POTENTIAL OF DEEP EUTECTIC SOLVENT PRETREATMENT FOR XYLOOLIGOSACCHARIDES PRODUCTION FROM CORNCOB

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ABSTRACT

POTENTIAL OF DEEP EUTECTIC SOLVENT PRETREATMENT FOR XYLOOLIGOSACCHARIDES PRODUCTION FROM CORNCOB

Enzymatic hydrolysis of xylan to produce xylooligosaccharides (XOS) requires pretreatment of the feedstock lignocellulosic biomass. The current pretreatments have some disadvantages, such as the use of harsh chemicals, high energy requirements, and impurity generation. This study aimed to develop a greener method for high-purity XOS production based on mild pretreatment of corncob and subsequent enzymatic hydrolysis. Both deep eutectic solvent (DES) and dilute alkali pretreatments provided approximately 65% lignin removal from the corncob, while 80% of the xylan remained in the solid. When corncob was treated with DES at 130 °C for 2 h, the total XOS yield in the hydrolysis was 44.1%, and the yield of xylotriose and xylobiose (LDP XOS) was 39.7% based on feedstock xylan. Dilute alkali treatment of corncob at 30 °C for 8 h, provided a total XOS yield and LDP XOS yields of 41.6% and 33.4%, respectively. The enzyme was removed from the XOS solution, while most of the LDP XOS was recovered, using the ultrafiltration system with a 10 kDa membrane. Fermentable sugars were produced from the spent solids as a secondary product using cellulase hydrolysis. This study suggested that DES and dilute alkali-based processes can be considered an eco-friendly approach to XOS production. Since XOS was mostly composed of LDP XOS, they can be preferred as food additives.

ÖZET

MISIR KOÇANINDAN KSİLOOLİGOSAKKARİT ÜRETİMİ İÇİN DERİN ÖTEKTİK ÇÖZELTİ ÖNİŞLEMİNİN POTANSİYELİ

Ksilooligosakkaritler (KOS) üretmek için ksilanın enzimatik hidrolizi, hammadde lignoselülozik biyokütlenin ön işleme tabi tutulmasını gerektirir. Mevcut önişlemler, sert kimyasalların kullanımı, yüksek enerji gereksinimleri ve safsızlık oluşumu gibi bazı dezavantajlara sahiptir. Bu çalışmada, mısır koçanına hafif önişlem ve ardından enzimatik hidroliz uygulanmasıyla yüksek saflıkta KOS üretimi için daha çevreci bir yöntem geliştirilmesi amaçlanmıştır. Hem derin ötektik çözücü (DES) hem de seyreltik alkali önişlemleri mısır koçanından yaklaşık %65 lignin uzaklaşması sağlarken, ksilanın %80'i katıda kalmıştır. Mısır koçanı, 130 °C'de 2 sa. boyunca DES ile muamele edildiğinde, hidrolizdeki toplam KOS verimi %44,1 ve ksilotrioz ve ksilobioz (LDP KOS) verimi, hammadde ksilanına dayalı olarak %39,7 idi. Mısır koçanına 30 ^{o}C'de 8 sa. boyunca uygulanan seyreltilmiş alkali muamelesi, sırasıyla %41.6 ve %33.4 toplam KOS ve LDP KOS verimi sağlamıştır. Enzim, KOS çözeltisinden uzaklaştırılırken, LDP KOS'un çoğu, 10 kDa'lık bir membran ile ultrafiltrasyon sistemi kullanılarak geri kazanılmıştır. Selülaz hidrolizi kullanılarak ikincil bir ürün olarak kullanılmış katılardan fermente edilebilir şekerler üretilmiştir. Bu çalışma, DES ve seyreltik alkali bazlı süreçlerin KOS üretimine çevre dostu bir yaklaşım olarak kabul edilebileceğini öne sürmüştür. KOS çoğunlukla LDP KOS'tan oluştuğu için gıda katkı maddesi olarak tercih edilebilir.

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CHAPTER 1

INTRODUCTION

Recently, the production of high-value-added compounds and biofuels has gained attention due to the sustainability and valorization of biomass from the perspective of the biorefinery concept. The use of lignocellulosic biomass instead of non-renewable resources can eliminate the competition in energy and food production areas. Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin.

In the processes in which lignocellulosic biomass will be used as a raw material, the first target has mostly been to provide hydrolysis of cellulose. In addition, it is of great importance to obtain products from xylan (a widely occurring hemicellulose) with high added value and market potential. It is possible to obtain monomers, oligomers, or polymers from xylan with appropriate pretreatments. For example, high-value-added products such as xylitol, furfural, and XOS can be obtained from xylan. Moreover, since cellulose and lignin are not damaged during the processing of xylan, it is possible to valorize these components in the next stages. With this approach, it is possible to maximize the use of lignocellulosic biomass and create a sustainable bioeconomy.

XOS, which have prebiotic activity, are used in various industrial areas, such as food, feed, and cosmetics. Considering the increasing market potential of prebiotics and the diversity of XOS usage areas, the demand for developing efficient production methods is increasing. XOS production methods can be classified as chemical, thermo-chemical, physical, and biological. Alkali extraction is the most widely used method of producing XOS. Firstly, xylan is extracted from the biomass using alkali. The use of acid neutralizes the alkaline environment, and then xylan is precipitated using ethanol and dissolved in an aqueous solution. In the second step, XOS is produced from xylan by

enzymatic hydrolysis or acid hydrolysis. Alkali extraction and the alternative methods have disadvantages in that they are harmful to the environment due to the use of chemicals, require high energy and specific equipment, and contain impurities in the final product. Considering these, a sustainable and environmentally friendly process should be developed for the production of XOS with the potential for prebiotics and industrial use to provide a xylan-priority biorefinery.

DES is an environmentally friendly, cheap, easy-to-prepare, and easy-to-use alternative used for the fractionation of lignocellulosic biomass. DES pretreatment is generally applied to remove lignin and hemicellulose, while enabling the cellulose remaining in the solid to be utilized. It has not been considered among the methods for XOS production.

Alkali is a chemical that has a high effect on the hemicellulose structure. It is not common to use dilute alkali instead of the traditional method, which is widely used in xylan extraction at high alkali concentrations.

The objective of this study was to develop a xylan-first biorefinery process that was targeting environmentally friendly, cost-effective, and high purity in the production of XOS. DES pretreatment was tested for retaining the hemicellulose in the solid and removing the lignin; subsequently, XOS was produced using enzymatic hydrolysis. Thus, thanks to the green method of DES solution and its application at relatively low temperatures, compared to other methods that required high energy, such as autohydrolysis, acid hydrolysis, and organosolv could be avoided limitations. In the other part of the study, alkali treatment was tested to obtain XOS from the solid as a result of enzymatic hydrolysis after the dilute alkali pretreatment, which removed lignin and left the hemicellulose remained in the solid.

CHAPTER 2

LITERATURE REVIEW

2.1. Biorefinery Concept

The approach of biorefinery first emerged in the late 1990s (Maity, 2015). For the first time in 1997, green biorefinery is defined as complex systems that utilization of biological raw materials (green and biomass residue) together with sustainable environmentally friendly technologies (Kamm et al., 2006). The concept of the biorefinery is stated that the concept of all processes involving the conversion of biomass feedstock into valuable products (Kamm & Kamm, 2004). Biorefinery is defined as a facility for the production of fuel, power, and chemicals from biomass together with equipment and processes (Fernando et al., 2006). It is the conversion of biomass into a wide range of products through the combination of different processes and technologies (FitzPatrick et al., 2010).

Biomass used in the biorefinery approach is classified depending on its origin as energy crops, agricultural residues, and waste, forestry waste and residues, and industrial and municipal wastes (de Lasa et al., 2011). Generations of biorefinery approaches have emerged with the development of various processes for the treatment of different biomass (Nigam & Singh, 2011). In the first generation, the main biofuels are bioethanol and biodiesel, which were produced from starch, sucrose, or oil-containing crops (Correa et al., 2017). In addition, barley, corn, sorghum, sugarcane, sunflower, and wheat are used as raw materials (Menon & Rao, 2012). Although it is easy to convert the high sugar and

oil content of raw materials into biofuels, the use of edible resources causes competition in terms of energy and food (Correa et al., 2017; Pinales-Márquez et al., 2021). For this reason, excessive demand for raw materials causes the expansion of agricultural lands, which leads to soil degradation, erosion, and an increase in water consumption (Pinales-Márquez et al., 2021). In the second generation, lignocellulosic biomass is used as a raw material. Various products such as biochemicals, biofuels, or biomaterials are obtained due to the lignocellulosic biomass structure containing cellulose, hemicellulose, and lignin (Ajao et al., 2018). Cellulose is consisted of glucose monomers and converted into liquid fuels such as bioethanol. Aromatic substances, syngas products, heavy metal sequestrants, and antimicrobial agents are produced due to the biopolymer structure of lignin consisting of aromatic components. From the hemicellulose part, xylitol, furfural, hydroxymethylfurfural (HMF), levulinic acid, and XOS with the high added value used in the food and pharmaceutical industries are obtained (Pinales-Márquez et al., 2021). Also, polymers are broken down into oligomers or monomers and converted into value-added products. In this step, the processes and technologies chosen due to the recalcitrant nature of the polymers play an important role (Mussatto & Dragone, 2016). The second generation is environmentally friendly, cost-effective, and socially more appropriate as there is no competition with food sources (Dutta et al., 2014). The third-generation approach is to obtain biofuels and products with high added value (proteins, polyunsaturated fatty acids, antioxidants, alginate, etc.) by using algae (Pinales-Márquez et al., 2021). Disadvantages are excessive energy requirements for algae cultivation and the cost of bioreactors (Dutta et al., 2014). The fourth generation is the approach using algae and other microorganisms that focus on ideal biomass. The fourth generation aims effectively produce bioenergy by combining genetically modified feedstock with microorganisms created by genomic syntheses, such as cyanobacteria. The fourth approach is still under development and research (Ale et al., 2019). Considering four different classifications, the second generation is preferred for industrial purposes in terms of being environmentally friendly, and cost–effective (Singh et al., 2020).

2.2. Lignocellulosic Biomass

The most prevalent type of biomass is lignocellulosic biomass. It is widely available because the lignocellulosic network provides structural integrity to plants so it is the most common (Alonso et al., 2010). Agricultural residues (e.g., wheat straw, sugarcane bagasse, corn cob), forest products (hardwood and softwood), and energy crops (switchgrass, miscanthus) are some examples of lignocellulosic feedstock (Kumar et al., 2009). Figure 2.1 shows the three main components of the lignocellulosic biomass: cellulose (35-50%), hemicellulose (20-35%), and lignin (10-25%) (Wei et al., 2017). In addition to these, there are small amounts of pectin, protein, extractives, and ash (Peng et al., 2010).

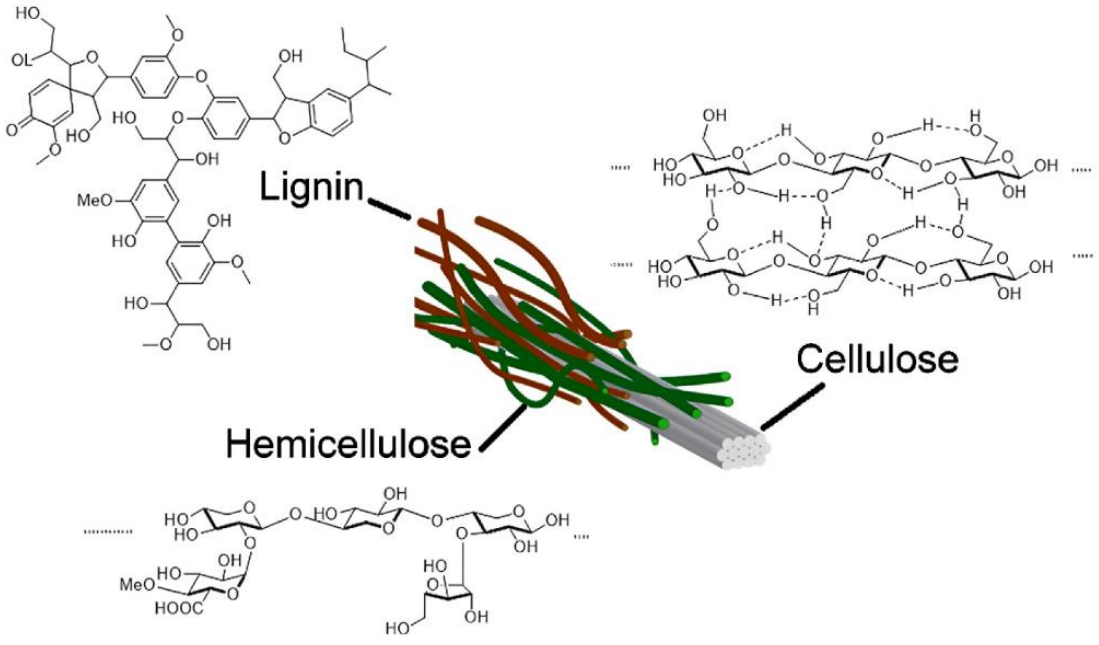

Figure 2.1. Chemical structure of lignocellulosic biomass (Source: Chai et al., 2022)

The distribution of lignin, cellulose, and hemicellulose within the cell walls varies. According to the species, tissues, and maturity of the plant cell wall, these components of the plant cell wall vary in structure and amount (Isikgor & Becer, 2015). Table 2.1 lists several lignocellulosic biomass types and their chemical contents.

Table 2.1. Compositions of lignocellulosic biomass[a] (Source: Cherubini, 2010; Menon & Rao, 2012)

Feedstocks	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Barley straw	36-43	36	19
Corncob	32.3-45.6	39.8	6.7-13.9
Corn stover	35.1-39.5	20.7-24.6	11.0-19.1
Cotton stalk	31	11	30
Eucalyptus	45-51	11-18	29
Rice husk	28.7-35.6	12-29.3	15.4-20
Poplar wood	45-51	25-28	10-21
Sugarcane bagasse	25-45	28-32	15-25
Sorghum straw	32-35	24-27	15-21
Switchgrass	35-40	25-30	15-20
Wheat straw	32.6	22.6	16.8

[a]Results are given as percent dry weight.

2.3. Cellulose

Cellulose, which consists of glucose units linked by β-1,4 glycosidic bonds, is the main component of the cell wall. It is a linear polymer with a molecular weight of 10^6kg $kmol^{-1}$ or more, with 5,000-10,000 glucose units (Maity, 2015). The cellulose chains are linked by Van der Waals and hydrogen bonds and are packed to form microfibrils (Kumar et al., 2009). In the interaction of cellulose and other components, hemicellulose and lignin surround the structure of cellulose through hydrogen bonds between cellulose-hemicellulose, and hydrogen bonds, and covalent bonds (mainly ether bonds) between cellulose-lignin (Zhang et al., 2011; Zhang et al., 2015). The crystalline and amorphous portions of cellulose are interwoven to create the cellulose structure. Cellulose has crystalline properties so; it is insoluble in an aqueous solution. Its interaction with other components and its crystalline structure makes cellulose resistant to biological and hydrolytic processes (Maity, 2015). Amorphous parts are vulnerable to enzymatic and chemical treatments (Singhvi & Gokhale, 2019).

Bioethanol, sorbitol, levulinic acid, succinic acid, levoglucosan, hydroxyacetaldehyde, ethylene glycol, acetic acid, lactic acid, glycerol, glycolic acid, etc. are the value-added products from cellulose (Devi et al., 2022). Various treatments are applied to lignocellulosic biomass by chemical (acid, alkali, ammonia steeping, inorganic salt-mediated pretreatment strategies, ozonolysis, ionic liquids (ILs), organosolv), physical (grinding and milling, irradiation-based processes, ultrasonication, thermal processes) and biological methods to enable the conversion of cellulose into products (Arumugam et al., 2020). For instance, ethanol (34.9 g/L) was produced by acid and alkali pretreatment and enzymatic hydrolysis applied to corn cob (Feng et al., 2016). Lactic acid (67 g/L) was obtained from sugarcane bagasse by steam and alkali pretreatment and simultaneous saccharification and fermentation using cellulase enzyme (Adsul et al., 2007). Succinic acid (47.4 g/L) was produced from corn straw by simultaneous saccharification and fermentation, after different pretreatments such as diluted acid, diluted alkaline, alkaline peroxide, and aqueous-ammonia soaking (Zheng et al., 2010).

2.4. Hemicellulose

Hemicellulose, the secondary polymer of lignocellulosic biomass, consists of various heteropolymers: xylan, galactomannan, arabinoxylan, and xyloglucan (Sharma & Saini, 2020). The heteropolysaccharide, which is rich in xylose, galactose, fructose, glucose, and mannose, has a low molecular weight of about 15 kDa (Cano et al., 2020). In addition, functional groups such as acetyl, methyl, glucuronic acid, and galacturonic acid make their structure branched via ether or ester bonds (Vázquez et al., 2000). The surface of the hemicellulose is covered with lignin, forming a lignin-carbohydrate complex. In addition, they attach to cellulose fibrils to form microfibrils by hydrogen bonding. This complex network of hemicellulose is embedded in the plant cell wall (Isikgor & Becer, 2015; Yang et al., 2021).

Xylan-type hemicellulose is widely distributed in plant cell walls. Polysaccharides known as xylans have their β-(1→4)-linked xylose residue-based backbone (Scheller & Ulvskov, 2010). Xylan has a branched structure with arabinose, 4-*O*-methyl glucuronic acid, ferulic acid, p-coumaric acid, or acetyl side groups (Figure 2.2). Xylan is a low molecular weight polymer found in all annual plants, hardwoods, softwoods, and seaweeds. The degree of polymerization (DP) is between 80-200 (Palaniappan et al., 2021). However, the DP of the isolated xylan varies according to the isolation method (Zhang et al., 2016a). Xylans that predominately contain α-(1→2)-linked 4-*O*-methyl glucuronosyl and glucuronosyl residues are referred to as glucuronoxylans (Scheller & Ulvskov, 2010). Arabinoxylans and glucuronoarabinoxylans contain xylose backbone and arabinose that are connected to the backbone, also minor amounts of glucuronic acid and 4-*O*-methyl derivative (Palaniappan et al., 2021). Positions 3 and/or both positions 2 and 3 are substitution points of L-arabinofuranose to xylose backbone (Ebringerová & Heinze, 2000).

Besides, there are many β-(1→4)-linked polysaccharides that include mannose. As in mannans and galactomannans, the backbones may be fully composed of mannose, or with mannose and glucose in a nonrepeating pattern as in glucomannans and

galactoglucomannans (Scheller & Ulvskov, 2010). Glucose attached to the xylan structure forms another type, gluco-xylans (Palaniappan et al., 2021).

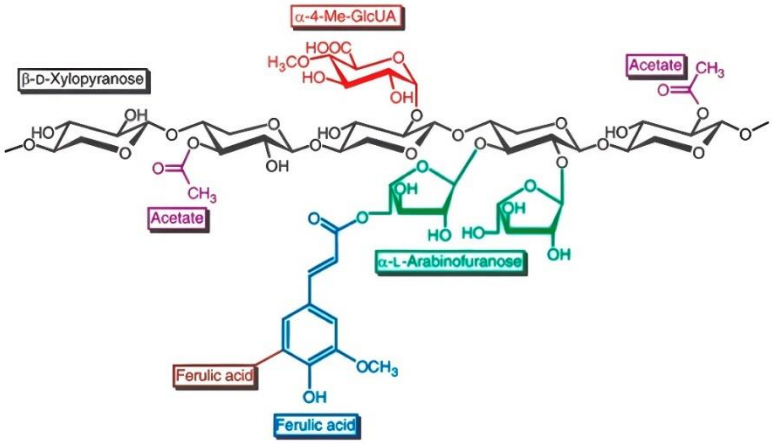

Figure 2.2. Xylan structure with functional groups (Source: Palaniappan et al., 2021)

Hemicellulose can be transformed into a variety of compounds with added value due to its amorphous and highly branching structure (Banerjee et al., 2019). XOS, xylose, xylitol, furfural, HMF, levulinic acid, pentane, etc. can all be produced from xylan (Pinales-Márquez et al., 2021).

2.5. Lignin

Lignin is a non-crystalline and complex structure with a three-dimensional network structure of phenolic polymers, with a molecular weight of 10,000 Da (Cao et

al., 2018; Limayem & Ricke, 2012). Lignin is mainly composed of C, H, and O elements, it is formed by oxidative radical polymerization of three main p-hydroxy cinnamyl alcohols (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, that are at the origin of the p-hydroxyphenyl, guaiacyl, and syringyl units) (Garedew et al., 2020). Lignin, which is formed by the combination of carbon-carbon and carbon-oxygen bonds between these units, has a complex amorphous polymer structure with a three-dimensional spatial structure (Zhu et al., 2016). Lignin constitutes a complex three-dimensional network structure via cross-linking with cellulose and hemicellulose by covalent bonding (Wang et al., 2021a). In particular, the hydrophobic combination of cell wall polysaccharides and lignin called the lignin-carbohydrate complex affects the use of lignocellulose by providing defense to the plant cell wall (Qing et al., 2013).

The majority of the lignin waste is disposed of and burned into low-energy fuels. This process causes a waste of resources and environmental pollution. Instead, lignin can be valorized using physical, biological, and chemical methods (Gadhave et al., 2018). Thermochemical processes such as hydrogenolysis, pyrolysis, and gasification are generally used for the production of fuels and chemicals from lignin (Cao et al., 2018). The products with high added value that can be produced from lignin are ethyl benzene, guaiacol, p-hydroxyl acetophenone, vanillin, and carbon materials, also lignin-derived chemicals are used in some industrial municipalities; such as asphalt emulsifiers, fuel dispersants, resin adhesive, soil amelioration agent, rubber reinforcing agent, water coal slurry additive, water reducing agent for concrete, viscosity reducer for heavy oil, a surface active agent for oil extraction (Cao et al., 2018).

2.6. Corncob

Corn, which is among the basic agricultural products around the world, is used in a wide range from animal feed to corn syrup in processed foods, from energy sources to basic foods (Mısır Bülteni, 2019). According to the data of the International Grain

Council (IGC), in the 2021-2022 marketing year, global corn production and consumption were 1,214 billion tons and 1,207 billion tons, respectively. In the marketing year of 2022-2023, global corn production is predicted to be 1,184 billion tons and consumption will be 1,200 billion tons (Mısır Bülteni, 2022). Since corn cob is the central kernel of corn, it becomes waste in parallel with corn production. Considering the amount of corn production and consumption, the waste corn cob causes environmental problems. To prevent this, corncob can be used as agricultural waste utilization in the biorefinery concept. The cellulose, hemicellulose, and lignin contents of the corncob are significant in terms of product selection. The compositions of corncob are illustrated in Table 2.2.

Table 2.2. Composition of corncob reported by other authors

Component (%)	(Pointner et al., 2014 et al., 2014)	(Zhang et al., 2014)	(Fan et al., 2013)	(Rofiqah et al., 2019)	(Buyukkileci & Temelli, 2023)
Cellulose	38.8	34.9	41.6	35.82	41.9
Hemicellulose	44.4	35.16	36.2	37.48	33
Lignin	11.9	10.10	16.1	15.59	18.8

There are products obtained from corncob such as biochar (Yu et al., 2014), activated carbon (Tsai et al., 2001), polyethylene composites (Chen et al., 2018a), bioethanol (Cai et al., 2016), microbial lipid (Cai et al., 2016), organic acids (Ruan et al., 2019), and glucose (Xing et al., 2016).

Corn cob is among the raw materials generally used for manufacturing high-value-added XOS (Vázquez et al., 2000). Biomass containing less lignin and more xylan is the better raw material for XOS production (Amorim et al., 2019). Considering its xylan lignin contents, corncob is regarded as a suitable feedstock. Two distinct structural categories can be used to describe corn cob xylan. One is a mainly water-insoluble low-

branched arabinoglucuronoxylan, and the other is a highly-branched, water-soluble heteroxylan (Oliveira et al., 2010). There are studies in which corncob is used as biomass for the production of XOS by alkaline method and then by enzymatic hydrolysis (Chapla et al. 2012, Samanta et al. 2015a, Aachary & Prapulla. 2009). Studies that obtained XOS as a result of acid hydrolysis and autohydrolysis pretreatments have chosen corncob as biomass (Zhang et al., 2017, Parajó et al., 2004). Han et al. (2020) applied the biorefinery approach by obtaining glucose with XOS and enzyme hydrolysis applied to the solid part after the gluconic acid treatment applied to the corn cob. However, since acid is used in this biorefinery approach, xylose, and furfural formation increased as XOS yield increased (Han et al., 2020). Especially, AIDP Inc. manufactures XOS through the enzymatic hydrolysis of corncob and offers it as PreticX (Michelin & Teixeira, 2020).

2.7. Xylooligosaccharides

Typically, XOS are oligosaccharides consisting of β-(1,4)-xylosidic linked xylose units. XOS can be branched by different side groups (α-d-glucuronic acid or its 4-O-methyl derivative, acetyl groups, or arabinofuranosyl residues) due to the substitutions in the xylan structure (Kumar et al., 2012). The chemical formula of the XOS can be simplified to $C_{5*n}H_{8*n} + 2O_{4*n+1}$. The range of XOS's DP is 2 to 12 (de Freitas et al., 2019; Palaniappan et al., 2021), and short XOS is named as xylobiose (2 monomers), xylotriose (3 monomers), xylotetraose (4 monomers), xylopentose (5 monomers), and xylohexose (6 monomers) (Samanta et al., 2015b). Figure 2.3. shows the structure of xylose and XOS (Kaprelyants et al., 2017).

XOS is present naturally in a variety of foods, including fruits and vegetables, milk, and honey (Mano et al., 2017). XOS is a crystalline solid that has a melting temperature of 134 ^{o}C, a decomposition temperature of 120 ^{o}C, and a solubility of 58% w/w at 21 ^{o}C (Samanta et al., 2015b).

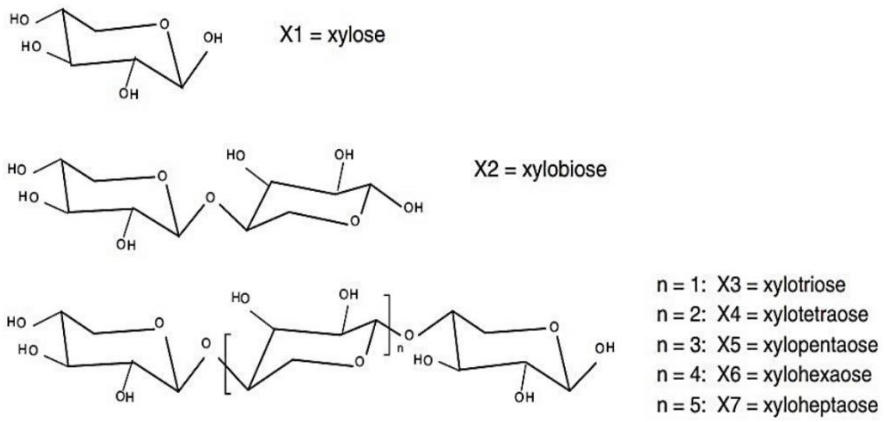

Figure 2.3. Structure of xylose and XOS components (Source: Kaprelyants et al., 2017)

XOS are stable in a wide pH range (2.5 to 8) and high temperatures (up to 100 ºC) (Amorim et al., 2019; Vázquez et al., 2000). Xylobiose has a sweetness that is 0.3-0.4 times sucrose, while another XOS has a modest sweetness and no off-taste (Vázquez et al., 2000; Park et al., 2017). Additionally, XOS presents a potential to be used as a stabilizer, emulsifier, and fat replacer in the food industry, thanks to its stability and organoleptic properties. XOS has been used for several purposes such as sucrose replacement in Chinese-type meatballs (Wu & Lin, 2011) and cookies (Ayyappan et al., 2015), fat replacer and sodium reduction in processed cheese with requeijão cremoso (Ferrão et al., 2018), chemical stabilizer in orange juice during heat treatment (Silva et al., 2020), and stabilizer in yogurt (Mumtaz et aL., 2008). XOS has an area of utilization in the cosmetics industry in terms of its capacity to restore microflora, its antioxidant, and its moisturizing properties (Amorim et al., 2019). In addition to these, XOS is applied to animal feeds and even odor-reducing feed for pigs (Hardy et al., 2017) has been patented.

In terms of health benefits, XOS has positive effects on gastrointestinal maintenance, blood cholesterol reduction, mineral absorption, immune stimulation, glucose-lowering capacity, antioxidant activity, and anticancer activity (Palaniappan et al., 2021). XOS demonstrates a range of biological functions, such as the ability to decrease inflammation, and possesses antioxidative, anticancer, and antibacterial

qualities (Chen et al., 2021). Furthermore, The International Scientific Association of Probiotics and Prebiotics defines prebiotics as "a substrate used selectively by host microorganisms that confer health benefits." (Gibson et al., 2017). Prebiotics reaches the colon as non-digestible food components, stimulating the numbers and/or activity of bacteria that benefit the host (Roberfroid, 2000). The mechanism of action of prebiotics is explained as follows: (1) It reduces adiposity by causing a decrease in concentration and thus lipolysis, by reducing the expression of the G protein-coupled receptor in the adipose tissue (Dewulf et al., 2011; Palaniappan et al., 2021). (2) Short-chain fatty acids are produced as a result of the fermentation of prebiotics by beneficial gut bacteria. Short-chain fatty acids affect the cellular integrity of the digestive system, glucose homeostasis, and immune function, and also reduce the pH value of the environment, that's why preventing the growth of pathogens, preventing the formation of phenolics and toxic substances, and reducing the activity of undesirable bacterial enzymes (Koh et al. 2016; Palaniappan et al., 2021). (3) It causes a decrease in energy intake by affecting satiety hormones (Parnell & Reimer, 2012; Palaniappan et al., 2021). Although inulin and fructooligosaccharides are the most well-known prebiotics, nowadays XOS has gained potential due to its applicability in foods and multidimensional health effects. (Samanta et al., 2015b; Cano et al., 2020). Among the XOSs, those with DP 2-4 are preferred in food applications because of their rapid assimilation in the gastrointestinal tract, easily metabolized by probiotics and higher prebiotic activity (Ho et al., 2018; Pinales-Márquez et al., 2021). In vitro studies have shown that several species of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* grow and produce short-chain fatty acids when the carbon source is XOS (Huang et al., 2019). In addition to its effect on the growth of *Bifidobacterium* species and *Lactobacillus brevis*, it has been observed that it inhibits pathogenic bacteria and prevents their adhesion to the intestinal epithelium (Crittenden et al., 2002; Ebersbach et al., 2012). Compared with galactooligosaccharides, fructooligosaccharides, and inulin, XOS is superior in terms of non-digestion during upper gastrointestinal and effects on *Bifidobacteria* and *Lactobacillus* species growths (Palaniappan et al., 2021). According to animal studies, feeding with oral XOS can considerably raise the amount of moisture in feces, the weight of the entire cecum, the *Bifidobacteria* population, and decrease the pH level (Aachary & Prapulla, 2010). Finegold et al. (2014), in their study with healthy adults, concluded that a dose of 2.8 g XOS per day increased the number of *Bifidobacterium*. Although studies on humans such

as this are available in the literature, the information is limited and needs further investigation.

The global prebiotic market was projected at $4.07 billion in 2017 and is estimated to increase to $7.37 billion by 2023 (Lan et al., 2021). The XOS market is expected to reach 130 million in 2023 and it is thought that this will contribute to the economic growth of the food industry (Poletto et al., 2020). The leading XOS manufacturers are Longlive, Kangwei, HFsugar, Henan, Shengtai, YIBIN YATAI, HBTX, YuHua, and ShunTian (Pinales-Márquez et al., 2021). The price of the Long Live prebiotic supplement, which is classified as GRAS by the FDA, varies between 12 and 22 €/100 g product (Michelin and Teixeira, 2020). XOS intake is regarded as the most competitive prebiotic component in terms of price per suggested dose since there is a lower requirement (1.4-2.8 g/day) to obtain the prebiotic effect (Amorim et al., 2019). Additionally, the application of XOS in food was developed in the Japanese market. Nowadays, XOS is used by around 60 companies in about 100 products, some of which have received FOSHU (Foods for Specific Health Use) within YOGHURINA produced by Suntory Ltd., MARUSHIGE GENKISU produced by Marushige Ueda Co., L-ONE produced by Enzamin Laboratory Inc., and SUKKIRI KAICHO produced by Lotte Co. (Vázquez et al., 2000).

2.8. XOS Production Methods

The production of XOS is important considering their use in industry, health effects, and prebiotic market potential. Generally, there are three different approaches to XOS generation. These are 1) direct enzyme hydrolysis to xylan-rich solid 2) enzyme hydrolysis after isolation of xylan from lignocellulosic biomass 3) hydrolytic degradation pathways such as steam or dilute acid treatments (Kumar et al., 2012; Vázquez et al., 2000). There are various pretreatments applied to lignocellulosic biomass with different techniques. Milling/grinding, alkali pretreatment, dilute acid pretreatment, steam pretreatment/ steam explosion, ILs, DES, organosolv, and biological treatment are among

the commonly used. Milling is the pretreatment in which the surface area is increased and the access is enhanced by particle size reduction. Sodium hydroxide, lime, or similar alkaline components provide lignin extraction in alkaline pretreatment. Dilute acid pretreatments cause hydrolysis of hemicellulose with the application of sulfuric acid, phosphoric acid, and other strong acids. In steam pretreatments, high-temperature steam hydrolyzes hemicellulose. In ILs, and DES applications that support the fractionation of polymers, large organic cations- small inorganic anions and Lewis and Brønsted acid-base mixtures are the effective solutions. Organic solvents such as ethanol and butanol extract lignin in the organosolv method. Finally, in biological processes: brown rot fungi degrade hemicelluloses and cellulose, white rot fungi break down lignin, and soft rot fungi break down cellulose (Galbe & Wallberg, 2019). The effect of pretreatments on the polymer is shown in Figure 2.4 (Ilanidis, 2021). As there are various modes of action, pretreatments should be chosen according to the biorefinery approach involving feedstock, and products.

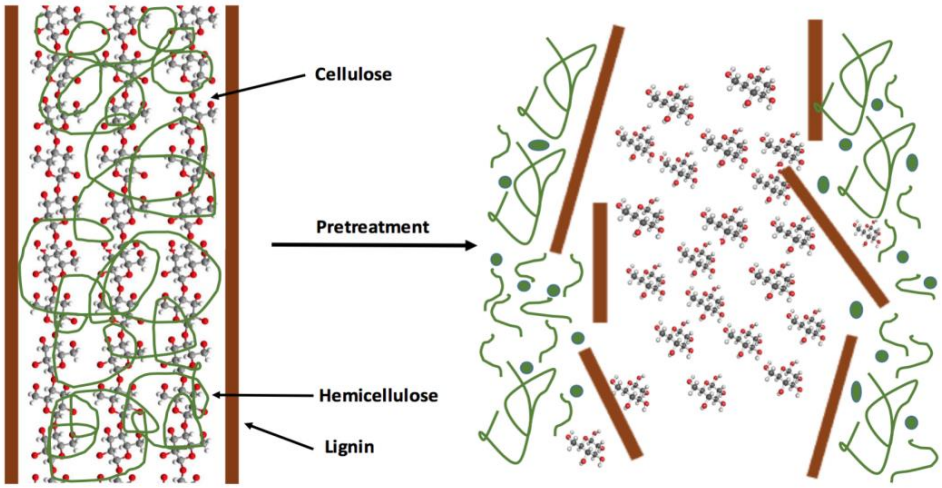

Figure 2.4. Deconstruction scheme of lignocellulosic biomass with pretreatment (Source: Ilanidis, 2021)

Considering to direct XOS production by enzymatic hydrolysis, enzyme-sensitive and accessible feedstock should be selected (Vázquez et al., 2000). Takao and Yoshio (1996) reported manufacturing XOS for this purpose using enzymatic techniques from the membranes of citrus fruit pulp.

Isolation of xylan by alkali treatment is one of the methods used. Until recently, the two-stage method combination of alkaline pre-treatment and enzymatic hydrolysis was the one that was the most frequently advised for producing XOS. Since a high concentration of alkali solvents successfully breaks the ester, aryl–ether, and C–C bond between lignin and carbohydrates as well as the hydrogen bonds between cellulose and hemicellulose by the presence of hydroxide ions from alkaline reagent, this alkali treatment enables the solubilization of hemicellulose (Pinales-Márquez et al., 2021). The soluble hemicellulose is recovered by precipitation with the addition of alcohol which changes the dielectric constant of the medium and causes polymer conformation differentiation (Santibáñez et al., 2021). Since acetyl groups are also cleaved during this process, it is advantageous that the xylan substitutions are already removed before enzymatic hydrolysis. On the contrary, acetyl groups reduce the solubility of hemicellulose, reducing yield (Gabrielii et al., 2000). Another disadvantage, the use of alkali causes corrosion, and salt formation so the extracted xylan must be purified (Poletto et al., 2020). Brienzo et al. (2010) solubilized xylan from sugarcane bagasse by chemical methods using alkaline hydrogen peroxide. Xylan was extracted with alkali peroxide (6%) and magnesium sulfate (0.5%) solution at 20 °C in 4 h and was converted to 39 g of xylose, 59 g of xylobiose, and 2 g to other XOS/100 g hemicellulose with enzymatic hydrolysis (Brienzo et al., 2010). Chapla et al. (2012) acquired xylan from corn cobs treated with 15% NaOH alkaline solution for 24 h at room temperature after acid soaking and delignification with sodium hypochlorite. XOS (6.73 mg/ml) was produced with maximum yield from corncob treated with enzyme hydrolysis (Chapla et al., 2012). Singh et al. (2018) tested obtaining XOS by enzyme hydrolysis after extracting xylan from areca nut husk using combined alkali and hydrothermal treatments under different conditions. As a result of enzymatic hydrolysis, xylobiose (25 g/100g xylan), xylotriose (9.2 g/100g xylan), and xylotetrose (0.9 g/100g xylan) was produced from the xylan extract obtained with 10% w/v NaOH at 65 °C for 8 h, followed by hydrothermal treatment at 121 °C for 1 h (Singh et al., 2018). In another study, where the biomass was sugar cane straw and

coffee husk after pretreatment with 24% KOH at 35 °C for 3h, 10.23g/L, and 8.45g/L XOS concentrate were formed from sugar cane straw and coffee husk, respectively, by xylanase hydrolysis (Ávila et al., 2020a).

The hydrothermal treatments are steam explosion and liquid hot water applications, also known as autohydrolysis. These processes require high pressure and temperature. Steam explosion is initiated at a temperature of 160–260°C and a pressure of 0.69–4.83 MPa to release the lignocellulosic biomass. It is then kept at a certain time atmospheric pressure, thus triggering hemicellulose hydrolysis (Aftab et al., 2019). Liquid hot water application is a process applied at 150-230°C, 4.9-20 bar pressures, and 10-50 min conditions (Pinales-Márquez et al., 2021). Hydrothermal processes cause the cellulose matrix to expand, changing the biomass's cell walls. As a result of cell wall conversion of the biomass, hemicellulose dissolves. During the autohydrolysis, the release of xylan and side groups to the medium is a result of the autoionization of water, OH^- and $(H_3O)^+$ ions, which act as catalysts. The decrease in pH as a result of the conversion of acetyl groups to acetic acid and the cleavage of uronic acids causes hydrolysis. Thus, XOS is produced by the depolymerization of hemicellulose. Despite it being a method that can be produced directly XOS and is eco-friendly, the harshness of the treatment itself poses a challenge because it can quickly produce degradation products such as HMF and furfural and low yield of extracting sugars (Singh et al., 2015). Surek and Buyukkileci (2017) obtained 62% of biomass xylan in liquor as XOS by autohydrolysis of hazelnut shells at 190 °C for 5 min. It was concluded that the concentrations of xylose, acetic acid, and furfural increased with the severity of the treatment (Surek & Buyukkileci, 2017). In another study, it was found that the XOS yield was proportional to the amount of acetyl after autohydrolysis at 179 °C for 23 min applied to six different biomasses (Nabarlatz et al., 2007a). The XOS yield on xylan was 60% for corn cob, 55% for almond shells, and 30% for rice husks. In another research, after biomass fractionation to oil palm frond fiber by autohydrolysis at 121°C for 60 min, a solution containing 17.5% XOS and 25.6% xylose was formed by enzymatic hydrolysis (Sabiha-Hanim et al., 2011).

Another widely used method for direct XOS production from feedstock is dilute acid pretreatment. This treatment acts on the hemicellulose, causing it to dissolve and hydrolyze. While hydrochloric acid and sulfuric acid are widely preferred for the process,

various organic acids such as acetic, phosphoric, and oxalic and inorganic acids such as nitric, maleic, and nitrous are used. The application temperature of dilute acid pretreatment ranges between 120- 200°C. Acid type and application temperature affect the formation of degradation products such as furfural and HMF. These chemicals have some industrial application, however, they are regarded as by-products in the production of XOS and must be removed from the treatment liquors. Other disadvantages are the need for special equipment for high temperatures and pressure and the formation of corrosion due to the use of acids (Kumar et al., 2020). To increase the yield of XOS, Han et al. (2020) subjected corncob to gluconic acid hydrolysis utilizing response surface methods. From this, a maximum yield of 56.2% of XOS was obtained using 0.6 mol/L gluconic acid at 154 °C for 47 min (Han et al., 2020). Ying et al. (2022a) produced XOS from poplar by combining acetic acid treatment and xylanase hydrolysis. 17.5% of XOS was obtained from the poplar treated with acetic acid for 30 min at 170 °C and hydrolyzed enzymatically. In another study in which poplar acid treatment was applied, 42.7% XOS was produced when 2% lactic acid was processed at 170 °C for 30 min (Yang et al., 2021).

Among other common treatments, organic solvents including methanol, ethanol, acetone, ethylene glycol, etc. are used in organosolv pretreatment, either alone or in combination with catalysts (organic or inorganic acids, or bases). The temperature range of organosolv application is between 100 °C and 250 °C. It causes fractionation by breaking -O-aryl, and 4-O-methyl glucuronic acid ester bonds in lignocellulosic biomass (Zhao et al., 2012). This method, which is used for the dissolution of lignin and hemicellulose, was used in the studies in our lab for the conversion of xylan to XOS by enzymatic hydrolysis. In the TUBITAK supported study (218M252), corncob was used as a model feedstock, and it was first treated with ethanol and MgO catalyst and then xylan in solid biomass was enzymatically converted to XOS, in the developed system. The temperature was kept relatively low (150°C-170°C) in contrast to the other applications of organosolv so that the lignin was partially removed and the xylan remained in the solid. As a result of the enzymatic application carried out in buffer solutions, a clean XOS solution was obtained that does not contain carbohydrate degradation products or extractive substances (Buyukkileci & Temelli, 2023). Although it is a method for obtaining XOS with high purity, the requirement of a reactor that can

work at high temperatures and pressures is a drawback for organosolv operation (Aftab et al., 2019).

Enzymatic hydrolysis is frequently used for XOS production because of its repeatability and high yield (Palaniappan et al., 2021). It is applied directly to the biomass or in combination with other pretreatments. Xylanase forms oligomers and monomers such as xylotriose, xylobiose, and xylose by cleaving the xylosidic bonds in the xylan backbone. Xylanase is either endo-acting (hydrolyzing different substituted xylan backbones) or exo-acting (xylan from reducing or non-reducing end gets hydrolyzed) on the 1,4- (or 1,3) linkages. The presence of two major enzymes, endo-xylanases (endo-1,4-β xylanase, E.C.3.2.1.8), and β-xylosidase, (xylan-1,4-β-xylosidase, E.C.3.2.1.37), can lead to the complete hydrolysis of the xylan backbone (Poletto et al., 2020; Vázquez et al., 2000). While endo xylanases cleave β-1,4-xylose linkages in the interior of the heteroxylan backbone, β-xylosidase releases monomeric xylose from the non-reducing ends of xylooligomers and xylobiose generated by endo-xylanase action on xylan (Juturu & Wu, 2012; Palaniappan et al., 2021). However, based on the primary xylanases' action modes, two critical points must be considered in the composition of the enzymes for XOS production purposes: 1) the release of xylose monomers should be minimal thanks to low β-xylosidase activity, 2) depending on substrate specificity, endo-xylanases should be able to release small molecules (Poletto et al., 2020). Based on similarities in their amino acid sequences and the results of hydrophobic cluster analysis, endo xylanases have been grouped into the glycoside hydrolase families 5, 8, 10, 11, and 43 (Kolenová et al., 2006). XOS is manufactured using the main endo xylanases glycoside hydrolase (GH) family 10- 11 enzymes. GH10 and GH11 enzymes are obtained from both bacteria and fungi. Endoxylanase from the GH10 family is preferred during hydrolysis since they show higher hydrolysis performance in terms of greater access to the acetyl-substituted xylan backbone compared to GH11 (Hu & Saddler, 2018). Endoxylanase from the GH10 family produces shorter XOS than the GH11 family, which is another positive case as low polymerization XOS is widely used in foods (Poletto et al., 2020). In addition, the GH10 family is more thermostable than the GH11 family considering the hydrolysis conditions (Hu & Saddler, 2018). GH10 xylanases have a molecular weight that varies between 32 and 39 kDa. As, the commercial example of the GH10 family enzyme Econase XT® by ABVista, is an endo-1,4-β xylanase produced by the strain *Trichoderma reesei* RF5427

(Lorentsen et al., 2019). In addition to these, enzymatic hydrolysis is improved with accessory enzymes. Auxiliary enzymes that operate on the side chains of xylan or the leading chains of various forms of xylan can be hydrolases or esterases (Pinales-Márquez et al., 2021). Table 2.3 summarizes some of the studies that used enzymatic hydrolysis in the production of XOS. Lastly, enzyme hydrolysis is usually preferred in the food sector, as it doesn't produce undesired byproducts and doesn't need specialized equipment to operate at high temperatures, and pressure and prevent corrosive properties. Nonetheless, enzymes for food applications must be generated by GRAS microorganisms to guarantee a safe product (Linares-Pasten et al., 2018).

Table 2.3. Summary of XOS yields of enzyme-used studies

Lignocellulosic Biomass	Pretreatment	Enzyme used	XOS Yield[a]	Reference
Almond shell	Hydrothermal pretreatment, 200 °C for 15 min	Endo-xylanase from *Thermomyces lanuginosus* (expressed in *A oryzae*)	55	Singh et al., 2019
Corncob	Dilute acid followed by hydrothermal treatment, 135 °C for 30 min	Xylanase from *Penicillium corylophilum* P-3-31	68	Yang et al., 2005
	KOH 5 % (w/v), 90 °C for 1 h	Endo-xylanase from *Streptomyces thermovulgaris*	30	Boonchuay et al., 2018
	Hydrothermal treatment, 190 °C for 13 min	GH10 Xylanases	49	Arai et al., 2019
	Steam explosion using acidic electrolyzed water, 165 °C for 35 min	Xylanase (PbXyn10A)	75	Liu et al., 2018
Pineapple peel	NaOH 15 % (w/v), 121 °C for 1.5 h	Endo-xylanase (Megazyme)	26	Banerjee et al., 2019
Reed scraps	Hydrothermal treatment, 170 °C for 30 min	Xylanase (Qingdao Blue Biological Technology)	68	Chen et al., 2019
Rice husk	NaOH 18 % (w/v), 120 °C for 45 min	Beta-xylanase (Pentopan™ MonoBG)	35	Khat-udomkiri et al., 2018
Sugarcane bagasse	H_2O_2 6% (w/v), 20 °C for 4 h	Xylanase from *Thermoascus aurantiacus*	22	Brienzo et al., 2009
	KOH 5 % (w/v), 121 °C for 30 min	Endoxylanase from *A. fumigatus* (M51) and *T. reesei* QM 9414	13	de Figueiredo et al., 2017
Wheat straw	Hydrothermal treatment, 180 °C for 40 min	Endo-xylanase	30	Huang et al., 2017
	Steam explosion, 200 °C for 4 min	Endo-xylanase (NS50030)/β-glucosidase (Novozym 188)	35	Álvarez et al., 2017

[a] g XOS/ 100 g xylan is represented by XOS Yield

An alternative approach for XOS production can be the using ILs pretreatment. Recently, interest in ILs treatments has been increasing for processing lignocellulose. ILs, whose components are cations or anions, are a new type of solvent with low melting points (<100 °C), negligible vapor pressure, and good thermal stability and polarity. Also, ILs are environmentally friendly solutions. ILs typically consist of large organic cations and small inorganic anions (Behera et al., 2014). The degree of anion charge delocalization and cation structure affect the physical, chemical, and biological properties of ILs. Temperature, cations and anions, and pretreatment time all have an impact on how ILs and biomass interact (Aftab et al., 2019). ILs compete with lignocellulosic components for hydrogen bonds, and this causes network disruption. The oxygen and hydrogen atoms in cellulose's hydroxyl groups play a role in the cellulose's dissolution interaction with ILs by forming an electron donor-electron acceptor. When cellulose-OH and ILs contact, the hydrogen bonds between the molecular chains of the cellulose are disrupted, which causes the cellulose to dissolve (Feng & Chen, 2008). Rice husk, water hyacinth, rice straw, kenaf powder, poplar wood, wheat straw, and pine were treated with ILs such as 1-Ethyl-3-methylimidazolium diethyl phosphate-acetate, 1-butyl-3-methylimidazolium-acetate, cholinium amino acids, cholinium acetate, 1-ethyl-3-methylimidazolium diethyl phosphate-acetate, and 1-allyl-3-methylimidazolium chloride for delignification and cellulose dissolution (Aftab et al., 2019). Recent investigations have demonstrated that ILs pretreatment can efficiently solubilize biomass and yield polysaccharides with decreased lignin contents. Using a combination of commercial hemicellulases, the biomass treated with this pretreatment was hydrolyzed into XOS effectively (Ávila et al., 2020b). Sugarcane bagasse was hydrolyzed with α-L-arabinofuranosidase and endo-xylanase after pretreatment with 1-ethyl-3-methylimidazolium acetate at 100 °C for 30 minutes. As a result, XOS was produced with a 53% yield (Ávila et al., 2020b). Although not yet commercially developed, ILs pretreatment is the focus of research as leads to highly selective delignification.

The most popular methods for producing XOS from lignocellulosic biomass are hydrothermal, chemical, and enzymatic hydrolysis. There is an uncontrolled release of xylose, and degradation products in chemical and hydrothermal approaches. In addition, chemicals such as acids and bases are harmful to the environment. Hydrothermal processes consume high energy as they require high temperatures and pressure. When only enzymatic hydrolysis is applied to obtain XOS, the yield is low. To overcome these

disadvantages, the search for a biorefinery-based method continues. Researchers have been focusing on merging and/or integrating methods that are both economically feasible and healthy to produce the product. Moreover, various raw materials, xylan extraction techniques, and enzyme sources for xylan hydrolysis continue to be studied for maximum effectiveness in the most environmentally friendly settings (Poletto et al., 2020).

2.9. Deep Eutectic Solvent

Nowadays, reducing energy use in production processes and utilizing "green" chemicals/solvents that do not affect the environment or the health of living things come to the forefront with the rising demand for bio-based products. Green solvents find scope for use in the separation of lignocellulosic biomass and as pretreatments in the production of bioproducts. As mentioned above, although ILs are of great interest as green solvents, they have disadvantages, such as high cost, difficulty in synthesis, toxicity, and non-biodegradability (Zhang et al., 2012; Smith et al., 2014). DESs, which have been used since the beginning of this century, are prepared by mixing two or three components in appropriate molar ratios to form eutectic mixtures with much lower freezing points than their components (Kalhor & Ghandi, 2019). DESs, which have the advantages of ILs, are also very attractive green solvents due to their short preparation time, biodegradability, low toxicity, and much lower cost than ILs (Zhang et al., 2012; Smith et al., 2014; Das & Biswas, 2015). For this reason, interest in DESs has been increasing rapidly in recent years; The exponential increase in the number of articles containing the term “DES” in the title can be seen in Figure 2.5.

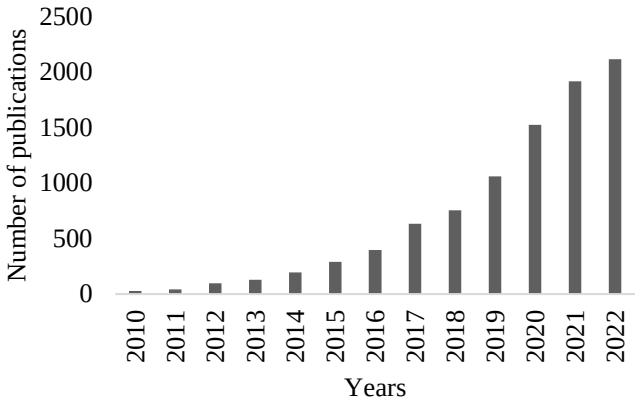

Figure 2.5. Distribution of articles with the phrase "DES" in the title according to years. (Source: Web of Science (www.webofknowledge.com)).

DESs are a particular mixture of two components: hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). The components of DESs can be extended to three or more components. While choline chloride (ChCl) is generally used as HBA, other HBAs such as imidazole, ethyl ammonium chloride, alanine, betaine, and proline have also been tested. ChCl is a highly affordable, biodegradable, and non-toxic quaternary ammonium salt, which can be easily produced from fossil sources (million metric tons) or extracted from biomass (Zhang et al., 2012). A wide variety of HBD appears to be used: lactic acid, malic acid, formic acid, acetic acid and other organic acids, urea, glycerol, ethylene glycol, various monosaccharides, and others (Satlewal et al., 2018; Zdanowicz et al., 2018; Kalhor & Ghandi, 2019). Natural substances such as carbohydrates, ChCl, glycols, and organic acids can be used as DES components. In some sources, such DESs are called "natural DES". Natural DESs have been described by the European Confederation of Paper Industries (CEPI) as the most promising platform for the fractionation of biomass in the future, and natural DESs are helping to increase added value and reduce CO_2 emissions, which are the key objectives of the industry to achieve a low-carbon bio-economy in Europe by 2050 (Morais et al., 2018).

DES compose of the proper combination of an HBA and an HBD with hydrogen bonds (Francisco et al., 2013). Three hydrogen bonds can be formed by one amine group (two hydrogen atoms on the nitrogen atom and a lone pair of nitrogen atoms). Carbonyl

oxygen can form two hydrogen bonds, while hydroxyl oxygen can form one. Figure 2.6 shows the structure of ChCl and different HBD. According to structure, one urea molecule with one carbonyl group and two amine groups can form eight hydrogen bonds. One oxalic acid molecule can form six hydrogen bonds because it has two hydroxyl groups and two carbonyl groups. Similarly, one glycerol molecule (with three hydroxyl groups) can form three hydrogen bonds, while one citric acid molecule (with four hydroxyl groups and three carbonyl groups) can form ten. ChCl can form three hydrogen bonds by accepting H^+. Considering Figure 2.7, the hydrogen bond formation mechanism between the chloride (from HBA), and the hydrogen of the hydroxyl group (from HBD), three, two, three, and three hydrogen bonds are formed between ChCl and HBD as urea, oxalic acid, citric acid, and glycerol, respectively (Zhang et al., 2020). In addition, the melting point of DESs is much lower than their components (HBD and HBA) due to the result of charge delocalization that occurred from the hydrogen bond between the halide ion and HBD (Smith et al., 2014). For instance, the melting points of ChCl and urea are 302°C and 133°C, respectively. When ChCl and urea are combined in a 1:2 molar ratio to form a solvent, they reached a melting point of 12°C (Kuehn et al., 2017).

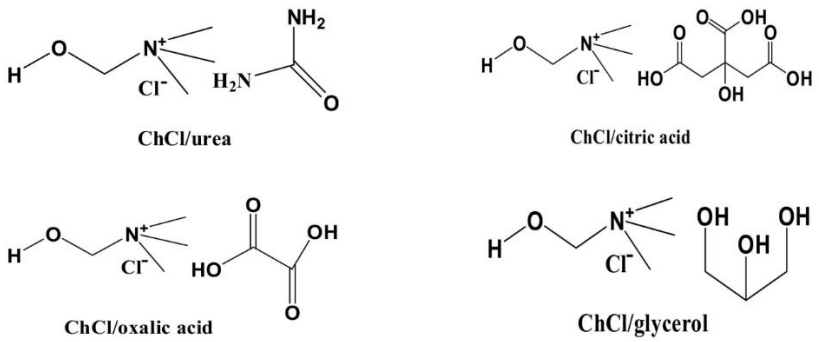

Figure 2.6. Structure of HBA (ChCl) and four different HBD (urea, citric acid, oxalic acid, and glycerol) components (Source: Zhang et al., 2020)

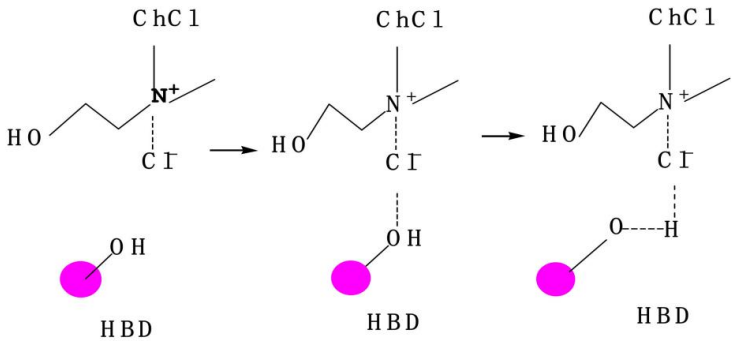

Figure 2.7. Hydrogen bond formation between ChCl and HBD (Source: Francisco et al., 2013)

Recently, various studies have been conducted to reveal the role of DES in the preparation of lignocellulosic biomass for various productions. Intermolecular hydrogen bonds in DESs increase the probability of breaking the strong hydrogen bonds in the lignocellulosic network, thus increasing the biomass solubility and conversion rate (Chen & Mu, 2019). These studies can be accessed from various review articles (Loow et al., 2017; Satlewal et al., 2018; Zdanowicz et al., 2018; Chen & Mu, 2019; Kalhor & Ghandi, 2019). Enzymatic degradation of cellulose in lignocellulosic biomass is often the first target of bioprocesses. To achieve this, lignin and hemicellulose must be separated from the biomass. Therefore, in previous studies, generally, the effect of DES on lignin and hemicellulose solubility was investigated, and then the saccharification efficiency of cellulolytic enzymes was determined. While DES generally does not dissolve cellulose, and remove lignin and hemicellulose from the solid to some extent, it does not cause cellulose loss during pretreatment and facilitates the enzymatic hydrolysis of the cellulose remaining in the solid. DES treatment could effectively break the ether bonds in lignin carbohydrate complexes and delignification occurs on lignocellulosic biomass structure (Ma et al., 2021). Some acidic DESs exhibit effective lignin extraction capabilities. Nonetheless, it has frequently been noted that glycerol-based DESs are less efficient for fractionating biomass (Xia et al., 2018). According to Liu et al (2017), due to competitive hydrogen-bond formation between the hydroxyl groups in the lignin carbohydrate complexes and the chloride ions of the DES, the strong hydrogen-bonding interactions in lignocellulosic biomass may be decreased. The breakage of lignin carbohydrates bonds

allows for the removal of lignin and hemicellulose using DES (Liu et al., 2017). On the other hand, DES affects cellulose dissolution. The interactions between the anions and the carbohydrate are the main cause of cellulose dissolution. Free chloride ions can cleave the hydrogen bonds within and between cellulose chains. The breakdown of cellulose can also be affected by other functional groups on the cation (imidazolium, pyridinium, ammonium, and phosphonium cations). The presence of a hydroxyl end-group in the cation decreases the solubility of cellulose. The hydroxyl group on the cation competes with the anion to form a hydrogen group with cellulose, which reduces the dissolution of the cellulose (Wang et al., 2012). The components of DES used and therefore their physicochemical properties change the dissolution mechanisms and efficiency of the processes.

It has been observed that some DESs selectively remove lignin at low temperatures. In the treatment of wheat straw with DES (ChCl -lactic acid) at 60 °C, the solubility of lignin increased with the amount of acid (Kroon et al., 2013). 60% of the lignin in rice straw was removed by low-temperature DES application and >90% pure lignin was obtained (Kumar et al., 2015). In tests using pure lignin, cellulose, and xylan, it was observed that lignin was selectively dissolved at 60 °C by using organic acids such as HBD (Lynam et al., 2017). Also, Kumar et al. (2015) investigated the effect of the amount of water added during DES application. The solubility of the lignin was 90% at 5% water addition. The solubility of lignin decreased and when 50% water was added, the solubility of lignin decreased to 5%. Hou et al. (2018) when they examined the effect of various HBDs together with ChCl on rice straw, observed that the amount of cellulose, lignin, and xylan in the treated solid was very different according to HBD and application temperature (80°C and 120°C). For example, when some HBDs are used, there is around 20% xylan in the solid, while some have no xylan left in the solid. Similarly, the amount of lignin was different and varied between 12% and 33%. The different compositions of the processed biomass showed its effect on the post-enzymatic hydrolysis, yielding hydrolysis efficiency of cellulose between 24% to 84% (Hou et al., 2018). Morais et al. (2018) in their study using pure xylan, found that the temperature, the amount of added water, and the HBA/HBD molar ratio were effective on the xylan solubility with DES prepared with ChCl-urea. In their tests with the *Eucalyptus globulus*, they were able to recover most of the xylan in the biomass in soluble form (Morais et al., 2018). For the HBA/HBD 1:2 molar ratio, by adding 20% water at 70^{o}C, 33.3% water at 80^{o}C, and 50%

water at 90^{o}C, the xylan concentrations were increased to a maximum of 300.45 mg/mL, 328.23 mg/mL, and 320.80 mg/mL, respectively. After the amount of water added exceeded 50%, there was a decrease in xylan solubility (Morais et al., 2018). Liang et al. (2020) observed that xylan solubility was adversely affected as the amount of water increased between 65-93% at DES 60 ^{o}C, which they formed with betaine and various amino acids. When the amount of water added to betaine-lysine increased from 65% to 93%, the xylan solubility decreased from 545 mg/g to 89 mg/g. In another study in the literature, fermentable sugars were obtained with the cellulase enzyme after DES treatment was applied to the corn cob (Procentese et al., 2015). When ChCl and urea were used as DES in the study, 22.6% and 26.8% of the initial xylan were converted to xylose at 80^{o}C and 115^{o}C, respectively.

In systems developed based on DES for XOS production, Isci et al. (2021), used ChCl and formic acid DES on the wheat straw with microwave with a different approach. As a result of the study, they found 32% of the initial xylan as XOS, 20% as xylose, and about 5% as furfural in the liquid part (Isci et al., 2021). Shen et al. (2021), since DES was not effective in XOS production, they tried to increase it by hydrothermal pretreatment. Generally, DES was applied for delignification after a different pretreatment was used for XOS production (Gong et al., 2022; Ma et al., 2021; Ying et al., 2022b).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The feedstocks barley stalk, corn cob, corn stover, and oat straw used in the study were provided by the Aegean Agricultural Research Institute (Turkey, Izmir). The Cellulast 1.5 L and Shearzyme 500L enzymes were obtained from Novozymes (Denmark). The Veron 191S and Econase XT enzymes were obtained from AB Enzymes (Germany). In this study, all chemicals were used in their analytical standards and no purification or other methods were performed. Chemicals were purchased in specified brands; ChCl (Solarbio), urea (Merck), glycerol (Merck), ethylene glycol (Merck), lactic acid (Carlo Erba), acetic acid (Merck), citric acid monohydrate (Merck), tri-sodium citrate dihydrate (Isolab), xylan from beechwood (Megazyme), D (+) glucose monohydrate (Sigma-Aldrich), D (+) xylose, D (-) arabinose (Sigma-Aldrich), xylobiose (Megazyme), xylotriose, (Megazyme), ethanol (Tekkim), toluene (Tekkim), 3,5 dinitro salicylic acid (Aldrich), potassium sodium tartrate tetrahydrate (Merck), phenol (Fluka), sodium metabisulfite (Merck), sodium hydroxide (Merck), sulphuric acid (Merck), calcium carbonate (Sigma-Aldrich),

3.2. Methods

The general flow diagram of mainly XOS production from corn cob following enzymatic hydrolysis after using DES treatment was shown in Figure 3.1. For the selection of the conditions described in the method section, the obtained solids were subjected to enzymatic hydrolysis, and LDP XOS concentrations were considered. Unless otherwise stated, all experiments were performed in duplicate. The results were reported as ± standard deviation.

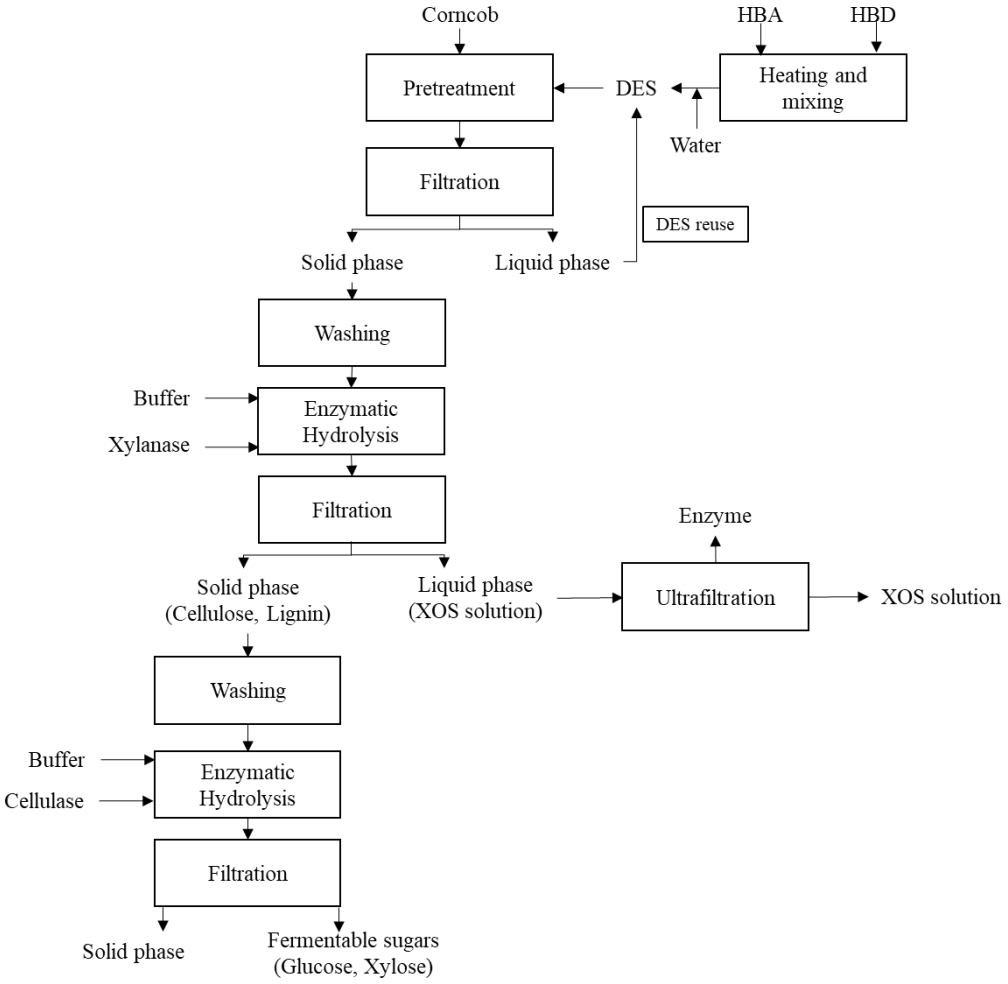

Figure 3.1. Flow diagram of primarily XOS production from corncob

3.2.1. DES Synthesis

Five different DES solutions were obtained by using ChCl as HBA; urea, glycerol, ethylene glycol, lactic acid, and acetic acid as HBD. The water in the beaker, which was placed on the heated magnetic stirrer (Velp Scientifica, Italy), was adjusted to a constant temperature of 70 ^{o}C using a digital thermometer, forming a water bath (Figure 3.2). HBA and HBD were mixed at a molar ratio of 1:2 in the Schott bottle. Then, it was immersed in the water bath and mixed at 100 rpm until a homogeneous and transparent liquid was obtained. Besides, after the HBD was selected according to the results of subsequent enzymatic hydrolysis, molar ratios of 1:4, 1:6, and several water addition rates were tested. Water was added to the DES mixture at 20, 40, 60, 80, 90, and 95% by volume and mixed with a magnetic stirrer. The solutions were stored in a desiccator until future use. DES preparation setup and DES solution were shown in Figure 3.2.

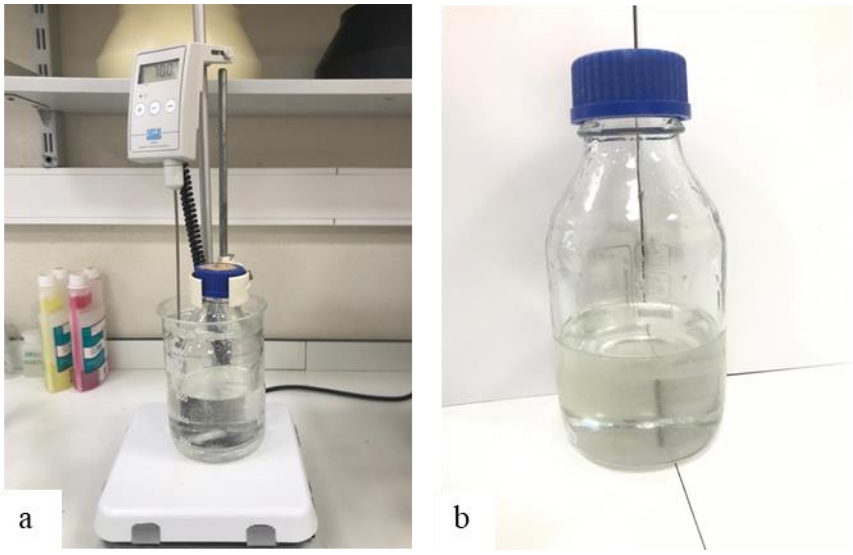

Figure 3.2. DES preparation a) DES preparation setup b) DES solution

3.2.2. DES Treatment

The corncob was dried in an oven (Memmert, Germany) at 40 ^{o}C overnight. Corncob was added to DES at a solid-to-liquid ratio of 1:10 (w:v) in the screw cap test tube. Different pretreatment conditions were tested. For this purpose, all HBA: HBD mixtures formed were subjected to two different temperatures and times, in an autoclave (Alp CL 32L, Japan) at 130 ^{o}C (Chen et al., 2018b; Chen et al., 2019) for 2 h and in a water bath (Thermal, Turkey) at 90 ^{o}C (Morais et al., 2018) for 24 h. After the application, the liquid and solid phases were separated by filtration with the aid of nylon cheesecloth with a pore diameter of 0.45 mm. The filtrate was stored in the refrigerator at 4 ^{o}C for DES reuse. The solid was washed three times with hot water to remove residual DES. The samples were dried in an oven at 40 ^{o}C overnight. Solids were characterized by two-step acid hydrolysis. In addition, for the DES selected according to the results of subsequent enzymatic hydrolysis, the pretreatment temperature of 110 ^{o}C (Chen et al., 2018b; Ren et al., 2016); solid-liquid ratio of 1:5, 1:20, and autoclave times of 1h, and 4h were applied as well. As a control, experiments were performed using only water or HBA, or HBD instead of DES. To determine the effect of DES treatment on XOS production, the solids that remained after all DES treatments were subjected to enzymatic hydrolysis. Considering the results of subsequent enzymatic hydrolysis, the HBD and DES conditions were selected. Lastly, the same process was applied to barley stalk, corn stover, and oat straw in selected HBD and DES conditions to examine the treatment effect for different biomass.

3.2.3. DES Reuse

The filtrate of the previous DES treatment was applied directly at the subsequent cycle of pretreatment without separation, purification, or addition of fresh DESs. This process was repeated for three cycles.

3.2.4. Alkaline-assisted DES Treatment

Trials were done by adding alkali (NaOH) to improve the low-temperature DES treatment. A ChCl-urea solution with a molar ratio of 1:2 was prepared and 40% v/v water was added. NaOH was added to make a total of 1% w/v (Teng et al., 2022; Li et al., 2023), taking into account the total liquid volume. The biomass was suspended in this solution at a 1:10 solid-liquid ratio and incubated at two different temperatures, 60 ^{o}C (Ma et al., 2023), and 90 ^{o}C (Okur & Koyuncu, 2020). For the control of the experiment, the biomass was treated with ChCl-urea and also with 1% NaOH solutions under the same conditions. After incubation, the solid part was washed and dried, and hydrolyzed with the enzyme, as described in the DES treatment section.

3.2.5. Alkali Treatment

NaOH was prepared at 0.10, 0.25, 0.75, 1, 3, 5, and 10 % w/v concentrations. Biomass was incubated in these alkaline solutions at a solid-liquid ratio of 1:10 (w:v) at

30 ºC and 60 ºC at 180 rpm. After incubation, the solid part was washed and dried, and enzymatically hydrolyzed, as described in the DES treatment section. According to the results of the enzymatic hydrolysis, the optimal NaOH concentration and treatment temperature values were determined. Then, different conditions were tested, such as application time 2, 4, 6, 8, and 18 h and solid-liquid ratio of 1:7, and 1:20.

3.2.6. Enzymatic Hydrolysis

The pretreated solid was hydrolyzed using xylanase enzyme and XOS was produced. Subsequently, the spent solid was hydrolyzed using cellulase enzyme to produce fermentable sugars.

3.2.6.1. Xylanase Hydrolysis

Firstly, enzymatic hydrolysis with three different commercial enzymes (Shearzyme 500L, Veron 191S, and Econase XT) was tested. Shearzyme 500L and Veron 191S enzymes were used together to improve XOS production (Kiran et al., 2013).

The pH of the buffer was adjusted to 5.5 due to; the endo-1,4-β-xylanases showing optimum activity between pH 5.0-5.5 (Guido et al., 2019). Besides, the optimum pH values of fungal xylanases for XOS production were reported to be between 5 and 6 (Zhu et al., 2006; Chidi et al., 2008; Aachary & Prapulla, 2009; Pal & Khanum, 2011; Goncalves et al., 2012; Peng et al., 2012; Guido et al., 2019). Accordingly, 50 mM citrate buffer was prepared using citric acid and sodium citrate adjusting the pH to 5.5. Veron 191S and Econase XT enzymes which were in powder form were mixed with the citrate buffer at 4% (w/v) concentration. The tubes were shaken gently by inverting them several

times. The insoluble components were separated by centrifugation at 6,000 rpm for 5 min. Supernatants were added to the hydrolysis medium.

Enzymatic hydrolysis was performed in 25 mL Erlenmeyer flasks with 5 mL working volume to ensure effective mixing. DES-treated solids were suspended in 50 mM citrate buffer (pH 5.5) at a solids-to-liquid ratio of 1:20 (w:v). Enzyme hydrolysis was first tested with 50 U/g biomass enzyme loading. Therefore, both Shearzyme 500L and Veron 191S enzymes were added as 25 U/g biomass, with a total of 50 U/g biomass xylanase. For the Econase XT enzyme, 50 U/g biomass was added. Flasks were incubated at 50 ^{o}C for Shearzyme 500L – Veron 191S and 70 ^{o}C for Econase XT in an incubator shaker (ZHWY-200B, Germany) at 180 rpm for up to 48 h (Guido et al., 2019; Buyukkileci and Temelli, 2023). The samples were centrifuged at 6,000 rpm for 5 min and the supernatants were kept at 100 ^{o}C for 20 min for enzyme inactivation. After cooling to room temperature, the hydrolysate was used for high-performance liquid chromatography (HPLC) analysis. The solid parts were washed three times with hot water and dried at 40 ^{o}C for characterization. Furthermore, after selecting the effective enzyme for XOS production, several enzyme dosages, such as 100, 200, 400, 800, and 1600 U/g biomass, several hydrolysis times, such as 6, 24, and 48h, hydrolysis temperatures such as 50 °C, 60 °C, and 70 °C, and several solid-liquid ratios such as 1:10, and 1:20 (w:v) were tested.

The yield of xylose or total XOS and xylan digestibility were calculated using equations 3.1, and 3.2, respectively. As an important point, in these calculations, the remaining liquid volume after enzymatic hydrolysis was measured thus, the liquid drawn by the raw material and evaporation were eliminated.

$$\text{Xylose or LDP XOS yield } (Y_{LDP\ XOS})\ (\%) = \frac{\text{xylose or xylobiose and xylotriose in hydrolysate (g)}}{\text{xylan in feedstock corncob (g)}} \text{x}100 \quad (3.1)$$

$$\text{Xylan digestibility} = \frac{\text{xylan converted to xylose or total XOS (g)}}{\text{xylan in pretreated corncob (g)}} \text{x}100 \quad (3.2)$$

3.2.6.2. Cellulase Hydrolysis

The solid remained after hydrolysis with xylanase was hydrolyzed with cellulase according to the National Renewable Energy Laboratory (NREL) NREL/TP-510-42629 method (Selig et al., 2008). Thus, a 50 mM citrate buffer at pH 4.8 was prepared using citric acid and sodium citrate. Solids were suspended in the citrate buffer in 25 mL Erlenmeyer flasks at a solids-to-liquid ratio of 1:20. Cellulast 1.5 L enzyme was added as 60 FPU/g cellulose. All flasks were incubated at 50 ^{o}C in an incubator shaker at 180 rpm for 48 h. The samples were centrifuged at 6000 rpm for 5 min and the supernatants were kept at 100 ^{o}C for 5 min for enzyme inactivation. After cooling to room temperature, the hydrolysate was analyzed in HPLC. The solid part was washed three times with hot water and dried at 40 ^{o}C for characterization. The yield of glucose, and glucan digestibility were calculated using equations 3.3, and 3.4 respectively. As mentioned in the section on xylanase hydrolysis, the post-hydrolysis liquid volume was taken into account in the calculations.

$$\text{Glucose } (Y_{Glucose})\ (\%) = \frac{\text{glucose in hydrolysate (g)}}{\text{glucan in feedstock corncob (g)}} \text{x}100 \tag{3.3}$$

$$\text{Glucan digestibility} = \frac{\text{glucan converted to glucose (g)}}{\text{glucan in pretreated corncob (g)}} \text{x}100 \tag{3.4}$$

3.2.7. Ultrafiltration

Enzymatic hydrolysate (500 mL) containing XOS and the enzyme was subjected to ultrafiltration. Cross-flow ultrafiltration system with a 10 kDa cut-off membrane was used for enzyme separation (Sartocon, Sartorius, Germany). For preliminary cleaning of the system, it was washed with 2L of deionized water. Then the sample feeding was started. The peristaltic pump speed was set at 40 rpm to keep the pressure between 1.5

and 2 bar. Additionally, the retentate was fed into the system and recycling was ensured, allowing all hydrolysate to pass through the membrane. The two permeate outlets were collected as filtered XOS solutions. The ultrafiltration system, feed, retentate, and permeate parts were shown in Figure 3.3. After the entire sample was passed through the system, the filter was again washed with 2L of distilled water. The system, which was cleaned by passing 1M NaOH at 50 ^{o}C for 1 h, was washed with 2L deionized water again and NaOH was removed. The system was washed with 1 L of 20–24% ethanol before being removed from the system and the filter was stored in its container with 20% ethanol at 4 ^{o}C. The filtered sample was stored in a refrigerator at 4 ^{o}C until XOS and protein were determined.

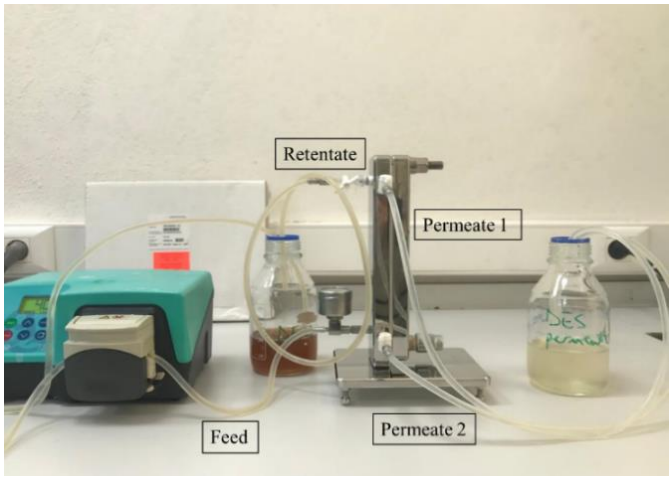

Figure 3.3. Cross-flow membrane filtration system

3.3. Analytical Methods

Several analytical methods were used for analysis throughout the study.

3.3.1. Moisture Analysis

According to the NREL/TP-510-42621 method, 1 g of biomass was placed onto the pre-dried aluminum pans and dried in the oven at 105 ºC, until constant weight, at least for 4 h (Sluiter et al., 2008a). The samples were kept in the desiccator before taking the weights. Constant weight is defined as less than a 0.1% change in the weight percent of solids, after re-heating the sample for 1 h (Sluiter et al., 2008a). The moisture content was calculated using Equation 3.5.

$$\text{Moisture \%} = 100 - \left(\frac{\left(\text{Weight}_{\text{dry pan plus dry sample}} - \text{Weight}_{\text{dry pan}} \right)}{\text{Weight}_{\text{sample as received}}} \text{ x } 100 \right) \quad (3.5)$$

3.3.2. Extractives Content

Corncob was extracted with toluene and ethanol (50:50 v/v) and at a 1:35 solid-to-liquid ratio in the Soxhlet apparatus (Isolab, Turkey) for 6 cycles (Macfarlane et al., 1999; Subroto & Hayati, 2020). The amount of extractive was determined by weight difference.

3.3.3. Structural Carbohydrate and Lignin Analysis

Cellulose, hemicellulose, and acid-insoluble lignin content in raw materials and treated solids were determined according to the NREL/TP-510-42618 method (Sluiter et al., 2008b). Solid samples were dried at 40 ºC overnight. According to the method, 6 ml of 72% (w/w) H_2SO_4 solution and 0.6 g solid samples were incubated in test tubes for 1 h at room temperature. During the incubation, tubes were mixed by vortexing at intervals. Then, the acid concentration was reduced to 4% by adding 168 ml of deionized water. The suspension was kept in the autoclave at 121 °C for 30 min. Thus, monosaccharides in the solid biomass were released and dissolved in the liquid phase by this two-stage acid hydrolysis After autoclaving, the samples were filtered through porcelain crucibles, and the filtrate is separated for HPLC analysis. The amounts of glucan, xylan, and arabinan were calculated by taking into account the amounts of glucose, xylose, and arabinose, the concentrations of which were determined in HPLC. For this, since one water molecule is added during hydrolysis of each glycosidic bond; glucose (6C sugar), xylose, and arabinose (5C sugars) multiplied by anhydrous factors which are 0.90 for 6C sugars and 0.88 for 5C sugars. Glucan, xylan, and arabinan were calculated using equations 3.6, 3.7, and 3.8, respectively. The solid part was first dried at 105ºC, then burned at 575ºC for 3 h in an ashing furnace (Carbolite, UK). After burning, the amount of ash was determined by weight measurement. Acid-insoluble lignin (AIL) was calculated using equation 3.9. Cellulose, xylan recovery, and lignin removal were calculated using equations 3.10, 3.11, and 3.12. The experiments in this section were performed in triplicate.

$$\text{Glucan (\%)} = \frac{\text{Glucose concentration } \left(\frac{g}{L}\right) \text{ x } 0.174 \text{ L x } 100}{0.6 \text{ g}} \text{x} 0.9 \tag{3.6}$$

$$\text{Xylan (\%)} = \frac{\text{Xylose concentration } \left(\frac{g}{L}\right) \text{ x } 0.174 \text{ L x } 100}{0.6 \text{ g}} \text{x} 0.88 \tag{3.7}$$

$$\text{Arabinan (\%)} = \frac{\text{Arabinose concentration } \left(\frac{g}{L}\right) \text{ x } 0.174 \text{ L x } 100}{0.6 \text{ g}} \text{x} 0.88 \tag{3.8}$$

$$\text{AIL (\%)} = \frac{(\text{Weight}_{\text{crucible plus AIR}} - \text{Weight}_{\text{crucible}} - \text{Weight}_{\text{ash}})}{\text{Weight}_{\text{sample as received}}} \text{x}100 \quad (3.9)$$

$$\text{Cellulose recovery} = \frac{\text{Cellulose in pretreated corncob (g)}}{\text{Cellulose in raw corncob (g)}} \text{x}100 \quad (3.10)$$

$$\text{Xylan recovery} = \frac{\text{Xylan in pretreated corncob (g)}}{\text{Xylan in raw corncob (g)}} \text{x}100 \quad (3.11)$$

$$\text{Lignin removal} = \frac{\text{Lignin in raw corncob (g)} - \text{Lignin in pretreated corncob (g)}}{\text{Lignin in raw corncob (g)}} \text{x}100 \quad (3.12)$$

3.3.4. Total XOS in Liquid Fraction Analysis

According to NREL/TP-510-42623 method, to determine the total XOS amount of the enzymatic hydrolysate, 174µl of 72% H_2SO_4 solution was added to 5 ml of hydrolysate (Sluiter et al., 2006). The solution was kept in the autoclave at 121 ºC for 30 min to convert XOS to xylose. The concentration of xylose produced was analyzed in HPLC. The total amount of XOS was calculated using equation 3.13. Since the xylose concentration was known, the total XOS was calculated by multiplying the anhydrous factor of 0.88 (for sugars with 5 carbons).

$$\text{XOS (g/L)} = (\text{Xylose concentration}_{\text{after acid hydrolysis}} - \text{Xylose concentration}_{\text{before acid hydrolysis}}) \text{ x } 0.88 \quad (3.13)$$

3.3.5. High-Performance Liquid Chromatography (HPLC) Analysis

The monomers released after two-stage hydrolysis of raw material, processed biomass and the monomers released as a result of acid hydrolysis applied to the enzyme

hydrolysate for total oligosaccharide were analyzed Aminex HPX-87H organic acid column (Bio-Rad, USA) in HPLC (Thermo Scientific, UltiMate 3000, US). Oligomers and monomers in the hydrolysate as a result of enzyme hydrolysis were analyzed by Rezex RPM-Monosaccharide column (Phenomenex, USA) in HPLC.

3.3.5.1. Monomers Analysis After Acid Hydrolysis

The solution obtained after the acid hydrolysis (5 mL) was mixed with 0.26 g of calcium carbonate ($CaCO_3$) for the neutralization process. Thus, the pH value was adjusted to be between 5-7 suitable for the column. The solution was centrifuged at 6,000 rpm for 5 min and then at 15,000 rpm for 30 min to precipitate $CaCO_3$. After dilution of supernatant 5 times with ultra-pure water, it was bottled in vials by passing it through a 0.45 µm syringe. The information about the analysis was as follows: micro-guard cation-H cartridge (Bio-Rad, USA), Aminex HPX-87H organic acid (Bio-Rad, USA) column, refractive index detector (RefractoMax, 521, Thermo Fisher Scientific, US), 5mM H_2SO_4 mobile phase, flow rate 0.6 ml/min, column temperature 65 ºC. Standard calibration curves for glucose, xylose, and arabinose were included in Appendix A.

3.3.5.2. Oligomers and Monomers Analysis After Enzyme Hydrolysis

After the enzyme was inactivated in the enzyme hydrolysate, the solution was centrifuged at 6,000 rpm for 5 min and then at 15,000 rpm for 30 min, respectively. Supernatants diluted 10-fold with ultrapure water were bottled in vials. The information about the analysis was as follows: security guard column (Phenomenex, USA), Rezex

RPM-Monosaccharide column (Phenomenex, USA) refractive index detector, ultra-pure water mobile phase, flow rate 0.6 mL/min, column temperature 80ºC. Standard calibration curves for xylotriose, xylobiose, and xylose were included in Appendix B.

3.3.6. Determination of Xylanase Enzyme Activity

Enzyme activity is determined by measuring the reduction of the substrate to xylose as a result of enzyme hydrolysis, and the reaction with dinitro salicylic acid (DNS), which changes color when reduced, by spectrophotometer (Bailey et al., 1992). The amount of reduced sugar is proportional to the color change. As a substrate, beechwood xylan at a concentration of 0.5% (w/w) was prepared using citrate buffer (pH 5.5). Substrate (900 µm) and several enzyme dilutions (100 µm) were added into the test tubes and incubated at 50 °C for 5 min. Then, DNS (1.5 mL) was added to stop the reaction, and it waited at 100 °C for 5 min to color change. Several xylose solutions such as 0.15, 0.3, 0.6, 1, 1.5, and 2 g/L were prepared using citrate buffer (pH 5.5) for the standard curve. The same procedure was followed by adding xylose solution instead of the enzyme. Absorbance values were measured at 540 nanometers in the spectrophotometer (T80+ UV-Vis PG Instruments, United Kingdom). Xylanase activity was calculated using equation 3.14. In the equation; X, 150.13, 1, 5, 0.1, and df represent the µg xylose obtained from the standard graph, xylose molecular weight, reaction volume, incubation time, amount of enzyme added, and dilution factor respectively. The xylose standard curve graph was in Appendix C. The experiments in this section were performed in triplicate.

$$\text{Activity (U/ml)} = \frac{X}{150.13} \times \frac{1}{5} \times \frac{1}{0.1} \times \text{df} \qquad (3.14)$$

3.3.7. Determination of Cellulase Enzyme Activity

Cellulase activity of Cellulast 1.5L enzyme was determined as FPU (filter paper units) according to the NREL/TP-510-42628 method (Adney & Baker, 2008). Whatman paper No. 1 (1x6 cm long roll) was used as a substrate. The substrate, pH 4.8 citrate buffer (1 mL), and several enzyme dilutions (500 µL) were incubated for 1 h at 50 °C in test tubes. DNS (3 mL) was added to the tubes to stop reactions and kept for 5 min at 100 °C for color changing. Several glucose solutions such as 2, 3.3, 5, and 6.7 g/L were prepared using citrate buffer (pH 4.8) for the standard curve. The same procedure was followed by adding glucose solution instead of the enzyme. Absorbance values were measured at 540 nanometers in the spectrophotometer. The absorbance values of the diluted enzymes were converted to the amount of glucose using the glucose standard curve. The enzyme dilution factor was found for 2 mg glucose using the equation obtained with the graph of enzyme dilution factors and glucose values. The calculation of FPU from the dilution and glucose concentration graph was using equation 3.15. The glucose standard curve graph was in Appendix C. The experiments in this section were performed in triplicate.

$$\text{Filter Paper Activity (units/mL)} = \frac{0.37}{\text{[enzyme] releasing 2.0 mg glucose}} \qquad (3.15)$$

3.3.8. Spectroscopic Analysis of the Solid Samples

Raw materials and treated biomasses were characterized using Fourier transform infrared spectroscopy (FTIR) (Spectrum 100, Perkin Elmer, Massachusetts, USA) analysis. To form a sample disk (pellet), the samples were thoroughly mixed with dried KBr (100:1, w: w), crushed, and pressed in a hydraulic press (Wir Sas, Camilla'95, Germany) at 200 bar for 3 min. The samples' spectra were recorded in the 4000-400 cm^{-1} range with a spectral resolution of 4 cm^{-1}. The number of scans and scan speed performed were 16, and 1 cm/s, respectively.

3.3.9. Soluble Protein Content

The amount of soluble protein in the samples before and after ultrafiltration was measured according to the Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard. The experiments in this section were performed in triplicate.

3.4. Statistical Analysis

One-way analysis of variance (ANOVA) and the Tukey test were used to evaluate the data ($p < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Feedstock Characterization

The cellulose, hemicellulose, lignin, and arabinan contents of the raw materials (corncob, barley stalk, corn stover, and oat straw) used in the study were determined. Among the raw materials, corncob had the highest cellulose and xylan content, while the least lignin content (Table 4.1). Having high xylan and low lignin content is most suitable for XOS production (Amorim et al., 2019). For this reason, mainly corncob was used throughout the study. The corncob content (41.2% cellulose, 32.7% xylan, 12.8% lignin, and 6.71% arabinan) was found to be close to the other studies in the literature. The corncob compositions reported in some previous studies were as follows: cellulose %38.8, hemicellulose %44.4, lignin 11.9% (Pointner et al., 2014); cellulose 35.8%, hemicellulose 37.5%, lignin 15.6 (Rofiqah et al., 2019); glucan 41.6%, hemicellulose 36.2%, xylan 33%, lignin 16.1% (Fan et al., 2013); glucan 41.9%, hemicellulose 33%, lignin 18.8% (Buyukkileci & Temelli., 2023); glucose 34%, xylose 28%, acid-insoluble lignin 17% (Van Dongen et al., 2011); glucose 34.6%, xylose 27.0, acid-insoluble lignin 9.4 (Wang et al., 2011); glucose 47.1%, xylose 28.0%, lignin 17.8% (Silva et al., 2015). Additionally, the extractive content of corn cob was determined by soxhlet analysis using toluene and ethanol as 6.19% by weight. The corn cob also contained 1.35% acetyl groups and 0.20% ash. In another study, similar results were obtained for extractive, acetyl group, and ash amounts of 7.86%, 3.76%, and 0.21%, respectively (Buyukkileci & Temelli, 2023). The compositions of the other lignocellulosic biomass tested in this study (Table 4.1) were similar to those reported in the other studies (Cherubini, 2010; Menon & Rao,

2012). In general, the results were in agreement with the literature. Minor differences in compositions could be caused by differences in analysis methods, the genotype of raw materials, and growing conditions (Keskin et al., 2018).

Table 4.1. Lignocellulosic contents of agricultural wastes used in the study[a]

Raw materials	Cellulose (%)	Xylan (%)	Lignin (%)[b]	Arabinan (%)
Corn cob	41.2±3.4	32.7±1.9	12.8±0.2	6.71±0.2
Barley stalk	31.4±1.8	19.9±1.3	25.6±0.7	3.27±0.5
Corn stover	28.2±1.0	18.2±1.1	12.9±1.4	5.44±1.0
Oat straw	26.95±2.3	18.61±1.3	17.37±1.7	5.26±0.3

[a]Results were expressed as a percentage of dry weight.
[b]Lignin values were measured as acid-insoluble lignin contents.

4.2. DES Treatment and Enzymatic Hydrolysis

In this study, it was aimed to apply enzymatic hydrolysis to the solid. Therefore, the DES pretreatment has been tried to be applied in such a way as to ensure that the xylan remains in the solid. Appropriate HBD and conditions were tested to be determined by considering LDP XOS and xylose. The molar ratio of ChCl (HBA) to each HBD tested was kept constant at 1:2 (Zdanowicz et al., 2018; Kalhor & Ghandi, 2019), DES treatment temperature and time were either 90 °C- 24 h or 130 °C- 2 h, while solid to liquid ratio was 1/10. Due to the high viscosity of DES containing urea and glycerol, 20% (v/v) water was added after DES formed. Subsequently, the treated biomass was hydrolyzed using Sheazyme 500L (activity 3025±91 U/mL), and Veron 191S (activity of 4% w/v solution: 42±7 U/mL) (Table 4.2).

Table 4.2. The effect of DES formed with HBA (ChCl) and different HBDs and treatment conditions on XOS production from corn cob using Shearzyme 500L- Veron 191S hydrolysis[a]

HBA-HBD[b]	**Water Percentage[c]**	**Temperature-Time**	**$C_{LDP\ XOS\ (g/L)}$**	**$C_{Xylose(g/L)}$**
ChCl- Urea	20	90 °C- 24 h	0.57±0.02	0.13±0.02
ChCl- Glycerol	20	90 °C- 24 h	0.12±0.00	0.06±0.00
ChCl- Ethylene glycol	0	90 °C- 24 h	0.13±0.00	0.05±0.01
ChCl- Lactic acid	0	90 °C- 24 h	0.41±0.07	0.20±0.02
ChCl- Acetic acid	0	90 °C- 24 h	0.65±0.02	0.17±0.02
Control (water)	-	90 °C- 24 h	0.16	0.03
ChCl- Urea	20	130 °C- 2 h	3.20±0.44	0.52±0.15
ChCl- Glycerol	20	130 °C- 2 h	0.78±0.43	0.19±0.14
ChCl- Ethylene glycol	0	130 °C- 2 h	0.36±0.04	0.09±0.02
ChCl- Lactic acid	0	130 °C- 2 h	0.90±0.04	0.08±0.01
ChCl- Acetic acid	0	130 °C- 2 h	0.72±0.07	0.11±0.01
Control (water)	-	130 °C- 2 h	0.67±0.04	0.10±0.00

[a] Enzymatic hydrolysis conditions: 25 U/g biomass Shearzyme 500L and 25 U/g biomass Veron 191S loading, solid-liquid ratio 1/20, 50 °C 48h, 180 rpm

[b] Molar ratio 1:2 (HBA: HBD)

[c] Percentage of water added by volume after obtaining DES.

In Table 4.2, it can be seen that a high XOS concentration was obtained when urea was used as the HBD at 90 °C- 24 h condition. When the treatment was applied at 130 °C- 2 h, the highest concentration was obtained, while the urea was the HBD. Comparing 90°C- 24 h and 130 °C- 2 h, the latter treatment temperature and time provided higher XOS production. It was observed that the concentration of LDP XOS increased as the DES treatment temperature increased. The highest XOS concentration (3.20 g/L) was

obtained when urea was used at 130 °C- 2 h. Concentration increased 4.8 times compared to treatment with water (negative control). In the literature, there were studies examining the effect of temperature on DES treatment. In the study examining the effect of DES application temperature on enzymatic saccharification, Nagoor Gunny et al. (2014) concluded that the reducing sugar produced increased as the temperature increased. The results were similar to this study. It was thought that high temperatures increased the biomass pore size and positively affected hydrolysis by lignin rebinding (Nagoor Gunny et al., 2014). Since the diffusion of ILs into biomass was accelerated at high temperatures, the increase in cellulose and lignin solubility may have been another reason for improved hydrolysis (Fu et al., 2010). In this study, the raising of both biomass pore size and diffusion due to high temperatures may have triggered the increase in XOS concentration. The low xylose concentration in all trials was positive concerning the purity of the obtained XOS. Due to the high concentration of XOS, it was decided to use urea as the HBD component in the following tests.

To examine the effect of biomass type on DES performance, DES was applied to corn stover, oat straw, and barley stalk using ChCl and urea as the DES components (Table 4.3). After the application of DES at low temperatures, very few amounts of XOS could be obtained in enzymatic hydrolysis, as in corn cobs. ChCl-urea application at 130 ^{o}C- 2 h provided a higher XOS concentration. XOS amounts and concentrations obtained with these raw materials were notably lower than those obtained with the corn cob. Therefore, the study continued with corn cob as the feedstock.

Table 4.3. Enzymatic XOS production from alternative DES-treated lignocellulosic biomass[a]

Feedstock	**Temperature-Time**	**$C_{LDP\ XOS\ (g/L)}$**	**$C_{Xylose(g/L)}$**
Barley stalk	90 °C- 24 h	0.14±0.01	
Barley stalk	130 °C- 2 h	1.59±0.02	0.23±0.01
Oat straw	90 °C- 24 h	0.08±0.01	
Oat straw	130 °C- 2 h	1.20±0.01	0.20±0.00
Corn stover	90 °C- 24 h	0.10±0.01	
Corn stover	130 °C- 2 h	1.10±0.13	0.20±0.02

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, Water ratio 20% (by volume), solid-liquid ratio 1/10; Enzymatic hydrolysis conditions: 25 U/g biomass Shearzyme 500L and 25 U/g biomass Veron 191S loading, solid-liquid ratio 1/20, 50 ºC 48h, 180 rpm

The properties of the enzyme used in the production of enzymatic XOS could have a significant effect on its production. In the next step, another enzyme called Econase XT was tested as an alternative to Shearzyme 500L- Veron 191S. Hydrolysis with Econase XT (activity of 4% w/v solution 1379±62 U/mL) was tested for all HBDs and conditions (Table 4.4).

Table 4.4. The effect of DES formed with HBA (ChCl), different HBDs, and treatment conditions on enzymatic XOS production from corn cob using Econase XT in the hydrolysis[a]

HBA-HBD[b]	**Water Percentage[c]**	**Temperature-Time**	**$C_{LDP\ XOS\ (g/L)}$**	**$C_{Xylose(g/L)}$**
ChCl- Urea	20	90 °C- 24 h	2.28±0.04	0.06±0.00
ChCl- Glycerol	20	90 °C- 24 h	0.62±0.06	0.03±0.01
ChCl- Ethylene glycol	0	90 °C- 24 h	0.83±0.01	0.03±0.00
ChCl- Lactic acid	0	90 °C- 24 h	2.12±0.18	0.11±0.02
ChCl- Acetic acid	0	90 °C- 24 h	2.19±0.10	0.13±0.02
Control (water)	-	90 °C- 24 h	0.91	0.01
ChCl- Urea	20	130 °C- 2 h	5.98±0.01	0.26±0.03
ChCl- Glycerol	20	130 °C- 2 h	1.28±0.11	0.03±0.00
ChCl- Ethylene glycol	0	130 °C- 2 h	1.43±0.00	0.03±0.00
ChCl- Lactic acid	0	130 °C- 2 h	0.88±0.04	0.04±0.01
ChCl- Acetic acid	0	130 °C- 2 h	1.19±0.01	0.04±0.01
Control (water)	-	130 °C- 2 h	3.04±0.04	0.13±0.01

[a] Enzymatic hydrolysis conditions: 50U/g biomass enzyme loading, solid-liquid ratio 1:20, 70 °C 48h, 180 rpm

[b] Molar ratio 1:2 (HBA: HBD)

[c] Percentage of water added by volume after obtaining DES.

Urea, lactic acid, and acetic acid yielded similar XOS concentrations at 90 °C- 24 h. When the temperature was increased (130 °C- 2 h studies), XOS concentration was enhanced in the use of urea, while it decreased in the case of lactic acid and acetic acid. In acid use, as the temperature increased, the decrease in concentration may have been caused by the probable decrease in solid recovery in the DES treatment step. Jiang et al. (2019) reported that when acid pretreatment was applied to sweet sorghum bagasse, solid

recovery decreased as temperature and acid use increased. When the solid obtained after ChCl-urea treatment was subjected to Econase XT hydrolysis, the highest XOS concentration (5.98 g/L) was obtained, which was two times that of the negative control. It was decided to use urea as an HBD component with Econase XT as well, due to the higher XOS concentrations. The improvement in concentration with temperature supported the interpretation of temperature for the results in the previous section. Considering the results in Table 4.4, the XOS concentrations obtained for all conditions were higher than those obtained using Shearzyme 500L- Veron 191S hydrolysis (Table 4.2). The low xylose concentration in all runs was also an advantage considering the purification of XOS. It was thought that the difference in the XOS concentration may have been related to the substrate specificity of the enzymes and the diversity of the auxiliary enzymes (β-xylosidase, acetyl esterase, arabinofuranosidase) they contain. Auxiliary enzymes cleave the bonds on the leading chains or side chains of different types of xylan (Pinales-Márquez et al., 2021). Auxiliary enzymes that improved the hydrolytic capacity of enzymes increased enzymatic saccharification (Brar et al., 2019). According to the information provided by the manufacturers, Shearzyme 500 L was developed for bioethanol production, Veron 191S for bread making, and Econase XT for feed production. The reason for different XOS amounts could be attributed to different usage areas of xylanase. Econase XT enzyme provided more promising hydrolysis for XOS manufacturing.

4.2.1. Effect of Water Addition on the DES

To demonstrate the hypothesis that the amount of water added to DES will affect the process between DES and raw material (Chen et al., 2018b; Huang et al., 2020; Liang et al., 2020; Morais et al., 2018), water was added to several levels (0, 20, 40%) to DES prepared with ChCl-urea. Besides, to examine the effect of temperature, the runs were performed at 90 ^{o}C- 24 h, 110 ^{o}C- 2 h, and 130 ^{o}C- 2 h (Table 4.5).

Table 4.5. The effect of water addition to DES (ChCl- urea) under different treatment conditions on enzymatic XOS production[a]

Molar Ratio	Water Percentage[b]	Temperature-Time	Enzyme[c]	$C_{LDP\ XOS\ (g/L)}$	$C_{Xylose(g/L)}$	$C_{XOS\ (g/L)}$
1:2	0	90 °C- 24 h	SV	0.37±0.08	0.06±0.07	-
1:2	20	90 °C- 24 h	SV	0.57±0.02	0.13±0.02	-
1:2	40	90 °C- 24 h	SV	0.67±0.01	0.12±0.01	-
1:2	0	110 °C- 2 h	SV	1.02±0.03	0.16±0.01	-
1:2	20	110 °C- 2 h	SV	1.10±0.01	0.17±0.01	-
1:2	40	110 °C- 2 h	SV	1.18±0.06	0.19±0.02	-
1:2	0	130 °C- 2 h	SV	2.70±0.33	0.36±0.04	3.47±0.45
1:2	20	130 °C- 2 h	SV	3.20±0.44	0.52±0.15	4.28±0.08
1:2	40	130 °C- 2 h	SV	3.37±0.19	0.46±0.03	4.39±0.03
1:2	0	90 °C- 24 h	E	0.47±0.04	0.06±0.00	-
1:2	20	90 °C- 24 h	E	1.28±0.04	0.06±0.00	-
1:2	40	90 °C- 24 h	E	1.67±0.09	0.08±0.00	-
1:2	0	110 °C- 2 h	E	1.58±0.12	0.08±0.00	-
1:2	20	110 °C- 2 h	E	2.38±0.03	0.11±0.01	-
1:2	40	110 °C- 2 h	E	2.67±0.27	0.11±0.01	-
1:2	0	130 °C- 2 h	E	4.36±0.31	0.25±0.02	5.81±0.01
1:2	20	130 °C- 2 h	E	5.98±0.01	0.26±0.03	9.19±0.59
1:2	40	130 °C- 2 h	E	7.70±0.66	0.30±0.04	10.35±0.70

[a] - not analyzed

[b] Percentage of water added by volume after obtaining DES.

[c] Enzymatic hydrolysis conditions: 25 U/g biomass Shearzyme 500L and 25 U/g biomass Veron 191S loading (SV), solid-liquid ratio 1:20, 50 °C 48h, 180 rpm or 50U/g biomass Econase XT loading (E), solid-liquid ratio 70 °C 48h, 180 rpm

Generally, a slight increase in LDP XOS concentrations was observed as the water ratio was increased from 0% (v/v) to 40% (v/v) at each temperature. When each water ratio and enzyme were examined separately, it was observed that the LDP XOS concentrations increased with the temperature increased. For the Shearzyme 500L- Veron 191S hydrolysis, the highest concentration was obtained at 130 ^{o}C with 40% (v/v) water addition and 3.37 g/L LDP XOS and 4.39 g/L total XOS were obtained. 77% of the total XOS consisted of LDP XOS. The low conversion to xylose stated that the amount of xylose in the hydrolysate obtained by enzymatic hydrolysis was low. Similar trends were observed in the previous set, higher concentrations were obtained with Econase XT. 7.70 g/L LDP XOS and 10.35 g/L total XOS concentration were obtained with both 40% water at 130^{o}C for Econase XT hydrolysis. 74% of the total XOS was LDP XOS.

As it was seen that the amount of water added to DES was effective on XOS production in the previous set of experiments, it was decided to test the water ratio in a wider range (0%-100%). In this test, a condition of 130 ^{o}C- 2 h, which provided high XOS production, in the DES treatment, and the pretreated biomass was hydrolyzed using Shearzyme 500L- Veron 191S and Econase XT. The results obtained were presented in Figure 4.1.

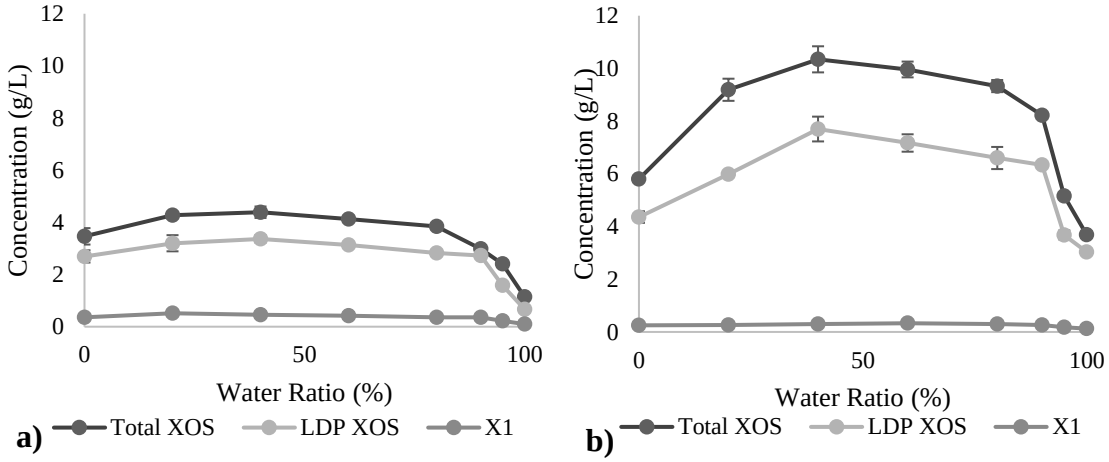

Figure 4.1. The effect of water percentage in DES pretreatment[a] on the enzymatic hydrolysis using a) Shearzyme 500L- Veron 191S hydrolysis b) Econase XT[b]

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, solid-liquid ratio 1/10, 130 ^{o}C- 2 h

[b] Enzymatic hydrolysis conditions: 25 U/g biomass Shearzyme 500L and 25 U/g biomass Veron 191S loading, solid-liquid ratio 1:20, 50 ^{o}C 48h, 180 rpm or 50U/g biomass Econase XT loading, solid-liquid ratio 1:20, 70 ^{o}C 48h, 180 rpm

The addition of water caused a significant difference in total XOS ($p=0.00<0.05$) and LDP XOS ($p=0.00<0.05$) concentrations as a result of enzyme hydrolysis using Shearzyme 500L- Veron 191S. The addition of water caused a significant difference in total XOS ($p=0.00<0.05$) and LDP XOS ($p=0.00<0.05$) concentrations as a result of enzyme hydrolysis using Econase XT. There was an increase in total XOS and LDP XOS concentrations when the amount of water was increased from 0% to 40% in Econase XT hydrolysis (Figure 4.1-b). A slight increase in concentrations was observed from 0% to 40% in Shearzyme 500L- Veron 191S hydrolysis (Figure 4.1-a). While the amount of water was increased from 40% to 90%, the XOS production decreased. There was a sharp decrease between 90% and 100% water addition. When the enzymes were compared, it was seen that the Econase XT enzyme provided higher XOS than the Shearzyme 500L-Veron 191S. When the Econase XT enzyme was used, the highest total XOS and LDP XOS concentrations were obtained with 40% water addition and were 10.35 g/L and 7.70 g/L, respectively. Considering the high results of 40% water addition, further studies were

carried out with 40% water. In addition, for control experiments of DES treatment, the use of HBA or HBD at the same concentration was tested instead of DES with water addition. When HBA (403g/L ChCl which equals DES with a 40% water ratio) was tested alone, a very low amount of LDP XOS (1.24 g/L), and xylose (0.08 g/L) was produced. When HBD (347 g/L urea which equals DES with a 40% water ratio) was tested alone, a low amount of LDP XOS (5.42 g/L), and xylose (0.23 g/L) were produced.

The high viscosity of DES solutions may lead to cause problems such as mixing and mass transfer in DES applications. To reduce this problem, usually water was added to DES (Shishov et al., 2017). For example, it has been reported that the viscosity was reduced to one-tenth by adding 15 mol% water to the ChCl- urea mixture (Xie et al., 2014). In parallel with lowering the viscosity, the polarity changed, the mass transfer improved and the extraction efficiency increased (Vilková et al., 2020). On the other hand, it has been reported that the effect of the interaction between DES components decreased with water (El Achkar et al., 2019; González et al., 2017). Although it varied according to the components used, it has been shown that 50% or more water amount eliminated the interaction between the components (Dai et al., 2015; Gabriele et al., 2019; Hammond et al., 2017). When the ChCl- urea mixture was applied to palm tree wastes, New et al. (2019) observed the highest delignification (17%) with 30% water, while when the water was increased to 50%, delignification decreased (11%). Additionally, studies observed DES in the presence of water content effect on the extraction of compounds. Wan et al. (2019) used ChCl - acetic acid to extract flavonoids from flowers, while the best results were obtained with 70% water. When ChCl- methacrylic acid mixture was used, a remarkable amount was observed as an extraction with 90% water. González et al. (2017) obtained similar results between 10% and 60% when the effect of water was examined up to 60% by volume in different DES mixtures for vanilla extraction. In this study, it was observed that the addition of water up to 40% increased the subsequent hydrolysis, on the other hand, it was observed that the hydrolysis of xylan was lower at higher water levels. Accordingly, it can be concluded that DES also provides an effect in dilute form for xylan hydrolysis.

The treated corn cobs obtained by applying DES containing 0% 40% (selected DES condition) and 90% water by volume were characterized by acid hydrolysis, and their compositions were determined (Tablo 4.6). There was no significant difference in the effect of water on glucan recovery ($p=0.333>0.05$) and xylan recovery

(p=0.841>0.05). The effect of adding water caused a significant difference in lignin removal (p=0.019<0.05). Glucan recovery was between 65% and 69%. Delignification was 40.4%, 65.5%, and 53.9% for biomass treated with DES containing 0% 40%, and 90% water, respectively. The concentrations obtained as a result of delignification and enzymatic hydrolysis overlapped with each other and it was evaluated that the hydrolysis efficiency increased as delignification increased. Since the xylan recovery was determined as approximately 80% in three different conditions, it can be interpreted that a xylan-rich treated biomass was obtained.

Table 4.6. Effect of DES (ChCl- urea) treatment[a] on cellulose, xylan, and lignin in corncob

Water percentage[b]	**Solid recovery (%)**	**Glucan recovery (%)**	**Xylan recovery (%)**	**Lignin removal (%)**
0	72.87	64.9±0.14	79.48±2.72	40.43±2.94
40	65.38	68.53±3.13	78.92±6.46	65.48±3.37
90	69.41	66.48±1.56	77.02±2.31	53.85±5.41

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, solid-liquid ratio 1/10, 130 ^{o}C- 2 h

[b] Percentage of water added by volume after obtaining DES.

Procentese et al. (2015), applied ChCl and imidazole to corn cob, and the highest saccharification rate was obtained while delignification was 55%. In another study, it was concluded that delignification which provided the availability of xylan increased the XOS yield since lignin covered the surface of xylan and bound to hemicelluloses as lignin-carbohydrate complexes (Yang et al., 2021). In the studies, it was concluded that lignin removal improves enzymatic hydrolysis. In this study, when delignification was compared at 0, 40, and 90% water addition, the ordering of the lignin removal rate as water addition was observed at 40% >%90 >0% (water ratio of DES) (Table 4.6). Considering the hydrolysis results for 0%, 40%, and 90% water addition (Figure 4.1-b),

the order of XOS production amount was 40%>90%>0% according to water addition. As delignification increased, XOS production increased. In this study, a similar delignification and hydrolysis relationship was concluded (Procentese et al., 2015; Yang et al., 2021). Lignin extractability was found to be between 43-93% with ChCl and monocarboxylic acids, 22-98% with ChCl and dicarboxylic acids, and 71.88% with ChCl glycerol and ChCl ethylene glycol, respectively (Zhang et al., 2016b). Zhang et al. (2016b) concluded that lignin extractability increased significantly as the temperature increased from 70^{o}C to 110^{o}C. The increase in the efficiency of enzymatic hydrolysis as the temperature increased in this study, could be attributed to the enhanced lignin removal at higher temperatures. Besides, DES was mostly used for delignification in the literature. Considering other studies, the extent of delignification achieved in this study (Table 4.6) could be considered successful: When rice straw was treated with ChCl and urea, lignin removal was increased up to 45% (Pan et al., 2017). In another study using rice straw, lignin removal was found to be 61% using ChCl and lactic acid (Hou et al., 2018). Kumar et al. (2015) removed 60% of lignin when ChCl and lactic acid DES was applied to rice straws. In the study in which different HBD was tested for delignification in wheat straw, the highest lignin removal was found to be 57.9%, since ChCl and oxalic acid dihydrate were used (Jablonský et al., 2015).

4.2.2. Effect of Enzyme Dosage

In the next step, the effect of enzyme amount on XOS production was investigated. Firstly, corncob was treated with a ChCl- urea solution containing 40% water at 130 °C for 2 h. Then, enzymatic hydrolysis was carried out by adding Shearzyme 500L- Veron 191S or Econase XT enzymes to the treated biomass at an enzyme dosage of 25-50-100-200-400 U/g biomass. The XOS concentrations obtained were shown in Figure 4.2-a for Shearzyme 500L- Veron 191S hydrolysis, while Figure 4.2-b is for Econase XT hydrolysis. Enzyme loading amount had a significant difference in total XOS ($p=0.000<0.05$) and LDP XOS ($p=0.000<0.05$) when using Shearzyme 500L- Veron

191S. Enzyme loading amount had a significant difference in total XOS ($p=0.001<0.05$) and LDP XOS ($p=0.002<0.05$) when using Econase XT. As in previous experiments, Econase XT provided higher concentrations than the Shearzyme 500L- Veron 191S. A similar trend was observed in this set. Since the Econase XT enzyme was more effective in the production of XOS, it was used for the hydrolysis process in the following tests. After a significant increase was seen when enzyme dosages were increased from 25 U/g to 50 U/g, the effect of the increase in dosage was less pronounced in the higher doses (Figure 4.2-b). It was concluded that 50- 100 U/g enzyme dosages were appropriate. It was seen that approximate concentrations of total XOS, and LDP XOS were 10.4 g/L, and 7.7 g/L, respectively at 50 U/g biomass Econase XT loading; while 11,77 g/L total XOS and 8,24 LDP XOS were produced at 100 U/g biomass Econase XT loading (Figure 4.2-b). Considering the high cost of enzymes, hydrolysis with 50U/g biomass Econase XT was selected for the following studies.

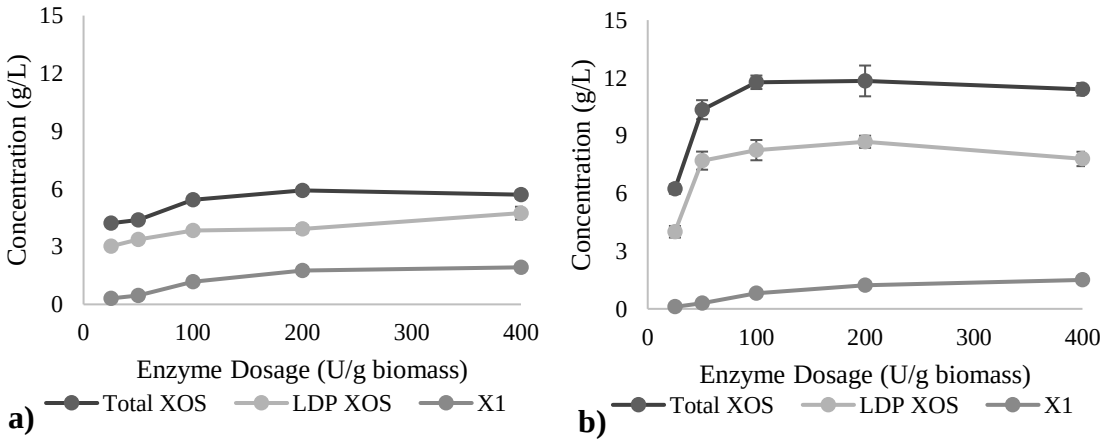

Figure 4.2. Effect of enzyme dosage on XOS concentration a) Shearzyme 500L- Veron 191S hydrolysis b) Econase XT hydrolysis[a]

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, solid-liquid ratio 1/10, 130 °C- 2 h; Enzymatic hydrolysis conditions: Shearzyme 500L and Veron 191S, solid-liquid ratio 1:20, 50 °C 48h, 180 rpm or Econase XT, solid-liquid ratio 1:20, 70 °C 48h, 180 rpm

4.2.3. Effect of DES Treatment Conditions

In another set of experiments, the effect of DES treatment time was examined. ChCl-Urea was applied at 130^{o}C for 1, 2, and 4 h and in the subsequent enzymatic hydrolysis, LDP XOS and total XOS concentrations were determined (Figure 4.3). DES pretreatment time caused a significant difference in terms of total XOS ($p=0.001<0.05$) and LDP XOS ($p=0.003<0.05$). It differed from the others when the pretreatment time was 1 h. Similar concentrations were obtained at 2 and 4 h. When the DES treatment time was reduced from 2 h to 1 h and increased from 2 h to 4 h, the LDP XOS concentrations also decreased. Total XOS increased very slightly when the time was increased from 2 h to 4 h. For this reason, the DES application for 2 h, with 10.35 g/L total XOS and 7.7 g/L LDP XOS, was selected to be the optimal treatment duration. It was shown in the literature, that the prolongation of the treatment can be reduced the reuse quality of DES and increased the energy input and biorefinery costs, which was a major barrier to its economic viability (Guo et al., 2019). Guo et al. (2019) concluded that the recalcitrant structure of the corn cob was released quickly and effectively by DES treatments, and the DES treatment was applied for 2 h at 100- 140 ^{o}C in the research study.

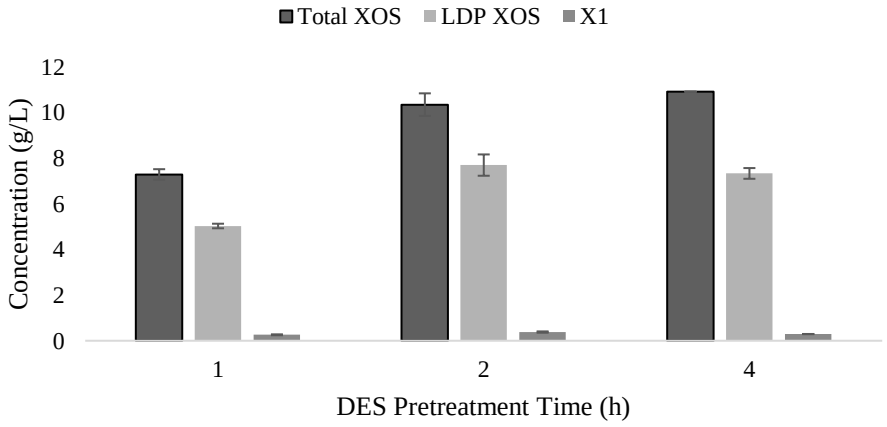

Figure 4.3. Effect of DES treatment time on enzymatic XOS production[a]

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, solid-liquid ratio 1/10, 130 ^{o}C; Enzymatic hydrolysis conditions: 50 U/g biomass Econase XT, solid-liquid ratio 1:20, 70 ^{o}C 48h, 180 rpm

Another DES treatment condition was the molar ratio. In the ChCl- urea DES application, it was planned to increase the molar ratio to 1:4 and 1:6, but the molar ratio was continued to be applied as 1:2 due to the increase in urea could not form transparent and homogeneous DES.

In the DES treatment, the solid-liquid ratio was also evaluated as a parameter and tests were carried out at 1/5, 1/10, and 1/20 (g/mL). In previous studies, the solid-liquid ratio of DES with biomass had applications as 1/5, 1/10, and 1/20 (Liang et al., 2020; Chen et al., 2018b; Fang et al., 2017; Liu et al., 2019). In this study, LDP XOS concentrations were found to be 7.61±0.23 g/L, 7.08±0.80 g/L, and 7.60±0.30 g/L for 1/5, 1/10, and 1/20 (g/mL), respectively (Figure 4.4.). Xylose concentrations were low for all solid-liquid ratios. There was no significant difference in the effect of solid-liquid ratios on LDP XOS ($p=0.643>0.05$) and xylose ($p=0.05$) production. The fact that the concentrations did not change considerably when the solid-liquid ratio was changed within the levels of this study showed that DES could give sufficient results even at the highest biomass loading (at solid to liquid ratio of 1/5). It had been observed that higher biomass loadings lead to less xylan removal in the treatment of DES processes (Huang et al., 2020). In another study aimed at extracting xylan from *Eucalyptus globulus* with DES (ChCl- urea) treatment, more xylan was extracted at a solid-to-liquid ratio of 1/25 than 1/10 (Morais et al., 2018).

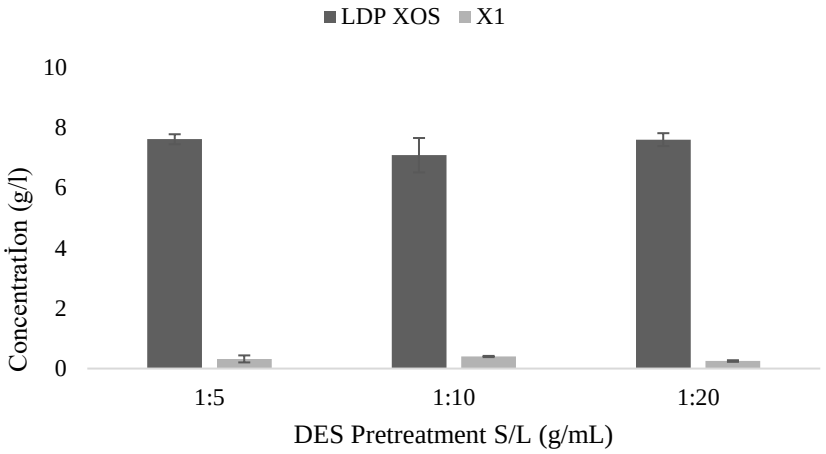

Figure 4.4. Effect of DES treatment solid to liquid ratio on enzymatic XOS production[a]

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, solid-liquid ratio 1/10, 130 °C; Enzymatic hydrolysis conditions: 50 U/g biomass Econase XT, solid-liquid ratio 1:20, 70 °C 48h, 180 rpm

Considering the relationship between xylan and biomass loading in the studies, high biomass loading should be preferred in this study, where it is aimed to keep xylan in a solid. According to the enzymatic hydrolysis results in this study, although the effect of the solid-liquid ratio was not significant, reducing the amount of DES was cost-effective. Further studies were continued with a 1/5 solid-liquid ratio.

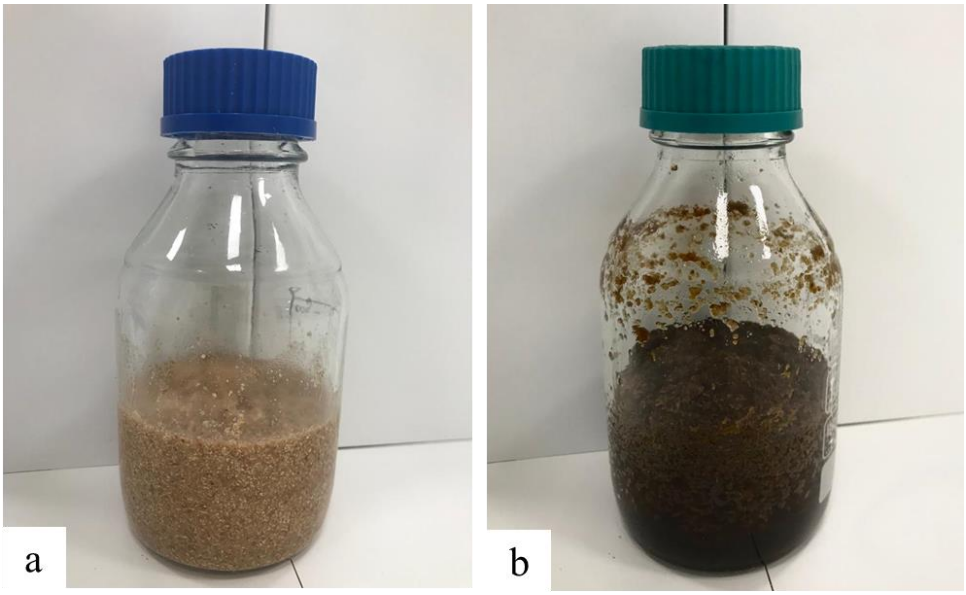

Figure 4.5. The appearance of corncob before (a) and after (b) DES treatment (ChCl: urea 1:2, solid-liquid ratio 1/5, 130 ^{o}C 2h)

The change in the appearance of the corncob with the DES treatment selected considering the effect of the treatment conditions on the enzymatic hydrolysis efficiency was observed in Figure 4.5. Corn cob was suspended in DES liquid (Figure 4.5-a). After DES treatment, the solution was absorbed by the solid. There was no DES liquid in the environment. The color of the corn cob changed from yellow to brown with DES treatment (Figure 4.5-b).

4.2.4. DES Reuse

The reuse of DES was tested with ChCl- urea with a molar ratio of 1:2, a solid-liquid ratio of 1/10, and at 130 ºC for 2 h, and the results in Table 4.7 were obtained. In the reuse experiment, the reason why 1/10 was used instead of 1/5 solid-liquid ratio was that when biomass loading increased, there was no liquid in the environment after DES treatment. Also, spent DES recovered by filtration after each treatment was used for the next treatment, without any addition of chemicals or modification. DES could be reused effectively and yielded close XOS concentrations in the subsequent enzymatic hydrolyses following each run. There was a significant difference between the cycles in terms of LDP XOS ($p=0.643<0.05$). The second cycle LDP XOS concentration result showed a significant difference compared to the others. Considering the bioprocess, although there was no drastic difference between LDP XOS concentration, the amount of liquid recovered decreased to approximately half in each use. At first, while 5 g of corn cob was processed with 50 mL of DES, 28 mL of DES was recovered for the second use, and 2.8 g of corn cob could be treated to keep the solid-to-liquid ratio at 1/10. After the second run, 16 mL of DES was recovered and 1.6 g of corn cob could be processed in the third cycle. For this reason, the number of cycles was limited to three under the conditions of this study. The reduction of DES liquid and corncob used as halved in each use decreased the efficiency of reuse. Instead, it had become a good alternative to reduce the solid-liquid ratio and apply DES without recovery. As mentioned at the beginning, when the biomass loading was increased, reuse could not be applied because there was no liquid in the environment after DES treatment. Reuse aimed to reduce the use of DES. When the solid-liquid ratio was applied as 1/5, less DES was used initially (without reuse).

Table 4.7. XOS and xylose concentrations in DES reuse[a]

Cycle	$C_{LDP\ XOS\ (g/L)}$	$C_{Xylose(g/L)}$
1st	7.70±0.66	1.03±0.00
2nd	7.32±0.12	0.95±0.01
3rd	7.73±0.09	1.05±0.06

[a] DES treatment conditions: ChCl- urea, Molar ratio 1:2, solid-liquid ratio 1/10, 130 °C; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1:20, 70 °C 48h, 180 rpm

4.2.5. Effect of Enzymatic Hydrolysis Conditions and Biomass Loading in DES Treatment Step

The effect of temperature (50- 60-70 °C) during enzymatic hydrolysis of the DES-treated corncob was investigated at two enzyme dosages, namely 50 and 400 U/g biomass (Figure 4.6). The optimal (50 U/g biomass), and previously tested highest (400 U/g biomass) enzyme dosages were chosen to observe the effect between hydrolysis temperature and enzyme loading. The temperature had a significant effect on LDP XOS in enzymatic hydrolysis ($p=0.000<0.05$). At 50 °C and 60 °C, XOS production increased with enzyme dosage (Figure 4.6-a). However, increasing the enzyme dosage from 50 to 400 U/g biomass did not affect the XOS concentration at 70 °C and the concentration at higher enzyme dosage was lower than the other two temperature values. With 400 U/g biomass enzyme loading, when the temperature was lowered from 70 °C to 60 °C, the concentration of LDP XOS increased by about 29%. With 50 U/g biomass enzyme loading, the concentration of LDP XOS was 5.94 g/L and 7.33 g/L at 50 °C and 60 °C, respectively. 400 U/g biomass enzyme loading at 60 °C showed a significant difference. The highest LDP XOS concentration (10.81 g/L) was obtained when the enzyme dosage was 400 U/g biomass and the hydrolysis temperature was 60 °C. Based on these results, the enzymatic hydrolysis process was conducted at 60 °C in the following tests. The effect of temperature and enzyme amount on the by-product (xylose) was shown in Figure 4.6-

b. At low enzyme dosage (50 U/g biomass), xylose formation remained at a low level (0.26-0.51 g/L). It was observed that xylose formation was significantly increased at high enzyme dosage (1.5-2.41 g/L). The xylose concentration was found to be relatively lower at 70 ºC.

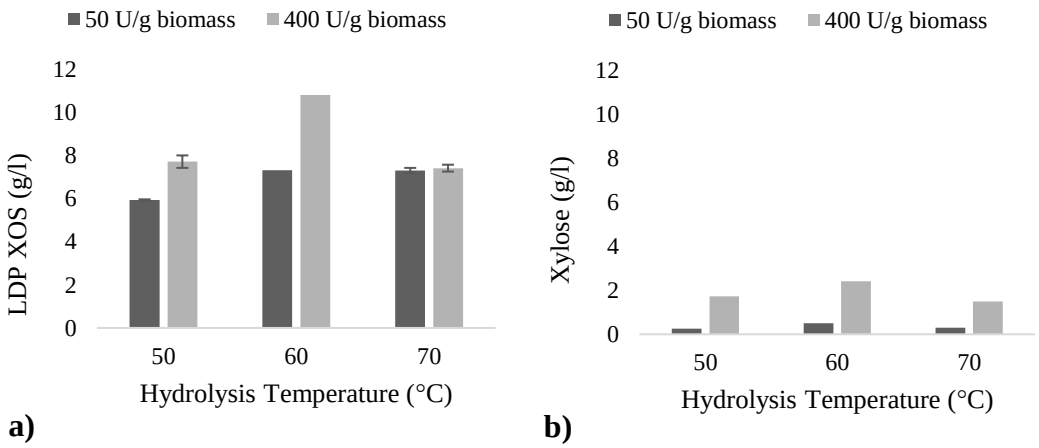

Figure 4.6. Effect of enzymatic hydrolysis[a] temperature on the formation of a) LDP XOS b) xylose

[a] DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/10, 130 °C - 2 h; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, 180 rpm, 48h

In the previous tests of this study, it was observed that the enzyme dosages at 60ºC affected the XOS formation. For this reason, enzyme dosages had been tested over a wider range (50- 100- 200- 400- 800- 1600 U/g biomass). In this test, the effect of biomass loading during DES treatment was also investigated. The treated corn cob samples obtained by DES application at 1/5 and 1/10 solid-to-liquid ratio (g corn cob/ ml DES) were examined at the selected enzyme dosages. It had been observed that two different solid-liquid ratios provided very similar LDP XOS concentrations (Figure 4.7).

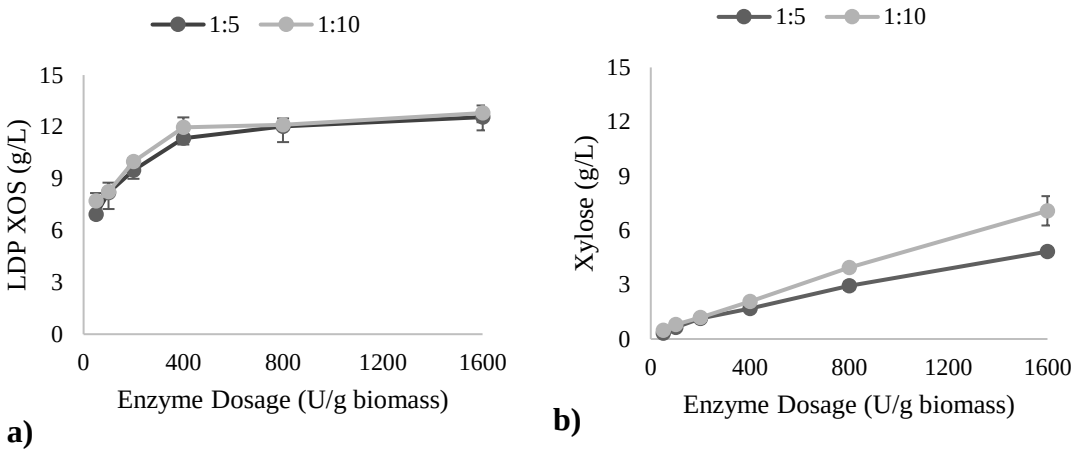

Figure 4.7. Effect of DES solid-liquid ratio and enzyme dosage[a] concentration of a) LDP XOS b) xylose

[a] DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, 130 °C- 2 h; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1:20, 60 °C 48 h, 180 rpm

Accordingly, it was concluded that the solid-liquid ratio selected in the DES treatment did not affect the subsequent enzymatic hydrolysis (Figure 4.7-a). When the biomass loading was increased (1/5 of the solid-liquid ratio) in DES treatment, similar XOS production was obtained and the use of DES was halved. Enzyme loading had a significant difference in LDP XOS production ($p=0.000<0.05$). As the enzyme amount was increased from 50U/g biomass to 400U/g biomass, LDP XOS concentration increased almost linearly (Figure 4.7-a). When the enzyme amount was increased further to 800 and 1600 U/g biomass, the concentrations were close to that obtained with 400 U/g biomass. The increase in concentration was only 1.2% and 6.8%, respectively, with 800 and 1600 U/g biomass enzymes (compared to 400 U/g). The reason XOS production plateaued after 400U/g biomass enzyme loading may have been that the capacity of enzyme bound to the substrate reached the plateau with 400 U/g biomass. The 400U/g enzyme dosage was concluded to be the most efficient level since the low increase in LDP XOS concentration for 800 and 1600 U/g biomass was not considerable. Besides, enzymatic hydrolysis was demonstrated as the most expensive step due to the high cost of the enzyme in the lignocellulosic biorefinery approach (Volynets & Dahman, 2011; Olivieri et al., 2021). Considering the bioprocess economy, enzyme loading affected the

cost of the whole process. Therefore, it was important to aim to minimize the amount of enzyme used while maximizing saccharification efficiency. When the enzyme amount was doubled and quadrupled, the slight increase in XOS production also supported the choice of 400U/g biomass when considering the enzyme loading by keeping the cost in the foreground. When the effect of enzyme dosage and solid-liquid ratio on the formation of xylose (by-product), was examined, it was seen that a similar conversion was obtained at the two solid-liquid ratios in the range of 50-400 U/g (Figure 4.7-b). On the other hand, a difference was observed at higher enzyme dosages. Additionally, it was investigated whether the enzyme dosage applied under 60 ºC temperature conditions could have an effect in mild DES conditions (90 ºC 24 h). According to the result, it was ensured that a high temperature (130 ºC- 2 h) was required for the production of XOS (Appendix D).

The effect of enzyme dose was also examined in literature studies. Akpinar et al. (2007) reported that hydrolysis yield and rate would increase with increasing enzyme dose while producing XOS from cotton stem xylan. In another study, in which XOS was produced from *Sehima nervosum* grass xylan, it was concluded that the hydrolysis yield and rate increased with increasing enzyme dose (Samanta et al., 2012a). Yamamoto et al. (2019) tested hydrolysis at two different enzyme dosages (13.5–54 U) after obtaining xylan from red algae dulse (*Palmaria sp.*). As a result, the increase in the amount of enzyme in 36 h hydrolysis time increased the amount of XOS in hydrolysates from 3.5 to 4.8 mg/ml and increased the hydrolysis rate from 60% to 81%. In another study, XOS was produced by enzymatic hydrolysis from xylan obtained from corncob (Chapla et al., 2012). Improvement in yield was found after prolonged incubation with a higher enzyme dose (Chapla et al., 2012). Wang et al. (2018) obtained XOS from hemicellulose obtained from soluble wood pulp by enzymatic hydrolysis. As a result of hydrolysis with different enzyme concentrations (50, 80, 120, and 150 IU/g substrate), it was observed that enzyme dosage had a positive effect on XOS production. The XOS yield were almost unchanged when the enzyme concentration increased from 120 to 150 IU/g substrate, possibly as the enzyme protein's capacity to bind to its substrate reached a plateau at a given enzyme concentration (Wang et al., 2018). The increase in XOS production as the enzyme dose increased and the absence of an increase after a certain amount of enzyme was similar in this study.

After the enzyme amount was determined as 400 U/g biomass and the solid-liquid ratio of DES treatment was 1/5, the effect of xylanase hydrolysis time was investigated

under this condition. Enzyme hydrolysis time had a significant effect on LD COS concentration ($p=0.000<0.05$). It was observed that the concentrations increased as the hydrolysis time was extended for 48 h (Figure 4.8). A rapid formation of XOS was observed in the first 6 h; 41% of LDP XOS was produced in the first 6 h. Most of the XOS release (85% of total LD concentration at 48 h) was completed within 24 h. There was another 17.3% concentration increase in the next 24 h, and XOS concentration (11.48 g/L) was achieved at 48 h. Approximately 1.69 g/L xylose was produced within 48 h. Accordingly, the ratio of LDP XOS/xylose in the hydrolysate was calculated to be approximately seven. For the enzymatic hydrolysis time of 48 h, the concentration of LDP XOS increased, while the concentration of xylose was almost unchanged. Based on these, 48 h was determined as the most suitable time for hydrolysis.

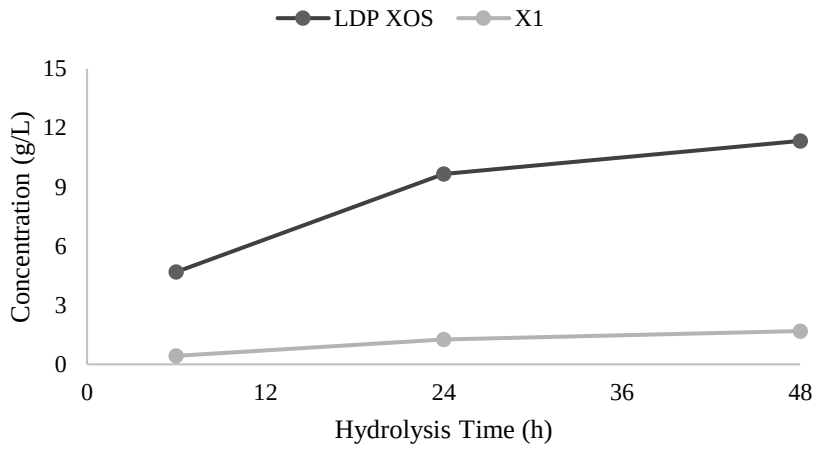

Figure 4.8. Effect of enzymatic hydrolysis time on LDP XOS and xylose

DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/5 ,130°C- 2 h; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, enzyme dosage 400U/g biomass, 60 ^{o}C, 180 rpm

The solid-liquid ratio in the hydrolysis process had been tested. Up to this point DES-treated corncob to citrate buffer ratio was kept at 1/20, in the enzymatic hydrolysis step. In addition to that, a solid-to-liquid ratio of 1/10 was performed. The motivation

behind increasing the solid-liquid ratio (increasing the biomass loading) was to ensure a high product concentration in the liquid obtained after enzymatic hydrolysis. High product concentration had the potential to reduce the cost of the downstream process, as it resulted in a low volume processed in product purification and a low probability of water being removed during drying. While doing this, the enzyme loading per biomass was kept constant at 400 U/g biomass. When the solid-liquid ratio was 1/10, it was observed that the XOS yield (based on xylan in the raw corncob) was reduced by about half (Table 4.8). The negative effect of high solids loading could be attributed to ineffective mixing and mass transfer. The findings of this study were similar to previous studies. In other studies, it had been concluded that high biomass loading reduced sugar conversion by causing reduced mass transfer while increasing viscosity, inefficient binding of the enzyme to the substrate, and product inhibition (Andrić et al., 2010; Gruno et al., 2004; Kristensen et al., 2009). Ioelovich and Morag (2012), tested cellulase hydrolysis of acid and alkali pretreated switchgrass with different biomass loading (50 g/L and 200 g/L). They concluded that as the biomass loading increased, the enzyme digestibility decreased due to the difficulty of mass transfer of the enzyme to the high-viscosity substrate slurry and the inhibitory property of the products as a result of hydrolysis (Ioelovich & Morag, 2012). In another study, wheat straw hydrolysis was tested with high solids loading, and it was found that the low amount of liquid in the hydrolysis limited the diffusion of the enzyme to the substrate and the yield decreased (Weiss et al., 2019).

Table 4.8. The effect of solid-liquid ratio (treated biomass loading) on yield in enzyme hydrolysis[a]

Solid-Liquid Ratio[b]	**$C_{LDP\ XOS\ (g/L)}$**	**$C_{Xylose(g/L)}$**	**$Y_{LDP\ XOS}$**	**Y_{Xylose}**
1/10	19.73±0.72	3.56±0.04	19.21±0.34	3.47±0.01
1/20	11.03±0.31	2.05±0.04	39.70±0.45	5.73±0.86

[a]DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/5 130°C- 2 h; Enzymatic hydrolysis conditions: Econase XT, enzyme dosage 400U/g biomass, 60 °C 48h, 180 rpm

[b] g biomass/ mL citrate buffer

Considering the results of this research, it was found appropriate to apply DES treatment to corncob with ChCl-Urea (1:2 molar ratio, and 40% water addition by volume) using 1/5 solid-liquid ratio at 130°C for 2 h. It was concluded that a high concentration of XOS would be produced with 400 U/g biomass Econase XT loading at a 1/20 solid-liquid ratio of the processed biomass for 48 h at 60 ^{o}C.

4.3. Alkaline-assisted DES Treatment

Increasing the acidity and alkalinity of DES also increased the efficiency of lignin removal (Teng et al., 2022). Mild acidic and alkaline DES affected delignification by breaking unstable ether bonds (Yue et al., 2020). In this study, it was observed that XOS production was low when DES-containing acid (HBD, such as lactic acid, and acetic acid) was used, so alkali addition was preferred. The addition of alkali (NaOH) was tested to increase the lignin removal rate of DES treatment. DES treatment was applied to corn cobs at low temperatures (60 ^{o}C, 90 ^{o}C) was conducted in the presence of NaOH. Alkaline-assisted DES treatment was compared with the treatments conducted with only DES, only alkali, as well as with water. Those applied to the corn cob at 60 ^{o}C and 90 ^{o}C and the XOS release in the subsequent enzymatic hydrolysis were determined in Table 4.9. Teng et al., (2022) observed that the addition of 1% NaOH to the DES treatment applied to wheat straw increased the lignin removal and did not make a significant difference in the hemicellulose content. Li et al. (2023), added NaOH to the organosolv treatment of poplar and observed lignin removal while hemicellulose and cellulose remained in the solid.

Table 4.9. The effect of alkali addition in DES treatment on XOS production[a]

Solvent	**NaOH (wt/v %)**	**Temperature-Time**	**$C_{LDP\ XOS\ (g/L)}$**	**$C_{Xylose(g/L)}$**
Alkali-assisted DES	1 %	60 °C- 18 h	6.09±0.08	0.76±0.03
Alkali-assisted DES	1 %	90 °C- 18 h	7.29±0.14	0.37±0.06
DES	-	60 °C- 18 h	0.82±0.16	0.31±0.00
DES	-	90 °C- 18 h	3.70±0.13	0.21±0.01
Alkali	1 %	60 °C- 18 h	8.73±0.39	2.51±0.42
Alkali	1 %	90 °C- 18 h	9.70±0.35	2.41±0.29
Water (control)	-	60 °C- 18 h	0.05	0.20
Water (control)	-	90 °C- 18 h	0.42	0.03

[a] DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/10; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, enzyme dosage 50U/g biomass, 60 ^{o}C 48h, 180 rpm

In all treatments, LDP XOS concentrations increased with increasing temperature. The addition of 1% alkali to the DES improved the concentrations. Alkali-assisted DES treatment at 60 ^{o}C yielded 6.09 g/L XOS in the enzymatic hydrolysis, which was 7.4 times higher than the one obtained in the hydrolysis after only DES (no alkali addition) treatment at the same temperature. At 90 ^{o}C, the effect of alkali addition was pronounced less and provided two times more XOS (7.29 g/L) compared to unassisted DES treatment. Alkaline addition at lower temperatures had a greater effect on the LDP XOS concentration. When the treatment was applied to corn cob by adding alkali to DES, it was thought to improve hydrolysis since it did not affect hemicellulose structure by increasing lignin removal (Teng et al., 2022). However, the alkali treatment itself yielded slightly higher XOS compared to alkali-assisted DES. The positive effect of the alkali-assisted DES treatment should be attributed to NaOH rather than DES. It was concluded that low-temperature alkaline treatment could be regarded as a promising pretreatment for XOS production. Additionally, xylose production increased only when alkali pretreatment was applied. The LDP XOS to xylose ratio was approximately 3.5-4, indicating that the xylose produced was relatively low when only NaOH was used.

4.4. Alkali Treatment

Besides DES treatment, alkali treatment as a pretreatment for enzymatic XOS production was tested. Alkali treatment was applied at a wide range of NaOH concentrations (0.1, 0.25, 0.5, 0.75, 1, 3, 5, 10 % w/v) and two temperatures (30 °C, 60 °C). The effect of pretreatment at low temperatures had eliminated the requirement for special equipment and high energy. The effect of conditions on LDP XOS concentration was determined (Figure 4.9).

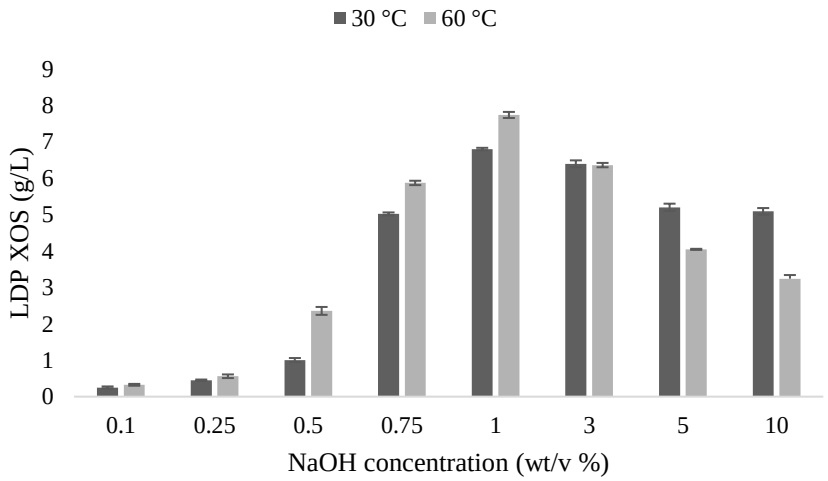

Figure 4.9. Effect of alkali concentration and temperature on LDP XOS production[a]

[a] Alkali treatment conditions: solid-liquid ratio 1/10, 18 h, 180 rpm; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, enzyme dosage 50U/g biomass, 60 ºC 48h, 180 rpm

As the amount of alkali increased up to 1% NaOH, the concentration of LDP XOS increased. At higher than 1% NaOH, the concentration decreased as the alkali content increased. There was an increase in LDP XOS concentration as the temperature increased up to 1% NaOH. The highest LDP XOS concentration (7.74 g/L) was obtained when 1%

NaOH was applied at 60 °C. At 5% and 10% alkali concentrations, the effect of temperature was harmed the LDP XOS concentration. It was thought that several alkaline concentrations caused different LDP XOS amounts, which may have been due to the difference in the effect on the corncob composition (cellulose, xylan, lignin, and arabinan content). The effect of the treatments using 0.75%, 1%, and 3% NaOH on cellulose, xylan, and lignin content was shown in Table 4.10. As the alkali concentration increased, the solid recovery decreased. Alkali concentration had no significant effect on glucan recovery ($p=0.590>0.05$). In general, it was observed that alkali pretreatment significantly affected xylan recovery ($p=0.004<0.05$) and lignin removal ($p=0.000<0.05$). This may have been because alkali pretreatment reacted with the ester bonds between lignin and hemicellulose (Modenbach & Nokes, 2014) so that some of the xylan and lignin can be separated from the biomass dissolved in the pretreatment liquor. Solid recovery, and cellulose, xylan, and arabinan contents were similar for 0.75%, and 1% alkali treatments, whereas the lignin content decreased 50%. Lignin removal at an alkali concentration of 1% was approximately twice that of an alkaline concentration of 0.75%. When the alkali concentration was 3%, the percentages of cellulose, xylan, arabinan, and lignin at the end of the treatment were similar to the alkaline concentration of 1%. Lignin removal was 65.24% at 1% NaOH, and 71.39% at 3% NaOH. In alkaline pretreatment applied to jute (*Corchorus olitorius L.*) biomass at 30 °C for 6 h, delignification was found to be 64.86% and 71.89% for alkaline concentrations of 1% and 3%, respectively (Sharma et al., 2023). The delignification found in the study was similar. Since solid recovery was low when 3% NaOH was used, there was a difference between the removal rates compared to 1%. In 3% alkali used, xylan recovery was approximately twice that of 1% used. An increase in LDP XOS production results from 0.75% alkali to 1% alkali may result in a doubling of lignin removal (Figure 4.9 and Table 4.10). Delignification led to the accessibility of xylan, increasing the XOS yield (Yang et al., 2021). According to Kusakabe et al. (1976), when a diluted alkali solution was treated with lignocellulosic biomass, xylan hydrolysis was improved as lignin is removed. In addition, the decrease in LDP XOS concentration between 1% and 3% alkaline could be due to the halving of xylan recovery (Figure 4.9 and Table 4.10). This study aimed to retain xylan in treated corncob. Cui et al. (2012) concluded that xylan removal increased with increasing alkali concentration in the treatment of corn stover. There were studies in which xylan is extracted at a high alkaline concentration (Hakala et al., 2013; Jayapal et al., 2013; Quintero et al., 2021; Sporck et al., 2017). As the concentration of alkali increased, the

xylan removal increased, and the decrease in LDP XOS concentration (Figure 4.9) was thought to be due to the removal of xylan from the solid between 1- 10% alkaline concentration.

Table 4.10. Effect of alkali concentration on cellulose, xylan, and lignin in corncob[a]

C_{alkali}[b] (wt/v %)	**Solid recovery (%)**	**Glucan recovery (%)**	**Xylan recovery (%)**	**Lignin removal (%)**
0.75	73.52	66.41±1.66	84.25±2.47	30.75±0.53
1	71.08	69.23±1.76	80.57±3.90	65.24±0.08
3	57.59	65.63±6.09	58.08±0.83	71.39±0.19

[a] Alkali treatment conditions: solid-liquid ratio 1/10, 30 ºC 18 h, 180 rpm

[b] C_{alkali} represents alkali concentration

Results in Figure 4.9 and Table 4.10 showed that a 1% alkali for the pretreatment was the most effective. Since the 30 °C treatment temperature required less energy, the tests continued at this temperature. The amount of LDP XOS was produced under these conditions was 6.8 g/L.

4.4.1. The Effect of Treatment Time and Solid-Liquid Ratio in Alkaline Treatment

The effect of treatment time in alkali treatment was investigated. The results of the experimental set in which the several treatment time (2 h, 4 h, 6 h, 8 h, and 18 h) were

tested were shown in Figure 4.10. The alkali pretreatment time had a significant effect on the LDP XOS concentration ($p=0.007<0.05$). As the time increased up to 8 h, LDP XOS production increased. LDP XOS concentration increased from 5.19 g/L to 6.89 g/L from 2 h to 8 h. There was a decrease in LDP XOS concentration was slight for 8 h to 18 h. There was no need to extend the time beyond 8 h because the change was in the decreasing direction and slight. The xylose concentration was low at all treatment times.

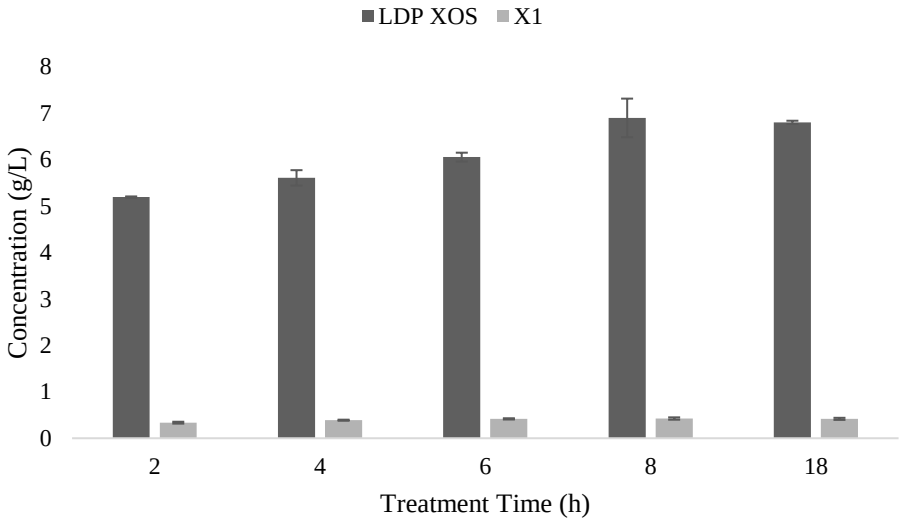

Figure 4.10. Effect of incubation time on XOS production[a]

[a]Alkali treatment conditions: 1% (wt/v) NaOH, solid-liquid ratio 1/10, 30 °C, 180 rpm; Enzymatic hydrolysis conditions: 50 U/g biomass Econase XT, solid-liquid ratio 1:20, 60 °C 48 h, 180 rpm

Since the reduction of the treatment time did not affect the production of LDP XOS drastically, the solid-liquid ratio was tested with both using 2 h and 8 h of treatment time (according to the result of the Tukey test). The effect of the solid-liquid ratio (1/7, 1/10, and 1/20) and the treatment time (2 h and 8 h) on XOS production were shown in Figure 4.11. The effect of solid-liquid ratio and pretreatment time on LDP XOS concentration was significant ($p=0.000<0.05$). As the solid-liquid ratio increased, the LDP XOS concentration increased. The low XOS concentration with a 1/7 solid-liquid

ratio may have been due to ineffective mixing. When the treatment times were compared, higher results were obtained in the 8 h application for all solid-liquid ratios. As biomass loading increased, the use of alkali decreased, and the environmentally harmful waste decreased in parallel. There were studies in which the alkali process was used at a ratio of 1/10 solid-liquid (Sun et al., 2018; Toma et al., 2021; Wang et al., 2021b). Hamid et al. (2023), applied low-temperature alkali pretreatment to wheat straw and optimized the solid-liquid ratio to be 1/10. In the following treatments, a solid-liquid ratio of 1/10 and an incubation time of 8 h were used.

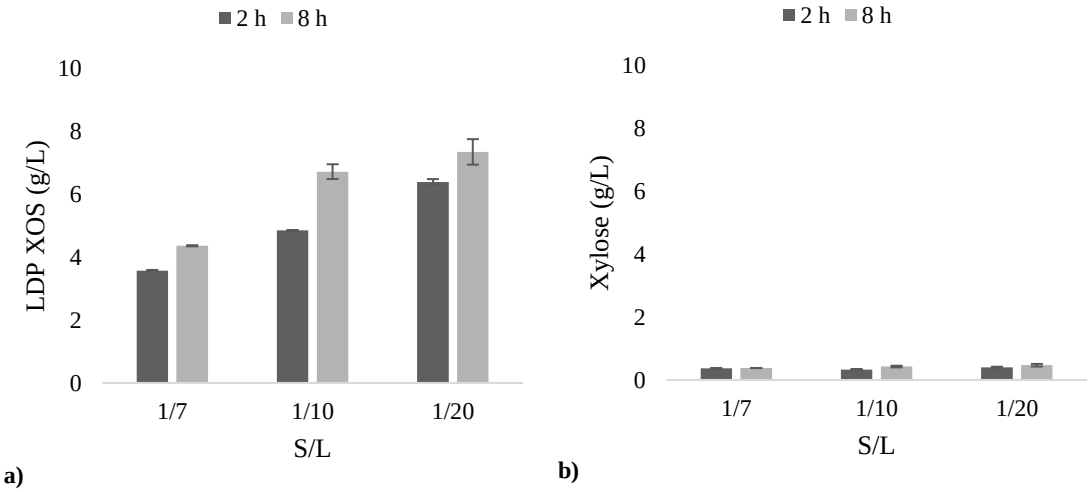

Figure 4.11. The effect of solid-liquid ratio and incubation time on XOS production[a]

[a] Alkali treatment conditions: 1% (wt/v) NaOH, 30 ºC, 180 rpm; Enzymatic hydrolysis conditions: 50 U/g biomass Econase XT, solid-liquid ratio 1:20, 60 ºC 48 h, 180 rpm

4.4.2. Effect of Enzymatic Hydrolysis Conditions on XOS Production from Alkali-treated Corncob

In this section, the effect of enzyme dosage, and hydrolysis time on XOS production were investigated. First of all, corncob was treated with 1% NaOH at a solid-

liquid ratio of 1/20 at 30 °C for 8 h. The treated corncob was hydrolyzed with several enzyme dosages (50- 100- 200- 400- 800- 1600 U/g biomass). The results were presented in Figure 4.12. The amount of enzyme had a significant effect on the LDP XOS concentration ($p=0.000<0.05$).

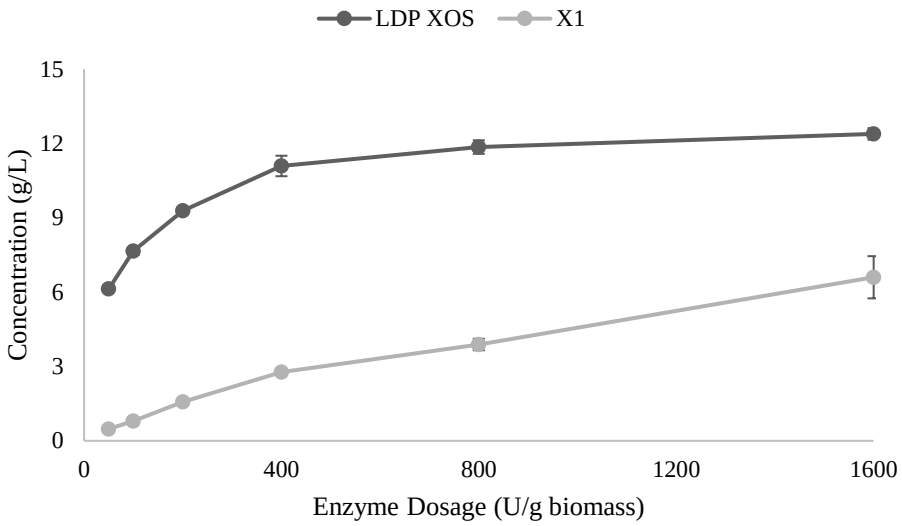

Figure 4.12. Effect of enzyme dosage on XOS production[a]

Alkali treatment conditions: 1% (wt/v) NaOH, solid-liquid ratio 1/10, 30 °C 8 h, 180 rpm; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1:20, 60 °C 48 h, 180 rpm

There was a drastic increase in LDP XOS between 50- 400 U/g biomass enzyme dosages. LDP XOS concentration was 11.09 g/L when 400 U/g biomass was used in enzyme hydrolysis. It was similar to the literature results in which XOS yields increased as the amount of enzyme increased (Akpinar et al., 2007; Samanta et al., 2012a; Yamamoto et al., 2019; Wang et al., 2018). The increase in LDP XOS concentration reached a plateau after 400 U/g biomass enzyme dose. With 800 and 1600 U/g biomass enzymes, 11.86 and 12.39 g/L XOS were produced, respectively. Wang et al. (2018) stated that there was no difference in the XOS yield since the binding capacity of the enzyme protein to its substrate reached a plateau at a certain enzyme concentration.

Xylose production also increased with increasing enzyme dose. Considering the enzyme cost, LDP XOS production, and xylose production, it was decided to produce XOS with 400 U/g biomass enzyme hydrolysis.

Enzymatic hydrolysis time was tested at 6- 24- 48 h (Figure 4.13). Enzyme hydrolysis time had a significant effect on LDP XOS ($p=0.002<0.05$) and xylose ($p=0.000<0.05$) concentration. As the hydrolysis time increased, LDP XOS and xylose production increased. Half of the LDP XOS concentration was achieved in approximately the first 6 h. There was a drastic increase between 6 h and 24 h. The LDP XOS concentration at 24 h (10.19 g/L) was 1.7 times higher than at 6 h. There was a slight increase between 24 h and 48 h. 11.09 g/L LDP XOS was produced in 48 h. The amount of xylose also increased with time. While 0.47 g/L xylose was produced in 6 h, it tripled in 24 h. The amount of xylose produced in 48 h (2.77 g/L) was about twice that produced in 24 h. When enzymatic hydrolysis was extended to 48 h, the increase in LDP XOS was slight, while the increase in xylose was drastic. Considering the difference between concentrations, a hydrolysis time of 24 h was preferred.

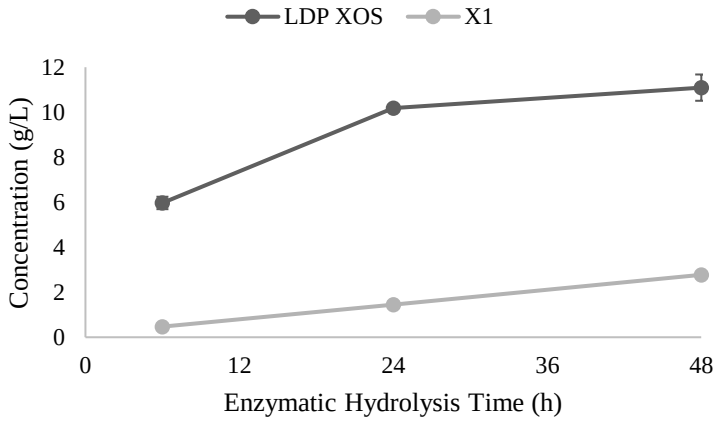

Figure 4.13. Effect of enzymatic hydrolysis time on LDP XOS and xylose[a]

[a]Alkali treatment conditions: 1% (wt/v) NaOH, solid-liquid ratio 1/10, 30 °C 8 h, 180 rpm; Enzymatic hydrolysis conditions: 400 U/g biomass Econase XT, solid-liquid ratio 1:20, 60 °C, 180 rpm

Considering the results of this research, it was found appropriate to apply alkali treatment to corncob with 1% (wt/v) NaOH concentration, using a 1/10 solid-liquid ratio at 30°C for 8 h. It was concluded that a high concentration of XOS would be produced with 400 U/g biomass Econase XT loading at a 1/20 solid-liquid ratio (5% biomass loading) for 24 h at 60 ^{o}C.

4.5. FTIR Analysis

Raw corncob and solids after pretreatment and enzymatic hydrolysis were characterized using FTIR (Figure 4.14). The specific bands ascribed to lignin were between 1100 cm^{-1} and 1050 cm^{-1} (Adapa et al., 2011). While lignin peak was found in 1047 cm^{-1} in corncob, it was not observed after treatments. The peaks around 1515 cm^{-1} indicated aromatic skeletal stretching in lignin (Pan et al., 2017). The peak on the cob in 1517 cm^{-1} decreased in the treated samples. The specific absorption band between 1650 cm^{-1} and 1600 cm^{-1} ascribed the presence of aromatic compounds and aromatic lignin (Adapa et al., 2011). While a significant peak was seen at 1640 cm^{-1} in corncob, it was less pronounced in others. It was showed that the lignin was removed as a result of mainly the pretreatments. The FTIR spectra were in agreement with the lignin removal observed after the treatments. For the cellulose peak, 1400 cm^{-1} and 1350 cm^{-1} absorption bands had been attributed. (Adapa et al., 2011). The presence of the peak at 1383 cm^{-1} in all solids was demonstrated resulting in most of the cellulose remaining in the solid after DES and alkali treatments and xylanase hydrolysis. The FTIR spectra were in agreement with the cellulose recovery observed after the treatments. There were 897 cm^{-1}, 1250 cm^{-1}, and 1735 cm^{-1} bands had been determined in the presence of characteristic β xylosidic bonds between sugars, acetyl hemicellulose, and hemicellulose, respectively (Adapa et al., 2011; Lun et al., 2017; Samanta et al., 2012b). In addition, the band between 1200 cm^{-1} and 1100 cm^{-1} characterized hemicellulose (Lun et al., 2017). The peaks at 900 cm^{-1}, 1250 cm^{-1}, 1734 cm^{-1}, 1110 cm^{-1} were apparent for the corncob. It decreased after

enzymatic hydrolysis of solids. It showed a decreased amount of xylan in the solid after enzymatic hydrolysis.

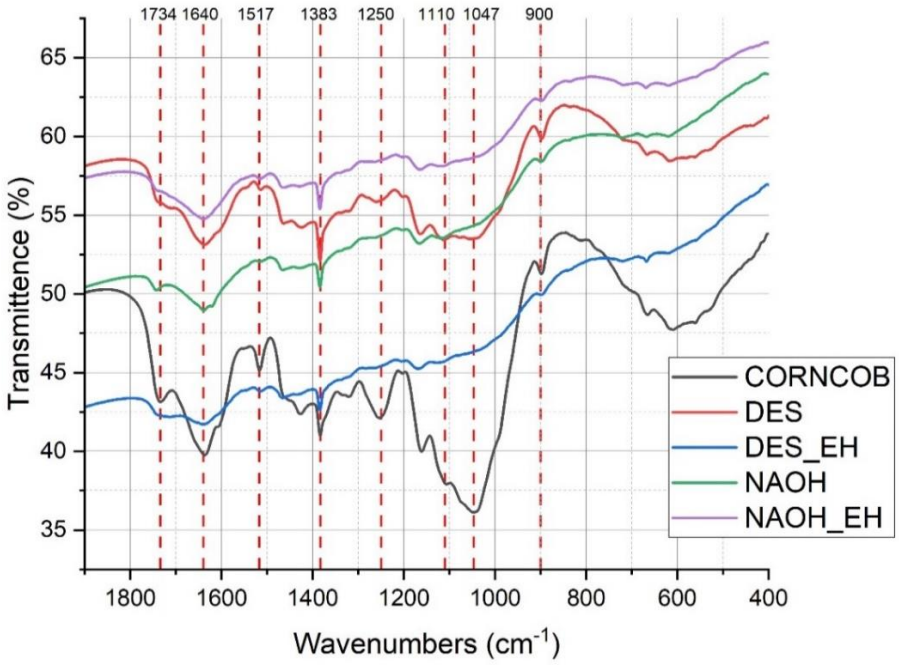

Figure 4.14. Characterization of solids in all processes[a]

[a] CORNCOB, DES, NAOH, DES_EH, NAOH_EH show the spectra of the raw material, solid after des treatment, solid after alkali treatment, solid after des and xylanase hydrolysis, and solid after alkali and xylanase hydrolysis, respectively.

4.6. Cellulose Hydrolysis

The solids obtained after DES and alkali treatments followed by xylanase hydrolysis were subjected to cellulose hydrolysis for fermentable sugar production. Glucose and xylose were produced by cellulase hydrolysis to evaluate the residue after xylanase hydrolysis. 60 FPU/g cellulase Cellulast 1.5L (activity 58.17 FPU/mL) enzyme

provided hydrolysis at 1/20 solid-liquid ratio, at 50°C for 48 h. Glucose and xylose released as a result of cellulase hydrolysis were given in Table 4.11.

Table 4.11. The concentration of fermentable sugar produced in cellulose hydrolysis of solid remaining after treatments and xylanase hydrolysis[a]

Treatment	**$C_{Glucose}$ (g/L)**	**C_{Xylose} (g/L)**	**$Y_{Glucose}$**	**Y_{xylose}**
DES	17.78±0.11	3.61±0.08	41.29±0.26	10.55±0.08
Alkali	18.10±0.03	4.77±0.06	44.28±0.07	14.67±0.06

[a] DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/5, 130 °C 2h; Enzymatic hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, enzyme dosage 400U/g biomass, 60 °C 48h, 180 rpm; alkali conditions: 1% (wt/v) NaOH, solid-liquid ratio 1/10, 30 °C 8 h, 180 rpm; Enzymatic hydrolysis conditions: 400 U/g biomass Econase XT, solid-liquid ratio 1:20, 60 °C 24 h, 180 rpm

As a result of cellulose hydrolysis of the spent corncob that was treated with DES-treated and subsequently hydrolyzed using xylanase, 17.78 g/L glucose, and 3.61 g/L xylose were produced. From the alkali-treated and enzymatically hydrolyzed corncob, 18.10 g/L glucose, and 4.77 g/L xylose were produced. Glucose yields (based on the cellulose content of the raw corncob) were 41.29 % for DES and 44.28% for alkali treatments while, xylose yields were 10.55 % and 14.67 %, respectively. There were studies on cellulase hydrolysis in the literature. The glucose and xylose yields were 93.3% and 82.1% when the corncob was hydrolyzed with cellulase after pretreatment with soaking in aqueous ammonia (Zhang et al., 2009). The glucose and xylose yields obtained by enzymatic hydrolysis after milling were 78.7% and 72.1% for sugarcane bagasse and 77.6% and 56.8% for sugarcane straw, respectively (da Silva et al., 2010). The glucose yield obtained from sugarcane bagasse as a result of subcritical carbon dioxide pretreatment and enzymatic hydrolysis was 93% (Zhang & Wu, 2014a). When dilute ammonia pretreatment and cellulase hydrolysis were applied to sugarcane bagasse, the glucose yield was 92% and the xylose yield was 54% (Zhang & Wu, 2014b). It was thought that there was a difference between the results in the literature and this study due to the difference in raw materials (da Silva et al., 2010; Zhang & Wu, 2014a; Zhang &

Wu, 2014b) and the use of multiple enzymes which contained cellulase enzyme with xylanase and pectinase (Zhang et al., 2009). Although the fermentable sugar yields in this study were lower than the literature, when considered as a coproduction with XOS, the waste solid was evaluated.

4.7. Mass Balance of XOS Production

The mass balance of all stages from raw materials to final products for DES-based and alkali-based processes was shown in Figure 4.15 and Figure 4.16, respectively.

In the DES-based process, XOS and xylose were produced with the xylanase enzyme. Total XOS yield (based on the xylan content of raw corncob) was 44.08%, LDP XOS yield was 39.71% and xylose yield was 5.71%. 90.09% of the total XOS was LDP XOS. Also, xylan digestibility (xylan converted to total XOS or xylose based on xylan in pretreated corncob) was 54.04% and 7%, respectively. While the glucose yield (based on the glucan content of raw corncob) was 41.3%, the glucan digestibility (glucan converted to glucose based on glucan in pretreated corncob) was 58.69%. The xylose yield (based on the xylan content of raw corncob) was 10.56%, while the xylan digestibility (xylan converted xylose based on xylan in pretreated corncob) was 33.69% (Figure 4.15).

In the NaOH-based process, XOS and xylose were also produced with the xylanase enzyme. Total XOS yield was 41.64%, LDP XOS yield was 33.36% and xylose yield was 2.56%. 80.13% of the total XOS was LDP XOS. Xylan digestibility was 51.84% and 3.19% according to total XOS and xylose, respectively. While the glucose yield was 44.28%, the glucan digestibility was 66.92%. The xylose yield was 14.68%, while the xylan digestibility was 38.88% (Figure 4.16).

When the pretreatments were compared, similar results were obtained in terms of XOS production. Also, the effects of the treatments on the raw material content (lignin removal, xylan recovery) were similar (Table 4.6 and Table 4.10). Enzymatic hydrolysis was thought to be effective, as DES pretreatment and alkaline pretreatment provided delignification. There was a relationship between the chemical properties of the

pretreatment solutions and the lignin fractionation (Yue et al., 2020). For this reason, it was considered that DES and alkali chemicals required different pretreatment conditions (130 °C- 2 h for DES, and 30 °C- 8 h for NaOH) due to their different characteristics.

In studies in the literature, when XOS was obtained using alkaline extraction pretreatment applied to corncob, the total XOS yield was 10% (Samanta et al., 2015a), 12% (Chapla et al., 2012), 20% (Boonchuay et al., 2018), 38% (Zhu et al., 2006). According to these studies, the result of this study was higher, which were 44.08% and 41.64% for DES and alkali, respectively. In addition, high xylobiose conversion (56%) was obtained in another study with 20% NaOH alkali pretreatment (Khangwal et al., 2021). It was important that the processes were not environmentally friendly since the alkali concentration was high and that the 28.07% xylobiose conversion obtained in this study was done with the environmentally friendly DES process. In this study, xylobiose conversion in the other pretreatment production using dilute alkali was 20.19% and it was relatively more environmentally friendly because it contained a small amount of alkali. In studies that produced XOS from corn cob with autohydrolysis; the total XOS yield was 60% (Nabarlatz et al., 2007a), 65% (Moura et al., 2007), 79% (Parajó et al., 2004), 49% (Chen et al., 2021). Also, XOS was produced with yields of 75% (Liu et al., 2018), 68% (Yang et al., 2005), and 29% (Teng et al., 2010) using steam explosion (one of the autohydrolysis processes). In acid hydrolysis studies applied to biomass, the total XOS yield was found to be 55% (Han et al., 2020), and 46% (Zhang et al., 2017). During autohydrolysis and acid hydrolysis, hemicellulose oligomers in the solution were partially hydrolyzed to xylose and sugar degradation products. Sugar degradation products included HMF, consisting of hexose sugars, and furfural and uronic acids, consisting of pentose sugars (Chen et al., 2010). Although the yield of DES and alkali pretreatment was lower than the results of autohydrolysis, degradation products were not formed thanks to the enzymatic hydrolysis applied to the solid part. The fact that the majority of the XOS formed was composed of LDP XOS, showed that they could be preferred in food applications due to their rapid assimilation in the gastrointestinal tract, being easily metabolized by probiotics and high prebiotic activities (Ho et al., 2018; Pinales-Márquez et al., 2021).

Isci et al. (2021) used ChCl and formic acid DES on the wheat straw with microwave with a different approach. As a result of the study, 32% (initial of xylan) XOS, 20% xylose, and about 5% of furfural in the liquid part was produced (Isci et al., 2021).

The presence of XOS, xylose, and furfural in the same liquid affected the purity of the XOS production. Also, the result of 32% XOS yield was lower than in this study. In another study in which only DES was used in the production of XOS when DES containing ChCl and lactic acid was applied to poplar sawdust at 110^{o}C and 130^{o}C, the yield was found to be 11% and 26%, respectively, compared to the highest initial xylan, and then this result was tried to be increased by hydrothermal pretreatment (Shen et al., 2021). Other studies, which obtained XOS from biomass with DES, were lower than the results of the total XOS yield in this study. In other studies, DES was applied for delignification after a different pretreatment was done for XOS production (Chang et al., 2023: Chen et al., 2023; Gong et al., 2022; Ma et al., 2021; Sun et al., 2023; Ying et al., 2022b).

Ai et al. (2005), were studied the production of XOS by using immobilized enzyme hydrolysis after alkaline pretreated corn cob powder using 2% NaOH. The specified condition was that the corncob was treated using 2% NaOH overnight at room temperature. After obtaining and immobilizing *Streptomyces olivaceoviridis* E-86 xylanase enzyme, provided hydrolysis. As a result, the total XOS yield was 84%. This result was two times higher than the production of XOS by xylanase hydrolysis following dilute alkali treatment in this study. It was thought that the different xylanase enzymes used may have affected the XOS yield. Additionally, the alkaline used in this study was more dilute (1% NaOH) and the treatment time (8 h) was shorter, which was a more environmentally friendly approach. Ai et al. (2005) did not correlate XOS production with changes in solid content. The solid content details in the bioprocess developed in this study were also examined.

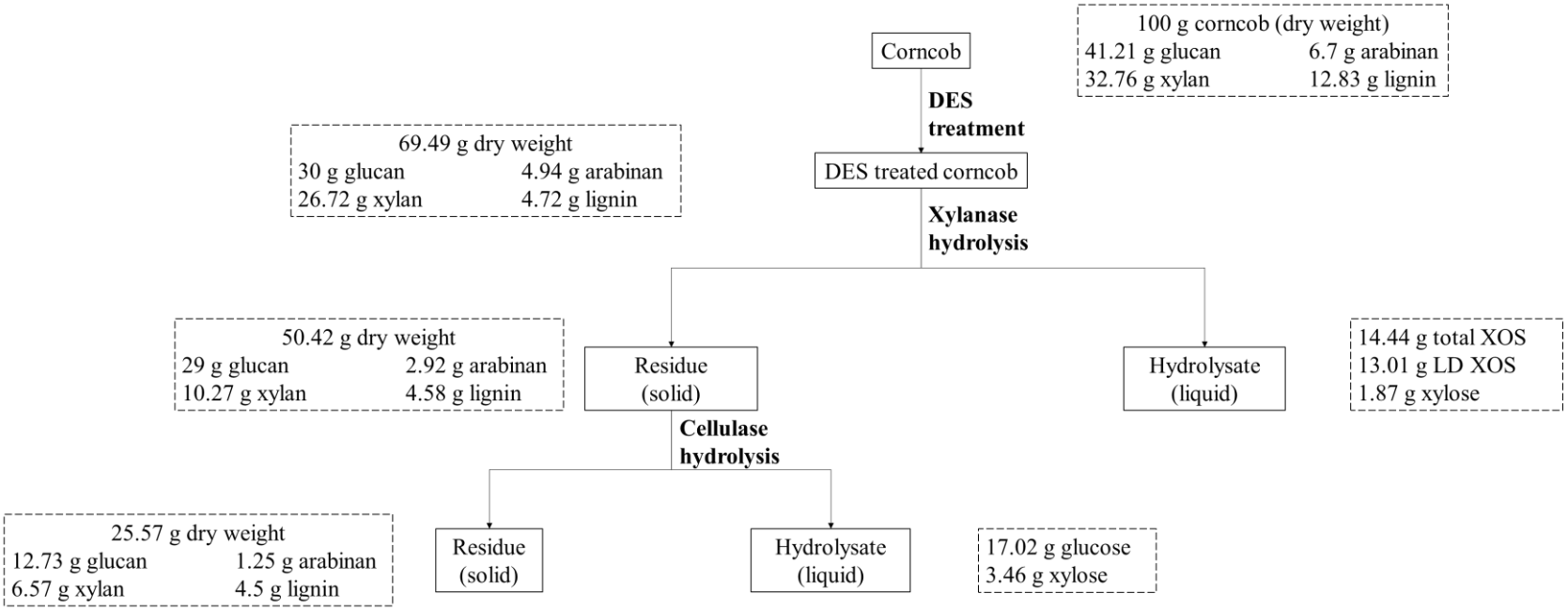

Figure 4.15. Mass balance of the DES treatment and subsequent enzymatic hydrolysis

DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/5, 130 ^{o}C 2h;

Xylanase hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, enzyme dosage 400U/g biomass, 5.5 pH, 60 ^{o}C 48h, 180 rpm

Cellulase hydrolysis conditions: Cellulast 1.5L, solid-liquid ratio 1/20, enzyme dosage 60FPU/g cellulase, 4.8 pH, 50 ^{o}C 48h, 180 rpm

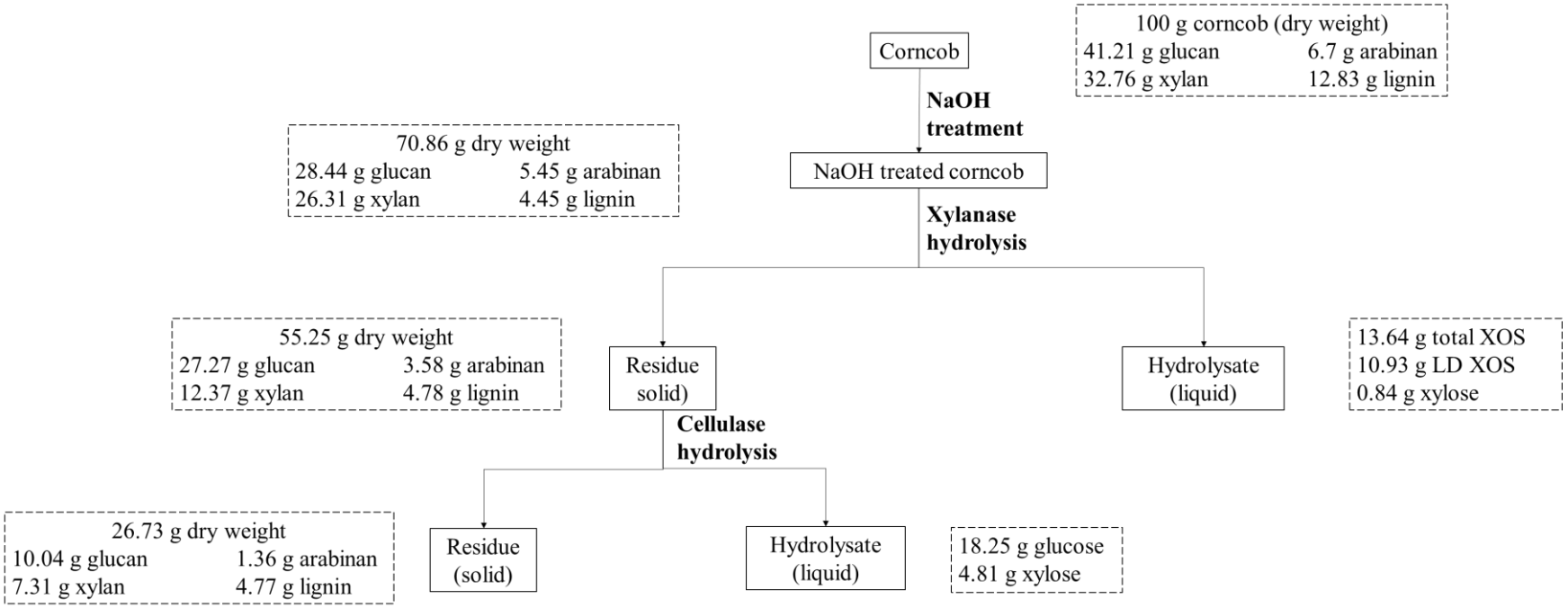

Figure 4.16. Mass balance of the alkali (NaOH) treatment and subsequent enzymatic hydrolysis

Alkali conditions: 1% (wt/v) NaOH, solid-liquid ratio 1/10, 30 ºC 8 h, 180 rpm

Xylanase hydrolysis conditions: Econase XT, solid-liquid ratio 1/20, enzyme dosage 400U/g biomass, 5.5 pH, 60 ºC 24h, 180 rpm

Cellulase hydrolysis conditions: Cellulast 1.5L, solid-liquid ratio 1/20, enzyme dosage 60FPU/g cellulase, 4.8 pH, 50 ºC 48h, 180 rpm

4.8. Ultrafiltration (UF)

UF was an effective process that provided purification and fractionation of XOS solutions (Nabarlatz et al., 2007b). Molecules with different molecular weights were filtered by the size-dependent membrane (Akpinar et al., 2007). UF was performed to purify the enzymatic hydrolysate containing XOS by removing the xylanase. The molecular weight of the GH10 family, which contains the Econase XT enzyme, was between 32-39 kDa (Lorentsen et al., 2019). The molecular weight of LDP XOS (xylobiose, xylotriose) was 282.24 and 414.4 Da. Since the 10kDa membrane was used, it was thought that while the xylanase with higher molecular weight was collected in the retentate, the XOS could pass through the filter and be collected in the permeate. The ultrafiltration process was completed by a recycling system until the feed passed into the permeate and retentate parts. The ultrafiltration process was taken approximately 8 h, including pre-washing, filtration, and shutting down the system. The protein and oligosaccharide results of the feed, permeate, and retentate fractions in the UF process were given in Figures 4.17 and 4.18.

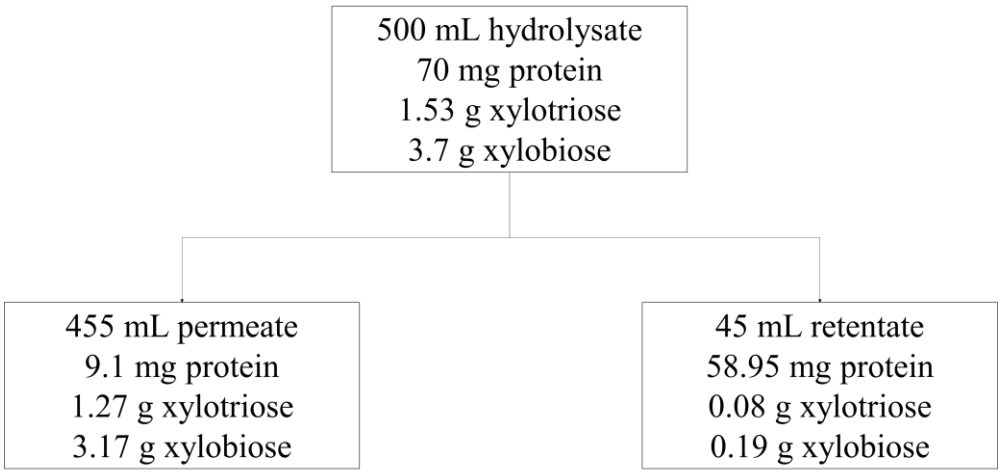

Figure 4.17. Protein and oligosaccharide amounts in the UF process of the hydrolysate in the DES-based process

As shown in Figure 4.17, 455 ml filtrate was collected, while the remaining volume was collected as a stream that could not pass through the filter, in the UF process. It was observed that 70 mg of protein (enzyme) in the feed fluid was largely (84%) separated by not passing through the filter. 85% of the LDP XOS in the feed were recovered in the filtrate. LDP XOS concentration in the filtrate was measured as 9.8 g/L. Approximately 5% of each of the initial LDP XOS was remained in the unfiltered liquid. According to mass balance, about 10% of XOS was not recovered either in the filtrate or the permeate. It can be concluded that the remaining XOS was retained by the filter.

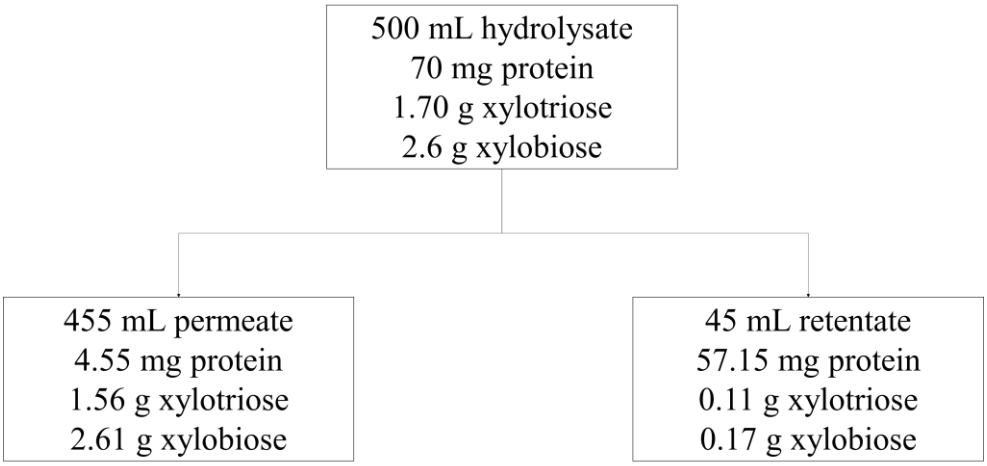

Figure 4.18. Protein and oligosaccharide amounts in the UF process of the hydrolysate in the alkali-based process

Considering Figure 4.18, 455 ml filtrate was collected, while the remaining volume was collected as a stream that could not pass through the filter, in the UF process. It was observed that 70 mg of protein (enzyme) in the feed fluid was largely (82%) separated by not passing through the filter. 97% of the LDP XOS in the feed were recovered in the filtrate. LDP XOS concentration in the filtrate was measured as 9.2 g/L. Approximately 6.5% of each of the initial LDP XOS was remained in the unfiltered liquid.

Akpinar et al. (2007) obtained XOS using alkali and enzymatic hydrolysis to the cotton stalk and filtered it with a 10kDa membrane. In another study, XOS was obtained using enzymatic hydrolysis to the palm leaf after alkali extraction and filtered with a 10kDa membrane (Saleh et al., 2016). As a result of two studies, filtrates containing mostly oligosaccharides were obtained, similar to this study.

CHAPTER 5

CONCLUSION

Prebiotics are components that have positive health effects on animals and humans and have recently attracted great interest. XOS has been drawing attention owing to its prebiotic activities, stability, organoleptic properties and applicability to food. Although there are various production methods, efforts to develop cost-effective and environmentally friendly methods continue. With the development of such processes, more consumers may have access to XOS thanks to its low cost, while biosustainability is ensured. If an economical and sustainable production process is developed, the usage of XOS may increase because of its technological properties and food compatibility.

The treatments developed in this study were aimed to produce high-purity XOS, considering the economic and the environmental concerns. It was crucial to establish a process that incorporated enzymatic hydrolysis for high purity. For efficient utilization of the xylan in the corncob, it was targeted to develop pretreatments that removed the lignin, while xylan was kept in the solid to a high extent, instead of extracting xylan. Two different processes was developed, namely DES and dilute alkali based pretreatments. In the processes, the xylan in the corncob remained in the solid, which was then subjected to enzymatic hydrolysis. The enzyme, xylanase, could act on the xylan in the pretreated corncob to yield XOS thanks to the lignin removal during pretreatment.

The most important aspect of using DES pretreatment for XOS production was that it was an environmentally friendly process, since DESs are biodegradable, green solvents, and have low toxicity. It was observed that the effect of adding water in DES pretreatment had a positive effect probably due to the improvement of mass transfer, while the addition of excess water had a negative effect due to the weakening of the interaction between DES components. DES pretreatment required elevated temperatures

for lignin removal. The requirement of a pressure reactor could be considered as the main disadvantage of this process. However, a relatively lower temperature was sufficient compared to processes requiring high temperature such as hydrothermal, acid hydrolysis and organosolv treatments. In the study where DES and enzymatic hydrolysis conditions were tested, the optimum values were determined as follows: DES treatment using ChCl-urea at 1:2 molar ratio as the DES components, 40% water addition by volume, 1/5 solid-liquid ratio, 130°C, and 2 h; enzymatic hydrolysis using Econase XT xylanase, 400 U/g biomass loading, 1/20 solid-liquid ratio, 60 ºC, and 48 h.

In alkaline pretreatment, xylan remained in the solid and lignin was removed similar to DES treatment. Since the increase in alkaline concentration negatively affects xylan recovery and lignin removal, it was used at low concentrations. Low treatment temperatures led to an economically feasibile process. Instead of the high alkali concentrations as used in alkali extraction, the use of dilute alkali made the process more environmentally friendly. The disadvantage of using various chemicals such as acid, and ethanol during alkaline extraction was eliminated by the development of this process. For the dilute alkali-based process, dilute alkali and enzymatic hydrolysis conditions were selected as: 1% (wt/v) NaOH concentration, 1/10 solid-liquid ratio at 30°C for 8 h for alkali pretreatment, and to use 400 U/g biomass Econase XT loading, 1/20 solid-liquid ratio for 24 h at 60 ºC for enzyme hydrolysis.

In the DES-based process, the total XOS yield (based on the xylan content of raw corncob) was 44.08%, and LDP XOS yield was 39.71%. In the NaOH-based process, the total XOS yield was 41.64%, LDP XOS yield was 33.36%. As a result of the two pretreatments, the fact that XOS was mostly composed of LDP XOS showed that these methods can be used to produce functional XOS suitable for food applications and nutraceuticals. Because, XOS with DP 2-4 have rapid assimilation in the gastrointestinal tract, easily metabolized by colon bacteria and show higher prebiotic activity. The application of enzymatic hydrolysis to the pretreated corncob subsequent to both pretreatments provided an additional advantage that xylose formation was low, and there were no sugar degradation products owing to comparably milder treatment conditions. However, as a result of enzymatic hydrolysis, all of the xylan could not be hydrolyzed. Still, the yield was not found to be lower than the most commonly used alkali extraction and subsequent enzymatic hydrolysis process. The reason for this is the low yield of xylan

obtained from the solid using alkali extraction. In addition, such xylan-first process may have allowed cellulose and lignin to be valorized using additional processes.

This study contributed to the literature in terms of developing a method for obtaining high-purity XOS using a sustainable process. Considering industrial production, the DES-based process was environmentally friendly, but it had economic disadvantages due to the requirement of high temperatures. The dilute alkali pretreatment was environmentally friendly compared to well-known alkali extraction treatment, and an economical method owing to its effectiveness at low temperatures. In terms of time, XOS could be produced in a shorter time when DES pretreatment processing was used.

In conclusion, high-purity XOS could be obtained with a greener approach, which was advantageous over other methods in terms of requiring either lower temperature or lower chemical use. It was considered that the XOS solution obtained in this approach can be preferred in terms of use in foods. Further studies are recommended for the determination of prebiotic activity and stability. In addition, it is recommended that the lignin removed during DES be recovered and evaluated.

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APPENDIX A

STANDARD CALIBRATION CURVES FOR AMINEX HPX-87H ORGANIC ACID COLUMN IN HPLC

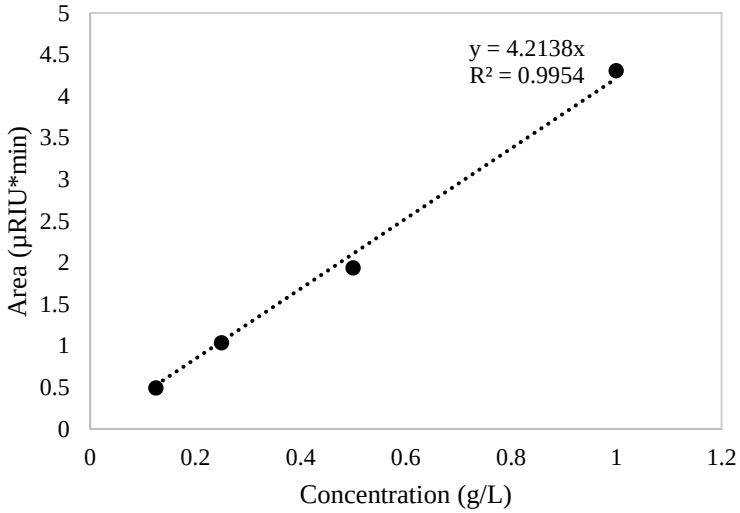

Figure A.1. Standard calibration curve of glucose.

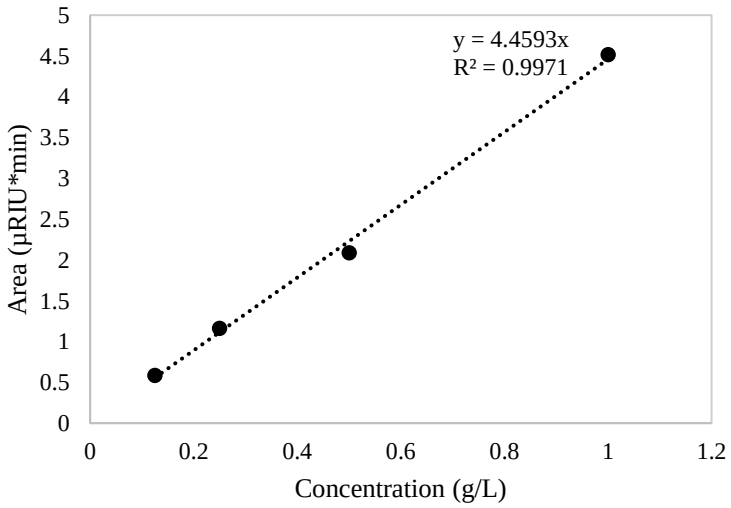

Figure A.2. Standard calibration curve of xylose.

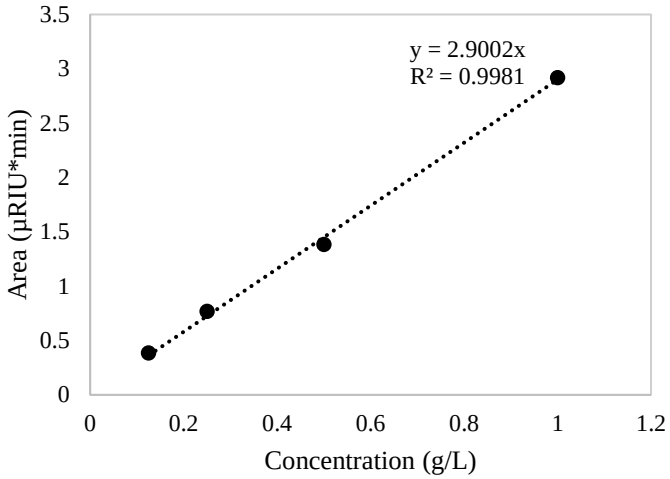

Figure A.3. Standard calibration curve of arabinose.

APPENDIX B

STANDARD CALIBRATION CURVES FOR REZEX RPM-MONOSACCHARIDE COLUMN IN HPLC

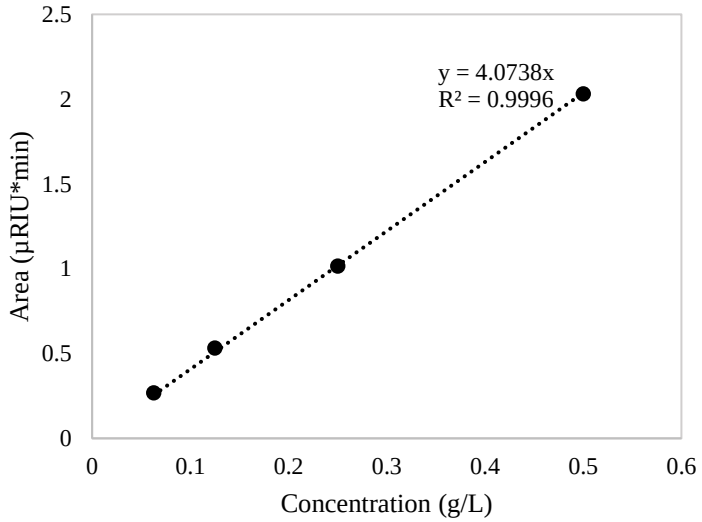

Figure B.1. Standard calibration curve of xylotriose.

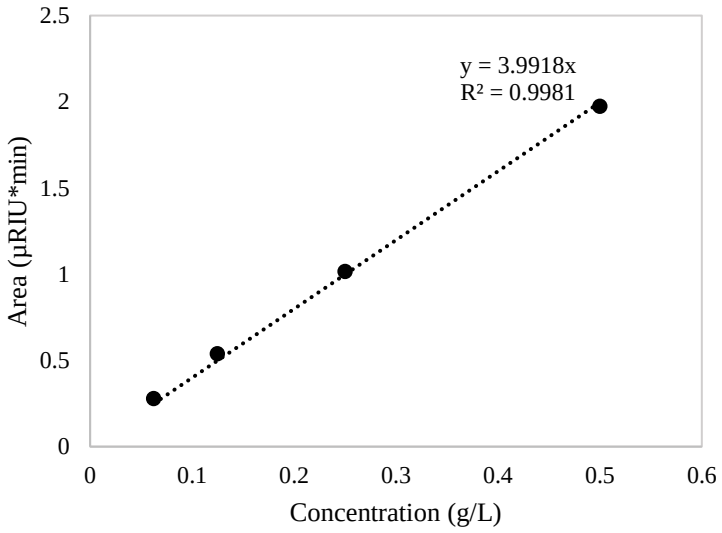

Figure B.2. Standard calibration curve of xylobiose.

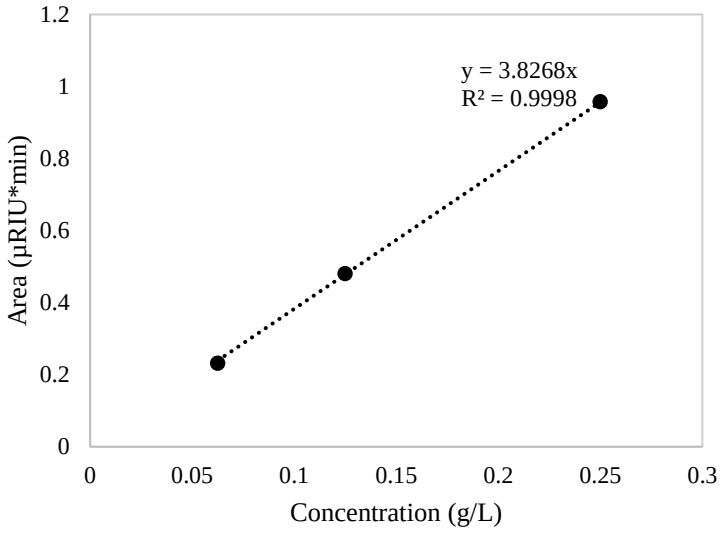

Figure B.3. Standard calibration curve of xylose.

APPENDIX C

STANDARD CALIBRATION CURVES FOR ENZYME ACTIVITY ASSAY

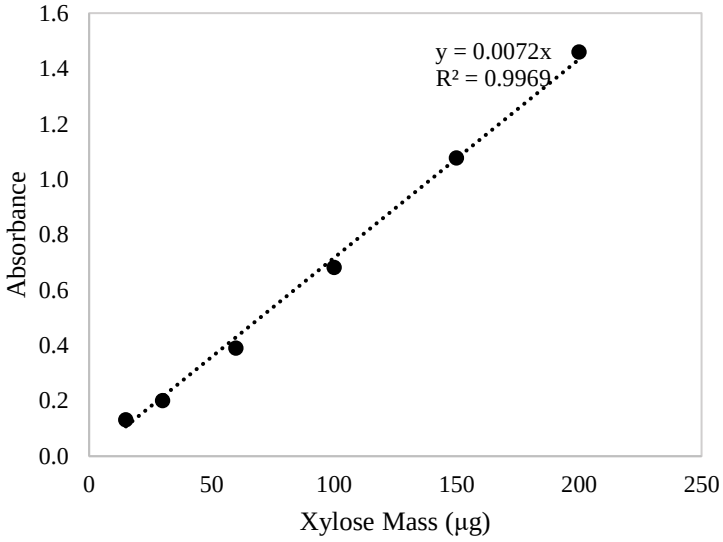

Figure C.1. Standard calibration curve of xylose for xylanase activity assay.

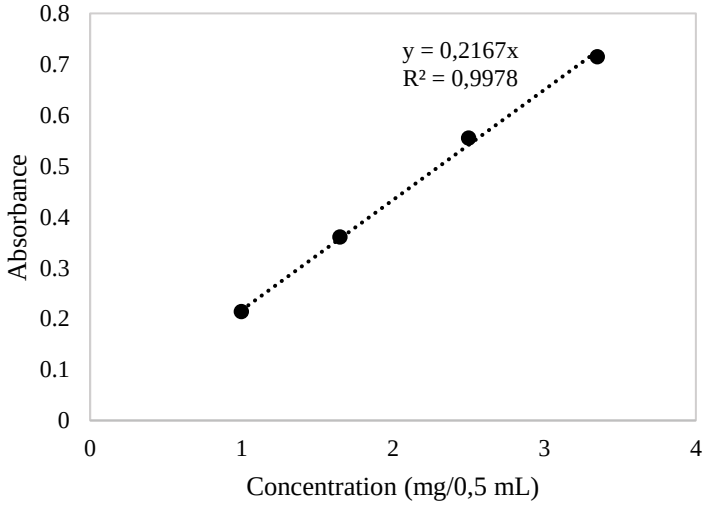

Figure C.2. Standard calibration curve of glucose for cellulase activity assay.

APPENDIX D

THE EFFECT OF DES TREATMENT AT DIFFERENT TEMPERATURES-TIMES, AND SEVERAL ENZYME DOSAGES ON XOS PRODUCTION

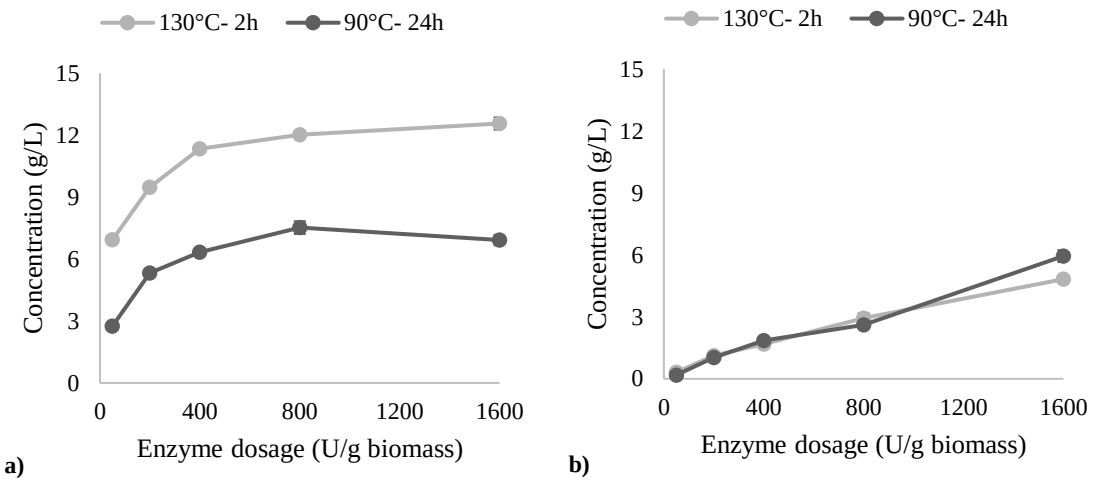

Figure D**.** The effect of DES treatment at different temperatures- times, and several enzyme dosages on XOS production

DES conditions: ChCl- Urea, 1:2 molar ratio, water addition 40%, solid-liquid ratio 1/5; Enzymatic hydrolysis conditions: Econase XT, 60 ºC 48h,180 rpm