	23.9	44.0	11.8	78.7	19.8	69.4	17.6	51.9	ND	79.0
13	26.8	44.6	12.7	81.8	25.3	71.6	20.2	53.0	ND	80.2
14	11.0	55.8	4.1	87.4	15.1	75.2	9.7	58.8	ND	69.3
15	10.1	37.2	3.7	60.9	14.9	60	9.1	42.0	ND	64.7
16	4.8	59.6	3.7	81.3	14.9	63.2	9.1	41.5	ND	71.9
17	25.0	60.5	12.1	62.9	22	56.3	21.1	50.7	ND	62.7
18	37.1	50.6	12.9	87.0	22.1	76.5	25.5	54.6	ND	88.1
19	39.0	35.5	4.9	93.9	25.1	77.1	27.8	66.0	ND	98.2
20	22.8	32.2	4.5	92.1	21.2	77.9	20.1	57.5	ND	93.8

ND: Not detectable

Table 2.4 : Activities of Viscozyme and Celluclast on different substrates (U/mL)

Substrate	Activity measured	Viscozyme U/mL	Celluclast
Polygalacturonic acid	Polygalacturonase	6516	66
Filter paper	Total cellulase	29.4	70
4-nitrophenyl- β -D-glucopyranoside	β -D-glucosidase	516	855
Arabinan	Endo-arabinase	180	64
4-nitrophenyl- α -L-arabinofuranoside	α -L-arabinofuranosidase	0.18	0.27

2.3.3 Effect of different independent variables on the production of oligosaccharides

The results of the RSM experiment were analyzed using Design expert 8.0 evaluation software using a quadratic model. For all responses, the factors and second order interactions were selected that were significant in the 95% confidence range. Thus, $p \leq 0.05$ was considered significant in the present study. The final response function to predict galacturonic acid, arabinose, rhamnose, galactose, glucose and xylose oligosaccharide concentration after eliminating the nonsignificant terms are presented in Eqs (2-6).

$$Y(\text{GalaOS}) = 16.1 - 11.5 X_1 - 3.9 X_2 + 2.3 X_3 - 0.8 X_2 X_3 + 9.8 X_1^2 + 0.9 X_2^2 - 0.4 X_3^2 \quad \text{Eqn 2}$$

$$Y(\text{AOS}) = 9.3 + 6.2 X_1 + 2.8 X_2 + 1.8 X_3 - 3.9 X_1 X_2 + 0.8 X_2 X_3 - 0.4 X_3^2 \quad \text{Eqn 3}$$

$$Y(\text{GalOS}) = 3.0 - 0.2 X_1 + 1.7 X_2 + 0.9 X_3 - 0.4 X_2^2 - 0.2 X_3^2 \quad \text{Eqn 4}$$

$$Y(\text{RhOS}) = 1.1 - 1.2 X_1 - 0.1 X_2 + 0.1 X_3 + 0.3 X_1 X_2 + 0.2 X_1 X_3 + 0.4 X_1^2 - 0.03 X_3^2 \quad \text{Eqn 5}$$

$$Y(\text{XOS}) = 0.9 + 0.9 X_1 + 0.1 X_2 + 0.2 X_3 + 0.08 X_2 X_3 - 0.7 X_1^2 - 0.04 X_3^2 \quad \text{Eqn 6}$$

Where Y, X₁, X₂ and X₃ represent yield, particle size, enzyme concentration and time, respectively.

The overall quadratic model was significant. The R² values for GalaOS, AOS, GAIOS, RhOS and XOS were 0.85, 0.79, 0.90, 0.82, 0.83 respectively. This indicates 85%, 79%, 90%, 82% and 83% of the total variation around the average could be explained by the regression analysis performed for the GalaOS, AOS, GalOS, RhOS and XOS production. In all cases, the lack of fit was not significant, indicating the fitness of the model for all five responses. The models show that the responses depend on the three parameters, i.e. size of the particles, the enzyme concentration and incubation time, but the extent and the details of the effect differ from the responses analyzed. Also in some cases, interaction between the parameters as well as second order effects were found to be relevant.

2.3.4. Model graphs and numeric optimization

The model graphs were plotted according to the model equations 2-6 to investigate the interaction between the independent variables and to determine the optimal value of each variable for a desired response. The response surfaces shown in Figure 1-4, were based on the final model in which two variables were kept constant at their optimum values and the other two were varied within their experimental range. It is clear from the model graphs responses that the interaction between time and enzyme concentration had significant effect on GalaOS concentration. As shown in Fig 2.1, as time progresses GalaOS production also increases (at minimum enzyme concentration of 0.7 FPU/ g d.m.). While at higher enzyme concentration of

2.0 FPU/ g d.m. increasing time had a reducing effect on GalAOS (Fig 2.1) which is corroborated with the increased galacturonic acid content of the hydrolysates (Table 2.3).

In case of AOS, at the lowest particle size 0.3 mm, with an increase in enzyme concentration from 0.7 to 2.0 FPU/ g d.m., there was a sharp increase in AOS production. While at maximum enzyme concentration of 2 FPU/ g d.m., with increase in particle size a slight increase in AOS production was seen (Fig 2.2a). The results in Fig 2.2b show that the interaction between time and enzyme concentration was found to be significantly affecting the AOS production (Fig 2.2b). With the increase in both, AOS production increased. Maximum AOS was produced at time of 3h and enzyme concentration of 2.0 FPU/ g d.m.

In case of RhOS, increase in enzyme concentration at lowest particle size *i.e.* 0.3 mm had a non-significant effect on the RhOS production. However, when particle size and enzyme concentration both increased, RhOS also increased from 0.94 g/L to 1.05 g/L (Fig 2.3a). In case of interaction between time and particle size, at a particle size between 0.3 – 0.8 mm, the increase in time led to the increase in RhOS production. Maximum RhOS of 1.08 g/L was observed at 3 h and particle size of 0.8 mm (Fig 2.3b).

In case of XOS production, at the lowest enzyme concentration (0.7 FPU/ g d.m.), with increase in time, XOS production increases while maximum XOS production was seen at 3h and enzyme concentration of 2.0 FPU/ g d.m. (Fig 2.4).

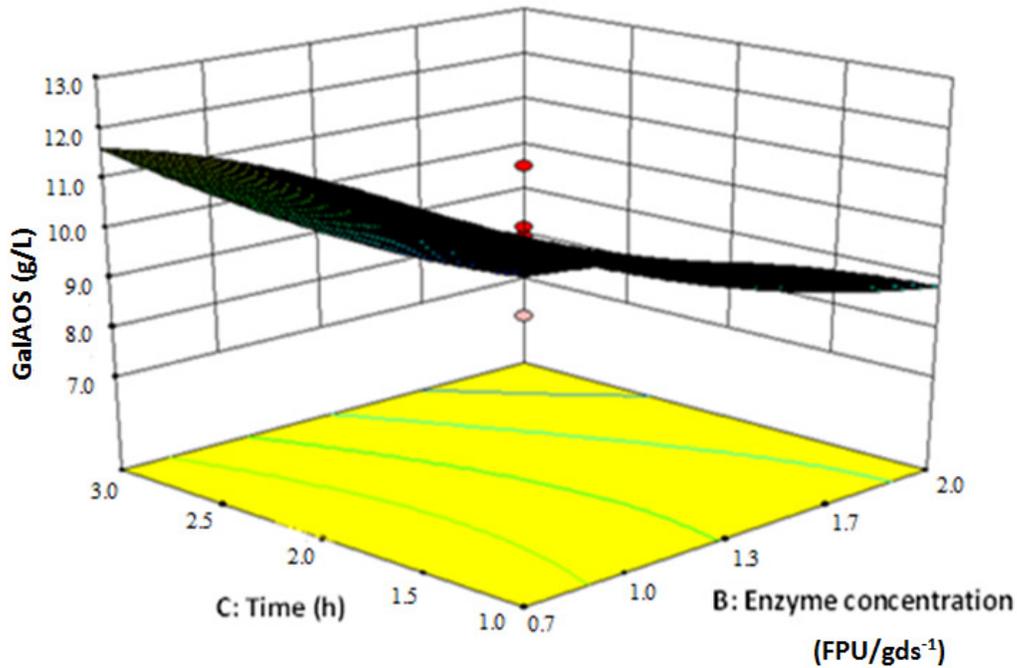


Fig 2.1- Response surface and contour plot showing the effect of interaction between enzyme concentration and time on galacturonic acid oligosaccharides (GalAOS) production from sugar beet pulp

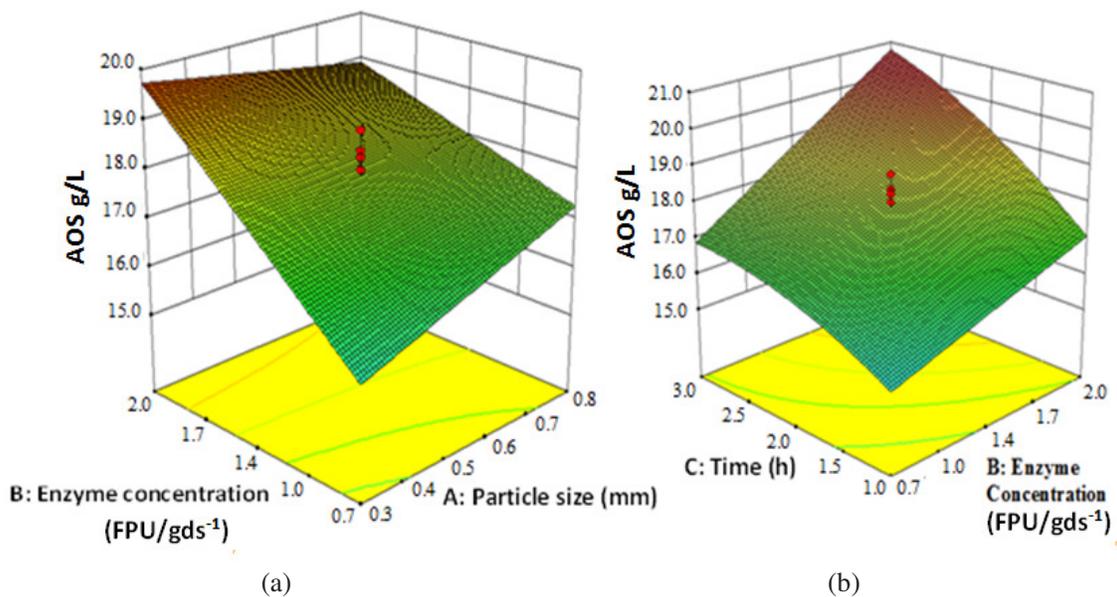


Fig 2.2- Response surface and contour plot showing effect of interaction between (a) particle size and enzyme concentration (b) enzyme concentration and time on arabinooligosaccharides (AOS) production from sugar beet pulp

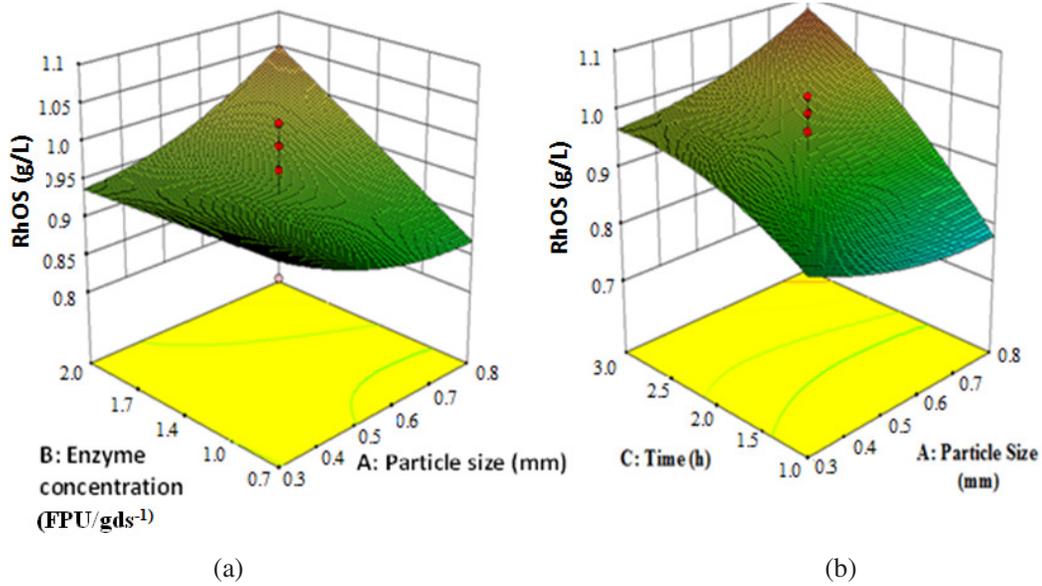


Fig 2.3- Response surface and contour plot showing the effect of interaction between (a) particle size and enzyme concentration (b) particle size and time on rhamno-oligosaccharides (RhOS) production from sugar beet pulp

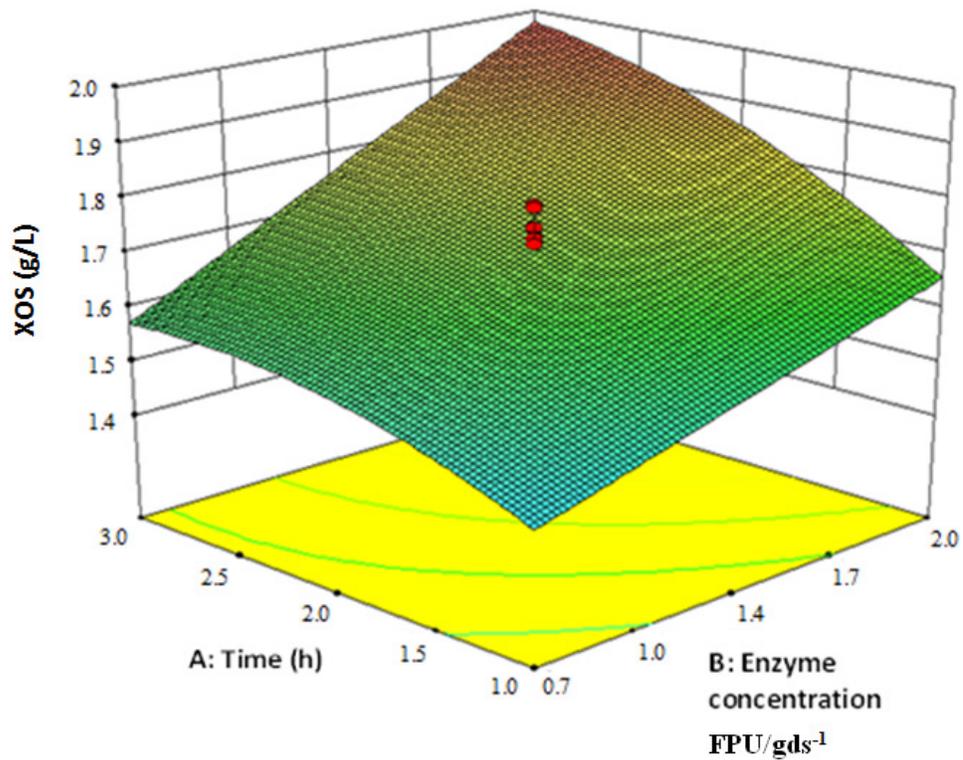


Figure 2.4- Response surface and contour plot showing effect of interaction between enzyme concentration and time on Xylo oligosaccharides (XOS) production from sugar beet pulp

2.3.5. Validation using optimized parameters

The numerical optimization package suggested 38 different combinations. We conducted an experiment with a particle size of 0.8 mm, Viscozyme at 0.75 FPU/ g d.m. for 3h. Under these conditions models predicted concentration of GalaOS 12.0 g/L, AOS 17.7 g/L, GalOS 5.1 g/L, RhOS 0.9 g/L and XOS as 1.6 g/L while the obtained values through validation were similar *i.e.* 13.2, 15.2, 5.1, 0.9 and 1.4 g/L respectively. Therefore Viscozyme used at 0.75 FPU/g d.m is the most appropriate concentration to obtain optimum concentration of pectic oligosaccharide.

2.4 CONCLUSIONS

In this study, a combination of cell wall degrading enzymes successfully produced pectic oligomers in a one-step approach. By using a statistical design based on RSM, the Viscozyme concentration, the hydrolysis time and particle size were optimized for oligosaccharide production. The effect on the various oligosaccharides was evaluated. A validation experiment was carried out using Viscozyme concentration 0.75 FPU/ g d.m., time 3h and particle size 0.8mm. Under these conditions, the rhamno-oligosaccharides (RhOS), arabino-oligosaccharides (AOS), galacto-oligosaccharides (GalOS), xylo-oligosaccharides (XOS) and galacturonic acid-oligosaccharides (GalaOS) concentration of 0.9, 15.2, 5.1, 1.4, 13.2 g/L respectively, obtained through the validation experiment were similar to the theoretical values for GalaOS, RhOS, lower for XOS and AOS and higher for GalaOS predicted by the software.

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3.EFFECT OF EXTRACTION CONDITIONS ON THE SACCHARIDE (NEUTRAL AND ACIDIC) COMPOSITION OF THE CRUDE PECTIC EXTRACT FROM VARIOUS AGRO-INDUSTRIAL RESIDUES

Based on Neha Babbar, Sandra Van Roy, Marc Wijnants, Winnie Dejonghe, Augusta Caligiani, Stefano Sforza, Kathy Elst. Effect of extraction conditions on the saccharide (neutral and acidic) composition of the crude pectic extract from various agro-industrial residues. *Journal of Agriculture and Food Chemistry*. <http://dx.doi.org/10.1021/acs.jafc.5b04394>

3.1 INTRODUCTION

During the production of agricultural commodities, various agro-industrial residues are generated that can be a potential source of valuable compounds, such as antioxidant, protein, pigment and starch. Some of these residues are rich in pectin- a carbohydrate polymer. The structure of pectin is hypothesized to be made of smooth and hairy regions. The smooth region, also known as homogalacturonan, is composed of (1,4)- linked galacturonic acid residues backbone while the hairy region, recognized as rhamnogalacturonan, comprises an alternating backbone of galacturonic acid and rhamnose residues ($\rightarrow 2$)- α -L-Rha- (1 \rightarrow 4)- α -D-GalA-($\rightarrow 1$)_n.¹ The hairy appearance of RG is due to the presence of branched arabinan, galactan, arabinogalactan chains at O-4 of rhamnose residues.²

Pectin is typically categorized into three classes: (1) soft pectins, extractable with dilute salt solutions; (2) chelator-soluble pectins, extractable with calcium-chelating solutions such as ethylenediaminetetraacetic acid, cyclohexanediaminotetraacetic acid and sodium hexametaphosphate, and (3) protopectin, extractable with alkali or hot dilute acid solutions.³ The widely used method for liberating pectin from plant materials is acidic extraction. The principle involves the hydrolysis of the protopectin which results in diffusion of pectin from the cell walls.⁴ The extraction with chelators, on the other hand, liberates calcium bound pectin by loosening the egg box structures formed by homogalacturonan and calcium complex.⁵

A major challenge in pectin recovery from residues concerns the extraction process used for its isolation. Previous studies have indicated that the extraction process can cause a high degree of pectin degradation, resulting in a low yield and a loss of pectin functionality *i.e.* gelling property.⁶ This can partially be due to the uncontrolled generation of pectic monomers, as a result of partial pectin hydrolysis, that fail to precipitate during purification.⁷ Knowledge on these monomers, present in the crude extract but lost during pectin purification, aids to design a high yield recovery of minimally damaged pectic polysaccharides.

Moreover, there is an increasing interest in pectin-based derivatives. This includes for instance the pectic oligosaccharides with a dedicated chain length that recently have gained interest as a potential prebiotic agent and are produced from the homogalacturonan and rhamnogalacturonan moieties.⁸ These type of products are in full exploration and are not expected to have the same requirements as that of pectin which is used for traditional applications. To reduce production costs, they are produced from a crude rather than from a purified pectin, and their compositional and molecular requirements, as galacturonan content or esterification degree, may be different. Therefore, also for this applications an accurate knowledge is needed on the influence of the extraction technology on the chemical composition of the crude extract.

For the production of these dedicated pectin-based derivatives, un-common agro-industrial by-products may become of value. This includes for instance by-products that are smaller in volume, different in composition or not having the required properties as the ones typically used for pectin production. Berry pomace (BP), onion hulls (OH) and pressed pumpkin (PP) are by-products generated during processing of berries, onions and pumpkin. These by-products are expected to have potential because they have not extensively been exploited for commercial purposes. Their properties can however not be assessed properly

since only scanty literature is available on their pectin content/pectic saccharide composition.⁹⁻¹² Sugar beet pulp (SBP), on the other hand, a by-product of sugar beet processing, is more widely studied and contains 12-13% pectin on dry matter basis.¹³ Nevertheless, the highly-branched pectin composition makes it less suitable for typical pectin applications, necessitating the search for other types of outlets.

It has been reported that different sources of pectin are affected differently by various extraction conditions.¹⁴ The main aim of the present study is therefore to study the effect of the extraction on the composition of neutral (arabinan, galactan side chains) and acidic (galacturonic acid polysaccharide) pectic polysaccharides and their degradation into low molecular weight sugars in the crude extract. Four different wastes are assessed, being BP, OH, PP and SBP, and the effect of different extraction methods, *viz.*, the use of acids (nitric acid), enzymes and chelators is analyzed on the recovery and composition of crude pectic polysaccharides.

3.2 MATERIAL AND METHODS

3.2.1 Raw materials

The agro-industrial residues *i.e.* onion hulls (OH) and sugar beet pulp (SBP) were provided in a dried condition by the Institut für Getreideverarbeitung (IGV GmbH), Germany. Dried and deseeded berry pomace (BP) was provided by Eco Treasures, Belgium. Pressed pumpkin cake (PP) was procured in a wet condition from Provalor, The Netherlands, and then dried for 24 h in a vacuum oven (Thermo Scientific) to a moisture content of 7-8%. The residues were milled with a laboratory blender (Kenwood), sieved to a particle size smaller than 0.8 mm, and stored in zip-lock bags at room temperature until use. Celluclast 1.5 L (C-2730), Viscozyme L (V-2010) and standards for rhamnose, arabinose, galactose, xylose, glucose, fructose and galacturonic acid were purchased from Sigma-Aldrich (St. Louis, MO,

USA) and Merck (Germany). The dextran oligomers (molecular mass 180-1223000 Da) and analytical grade chemicals used during extraction were also purchased from Sigma-Aldrich.

3.2.2 Total sugar composition of the raw material

The total sugar composition of the raw materials was determined by performing two types of hydrolysis followed by the analysis of the monosaccharides. In the first, ground BP, PP, SBP and OH residues (1 g each) were subjected to enzymatic hydrolysis at a substrate concentration of 5% (w/v) in 100 mL erlenmeyer flasks. An enzyme cocktail of Celluclast 1.5L and Viscozyme L, each at 20 U/g d.m. (based on filter paper activity, FPU/ mL), was added to the biomass suspended in 50 mmol/L citrate buffer (pH 4.8). The filter paper activity of the enzymes was analyzed using laboratory analytical procedure of National Renewable Energy Laboratory.¹⁵ The flasks were incubated in an incubator shaker (Innova, New Brunswick, USA,) at 48 °C and 150 rpm for 48 h. The hydrolyzed biomass was centrifuged at 5000 xg for 10 min. The supernatant (containing the monosaccharides) was assayed for its monosaccharide composition by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, see Section 2.4). Fructose and glucose were corrected for the amounts of sucrose present in the enzyme cocktail. In comparison, also a seamans hydrolysis was performed. In short, it comprises of treatment of biomass with 11.5 M H₂SO₄ at 20 °C for 2 h followed by dilution of 11.5 M H₂SO₄ to 1 M and further keeping at 100 °C for 2h.¹⁶ The hydrolyzed biomass was centrifuged at 5000 xg and analyzed on HPAEC-PAD (HPAEC-PAD, see Section 2.4).

3.2.3 Extraction of pectin from agricultural residues

To extract intact pectic polysaccharides from the four agro-industrial residues, three types of extraction methods were investigated. All the extraction processes were carried out in 100 mL conical flasks using 1g of biomass at 5% (w/v) substrate loading. Enzymatic

hydrolysis was carried out using Celluclast 1.5 L which primarily contains cellulases (endo-glucanase units) and has been found to be effective in extracting pectin from plant cell walls.¹⁷ While acid extraction was carried out using dilute nitric acid.¹⁸ For the extraction of pectin from OH, in addition to acid and enzyme extraction, chelator based extraction was carried out using ammonium oxalate¹¹ and sodium hexametaphosphate.¹⁹ The selection of extractant was based on the inefficiency of conventional extractant *i.e.* enzyme and nitric acid to release pectin from OH stated elsewhere in the article. After each extraction, the biomass was centrifuged at 5000 x g for 10 min and the supernatant was assayed for sugars on HPAEC-PAD (see Section 2.4). The extraction conditions for different agro-industrial wastes is given in Table 3.1.

Also, the monosaccharide and polysaccharide composition of each biomass was determined by dissolving the sample (1g) at 5% (w/v) in distilled water at 48 °C for 4h.

3.2.4 Analysis of total free monosaccharides and pectic polysaccharides by HPAEC-PAD

All extracts were analyzed on HPAEC-PAD in two ways, *i.e.*, without further treatment to assess its monosaccharide content, and after complete hydrolysis to determine its total saccharide content. 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 of galacturonic acid and the neutral sugars, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h.²⁰ Viscozyme L is a multienzyme complex composed of pectinases, hemicellulases and arabinases.²¹

Table 3.1: Summary of extraction conditions used for extracting pectin from agro-industrial residues.

Extractant	Hydrolysis medium	pH during hydrolysis	Concentration of extractant	Incubation conditions	Time (h)	Neutralization	Enzyme inactivation
Nitric acid	Water	1.4	0.4% w/v	48 °C, 150 rpm	12-48	10 M NaOH to pH 4.8	-
Enzyme (Celluclast L)	Citrate buffer 50 mmol	4.8	20 FPU/g d.m	80 °C, 125 rpm	2-8	-	100 °C, 5 min
Ammonium* Oxalate	Water	4.8	0.5 and 1% w/v	90 °C**	0.5-2	-	-
Sodium hexameta phosphate*	Water	4.8	1-5% w/v	95 °C**	0.5-1	-	-

* used only for onion hulls

** without stirring

The hydrolysis method is based on the work of Martinez and coworkers.²² It is worth mentioning that the commonly used acid hydrolysis was not suitable for these pectin rich extracts, even when applying the mild conditions of the seamans hydrolysis. In all cases low amounts of galacturonic acid were found due to degradation (data not given). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 5000 x g for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into a high performance anion exchange chromatography with pulsed amperometric detection (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 with a Carbpac PA -1 (4mm X 250mm X 4 mm) column coupled to a guard column Carbpac 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 mobile phase was used at a flow rate of 1 mL/min for 46 min. The monosaccharides were quantified by comparing them with the concentration of known standard solutions (ranging from 10 mg/L to 1000 mg/L) of rhamnose, arabinose, galactose, glucose, xylose, fructose and galacturonic acid.

3.2.5 Analysis of hydroxymethylfurfural (HMF) and furfural.

The formation of HMF and furfural in acid treated samples were determined by UPLC. The system used was from Waters coupled with a PDA UV detector, a column, Kinetex 2.6 μ biphenyl 100A with dimensions of 100 mm X 2.1 mm. The flow rate was maintained at 0.4 mL/min. The integration of the peaks were done on the basis of wavelength at 240 nm. The temperature of the column was set at 40 °C. The system was eluted isocratically with a mixture of 99% water and 1% acetonitrile.

3.2.6 Analysis of the methylation and acetylation degree.

Methylation and acetylation degrees were determined as previously reported.²³ Briefly, 30 mg of freeze dried crude extracts containing pectic material were incubated (2 h, room temperature) with 1 mL 0.4 M NaOH in D₂O and 0.1 mL of internal standard (TSP, 2 mg/mL in D₂O). The supernatant was centrifuged (Centrifuge eppendorf 5810R, EPPENDORF Augsburg, Germany), clarified (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 collected at 298 K, with 32K complex points, using a 90° pulse length. 128 scans are 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 quantitative determination of acetic acid, methanol and ferulic acid was obtained by manual integration of the corresponding signals (1.920 ppm for acetic acid and 3.358 for methanol) and the comparison with the TSP area.

3.2.7 Assessment of the molecular weight.

The molecular weight (MW) of pectic polysaccharides from BP, SBP, PP and OH was determined by gel permeation chromatography against dextran standards. In this system, the pectic polysaccharides are separated based on their different hydrodynamic volumes (radius of gyration). Since the MW estimations were done using dextran standards which have different hydrodynamic volumes as compared to pectic polysaccharides, only approximate estimations of the molecular weight were obtained. All the samples were filtered through a 0.45 µm membrane filter prior to analysis. A gel permeation chromatography system from Shimadzu, Japan was used and consisted of a solvent degasser (DGU-20 A), quaternary pump (LC-20AT), refractive index detectors (RID-10A), auto sampler (SIL-20A) and column

oven (CTO-20AC). An isocratic method using 0.1% sodium nitrate as eluent was applied. The flow rate was maintained at 0.6 mL/min and the temperature of the columns was maintained at 40° C. The system contained a guard column followed by two separation columns, *i.e.* Ultrahydrogel column 120 and a Ultrahydrogel column 500, both with dimension of 7.8 × 300 mm (Waters) and an exclusion limit of respectively 5x10³ and 4x10⁵. All the chromatograms were corrected for the contribution of the enzyme/ acid/ chelator by subtracting the corresponding peaks.

3.2.8 Statistical analysis and assessment of the extraction efficiency

All the experiments were conducted in triplicate 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).

The percent extraction efficiency of pectic polysaccharides extracted from agro-industrial residues was determined by the following formula:

$$\text{Extraction efficiency (\%)} = \frac{(C_{total,extr} - C_{mono,extr})}{C_{total,biomass}} \times 100$$

$C_{total, extr}$ = total concentration of galactose, arabinose and galacturonic acid found in the extract after post-hydrolysis. Expressed relative to the biomass treated (in w/w DM).

$C_{mono, extr}$ = concentration of free galactose, arabinose and galacturonic acid found in the extract by direct analysis. Expressed relative to the biomass treated (in w/w DM).

$C_{total, biomass}$ = concentration of galactose, arabinose, and galacturonic acid found after hydrolysis of the biomass. Expressed relative to the biomass treated (in w/w DM).

The extraction efficiency of rhamnose is not shown owing to its very low concentration in the biomass considered.

3.3 RESULTS AND DISCUSSION

3.3.1 Total sugar (galacturonic acid and neutral sugar) composition of berry pomace (BP), pressed pumpkin (PP), sugar beet pulp (SBP) and onion hulls (OH)

To determine the composition of pectic polysaccharides, the biomass was hydrolyzed and analyzed for monosaccharides. In particular, these are galacturonic acid as acidic sugar, and arabinose, galactose and rhamnose as neutral sugars. In the present study, acid hydrolysis (Seamans hydrolysis) was initially used as a first choice to completely hydrolyse the biomass and determine constituent sugars. It was found that in all cases significantly low amounts of galacturonic acid were obtained (data not shown), which relates to rapid breakdown to other degradation products. Therefore second method of hydrolyzing the biomass with enzyme containing cellulolytic, pectinolytic and hemicellulolytic activities was then evaluated. Both Viscozyme and celluclast contains several activities in the form of endoglucanase, endoxylanase, pectinase, endomannase, polygalacturonase, total cellulase, cellobiohydrolase, β -D-glucosidase, β -D-mannosidase, β -D-xylosidase, α -L-arabinofuranosidase in various proportions.²⁴ Using enzyme cocktail, the obtained values of glucose and xylose were lower, which could be due to lesser β -glucosidase and β -xylosidase activity of enzyme cocktail. Therefore, to obtain the most representative estimation, the values of galacturonic acid, galactose, fructose, rhamnose and arabinose are taken from enzymatic hydrolysis while glucose and xylose were obtained from acid hydrolysis. As also stated in the review of Sluiter et al., each method has its limitations and there is still a need to develop methods for complete hydrolysis of the feedstocks.²⁵ Moreover, it is possible that the standard method like NREL and Seamans hydrolysis which are mainly developed for lignocellulosic biomass are not completely suitable for pectin containing biomass due to sensitivity of galacturonic acid moiety. Table 2 shows the constituent sugars present in various agro-industrial residues. The results are expressed as % (w/w) d.m. The galacturonic acid content of OH is the highest

(19.3 ± 0.9 %) followed by SBP ($17.3 \pm 0.9\%$), PP (5.6 ± 0.1 %) and BP (5.9 ± 0.6 %), respectively. The value of galacturonic acid obtained for SBP in the present study is slightly lower than the 21.9% (w/w) d.m. found by Leijdekkers et al.²⁶ This can be due to a different source of the sugar beet pulp starting material.

In addition to acidic sugars, some agricultural residues contain significant amounts of the pectin-related neutral sugars *i.e.* rhamnose, arabinose and galactose.²⁷ In the present study, the total neutral sugar composition calculated as the sum of the individual neutral sugars, varied from 25.2% to 44.1% for PP and SBP respectively (Table 3.2). Whereas, the neutral sugar content of both BP and OH are 39.1% and 36.3%, respectively. The arabinose content is the highest in SBP ($16.6 \pm 0.6\%$) while the other residues contains low amounts ($0.3 \pm 0.1\%$ to $0.9 \pm 0.1\%$). The presence of minor amount of arabinose in OH in this investigation is in line with the results obtained by Matsura et al., who also found less arabinose in OH.²⁸ The rhamnose content of SBP is the highest ($1.2 \pm 0.4\%$) followed by PP ($0.6 \pm 0.4\%$), OH ($0.4 \pm 0.1\%$) and BP ($0.4 \pm 0.5\%$), respectively. The highest galactose content of 7.0 ± 0.9 % is found in OH followed by SBP ($5.7 \pm 0.1\%$), BP ($4.7 \pm 0.1\%$), and PP ($3.7 \pm 0.2\%$).

In the residues other neutral sugars such as xylose, glucose and fructose are also found and considered to be primarily originating from the hemicellulose, cellulose and residual sucrose.⁸ The glucose content of residues range from $15.2 \pm 0.3\%$ to $26.1 \pm 0.1\%$ for PP and OH, respectively (Table 3.2). In case of BP and SBP, the glucose content account for 18.9 ± 0.5 and 18.8 ± 0.2 , respectively. A significant amount of fructose up to $8.9 \pm 0.1\%$ and 4.3 ± 0.2 % is present respectively in BP and PP. Both OH and SBP contains negligible amount of fructose. Also, Chandrasekaran & Bahkali found that some fruit processing residues contain significant amounts of inverted sugars.²⁹ The remaining constituents of the biomasses were not analysed and can comprise of protein, ash, unhydrolysed fiber etc. There is still a need to develop methods for complete biomass hydrolysis of the feedstocks.²⁹

Thus, both SBP and OH contain pectic polysaccharides in a reasonable amount, while relatively low amounts of pectic polysaccharides are found in BP and PP. Also, based on the results, the highest rhamnogalacturonan sugars are found in SBP amongst the other residues. In the following sections the impact of different extractants on the extraction of pectic polysaccharides from the four residues are evaluated.

3.3.2 Effect of different methods on the extraction of pectic polysaccharides from agro-industrial residues

The effect of the hydrolysis time on the extraction of pectic polysaccharides from BP, PP, SBP and OH by both enzymatic and nitric acid treatment was evaluated. In addition, the extraction of pectic material from onion hulls was also studied with the chelators mentioned earlier in the paper. In the following section, the polysaccharides relevant to pectin are described and the results are expressed as extraction efficiency and yield as % (w/w) on dry matter for all the residues.

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3.3.2.1 Berry pomace (BP)

The pectin composition of BP is shown in Table 2, highlighting the presence of galacturonic acid and galactose as major pectic sugars. The data in Fig 3.1a shows that time has a significant effect on the recovery of galacturonic acid polysaccharides. A rapid increase in the galacturonic acid polysaccharides is seen in the first 24 h with an extraction efficiency of 59.0% and a yield of 3.5% (Fig 3.1a). It has been reported that cellulase breaks down the

Table 3.2: Galacturonic acid and neutral sugar composition of different agro-industrial residues

Residue	Galacturonic acid	Rhamnose	Arabinose	Galactose	Xylose*	Glucose*	Fructose
	% (w/w) d.m						
BP	5.9 ± 0.6	0.4 ± 0.1	0.9 ± 0.1	4.7 ± 0.1	5.2 ± 0.3	18.9 ± 0.5	8.9 ± 0.1
PP	5.6 ± 0.1	0.7 ± 0.4	0.6 ± 0.4	3.7 ± 0.2	1.4 ± 0.1	15.2 ± 0.3	4.3 ± 0.2
SBP	17.3 ± 0.9	1.2 ± 0.4	16.6 ± 0.6	5.7 ± 0.1	2.1 ± 0.2	18.8 ± 0.2	0.9 ± 0.1
OH	19.3 ± 0.9	0.4 ± 0.5	0.3 ± 0.1	7.0 ± 0.9	2.9 ± 0.2	26.1 ± 0.1	N.D

Non-hydrolysed cellulose, lignin, protein, hemicellulose and other extractives make up for the remainder of the composition.

Values are mean ± SD, *n*= 3.

BP: Berry pomace; PP: pressed pumpkin ; SBP: sugar beet pulp OH: onion hulls.

N.D- Not detectable

cellulosic material and β -glucanase further converts cellobiose to glucose, thereby liberating pectin during hydrolysis.¹⁶

Simultaneously, also increasing amounts of free galacturonic acid is formed during the extraction, accounting for 20.0% at 24h and additionally also doubles by the end of 48h. Celluclast 1.5L is a multi-enzyme complex composed of different enzyme sub components in different titres. In addition to the cellulase activity, also polygalacturonase and arabinase activity are present.²² These side-activities are considered to be responsible for increased monosaccharide concentration at extended extraction times. At the optimal extraction time of 24h, the extraction efficiency of galacturonic acid comprises of 59.0% polysaccharides and 20.5% monosaccharides which indicates that 20.0% of galacturonic acid remains unextracted. Ptichkina et al., studied the recovery of pumpkin pectin and demonstrated that the use of an enzyme from *Aspergillus awamori* having higher cellulase activity (β -glucanase) gave higher pectin recovery than the enzyme prepared from *Trichoderma viridae*.³⁰ In the present study, enzyme from *Trichoderma viridae* was used which has multi-component enzyme activity as mentioned earlier and therefore could be responsible for lower activity and hence lesser yield. For the treatment with nitric acid, time has insignificant effect on the galacturonic acid polysaccharide extraction from BP (Fig 3.1b). The highest extraction efficiency of 62.9% with a corresponding yield of 3.7% is obtained at 2 h with a levelling off of the concentration after this time. Negligible amounts of monosaccharides are formed (Fig 3.1b). A comparison of the results of Table 4.3 with those in Fig 3.1b, suggests that at an ideal extraction time with nitric acid solution, still 37.1% of galacturonic acid polysaccharides remains unextracted. For neutral sugars, the enzymatic extraction at 24h results in highest extraction and yield of galactose polysaccharides *i.e.* 85.9% and 4.0% respectively (Fig 3.1a). Also, the presence of 0.025% (w/w) HMF is observed at the end of hydrolysis, which is likely from the degradation of monosaccharides (Fig 3.2 a).

Based on the results obtained it can be inferred that nitric acid extraction seems to be more effective for the extraction of galacturonic acid polysaccharides and enzymatic extraction more suitable for galactose polysaccharides extraction from BP which can be explained owing to structure of the raw material. It is very likely that the source of enzyme and the nitric acid concentration play a significant role in the extraction. Nevertheless, further investigation with other extractants is needed to recover all the galacturonic acid polysaccharides from BP.

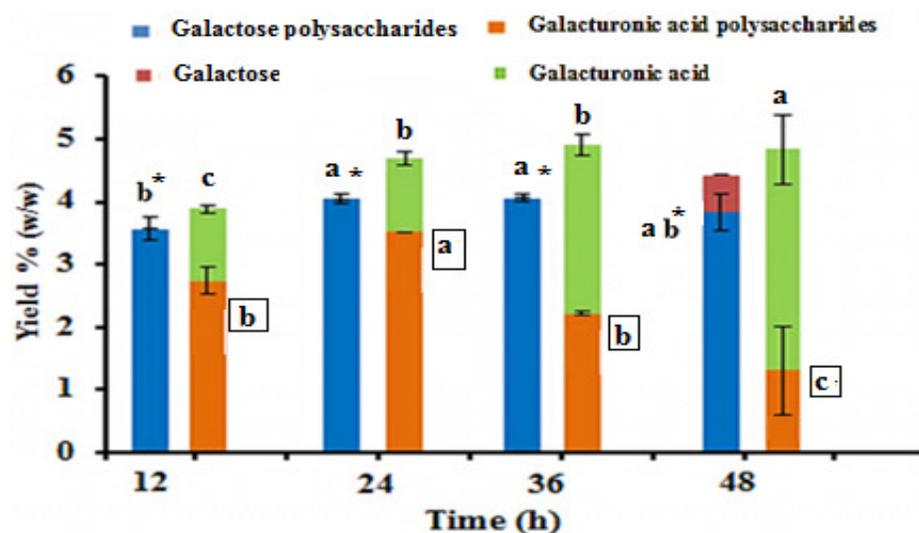


Fig 3.1 (a)

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides, and galacturonic acid are 0.34, 0.76 and 0.56, respectively.

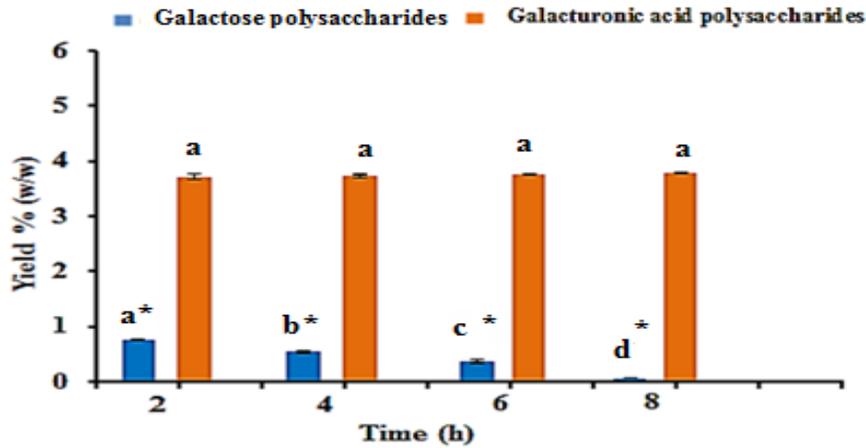


Fig 3.1(b)

LSD ($p < 0.05$) values for galactose polysaccharides and galacturonic acid polysaccharides are 0.05, 0.08 respectively.

Fig 3.1: Effect of time on the extraction of neutral and acidic pectic polysaccharides from berry pomace by (a) enzymatic and (b) chemical pretreatment

Mean values having the same superscript are not significantly different ($P < 0.05$).

Control: galacturonic acid polysaccharides is 1.1% w/w on d.m.

LSD: Least significant difference Values are reported on the basis of dry matter

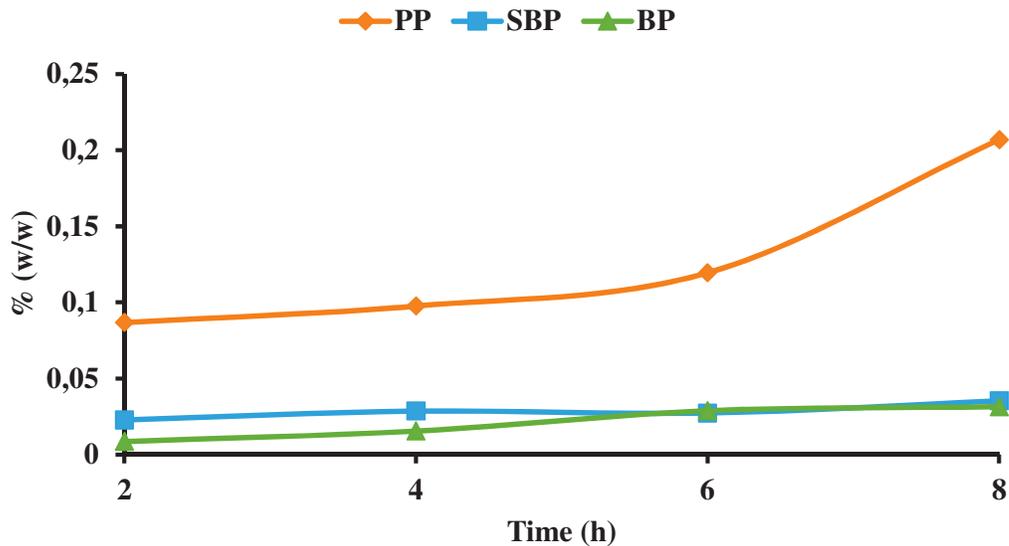


Fig 3.2 (a)

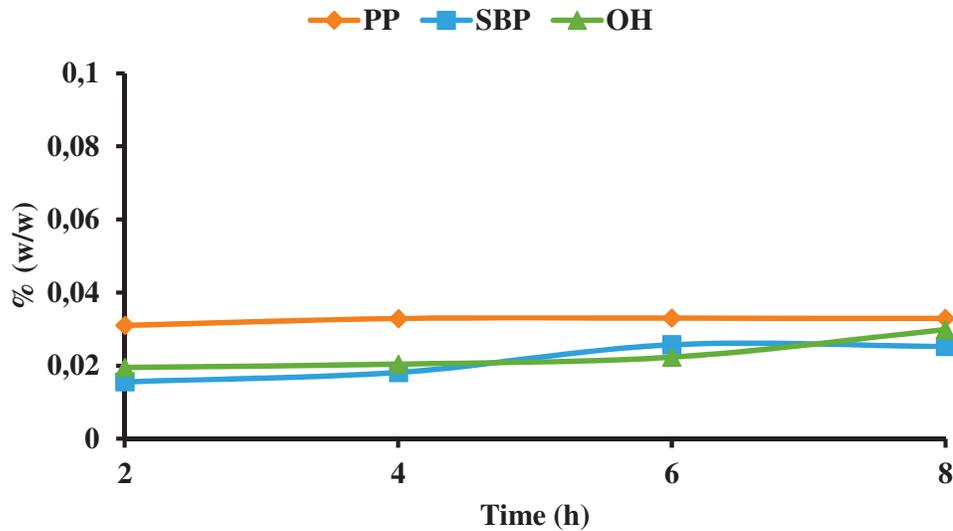


Fig 3.2 (b)

Fig 3.2 : Effect of time on the production of (a) HMF and (b) furfural during nitric acid extraction of pectic polysaccharides

BP: Berry pomace SBP: Sugar beet pulp; OH : Onion hulls; PP: pressed pumpkin

3.3.2.2 Pressed pumpkin (PP)

Pumpkin cake contains mainly galacturonic acid, galactose and smaller amount of arabinose (Table 3.3). It is evident from Fig 3.3a, that hydrolysis time in enzymatic extraction has insignificant effect on the extraction of galacturonic acid polysaccharide. An extraction efficiency of 75.0% with corresponding yield of 4.3% is obtained after 12 h of hydrolysis. Production of insignificant amounts of monosaccharides of 0.3% and 0.6% are seen at 36 and 48 h respectively with enzymatic extraction, which can probably be due to polygalacturonase activity of cellulase.²² In case of nitric acid extraction, at 6 h, an extraction of 59.2% galacturonic acid polysaccharides are obtained (Fig 3.3b). The extraction behavior of the polysaccharides in case of BP was different as the time of hydrolysis (with nitric acid) has insignificant effect on the yield. Differences in the structure of plant material are likely to have a significant effect on the extraction processes.³¹

For neutral sugars, the highest extraction of galactose polysaccharides is 44.3% and 82.2% at 12 h and 6 h hydrolysis with enzymatic and nitric acid extraction, respectively (Fig 3.3a and 3.3b). Small amount of arabinose polysaccharides (< 1%) are also extracted by both the extractants (Fig 3.3a and 3.3b). It should be noted that in PP, about 18.9% monosaccharides are already present in the control extraction. It is worth mentioning here that some HMF is formed in the beginning of hydrolysis which is due to presence of monosaccharides of galactose and a further increase in HMF content after 6 h is noted (Fig. 3.2a). Formation of furfurals were also observed in the beginning of hydrolysis and their content levelled off thereafter (Fig 3.2b). Presence of furfurals indicates the degradation of pentose sugars *i.e.* arabinose also took place during hydrolysis.

Enzymatic extraction results in highest galacturonic acid polysaccharides yield, indicating the possible hydrolysis of cellulose which led to solubilization of pectin from cell wall. The extraction of galactose polysaccharides is higher with nitric acid which is in contrast with the results obtained for BP, clearly highlighting the differences in the structure of plant materials.

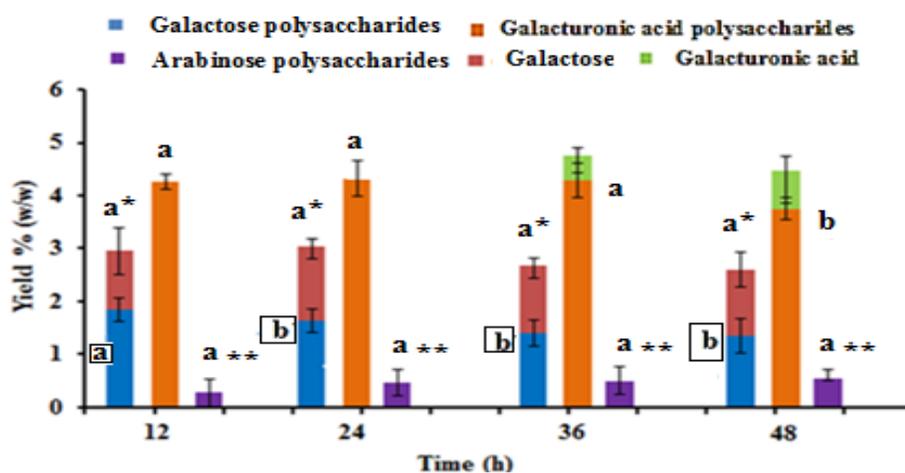


Fig 3.3 (a)

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides, galactose and arabinose polysaccharides are 0.98, 0.85, 0.80 and 0.61, respectively.

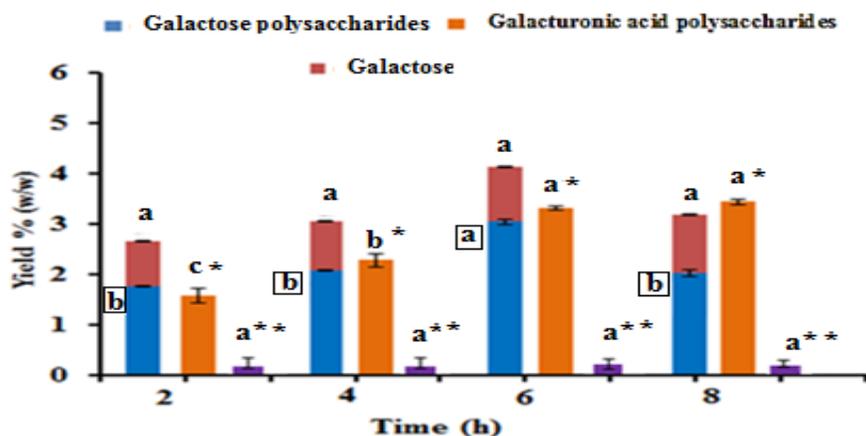


Fig 3.3 (b)

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides and galactose are 0.09, 0.20 and 0.05, respectively.

Fig 3.3: Effect of time on the extraction of neutral and acidic pectic polysaccharides from pressed pumpkin by (a) enzymatic and (b) chemical pretreatment

Mean values having the same superscript are not significantly different ($P < 0.05$).

Control : galactose 0.7% w/w ; galactose polysaccharides is 1.0 % w/w on d.m.

LSD: Least significant difference

Values are reported on the basis of dry matter

3.3.2.3 Sugar beet pulp (SBP)

The presence of both neutral and acidic sugars in sugar beet pulp indicates a substantial amount of neutral side chains and acidic backbone of pectic polysaccharides (Table 3.2). Fig 3.4a, shows that with the time of incubation in enzymatic extraction, a linear increase in galacturonic acid polysaccharides occurs. The highest extraction efficiency of 89.8% and corresponding yield of 15.5% is obtained at 48 h. Throughout the enzymatic hydrolysis, monosaccharide production is not observed. These results are in contrast to the results obtained for BP and PP, wherein a degradation of acidic polysaccharides is noted with a simultaneous increase in monosaccharides. In case of nitric acid, an extraction of galacturonic acid polysaccharides is highest at 4 h with an extraction efficiency of 76.5% and yield of 13.2%, after which it levelled off (Fig 3.4b).

Amongst the neutral sugars present in SBP, the most important neutral sugar is arabinose followed by galactose (Table 3.2). The enzymatic extraction of arabinose polysaccharides does not result in any monosaccharide production (Fig 3.4a). The highest extraction efficiency of 97.5% and yield of 16.2% is obtained at 48h. However, a continuous decline of 1.2 and 1.6 fold arabinose polysaccharide at 6h and 8h is noted with nitric acid extraction (Fig 3.4b). Formation of furfural arising from the degradation of arabinose monosaccharides is also observed in SBP and their concentration increases with the increase in incubation time (Fig 3.2b). Also a slight production of HMF is observed in the beginning of hydrolysis and their concentration levelled off thereafter (3.2a). The enzymatic extraction of galactose polysaccharides was highest at 48 h with an extraction efficiency of 98.4% (Fig 3.4a). As observed with BP, nitric acid is also not suitable for the extraction of galactose polysaccharides from SBP.

From the above results, it is clear that both enzymatic and nitric acid extraction are suitable for pectic polysaccharide extraction from SBP with enzymatic extraction giving better efficiencies and yield. The cost of nitric acid is significantly lower than enzyme, therefore nitric acid can be considered as a cheaper extractant for pectic polysaccharide extraction from SBP, since extraction times are much shorter.

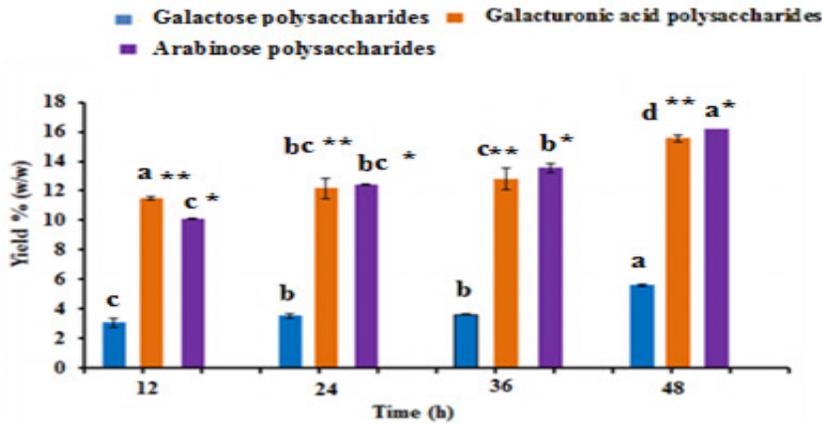


Fig 3.4 (a)

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides and arabinose polysaccharides are 0.99, 0.99 and 0.09 respectively.

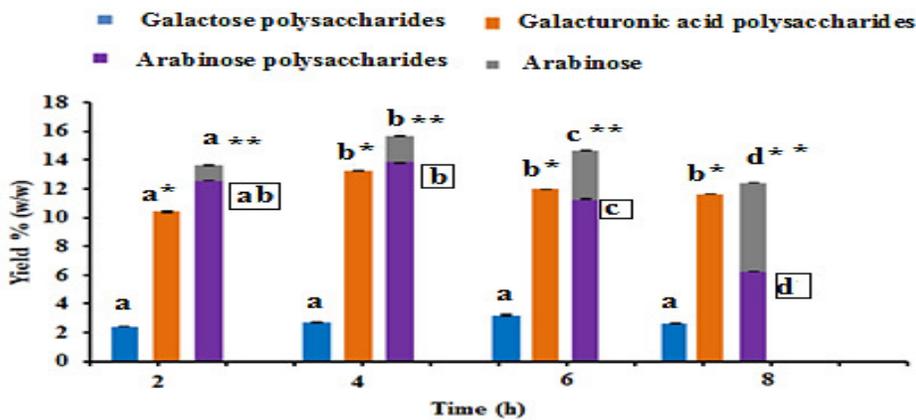


Fig 3.4 (b)

LSD ($p < 0.05$) values for galacturonic acid polysaccharides, arabinose polysaccharides and arabinose are 0.06, 0.08 and 0.08, respectively.

Fig 3.4: Effect of time on the extraction of neutral and acidic pectic polysaccharides from sugar beet pulp by (a) enzymatic and (b) chemical pretreatment

Mean values having the same superscript are not significantly different ($P < 0.05$).

Control: Arabinose polysaccharides is 5.5 % w/w; galactose polysaccharides is 1.4 % w/w; galacturonic acid polysaccharides is 6.6 % w/w

LSD: Least significant difference

3.3.2.4 Onion hulls (OH)

Onion hulls have a good potential for pectic polysaccharide extraction because they contain a reasonable quantity of galacturonic acid (Table 3.2). As evident from the results given in Fig 4.4a, enzymatic extraction did not facilitate the extraction of galacturonic acid polysaccharides from OH and a yield of 2.1% was obtained at 12 h without further increase. It is likely that the pectin was strongly bound through calcium cross links and could therefore not be extracted by simple enzymatic hydrolysis (Fig 3.5a). To the best of our knowledge, this is the first time that the extraction of pectic polysaccharides from OH with enzymes is described. With nitric acid, although, an increase in hydrolysis time has a significant effect on the extraction, but the overall yield of galacturonic acid polysaccharides remains low. The highest galacturonic acid polysaccharide yield of 3.1% is achieved at 8 h (Fig 3.5b). A slight increase in furfurals are observed in onion hulls (3.2b).

Kalapathy & Procter³² reported that acid strength used for extracting pectin from soy hull has a significant effect on pectin extraction while a longer time and higher extraction temperature does not affect the pectin yield. It is therefore likely that further lowering of pH can help in breaking the recalcitrant bonds to liberate pectin, but at the same time further lowering of pH can damage the neutral side chains.⁸ The inefficient extraction of galacturonic acid polysaccharides by both acid and enzyme suggests that pectic substances in OH are tightly bound within the cell wall, probably *via* Ca²⁺ cross bridges between adjacent galacturonic acid sequences within pectin polymers.¹¹ Therefore, extraction with two different chelators *i.e.* ammonium oxalate and sodium hexametaphosphate was investigated. The results in Fig 3.5c shows that the variation in galacturonic acid polysaccharide content is insignificant with respect to the concentration and time of ammonium oxalate extraction. The galacturonic acid polysaccharide are completely extracted with yield of 22.3% with 0.5% ammonium oxalate at 0.5h. For sodium hexametaphosphate, on the other hand, a significant

interaction between extraction time and concentration of the chelator on galacturonic acid polysaccharide formation is observed (Fig 3.5d). Full extraction and a yield of 23.1% is obtained with 2% sodium hexametaphosphate at 0.5h with a marginal decline after 3% concentration. At sodium hexametaphosphate concentration of 4% and 5%, the pectin formed a highly viscous mass. This viscous mass may have induced diffusion limitation in the enzymatic post-hydrolysis which could be responsible for an underestimation. A lower extraction of galacturonic acid polysaccharide with sodium hexametaphosphate at 1 h in comparison to 0.5 h is observed (Fig 3.5d). However, it is likely that this low value is again due to an underestimation of galacturonic acid polysaccharide as explained previously. Yamaguchi et al., obtained maximum pectic polysaccharides from soy hull at sodium hexametaphosphate concentration of 2%. These results are in line with the results obtained in the present study, except that a slightly longer extraction time of 3.5 h was needed.³³ The scarce availability of literature on the extraction of pectic polysaccharide from OH restricts the discussion about this specific residue. However, the results clearly demonstrate that the sodium hexametaphosphate and ammonium oxalate treatment solubilized most of the polygalacturonic acid from OH without production of monosaccharides. These results are in line with the results obtained by Kratchanova et al., for leek.³⁴ They also found that the extraction of pectic polysaccharides by chelators is less destructive. Furthermore, the remarkably high extraction obtained with sodium hexametaphosphate and ammonium oxalate confirms that most pectic substances are bound within the cell walls of OH *via* Ca²⁺ cross bridges between adjacent galacturonate sequences within pectin polymers.

Table 3.3 presents a brief summary of the results obtained at the best optimized conditions of extractants for polysaccharides extraction from all the four agro-industrial residues. Based on the detected amounts of free galacturonic acid and galactose, it is concluded that there are some losses in the quality of pectic polysaccharides during extraction

at the best optimized condition. Especially, in PP, there is a loss of about 45.8% of galactose-containing polysaccharides, in BP of 20% galacturonic acid- containing polysaccharides and in SBP of 10.1% of arabinose- containing polysaccharide, respectively, with enzymatic and nitric acid extraction. In SBP, the extraction with enzymes and in OH, extractants, sodium hexametaphosphate and ammonium oxalate resulted in pectic polysaccharides without any degradation. Further studies on MW of pectic polysaccharides obtained at the optimized extraction conditions is given in section 3.3.4.

3.3.3 Degree of Methylation (DM) and Acetylation (DAc)

The degree of methylation (DM) corresponds to the percentage of carboxyl groups esterified with methanol. The degree of acetylation (DAc) on the other hand, is the percentage of galacturonosyl residues esterified with one acetyl group.³⁵ The results for the DM and DAc are shown in Table 3.3. As expected the SBP exhibits highest DAc (59.1%) as also found in the literature.³⁶ In general, the DAc is high in pectin extracted by enzymatic extraction for SBP, PP and BP. Low values of DAc of pectin extracted by nitric acid from these residues could be due to an increased hydrolysis of acetyl esters by nitric acid. Some exceptions are found in OH, where in the highest DAc (21.0%) is exhibited by nitric acid extracted pectin (Table 3.3). On the other hand, the overall trend of increased DM is found in the samples treated with nitric acid in comparison to enzymatic extraction. This effect could partly be due to the inactivation of demethylase enzyme by nitric acid resulting in higher DM of the samples. The highest value (> 50%) of DM is found in BP followed by PP, SBP and OH (Table 3.3).

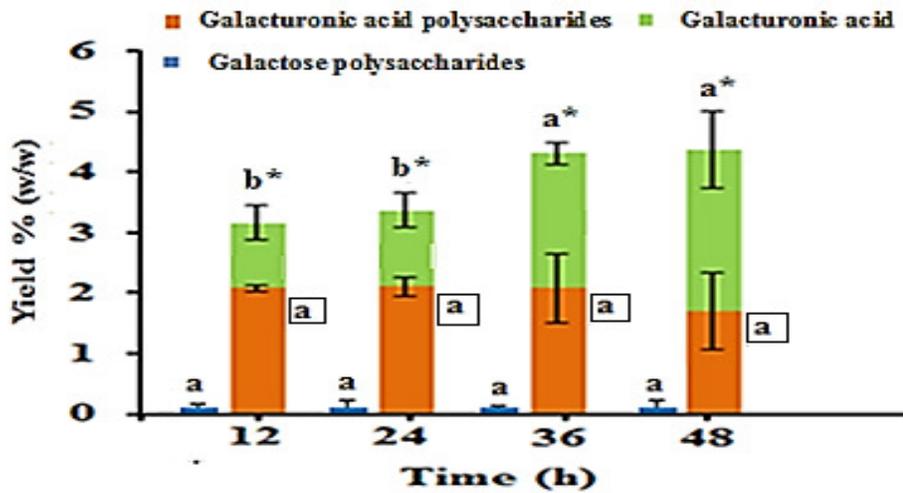


Fig 3.5 (a)

LSD ($p < 0.05$) values for galacturonic acid polysaccharides and galacturonic acid are 0.77 and 0.82, respectively.

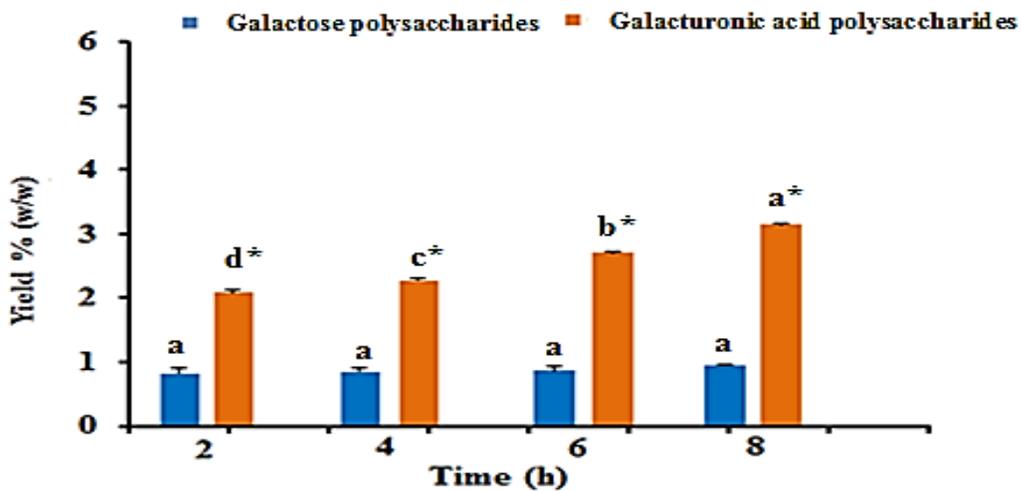


Fig 3.5 (b)

LSD ($p < 0.05$) values for galactose polysaccharides, galacturonic acid polysaccharides are 0.13, 0.01 respectively.

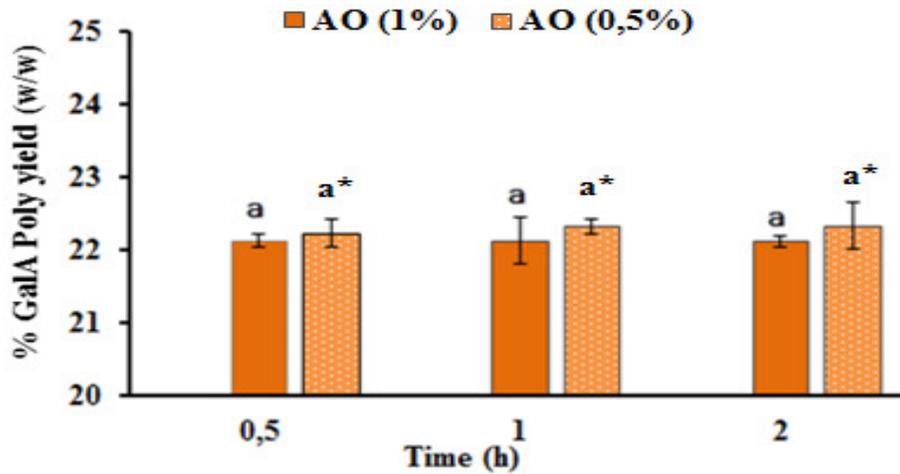


Fig. 3.5 (c)

LSD ($p < 0.05$) values for ammonium oxalate (1%) and (0.5%) are 0.39 and 0.45, respectively.

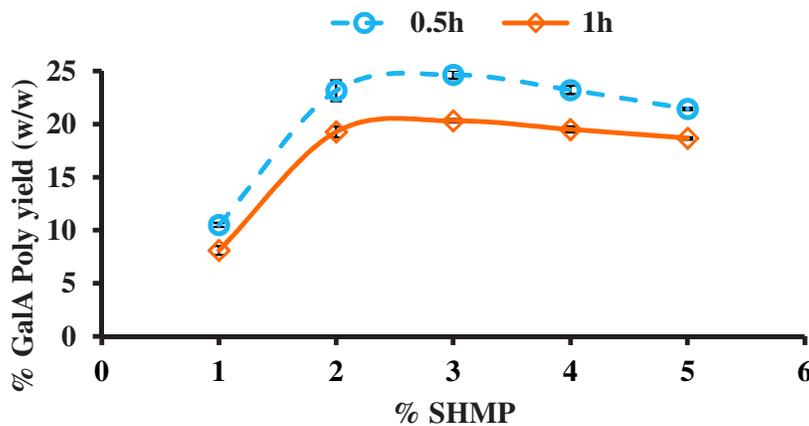


Fig. 3.5 (d)

LSD ($p < 0.05$) values for galacturonic acid polysaccharides (0.5 h) and (1 h) are 0.48 and 0.30 respectively.

Fig 3.5: Effect of time on the extraction of neutral and acidic pectic polysaccharides from onion hulls by (a) enzymatic and (b) chemical pretreatment (c) ammonium oxalate and (d) sodium hexametaphosphate

Mean values having the same superscript are not significantly different ($P < 0.05$). The superscripts of different sugars are marked with asterisk sign to depict the difference.

Control: Did not show extraction of any monomer /polymer.

LSD: Least significant difference

Values are reported on the basis of dry matter.

Table 3.3: Effect of best pretreatment on the extraction efficiency of polysaccharides (POLY) and monosaccharides (MONO) and degree of acetylation & methylation of pectin extracted from various agro-industrial residues

Residue	Extraction process	Conditions	Extraction efficiency %							
			Galactose		Arabinose		Galacturonic acid		DAc (%)	DM (%)
			POLY	MONO	POLY	MONO	POLY	MONO		
BP	Enzymatic	24 h	85.9	N.D	N.D	N.D	59.0	20.0	6.4 ± 0.4	32.7 ± 1.7
	Nitric acid	2 h, pH 1.4	15.9	N.D	N.D	N.D	62.9	N.D	4.7 ± 0.7	72.5 ± 5.1
PP	Enzymatic	12 h	44.3	37.7*	N.D	N.D	75.0	N.D	58.3 ± 5.4	57.6 ± 5.8
	Nitric acid	6 h, pH 1.4	82.2	N.D	N.D	N.D	59.2	N.D	16.3 ± 0.5	72.1 ± 1.6
SBP	Enzymatic	48 h	98.4	N.D	97.5	-	89.8	N.D	59.1 ± 0.9	51.9 ± 0.9
	Nitric acid	4 h, pH 1.4	53.9	N.D	83.2	10.9	76.5	N.D	48.5 ± 0.4	62.6 ± 0.8
OH	Enzymatic	12 h	N.D	N.D	N.D	N.D	10.8	15.5	8.1 ± 0.4	29.2 ± 1.7
	Nitric acid	8 h, pH 1.4	13.4	N.D	N.D	N.D	16.3	N.D	20.6 ± 6.9	56.3 ± 3.4
	Ammonium oxalate 0.5% (w/v)	0.5 h, 95 °C	N.D	N.D	N.D	N.D	100	N.D	3.7 ± 0.8	18.6 ± 0.4
	Sodium Hexameta phosphate 2.0% (w/v)	0.5 h, 95 °C	N.D	N.D	N.D	N.D	100	N.D	4.0 ± 0.3	23.8 ± 1.3

* 18.9 % of galactose monosaccharides were already present in the control sample BP: berry pomace; PP: pressed pumpkin; SBP: sugar beet pulp; OH: onion hulls; DAc: Degree of acetylation; DM: Degree of methylation

3.3.4 Molecular weight distribution of pectic polysaccharides extracted from agro-industrial residues

The chromatogram showing the molecular weight distribution of polysaccharides obtained from agro-industrial residues at their best optimized conditions is shown in Fig 3.6. Since, no further purification was performed, the relative abundance of log MW values derived from size exclusion chromatogram constitutes information on the crude pectin. The crude pectin also contains contaminants like degradation products of cellulose and hemicellulose. The HPSEC chromatograms (Fig 3.6 a) show the MW distribution of polysaccharides extracted from BP with enzymatic and nitric acid extraction at their optimized levels. With nitric acid, higher molecular weight (MW) fractions *i.e.* 40-5371981 Da are extracted in comparison to enzymatic extraction. Formation of lower MW fractions in enzymatic extraction is in agreement with the detection of higher amount of galacturonic acid monomers during extraction. Also, the presence of low molecular weight fractions in both enzymatic and nitric acid could be from the hydrolysis of cellulose. In case of PP, the higher MW fractions *i.e.* 31- 1461234 Da extracted with nitric acid show that there is less degradation of pectic polysaccharides in comparison to enzymatic extraction (Fig 3.6b). In case of SBP, the fractions obtained with enzymatic extraction have higher MW 54- 2299596 Da in comparison to nitric acid (Fig 3.6c). This indicates higher degradation of pectic polysaccharides with nitric acid resulting in smaller MW fractions, which is evident from the degradation of arabinose polysaccharide (Fig 4.3b). From the results of Fig 3.6d it can be derived that in case of OH, fractions of lower MW are formed with both enzymatic and nitric acid extraction. In comparison, pectic polysaccharides extracted by chelators, reveal the presence of one significant peak in ammonium oxalate (961-145352 Da) and sodium hexametaphosphate (1814-119497 Da) extracted pectic polysaccharides with few smaller

peaks (Fig 3.6e). Therefore, both sodium hexametaphosphate and ammonium oxalate can be considered as smooth extractant due to solubilization of higher molar mass.

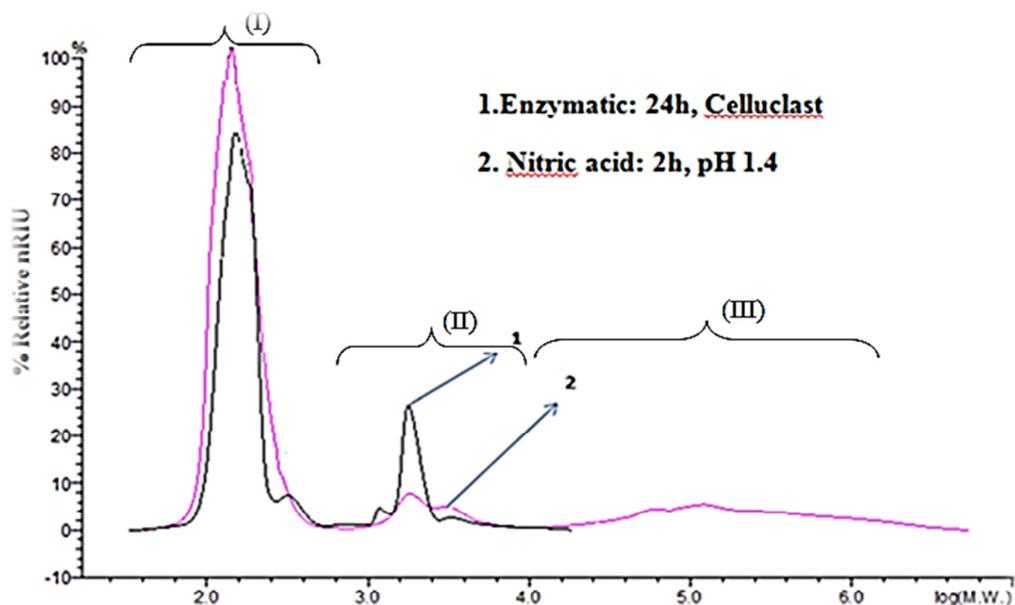


Fig 3.6(a)

1. EAE: Peaks of MW (I) 18479-2604 (II) 2604-507 (III) 507-33
2. NAE: Peaks of MW (I) 5371981-10554 (II) 10554-702 (III) 702-40

The highest peak is put on 100%

Fig 3.6 (a): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from BP by enzymatic and nitric acid

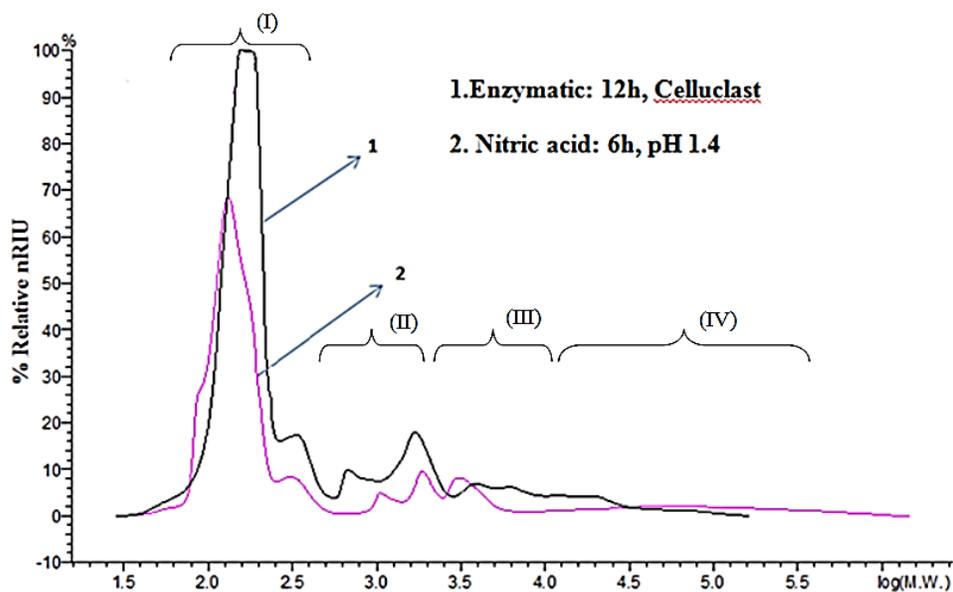


Fig 3.6 (b)

1. EAE: Peaks of MW (I) 163374-30252 (II) 30252-2766 (III) 2766-540 (IV) 540-28
 2. NAE: Peaks of MW (I) 1461234-6079 (II) 6079-647 (III) 647-31

The highest peak is put on 100%

Fig 3.6 (b): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from SBP by enzymatic and nitric acid

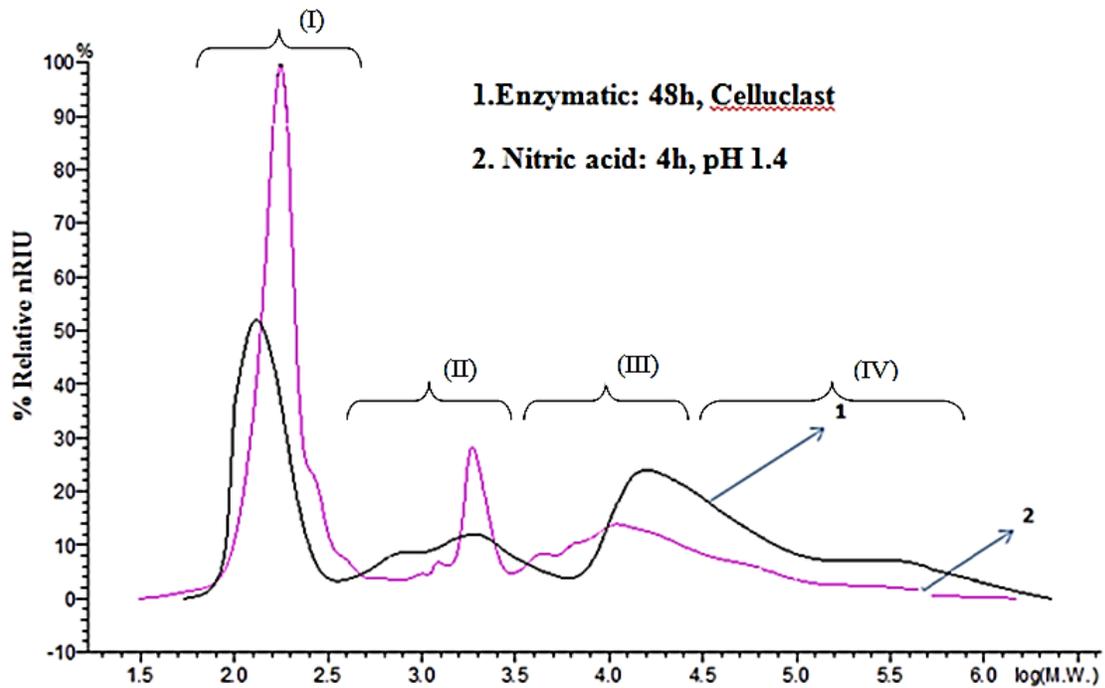


Fig 3.6 (c)

1. EAE: Peaks of MW (I) 2299596-167967 (II) 167967-5963 (III) 5963-341 (IV) 341-54
2. NAE: Peaks of MW (I) 1513202-118702 (II) 118702-2939 (III) 2939-476 (IV) 476-31

The highest peak is put on 100%

Fig 3.6 (c): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from PP by enzymatic and nitric acid

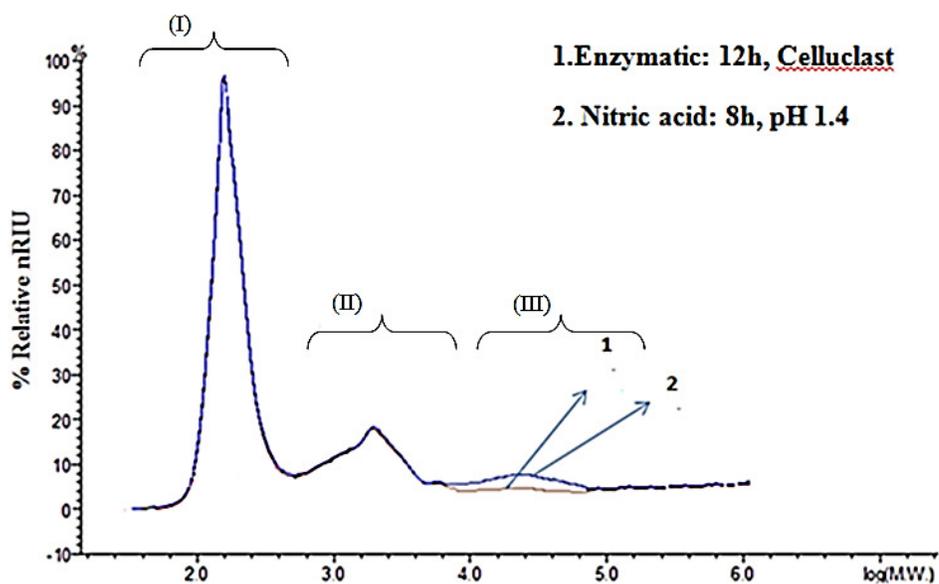


Fig 3.6 (d)

- 1. EAE : Peaks of MW (I) 224565-9567 (II) 9567- 444 (III) 444-45
- 2. NAE: Peaks of MW (I) 295827- 9682 (II) 9682- 453 (III) 453- 49

The highest peak is put on 100%

Fig 3.6 (d): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from OH by enzymatic and nitric acid

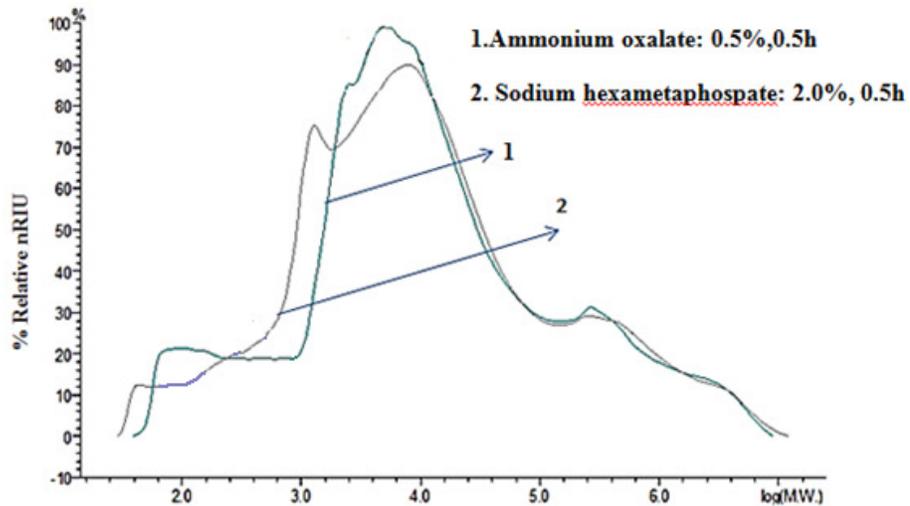


Fig 3.6 (e)

1. AO: Peaks of MW (I) 145352-961 (II) 961-39
2. SHMP: Peaks of MW (I) 119497-1814 (II) 1814-588 (IV) 588-29

The highest peak is put on 100%

Fig 3.6 (e): Relative abundance of log MW values derived from Size exclusion chromatograms of polysaccharides extracted from OH by Ammonium oxalate and sodium hexametaphosphate

MW: molecular weight; BP: berry pomace; PP: pressed pumpkin; SBP: sugar beet pulp; OH: onion hulls

3.4 CONCLUSION

This study shows that out of the four residues *i.e.* sugar beet pulp (SBP), onion hulls (OH), berry waste (BW) and pressed pumpkin (PP), substantial amounts of galacturonic acid polysaccharides and both galacturonic and arabinose polysaccharides are present respectively in OH and SBP. This study provides information on the best suitable extraction method for producing a crude pectin extract causing minimal damage to pectic polysaccharides from the agro-industrial residues under study. The present research further broadens the possibilities for the production of pectic oligosaccharides and partly for producing minimally damaged pectin at an industrial scale especially useful for gelling properties.

3.5 ACKNOWLEDGMENT

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4. CONTINUOUS PRODUCTION AND SEPARATION OF PECTIC OLIGOSACCHARIDES (POS) FROM CRUDE PECTIC EXTRACT OF SUGAR BEET PULP (SBP) IN A CROSS FLOW CONTINUOUS ENZYME MEMBRANE REACTOR

4.1 INTRODUCTION

Agro-industrial residues in the form of pulp, peel and seed are discarded after the processing of agricultural commodity. The residues, although, a waste for the processor can be a useful substrate for the production of other valuable products. These valorization practices not only reduces the environmental pollution but also help in production of valuable products from cheaper sources. Most of the agro-wastes are rich in compounds such as cellulose, hemicellulose, pectin, gums, colorants and bioactive compounds. Amongst the current valorization practices, the production of pectic oligosaccharides (POS) from pectin rich waste is a recent development. In general, oligosaccharides have found applications in various fields, notably because of their specific biological activities such as antifungal, phytoalexin-elicitors in plants antibacterial agents.¹⁻³ Also, the prebiotic potential of POS has been reported as they selectively increase the population of beneficial bacteria such as *bifidobacteria* and *Eubacterium rectale* in human gastrointestinal tract.⁴ Some of the pectin rich agro waste like sugar beet pulp, orange peel waste and olive pomace are already being investigated for POS production.⁵⁻⁷ Amongst all, sugar beet pulp is considered as a very interesting substrate for POS production owing to the presence of abundant neutral sugars in addition to acidic homogalacturonan in the pectin.^{4,8,9} Sugar beet pulp is a lignocelluloic by-product of the sugar refining industry and is mostly used as animal feed. It is mainly composed of 22-24% cellulose, 30 % hemicellulose, 15-25% pectin and 10% of proteins, in addition of remaining small amounts of fat, ash and lignin at 1%, 4% and 6% (w/w).¹⁰ The typical structure of pectin extracted from sugar beet pulp has been found to contain galacturonic acid, rhamnose, arabinose, galactose and several other sugars. The structure of

pectin has been hypothesized to be consisting of a pattern of “smooth” homogalacturonic acid and ramnified “hairy” rhamnogalacturonic regions in which most of the neutral sugars are located.¹¹ Homogalacturonan is the most abundant pectic polysaccharide and is made of GalpA residues with α -1,4-linkages and comprises more than 65% of pectin.¹¹ The RGI backbone is composed of [\rightarrow 2)- α -L-Rhap- (1 \rightarrow 4)- α -D-GalpA-(\rightarrow 1] repeats.¹² Rhamnose is a minor component of the pectin backbone and introduces a kink into the straight chain.¹³ The production of POS involves tailoring of pectin to smaller chain of oligomers with varying degree of polymerization. Most food grade oligosaccharides are manufactured using enzymatic tailoring of the pectin. So far, the work reported on POS production from agro – waste has been carried out at shake flask level. Some scanty literature on the continuous production of POS from pure polygalacturonic acid in a dead end set up has been reported by Olano martin et Al.¹⁴ However, we have not come across any literature reporting the continuous production of POS from sugar beet pulp pectin. Therefore, the present study was conducted in order to produce POS in a continuous cross flow enzyme membrane reactor (EMR). Membrane reactors with cross flow filtration was introduced in 1970. Enzyme membrane bioreactors have some potential advantages over classical batch wise production. This type of set-up allows (i) continuous production of the product of interest with smaller downtime and reduced cleaning sessions of reactor, (ii) prevents inhibition of the enzyme because of washing out of sugars and other inhibitors¹⁵⁻¹⁶ (iii) higher productivities per unit enzyme used (iv) control of the molecular weight distribution of fractions in the extract within the limits by the appropriate membrane used.

In the present study a new process to produce tailored POS in a continuous way by combining hydrolysis with in-situ separation in a membrane enzymatic reactor has been developed. In order to produce POS in a continuous set up, a thorough study comprising of various processes *i.e.* batch and semi continuous were conducted in order to obtain the right

amount of enzyme concentration, residence time for further production of POS in a continuous system..

4.2 MATERIALS AND METHOD

4.2.1 Raw Materials

Sugar beet pulp (SBP) was provided in a dried condition by the Institut für Getreideverarbeitung (IGV GmbH), Germany. Celluclast 1.5 L (C-2730), Viscozyme L (V-2010) and standards of rhamnose, arabinose, galactose, xylose, glucose, fructose and galacturonic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Germany). All other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). 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 was obtained from Koch membrane systems, Inc. (GB). The total filtration surface of these membranes is 0.093 m². P3-Oxonia active and Ultrasil 115 are liquid disinfectants, used for the cleaning of the reactor and membrane modules, were purchased from ECOLAB bvba (Belgium). Polygalacturonic acid was obtained from Megazyme International (Ireland). Viscozyme L (V-2010- a multizyme complex) was obtained from Sigma Aldrich (USA).

4. 2.2 Extraction of pectin from Agricultural residues

The sugar beet pulp residue at 5% (w/v) substrate loading was subjected to pretreatment with dilute nitric acid at pH 1.4.¹⁷ 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 x g for 10 min and the supernatant was assayed for sugars on HPAEC-PAD (see Section 2.6).

4.2.3 Enzyme activity measurements

The endo-polygalacturonase activity of Viscozyme was determined on polygalacturonic acid by following the protocol provided by Megazyme International (Ireland). The activity of the enzyme was determined as 4135 U/mL.

4.2.4 Tailoring of pectin to POS

Enzyme catalyzed POS production from sugar beet pulp pectin was performed at shake flask and in EMR as discussed below.

A. POS production in a shake flask

Hydrolysis of crude pectin obtained from sugar beet pulp was done with three different concentrations of viscozyme. Viscozyme used here is known for its diverse pectinase and other side chain activity.¹ To simplify, the units of viscozyme in the present study are reported in terms of Endo-pg units/mL. The activity of the enzyme was found to be 4135 U/mL. The Enzyme was diluted 50x, 30x and 10x which corresponds to Endo-pg units of 83, 138 and 414 U/mL. Further the substrate solution (crude pectin) was hydrolyzed at 10% v/v of the above concentration/ dilution of enzyme. The hydrolysis was done in shake flask (100 mL) at 45 °C for 2h in an incubator shaker (Brunswick) at 150 rpm. Samples were collected at a regular interval of 10 til 120 min. The enzyme was inactivated by thermal treatment at 100 °C for 5 min and further analyzed on HPAEC –PAD as stated elsewhere in the paper.

B. POS production in a continuous EMR

For the POS production in an EMR, firstly optimization studies were conducted by batch wise loading of substrate followed by washing of POS with water (Semi-continuous process). Information obtained from the semi continuous process was further used in the continuous process aimed at higher POS production in a steady state.

(i) Semi continuous POS production

In the semi continuous mode, the test was performed in an enzyme membrane reactor as outlined in Fig 1. The whole system was sterilized with P3-Oxonia active and ultrasil 115. The semi continuous mode of feeding consisted of filling the double jacketed reactor vessel with 600 mL sugar beet pectin extract at a substrate concentration of 50 g/L, pH 4.8. The vessel was further connected to two KOCH membrane assemblies with a membrane surface of $2 \times 0.093 \text{ m}^2$ (Fig 4.1). The system was thermostated at 45°C . Based on the inputs (enzyme concentration) from the shake flask experiments, two tests were conducted at a fixed Residence time (RT) of 30 min but varied enzyme (viscozyme) concentration of 83 and 138 U/mL. Here, also the substrate solution was hydrolyzed at 10% (v/v) with the two different enzyme concentrations. In semi continuous mode of feeding, during the entire process, a continuous flushing with water was done into the feed tank. A cross flow velocity of 1.35 m/s was maintained over the membrane surface with a peristaltic pump (Watson-Marlow 620U, 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). After every 10 min, permeate samples were taken. Since initial tests

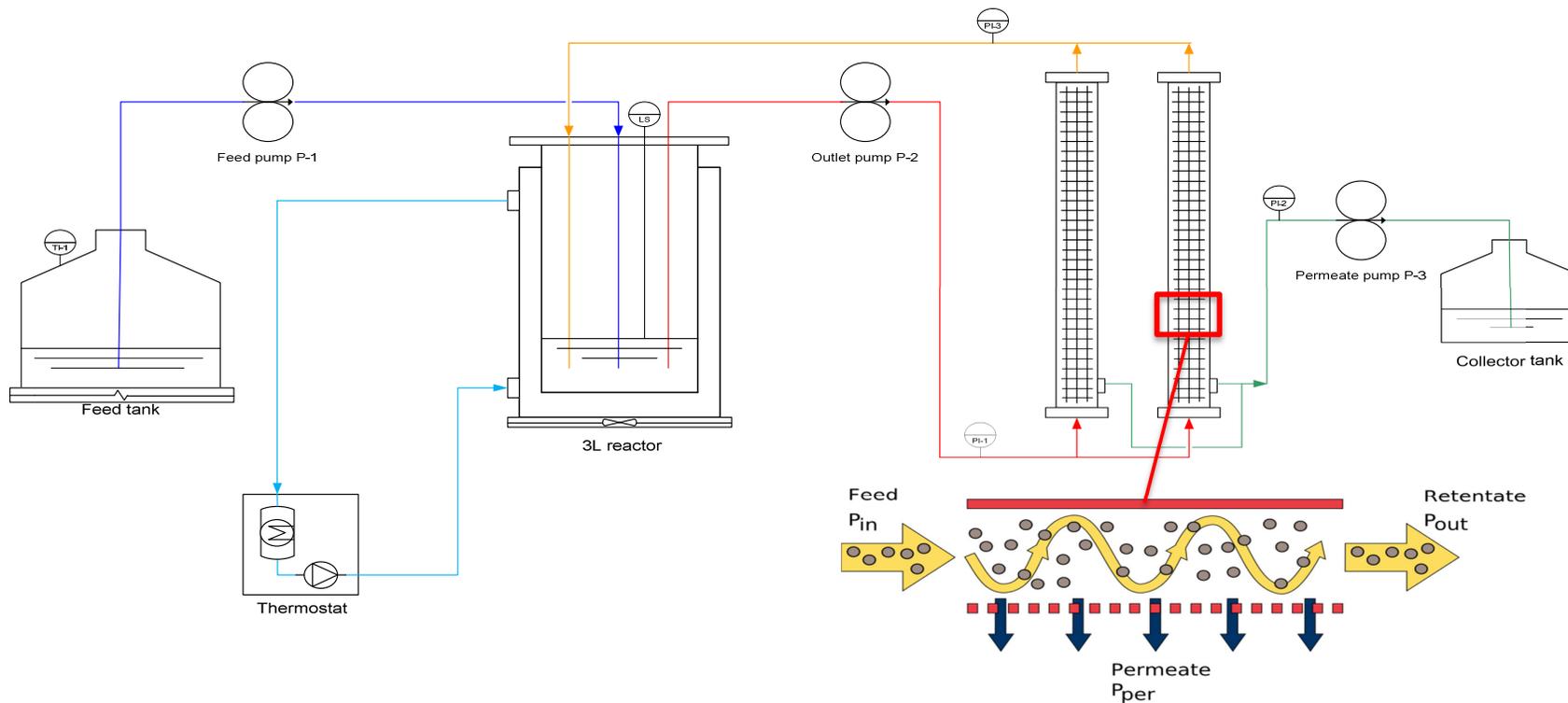


Fig 4.1a: Experimental set-up of the enzyme membrane reactor (EMR).

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.

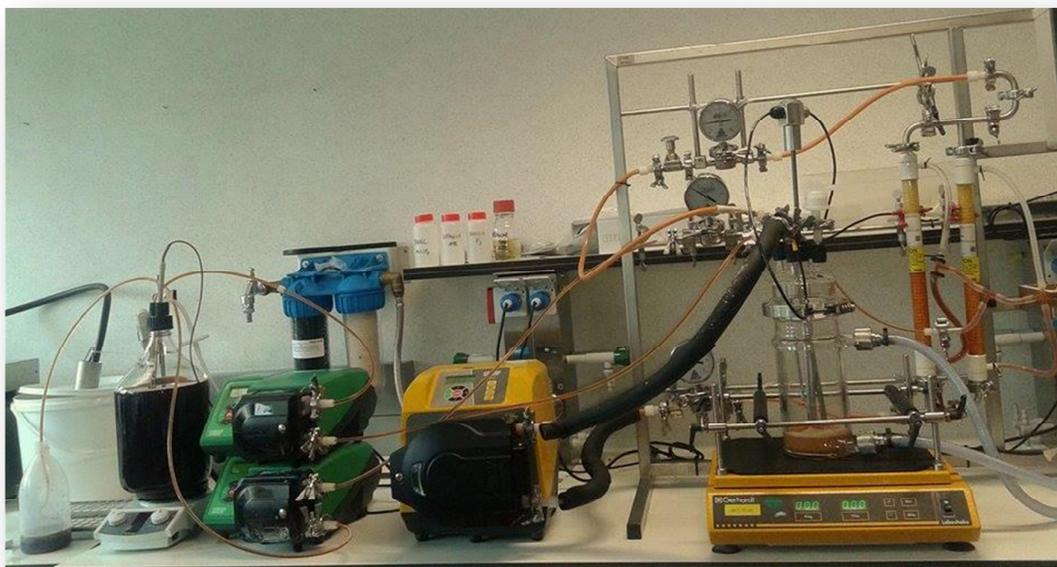


Fig 4.1b: Experimental set-up of the enzymatic membrane reactor with crossflow continuous ultrafiltration system for the production of POS.

indicated that the enzyme was completely retained in the reactor, no inactivation step was performed for the permeates. At the end of the experiment, the residue was recovered and the enzyme was inactivated by heating at 95°C for 10 min and the whole system was rinsed with MilliQ water and thoroughly cleaned.

(ii) POS production in a continuous EMR

In the process of continuous filtration, the same assembly was used as that for semi continuous process with an exception of a continuous substrate feeding. In the continuous process, the tests were conducted using one concentration of enzyme optimized in the semi-continuous test *i.e.* 83 U/mL at 10% v/v of substrate solution. The effect of different substrate loadings along with different RT were studied. In the first set, three different RT *i.e.* 13, 20 and 30 min were studied with a continuous feeding of sugar beet pulp pectin extract at a substrate concentration of 25 g/L (diluted). While the second series of tests were performed at RT of 30, 40 and 60 min with a continuous feeding of sugar beet pulp pectin extract at a substrate concentration of 50 g/L (undiluted). With both feeds *i.e.* diluted and undiluted respectively, the minimal RT than 13 min and 30 min was difficult to achieve, likely due to fouling or insufficient membrane surface area. At the start, the hydrolysis was performed in a batch mode, without substrate feeding or product filtration. Only after a fixed time (equal to the RT), the feeding of the substrate (diluted or undiluted) and the filtration of the products was started (see section 2.4). The filtration flux was set to match the average RT of the substrate in the reactor with the requested RT.

4.2.5 Start up and reactor operation

The reactor vessel was filled with 600 mL sugar beet pulp pectin extract. The reactor temperature was set at 45°C and the reaction was initiated by adding the enzyme solution (viscozyme). The substrate /enzyme solution was continuously circulated over the membrane

with a peristaltic pump P-2 (cross flow velocity of 1.35 m/s) and filtrated with a fixed flux monitored by P-3 to obtain different RT. The level in the reactor vessel was controlled with a level sensor by adding substrate into the reactor vessel using a peristaltic pump (p-1) (Fig 4.1a). Figure 4.1b shows the picture of reactor. During the hydrolysis of sugar beet polysaccharides into POS fractions (which occurred in both reactor vessel and recirculation line), enzyme and POS fractions larger than 10 kDa were retained in the system by the membrane while low molecular weight products (smaller than 10 kDa) were continuously separated from the system.

4.2.6 Analysis of total free monosaccharides and pectic polysaccharides by HPAEC-PAD

The POS fractions were analyzed on HPAEC-PAD in two ways, *i.e.*, without further treatment to assess its monosaccharide content, and after complete hydrolysis to determine its total saccharide content. 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 of galacturonic acid and the neutral sugars, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h.⁵ Viscozyme L is a multienzyme complex composed of pectinases, hemicellulases and arabinases.¹⁸ It is worth mentioning here that the commonly used acid hydrolysis was not suitable for these pectin rich extracts, even when applying the mild conditions of the seamans hydrolysis. In all cases low amounts of galacturonic acid were found due to degradation (data not given). Martinez and coworkers also used viscozyme for the post hydrolysis of pectin containing samples.⁶ After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 5000 x g for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into a high

performance anion exchange chromatography with pulsed amperometric detection (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 with a Carbpac PA -1 (4mm X 250mm X 4 mm) column coupled to a guard column Carbpac 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 mobile phase was used at a flow rate of 1 mL/min for 46 min. The monosaccharides were quantified by comparing them with the concentration of known standard solutions (ranging from 10 mg/L to 1000 mg/L) of rhamnose, arabinose, galactose, glucose, xylose, fructose and galacturonic acid.

4.2.7 Assessment of the yield, ratios and productivities

The yield (%) of POS, monosaccharides, arabino-oligosaccharides (AOS) and galacturonic acid oligosaccharides (GalaOS) (wherever reported) is calculated by:

$$Yield (\%)_{total\ POS/monosaccharides} = \frac{Total\ POS/Total\ monosaccharides}{Total\ sugars\ in\ feed} \times 100$$

$$Yield (\%)_{GalaOS/AOS} = \frac{Total\ GalaOS/AOS}{Total\ sugars\ in\ feed} \times 100$$

*for semi continuous process, yield % is depicted by cumulative of POS/monosaccharides in the subsequent permeate

The volumetric productivity (wherever reported) is calculated by:

$$\text{Volumetric Productivity (total POS) (g/l/h)} = \frac{\text{total POS g/l}}{\text{RT (min)}} \times 60$$

The specific productivity (wherever reported) is calculated by:

$$\text{Specific Productivity (total POS) g/g (enzyme)} = \frac{\text{total POS g/l}}{\text{RT (min)}} \times 60 \times \text{g/l (enzyme used)}$$

The ratios of POS/monosaccharides (wherever reported) is calculated by:

$$\text{Ratio} = \frac{\text{GalAOS/AOS}}{\text{Galacturonic acid/Arabinose}}$$

4.3 RESULTS AND DISCUSSION

4.3.1 Extraction of crude pectin from sugar beet pulp

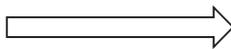
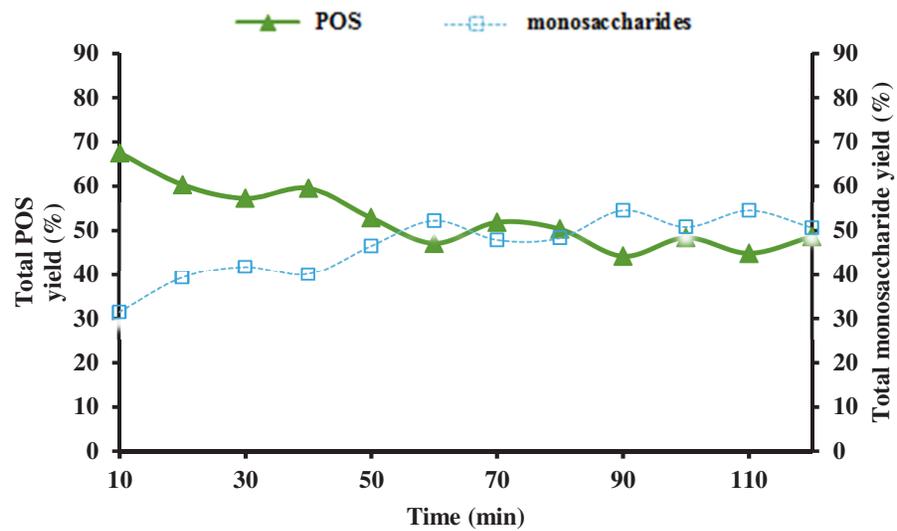
The aim of the extraction process was to release the pectic polysaccharides as intact as possible. Optimization of the extraction conditions were done in our previous work.¹⁹

The crude pectic extract after nitric acid extraction is found to contain both neutral and acidic sugars in the form of polysaccharides. The galacturonic acid (after the hydrolysis of polysaccharides) obtained in the crude pectic extract represents 13% (w/w) on d.m basis. Amongst the neutral sugars present in sugar beet pulp, the most important is arabinose followed by galactose. The extraction of 14% (w/w) arabinose on d.m basis (in its polymeric form) is obtained along with small amount of free monosaccharides accounting for 2% (w/w) on d.m basis. Previous papers reported that a considerable degradation of neutral side chains can occur at pH below 2 at longer incubation times as they are more prone to acid hydrolysis (Yapo et al).²⁰ Other neutral sugars in the form of galactose, rhamnose, xylose are also found which accounts for 4.5%, 1.5% and 1.2% (w/w) d.m respectively. Cellulosic sugar *i.e.* glucose

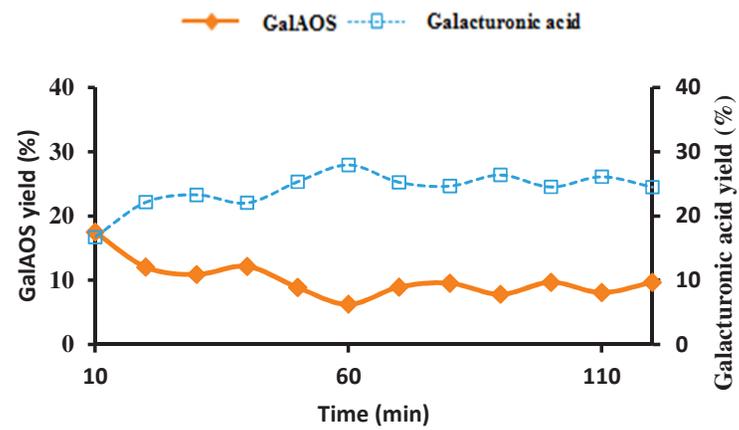
and some amount of fructose are also present amounting to 15% and 7% (w/w) d.m respectively. The fructose present in sugar beet pulp is probably from the inversion of sucrose during sugar production from sugar beet processing. The crude pectic extract was used for the production of pectic oligosaccharides (POS) in the following sections.

4.3.2 POS production from sugar beet pulp in a shake flask experiment

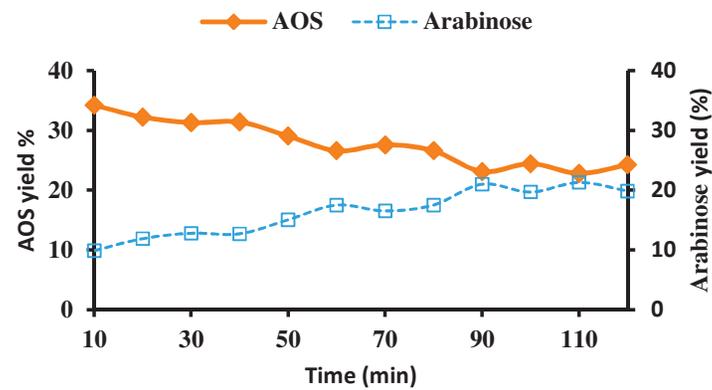
The results shown in Fig 4.2 clearly depicts that both reaction time and enzyme concentration has significant effect on the POS and monosaccharide formation. It should be noted that since there is no real separation of formed POS from the feed (pectin extract), the term POS used here is not a true POS but can be remnants of polysaccharides as well. As expected, the higher the enzyme concentration the more the monosaccharide formation (Fig 4.2). As depicted in Fig 4.2a, within the first 10 min of reaction, a simultaneous formation of 68% POS along with 30% monosaccharides is obtained at the highest enzyme dosing (414 U/mL). Thereafter, a gradual decrease of 20% POS and concomitant increase in monosaccharide is noted in the first 60 min. Later, a slow conversion of POS to monosaccharides is seen which can be due to the inhibition of enzyme in the presence of monosaccharides. Bako et al., observed that galacturonic acid formed in big amounts have significant inhibition effect on the reaction.²⁰ Fig 4.2(a-i) and 4.2(a-ii) shows that the major POS formed are



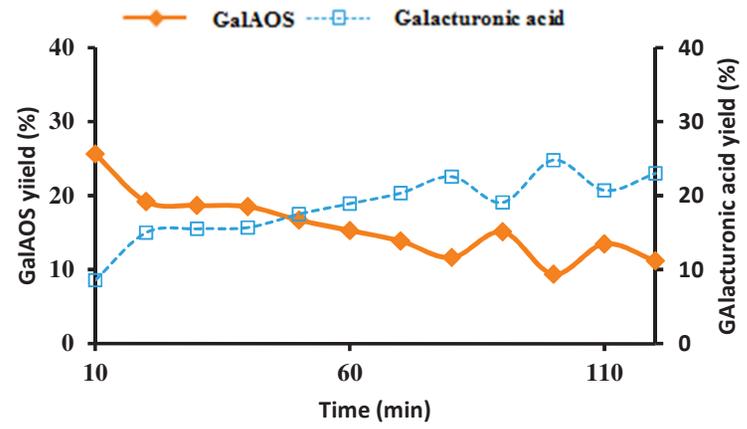
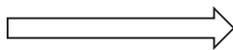
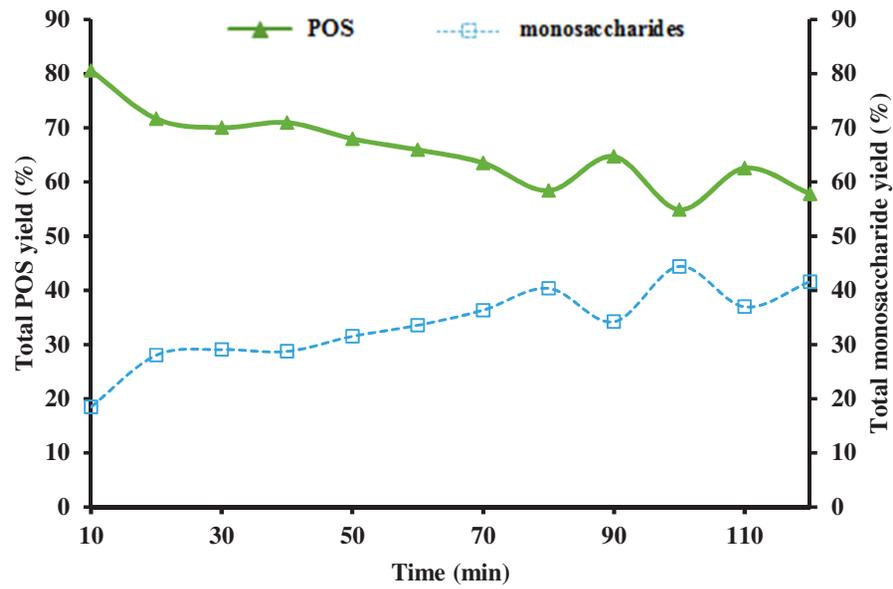
(a)



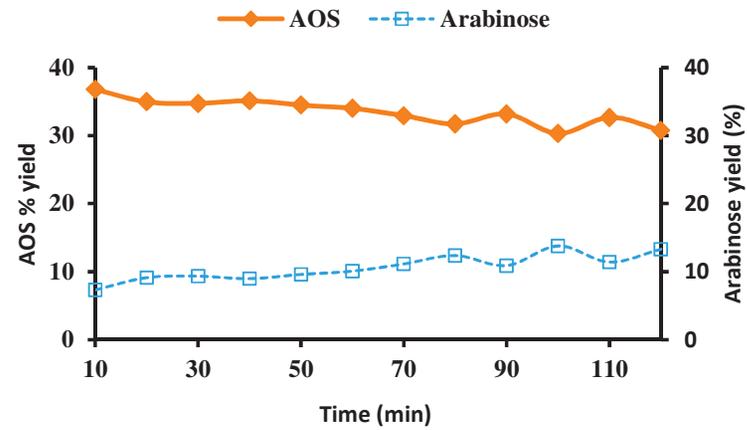
(i)



(ii)

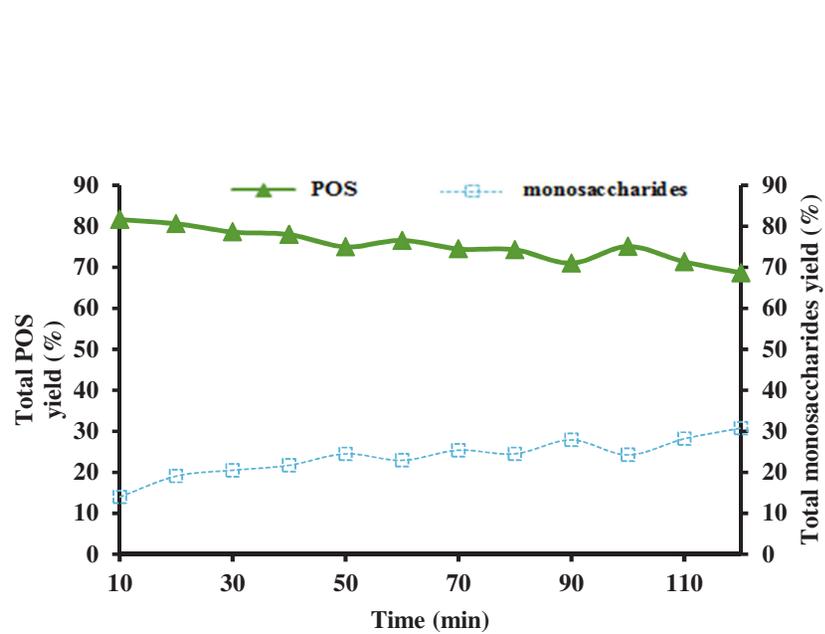


(i)

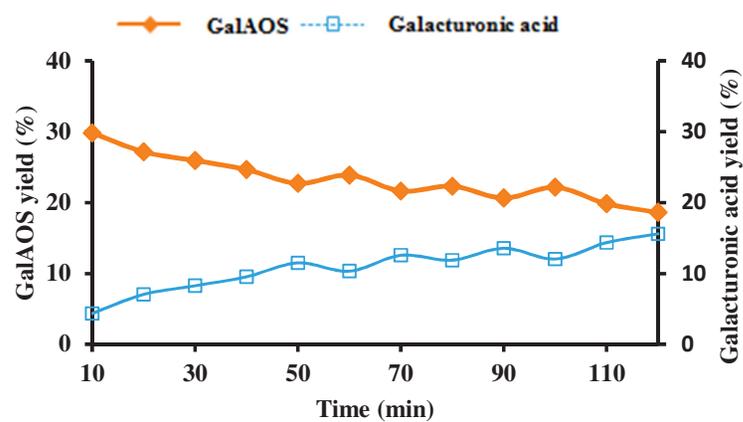
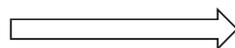


(ii)

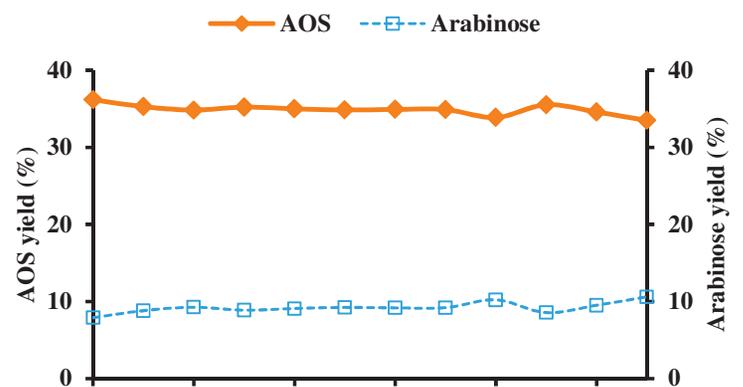
(b)



(c)



(i)



(ii)

Fig 4.2: Effect of different concentrations of enzyme (a) 414 U/mL (b) 138 U/mL (c) 83 U/mL on the yield (%) of POS and monosaccharides in a shake flask. POS: Pectic oligosaccharides; AOS: Arabino-oligosaccharides; GalAOS: Galacturonic acid- oligosaccharides

AOS and GalaOS accounting for total 50% POS fractions in the beginning of reaction. Thereafter the rate of degradation of AOS to arabinose and GalaOS to galacturonic acid is observed. As depicted in the graphs, during the first 60 min, the rate of conversion of AOS to arabinose is 10% which is comparatively slower than GalaOS conversion to galacturonic acid accounting for 50%.

On the other hand, when lower concentration of enzyme (138 U/mL) is used, the resultant fractions comprised of 80% POS and 20% monosaccharides) formed in the first 10 min (Fig 4.2b). After that, a decline of total 12% POS and concomitant increase of monosaccharides is seen in first 60 min. Further decrease of 8% POS at the end of 120 min is observed which is in contrast to the results obtained for enzyme concentration of 83 U/mL. Further the contribution of GalaOS and AOS accounted for 62% of total POS fractions in the beginning (Fig 4.2b-i and Fig 4.2b-ii). A total decline of 13% GalaOS to galacturonic acid and 4% AOS to arabinose in comparison to what was observed previously with (138 U/mL) indicates a slower conversion to monosaccharides. In case when lowest enzyme concentration (83 U/mL) is used to hydrolyze pectin, the rate of formation of POS and monosaccharides is 81% and 13% respectively (Fig 4.2c). The GalaOS and AOS accounts for total of 66% POS fraction in the beginning (Fig 4.2c-i and Fig 4.2c-ii). A total conversion of 11% GalaOS to galacturonic acid while only 2.7% AOS to arabinose is observed.

This shows that using the lowest concentration of enzyme gives higher GalaOS and AOS. Therefore, further lowering of enzyme concentration was not done because it is possible that AOS production remains unaffected which means that only longer AOS fractions will be present. At the same time, it should be noted that there are not big differences in the POS and monosaccharides formation when 138 U/mL and 83 U/mL of enzyme is used. Based on this, further optimization of POS production in EMR was done with 138 U/mL and 83 U/mL of viscozyme. Although enzyme concentration of 83 U/mL gives best results in shake flask

experiments, but this was the first time we were operating EMR so both concentration of enzymes *i.e.* 138 U/mL and 83 U/mL were used so as to avoid any fouling associated with membranes.

4.3.3 Production of POS from sugar beet pulp in an EMR

4.3.3.1 Semi continuous process

The semi continuous process of POS production consisted of a first hydrolysis with a set of RT, followed by a regular washing of the POS fractions by water. It was assessed that an average RT of 30 min would be needed to influence the hydrolysis (data not shown). Therefore, the RT was set at 30 min, to keep replenishment rates at minimum and to avoid monosaccharides formation. The different POS fractions were continuously removed from the reactor vessel by a crossflow filtration system using a 10 kDa ultrafiltration membrane. Fig 4.3 shows the cumulative distribution of POS and monosaccharides produced at an enzyme concentration of 138 U/mL and 83 U/mL over the period of 200 min. The total POS permeation accounts for 88% and 70% when 83 U/mL and 138 U/mL of enzyme is used respectively. The monosaccharides produced in the test performed with enzyme loading of 138 U/mL and 83 U/mL respectively is 36% and 34. Further the distribution of major POS *i.e.* GalaOS and AOS represents 8% & 31% and 12% & 34% respectively from test performed with enzyme concentration of 138 U/mL and 83 U/mL (Fig 4.3-i and Fig 4.3-ii).

The results also show that when lowest concentration of enzyme *i.e.* 83 U/mL is used, higher amount of GalaOS could be measured in the permeates. The impact of viscozyme dilution on AOS formation is not so significant. However, during both reactor tests, 2 to 3 times more AOS is formed in comparison to GalaOS. The rest of the POS fractions present in the permeates obtained from EMR are rich in galactose-oligosaccharides (GalOS) and xylose-oligosaccharides (XOS) with small amounts of rhamnose-oligosaccharides (ROS) (data not

shown). Since, the mass balance indicates 100% permeation from both the tests which further clarifies that all the pectin was converted to POS fractions lesser than 10 kDa. Comparing the reaction kinetics of both semi continuous and shake flask process at the same time and enzyme concentration, it can be seen that due to washing out of POS, a lesser conversion to monosaccharides occurs when membrane is coupled (Fig 4.2c and Fig 4.3).

The results in Fig 4.3, depicts that in the first 60 min, a linear increase in POS formation could be observed and thereafter only a slight increase is noted (Fig 4.3). Looking at the reaction kinetics, it was obvious that 60 RT is the most optimum time for POS production for continuous process. Since the continuous process was performed for the first time, so we decided to study different RT as well *i.e.* 40 and 30 using undiluted feed (50 g/L). However, it was observed that use of undiluted feed can cause fouling of the membrane if operated for longer duration. Therefore, effect of diluted feed *i.e.* 25 g/l on the POS production at shorter RT *i.e.* 13, 20 and 30 min was also investigated, which is theoretically the same when undiluted substrate at RT 30, 40 and 60 min is used.

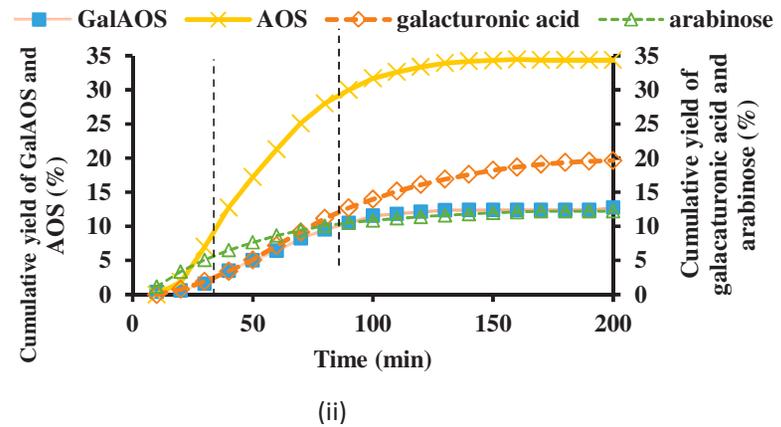
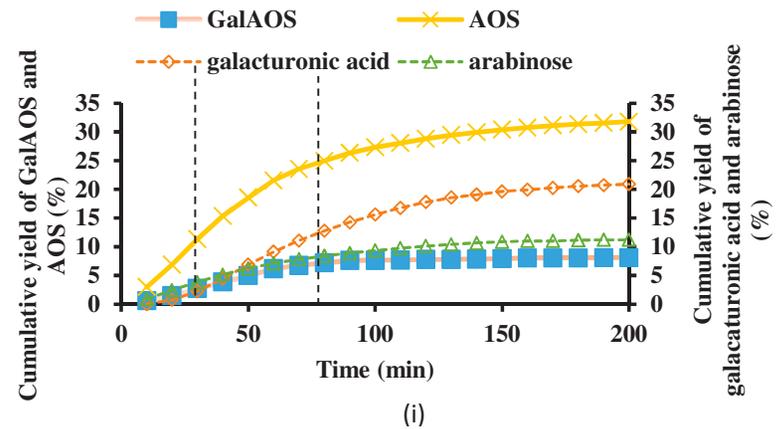
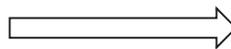
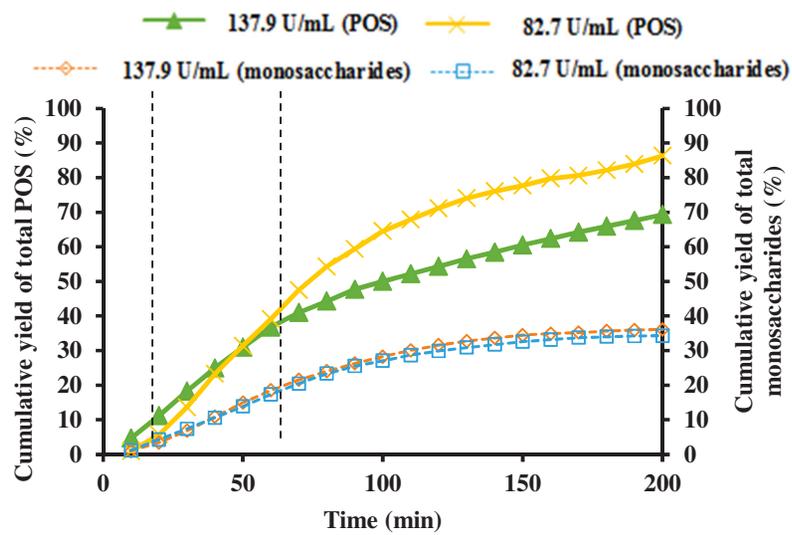
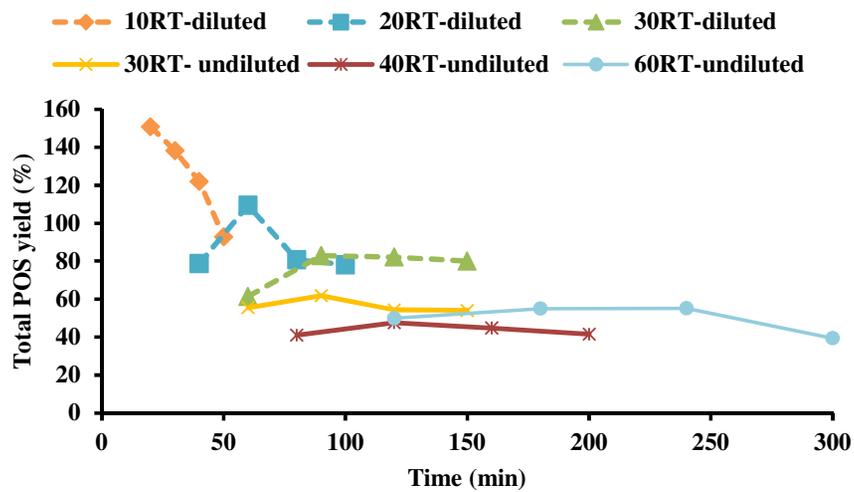


Fig 4.3: Effect of different concentration of enzyme (i) 138 U/mL (ii) 83 U/mL on the cumulative yield (%) of POS and monosaccharides in a semi-continuous test

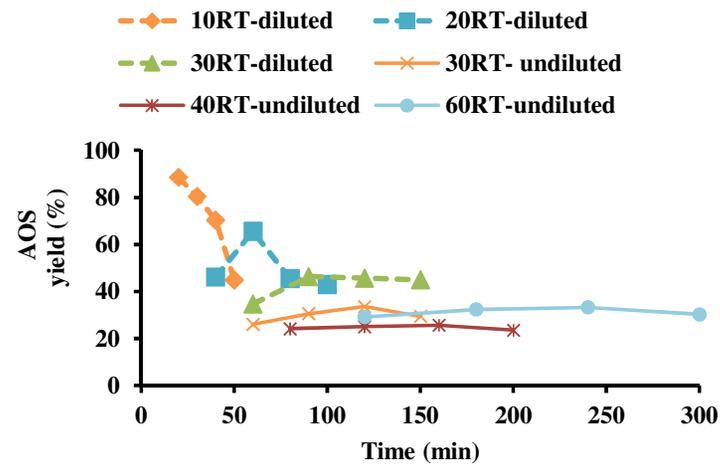
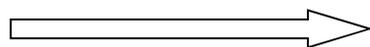
4.3.3.2 Continuous process

In order to overcome problems like cost of enzyme and monosaccharide generation in shake flask experiments, a continuous process for the enzymatic hydrolysis of pectin to POS using an EMR was introduced.

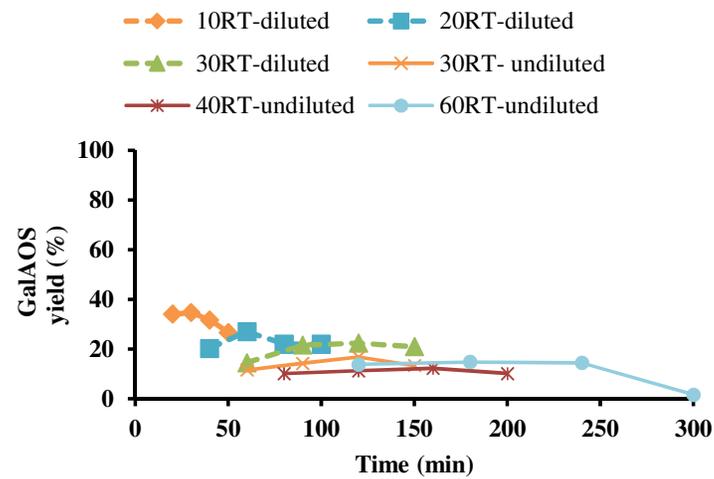
The continuous process was aimed at obtaining higher and stable POS production. Fig 4.4(a) presents recovery yield (%) of total POS at different RT and substrate concentration. In all the cases, the first permeate was discarded because it is expected to be significantly different due to start up of the reactor. The results indicates that permeates obtained with a regular dosing of diluted feed (25 g/L) shows higher POS (70-150%) and low monosaccharides (< 20%) yields (Fig 4.4a and 4.4b). It should be noted that at RT 13 min, POS yield is even more than 100% which further decreases with time (Fig 4.4a). This could be due to very short RT which resulted in a less stable system. As shown in Figure 4, except for the process conducted with feeding of diluted substrate (25 g/L) at RT- 13 min, all other conditions operated in EMR reached a steady state. As stated by Alam et al., a steady state is reached when there is a balance between the amount of substrate pumped into the system, the substrate degradation rate promoted by the enzyme and the mass transport rate through the membrane.²¹ Fig 4.4(a-i) and Fig 4.4(a-ii) presents the production yield of GalAOS and AOS which are higher when diluted substrate (25g/L) is pumped. The rest of POS are present in the form of GalOS and XOS (data not shown). On the other hand, the permeates obtained with feeding of undiluted substrate (50 g/L) have lower POS (40-60%) and higher monosaccharides (30-50%) yield (Fig 4.4a and 4.4b). It should be noted that fouling of the membrane was observed when undiluted feed was pumped. The foulant could be proteins, or even the longer chain oligomers which resulted in underpressure (data not shown). This



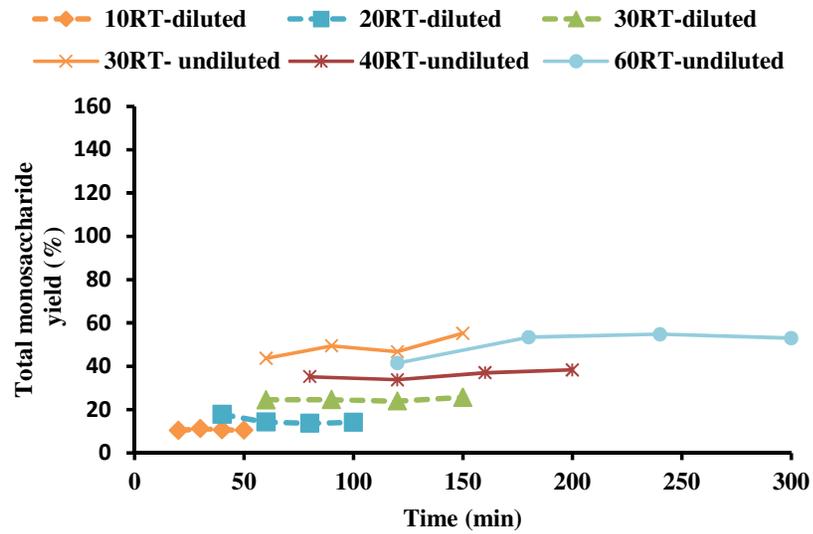
(a)



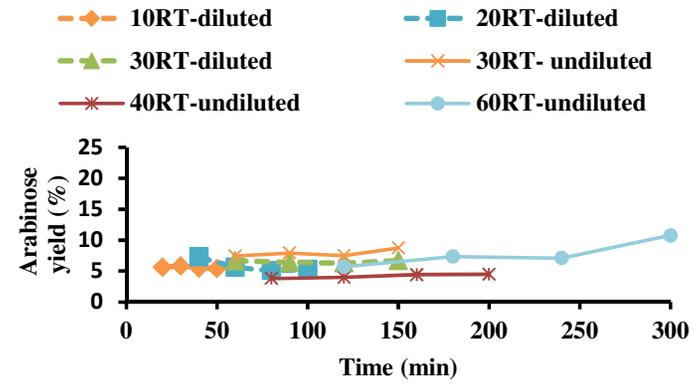
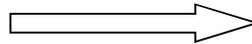
(i)



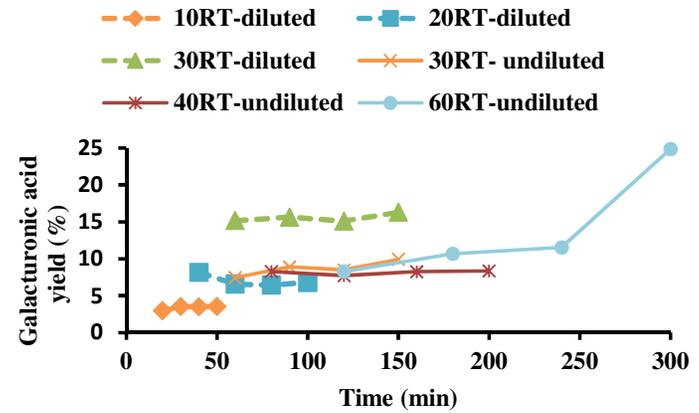
(ii)



(b)



(i)



(ii)

Fig 4.4: Effect of different substrate loading and residence time on the yield of (a) total POS: (i) AOS (ii) GalaOS and (b) total monosaccharides: (i) Arabinose (ii) Galacturonic acid in a continuous EMR

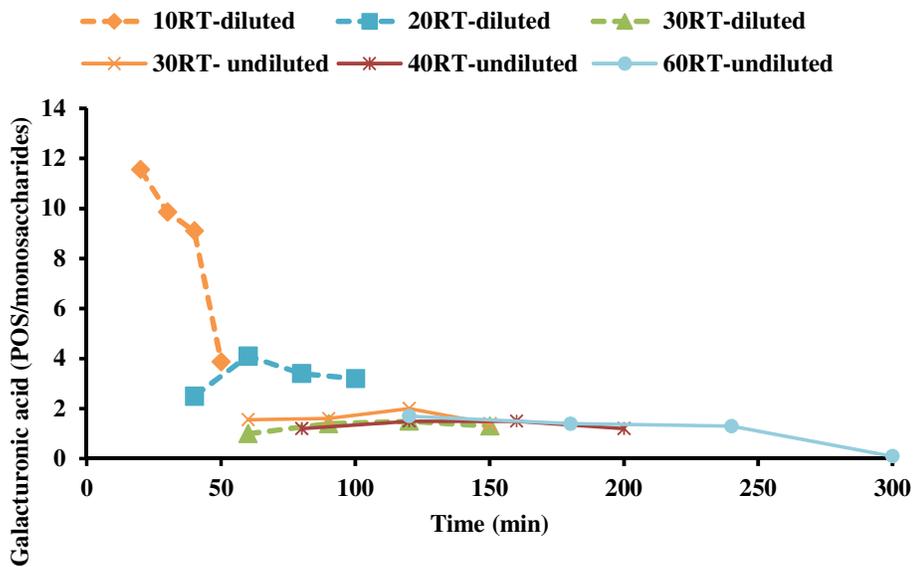
POS: Pectic oligosaccharides; AOS: Arabino-oligosaccharides; GalaOS: Galacturonic acid-oligomers; RT: Residence time

Substrate concentration- Diluted: 25 g/L; Undiluted: 50 g/L

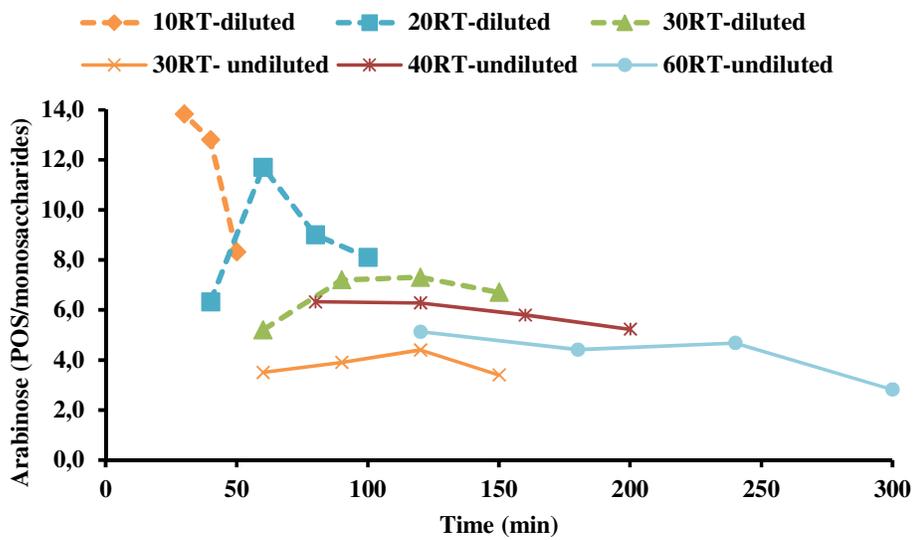
could therefore be responsible for slow permeation and hence more tailoring of POS to monosaccharides.

Overall in all the tests, some retention of saccharides (5-15%) were observed due to continuous feeding of the substrate (data not shown). The formation of monosaccharides in the form of arabinose and galacturonic acid is shown in Fig (4.4b-i) and Fig (4.4b-ii). Comparing the overall results, experiments performed with feeding diluted substrate (25g/L) at RT 20 min resulted in stable and higher POS production (80%) with lesser monosaccharide formation. Also, the highest POS to monosaccharides ratio of 3 and 6 is respectively obtained for GalaOS/ galacturonic acid and AOS/ Arabinose at RT 20 min (Fig 4.5).

The volumetric and specific productivities of POS produced in a continuous EMR is shown in Fig 4.6. The steady state as indicated by stable productivity yields are obtained at RT 40 and 60 min when undiluted substrate (50g/L) is fed and at RT 20 and 30 min when diluted substrate is used (Fig 4.6). Contrary, the volumetric and specific productivity of the reactor test performed with diluted feed (25 g/L) at RT 13 min, a decrease in POS production in function of time is noted. As stated elsewhere in the article, RT 13 min is too short for a stable POS production. Fig 4.6 also show that the continuous production of POS with diluted substrate (25 g/L) at RT 30 min results in volumetric and specific productivity of 15 g/L/h and 8 g/g E/h respectively and is half the volumetric and specific productivity of 28 g/L/h and 15 g/g E/h obtained with undiluted substrate (50g/L) feeding at RT 30 min. Also, the volumetric and specific productivity of the POS produced with feeding of diluted substrate (25 g/L) at RT 30 min is 15 g/L/h and 8 g/g E/h respectively and as expected, is almost same as that of continuous reactor test with undiluted substrate at RT 60 min resulting in 15 g/L/h and 8 g/g E/h POS respectively (Fig 4.6). It could be expected that the volumetric and specific productivity of the continuous reactor test with diluted substrate



(a)

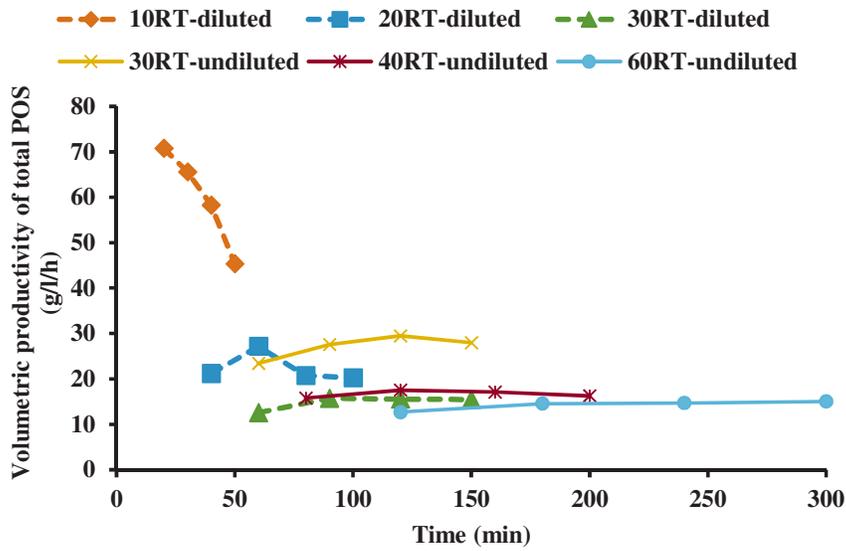


(b)

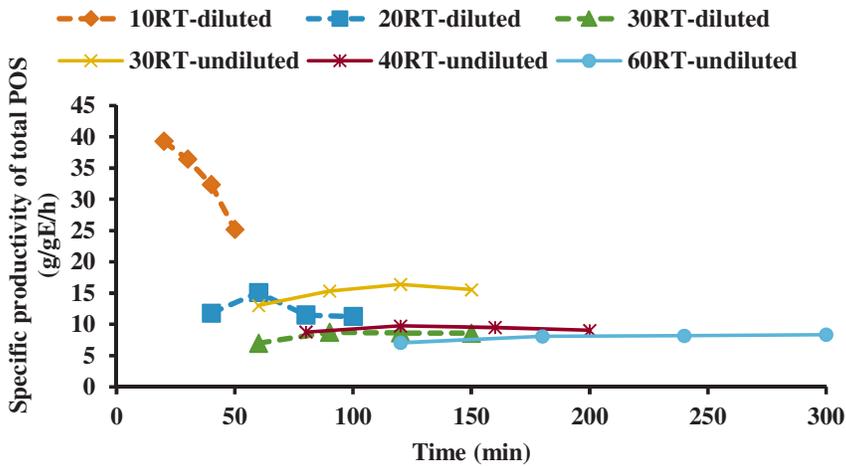
Fig 4.5: Effect of different substrate loading and residence time on ratio of POS to monosaccharides (a) GalAOS to monosaccharides (b) AOS to monosaccharides in a continuous EMR

Diluted: refers to continuous feeding at a substrate concentration of 25 g/L

Undiluted: refers to continuous feeding at a substrate concentration of 50 g/L



(a)



(b)

Fig 4.6: Effect of different substrate loading and residence time on (a) Volumetric productivity (b) Specific productivity in a continuous EMR

Diluted: refers to continuous feeding at a substrate concentration of 25 g/L

Undiluted: refers to continuous feeding at a substrate concentration of 50 g/L

RT: Residence time; POS: pectic oligosaccharides

feeding at RT 20 min is almost same as the volumetric and specific productivity of the continuous reactor test with undiluted substrate at RT 40 min. However, as depicted in Fig

(6a) and Fig (6b) there is a small deviation (10-15%) in the productivity yields. The volumetric and specific productivity of the reactor test with diluted substrate at RT 20 min is respectively 20 g/L/h and 11 g/g E/h while undiluted substrate (50g/L) feeding at RT 40 min resulted in 17 g/L/h and 9 g/g E/h respectively. This difference could be related to the uncertainty that is inherent of the analysis. Based on productivities, it can be concluded that using diluted substrate (25g/L) at RT 20 min and undiluted substrate (50 g/L) at RT-40 min results in the highest stable productivities.

The permeates in all runs were contaminated by neutral saccharides mainly glucose and fructose that arise from the raw materials (data not shown). Obtaining a pure pectate oligosaccharide solution would therefore require separation of these substrates before or after the experiment. The steady state reactions also shows that the enzyme did not lose activity over the period of time. The enzyme activity was not assessed during the operation due to limitations like presence of substrate already in the reactor which could interfere with the activity measurements.

4.3.4 Large scale production of pectic oligosaccharides in a cross flow continuous enzyme membrane reactor

In order to conduct animal feed trial experiments (by NOSHAN partner), atleast 1 kg of POS fractions were needed. The production of 1 kg POS fractions in one continuous reactor test was not feasible because the membranes were subjected to fouling which resulted in a decrease of permeability in function of time. The regular bleeding of the reactor could help but we choose for a full restart of the system. This was likely due to an accumulation of constitutnts of the feed that were not permeated. For this, six consecutive continuous reactor tests using the optimized conditions (*i.e.* 20 RT, ½ diluted feed) were performed. During the continuous membrane reactor tests total 106 L feed (2.987 kg raw material) was treated to produce about 1 kg freeze dried POS fractions for the feed trial (Table 4.1). Table 4.1 also

shows that 2.987 kg pretreated raw material contains 0.958 kg (32.1%) sugars of which 0.908 kg and 0.050 kg are polysaccharides and monosaccharides respectively. During the reactor tests, the sugars of the pretreated feed were continuously converted into POS and monosaccharides. The permeate of the different reactor tests contains 0.843 kg sugars of which 0.700 kg are POS fractions and 0.147 kg monosaccharides (Table 4.1). Besides, the total run time for reactor test 4 was 30 hours and during this time the enzyme didn't lose any activity, moreover, the enzymes produced 60-80% POS (Fig 4.7). The retention of activity of the enzymes also indicates that no inhibiting by-products are formed during POS production and are accumulated in the reactor vessel. The POS and monosaccharide yield was determined relative to the total sugars present in the raw material. Fig 4.7 shows that the POS yield varies between 60-80%, except for reactor test 3 while the monosaccharides yields varies between 8-20%. The variation between the different reactor tests can be explained by the difference in the different lots used as feed. In addition to the variability in feed also a small fluctuation of residence time occurred. Fig 4.7 also shows that a significant difference between POS and monosaccharide yields which indicates that a biocatalytic, continuous membrane reactor used for pretreated sugar beet pulp is very efficient for POS production and can suppress the production of monosaccharides at optimum conditions. Fig 4.7 also shows that in steady state the productivity of POS for the 6 different reactor tests was about 20 g /l /h, except for reactor test 3.

Table 4.1: Overview of the feed and permeates of the different reactor tests

No of Tests	Raw Material (g/L)	Feed Volume (L)	Raw material (g)	Total sugars in feed (g)	Total polysaccharides feed (g)	Total monosaccharides feed (g)	Total POS in permeate (g)	Total monosaccharides in permeate (g)
1.0	28.2	11.9	334.3	108.3	102.4	5.9	60.8	21.3
2.0	28.1	10.8	305.3	123.5	116.0	7.5	74.5	23.7
3.0	28.0	3.06	85.7	21.3	20.2	1.1	9.3	4.0
4.0	28.1	57.5	1614.1	487.1	471.0	16.1	397.0	68.9
5.0	28.2	13.8	387.2	114.5	112.3	2.2	92.9	7.4
6.0	28.1	9.3	261.3	103.0	85.9	17.1	62.3	21.2
Total	-	106.4	2987.8	957.8	907.8	49.9	696.9	146.5
Percentage in raw material	-	-	-	32.1	30.4	5.2	23.3	4.9

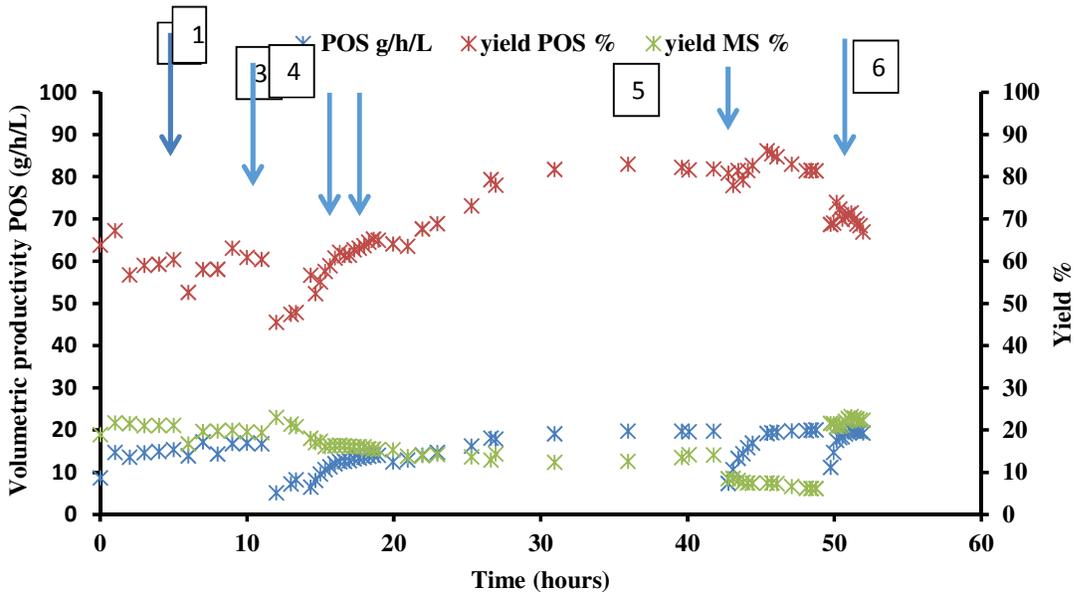


Fig 4.7: POS productivity and yield of POS and monosaccharides in the permeate of 6 reactor tests with diluted feed.

**arrows: show the start of each reactor test 1-6*

4.4 CONCLUSION

Sugar beet is an ideal substrate for the production of pectic oligosaccharides (POS) in a continuous cross flow enzyme membrane reactor (EMR). The continuous POS production using EMR indicated that dosing of diluted substrate (25 g/L) at residence time of 20 min results in a higher POS and lower monosaccharides yield of 80% and 20% respectively. The volumetric and specific productivities of 20 g/L/ h and 11 g/g E/h respectively was obtained using these conditions. The longest run was more than 36 h with stable POS and monosaccharides yield. Further experiments will be conducted to characterize and study the prebiotic activity of POS fractions.

4.5 ACKNOWLEDGMENT

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

Based on: Neha Babbar, Stefania Baldassarre, Winnie Dejonghe, Stefano Sforza and Elst Kathy. Enzymatic production of pectic oligosaccharides (POS) from onion skins. Submitted with Carbohydrate Polymers.

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.¹ 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.² 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.³ 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.⁴

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.⁵ 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.⁶ 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.⁷⁻¹³ Some scanty studies are available for POS production from potato pulp.^{14,15} Nevertheless, the search for new resources and alternatives continues.

Onion skins are known to be very rich in pectin.¹⁶ In our previous work, onion skins were found to contain around 20% (w/w) d.m of galacturonic acid.¹⁷ The waste of onion in the form of dried, whole, cut, sliced and broken pieces is significant and accounts for 8.5 x 10⁴ tons in Europe.¹⁸ 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.¹⁹ 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.^{20,21} 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 MATERIAL 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.¹⁷ Based on our preliminary tests, sodium hexametaphosphate was selected as an extractant for onion skins;¹⁷ 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 °C and measured photospectrometrically at 520 nm using the Nelson-Samogvi method.²² 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.⁹ 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.⁹ 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-DP10.⁹ 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 DP x 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.

$$R_M(DPx) = 2.23 * 10^4 * \sqrt{1/MW} \quad (\text{equation 1})$$

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.²³ The distribution of the different

oligomers was determined on the basis of retention time, molecular weight and in source fragmentation.²³ 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 (H₂O:CH₃CN 80:20, 10 mM HCOONH₄ + 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 H₂O:CH₃CN 20:80, 10 mM HCOONH₄ + 0.2% HCOOH, eluent B was H₂O:CH₃CN 80:20, 10 mM HCOONH₄ + 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 [$Y_{GalAc}^{mono}(pectin)$] 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 [$Y_{GalAc}^{total}(pectin)$] 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 [$Y_{GalAc}^{poly}(pectin)$] was calculated by correcting the total saccharide yield by the monosaccharide yield, i.e.,

$$Y_{GalAc}^{poly}(pectin) = Y_{GalAc}^{total}(pectin) - Y_{GalAc}^{mono}(pectin)$$

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

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

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

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

$$\% \text{ conversion } (DP) = \frac{Y_{GalAc}^{DP}(POS)}{Y_{GalAc}^{poly}(pectin)} * 100$$

Where $Y_{GalAc}^{poly}(pectin)$ and $Y_{GalAc}^{DP}(POS)$ 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 $21.1 \pm 0.2\%$ (w/w) on d.m. forming a significant part of the total sugars. The other sugars are galactose, glucose, rhamnose and arabinose accounting for $6.9 \pm 0.1 \%$, $14.7 \pm 0.2\%$, 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^{8, 24, 25}. 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 who found 11-12% of pectin in onion skins of which galacturonic acid accounted for 80%.¹⁶

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.¹⁷ A new extraction method was developed based on sodium hexametaphosphate. This chelator based extraction frees calcium bound pectin by loosening the egg box structures formed by the homogalacturonan and calcium complex.²⁶

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

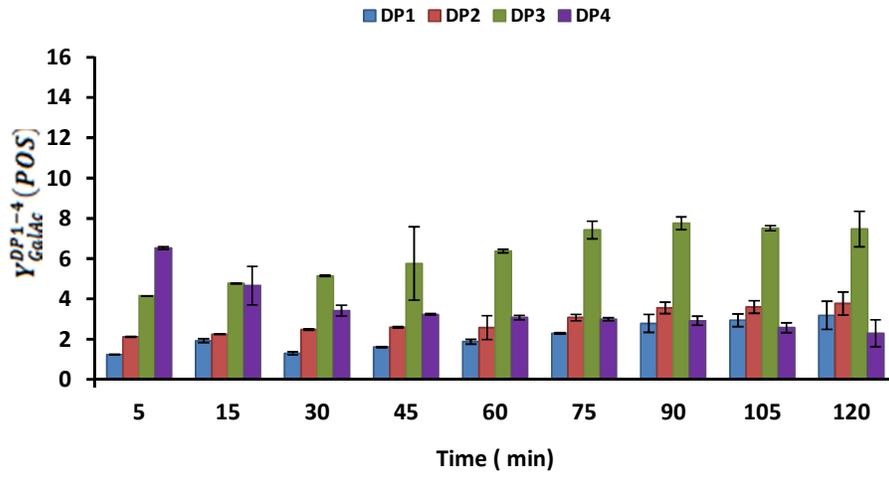
5.3.2 Pectic oligosaccharide production using EPG-M2, Pectinase and Viscozyme

The effect of different enzymes *viz.* Pectinase, Viscozyme and EPG-M2 on POS production from onion skins pectin 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.

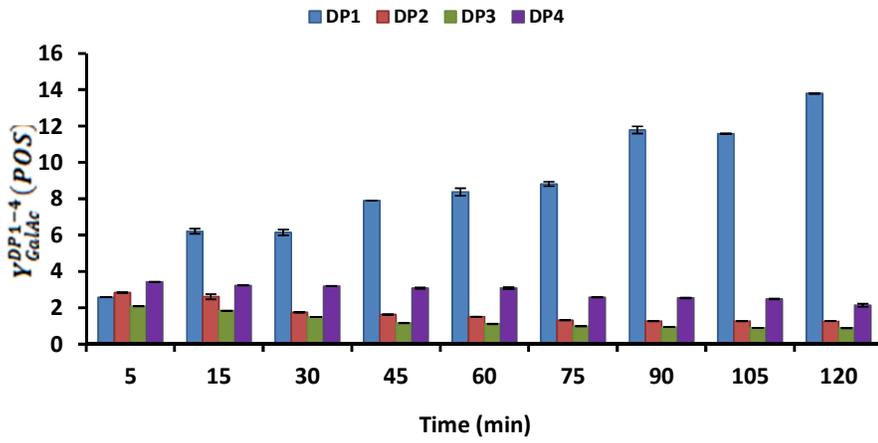
Already after 5 min of reaction, all enzyme preparations started converting pectin to monosaccharides and oligosaccharides in varying amounts indicating the presence of both exo and endo activity of the enzymes. Fig 5.1 illustrates the conversion of pectin to free galacturonic acid (DP1). It reveals an increase in DP1 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 which both almost double 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.

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

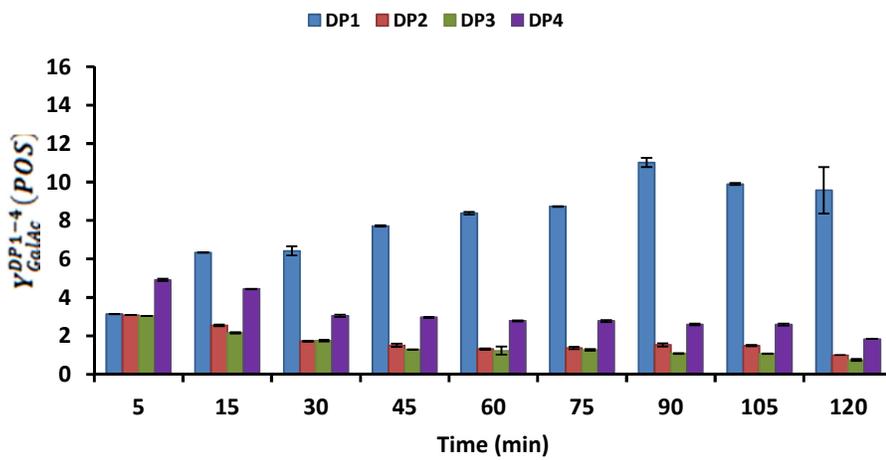
hydrolysis. The monosaccharide content further increases to 11% and 6.4% respectively, by the end of 120 min. At the same time, a significant decrease in DP2, DP3 and DP4 is observed indicating predominantly exo activity of the latter enzymes.⁹ Fig 5.2, shows the overall conversion of the onion skins 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, who also obtained similar trends of oligomer formation from pure polygalacturonic acid using different enzymes.⁹ This means that tailoring of pure polygalacturonic acid and that of crude pectic extract from onion skin progresses the same way.



(a)

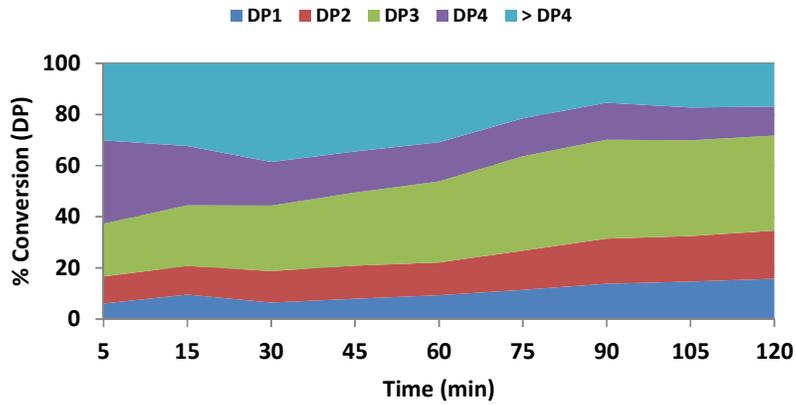


(b)

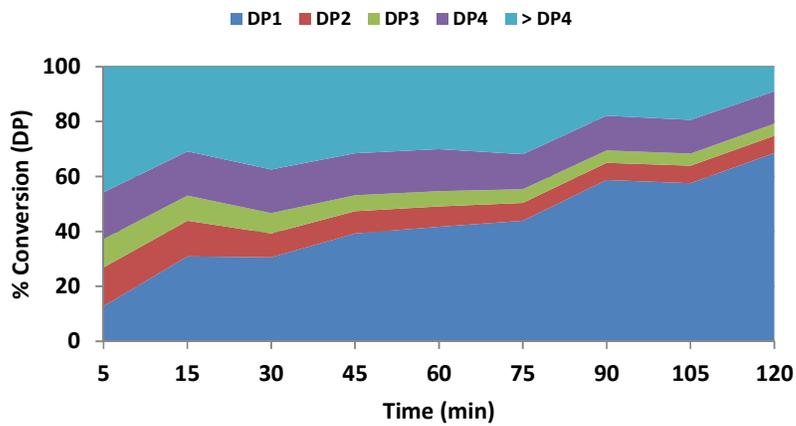


(c)

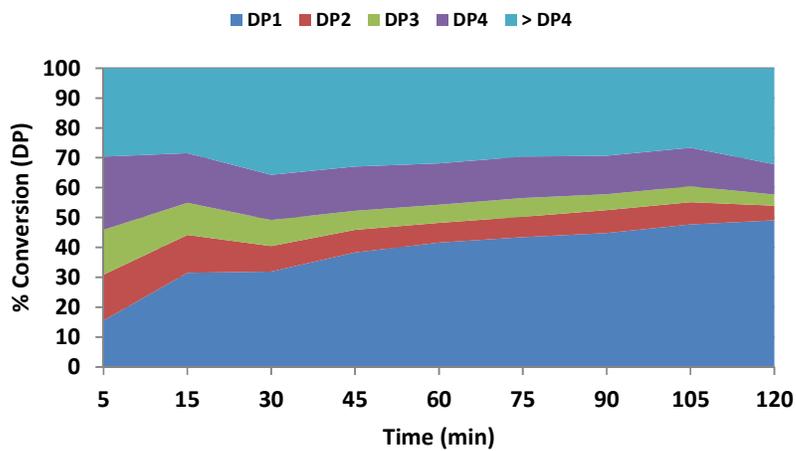
Fig 5.1: Production yield (% w/w d.m) of POS (DP1-DP4) from onion skins pectin by (a) EPG-M2 (b) Pectinase and (c) Viscozym



(a)



(b)



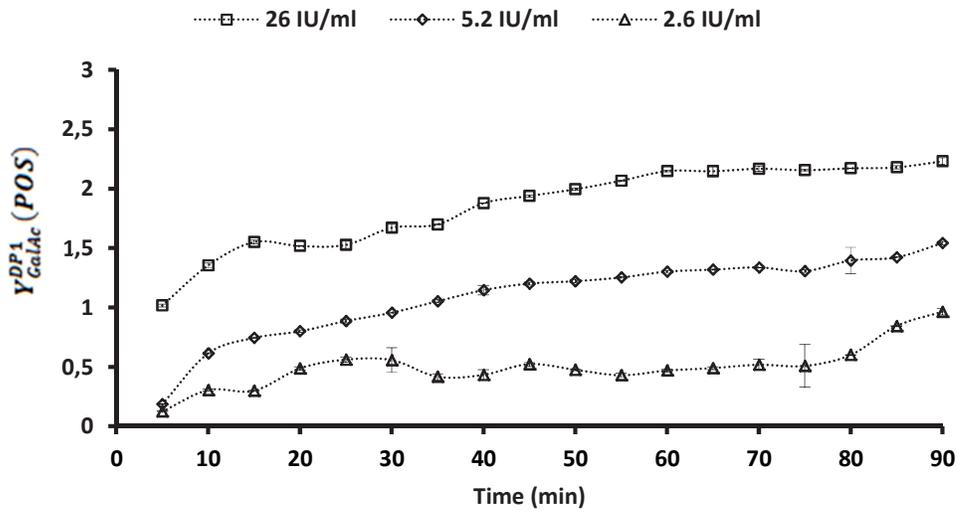
(c)

Fig 5.2: Conversion (%) of onion skins pectin to various POS fractions by (a) EPG-M2 (b) Pectinase and (c) Viscozyme

5.3.3 POS production using different concentration of EPG-M2

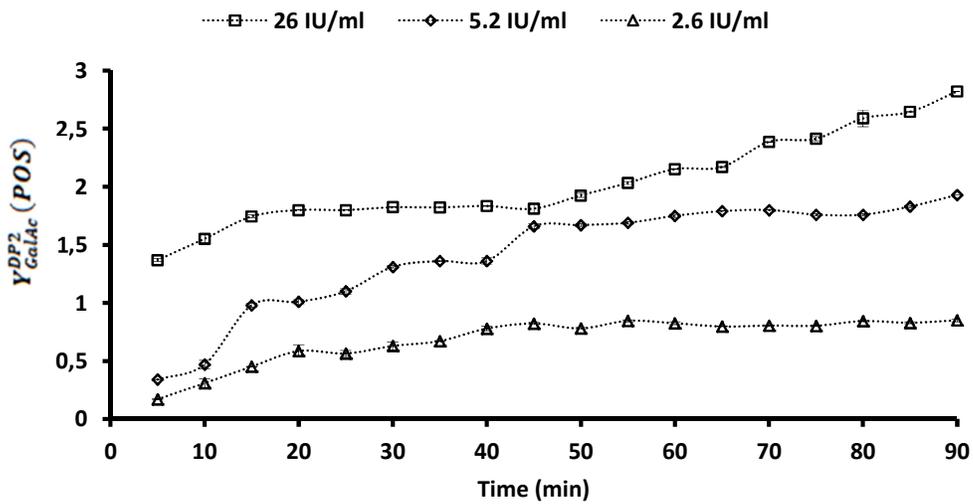
Since the enzyme EPG-M2 gave the most promising results, the POS production with this enzyme was further optimized. Three different concentrations of EPG-M2 *i.e.* 26.5, 5.2 and 2.6 IU/mL were used and the products of hydrolysis were characterized on HPAEC-PAD. The results are shown in Fig 5.3, whereby 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,



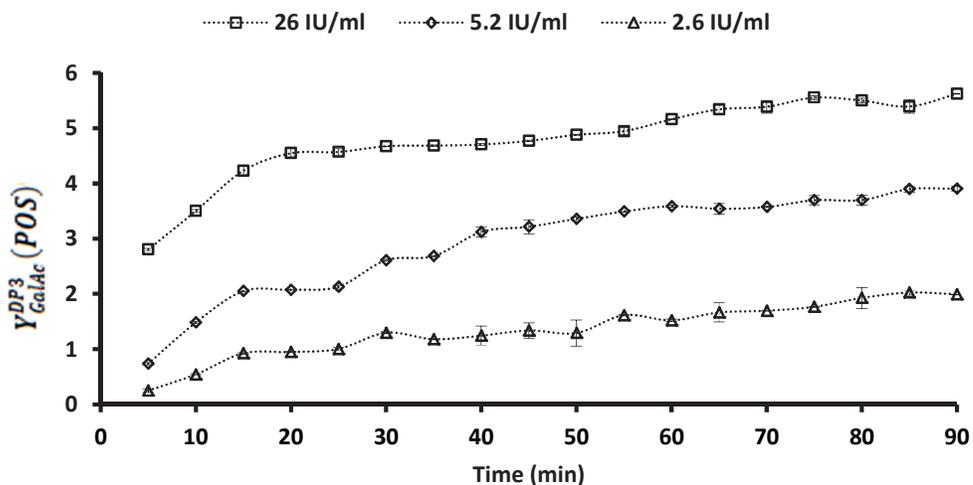
(a)

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.



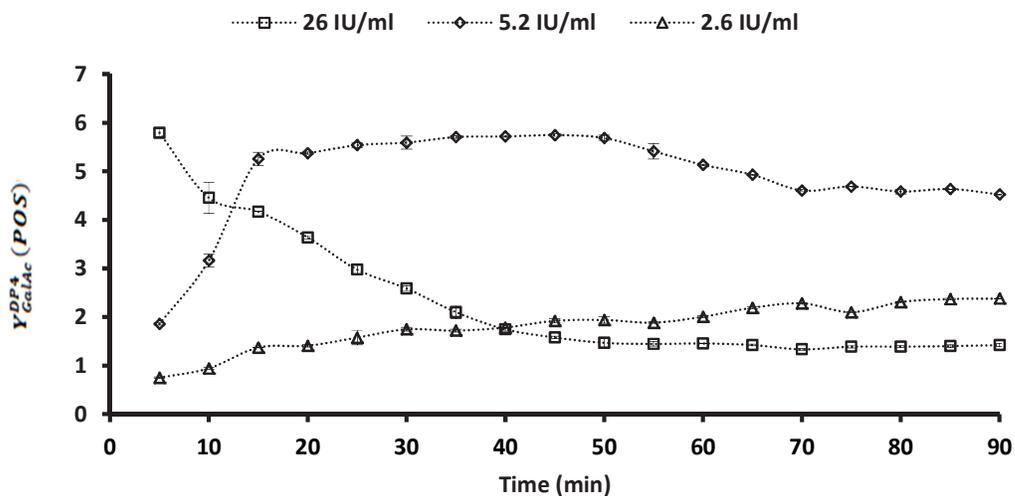
(b)

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.



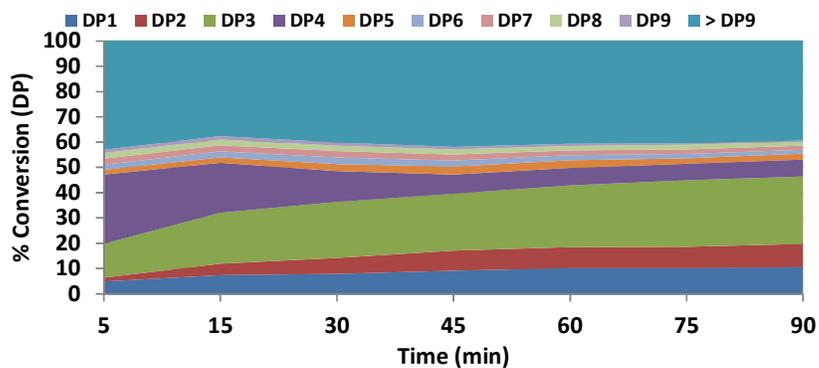
(d)

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.

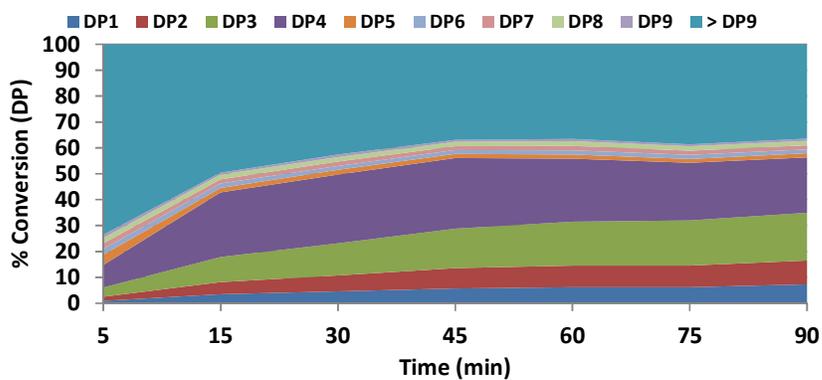
Fig 5.3: Effect of different concentrations of EPG-M2 on the production yield (% w/w d.m) of (a) DP1 (b) DP2 (C) DP3 and (d) DP4 oligosaccharides from onion skins pectin

LSD: Least significant difference

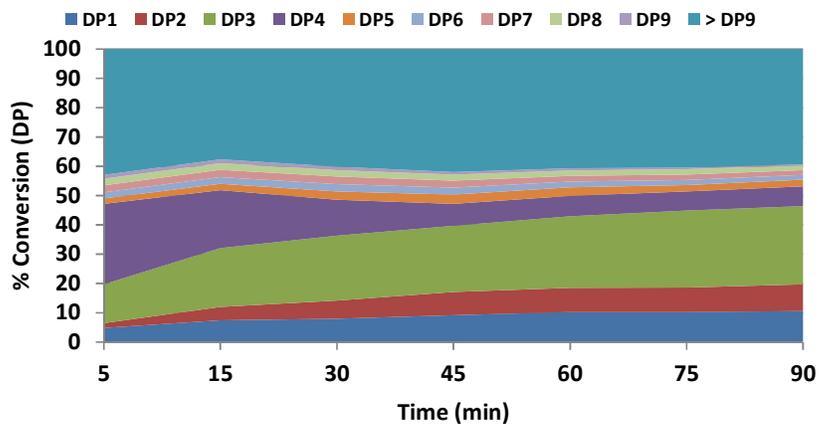
Matsubara is the approximate time required for sufficient conversion.¹⁰ This can be due a continuous conversion of DP4 and other higher oligomers to DP3 with higher enzyme concentration (Fig 5.4a). Our results are in line with the results obtained by Combo, Aguedo, Goffin, Wathelet & Paquot, who also obtained high DP3 from pure polygalacturonic acid catalyzed with EPG-M2.⁹ As mentioned elsewhere in the article, 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)



(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

5.3.3 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.¹⁷ 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, our future studies will be focused on the continuous production of POS from onion skin pectin using a membrane enzyme reactor. Also evaluation of prebiotic activity will be done for the POS obtained from onion skin pectin.

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

ID		MW (Da)	Trace (m/z)	RT (min)
GalA2	●●	370	369.3	22.10
GalA3OMe	●●●OMe	560	559.4	24.03
GalA3OAc	●●●OAc	588	587.4	28.46
GalA3	●●●	546	545.4	32,15
GalA4OMe	●●●●OMe	736	735.5	35,34
GalA4OAc	●●●●OAc	764	763.6	37.10
GalA4	●●●●	722	721.5	41,50
GalA5OMe2	●●●●●●OMe2	926	925.5	36,85
GalA5OMe	●●●●●●OMe	912	911.6	43,47
GalA5OAc	●●●●●●OAc	940	939.5	44,48
GalA5	●●●●●●	898	897.6	49,12
GalA6OMe2	●●●●●●●●OMe2	1102	1101.5	45,18
GalA6OMe	●●●●●●●●OMe	1088	1087.5	50,98
GalA6	●●●●●●●●	1074	1073.5	56,95
GalA7OMe3	●●●●●●●●●●OMe3	1078	1277.5	38,46
GalA7OMe2	●●●●●●●●●●OMe2	1292	1291.5	54,40
GalA8OMe3	●●●●●●●●●●●●OMe3	1468	1467.5	51,75
GalA8OMe2	●●●●●●●●●●●●OMe2	1454	1453.5	52,37

RT: Retention time

MW: Molecular weight

●: Galacturonic acid

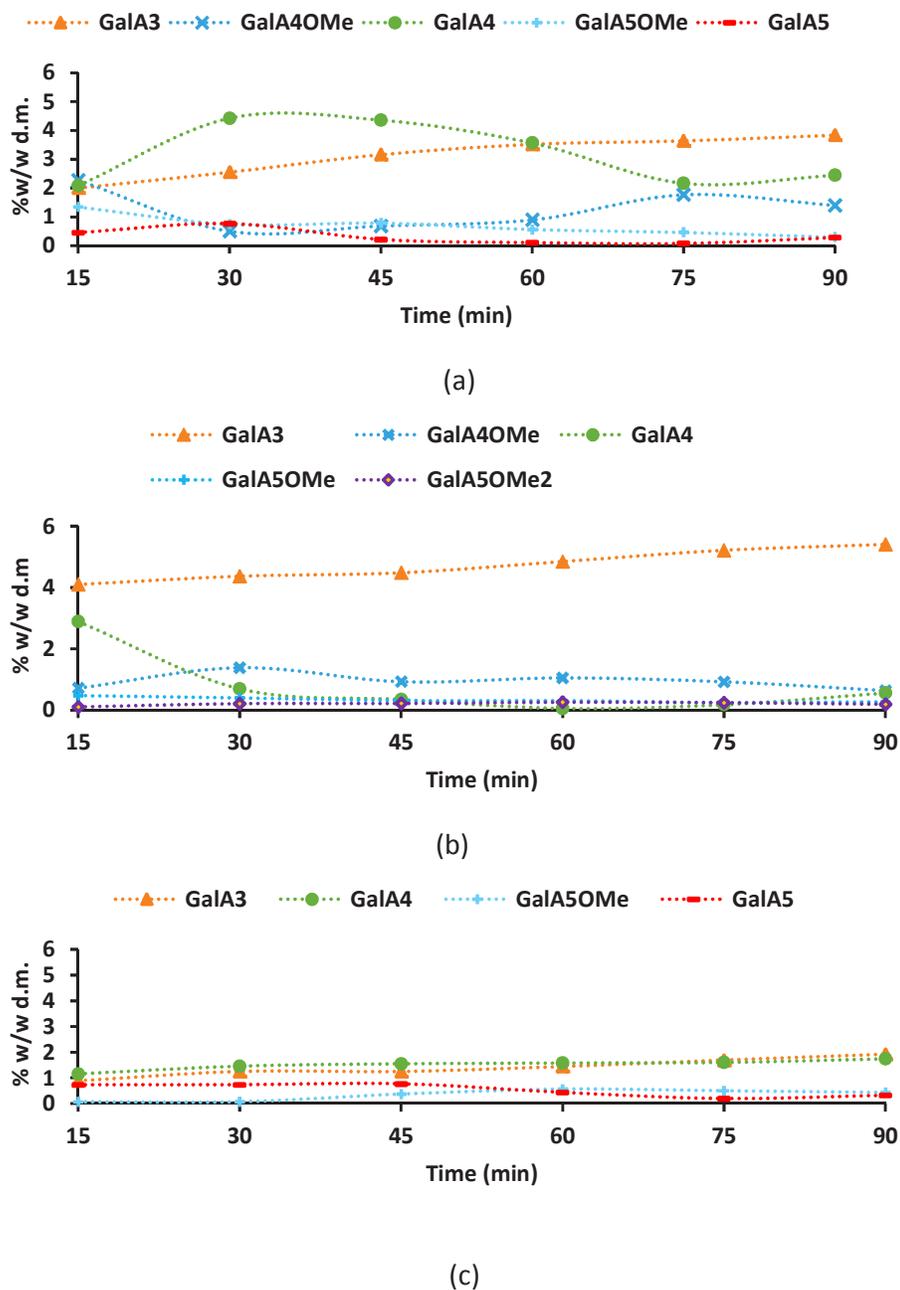


Fig 5.5: Digestion pattern of unsubstituted and substituted galacturonic acid oligomers by different concentration of EPG-M2 (a) 26 IU/mL (b) 5.2 IU/mL and (c) 2.6 IU/mL

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.

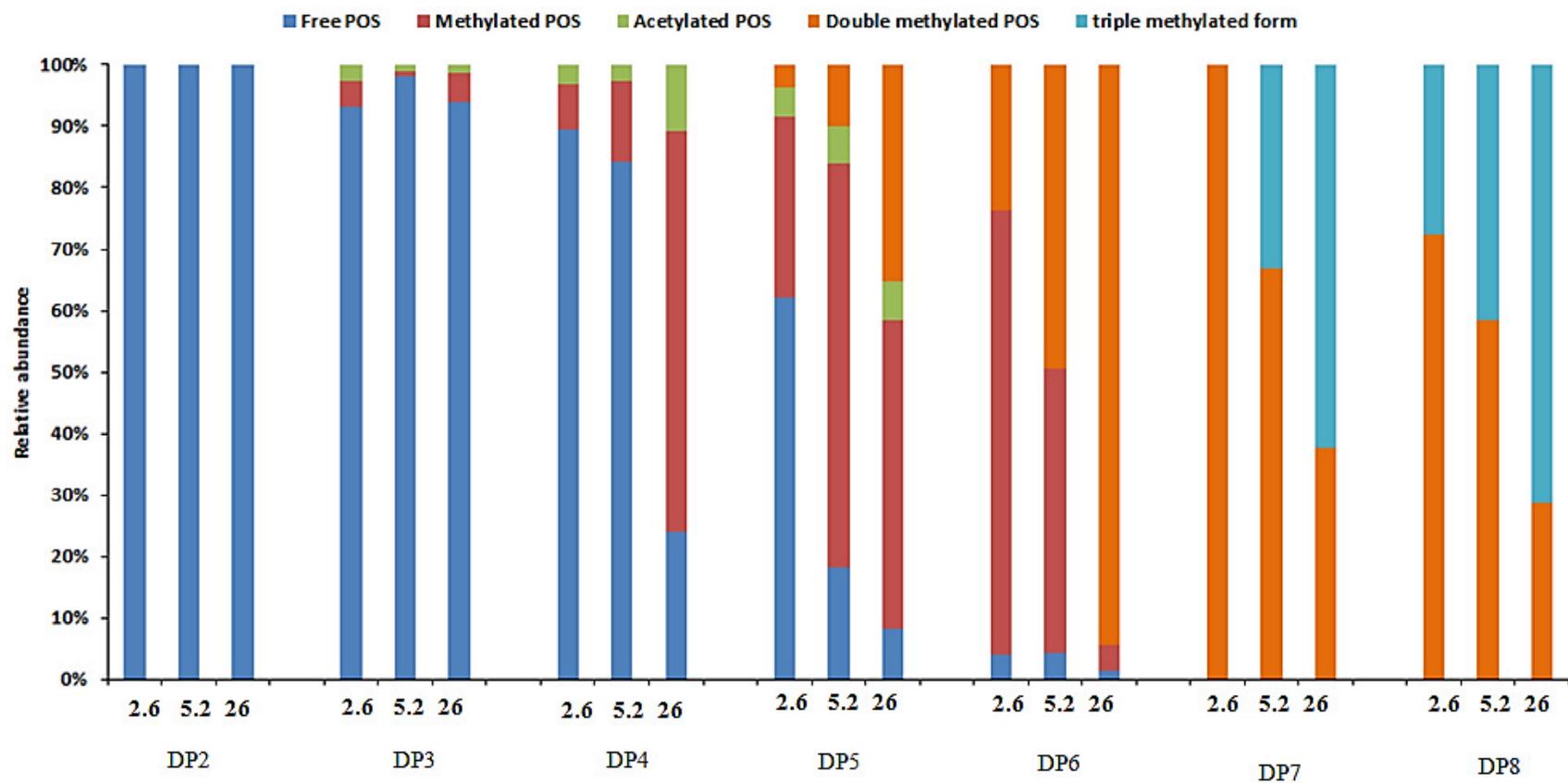
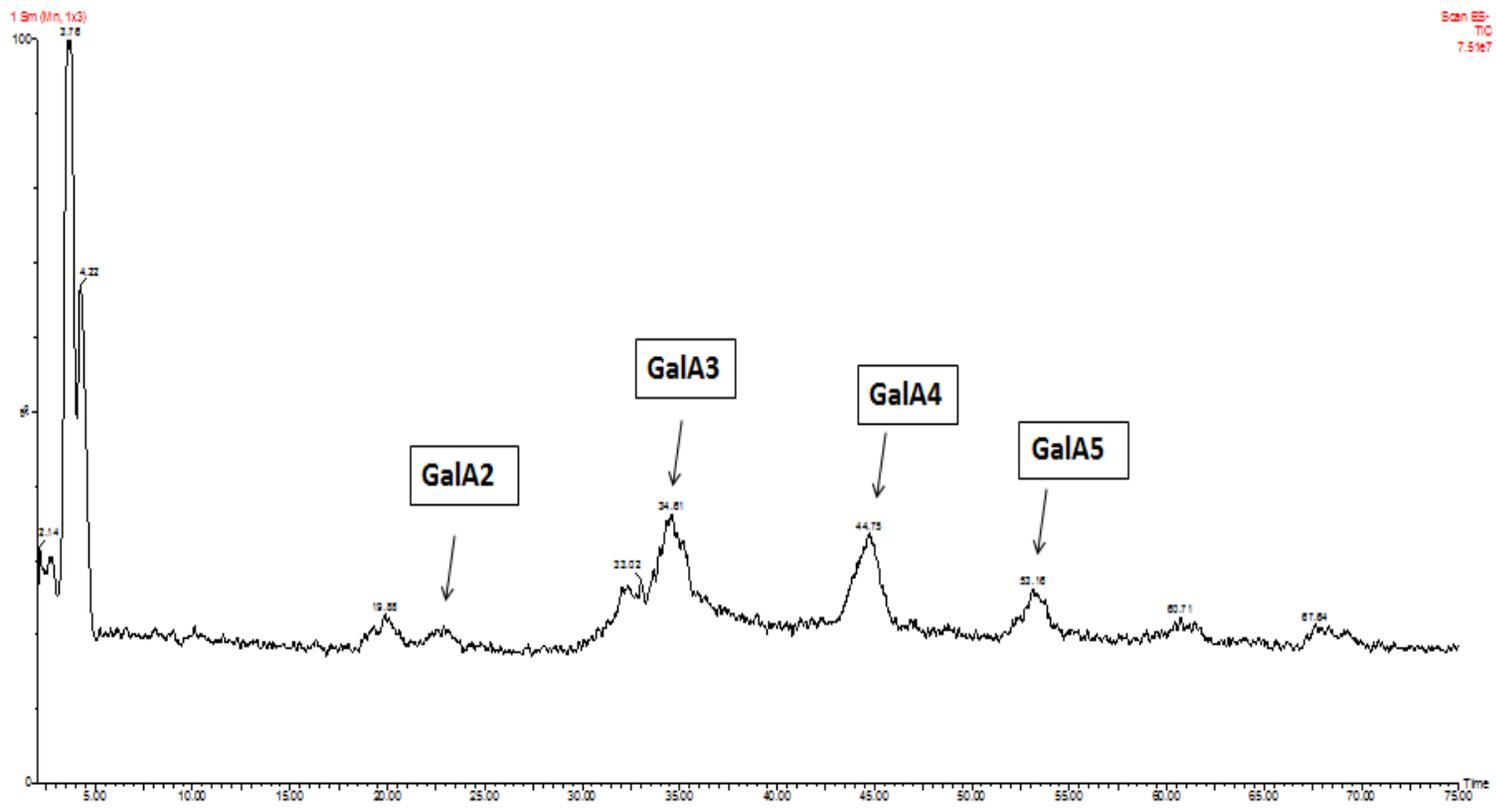
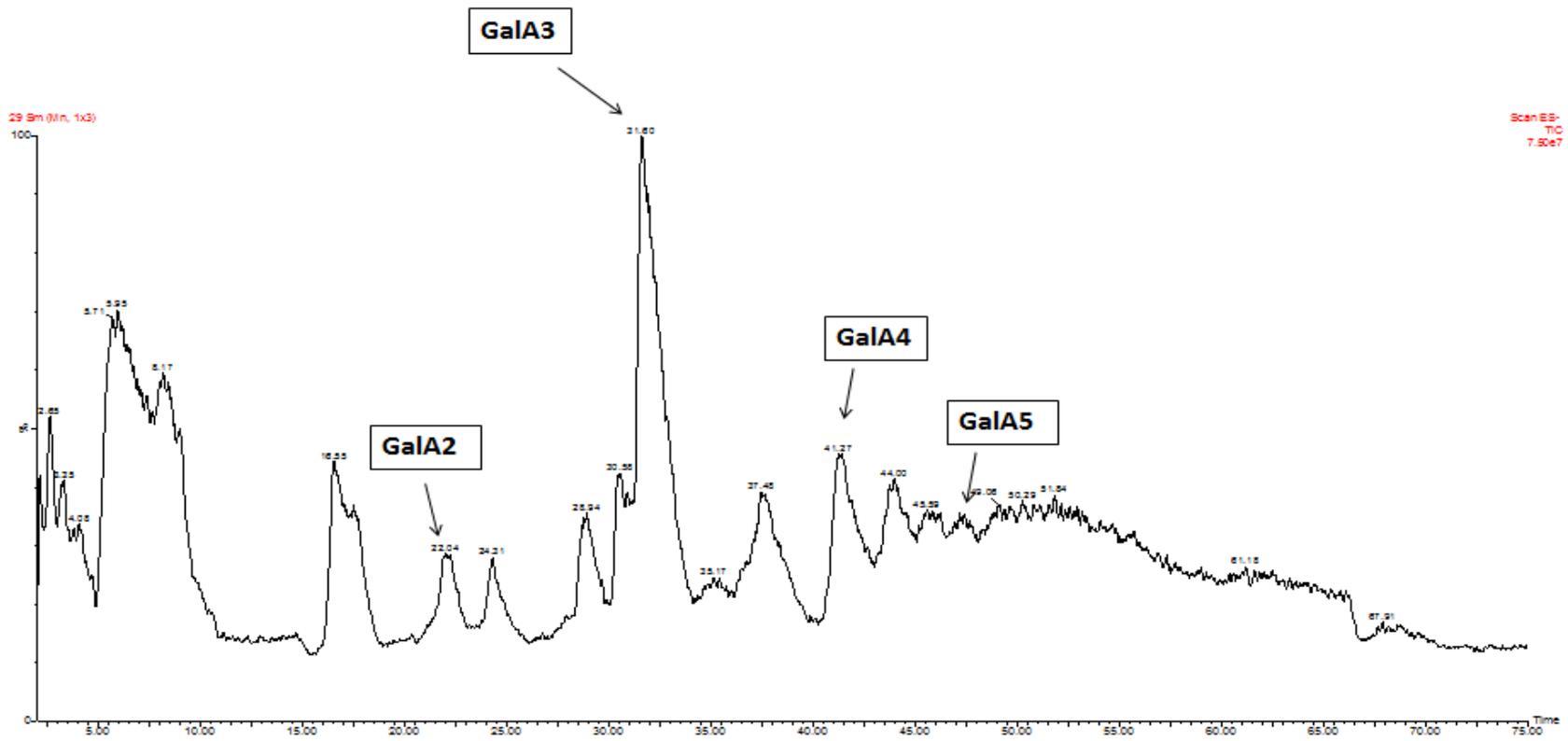


Fig 5.6: Relative abundance of different POS fractions obtained at 45 min.

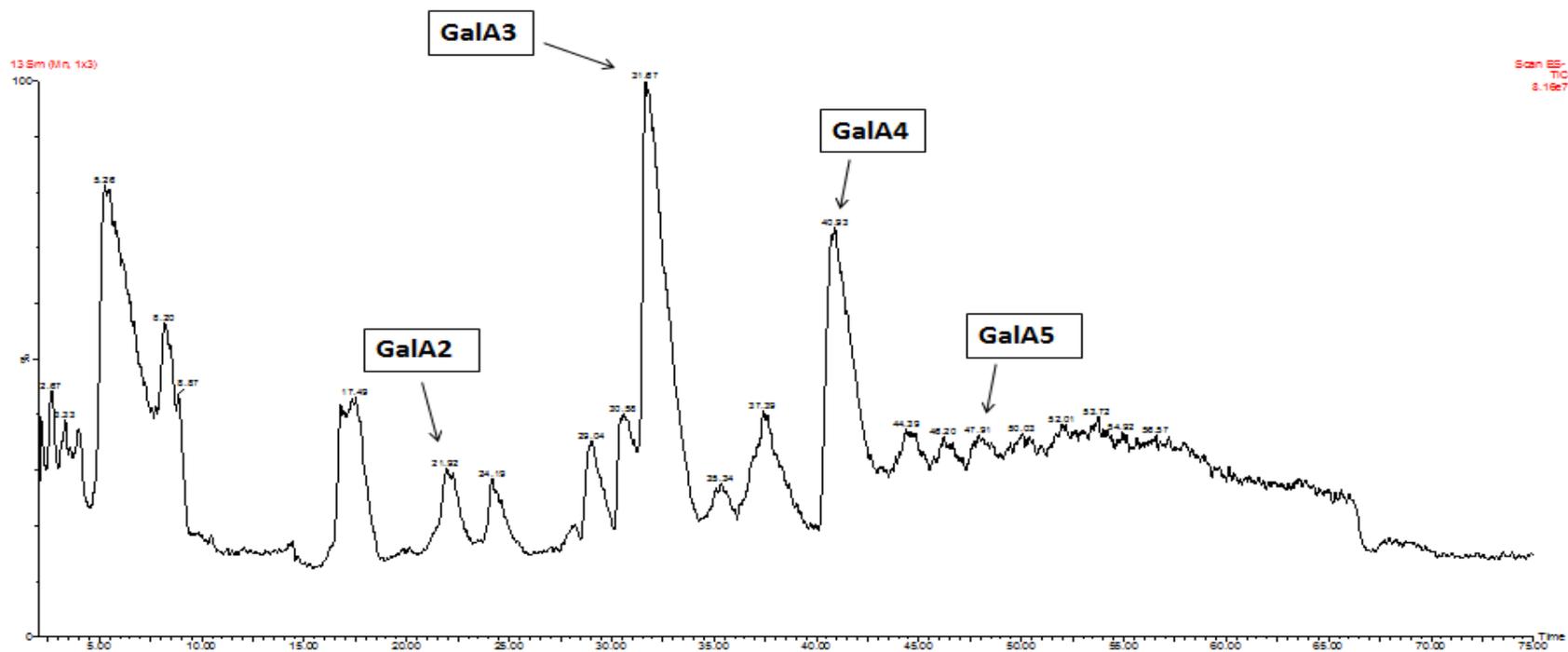
0.5, 1 and 5 indicates enzyme concentration in IU/mL



(a)

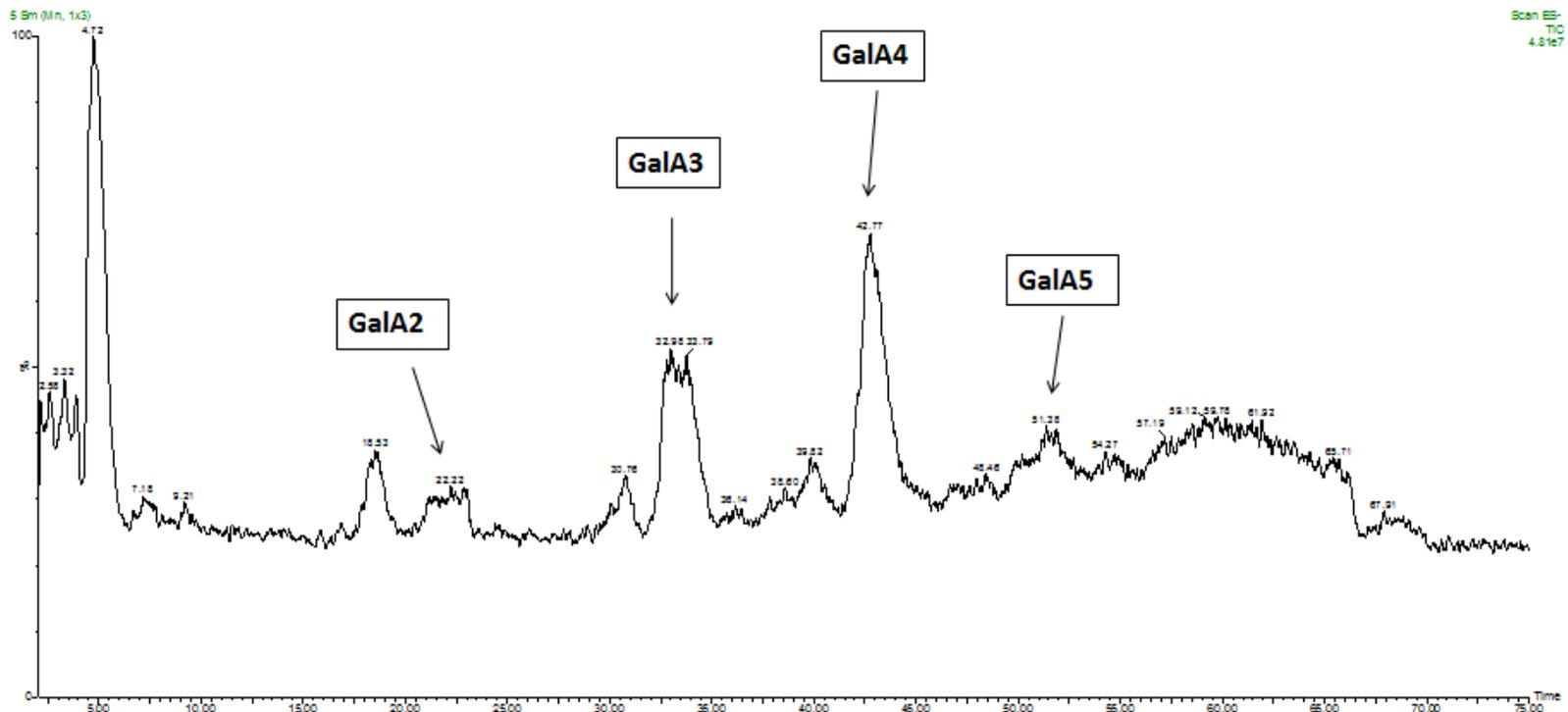


(b)

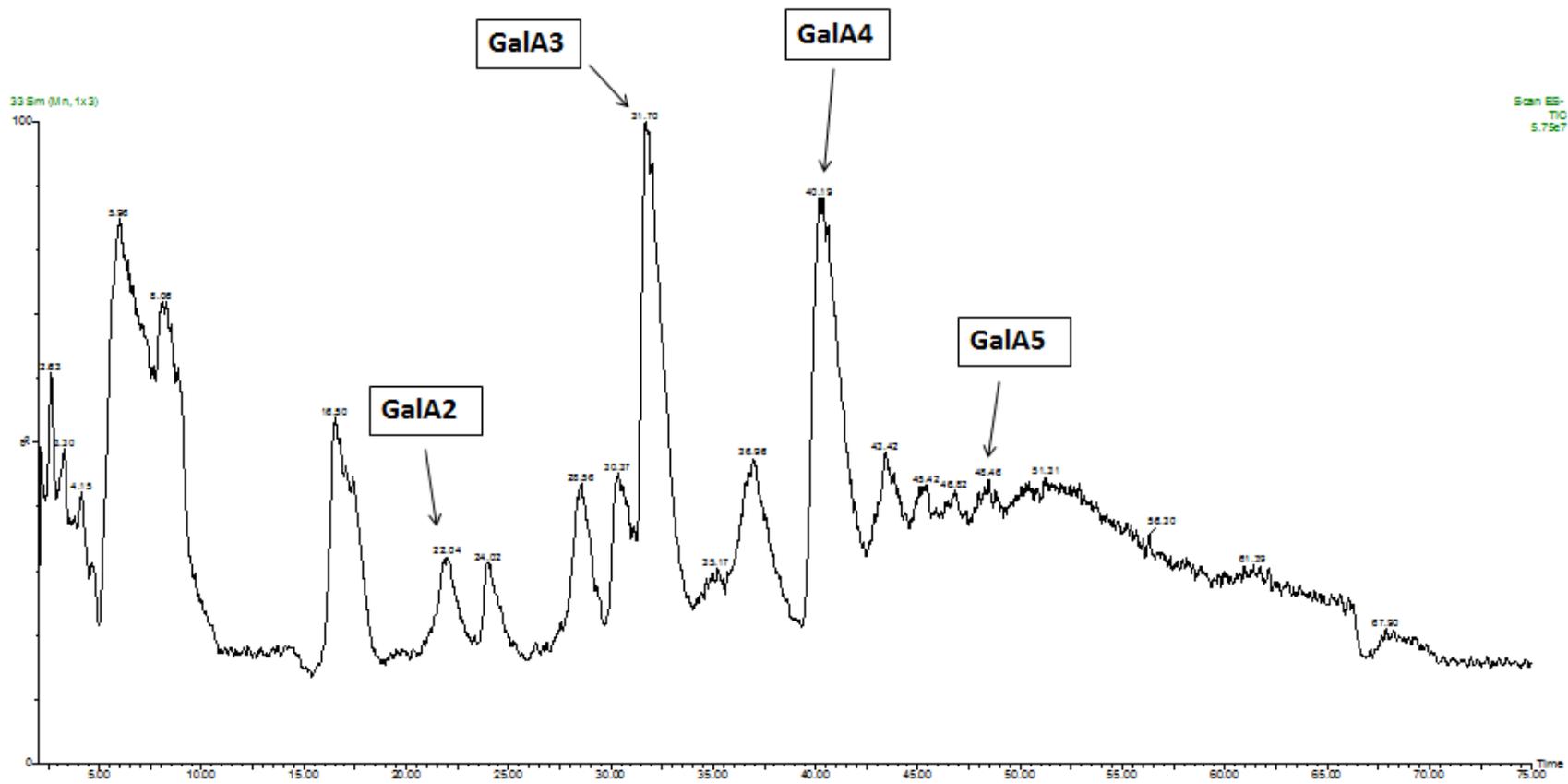


(c)

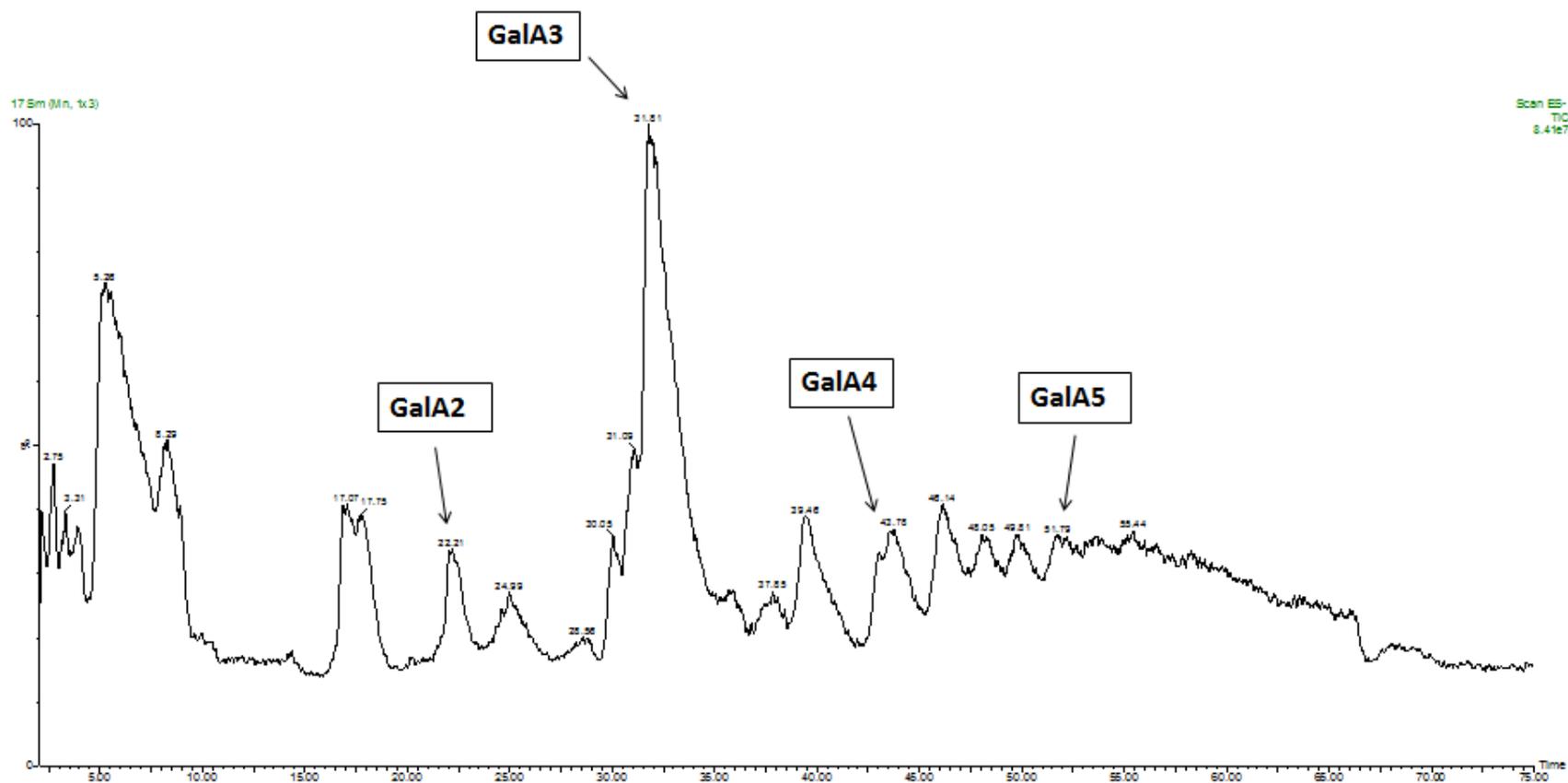
Fig 5.7: ESI-Scan of onion pectin oligomers at 15 min with EPG-M2 concentration of (a) 2.6 IU/ mL (b) 5.2 IU/ mL and (c) 26 IU/ mL



(a)



(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

5.4 CONCLUSION

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.

5.5 ACKNOWLEDGMENT

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6. CONCLUSION AND FUTURE PERSPECTIVES

Pectic oligosaccharides (POS) is an emerging topic, as some experiments have shown the prebiotic potential of these compounds. Pectin is a complex and heterogenous polysaccharide present within the primary cell wall and intercellular regions of higher plants. Pectin comprises a family of acidic polymers, known as homogalacturonan (HG) and rhamnogalacturonan (RG) with several neutral sugars/polymers such as arabinans, galactans and arabinogalactans (attached as side chains). The extraction of these neutral and acidic polymers in the form of pectic oligosaccharide (POS) is a promising step towards the manufacture of prebiotics from agricultural by-products.

Scanty information on the production and almost no literature on the continuous production of pectic oligosaccharides was the basis for this PhD. The safest and greener way of producing POS is by means of enzymatic tailoring of long pectic chain to smaller POS fractions. Since the pectic molecules comprises of different sugars polymers, it necessitates the use of enzymes (having that specific activity) to produce oligomers. The main drawback of using commercial enzyme having specific activities is the cost of these enzymes. Therefore we decided to use only one commercial enzyme mixture (Viscozyme) having a cocktail of diverse activities. But the drawback of using Viscozyme is the presence of some exo-activities which lead to generation of monosaccharides (undesirable) from POS fractions. Considering these two issues *i.e.* cost of enzyme and generation of monosaccharides, the use of enzyme membrane reactor was the most appropriate choice which allows the use of such enzyme as well as separation of product (preventing its further conversion to monosaccharides).

In this PhD thesis, four different agro-industrial residues were evaluated as source of pectin, with the final focus on the continuous production of pectic oligosaccharides (POS).

The initial study optimized the parameters *i.e.* particle size, enzyme concentration and time in one stage-shake flask level (directly from sugar beet pulp) for POS production from sugar beet pulp, using response surface methodology. Although promising results were obtained with this experiment it was realized that a continuous operation would be difficult using this approach due to membrane associated fouling. Hence, one stage POS production was not continued further. Then two stage study including crude pectin extraction followed by POS production in an enzyme membrane reactor was planned. For this, screening based on pectin content was done for the selection of most appropriate agro-residue. Out of the four residues *i.e.* sugar beet pulp (SBP), onion hulls (OH), berry waste (BW) and pressed pumpkin (PP), substantial amounts of galacturonic acid polysaccharides and both galacturonic and arabinose polysaccharides were present respectively in OH and SBP. An important information on the best suitable extraction method for producing a crude pectic extract (which is to be used as a feed to continuous enzyme membrane reactor) causing minimal damage to pectic polysaccharides was obtained. Nitric acid at pH 1.4 for 4h and sodium hexametaphosphate 2% at 95 °C for the extraction of pectin from sugar beet pulp and onion hulls respectively are the appropriate extractants.

The first substrate to be evaluated for POS production in a continuous enzyme membrane reactor was sugar beet pulp. The continuous POS production using EMR indicated that dosing of substrate (25 g/L) at residence time of 20 min results in a higher POS and lower monosaccharides yield of 80% and 20% respectively under steady state. The volumetric and specific productivities of 20 g/L/h and 11 g/g E/h respectively was obtained using these conditions. Further, the experiments on sugar beet pulp were done (using optimized parameters) with the aim of producing 1 kg of POS for animal feed trials by a project partner based in Gent, Belgium (Nutrition Sciences). Owing to the fouling associated with the membranes, it was difficult to produce 1 kg in one run, so six successive experiments were

conducted with the longest run of 36 h. During these experiments the steady state POS production was obtained indicating no enzyme inactivation.

Further for the future studies, it is necessary to look for strategies to reduce fouling, which could be partial bleeding and intermediate cleaning cycles or use of different type of membranes. A techno-economic evaluation is also needed to assess its performance against batch tests.

Some preliminary studies on the production of POS from onion hulls was also conducted at shake flask level. Production of POS from onion hulls was evaluated using endo-polygalacturonase M2 due to the presence of only one type of sugar polymer in onion hulls *i.e.* galacturonic acid. Several different polymers with DP from 1-10 were obtained from onion hulls. Molecular characterization of onion hulls digests revealed the presence of both substituted and unsubstituted form of POS. Further studies are needed to evaluate the continuous POS production from onion hulls in an enzyme membrane reactor.

7. CURRICULUM VITAE

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Work Experience:

2009 Satnam Agro processing, India

Position- Quality Control Analyst

Duration- January'15- April'15

2009 Central Institute of Post Harvest Engineering and Technology, India

Position- Senior Research fellow

Duration- April'07-June'05.

- Worked in a project on ' Value addition to non edible parts of fruits and vegetables'.

2009 Central Institute of Post Harvest Engineering and Technology, India

Position- Research Associate

Duration- June'15- Jan'15 2013

- Worked in world bank sponsored NAIP project on " Novel biotechnological process for production of high value products from Bagasse and Rice straw"
- Currently doing PhD at University of Parma, Italy and Flemish Institute of Technology, Belgium. Involved in European Union sponsored project " safe and functional feed ingredients "

Trainings:

2005 M/S Nijjer Agro Foods Ltd., Jandiala Guru, Distt Amritsar, India

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ACADEMIC QUALIFICATION:

Jan 2013- Jan 2016 PhD from University of Parma, Italy and Flemish Institute of Technological Research, Belgium.

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2006-2009 MSc in Food Technology: Fruits and vegetables (First Class 7.00/10.00)
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Thesis: To develop cloud stable juices and nectar from litchi and pear

2002-06 B.Sc in Food Science and Technology (First Class 76.45%)
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2000 Matric from A.V. Public school, India (First class 81 %)

LIST OF PUBLICATIONS

Published:

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Submitted:

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- **Babbar N**, Dejonghe W, Elst K and Sforza S. Enzymatic pectic oligosaccharides (POS) production from sugar beet pulp using response surface methodology. *Submitted with LWT Food Science and Technology*

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- **Babbar N**, Sandra VR Dejonghe W, Sforza S, Elst K. Continuous production of pectic oligosaccharides from sugar beet pulp.

Popular Articles

- **Babbar N**, Aggarwal P and Kaur A (2009) Understanding Umami and its applications. *Beverage and Food World* 36 (7), pp 42-45.

Book chapters

- Oberoi H S, Sandhu S K, **Babbar N**, Dhaliwal S S and Kaur U (2011). Ethanol production from fruit residues-Prospects and Problems. In **Bio-Processing of Foods**, Chapter 23, pp 350-358, Asia tech Publishers, New Delhi, India.
- Uppal DS and **Babbar N** (2011). Co-product utilization of Kinnow processing for food and Industrial use. In **Food processing technologies, Co-product utilization and quality assurance**, Chapter 20, pp 291-296, Satish serial publishing house, New Delhi.

- Oberoi HS and **Babbar N** (2012). Health foods based on nutraceuticals extracted from horticultural crop residues. In **Health Foods: Concept, Tech & scope**, chapter 7, pp 147-161. Biotech Books, New Delhi.
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- Participated in the 50th annual AMI conference at NCL, Pune, India
- Participated in the 51st annual AMI conference at BIT, Ranchi, India
- Participated in the 5^{2nd} annual AMI conference at PU, Chandigarh, India
- Participated in Punjab Science Congress at PAU, Ludhiana, India
- Participated in 4th Indian Horticultural Congress at New Delhi, India
- Participated in International Conference on sustainable Agriculture for Food and Livelihood security at PAU from 27-29 Nov 2011, India.

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

- 27th International Carbohydrate Symposium, Indian Institute of Science, India. Jan 12-17, 2014 (Abstract published).
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- 9th Biotechnology Conference, Orlando, Florida. Aug 31-Sep 02, 2015 (Abstract published).
- 11th European symposium on Biochemical Engineering Sciences, Lille, France. Sept 27- Oct 01, 2015 (Poster presented).
- 28th Euro Food Chem, Madrid, Spain. Oct 13- 15, 2015 (Poster presented).
- 28th Euro Food Chem, Madrid, Spain. Oct 13-15, 2015 (Oral Communication presented by Kathy Elst, abstract published).
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 - *Noshan Annual Meeting, Istanbul, Turkey 06-07/02/2014*
 - *Noshan Annual Meeting, ILVO-Gent, Belgium*

SKILLS

Instruments handled: : HPLC, ELISA reader, Ethanol biosensor, Hunter colour Meter, freeze dryer, vaccum evaporator, Texture analyser, turbidimeter, tintometer, Fermenter Electrophoretic instrument, Gel Doc, gel permeation chromatography, high permformance anion exchange chromatography.
