Continuous production of pectic oligosaccharides from onion skins with an enzyme membrane reactor

***Highlights (for review)**

- Pectic oligosaccharides produced from onion skins by an enzymatic process
- Continuous production achieved by using a cross flow enzyme membrane reactor
- Effect of process conditions investigated and optimized
- Stable production of pectic oligosaccharides with high volumetric productivity achieved
- Tailored production of pectic oligosaccharides having low degree of polymerisation

Continuous production of pectic oligosaccharides from onion skins with an enzyme membrane reactor

Stefania Baldassarre[a,b], Neha Babbar[a,b], Sandra Van Roy[a], Winnie Dejonghe[a], Miranda Maesen[a], Stefano Sforza[b] and Kathy Elst[a,*]

[a] Separation and Conversion Technology, Flemish Institute for Technological Research, VITO, Mol 2400, Belgium.

[b] Department of Food and Drug Science, University Campus, University of Parma, 43124, Parma, Italy.

*Corresponding author at Separation and Conversion Technology, Flemish Institute for Technological Research, VITO, Mol 2400, Belgium. E-mail address: Kathy.elst@vito.be; Telephone number: 003214335617.

Abstract

The aim of this research was to valorize onion skins, an under-utilized agricultural by-product, into pectic oligosaccharides (POS), compounds with potential health benefits. To achieve high hydrolysis performance with the multi-activity enzyme Viscozyme L, an innovative approach was investigated based on a cross-flow continuous membrane enzyme bioreactor (EMR). The influence of the various process conditions (residence time, enzyme concentration, substrate concentration) was investigated on productivity and yield. The composition of the POS mixtures in terms of mono- and oligosaccharides was assessed at the molecular level. At optimized conditions, a stable POS production with 22.0 g/L/h volumetric productivity and 4.5 g/g POS/monosaccharides was achieved. Compared to previous results obtained in batch for the enzyme Viscozyme L, EMR provided a 3-5x higher volumetric productivity for the smallest POS. Moreover, it gave competitive results even when compared to batch production with a pure endo-galacturonase enzyme, demonstrating its feasibility for efficient POS production.

Keywords: pectin; pectic oligosaccharides (POS); onion skin; enzyme membrane reactor; continuous process, molecular analysis.

1 Introduction

Pectin is one of the most complex carbohydrates present in nature and it is mainly found in the cell wall of higher plants. It is formed by "smooth" regions, called homogalacturonan, made by D-galacturonic acid units, and "hairy" regions, in which the galacturonic acid is interspersed with rhamnose residues linked to neutral sugar side chains. The complexity of the polymer is increased by its variability, whereby the exact composition and structure depends on the source considered (Caffall & Mohnen, 2009). Starting from pectin, by performing an enzymatic hydrolysis, it is possible to obtain pectic oligosaccharides (POS) with different structures and different degree of polymerization (DP) (Babbar *et al*, 2015). Given the natural variability of pectin, POS can also be very variable from the structural point of view, including arabinogalacto-oligosaccharides, arabinoxylo-oligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, oligo-galactouronides and rhamnogalacturonan-oligosaccharides (Concha-Olmos & Zùniga, 2012). In recent years, the research on POS has increased since these oligosaccharides have shown interesting biological properties. Some *in vitro* studies demonstrated that they are not digestible by animal digestive enzymes, they can induce changes in the activity and composition of the gastrointestinal microbiota, stimulating the growth of probiotic bacteria such as *Lactobacilli* or *Bifidobacteria* and preventing the growth of pathogenic strains such as *E.coli* (Gullòn, Gullòn, Sanz, Alonso & Parajo, 2011).

For the production of pectin-derived products, including POS, several agricultural and agro-industrial by-products, such as sugar beet pulp and citrus and apple peels have already been investigated (Babbar, Dejonghe, Gatti, Sforza & Elst, 2015). Nevertheless, in order to have a more cost-effective solution or to be able to produce products with different characteristics, the search for alternative underutilized sources, rich in pectin, continues. Among the possible sources of pectin, onion skins are a consistent waste stream in the European Union (EU). In 2014/2015 the EU bulb onion crop was estimated at 5.9 million tons which was 13 % more than in the year before. The

huge production of onions implies as well a significant production of wastes. Indeed, approximately 450 000 tons of onion waste are produced annually, mainly in the UK, the Netherlands and Spain. The major by-products resulting from industrial peeling of onion bulbs are the brown skins, the outer two fleshy leaves and the top and bottom bulbs (EURONION, 2016).

In previous works, onion skins have been assessed for their pectin content (Muller-Maatsch *et al*, 2016) and their possible utilization as pectin and pectin-derived products (Babbar, Baldassarre, Maesen, Prandi, Dejonghe, Sforza & Elst, 2016b).

Batch-wise enzymatic hydrolysis (also indicated as depolymerization) is the main approach used to prepare large amount of carbohydrate oligomers starting from a polymeric sugar (Munoz et al, 2012) and several types of enzymes, such as pectinases, pectate lyases and other polygalacturonases are applied to generate oligogalacturonides from pectin cleavage (Jayani, Saxena & Gupta, 2005). Batch preparations, especially when using crude multi-enzyme preparations with endo- and exo-activity, typically yield POS mixtures quite high in monosaccharide content, mostly galacturonic acid, the end-product of the pectin hydrolysis (Babbar, Baldassarre, Maesen, Prandi, Dejonghe, Sforza & Elst, 2016b).

In order to be able to minimize monosaccharide formation as much as possible, an enzyme membrane reactor (EMR) could be used: indeed, the integrated use of a biocatalytic reactor and a membrane process such as ultrafiltration allows the continuous removal of products as soon as they are small enough to permeate, protecting the POS from further hydrolysis and preventing monosaccharide formation. In this way, enzyme inhibition is also avoided and higher productivity is achieved (Kiss, Nemestothy, Gubicza, Bélafi-Bako, 2008). More advantages of such technology include (i) the targeted production of tailored products by choosing an appropriate membrane cut-off, (ii) a reduction of costs owing to a re-use of the enzyme and a more continuous way of operation (Giorno & Drioli, 2000; Béfali-Bako, Nemestothy, Vladan & Gubicza, 2002; Bèfali-Bako, 2006), (iii) no need for inactivation of enzyme by acid or high temperature treatment avoiding side reactions and (iv) no contamination of the end-product by the enzyme. The

use of EMR for the hydrolysis of pectin has already been studied by Kulbe, Heinzler & Knopki (1987) and Olano-Martin, Mountzouris, Gibson & Rastall (2001) but in set-up that did not allow a continuous production with simultaneous feeding and product removal. Moreover, with the dead end filtration applied, large amounts of insoluble materials present in the substrate could lead to clogging problems: the accumulation of particles onto the membrane might indeed result in an increased resistance to filtration and a decline in permeate flux, requiring frequent cleaning steps and ultimately the replacement of the membrane (Pinelo, Jonnson and Meyer, 2009). Moreover, in these studies, pure commercial pectin was used as starting material, whereas the interest lies in systems able to work with industrial byproduct stream also containing other compounds.

The aim of the present work was to valorize onion skins towards enzymatic POS production with high hydrolysis performance by using a cross-flow continuous membrane bioreactor process at lab-scale. To our knowledge, there is no literature so far dealing with the continuous production of POS from pectin of onion skins. Key to this approach is the combination of the hydrolysis with the simultaneous *in-situ* POS-removal through cross-flow membrane filtration, allowing the continuous production of POS and providing options for significantly increased productivity. Moreover, the process is based on the use of an enzyme complex, Viscozyme L, having a lower cost as compared to more specific enzymes, nevertheless avoiding high galacturonic acid production. The influence of various process conditions (residence time, enzyme and substrate concentration) was studied, both on productivity and yield. In addition, also a detailed characterization of the POS products at the molecular level was performed by High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection (HPAEC-PAD). Optimal conditions for high POS yield and low monosaccharides yield were defined, together with a stable production of POS with low degree of polymerization, fully demonstrating the feasibility of this technique for efficient POS production starting from onion skins.

2 Material and methods

2.1 Materials

Onion skins were provided by the Institut fur Getreideverarbeitung (IGV, GmbH), Germany. The skins were milled with a laboratory blender, sieved to a particle size (< 1 mm) and stored in ziplock bags at room temperature until use. Viscozyme L (V-2010) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Viscozyme L is a multienzyme complex composed of pectinases, hemicellulases and arabinases (Foulk, Akin & Dodd, 2008). The endo-galacturonase activity of the undiluted Viscozyme, determined by Babbar *et al* and expressed as EPG units, was determined to be 4135 U/mL. (Babbar, 2016a)

Romicon 1-hollow fiber membrane cartridges (Type HF 1018-1.0-43-PM10 and PM5), containing polysulfone ultrafiltration membranes with a molecular weight cut-off of 10 kDa, were obtained from Koch membrane systems, Inc. (Stafford, GB). The total filtration surface of these membranes was 2 X 0.093 m^2. P3-Oxonia active and Ultrasil 115, the liquid disinfectants used for cleaning the reactor and the membrane modules, were purchased from ECOLAB bvba Zellik,Belgium). Polygalacturonic acid was obtained from Megazyme International (Bray, Ireland).

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

2.2 Extraction and analysis of crude pectin from onion skins

Based on our preliminary study (Babbar *et al*, 2016a), sodium hexametaphosphate (SHMP) was selected as pectin extractant for onion skins. Onion skins were grinded to a particle size < 1

mm using a laboratory blender and extracted at a substrate concentration of 50 g/L with 2% sodium hexametaphosphate solution at 95 °C for 0.5 h in a hot water bath. The biomass was then centrifuged at 4500 x g for 10 min and the supernatant containing the crude pectin was collected. This crude pectin extract was diluted by a factor of 2, for obtaining a feed having a substrate loading of 25 g/L, or utilized as such (for the feed having a substrate loading of 50 g/L). Both solutions were analyzed for their free monosaccharide as well as total pectic saccharide composition as described in section 2.3.

2.3 *Analysis of crude pectin extract*

HPAEC-PAD analysis of the crude extract was used in order to quantify the galacturonic acid and neutral sugars present in the pectin, in particular rhamnose and arabinose. In order to achieve a complete hydrolysis and to recover all pectic saccharides in the form of monomers, the extraction fluid was post-hydrolyzed by digestion with 5% (v/v) of Viscozyme L at 45 °C for 24 h (Martinez, Gullòn, Schols, Alonso & Parajo, 2009; Babbar *et al*, 2016 a). After hydrolysis, the enzyme was inactivated by a thermal treatment at 100 °C for 5 min and the liquid was centrifuged at 2040 x g (Eppendorf centrifuge 5415 C) for 10 min to get a clear supernatant. Samples of the extraction fluid were adequately diluted and injected into HPAEC-PAD. The HPAEC-PAD used for analytical purpose was a Dionex ICS-5000 model (Thermo Scientific, Inc., USA) equipped with an ED-5000 electrochemical detector. (Lee, 1996). The separation of monosaccharides was carried out with a Carbopac PA -1 (4 mm X 250 mm) column coupled to a guard Carbopac PA- 1 (4 mm X 50 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, A:B at 25:75 (start cleanup); at 10 min, B:C:D at 6:0:47 (re-equilibration); at 30min, B:C:D at 6:15:39.50 (stop re-equilibration and start acquisition); at 35 min, B:C:D at 50:50:0; at 36 min, B:C:D at 6:0:47; and at 46 min B:C:D at 6:0:47 (stop acquisition). The mobile

phase was used at a flow rate of 1 mL/min for 46 min and the injection volume was 5 microliters. Galacturonic acid, rhamnose and arabinose were quantified by comparing them against 9 standard solutions with known concentrations (ranging from 5 mg/L to 1000 mg/L).

For extensive measurement series covering several days, the measurement of the standard solutions was repeated several times to monitor the performance of the instrument throughout the analysis.

As an additional check, two fully independent, representative, quality control solutions of 75 mg/L and 260 mg/L were measured after every cycle of ~15 measurements. They were quantified against the standard solution and compared to with their expected concentration. On average their recovery was 102.9+/-3.4% (75 mg/L) and 100.5+/-4.0% (260 mg/L),

2.4 Enzymatic hydrolysis of onion pectic polysaccharides to POS in an EMR

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

2.4.1 General EMR operation

The general scheme of the EMR is given in Figure 1. It consists of a thermostated feed tank with fresh substrate, a thermostated reactor containing the reaction mixture, a cross flow membrane module coupled to the reactor and a product tank collecting the final product. The reaction mixture, composed of the enzyme and the hydrolyzing substrate, is continuously circulated with a pump over a cross flow membrane unit of 10 kDa. The POS (and other solutes) with a molecular weight lower than 10 kDa are (partially) removed from the reaction mixture by permeation and led to the product tank, while the retentate is recycled back to the reactor for further hydrolysis. The permeation flux is fixed throughout the experiment and pre-set to guarantee the specified average residence time of the reaction mixture in the reactor. The product removal is compensated by the

continuous addition of fresh substrate from the feed tank providing in this way a continuous operation.

2.4.2 Detailed POS production process

The 3 L reactor was filled with 600 mL (±5 mL) crude pectin solution obtained from the extraction process. Two different types of feed were used, i.e., either the crude pectin extract as such corresponding to an onion skin substrate ratio of 50 g/L, or the 2x diluted crude pectin extract corresponding to an onion skin substrate ratio of 25 g/L. The system was thermostated at 45°C (optimum temperature of the enzyme). After reaching the temperature, 60 mL of a diluted solution of Viscozyme L at pH 4.5 was added to the reactor vessel. The dilution was chosen to achieve the enzyme activity as reported below.

Before starting the full operation of the reactor, i.e. continuous feeding and filtration, first a (static) pre-hydrolysis was performed while stirring the vessel at 200 rpm. The pre-hydrolysis time was taken equal to the residence time (RT) applied during full reactor operation to generate a reaction mixture resembling the actual operation. This was done to facilitate the start-up of the reactor, avoid initial filtration problems, and achieve steady state as quickly as possible. After pre-hydrolysis, the continuous feeding of the substrate and the simultaneous filtration of the products were started. The permeate pressure was monitored along the experiment and was found to stabilize at 0.5 ±0.1 bar. The permeates were collected every 15 min (in case of a residence time of 15 min - RT15) or every 30 min (in case of 30 min - RT30) until the end of the experiment. These time intervals corresponded to the time needed to obtain a new replenishment of the reactor content. The volume and the pH of the permeates collected were monitored. Since preliminary tests indicated that the enzyme was completely retained in the reactor, no deactivation step was performed on the permeates. At the end of the experiment, the reactor content (residue)was recovered as well. This residue was immediately heated at 95^{o}C for 10 minutes to inactivate the enzyme and avoid further hydrolysis. The residue was weighted as well in order to make mass balances.

At the end of the experiment, the whole system was rinsed with MilliQ water and thoroughly cleaned and disinfected by P3-Oxonia active and Ultrasil 115.

Seven experiments were conducted testing three enzyme dilutions with different endo-polygalacturonase activities (determined as reported in Babbar, 2016a), i.e. 82.7 U/mL, 41.4 U/mL and 20.7 U/mL, two substrate concentrations, i.e. the equivalent of 25 g/L and 50 g/L onion skins, and two residence times of 15 (RT15) and 30 minutes (RT30). The range in residence time and enzyme concentration was selected based on the kinetic information obtained in previous research on the hydrolysis of onion skins in a batch reactor (Babbar *et al*, 2016b). The substrate concentrations were based on earlier research that was performed on the hydrolysis of sugar beet pectin in a similar EMR set-up.

2.5 Analysis and characterization of the POS mixtures

The permeates obtained during reactor operation and the residue were analyzed on their POS-content and composition.

Free monosaccharides (galacturonic acid, arabinose and rhamnose) were determined by HPAEC-PAD as already described in section 2.2. Total saccharides, measured as total galacturonic acid, arabinose and rhamnose, were measured by performing a complete digestion with Viscozyme L, and then determining the free monosaccharides as reported above. The analyses were done in duplicate, unless specified otherwise.

The concentration of free and total galacturonic acid obtained were further converted to total mass by taking into account the collected volume of permeate or retentate under consideration. Based on these results, the mass of oligo- and polygalacturonic acid ($(m_{GalA}^{POS}(\text{permeate t})$) in the permeate collected at time t (permeate t) was then calculated by subtracting the total mass of free galacturonic acid ($m_{GalA}^{mono}(\text{permeate t})$) measured as such from the total mass of galacturonic acid detected after post hydrolysis ($m_{GalA}^{total}(\text{permeate t})$).

Individual oligosaccharides were determined by the same HPAEC-PAD system as above, equipped with a different column and using a different elution system. The column was a Dionex CarboPac PA-100 (4 mm × 250 mm) coupled to a CarboPac guard column (4 mm × 40 mm). The mobile phase consisted of 100 mM sodium hydroxide (eluent A), 600 mM sodium acetate in 100 mM sodium hydroxide (eluent B) and 500 mM sodium hydroxide (eluent C). Elution conditions were as follows: A:B as 95:5 over 0–5 min, A:B 50:50 at 10 min, A:B 20:80 over 15–35 min, B:C 50:50 over 36–43 min and A:B 95:5 over 44–50 min. The flow-rate was 1 mL/min and the injection volume was 25 microliters. The identification of retention time of the different oligomers was performed based on a galacturonan oligosaccharide mixture DP1-DP10 (DP = degree of polymerization).

The quantification of DP2-DP4 was done against standard solutions of DP2, DP3 and DP4 oligomers, prepared in the range of 5 to 150 ppm and measured independently in each experimental series. The (absolute) areas obtained for the standards relevant for the quantification were compared among the different measurement series done in different days. They were found to vary less than 10% for DP2 and DP3 and less than 15% for DP4, indicating a high instrumental stability. Variability due to sample preparation was also determined based on the free monosaccharide analysis (done on the same samples) The combined contribution on the overall variability of the oligosaccharide analysis was estimated to be 15%. Duplicate analysis of the last permeates of the various experiments, confirmed the estimated variation.

Due to a lack of standards, the quantification of DP5 to DP9 was performed by using an estimated molar response factor R_M, essentially as reported in Babbar et al (2016b). In the present paper, the molar response factor was calculated on areas rather than peak heights, in order to increase the accuracy of the quantification of the smallest peaks. The fitting was done separately for every experimental series providing 6 independent fitting results with an r^2 ranging between 0.93 and 0.98, and having as overall equation $R_M(DPx) = a\sqrt{\frac{1}{MW}}$ with a=2530±180. To make a

274 comparison with the earlier work, the fitting was repeated for the peak height and a very good
275 agreement was.
276
277 *2.6 Assessment of the oligomer/monosaccharide ratio, yield and productivity*
278 After the quantification, each permeate collected during a reactor refreshment cycle at time t
279 was characterized by the ratio between its mass of dissolved galacturonan oligomers over its mass
280 of dissolved galacturonic acid monosaccharides (in g/g), which was calculated by formula (1):
281 $$Ratio(t) = \frac{m_{GalA}^{POS}(\text{permeate t})}{m_{GalA}^{mono}(\text{permeate t})}, (1)$$
282 With permeate t = the permeate collected at time t (in min); $m_{GalA}^{POS}(\text{permeate t})$ = mass of
283 galacturonan oligomer (in g) in the permeate collected at time t; $m_{GalA}^{mono}(\text{permeate t})$ = mass of
284 monomeric galacturonic acid (in g) in the permeate collected at time t.
285 The yield Y^{POS} of the POS (in % g/g) was calculated in a cumulative way as a function of
286 the collection time t, by dividing the total mass of POS recovered in all permeates collected up to
287 time t by the total mass of saccharides already added to the reactor at time t by the feed, by using
288 the following formula:
289 $$Y^{POS}(t) = \frac{\sum_0^t m_{GalA}^{POS}(\text{permeate t})}{\sum_0^t m_{GalA}^{total}(\text{feed,t})} x100, (2)$$
290 where $\sum_0^t m_{GalA}^{POS}(\text{permeate t})$ = total mass of galacturonan oligomers (in g) obtained in all
291 permeates collected up to time t; $\sum_0^t m_{GalA}^{total}(\text{feed, t})$ = total mass of galacturonic saccharides (in g),
292 in the form of free, oligo and polysaccharides, already added to the reactor at time t as the feed.
293 Very similarly also the yields of each DP were calculated with equation (3):
294 $$Y^{DP}(t) = \frac{\sum_0^t m_{GalA}^{DP}(\text{permeate t})}{\sum_0^t m_{GalA}^{total}(\text{feed,t})} x100, (3)$$
295 where $\sum_0^t m_{GalA}^{DP}(\text{permeate t})$ = total mass of oligomers with a specific DP (in g) obtained in all
296 permeates collected up to time t.
297 The volumetric productivity of POS (in g/L/h) was calculated in a cumulative way as a

function of the collection time t by considering all permeates collected up to the time under consideration by formula (4):

$$Volumetric\ Productivity\ (t) = \frac{\sum_0^t m_{GalA}^{POS}(\text{permeate t})}{0{,}66\ L} \times \frac{60\ min/h}{t}, \quad (4)$$

where $\sum_0^t m_{GalA}^{POS}(\text{permeate t})$ = total mass of galacturonan oligomers (in g) obtained in all permeates collected up to time t (in g); t= collection time under consideration (in min); 0.6 L = total volume of the reaction mixture including enzyme.

Very similarly, the specific productivity (in g/g enzyme/h) was calculated by using the following formula:

$$Specific\ Productivity\ (t) = \frac{\sum_0^t m_{GalA}^{POS}(\text{permeate t})}{m_{enzyme}} \times \frac{60\ min/h}{t}, \quad (5)$$

where m_{enzyme} = the mass of pure enzyme added to the reaction mixture. This was calculated from the mass of enzyme solution added corrected for the dilution applied when dissolving the enzyme.

3 Results and Discussion

3.1 Composition of the crude pectic extract

The crude pectic extract, obtained by SHMP extraction from onion skins (see experimental section) and used as a substrate for the process, was analyzed for its total saccharide composition by using the procedure described in section 2.3. The extracted galacturonic acid content accounted for 19.9 ±2.0 % (w/w) of the onion skins on dry matter base. Rhamnose and arabinose accounted for 0.226±0.028 % (w/w) and 0.0932±0.0078 % (w/w), respectively. The low content of arabinose and rhamnose is in agreement with previous results which indicated that onion skin pectin was mostly made of homogalacturonan, and very poor in rhamnogalacturonan regions (Babbar *et al*, 2016b). To prepare the feed for the hydrolysis, the crude pectic extract was standardly diluted two times, corresponding to an onion skin equivalent of 25 g/L. This feed contained 4.33±0.43 g/L of galacturonic acid, 0.0203±0.0017 g/L of arabinose and 0.0492 ±0.0060 g/L of rhamnose. In a last

experiment also the undiluted crude extract was used, corresponding to an onion skin equivalent of 50 g/L. The galacturonic acid was found to be mostly present as polysaccharide with a free galacturonic acid content of less than 1% of the total galacturonic acid measured. No oligomers were detected in the DP2-DP9 range.

3.2 POS production: effect of process conditions

As already mentioned, the main aim of the present work was to valorize onion skins towards POS through enzymatic hydrolysis by using an innovative approach based on a continuous cross-flow membrane bioreactor. It was anticipated that the combination of enzymatic hydrolysis with *in-situ* membrane based separation might allow to increase productivity as well as to obtain more tailored POS-products, when using a multi-activity enzyme. In order to determine the best process window of operation, in first instance the influence of residence time as well as enzyme concentration (and thus activity) was investigated. More specifically, six tests were carried out at a fixed substrate concentration of 25 g/L (as onion skin equivalent), but with varying residence time, i.e., 15 and 30 minutes, and enzyme concentration, i.e., 82.7 U/mL, 41.4 U/mL and 20.7 U/mL. For all tests the mass balance was made for the galacturonic acid. For each in-going (total feed of the reactor) and out-going (all permeates and residue in reactor at the end of the experiment) stream, the total galacturonic acid content was determined based on its concentration and volume. The mass balance, as averaged over all experiments, was found to be 101.3 ±7.9%.

The process was first evaluated on a more global level, whereby the POS production was assessed in time on its yield and productivity and on its ratio against the galacturonic monosaccharides present. The results are displayed in Fig.2 (POS-volumetric productivity, eq.4), Fig.3 (ratio POS/monosaccharides, eq.1) and Fig.4 (POS-yield, eq.2). An important indicator of the POS-production performance is given by the volumetric productivity, which is defined as the amount of POS that can be produced per unit of time and per unit of reaction volume and is affected by the process parameters (the process level) as well as the hydrolysis performance (the reaction

level), which should lead to high amount of POS production, but not to monosaccharide production. As an example, if the residence time of the enzyme can be halved without affecting the hydrolysis performance of the reaction, a double amount of POS can be produced per hour with a given reactor capacity, giving a doubling of the volumetric productivity. If, on the other hand, reaction conditions are selected that lead to more extensive monosaccharide formation, POS production and thus also volumetric productivity will be lower than expected for a specific reactor volume.

Fig. 2 shows the volumetric productivity of POS (expressed in gram POS per liter per hour) in function of the residence time and enzyme concentration applied. When comparing the steady state results at RT15, the POS-volumetric productivity increases from 10.3±0.3 g/L/h at the lowest (20.7 U/mL, Fig 2a) up to 12.8 ± 0.6 g/L/h at the intermediate and similarly 11.8 ± 1.3 g/L/h at the highest enzyme concentration (41.4 U/mL and 82.7 U/mL, Fig.2a). This indicates that for a residence time of 15 minutes, at least an enzyme concentration of 41.4 U/mL is needed to convert the polysaccharides fast enough to POS in order to achieve the highest volumetric productivity of 13 g/L/h. At this condition the steady state is also reached faster (75 min) as compared to the 105 min needed at the lowest enzyme amount.

For RT30, the POS-volumetric productivity is typically lower. An optimal volumetric productivity of 5.5 ± 0.8 g/L/h was found at the intermediate enzyme concentration (41.4 U/ml, Fig.2b). Increasing the enzyme concentration (82.7 U/ml) lowers the POS volumetric productivity (3.83 ± 0.03 g/L/h), indicative for losses through more extensive monosaccharide formation. At RT30 consequently a lower enzyme concentration has to be applied to achieve optimal POS-production, since the high enzyme concentration more easily leads to monosaccharides, an effect which is less present at RT15 as compared to RT30.

When comparing the volumetric productivities obtained at the two residence times for each enzyme concentration, a doubling of volumetric productivity is noted between RT15 and RT30 at the lowest enzyme concentration (10.3 g/L/h vs 5.2 g/L/h). This result is in full agreement with the expectations at a process level for the change in residence time, i.e., a doubling of volumetric

productivity when halving the residence time, and indicates that the POS production itself is not altered by the shorter contact times applied. At the highest enzyme concentration, the volumetric productivity increases with a factor 3 from RT30 to RT15 (3.8 g/L/h vs 11.8 g/L/h, respectively). In this case, the POS-production at RT30 is lower than expected, likely due to more extensive monosaccharide formation as mentioned earlier.

By following the method reported in section 2.5, the amount of POS related to the amount of free galacturonic acid (POS/mono ratio) and the POS yield were determined. Figure 3 shows the ratio between the total POS and the total monosaccharides (not cumulative) collected in the permeates in function of reaction time as determined by equation 1. The graphs in the Fig. 4 show the yield of POS and monosaccharides in function of the collection time on percentage weight by weight-basis. Clear differences are seen between the various production conditions. At low enzyme concentration, the permeates are typically very rich in POS with little monosaccharide formation. However, at the same time, the ratio between POS and monos keeps on increasing in time, indicating an instable process and possibly an accumulation of saccharides in the reactor As can be expected, the effect is more pronounced at RT15 than at RT30 when considering the same reactor replenishment. The two highest enzyme concentrations, on the other hand, lead to more stable POS/mono ratios indicating a more stable process. Within this operational area, the shortest RT15 as well as the middle enzyme concentration give the best results, whereby longer residence times as well as higher enzyme concentrations lead to more extensive monosaccharide formation. In all the cases the POS/mono ratio is higher than 1, hence the process allowed the production of mixtures rich in POS. As already mentioned for Fig. 2, monosaccharide formation is indeed higher at the 82.7 U/mL than at the 41.4 U/mL condition.

The present observations were confirmed by the behavior of the yield of POS and the monomeric galacturonic acid. Indeed, as depicted in the graphs (Fig. 4), at the lowest enzyme concentration, 20.7 U/mL, the substrate is not completely hydrolyzed in comparison with the higher enzyme concentrations. In fact, until 70 and 120 minutes of reaction at respectively RT 15 (Fig. 4e)

and RT 30 (Fig. 4f), the total percentage of permeation is lower than 70%, suggesting an accumulation of the long pectic chains in the reactor. Moreover, the low monosaccharides formation (about 20-30%) is confirmed, and a concomitant increase in POS yield is observed even after multiple reactor replenishments indicating a lower stability of the process.

When the enzyme is more concentrated, most of the substrate fed into the reactor is hydrolyzed into POS fractions lower than 10 kDa and filtered, especially at 82.7 U/mL and at RT30 (100% permeation in steady state) (Fig. 4b). However, according to the aim of the work, this latter result is not acceptable since the amount of the monosaccharides produced is too high (50%).

A very good compromise between POS yield and monosaccharides formation can be noticed using the middle enzyme concentration and the short residence time (Fig. 4c). Indeed, the hydrolysis results in a POS yield of 60% and only a monosaccharides yield of 25%. The built-up of long pectic chains inside the reactor can still not fully be excluded since the pectin feed does not seem fully converted. Nevertheless, compared to other conditions, this condition lead to a limited formation of monosaccharides, and a stable POS production already after 45 to 60 minutes. In order to evaluate the percentage of conversion of the galacturonan polysaccharides into POS, the oligosaccharides were quantified as outlined in section 2.6. The quantification of the higher DPs (DP>4) was done using the corresponding response factors from the fitting. Based on these results the DP-yields were calculated on mass/mass percent for each condition in function of time by using equation (3) (see Fig. 5, a-f).

The DP-yield in function of the process time immediately shows an instability of the hydrolysis at lower enzyme concentration (Fig 5 e and f). This confirms the earlier observations made based on the POS/mono ratio (Fig. 3) and the POS yield (Fig. 4). In fact, in function of time the production of small POS seems to decrease, in favor of the longer POS (DP>4) indicating an accumulation of long pectic chains inside the reactor.

A more stable production of short POS (DP2, DP3 and DP4) in function of time is observed at middle enzyme concentration and short residence time. As already observed in Fig.4, at this

condition the POS production seems to stabilize after 45 min process time in a POS product rich in the lower DPs, with only minor fractions of DP6 and higher. Changing the residence time to 30 minutes, gives overall a lower amount of POS (especially DP3 and DP2) concomitant with the higher degree of monosaccharides observed in Fig. 3 d. Higher enzyme concentrations provide, on the other hand, POS-products that are enriched in the smaller DP2 and DP3 -oligomers (Fig 5 a and b).

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

The results from tests performed at a substrate concentration at 25g/L demonstrated that it is possible to achieve a good yield of pectic oligosaccharides (60% w/w), especially using the middle enzyme concentration (41.4 U/mL) and the short residence time (15 minutes). The process leads to a POS-product composed mostly of DP2 to DP4, with minor amounts of oligomers with a higher DP. The enzyme was found to be stable during the applied production times.

3.2 POS production: effect of substrate concentration

In order to optimize the continuous production of POS from onion skins, also the possibility to work at higher substrate concentration was studied, i.e. 50 g/L. The hydrolysis was performed using the residence time of 15 minutes and the enzyme concentration of 41.4 U/mL, since the latter conditions gave the most promising results in the previous sections.

The ratio POS/monos and the volumetric productivity (g/L/h) were calculated and they accounted for about 4.1 and 22.0 g/L/h in the steady state respectively (data not shown).

Fig. 5 shows the yield (% w/w) of POS and monosaccharides, as well as the yield (% w/w) of POS with DP2-DP9 in function of the process time.

As depicted in Fig. 6a, the hydrolysis of crude pectin extract provides a good compromise between the POS yield, which accounts for 60%, and the monos yield, which is found to be around 12% in

452 the steady state. The process reaches the steady state already after 45 minutes and a stable
453 conversion into low DP POS (DP2, DP3, DP4) is observed. The volumetric productivities of the
454 low DP POS are respectively 3.2 (DP2), 5.7 (DP3) and 4.2 g/L/h (DP4). Compared to the POS
455 composition obtained at the lower substrate concentration (25 g/L) at the same conditions (Fig. 5c),
456 Fig.6a suggests that in this case the POS mixture is enriched in DP4 at the expense of DP2 and
457 DP3. As expected, the higher substrate concentration also leads to a higher productivity. In
458 particular, the specific productivity, i.e, the POS productivity per mass of enzyme used (eq. 5),
459 reached 20.4 g/gE/h as compared to 12.2 ± 0.9 g/gE/h for the lower substrate concentration (see
460 Fig 6c). It is also higher than the specific productivity reported in literature for continuous citrus
461 pectin hydrolysis, i.e. 9.7 g/gE/h (Bèlafi-Bako, Eszterle, Kiss, Nemastòthy & Gubicza, 2007).
462 Table 1 compares the results of this work in an EMR with the results obtained at similar
463 conditions in a batch set-up and discussed by Babbar et al (2016b). The batch experiments were
464 performed both with the multi-activity enzyme Viscozyme L as well as the specific endo-
465 polygalacturonase enzyme EPG-M2. In order to assess the potential of EMR, it was chosen to make
466 the comparison based on the optimal results achieved in each of the set-ups. Several parameters
467 were selected as indicator of the performance. The table gives an overview of the yield in which a
468 specific class of POS is produced from the crude pectin extract, the corresponding volumetric
469 productivity, and a measure for enzyme utilization given by the POS/enzyme activity ratio. Since
470 the details of operation differs among the two set-ups, some assumptions were made to assess the
471 productivity. For EMR, the volumetric productivity was determined as outlined in eq. 4 but then
472 separately for each specified POS-class. In the case of batch, the productivity was estimated based
473 on the POS yield reported by Babbar et al (2016) at the chosen reaction time (end time), substrate
474 loading and substrate composition. The reaction time chosen for the batch operation was rather
475 short (15 and 30 min), since larger reaction times were found to lead to significant monosaccharide
476 formation in the case of Viscozyme. Downtime in reactor operation, such as for instance due to
477 discharging of the hydrolysate or the loading of fresh substrate, was neglected. The POS/enzyme

activity ratio was determined in a similar way. For EMR is was calculated based on the volume (and EPG activity) of the enzyme added at the start and the total POS produced at the end of the experiment. In the case of batch, it was estimated assuming that for each new batch fresh enzyme is added to the reactor.

The table shows that EMR has a clear advantage over the batch process in terms of POS-yield and POS-volumetric productivity when the enzyme Viscozyme L is used in both cases. Both the yield as the volumetric productivity of the POS(DP2-DP4)-fraction is a factor of 3-5 higher in the EMR as to the batch. For the latter, the POS yield is hampered by extensive monosaccharide formation limiting the optimization options of the process towards POS, which is reduced when applying the EMR set-up. When comparing the best EMR-results of this work with the batch hydrolysis based on the specific enzyme EPG-M2, the yield and volumetric productivity are still somehow lower. But as mentioned above, in the calculation of the volumetric productivity of the batch system, the downtime related to the emptying and refilling of the reactor was not considered. The real productivity is therefore expected to be significantly lower and likely more close to the EMR-performance. The most striking difference between the two set-ups concerns the POS that can be produced per EPG-unit of the enzyme. Thanks to the continuous recycling of the enzyme and its stable performance, 2-3 times lower enzyme consumption (expressed as EPG-units) is observed in these tests.

It is clear that the EMR hydrolysis of undiluted crude extract from onion skins (50 g/L) results in a competitive process as compared to batch hydrolysis, even when the specific enzyme EPG-M2 is used. Of course, long-term tests are needed to possibly extend the enzyme recycling as well as determining the impact on the membrane performance. Nevertheless, the values reached in the present study starting from onion skins waste can be considered as very promising results.

4. Conclusions

The continuous enzyme membrane reactor for the pectin-oligosaccharide (POS) production, in which conversion is combined with separation, was found to be very promising for the continuous production of tailor made pectin oligosaccharides from onion skins.

The present study showed that an efficient separation of POS can be achieved using the 10 kDa MW cut-off membranes. Moreover, working with Viscozyme L at a residence time of 15 minutes, enzyme concentration of 41.4 U/ml and substrate concentration of 50 g/L, resulted in the highest POS volumetric productivity (22.0 g/L/h) and yield as well as the lowest POS/mono-saccharide ratio (4.5 g/g). At these conditions, a stable production of mainly short POS (DP2, DP3 and DP4) was also observed in function of time.

By comparing the present data for the low molecular POS-fraction with those obtained by Babbar et al (2016b) in a batch process using the crude pectin obtained from 50 g/L of onion skins as a substrate, the volumetric productivity of POS with DP2-DP4 and total POS was respectively g/L/h for the batch production and 3.2, 5.7 and 4.2 g/L/h for the present continuous production (RT15, 41.1 U/ml). Moreover, the POS (DP2-DP4)/monosaccharide ratio was 1.3 for the batch and 2.3 for the EMR-process. Thus, the use of the EMR provided a higher POS productivity and the suppression of the monosaccharides formation, fully demonstrating the feasibility of this technique for efficient POS production starting from onion skins.

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Figures captions

- **Figure 1:** Schematic diagram of the enzyme membrane reactor used.

- **Figure 2:** Volumetric productivity (g/L/h) of POS for RT of 15 (a) and 30 minutes (b), and at an enzyme concentration of 20.7 U/mL, 41.4 U/mL and 82.7 U/mL, using a substrate concentration of 25 g/L.

- **Figure 3**: Ratio of oligosaccharides to monosaccharides calculated for the residence times 15 (a) and 30 minutes (b) for an enzyme concentrations of 20.7 U/mL, 41.4 U/mL and 82.7 U/mL, and using a substrate concentration of 25 g/L.

- **Figure 4:** Yield (% w/w) of the POS and monosaccharides at a substrate concentration of 25 g/L in the following conditions: 82.7 U/mL at RT 15 (a) and RT 30 (b); 41.4 U/mL at RT15 (c) and RT 30 (d); 20.7 U/mL and RT 15 (e) and RT 30 (f).

- **Figure 5:** Yield (% w/w) of POS for the individual DPs for the hydrolysis at a substrate concentration of 25 g/L at the following conditions: 82.7 U/mL and RT 15 (a) and RT 30 (b); 41.4 U/mL and RT15 (c) and RT 30 (d); 20.7 U/mL and RT 15 (e) and RT 30 (f).

- **Figure 6:** Results obtained at a substrate concentration of 50 g/L at an enzyme concentration of 41.4 U/mL and RT 15. Yield (% w/w) of POS and monosaccharides (a); yield (% w/w) of POS for the individual DPs (b) and specific productivity of the POS-production in comparison to a low substrate concentration (c).

- **Table 1:** Comparison of the present EMR-results with the batch results of Babbar et al. (2016) obtained on the hydrolysis of pectin extracts from onion skins at similar conditions. A comparison is made between the volumetric productivity and yield for the DP2-DP4 fraction as well as the DP2-DP8-fraction. Also the enzyme consumption at the end of the experiment is given, expressed as g POS

620 produced per unit of endo-poly galacturonase (EPG) activity of the enzyme added. EPG-M2 is a pure
621 endo-polygalacturonase enzyme (Megazyme, Ireland).

Table(s)

Process	Enzyme	Activity enzyme solution	Substrate concentration (onion skin eq.)	Time		Yield		Volumetric productivity			POS/enzyme (end)	
		(EPG U/ml)	(g/L)	(min)		% (w/w)		(g/L/h)			g/EPG U enzyme x 10^3	
				RT	End	DP2-DP4	DP2-DP8	DP2-DP4	DP2-DP8	Total	DP2-DP4	Total
Batch	Viscozyme	82.7	50		15	8.3 ± 0.5		3.35 ± 0.04			0.102± 0.001	
Batch	Viscozyme	82.7	50		30	6.0 ± 0.7		1.20 ± 0.02			0.073± 0.001	
Batch	EPG M2	26	50		15	45	70	18.0	28.0		1.73	
Batch	EPG M2	26	50		30	41	59	8.1	11.8		1.57	
EMR	Viscozyme	41.4	25	15	105	34 ± 5*	60	7.1 ± 1.3*	11.0	12.8 ± 0.6	3.7 ± 0.7*	6.2 ± 0.8
EMR	Viscozyme	82.7	25	15	120	46 ± 7*	58	10.0 ± 1.8*	13.2	11.9 ± 0.6	2.6 ± 0.5*	3.0 ± 0.4
EMR	Viscozyme	41.4	50	15	120	32	57	11.9	18.1	22.0	6.3	10.3

*The estimated error is 15%, as outlined in section 2.5

Figure(s)

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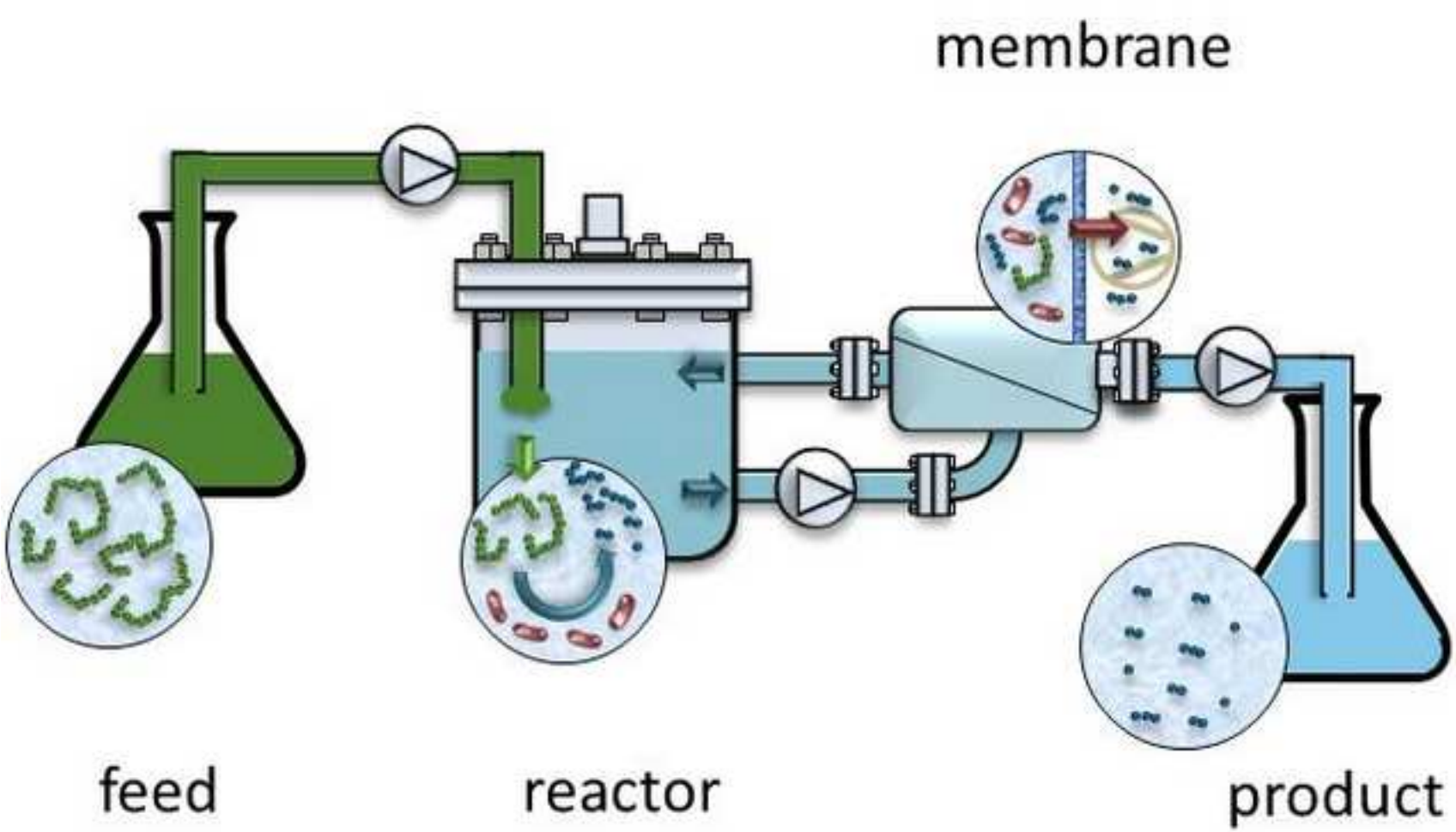

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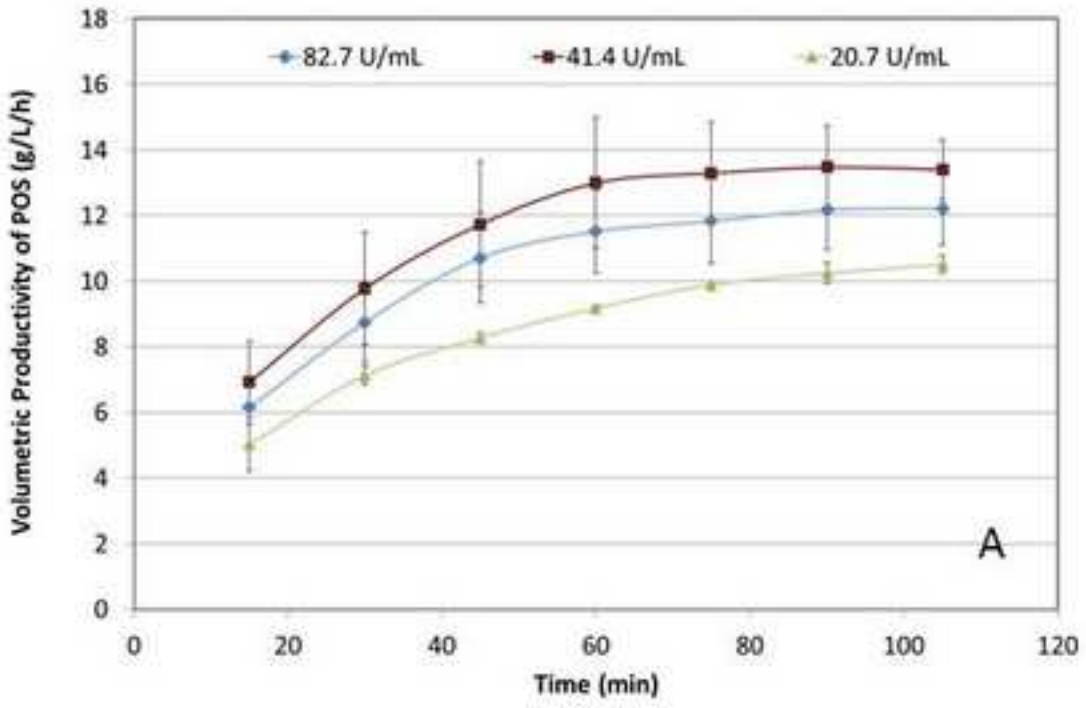

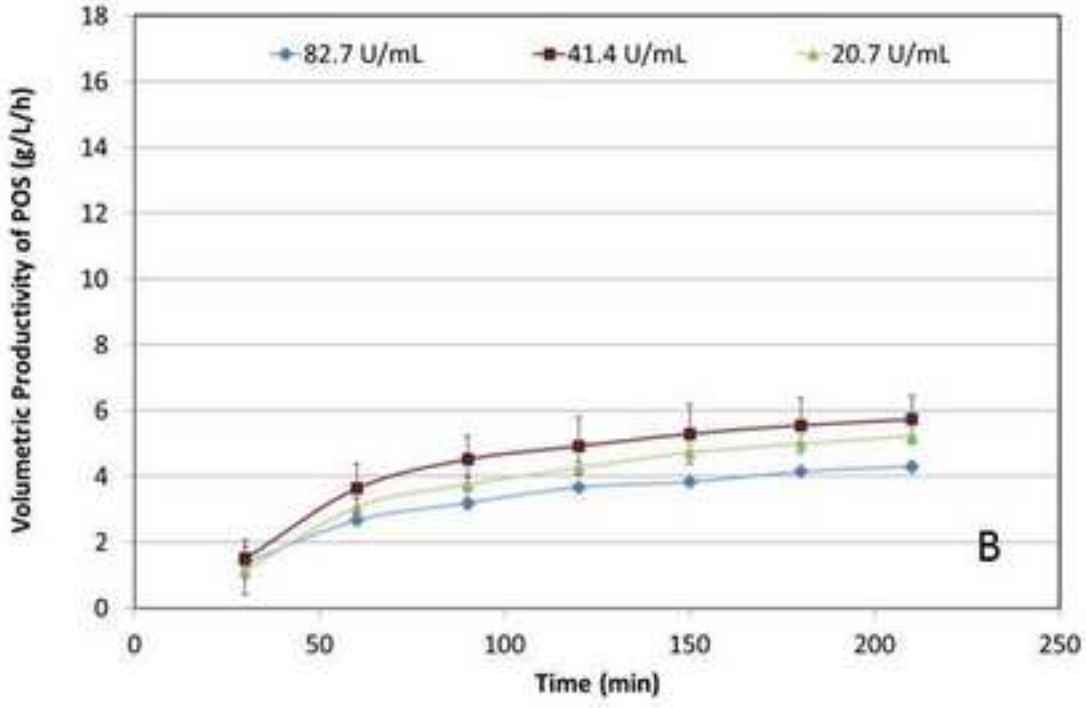

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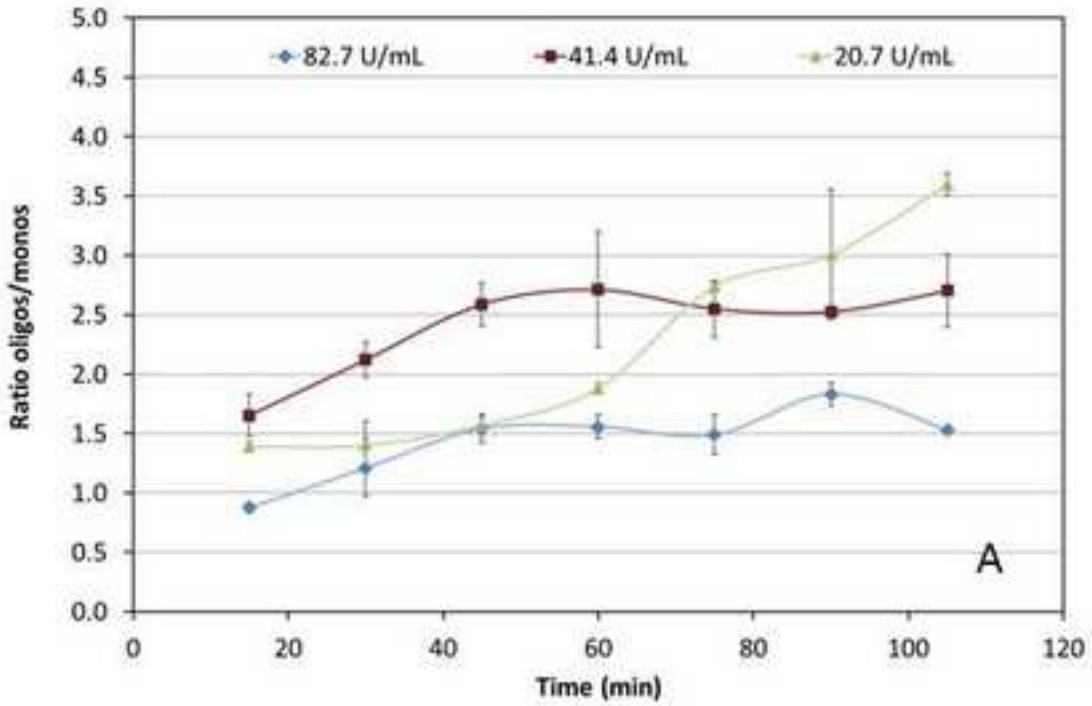

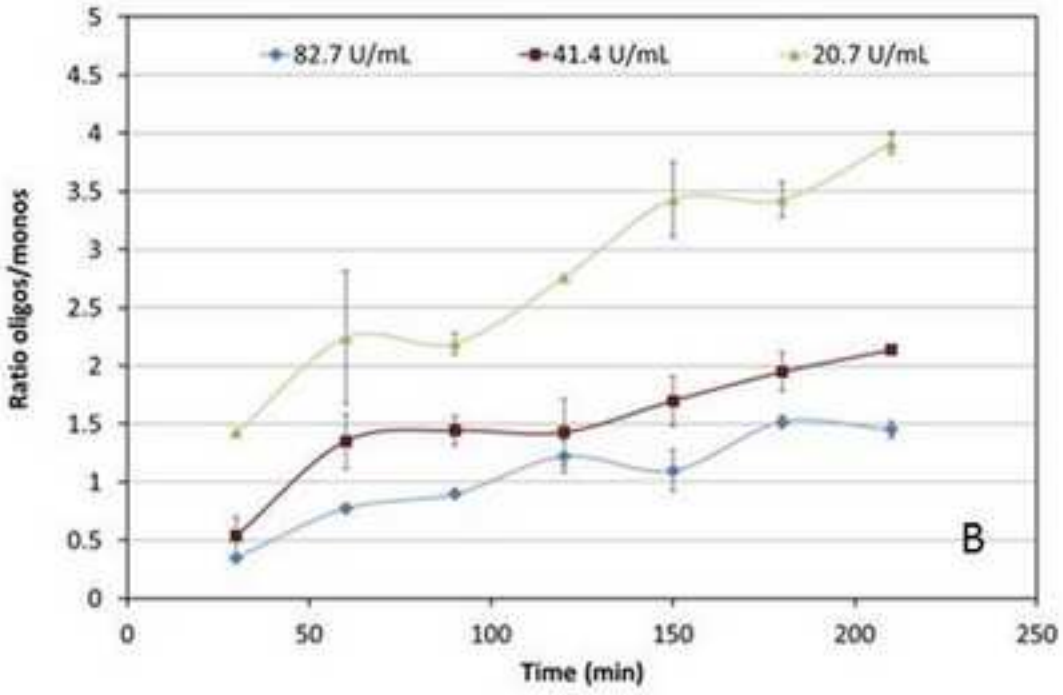

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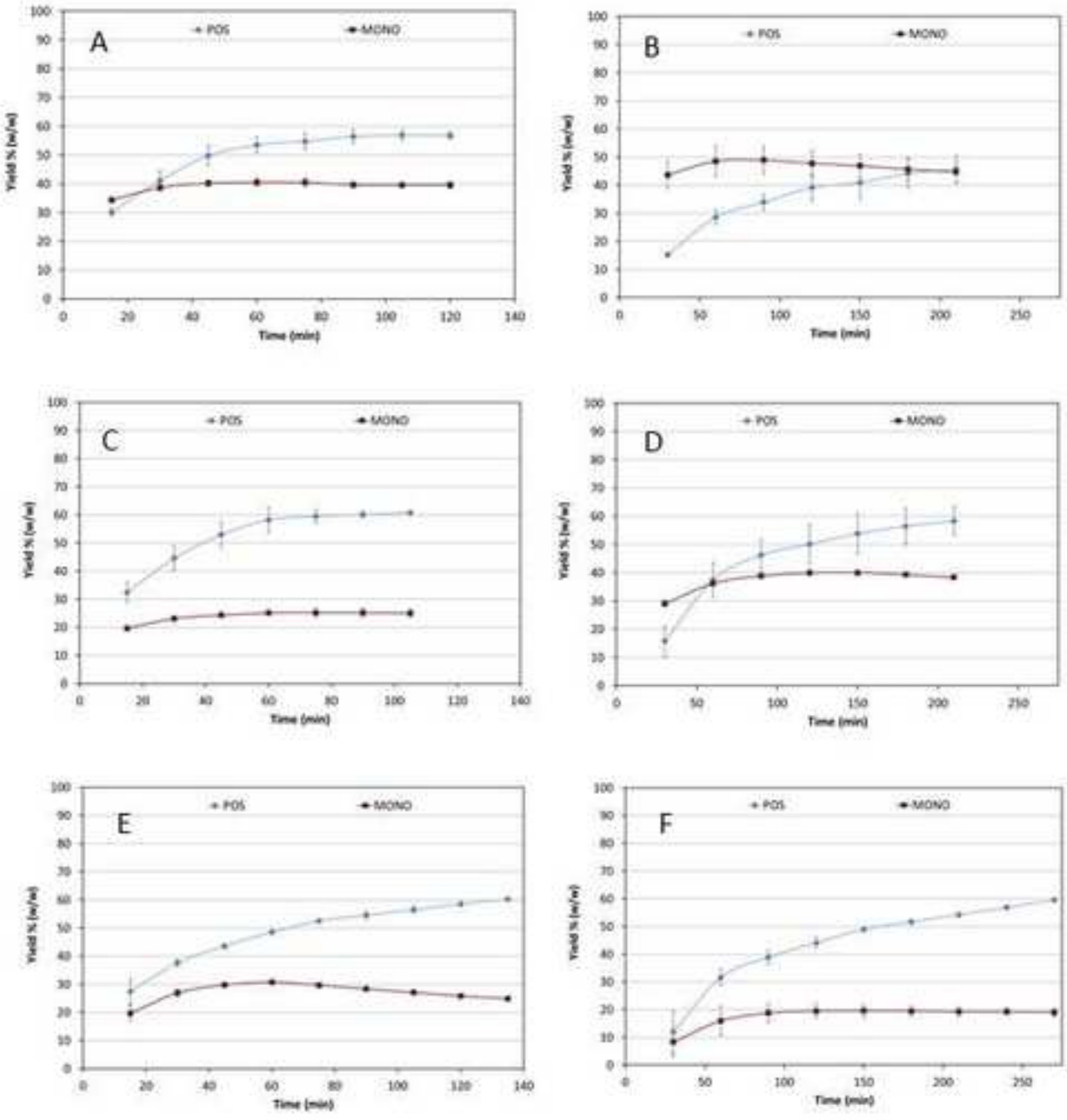

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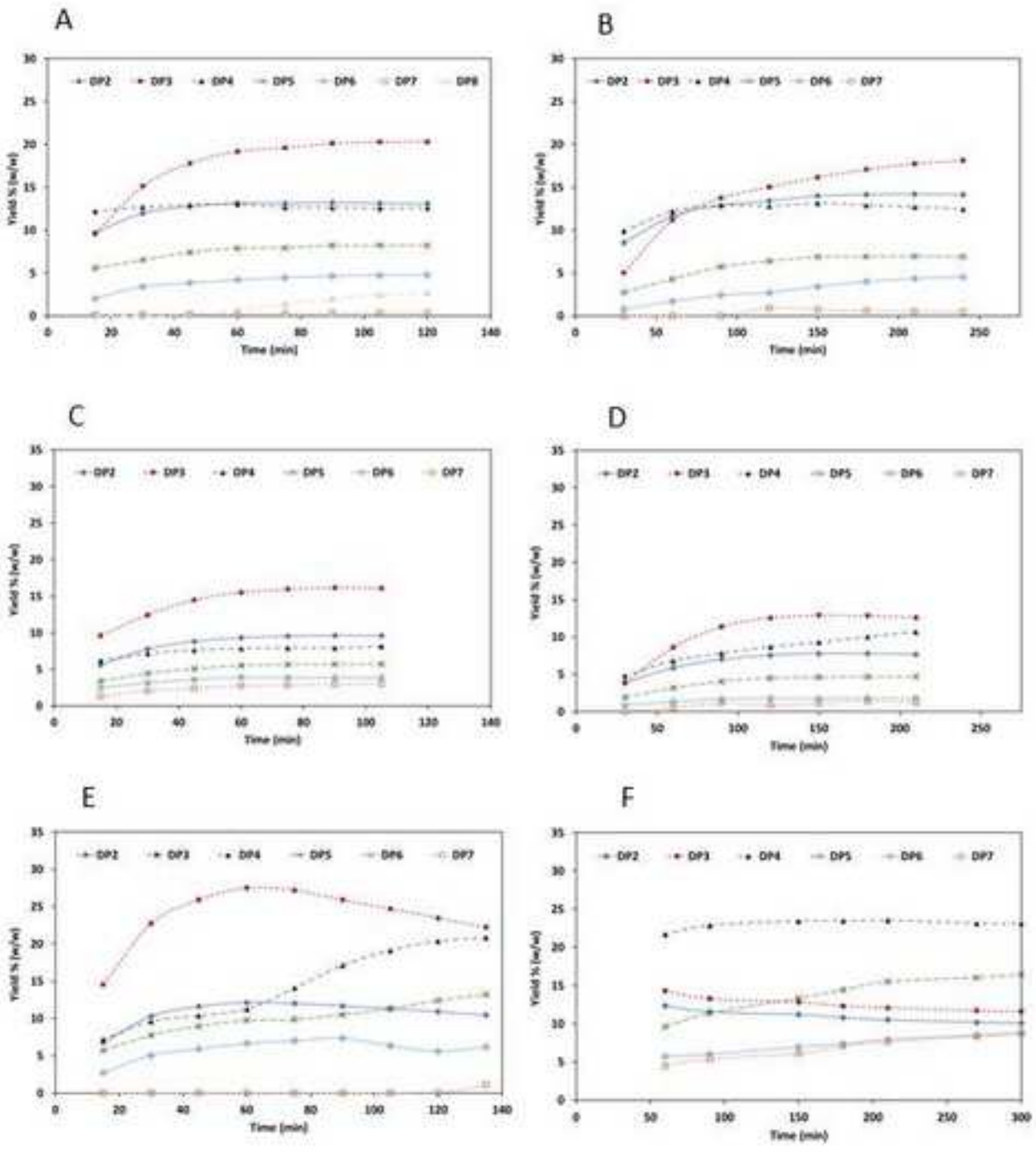

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