ENHANCEMENT OF XYLANASE ACTIVITY IN XYLOOLIGOSACCHARIDE PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

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ABSTRACT

ENHANCEMENT OF XYLANASE ACTIVITY IN XYLOOLIGOSACCHARIDE PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

Xylo-oligosaccharides (XOS) with prebiotic properties are obtained through xylan hydrolysis. Previously organosolv was found to be an effective pretreatment for XOS production from corncob. However, this process suffered from low XOS yields because of the limited hydrolysis of xylan in the pretreated biomass. This study was designed to test some approaches toward increasing the efficiency of xylanases on the organosolv-treated corncob.

The two commercial enzymes (Shearzyme 500L and Veron 191S) used in this study showed a synergistic effect yielding higher XOS compared to single enzyme application. Partial removal of acetyl groups on the xylan in organosolv-treated corncobs enhanced XOS production significantly. The solid loading above 10% decreased XOS yield as it resulted in a highly viscous slurry that may have limited heat and mass transfer. Fed-batch addition of enzyme and biomass did not play a role in the improvement of hydrolysis. In sequential batch mode, the residual enzyme activity from the previous batch could release more XOS from the fresh biomass, though the yield was low. Addition of fresh enzymes to the previous hydrolysate together with biomass provided a more concentrated XOS solution (15.4 g/l) after the second batch. The addition of surfactants into the hydrolysis media to prevent enzyme binding to lignin did not improve XOS production. This study showed that organosolv could be considered an effective treatment for XOS production from corncob and the enzymatic hydrolysis could be improved by optimizing the conditions.

ÖZET

LİGNOSELÜLOZİK BİYOKÜTLEDEN KSİLOOLİGOSAKKARİT ÜRETİMİNDE KSİLANAZ AKTİVİTESİNİN ARTTIRILMASI

Prebiyotik özelliklere sahip ksilo-oligosakkaritler (KOS), ksilan hidrolizi yoluyla elde edilir. Daha önce organosolv metodunun mısır koçanından KOS üretimi için etkili bir ön işlem olduğu bulunmuştur. Bununla birlikte, bu işlem, önceden işlenmiş biyokütlede ksilanın sınırlı hidrolizinden dolayı düşük KOS verimlerine sebep olduğu bilinmektedir. Bu çalışma, organosolv uygulanmış mısır koçanı üzerinde ksilanazların etkinliğini arttırmaya yönelik bazı yaklaşımları test etmek için tasarlanmıştır.

Bu çalışmada kullanılan iki ticari enzim (Shearzyme 500L ve Veron 191S),birlikte kullanıldığı koşullarda tek enzim uygulamasına kıyasla daha yüksek KOS konsantrasyonları ile sinerjik bir etki göstermiştir. Organosolv ile işlenmiş mısır koçanlarında ksilan üzerindeki asetil gruplarının kısmen uzaklaştırılması, KOS üretimini önemli ölçüde arttırdı. %10'un üzerindeki katı yükleme, sınırlı ısı ve kütle transferine sahip olabilen oldukça viskoz bir bulamaçla sonuçlandığı için KOS verimini azalttı. Enzim ve biyokütlenin kesikli ilavesi, hidrolizin iyileştirilmesinde bir rol oynamadı. Ardışık parti modunda, önceki partiden kalan enzim aktivitesi, verim düşük olmasına rağmen taze biyokütleden daha fazla KOS üretimini teşvik edebilir. Önceki hidrolizata biyokütle ile birlikte taze enzimlerin eklenmesi, ikinci gruptan sonra daha konsantre bir KOS çözeltisi (15,4 g/l) sağladı. Enzimin lignine bağlanmasını önlemek için hidroliz ortamına sürfaktanların eklenmesi, KOS üretimini iyileştirmedi. Bu çalışma, organosolv yaklaşımının mısır koçanından KOS üretimi için etkili bir işlem olarak kabul edilebileceğini ve enzimatik hidrolizin koşulları optimize ederek iyileştirilebileceğini gösterdi.

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CHAPTER 1

INTRODUCTION

Xylooligosaccharides (XOS) are sugar oligomers with xylose units linked together through β (1→4)-xylosidic linkages. These sugars occur naturally in various sources, such as bamboo shoots, fruits, vegetables, milk, and honey. They are industrially produced by hydrolyzing xylan, a component of lignocellulosic biomass (LCB). XOS has gained significant market value in recent years due to its prebiotic effects in humans and animals, even at low doses. It has been associated with various health benefits, including reduced blood cholesterol, increased calcium absorption, antioxidant effects, maintenance of gastrointestinal health, decreased risk of colon cancer, cytotoxic effects on leukemia cells, and potential benefits for individuals with type two diabetes mellitus.

In 2017, the primary use of XOS was in the feed industry, followed by health and medical products, food and beverages, and other applications. The growing interest in XOS is reflected in the increasing number study about this field. Additionally, utilizing lignocellulosic biomass for XOS production can contribute to sustainable development and the economy, considering the abundance of agricultural residues available worldwide. The prebiotics market, including XOS, will expand significantly in the coming years. Various companies have obtained safety certifications for XOS production, and the market price ranges from US $25,000 to US $50,000 per ton, depending on purity levels.

XOS production commonly involves hydrolyzing xylan from lignocellulosic biomass. However, the best methodologies, catalysts, and substrates for XOS production need investigation. Lignocellulosic biomass has a complex structure, so it needs pretreatment. Developing and selecting a pretreatment method play a critical role in the production process, as they must be feasible and cost-effective (Koppram et al., 2014).

These methods are typically used to disrupt the cell structure and make the substrate more accessible for subsequent enzymatic hydrolysis processes. Xylanase enzymes, which hydrolyze the bonds in xylan, are preferred for hydrolysis. Due to the complex structure of xylan polymer, multiple hydrolytic enzymes with different modes of action are required. These enzymes are commercially produced as a complex because they synergize. Therefore, the enzymatic hydrolysis conditions should be optimized for the desired product (xylose, short-chain XOS, long-chain XOS). For example, the efficiency of XOS production through enzymatic hydrolysis can be enhanced by optimization.

Within this project's scope, considering the limitations of alkaline and autohydrolysis methods in XOS production preferred, the organosolv method. Corn cob subjected to the organosolv process was further treated with deacetylation to remove acetyl groups, and efficient and high-concentration XOS production through enzymatic hydrolysis was investigated. Several factors that affect the optimization of the hydrolysis process, such as substrate loadings, enzyme loadings, inhibitors, and surfactants, were evaluated in this study.

CHAPTER 2

LITERATURE REVIEW

2.1. Lignocellulosic Biomass

Biomass is a renewable energy source that is sustainable, readily available, and environmentally friendly. Due to these benefits, the interest in obtaining energy and value-added products from biomass has increased in recent years. Agricultural food and forestry wastes are a potential source for obtaining value-added products because they are formed in high amounts. These materials can be used to obtain useful bio-based chemicals and fuels by going through various biotechnological processes (Arthur et al.,2018; Routledge et al., 2011; Silva et al., 2013; Saini et al., 2015)

Food and agricultural wastes are used in many ways, such as animal feed, fertilizer, energy, and heat. Using lignocellulosic wastes in producing value-added products creates an environmentally friendly and sustainable resource potential (Anwar et al., 2014). Global annual lignocellulosic biomass production is approximately 181.5 billion tons (Paul & Dutta,2018). The use of agricultural wastes consisting of lignocellulosic materials as biomass has advantages such as abundant raw material, a renewable resource, and cost-effective production (Brinchi, 2013). LB can be used second in the generation of biofuel productions. Some of these LB are rice straw, sugarcane bagasse, wheat straw, cotton stalk, switchgrass, bamboo, sugarcane tops, and paper waste.

Lignocellulosic biomass is an abundant and sustainable resource. Bioethanol, biomethane, biohydrogen, organic acids, lignocellulolytic enzymes, and other value-

added products have considerable potential for production in lignocellulosic biomass. Various agricultural residues such as deciduous and coniferous trees, paper industry waste, and energy crops are called lignocellulosic biomass.

Lignocellulosic biomass was composed of three natural structures cellulose (a carbohydrate polymer), hemicelluloses (another carbohydrate polymer), and lignin (an aromatic polymer). These three polymers are in tight and compact associations in the plant cell walls. The remaining content of lignocellulosic biomass includes proteins, fats, and ash (Peng et al., 2010). These polymers are the building blocks for various industrially highly valuable products because of their composition and structure differences. (Von Schenck et al., 2013; Schutyser et al., 2018; Yao et al., 2018; Lee et al., 2019; Collett et al., 2019); Zhao et al., 2020).

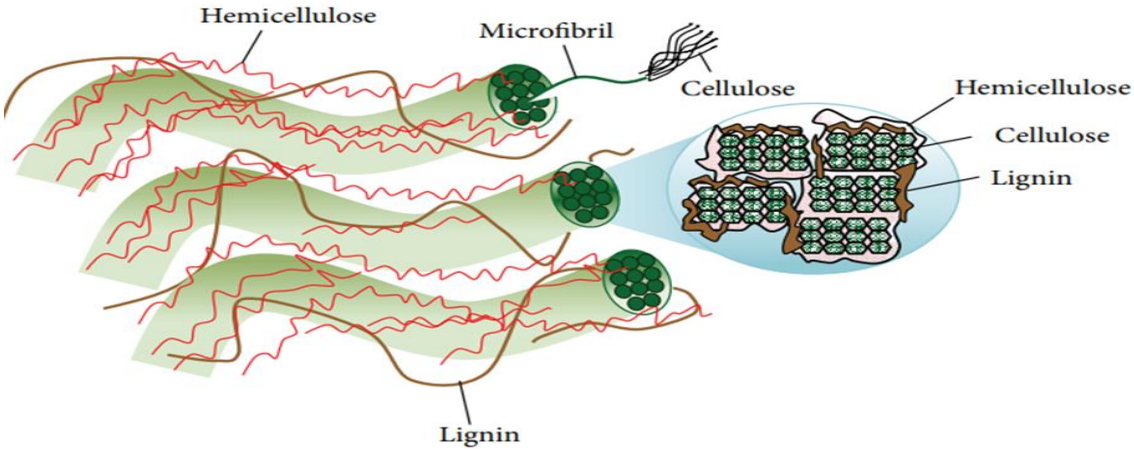


Figure 2.1. Lignocellulosic biomass structure (Doherty et al., 2011)

Technology for producing biofuels (such as ethanol, butanol, or various hydrocarbons) and biobased chemicals from lignocellulosic material is experiencing significant advances to meet global energy and chemical needs. Examples of lignocellulosic biomass materials considered feedstocks for bioethanol production include crop residues such as corn stover and wheat straw, woody residues from forest

thinning and paper production, cool and warm season grasses such as switchgrass and fescue, and crops such as sorghum (Sticken, 2008).

Enzymatic hydrolysis provides to convert lignocellulosic materials to fermentable sugar. The fermentable sugar is derived from cellulose and hemicelluloses in lignocellulosic materials.

2.1.1. Cellulose

Cellulose is an organic compound that is the most abundant polymer. It comprises unbranched chains of glucose molecules linked via β-(1→4) glycosidic bonds. Cellulose has an important role in plant wall structure and strength.

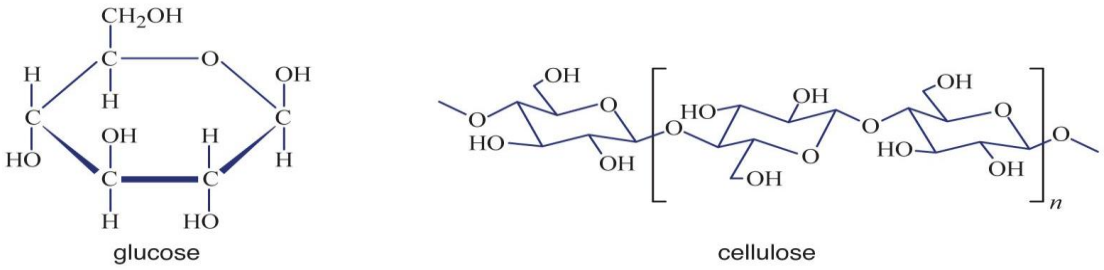


Figure 2.2. Cellulose structure

Cellulose is non-digestible for the human body and food for herbivores like cows, horses, and deer. They keep it long enough to be digested by microorganisms in the digestive tract; Like termites, protozoans in the guts of insects also digest cellulose. It is used in many applications in industry. Some of these are listed below. Cellulose has great economic importance. It is processed to produce paper and paper products and chemically modified to obtain substances used in the manufacture of products such as plastics,

photographic film, insulation paper, and biofuel. Other cellulose derivatives are used in food adhesives, explosives, thickening agents, and moisture-resistant coatings.

2.1.2. Hemicellulose

Hemicelluloses are heteropolysaccharides and have an amorphous structure containing pentoses (xylose, arabinose) and hexoses (glucose, mannose, galactose) that can be substituted by phenolic, uronic, or acetyl groups (Saha, 2003; Gong et al., 1981; Gírio et al., 2010).

The most common hemicelluloses are xylans and glucomannans; Hardwood hemicelluloses mostly contain xylan, while softwood hemicelluloses mostly contain glucomannans (Gírio et al., 2010). Xylans and mannans can be used to develop bioproducts from lignocellulosic biomass. Xylans are the main structure of the secondary cell walls found in herbaceous and hardwood plants. Xylan is made up of a xylose backbone with different substitutions. Xylan is composed of β-(1→4) D-xylopyranose with short branches of D-glucuronic acid, L-arabinose, D-galactose, L-galactose along with 4-O-methyl-ether-modified glucuronic acid. (Ebringerová et al., 2005; Saha, 2003; Ji et al., 2012; Gírio et al., 2010). While glucomannans have a backbone made up of both D-glucopyranose and D-mannopyranose, and both have a variety of possible branching carbohydrates, galactomannans, for example, only have a backbone made up of D-mannopyranose linked by β-(1→4) bonds. (Ebringerová et al., 2005; Willför et al., 2008).

Furthermore, galactomannans are typically branched, with galactose as the main branching component. Individual chains behave as random coils and, within lignocellulose, produce an aggregate network structure regardless of these monomeric variances. Large hemicellulose polymers (70 to 200 monomers, change to species) that establish interpolymer connections make up this network structure. The cellulose and lignin fractions of lignocellulose are also embedded in the network.

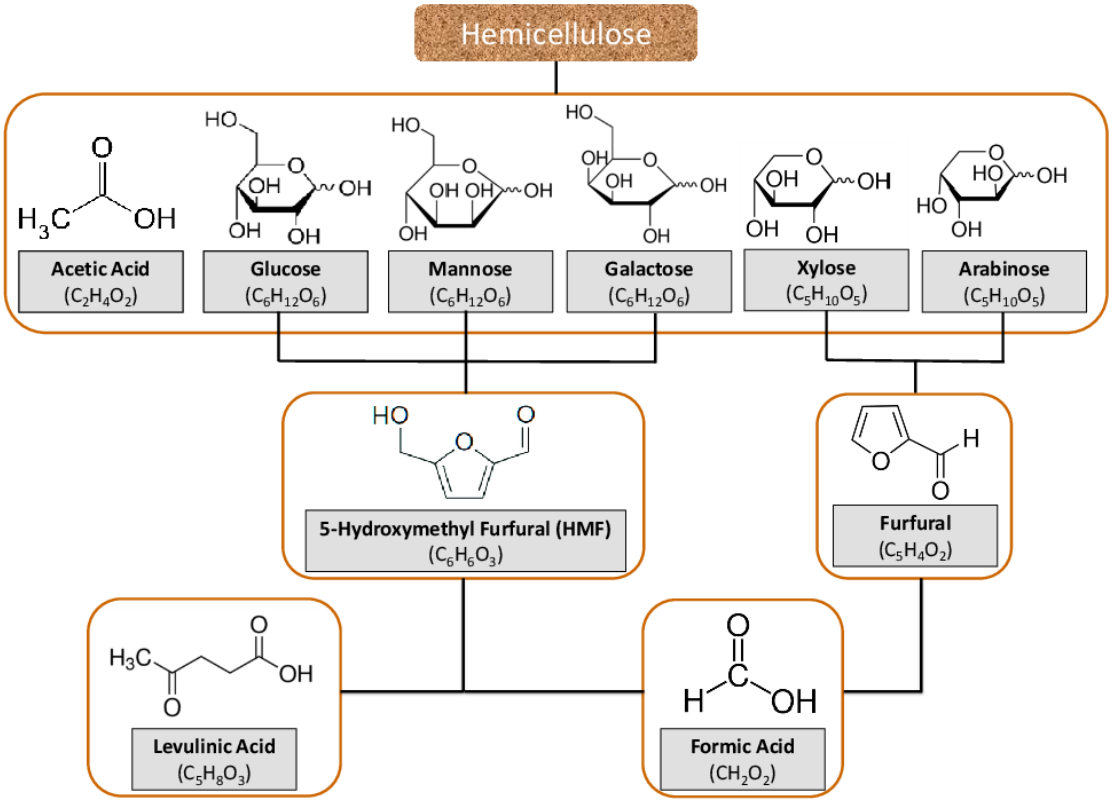


Figure 2.3. Products derived from hemicellulose degradations (Harahap, 2020).

2.1.3. Lignin

Lignin is a phenolic polymer that provides structural strength to the plant. Its structure is made of the condensation of phenylpropene units. P-hydroxyphenyl alcohol, guacyl alcohol, and syringyl alcohol are lignin precursors. Lignin holds the cellulose and hemicellulose fibers together, which functions as a glue in the cell wall structure . The key factor preventing biomass resistance during the separation process is the presence of lignin in lignocellulosic biomass. Lignin functions as a barrier to protect plant cells from microbial attack and permeability, preventing the destruction of plant cells (Sixta, H., 2006). Due to its complex structure of lignin isolation, characterization, and analysis

methods, it is not defined as a clearly defined compound like other carbohydrates, named as a class of natural phenolic polymers (Ghaffar & Fan, 2014).

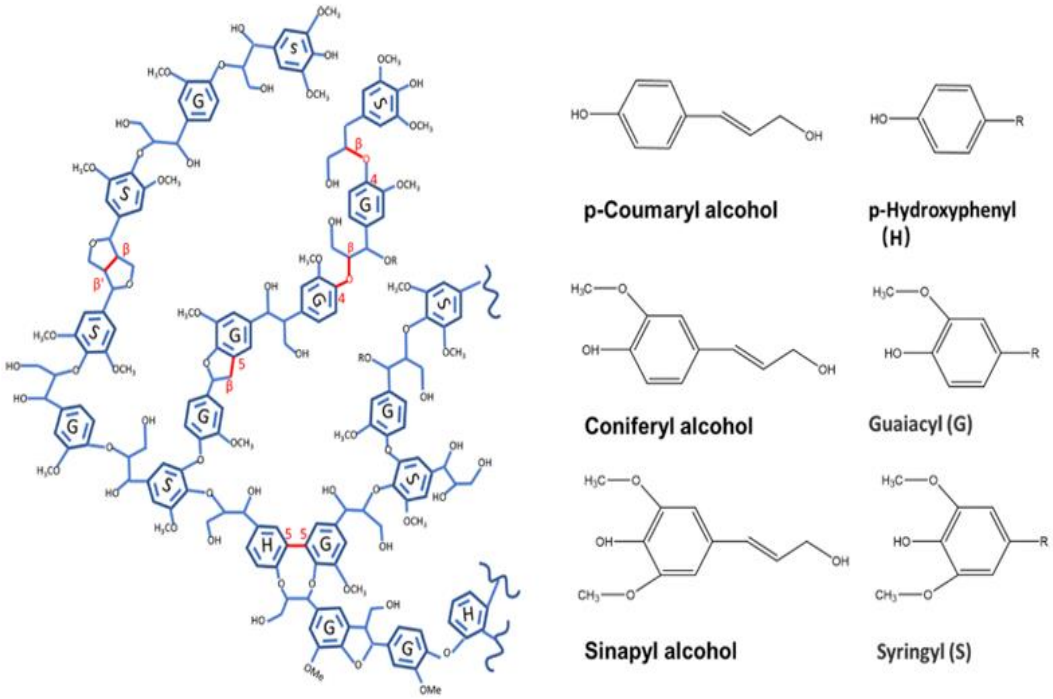


Figure 2.4. Chemical structure of lignin modified from Prieur et al., 2017 red linkage shows the β -O-4 , β -5, β – β'.

Agricultural wastes are among the lignocellulosic materials containing the lowest (3-15%), conifer bark the highest (30-60%) lignin. With the lignin structure, different products can be obtained like these (Holloday et al., 2007; Zhang et al., 2011). power, fuel, and syngas products, macromolecules, and low-molecular-weight aromatic or phenolic compounds. Lignin products' quality and parameters standards and control are difficult because lignin has a heterogeneous polymer.

2.2. Pretreatments

Lignocellulosic materials have complicated structures, with highly crystalline cellulose coated by lignin and hemicellulose. Due to cellulose's rigid affiliation with a three-dimensional complex lignin biopolymer, one of the issues with employing lignocellulosic materials for the value-added material is the low accessibility of cellulose to enzymes. Only up to 20% of the native cellulose (not altered or damaged by any pretreatment operations) is converted to sugars by these barriers, hemicellulose and lignin. (Salehi et al., 2012; Mood et al., 2013; Kumar et al., 2009).

Pretreatment is crucial for the efficient release of sugars from the lignocellulosic material. Pretreatment breaks down the lignin structure and disrupts the crystalline structure of the cellulose fibers, thereby making biomass accessible to hydrolyzing chemicals or enzymes. Hemicellulose can be hydrolyzed to sugar components by physical, chemical, and enzymatic processes. Chemically acting on hemicelluloses is easier than hydrolysis of cellulose due to the heterogeneous structure and composition, low degree of polymerization, and low crystallinity. The first step of hemicellulose-based fuel or chemical production is lignocellulose pretreatment, and different pretreatment methods have been developed.

The pretreatment process aims to modify these properties to prepare enzymatic degradation materials. The pretreatment required is important because lignocellulose is so complex (Kristiani et al., 2013).

Various pretreatments exist for the selective fractionation of hemicelluloses from biomass. These pretreatments can be classified into four main groups based on physical, chemical, physicochemical (combined), and biological techniques. These can be used individually or in combination (Beheraet et al., 2014). These techniques include the use of acids, water (liquid or steam), organic solvents, and alkaline agents (the techniques used are listed in the figure) (Lee et al., 1999; DOE US 2006; Mosier et al., 2005).

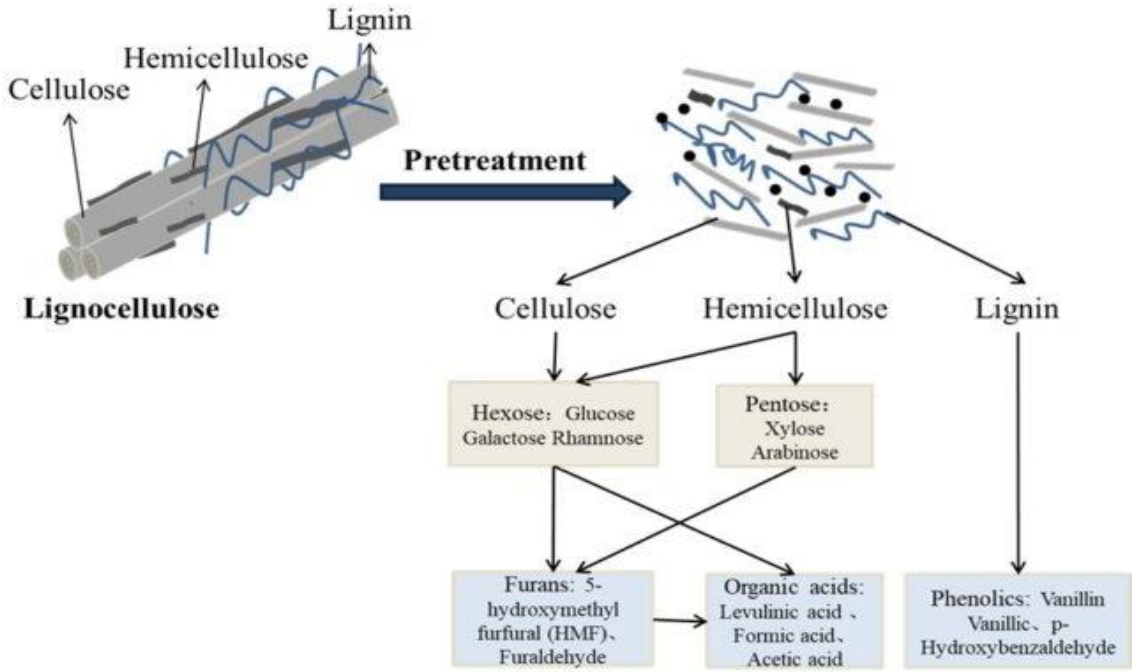
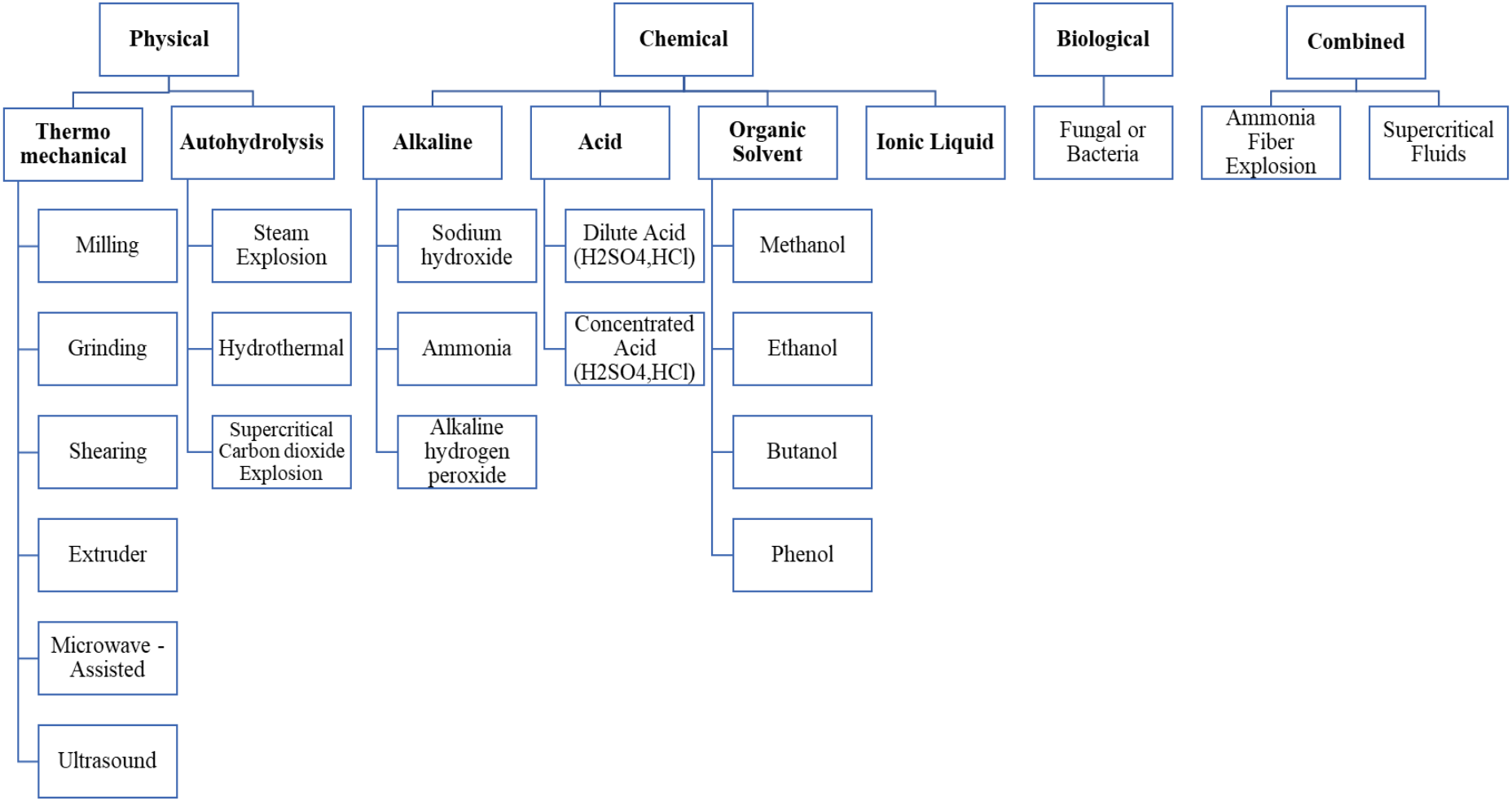


Figure 2.5. Pretreatment of lignocellulosic material, schematic representation of lignocellulosic biomass pretreatment (adapted from Luo et al., 2022).

Alkaline and organic solvent approaches are not selective towards hemicellulose because they also remove lignin, which can inhibit the fermentation or bioconversion process of hemicellulose sugars. Determining the process to be used has great importance on the efficiency and productivity of the process. Depending on the pretreatment and application conditions, hemicellulose sugars can be broken down into weak acids, furan derivatives, and phenolics during pretreatment (Larsson et al., 1999). These compounds can hinder further fermentation, leading to lower yields and productivity. Raw material characteristics, such as the proportions of cellulose, hemicellulose, and lignin, production capacity, and intended product types, are considered when choosing these pretreatment techniques (Wang et al., 2012).

Pretreatment strongly affects downstream costs by determining fermentation toxicity, enzymatic hydrolysis rates, enzyme loadings, and other process variables (Wyman et al., 2005). Therefore, it is important to consider the effect of the selected process on the whole condition and the key step limiting the process.

Figure 2.6. Pretreatments methods (Adapted from Volynets et al., 2017).



2.2.1. Milling

This pretreatment is a mechanical pretreatment in which milling breaks the lignocellulosic biomass into smaller pieces. A mechanical pretreatment aims to reduce particle size and crystallinity. Reducing the particle size increases the available specific surface and decreases the degree of polymerization (DP) (Palmowski and Muller, 2000) Milling also causes the shearing of biomass. Depending on the type of biomass, grinding type, and processing time, an increase in specific surface area and a decrease in DP are the factors that increase the total hydrolysis efficiency of lignocellulose (Hendriks & Zeeman, 2009). It also reduces the technical degradation time during hydrolysis by 23-59% (Delgenés et al., 2003; Hartmann et al., 2000). Thus, the rate of hydrolysis is increased.

2.2.2. Acid Pretreatment

Acid pretreatment can occur at a high temperature and low or low temperature and high acid concentration. While acid pretreatment can be used for inorganic acids such as nitric, hydrochloric, and phosphoric, the most common is sulfuric acid. Temperature, acid type, concentration, solid-liquid ratio, and other factors affect acid pretreatment. High-temperature pretreatment of biomass can enhance enzymatic hydrolysis (Taherzadeh & Karimi, 2007). However, pretreatments at high acid concentrations are important, as this operation can be corrosive and dangerous. Therefore, special construction or expensive equipment is needed. (Wyman, 1996; Sun et al., 2004).

Diluted acid hydrolysis is one of the most widely used methods. This method is preferred for large-scale production as diluted acid pretreatment produces less degradation than concentrated acid pretreatments (Wyman et al., 2005; Pingali et al.,

2010). There are some restrictions like the formation of undesirable and inhibitory by-products such as furanic aldehydes, weak acids, and phenolic compounds (Palmqvist & Hahn-Hagerdal, 2000b; Thomsen et al., 2009; Klinke et al., 2004). These undesirable by-products inhibit the hydrolytic enzymes that release sugars in the hemicellulose structure and the growth and metabolism of microorganisms used in the subsequent fermentation process. (Heer & Sauer, 2008; Jing et al., 2009; Klinke et al., 2004; Palmqvist & Hahn-Hagerdal, 2000b).

These compounds have antagonistic roles in the downstream processes. Furfural and 5-hydroxymethyl furfural (HMF) cause reduce the process yield and productivity due to lignocellulosic hydrolysate (Almeida et al., 2007; Thomsen et al., 2009). The acid dose, application temperatures, and process time affect the formation of by-products. Therefore, preventing inhibitor formation during biomass pretreatment is vital (Wierckx et al., 2011; Kumar et al., 2009).

2.2.3. Organosolv Pretreatment

Organosolv pretreatment is extracting lignin from lignocellulosic raw materials using organic solvents or their aqueous solutions. Pretreatment with an organic solvent ensures that high-purity cellulose is isolated with minimal degradation (Zhang et al., 2016). While most lignin and hemicellulose are dissolved in organic solvents, cellulose is recovered as solids. As a result, removing lignin and hemicellulose increases cellulose surface area, reduces lignin resistance, and increases enzymatic accessibility to hydrolysis, thus improving the yield of bioethanol produced during fermentation (Koo et al., 2011). Organosolv pretreatment is an environmentally friendly technology that shows great promise in biomass processing. It involves using solvents to penetrate the pores of biomass, resulting in the solubility of lignin and facilitating the physical breakdown of the lignocellulosic structure (Taherzadeh & Karimi, 2008; Zhang, Pei & Wang, 2016). When compared to acid-catalyzed saccharification, Pye et al., (2008) research showed

that pretreatment with organic solvents increased cellulose saccharification yields and conversion rates with the application of organosolv, the main components of the biomass can be efficiently fractionated and recovered in separate process streams. The cellulose in the biomass remains in the solid phase and can be converted into biofuels and other valuable chemicals. Hemicellulose, which can be converted to bioethanol, biobutanol, and xylitol, is partially hydrolyzed. Phenolic and/or epoxy resins, polyurethane and polyurethane foam, and antioxidants can be developed by precipitating high-purity lignin products from the liquor (Carvalho et al., 2013). In addition, high-quality lignin contains superior properties such as no sulfur, narrow molecular weight, and water repellency. Because of these properties, they can be turned into high-value products such as veneers, construction, plywood, specialty adhesives, and resins (Arato et al., 2005; Zhang et al., 2016).

Organosolv pretreatment using various organic solvents has been developed to improve the enzymatic digestibility of lignocellulosic biomass (Zhang et al., 2016; Zhang et al., 2016b). Some of these solvents are classified into two groups. Lower boiling points of methanol and ethanol, and higher boiling points of ethylene glycol, glycerol, tetrahydrofurfuryl alcohol, and other classes of organic compounds, dimethyl sulfoxide, and ethers, can be used as solvents in the process. The most common solvent is ethanol for the pretreatment of biomass because ethanol is low price, has good solubility of lignin, no toxicity, miscibility with water, and recovers easily. (Zhao, et al., 2017; Yáñez et al., 2013; Yáñez et al., 2014).

Lignins and hemicelluloses are typically selectively removed during ethanol-organosol pretreatment, but most cellulose is kept in the substrate for enzymatic hydrolysis. (El Hage et al., 2010; Hallac et al., 2010; Yáñez et al., 2013) Organic solvent pretreatment shows higher efficiency for hemicellulose fractionation compared to conventional processes. (Zhao et al., 2009).

Organosolv pretreatment can be performed with or without additional catalysts in the 100–250°C (Muurinen, 2000). If pretreatment is done at high temperatures (185–210°C), adding acid is unnecessary as it is believed to act as a catalyst for lignin-degradation of organic acids released from the biomass. Carbohydrate complex (Duff & Murray, 1996). If acid catalysts are added, the delignification rate increases, and higher xylose yields are obtained. In addition, the organic solvents used can be recycled to reduce

costs. In addition, solvents must be removed from the system because solvents can inhibit the growth of organisms through enzymatic hydrolysis and fermentation. Therefore, most processes involve the washing step (Zhao et al., 2009).

Organosolv pretreatment advantages and disadvantages showed in the Figure 2.8.

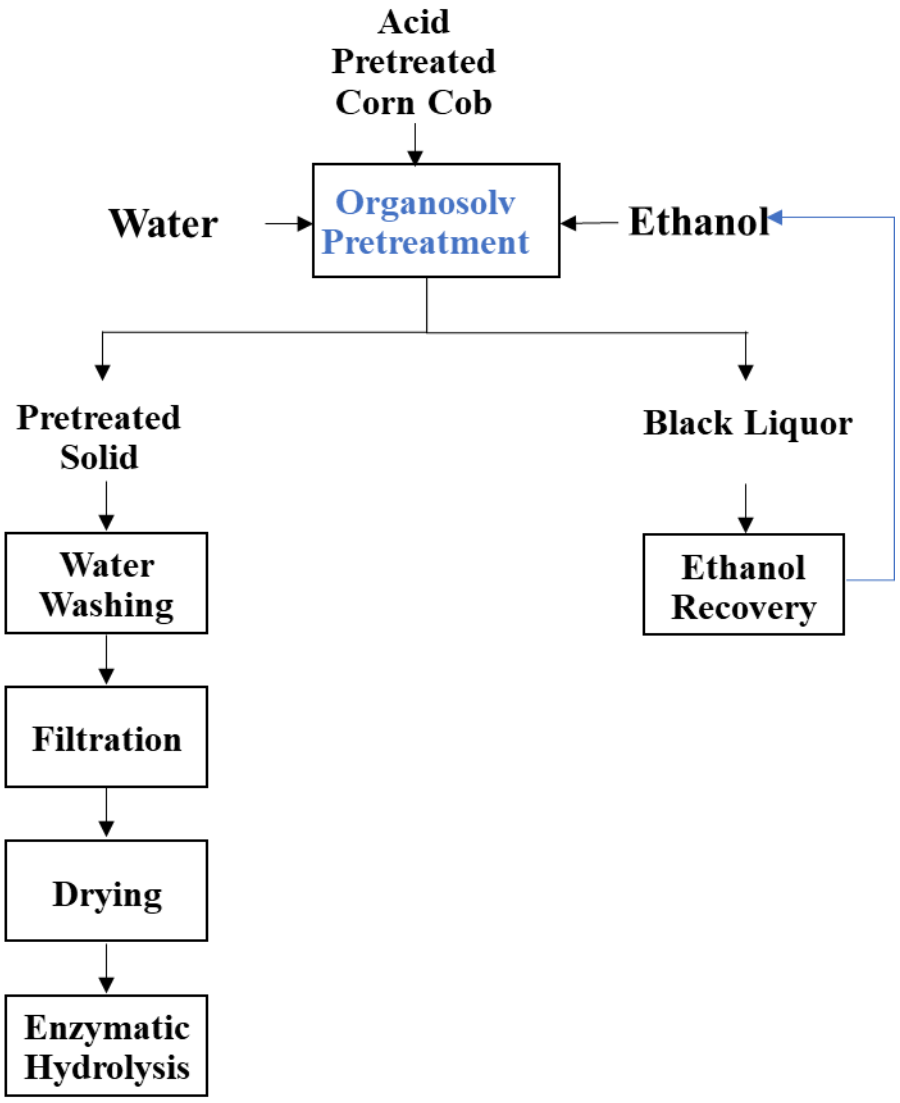


Figure 2.7. Organosolv pretreatment procedure

Advantages	Disadvantages
- Woody and nonwoody biomass could be used - Lignin removing increases the enzymatic hydrolysis yield - Water, energy, and reagent requirement is less than other pretreatment methods	- High solvent consumption lead to solvents have to be recovered - Volatile materials used so dangerous process, attention need for exploison and fire - Energy consumption for recovery

Figure 2.8. Organosolv pretreatment advantages and disadvantages

2.2.4. Deacetylation Pretreatment

Deacetylation is a reverse reaction that removes the acetyl group from the molecule. Since acetyl groups may decrease the activity of the xylanase enzyme, it may cause a decrease in the efficiency of enzymatic hydrolysis (Chang & Holtzapple, 2000). Therefore, removing acetyl groups from the backbone of xylan in the feedstock by deacetylation can increase xylan digestibility and enzymatic hydrolysis of cellulose (Chen et al., 2012). A deacetylation step using a diluted alkali solution is advised to release acetyl groups from the xylan backbone and facilitate enzymatic hydrolysis (Li et al., 2016; Chen et al., 2019). Reasons for improved biomass saccharification by deacetylation are given below.

First, deacetylation reduces the degradation of hemicellulose and the formation of inhibitors (Chen et al., 2019; de Assis Castro et al., 2017). Thus, less inhibition of cellulose hydrolysis occurs. Deacetylation increases the susceptibility of xylan to enzymatic degradation (Wu et al., 2020; Wang et al., 2019; Hu et al., 2018), which may reduce biomass resistance and plays a role in deacetylation partially removing lignin and ash, increasing biomass-specific surface area, or introducing significant nanoscale porosity to biomass, Tang et al., 2018; Wang et al., 2019; Lima et al., 2019) thereby promoting the accessibility of cellulose. Deacetylation reduces acetic acid production

during the pretreatment (Kuhn et al., 2020), which lowers the production of pseudo-lignin and lignin droplets on the fiber surface.

2.3. Enzymatic Hydrolysis

A complex heterogeneous catalytic process, enzymatic hydrolysis involves several processes, such as reaction kinetics and molecular mass transfer kinetics (Zhang et al., 2021). Numerous experimental studies have been conducted by researchers to optimize the conditions to increase conversion efficiency and lower cost. Many factors affect enzymatic hydrolysis. These can be diversified as enzyme-related, substrate-related, hydrolysis conditions, inhibitory effects, and surface-active additives. (Amit et al., 2018). These factors significantly affect the rate and effect of hydrolysis, so optimizing these parameters determines the effect of enzymatic hydrolysis. Because these factors are all interrelated, repeated testing is needed to identify conditions.

Enzymatic hydrolysis is a more popular method in the food industry because it provides a controlled process and it, does not produce undesirable by-products, or produce high amounts of monosaccharides, and does not require special equipment that can be operated under high temperatures or pressure (Aachary & Prapulla, 2011; Akpinar et al., 2009). Research on XOS production to improve the existing systems has focused on applications with enzymatic hydrolysis (Amorim et al., 2019). The digestibility of enzymatic hydrolysis can be improved using various pretreatment systems (Pihlajaniemi et al., 2016). Enzyme loading plays an important role in influencing enzymatic hydrolysis. However, the high cost of enzymes has prompted research to explore techniques that can adequately activate the enzyme or reduce enzyme load while keeping enzymatic digestibility at the same level (Zhang et al., 2018).

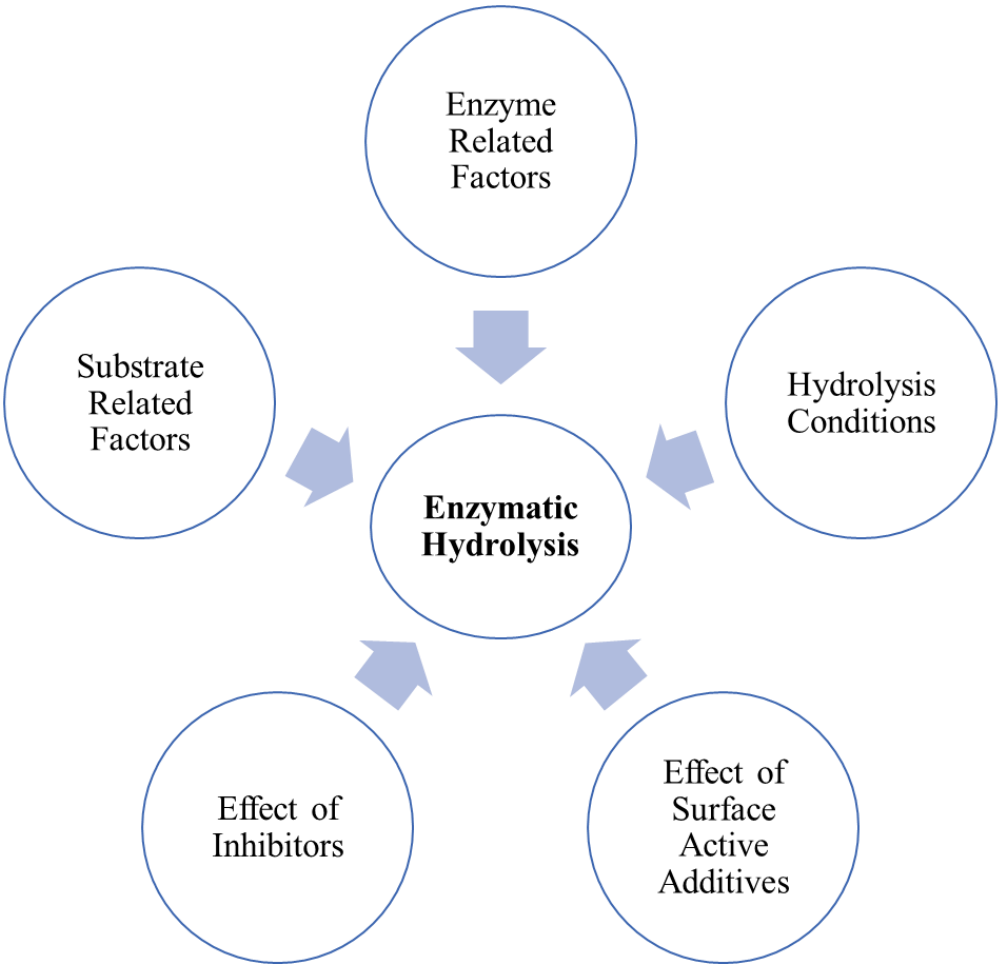


Figure 2.9. Effecting Factor of Enzymatic Hydrolysis

Xylanase enzymes play a vital role in xylan hydrolysis to produce XOS. Literature studies focus on selecting suitable xylanase enzymes, optimizing enzyme dosage and reaction conditions (pH, temperature, substrate concentration), and investigating the hydrolysis kinetics. Endo-xylanases, which hydrolyze the β-1,4 glycosidic links in xylan, are responsible for the enzymatic synthesis of XOS. To prevent the release of xylose, which can limit endo-xylanase activity, xylanase preparations with low exo-xylanase activity and/or β-xylosidase activity are desirable (Vázquez et al., 2000; Escarnot et al., 2012). To obtain a good XOS yield and enable large-scale production, it is crucial to evaluate its use in commercial xylanase hydrolysis of xylan and to understand its xylanolytic activities, optimum pH and temperature, thermal stability, and kinetic properties.

2.3.1. Batch Hydrolysis

Batch enzymatic hydrolysis is a process used to break down complex biomaterials, such as cellulose or hemicellulose by enzymes. It involves mixing the biomaterial with an enzyme solution in a batch reactor and allowing the enzymatic reaction to occur over a specified period. During the process, the enzymes catalyze the hydrolysis of the biomaterial into smaller parts. Thus, they break down into smaller components such as sugars or oligomers. The reaction conditions like pH, temperature, and reaction time, are carefully controlled to optimize the efficiency of the enzyme and achieve the desired level of hydrolysis.

Batch enzymatic hydrolysis has some advantages. It is a relatively simple and cost-effective method that can be easily scaled up for large-scale production. It allows for precise control over reaction conditions, enabling researchers to study the effects of different parameters on the hydrolysis process. Additionally, batch hydrolysis can be performed with a variety of enzymes, allowing for customization based on the specific material and end products. However, batch enzymatic hydrolysis has some limitations. The reaction progress is typically slower compared to other hydrolysis methods, which can prolong the overall processing time. The presence of inhibitory compounds or enzyme deactivation over time may also affect the efficiency of the hydrolysis reaction. Therefore, optimization of enzyme dosage, reaction conditions, and process parameters is necessary to maximize the yield and efficiency of the hydrolysis process.

Solid loading in the process has a significant impact on capital and operating costs (Modenbach & Nokes, 2013). High doses of expensive commercial enzymes are often required to achieve high conversion efficiencies from biomass (Leathers, 2003; Chundawat et al., 2008; Qing et al., 2010). Therefore, optimization of the enzymatic hydrolysis step is a key goal in the cost-effective production of value-added from lignocellulosic source. With high solids loading in the process, it serves to cost-effectively produce high XOS concentrations and minimize water usage (Hodge et al, 2008; Kristensen et al., 2009; Leathers, 2003; Chundawat et al.,2008; Qing et al., 2010; Hodge et al., 2008; Kristensen et al., 2009).

Considering all these reasons, the advantages and disadvantages of high solid loading are listed below.

Low solids loadings cause losses in terms of production capacity, time and cost to produce the product at low concentrations. On the other hand, running the hydrolysis at high solids loading may create some technical difficulties due to the high initial viscosity of the material, which makes mixing difficult and causes mass and heat transfer problems (Hoyer et al., 2013; Joy et al.,2022).

2.3.2. Fed-Batch Hydrolysis

Fed-batch enzymatic hydrolysis involved the incremental addition of biomass and enzyme throughout the reaction to maintain optimal conditions and improve the efficiency of the hydrolysis process. In contrast to conventional batch hydrolysis, where all components are added at the start, fed-batch hydrolysis enables controlled addition of the biomass and enzyme during the reaction. This approach overcomes challenges such as substrate inhibition and enzyme deactivation that can occur in the early stages of the process (Gong et al., 2020; Xue et al., 2015). By adding the biomass and enzyme incrementally and in a controlled manner, the reaction conditions remain favorable, allowing the hydrolysis process to proceed at the desired rate. This method improves enzyme utilization and enhances the overall yield of desired products, such as sugars or oligomers. Fed-batch enzymatic hydrolysis offers several benefits. It mitigates the negative impacts of substrate inhibition and enzyme deactivation, resulting in enhanced efficiency and higher yields. Moreover, it provides flexibility in controlling reaction parameters and facilitates process optimization. However, the successful implementation of fed-batch hydrolysis requires careful monitoring and control of addition rates to maintain desired conditions throughout the reaction. The complexity of the process and the need for precise control may present challenges in terms of process optimization and scalability (Liu et al., 2015; Gong et al., 2020).

2.3.3. Xylanases

Xylanase enzymes are classified as endo-acting enzymes and belong to the glycoside hydrolase enzyme family. Xylanase enzymes play a crucial role in the hydrolysis of xylan, a polysaccharide found abundantly in lignocellulosic materials. Their enzymatic action breaks down the β-1,4 glycosidic bonds of xylan molecules, producing xylooligosaccharides (Carvalho et al., 2013).

The catalytic mechanism of xylanase enzymes involves binding the enzyme to the xylan substrate and the subsequent hydrolysis of the glycosidic bonds. This process occurs within the enzyme's three-dimensional structure, where a catalytic site facilitates the cleavage reaction, enabling the internal breakdown of xylan (Carvalho et al., 2013). The optimal activity of xylanase enzymes often requires the presence of cofactors, such as calcium or magnesium ions. Researchers have explored different sources of xylanase enzymes, such as bacteria, fungi, and plant tissues, for their effectiveness in breaking down xylan chains. These enzymes are commonly isolated from microbial sources that can degrade plant cell walls. The production of xylanase enzymes can be influenced by factors such as the type of microorganism, cultivation conditions, and the presence of inducers in the growth medium. Based on the biochemical classification, xylanolytic enzymes primarily belong to the glycosidase hydrolases (GH) group, with some enzymes classified as esterases. Furthermore, these enzymes can be categorized based on their substrate specificity.

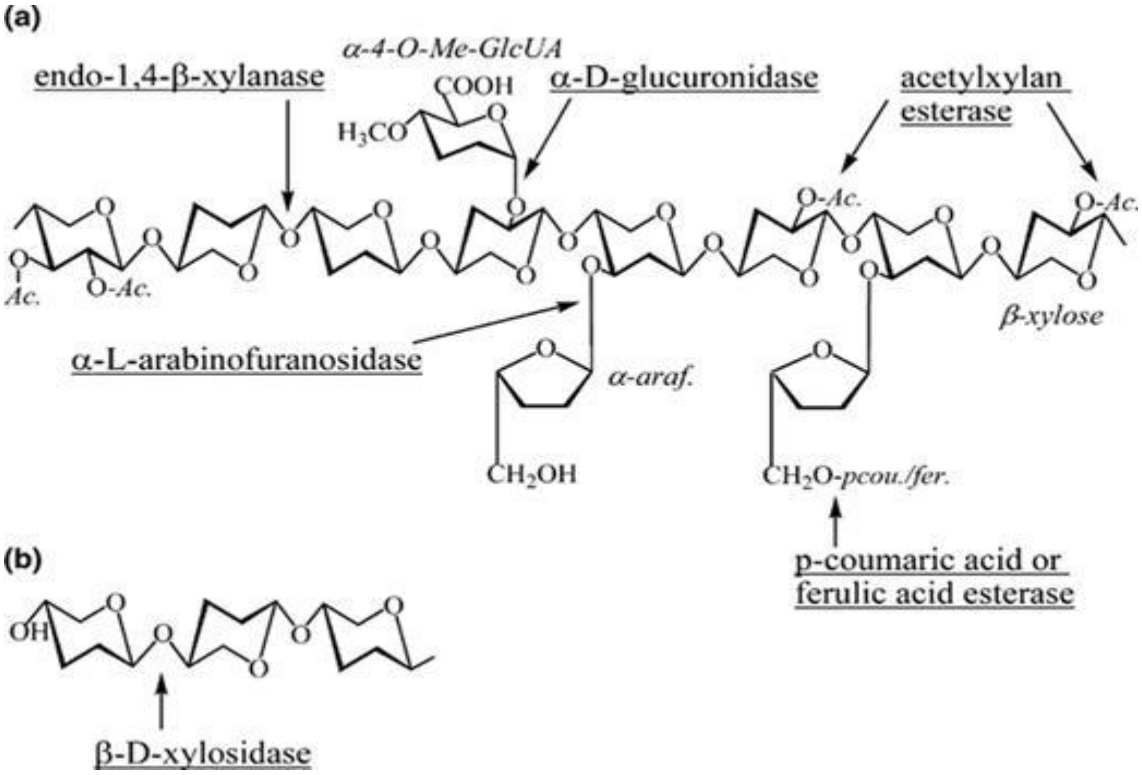


Figure 2.10. (a) Xylan, a polysaccharide, has a structure consisting of xylose residues linked together through 1,4-β bonds. Xylanolytic enzymes target specific sites on the xylan molecule, such as the acetyl group, α-arabinofuranose, α-4-O-Me- GlcUA (α-4-O-methylglucuronic acid), p-coumaric acid, and ferulic acid. (b) The enzyme β-xylosidase facilitates the process of hydrolyzing xylo-oligosaccharides. This enzyme breaks down the xylooligosaccharides molecule into its constituent parts through enzymatic action (Collins et al., 2005).

Endo-1, 4-β-xylanase, also known as 1, 4-β-D-xylan xylanohydrolases (EC 3.2.1.8), is an enzyme that plays a crucial role in the degradation of xylan, a complex polysaccharide found in plant cell walls. This enzyme acts on the interior xylose β (1,4) linkages within the xylan backbone, leading to the hydrolysis of these bonds and the generation of short chain xylooligomers. The effectiveness of endo-1, 4-β-xylanase depends on the presence of a xylan backbone consisting of consecutively unsubstituted xylopyranose units. The unique hydrolytic activity of endo-1, 4-β-xylanase makes it an essential tool in various biotechnological applications, including producing xylooligosaccharides (XOS) with

potential prebiotic properties. The ability of this enzyme to selectively hydrolyze the xylan backbone into shorter oligomers contributes to the production of XOS with different degrees of polymerization (DP), such as xylobiose (DP2) and xylotriose (DP3).

Exo-1,4-β-xylanase is an enzyme that is involved in the degradation of xylan, a complex polysaccharide present in plant cell walls. It specifically acts on the exterior xylose β (1,4) linkages of xylan, hydrolyzing them and producing shorter-chain xylooligosaccharides. The activity of Exo-1,4-β-xylanase is dependent on the structure of xylan, including its length and branching patterns.

β-Xylosidase (1,4-β-D-xylanohydrolase, EC 3.2.1.37) hydrolyzes short-chain oligosaccharides or xylobiose into xylose monomers, starting from the non-reducing end. It may not be effective on polymeric xylans and can be inhibited by the presence of xylose end-products.

Acetyl xylan esterase (EC 3.1.1.6) removes O-acetyl groups from β-D-pyranosyl residues in acetylated xylan. This enzyme plays a significant role in the hydrolysis of xylan, as acetyl side chains can impede the action of other enzymes targeting the backbone, such as endo-xylanase.

Other xylanolytic enzymes include α-arabinofuranosidase (EC 3.2.1.55), which removes non-reducing α-arabinofuranose residues from arabinoxylans, and α-glucuronidase (EC 3.2.1.131), which hydrolyzes α-1,2 glucosidic linkages between D-glucuronic acid and β-D-xylopyranosyl in glucuronoxylan, releasing D-glucuronic acid (Marais, 2008; Collins et al., 2005; Hu et al., 2016; Sørensen et al., 2007).

2.3.4. Surfactants and Non-Catalitic Proteins Effect on Enzymatic Hydrolysis

Lignin increases the mechanical strength of the plant cell wall and makes the plant durable (Ludwig & Sarkanen, 1971). It is known that lignin is one of the most important factors limiting the hydrolysis of biomass by cellulolysis and hemicellulolytic enzymes (Dijkerman et al., 1997; Jung et al., 2000; Mussatto et al., 2008). The negative effect of lignin on cellulase activity has been investigated in various studies (Berlin et al., 2005; Berlin et al., 2006; Pan et al., 2004; Pan, 2008). Binding enzymes on lignin is the main inhibitory mechanism for enzyme action on biomass and reduces hydrolysis rates and efficiency. Lignin in the pretreated biomass disrupts hydrolysis by physically blocking the enzyme's access to polysaccharides and enzymes (Palonen, 2004; Várnai et al., 2010). In addition, soluble phenolic compounds released in the pretreatment can affect the activity of enzymes (Kellock et al., 2017). It has been mentioned in the literature that cellulase prevents specific binding to cellulose and that lignin can directly inhibit hydrolytic enzymes (Yoon et al., 2006; Jung et al., 2000; Qi et al., 2011; Tu et al., 2009; et al., 1984; Morrison et al., 2011). The choice of the pretreatment method also has a significant effect on enzymatic hydrolysis since removing lignin can provide the removal of barriers on hydrolysis. On the other hand, organosolv and other lignin-efficient pretreatments to be applied in this project did not completely remove lignin; part of its effect is due to the displacement of lignin and the breaking of its bonds with polysaccharides (Van & Pletschke, 2012). Based on the hypothesis that lignin inhibits the enzyme's binding to the substrate, various methods have been applied to prevent this. Exogenous proteins such as bovine serum albumin (BSA), peptides, and soybean protein, as well as Tween 20 and Tween 80, Triton X100, polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), and lignosulfonates are effective in improving the enzymatic hydrolysis of pre-processed lignocellulosic biomass (Tejirian & Xu,2011; Eckard et al.,2012). Some of these proteins showed the Fig. 2.3.4. Eriksson et al. (2002) added dodecyl-trimethyl-ammonium-bromide (DoTAB), Triton X-100, Triton X-114, sodium dodecylsulfate (SDS), Tween 20 and Tween 80 to steam-treated spruce, Tween 20 and Triton provide the best cellulase conversion. However, Triton is both

environmentally harmful and unsuitable for large-scale processes. Surfactants interact hydrophobicity with the lignin portion of the substrate, thereby releasing the non-specifically bound enzyme (Eriksson et al., 2002).

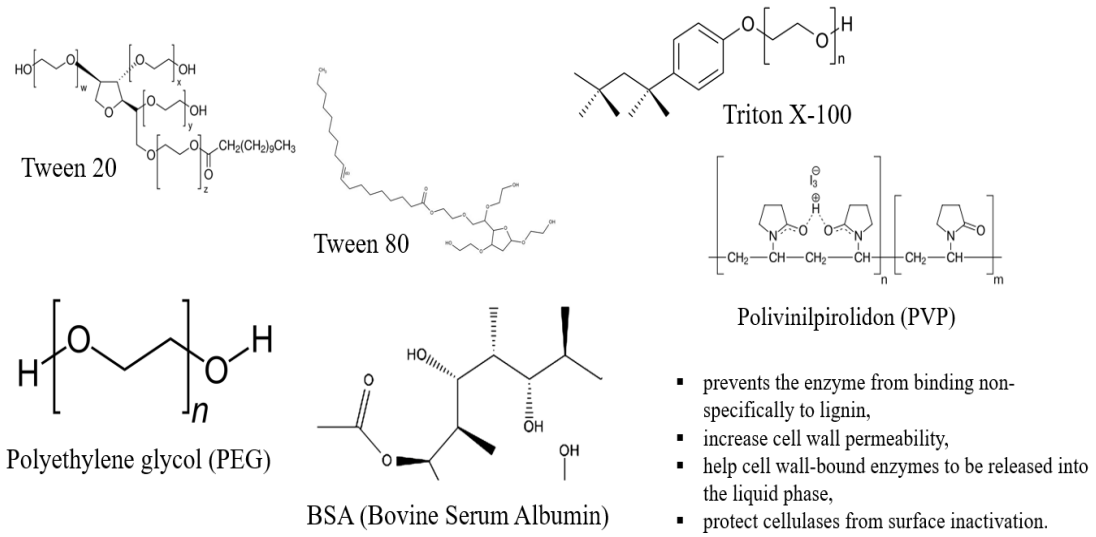


Figure 2.11. Chemical formulas of surfactants

The purpose of these substances is to prevent the non-specific binding of the enzyme to lignin. In addition, these substances increase cell wall permeability, help cell wall-bound enzymes to be released into the liquid phase, and protect cellulases from surface inactivation. In previous studies, the effect of various additives on cellulase conversion and enzyme adsorption was investigated, and it was observed that non-ionic surfactants had the most positive effect (Tejirian & Xu,2011; Sipos et al.,2011). It has been stated that Tween 20 and 80 swell the fiber and increase the surface area, improve the adsorption of the enzyme to cellulose, and thus enable the development of enzymatic hydrolysis (Bhagia et al., 2017; Alkasrawi et al., 2003). Qing et al. (2010) showed that pretreatment with Tween-80 makes the surface of solids more hydrophilic. Kaar & Holtzapple (1998) treated corn straw with Tween 80 and Tween 20 and found that it improved enzymatic hydrolysis. Tu et al. (2009) concluded that Tween 80 exhibits

competitive binding with cellulases, thereby reducing cellulase adsorption to lignin. They stated that this decreases the amount of enzyme required during hydrolysis. Also, Chen et al. (2008) found that adding Tween 80 improved hydrolysis, while Jeya et al. (2010) achieved the best results with Tween 20. Kumar & Wyman (2009) examined the effect of BSA, Tween 20, and PEG 6000 and stated that hydrolysis efficiency increased with all these additives, and Tween 20 showed the best performance. Kristensen et al. (2007) treated wheat straw with five different pretreatment techniques and added Tween 80, PEG (2000-4000-6000), Berol 08, and BerolOX-91-8. They continued their studies with PEG 6000, which has the highest molecular weight among PEG 2000, 4000, and 6000. This was explained as the increase in cellulose conversion with increasing molecular weight (Börjesson et al., 2007). In the Kristensen study, it was found that PEG 6000 improved enzymatic hydrolysis better than Berol 08, and it was stated that the effect of surfactants on xylan conversion was not as pronounced as the effect of increasing glucose (Kristensen et al., 2007).

Zhang et al. (2018) added BSA, Tween 80, organosolv lignin, sulfonated lignin, and PEG 4000 to the medium during hydrolysis and observed an increase in glucose yields, excluding organosolv lignin. Among these, the best results were obtained when using PEG 4000 (44.6% increase in yield). This is due to the hydrophobic interactions and hydrogen interaction between lignin and PEG, and it has been stated that PEG forms a hydrated layer on the lignin surface and prevents the inefficient binding of cellulase to lignin. The 29.5% increase in yield with BSA was due to the lignin coating of BSA. As the hydrolysis time increased, all glucose yields gradually increased. When the hydrolysis time reached 6 h, glucose yields with the addition of BSA, Tween 80, organosolv lignin, sulfonated lignin, and PEG 4000 were observed as 85.9%, 92.5%, 88.4%, 87.4%, and 83.3%, respectively. Tween 80 presented the equivalent of glucose (93.8%) obtained after 72 h without additives in 6 h, and it was also observed that similar yield values were achieved with fewer enzymes (Zhang et al., 2018). It has been said that the non-specific competitive binding of BSA on lignin can be a good strategy to reduce its irreversible adsorption and enzyme requirements for cellulose hydrolysis. It has also been said that solids with high lignin content have a greater effect when BSA is added than those with reduced lignin content (Yang & Wyman, 2006; Yang et al., 2009). It has been observed that Tween 20 increases the hydrolysis efficiency of wheat straw by blocking the lignin surface (Qi et al., 2010). Another study reported that bovine serum albumin (BSA) had a

significant effect on dilute acid-treated solids but had a low effect on hot water-treated solids, probably due to differences in lignin hydrophobicity (Bhagia et al., 2017). Also, Ouyang et al. (2010) used PEG 4000 as an additive and found that in addition to a higher enzyme recovery, hydrolysis was improved by 91%, and the addition of PEG stabilized the enzymes. They observed no effect and could not explain why it only affected glucan yield. Sipos et al. (2011) showed that the varying degrees of increased free cellulase activity obtained by the addition of PEG were based on various lignin structures in the substrates. It was concluded that phenolic hydroxyl groups exposed on the lignin surface interact with PEG via hydrogen bonding, forming a PEG layer on the lignin surface that prevents the inefficient binding of cellulases on lignin. Other studies have also shown that adding surfactants prevents cellulase from sticking to lignin (Kumar et al., 2012; Harrison et al., 2014).

2.4. Xylooligosaccharides (XOS)

Xylooligosaccharides (XOS) derived from lignocellulosic materials have gained significant attention in the health supplement industry due to their beneficial prebiotic effects on humans and animals (Ma et al., 2019). The global XOS market has experienced consistent growth in recent years, with a substantial annual production volume. In 2019, the global XOS market was valued at $240 million, and it is projected to reach $410 million by 2026, demonstrating a compound annual growth rate (CAGR) of 1.5% (Liao et al., 2021). The production of XOS is a significant investment for the food industry, given the high market value of these manufactured products. As the functional ingredient market continues to evolve rapidly, there is a growing demand for research on various microbial xylanases, xylan species, and novel technologies to obtain XOS with high purity and low degree of polymerization (DP) (Moure et al., 2006; Vegas et al., 2008; Samanta et al., 2015).

Xylooligosaccharides (XOS) are xylose-based oligosaccharides that may have varying proportions of substituents, such as acetyl groups and uronic acids, depending on the xylan structure of the raw material and the production process (Kabel et al., 2002). XOS with a degree of polymerization (DP) between 2 and 6 have higher prebiotics in conditions where xylobiose (DP2) and xylotriose (DP3) are more abundant. Xylooligosaccharides (XOS) are preferred in functional food production due to their consumption by probiotic bacteria (Kiran et al., 2013; Jiang et al., 2004; Gullón et al., 2011; Seesuriyachan et al., 2017). Despite being a sugar-free compound, the sweetness of xylobiose is 30% of sucrose, allowing it to be used in anti-obesity diets (Goldman, 2009).

2.4.1. Chemical Structure of XOS

Xylooligosaccharides are sugar oligomers of xylose units linked via β (1 → 4) xylosidic bonds. XOSs formula is C5nH8n + 2O4n + 1, degree of polymerization 2 to 6, which is obtained by xylan hydrolysis (Kumar & Satyanarayana, 2011; Moniz et al., 2014; Samanta et al. 2015).

XOS production typically involves extracting xylan from lignocellulosic biomass using an alkali agent, followed by xylanase hydrolysis (Moure et al., 2006; Rajagopalan et al., 2017). This method offers several advantages, including relatively high XOS purity and low levels of by-products (Rajagopalan et al., 2017). XOSs are named according to the number of xylose monomers they contain, such as xylobiose (two monomers), xylotriose (three monomers), xylotetraose (four monomers), xylopentaose (five monomers), xylohexose (six monomers), etc. (Kumar & Satyanarayana, 2011). Additionally, according to some authors, molecules with a xylose polymerization degree of 20 or less (DP) ≤ 20 can also be classified as XOS, broadening the chemical variety of these substances (Mäkeläinen et al., 2010).

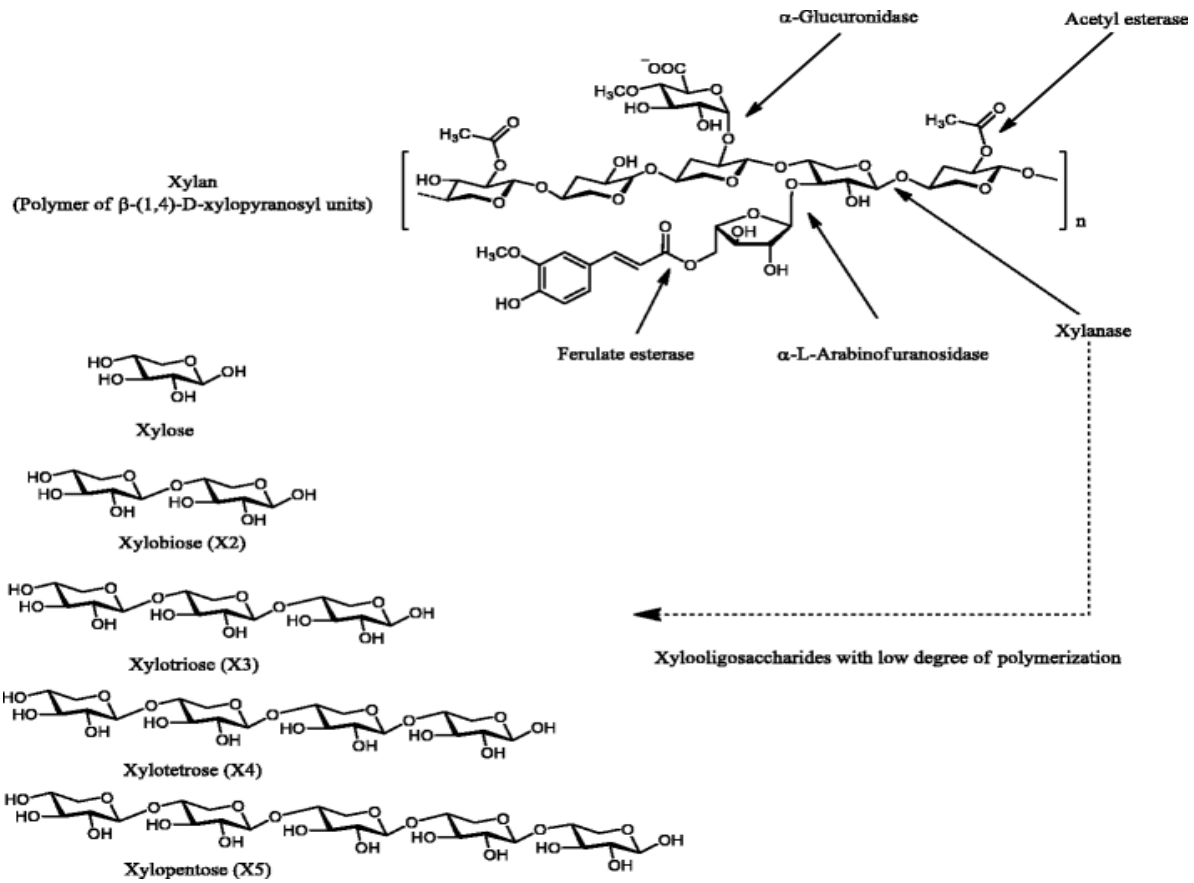


Figure 2.12. Oligosaccharide structures taken from Mano et al., 2018.

The types of oligosaccharide and sugar residues, interactions, ring topologies, and anomeric arrangements all have an impact on how stable XOS is. In general, hexoses are more tightly bound than pentoses (Carvalho et al., 2013).

2.4.2. Prebiotics and Functional Properties of XOS

The xylan in waste lignocellulosic materials, which are abundant and not suitable for human consumption, can be used as raw materials to produce value-added products, xylooligosaccharides (XOS) and xylose. When XOS is consumed by humans and animals

with food, it passes through the digestive system without being broken down and absorbed, and then reaches the large intestine. It has a prebiotic effect by being metabolized by beneficial bacteria such as Bifidobacterium species. Gibson and Roberfroid defined prebiotics as “an indigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thereby improving host health” (Gibson & Roberfroid, 1995). A prebiotic substrate is selectively utilized by beneficial components of the intestinal flora but does not favor potential pathogens such as toxin-producing Clostridia, proteolytic bacterioids, and toxigenic Escherichia coli. In this way, a "healthier" microflora composition is obtained, in which *Bifidobacteria* and/or *Lactobacteria* predominate in the gut and exert possible health-promoting effects (Manning & Gibson, 2004). Not all dietary carbohydrates are prebiotic; prebiotic food ingredients must resist hydrolysis by mammalian enzymes, gastrointestinal absorption, fermentation with intestinal microflora, and specifically stimulate the growth and/or activity of gut bacteria that promote health and well-being.

Inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), isomalto-oligosaccharides, soybean oligosaccharides and lactulose are common prebiotics (Macfarlane et al., 2006). Xylo-oligosaccharides (XOSs) produced from a wide variety of raw materials containing xylan are recommended as excellent candidates for new generation prebiotics (Moure et al., 2006). XOSs cannot be digested by stomach or pancreatic enzymes but can be used by a certain group of beneficial intestinal microflora. It has many beneficial physiological properties such as improving bowel function, increasing mineral absorption, reducing the risk of colon cancer and helping to control type 2 diabetes (Scheppach et al., 2001). In addition, it exhibits effects such as antioxidant, immunomodulation, antimicrobial, anti-inflammatory, anti-carcinogenic effects and reducing high cholesterol levels and triglycerides in blood plasm (Kaprelyants et al., 2017; Aachary & Prapulla, 2011).

Figure 2.13. Xylooligosaccharides benefits on health

Since it has a lower requirement (1.4-2.8 g/day) than other prebiotic oligosaccharides to exert its prebiotic effect, the price of XOS is advantageous and competitive (Amorim et al., 2019). However, the cost of XOS is high in terms of price per unit weight, so there is a need to develop new methods for cost-efficient production. XOSs are used in the animal feed, pharmaceutical, cosmetic, and functional food industries (Vazquez et al., 2000; Gupta et al., 2016). The monomer xylose, obtained by the complete hydrolysis of xylan, is used as a sweetener by individuals who are diabetic or want to make healthy dietary choices (Chattopadhyay et al., 2014). In addition, xylitol obtained by hydrogenating xylose is used as a food, cosmetic and pharmaceutical

additive. Xylitol is a popular sweetener for its anti-caries properties (Sugar Alcohols (Polyols) and Polydextrose Used as Sweeteners in Foods - Food Safety - Health Canada, 2005). As a result, the conversion of xylan to KOS and xylose to obtain value-added products can be beneficial both in terms of human and animal health and economically (Samanta et al., 2015).

2.4.3. XOS Production Methods

With the increasing demand for prebiotics, researchers are working to develop an economical and efficient process to produce XOS from various plant biomass. After extracting xylan from agricultural biomass, XOS can be obtained by methods such as chemical (Samanta et al., 2012a), enzymatic, or a combination of chemical and enzymatic (Jayapal et al., 2013; Samanta et al.,2015).

The degradation of xylan polymer obtains XOS, one of the structural polysaccharides of the cell wall of many plant species. Lignocellulosic biomass left over from agriculture, forestry and some food industry activities is a cheap and sustainable source of xylan. However, lignocellulosic biomass cannot be directly hydrolyzed due to its complex structure. Studies on XOS production mainly focused on alkali extraction followed by enzyme hydrolysis. Other than these, autohydrolysis and acid hydrolysis are thermal processes used for XOS production. Experiments include enzymatic hydrolysis following microwave pretreatment and direct fermentation of brewery waste grains by *Trichoderma reseei* (Wang et al., 2013; Amorim et al., 2019).

However, these methods have some disadvantages. After the xylan extraction from biomass with alkali, high amounts of alkali and acid are used to produce XOS by hydrolysis with xylanases. In autohydrolysis, which is another method, biomass is treated with water under high temperature and pressure, and high energy demand and release of sugar degradation products into the environment are important disadvantages of this method. XOS production with ethanol-based organosolv process has advantages such as recycling, low toxicity, and obtaining digestible pre-treated solid. In the process of

obtaining xylose or XOS, xylan is separated from the biomass by pretreatment and then hydrolyzed. In another approach, pretreatments that leave the xylan in the solid are applied, and hydrolysis of the xylan in the solid is achieved. The enzyme xylanase, which hydrolyzes the bonds in xylan, is preferably used for hydrolysis. Due to the complex structure of the xylan polymer, many hydrolytic enzymes with various modes of action are needed (Polizeli et al., 2005). These enzymes are commercialized as complexes because they have a synergistic effect together. Therefore, enzymatic hydrolysis conditions should be optimized for the target product (xylose, short XOS, long XOS). For example, to increase the efficiency of XOS production by enzymatic hydrolysis, conditions must be found where the endo-xylanase activity is maximum, and the exo-xylanase and beta-xylosidase activity is minimum. On the other hand, environmental factors such as pH and temperature should be adjusted to obtain high XOS production efficiency (Goswami & Rawat, 2015; Aachary & Prapulla, 2011).

2.5. Agricultural Wastes and Corn Cob

Recently, the world has set goals to achieve carbon neutrality, which includes reducing greenhouse gas emissions from human activities as much as possible and absorbing and eliminating the remaining greenhouse gases to reduce actual emissions to zero. More than 100 countries have committed to take action to achieve carbon neutrality by 2020. (Wu et al., 2021). Globally, billions of tons of agricultural residues (for example, cereal straw, corn residues, and wheat straw) are produced each year. Such residues can harm the environment when disposed of (Jittin et al., 2021). The remains are typically incinerated, buried and recycled. Incineration of agricultural wastes can have adverse effects on humans and the environment. Outdoor burning releases large amounts of pollutants into the atmosphere, including particulate matter (PM), CO and hydrocarbons, causing severe local and regional environmental impacts (Li et al., 2007; Tiammee & Likasiri, 2020). Such reasons have led many researchers to focus on using abundant and inexpensive agricultural residues as sustainable materials (Tarrés et al., 2017). Non-edible

residues are formed during the cultivation and processing of agricultural products (Pagano et al., 2021). Agricultural residues form vegetable residues during the primary growing process and secondary residues that are disposed of after food processing (Santana-Méridas et al., 2012). Various types of agricultural residues produce 140 billion tons of biomass per year (Zuin & Ramin, 2018).

Corncob is a lignocellulosic material that contains approximately 40%–45% cellulose, 25%–35% hemicelluloses, 17%–20% lignin, and small amounts of ash and extractives. Due to its high xylan content (>30%), corncob is commonly used as a valuable resource for producing xylooligosaccharides (XOS) (Han et al., 2020; Ling et al., 2017; Boonchuay et al., 2014; Kawee-ai et al., 2016, Yang et al., 2005).

Figure 2.14. Corn cob and grains

XOS is generally produced in lignocellulosic material by hydrolysis of xylan, and corn cob is currently one of the primary raw materials due to its high hemicellulose content and widespread availability (Poletto et al., 2020; Kadam & McMillan, 2003). Over the last ten years, the amount of corn grain produced worldwide has increased by 40%. 47–50% of the residue is made up of the stems, leaves, quick balls, husks, and corncobs that are produced by corn. Since there are typically 18 kg of corn cobs produced for every 100 kg of maize grain produced, corn cobs are the primary inedible residues left

over from maize production and contain significant amounts of maize residues globally each year (Tsai et al., 2001). Corn waste is a large volume of solid waste from the sweet corn processing industry. According to 2017-18 International Grain Council (IGC) data, corn is the most produced grain, with a production amount of 1.045 million tons, and is grown in vast geography (International Grain Council, 2018). Corn is the second largest biotech crop after soybean (Torney et al., 2007). In Turkey, approximately 71% (11.1 million hectares) of total agricultural land has grain. According to TUIK, wheat ranks first at 69%, barley ranks second at 22%, and corn ranks third with a share of 6% (Toprak Mahsulleri Ofisi, 2018). In Turkey, 6.9 million tons of corn waste (stalk, cob) is produced annually (Sumer et al., 2016). Although Turkey ranks 21st in corn production, it has high yields. While the world corn yield is 580 kg/da, Turkey's average yield varies between 700-1000 kg/da .

According to numerous research (Czajkowski et al., 2019; Menardo et al., 2015; Takada et al., 2018), corn cob is a rich source of biomass. The corncob has cellulose, hemicellulose, and lignin are porous sponge structures (Takada et al., 2018). Thanks to their chemical composition, corn residues show great potential as a renewable raw material to produce various value-added chemicals (Rivas et al., 2004). As a result of these informations, corn cob is a precious and widely recognized resource due to its abundance, availability, and significant waste utilization potential.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The chemicals used in this study and their sources are listed below.

- Corncob (Ministry of Agriculture and Forestry, Aegean Agricultural Research Institute)
- Ethanol (96 %, Isolab)
- Veron 191S (AB Enzymes)
- Shearzyme 500L (Novozymes)
- Trisodium Citrate Dihydrate (Isolab)
- Citric Acid Monohydrate (Sigma Aldrich)
-Magnesium Sulfate Heptahydrate (Sigma-Aldrich)
- Sulphuric Acid (95-98%)(Sigma)
-Methanol (99%,HPLC Grade Isolab)
- Calcium Carbonate (99%)(Sigma-Aldrich)
- 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)

- Phenol (Fluka)

- Polyethylene Glycol (Sigma-Aldrich)

3.2. Methods

Pretreatments applied to lignocellulosic material and xylooligosaccharide production methods and analysis are listed in this section.

3.2.1. XOS Production from the Corn Cob

Corn cob was obtained from the Aegean Agricultural Research Institute (İzmir, Türkiye) in ground form. It was stored in airtight packages at room temperature. To ensure the corncob was dry, it was kept in an oven at 60 degrees overnight. It was ensured that it reached a constant weight. Raw corncob, with a particle size lower than 1 mm, was put in the 1g/l H_2SO_4 solution, and acid pretreatment was applied on a water bath at 60 °C for 14 h. Detailed information given in the Chapter 3.2.1.1. The acid-pretreated solid was dried to constant weight. Then, organosolv pretreatment was applied. For the organosolv treatment twenty-five grams of acid-pre-treated corn cob was mixed with 250 ml of 70% ethanol. This mixture was kept in a pressurized reactor at 170 degrees for 1 hour. After the organosolv pretreatment, this solid was deacetylated. 0.4 mM NaOH solution was added to the solid, and the application was carried out as described in Section 4. The solids obtained from the pretreatments were added to 50mM citrate buffer at optimum pH (5.5) and subjected to enzymatic hydrolysis at the selected enzyme

concentrations. Enzymatic hydrolysis was carried out at different temperatures, times, and solid-to-liquid ratios to optimize the XOS production conditions. In Figure 3.3., XOS production steps are explained in detail.

3.2.1.1. Acid Pretreatment

Dilute acid pretreatment was applied to ground dry corn cobs. In this step, H_2SO_4 solution (1.0 g/l) was mixed with the corn cob at a solid-to-liquid ratio of 1:8 (200 ml solution was prepared in a 400 ml shot bottle using 25 g of corn cobs.). This mixture was incubated in a water bath (Termal, Turkey) at 60 °C for 14 h. The pre-treated solid was washed with distilled water. The washing liquor pH was measured with a pH meter (Hannah Instrument), and washing was continued until the pH was 6-7. It was then, filtered through cheesecloth and then filter paper under vacuum with the help of a vacuum pump. The solid was dried at 60 °C in an oven (Memmert) until it reached constant weight.

3.2.1.2. Organosolv Pretreatment

The process to be carried out in the 600ml pressure reactor (Berghof, Germany) (Figure 3.2.1.2) at a solid-liquid ratio of 1:10. Twenty-five grams of acid-pre-treated corn cob was mixed with 250 ml of 70% ethanol. After the biomass-liquid mixture was placed in the reactor, the reactor cover was closed, and the heating was started. The reaction took place under isothermal conditions when the temperature of 170 °C was reached. (Temelli, 2020). The pedal mixer was operated at 300 rpm for 1 h excluding heating and cooling periods, which took about 1 h (Fig.3.1.) After the reaction time was completed, the tap

water, which circulated in the cooling coil, provided rapid (15- 20 min) cooling. When the reactor temperature dropped to 60ºC, the pressure valves were opened and the lid was opened.

The product, which comes out of the reaction in solid and liquid phases, was subjected to filtration for phase separation. The product was passed through four layers of cheesecloth and filtered with a vacuum pump to separate the solid phase. The volume of the liquid fraction (liquor) was measured. The solid phase remaining on the cheesecloth was washed with water to remove the ethanol, which may otherwise have a negative effect on the enzymatic action.

Figure 3.1. High-Pressure Reactor and Data Logger Berghof (Germany)

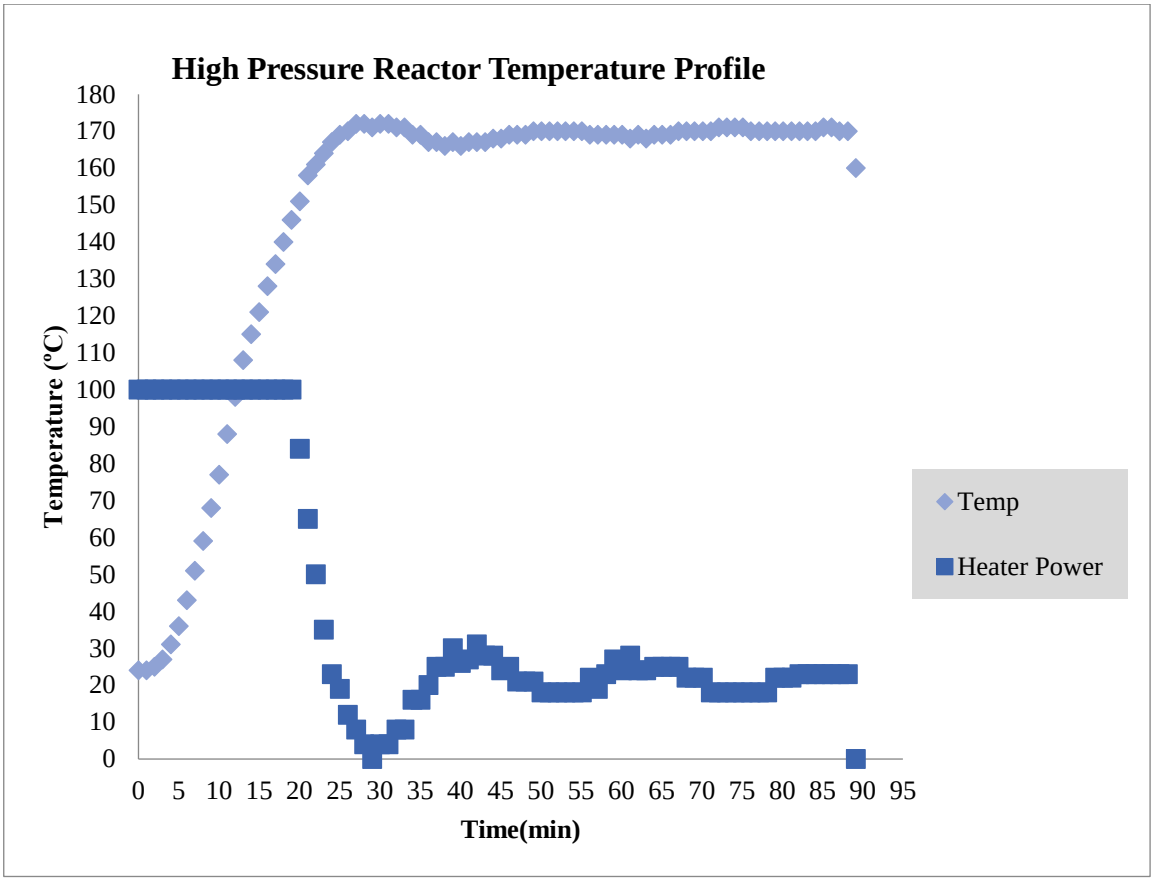


Figure 3.2. The temperature profile of organosolv pretreatment (data taken from the data logger software BTC 3000)

The solid was filtered through coarse filter paper under a vacuum to remove the remaining liquid in the biomass. The solid remaining on the filter paper was dried at 60°C overnight. The dried solid was taken into a desiccator and weighed after cooling. After weighing, the amount of dissolved solid was compared with the solid put into the reactor. The dried biomass was milled and homogenized with a kitchen-type grinder (Sinbo, Turkey). The ground pre-treated solid was stored in airtight polyethylene bags at room temperature.

3.2.1.3. Deacetylation Pretreatment

In this study, experiments were carried out at different NaOH concentrations (0.2, 0.4, 0.6 ,0.8 mM) on the organosolv treated solid, and it was aimed to increase the enzymatic hydrolysis efficiency by removing the acetyl groups from the solid. (Zhang, 2014). Deacetylation was carried out in a 50 ml flask containing 24 ml NaOH solution and 2 g of organosolv-treated corncob in a shaking incubator (ZHWY-200-B, Zhicheng, China) at 160 rpm at two temperature values (30℃ and 60℃) for 3h. The deacetylated solid was washed with distilled water until the washing liquid reached neutral pH. The washed solid was filtered through filter paper under a vacuum. The solid was dried overnight at 60℃. The dried solid was taken into a desiccator and weighed after cooling. The amount of dissolved solid was compared with the solid put into the reactor. The dried biomass was milled and homogenized with a kitchen-type grinder (Sinbo, Turkey). The ground pre-treated solid was stored in airtight polyethylene bags at room temperature. In order to control the removal of the acetyl group in the treated solid, acid hydrolysis was applied to the solid and acetic acid released was analyzed in HPLC. The acetate was calculated according to the equation 1.

$$\%\ Acetate = \frac{CAA,HPLC\ x\ Volume\ x\ Conversion\ factor}{ODWsample}\ x\ 100 \quad \text{Eqn 1}$$

Where:

$C_{AA,HPLC}$=concentration in mg/ml of acetic acid determined by HPLC

Volume= volume of filtrate, 87 ml

Conversion factor= (59/60): 0.983, the conversion from acetic acid to acetate in biomass

ODW_{sample} = oven dry weight, the weight of biomass mathematically corrected for the amount of moisture present in the sample at the time of weighing.

3.2.1.4. Enzymatic Hydrolysis

Enzymes are active at wide pH ranges and show significant activity at temperatures above those experienced under natural conditions (Pavasovic et al., 2004). However, optimum pH and temperature conditions in which specific enzymes can work are significant for enzymatic hydrolysis. In this study, two different commercial xylanase enzymes, namely Shearzyme 500L (Novozymes, Denmark) originated from *Aspergillus oryzae* and Veron 191S (AB Enzyme, Finland) originated from *Aspergillus niger* were used. *Aspergillus oryzae* and *Aspergillus niger* xylanase enzymes generally have the highest activity level at slightly acidic pH conditions (pH 4-6). However, they can also operate between pH 2 and 9 and generally show the highest catalytic activity at operating temperatures between 40-60°C. The working temperatures for the main activities for Shearzyme 500L is 35–75 °C and Veron 191 is 40–60 °C according to information supplied by product sheets. In this study, enzymatic hydrolysis for Veron 191S and Shearzyme 500 L enzymes was adjusted using 50 mM citrate buffer at pH 5.5 and temperature at 50 ºC. According to Guido et al. (2019) supported that 5.5 is the optimum pH for Shearzyme 500L. For this study, enzymatic hydrolysis conditions changed for the different experiments. For the Shearzyme 500L and Veron 191S hydrolysis was conducted at 50ºC, 180 rpm in a 25 ml flask with 10 ml working volume. These experiments carried out 5% solid- liquid ratio. Hydrolysis was also carried out at 10%, 12.5% and, 16.67% solid loadings. For these experiments, two biomass samples namely organosolv-treated corn cob and deacetylated organosolv-treated corn cob were used. The activity of Shearzyme 500L and Veron191S enzymes in the samples from the hydrolyses during the 48-h incubation period were stopped by keeping the sample tubes in a 100ºC water bath (Termal, Turkey) for 5 min (Büyükkileci & Sürek, 2017). The tubes were cooled in a cold-water bath and then centrifuged. The supernatant was taken and put in the new tube. Samples were stored at -20ºC until analysis.

HPLC was used to determine the amounts of XOS and xylose in the hydrolysis samples. According to the values, the best conditions, where the amount of XOS is high, and the amount of xylose is low, were determined. The parameters to produce XOS were selected by calculating the following yield values.

$$\% \text{ Conversion} = \frac{\text{Amount of released XOs and xylose (g)}}{\text{Amount of xylan in pretreated biomass(g)}} \times 100 \quad \text{Eqn.2}$$

$$\% \text{ XOS Yield} = \frac{\text{(Released XOs in hydrolysate)(g)}}{\text{Amount of xylan in pretreated biomass(g)}} \times 100 \quad \text{Eqn.3}$$

$$\% \text{ Xylose Yield} = \frac{\text{(Released X1 in hydrolysate)(g)}}{\text{Amount of xylan in pretreated biomass(g)}} \times 100 \quad \text{Eqn.4}$$

$$\text{XOS} = X2+X3+X4+X5+X6 \quad \text{Eqn.5}$$

where, X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose, X5: xylopentaose, X6: xylohexaose.

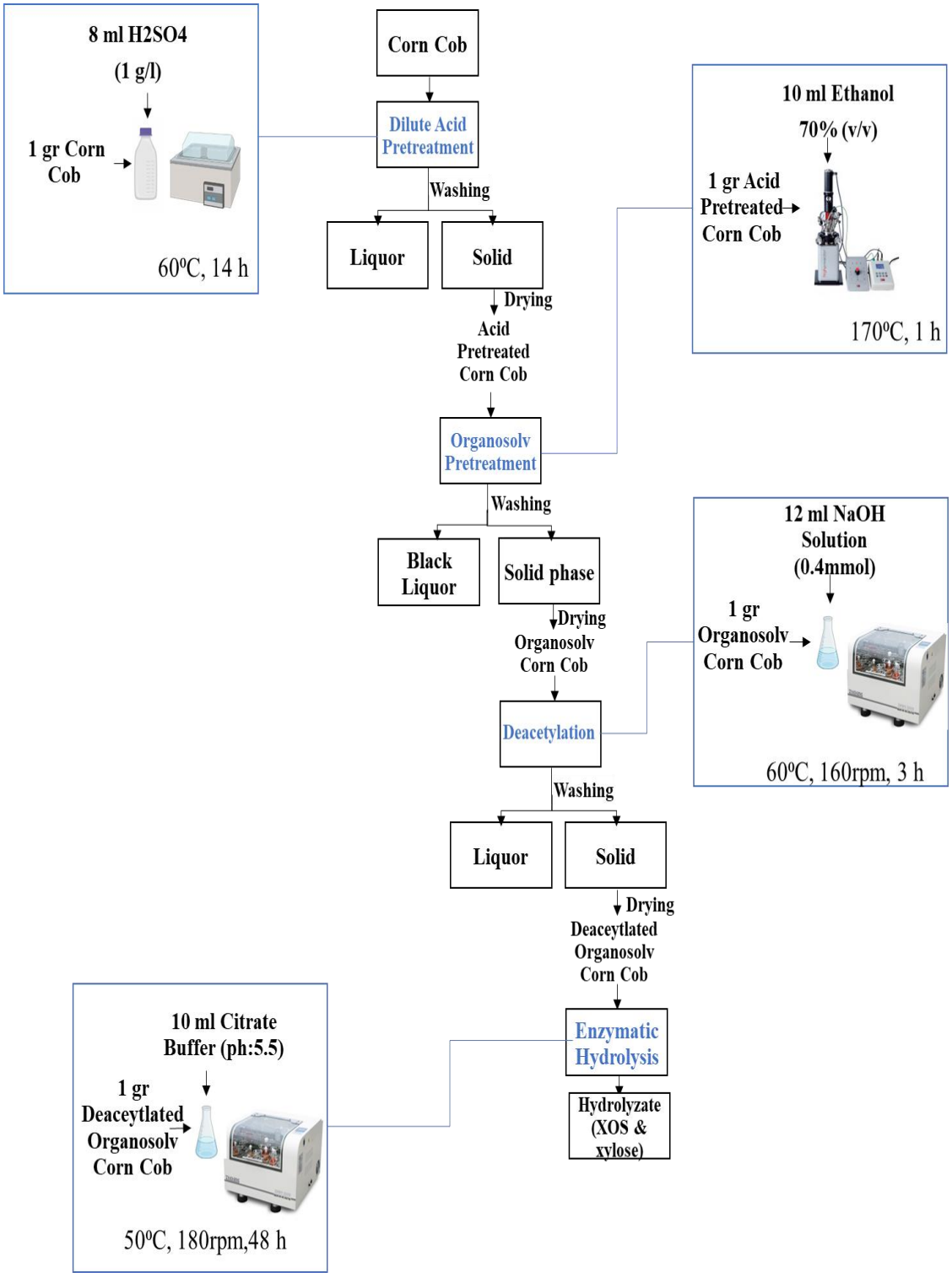


Figure 3.3. Optimized XOS Production Steps (all conditions given for the 1 gr biomass)

3.2.1.4.1. Sequential Batch Hydrolysis

Deacetylated organosolv-treated corn cob hydrolysis experiments were performed in 50 mM citrate buffer at pH 5.5 (Fig. 3.3). The reactions were initiated by mixing the buffer and biomass with the enzyme in an Erlenmeyer flask. The solid-liquid ratio of this hydrolysis is 1:10. The experiments were performed in an incubator shaker at 50 °C with gentle agitation inverting the tubes at 180 rpm. As detailed in Fig. 3.3, the deacetylated organosolv solid was hydrolyzed with 26.5U/g per biomass Shearzyme 500L and 26.5U/g per biomass Veron 191S for 24 h. After hydrolysis, the solid-liquid solution was separated into solid and liquid phases with the help of a vacuum pump and filter. A part of the liquid was taken for analysis, boiled at 100ºC for 5 min, and then cooled in a cold-water bath. This liquid was diluted 10-fold and analyzed by HPLC .The hydrolysate was divided into equal volumes without stopping the remaining enzyme activity. To half of this liquid, enzyme (26.5U/g per biomass Shearzyme 500L and 26.5U/g per biomass Veron 191S) and deacetylated organosolv-treated corn cob were added. This mixture was subjected to hydrolysis for 24 h. Then, this hydrolysate was filtered and analyzed in HPLC. The remainder of the divided liquid was subjected to hydrolysis for 24 h by adding only fresh deacetylated organosolv solid. Solid and liquid were also separated by vacuum filtration. The remaining liquid and solid phases were separated. Furthermore, a sample was taken from the filtered liquid for condition G. New solid was added to the separated liquid phase. Hydrolysis was carried out with this liquid for 24 h, and the sample was taken for condition H. Apart from that, the solid and liquid phases separated after hydrolysis, solid phase was washed with pure water. In this process, which was done with a vacuum pump and filter, washing continued until the liquid's pH was neutral. With this process, the enzyme and buffer attached to the solid were removed. Then, the liquid and the solid removed from the enzyme were dried in an oven at 60 °C until constant weight.

3.2.1.4.2. Investigation of Synergistic Activity of Enzymes

Two commercial enzymes at 5.5 pH and 50 °C temperature values were used for the enzymatic hydrolysis. Enzymes were tested at different concentrations when used individually and mixed. These mixed forms was Shearzyme 500L-Veron191S used together. The enzyme concentration at which the best XOS production was achieved was determined.

3.2.1.4.3. Addition of Surfactant and Non-Catalytic Proteins

Organosolv treated corn cob hydrolysis experiments were performed in 50 mM citrate buffer at pH 5.5 in the presence of several additives. The reactions were initiated by mixing the buffer and biomass with the additives in a 25 ml Erlenmeyer flask with a 10 ml working volume. The solid-liquid ratio of this hydrolysis was 1:20. The experiments were performed in an incubator shaker at 50 °C with gentle agitation inverting the tubes at 180 rpm. Tween 20, Tween 80 and polyethylene glycol (PEG6000), and bovine serum albumin (BSA), these materials were added to be 0,5 % of the hydrolysis medium. The biomass, buffer solution, and additives were mixed in a 50°C incubator for 15 minutes. Additives were added before the enzymes were added to ensure the samples were thoroughly mixed and partially heated to the hydrolysis temperature. In order not to give heat shock to the enzymes, the enzyme was added before the samples reached 50 °C in the first 15 minutes. (Sipos et al., 20011; Helle et al., 1993; Börjesson et al., 2007; Kristensen et al., 2007; Ooshiama et al., 1986: Seo et al., 2011; Kaar & Holtzapple, 1998) After warming up for 15 min, Tween 20, Tween 80, BSA and PEG were added to three parallel flasks. The concentration of additives was adjusted to be 10%

biomass. For each one, 0.1 g of additive was added to 1 g of biomass and subjected to enzymatic hydrolysis in 10 ml buffer. Experimental explanation was visualized in detail in the Fig.3.4. The samples were subjected to hydrolyses for 24 h, and the samples were analyzed in HPLC. The effect of improving the enzymatic hydrolysis of lignocellulosic biomass was investigated by comparing the additives with the control.

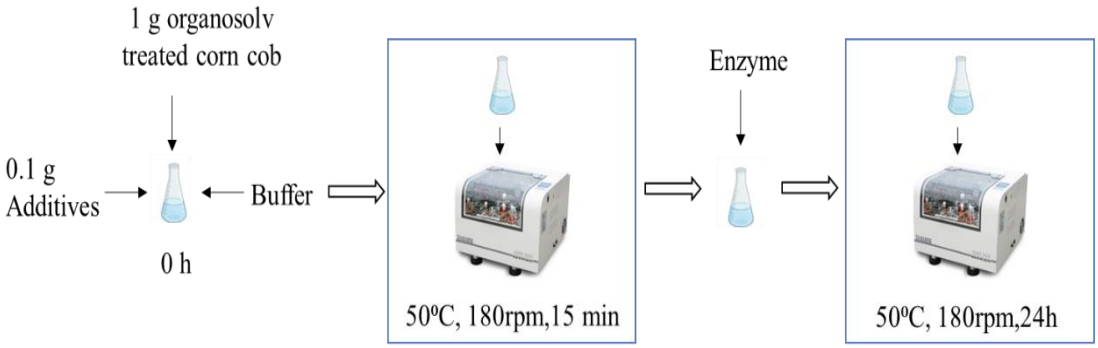


Figure 3.4. Additives Addition Procedure

3.3. Analyses

This section describes the analysis methods used in this study.

3.3.1. Moisture Content

According to National Renewable Energy Laboratory (NREL) NREL/TP-510-42621 method, corncobs were dried at 105ºC overnight in an oven (Memmert, Germany).

They were kept in the desiccator until they reached constant weight. Considering the equation, the moisture content was calculated by weighing it on the analytic balance.

$$Moisture\ Content\ (\%) = 100 - \frac{Weight\ of\ Dried\ Sample\ (g)}{Weight\ of\ Analyzed\ Sample\ (g)} \times 100 \qquad \text{Eqn.6}$$

3.3.2. Ash Content

The ash content of corncob and pretreated solids was measured by burning the biomass in an ashing furnace (Carbolite, UK) at 575 °C according to the American Society for Testing and Materials (ASTM) E 1755-01 (ASTM, 2001).

3.3.3. Characterizations of Structural Component

Determination of the cellulose, hemicellulose and lignin of raw biomass and pretreated solids were performed using the NREL/TP-510-42618 method (Sluiter et al., 2012). According to the method, 3 ml of 72% (w/w) H_2SO_4 was added to 10 ml glass tubes containing 0.3 g solid sample and incubated for 1 h under room temperature by frequent vortexing to increase solid-liquid interaction. Then, this mixture and 84 ml of water were added to a 250 ml schot bottle, so that the acid concentration was diluted to 4%. Then diluted solution was autoclaved at 121°C for 30 min. After the autoclave, solution pH was increased to 5-7 with $CaCO_3$, the samples were centrifuged at 4 ºC and 6000 rpm for 20min (Centurion K241R, UK) to remove solids. It was then filtered with 0.45 μm PTFE membrane filters. The sample was diluted and analyzed in the HPLC system. Glucose, xylose, arabinose and acetic acid concentrations of the filtered samples were determined. Acid hydrolysis breaks down cellulose and hemicellulose in the biomass into their

monomers, which were determined by the HPLC For the calculation of the concentration of cellulose and xylan, glucose six was multiplied by the anhydro factor 0,9 (162/180) while xylose and arabinose by 0,88 (132/150) (Sluiter et al., 2008). To calculate the acetyl content, acetic acid released upon acid hydrolysis was multiplied by 0,983(59/60) is used.

$$Concentration\ of\ Sample\ (\%) = \frac{Concentration\ of\ hyrolyzed\ sample\ determined\ by\ HPLC\ \left(\frac{g}{l}\right) \times volume\ of\ filtrate\ (l) \times converison\ factor}{Hydrolyzed\ dry\ sample(g)} \times 100 \quad \text{Eqn.7}$$

$$Glucan\ (\%) = \frac{Concentration\ of\ glucose\ determined\ by\ HPLC\ \left(\frac{g}{l}\right) \times 0{,}087\ (l) \times 0{,}90}{0{,}3\ (g)} \times 100 \quad \text{Eqn.8}$$

$$Xylan - Arabinan\ (\%)\ \frac{Concentration\ of\ xylose-arabinose\ determined\ by\ HPLC\ \left(\frac{g}{l}\right) \times 0{,}087\ (l) \times 0{,}88}{0{,}3\ (g)} \times 100 \quad \text{Eqn.9}$$

$$Acetyl\ group\ (\%) = \frac{Concentration\ of\ acetic\ acid\ determined\ by\ HPLC\ \left(\frac{g}{l}\right) \times 0{,}087\ (l) \times 0{,}983}{0{,}3\ (g)} \times 100 \quad \text{Eqn.10}$$

Solid particles were filtered through the porcelain crucibles under a vacuum. The remaining part of the solid included lignin and ash. The ash content was determined gravimetrically. The crucibles were firstly dried overnight at 105ºC and weighed. Then they were incubated at 525ºC for 3 h. Using equation 11, the amount of acid-insoluble lignin was calculated.

$$Acid\ insoluble\ lignin = \frac{Weight\ of\ the\ Dried\ Samples\ after\ Acid\ Hydrolysis\ (g) - Weight\ of\ Ash\ (g)}{0{,}3\ g} \times 100 \quad \text{Eqn.11}$$

3.3.4. High-Performance Liquid Chromatography (HPLC) Analysis

HPLC analyzes for characterization of solid composition and enzymatic hydrolysis liquid are given below.

3.3.4.1. Determination of Biomass Structure

Analyzes were performed on a Thermo Fisher Scientific HPLC system using a BIORAD Aminex HPX-87H (300 x 7.8 mm) column. (HPLC is visualized given in figure 3.2.1.4.3). Micro-guard columns (Biorad) were used to protect the column during the analysis. When the resolution starts to drop, the cartridge has been washed or regenerated.

For carbohydrate structure, Injection volume: 20 µL, Flow rate of mobile phase (5mM H_2SO_4): 0.6 mL min^{-1}, Column temperature: 65 ºC, Detector: RID, Analysis Time: 20 min

For the acetyl group, HMF and furfural, Injection volume: 20 µL, Flow rate of mobile phase (5mM H_2SO_4): 0.6 mL min^{-1}, Column temperature: 65 ºC, Detector: RID and UV (210nm), Analysis Time: 50 min

Figure 3.5. The HPLC equipment, images show the pump, autosampler, oven and UV detector respectively (Thermo Fisher Scientific).

Calibration curves given in Appendix A were obtained using standard solutions at certain concentrations. With these calibration curves concentrations of the samples were calculated from the peak areas in the chromatogram.

3.3.4.2. Determination of Xylooligosaccharides

Analyzes of enzymatic hydrolysates were done using HPLC. To determine xylose and XOS content lead ionic form column Rezex RPM-monosaccharides Pb+2 300 x 7.8 mm column (Phenomenex, USA) was used. The samples were filtrated through 0.45 μm PTFE membrane filters and then analyzed by HPLC (Thermo Fisher Scientific) with an RI detector system. For this analysis, the security guard column Rezex RPM-Monosaccharide Pb+2 50 x 7.8 mm (Phenomenex) is used to protect the column. This security guard provides to extend the life of the column by capturing sample contaminants that can cause an increase in backpressure and affect baseline noise. When the resolution starts to drop or backpressure is increased, the cartridge was washed or regenerated.

For this column, filtrated ultrapure water was used as the mobile phase. P column with guard analysis conditions is given below.

For xylose and XOS structure, the injection volume is 20 μL, the flow rate of the mobile phase is 0.6 mL min^{-1}, Column temperature is 80 ºC, the detector is RID, Analysis time is 20 min.

For calibration curves of xylose and xylobiose, xylotriose, xylotetraose, and xylopentaose for low-DP oligosaccharide analysis were prepared for different concentrations. Calibration curves given in Appendix A were obtained using standards at specific concentrations. With these calibration curves, concentrations of the samples were calculated from the peak areas in the chromatogram for the sample preparation hydrolysate diluted ten times with filtered ultra-pure water.

3.4. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Mid-infrared spectroscopic profiles were collected with a Fourier transform infrared spectrometer (Spectrum 100, Perkin Elmer, USA). Raw and pretreated samples were analyzed by a DTGS-FTIR spectrophotometer (Spectrum 100, Perkin Elmer, USA) for their characterization. Spectra were recorded in the 4000–400 cm^{-1} range, with 128 scans at 4 cm^{-1} resolution taken per sample. For this purpose, all solid samples were mixed with KBr (3%), and mid-infrared spectra of corn cob-KBr and pretreated solids-KBr pellets were obtained with 128 scans at 4 cm^{-1} resolution.

3.5. Xylanase Enzyme Activity Assay

The xylanase activity was measured using the dinitro salicylic acid (DNS) method (Bailey et al., 1992; Yeğin, 2017). Beechwood xylan was used as a substrate. Beechwood xylan was soluted in the 50mM citrate buffer (pH 5.5). The concentration of this solution is 0.5% (w/v). 900 ml of xylose stock solution at different concentrations was prepared in 10 ml glass tubes, then 100 ml of the enzyme was added. This mixture was incubated in a water bath at 50ºC for 5 min. After the incubation of these samples, 1500 µl DNS solution was added, and the sample was kept for 5 min in the water at 100ºC bath, which changed the color and stopped the enzyme activity. After stopping the activity, these tubes were cooled for 1 min before the analysis. By introducing the enzyme before the boiling phase rather than before incubation, blank samples for each enzyme concentration were created. This indicates that there was no response in these samples and that the enzyme's lack of an impact on color is eliminated in the spectroscopic results (Yeğin, 2017).

The color change formed by the reaction of the reducing sugar and DNS formed by the degradation of the substrate after enzymatic hydrolysis was read absorbance by the spectrophotometer against water at a wavelength of 540 nm. By removing the blank, the

enzyme-dependent absorbance change was obtained. Determination of the concentration for the standard calibration curve, xylose stock solution was prepared as 2 mg /ml. Xylose stock solution was diluted 0-200 µg with the pH 5.5, 50 mM citrate buffer. As a blank, only buffer solution was used instead of xylose solution. After removing the absorbances of the samples from the blank, the color change depending on the xylose concentration was obtained, and the standard graph was drawn with these absorbance values. The amount of reducing sugar resulting from the enzyme effect was found in free xylose using the standard graph.

The enzyme activity unit (U/ml) is 1 µmol xylose per minute at 50ºC and pH 5.5 conditions. It was defined as the amount of enzyme that liberates equivalent reducing sugars. Enzyme activity was calculated with equations 12-13 below.

$$Xylanase\ Acivity(\frac{U}{ml}) = \frac{mg\ xylose}{MW\ of\ xylose} \times \frac{1}{Incubation\ time(\text{min})} \times \frac{1}{Addition\ of\ enzyme(ml)} \times Dilution\ factor \quad \text{Eqn.12}$$

$$Xylanase\ Acivity\left(\frac{U}{ml}\right) = \frac{mg\ xylose}{150{,}13} \times \frac{1}{5} \times \frac{1}{0{,}1} \times Dilution\ factor \quad \text{Eqn.13}$$

3.6. Statistical analysis

All experiments were performed in duplicate ANOVA was performed using Minitab 17.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Effect of pretreatments on the chemical composition of corn cob

The images of all solids are shown in Fig. 4.1., indicating that the applied processes have caused changes in the physical structures of the materials. The structural carbohydrates, acetyl groups, lignin, and ash levels of raw and treated corn cobs were determined. The primary components of the raw biomass were found to be cellulose and xylan as 42.1% and 26.1%, respectively. The impacts of a 1% sulfuric acid treatment applied to corn cob at 60 ºC for 14 h at solid to liquid ratio of 1:8, the effect of an organosolv treatment using 70% ethanol at 170 ºC for 1 hour on an acid-pretreated corn cob at a solid to liquid ratio of 1:10, and the effect of the deacetylation process conducted with 0.4 mM NaOH at a solid-to-liquid ratio of 1:12 on the biomass composition are given in the Table 4.1.

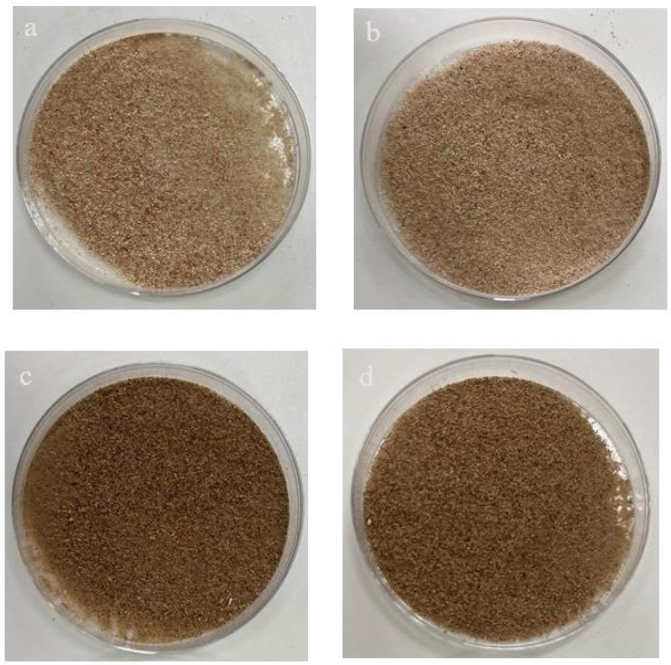


Figure 4.1. Photographs of ground corn cob biomass, a) Raw corn cob, b) Acid-treated corn cob, c) Acid and organosolv-treated corn cob d) Deacetylated acid and organosolv-treated corn cob

Table 4.1. Lignocellulosic composition of raw and pretreated biomass solids (% of dry matter, mean value of triplicates ± standard deviations)

Material	**Composition of Solid (%)**			
	Glucan	**Xylan**	**Lignin (acid insoluble)**	**Acetyl Group**
Corncob	42.0 ± 1.5	26.13 ± 0.90	17.66 ± 1.13	4.46 ± 0.27
Organosolv- treated corn cob	48.31 ± 0.75	34.56 ± 0.75	12.88 ± 0.79	4.01± 0.46
Deacetylated organosolv-treated corn cob	45.40± 0.83	34.50 ± 0.90	12.40 ± 1.10	2.38 ± 0.15

Raw corn cob was composed of approximately 42.0% glucan, 26.1% xylan, 17.7% acid-insoluble lignin, and 4.5% acetyl group. The acid-treated corn cob was treated with organosolv treatment. Based on Hildebrand and Scott's theory of polymer solubility modified by Hansen solubility parameters, the addition of an organic solvent to water by organosolv pretreatment makes the solvent mixture dissolve lignin due to chemical similarities between the aromatic macromolecule and organic solvents (Thoresen et al., 2020). According to Bhutto et al. (2017), hemicellulose and cellulose-rich solid are obtained by removing lignin in the organosolv process. Lignin is a complex polymer that inhibits the enzymatic hydrolysis of xylan, and its removal helps improve the efficiency of xylan conversion (Kellock et al., 2022). The organosolv-treated con cob composition showed glucan and xylan content up to approximately 48.3% to 34.6%, respectively. The acid-insoluble lignin decreases to 12.9% and the acetyl group to 4.0%.

Furthermore, a deacetylation process was carried out on the organosolv-treated material. The composition of the deacetylated organosolv-treated corn cob revealed a glucan content to around 45.4%, xylan remains relatively unchanged at 34.5%. The acetyl group content decreases significantly to 2.4%. In summary, the Table 4.1. illustrates the composition changes for each treatment stage. The acid pretreatment leads to a reduction in glucan while increasing the acetyl group content. The organosolv treatment increases the glucan and xylan but decreases acid-insoluble lignin (Li et al., 2023; Buyukkileci et al, 2023).

Solid samples were analysed by FTIR for detailed examination of the chemical content of the biomass. FTIR spectra have characteristic vibrational bands of different chemical bonds. Therefore, the FTIR spectrum of the solid was used as a tool to determine the chemical bonds it contains and the changes in structure with the treatments. The Fig. 4.1.2. shows the FTIR frequency spectra representing the functional group and compositional analysis of the solid samples collected for corn cob, acid-pretreated corn cob, organosolv corn cob, deacetylated corn cob, and enzymatic hydrolysis solids.

The cellulose spectrum have five distinct peaks at 1431, 1373, 1338, 1319, and 1203 cm^{-1} (Adapa et al,2011). Similarly, xylan have prominent peaks at wavenumbers of 1606, 1461, 1251, 1213, 1166, and 1050 cm^{-1}. The lignin spectrum showed characteristic peaks at a wavenumber of 1599, 1511, 1467, 1429, 1157 and 1054 cm^{-1}. In single-band chemical imaging, specific vibrational frequencies are associated with certain

components in cellulose, hemicellulose, and lignin. The C–O–C vibration in cellulose and hemicellulose is detected at 1157 cm^{-1}, while the aromatic skeleton in lignin is observed at 1504 cm^{-1} (Tomak, 2014). Additionally, the non-conjugated C=O in hemicellulose appears at 1734 cm^{-1}. The presence of ketones can be indicated by the carboxyl C=O stretching vibrations, which exhibit absorbance within the range of 1640–1714 cm^{-1}. Similarly, the presence of benzene substitutes can be identified by a prominent absorbance peak between 1014 and 1088 cm^{-1}. Furthermore, the absorbance peak between 1250 and 1390 cm^{-1} suggests the presence of C-N stretching vibrations in amines (secondary and tertiary amines), C-O stretching vibrations in ethers and esters, C-S stretching vibrations in sulfides, C-H bending vibrations in alkanes and alkyl groups. When examining these spectra, it becomes evident that they contain similar chemical bonds. At a wavelength of 1200 cm^{-1}, there are O-H groups present, which are characteristic of cellulose and hemicellulose polymers. The absence of this peak in the enzymatic hydrolysis solid further supports the findings from the acid hydrolysis characterization, indicating a decrease in the hemicellulose and cellulose structure of the hydrolysis solid. An increase in guaiacyl lignin is observed in the 1270 band, suggesting a decrease in lignin content in the deacetylated organosolv solid and the enzymatic hydrolysis layer. Considering all the peaks, it can be concluded that corn cob, acid-pretreated corn cob, and organosolv solids exhibit similar profiles, while the deacetylated organosolv solid and enzymatic hydrolysis active layer share similar functional groups furans.

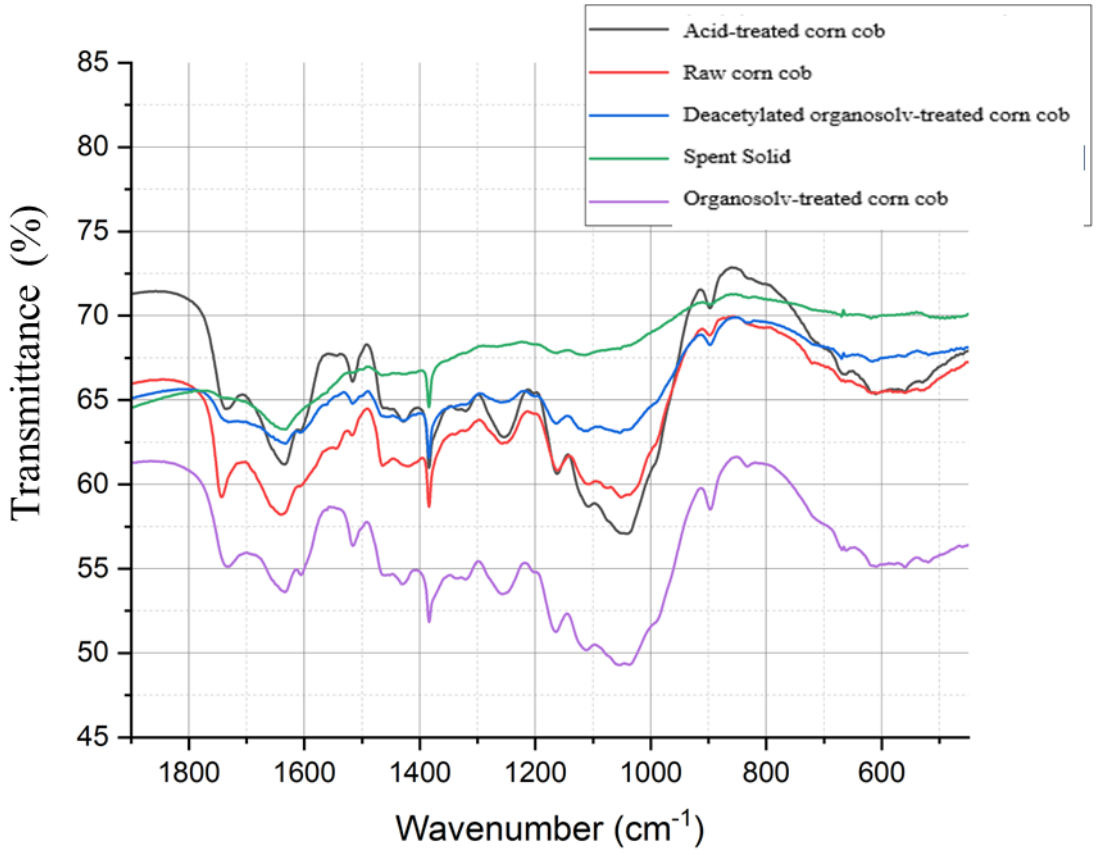


Figure 4.2. FTIR spectra of raw corn cob and pretreated corn cobs

It is worth noting that there are variations in the intensities of these peaks. For the determination of acetyl group removal FTIR spectra especially related with 1730 cm−1, 1372 cm−1, and 1237 cm−1 delegated for C double bond O, –C–CH3, –C–O–, respectively (Zhao et al., 2008). When these wavelengths are closely analysed, the deacetylated organosolv solid and it is observed that the solid remaining after hydrolysis performed under the condition where Shearzyme 500L 26.5 U/g biomass and Veron 191S 26.5 U/g biomass, which are called spent solid, are used together, does not give peaks in these areas. These results support the decrease in the acetyl group ratio in structural carbohydrate analyses, which should be theoretically expected.

4.2. Enzymatic XOS Production from Organosolv-treated Corn Cob

Enzymatic hydrolysis is one type of hydrolysis widely used in producing value-added products from lignocellulosic materials, mostly to degrade polysaccharides into smaller units. Generally, enzymatic hydrolysis is affected by various factors such as pH, temperature, time, enzyme concentration, substrate loading and additives (Viera et al., 1995; Liaset et al., 2000; See. et al., 2011; Prabha et al., 2013). Optimization of these parameters is necessary to obtain maximize process efficiency. In the following sections, information about the efforts to optimize the hydrolysis conditions is given.

4.2.1. Synergistic Effect of Xylanases

Determining enzyme type and loading is one of the most critical parameters affecting hydrolysis efficiency. Xylanolytic enzyme systems comprise endo-xylanase, exo-xylanase, β-xylosidase, and debranching enzymes (Aachary et al., 2011). Most endo-xylanases are hindered by substituents present in xylan, and they preferentially randomly cleave unsubstituted xylan, producing unsubstituted and branched XOS (Katapodis et al., 2008). Although the presence of side chain groups partially hinders the enzyme from binding to the substrate, it enhances the solubility of polysaccharides (Puls, 1997). Consequently, the complete enzymatic degradation of complex hemicelluloses relies on the cooperative action of different enzymes (Den Haan et al., 2003). When the literature is searched for obtaining less xylose and more low DP XOS with this approach, xylanase preparations with low exo-xylanase activity and/or β-xylosidase activity were preferred (Vázquez et al., 2000; Escarnot et al., 2012).

In this study, two different commercial xylanase enzymes, namely Shearzyme 500L (Novozymes, Denmark) originated from *Aspergillus oryzae* and Veron 191S (AB Enzyme, Finland) originated from *Aspergillus niger* were applied at different enzyme dosages. Shearzyme 500L and Veron 191S were used in pH 5.5 and 50 ºC enzyme dosage tests. It was studied over 3.5-138 U/g biomass (organosolv-treated corn cob). Table 4.2. shows that organosolv-treated corn cob solid was loaded with Shearzyme 500L and Veron 191S enzymes at different enzyme dosages, and their effect on xylooligosaccharides and xylose formation was investigated. According to the data in the table, two different enzymes were used, Veron 191S and Shearzyme 500L, and different dosages were tested for each enzyme. Also, in some cases, combinations of two enzymes were included in the experiments. First, when the conditions under which enzymes are added individually are examined; It was observed that the concentration of XOS and xylose products increased with increasing enzyme dosage. For example, for the Veron 191S enzyme, at 35 U/g biomass dosage, the total low DP XOS concentration was 1.37 g/l, while it increased to 1.69 g/l at 53 U/g biomass dosage. Similarly, for the Shearzyme 500L enzyme, it was observed that increasing the dosage increased XOS and xylose products. For example, while the total low DP XOS concentration was 1.41 g/l at 35 U/g biomass dosage, it increased to 1.74 g/l at 70 U/g biomass dosage. Enzyme loading is an essential factor in the rate of enzymatic reaction and product concentrations. According to the data in the Table 4.2., XOS and xylose concentrations generally increase as the enzyme loading increases. However, this increase was only sometimes linear, and a complex relationship between enzyme loading and the resulting concentrations may exist. Here, after a specific dosage for Shearzyme 500L, enzyme loading did not have a hydrolysis-enhancing effect. In experiments using Veron 191S, the XOS and xylose concentrations appear low. On the other hand, higher concentrations of XOS and xylose were obtained in experiments using Shearzyme 500L. Maximum product formation was obtained with Shearzyme 500L enzyme at 53U/g biomass enzyme dosage. Under this condition, 1.98 g/l total low DP XOS was accepted, and when Veron 191S was loaded with the same enzyme dosage, the total low DP XOS amount was less with 1.69 g/L. The findings of Kiran et al. (2013) supported these results. They showed that Shearzyme 500L showed better activity on xylan backbones than Veron 191S. In their study, Kiran et al. evaluated the synergistic effects of enzymes in lignocellulosic material extracted with alkali, while in this study, the synergistic effects of enzymes were evaluated in organosolv-treated corn cob, as it offers an approach where xylan remains solid.

Many studies in the literature describe the improvement in hydrolysis by the cooperation or synergistic effect of enzymes (Wong and Maringer, 1999; Sorensen et al., 2003; Raweesri et al., 2008). When the studies were examined, it was revealed that while higher concentrations should be used per biomass in conditions where enzymes were used individually, using fewer enzymes combined could achieve the same efficiency. Thus, for in-depth evaluation, a matrix with two enzymes combined, and control groups were formed to evaluate the synergistic effects of enzymes (Table.4.2.). Shearzyme 500L and Veron 191S were used together to investigate the synergistic effect of these two enzymes' different loading. Table 4.2. shows the results of a series of experiments where different combinations and enzyme loading were tested to increase the yields of XOS and xylose from organosolv-treated corn cob. The combination of Shearzyme 500L and Veron 191S was also effective and increased the concentration of XOS and xylose products when used at different dosages (17.5-17.5, 26.5-26.5 and 35-35). When these combinations were compared with the conditions in which the enzymes were used individually, the synergitstic usages were found to be more successful. When the same enzyme concentration was used in total, the conditions in which the two enzymes were used together gave better results in indiviaidual all enzyme loadings. This was because two xylanases may have acted on different parts of the biomass, thus increasing the hydrolysis efficiency. When the studies are examined, it has been revealed that while higher concentrations should be used per biomass in conditions where enzymes are used individually, it can be achieved by using fewer enzymes in with the synergistic effect of enzymes to provide the same concentration. Thus, for in-depth evaluation, two enzymes combined, and control groups were formed to evaluate the synergistic effects of enzymes. The conditions under which maximum XOS production and transformation occur, keeping the total enzyme unit low in this matrix, were chosen for further analysis. Furthermore, other experiments were continued on these determined conditions. Consequently, the combination of Veron 191S and Shearzyme 500L, where enzymes may have acted synergistically, was identified as the most suitable condition. Other studies in the literature provide supporting evidence for this (Wong and Maringer, 1999; Sorensen et al., 2003; Raweesri et al., 2008). The simultaneous use of different xylanase preparations has been found to enhance the breakdown of complex xylan structures and reduce the formation of unwanted xylose (Kiran et al., 2013). Two enzymes were founded to improve the hydrolysis rate of corn stalks effectively pretreated with sodium hydroxide using xylanase and β-xylosidase from white rot fungus (Zhuo et al., 2018). Moreover,

the combined use of xylanase preparations with different specificities can target and hydrolyze specific regions of the polymer more effectively, thereby facilitating the production of xylo-oligosaccharides (XOS). These results show that the use of two different enzymes together is more effective in obtaining XOS and xylose products by creating a synergistic effect. From the table, it is seen that the best enzyme condition is "Shearzyme 500L & Veron 191S (26.5-26.5 U/g Biomass) combination". The total low DP XOS concentration obtained in this combination is 3.51 g/l, which is the highest value compared to the other conditions. Some of the benefits of using two enzymes together compared to using a single enzyme likes the use of different enzymes together creates a synergistic effect. This can result in an effect that cannot be achieved when each enzyme is used separately. The synergistic effect allows the substrate to be degraded more efficiently and higher product concentrations to be obtained. As a result, the use of a combination of enzymes has increased the efficiency of the hydrolysis process of XOS and xylose products and higher product concentrations can be obtained. Utilizing low enzyme dosages, the optimal condition for achieving low DP XOS content and low xylose content was determined to be Veron 191S at 26.5 U/g biomass along with Shearzyme 500L at 26.5 U/g biomass. Under this condition, xylobiose content was high , which was desirable,since XOS with lower DP can be more quickly consumed by colon bacteria and exhibit higher prebiotic activity (Ghosh, 2012; Remon et al., 2019). When all these results were evaluated, it was decided to use Veron 191S and Shearzyme 500L condition of 26.5-26.5U/g biomass in the following experiments.

Table. 4.2. The effects of enzyme dosages on XOS and xylose concentration (g/l) under 5% (w/v) solid loading at 50 °C, 5.5 pH 48h hydrolysis with organosolv treated corn cob. Each data point is the average of at least three replicate measurements.

Enzyme	**Enzyme Loading (U/g Biomass)**	**X4**	**X3**	**X2**	**X1**	**Total Low DP XOS**
Veron 191S	35	0.08	0.54	0.75	0.22	1.37
Shearzyme 500L	35	0.24	0.42	0.75	0.17	1.41
Veron 191S	53	0.61	0.04	1.04	0.38	1.69
Shearzyme 500L	53	0.61	0.04	1.33	0.38	1.98
Shearzyme 500L	70	0.19	0.29	1.26	0.27	1.74
Shearzyme 500L & Veron 191S	17.5-17.5	0.40	0.98	1.67	0.44	3.05
Shearzyme 500L & Veron 191S	26.5-26.5	0.49	0.99	2.03	0.57	3.51
Shearzyme 500L & Veron 191S	35-35	0.48	0.62	1.04	0.67	2.14

*X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetraose

4.2.2 Enhancement of Enzymatic Hydrolysis Efficiency by Deacetylation

Deacetylation is a reverse reaction that removes the acetyl group from the molecule. Since acetyl groups may decrease the activity of the xylanase enzyme, it may cause a decrease in the efficiency of enzymatic hydrolysis (Chang & Holtzapple, 2000). The presence of an acetyl group on the substrate might result in structural and chemical alterations that disrupt the interaction between the enzyme and the substrate and impair the efficiency of the hydrolysis reaction, decreasing the reaction's yield. The acetyl group can change the substrate's electrical characteristics, which can change how the substrate interacts with the enzyme's active site. As a result, the rate of enzymatic hydrolysis may slow down, which could lower the reaction's overall yield. Therefore, removing acetyl groups from the backbone of xylan in the feedstock by deacetylation can increase xylan digestibility and enzymatic hydrolysis. For this purpose, it was aimed to remove acetyl groups, which are constraints to enzymatic hydrolysis, and to increase the enzymatic hydrolysis of xylan.

In this study, 0.8, 0.6, 0.4 and 0.2 mM NaOH solutions were added to the organosolv-treated corn cob to perform deacetylation and these solids were treated at two different temperatures at 30 and 60º C. (Detailed information is given in Section 3.2.1.3.). The aim was to remove acetyl groups from the solid. The results of the acetyl groups remaining in the solid are shown in Table 4.3. According to the table, the effect of deacetylation treatment on XOS and xylose production on organosolv-treated corn cob was studied. The deacetylation process was carried out at different NaOH concentrations and temperature conditions and the results were obtained after 48 hours. There was a correlation among NaOH concentration, temperature, and acetyl percentage .Temperature is one of the important factors that determine the rate of chemical reaction. In general, the higher the reaction rate, the higher the temperature. That is, the deacetylation reaction occurs faster at higher temperatures and proceeds more slowly at lower temperatures. For this, the effect of two different temperatures on deacetylation was investigated. The acetyl group amount, which was 4.46% before the treatment, decreased with the applied deacetylation processes. Deacetylation reduced the amount of residual acetyl groups in

organosolv-treated corn cob. The decrease in residual acetyl groups increased the production of XOS and xylose. Acetyl groups were able to be removed 1.5% in the organosolv treated corn cob sample, which was exposed to 0.2 mM NaOH concentration at 30 ºC. It was revealed that the deacetylation process at this temperature and concentration did not make a significant difference in acetyl group removal. When the NaOH concentration was increased to 0.4 mM at 30 ºC, the removal rate of acetyl groups increased to 42%. Acetyl group removal increased to 58% and 82% when the temperature was kept constant and the NaOH concentration was increased to 0.6 mM and 0.8 mM, respectively. This indicates that the NaOH concentration plays an important role in the deacetylation process. As NaOH acts as a base in the deacetylation reaction and reacts to remove acetyl groups, it increases the reaction rate by providing more OH- ions at higher NaOH concentrations. This means a faster deacetylation process. The acetate percentage of the organosolv solid treated with 0.8 mM NaOH concentration and 60 ºC temperature decreased from 4.46% to 0.41%, and acetyl groups were 90% removed. The percentage of acetyl group was 0.80% at 0.8 mM NaOH concentration 90% removal, but at a temperature reduced to 30 ºC with this conditions 82% acetyl group removal. In this case, the effect of temperature on the removal of acetyl from biomass was evident. Similarly, when the organosolv treated corn cob was exposed to different NaOH concentrations (0.6, 0.4 and 0.2 mM) at 60 ºC, it was observed that the acetyl group became more difficult to remove as the NaOH concentration decreased. For example, at 0.2 mM NaOH concentration and 60 ºC, the percentage of acetate was 3.04%, while the removal of acetyl group increased as the NaOH concentration increased while the temperature remained the same. In this study, the highest removal of acetyl group was obtained by hydrolyzing the solid treated with 0.8 mM NaOH at 60 ºC.

After the removal of acetyl groups in the solids with deacetylation process, these solids were subjected to enzymatic hydrolysis to investigate whether acetyl groups are an obstacle to enzymatic hydrolysis or have a role in the production of XOS. In the Table 4.4. the results of enzymatic hydrolysis of solids subjected to deacetylation under different NaOH concentrations and temperatures are provided. An overall increase in the concentrations of xylotetraose, xylotriose, xylobiose, and xylose compounds were observed with an increase removal of acetyl groups. Similarly, temperature also has an impact on this increase. High temperature and high NaOH concentrations provide the removal of acetyl group of the xylan backbone and promote the formation of low-DP

XOS. It was observed that acetyl groups were more effectively removed at high temperatures and high NaOH concentrations (Table 4.3.).

Table. 4.3. Effect of deacetylation of organosolv-treated corn cob on XOS and xylose production (5% (w/v) solid loading, 26.5 U/ g organosolv-treated corn cob Shearzyme 500L & 26.5 U/ g organosolv-treated corn cob Veron191S used together, 48h results)

NaOH (mM)	**Temperature (ºC)**	**Remaining Acetyl Group (w/w%)**	**Concentration (g/l)**				
			X4	**X3**	**X2**	**X1**	**Total Low DP XOS**
0.8	60	0.41	0.33	0.86	5.25	0.59	6.45
	30	0.80	0.38	1.04	5.13	0.59	6.55
0.6	60	1.48	0.47	1.37	5.53	0.66	7.37
	30	1.83	0.56	1.48	3.97	0.68	6.01
0.4	60	2.38	0.69	1.63	4.50	0.61	6.83
	30	2.59	0.57	0.88	1.60	0.06	3.04
0.2	60	3.04	0.15	0.78	1.78	0.41	2.72
	30	4.39	0.19	0.80	1,67	0.64	2.66
0	60	4.46	0.25	0.70	1.70	0.59	2.65

*X1: xylose, X2: xylobiose, X3: xylotriose, X4: Xylotetraose

When solid samples were deacetylated at concentrations of 0.8, 0.6, 0.4, and 0.2 mM at 60ºC, enzymatic hydrolysis resulted in XOS concentrations of 6.45, 7.37, 6.83, 3.04 and 2.72g/l after 48 h, respectively. For instance, enzymatic hydrolysis of the solid treated with 0.8mM NaOH concentration at 60°C resulted in a low-DP XOS

concentration of 6.45 g/l. Similarly, at 60°C in reactions carried out with 0.6 mM NaOH concentration, a low DP XOS concentration of 7.37 g/l was obtained. Temperature also plays a role in the low DP XOS concentrations. For instance, in reactions conducted at 0.4mM NaOH concentration, the low DP XOS concentration decreased to 3.04 g/l at 30°C. Considering the result given in the Table 4.3. the effect of solids deacetylated at higher temperature on enzymatic hydrolysis was higher in solid processes performed at 60 ºC and 30ºC. While there was no improvement in enzymatic hydrolysis with 0.2mM NaOH compared to the control, an increase in XOs concentration from 2.65 g/l to 6.83 g/l was observed with 0.4mM NaOH at 60ºC.

Similar results to these results have also been reported in the literature. According to the literature, the removal of acetyl groups during pretreatment has a notable impact on enzymatic saccharification (Chen et al., 2012). Wu et al., (2020) found that deacetylation significantly enhanced the hydrolysis of both cellulose and hemicellulose with both NaOH and mild KOH treatments. In this study corn stover was treated with alkali of different strengths. According to the study, the addition of either NaOH (at 80 °C) or mild KOH (at 25 °C) to corn stover prior to mechanical refining led to greater than 80% deacetylation. Hemicellulose deacetylation increases the exposure of xylan to enzymes, which enhances the hydrolysis of both cellulose and hemicellulose (Wu et al., 2020). Grohmann et al., (1989) demonstrated that removing 75% of the acetyl groups from xylan before pretreatment significantly improved xylan digestibility compared to native xylan, increasing it by 5-7 times. Additionally, deacetylation enhanced the digestibility of the remaining cellulose fraction by 2-3 times by improving enzyme accessibility. In addition to this study according to Chen et al., (2012) the deacetylated corn stover feedstock is approximately 20% more digestible compared to pretreated corn stover controls and this indicates that eliminating acetyl groups improves the degradation of cellulose and xylan, which are the primary constituents of corn stover. As a result, the removal of acetyl groups enhances the accessibility of cellulose and xylan for enzymatic saccharification.

As a result, as the NaOH concentration increased (from 0.2 mM to 0.8 mM), the total low DP XOS concentration increased (from 2.72 g/l to 7.37 g/l). The combination of high temperature (60 °C) and high NaOH concentration (0.6 mM) gave the highest total low DP XOS concentration (7.37 g/l). However, since there was no significant difference with 0.4 mM NaOH, the 6.83 g/l condition was chosen as a model for further

experiments. Thus, to achieve similar results with reduced chemical usage and environmental considerations. The concentrations of XOS and xylose products were generally lower in deacetylation processes carried out at low temperature (30 °C). The reduction of acetyl groups remaining in the organosolv-treated corn cob as a result of the deacetylation process increased the production of XOS and xylose. According to Table 4.4., deacetylation, the process of removing acetyl groups from organosolv-treated corn cob feedstock, has been demonstrated to significantly enhance the digestibility of the biomass following pretreatment. A study revealed that deacetylated organosolv-treated corn cob feedstock exhibited higher digestibility compared to untreated organosolv controls. This indicates that the elimination of acetyl groups improves the breakdown of cellulose and xylan, the primary constituents of corn cob, thereby rendering them more accessible for enzymatic hydrolysis Overall, deacetylation plays a crucial role in enhancing the XOS and xylose production from the organosolv-treated corn cob feedstock. Consequently, it can be observed that the deacetylation and hydrolysis processes applied to the treated solid were strongly influenced by the factors of NaOH concentration and temperature, leading to a significant impact on the formation of xylo-oligosaccharide compounds. These findings indicate the potential to establish an optimized reaction process that can yield a higher production of xylo-oligosaccharides under these specific conditions.

In addition to the above studies (Section 4.2), increasing the enzyme dosage was tested to increase the enzyme loading for deacetylated organosolv-treated corn cobs of Shearzyme 500L and Veron 191S enzyme. The reason for this additional study was that enzymatic hydrolysis improved with deacetylation and it was investigated whether better conditions could be achieved with higher enzyme loadings with the deacetylated solid. These experiments were carried out at 10% solid loading. Since no changes were observed 48 hours after hydrolysis, the reaction was terminated at 24 hours and the results are given in Table 4.4. In these experiments, it was observed that increasing the enzyme dosage beyond a certain value did not increase the XOS concentration and conversion. For this evaluation, enzymes were loaded at 2, 3, and 4 times the optimum enzyme dosage, as a result of which the effect of increasing the synergistic enzyme dosage is shown in the table over the total low DP XOS.

In the deacetylated organosolv treated (DO)solid with 53U/g biomass enzyme for 24 h, the X4 content was 0.552 g/l, X3 content was 1.066 g/l, X2 content was 6.844 g/l, and X1 content was 0.774 g/l, resulting in a total XOS content of 8.461 g/l. For the DO solid treated with 106 U/g biomass enzyme for 24 h, the X4 content was 0.524 g/l, X3 content was 0.804 g/l, X2 content was 6.726 g/l, and X1 content was 1.053 g/l, resulting in a total XOS content of 8.054 g/l. In the DO solid treated with 159U enzyme for 24 h, the X4 content was 0.548 g/l, X3 content was 0.508 g/l, X2 content was 6.484 g/l, and X1 content was 1.408 g/l, resulting in a total XOS content of 7.540 g/l. For the DO solid treated with 212 enzymes for 24 h, the X4 content was 0.530 g/l, X3 content was 0.330 g/l, X2 content was 5.585 g/l, and X1 content was 1.475 g/l, resulting in a total XOS content of 6.445 g/l. These results indicate that increasing the enzyme dosage led to a decrease in XOS content, particularly in the higher molecular weight XOS compounds (X4 and X3).

When look at the table, higher total low DP XOS concentration was obtained at 24 hours at 10% solid loading at the same enzyme dosage compared to 48h results at 5% solid loading. With the systematic increase of the enzyme dosage, it was observed that the amount of xylose increased, but xylotriose and xylobiose concentrations decreased. There was no particular difference in xylotetraose concentrations. Here, increasing the enzyme dosage had a limiting effect for xylobiose and xylotriose. Excessive presence of xylanase enzyme in the medium may cause product inhibition. Xylanase is an enzyme that breaks down the polysaccharide called xylan found in lignocellulosic materials. This enzyme converts β-1,4 glycosidic bonds attached to xylose. The xylose formed because of the reaction can stop or slow down the catalytic activity of xylanase. Here, as the amount of xylose formed in the medium increased, it may have prevented xylanase from working, which may have caused a decrease in the total amount of low DP XOS.

Table 4.4. Effect of enzyme loading on XOS production with deacetylated organosolv treated corn cob (Enzymatic hydrolysis conditions was 50ºC, %10 solid loading and conditions was designed Shearzyme 500L & Veron 191S used together)

Conditions				**Concentration (g/l)**				
Solid Type	**Time (h)**	**Enzyme**	**Enzyme Loading (U/g biomass)**	**X4**	**X3**	**X2**	**X1**	**Total Low DP XOS**
Deacetylated organosolv treated corn cob	24	Shearzyme 500L & Veron 191S	26.5 & 26.5	0.55	1.07	6.84	0.77	8.46
Deacetylated organosolv treated corn cob	24	Shearzyme 500L & Veron 191S	53 & 53	0.52	0.80	6.73	1.05	8.05
Deacetylated organosolv treated corn cob	24	Shearzyme 500L & Veron 191S	80 & 80	0.55	0.51	6.48	1.41	7.54
Deacetylated organosolv treated corn cob	24	Shearzyme 500L & Veron 191S	106 & 106	0.53	0.33	5.59	1.48	6.45

***** X4: xylotetraose, X3: xylotriose, X2: xylobiose and X1: xylose

4.2.3. Effect of Solids Loading

In this part of the study, solid material pretreated with 0.4 mM NaOH at 60°C as described in Section 4.3. was used. The hydrolysis conditions that gave the best results at 5% solid loading in Table 4.4., where Shearzyme 500L and Veron 191S were used together, were selected to observe the effect of solid loading. In order to examine the

hydrolysis in detail, hydrolysis samples were taken at 6, 12, 24, 36 and 48 hours and analyzed. These results shown in Table 4.5. When the enzyme efficiency was analyzed, the highest efficiency was observed in the first 6 hours at 1:20, 1:10, 1:8 and 1:6 solid-liquid ratios, and the efficiency decreased as the hydrolysis progressed. A significant decrease in efficiency was observed especially after 24 hours (Appendix B). Looking at the results, the highest total low DP was obtained at XOS 10% solids loading. In this condition, 8.25 g/l of low DP XOS was obtained at 24 hours and increased to 11.34 g/l at 48 hours. At 12.5% (w/v), the low DP XOS concentration increased from 8.43 g/l to 9.98 g/l in 24 hours. Although the 24 h result was higher at 12.5%, no increase in concentration was observed after 36 h. At 12.5% (1:8) and 10% (1:10), no significant difference was observed in the amount of final product obtained in 48 hours. Some difficulties were encountered in hydrolysis at 12.5% (1:8) and 16.67% (1:6) conditions. These are difficulties in mixing, heat and mass transfer (Chen et al., 2017; Hernandez et al., 2018). Considering the conversions, 33.8% of xylan was converted to low DP XOS with 5% solid loading, while 63.8% with 10%, 44.56% with 12.5% and 28.14% with 16.67% were obtained. More substrate loading leads to the formation of more enzyme-substrate complexes and therefore more product formation. However, at some point all active sites of the enzyme reach saturation and further substrate addition cannot increase the reaction rate. In this case, the conversion rate is no longer dependent on the substrate concentration and becomes a limiting factor. At conditions above 10%, the conversion rate did not increase with increasing solid loading. The reason for the decrease in solid conversion could be the low working volume, mixing problems due to less liquid in the medium and adhesion of the enzyme to the solid could be an option. In practice this problem was encountered because the mixture was not sufficiently mixed as it attracted the solid-liquid and reduced the buffer in the medium. Since these conditions are difficult to apply on a large scale, it was decided to continue with the optimum condition of 10% solids loading. The decrease in hydrolysis efficiency in case of high solid:liquid ratio can explain the decrease in 16.67% solid loading. High solids loading in enzymatic hydrolysis can cause a drop as it results in a highly viscous slurry that can cause heat and mass transfer limitations when operated in a reactor. This decrease can generally be explained by lignin binding to the enzyme or low mass transfer rate and solubility (Gatt et al., 2019; Quiñones et al., 2015; Akpınar et al., 2009). However, high solids loading is preferred to achieve higher product concentration as it will reduce the distillation cost and make the process economical (Chen et al., 2017; Hernandez et al., 2018).

Table. 4.5. Solid loading effect on XOS Production (26.5 U/g-26.5 U/g Shearzyme 500L & Veron 191S deacetylated organosolv-treated corn cob hydrolyzed at 50°C)

Solid Loading (%)	**Time(h)**	**Concentration (g/l)**				
		Xylotetraose	**Xylotriose**	**Xylobiose**	**Xylose**	**Total Low DP XOS**
5	6	0.13 ± 0.01	0.69 ± 0.02	1.39 ± 0.27	0.10 ± 0.01	2.20 ± 0.3
	12	0.17 ± 0.01	1.09 ± 0.07	2.42 ± 0.2	0.08 ± 0.01	3.68 ± 0.2
	24	0.11 ± 0.08	1.10 ± 0.06	2.97 ± 0.13	0.22 ± 0.02	4.21 ± 0.2
	36	0.14 ± 0.0	1.08 ± 0.08	3.22 ± 0.20	0.33 ± 0.02	4.44 ± 0.2
	48	0.14 ± 0.01	1.23 ± 0.04	3.87 ± 0.21	0.47 ± 0.07	5.24 ± 0.3
10	6	0.33 ± 0.01	1.40 ± 0.012	3.04 ± 0.03	0.08 ±0.04	4.77 ±0.08
	12	0.40 ± 0.04	1.89 ± 0.04	4.86 ± 0.03	0.26 ± 0.08	7.14 ± 0.09
	24	0.33 ± 0.01	1.99 ± 0.04	5.93 ± 0.07	0.60 ± 0.01	8.25 ±0.13
	36	0.35 ± 0.01	2.19 ± 0.01	7.14 ± 0.01	1.10 ± 0.11	9.68 ± 0.04
	48	0.38 ± 0.07	2.44 ± 0.22	8.53 ± 0.31	1.44 ± 0.12	11.34 ± 0.4
12.5	6	0.21 ± 0.06	1.35 ± 0.04	3.78 ± 0.3	0.30 ± 0.2	5.35 ± 0.56
	12	0.25 ± 0.04	1.65 ± 0.2	4.89 ± 0.01	0.44 ± 0.01	6.79 ± 0.5
	24	0.27 ± 0.0	1.76 ± 0.05	6.40 ± 0.2	1.05 ± 0.01	8.43 ± 0.2
	36	0.33 ± 0.03	1.81 ± 0.013	7.84 ± 0.04	1.68 ± 0.05	9.98 ± 0.5
	48	0.31 ± 0.02	1.72 ± 0.01	7.88 ± 0.09	1.89 ± 0.09	9.98 ± 0.2
16.67	6	0.21 ± 0.03	1.09 ± 0.01	2.78 ± 0.04	0.13 ± 0.03	4.07 ± 0.06
	12	0.24 ± 0.02	1.28 ± 0.05	3.89 ± 0.5	0.35 ± 0.07	5.41 ± 0.5
	24	0.22 ± 0.09	0.77 ± 0.06	4.06 ± 0.07	0.87 ± 0.4	5.06 ± 0.6
	36	0.22 ± 0.01	1.15 ± 0.02	4.86 ± 0.03	1.01 ± 0.04	6.23 ± 0.03
	48	0.27 ± 0.05	1.37 ± 0.064	6.72 ± 0.3	1.65 ± 0.03	8.36 ± 0.09

The concentration of XOS (g/L). Different letters indicate statistically significant difference. ($p < 0.05$, One-way ANOVA and Tukey test.

4.2.4. Other Operational Modes of Enzymatic XOS Production

In this section, fed batch and sequential batch approaches are investigated for the improvement of the batch hydrolysis conditions.

4.2.4.1. Fed Batch

Fed-batch strategies have the advantages of mass transfer of substrates and enzymes, overcoming mixing difficulties due to maintaining low viscosity in the hydrolysis system and decreasing yields of high solids enzymatic hydrolysis. Compared to the single-step batch, the mixing power input requirement is lower (Mukasekuru et al., 2018 ; Xu et al., 2019 ; Gao et al.,2014). Fed-batch enzymatic hydrolysis involved the incremental addition of biomass and enzyme throughout the reaction to maintain optimal conditions and improve the efficiency of the hydrolysis process. Fed-batch hydrolysis provides an ongoing supply of substrate, allowing enzymes to function at their best for more extended periods. Higher yields of the intended product are produced as a result, improving production. This approach overcomes challenges such as substrate inhibition and enzyme deactivation that can occur in the early stages of the process (Gong et al., 2020; Xue et al., 2015). In the batch enzymatic hydrolysis tests, the optimum solids loading was found as 10%, which was set as the target for the fed-batch type hydrolysis. The pretreated corn cob and/or the enzymes were fed into the hydrolysis medium in three different approaches (detailed infromation given in the Table 4.6. this table shows the addition time of biomaas and enzyme) and they were compared with the batch hydrolysis.

In Fig. 4.3., a batch hydrolysis condition was created by adding biomass and enzyme to the buffer at the beginning of the reaction, which were targeted to react in condition A. And the reaction started with the initial addition of all the inputs. In condition B, the amount of enzyme included in batch hydrolysis was added to the buffer at the beginning of the reaction. Solid was added in batches in 4 different time periods, in total the same amount as batch hydrolysis. All enzymes and biomass that were react at the end of 24 hours have been completed in the hydrolyzate. In the C condition, the enzyme and biomass batch were added in 4 time periods to provide the amount of hydrolysis. As in condition B, the amount entering the batch condition was provided in 24 hours. In the D condition, it was worked with 5% solid loading. Here, batch hydrolysis was applied by adding enzyme and biomass at the beginning of the reaction. Here, the reaction started as a batch, and after 24 hours, the solid was washed and a fresh buffer was added. The reason for washing the solid here is to remove the enzymes attached to the solid and the remaining ions from the buffer. Fresh buffer was added to the washed solid in such a way that 5% solids were liquid to meet the initial condition. And the enzyme was calculated according to the amount of added solid. The results of these experiments are given in Table 4.7. In order to compare the batch conditions and fed batch conditions, 10% solid loading conditions and 5% solid loading batch conditions and the condition with fresh buffer & enzyme added after 24 h were added to the table.For the 5% solid loading condition in batch hydrolysis, the concentration of xylotetrose, xylotriose, xylobiose, xylose, and low DP XOS increases with increasing hydrolysis time. The highest concentration of low DP XOS is observed after 48 h of hydrolysis. After 48 h of hydrolysis, the xylobiose concentration increases to 3.9 g/l, and the low DP XOS concentration increases to 5.24 g/l. When 5% solid loading and D condition were compared, 5.24 g/l low DP XOS was obtained in batch hydrolysis, while 5.62 g/l low DP XOS was obtained by adding fresh buffer and enzyme in D condition. The aim of this approach was to maintain the hydrolysis of unhydrolyzed xylan in the processed corn cob by changing buffer and adding fresh enzyme. Thus, the questions of whether the product in the environment creates an obstacle to hydrolysis were investigated. While 4.39 g/l total low DP XOS was obtained in 24h with 5 grams of deacetylated organosolv solid, 1.23 g/l total low DP XOS was obtained with 4 grams of spent solid. After this approach was observed to be promising, an experimental model was created by evaluating spent solid in further experiments. This experimental model is given in Section 4.5.3.In the batch hydrolysis with 10% solid loading in, after 24 h of hydrolysis, the xylobiose

concentration is 3.17 g/l, and the low DP XOS concentration is 4.51 g/l. After 48 h of hydrolysis, the xylobiose concentration further increases to 8.526 g/l, and the low DP XOS concentration increases to 11.34 g/l. Comparing 5% solid loading and 10% solid loading, 33.2% conversions and 63% conversions were made. In the condition of adding fresh buffer, a total of 35.3 % conversion was achieved. For the 10% solid loading in fed-batch biomass hydrolysis condition, after 24 h of hydrolysis, the xylobiose concentration is 2.62 g/l, and the low DP XOS concentration is 3.86 g/l. After 48 h of hydrolysis, the xylobiose concentration increases to 4.80 g/l, and the low DP XOS concentration increases to 6.53 g/l. For the 10% solid loading in fed-batch biomass with enzyme hydrolysis condition, after 24 h of hydrolysis, the xylobiose concentration is 2.80 g/l, and the low DP XOS concentration is 4.08 g/l. After 48 h of hydrolysis, the xylobiose concentration further increases to 4.90 g/l, and the low DP XOS concentration increases to 7.27 g/l. Higher concentrations were obtained at the 6th and 12th hours, since higher enzyme was provided in the hydrolysate at the zero hour in the B condition. In the C condition, however, the enzyme was added in 4 different hours and hydrolysis therefore took place more slowly. However, after a total of 48 hours, 0.73 g/l more total low DP XOS was obtained in the C condition. It can be interpreted that the act of the enzyme on the solid becomes easier. From the comparisons, it can be observed that higher solid loading generally leads to higher concentrations of xylobiose and low DP XOS. Additionally, the presence of enzymes in the hydrolysis process (C condition at Figure 4.3.) results in higher concentrations compared to the hydrolysis without enzyme addition (fed-batch biomass). The solid loading and presence of enzymes in the hydrolysis process also play a role in determining the concentrations of these components. Based on the results obtained under these conditions, it has been decided to select the 10% batch condition for further experiments.

Table 4.6. Fed-Batch Enzymatic Hydrolysis Addition Procedures

Total Solid Loading (%, w/v)	**Hydrolysis Conditions**	**Time(h)**	**Addition Step**
10	Fed-Batch Biomass	0	2.5 gram Biomass
		6	2.5 gram Biomass
		12	2.5 gram Biomass
		24	2.5 gram Biomass
10	Fed-Batch Biomass & Enzyme	0	2.5 gram Biomass + 132.5U Enzyme
		6	2.5 gram Biomass + 132.5U Enzyme
		12	2.5 gram Biomass + 132.5U Enzyme
		24	2.5 gram Biomass + 132.5U Enzyme
5	Buffer & Enzyme Change	0	5 gram Biomass + 265U Enzyme
		24	Solids wash fresh buffer & enzyme

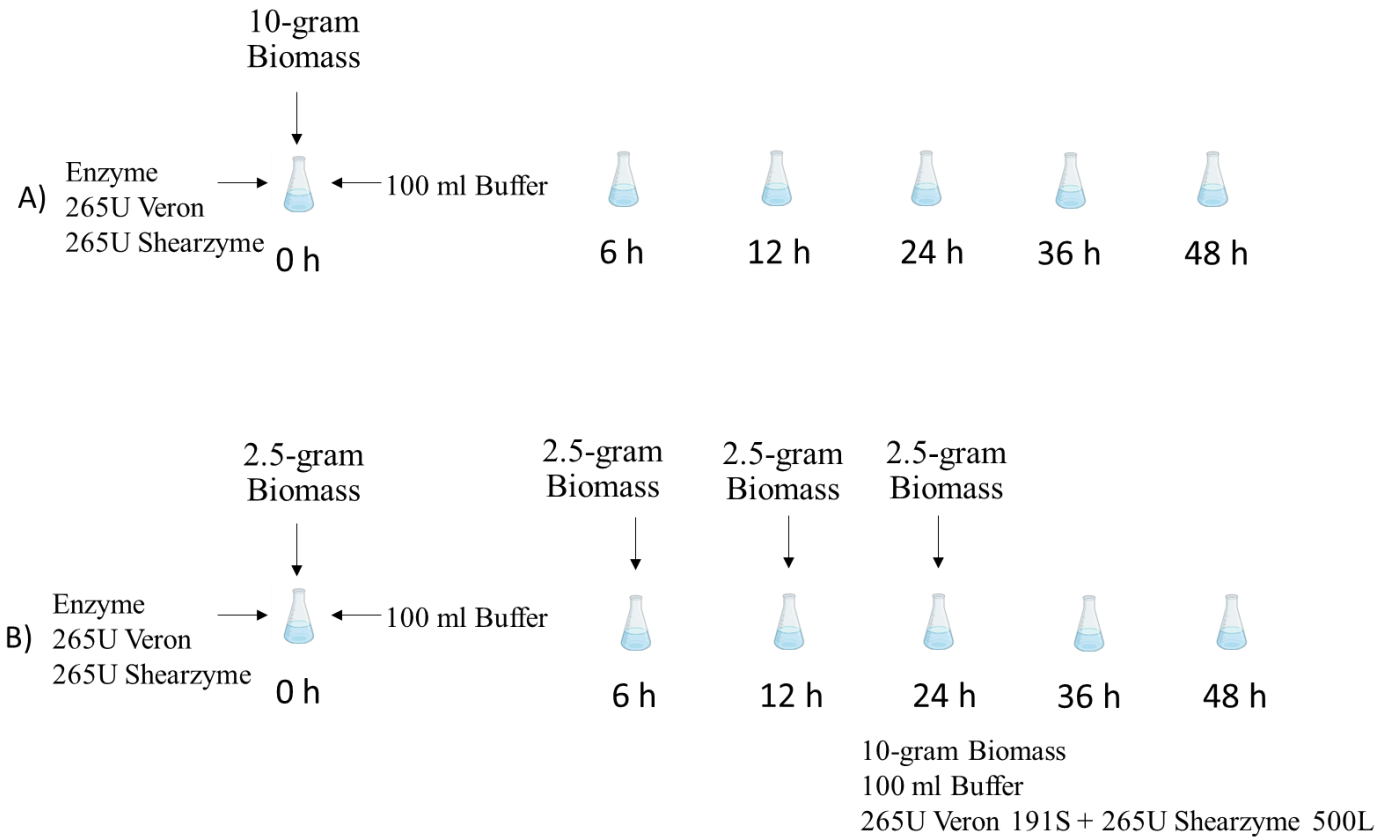


Figure 4.3. Enzymatic hydrolysis step A) Batch-type enzymatic hydrolysis 1:10 solid-liquid ratio, B) Fed-batch type biomass addition enzymatic hydrolysis 1:10 solid-liquid ratio, C) Fed-batch type biomass and enzyme addition enzymatic hydrolysis 1:10 solid-liquid ratio, D) Buffer change type enzymatic hydrolysis 1:10 solid-liquid ratio.

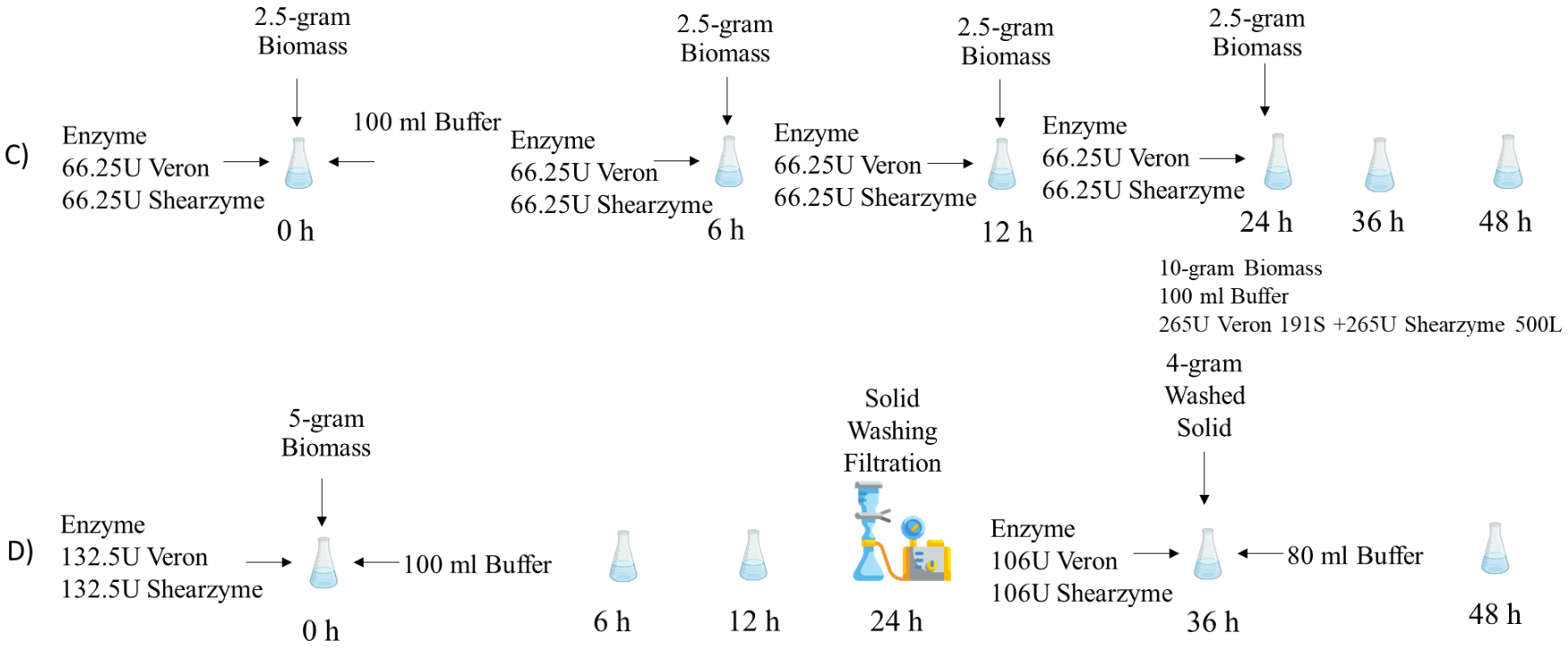


Figure 4.3. (cont.)

Table 4.7. Batch, Fed-Batch & Sequential Enzymatic Hydrolysis Effect on XOS

Solid Loading (w/v)	Hydrolysis Conditions	Time (h)	Concentration (g/l)				
			Xylotetraose	Xylotriose	Xylobiose	Xylose	Low DP XOS
5%	Batch	6	0.125	0.688	1.389	0.100	2.201
		12	0.169	1.093	2.415	0.077	3.677
		24	1.108	1.229	3.170	0.222	4.506
		36	0.135	1.085	3.219	0.329	4.839
		48	0.143	1.230	3.866	0.470	5.239
10%	Batch	6	0.330	1.401	3.043	0.078	4.774
		12	0.397	1.888	4.857	0.256	7.143
		24	0.328	1.993	5.928	0.598	8.248
		36	0.346	2.190	7.144	1.099	9.680
		48	0.376	2.440	8.526	1.442	11.341
10%	Fed-Batch Biomass	6	0.095	0.420	0.878	0.052	1.393
		12	0.113	0.703	1.605	0.096	2.422
		24	0.155	1.081	2.620	0.288	3.856
		36	0.174	1.303	3.445	0.431	4.923
		48	0.209	1.522	4.801	0.509	6.532
10%	Fed-Batch Biomass & Enzyme	6	0.038	0.346	0.616	0.036	1.000
		12	0.111	0.676	1.285	0.043	2.072
		24	0.149	1.138	2.797	0.143	4.084
		36	0.174	1.321	3.534	0.244	5.030
		48	0.215	1.504	4.901	0.649	7.269
5%	After 24h Fresh Buffer	6	0.138	0.795	1.593	0.045	2.525
		12	0.143	0.969	2.169	0.078	3.281
		24	0.157	1.167	3.065	0.220	4.390
		36	0.019	0.140	0.294	0.057	0.453
		48	0.028	0.719	0.479	0.058	1.226

4.2.4.2. Sequential Batch

Sequential batch is used to express that the hydrolysis process is carried out step by step or sequentially. This means that different hydrolysis reactions or steps are carried out one after the other. A new experiment was designed to reuse the enzymatic hydrolysis liquid and solid and to obtain XOS from this solid and liquid part. The constructed experiment is shown in Fig. 4.4. and explained in detail conditions in Section 3.2.1.5. These experimental results are shown in Fig. 4.4. was lettered, and the results are given in Table 4.8. Sustainable enzymatic hydrolysis conditions for both solid and liquid were investigated with the sequential batch. With one of these approaches, it is aimed to reuse the active enzyme remaining in the hydrolysate. By removing the solid residue from the hydrolysate, new biomass was added to the liquid hydrolysate containing the enzyme, and by this means, the continuity of the hydrolysis was tried to be ensured by utilizing the active enzyme in the liquid. In this study, experiments were performed using Veron 26.5U/g biomass and Shearzyme 500L 26.5U/g biomass enzyme mixture at 10% solid-liquid ratio at 50ºC. In condition E, 6.84 g/l xylobiose and 8.46 g/l total low DP XO were obtained after 24 hours. The solid and liquid coming out of the hydrolysis were separated by the filter and the liquid volume was divided into 2 equal parts. By adding only solid biomass to the first liquid to provide 10% solid liquid ratio, the continuity of the hydrolysis was tried to be ensured by making use of the active enzyme in the liquid (Condition G). In this way, the active enzyme in the liquid was able to continue the hydrolysis without the need to add new enzymes. In addition, it is aimed to prevent the accumulation of solids and the adhesion of enzymes to the biomass, thus reducing the enzyme activity and slowing the reaction rate by separating the non-hydrolyzed solid in the medium. When only the solid was added, 2.49 g/l of xylobiose and 3.5 g/l of low DP XOS were obtained. This condition indicates that the active enzyme was still present in the liquid and could act on the xylan in the new solid. And in this way, the total low DP XOS in the liquid has increased to 11.9 g/l. In condition H, after the initial hydrolysis, a new solid was added 3.29 g/l of xylobiose and 4.93 g/l of low DP XOS were obtained. When solid was added to the liquid again, the total low DP XOS concentration increased

up to 13.39 g/l. This allowed us to conclude that the activity of the enzyme continues and the xylan in the solid hydrolyzes to obtain a higher concentration hydrolysate. In this case, the volume can be reduced to obtain concentrated liquid and to reduce the required equipment size. The purification process is also facilitated by the highly concentrated liquid. However, the product concentration facilitating purification cannot be understood by evaluating the product concentration in the reaction medium alone. Other factors affecting purification processes should also be evaluated. Besides the XOS obtained because of the reaction, by-products and contaminants may also be formed. These by-products and contaminants can complicate the purification process and require unnecessary processing. However, since organosolv treated corn cob is used in this process, in which xylan remains solid, there are no by-products such as furans, aliphatic acids, and HMF in the hydrolysis medium, so the obstacles in the purification process are removed.

Another reason for the decrease in yield may be xylan derivatives produced in the environment. Studies for cellulase have shown that xylan derivatives inhibit cellulase. Qing et al. (2010) observed the effects of xylose and xylan derivatives on the initial hydrolysis rates of enzymatic hydrolysis by adding different concentrations (1.67 mg/ml, 12.5 mg/ml) of xylan, xylose and xylo-oligomer to cellulase. As the concentration of added xylan, xylose and XOS increased, the initial rate of enzymatic hydrolysis decreased and an inhibitory effect was observed. Kont et al. (2013) proposed two scenarios for the inhibition of cellulose hydrolysis by polymeric xylans and KOS: (i) xylans bind to the cellulose surface, limiting the accessibility of cellulose to cellulases, and (ii) xylans bind to the active sites of cellulases, competing with the binding of the cellulose chain. Also, Kont et al. (2013) emphasized that oligosaccharides (GOS, XOS) are 100 times more potent inhibitors than cellobiose. Zhang & Viikari (2012), in their article examining the effects of XOS and xylose on the activity of cellulase, stated that xylobiose and xylotrioses, which bind to the active site of cellobiohydrolase I, reduce the activity of cellulases and that XOS are potent inhibitors. In another study supporting these studies, the inhibitory effect of XOS for cellulases was mentioned and the effect of XOS lengths on binding was also examined (Baumann et al., 2011). Also, Kim et al. (2011) observed that XOS and xylose instantly inhibit cellulases, especially in the early stages of enzymatic hydrolysis. As a result of these studies with cellulase enzyme in the literature, it has been stated that xylan derivatives, xylose and xylooligosaccharides cause product

inhibition and decrease hydrolysis efficiency. In this study, in order to investigate whether components such as xylooligosaccharides and xylose in the hydrolysate are an obstacle to the hydrolysis of xylan, the condition in which the other half of the separated liquid is added together with the enzyme and biomass was created. When the enzyme and new solid were added to this liquid, the concentrations increased to 12.81 g/l of xylobiose and 15.43 g/l of total XOS. It was determined that there was no product inhibition in sequential hydrolysis, almost the same amount of product was obtained compared to batch hydrolysis. . With the help of the same liquid, higher concentration XOS could be obtained. In this case, it facilitated purification.

In contrast, 15.43 g/l of total XOS could be obtained from 2 grams of biomass under the experimental condition with enzyme supplementation. The enzyme addition was adjusted based on the amount of xylanase added per xylan in the deacetylated biomass, as the xylan content of the solid resulting from enzymatic hydrolysis decreased. It was observed that the enzyme was still active even though less increase in concentration was observed compared to the first hydrolysis. 13.39 g/l low DP XOS could be obtained without the addition of enzyme. Since non-hydrolyzed xylan remains in solid after hydrolysis, it was aimed to use this xylan in the hydrolysis again. And this solid (spent solid), which came out of hydrolysis, was subjected to hydrolysis by adding enzyme and a new buffer. For the investigate spent solid, under condition I, 2.56 g/l of xylobiose and 3.02 g/l of total low DP XOS were obtained in 24 h, while 3.54 g/l of xylobiose and 4.1 g/l of total low DP XOS were obtained in 48 h.

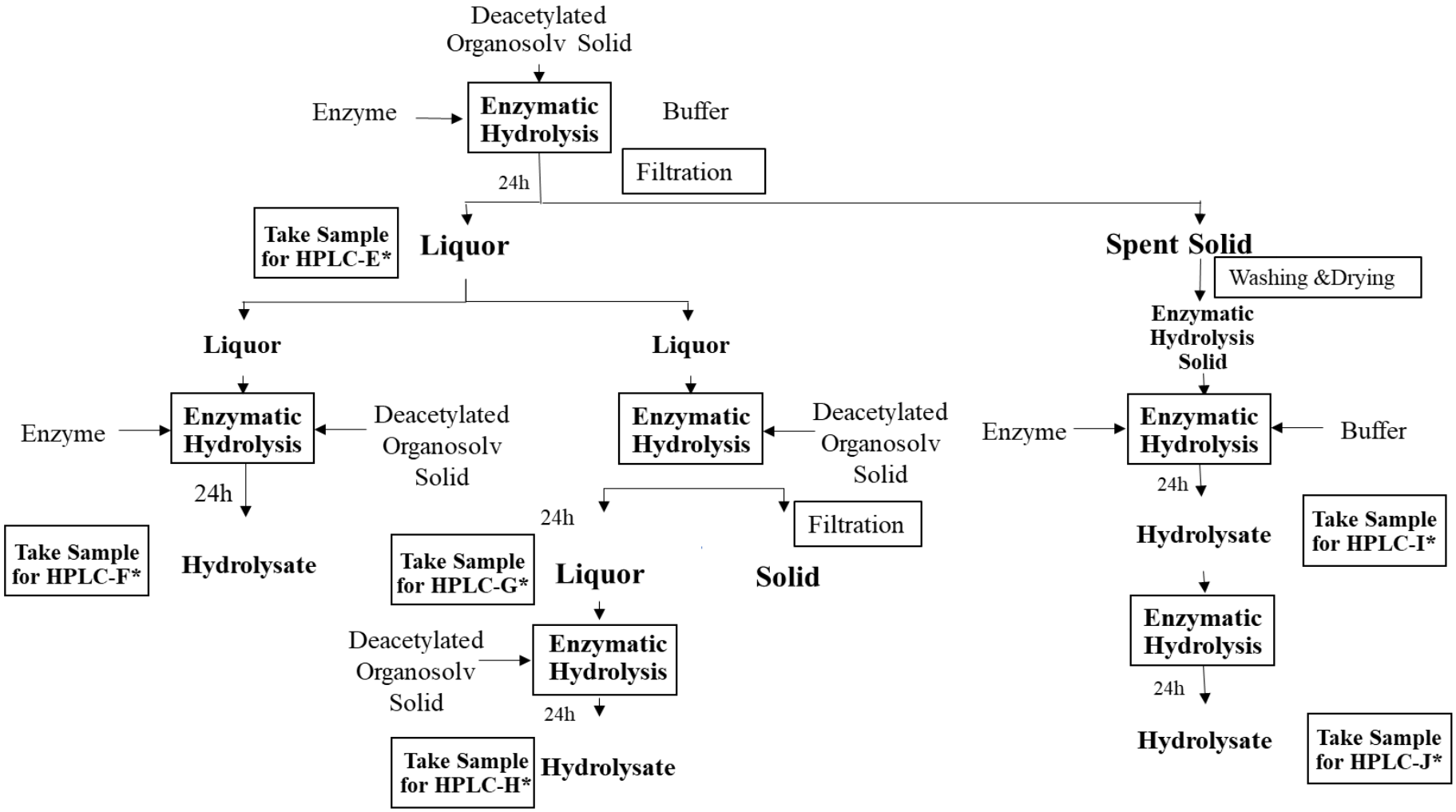


Figure 4.4. Sequential Batch Enzymatic Hydrolysis Scheme

Table 4.8. Sequential Batch (Figure shows the means of E, F, G, H, I, J)

Condition	**Concentration (g/l)**				
	Xylotetraose	**Xylotriose**	**Xylobiose**	**Xylose**	**Total Low DP XOS**
E	0.55	1.07	6.84	0.77	8.46
F	0.82	1.80	12.81	2.04	15.43
G	0.73	1.91	9.33	1.16	11.96
H	1.08	2.18	10.13	1.36	13.39
I	0.13	0.33	2.56	0.54	3.02
J	0.24	0.32	3.54	1.01	4.10

As a result, when comparing conditions F and G, it can be observed that both processes lasted 48 h and utilized the same amount of biomass. However, in condition F, a higher amount of enzyme was added, resulting in a 4 g/l increase in the concentrations. In condition I, although the hydrolysis also lasted 48 h, the drying of the solid took additional time. However, if the weight of the solid is known, it can be hydrolyzed without the need for drying. In this condition, the concentration of XOS obtained from hydrolysis decreased due to the reduced amount of xylan available for the enzyme to act upon. This suggests that while a portion of xylan was hydrolyzed, another portion remained unhydrolyzed. There is unhydrolyzed xylan in the biomass and this xylan can be used. In condition H, the addition of a new solid along with a new enzyme and buffer after the initial hydrolysis is not logical. It would be more efficient to use a new solid with fresh enzyme and buffer to achieve better results. In order to benefit from sequential batch in industrial XOS production, cost calculation for biomass, enzyme and buffer is important for the condition to be selected.

4.2.5. Effect of Surface-active Additives and Non-Catalytic Proteins on Enzymatic Hydrolysis

In previous studies in the literature, surfactants and BSA were shown to exert a positive effect on cellulase, as they prevent the non-specific binding of the enzyme to lignin, increase cell wall permeability, help cell wall-bound enzymes to be released into the liquid phase, and protect cellulases from surface inactivation. (Tejirian & Xu ,2011; Sipos et al.,2011 Bhagia et al.,2017). It has been stated that Tween 20 and 80 swell the fiber and increase the surface area, improve the adsorption of the enzyme to cellulose, and thus enable the development of enzymatic hydrolysis (Bhagia et al., 2017; Alkasrawi et al., 2003). PEG, lignosulfonate, BSA, Tween 20 and Tween 80, which were shown previously to prevent cellulase enzyme from adhering to biomass, were tested to increase the effect of xylanase on solid biomass. Although there are studies on cellulase enzyme in the literature on this subject, there is limited information about the effect of xylanase enzyme with additives on lignin and its effect on improving the benefit of enzymatic hydrolysis. In order to improve this limited information, the effect of surfactant and non-catalytic proteins on xylanase enzyme and biomass was investigated in this study. The effect of additives on enzymatic hydrolysis of lignocellulosic biomass was investigated by comparing the additives with the control. These experiments were carried out using 70U/g biomass Shearzyme 500L with 5% solid loading at 50°C, pH 5.5 conditions as a substrate organosolv treated corn cob used. The results are presented in Fig. 4.5. and 4.6. showing the concentrations of xylobiose and xylose, respectively.

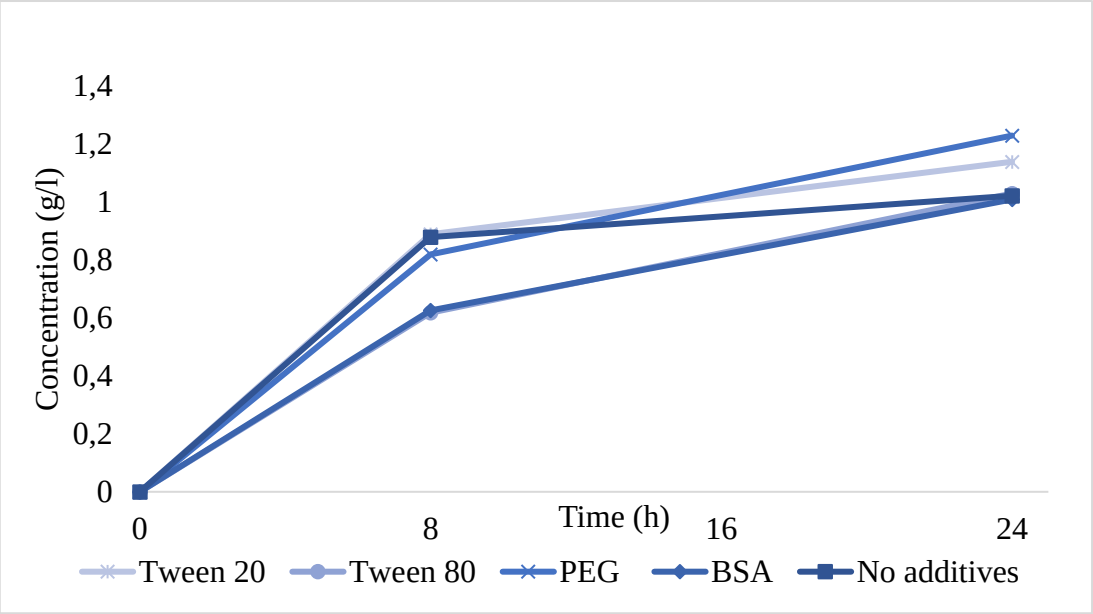


Figure 4.5. Effect of Surfactant on Xylobiose Concentration at 50°C, 5% solid loading (w/v)

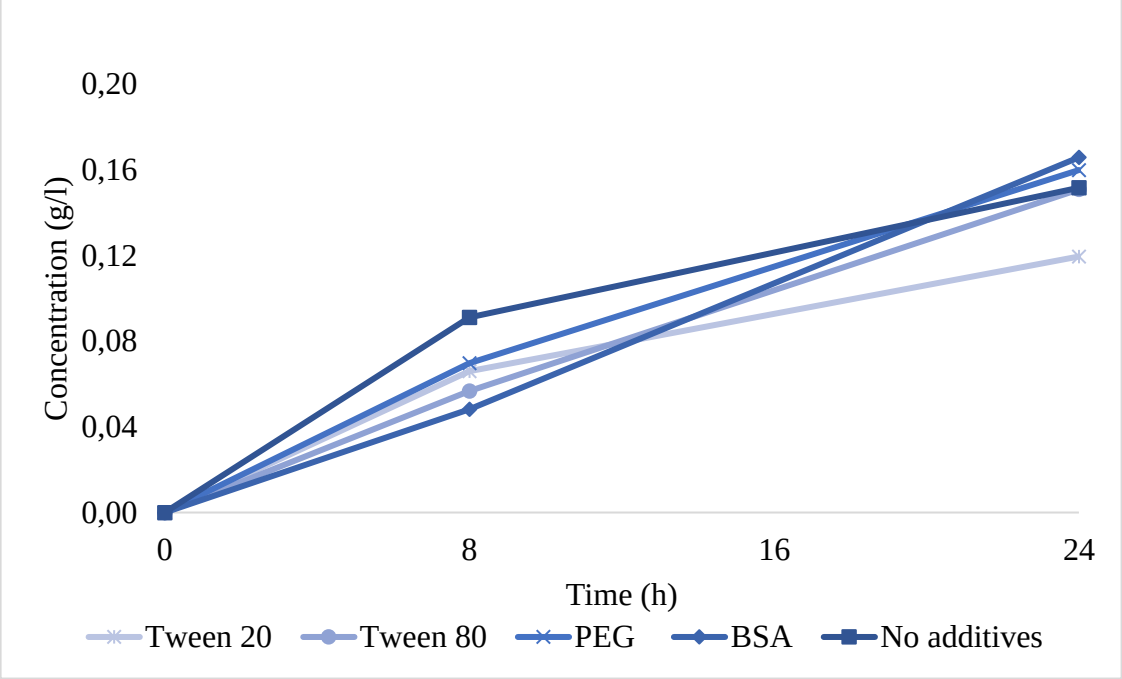


Figure 4.6. Effect of Surfactant on Xylose Concentration at 50°C, 5% solid loading (w/v)

It was decided not to use surfactants in further experiments, as they did not represent a significant value for development. Therefore, other approaches have been sought to improve enzymatic hydrolysis. These results showed that additives that showed a positive effect for the cellulase enzyme did not show the same effect for the xylanase

enzyme. Bhagia et al (2017) mentioned that BSA and Tween 20 were used to block the lignin surface. No effect of BSA on xylan yield was observed, and it is not known why it only affects glucan yield. In another study by Kristensen et al. (2007), surfactants' effect on xylan conversion was not as pronounced as the effect of increasing glucose. As a result, the positive effects of additives on xylanases could not be observed in the literature and this study.

CHAPTER 5

CONCLUSIONS

Xylo-oligosaccharides (XOS) demonstrate significant potential for various industries such as food, pharmaceuticals, feed formulations, and agriculture. In this field, XOS offers distinct advantages over other oligosaccharides in terms of their positive health effects and optimal concentration levels. Alkaline extraction, autohydrolysis and acid hydrolysis are the commonly used methods for XOS production. The use of strong bases and chemicals such as ethanol is quite high for XOS obtained by alkaline extraction and enzymatic hydrolysis. At the same time, extraction methods are multi-step and take a long time. In autohydrolysis, sugar degradation products are formed at high temperatures and hydrolysate with mixed degree of polymerization is formed and different degradation products are present in the hydrolysate. Nevertheless, the relatively higher costs associated with their production indicate the need for new approaches in this regard on the market. Therefore, additional enhancements in processing technology are required to facilitate a wider adoption of XOs and accelerate market development.

The main approach in this work was to use the organosolv method, where the xylan was retained in the solid part and then hydrolyzed enzymatically, thus avoiding the need for extraction and the formation of degradation products. In this study, various approaches were used to increase xylanase enzyme activity and develop efficient processes for XOS production. Commercial xylanases (Shearzyme 500L and Veron 191S) were evaluated separately and in combination, and it was revealed that they allowed higher XOS concentrations and conversions when used together. This means that the two enzymes have different modes of action and with the synergistic effect can enhance enzymatic hydrolysis. Deacetylation of organosolv-treated corn cob was performed to remove acetyl groups and the effect on hydrolysis efficiency of deacetylated

biomass at different NaOH concentrations and temperatures was evaluated. The presence of an acetyl group on the substrate can cause structural and chemical changes that impair the interaction between the enzyme and the substrate and reduce the efficiency of the hydrolysis reaction, in which case the removal of acetyl groups may be associated with increasing the efficiency of hydrolysis. Different solid loadings were tested to improve the enzymatic hydrolysis efficiency by utilizing the synergistic effects of enzymes and deacetylated organosolv-treated corn cob. In these experiments, hydrolysis efficiency was improved when the solid loading was increased from 5% to 10%, while a decrease in hydrolysis efficiency was observed when the solid loading was increased to 12.5% and 16.6%. The decrease in solid conversion may be due to the low working volume, mixing problems caused by the low amount of liquid in the medium and the adhesion of the enzyme to the solid. Here, it can be said that mass and heat transfer may have become difficult due to high viscosity in the hydrolysate, therefore, xylan in the solid could not be efficiently converted to XOS. Hydrolysis was tested with fed and sequential batch approaches with 10% solid loading, which has the highest efficiency in batch. In these experimental conditions in which enzyme and biomass were added intermittently, no increase in efficiency was achieved, and it was revealed that there was no substrate inhibition in batch hydrolysis with these experiments. The conversion of xylan to XOS with fed-batch addition of biomass was slower than in batch conditions, which may be due to the inability to form an enzyme-substrate complex because the xylan ratio in the medium was limited while the initial enzyme concentration was high. Another approach was to sustain the hydrolysis of xylan in pretreated corncobs by changing the buffer and adding fresh enzymes. With this approach, the same amount of XOS obtained in batch hydrolysis. The sequential batch is like a fed-batch, except that the fresh solid and enzyme were added in bulk. New enzyme and solid were added and no product inhibition was observed. New solid was added and production continued, indicating that the enzyme was still active. In the other approach, the presence of non-hydrolyzed xylan in the biomass was proven and it was observed that this spent solid was reusable. However, we can also conclude that a small part of the solid was not used. As a result, high XOS concentration was obtained in this case, thus facilitating purification. In the Sequential Batch approach, it has been shown that since the enzyme and buffer can be reused, the cost reduction and the convenience of working with lower-volume equipment can be achieved. Another approach for the enhancement of xylanase activity for XOS production was using surface active materials. Surfactants known to enhance cellulase enzyme performance were tested

to overcome limitations in enzymatic hydrolysis. However, these additives did not demonstrate a positive effect on the xylanase enzyme.

In conclusion, the approach to XOS production with deacetylated organosolv-treated corn cob can contribute to the development of a sustainable process for industrial production by enabling efficient use of the substrate and generating product-rich solutions. With this approach, other lignocellulosic materials can also be expanded.

REFERENCES

Aachary, Ayyappan Appukuttan, and Siddalingaiya Gurudutt Prapulla. 2009. “Value Addition to Corncob: Production and Characterization of Xylooligosaccharides from Alkali Pretreated Lignin-Saccharide Complex Using *Aspergillus oryzae* MTCC 5154.” *Bioresource Technology* 100 (2): 991–95. https://doi.org/10.1016/j.biortech.2008.06.050.

Aachary, Ayyappan Appukuttan, and Siddalingaiya Gurudutt Prapulla. “Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications.” Comprehensive Reviews in Food Science and Food Safety 10, no. 1 (2010): 2–16. https://doi.org/10.1111/j.1541-4337.2010.00135.

Akpinar, Ozlem, Kader Erdogan, and Seyda Bostanci. 2009a. “Enzymatic Production of Xylooligosaccharide from Selected Agricultural Wastes.” *Food and Bioproducts Processing* 87 (2): 145–51. https://doi.org/10.1016/j.fbp.2008.09.002.

Alkasrawi, Malek, Torny Eriksson, Johan Börjesson, Anders Wingren, Mats Galbe, Folke Tjerneld, and Guido Zacchi. 2003. “The Effect of Tween-20 on Simultaneous Saccharification and Fermentation of Softwood to Ethanol.” *Enzyme and Microbial Technology* 33 (1): 71–78. https://doi.org/10.1016/S0141-0229(03)00087-5.

Almeida, João R M, Tobias Modig, Anneli Petersson, Bärbel Hähn-Hägerdal, Gunnar Lidén, and Marie F Gorwa-Grauslund. 2007. “Increased Tolerance and Conversion of Inhibitors in Lignocellulosic Hydrolysates by *Saccharomyces cerevisiae*.” *Journal of Chemical Technology & Biotechnology* 82 (4): 340–49. https://doi.org/https://doi.org/10.1002/jctb.1676.

Amorim, Cláudia, Sara C Silvério, Kristala L J Prather, and Lígia R Rodrigues. 2019. “From Lignocellulosic Residues to Market: Production and Commercial Potential of Xylooligosaccharides.” *Biotechnology Advances* 37 (7): 107397. https://doi.org/https://doi.org/10.1016/j.biotechadv.2019.05.003.

Anwar, Zahid, Muhammad Gulfraz, and Muhammad Irshad. 2014. “Agro-Industrial Lignocellulosic Biomass a Key to Unlock the Future Bio-Energy: A Brief Review.” *Journal of Radiation Research and Applied Sciences* 7 (2): 163–73. https://doi.org/https://doi.org/10.1016/j.jrras.2014.02.003.

Arato, Claudio, E Kendall Pye, and Gordon Gjennestad. 2005. “The Lignol Approach to Biorefining of Woody Biomass to Produce Ethanol and Chemicals.” *Applied Biochemistry and Biotechnology* 123 (1): 871–82. https://doi.org/10.1385/ABAB:123:1-3:0871.

Assis Castro, Rafael Cunha de, Bruno Guedes Fonseca, Hilton Túlio Lima dos Santos, Isabela Silveira Ferreira, Solange Inês Mussatto, and Inês Conceição Roberto. 2017. “Alkaline Deacetylation as a Strategy to Improve Sugars Recovery and Ethanol Production from Rice Straw Hemicellulose and Cellulose.” *Industrial Crops and Products* 106: 65–73. https://doi.org/https://doi.org/10.1016/j.indcrop.2016.08.053.

Behera, Shuvashish, Richa Arora, N Nandhagopal, and Sachin Kumar. 2014. “Importance of Chemical Pretreatment for Bioconversion of Lignocellulosic Biomass.” *Renewable and Sustainable Energy Reviews* 36: 91–106. https://doi.org/https://doi.org/10.1016/j.rser.2014.04.047.

Berlin, Alex, Neil Gilkes, Arwa Kurabi, Renata Bura, Maobing Tu, Douglas Kilburn, and John Saddler. 2005. “Weak Lignin-Binding Enzymes.” *Applied Biochemistry and Biotechnology* 121 (1): 163–70. https://doi.org/10.1385/ABAB:121:1-3:0163.

Bhagia, Samarthya, Rajeev Kumar, and Charles E Wyman. 2017. “Effects of Dilute Acid and Flowthrough Pretreatments and BSA Supplementation on Enzymatic Deconstruction of Poplar by Cellulase and Xylanase.” *Carbohydrate Polymers* 157: 1940–48. https://doi.org/https://doi.org/10.1016/j.carbpol.2016.11.085.

Blaut, Michael. 2002. “Relationship of Prebiotics and Food to Intestinal Microflora.” *European Journal of Nutrition* 41 (1): i11–16. https://doi.org/10.1007/s00394-002-1102-7.

Boonchuay, Pinpanit, Charin Techapun, Phisit Seesuriyachan, and Thanongsak Chaiyaso. 2014. “Production of Xylooligosaccharides from Corncob Using a Crude Thermostable Endo-Xylanase from *Streptomyces thermovulgaris* TISTR1948 and Prebiotic

Properties.” *Food Science and Biotechnology* 23 (5): 1515–23. https://doi.org/10.1007/s10068-014-0207-0.

Börjesson, Johan, Ragna Peterson, and Folke Tjerneld. 2007. “Enhanced Enzymatic Conversion of Softwood Lignocellulose by Poly(Ethylene Glycol) Addition.” *Enzyme and Microbial Technology* 40 (4): 754–62. https://doi.org/https://doi.org/10.1016/j.enzmictec.2006.06.006.

Brinchi, L, F Cotana, E Fortunati, and J M Kenny. 2013. “Production of Nanocrystalline Cellulose from Lignocellulosic Biomass: Technology and Applications.” *Carbohydrate Polymers* 94 (1): 154–69. https://doi.org/https://doi.org/10.1016/j.carbpol.2013.01.033.

Brosse, Nicolas, Poulomi Sannigrahi, and Arthur Ragauskas. 2009. “Pretreatment of Miscanthus x Giganteus Using the Ethanol Organosolv Process for Ethanol Production.” *Industrial & Engineering Chemistry Research* 48 (18): 8328–34. https://doi.org/10.1021/ie9006672.

Buyukkileci, Ali Oguz, and Nuran Temelli. 2023. “Organosolv Pretreatment of Corncob for Enzymatic Hydrolysis of Xylan.” *Biomass Conversion and Biorefinery* 13 (7): 6385–94. https://doi.org/10.1007/s13399-023-03786-w.

Carvalho, Ana Flávia Azevedo, Pedro de Oliva Neto, Douglas Fernandes da Silva, and Gláucia Maria Pastore. 2013a. “Xylo-Oligosaccharides from Lignocellulosic Materials: Chemical Structure, Health Benefits and Production by Chemical and Enzymatic Hydrolysis.” *Food Research International* 51 (1): 75–85. https://doi.org/https://doi.org/10.1016/j.foodres.2012.11.021.

Cebreiros, Florencia, Leonardo Clavijo, Elzeario Boix, Mario Daniel Ferrari, and Claudia Lareo. 2020. “Integrated Valorization of Eucalyptus Sawdust within a Biorefinery Approach by Autohydrolysis and Organosolv Pretreatments.” *Renewable Energy* 149: 115–27. https://doi.org/https://doi.org/10.1016/j.renene.2019.12.024.

Chapla, Digantkumar, Pratima Pandit, and Amita Shah. 2012. “Production of Xylooligosaccharides from Corncob Xylan by Fungal Xylanase and Their Utilization by Probiotics.” *Bioresource Technology* 115: 215–21. https://doi.org/https://doi.org/10.1016/j.biortech.2011.10.083.

Chattopadhyay, Sanchari, Utpal Raychaudhuri, and Runu Chakraborty. 2014. “Artificial Sweeteners – a Review.” *Journal of Food Science and Technology* 51 (4): 611–21. https://doi.org/10.1007/s13197-011-0571-1.

Chen, Hong-Zhang, and Zhi-Hua Liu. 2017. “Enzymatic Hydrolysis of Lignocellulosic Biomass from Low to High Solids Loading.” *Engineering in Life Sciences* 17 (5): 489–99. https://doi.org/https://doi.org/10.1002/elsc.201600102.

Chen, Ming, Jing Zhao, and Liming Xia. 2008. “Enzymatic Hydrolysis of Maize Straw Polysaccharides for the Production of Reducing Sugars.” *Carbohydrate Polymers* 71 (3): 411–15. https://doi.org/https://doi.org/10.1016/j.carbpol.2007.06.011.

Chen, Xiaowen, Nathan Crawford, Wei Wang, Erik Kuhn, David Sievers, Ling Tao, and Melvin Tucker. 2019. “Kinetics and Rheological Behavior of Higher Solid (Solids >20%) Enzymatic Hydrolysis Reactions Using Dilute Acid Pretreated, Deacetylation and Disk Refined, and Deacetylation and Mechanical Refined (DMR) Corn Stover Slurries.” *ACS Sustainable Chemistry & Engineering* 7 (1): 1633–41. https://doi.org/10.1021/acssuschemeng.8b05391.

Chen, Xiaowen, Joseph Shekiro, Rick Elander, and Melvin Tucker. 2012. “Improved Xylan Hydrolysis of Corn Stover by Deacetylation with High Solids Dilute Acid Pretreatment.” *Industrial & Engineering Chemistry Research* 51 (1): 70–76. https://doi.org/10.1021/ie201493g.

Chen, Xiaowen, Joseph Shekiro, Mary Ann Franden, Wei Wang, Min Zhang, Erik Kuhn, David K Johnson, and Melvin P Tucker. 2012. “The Impacts of Deacetylation Prior to Dilute Acid Pretreatment on the Bioethanol Process.” *Biotechnology for Biofuels* 5 (1): 8. https://doi.org/10.1186/1754-6834-5-8.

Chundawat, Shishir P S, Venkatesh Balan, and Bruce E Dale. 2008. “High-Throughput Microplate Technique for Enzymatic Hydrolysis of Lignocellulosic Biomass.” *Biotechnology and Bioengineering* 99 (6): 1281–94. https://doi.org/https://doi.org/10.1002/bit.21805.

Colakoğlu, B. “Tarımsal Atıkların Alternatif Kullanım Alanları Konusunda Üretici Eğilimleri .” (Master’s thesis, Namık Kemal Üniversitesi)., 2018.

Czajkowski, Łukasz, Dawid Wojcieszak, Wiesław Olek, and Jacek Przybył. 2019. “Thermal Properties of Fractions of Corn Stover.” *Construction and Building Materials* 210: 709–12. https://doi.org/https://doi.org/10.1016/j.conbuildmat.2019.03.092.

Dijkerman, Rembrandt, Diana C P Bhansing, Huub J M Op den Camp, Chris van der Drift, and Godfried D Vogels. 1997. “Degradation of Structural Polysaccharides by the Plant Cell-Wall Degrading Enzyme System from Anaerobic Fungi: An Application Study.” *Enzyme and Microbial Technology* 21 (2): 130–36. https://doi.org/https://doi.org/10.1016/S0141-0229(96)00251-7.

Ding, Changhe, Mengxing Li, and Yuqi Hu. 2018. “High-Activity Production of Xylanase by Pichia Stipitis: Purification, Characterization, Kinetic Evaluation and Xylooligosaccharides Production.” *International Journal of Biological Macromolecules* 117: 72–77. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2018.05.128.

Doherty, William O S, Payam Mousavioun, and Christopher M Fellows. 2011. “Value-Adding to Cellulosic Ethanol: Lignin Polymers.” *Industrial Crops and Products* 33 (2): 259–76. https://doi.org/https://doi.org/10.1016/j.indcrop.2010.10.022.

Duff, Sheldon J B, and William D Murray. 1996. “Bioconversion of Forest Products Industry Waste Cellulosics to Fuel Ethanol: A Review.” *Bioresource Technology* 55 (1): 1–33. https://doi.org/https://doi.org/10.1016/0960-8524(95)00122-0.

Dyk, J S Van, and B I Pletschke. 2012. “A Review of Lignocellulose Bioconversion Using Enzymatic Hydrolysis and Synergistic Cooperation between Enzymes—Factors Affecting Enzymes, Conversion and Synergy.” *Biotechnology Advances* 30 (6): 1458–80. https://doi.org/https://doi.org/10.1016/j.biotechadv.2012.03.002.

Ebringerová, Anna, Zdenka Hromádková, and Thomas Heinze. “Hemicellulose.” Polysaccharides I, n.d., 1–67. https://doi.org/10.1007/b136816.

Ebringerová, Anna. “Structural Diversity and Application Potential of Hemicelluloses.” Macromolecular Symposia 232, no. 1 (2005): 1–12. https://doi.org/10.1002/masy.200551401.

Eckard, Anahita Dehkhoda, Kasiviswanathan Muthukumarappan, and William Gibbons. 2012. “Pretreatment of Extruded Corn Stover with Polyethylene Glycol to Enhance Enzymatic Hydrolysis: Optimization, Kinetics, and Mechanism of Action.” *BioEnergy Research* 5 (2): 424–38. https://doi.org/10.1007/s12155-011-9162-2.

Eriksson, Torny, Johan Börjesson, and Folke Tjerneld. 2002. “Mechanism of Surfactant Effect in Enzymatic Hydrolysis of Lignocellulose.” *Enzyme and Microbial Technology* 31 (3): 353–64. https://doi.org/https://doi.org/10.1016/S0141-0229(02)00134-5.

Gao, Yueshu, Jingliang Xu, Zhenhong Yuan, Yu Zhang, Yunyun Liu, and Cuiyi Liang. 2014. “Optimization of Fed-Batch Enzymatic Hydrolysis from Alkali-Pretreated Sugarcane Bagasse for High-Concentration Sugar Production.” *Bioresource Technology* 167: 41–45. https://doi.org/https://doi.org/10.1016/j.biortech.2014.05.034.

Gatt, Etienne, Vinay Khatri, Julien Bley, Simon Barnabé, Virginie Vandenbossche, and Marc Beauregard. 2019. “Enzymatic Hydrolysis of Corn Crop Residues with High Solid Loadings: New Insights into the Impact of Bioextrusion on Biomass Deconstruction Using Carbohydrate-Binding Modules.” *Bioresource Technology* 282: 398–406. https://doi.org/https://doi.org/10.1016/j.biortech.2019.03.045.

Ghosh, A.R. “Appraisal of Probiotics and Prebiotics in Gastrointestinal Infections Appraisal of Probiotics and Prebiotics in Gastrointestinal Infections. .” WebmedCentral Gastroenterol. 3, 1–27. , 2012. https://doi.org/https://doi.org/10.9754/journal.wmc.2012.003796.

Ghosh, Arabinda, Saikat Sutradhar, and Debabrat Baishya. 2019. “Delineating Thermophilic Xylanase from *Bacillus licheniformis* DM5 towards Its Potential Application in Xylooligosaccharides Production.” *World Journal of Microbiology and Biotechnology* 35 (2): 34. https://doi.org/10.1007/s11274-019-2605-1.

Goldman, N. 2009. “Methods for optimizing enzymatic hydrolysis of xylan to improve xylooligosaccharide yield.” Basic Biotechnology Journal 5: 31–36.

Gong CS, Chen LF, Flickinger MC, Tsao GT (1981).” Conversion of hemicellulose carbohydrates.” Adv Biochem Eng Biotechnology 20:93–118

Gírio, F M, C Fonseca, F Carvalheiro, L C Duarte, S Marques, and R Bogel-Łukasik. 2010. “Hemicelluloses for Fuel Ethanol: A Review.” *Bioresource Technology* 101 (13): 4775–4800. https://doi.org/https://doi.org/10.1016/j.biortech.2010.01.088.

Grohmann, K, D J Mitchell, M E Himmel, B E Dale, and H A Schroeder. 1989. “The Role of Ester Groups in Resistance of Plant Cell Wall Polysaccharides to Enzymatic

Hydrolysis." *Applied Biochemistry and Biotechnology* 20 (1): 45–61. https://doi.org/10.1007/BF02936472.

Grootaert, Charlotte, Jan A Delcour, Christophe M Courtin, Willem F Broekaert, Willy Verstraete, and Tom Van de Wiele. 2007. "Microbial Metabolism and Prebiotic Potency of Arabinoxylan Oligosaccharides in the Human Intestine." *Trends in Food Science & Technology* 18 (2): 64–71. https://doi.org/https://doi.org/10.1016/j.tifs.2006.08.004.

Gullón, Patricia, Patricia Moura, María Paula Esteves, Francisco Manuel Girio, Herminia Domínguez, and Juan Carlos Parajó. 2008. "Assessment on the Fermentability of Xylooligosaccharides from Rice Husks by Probiotic Bacteria." *Journal of Agricultural and Food Chemistry* 56 (16): 7482–87. https://doi.org/10.1021/jf800715b.

Haan, R Den, and W H Van Zyl. 2003. "Enhanced Xylan Degradation and Utilisation by *Pichia stipitis* Overproducing Fungal Xylanolytic Enzymes." *Enzyme and Microbial Technology* 33 (5): 620–28. https://doi.org/https://doi.org/10.1016/S0141-0229(03)00183-2.

Haghighi Mood, Sohrab, Amir Hossein Golfeshan, Meisam Tabatabaei, Gholamreza Salehi Jouzani, Gholam Hassan Najafi, Mehdi Gholami, and Mehdi Ardjmand. 2013. "Lignocellulosic Biomass to Bioethanol, a Comprehensive Review with a Focus on Pretreatment." *Renewable and Sustainable Energy Reviews* 27: 77–93. https://doi.org/https://doi.org/10.1016/j.rser.2013.06.033.

Han, Jian, Rou Cao, Xin Zhou, and Yong Xu. 2020. "An Integrated Biorefinery Process for Adding Values to Corncob in Co-Production of Xylooligosaccharides and Glucose Starting from Pretreatment with Gluconic Acid." *Bioresource Technology* 307: 123200. https://doi.org/https://doi.org/10.1016/j.biortech.2020.123200.

Harahap, Budi Mandra. 2020. "Degradation Techniques of Hemicellulose Fraction from Biomass Feedstock for Optimum Xylose Production: A Review." *Jurnal Keteknikan Pertanian Tropis Dan Biosistem* 8 (2): 107–24. https://doi.org/10.21776/ub.jkptb.2020.008.02.01.

Harrison, Mark D, Zhanying Zhang, Kylie Shand, Barrie Fong Chong, Jason Nichols, Paul Oeller, Ian M O'Hara, William O S Doherty, and James L Dale. 2014. "The Combination of Plant-Expressed Cellobiohydrolase and Low Dosages of Cellulases for

the Hydrolysis of Sugar Cane Bagasse." *Biotechnology for Biofuels* 7 (1): 131. https://doi.org/10.1186/s13068-014-0131-9.

Harrow, B., and Mazur, A."Textbook of Biochemistry" (1958), 109, Saunders, *Philadelphia*

Heer, Dominik, and Uwe Sauer. 2008. "Identification of Furfural as a Key Toxin in Lignocellulosic Hydrolysates and Evolution of a Tolerant Yeast Strain." *Microbial Biotechnology* 1 (6): 497–506. https://doi.org/10.1111/j.1751-7915.2008.00050.x.

Helle, Steve S, Sheldon J B Duff, and David G Cooper. 1993. "Effect of Surfactants on Cellulose Hydrolysis." *Biotechnology and Bioengineering* 42 (5): 611–17. https://doi.org/https://doi.org/10.1002/bit.260420509.

Hendriks, A.T.W.M., and G Zeeman. 2009. "Pretreatments to Enhance the Digestibility of Lignocellulosic Biomass." *Bioresource Technology* 100 (1): 10–18. https://doi.org/https://doi.org/10.1016/j.biortech.2008.05.027.

Hernández-Beltrán, Javier Ulises, and Héctor Hernández-Escoto. 2018. "Enzymatic Hydrolysis of Biomass at High-Solids Loadings through Fed-Batch Operation." *Biomass and Bioenergy* 119: 191–97. https://doi.org/https://doi.org/10.1016/j.biombioe.2018.09.020.

Hidaka, H, T Takizawa, H Fujikawa, T Ohneda, and T Fukuzumi. 1984. "A Study on the Inhibition of Cellulolytic Activities by Lignin." *Applied Biochemistry and Biotechnology* 9 (4): 367. https://doi.org/10.1007/BF02798979.

Hodge, David B, M Nazmul Karim, Daniel J Schell, and James D McMillan. 2008. "Soluble and Insoluble Solids Contributions to High-Solids Enzymatic Hydrolysis of Lignocellulose." *Bioresource Technology* 99 (18): 8940–48. https://doi.org/https://doi.org/10.1016/j.biortech.2008.05.015.

Holladay, J E, J J Bozell, J F White, and D Johnson. 2007a. "Top Value-Added Chemicals from Biomass - Volume II—Results of Screening for Potential Candidates from Biorefinery Lignin." http://www.ntis.gov/ordering.htm.

Hoyer, Kerstin, Mats Galbe, and Guido Zacchi. 2013. "The Effect of Prehydrolysis and Improved Mixing on High-Solids Batch Simultaneous Saccharification and

Fermentation of Spruce to Ethanol." *Process Biochemistry* 48 (2): 289–93. https://doi.org/https://doi.org/10.1016/j.procbio.2012.12.020.

Hu, Jinguang, Joshua Davies, Yiu Ki Mok, Bryan Gene, Quak Foo Lee, Claudio Arato, and Jack N Saddler. 2016. "Enzymatic Hydrolysis of Industrial Derived Xylo-Oligomers to Monomeric Sugars for Potential Chemical/Biofuel Production." *ACS Sustainable Chemistry & Engineering* 4 (12): 7130–36. https://doi.org/10.1021/acssuschemeng.6b02008.

Hu, Jinguang, and Jack N Saddler. 2018. "Why Does GH10 Xylanase Have Better Performance than GH11 Xylanase for the Deconstruction of Pretreated Biomass?" *Biomass and Bioenergy* 110: 13–16. https://doi.org/https://doi.org/10.1016/j.biombioe.2018.01.007.

Jayapal, Natasha, A K Samanta, Atul P Kolte, S Senani, Manpal Sridhar, K P Suresh, and K T Sampath. 2013. "Value Addition to Sugarcane Bagasse: Xylan Extraction and Its Process Optimization for Xylooligosaccharides Production." *Industrial Crops and Products* 42: 14–24. https://doi.org/https://doi.org/10.1016/j.indcrop.2012.05.019.

Jeya, Marimuthu, Ngoc-Phuong-Thao Nguyen, Hee-Jung Moon, Sang-Hwan Kim, and Jung-Kul Lee. 2010. "Conversion of Woody Biomass into Fermentable Sugars by Cellulase from Agaricus Arvensis." *Bioresource Technology* 101 (22): 8742–49. https://doi.org/https://doi.org/10.1016/j.biortech.2010.06.055.

Jiang, Z Q, W Deng, Y P Zhu, L T Li, Y J Sheng, and K Hayashi. 2004. "The Recombinant Xylanase B of *Thermotoga maritima* Is Highly Xylan Specific and Produces Exclusively Xylobiose from Xylans, a Unique Character for Industrial Applications." *Journal of Molecular Catalysis B: Enzymatic* 27 (4): 207–13. https://doi.org/https://doi.org/10.1016/j.molcatb.2003.11.012.

Ji, X. J., Huang, H., Nie, Z. K., Qu, L., Xu, Q., & Tsao, G. T. (2012). "Fuels and chemicals from hemicellulose sugars." Biotechnology in China III: biofuels and bioenergy, 199-224.

Jing, Xinyun, Xiaoxi Zhang, and Jie Bao. 2009. "Inhibition Performance of Lignocellulose Degradation Products on Industrial Cellulase Enzymes During Cellulose Hydrolysis." *Applied Biochemistry and Biotechnology* 159 (3): 696–707. https://doi.org/10.1007/s12010-009-8525-z.

Jiang, Z. Q., Deng, W., Zhu, Y. P., Li, L. T., Sheng, Y. J. and Hayashi, K. 2004. The recombinant xylanase B of *Thermotoga maritima* is highly xylan specific and produces exclusively xylobiose from xylans, a unique character for industrial applications. Journal of Molecular Catalysis B: Enzymatic 27(4-6): 207–213.

Joy, Shereena P, and Chandraraj Krishnan. 2022. "Modified Organosolv Pretreatment for Improved Cellulosic Ethanol Production from Sorghum Biomass." *Industrial Crops and Products* 177: 114409. https://doi.org/https://doi.org/10.1016/j.indcrop.2021.114409.

Jung, H. J. G., Jorgensen, M. A., Linn, J. G., & Engels, F. M. (2000)." Impact of accessibility and chemical composition on cell wall polysaccharide degradability of maize and lucerne stems." *Journal of the Science of Food and Agriculture*, 80(3), 419-427.

Kadam, Kiran L, and James D McMillan. 2003. "Availability of Corn Stover as a Sustainable Feedstock for Bioethanol Production." *Bioresource Technology* 88 (1): 17–25. https://doi.org/https://doi.org/10.1016/S0960-8524(02)00269-9.

Katapodis, Petros, and Paul Christakopoulos. 2008. "Enzymic Production of Feruloyl Xylo-Oligosaccharides from Corn Cobs by a Family 10 Xylanase from *Thermoascus aurantiacus*." *LWT - Food Science and Technology* 41 (7): 1239–43. https://doi.org/https://doi.org/10.1016/j.lwt.2007.08.004.

Kellock, Miriam, Jenni Rahikainen, Anna S Borisova, Sanni Voutilainen, Anu Koivula, Kristiina Kruus, and Kaisa Marjamaa. 2022. "Inhibitory Effect of Lignin on the Hydrolysis of Xylan by Thermophilic and Thermolabile GH11 Xylanases." *Biotechnology for Biofuels and Bioproducts* 15 (1): 49. https://doi.org/10.1186/s13068-022-02148-4.

Kellock, Miriam, Jenni Rahikainen, Kaisa Marjamaa, and Kristiina Kruus. 2017. "Lignin-Derived Inhibition of Monocomponent Cellulases and a Xylanase in the Hydrolysis of Lignocellulosics." *Bioresource Technology* 232: 183–91. https://doi.org/https://doi.org/10.1016/j.biortech.2017.01.072.

Kim, Da-Eun, and Xuejun Pan. 2010. "Preliminary Study on Converting Hybrid Poplar to High-Value Chemicals and Lignin Using Organosolv Ethanol Process." *Industrial & Engineering Chemistry Research* 49 (23): 12156–63. https://doi.org/10.1021/ie101671r.

Klinke, H B, A B Thomsen, and B K Ahring. 2004. "Inhibition of Ethanol-Producing Yeast and Bacteria by Degradation Products Produced during Pre-Treatment of Biomass."

Applied Microbiology and Biotechnology 66 (1): 10–26. https://doi.org/10.1007/s00253-004-1642-2.

Koo, Bon-Wook, Ho-Yong Kim, Nahyun Park, Soo-Min Lee, Hwanmyeong Yeo, and In-Gyu Choi. 2011. "Organosolv Pretreatment of Liriodendron Tulipifera and Simultaneous Saccharification and Fermentation for Bioethanol Production." *Biomass and Bioenergy* 35 (5): 1833–40. https://doi.org/https://doi.org/10.1016/j.biombioe.2011.01.014.

Koppram, R., Tomas-Pej ´ o, ´ E., Xiros, C., Olsson, L., 2014. "Lignocellulosic ethanol production at high-gravity: challenges and perspectives." Trends Biotechnol. 32 (1), 46–53. https://doi.org/10.1016/j.tibtech.2013.10.003.

Kristensen, Jan B, Johan Börjesson, Maria H Bruun, Folke Tjerneld, and Henning Jørgensen. 2007. "Use of Surface Active Additives in Enzymatic Hydrolysis of Wheat Straw Lignocellulose." *Enzyme and Microbial Technology* 40 (4): 888–95. https://doi.org/https://doi.org/10.1016/j.enzmictec.2006.07.014.

Kristensen, Jan B, Claus Felby, and Henning Jørgensen. 2009. "Yield-Determining Factors in High-Solids Enzymatic Hydrolysis of Lignocellulose." *Biotechnology for Biofuels* 2 (1): 11. https://doi.org/10.1186/1754-6834-2-11.

Kristiani, A., Abimanyu, H., Setiawan, A. H., and Aulia, F. (2013). "The effect of pre-treatment using diluted acid on the properties of oil palm leaves." Energy Procedia, 32, 183-189.

Kuhn, Erik M, Xiaowen Chen, and Melvin P Tucker. 2020. "Deacetylation and Mechanical Refining (DMR) and Deacetylation and Dilute Acid (DDA) Pretreatment of Corn Stover, Switchgrass, and a 50:50 Corn Stover/Switchgrass Blend." *ACS Sustainable Chemistry & Engineering* 8 (17): 6734–43. https://doi.org/10.1021/acssuschemeng.0c00894.

Kumar, Amit, Nakachew Minuye, Yilkal Bezie, Ayele Bahir, Mukesh Yadav, Kumar Amit, Minuye Nakachew, Bezie Yilkal, and Yadav Mukesh. 2018a. "A Review of Factors Affecting Enzymatic Hydrolysis of Pretreated Lignocellulosic Biomass Bioecology of Model Organism for Societal Welfare and Conservation of Nature and Natural Resources View Project Green Pulp and Paper Industry View Project A Review of Factors Affecting Enzymatic Hydrolysis of Pretreated Lignocellulosic Biomass." *Research Journal of Chemistry and Environment*

Kumar, Linoj, Valdeir Arantes, Richard Chandra, and Jack Saddler. 2012. “The Lignin Present in Steam Pretreated Softwood Binds Enzymes and Limits Cellulose Accessibility.” *Bioresource Technology* 103 (1): 201–8. https://doi.org/https://doi.org/10.1016/j.biortech.2011.09.091.

Kumar, Parveen, Diane M Barrett, Michael J Delwiche, and Pieter Stroeve. 2009. “Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production.” *Industrial & Engineering Chemistry Research* 48 (8): 3713–29. https://doi.org/10.1021/ie801542g.

Kumar, Rajeev, and Charles E Wyman. 2009. “Effect of Additives on the Digestibility of Corn Stover Solids Following Pretreatment by Leading Technologies.” *Biotechnology and Bioengineering* 102 (6): 1544–57. https://doi.org/https://doi.org/10.1002/bit.22203.

Kumar, Vikash, and T Satyanarayana. 2011. “Applicability of Thermo-Alkali-Stable and Cellulase-Free Xylanase from a Novel Thermo-Halo-Alkaliphilic *Bacillus halodurans* in Producing Xylooligosaccharides.” *Biotechnology Letters* 33 (11): 2279–85. https://doi.org/10.1007/s10529-011-0698-1.

Kundu, Pranati, Sandeep Kumar, Vivek Ahluwalia, Sushil K Kansal, and Sasikumar Elumalai. 2018. “Extraction of Arabinoxylan from Corncob through Modified Alkaline Method to Improve Xylooligosaccharides Synthesis.” *Bioresource Technology Reports* 3: 51–58. https://doi.org/https://doi.org/10.1016/j.biteb.2018.01.007.

L., Shuler M. 1992. “Bioprocess Enzineering.” *Prentice-Hall*, 412–20. https://cir.nii.ac.jp/crid/1573668924360652928.bib?lang=ja.

Larsson, Simona, Eva Palmqvist, Bärbel Hahn-Hägerdal, Charlotte Tengborg, Kerstin Stenberg, Guido Zacchi, and Nils-Olof Nilvebrant. 1999. “The Generation of Fermentation Inhibitors during Dilute Acid Hydrolysis of Softwood.” *Enzyme and Microbial Technology* 24 (3): 151–59. https://doi.org/https://doi.org/10.1016/S0141-0229(98)00101-X.

Li, Caiqun, Meishan Fan, Jun Xie, and Hongdan Zhang. 2023. “Effect of NaOH-Catalyzed Organosolv Pretreatment on the Co-Production of Ethanol and Xylose from Poplar.” *Industrial Crops and Products* 200: 116774. https://doi.org/https://doi.org/10.1016/j.indcrop.2023.116774.

Li, Mei-Chun, Qinglin Wu, Kunlin Song, H N Cheng, Shigehiko Suzuki, and Tingzhou Lei. 2016. “Chitin Nanofibers as Reinforcing and Antimicrobial Agents in Carboxymethyl Cellulose Films: Influence of Partial Deacetylation.” *ACS Sustainable Chemistry & Engineering* 4 (8): 4385–95. https://doi.org/10.1021/acssuschemeng.6b00981.

Liao, Hong, Yong Xu, and Junhua Zhang. 2021. “Efficient Production of Xylooligosaccharides and Fermentable Sugars from Corncob by Propionic Acid and Enzymatic Hydrolysis.” *Bioresource Technology* 342: 125680. https://doi.org/https://doi.org/10.1016/j.biortech.2021.125680.

Lima, Cleilton S., Sarita C Rabelo, Peter N Ciesielski, Inês C Roberto, George J M Rocha, and Carlos Driemeier. 2018. “Multiscale Alterations in Sugar Cane Bagasse and Straw Submitted to Alkaline Deacetylation.” *ACS Sustainable Chemistry & Engineering* 6 (3): 3796–3804. https://doi.org/10.1021/acssuschemeng.7b04158.

Lin, Yu-Sheng, Min-Jen Tseng, and Wen-Chien Lee. 2011. “Production of Xylooligosaccharides Using Immobilized Endo-Xylanase of *Bacillus halodurans*.” *Process Biochemistry* 46 (11): 2117–21. https://doi.org/https://doi.org/10.1016/j.procbio.2011.08.008.

Lin, C. S. K., Pfaltzgraff, L. A., Herrero-Davila, L., Mubofu, E. B., Abderrahim, S., Clark, J. H., & Thankappan, S. (2013). “Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective.” Energy & Environmental Science, 6(2), 426-464.

Ling, HongZhi, KeKe Cheng, JingPing Ge, and WenXiang Ping. 2017. “Corncob Mild Alkaline Pretreatment for High 2,3-Butanediol Production by Spent Liquor Recycle Process.” *BioEnergy Research* 10 (2): 566–74. https://doi.org/10.1007/s12155-017-9822-y.

Liu, Yunyun, Jingliang Xu, Yu Zhang, Zhenhong Yuan, and Jun Xie. 2015. “Optimization of High Solids Fed-Batch Saccharification of Sugarcane Bagasse Based on System Viscosity Changes.” *Journal of Biotechnology* 211: 5–9. https://doi.org/https://doi.org/10.1016/j.jbiotec.2015.06.422.

Manning, Thea Scantlebury, and Glenn R Gibson. 2004. “Prebiotics.” *Best Practice & Research Clinical Gastroenterology* 18 (2): 287–98. https://doi.org/https://doi.org/10.1016/j.bpg.2003.10.008.

Mano, Mario Cezar Rodrigues, Iramaia Angélica Neri-Numa, Juliana Bueno da Silva, Bruno Nicolau Paulino, Marina Gabriel Pessoa, and Gláucia Maria Pastore. 2018. "Oligosaccharide Biotechnology: An Approach of Prebiotic Revolution on the Industry." *Applied Microbiology and Biotechnology* 102 (1): 17–37. https://doi.org/10.1007/s00253-017-8564-2.

Maulana Hidayatullah, Ibnu, Tjandra Setiadi, Made Tri Ari Penia Kresnowati, and Ramaraj Boopathy. 2020. "Xylanase Inhibition by the Derivatives of Lignocellulosic Material." *Bioresource Technology* 300: 122740. https://doi.org/https://doi.org/10.1016/j.biortech.2020.122740.

Mäkeläinen H, Forssten S, Saarinen M, Stowell J, Rautonen N, Ouwehand AC (2010) "Xylo-oligosaccharides enhance the growth of *bifidobacteria* and *Bifidobacterium lactis* in a simulated colon model." Benefic Microbes 1:81–91. https://doi.org/10.3920/BM2009.0025

Mbithi-Mwikya, Stephen, Wilfried Ooghe, John Van Camp, Delphin Ngundi, and Andre Huyghebaert. 2000. "Amino Acid Profiles after Sprouting, Autoclaving, and Lactic Acid Fermentation of Finger Millet (Eleusine Coracan) and Kidney Beans (Phaseolus Vulgaris L.)." *Journal of Agricultural and Food Chemistry* 48 (8): 3081–85. https://doi.org/10.1021/jf0002140.

Menardo, Simona, Gianfranco Airoldi, Vincenzo Cacciatore, and Paolo Balsari. 2015a. "Potential Biogas and Methane Yield of Maize Stover Fractions and Evaluation of Some Possible Stover Harvest Chains." *Biosystems Engineering* 129: 352–59. https://doi.org/https://doi.org/10.1016/j.biosystemseng.2014.11.010.

Millati, Ria, Rochim Bakti Cahyono, Teguh Ariyanto, Istna Nafi Azzahrani, Rininta Utami Putri, and Mohammad J Taherzadeh. 2019. "Chapter 1 - Agricultural, Industrial, Municipal, and Forest Wastes: An Overview." In *Sustainable Resource Recovery and Zero Waste Approaches*, edited by Mohammad J Taherzadeh, Kim Bolton, Jonathan Wong, and Ashok Pandey, 1–22. Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-444-64200-4.00001-3.

Modenbach, Alicia A, and Sue E Nokes. 2013. "Enzymatic Hydrolysis of Biomass at High-Solids Loadings – A Review." *Biomass and Bioenergy* 56: 526–44. https://doi.org/https://doi.org/10.1016/j.biombioe.2013.05.031.

Moniz, Patrícia, Helena Pereira, Luís C Duarte, and Florbela Carvalheiro. 2014. "Hydrothermal Production and Gel Filtration Purification of Xylo-Oligosaccharides from Rice Straw." *Industrial Crops and Products* 62: 460–65. https://doi.org/https://doi.org/10.1016/j.indcrop.2014.09.020.

Morrison, David, J Susan Van Dyk, and Brett Ivan Pletschke. 2011a. "Effect of Chemicals on XynA." *BioResources*. Vol. 6.

Mosier, Nathan, Charles Wyman, Bruce Dale, Richard Elander, Y Y Lee, Mark Holtzapple, and Michael Ladisch. 2005. "Features of Promising Technologies for Pretreatment of Lignocellulosic Biomass." *Bioresource Technology* 96 (6): 673–86. https://doi.org/https://doi.org/10.1016/j.biortech.2004.06.025.

Moure, Andrés, Patricia Gullón, Herminia Domínguez, and Juan Carlos Parajó. 2006. "Advances in the Manufacture, Purification and Applications of Xylo-Oligosaccharides as Food Additives and Nutraceuticals." *Process Biochemistry* 41 (9): 1913–23. https://doi.org/https://doi.org/10.1016/j.procbio.2006.05.011.

Mukasekuru, Marie Rose, Jinguang Hu, Xiaoqin Zhao, Fubao Fuelbiol Sun, Kaneza Pascal, Hongyan Ren, and Junhua Zhang. 2018. "Enhanced High-Solids Fed-Batch Enzymatic Hydrolysis of Sugar Cane Bagasse with Accessory Enzymes and Additives at Low Cellulase Loading." *ACS Sustainable Chemistry & Engineering* 6 (10): 12787–96. https://doi.org/10.1021/acssuschemeng.8b01972.

Mulchandani, Ashok, Howard H WeetaU, Founding Editor, David R Walt, Former Editor, Isao Karube, Klaus Mosbach, et al. 2008a. "Applied Biochemistry and Biotechnology Part A: Enzyme Engineering and Biotechnology Advisory Board Associate Editors Assistant Editor." *Hatfield· Herts • UK* 145: 1–3.

Mussatto, Solange I, Marcela Fernandes, Adriane M F Milagres, and Inês C Roberto. 2008. "Effect of Hemicellulose and Lignin on Enzymatic Hydrolysis of Cellulose from Brewer's Spent Grain." *Enzyme and Microbial Technology* 43 (2): 124–29. https://doi.org/https://doi.org/10.1016/j.enzmictec.2007.11.006.

Mussatto, Solange I, and Ismael M Mancilha. 2007. "Non-Digestible Oligosaccharides: A Review." *Carbohydrate Polymers* 68 (3): 587–97. https://doi.org/https://doi.org/10.1016/j.carbpol.2006.12.011.

Muurinen, Esa. 2000a. *Organosolv Pulping : A Review and Distillation Study Related to Peroxyacid Pulping*. Oulun yliopisto.

Ooshima, Hiroshi, Masaru Sakata, and Yoshio Harano. 1986. “Enhancement of Enzymatic Hydrolysis of Cellulose by Surfactant.” *Biotechnology and Bioengineering* 28 (11): 1727–34. https://doi.org/https://doi.org/10.1002/bit.260281117.

Temelli, N. 2020 “ORGANOSOLV TREATMENT FOR PREBIOTIC OLIGOSACCHARIDE PRODUCTION FROM AGRO-FOOD WASTE.”

Ouyang, Jia, Zhenwei Dong, Xiangyang Song, Xin Lee, Mu Chen, and Qiang Yong. 2010. “Improved Enzymatic Hydrolysis of Microcrystalline Cellulose (Avicel PH101) by Polyethylene Glycol Addition.” *Bioresource Technology* 101 (17): 6685–91. https://doi.org/https://doi.org/10.1016/j.biortech.2010.03.085.

P., Delgenes J. 2003. “Pretreatments for the Enhancement of Anaerobic Digestion of Solid Wastes.” *Biomethanization of the Organic Fraction of Municipal Solid Wastes*. https://cir.nii.ac.jp/crid/1573105975535380224.bib?lang=ja.

Palmqvist, Eva, and Bärbel Hahn-Hägerdal. 2000. “Fermentation of Lignocellulosic Hydrolysates. II: Inhibitors and Mechanisms of Inhibition.” *Bioresource Technology* 74 (1): 25–33. https://doi.org/https://doi.org/10.1016/S0960-8524(99)00161-3.

Palonen, Hetti., and Valtion teknillinen tutkimuskeskus. 2004. *Role of Lignin in the Enzymatic Hydrolysis of Lignocellulose*. VTT Technical Research Centre of Finland.

Park, Jin Won, Yasuyuki Takahata, Toshio Kajiuchi, and Takashi Akehata. 1992. “Effects of Nonionic Surfactant on Enzymatic Hydrolysis of Used Newspaper.” *Biotechnology and Bioengineering* 39 (1): 117–20. https://doi.org/https://doi.org/10.1002/bit.260390117.

Paul, Subhash, and Animesh Dutta. 2018. “Challenges and Opportunities of Lignocellulosic Biomass for Anaerobic Digestion.” *Resources, Conservation and Recycling* 130: 164–74. https://doi.org/https://doi.org/10.1016/j.resconrec.2017.12.005.

Pavasovic, M, N A Richardson, A J Anderson, D Mann, and P B Mather. 2004. “Effect of PH, Temperature and Diet on Digestive Enzyme Profiles in the Mud Crab, Scylla Serrata.” *Aquaculture* 242 (1): 641–54. https://doi.org/https://doi.org/10.1016/j.aquaculture.2004.08.036.

Pan, X. (2008). “Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose.”Journal of Biobased Materials and Bioenergy, 2(1), 25-32.

Pan, X., Zhang, X., Gregg, D. J., & Saddler, J. N. (2004). “Enhanced enzymatic hydrolysis of steam-exploded Douglas fir wood by alkali-oxygen post-treatment.”In Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO (pp. 1103-1114). Humana Press, Totowa, NJ.

Pfeiffer, J. “ Enzymes, the Physics and Chemistry of Life”, pg 171-173, Simon and Schuster,NY (1954)

Pingali, Sai Venkatesh, Volker S Urban, William T Heller, Joseph McGaughey, Hugh O’Neill, Marcus Foston, Dean A Myles, Arthur Ragauskas, and Barbara R Evans. 2010. “Breakdown of Cell Wall Nanostructure in Dilute Acid Pretreated Biomass.” *Biomacromolecules* 11 (9): 2329–35. https://doi.org/10.1021/bm100455h.

Polizeli, Mltm, A C S Rizzatti, R Monti, H F Terenzi, J A Jorge, and D S Amorim. 2005. “Xylanases from Fungi: Properties and Industrial Applications.” *Applied Microbiology and Biotechnology* 67 (5): 577–91. https://doi.org/10.1007/s00253-005-1904-7.

Poutanen, Kaisa, Marjaana Rättö, Jürgen Puls, and Liisa Viikari. 1987. “Evaluation of Different Microbial Xylanolytic Systems.” *Journal of Biotechnology* 6 (1): 49–60. https://doi.org/https://doi.org/10.1016/0168-1656(87)90045-9.

Prabha, J., Narikimelli, A., Sajini, M. I. and Vincent, S. 2013. “Optimization for autolysis assisted production of fish protein hydrolysate from underutilized fish *Pellona ditchela.”*International Journal of Scientific and Engineering Research 4(12): 1863-1869.

Puls, Jürgen. 1997. “Chemistry and Biochemistry of Hemicelluloses: Relationship between Hemicellulose Structure and Enzymes Required for Hydrolysis.” *Macromolecular Symposia* 120 (1): 183–96. https://doi.org/https://doi.org/10.1002/masy.19971200119.

Samanta, A. K., Kolte, A. P., Chandrasekhariah, M., Thulasi, A., Sampath, K. T., & Prasad, C. S. (2007). “Prebiotics: The rumen modulator for enhancing the productivity of dairy animals.” Indian Dairyman, 59, 58-61.

Sixta, H.” Handbook of Pulp” 2006. Wiley-VCH: Weinheim, Germany.

Sugar Alcohols (Polyols) and Polydextrose Used as Sweeteners in Foods - Food Safety – Health Canada". (2005). Retrieved from Canada.ca: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/sugar-substitutes/sugar-alcohols-polyols-polydextrose-used-sweeteners-foods-food-safety.html

http://www.tmo.gov.tr/Upload/Document/hububatsektorraporu2017.pdf

Qi, Benkun, Xiangrong Chen, Yi Su, and Yinhua Wan. 2011. "Enzyme Adsorption and Recycling during Hydrolysis of Wheat Straw Lignocellulose." *Bioresource Technology* 102 (3): 2881–89. https://doi.org/https://doi.org/10.1016/j.biortech.2010.10.092.

Qi, Benkun, Xiangrong Chen, and Yinhua Wan. 2010. "Pretreatment of Wheat Straw by Nonionic Surfactant-Assisted Dilute Acid for Enhancing Enzymatic Hydrolysis and Ethanol Production." *Bioresource Technology* 101 (13): 4875–83. https://doi.org/https://doi.org/10.1016/j.biortech.2010.01.063.

Qing, Qing, Bin Yang, and Charles E Wyman. 2010. "Xylooligomers Are Strong Inhibitors of Cellulose Hydrolysis by Enzymes." *Bioresource Technology* 101 (24): 9624–30. https://doi.org/https://doi.org/10.1016/j.biortech.2010.06.137.

Quiñones, Teresa Suárez, Andy Retter, Phil J Hobbs, Jörn Budde, Monika Heiermann, Matthias Plöchl, and Sreenivas Rao Ravella. 2015. "Production of Xylooligosaccharides from Renewable Agricultural Lignocellulose Biomass." *Biofuels* 6 (3–4): 147–55. https://doi.org/10.1080/17597269.2015.1065589.

Raweesri, P, P Riangrungrojana, and P Pinphanichakarn. 2008. "α-l-Arabinofuranosidase from Streptomyces Sp. PC22: Purification, Characterization and Its Synergistic Action with Xylanolytic Enzymes in the Degradation of Xylan and Agricultural Residues." *Bioresource Technology* 99 (18): 8981–86. https://doi.org/https://doi.org/10.1016/j.biortech.2008.05.016.

Remón, Javier, Tianzong Li, Christopher J Chuck, Avtar S Matharu, and James H Clark. 2019. "Toward Renewable-Based, Food-Applicable Prebiotics from Biomass: A One-Step, Additive-Free, Microwave-Assisted Hydrothermal Process for the Production of High Purity Xylo-Oligosaccharides from Beech Wood Hemicellulose." *ACS Sustainable*

Chemistry & Engineering 7 (19): 16160–72. https://doi.org/10.1021/acssuschemeng.9b03096.

Rivas, Beatriz, Ana B Moldes, José M Domínguez, and Juan C Parajó. 2004. "Lactic Acid Production from Corn Cobs by Simultaneous Saccharification and Fermentation: A Mathematical Interpretation." *Enzyme and Microbial Technology* 34 (7): 627–34. https://doi.org/https://doi.org/10.1016/j.enzmictec.2004.01.011.

Ruiz, Héctor A, Mats Galbe, Gil Garrote, Diana M Ramirez-Gutierrez, Eduardo Ximenes, Shao-Ni Sun, Daniel Lachos-Perez, et al. 2021. "Severity Factor Kinetic Model as a Strategic Parameter of Hydrothermal Processing (Steam Explosion and Liquid Hot Water) for Biomass Fractionation under Biorefinery Concept." *Bioresource Technology* 342: 125961. https://doi.org/https://doi.org/10.1016/j.biortech.2021.125961.

Ruiz, Héctor A, Denise S Ruzene, Daniel P Silva, Fernando F Macieira da Silva, António A Vicente, and José A Teixeira. 2011. "Development and Characterization of an Environmentally Friendly Process Sequence (Autohydrolysis and Organosolv) for Wheat Straw Delignification." *Applied Biochemistry and Biotechnology* 164 (5): 629–41. https://doi.org/10.1007/s12010-011-9163-9.

Saini, Jitendra Kumar, Reetu Saini, and Lakshmi Tewari. 2015. "Lignocellulosic Agriculture Wastes as Biomass Feedstocks for Second-Generation Bioethanol Production: Concepts and Recent Developments." *3 Biotech* 5 (4): 337–53. https://doi.org/10.1007/s13205-014-0246-5.

Salehi, S M Amin, Keikhosro Karimi, Tayebeh Behzad, and Nafiseh Poornejad. 2012a. "Efficient Conversion of Rice Straw to Bioethanol Using Sodium Carbonate Pretreatment." *Energy & Fuels* 26 (12): 7354–61. https://doi.org/10.1021/ef301476b.

Samanta, A K, Natasha Jayapal, C Jayaram, Sohini Roy, A P Kolte, S Senani, and Manpal Sridhar. 2015a. "Xylooligosaccharides as Prebiotics from Agricultural By-Products: Production and Applications." *Bioactive Carbohydrates and Dietary Fibre* 5 (1): 62–71. https://doi.org/https://doi.org/10.1016/j.bcdf.2014.12.003.

Samanta, A. K., Natasha Jayapal, C. Jayaram, Sohini Roy, A. P. Kolte, S. Senani, and Manpal Sridhar. 2015b. "Xylooligosaccharides as Prebiotics from Agricultural By-Products: Production and Applications." *Bioactive Carbohydrates and Dietary Fibre* 5 (1): 62–71. https://doi.org/10.1016/J.BCDF.2014.12.003.

Sarkanen, K. V., and Charles Heberle Ludwig. 1971. Lignins : Occurrence, Formation, Structure and Reactions. *Wiley-Interscience.* https://cir.nii.ac.jp/crid/1130000797281707008.bib?lang=ja.

Seesuriyachan, Phisit, Arthitaya Kawee-ai, and Thanongsak Chaiyaso. 2017. "Green and Chemical-Free Process of Enzymatic Xylooligosaccharide Production from Corncob: Enhancement of the Yields Using a Strategy of Lignocellulosic Destructuration by Ultra-High Pressure Pretreatment." *Bioresource Technology* 241: 537–44. https://doi.org/https://doi.org/10.1016/j.biortech.2017.05.193.

Seo, Dong-June, Hirotaka Fujita, and Akiyoshi Sakoda. 2011. "Effects of a Non-Ionic Surfactant, Tween 20, on Adsorption/Desorption of Saccharification Enzymes onto/from Lignocelluloses and Saccharification Rate." *Adsorption* 17 (5): 813–22. https://doi.org/10.1007/s10450-011-9340-8.

Sharma, Manju, Bhupinder Singh Chadha, and Harvinder Singh Saini. 2010. "Purification and Characterization of Two Thermostable Xylanases from *Malbranchea flava* Active under Alkaline Conditions." *Bioresource Technology* 101 (22): 8834–42. https://doi.org/https://doi.org/10.1016/j.biortech.2010.06.071.

Shivakumaraswamy, Vidyashree, Gupta P K, and Singh S A. n.d. "XYLOOLIGOSACCHARIDE-A VALUABLE MATERIAL FROM WASTE TO TASTE : A REVIEW Nanotechnology Based Sustained Action Drug Delivery System View Project A Review on Xylooligosaccharides View Project XYLOOLIGOSACCHARIDE-A VALUABLE MATERIAL FROM WASTE TO TASTE : A REVIEW." *J. Environ. Res. Develop. Journal of Environmental Research And Development*. Vol. 10. https://www.researchgate.net/publication/311323507.

Silva, João Paulo Alves, Livia Melo Carneiro, and Inês Conceição Roberto. 2013. "Treatment of Rice Straw Hemicellulosic Hydrolysates with Advanced Oxidative Processes: A New and Promising Detoxification Method to Improve the Bioconversion Process." *Biotechnology for Biofuels* 6 (1): 23. https://doi.org/10.1186/1754-6834-6-23.

Sipos, Bálint, Mátyás Szilágyi, Zoltán Sebestyén, Raffaella Perazzini, Dóra Dienes, Emma Jakab, Claudia Crestini, and Kati Réczey. 2011. "Mechanism of the Positive Effect of Poly(Ethylene Glycol) Addition in Enzymatic Hydrolysis of Steam Pretreated

Lignocelluloses." *Comptes Rendus Biologies* 334 (11): 812–23. https://doi.org/https://doi.org/10.1016/j.crvi.2011.06.005.

Sluiter, A, B Hames, R Ruiz, C Scarlata, J Sluiter, D Templeton, and D Crocker. 2008. "Determination of Structural Carbohydrates and Lignin in Biomass Laboratory Analytical Procedure (LAP) Issue Date: 7/17/2005." www.nrel.gov.

Sørensen, Hanne R, Anne S Meyer, and Sven Pedersen. 2003. "Enzymatic Hydrolysis of Water-Soluble Wheat Arabinoxylan. 1. Synergy between α-L-Arabinofuranosidases, Endo-1,4-β-Xylanases, and β-Xylosidase Activities." *Biotechnology and Bioengineering* 81 (6): 726–31. https://doi.org/https://doi.org/10.1002/bit.10519.

Sørensen, Hanne R, Sven Pedersen, Christel T Jørgensen, and Anne S Meyer. 2007. "Enzymatic Hydrolysis of Wheat Arabinoxylan by a Recombinant 'Minimal' Enzyme Cocktail Containing β-Xylosidase and Novel Endo-1,4-β-Xylanase and α-l-Arabinofuranosidase Activities." *Biotechnology Progress* 23 (1): 100–107. https://doi.org/https://doi.org/10.1021/bp0601701.

Sticklen, Mariam B. 2008. "Plant Genetic Engineering for Biofuel Production: Towards Affordable Cellulosic Ethanol." *Nature Reviews Genetics* 9 (6): 433–43. https://doi.org/10.1038/nrg2336.

Sun, X F, F Xu, R C Sun, Y X Wang, P Fowler, and M S Baird. 2004. "Characteristics of Degraded Lignins Obtained from Steam Exploded Wheat Straw." *Polymer Degradation and Stability* 86 (2): 245–56. https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2004.05.003.

Surek, Ece, Ali Oguz Buyukkileci, and Sirma Yegin. 2021. "Processing of Hazelnut (*Corylus avellanal l.*) Shell Autohydrolysis Liquor for Production of Low Molecular Weight Xylooligosaccharides by Aureobasidium Pullulans NRRL Y–2311–1 Xylanase." *Industrial Crops and Products* 161: 113212. https://doi.org/https://doi.org/10.1016/j.indcrop.2020.113212.

Taherzadeh, Mohammad J, and Keikhosro Karimi. 2007a. "Bioethanol Review." *BioResources*. Vol. 2.

Takada, Masatsugu, Rui Niu, Eiji Minami, and Shiro Saka. 2018. "Characterization of Three Tissue Fractions in Corn (*Zea mays*) Cob." *Biomass and Bioenergy* 115: 130–35. https://doi.org/https://doi.org/10.1016/j.biombioe.2018.04.023.

Tang, Yong, Xiaoli Dou, Jinguang Hu, Jianxin Jiang, and Jack N Saddler. 2018. “Lignin Sulfonation and SO2 Addition Enhance the Hydrolyzability of Deacetylated and Then Steam-Pretreated Poplar with Reduced Inhibitor Formation.” *Applied Biochemistry and Biotechnology* 184 (1): 264–77. https://doi.org/10.1007/s12010-017-2545-x.

Tejirian, Ani, and Feng Xu. 2011. “Inhibition of Enzymatic Cellulolysis by Phenolic Compounds.” *Enzyme and Microbial Technology* 48 (3): 239–47. https://doi.org/https://doi.org/10.1016/j.enzmictec.2010.11.004.

Templeton, David W, Christopher J Scarlata, Justin B Sluiter, and Edward J Wolfrum. 2010. “Compositional Analysis of Lignocellulosic Feedstocks. 2. Method Uncertainties.” *Journal of Agricultural and Food Chemistry* 58 (16): 9054–62. https://doi.org/10.1021/jf100807b.

Thomsen, Mette Hedegaard, Anders Thygesen, and Anne Belinda Thomsen. 2009. “Identification and Characterization of Fermentation Inhibitors Formed during Hydrothermal Treatment and Following SSF of Wheat Straw.” *Applied Microbiology and Biotechnology* 83 (3): 447–55. https://doi.org/10.1007/s00253-009-1867-1.

Thoresen, Petter Paulsen, Leonidas Matsakas, Ulrika Rova, and Paul Christakopoulos. 2020. “Recent Advances in Organosolv Fractionation: Towards Biomass Fractionation Technology of the Future.” *Bioresource Technology* 306: 123189. https://doi.org/https://doi.org/10.1016/j.biortech.2020.123189.

Torney, François, Lorena Moeller, Andréa Scarpa, and Kan Wang. 2007. “Genetic Engineering Approaches to Improve Bioethanol Production from Maize.” *Current Opinion in Biotechnology* 18 (3): 193–99. https://doi.org/https://doi.org/10.1016/j.copbio.2007.03.006.

Toprak Mahsulleri Ofisi. “2017 Hububat Sektör Raporu”.

Tsai, W T, C Y Chang, S Y Wang, C F Chang, S F Chien, and H F Sun. 2001. “Cleaner Production of Carbon Adsorbents by Utilizing Agricultural Waste Corn Cob.” *Resources, Conservation and Recycling* 32 (1): 43–53. https://doi.org/https://doi.org/10.1016/S0921-3449(00)00093-8.

Tu, Maobing, Xuejun Pan, and Jack N Saddler. 2009. “Adsorption of Cellulase on Cellulolytic Enzyme Lignin from *Lodgepole pine*.” *Journal of Agricultural and Food Chemistry* 57 (17): 7771–78. https://doi.org/10.1021/jf901031m.

Uçkun Kiran, Esra, Ozlem Akpinar, and Ufuk Bakir. 2013. “Improvement of Enzymatic Xylooligosaccharides Production by the Co-Utilization of Xylans from Different Origins.” *Food and Bioproducts Processing* 91 (4): 565–74. https://doi.org/https://doi.org/10.1016/j.fbp.2012.12.002.

Várnai, Anikó, Matti Siika-aho, and Liisa Viikari. 2010. “Restriction of the Enzymatic Hydrolysis of Steam-Pretreated Spruce by Lignin and Hemicellulose.” *Enzyme and Microbial Technology* 46 (3): 185–93. https://doi.org/https://doi.org/10.1016/j.enzmictec.2009.12.013.

Vázquez, M J, J L Alonso, H Domínguez, and J C Parajó. 2000. “Xylooligosaccharides: Manufacture and Applications.” *Trends in Food Science & Technology* 11 (11): 387–93. https://doi.org/https://doi.org/10.1016/S0924-2244(01)00031-0.

Vegas, Rodolfo, José Luis Alonso, Herminia Domínguez, and Juan Carlos Parajó. 2008a. “Enzymatic Processing of Rice Husk Autohydrolysis Products for Obtaining Low Molecular Weight Oligosaccharides.” *Food Biotechnology* 22 (1): 31–46. https://doi.org/10.1080/08905430701863811.

Vieira, Gustavo H F, Antonio M Martin, Silvana Saker-Sampaiao, Semir Omar, and Raimunda C F Goncalves. 1995. “Studies on the Enzymatic Hydrolysis of Brazilian Lobster (*Panulirus spp*) Processing Wastes.” *Journal of the Science of Food and Agriculture* 69 (1): 61–65. https://doi.org/https://doi.org/10.1002/jsfa.2740690110.

Waltmann, Curt, Carolyn E Mills, Jeremy Wang, Baofu Qiao, John M Torkelson, Danielle Tullman-Ercek, Monica Olvera De La Cruz, M O D L C Designed, and J W Performed. 2022a. “Functional Enzyme-Polymer Complexes.” https://doi.org/10.1073/pnas.

Wang, Jing, Xiaoping Yuan, Baoguo Sun, Yanping Cao, Yuan Tian, and Chengtao Wang. 2009. “On-Line Separation and Structural Characterisation of Feruloylated Oligosaccharides from Wheat Bran Using HPLC-ESI-MSn.” *Food Chemistry* 115 (4): 1529–41. https://doi.org/https://doi.org/10.1016/j.foodchem.2009.01.058.

Wang, Kun, Haiyan Yang, Shihuai Guo, Yong Tang, Jianxin Jiang, Feng Xu, and Run-Cang Sun. 2012. “Organosolv Fractionation Process with Various Catalysts for Improving Bioconversion of Triploid Poplar.” *Process Biochemistry* 47 (10): 1503–9. https://doi.org/https://doi.org/10.1016/j.procbio.2012.06.002.

Wang, Tseng-Hsing, and Shin Lu. 2013. “Production of Xylooligosaccharide from Wheat Bran by Microwave Assisted Enzymatic Hydrolysis.” *Food Chemistry* 138 (2): 1531–35. https://doi.org/https://doi.org/10.1016/j.foodchem.2012.09.124.

Wang, Zhaoqin, Bruce S Dien, Kent D Rausch, M E Tumbleson, and Vijay Singh. 2019. “Improving Ethanol Yields with Deacetylated and Two-Stage Pretreated Corn Stover and Sugarcane Bagasse by Blending Commercial Xylose-Fermenting and Wild Type *Saccharomyces yeast*.” *Bioresource Technology* 282: 103–9. https://doi.org/https://doi.org/10.1016/j.biortech.2019.02.123.

Wierckx, Nick, Frank Koopman, Harald J Ruijssenaars, and Johannes H de Winde. 2011. “Microbial Degradation of Furanic Compounds: Biochemistry, Genetics, and Impact.” *Applied Microbiology and Biotechnology* 92 (6): 1095–1105. https://doi.org/10.1007/s00253-011-3632-5.

Willför, Stefan, Kenneth Sundberg, Maija Tenkanen, and Bjarne Holmbom. 2008. “Spruce-Derived Mannans – A Potential Raw Material for Hydrocolloids and Novel Advanced Natural Materials.” *Carbohydrate Polymers* 72 (2): 197–210. https://doi.org/https://doi.org/10.1016/j.carbpol.2007.08.006.

Wong, Ken. K Y, and Ute Maringer. 1999. “Substrate Hydrolysis by Combinations of *Trichoderma* Xylanases.” *World Journal of Microbiology and Biotechnology* 15 (1): 23–26. https://doi.org/10.1023/A:1008865100607.

Wu, Jie, Richard P Chandra, Kwang Ho Kim, Chang Soo Kim, Yunqiao Pu, Arthur J Ragauskas, and Jack Nicholas Saddler. 2020. “Enhancing Enzyme-Mediated Hydrolysis of Mechanical Pulps by Deacetylation and Delignification.” *ACS Sustainable Chemistry & Engineering* 8 (15): 5847–55. https://doi.org/10.1021/acssuschemeng.9b07226.

Wyman, Charles E, Bruce E Dale, Richard T Elander, Mark Holtzapple, Michael R Ladisch, and Y Y Lee. 2005. “Coordinated Development of Leading Biomass Pretreatment Technologies.” *Bioresource Technology* 96 (18): 1959–66. https://doi.org/https://doi.org/10.1016/j.biortech.2005.01.010.

Xu, Chao, Jun Zhang, Yu Zhang, Ying Guo, Huijuan Xu, Jingliang Xu, and Zhongming Wang. 2019. “Enhancement of High-Solids Enzymatic Hydrolysis Efficiency of Alkali Pretreated Sugarcane Bagasse at Low Cellulase Dosage by Fed-Batch Strategy Based

on Optimized Accessory Enzymes and Additives." *Bioresource Technology* 292: 121993. https://doi.org/https://doi.org/10.1016/j.biortech.2019.121993.

Xue, Saisi, Nirmal Uppugundla, Michael J Bowman, David Cavalier, Leonardo Da Costa Sousa, Bruce. E Dale, and Venkatesh Balan. 2015. "Sugar Loss and Enzyme Inhibition Due to Oligosaccharide Accumulation during High Solids-Loading Enzymatic Hydrolysis." *Biotechnology for Biofuels* 8 (1): 195. https://doi.org/10.1186/s13068-015-0378-9.

Yáñez-S, Mauricio, Betty Matsuhiro, Carolina Nuñez, Shaobo Pan, Christopher A Hubbell, Poulomi Sannigrahi, and Arthur J Ragauskas. 2014. "Physicochemical Characterization of Ethanol Organosolv Lignin (EOL) from *Eucalyptus globulus*: Effect of Extraction Conditions on the Molecular Structure." *Polymer Degradation and Stability* 110: 184–94. https://doi.org/https://doi.org/10.1016/j.polymdegradstab.2014.08.026.

Yáñez-S, Mauricio, Jonathan Rojas, Jean Castro, Arthur Ragauskas, Jaime Baeza, and Juanita Freer. 2013. "Fuel Ethanol Production from *Eucalyptus globulus* Wood by Autocatalized Organosolv Pretreatment Ethanol–Water and SSF." *Journal of Chemical Technology & Biotechnology* 88 (1): 39–48. https://doi.org/https://doi.org/10.1002/jctb.3895.

Yang, Bin, and Charles E Wyman. 2006. "BSA Treatment to Enhance Enzymatic Hydrolysis of Cellulose in Lignin Containing Substrates." *Biotechnology and Bioengineering* 94 (4): 611–17. https://doi.org/https://doi.org/10.1002/bit.20750.

Yang, Si, Wen-Zhi Jia, Qing-Yun Qian, Yi-Ge Zhou, and Xing-Hua Xia. 2009. "Simple Approach for Efficient Encapsulation of Enzyme in Silica Matrix with Retained Bioactivity." *Analytical Chemistry* 81 (9): 3478–84. https://doi.org/10.1021/ac802739h.

Yegin, Sirma. 2017. "Single-Step Purification and Characterization of an Extreme Halophilic, Ethanol Tolerant and Acidophilic Xylanase from *Aureobasidium pullulans* NRRL Y-2311-1 with Application Potential in the Food Industry." *Food Chemistry* 221: 67–75. https://doi.org/https://doi.org/10.1016/j.foodchem.2016.10.003.

Yoon, Kyung Young, Edward E Woodams, and Yong D Hang. 2006. "Enzymatic Production of Pentoses from the Hemicellulose Fraction of Corn Residues." *LWT - Food Science and Technology* 39 (4): 388–92. https://doi.org/https://doi.org/10.1016/j.lwt.2005.02.005.

Zhang, Haiyan, Lujia Han, and Hongmin Dong. 2021. “An Insight to Pretreatment, Enzyme Adsorption and Enzymatic Hydrolysis of Lignocellulosic Biomass: Experimental and Modeling Studies.” *Renewable and Sustainable Energy Reviews* 140: 110758. https://doi.org/https://doi.org/10.1016/j.rser.2021.110758.

Zhang, Hongdan, Shuaishuai Zhang, Hongyou Yuan, Gaojin Lyu, and Jun Xie. 2018. “FeCl3-Catalyzed Ethanol Pretreatment of Sugarcane Bagasse Boosts Sugar Yields with Low Enzyme Loadings and Short Hydrolysis Time.” *Bioresource Technology* 249: 395–401. https://doi.org/https://doi.org/10.1016/j.biortech.2017.10.053.

Zhang, Junhua, Matti Siika-aho, Maija Tenkanen, and Liisa Viikari. 2011. “The Role of Acetyl Xylan Esterase in the Solubilization of Xylan and Enzymatic Hydrolysis of Wheat Straw and Giant Reed.” *Biotechnology for Biofuels* 4 (1): 60. https://doi.org/10.1186/1754-6834-4-60.

Zhang, Ke, Zhijian Pei, and Donghai Wang. 2016. “Organic Solvent Pretreatment of Lignocellulosic Biomass for Biofuels and Biochemicals: A Review.” *Bioresource Technology* 199: 21–33. https://doi.org/https://doi.org/10.1016/j.biortech.2015.08.102.

Zhang, Xiao, Maobing Tu, and Michael G Paice. 2011. “Routes to Potential Bioproducts from Lignocellulosic Biomass Lignin and Hemicelluloses.” *BioEnergy Research* 4 (4): 246–57. https://doi.org/10.1007/s12155-011-9147-1.

Zhao, Xuebing, Keke Cheng, and Dehua Liu. 2009. “Organosolv Pretreatment of Lignocellulosic Biomass for Enzymatic Hydrolysis.” *Applied Microbiology and Biotechnology* 82 (5): 815–27. https://doi.org/10.1007/s00253-009-1883-1.

Zhao, Xuebing, Siming Li, Ruchun Wu, and Dehua Liu. 2017. “Organosolv Fractionating Pre-Treatment of Lignocellulosic Biomass for Efficient Enzymatic Saccharification: Chemistry, Kinetics, and Substrate Structures.” *Biofuels, Bioproducts and Biorefining* 11 (3): 567–90. https://doi.org/https://doi.org/10.1002/bbb.1768.

Zhao, Xue-Bing, Lei Wang, and De-Hua Liu. 2008. “Peracetic Acid Pretreatment of Sugarcane Bagasse for Enzymatic Hydrolysis: A Continued Work.” *Journal of Chemical Technology & Biotechnology* 83 (6): 950–56. https://doi.org/https://doi.org/10.1002/jctb.1889.

Zhao, Yong, Usama Shakeel, Muhammad Saif Ur Rehman, Hongqiang Li, Xia Xu, and Jian Xu. 2020. “Lignin-Carbohydrate Complexes (LCCs) and Its Role in Biorefinery.”

Journal of Cleaner Production 253: 120076.
https://doi.org/https://doi.org/10.1016/j.jclepro.2020.120076.

APPENDICES

APPENDIX A

CALIBRATION CURVES

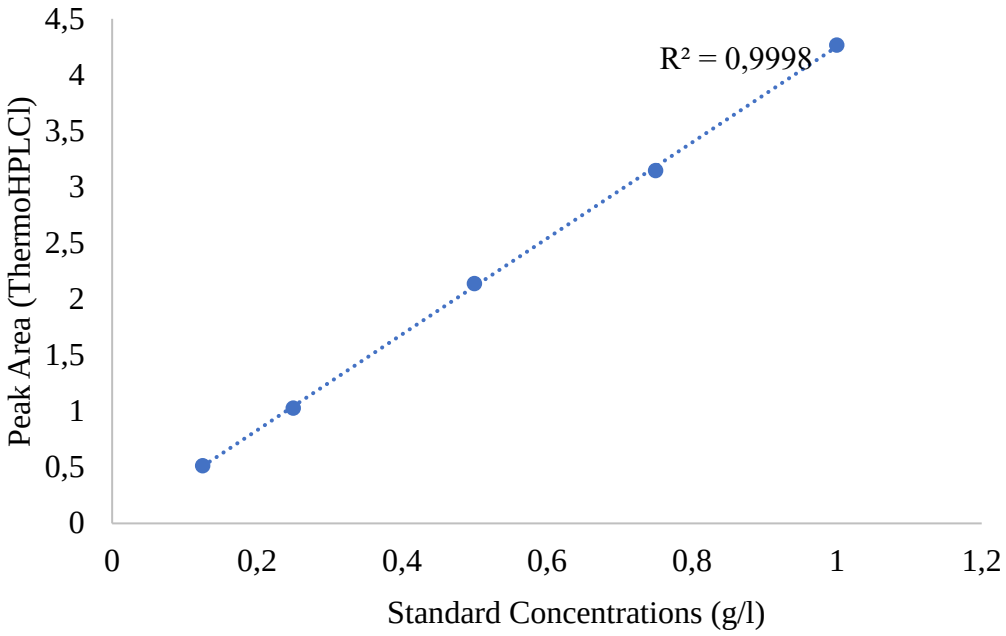


Figure A1. Standard calibration curve of xylose for HPLC analysis.

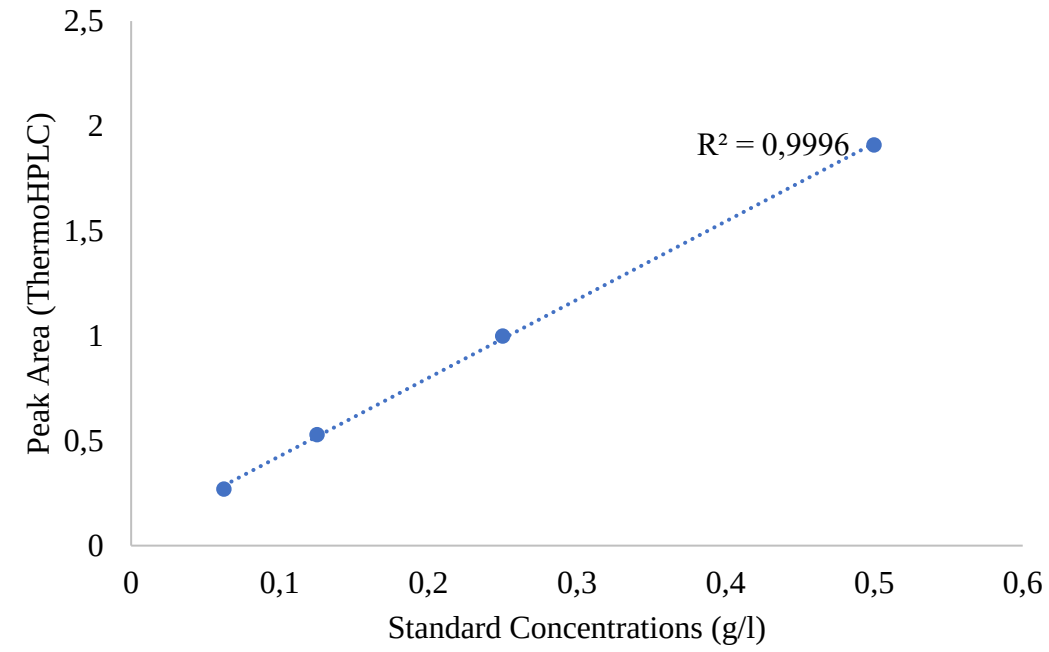


Figure A2. Standard calibration curve of xylobiose for HPLC analysis.

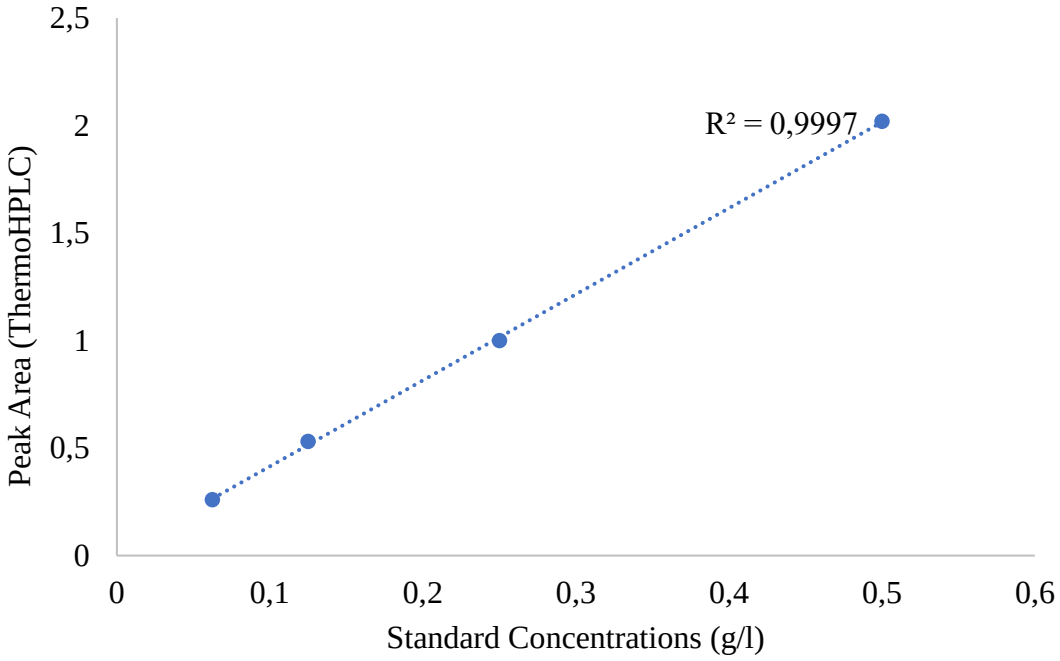


Figure A3. Standard calibration curve of xylotriose for HPLC analysis.

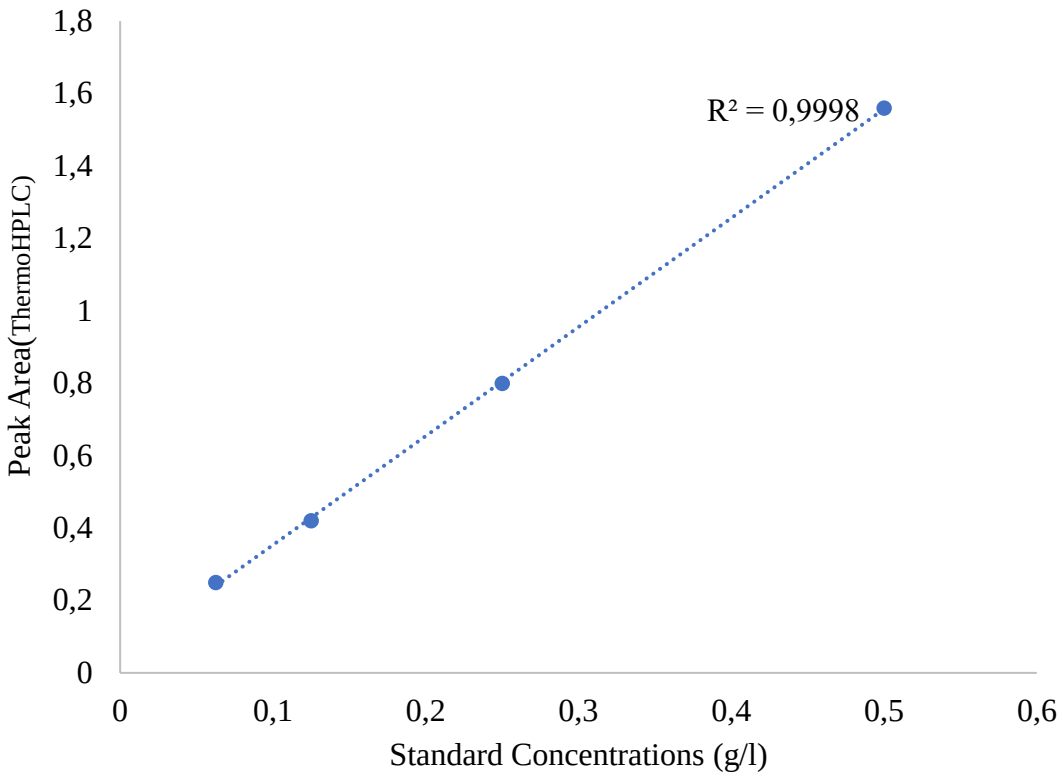


Figure A4. Standard calibration curve of xylotetrase for HPLC analysis.

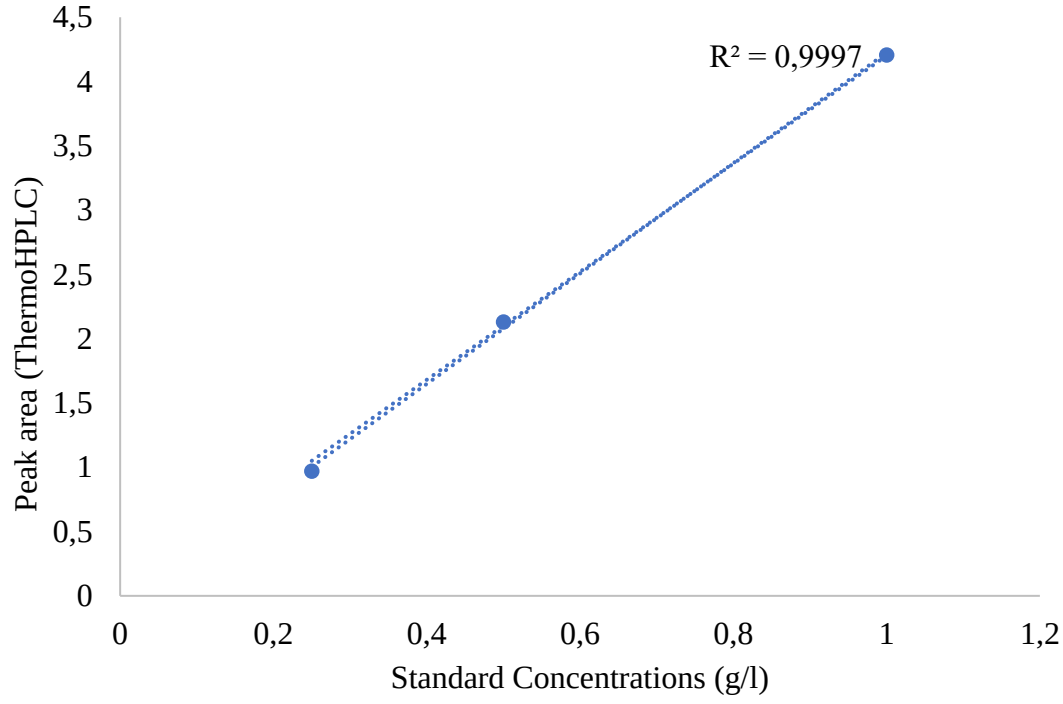


Figure A5. Standard calibration curve of glucose for HPLC analysis.

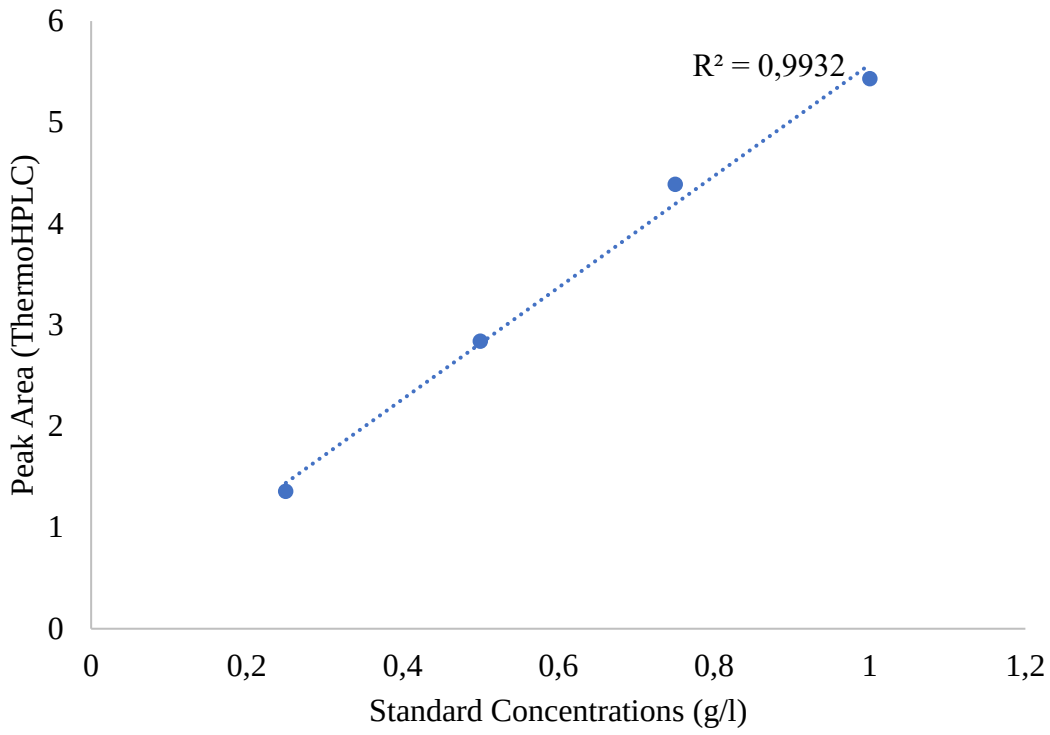


Figure A6. Standard calibration curve of arabinose for HPLC analysis.

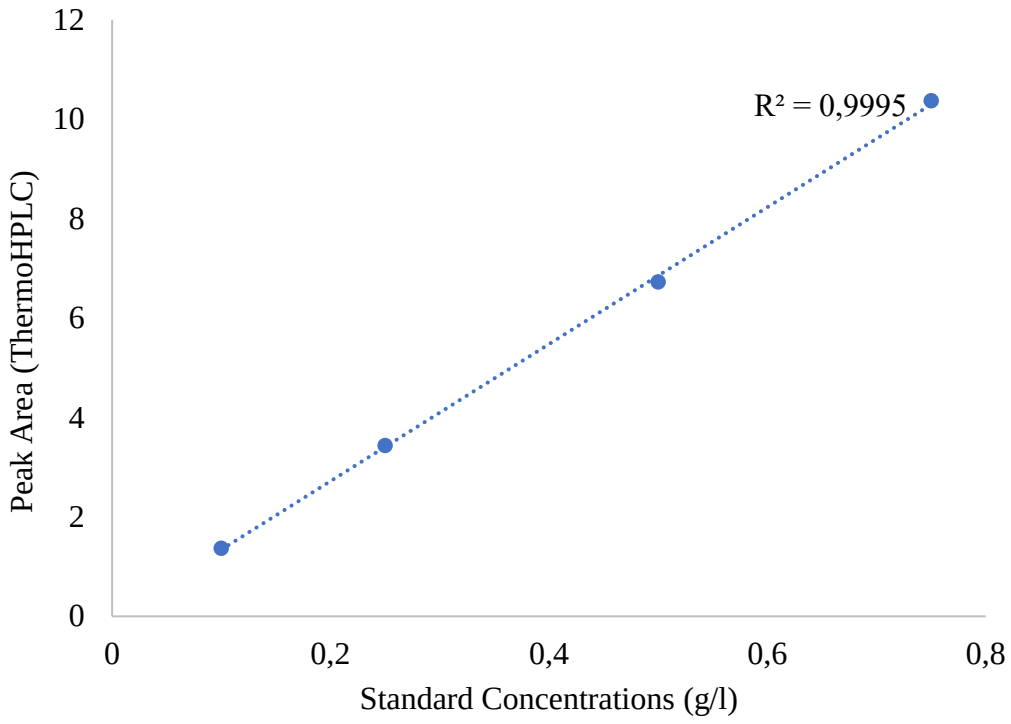
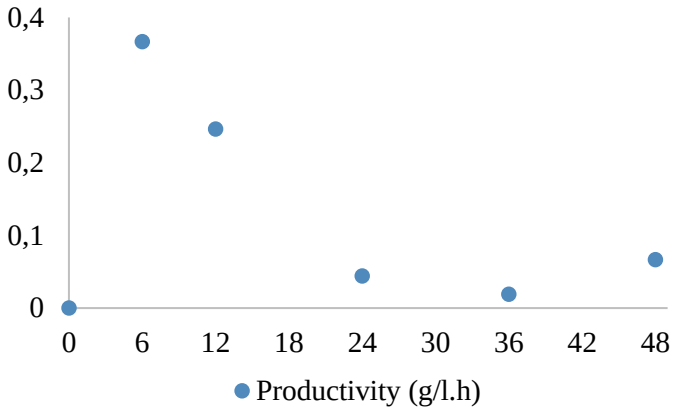


Figure A7. Standard calibration curve of acetic acid for HPLC analysis.

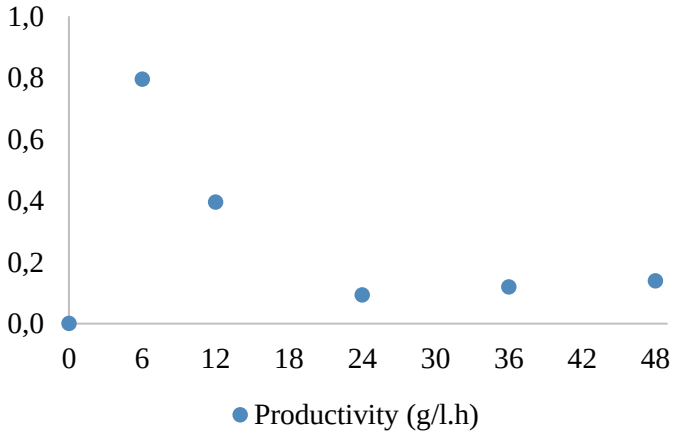
APPENDIX B

PRODUCTIVITY GRAPH

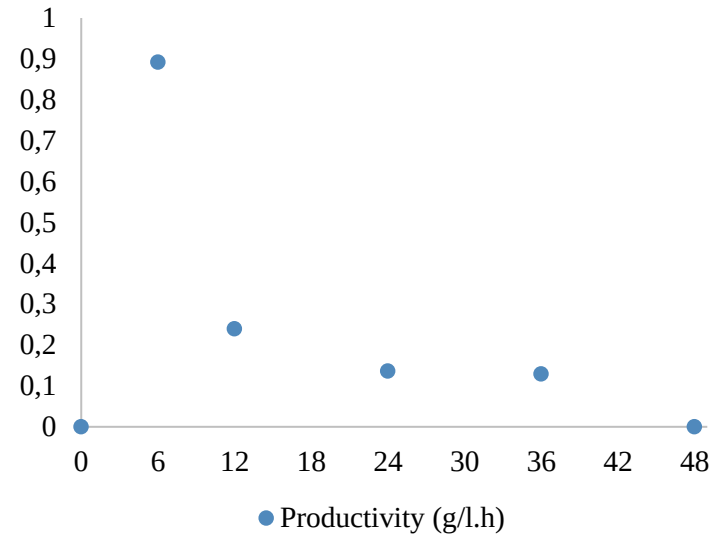
The productivity graphs show solid loading effect on XOS production.



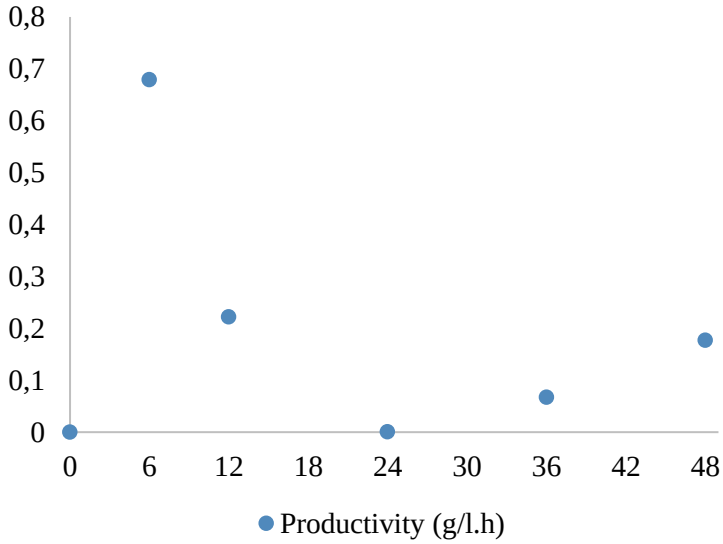
(a)



(b)



(c)



(d)

Figure B.1. Solid Loading effect on Productivity at 50°C a) 5%, b) 10%, c) 12.5%, d) 16.67% (All productivity values are calculated based on total low DP XOS concentrations, total low DP XOS values included xylotetraose, xylotriose and xylobiose concentrations)