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Short Communication

Continuous flow production of xylooligosaccharides by enzymatic hydrolysis

Debjani Ghosh [a], Anil B. Vir [b], Gil Garnier [b], Antonio F. Patti [a], Joanne Tanner [b,*]

[a] School of Chemistry, Monash University, Clayton, VIC 3800, Australia
[b] Bioresource Processing Research Institute of Australia, Department of Chemical Engineering, Monash University, Clayton, VIC 3800, Australia

HIGHLIGHTS

- Continuous-flow system demonstrated for enzymatic hydrolysis of xylan.
- Effect of pH, flow rate ratio and concentration on the yield of low DP XOS studied.
- Continuous-flow xylan hydrolysis is more efficient than batch system.
- More than 80% conversion of xylan to xylooligosaccharides (XOS) was achieved at optimized enzyme: substrate flow.

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ABSTRACT

Xylooligosaccharides (XOS) are naturally occurring high value biochemicals with potential applications in the food, pharmaceutical and fine chemical industries. They are typically produced and purified from a variety of biomass resources in expensive and inefficient multi-step batch processes, and this high cost of production is a significant barrier to their commercial adoption. A flow-type microreactor was used to intensify and increase the efficiency of enzymatic XOS production from beechwood xylan. The xylan hydrolysis performance was compared with the same reaction performed under typical batch conditions. Over 80 g XOS per 100 g xylan was obtained in less than one minute of residence time at the optimum conditions tested in continuous flow experiments. The enzymatic xylan hydrolysis performance was improved and xylan to XOS conversion was three times higher under optimised flow conditions compared to a conventional batch process.

1. Introduction

With the growing interest of consumers in an active and healthy lifestyle, the demand for high quality dietary products is ever increasing. Xylooligosaccharides (XOS) are oligomers of xylose recognised for their potential health benefits (Amorim et al., 2019) and biological applications, including cancer treatment and prevention of neuro-toxicity (Aachary and Prapulla, 2011; Fei et al., 2020). Their unique physio-chemical properties can be utilized to improve the textural and rheological attributes of formulated products for food and personal care products (Xu et al., 2009; Mikkonen et al., 2016; Lahtinen et al., 2019). XOS are newly recognized and increasingly popular for their application in the area of prebiotics. When consumed, these non-digestible oligosaccharides have been shown to selectively stimulate the growth of good bacteria present in the host system, helping to boost the overall immunity of the host (Yang et al., 2015). The global market for prebiotics in 2017 was estimated around USD 4.07 billion and XOS alone are expected to reach a value of USD 130 million by the year 2023 (Amorim et al., 2019). To cope with this ever-increasing demand, continuous and sustainable production of XOS will be a crucial factor going forward.

XOS are produced by chemical or enzymatic hydrolysis of xylan, a major constituent of the hemicellulose fraction of lignocellulosic biomass, including residues from the agricultural (Samanta et al., 2012; Gullón et al., 2010) and timber industries (Nitsos et al., 2016). These biomass resources are potentially recoverable, renewable and readily available at large-scale, meaning that their xylan content can be sustainably utilised for value addition or new product development via XOS production.

In the native biomass, xylan exists as a complex with lignin, a structural component of the plant cell wall, which hinders xylan recovery and purification. Various methods including alkaline

* Corresponding author.
E-mail address: joanne.tanner@monash.edu (J. Tanner).

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hydrolysis (Huang and Ragauskas, 2013; Samanta et al., 2012) acid hydrolysis (Carvalho et al., 2018), autohydrolysis (Huang et al., 2020), direct enzymatic hydrolysis (Sinner et al., 1979), and combinations of these have been used for the sequential or simultaneous extraction of xylan and production of XOS. Owing to the inherent difficulties associated with direct XOS production from feedstock sources, a two-stage strategy is often adopted where xylan is first isolated from the feedstock using an alkali treatment and subsequently hydrolysed to produce XOS (Samanta et al., 2012; Hakala et al., 2013).

Hydrolysis of xylan to XOS can be achieved using either dilute acid or enzymes (Wang et al., 2018); (Sepulchro et al., 2020). Acid hydrolysis is typically quicker and cheaper, but requires high temperature (120–150 °C). Furthermore, the product is usually a mixture of XOS, monosaccharides and sugar byproducts (Akpinar et al., 2010). Enzymatic hydrolysis offers significant advantages over chemical hydrolysis. It is product specific, does not produce undesirable by-products, can be performed under milder conditions, and with careful enzyme selection can therefore generate an oligosaccharide-rich hydrolysate with minimal impurities (Bhardwaj et al., 2019). However, the reaction time required for enzymatic methods is typically longer, and the cost of enzyme replacement or recovery and recycling needs to be considered for large scale applications (Akpinar et al., 2010).

The goal of reducing cost and improving efficiency drives the current and continued research into greener and more sustainable XOS production processes. The ubiquitous commercial use of stirred-batch reactors represent a barrier to increasingly efficient production of fine chemicals, food and pharmaceutical products, including XOS. However, continuous reactors have been more difficult to optimize in laboratory than batch reactors, as specific systems had to be built, often requiring high quantities of reactants. Continuous flow microfluidic devices offer several advantages over traditional batch modes of reaction. Their high surface area to volume ratio and shorter diffusion path length provides more efficient mass transfer, and their low processing volume improves inherent safety and waste minimization, reflecting the principles of green chemistry (Vaccaro et al., 2014; Wiles and Watts, 2012; Pastre et al., 2013).

Given the growing market value and the increasing interest of xylan and XOS, enzymatic hydrolysis coupled with continuous flow systems represents a potentially efficient method for increased conversion of xylan to high value XOS as chemical intermediates and products. Aragon et al. (2013) have reported the continuous production of XOS using birchwood xylan and xylanase from *Aspergillus versicolor* immobilised on glyoxyl–agarose. Hydrolysis was performed at pH 5 and 60 °C resulting in 18% XOS (X2-X6) with the advantage of enzyme reusability for 10 continuous cycles (Aragon et al., 2013). Another step towards process intensification was recently reported by M. Romero-Fernández et al. (2018) where a packed bed reactor was used for continuous conversion of corn cob xylan to XOS using xylanase from *Streptomyces halstedii* JM8, which was immobilized on a methacrylate-based resin. The key advantages in this study included hydrolysis at low temperature (30 °C and pH 5), high flow rates (10 mL/min) in the reactor bed and high enzyme efficiency (retaining 90% of the initial activity) for a continuous operation of 120 h. Xylan conversion reached a maximum value of 86%. However, xylose was the major product (71%) with the balance of XOS (X2, X3, X4) (Romero-Fernández et al., 2018). In a recent study, Birchwood xylan was hydrolysed using xylanase immobilised in a sol–gel matrices and it was observed that xylan hydrolysis was improved when microreactors were used producing 18% xylose equivalent sugars in the hydrolysate for 40 h (Pirozzi et al., 2019). Furthermore, enzyme immobilization compromises reaction efficiency, and is frequently impermanent, necessitating frequent enzyme rejuvenation or replacement of the microreactors.

In this study, a continuous flow microreactor was used to investigate and optimise the enzymatic hydrolysis of beechwood xylan to commercially attractive XOS. The current approach represents a green alternative to traditional XOS production methods. In this paper, a sequential optimisation of the enzymatic hydrolysis process is presented considering the effect of pH, reagent stoichiometry, and residence time on xylan conversion, and XOS yield and composition. It is the objective of this study to investigate micro flow-reactors as mean to optimize a bioprocess and to replace batch reactors by efficient continuous methods to produce XOS via an enzymatic process.

2. Materials and methods

2.1. Materials

Beechwood xylan (≥90% xylose residues) and *endo*-β-(1,4)-xylanase from *Thermomyces lanuginosus* (≥2500 U/g) were obtained from Sigma-Aldrich (St Louis, USA), XOS standards were obtained from Megazyme (USA). All other chemicals were of analytical grade and used as received.

2.2. Preparation of xylan solution

Xylan solutions were prepared at two different pH conditions i.e. pH 4 and pH 10 by mixing 0.2 g of beechwood xylan with 12 mL of (i) pH 4 50 mM citrate buffer (pH 4 xylan solution) (ii) DI water and adjusting to pH 10 with 5 M sodium hydroxide (pH 10 xylan solution). The resulting xylan suspensions were equilibrated for 16 h at 45 °C and 100 rpm in a shaker incubator. Post incubation, the suspensions were centrifuged to remove any insoluble materials and the supernatant solutions were stored at 10 °C prior to further processing and analysis. The xylan concentration in the supernatant was 1.00% (w/v) and 1.25% (w/v) at pH 4 and 10 respectively, determined gravimetrically (Table 1, supporting document). These stock solutions were prepared fresh, stored in sealed containers at 10 °C and used for all batch and continuous flow microreactor experiments. Prior to enzymatic hydrolysis, the prepared xylan stock solutions were always analysed by HPLC to confirm no degradation of xylan to XOS had occurred during the storage.

2.3. Preparation of xylanase solution

The xylanase solution was prepared with purified *endo*-β-(1,4)-xylanase obtained from *Thermomyces lanuginosus* at an enzyme dosage of 2.5 U/mL (50 mM citrate buffer, pH 4) and equilibrated for 16 h at 45 °C and 100 rpm in a shaker incubator. Post incubation, the suspensions were centrifuged and the supernatant solutions were stored at 10 °C prior to further processing and analysis. The same stock solution was used for all batch and microreactor experiments. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μ mol of xylose equivalent per minute.

2.4. Enzymatic hydrolysis of xylan in a batch process

In typical batch experiment a 10 mL of xylan solution and 10 mL of enzyme solution were mixed and incubated at 25 °C and 100 rpm in a shaker incubator. The xylan hydrolysis was monitored by taking aliquots at 10, 15, 20, 40 and 60 min. The enzymatic reaction was quenched by heating the aliquot at 100 °C for 15 min. The residual enzyme activity was <15%, was validated by monitoring the enzyme activity at 100 °C (Fig. 1, supporting document). The samples were stored at 4 °C prior to analysis by HPLC. All the reactions were carried out at least in duplicate.

2.5. Continuous enzymatic hydrolysis of xylan in a microreactor

The continuous enzymatic hydrolysis of xylan were carried out in the microreactor and the prepared xylan and xylanase solutions were fed separately to the microreactor via syringe pumps (Fig. 1). The microfluidic experimental set-up consisted of a Y-Junction serpentine glass microchannel (Micronit Microtechnologies, The Netherlands) with a rectangular cross-section having two inlets and one outlet. The width, height, and volume of the microchannel were 150 μm, 300 μm and 18.7 μL, respectively. Flow rates of the enzyme and substrate solutions were manipulated using the syringe pumps to achieve the different residence time (Table 2, supporting document). The hydrolysis was performed at ambient temperature (23–25 °C). The enzymatic reaction was quenched by heating the hydrolysate at a constant temperature of 100 °C for 15 min, and the product samples stored at 4 °C prior to analysis. All the experiments are repeated 3 times to confirm the reproducibility of the results, and average values along with standard deviations are reported.

2.6. Carbohydrate analysis

The produced XOS in the hydrolysis product samples were quantified using a high-performance liquid chromatograph (HPLC) (Agilent infinity 1290) coupled with a refractive index detector (RID) and a UV detector set at 280 nm. A Biorad Aminex HPX-87P column (300 × 7.7 mm) was used for the oligo- and monosaccharide analysis. The column was operated at 80 °C, using deionized water as the mobile phase at a constant flow of 0.5 mL min^{-1}. Calibration was performed using a range of XOS standards (xylobiose, xylotriose, xylotetraose), monomeric sugars (xylose, glucose, arabinose) and acetic acid. Based on the composition of the xylan feedstock and the specificity of the xylanase, the majority of the products were expected to be short, linear *xylo*-oligosaccharides.

2.7. Calculation of parameters described

The conversion of individual XOS (w/w %) was calculated based on the initial amount of xylan (mg) using the following equation:

$$\boldsymbol{Conversion\ to\ XOS}\ (\%) = \frac{\boldsymbol{Amount\ of\ individual\ XOS\ component\ produced}\ (\boldsymbol{mg})}{\boldsymbol{Initial\ amount\ of\ xylan}\ (\boldsymbol{mg})} \times 100 \tag{1}$$

$$\boldsymbol{Conversion\ to\ xylose}\ (\%) = \frac{\boldsymbol{Amount\ of\ xylose\ produced}\ (\boldsymbol{mg})}{\boldsymbol{Initial\ amount\ of\ xylan}\ (\boldsymbol{mg})} \times 100 \tag{2}$$

The total conversion to XOS was calculated by summing the conversion to each individual XOS component, i.e. xylobiose (X2), xylotriose (X3) and xylotetraose (X4), as calculated using Equation (1):

$$\text{Overall conversion to XOS } (\%) = X2(\%) + X3(\%) + X4(\%)$$

In the continuous flow process the residence time was used for the analysis of xylan hydrolysis and is defined as the time spent by the enzyme (xylanase) and substrate (xylan) in the reactor. The residence time was calculated based on the total combined flow-rate of the xylan and the xylanase phases as:

$$\boldsymbol{Residence\ time}\ (\boldsymbol{min}) = \frac{\boldsymbol{Volume\ of\ microreactor}\ (\boldsymbol{\mu L})}{\boldsymbol{total\ volumetric\ flow\ rate}\ (\boldsymbol{\mu L\ min^{-1}})} \tag{3}$$

For the batch system, reaction time was used for the analysis of xylan hydrolysis performance and was taken as the sampling time as described above.

3. Results and discussion

Enzymatic hydrolysis of xylan to XOS is known to proceed via selective cleavage of the main chain β-1,4 glycosidic bonds, generating smaller chains of xylose repeating units (Fig. 2). Many native xylan exhibit side chain substitution, the patterns of which are biomass dependent. These side chains can interfere with the action of the enzyme leading to the production of oligomers greater than

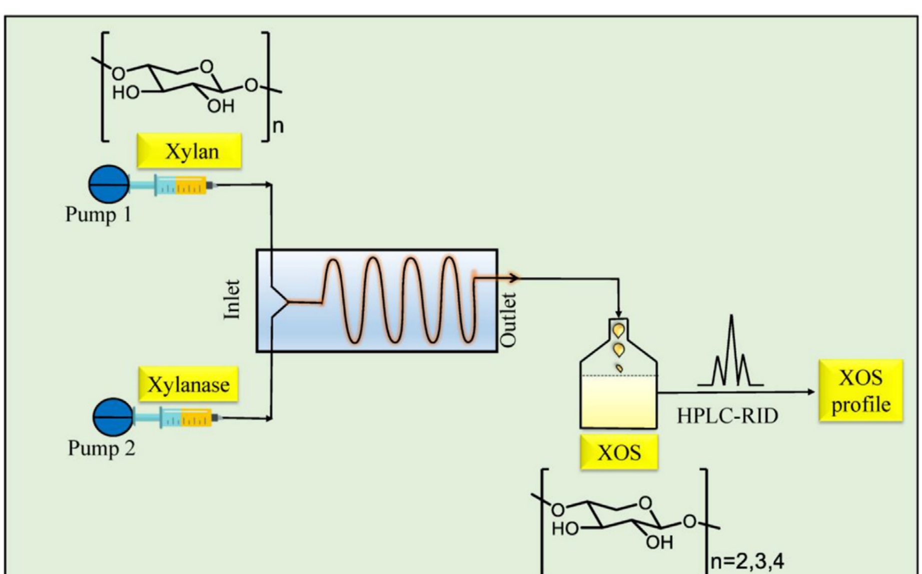

Fig. 1. Schematic illustration of the continuous flow system used for the enzymatic hydrolysis of xylan producing XOS.

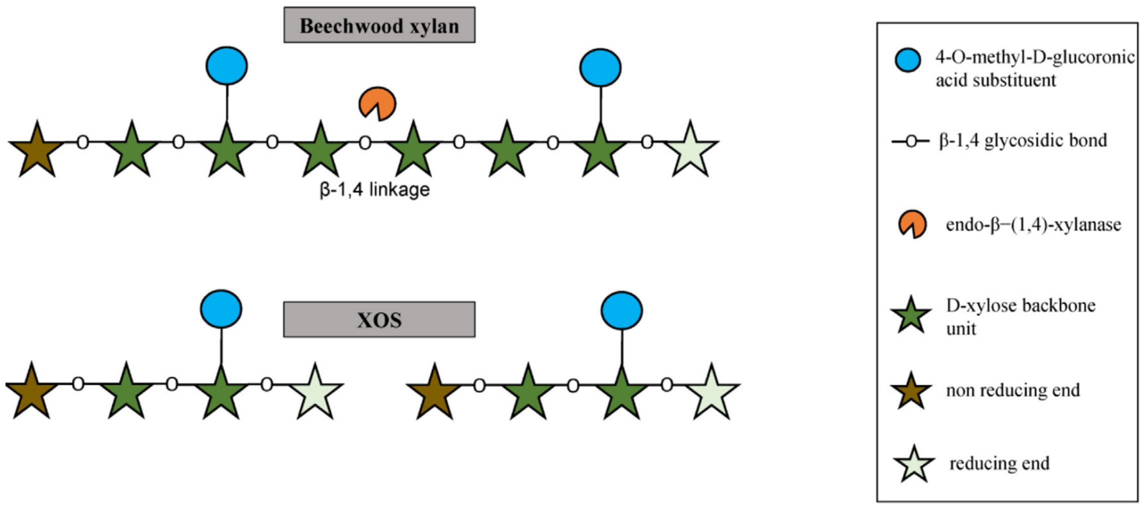

Fig. 2. Schematic representation of xylan depolymerisation by *endo*-β-(1,4)-xylanase to produce xylooligosaccharides (Smith et al., 2017).

degree of polymerization (DP) 4 with either a 4-O-methyl glucuronic acid or acetyl group as substituent. In beechwood xylan, which was used in this study, approximately one in ten xylose units is substituted with 4-O-methyl glucuronic acid (Smith et al., 2017). Xylan hydrolysis always proceeds preferentially from the non-reducing end of the main chain, generating both unsubstituted and branched XOS in the process (Polizeli et al., 2005). The endoxylanase is known to prefer long substrates when accessible, particularly the internal glycosidic bonds where three consecutive unsubstituted xylose units are available along the polymer chain (Bhardwaj et al., 2019) (Fig. 2). The cleaved oligosaccharides can be seen as transient species that in turn becomes the substrate and are further depolymerised to smaller oligomers. Apart from these molecular composition considerations, the activity or the efficiency of the enzyme in producing a particular XOS compound largely depends on the substrate biomass and the applied process conditions. The process parameters known to affect enzyme efficiency in xylan hydrolysis includes pH, reaction time, enzyme to substrate ratio, and temperature (Santibáñez et al., 2021).

3.1. Production of XOS in continuous flow and batch process

Enzymatic hydrolysis of xylan at pH 4 and pH 10 was performed in continuous flow and batch process. The batch reactions were performed in sealed vials with continuous stirring, and the reaction was initiated as soon as the enzyme and substrate solution first came into contact. In the continuous flow experiments, the enzyme and substrate were introduced via separate inlets and mixed at the inlet junction and proceeding under single-phase laminar flow through the channel. The current setup allows to vary the volume ratio of enzyme to substrate (E:S) as well as the residence time by changing the individual flow rates. Note that XOS in this study refers to the sum of X2, X3 and X4 oligomers produced for each reaction condition, and that higher DP oligomers were not determined in this study.

The effects of system configuration and xylan solution pH were compared and the results are shown in Fig. 3. The choice of preparing xylan solution at two different pH i.e., pH 4 and 10 was made since higher pH is helping in better dissolution of xylan reducing the internal hydrogen bonds which holds the xylan chains together (Laine et al., 2013). Due to the use of two different pH preparations on xylan, the reaction mixtures were sometimes at pH 4, sometimes slightly higher. Mixtures of xylan solution prepared at pH 4 and enzyme solution (always prepared at pH 4) resulted in a reaction mixture with a pH of 4, as expected. However, mixtures of xylan solution prepared at pH 10 and enzyme solution (pH 4) resulted in a buffering and dilution effect, and the overall pH of the reaction mixtures made with high pH xylan solution were between 4.5 and 5.5. This range falls within the operating range for endoxylanase (Akpinar et al., 2010).

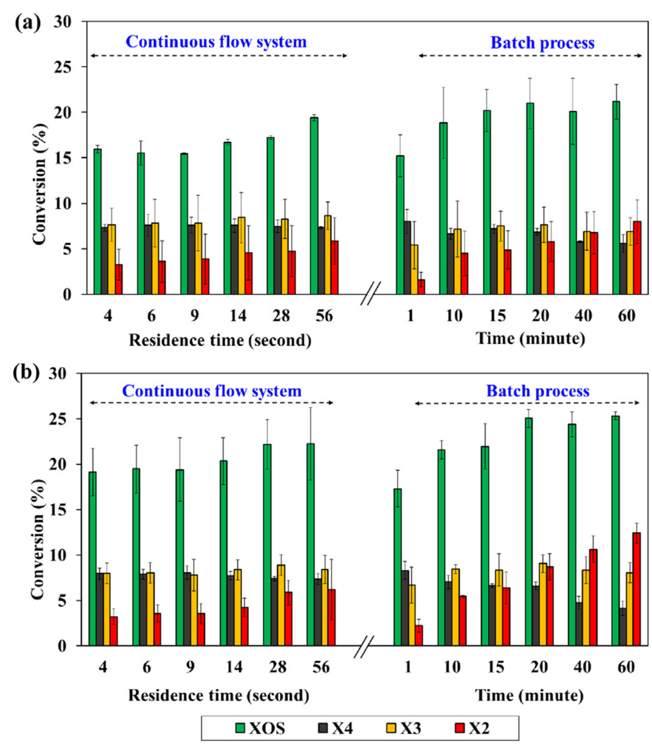

Fig. 3. Xylan hydrolysis performance with **(a)** xylan solution prepared at pH 4 [Xylan conc. = 1% (W/V)] and **(b)** xylan solution prepared at pH 10 [Xylan conc. = 1.25% (W/V)] in continuous flow and batch process [xylanase conc. = 2.5 U/mL, Tempt. = 25 °C, Enzyme: Substrate volume ratio = 1; Batch expt. stirring speed = 100 rpm, Flow rate range = 5–120 μL per min, pH of xylanase phase = 4].

The xylan hydrolysis performance for both batch and continuous mode are shown in Fig. 3 at two different pH conditions of the substrate xylan i.e. pH 4 (Fig. 3-a) and pH 10 (Fig. 3-b). In both flow and batch systems, conversion of xylan to XOS increases with increase in the reaction time. At the same reaction time i.e., 1 min in both the systems, the conversion of xylan to XOS was similar (not statistically different) in the continuous flow system and the batch process. However, Fig. 3 also shows that this conversion did not take the entire minute to achieve, and was in fact achieved in less than 5 s, in the continuous flow system. Due to the significantly larger volume and mechanical stirring, it was not possible to achieve sufficiently good mixing in the batch system to give consistent results with a residence time below 1 min. It is precisely this difference in volumes and mixing efficiency that results in the observed 12-fold difference in reaction time scale between the two systems due to the combined effect of faster diffusion (Swarts et al., 2010) and efficient mass transfer (Vaccaro et al., 2014) in the micro-channel of the continuous flow system. These results confirm that substantial efficiency increase can be realised by using continuous flow systems to perform this enzymatic reaction.

Hydrolysis of xylan solution prepared at pH 4 (Fig. 3-a) and pH 10 (Fig. 3-b) resulted in a mixture of XOS including xylobiose (X2), xylotriose (X3) and xylotetraose (X4) and higher oligomers of xylose (DP > 4, not quantified). X1 was not detected under any condition or time scale used in this work since the enzyme *endo*-β-1,4-xylanase preferentially targets the internal glycosidic bonds between non-terminal xylose units along the polymer chain (Bhardwaj et al., 2019). X3 and X4 were the initial dominant products of the hydrolysis and conversion to X2 proceeded more slowly. With increasing residence time, X2 and X3 fractions also generally increased while X4 decreased in both the batch and continuous flow systems. This trend was more apparent for the hydrolysis of xylan solution prepared at pH 10 (Fig. 3-b). Furthermore, the total XOS produced upon hydrolysis of xylan solution prepared at pH 10 was higher than obtained for xylan solution prepared at pH 4. This result highlights the influence of pH in the xylan preparation phase on the overall hydrolysis process. As higher pH allows better dissolution of xylan resulting in better availability of the substrate molecules in the reaction medium (Heinze & Daus, 2011), which may help in higher xylan to XOS conversion.

3.2. Effect of flow rate on xylan conversion to XOS in the continuous flow system

The comparison between the continuous flow and batch process (Fig. 3) has demonstrated that residence time is a crucial factor in xylan to XOS conversion, where transient species (intermediate shorter chain of xylan or XOS compounds) that are generated during the course of the reaction are further depolymerised to smaller molecules (X4, X3, X2). Residence time in continuous flow system can be further optimised by manipulating the individual enzyme or substrate flow rate, which also affects the enzyme to substrate (E:S) volume ratio. The current microreactor setup allows for the variation of flow rate and hence the volume ratio of E:S. The different residence time was achieved by changing the flow rate of either the xylan and xylanase phase keeping the other constant. The effect of varying enzyme or substrate solution flow rates on xylan to XOS (total and component) conversion was investigated at both low pH (pH = 4) and high pH (pH = 10) of the xylan solution.

In a series of discrete trials, the flow rate of xylan solution (pH 4) was fixed at 0.1 mL/min and the enzyme flow rate was varied from 0.01 to 0.3 mL/min (Fig. 4-a). This equates to a residence time ranging from 2.8 to 10.1 s. The maximum conversion of xylan to XOS, approximately 15% occurred between flow rate 0.08 – 0.15 mL/min, which corresponds to E:S volume ratio of 0.8 –1.5, and a residence time of 6.2–4.5 s respectively. At higher flow rates (0.25–0.3 mL/min), a drop in XOS yield was observed. The increased velocity of the enzyme solution increases the total system velocity

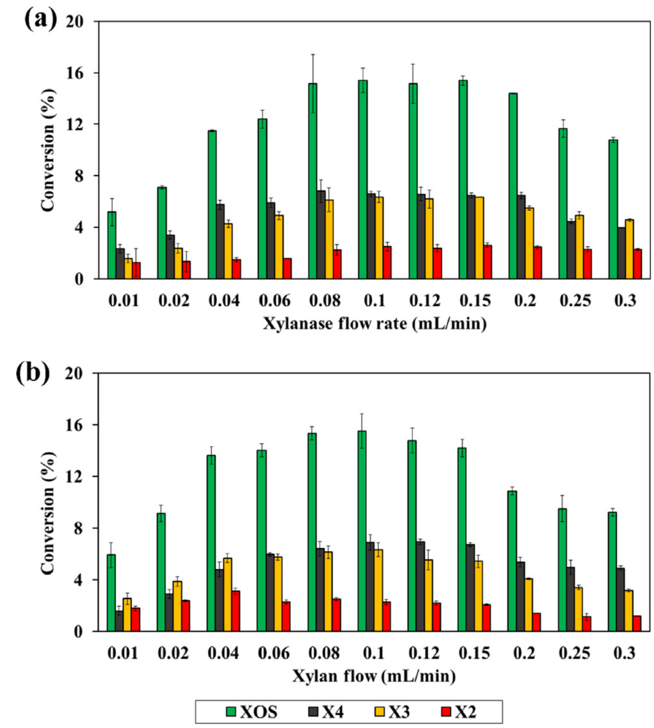

Fig. 4. Xylan hydrolysis performance in continuous flow microreactor with xylan solution prepared at pH 4, **(a)** at fixed xylan flow rate 0.1 mL/min and **(b)** fixed xylanase flow rate 0.1 mL/min [Xylan conc. = 1% (W/V), xylanase conc. = 2.5 U/mL, Tempt. = 25 °C, Flow rate range = 10–300 μL per min, pH of Xylanase phase = 4].

and reduces residence time, resulting in decreased overall xylan conversion. At lower flow rates, the enzyme concentration is lower, which slows the reaction rates and hence lowering the total conversion despite the longer reaction time. A similar trend was observed when the substrate flow rate was varied from 0.01 to 0.3 mL/min while keeping the enzyme flow rate fixed at 0.1 mL/min (Fig. 4b). The maximum total conversion at this condition was approximately 15% at E:S volume ratios 0.8–1.2. Both experimental conditions i.e. (i) constant enzyme flow rate and (ii) constant substrate flow rate results show that the maximum conversion (∼15%) was achieved with E:S volume ratio varying between 0.8 and 1.5. Also, the lower (<0.8) and higher (>1.5) E:S volume ratios resulted in lower conversion to XOS. This was observed since at lower (<0.8) E:S volume ratio, there is insufficient enzyme available for reaction despite of higher residence time whereas at higher (>1.5) E:S volume ratio the reaction mixture flows with higher velocity and does not spend sufficient time needed for the reaction to progress.

In terms of the XOS composition, with a fixed substrate flow rate and low enzyme flow rates, i.e. smaller E:S volume ratios and longer residence times, the composition trend of the hydrolysate was X4 > X3 > X2 (Fig. 4a). At higher enzyme flow rates, i.e. larger E:S volume ratios and shorter residence times, the trend shifted to X3 > X4 > X2. While this change in XOS composition with changing volume ratio is different from that obtained at fixed volume ratio 1:1 (c.f. Fig. 3). There is also an aspect of selectivity to enzymatic xylan hydrolysis as the terminal glycosidic bonds are not targeted, as indicated by the absence of X1 in the hydrolysis products and confirmed in literature (Saha and Bothast, 1999). There may also be an initial preference of the enzyme for the xylan bonds that produce X3 and X2, as indicated by the relative abundance of X2 and X3 in comparison to X4 for different residence times. When the reaction time is long, X3 dominates and X2 is higher than it is in the product composition from a shorter reaction time, where X4 production has more time to increase, and subsequent conversion of X4 to X2 is lower.

A dissimilar trend in XOS composition was observed when operating with fixed enzyme flow rate and variable substrate flow rates. At low substrate flow rate, i.e. higher E:S volume ratio and longer residence times, the composition of X3 was greater than X4, whereas at high substrate flow rate, X4 dominated. These results clearly indicate the importance and interplay of the E:S volume ratio and the residence time on the xylan conversion and overall XOS composition.

For xylan solution prepared at pH 10, the maximum xylan to XOS conversion with the varying enzyme flow rate experimental series occurred at a slightly lower E:S volume ratio, between 0.1 and 0.2 in comparison to the analogous pH 4 series. When varying the enzyme flow rate at a fixed xylan flow rate (0.1 mL/min), the maximum conversion of xylan to XOS was higher than the equivalent pH 4 series, reaching almost ~65% conversion (Fig. 5a). The E:S ratio for the corresponding maximum was 0.1 and a residence time of 10 s.

This difference in conversion with pH was not observed in the earlier comparison between batch and flow systems where the E:S volume ratio was 1 and total conversion was ~20% regardless of the substrate pH (c.f. Fig. 3). The higher conversion to XOS at high substrate pH can be attributed to a combination of 1) increased concentration of xylan due to increased solubility during solution preparation at high pH 2) slightly elevated reaction pH (5 to 5.5.) and 3) mixing ratio. As shown in Fig. 5a, at slower enzyme flow rates (<0.02 mL/min), X2 was the predominant XOS, a result of increased residence time leading to continuous depolymerisation of transient species (higher DP XOS, including X4) to X2. These results indicate that lower E:S and longer residence times produce the most favourable reaction conditions for total conversion of xylan to XOS. At higher enzyme flow rates, a drop in total conversion was observed, indicating that increased enzyme amount alone does not necessarily promote higher reaction rates, and further highlighting the importance of residence time. This relationship between enzyme availability and activity vs reaction inhibition is well known (Zheng et al., 2009).

The XOS composition generated under these conditions was also significantly different to previous results. At high substrate pH and low E:S, a significant increase in the proportion of X2 compared with previous experiments is observed, with a corresponding decrease in X4. This indicates that one potential reason for low E:S and longer residence time increasing overall yield is with that increased residence time higher XOS compounds are being further depolymerised to X2. Comparison of the X3 component of the two lowest substrate flowrate experiments, coupled with the absence of X1, indicates that X3 is not being depolymerised to X2 and X1 under these circumstances. This result further confirms that this xylanase preferentially targets non-terminal glycosidic bonds.

When operating with fixed enzyme flowrate (0.1 mL/min) and variable substrate flowrates at higher pH (Fig. 5b) the trend for total XOS was similar for the equivalent reaction using substrate at pH 4 (Fig. 4b). At low substrate flowrate, i.e. higher E:S ratio and longer contact times, the relative composition of X3 was greater than X4, whereas at high substrate flowrate, X4 dominated.

3.3. Process intensification

As shown in Fig. 5a, slower enzyme flow rates favour enzyme - substrate interaction. Therefore, two additional experimental series were conducted with fixed low enzyme flow rates at 0.01 and 0.02 mL/min (Fig. 6 a and b). In both of these series, the substrate flow rate (xylan solution prepared at pH 10) was varied from 0.02 to 0.15 mL/min. A significant increase in conversion to over 80% was observed. As xylan is a heterogeneous polymer with different

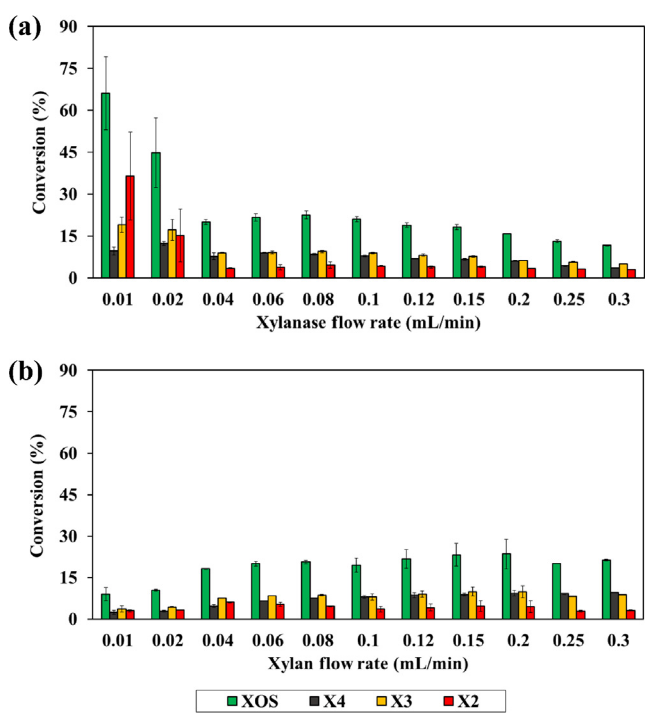

Fig. 5. Xylan hydrolysis performance in continuous flow microreactor with Xylan solution prepared at pH 10, (**a**) at fixed xylan flowrate 0.1 mL/min and (**b**) fixed xylanase flowrate 0.1 mL/min in [Xylan conc. = 1.25% (W/V), xylanase conc. = 2.5 U/mL, Tempt. = 25 °C, Volume ratio = 1:10–3:1, Flow rate range = 10–300 μL/min, pH of Xylanase phase = 4, pH of Xylan phase = 10].

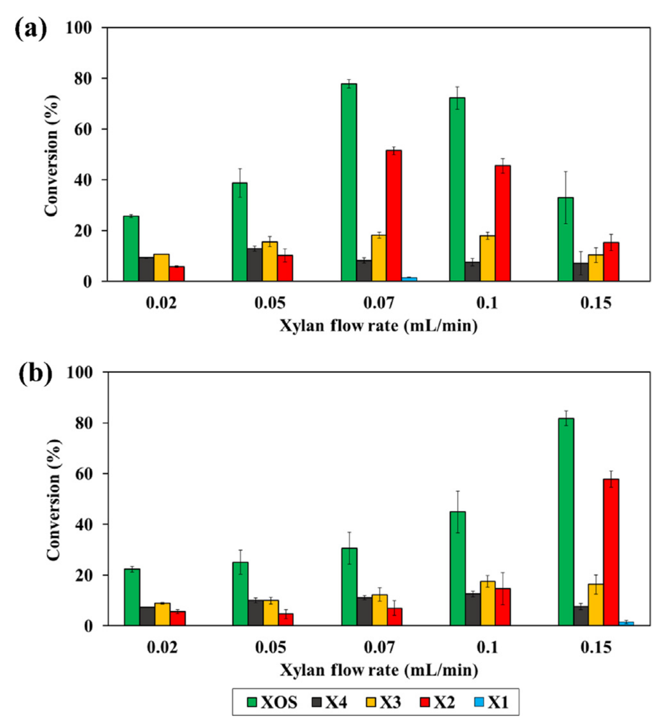

Fig. 6. Xylan hydrolysis performance in continuous flow microreactor with xylan solution prepared at pH 10, (**a**) at fixed xylanase flowrate 0.01 mL/min and (**b**) at fixed xylanase flowrate 0.02 mL/min with varying xylan flowrate [Xylan conc. = 1.25% (W/V), xylanase conc. = 2.5 U/mL, Tempt. = 25 °C, Volume ratio = 0.07–0.5 for a and 0.13–1 for b, Flow rate range = 20–150 μL/min, pH of Xylanase phase = 4, pH of Xylan phase = 10].

substituents along the polymer chain, complete analysis of the product stream would also include non-XOS compounds (e.g. xylose, hetero-oligosaccharides) that are not included in the XOS conversion figures reported here. Furthermore, complete degradation of xylan also requires a combination of different enzymes, which can effectively cleave the substituents from the xylose backbone (Aragon et al., 2013).

The maximum conversion under low enzyme flow conditions still appears to occur at an E:S of approximately 0.1–0.3 corresponding to residence time 14 and 10 s (Fig. 6 a and b), despite the order of magnitude lower flowrate of each solution. This confirms that both residence time and volume ratio are the important process parameters with the most effect on xylan to XOS conversion. In both series, the composition of the XOS product from the highest conversion experiment (>80%) was dominated by X2 ($\sim$60%) followed by X3 ($\sim$20%) and X4 ($\sim$10%). Xylose (X1) was also observed (<1%) only under the highest conversion reaction conditions in each series. The eventual generation of X1 is attributed to the extended residence time and higher reaction rate leading to exhaustion of reagents, i.e., once the enzyme has cleaved all preferred non-terminal glycosidic bonds, it will start to act on the terminal bonds, producing X1 (Fig. 6a and b). The XOS profile also showed a distinct transition with the changing xylan flow rate, a result of changing residence time.

This study has shown that a synergistic effect of pH, residence time and volume ratio can be used to maximise overall XOS production. Furthermore, high xylan to XOS conversion has been achieved at an ambient temperature while keeping the monosaccharide (X1) formation to minimum.

4. Conclusions

The conversion of xylan to XOS by enzymatic hydrolysis in a continuous flow system was analysed using a microreactor and the performance compared with conventional batch process at different experimental conditions. Use of the microreactor for xylan to XOS conversion was found to be advantageous, resulting in a 12-fold difference in reaction time scale between the flow and batch system. Subsequent optimisation of this enzymatic system using microreactors was highly efficient as very low reactant volumes were required. The effect of substrate pH, residence time and enzyme to substrate mixing ratio in the flow system were investigated. High substrate pH, low E:S of approximately 0.3 to 0.4, and longer residence time represented the optimum conditions for total conversion of xylan to XOS in the flow system. At higher E:S, a drop in total conversion was observed, indicating that increased enzyme concentration does not promote higher reaction rates, and highlighting the effect of residence time. The composition of the XOS in the hydrolysate was also dependent on the reaction conditions and product selectivity was observed. A mixture of X2, X3 and X4 oligomers was typically produced, with relative concentration depending on the reaction conditions. X1 (xylose) was produced only under the longest residence times and E:S ratio of 0.1–0.3. Thus the desired ratio of X2, X3, X4 can be controlled and X1 excluded, if undesirable, by tuning the operating conditions in the continuous flow microreactor system. With the increasing commercial development of high-throughput systems, microreactor therefore represent a continuous, easily controlled, scalable process to deliver sustainable, high quality, X1-free oligosaccharides (XOS).

CRediT authorship contribution statement

Debjani Ghosh: Investigation, Writing - original draft. **Anil B. Vir:** Investigation, Writing - review & editing. **Gil Garnier:** Supervision, Writing - review & editing. **Antonio F. Patti:** Supervision, Writing - review & editing. **Joanne Tanner:** Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ces.2021.116789.

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