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Processing of hazelnut (*Corylus avellana* L.) shell autohydrolysis liquor for production of low molecular weight xylooligosaccharides by *Aureobasidium pullulans* NRRL Y–2311–1 xylanase

Ece Surek [a], Ali Oguz Buyukkileci [a], Sirma Yegin [b,*]

[a] Department of Food Engineering, Izmir Institute of Technology, 35430, Gulbahce, Urla, Izmir, Turkey
[b] Department of Food Engineering, Ege University, 35100, Bornova, Izmir, Turkey

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ABSTRACT

In this study, a versatile process for the production of xylooligosaccharides (XOS) with a low degree of polymerization (DP 2–6) from hazelnut shells was designed. This process included autohydrolysis integrated with sequential enzymatic hydrolysis by crude xylanase produced with *Aureobasidium pullulans* NRRL Y–2311–1 from wheat bran. Autohydrolysis of hazelnut shells was carried out at a solid:liquid ratio of 1:6 (w/w) and 190 °C non-isothermally. The effects of several parameters on enzymatic hydrolysis of the autohydrolysis liquor were determined. The maximum XOS (DP 2–6) production was 22.5 g/L which was obtained at pH 5.0 and 40 °C using enzyme concentration of 240 U/g XOS and substrate concentration of 72 g/L. Under these conditions, 31.29 % of the substrate (total XOS) was converted to low-DP-XOS; xylobiose and xylotriose are being the major oligomers. This is the first study on the application of *A. pullulans* xylanase in production of xylooligomers from hazelnut shells.

1. Introduction

Lignocellulosic biomass is one of the cheapest, abundant and energy-rich renewable resources for the production of several value-added materials with economical significance (Jayapal et al., 2013). Among these value added materials, oligosaccharides gain much attention due to their prebiotic function. Currently, fructooligosaccharides, galactooligosaccharides and inulin are the most commonly used prebiotics. On the other hand, pectooligosaccaarides (Miguez et al., 2020) and xylooligosaccharides (XOS) are considered as emerging oligomers among the prebiotics. XOS have additional benefits within the oligosaccharides. XOS are more stable over a wide pH range (2.5–8.0), even at relatively low pH of the gastric juice, as compared to other oligosaccharides. They are also stable at high temperatures (up to 100 °C) (Vazquez et al., 2000). Moreover, the recommended dietary daily intake of XOS (2.1 g) is lower than the other oligosaccharides (Amorim et al., 2018). XOS are naturally present in honey, milk and, many fruits and vegetables but their concentrations in these products are very low (Jayapal et al., 2013). Lignocellulosic feedstocks which are rich in hemicellulose are the primary source for XOS production. Several agricultural wastes and by-products such as corn cob (Chapla et al., 2012) barley straw (Nabarlatz et al., 2007), almond shell (Nabarlatz et al., 2005), wheat straw (Carvalheiro et al., 2009), wheat and rye bran (Radenkovs et al., 2018), rice husk (Vegas et al., 2004), cotton stalk and tobacco stalk (Akpinar et al., 2009) have been used as raw materials for XOS production. The structure of XOS varies depending on the xylan source and the production method. Different strategies have been followed for the production of XOS from lignocellulosic feedstocks: (i) direct enzymatic hydrolysis, (ii) chemical extraction, (iii) hydrothermal treatments (using water, steam or dilute acids under pressure), (iv) chemical extraction followed by enzymatic hydrolysis, (v) hydrothermal treatments followed by enzymatic hydrolysis (Sabiha-Hanim et al., 2011). Autohydrolysis is one of the most effective methods for the production of XOS from hemicellulose-rich biomass where water is used as the sole reagent (Garrote et al., 2002; Aachary and Prapulla, 2011). The increase in hydronium ion concentration due to the water autoionization during autohydrolysis results in depolymerisation of xylan into xylooligomers and xylose, while cellulose and lignin retain in the solid phase (Garrote et al., 2002). Furthermore, the acetic acid generated due to the release of acetyl groups from xylan also catalyzes the cleavage of the exocyclic ethers of the hemicelluloses together with the hydronium ions in the later phase of the autohydrolysis (Vazquez et al.,

* Corresponding author at: Department of Food Engineering, Ege University, 35100, Bornova, Izmir, Turkey.
E-mail address: sirma.yegin@ege.edu.tr (S. Yegin).

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2001). During autohydrolysis, production of undesirable monosaccharide degradation products (e.g. furfural, 5-hydroxymethyl-2-furaldehyde (HMF)) and lignin-derived phenolics are also released. However, these types of compounds can be separated from the liquors by simple purification techniques such as activated charcoal treatment (Carvalheiro et al., 2005) and ultrafiltration (Akpinar et al., 2010). It is important to note that the degree of polymerisation (DP) of the xylooligomers produced by this method is usually high (Vegas et al., 2008). Earlier studies showed that XOS with low DP (2–3) are more selective to beneficial bacteria in the colon than the XOS with high DP (Ho et al., 2018). Therefore, it is of great importance to produce XOS with low DP. Low-DP-XOS can be produced using autohydrolysis by applying high treatment severity (high temperature and long holding time). However, this enhances the formation of undesirable compounds (xylose, monosaccharide degradation products and phenolics) (Carvalheiro et al., 2004; Surek and Buyukkileci, 2017) which complicates XOS purification. In order to alleviate this disadvantage, low-DP-XOS can be produced by autohydrolysis under mild treatment conditions accompanied by subsequent enzymatic hydrolysis. This is an effective strategy when utilizing recalcitrant biomass for XOS production since the application of direct enzymatic methods is limited to non-recalcitrant biomass such as fruit peels and citrus fruit pulps (Parajo et al., 2004; Vazquez et al., 2000).

Hazelnut (*Corylus avellana* L.) is one of the most popular nut crops worldwide. Turkey is the world leader in hazelnut production with annual production of 515,000 t for 2018 (Food and Agriculture Organization of the United Nations (FAO, 2018). Significant amount of wastes such as hazelnut skins, hard shells, husks and pruning residues are generated during its harvesting and processing. These wastes have the potential for the production of carbohydrate and lignin-derived platform chemicals, bioactive compounds and biofuels (Licursi et al., 2017). In previous studies; levunilic acid and hydrochar (Licursi et al., 2017), ethanol and fermentable sugars (Arslan and Eken-Saracoglu, 2010; Sabanci and Buyukkileci, 2018; Uyan et al., 2020), antioxidant compounds (Shahidi et al., 2007) and pectinase (Uzuner and Cekmecelioglu, 2015) were produced or recovered from hazelnut residues.

In our previous study, XOS production by autohydrolysis of hazelnut shells was performed under various conditions, and the highest XOS yield (62 % of the feedstock xylan) was obtained in a process carried out at 190°C for 5 min. The DP of the most of the XOS obtained under this condition was greater than 6 (Surek and Buyukkileci, 2017). Consequently, in the present study, a new process including autohydrolysis integrated with sequential enzymatic hydrolysis was designed in order to produce XOS with low DP. The crude xylanase preparation produced by *Aurebasidium pullulans* NRRL Y–2311–1 from wheat bran (Yegin et al., 2017) was comparatively used with commercial xylanase preparation for enzymatic hydrolysis of hazelnut shell autohydrolysis liquor. This is the first study on applications of *A. pullulans* xylanase for production of XOS from hazelnut shell.

2. Materials and methods

2.1. Materials

Hazelnut shells were kindly supplied by Gursoy Agricultural Products, Food Industry and Trade Inc. (Ordu, Turkey) and dried in an oven at 60 °C for 24 h. They were milled in a laboratory type plant grinder to a particle size smaller than 2 mm and stored in airtight packages at room temperature. Commercial accessory xylanase complex for biomass hydrolysis, produced with a genetically modified strain of *Trichoderma reesei* (Accellerase XY), was a gift from DuPont-Genencor (Hanko, Finland). *A. pullulans* NRRL Y-2311-1 was supplied from US Department of Agriculture, Agricultural Research Service Culture Collection (Peoria, IL, USA). The microbial medium components for xylanase production by *A. pullulans* NRRL Y–2311–1 were obtained either from Difco (BD Detroit, MI, USA) or Sigma-Aldrich (Steinheim, Germany). Beechwood xylan was purchased from BOC Science (Shirley, NY, USA). The xylooligosaccaharide standards; xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6), were purchased from Megazyme (Bray, Ireland). All other chemicals were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany) or Merck Millipore (Darmstadt, Germany).

2.2. Production of xylanase by A. pullulans NRRL Y–2311–1

The production of xylanase from wheat bran by *A. pullulans* NRRL Y–2311–1 was performed under the conditions as described before (Yegin, 2017a; Yegin et al., 2017): The strain was propagated on yeast-mold media containing (g/L): yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0 and agar 20.0. The inoculated plates were incubated at 24 °C for 3 days. Seed culture was obtained by inoculation of two loops of *A. pullulans* NRRL Y–2311–1 cells from agar plates into 250 mL Erlenmeyer flasks containing 50 mL of growth medium (pH 5.0). The growth medium was composed of (g/L): xylose 10.0, yeast nitrogen base, 6.7; asparagine 2.0, KH_2PO_4 5.0. Seed culture was kept at 28 °C and 150 rpm for 1 day on an orbital shaker. Enzyme production was carried out in a Bio-Flo 110 Modular Benchtop Fermentor (New Brunswick Scientific Corporation, NJ, USA) with 3.0 L vessel. The working volume was 1.5 L. The fermentation medium for xylanase production composed of (g/L): wheat bran 10.0, yeast extract 1.0, $(NH_4)_2SO_4$ 2.5, KH_2PO_4 5.0. The pH of the medium was adjusted to 4.24 with HCl and NaOH before sterilization (121 °C for 20 min). The bioprocess parameters for xylanase production were as follows: agitation, 200 rpm; aeration, 1.5 vvm, temperature, 30 °C; and incubation time, 126 h. The xylanase production medium was inoculated with 2% of the inoculum. After 126 h of fermentation, the cells were harvested by centrifugation (21500x*g*) at 4 °C for 15 min. The supernatant was referred to the crude enzyme (Yegin, 2017a).

The crude xylanase was used for xylooligomer production without purification. Xylanase activity of in-house-produced xylanase and commercial xylanase preparation was determined according to Bailey et al. (1992) with slight modifications (Yegin et al., 2017). Beechwood xylan solution (0.5 %, w/v) prepared in 0.05 M citrate buffer (pH 5.2) was used as the substrate. For enzyme assay, 0.9 mL of substrate solution was mixed with 0.1 mL of suitably diluted xylanase preparation and the mixture was incubated at 50 °C for 5 min. After incubation, the reaction was terminated by the addition of 1.5 mL 3,5-dinitro salicylic acid reagent. The content was boiled for 5 min at 100 °C followed by cooling in ice-cold water for 1 min. The amount of reducing sugars released was estimated by measuring the absorbance at 540 nm. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol of xylose equivalent per min under the assay conditions (pH 5.2 and 50 °C).

2.3. Autohydrolysis of hazelnut shells

The autohydrolysis of hazelnut shells was performed in a stainless steel high-pressure reactor (600 mL) (Berghof BR-500, Eningen, Germany). Hazelnut shell was mixed with deionized water at a ratio of 1:6 (solid:liquid). The mixture was heated to 190 °C with continuous stirring at 300 rpm. The pressure was 1.0 MPa during autohydrolysis. After reaching to the set temperature, the reactor was cooled to 60 °C with tap water circulating in the cooling coil. The autohydrolysis liquor was recovered by filtration of the mixture through cheesecloth. The liquor was further clarified by a consecutive filtration through coarse filter paper under vacuum.

2.4. Enzymatic hydrolysis

The autohydrolysis liquor was treated with activated charcoal (1% w/v) in order to partly remove the lignin-derived phenolics and the impurities, as described by Carvalheiro et al. (2005) with slight

modifications. The activated charcoal-liquor mixture was shaken at 25 °C and 100 rpm for 60 min on an orbital shaker. The liquor was recovered by centrifugation (4800 *g*) at 25 °C for 10 min and filtered through polytetrafluoroethylene (PTFE) filter with a pore size of 0.22 μm. The pH of the filtrates was adjusted to the desired value by 1% NaOH (w/v). The hydrolysis by commercial xylanase preparation was carried out at pH 6.0 and 60 °C (optimum parameters indicated by the supplier) while the hydrolysis by *A. pullulans* Y–2311–1 xylanase was performed at pH 5.0 and 50 °C. Enzymatic hydrolyses were performed in screw cap tubes with total volume of 8 mL on an orbital shaker at 100 rpm. In order to prevent microbial growth during enzymatic hydrolysis, 100 μL of 2% (w/v) sodium azide solution was also added into each hydrolysis mixture. Samples were taken from the reaction mixtures after 0, 2, 8 and 24 h of incubation and the xylanase activity was stopped by keeping the samples in boiling water at 100 °C for 5 min. The samples were stored at −20 °C until use. The effects of various parameters (enzyme dosage, incubation temperature, reaction pH, and substrate concentration) on enzymatic hydrolysis were examined in order to increase the production of low-DP-XOS. All enzymatic hydrolyses were performed in duplicates and the results were given as mean ± SD.

2.5. Analytical methods

The concentrations of xylose and XOS with DP of 2–6 in the liquors were determined by high performance liquid chromatography (HPLC) system (Perkin Elmer, Waltham, MA) equipped with refractive index detector. Rezex RPM-Monosaccharide column (Phenomenex, Torrance, CA) and Aminex HPX-42A column (Bio-Rad, Hercules, USA) were used for quantification of xylose and XOS, respectively. Both columns were maintained at 80 °C and ultra-pure water was used as the mobile phase at a flow rate of 0.6 mL/min.

Total XOS contents of the liquors were determined after acid hydrolysis with 4% H_2SO_4 at 121 °C for 1 h, according to the standard laboratory analytical procedures provided by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). The increase in the xylose concentration in liquors was expressed as the total XOS content of the liquors after multiplying by the anhydro correction factor (0.88). Total low-DP-XOS concentration indicates the total concentration of X2-X6 oligomers. In order to increase resolution of the xylooligomers, samples were treated with NaOH prior to HPLC analysis. Equal volumes of the sample and NaOH (10 g/L) were mixed thoroughly and the mixture was kept at 25 °C for 15 min. Afterwards it was neutralized using HCl. All samples were filtered through 0.45 μm PTFE membrane filters before HPLC analysis.

The acetic acid, furfural and HMF concentration of the liquors were determined by HPLC system equipped with a diode-array detector using Aminex HPX-87H column (Bio-Rad, Hercules, USA).The column temperature was kept at 65 °C during the analysis. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min.

3. Results and discussion

3.1. Properties of the autohydrolysis liquor of the hazelnut shells

The majority of the by-products of hazelnuts are the shells since the kernel of this nut occupies less than 50 % of the total weight (Uzuner and Cekmecelioglu, 2015). Therefore, the shells are of special interest for the production of value-added materials among the hazelnut residues. In a previous study of our group, hazelnut shells, husks and pruning residues were characterized and evaluated for XOS production by autohydrolysis (Surek and Buyukkileci, 2018). Among the hazelnut residues tested, the shells contained the highest amount of xylan (18.7 %) as compared to the other residues of hazelnut processing thereby yielding higher XOS production. The autohydrolysis of hazelnut shells (solid: liquid ratio of 1:10) at 190 °C for short times (0−15 min) maximized the xylan solubilization, whereas it did not produce high amount of low-DP-XOS. In the present study, autohydrolysis liquor of the hazelnut shells was further subjected to enzymatic hydrolysis in order to increase the fraction of low-DP-XOS. Autohydrolysis was carried out at a solid:liquid ratio of 1:6 (w/w) and 190 °C non-isothermally. The total concentration of xylooligomers and xylose were 18.90 g/L and 0.91 g/L, respectively, while the total low-DP-XOS concentration was 2.57 g/L. The concentrations of each low-DP-oligomers were as follows: X2: 0.72 g/L, X3: 0.65 g/L, X4: 0.68 g/L, X5: 0.34 g/L and X6: 0.18 g/L. The fraction of total low-DP-XOS was only 13.60 % of the total XOS. The amount of inhibitors generated during autohydrolysis is important as well as the amount of xylan extracted. It is well known that a minor portion of the lignin is solubilized during the autohydrolysis of lignocellulosic feedstocks, therefore; some phenolics and other degradation compounds can be found in the autohydrolysis liquors (Alvarez et al., 2017). The formation of degradation products as a result of autohydrolysis may result in inhibition of the xylanase activity during the enzymatic hydrolysis (Akpinar et al., 2010). Therefore, their concentrations should be taken into consideration. The acetic acid, furfural and HMF concentrations of the autohydrolsis liquor of the hazelnut shells were 1.83 g/L, 0.29 g/L and 0.075 g/L, respectively. It can be clearly observed that the autohydrolysis conditions chosen for the hydrolysis of hazelnut shell provided recovery of xylooligomers with high DP and low concentration of the degradation products. Earlier studies also indicated that hydrothermal treatments resulted in the production of xylooligomers with high DP (>6). In a study performed by Akpinar et al. (2010), the initial xylooligomer concentrations for autohydrolysis liquors (160 °C for 1 h) of wheat straw and sunflower stalk were determined as 2.733 g/L and 2.068 g/L, respectively. Furfural concentrations were 0.252 g/L and 0.202 g/L for the liquors of wheat straw and sunflower stalk, respectively. In general, acetic acid concentrations were low in the liquors of these feedstocks (wheat straw: 0.061 g/L; sunflower stalk: 0.179 g/L). The steam explosion route (200 °C for 4 min) developed by Alvarez et al. (2017) for the hydrolysis of wheat straw resulted in the production of total xylooligomers (X2-X13), furfural, HMF and acetic acid at concentrations of 28.5 g/L, 0.3 g/L, 0.06 g/L and 3.9 g/L, respectively. The total concentration of X2-X6 oligomers was 6.22 g/L. Although, steam explosion of wheat straw provided high concentration of xylooligomers, the concentration of acetic acid and furfural were high. Autohydrolysis of almond shell (200 °C for 5 min) led to production of low-DP-XOS (X2 and X3) at a concentration of only 3.5 % (w/w of biomass) (Singh et al., 2019). In all of these studies, enzymatic hydrolysis was consequently applied after hydrothermal treatments of the biomass in order to increase the concentration of low-DP-XOS.

3.2. Production of low DP-XOS by enzymatic hydrolysis

To date, the majority of the studies on enzymatic production of xylooligomers from lignocellulosic biomass have been carried out by commercial xylanase preparations. The numbers of the studies considering application of indigenous microbial xylanases are scarce (Chapla et al., 2012). Introduction of novel and versatile enzyme systems to the use of different industrial sectors has always been important. In previous studies of our group members, the xylanase from *A. pullulans* Y–2311–1 was produced from various lignocellulosic feedstocks and was purified and characterized (Yegin et al., 2017; Yegin, 2017a, b). Furthermore, it was successfully applied in bread making (Yegin et al., 2018). The xylanase from *A. pullulans* exhibited significant improvements in bread quality attributes as compared to the commercial counterparts. In the present study, *A. pullulans* xylanase was applied in the production of low-DP-XOS from hazelnut autohydrolysis liquor in order to widen the industrial application of this enzyme. Enzymatic hydrolysis of hazelnut autohydrolysis liquor by *A. pullulans* xylanase was comparatively performed with the commercial xylanase preparation. At the initial stage, effects of different concentrations (30, 120, 500 U/g XOS) of commercial xylanase preparation and *A. pullulans* xylanase on the production of low-DP-XOS were tested and the results are shown in Fig. 1. As expected,

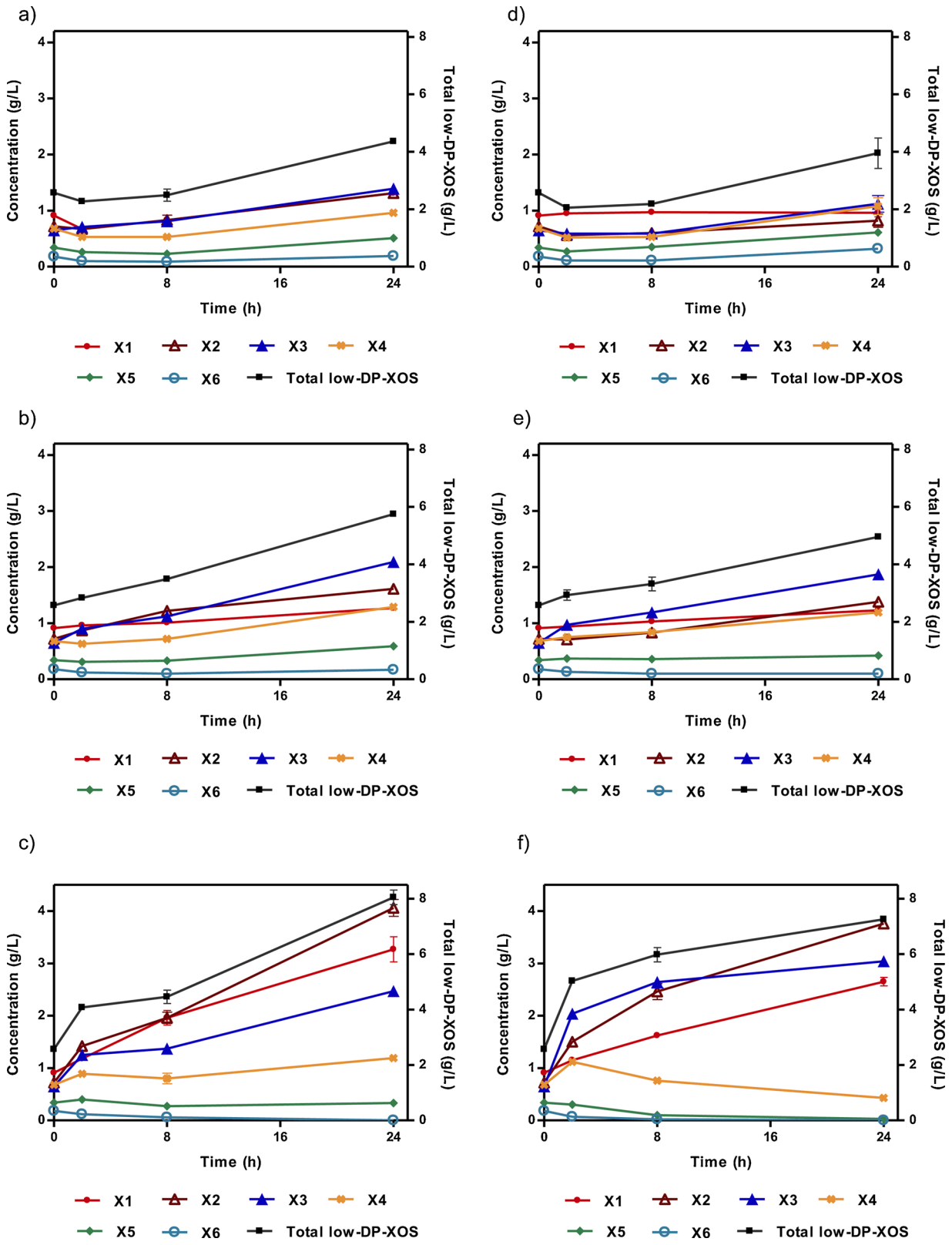

Fig. 1. The effects of enzyme concentration on xylose and low-DP-XOS production from hazelnut autohydrolysis liquor: (a) 30 U/g XOS, (b) 120 U/g XOS, (c) 500 U/g XOS for commercial xylanase preparation and (d) 30 U/g XOS, (e) 120 U/g XOS, (f) 500 U/g XOS for xylanase from *A. pullulans* NRRL Y–2311–1.

the fraction of shorter oligomers was enhanced after the enzymatic treatment with both of the enzymes. 30 U/g XOS and 120 U/g XOS enzyme concentrations seemed inadequate for the production of high concentration of low-DP-XOS by *A. pullulans* xylanase and commercial xylanase since maximum low-DP-XOS was obtained at concentration of 500 U/g XOS in each case. After 24 h of incubation, the maximum total low-DP-XOS production with the commercial xylanase was 4.36 g/L and 5.75 g/L for 30 U/g XOS and 120 U/g XOS, respectively (Fig. 1a–b).

After the same period of incubation, the maximum total low-DP-XOS production by *A. pullulans* xylanase was 3.95 g/L and 4.96 g/L for 30 U/g XOS and 120 U/g XOS, respectively (Fig. 1d–e). The concentrations of total low-DP-XOS and xylose increased with the increase in enzyme concentration for both of the enzymes. In the case of commercial xylanase, maximum low-DP-XOS (8.05 g/L) was obtained when 500 U/g XOS was used after 24 h. However, the xylose concentration was also increased considerably and reached its maximum value (3.27 g/L) under these conditions (Fig. 1c). The increase in the total low-DP-XOS could be ascribed to X2, X3, and X4 concentrations since X5 and X6 did not change notably. This indicated that the oligomers having DP greater than six in the autohydrolysis liquor were hydrolyzed into shorter fragments by commercial xylanase preparation. When xylanase from *A. pullulans* was used, the hydrolysis with 500 U/g XOS resulted in 7.26 g/L total low-DP-XOS and 2.65 g/L xylose after 24 h (Fig. 1f). These results were comparable to the ones obtained with the commercial xylanase preparation at the same enzyme concentration. In spite of the similar low-DP-XOS profiles after 24 h, *A. pullulans* xylanase released higher amount of low-DP-XOS (5.98 g/L) in the first 8 h at the highest enzyme concentration tested (Fig. 1f) as compared to the commercial xylanase preparation (4.46 g/L) (Fig. 1c). The xylose concentrations after 8 h were 1.96 g/L and 1.62 g/L for commercial xylanase preparation and *A. pullulans* xylanase, respectively. Similarly, the rate of XOS production was decreased in the later phase of the enzymatic hydrolysis in previous studies (Chapla et al., 2012; Akpinar et al., 2009). It has been attributed to the decreased endoxylanase activity due to inhibition by end-product accumulation in these studies. Another possible explanation is that the level of easily accessible hydrolytic sites in xylan chain may have decreased after 8 h (Chapla et al., 2012). The formation of X2-X4 XOS was high in the earlier phase followed by a decreasing trend in the later phase of the hydrolysis for both enzymes. Xylose was formed at almost a linear rate for 24 h. The X2 and X3 concentrations after 8 h with *A. pullulans* xylanase were 2.46 g/L and 2.64 g/L, respectively. Lower concentrations of X2 (1.97 g/L) and X3 (1.37 g/L) were noted with the commercial xylanase preparation for the same period of time. Previous studies indicated that XOS with DP less than four were more beneficial for the proliferation of probiotic bacteria (Ho et al., 2018). From those perspectives, *A. pullulans* xylanase seems more advantageous. It is also important to emphasize that the indigenous xylanase produced by *A. pullulans* resulted in lower xylose formation than the commercial xylanase (from *T. reesei*) utilized in the present study for all the enzyme concentrations tested. *A. pullulans* strains are known to produce xylanases belonging to glycosyl hydrolyase family 11 (Christov et al., 1999), while *T. reesei* strains are known to produce xylanases belonging to glycosyl hydrolyase family 10 (Akpinar et al., 2010). The enzymes of these two families exhibit different physicochemical properties (molecular weight, pI, thermostability, etc.) and specificities towards different types of xylans (Akpinar et al., 2010). Production of high concentration of xylose and shorter oligomers during hydrolysis of xylan is a characteristic behavior of family 10 xylanases. In addition, these xylanases are known to utilize low molecular weight celluloses as the substrates (Collins et al., 2005). However, *A. pullulans* strains are known to produce xylanase without any cellulase activity which is the characteristic behavior of family 11 xylanases (Christov et al., 1999). All of these indicate the superior properties of *A. pullulans* xylanase over the commercial xylanase preparation. Therefore, indigenous xylanase obtained by *A. pullulans* was used for the rest of the study.

An attempt was further made to decrease the concentration of *A. pullulans* xylanase utilized for the hydrolysis of hazelnut shell autohydrolysis liquor since the cost of enzyme is a critical parameter in terms of bioprocess economics. In addition, utilization of high concentration of xylanases usually results in formation of high concentration of xylose which is an undesirable property for commercial xylooligomer production. Therefore, the optimum enzyme concentration providing maximum low-DP-XOS production and minimum xylose formation has to be figured out. For that purpose, *A. pullulans* xylanase at concentrations of 240 U/g XOS and 360 U/g XOS were also applied for hydrolysis of hazelnut shell liquor. Both concentrations exhibited similar low-DP-XOS formation efficiencies to the one obtained with the concentration of 500 U/g XOS (Figs. 2a–b and 1 f). The total low-DP-XOS concentrations were 7.77 g/L, 7.90 g/L and 7.26 g/L for 240, 360 and 500 U/g XOS, respectively. The xylose concentrations were 1.84 g/L, 2.40 g/L and 2.65 g/L for 240, 360 and 500 U/g XOS, respectively. It is important to note that the xylose concentration increased with the increase in enzyme concentration for these three enzyme concentrations. Consequently, the xylanase concentration of 240 U/g XOS was chosen because of providing the lowest xylose formation and very similar low-DP-XOS production trend to the higher enzyme concentrations. The percentage of low-DP-XOS fraction in the autohydrolysis liquor was increased to 41.20 % from its initial value of 13.60 % after the enzymatic hydrolysis with *A. pullulans* xylanase at a dosage of 240 U/g XOS.

The concentration of enzyme required for hydrolysis can change depending on the biomass source and the preceding pretreatment method (autohydrolysis, alkali extraction, etc.) chosen for the extraction of xylan. The effectiveness of each pretreatment technique also varies for each biomass due to the structural and compositional differences. As comparison with the results of the current study, the optimum concentration of commercial xylanase was determined as 8 U / 100 mg liquor (535 U/g XOS) for the enzymatic hydrolysis of the oil palm frond fibers autohydrolysis liquor at pH 5.0 and 40 °C for 24 h. The final enzymatic hydrolysis solution contained 17.5 % of XOS and considerably high amount of xylose (25.6 %) (Sabiha-Hanim et al., 2011). Enzymatic hydrolysis of autohydrolysis liquors of wheat straw and sunflower stalk required commercial xylanases at concentrations of 293 U/ g XOS and 387 U/g XOS, respectively (Akpinar et al., 2010). In some of the studies focusing on enzymatic processing of autohydrolysis liquors (Sing et al., 2020) and alkali extracted xylan solutions (Chapla et al., 2012), the enzyme concentrations have not been provided clearly. Therefore, it was not possible to calculate the units required per g of total XOS in the liquors to compare with the present study.

3.3. Effect of process parameters on XOS production by A. pullulans NRRL Y–2311–1 xylanase

Previous studies have proven that enzymatic hydrolysis efficiency is closely related to the substrate structure and environmental conditions, such as enzyme specificity, reaction time, pH and temperature (Yang et al., 2011). Therefore, the influence of these parameters on enzymatic hydrolysis of hazelnut shell autohydrolysis liquor by *A. pullulans* xylanase was examined. The effect of different temperatures on the production of low-DP-XOS and xylose by *A. pullulans* xylanase was investigated at pH 5.0. The XOS profiles were very similar at temperature values of 30 °C, 40 °C and 50 °C (Fig. 3). X2 and X3 were the major oligomers. Xylose concentrations were also similar in the hydrolysates at these temperature values. At 60 °C, there was no release of any low-DP-XOS or xylose. These results are compatible with the previous studies focusing on the characterization of the crude and purified enzymes from the same strain. The optimum temperature for *A. pullulans* NRRL Y–2311–1 xylanase was previously determined as 30−50 °C by utilizing beechwood xylan in the enzyme assay (Yegin, 2017a, 2017b). It has also been indicated that exposure of the purified enzyme to heat at 60 °C for 90 min completely abolished the enzyme activity (Yegin, 2017a). However, the crude enzyme obtained by the same strain was more stable against heat since it exhibited ~ 33 % of the initial activity at 60 °C (Yegin, 2017b). The optimum temperature and thermal stability of the enzymes may change depending on the substrate (xylan source) used and the composition of the hydrolysis mixture since some of the components can protect the enzyme against heat.

The effect of pH was investigated in the range of pH 3.0–6.0 at 40 °C (Fig. 4). The pH of the each liquor was adjusted to the desired value by using NaOH and HCl. The maximum XOS yield was obtained at pH 4.0

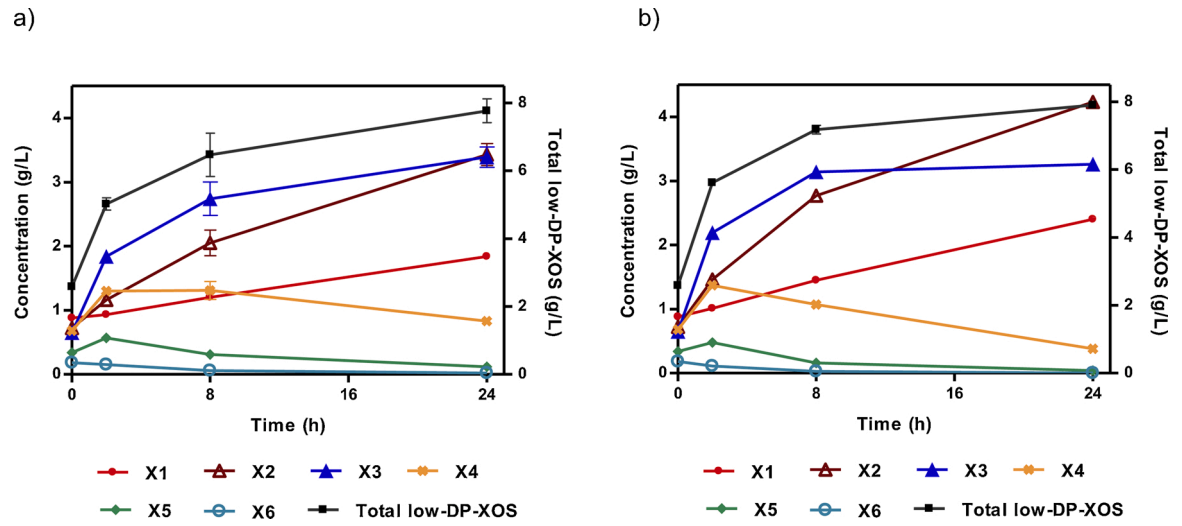

Fig. 2. The effects enzyme concentration on xylose and low-DP-XOS production from hazelnut autohydrolysis liquor with *A. pullulans* NRRL Y–2311–1 xylanase: (a) 240 U/g XOS, (b) 360 U/g XOS.

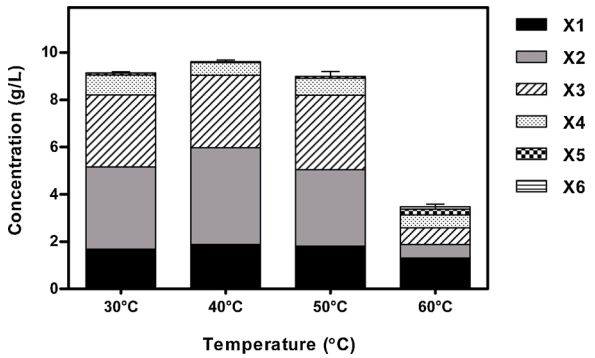

Fig. 3. The effects of reaction temperature on xylose and low-DP-XOS production from hazelnut autohydrolysis liquor with *A. pullulans* NRRL Y–2311–1 xylanase.

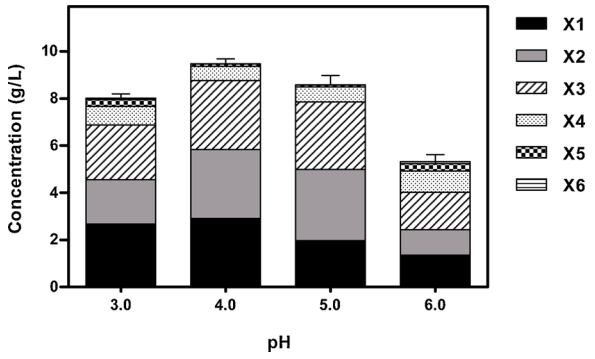

Fig. 4. The effects of reaction pH on xylose and low-DP-XOS production from hazelnut autohydrolysis liquor with *A. pullulans* NRRL Y–2311–1 xylanase.

and 5.0 with similar total low-DP-XOS concentrations in the hydrolysates, followed by pH 3.0. The distribution of low-DP-XOS was also similar at pH 4.0 and 5.0. Hydrolysis was relatively poor at pH 6.0, and the fraction of X2 was lower, while X5 and X6 fractions were higher as compared to other pH values. The optimum pH of the crude and purified xylanase from *A. pullulans* NRRL Y–2311–1 was previously determined as 4.0 by utilizing beechwood xylan in the enzyme assay (Yegin, 2017a, 2017b). In the present study, the effect of pH on the distribution of low-DP-XOS from hazelnut autohydrolysis liquor has also been elucidated. In general, the optimum pH and the temperature for most of the xylanases utilized in the production of xylose and xylooligomers have been reported as pH 4.5–6.5 and 50−65 °C, respectively (Yoon et al., 2006). The xylanase from *A. pullulans* NRRL Y–2311–1 also exhibited similar characteristics. Moreover, it provided maximum low-DP-XOS production in a wide temperature range (30−50 °C) which could be translated into lower energy requirement for prebiotic xylooligomer production.

Substrate concentration is a critical parameter in enzymatic hydrolysis. Product concentrations and productivity values can be enhanced with high initial substrate concentrations, whereas that may also exert a negative impact on the enzyme activity. In order to investigate the effect of substrate concentration on enzymatic hydrolysis, the autohydrolysis liquor was lyophilized and then reconstituted in water to obtain varied concentrations of XOS. Enzymatic hydrolyses were performed at pH 5.0 and 40 °C with a total volume of 16 mL using *A. pullulans* xylanase at a concentration of 240 U/g XOS. Samples were taken from hydrolysis reactions after 24 h and 48 h. After 24 h, the release of low-DP-XOS increased as the initial substrate concentration increased (Fig. 5a). The total low-DP-XOS concentrations were 6.18 g/L, 9.00 g/L and 10.20 g/L for the substrate concentrations of 18 g/L, 36 g/L and 72 g/L (total XOS basis), respectively. The xylose formation was also increased with the increase in substrate concentration after 24 h. The xylose concentrations were 1.31 g/L, 2.12 g/L and 3.23 g/L for the substrate concentrations of 18 g/L, 36 g/L and 72 g/L, respectively (Fig. 5a). When the enzymatic hydrolysis time was extended to 48 h, the concentration of low-DP-XOS increased for all of the substrate concentrations tested as compared to the values obtained after 24 h (Fig. 5a–b).The maximum low-DP-XOS concentration (22.5 g/L) was obtained with the highest initial substrate concentration (72 g/L). The xylose concentration was 4.76 g/L at this substrate concentration after 48 h (Fig. 5b). However, the fraction of the low DP-XOS within the total XOS supplied decreased with the increase in substrate concentration. The fraction of low-DP-XOS within the total XOS after 48 h was 43.20 %, 37.69 % and 31.29 % for the substrate concentrations of 18 g/L, 36 g/L and 72 g/L, respectively. The percentage of low-DP-XOS was 1.4 times higher at 18 g/L substrate concentration than the substrate concentration of 72 g/L. Concentrations of X2, X3 and X4 oligomers increased as the substrate concentration increased after 24 h of incubation. Slight increase in the concentrations of X5 and X6 was also observed with the increase in substrate concentration after 24 h. This indicates that the break down of XOS with high DP (> 6) mostly into X2-X4 oligomers after 24 h. When

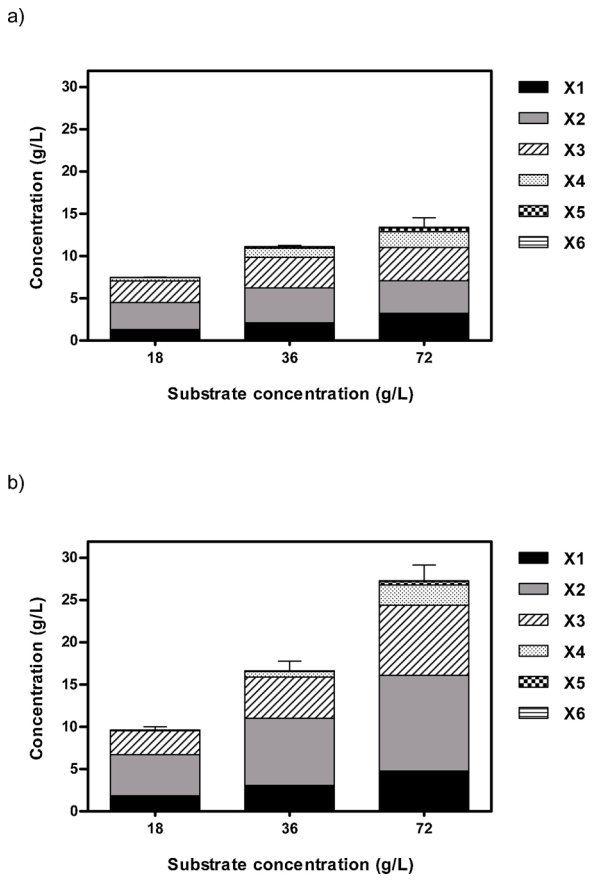

Fig. 5. The effects of substrate concentration on xylose and low-DP-XOS production from hazelnut autohydrolysis liquor with *A. pullulans* NRRL Y–2311–1 xylanase: (a) 24 h, (b) 48 h.

the time was extended to 48 h, increases in the formation of X2-X4 oligomers continued for all of the substrate concentrations tested. However, the concentration of X5 and X6 decreased for the substrate concentrations of 18 g/L and 36 g/L while the concentration of these two oligomers stayed almost constant for the substrate concentration of 72 g/L after 48 h of hydrolysis. It was obvious that the break down of X5-X6 oligomers into shorter oligomers by enzyme action occurred at lower substrate concentrations. In contrast to our results, Chapla et al. (2012) observed that the concentration of XOS obtained after the enzymatic hydrolysis of alkali pretreated corn cob decreased with the increase in substrate concentration. Increasing the substrate concentration from 1% to 3% did not provide a substantial increase in XOS concentration. However, reduction in substrate concentration has lead to a remarkable decrease in XOS production. The concentrations of total XOS were 0.10, 1.11, 6.73, 7.84 and 7.89 mg/mL for the substrate concentrations of 0.1, 0.2, 1.0, 2.0 and 3.0 %, respectively (Chapla et al., 2012). Reduction in pentose production from enzymatic hydrolysis of alkali treated corn husk and corn cob solutions with the increase in substrate concentration was also observed by Yoon et al. (2006). The decrease in product concentration at higher substrate concentration has been linked to reduction of water content in aqueous medium in those studies (Yoon et al., 2006; Chapla et al., 2012). However, substrate inhibition of the enzymes may be another reason for the reduction in oligomer concentration at higher substrate concentrations in these studies.

To date, no previous study utilized *A. pullulans* xylanase for the production of xylooligomers. In a study performed by Gauterio et al. (2018), xylanase production by *A. pullulans* sp. from different agricultural wastes has been carried out. The main focus of this study was the optimization of the enzyme production from pretreated and untreated biomass sources. In addition, low-DP-XOS (X2-X5) concentration in fermentation medium was also determined. The maximum total low-DP-XOS concentration was 1.68 mg/mL which mainly composed of X2 and X3. The amount of low-DP-XOS was extremely low as compared to the result of the present study (22.5 g/L). Fermentative production of XOS does not seem an attractive method since the strain can utilize the XOS as the carbon source when the depletion of carbon source occurs. Therefore, utilization of cell free enzymes for production of oligomers is more advantageous.

4. Conclusion

Agricultural waste and by-products are cheap, abundant and renewable raw materials that can serve as a xylan source for sustainable production of prebiotic xyooligomers. In this study, a new process including sequential application of autohydrolysis and enzymatic hydrolysis was introduced for the production of low-DP-XOS from hazelnut shell by *A. pullulans* xylanase. Under the optimum conditions, the maximum low-DP-XOS concentration reached to 22.5 g/L. To the best of our knowledge, this is the highest concentration reported up to now. This study demonstrated that the *A. pullulans* xylanase possess a high potential for commercial production of xylooligomers from agricultural wastes.

Declaration of Competing Interest

The authors report no declarations of interest.

CRediT authorship contribution statement

Ece Surek: Conceptualization, Investigation, Formal analysis. **Ali Oguz Buyukkileci:** Conceptualization, Supervision, Investigation. **Sirma Yegin:** Conceptualization, Supervision, Investigation, Writing - original draft.

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