EFFECTS OF HIGH HYDROSTATIC PRESSURE (HHP) ON CELLULOSE HYDROLYSIS AND CELLULASE ACTIVITY, AND ITS USE FOR PEANUT HULLS HYDROLYSIS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

EYLÜL ÖZTÜRK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

JANUARY 2019

Approval of the thesis:

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ABSTRACT

EFFECTS OF HIGH HYDROSTATIC PRESSURE (HHP) ON CELLULOSE HYDROLYSIS AND CELLULASE ACTIVITY, AND ITS USE FOR PEANUT HULLS HYDROLYSIS

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January 2019, 117 pages

High hydrostatic pressure (HHP) technique has potential for food preservation purposes and HHP treatment can inactivate enzymes and/or increase their activity. Avicel, the commercial name of microcrystalline cellulose, is a commonly used substrate to observe the activity of cellulase. Cellulase activity is a crucial parameter for the production of alternative renewable fuels. Celluclast 1.5L, a mixture of enzymes, are used for the hydrolysis of cellulose and hemicellulose containing substances. Peanut hull (35% cellulose) as a lignocellulosic biomass is an appropriate material for conversion of the chemical feedstocks. The aim of this study is to observe different effects of HHP on substrate hydrolysis, and cellulose-hemicellulase (Celluclast) activity. Pressure and time were changed for HHP at the ranges of 0.1-500MPa, and 5-15min, respectively, at 30°C. The results showed that hydrolysis of HHP-treated enzyme and HHP-treated enzyme-substrate solutions gave significantly higher reducing sugar content than the non-HHP-treated solution ($p<0.05$). However, no significant effect was observed between applied pressure levels of 100 and 500MPa ($p>0.05$). Additionally, the hydrolysis results demonstrated that pretreatment process was a necessary step to increase hydrolysis efficiency due to high lignin content of

biomass. According to the Nuclear Magnetic Resonance (NMR) Relaxometry results, significant increases of T_2 relaxation were observed with HHP-treated (100 MPa-5 min-30°C) samples due to an increase in hydrophilicity of the sample surface ($p<0.05$). Additionally, enzymatic hydrolysis led significant increase on the relaxation time ($p<0.05$). The effects of HHP, alkaline pretreatment and enzymatic hydrolysis were observed with the changes Fourier Transform Infrared (FTIR) spectra.

Keywords: Peanut hulls, Enzymatic hydrolysis, HHP, NMR, FTIR

ÖZ

YÜKSEK HİDROSTATİK BASINCIN (YHB) SELÜLOZ HİDROLİZİ VE SELÜLAZ AKTİVİTESİ ÜZERİNE ETKİLERİ VE YERFISTIĞI KABUKLARININ HİDROLİZİ İÇİN KULLANIMI

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Ocak 2019, 117 sayfa

Yüksek hidrostatik basınç (YHB) tekniği besinleri korumak için kullanılmaktadır ve YHB uygulaması enzimleri de inaktive edebilir ya da onların aktivitesini artırabilir. Ticari olarak mikrokristalin selüloz olarak bilinen Avicel, genel olarak selülazın aktivitesini gözlemlemek için kullanılmaktadır. Bir enzim karışımı olan selülaz selüloz içerin malzemelerin hidrolizinde kullanılmaktadır ve selülaz aktivitesi, alternatif yenilenebilir yakıtların üretimi için çok önemli bir parametredir. Lignoselülozik biyokütle olarak yer fıstığı kabukları (%35 selüloz) kimyasal hammaddelere dönüştürülmek için uygun bir malzemedir. Bu çalışmanın amacı; YHB uygulamasının substrat hidrolizi ile selülaz-hemiselülaz (Celluclast) aktivitesi üzerindeki artıcı veya azaltıcı etkisini gözlemlemektir. Basınç ve zaman değerleri YHB için sırasıyla 0.1-500 MPa ve 5-15 dk aralıkları içerisinde değiştirilmiş, sıcaklık 30°C olarak ayarlanmıştır. Sonuçlar, YHB uygulanmış enzim çözeltileri ve uygulanmış enzim-substrat çözeltileri ile gerçekleştirilen hidrolizin, YHB uygulanmamış çözeltiler ile gerçekleştirilen hidrolizden önemli ölçüde ve daha fazla miktarda indirgen şeker ortaya çıkardığı gözlemlenmiştir ($p<0.05$). Ek olarak, uygulanan basınç değerleri 100 ve 500 MPa arasında önemli ölçüde artırıcı etki gözlemlenmemiştir ($p>0.05$). Ayrıca; hidroliz

sonuçları, biyokütlenin yüksek lignin içeriği nedeniyle alkalin ön işlemin hidroliz verimliliği için gerekli bir aşama olduğunu göstermiştir. Nükleer Manyetik Rezonans (NMR) Relaxometri sonuçlarına göre, yüzeyindeki hidrofilisitede artış nedeniyle YHB uygulanan (100 MPa-5 dk-30°C) örneklerin T_2 rahatlama sürelerinde artış gözlemlenmiştir ($p<0.05$). Bununla birlikte; enzimatik hidroliz, örneklerin T_2 rahatlama sürelerinde artışa yol açmıştır ($p<0.05$). Fourier Dönüşümlü Kızılötesi (FTIR) spektrumunda; YHB, alkali ön işlem ve enzimatik hidroliz etkileri piklerin şiddetleri, dalga boyları ve absorbans değerlerinde meydana gelen değişikliklerle gözlemlenmiştir.

Anahtar Kelimeler: Yerfıstığı kabukları, Enzimatik hidroliz, YHB, NMR, FTIR

To my family…

ACKNOWLEDGMENTS

I would you like to indicate my deepest appreciation to my supervisor Prof. Dr. Hami ALPAS for his endless patience, scientific guidance and support during my thesis work. It was a great pleasure working with him.

I would like to express my gratitude to Assoc. Prof. Dr. Mecit ÖZTOP for his valuable suggestions, interest, help and scientific guidance through my thesis. It would be much more difficult for me to deal with some problems faced without his support and understanding.

I want to say thank to Pressure Laboratory members; İlhami Okur, Pürlen Sezer, Burcu Kaya, Eda Ceren Kaya, Ufuk Yorgancı, Bige Tırpancı, Berkay Bolat, and Alperen Köker. It was so was quite enjoy and funny working with you in the same laboratory. I am very happy to share unforgettable memories with you and to have grateful friends. Thank you very much for your friendship. In addition, I also would like to thank to especially İlhami Okur, Pürlen Sezer and Berkay Bolat for their help for my experiments.

I would like to say thank to Assist. Prof. Dr. Sezen Sevdin for her help. With her guidance for laboratory, I worked in our laboratory easily. I also want to state my thanks to Barış Özel for his help for my experiments.

I also would like to indicate my gratitude to my all teachers at food engineering department. In addition, I am thankful to workers at our department especially for Mukaddes Ünver. During both undergraduate and master education, her support and help were always accompanied to me.

I would like to say thank to my workplace, Food Engineering department at Yıldız Technical University, for their support. I also want to state my thanks to Prof. Dr. Muhammet Arıcı for his understanding and patience in the period of writing my thesis.

I want to indicate my deepest thanks to Tolga Cantürk for his support, help and especially patience. I also want to say thank to all my loved ones who help me whenever I need and are always with me.

Lastly, I want to express my deepest gratitude to all members of my dearie family; my mother Ayten Öztürk and my father Metin Öztürk. Through all my life, whatever my decision is, they have been always on side of me and always support my decision. I feel very lucky to have such a family. Without their support and help this work could not come to exist. Thank you very much for all things that you have done for me. This work is dedicated to them.

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CHAPTER 1

INTRODUCTION

1.1 The Need of Lignocellulosic Biomass Conversion

Limited current fossil fuel resources have significant potential in terms of energy and materials for a while. Petroleum resources, one of the fossil fuels, are raw materials of many industrial materials such as plastics and of fuel element such as petrol or diesel. In the 21st century, petroleum price and cost of derived products of petroleum such as fuels and industrial materials are estimated to be high due to depletion of petroleum resources (Mosier et al., 2005). In 2015, energy consumption was supplied 78.4% from fossil fuel, 2.3% from nuclear power and the rest part, 19%, from renewables/ renewable energy. The renewable energy includes 9.1% traditional biomass (wood, charcoal, leaves, agricultural residue, etc.) and 10.2% modern renewables that consist of biomass, geothermal, solar, wind, hydropower and biofuels according to the annual report of Renewable Energy Policy Network (*Annual Report*, 2017) (Figure 1.1).

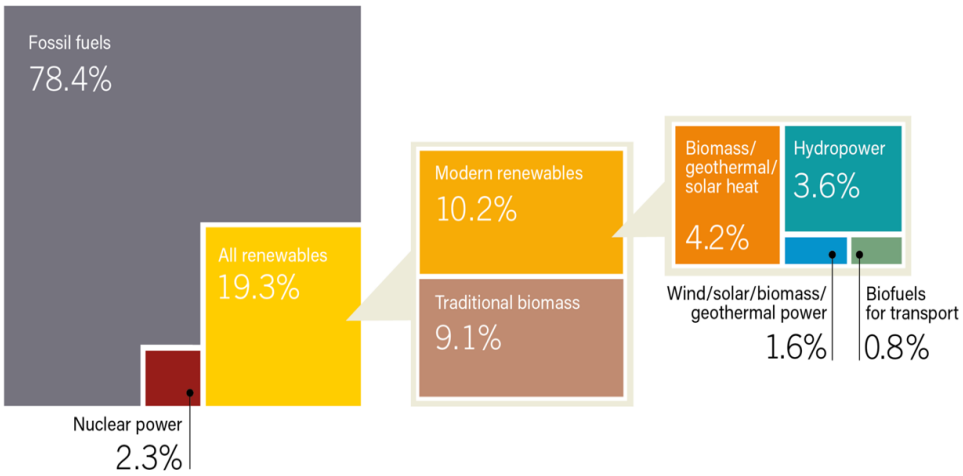

Figure 1.1: Estimated renewable energy share of total final energy consumption, 2015 (*World Energy Outlook*, 2018)

In the last two decades, approximately 85 million barrels of crude oil was needed for energy consumption of the world and it is estimated as 116 million barrels by 2030 (*World Energy Outlook*, 2007). In addition to the rise of fossil fuel consumption, according to the International Energy Agency's (IEA) World Energy Outlook 2018 report, demand for renewable energy resources will increase in 2040 for the energy industry (*World Energy Outlook*, 2018).

Biomass processing show similarities with petroleum refineries such as crude oil refined into several products of fuels (petrol, diesel, and kerosene)(Jones, Pujadó, & Pujadó, 2006). Developing biofuels/ bioenergy/ biorefining and biomass processing technologies promotes the tendency of sustainable replacements for petroleum resources. Together with the shift from petroleum- to biomass-derived materials, many advantages of obtaining biofuels and biochemicals come up in terms of economy, environment and industry. Production of them by using nonedible feedstock helps being renewable and sustainable, to create new job opportunities, improve development of local economy and reduce the environmental pollutions, especially air pollution (Balan, 2014; Greenwell, Lyold-Evans, & Wenner, 2013).

Biorefinery process, backbone of the biomass economy is the combination of biomass conversion processes that are alternative for traditional energy generation to produce fuels, power, material and chemicals (FitzPatrick, Champagne, Cunningham, & Whitney, 2010). Bioethanol production is one of the good examples and bioethanol has been used generally in fuel industry as an additive for a long time. This process depends the hydrolysis of starch; glucose obtained from hydrolysis and ethanol formed by the fermentation of the glucose. Therefore, ethanol production is based on derived sugars that are obtained from starch-containing grain, sugar beet and sugarcane. Another developed process of ethanol production from biomass is fermentation of cellulose to lignocellulosic biomass. Cellulose is found in large amounts on earth, and can be obtained from plant structural materials such as cotton straw, wood, paper waste, nut and ground nut hulls. (Tatlı, 2013).

Lignocellulosic biomass, nowadays, is a usable source as a renewable feedstock although it gives less bioenergy than sugarcane or starch as the feedstock. Some of the important challenges of the biorefinery process are; lignocellulosic biomass has a rigid polymer structure which requires some pretreatment steps; enzymatic hydrolysis efficiency is low (Den, Sharma, Lee, Nadadur, & Varma, 2018). As a result, pretreatment is a crucial step to remove lignin, isolate cellulose and promote enzymatic hydrolysis of cellulose (Tatlı, 2013).

Another important point of the process is the different pretreatment steps such as alkaline or acid pretreatment which also cause different structural changes and consequently enzymatic hydrolysis ends with different glucose and xylose amounts from cellulose and hemicellulose, respectively. After the whole process, ethanol yields change according to the pretreatment type (Wyman et al., 2005). In addition, the process may be more effective and feasible when lignocellulose parts (lignin and hemicellulose) are benefited and more than one products are produced in one cycle process (Bahcegul, Toraman, Ozkan, & Bakir, 2012). In Figure 1.2, biorefinery schematic is shown from the lignocellulosic biomass to the final products.

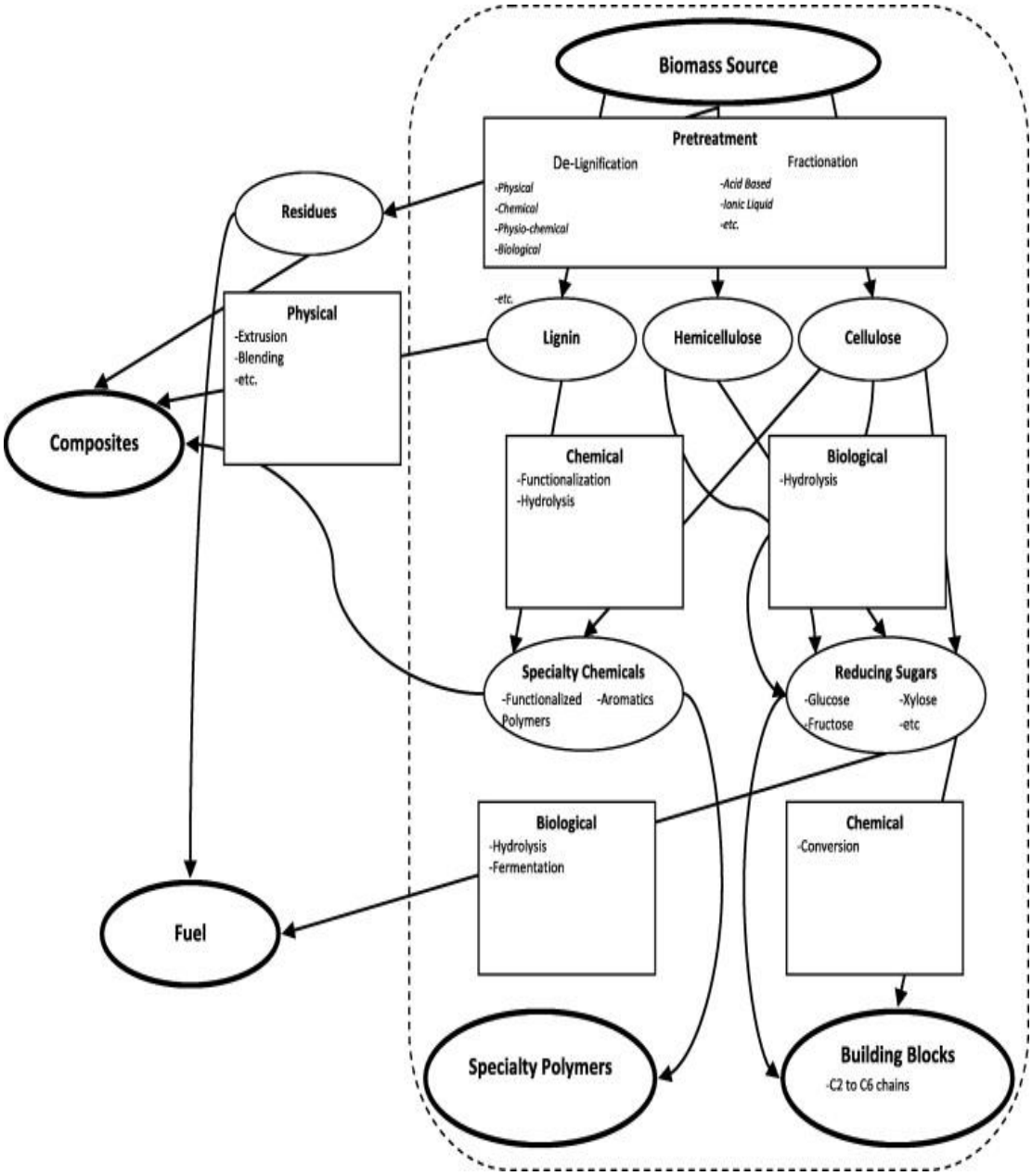

Figure 1.2: Biorefinery schematic (FitzPatrick et al., 2010)

1.2 Importance of Lignocellulosic Biomass

Lignocellulosic biomass is classified in four groups including agricultural residues such as sugarcane, woody materials, energy crops and cellulosic wastes. In addition, sources of lignocellulose are divided into two: hardwood and softwood. While hardwood materials can be exemplified with flowering plants, softwood can be identified as non-flowering plants and seed-producing plants (Kim, Lee, & Kim, 2016). Sugarcane bagasse and straw, corn stover, cotton stalks, corncob, wheat straw, rice straw, rice husk, wood chips, waste paper, cotton and linen fabrics are good examples of lignocellulosic material. All these materials have low cost and are efficient raw materials to produce biofuel (Seidl & Goulart, 2016).

Lignocellulosic materials have a complex structure that is formed with the combination of cellulose, hemicellulose, lignin and pectin and also have some monosaccharides, protein, or oils. (Wu, McLaren, Madl, & Wang, 2009).

The structure of lignocellulose and the polymers forming this structure are illustrated in Figure 1.3.

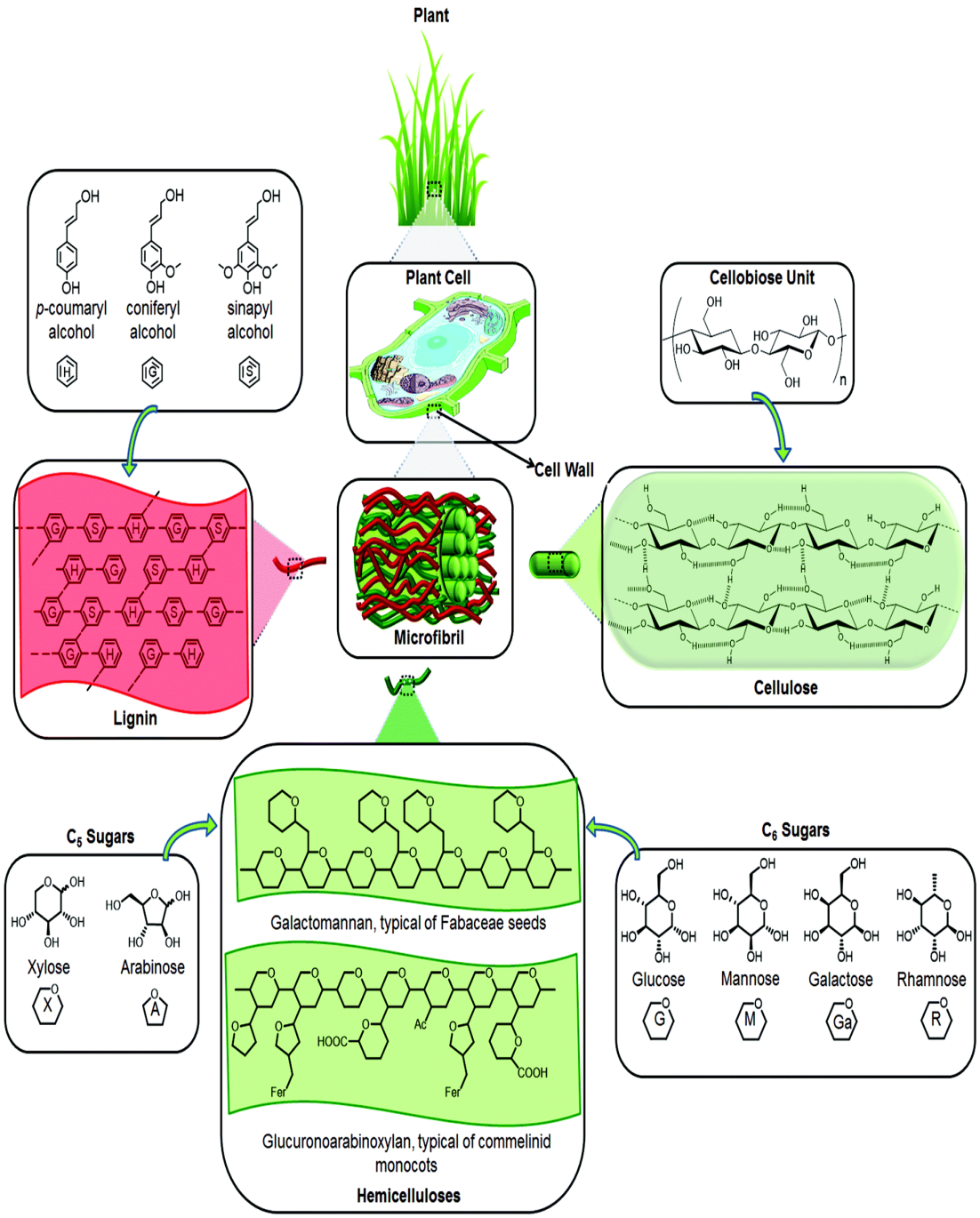

Figure 1.3: Lignocellulosic cell wall structure (Isikgor & Becer, 2015)

On a dry weight basis, lignocelluloses contain 40-50% cellulose, 20-30% hemicellulose and 15-25% lignin. These materials constitute approximately 90% of the dry weight of most plant. Ash and extractives such as waxes also exist in the lignocellulose structure with a small percentage (El-Naggar, Deraz, & Khalil, 2014). In Figure 1.4, main components of the structure are given. In addition, some types of lignocellulose and their chemical composition are given in Table 1.1.

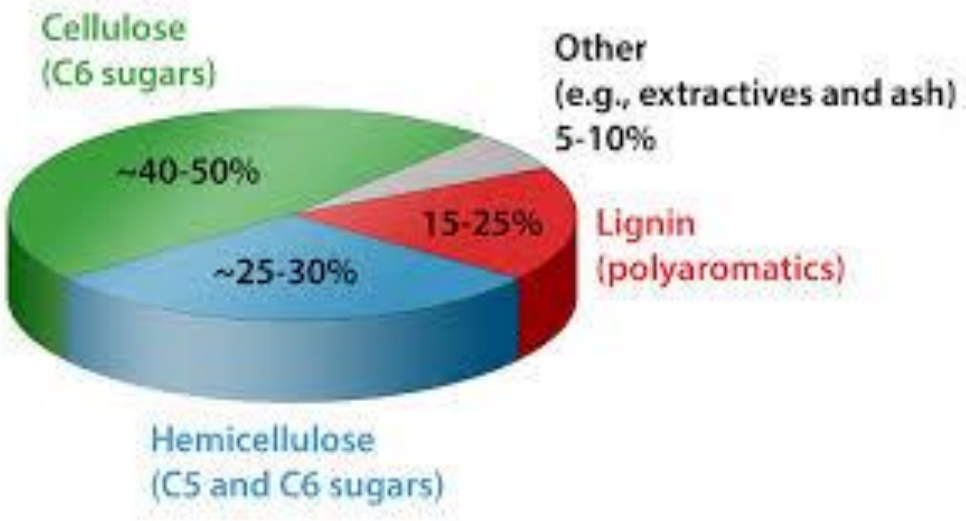

Figure 1.4: The composition of lignocellulosic biomass (*Lignocellulosic Biomass for Advanced Biofuels and Bioproducts*, 2015)

Table 1.1: Lignocellulosic Biomass Types and Their Chemical Composition (Isikgor & Becer, 2015)

Lignocellulosic Biomass		**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hardwood	Poplar	50.8-53.3	26.2-28.7	15.5-16.3
	Oak	40.4	35.9	24.1
	Eucalyptus	54.1	18.4	21.5
Softwood	Pine	42.0-50.0	24.0-27.0	20.0
	Douglas fir	44.0	11.0	27.0
	Spruce	45.5	22.9	27.9
Agricultural waste	Wheat Straw	35.0-39.0	23.0-30.0	12.0-16.0
	Barley Hull	34.0	36.0	13.8-19.0
	Barley Straw	36.0-43.0	24.0-33.0	6.3-9.8
	Rice Straw	29.2-34.7	23.0-25.9	17.0-19.0
	Rice Husks	28.7-35.6	12.0-29.3	15.4-20.0
	Oat Straw	31.0-35.0	20.0-26.0	10.0-15.0
	Ray Straw	36.2-47.0	19.0-24.5	9.9-24.0
	Corn Cobs	33.7-41.2	31.9-36.0	6.1-15.9
	Corn Stalks	35.0-39.6	16.8-35.0	7.0-18.4
	Sugarcane Bagasse	25.0-45.0	28.0-32.0	15.0-25.0
	Sorghum Straw	32.0-35.0	24.0-27.0	15.0-21.0
Grasses	Grasses	25.0-40.0	25.0-50.0	10.0-30.0
	Switchgrass	35.0-40.0	25.0-30.0	15.0-20.0

1.2.1 Cellulose

The most abundant polysaccharide is cellulose that is the major component of lignocellulosic biomass with 40-50% of the dry weight. Also cellulose is entrapped into the lignin-hemicellulose matrix (Isikgor & Becer, 2015). Cellulose is a linear polysaccharide and consists glucose disaccharide with β-1,4-glucosidic linkages unlike α-1-4-glucosidic bonds that exist in starch and glycogen. Cellulose has a sheet like structure and is formed with a few thousands of tightly binding glucose monomers (Figure 1.5) (Den et al., 2018). The repeating unit of cellulose is named as cellobiose disaccharide and it is represented in Figure 1.6.

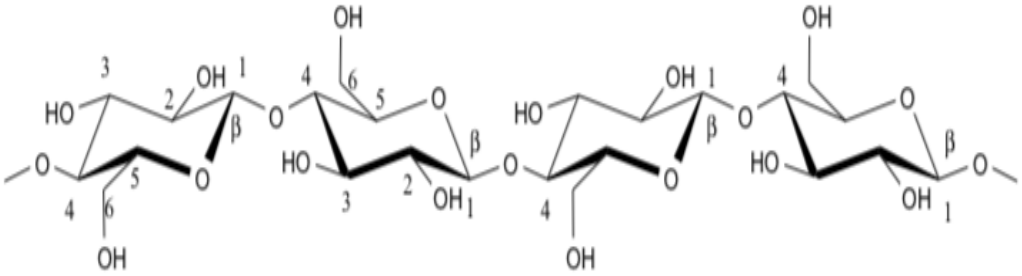

Figure 1.5: Cellulose structure (Laine, 2005)

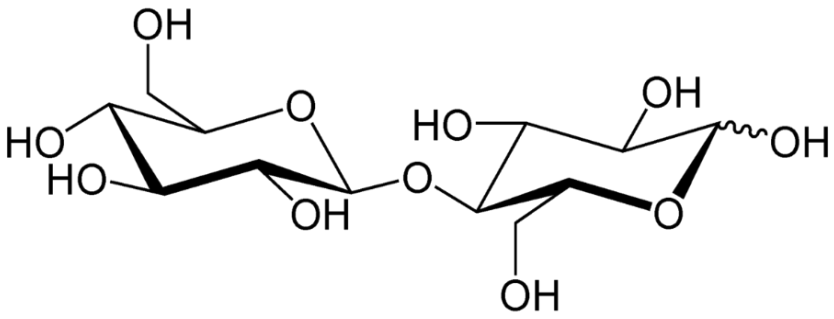

Figure 1.6: Cellobiose unit of cellulose (Isikgor & Becer, 2015)

Cellulose have both crystalline and amorphous regions. Cellulose molecules generate the linear chain structures by forming intramolecular and intermolecular hydrogen bonds and thus micro fibrils (Laine, 2005). These microfibrils that are generated by the cross linkages of hydroxyl groups give a pact and stronger property to the molecule. Hydrogen bonds between the molecules gives crystallinity; therefore, it has stronger matrix structure (Tatlı, 2013).

1.2.2 Hemicellulose

Hemicellulose is generally considered as the second most abundant polysaccharide. It has branched and amorphous structure in contrast to the crystalline cellulose and is formed by pentoses (5-carbon sugar unit) such as xylose, and hexoses (6-carbon sugar unit) such as glucose and mannose and acetylated sugars (Isikgor & Becer, 2015).

It can be defined as a branched heteropolymer of D-glucose, D-xylose, D-glucuronic acid, D- mannose, D-galactose and L-arabinose (Menon & Rao, 2012). These monomers may be contained in hemicellulose at different ratios and it results in different types of hemicellulose. Hardwood hemicelluloses have mostly xylans in its composition while softwood hemicelluloses have mostly glucomannans (Isikgor & Becer, 2015). This linear sheet polymer of β-D-xylopyranosyl units are linked with β-1-4- glycosidic bonds. In Figure 1.7 and 1.8, structures of xylan and glucomannan are shown.

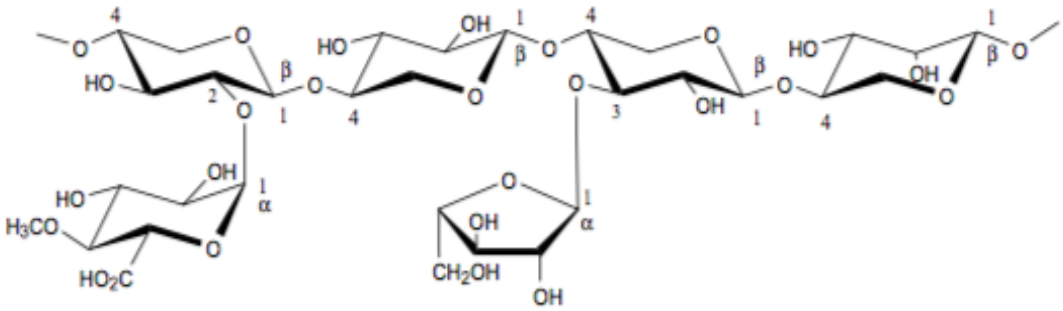

Figure 1.7: Xylan structure that is one of the hemicellulose (Laine, 2005)

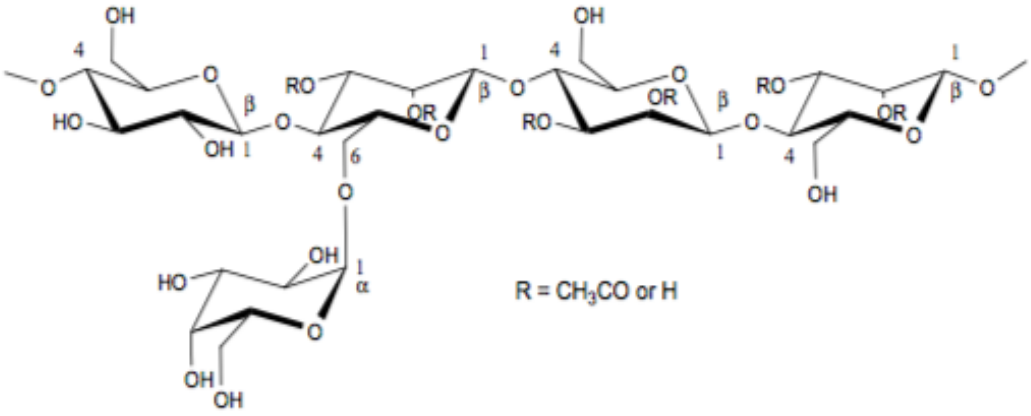

Figure 1.8: Glucomannan structure that is one of the hemicellulose (Laine, 2005)

Hemicellulose makes hydrogen bonds that provide structural strength by linking cellulose fibrils and cross-linking with lignin. By this way, it forms a network which provide structural stability and durability for the plant cell wall (Isikgor & Becer, 2015; Mosier et al., 2005; Tatlı, 2013).

1.2.3 Lignin

Lignin has 15-25% part in total biomass and is also one of the most abundant biopolymers (Den et al., 2018). Lignin has three different phenolic components in its structure and is formed by linking of monolignols that are p-coumaryl, coniferyl and sinapyl alcohols (Figure 1.9 and 1.10). The identic phenylpropanoid monomeric units are mentioned as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively (Isikgor & Becer, 2015). Lignin has various intermolecular linkages, but arylgycerol-β-O-4-aryl ether linkage is found in the structure mainly with 48-60% of total intermolecular linkages (Braun, Holtman, & Kadla, 2005; Tatlı, 2013). This linkage may be easily broken chemically for industrial processes and several analytical methods. In addition, the other linkages are β–5, β–β, 5–5 , 5–O–4 , and β–1. Unlike the arylgycerol-β-O-4-aryl ether linkage, they are all more resistant to chemical and biological processes (Boerjan, Ralph, & Baucher, 2003).

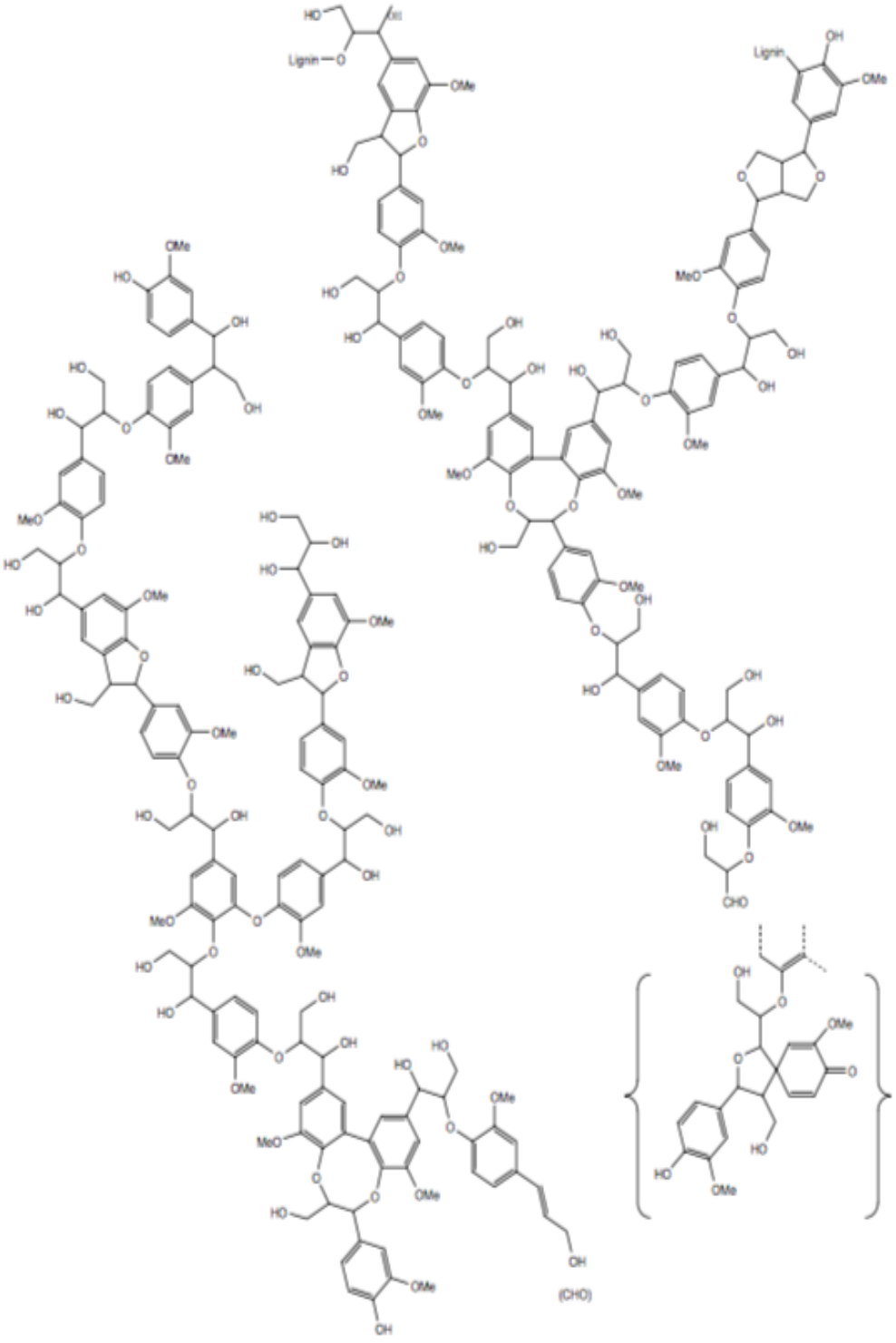

Figure 1.9: Structural schematic of softwood lignin (Laine, 2005)

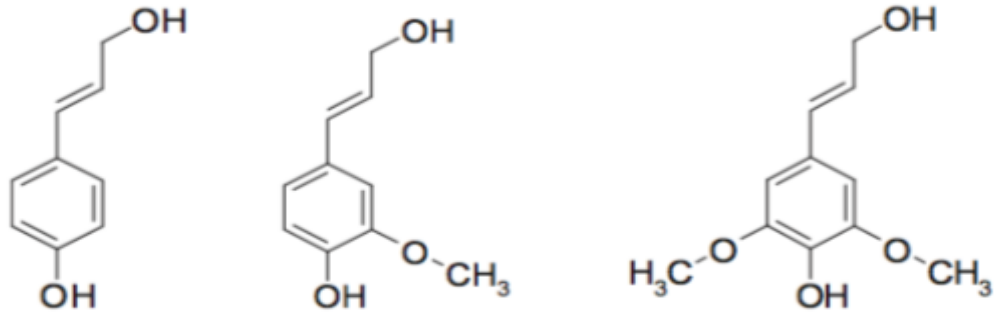

Figure 1.10: Monolignol monomers: p-Coumaryl, Coniferyl and Sinapyl alcohols respectively (Tatlı, 2013)

For enzymatic hydrolysis of cellulosic materials, it is needed to break the cellulose chains. However, it is resistant to biological conversion processes and that is the major reason of inefficient use of lignocellulosic biomass. Cellulose have tight and packed microfibrils encapsulated by hemicellulose and lignin. Under these conditions, enzyme ability on fiber wall is so limited and hydrolysis can be conducted only on the surface layers of microfibrils. The lignocellulosic materials have essentially non-biodegradable property without any pretreatment that broke the lignin matrix of biomass (Arantes & Saddler, 2010; Den et al., 2018).

1.3 Cellulose Sources for Enzymatic Hydrolysis

1.3.1 Avicel

Avicel is the commercially named microcrystalline cellulose (MCC) material. Cellulose is the most abundant natural polymer on earth with an annual biomass production of 50 billion tons (Thoorens, Krier, Leclercq, Carlin, & Evrar, 2014). More than 50 years later, MCC is manufactured globally by more than 10 suppliers (Thoorens et al., 2014). MMC is widely used in food industry for meringue and ice cream, confection industry, pharmaceutical industry for tablets as binder and cosmetic industry. Avicel has important roles on a stabilizing foams and controlling ice crystal formation (Holtzapple, 2003).

MCC is a purified, partially depolymerized cellulose prepared by treating alpha cellulose (type I_β), obtained as a pulp from fibrous plant material, with mineral acids. Cellulose has linear chains including β-1,4-D anhydroglucopyranosyl units and its chains are packed in layers that are held together by a cross-linking polymer (lignin) and strong hydrogen bonds. Cotton has also been mentioned as a possible cellulose source for MCC (Shlieout, Arnold, & Müller, 2002; Suzuki & Nakagami, 1999). Both softwoods (evergreen conifer) and hardwoods (deciduous broadleaf) can also be used for MCC production (Landín et al., 1993). These woods are divided considerably according to chemical composition such as proportions of cellulose, hemicelluloses and lignin and structural organization such as regions which are relatively more crystalline or amorphous. The amorphous regions are more inclined to hydrolysis so partial depolymerization by acid hydrolysis and it results in shorter and more crystalline fragments such as microcrystalline cellulose. In addition, microcrystalline cellulose (Avicel) is also used as substrate to observe the activity of cellulase in several studies (Peña & York, 2012).

1.3.2 Peanut Hulls

Peanut (*Arachis hypogaea L.*) is an agricultural plant and grown in tropical, subtropical, and warm temperate regions in Asia, Africa, Oceania, North and South America, and Europe. Its fruits or nuts, important raw materials, are used in food industry for many different purposes (Figure 1.11 and 1.12) (Freeman, Nigam, Kelley, & Ntare, 1999; Tatlı, 2013).

The edible part of the peanut is covered with an external hull or shell (21-29%) that surrounds the nut (79-71%). Peanut hulls, different from peanut skins that is the thin paper-like seed coats enclosing the kernel, are a by-product of peanut processing (Davis & Dean, 2016; van Doosselaere, 2103). The total peanut production with shells was approximately 43 million tons in 2017 (USDA, 2018). In Turkey, peanut cultivation area and production was 26.000 ha, and 80.000 tons in 2005, respectively. Approximately 0.1 % of Turkey's agricultural land is used to plant for peanut. In

addition, only three proveniences (Icel, Adana and Osmaniye) provide 80% of Turkey's peanut production (Alemdar & Isik, 2008). In the developing countries, groundnut becomes important for production of both oil and food crop. About 25% of the total produced mass consists of peanut hulls and their utilization is so important. In other words, peanut hulls generate large-amount bulky waste. Groundnut hulls are generally burned, sometimes left in forest areas to deteriorate naturally. In some cases, groundnut hull may be used as cattle feed, in the manufacture of logs and production of pulp and as a fiber component in human diet despite low digestibility (Nautiyal, 2002). In recent times, using peanut shells for a variety of purpose such as biofuel or bioethanol production becomes one of the agenda topics of environmental concerns (Hill, 2002).

Figures 1.11 & 1.12: Peanut fruits/seeds with shells

Chemical composition of peanut is shown in Table 1.2.

Table 1.2: Chemical composition of peanut hulls (Punnadiyil, P, & Purushothaman, 2016)

Component	Content (wt %)
Cellulose	35.7
Hemicellulose	18.7
Lignin	30.2
Ash	5.9

1.4 Enzymes Used for Lignocellulosic Biomass Hydrolysis

For efficient lignocellulose hydrolysis, collaborative work of different enzymes was required. Degradation of biomass includes four enzyme groups that are cellulases, hemicellulases, pectinases and ligninases. These groups are formed by both endo-enzymes and exo-enzymes. While endo-enzymes broke the linkages inside the cellulose chain and so molecular weight is reduced, exo-enzymes cleavage the ends of cellulose chain and release monomers (Henrissat & Davies, 1997).

Most of the lignocellulosic enzymes are obtained from cellulolytic bacteria and fungi. Cellulase and hemicellulase enzyme mixtures are used by a great number of the industrial sector, such as pulp and paper, textile, wine and beer, food, animal feed, agricultural, detergent, waste recycling and biofuel production industries. For these commercial purposes as industrial sources, enzymes or enzyme cocktails can be produced via some microorganisms that are *Trichoderma longibrachiatum, Humicola insolens, Termomonospora fusca, T. reesei, Aspergillus niger* and *T. koningii* (Himmel et al., 2010; Menon & Rao, 2012).

1.4.1 Cellulase

For cellulose hydrolysis of lignocellulosic biomass, there are three main types of cellulase that are endoglucanases, exoglucanases and β-glucosidases. Endo-1,4-β-D-glucanases (EG, EC 3.2.1.4) digests the internal β-1,4 bonds of the amorphous cellulose polymer randomly. One of the exoglucanase enzymes are cellobiohydrolases (CBH, EC 3.2.1.91) that attacks the ends of cellulose, hydrolyzes the most distant β-1,4 bonds and releases the cellobiose. In addition, CBHI acts on reducing ends CBHII act on non-reducing ends of cellulose. Other ones are glucanohydrolases (EG, EC 3.2.1.74) that releases glucose monomers from the ends of the chain. β-glucosidase (BG, EC 3.2.1.21) catalyzes the degradation of cellobiose and cellulo-oligomers. As a result, glucose monomers are released after hydrolyzation of cellobioses (Toth, 2014; Tsai & Meyer, 2014). Hydrolysis mechanism is summarized in detail (Figure 1.13).

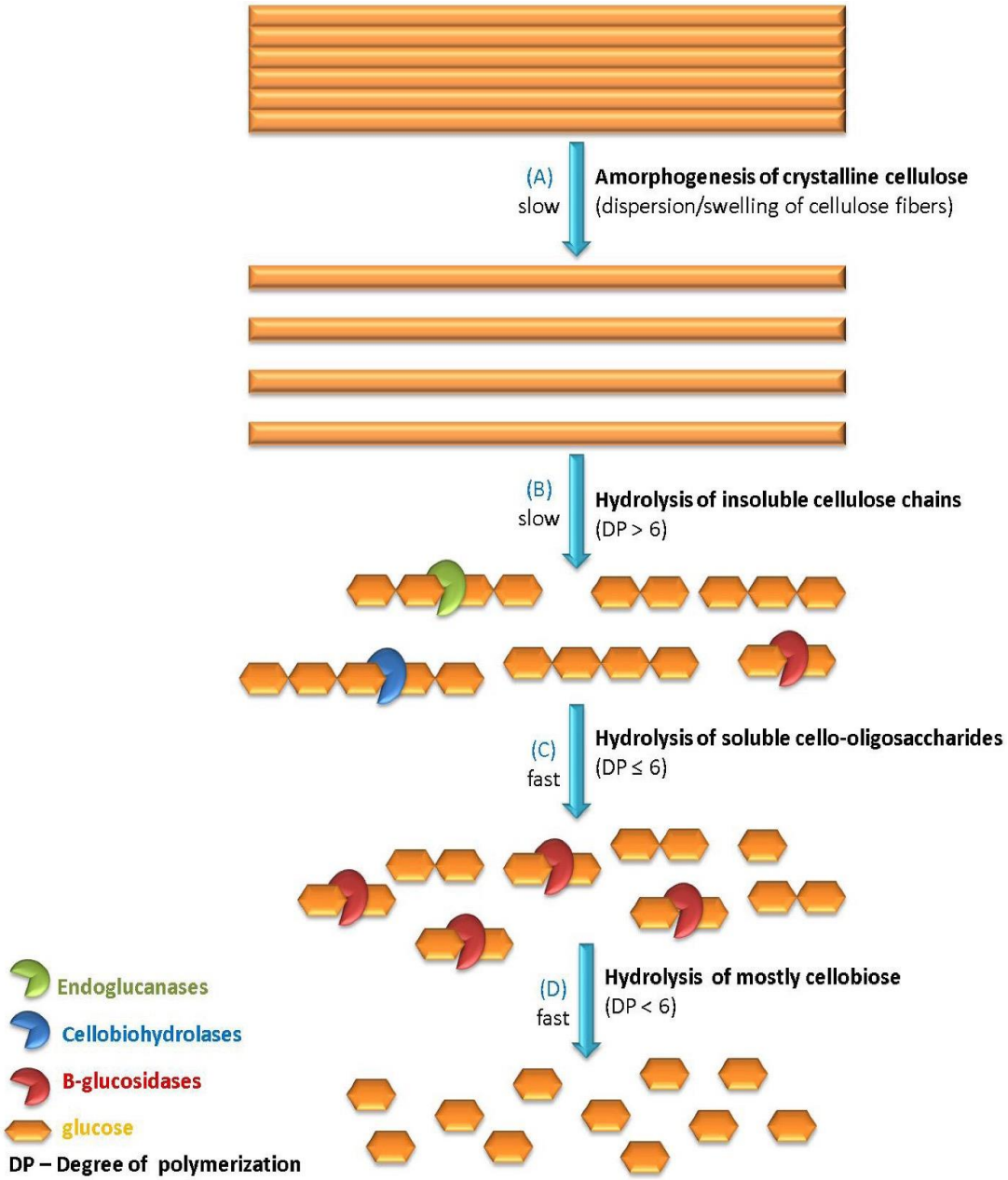

Figure 1.13: Mechanism of cellulose hydrolysis and cellulase system (Arantes & Saddler, 2010)

Cellulase, the most relevant used enzyme in industry, is generally obtained by being produced with saprophytic mesophilic fungus *Trichoderma reesei.* In addition, one of reasons for using fungus *Trichoderma reesei* to produce cellulase is that this strain is an efficient producer of cellulase enzymes (Toth, 2014).

1.4.2 Hemicellulase

Hemicellulose degradation requires synergistic effect of many different types of enzymes. According to their actions, hemicellulases are divided into three groups and they are endo-acting enzymes, exo-acting enzymes and further accessory enzymes. The endo-acting enzymes are responsible for cleaving of short oligomers by attacking hemicellulose chains internally, but it has low efficiency. However, exo-acting enzymes digest both short and long chain hemicellulose and attack from the reducing and non-reducing ends of the chain. Accessory enzymes, additionally, act on lignin-linked glycoside bonds of the hemicellulose structure in native plant tissue (Himmel et al., 2010; Toth, 2014).

Xylan, commonly known, is one of the most abundant hemicellulose and most studies including hemicellulases are about degradation of xylan (Menon & Rao, 2012). To obtain complete degradation, xylan chains are required to liberate from whole structure firstly and then the bonds of xylan chains are broken. These complex hydrolysis steps are also performed with synergistic working enzymes. Some of accessory enzymes play a role for debranching of side chains from backbone and these enzyme group contains α-L- arabinofuranosidases, α-glucuronidases (EC 3.2.1.139), ferulic acid esterase (EC 3.1.1.73), and acetyl xylan esterases (EC 3.1.1.72). For backbone hydrolysis, endo-xylanases (E.C. 3.2.1.8) (or β- xylanases) and β-xylosidases (E.C. 3.2.1.37) are needed. While endo-xylanases attack randomly the backbone of xylan and reveal xylooligosaccharides(xylotriose, xylobiose), β-xylosidases release xylose monomers by acting on non-reducing ends (Menon & Rao, 2012; Saha, 2003; Toth, 2014).

Xyloglucans and xylomannans are other sugar polymers in hemicellulose structure. Complete degradation of these polymers requires synergistic act of some enzymes that are xyloglucanases (EC 3.2.1.151), β- glucosidases, endo-mannases (EC 3.2.1.78) and β–mannosidases (EC 3.2.1.25) to break the polymer backbone. In addition, α–xylosidases (EC 3.2.1.177), α-galactosidases (EC 3.2.1.22), α-arabinofuranosidases

(EC 3.2.1.55) and acetyl esterases (EC 3.2.1.6) are additional enzymes to cleavage side chain sugars (Menon & Rao, 2012; Toth, 2014).

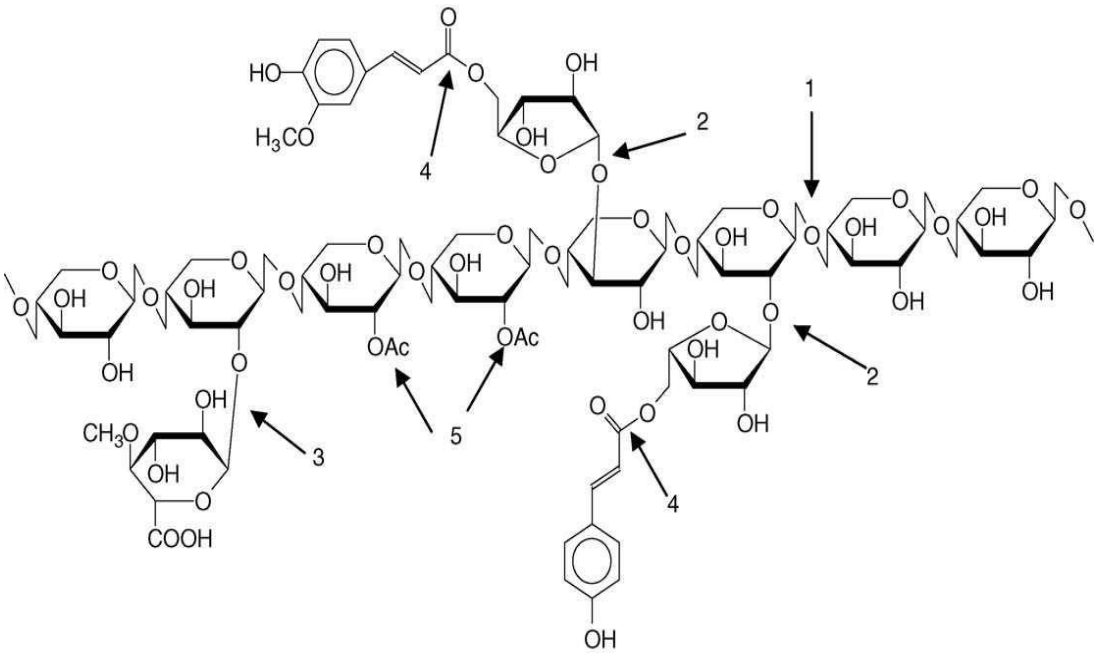

Figure 1.14: Xylan structure and action points of enzymes to the xylanase complex. (1: endoxylanases; 2: α-L- arabinofuranosidases; 3: glucuronidases; 4: feruloyl and coumaroyl esterases; 5: acetyl xylan esterases)(Chavez, Bull, & Eyzaguirre, 2006)

As shown in the Figure 1.14, endoxylanases (EC 3.2.1.8) hydrolyze the main chain of xylan randomly and release a mixture of xylooligosaccharides. β-Xylosidases (E.C. 3.2.1.37) release xylose from short oligosaccharides, while α-L- arabinofuranosidases (E.C. 3.2.1.55) remove L-arabinofuranose side chains. In addition, α-D-glucuronidases (E.C.3.2.1.139) hydrolyze the methyl glucuronate residues and acetyl xylan esterases (E.C. 3.1.1.72) hydrolyze acetate groups from the main chain. Feruloyl (E.C. 3.1.1.73) and coumaroyl esterases (E.C. 3.1.1.-) also liberate the respective aromatic acids linked to the arabinofuranoside residues. Their act point are marked in the Figure 1.14 (Chavez et al., 2006).

Hemicellulases are obtained by fungi, bacteria, yeast. Filamentous fungi are generally used for xylanases production because especially their enzyme level is much higher (Chavez et al., 2006). Due to many commercial purposes as paper producing, bread-making, juice and wine industries, xylanases can be produced from fungi of the genera *Trichoderma, Aspergillus,* and also *Penicillia*. Some of the examples of the other producer microorganisms are *Termomonospora fusca, T. reesei, Trichoderma longibrachiatum, Aspergillus niger*, bacteria such as *Bacillus sp*. (Menon & Rao, 2012).

1.4.3 Commercial Enzyme Mixture (Celluclast 1.5L)

Celluclast 1.5L, enzyme complex, obtained from *Trichoderma reesei* and has both cellobiohydralases and endo-glucanase activity. Cellulose sheet is degraded to oligosaccharide, cellobiose and glucose. It includes not only cellulase activity but also hemicellulase activity. It also includes β-xylosidase that plays a role of debranching xylobiose and short chain xylo-oligosaccharides. The Celluclast 1.5L has high activity at the pH range of 5–6 and at 40–50 °C (Marcos et al., 2013).

Celluclast 1.5L has an activity of 65 FPU/mL (FPU = filter paper unit) and 10 CBU/mL (CBU = cellobiose units). In addition, the activity is also mentioned as 700 EGU/g (EGU = endoglucanase) in the product data sheet by Novozyme (Ghose, 1987; *Product Data Sheet*, n.d.; Rosgaard, Pederse, Cherry, Harris, & Meyer, 2008)

1.5 Pretreatment Methods

Degradation of lignocellulosic biomass is more complicated processes and requires synergistic enzymatic actions. However, additional processes such as different pretreatment methods are crucial before enzymatic hydrolysis of biomass. Most pretreatment methods are applicable in industry to separate the components of lignocellulose (cellulose, hemicellulose, and lignin) (Saha, 2003; Sweeney & Xu, 2012). Lignin removing and decreasing the cellulose crystallinity are accepted as the main purposes of the pretreatment because the crystallinity of cellulose and the existence of rigid structure of lignin and hemicellulose have a characteristic of physical barriers and this property leads to the resistance for hydrolysis of lignocellulose. (Mosier et al., 2005). In industry, the application of more efficient and economical pretreatments breaking the rigid structure of the lignocellulosic matrix are needed. The pretreatment has some important functions that are to increase the formation of sugars, to improve the ability of sugar formation by enzymatic hydrolysis, to prevent the degradation or loss of carbohydrate and to prevent the formation of inhibitor byproducts to hydrolysis. (Galbe & Zacchi, 2007; Toth, 2014).

In addition to the destruction of the rigidity of biomass and cell wall, pretreatment increases the efficiency of enzymatic hydrolysis rate. As shown in the Figure 1.15, breaking down of the crystallinity, removing of lignin and hemicellulose structure, and decreasing of polymerization degree of cellulose lead to an increase in the amorphous structure of cellulosic biomass (also increase accessibility) and then surface area of substrate also increases. As a result, enzyme attack and so glucose yield are improved due to increased surface area (FitzPatrick et al., 2010; Tatlı, 2013).

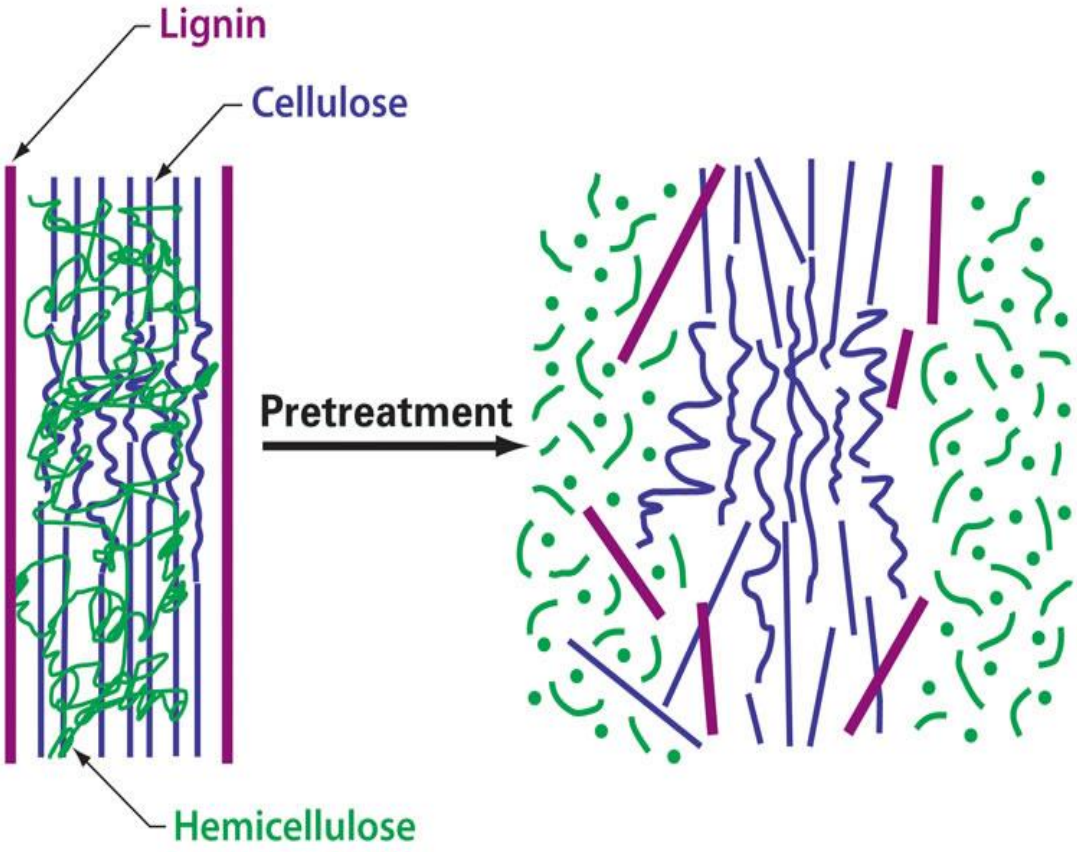

Figure 1.15: Results of pretreatment step (taken from https://public.ornl.gov/site/gallery/detail.cfm?id=248)

Pretreatment methods can be grouped to different categories and they are physical, chemical, physicochemical and biological methods. In addition, combination of these methods can be used if it is necessary (Isikgor & Becer, 2015). Some of the methods are summarized in Table 1.3.

Table 1.3: Some pretreatment methods and its effects on biomass (Taherzadeh & Karimi, 2008)

Pretreatment Methods	Processes	Study Application	Possible changes in biomass	Notable marks
Physical pretreatments	Milling Irradiation Others (Hydrothermal, Extrusion, etc.)	Ethanol Ethanol & Biogas Ethanol & Biogas	- Increase in accessible surface are and pore size - Decrease in cellulose crystallinity - Decrease in degrees of polymerization	-Most of the methods are highly energy-demanding - Most of them cannot remove the lignin - It is preferable not to use these methods for industrial applications - No chemicals are generally required for these methods

Pretreatment Methods	Processes	Study Application	Possible changes in biomass	Notable marks
Chemical and physicochemical pretreatments	Explosion	Ethanol & Biogas	- Increase in accessible surface area - Partial or nearly complete delignification - Decrease in cellulose crystallinity - Decrease in degrees of polymerization - Partial or complete hydrolysis of hemicellulose	-These methods are among the most effective and include the most promising processes for industrial applications - Usually rapid treatment rate -Typically need harsh conditions -There are chemical requirements
	Alkali	Ethanol & Biogas		
	Acid	Ethanol & Biogas		
	Gas	Ethanol & Biogas		
	Solvent extraction of lignin	Ethanol		
Biological pretreatments	Fungi and actinomycetes	Ethanol & Biogas	-Delignification - Reduction in degree of polymerization of cellulose - Partial hydrolysis of hemicellulose	- Low energy requirement - No chemical requirement - Mild environmental conditions - Very low treatment rate - Did not consider for commercial application

1.5.1 Milling

Milling is one of the most preferred pretreatment processes. It helps to improve enzymatic hydrolysis via reducing the size of substrate and the degree of crystallinity of lignocelluloses. Therefore, ethanol or biogas production increases with improved enzymatic degradation. Milling and size reduction have been applied before enzymatic hydrolysis, or other pretreatment processes to increase efficiency of enzymatic activity. However, it is not a sufficient pretreatment because lignin and hemicellulose components still exist in the material and so another pretreatment process is needed to remove the resistance for lignocellulose hydrolysis (Taherzadeh & Karimi, 2008; Tatlı, 2013).

1.5.2 Alkaline pretreatment

Alkali is known to break the linkages of lignin and glyosidic bonds of lignocellulose and it leads a reduction in the degree of polymerization and crystallinity of cellulose, swelling of the fibers. As a result, lignin in the lignocellulosic structure is cleaved. This type pretreatment method includes alkaline reagents that are sodium hydroxide, calcium hydroxide, potassium hydroxide, aqueous ammonia hydroxide or combination of them with hydrogen peroxide (Chen, Stevens, Zhu, Holmes, & Xu, 2013). Process can be applied at low temperatures but it requires relatively long time and high concentration of the base (Taherzadeh & Karimi, 2008).

The main effect of sodium hydroxide pretreatment on lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity the of biomass (Mosier et al., 2005; Sun & Cheng, 2002). Zhao et al. (2008) reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose compared with H_2SO_4 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, and avoiding fragmentation of the hemicellulose polymers (Taherzadeh & Karimi, 2008; Zhao, Zhang, & Liu, 2008).

1.6 High Hydrostatic Pressure (HHP) Treatment

High hydrostatic pressure (HHP) treatment is known as a non-thermal processing and cold pasteurization technique. This method can be used to destroy microorganisms and also used to inactivate enzymes in order to enhance safety and shelf-life of foods. Therefore, HHP has become a reality in the food industry and has spread worldwide (Alpas, Kalchayanand, Bozoglu, & Ray, 2000; Buzrul, 2015). After 2000, the prevalence of HPP application in the food industry increased exponentially (Buzrul, 2015).

First of all, the research of HHP technology was conducted for microbial inactivation to be more effective. In addition, enzyme inactivation with HHP has become the second important challenge of researchers; therefore, numerous results have been found proving that enzyme inactivation/activation depends on the process conditions and treatment medium (Bermudez-Aguirre & Barbosa-Canovas, 2011). New or improved characteristics of both foods and ingredients have been a research topic after high pressure pressurization and this case leads to the development of new value-added products. To extend the shelf lives of several high-acid foods, high pressure also has been another effective choice for pasteurization. Important findings in terms of microbial and enzymatic inactivation with HHP continue to be studied and many new research projects have been undertaken (Bermudez-Aguirre & Barbosa-Canovas, 2011).

HHP application is also known as the effective method for protein disruption. Thanks to the feature of high pressure, it is possible to protect foods against pathogens and microorganisms by denaturing the protein structure. (Murao et al., 1992). In the case of absence of temperature changes and complex experimental system, HHP application can change the protein dynamics and affect protein-protein interaction (Ohmae, Murakami, Gekko, & Kato, 2007). Alpas et al. (2000) reported that *S. aureus* 485, *L. monocytogenes* CA, *E. coli* O157:H7 933 and *S. enteritidis* FDA are more pressure-resistant than the respective strain of the same species. In general, viability

loss of all pathogens was improved significantly while the level of pressure and temperature were increased. All the strains except *S. aureus* 485 illustrated more than 8 log cycle reduction after pressurized at 345 MPa at 50°C for 5 min. Moreover, bacterial cells were killed by hydrostatic pressure mainly due to destabilization of the membrane functions (Alpas et al., 2000).

The effects of pressure on enzymatic reactions can be complicated in terms of the possibility of denaturing the enzyme protein and unfolding of the protein and dissociation of the subunits (Northrop, 2002). However, the conformational changes, unfolding of enzymes, changes in protein solubility can cause both enzyme activation and inactivation. HHP increasing the activity of some enzymes was mentioned while other studies showed that other enzymes such as endoglucanase, glucosidase and hydrolases were inactivated by this treatment (Eisenmenger & Reyes-De-Corcuera, 2009). In the field of enzymology, high pressure can be used to alter both the stability and activity of several enzymes by leading to potential applications. HHP affects enzymatic activity by changing protein structure and so it could either activate or inhibit enzymatic activities, depending on the proteins and conditions (Vila Real, Alfaia, Calado, & Ribeiro, 2007).

High hydrostatic pressure more likely affects the non-covalent bonds like hydrogen bond and hydrophobic interactions (Chakraborty & Rao, 2015). Moreover, Albuquerque et al. (2016) reported that hydrostatic pressure broke the hydrogen bonds and so ruptures and porous areas occurred on coconut fibers after a pressure treatment in the range of 131–175 MPa. Both ruptures and porous areas were enhanced by pressure exposure new hydrophobic parts of the lignocellulosic material (Albuquerque et al., 2016). Ding et al. (2012) reported that hydrophobic parts of cellulose elementary fibers (CEF) were important for cellulose digestibility and they could bind to the carbohydrate binding module (CBM) or to the hydrophobic amino acids of enzymes thanks to hydrophobic interactions. As a result, increase in cellulase activity was obtained.

As mentioned above, HHP directly affected the hydrogen bonds in molecular structures. Kunugi (1992) reported that hydrogen bonding might also be slightly strengthened by an increase in pressure, because of the decrease in the inter-atomic distance leading to a smaller molecular size. After that, the lengths of existing hydrogen bonds within proteins were observed to get shorter under high pressure (Linowski, Liu, & Jonas, 1976).

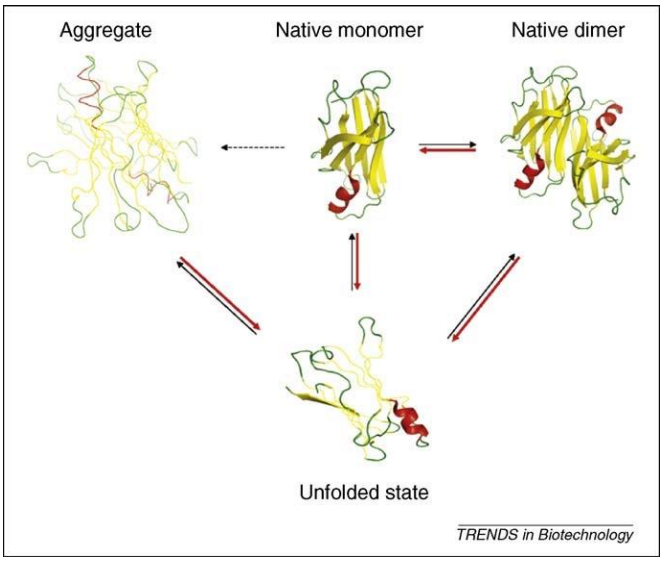

Figure 1.16: Pressure effects on proteins and protein assemblies (Aertsen, Meersman, Hendrickx, Vogel, & Michiels, 2009)

In Figure 1.16, red arrows show the direction of shifts in reaction via pressure increase. Pressure can unfold proteins and dissociate native oligomers with or without subsequent unfolding. Pressure can also decompose some aggregates into their monomeric species. The possibility of pressure-induced aggregation is shown with dashed arrow (Aertsen et al., 2009).

High hydrostatic processing consists of subjecting food, previously sealed in flexible and water-resistant packaging (Figure 1.17). A high level of hydrostatic pressure (pressure transmitted by water) up to 600 MPa / 87,000 psi can be applied for a few seconds to a few minutes. The temperature of the product in the pressure chamber can

rise by 3-6°C for every 100 MPa increase in pressure, but this situation can change depending on the composition of the product (Erkan et al., 2010). This phenomenon is known as adiabatic heating. Due to the compressive work against intermolecular forces, HHP causes temperatures to increase in the pressure vessel (Elamin, Endan, Yosuf, Shamsudin, & Ahmedov, 2015).

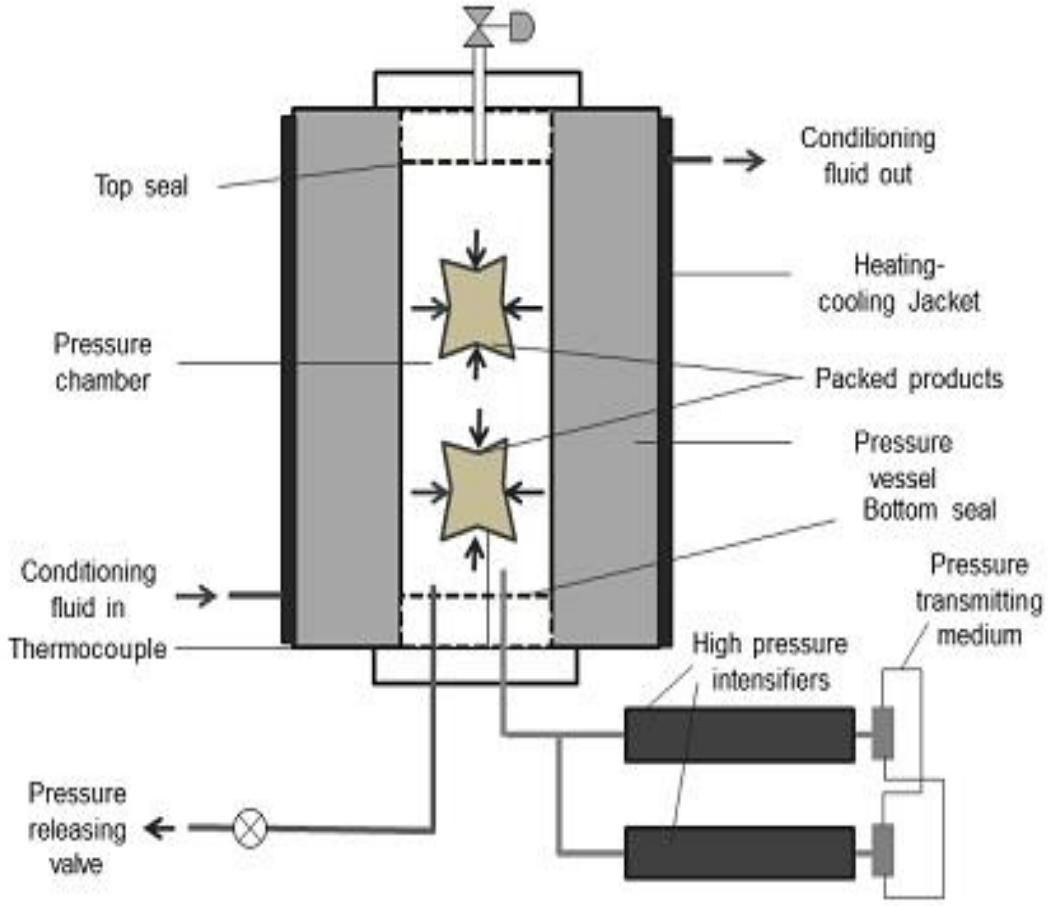

Figure 1.17: Schematic of high pressure processing (Chakraborty & Rao, 2015)

The behavior of foods under effects of high pressure can be explained with the general principles that are the Le Chatelier, Isostatic pressing, and microscopic-ordering principles. Le Chatelier's Principle states that a chemical system under equilibrium condition and a reaction change, accompanied by a decrease in volume when enhanced by pressure is experienced. Secondly, in the case of isostatic pressing (Pascal's

Principle), the pressure is transmitted in a uniform manner in all directions. Following the decompression, the material returns to its initial shape. Lastly, microscopic ordering principle emphasize that increase in the pressure mutually has increasing effect of the degree of ordering of the molecules of a substance at a constant temperature. As a result, pressure, as well as temperature, exert antagonistic forces on molecular structure (Elamin et al., 2015).

1.7 Characterization Tools for Enzymes and Substrates

Several methods are available for determination of some characteristic properties (physical or chemical) of enzymes and substrates. These methods can be divided depending on the properties that are thermostability, intermolecular or molecular interactions, linkages between molecules and structural adjustment.

1.7.1 Differential Scanning Calorimetry (DSC)

Calorimetry is an important technique in order to determine the thermal properties of materials for establishing a relation between temperature and specific physical properties of substances. The technique is the only method to determine the enthalpy associated with the process of interest directly. Differential scanning calorimetry (DSC) is one of the thermo-analytical techniques. This technique is the most widely used amongst various types of calorimeters (Gill, Moghadam, & Ranjbar, 2010; Kodre, Attarde, Yendhe, Patil, & Barge, 2014). In addition, DSC is a very relevant tool in order to investigate the thermodynamic properties of various products, such as, biopolymers, proteins, peptides, and lipid carriers (Chiu & Prenner, 2011). DSC, a thermal analysis apparatus, measures how physical properties of a sample change, along with temperature against time. When temperature changes, DSC measures a heat quantity that the samples radiate or absorb excessively on the basis of a temperature difference between the sample and the reference material (typically an empty sample pan) (Gill et al., 2010).

The DSC is particularly used to monitor the changes of phase transitions. When a sample exposed to a physical transformation, heat will need to flow to it than to the reference to maintain both at the same temperature. Heat flow between the sample and reference material depends on whether the process is exothermic or endothermic (Chiu & Prenner, 2011; Gill et al., 2010). For example, a solid sample melts to a liquid phase and this process require more heat flowing to the sample. It is needed in order to increase its temperature at the same rate as the reference. This situation is due to the absorption of heat by the sample as it undergoes the endothermic phase transition from solid to liquid. Likewise, as the sample passes through exothermic processes such as crystallization, less heat is required to raise the sample temperature. DSC can measure the amount of heat absorbed or released during such transition, due to the difference between heat flows of the sample and reference (Iwasa, 2011).

DSC measurements can estimate the free energy of enzyme denaturation. Denaturation of enzyme may not be reversible generally; the denaturation transition may be too broad or fairly sharp (Bhambhani & Kumar, 2008). Both denaturation and degradation of enzymes at high temperatures are important to be understand the inter-relationship of with each other and with enzyme activity and degradation is likely to play a major role in the loss of enzyme activity. In addition, denaturation is defined as loss of tertiary (and often secondary) protein structure not involving covalent bond cleavage and being reversible while degradation is the loss of primary structure with associated covalent bond cleavage. Degradation refers to irreversible changes (Daniel, Dines, & Petach, 1996). The tertiary structures of enzymes, which are vital for their physiological functions, are related to amino-acid sequences and stabilized by thermodynamic rules. For hyper thermostable enzymes, thermodynamics of irreversible protein denaturation can be expected at temperatures over 100 °C (Matsuura et al., 2015).

1.7.2 Fourier Transform Infrared (FTIR) Spectrometer

FTIR spectrometers (Fourier Transform Infrared Spectrometer) are widely used in organic synthesis, polymer science, petrochemical engineering, pharmaceutical industry and food analysis. In addition, the mechanism of chemical reactions and the detection of unstable substances can be investigated with FTIR instruments.

The range of infrared region is 12800 ~ 10 $cm^{-1}$and can be divided into three parts that are near-infrared region (12800 ~ 4000 cm^{-1}), mid-infrared region (4000 ~ 200 cm^{-1}) and far-infrared region (50 ~ 1000 cm^{-1}). Infrared absorption spectroscopy is the method in order to determine the structures of molecules with the molecules' characteristic absorption of infrared radiation. Infrared spectrum refers to molecular vibrational spectrum. When exposed to infrared radiation, sample molecules selectively absorb radiation of specific wavelengths. It leads to causes the change of dipole moment of sample molecules. The vibrational energy levels of sample molecules transfer from ground state to excited state. The frequency of the absorption peak is determined by the vibrational energy gap. As a result, the number of absorption peaks is linked to the number of vibrational freedom of the molecule. The intensity of absorption peaks is related to the change of dipole moment and the possibility of the transition of energy levels (How an FTIR Spectrometer Operates, 2015) (Figure 1.18).

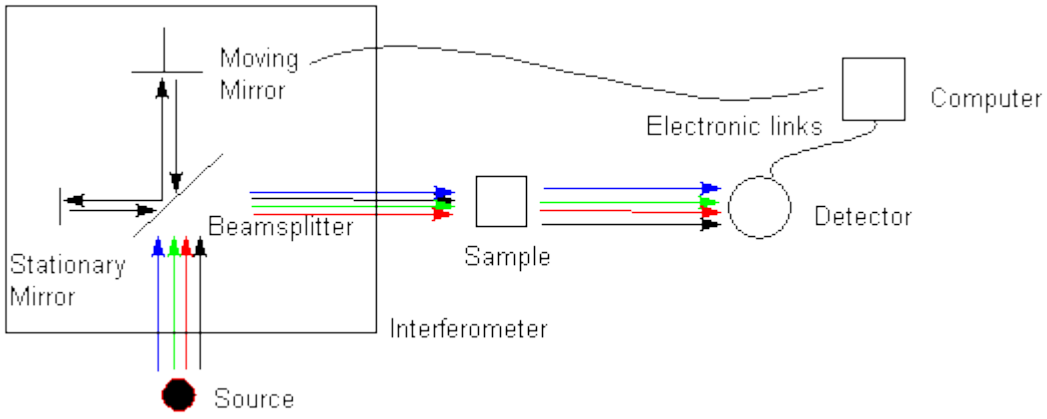

Figure 1.18: Working mechanism of FTIR spectroscopy

Like all materials, lignocellulosic biomass can be investigated by using FTIR spectroscopy to determine structural changes after different applications. Within the range of 1800 to 800 cm-1, cellulose, hemicelluloses, and lignin have their own characteristic absorption peaks (Cheng, Huang, Wang, & Zhang, 2016). In Figure 1.19, these peaks are illustrated with FTIR spectra.

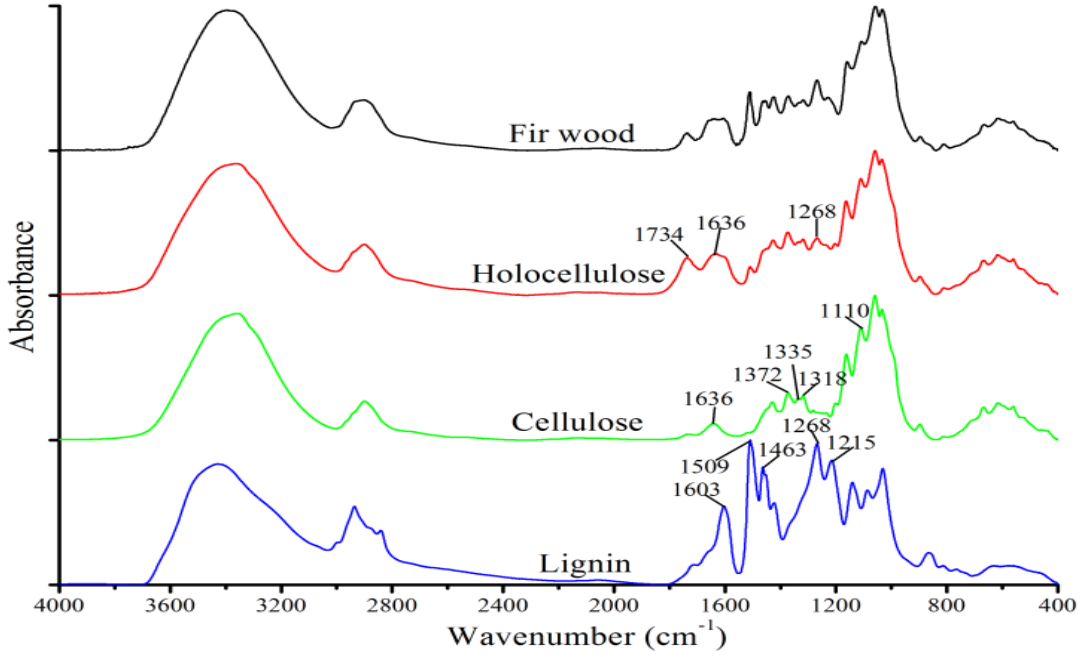

Figure 1.19: FTIR spectra of lignocellulosic material and some contents of it (Cheng et al., 2016)

1.7.3 Nuclear Magnetic Resonance (NMR) Relaxometry

Nuclear Magnetic Resonance (NMR) technique is an analytical chemistry technique used in quality control and research for determining the content and purity of a sample as well as its molecular structure. NMR can be used to determine molecular conformation in solution as well as studying physical properties at the molecular level such as conformational exchange, phase changes, solubility, and diffusion. In order to achieve the desired results, a variety of NMR techniques are available (Huntley, Crews, & Curry, 2015).

The technology and the method's promising results have increased the popularity of low field benchtop H^1 Nuclear Magnetic Resonance devices that can give invaluable information on samples via analysis of magnetic relaxation times, longitudinal relaxation time T_1 and Transverse relaxation time T_2, which are intrinsic properties of substances (Kirtil et al., 2017).

Mobility and distribution of protons can give information about the internal structure of a sample. T_2 is described as spin-spin relaxation time. It gives information about the relaxation and mobility of hydrogen molecules. Short spin-spin relaxation times are attributed to the hydrogen nuclei in an immobile structure while long spin-spin relaxation times belong to hydrogen nuclei in mobile structures (Karuna et al., 2014; Ozel, Uguz, Kilercioglu, Grunin, & Oztop, 2016) (Figure 1.120). The tight bonding and small compartments shorten the spin–spin relaxation time of hydrogen nuclei (Felby, Thygesen, Kristensen, Jørgensen, & Elder, 2008).

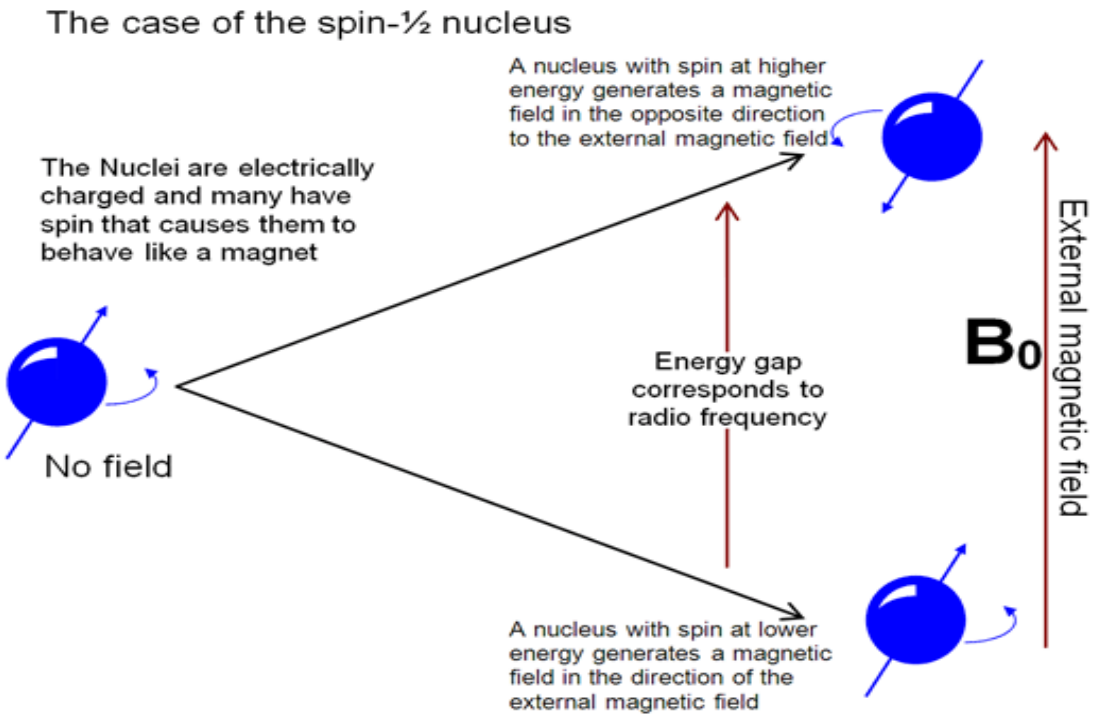

Figure 1.20: Working mechanism of external magnetic field in NMR spectrometer (Image taken from http://chem.ch.huji.ac.il/nmr/whatisnmr/whatisnmr.html)

The atomic nucleus is a spinning charged particle and so it generates a magnetic field. When an external applied magnetic field does not conduct, the nuclear spins are random and spin in random directions. However, when an external magnetic field is present, the nuclei align themselves either with or against the field of the external magnet and they start to spin at the frequency that is proportional to magnetic field strength. A radio frequency (RF) is applied and the protons come down the lowest energy state when RF is turned off. As a result, the relaxation signal is obtained with this way (Akoka, Franconi, Seguin, & Le Pape, 1993; Huntley et al., 2015; Karuna et al., 2014).

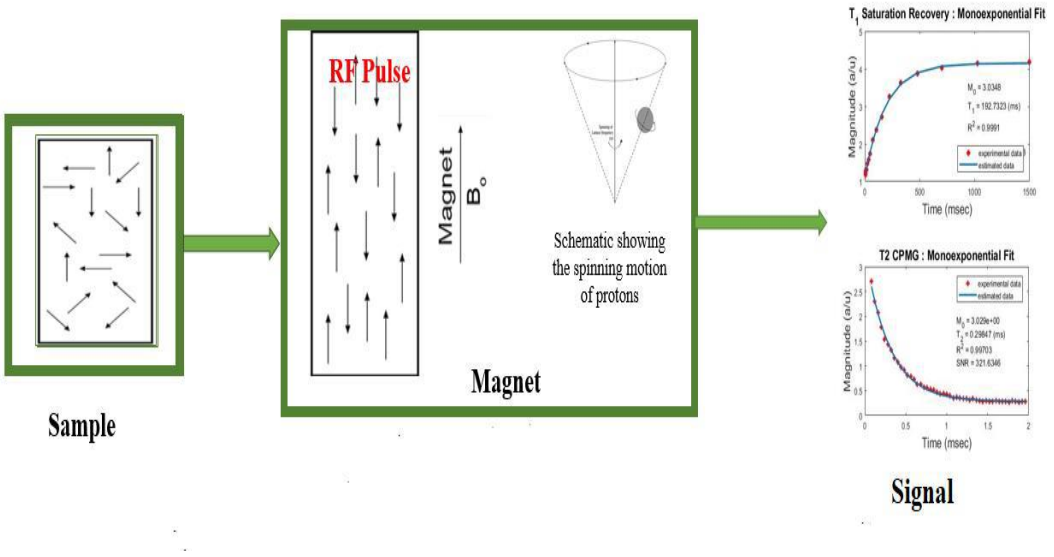

Figure 1.21: Schematic showing of NMR mechanism

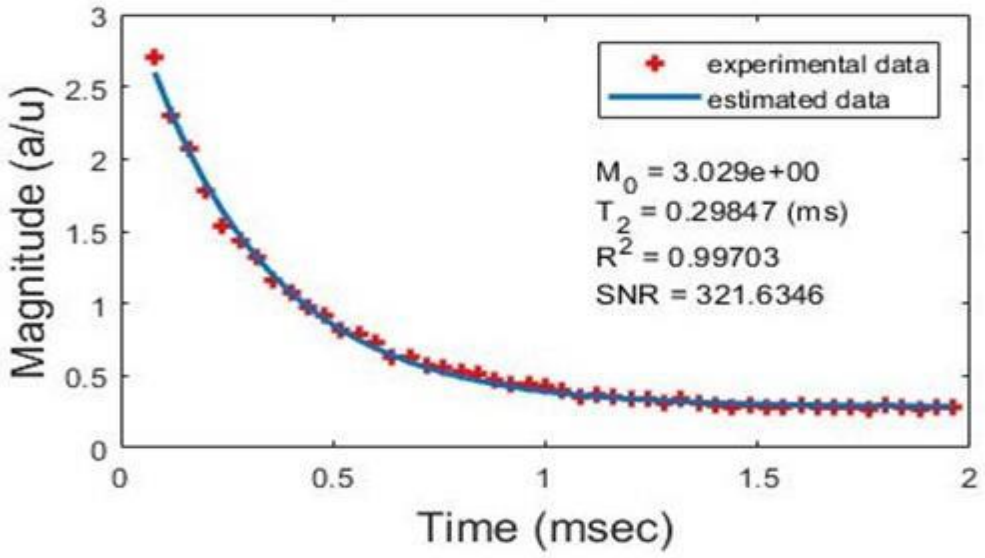

Figure 1.22: Representative T_2 relaxation curve

The rate differences of decay of the proton signal refer to differences in chemical and physical interactions of water in the samples. The processed T_2 spectra from the decay curves yield characteristic T_2 relaxation times and peak amplitudes. All these parameters give some information about the contributions of the biomass surface chemical and physical properties.

Dourado et al. (1999) suggested that cellulase treatment of cellulose increases the water holding capacity. The longer relaxation time can be attained with the structure loosening at the earliest point of cellulose breakdown. Thus, the initial action of the endo-glucanase break into pieces the cellulose chains and may also reveal water into the cellulose structure by the formation of cavities and micro pores (Felby et al., 2008). Additionally, increasing in water-accessible specific surface area of the biomass due to the alkali pretreatment is also increase the substrate porosity. Substrates with smaller pores lead rapid decay of the water proton signal associated with shorter T_2 relaxation time, while substrates with larger pores resulted in slower decay of the water proton signal and longer T_2 relaxation time cab be obtained (Karuna et al., 2014). Moreover, an increase in the amount of water at the biomass surface may also indicate an increase in the hydrophilicity of the surface and also longer relaxation time.

1.8 Aim of the Study

This comparative study investigates the effects of different HHP parameters (100-500 MPa at 30°C for 5-15 min) on cellulose-hemicellulase activity and on enzymatic hydrolysis (incubation with 150 rpm at 50°C for 24 hours) of microcrystalline cellulose (Avicel) and lignocellulosic biomass at different conditions. Effect of alkaline pretreatment and different enzyme loadings (50, 75,100,125µl enzyme amount) on hydrolysis was examined. In addition, effect of HHP application (100-500 MPa at 30°C for 5-15 min) on Celluclast, microcrystalline cellulose and peanut hulls was observed. The hydrolysis efficiency was determined by using DNS (Dinitrosalicylic acid) method. Changes in physicochemical properties of enzyme mixture and biomass were interpreted by using Nuclear Magnetic Resonance (NMR) Relaxometry and Fourier transform infrared (FTIR) spectroscopy.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Peanut hulls were supplied as whole raw peanut from local markets in Ankara (Unal Kuruyemis, 2018). Hulls and peanuts were separated from each other by hand and peanut hulls were milled to obtain proper particle sample size (10µm-2mm) by using Laboratory mill (Philadelphia, USA) before hydrolysis and alkaline pretreatment.

3-5 Dinitrosalicylic acid, phenol, Rochelle salt (Sodium potassium tartrate), Avicel were purchased from Sigma-Aldrich (St. Lois, MO, USA). Tri-sodium citrate dehydrate, citric acid monohydrate, sodium sulfate, sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany).

Cellulase as enzyme, prepared from *Trichoderma reesei* (Celluclast 1.5L), was kindly requested from Novozyme (Bagsvaerd/Copenhagen, Denmark).

2.2 Experimental Design

The sample of Cellucalst 1.5L was prepared and DSC analysis was conducted to determine denaturation temperature. Three different solutions were prepared with enzyme, enzyme-avicel, enzyme-pretreated peanut pull solutions. HHP treatment conditions were selected according to the results of preliminary works. 30°C was selected as HHP temperature for pressure application on three different solutions based on literature findings (Albuquerque et al., 2016; Murao et al., 1992). Pressure levels

were selected to be one low and one high level as 100 and 500 MPa. Pressure application time was arranged as 5 and 15 min. For peanut hull samples, NMR and FTIR analysis were done in duplicate. Applied independent variables are given at Table 2.1.

Table 2.1: Independent variables of the study

HHP-treated Solution	Pressure (MPa)	Time (min)
Enzyme Solution	100	5
Enzyme-avicel Solution	500	15
Enzyme-Pretreated Peanut Hull Solution		

2.3 Alkaline Pretreatment

Alkaline pretreatment was applied to milled peanut hulls in order to obtain efficient hydrolysis of cellulose due to high lignin content. Removing lignin compounds of peanut hulls specifically was aimed for of alkaline pretreatment. The revised procedure of Bahcegul et al. (2011) for the alkaline pretreatment was carried out. 1L of 2% NaOH (w/v) solution was prepared for the first step of pretreatment. The autoclaving step at 121˚C for 1 hour is applied to a hundred grams of milled peanut hulls by using prepared 2% NaOH(w/v). In further steps of the pretreatment, the autoclaved solution was cooled for filtration using ice bath or cold water. To remove the solid particles from the suspension, the solution was filtered by using a filter paper. Washing steps were applied to filtered solid part with 200 ml distilled water three times. Between each washing steps, the solid particles were filtered with filter paper. Filtered powder peanut hulls were put into a beaker after three washing steps and 200 ml of distilled water was added. pH of the suspension was adjusted to approximately 4.8 with acetic acid. After

pH adjustment step, the solid particles were rinsed with distilled water and filtered again. The obtained biomass was dried in an incubator at 60˚C overnight. Finally, the remaining solid part was weighed. After alkaline pretreatment, the percentage of the biomass recovery was calculated as follows (Bahcegul et al., 2011);

$$Biomass\ recovery\ \% = \frac{W_{PT}}{W_{UT}} x100 \tag{2.1}$$

W_{PT} : The weight of the biomass recovered after alkaline pretreatment
W_{UT}: The weight of the untreated biomass

Non-alkaline-pretreated peanut hulls (milled) and alkaline pretreated peanut hulls were shown in Appendix E.

2.4 Enzymatic Hydrolysis of Avicel and Peanut Hulls

Enzymatic hydrolysis was performed in 25 ml of 0.05 M sodium citrate buffer (pH of 4.8) by using a shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland). The conditions that are 50˚C, 150 rpm and 24 hours were applied for incubation. According to Pryor and Nahar (2015), 24-hour was suitable for the observation of hydrolysis. Therefore, 24 hours (1 day) period for enzymatic hydrolysis was considered. The suitable enzyme loadings for hydrolysis were obtained by some preliminary hydrolysis trials with different enzyme amounts. These trials will be explained later. With the chosen enzyme amounts, 0.9 g of biomass was hydrolyzed under determined conditions by using cellulose (Celluclast 1.5L) (Bahcegul et al., 2011). The amount of biomass was kept constant as 0.9 g to investigate the effect of the enzyme loadings on hydrolysis.

Same procedure of enzymatic hydrolysis was applied for all Avicel and untreated and pretreated peanut hulls.

$$Conversion\ of\ glucose\ (\%) = \frac{C_G\ x\ V}{W_B}\ x\ 100$$

(2.2)

$$Glucose\ yield\ (\%) = SR\ x\ \frac{C_G}{100}$$

(2.3)

According to Bahcegul et al. (2011), the conversion of biomass to reducing sugar amounts and their yield is defined as the amount of glucose obtained from the initial amount of peanut hulls on dry weight basis upon pretreatment and subsequent enzymatic hydrolysis and were also calculated via the equations above. Pretreatment step is applied to samples, second formula should be used for calculation. CG is the glucose concentration in the enzymatic hydrolysate, V is the volume of the enzymatic hydrolysis system, WB is the weight of biomass subjected to enzymatic hydrolysis, SR is the solid recovery (%) and CG is the conversion to glucose (%).

2.5 Determination of Enzyme Loading

To determine the enzyme loading, hydrolysis trials were first carried out at ten enzyme concentrations between 50µl and 500µl in 25 ml citrate buffer by using both Avicel and milled peanut hulls (0.9g) as substrates. However, the further increase in the enzyme loadings did not cause any significant improvement in terms of the enzymatic hydrolysis (Bahcegul et al., 2011). In other words, more enzyme amounts do not have increasing effect on the hydrolysis efficiency and the final glucose amount. In the light of this information and our trials, to avoid the effect of the excess enzyme concentration, only four (50, 75, 100 and 125µl) of the enzyme amounts below 150µl were decided to be used rather than 150µl and more. These preliminary trials were conducted in duplicates. The results of these trials will be explained in further sections.

2.6 High Hydrostatic Pressure (HHP) Application

HHP applications were performed in a designed and constructed laboratory-scale unit, SITEC-Sieber Engineering AG, Zurich, Switzerland (type 760.0118), and experiments was carried out in Food Engineering Department, METU. The vessel had a volume of 100 ml with internal diameter (ID) 24 mm and length 153 mm (Figure 2.1). A built- in heating-cooling system (Huber Circulation Thermostat, Offenburg, Germany) was used to maintain and control required temperature, which is measured by a thermocouple type K in the vessel. The vessel was filled with a pressure transmitting medium consisting of distilled water. Pressurization rate was 75 MPa/min for 100 MPa and 300 MPa/min for 500 MPa. Pressure release times were less than 20s.

Figure 2.1: HHP equipment (SITEC-Sieber Engineering AG, Zurich, Switzerland)

Samples were prepared according to enzymatic hydrolysis conditions; however, some preparational differences of sample were conducted for HHP application. While applying HHP, two different sample preparation methods were followed for each type of substrate that are Avicel, peanut hulls (only milled) and pretreated peanut hulls. These differences arose from the application of high pressure at different stages of the hydrolysis and to different solutions that contain at least one of citrate buffer, enzyme and substrate.

One of HHP application method was performed by applying pressure only to the enzyme and citrate buffer solution. Enzyme-citrate buffer solution samples were prepared in 25ml polyethylene bottles by using four different enzyme concentration and citrate buffer. However, it is very important that there is no air bubble when filling the bottles. First of all, a quantity of citrate buffer solution was put in the plastic bottles. The above-mentioned amounts of enzyme that were obtained from the experiments were added to the bottles and the bottles were shaken gently without agitating to mix enzyme into buffer. Bottles and caps were filled with citrate buffer and closed to prevent air bubbles as much as possible. Prepared sample bottles were placed into the pressure vessel and the lid of machinery was closed. Intensifier was operated and desired pressures, 100 - 500 MPa, were obtained. After that, the valve of the vessel was opened and the processing pressure was obtained to apply to the samples for the desired time (5-15min and temperature of 30°C). After the pressure application, the pressure was released and then the samples were taken. The sample bottles were uncapped and each pressurized enzyme-citrate buffer solutions were poured into separate beakers. Required amount of substrate (Avicel or peanut hulls or pretreated peanut hulls) for hydrolysis was put into each beaker. Samples were placed in the incubator and enzymatic hydrolysis was conducted with the afore mentioned conditions.

Second method was performed by applying pressure just before enzymatic hydrolysis; in other words, high hydrostatic pressure was applied to final solution that contained enzyme, citrate buffer and substrate. Like the first method, a fair amount of citrate buffer solution was put in the plastic bottles and mentioned stated enzyme amounts were added to the bottles. The bottles were shaken gently. After that, required amount of substrate (Avicel or peanut hulls or pretreated peanut hulls) for hydrolysis was put, at this stage, into bottles containing enzyme-citrate buffer solution. The bottles were shaken gently again to mix whole substrate with solution. Bottles and caps were filled with citrate buffer and closed to prevent air bubbles. Prepared sample bottles were placed into the pressure vessel and lid of machinery was closed. Desired pressure, 100 - 500 MPa, was applied for the desired time (5-15min and temperature of 30°C) by following the same method. After the pressure application, the sample bottles were uncapped and each pressurized enzyme-citrate buffer-substrate solutions were poured into separate beakers. Samples were placed in the incubator and then enzymatic hydrolysis was conducted with mentioned conditions.

2.7 Determination of Thermostability of Celluclast

Differential scanning calorimetry (DSC) method was used to determine the thermostability and denaturation temperature of Celluclast (enzymes). For DSC measurements, Perkin Elmer DSC 4000 System and required equipment were used in Food Engineering Department, METU. Samples were prepared only with Celluclast 1.5L. Approximately 5 mg of Celluclast samples were put into DSC pans. The pans were sealed tightly with crimper press. To use as reference pan, an empty DSC pan was sealed. When starting the DSC measurements, initial temperature was arranged at 40°C. Measurements were conducted by heating from 40°C to 280°C with 5°C/min heating rate. When reached to 280°C, measurements stopped automatically.

2.8 Determination of Reducing Sugar Content

DNS (Dinitrosalicylic acid) method (Miller,1959), one of the traditional methods for sugar analysis, was used to determine reducing sugar content. Firstly, DNS reagent was prepared by using sodium hydroxide, 3,5-dinitrosalicyclic acid, Rochelle salt (sodium potassium tartrate), sodium sulfate and phenol. In sample preparation, filtrated fluids of enzymatically hydrolyzed samples were diluted by using distilled water at a certain dilution ratio and samples in tubes were mixed by using vortex at 25°C and 150 rpm for 3 min approximately. DNS regents were added into each of these solutions as 1:1.5 v/v or ml/ml and solutions were mixed again. Mixed solutions were put into water bath at 90-100°C for 5-8 min. Desired color change (yellow color need to turn to orange or dark orange color) was obtained for all mixtures. Then they were put into ice bath or cold-water bath for 5 min to cool the mixtures and mixed with vortex again. After all preparation steps, absorbance measurements were carried out by using a UV Spectrophotometer (UV-1700 PharmaSpec Shimadzu, Japan) at 540 nm.

1g/L of glucose stock solution was prepared and diluted again at different concentrations using distilled water while obtaining the DNS calibration curve given in Appendix A. Diluted glucose stock solutions were mixed by using vortex. Like whole sample preparation procedure, DNS regents were added into each of these solutions as 1:1.5 v/v or ml/ml and solutions were mixed again. Mixtures were put in water bath at 90-100°C for 5-8 min and desired color change (orange color) was obtained. Then, mixtures were put into ice bath or cold-water bath for 5 min in order to cool and mixed again. By using a UV Spectrophotometer at 540 nm, absorbance values were measured. In the light of these results, absorbance versus concentration (concentration of diluted glucose stock solution) graph was obtained as calibration curve.

Reducing sugar content of hydrolyzed samples were calculated by using measured absorbance values and the equation of the prepared DNS calibration curve.

2.9 Determination of Moisture Content

Moisture content of samples that is needed for experiments was measured by using an infrared moisture analyzer (Radwag, MAC 50) in the Food Engineering Department, METU.

2.10 Nuclear Magnetic Resonance (NMR) Spectroscopy

Cellulose-water interactions were explored by measuring the T_2 relaxation times by using and NMR spectrometer. Samples to be measured consisted of control samples including untreated and alkaline pretreated peanut hulls, high-hydrostatic-pressure-treated hulls and hydrolyzed hulls with different enzyme concentration solution (75 and 125 μl enzyme amounts). All of these samples (whether or not any treatment was performed) were dried in incubator at low temperature (20-30°C) prior to NMR measurement. When NMR measurements was conducted, moisture content of sample were arranged at 70-76% by adding 2.5ml citrate buffer. Moist samples were prepared and then the relaxation times were measured.

Spin-spin relaxation time experiments (T_2) were carried out using a 0.5 T NMR spectrometer operating at a Larmor frequency of 23.2 MHz, equipped with a 10-mm diameter radio frequency coil (SpinCore Inc., Gainsville, FL, USA). Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to record relaxation data with 3.5 ms echo time, 750 echoes, 8 scans and 3s repetition time.

Figure 2.2: NMR relaxometer (SpinCore Inc., Gainsville, FL, USA)

2.11 Fourier Transform Infrared (FTIR) Spectrometer

Fourier Transform Infrared spectra of peanut hull samples were analyzed by IR Affinity-1 Spectrometer with Attenuated Total Reflectance (ATR) attachment (Shimadzu Corporation, Kyoto, Japan). The measurements were recorded in 4000 – 500 cm-1 with 4 cm-1 resolution and 32 scans. For each peanut hull sample, the measurements were replicated for three times. Samples to be measured consist of control samples including untreated and alkaline pretreated peanut hulls, high-hydrostatic-pressure-treated hulls and hydrolyzed hulls with different enzyme concentration solution (75 and 125 μl enzyme amounts). All samples were dried in incubator at low temperature (20-30°C) prior to FTIR analysis.

Figure 2.3: Fourier Transform Infrared (FTIR) Spectrometer (Shimadzu Corporation, Kyoto, Japan)

2.12 Statistical Analysis

In order to analyze the obtained results, Minitab 16 (Minitab Inc., Penn State, USA) was used. ANOVA analysis was carried out with Tukey multiple comparison test at 95% confidence interval to see the similarity or differences between the samples. All statistical data results were given in Appendix C.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Determination of Thermal Stability of Celluclast

The thermal denaturation of Celluclast 1.5L was conducted by using differential scanning calorimetry (DSC). According to the results shown in Figure 3.1, two small peaks and one huge peak were observed approximately at 70, 130 and 150°C, respectively and these peaks are critical temperatures for the enzyme mixture after DSC experiment was performed for the enzyme mixture Celluclast including different types of endo- and exo-glucosidases.

According to experimental work done by Dourado et al. (2002), the denaturation of Celluclast is nearly at 70°C when the experiment was conducted between 5-110°C with a heating rate 1 K min^{-1}. Additionally, Sichina (2000) reported that the protein denaturation transition was observed as a very small endothermic peak and in the range 65.9 and 82.5°C with peaks observed at 71.4 and 78.9°C when DSC experiments were performed 5 mg protein/mL of solutions. In our experiment, the peak at 70°C indicates the denaturation of some thermophilic protein structures of enzymes in cellulase mixture (Celluclast).

The peak approximately at 130°C indicates that thermostable proteins of enzymes may be in existence in the enzyme mixture Celluclast. β-glucosidase from *T. reesei* having an optimum temperature at 70°C and also its stability is maintained nearly up to 135°C (Murray et al., 2004). Enhanced thermal stabilities of enzymes might be allow the enzymatic reactions to be carried out at high temperatures.

In theoretical case, the high degree of chemical stability of the peptide bonds of enzymes would permit activities at temperatures > 150°C although most enzymes denature at moderate temperatures and unwind their secondary or tertiary structures (Bhambhani & Kumar, 2008). As a result, the huge peak at 150°C may refer to the breaking of peptide bonds between amino acids and the degradation of all chemical structures. Therefore, it was ensured that the temperature value to be applied (up to 50°C) in subsequent experiments had no adverse/denaturizing effect on the enzyme activity.

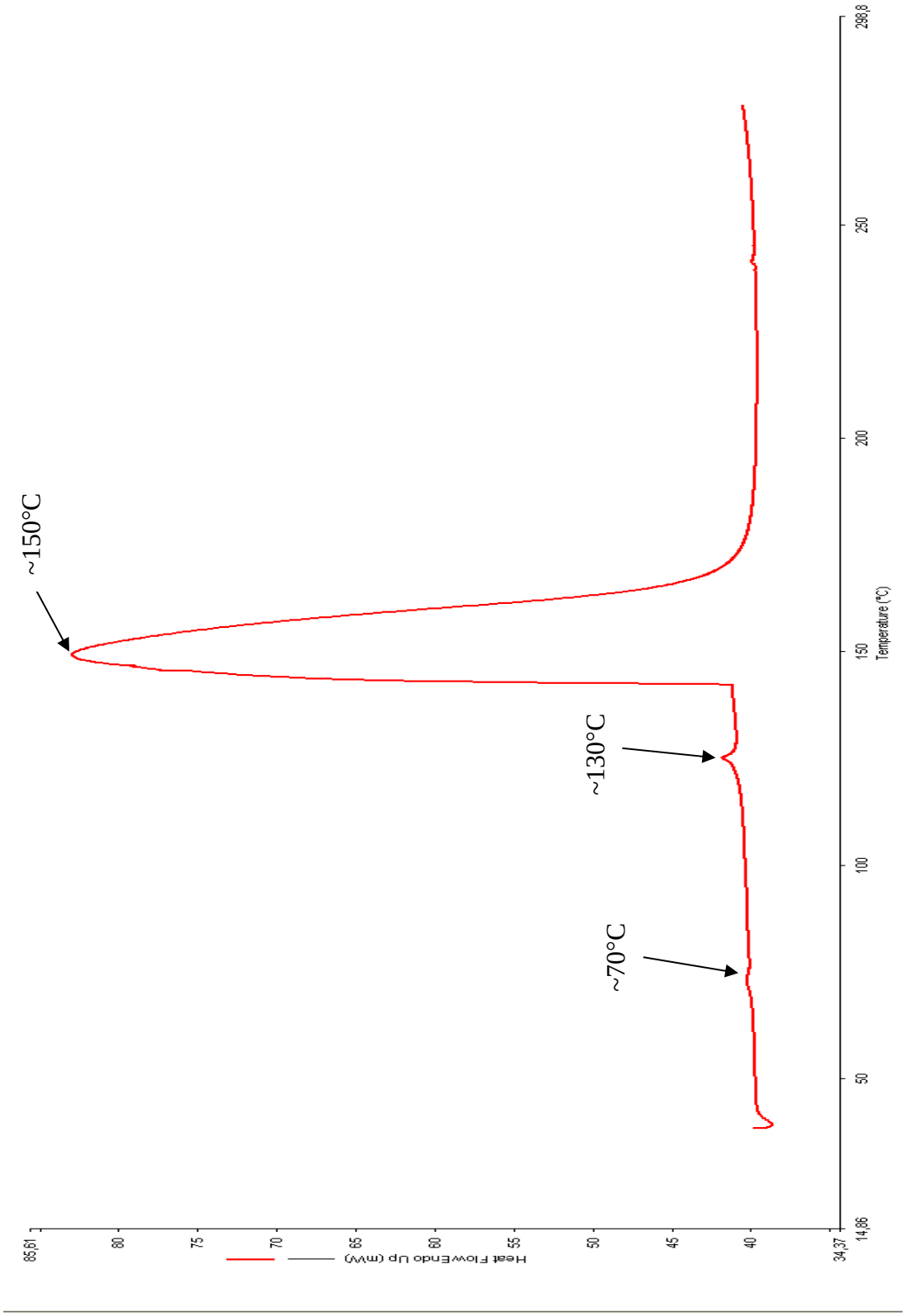

Figure 3.1: DSC thermogram of Celluclast 1.5L

3.2 Effect of Different Enzyme Loadings on Enzymatic Hydrolysis

Hydrolysis trials, using both Avicel and peanut hulls, with different concentrations of enzyme were shown in Figure 3.2 as two parallel sets. Numerical values of these results were given as tables in Appendix B. When the enzyme concentrations were used within a range of 50-500 µL, the reducing sugar amounts for Avicel were obtained as between 6.2 and 13.2 g/L and for peanut hulls as between 2.5 and 3.4 g/L. The amounts of reducing sugar produced by enzymatic hydrolysis did not show a constant increase.

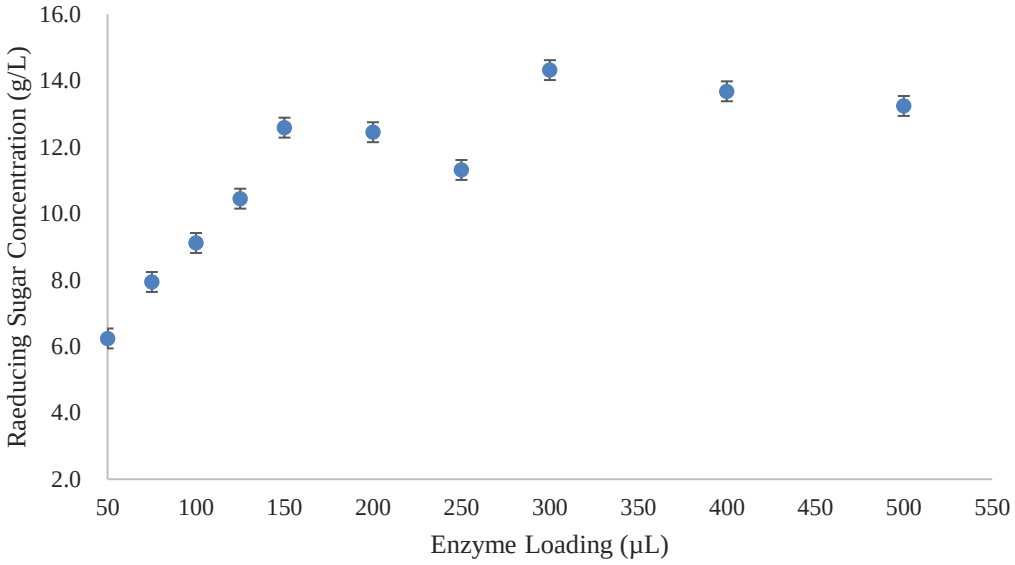

Figure 3.2: Total reducing sugar concentrations for Avicel hydrolysis

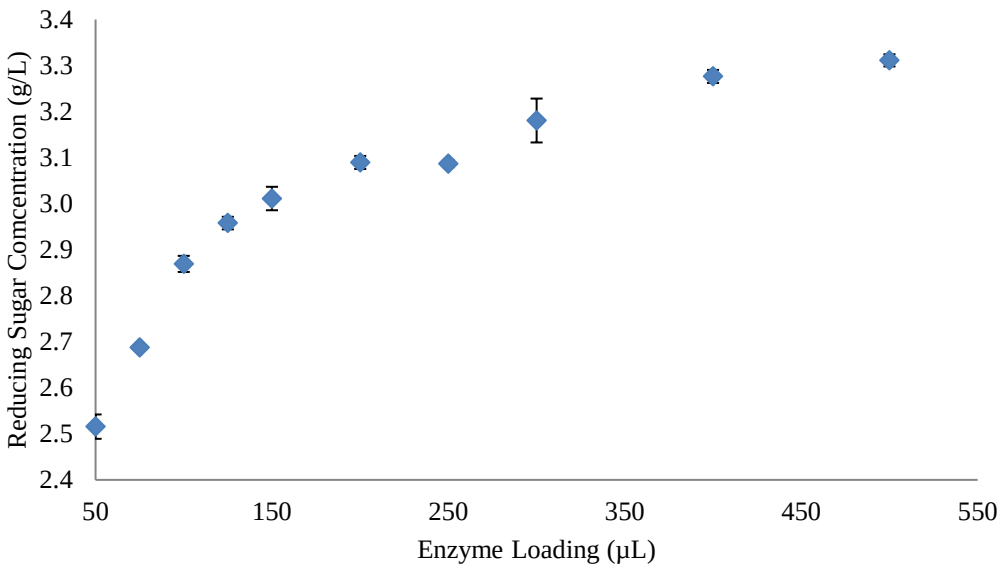

Figure 3.3: Total reducing sugar concentrations for peanut hulls hydrolysis

According to Martín et al. (2012), glucose concentrations increased when the enzyme loading also increased; however, if enzyme loadings were higher than 15 FPU/g, both increasing and decreasing results were obtained in terms of glucose concentration after hydrolysis. Similar results were obtained in our trials. While reducing sugar concentration after hydrolysis increased straightly up to 150 µL, approximately same or a slight increase of the values was observed (Fig 3.3).

The increase rate in the yields were decreased due to blocking excess enzyme adsorption on the biomass and by restricting the diffusion through the structure (Martín et al., 2012). Also, the oscillation after 150 µL was observed. These oscillations can be caused by limited glucose release. If cellobiase activity was effective, the increase in glucose release was expected. However, the cellobiose cannot be broken into glucose and the cellobiose inhibition could not be removed. In addition, the reason why the reducing sugar amount remains at a certain level may be the competition by active site of either glucose or cellobiose. The inhibition by glucose also reported for commercial enzymes in previous studies (Albuquerque et al., 2016; Andrie et al., 2010). Another reason can be the initially adsorbed enzyme on the fiber surface. It

forms just a single layer in such a way that the excess of enzyme would be adsorbed forming additional layers. This superficially adsorbed enzyme on the first layer of the fiber would play a significant role in the hydrolysis, but will also restrict the diffusion process of enzyme through the structure of the substrate, and most likely impact the effectiveness of further loaded enzymes (Martín et al., 2012).

In the light of these information, to avoid the deviations in the results, four of these enzyme concentrations below 150 µL (50, 75,100, and 125 µL) that give reliable straight increasing values was decided to be suitable for our study.

3.3 Effect of Alkaline Pretreatment on Enzymatic Hydrolysis

The reducing sugar concentrations at the end of hydrolysis by using untreated and alkaline pretreated peanut hulls were shown in Figure 3.4. When comparing to reducing sugar yields of alkaline-treated and untreated peanut hulls, reducing sugar yields of alkaline pretreated peanut hulls were significantly higher due to degradation of lignin and hemicellulose components ($p<0.05$). In addition, while reducing sugar yield is significantly increased according to increases in enzyme concentrations ($p<0.05$), interaction of reducing sugar yield and enzyme concentration was not significant ($p>0.05$). Therefore, the increase approximately 15% in yield values confirmed that hydrolysis efficiency could be increased clearly by alkaline pretreatment for all enzyme concentrations. For the case of untreated biomass, rigid crystalline structure obstructs the enzymatic hydrolysis and thus decreases the digestibility of enzyme. Yan et al. (2015) suggested that lignin content of lignocellulosic biomass allows maximum 10-15% of penetration of enzymes trough the cell wall. Increase of the accessibility of cellulose in the interior of the structure and increase of enzyme permeability of degraded lignocellulose leads an increase on the reducing sugar amount after hydrolysis (FitzPatrick et al., 2010). Cellulose and hemicellulose are easily degraded into reducing sugars that are glucose, xylose, arabinose, mannose and galactose (Rosgaard, 2007). Our results also confirmed this information about pretreatment as depicted in Figure 3.4.

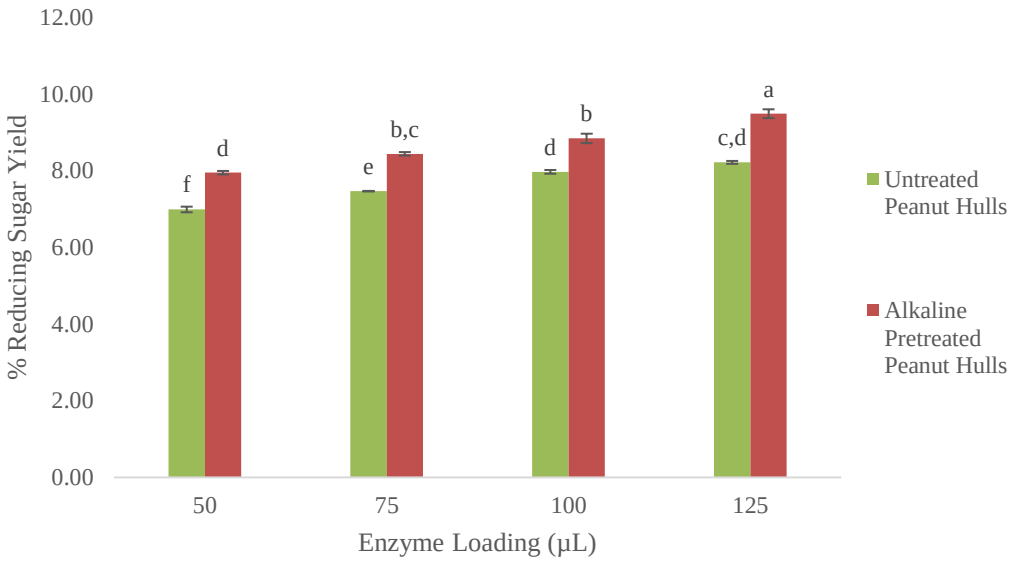

Figure 3.4: Reducing sugar yield percentages of untreated and alkaline pretreated peanut hulls. Different small letters represent significant differences ($p<0.05$)

3.4 High Hydrostatic Pressure (HHP) Application for Enzymatic Hydrolysis of Avicel

High hydrostatic pressure (HHP) were has an increasing effect on enzymatic hydrolysis. In Figure 3.5 & 3.6, the reducing sugar concentrations after hydrolysis using non-HHP-treated enzyme solution and HHP-treated enzyme solutions (100 and 500 MPa for 5 and 15 min) were shown. The reducing sugar concentrations obtained after hydrolysis with enzyme solutions that were exposed 100 and 500 MPa pressure for 5 and 15 minutes were compared with hydrolysis using non-high-pressure-treated enzyme solution (control sample).

Murao et al. (1992) reported that the cellulase and avicelase activities were not improved in their experiments although the avicelase activity of enzyme were expected about a 1.6-fold increase with high pressure treatment. In addition, Albuquerque et al. (2016) reported that cellulase activity was not increased when both substrate and cellulases were separately treated with HHP (300 MPa at 20 and 50°C) before the

hydrolysis reaction. On the contrary, hydrolysis experiments with HHP-treated enzyme solution (both 100 and 500 MPa for 5 and 15 min) yielded higher amounts of reducing sugar than hydrolysis with non-pressure-treated enzyme solution.

For the case of 5 min application, high pressure treatments (both 100 and 500 MPa) on enzyme solution provided significant improvement for hydrolysis of Avicel ($p<0.05$). On the other hand, no significant difference in hydrolysis yield was observed between 100 and 500 MPa applications ($p>0.05$). The reason of these enhancements may be the conformational changes, unfolding of enzymes, changes in protein solubility (Albuquerque et al., 2016). Pressure can unfold proteins and dissociate native oligomers with or without subsequent unfolding (Aertsen et al., 2009). According to Albuquerque et al. (2016) and Sousa & Parodi (1995), many disordered conformations (exposing more hydrophobic amino acids) of cellulase enhance to interact through hydrophobic part of cellulose. As a result, HHP has an effect as promoting hydrophobic property of cellulase and so cellulase activity can be improved by increasing the proximity of enzymes to their substrate (Sousa & Parodi, 1995). While approximately 17% increase was achieved by hydrolysis with high-hydrostatic-pressure-treated enzyme solution at 100MPa, 19% increase was obtained by 500 MPa application by comparison with hydrolysis using non-HHP-treated enzyme solutions. Therefore, changes were so close to each other for two magnitudes of HHP application.

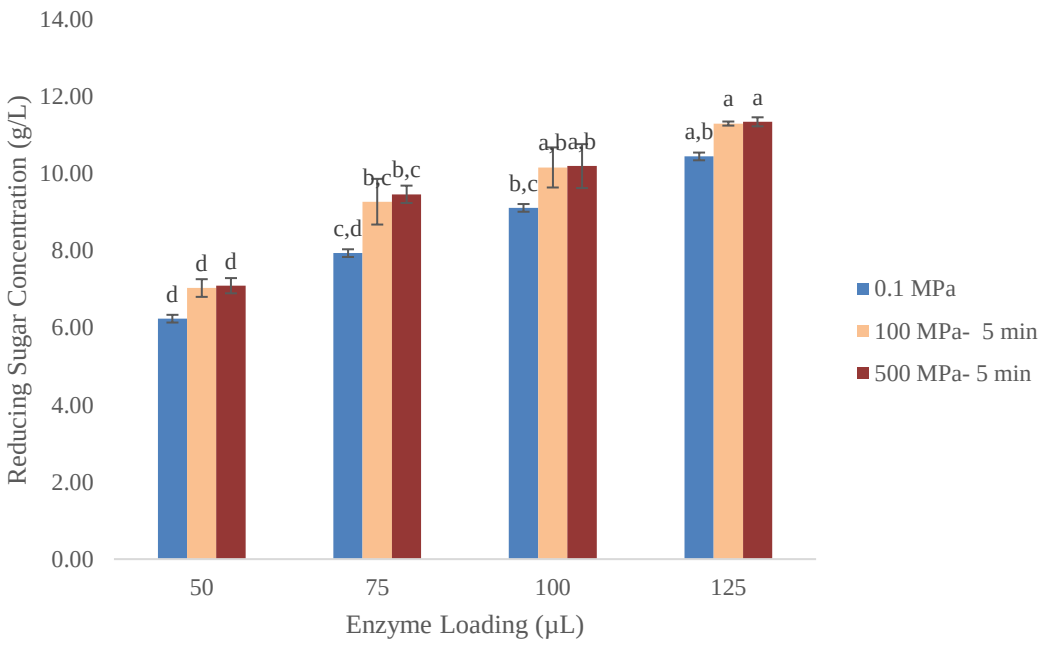

Figure 3.5: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with HHP-treated enzyme solution at 100 & 500 MPa for 5 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$).

Congruent with the results of 5-min HHP treatment, 15-min high pressure treatments (both 100 and 500 MPa) significantly increased reducing sugar amount obtained by hydrolysis of Avicel when compared with hydrolysis using non-HHP-treated enzyme solutions ($p<0.05$). Parallel with 5-min pressure application, the conformational changes enhanced hydrophobic property of cellulose-hemicellulase mixture by HHP led to increase Celluclast activity for hydrolysis with 15-min-HHP-treated enzyme solution. But, hydrolysis yields did not increase or change significantly with increase of applied pressure magnitude ($p>0.05$). By hydrolysis with 100 and 500 MPa HHP applied Celluclast solution, 27 and 23% increases were achieved respectively in comparison with hydrolysis using non-HHP-treated Celluclast solution.

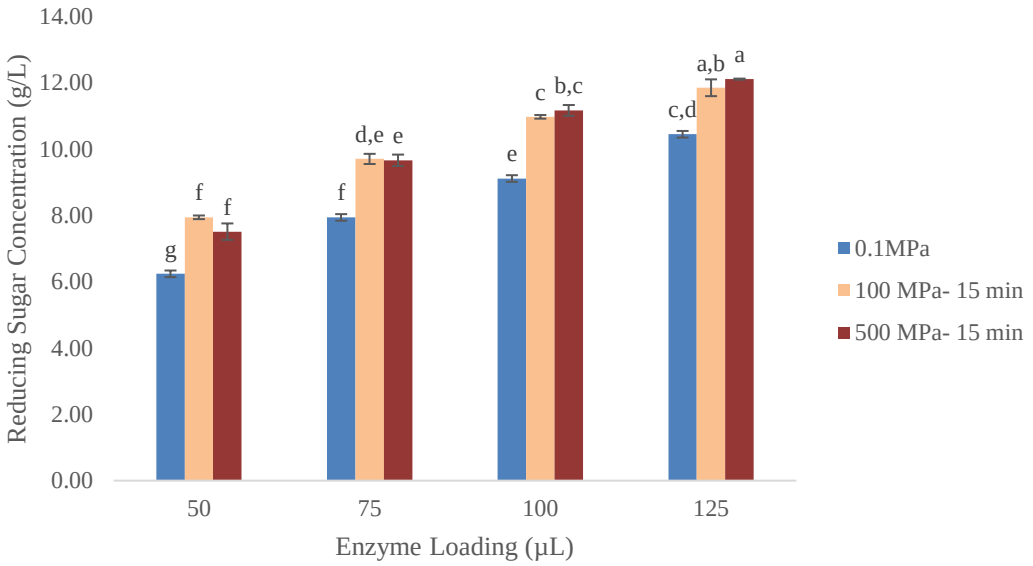

Figure 3.6: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with HHP-treated enzyme solution at 100 & 500 MPa for 15 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

In order to better examine the effect of the application time of the high hydrostatic pressure, the obtained results were re-grouped according to the applied pressure magnitude. In Figure 3.7, the reducing sugars belonging to hydrolysis with non-HHP-treated enzyme solution and 100 MPa-high-pressure-treated enzyme solution were illustrated. Rather than the level of the pressure, pressure duration was significantly effective in terms of enzyme activity ($p<0.05$). HHP-treated enzyme solution for 15 minutes gave relatively high reducing sugar yield than 5-minute treatment by comparison with non-pressure-treated control solution ($p<0.05$). 15-min HHP treatment provided additional 13% increase of yield compared to 5 min application for the case of 100 MPa pressure.

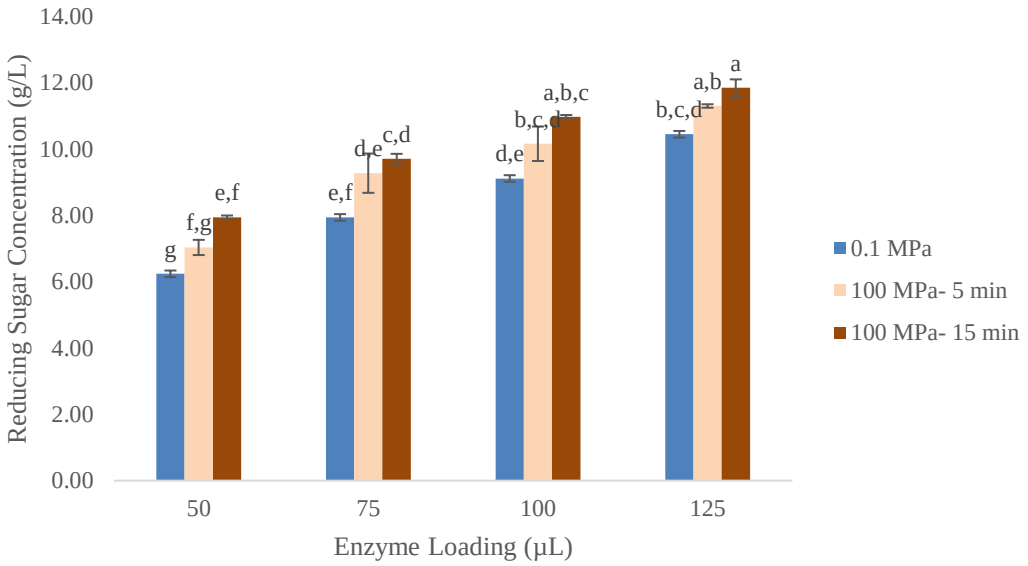

Figure 3.7: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with HHP-treated enzyme solution at 100 MPa for 5 and 15 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

Group of 500 MPa high hydrostatic pressure was also compared according to time values 5 and 15 minutes. As is the case with 100 MPa high pressure, 500 MPa HHP application had a similar effect. 15-min HHP treatment provided additional 10% increase of yield to 5 min application, approximately. With increasing HHP treatment time, reducing sugar amount obtained at the end of hydrolysis also increases significantly ($p<0.05$). Longer HHP treated enzyme solution generally gave more sugar yield as shown in Figure 3.8.

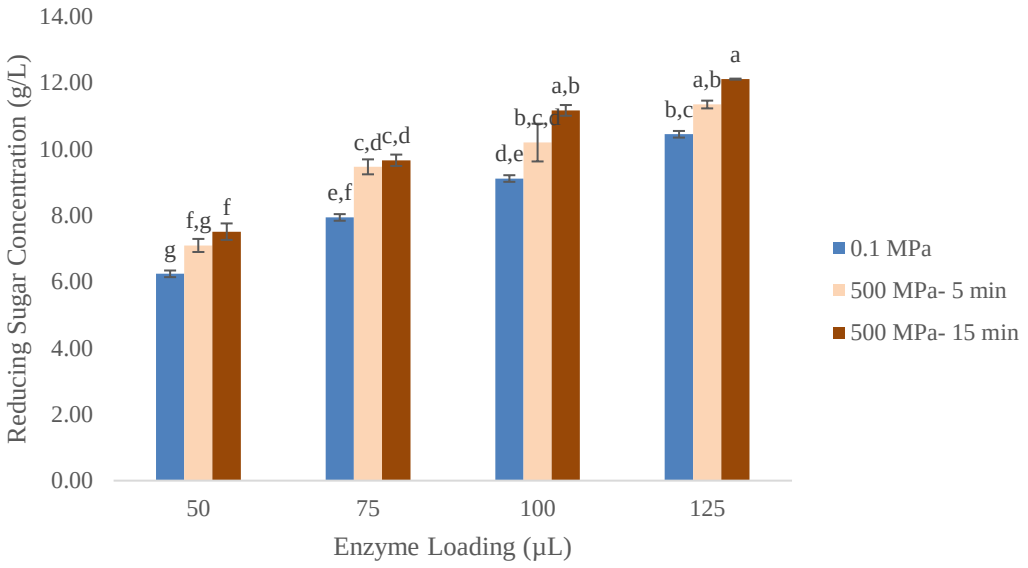

Figure 3.8: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with HHP-treated enzyme solution at 500 MPa for 5 and 15 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

Another investigation step of lignocellulose hydrolysis includes application of HHP treatment on enzyme and Avicel as substrate together at the same time. The improvement of enzymatic activity and efficiency of hydrolysis was generally observed in the case of HHP treatment on substrate and cellulase together at around 300MPa (Albuquerque et al., 2016). When cellulase was reacted with high pressure under proper condition for hydrolysis, Celluclast activity were generally enhanced. As shown in Figure 3.9, HHP treated solutions of enzyme and Avicel (100 and 500 MPa for 5 min) gave significantly higher reducing sugar amounts than the control solution that was not HHP-treated ($p<0.05$). Parallel with our previous results, effect of different pressure levels was not significant and increasing pressure did not improve the yields of Avicel hydrolysis ($p>0.05$). For both pressure levels, the reducing sugar yield increased with almost 37%. Therefore, our results of Avicel hydrolysis with HHP treated enzyme-avicel solution also confirmed the previous studies in literature reporting similar results (Albuquerque et al. (2016); Murao et al. (1992)).

Rupture and porous areas promoted by HHP leads to forming new hydrophobic parts of the lignocellulosic material which in turn increases the reducing sugar yield (Albuquerque et al., 2016). Additionally, Ding et al. (2012) emphasized the importance of hydrophobic parts of cellulose elementary fibers (CEF) for cellulose digestibility. Cellulase activity is enhanced due to the hydrophobic side of CEF being able to bind to the carbohydrate binding module (CBM) or to the hydrophobic amino acids of enzymes via hydrophobic interactions. Considering the conformational change(s) in the enzyme structure under HHP, highly satisfactory results in hydrolysis yield was observed. In addition, Albuquerque et al. (2016) reported a similar case where the amounts of reducing sugars and glucose released at atmospheric pressure were lower than observed at 300 MPa for the entire period of enzymatic hydrolysis of coconut husk. The increased glucose release demonstrates an increase of cellobiase activity under pressure. It is highly possible that the reducing sugars released (cellobiose) had been broken into glucose, which removes cellobiose inhibition and this effect was not observed at atmospheric pressure.

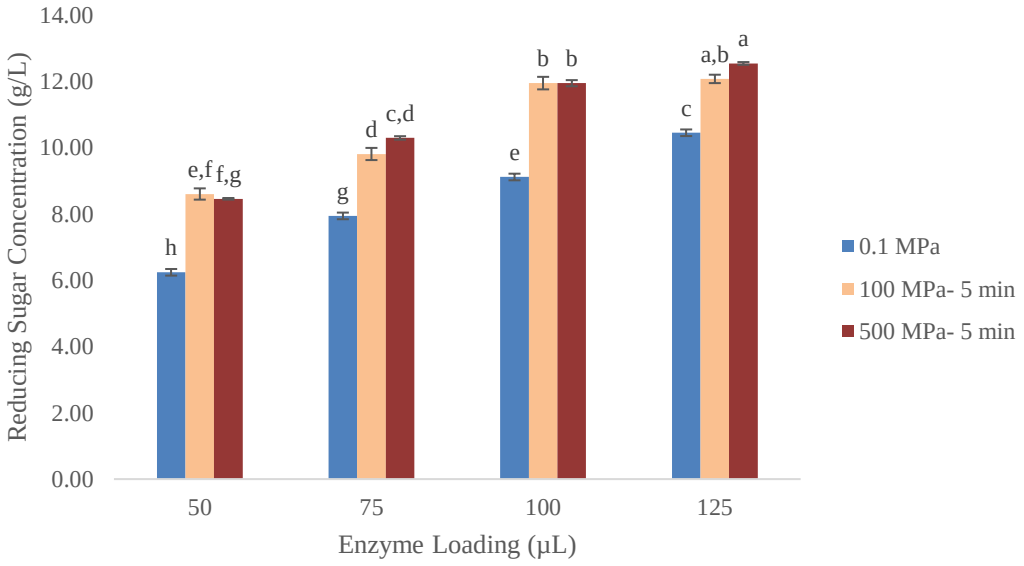

Figure 3.9: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with HHP-treated enzyme-avicel solution at 100 & 500 MPa for 5 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

HHP- treated (100 and 500 MPa for 5 minutes) enzyme solutions and enzyme-avicel solutions used in hydrolysis are compered in Figures 3.10 and 3.11. Both HHP treated solutions with different contents had improving effect on hydrolysis reaction by comparison with non-HHP-treated samples. However, a higher yield in reducing sugar content after hydrolysis by using HHP treated enzyme-avicel solution were obtained. Hydrolysis efficiency could be enhanced by approximately 22 % when enzyme-avicel solution was pressurized at 100MPa for 5 min ($p<0.05$) with regard to only pressure-treated enzyme solution. For the case of 500 MPa with same time period of application, 19 % increase was calculated.

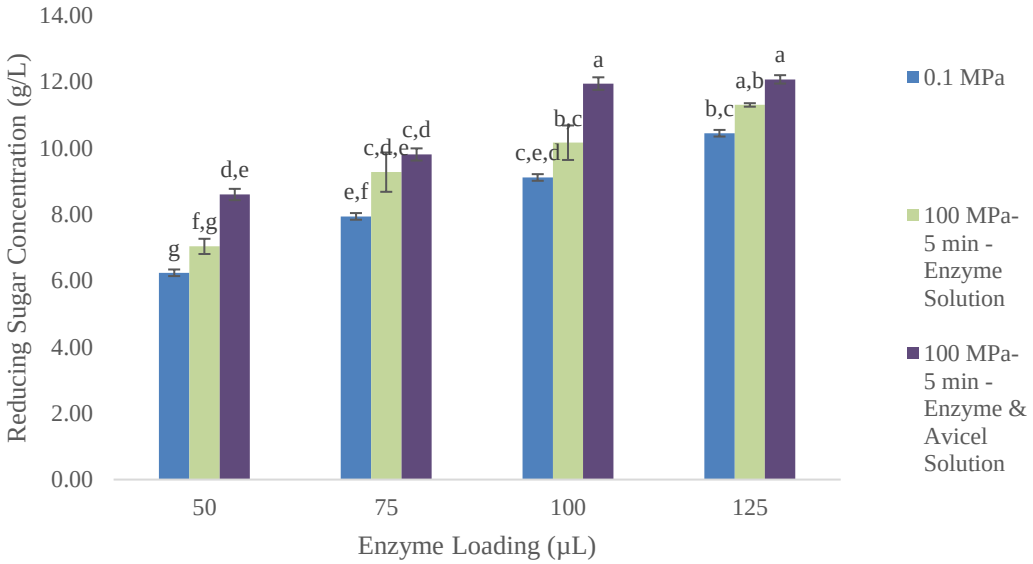

Figure 3.10: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with both HHP-treated enzyme solution and HHP-treated enzyme-avicel solution at 100 MPa for 5 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

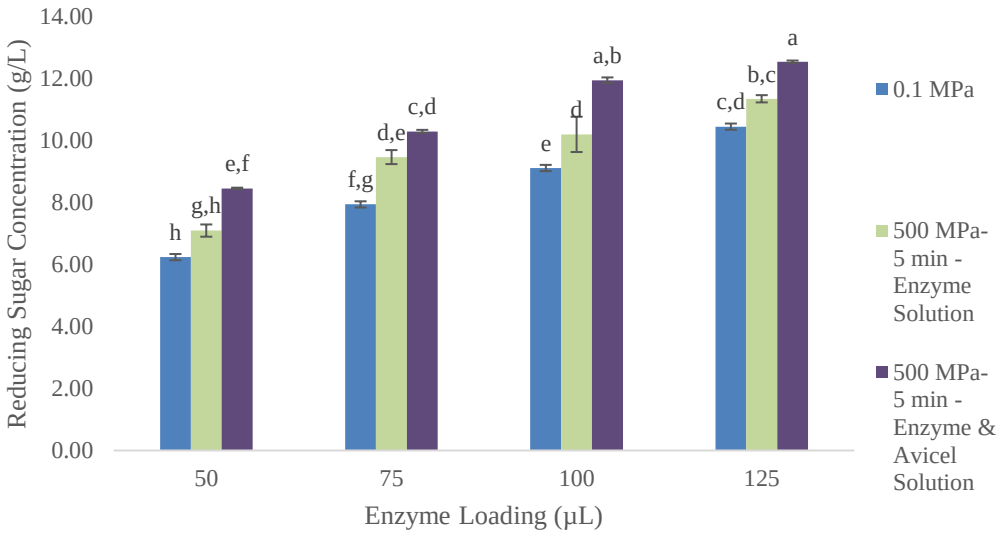

Figure 3.11: Reducing sugar concentrations: Enzymatic hydrolysis of Avicel with both HHP-treated enzyme solution and HHP-treated enzyme-avicel solution at 500 MPa for 5 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

The pressure of 100 MPa had the same effect of 500 MPa on improving the Celluclast activity. Thus, it was decided that it would be reasonable to use 500 for the peanut shell.

3.5 High Hydrostatic Pressure (HHP) Application for Enzymatic Hydrolysis of Pretreated Peanut Hulls

In parallel with Avicel results, there was significant differences between the reducing sugar concentrations of HHP-treated enzyme and HHP-treated enzyme-peanut hull solutions ($p<0.05$). While 5 min application of high hydrostatic pressure application led to the improvement with 10% in comparison with non-pressure-treated enzyme solution, 20% increase of hydrolysis efficiency was attained with 15-minute HHP operation. Figure 3.12 details these results. As mentioned above, promoted hydrophobic interactions by conformational changes and unfolding of enzyme, changes in protein solubility can explain the reasons of enzyme activation and

hydrolysis efficiency. In addition, ruptures and porous areas were observed after alkaline pretreatment. Combination of porosity and activated hydrophobicity of enzyme provided enhancing effect on enzymatic hydrolysis (Albuquerque et al., 2016; Sousa & Parodi, 1995).

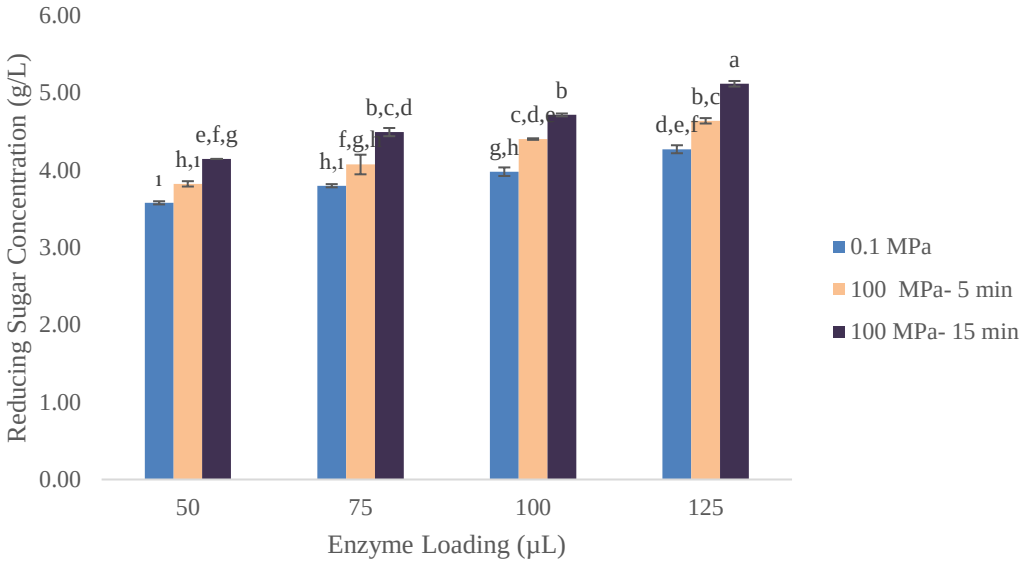

Figure 3.12: Reducing sugar concentrations: Enzymatic hydrolysis of pretreated peanut hulls with HHP-treated enzyme solution at 100 MPa for 5 and 15 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

For HHP-treated enzyme-peanut hull solutions, the reducing sugars contents at the end of hydrolysis significantly increased with longer treatment time ($p<0.005$). As illustrated in Figure 3.13, 20 % increase was obtained with 5-minute-HHP treatment of enzyme-peanut hull solution. Additionally, for 15 min application, the enhancement with 24% increase was observed.

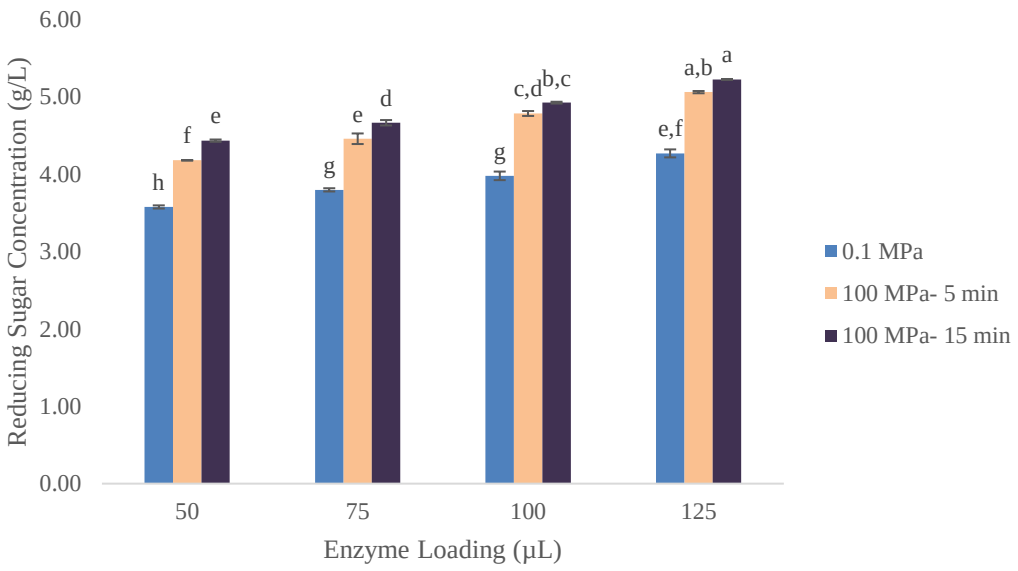

Figure 3.13: Reducing sugar concentrations: Enzymatic hydrolysis of pretreated peanut hulls with HHP-treated enzyme-peanut hull solution at 100 MPa for 5 and 15 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa).. Different small letters represent significant differences ($p<0.05$)

If the results obtained are to be looked from a different standpoint, the effects of high hydrostatic pressure-improved solutions on enzymatic hydrolysis should also be compared. Like in the case of Avicel hydrolysis, the pressurization of the enzyme and the substrate together had a significant positive effect compared to the pressurization of the enzyme solution. For both 5- and 15-min periods of times, 100 MPa treatment improved the activity of Celluclast and cellulose hydrolysis efficiency significantly ($p<0.05$). Bar graphs represented the mentioned results in Figure 3.14 & 3.15. In Figure 3.14, HHP-treated enzyme-peanut shell solution, which was observed to increase by 20 % in total, showed an approximately 9% extra increase in hydrolysis efficiency compared to HHP-treated enzyme solution for 5 min. Moreover, as represented in Figure 3.15, 6% additional improvement was gained due to hydrolysis by using high-pressure treated enzyme-peanut hull solution for 15 min when compared with hydrolysis with HHP-treated enzyme solution. Ruptures and porous areas were formed after a pressure treatment; as a result, high hydrostatic pressure can break

hydrogen bonds (Albuquerque et al., 2016). Pressure pretreatment also activates oxidation of the lignocellulose fibers. Furthermore, both ruptures and porous areas promoted by pressure exposed new hydrophobic parts of the lignocellulosic material, and so hydrolysis was increased. As mentioned above, that hydrophobic parts of cellulose elementary fibers (CEF) are important for cellulose digestibility in terms of being able to bind to the carbohydrate binding module (CBM) or to the hydrophobic amino acids of enzymes in order to enhance cellulase activity (Albuquerque et al., 2016; Ding et al., 2012).

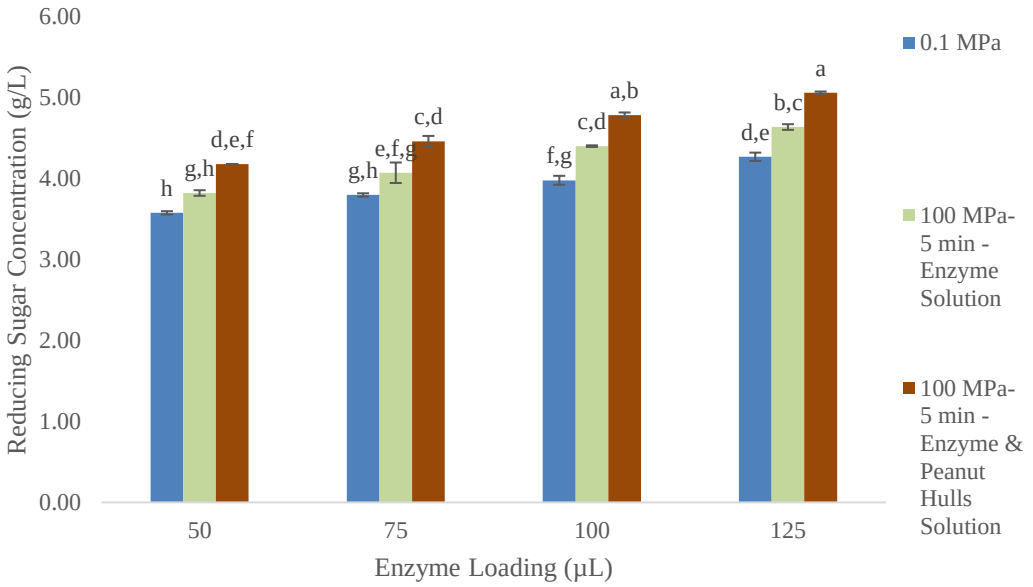

Figure 3.14: Reducing sugar concentrations: Enzymatic hydrolysis of pretreated peanut hulls with both HHP-treated enzyme solution and HHP-treated enzyme-peanut hull solution at 100 MPa for 5 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

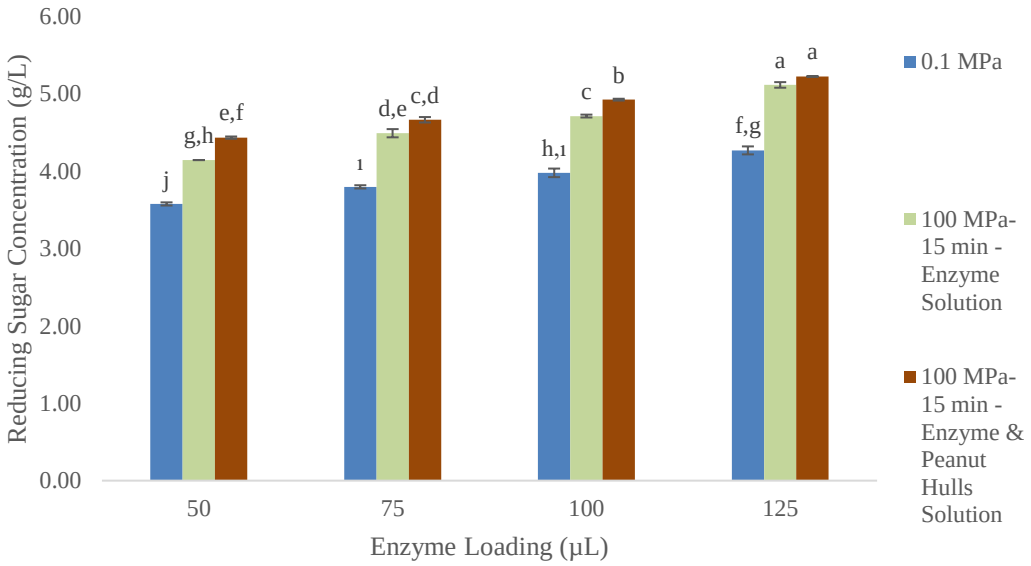

Figure 3.15: Reducing sugar concentrations: Enzymatic hydrolysis of pretreated peanut hulls with both HHP-treated enzyme solution and HHP-treated enzyme-peanut hull solution at 100 MPa for 15 min and with non-HHP-treated-enzyme solution as control samples (0.1 MPa). Different small letters represent significant differences ($p<0.05$)

3.6 Nuclear Magnetic Resonance (NMR) Relaxometry

In this study, the states of water in a cellulose-water system exposed to alkaline pretreatment and high hydrostatic pressure (HHP) hydrolysis with different enzyme concentration were examined and effects of these different conditions were observed. In Table 3.1, T_2 results of different samples were given and divided into five main groups. Each group had untreated and alkaline pretreated peanut hulls and they were labeled with subjected conditions (HHP and hydrolysis with 75 and 125 µl enzyme amounts) except for control samples that are not exposed to any operation. Graphical representations of all NMR results are given in Appendix D.

Table 3.1: The relaxation times (T_2) of prepared different samples

Sample	**T_2 (± 0.60 ms)**
Untreated Peanut Hulls	62.38
Treated Peanut Hulls	151.32
Untreated- 100 MPa - Unhydrolyzed	113.39
Treated- 100 MPa - Unhydrolyzed	302.09
Untreated - 125µl enzyme - 100 MPa - Unhydrolyzed	137.23
Treated - 125µl enzyme - 100 MPa - Unhydrolyzed	340.74
Untreated - 75µl enzyme - Hydrolyzed	118.17
Treated - 75µl enzyme - Hydrolyzed	376.46
Untreated - 125µl enzyme - Hydrolyzed	154.43
Treated - 125µl enzyme - Hydrolyzed	405.83

For detailed observation, obtained T_2 values were divided into two part. Firstly, untreated and alkaline pretreated peanut hulls as also control samples, untreated and alkaline pretreated peanut hulls hydrolyzed with HHP treated (100 MPa) enzyme solution, and untreated and pretreated peanut hulls hydrolyzed after HHP treated (100MPa) enzyme-peanut hull solution together. All results of six samples were illustrated in Figure 3.16. When the results of untreated and alkaline pretreated peanut hulls were compared, T_2 values of pretreated samples were found to be significantly higher than untreated hulls ($p<0.05$). Approximately 1.5-2-fold longer relaxation times were obtained for alkaline pretreated samples. These chances can be caused by the cell wall swelling due to the moisture content is increasing up to 76 %, and thus increasing the relaxation times. Higher amounts of water on the pretreated peanut hull surfaces than on the untreated peanut hull surfaces are caused by an increasing in water-accessible specific surface area of the peanut hulls due to the alkali pretreatment, increasing the substrate porosity. The reason of this approach is that samples with larger pores resulted in slower decay of the water proton signal associated with longer

T_2 time while smaller pores resulted in rapid decay of the water proton signal associated with shorter T_2 relaxation time (Coates, Xiao, & Prammer, 1999). A second factor that may have impacted the amount of water at the surface was the surface chemistry such as hydrophilicity or hydrophobicity of the surface. An increase in the amount of water at the peanut hull surface may also indicate an increase in the hydrophilicity of the surface (Karuna et al., 2014).

When 100 MPa was applied on peanut hulls, the relaxation times of both untreated and alkaline treated hulls were found to be quite longer ($p<0.05$). In the case of untreated peanut hulls, 1.8 times longer T_2 values were attained while T_2 of alkaline pretreated hulls reached to 1.9 times higher value according to control samples. In terms of numerical comparison, T_2 values for both samples are nearly doubled by pressure application. Like alkaline pretreatment, ruptures and porous areas occurred on coconut fibers after a pressure treatment (Albuquerque et al., 2016). Non-HHP-treated samples were expected to contain the smaller pores and HHP-treated samples were expected to contain the larger pores. In the light of this information, smaller pores resulted in rapid decay of the water proton signal and thus shorter T_2 relaxation time was obtained for non-HHP-treated samples. On the contrary, samples with larger pores (HHP-treated samples) resulted in slower decay of the water proton signal and thus longer T_2 relaxation time was observed (Coates et al., 1999; Karuna et al., 2014).

In Figure 3.16, the obtained results showed T_2 value of hydrostatic pressure application to both enzyme and peanut hulls together giving the longest relaxation time. When the effect of hydrolysis using only HHP-treated enzyme solution was examined, the T_2 results belonging to both untreated and alkaline pretreated peanut hulls are significantly longer than others ($p<0.05$). Untreated and alkaline pretreated samples have 2.2 and 2.3 times longer relaxation times when compared to control untreated and pretreated hulls. One of the possible reasons was that similar to the effect of high hydrostatic pressure on the enzyme, it also caused structural changes on the cellulose. Hydrostatic pressure can affect and break hydrogen bonds. As a result, ruptures and porous areas formed after HHP treatment promoted by pressure subject

new hydrophobic parts of the lignocellulosic material and they lead to increased hydrolysis efficiency. Cellulose digestibility is improved by being able to bind to the carbohydrate binding module (CBM) or to the hydrophobic amino acids of enzymes due to hydrophobic interactions and then cellulase activity also enhanced (Albuquerque et al., 2016; Ding et al., 2012). Corresponding with previous results, increase in hydrophilicity of the surface leads to increase in the water amount due to hydrophobic interaction between amino acids and enzymes (cellulase and hemicellulase mixture) and then increase in the relaxation time also observed.

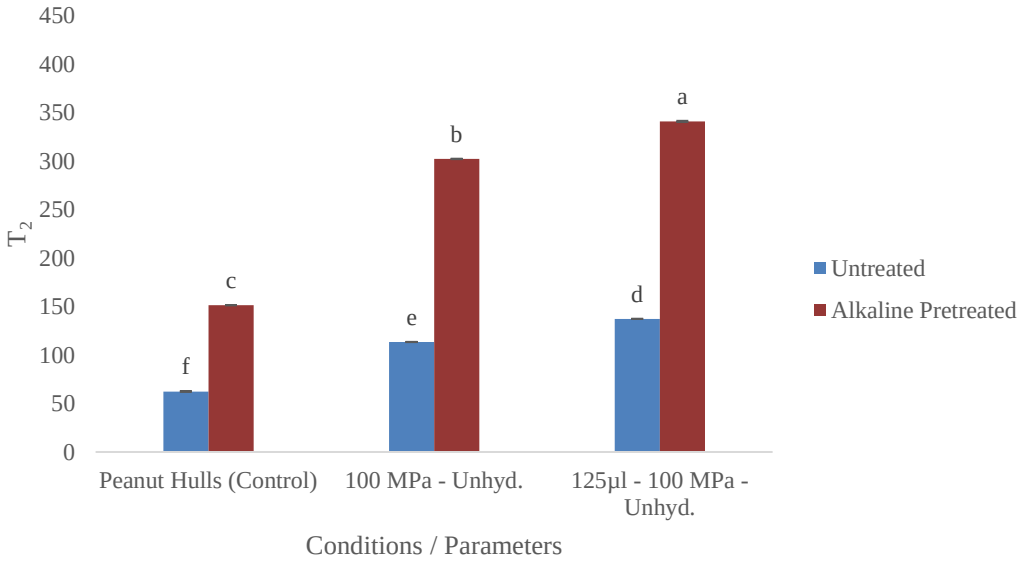

Figure 3.16: The relaxation time values: Control samples (non-HHP-treated peanut hulls), 100MPa-HHP-treated peanut hulls and 100 MP-HHP-treated enzyme-peanut hulls together according to untreated and alkaline pretreated peanut hull types. Different small letters represent significant differences ($p<0.05$)

The increased relaxation time for the main part of the cell wall water was associated with a significant loosening or fragmentation of the whole cell wall matrix. Thus enzymatic drilling was associated with a loosening of the cellulose matrix structure creating cavities and micropores, but still maintaining the overall structural characteristics (Dourado et al., 1999). In addition, according to Felby et al. (2008), a

clear effect of increasing relaxation times, a degradation or loss of cellulose matrix structure can be seen in terms of cell wall water for Celluclast 1.5 L. The lumen water shows similar behavior for endo-glucanase (EG) and cellobiohydrolases (CBH) with slightly increasing relaxation times caused by the swelling of the cellulose cell wall structure. Celluclast 1.5 L causes the most pronounced changes on the cellulose matrix and the cellulose-water interactions, it also increases the porosity and water bonding capacity of the cell wall, which counteracts the effect of swelling and loosening of the cell wall. The breakdown of cellulose should result in a less organized structure and thus longer relaxation times. Using both 75 and 125µl enzyme amount for hydrolysis led significant enhancement on the relaxation time like enzymatic hydrolysis efficiency when they were compared with control samples ($p<0.05$). For the case of untreated peanut hulls, hydrolysis with 125µl enzyme amount represents 1.5-fold higher T_2 value while 75µl enzyme amount had approximately one-fold longer T_2. For treated peanut hulls, hydrolysis with 75µl enzyme amount provided approximately 1.5-fold increase of relaxation time and hydrolysis with 125µl enzyme amount represents 1.7-fold increase. Another reason of longer relaxation time after enzymatic hydrolysis is that cellulose-hemicellulase treatment of cellulose increases the water holding capacity. That using 125µl enzyme amount for cellulose hydrolysis had much longer relaxation times than using 75 µl enzyme amount was observed for both untreated and pretreated hulls, but increase of T_2 belongs to alkaline pretreated hulls between 75 and 125µl enzyme amount was not as much as T2 belongs to untreated hulls. Reason of this difference can be caused by alkaline pretreatment effect. Alkaline pretreated sample has already structural deformation and hydrolysis also has sustained effect in addition to pretreatment effect. Therefore, enzyme concentration did not have same improving effect on alkaline pretreated peanut hull samples.

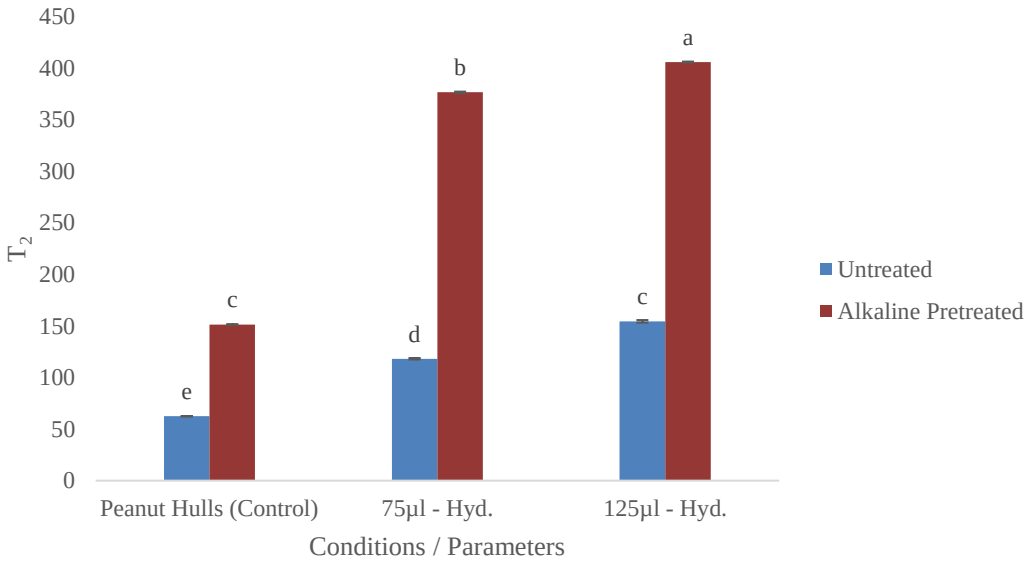

Figure 3.17: The relaxation time values: Control samples (non-HHP-treated peanut hulls), hydrolyzed peanut hulls with 75 and 125 µl enzyme according to untreated and alkaline pretreated peanut hull types. Different small letters represent significant differences ($p<0.05$)

3.7 Fourier Transform Infrared (FTIR) Spectroscopy

In order to interpret the FTIR spectra, the ranges 500-900, 900-1800 and 2070-3700 cm^{-1} ranges were investigated. 3700 to 3000 cm^{-1} band refers to O-H stretching frequencies and also to water. The nearest peak is in the range of 2980 and 2850 cm^{-1} assigns to -C-H stretching vibrations or symmetric and asymmetric linkage vibrations of CH_2 groups. The 1700-1500 cm^{-1} range corresponds to C=O stretching and carbonyl-specific absorptions (Adina, Florinela, Abdelmoumen, & Carmen, 2010). Moreover, deformation vibrations of CH_2 and CH groups and angular deformation vibrations of C-O-H are determined by frequencies between 1650-1350 cm^{-1} (Huntley et al., 2015). While 1000-1150 cm^{-1} range refers to C-C and C-H ring structures, the band 890-1200 cm^{-1} assigns to C-H deformation vibration (Lokshina et al., n.d.; Tatlı, 2013).

In Figure 3.18, nearly whole of the characteristic peaks of lignocellulosic materials (untreated peanut hulls) are illustrated. The peaks at 3337 and 2913 cm^{-1} correspond to OH and CH stretching vibrations, respectively. The peak at 1734 cm^{-1} only existed in untreated peanut hulls spectra due to unconjugated C=O stretching vibrations of hardwood lignin (Tatlı, 2013). In between 1640-1625 cm^{-1} band, there were a group of peaks that correspond to deformation of CH_2 and CH groups and to angular deformation of C-O-H. In addition, the peak at 1606 cm^{-1} that is only represented in untreated peanut hulls spectra demonstrates the lignin aromatic skeletal vibration (Huntley et al., 2015; Tatlı, 2013). In the pretreated spectra, the lack of peaks at 1734 cm^{-1}, 1607 cm^{-1}, 1419-1456 cm^{-1}, and 1384–1346 cm^{-1} prove the effect of alkaline pretreatment due to removal of hemicelluloses and lignin. The characteristic band at 1026 cm^{-1} is the most intense peaks for both untreated and pretreated peanut hulls. This peak originated from C-C and C-H ring and side group vibrations. The 893 cm^{-1} band was observed on only pretreated spectra and referred to the glucose ring vibrations in cellulose. In this case, this may be caused by effect of the alkaline pretreatment (Tatlı, 2013). Main reason of this peak is the deformation and stretching of COC and CCH.

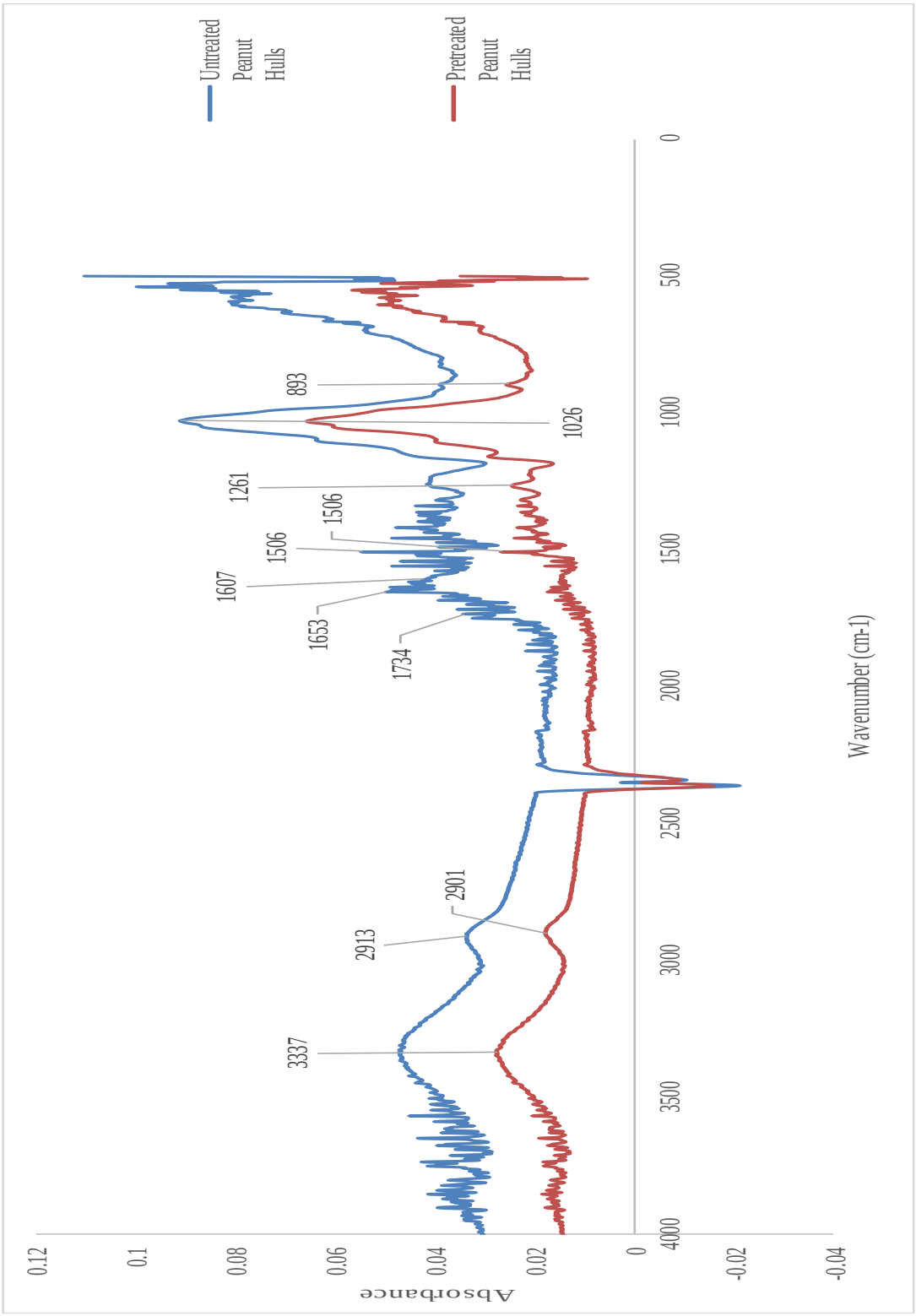

Figure 3.18: FTIR spectra of untreated and alkaline pretreated peanut hulls

Compared with untreated peanut hull samples that were prepared at different conditions, FTIR spectra of these three samples in Figure 3.19 were examined in detail. Two of the characteristic peaks are O-H stretching peak at 3337 cm^{-1} and C-H stretching vibration at 2914 cm^{-1}. For both bands, these peaks become more explicit when 100 MPa high hydrostatic pressure were applied to peanut hull solution and to enzyme-peanut hulls (there was no hydrolysis step after HHP treatment). In the case of HHP-treated enzyme-peanut hulls, the highest peaks were observed by comparison with the samples prepared by using HHP-treated peanut and only peanut hulls (control sample). Only untreated peanut hulls gave the smoothest result among other samples. At 1732 and 1607 cm^{-1}, HHP-treated enzyme-peanut hulls and HHP-treated peanut hulls have more clear peaks that refer to the lignin aromatic skeletal vibration. However, there are also band shifts with reference to only untreated peanut hull sample. Lignin-vibration peaks at 1732 cm^{-1}, 1606-1593 cm^{-1}, 1418 cm^{-1}, and 1384–1346 cm^{-1} existed in all samples' spectra because all of the samples in this graph are untreated (Huntley et al., 2015). Each peak of three samples at 1026 cm^{-1} indicates C-C and C-H ring and side group vibrations. HHP-treated peanut hulls and HHP-treated enzyme-peanut hulls had more clear sharp peaks than the control sample. Unlike untreated peanut hulls, there are peaks of the deformation and stretching of COC and CCH at 895 cm^{-1} for HHP-treated peanut hulls and HHP-treated enzyme-peanut hull. This may be caused by HHP and enzyme-HHP factors leading glucose vibration (Tatlı, 2013).

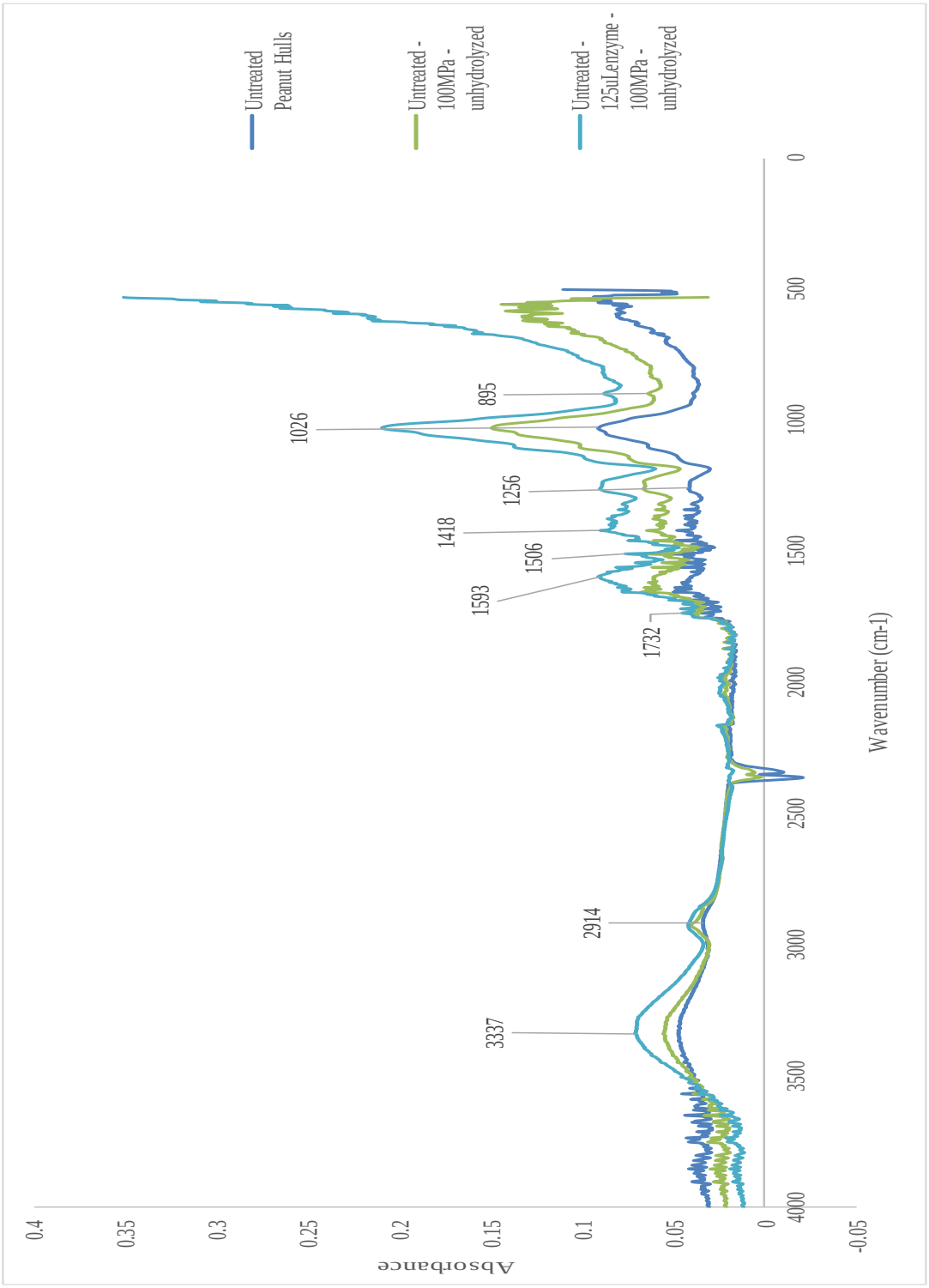

Figure 3.19: FTIR spectra of non-HHP-treated (non-alkaline-pretreated) peanut hulls as control sample, 100 MPa-HHP-treated peanut hulls and 100 MPa-HHP-treated enzyme-peanut hulls together

In Figure 3.20, O-H stretching peak at 3337 cm^{-1} was obtained for all alkaline pretreated samples. The C-H stretching-vibration peaks also existed for each sample, but it locates at 2914 cm^{-1} for both HHP-treated peanut hulls and HHP-treated enzyme-peanut hulls while alkaline pretreated peanut hulls (control sample) locates at 2901 cm^{1}. In this case, little bend shift occurred. Like untreated samples, the peaks of pretreated peanut hulls were smoother than others at these two bands. There was no explicit peak that belonged to lignin aromatic skeletal vibration at the range of 1725-1730 or 1417-1420 cm^{-1}. At 1026 cm^{-1}, the most intense characteristic peaks were assigned to C-C and C-H ring and side group vibrations. HHP-treated peanut hulls and HHP-treated enzyme-peanut hulls had more clear sharp peaks than control sample. For all three samples, there are bigger peaks of the deformation and stretching of COC and CCH at 893 cm^{-1}.

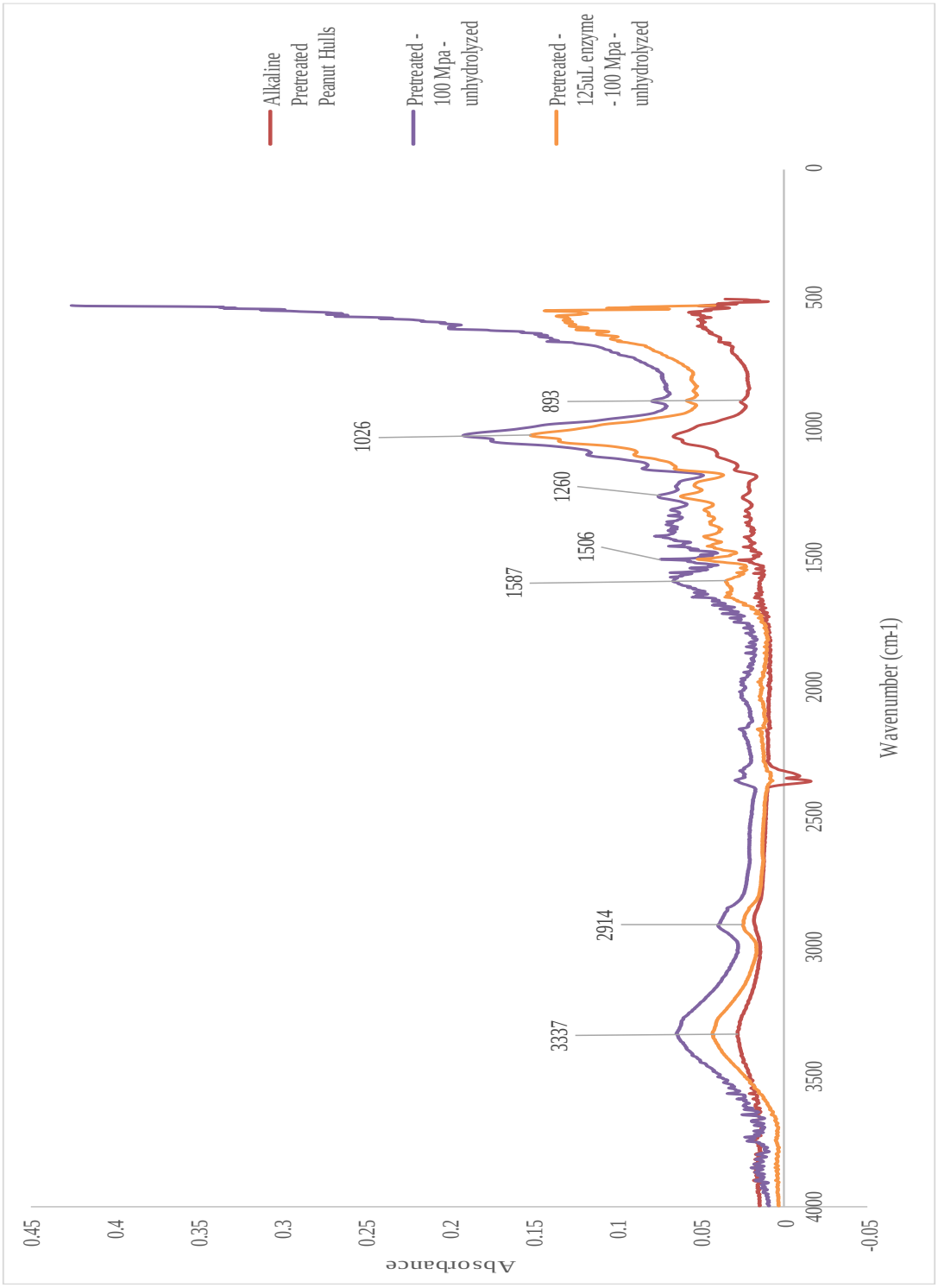

Figure 3.20: FTIR spectra of non-HHP-treated alkaline-pretreated peanut hulls as control sample, 100 MPa-HHP-treated peanut hulls and 100 MPa-HHP-treated enzyme-peanut hulls together

To compare the effect of enzyme amount/concentration on hydrolysis, untreated peanut hulls as control sample, hydrolyzed untreated-peanut hulls with 75μL enzyme amount and hydrolyzed untreated-peanut hulls with 125μL enzyme amount were used for FTIR experiment. As shown in Figure 3.21, there is hardly any difference between the hydrolysis results conducted by using different enzyme concentration. Characteristic peaks of lignocellulose biomass were observed at 3327 cm^{-1} and at 2924 cm^{-1}. These bands refer to O-H stretching and C-H stretching vibration peaks. At 1732 cm^{-1}, these three peaks are originated by the lignin aromatic skeletal vibration. Additionally, at 1732 cm^{-1}, 1601 cm^{-1}, 1418 cm^{-1} and 1384–1346 cm^{-1}, all peaks prove the lignin aromatic skeletal vibration because all of our samples in this graph are untreated. Each peak of three samples at 1026 cm^{-1} indicates C-C and C-H ring and side group vibrations. Hydrolyzed untreated peanut hulls with both 75 and 125μL enzyme amounts have slightly larger peaks when they are compared with control sample. Likely, 75 and 125μL enzyme amounts gave nearly same results. At 899 cm^{-1}, each sample, except control sample, has peak that are originated the deformation and stretching of COC and CCH.

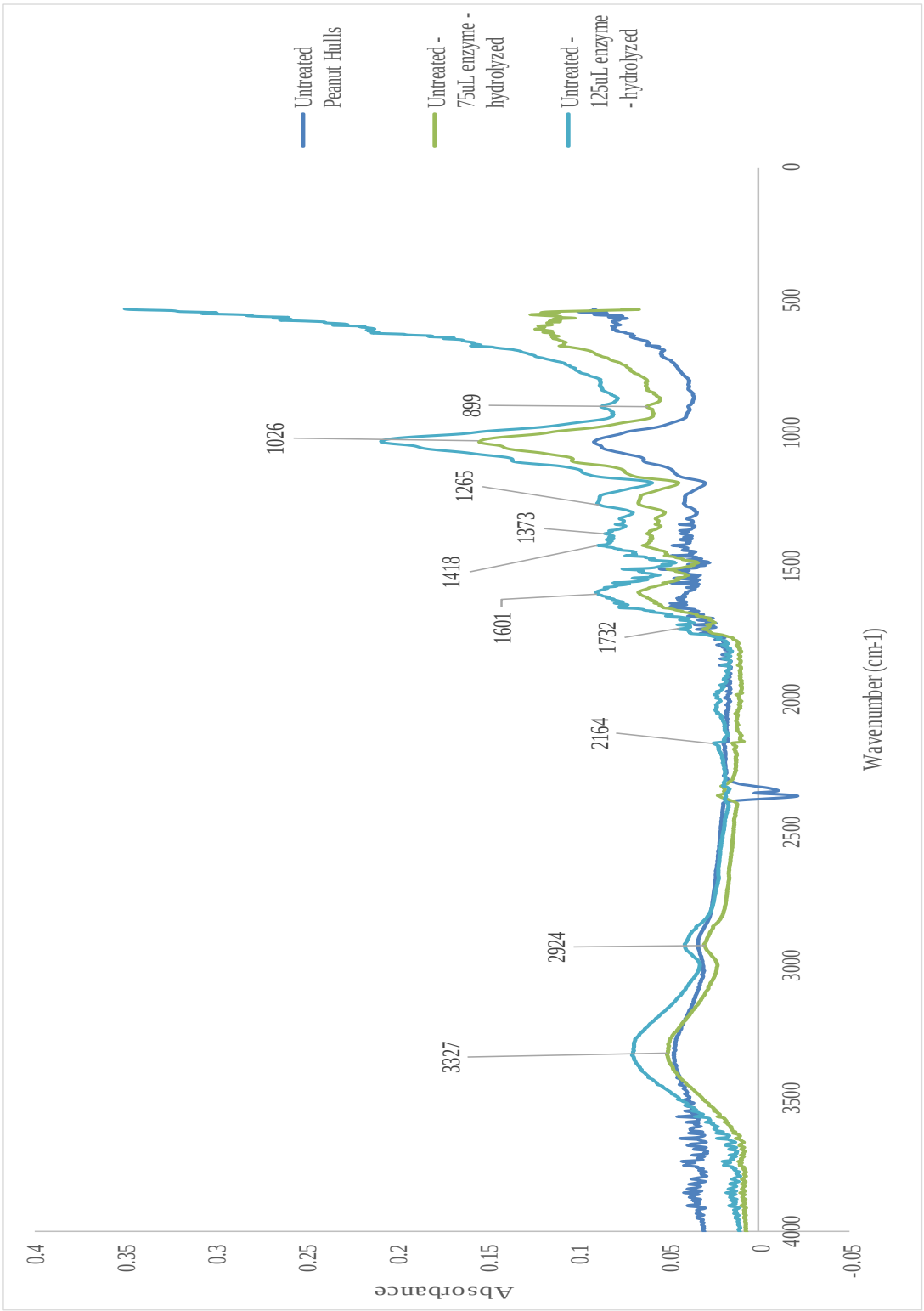

Figure 3.21: FTIR spectra of non-HHP-treated (non-alkaline-pretreated) peanut hulls as control sample, hydrolyzed peanut hulls with 75 and 125 µl enzyme

Parallel with untreated peanut hulls, alkaline-treated peanut hulls as control sample, hydrolyzed treated-peanut hulls with 75μL enzyme amount and hydrolyzed treated-peanut hulls with 125μL enzyme amount were used for FTIR experiment. In Figure 3.22, as in other FTIR graphs, the O-H stretching peak corresponds to the band 3331 cm^{-1}. Hydrolyzed treated-peanut hulls with both 75 and 125 μL enzyme concentration have different absorbance values that refer to C-H stretching vibration at 2924 cm^{-1}. However, control sample, pretreated hulls, has wave number value as 2903 cm^{-1}. Effect of hydrolysis of peanut hulls can cause slight band shift. Explicit peak belongs to lignin aromatic skeletal vibration did not observed nearly at 1700 cm^{-1}, 1600 cm^{-1}, 1418 cm^{-1}, and 1384–1346 cm^{-1} because whole samples are alkaline pretreated in this part. The peaks of these three samples at 1030 cm^{-1} represent C-C and C-H ring and side group vibrations. Similarly, with untreated peanut hull sample, the peak of hydrolyzed untreated peanut hulls with both 75 and 125μL enzyme amounts have significantly higher than the control sample's. When the peaks belonging to 75 and 125 μL enzyme amounts, there is also difference from each other and even hydrolysis with 125 μL enzyme has highest absorbance value. Lastly, values of these peaks referring the deformation and stretching of COC and CCH were obtained at 893 cm^{-1}.

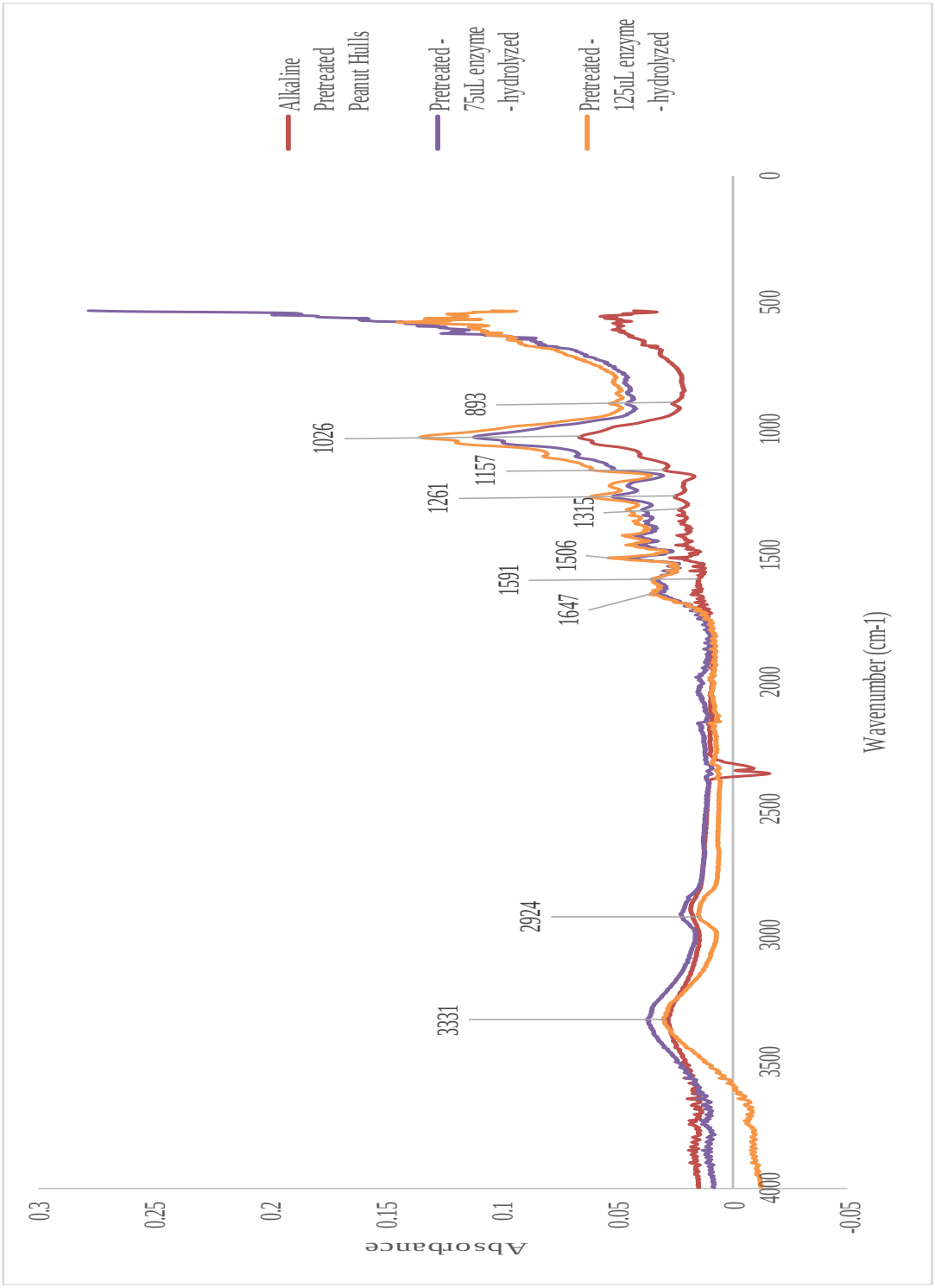

Figure 3.22: FTIR spectra of non-HHP-treated alkaline-pretreated peanut hulls as control sample, hydrolyzed peanut hulls with 75 and 125 µl enzymes

CHAPTER 4

CONCLUSION AND RECOMMENDATION

HHP is reported as an effective method to enhance the efficiency biomass hydrolysis, and this claim is also shown with the results of this study. For Avicel, hydrolysis with HHP-treated enzyme solutions and HHP-treated enzyme-avicel solution had significantly higher reducing sugar content than those of non-HHP-treated solution (control sample) ($p<0.05$). Additionally, hydrolysis with HHP-treated enzyme-avicel solution had the highest reducing sugar concentration and this situation proved the enhancing effect of HHP application; however, no significant improving effect was observed between applied pressure levels of 100 and 500 MPa ($p>0.05$). Longer HPP application led to significant improvement of the hydrolysis of cellulose and Celluclast activity for all conditions ($p<0.05$). For peanut hull hydrolysis, similar and confirming results were obtained and also effect of HHP application was observed. Hydrolysis with HHP-treated enzyme solutions and HHP-treated enzyme-pretreated peanut hull solution led to significant increase of reducing sugar amount obtained after enzymatic hydrolysis. In addition, hydrolysis of peanut hulls with HHP-treated enzyme-pretreated peanut hull solution had the highest reducing sugar concentration ($p<0.05$). The improving effects of HHP in terms of hydrolysis efficiency and Celluclast activity were clearly observed in this part. However, a significant change was not observed between different pressure levels such as 100 and 500 MPa for the case of hydrolysis of peanut hulls ($p>0.05$). In addition, requirement of pretreatment process as a necessary step for hydrolysis of lignocellulosic biomass was also demonstrated with results.

According to the NMR results, like alkaline pretreatment, significant increase of T_2 values were observed when HHP was applied to samples due to increase in hydrophilicity of the surface leading to increase in resulting from the hydrophobic interaction between amino acids and cellulose ($p<0.05$). Additionally, enzymatic hydrolysis led significant enhancement on the relaxation time like HHP treatment ($p<0.05$).

The effects of HHP, alkaline pretreatment and enzymatic hydrolysis were also observed with the changes in the intensity, wavenumbers and absorbance values of FTIR spectra.

To better understand the effects of high hydrostatic pressure on both the enzyme and substrate, as a future study, HHP treatment can be performed using different conditions in terms of pressure levels, different application times and temperatures. In addition, a more detailed investigation of the effect of HHP using different substrates can be performed. By using other methods, the effects of HHP on the chemical structure of the substrate and the enzyme can be investigated in more detail.

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APPENDIX A

STANDARD CURVE FOR DINITROSALICYLIC ASID (DNS) ANALYSIS

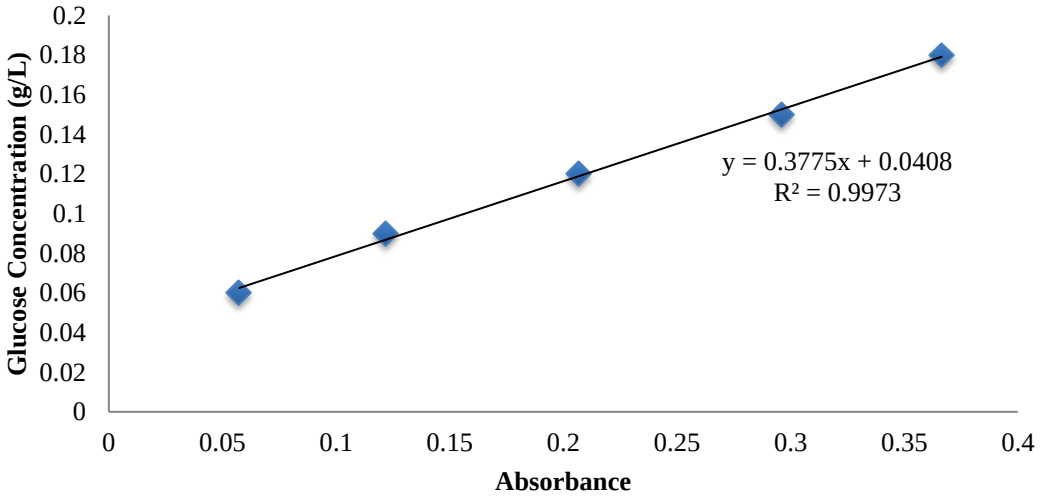

Figure A.1: The standard curve of DNS method

APPENDIX B

NUMERIC-VALUES USED IN GRAPHS

Table B.1: Total reducing sugar amounts for Avicel

Enzyme Amount (µL)	Reducing Sugar Conc. (g/L)
50	6.24
75	7.94
100	9.11
125	10.45
150	12.59
200	12.45
250	11.31
300	14.32
400	13.68
500	13.24

Table B.2: Total reducing sugar amounts for peanut hulls

Enzyme Amount (µL)	Reducing Sugar Conc. (g/L)
50	2.52
75	2.69
100	2.87
125	2.96
150	3.01
200	3.09
250	3.09
300	3.18
400	3.28
500	3.31

Table B.3: Total reducing sugar amounts for untreated and pretreated peanut hulls

	Reducing Sugar Conc. (g/L)	
Enzyme Amount (µL)	**Untreated Peanut Hulls**	**Pretreated Peanut Hulls**
50	2.52	3.58
75	2.69	3.80
100	2.87	3.98
125	2.96	4.27

Table B.4: Glucose yields percentages for untreated and pretreated peanut hulls

	% Glucose Yield	
Enzyme Amount (µL)	**Untreated Peanut Hulls**	**Pretreated Peanut Hulls**
50	6.99	7.95
75	7.47	8.44
100	7.97	8.84
125	8.22	9.49

Table B.5: Reducing sugar concentrations after hydrolysis of Avicel using non-HHP-treated and HHP-treated enzyme (100 & 500 MPa for 5 & 15 min) solutions

	Reducing Sugar Concentration (g/L)				
Enzyme Amount (µL)	**0.1 MPa**	**100 MPa 5 min**	**500 MPa 5min**	**100 MPa 15 min**	**500 MPa 15 min**
50	6.24	7.03	7.09	7.94	7.51
75	7.94	9.27	9.46	9.70	9.66
100	9.11	10.16	10.20	10.97	11.17
125	10.45	11.30	11.35	11.85	12.11

Table B.6: Reducing sugar concentrations after hydrolysis of Avicel using non-HHP-treated and after hydrolysis with HHP-treated enzyme-avicel (100 & 500 MPa for 5 min) solutions

	Reducing Sugar Concentration (g/L)		
Enzyme Amount (µL)	**0.1 MPa**	**100 MPa 5 min**	**500 MPa 5 min**
50	6.24	8.60	8.45
75	7.94	9.81	10.29
100	9.11	11.94	11.94
125	10.45	12.07	12.54

Table B.7: Reducing sugar concentrations after hydrolysis of alkaline pretreated peanut hulls using non-HHP-treated and HHP-treated enzyme (100 MPa for 5 & 15 min) solutions

	Reducing Sugar Concentration (g/L)		
Enzyme Amount (µL)	**0.1 MPa**	**100 Mpa 5 min**	**100 Mpa 15 min**
50	3.58	3.82	4.14
75	3.80	4.07	4.49
100	3.98	4.40	4.71
125	4.27	4.64	5.12

Table B.8: Reducing sugar concentrations after hydrolysis of alkaline pretreated peanut hulls using non-HHP-treated and after hydrolysis with HHP-treated enzyme-pretreated peanut hull (100MPa for 5 & 15min) solutions

	Reducing Sugar Concentration (g/L)		
Enzyme Amount (µL)	**0.1 MPa**	**100 MPa 5 min**	**100 MPa 15 min**
50	3.58	4.18	4.43
75	3.80	4.46	4.67
100	3.98	4.78	4.92
125	4.27	5.06	5.22

APPENDIX C

STATISTICAL ANALYSIS

Table C.1: Two way ANOVA for alkaline pretreatment effect on reducing sugar yield of peanut hulls

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz Cons.	3	4.24	4.24	1.41	135.68	0.000
Pret.	1	4.15	4.15	4.15	39877	0.000
Enz Cons.*Pret.	3	0.09	0.09	0.03	2.90	0.102
Error	8	0.08	0.08	0.01		
Total	15	8.57				

Table C.2: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel with HHP-treated enzyme solution at 100 & 500 MPa for 5 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	57.80	57.80	19.26	104.68	0.000
Pres.	2	5.90	5.90	2.95	16.03	0.000
Enz. Cons.*Pres.	6	0.31	0.31	0.05	0.29	0.932
Error	12	2.20	2.20	0.18		
Total	23	66.23				

Table C.3: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel with HHP-treated enzyme solution at 100 & 500 MPa for 15 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	60.11	60.11	20.03	568.05	0.000
Pres.	2	15.06	15.06	7.53	213.54	0.000
Enz. Cons.*Pres.	6	0.48	0.48	0.08	2.27	0.107
Error	12	0.42	0.42	0.03		
Total	23	76.08				

Table C.4: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel with HHP-treated enzyme solution at 100 MPa for 5 and 15 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	55.73	55.73	18.57	144.68	0.000
Time	2	11.48	11.48	5.74	44.70	0.000
Enz. Cons.*Time	6	0.29	0.29	0.04	0.38	0.876
Error	12	1.54	1.54	0.12		
Total	23	69.05				

Table C.5: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel with HHP-treated enzyme solution at 500 MPa for 5 and 15 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	62.05	62.05	20.68	227.50	0.000
Time	2	11.60	11.60	5.80	63.82	0.000
Enz. Cons.*Time	6	0.63	0.63	0.10	1.16	0.387
Error	12	1.09	1.09	0.0909		
Total	23	75.38				

Table C.6: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel with HHP-treated enzyme-avicel solution at 100 & 500 MPa for 5 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	55.54	55.54	18.51	868.57	0.000
Pres.	2	27.68	27.68	13.84	649.36	0.000
Enz. Cons.*Pres.	6	0.99	0.99	0.16	7.76	0.001
Error	12	0.25	0.25	0.02		
Total	23	84.48				

Table C.7: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel both HHP-treated enzyme solution and HHP-treated enzyme-avicel solution at 100 MPa for 5 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	54.54	54.54	18.18	137.84	0.000
Soln.	2	18.89	18.89	9.44	71.61	0.000
Enz. Cons.*Soln.	6	1.42	1.42	023	1.80	0.182
Error	12	1.58	1.58	0.13		
Total	23	76.44				

Table C.8: Two way ANOVA for reducing sugar concentrations after hydrolysis of Avicel both HHP-treated enzyme solution and HHP-treated enzyme-avicel solution at 500 MPa for 5 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	58.00	58.00	19.33	263.13	0.000
Soln.	2	22.55	22.55	11.27	153.49	0.000
Enz. Cons.*Soln.	6	0.68	0.68	0.11	1.56	0.241
Error	12	0.88	0.88	0.07		
Total	23	82.13				

Table C.9: Two way ANOVA for reducing sugar concentrations after hydrolysis of pretreated peanut hulls with HHP-treated enzyme solution at 100 MPa for 5 and 15 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	2.22	2.22	0.74	150.64	0.000
Time	2	2.02	2.02	1.01	204.96	0.000
Enz. Cons.*Time	6	0.05	0.05	0.01	1.78	0.185
Error	12	0.05	0.05	0.01		
Total	23	4.36				

Table C.10: Two way ANOVA for reducing sugar concentrations after hydrolysis of pretreated peanut hulls with HHP-treated enzyme solution-peanut hull solution at 100 MPa for 5 and 15 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	2.06	2.06	0.68	292.72	0.000
Time	2	3.65	3.65	1.82	777.29	0.000
Enz. Cons.*Time	6	0.03	0.03	0.01	2.14	0.123
Error	12	0.02	0.02	0.01		
Total	23	5.77				

Table C.11: Two way ANOVA for reducing sugar concentrations after hydrolysis of peanut hulls with both HHP-treated enzyme solution and HHP-treated enzyme-peanut hull solution at 100 MPa for 5 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	2.13	2.13	0.71	137.47	0.000
Soln.	2	2.05	2.05	1.02	198.04	0.000
Enz. Cons.*Soln.	6	0.03	0.03	0.01	1.12	0.407
Error	12	0.06	0.06	0.01		
Total	23	4.28				

Table C.12: Two way ANOVA for reducing sugar concentrations after hydrolysis of peanut hulls with both HHP-treated enzyme solution and HHP-treated enzyme-peanut hull solution at 100 MPa for 15 min

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Enz. Cons.	3	2.16	2.16	0.72	342.69	0.000
Soln.	2	3.63	3.63	1.81	865.47	0.000
Enz. Cons.*Soln.	6	0.04	0.04	0.01	3.43	0.033
Error	12	0.02	0.02	0.00		
Total	23	5.86				

Table C.13: Two way ANOVA for the relaxation time values of non-HHP-treated peanut hulls, 100 MPa-HHP-treated peanut hulls and 100 MPa-HHP-treated enzyme-peanut hulls together according to untreated and alkaline pretreated peanut hull types

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Un/Tre	1	76978	76978	76978	519575.17	0.000
Soln.	2	37954	37954	18977	128086.88	0.000
Un/Tre*Soln.	2	7703	7703	3852	25997.80	0.000
Error	6	1	1	0		
Total	11	122637				

Table C.14: Two way ANOVA for the relaxation time values of non-HHP-treated peanut hulls, hydrolyzed peanut hulls with 75 and 125 µl enzyme according to untreated and alkaline pretreated peanut hull types

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Un/Tre	1	119815	119815	119815	124195.37	0.000
Soln.	2	67066	67066	33533	34758.91	0.000
Un/Tre*Soln.	2	18447	18447	9224	9560.91	0.000
Error	6	6	6	1		
Total	11	205335				

APPENDIX D

NMR RESULTS

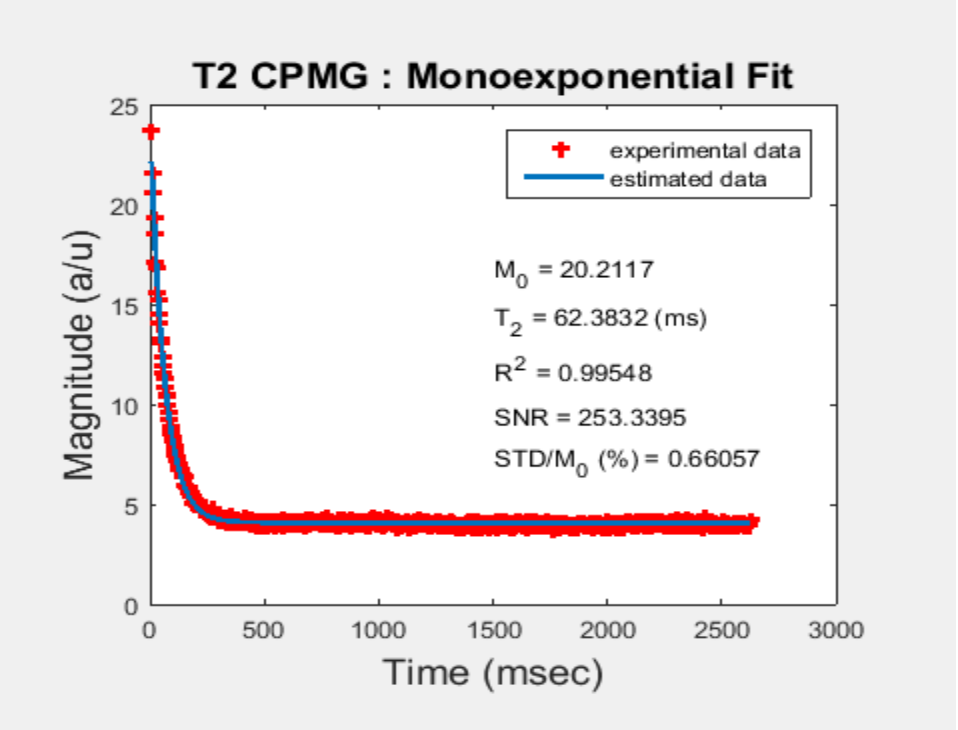

Figure D.1: The relaxation time (T_2) of untreated peanut hulls

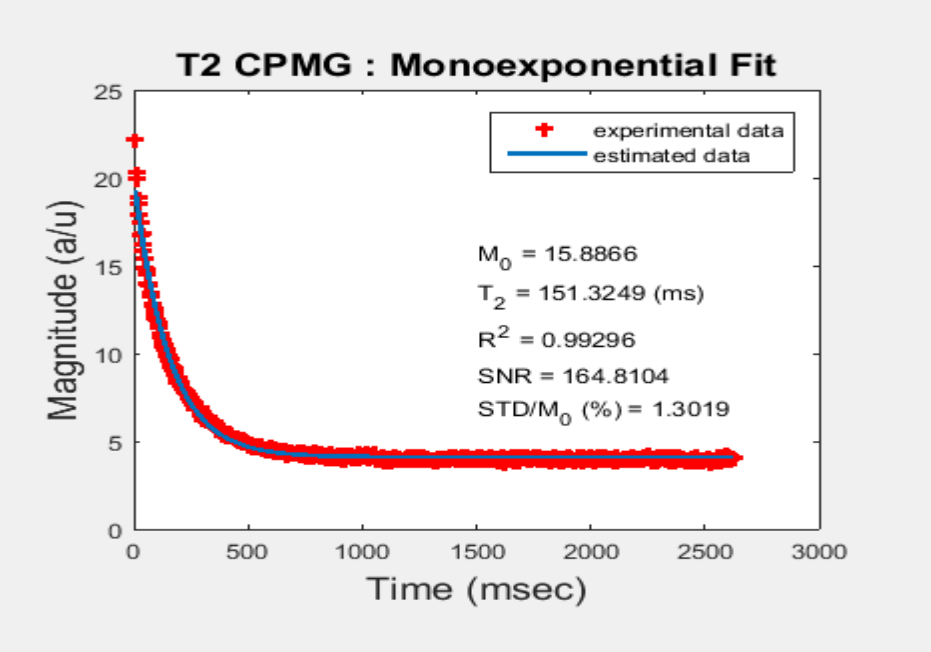

Figure D.2: The relaxation time (T_2) of alkaline pretreated peanut hulls

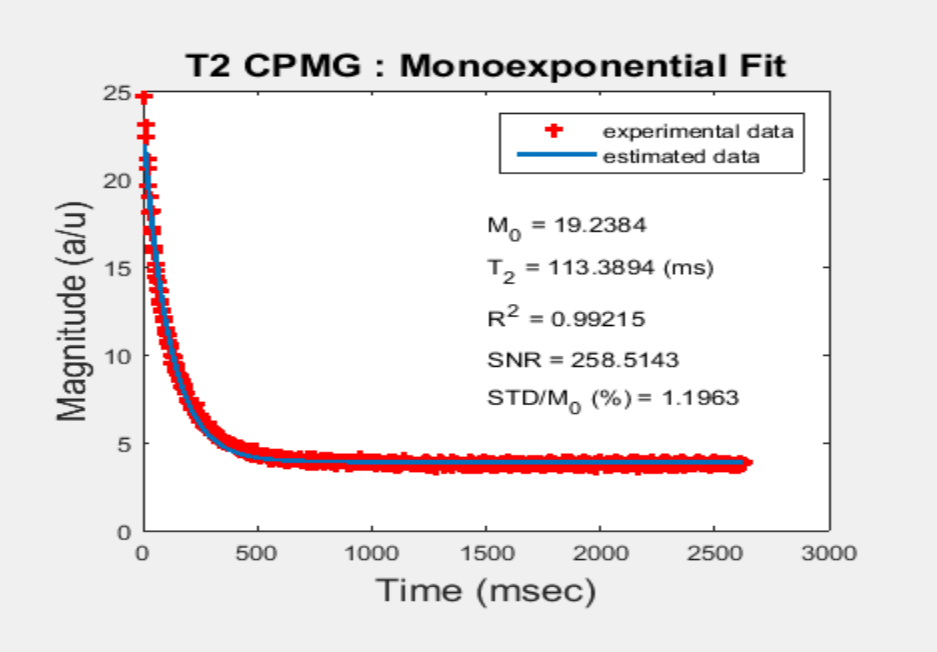

Figure D.3: The relaxation time (T_2) of 100 MPa-HHP-treated (non-alkaline pretreated) peanut hulls

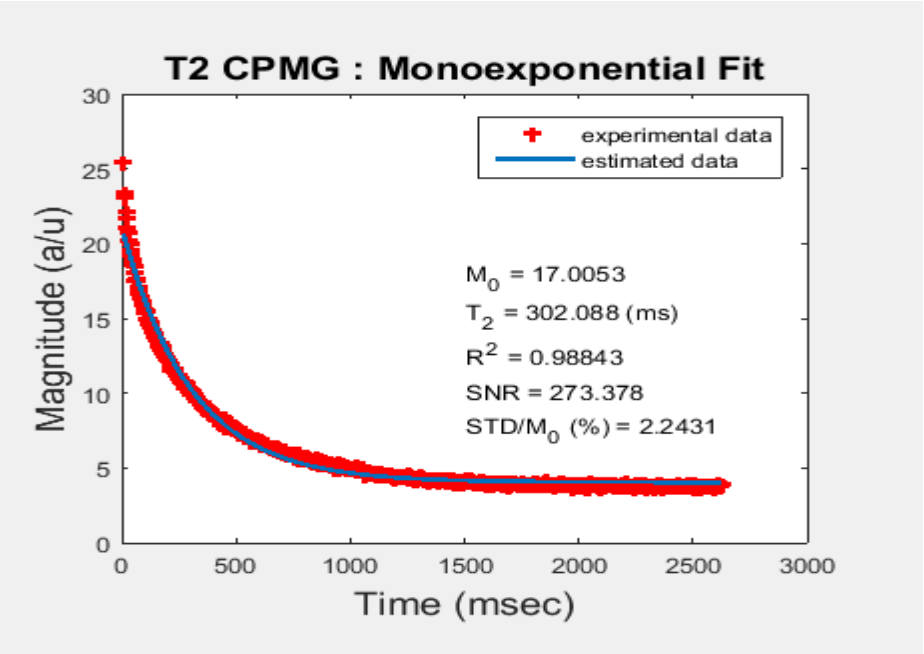

Figure D.4: The relaxation time (T_2) of 100 MPa-HHP-treated alkaline-pretreated peanut hulls

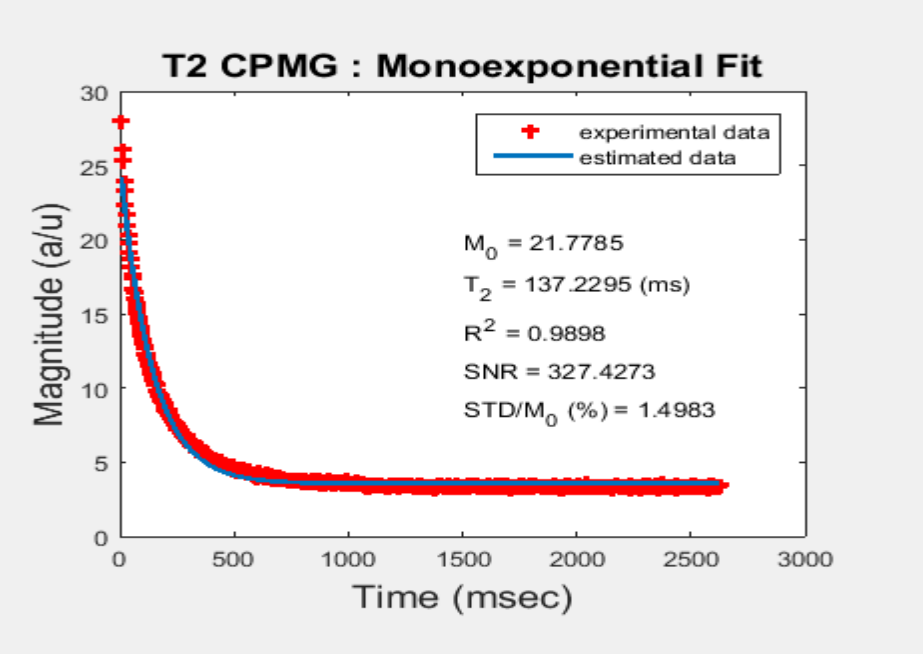

Figure D.5: The relaxation time (T_2) of 100 MPa-HHP-treated peanut hulls together with 125µl enzyme

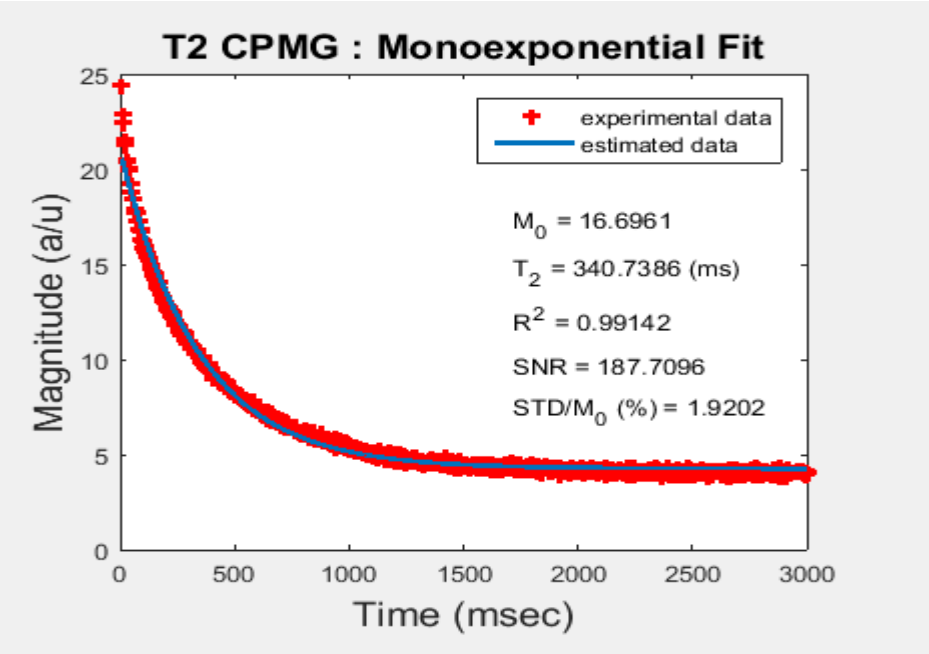

Figure D.6: The relaxation time (T_2) of 100 MPa-HHP-treated alkaline-pretreated-peanut hulls together with 125µl enzyme

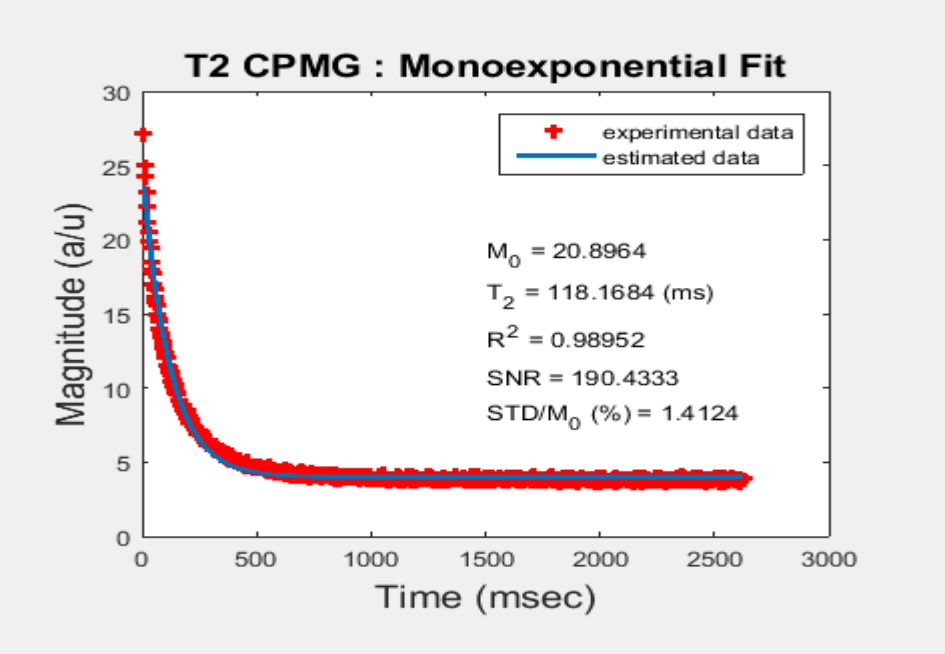

Figure D.7: The relaxation time (T_2) of hydrolyzed (non-alkaline-pretreated) peanut hulls with 75µl enzyme

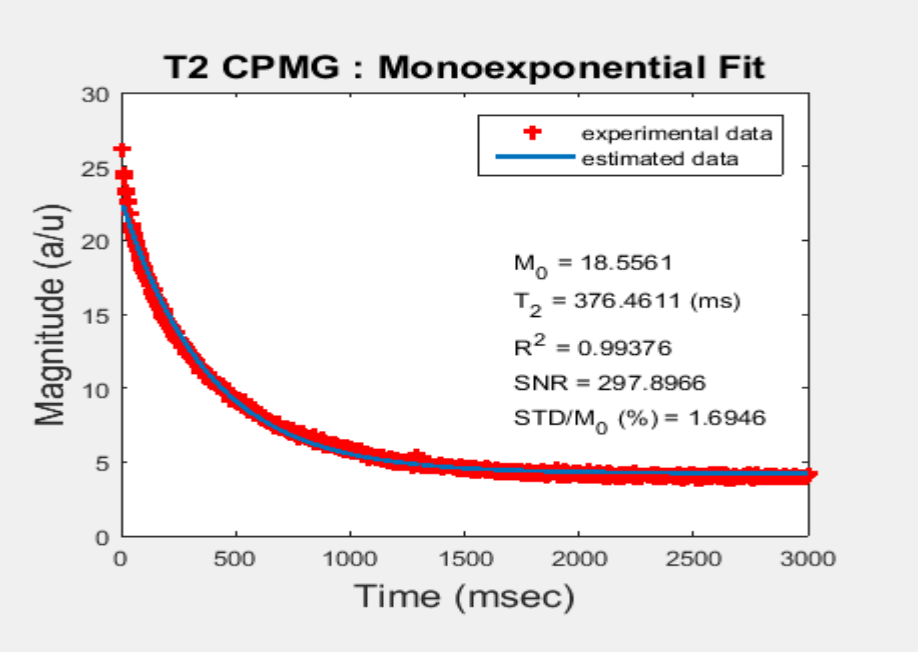

Figure D.8: The relaxation time (T_2) of hydrolyzed alkaline pretreated peanut hulls with 75µl enzyme

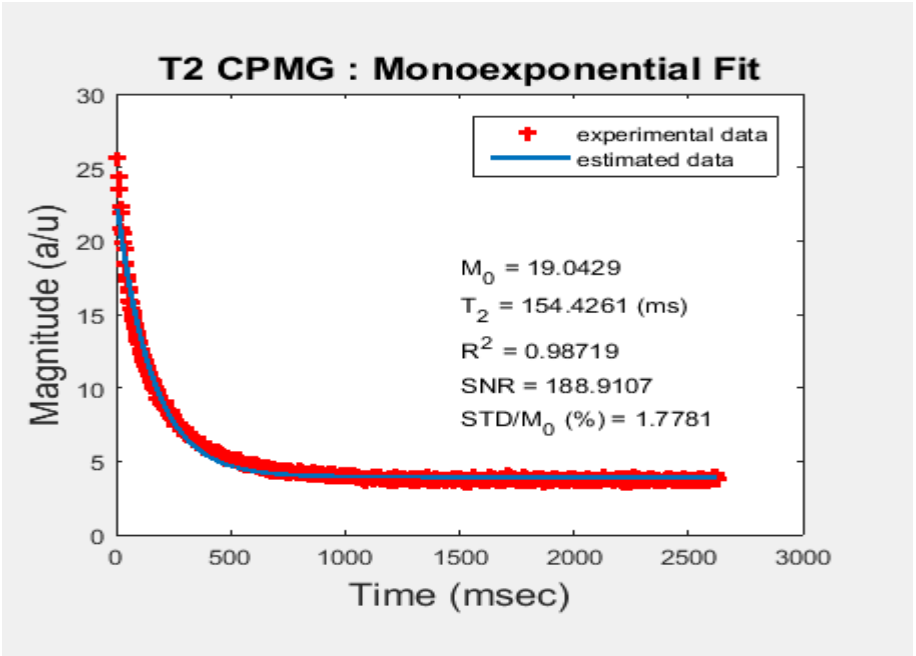

Figure D.9: The relaxation time (T_2) of hydrolyzed (non-alkaline-pretreated) peanut hulls with 125µl enzyme

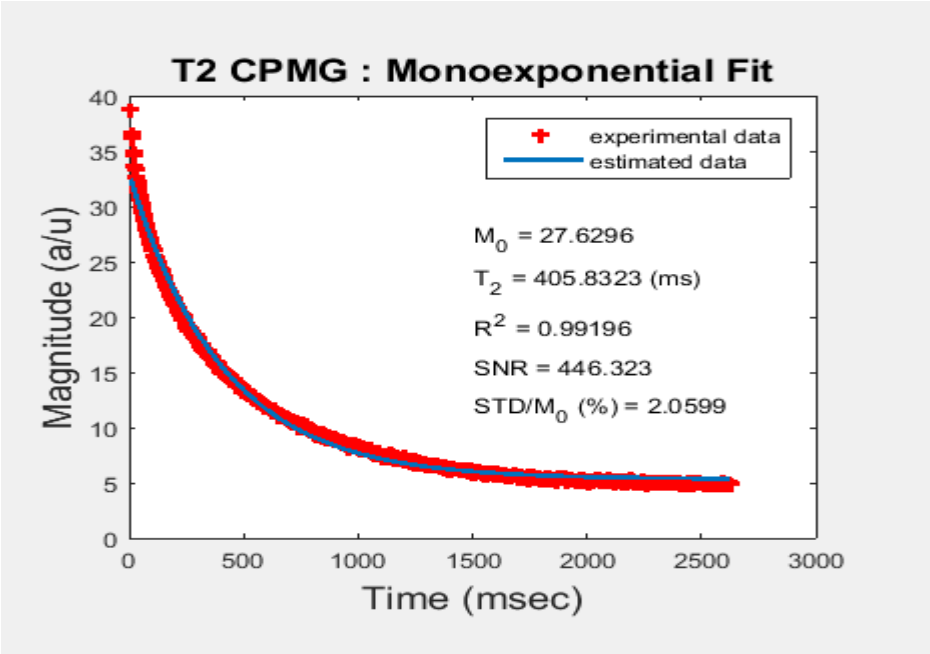

Figure D.10: The relaxation time (T_2) of hydrolyzed alkaline pretreated peanut hulls with 125µl enzyme

APPENDIX E

IMAGES OF SUBSTRATES

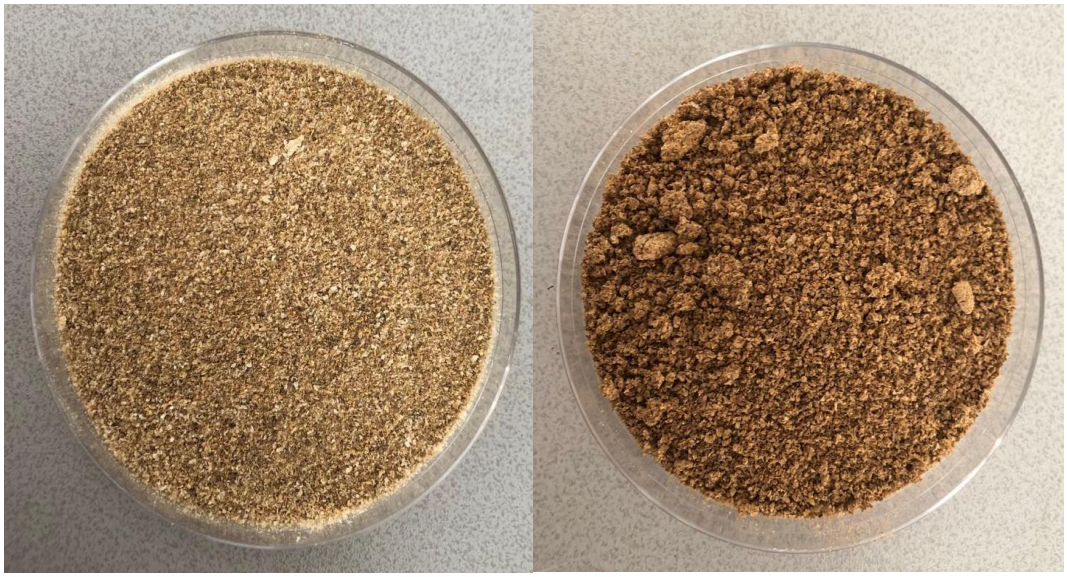

Figure E.1 & E.2: Non-alkaline pretreated (left) and alkaline pretreated (right) peanut hulls