PREBIOTIC OLIGOSACCHARIDE PRODUCTION FROM HAZELNUT WASTES

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ABSTRACT

PREBIOTIC OLIGOSACCHARIDE PRODUCTION FROM HAZELNUT WASTES

Turkey is the world leader in hazelnut production and a large amount of wastes is produced during its harvesting and processing. Obtaining valuable products from hazelnut by-products can add value to those. In this study, xylooligosaccharide (XOS), which has prebiotic potential and not manufactured in Turkey, was selected as the target product. Hazelnut wastes (shell, green leafy cover and pruning wastes) are suitable sources for production of XOS. Although there are some studies on valorization of hazelnut wastes, there is no study about XOS production.

In this study, xylan in the shell was hydrolyzed to XOS by autohydrolysis and enzymatic hydrolysis at different conditions. The highest XOS yield was obtained as 62.93% after autohydrolysis at 190 ^{o}C for 5 min. The content of XOS with low degree of polymerization (DP<6) increased from 10.13 to 32.74% after hydrolysis with xylanase. XOS from enzymatic hydrolysis was purified by ultrafiltration through 10 and 2 kDa size membranes. The percent of low DP-XOS in liquor was increased after ultrafiltration; however monomers could not be separated.

Prebiotic activity and stability of XOS were determined under different conditions. The growth of probiotic bacteria was observed after in vitro fermentation of XOS. XOS showed a good stability to food production and gastrointestinal digestion conditions. The objective of this study was to produce of a high value product such as XOS with prebiotic potential from hazelnut wastes by autohydrolysis and enzymatic hydrolysis. This study was highly original since XOS has been produced from hazelnut wastes for the first time.

ÖZET

FINDIK ATIKLARINDAN PREBİYOTİK OLİGOSAKKARİT ÜRETİMİ

Türkiye fındık üretiminde dünya lideri olup bu ürünün hasadı ve işlenmesi sırasında büyük miktarda atık açığa çıkmaktadır. Bu atıklardan değerli ürünler elde edilmesi katma değer yaratacaktır. Bu çalışmada, hedef ürün olarak ülkemizde üretimi olmayan, prebiyotik özellik taşıyan ksilooligosakkarit (KOS) seçilmiştir. Fındık tarımı ve işleme endüstrilerinin atıkları (kabuk, zuruf ve budama atıkları) prebiyotik özellikli KOS eldesi için iyi birer kaynaktır. Fındık atıklarının değerlendirilmesi ile ilgili çalışmalar olsa da fındık atıklarından KOS üretimi konusunda herhangi bir çalışma bulunmamaktadır.

Bu çalışmada kabukta bulunan ksilan otohidroliz ve enzimatik hidroliz kullanılarak farklı koşullarda hidroliz edilmiştir. En yüksek KOS verimi %62.93 olarak 190 ^{o}C'de 5 dk. otohidroliz ile elde edilmiştir. Düşük polimerizasyon dereceli (DP<6) XOS içeriği ksilanaz ile hidroliz sonrası %10.13'den 32.74'e arttırılmıştır. Enzimatik hidroliz sonrası KOS, 10 ve 2 kDa ölçülerindeki membranlardan ultrafiltrasyon ile saflaştırılmıştır. Ultrafiltrasyon sonrası KOS'un düşük DP yüzdesi arttırılırken; monomerlerin ayrımı sağlanamamıştır.

KOS'un çeşitli şartlardaki prebiyotik aktivitesi ve stabilitesi ölçülmüştür. KOS'un in vitro fermentasyonu sonrası probiyotik bakterilein gelişimi gözlemlenmiştir. KOS, gıda üretimi ve mide-bağırsak sindirim koşullarına iyi bir dayanıklılık göstermiştir. Bu çalışmanın amacı fındık atıklarından otohidroliz ve enzimatik hidroliz ile prebiyotik potansiyeli olan XOS gibi yüksek değerde ürün üretmektir. Bu çalışma, fındık atıklarından KOS üretimini gerçekleştirmiş olması nedeniyle yüksek bir özgün değer taşımaktadır.

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CHAPTER 1

INTRODUCTION

In recent years, one of the greastest problems in the world is to increase the production of healthy and economic foods, which are available for a big proportion of the world's population and decrease consumption of fossil, non-renewable resources such as petroleum, natural gas, coal and minerals. In order to prevent deforestation and destruction of natural sources, agro-industrial wastes, which are eliminated in a huge amount annually, should be valorized (Carvalho, Neto, da Silva, & Pastore, 2013; Kamm & Kamm, 2004). New approaches are needed for valorization of biological raw materials. Biorefinery is expressed as "An overall concept of an integrated and diversified processing plant where biomass feedstocks are converted into a wide range of valuable products, much likewise to petroleum refineries" (Carvalheiro, Duarte, & Gírio, 2008) or "The concept of converting biomass materials into biofuels or bio-based chemicals via the fusion of biotechnology and physico-chemical technology" (Park, Kondo, Chang, Perry Chou, & Monsan, 2013). Biorefineries contain the combination of biological raw materials, industrial intermediates and final products. "Whole crop biorefinery", "green biorefinery" and "lignocellulose-feedstock biorefinery" are the systems used in research and development. The main purpose should be access to carbohydrates and their final products, efficiently (Kamm & Kamm, 2004). Carbohydrates, oils, lignin, and other materials are extracted from biomass and converted into fuels, high value chemicals and other materials, with a zero waste approach (Carvalheiro et al., 2008). Glucose is a basic chemical to obtain many biotechnological or chemical products. It can be accessible from starch, sugar or cellulose by chemical or microbial procedures. Enzymatic hydrolysis of starch is more advantegous than chemical hydrolysis because of including no chemicals. On the other hand, pretreatments are necessary to disintegrate cellulose-hemicellulose-lignin structure to use cellulose hydrolyzing enzymes efficiently. Therefore, extra energy is required for the pretreatments, which are usually thermal, thermo-mechanical or thermo-chemical (Kamm & Kamm, 2004).

Bioethanol can be produced by fermentation from foods such as corn; however gasification or hydrolysis is needed to use lignocellulosic part of the corn. Using food

stock for energy production can not be renewable because of the economic, social and political effects. If nonfood lignocellulosic sources are used more to produce ethanol, the world's food sources are not spent for enery needs. Cellulosic part of biomass is still unused source of fermentable sugars for important industrial use. Lignocellulosic biomass such as wood, straw and corn stover can be used as a lignocellulosic biorefinery approach to be fractionated and processed into many enery and chemical products like cellulose and hemicellulose (Guney, 2013). Hemicellulosic oligomers are valuable products that can be obtained from lignocellulosic materials. The interest to functional oligomer production has been increased in recent years. Oligomers such as fructo-, galacto- and xylo-oligosaccharides (XOS) have prebiotic properties. Fructooligosaccharides are the market leader among prebiotics; however XOS are claimed to have shown better properties (Otieno & Ahring, 2012).

Autohydrolysis is one of the pretreatments that can be used prior to ethanol production. It is a hot water treatment and can be carried out at high temperatures in a pressurized reactor. Hemicellulose is hydrolzed and cellulose remained in residual solid becomes hydrolyzable (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010; Hendriks & Zeeman, 2009). XOS production has a growing interest and importance in recent years and there are many studies in literature on autohydrolysis of different lignocellulosic biomass to obtain XOS. To our knowledge, however, there has been no research related to production of XOS from hazelnut wastes, which are among the most important products of Turkey. Turkey is the largest hazelnut producer and exporter in the world. A large amount of wastes like hazelnut hard shell, green leafy cover and pruning wastes are discarded during harvesting or processing (Guney, 2013).

The objectives of this thesis study were to valorize hazelnut waste products which are abundant agricultural residues in Turkey and obtaining a prebiotic product from a novel source. It is aimed to expand the use of prebiotics in the food industry and reduce dependence of this product group on import. The main purpose is to produce XOS which is well known as a prebiotic. This study is the first in which hazelnut wastes were used for production of XOS. Autohydrolysis and enzymatic hydrolysis methods were used for the first time for production of XOS from hazelnut wastes and method conditions were optimized. Prebiotic activity and the resistance to food production and digestion conditions of XOS were determined.

CHAPTER 2

LIGNOCELLULOSIC BIOMASS

The main objective of biorefinery is to increase the use of bioenergy and bio-based products by using environmentally friendly and cost effective technologies. All components of biomass should be used, in order to improve economic feasibility of the waste. Therefore, the targets of biorefinery approach should be utilization of hemicellulose by conversion to xylan or high value-added products, and valorization of lignin by conversion to valuable chemicals (Sarkar, Ghosh, Bannerjee, & Aikat, 2012). Lignocellulose is a complex carbohydrate polymer of cellulose, hemicellulose and lignin. Lignin is hydrophobic and linked to hemicellulose and cellulose to protect polymers from microbial attack (Sarkar et al., 2012).

Lignocellulosic biomasses are important substrates for bioethanol production and contribute to environmental sustainability. They can be obtained from low cost raw materials such as industrial and agricultural residues. Corn stover, sugarcane bagasse, rice and wheat straws are the most abundant cellulosic raw materials. They can be obtained as large amounts; however high labor and capital cost is needed for processing, which is not economic.

Lignocellulosic materials can be decomposed by enzymatic or chemical procedures. Lignocellulosic-based ethanol production contains four steps as pretreatment for delignifications, hydrolysis of cellulose and hemicellulose to produce fermentable sugars such as glucose, xylose, arabinose, galactose, mannose; fermentation and distillation, respectively (Limayem & Ricke, 2012; Sarkar et al., 2012). Xylose is the second important sugar of hemicellulosic part of the cell wall; however *Saccharomyces cerevisiae*, the main yeast used in bioethanol production is able to use only hexose sugars such as glucose (Limayem & Ricke, 2012).

2.1. Cellulose

Cellulose is a huge amount of sustainable and biodegradable resource for raw materials due to being one of the most abundant organic compound and carbohydrate on earth. It is the main cell-wall component of plant.

It has a linear structure and high molecular weight. Its linear polymer is formed by aldehyde sugars joined by β-(1→4) glycosidic linkages (between the C-4 of one sugar unit and the anomeric C-1 of a second sugar), so called D-anhydroglucopyranose units, combination of 2 groups of glucose units as "cellobiose", which is shown in Figure 2.1 (Fennema, 1996; Huber et al., 2012).

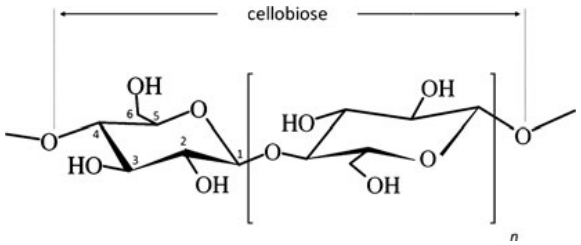

Figure 2.1. The typical molecular structure of a cellobiose unit
(Source: Huber et al., 2012).

Cellulose is usually present in association with lignin and hemicellulose in the cell walls of plants, which makes it difficult to separate from other compounds and obtain pure cellulose (Rachtanapun, Luangkamin, Tanprasert, & Suriyatem, 2012). Several methods can be used to extract cellulose from the cell wall. Bleaching treatment with sodium chlorite or hydrogen peroxide to degrade lignin and using alkaline solution such as sodium hydroxide to solubilize pectins and hemicelluloses are the most common methods.

Cellulose can be used in original form or with derivatives such as cellulose nitrate and carboxymethyl cellulose for textile and papers or film packaging (Rachtanapun et al., 2012).

Glucose is produced by enzymatic hydrolysis of biomass to obtain liquid fuel ethanol. Cellulase, xylanase, ligninase, pectinase are the common enzymes used for biomass hydrolysis. Cellulase is the most important enzyme because it hydrolyzed cellulose, which is 40% or above of biomass. It is a complex enzyme and contains three different enzymes such as exoglucanase, endoglucanase and beta-glucosidase. Cellulose

is firstly separated by endoglucanase and small cellulose parts with free reducing and non reducing ends are released. Small oligosaccharides can be released by exoglucanase. Beta-glucoside finally hydrolyzes cellobiose into glucose units (Singhania, Patel, Sukumaran, Larroche, & Pandey, 2013).

2.2. Hemicellulose

Hemicelluloses are types of plant polysaccharide and the most abundant sustainable polymers after cellulose in lignocellulosic materials in the world. They are branched polymers and low-molecular-weight heteropolysaccharide with a degree of polymerization of 80-200 (Peng, Peng, Xu, & Sun, 2012). Their complex structure includes monosaccharides such as glucose, xylose, mannose, galactose, arabinose, rhamnose, glucuronic acid and galacturonic acid with variable amounts in different biomass. Hemicellulose content depends on source and part of the plant such as stem, branches, roots, and bark. Maize stem, barley, wheat and rye straws contain 28.0%, 34.9%, 38.8%, and 36.9%, respectively. Hemicelluloses are characterized by being neither cellulose nor pectin and by having β-(1→4) linked backbones of glucose, mannose, or xylose (Peng et al., 2012). Xylans are the most abundant of the hemicelluloses present in the cell walls of plants, of which they are up to 30% of the dry weight (Sun, Sun, Sun, & Su, 2004). Hemicelluloses also include arabinoxylans, xyloglucans, β-(1→3, 1→4)-glucans, mannans and glucomannas (Peng et al., 2012).

Hemicelluloses can be extracted from plant sources by mainly hot liquid water, aqueous alkali extraction, alkaline peroxide extraction, dimethyl sulfoxide or methanol/water, steam explosion-based and microwave treatments; however the extraction methods should be improved for hemicellulose to be used as commercial (Peng et al., 2012; Celebioglu, Cekmecelioglu, Dervisoglu, & Kahyaoglu, 2012).

Figure 2.2 shows the potential applications of hemicellulose. They can be hydrolyzed into XOS, pentose (xylose and arabinose), hexose (glucose, galactose, and mannose), and can be transformed into ethanol and other chemicals, such as 5-hydroxymethylfurfural (HMF), furfural, levulinic acid, and xylitol (Peng et al., 2012).

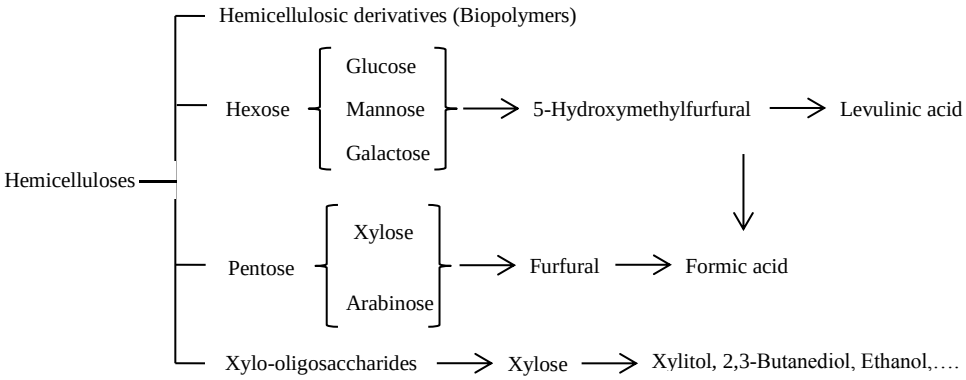

Figure 2.2. The potential products from hemicellulose
(Source: Peng et al., 2012).

The interest in hemicellulose for medical and pharmaceutical applications has increased in recent years (Liu et al., 2017). They can be used in food packaging films and pharmaceutical applications because of their possible uses as ulcer protective, antitussive, immunostimulatory, and antitumor properties (Peng et al., 2012). Due to their gel and film forming properties, biocompatibility and biodegradability, they have been used in cellular therapy and encapsulation (Celebioglu et al., 2012).

2.3. Lignin

Lignin is tightly linked to hemicellulose and cellulose with covalent bonds (Sarkar et al., 2012). It is an aromatic and hard biopolymer with a molecular weight of 10000 Da (Limayem & Ricke, 2012). Lignin is a three dimensional phenolic polymer derived mainly three phenolic monomers of phenyl propionic alcohol namely, p-coumaryl, coniferyl and sinapyl alcohol by free radical generation followed by chemical coupling process (Ghaffar & Fan, 2014; Limayem & Ricke, 2012). On the other hand, there has been no definition of lignin as clear as other carbohydrates because of its highly complicated isolation and characterizaton. It is not expressed as a compound since it is a class of phenolic natural polymer (Ghaffar & Fan, 2014). Therefore, phenolic groups are obtained from the degradation of lignin (Limayem & Ricke, 2012). Agricultural wastes and softwood barks have the lowest and highest levels of lignin, respectively. The interest in potential applications of lignin in the engineering industry such as the development of construction materials or using as a natural antioxidant is increasing (Ghaffar & Fan, 2014).

CHAPTER 3

PREBIOTIC OLIGOSACCHARIDES

3.1. Prebiotics

Prebiotic is described as “nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves the host’s health” (Gibson & Roberfroid, 1995) or “selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits” (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). A food ingredient should have some specific properties to be accepted as a prebiotic. It should have resistance to gastric acidity and not be hydrolyzed by gastrointestinal tract enzymes, not be absorbed in the upper gastrointestinal tract, be used by intestinal microorganisim, and stimulate the growth or activity of intestinal bacteria (Aachary & Prapulla, 2011). Galactooligosaccharides, fructooligosaccharides, inulin and its hydrolysates, maltooligosaccharides, and resistant starch are prebiotics the most commonly known (Al-Sheraji et al., 2013).

The live organisms which use prebiotics to release energy, and produce metabolic subtstrates and lactic acid are called as probiotics. Probiotics such as bifidobacteria and lactobacilli prevent growth of pathogenic microbes by decreasing pH of the large intestine, produce B vitamins and stimulate the immnune system (Patel & Goyal, 2011).

Prebiotics can be produced by isolation from plant resources, microbiological production or enzymatic synthesis, and enzymatic degradation of polysaccharides. They are effective on reduction of cancer risk and mineral absorption besides being important for gastrointestinal health (Al-Sheraji et al., 2013).

The carbohydrates can be classified as monosaccharides, oligosaccharides or polysaccharides according to molecular size or degree of polymerization. Oligosaccharides are low molecular weight carbohydrates having the degree of polymerization of 2-10. They can be present free or in combined forms in all living

organisms. 2-10 monosaccharides are bonded by glycosidic linkages, which can be hydrolyzed by acids or enzymes (Mussatto & Mancilha, 2007; Nakakuki, 2002).

Oligosaccharides can be found as natural component in several foods such as milk, honey, sugarcane juice, soybean, mustard, onion, asparagus, sugar beet, artichoke, chicory, garlic, banana, rye, barley, wheat (Nakakuki, 2002; Patel & Goyal, 2011). They are water soluble and 0.3-0.6 times as sweet as sucrose. Oligosaccharides are preffered as a low calorie bulking agent in food production due to their low sweetness, which is desired to improve other favors (Mussatto & Mancilha, 2007). They can be used as humectants due to their high moisture keeping capacity without increasing water activity. They can be used in animal feeds and cosmetics as stabilizers and bulking agents and in probiotic yoghurts as prebiotic for gut health (Patel & Goyal, 2011). They are functional food ingredients and used to develop the physicochemical properties of foods. They also have important prebiotic properties as the improvement of intestinal microflora, plasma cholesterol and blood glucose level and stimulation of mineral absorption (Nakakuki, 2002).

The functional oligosaccharides which act as nutrient for probiotic organisms are fructooligosaccharides, glucooligosaccharides, mannan oligosaccharides, soybean meal oligosaccharides, galactooligosaccharides, gentiooligosaccharides, isomaltulose, lactosucrose, maltooligosaccharides, xylooligosaccharides, pectin-derived acidic oligosaccharides and cyclodextrins (Patel & Goyal, 2011). Xylooligosaccharides (XOS), agarooligosaccharides, mannooligosaccharides, and chitin/chitosan oligosaccharides are produced from different polysaccharides such as xylan, agar, mannan, chitin, and chitosan as the raw materials (Nakakuki, 2002).

Oligosaccharides can be extracted from natural sources by enzyme processing or chemical production. Acid hydrolysis, oxidative degradation, thermomechanical degradation including ultrasonication and extrusion are the chemical methods used for oligosaccharide production. Autohydrolysis can also be used as a more enviromentally friendly technology. Autohydrolysis has good advantages such as using only water, having limited problems about equipment corrosion because of the mild pH of reaction media and low operational costs (Swennen, Courtin, & Delcour, 2006).

3.2. Xylooligosaccharides

XOS is a prebiotic which is found in bamboo shoots, fruits and vegetables. The structures of XOS can be different according to xylan source, degree of polymerization (DP), monomeric units, and types of linkages. They are composed of two to ten xylose units (xylobiose, xylotriose and so on) linked through β-(1→4) bonds. The structures of xylose and XOS are shown in Figure 3.1.

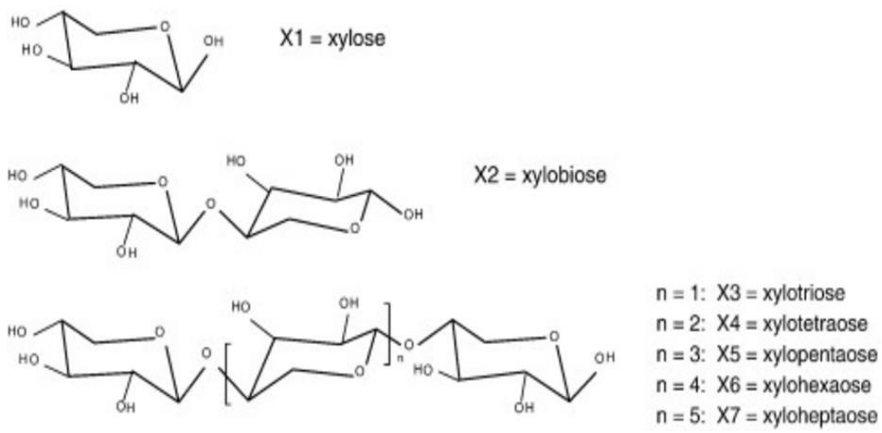

Figure 3.1. Schematic structure of xylose and XOS
(Source: Carvalho et al., 2013).

Hardwoods (e.g., birchwood, beechwood), corn cobs, straws, bagasse, rice hulls, barley hulls, almond shells and wheat bran have been used as raw material for XOS production (Patel & Goyal, 2011; Qing, Li, Kumar, & Wyman, 2013). Renewable and cheap xylan sources such as agricultural wastes (cotton stalks, wheat straw, tobacco stalks) are desired for XOS production instead of hardwood xylan in recent years (Qing et al., 2013). XOS can be used in medical, food, and health products because of their prebiotic properties. They have been shown to have economic use in the pharmaceutical industry for applications such as treating viral and cancer processes in human body (Peng et al., 2012). In human body, XOS are not digested or absorbed in the gastrointestinal tract and reaches colon, where they selectively stimulate the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* species. They enhance short chain fatty acid production, decrease colonic pH, increase faecal weight and mineral absorption (Aachary & Prapulla, 2011). XOS also have antioxidant, antimicrobial, anticancerous, antiallergenic, antiinfection, anti-inflammatory and antihyperlipidemic properties (Samanta et al., 2015; Singh, Banerjee, & Arora, 2015).

CHAPTER 4

HAZELNUT AND HAZELNUT WASTES

4.1. Properties of Hazelnut and Hazelnut Wastes

Hazelnut is the fruit of plants (*Corylus avellana* L.) belongs to Betulaceae family and generally used as an ingredient in several processed foods such as bakery and confectionery products including pastry, chocolate spread, ice cream, cereal bar, cookie; in cooking oil production and consumed as the whole nut (raw or roasted (Demirbas, 2008; Özdemir, Yılmaz, Durmaz, & Gökmen, 2014; Shahidi, Alasalvar, & Liyana-Pathirana, 2007). Hazelnut plays a major role in human nutrition and health cause of its B vitamins, minerals, phenolic content, fat, protein, carbohydrate and dietary fiber (Altun et al., 2013).

Shelled hazelnuts can be classifed into different groups according to the shape and structure of their husks (green leafy cover) or commercial names. According to Turkish Standards, shelled hazelnut varieties are categorized as; Shelled Chobby Hazelnuts ("Tombul", "Palaz", "Mincane", "Çakıldak", "Foşa", "Kalınkara", "Kan", "Cavcava", "Delisava"), Shelled Pointed Hazelnuts ("İncekara", "Sivri", "Kuş") and Other Shelled Hazelnuts ("Kargalak", "Badem", "Orduikizi") (TS 3074, 2001). Among these types, "Tombul" is worldwide known Turkish hazelnut, which can also be divided into 2 groups as "Giresun" and "Levant". Giresun hazelnuts are grown in all towns of the province of Giresun and certain towns of the province of Trabzon. The fat content of Levant hazelnut is lower than that of Giresun but, higher than similar types grown in other countries (Köksal, Artik, Şimşek, & Güneş, 2006). Harvesting is performed between the middle of August to the end of September. Ripining of hazelnut depends on climate, height above sea level and variety of the hazelnut. (Guney, 2013).

Hazelnut and its by-products are presented in Figure 4.1. The hazelnuts have tough and smooth shell. The green leafy cover (or named as "husk") is found together with hazelnut tree leaves. Its color is yellowish before harvesting and brown after harvesting and drying (Guney, 2013). After harvesting, they are eliminated mechanically. The hazelnut kernel can be consumed as raw, with skin or without skin

after removal of the hard shell. Skin, hard shell and green leafy cover are obtained from roasting, cracking, shelling and harvesting (Shahidi et al., 2007).

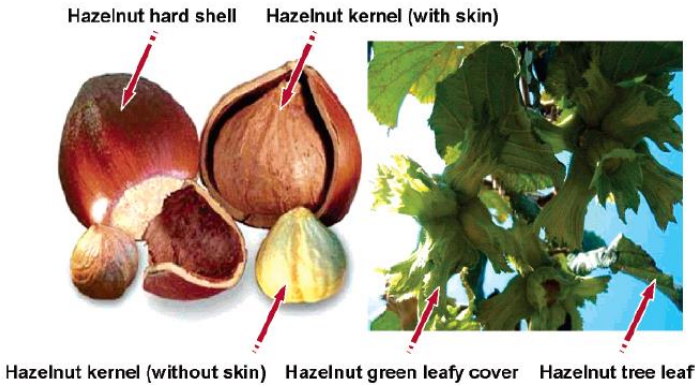

Figure 4.1. Hazelnut shell and hazelnut by-products
(Source: Shahidi et al., 2007).

Turkey is the biggest hazelnut producer (450000 tonnes in 2014, FAOSTAT) and exporter in the world producing 65.4 % of the hazelnut production according to average hazelnut production in 2010-2014 period as presented in Figure 4.2.

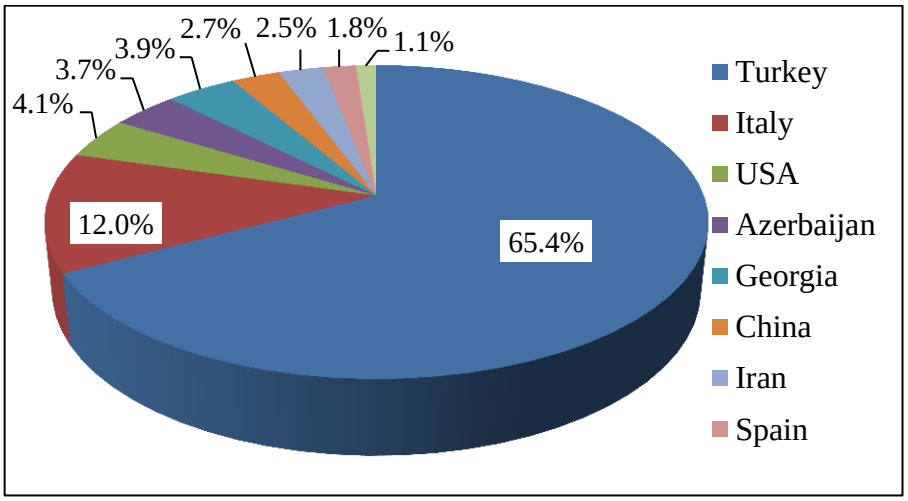

Figure 4.2. The hazelnut production (%) in the world.
(Source: FAOSTAT, 2017).

The hazelnut production of Turkey was 537800 tonnes and followed by Italy, USA, Azerbaijan, Georgia, China, Iran, Spain and France, respectively, for the average hazelnut production in 2010-2014 period as shown in Table 4.1.

Table 4.1. The amount of production (tonnes) of hazelnut between 2010 and 2014 (Source: FAOSTAT, 2017).

Country\Year	2010	2011	2012	2013	2014	Average
Turkey	600000	430000	660000	549000	450000	537800
Italy	90270	128940	85232	112650	75456	98510
USA	25401	34927	35500	40823	32659	33862
Azerbaijan	29454	32922	29624	31202	29796	30600
Georgia	28800	31100	24700	39700	37400	32340
China	19500	22000	23000	23000	23520	22204
Iran	20525	20244	20275	20756	21238	20608
Spain	15086	17590	14406	15302	13542	15185
France	10073	7337	10030	8104	11053	9319
World	857179	746596	925092	869159	713451	822295

The Black sea region, which has a production percentage higher than 50 of the world, contains the main hazelnut-producing provinces (Ordu, Giresun, Samsun, Sakarya and Düzce) (Table 4.2). Many other crops cannot be grown in Black Sea region since the soil is sharply sloping and not suitable. Hazelnut growing areas with high rainfall prevent soil erosion (Demirbas, 2008).

Table 4.2. The amount of the hazelnut production (tonnes) of some provinces (Source: TURKSTAT, 2017).

Country\Year	2012	2013	2014	2015	2016	Average
Ordu	145353	178357	84874	200938	93030	140510
Giresun	118057	64540	94895	82708	77279	87496
Samsun	88392	69392	73544	90857	67855	78008
Sakarya	101532	81342	31567	105023	37591	71411
Düzce	81278	48295	69503	69344	54493	64583

4.2. Valorization of Hazelnut Wastes

The high production amount shows the importance of hazelnut shell, hazelnut husk (green leafy cover) and hazelnut pruning wastes obtained during cultivation and processing and hazelnut pulp removed during oil production as raw material, clearly (Çöpür, Güler, Akgül, & Taşçioğlu, 2007).

4.2.1. Fuel Source

Hazelnut shell is one of the important agricultural waste products from hazelnut processing and large quantities of material are removed. Hazelnut shell is generally used as a valuable and high calorie fuel source for burning, mulching and as raw material for furfural production in the dye industry (Stevigny, Rolle, Valentini, & Zeppa, 2007). On the other hand, other hazelnut by-products have no commercial value. Hazelnut husk (green leafy cover) is a renewable natural resource, which has not been used in the industry. For this reason, there are very limited research about potential utilization of hazelnut shell and husk in the forest industry. Hazelnut husk is burned for house heating that causes pollution or left for soil improvement after harvesting (Guney, 2013).

4.2.2. Natural Antioxidants

Hazelnut is a rich source of polyphenolic components such as, flavan-3-ols, benzoic acids, and flavonols (Özdemir et al., 2014). There are some publications showing antioxidant activity and phenolic content of hazelnut kernel, brown skin, green leafy cover and hard shell (Contini, Baccelloni, Massantini, & Anelli, 2008; Shahidi et al., 2007; Stevigny et al., 2007). Shahidi et al. (2007) and Contini et al. (2008) researched antioxidant compounds in European hazelnuts and determined that hazelnut by-products showed stronger antioxidant activity than the kernel (Xu, Sismour, Parry, Hanna, & Li, 2012). Shahidi et al. (2007) reported that hazelnut wastes, especially, skin and hard shell, could be a reliable source of new and efficient natural antioxidants (Contini et al., 2008).

The studies are generally about characterization of antioxidant phenolic compounds present in hazelnut hard shell. Shahidi et al. (2007) determined that hazelnut by-products had higher antioxidant activity than hazelnut seed. The highest total phenolic content was observed in hazelnut kernel as 577.7 mg catechin equivalent/g ethanolic extract and hazelnut seed showed the lowest value as 13.7 mg catechin equivalent/g ethanolic extract. They determined contents of phenolic acids in extracts of hazelnut kernel and hazelnut by-products. The order of total phenolic acid concentration was as follows: hard shell > husk > tree leaf > skin > kernel. Different phenolic acids were dominant in each part. Gallic acid was the most abundant in hazelnut skin and

shell. P-coumaric acid was the most abundant in kernel, husk and tree leaves (Shahidi et al., 2007).

In another study, the highest total antioxidant activity was shown by hazelnut tree leaves (148 μmol Trolox equivalent/g ethanolic extract) and edible part of hazelnut showed the lowest activity (29 μmol Trolox equivalent/g ethanolic extract) (Alasalvar, Hoffman, & Shahidi, 2008) . Four hydroxycinnamic acid (caffeic, p-coumaric, ferulic and sinapic acid) and one hydroxybenzoic acid (gallic acid) were identified in hazelnut seed and its by-products. Besides phenolic acids, hazelnut also contains cyanidin anthocyanin, (-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin 3-gallat, (+)-catechin flavonol-3-ol (Alasalvar et al., 2008). Contini et al. (2008) measured natural antioxidants in hazelnut hard shell and kernel and determined that extracts from kernel showed the highest antioxidant activity similar to BHA, BHT, α-tocopherol, synthetic antioxidants in the same concentration.

4.2.3. Production of Ethanol

According to previous reports, the cellulose content of hazelnut shell and hazelnut husk were 25-27% (Arslan & Eken-Saraçoğlu, 2010; Cimino, Passerini, & Toscano, 2000; Demirbas, 2008; Demirbas, 2006) and 24.2% (Çöpür, Tozluoglu, & Özkan, 2013), respectively. Arslan & Eken-Saracoglu (2010) and Çöpür et al. (2013) studied on conversion of cellulose in hazelnut by-products to ethanol.

Arslan & Eken-Saracoglu (2010) treated hazelnut shell with diluted acid directly and observed that furfural, which was toxic for the organism produces ethanol, was formed with reducing sugar. Therefore, they added toxin separation section before acid hydrolysis after delignification and produced 16.79 g/L ethanol by fermentation in which *Pichia stipitis* was used. In other study, hazelnut husk was first delignified with 4% $NaBH_4$ in order to produce ethanol, xylan was separated with 2% NaOH and then cellulose was enzymatically treated. 52.6 g ethanol/kg husk was obtained by fermentation of this hydrolyzate (Çöpür et al., 2013).

4.2.4. Others

Xylose is converted to xylitol, which is used as sweetener, by fermentation. It can also be used as a raw material in furfural production. Özay, Küçükada, & Ceylan (2011) used hazelnut husk in production of xylose. Xylose concentrations obtained as a result of reactions performed with different acid type and concentrations and at different temperature, time; and optimum conditions were researched with a model (Özay et al., 2011).

Hazelnut wastes have also been valorized by applying pyrolysis (Aydinli & Caglar, 2012; Ceylan & Topçu, 2014; Haykiri-Acma, Yaman, & Kucukbayrak, 2012; Pütün, Özean, & Pütün, 1999).

Çöpür et al. (2007) examined some chemical properties of hazelnut husk and evaluated its suitability to produce composite panels. They showed that hazelnut husks could be used as a valuable material for the production of particleboards (Çöpür et al., 2007).

There is no study in literature on production of prebiotic oligomers from hazelnut shell, husk and pruning wastes. In this thesis, hemicellulose in hazelnut wastes was used in order to obtain prebiotic source.

CHAPTER 5

PRODUCTION OF XYLOOLIGOSACCHARIDES BY AUTOHYDROLYSIS OF HAZELNUT WASTES

5.1. Introduction

Autohydrolysis (hot water treatment) of lignocellulosic biomass is an effective and environmentally friendly method to produce XOS with an acceptable yield and different compositions (Nabarlatz, Farriol, & Montané, 2005). Autohydrolysis, in which biomass is treated with only water at elevated temperatures, leads hydrolytic depolymerization of xylan backbone and production of XOS (Parajó, Garrote, Cruz, & Dominguez, 2004; Samanta et al., 2015).

It is advantageous method to eliminate chemicals for extraction by using only hot water as agent; however it needs a specialized equipment, which can be operated at high temperatures and pressures as high as or higher than acid or alkali treatments. Biomass is mixed with water in pressurized reactor and heated to temperatures between 160-240 ^{o}C. After holding for a certain time, the reactor is cooled and autohydrolysis liquor is evacuated (Alvira et al., 2010; Hendriks & Zeeman, 2009).

Autohydrolysis is one of the first steps in the fractionation process for lignocellulosic materials. The liquid phase contains hemicellulose and can be decomposed into soluble oligosaccharides, sugars and aldehydes. The precipitated phase is rich in cellulose and lignin, which can be separated further processing. XOSs are major products of the hydrolysis of hemicellulose (Carvalho et al., 2013).

Autohydrolysis produces a liquid phase (liquor) including xylooligomers, monosaccharides, sugar decomposition products, acetic acid, fractions derived from extractives and acid-soluble lignin. Ethyl acetate is used to extract extractives and compounds from acid-soluble lignin (Conde, Moure, Domínguez, & Parajó, 2008). High temperature during autohydrolysis causes autoionization of water and conversion of acetyl groups in hemicellulose chain to acetic acid (deacetylation). Hydronium ions and acetic acid catalyze the removal of xylan from the lignocellulosic network and degradation into high or low molecular weight XOS and xylose (Aachary & Prapulla,

2011). They can be utilized in polymeric form to be used as hydrogel or biodegradable barrier films for food packaging, in oligomeric from to be used as functional food ingredients and in monomeric from to be fermented to ethanol or xylitol (Aachary & Prapulla, 2011; Otieno & Ahring, 2012; Ruiz et al., 2013). It is not a selective reaction and other extractive compounds, acid soluble lignin and proteins also released besides hemicellulose hydrolysis (Garrote, Falqué, Domínguez, & Parajó, 2007). Those compounds can be eliminated by membran filtration or organic solvent extraction.

Autohydrolysis has been applied to obtain XOS from various lignocellulosic biomass such as wheat straw (Carvalheiro, Silva-Fernandes, Duarte, & Gírio, 2009), almond shells (Nabarlatz et al., 2005), olive pruning wastes (Cara et al., 2012), brewery's spent grain (Carvalheiro, Esteves, Parajó, Pereira, & Gírio, 2004), corn stalk (Egüés, Sanchez, Mondragon, & Labidi, 2012), sunflower stalk (Akpinar, Gunay, Yilmaz, Levent, & Bostanci, 2010), corncobs, olive stones, barley straw (Nabarlatz, Ebringerová, & Montané, 2007) and rice husk (Vegas, Alonso, Domínguez, & Parajó, 2004). Due to differences in the structure of the raw materials and the conditions of the production process, the composition and the structure of the XOS can be different (Nabarlatz, Ebringerová, et al., 2007). The reaction conditions such as temperature and time should be optimized for each waste product. Degree of polymerization decreases when the conditions become harder; however monosaccharides as xylose can be formed. Furfural, hydroxymethylfurfural (HMF), acetic, formic and some other acids are also produced as a result of degradation of carbohydrates (Aachary & Prapulla, 2011). Therefore, reaction conditions should be observed to obtain oligomer or another target product. Autohydrolysis experiments should be carried out in different conditions.

Nabarlatz et al. (2005) autohydrolyzed almond shells at 150-190 ^{o}C and reported XOS yields at 150 ^{o}C for 300 minutes and 190^{o}C for 19 minutes as 42% and 63%, respectively. They also obtained a product, which was 24% of dry almond shell and had 58.3% XOS by drying autohydrolysis liquor in spray dryer.

Cara et al. (2012) treated olive pruning wastes at 170-230 ^{o}C for 10 minutes and obtained the highest XOS yield as 60 g/kg at 180^{o}C. Furfural and acetic acid production increased while temperature increased; however xylose production increased until 200 ^{o}C and then decreased.

Carvalheiro et al. (2004) obtained the highest yield of autohydrolyzation of beer wastes at 190 ^{o}C for 5 minutes. They reported that time should be extended to increase amount of soluble xylan at low temperature.

Nabarlatz et al. (2007) used six agricultural residues (corncobs, almond shells, olive stones, rice husks, wheat straw and barley straw) to produce XOS by autohydrolysis at 179 °C for 23 min. The xylan content, its accessibility and the acetyl content of the raw materials influenced the yield of XOS. Corncob (60%) and almond shells (55%) showed had the highest yield, while it was the lowest for rice husk (30%), which also had the lowest acetyl content.

Vegas, Alonso, Domínguez, & Parajó (2005) treated waste solids from the malting industry with autohydrolysis reaction. The amount and composition of the phases were determined as 11.27 kg of monosaccharides, 70.80 kg of oligosaccharides and oligosaccharide substituents in nonvolatile components, and 17.93 kg of other nonvolatile components.

Rice husk was treated with hot and compressed water for hydrolytic degradation of arabinoxylan by Vegas et al. (2004). After ethyl acetate extraction of autohydrolysis liquors, they used different alternative treatments such as solvent precipitation, freze-drying solvent extraction and ion excange to decrease the content of other nonvolatile solutes, which were not hemicellulose-derived. They obtained the highest yield as 92% of the nonvolatile compounds by ion exchange (Vegas et al., 2004).

Studies in literature have showed that autohydrolysis is a suitable process to fractionate lignocellulosic wastes to their compounds. It is possible to obtain a valuable prebiotic such as XOS by that procedure; however there is no research in literature on utilization of hazelnut wastes in autohydrolysis. Therefore, this thesis is the first study related to autohydrolysis of hazelnut wastes and production of XOS from them.

The aim of this study was to determine the potential of hazelnut wastes as a feedstock for production of XOS by autohydrolysis. The effects of autohydrolysis temperature and holding time on XOS yield from xylan, by-product formation and the distribution of molecular weight of the XOS were investigated.

Severity factor was used to evaluate the combined effect of temperature and holding time on the reaction products.

5.2. Materials and Methods

Hazelnut wastes were obtained from hazelnut producers in Ordu (Gürsoy Agricultural Products Food Industry and Trade Inc.) and Çaycuma (Zonguldak) in hazelnut harvesting and processing time. Hazelnut shell, green leafy cover (husk) and pruning wastes were dried in oven at 60^{o}C for 24 h and milled in a plant grinding mill as particles passing from 2 mm screen. The dry ground samples were stored in airtight packages at room temperature until they were used. Hazelnut wastes were obtained after milling as shown in Figure 5.1.

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany) and Merck Millipore (Darmstadt, Germany). Xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose obtained from Megazyme (Ireland) were used as external standards for XOS analysis.

Figure 5.1. Hazelnut wastes.

5.2.1. Moisture Content

Moisture content of hazelnut wastes and processed solids obtained after autohydrolysis was determined according to National Renewable Energy Laboratory (NREL) NREL/TP-510-42621 method (NREL, 2008b). 2 g of each sample were weighed and placed in disposable aluminum containers. They were dried overnight in an oven (Memmert, Germany) at 105 ^{o}C.

5.2.2. Protein Content

The total protein content of hazelnut wastes was determined by the Kjeldahl method (protein=6.25xN) using Kjeldatherm digestion and Vapodest 50s distillation units (Gerhardt, Germany).

5.2.3. Ash Content

Ash content of hazelnut wastes and processed solids obtained after autohydrolysis was measured by igniting the biomass at 575 ^{o}C in an ashing furnace (Carbolite, UK) according to American Society for Testing and Materials (ASTM) E 1755-01 (ASTM, 2001).

5.2.4. Total Extractives

Total extractives content was calculated after treating the biomass sequentially with ultra-pure water and ethanol 96% in a Soxhlet apparatus.

5.2.5. Uronic Acid Content

Uronic acid content of hazelnut wastes was determined spectrophotometrically at 525 nm using m-hydroxydiphenyl method (Melton & Smith, 2001). The calibration curve was prepared by using D-galacturonic acid standard solutions with different concentrations (0.01 - 0.1 mg/mL) as presented in Appendix C.

5.2.6. Structural Carbohydrate Content

Cellulose, xylan, galactan, arabinan, acid soluble lignin and Klason lignin contents of hazelnut wastes were determined by NREL/TP-510-42618 method (NREL, 2011). Alcohol soluble compounds such as chlorophyll, wax and others were removed by ethanol extraction in Soxhlet apparatus. After Soxhlet extraction, extractive-free biomass was dried overnight at 45 ^{o}C and 0.3 g dry biomass was treated with 3 mL of 72% H_2SO_4 (w/w) at room temperature. After 60 min, the mixture was diluted to 4% by

using water. The diluted solution was kept at 121 ^{o}C for 1 h in an autoclave (Alp, Japan).

The solid part was filtered through porcelain filter crucibles under vacuum and dried at 105 ^{o}C. The weight of this solid was reported as Klason lignin after subtracting the ash content.

Acid soluble lignin was determined by measuring absorbance of filtrate at 240 nm after hydrolysis and it was calculated by using the following formula (5.1);

$$ASL\% = \frac{UVabs * Filtrate\ Volume * DF * 100}{\varepsilon * Dry\ weight} \quad (5.1)$$

UVabs: Absorbance at 240 nm
Filtrate Volume: Volume of acid hydrolysis (87 mL)
ε: Absorptivity value (25 L/g cm)
DF: Dilution Factor

After increasing pH of filtrate samples obtained from acid hydrolysis to 5-7 by $CaCO_3$, samples were centrifugated at 4 ^{o}C for 15 min at 4800 g (Centurion Scientific, England) and filtrated by 0.45 μm PTFE membrane filters. Glucose, xylose, galactose and arabinose concentrations of filtrated samples were analyzed by HPLC system. Acid hydrolysis disintegrated cellulose and hemicellulose in biomass to its monomers. Structural carbohydrate% in biomass was calculated by multiplying the glucose, xylose, galactose and arabinose concentrations of filtrate measured by HPLC, respectively by anhydro correction factors (0.90 for hexoses and 0.88 for pentoses). Acetyl content% was calculated by multiplying acetic acid concentration by 0.717.

Cellulose, xylan, galactan, arabinan and Klason lignin contents of processed solids after autohydrolysis were also determined by the same procedure without any treatment for removing extractives.

5.2.7. Autohydrolysis

The autohydrolysis treatmets were carried out in a 600 mL stainless steel high pressure reactor as shown in Figure 5.2 (Berghof, Germany). The biomass (35 g) was

mixed with distilled water as a 1:10 (solid/liquid) ratio and placed into the reactor. The mixture was heated to selected temperature with continuous stirring at 300 rpm. At the end of the holding time, the reactor was cooled to 60 ^{o}C with tap water circulating in the cooling coil. The solid and liquid phases were separated by cheesecloth and vacuum filtration, respectively. The processed solids were washed, dried at 45 ^{o}C overnight and stored at room temperature in airtight packages until analysis. The filtrates which were named as autohydrolysis liquors in this study were kept at -20 ^{o}C.

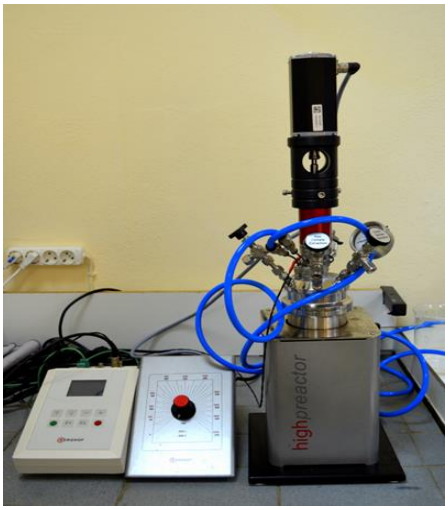

Figure 5.2. High pressure reactor.

In order to evaluate the combined effect of temperature (T) and time (t) on thermal treatment efficiency in one variable, severity factor (R_o) was calculated for each treatment condition. R_o can be defined as the following formula (Overend, Chornet, & Gascoigne, 1987).

$$\mathrm{Ro} = \int_0^{\mathrm{t}} \exp\left[\frac{T(t)-100}{14.75}\right] \mathrm{dt} \qquad (5.2)$$

t: Treatment time (min)

T: Selected reaction temperature (^{o}C)

14.75: Empirical value related to activation energy of the reaction energy of the reaction.

100: Reference temperature (^{o}C).

Autohydrolysis of hazelnut wastes was performed at different severity factors. Logarithmic values of severity factor (log R_o) were calculated for each autohydrolysis treatment. The main aim of this evaluation was to determine the optimum time and temperature combination or optimum log R_o having the highest XOS% yield in liquor.

The set temperature was adjusted to 2^{o}C above reaction temperature. After waiting at selected temperature during holding time, the reactor was stopped and cooled. Temperature changes during heating to selected temperature, holding time and and cooling to 60 ^{o}C were taken into consideration in the log R_o calculation.

Log R_o values were calculated for each condition as presented in Figure 5.3 according to integration by "Rectangle Midpoint Rule", which gives the best approximation in most cases. The reactor temperature was measured at regular intervals, 1 min. Therefore, difference in time (Δt) was 1 min. The value, exp[(T(t)-100)/14.75] was calculated for each measured temperature. The average of exponential values of 2 sequential measured temperature was calculated. R_o was obtained by multiplying time difference and average temperature difference values for each measured temperature.

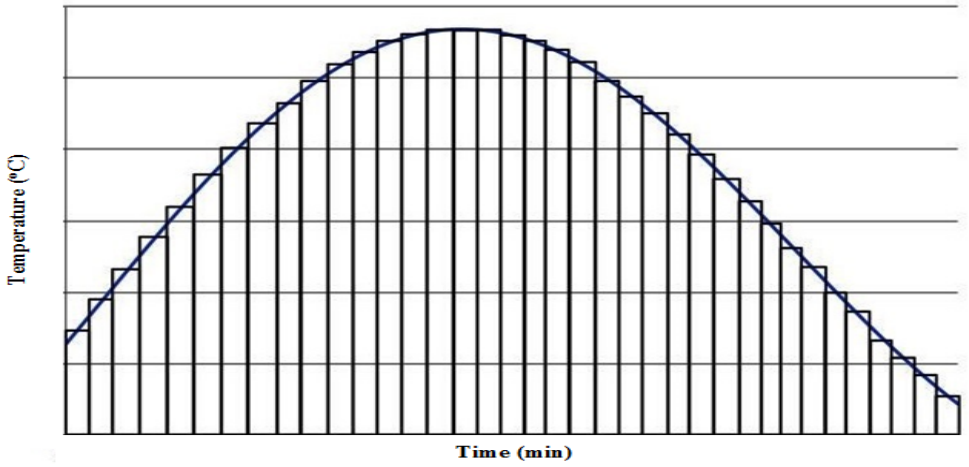

Figure 5.3. Rectangle midpoint rule for log R_o calculation.

Autohydrolysis was firstly applied to hazelnut shell, husk and pruning wastes at 190 ^{o}C for 15 min as holding time. Then, due to having the highest XOS% yield value in autohydrolysis liquors, the effect of autohydrolysis treatment conditons on formation XOS and other products from hazelnut shell was investigated at different temperatures (150 – 200 ^{o}C) and holding times (0 - 45 min).

5.2.8. HPLC Analysis

Monosaccharide, oligosaccharide, acetic acid and formic acid concentrations of samples were measured by high performance liquid chromatography (HPLC) system (Perkin Elmer, USA). Autohydrolysis liquors were directly analyzed by HPLC after filtration through 0.45 µm PTFE membrane filters. Total oligosaccharide content of liquors and acetyl groups bound to XOS were determined indirectly after acid hydrolysis with 4% H_2SO_4 at 121 ^oC for 1 h, according to NREL/TP-510-42623 method (NREL, 2008a). The increase in the monomeric sugar concentrations in liquors was expressed as the corresponding oligosaccharide content after multiplying by the anhydro correction factor (0.90 for hexoses and 0.88 for pentoses). Acetyl groups bound to XOS were also quantified by the increase in acetic acid concentration after acid hydrolysis. After neutralizing pH of the liquor hydrolyzed as mentioned before, liquors were analyzed by HPLC.

Monomeric sugars such as glucose, xylose, galactose and arabinose were detected using a Rezex RPM-Monosaccharide column (Phenomenex, USA) and a refractive index (RI) detector at 80 ^oC. Ultra-pure water was used as mobile phase and flow rate was 0.6 mL/min. "Deashing Protective Column" (Bio-Rad, USA) was used to hold cation and anions by cartridges in H^+ ve CO_3^{-2} form for samples obtained from acid hydrolysis. "Security Guard Column" (Phenomenex, USA) was used as pre-column for samples analyzed directly.

The liquors were analyzed for furfural and 5-hydroxymethylfurfural (HMF) using HPLC system (Agilent Technologies, USA) coupled with diode-array detection (DAD) using Aminex HPX-87H column. Analysis was performed at 285 nm for furfural and 210 nm for HMF using 5mM H_2SO_4 as mobile phase (flow rate: 0.6 mL/min) at 65 ^oC.

The degree of polymerization (DP) of the XOS was measured by HPLC (Perkin Elmer, USA) with a RI detector and an Aminex HPX-42A column (Bio-Rad, USA) at 80 °C using ultra-pure water as the mobile phase and a flow rate of 0.6 mL/min. "Deashing protective column" was installed as pre-column. In order to increase resolution and measure the concentrations accurately, deacetylation (removing of acetyl groups in xylan structure by alkali treatment) was performed for autohydrolysis liquors as presented in Figure 5.4. For deacetylation, pH of liquors was increased (to 12-13) by

using NaOH. Different amounts of NaOH solution was added in autohydrolysis liquors to the final concentrations of 0.1, 0.25, 0.5, 1, 2 and 4% NaOH (w/v).

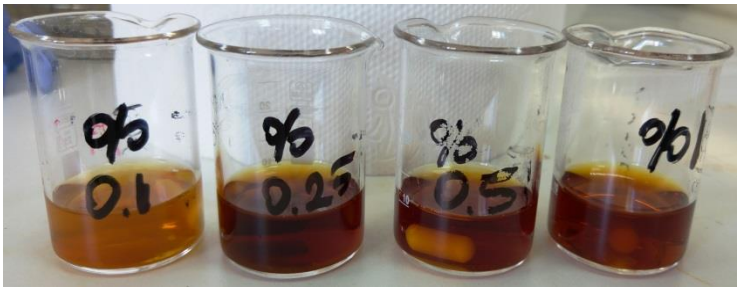

Figure 5.4. Deacetylation of autohydrolysis liquors with different NaOH solutions.

The solutions were mixed for 15 min at room temperature. Neutralization was performed to prepare the solutions for HPLC. After mixing, pH of the mixture was decreased to 6-7 by adding HCl with different concentrations. All liquors were centrifuged at 4 ^{o}C for 15 min at 22000 g (Centurion Scientific, England) and filtered through 0.45 μm PTFE membrane filters (Isolab, Germany) before HPLC analysis. According to HPLC chromatograms of liquors (Appendix A), alkali treatment with 0.5% NaOH (w/v) (as the final concentration in the mixture) was successful to improve resolution of the peaks of low-DP XOS (xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose). The differences in resolution of peaks were observed as presented in Figure A.1.

HPLC calibration curves of glucose, xylose, galactose and arabinose for monosaccharide analysis (Figure B.1-B.4); acetic acid, HMF and furfural for by-product analysis (Figure B.5-B.7); and xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose for low- DP oligosaccharide analysis (Figure B.8-B.12) were prepared for different concentrations.

HPLC chromatograms of standard monosaccharides (Figure A.2), processed solids (Figure A.3) and liquor obtained after autohydrolysis (Figure A.4); and autohydrolysis liquor after acid hydrolysis (Figure A.5) were presented in Appendix A.

5.2.9. Color Analysis

Color of autohydrolysis liquors were measured as described by (Cemeroglu, 2007) with some modifications. Liquors were diluted with absolute ethanol and 80%

ethanol-water mixture including 1% HCl. After centrifiguation at 4 ^{o}C for 4800 g, the liquors were filtrated by 0.45 µm PTFE membrane filter. Absorbances were measured against distilled water at 420, 520 and 620 nm by UV-Visible spectrophotometer (T80+, PG Instruments Ltd., England). Hazelnut shells were added to water with 1:10 solid:liquid ratio as used in reactor during autohydrolysis. The absorbance obtained from shell-water mixture was used as blank. The highest absorbance values were obtained at 420 nm, and there was no difference between liquors diluted with absolute ethanol or acidic solution. Therefore, absorbances of liquors diluted with absolute ethanol were measured at 420 nm and the values were expressed after multiplying with dilution factor.

5.2.10. Total Phenolic Content and Total Antioxidant Activity

Dry de-fatted hazelnut waste samples and hazelnut shell autohydrolysis liquors were analyzed for total phenolic content (TPC) and total antioxidant activity (TAA). Hazelnut shell was de-fatted by treating in Soxhlet apparatus for 6 h. 25 g of hazelnut shell was weighed in a filter paper and it was placed into 250 mL extraction chamber. 350 mL of hexane was added in 500 mL boiling flask. After 6 h, de-fatted sample was removed and it was dried in oven (Termal, Turkey) at 60 ^{o}C.

Extraction was performed as described by Xu et al. (2012) with some modifications. Three different types of solvents such as water, 80% methanol-water and 80% acetone-water solutions were used for extraction. 0.4 g of de-fatted sample was put in test tubes and 5 mL of solvent was added. The sample was sonicated for 15 minutes at room temperature in ultrasonic bath (Elma, Transsonic 780/H, Germany). After sonication, they were centrifuged 4800 g under 4^{o}C and the centrifugates were collected. This procedure was repeated 4 times with fresh solvent until 20 mL of solvent was used. Sample extracts were stored at -20^{o}C until TPC and TAA analysis.

Folin-Ciocalteu method which was used by Singleton & Rossi (1965) was modified to measure TPC of autohydrolysis liquors and hazelnut shells extracted with different solvents. Hazelnut shell extract, autohydrolysis liquor or standard solution (500 µL) was vortexed (Isolab, Germany) with 2.5 mL of Folin-Ciocalteu reagent (diluted 10 times with distilled water) and then 2 mL of Na_2CO_3 solution (75 g/l) was added to the mixture. After incubating for 120 min at room temperature by protecting

from light, absorbance was measured against distilled water at 765 nm using a UV-Visible spectrophotometer (T80+, PG Instruments Ltd., England). The calibration curves were prepared as presented in Appendix C by using gallic acid standard solutions in water (0.01-0.40 g/L), 80% methanol-water (0.01-0.20 g/L) and 80% acetone-water (0.01-0.20 g/L). The results were expressed in mg gallic acid equivalents (GAE) per 100 g of dry weight (DW) for hazelnut shell extracts and mg GAE/mL for autohydrolysis liquors.

TAA of autohydrolysis liquors and hazelnut shells extracted with different solvents was determined by 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method as described by Miller & Rice-Evans (1997). ABTS method is based on the production of a blue/green $ABTS^{\bullet+}$ that is useful for hydrophilic and lipophilic antioxidant systems. ABTS reagent solution was prepared by dissolving 200 mg of ABTS in 200 mL water. 38 mg of potassium persulphate ($K_2S_2O_8$) was added in 2 mL water. ABTS reagent and potassium persulphate solutions were mixed and left overnight by protecting from light to form the radical. 0.05 M potassium dihydrogen phosphate (KH_2PO_4) and 0.05 M dipotassium hydrogen phosphate (K_2HPO_4) solutions were used to prepare a buffer solution at pH 8. ABTS analysis solution was prepared by adding ABTS reagent mixture into buffer solution until the absorbance of the solution is 0.9 ± 0.2 at 734 nm. 100 µL of hazelnut shell extract, autohydrolysis liquor or standard solution was taken into tubes, 1 mL of prepared ABTS analysis solution was added and vortexed for 15 seconds. After waiting for 45 seconds, absorbance was read against distilled water at 734 nm using a UV-Visible spectrophotometer (Carry 100 BIO, Varian, USA).

Standard calibration curves were prepared with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in water (0.04-0.4 g/L), 80% methanol-water (0.01-0.1 g/L) and %80 acetone-water (0.01-0.1 g/L) as presented in Appendix C. The results were expressed in mg Trolox equivalents (TEAC) per 100 g of dry weight (DW) for hazelnut shell extracts and mg TEAC/mL for autohydrolysis liquors.

5.2.11. Statistical Analysis

Autohydrolysis treatments were performed in duplicate. Characterization analysis of hazelnut wastes were carried out in triplicate. The results were given as mean ± SD.

Total XOS concentration of autohydrolysis liquors of hazelnut shell were evaluated statistically by using one way analysis of variance (ANOVA). Tukey test was used to analyze differences between treatments ($p < 0.05$). Analysis was performed using Statistical Package for the Social Sciences software as shown in Appendix D (version 16.0, SPSS Inc., Chicago, IL).

5.3. Results and Discussion

Compositional analysis results of hazelnut wastes from Ordu (shell, husk and pruning wastes) were expressed as g per 100 g dry raw material as shown in Table 5.1.

Table 5.1. Compositional analysis of hazelnut wastes obtained from Ordu.

Components	**Content (g/100 g dry raw material)**		
	Shell	**Husk**	**Prunings**
Moisture	9.7 ± 0.1	8.8 ± 0.1	6.9 ± 0.2
Cellulose	18.7 ± 0.5	15.4 ± 1.5	34.7 ± 4.3
Klason lignin	46.4 ± 0.2	25.9 ± 0.7	25.4 ± 0.8
Acid soluble lignin	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
Xylan	18.7 ± 0.1	5.8 ± 0.2	12.8 ± 1.6
Galactan	1.6 ± 0.3	2.6 ± 0.1	1.3 ± 0.5
Arabinan	0.3 ± 0.0	1.7 ± 0.2	1.0 ± 0.0
Acetyl groups	2.6 ± 0.3	1.2 ± 0.1	2.0 ± 0.1
Acetyl/xylose[b]	0.4	0.5	0.5
Uronic acids	5.3 ± 1.7	10.6 ± 2.4	9.4 ± 1.1
Ash	0.9 ± 0.1	5.0 ± 0.3	1.0 ± 0.0
Protein	2.8 ± 0.1	8.0 ± 0.2	4.5 ± 0.1
Extractives	1.2 ± 0.3	24.6 ± 1.7	4.0 ± 0.3
Total	102.3	103	98.6

[b]expressed as molar ratio.

Moisture, protein, structural carbohydrate, acetyl groups, lignin, ash and total extractive contents were determined. Major components in hazelnut wastes were lignin, cellulose followed by xylan, except husk, which was also rich in extractives. The

protein and ash contents were also higher for husk than other wastes. On the other hand, husk had the lowest acetyl groups and total carbohydrate contents. Pruning wastes were rich in cellulose (34.7%) while shell had the highest total lignin concentration (46.7%). Klason lignin was higher than acid soluble lignin content for all wastes. The content of other hemicellulosic carbohydrates such as galactan and arabinan were lower than xylan. The highest xylan, galactan and arabinan contents were determined as 18.7 g/100g for shell, 2.6 g/100g for husk and 1.7 g/100g for husk, respectively. Shell also had the highest acetylated xylan and uronic acid content. The determination of xylan content of hazelnut wastes was important for this study, since one of the main objectives was producing XOS after hydrolysis of hemicellulose.

Xu et al. (2012) determined protein and ash contents of hazelnut shells from US-grown cultivars as 2.1-4 and 0.8-2.0 g/100 g dry shells, respectively, which were consistent with the results in this study. Çöpür et al. (2007) determined higher lignin, ash and alcohol-benzene soluble content of husk as 35.1%, 8.22% and 1.63%, respectively as compared to this study. In this study, lignin and ash contents of husk were less than values obtained by Çöpür et al. (2007); however water-ethanol soluble content was calculated as 24.6%. Haykiri-Acma, Baykan, Yaman, & Kucukbayrak (2013) found less lignin content (32.7%) than in this study. Cellulose and hemicellulose contents of shell used in this study were lower than reported values in previous studies; however lignin content was consistent with their values (Caglar & Aydinli, 2009; Çepelioğullar & Pütün, 2014; A Demirbas, 2008). The reason of differences in results was probably due to using different sources for feedstocks or applying different methods for characterizaton.

To our knowledge, there is no report in literature showing the composition of hazelnut pruning wastes.

Structural carboyhdrate analysis of hazelnut wastes were also carried out for hazelnut wastes such as shell and husk obtained from Çaycuma, Zonguldak. The analysis results were expressed as per 100 g dry sample as presented in Table 5.2. Cellulose, xylan and galactan arabinan contents of hazelnut shell obtained from Ordu were higher than shell from Çaycuma. Therefore, autohydrolysis experiments with different conditions were performed for hazelnut waste samples received from Ordu, which had higher xylan content in the shell. On the other hand, husk from Çaycuma contained higher cellulose, xylan and galactan contents than Ordu.

Table 5.2. Structural carbohydrate analysis of hazelnut wastes obtained from Çaycuma.

Carbohydrates	Content (g/100 g dry raw material)	
	Shell	Husk
Cellulose	13.8 ± 0.5	18.6 ± 1.1
Xylan	14.3 ± 0.3	7.9 ± 0.1
Galactan	1.4 ± 0.0	3.3 ± 0.5
Arabinan	0.4 ± 0.0	1.1 ± 0.1
Total	29.9	30.9

5.3.1. Autohydrolysis of Hazelnut Wastes at Different Conditions

Autohydrolysis was applied to shell, husk and pruning wastes at 190^{o}C for 15 min (excluding heating and cooling periods). In order to maximize XOS yield and minimize by-product formation, the effect of autohydrolysis conditions on formation of XOS and by-products from shell were investigated at selected temperatures (150-200 ^{o}C) and holding times (0-45 min). Reactor temperature profiles of each autohydrolysis treatment were obtained for severity factor calculation as as shown in Figure 5.5 as an example. In Figure 5.5, reaction temperature profile was obtained from time and temperature changes of autohydrolysis treatments at 150, 160, 170, 180, 190 and 200^{o} C for 15 min including heating, holding and cooling steps. Log R_o value was calculated for each temperature-time condition as shown in Table 5.3.

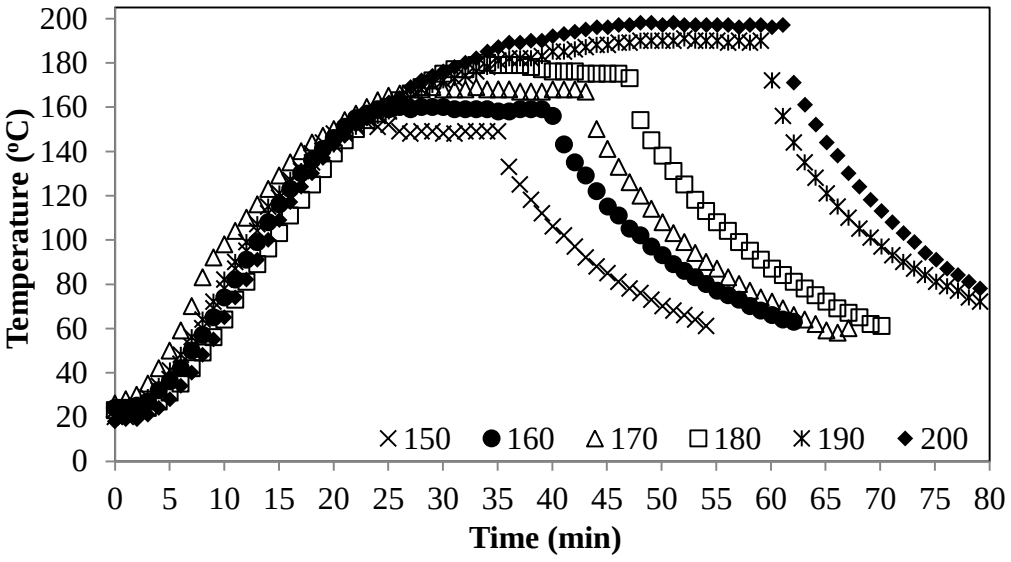

Figure 5.5. Reactor temperature profiles including heating, holding and cooling for autohydrolysis at different temperatures for 15 min.

Table 5.3. Log R_o values calculated for each autohydrolysis condition.

Biomass	Temperature (^oC)	Time (min)	Log R_o
Husk	190	15	4.02
Prunings	190	15	4.02
Shell	150	15	2.73
Shell	160	15	3.09
Shell	170	15	3.36
Shell	180	5	3.53
Shell	180	15	3.63
Shell	180	30	3.91
Shell	180	45	4.03
Shell	190	0	3.64
Shell	190	5	3.92
Shell	190	15	4.02
Shell	190	30	4.15
Shell	200	0	3.93
Shell	200	15	4.29

Minimum and maximum log R_o values were obtained as 2.73 and 4.29 for 150^{o}C-15 min and 200^{o}C-15 min for autohydrolysis of shell. Log R_o values of autohydrolysis of husk and pruning wastes were calculated as 4.02.

5.3.2. Composition of Processed Solids of Hazelnut Wastes

Liquors and processed solids obtained after autohydrolysis of hazelnut husk and pruning wastes at 190 ^{o}C for 15 min and hazelnut shell at different conditions (150-200 ^{o}C, 0-45 min) were analyzed by HPLC. Celluose, Klason lignin, xylan, galactan, acetyl groups, and ash contents of processed solids were determined as presented in Table 5.4.

For all three processed solids (husk, shell and pruning wastes) obtained from autohydrolysis at 190 ^{o}C for 15 min, more than one third of the raw material dissolved during autohydrolysis. Autohydrolysis generated solids with increased lignin and cellulose content and it was an effective process to remove xylan from the lignocellulose network. The cellulose content of processed solids of pruning wastes was as high as 57.7%, thus this biomass can be considered as a potential source of glucose for bioethanol production.

Table 5.4. Composition of processed solids from autohydrolysis of hazelnut wastes.

Biomass	Treatment conditions			Residual solids (g/100 g dry raw material)	Component (g/100 g dry processed solid)					
	Temperature (^{o}C)	Time (min)	log R_o		Cellulose	Klason lignin	Xylan	Galactan	Acetyl groups	Ash
Husk	190	15	4.02	60.9 ± 2.7	31.1 ± 1.4	53.1 ± 0.5	5.7 ± 0.1	2.7 ± 0.1	0.40 ± 0.05	2.8 ± 0.0
Prunings	190	15	4.02	64.3 ± 2.8	57.7 ± 2.3	39.6 ± 1.2	5.3 ± 0.1	0.7 ± 0.2	0.79 ± 0.05	0.9 ± 0.3
Shell	150	15	2.73	87.7 ± 0.7	24.2 ± 0.9	46.8 ± 0.1	21.6 ± 0.5	0.8 ± 0.1	4.23 ± 0.38	0.8 ± 0.0
Shell	160	15	3.09	87.5 ± 1.6	22.5 ± 1.4	52.1 ± 0.3	14.6 ± 2.6	0.6 ± 0.1	3.57 ± 0.35	0.8 ± 0.0
Shell	170	15	3.36	77.4 ± 0.1	25.0 ± 0.6	50.8 ± 2.6	15.6 ± 1.1	0.6 ± 0.0	2.58 ± 0.20	0.7 ± 0.0
Shell	180	5	3.53	71.4 ± 0.7	26.8 ± 1.4	55.4 ± 1.3	12.2 ± 0.7	0.3 ± 0.0	2.14 ± 0.18	0.7 ± 0.0
Shell	180	15	3.63	70.5 ± 0.5	27.2 ± 1.1	54.9 ± 1.5	11.2 ± 0.7	0.4 ± 0.1	1.64 ± 0.37	0.6 ± 0.0
Shell	180	30	3.91	68.1 ± 2.6	27.8 ± 0.9	58.7 ± 0.3	4.5 ± 0.5	0.3 ± 0.0	0.95 ± 0.01	0.7 ± 0.0
Shell	180	45	4.03	68.2 ± 1.8	28.4 ± 1.3	62.7 ± 2.8	5.4 ± 0.2	0.2 ± 0.0	0.90 ± 0.11	0.6 ± 0.0
Shell	190	0	3.64	70.3 ± 1.4	22.4 ± 2.5	61.3 ± 1.7	10.3 ± 3.0	0.3 ± 0.0	1.09 ± 0.14	0.5 ± 0.0
Shell	190	5	3.92	66.5 ± 1.3	27.9 ± 2.3	58.7 ± 0.9	9.2 ± 0.2	0.2 ± 0.0	1.21 ± 0.09	0.6 ± 0.0
Shell	190	15	4.02	64.7 ± 0.2	29.3 ± 0.2	61.4 ± 0.6	6.5 ± 0.5	0.1 ± 0.0	0.66 ± 0.25	0.6 ± 0.0
Shell	190	30	4.15	64.6 ± 0.3	30.4 ± 1.1	63.1 ± 1.5	2.2 ± 0.2	0.2 ± 0.0	0.57 ± 0.02	0.7 ± 0.1
Shell	200	0	3.93	65.6 ± 1.3	30.4 ± 0.4	59.4 ± 0.7	5.9 ± 0.9	0.0 ± 0.0	0.84 ± 0.14	0.7 ± 0.0
Shell	200	15	4.29	61.8 ± 0.6	28.3 ± 1.3	67.9 ± 0.6	1.7 ± 0.1	0.1 ± 0.0	0.55 ± 0.08	0.6 ± 0.0

According to compositon analysis of processed solids from autohydrolysis of hazelnut shell at different conditions (Table 5.4), the highest xylan content was determined from autohydrolysis at 150^{o}C for 15 min. Xylan and cellulose concentrations were higher than galactan and arabinan. Arabinan concentrations could not be calculated because they were below detection limits. It can be reported that galactan and arabinan (%) values in processed solids were generally lower than 0.5. Klason lignin was generally more than 50% of the dry processed solids. Therefore, it can be reported as solubilization of hemicellulose produced processed solids rich in cellulose and lignin. Autohydrolysis at 200 ^{o}C for 0 min and 190 ^{o}C for 30 min resulted higher cellulose (30%) content in processed solids. There was no significant change in cellulose content as due to its firmly packed structure and resistance to autohydrolysis (Ertas, Han, Jameel, & Chang, 2014). Lignin also showed resistance to autohydrolysis, so 85-90% of the initial amount remained in processed solids after autohydrolysis. Processed solids can be valorized as feedstocks for production of other bio-based materials. As the severity factor increased, xylan in processed solids showed a general decrease up to 5.74% of the initial xylan in raw shell (log R_o 4.29, 200 ^{o}C–15 min, respectively), similar to the trends observed in the previous studies (Carvalheiro et al., 2009; Moniz, Pereira, Duarte, & Carvalheiro, 2014; Moniz, Pereira, Quilhó, & Carvalheiro, 2013). The increase in solubilization of xylan as temperature and time increased, showed that hemicellulosic carbohydrates were susceptible to hydrothermal treatments (Aachary & Prapulla, 2011; Carvalheiro et al., 2008, 2009). The change in solubilization of hazelnut shell according to severity factor (log R_o) can also be observed as shown in Figure 5.6. Solubilization increased up to 38.2% of the raw shell as severity factor increased from 2.73 to 4.29. Therefore, a general decrease in processed solid yield was observed (Table 5.4).

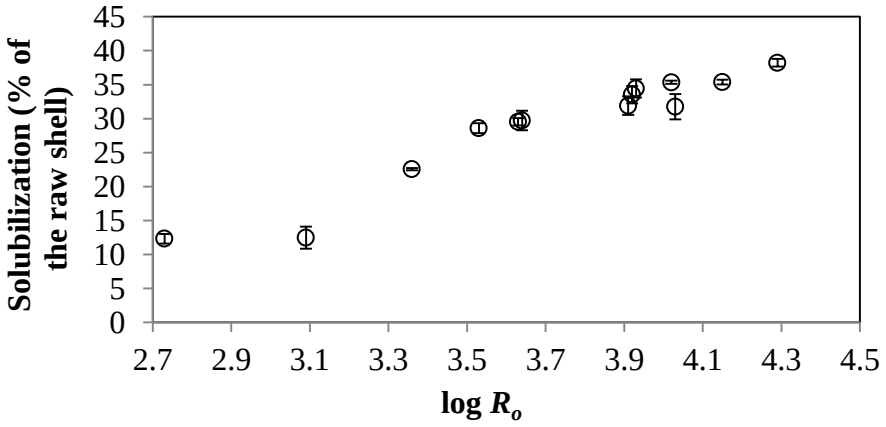

Figure 5.6. The effect of the severity factor (log R_o) on solubilization.

5.3.3. Composition of Autohydrolysis Liquors of Hazelnut Wastes

Autohydrolysis liquors from different agricultural wastes contain monomeric and oligomeric carbohydrates, degradation products such as furfural, HMF, acetic acid and formic acid; and phenolic compounds or other extractives (Carvalheiro et al., 2004). Monosaccharides, oligosaccharides and sugar degradation products in liquors after autohydrolysis of hazelnut wastes were measured. The monosaccharide (glucose, xylose, galactose and arabinose) and oligosaccharide (GOS, GaOS and XOS) concentrations in liquors after autohydrolysis of hazelnut husk and pruning wastes at 190 ^{o}C for 15 min and hazelnut shell for different conditions (150-200 ^{o}C) for 0-45 min were measured by HPLC as presented in Table 5.5.

Glucose could not be detected in liquors obtained after autohydrolysis of pruning wastes at 190 ^{o}C for 15 min and hazelnut shell at higher tempreatures (> 170 ^{o}C). The concentrations of other monosaccharides, such as galactose and arabinose were relatively low and their concentrations were generally lower than 0.5 g/L. The highest xylose concentration was obtained as 2.79 g/L after autohydrolysis of hazelnut shell at 200 ^{o}C for 15 min. The effect of severity factor (log R_o) on xylose; and galactose and arabinose was also observed as shown in Figure 5.7 and 5.8.

According to Table 5.5., XOS was the dominant oligosaccharide in liquors and obtained from autohydrolysis of shell, husk and pruning wastes. GOS and GaOS concentrations in liquors were also determined; however arabinooligosaccharide could not be obtained after autohydrolysis. The effect of severity factor on GOS and GaOS concentrations and yield (%) in liquors from autohydrolysis of hazelnut shell were also researched as presented in Figure 5.9 and 5.10.

Shell was the best hazelnut waste for XOS production due to its higher xylan content as raw material and higher XOS concentration in its liquor (8.7 g/L) after autohydrolysis compared to husk (3.3 g/L) and pruning wastes (7.3 g/L). Therefore, in order to maximize XOS yield and determine optimum temperature and time conditions for production, shell was autohydrolyzed at different conditions and severity factor (2.73-4.29). The concentration of XOS for hazelnut shell changed between 0.16 and 10.15 g/L as severity factor increased.

Table 5.5. Composition of liquors from autohydrolysis of hazelnut wastes.

Biomass	Treatment Conditions			Component (g/L)						
	Temperature (^{o}C)	Time (min)	log R_o	Glucose	Xylose	Galactose	Arabinose	GOS	XOS	GaOS
Husk	190	15	4.02	0.03 ± 0.00	0.07 ± 0.00	1.15 ± 0.12	0.51 ± 0.13	1.72 ± 0.10	3.30 ± 0.10	0.69 ± 0.25
Prunings	190	15	4.02	0.00 ± 0.00	1.26 ± 0.00	0.38 ± 0.01	0.00 ± 0.00	0.71 ± 0.15	7.30 ± 0.10	0.79 ± 0.08
Shell	150	15	2.73	0.03 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.07 ± 0.00	0.04 ± 0.01	0.16 ± 0.01	0.12 ± 0.01
Shell	160	15	3.09	0.03 ± 0.00	0.02 ± 0.00	0.05 ± 0.01	0.20 ± 0.00	0.11 ± 0.03	1.66 ± 0.08	0.35 ± 0.05
Shell	170	15	3.36	0.03 ± 0.00	0.14 ± 0.04	0.09 ± 0.01	0.30 ± 0.01	0.22 ± 0.01	5.40 ± 0.12	0.49 ± 0.03
Shell	180	5	3.53	0.00 ± 0.00	0.28 ± 0.13	0.13 ± 0.02	0.35 ± 0.02	0.16 ± 0.02	8.01 ± 0.78	0.46 ± 0.07
Shell	180	15	3.63	0.00 ± 0.00	0.43 ± 0.08	0.14 ± 0.01	0.38 ± 0.02	0.19 ± 0.01	8.56 ± 0.38	0.52 ± 0.05
Shell	180	30	3.91	0.00 ± 0.00	1.24 ± 0.18	0.28 ± 0.04	0.57 ± 0.01	0.17 ± 0.01	8.77 ± 0.16	0.44 ± 0.00
Shell	180	45	4.03	0.00 ± 0.00	2.41 ± 0.14	0.35 ± 0.00	0.38 ± 0.01	0.12 ± 0.01	8.04 ± 0.05	0.33 ± 0.02
Shell	190	0	3.64	0.00 ± 0.00	0.38 ± 0.11	0.20 ± 0.03	0.44 ± 0.01	0.19 ± 0.01	9.20 ± 0.15	0.68 ± 0.03
Shell	190	5	3.92	0.00 ± 0.00	0.88 ± 0.08	0.27 ± 0.04	0.52 ± 0.03	0.20 ± 0.01	10.1 ± 0.17	0.56 ± 0.03
Shell	190	15	4.02	0.00 ± 0.00	1.88 ± 0.39	0.31 ± 0.01	0.38 ± 0.02	0.15 ± 0.01	8.72 ± 0.34	0.44 ± 0.00
Shell	190	30	4.15	0.00 ± 0.00	2.24 ± 0.27	0.19 ± 0.02	0.28 ± 0.02	0.15 ± 0.01	4.65 ± 0.79	0.00 ± 0.00
Shell	200	0	3.93	0.00 ± 0.00	0.83 ± 0.00	0.22 ± 0.04	0.39 ± 0.01	0.16 ± 0.01	7.89 ± 0.03	0.69 ± 0.03
Shell	200	15	4.29	0.00 ± 0.00	2.79 ± 0.06	0.20 ± 0.02	0.23 ± 0.02	0.13 ± 0.01	3.12 ± 0.81	0.00 ± 0.00

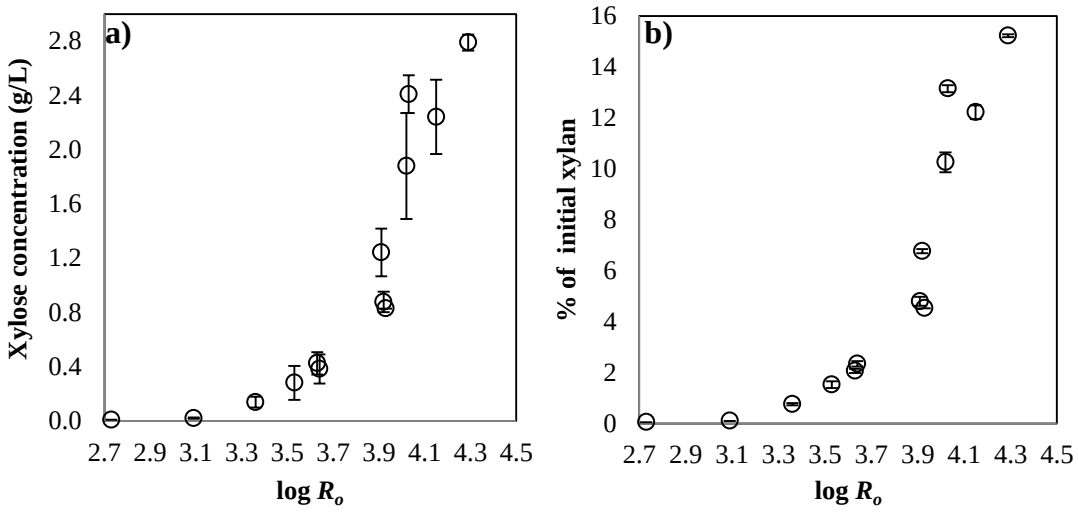

Figure 5.7. The effect of severity factor (log R_o) on a) xylose concentration (g/L) and b) % of initial xylan in liquors from autohydrolysis of hazelnut shell.

Xylose concentration in liquors from autohydrolysis of hazelnut shell increased exponentially as severity factor increased. While the severity factor increased, xylose (% of initial xylan) increased and reached the maximum value, 15.22% for 4.29 (200 ^{o}C-15 min). At medium treatment severity ($3.53 \leq \log Ro \leq 4.03$), where XOS yields were similar (Table 5.5), the effect of severity factor was significant. After autohydrolysis at 190 ^{o}C for 5 min (log R_o=3.92), the conditions which XOS was obtained as the highest yield (%), xylose concentration was 0.88 g/L and theis was 4.1% of the initial xylan (Figure 5.7-b). This value was less than the xylose yields reported for rice straw (Moniz et al., 2014), wheat straw (Carvalheiro et al., 2009), corn straw (Moniz et al., 2013), oil palm fruit brunch fiber (Ho et al., 2014) and brewery's spent grain (Carvalheiro et al., 2004), while close to ones reported for sweet sorghum stems (Sun, Wen, Sun, & Sun, 2015), bamboo culm (Xiao et al., 2013) and olive tree pruning (Cara et al., 2012). When the holding time was increased to 30 min at 190 ^{o}C, in order to maximize low-DP XOS content of the liquor, the xylose concentration increased to 2.24 g/L, which was 10% of the initial xylan. The liquors in which XOS concentration is high and xylose concentration is low can be used for further prebiotic activity tests.

The effect of severity factor on the concentrations of galactose and arabinose was different from xylose. As severity factor increased, galactose and arabinose concentrations showed an increase and reached their maximum values as 0.35 g/L (for 4.03) and 0.57 (for 3.91), respectively and then they decreased sharply. Galactose and arabinose may have been more susceptible to high severity factor values and their

degradation may have started before xylose. On the other hand, the reason of the decrease in xylose concentration while severity factor increased may be due to conversion of XOS into xylose at harder treatment conditions (Garrote & Parajó, 2002; Gullón et al., 2009).

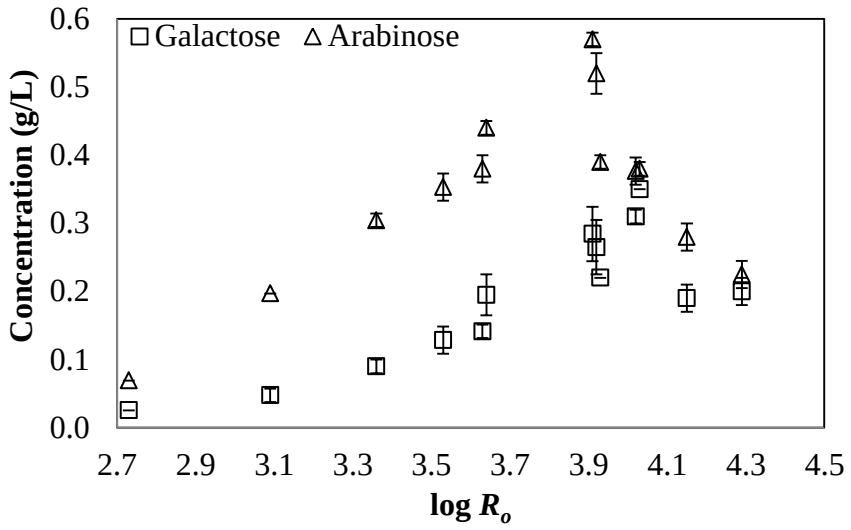

Figure 5.8. The effect of severity factor (log R_o) on galactose and arabinose concentrations (g/L) in liquors from autohydrolysis of hazelnut shell.

GOS, XOS, and GaOS concentrations were calculated as previously reported for other lignocellulosic wastes in literature (Akpinar et al., 2010; Carvalheiro et al., 2009; Gullón et al., 2008; Vázquez, Alonso, Domínguez, & Parajó, 2006; Xiao et al., 2013). A sample from liquor was subjected to quantitative post-hydrolysis (121^{o}C for 60 min, 4% H_2SO_4) and analyzed by HPLC to measure total oligosaccharide concentration. The increase in monosaccharide concentration caused by post-hydrolysis provided a measure of total oligosaccharide concentration. For instance, XOS concentration was calculated as shown in equation 5.3. The difference between monosaccharide concentration in liquor after post-hydrolysis and before was multiplied by anhydro factor, which was 0.88 for xylan and arabinose; and 0.90 for glucose and galactose.

$$\text{XOS} = [(\text{Xylose concentration in liquor after hydrolysis}) - (\text{Xylose concentration in liquor})] * 0.88 \quad (5.3)$$

The effect of severity factor on GOS and GaOS concentrations and yield (%) in liquors of hazelnut shell were determined as presented in Figure 5.9 and 5.10. Actually, oligosaccharides (GOS, GaOS and XOS) showed a similar trend as severity factor increased; however their values were different from each other. Glucose could not be

determined in liquors, thus GOS concentrations in liquors were too low, as expected. It can be reported as autohydrolysis or hydrothermal treatment was not effective on cellulose and production of other oligosaccharides (Ho et al., 2014; Parajó et al., 2004).

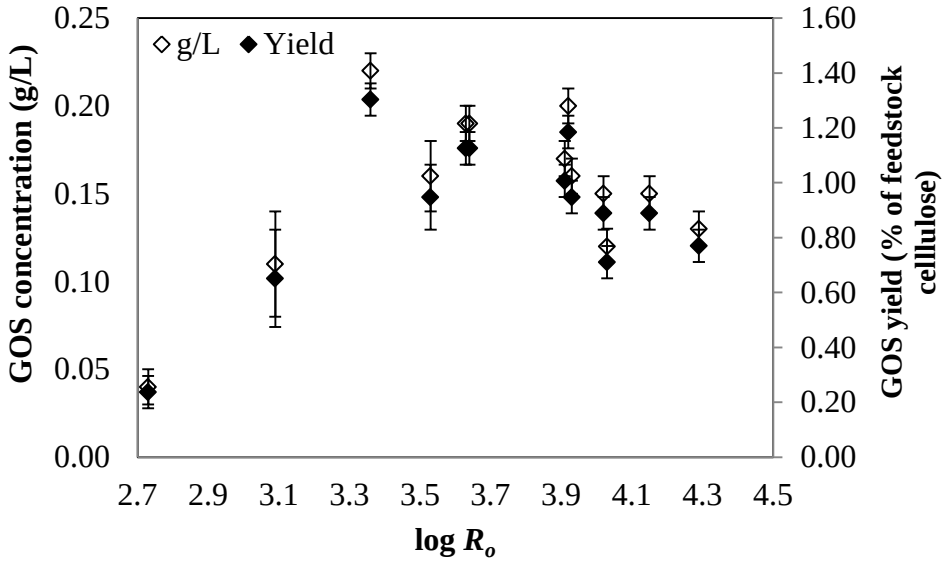

Figure 5.9. The effect of severity factor (log R_o) on GOS concentration and yield (%) in liquors from autohydrolysis of hazelnut shell.

GOS yield was relatively low and the concentrations changed between 0.04 g/L and 0.22 g/L. On the other hand, GaOS concentrations were between 0.12 and 0.69 g/L and the yield reached 47% of the initial galactan at medium conditions (3.93, 200 ^{o}C- 0 min). At higher severity factor values (> 4.15), GaOS was not detected in liquors.

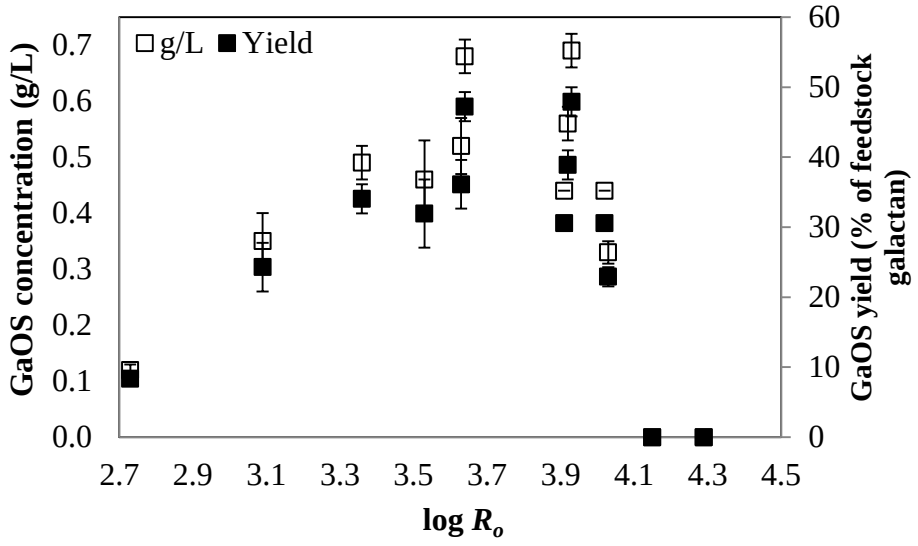

Figure 5.10. The effect of severity factor (log R_o) on GaOS concentration and yield (%) in liquors from autohydrolysis of hazelnut shell.

The change in XOS yield (%) according to different autohydrolysis temperatures (150, 160, 170, 180, 190 and 200 ^{o}C) for the same holding temperature, 15 min was obtained as shown in Figure 5.11. The highest XOS yields (%) were obtained at 180 and 190 ^{o}C. Therefore, the effect of different holding time for 180^{o}C and 190^{o}C on XOS yield (%) was also researched as presented in Figure 5.12.

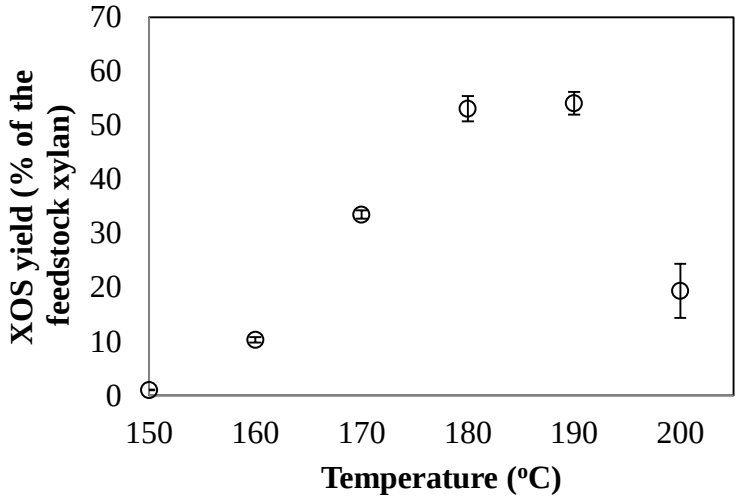

Figure 5.11. The effect of temperature for 15 min on XOS yield.

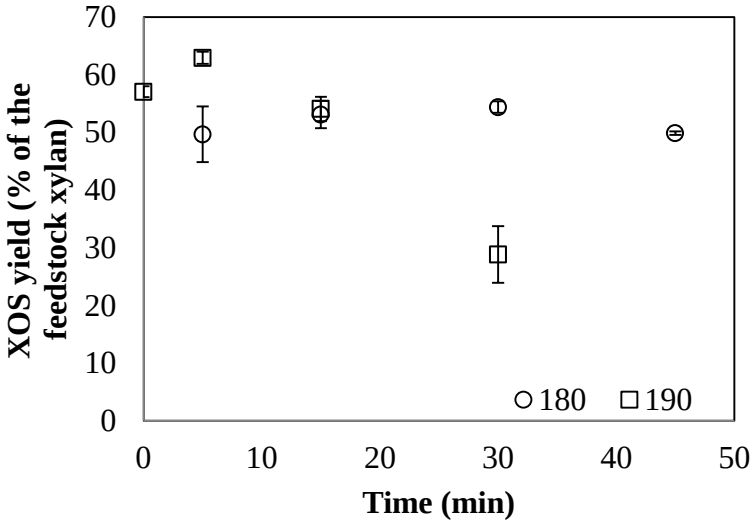

Figure 5.12. The effect of time for 180^{o}C and 190^{o}C on XOS yield.

According to Figure 5.11, for the same holding time (15 min), when temperature increased from 150 ^{o}C to 180 ^{o}C and 190 ^{o}C, there was an increase in XOS yield, but it decreased from 190 ^{o}C to 200 ^{o}C.

The effect of time and temperature on XOS yield was researched individually before using severity factor for combined effect. The effect of holding time on XOS formation was different for 180 ^{o}C and 190 ^{o}C (Figure 5.12). For 180 ^{o}C, there was no

effect of holding time (ranging between 5 and 45 min) on XOS yield. The highest yield values were obtained for 0 and 15 min at 190 ^{o}C; however when the holding time increased to 30 min, there was a certain decrease in XOS yield. Therefore, increase in either temperature or holding time, above the optimum conditions, caused a decrease in XOS yield. That result was consistent with the previous studies, which reported that when temperature was increased, the optimum holding time decreased (Cara et al., 2012; Carvalheiro et al., 2004; Xiao et al., 2013). On the other hand, Ligero, Kolk, Vega, & Dam (2011) obtained maximum XOS yield from *Miscanthus x giganteus* for longer holding times (60 min) and low temperature (160 ^{o}C).

The change in XOS concentration (g/L) and yield (%) in liquor according to severity factor was observed as shown in Figure 5.13. XOS yield showed a parabolic trend as severity factor increased. The highest values were obtained between 3.53 and 4.03. XOS yield showed a continuos increase until 3.92 but then started to decrease and reached its minimum value as 19.34% (log R_o = 4.29) for autohydrolysis at 200^{o}C for 15 min.

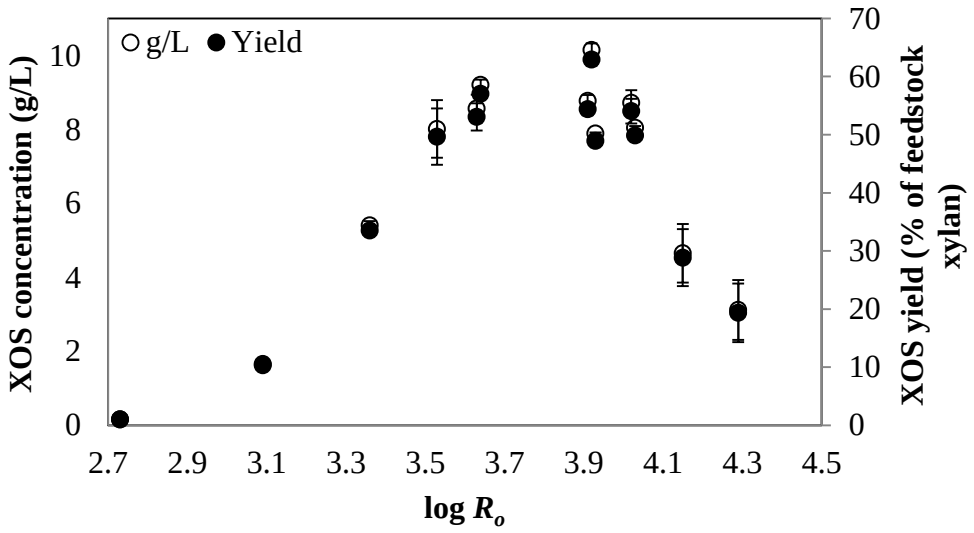

Figure 5.13. The effect of severity factor (log R_o) on XOS concentration and yield (%) in liquors from autohydrolysis of hazelnut shell.

The highest XOS concentration and yield (%) were obtained as 10.1 ± 0.2 g/L and 62.93 ± 0.17% (log R_o=3.92), respectively, from autohydrolysis at 190 ^{o}C for 5 min; and 32.7% of initial xylan did not dissolve and remained in the processed solid. This value was in accordance with other XOS yields (%) obtained from autohydrolysis of different lignocellulosic wastes such as corncobs (60%), almond shells (61.2%), olive stones (53.5%), wheat straw (41.2%), barley straw (47.1%) and rice husk (42.8%) (179

^{o}C-23 min) (Nabarlatz, Ebringerová, et al., 2007), olive tree pruning (55%, 180 ^{o}C-10 min) (Cara et al., 2012), rice husk (64.3%, 205 ^{o}C-0 min) (Vegas et al., 2004), brewer's spent grain (61%, 190 ^{o}C-5 min) (Carvalheiro et al., 2004); oil palm empty fruit bunch fiber (63%, 210 ^{o}C) (Ho et al., 2014).

The XOS yield was low below 3.53 due to low solubility of xylan in the raw material, as it was reported previously.On the other hand, the increase in severity factor to 4.15 and 4.29 decreased the yield, although the solubility of xylan increased. This decrease can be explained by the increase in the by-product such as acetic acid, HMF and furfural formation at high severity factor values since XOS obtained from the feestock xylan can be degraded into xylose and then sugar degradation products (Garrote & Parajó, 2002; Gullón, Pereiro, Alonso, & Parajó, 2009).

According to Figure 5.13, it can be reported as severity factor, which was used in order to research the combined effect of temperature and time, could explain the effecton XOS yield (%), successively. Different time and temperature couples which had similar severity factor values also had close XOS yields. Severity factor has been used by other researchers in literature and the optimum values found in this study are close to their values. Moniz et al. (2013) obtained the highest XOS yields from corn straw between 3.60 and 3.75 and the yield decreased except that range. They also determined a similar trend in autohydrolysis of rice straw in 2014 and 3.59 was reported as the severity factor giving the highest XOS yield (Moniz et al., 2014). The maximum XOS yield from wheat straw was determined at 215 ^{o}C (log R_o=3.96) by Carvalheiro et al. (2009). On the other hand, Ligero et al. (2011) reported the optimum severity factor value as around 3.5 for production of XOS from *Miscanthus x giganteus* at 160 ^{o}C for 60 min, which was a longer holding time compared to the ones used this study.

The DP of XOS obtained after autohydrolysis was also researched since it was effective on fermentability of XOS in the gut. XOS with low-DP or having shorter chain lengths are consumed by the gut bacteria at a faster rate (Gullón, Salazar, et al., 2011; Moura et al., 2008). Low-DP XOS was described as the XOS with DP between 2 and 6 by many researchers (Sun et al., 2015; Xiao et al., 2013). The DP of XOS from autohydrolysis of hazelnut shell at different conditions was determined by HPLC as shown in Figure 5.14.

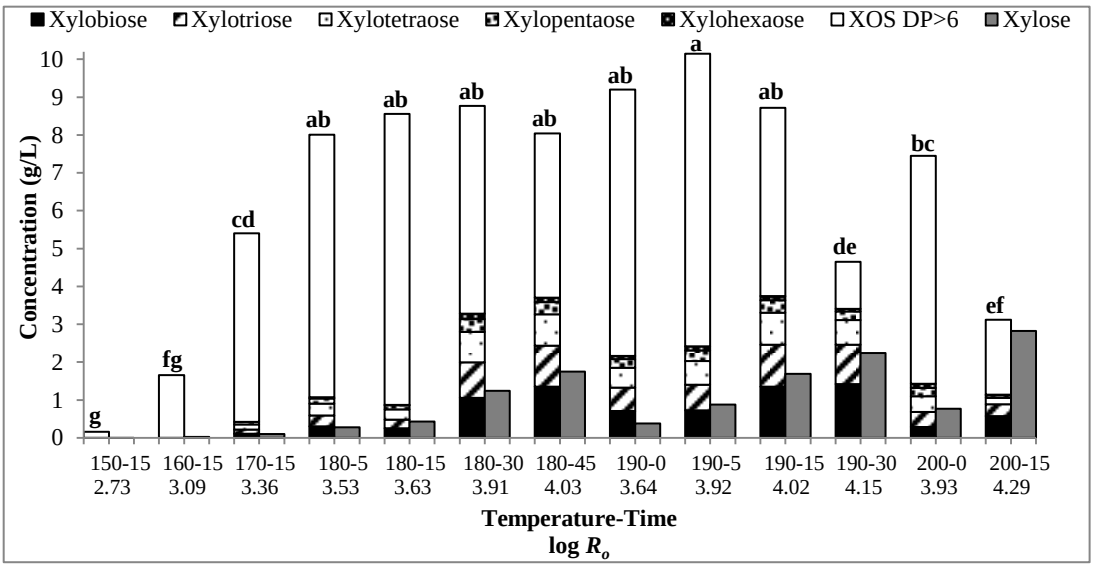

Figure 5.14. The concentration of XOS (g/L) with different DP. Different letters indicate statistically significant difference. ($p < 0.05$, one-way ANOVA and Tukey test)

The liquors were mainly composed of xylobiose, xylotriose and xylotetraose. Low-DP XOS can not be detected at low temperatures such as 150 and 160 ^{o}C. At higher temperatures, except 200 ^{o}C, total low-DP XOS increased as severity factor increased. The effect of severity factor on xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose concentrations in liquors was also researched separately as shown in Figure 5.15. Xylobiose, xylotriose and xylotetraose showed a similar trend to total low DP-XOS; however for log R_o values higher than 4.03, deviations from linearity were observed. The highest low-DP XOS concentrations could not be obtained at the conditions at which the highest total XOS yields were observed. The autohydrolysis at 190 ^{o}C for 0 and 5 min produced the highest total XOS concentrations; however the low-DP XOS concentrations at those conditions were low as 2.16 g/L and 2.42 g/L, respectively. The highest low-DP XOS concentrations (3.70-3.75 g/L, approximately 45% of the total XOS) were measured for 180 ^{o}C for 45 min (log R_o=4.03) and 190 ^{o}C for 15 min (log R_o=4.02); however total XOS concentrations were the higher for 190 ^{o}C for 0 min, 190 ^{o}C for 5 min and 180 ^{o}C for 30 min. Lower amount of high DP XOS (DP>6), but less low-DP XOS (3.41 g/L) was obtained in liquor after autohydrolysis at 190 ^{o}C for 30 min. Therefore, the highest low-DP XOS%, 73%, was determined at these conditions. Xylobiose, xylotriose and xylotetraose fractions were 30%, 22% and 14% of the total low-DP XOS, respectively. Major fraction was generally high DP XOS for other treatments.

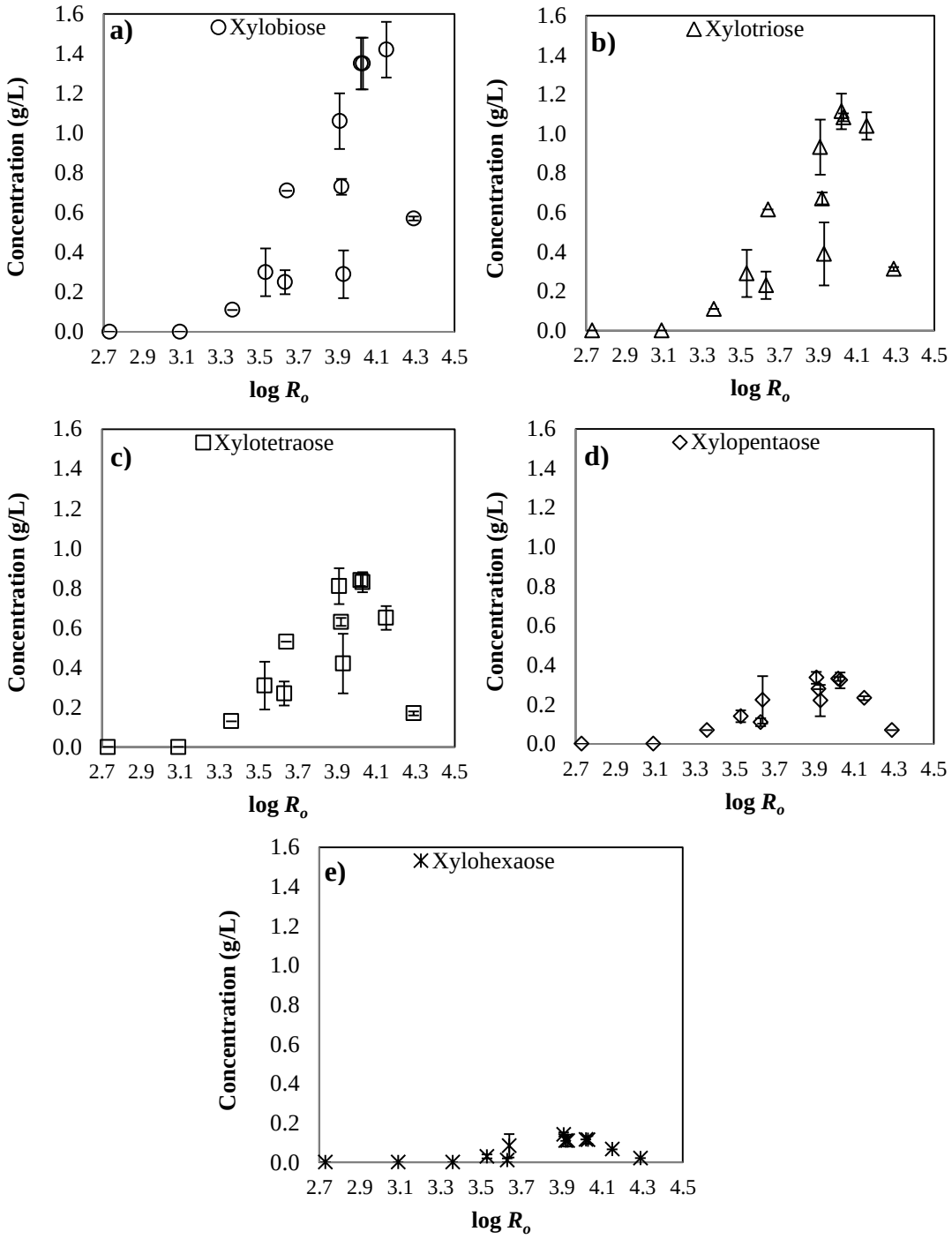

Figure 5.15. The effect of severity factor (log R_o) on the (a) xylobiose, (b) xylotriose, (c) xylotetraose, (d) xylopentaose and (e) xylohexaose concentrations in liquors from autohydrolysis of hazelnut shell.

When the effect of conditions on low-DP XOS yield was researched for the same holding time for different temperatures, it can be claimed that low-DP XOS% increased as the time generally increased for 180 ^{o}C, 190 ^{o}C and 200 ^{o}C. At 180 ^{o}C, although there was no significant difference in total XOS concentrations for different

times ($p>0.05$), lower low-DP XOS concentrations were obtained for 5 and 15 min then 30 and 45 (Figure 5.14). On the other hand, this effect was not certain for 190 ^{o}C as 180. Carvalheiro et al. (2004) reported a similar effect for autohydrolysis of brewery's spent grain. According to their results, DP of XOS mostly decreased when the holding time increased; however the highest total XOS concentration was obtained at moderate holding times, as observed in this study. For autohydrolysis of mixed herbs and sunflower seed shells, the fraction of low-DP XOS increased with reaction times (Gullón et al., 2009). Sun et al. (2015) and Xiao et al. (2013) also obtained increased low-DP XOS generation at higher temperatures for sweet sorghum stems and bamboo culm, respectively.

In order to increase low-DP XOS concentration, liquor can be enzymatically hydrolyzed to xylooligomers between xylobiose (X2) and xylohexose (X6).

Formic acid was not detected in liquors. The highest pH value was 4.25 for liquor from 150 ^{o}C-15 min. It showed lower values between 3.44 and 4.02 for other conditions due to probably the increase in acetic acid concentration during autohydrolysis. Acetic acid concentrations of post-hyrolyzed liquors, which were obtained after post-hydrolysis (121^{o}C for 60 min, 4% H_2SO_4) of liquor, were also calculated. XOS obtained after autohydrolysis was partly acetylated as xylan in the raw material. Under the conditions giving higher XOS yield, molar ratio of acetyl groups to xylose in the XOS structure was generally between 0.25 and 0.35. Some of the acetyls of the raw material xylan can remain in the XOS obtained after autohydrolysis (Garrote, Domínguez, & Parajó, 1999; Ho et al., 2014; Nabarlatz et al., 2005; Xiao et al., 2013). The degree of acetylation is important since it can influence the solubility and the prebiotic activity of XOS. Kabel, Linda Kortenoeven, Henk A. Schols, & Voragen (2002) reported that in vitro fermentation model of acetylated and non-acetlylated XOS by the fecal inocula was different for their short chain fatty acid and lactate formations. On the other hand, acetyl groups in the xylan structure can be removed by extraction with alkali solution due to saponification of ester bonds (Nabarlatz et al., 2005).

The effect of severity factor (log R_o) on the liquor pH and the by-product concentration was observed as presented in Figure 5.16. Acetic acid was the main by-product and it showed an exponential increase as severity factor increased, thus lower pH values were recorded as log R_o increased. The conditions at which the highest XOS yield was obtained ($3.53 \leq \log R_o \leq 4.03$), the concentration of acetic acid was between 0.5 g/L and 1.5 g/L. When the severity factor increased up to 4.29, the concentration

decreased to 2.78 g/L. These results were in accordance with other studies reported for autohydrolysis of almond shells (Nabarlatz et al., 2005), brewery's spent grain (Carvalheiro et al., 2004), bamboo culm (Xiao et al., 2013) and sweet sorghum stems (Sun et al., 2015). Acetyl groups bonded to the xylan structure by ester bonds are released during autohydrolysis and acetic acid concentration in the reaction medium increases. Acetic acid in the medium can act as a catalyst and help the hydrolysis of xylan into XOS. Although it is good for the production of XOS, its presence is undesirable due to being toxic to microorganisms and inhibiting their prebiotic activity. Before autohydrolysis, pH of the hazelnut waste-water mixture was mesaured as 4.91, 5.33 and 5.41 for shell, husk and pruning wastes, respectively. After autohydrolysis, the generation of acetic acid in the medium reduced pH of the all liquor samples as expected due to formation of acetic acid. The values decreased to 3.67, 4.15 and 3.60 for shell, husk and pruning wastes, respectively.

The concentration of furfural, which was formed by dehydration of pentoses, was between 0.01 and 0.95 g/L and followed an exponential trend similar to xylose and acetic acid. HMF, which was formed by dehydration of hexoses, levels were relatively low (<0.1 g/L) and it also showed an exponential increase as log R_o increased.

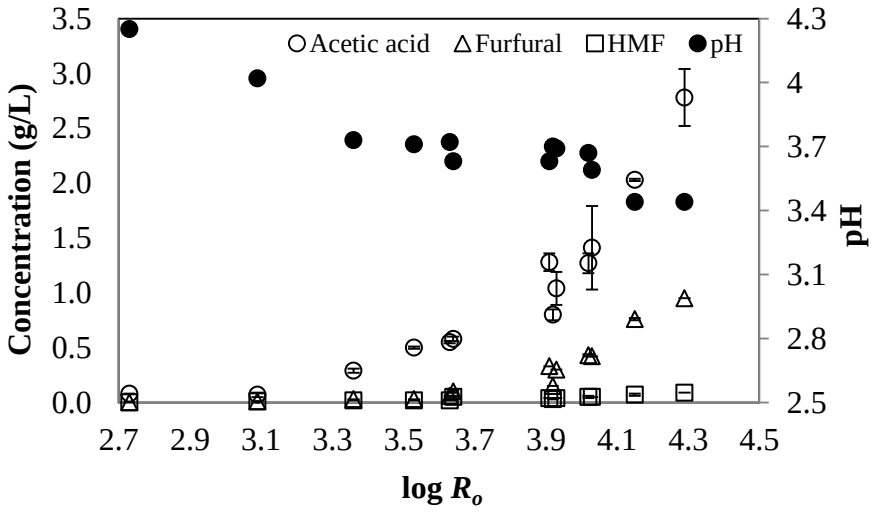

Figure 5.16. The effect of severity factor (log R_o) on the liquor pH and by-product concentrations.

The color of autohydrolysis liquors was measured between 1.08 ± 0.25 (190 ^oC for 15 min) and 3.76 ± 0.19 (170 ^oC for 15 min) Abdorbance (Abs) as shown in Figure 5.17. There was an increase in absorbance values until 170 ^oC (3.36); but then it started to decrease and reached to 1.35 Abs at 3.64. There was no important change while log R_o value increased from 3.64 to 4.29 (200 ^oC for 15 min). Therefore, it can reported as

increasing temperature or severity factor at lower values (150-170 ^{o}C) was effective on Abs of liquors; however for higher R_o values, there was no effect.

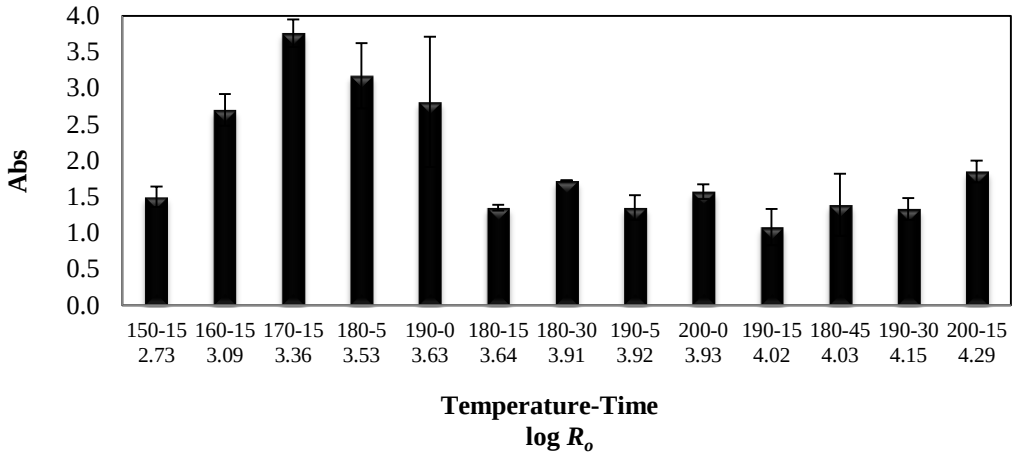

Figure 5.17. The color of liquors from autohydrolysis of hazelnut shell.

5.3.4. Total Phenolic Content and Total Antioxidant Activity of Liquors

Total phenolic content (TPC) and total antioxidant activity (TAA) of hazelnut waste extracts extracted by different solvents (water, 80% methanol-water and 80% acetone-water) were determined as presented in Table 5.6 and Table 5.7, respectively. Xu et al. (2012) determined TPC of hazelnut shell extracts (extracted by 80% acetone-water) as 4.5-14.5 mg tannic acid equivalents/g hazelnut shell, which was consistent with this study; and TAA as 35.7-169.7 μmol Troloks/g hazelnut shell. Alasalvar et al. (2009) reported in a study, in which they investigated TAA of hazelnut kernel phenolics, that extraction with 80% methanol-water and 80% acetone-water were effective solvents in order to extract phenolic compounds from hazelnut wastes. Similar to their research, hazelnut shell extract obtained from extraction with 80% acetone-water had the highest TPC and TAA.

Table 5.6. Total phenolic content (TPC) of hazelnut wastes extracted with different solvents.

Solvent	**TPC (mg GAE/100 g raw material)**		
	Shell	**Husk**	**Prunings**
Water	158.8 ± 25.6	1274.7 ± 70.8	302.8 ± 26.8
80% Methanol-water	407.8 ± 32.9	1208 ± 24.9	950.6 ± 15.1
80% Acetone-water	666.6 ± 26.6	1683.7 ± 41.2	1354.9 ± 50.3

Table 5.7. Total antioxidant activity (TAA) of hazelnut wastes extracted with different solvents.

Solvent	TAA (mg TEAC/100 g raw material)		
	Shell	**Husk**	**Prunings**
Water	2077.4 ± 148.2	15720.5 ± 432.8	7011.9 ± 165.9
80% Methanol-water	1148 ± 97.7	3305.8 ± 88.3	3467.7 ± 103.8
80% Acetone-water	2674.3 ± 7.2	5622 ± 164.2	5167.7 ± 191.5

TPC of the liquors of hazelnut husk and pruning wastes from autohydrolysis at 190 ^{o}C for 15 min were 1.25 (1364) and 1.31 mg GAE/mL (1394 mg GAE/100g dry raw material), respectively. TAA of those liquors were 12.28 (13455) and 9.67 mg GAE/mL (10310 mg GAE/100g dry raw material), respectively. In this study, the effects of hydrothermal treatment on TPC or TAA of autohydrolysis liquors of hazelnut wastes were investigated for the first time. The effect of severity factor on TPC and TAA of liquors obtained after hydrothermal treatment was observed (in Figure 5.18).

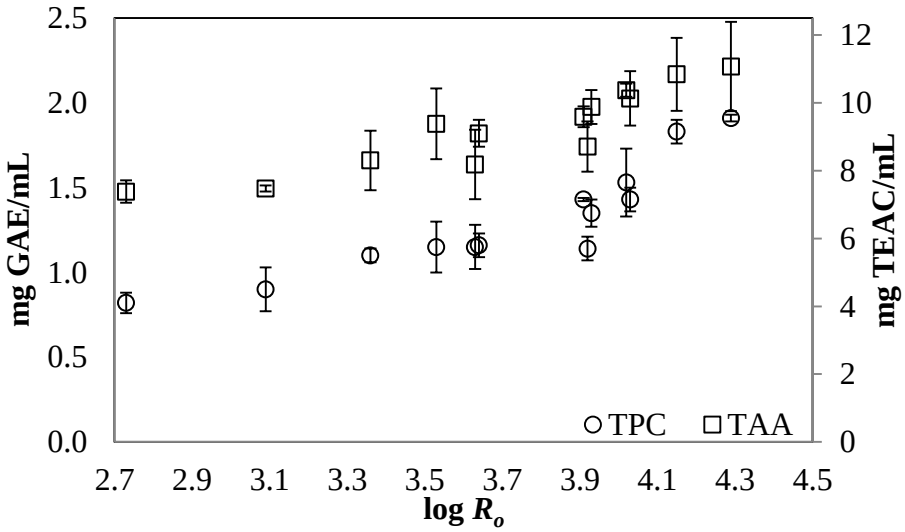

Figure 5.18. The effect of severity factor (log R_o) on total phenolic content (TPC) and total antioxidant activity (TAA) of liquors from autohydrolysis of hazelnut shell.

TPC and TAA of liquors increased from 0.82 to 1.91 mg GAE/mL (905.3-2115.7 mg GAE/100 g hazelnut shell) and 7.38 to 11.07 mg TEAC/mL (8163.9-12261.5 mg TEAC/100 g hazelnut shell) as severity factor increased from 2.73 to 4.29. Increasing temperature and time were effective on increasing TPC and TAA of liquors. Autohydrolysis is a technology leading to liquor including fractions derived from the extractives and compounds derived from acid-soluble lignin (Conde et al., 2009). Lignin and phenolic compounds produced during the fractionation can be considered as

high added-value compounds (Amendola et al., 2012). Extracts with activity comparable to that of synthetic antioxidants can be obtained by extraction of liquor with ethyl acetate (Conde et al., 2009). Two-step extraction process, consisting of an autohydrolysis pretreatment followed by ethanol organosolv process can be applied for selectively separating phenolic compounds and lignin (Amendola et al., 2012).

Amendola et al. (2012) determined TPC of liquor from autohydrolysis of grape stalk at 180 ^{o}C for 30 min as 2.54 g GAE/L. For the same conditions, it was differently 1.35 g GAE/L for hazelnut shell in this study. Conde et al. (2009) reported that higher temperatures of hydrothermal treatment of olive tree prunings increased the release of phenolic compounds in liquors as observed in this study. They found TPC of liquors from autohydrolysis at 170, 190, 210 and 230 ^{o}C for 10 min as 1460, 1760, 2030 and 2290 mg GAE/100 g pruning wastes. Similar to Conde et al. (2009), it was determined as 1219, 1697 and 2115 mg GAE/100 g hazelnut shell in liquors of hazelnut shell from autohydrolysis at 170, 190 ve 200 ^{o}C for 15 min.

5.4. Conclusions

Hazelnut wastes such as shell, husk and prunings were characterized and evaluated for XOS production by autohydrolysis. Autohydrolysis was applied to hazelnut wastes at 190 ^{o}C for 15 min. Shell contained the highest amount of xylan and higher XOS yield was obtained compared to other hazelnut wastes. Therefore, the effect of autohydrolysis conditions such as temperature (150-200 ^{o}C) and holding time (0-45 min) on XOS and by-product formation from shell were evaluated. In order to observe the combined effect of temperature and time, severity factor was calculated for each treatment. The temperature and holding time were effective on composition of the liquor and the processed solids. As severity factor increased, solubilization of hazelnut shell showed a general increase. Solubilization of xylan enhanced the cellulose and lignin contents in processed solids. The maximum total XOS yields were obtained at moderate treatment conditions (at 190 ^{o}C for 5 min, 62% of the original xylan). On the other hand, maximum low-DP XOS yield was obtained at higher severity factor values. At 190 ^{o}C, 30 min of holding time was required to maximize low-DP XOS yield. The results showed that autohydrolysis temperature and holding time were also effective on DP of XOS generated. The severity factor was successful in describing the trends of

oligosaccharides (GOS, GaOS and XOS), monosaccharides (xylose, galactose and arabinose), by-products (acetic acid, furfural, HMF) and phenolic compounds in liquors after autohydrolysis. The concentration of by-products in liquor showed an exponential increase as severity factor increased.

CHAPTER 6

ALKALI EXTRACTION OF XYLAN FROM HAZELNUT WASTES

6.1. Introduction

Alternative to autohydrolysis, XOS can also be produced using combined chemical-enzymatic methods. Xylan is extracted from lignocellulosic biomass with an alkali solution such as NaOH, KOH, $Ca(OH)_2$, ammonia or a mixture of these, and then xylan is hydrolyzed to XOS by xylanase (having low exo-xylanase or β-xylosidase activity) (Aachary & Prapulla, 2011; Akpinar, Ak, Kavas, Bakir, & Yilmaz, 2007; Vázquez, Alonso, Domínguez, & Parajó, 2001b).

Many agricultural wastes have been used to extract xylan by various methods as presented in Table 6.1; and 90% of the xylan in the raw material was recovered by alkali extraction with steam treatment (Samanta et al., 2015). The recovery of original xylan can be different according to type and concentration of alkali, the presence of lignin, hydrogen bonds and cellulose contents (Doner & Hicks, 1997). This method is more desirable than autohydrolysis due to not requiring a reactor working at high pressure and temperatures., not producing unwanted by-products or high level monosaccharides (Aachary & Prapulla, 2011). On the other hand, alkali extraction is more hazardous to environment due to using chemicals. The xylan-lignin complex structure makes xylan to resistant to hydrolysis, thus XOS production has to be performed in two sequential processes: alkali extraction of xylan from agricultural waste and enzymatic or acidic hydrolysis of xylan (Akpinar, Erdogan, & Bostanci, 2009).

Xylan is the major component of hemicellulose fraction (20-35% of the lignocellosic biomass) and the precursor of XOS. Hemicellulose is associated with cellulose and lignin and the bonding between cellulose, hemicelluloe and lignin is different. Cellulose and hemicellulose or lignin molecules are linked via hydrogen bonds and in addition to the hydrogen bond, there is covalent bonding between hemicellulose and lignin (Chen, 2014; Jayapal et al., 2013). Alkali treatments are based

on the saponification of ester bonds between lignin and hemicelluloses. They decompose the cell wall of lignocellulosic biomass by dissolving lignin and hemicellulose and breaking the ester linkages between hemicellulose and lignin (Qing et al., 2013). Acetyl groups and uronic acids in the xylan structure are released by saponification during extraction. Alkali processing of xylan-containing materials is supported by the pH stability of this polymer, and solubilized xylan and xylan degradation products can be recovered by precipitation with organic compounds (including acids, alcohols or ketones). DP of the recovered xylan can be reduced by hydrolysis with xylanases (Vázquez et al., 2001b).

In order to increase the yield of the hemicellulose extraction, delignification is performed to break the strong interaction between hemicellulose and lignin. The lignocellulosic biomass can be treated with salts, alcholos or oxidizing agents to remove lignin before alkali extraction. Delignification with acidified sodium chlorite or alkali extraction with H_2O_2 is the method that can be used to perform delignification more efficiently (Rabetafika, Bchir, Blecker, Paquot, & Wathelet, 2014; Vázquez et al., 2001b).

On the other hand, one-step method is more desirable for industrial production. In contrast to autohydrolysis, alkali treatment is more hazardous to environment due to using chemicals (Rabetafika et al., 2014).

The purposes of the alkali extraction experiments of hazelnut wastes were to determine the effect of alkali extraction on XOS yield, investigate the possibility of having XOS yield more than obtained from autohydrolysis.

Table 6.1. Extraction of xylan from different agricultural wastes with different methods (Source: Samanta et al., 2015).

Raw material	Extraction with	Yield	Reference
Cotton stalks	24% KOH + 1% $NaBH_4$	0.4g/2 g of cotton stalks	(Akpinar et al., 2007)
Corn cobs	12% NaOH with steam	83% of original xylan	(Samanta, 2012)
Corn stalks	10% NaOH 20 ^oC	54% of original xylan	(Egüés et al., 2012)
Wheat straw	0.5 M NaOH at 55 ^oC	49.3% of original xylan	(Ruzene, Silva, Vicente, Goncalves, & Teixeira, 2008)
Poplar wood	NaOH coupled with sonication	75.5% of original xylan	(Yuan, Xu, He, & Sun, 2010)
Sugarcane bagasse	3% NaOH at 50 ^oC	74.9% of original xylan	(Peng et al., 2009)
	12% NaOH coupled with steam	85% of original xylan	(Jayapal et al., 2013)
	Two stage extraction with water and 1% NaOH	49.5% of original xylan	(Peng et al., 2010)
Tobacco stalks	24% KOH + 1% $NaBH_4$	21.8% of total dried raw materials	(Akpinar et al., 2009)
Sunflower stalks	24% KOH + 1% $NaBH_4$	18.9% of total dried raw materials	(Akpinar et al., 2009)
Wheat straw	24% KOH + 1% $NaBH_4$	20.6% of total dried raw materials	(Akpinar et al., 2009)
Natural grass	12% NaOH coupled with steam	98% of original xylan	(Samanta, et al., 2012)
Pigeon pea stalks	12% NaOH coupled with steam	96% of original xylan	(Samanta et al., 2013)
Green coconut husks	4% NaOH coupled with steam	84% of original xylan	(Jayapal et al., 2014)

6.2. Materials and Methods

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany) and Merck Millipore (Darmstadt, Germany). Ethanol used in alkali extraction was purchased from (Tekkim, Turkey). Ground hazelnut shell and pruning wastes were used for alkali extraction of xylan.

6.2.1. Alkali Extraction

Wax, chlorophyll and other extractable components in lignocellulosic biomass were separated by Soxhlet apparatus using ultra pure water and ethanol 96%. After drying at 60 ^{o}C, biomass was used for alkali extraction. Alkali extraction of hazelnut wastes was performed by using different alkali solutions with different concentrations for different extraction times.

The method described by Zilliox & Debeire (1998) for xylan extraction from wheat straw was used for alkali extraction of xylan from hazelnut wastes with some modifications. This method was also used in several studies for alkali extraction of xylan from such as cotton stalk (Akpinar et al., 2007), tobacco stalk, sunflower stalk, wheat straw (Akpinar et al., 2009) and corn cobs (Bahcegul et al., 2013). Firstly, 2 g of hazelnut waste was kept in distilled water at 60 °C for 16 h for the process which was named as swelling. After separation the pellet by centrifugation at 4800 g for 15 min, the swollen sample was extracted in a 17 mL solution containing 24% KOH and 1% $NaBH_4$ (w/v) in an incubator shaker (ZHWY-200B, Germany) at 120 rpm and room temperature for 3 h. At the end of the alkali treatment, liquid (hemicellulose and lignin) and solid (cellulose) phases were separated by vacuum filtration. After adjusting pH of the filtrate to 5 by HCl, 2 volumes of cold ethanol (96%) was added to precipitate xylan. After centrifugation at 4 ^{o}C for 10 min at 4800 g, the precipitated solid was dried overnight in an oven at 60 ^{o}C. The dry solid was weighed and dissolved in 10 mL distilled water. The mixture was hydrolyzed by 4% (w/v) H_2SO_4 at 121 ^{o}C for 1 h in order to determine the amount of extracted xylan. The xylan concentration of the precipitated solid was calculated by measuring the amount of xylose released after hydrolysis by HPLC as mentioned in previous chapter. The same alkali extraction procedure was performed with 10% KOH instead of 24% KOH.

Alkali extraction of hazelnut wastes was also carried out without swelling and using NaOH solutions with different concentrations as described by other researchers, previously (Bian et al., 2013; Chapla, Pandit, & Shah, 2012a; Faryar et al., 2015; Jayapal et al., 2013; Rabetafika et al., 2014). After mixing the biomass with 5 or 10% NaOH (w/v) solution at a solid:liquid ratio 1:10, the mixture was stirred at 120 rpm at room temperature in the incubator shaker for 16 h. The separation of phases and precipitation of the solid were carried out as explained above. The xylan concentration of the precipitated solids and the xylose concentration of the filtrated liquor were determined by HPLC. Additionally, pruning wastes were extracted with 1% and 2% NaOH (w/v) solutions for 16 h.

6.3. Results and Discussion

After Soxhlet extraction, hazelnut shells were treated with alkali solutions listed in Table 6.2 and the amount of precipitated solids were calculated. The xylan content of hazelnut shell was 18.7%. Although the highest precipitated solid was obtained with 10% KOH ± 1% $NaBH_4$, it was 8.03% of the raw hazelnut shell. The amount of xylan in the precipitated solid from this treatment was 18.9% and xylan concentration in liquor was 0.39 g/L (2% of the initial xylan). Increasing alkali concentration (NaOH or KOH) caused a negative effect on extracton yield.

Table 6.2. Alkali extraction of xylan in hazelnut shell.

Alkali Solutions	**Time (h)**	**The amount of precipitated solid (% of raw hazelnut shell)**
5% NaOH	16	6.09 ± 0.29
10% NaOH	16	5.45 ± 0.73
10% KOH + 1% NaH_4	3	8.03 ± 0.23
24% KOH +1% NaH_4	3	6.12 ± 0.12

Xylan in hazelnut pruning wastes was extracted with the same alkali extraction methods and also using NaOH concentrations lower than 5% (1% and 2%) as listed in Table 6.3. The xylan content of the hazelnut pruning wastes was 12.8%. The highest xylan% in precipitated solid was 39.6% and this value was 27.1% of initial xylan. The highest xylan content in solids was obtained from alkali extraction with 5% NaOH for 16 h.

Table 6.3. Alkali extraction of xylan in hazelnut pruning wastes.

Alkali Solutions	Time (h)	The amount of precipitated solid (% of raw material)	Xylan% in precipitated solid	Recovery% of initial xylan
1% NaOH	16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2% NaOH	16	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5% NaOH	16	9.90 ± 1.05	39.6 ± 2.70	27.1 ± 2.14
10% NaOH	16	13.1 ± 0.13	22.3 ± 0.28	18.4 ± 1.73
24% KOH +1% NaH_4*	3	19.16	4.46	14.8*

*This was an unrepeated experiment.

According to analysis of the precipitated solids from alkali extraction, the highest xylan recovery was obtained by alkali extraction of hazelnut pruning wastes with 5% (w/v) NaOH as 27.1%. Lower than 5% NaOH concentration was not enough to extract xylan in hazelnut pruning wastes, efficiently. The results of extraction with 24% KOH + 1%$NaBH_4$ could not be expressed as mean ± SD since the xylose concentration in the precipitated solid was too low and it was not detected in one of the samples. Increasing alkali (NaOH or KOH) concentration decreased the extraction yield, thus extraction with 5% NaOH was concluded to be the most suitable method to recover xylan in hazelnut pruning wastes.

Zilliox & Debeire (1998) recovered 23% of the xylan in wheat straw by alkali extraction with 24% KOH + 1% $NaBH_4$ for 3 h. Although their initial xylan content was 30.9% (Zilliox & Debeire, 1998) and higher compared to hazelnut pruning wastes in this study (12.8%), higher recovery (27.1%) was obtained by extraction with only 5% NaOH for 16 h. Moreover, Faryar et al. (2015) extracted 4.25 g xylan from 50 g wheat straw (including 20.1% xylan) with 2% NaOH at 80 ^{o}C for 90 min. The xylose content of the precipitated xylan was 55.3%, thus 23.5% of the initial xylan was recovered with lower alkali concentration in a shorter extraction time (Faryar et al., 2015).

On the other hand, recovery of initial xylan from hazelnut wastes was lower than recovery of xylan from alkali extraction of other lignocellulosic biomass in literature. Bahcegul et al. (2013) extracted arabinoglucoxylan with 24% KOH + 1% $NaBH_4$ as 29% of the initial corn cob. The xylose and arabinose content of the extracted arabinoglucoxylan was calculated as 65% and 15%, respectively. Akpinar et al. (2007) extracted 0.4 g solids from 2 g of cotton stalk containing 21.42% xylan with the same method. The xylose content of the extracted cotton stalk xylan was 83.6%. Akpinar et al. (2009) applied the same extraction method to tobacco stalk, cotton stalk, sunflower stalk and wheat straw including 21.8, 21.3, 18.9 ve 20.6 % xylan, respectively. The

xylose content of the extracted xylan was between 77-87%. The extracted xylan was about 20% of the total weight, thus they recovered 78.5% of the inital xylan.

Jayapal et al. (2013) extracted xylan from sugarcane bagasse in which they reported 23.2% hemicellulose, by NaOH or KOH at different concentrations (2, 4, 8 and 12%) and 25 ^{o}C for 16 h. The xylan dissolved in alkali solution was precipitated by 3 volume cool 95% ethanol solution and it was dried at 60 ^{o}C. Contrary to this study, the increase in alkali concentration, efficiently increased the extraction of xylan for both of two alkali solution. The precipitated solid ratio was 11.91% and 8.87% after extraction with 12% NaOH and KOH, respectively. Recovery of xylan was 7-38% for KOH; however it was 9-51% for NaOH (Jayapal et al., 2013).

Bian et al. (2013) extracted hemicellulose containing 83.1% xylose from sugarcane bagasse by 10% KOH at 25 ^{o}C for 10 h (Bian et al., 2013).

Rabetafika, Bchir, Blecker, Paquot, & Wathelet (2014) extracted pear pomace by 4 M NaOH at 60 ^{o}C for 18 h. The initial xylan ratio was 20.2%. They recovered 61.7% of the inital xylan. They were also able to increase the yield to 80.2% and 94.5% by using sodium chlorite or hydrogen peroxide, respectively (Rabetafika et al., 2014).

Chapla, Pandit, & Shah (2012) extracted corn cob by 1.25 M NaOH for 3 h. They indicated that they extracted 17.9% of the total weight as xylan; however they did not analyze the precipitated xylan and determine the its xylose content (Chapla et al., 2012)

6.4. Conclusions

The xylan recovery by alkali extraction of hazelnut shell was 18.9%; however 62.9% of the original xylan was recovered in liquor after autohydrolysis at 190 ^{o}C for 5 min (Figure 5.13). Therefore, alkali extraction was not enough to extract xylan from hazelnut wastes as efficiently as autohydrolysis. Therefore, autohydrolysis liquor of hazelnut shell was used for enzymatic hydrolysis to produce XOS with low DP in the next step of the study.

Alkali extraction can be a better method for extraction of xylan from lignocellulosic biomass with higher xylan content. The efficiency of alkali extraction depends on type and concentration of the alkali and extraction time. If the combination effects of those parameters are considered, the extraction yield may be increased.

CHAPTER 7

ENZYMATIC HYDROLYSIS OF AUTOHYDROLYSIS LIQUOR

7.1. Introduction

Enzymatic hydrolysis is one of the methods to produce oligosaccharides for the food industry. Thermal processes produce undesirable by-products and high level of monosaccharides; while there are no by-products formation or need for special equipment working at high pressure and temperatures in enzymatic hydrolysis (Aachary & Prapulla, 2011; Qing et al., 2013). On the other hand, a longer time than other treatments is needed to complete hydrolysis. Furthermore, undesirable end-products can be generated by hydrolysis with different type of xylanases and production of xylooligosaccharides (XOS) with desired degree of polymerization (DP) becomes more difficult (Qing et al., 2013).

Extracted xylan from lignocellulosic biomass with alkali or any other methods such as autohydrolysis is hydrolyzed into XOS by xylanases. Endo-xylanase is responsible for the degradation of xylan by hydrolyzing β-1,4 bonds on the main chain of xylan between xylose units and produces XOS with low-DP (Aachary & Prapulla, 2011; Carvalho et al., 2013; Qing et al., 2013). Low-DP XOS is hydrolyzed into xylose with β-xylosidase because it acts on the non-reducing end of xylobiose or other oligomers. Therefore, XOS production can be maximized and xylose production can be minimized by using low exo-xylanase and/or β -xylosidase activity. Synergistic effect of different enzymes can be beneficial to complete the hydrolysis of the xylan structure (Qing et al., 2013). The structure of xylan and site of action of the enzymes of the xylanase complex are shown in Figure 7.1.

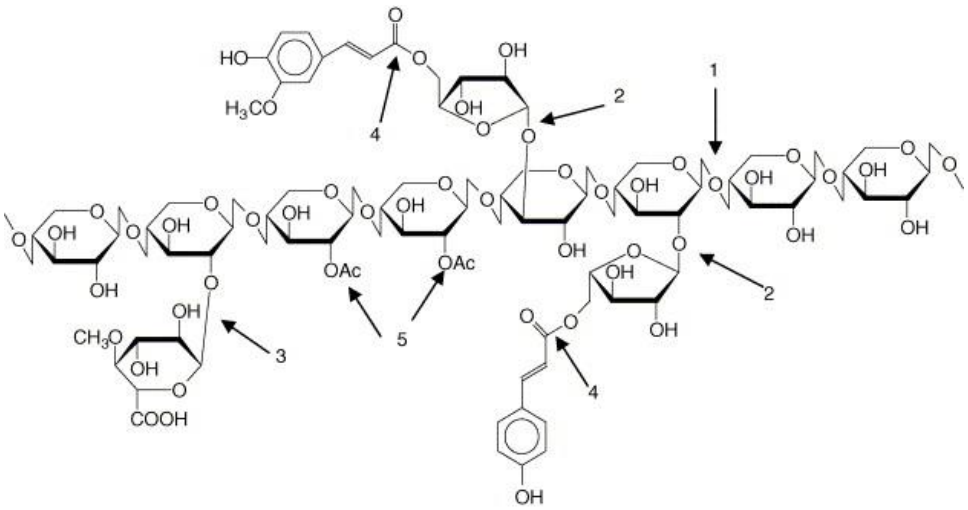

Figure 7.1. The structure of xylan and site of action of the enzymes of the xylanase complex. 1: endoxylanases; 2: α-L-arabinofuranosidases; 3: glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases (Source: Chávez, Bull, & Eyzaguirre, 2006).

Although XOS has been produced by enzymatic hydrolysis from many feedstock, such as oat spelt, beech wood, birchwood, corncob, wheat straw and hardwood (Aachary & Prapulla, 2011); this was the first study about production of XOS from hazelnut wastes by enzymatic hydrolysis.

XOS with low-DP are used by probiotic bacteria, thus enzymatic hydrolysis or acid treatment can be used after autohydrolyisis, in order to obtain liquors with low DP-XOS (Akpinar et al., 2010). In this study, XOS in autohydrolysis liquor was enzymatically hydrolyzed into XOS with low DP, since higher amounts of the original xylan in hazelnut shell was recovered by autohydrolysis compared to alkali extraction. The aim of enzymatic hydrolysis was to reduce DP of XOS from autohydrolysis and convert XOS to a form that can be efficiently used as a prebiotic by beneficial bacteria in the human gut.

7.2. Materials and Methods

Accellerase XY (Accessory Xylanase Enzyme Complex for Biomass Hydrolysis, produced with a genetically modified strain of *Trichoderma reesei*) was purchased from DuPont-Genencor (Finland) and non-commercial, in-house-made xylanase prepared from *Aureobasidium pullulans* (*A. pullulans* XY*)* NRRL Y-2311-1 was kindly supplied by Dr. Sirma Yegin from Ege University, Department of Food

Engineering (Izmir, Turkey). The production of that enzyme was developed in the project entitled "Development of bioprocess strategies for production of xylanase on agro-residual products with *Aureobasidium*", which was funded by TÜBİTAK (project no: 112O521). According to the manufacturer, Accellerase XY contained 20000-30000 ABXU/g xylanase activity (ABXU: Acid Birchwood Xylanase Units). According to enzyme information sheet, Accellerase XY had an optimum range of 50 and 75 ^{o}C and an optimal pH of 4.5-7.0. Optimum hydrolysis conditions of *A. pullulans* XY had already been determined as 50 ^{o}C and pH 5 before, thus the first experiments were performed at these conditions.

Activity of enzymes was also measured to determine the amount would be added in liquors for hydrolysis.

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Steinheim, Germany).

7.2.1. Xylanase Activity

Enzyme activity was determined by measuring xylose as reducing sugar after incubation enzyme mixture and subtsrate (xylan) for a certain time (Bailey, Biely, & Poutanen, 1992). Beechwood xylan (0.5% (w/w)) was used as substrate by dissolving in 50 mM citrate buffer (pH: 5.2). One hundred µL of enzyme solution was mixed with 900 µL substrate. After waiting at 50 ^{o}C for 5 minutes in water bath, 1.5 mL of 3,5-dinitrosalicylic acid (DNS) solution was added and the mixture was kept for 5 min in a boiling water bath (Termal, Turkey). After 5 min, the absorbance of cool solution was read at 540 nm against reagent blank. Reducing sugar released during incubation was calculated by using standart curve prepared by xylose (500-2000 µg/mL) as shown in Appendix C (Figure C.8). One unit of enzyme activity (U/mL) was expressed as the amount of enzyme that released reducing sugars which is equal to 1 µmol for 1 min. Enzyme activity of Accellerase XY and *A. pullulans* XY were determined as 30011 and 278.46 U/mL in this study. Enzyme activity was calculated by the following equation.

$$\text{Activity (U/mL)} = (X/150.13)*(1/5)*(1/0.1).DF \quad (7.1)$$

X: µg xylose (obtained from the standart curve)

DF: Diluton Factor

150.13: Molecular weight of xylose

5: Incubation time (min)

1: Reaction volume (1000 µL)

0.1: Volume of added enzymatic solution (100 µL)

7.2.2. Enzymatic Hydrolysis

By-product, xylose, total XOS, total low-DP XOS concentrations and the fraction of low DP-XOS of liquors obtained under moderate autohydrolysis conditions, which yielded high total XOS, were compared (Table 7.1). According to this comparison, liquor from autohydrolysis at 190 ^{o}C for 0 min was selected for further enzymatic hydrolysis experiments due to having low acetic acid, HMF, furfural and xylose concentrations; and the highest total XOS concentration.

Table 7.1. Liquors for enzymatic hydrolysis.

Treatment Conditions		**Concentration (g/L)**			**Total low DP-XOS ratio (%)**	**Concentration (g/L)**		
(^{o}C-min)	**log R_o**	**Xylose**	**Total XOS**	**Total low-DP XOS**		**Acetic Acid**	**Furfural**	**HMF**
180-15	3.63	0.43	8.56	0.87	10.2	0.55	0.06	0.02
190-0	**3.64**	**0.38**	**9.20**	**2.16**	**23.5**	**0.58**	**0.10**	**0.05**
180-30	3.91	1.24	8.77	3.28	37.4	1.28	0.33	0.04
190-5	3.92	0.88	10.1	2.42	23.8	0.80	0.15	0.03
190-15	4.02	1.88	8.72	3.75	43.0	1.27	0.43	0.05
180-45	4.03	2.41	8.04	3.70	46.0	1.41	0.42	0.05
190-30	4.15	2.24	4.65	3.41	73.3	2.03	0.76	0.07

In order to have a higher amount of XOS (g) in liquor, liquor obtained after autohydrolysis with 1/6 solid/liquid ratio was used for enzymatic hydrolysis and further analyses. The total XOS and xylose contentration of liquor from 190^{o}C for 0 min with 1/6 solid/liquid ratio were 15.71 and 1.39 g/L, respectively.

The main purpose of the enzymatic hydrolysis was increasing xylooligomer concentration without increasing xylose concentration. For the first experiments, pH of liquors (5 mL) was adjusted to 6.0 by 1% NaOH (w/v) before enzymatic hydrolysis by Accellerase XY. In order to prevent the growth of microorganisms, 2% (w/v) sodium azide solution was added to liquors. The liquors were placed into shaking incubator and

temperature was adjusted to 60^{o}C. Temperature was controlled during shaking at 140 rpm and when the temperature reached to 60^{o}C, 87 or 43 U/mL xylanase was added to liquors. After 1, 2, 4, 6, 8 and 24 h of incubation, 1 mL sample was taken and it was kept at 100 ^{o}C for 5 min to inactivate the enzyme. 2 mL of water was added for dilution.

After analyzing the results of the first experiments, enzymatic hydrolysis was performed for Accellerase XY and *A. pullulans* XY in parallel. The liquor from autohydrolysis at 190 ^{o}C for 0 min was treated with 1% (w/v) activated charchoal at room temperature for 1 h for removing phenolics before enzymatic hydrolysis (This treatment was explained in Chapter 8). After filtration, pH of 10 mL of filtrates was increased to 5.0 and 6.0 by adding from 1% NaOH (w/v) solution for *A. pullulans* XY and Accellerase XY, respectively. After adding 100 µL of 2% (w/v) sodium azide solution, enzymes were added to liquors. Accellerase XY was added as 30, 120 and 500 U/g XOS at 60 ^{o}C. *A. pullulans* XY was added as 30, 120, 240, 360 and 500 U/g XOS at 50 ^{o}C. 2 mL of samples were taken from the mixture after 0, 2, 8 and 24 h incubation and enzyme activity was stopped by boiling for 5 min.

After determining the optimum enzyme activity, the effects of temperature, pH and substrate concentration were investigated.

The samples were stored at -20 ^{o}C until analysis. The change in xylooligomers and xylose concentration with hydrolysis in these samples was monitored by HPLC analyses, directly as described previously in section 5.2.8. All enzymatic hydrolysis treatments were performed in duplicates and the results were given as mean ± SD.

7.3. Results and Discussion

Liquor from autohydrolysis at 190 ^{o}C for 0 min was hydrolyzed with Accellerase XY enzyme (with different amounts as 87 and 43 U/mL) after treated with 1% activated charchoal. According to Figure 7.2a, total low-DP XOS concentration increased until 2 h and then started to decrease for hydrolysis with 87 U/mL of Accellerase XY enzyme. Xylobiose showed an important increase from 0.34 (0 h) to 5.93 g/L (2 h). Xylopentaose (0.74 g/L) and xylotetraose (2.25 g/L) concentrations increased up to 1 h and than started to decrease. Xylotriose also decreased after 2 h. On the other hand, total low DP-XOS concentration after hydrolysis with 43 U/mL of enzyme for 24 h (Figure 7.2b) did not increase as much as for 87 U/mL. The highest

xylobiose concentration was measured as 4.66 g/L after 4 h. Xylopentaose (0.51 g/L) and xylotetroase (1.84 g/L) showed similar changes as in Figure 7.2a.

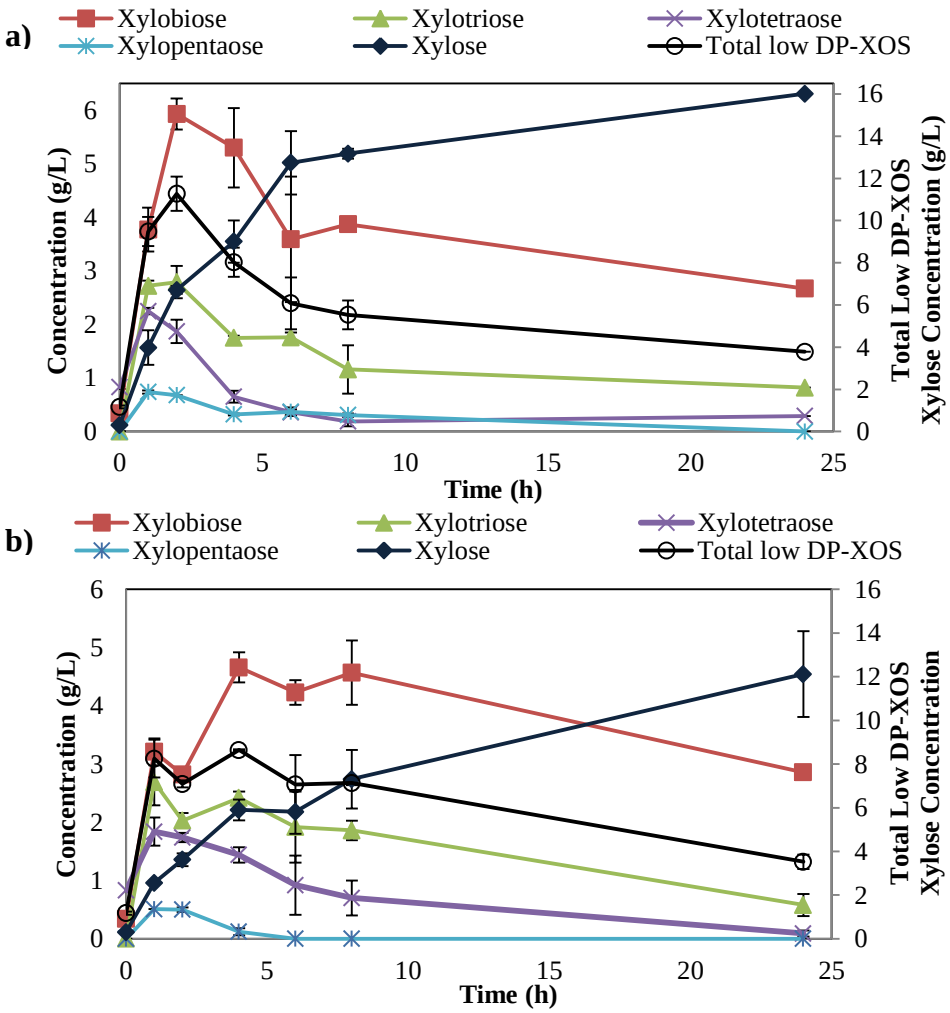

Figure 7.2. The effect of enzyme dose on xylose and xylooligomer concentration during hydrolysis: a) 87 U/mL and b) 43 U/mL for Accellerase XY.

Enzymatic hydrolysis of liquor with 87 U/mL and 43 U/mL of Accellerase XY resulted higher concentration of xylose compared to total low DP-XOS. Therefore, in order to investigate decreasing DP of XOS in liquor without increasing xylose concentration, *A. pullulans* XY and Accellerase XY were used with different amounts such as 30, 120, and 500 U/g XOS. Total low DP-XOS, xylooligomers (between xylobiose and xylohexaose) and xylose concentrations after hydrolysis with different amounts of Accellerase XY and *A. pullulans* XY enzymes (30, 120, and 500 U/g XOS) for 24 h were measured (Figure 7.3). Before enzymatic hydrolysis, xylose, total XOS, total low DP-XOS concentration of liquor were 0.91, 18.86, 2.57 g/L, respectively. 30

U/g XOS xylanase increased total low DP-XOS concentration to 4.36 and 3.95 g/L, for Accellerase XY and *A. pullulans* XY, respectively, after 24 h; however there was no change in xylose concentration. At the end of the incubation, xylose concentration increased to 1.2 g/L for 120 U/g xylanase and total low DP-XOS increased to 5.75 and 4.96 g/L for Accellerase XY and *A. pullulans* XY, respectively. For 500 U/g XOS xylanase, the final xylose and total low DP-XOS concentrations were 3.27 and 8.05 g/L for Accellerase XY; and 2.65 and 7.26 g/L for *A. pullulans* XY.

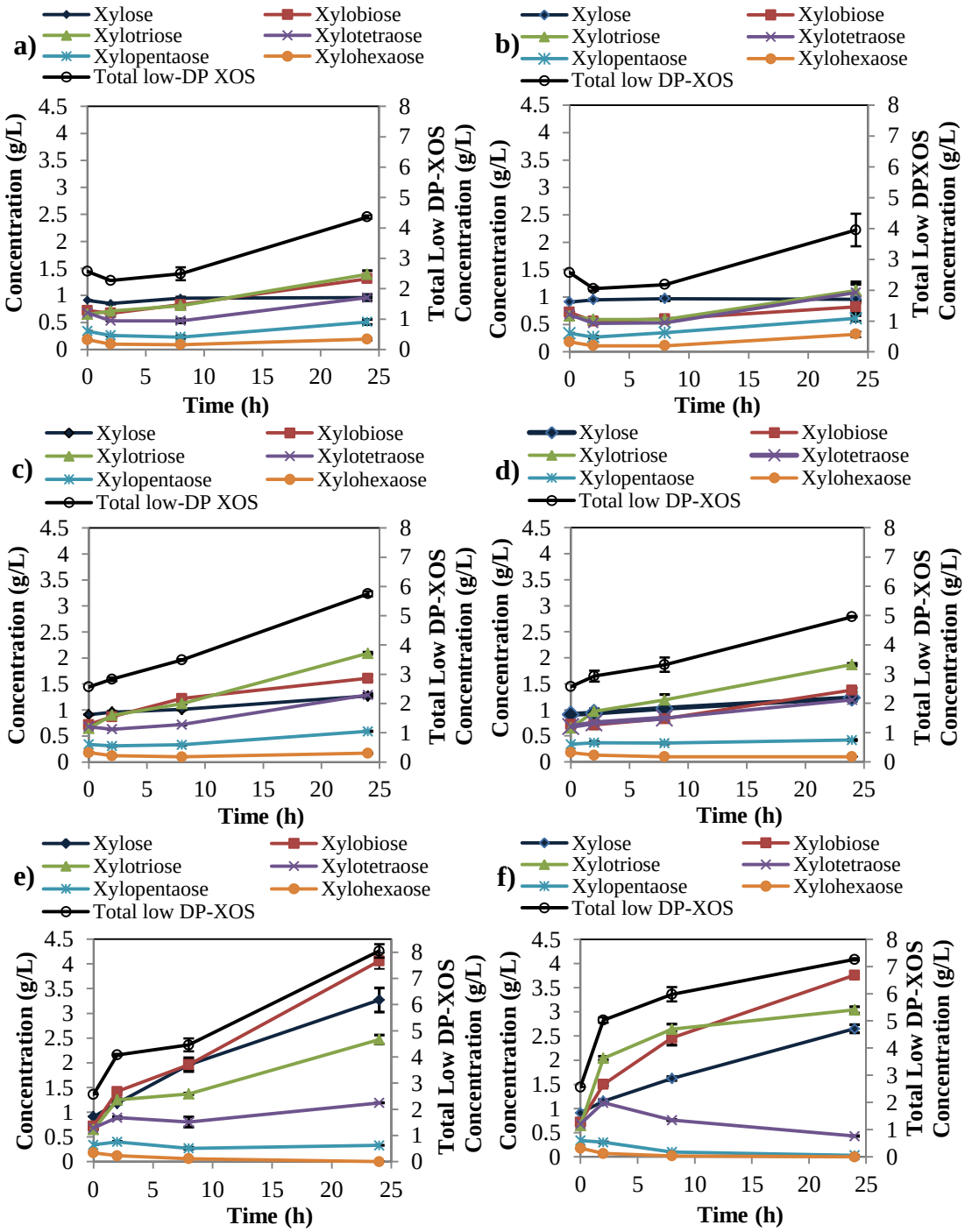

Figure 7.3. The effect of enzyme dose on xylose and xylooligomer concentration during hydrolysis: a) 30, c) 120, e) 500 U/g XOS for Accellerase XY and b) 30, d) 120, f) 500 U/g XOS for *A. pullulans* XY.

There was no significant difference between xylanases for total low DP-XOS formation. Therefore, *A. pullulans* XY was used for further enzymatic hydrolysis due to being a house-made enzyme. Moreover, the effect of using *A. pullulans* XY with lower than 500 U/g XOS activity on low DP-XOS concentration was investigated. The amount of enzyme was decreased to 240 and 360 U/g XOS but the other conditions were not changed. According to results shown in Figure 7.4, xylose concentration was 1.84 and 2.40 g/L for 240 and 360 U/g XOS, respectively; however there was no marked difference in total low DP-XOS concentration (7.77 and 7.90 g/L). The results showed that 240 U/g XOS xylanase was the optimum enzyme dose for hydrolysis for 24 h, at pH 5.0 and 50 ^{o}C.

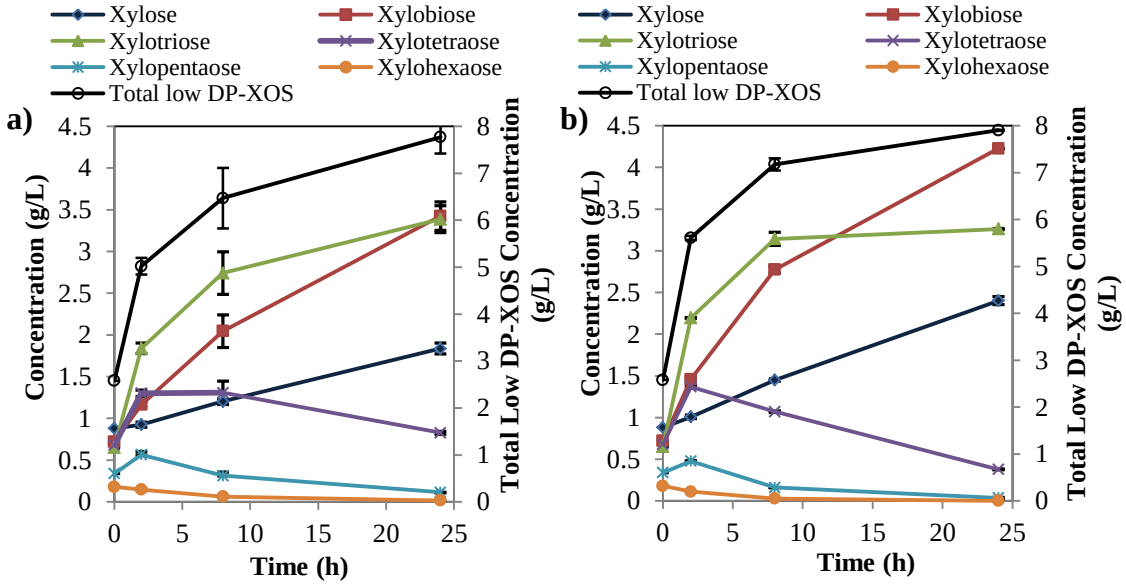

Figure 7.4. The effect of enzyme dose on xylose and xylooligomer concentration during hydrolysis: a) 240 and b) 360 U/g XOS for *A. pullulans* XY.

The effect of temperature and pH on XOS production by hydrolysis with *A. pullulans* XY was investigated. The change in total low DP-XOS, xylooligomer and xylose concentrations were observed in Figure 7.5 and 7.6 for different hydrolysis conditions such as different temperatures (at pH 5) and different pH (at 40 ^{o}C), respectively. The effects of temperature and pH on xylooligomer (xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose) concentrations were also observed in Figure E.1 and E.2, respectively.

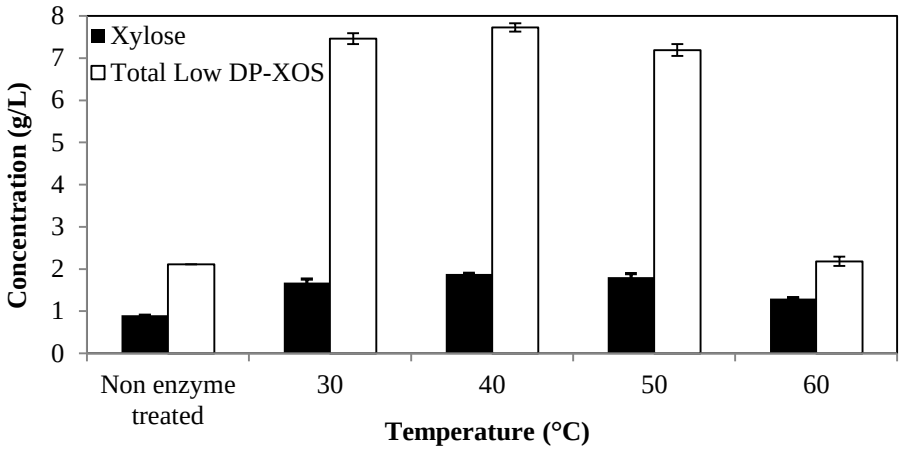

Figure 7.5. The effect of temperature on xylose and total low DP-XOS concentration after hydrolysis at pH 5.

According to Figure 7.5, it was clear that the hydrolysis at different temperatures was more effective on the concentration of total low DP-XOS than xylose. Increasing temperature from 30 to 50 ^{o}C did not have an important effect on total low DP-XOS concentration; however there was a sharp decrease while it was increased to 60 ^{o}C. The hydrolysis temperature was selected as 40 ^{o}C for further experiments due to giving the highest low DP-XOS concentration.

The hydrolysis was carried out at different pH (3, 4, 5 and 6) and 40 ^{o}C (Figure 7.6). Higher concentrations of total low DP-XOS were obtained after hydrolysis at pH 4 and 5. Due to yielding less xylose, pH 5 was concluded to be the optimum pH.

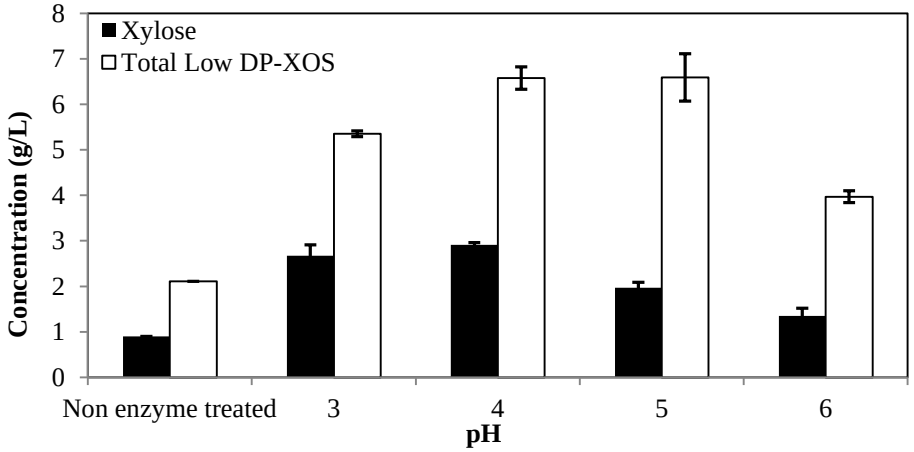

Figure 7.6. The effect of pH on xylose and total low DP-XOS concentration after hydrolysis at 40 ^{o}C.

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The effect of the amount of substrate (total XOS) on hydrolysis was also researched by conducting hydrolysis started with 18, 36 and 72 g/L of XOS. The change in xylose and total low DP-XOS concentration and fraction after hydrolysis at 40 ^{o}C and pH 5 were measured after 24 and 48 h (Figure 7.7). The effect of substrate concentration on xylooligomer (xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose) concentrations was also investigated in Figure E.3. When the substrate concentration increased, the amount of xylose and total low DP-XOS concentration also increased after 24 and 48 h, as expected. On the other hand, the fraction of low DP-XOS (% values) showed a sharp decrease when the substrate concentration increased to 2 and 4 fold. Increasing the incubation time from 24 h to 48 h did not create an important difference in those fractions. Therefore, the optimum conditions of the enzymatic hydrolysis of autohydrolysis liquor were determined as 40 ^{o}C, pH 5 with 240 U/g XOS of *A. pullulans* XY, 18 g/L of XOS and 24 h of autohydrolysis time.

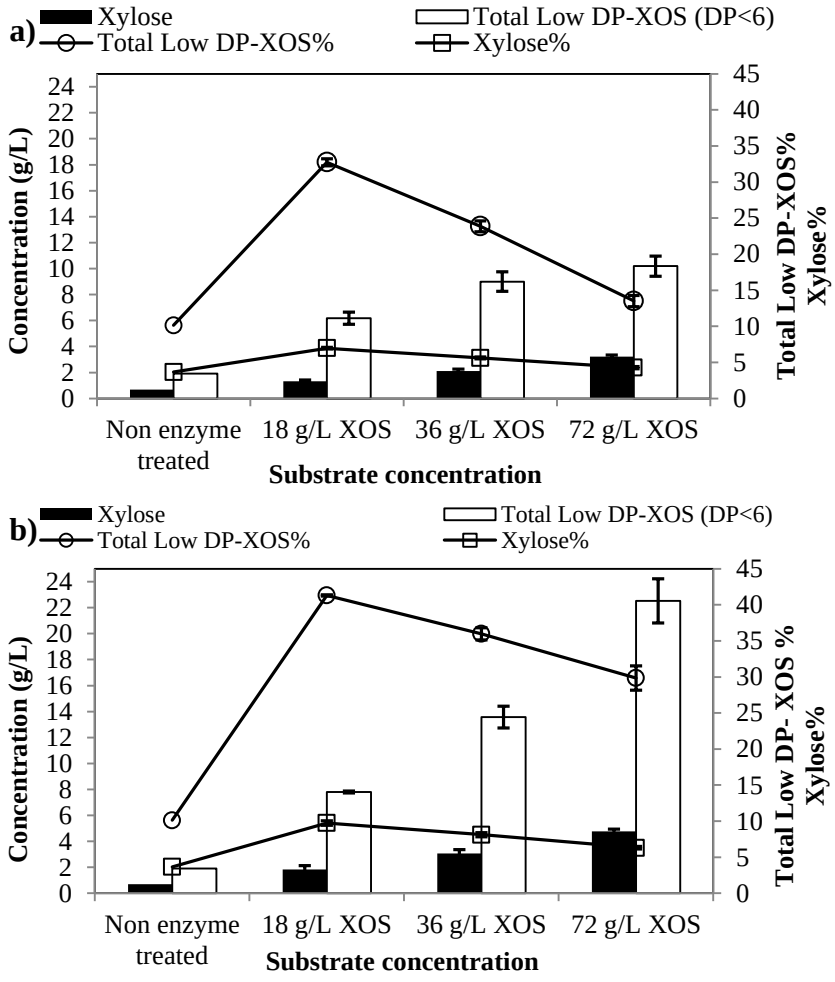

Figure 7.7. The effect of substrate concentration on xylose and total low DP-XOS concentration after hydrolysis for a) 24 h and b) 48 h.

The conversion of XOS in liquor from heat treatment into low DP-XOS or xylose by enzymatic hydrolysis was performed for other studies previously. Low DP-XOS from corn cob (Vázquez, Alonso, Domínguez, & Parajó, 2001a), *Eucalyptus* wood (Vazquez, Alonso, Dominguez, & Parajo, 2002), oil palm frond fibres (Sabiha-Hanim, Noor, & Rosma, 2011), rice husk (Vegas, Alonso, Domínguez, & Parajó, 2008), wheat straw and sunflower stalk (Akpinar, Gunay, et al., 2010) were obtained after autohydrolysis followed by enzymatic hydrolysis.

Vázquez et al. (2001) applied enzymatic post-hydrolysis of liquor produced from autohydrolysis of corncob at 175 ^{o}C for 20 min. The initial concentrations of XOS and xylose in the liquor were 28.1 and 1.29 g/L, respectively. Enzymatic hydrolysis was performed with commercial xylanolytic complex at pH 4, 5 and 6 (by NaOH addition); and 30, 45 and 60 ^{o}C. The amount of enzyme was 0.22-0.33 g/L hydrolysate for solids; and 0.33 mL formulation/L hydrolysate for liquids. They followed a similar experimental order to this study. They firstly changed the hydrolysis temperature at pH 5 and then they adjusted pH of liquor to different pH as 4, 5 and 6. According to their results, they determined optimum hydrolysis conditions as 45 ^{o}C and pH 4, close to values in this study (40 ^{o}C, pH 5), and finally they used different amounts of substrate. Similiarly, they reported that the increase in the amount of enzyme increased the xylose concentration and the conversion ratio of xylan into xylose was 80%. On the other hand, their purpose was not to produce XOS; it was production of xylose-containing fermeation media. (Vázquez et al., 2001a)

Vazquez et al. enzymatically hydrolyzed autohydrolysis liquor of *Eucalyptus* wood obtained from 195 ^{o}C with different type of commercial enzymes at pH 4 and 45 ^{o}C in 2002. Initial concentrations of XOS were 14.23 g/L, which was close to the value in this study (18.86 g/L); xylose was 2.79 g/L, which was much higher than in this study (0.90 g/L). At the end of the hydrolysis, the xylose concentration increased to 17 g/L. The conversion ratio of XOS into DP 2 was 50-58% and xylobiose concentration increased from 0.18 to 8.8 g/L. On the other hand, conversion ratio to xylotriose was lower (8.8-16.7%) and the concentration increased to 3.3 g/L. They indicated that 67% of the initial oligomers can be recovered as products recommended for food applications and increasing the amount of enzyme did not increase the conversiton ratio of XOS to xylose, differently from their study in 2001. The enzymes could convert 90% of XOS into xylose (Vazquez et al., 2002).

Sabiha-Hanim et al. (2011) hydrolzed autohydrolysis liquor of oil palm frond fibres from 121 ^{o}C for 60 ^{o}C with mixed endo-(1,4)-β-xylanase. They used 4 U xylanase per 100 mg autohydrolysate. The xylose and xylobiose concentrations of liquor were 4.30% and 1.55% (w/w), respectively. They dissolved 1% (w/v) freze dried liquor in 0.05 M sodium acetate buffer; and hydrolyzed at pH 5 and 40 ^{o}C for 48 h. According to reducing sugar analysis, the concentration increased from 0.82 to 2.04 g/L after 48 h; however there was no difference between 48 and 24 h. After 24 h, the hydrolysis ratio was 19.2%. They increased the amount of enzyme from 4 to 8, 12 and 16 U/100 mg liquor. They determined the same hydrolysis conditions as optimum; however the optimum enzyme amount was 8 U/100 mg liquor (534 U/g XOS), which was 2 fold of the amount of enzyme used in this study (278 U/g XOS). There was no important increase in reducing sugar concentration when the amount of enzyme increased from 8 to 16 U, thus they indicated that the activity of enzyme was depended on the availability of substrate. They determined hydrolysis with 8 U/100 mg liquor for 24 h as the best conditions. The hydrolysis ratio was 31.4% at those conditions; however total sugar concentrations did not change (4.74 g/L). They obtained as a liquor containing 25.6% xylose and 17.5% XOS after hydrolysis. The increase in xylose concentration was not a problem for them since they aimed to produce xylose and XOS not only XOS. The initial total shot XOS concentration in this study was 2.11 g/L and after hydrolysis it was increased to 3.3-3.4 fold; there was 2 fold increase in xylose concentration. At optimum conditions determined by Sabiha-Hanim et al. (2011), 1.63 fold increase in reducing sugar concentration and a significant increase in xylose concentration (from 4% to 25%) were obtained (Sabiha-Hanim et al., 2011).

Vegas et al. (2008) hydrolyzed autohydrolysis liquor of rice husk after autohydrolysis at 205 ^{o}C with different type of enzymes (Shearzyme 2x: pH 4.5, 40 ^{o}C, 96 h, 159, 311 and 481 XU/kg liquor; Pentopan Mono BG: pH 6.0, 40 ^{o}C, 48 h, 117, 234 and 350 XU/kg liquor and; Pulpzyme HC: pH 6.0, 40 ^{o}C, 48 h, 137, 334 and 685 XU/kg liquor).The initial XOS concentration was 11.57 g/L, which was lower than in this study; however the initial xylose concentration was same as (1.03 g/L) in this study (0.90 g/L). Their amount of enzyme was lower as 41-59 XU/g XOS, and probably due to using lower amount, they completed the hydrolysis after 48 or 96 h. They reported their results as xylose equivalent after reducing sugar analysis. The xylose equivalents concentration increased from 0 to 1-2 g/L for Pentopan Mono BG and Pulpzyme HC and 3-4 g/L for Shearzyme 2x. The xylose concentration did not change significantly

for any enzyme (1.6-1.8 g/L). They indicated that there was an increase in the xylose equivalent concentration as the amount of enzyme was increased and there was no important increase in that concentration when they exceeded the maximum amount of enzyme they used. They calculated DP% ratio (2-3, 4-5, 6-7 and >7) for each enzyme. The highest concentration of DP 2-3 was obtained by Shearzyme 2x enzyme with 159/kg liquor (Vegas et al., 2008).

Akpinar et al. (2010) enzymatically hydrolyzed autohydrolysis liquors of wheat straw and sunflower stalk (raw or refined by ultrafiltration through 1 kDa membrane) obtained from autohydrolysis at 160 ^{o}C for 1 h by an endo-xylanase from *Trichoderma reseei* (pH:4.6, 50 ^{o}C, 24 h) and *Aspergillus niger* (pH:5.5, 40 ^{o}C, 24 h). Initial XOS concentrations of liquors were between 2.07 and 4.59 g/L, which were very low compared to XOS in this study (18.86 g/L). Xylose concentrations were ranged from 0.01 to 0.09 g/L; however it was higher as 0.90 g/L in this study. They treated 10 mL of liquor with xylanase at a loading of 8 U/mL, which was between 1745-3865 U/g XOS. In this study, enzymatic hydrolysis experiments were performed with xylanase at a loading of a lesser amount as 30-500 U/g XOS. They analyzed liquors after enzymatic hydrolysis by thin layer chromatography and XOS concentrations were determined and expressed as xylose equivalent after reducing sugar analysis. In this study, the initial low DP-XOS concentration was 2.11 g/L and it was increased to 3.3-3.4 fold at the end of the enzymatic hydrolysis for 24 h at optimum conditions. The xylose concentration was 2 fold (1.8 g/L) of the initial concentration (0.90 g/L). On the other hand, the xylose equivalent concentration was increased to 4.5-5.5 and 7-9 fold for raw and refined autohydrolysis liquors, respectively.

Teng, Yan, Jiang, Fan, & Shi (2010) also hydrolyzed steam explosion liquor of corncobs from 196 ^{o}C for 5 min with a thermostable xylanase. The hydrolysis was performed at 70 ^{o}C and pH 7 for 0-4 h with 2-12 U/mL for 1 L liquor. The optimum conditions were determined as 7.5 U/mL and 2.5 h. Due to using a thermostable enzyme (hydrolysis at 70 ^{o}C), hydrolysis time was short as 2.5 h, thus traditional enzymatic hydrolysis was a time consuming process for hydrolysis with othe mesophilic enzymes (40-60 ^{o}C). In those conditions, 28.6% of initial xylan was obtained as XOS, which contained more than 90% xylobiose and xylotriose. Increasing the amount of enzyme from 7.5 to 10 provided a little increase in XOS and xylobiose concentrations, as observed when it was increased to 360 from 240 U/g XOS in this study. The highest increase in low DP-XOS was observed after the first 0.5 h, and then xylobiose increased

slowly; however xylotriose started to decrease. The initial low DP-XOS concentration increased from 4 to 8.3 g/L and xylobiose from 2 to 4.3 g/L. At the optimum conditions, xylose concentration was 2.3 g/L. On the other hand, xylobiose increased from 0.67 to 3.19 g/L, there was a 3-3.5 fold increase in XOS concentration; and xylose concentration was lower at optiumum conditions (1.8 g/L) in this study (Teng et al., 2010).

7.4. Conclusions

Hazelnut shell was used to produce low DP-XOS by autohydrolysis followed by enzymatic hydrolysis. Enzymatic hydrolysis of liquor from autohydrolysis of hazelnut shell at 190 ^{o}C was carried out by commercial and non-commercial xylanases. The results showed that DP of XOS in autohydrolysis liquors can be reduced to the range (DP<6) acting as prebiotics by subsequent enzymatic hydrolysis. Total low DP-XOS concentration in liquor was increased from 2.58 to 7.77 g/L by hydrolysis with 240 U/g XOS of non-commercial xylanase (*A. pullulans* XY) at 40 ^{o}C, pH 5 for 24 h. Autohydrolysis liquor containing low DP-XOS as 10.7% of total XOS was converted to a liquor with 32.7% low DP-XOS, which was mainly xylobiose and xylotriose. On the other hand, xylose content of the liquor was increased from 3.66 to 6.97%, but this was an acceptable value compared to results of other studies in literature. Purification of low DP-XOS obtained from enzymatic hydrolysis of autohydrolysis liquor was required as a further study.

CHAPTER 8

PURIFICATION OF XOS

8.1. Introduction

XOS are obtained from lignocellulosic biomass by hydrothermal treatment or chemical and enzymatic methods. Although XOS are the main product of autohydrolysis liquor, many undesired compounds are also produced as by-product during production of XOS. XOS produced by autohydrolysis are present with other components such as ferulic acids, uronic acids, monosaccharides, lignin-related phenolics, carbohydrate dehydration products, ashes and other impurities from present in lignocellulosic biomass. The impurities can be protein, tannin, pectin and starch; however lignin-derived products are the main impurities present with XOS (Montane, Martorell, Torne, & Fierro, 2006; Nabarlatz, Torras, Garcia-Valls, & Montané, 2007; T. Wang & Lu, 2013). For instance, phenolic compounds in liquor can decrease purity of XOS and inhibit the microbial growth (Akpinar, Gunay, et al., 2010). Therefore, liquors rich in XOS should be processed in order to obtain a homogenous, well characterized and food-grade XOS (Soto, Moure, Domínguez, & Carlos, 2011).

The purification of XOS solution obtained from hydrothermal processing is an important problem. High molecular weight polysaccharides and low molecular weight sugars are separated from the product by purification. Several physicochemical treatments such as vacuum evaporation, solvent extraction, adsorption by surface active materials, chromatographic techniques, membrane separation and centrifugal partition chromatography are used for to remove unwanted compounds or have XOS within a desired DP range XOS separation and purification (Akpinar et al., 2007; Qing et al., 2013; Vázquez et al., 2001b). Purification treatments can be different according to the desired purity level. Vacuum evaporation can be used as a first step for the removal of acetic acid and flavours. The solubility of impurities is decreased by reducing water concentration and organic solvents such as acetone and alcohols are used for the recovery of hemicellulose-degradation products (Vázquez et al., 2001b). On the other hand, practical and economical alternative methods such as ultrafiltration and nanofiltration are preferred for large-scale production of XOS (Akpinar et al., 2007).

Membrane technology is used, in order to separate XOS within desired DP range and eliminate non-saccharide compounds (Vázquez et al., 2001b). The size-dependent mechanism helps the separation of molecules according to different molecular weights. Membrane separations have been used for the preparation of many oligosaccharides such as fructooligosaccharides, chitooligosaccharides, pectic oligosaccharides, soybean oligosaccharides, and maltooligosaccharides (Akpinar et al., 2007). XOS produced by autohydrolysis and enzymatic hydrolysis was purified by membrane technologies in previous studies (Nabarlatz et al., 2005; Swennen, Courtin, Bruggen, Vandecasteele, & Delcour, 2005; R Vegas, Luque, Alvarez, & Alonso, 2006; Q. P. Yuan, Zhang, Qian, & Yang, 2004). Membrane technology has advantages such as reduced fresh water need, creating of new products, decreased waste treatment volume and cost, low space and capital need. On the other hand, obtaining approval for utilization of new membrane filters in food processing can be difficult. There can be some problems such as limited operating pressure, fouling, pH sensitivity and replacement costs (Akin, Temelli, & Köseoğlu, 2017).

Purification of liquors including XOS has been carried out by adsorption using such as activated charcoal (AC), acid clay, bentonite, diatomaceous earth, aluminium hydroxide or oxide, titanium, silica and porous synthetic materials or chromatographic separation using anion and cation exchangers for removal of coloring compounds and salts (Soto et al., 2011; Vázquez et al., 2001b). In order to remove lignin-related impurities from XOS, adsorption on activated carbon and solvent extraction have been used (Nabarlatz, Torras, et al., 2007). Activated carbon treatment is reported as an effective process for removal of impurities from carbohydrate products (Montane et al., 2006). Activated carbons are composed of small hydrophobic graphite layers with irregular and heterogeneous surfaces. It is preferred due to its simple design, operation, scale up, high capacity, selectivity, regenerability, low cost, ease of activation, insensivity to toxic materials, no need for toxic chemicals and decreasing degradation (Soto et al., 2011).

Alternatively, XOS can also be removed and separated by activated carbon adsorption method. Activated charcoal treatment followed by ethanol elution is a process that can be used for purification of XOS. Firstly, XOS are adsorped by charcoal and then XOS can be fractionated by ethanol elution according to alcohol concentration (Vázquez et al., 2001b).

Ultrafiltration is a process used in biotechnology and fermentation industries to separate oligosaccharides and polysaccharides (Swennen et al., 2005). Several industries have accepted cross-flow filtration such as microfiltration, ultrafitration, nanofiltration, and reverse osmosis, as standard methods for concentration or clarification. More than one type of those processes can also be used in series. In the cross-flow system, the liquid flows across the membrane surface at a constant velocity when the filtrate is passing through the membrane and the retentate is removed from the membrane. Ultrafiltration is used in order to separate large molecules like proteins, polysaccharides and enzymes at operating pressures between 2 and 15 bar (Akin et al., 2017).

In this study, the aim of purification process including activated charcoal treatment followed ultrafiltration, was to remove impurities such as phenolics, lignin and organic acids from XOS; and to fractionate XOS into desirable DP range in which prebiotic activity is expected to be higher.

8.2. Materials and Methods

AC used in experiments was purchased from Merck (Germany) as powdered form. Liquors after AC treatment and enzymatic hydrolysis were purified by cross-flow membrane filtration system and 10 and 2 kDa size membrane filters (Sartorius, Germany). Technical grade ethanol and sodium hydroxide purchased from (Tekkim, Turkey) were used for cleaning of membrane filters.

8.2.1. Activated Charcoal Treatment

After the first enzymatic hydrolysis experiments, the final xylose concentration in liquor was too high and DP of XOS could not be reduced efficiently. Therefore, in order to increase the effect of enzymatic hydrolysis, AC treatment was decided to be tried to remove non saccharide compounds that can possibly prevent the action of xylanase enzyme. Purification of autohydrolysis liquor by activated charcoal treatment was performed as shown in Figure 8.1. Liquor obtained from autohydrolysis at 190 ^{o}C for 5 min (solid/liquid ratio: 1/6), which were the conditions giving the highest total XOS yield, was treated with AC at different concentrations such as 1, 2, 5 and 10% (w/v) in duplicates. The concentrations of AC were determined according to previous

studies (Carvalheiro, Duarte, Lopes, Parajó, & Pereira, 2005; Montane, Martorell, Torne, & Fierro, 2006; Mussatto & Roberto, 2004; Parajó, Dominguez, & Dominguez, 1996). The researchers indicated that 5% (w/v) AC was the maximum level for removing impurities and lignin; however concentrations higher than 5% were effective on adsorption of XOS. Therefore, in order to observe the effect of the concentration of AC on the composition on the liquor clearly, the range was extended to 10%. The other treatment conditions were modified from Carvalheiro et al. (2005); and Mussatto & Roberto (2004). The AC-liquor mixture was stirred in incubator shaker at 25 ^{o}C for 1 h. The liquor was recovered by vacuum filtration using Whatman no.1 paper followed by centrifugation at 4800 g for 10 min at 25 ^{o}C and filtration by 0.45 µm PTFE syringe filter to remove all remaning AC particles. The effect of AC on total phenolic content (TPC), acetic acid, xylose and XOS concentration of the liquor was investigated. TPC of liquors were measured spectrophotometrically as described in section 5.2.10. The concentrations of acetic acid, xylose and total XOS of all liquors were measured by HPLC as explained in section 5.2.8.

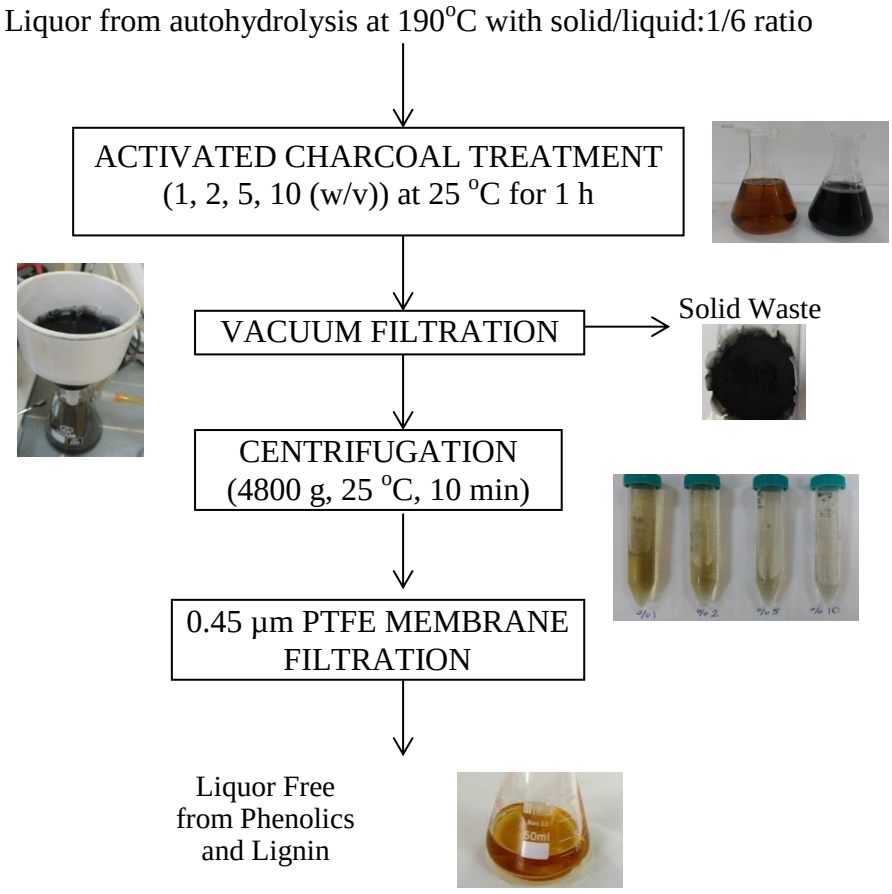

Figure 8.1. Purification of autohydrolysis liquor by activated charcoal treatment.

8.2.2. Ultrafiltration

After determination of the amount of AC for removal of lignin and phenolics from liquor as 1%, the liquor from isothermal (0 min of holding time) autohydrolysis at 190 ^{o}C, which was selected for enzymatic hydrolysis, was treated with 1% (w/v) AC. The liquor from AC treatment was hydrolyzed by *A. pullulans* xylanase. Ultrafiltration was applied both to autohydrolysis liquor and to enzyme treated autohydrolysis liquor. The cross-flow filtration system used for ultrafiltration of liquors is composed of a filter cassette holder, 1 outlet for retentate phase and 2 outlets for permeate phase (Figure 8.2). The pump was run at 75 rpm and the feed pressure was adjusted manually. The operating pressure was about 1.5-2 and 1 bar for flow through 10 and 2 kDa, respectively.

Before starting ultrafiltration process, the filter was placed into the system and it was washed with 2 L distilled water. The valves of inlet and outlets were open during washing. Then, one of the valves of permeates (Permeate-2) were closed and liquor was fed into the system. The remaining water in the system was removed until permeate and retentate phases of liquor began to be separated.

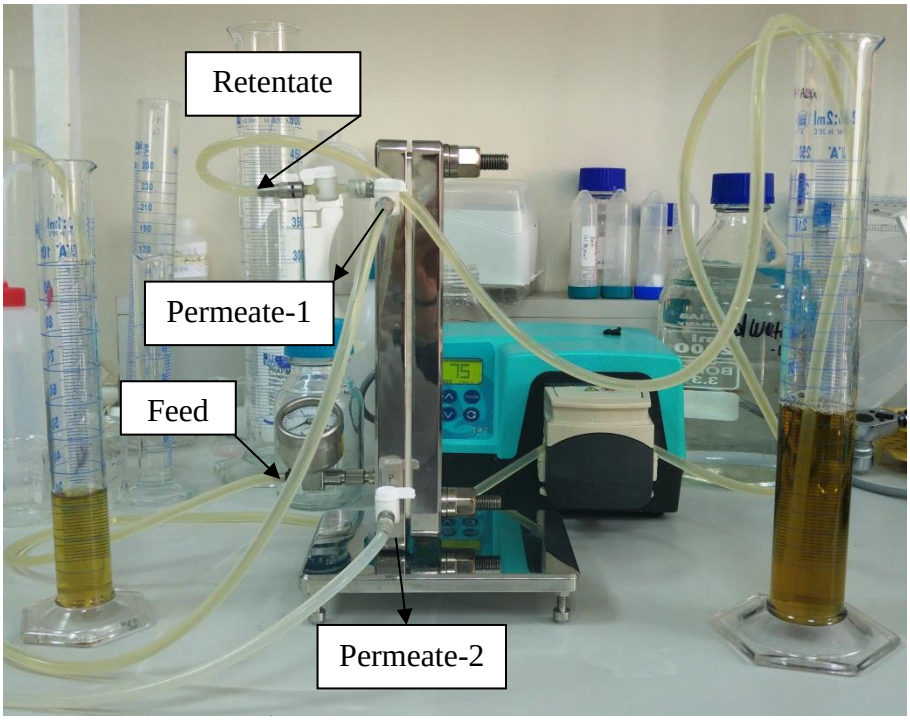

Figure 8.2. Cross-flow membrane filtration system.

After all the liquor was fed to the system, accumulated retentate phase was fed to the system as the feed and this recycle process was performed until volume of the liquid in the measuring cylinder of the feed (R-1) was constant. Measuring cylinders were used to observe the changes in volumes of phases, clearly. For ultrafiltration via 2 kDa, the volume of R-1 was measured and it was diluted to 3 volumes of that value.The diluted liquid was fed to the system and the process was repeated as described below. Constant volume of the liquid in the cylinder (R-2) was measured and diluted once again to 3 volumes. The same procedure was performed for R-2 and constant volume of the liquid in the cylinder (R-3) was measured. Liquid from permeate outlet after each step of filtration was collected and named as P-1, P-2 and P-3, respectively. The filter was washed with 2 L distilled water at the end of the filtration. Then, the filter was cleaned with 1 L of 1 M NaOH at 50 ^{o}C for 1 h. Firstly, 500 mL of NaOH solution was fed to the system and then solution coming from retentate was combined with the remaining solution as feed and the recycle process was carried out for 1 h. After 1h, the filter was washed again with 2 L distilled water. The filters were washed with 1 L of 20-24% ethanol before removing from the system since the filters were stored in their own packages including 20% ethanol at 4 ^{o}C. All permeate valves were open during sampling and washing processes. Samples (P-1, P-2, P-3, R-1, R-2, R-3) from filtration through 10 and 2 kDa were analyzed by HPLC as explained in section 5.2.8 for determination of their xylose and xylooligomer concentration.

8.2.3. Statistical Analysis

Acetic acid, xylose and total XOS concentrations of liquors before and after AC treatment at different concentrations were evaluated by using one way analysis of variance (ANOVA), statistically. The differences between AC concentrations were analyzed by Tukey test (α= 0.05). Statistical analysis was performed using Statistical Package for the Social Sciences software as shown in Appendix D (version 16.0, SPSS Inc., Chicago, IL).

8.3. Results and Discussion

TPC, acetic acid, xylose and XOS concentration of liquors after AC treatment were compared with the initial liquor (without AC) (Figure 8.3).

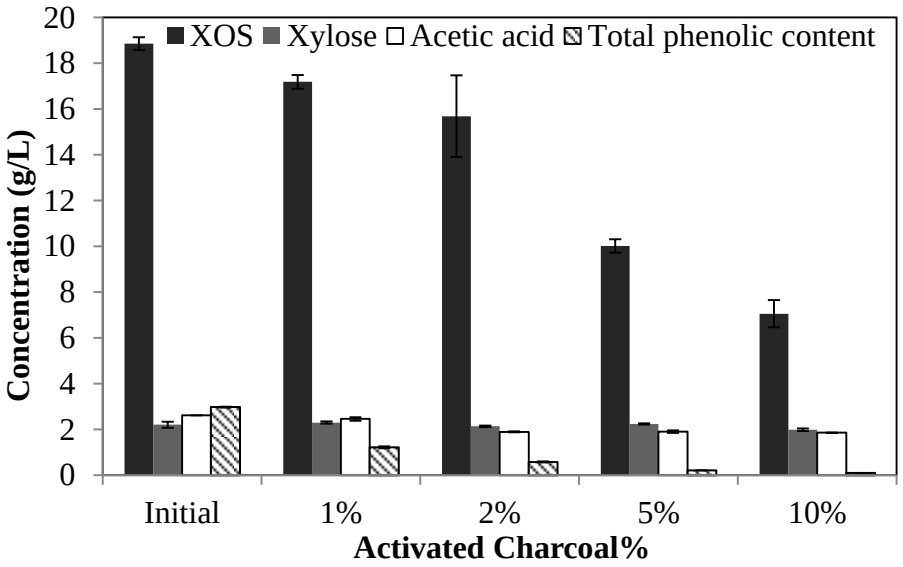

Figure 8.3. The effect of AC treatment at 1%, 2%, 5% and 10% (w/v liquor) on the composition of autohydrolysis liquor. (TPC: mg GAE/mL)

AC was effective to remove phenolics because 59.04% of phenolics were removed after AC treatment at 1%. This value was remarkably higher than values, 48.9 and 27%, reported by Mussatto & Roberto (2004) and (Carvalheiro et al., (2005), respectively. Adsorption of phenolics was markedly higher than XOS, xylose and acetic acid. Although the phenol adsorption mechanism is still not well known and there are many factors such as interactions among several chemical groups, small surface area, poor porosity, and possible interactions with other compounds, phenol molecules form hydrogen bonds with surface functional groups (Soto et al., 2011). Furthermore, removal of lignin derived products can be limited due to being linked to XOS (Montane et al., 2006). AC treatment at 1% and 2% caused 8.83 and 16.79% loss in XOS, respectively; however they were not statistically significant ($p>0.05$). On the other hand, there was an import loss in XOS at 5 and 10% AC as 46.84 and 62.58%. The concentration of xylose did not change significantly as AC concentration increased compared to initial liquor. Xylose was not adsorbed by charcoal due to probably little interaction with carbon. There was a stronger adsorption between higher DP-XOS and charcoal (Zhu, Kim, Lee, Chen, & Elander, 2006). Acetic acid concentration reduced

when AC concentration was higher than 1%. According to Figure 8.3., treatment with 1% AC was enough to remove phenolics and prevent loss of XOS, thus the initial purification step of liquors before ultrafiltration was accomplished by treatment with 1% AC.

The reason of the differences in the results of studies on removal of phenolics or other components was probably due to difference in AC treatment conditions. The adsorbent properties of activated carbons depend on their composition, physicochemical properties and mechanical strength; and the adsorption can also be affected by surface area, functionality, porosity, irregularities, strongly bound impurities, internal porous structure and particle size (Soto et al., 2011). Temperature, pH and stirring rate were effective on the treatment (Mussatto & Roberto, 2004). Mussatto & Roberto (2004) determined the optimum conditions of AC treatment of liquor from diluted acid hydrolysis of rice straw (121 ^{o}C, 27 min with 0.1 M H_2SO_4) as treatment with 2.5% (w/w) at pH 2.0 and 45 °C for 150 rpm and 60 min. Carvalheiro et al. (2005) stirred the liquor from autohydrolysis of brewery's spent grain at 190 ^{o}C for 2.5 min (with pH adjusted to 5.5) with 10% AC for 1 h at room temperature. Montane et al. (2006) investigated purifaction of XOS from autohydrolysis of almond shells by activated carbon at different loads from 1.5 to 50 g/L, in order to remove lignin and associated impurities. They placed the XOS-activated carbon mixture in a water bath at 30 °C, differently, for 24 h and after precipitation of AC by centrifugation at 4000 rpm, they filtered the liquor through a 0.22 µm syringe filter similar to this study (Montane et al., 2006). The average retention was 64% and 21% for lignin products and carbohydrates, respectively.

Zhu et al. (2006) purified XOS to food-grade by charcoal adsorption followed by ethanol elution. Activated carbon powder was added into the liquid with in the range between 1 and 10 % of the liquid weight. At room temperature, the mixtures were mixed at 200 rpm for 30 min. After the mixtures were filtered and washed, most of the XOS adsorbed on charcoal were recovered with 15% and the remaining was recovered by 30% ethanol elution (Zhu et al., 2006).

Wang & Lu (2013) used ethanol precipitation or activated carbon adsorption method as described by Zhu et al. (2006) before in order to remove these impurities for production of XOS from wheat bran by microwave assisted enzymatic hydrolysis. They reported that activated carbon adsorption was better than ethanol precipitation since XOS generated by activated carbon adsorption were low DP-XOS containing xylobiose,

xylotriose ans small amount of xylotetraose (Wang & Lu, 2013). Chen et al. (2014) added different amounts of activated carbon powder as 1%, 5%, 10% or 20% (w/v) in liquor from autohydrolysis of *Miscanthus x giganteus* and stirred the mixture at 100 rpm for 1 h, in order to recover XOS. The XOS enriched activated carbon was eluted with aqueous ethanol. They indicated that activated carbon ratios lower than 10% were not enough for XOS recovery. The highest recovery was obtained as 47.9% (w/w) of XOS from liquor of using 10% activated carbon (w/v) with ethanol/water elution (Chen et al., 2014).

The composition of retentate and permeate phases of two treatments were determined as presented in Table 8.1 and Table 8.2. The changes in components were also showed as a diagram in Figure 8.4 and Figure 8.5. Performing ultrafilration process as sequential three step procedure was effective to recover components remaining in retentate phases; however higher amounts of XOS and low DP-XOS were obtained after the first step of ultrafiltration, as expected. According to Table 8.1, total XOS and low DP-XOS were recovered efficiently after ultrafiltration with 10 kDa. Total low DP-XOS% in liquor was increased after ultrafilration with 2 kDa (Figure 8.4 and 8.5). It was increased up to 76% for enzymatically hydrolyzed liquor. On the other hand, xylose could not be removed after filtration through 10 and 2 kDa.

Table 8.1. Composition of feed, permeate and retentate of 10 kDa ultrafiltration of liquor and enzymatically hydrolyzed liquor after treatment with AC (X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose, X6: xylohexaose).

	Liquor treated with AC			**Enzymatically hydrolyzed liquor after treated with AC**		
	Feed (Initial)	**Permeate**	**Retentate**	**Feed (Initial)**	**Permeate**	**Retentate**
Volume (mL)	340	404	26	290	360	23
Total XOS (g/L)	14.88	7.33	37.22	11.57	9.76	3.58
Total XOS (g)	5.06	2.96	0.97	3.35	3.51	0.08
Total low DP-XOS (g/L)	0.46	0.77	0.00	5.81	5.69	1.22
X6	0.04	0.06	0.00	0.02	0.00	0.00
X5	0.04	0.08	0.00	0.24	0.03	0.00
X4	0.11	0.19	0.00	0.27	0.32	0.1
X3	0.14	0.23	0.00	2.77	2.8	0.59
X2	0.13	0.21	0.00	2.51	2.54	0.54
X1	0.46	0.35	0.00	1.89	1.15	0.06
Total low DP-XOS (g)	0.16	0.31	0.00	1.69	2.05	0.03
Total low DP-XOS%	3.08	10.51	0.00	50.25	58.31	34.23

Table 8.2. Composition of permeate and retentate of 2 kDa ultrafiltration of liquor and enzymatically hydrolyzed liquor after treatment with AC (X2: Xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentasoe, X6: xylohexaose, P: Permeate, R: Retentate).

	Liquor treated with AC					**Enzymatically hydrolyzed liquor after treated with AC**					
	P-1	**P-2**	**P-3**	**R-1**	**R-3**	**P-1**	**P-2**	**P-3**	**R-1**	**R-2**	**R-3**
Volume (mL)	350	64	72	40	34	322	54	52	28	26	35
Total low DP- XOS (g/L)	0.49	0.36	0.28	1.82	0.75	4.92	2.73	1.55	2.62	4.67	2.09
X6	0.01	0.01	0.03	0.26	0.23	0.00	0.00	0.00	0.00	0.00	0.00
X5	0.03	0.03	0.04	0.27	0.19	0.00	0.00	0.00	0.00	0.16	0.06
X4	0.09	0.1	0.09	0.55	0.18	0.2	0.23	0.09	0.24	0.53	0.35
X3	0.19	0.11	0.07	0.39	0.08	2.47	1.31	0.77	1.25	2.09	0.88
X2	0.17	0.1	0.06	0.35	0.07	2.24	1.19	0.69	1.13	1.89	0.8
Total low DP- XOS (g)	0.17	0.002	0.002	0.07	0.03	1.58	0.15	0.08	0.24	0.12	0.07

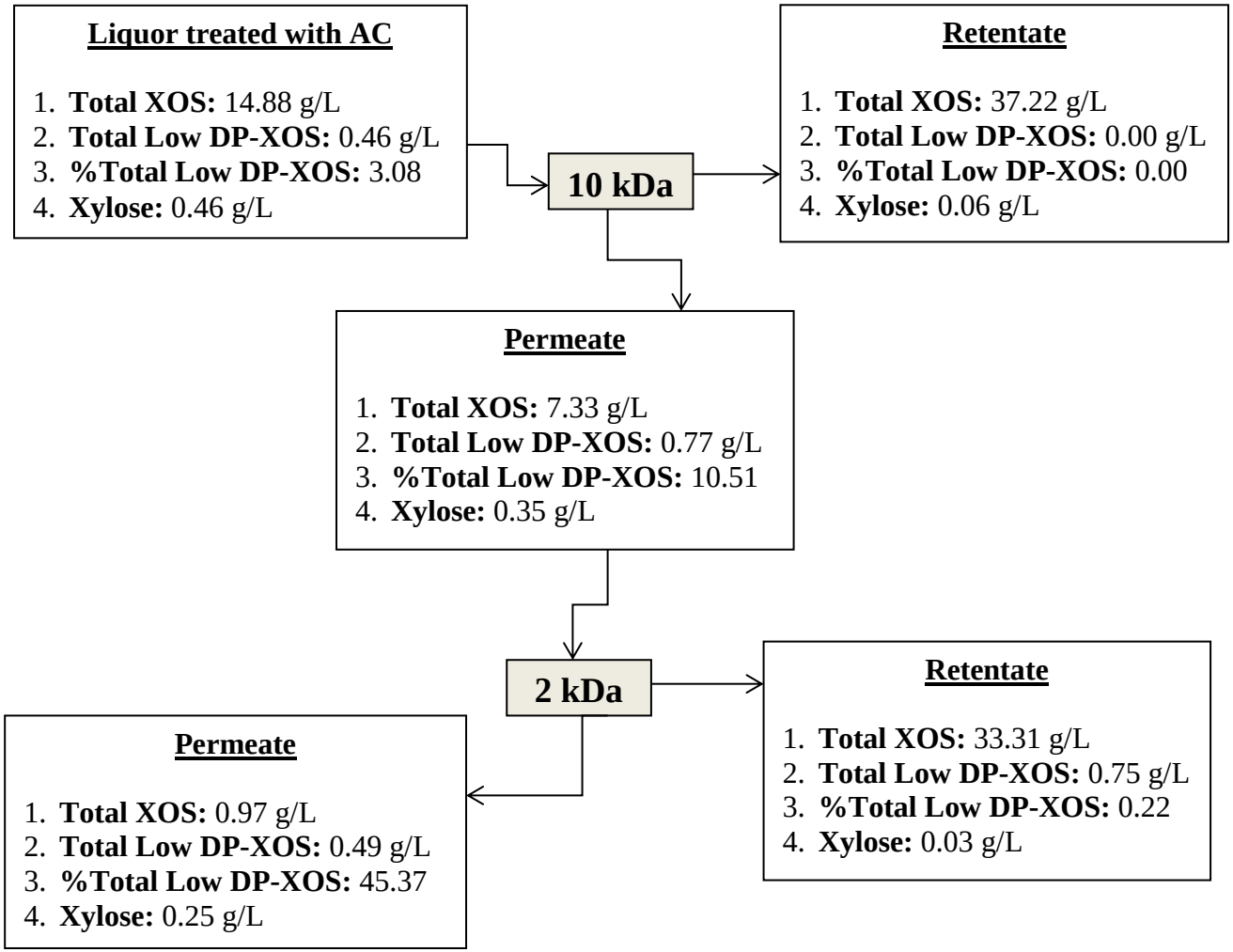

Figure 8.4. Ultrafiltration of autohydrolysis liquor treated with AC.

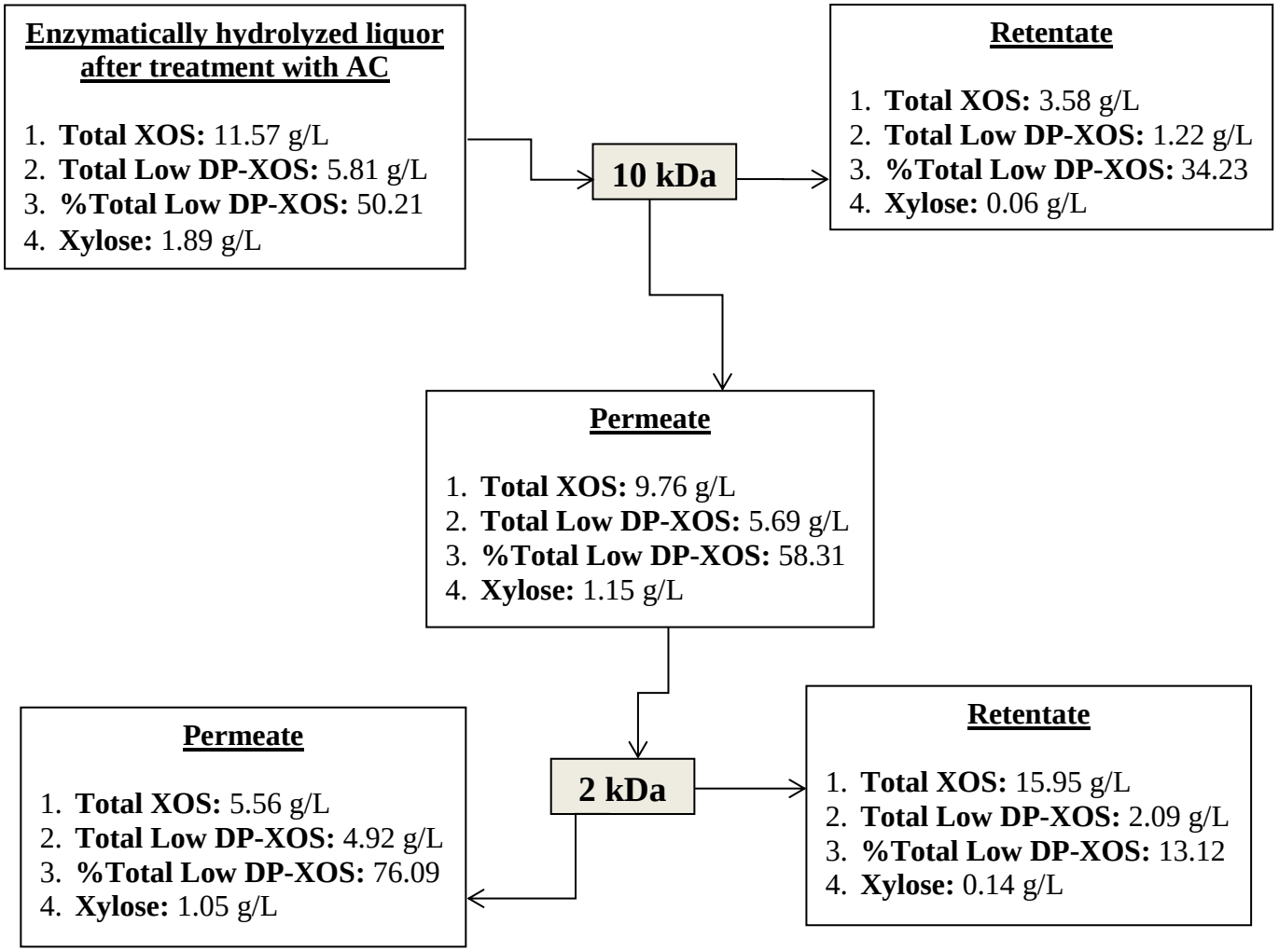

Figure 8.5. Ultrafiltration of enzymatically hydrolyzed liquor after treatment with AC.

The liquors (permeate and retentate) obtained after ultrafiltration through 2 kDa were also lyophilized to be analyzed separately. Lyophilized samples were dissolved in water (20 g/L) and analyzed by HPLC for determination of their by-product, xylose and XOS concentrations. According to by-product analysis, HMF and furfural were not dedected in samples. On the other hand similar to xylose, acetic acid concentrations of permeates were higher as 2.73 and 0.90 compared to retentates, which were 0.17 and 0.05 g/L for obtained from liquor and enzymatically hydrolyzed liquor, respectively. Total low DP-XOS concentrations in permeates was higher than retentates; however total XOS was higher in retentates. Therefore, ultrafiltration was not effective to remove acetic acid and xylose in liquors, but the percent low DP-XOS% in samples could be increased up to 45.37 and 76.09 for samples obtained from liquor and enzymatically hydrolyzed liquor, respectively. The separation of low molecular weight compounds by ultrafiltration through smaller size membranes such as 1 kDa can be researched. Akpinar et al. (2007) fractionated xylan of cotton stalk obtained from alkali extraction and followed by enzymatic hydrolysis, via ultrafiltration by using 10, 3 and 1 kDa membranes. They reported no difference between composition of permeate and retentate phases from 3 and 1 kDa, thus they preferred using 3 kDa for ultrafiltration of permeate from 10 kDa in their next study in 2010 (Akpinar, Erdogan, Bakir, & Yilmaz, 2010).

Akpinar, Erdogan, Bakir, & Yilmaz (2010) ultrafiltrated xylan of tobacco stalk obtained from alkali extraction followed by enzymatic hydrolysis through 10 and 3 kDa. They reported that permeate from 2 step-ultrafiltration consisted of mostly oligosaccharides. They used 10 kDa membrane filter, in order to separate high molecular weight polysaccharides and proteins; and they recovered 90% of oligosaccharides, similar to this study. The permeate was 20% high DP-XOS, and 1% xylose. After ultrafiltration through 3 kDa the composition of the permeate was 8% high DP-XOS and 2% xylose. The high DP-XOS content of retentate phases were 48% and 76% after ultrafiltration with 10 and 3 kDa, respectively. They recovered 71% of XOS at the end of the filtration through 3 kDa.

Akpinar, Gunay, et al. (2010) indicated that the presence of phenolic compounds in autohydrolysis liquors can inhibit microbial metabolism; however they could not determine a significant reduction after ultrafiltration with 1 kDa. Therefore, they indicated that ultrafiltration was not sufficient process to produce prebiotics (Akpinar, Gunay, et al., 2010).

Akpinar, Erdogan, et al. (2010) applied 1 kDa ultrafiltration to XOS obtained from acid hydrolysis and filtrated with 10 kDa, in order to reduce monosaccharide level. They determined that monosaccharides could not be separated effectively through 1 kDa, therefore they suggested nanofiltration or reverse osmosis after ultrafiltration with 1 kDa.

Akpinar, Gunay, et al. (2010) ultrafiltrated raw and enzymatically hydrolyzed autohydrolyis of liquors of sunflower stalk and wheat straw by 1 kDa membrane filter. They removed more than 90% of monosaccharides (from 0.33-0.11 to 0.04-0.02 g/L) and 50% of furfural (from 0.25-0.20 to 0.11-0.13 g/L) from autohydrolysis liquors. The concentration of XOS, which was the target product, was increased. Vegas et al. (2006) purified XOS from autohydrolysis of rice husks by using different membranes. By increasing the pressure to 14 from 5 bar, they were able to recover 90% of the oligosaccharides; however 58.6% of the nonsaccharide components and 20.9-46.9% of monosaccharides were kept in retentate (Vegas et al., 2006).

Zhao & Dong (2016) ultrafiltrated xylan of wheat bran through 20 and 1 kDa. After adding water into permeate from 20 kDa, in order to remove macromolecules such as protein, starch, pectin, pigment and decrease loss of xylan, they filtrated again through the same filter similar to this study. They evaporated the final permeate before filtration via 1 kDa. They performed the same filtration procedure for three times through filtration by 1 kDa and they analyzed all permeates by HPLC. XOS content of the final product was 57.5%. They finally reported that DP of XOS should be between 2 and 4 to be used in foods, and nanofiltration can be applied after filtration by 1 kDa in order to increase low DP-XOS concentration.

Swennen et al. (2005) researched ultrafiltration (with 5, 10 and 30 kDa) as an alternative method to ethanol precipitation (ethanol concentrations of 60%, between 60 and 90%, and above 90%) for purification of arabinoxylooligosaccharides from wheat flour. XOS fractions with similar structure were obtained with both methods. They suggested using ultrafiltration membranes with narrow pore sizes however it was not clear ethanol precipitation was an applicable on industrial scale (Swennen et al., 2005).

Nabarlatz, Torras, et al. (2007) purified XOS from autohydrolysis of almond shells at 179 ^{o}C for 23 min. They reported that lignin related low molar mass compounds products were about half of the impurities and continuous diafiltration with 1 kDa allowed purification of XOS (Nabarlatz, Torras, et al., 2007).

8.4. Conclusions

AC treatment of liquor was performed at different concentrations in order to remove impurities such as phenolics. Treatment with 1% was found as effective to remove phenolics without loss of XOS. Liquors from autohydrolysis and autohydrolysis followed by enzymatic hydrolysis treated with AC, were ultrafiltrated through 10 and 2 kDa size membranes. High amount of XOS was recovered after ultrafiltration with 10 kDa. On the other hand, XOS with higher DP was separated in retentate phases after ultrafiltration with 2 kDa; and xylose and acetic acid could not be separated from the liquors.

Ultrafiltration with 1 kDa size membrane or utilization of other technologies such as nanofiltration is suggested to increase purifity of XOS before utilization for prebiotic activity tests or food production.

CHAPTER 9

PREBIOTIC ACTIVITY AND STABILITY OF XYLOOLIGOSACCHARIDES

9.1. Introduction

Various bacteria living in human intestine such as *Bifidobacterium* spp, *Bacteroides* spp, *Clostridium* spp, use different type of sources for providing energy. Bifidobacteria and *Bacteroides* utilize xylan with different degree as substrate (Singh et al., 2015). Increasing the growth of bifidobacteria is one of the most important characteristics of XOS as food ingredients (Vázquez et al., 2001b). Bifidobacteria can generate a wide range of glycosidases and use complex oligosaccharides such as FOS and XOS. Lactobacilli, except *Lactobacillus brevis*, can not use XOS as an only carbon source. On the other hand, when *L. brevis* was fed with XOS, it showed a mild increase in growth. When XOS was fed with FOS, differences in growth of probiotic bacteria can be observed. FOS increased the growth of lactobacilli; however XOS was more bifidogenic (Singh et al., 2015).

XOS are utilized as food ingredients due to their healthy effects and functional properties. Xylobiose is accepted as XOS for food applications; however XOS with higher DP is considered as "oligo" for other objectives (Vázquez et al., 2001b). Xylobiose, the main compound of XOS is utilized by *Bifidobacterium*, especially by *B. adolescentis*, which is present in most adults' intestines (Okazaki, Fujikawa, & Matsumoto, 1990). Xylooligomers, raffinose and fructooligomers are preferred to hexose by *Bifidobacterium* spp. Furthermore, xylooligomers are nearly effective as raffinose and better than FOS to stimulate in vitro growth of those bacteria (Vázquez et al., 2001b). In vitro studies showed that *Bifidobacterium* spp. and *B. adolescentis* have the ability to utilize xylobiose and xylotriose; however a mixture including xylobiose as the major compound was utilized by *B. adolescentis*, *B. infantis*, and *B. longum*. Most *Lactobacillus* species, except *L. fermentum*, hardly utilized XOS (Gullón et al., 2008). *Bacteroides* utilize XOS; however to a smaller extent compared with glucose (Vázquez et al., 2001b).

The human gut microflora contains more than 400 bacterial species. Non-digested dietary carbohydrates such as resistant starch, non-starch polysaccharides, non-digestible oligosaccharides are utilized by this microflora and many products such as short-chain fatty acid (butyric acid, propionic acid and acetic acid) and other organic acids (pyruvic acid, succinic acid and lactic acid) and gases (CH_4, CO_2, H_2S and H_2) are produced (Wang, Sun, Cao, & Wang, 2010). The fermentation of oligosaccharides is dependent on DP, sugar and glycosidic linkage, synergy between bacteria during fermentation, relationship between substrate bacteria and fermentation products (Mussatto & Mancilha, 2007).

Several healthy effects of bifidobacteria have been reported as inhibiting of the formation of products such as toxic amines, preventing the growth of pathogenic bacteria by short-chain organic acid production and decreasing pH, decreasing cholesterol levels, production of vitamin, protection against infections and supporting the digestion of nutrients. They can prevent gastrointestinal infections by reducing the period of diarrhea section and preserving the fecal water content within normal levels (Vázquez et al., 2001b; Wang et al., 2010).

Prebiotic activity is defined as "the ability of a given substrate to support the growth of an organism relative to other organisms and relative to growth on a non-prebiotic substrate, such as glucose". If carbohydrates are metabolized as well as glucose by probiotic bacteria and selectively metabolized by probiotics but not other intestinal bacteria, they are considered as having a positive prebiotic activity score (Huebner, Wehling, & Hutkins, 2007). Prebiotics are functionally stable when their prebiotic activity remains the same or shows an increase before and after food processing conditions (Huebner, Wehling, Parkhurst, & Hutkins, 2008).

There are many in vivo and in vitro studies on the effect of prebiotics on the growth and activity of probiotic bacteria (Mumcu & Temiz, 2014). Many studies showed that the ability of bifidobacteria for growing on XOS depended on the considered strain. In some studies, XOS were not selective for bifidobacteria especially, because *Bacteroides* spp., *Clostridium* spp., *Lactobacillus acidophilus*, and *Klebsiella pneumoniae* also showed moderate growth on these substrates (Aachary & Prapulla, 2011).

Mumcu & Temiz (2014) reported that a suitable prebiotic should be selected for each bacterial strain in order to obtain its good growth and acidifying performance. They researched in vitro effects of six prebiotics including XOS at different

concentrations on growth and acidifying activity of *L. acidophilus* and *Bifidobacterium* spp. Type and concentration of the prebiotics were effective on growth and acidifying activity. There was no significant difference in the strains of *L. acidophilus*; however the strains of *Bifidobacterium* had significantly different growth and acidifying activity (Mumcu & Temiz, 2014).

Moura et al. (2008) compared in vitro fermentability of XOS from brewery's spent grain, corn cobs and *Eucalyptus globulus* wood with different DP (2-5, 2-14 and 2-25) by the intestinal digesta collected in three different sections (ileum, caecum, and distal colon) of the porcine intestinal tract. They reported that XOS with DP up to 25 could be fermented in vitro by three sections (Moura et al., 2008).

Kallel et al. (2014) evaluated prebiotic effect of XOS from hydrolysis of garlicstraw xylan by in vitro fermentation using *B. adolescentis* and *L. acidophilus*. They reported that both strains utilized XOS; but the efficiency was different due to absence of β-xylosidase activity in *Lactobacillus* spp. which was effective on utilization of XOS (Kallel et al., 2014).

Kontula, von Wright, & Mattila-Sandholm (1998) investigated the effect of oat bran of three different lactic acid bacteria (*L. rhamnosus, L. plantarum and Lactococcus lactis*). The results showed that all organisms utilized β-glucooligosaccharides; however only *L. plantarum* utilized XOS. Oat bran oligosaccharides influenced both qualitatively and quantitatively the end-products of lactic acid bacteria. Therefore, end-products should be considered before selecting strains for new fermented cereal based food products (Kontula et al., 1998).

Gullón et al. (2008) researched the the fermentability of XOS with DP in the range of 2-6 from autohydrolysis of rice husks by *Bifidobacterium* spp. The growth rate of *B. adolescentis* was higher than the ones of *B. longum*, *B. infantis*, and *B. breve*. The XOS consumption by *B. adolescentis* was 77% after 24 h, and the highest amount of utilization was xylotriose (90%), xylobiose (84%), xylotetraose (83%), and xylopentaose (71%) (Gullón et al., 2008).

The human studies on XOS are limited and there are a few studies on the prebiotic effect of XOS. Animal studies showed that consumption of XOS increased the moisture content of feces and reduced the pH level of feces in mice. Moreover, XOS was more effective than FOS on increasing the number of bifidobacteria (Aachary & Prapulla, 2011). According to study on the effect of XOS by Howard, Gordon, Garleb, & Kerley (1995) on the colonic microflora of mice, there was no increase in population

of bifidobacteria; however they determined an important increase in the same bacteria level in another study on utilization of XOS in human volunteers. Therefore, they reported that the difference in bifidobacterial species inhabiting the gastrointestinal tract of humans and mice can be the reason of the absence of prebiotic activity (Aachary & Prapulla, 2011).

Okazaki, Fujikawa, & Matsumoto determined positive effect of XOS on human intestinal flora in 1990. They researched the potential utilization of XOS as an energy source by intestinal bacteria in their in vitro and in vivo studies. In vitro studies showed that bifidobacteria utilized XOS; however they were not utilized by *Escherichia coli* and *Clostridium* spp. XOS was very stable at pH 7 or low gastric pH and it was degraded by human and animal digestive enzymes. In vivo studies, XOS (5 g/day) increased the population of *Bifidobacterium* spp. from 10% pre-administration level to 32% after 2 weeks, decreased fecal pH and preserved the fecal water content within normal range (Okazaki et al., 1990).

Chung, Hsu, Ko, & Chan (2007) researched the effect of XOS on the intestinal microbiota, gastrointestinal function, and nutritional parameters of the elderly who did not have recent history of gastrointestinal disease. The treatment and the control group consumed 4 g/day XOS and placebo, respectively, for 3 weeks. The results showed that XOS increased the population of bifidobacteria and the fecal moisture content; and decreased the fecal pH value. Consequently, XOS was effective on improving the intestinal health (Chung et al., 2007).

Finegold et al. (2014) researched effects of the prebiotic XOS on the composition of human colonic microbiota, pH and fecal short chain fatty acids to determine the tolerance of XOS by 32 healthy adults. Subjects consumed 1.4 g XOS, 2.8 g XOS or placebo in daily doses. XOS increased the counts of *Bifidobacterium* but not *Lactobacillus* in healthy adults. *Bifidobacterium* increased in both XOS groups; however greater increase was observed in 2.8 g/day group than the 1.4 g/day group. There were no important differences in the counts of *Lactobacillus,* Enterobacteriaceae and *Clostridium* between the groups. The results showed the possibility of XOS to be used as a food supplement (Finegold et al., 2014).

XOS are stable over a pH range of 2.5-8.0 and temperatures up to 100 ^{o}C; however XOS with DP between 3 and 5 are more sensitive to alkali decomposition than XOS with higher DP. XOS has been claimed as having better heat stability compared to FOS during pasteurization and sterilization at low pH (Singh et al., 2015; Vázquez et

al., 2001b). It is also more advantegous than inulin in terms of acids and heat, allowing their utilization in low pH juices and carbonated drinks (Vázquez et al., 2001b).

Lactobacilli and bifidobacteria are the intestinal bacteria added to foods such as yogurt and other fermented dairy products as probiotic cultures. Prebiotics show resistance to food processing conditions and thus allow them to reach the colon intact. To be used as a functional food ingredient, prebiotics must have stability to food processing conditions such as heat, low pH, and Maillard reaction conditions. The stability of prebiotics to heat and acidic conditions has been researched in many studies (Huebner et al., 2008).

The stability of oligosaccharides can be different according to the sugar residues present, their ring form and anomeric configuration linkage types. Hexoses are more firmly bonded than pentoses and β-linkages are stronger than α –linkages. Even though their strong structure, treatments at pH lower than 4.0 and high temperatures or storage at room conditions for a long time can cause loss of nutritional and physicochemical properties of oligosaccharides present in food (Mussatto & Mancilha, 2007). Moreover, undesired conditions can cause the hydrolysis of saccharides. Therefore, extreme conditons are applied in order to determine stability of XOS (Chapla et al., 2012).

The main objective was to assess the suitability of XOS obtained after autohydrolysis and enzymatic hydrolysis as a prebiotic source for selected probiotic bacteria. In vitro growth of those bacteria by utilization of XOS was evaluated. The stability of XOS to food production conditions such as low pH and high temperature; and in vitro gastrointestinal conditions was researched.

9.2. Materials and Methods

Lactobacillus brevis NRLL B-4527, *B. bifidum* NRLL B-41410, *B. breve* NRLL B-41408, *B. infantis* NRLL B-41661 and *B. longum* NRLL B-41409 used in this study was supplied by the US Department of Agriculture, Agricultural Research Service and *Bifidobacterium animalis* subsp. *lactis* DSM-10140 was obtained from Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures. Strains were grown anaerobically in MRS broth consisting cysteine and stored in eppendorf tubes including the medium supplemented with 20% glycerol as a cryoprotectant at -80°C until fermentation.

E.coli obtained from SHAM National Collection of Type Cultures was used as enteric bacteria (RSHM number: 4024).

Liquor from autohydrolysis at 190 ^{o}C for 5 min (for obtaining the highest XOS yield) was utilized for prebiotic activity and stability tests after treatment with 1% activated charcoal and lyophilization at -60 ^{o}C in a freeze dryer (Labconco, USA). Liquor from 190 ^{o}C for 0 min was hydrolyzed by *A. pullulans* XY enzyme at optimum conditions (pH 5, 40 ^{o}C, with 240 U/g XOS xylanase). After treating with activated charcoal, it was lyophilized at the same conditions; and utilized for tests.

9.2.1. Prebiotic Activity Assay

Prebiotic activity of XOS was determined by observing their effects on the growth of potential probiotic bacteria (*B. lactis, B. bifidum, B. breve, B. infantis, B. longum* and *L. brevis*). Moreover, XOS was also tested on *E. coli*. The assay was carried out in duplicate. Probiotic bacteria were activated at 37 ^{o}C for 24 h. Reinforced Clostridial Medium (RCM), which was a semi-solid medium for the enumeration and cultivation of anaerobes occurring in food and pathological samples, was used for the growth of *Bifidobacterium* species. The composition (g/L) of RCM (Oxoid, UK) was: Yeast extract 13.0, peptone 10.0, glucose 5.0, soluble starch 1.0, sodium chloride 5.0, sodium acetate 3.0, cysteine hydrochloride 0.5 and agar 0.5. Basal Medium (BM) (Palframan, Gibson, & Rastall, 2003) was used as the fermentation medium for probiotic bacteria. The composition of Basal medium was (g/mL): Peptone water 2, yeast extract 2, sodium chloride 0.1, potassium phospate dibasic 0.04, potassium phospate monobasic 0.04, magnesium sulfate heptahydrate 0.01, calcium chloride hexahydrate 0.01, sodium bicarbonate 2, , heamin 0.05, L-cysteine HCl 0.5, resazurin 0.001 and bile salt 0.5. There were also 2 µL Tween 80 and 10 µl vitamine K in 1 L of Basal Medium.

Liquid BM was prepared without adding heat sensitive components such as vitamine K1 and heamin. Resazurin as an oxygen indicator was added in the medium as the concentration would be 0.001 g/L. Resazurin was blue in the powder form and its aqueous solution was pink if there was oxygen in the medium. Therefore, the medium should be colorless in the absence of oxygen. After addition of resazurin, the medium

was heated for 20-30 min on a magnetic stirrer. When the color of medium turned from blue to pink, L-cysteine HCl (0.5g/L) was added and the medium was heated for 5 min. There was no more change in the color of the medium, thus it was taken from the strirrer and treated with high purity of N_2. The medium was added in the hungate tubes (15 ml) under N_2 and they were covered with septum and screw caps. Hungate tubes including the medium were autoclaved at 121 ^oC for 15 min. Vitamine K1 and heamin were sterilized by steril syringe filter (0.22 µm) and added into tubes by using sterile syringes (1 µL) at the end of the sterilization of the medium. The sterile syringe was used for inoculation.

E. coli activated in TSB (tryptic soy broth) agar including glucose at 37 ^oC for 24 h was. The fermentation medium for *E. coli* was prepared with (g/L) tryptone 17.0, soy peptone 3.0, sodium chloride 5.0, and potassium phospate dibasic 2.5. The growth conditions were aerobic for *E. coli*, thus the basal medium was filled in glass tubes. The tubes were sterilized at 121 ^oC for 15 min.

1% liyophilized XOS and glucose (as control) were prepared and sterilized by using 0.22 µm steril syringe filter. Active cultures were added to each tube (one of them is control and 2 of them are including XOS) as their concentrations were 1%. Samples were taken prior to incubation and after fermentation at 37 ^oC for 24 h in in a cooled incubator (Termal, Turkey); for HPLC analysis of xylose and xylooligomer concentrations. The optical density (OD) values of the mediums were measured at 600 nm (OD_{600}) by multimode microplate reader (Varioskan Flash, Themo Fisher Scientific, USA). The differences in absorbances were used to determine the amount of growth of bacteria. The growth of *E. coli* and probiotic bacteria in basal medium containing XOS instead of glucose and medium including glucose were measured.

9.2.2. The Effect of Food Production Conditions

The utilization of prebiotics in food products depend on their resistance to production conditions of those products. Therefore, XOS obtained in this study was tested on low pH and high temperature conditions that can be responsible for deterioration of oligomers. The method recommended by Huebner et al. (2008) was used in duplicate. Their study was a basis study, in order to evaluate functional stability of prebiotics to food processing conditions. XOS (2% (w/v)) was dissolved in 20 mM

citrate-phosphate buffer solution and held at various pH (3, 4, 5, 6) and temperature values. XOS solutions with different pH were incubated at 25 ^{o}C for 24 h as low pH treatment; and 85 ^{o}C for 30 min as high temperature and different pH treatment. Samples were taken before and after incubation. At the end of the incubation, pH of XOS solution was increased to 7 by NaOH and the solutions were stored at -20 ^{o}C until HPLC analysis of xylose and xylooligomers. In order to determine the effect of Maillard reaction conditions, 2% XOS and 1% glycine were added to 20 mM citrate-phosphate buffer solution (pH: 7). The solution was incubated at 85^{o}C for 3 h in a water bath. Samples were taken for each hour and stored at -20 ^{o}C until HPLC analysis. The color change as a result of Maillard reaction was determined by absorbance measurements at 420 nm (ABS_{420}). The citrate-phosphate buffer solution (at pH 7) including 2% glucose and 1% glycine incubated at the same conditions were used as reference.

9.2.3. The Effect of Digestive System Conditions

Prebiotics should pass previous steps of digestive system without deterioration before reaching the large intestine. Therefore, XOS was tested in gastric-intestinal conditions in duplicate. 1% (w/v) XOS and 0.3% pepsin were added in HCl buffer solution (g/L) including NaCl 8.0, KCl 0.2, $Na_2HPO_4.2H_2O$ 8.25, NaH_2PO_4 14.35, $CaCl_2.2H_2O$ 0.1, $MgCl_2.6H_2O$ 0.18 (Hongpattarakere, Cherntong, Wichienchot, Kolida, & Rastall, 2012; Korakli, Gänzle, & Vogel, 2002). After adjusting pH of the buffer solution to 1, the solution was incubated at 37 ^{o}C for 4 h in the incubator. After 4 h, pH was increased to 8 by NaOH and 0.1% trypsin was added to the solution. The solution was incubated at 37 ^{o}C for 6 h. Samples were taken before the treatment and after incubation at gastric and intestinal conditons. They were analyzed by HPLC to measure the concentrations of xylose and XOS.

9.2.4. HPLC Analysis

The samples were filtrated through 0.45 µm PTFE membrane filters and analyzed by HPLC (Perkin Elmer, USA) coupled with RI dedector system as decribed before in section 5.2.8. The concentrations of xylose, total low DP-XOS, xylooligomers (xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose) were determined.

9.3. Results and Discussion

After fermentation for 24 h, the microbial growth in Hungate tubes were visually monitored (Figure 9.1). No growth was observed visually in tubes including XOS from autohydrolysis or XOS after enzymatic hydrolysis for *B. breve*, *B. infantis*, *B. longum* and *L. brevis*.

Figure 9.1. Hungate tubes consisting XOS after fermentation by *B. lactis* and *B.bifidum*.

Therefore the changes in OD values were researched for only *B. lactis* and *B. bifidum*. There are many studies in litreture showing that probiotic bacteria of the same species can grow at different conditions. For instance, Wang et al. (2010) researched the utilization of XOS from wheat bran dietary fiber by *B. strains. B. adolescentis* showed the highest growth on XOS; however *B. breve* did not grow. The highest consumption rate of XOS was observed for DP 3, followed by DP 2, and DP 4–7 (Wang et al., 2010).

The increase in OD values for *B. bifidum*, *B. lactis* and *E. coli* after fermentation was obtained as presented in Table 9.1.

Table 9.1. The increase in OD_{600} values after incubation for 24 h in medium consisting glucose, XOS from autohydroysis (XOS-1) and autohydrolysis followed by enzymatic hydrolysis (XOS-2).

Bacterial Culture	**The increase in OD_{600}**		
	Glucose	**XOS-1**	**XOS-2**
B. lactis	0.26	0.07	0.08
B. bifidum	0.37	0.12	0.10
E.coli	0.22	0.00	0.17

The increase in OD values of probiotic bacteria after fermentation for 24 h was low and not higher than glucose. On the other hand, OD values of *E. coli* did not show an increase after fermentation in medium including XOS from autohydrolysis. It can be reported as higher increase was obtained for *B. bifidum* after fermentation for 24 h in

medium containing XOS. On the other hand, similar change was obtained for the same bacteria after fermentation in medium including XOS from autohydrolysis and XOS from autohydrolysis followed by enzymatic hydrolysis.

The scores can also be different according to bacterial strain tested and the type of prebiotic carbohydrate utilized.

The change in xylose and XOS concentrations (g/L) were also analyzed by HPLC as shown in Table 9.2 and Table 9.3 for XOS from autohydrolysis and XOS from autohydrolysis followed by enzymatic hydrolysis, respectively.

Table 9.2. The change in XOS concentrations after fermentation for 24 h with XOS from autohydrolysis for *B. bifidum* and *B. lactis*.

	Concentration (g/L)						
	Xylohexaose	**Xylopentaose**	**Xylotetraose**	**Xylotriose**	**Xylobiose**	**Xylose**	**Total Low-DP XOS**
Initial	0.06	0.10	0.21	0.33	0.39	0.22	1.10
B. bifidum	0.10	0.15	0.34	0.55	0.58	0.36	1.73
B. lactis	0.09	0.15	0.25	0.42	0.55	0.40	1.46

Table 9.3. The change in XOS concentrations after fermentation for 24 h with XOS from autohydrolysis followed by enzymatic hydrolysis for *B. bifidum* and *B. lactis*.

	Concentration (g/L)						
	Xylohexaose	**Xylopentaose**	**Xylotetraose**	**Xylotriose**	**Xylobiose**	**Xylose**	**Total Low-DP XOS**
Inital	0.00	0.01	0.17	1.48	1.88	0.33	3.54
B.bifidum	0.00	0.00	0.16	1.59	2.34	0.53	4.10
B. lactis	0.00	0.00	0.04	1.43	1.94	0.51	2.61

According to Table 9.2 and 9.3., XOS concentrations after 24 h fermentation were compared to initial conditions. The change in XOS concentration measured by HPLC was not consistent with the change obtained by OD measurements. Similar total low DP-XOS concentration values were obtained after fermentation in medium containing XOS from autohydrolysis. On the other hand, there was an increase in some xylooligomer concentrations after fermentation in medium containing XOS from autohydrolysis followed by enzymatic hydrolysis. The reason of that increase can be degradation of XOS with higher DP (DP>6) to low-DP XOS or monomers. The prebiotic activity was based on the change in cell biomass after 24 h of growth of the probiotics on 1% prebiotic or 1% glucose compared to the change in cell biomass of a mixture of enteric strains grown under the equivalent conditions (Huebner et al., 2007),

thus the reason of the change in cell biomass should be researched. Although Gullón, González-Muñoz, et al. (2011) determined that the purity of XOS (different molecular weight) from autohydrolysis of industrial solid wastes was not effective on prebiotic potential of XOS, the impurities such as by-products can prevent the growth of organisims.

The effect of food production conditions such as low pH and high temperature on stability of XOS from autohydrolysis was observed as presented in Figure 9.2.

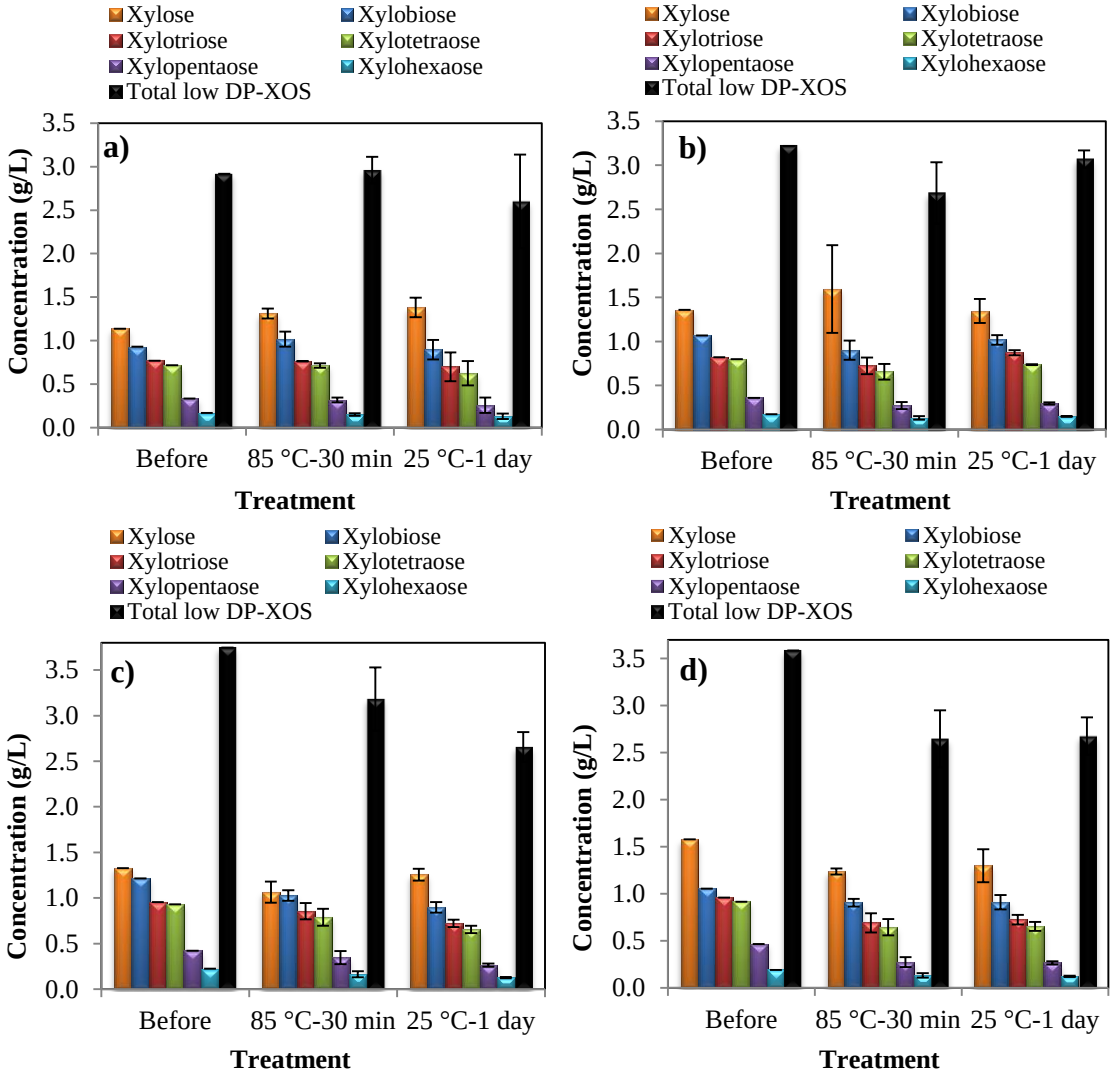

Figure 9.2. The effect of food production conditions on stability of XOS from autohydrolysis (a: pH 3, b: pH 4, c: pH 5, d: pH 6).

Holding the XOS solution at different pH values at 25 ^{o}C for 1 day and at 85 ^{o}C for 30 min did not cause any difference in xylose concentration. On the other hand, there was a decrease in low DP-XOS concentrations and total low DP-XOS

concentration at higher pH such as 4 and 5 for both of two treatments. Huebner et al. (2008) researched also the prebiotic stability of FOS and inulin by using the same treatments; however the processing conditions did not cause a significant change in prebiotic activity (Huebner et al., 2008). Böhm, Kleessen, & Henle (2006) investigated the effect of inulin from Jerusalem artichoke heated at 165 and 195 ^{o}C for 30 min on selected intestinal bacteria. Heating at 195 °C for 30 min caused degradation of the fructan chains and and the formation of new low-molecular weight products. On the other hand, when heated inulin was added to a mixed fecal culture, it showed significant stimulation of the growth of bifidobacteria and Enterobacteriaceae; and a significant decrease in growth of pathogenic bacteria. This study showed that processing of oligosaccharides or its degradation products can also improve gut microflora (Böhm et al., 2006).

The effect of food production conditions such as low pH and high temperature on stability of XOS from autohydrolysis followed by enzymatic hydrolysis was observed as presented in Figure 9.3.

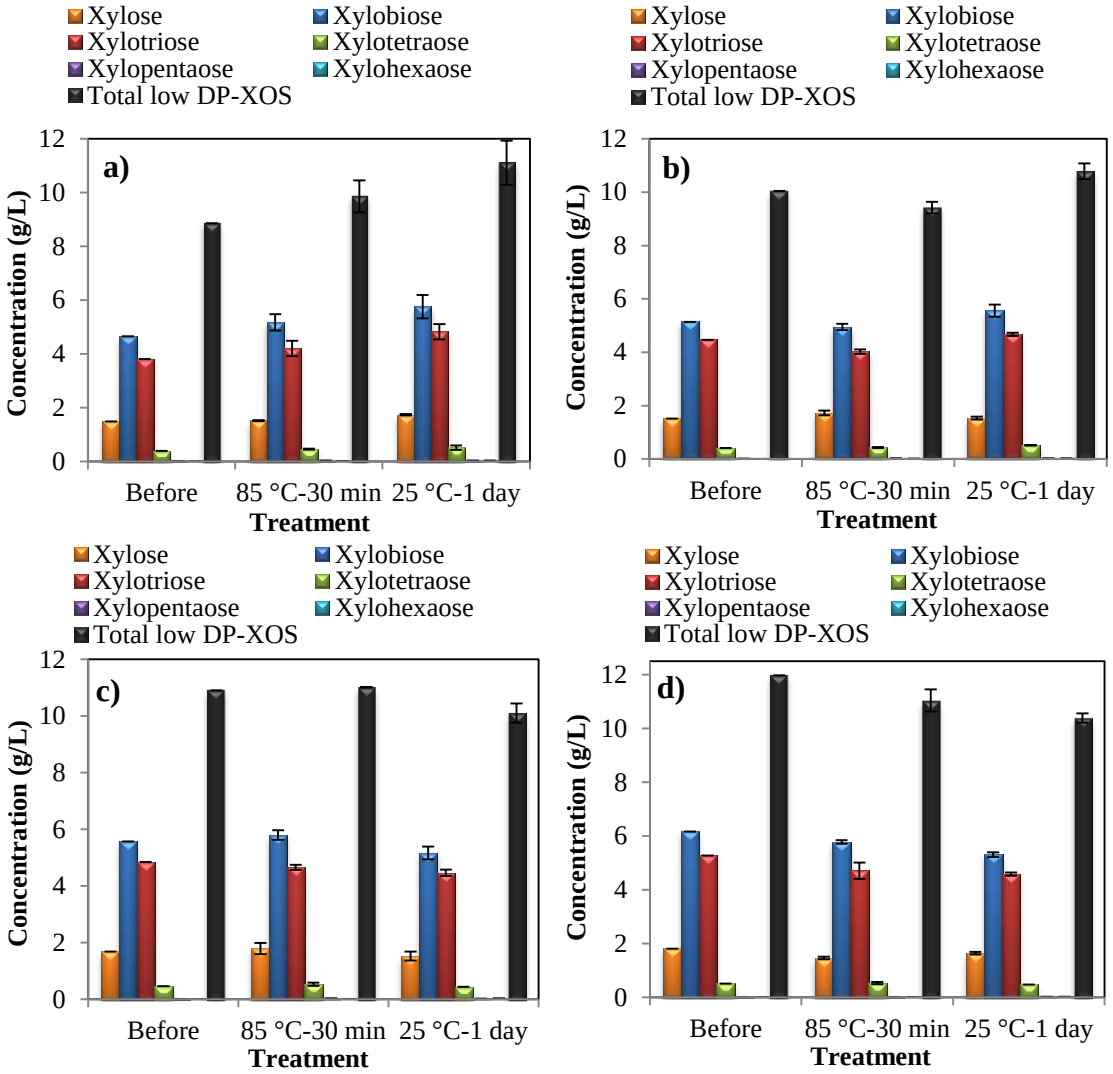

Figure 9.3. The effect of food production conditions on stability of XOS from autohydrolysis followed by enzymatic hydrolysis (a: pH 3, b: pH 4, c: pH 5, d: pH 6).

According to Figure 9.3, there were no changes in xylose, low DP-XOS and total low DP-XOS concentrations after treatment at low pH and high temperature. Therefore, it can be reported as XOS from autohydrolysis followed by enzymatic hydrolysis had higher stability to food production conditions than XOS from autohydrolysis in terms of low DP-XOS. Chapla et al. (2012) evaluated stability of XOS produced by mild alkali treatment followed by enzymatic hydrolysis by heating at different temperatures (60, 80, 100 ^{o}C) for 30 min and sterilization at different pH (2-5) for 15 and 30 min. Similarly, XOS showed high thermal stability and did not hydrolyze to monomers at low pH and after sterilization (Chapla et al., 2012).

Browning intensities of XOS from autohydrolysis and autohydrolysis followed by enzymatic hydrolysis were calculated as presented in Table 9.4. While time increased from 0 to 3 h, browning values showed an increase, as expected. Browning values of XOS from enzymatic hydrolysis were generally lower than XOS from autohydrolysis.

Table 9.4. Browning of XOS from autohydroysis (XOS-1) and autohydrolysis followed by enzymatic hydrolysis (XOS-2).

Sample	Time (h)			
	0	1	2	3
Glucose	0.01 ± 0.00	0.27 ± 0.07	0.92 ± 0.12	1.67 ± 0.23
XOS-1	6.05 ± 0.00	8.45 ± 0.11	10.1 ± 0.09	14.8 ± 0.33
XOS-2	4.81 ± 0.38	8.23 ± 1.06	8.32 ± 1.48	10.6 ± 0.54

The effect of gastrointestinal digestion conditions on stability of XOS were evaluated as shown in Table 9.5 and Table 9.6.

Table 9.5. The effect of digestion conditions on stability of XOS from autohydrolysis.

Time (h)	Concentration (g/L)					
	Xylohexaose	Xylopentaose	Xylotetraose	Xylotriose	Xylobiose	Total Low-DP XOS
0	0.30 ± 0.00	0.58 ± 0.04	1.15 ± 0.06	1.17 ± 0.09	1.05 ± 0.00	4.25 ± 0.02
4	0.10 ± 0.00	0.21 ± 0.01	0.42 ± 0.03	0.45 ± 0.04	2.58 ± 0.12	3.78 ± 0.20
10	0.09 ± 0.00	0.17± 0.01	0.34 ± 0.01	0.33 ± 0.01	1.99 ± 0.07	2.92 ± 0.69

Table 9.6. The effect of digestion conditions on stability of XOS from autohydrolysis followed by enzymatic hydrolysis.

Time (h)	Concentration (g/L)					
	Xylohexaose	Xylopentaose	Xylotetraose	Xylotriose	Xylobiose	Total Low-DP XOS
0	0.00 ± 0.00	0.02 ± 0.00	0.41 ± 0.00	3.60 ± 0.00	4.58 ± 0.01	8.62 ± 0.01
4	0.00 ± 0.00	0.01 ± 0.00	0.18 ± 0.00	1.67 ± 0.01	4.93 ± 0.02	6.79 ± 0.03
10	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.01	1.53 ± 0.08	4.78 ± 0.31	6.49 ± 0.40

There was a decrease in low DP-XOS and total low-DP XOS concentration after digestion at gastric conditions. Adding pepsin-HCl solution in liquor for gastric digestion probably caused degradation of high DP-XOS and the decrease in xylooligomer concentrations. On the other hand, no significant change was observed after intestinal digestion conditions. The results showed that gastric conditions caused an important loss of low DP-XOS due to the effect of acidic conditions; however XOS

can be stable after intestinal conditions. The change in XOS concentrations can be dependent on the source of XOS. Therefore, the stability of XOS to food production and digestive system conditions should be researched in order to determine its feasibility for food production.

9.4. Conclusions

In vitro fermentability of XOS from autohydrolysis and XOS from autohydrolysis followed by enzymatic hydrolysis of hazelnut shell by probiotic bacteria such as *B. lactis*, *B. bifidum*, *B. breve*, *B. infantis*, *B. longum* and *L. brevis* were researched after lyophilization of XOS. The growth in medium containg XOS was obtained for *B. lactis* and *B. bifidum*. The changes in optical density of samples were measured after fermentation by XOS from autohydrolysis and XOS from autohydrolysis followed by enzymatic hydrolysis for 24 h and positive differences were obtained. On the other hand, no important change in low DP-XOS and total low DP-XOS concentration were determined by HPLC.

The stability of XOS to gastrointestinal conditions was evaluated. Low DP-XOS concentration decreased after gastric digestion due to the effect of acidic pH; however no change was determined after intestinal digestion. XOS from autohydrolysis followed by enzymatic hydrolysis, in which low DP-XOS% was more, showed higher stability to food production conditions such as low pH (3-6) and high temperature (85 ^{o}C) compared to XOS from only autohydrolysis. Therefore, prebiotic activity and stability of prebiotics to food processing conditions such as heat, low pH, and Maillard reaction conditions should be researched for each type of XOS.

CHAPTER 10

CONCLUSION

Interest in prebiotics has been increasing due to their positive effects on human health and several oligosaccharides are used in food formulations. Using agricultural by-products as the source of prebiotics can provide economically feasible processes for prebiotic products. A large amount of hazelnut wastes is discarded during its harvesting and processing. Although there were some studies on valorization of hazelnut wastes for different purposes, there was no study about XOS production. The production of XOS from hazelnut wastes was investigated for the first time in this study.

The results obtained from the present study clearly showed the potential utilization of hazelnut wastes, which are produced in a large amount in Turkey, for the production of XOS with prebiotic potential and resistant to food production conditions. There were two different approaches in this study for production of XOS: autohydrolysis and enzymatic hydrolysis. They were used for valorization of hazelnut wastes for the first time. Alkali extraction was also performed to obtain xylan from hazelnut wastes; however the extracted xylan was not utilized for enzymatic hydrolysis due to having lower yields than autohydrolysis. According to characterization analysis of hazelnut wastes such as shell, husk and pruning wastes; shell had the highest xylan content (18.7%). Therefore the combined effect of autohydrolysis temperature (150-200 ^{o}C) and time (0-45 min) on XOS yield was researched using severity factor for shell. Autohydrolysis at 190 ^{o}C for 5 min were the mild conditions at which the highest yield was obtained as 62.93%. On the other hand low DP-XOS% of the liquor increased as severity factor increased and reached the maximum value as 73% at 190 ^{o}C for 30 min. In order to decrease DP of XOS, enzymatic hydrolysis was performed with a commercial xylanase (Accellerase XY) and a non-commercial xylanase from *A. pullulans*. The optimum hydrolysis conditions were determined as hydrolysis with 240 U/g XOS non-commercial xylanase at 40 ^{o}C and pH 5 for 24 h. After hydrolysis, the low DP-XOS content of the liquor was increased from 10.7 to 32.7%. In order to remove phenolics and lignin, autohydrolysis liquor was treated with activated charchoal at different concentrations such as 1, 2, 5 and 10% (w/v). Treatment with 1% activated

charchoal was enough to remove phenolics according to total phenolic content analysis of treated liquors. XOS from autohydrolysis and autohydrolysis followed by enzymatic hydrolysis were ultrafiltrated through 10 and 2 kDa size membranes after treated with activated charchoal. XOS was recovered after ultrafiltration with 10 kDa. Acetic acid and xylose could not be removed after ultrafiltration; however the fraction of low DP-XOS in total amount of XOS in liquor could be increased after ultrafilration with 2 kDa. XOS from autohydrolysis and autohydrolysis followed by enzymatic hydrolysis showed prebiotic effect on *B. bifidum* and *B. lactis* and a good stability to food production conditions such as low pH (3-6) and high temperature (85 ^{o}C). Prebiotic activity of XOS can be improved by purification of XOS with lower size membranes and other technologies. The prebiotic activity and stability of purified XOS should also be researched. Prebiotic activity of XOS should be investigated by in vivo experiments.

Production of a high value product from a low value raw material can contribute to Turkish economy and dependence on import for prebiotics may decrease. This study showed that hazelnut wastes could be valorized to produce XOS with prebiotic potential and if the activity is improved, it can be used in food applications.

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APPENDIX A

HPLC CHROMATOGRAMS

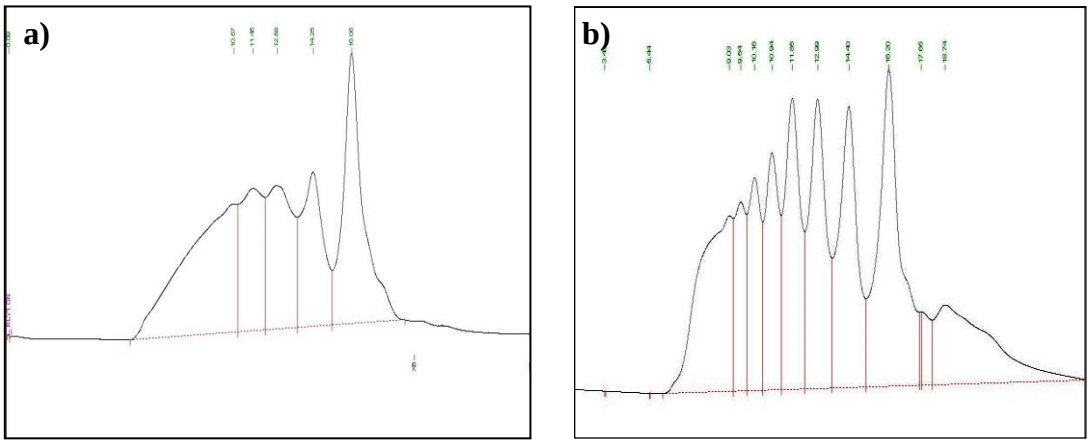

Figure A.1. Chromatograms of liquor from autohydrolysis at 180 ^{o}C for 45 min without deacetylation (a) and treated with 0.5% (w/v) NaOH (b).

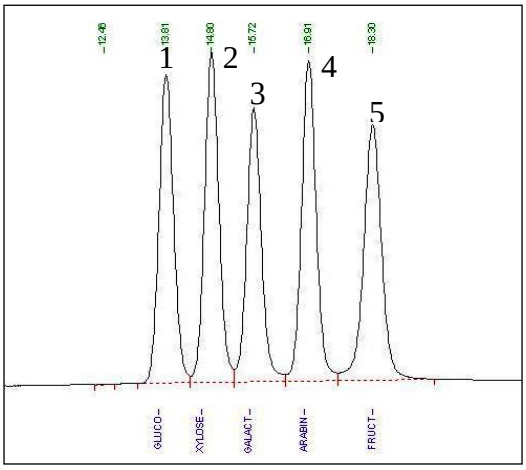

Figure A.2. Chromatogram of standard monosaccharides (1 g/L) (1: glucose, 2: xylose, 3: galactose, 4: arabinose, 5: fructose).

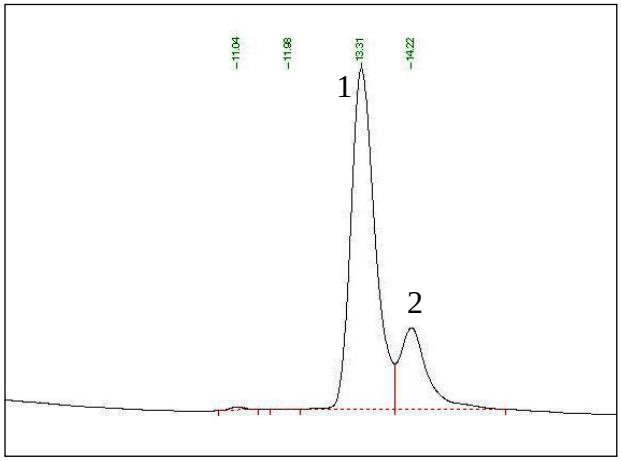

Figure A.3. Chromatogram of processed solids from autohydrolysis at 190 ^{o}C for 5 min (1: glucose, 2: xylose).

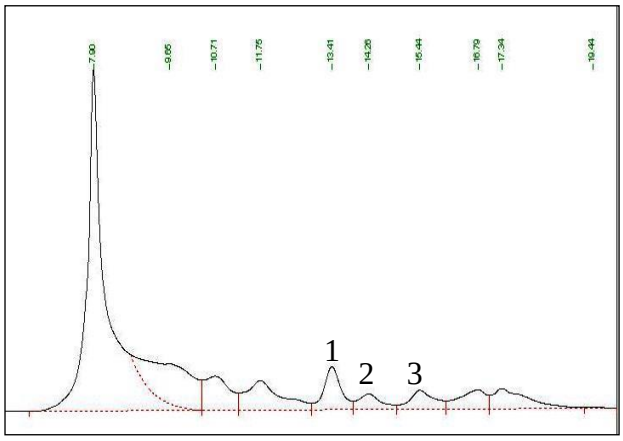

Figure A.4. Chromatogram of liquor from autohydrolysis at 190 ^{o}C for 5 min (1: xylose, 2: galactose, 3: arabinose).

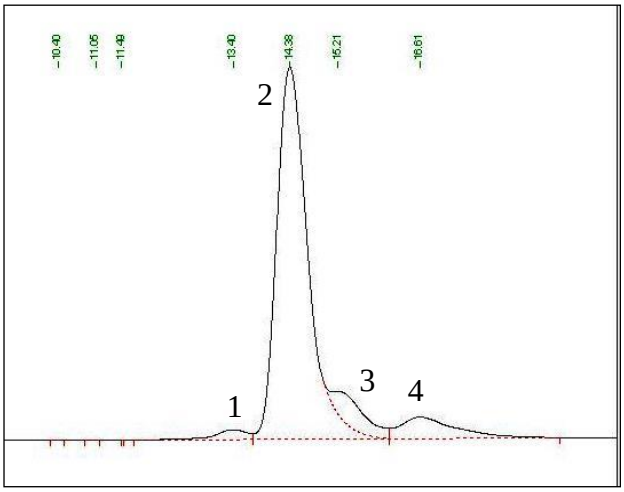

Figure A.5. Chromatogram of acid hydrolyzed liquor from autohydrolysis at 190 ^{o}C for 5 min (1: glucose, 2: xylose, 3: galactose, 4: arabinose).

APPENDIX B

CALIBRATION CURVES FOR HPLC ANALYSIS

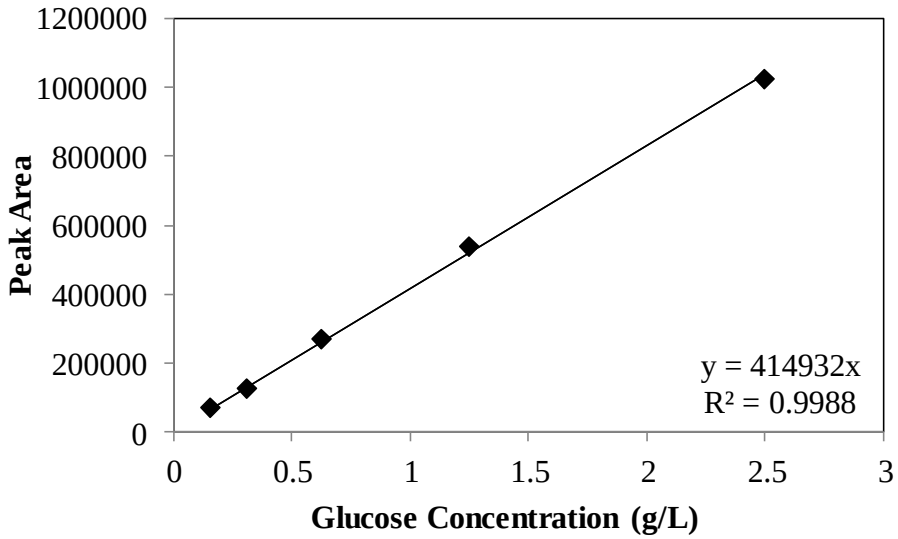

Figure B.1. Standard calibration curve of glucose for HPLC analysis.

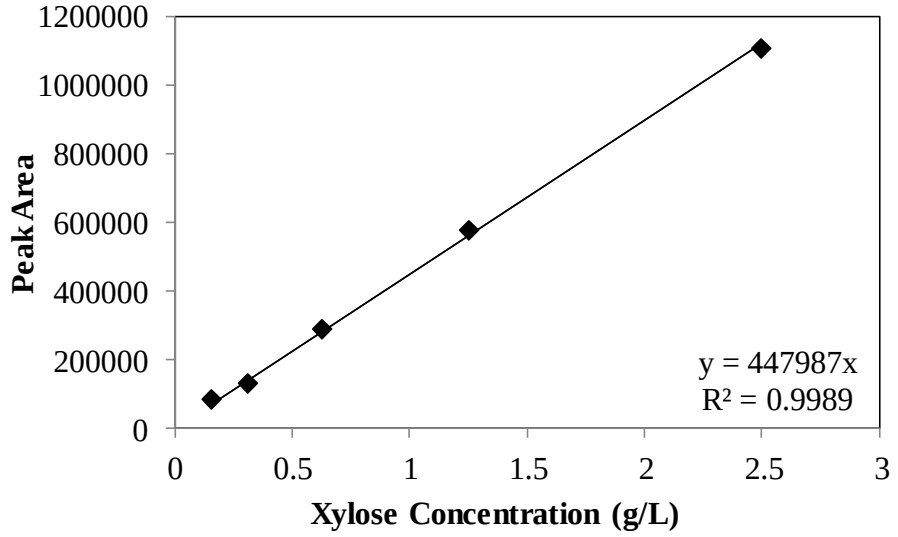

Figure B.2. Standard calibration curve of xylose for HPLC analysis.

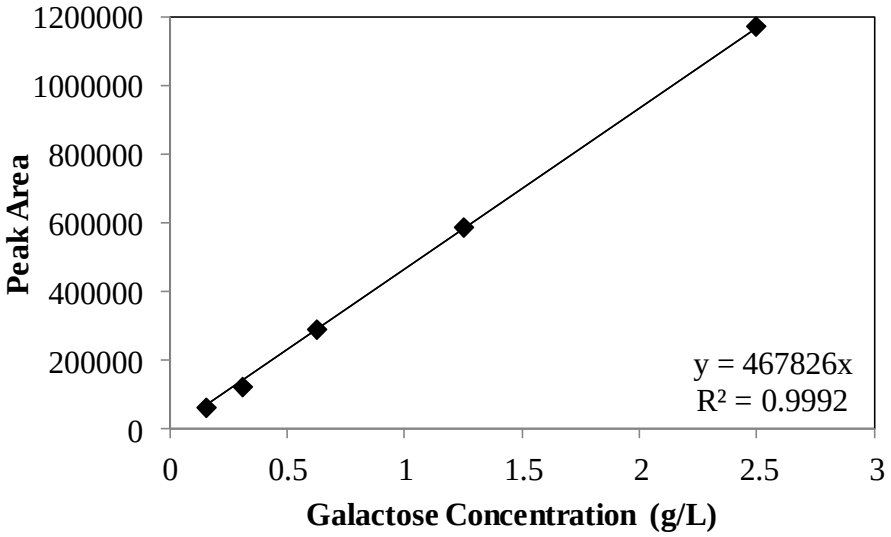

Figure B.3. Standard calibration curve of galactose for HPLC analysis.

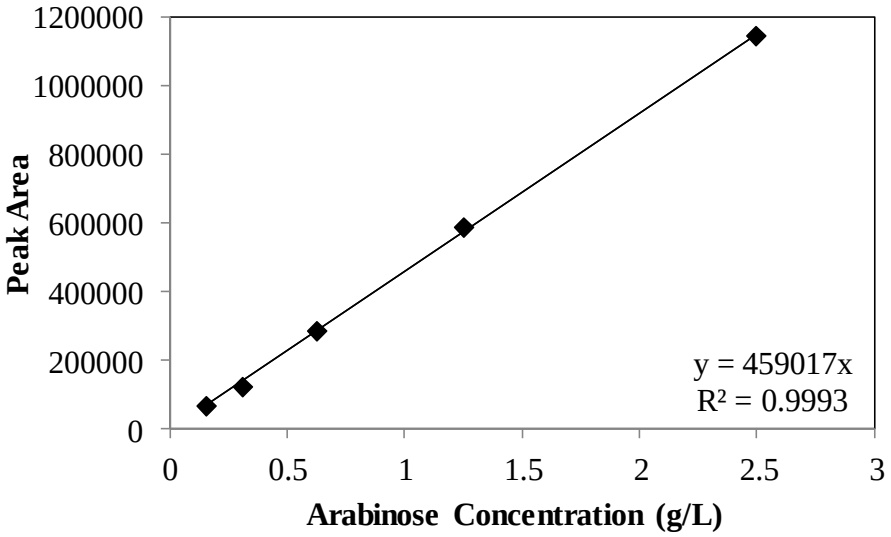

Figure B.4. Standard calibration curve of arabinose for HPLC analysis.

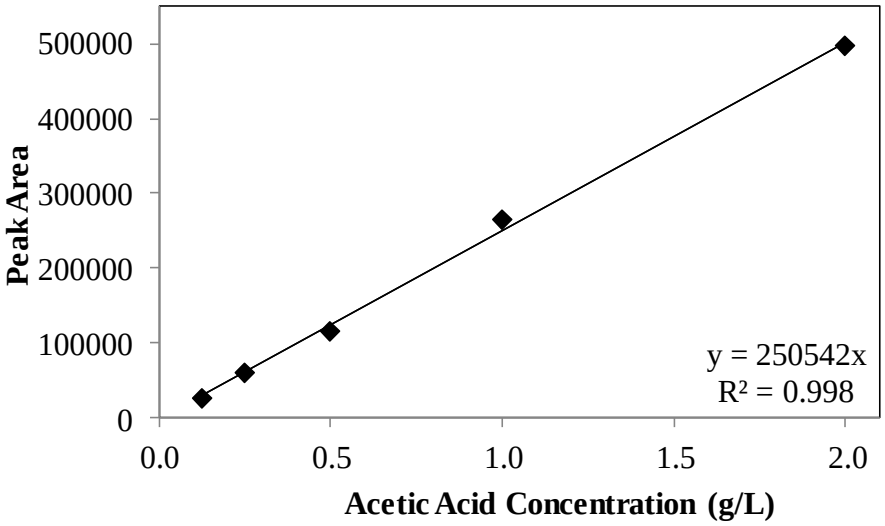

Figure B.5. Standard calibration curve of acetic acid for HPLC analysis.

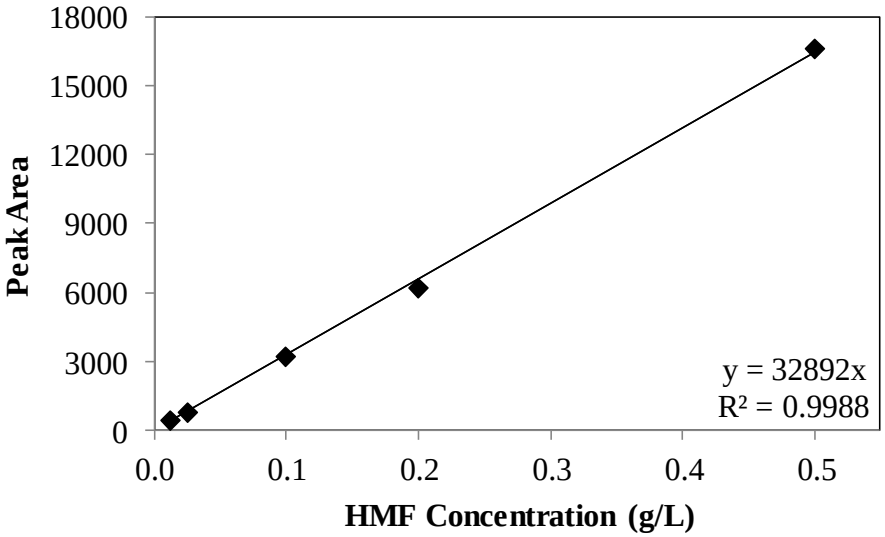

Figure B.6. Standard calibration curve of HMF for HPLC analysis.

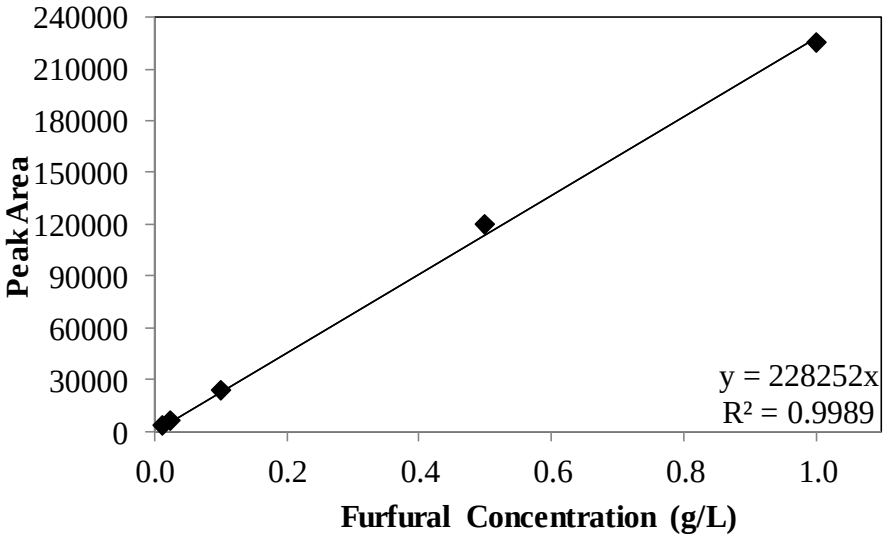

Figure B.7. Standard calibration curve of furfural for HPLC analysis.

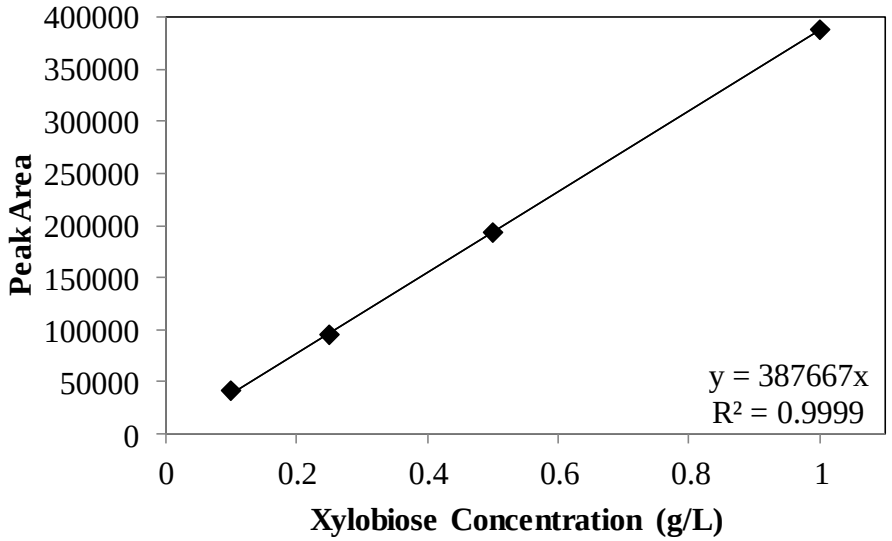

Figure B.8. Standard calibration curve of xylobiose for HPLC analysis.

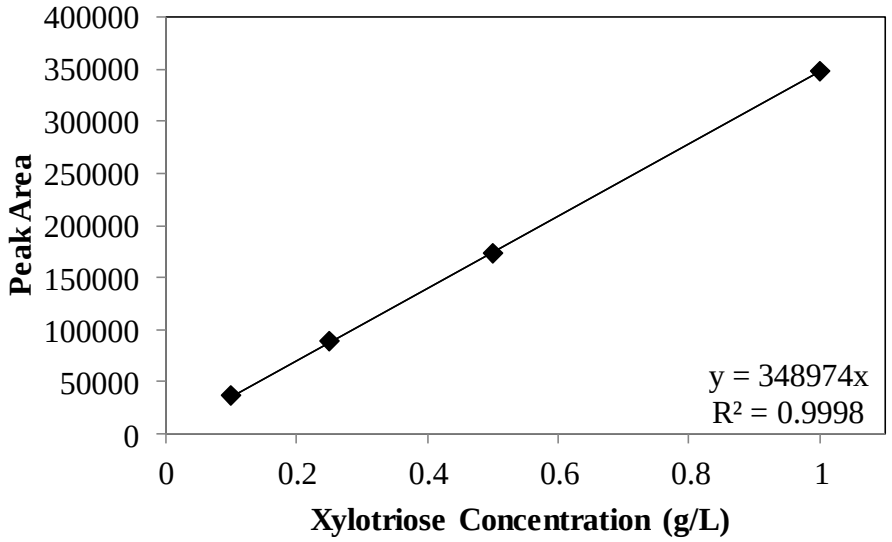

Figure B.9: Standard calibration curve of xylotriose for HPLC analysis.

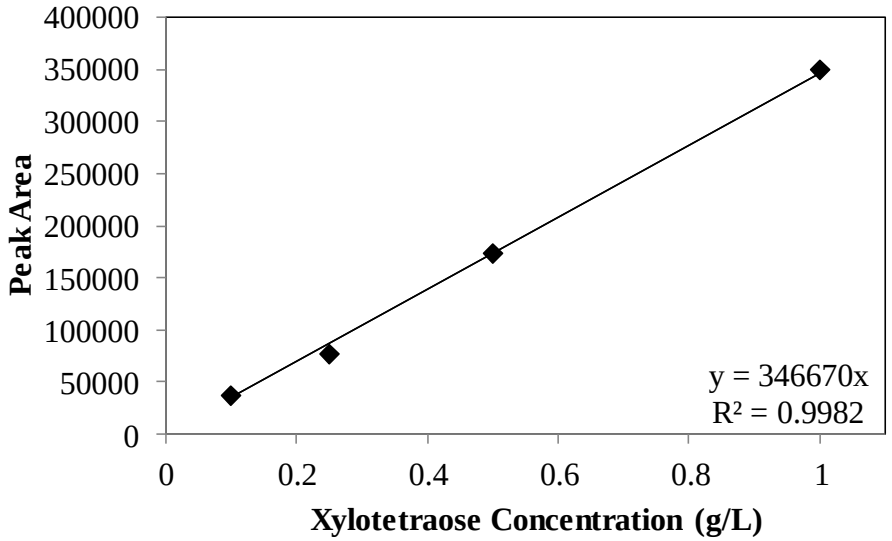

Figure B.10. Standard calibration curve of xylotetraose for HPLC analysis.

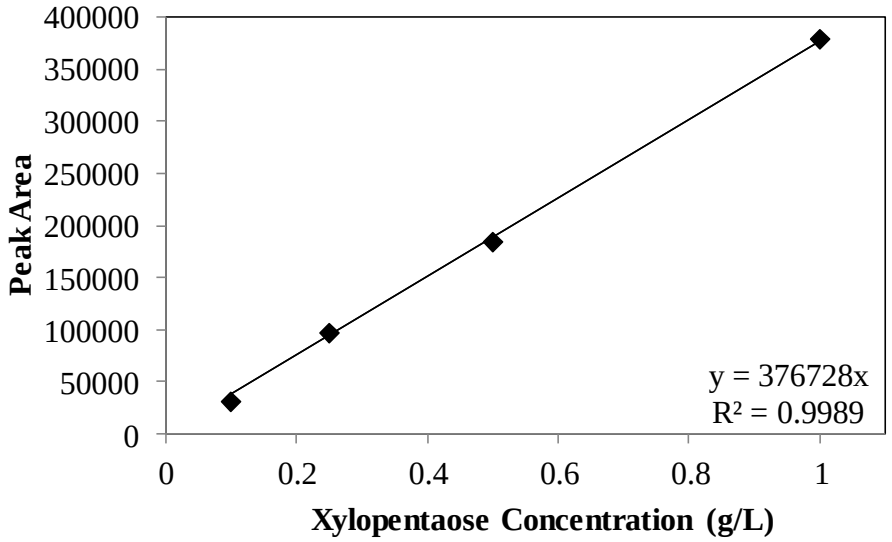

Figure B.11. Standard calibration curve of xylopentaose for HPLC analysis.

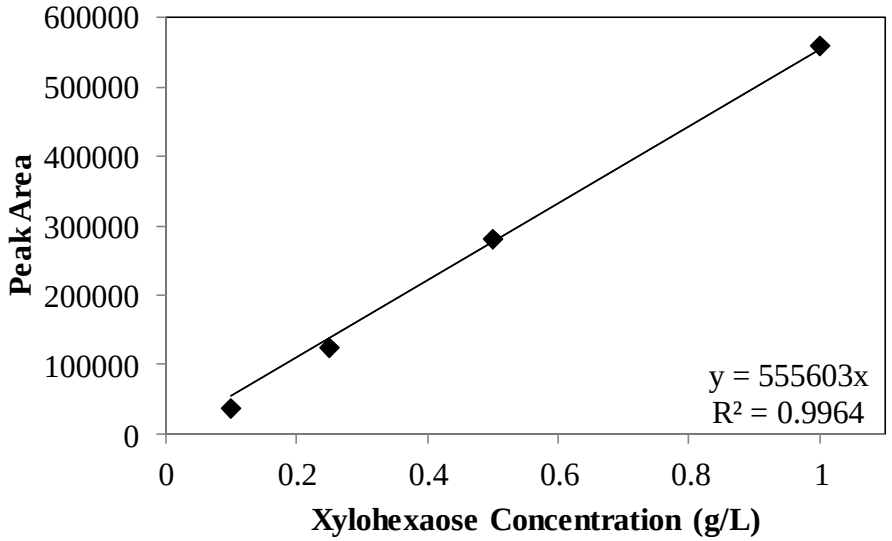

Figure B.12. Standard calibration curve of xylohexaose for HPLC analysis.

APPENDIX C

CALIBRATION CURVES FOR SPECTROPHOTOMETRIC ASSAYS

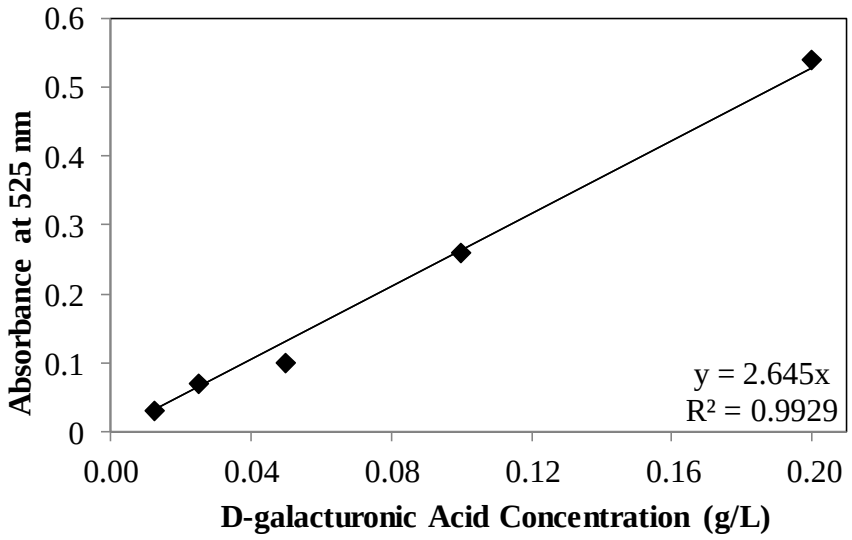

Figure C.1. Standard calibration curve of D-galacturonic acid for uronic acid analysis.

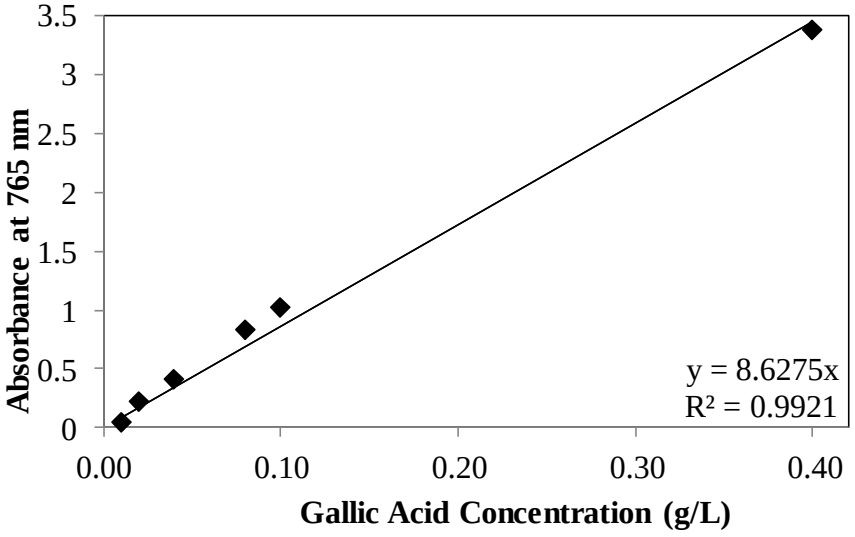

Figure C.2. Standard calibration curve of gallic acid in water for TPC analysis.

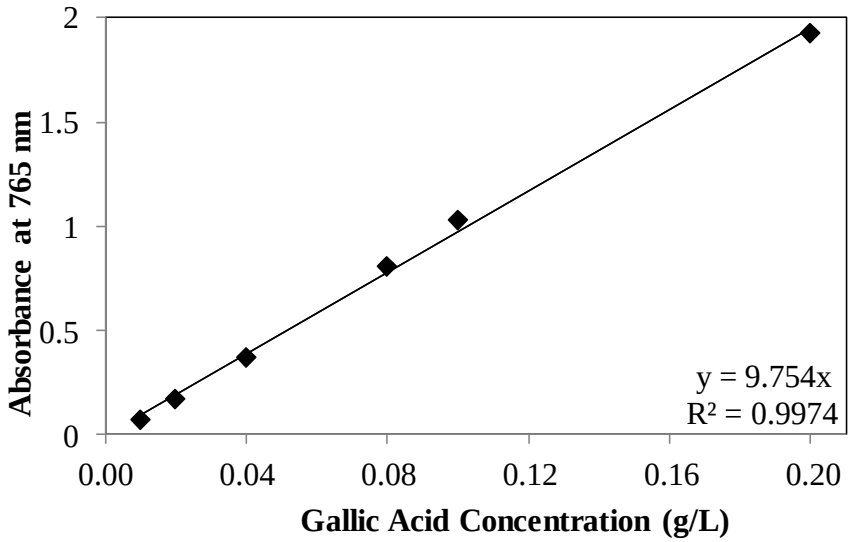

Figure C.3. Standard calibration curve of gallic acid in 80% methanol-water for TPC analysis.

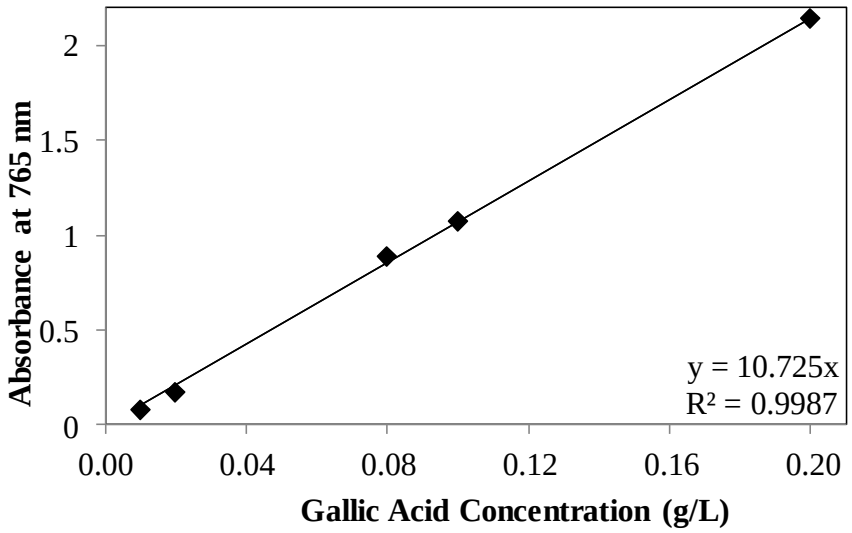

Figure C.4. Standard calibration curve of gallic acid in 80% acetone-water for TPC analysis.

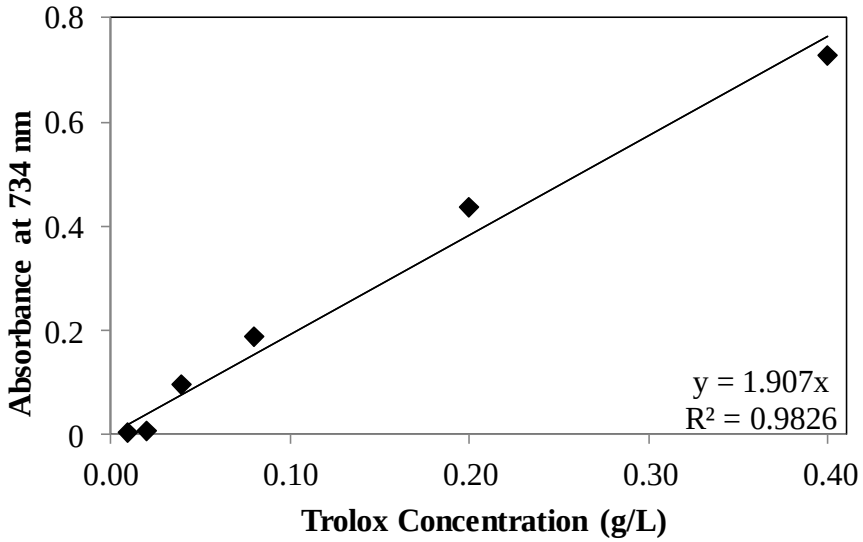

Figure C.5. Standard calibration curve of Trolox in water for TAA analysis.

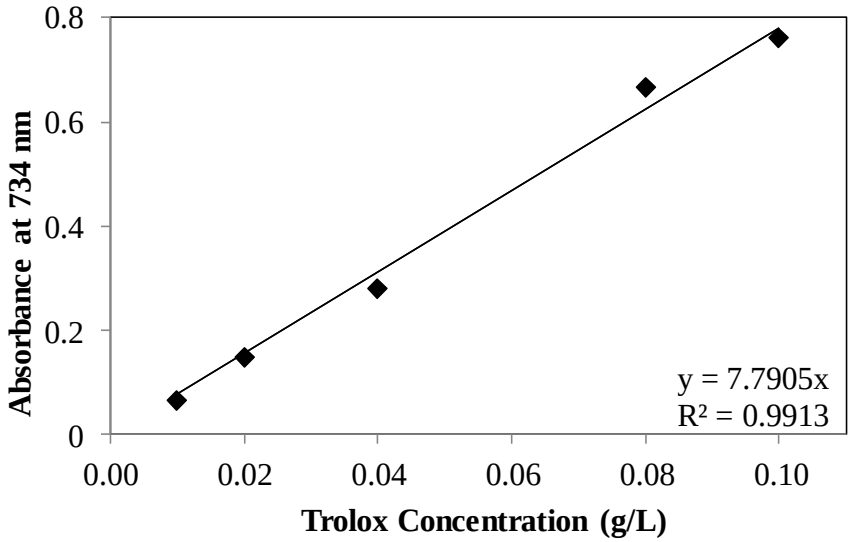

Figure C.6. Standard calibration curve of Trolox in 80% methanol-water for TAA analysis.

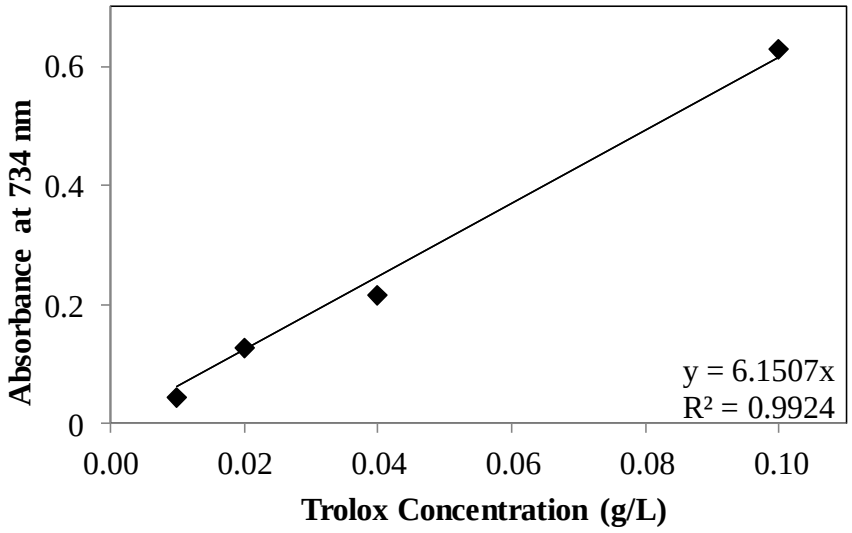

Figure C.7. Standard calibration curve of Trolox in 80% acetone-water for TAA analysis.

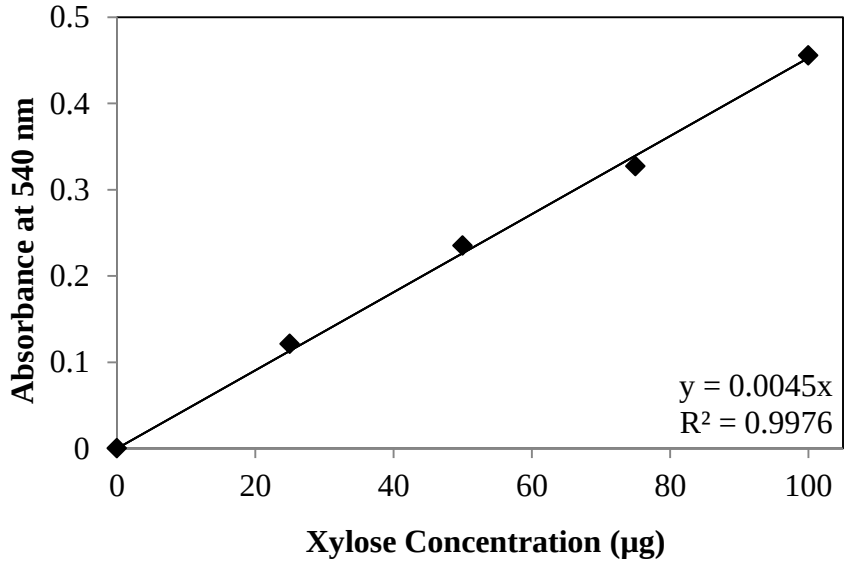

Figure C.8. Standard calibration curve of xylose for enzymatic activity analysis.

APPENDIX D

SPSS TABLES FOR STATISTICAL ANALYSIS (α =0.05)

Table D.1. ANOVA Table for total XOS concentrations in liquors.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	239.699	12	19.75	63.099	0.000
Within Groups	4.115	13	0.317		
Total	243.814	25			

Table D.2. Statistical comparison of total XOS concentrations in liquors by Tukey test.

Samples	N	Subset for alpha = 0.05						
		1	2	3	4	5	6	7
15015	2	0.163						
16015	2	1.675	1.675					
20015	2		3.121	3.121				
19030	2			4.651	4.651			
17015	2				5.398	5.398		
2000	2					7.447	7.447	
1805	2						8.007	8.007
18045	2						8.040	8.040
18015	2						8.560	8.560
19015	2						8.720	8.720
18030	2						8.768	8.768
1900	2						9.200	9.200
1905	2							10.14
Sig.		0.351	0.407	0.337	0.969	0.086	0.195	0.068

Means for groups in homogeneous subsets are displayed.

Table D.3. ANOVA Table for TPC (mg GAE/mL), acetic acid, total XOS and xylose concentrations (g/L) in liquors after activated charchoal treatment with different concentrations.

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Groups	11.126	4	2.781	5.057E3	0.000
	Within Groups	0.003	5	0.001		
	Total	11.129	9			
Acetic acid	Between Groups	1.027	4	0.257	109.240	0.000
	Within Groups	0.012	5	0.002		
	Total	1.039	9			
XOS	Between Groups	203.411	4	50.853	63.970	0.000
	Within Groups	3.975	5	0.795		
	Total	207.386	9			
Xylose	Between Groups	0.116	4	0.029	5.693	0.042
	Within Groups	0.025	5	0.005		
	Total	0.142	9			

Table D.4. Statistical comparison of TPC (mg GAE/mL), acetic acid, total XOS and xylose concentrations (g/L) in liquors after activated charchoal treatment with different concentrations by Tukey test.

TPC

Samples	N	Subset for alpha = 0.05				
		1	2	3	4	5
10	2	0.095				
5	2		0.210			
2	2			0.585		
1	2				1.220	
0	2					2.975
Sig.		1.000	1.000	1.000	1.000	1.000

Acetic acid

Samples	N	Subset for alpha = 0.05	
		1	2
10	2	1.865	
2	2	1.905	
5	2	1.915	
1	2		2.460
0	2		2.620
Sig.		0.832	0.100

XOS

Samples	N	Subset for alpha = 0.05	
		1	2
10	2	6.955	
5	2	10.025	
2	2		15.690
1	2		17.190
0	2		18.855
Sig.		0.086	0.078

Xylose

Samples	N	Subset for alpha = 0.05	
		1	2
10	2	1.985	
2	2	2.140	2.140
0	2	2.205	2.205
5	2	2.240	2.240
1	2		2.300
Sig.		0.076	0.298

Means for groups in homogeneous subsets are displayed.

APPENDIX E

THE EFFECT OF ENZYMATIC HYDROLYSIS CONDITIONS ON XYLOOLIGOMER CONCENTRATION

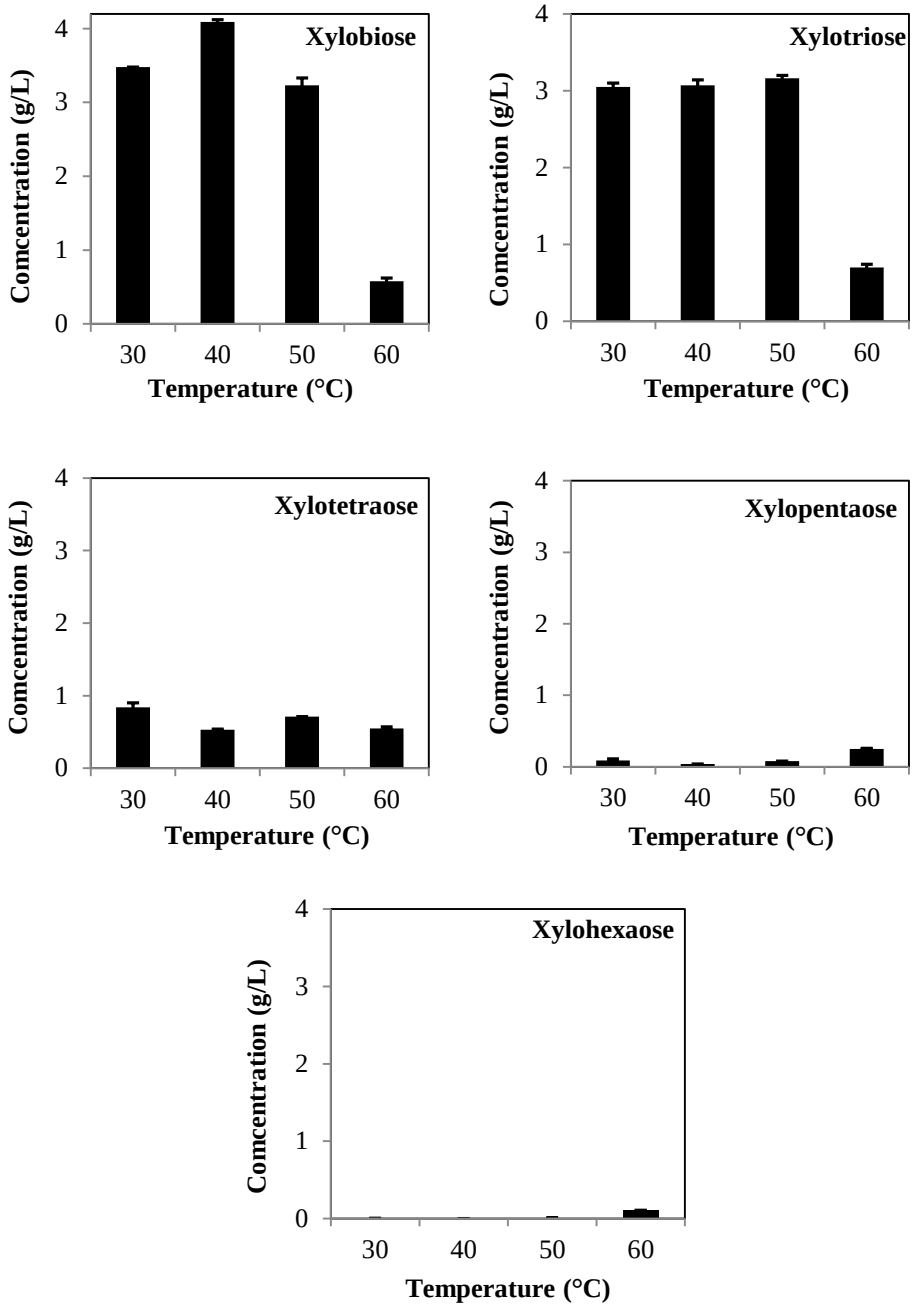

Figure E.1. The effect of temperature on xylooligomer concentrations.

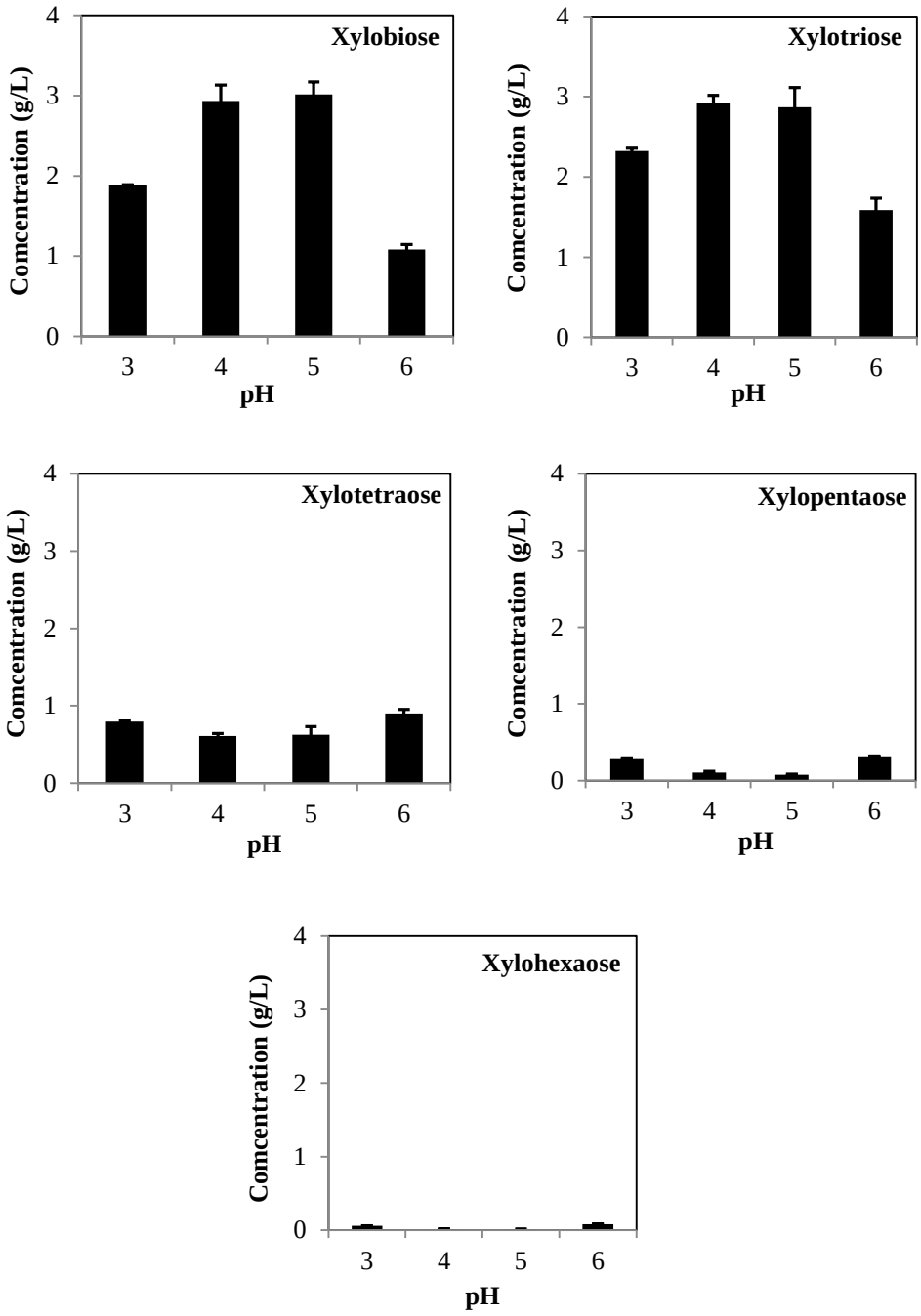

Figure E.2. The effect of pH on xylooligomer concentrations.

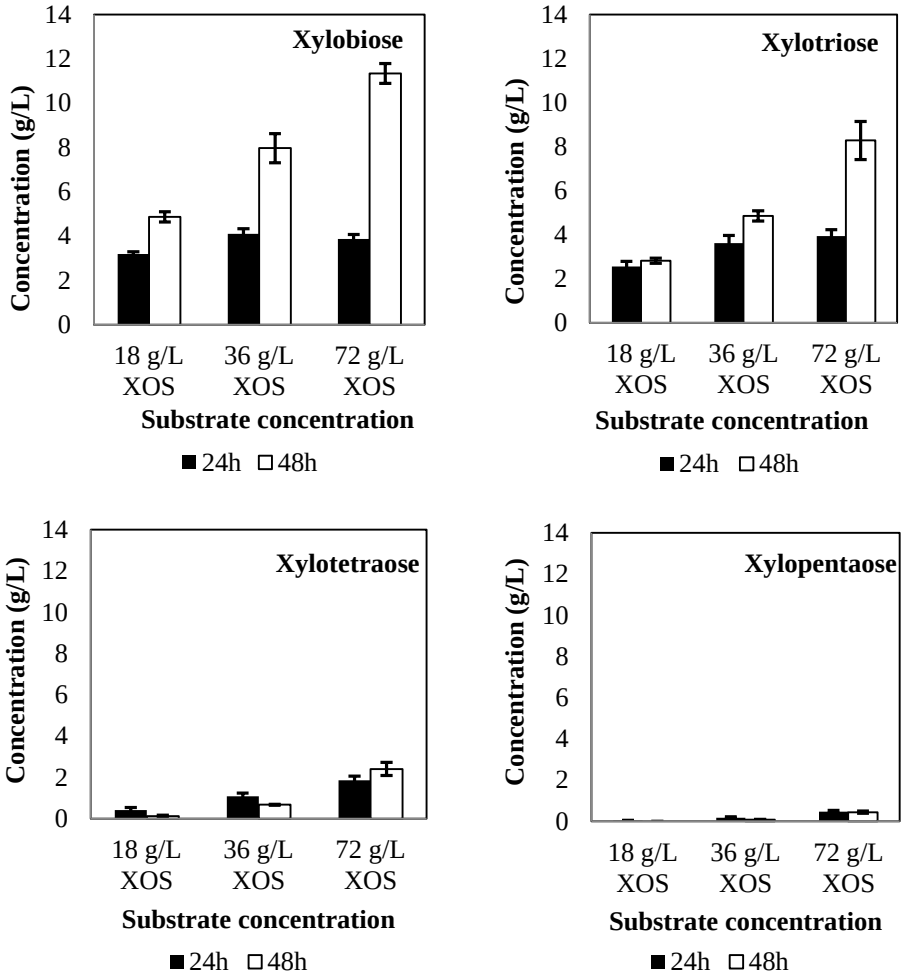

Figure E.3. The effect of substrate concentration on xylooligomer concentrations.

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Publications/Presentations on the Thesis:

- **Surek E.,** Buyukkileci, A. O., 2017. Production of xylooligosaccharides by autohydrolysis of hazelnut (*Corylus avellana* L.) shell. *Carbohydrate Polymers*, 174: 565-571.
- Buyukkileci, A.O., **Surek E.,** Dogru, K., Yegin, S., 2016. Valorization of hazelnut wastes by production of prebiotic oligosaccharide and bioethanol. Abstract book of *The Food Factor I Barcelona Conference*, November, 2-4, Barcelona, Spain, p. 287 (Oral).
- **Surek E.,** Buyukkileci, A. O., 2015. Autohydrolysis of hazelnut shell for xylooligosaccharides production. *EURO FOOD CHEM XVIII*, October 13-16, Madrid, Spain (Oral and Poster).
- **Surek, E.,** Dogru, K., Buyukkileci, A. O., 2014. Prebiotic xylooligosaccharide production from agricultural wastes. Abstract book of *International Conference and Exhibition on Nutraceuticals, Natural Health Products and Dietary Supplements*, October 14-17, Istanbul, Turkey, p. 252 (Poster).

Other International Publications:

- **Surek, E.,** Nilufer-Erdil, D., 2016. Phenolic contents antioxidant activities and potential bioaccessibilities of industrial pomegranate nectar processing wastes. *International Journal of Food Science & Technology*, 51(1), 231-239.
- Oksuz, T., **Surek, E.**,Tacer, C. Z., Nilufer-Erdil, D., 2015. Phenolic contents and antioxidant activities of persimmon and red beet jams produced by sucrose impregnation. *Food Science and Technology*, 3(1), 1-8.
- Sengul, H., **Surek, E.,** Nilufer-Erdil, D., 2014. Investigating the effects of food matrix and food components on bioaccessibility of pomegranate (*Punica granatum*) phenolics and anthocyanins using an *in-vitro* gastrointestinal digestion model. *Food Research International*, 62, 1069-1079.
- **Surek, E.,** Nilufer-Erdil, D., 2014. Changes in phenolics and antioxidant activity at each step of processing from pomegranate into nectar. *International Journal of Food Sciences and Nutrition*, 65(2), 194-202.