ORGANOSOLV TREATMENT FOR PREBIOTIC OLIGOSACCHARIDE PRODUCTION FROM AGRO-FOOD WASTE

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ABSTRACT

ORGANOSOLV TREATMENT FOR PREBIOTIC OLIGOSACCHARIDE PRODUCTION FROM AGRO-FOOD WASTE

Xylooligosaccharides (XOS), which are among the prebiotic carbohydrates, are produced by hydrolysis of xylan in the lignocellulosic agricultural and food wastes. Production processes, such as autohydrolysis and enzymatic hydrolysis following alkali extraction, have some limitations. In this study, it was aimed to develop a process that could overcome those limitations. Corn cob was used as the model biomass and it was pretreated with organosolv. The organosolv pretreatment conditions (solvent concentration, time, temperature, catalyst addition) were adjusted to maximize the lignin removal from the biomass while recovering xylan in the biomass. Delignification could be achieved with 40%-50% lignin removal, and around 85% of the xylan was retained in the biomass. The effect of the organosolv conditions on XOS formation was investigated by the hydrolysis of the pretreated biomass using three commercial xylanases. The organosolv and the enzymatic hydrolysis conditions influenced the XOS formation. The maximum XOS production was observed with the biomass pretreated at 150°C for 1 h with 70% ethanol and 0.1 M MgO. That biomass was enzymatically hydrolyzed at 70°C with 0.6 U/ml xylanase and 70% of the xylan was converted to XOS yielding a hydrolysate containing 9.5 g/l XOS. With the process developed in this study, the need for the application of concentrated alkaline and acidic solutions can be eliminated since the xylan extraction step is not needed. In addition to that, the formation of carbohydrate degradation products can be avoided due to the lower treatment temperatures compared to autohydrolysis. These can simplify the downstream processing following the production of XOS and minimize the environmentally harmful chemical wastes. In this process, a liquid stream rich in lignin and a solid stream rich in cellulose were released. Following a biorefinery concept, these streams can potentially be valorized together with the xylan, so that lignocellulosic wastes can have an added value. This study will be followed by related projects on XOS purification and determination of the prebiotic potential of XOS. In addition to that, the process developed will be tested on other lignocellulosic wastes.

ÖZET

TARIM-GIDA ATIKLARINDAN PREBİYOTİK OLİGOSAKKARİT ÜRETİMİ İÇİN ORGANOSOLV İŞLEMİ

Prebiyotik karbonhidrat olan ksilooligosakkaritler (KOS), lignoselülozik tarım ve gıda atıklarındaki ksilan polisakkaritinin hidroliz edilmesiyle üretilir. Alkali ekstraksiyona dayanan enzimatik hidroliz ve otohidroliz kullanılan yöntemler çeşitli kısıtlara sahiptir. Bu çalışmada bu kısıtları aşabilecek olan bir yöntem geliştirilmesi hedeflenmiştir. Model olarak kullanılan mısır koçanı organosolv önişleminden geçirilmiştir. Organosolv şartları (çözgen derişimi, süre, sıcaklık, katalizör ilavesi) biyokütledeki lignini uzaklaştıracak ancak ksilanın biyokütlede kalmasını sağlayacak şekilde ayarlanmıştır. Uygun şartlarda ligninin %40-%50'si ayrılabilirken ksilan yaklaşık %85 oranında katıda kalmıştır. İşlem görmüş biyokütle üç ticari ksilanaz ile hidroliz edilerek organosolv işleminin KOS üretimine etkisi belirlenmiştir. Organosolv ve enzimatik hidroliz şartlarının toplam KOS üretiminde etkili olduğu görülmüştür. En verimli KOS üretimi 150°C'de 1 sa %70 etanol ve 0,1M MgO varlığında organosolv işlemi görmüş biyokütle ile elde edilmiştir. Bu biyokütlenin 70°C'de 0,6 U/ml ksilanaz ile geçekleştirilen enzimatik hidrolizi sonucu ksilanın %70'si dönüştürülerek 9,5 g/l KOS içeren hidrolizat elde edilmiştir. Geliştirilen yöntem sayesinde ksilanın ekstrakt edilmesine ihtiyaç duyulmadığı için derişik alkali ya da asit gibi kimyasallar kullanılmayacak ve otohidroliz yöntemindeki gibi çok yüksek sıcaklıklar kullanılmadığı için karbonhidrat bozunma ürünleri oluşmayacaktır. Bunlar ürün saflaştırma aşamalarını kolaylaştıracak ve çevreye zararlı kimyasal atık miktarını azaltacaktır. Süreçte lignince zengin bir sıvı faz ve selülozca zengin katı faz oluşmaktadır ve ksilan ile beraber bunlar da değerlendirilerek biyorafineri yaklaşımıyla lignoselülozik atıklara katma değer kazandırılabilir. Bu çalışmanın devamında ürünün saflaştırılması ve prebiyotik etkilerinin belirlenmesi için projeler geliştirilmesi planlanmaktadır. Ayrıca geliştirilen süreç diğer lignoselülozik atıklar üzerinde de test edilecektir.

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CHAPTER 1

INTRODUCTION

In today's society, boarding healthy and affordable food production systems is one of the primary goals. In line with this purpose, the sustainability of this increase and the establishment of eco-friendly systems are the benchmarks. Thereby, agricultural food wastes have become prominent. Despite the consumption of this biomass as animal feed and fertilizer, most of them are disposed of and cause environmental issues.

Agricultural food wastes in the form of lignocellulose have been regarded as potential raw material for many valuable products due to cellulose, hemicellulose, and lignin polymers in its structure. Currently, while fuels obtained from a cellulose like bioethanol and biobutanol have a chance to replace with conventional fuels, these fermentable carbohydrates have been already employed in many bioproducts as carbon source. However, the pretreatment requirement of lignocellulose before its use causes high production costs. That’s why obtaining more than one valuable product from lignocellulosic biomass is obligatory. Thus, the valorization of hemicellulose and lignin parts beside cellulose has become an important point.

Xylooligosaccharides (XOS), naturally found in sources like honey and bamboo shoot (sprouts), pass through the digestive system without degradation or absorption and reach the large intestine. Here, they are metabolized by beneficial bacteria such as *Bifidobacterium* species and show prebiotic effect. However, they are naturally found in low concentrations and insufficient to show their effects. Because of that, XOS is commercially obtained by enzymatic hydrolysis of the xylan polymer in lignocellulosic material.

Xylan is obtained as a polymer in different purities and yields after dissolving with alkaline chemicals and precipitating with alcohol. The extracted xylan polymer is then hydrolyzed in aqueous solutions with xylanase enzymes. Since excessive and corrosive chemicals cause damage in equipment, an increase in costs, and environmental pollution, alternative methods have been sought. In autohydrolysis, which has been developed as an alternative XOS production method, biomass is treated with sub-critical water and xylan is hydrolyzed into soluble XOS at high temperatures. However, thermal degradation products, such as furfural, hydroxymethylfurfural, and acetic acid released during autohydrolysis, might exert toxic effects on humans and animals. This as well as the high energy demand and the requirement of special equipment are the main drawbacks of the KOS production using autohydrolysis.

Organosolv is based on an alternative approach to disrupting the lignocellulosic structure. In this method, hemicellulose and lignin are removed via organic solvents to obtain cellulose-rich solid. Afterward, saccharification with cellulase enzyme is performed and a pure glucose solution is obtained for later use. Despite the high-temperature requirement, the recovery of used solvents is an important advantage in terms of both cost and environmental problems.

In this study, it is aimed to develop a method that can exceed the limits and disadvantages of current XOS production methods. Excessive chemical use in the method based on the extraction of xylan and the formation of sugar-degradation products in autohydrolysis are important problems of known methods. The main approach in this study is to enable the hydrolysis of the xylan directly in solid part by enzymatic hydrolysis. Lignocellulosic biomass in its raw state prevents the hydrolytic enzymes to show activity. In the study, the organosolv pretreatment method was employed to weaken the structure, but the pretreatment conditions were adjusted to avoid the xylan separation from solid. The pretreatment was focused particularly on lignin removal, thereby suitable treated biomass for the following enzymatic hydrolysis was obtained.

In the first part of the study, pretreatment conditions such as temperature, time, and solvent concentration were adjusted for the highest lignin removal and hemicellulose recovery. In the second part, pretreated biomasses were subjected to enzymatic hydrolysis to obtain XOS in the hydrolysis liquor. Three commercial enzymes were used, and the highest XOS concentration with the lowest xylose production was sought.

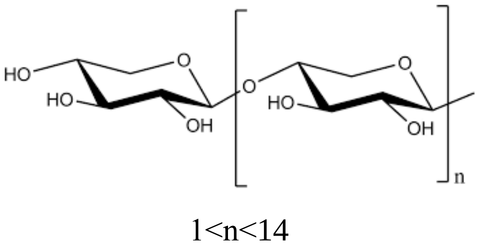
In this recommended method, there is not hazardous or alkaline chemical use; energy consumption is lower than autohydrolysis; there are no sugar degradation products in the XOS solution.

CHAPTER 2

LITERATURE REVIEW

2.1. Xylooligosaccharides (XOS)

As sugar oligomers, xylooligosaccharides (XOS) consist of β (1-4) covalent xlyosidic bonds of five carbon xylose monomers. As a result of d-xylopyranose group polymerization as seen in Figure 2.1, the molecular formula of xylooligosaccharides is expressed as $C_{5a}H_{8a+2}O_{4a+1}$ where a is from 2 to 6. Although molecules from 2 to 15 units are accepted as oligomers, generally a degree of polymerization of 2-6 is preferred in applications due to the susceptibility to alkaline decomposition (Childs et al., 2014). Their molecular weights vary between 282 to 810 g /mol from xylobiose to xylohexaose. Physio chemically, XOS is stable up to 100°C and in a broad pH range of 2.5-8.0 (Samantha et al., 2015). The solubility of XOS in the water at 21°C is 58% (w/w). While they provide 1.5 kcal per gram, they are 18% less sweet rather than sucrose in the comparison of 10% solutions (Samantha et al.,2015).



Molecular formula	$C_{5a}H_{8a+2}O_{4a+1}$(a=2 to 6)
Molecular weight	282 to 810 (X_2 to X_6)
Melting temperature	134°C
Water solubility	58% (w/w) at 21°C
pH stability	2.5 to 8
Energy value	1.5 kcal/g
Relative sweetness	92% of sucrose (compared to10% solutions)

Figure 2.1. Structure and physicochemical properties of xylooligosaccharides (adapted from (Samanta et al.,2015)).

Honey, bamboo sprouts, some fruits, and vegetables are natural sources of XOS, but they are insufficient to exhibit their effect because of the low concentration. Thus, XOS is commercially obtained by the hydrolysis of xylan polymer in lignocellulosic material (Singh et al., 2015). According to the source of xylan, some side groups like acetyl, glucose, and arabinose might be found in the structure of XOS.

Together with a rapid increase in the global population and demand to health-supporting products, prebiotics, which are defined as ‘substrates that are selectively utilized by host microorganisms conferring a health benefit’, take place in the market as a derived functional food ingredient (Gibson et al., 2017).

XOS is classified as prebiotics and can be considered suitable for health-related applications because they are nondigestible oligosaccharides, passing through the gastrointestinal tract without digested. That’s why, they reach the lower intestine and metabolized by beneficial microorganisms (Aachary and Prapulla, 2011a; Gibson et al., 2017; Amorim et al.,2019a). Compared to other well-established choices, prebiotic XOS draws attention in terms of higher stability and heat resistance at low pH; low digestibility; selectively stimulation in the growth of bifidobacteria; and higher total short-chain fatty acid (SCFA) concentrations (Otieno and Ahring, 2012b; Samanta et al., 2015; Singh et al.,2015; Fehlbaum et al., 2018). As a prebiotic, XOS potentially shows positive effects on colon cancer, neurotoxicity, and diabetes prevention (Aachary and Prapulla, 2011; Krishna et al., 2015).

Currently, in the global market, XOS has been accepted as an emerging food ingredient rather than other established competitors. Although XOS has been used in the Asia-Pacific region for a while, it was firstly valued by Food and Drug Administration (FDA) in 2010 and obtained generally recognized as safe (GRAS) status in 2013 in the USA (FDA, 2010; 2013). Finally, in 2018, enzymatically produced Longlive labeled XOS was evaluated by the European Food Safety Authority (EFSA) and accepted as a novel food (EFSA, 2018). These given status have provided high R&D investment for XOS production and application studies. According to reports, expansion of the XOS market worldwide is expected as 5.3% from 2017 to 2023, namely from 93 million US$ in 2017 to 130 million US$ (The Market Reports, 2018). Since it has a lower requirement rather than peers to show its prebiotic effect (1.4-2.8 g/day), the price of XOS is also advantageous and open to competing for the recommended dose (Amorim et al., 2019a).

However the cost of XOS is extremely high in terms of price per kilogram, thus new methodologies are urgent for cost-efficient production.

In today's market, XOS is valorized only for their biological properties as a prebiotic supplement. For example, Jarrow Formulas Prebiotics are combination XOS plus Acacia Gum that support the growth of *Bifido* and *Lactobacilli* and conditionally intestinal-immune function. Nutrasumma XOS prebiotic powder supports the immune system, aid efficient nutrient absorption, and control blood sugar level (Mohanty et al, 2018). Apart from the biological benefits, XOS can be considered as a food ingredient in many areas namely as a stabilizer, emulsifier, or fat replacer. In recent studies, XOS is studied for both to pursue its technological properties and to develop its applications in products (Poletto et al., 2020).

In one of the first studies, 'how the features of emulsified frozen meat are affected if ingredient sucrose is replaced with prebiotic XOS' was examined in terms of sensorial, rheological, and quality parameters (Wu et al., 2011; Xu et al., 2019). It was found that XOS use has no diverse effect on product design in addition to its 'healthy food' logo (Wu et al., 2011). Similar principles were also applied to various products like hemicellulose/chitosan film production (Xu et al., 2019). In another study group, 'how XOS is affected during food processes and how it adds value to the product parameters as brand new ingredient' concept was examined (Fernandes et al., 2020; Aachary and Prapulla, 2011b; Souza et al., 2019; Silva et al., 2020). As a result of studies with whey protein films (Fernandes et al., 2020), cookies (Aachary and Prapulla, 2011b), strawberry-flavored beverage (Souza et al., 2019) and orange juice (Silva et al., 2020), it was shown that XOS is quite resistant to conditions of food unit operations and in addition to its prebiotic effect, it also provides significant changes to their physiochemical properties (Fernandes et al., 2020; Aachary and Prapulla, 2011b; Souza et al., 2019; Silva et al., 2020). In the last group of studies, XOS was added with predetermined objectives and its evaluation was examined in addition to physiochemical and prebiotic advantageous. For example in cheese production, the fat-replacer property of XOS was investigated and successful characteristics were obtained without any disadvantages compared to full-fat products (Ferrão et al., 2018). The cryoprotective property of XOS on frozen shrimps was evaluated (Zhang et al., 2018a). Denaturation of proteins and crystal growth were hindered with hydrogen bonds between proteins and XOS as expected from cryoprotectants (Zhang et al., 2018a).

2.2. Biorefinery Concept

The valorization of biomass is quite popular in the last decade. Produced energy and chemicals from biomass can be replaced with the current fossil-based industry. Combustion is a traditional method to produce heat and energy. Nowadays biomass has been combusted to produce them instead of coal. Biogas from lignocellulosic biomass can be produced in several ways. It depends on the desired last product, budget for the system, and raw materials. It can be produced via partial combustion, anaerobic digestion, or supercritical water treatment. Pyrolysis is another way to produce biogas and other products. Pyrolysis is a thermal process to crack biomass in anaerobic conditions. It produces solid biochar, liquid bio-oil, and biogas (Briens et al., 2008). The final product is affected by operating conditions, gasifying agents, and design. Syngas, which is a mixture of CO and H_2 gases, is one of the desired products in this process. Syngas is an ideal source for electricity production to utilize it as a fuel (Thomsen et al., 2020). Solid biochar can be valued as fuel, as a carbon sink to capture carbon in the atmosphere or as fertilizer. In pyrolysis, the most valuable product is liquid bio-oil. When it be refined and upgraded, it can be easily used in modern engines as a transport fuel.

The biorefinery is an integrated system to process a wide spectrum of marketable bio-based products and bioenergy. In order to sustain a more circular economy, the source of biorefineries has been changed. In that concept, the valorization of lignocellulosic biomass has been taking attention in terms of environmental, economic, and societal aspects. However, some strategies are needed to design these sustainable biorefineries. In terms of environmental aspects, existing and new value chains should be promoted to supply sustainable raw materials, which are produced with responsible land use, minimized soil depletion, and balanced greenhouse gas emissions (Dragone et al., 2020). Also, the uptaking of biobased products in the market should be accelerated with market analysis, collaborations, and removing hurdles for the use of side products. Supportive policies might also be created to improve rural areas and to add value to farmers in terms of societal concepts. In addition to all of these strategies, technical requirements are the key point for sustainable and circular biorefineries. The valorization of all components of biomass with cost-effective pretreatments and with improved selectivity of processes in right operation scales are the main requirements through the research and development studies in that aspect (Dragone et al., 2020).

In valorization of second-generation feedstocks (nonfood crops, lignocellulosic biomass) a pretreatment process is a mandatory step for the fractionation of biomass in order to utilize each component in further stages. The cost and efficiency of this fractionation are affected by the type of applied methods, solvents used, and temperature. The combined hydrolysis factor is a measure to determine the severity of a pretreatment (Ji et al., 2017). The pretreatment severity depends on the molar concentration of chemicals used in the reaction, reaction time, activation energy, and temperature. While low pretreatment severity is not effective in enzymatic digestibility, very high pretreatment severity negatively affects the biorefinery concept because of the conversion of raw material carbohydrates into the inhibitory products (Jung et al., 2016).

The total cost of pretreatments is the total of fixed and operational costs. While costs of energy, reagents, catalysts are classified as operational costs, the establishment of systems and costs of equipment are classified as fixed costs. All of these costs are quite variable to process and its severity (da Silva et al., 2016). For example, while there is not a high operational cost due to the lack of complex reagents and recycling, fixed costs are higher for the corrosion-resistant equipment.

In the comparison of several pretreatment methods, organosolv is an effective pretreatment method than, autohydrolysis, dilute acid hydrolysis, and SPORL(sulfite pretreatment to overcome recalcitrance of lignocellulose). Additionally, while organosolv has much lower water consumption during treatment and washing steps than alkali treatment, there is a small gap in terms of water consumption between dilute acid and organosolv. On the other hand, ethanol use during pretreatment provides considerable high energy savings. Ethanol is the lowest energy-consuming chemical among THF, SPORL, diols, and acids. Apart from them, ethanol is the safest and environmentally friendly reagent after the water (Islam et al., 2020).

After fractionation, each component of lignocellulose can be valued as a high value-added product in a wide-range. Hemicellulose part can be used directly in hemicellulose based biofilms; can be converted to oligosaccharides with xylanolytic enzymes, can be fermented to xylitol with microorganisms like *Candida tropicalis*, or can be utilized with catalytic conversions to furfural and furfural based chemicals such as furfuryl alcohol, levulinic acid, maleic acid, cyclopentanone. With a similar approach, glucose from the cellulosic part can be fermented to ethanol, lactic acid, succinic acid, acetic acid, malic acid, etc., with various microorganisms. It also can be converted to HMF or biohydrogen with catalytic conversion (Singhvi and Gokhale, 2019).

Apart from carbohydrates, lignin is the third most abundant structure in lignocellulose and it has a unique structure. It is a complex aromatic biopolymer consisting of p-coumaryl, coniferyl and sinapyl alcohols. Heterogeneous structure, low solubility, and reactivity are the most apparent obstacles in conversion to value-added products. However anaerobic digestion of remaining lignin is one of the old and promising technology for biogas and biohydrogen production. Biohydrogen is an energy source and a raw material used in chemical industries (Millati et al., 2020). In order to valorize lignin in that concept, other fractions of lignocellulose are generally separated with various pretreatment methods, like organosolv, alkaline, steam explosion. However, recent studies have been focused to convert this material to value-added chemicals via biological conversions. In that regard, *Rhodococcus opacus* and *Pseudomonas putida* are the most promising microorganisms (Dragone et al., 2020). Apart from them, a few lignin modifying enzymes can be used in the modification of lignin complexes. These enzymes are secreted by fungi like *Phanerochaete chrysosporium*, and classified in oxidative group enzymes namely, lignin peroxidases, manganese peroxidase, or phenoloxidases (Singhvi and Gokhale, 2019). After pretreatment, modified lignin is demethoxylated and aromatic rings in the structure are obtained. Biogas and biohydrogen are produced with anaerobic oxidation. From lignin part of lignocellulose, chemicals like phenols, lignin monomers, vanillin, vanillic acids, quinones, methane, aromatic acids, etc. can be obtained. These chemicals can be applied in the production of insulation material, rubber processing, polyurethane foams, etc (Ruiz et al., 2020).

2.3. Lignocellulosic Biomass

Lignocellulose, apart from being the main source of commercial XOS, is one of the thriving pioneers of the biobased economy. Biomasses are valued in the production of biofuels, biopower, and bioproducts including chemicals and materials. Since replacing petrol into biofuel plays a vital role in the reduction of greenhouse gas emissions; energy security can be obtained with the use of biopower instead of outer sources; bioproducts, which are produced within the biorefinery concept, provide economic value to the wastes, the valorization of this available resource has positive impacts on a diverse range (DOE, 2016).

The structure of lignocellulose consists of a complex carbohydrate polymer of cellulose, hemicellulose, and lignin (Figure 2.2). In this composition, cellulose is comprised of long chains of β (1-4) linkages of glucose linkage. Since it has a linear arrangement, many hydrogen bonds among cellulose fibrils arise and a rigid structure is seen. Hemicellulose, which is an amorphous polymer consisting of a xylan backbone and various substitutions, interacts with cellulosic microfibrils via noncovalent interactions, mainly hydrogen bonds. The strength of the interactions increases according to the decrease in the degree of side-chain substitution. Also, macromolecular interactions are effective between acidic hemicellulose and microfibrils. Lignin, formed as a result of polymerization of phenolic structures, covers carbohydrate polymers with covalent interactions and provides resistance to this complex structure towards degradations. As a covalent bond, ester linkages is most demonstrated one between lignin and hemicellulose, where the phenolic unit of lignin methylated to the glucose subunit of xylan backbone. Ether linkages are the second commonly seen one between lignin and hemicelluloses where lignin bonded from xylose and arabinose units of xylan.

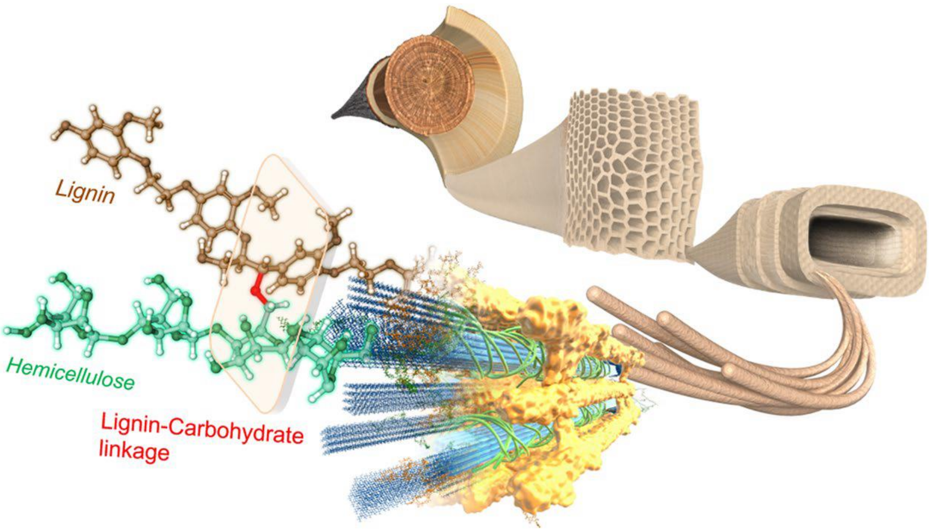


Figure 2.2. Structure of lignocellulosic biomass and its compositions (Nishimura et al., 2018).

The composition of lignocellulosic biomass is highly variable with respect to the source and harvest time, but it roughly accepted as 10-25% lignin, 40-50% cellulose, and 5-30% hemicellulose (Kumar and Sharma, 2017). When the optimum source for XOS production is pursued, a high ratio of hemicellulose becomes an important parameter to obtain high efficiency. Besides, the lignin ratio should be low as much as possible to ease the hydrolysis. As seen in Table 2.1., corncob is one of the suitable candidates for this process. Corn cob, which belongs to cereal family, can be chosen as model raw material for XOS production due to its suitableness to parameters, containing particularly high amount xylan, and studying previously.

Table 2.1. Composition of lignocellulosic biomasses (Biswas et al., 2015).

Agricultural Waste	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Wheat Straw	35.0-39.0	23.0-30.0	12.0-16.0
Barley Hull	34	36	13.8-19.0
Barley Straw	36.0-43.0	24.0-33.0	6.3-9.8
Rice Straw	29.2-34.7	23.0-25.9	17.0-19.0
Rice Husks	28.7-35.6	12.0-29.3	15.4-20.0
Oat Straw	31.0-35.0	20.0-26.0	10.0-15.0
Ray Straw	36.2-47.0	19.0-24.5	9.9-24.0
Corn Cobs	33.7-41.2	31.9-36.0	6.1-15.9
Corn Stalks	35.0-39.6	16.8-35.0	7.0-18.4
Sugarcane Bagasse	25.0-45.0	28.0-32.0	15.0-25.0
Sorghum Straw	32.0-35.0	24.0-27.0	15.0-21.0

In a widespread geographical area, harvested maize (corn) is the most produced grain worldwide with annually one billion tons amount. While the US is the top producer of maize with a 38% market ratio, China and Brazil follow the US respectively with 21% (216 million tons) and 10% (88 million tons) share (IGC, 2018). Turkey is at the 10th place in the FAO maize producers' list with an annual production of 5.8 million tons. Despite the producers' list, Turkey has the highest yield in the production of maize followed by the US and Canada (FAO, 2018). In Turkey, the grain planted area has been covered 71% (11.1 million hectares) of the total agricultural area. According to TÜİK data, grain harvesting area in Turkey has been sharing among wheat with a 69% ratio;

barley with 22% ratio; and maize with a 6% ratio (TMO, 2018). In addition to the high production rate of maize, the amount of waste obtained from this grain is also higher than wheat, barley, and other grains (DOE, 2018). When considering all evaluation, maize (corn) is such a suitable model for XOS production due to its availability, quantity, and high waste rate. Also, it gives a promising and easy application rather than other raw materials.

2.4. Hemicellulose

Hemicellulose, which is a binding element between cellulose and lignin, is the second most abundant polymer in nature following the cellulose. Hemicellulose is an enormous substantial source of many sustainable products. It can be hydrolyzed into XOS. In addition to that, hemicellulose can be transformed into ethanol and other chemicals like xylitol, furfural, formic acid, lactic acid, etc. (Arcañoa et al., 2018). End products can be employed in the industry due to their biological or technological properties. They can be used as food ingredients, packaging materials, encapsulation materials, and pharmaceuticals (Amorim et al., 2019a).

These shortly branched hetero-polysaccharides generally have low molecular weight and degree of polymerization, which is between 80 and 200 on average (Isıkgor and Becer, 2015). In the lignocellulosic material, the hemicelluloses' role is to interact with cellulose and lignin to support the structure of the cell wall. According to the type of biomass, which are hardwood, softwood, and cereals; hemicellulose composition and characteristics differ. In terms of composition, softwoods, hardwoods and cereals consist of 25-30%; 20-25% and 20-25% hemicelluloses, respectively (Peng et al., 2012). In Table 2.2., the hemicellulose is classified according to this characterization. Although most of the biopolymers follow this classification, some of them belong to two of the classes. For example, arabinogulucuronoxylan is mostly in the structure of cereal plants, but it also found in softwoods. Or, galactoglucomannan can be seen in both hardwood and softwoods.

The structure of hemicellulose consists of β (1-4) linked backbones of main xylose (xylp), in rare cases mannose (manp), glucose (glcp), and galactose (galp). Additionally, many saccharide or non-saccharide units are included depending on the source.

Backbones of hemicelluloses are arisen by one unit and various side chains bounded with linkages.

Table 2.2. Characterization of hemicelluloses according to their structures (adapted from Grio et al., 2010 and Peng et al., 2012).

Source	**Polysaccharide**	**Abbr**	**DP**	**Backbone**	**Backbone glycosidic linkage**	**Branch**	**Branch glycosidic linkage**
Hardwood	Glucuronoxylan	GX	100-200	β-D-Xylp	β (1→4)	4-o-Me-α-Glc*p*A Acetyl	α (1→2)
	Glucomannan	GM	40-70	β-D-Manp β-D-Glcp	β (1→4)		
	Xyloglucan	XG		β-D-Glcp β-D-Xylp	β (1→4)	β-D-Xyl*p* β-D-Gal*p* α-L-Ara*f* α-L-Fuc*f* Acetyl	β (1→4) α (1→3) β (1→2) α (1→2) α (1→2)
Softwood	Galactoglucomannan	GGM	40-100	β-D-Manp β-D-Glcp	β (1→4)	β-D-Gal*p* acetyl	α (1→6)
	Arabinogalactan	AG	100-600	β-D-Galp	β (1→3)	β-D-Gal*p* α-L-Ara*f* α-L-Ara*p*	β (1→6) α (1→3) β (1→3)
Cereal (Graminea)	Arabinoxylan	AX		β-D-Xylp	β (1→4)	α-L-Ara*f* Feruloyl	α (1→3) α (1→2)
	Arabinogulucuronoxylan-Glucuronoarabinoxylan	AGX-GAX		β-D-Xylp	β (1→4)	4-o-Me-α-Glc*p*Aβ-L-Ara*f* α-L-Ara*f* acetyl	α (1→2) α (1→3)

On the other hand, two different monomers can be repeated in the backbone and again many side chains can be bounded to the backbones like in glucomannan and galactoglucomannan. Sidechains extremely depend on the source of lignocellulose. Generally, glucuronic acid (4-o-Meα-GlcpA) and arabinose (α-L-Araf) (α-L-Arap) are terminal single units, these unit are bounded from first carbon to the second or third. Arabino glucuronic acid (4-o-Me-α-GlcpAβ-L-Araf) is one of the complex oligosaccharide side-chain units and is linked from first to third carbon. Lastly, acetyl and feruloyl units are non-saccharide side chains and linked from first to second or third carbon. The linkage of the acetyl group to the backbone is named as acetylation. This provides complexity and prevents easily depolymerization of hemicelluloses (Grio et al., 2010).

2.5. XOS Production Methods

Despite the high potential of lignocellulosic biomass as a source of XOS production, it can’t be directly hydrolyzed due to its complex structure. It requires a pretreatment to weak the structure (Figure 2.3.). Studies on XOS production mainly focus on enzymatic hydrolysis of alkali extracted xylan or hydrothermal treatments, which are specifically autohydrolysis and acid hydrolysis (Table 2.3)(Appendix B). In addition to those, there are a few unusual methods applied to produce XOS. For example, the microwave was employed as pretreatment and pretreated substrate was hydrolyzed enzymatically (Wang et al., 2013). In another study, brewer’s spent grain was directly fermented into XOS by *Trichoderma reseei* (Amorim et al., 2019b).

As a traditional and commercial method, alkali extraction followed by enzymatic hydrolysis is the most studied method. In this method, xylan in the lignocellulosic structure is extracted via alkali solutions, and arisen xylan polymer is partially hydrolyzed to oligosaccharides. In alkali extraction, Hydroxyl ions (-OH) in high concentration alkali solutions, like NaOH and KOH, act on the ester and ether bonds as well as hydrogen bonds and provide removal of hemicellulose (Rabetafika et al., 2013). Removed xylan remains solubilized in the alkaline medium. This treatment is carried out around room temperature, or maximum at 50 or 60°C. After this step, the high alkali medium is neutralized by the addition of a strong acid. The xylan polymer is precipitated with the addition of alcohol because alcohol decreases the dielectric constant of medium and polymer changes its confirmation (Akpınar et al., 2007). After extraction, xylan is solubilized in a buffer solution and hydrolyzed to oligosaccharides with a xylanase enzyme (Yang et al., 2005; Akpinar et al., 2007; Lin et al., 2011, Boonchuay et al., 2014). Corncob, cotton stalk, grass are some of the studied raw materials. While alkali concentrations between 12-24%, XOS conversion yields vary depending on both pretreatment conditions and raw materials (Quinones et al., 2015; Kiran et al., 2013). The main drawback of the alkali extraction method for XOS production is the excess consumption of chemicals during treatment, neutralization, and precipitation. While the use of chemicals causes economic problems because of the costs of chemicals and the requirement for corrosion-resistant equipment, there are also significant environmental aspects because of non-sustainable wastes. Although studies turn to apply mild conditions like treatment of corncob with 2 M NaOH and NH_4OH or sugarcane bagasse with 1% NaOH and 2.5% H_2O_2, these treatments have relatively low yields and there is no absolute solution to excess use of chemicals (Kundu et al., 2018; Li et al., 2019).

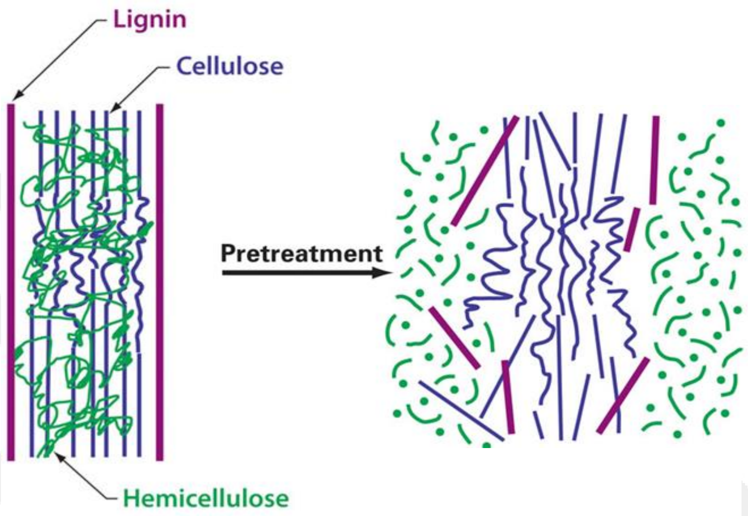


Figure 2.3. Effect of pretreatment on lignocellulose (adapted from Moiser et al., 2015).

To deal with the negative aspects of alkali treatment, hydrothermal treatments have been assessed as an alternative method for XOS production. In autohydrolysis (also known as liquid hot water (LHW)) at high temperature and pressure, water acts as a catalyst via ionization into H^+ ions, at the same time acetyl groups in lignocellulose easily split and increase the effect of ionized water. This auto ionized solution acts on carbohydrate lignin linkages and provides removal and fragmentation of xylan (Otieno and Ahring, 2012a). In some of the studies, to increase the severity of treatment, several dilute acid solutions are also added to autohydrolysis conditions. This application is called (dilute) acid hydrolysis (Samanta et al., 2015). Unlike the alkali based method, enzymatic hydrolysis is not a mandatory procedure in the hydrothermal process because of oligosaccharides formed by the acid-catalyzed hydrolysis during the process. In the process, biomass is continuously stirred and heated to 160-240 °C in a high-pressure reactor; after holding under these conditions for a specified period, the reactor is cooled and liquor containing the XOS is obtained (Otieno and Ahring, 2012a). Since each raw material has different structural properties, treatment conditions should be optimized accordingly. Thus in a wide range of raw materials; such as; sugarcane bagasse, corncobs, brewers spent grain, straw pulping studied with this method (Avila et al., 2019a; Wu et al., 19; Carvelheiro et al., 2004; Huang et al., 20117). During the process, as conditions get more severe, the degree of polymerization of XOS decreases, but at the same time monosaccharides and other carbohydrate degradation products such as hydroxymethylfurfural, furfural, and formic and acetic acids are formed in the liquid phase (Surek and Buyukkileci, 2017). At the end of the hydrothermal process, while oligosaccharides, monosaccharides, sugar degradation products, and acid-soluble lignin are obtained in the liquor, cellulose and acid-insoluble lignin remain in the solid (Petersen et al., 2009). For example, after the autohydrolysis of hazelnut shell at 190°C for 5 minutes, while XOS was obtained with a 63% conversion yield with respect to total xylan in biomass, furfural, and acetic acid are also produced during the process (Surek and Buyukkileci, 2017). Additionally, the concentrations of sugar degradation products and xylose monomer increase with increasing time and temperature. When this process is compared to the alkali extraction method, the conversion yield of xylan into XOS is moderately lower. In a study of Zhou, et al. (2019) after dilute acid treatment of sugarcane bagasse with 5% glucuronic acid at 150°C for 60 minutes, 53% of xylan was converted to XOS. In another study, a 40% yield from total xylan was obtained from the autohydrolysis of miscanthus at 190°C for 10 minutes (Hong et al., 2019). Although XOS is directly hydrolyzed in hydrothermal processes, in some of the studies enzymatic hydrolysis step is added to decrease the degree of polymerization of XOS because of the prebiotic activity of oligomers is higher in short-chain oligomers (Singh et al., 2019). Compared to the alkali extraction method, the most important advantage of hydrothermal treatments is that chemical use in this method is very low. Thus, it is more environmentally friendly and there is no need for use of corrosion-resistant equipment. However this method still has some drawbacks; high energy requirement, pressure-temperature resistant equipment, and difficulties in removals of undesired byproducts (Hendriks and Zeeman, 2009). For all these reasons, a more sustainable and economic production method for XOS is still a need.

Table 2.3. XOS Production with various processes.

Biomass	**Method**	**Reaction Conditions**	**XOS Yield (%) (based on total xylan)**	**Ref.**
Sugarcane bagasse	Dilute acid hyd.	0.5% glucuronic acid at 150°C for 60 min	53	Zhau et al., 2019
Almond shell	LHW + e.h.	200°C for 5 min	54	Singh et al., 2019
Sugarcane bagasse	LHW	0.05M ($FeCl_2$ + $MgCl_2$) at 140°C for 30 min	55	Zhang et al., 2019
Sugarcane bagasse	Alkali + e.h.	1% NaOH + 2.5% H_2O_2	-	Li et al., 2019
Brewer’s spent grain	Direct fermentation	*Trichoderma reesei*	38.3 mg/g BSG	Amorim et al.,2019b
Miscanthus	LHW	190°C for 10 min	40	Hong et al.,2019
Corncob	LHW	180°C for 30 min	14 of total biomass	Wu et al., 2019
Corncob	Alkali + Dilute acid hyd.	0.25-2.5M (NaOH + NH_4OH) + H_2SO_4	69	Kundu et al,.2018
Corncob	Dilute acid hyd.	pH: 2 adjusted	75	Liu et al.,2018
Straw pulping	LHW + e.h.	180°C for 40 min	73 mg/g	Huang et al,.2017
Hazelnut shell	LHW	190°C for 5 min	62	Surek and Buyukkileci,2017
Kenaf	Alkali + e.h.	7% $Ca(OH)_2$	95	Azelee et al.,2016
Corncob	Alkali + e.h.	10% KOH	752mg/g xylan	Boonchuag et al.,2014
Mischanthus giganteus	LHW	200°C for 5 min	69	Chen et al.,2014
Grass	Alkali + e.h.	12% KOH	11	Samantha et al.,2012
Bagasse	Dilute acid hyd. + LHW	0.1% H_2SO_4 at 60°C for 12h + 145°C for 60 min	92.3	Otieno and Ahring, 2012a
Corncob	Alkali + e.h.	15% NaOH	25.2	Lin et al., 2011
Corncob	Dilute acid hyd.	0.25M H_2SO_4 at 140°C for 30 min	13 of the total biomass	Akpinar et al.,2009a
Cotton stalk	Alkali + e.h.	24% KOH	53	Akpinar et al., 2007
Corncob	Dilute acid hyd. +LHW	1g/l H_2SO_4 at 60°C for 12h + 135°C for 30 min	67.7	Yang et al., 2005
Brewer’s spent grain	LHW	190°C for 5 min	61	Carvalheiro et al., 2004

2.6. Organosolv

Organosolv treatment includes the action of an organic solvent-water mixture on lignin-lignin and lignin-carbohydrate bonds on lignocellulosic materials at high temperatures (Bhutto et al., 2017). With the removal of lignin and hemicellulose, cellulose-rich solid is obtained. That’s why cellulolytic enzymes can easily hydrolyze the polymer into its glucose monomers. For this reason, organosolv is valued as a pretreatment method before enzymatic hydrolysis (Sun et al., 2016). In the principle of enzymatic hydrolysis for XOS production, a pretreatment method is also required to eliminate the protective lignin and to obtain a more porous structure, which is ready for the hydrolysis (Carvalho et al., 2013). Thus organosolv, which has already been used as a pretreatment method on the conversion of cellulose to glucose, can be a possible pretreatment method for XOS production, as well.

Although this method has been frequently employed in cellulose targetting studies, there is not an effective use for XOS production. That’s why this method has to be fitted for this purpose based on previous works. Many lignocellulosic biomasses like oat, sugarcane bagasse, or wheat straw, has already been studied as a raw material in bioethanol production studies (Cebeiros et al., 2020; Mataskas et al., 2019). According to these studies, the action of organosolv is variable according to solvent, time, and temperature as well as raw material. In Table 2.4 the effect of organosolv on lignin and xylose removal from the lignocellulosic biomass. Delignification is around 65-70% when glycerol used as a solvent for organosolv on wheat straw pretreatment. Delignification and xylan amount of solid increase with only a change in solvent type without any change in treatment time of temperature (Jiang et al., 2018). Apart from these variables, the addition of a catalyst also affects the action of organosolv (Jiang et al., 2018). While delignification and xylose amount in the liquid are 39 and 16% in orgonasolved corn stover with ethanol, respectively, these values jump to 89 and 88% with the addition of oxalic acid as catalyst (Table 2.4). Besides, these parameters might have divergent effects on different building blocks of the same raw materials. For example, when sugar cane bagasse is treated under the same conditions with different catalysts, there is no difference in delignification but xylose amount in the liquid is different; 20 and 50% (Zhang et al., 2016a).

Table 2.4. Organosolv treatments studied in various conditions. (Adapted from Zhang et al., 2015 and Jiang et al.,2018)

Biomass	**Solvent/Catalyst**	**Temperature (ºC)**	**Time (min)**	**Delignification (%)**	**Xylose removal (%)**
Wheat straw	50% ethanol	210	90	85	95
Wheat straw	60% ethanol/ H_2SO_4	190	60	60	81
Wheat straw	70% glycerol	220	240	65	70
Sugarcane bagasse	80% glycerol	190	240	80	-
Sugarcane bagasse	50% ethanol/ H_2SO_4	175	60	10	50
Sugarcane bagasse	50% ethanol/ NaOH	175	60	10	20
Corn stover	50% ethanol	140	60	39	16
Corn stover	50% ethanol/ oxalic a.	140	60	89	88

In this method, various alcohols, esters, ketones, organic acids, ethers, or phenols can be employed as an organic solvent (Zhang et al., 2016a). The selection of solvent should depend on its price and recyclability as much as its effectiveness. For this reason alcohols, especially ethanol, are one step ahead; they have low boiling points, easy to separate from other molecules, harmless, and cheap (Zhang et al., 2016b). As mentioned in Table 2.5, each method used and proposed in the XOS production method has a different mechanism, drawbacks, and advantages. Although there is still an energy requirement in organosolv pretreatment as the XOS production method, it is lower than autohydrolysis. Additionally, this method is more appropriate than autohydrolysis because of recyclability, less water consumption, and lower costs. Besides, organosolv pretreatment is cleaner technology because the solid part, where there are no degradation or inhibition products, is used as a substrate for enzymatic hydrolysis. Thus, there is no need for an additional purification step.

Tablo 2.5. Mode of actions of XOS production methods.

Method	**Mechanism**	**Notes**
Alkali Extraction + Enzymatic Hydrolysis	Biomass+alkali→ Xylan dissolves in liquor Xylan+EtOH → Xylan precipitation Xylan polymer is enzymatically hydrolyzed into XOS	-Corrosion in equipment -High chemical use
Hydrothermal Treatments	Ionization of water → Action of ions as catalyzer → Removal of carbohydrates	- High energy req. -Degradation products
Mild Organosolv + Enzymatic Hydrolysis	Organic solvent+Water → Partial removal of lignin Xylan in the biomass is hydrolyzed enzimatically.	-High energy req.

2.7. Enzymatic Hydrolysis

Enzymatic hydrolysis in XOS production has been always the preferred choice because it provides a controlled process, while it does not produce undesirable by-products and does not require any special equipment. To improve the current systems, researches on XOS production have focused on applications with enzymatic hydrolysis (Amorim et al., 2019). In this process after obtaining a carbohydrate-rich porous structure with pretreatments, XOS is produced with partial hydrolysis of xylan with xylanolytic enzymes. Due to the complex structure of xylan polymer, there is a need for a complex of several hydrolytic enzymes with diverse modes of actions. For absolute breakdown and metabolism, microorganisms naturally secrete these enzymes in a complex depending on the type of microorganism (Beg et al., 2001). Additionally, since these enzymes have a synergistic effect together, they are commercialized as a complex. That's why enzymatic hydrolysis conditions should be optimized for the intended system. To increase the yield of XOS production with enzymatic hydrolysis, conditions should be optimized that endo xylanase activity is maximum, while exo-xylanase and beta-xylosidase are minima. On the other hand, environmental factors like pH, the temperature should be adjusted for high XOS production yield (Aachary and Prapulla, 2011b).

Based on the biochemical classification, xylanolytic enzymes mostly belong in glycosidase hydrolases (GH) and some of them are in the esterases group. Apart from that, these enzymes can be classified depending on their substrate. While beta-xylosidase, endo, and exo-xylanases depolymerize backbone, other accessory xylanolytic enzymes act on side chains (Figure 2.4) (Marais, 2008).

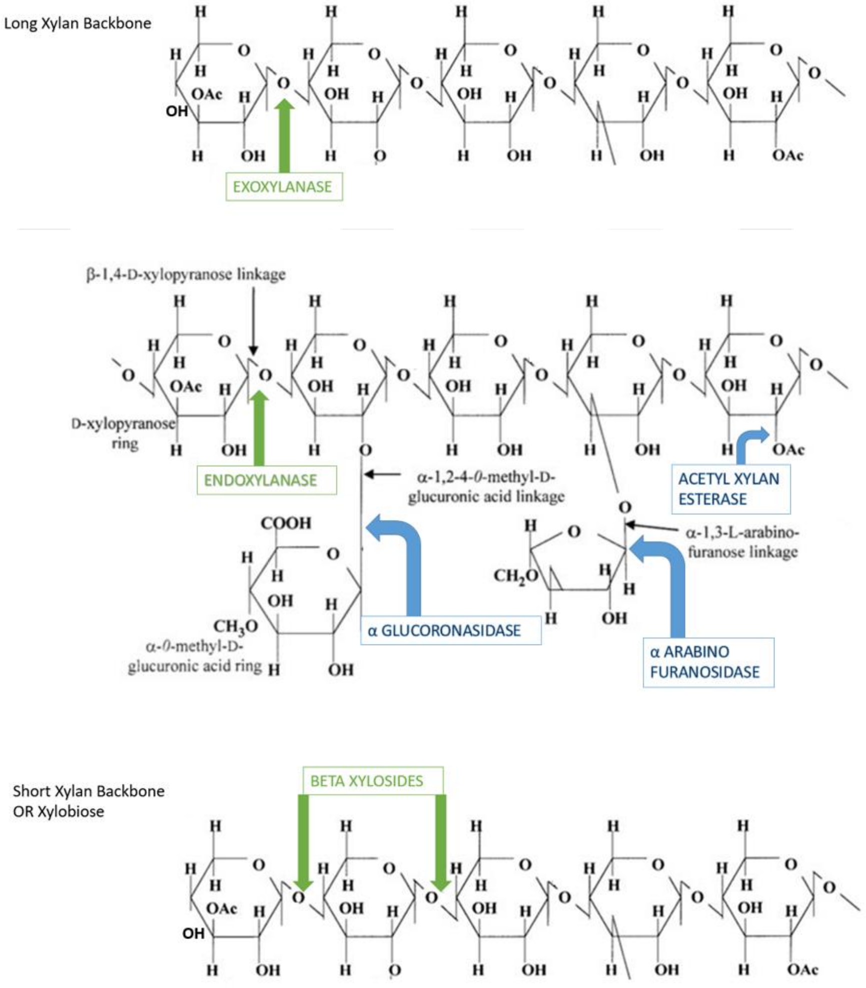


Figure 2.4. Action sites of different types of xylanases (adapted from Beg et al., 2001).

- **Endo-1, 4-β-Xylanase** (1, 4-β-D-xylanxylanohydrolases, EC 3.2.1.8): interior xylose β (1,4) linkages are hydrolyzed from the xylan backbone into the short-chain xylooligomers. Backbone should be consist of the long chain of consecutively unsubstituted xylopyranose units to see the endo xylanase effect. The average molecular masses of this group enzymes are approximately 40 kDa.

- **Exo-1, 4-β-Xylanase**: act from the non-reducing end of xylan backbone, hydrolase interior β-1,4-xylose linkages, and produce xylose units.
- **β-Xylosidase** (1,4-β-D-xylanohydrolase, EC 3.2.1.37): short-chain oligosaccharides or xylobiose are hydrolyzed into the xylose monomers which is β-D-xylopyranosyl, from non-reducing end. This enzyme might not act on polymeric xylans. Also, it might be inhibited from xylose end-product.
- **Acetyl Xylan Esterase** (EC 3.1.1.6): O-acetyl groups are removed from β-D-pyranosyl residues in acetylated xylan. This enzyme has an important role in the hydrolysis of xylan because acetyl side chains prevent the effect of other enzymes that act from the backbone, like endoxylanase.
- **α-Arabinofuranosidase** (EC 3.2.1.55): non-reducing α-arabinofuranose is removed from arabinoxylans.
- **α- Glucuronidase** (E.C.3.2.1.131): α-1,2 glucosidic linkages are hydrolyzed between D-glucuronic acid and β-D-xylopyranosyl in glucuronoxylan and release D-glucuronic acid.

The Aim of the Thesis

This study aimed to assess the potential of organosolv treatment as a pretreatment method before the enzymatic hydrolysis of xylan in the XOS production process from lignocellulosic biomass. The hypothesis was that mild organosolv pretreatment could keep the xylan in the solid phase, while removing lignin partially, so that, xylan could be rendered amenable to enzymatic hydrolysis. This approach was tested to obtain clean XOS solutions without the formation of toxic products. Furthermore, optimization of enzymatic hydrolysis conditions to maximize XOS yield (XOS/xylan), maximize concentration in hydrolysis liquor, and minimize xylose formation, were targeted.

CHAPTER 3

MATERIAL METHODS

3.1. Materials

The materials used in the study and their sources are listed below.

- Corncob (41.92% Cellulose, 33.02% Hemicellulose (26.83% Xylan, 3.76% Acetyl Groups, 2.43% Arabinan), 18.78% Lignin, 7.86% Extractives, 0.21% Ash) (Ministry of Agriculture and Forestry, Aegean Agricultural Research Institute)
- Ethanol (96 %, technic ethanol, Isolab)
- Accelerase XY (Genecor, Danisco, Finland)
- Veron 191S (AB Enzymes)
- Econase XT (AB Enzymes)
- Sodium Citrate (99%)(Sigma-Aldrich)
- Citric Acid (99%)(Sigma-Aldrich)
- Sulphuric Acid (96%)(Sigma)
- Calcium Carbonate (99%)(Sigma-Aldrich)
- Acetone (98%, technic acetone, Isolab)
- Acetic acid, Glucose Monohydrate, Xylose, Arabinose (99%)(Sigma-Aldrich)
- Xylobiose, Xylotriose, Xylotetarose, Xylopentaose, Xylohexaose (99%) (Megazyme)
- Xylan from beechwood (>90%)(Megazyme)
- DNS (3,5 dinitro salicylic acid) (99%)(Sigma-Aldrich)
- Magnesium Oxide (Feed Grade) (Kimetsan. Turkey)

3.2. Methods

3.2.1. XOS Production Process

At the beginning of the study, the composition of the raw material ‘corn cob’ was determined. Afterward, organosolv pretreatment under various conditions was applied and XOS from selected solids was obtained via enzymatic hydrolysis using commercial xylanases (Figure 3.1). In the organosolv treatment step, temperature, time, ethanol concentration, and catalyst addition were tested. Enzyme amount, hydrolysis time, solid: liquid ratio, and temperature were parameters tested in enzymatic hydrolysis.

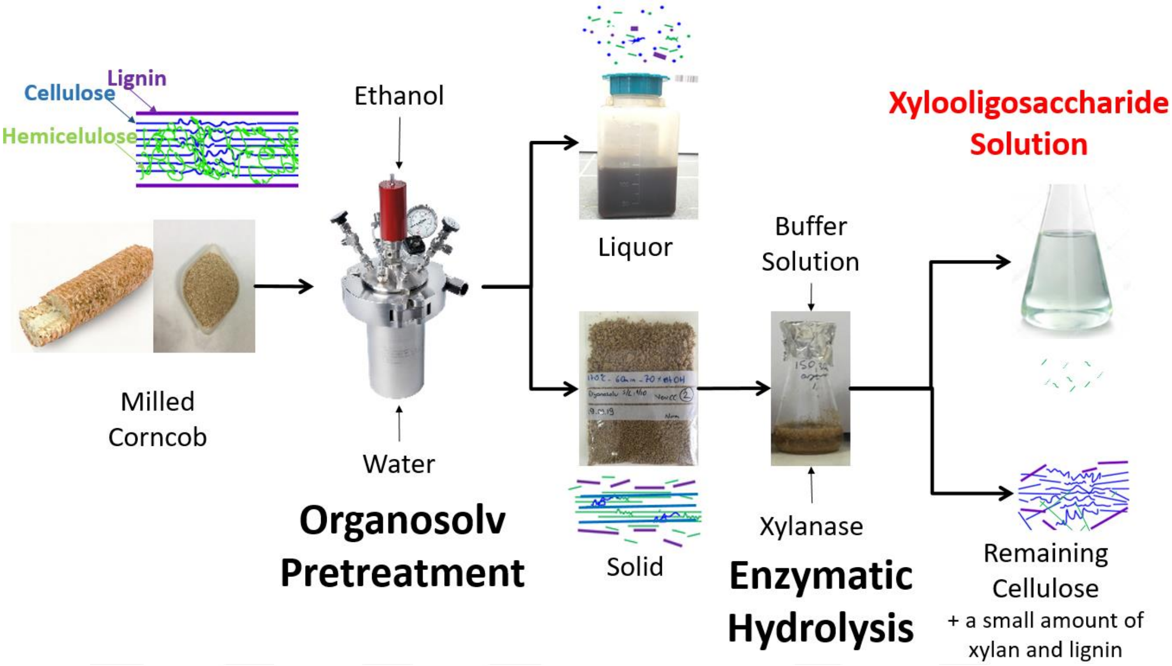


Figure 3.1. XOS production flowchart.

3.2.1.1. Supply and Storage of Corncob

The corn cob was supplied from the Ministry of Agriculture and Forestry, Aegean Agricultural Research Institute as dried in the open air and milled in a hammer mill (grain size <2 mm). It was stored in airtight plastic containers until use.

3.2.1.2. Organosolv Pretreatment

To set solid: liquid ratio to 1: 10, 25 g milled corncob, and 250 ml total liquid were put into the stainless steel tank (600 ml total volume) of the high-pressure reactor (Berghof BR 600, Germany) and the reactor was closed tightly. The tank was placed in the heating chamber of the reactor and the heating was started. During the reaction, the pedal mixer was run 300 rpm for the mixing of the contents. After the temperature reached the desired level, the reaction time was started. At the time end of the time set, the heating was turned off and the cooling was started. The reactor was cooled with tap water circulating in the cooling coil for approximately 20 min and it was opened after temperature decreased to 60ºC (Sabanci and Buyukkileci, 2018).

The reaction temperature was between 130 and 190ºC, and the reaction time was set as 0.5, 1, and 2 h. Ethanol solutions (50% and 70%) were employed as the treatment liquid in the organosolv pretreatments. Additionally, autohydrolysis was also conducted to compare with organosolv pretreatments. Magnesium oxide (MgO) added into the mixture at a concentration of 0.1 mol/g in the tests with catalysts.

Following the organosolv pretreatment, solid was prepared for enzymatic hydrolysis (Figure 3.2). The mixture composed of ethanol, water, and biomass was filtered with a cloth, thus solid and liquor separated from each other. The pH of the liquor phase was measured with a pH meter (Selecta, Spain). Solid-phase was rinsed with distilled water and filtered with a filter paper under vacuum. The remaining solid on the filter paper was dried at 60°C until it reached a constant weight. The dried biomass was milled and homogenized using a kitchen type grinder (Sinbo, Turkey) (Kapoor et al., 2019). Milled biomass was stored in labeled plastic bags at room temperature for enzymatic hydrolysis and further analysis.

Figure 3.2. Preparation of organosolved solid to the enzymatic hydrolysis.

a: filtration of the mixture from organosolv pretreatment through a cloth

b: washing of the filter cake

c: removing the excess water in the filter cake

d: wet treated solid

e: solid ready for the enzymatic hydrolysis

3.2.1.3. Enzymatic Hydrolysis

Three xylanases (Accelarase XY, Veron 191S, and Econase XT) were used in the enzymatic hydrolysis step. Pretreated corn cobs were suspended in a 50mM citrate buffer solution composed of citric acid and sodium citrate. According to the enzyme manufacturers' recommendations, the pH of the buffer was set as 5.5 for Accelarase XY and Veron 191S, and 6.0 for Econase XT. The solid-liquid ratio was set to 1:20 unless otherwise stated. To prevent biases in solid-liquid ratio due to improper sampling and evaporation, separate 10 ml tubes containing 0.1g biomass in 2ml buffer were prepared for each sampling point. Solid Econase XT and Veron 191S enzymes were suspended in respective buffers at a concentration of 4% (w/v). After mixing gently for 5 min with vortex, the insoluble part was separated by centrifugation at 5000 rpm for 4 min. The soluble proteins (enzyme) in the supernatant were used in hydrolysis. The tubes were statically incubated in water baths at 55, 70, and 85°C respectively, and mixed manually at regular intervals. To stop the activity of Accelerase XY and Veron enzymes in sampling, the test tubes were centrifuged (25000 g) to remove solids and the supernatant was kept in a water bath at 100°C for 5 min. For the inactivation of the thermophilic Econase XT enzyme in sampling, the solids were removed by centrifugation (25000 g) for 5 min, and then 4 ml of concentrated acetone was added to 1 ml of the sample (supernatant). After overnight incubation of samples in acetone at -20°C for the precipitation of the proteins, acetone was evaporated by keeping the tubes in a water bath at 100°C for 20 min. At the end of the process, the total volume completed to 7 ml in a falcon tube. Thus both fixed volume and dilution were obtained. The XOS production was optimized for the amount of enzyme, temperature, and time. Afterward, the concentration of corncob in the buffer solution was increased from 5% (solid: liquid ratio of 1:20) to 10% and 20%. The effect of an increase in solid loading was investigated in terms of yield.

Conversion and total yields were calculated using XOS and xylose concentrations obtained after enzymatic hydrolysis.

$$\text{conversion \%} = \frac{\text{XOS or xylose released(g)}}{\text{Xylan in pretreated biomass (g)}} \times 100 \qquad \text{Equation 1}$$

$$\text{yield } \% = \frac{\text{XOS released (g)}}{\text{Raw biomass (g)}} \text{ x } 100 \qquad \text{Equation 2}$$

3.2.2. Analyses

3.2.2.1. Moisture Analysis

After corncobs were dried overnight in an oven (Memmert, Germany) at 105ºC according to National Renewable Energy Laboratory (NREL) NREL/TP-510-42621 method, they were kept in a desiccator until they about 2 hours. They were weighed in an analytical balance (Shimadzu, Japan) and moisture content was calculated using Equation3.

$$\text{Moisture } \% = 100 - \frac{\text{Dried Sample (g)}}{\text{Analyzed sample (g)}} \times 100 \qquad \text{Equation 3}$$

3.2.2.2. Structural Carbohydrate Analysis in Solid Samples

Before the structural carbohydrate analysis, corncob was extracted respectively with water and ethanol (96%) in the Soxhlet apparatus (Isolab, Germany) for 4 h in order to determine extractives. Unpretreated corncob and pretreated biomass were analyzed according to the NREL / TP-510-42618 method (Sluiter et al., 2012) (Figure 3.3). Thus, 3 ml of 72% sulphuric acid (H_2SO_4) solution was added on 0.3 g dry biomass and incubated for one hour at room temperature. After acid concentration was decreased to 4% with the addition of 84ml water, the solution was autoclaved at 121ºC for 1 h. In order to analyze the remaining solid consisting of lignin and ash, the mixture was cooled down to room temperature and filtered through porcelain filtering crucibles under vacuum. The crucibles containing samples were firstly dried overnight at 105ºC and weighed. Then the ash amount was determined gravimetrically after crucibles were incubated at 525ºC for 3 h. Acid insoluble lignin content was calculated using equation 4.

$$\text{Acid insoluble lignin} = \left(\frac{\text{dried samples after acid hydrolysis (g) - ash (g)}}{0.3 \text{ g}}\right) \times 100 \qquad \text{Equation 4}$$

Figure 3.3. Steps of structural carbohydrate analysis.

a: incubated biomass at 72% H_2SO_4 for 1h at room temperature

b: Mixture after autoclave

c: filtering of the mixture in crucible under vacuum

d: dried lignin and ash

e: ash

For the determination of the carbohydrate concentrations of the samples, the released monomers as a result of acid hydrolysis were analyzed in HPLC. After concentrations of the monomers were determined, polymer quantities in solids were calculated using equation 5 for cellulose (glucan) and equations 6 and 7 for hemicellulose (xylan, arabinan, and acetic acid). Since water molecules bonded to monomers during hydrolysis, values were multiplied with correction factors. Correction factor is 0.88 for five-carbon sugars; 0.9 for six-carbon sugar; and 0.72 for acetyl groups.

$$\text{Glucan\%} = \frac{\text{Glucose concentration (g/l)} \times 100 \times 0{,}90}{\text{Anaylzed dry matter (g) x } 0{,}087} \qquad \text{Equation 5}$$

$$\text{Xylan\% - Arabinan\%} = \frac{\text{Xylose-arabinose concentration (g/l)} \times 100 \times 0{,}88}{\text{Anaylzed dry matter (g)} \times 0{,}087} \qquad \text{Equation 6}$$

$$\text{Acetyl\%} = \frac{\text{Acetic acid concentration (g/l)} \times 100 \times 0{,}72}{\text{Analyzed dry matter (g)} \times 0{,}087} \qquad \text{Equation 7}$$

3.2.2.3. Carbohydrate Analysis in Enzyme Hydrolysate

After enzymatic hydrolysis, hydrolysates were analyzed without further processing in HPLC to determine the amounts of the xylose and the XOS with a low degree of polymerization (DP). To determine the total amount of XOS (sum of low and high DP XOS) the liquid samples were acid-hydrolyzed and the xylose formed was quantified to calculate the XOS concentration. 174 µl of 72% H_2SO_4 solution was added into 5 ml liquid samples and the mixture was autoclaved at 121ºC for 1 h (NREL, 2018). The total XOS concentration was calculated using equation 8.

$$\text{Total XOS} = \text{xylose after postacid hydrolysis (g/l)} - \text{xylose before postacid hydrolysis (g/l)} \quad \text{Equation 8}$$

3.2.2.4. High-Performance Liquid Chromatography (HPLC) Analysis

All monomers, oligomers, and polymers (cellulose and xylan) in the samples from pretreatment and enzymatic hydrolysis were analyzed in high-pressure liquid chromatography (HPLC). Samples, which are acid hydrolyzed, were prepared to fit in column conditions. To calculate the concentration of analyzed samples, calibration curves were used. Curves prepared using standard solutions of analytes with known concentrations.

- **Monosaccharide Analysis:** Samples from acid hydrolysis were subjected to a neutralization process to fit column conditions. Thus, 0.26 g calcium carbonate ($CaCO_3$) was added into 5 ml of sample, so thatpH increased to 5-7. To remove excess calcium carbonate, samples were centrifuged for 5 min at 6000 rpm. Supernatants were diluted with distilled water. Samples were filtered through 0.45 µm syringe filters to eliminate any impurities. To determine the xylose concentration formed during enzyme hydrolysis, liquors were analyzed directly, without acid hydrolysis.

  Analyses performed in HPLC (Perkin Elmer Series 200, USA) using a refractive index (RI) detector. Rezex RPM-Monosaccharide column (Phenomenex, USA), which is in a lead ionic form, was used in the analysis of oligomers and polymers which were broken down into their monomers as a result of the acid hydrolysis. During analysis, while ultra-pure water used as a mobile phase, the flow rate was 0.6 ml/min and the column temperature was 80ºC.

  In order to protect column from excessive ions originating from acid hydrolysis and post-treatments, H^+ and CO_3^- containing deashing column (Biorad, USA) was placed before the main column. Also, the guard column (Phenomenex, USA) used before the main column in the analysis of the liquor to prolong column life.

- **Oligosaccharide Analysis:** After enzymatic hydrolysis samples were filtered through 0.45 µm filter membranes, they were analyzed in the same HPLC system and the detector using Aminex HPX-42A oligosaccharide column (Bio-Rad, USA). As a result of the analysis, oligomers with a DP of 2-6 were determined. During analysis, the mobile phase was ultrapure water, the flow rate was 0.6ml/min and the column temperature was 80ºC. Also, to prevent shifts in chromatograms, again deashing column (Biorad, USA) was used before the main column in this analysis.
- **Acetic Acid Analysis:** During structural analyses of corncob, after monosaccharides analyses, the same samples were subjected to acetic acid analysis. The analysis was carried out in the same system with Aminex HPX-87H organic acid (Bio-Rad, USA) column. During analysis 5mM H_2SO_4 was used as the mobile phase. The flow rate was set to 0.6 ml/min and the temperature of the column was set to 60ºC.

3.2.2.5. Xylanase Enzyme Activity Assay

The xylanase activity was measured based on the dinitro salicylic acid (DNS) method (Bailey et al., 1992; Yegin, 2017). Xylan from beech wood was used as the substrate (0.5% (w/v)) in 50mM citrate buffer (pH 5.5). 100 µl enzyme at different concentrations was added to 900 µl substrate and the mixture was incubated in a water bath at 50ºC for 5 min. At the end of the period, 1500 µl DNS solution was added and the sample was kept in a boiling water bath for 5 min, which developed the color and stopped the enzyme activity. The tubes were cooled for 1 min before analysis. Blank sample for each enzyme concentration was formed adding enzyme just before the boiling step instead of before incubation. Thus, there was no reaction in these samples, so that the effect of the enzyme on the color could be eliminated in the spectroscopic readings (Yeğin, 2017). The color change as a result of the reaction between the reducing sugar coming from the hydrolyzed substrate and DNS was determined using a spectrophotometer (Shimadzu, Japan). Absorbance values of both samples and blanks were measured against water at

540 nm. After the measurement, the color change of samples due to the enzymatic activity determined by subtracting the absorbance of blanks from that of the samples.

To prepare a standard graph for the determination of the reducing sugar concentrations, thus the xylanase activity, 2g/l xylose stock solution was diluted with 50mM (pH: 5.5) citrate buffer to the concentrations of 0-75 µg xylose. The color change formed by the xylose solutions in various concentrations was determined spectrophotometrically following the same procedure described above. The blank was obtained by adding a buffer solution instead of a xylose solution. The standard graph was prepared by drawing a curve using the xylose concentrations and the corresponding absorbance values. The amount of reducing sugar formed as a result of the enzyme activity was found in terms of xylose using this standard graph.

One unit of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose equivalent per min under standard assay conditions (for Veron 191S and Accelerase XY pH 5.5 and 50 °C, for Econase XT pH 6.0 and 70 °C). The enzyme activity of the samples was calculated using equation 9 given below.

$$\text{Activity(U/ml)}=\frac{X}{150.13}\times\frac{1}{5}\times\frac{1}{0.1}\times\text{Dilution Factor} \qquad \text{Equation 9}$$

X: µg xylose (from the standard graph)

150.13: Molecular weight of xylose

5: Incubation time (min)

0.1: Enzyme amount (ml)

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Characterization of Corn Cob

Xylan, which is the main carbohydrate of hemicellulose in lignocellulosic biomass, is the substrate for XOS production. That’s why food-agricultural wastes with high xylan contents are possible candidates for XOS production (Hamsen et al., 2010; Dongen et al., 2011; Isıkgor et al., 2015). Additionally, lignin has a critical role in the prevention of lignocellulose hydrolysis. Thus, the low lignin content in the raw material is another critical point. Satisfying these requirements, corn cob was selected as a suitable substrate for XOS production in this study.

Characterization of raw material is a vital step for the survey of the effect of the further process on the raw material and the efficiency of the XOS production. It is also important for the comparison of the composition of the raw material with the data in the literature.

According to the characterization results given in Table 4.1, cellulose had the highest portion (41.9% in terms of glucan) in the raw material. Xylan was found to be the second most abundant polymer with a concentration of 26.8% and lignin is the third one (18.8%). In the previous studies, xylan content of corn cob was measured as 20, 25, and 25%, respectively (Liujan et al., 2016; Dongen et al., 2011; Fialho et al., 2015). Hemicellulose consists of a xylan backbone and side chains with five or six carbon monosaccharides and acetyl groups. In this study, corncob has 26.8% xylan, 2.43% arabinan, and 3.76% acetyl groups, so the hemicellulose content of raw material equals 33%. In the previous studies, hemicellulose content of corn cobs was reported as 44% (Pointer et al., 2014); 44% (Hamsen et al.,2010); 35% (Bajpai et al., 2016) and 26-31% (Isıkgor et al., 2015). According to the same studies, the lignin amount was 6-17%. Generally, xylan and lignin contents of the corncob used in this study were in good agreement with the literature. While the minor differences might be due to the analysis methods applied, the variety and the physiological state of the plant could have been different in the studies (Keskin et al., 2018).

Table 4.1. Composition of the corncob.

Component	**Concentration**[a] **(g/100 g Dry Matter)**
Ash	0.21 ±0.04
Cellulose (as glucan)	41.9 ±1.75
Xylan	26.8 ±1.12
Arabinan	2.43 ±0.17
Acetyl Group	3.76 ±0.43
Lignin (acid insoluble)	18.8 ±1.60
Extractives (water and alcohol soluble)	7.86 ±1.59
Total	101.8

[a] Values are the average of two measurements. ± Standard deviation

4.2. Organosolv Pretreatment

In XOS production, cellulose, hemicellulose, and lignin should be partially or completely separated from each other by the application of some pretreatment methods. Since the enzymatic hydrolysis carries out on solid substrate in this study, xylan should be kept in solid; while lignin should be removed as much as possible. For this purpose, organosolv employed in this study as a pretreatment method, and conditions were optimized. In order to obtain the desired effect, treatment temperature was set between 130 and 190°C; time was set between 0.5 and 2h; ethanol concentration in the liquid phase was 50% or 70% (Table 4.2). These values were adapted according to the previous studies in the literature. Additionally, MgO was tested as a catalyst. Autohydrolysis was used as control and compared with organosolv pretreatment. After the process, the amount of insoluble solid parts, and the pH of the liquors were measured, and the structural composition of the solid part was determined.

Total solid recovery decreased with increasing temperature at a constant time (1 h) and ethanol concentration (70%) (Table 4.2). While 20% of the solid was dissolved at the liquid phase in organosolv at 150°C, it was 29% at 170°C. The amount of cellulose in the solid-phase remained the same at around 83-85% despite the increase in temperature (Figure 4.1.a). This is because of the nature of the organosolv pretreatment; this method originally aims to keep cellulose in the solid phase for further cellulose hydrolysis. While xylan recovery values were 85.4 and 80.9% at 150°C and 170°C, respectively, it was only

35.5% at 190°C. Moreover, lignin removal was improved with an increase in temperature. 76% lignin removal at 190°C could be considered as favorable in terms of cellulose and xylan hydrolysis. However, this condition was not suitable for XOS production, due to the low xylan recovery at that temperature. On the other hand, this pretreatment can be used for cellulose hydrolysis due to the considerable removal of both lignin and xylan. That's why this condition has the potential for the production of other bio-based products.

When pretreatment was applied in the form of autohydrolysis using %100 H_2O (without incorporation of ethanol) at 170°C for 1 h, xylan solubilized in liquor substantially, and xylan recovery in solid part was only 23.5% (Figure 4.1.b and Table 4.2). In autohydrolysis, H_3O^+ from water ionization and the acetic acid released by the removal of acetyl groups (C_2H_3O) from hemicellulose decrease the pH and act as acid catalysts, which carry out both xylan removal and hydrolysis (Zhao et al., 2009). Hemicellulose is susceptible to acidity and removal or recovery of this component depends on the pH of the medium. In organosolv with 50% ethanol concentration xylan recovery was lower compared to organosolv with 70% ethanol concentration. This is again may have been due to the higher water content in the organosolv medium, thus ionization ratio and autohydrolysis effect were notable(Mataskas et al., 2019; Nitsos et al., 2018). In the comparison of pretreatments with 50% and 70% ethanol concentrations to the autohydrolysis (0% ethanol), cellulose recovery is lower in autohydrolysis because of the same reason. That is, a decrease in alcohol concentration resulted in an increase in the solubilization and a decrease in the recovery of carbohydrates.

The tests at 150°C with 70% ethanol concentration showed that processing time on pretreatments was an effective parameter, although the effect was not drastic (Table 4.2). An increase in pretreatment time decreased the xylan recovery (Figure 4.1.d and Table 4.2). Additionally, lignin removal was lower when pretreatment applied for 0.5 h than when the same conditions applied for 1 h or 2 h. As a result, 1 h chosen as the optimum time interval for this study.

Acidic catalysts or metal salts such as $FeCl_3$ and $MgCl_2$ have been used to improve the efficiency of both organosolv and autohydrolysis. However, these catalysts lead to hemicellulose degradation, while increasing the lignin removal (Nitsos et al., 2018; Huijigen et al., 2011; Matsakas et al., 2019; Farmanbordar et al., 2018, Zhang et al., 2018b; Cuilty et al., 2018). At this point, alkali catalysts instead of acidic ones could be the solution to this problem. It was predicted that hydroxyl molecules in catalyst would neutralize the decreasing pH during the reaction, thereby degradation of hemicellulose due to low pH could be prevented. Since metal hydroxyls are very reactive compounds, more stable ‘metal oxides’ can provide the ease of use (Li et al., 2018a). In previous studies, where MgO was used as a catalyst, its effect on cellulase enzyme was followed, and successful results were obtained in terms of cellulose saccharification (Deng et al., 2015, Li et al., 2018b, You et al., 2019). In the study as predicted, the addition of MgO prevented the decrease in the pH of the organosolv medium (Table 4.2 and Figure 4.1c). When organosolv conditions for lignin removal in 70% ethanol for 1 h at 150, 170, and 190°C with MgO catalyst compared to catalyst-free process under the same conditions, there was not a substantial difference. While there was no notable effect on hemicellulose recovery with the addition of MgO at 150°C, there was a gradual increase in hemicellulose recovery at 170 and 190°C. Hemicellulose recovery increased from 80.86% to 87.17% at 170°C and from 35.55% to 70.70% at 190°C with MgO addition. Additionally, 23.55% hemicellulose recovery in autohydrolysis at 170°C almost tripled to 69.53% with the addition of MgO (Figure 4.1 a and c).

The results showed that hemicellulose recovery was affected by the pH of the medium. When it was possible to keep the pH of medium near-neutral value by using a lower temperature, lower water content, or by the addition of MgO, hemicellulose was recovered in the biomass (the solid phase) to a great extent. On the other hand, lignin removal was affected mainly by the increase in temperature. Other factors had minor effects on lignin removal. Besides, there was an increase in the total of polymer (cellulose, hemicellulose, and lignin) concentration, with an increase in temperature. It can be concluded, extractives and other small groups like acetyl and arabinan remained in the solid, while they were solubilized by gradually as an increase in temperature.

In the following part of this study, the effect of various organosolv pretreatment conditions on the action of xylanase enzyme and efficiency of xylanase hydrolysis was investigated. Since their effects were similar in terms of lignin removal and cellulose/hemicellulose recovery, pretreatments at 150 and 170°C at constant ethanol concentration (70%) and time (1h), with or without the addition of MgO were selected for the next step.

Table 4.2. Effect of organosolv pretreatment conditions on biomass composition[a].

Organosolv Conditions (temperature-time-ethanol%-catalyst)	**Solid recovery (g)**	**Solubilization (%)**	**Liquor pH**	**Composition of Solid (%)**			**Recovery[c] (%)**		**Lignin Removal[c] (%)**
				cellulose	**Xylan**	**Lignin**	**cellulose**	**xylan**	
Untreated Corncob				41.91 ±1.75	26.83 ±1.12	18.78 ±1.60			
170°C-1 h-0% (autohydrolysis)	11.1	55.6	3.83	63.20 ±0.06	13.99 ±0.24	23.75 ±0.07	67.02 ef	23.55 g	41.42 de
170°C-1 h-0%-MgO (autohydrolysis + catalyst)	15.4	36.6	6.33	52.58 ±1.04	29.40 ±1.79	21.86 ±0.11	79.60 bc	69.53 d	25.04 f
130°C-0.5 h-50%	19.2	23.2		36.81 ±0.40	27.54 ±0.05	13.88 ±0.75	67.46 ef	78.82 b	40.78 de
130°C-1 h-70%	23.03	7.88	5.28	27.57 ±1.24	24.20 ±1.17		65.78 ef	90.25 ab	
150°C-0.5 h-70%	20.0	20.0	5.54	37.02 ±0.59	34.56 ±0.35	16.33 ±0.35	70.70 de	103.10 a	27.39 f
150°C-1 h-70%	19.9	20.2	5.33	43.59 ±1.24	28.71 ±1.17	13.68 ±0.47	82.97 ab	85.39 b	39.37 de
150°C-2 h-70%	19.1	23.7	5.26	35.70 ±0.48	27.33 ±1.17	14.93 ±0.30	65.01 f	77.74 bcd	36.70 e
170°C-1 h-50%	14.8	40.8	4.62	53.15 ±0.31	24.72 ±0.25	14.18 ±0.60	75.13 cd	54.59 e	53.33 b
170°C-1 h-70%	17.8	29.0	5.15	50.08 ±0.64	30.56 ±2.00	12.78 ±0.52	84.83 a	80.86 bc	49.59 cd
190°C-1 h-70%	12.3	50.8	4.81	70.62 ±1.44	19.39 ±0.79	8.84 ±0.10	82.90 ab	35.55 f	75.86 a
150°C-1 h-70%-MgO	20.2	19.3	7.65	42.75 ±2.12	28.15 ±1.15	13.12 ±0.87	82.34 ab	84.68 b	41.16 de
170°C-1 h-70%-MgO	19.3	22.7	6.86	46.22 ±0.72	30.25 ±2.48	11.21 ±0.71	85.27 a	87.17 b	51.55 bc
190°C-1 h-70%-MgO	14.9	40.4	6.45	58.31 ±0.03	31.84 ±0.81	9.65 ±0.02	82.89 ab	70.70 cd	68.06 a

[a] Values are the average of two measurements. ± Standard deviation

[b] Initial corncob mass =25 g

[c] (Cellulose and xylan recovery and lignin separation were compared using one-way analysis of variance ($p < 0.05$) and Tukey test. Letters in the same column represent statistical similarities and differences.

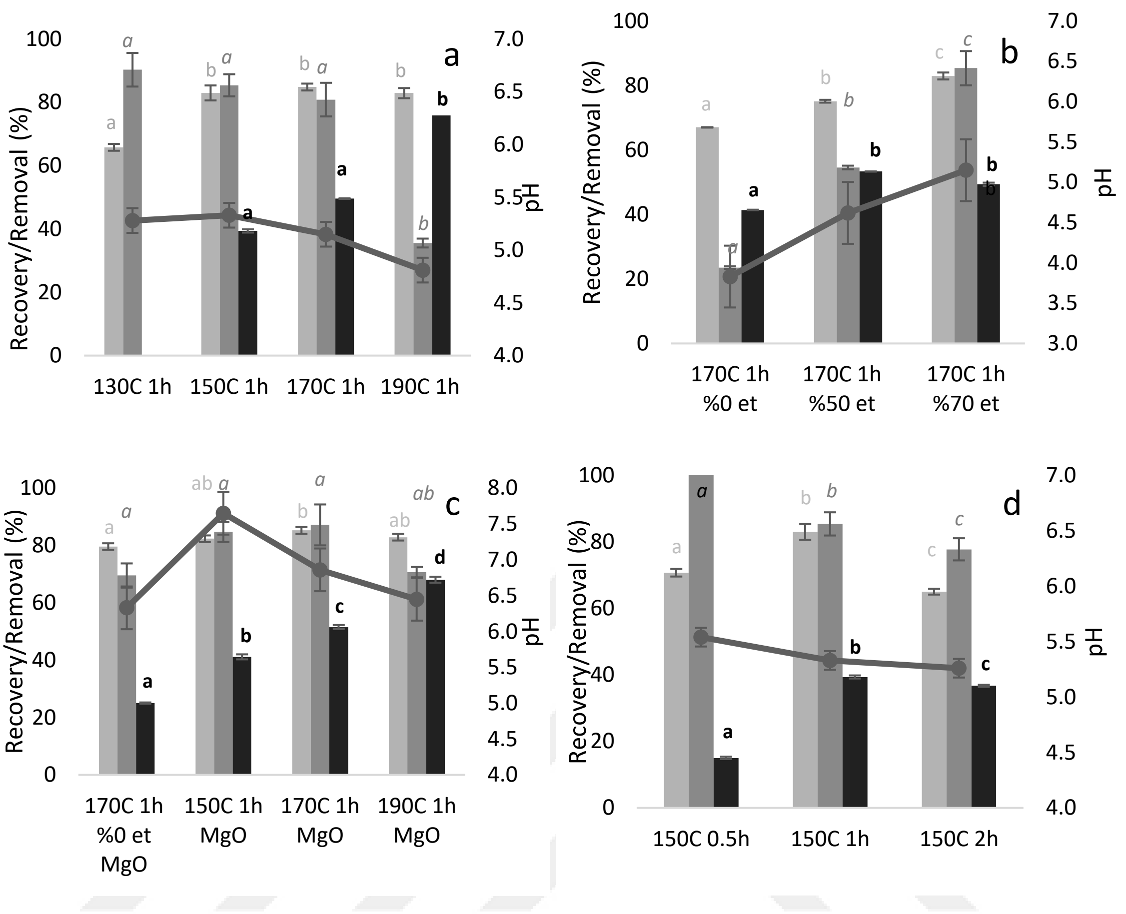


Figure 4.1. Effects of organosolv pretreatment conditions on lignin removal, cellulose and xylan recovery, and liquor pH.

a: Temperature; b: Ethanol concentration; c: MgO (0.1 mole/L); d: Time effect

(Unless otherwise, specified ethanol concentration is %70.)

Light grey bar: cellulose recovery, dark grey bar: hemicellulose recovery, black bar: lignin removal, line: pH

Letters express statistical differences and similarities ($p < 0.05$, one-way variance analysis and Tukey test were applied).

4.3. Enzymatic Hydrolysis

Three commercial enzymes were used for the production of XOS under the conditions recommended by manufacturers for xylanase activity and additionally at different temperatures, enzyme dosage, and solid: liquid ratios were applied. After pre-treated biomasses were suspended in buffer solutions and the enzymes were added, total XOS and xylose released were checked periodically. The polymerization degree distribution of XOS was also determined in the selected samples.

Previous studies have shown that there was not an efficient sugar conversion in enzymatic hydrolysis of untreated lignocellulosic biomass (Jiang et al., 2018). In this study, that was confirmed and only 0.96 g/l total XOS was released as a result of the enzymatic hydrolysis of the unpretreated corncob with Accelerase XY enzyme at 55°C for 48 h.

4.3.1. The Effect of Temperature on XOS Production on Enzymatic Hydrolysis

Temperature is one of the most important factors in enzymatic hydrolysis. Also, temperature influences the solubility of the substrates and the viscosity of the medium. So, mixing and mass transfer rates are more efficient at high temperatures (Gatt et al., 2019). At high temperatures, the accessibility of the enzyme to xylan or other polymers in solid biomass can be improved. Thus the efficiency of hydrolysis also increases. Besides, high temperature may eliminate the need for antimicrobials because it prevents/delays microbial contamination during the enzymatic hydrolysis process.

In this part of the study, three different enzymes (Econase XT, Accelerase XY, and Veron 191S) were applied at three different temperatures (55°C, 70°C and 85°C). In this test, one of the pretreated solids was used as the model biomass; corn cob treated with 70% ethanol at 170°C for 1 h was chosen for this test because of the high xylan recovery and lignin removal obtained under these conditions.

According to the manufacturer’s information, the optimum temperature for the Accelerase XY enzyme is between 55-75°C. In the case of the enzymatic hydrolysis at 55°C, both xylose and XOS formations were observed at the end of the hydrolysis (Figure 4.2 a). After 44% of the xylan in the treated biomass was converted rapidly in the first 4 h of hydrolysis, hydrolysis continued at a lower rate for the next 20 h. In 24 h, 90% of total xylan in the solid solubilized in the liquid part, but only 60% of it obtained as XOS. Since enzymes are produced as a complex by microorganisms and they have a synergistic effect together. Endo- and exo-xylanases, and beta-xylosidases coexist in commercial xylanases (Dhillon et al., 2011). That's why due to the probable high exo-xylanase activity at 55°C, there was substantial xylose conversion. When hydrolysis was performed at 70°C, there was a noticeable reduction in xylanase activity. In 8 h of the hydrolysis, while 30% of xylan converted to XOS, 8.37% of xylan converted to xylose. Although there was a general trend as a reduction in the xylanase activity, it was seen that exo-xylanase and beta-xylosidase activities were affected more than endo-xylanase by the increase in temperature. After 8h hydrolysis, the conversion rate stayed constant until the end of the hydrolysis at 24 h. The decrease in the activity was more drastic at 85°C, which was already out of the range that the manufacturer recommended for this enzyme. Only 21% of the xylan was converted to XOS in 8 h of hydrolysis. Additionally, there was no xylose conversion throughout the hydrolysis at 85°C. In conclusion, the best result for pure XOS production with Accelerase XY was achieved at 70°C n 24 h.

Veron 191S enzyme was used in a previous study and it was reported to form a low amount of xylose in XOS production (Kiran et al., 2013). Similarly, in this study, the xylose formation was very low at all three temperatures tested (Figure 4.2 b, d, g). However, XOS production was also comparably lower. The highest conversion was 50%, which was obtained at 70°C.

Econase XT is a thermophilic enzyme that is optimally active at high temperatures. According to the manufacturer, it preserves its activity between 65°C and 95°C. In the preliminary tests of this study, it was observed that the enzyme showed low activity at 55°C. That's why enzymatic hydrolysis was carried out at 70°C and 85°C considering the previously mentioned benefits of high temperature. In 6 h, it was observed that the conversion rate of Econase XT is higher than the other two enzymes. Xylan conversion reached to 70% at 70°C. Also, xylose formation was relatively low (3%). At this conversion rate, the concentration of the XOS in the hydrolysis medium was 7.64 g/l. XOS conversion was 38% at 85°C with almost no conversion to xylose.

As a result, enzymatic hydrolysis at 70°C was concluded as the most favorable temperature for Accelerase XY and Econase XT enzymes due to both high XOS and low xylose formations. Although exo-xylanase in Accelerase XY enzyme complex shows low activity under optimum conditions (70°C), there was still notable xylose conversion and more importantly, there was a higher XOS production with Econase XT. The same conclusion could be reached in the comparison of Veron 191S and Econase XT. As a result, the Econase XT enzyme at 70°C shows promising results for XOS production. However, the action of Accelerase XY enzyme should be surveyed in different biomass at 70°C. On the other hand, the Accelarase XY enzyme can also be considered as a potential enzyme in xylose conversion studies.

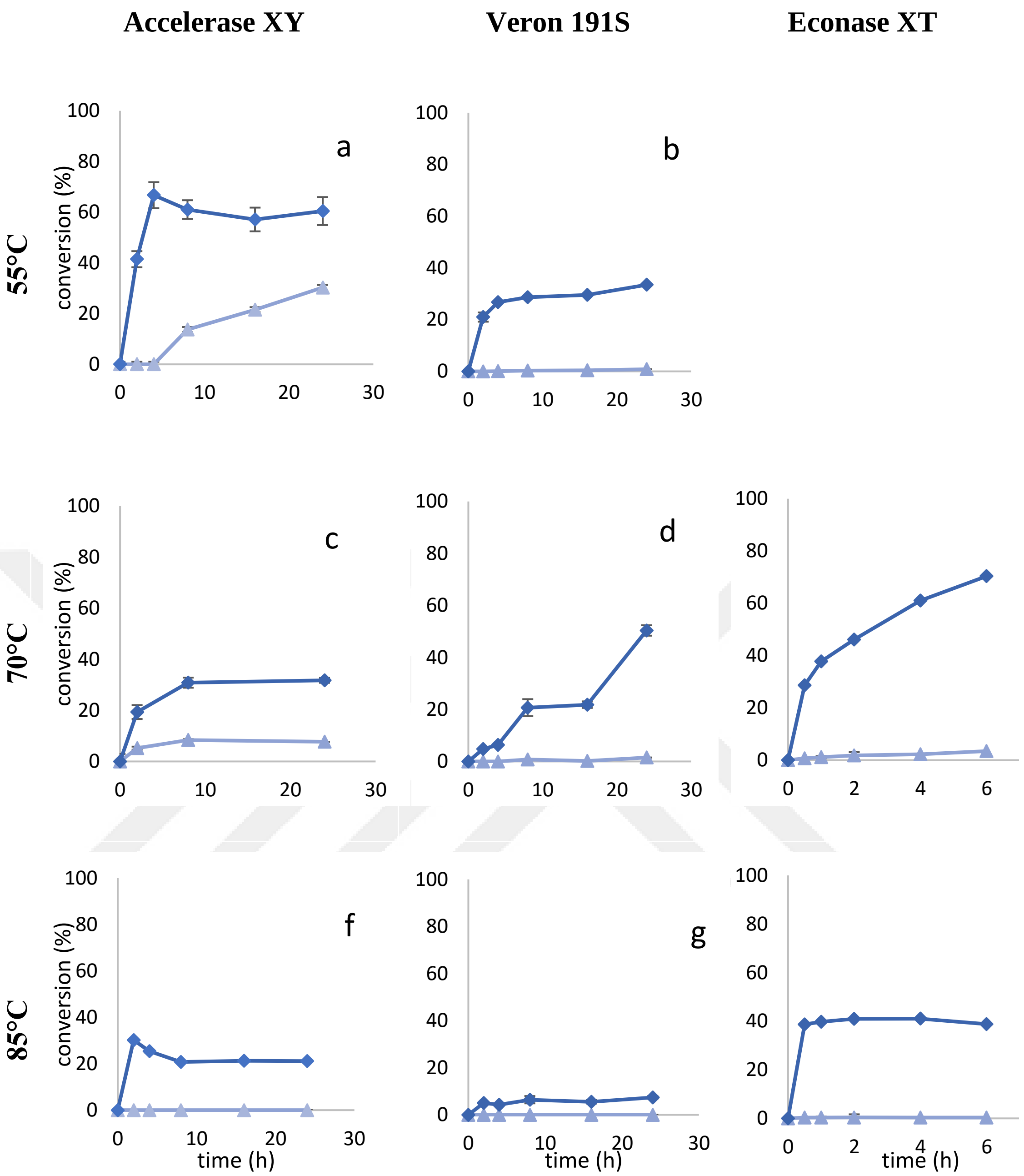


Figure 4.2. The effect of temperature on the XOS production and xylose formation in the enzymatic hydrolysis. ▲: xylose; ♦: XOS

Enzymes: left column: Accelerase XY; middle column: Veron 191S; right column: Econase XT

Temperature: First line: 55°C, Second line: 70°C, Third line: 85°C

4.3.2. The Effect of Pretreatment Conditions on Enzymatic Hydrolysis

There are several studies on how the parameters in the organosolv process affect the activity of cellulase and how the cellulose conversion yield can be increased (Kalogiannis et al., 2018; Jang et al., 2016; Milling et al., 2018; Kim et al., 2019; Huijgen et al., 2011; Cebreiros et al., 2020; Matsakas et al., 2019; Park et al., 2010). In some studies, the condition of organosolv was adapted to keep hemicellulose in the biomass, and the hydrolysis mechanism of all carbohydrates by cellulase enzyme was examined (Li et al., 2018a, 2018b 2019a, 2019b). On the other hand, there is no previous study on how organosolv pretreatment affects the activity of the xylanases and the XOS production. In this part of the study, hydrolysis of selected pretreated biomass was carried out with three different enzymes at specific temperatures, in order to see the effect of organosolv pretreatment on the production of XOS

When the biomass subjected to organosolv at 130°C for 1 h in 70% ethanol was used for enzymatic hydrolysis, the result obtained was similar to the untreated sample. It showed that the treatment performed at this temperature was ineffective, therefore, the organosolv sample at 130°C was not used in the design of the subsequent trials. Apart from that, enzymatic hydrolysis (at 70°C) of biomass samples treated under various conditions is presented in Figure 4.3.

Accelerase XY enzyme succeeded in a certain amount of XOS conversion rate with all biomass samples. For 150°C, 1 h, 70% ethanol pretreatment, there was no xylose conversion in 24 h, as desired. After that point, a slight xylose formation (around 5%) was observed (Figure 4.3.a). Despite this favorable low xylose yield, XOS conversion was not substantial. 33% of the xylan was converted to XOS in 24h, then a small part of it it was degraded to xylose. When MgO added to the organosolv pretreatment under the same condition, there was a noticeable xylose formation from the beginning to the end of the hydrolysis, in addition to the around 30% xylan to XOS conversion (Figure 4.3.b). The difference in the xylose conversion yields between two conditions (with MgO vs without MgO) was not associated with the actionability of exo-xylanase activity because there were also xylose conversions in the same samples when they were hydrolyzed by Veron 191S and Econase XT. So, it could have been related to the action of Accelerase XY on this particular pretreatment condition. When pretreatment temperature increased to 170°C subsequent enzymatic hydrolysis yielded in 58% xylan to XOS conversion was observed in 24h. Additionally, there was a 12% conversion to xylose (Figure 4.3.g). MgO addition at 170°C had a negative effect on conversion to XOS for all three enzymes. For Accelerase XY enzyme, conversion to XOS was around 26% in 24h. Although there was an increase to 40%, a slow conversion rate in the first 8h of this condition showed the difficulty in the hydrolysis of this condition.

Veron 191S enzyme did not have high conversion rates on various biomass at 70°C, so the action of this enzyme is not effective on these conditions. The xylan conversion was under 5% with the biomass treated at 150°C for 1 h in the presence of 70% ethanol and the biomass treated under the same conditions but with MgO (Figure 4.3 b and e). On the other hand, there is a fair increase in XOS conversion when hydrolysis carried out with the biomass treated under 170°C, 1 h, 70% ethanol condition (Figure 4.3 k). The conversion to XOS reached 8 h was 47.5% and there was additionally 4% conversion to xylose. After that period, when the maximum conversion was achieved, XOS started to degrade slowly into xylose. However, the conversion to XOS was dropped to 8.36% when MgO was incorporated into the organosolv medium under the same condition (Figure 4.3 k). Despite this unexpected decrease in XOS conversion, its conversion pattern was similar to the one with no MgO added pretreatment. Eventually, this clear conversion difference between pretreatments at 150°C and 170°C showed how affective the lignin removal was on xylanase enzyme activity and xylan conversion to XOS and xylose.

Econase XT enzyme generally showed the highest conversion on all selected pretreated biomasses. While enzymatic hydrolysis patterns of pretreatments at 150°C with or without MgO addition showed slow hydrolysis, pretreatments at 170°C with or without MgO resulted in a quite fast hydrolysis (Figure 4.3.c, f, i and l). For 150°C, 1h, 70% ethanol condition, the conversion rate was only 2% in the first 2h. Then, it reached 28% in 8h and reached to a maximum conversion rate of 52% in 24 h. After that point, the conversion level remained the same. Although MgO added sample was yielded faster XOS formation than the not added sample, it was still slow. The conversion to XOS reached 70% in 24 h after reaching 29% in 2 h and 48% in 8 h. The conversion to xylose followed a similar pattern. For the sample treated under 150°C, 1h, 70% ethanol, with MgO, the conversion to xylose was 16% in 24h. Conversely, for 170°C, 1h, 70% ethanol condition, hydrolysis was fast. The conversion to XOS was 25% even in the first 2 h. Then it was followed by 63%, 73%, and 84% conversions in 8, 24, and 48 h, respectively.

Despite the same resistance in 170°C, 1h, 70% ethanol, MgO condition, it was still fast. The conversion to XOS was around 47% in 2 h and 8 h, then it became 52 and 62% in 24 and 48 h. The conversion to xylose was not fast for the samples treated at this temperature, but there was a consistent increase. Since there was no degradation from XOS to xylose monomer after achieving the highest XOS conversion, it can be concluded that the Econase XT enzyme did not show notable beta-xylosidase activity. This conclusion can be generalized for Econase XT in all conditions because the same result was also observed in other tests. On the other hand, exo-xylanase activity may have existed. There was not a distinctive difference in pretreated biomass when MgO added to the treatment at 150°C (approximately 40% lignin removal, 85% xylan recovery under both conditions). But, due to the difference at 150°C, it can be concluded that MgO addition affects the accessibility of the enzyme without dependency on xylan recovery or lignin removal. This phenomenon can be explained by the degradation of remaining cellulose crystals and cellulose-hemicellulose interaction after a certain amount of lignin removal (Avila et al., 2020).

As a result of this section, it was shown that commercial enzymes in various structures could produce XOS in different amounts from the same pretreated biomass. An increase in temperature in the pretreatments extended XOS production by each enzyme. The action of the MgO catalyst also depends on the effect of temperature on biomass. While xylose conversion was reduced with an increase in temperatures of pretreatment conditions, it was affected by different enzyme brands.

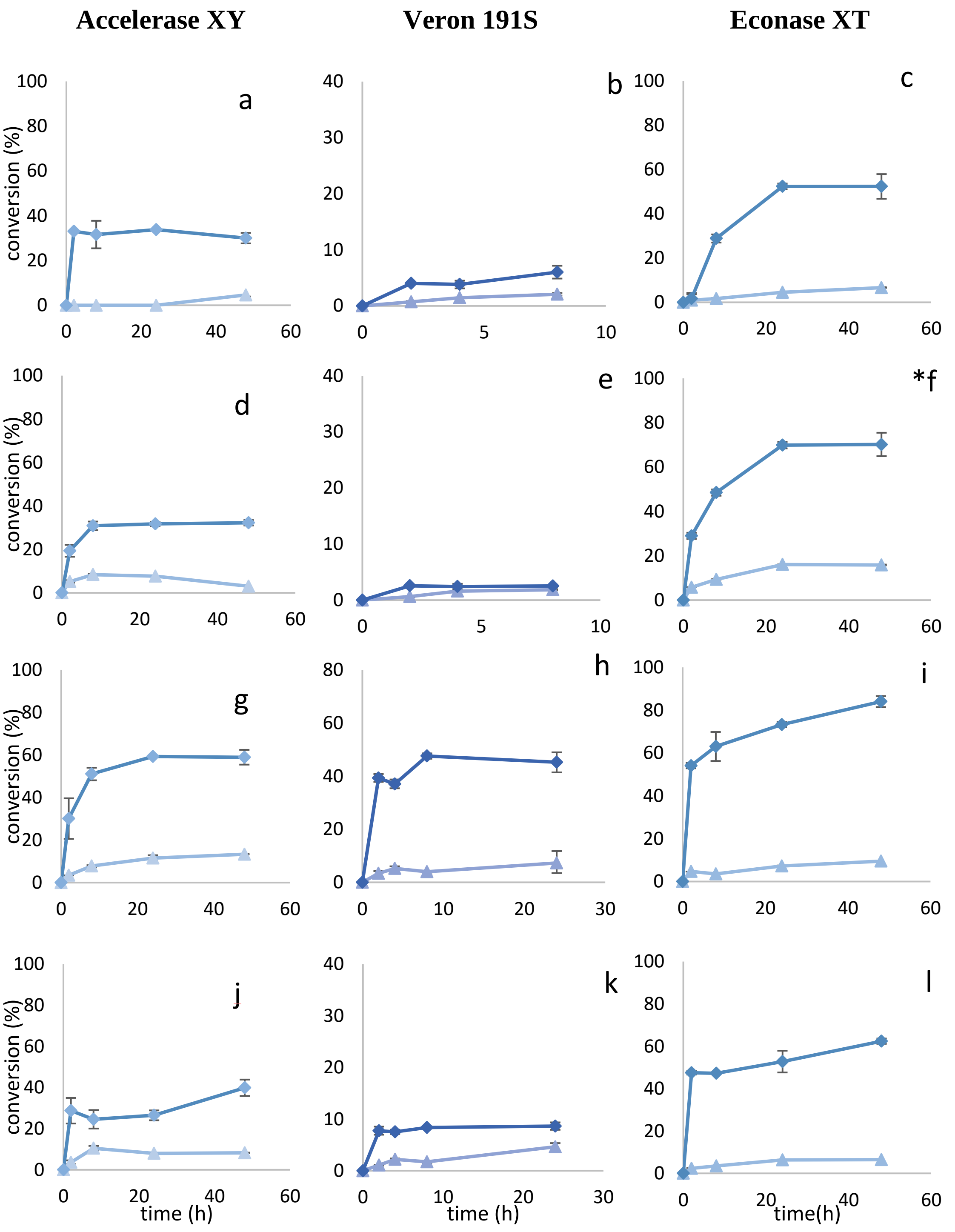


Figure 4.3. Effect of pretreatment conditions on enzymatic hydrolysis (T = 70 ° C).

Enzymes: left column: Accelerase XY; middle column: Veron 191S; right column: Econase XT

* is 5 replicated, others 2 replicated

▲: xylose; ♦: XOS; x: glucose. Pretreatment conditions:

a, b,c: 150°C 1h 70% ethanol;

d, e, f: 150°C 1h 70% ethanol-MgO;

g, h, j: 170°C 1h 70% ethanol;

j, k, l: 170°C 1h 70% ethanol- MgO

4.3.3. The Effect of Pretreatment Conditions on XOS Polymerization Degree

As examined in the last part, in addition to the effect of organosolv conditions on xylan recovery and lignin removal, variations in organosolv conditions also affected enzyme accessibility on lignocellulosic biomass and XOS production. In order to see this effect in more detail, the polymerization degrees of XOS solutions were determined for the selected conditions. The polymerization degree on XOS solutions is an important parameter because it has an impact on prebiotic activity. While oligosaccharides with a polymerization degree of 1-6 are considered as a low polymerization degree, it was observed that the prebiotic effect of low polymerization degree XOS was higher than high polymerization degree XOS (Childs et al., 2014).

When the biomass pretreated under 150°C, 1h, 70% ethanol, MgO condition was hydrolyzed with Accelarase XY at 70°C, a total of 2.73g/l of XOS was obtained in 24 h (Figure 4.4.a). Also, 0.72g/l of XOS was obtained as a low degree of polymerization (LDP) XOS, which was equal to 26% of total XOS. In the case of 170°C, 1h, 70% ethanol condition with the same enzyme, time and temperature, while 5.18 g/l total XOS was containing in liquor, 1.88 g/l of this XOS was LDP (36% of total XOS) (Figure 4.4.c). While 0.88g/l xylose was formed under 150°C, 1 h, 70% ethanol, MgO condition, 1.25 g/l xylose was formed under 170°C, 1 h, 70% ethanol condition.

When the same biomass was hydrolyzed with the Econase XT enzyme at 70°C, there was an obvious difference in XOS concentrations compared to Accelerase XY. XOS concentrations were around 10g/l under both of the conditions (Figure 4.4. b and d). The highest concentration was obtained in 24 h of hydrolysis as 10.9 g/l total XOS with the biomass treated under 170°C, 1h, 70% ethanol condition. While 64% (6.4 g/l) of XOS had a low degree polymerization, xylose concentration was 1.8 g/l. Total XOS was 9.5 g/l using the biomass treated under 150°C, 1h, 70% ethanol, MgO condition with 85% low degree polymerization (8.1 g/l). For this condition xylose amount was also 1.8g/l, which could be considered low compared to total oligomers.

In the comparison of Econase XT and Accelerase XY enzymes, it was concluded that Econase XT was capable of hydrolyzing xylan into a lower degree of oligosaccharides, whereas Accelerase XY yielded oligosaccharides with higher than six units. Econase XT enzyme specifically produced xylobioses as the oligomers. Almost all low degree oligomers were produced in the form of xylobiose in both the biomass pretreated under 150°C, 1h, 70% ethanol, MgO, and the biomass pretreated under 170°C, 1h, 70% ethanol conditions. Despite the xylobioses had also high ratio among low degree oligomers in Accelerase XY enzyme, there was an even distribution among low degree oligomers. The hydrolysis kinetics showed that all oligomers (xylobiose to xylohexaose) were formed simultaneously. In other words, XOS with a low degree of polymerization was not formed by the hydrolysis of XOS with a high degree of polymerization. This indicated that endo-xylanase was the main form of the xylanase active under the hydrolysis condition. For Accelerase XY enzyme, the biomass pretreated under 170°C, 1h, 70% ethanol condition provided a better XOS production mechanism. In addition to higher total XOS concentration, a low degree oligomer ratio was also more than the other conditions. Conversely, for the Econase XT enzyme, the biomass pretreated under 150°C, 1h, 70% ethanol condition showed more optimistic results.

Although total XOS concentration was slightly lower than other conditions, a high amount of low degree oligomers (85% of total oligomer) put forward150°C, 1h, 70% ethanol, MgO condition. Besides, since 150°C is more affordable in terms of energy consumption and equipment strength (because while pressure is around 5 bar in 150°C, it is around 10 bar in 170°C) than 170°C during organosolv treatment, there is one more reason to determine the biomass pretreated under 150°C, 1h, 70% ethanol, MgO condition for model XOS production.

There is more than one optimum case in all systems. In 150°C, 1h, 70% ethanol, MgO condition, hydrolysis rate is slower compared to 170°C, 1h, 70% ethanol condition. While 2.62 g/l/h per hour XOS was producing in the first two 2 hours with 150°C,1h,70% ethanol, MgO condition, this rate is almost double, 4.44 g/l/h per hour with 170°C,1h,70% ethanol condition. These rates were reduced at the end of 8h to 0.97 g/l and 1.2 g/l/h per hour, respectively. After that point hydrolysis rates reduced under 1g/l for both cases. At that point, 170°C, 1h, 70% ethanol condition also took attention due to total concentration and the speed of hydrolysis.

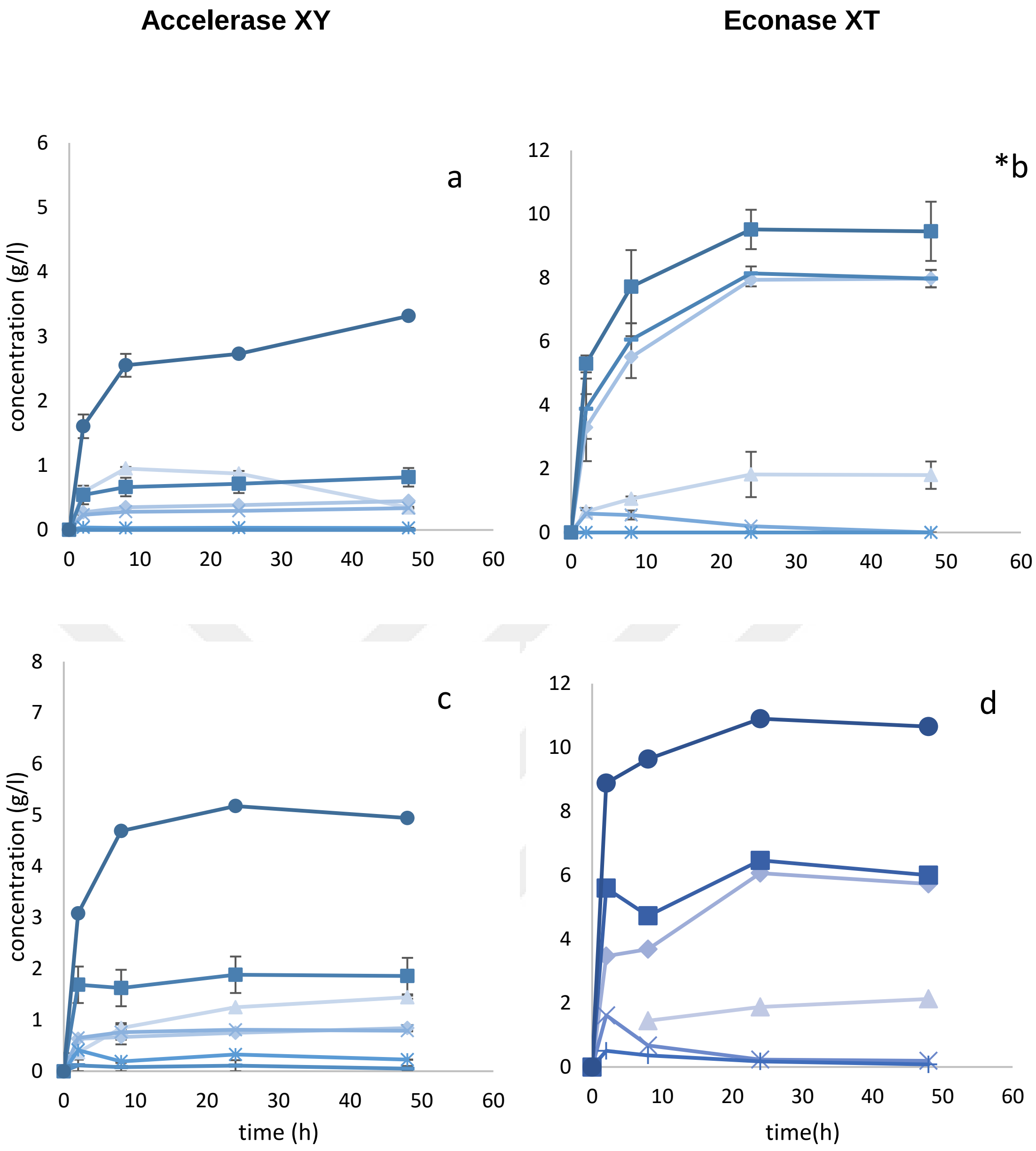


Figure 4.4. The effect of organosolv pretreatment conditions on the XOS polymerization degree.

Enzymes: left column: Accelerase XY; middle column: Veron 191S; right column: Econase XT

Pretreatment conditions: a, b: 150°C 1h 70% ethanol-MgO;

c, d, e: 170°C 1h 70% ethanol

•: total XOS; ■: total LDP XOS; ▲: xylose; ♦: xylobiose; x: xylotriose; │: xylotetraose

4.3.4. The Effect of Solid Loading on XOS Production

In many studies, 5% (w/v) (solid: liquid ratio of 1:20) or below solid loading has been used in the enzymatic hydrolyses (Quiñones et al., 2015; Akpinar et al., 2009b). An increase in solid loading can provide higher product concentrations in the enzymatic hydrolysate, in addition to a reduction in operational costs and energy consumption. However, hydrolysis efficiency might decrease in the case of high solid: liquid ratio. The reason for this reduction is generally related to the binding of lignin on the enzyme or low mass transfer rate and solubility (Gatt et al., 2019).

In this study, the biomass from the organosolv pretreatment at 150°C for 1 h in the presence of 70% ethanol and MgO was used to investigate the effect of solid loading. This biomass was loaded at 5%, 10%, and 20% and hydrolyzed at 70°C with the Econase XT enzyme. In 5% solid loading, 9.5 g/l XOS (70% conversion) was produced with 8.1 g/l low degree polymerized XOS, while 1.8 g/l xylose was released (Figure 4.5 a). When solid loading was increased to 10%, the total XOS was 8.3 g/l and xylose was 2.3 g/l. (Figure 4.5. b). That is, doubling in solid loading didn't provide an increase in product concentration. Since the XOS concentration was similar although the substrate concentration was two-fold, the conversion rate was almost half of the one obtained in the former case. Only a 30% conversion was achieved. Additionally, xylose concentration increased significantly. A decrease in mass transfer rate and the amount of available water might be the reason for this situation (Hodge et al., 2008; Kim et al., 2008). Similarly, in the case of increasing solid loading to 20%, 7.4 g/l total XOS was obtained, which equals to 13% XOS conversion. Also, xylose was produced in higher concentrations (3.1 g/l) in 24 h. Apart from those, the hydrolysis pattern was the same in all three conditions. A low degree of polymerization XOS dominated (mainly xylobiose) XOS solution was produced in all cases.

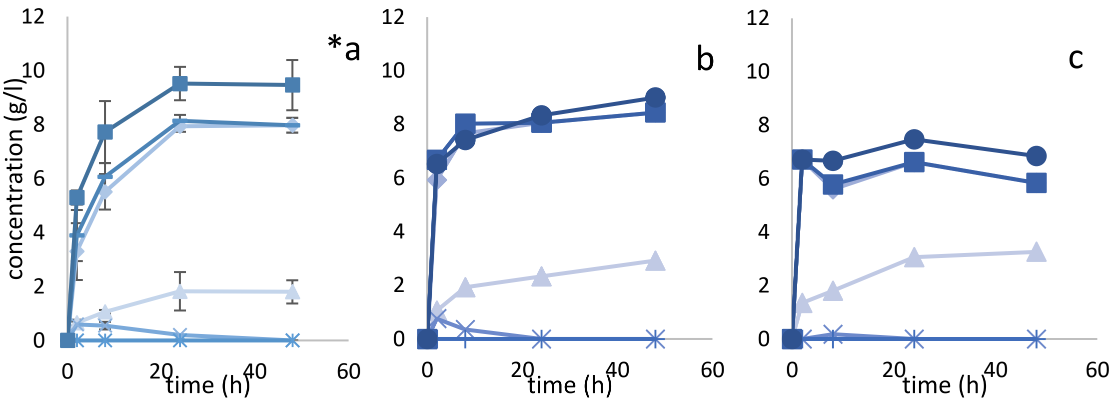


Figure 4.5. The effect of solid loading on XOS production.

(Hydrolysis of 150°C 1h 70% ethanol organosolved solid at 70°C with Econase XT enzyme)

Solid loading rates: a: 5%; b: 10%; c: 20%

* is 5 replicated, others 2 replicated

•: total XOS; ■: total LDP XOS; ▲: xylose; ♦: xylobiose; x: xylotriose; │: xylotetraose

4.3.5. The Effect of Enzyme Concentration on XOS Production

In order to examine the effects of the enzyme concentration on XOS production, 10% solid loading was used with the biomass which was organosolved in at 150°C for 1 h with 70% ethanol and 0.1 mol/g biomass MgO.

Econase XT enzyme was added to the xylanase activity of 0.1, 0.6, and 1 U/ml at 70°C. With 0.6 U/ml enzyme, which was the dose used in the previous tests in this study, 7.6 g/l LDP XOS and 2.2 g/l xylose, were produced in 24 h. The concentrations remained the same in the next 24 h (Figure 4.6.a). When the enzyme concentration was reduced six times (0.1 U/ml), 3 g/l LDP XOS U/ml (7.5 g/l total XOS) was obtained. While XOS concentration was 31% of the total solution, 27% of this volume was LDP. In this condition, although there is a low xylose concentration (0.84 g/l) as desired, low concentration in total XOS solution shows insufficient enzyme amount in the medium.

When the enzyme amount was increased to 1 U/ml, total XOS solution concentration remained at similar levels with 0.6 U/ml despite there was an increase in the conversion rate. Compared to 0.6 U/ml, LDP XOS concentration was slightly higher (7.93 g/l in 24 h) with 1 U/ml, while there was not a notable difference in xylose concentration. While conversion to total XOS was 43%, 80% of the total XOS consisted of oligomers with low polymerization degrees. Thus, increasing the xylanase concentration did not result in a considerable increase in XOS production under the conditions used in this study.

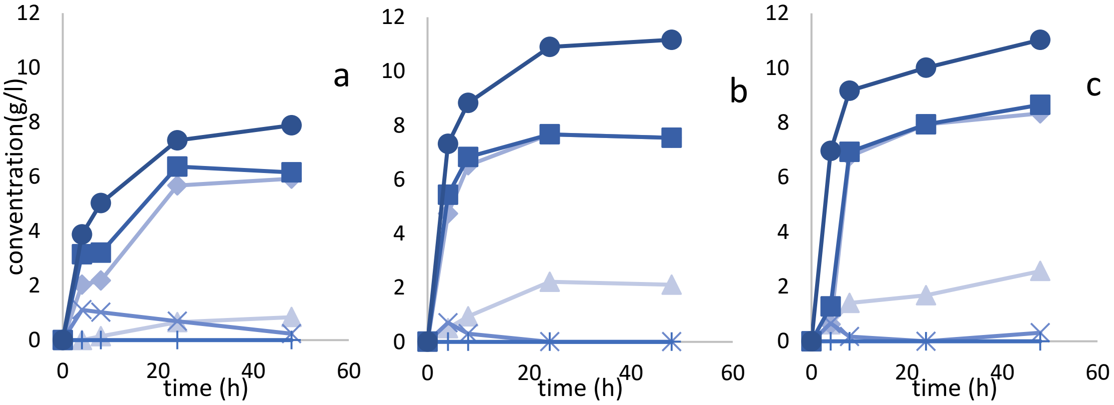


Figure 4.6. The effect of enzyme amount on XOS production.

(Hydrolysis was performed with Econase XT enzyme at 70°C on 150°C 1h 70% ethanol organosolved biomass.)

Enzyme activity: a: 0.1 U/ml; b: 0.6 U/ml; c: 1 U/ml

•: total XOS; ■: total LDP XOS; ▲: xylose; ♦: xylobiose; x: xylotriose; │: xylotetraose).

4.3.6. One Step Further: Xylose Production

D-xylose is the predominant pentose sugar found in the form of xylan polymer in lignocellulosic agricultural and food wastes (Kumar et al., 2018). Xylose is utilized for the production of industrial chemicals, such as xylitol and furfural. In addition to that, rare microorganisms and some genetically modified platform organisms could metabolize xylan into value-added bioproducts (Kwak et al., 2019). Thermal and chemical pretreatments, such as autohydrolysis, steam explosion, and dilute acid treatments break the majority of xylan in the lignocellulosic biomass into xylose (Venkateswar, et al., 2016). However, these methods suffer from the formation of carbohydrate degradation products and the release of extractives from the biomass. Thus, xylose containing streams are subjected to purification processes, when high purity xylose is required (Torres-Mayanga et al., 2019). Besides, the enzymatic approach is generally applied following alkali extraction of xylan, however, the requirement of alkali at high concentrations (5-10%) is the main drawback of that method.

Since pure XOS can be obtained with the improved method in this study, it is adapted for pure xylose production. That’s why a two-step xylose production method was repeated, based on organosolv pretreatment of biomass followed by enzymatic hydrolysis of xylan.

In this part, only Accelerase XY enzyme was employed at 55°C due to higher xylose production potential. The experiments were set with the same logic. First, determined pretreatments subjected to enzymatic hydrolysis. Then, successful pretreated biomass used in enzyme amount and solid loading optimizations.

As expected, biomasses had also different potential in xylose production. The different concentrations depend on temperature differences (Figure 4.7.a). But, MgO addition could create some minor differences in concentrations. On the other hand, reduction in conversion in XOS production due to the MgO addition at 170°C was not observed in this method. The highest concentration obtained from the biomass pretreated under 170°C, 1 h, 70% ethanol, MgO condition was 9 g/l xylose concentration in 24 h. Non- MgO added version was reached 8 g/l. Xylose production was gradually reduced with temperature and MgO addition. 6 g/l xylose was obtained using the biomass pretreated under 150°C, 1h, 70% ethanol, MgO; 4 g/l using the biomass pretreated under 150°C, 1h, 70% ethanol; and 3 g/l using the biomass pretreated under 130°C, 1h, 70%

ethanol. The condition that yielded the highest xylose concentration was selected for other steps.

Xylose concentration of 9 g/l obtained using the biomass pretreated under 170°C, 1h, 70% ethanol, MgO condition corresponded to 65% conversion (Figure 4.7.b). In an effort to maximize conversion, the enzyme amount was increased. However,), it caused to decrease in conversion Figure (4.7.b). When solid loading was increased to obtain a concentrated xylose solution, a similar decrease observed, as well. Minimizing in mass transfer and available water might be the reason for this reduction as mentioned in the XOS production part.

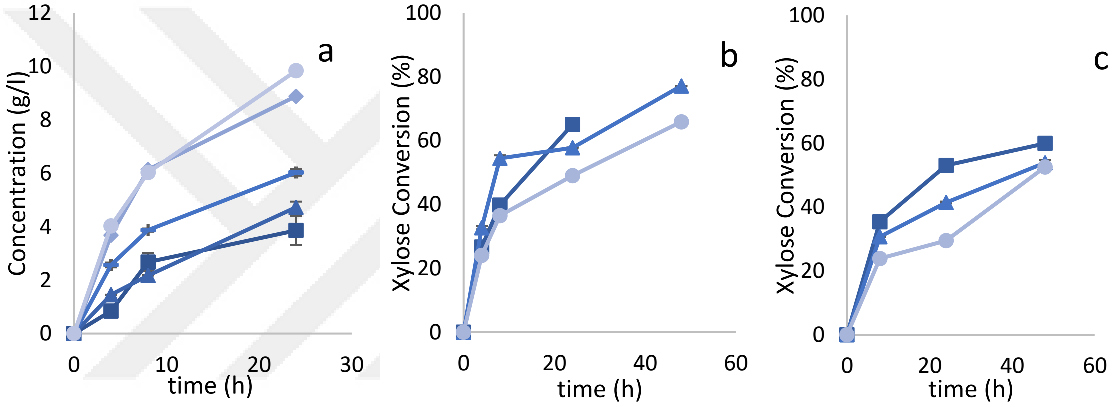


Figure 4.7. Xylose production.

(Hydrolysis was performed with Accelerase XY enzyme at 55°C.)

a: pretreatment effect: ■: 130°C 1h 70% ethanol,

-: 150°C 1h 70% ethanol-MgO,

•: 170°C 1h 70% ethanol- MgO

▲: 150°C 1h 70% ethanol,

♦: 170°C 1h 70% ethanol

b: enzyme loading effect

■: 250 U/ml; ▲: 500 U/ml; •: 1000 U/ml

c: solid loading effect

■: 5%; ▲:7%; •: 10%

CHAPTER 5

CONCLUSION

In this study, we applied the organosolv pretreatment method, which has been used to facilitate the enzymatic saccharification of cellulose, to the production of XOS. In this approach, the hydrolysis of xylan without extraction from biomass was aimed. In order to sustain this, partially disruption of lignocellulose was achieved with organosolv pretreatment.

Since the liquid phase used in the organosolv process consists of a high percentage (70%) of ethanol, recovery, and reuse of the solvent can be achieved with low energy consumption. This pretreatment could be applied at lower temperatures since it is more effective on biomass than autohydrolysis. Thus energy consumption and carbohydrates degradation are reduced. Indeed, in this study, organosolv was effective between 150°C and 170°C. Under these conditions, while lignin is partially separated from biomass, xylan and cellulose remained in biomass to a great extent. Thus, the lignin barrier, which is one of the obstacles to the subsequent enzymatic hydrolysis, was weakened. During the organosolv process, by adding MgO to the medium, it was possible to prevent the decrease in the pH of the medium. Thus, an additional barrier has been provided to prevent the removal and degradation of the xylan due to the acid catalysis at low pH. The effect of MgO was apparent especially in organosolv at 190°C. When ethanol concentration was reduced, xylan loss increased together with an increase in acidity due to an increased ionization rate of water.

The pretreatment results were examined in terms of delignification and xylan recovery, and potential pretreated biomasses were selected to be used in further enzymatic hydrolysis. They were treated with three commercial enzymes and the effect of temperature, time, enzyme amount, and solid loading were tested. As a result of enzymatic hydrolysis, the xylan in solid biomass was substantially converted to XOS. Thus, XOS production, the main target of the study, could be reached. Each enzyme was tested at different temperatures and the most favorable conditions for XOS production were sought. Econase XT enzyme efficiently increased the conversion yield of xylan to XOS. The temperature of organosolv pretreatment and the use of catalysts in the organosolv was shown to affect the functioning of the enzyme. This enzyme is advantageous for industrial processes because it is thermally resistant and can operate at high temperatures. Also, the antimicrobial requirement can be reduced during enzymatic hydrolysis since high temperature generally prevents microbial growth.

The xylose monomers present in the XOS solution obtained are considered as undesirable units because they do not show a prebiotic effect. Xylose units are released by the hydrolysis of XOS and/or xylan, so it reduces the yield of XOS production. Since LDP XOS has a molecular weight close to xylose, they require a complex downstream process for separation from the medium. The absence of xylose or presence at a low amount in the XOS solution eliminates this requirement. As a result of studying three different enzymes, the low xylose formation was shown and it was considered as a promising result for the further steps. Besides, the effect of temperature on xylose formation was also observed. Apart from that, finding xylanases that do not have exo-xylanase and β-xylosidase activities or development of xylanase complexes without these activities by synthetic biology methods may prevent the formation of xylose in XOS production.

In this study, after suspending biomass in the buffer, the xylanase enzyme was added and hydrolysis was performed. Since the substances that can be dissolved in water or alcohol in the biomass, should have been removed during the organosolv process, no extractives were expected to be present in the enzymatic hydrolysate. Thus, it was assumed that there were few or no by-products in the hydrolysate, which were needed to be separated by downstream processes. This is an important advantage of the process developed in this study. In the purification of XOS, the substances to be separated from the environment will be the protein (enzyme) and components of the buffer solution. When autohydrolysis is used for the production of XOS, degradation products such as furfural, hydroxymethylfurfural, acetic and formic acids are formed. Also, due to the high temperature, XOS is formed in a wide range of polymerization degrees. For these reasons, the purification step becomes difficult and costly.

Since cellulose and xylan remained largely in solid biomass during the organosolv conditions used in this study, the majority of the substance that passed into the liquid medium should have been lignin. When the ethanol in the medium is recycled, lignin can be concentrated in the remaining aqueous solution. In this way, lignin can be separated from this medium and purified. During enzymatic hydrolysis, xylan was separated substantially from the biomass. Thus, cellulose-rich solid biomass remains. Thanks to the good separation of lignin and xylan, it is possible to hydrolyze cellulose to glucose with cellulolytic enzymes. The process developed in the study by evaluating lignin and cellulose valorizations can be evolved into a bio-refinery approach.

It is possible to reveal new projects in light of the information obtained from this study. Firstly, the purification and characterization of the XOS produced under optimized conditions can be carried out. Obtained pure XOS can be added to the growth media of *Bifidobacterium* species to test the prebiotic potentials. Organosolv-Enzymatic hydrolysis system might be tested on other lignocellulosic biomass. Since each biomass has a different structure, environmental conditions are needed to be optimized.

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Using Cellulosic Derived Gluconic Acid as Efficient Catalyst. *Bioresource Technology* 289(159):4–7.

APPENDICES

Appendix A

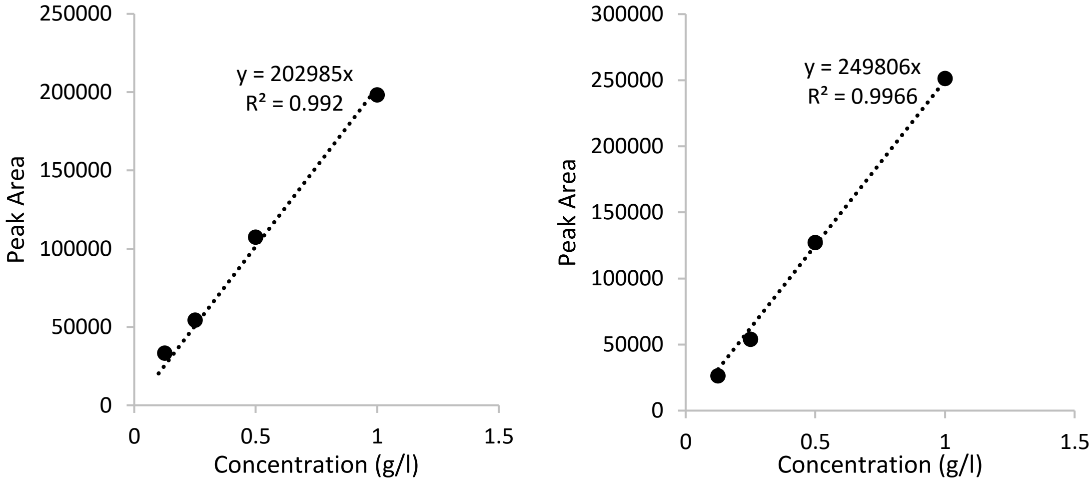

Figure A.1. Standard calibration curve of xylose and glucose in monosaccharide column for HPLC analysis.

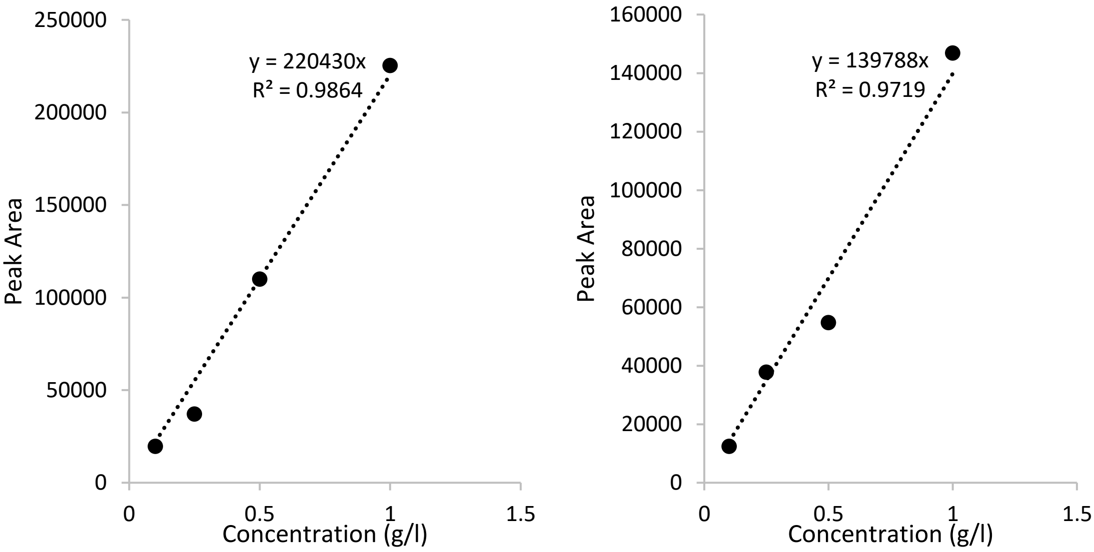

Figure A.2. Standard calibration curve of xylobiose and xylotriose in 42-A oligosaccharide column for HPLC analysis.

Appendix B: Comparision of XOS Production Methods

When this new method is compared to the current alkali treatment and autohydrolysis methods in terms of costs, environmental impact, and process time, the organosolv pretreatment method appears as a strong alternative method (Appendix B). Equipment cost is an important issue in alkali treatment because alkali-resistant equipment is required not only in reaction tank but also in all assistive equipment until neutralization of medium. Costs of chemicals and not reusability of these chemicals also increase the costs in alkali treatment. On the other hand, a pressure-resistant reaction tank increases the costs of autohydrolysis. Energy consumption is one other important cost in autohydrolysis. In organosolv pretreatment, there is still a certain amount of costs due to the pressure-resistant equipment and energy consumption during the process. However, these costs are relatively lower than the autohydrolysis due to the lower temperature and pressure. In alkali treatment, byproduct chemicals are a vital environmental problem. In autohydrolysis, there is a need for an additional purification step to separate toxic degradation products. This step both increases the costs of the process and prolongs the processing time. On the other hand in organosolv, there is not an additional step. This provides both lowers costs and decrease in process time. After this step, all three methods almost the same, intermediate products are washed to remove residues, that prevent enzymatic hydrolysis, and dried. Enzymatic hydrolysis is carried out in a shorter time at autohydrolysis because there are already long oligosaccharides obtained during treatment. This step is required to produce low degree oligomers, which have prebiotic potential. Then, enzymes are eliminated with a short ultrafiltration process and XOS solutions are obtained as the last product. At the end of the process, all three methods complete almost at the same time. While autohydrolysis and organosolv carry out in approximately 2 days, it is almost 2.5 days in the alkali treatment method.

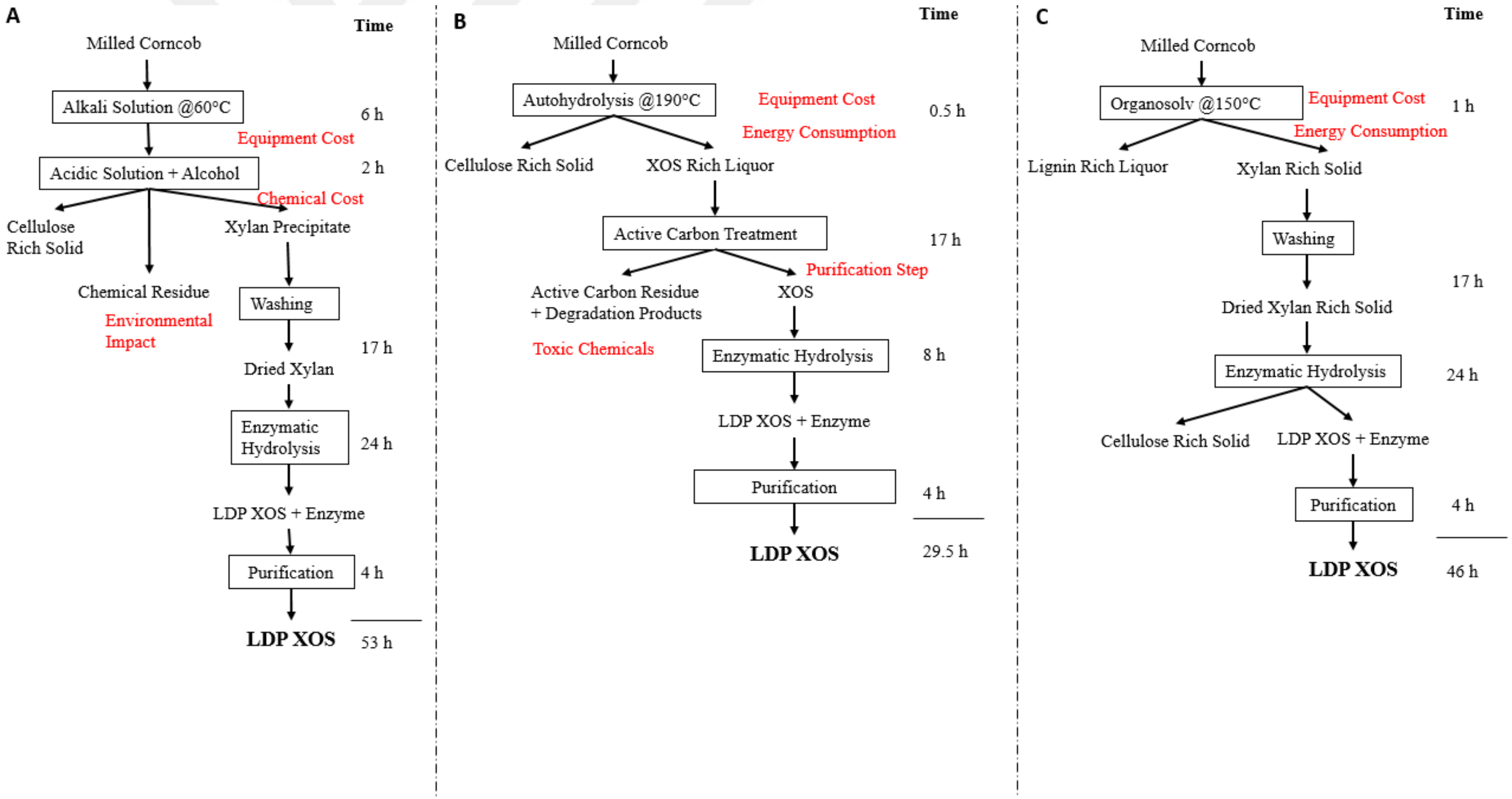

Figure B.1. Flowcharts of alkali extraction-enzymatic hydrolysis (A), autohydrolysis (B), and organosolv pretreatment-enzymatic hydrolysis (C) approaches for XOS production.