

**PRETREATMENT METHODS
FOR VALORIZATION OF
HAZELNUT PRUNING WASTES**

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ABSTRACT

PRETREATMENT METHODS FOR VALORIZATION OF HAZELNUT PRUNING WASTES

Turkey is the world leader in hazelnut production and a large amount of residues is produced during its harvesting and processing. So far, the residues of hazelnut production had no economic value and usually burned in the fields. Obtaining valuable products such as ethanol from hazelnut pruning waste (HPW) can add value to those.

Ethanol produced by microorganisms via fermentation is a promising alternative biofuel. Ethanol has been produced for a long time from sugary substances, while lignocellulosic biomasses (LCBs) are interesting alternative to fossil fuel based resources in order to have a sustainable production process.

Liquid hot water (LHW) treatment is one of the pretreatment processes necessary to facilitate enzymatic hydrolysis of cellulose into glucose before ethanol fermentation. Organosolv is similar to LHW treatment except that ethanol-water mixture is generally used is the liquid part instead of only water. LHW could remove the hemicelluloses from the lignocellulosic matrix to some extent, while adding H_2SO_4 improved the hemicellulose removal. Organosolv was effective on removal of lignin as well as of hemicellulose. Acid catalysis improved the hemicellulose solubilization in organosolv, like in LHW treatment. After acid catalyzed organosolv, cellulose content of the HPW was increased to 67.91%. This sample was hydrolyzed with a conversion efficiency of 87.32%. Hydrolysate containing 60.63 g/L glucose was used as the medium for ethanol production using *Saccharomyces cerevisiae*. At an 83.49% theoretical yield, 22.2 g/L ethanol was obtained after 6 h. These results demonstrated that hazelnut pruning waste has potential to be used as a feedstock for ethanol production.

ÖZET

FINDIK BUDAMA ATIKLARININ DEĞERLENDİRİLMESİ İÇİN ÖN İŞLEM YÖNTEMLERİ

Türkiye, fındık üretiminde dünya lideri olup bu ürünün hasadı ve işlenmesi sırasında büyük miktarda atık açığa çıkmaktadır. Fındık budama atıklarının büyük bölümü tarlalarda sadece yakılarak değerlendirilmektedir. Bu atıkların değerlendirilmesi ve etanol gibi katma değeri yüksek ürünlere dönüştürülmesi ekonomiye fayda sağlayabilir.

Mikroorganizmalar tarafından fermentasyon yolu ile üretilen etanol ümit verici bir alternatif biyoyakıttır. Üretiminde genellikle kolay fermente edilebilen karbonhidratlar kullanılırken, atık bitkisel biyokütlenin kullanılmasına yönelik bir eğilim vardır.

Lignoselülozik biyokütledeki selüloz, hemiselüloz ve lignin ile karmaşık ve kuvvetli bir yapı oluşturduğundan, selüloz erişilebilirliğinin arttırılması için ön işlem gereklidir. Sıvı sıcak su (SSS) işlemi, etanol fermentasyonundan önce selülozun glikoza enzimatik hidrolizini kolaylaştırmak için gerekli olan ön işlem proseslerinden biridir. Organosolv, SSS ön işlemine benzer olmakla birlikte ortama sadece su yerine etanol-su karışımı eklenerek yapılan bir ön işlemdir. SSS hemiselülozları lignoselülozik yapıdan bir miktar uzaklaştırabilirken, H₂SO₄ ilavesi hemiselülozun uzaklaşmasında daha etkili olmuştur. Organosolv, hemiselülozun yanı sıra ligninin de uzaklaştırılmasında etkili olmuştur. Asit katalizörü, SSS uygulamasında olduğu gibi organosolv uygulamasında da hemiselülozun çözünürlüğünü geliştirmiştir. Asit katalizörü eklenerek uygulanan organosolv ile, fındık budama atıklarının selüloz içeriği %67.91'e yükselmiştir. Bu örnek %87.32'lik glikoz dönüşüm verimliliği ile hidroliz edilmiştir. *Saccharomyces cerevisiae* kullanılarak etanol üretimi için ortam olarak 60.63 g/L glikoz içeren sıvı kullanılmıştır. Fermentasyon işleminde 6 saat sonra 22.2 g/L etanol elde edilerek teorik verim 83.49% olarak hesaplanmıştır. Bu sonuçlar, fındık budama atığının etanol üretimi için bir hammadde olarak kullanılma potansiyeline sahip olduğunu ortaya koymuştur.

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CHAPTER 1

INTRODUCTION

Recently, rapid growth of human population, excessive consumption of fossil fuels, especially in large urban areas and the world energy issues have driven the search for alternative and environmentally friendly renewable energy sources (Chen & Fu, 2016). Alternative energy sources that are made from byproducts are known as biomass fuels (Biofuels). Paper waste, animal fat, rapeseed, sugar cane, soy, corn, algal oil and cellulose are some of the most popular sources of biofuel (Gupta & Verma, 2015). By the whole of liquid biofuels, ethanol has been advocated as a sustainable option to tackle the problems in question.

Ethanol is a kind of biofuels and recently used as a gasoline additive to improve burning of gasoline. Ethanol has increased its popularity due to rising crude oil price and the need for energy security. Moreover, ethanol is the most common biofuel and can be produced from a variety of cheap substrates (Tesfaw & Assefa, 2014). Ethanol is made biologically by fermentation of sugars derived from a variety of sources. “First generation ethanol” is made from sugar feedstock such as cane juice and molasses or from starch-rich materials such as corn. Although ethanol production from first generation is predicted to grow to more than 100 billion liters by 2022, these raw materials participate in food, are deficient to meet the increasing demand for fuels, have unpleasant effect on biodiversity and may even give rise to deforestation to gain more farmland (Bangaraiah & Kumar, 2014). The cumulative impacts of these worries have increased the interests in developing “second generation ethanol” (from biomass to liquid) from plant biomass refers largely to lignocellulosic materials, as this creates the majority of the abundant non-food materials available from plants. Plant biomass (or “lignocellulose”) is one of the greatest reserves and is mostly composed of cell walls. Basically, plant biomass can simply be burned in order to produce heat and electricity. However, there is a great potential in the use of plant biomass to produce liquid biofuels. (Naik, Goud, Rout, & Dalai, 2010). Hazelnut is one of the main agricultural products in Turkey and huge amount of waste which is lignocellulosic material is produced during its agriculture and processing. So far, the residues of hazelnut production had no economic value that is they

are not utilized for production of value added chemicals and materials and usually burned in the fields or in heaters like other wastes (Monarca et al., 2012). Obtaining valuable products such as ethanol from HPW can add value to those.

Lignocellulosic biomass is the most abundant of all renewable materials on earth, and this makes it very interesting as raw material for ethanol production (Domínguez-Bocanegra, Torres-Muñoz, & López, 2015). Polymeric carbohydrates in lignocellulosic biomass are hydrolyzed enzymatically into their monomers to be used as a carbon source in ethanol fermentations by microorganisms. However, lignocellulosic materials are recalcitrant therefore pretreatments should be applied before the enzymatic hydrolysis. There are various physical, chemical and biological pretreatment methods. Among the various methods for pretreatment, liquid hot water treatment under subcritical conditions draws attention due to not requiring acid or alkaline catalyst for decreasing the recalcitrance and/or fractionation of lignocellulosic biomass. Therefore, it can be considered as an environmentally friendly technology. Organosolv pretreatment provides an alternative for effectively increasing the enzymatic digestibility of lignocellulosic biomass using solvent-water mixture instead of only water without any solvent.

The objective of this thesis is valorization of hazelnut tree woods left after the pruning by using them as feedstock for ethanol production. Accordingly, production of a high value product from a low value raw material can contribute to Turkish economy. During this study, hazelnut prunings were treated with liquid hot water, alkali and organosolv. After the pretreatments, cellulose was hydrolyzed to glucose using commercial cellulase and subsequently glucose was fermented to ethanol using *Saccharomyces cerevisiae*. In these steps, the effects of several conditions were tested in an effort to increase yield and productivity values.

The outputs of this thesis can contribute to Turkish economy and the hazelnut farmers and this is the first effort for valorization of hazelnut pruning residues to our knowledge.

CHAPTER 2

LITERATURE REVIEW

2.1. Hazelnut

The hazelnut is also known as cobnut or filbert nut belongs to the *Betulaceae* or Birch family. Hazelnut is ordinarily used in confectionery to make pralines, cakes, cookies and chocolate truffles (Belviso et al., 2017). Hazelnut trees and hazelnut are shown in Figure 2.1.



Figure 2.1. Hazelnut trees and hazelnut

Harvest of hazelnut is by hand in most of the world, but automated in the US. Hazelnut drops naturally during a 6-week period between the middle of August to the end of September (Beyhan & Marangoz, 2007).

Almost 90% of world crop is shelled and sold as kernels with the remaining 10% utilized in shell for fresh consumption. The highest quality nuts, which command the highest prices, are sold unshelled. The most important market for these nuts is the snack food industry. A quarter of Turkey's total hazelnut production is processed by European chocolate giant Ferrero, which is famous for its Nutella brand. Besides providing desirable flavor and texture, hazelnuts can play a significant role in human nutrition and health due to their high vitamin, mineral, fat, protein, carbohydrate and dietary fiber content (Kris-Etherton, Hu, Ros, & Sabaté, 2008).

Hazelnut plays a major role in human health due to their special nutritional value. One hundred grams of hazelnut ensure 600-630 kcal, especially owing to the fat (61%), protein (15%) and carbohydrate (17%) content. Table 2.1 shows nutrient values of hazelnut and % of recommended daily allowance set by FDA.

Table 2.1. Nutrient content of hazelnut (cont. on next page)
(Source: USDA Nutrient Database)
Hazelnuts or filberts, raw

Nutritional value per 100 g	
Energy	2,629 kJ (628 kcal)
Carbohydrates	16.70 g
Sugars	4.34 g
Dietary fiber	9.7 g
Fat	60.75 g
Protein	14.95 g

Table 2.1. (cont.)

Vitamins	
Vitamin A equiv.	1 µg (0%)
beta-carotene	11 µg
lutein zeaxanthin	92 µg
Thiamine (B1)	0.643 mg (56%)
Riboflavin (B2)	0.113 mg (9%)
Niacin (B3)	1.8 mg (12%)
Pantothenic acid (B5)	0.918 mg (18%)
Vitamin B6	0.563 mg (43%)
Folate (B9)	113 µg (28%)
Vitamin C	6.3 mg (8%)
Vitamin E	15.03 mg (100%)
Vitamin K	14.2 µg (14%)
Minerals	
Calcium	114 mg (11%)
Iron	4.7 mg (36%)
Magnesium	163 mg (46%)
Manganese	6.175 mg (294%)
Phosphorus	290 mg (41%)
Potassium	680 mg (14%)
Sodium	0 mg (0%)
Zinc	2.45 mg (-26%)
Other constituents	
Water	5.31 g
Units	
µg = micrograms • mg = milligrams	
IU = International units	
Percentages are roughly approximated using US recommendations for adults. (Source: USDA Nutrient Database)	

* Percent of recommended daily allowance set by FDA, assuming 2700 calories per day.

Besides its nutritional characteristics, hazelnut provides a unique and distinctive flavour as an ingredient in a variety of food products (Seyhan et al., 2007)

2.1.1. Turkey: The Leader in Hazelnut Production

Turkey (along the Black Sea) is one of the few countries in the world having suitable weather conditions for hazelnut production and it accounts for around 60% of the global production. It is followed by Italy with almost 12% in production. Actually, world hazelnut production has increased in parallel with Turkey's production as a result of convenient climatic conditions. The other countries producing hazelnut are USA, Georgia, Azerbaijan and Greece.

Turkish hazelnut trade comes about on the Giresun Commodity Exchange in Turkey, which makes it the heart of the global hazelnut trade. In recent years, Turkey's average shelled hazelnut production has reached up 549,000 metric tonnes for the year (m/t). Table 2.2 indicates the top 5 hazelnut producing countries in metric tonnes for the year 2013 which is the latest available data as of October, 2016. Production data shows hazelnut in shell by the Food and Agriculture Organization of the United Nations.

Table 2.2. The top five hazelnut producing countries
(Sources: FAOSTAT, 2016)

	Country	Hazelnut Production	% of World Total
1	Turkey	549,000 m/t	59.90%
2	Italy	112,643 m/t	12.20%
3	United States	40,500 m/t	4.40%
4	Georgia	39,700 m/t	4.30%
5	Azerbaijan	31,202 m/t	3.40%

Hazelnuts, also known as filberts, have been grown in the Black Sea region for at least 2300 years. The Black sea region, which has a production percentage higher than 50 of the world, contains the main hazelnut-producing provinces (Ordu, Sakarya, Giresun, Düzce, Samsun and Trabzon) as shown in Table 2.3 (Taş & Gökmen, 2015).

Table 2.3. Hazelnut production in Turkey
(Source: Taş & Gökmen, 2015)

Hazelnut growing areas in Turkey		
Western Black Sea (New Developing Region)	Sakarya	16.8%
	Düzce	15.1%
	Samsun	14.3%
Eastern Black Sea (Traditional Producing Region)	Ordu	18.4%
	Giresun	15.3%
	Trabzon	7.9%

2.1.2. Valorization of Hazelnut Wastes

During hazelnut agriculture and processing hard shells, husks and pruning wastes are discarded as waste or residues. Figure 2.2 shows hazelnut wastes and their milled forms.



Figure 2.2. Hazelnut wastes

Biomass characterization of hazelnut wastes has obtained the following results, considering moisture and ash, determined on weight fraction on dry basis. Carbon (C), hydrogen (H), oxygen (O) and nitrogen (N) contents were determined using fraction on mass as it can be seen in Figure 2.3.

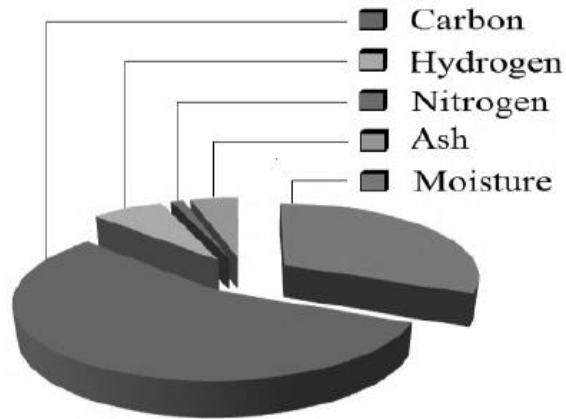


Figure 2.3. Hazelnut biomass composition
Source: (Monarca et al., 2012)

Hazelnut residues in Turkey can be divided into three groups: annual crop, long-lived (after pruning) and agro-industrial residues. These residues are actually treated in an uncontrolled manner in Turkey, they are either burnt in open-air fires or disposed of to decay, giving rise to many environmental issues. Biomass as a yield of pruning crops operation represents an interesting and attractive resource to be utilized in different ways especially as fuel for energy production (Monarca et al., 2012).

Hazelnut is a valuable and sensitive product at international levels. Turkey should focused on income enhancement via shell and pruning waste valorization along with certain growing conditions/cultural management techniques affecting both nutritional value of hazelnut varieties and economic income (Bilgen et al., 2008).

The annual amount of HPW is reported to be 2.177.986 tons (Demirbas, 2008). Finally, it is clear that HPW in Turkey have the potential to produce ethanol.

2.2. Ethanol Fuel - Alternative Energy

2.2.1. Ethanol as an Eco-Friendly Biofuel

It is evidently known that, fossil fuels cause rising temperatures in the Earth's atmosphere which is global warming (Sarkar, Ghosh, Bannerjee, & Aikat, 2012). The use of alternative fuels greatly reduces detrimental exhaust emissions such as carbon dioxide, carbon monoxide, particulate matter and sulfur dioxide (Lu, Li, Zhao, & Qu, 2012).

Therefore, discovering sources of cleaner fuel is an essential step to enhance the quality of our environment.

Furthermore, global depletion of fossil fuels, rising fuel prices and environmental concerns are determining a new solution with respect to create new fuels would reduce the conflict resulting from the world's dependence on fuel supply (Mahamud & Gomes, 2011). Ethanol is regarded as the most promising alternative to fossil fuels owing to the fact that renewable fuel in terms of unique transportation fuel with powerful economic, environmental and strategic attributes (Lennartsson, Erlandsson, & Taherzadeh, 2014). Moreover, ethanol as alternative fuels can be advanced domestically, utilizing a country's resources and thereby reinforcement the economy (Foust, Aden, Dutta, & Phillips, 2009).

Ethanol (C_2H_5OH) which is a clear colourless liquid with mild characteristic odor that boils at $78^{\circ}C$ and freezes at $-112^{\circ}C$ and the molecular structure is shown in Figure 2.4. The basic formula for making ethanol from sugar glucose is as follows:

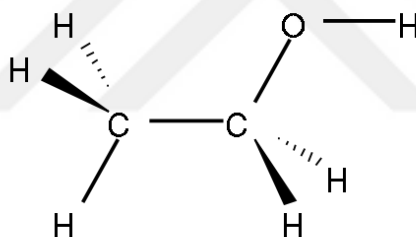


Figure 2.4. Molecular structure of ethanol

Ethanol has a lot of advantages in comparison with conventional fuel sources. First of all, using ethanol has positive environmental impacts (Budsberg, Crawford, Gustafson, Bura, & Puettmann, 2015). For example, it is much cleaner which is less toxic when compared to petroleum sources and is not a water-contaminant, so it burns more cleanly. Therefore, using ethanol can reduce the net emissions of greenhouse gases. Moreover, it has positive health impacts which replaces dangerous gasoline additives to human health. When it comes to the political impacts, it potentially replaces crude oil, which is a finite, non-renewable resource and it can be domestically produced, thus reducing dependence on oil imports. In addition, it has a positive socio-economic impact. For instance, ethanol uses agri-products as a feed-stock. It is a renewable source of energy,

which can replace fossil fuel in the future. Also it creates more jobs in the rural sector and strengthens rural economies.

Ethanol is an oxygenated fuel that contains 35% oxygen, which reduces the net emissions of greenhouse gases. Furthermore, ethanol has a higher octane number (108), broader flammability limits, higher flame speeds and higher heats of vaporization. Table 2.4 shows some properties of fuel ethanol compared to gasoline (Balat, Balat, & Öz, 2008).

In summary, ethanol as alternative fuel has, compared to gasoline,

- ✓ a higher natural octane rating,
- ✓ broader flammability limits,
- ✓ higher flame speeds,
- ✓ higher heats of vaporization,
- ✓ a higher compression ratio,
- ✓ a shorter burn time.

Therefore, changing consumer choice to ethanol can:

- ✓ reduce dependence on foreign oil,
- ✓ reduce local pollution and clean the atmosphere,
- ✓ slow climate change,
- ✓ provide a more renewable fuel source.

Table 2.4. Properties of ethanol compared to gasoline
(Source: Balat, 2008)

Parameters	Ethanol	Gasoline
Octane rating	108	87.5
Oxygen content	35%	0%
Density (g/mL)	0.79	0.72-0.78
Flammability Limits	3-19%	1-8%

2.2.2. Feedstocks for Ethanol Production

Ethanol is made biologically by fermentation of sugars derived from a variety of sources (Tan & Lee, 2014). Nowadays, the varied sources used in the manufacture of ethanol are conveniently classified into three main types: sugars, starches, and lignocellulosic raw materials (Lennartsson et al., 2014). For “first generation” approach which is sugars such as sugarcane or sweet sorghum might be used directly for ethanol production via fermentation. Starches from corn and cassava must first be hydrolyzed to fermentable sugars and following production of ethanol (Dias et al., 2012). However, this “first generation” approach led to the “food versus fuel” conflict leading to search for alternative biomass sources for the “second generation biofuels” mostly based on cellulose (Balat et al., 2008). The second generation production of ethanol derived from lignocellulosic materials from wood and agricultural residues must be converted into sugars, usually by the action of acids or cellulolytic enzymes (Naik et al., 2010). Some raw materials and their potential for bioethanol production is outlined in Table 2.5.

Table 2.5. Different feedstocks for bioethanol production and their comparative production potential (Source: Balat et al., 2008)

Raw material	Bioethanol production potential (l/ton)
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulosic biomass	280

Ethanol, which can be produced from various lignocellulosic raw materials, is a renewable and biodegradable liquid fuel. Moreover, lignocelluloses are the most promising feedstock as natural, abundant, and can potentially provide a long term sustainable fuel supply (Anwar, Gulfranz, & Irshad, 2014). Cellulosic feedstocks have the

potential to greatly improve the benefits of fuel ethanol which is significantly displace petroleum demand (Wanderley, Martin, Rocha, & Gouveia, 2013).

Agricultural crop residues comprise field residues and processing residues. Field residues such as stalks and stubble (stems), leaves and they represent materials left in an agricultural field after the crop has been harvested. Processing residues, such as husks, seeds, bagasse, and roots, are those materials left after the processing of the crop into a usable resource.

Rice straw, wheat straw, corn stover, and sugarcane bagasse are the primary agricultural wastes in terms of quantity of biomass availability. About 491×10^9 L of ethanol might be produced from the wasted crops and their associated lignocellulosic raw materials, about 16 times higher than the current world ethanol production (31×10^9 L). Crop residues are responsible for 90% of the total potential bioethanol production. The potential bioethanol production can replace 353×10^9 L of gasoline, which is equivalent to 32% of the total gasoline (Kim & Dale, 2004).

Faraco and Hadar (2011) studied the potential of ethanol production from lignocellulosic wastes in the Mediterranean Basin. Wastes from cereal, crops, olive trees and grape processing are abundant lignocellulosic wastes in France, Italy, Spain, Turkey, and Egypt, and using them as raw materials for ethanol production could bring about a potential production of ethanol. A maximum potential production capacity could be achieved from 50 % of the 180 million tons of waste currently produced in the Mediterranean Basin (Faraco & Hadar 2011).

Puri et al. (2012) found the largest renewable resources for biofuel production revealed to be forest plantations, based on Eucalyptus trees and agricultural residues for the prospects, challenges, and feedstock for biofuel production in Australia.

Another study focused on characterization of three plant species such as nut husk, moj, and bonbogori, which are available unique lignocellulosic biomass in North–East India. According to physical and chemical analysis of these lignocellulosic biomass samples, potential sources for biofuel production can be served (Sasmal, 2012).

The use of cellulosic feedstocks such as wastes could significantly decrease the energy (from all sources) required to produce the fuel, as well as decreasing associated greenhouse gases. However, cellulose forms a majority of plant matter and it is generally fibrous, therefore it cannot be directly fermented. It must be broken down into simpler molecules by pretreatments and enzymatic hydrolysis, which are currently expensive (Li, Gao, Demartini, Kumar, & Wyman, 2013).

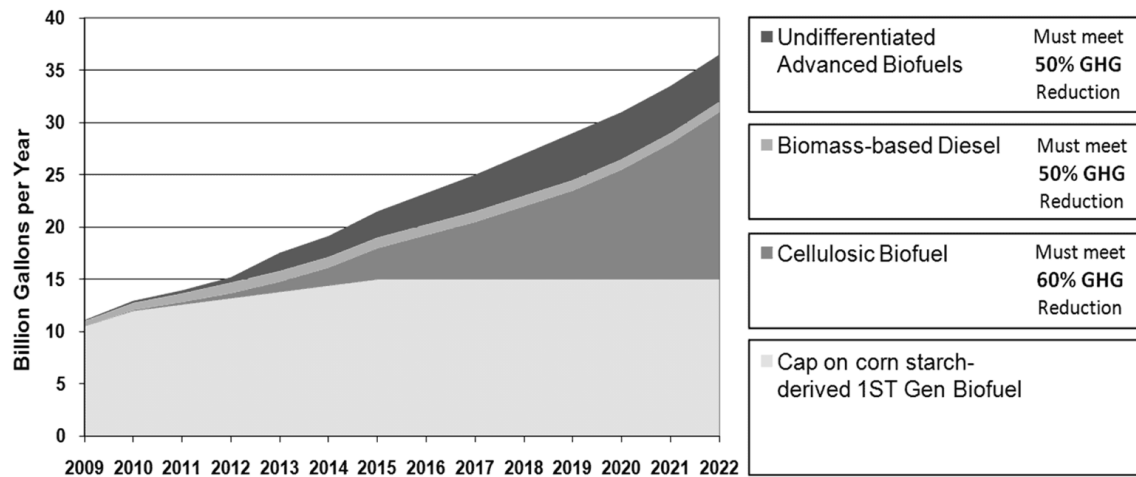


Figure 2.5. Renewable fuel standard requirements through 2022 (GHG: Greenhouse gas)
(Source: EPA, 2015)

Figure 2.5 indicates that conventional biofuels from especially corn constitutes great part of production. However, cellulosic biofuel has dramatically improved in recent years (EPA, 2015).

As a result, bioethanol produced from non-food cellulose is an attractive alternative because of its sustainability, lower-cost, efficient availability of feedstock reserves, renewable clean energy and reduction of greenhouse gas emission. Bioethanol has increased in popularity due to oil price rising and the need for energy security (Santos, Kawase, & Coelho, 2011).

2.2.3. Current Status of Ethanol Production Worldwide

Recently, ethanol has been produced in a large scale in the USA, Brasil and some European countries. It is expected to become one of the dominant renewable biofuels in the transportation sector within the next 20 years.

Generally, ethanol is produced by different raw materials such as corn and sugarcane in the worldwide. While Brazil has produced ethanol from sugarcane, the USA has manufactured ethanol from corn (Elfasakhany, 2016).

Ethanol which is high octane fuel and water free alcohol has displaced lead as an octane improver in petrol especially in the United States. Ethanol fuel blends are widely sold and the most familiar blend is 10% ethanol and 90% petrol (E10). Vehicle engine does not require any modifications to run on E25. Only special vehicles can run on up to 85% ethanol and 15% petrol blends (E85). The use of ethanol-blended fuels such as E85

can reduce the net emissions of greenhouse gases by as much as 37.1%, which is a significant amount (Bisig et al., 2016). Figure 2.6 shows ethanol blends used in the world.

Common ethanol fuel mixtures

Code	E5	E10	E15	E25	E85	E100
Composition	<div><div>max 5% anhydrous ethanol</div><div>min 95% gasoline</div></div>	<div><div>max 10% anhydrous ethanol</div><div>min 90% gasoline</div></div>	<div><div>max 15% anhydrous ethanol</div><div>min 85% gasoline</div></div>	<div><div>max 25% anhydrous ethanol</div><div>min 75% gasoline</div></div>	<div><div>max 85% anhydrous ethanol</div><div>min 15% gasoline</div></div>	<div><div>~5.3% water</div><div>100% Brazilian hydrous ethanol (contains on average 5.3 vol.% water)</div></div>
Countries	Western Europe today	USA today (Western Europe in near future)	USA EPA approval cars > 2000	Brazil	USA / Europe	Brazil

Gasoline blends for use in regular cars

Flex Fuel Vehicles

Figure 2.6. Summary of the main ethanol blends used around the world (Source: Hanskeuken, 2011)

The global production of ethanol showed a distinctly increase over the last 25 years. Worldwide annual ethanol production capacity in 2005 and 2006 were about 45 and 49 billion litres, respectively and reach over 115 billion litres in 2015. Although Brazil was for a time the largest bioethanol producing country until 2005, the United States passed Brazil and became the world's number one ethanol producer (Zhang, Asche, & Oglend, 2014).

Ethanol producers can be seen in Figure 2.7. The areas with more intense green color indicates higher production. Global ethanol production currently concentrated in two countries (United States and Brazil). Turkey is also one of the bioethanol producers (Olgun, 2009).

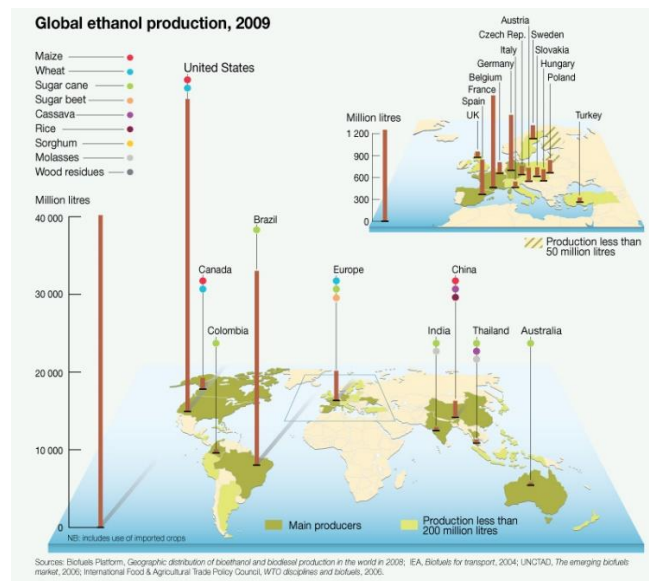


Figure 2.7. Global distribution of ethanol production geographically in 2009
(Source: Biofuels Platform, 2012)

World production of ethanol based by country is shown in Figure 2.8. The US produces the most ethanol worldwide (57%), primarily from corn. Brazil is the second largest producer with 27%, primarily from sugarcane. Other countries, including China, India, Australia, Ethiopia, Vietnam, and Zimbabwe, are also beginning to produce ethanol from sugarcane.

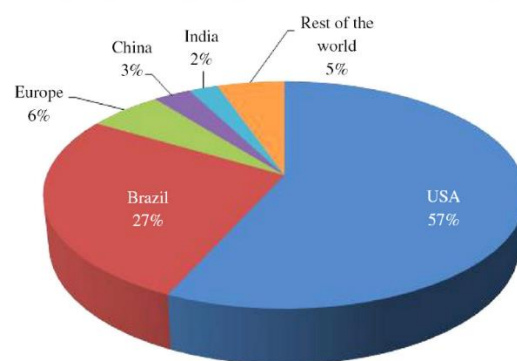


Figure 2.8. World ethanol production by country, in percent
(Source: Renewable Fuels Association, 2015)

Corn constitutes about 95% of the feedstock for ethanol production in the United States. The other 5% is composed by grain sorghum, barley, wheat, cheese whey and potatoes. According to the Renewable Fuels Association, about 79% of the corn used for ethanol production and the other 21% is processed by chemical extraction process. However, the US has a purpose of 136.260 million litres per year of renewable fuels production by 2022 and calls for 60.560 million liters to come from lignocellulosic

sources. The primarily driving force for this requirement is energy independence (Perrin, Fretes, & Sesmero, 2009).

The potential for expanding production of ethanol is one motivation behind research on cellulosic ethanol resources as feedstock is of particular relevance in countries with large populations and growing gasoline consumption such as Brazil, Egypt, China and India. About 3.9 billion litres of ethanol can be produced from rice straw and bagasse in China and India (Abdullah, Shirai, Ali, Mustapha, & Hassan, 2016).

The largest and the most efficient bioethanol plant which has 400.000 m³ production capacity annually in Europe is located in Germany (in Zeitz). Grain and sugar beet are used for the bioethanol production (Köhler, Walz, Marscheder-Weidemann, & Thedieck, 2014).

As stated above, the primary feedstock for ethanol in the US and worldwide has been corn. The increase in ethanol production in the next 10 years is expected to be from sugar-based ethanol (cane, beets). It is expected that second generation biofuel production (from cellulosic feeds) will increase after 2015.

2.2.4. Current Status of Bioethanol Production in Turkey

According to the Ministry of Energy and Natural Resources (MENR) statistics (2011), petroleum products consist of the bulk of fundamental energy consumption in Turkey. Approximately 90% of the petroleum demand is supplied with imported oil. Therefore, ethanol production is great significant in Turkey (Yousefi-Sahzabi et al., 2017).

Sugar beets are the main source of bio-ethanol production in Turkey, followed by corn and wheat. Once the sugar is extracted from beets, the alcohol remaining in the molasses is converted into ethanol (Melikoglu, 2016). There are four factories which are located in Eskişehir, Turhal, Malatya and Erzurum with the theoretical production capacity of 20,000 m³/year, 14,000 m³/year, 12,500 m³/year, and 12,500 m³/year, respectively. There are also three other private factories which are called Çumra Sugar Factory, Tarkim and Tezkim with the capacity of 80,000 m³/year, 40,000 m³/year and 40,000 m³/year, respectively (Olgun, 2009). Factories which produce ethanol in Turkey are shown in Table 2.6 below.

Table 2.6. Bioethanol production in Turkey
(Source: Olgun, 2009)

Factory	Feedstock	Production Capacity (million liters/year)
Eskişehir Sugar Factory	Sugar Beet	20
Çumra Sugar Factory	Sugar Beet	80
Turhal Sugar Factory	Sugar Beet	14
Malatya Sugar Factory	Sugar Beet	12.5
Erzurum Sugar Factory	Sugar Beet	12.5
Tarkim (Bursa)	Corn	40
Tezlim (Adana)	Wheat – Corn	40

2.3. Bioethanol Production Processes from Lignocellulosic Biomass

Biomass conversion involves three basic steps (Gao et al., 2013):

1. Pretreatment - the usefulness of cellulose as a feedstock has been limited by its rigid structure and difficulty to breakdown into simple sugars
2. Hydrolysis - Cellulose is broken down into individual glucose units by cellulase enzymes
3. Fermentation - Glucose is fermented by microorganisms, for production of ethanol

Ethanol production from lignocellulosic biomass using enzymatic hydrolysis and fermentation can be improved by (Zabed, Sahu, Boyce, & Faruq, 2016):

1. development of effective pretreatment technologies;
2. maintaining a high density of cells within the reactor to convert sugars to ethanol quickly;
3. integrating enzymatic hydrolysis of cellulose and hemicellulose;
4. converting both the cellulose (glucose) and hemicellulose (xylose) to ethanol to increase the overall ethanol yield.

2.3.1. Lignocellulosic Biomass

Lignocellulosic biomass comprising forestry, agricultural and agro-industrial wastes are clean, abundant, renewable, cost effective and inexpensive energy sources. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane bagasse, waste paper, brewer's spent grains, switchgrass, and straws, stems, stalks, leaves, husks, shells and peels from cereals like rice, wheat, corn and barley, among others. For second-generation biofuel production, valorization of lignocellulosic wastes has received major focus in the world (Bangaraiah & Kumar, 2014).

Lignocellulosic biomass is the common name for all material primarily contain cellulose ($C_6H_{10}O_5$)_x, lignin [$C_9H_{10}O_3.(OCH_3)_{0.9-1.7}$]_n, hemicellulose such as xylan ($C_5H_8O_4$)_m, and extractives. Lignocellulosic agricultural wastes have cellulose as a major component, but their chemical composition varies considerably (Adaganti, 2014). Basically, cellulose forms a skeleton which is surrounded by hemicellulose and lignin as it is clearly demonstrated in Figure 2.9.

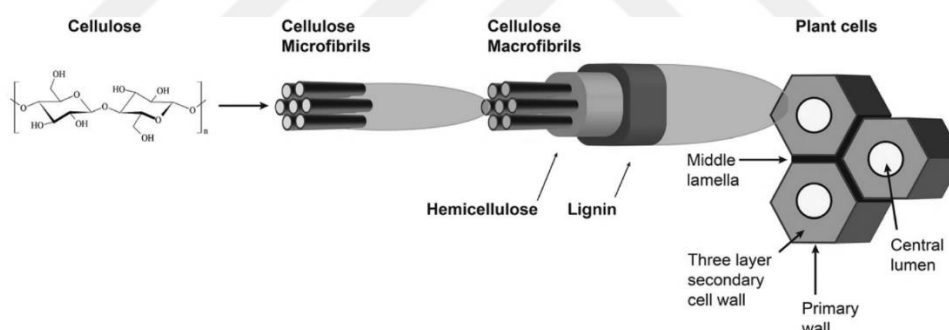


Figure 2.9. Schematic illustration of lignocellulosic structure showing cellulose, hemicellulose and lignin (Source: Zhang et al., 2016)

2.3.1.1. Cellulose

Cellulose is a huge amount of sustainable and biodegradable resource for raw materials. Cellulose makes up approximately half of the mass of wood and it is a linear homo-polysaccharide consisting of long glucose units that are linked by β-1,4 glucosidic bonds. Linking just two of these sugars produces a disaccharide called cellobiose. The β-D-glucose units result in the potential formation of intermolecular hydrogen bonds, which make native cellulose highly crystalline, insoluble, and resistant to enzyme attack (Figure 2.10) (Wu, Shen, Hu, Zhang, & Xiao, 2016). Cellulose molecular weight can reach to

50000-2500000 which is equivalent to 500-15000 glycosylic groups. Commercially, it has become the most important feedstock after fossil fuel. A variety of other polysaccharides such as hemicellulose and lignin are associated with cellulose in nature.

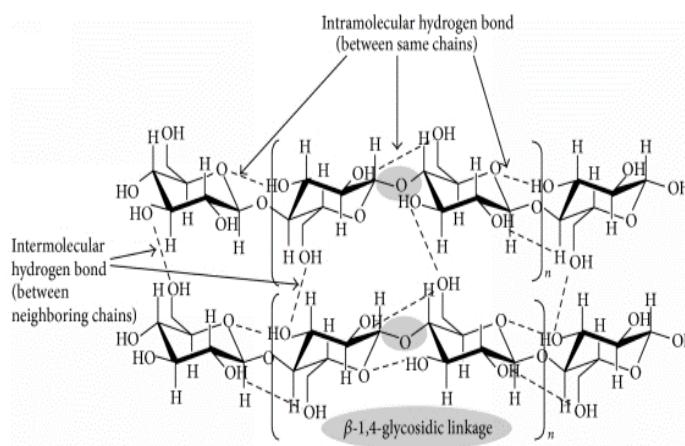


Figure 2.10. Chemical structure of cellulose compounds

2.3.1.2. Hemicellulose

Hemicellulose is a short, highly branched polymer of pentoses (e.g. D-xylose and L-arabinose) and hexoses (e.g. D-mannose, D-galactose, and D-glucose) with 50–200 units, unlike cellulose. The dominant sugars in hemicelluloses is xylose in hardwoods and agriculture residues followed by mannose, glucose, galactose, with small amount of arabinose and rhamnose.

The hemicellulose backbone chains can be comprising generally single sugar with repeat unit or mixture of different sugars. According to the main sugar residue, hemicellulose structure has different classifications such as xylans, mannans, glucans, glucuronoxylans, arabinoxylans, glucomannans, galactomannans, galactoglucomannans, β -glucans, and xyloglucans (Barana, Salanti, Orlandi, Ali, & Zoia, 2016). Figure 2.11 shows chemical structure of hemicellulose compounds as xylan and glucomannan which are the most existing biopolymer.

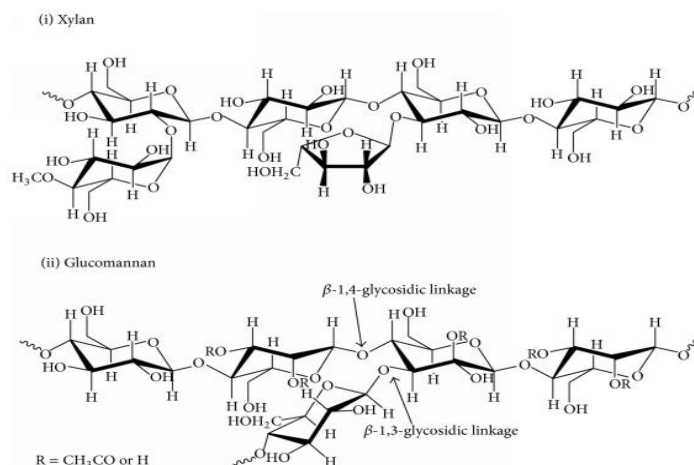


Figure 2.11. Chemical structures of xylan and glucomannan

Compared to cellulose with hemicelluloses differ by composition of monosaccharides by presence of smaller chains, and to be amorphous, which made its structure easier to hydrolyze than cellulose. The role of hemicellulose is to provide a linkage between lignin and cellulose.

2.3.1.3. Lignin

Lignin is a complex molecular structure containing cross-linked polymers of phenolic monomers especially p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2.12). The cellulose and hemicellulose are cemented together by lignin. Lignin is responsible for integrity, structural rigidity, and prevention of swelling of lignocelluloses, therefore the delignification processes can improve the rate and extent of enzymatic hydrolysis (Miyafuji, Komai, & Kanbayashi, 2017).

The presence of lignin in lignocellulosic biomass is the main disadvantage of biomass recalcitrance during separation process because of acting protective barrier for plant cell. As a result, removal of lignin is necessary to enhance biomass digestibility for bioethanol production.

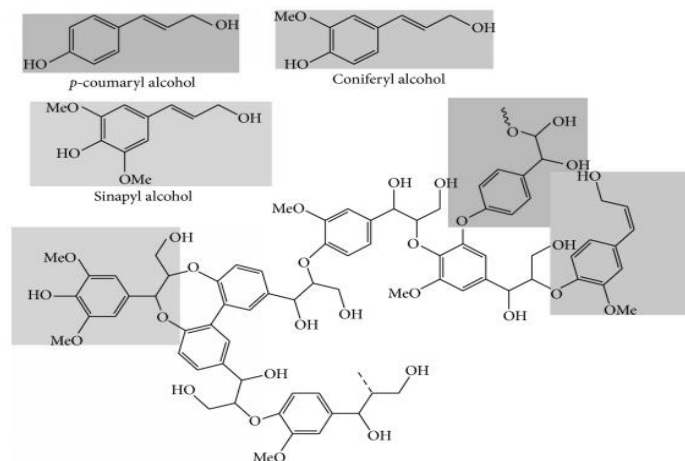


Figure 2.12. Chemical structure of lignin (p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol).

Overall components of the cellulosic biomass are shown in Figure 2.13.

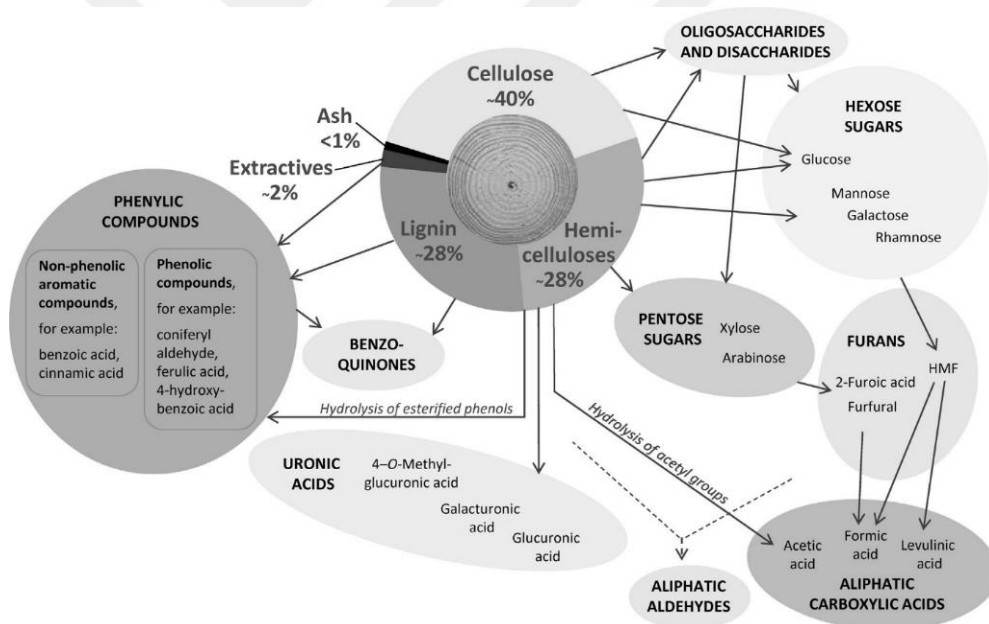


Fig. 2.13. Overall components of lignocellulosic materials (Source: Taherzadeh and Karimi, 2008)

The amounts of cellulose, hemicellulose and lignin diversify from one plant specie to another. However, cellulose is usually the predominant structural polysaccharide of plant cell walls (30–50%), followed by hemicellulose (20–35%) and lignin (10–25%).

The amount of carbohydrate polymers and lignin can vary from one type to another type of lignocellulosic material. Garrote and Wyman (1996) found the compositions of some different lignocellulosic feedstocks and they found that the hardwoods such as Eucalyptus, oak and white birch contained 39 – 54% cellulose, 14 – 37% hemicellulose, 17 – 30% lignin. Main components in some agricultural and forestry wastes are shown in Table 2.7.

Table 2.7. Chemical composition of common agricultural residues and wastes.
(Source: Balat, 2008)

Types of biomass	Lignocellulosic substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Agriculture waste	Corn stover	31-42	25-40	13-22
	Wheat straw	30-32	41-50	15-16
	Barley straw	33-40	20-35	8-17
	Rice straw	32	27	18
	Cereal straw	35-40	26	15-20
	Nut shells	25-30	25-30	30-40
Forestry waste	Hardwood stems	40-55	25-35	20-25
	Softwood stems	45-50	25-30	25-35

2.3.2. Bioethanol from Lignocellulosic Materials via Biochemical Pathway

The bioconversion of cellulose and hemicellulose to monomeric sugars is presently applied for bioethanol production. There are several options for a lignocellulose-to-bioethanol process, the following features must be assessed (Hahn-Hagerdal, Galbe, Gorwa-Grauslund, Liden, & Zacchi, 2006):

- Efficient degradation of cellulose and hemicellulose into fermentable sugars
- Efficient fermentation of sugars (both six- and five-carbon sugars)
- Advanced process integration in order to minimize process energy demand
- Using feedstocks with low lignin content in order to decrease production cost

Biochemical conversion of lignocellulosic materials contain three major steps (Balat et al., 2008):

- 1) **Pretreatment:** to alter or remove structural barriers to hydrolysis in order to improve the enzyme hydrolysis rate and increase yields of fermentable sugars (mainly glucose and xylose) from cellulose or hemicellulose by degradation of the lignocellulosic structure.
- 2) **Enzymatic hydrolysis:** chemical and physical changes in the residual solid-phase cellulose with the help of enzymes and occur ultimately to glucose.
- 3) **Fermentation:** conversion of fermentable sugars to bioethanol by microorganisms.

Conversion process of lignocellulosic biomass to bioethanol is summarized in Figure 2.14.

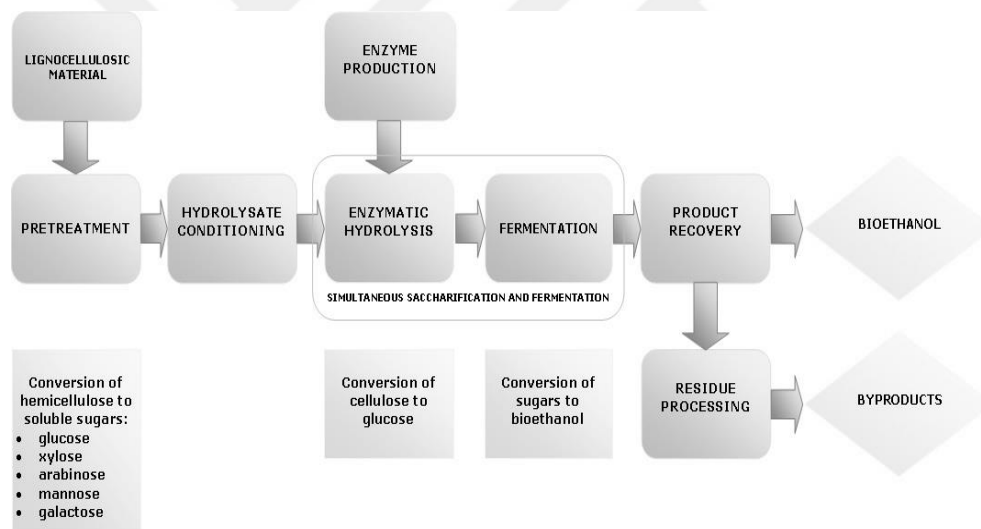


Figure 2.14. Schematic flowsheet for the conversion of biomass to ethanol (Source: Foust et al., 2009)

2.3.2.1. Pretreatments

Pretreatment is required in order to alter the recalcitrance of lignocellulosic biomass to improve cellulose more accessible to enzymatic hydrolysis and to achieve a greater glucose yield in the bioconversion processes for bioethanol production (Ma et al., 2016). This effect is achieved by increasing cellulose surface area through solubilization of hemicelluloses and lignin.

Pretreatment strategies are currently available with variation in terms of pH, temperature, types of catalyst and residence time. These variations affect strongly the severity of the pretreatment and the lignocellulosic biomass composition during biomass degradation. An effective pretreatment is defined by several criteria including low pretreatment catalyst usage or inexpensive catalyst recycle, and generation of higher value lignin co-product from a basis of comparison for various pretreatment options.

The optimum pretreatment method and conditions depend on the type of lignocelluloses including of surface morphology and substrate pore structure. Due to the structural differences among these fractions, separation of cellulose, hemicellulose and lignin from lignocellulose biomass requires the use of specific processes, which may be physical, physico-chemical, chemical or biological. Many industrial pretreatments (liquid hot water, acid and alkaline pretreatment, organosolv, steam-explosion, milling, irradiation, microwave, ammonia fiber explosion (AFEX), wet oxidation, ozonolysis etc.) have been developed to remove lignin and hemicelluloses from cell walls and to expose cellulose to hydrolytic enzymes. A brief discussion on the most commonly used pretreatment techniques, as summarized in Table 2.8.

Table 2.8. Overview of pretreatment methods for lignocellulosic feedstocks prior to enzymatic hydrolysis of cellulose (Source: Jönsson et al., 2016)

Pretreatment methods	Used chemicals	Main effect
Acid-based methods	Involve catalysts such as H_2SO_4 , HCl , H_3PO_4	Partial or complete hydrolysis of hemicelluloses to monosaccharides
Hydrothermal processing	No additives	Increase in accessible surface area and pore size, Solubilization of hemicelluloses without complete hydrolysis
Mild alkaline methods	Involve alkali such as NaOH , $\text{Ca}(\text{OH})_2$, NH_3	Partial or nearly complete removal of lignin and a minor part of hemicelluloses
Oxidative methods	Involve oxidants such as H_2O_2 and O_2 (alkaline conditions), and O_3	Removal of lignin and part of hemicelluloses
Alternative solvents	Ethanol, Methanol, Propanol, Isopropanol, Butanol	Dissolution of specific lignocellulosic components or the whole biomass

The main factors considered as affecting the rate of biological degradation of lignocelluloses by the enzymes are the crystallinity of cellulose and protection by lignin and hemicellulose, degree of cellulose polymerization, and degree of acetylation of hemicelluloses. These factors are shown briefly in Figure 2.15.

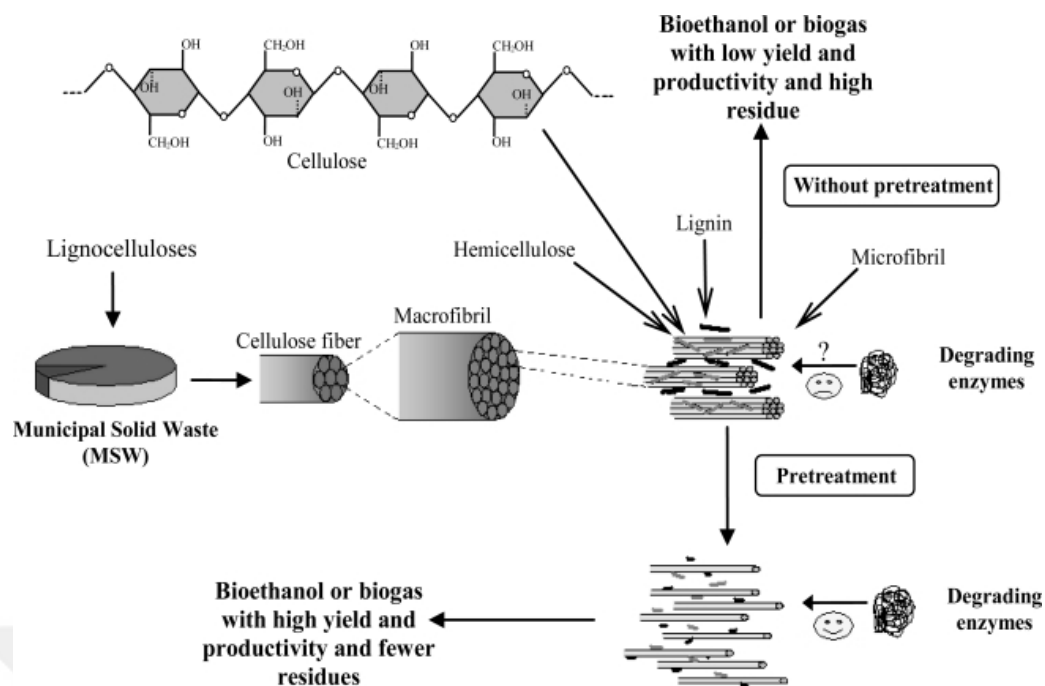


Figure 2.15. Effect of pretreatment on accessibility of degrading enzymes
(Source: Taherzadeh and Karimi, 2008)

Pretreatment process is necessary to achieve enzymatic hydrolysis. The choice of an appropriate pretreatment method plays an important role to increase the efficiency of enzymatic digestibility of the lignocellulosic biomass. An effective and economical pretreatment should meet the following requirements:

- production of reactive cellulosic fiber for enzymatic attack,
- avoiding formation of possible inhibitors for hydrolytic enzymes
- minimizing the energy demand,
- producing less residues,
- consumption of little or no chemical and using an inexpensive chemical (Sun, 2002).

2.3.2.1.1. Liquid Hot Water

Liquid hot water (LHW) pretreatment is a hydrothermal treatment using only liquid water at high temperatures under pressure. The major advantage of this method is that it requires no chemicals for hydrolysis reactorion and consequently does not cause any significant corrosion problems in this process. Therefore, it can be considered as an environmentally friendly technology.

Under high pressure water passes through into the biomass, hydrates cellulose, and removes hemicelluloses mainly and lignin partly. LHW can develop the accessible and sensitive surface area of the cellulose and make it more accessible to hydrolytic enzymes. Hydrothermal pretreatment is usually carried out at relatively high temperature (140-220°C) in a pressure reactor. After waiting for a certain time, the reactor is cooled and liquid phase is drained off. LHW is one of the first steps in the fractionation process for lignocellulosic materials (LCM). The precipitated solid phase is rich in cellulose and lignin and they can be separated by further processing.

The processing temperature and time should be controlled in order to optimize the enzymatic digestibility by LHW pretreatment. Moiser et al. (2005) found that an optimized condition for LHW pretreatment of corn stover was reported to be 190°C for 15 min, in which 90% of the cellulose conversion was observed by subsequent enzymatic hydrolysis. Laser et al. (2002) compared the performance of LHW and steam pretreatments of sugarcane bagasse, which was subsequently used in ethanol production by SSF. They performed the treatments in a reactor at 170-230°C with 1% to 8% solids concentration. The results showed that both methods can significantly develop the hydrolysis; however, the LHW resulted in much better xylan recovery compared to steam pretreatment.

The LHW processing removes mainly hemicellulose. A two-stage process which combines the liquid hot water for hemicellulose removal and a treatment for delignification (e.g. ammonia treatment or alkaline) was also suggested for further improvement of enzymatic hydrolysis (Kim et al., 2006).

As a result, LHW has been demonstrated to be a successful method to remove up to 80% of the hemicellulose in for pretreatment of different kinds of lignocellulosic materials including sugar cane bagasse (Laser et al. 2002), corn stover (Mosier, Wyman, et al., 2005), wheat straw (Pérez et al., 2008), and sunflower stalks (Monlau, Barakat, Steyer, & Carrere, 2012). In fact, after LHW pretreatment, the solid fractions are more susceptible to enzymatic digestion (Zeng, Mosier, Huang, Sherman, & Ladisch, 2007).

After enzymatic hydrolysis, LHW shows high cellulose solubilization and producing less inhibitor compounds, compared with acid catalyst. Actually, during LHW pretreatment, the cleavage of O-acetyl and uronic acid produces acetic acid that help to improve the hydrolysis of polysaccharides into soluble monosaccharides (Dien et al., 2006).

LHW pretreatment is appealing because it does not require addition of chemicals and it produces less inhibitor compounds than other pretreatments, while keeping sugar yields high. The main drawbacks of LHW are the high energy demand due to the high pressure and the large amount of water required by the system. However, this process is attractive for large-scale operations because LHW reactor systems are not expensive as well as reactor systems resistant to corrosive chemicals (Petersen, Larsen, & Thomsen, 2009). Practically, LHW pretreatment has been performed and optimized on pilot scale at DONG Energy facility in Denmark after indeep economic feasibility studies (Capolupo & Faraco, 2016).

2.3.2.1.2. Dilute Acid

Dilute acid pretreatment has been considered to be the most promising pretreatment technologies that can enhance biomass sugar release (Qureshi et al., 2016). Dilute acid pretreatment includes the treatment of biomass with heat, pressure and residence times a combination of an acidic pH, which is generally carried out using 0.2-2.0% sulphuric acid, at 121-220°C (Lee et al., 2015).

Dilute acid pretreatment of lignocellulose serves two important functions in the conversion process (Hendriks et al., 2009):

1. hydrolysis of the hemicellulose components to produce monomeric sugars and
2. exposure of cellulose for enzymatic digestion by removal of hemicellulose mainly and part of the lignin.

Generally, sulphuric acid (H_2SO_4), hydrochloric acid (HCl), boric acid (H_3PO_4), and nitric acid (HNO_3) are used as acid reagents (Sadasivam et al., 1996).

Dilute acid effectively removes and recovers most of the hemicellulose in liquid phase, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose hydrolysis. High temperature in the dilute-acid treatment is convenient for cellulose hydrolysis (Hendriks et al., 2009).

Acid pretreatment has also some drawbacks, such as high cost of the materials used for construction of the reactors. Acids such as H_2SO_4 and HCl have also been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis and hemicelluloses solubilization, these acids are toxic, corrosive, hazardous,

and thus require reactors that are resistant to corrosion (Alvira et al., 2010). Moreover, during dilute acid pretreatments, byproducts such as furfural and hydroxy methyl furfural, which are toxic to the following saccharification and fermentation processes, are formed. Therefore, detoxification process may be necessary, which increases the pretreatment cost (Larsson et al., 1999).

Two types of dilute-acid pretreatment processes are typically used: a high-temperature ($T > 160^{\circ}\text{C}$), continuous-flow process with low solids (5-10%) and a low-temperature ($T < 160^{\circ}\text{C}$), batch process for high solids loadings (10-40%) (Sun & Cheng, 2002). Very dilute acid (VDA) addition (about 0.1% versus the 0.7–3.0% typical for the dilute acid technology described) in a reactor is effective at very low acid levels (Kumar et al., 2009).

Dilute acid pretreatment has been studied for a wide range of lignocellulosic biomass, including corn (husks, cobs, and stover), hardwood bark from aspen, poplar, and sweet gum and agricultural residues (Kumar et al., 2009).

Cao et al. (2009) reported that corn stover pretreated with dilute H_2SO_4 (0.25–4%, w/v) at 121°C for 30 and 180 min could improve reduction of hemicellulose and lignin at H_2SO_4 concentration of 1.69% and reaction time of 117 min.

Varga et al. (2002) used sulphuric acid for pretreatment of corn stover under mild conditions (121°C , 1 h). Pretreatment with 5% H_2SO_4 solubilized 85% of the hemicellulose fraction, however the enzymatic conversion increased only two times compared to untreated stover.

Ishizawa et al. (2007) evaluated that corn stover was subjected to dilute H_2SO_4 pretreatment in a reactor at temperatures ranging from 180 to 200°C , solid loadings between 25% and 35% (w/w), and acid loadings of 0.03-0.06 g of acid/g of dry biomass. All of the pretreated samples demonstrated higher pore volumes than untreated corn stover. The authors determined that porosity might be a factor for enzymatic digestibility.

Lu et al. (2007) carried out pretreatment of corn stover for sulphuric acid concentrations of 2%, 4%, and 6% at 80, 100, and 120°C . The optimum conditions for corn stover pretreatment were a H_2SO_4 concentration of 2% and a reaction time of 43 min at 120°C . As a result, 77% xylose yield was obtained, and thus it showed good susceptibility toward enzymatic hydrolysis, leading up to 42.1 g of glucose/100 g of substrate, equivalent to a conversion yield of 70% under the optimum conditions.

Cara et al. (2008) studied the pretreatment of olive-tree biomass by dilute-acid and further enzymatic saccharification. Pretreatment was performed at 0.2%, 0.6%, 1.0%,

and 1.4% (w/w) sulphuric acid concentrations, and the temperature was varied in the range of 170-210°C. A maximum of 83% of hemicellulosic sugars was recovered in the liquid phase at 170°C and 1% H₂SO₄ concentration, but the enzyme accessibility of the pretreated solid was not sufficient at this condition. A maximum enzymatic hydrolysis yield of 76.5% was obtained from a pretreated solid at 210°C and 1.4% acid concentration. The maximum value of 36.3 g of glucose/100 g of raw material (75%) was obtained for the pretreatment of olive-tree biomass at 180 °C and 1% H₂SO₄ concentration. As it is clearly depicted that dilute-acid pretreatment improved the enzymatic hydrolysis process compared to water pretreatment.

2.3.2.1.3. Alkaline

Alkali pretreatment refers to the implementation of alkaline solutions, such as sodium hydroxide, calcium hydroxide or ammonia for the treatment of biomass, in order to remove high amount of lignin and part of hemicellulose and to efficiently increase the accessibility of cellulose: it is basically a delignification process, where a significant amount of hemicellulose is also solubilized. The concentration of alkaline solution should be optimized in accordance with different substrate to avoid hemicellulose loss (Taherzadeh & Karimi, 2008).

The major strategy of alkaline pretreatment is to disrupt the lignin structure in lignocellulosic biomass. Under alkaline conditions, the ester linkages in hemicelluloses and lignin are easily broken down, thus significantly promote the solubilization of hemicelluloses and lignin, resulting in the exposure of cellulose to enzymes. Pretreatment might be performed at low temperatures but with a relatively long time and high concentration of the raw material (Zheng et al., 2009).

Dilute NaOH, which is the most common agent used for alkali pretreatment of lignocellulosic materials has been found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. The digestibility of NaOH-treated hardwood was reported to increase from 14% to 55% with a decrease of lignin content from 55% to 20% (Chiaramonti et al., 2012).

Vaccarino et al. (1987) studied the effects of different alkali pretreatments, such as SO_2 , Na_2CO_3 , and NaOH pretreatments, on the enzymatic digestibility of grape pomace, and the highest degrading effects were obtained by pretreatment with 1% NaOH solution at 120°C .

Silverstein et al. (2007) studied the effectiveness of NaOH , H_2O_2 and O_3 pretreatments for enzymatic conversion of cotton stalks. They found that sodium hydroxide pretreatment resulted in the highest level of delignification as 65% with 2% NaOH in 90 min at 121°C and cellulose conversion was 60.8%.

Zhao et al. (2007) reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose in comparison with dilute acid pretreatment. Compared with acid or oxidative reagents, alkali treatment appears to be the most effective method in breaking the ester bonds between lignin hemicellulose and cellulose (Gaspar et al., 2007).

Chang et al. (1998) investigated that pretreatment of wheat straw by calcium hydroxide under recommended conditions of short treatment times (1–3 h) and high temperatures (85 – 135°C) or long treatment times (24 h) and lower temperatures (50 – 65°C) and the yield of reducing sugars was increased 10 times compared with untreated sample.

Consequently, lignin removal by alkali treatments increases enzyme effectiveness by increasing access to cellulose and hemicellulose. Therefore, alkaline pretreatment can play a meaningful role in revealing the cellulose to enzymatic hydrolysis.

2.3.2.1.4. Organosolv

Organosolv can be used to provide treated cellulose appropriate for enzymatic hydrolysis, using an organic or aqueous organic solvent to take out or decompose the network of lignin and possibly a part of the hemicellulose. For organosolv pretreatment, lignocellulosic biomass is mixed with solvent and water and heated to dissolve the lignin and part of the hemicellulose, leaving reactive cellulose in the solid phase. Moreover, a catalyst such as acid may be added either to reduce the operating temperature or to enhance the delignification process (Alvira et al., 2010).

In this process, several solvents at temperatures of 150 – 200°C can be used with or without addition of catalysts such as acid. Furthermore, the solvent may accompany acetic acid released from acetyl groups improved by hydrolysis of hemicelluloses. A variety of

organic solvents such as alcohols, esters, ketones, glycols, organic acids, phenols, and ethers have been used. However, the price of solvent and convenience for recovery of solvent should also be considered. The applied solvents should be separated by evaporation and condensation, and recycled to reduce the process cost (Sun & Cheng, 2002).

Araque et al. (2007) studied the organosolv acetone-water for pretreatment and found the highest ethanol yield to be 99.5% after pretreatment at 195°C, 5 min, pH 2, and with 50% (v/v) of acetone-water. For economic reasons, the use of low-molecular-weight alcohols such as ethanol and methanol has been preferential.

Ethanol pretreatment is a broadly studied organosolv pretreatment method. This is primarily because that ethanol has the advantages of low toxicity, low boiling point, the attribute of volatility results in the easy recovery of solvent after pretreatment, which benefits the reduction of energy consumption. Generally, ethanol pretreatment technologies have been developed with respect to the catalysts used, such as acid-catalyzed, autocatalyzed and alkali-catalyzed processes (Zhao et al., 2009).

Organosolv can be used together with acid hydrolysis to separate hemicellulose and lignin. Several reports suggested such a system for pretreatment of biomass. Firstly, lignocellulosic raw material can be treated with dilute acid at low temperature about 100°C for 10-60 min in order to selectively hydrolyze the hemicellulosic part. The aim of the second stage of the process is delignification of the pretreated lignocellulose. In this stage, ethanol is added to the system and treated at 81°C for 90 min to provide the medium for dissolving of lignin. Negligible cellulose loss (less than 2% w/w of original cellulose) and high lignin removal (more than 70% w/w of original lignin) makes the two-stage low-temperature organosolv and acid-catalyzed process amazing for laboratory pretreatment of lignocellulosic material before enzymatic hydrolysis (Taherzadeh & Karimi, 2008).

It should be known that for all the pretreatment methods, increasing the surface area is one of the major approaches, which can be achieved by solubilization of the hemicellulose and lignin with separation of cellulose. For most of organosolv processes, lignin and hemicellulose are solubilized. As a result, organosolv pretreatment provides an alternative for effectively increasing the enzymatic digestibility of lignocellulosic biomass (Binod et al., 2010).

2.3.3. Enzymatic Hydrolysis

Enzymatic hydrolysis using optimized pretreatment parameters is performed to ensure maximum cellulose conversion. In addition, varying pretreatment time, solid and enzyme loading to obtain maximum glucose yields while minimizing costs could optimize enzymatic hydrolysis (Kaar & Holtzapple, 2000). Therefore, an effective pretreatment is fundamental to a successful enzymatic hydrolysis.

Enzymatic hydrolysis provides a method to convert cellulose to glucose at high yields. Enzymatic hydrolysis of cellulose assists to break glycosidic bonds by the use of cellulase enzymes. Factors effecting hydrolysis of cellulose include type of substrate, cellulase loading and reaction conditions such as temperature and pH (Hahn-Hägerdal et al., 2006).

The characteristics of the biomass substrate are of great importance to hydrolysis optimization. The susceptibility of cellulosic substrates to cellulases mainly depends on the degree of crystallinity and polymerization of cellulose, availability of the surface area as well as lignin content (Sun et al., 2015).

Enzyme loading is another factor which is critical to hydrolysis efficiency. Because the amount of enzyme directly affects the operating cost, simply increasing enzyme loading is not a reasonable approach to facilitate the reaction and improve sugar yields (Mosier et al., 2005).

The efficient enzymatic hydrolysis means that the cellulase and hydrolysate can transfer effectively within the porous structure. Thus the porous structure, especially pore size distribution is largely responsible for the efficiency of enzymatic hydrolysis. To improve the efficiency of enzymatic hydrolysis, the relevant literatures focus on optimizing the hydrolysis process and enhancing the cellulase activity (Chen & Fu, 2016).

2.3.4. Fermentation

Nowadays, ethanol fermentation has become one of the most challenging biotechnological processes (Zaldivar et al., 2001).

There are four approaches for optimizing fermentation including physiological, biological, genetic, and engineering. The physiological approach involves in environmental factors including pH and temperature, the chemical composition of the

fermentation medium, and the concentration of essential nutrients or inhibitory compounds. The biological approach replaces the more traditional alcohol producing microorganism, yeast, with more efficient and productive species. The aim of genetic approach improves the metabolic characteristics of the microorganism. At last one, the engineering approach aims to increase productivity by using fermentors (Park et al., 2008).

There are three main ethanol fermentation processes that are used: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and direct microbial conversion (DMC) (Talebnia et al., 2010).

Traditionally, separate hydrolysis and fermentation (SHF) process steps performed separately which are pretreatments and enzymatic hydrolysis. The premier advantage of this approach is that by separating these steps, undesirable interactions are not permitted. Using separate hydrolysis and fermentation allows each step to be carried out at its optimum temperature 45-50°C for enzymatic hydrolysis and 30°C for fermentation, respectively (Merino & Cherry, 2007).

SSF process combines the enzymatic hydrolysis with the fermentation medium. The optimum temperature for the reaction (37-38°C) is a compromise between the optimum temperatures for the enzymes in hydrolysis and the yeast in fermentation (Olofsson, Bertilsson, & Liden, 2008).

Direct microbial conversion (DMC) is one of the alternative ways to conventional bioethanol production processes. In this process, cellulase production, cellulose hydrolysis and fermentation is carried out in a single step mediated by a single organism or microbial consortium (Sarkar et al., 2012).

Saccharomyces cerevisiae is the most commonly used microorganism for bioethanol production (Galbe & Zacchi, 2002). Latif et al. have been reported to produce maximal ethanol concentration of 21 g/L (w/v), using dry corn corbs after 96 h of fermentation using *S. cerevisiae* (Latif & Rajoka, 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1. Hazelnut Pruning Wastes (HPW)

HPW was obtained from hazelnut producers in Ordu in hazelnut harvesting and processing time. HPW was dried in oven at 60°C and milled in plant grinding mill to particles passing from 2 mm screen. The dry samples stored at room temperature until use.



Figure 3.1. Hazelnut pruning wastes

The ground HPW was treated for the degradation of lignocellulosic network, cellulose was hydrolyzed with cellulolytic enzymes (cellulase), and released glucose was converted to bioethanol by *S. cerevisiae*. Pretreatments (liquid hot water, very dilute acid, alkali and organosolv), enzymatic hydrolysis and fermentation steps implemented are shown Figure 3.2 , and followed by the methods used.

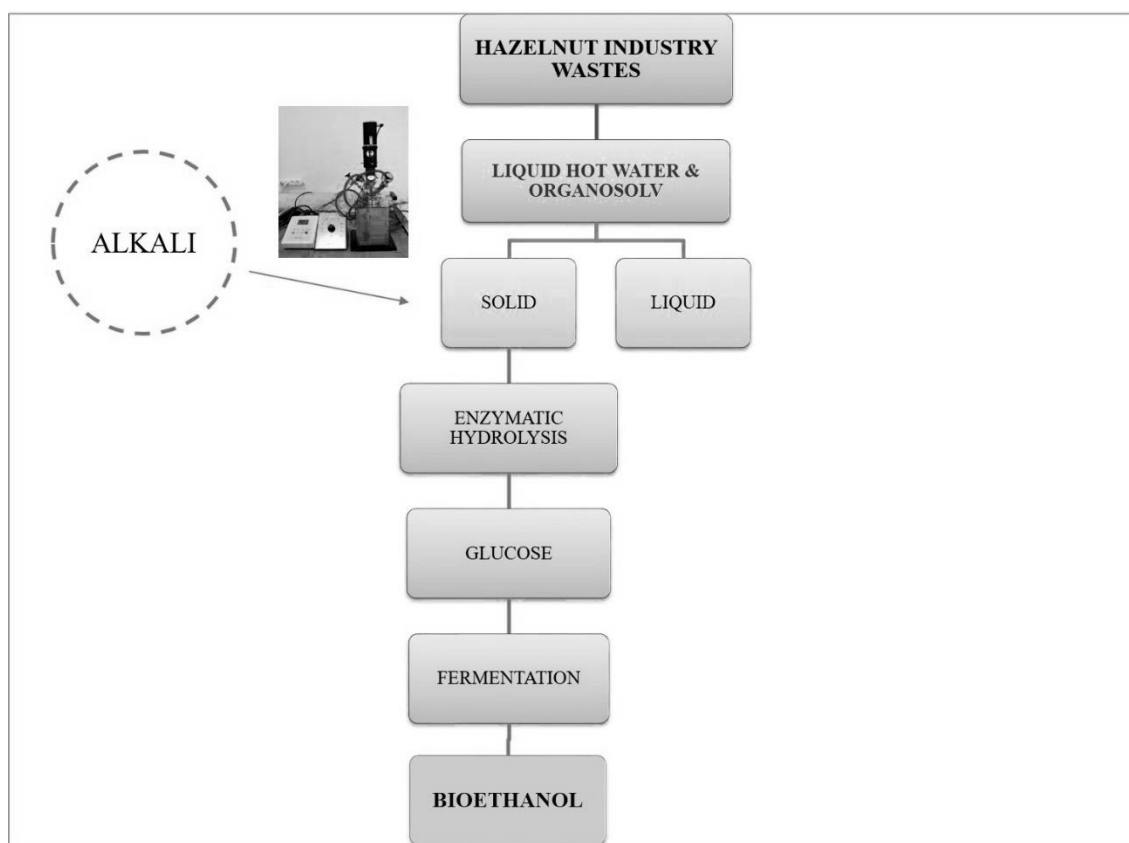


Figure 3.2. Flow chart of bioethanol production from HPW

3.2. Characterization of HPW

3.2.1. Moisture Content

Moisture content of ground HPW was determined by drying at 104°C for 24 h according to the method of American Society for Testing and Materials (ASTM) E1756-01 described by Sluiter et al. (2004b). All results in the study are reported on a dry weight basis.

3.2.2. Structural Carbohydrates and Lignin Analysis

Cellulose, hemicellulose and lignin contents of hazelnut industry wastes were determined by NREL/TP-510-42618 method described by Sluiter et al., (2010). The samples were hydrolyzed with 72% sulphuric acid for 1 h and then autoclaved after dilution to 4% sulphuric acid, with addition of water. 0.3 g biomass was treated with 3 ml

of 72% H_2SO_4 in 60 minutes and diluted to 4% by using water in 10 ml glass tube. The diluted solution was kept at 121°C for 60 minutes. After increasing pH to 5-7 by CaCO_3 , cleaned sample was analyzed by HPLC (High Pressure Liquid Chromatography).



Figure 3.3. The HPLC equipment for carbohydrate analysis

Acid hydrolysis disintegrates cellulose and hemicellulose in biomass to its monomers. Cellulose and hemicellulose percentages in the biomass were calculated by using monomer concentrations measured by HPLC.

Monosaccharides in samples was analyzed by high performance liquid chromatography system (HPLC). Samples will be centrifugated and filtrated by $0.45\ \mu\text{m}$ membrane. Monosaccharide analyses was carried out by lead ionic column and refractive index (RI) detector at $80\text{--}85^\circ\text{C}$. Mobil phase was used an ultra-pure water and flow rate was adjusted to $0.6\ \text{mL/min}$. The temperature of column was 80°C .

The carbohydrates in wood and pulp are hydrolyzed and solubilized by H_2SO_4 ; the acid-insoluble lignin is filtered off, dried, and weighed. Acid insoluble lignin (AIL) was determined by measuring weight the residue remaining on a medium porosity filtering crucible after a two-step hydrolysis. In this method of determination, lignin (also known as “Klason lignin”) is defined as a wood or pulp constituent insoluble in 72% sulfuric acid (Sluiter et al., 2010).

3.3. Pretreatments

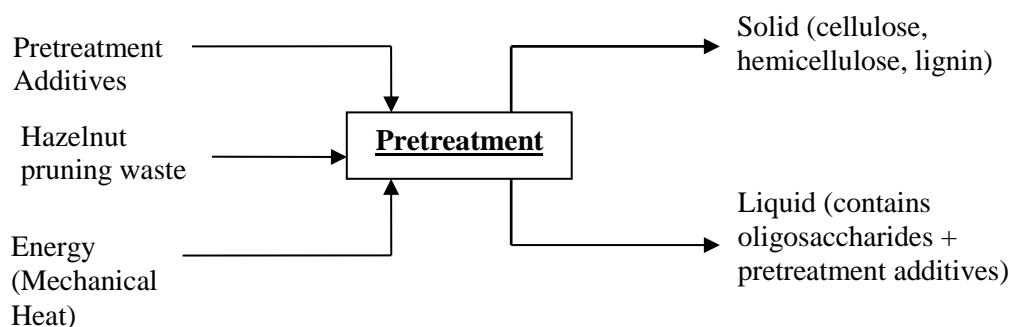


Figure. 3.4. Schematic representation of disintegration of biomass by pretreatment

3.3.1. LHW Pretreatment

HPW was treated with water at high temperature for convenience of the enzymatic saccharification step, and the analysis of the treated solids were carried out for characterization.

In this study, this treatment was carried out at 170°C, 190°C and 210°C for 15 and 45 minutes with 25 g HPW and 250 ml water in a 600 mL laboratory batch reactor (Berghof, Germany) in order to solubilize hemicelluloses thus decreasing the recalcitrance of the lignocellulosic network of the wood (Figure 3.5). The pressure reactor is locked with a quick-lock clamp that is manually applied, without the need for tools. All process parameters are easily accessible. The built-in data logger allows the parameter to be documented on a PC. The reaction mixture was heated at a rate of ~5°C/min, with continuous stirring. Pressure was increased to 15-20 bars, as a function of temperature. After each run, the reactor was cleaned and well washed. All treatments were performed for 15 and 45 minutes from the time of reaching the desired temperature. Preheating time was up to 30-40 min. Solid:liquid ratio was 1:10 and runs were performed in duplicate. At the end of each run, the reactor was rapidly cooled to 60°C and pressure released, then the reactor content was collected at room temperature and filtered by Whatman No.1 filter paper under vacuum. The solid phase was thoroughly washed with distilled water until the rinse water obtained was neutral. After washing, the solids were dried at 60°C and

solid recovery was measured gravimetrically. Moreover, the solid phase was characterized for cellulose, hemicellulose and klason lignin content as described above

The efficiency of the pretreatment was tested by enzymatic hydrolysis of the solid residue using HPLC.

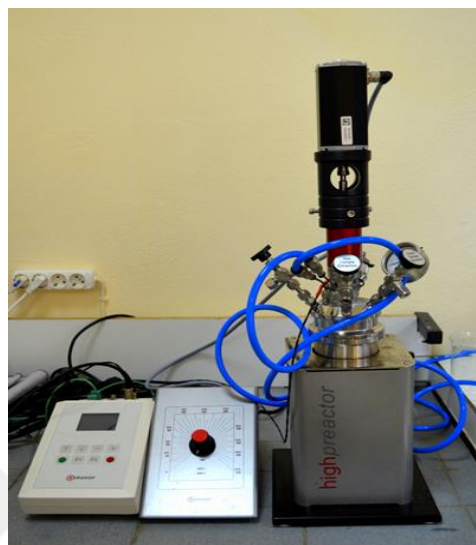


Figure 3.5. Laboratory batch reactor (Berghof, Germany)

3.3.2. VDA Pretreatment

Milled pruning waste was slurried with water and 0.1 % H_2SO_4 (w/v) (very dilute acid concentration) in order to decrease pH to 2 and treated at 130, 150, 170, 190°C for 15 min in the pressure reactor. The pretreated pruning waste was washed thoroughly with distilled water until neutral pH. After washing, the solids were dried at 60°C and solid recovery was measured gravimetrically. Structural carbohydrates and lignin composition of the solids were determined.

3.3.3. Alkaline Pretreatment

Alkaline pretreatment with NaOH under mild conditions was used to increase cellulose content and remove lignin. The pretreatments were carried out at different concentration of NaOH solution (2, 1, 0.5, 0.25%; w/v) for different samples which were HPW treated with LHW at 190°C for 45 min and HPW treated with dilute acid at 170°C for 15 min as well as the raw HPW. Solid:liquid ratio was 1:10 and runs were performed

in duplicate. The suspensions were autoclaved at 121°C for 1 h. The samples obtained after alkali treatment was dark in colour which was then filtered through cheesecloth and washed distilled water until no colour was visible in the wash water. The neutralized residue was pressed to remove excess water and used for the enzymatic hydrolysis. Treated biomasses were dried in the oven at 60°C for 24 h and solid recovery was measured gravimetrically. Structural carbohydrates and lignin composition of the solids were determined.

3.3.4. Organosolv Pretreatment

HPW was treated with 50% and 70% (v/v) EtOH in water in the presence or absence of 0.1% sulphuric acid (w/v) at 190°C for 15 and 45 min. The solid-to-liquid ratio applied was 1:10. The pretreatments were carried out in a 600 ml laboratory batch reactor with a temperature controller which was used in LHW and VDA treatments. The pretreated HPW was washed with water until neutral pH and then was dried at 60°C overnight. The solid and liquid was separated and solid recovery was measured gravimetrically. Besides, the solid phase was characterized for cellulose, hemicellulose and klason lignin content.

3.4. Solid and Cellulose Recovery of Biomass After Pretreatments

Solid and cellulose recovery calculated according to the following formulas in order to determine pretreatment and enzymatic hydrolysis efficiency.

$$\text{Solid recovery (\%)} = \frac{\text{Amount of insoluble solid after pretreatment (g)}}{\text{Initial amount of biomass before pretreatment (g)}} \times 100 \quad (3.1)$$

$$\text{Cellulose recovery (\%)} = \frac{\text{Amount of cellulose after pretreatment (g)}}{\text{Initial cellulose content of biomass (g)}} \times 100 \quad (3.2)$$

3.5. Enzymatic Saccharification

1 g lignocellulosic residues obtained from various pretreatments were placed in 25 mL Erlenmeyer flask on a rotary shaker. 10 mL reaction mixture in 50 mM sodium citrate buffer (pH 4.8) was prepared. In order to prevent microbial contamination 100 μ L of sodium azide (2%) was added. Saccharification of pretreated substrate was carried out as described in NREL Laboratory Analytical Procedure (NREL/TP-510-42629) (Selig et al., 2008). Cellulose, was hydrolyzed to glucose with cellulase. For enzymatic hydrolysis, Accellerase 1500 (Cellulase) (DuPont, Finland) was used. The enzyme was a gift from the company. Activities of enzyme was measured before using. While 15, 30 and 60 FPU/g biomass were treated for the residues after LHW pretreatments, 10, 20, 30 FPU/g biomass were preferred for the residues after organosolv pretreatments. Moreover, 30 FPU/g biomass were treated for the solid after VDA and alkali pretreatments. During the enzymatic hydrolysis, the samples were shaken in a rotary shaker (Zhicheng, China) at 150 rpm at 50°C for 72 h. Samples were taken from the reaction mixture at different times (0, 8, 24, 48, 72 h) and the enzymatic activities were stopped by placing the sample tubes in a boiling water bath for 10 min. Hydrolysates were clarified by centrifuging at 5000 rpm for 5 min. The supernatants were analysed for glucose and xylose released by enzymatic hydrolysis using HPLC. All enzymatic hydrolysis experiments were performed in duplicates, and the average results were determined.

Monosaccharides in aqueous samples were analyzed by high performance liquid chromatography system (HPLC). Samples were centrifugated and filtrated by 0.45 μ m membrane. Monosaccharide analysis were carried out by lead ionic column (Phenomenex Rezex RPM-Monosaccharide) and refractive index (RI) detector at 80-85°C. Ultra-pure water was used as the mobile phase at 0.6 ml/min. Standard calibration curves were prepared using five different concentrations of the monosaccharides.

$$\text{Cellulose to glucose conversion} = \frac{\text{Amount of glucose produced} \times 0.9}{\text{Amount of cellulose in pretreated solid}} \times 100 \quad (3.3)$$

The contents of glucose was multiplied by a factor 0.9 because of addition of a water molecule to each broken glycosidic bond during hydrolysis of cellulose.

3.5.1. Determination of Cellulase Activity

Enzyme activity was determined by measuring glucose as reducing sugar after incubation enzyme mixture and substrate for a certain time (Adney et al., 2012).

Filter-paper strip (1.0 x 6.0 cm) was used as substrate by saturating in 1.0 mL 50 mM sodium citrate buffer (pH 4.8). After the temperature equilibration of buffer and substrate mixture at 50°C, 0.5 mL enzyme diluted to the desired activity was added. The tubes were incubated at 50°C for 60 min. At the end of the incubation period, each assay tube was removed from the 50°C bath and the enzyme reaction was stopped immediately by the addition of 3.0 mL DNS reagent and mixing. The absorbance of cooled solution was read at 540 nm. For blank, DNS was added to the substrate solution before incubation and then enzyme will be added. Reducing sugar released during incubation was calculated by glucose standard curve using the absolute amounts of glucose (mg/0.5 mL) plotted against A₅₄₀.

One unit of enzyme activity unit (U/ml) is expressed as the amount of enzyme that released reducing sugars which is equal to 1 µmol for 1 minute. Enzyme activity can be calculated by the following equation:

$$\text{Filter Paper Activity} = \frac{0.37}{(\text{enzyme})\text{releasing 2.0 mg glucose}} \text{ units/ml} \quad (3.4)$$

Where [enzyme] represents the proportion of original enzyme solution present in the directly tested enzyme dilution. The numerator (0.37) in the equation is derived from the factor for converting the 2.0 mg of "glucose-equivalents" generated in the assay to mmoles of glucose ($2.0 \div 0.18016$), from the volume of the enzyme being tested that is used in the assay (0.5 mL), and from the incubation time (60 minutes) required for generation of the reducing equivalents.

$$\frac{(2.0 \text{ mg} \frac{\text{glucose}}{0.18016 \text{ mg} \frac{\text{glucose}}{\mu\text{mol}}})}{(0.5 \text{ mL enzyme dilution} \times 60 \text{ minutes})} = 0.37 \frac{\mu\text{mol}}{\text{minute}} \quad (3.5)$$

3.6. Ethanol Fermentation

After enzymatic saccharification, hydrolyzates were centrifuged at 5000 rpm for 10 min. For the ethanol fermentation, 5 g/L of yeast extract, 3.75 g/L of $(\text{NH}_4)_2\text{SO}_4$, 2.1 g/L of K_2HPO_4 , 0.375 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5 g/L of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were added into saccharification solution and then the mixture was autoclaved at 121°C for 15 minutes (Lu et al., 2012). After the medium was cooled to 30°C, 1 g/L baker's yeast (Pakmaya, Turkey) was added. The flasks were incubated in a rotary shaker at 150 rpm for 48 h at 32°C and 37°C. Periodically taken samples (0, 4, 6, 8, 12, 24 and 48 h) were centrifuged at 5000 rpm for 10 min and then the supernatants were filtered through 0.45 μm pore-sized filters and stored at 4°C until HPLC analysis (Puspawati et al., 2015). The supernatants were analysed for ethanol and glucose in HPLC. All enzymatic hydrolysis experiments were performed in duplicates, and the average results were determined.

Aminex HPX-87H column and RI detector was used for ethanol determination. HPLC conditions were 20 μL of injection volume, 60°C of column temperature and flow rate set to 0.6 ml/min. The mobile phase was 5 mM H_2SO_4 filtered through 0.45 μm filter and degassed. Ethanol standard solutions of known concentrations were used for calibration.

3.7. Statistical Analysis

The results were analyzed statistically by using one way analysis of variance (ANOVA), Tukey–Kramer test 16.0 version at significant level as 0.05. All experiments were carried out as 2 replicates. Means with the same letter are not significantly different from each other ($P>0.05$). Different letters indicate the results are significantly different at a level of 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Characterization of HPW

The chemical composition of raw HPW used in this study was determined and shown in Figure 4.1. HPLC results showed that the carbohydrate fraction of the material was nearly 50% of the dry biomass. Cellulose, the main component, accounted for $35.01 \pm 0.19\%$ of raw HPW. Among hemicellulose-derived sugars xylan, which is the main constituent found in the hardwoods structure, was $16.45 \pm 0.18\%$ of dry weight.

Nasser et al. (2016) measured the chemical composition of palm pruning wastes as 36% cellulose, 29% hemicellulose and 17% lignin. In another study on olive tree prunings, Cara et al. (2008) reported that it has 25% cellulose, 15.8% hemicellulose and 18.8% lignin. HPW consisted of more glucan and xylan, which demonstrated that more fermentable sugars could be produced by means of enzymatic saccharification and that hazelnut residues can be used in bioethanol production. Lignin is one of the drawbacks of using lignocellulosic materials in enzymatic hydrolysis and fermentation, as it makes lignocellulose resistant to chemical and biological degradation (Taherzadeh and Karimi, 2008). Klason lignin content was measured as $28.45 \pm 0.03\%$ of the biomass.

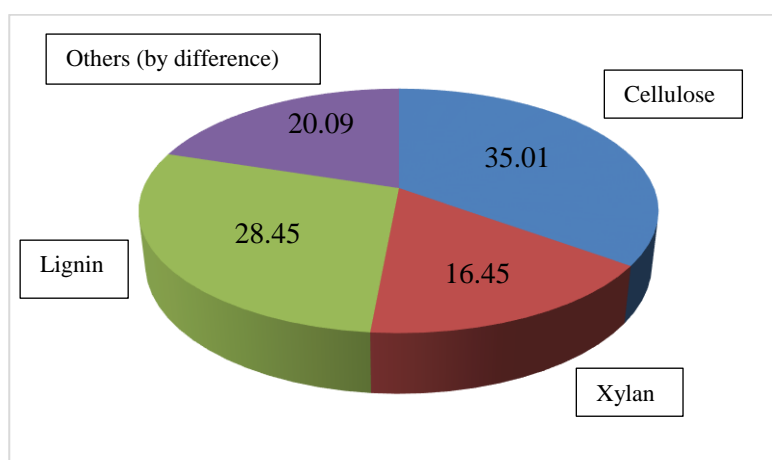


Figure 4.1. Chemical composition of untreated HPW (% on dry basis)

4.2. Pretreatments and Enzymatic Hydrolysis

Pretreatment is required in order to decrease the recalcitrance of cellulose to improve enzymatic hydrolysis and to achieve a greater glucose yield in the bioconversion processes for bioethanol production.

Enzymatic hydrolysis provides a method to convert cellulose to glucose at high yields. Enzymatic hydrolysis of cellulose proceeds in several steps to break glycosidic bonds by the use of cellulase enzymes. Factors effecting hydrolysis of cellulose include type of substrate, cellulase loading, reaction conditions such as temperature and pH, and end-product inhibitors (Kreith & Krumdieck, 2013).

Enzymatic digestibility is one of the major point for evaluating pretreatment efficiency. Enzyme activity was calculated as 46 FPU/ml before enzymatic saccharification.

For all enzymatic hydrolysis results, standard deviation for 2 replicates were too small and maximum level was 0.96 g/L.

4.2.1. Liquid Hot Water (LHW) Pretreatment

Liquid hot water (LHW) pretreatment is a hydrothermal treatment using only liquid water at high temperatures under pressure. In this study, this treatment was carried out at 170°C, 190°C and 210°C for 15 and 45 minutes in a 600 mL laboratory batch reactor (Berghof, Germany) in order to decrease recalcitrance of the cellulose.

4.2.1.1. Biomass Compositions After LHW

Figure 4.2 shows the chemical compositions after LHW pretreatment of dried HPW for 15 and 45 min at 170, 190 and 210°C in the reactor. As indicated in the method part, structural carbohydrates (cellulose, xylan) and klason lignin in the residual solids were determined according to the analytical procedure of the National Renewable Energy Laboratory (NREL) using the two-stage H₂SO₄ digestion protocol. The cellulose contents of all LHW treated-solids were higher than untreated sample (35.01±0.19%). The cellulose contents of samples ranged from 40.62±1.17% to 51.88±0.15%, and these values depended on the pretreatment temperature and residence time.

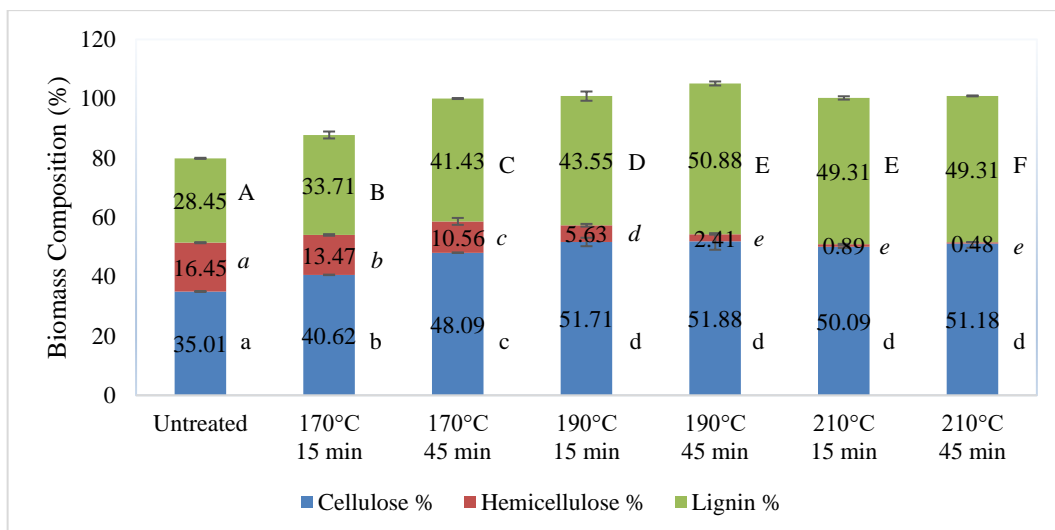


Figure 4.2. Chemical compositions of HPW after LHW treatment (% , on dry basis). Lower case letters: cellulose %; italic lower case letters: hemicellulose %; capital letter: lignin %. Tukey significant difference test was applied.

Cellulose, hemicellulose (as xylan) and lignin accounted for composition of the untreated HPW as total 79.91% (on dry basis). The remaining undefined portion of about 20% in the HPW could possibly be organic compounds such as uronic acid, acetyl groups, and several other minerals and residual extractives such as waxes, fats, gums, starches, resins, and essential oils (Kuhad & Singh, 1993). Similar result was achieved at low temperature and time LHW condition which was 170°C and 15 min. It was concluded that there were substances which were insoluble at low temperature and short time condition as in the untreated HPW. In this study, extractives and organic compounds removed simultaneously with hemicellulose during pretreatments and these compounds remaining in the pretreated solids were not quantified under such severe conditions. Moreover, as apparent from the post-pretreatment composition analysis, the sum of lignin, cellulose and hemicellulose approximately equaled the total solids (after pretreatment) as treatment conditions became more intense.

The cellulose contents of LHW treated-samples increased with increasing temperature until 190°C, to almost 51% (on dry basis). Cellulose composition of LHW-treated samples were analyzed statistically using one-way analysis of variance (ANOVA). Figure 4.2 shows that increasing temperature to 190 °C and 210°C for LHW treatment did not increase further the cellulose content of the samples. However, untreated HPW and lower temperature (170°C) treated HPW don't have the same letter, so they are significantly different. The increase in pretreatment time increased cellulose contents at

lower temperature values. However, changing pretreatment time did not significantly affect on cellulose enhancement at high temperature in LHW treatment for statistically.

The hemicellulose composition of LHW-treated samples were analyzed statistically using one-way ANOVA (Tukey–Kramer test, $P>0.05$). Increasing temperature and time had a significant effect on reducing xylan content. Unlike cellulose and lignin, the amount of hemicellulose in pretreated biomass samples decreased as intensity of pretreatments (higher temperature and/or longer residence time) increased. Xylan dissolved up to 97% (210°C, 45 min). This study suggested that, moderately high temperature and time were effective for hemicellulose reduction in HPW, without the presence of a catalyst such as acids.

Klason lignin content, representing between $33.71\pm0.08\%$ and $50.88\pm1.44\%$ of the pretreated solids, increased with pretreatment temperature and time.

The total gravimetric recovery (solids remaining after pretreatment) and cellulose recovery resulting from pretreatment at the different LHW conditions are shown in Table 4.1. As expected, total gravimetric recoveries decreased with higher pretreatment time and temperature.

For LHW pretreatment, the solid recovery of HPW ranged from $85.96\pm0.22\%$ at 170°C 15 min to $65.48\pm0.73\%$ at 210°C 15 min. At the harshest LHW treatment, $81.02\pm0.16\%$ cellulose recovery was obtained at 210°C for 45 min.

Table 4.1. Solid and cellulose recovery of LHW treated HPW

LHW Treatments	Solid recovery %	Cellulose Recovery %
170°C 15 min	85.96 ± 0.22	88.32 ± 0.45
170°C 45 min	74.99 ± 0.16	88.88 ± 0.82
190°C 15 min	73.12 ± 0.10	87.50 ± 0.23
190°C 45 min	66.41 ± 0.31	80.23 ± 0.47
210°C 15 min	65.48 ± 0.73	82.58 ± 0.33
210°C 45 min	64.00 ± 0.07	81.02 ± 0.16

4.2.1.2. Enzymatic Saccharification of LHW-treated HPW

All LHW treated samples were enzymatically hydrolyzed with different cellulase loadings. Glucose was the dominant monosaccharide in enzymatic saccharification. Very small amount of xylose was detected in the hydrolysates. The concentrations of arabinose and galactose were not reported in this study because their concentrations were below the detection limit.

Enzymatic hydrolysis yields were evaluated from glucose concentrations in saccharification media and were expressed as a percentage of the potential glucose in pretreated materials, after 0, 24, 48, 72 and 96 h of enzymatic attack. Increasing either the pretreatment temperature and time of LHW treatment had a positive effect on the enzymatic saccharification of pretreated HPW's cellulose fraction. Increasing cellulase loading from 15 to 30 and 60 FPU/g biomass greatly improved enzymatic hydrolysis efficiency.

The cellulose conversion of LHW-treated samples was between $11.22 \pm 0.65\%$ and $69.55 \pm 0.23\%$. The untreated sample was resistant to enzymatic hydrolysis and maximum conversion) of cellulose to glucose was only $4.15 \pm 0.63\%$ at 96 h of enzymatic hydrolysis at 60 FPU/g biomass enzyme loading.

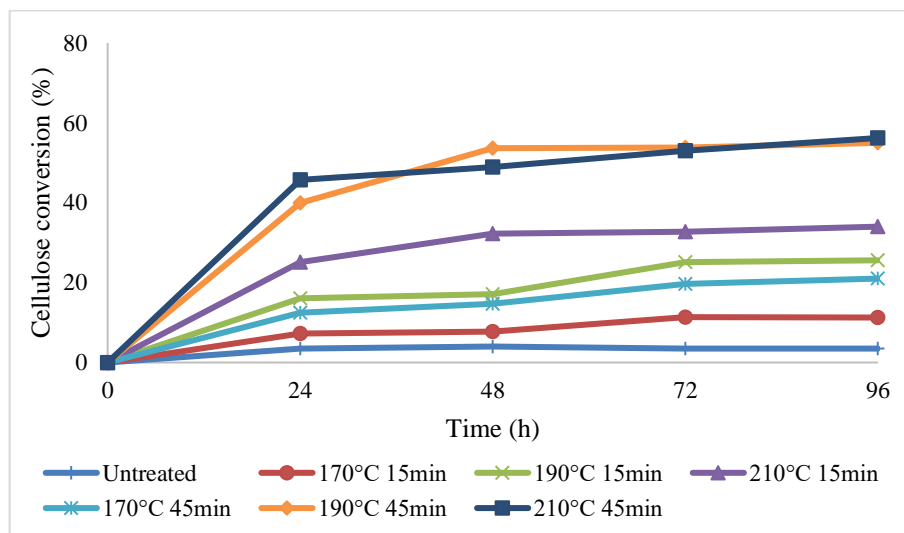


Figure 4.3. Conversion of cellulose to glucose in the enzymatic hydrolysis of HPW from LHW-treated with different pretreatment temperatures and times. Enzymatic hydrolysis conditions: 15 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 96 h.

Figure 4.3 shows cellulose conversion of HPW treated at different pretreatment temperatures and times using 15 FPU/g biomass cellulase. The enzymatic conversion of LHW-treated samples was between 11.22 ± 1.21 and $56.19 \pm 0.89\%$ (210°C 45 min) while conversion yield was $3.49 \pm 0.07\%$ in untreated sample. As it is clearly depicted in Figure 4.3, increasing either pretreatment temperature or time have a positive effect on the enzymatic saccharification of pretreated HPW.

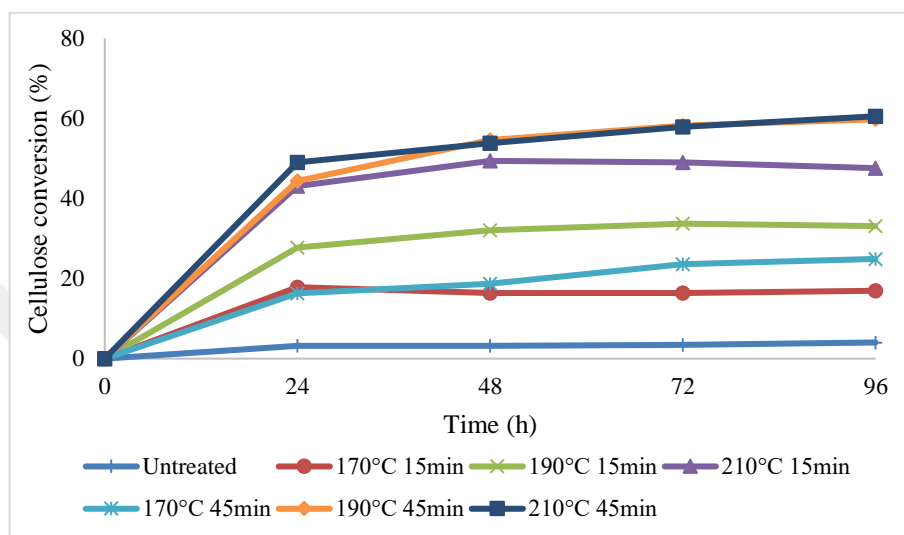


Figure 4.4. Conversion of cellulose to glucose in the enzymatic hydrolysis of pruning waste of hazelnut from LHW-pretreated with different pretreatment temperatures and times. Enzymatic hydrolysis conditions: 30 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 96 h.

Figure 4.4 indicates cellulose conversion of HPW with different pretreatment temperatures and times using 30 FPU/g biomass cellulase. The enzymatic conversion of LHW-treated samples was between $17.84 \pm 0.93\%$ and $60.54 \pm 1.74\%$ (210°C 45 min) while conversion yield was $4.03 \pm 1.26\%$ in untreated sample. As it is clearly represented in Figure 4.4, increasing cellulase loading improved enzymatic saccharification efficiency.

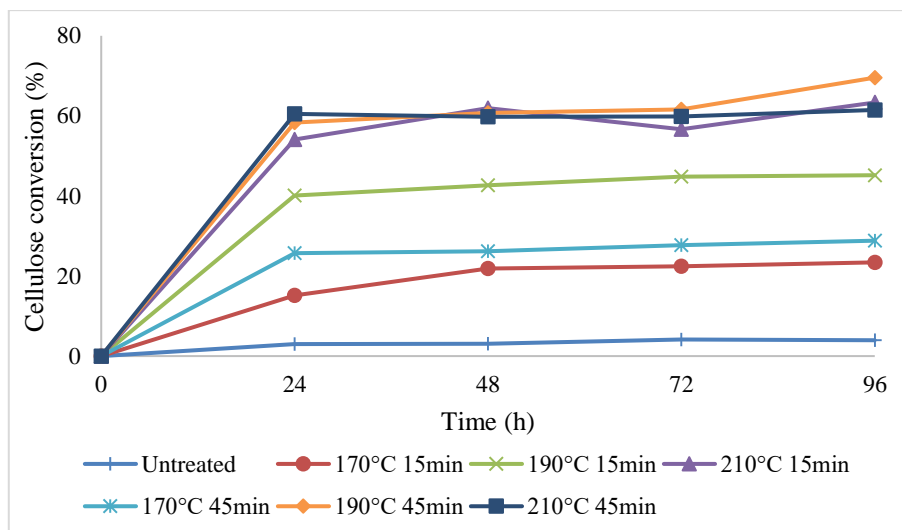


Figure 4.5. Conversion of cellulose to glucose in the enzymatic hydrolysis of pruning waste of hazelnut from LHW-pretreated with different pretreatment temperatures and times. Enzymatic hydrolysis conditions: 60 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 96 h.

Although the cellulase loading was relatively high (60 FPU/g biomass), HPWs treated at 170°C for 15 or 45 min resulted in lower cellulose conversion compared to higher temperature in enzymatic saccharification (Figure 4.5). At the same and relatively high cellulase loading, when the LHW pretreatment temperature increased, cellulose conversion for enzymatic digestibility was greatly improved.

When the LHW temperature was increased from 190°C to 210°C and residence time was decreased from 45 min to 15 min, adding 2 times of cellulase (60 FPU/g biomass instead of 30 FPU/g biomass) lead to the similar results of enzymatic digestibility. Considering the price of enzyme, changing pretreatment temperature and time had a significant role for glucose release and cellulose conversion improving in enzymatic digestibility to reduce enzyme loading.

Optimum reaction time for enzymatic digestibility changed with cellulase loadings. When 60 FPU/g cellulase was used, enzymatic hydrolysis was completed in 24 h, while enzymatic hydrolysis with lower cellulase loadings continued after 24 h. Therefore, when the cellulase loading was increased from 15 FPU/g biomass to 60 FPU/g biomass on pretreated HPW at all LHW temperature and time conditions, the reaction time for enzymatic digestibility of treated-samples was greatly decreased. It was concluded that cellulase loading of 30 FPU/g biomass was the optimum level for LHW pretreated samples.

The cellulose conversion yields obtained in this study for LHW treated samples were generally lower than the ones reported in literature for the similar processes. Cara et al. (2007) investigated the LHW treatment for olive tree prunings at 220°C for 10 min and obtained 75% cellulose conversion. Another study about LHW by Kim et al. (2009) for hybrid poplar at 200°C for 10 min reported 76.7% cellulose to glucose conversion.. Laser et al. (2002) optimized the pH-controlled LHW pretreatment of corn stover and they observed 90% cellulose conversion after treatment at 190°C for 15 min. Perez et al. (2008) also studied LHW for treating wheat straw and reported parallel findings under optimized condition when they achieved considerable glucose yield which is 92%.

4.2.1.2.1. Effect of β -glucosidase Addition

In this study, β -glucosidase was added in order to increase cellulose conversion. However, addition of this enzyme did not any improve for cellulose conversion. This could be because the cellulase enzyme complex contains sufficient β -glucosidase activity (DuPont, 2013). These result has been approved by cellulase assay. Also, several enzymatic treatments tried with adding β -glucosidase to see effect of enzymatic hydrolysis.

4.2.1.2.2. Effect of Intermittent Cellulase Addition

An approach was taken to determine the inhibition effects of glucose during cellulase hydrolysis of cellulosic material. The increased glucose content in the hydrolysate resulted in a dramatic increase in the degrees of inhibition on cellulase activities (Xiao et al., 2004). In this study, cellulase was added intermittently in order to observe inhibition effect.

In the first run which was started with 15 FPU/g biomass cellulase, an additional 15 FPU/g biomass was added after 8 h. This system was compared with the run which was started with 30 FPU/g biomass. Figure 4.6 depicts that while there was a difference between adding directly or intermittently for first 24 h, but the difference has disappeared with time.

Secondly, cellulase enzyme was added during enzymatic hydrolysis as 15 FPU/g biomass every 8 hour for 4 times to compare with direct addition of 60 FPU/g biomass

at time zero. The enzymatic conversion was 51% for two process at the end of the hydrolysis (Figure 4.7). According to one way ANOVA result, there was no difference intermittent addition of cellulase ($P>0.05$).

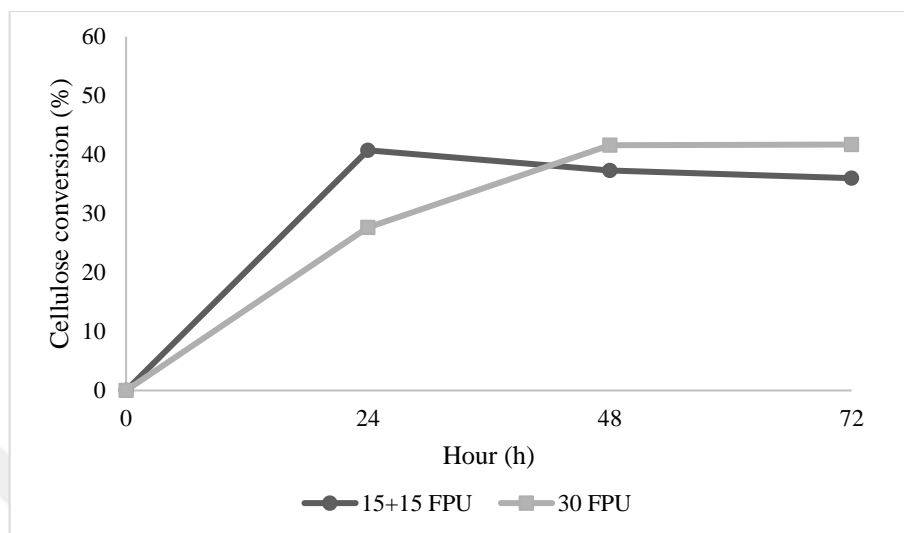


Figure 4.6. Effect of adding cellulase in time interval on enzymatic hydrolysis of LHW-pretreated HPW. LHW pretreatment: 190°C for 45 min. Enzymatic hydrolysis conditions: 15+15 FPU/g biomass and 30 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 72 h.

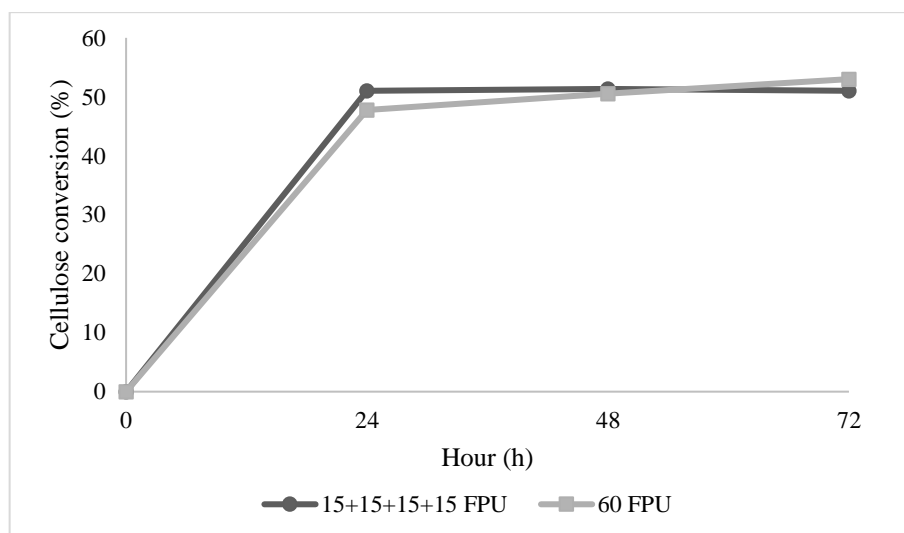


Figure 4.7. Effect of adding cellulase in time interval on enzymatic hydrolysis of LHW-pretreated HPW. LHW pretreatment: 190°C for 45 min. Enzymatic hydrolysis conditions: 15+15+15+15 FPU/g biomass and 60 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 72 h.

4.2.1.2.3. Effect of Solid-to-Liquid Ratio

The solid-to-liquid (S:L) mass ratio was the ratio between the LHW-treated solid and the entire liquid volume in the enzymatic saccharification. Unless otherwise stated, S:L was used as 1:10. Only in some cases the S:L ratio was 1:50.

In the literature, the solid to liquid ratio is generally low (1:50) (Lu et al., 2012). In the current study, S:L ratio was kept relatively high in order to obtain a solution with high glucose content at the end of the saccharification process. Otherwise, ethanol concentration after the fermentation would be too low, which is not desired in industrial processes.

When the S:L ratio was changed from 1:10 to 1:50 for sample LHW-treated at 190°C, 45 min; the cellulose conversion yield did not change in the enzymatic hydrolysis period. The cellulose conversion with 1:50 S:L ratio was 53.37 ± 1.02 compared with $58.2 \pm 0.12\%$ with 1:10 S:L ratio (Figure 4.8).

The glucose concentration at high S:L ratio was 33.5 ± 0.67 g/L, while it was 6.15 ± 0.63 g/L at low glucose concentration. When everything is taken into consideration, high glucose concentration is necessary to achieve high ethanol concentration by fermentation. Therefore, the increase in the glucose concentration by increasing biomass in the liquid part was beneficial in the subsequent ethanol fermentation.

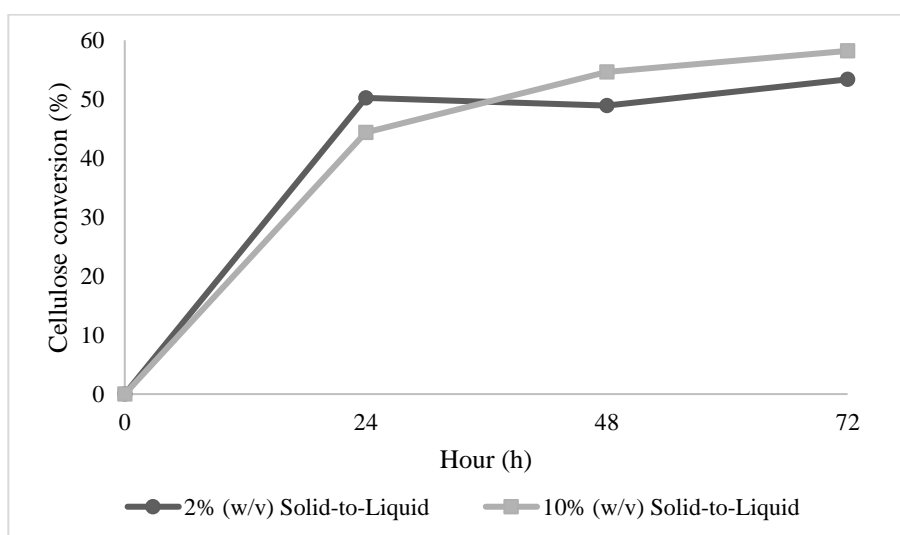


Figure 4.8. Effect of solid:liquid ratio on enzymatic hydrolysis of LHW-pretreated HPW. LHW pretreatment: 190°C for 45 min. Enzymatic hydrolysis conditions: 30 FPU/g biomass, pH 4.8, and 50°C for 72 h.

4.2.1.2.4. Effect of Cellulase Loading

The aim of this part of the study was to evaluate the influence of cellulase loading for enzymatic saccharification. In addition to the enzymatic temperature and pH, cellulase loading is also an important factor for the enzymatic saccharification. Increasing the cellulase loading generally resulted in an increase in cellulose conversion and glucose release. The enzymatic hydrolysis with 30 and 150 FPU/g biomass cellulase loadings are shown in Figure 4.9.

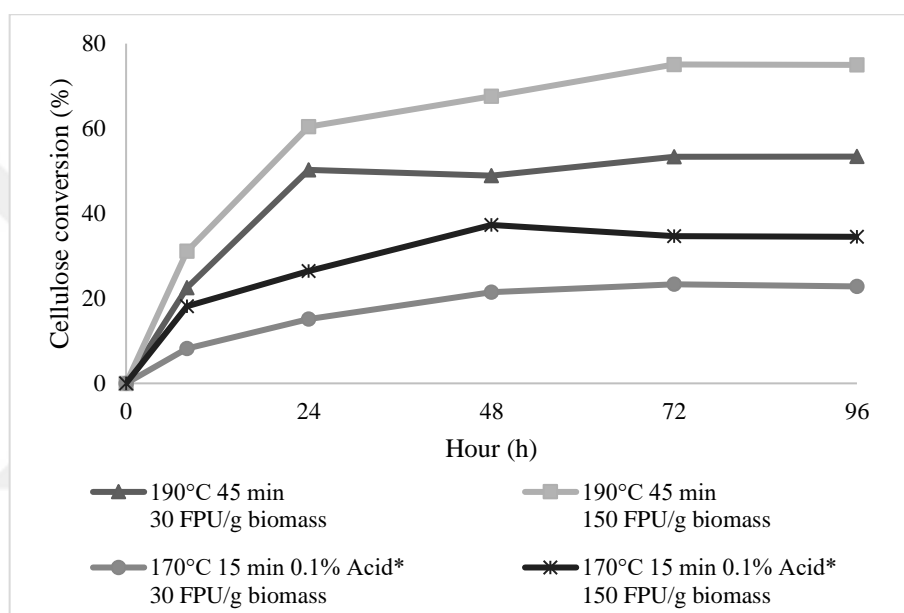


Figure 4.9. Enzymatic hydrolysis of HPW in the LHW and VDA pretreatment at 190°C, 45 min and 170°C, 15 min and 0.1% H₂SO₄ with respect to different cellulase loadings. Enzymatic hydrolysis conditions: 30 and 150 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:50 (w/v) and 50°C for 96 h.
*Acid: H₂SO₄

Cellulase loading had a significant effect on cellulose conversion in enzymatic hydrolysis. When the cellulase loading was increased from 30 FPU/g biomass to 150 FPU/g biomass, the glucose release was greatly improved in the enzymatic hydrolysis period. The conversion of cellulose to glucose increased from 53.37±0.66% to 75.10±0.82% for LHW-treated HPW (190°C, 45 min) when 150 FPU/g biomass cellulase was used in enzymatic hydrolysis (Figure 4.9).

4.2.2. Very Dilute Acid (VDA) Pretreatment

Hazelnut pruning waste was mixed with water and 0.1 % H_2SO_4 (w/v) (very dilute acid concentration) in order to decrease pH from 5 to 2 in a reactor at 130, 150, 170, 190°C for 15 min. Very dilute sulphuric acid addition (about 0.1% versus the 0.7–3.0% typical for the dilute acid technology described) in a reactor is effective at very low acid levels (Kumar et al., 2009)

4.2.2.1. Biomass Composition After VDA

Carbohydrate and klason lignin contents are given in Figure 4.10. The cellulose contents of VDA-pretreated samples were not statistically different with 98.48% confidence level. They ranged from $45.99 \pm 0.21\%$ (130°C, 15 min) to $53.90 \pm 1.23\%$ (190°C, 15 min).

Since hemicellulose (as xylan) reduction was the major target of VDA-pretreatment (Tsoutsos & Bethanis, 2011), xylose was the most abundant monomeric sugar in the prehydrolysate due to the increased xylan degradation at high temperature with acid catalyst. The hemicellulose content of VDA-pretreated solids ranged from $14.89 \pm 0.15\%$ (the lowest temperature; 130°C, 15 min) to $0.75 \pm 0.56\%$ (the highest temperature; 190°C, 15 min). Results showed that increasing temperature had significant effect on xylan reduction statistically and decreasing pH to 2 dissolved up to 95.44% (190°C, 15 min, 0.1% H_2SO_4) xylan from the HPW (Figure 4.10).

Very dilute sulphuric acid pretreatment (0.1%) did not sufficiently remove lignin from HPW even under high pretreatment temperature. Lignin has been reported to be very sensitive to sulphuric acid and accumulation and degradation can occur simultaneously when lignin interacts with sulphuric acid (Yang & Wyman, 2008).

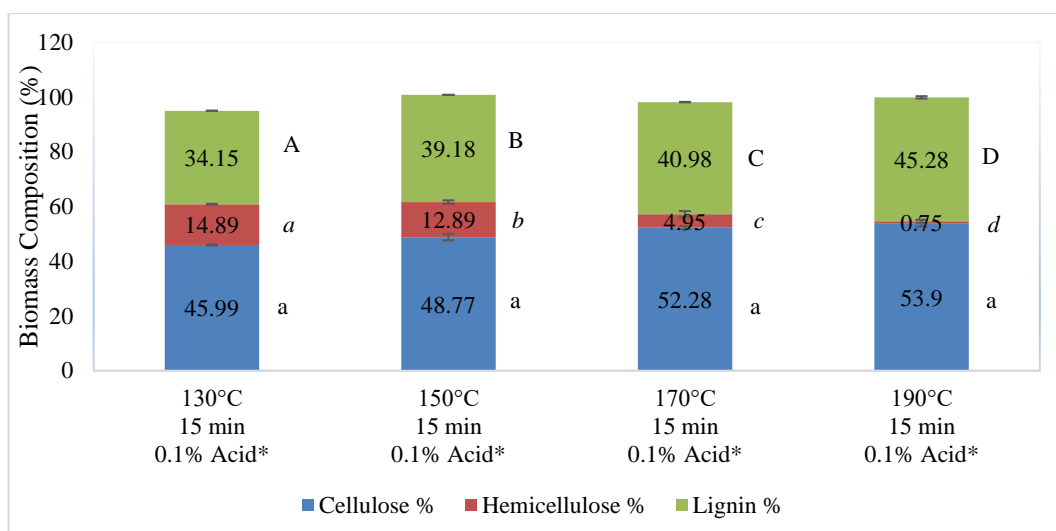


Figure 4.10. Chemical compositions of HPW after VDA pretreatment (% , on dry basis)

*Acid: H₂SO₄.

Lower case letters: cellulose %; italic lower case letters: hemicellulose %; capital letter: lignin %.

The total gravimetric recovery and cellulose recovery resulting from pretreatment at the different VDA conditions assayed were summarized in Table 4.2. As expected, total gravimetric recoveries decreased with pretreatment temperature or using acid catalyst.

VDA pretreatment resulted in 84.21±0.53-59.44±0.09% solid recovery for the four different temperatures. When the temperature increased, more hemicellulose and lignin were removed from the solid part. For this reason, the cellulose content was higher in the remaining solids. This might have decreased the recalcitrance of cellulose. Cellulose recovery was lowered with increasing reaction temperature as well as using acid-catalyst.

Table 4.2. Solid and cellulose recovery of VDA treated HPW

VDA Treatments	Solid recovery %	Cellulose Recovery %
130°C 15 min 0.1% H ₂ SO ₄	84.21±0.53	98.57±0.79
150°C 15 min 0.1% H ₂ SO ₄	79.41±0.63	98.56±0.42
170°C 15 min 0.1% H ₂ SO ₄	67.55±0.22	89.88±0.93
190°C 15 min 0.1% H ₂ SO ₄	59.44±0.09	81.54±0.41

4.2.2.2. Enzymatic Saccharification of VDA-treated HPW

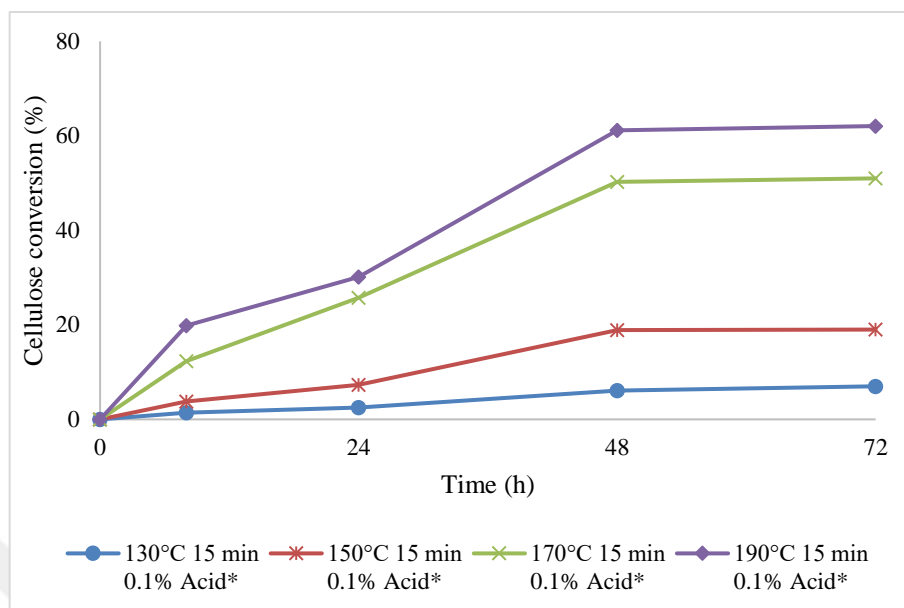


Figure 4.11. Conversion of cellulose to glucose in the enzymatic hydrolysis of pruning waste of hazelnut from VDA-pretreated with different pretreatment temperatures. Enzymatic hydrolysis conditions: 30 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 72 h. *Acid: H₂SO₄

Enzymatic hydrolysis yields for VDA-treated samples were evaluated from glucose concentrations in saccharification medium as the assessment of all enzymatic hydrolysis. VDA-treatment which decreasing pH to 2 of the medium in a reactor had a positive effect on the enzymatic saccharification of pretreated HPW (Figure 4.11). Milder pretreatment temperatures resulted in relatively poorer performance in terms of enzymatic hydrolysis yields. For example, after VDA treatment at 130°C for 15 min, enzymatic hydrolysis yield was only 7.00±0.85% after 72 h. On the other hand, enzymatic hydrolysis of the biomass treated at 190°C for 15 min resulted in 62.07±1.34% saccharification of the cellulose. Thus, it was concluded that VDA treatment at relatively high temperatures are necessary for sufficient cellulose hydrolysis.

Rice straw was pretreated with 1% (w/w) H₂SO₄ for 160-180°C with 1–5 min retention time followed by enzymatic hydrolysis, which resulted in the maximal sugar yield of 83%. In a different investigation using the same acid, rapeseed straw was pretreated for 10 min at 180°C and 63.17% of glucan was converted into glucose through enzymatic hydrolysis. Compared with the literature, our results also came out at similar range.

The improvement in enzymatic hydrolysis yields was detected with increasing pretreatment temperature and time in the presence of acid catalyst. The maximum result of enzymatic hydrolysis was 48 h for VDA-treated samples at 190°C, 15 min with 0.1% H₂SO₄ using 30 FPU/g biomass.

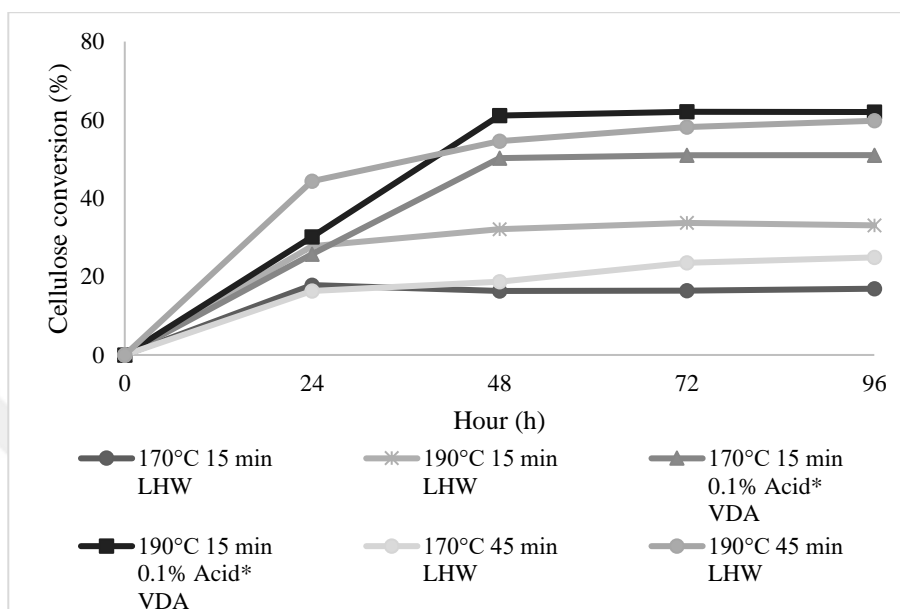


Figure 4.12. Effect of acid on the enzymatic saccharification at the constant treatment time (15 min) *Acid: H₂SO₄

The enhancement detected in cellulose conversion was mainly attributable to a significant increase of hemicellulose-derived sugars in prehydrolysates when decreasing pH of medium with using dilute sulphuric acid (Varga et al., 2002).

It was clearly observed that VDA treatment had a positive impact on enzymatic saccharification. The VDA-treated sample at 170°C, 15 min had 3.012 times higher cellulose conversion compared to the LHW-treated samples (without acid catalysis) at the same temperature and time. For 190°C, 15 min VDA-treated sample had 1.88 times higher cellulose conversion compared to the LHW-treated sample (Figure 4.12). This indicated that xylan removal had a great effect on the enzymatic digestibility of the HPW.

The VDA-treated sample at 170°C, 15 min provided 2.05 times higher cellulose conversion compared to the LHW-treated samples at the same temperature, but for a longer time (45 min). Using acid catalyst (0.1% H₂SO₄) even at lower temperature for the pretreatment strongly affected on enzymatic hydrolysis. However, the sample treated with VDA at 190°C, 15 min, cellulose conversion was 1.13 times higher compared to the

LHW-treated samples at the same temperature, but at 45 min (Figure 4.12). This indicated that pretreatment time had a significant effect on the enzymatic digestibility as well as decreasing pH to 2 of the HPW.

4.2.3. Alkali Pretreatment

After LHW and VDA treatments, an additional alkali (NaOH) treatment step was applied in an effort to increase the enzymatic hydrolysis efficiency. Alkali treatment can remove more lignin and hemicellulose so that it can render the biomass amenable to enzymatic hydrolysis. Alkali pretreatment was conducted at lower temperature and pressure but the duration is longer than those applied in the previous treatment methods. This pretreatment was performed at 121°C for 1 h in an autoclave.

4.2.3.1. Biomass Compositions After Alkali Pretreatment

Untreated, LHW-treated and VDA-treated biomasses were added 0.5% or 2% (w/v) NaOH and treated at 121°C for 1 h. Figure 4.13 summarizes the compositional change of HPW following alkali pretreatment.

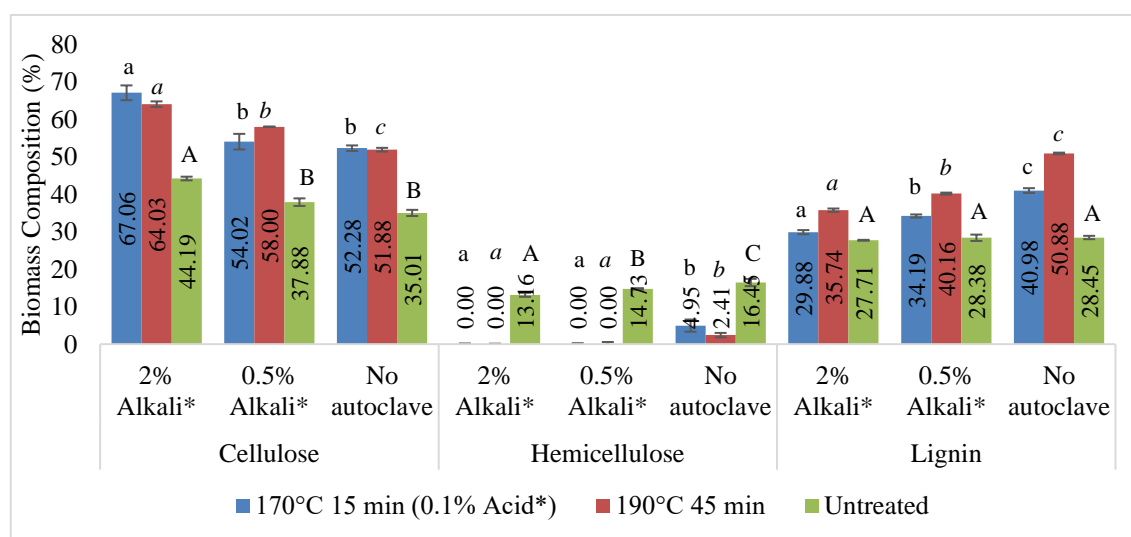


Figure 4.13. Chemical compositions of HPW after alkali pretreatment (%, on dry basis)

*Alkali: NaOH, *Acid: H₂SO₄

Cellulose, hemicellulose and lignin parts were evaluated statistically separately. Lower case letters: 170°C 15 min 0.1% H₂SO₄ treated HPW; italic lower case letters: 190°C 45 min treated HPW; capital letter: Untreated HPW.

The cellulose contents gradually increased as NaOH concentrations increased and it reached the highest amount of $67.06 \pm 1.22\%$ when 2% NaOH was used. At this alkali concentration, no hemicellulosic sugars were detected in the solid residue at any pretreatment condition (Figure 4.13). Moreover, alkali treatment greatly removed lignin after LHW and VDA pretreatment.

Dissolution of lignin for differently treated samples was clearly observed depending on NaOH concentration. Figure 4.13 shows the effect of the NaOH concentration on lignin reduction (delignification). For example, the amount of klason lignin in the solids at the beginning was $50.88 \pm 0.46\%$ for 190°C 45 min, after NaOH pretreatment it was $40.16 \pm 0.85\%$ (0.5% NaOH) or $35.74 \pm 1.12\%$ (2% NaOH). The results indicated that the delignification of HPW was up to 30%. However, the lignin content of untreated HPW did not statistically change upon 2% NaOH treatment. The reduction was only $2.6 \pm 0.05\%$. Results of this study demonstrated that the mild NaOH pretreatment was effective on removing lignin from the pretreated HPW.

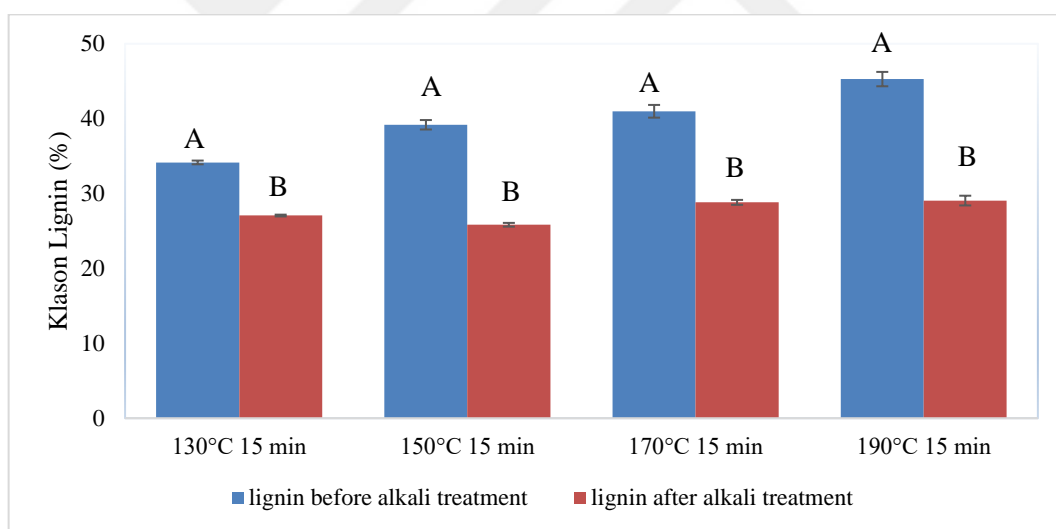


Figure 4.14. Effect of alkali treatment on Klason lignin in VDA-treated HPW.

*Alkali: NaOH, *Acid: H_2SO_4

Capital letter: lignin %. Every part was evaluated separately.

The effect of alkali treatment was investigated further using VDA-treated biomass at different temperatures. NaOH concentration was fixed at 2% for this study. Alkaline treatment with 2% (w/v) NaOH removed lignin significantly from VDA-treated HPW. The amount of lignin in the solids before alkali treatments was $45.28 \pm 0.96\%$ in VDA-treated sample, while further treatment 2% NaOH decreased lignin to $29.06 \pm 0.65\%$

(Figure 4.14). The mild alkaline pretreatment was an effective method to remove lignin from pretreated HPW.

Alkali-treated samples resulted in lower solid recovery with increasing NaOH concentration (Table 4.3). For example for untreated HPW, solid recovery was $86.45 \pm 1.22\%$ after 0.5% NaOH (w/v) treatment and $71.27 \pm 0.63\%$ after 2% NaOH (w/v) treatment.

The solid recovery of alkali-treatment after VDA of HPW ranged from $75.37 \pm 0.23\%$ (130°C 15 min 0.1% H_2SO_4 +2% NaOH) to $60.22 \pm 0.37\%$ (190°C 15 min 0.1% H_2SO_4 +2% NaOH).

Table 4.3 shows all of the solid and cellulose recovery after alkali treatments. Results indicated that the major solid loss during NaOH pretreatment was due to the delignification and xylan degradation of the HPW.

Table 4.3. Solid and cellulose recovery of alkali-treated HPW

Alkali pretreatments on VDA-treated HPW	Solid recovery %	Cellulose recovery %
2% NaOH (after 130°C 15 min 0.1% H_2SO_4)	75.37 ± 0.23	91.27 ± 0.53
2% NaOH (after 150°C 15 min 0.1% H_2SO_4)	67.24 ± 0.49	84.35 ± 0.21
2% NaOH (after 170°C 15 min 0.1% H_2SO_4)	64.21 ± 0.52	82.35 ± 0.43
2% NaOH (after 190°C 15 min 0.1% H_2SO_4)	60.22 ± 0.37	80.88 ± 0.12
Alkali pretreatments on untreated and VDA & LHW-treated HPW		
2% NaOH (Untreated)	71.27 ± 0.63	80.98 ± 0.73
2% NaOH (after 170°C 15 min 0.1% H_2SO_4)	64.21 ± 0.52	82.35 ± 0.43
2% NaOH (after 190°C 45 min)	58.59 ± 0.12	89.88 ± 0.11
0.5% NaOH (Untreated)	86.45 ± 1.22	96.76 ± 0.03
0.5% NaOH (after 170°C 15 min 0.1% H_2SO_4)	93.64 ± 0.22	91.95 ± 0.96
0.5% NaOH (after 190°C 45 min)	82.25 ± 0.68	93.45 ± 0.55

4.2.3.2. Enzymatic Saccharification of Alkali-treated HPW

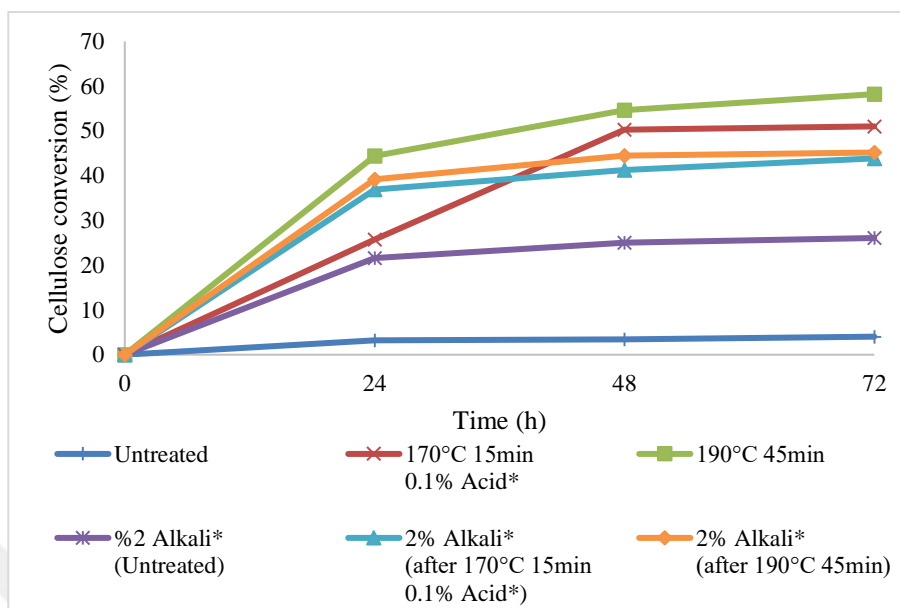


Figure 4.15. Conversion of cellulose to glucose in the enzymatic hydrolysis of pruning waste of hazelnut from Alkali-treated which was before LHW and VDA treated and untreated. Enzymatic hydrolysis conditions: 30 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 72 h.

*Alkali: NaOH, *Acid: H₂SO₄

Alkaline treatment can be used for removing lignin partly and thereby increasing the digestibility of cellulose for enzymatic hydrolysis (Schell et al., 1998). In this study, enzymatic hydrolysis was done for selected samples which were based on the highest cellulose to lignin ratio (2% NaOH; after 190°C 45 min and 170°C 15 min with 0.1% H₂SO₄). The cellulose conversion of each hydrolysis treatment is shown in Figure 4.15. Alkali treatment could not improve the cellulose conversion yield. However, Wang et al. (2010) reported that 91.7% cellulose conversion for 3% NaOH-treated bermuda grass.

This experiment was also performed to untreated HPW in order to compare the efficiency of alkaline treatment. Enzymatic conversion yield improved with alkaline treatment from 4.03±1.26% (untreated HPW) to 26.07±0.53% (2% (w/v) NaOH-treated HPW).

4.2.4. Organosolv Pretreatment

Organosolv pretreatment was performed in order to remove lignin in the solid residue. LHW, VDA and alkali treatments were successful in partial removal of hemicellulose and lignin, however enzymatic saccharifications were relatively low (Capolupo & Faraco, 2016). Cellulose could not be converted to glucose totally, probably due to the remaining lignin after the treatments. Hemicellulose could be removed totally in some of the treatments, however most of the lignin remained bounded to cellulose in the solid part. In an effort to increase lignin removal, organosolv treatment was applied on the HPW. Biomass was treated with ethanol-water mixture at 190°C in the pressure reactor. After the organosolv pretreatment, solids (cellulose-enriched part) were separated from the hemicellulose and lignin, relatively.

4.2.4.1. Biomass Compositions After Organosolv Pretreatment

HPW was treated at various conditions to select the appropriate condition. For the organosolv process with and without 0.1% H₂SO₄ (w/v) catalyst in the presense of 50% and 70% (v/v) EtOH. This process was conducted at 190°C for 15 and 45 min.

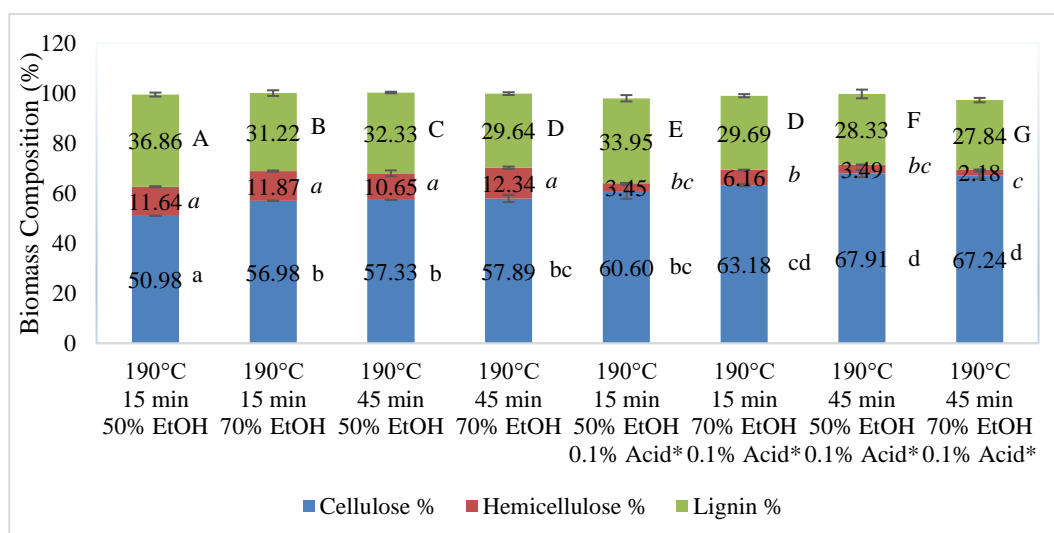


Figure 4.16. Chemical compositions of HPW after organosolv pretreatment (% , on dry basis). *Acid: H₂SO₄
Lower case letters: cellulose %; italic lower case letters: hemicellulose %; capital letter: lignin %.

Figure 4.16 represents the effects of the organosolv pretreatment and H₂SO₄ (0.1%, w/v) catalyzed organosolv treatment on cellulose, hemicellulose and lignin content of HPW. The maximum cellulose content was 67.91% at 190°C for 45 min with digesting solvent containing 50% ethanol (v/v) and 0.1% H₂SO₄ (w/v). Adding 0.1% H₂SO₄ (w/v) as a catalyst in the digesting solvent was observed to promote cellulose content. Acid catalysed organosolv was applied on lodgepole pine with 1.1% H₂SO₄ at 170°C, 60 min and 71.5% cellulose yield was provided by Del Rio et al. (2010). In another study, acid catalysed organosolv for loblolly pine was reported to yield 79.3% cellulose (Sannigrahi et al., 2010). Although the acid is used at a very low concentration in this study, the results were similar to the ones in the literature. Similar studies have obtained similar amounts of cellulose with higher acid concentrations.

Figure 4.16 showed that organosolv pretreatment with 0.1% H₂SO₄ (w/v) assisted degradation significantly higher amount of hemicellulose component with a removal >85% of the xylans from the solid residues.

Organosolv process permitted an efficient removal of lignin from the solid residues. Increasing the pretreatment time from 15 to 45 min at 190°C resulted in higher lignin removal. While the organosolv pretreatment at 190°C for 45 min without 0.1% H₂SO₄ resulted in 37.6% lignin removal, organosolv with 0.1% H₂SO₄ resulted in higher lignin removal with 45.4%. Furthermore, increasing EtOH concentration from 50% to 70 % (v/v) yielded the highest lignin removal (46.3%), achieving a 27.84% Klason lignin (Figure 4.16). Therefore, addition of 0.1% sulphuric acid as a catalyst revealed a positive impact on delignification through the current process. Contrarily, increasing EtOH concentration in the process at 190°C for 45 min had no significant effect on lignin removal. Klason lignin in solid parts after organosolv pretreatment was lower than all other pretreatments even in the raw material, which confirmed the significant elimination of lignin during the organosolv delignification process.

Table 4.4. Solid and cellulose recovery of organosolv treated HPW

Organosolv Treatments	Solid recovery %	Cellulose Recovery %
190°C 15 min 50% EtOH	67.99±0.52	105.43±0.27
190°C 15 min 70% EtOH	64.11±0.11	104.16±0.36
190°C 45 min 50% EtOH	57.29±0.95	94.98±0.06
190°C 45 min 70% EtOH	49.75±0.55	72.76±0.20
190°C 15 min 50% EtOH + 0.1% H₂SO₄	42.99±0.63	74.78±0.58
190°C 15 min 70% EtOH + 0.1% H₂SO₄	49.82±0.35	81.28±0.05
190°C 45 min 50% EtOH + 0.1% H₂SO₄	44.12±0.08	79.92±0.37
190°C 45 min 70% EtOH + 0.1% H₂SO₄	41.04±0.74	80.20±0.50

The total gravimetric recovery and cellulose recovery resulting from pretreatment at the different organosolv conditions assayed were summarized in Table 4.4. For organosolv pretreatment, the solid recovery of HPW ranged from 67.99±0.52% at 190°C 15 min 50% (v/v) EtOH to 41.04±0.74% at 190°C 45 min 50% (v/v) EtOH with H₂SO₄-catalyst. At the most severe organosolv treatment, 80.20±0.50% cellulose recovery was obtained as the Table 4.4 clearly shows.

In the case of the organosolv pretreatment, the conditions used allowed a very good recovery of cellulose in the solid. Solid recovery after organosolv treatment was lower compared to other pretreatments used in this study, because lignin solubilisation and xylan reduction increased with especially using solvent and acid-catalyzed pretreatment.

4.2.4.2. Enzymatic Saccharification of Organosolv-treated HPW

After organosolv pretreatment, the pretreated HPWs with high cellulose contents were subjected to enzymatic hydrolysis for glucose production. The enzymatic cellulose conversion after different ethanol organosolv pretreatment is presented in Figure 4.17. It was observed that; hemicellulose and lignin removal was an effective approach for improving cellulose conversion efficiency during enzymatic hydrolysis of HPW.

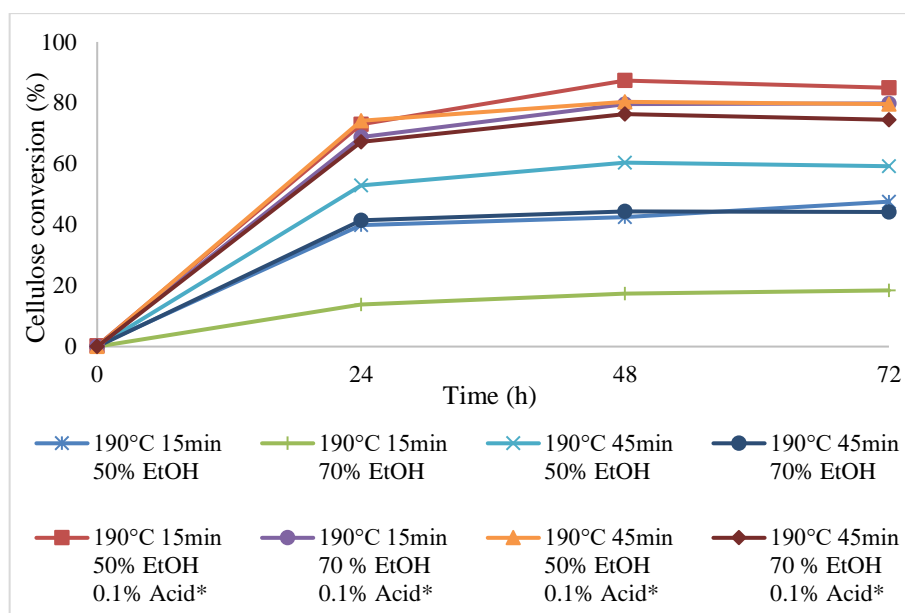


Figure 4.17. Conversion of cellulose to glucose in the enzymatic hydrolysis of pruning waste of hazelnut from Organosolv-pretreated with different pretreatment time and EtOH concentrations. Enzymatic hydrolysis conditions: 30 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v), and 50°C for 72 h. *Acid: H₂SO₄

Particularly, the enzymatic cellulose conversion quickly increased to 72.98±0.93% at the 24 h. This pretreated solids showed the highest hydrolysis rate among all experiments with 87.32±0.62% conversion after 48 h for 190°C, 15 min, 50% EtOH (v/v) and 0.1% H₂SO₄ (w/v) experimental condition. This was because that, cellulose-enriched part of solids was considerably separated from the hemicellulose, and lignin after organosolv treatments.

In the literature, Araque et al. (2007) studied the organosolv acetone-water for pinus radiata and found the highest enzymatic hydrolysis yield to be 99.5% after pretreatment at 195°C, 5 min. Pan et al. (2008) investigated organosolv at 187°C, 60 min for pine beetle killed and they reported 100% cellulosic conversion. Another study from Pan et al. (2008) was about lodgepole pine on organosolv treatment at 187°C, 60 min and reported 100% cellulose conversion. Brosse et al. (2009) examined *Miscanthus giganteus* in organosolv treatment at 170°C, 60 min retention time followed by enzymatic hydrolysis, which resulted in the 100% glucose yield.

Addition of H₂SO₄ to organosolv pretreatment facilitated enzymatic hydrolysis because of the increasing delignification. After uncatalyzed organosolv pretreatment, cellulose conversion was provided as 18.44±1.29% at 190°C, 15 min, 50% EtOH (v/v). However, cellulose conversion was greatly increased with 0.1% (w/v) H₂SO₄-catalyzed

as $87.32 \pm 0.62\%$ under the same conditions. Sannigrahi et al. (2010) treated loblolly pine with 1.1% H_2SO_4 catalyzed organosolv at 170°C , 60 min and 67.9% cellulose conversion was provided. Another study under the same conditions, which is about acid catalysed organosolv for lodgepole pine, reported 100% cellulose conversion (Del Rio et al., 2010). Park et al. (2010) studied acid catalysed organosolv for treating pitch pine at 180°C , 0 min and found that 80% enzymatic hydrolysis yield. Compared to similar studies, our study achieved better results even at lower acid concentrations.

Huijgen et al. 2011 found that the maximum cellulose conversion of wheat straw was as high as 99% for pretreated by 0.02 M HCl-catalyzed ethanol pretreatment, but only 44% for non-catalytic organosolv pretreatments in enzymatic hydrolysis. It could be emphasized that acid-catalyzed ethanol pretreatment was greatly developed for enzymatic conversion and thus facilitated following fermentation process.

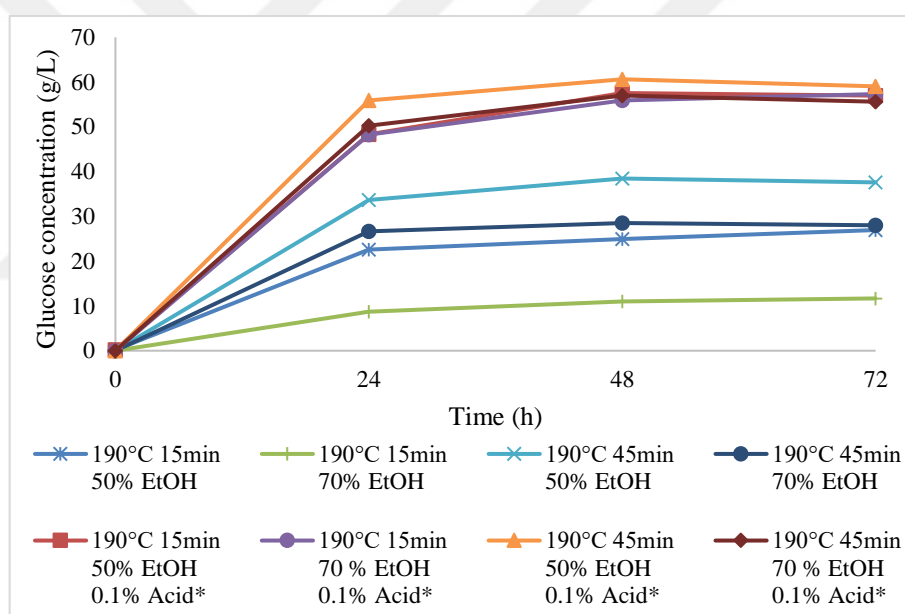


Figure 4.18. Saccharification of Organosolv-pretreated HPW. *Acid: H_2SO_4

The glucose concentration (g/L) obtained after enzymatic saccharification of organosolv-treated HPW is demonstrated in Figure 4.18. The best saccharification performance was obtained as 60.63 ± 0.87 g/L using HPW treated at 190°C for 45 min in the presence of 50% EtOH (v/v) and 0.1% H_2SO_4 (w/v).

4.2.4.2.1. Effect of Cellulase Loading on Enzymatic Saccharification for Organosolv Pretreatment

Several factors affect enzymatic saccharification such as temperature and pH. In addition to these, cellulase loading is also an important factor for the enzymatic saccharification. The aim of the study was to evaluate cellulase loading in enzymatic hydrolysis.

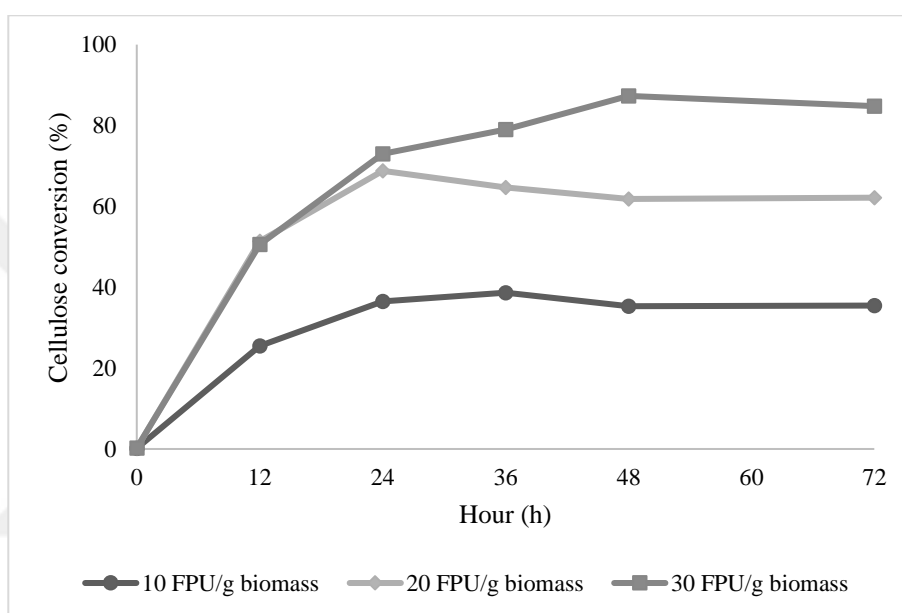


Figure 4.19. Conversion of cellulose to glucose in the enzymatic hydrolysis of pruning waste of hazelnut from Organosolv-pretreated with different cellulase loading. Organosolv pretreatment: 190°C for 15 min, 50% (v/v) EtOH with 0.1% H₂SO₄-catalyzed. Enzymatic hydrolysis: 10, 20 and 30 FPU/g biomass, pH 4.8, solid-to-liquid ratio 1:10 (w/v) and 50°C for 72 h.

Figure 4.19 shows cellulose conversion of 190°C for 15 min, 50% (v/v) EtOH with 0.1% H₂SO₄-catalyzed HPW with different cellulase loading as 10, 20 and 30 FPU/g biomass. Increasing the cellulase loading lead to increasing enzymatic conversion from 35.50±0.66% (10 FPU/g biomass) to 87.32±0.62% (30 FPU/g biomass). As it is depicted in Figure 4.19, when 20 FPU/g biomass cellulase loading preferred, it was similar cellulose conversion in comparison with 30 FPU/g biomass at the first hours (until 24 h). However, the maximum cellulose conversion was 62.12±1.32% for 20 FPU/g biomass at 72 h and this value could not increase in time.

4.3. Fermentation

Fermentation process is carried out for selected pretreated samples which are LHW-treated sample (190°C 45 min) and organosolv- treated sample (190°C, 15 min with 50% EtOH and 0.1% H₂SO₄) after 30 FPU/g biomass enzymatic saccharification. The maximum ethanol yield was observed when the hazelnut pruning wastes were treated at organosolv-treated one which was 190°C, 15 min with 50% EtOH (v/v) and 0.1% H₂SO₄ (w/v). Pretreatment had a significant effect on ethanol yield.

In this study, ethanol yields (of theoretical maximum) ranging from 64.01% (LHW) to 83.49% (organosolv) were obtained from hydrolyzates of pretreated HPW. Ethanol production using substrates increased with time until 6 h and the highest ethanol concentration of 22.2 g/L was observed using nutrient medium.

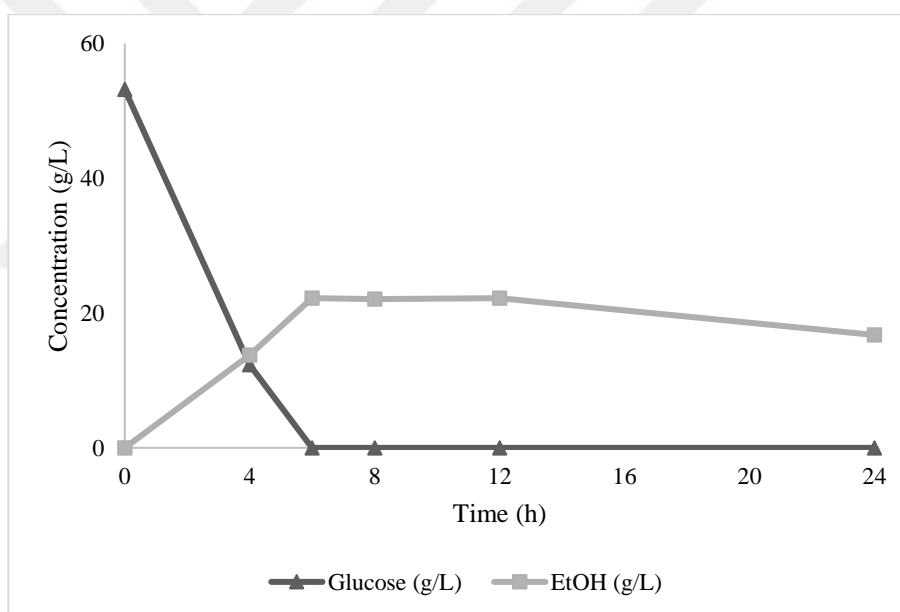


Figure 4.20. Production of ethanol by *S.cerevisiae*

To optimize the fermentation time for ethanol production, fermentation was conducted at 32°C or different time periods (0, 4, 6, 8, 12 h). Glucose released during enzymatic hydrolysis of selected pretreated samples was almost completely consumed by yeast within 6 h of fermentation. Ethanol production for organosolv-treated sample at 32°C, 150 rpm of fermentation condition is demonstrated in Figure 4.20.

CHAPTER 5

CONCLUSION

Lignocellulosic biomasses are interesting alternative to fossil fuel based resources and are the prime sources utilized in green biorefineries for the production of bioenergy. HPWs are the promising lignocellulosic biomass for their ethanol production potential because of containing 35.01% cellulose, 16.45% xylan, and 28.45% lignin.

Some steps required in lignocellulosic bioconversion because of its structure, the pretreatment is crucial for enhancing cellulose accessibility to the enzymes. According to the several reports, pretreatments at higher temperature and time give rise to a higher enzymatic digestibility than the untreated sample (Lu et al., 2012). The cellulose contents of all treated-samples were greater than that of the raw material and ranged from $40.62 \pm 1.17\%$ to $51.88 \pm 0.15\%$ by LHW, from $45.99 \pm 0.21\%$ to $53.90 \pm 1.23\%$ by very dilute acid hydrolysis, from $37.88 \pm 1.46\%$ to $67.06 \pm 1.22\%$ by alkaline and from $50.98 \pm 0.53\%$ to $67.91 \pm 1.06\%$ by organosolv treatments. The cellulose content increased with increasing pretreatment time and temperature. Meanwhile, the cellulose content also increased with presence of alkaline (NaOH), ethanol and very dilute acid concentration. For all the conditions, the cellulose content generally increased with increasing length of the pretreatment. Based on the obtained results, the best performance on cellulose content was obtained at 190°C for 45 min with 0.1% H_2SO_4 (w/v) and ethanol–water mixture (50%, v/v) (Organosolv). In addition to this, relatively high amount of hemicellulose was dissolved in treated samples. Hemicellulose content of solid part of the treated samples gradually decreased with increase temperature and time. Results showed that more severe pretreatment conditions dissolved more xylan from the pruning waste structure. Among LHW, very dilute acid hydrolysis, organosolv and alkaline (NaOH) pretreatments, NaOH resulted in the highest xylan solubility (almost all). The range of Klason lignin was 27-51% of treated HPW. In particular, the highest lignin removal rate 46.3%, achieving a $27.84 \pm 1.33\%$ Klason lignin was observed using organosolv condition, which might be enough to deconstruct the lignocellulosic structure.

Under LHW, VDA, alkaline and organosolv pretreatment conditions, solid recovery decreased with increasing reaction temperature and residence time. Furthermore,

solid recovery highly decreased in presence of acid or alcohol. On the other hand, cellulose recovery increased with increasing severe conditions of pretreatment because of the removal of hemicellulose and lignin.

Pretreatment conditions affect substrate characteristics, resulting in different enzymatic hydrolysis performances at different enzyme loadings for HPW. It can be concluded that greater level of hemicellulose and lignin removal in the HPW, better the digestibility performance of cellulose in residual solids, thus increasing the enzymatic conversion efficiency. Nevertheless, it was not necessary to promote a complete removal of hemicellulose and lignin to achieve high cellulose conversion ratio during the enzymatic hydrolysis of HPW. Cellulose conversion might be significantly enhanced by pretreatments.

The condition of enzymatic hydrolysis that yielded the highest cellulose conversion ($87.32 \pm 0.62\%$) was enzyme load of 30 FPU/g biomass, hydrolysis time of 48 h, and solid percentage of 10% (w/v), for the biomass treated at 190°C for 15 min with 50% EtOH (v/v) and 0.1% H₂SO₄ (w/v). Addition of H₂SO₄ even at small amount (0.1%), facilitated enzymatic saccharification because of the increasing delignification. Based on all obtained results, the best performance on glucose concentration was obtained as 60.63 ± 0.87 g/L at 190°C for 45 min with 0.1% H₂SO₄ (w/v) and 50% EtOH (v/v) experimental condition (organosolv) using 30 FPU/g biomass.

Ethanol yield is a significant process parameter on account of economy and because of the cost of the raw material, which composes a considerable part of the total production cost, and also because the processing costs are typically associated with the amount of material passing through the process. It is possible to conclude that the hydrolyzate produced in the enzymatic hydrolysis of HPW is easily fermented by *S. cerevisiae* (baker's yeast) to ethanol, resulting in the highest concentration of 22.2 ± 0.93 g/L in 6 h of fermentation at pH 4.8 and 32°C. Ethanol yields ranging from $64.01 \pm 0.11\%$ to $83.49 \pm 0.93\%$ of the maximum theoretical yield were obtained with LHW-treated and organosolv-treated of HPW, respectively.

These results demonstrate that HPW can be potential for producing ethanol. The outputs of this thesis can contribute to Turkish economy and the hazelnut farmers.

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APPENDIX A

CELLULASE ASSAY

Cellulase activity was determined by measuring glucose as reducing sugar after incubation enzyme mixture and substrate for a certain time. Linear glucose standard curve was constructed using the absolute amounts of glucose (mg/0.5 mL) plotted against A540.

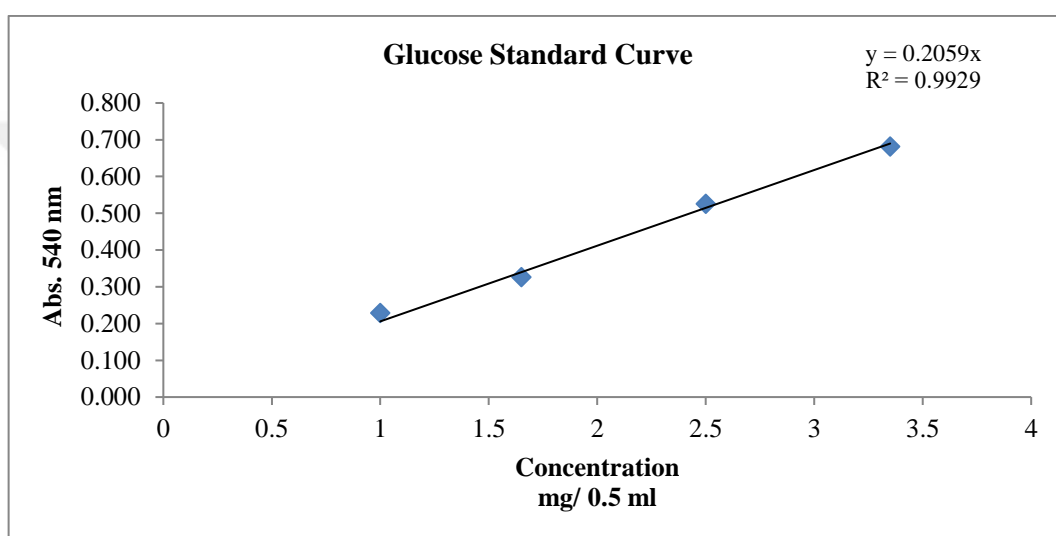


Figure A.1. Glucose Standard Curve for cellulase assay

Determination of cellulase activity in a commercial enzyme preparation. All enzyme dilutions were made in citrate buffer, pH 4.8, as indicated in the following Table A.1 from a working enzyme stock solution that had been diluted 1:100 in citrate buffer.

Table A.1. Numerical Values Used to Calculate Filter Paper Activity

Concentration *	Ave. Abs. / Glucose Standard Slope (mg/0.5ml)	Glucose g/L	AVE. ABS
0.003	1.011	2.021	0.2081
0.004	1.402	2.804	0.2887
0.005	1.526	3.052	0.3142
0.006	1.666	3.333	0.3431
0.007	1.891	3.782	0.3894
0.008	2.065	4.129	0.4251
0.009	2.428	4.855	0.49985

*The term "concentration" was used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture.

$$\text{FPU/mL} : 0.37/0.008 = 46 \text{ FPU/mL}$$

The numerator (0.37) in the equation was derived from the factor for converting the 2.0 mg of "glucose-equivalents" generated in the assay as indicated in the method.



APPENDIX B

STANDARD CALIBRATION GRAPH FOR GLUCOSE

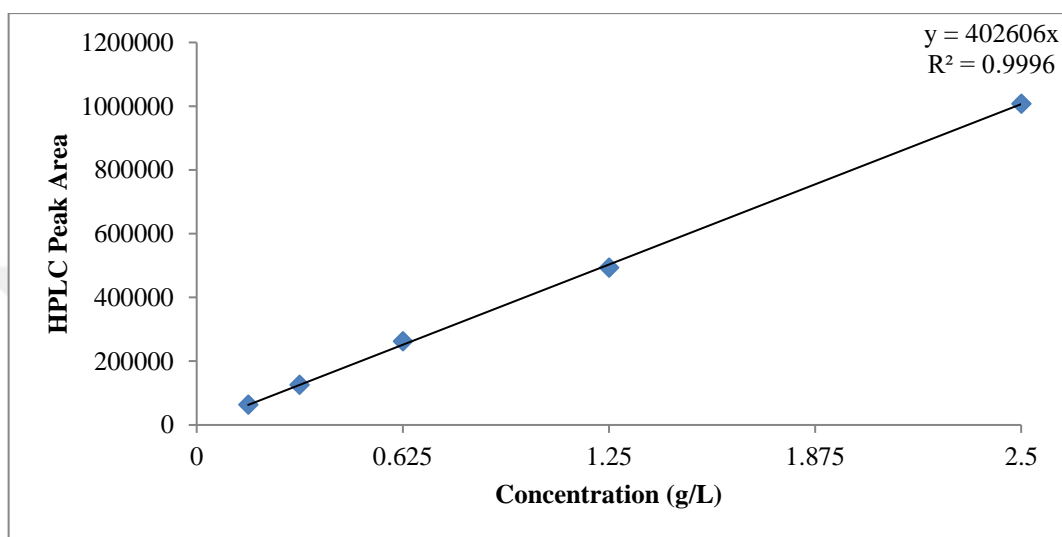


Figure B.1. Glucose standard curve for HPLC

Standard calibration curve was prepared for 5 different concentrations.

APPENDIX C

HPLC CHROMATOGRAM FOR STANDARDS

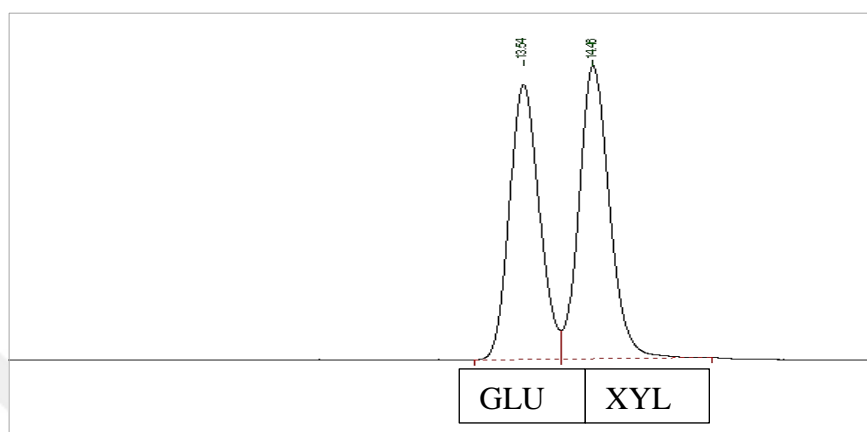


Figure C.1. Representative HPLC chromatogram of glucose and xylose standards