ENZYMATIC HYDROLYSIS OF FRUIT PEELS AND OTHER LIGNOCELLULOSIC BIOMASS AS A SOURCE OF SUGAR FOR FERMENTATION

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ABSTRACT

ENZYMATIC HYDROLYSIS OF FRUIT PEELS AND OTHER LIGNOCELLULOSIC BIOMASS AS A SOURCE OF SUGAR FOR FERMENTATION

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In this study, enzymatic hydrolysis of corn cobs, orange and pomegranate peels were evaluated. For the hydrolysis of corn cobs, effect of alkaline pretreatment was examined and it was found that glucose and reducing sugar yield (%) for the pretreated corn cobs increased from 7% to 21.5% and 14% to 33.6% respectively. Effect of cellobiase loading on hydrolysis efficiency was also investigated. It was observed that when cellobiase was added to hydrolysate in addition to cellulase, glucose and total reducing sugar yield increased from 11% to 20% and 21% to 33.5% respectively. On the other hand, although cellulase amount was increased successively while cellobiase was kept at constant load, glucose and total reducing sugar yield did not change and was constant at around 20% and 35% respectively. Due to their either low lignin content or high sugar content, alkaline pretreatment was not preferred for the pomegranate and orange peels. For the hydrolysis of these substrates, in addition to cellulolytic enzymes pectic enzymes were also used. It was found that when pectinase was used in addition to cellulase, conversion to glucose and total reducing sugar (%) increased significantly.

Keywords: Enzymatic hydrolysis, alkaline pretreatment, blood glucose monitor (BGM), corn cob, orange peel, pomegranate peel

ÖZ

FERMENTASYON İÇİN ŞEKER KAYNAĞI OLARAK MEYVE KABUKLARININ VE DİĞER LİGNOSELÜLOZİK BİYOKÜTLELERİN ENZİMATİK HİDROLİZİ

Poçan, Pelin
Yüksek Lisans, Gıda Mühendisliği Bölümü
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Bu çalışmada portakal kabuğu, nar kabuğu ve mısır koçanı gibi farklı substratların enzimatik hidrolizi incelenmiştir. Mısır koçanlarının enzimatik hidrolizinde alkali ön işleminin etkisi incelenmiş ve alkali ön işlem yapılan mısır koçanları hidroliz edildiğinde glikoz veriminin % 7 den %21.5'a, toplam indirgen şeker veriminin ise %14'den %33.6'ya arttığı gözlenmiştir. Ayrıca substratlar üzerinde sellobiaz enziminin etkisi de incelenmiştir. Selülaz enziminin yanısıra sellobiaz enzimi de eklenen hidrolizatlarda glikoz veriminin 11%'den 20% ye, toplam indirgen şeker miktarının ise %21'den %33.5'a yükseldiği gözlenmiştir.. Öte yandan sellobiaz enzimi sabit miktarda tutulup, selülaz enzimi artırıldığında şeker verimlerinde değişim gözlenmemiştir.Glikoz verimi %20, toplam indirgen şeker verimi ise %35 civarında sabit kalmıştır. Düşük lignin oranlarından dolayı, alkali ön işlem portakal ve nar kabuklarına uygulanmamıştır. Bu substratların enzimatik hidrolizi sırasında, selülolitik enzimlerin yanı sıra pektik enzimler de kullanılmıştır. Pektik enzimler de kullanıldığında glikoz ve toplam indirgen şeker veriminin, kullanılmayan hidrolizatlarda oranla arttığı görülmüştür.

Anahtar Kelimeler: Enzimatik Hidroliz, Alkali ön işlem, mısır koçanı, portakal kabuğu, nar kabuğu

In Memory of my Grandfather…

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INTRODUCTION

1.1 Need of Lignocellulose Conversion

Conversion of lignocellulosic biomass into valuable products such as biofuel has a significant effect in worldwide (Menon and Rao, 2012). Lignocellulosic biomass has an important role in the production of biofuel and reducing the world's dependency in consuming fossil fuel (Meng and Ragauskas, 2014). Utilizing fermentable sugars from lignocellulosic biomass which is known as the largest renewable carbohydrate source has become an important issue due to the economic dependency on fossil fuels and adverse effects of these fuels on environment and climate (Kristensen, 2009). Therefore, use of biomass will be a major contributor in the future supply of not only energy but also chemicals and materials (Kristensen, 2009).

As an example, lignocellulosic biomass can be used in lactic acid production. Cost of the raw material used in lactic acid fermentation is very important since it directly affects the economy of process (Abdel-Rahman et al., 2010). Use of refined sugars such as glucose, sucrose or starch can be very expensive when they are used as the feedstock for lactic acid fermentation (Abdel-Rahman et al., 2010). Thus, utilization of lignocellulosic biomass is a promising way to contribute the economy of fermentation process in terms of their high availability, sustainability and low cost (Abdel-Rahman et al., 2010).

In addition to production of biofuels and lactic acid, by-products could be used in different industrial areas too. Pectin obtained from citrus peel can be used as a gelling agent in food industry (Kuivanen et al., 2012). In the same way, limonene extracted

from citrus peel could be used as an antimicrobial and flavor compound in food industry (Kuivanen et al., 2012).

1.2 Characteristics of Lignocellulosic Materials

Plant biomass consists of mainly cellulose, hemicelluloses, lignin and smaller amounts of pectin, protein, ash and extractives (Kristensen, 2009). Cellulose, hemicellulose and lignin distribution show changes within different plants together with content of different sugars of hemicelluloses (Kristensen, 2009). Corn stover, straw, grass bagasse, rice straw, olive tree, softwood and hardwood are the examples of lignocellulosic feedstock (Rosgaard, 2007). The secondary cell wall of these feedstock is a dense network of polysaccharides (Rosgaard, 2007).

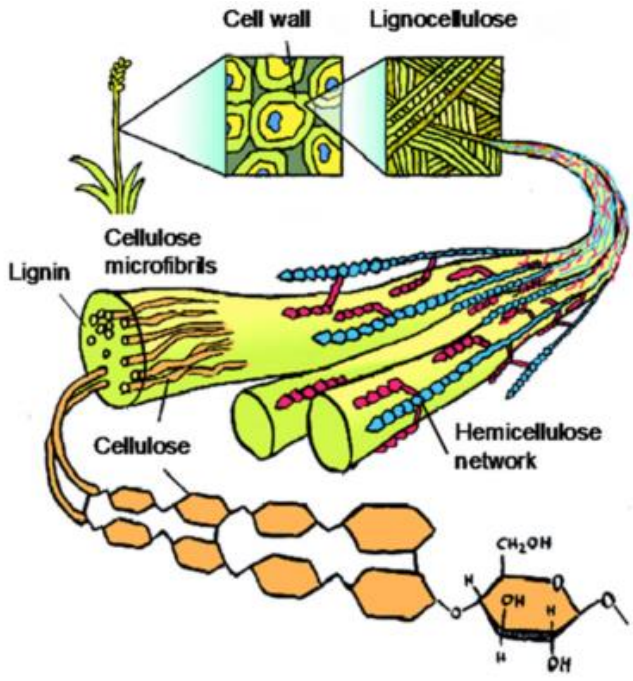

Figure 1.1 The structure of lignocellulose. (Hemicellulose is shown in blue and red, cellulose fibrils are shown in orange and lignin is shown as embedded in cellulose fibrils. (Rosgaard, 2007).

1.2.1 Cellulose

Cellulose is the most abundant polysaccharide since most of the lignocellulosic biomass is composed of 40-50% cellulose (Yavaş 2010). It is the main component of the plant cell wall and has a regular, fibrous structure (Kumar et al., 2009). It has a linear structure and consists of glucose monomers linked by ß–(l–4)-glycosidic bonds (Menon and Rao, 2012). It can be degraded into glucose and cellobioses through enzymatic hydrolysis. The formation of intra- and intermolecular hy

drogen bonds is enabled by the cellulose chains which have linear structure and formation of these bonds causes the aggregation of chains into elementary fibrils having a crystalline structure (Kristensen, 2009). Although elementary fibrils are crystalline, surface could have an amorphous structure (Kristensen, 2009). Moreover, it has been stated that amorphous cellulose could be degraded to cellobioses rapidly, while the hydrolysis of crystalline cellulose occurs slowly since the rate of hydrolysis is related with the crystallinity of cellulose and its polymerization degree (Mansfield et al., 1999).

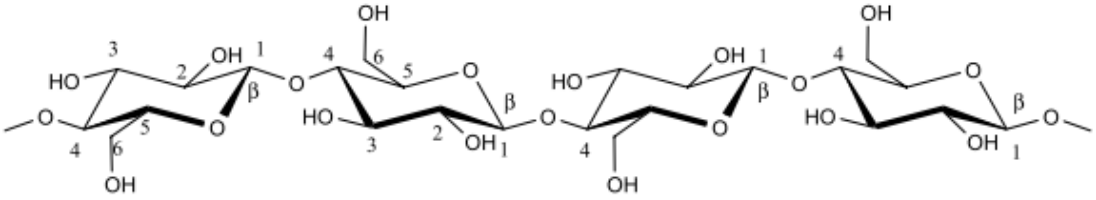

Figure 1.2 Structure of cellulose. (Laine, 2005)

1.2.2 Hemicellulose

Hemicellulose is different from cellulose since it does not have a homogeneous structure. Hemicellulose is a branched polymer containing D-glucose, D-xylose, L-arabinose, D-mannose, D-galactose and D-glucuronic acid (Menon and Rao, 2012). It is a linear polymer consisting of β-D-xylopyranosyl units linked by (1 - 4) glycosidic bonds (Polizeli et al., 2005). Hemicelluloses are classified according to main sugar unit so when a hemicellulose is hydrolyzed and produces mostly xylose, it is called as xylan; similarly hemicelluloses can be named as glucan, arabinan, mannans and galactans (Polizeli et al., 2005). The hemicellulose layer covers the cellulose fibrils and hemicellulose could be considered as the second abundant polysaccharide since plant cell wall is composed of nearly 20-40% hemicellulose (Yavaş, 2010). Unlike cellulose, hemicellulose does not have a rigid structure due to the existence of side chains which prevents the formation of semi-crystallinity (Yavaş, 2010).

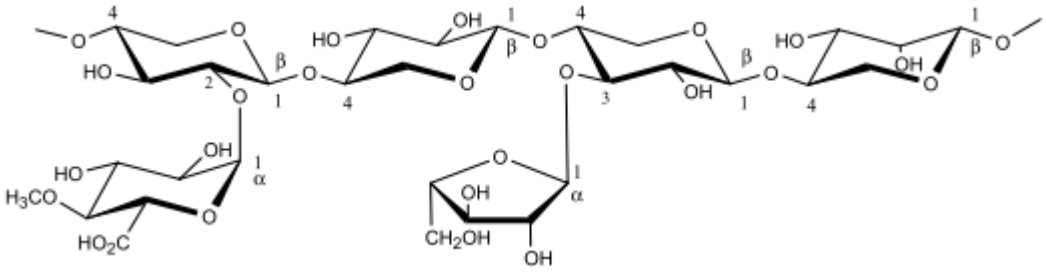

Figure 1.3 Structure of xylan known as major hemicellulose. (Laine, 2005)

1.2.3 Lignin

Lignin is the most abundant non-polysaccharide compound of lignocellulosic biomass and it has a complex network produced by polymerization of phenyl propane units

(Kristensen, 2009). It is a jelly-like compound and it is embedded in three dimension structure of cellulose-hemicellulose polysaccharides (Yan et al., 2015). It constitutes phenyl propane units such as syringyl, guaiacyl and p-hydroxyphenil units. (Yavaş, 2010). β–O–4 (β-aryl ether) linkage is the most frequently seen linkage in lignin. Cleaving of this bond is easier relative to other bonds found in lignin. On the other hand, β–5, β–β, 5–5, 5–O–4, and β–1 linkages are very resistance to chemical degradation so cleaving of these bonds is very difficult (Boerjan et al., 2003). Moreover, cellulose, xylose and lignin are linked to each other by ester, phenyl and covalent bonds and these bonds are also resistant to cleavage (Yavaş, 2010). Generally, herbaceous plants like grass have the lowest lignin content relative to hardwood and softwood (Kristensen, 2009). Lignin content of softwoods is higher than hardwoods (Rosgaard, 2007). Citrus peels were also reported as having low amount of lignin (Orozco et al., 2014). Plant cell wall contains nearly 20-30% lignin (Yavaş, 2010). Since lignin is a complex compound chemically related with benzene and its main role is to give rigidity to plant cell wall, it is considered as the most resistant portion of lignocellulosic biomass to hydrolysis.

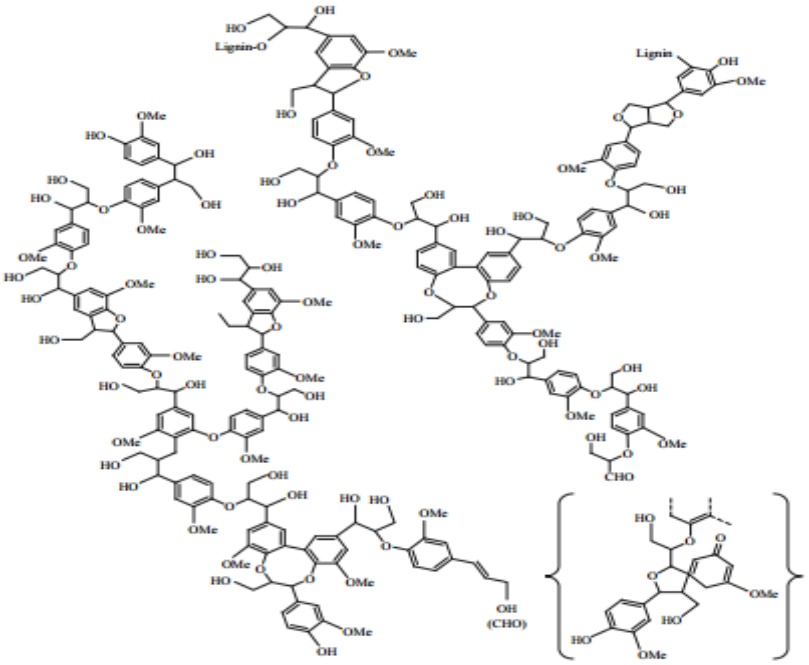

Figure 1.4 Structural model of a cork lignin. (Chen, 2014)

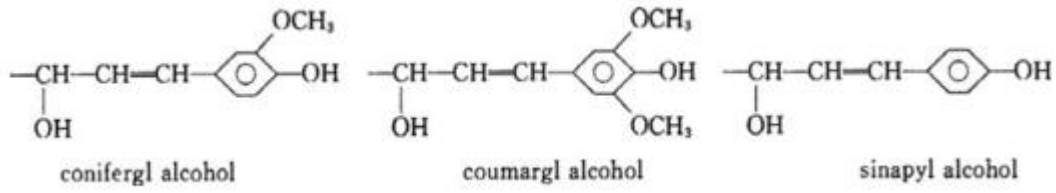

Figure 1.5 Basic structure of lignin. (Chen, 2014)

1.2.4 Pectin

Pectin is a complex natural molecular compound which is found highly in cell wall and middle lamella of higher plants (Wang et al., 2014). It is composed of α-1,4-linked D-galacturonic acid, which is methyl esterified and also contains side chains consists of several neutral sugars such as a L-arabinose, L-rhamnose, and D- galactose (Wang et al., 2014). It is usually used as the gelling and stabilizing agent in food industry like jam or yoghurt production (Yuliarti et al., 2015). Pectin is mainly obtained from apple pomace and citrus peel (Wang et al., 2014) . Pectin could be isolated and extracted by using different methods such as chemical, physical or enzymatic (Yuliarti et al., 2015). The use of chemical treatment like using dilute acid is generally not preferred. Therefore, enzymatic treatments using specific enzymes become advantageous due to formation of less chemical waste (Yuliarti et al., 2015). Pectin could be hydrolyzed to galacturonic acid units by pectinase enzyme.

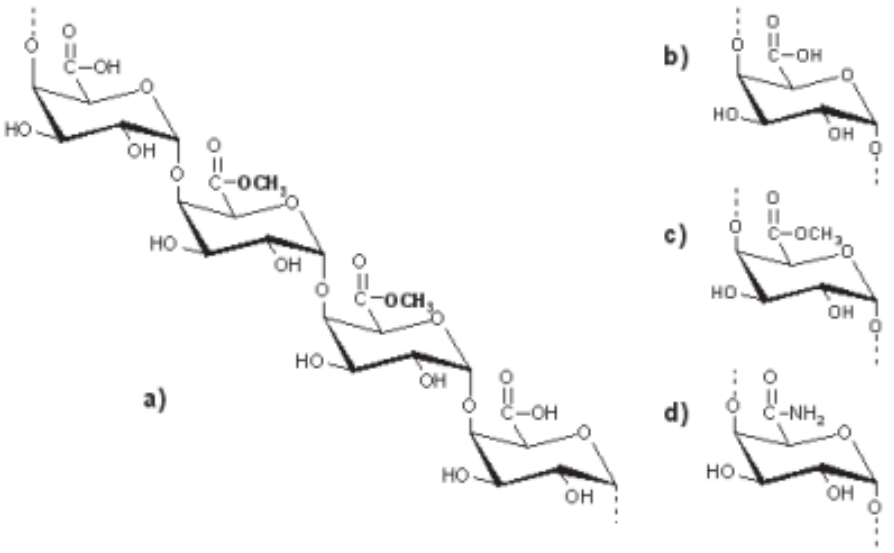

Figure 1.6 (a) A repeating segment of pectin molecule and functional groups: (b) carboxyl; (c) ester; (d) amide in pectin molecule. (Sriamornsak, 2003)

1.3 Examples for Lignocellulosic Feedstock Used In Enzymatic Hydrolysis

1.3.1 Sawdust

Sawdust is a byproduct of woody substances obtained as a result of the cutting or grinding process of woods. It could be obtained either from hard woods (Aspen, Beech, Poplar, etc.) or soft woods (Spruce, Pine, etc.) (Rosgaard, 2007). Rosgaard (2007) has mentioned that lignin content of soft woods is higher than the lignin content of hard woods. In previous studies compositional analysis of Meranti wood sawdust obtained from red *Meranti* species which was a well-known hard wood in Malaysia was performed prior to enzymatic hydrolysis (Rafiqul and Mimi Sakinah, 2012) and the composition is given in Table 1.1.

Table 1.1 Composition of Meranti wood sawdust (Rafiqul and Mimi Sakinah, 2012).

Constituents	**Content (% w/w)**
Cellulose (glucan)	41.06
Hemicellulose	30.64
Lignin (acid insoluble)	25.22
Extractives	3.08
Ash	0.43

.

1.3.2 Sun Flower Seed Husk

Sun flower seed husks are the byproducts of de-hulling process which is the critical economic process (Kamireddy et al., 2014). Hulls are lignocellulosic feedstock that contains cellulose, hemicellulose, lignin and organics forming ash (Kamireddy et al., 2014). Compositional analysis of sun flower seed husks was performed by Kamireddy et al. (2014) and results are given in table 1.2 as dry weight (%).

Table 1.2 Composition of Sun Flower Seed Husks (Kamireddy et al., 2014)

Constituents	**Content (% w/w)**
Cellulose	34
Hemicellulose	31
Lignin	22
Extractives	13
Ash	0.4

1.3.3 Corn Cobs

Corn cobs are lignocellulosic materials consisting of mostly arabinoglucuronoxylan (AGX) type of hemicelluloses (Bahcegul et al., 2013). It gains importance as a renewable raw material since it is a potential feedstock for the production of bioethanol, biodiesel and biogas (Pointner et al., 2014). Its carbohydrate composition is given in Table 1.3. However, it should be considered that different corn cobs which are harvested from different regions could have considerable changes in content.

Table 1.3 Carbohydrate Composition of Corn Cob (% dry weight) (Menon and Rao, 2012)

Constituents	**Content (% w/w)**
Cellulose	32.3-45.6%
Hemicellulose	39.8%
Lignin	6.7-13.9 %

1.3.4 Orange Peels

Orange juice production during year 2007-2008 in major producing countries was found as 2.3 million metric tons and amount of citrus peel was estimated as more than 15 million tones (Boluda-Aguilar et al., 2010). Citrus peel waste can be used as cattle feed either fresh or dried (Boluda-Aguilar et al., 2010). Moreover, it is a suitable substrate for the enzymatic hydrolysis reactions due to its low lignin content (Choi et al., 2013). Its carbohydrate content is given in Table 1.4.

Table 1.4 Carbohydrate Composition of Orange Peel (% dry weight) (Boluda-Aguilar and López-Gómez, 2013).

Constituents	**Content**
Cellulose	37.1
Hemicellulose	11
Lignin	7.5
Pectin	23

1.3.5 Pomegranate Peel

Pomegranate fruits contains mainly peel, seeds and the arils. Peels make up 50% of the fruit. (Alaa I, 2013). The peel consists of mainly phenolics, flavonoids, ellagitannins, and proanthocyanidin, complex polysaccharides and lots of minerals like potassium, nitrogen, calcium, magnesium, phosphorus and sodium. Its carbohydrate composition is given in Table 1.5

Table 1.5 Carbohydrate Composition of Pomegranate Peel (% dry weight)

(Alaa I, 2013)

Constituents	**Content**
Pectin	27.9
Hemicellulose	10.8
Cellulose	26.2
Lignin	5.7

Table 1.6 Comparative Constituent Content for Different Lignocellulosic Biomass (% dry weight)

Constituents	Cellulose	Hemicellulose	Lignin	Pectin	Reference
Saw dust	41.06	30.64	25.22	-	Rafiqul and Mimi Sakinah, 2012)
Corn cob	32.3-45.6	39.8	6.7-13.9	-	(Menon and Rao, 2012)
Sun flower seed husk	34	31	22	-	Kamireddy et al., 2014)
Orange peel	37.1	11	7.5	23	(Boluda-Aguilar and López-Gómez, 2013).
Pomegranate peel	26.2	10.8	5.7	27.9	(Alaa I, 2013

1.4 Pretreatment Methods for Lignocellulosic Biomass

Since lignocellulosic biomass has recalcitrance structure, prior to enzymatic hydrolysis, it is necessary to apply pretreatment methods to make them more susceptible to enzymes. With the help of pretreatment methods, solubilization or separation of the main components like cellulose, hemicellulose and lignin is achieved (Menon and Rao, 2012). The main aim of the pretreatment methods is to change or remove lignin and reduce the degree of crystallization of cellulose (Kristensen, 2009). According to Kristensen (2009), in cellulose hydrolysis, removal of lignin and in some cases removal of hemicellulose is vital to increase the hydrolysis efficiency. Ideal pretreatment method should degrade cellulose-hemicellulose matrix successfully, decrease crystallinity of cellulose leading to increase in amount of amorphous cellulose, release lignin from lignocellulose structure, increase porosity of biomass to achieve enzyme accessibility successfully (Yavaş, 2010). Moreover, formation of inhibitory compounds like hydroxymethylfurfural (HMF) and luvilinic acid should be prevented during the pretreatment (Yavaş, 2010).

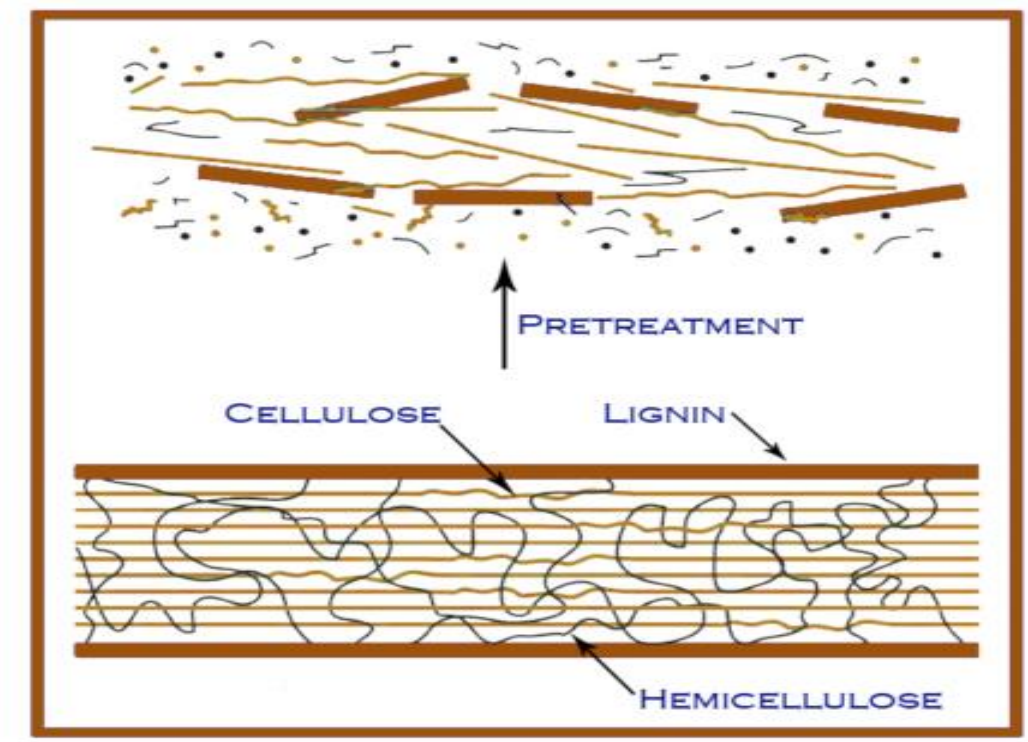

Figure 1.7 Pretreatment effect on the structure of lignocellulose (Perruzza, 2010)

Some of the most common pretreatment methods which can be commercialized are given in Table 1.6 (Menon and Rao, 2012).

Table 1.7 The Most Common Pretreatment Methods (Menon and Rao, 2012

Pretreatment method	**Sugar Yield**	**Inhibitor Formation**	**Advantages**	**Disadvantages**
Mechanical	Low	-	Decrease cellulose crystallinity	High power consumption
Acid	High	High	Cellulose & hemicellulose hydrolysis, changes lignin structure	Hazardous, corrosive and toxic
Alkali	High	High	Removal of hemicellulose and lignin, increases surface area for enzyme accessibility	Long residence time, irrecoverable salt formation
Organosolv	High	High	Hydrolysis of lignin and hemicellulose	Solvents need to be drained and evaporated
Wet oxidation	High or Low	-	Lignin removal, hemicellulose dissolution & cellulose decrystallization	-
Ozonolysis	High	Low	Reduction in lignin content,	Large amount of ozone is needed
CO_2 Explosion	High	Low	Decrystallization of cellulose and hemicellulose removal	No lignin modification
Steam Explosion	High	High	Hemicellulose removal and alteration in lignin structure	Incomplete destruction lignin-carbohydrate matrix
Ionic Liquids	High or Low	Low	Cellulose dissolution, increased accessibility to cellulase	Still in initial stages
Ammonia Fiber Exclusion(AFEX)	High	Low	Lignin & hemicellulose removal	Not efficient for lignin-rich biomass

Type of pretreatment method could change depending on the type of the feedstock, environmental impact and economy of the process. Pretreatment methods are classified as physical, physico-chemical, chemical and biological pretreatment methods.

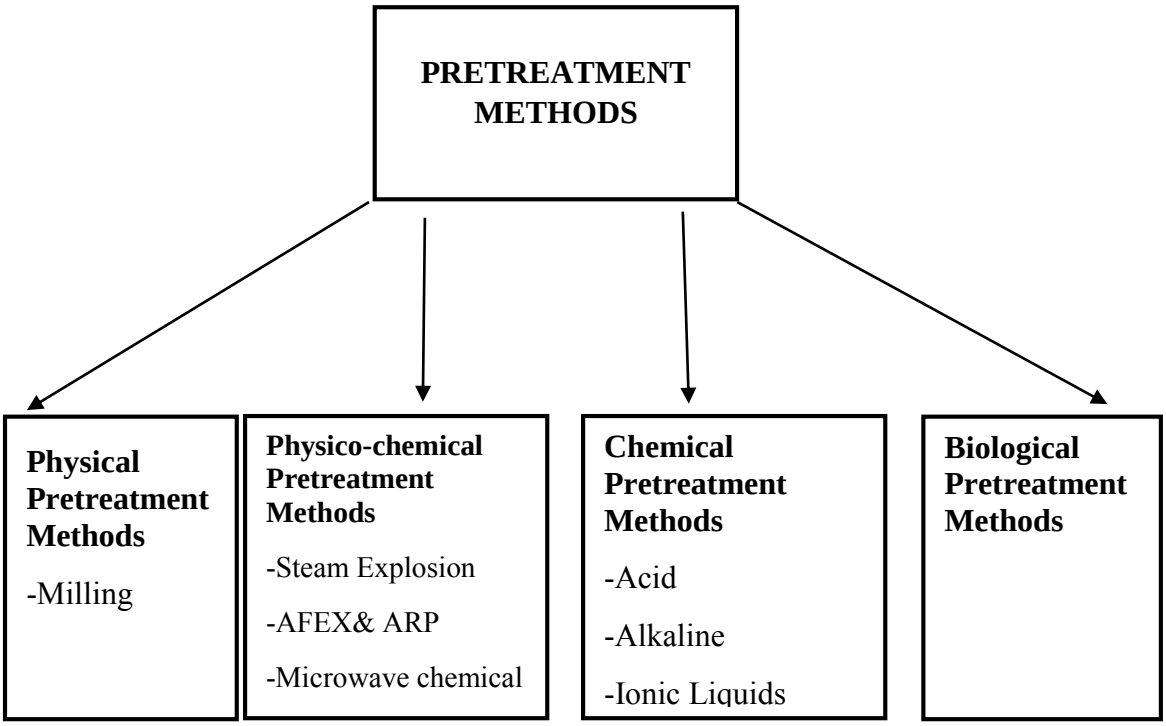

Figure 1.8 Some examples of pretreatment methods

1.4.1 Physical Pretreatment Methods

Particle size reduction could be classified as a physical pretreatment method. It is an important step in order to convert lignocellulosic biomass to valuable products. Most of lignocellulosic biomass requires mechanical size reduction like grinding and milling (Menon and Rao, 2012). According to Menon (2012), reduction in particle size increases the hydrolysis efficiency since it decreases the crystallinity degree and improves the characteristics of mass transfer. Furthermore, affinity between cellulose and enzymes could be improved with the help of particle size reduction leading to increase in hydrolysis rate (Yeh et al., 2010). Although it is an advantageous method because of high hydrolysis rates, it is not desirable in some cases since it requires high energy. As the particle size decreases, energy requirement increases for the lignocellulosic biomass (Miao et al., 2011).

1.4.2 Physico-chemical Pretreatment Methods

Physico-chemical pretreatment methods combine both chemical and physical pretreatment methods and steam explosion, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP) and microwave chemical pretreatment are examples for the physico-chemical pretreatment methods (Menon and Rao, 2012).

In steam explosion, biomass exposed to high pressure saturated steam and then pressure is decreased suddenly (Menon and Rao, 2012). In this method, biomass is treated with temperature in the range of 160-260ºC with a corresponding pressure, 0.69-4.83 MPa leading to hemicellulose degradation and lignin transformation due to high pressure and temperature and then biomass is exposed to atmospheric pressure (Menon and Rao, 2012). The sudden decrease in pressure cause defibrillation of cellulose bundles leading to better accessibility of cellulose to enzymes (Stelte, 2013).

Ammonia fiber explosion method (AFEX) is very similar to steam explosion since for both methods high pressure and high temperature are important parameters (Menon and Rao, 2012). However, in AFEX , biomass was exposed to liquid ammonia at high pressure and temperature for certain period of time (Menon and Rao, 2012). AFEX is a promising pretreatment method since it enhances decrystallization of cellulose, partial hemicellulose depolymerization and decreases the lignin recalcitrance of the substrates (Kim et al., 2009).

Another physico-chemical pretreatment method is ammonia recycle percolation method (ARP). Although it is a similar method with ammonia fiber explosion method (AFEX), in this process, aqueous ammonia (10-15 wt. %) is used instead of liquid ammonia and after the pretreatment, ammonia is recycled (Menon and Rao, 2012).

Microwave chemical pretreatment method is also classified as a physico-chemical pretreatment method. It is a more effective method than conventional heating chemical pretreatment methods due to reduced reaction time (Menon and Rao, 2012). Carbon materials can absorb microwave energy easily thus microwave heating is a suitable for the pretreatment of lignocelluloses (Diaz et al., 2015). It is possible to use different solvents in microwave pretreatment although solvents which have high boiling point

like glycerol, tetrahydrofurfuryl alcohol, ethylene glycol are preferable since they improve delignification (Diaz et al., 2015). Diaz et al. (2015) has mentioned that microwave pretreatment is a promising method in biomass studies since it decreases polymerization and crystallinity of cellulose, causes lignin depolymerization and increases specific surface area in a very short time.

1.4.3 Chemical Pretreatment Methods

Chemical pretreatment methods also takes an important role in the conversion of lignocellulosic biomass into valuable products. According to the study of Menon & Rao (2012), main aim of the chemical pretreatment methods is to remove lignin and/or hemicellulose and decrease the crystallization degree of cellulose. The most well-known chemical pretreatment methods are acid and alkaline pretreatment. Acid pretreatment could be achieved by using dilute acid or concentrated acid (Menon and Rao, 2012). Rosgaard (2007) indicated that by using acid pretreatment hemicellulose part of lignocellulosic biomass becomes soluble and remaining fraction is composed of mainly cellulose and lignin. Acid pretreatments could be used as a part of whole process to fractionate the lignocellulosic biomass because of its ability to remove hemicellulose (Menon and Rao, 2012).

Alkaline pretreatment is similar to acid pretreatment due to use of dilute or concentrated base (Yavaş, 2010). Sodium hydroxide and calcium hydroxide are the common bases used in alkaline pretreatments. It was indicated that sodium hydroxide is capable to disrupt lignin structure of lignocellulosic biomass and improves the accessibility of enzymes to hemicellulose and cellulose (Kristensen, 2009). It was found that alkaline pretreatment causes chemical and morphological changes in the structure of rice straws leading to increase in enzyme accessibility as seen from SEM images (Fig 1.9) (Remli et al., 2014). Moreover, it was found that sodium hydroxide loading is the most important parameter affecting enzymatic digestibility (Chen et al., 2013). According to findings of Wang et al. (2012), relative to untreated corn stover, nearly 4-fold increase had been observed in conversion of cellulose to glucose in

alkaline pretreated corn stovers. In some cases, combination of different pretreatment methods becomes more effective to increase hydrolysis yield. For instance, if acid pretreatment (removal of hemicellulose) is used together with alkaline pretreatment (removal of lignin), relatively poor cellulose which is very susceptible to enzymatic hydrolysis could be obtained (Menon and Rao, 2012).

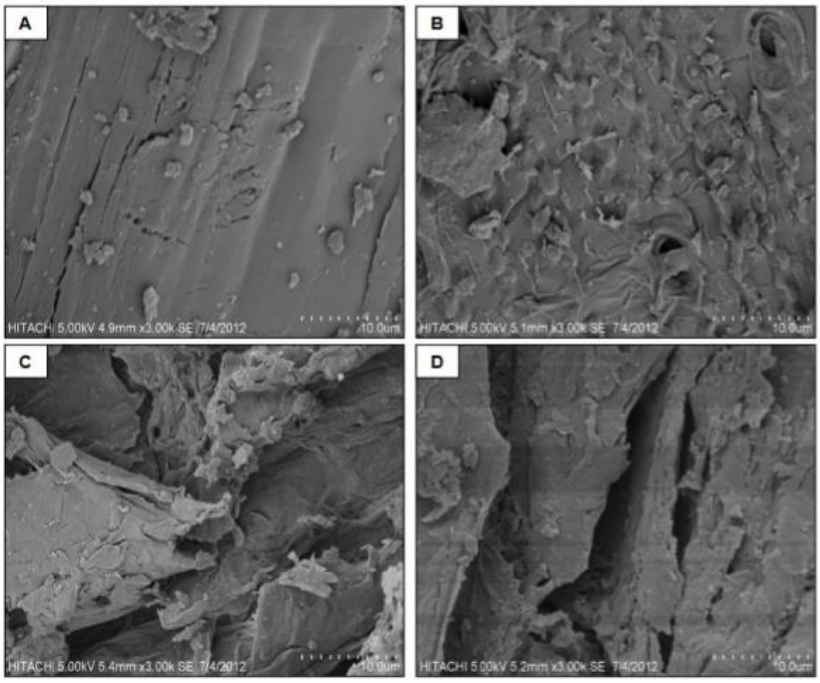

Figure 1.9 Scanning electron microscope images of rice straw (3000×). (A) Untreated rice straw; (B) pretreated with NaOH; (C) pretreated with KOH; (D) pretreated with $Ca(OH)_2$ (Remli et al., 2014)

In addition to these well-known chemical pretreatment methods, new class of solvent which is known as ionic liquids/green solvents has emerged recently (Menon and Rao, 2012). Ionic liquids have some advantages such as high thermal stability and low volatility which give them superiority over other conventional solvents that are toxic

and instable and work at severe conditions (Haykir et al., 2013). Menon & Rao (2012) has reported that use of 1-butyl- 3-methylimidazolium chloride (BMIMCl) which is known as ionic liquid for pretreatment, enzymatic hydrolysis rate of pretreated Avicel-PH-101 increased by 50 fold compared to untreated Avicel. Ionic liquid pretreatment has also important effect on crystallinity of cellulose since it is capable to convert crystalline region to amorphous region so it can ease the accessibility of cellulase enzymes (Haykir et al., 2013). Haykir et al. (2013) has mentioned that according to XRD patterns of the samples, for the cotton stalks pretreated with 1-ethyl-3-methyl imidazolium acetate (EMIMAc) ionic liquid ,the peaks were shifted to lower Bragg angles and got weaker compared to untreated cotton stalks indicating that treated samples gained amorphous property which has more susceptible to enzymatic hydrolysis.

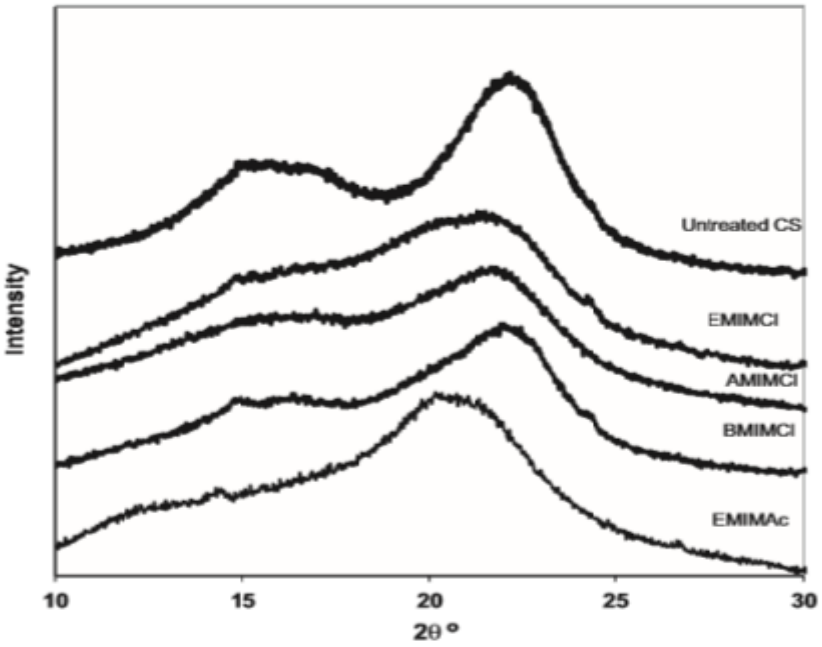

Figure 1.10 XRD patterns for the untreated cotton stalk and cotton stalk pretreated with different ionic liquids (Haykir et al., 2013).

1.4.4 Biological Pretreatment Methods

Biological pretreatment method uses microorganisms such as white- brown- soft-rot fungi and bacteria which are able to degrade woody substances in order to change the chemical structure of lignocelluloses and make them more susceptible to enzymatic hydrolysis (Menon and Rao, 2012). It has been reported that while brown rot fungi can degrade only cellulose , white-rot fungi are able to degrade cellulose and lignin (Sun and Cheng, 2002). Biological pretreatment methods are favorable since they need low energy and they do not need severe conditions like chemical pretreatments. (Yavaş, 2010).

1.5 Enzymatic Hydrolysis of Lignocellulosic Biomass

After applying necessary pretreatment methods to lignocellulosic biomass, hydrolysis is conducted to release the monomeric sugars for the subsequent fermentation step. For that reason, specific hydrolytic enzymes are used to degrade the polymers into their monomers. Since efficiency of the enzymes are strongly related with the process parameters like pH, temperature and time, control of these variable is vital during the hydrolysis. Optimum temperature and pH for the enzymatic hydrolysis of sugar cane leaves were determined as 50 ºC and 4.5 respectively (Hari Krishna et al., 1998). In the same study, optimum reaction time was found as 48 hours. However, in another study, it was indicated that for the corn stover hydrolysis, 1 day (24 hour) hydrolysis time was particularly chosen since this time interval was more representative for the hydrolysis rate (Pryor and Nahar, 2015). Substrate and enzyme loadings are other important factors affecting the hydrolysis efficiency. Generally, enzymatic hydrolysis of lignocellulose is preferred by using high solid load industrially, since it decreases the production cost (Olsen et al., 2014). On the other hand, working with high solid loads have some drawback affecting hydrolysis efficiency. Krishna et al. (1998) indicated that maximum solid load should not exceed 10% due to problems in proper mixing of hydrolysates which caused the reduction in enzyme accessibility to the substrates. Furthermore, Kristensen et al. (2009) stated that as the solid load increases,

product inhibition problem also increased leading to decrease in efficiency of the enzymes. Enzyme load is another important parameter affecting the enzymatic hydrolysis efficiency.

The enzymatic hydrolysis rate of lignocellulosic materials is lower than hydrolysis rate of other substrates like starch. This could be explained mainly by three reasons. (Yavaş, 2010). Firstly, in the structure of lignocellulosic materials β (1, 4) linkages are present while α (1, 4) linkages are present in the structure of starch and it is known that β (1, 4) linkages are more resistant to hydrolysis than α (1.4) linkages. Secondly, lignin that surrounds the hemicellulose and cellulose fraction of lignocelluloses poses an obstacle for the enzymatic hydrolysis. On the other hand, lignin does not exist in starch structure. And lastly, lignocellulosic material have more complex structure than starch. This 3-D structure prevents the accessibility of enzymes to lignocellulose matrix. On the other hand, linear structure of starch molecule ensures accessibility of the enzymes (Yavaş, 2010).

Presence of lignin in lignocellulosic material has an adverse effect like unproductive enzyme adsorption to lignin. (Kristensen, 2009). Unproductive binding is a big problem for the cellulase enzymes decreasing the rate of hydrolysis (Bahçegül, 2013). Unproductive binding means no product formation occurs as a result of binding of lignin to the cellulase enzymes and results in slower hydrolysis rates and lower glucose yields. (Bahçegül, 2013).

In some cases, addition of non-ionic surfactants like Tween 80 or Tween 20 can decrease the possibility of unproductive enzyme adsorption by binding lignin and preventing its binding to lignocellulosic substrate (Kristensen, 2009). Kristensen (2009) has reported that ethylene oxide polymers like polyethylene glycol (PEG) is also capable to bind lignin like non-ionic surfactants. PEG can bind to lignin with the help of hydrogen and hydrophobic bonding thus it prevent lignin binding to the enzymes. Moreover, it is a cheaper commodity product compared to non-ionic surfactants like Tween 80 or Tween 20.

1.6 Enzymes Used in Hydrolysis of Lignocellulosic Biomass

Enzyme production and utilization of these enzymes for the purpose of hydrolysis of lignocellulosic biomass for industrial applications is a relatively new and promising field in biotechnology. The most commonly used enzymes for this purpose is cellulase and hemicellulase (Yavaş, 2010). Depending on the composition of lignocellulosic biomass, enzymes that will be used in hydrolysis differ. For citrus peel hydrolysis, in addition to cellulase and hemicellulose enzymes, pectinase is also required. Therefore, both cellulolytic and pectic degradation is carried out together.

1.6.1 Cellulase

Cellulase is known as the cellulose hydrolyzing enzyme and it could be divided into three main groups as endoglucanases (EC 3.2.1.4), cellobiohydrolases (exoglucanases) (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Yeh et al., 2010). The endoglucanases cleaves the internal bonds of cellulose. On the other hand, cellobiohydrolases cleaves the chains of cellulose from its end point and releases the cellobioses. The β-glucosidase enzyme is only active on cellobiose and degrades cellobiose to glucose units (Yeh et al., 2010).

Cellulase could be obtained from some aerobic bacteria, anaerobic bacteria (e.g. *Clostridium thermocellum*) and mostly some types of fungi such as *Trichoderma resei* and *Aspergillium niger* which are capable to convert cellulose to its monomers (Yavaş, 2010). It was stated that *Trichoderma resei* is the most widely used microorganism for industrial applications since it contains plentiful cellulase enzymes. Its cellulase contains two endoglucanases, two cellobiohydrolases and cellobiase (β-glucosidase).

1.6.2 Hemicellulase

Hemicellulase enzyme is also known as xylanase enzyme. Kristensen (2009) indicated

that since hemicelluloses are heterogeneous polymers with plentiful side groups, hemicellulolytic system is more complex. Hemicellulase consists of endoxylanase (EC 3.2.1.8) which is capable to hydrolyze internal bonds found in xylan chain, xylosidase (EC 3.2.1.37) which attacks xylooligosaccarides and produces xylose, endomannases (EC 3.2.1.78) which breaks internal bonds in mannan and mannosidases (EC 3.2.1.25) which degrades monooligosaccarides to mannose. In addition to this, side groups are also degraded by several enzymes such as galactosidases (EC 3.2.1.22), arabinofuranosidases (EC 3.2.1.55) (Kristensen, 2009) . All the xylanolytic enzymes could be produced by certain microorganisms such as fungi, bacteria and actinomycetes (Motta et al., 2013). The most common xylanase producer microorganisms are known as *Aspergillus, Trichoderma, Streptomyces, Fibrobacters, Clostridia* and *Bacillus* (Motta et al., 2013).

1.6.3 Pectinase

Pectinase (E.C.3.2.1.15) is a commonly used commercial enzyme breaking down pectin which is a polysaccharide found in plant cell walls. It converts polygalacturonic acid units to monogalacturonic acid by opening glycosidic bonds. It could be obtained from both plants and microorganisms. The strains of *Penicillium* spp and *Aspergillus* sps are good examples to pectinase producer microorganisms (V and V, 2014). Pectinolytic enzymes could be classified in three main groups as protopectinases, esterases and depolymerases (Jayani et al., 2005). Protopectinases hydrolyzes protopectin which has insoluble form and produces soluble polymerized pectin while esterases performs de-esterification of pectin by removing the methoxy esters (Jayani et al., 2005). Depolymerizing enzymes breaks the glycosidic bonds of pectin with the help of hydrolysis (hydrolase) or by using β-elimination (lyases) (Kumar et al., 2012). Depolymerases could be examined in four different groups according to substrate preference of enzymes and cleavage mechanism. Polygalacturonase and polymethylgalacturonase degrades pectin and pectate with the help of hydrolysis mechanism while polygalacturonate lyase and poly-methylgalacturonate lyase degrade

pectate and pectin by β elimination, respectively (Jayani et al., 2005). Jayani et al. (2005) has mentioned that Polygalacturonase is one of the most well-known pectinolytic enzyme which is responsible from the cleavage of polygalacturonic acid chain with the help of water introduction across the oxygen bridge.

Since cellulose microfibrils in primary cell wall are bound to hemicellulose-pectin network, enzyme complexes that contain pectinase has a vital role to conduct total hydrolysis of plant polysaccharides since they achieve cell separation and allows access of cellulase to cellulose (Haltmeier et al., 1983).

1.7 Commercial Enzymes Used in Lignocellulose Hydrolysis

1.7.1 Celluclast 1.5 L

Celluclast 1.5 L is a commercial enzyme preparation that contains mainly cellulase (endo-glucanase unit). Endo-glucanase degrades the cellulose chain internally and oligosaccharides, cellobiose and glucose are produced (Yuliarti et al., 2011). Its declared activity is 700 EGU/g and it is produced from *Trichoderma Reesei*. (Product Data Sheet Celluclast 1.5 L, Novozymes). In addition to cellulase activity, this enzyme also has xylanase activity but it is very small compared to cellulase activity (Khan, 2010). Moreover, as well as endoglucanase activity it has exoglucanase activity (Khan, 2010).

1.7.2 Novozymes 188

Novozymes 188 is a widely used commercial enzyme mixture from Novozymes Company(Denmark) as the source of β-glucosidase mainly (Rosales-Calderon et al., 2014). In most of the hydrolysis reactions use of Celluclast 1.5L and Novozymes 188 together is necessary due to their complementary action (Rosales-Calderon et al., 2014).

It is produced from *Aspergillus Niger* and its activity) was reported as 626.4 CBU/ml (Rosales-Calderon et al., 2014).

1.7.3 Cellic CTec 2

Cellic CTec 2 is one of the new enzyme cocktails of Novozyme Company (Denmark) with higher enzyme activities. In addition cellulase enzymes it contains increased concentration of β-glucosidase with higher tolerance for product inhibition and with the addition of xylanase to enzyme mixtures hemicellulose degradation was also performed successfully (Novozymes, 2010). Its activity was determined as 168.8 FPU/ml (Kodaganti, 2011).

1.7.4 Biogazyme 2x

Biogazyme 2x is a cocktail enzyme mixture that is in powder form produced from ASA (Germany). It has high activities of cellulase and hemicellulase like mannanase, xylanase and ß-glucanase for the degradation of cellulose and hemicellulose. In addition to exo-cellulase and endo-cellulase activity it has also high cellobiase (β-glucosidase) activity. It is produced from *Trichoderma spp*. (Product Data Sheet Biogazyme 2x, ASA).

Table 1.8 Enzyme Activities of Commercial Enzyme Products

(Enzyme activities were shown in units/ml)

Name	**Endo-cellulase**	**Exo-cellulase**	**Cellobiase (β-glucosidase)**	**Xylanase**	**β-1,3 (4) Glucanase**
Celluclast 1.5 L	38.100	141	22	6.21	72.5
Novozyme 188	202	12	189	527	204
Biogazyme 2x	30.0000	840	225	311.000	36.700

(Product Data Sheet Biogazyme 2x, ASA)

1.7.5 Pectinex Ultra SP-L

Pectinex Ultra SP-L is a pectinase enzyme produced by Novozyme Company. Its declared enzyme is Polygalacturonase and its declared activity is 9500 PGU/ ml. It is produced from *Aspergillus aculeatus* (Product Data Sheet Pectinex Ultra SP-L, Novozyme). In previous studied it was also reported that Pectinex Ultra SP-L has 8.4 IU/ml β-galactosidase activity (One IU is defined as the amount of enzyme producing 1 mol of 6-galactosyl lactose per minute from lactose (5%, w/v) at pH 4.5 at 60 ºC.) (Aslan and Tanriseven, 2007).

1.8 Sugar Analysis of the Hydrolysates by Using Different Methods

As a result of the enzymatic hydrolysis, fermentable sugars are released into the hydrolysates. Measurement of the concentration of these sugars could be determined by using different methods like HPLC (High Pressure Liquid Chromatography) DNS

(Dinitrosalicylic acid), BGM (Blood glucose Monitor) and Nelson-Somogyi method.

1.8.1 DNS (Dinitrosalicylic acid)

For the determination of reducing sugar content of hydrolysates, 3-5-Dinitrosalicylic acid is widely used reagent (Saqib and Whitney, 2011).For the determination of reducing sugar, dinitrosalicylic acid reagent which is composed of dinitrosalicylic acid, Rochelle salt, phenol, sodium bisulfite and sodium hydroxide were used (Miller,1959). Use of DNS is not only a common method to monitor the reducing sugar content of hydrolysates, but also it is a recommended assay by the International Union of Pure and Applied Chemistry (IUPAC) (Saqib and Whitney, 2011).

Sugars could be classified as reducing sugar if they have an aldehyde group and this group exists in an open chain structure of the sugar molecule (Saqib and Whitney, 2011). Since, DNS reagent only reacts with aldehyde groups of the sugars, it is used in determination of reducing sugar concentration. According to study of Saqib and Whithey (2011), it was stated that the basis of DNS method depends on the reduction of 3,5- dinitrosalicylic acid (DNS) to 3-amino-5-nitro-salicylic acid (ANS) and during the test, aldehyde group of the sugars is oxidized to carboxylic acid (Saqib and Whitney, 2011). As a result of these reactions, the yellow color of DNS reagent turns into reddish color and reducing sugar concentration is determined by using a spectrophotometer.

1.8.2 BGM (Blood Glucose Monitor)

DNS method is capable to measure concentration of total reducing sugar content. However, it is a less sensitive method compared to HPLC (High Pressure Liquid Chromatography) since it cannot measure the concentration of sugars separately. In addition to these well-known methods, it is possible to measure the glucose amount of hydrolytes with the help of BGM (Blood Glucose Monitor). Since it’s a rapid (possible

to measure glucose amount of sample within 5-8 seconds) and low cost method, it is accepted as a practical step on lignocellulosic biomass research (Bahcegul et al., 2011).

Bahçegül et al. (2011) stated that although this device was designed to measure glucose level in human blood, it was also applicable to measure the glucose concentration of hydrolysates after enzymatic hydrolysis. It was also reported that when this device was used to monitor the in vitro digestion of starch, it was found that BGM gives very similar results with spectrophotometric methods which was based on glucose oxidase (Bahcegul et al., 2011).

1.9 Aim of the Study

This is a comparative study that investigates the enzymatic hydrolysis of different biomass at different conditions. Effect of alkaline pretreatment on different lignocellulosic biomass like corn cob and sawdust was explored. Effect of different enzyme loadings and synergistic effects of different enzymes (cellulase and β-glucosidase) were examined on alkali pretreated corn cobs. Effect of Tween 80 and product inhibition was also tested for alkaline pretreated corn cobs. In addition, enzymatic hydrolysis of agro-industrial waste like orange and pomegranate peels were examined. Due to their low lignin content, alkaline pretreatment was not applied for fruit peels. For these substrates, effect of pectinolytic enzymes together with cellulolytic enzymes were determined. Among these substrates, the most suitable one for enzymatic hydrolysis was tried to found considering process economy. Efficiency of the hydrolysis was determined using DNS (Dinitrosalicylic acid) and BGM (Blood glucose monitor) methods. DNS method measured the total reducing sugar concentration; BGM method detected the glucose amount of the hydrolysate.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Corn cobs, sun flower seed husks, saw dust and pomegranate peels were obtained from local markets in Ankara, Turkey, dried at 100ºC and milled into a proper particle size (10 μm-2mm) (Laboratory mill, Philadelphia, USA.) prior to pretreatment. Fresh orange peels obtained from Biopolis Valencia, Spain were grinded by a food processor (K 1190 Arçelik-Robolio, Turkey). Tri-sodium citrate dihydrate, citric acid monohydrate, sodium potassium tartarate, sodium hydroxide (NaOH), D-glucose and Tween 80 were purchased from Merck (Darmstadt, Germany). 3-5 Dinitrosalicylic acid, sodium sulfate and phenol were purchased from Sigma-Aldrich (St. Lois, MO, USA). One Touch Select blood glucose monitor and One Touch Select test strips (Life Scan, Inc. Milpitos, CA 95035) were used for BGM experiments.

Enzymes Celluclast1.5L (Cellulase), Novozyme 188 (β-glucosidase), Ctec2 and Pectinex Ultra SP-L (PC) were kindly provided by Novozymes (Bagsvaerd, Denmark). Biogazyme 2x (BG) was kindly provided by ASA Spezialenzyme GmbhH (Wolfenbüttel, Germany).

2.2 Moisture Content Determination

Moisture content of the samples used in the study was determined by using an infrared moisture analyzer (Radwag, MAC 50).

2.3 Alkaline Pretreatment

Alkaline pretreatment was conducted for corn cobs and saw dust due to their high lignin content. For alkaline pretreatment, the procedure by Bahcegul et al., (2011) was followed. Five grams of milled sample was autoclaved at 121ºC for 1 hour using 2% NaOH (w/v) solution with 10% solid loading. Following the pretreatment, the suspension was cooled and the solid part was filtered. Remaining biomass was washed with 200 ml distilled water twice and filtered after each washing step. After last washing, remaining solid part was again put into distilled water and pH of the suspension was adjusted to 4.8 by using acetic acid. Following pH adjustment, the solid part was again filtered and separated from the suspension. Before the enzymatic hydrolysis, remaining biomass was dried in an incubator at 60ºC for 16 hours and weighed for the desired amount for the hydrolysis experiments. Biomass amount recovered after pretreatment was calculated as follows:

Solid recovery (%) = $\frac{\mathrm{WPRT}}{WUT} \times 100$ (Bahcegul et al., 2011) (**1**)

W_{PRT} indicated the weight of biomass recovered after pretreatment and W_{UT} denoted the weight of the untreated biomass exposed to pretreatment.

Appearance of control (untreated) and alkaline pretreated corn cobs and saw dust were shown in Appendix D as Fig D.1 and Fig. D.2 respectively.

2.4 Limonene Removal

Liquid boiling water extraction was used to remove limonene from the orange peels. 100 grams of orange peel was soaked in 500 ml water at 95ºC for 24 hours. Peels were dried after extraction. Appearance of control (untreated) and liquid boiling water treated orange peels was shown in Appendix D as Fig D.3.

2.5 Enzymatic Hydrolysis

For all substrates, enzymatic hydrolysis was conducted in a shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) at 50ºC, 150 rpm for 24 hours using 0.05 M sodium citrate buffer solution. 1 day (24 hour) hydrolysis time was particularly chosen since this time interval was more representative for the hydrolysis rate (Pryor and Nahar, 2015). Time dependent hydrolysis experiments were also conducted. As will be seen, for the substrates studied, different loadings were used. Solid/liquid ratios for the substrates were determined based on preliminary trials. The maximum solid loadings in which the experiments could be conducted appropriately (proper mixing, insignificant swelling etc.) were chosen.

For corn cob and saw dust hydrolysis, Celluclast 1.5 L and Novozyme 188 were used whereas for the pomegranate and orange peels hydrolysis was conducted using Biogazyme x2 and Pectinex Ultra SP-L. Biogazyme x2 is a cellulolytic enzyme can be used for fruit peels as well as other lignocellulosic feedstock. Ctec2 enzyme was also used for the enzymatic hydrolysis of corn cobs. Enzymatic hydrolysis was stopped by immersing the samples in boiling water for 5 min. Following hydrolysis; samples were centrifuged at 5000 rpm for 3 minutes. After centrifugation, supernatants were taken for sugar analysis. Enzymatic hydrolysis was conducted in duplicates. Experiments were repeated if the coefficient of variation in the results were higher than 5%.

Glucose and reducing sugar yield (%) defined as the amount of glucose and total reducing sugar respectively obtained from initial amount of corn cobs on dry weight basis upon pretreatment and following enzymatic hydrolysis was calculated for alkaline pretreated samples (Bahcegul et al., 2011). For orange and pomegranate peel hydrolysis, since no pretreatment was applied, *conversion to glucose and reducing sugar* (%) which was defined as the amount of glucose and reducing sugar obtained upon enzymatic hydrolysis were calculated. Conversion to sugar (glucose or total reducing sugar) (%) and sugar yield were calculated as follows:

$$\%\ Glucose/reducing\ sugar\ yield = SR \times \frac{C}{100} \qquad (2)$$

$$\%\ Conversion\ to\ glucose/reducing\ sugar = \frac{C \times V}{W} \times 100 \quad (\mathbf{3})$$

C is the glucose/ reducing sugar concentration in the enzymatic hydrolysate; V is the total volume of the hydrolysis solution; W represents the weight of biomass subjected to enzymatic hydrolysis; SR is the solid recovery (%) calculated from Eq. 1.

2.5.1 Enzymatic Hydrolysis of Corn Cobs and Saw Dust

To confirm the effect of alkaline pretreatment on enzymatic hydrolysis of corn cobs and saw dust and to find the most suitable substrate for the alkaline pretreatment, untreated (control) and alkaline pretreated milled samples were hydrolyzed at fixed enzyme volumes of: 150 ul Celluclast 1.5 L and 150 ul Novozyme 188 at a solid/liquid ratio of 3%. As will be explained in the results and discussion section, due to low sugar yields sun flower seed husks and saw dust were not used for further experiments.

2.5.2 Enzymatic Hydrolysis of Corn Cobs

Alkaline pretreatment was applied to all corn cobs prior to enzymatic hydrolysis.

Celluclast 1.5 L is a cellulase obtained from *Trichoderma reesei* ATCCC 26921 whereas Novozyme 188 is a cellobiase obtained from *Aspergillus Niger*. Celluclast 1.5 L is the main enzyme used in enzymatic hydrolysis of cellulose rich biomass. To observe the effect of cellobiase on hydrolysis, reaction was carried out using a fixed a volume of Celluclast1.5 L (150 ul) with and without Novozyme 188 (150 ul). Hydrolysis was again conducted at a fixed solid/liquid ratio (3%).

In order to see the effect of cellulase loading on alkali-pretreated corn cobs, Celluclast 1.5 L was used at different volumes ranging from 150-600 ul and Novozyme 188 was used at a fixed volume of 200 ul at a constant solid/liquid ratio (3%).

Cellic Ctec2 which is a cocktail enzyme (enzyme mixture with a high activity of cellulase, β-glucosidase and xylanase) was also used at different loads ranging from

10-300 ul to test its effect on enzymatic hydrolysis efficiency of alkali pretreated corn cobs with a fixed solid/liquid ratio (3%).

In order to find the time in which sugar yield reaches a plateau, time dependent data were also taken for every 3 hours for the alkali pretreated corn cobs which were hydrolyzed by using fixed load of (50 ul Ctec2) at a constant solid/liquid ratio (3%).

2.5.3 Effect of Surfactant (Tween 80) on Corn Cob Hydrolysis

To understand the effect of Tween 80 on corn cob hydrolysis, firstly tween 80 was added to citrate buffer and mixed by using magnetic stirrer at 750 rpm during 1 hour. Ctec2 at a fixed load of 50 ul Ctec2 and alkali pretreated corn cobs were added to this mixture at a fixed solid/liquid ratio (3%). Since in a previous study it was observed that when Tween 80 load was increased from 0 to 0.06 g/g dry solid (~ 56 ul), glucose yield increased from 418 to 486 g/kg dry solid for the organosolv pretreated wheat straw (Cui et al., 2011), this load was decided to use as initial amount to see its impact on hydrolysis. To see the effect of high load surfactant on sugar yield, 500 ul of Tween 80 loading was also tested.

2.5.4 Testing Product Inhibition on Corn Cob Hydrolysis

As will be explained later in the discussion section, when substrate loading doubled, sugar yield did not increase in the same amount. To see whether the reason was product inhibition on the enzyme, three successive experiments were conducted. Firstly, pretreated corn cobs were hydrolyzed by using 150 ul Celluclast 1.5 L and 150 ul Novozyme 188 at 6% substrate loading. Secondly, hydrolysate (including the active enzymes) obtained from the first experiment was taken and mixed with the same amount of pretreated corn cobs without any new addition of enzyme to double the substrate concentration to 12%. For the third hydrolysis, glucose concentration of the hydrolysate obtained from the first experiment was measured by using blood glucose monitor and at the same concentration glucose solution was prepared. Same amount

of pretreated corn cobs and same amount of enzyme with the first experiment (150 ul from Celluclast 1.5 L and Novozyme 188) were added to this glucose solution. All hydrolysis reactions were performed for 24 hours and sugar concentrations of the hydrolysate were compared.

2.5.5 Enzymatic Hydrolysis of Orange Peels

Due to low lignin content alkaline pretreatment was not applied for orange peel. For the enzymatic hydrolysis of fresh orange peels, 10% solid/liquid ratio on dry basis was used. Fresh orange peels having 80% moisture content were used without using any pretreatment method. Before enzymatic hydrolysis, glucose and reducing sugar contents of the pomegranate peels were found to be 4 g/l and 18.2 g/l respectively. Biogazyme 2x from ASA and Pectinex Ultra SP-L from Novozymes were used for hydrolysis. After the addition of the enzymes, pH was adjusted to 4.8 before the hydrolysis begins.

To see the effect of solid load on orange peel hydrolysis, solid load was changed ranging from 2-10% by using constant load of enzyme (0.5 g Biogazymex2).

Different amount of pectinase and cellulase enzymes were used for the hydrolysis. In order to see the effect of low load enzyme on hydrolysis efficiency Biogazyme 2x was used at the range of 0.005g-0.25 g and pectinase was used ranging from 5 ul-250 ul.

Moreover, use of high load enzyme was also tested to see its effect on hydrolysis. For that reason, 0.25 g and 0.50 g Biogazyme 2x was used with 250 ul, 500 ul and 1000 ul pectinase.

To observe the effect of limonene removal on enzymatic hydrolysis, after hot water extraction, 5 grams of dried orange peel (moisture content 6 % wb) was hydrolyzed using 2.5 g Biogazyme 2x and 1250 ul pectinase. Solid/ liquid ratio was kept at 10%.

2.5.6 Testing Product Inhibition on Orange Peel Hydrolysis

As will be explained in later in the discussion section, when substrate loading doubled, sugar yield did not increase in the same amount. To see whether the reason was product inhibition on the enzyme, three successive experiments were conducted. Firstly, orange peels were hydrolyzed by using 0.5 gram Biogazyme 2x at 10% substrate loading. Secondly, hydrolysate (including the active enzymes) obtained from the first experiment was taken and mixed with the same amount of orange peels without any new addition of enzyme to double the substrate concentration to 20%. For the third hydrolysis, glucose concentration of the hydrolysate obtained from the first experiment was measured by using blood glucose monitor and at the same concentration glucose solution was prepared. Same amount of orange peels and same amount of enzyme with the first experiment (0.5 gram Biogazyme2x) were added to this glucose solution. All hydrolysis reactions were performed for 24 hours and sugar concentrations of the hydrolysate were compared.

2.5.7 Enzymatic Hydrolysis of Pomegranate Peels

Since the fruit peels had the potential to include sugar, alkaline pretreatment was not applied to prevent any undesirable products that could form from accelerated Maillard Browning rates at higher pHs. Before enzymatic hydrolysis, glucose and reducing sugar contents of the pomegranate peels were found to be 10.5 g/l and 16.6 g/l respectively. Dried and milled pomegranate peels having 8.6% moisture content were exposed to enzymatic hydrolysis by using varying amounts of Biogazyme 2x (0.03, 0.06, 0.12, 0.25, 0.5 g) and fixed volume of pectinase (250 ul) at a 6% solid/liquid ratio.

2.6 Determination of Reducing Sugar Content

Reducing sugar content of the samples was determined using the DNS method (Miller, 1959). For the method, D-glucose was used as a standard. Supernatants obtained from the enzymatic hydrolysis were diluted with distilled water and then DNS reagent was added (1:1.5 v/v (mL/mL)). After keeping the solution in water bath at 100ºC for 5 minutes, color change was observed. Absorbance of the samples was measured at 540 nm by using a spectrophotometer (U-1800 spectrophotometer, HITACHI). Reducing sugar concentrations of the samples were calculated using the calibration curve prepared.

2.7 Determination of Glucose Content

Glucose content of the samples was determined by using a blood glucose monitor (BGM) and its test strips (Bahcegul et al., 2011). D-glucose was used as standard. A calibration curve was prepared by using different amount of glucose stock solutions and citrate buffer (0.05 M, pH=4.8). Supernatants were diluted with citrate buffer and put into 1.5 ml centrifugal tubes and agitated by using a vortex (Vortex (ZX3, VELP Scientifica, Usmate, MB, Italy) for 10 seconds. Finally, with the help of test strips (Life Scan, Inc. Milpitas, CA 95035), value on the screen was read and using the calibration curve prepared, glucose concentrations of the samples were calculated.

2.8 Data Analysis

The results reported were the averages of two measurements. Data were reported as mean values. Analysis of variance (ANOVA) was conducted by using Minitab (ver.16.2.0.0, Minitab Inc., United Kingdom) and Tukey Test was used for multiple comparison at 95% significance level.

CHAPTER 3

RESULTS AND DISCUSSION

This chapter will focus on the results and interpretation of hydrolysis experiments for different substrates. The following list summarizes the outline of this chapter. Each bullet point will be discussed in detail on a separate section.

- In order to find the most suitable substrate for enzymatic hydrolysis, different biomass such as saw dust and corn cobs were hydrolyzed by using Celluclast 1.5 L and Novozyme 188 enzymes.
- Since these substrates are lignin-rich biomass, for delignification, alkaline pretreatment was applied to these lignocellulosic biomass and the most suitable substrate for alkaline pretreatment was determined.
- Based on the findings of these experiments, the most suitable substrate for alkaline pretreatment was found as corn cob and remaining experiments were performed on using this substrate.
- To see the effect of β-glucosidase (cellobiase) addition on hydrolysis of alkaline pretreated corn cobs, corn cobs hydrolyzed with and without Novozymes 188 (commercial cellobiase enzyme).
- To see the effect of different cellulase loading on hydrolysis of alkaline pretreated corn cobs, different load of cellulase enzymes were used by using Celluclast 1.5 L (commercial cellulase enzyme).
- To see the effect of Ctec2 enzyme which was a mixed enzyme cocktail with a high activity, alkaline corn cobs were hydrolyzed with this enzyme and similar sugar yields with lower enzyme dosage were obtained.
- Hydrolysis experiments with time was conducted and surfactant (Tween 80) effect was examined on alkaline pretreated corn cobs by using CTec2 enzyme.

- After corn cobs hydrolysis, within the scope of the project, hydrolysis of lignin-poor biomass such as orange peel and pomegranate peel were performed by using ASA Biogazymex2 enzyme.
- Since these substrates contain lignin in lower amounts, pretreatments for lignin removal like alkaline pretreatment was not needed. Therefore, these biomass was directly hydrolyzed.
- Since these fruit wastes also contain high amount of pectin, in addition to cellulolytic enzymes (Biogazymex2), pectinase (Pectinex Ultra SP-L) was also used for their hydrolysis.
- To determine the effects of enzyme load on hydrolysis of orange peels and pomegranate peels, Pectinex Ultra SP-L and Biogazymex2 were used at different concentrations.
- For orange peel hydrolysis, effect of different solid loads was also tested.

3.1 Effect of Alkaline Pretreatment on Enzymatic Hydrolysis of Lignin Rich Biomass

Figure 3.1 and 3.2 shows, glucose yield (%) and total reducing sugar yield (%) respectively for the untreated (control) and alkaline pretreated saw dust and corn cob. As seen from Fig. 3.1, for the corn cobs, glucose yield increased from 6.9% to 21.5% when the alkaline pretreatment was applied before the enzymatic hydrolysis. There was significant difference between control and pretreated corn cobs ($p<0.05$). Total reducing sugar yield was 14.1% for the control corn cobs. As seen in Fig. 2, after the alkaline pretreatment yield increased to 33.6%. Similar to glucose yield, total reducing sugar yield between control and pretreated corn cob samples was found to be significant ($p<0.05$). On the other hand, for the saw dust hydrolysis glucose and total reducing sugar yield between control and pretreated saw dust samples were found be insignificant ($p>0.05$).

In Figures 3.1 and 3.2, experiment results clearly indicated that alkaline pretreatment enhanced the efficiency of enzymatic hydrolysis of corn cobs. It was found that glucose and total reducing sugar yield (%) of alkali pretreated corn cobs are

significantly different from pretreated saw dust ($p<0.05$). It could be concluded that for the same substrate loading (3%) alkaline pretreatment is more suitable for corn cobs than sawdust in terms of the enzymatic hydrolysis efficiency.

Alkaline pretreatment is known as a mild thermochemical pretreatment that enhances enzymatic saccharification of lignocelluloses biomaterials and it is also very effective on cellulolytic hydrolysis of corn stovers (Karuna et al., 2014). For the untreated samples, enzymatic digestibility is very low because of their recalcitrant crystalline structure. Due to that, sugar yield was found to be low for the control samples. These samples contain high amount of lignin, which could interfere with the efficiency of enzymatic hydrolysis due to its complex chemical structure. It has been reported that 10-15% lignin content should not exceed 10-15% for the penetration of cellulolytic enzymes into the cell wall (Yan et al., 2015). Bahcegul et al. (2011) also evaluated the effect of alkaline pretreatment on glucose yield after enzymatic hydrolysis by using blood glucose monitor and DNS method for corn cobs. In their previous work, it was found that, as a result of the alkali pretreatment glucose yield increased from 6.8% to 34.3%.

Saw dust could be classified in wood category among lignocellulosic biomass. Their lignin content varies between 17-32% (Rosgaard, 2007). On the other hand, Menon et.al. (2012) reported that lignin content of corn cobs varies between 6.7-13.9%. Effect of alkaline pretreatment on saw dust and corn cobs could be related with the lignin content of these substrates. Since lignin content of the saw dust is higher than the other substrates, removal or redistribution of lignin could be more difficult relative to corn cobs. Low glucose and total reducing sugar yield for the pretreated saw dust samples could be explained by insufficient lignin removal leading to insufficient enzymatic hydrolysis efficiency.

Total reducing sugar yield was found to be higher than glucose yield for all the substrates. This could be explained by the carbohydrate composition of these substrates. Corn cob is composed of 32.3-45.6% cellulose, 39.8% hemicellulose and saw dust contains 40-48% cellulose and 19-26% hemicellulose (Rosgaard, 2007). Therefore, at the end of enzymatic hydrolysis, degradation of cellulose to glucose is expected. Hemicellulose unit was also degraded into xylose, arabinose, mannose and

galactose which were also known as reducing sugars due to the hemicellulase activity of commercial enzymes (Kristensen, 2009).

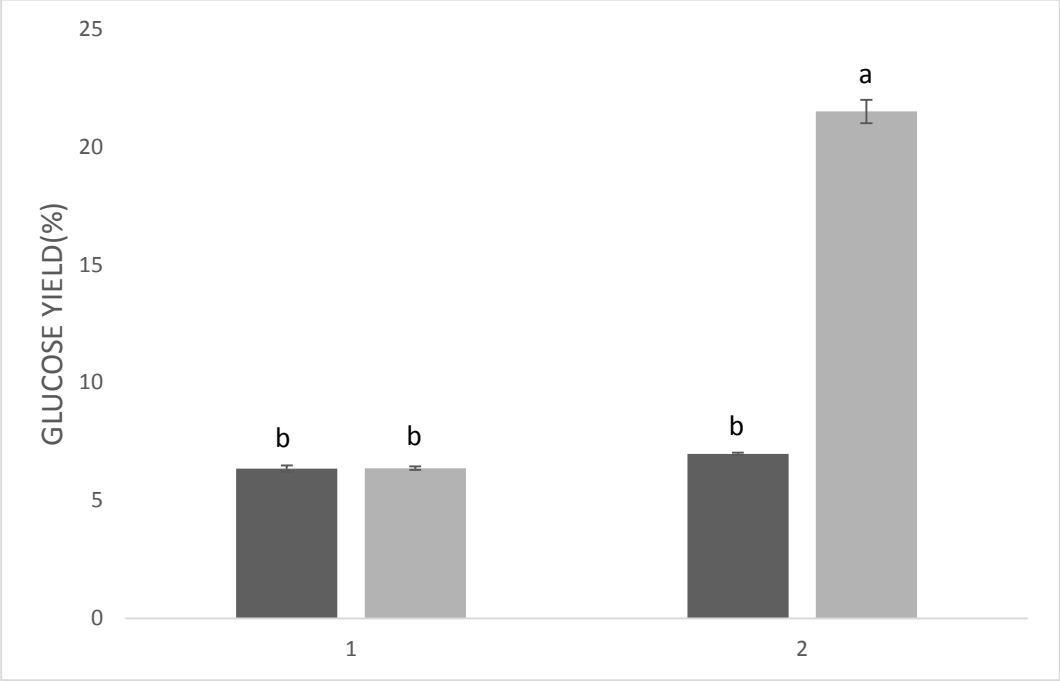

Figure 3.1 Glucose yield (%) values untreated (control) (■) and alkali pretreated (■) substrates at the end of enzymatic hydrolysis. (1: sawdust, 2: corn cob). Different letters represent significant difference ($p \leq 0.05$)

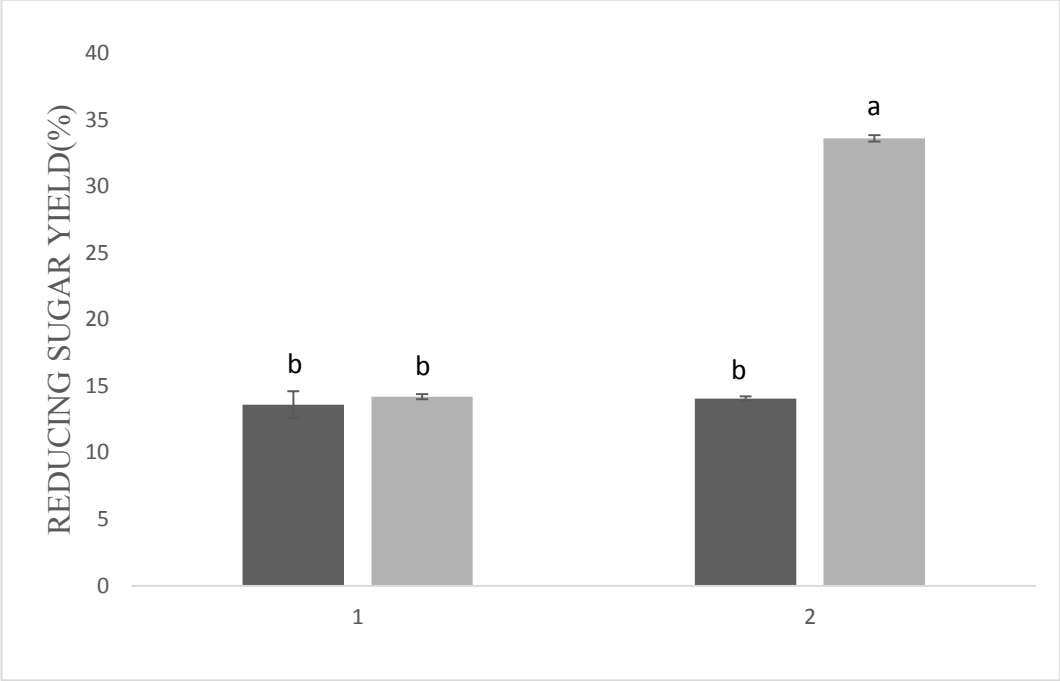

Figure 3.2 Total reducing sugar yield (%) values untreated (control) (■) and alkali pretreated (■) substrates at the end of enzymatic hydrolysis. (1: sawdust, 2: corn cob). Different letters represent significant difference ($p \leq 0.05$).

3.2 Effect of cellobiase (β- Glucosidase) on enzymatic hydrolysis of pretreated corn Cob

Fig. 3.3 shows glucose yield (%) and total reducing sugar yield (%) for the alkali pretreated corncobs with and without the cellobiase enzyme: Novozyme 188. Percent glucose yield increased from 11% to 20% and total reducing sugar yield increased from 21% to 33.5%. It was clear from the results that there was significant difference in glucose and total reducing sugar yield (%) when 150 ul Novozyme 188 was used in addition to 150 ul Celluclast 1.5 L ($p<0.05$). It was also observed that total reducing sugar yield (%) for the samples hydrolyzed without cellobiase is similar with glucose yield (%) of the samples hydrolyzed with cellobiase ($p>0.05$). It was clear that, addition of cellobiase (β-glucosidase) to the hydrolysate increased the sugar yield (%).

During enzymatic hydrolysis, substrates are firstly degraded into their monomers with the action of exoglucanases and endoglucanases. Exoglucanase and endoglucanase activity is higher for the Celluclast 1.5 L, which is a main cellulase enzyme that is used in the hydrolysis. The reported values by Novozyme are 700 u/g for Celluclast 1.5 L and 250 u/g for Novozyme 188. In addition to glucose, cellobiose, which is the dimer form of the glucose in cellulose, and the main disaccharide of cellulose, also forms during hydrolysis. At that point, use of β-glucosidase mainly found in Novozyme 188 is vital to hydrolyze cellobiose to glucose and increase the sugar yield.

Pryor et al. (2015) reported that β-glucosidase addition during the hydrolysis of corncobs could decrease to 10% and 20% of typical loadings. It was also stated that, further reductions could have negative impacts on hydrolysis rates. Furthermore, it was indicated when β-glucosidase added to microfluidized wheat bran wheat straw and corn bran samples in addition to cellulase, 1-2 fold increase was observed in total reducing sugar yield (%) while there was no significant change in untreated samples (Yavas et al., 2010). Similar results were also obtained in our study. For the alkali-pretreated corncobs, addition of cellobiase caused a significant change in both glucose and total reducing sugar yield (%) (Fig. 3.3).

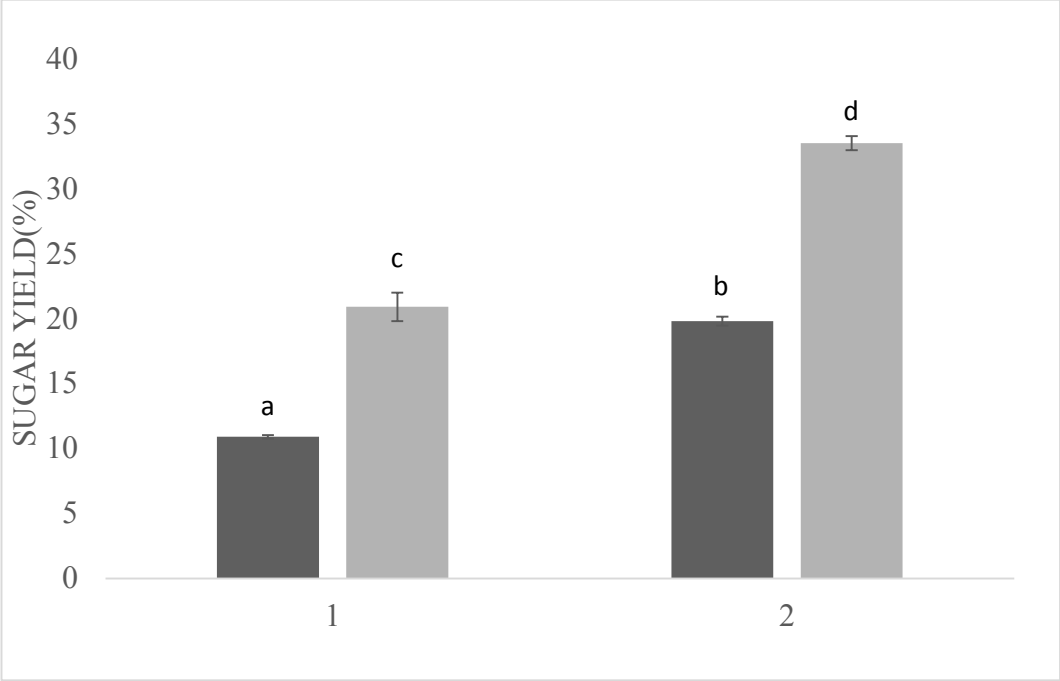

Figure 3.3 Glucose yield (%) (■) and total reducing sugar yield (%) values (■) of alkali pretreated corn cobs (1: without cellobiase, 2: with cellobiase). Different letters represent significant difference ($p \leq 0.05$). (For the glucose yield and total reducing sugar yield (%), one-way ANOVA was applied separately).

3.3 Effect of Cellulase Loading on Enzymatic Hydrolysis of Corn Cobs

To find the minimum concentration of cellulase, its concentration was changed between 150-600 ul. As seen in Figure 3.4, despite the 2- fold, 3-fold and 4-fold reduction in cellulase amount a significant difference in glucose yield and total reducing sugar yield was not observed ($p>0.05$).

This is an important finding since required enzyme dosage is a very important parameter in terms of the economy and the efficiency of the process. Similar results related with enzyme loadings were also reported in previous studies. During the hydrolysis of cardboard waste, hemicellulase loading (volume of enzyme/mass of biomass) was kept at constant (40 cm^3/ kg) and effect of cellulase loading was

examined (Kinnarinen and Häkkinen, 2014). It was observed that enzyme consumption increased more sharply than the obtained concentration of glucose indicating that doubling the enzyme concentration did not lead to doubling of glucose concentration. Although cardboard waste contains high cellulose and low lignin, Kinnarinen et al. (2014) explained that these results are related with reduction in water binding capacity of fibers resulting from drying causing to decrease in enzyme accessibility Similar results were also found in our study. When the cellulase amount decreased from 600 ul to 300 ul per gram biomass, no significant change in sugar yield was observed ($p>0.05$). However, in our case these results could also be associated with the higher lignin and lower cellulose content of corn cobs relative to cardboard waste as well as drying effect on enzyme accessibility.

There are other factors that could affect the efficiency of the hydrolysis other than enzyme dosage. Adsorption of cellulase which is an important step for the hydrolysis is an important issue. In some conditions, there could be limitations in the adsorption of enzyme to the substrate (Mansfield et al., 1999). These limitations could be expressed as the chemical composition like lignin content and crystallinity of the substrates. According to Mansfield et al. (1999), amorphous cellulose could be easily hydrolyzed to its monomers relative to crystalline cellulose. If the substrate used in hydrolysis is composed of mostly crystalline cellulose rather than amorphous, enzyme accessibility to these crystalline regions could decrease. Therefore, although high loads of enzyme was used, efficient hydrolysis could not be achieved. The crystallinity degree of cellulose on different substrates was not examined in this study. That would give important information about the interaction of substrates with the enzymes.

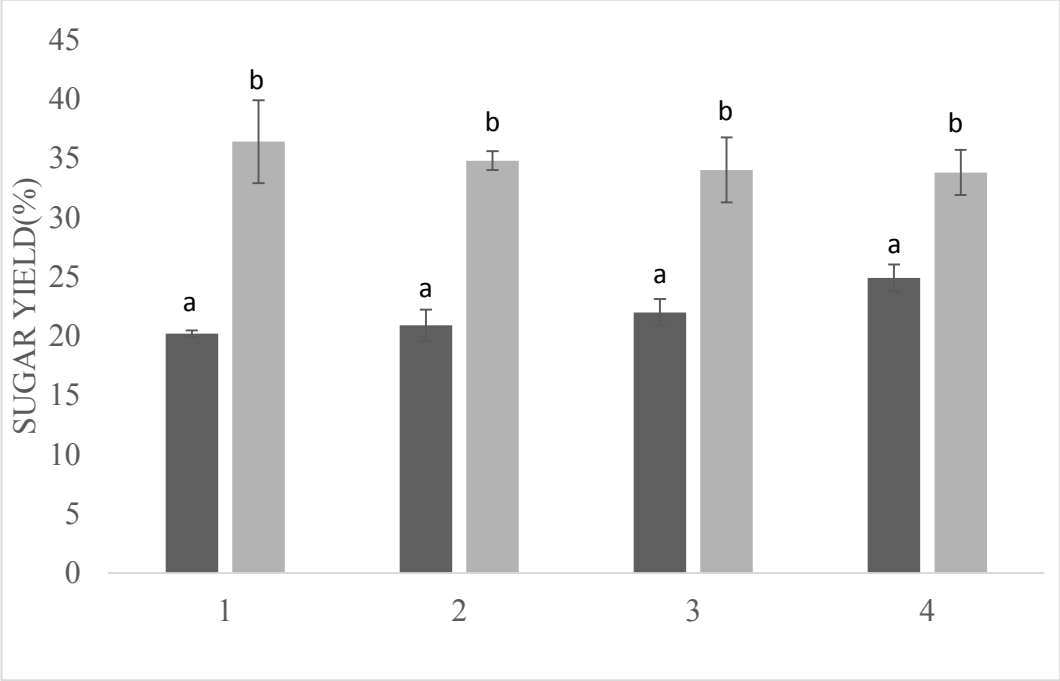

Figure 3.4 Glucose yield (%) (■) and total reducing sugar yield (%) values (■) of alkali pretreated corn cobs by using fixed volume (200ul) of cellobiase (Novozymes 188) and different load of cellulase (Celluclast 1.5L) (1: 600 ul Celluclast 1.5 L, 2: 300 ul Celluclast 1.5 L , 3: 200 ul Celluclast 1.5 L, 4:150 ul Celluclast 1.5 L. Different letters represent significant difference ($p \leq 0.05$)

3.4 Effect of Product Inhibition on the Enzymatic Hydrolysis of Corn Cobs

Fig. 3.5 shows the sugar yields at different substrate loadings as explained in section 2.5.4. It is clearly seen that if total amount of substrate increased 2-fold between the first and second hydrolysis, glucose amount also increased significantly ($p<0.05$). However, while it was expected to observe 2-fold increase in glucose concentration, only 33% increase was observed. Similar to glucose concentration, total reducing sugar content also increased significantly ($p<0.05$). The insufficient change in sugar yield was attributed to product inhibition and to test the reason behind, another hydrolysis experiment was conducted. Glucose concentration of the first cob hydrolysate was mimicked by pure glucose solution and then mixed with the same

amount pretreated corn cobs and hydrolysis was carried out. Glucose concentration of the third solution was significantly different from the first and the second one ($p<0.05$). Nearly, 2-fold increase was observed for this hydrolysis indicating that product inhibition was not present. These results indicated that efficiency of the hydrolytic enzymes decreased when they are reused in reactions. Therefore, rather than product inhibition, adsorption of enzymes to lignin (Pareek et al., 2013) or lower efficiency of the enzymes when they are reused (Romo-sánchez et al., 2014) could be the reason for low yield. Total reducing sugar concentration was found significantly different for all the hydrolysis reactions.

In Pareek et al. (2013)'s study, adsorption kinetics of cellulase, xylanase and β-glucosidase were found by using enzyme activity measurements and it was found that affinity constant, binding strengths and adsorption capacities were higher for the lignin relative to other carbohydrates. In our study, smaller increase in the total reducing sugar yield for the second hydrolysis (6% solid loading) could be associated with the lignin content and unproductive enzyme adsorption (Kristensen, 2009) on lignocelluloses.

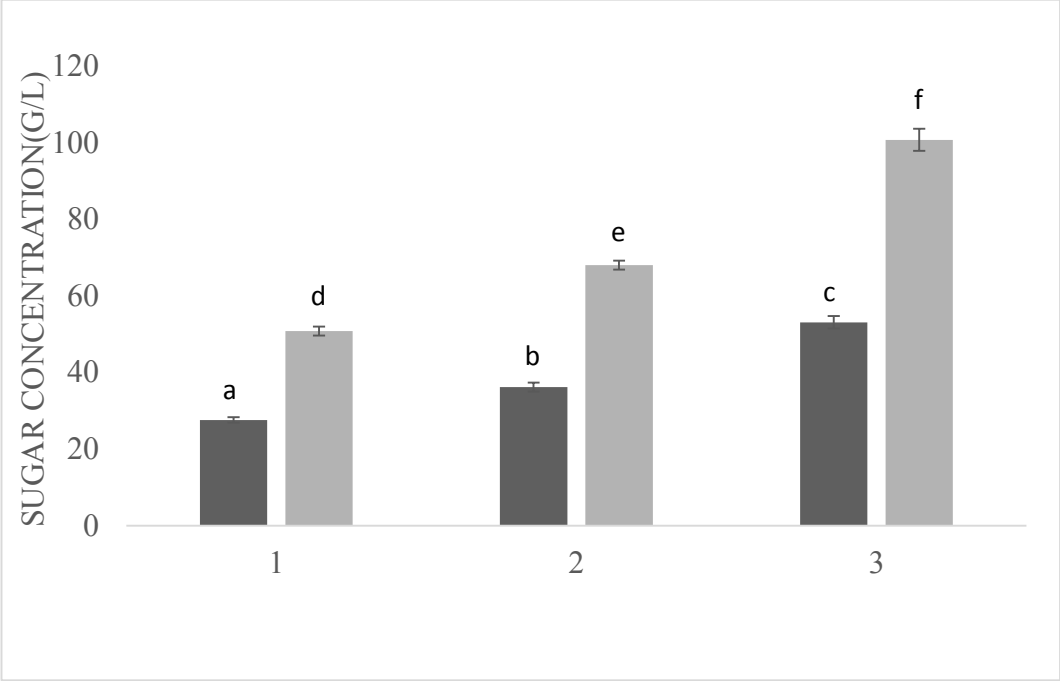

Figure 3.5 Glucose concentration/g/l) (■) and total reducing sugar concentration (g/l) (■) values of alkali pretreated corn cobs hydrolyzed by 150 ul Celluclast 1.5 L and 150 ul Novozymes 188. (1: 1st hydrolysis using only alkali pretreated corn cobs (6% solid load), 2:2nd hydrolysis by using hydrolysates obtained from 1st one and with the addition of new alkali pretreated corn cobs (12% solid load), 3: 3rd hydrolysis by using glucose solution with the same concentration with 1st hydrolysates, newly added alkali pretreated corn cobs and enzyme. Different letters represent significant difference ($p \leq 0.05$).

3.5 Effect of Ctec2 (commercial enzyme mixture with cellulase and β-glucosidase activity) on Enzymatic Hydrolysis of Corn Cobs

Figure 3.6 shows the glucose yield and total reducing sugar yield (%) of alkali pretreated corn cobs hydrolyzed by using different loads of Ctec2 enzyme. These experiments were conducted to find the optimum enzyme dosage. Since Ctec2 is a cocktail enzyme with increased concentration of β-glucosidase and high xylanase

activity (Novozymes, 2010), by using only this enzyme desirable sugar yields could be achieved at the end of enzymatic hydrolysis.

In order to find the optimum enzyme load to reach desirable sugar yield, Ctec2 concentration was changed between 10-300 ul. As seen from Fig. 3.6, despite 2-fold, 3-fold and 6-fold reduction in enzyme concentration, change in glucose and total reducing sugar yield were not significant up to 50 ul ($p>0.05$). However, if 50 ul enzyme was decreased 2-fold and 5-fold, significant decrease was observed in glucose yield ($p<0.05$). The reason for that could be associated with the maximum enzymatically accessible cellulose content (Novozymes, 2010). Most probably, by using 50 ul enzyme, maximum enzymatically accessible fiber content was nearly reached so glucose and total reducing sugar yield did not change significantly for the higher enzyme concentrations. Recommended dosage of Ctec2 enzyme is 3% w/w (g enzyme/g cellulose) and this was equivalent to 10 ul Ctec2 in our case. However, it was found that by using 10 ul Ctec2, glucose yield and total reducing sugar yield was 11.8% and 22.5% respectively while these values were 25.3% and 32.3% when 50 ul enzyme was used. Therefore, it could be concluded that by using recommended enzyme dosage, desirable sugar yields for further applications such as fermentation could not be reached. The main reason for this inconsistency was the use of pure cellulosic substrates like filter paper or avicel for the recommended dosages. Since, enzyme manufacturers determines the minimum required enzyme dosage by using these “pure” cellulosic substrates, unexpected results could appear when “real” lignocellulosic materials were used (Mansfield et al., 1999). According to Mansfield’s study (1999), existence of compositional constituents like lignin and hemicellulose in lignocellulosic biomass directly affects enzyme accessibility leading to production of low sugar at the end of enzymatic hydrolysis.

As seen in Fig. 3.6, when enzyme loading decreased from 50 to 25 ul, significant decrease was observed in glucose yield while decrease in total reducing sugar yield was not significant ($p>0.05$).

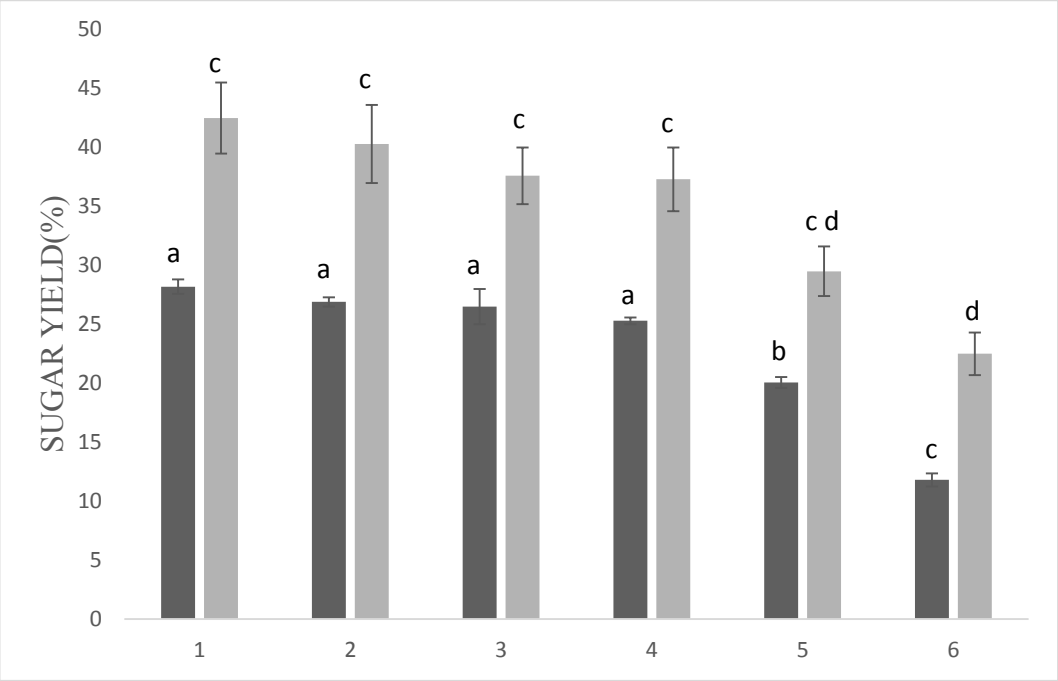

Figure 3.6: Glucose yield (%) (■) and total reducing sugar yield (%) values (■) of alkali pretreated corn cobs by using different enzyme loads. (1: 300 ul Ctec2, 2: 150 ul Ctec2, 3: 100 ul Ctec2, 4: 50 ul Ctec2, 5: 25 ul Ctec2, 6: 10 ul Ctec2). Different letters represent significant difference ($p \leq 0.05$)

3.6 Determination of Enzymatic hydrolysis time for Alkali Pretreated Corn Cobs

According to the study of Pryor and Nahar (2015), 1 day (24 hour) hydrolysis time was particularly chosen since this time interval was more representative for the hydrolysis rate for the corn stover hydrolysis. 24 hour hydrolysis time was mostly chosen for most of the enzymatic hydrolysis reactions like Pitch pine wood dust hydrolysis (Kwon et al., 2015), corn cob hydrolysis (Bahcegul et al., 2011) and marine macro alga hydrolysis (Yazdani et al., 2011). However, it is also important to know in which time interval glucose yield and total reducing sugar reaches a plateau. For that

reason, time dependent experiment was conducted for the alkali pretreated corn cobs. Glucose yield and total reducing sugar yield were determined at every 3 hours. As seen from Fig. 3.7, at the end of 3 hours, glucose and total reducing sugar yield were found 2.7% and 6.9% respectively. Up to 12 hours, there was a sharp increase in both glucose and total reducing sugar yield. At the end of 12 hours, glucose yield and total reducing sugar yield reached to 34.06% and 61.4% respectively. After that, very small changes was observed for both glucose and total reducing sugar yield. To conclude, it was possible to say that at the end of 12 hours, glucose and total reducing sugar yield reached the plateau. Similarly, in previous studies (Perruzza, 2010), during the hydrolysis of oat hulls the concentration of glucose plateaued nearly after 12 hours. At the end of 24 hours, glucose yield was found 37.7% while total reducing sugar yield was found 64.3%.

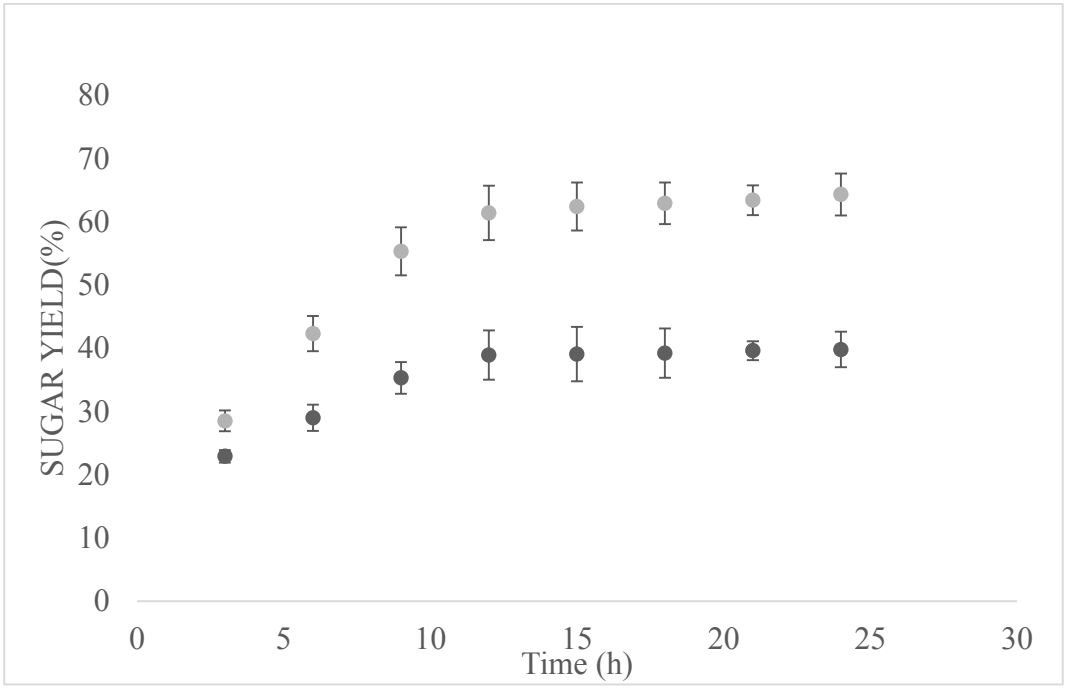

Figure 3.7: Glucose yield (%) (■) and total reducing sugar yield (%) values (■) of alkali pretreated corn cobs hydrolyzed by using 50 ul Ctec2 at different time intervals

3.7 Effect of Non-Ionic Surfactant (Tween 80) on Enzymatic Hydrolysis of Alkali Pretreated Corn Cobs

Addition of several chemicals to the biomass slurry before the hydrolysis has been reported to enhance enzymes performance by decreasing unproductive adsorption of enzymes to lignin (Kristensen, 2009). Surfactants could be used in order to increase enzymatic hydrolysis efficiency. Kristensen (2009) reported that addition of non-ionic surfactants like Tween 20 or Tween 80 could also decrease the unspecific binding of enzymes leading to enhance hydrolysis rate so by using lower enzyme loadings, same degree of conversion could be obtained with the help of surfactants. It was found in a study by Cui et al (2011) , for the organosolv pretreated wheat straw samples, as the surfactant concentration was increased from 0 to 0.06 g/g dry solid, the glucose yield increased from 418 to 486 g/kg dry solid (Cui et al., 2011)

For this study, to find the effect of Tween 80 on enzymatic hydrolysis of alkali pretreated corn cobs, corn cobs hydrolyzed with Tween 80 (concentration was changed between 56-500 ul) and without Tween 80 by using fixed enzyme concentration (50 ul cellulase). As seen in Fig.3.8, increase in glucose and total reducing sugar yield was insignificant when 56 ul Tween 80 was used in the hydrolysis ($p>0.05$).When this concentration was increased to 500 ul, no significant change was observed again ($p>0.05$). The reason for this result could be the use of high enzyme loading. According to most of the studies, it was reported that when surfactants were used together with low enzyme concentration, improvement in hydrolysis yields was achieved. However, when high concentration enzyme was used, this high load could mask the improvement effect which could be obtained from the use of surfactants by producing high amount of sugar and leave no room for enhancement of hydrolysis efficiency (A. Eckard et al., 2013). For instance, in previous study (A. D. Eckard et al., 2013) when extrusion pretreated corn stovers was hydrolyzed with the help of surfactants, it was found that glucose and xylose yield was increased 10% to 38.5% and 3.1% to 26.7%, respectively. On the other hand, if the enzyme amount was increased 2-fold, no significant increase was observed in sugar yields related with surfactant dosage. Furthermore, surfactant type is also an important factor effecting

hydrolysis efficiency. For instance, it has been reported that PEG (Polyethylene glycol) has an important effect in the increase in enzymatic hydrolysis efficiency (Kristensen, 2009). Moreover, it has been reported that use of PEG-6000 and PEG-4000 for the ammonia pretreated sugarcane bagasse showed higher delignification leading to higher hydrolysis rates compared to Tween 80 used ammonia pretreated sugarcane bagasses (Cao, 2012). Eckard et al. (2013) has reported that glucose yield was increased 7% when PEG concentration was increased up to 0.5 g/g glucan with the use of 14.2 mg/g glucan of Cellic CTec2 was used. To conclude, use of Tween 80 instead of PEG could be responsible from insignificant change in glucose and total reducing sugar yield.

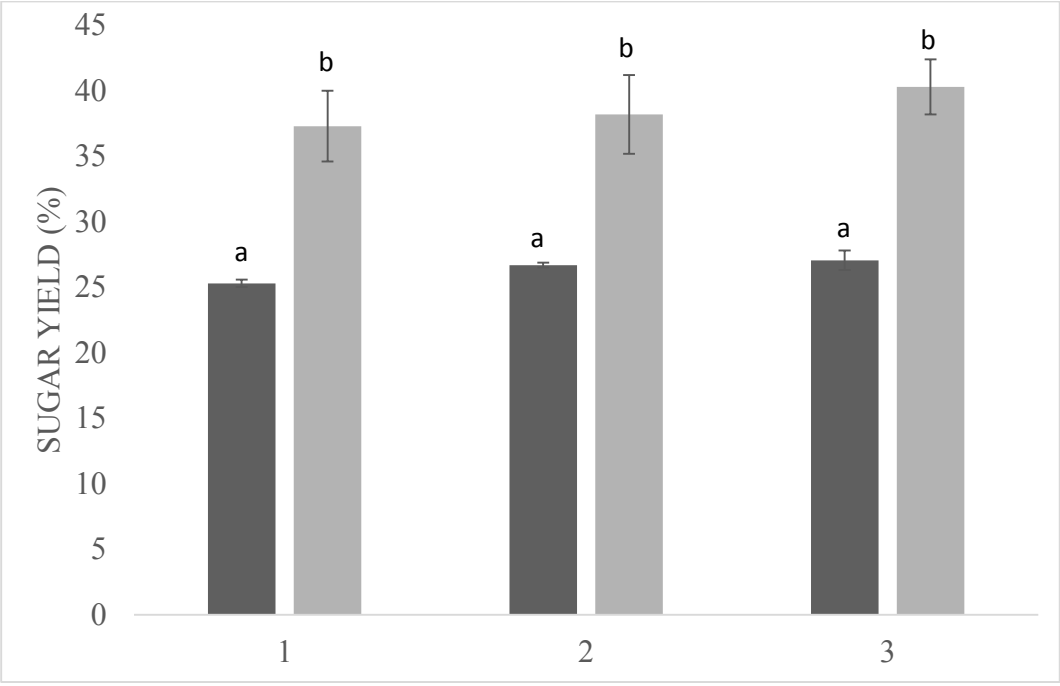

Figure 3.8: Glucose yield (%) (■) and total reducing sugar yield (%) values (■) of alkali pretreated corn cobs hydrolyzed with 50 ul Ctec2& different load of TW 80 (1: without TW 80, 2: 56 ul TW 80, 3: 500 ul TW 80). Different letters represent significant difference ($p \leq 0.05$). (For the glucose yield and total reducing sugar yield (%), one-way ANOVA was applied separately).

3.8 Effect of Solid Load on the Hydrolysis of Orange Peel Hydrolysis

In order to see the effect of solid loading on orange peel hydrolysis, 2%, 6% and 10% solid loads (% dry basis) were tried with fixed enzyme concentration. (0.5 g Biogazymex2) Krishna et al. (1998) indicated that maximum solid load should not exceed 10% due to problems in proper mixing of hydrolysates causing the reduction in enzyme accessibility to the substrates maximum solid load was chosen as 10%.

Figure 3.9 shows sugar conversion of orange peels which hydrolyzed by using different solid loads. As seen in the figure, glucose conversion was found 16.5%, 23.3% and 30.7% when 2%, 6% and 10% solid loads were used respectively in the hydrolysis. Glucose conversions were significantly different for each solid load ($p<0.05$). Total reducing sugar conversion was found 43.7%, 51.6% and 60.2 % for 2%, 6% and 10% solid load respectively. Similar to glucose conversion, total reducing sugar conversion was also significant ($p<0.05$).

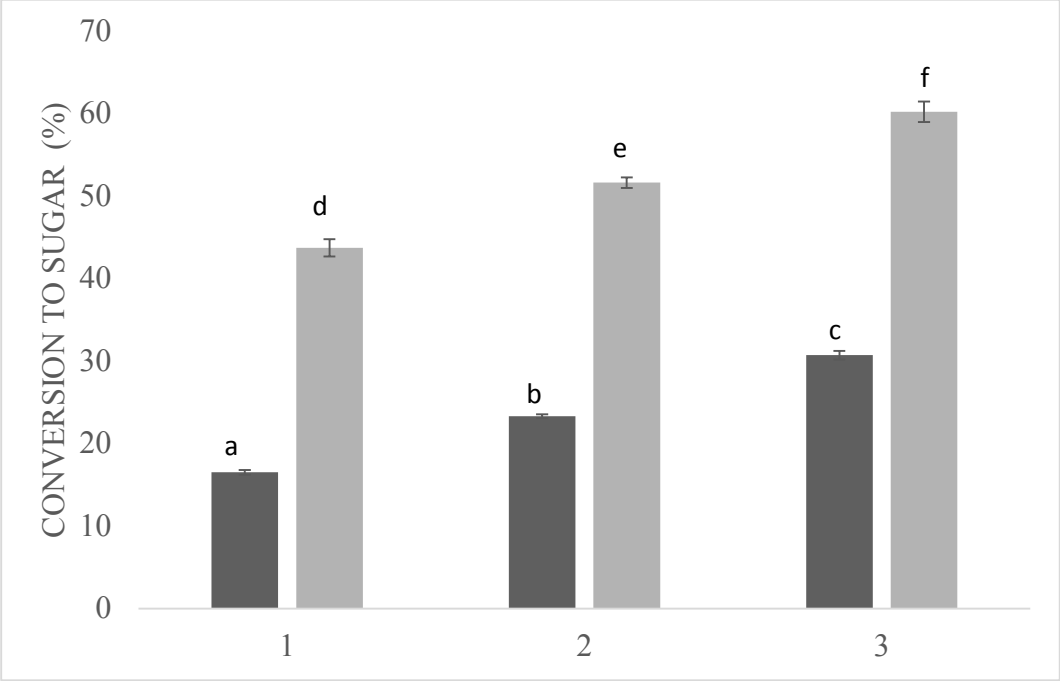

Figure 3.9: Conversion to glucose (%) (■) and conversion to total reducing sugar (%) values (■) of fresh orange peels hydrolyzed by 0.5 g cellulase (Biogazyme 2x) (BG) by using different solid loads. (1: 2% solid load, 2: 6% solid load, 3: 10 % solid load) Different letters represent significant difference ($p \leq 0.05$).

3.9 Effect of Pectinase (Low Loading) and Cellulase Loading on Enzymatic Hydrolysis of Fresh Orange Peels

In a previous study, it was indicated that the synergic activities of cellulase and β-glucosidase enzymes are needed to degrade cellulose and hemicellulose (Talebnia, 2008). In the same study, it was also showed that, in addition to these enzymes pectinase addition was needed for the whole degradation of citrus peels. For this experiment, instead of Novozymes enzymes, ASA Biogazyme 2x (BG) cocktail enzyme involving cellulase, β-glucosidase and xylanase together was used. In addition to cellulolytic enzyme mixture, Pectinex Ultra SP-L (PC) from Novozymes was used to hydrolyze pectin which was a complex organic polymer found in orange peels.

After the addition of enzymes and after waiting 30 minutes, especially for the samples which pectinase was added, it was observed that pH of the hydrolysis solution decreased drastically (from 4.8 to 3.5). This was due to degradation of pectin to galacturonic acid monomers in the presence of pectinase. Since pH is an important factor affecting enzyme activity directly, for the orange peel hydrolysis pH of the solution was adjusted to 4.8 by using 1 M NaOH just after addition of the enzymes. At the end of enzymatic hydrolysis pH was decreased to 4.3.

Different type of enzyme and enzyme loads were used in order to see the effect of on enzymatic hydrolysis efficiency of orange peels (Table 3.1). In previous studies it was also mentioned that for the orange peel hydrolysis, conversion to monomeric sugar was observed in high levels after treatment with pectinase enzyme (Haven, 1992).

As the enzyme load increased, glucose and total reducing sugar yield increased for all the samples. Up to 8^{th} sample, use of pectinase together with cellulase did not increase the glucose conversion significantly ($p>0.05$). Similarly, total reducing sugar concentration did not increase significantly ($p>0.05$) up to 8^{th} sample except for the 6^{th} sample. For this sample, since 150 ul pectinase was used, total reducing sugar conversion increased significantly ($p<0.05$) while the increase in glucose conversion was insignificant ($p>0.05$). The reason for that could be associated with galacturonic acid which is also a reducing sugar release as a result of the sufficient action of pectinase enzyme. Due to the low amount of pectinase enzyme usage, for the second and fourth samples increase in total reducing sugar yield were insignificant ($p>0.05$). For the 8^{th} and 10^{th} sample, as a result of the sufficient synergistic effect of cellulase and pectinase enzymes, increase in both glucose and total reducing sugar yield were found to be significant ($p<0.05$).

Table 3.1 Amount and Type of Enzymes Used in Hydrolysis of Orange Peels

1	0.005 g BG
2	0.005 g BG+ 5 ul PC
3	0.1 g BG
4	0.1 g BG+100 ul PC
5	0.15 g BG
6	0.15 g BG+150 ul PC
7	0.2 g BG
8	0.2 g BG+ 200 ul PC
9	0.25 g BG
10	0.25 g BG+250 ul PC

BG: Biogazyme 2x

PC: Pectinex Ultra SP-L

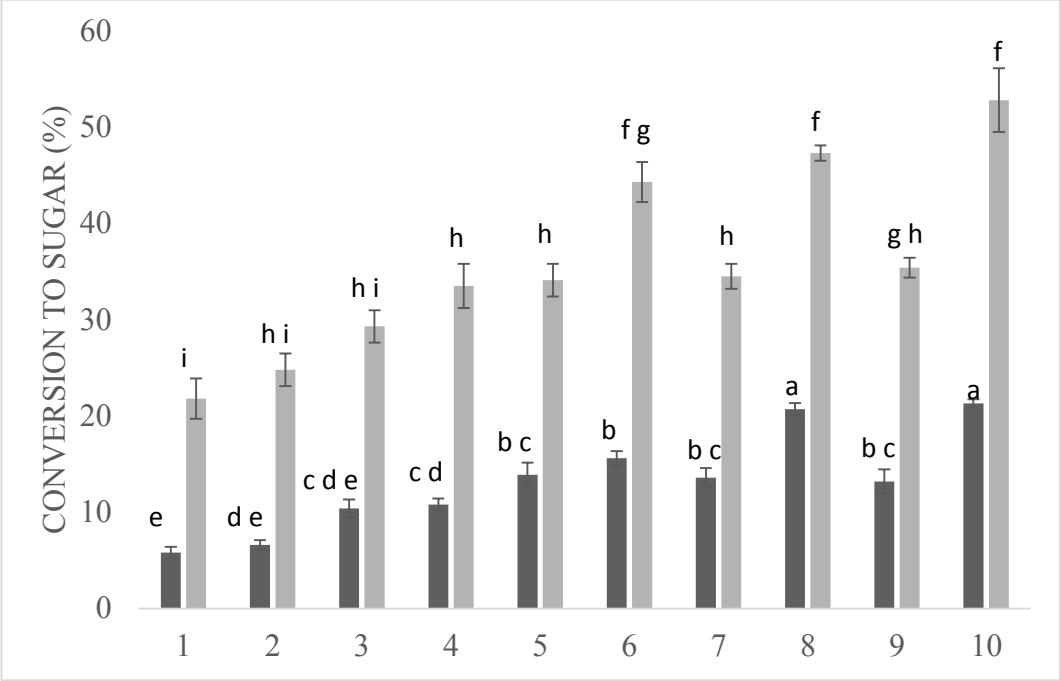

Figure 3.10 Conversion to glucose (%) (■) and conversion to total reducing sugar (%) values (■) of fresh orange peels hydrolyzed by different load of pectinase (Pectinex Ultra SP-L) (PC) and cellulase(Biogazyme 2x) (BG)) Different letters represent significant difference ($p \leq 0.05$)

3.10 Effect of High Load Pectinase and Cellulase Loading on Enzymatic Hydrolysis of Fresh Orange Peels

According to results shown in Figure 11, while there was no significant difference in glucose and total reducing sugar conversion when BG load was kept at constant (0.25 gram) and PC load was increased gradually from 250 ul to 1000 ul ($p>0.05$). However, when 250 ul PC was used together with 0.25 g BC, 8% increase in glucose conversion was observed relative to samples without pectinase. If BG amount was increased 2-fold and PC amounts were increased gradually from 250 ul to 1000 ul, no significant difference was observed again in glucose and total reducing sugar conversion ($p>0.05$). However, when BG amount was increased from 0.25 gram to 0.5 gram with a constant load of PC significant increase in glucose and total reducing sugar

conversion was achieved ($p<0.05$) due to increasing load of cellulolytic and pectolytic enzymes.

Table 3.2 Amount (High Loading) and Type of Enzymes Used in Hydrolysis of Orange Peels

1	0.25 g BG
2	0.25 g BG+ 250 ul PC
3	0.25 g BG+500 ul PC
4	0.25 g BG+1000 ul PC
5	0.5 g BG
6	0.5 g BG+250 ul PC
7	0.5 g BG+500 ul PC
8	0.5 g BG+ 100 ul PC

BG: Biogazyme 2x

PC: Pectinex Ultra SP-L

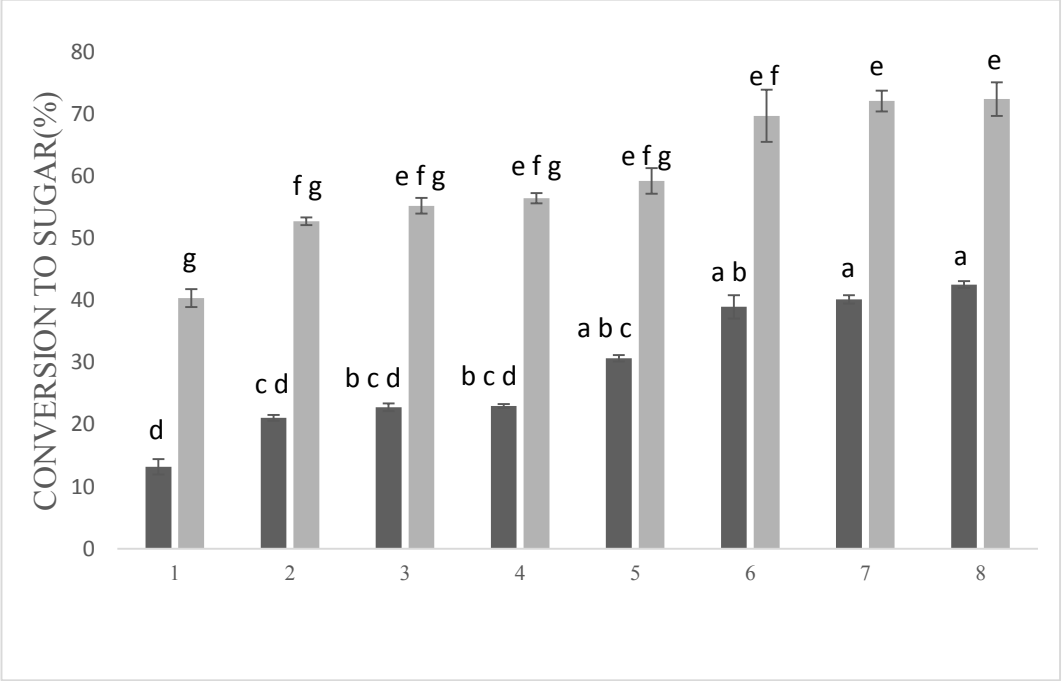

Figure 3.11 Conversion to glucose (%) (■) and conversion to total reducing sugar (%) values (■) of fresh orange peels hydrolyzed by different load of pectinase (Pectinex Ultra SP-L) (PC) and cellulase(Biogazyme 2x) (BG)) Different letters represent significant difference ($p \leq 0.05$) . (For the Conversion to glucose (%) and conversion to total reducing sugar (%) values, two-way ANOVA was applied separately).

3.11 Effect of Liquid Boiling Water Extraction to Remove Limonene on Enzymatic Hydrolysis of Orange Peels

Due their low lignin content of orange peels (7.5%) (Boluda-Aguilar and López-Gómez, 2013), pretreatment methods were not performed to remove the lignin for the orange peels.

As it was previously mentioned, threshold value for the lignin varies between 10-15% (Yan et al., 2015) to perform the cellulolytic degradation efficiently. Since the lignin content of orange peels is below this value, pretreatment methods which aimed to

remove lignin was not preferred for the fruit peels generally. Instead of removing lignin, pretreatment methods are generally based on the removing D-limonene.

As Talebnia (2008) explained , in orange peels , following enzymatic hydrolysis, mixture of pentose hexose sugars like glucose, galacturonic acid, galactose, arabinose and xylose are released in addition to peel oil having composition of 95% D-limonene which is an antimicrobial compound. Therefore, it has a high toxic effect on fermenting microorganisms. For that reason, in some applications such as lactic acid or ethanol production, enzymatic hydrolysis of orange peels was performed not only to produce sugars but also to remove D-limonene for the subsequent fermentation step.

In this study, liquid boiling water extraction was used to remove limonene. Fig. 3.12 showed that there was no significant difference in glucose conversion between the control (without pretreatment) and pretreated samples ($p>0.05$). Same results were also obtained for the total reducing sugar yield. The differences between control and pretreated samples' reducing sugar yield were also not significantly different ($p>0.05$).

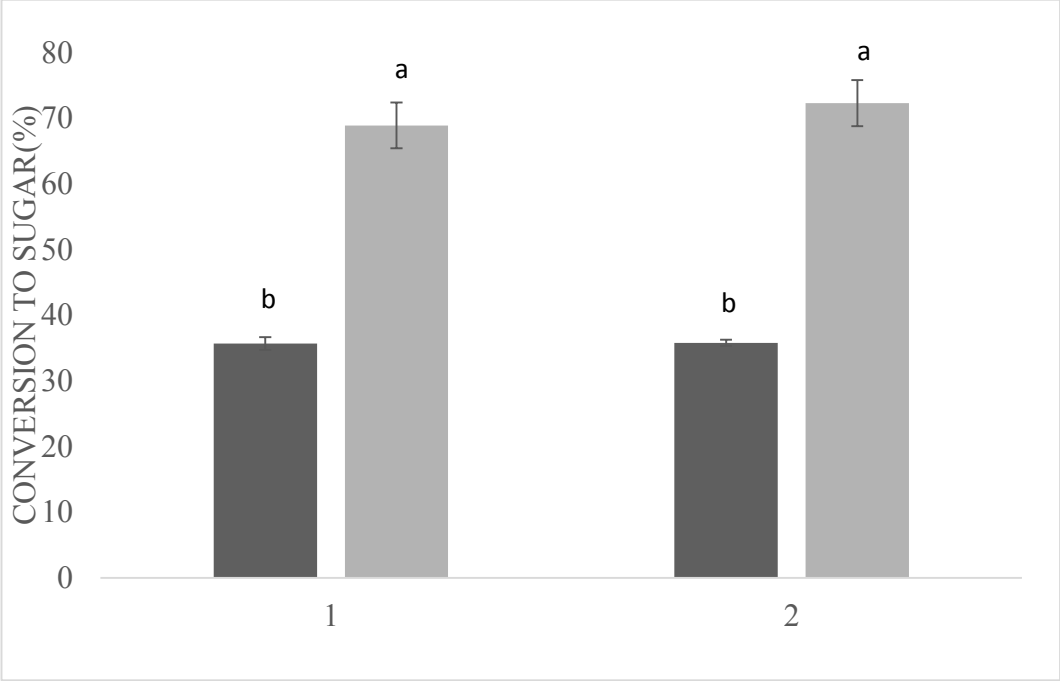

Figure 3.12 Conversion to glucose (%) (■) and conversion to total reducing sugar (%) values (■) of control (without limonene pretreatment) and pretreated fresh orange peels

3.12 Effect of Product Inhibition on Hydrolysis of Orange Peels

Fig. 3.13 shows the sugar yields at different substrate loadings as explained in Section 2.5.7. It is clearly seen that although there is 2-fold increase in total amount of substrate between the first and second hydrolysis, there was no significant difference in glucose amount ($p>0.05$). While it was expected to observe 2-fold increase in glucose concentration, only 11% increase was observed ($p>0.05$). Similarly, the change in total reducing sugar content was also insignificant ($p>0.05$). The insignificant change in sugar yield was attributed to product inhibition and for testing, another hydrolysis experiment was conducted. For the third solution, same glucose concentration of the first orange peel hydrolysate was mimicked by glucose solution and then mixed with the same amount fresh orange peels. Glucose concentration of the third solution was

significantly different ($p<0.05$) from the first solution while it was insignificantly different ($p>0.05$) from the second one. While 2-fold increase was expected for the third hydrolysis relative to first one, only 45% increase was observed indicating product inhibition was present. The results indicated that high solid load (10%) could be the reason for the product inhibition since high solid load is an important factor causing product inhibition. Kristensen et al. (2009) stated that as the solid load increases, product inhibition problem also increases leading to decrease in efficiency of the enzymes. Although other factors discussed in section 3.4 such as adsorption of enzymes to lignin (Pareek et al., 2013) or lower efficiency of the enzymes when they are reused (Romo-sánchez et al., 2014) could also be a reason for these results, product inhibition seems to be dominant this time.

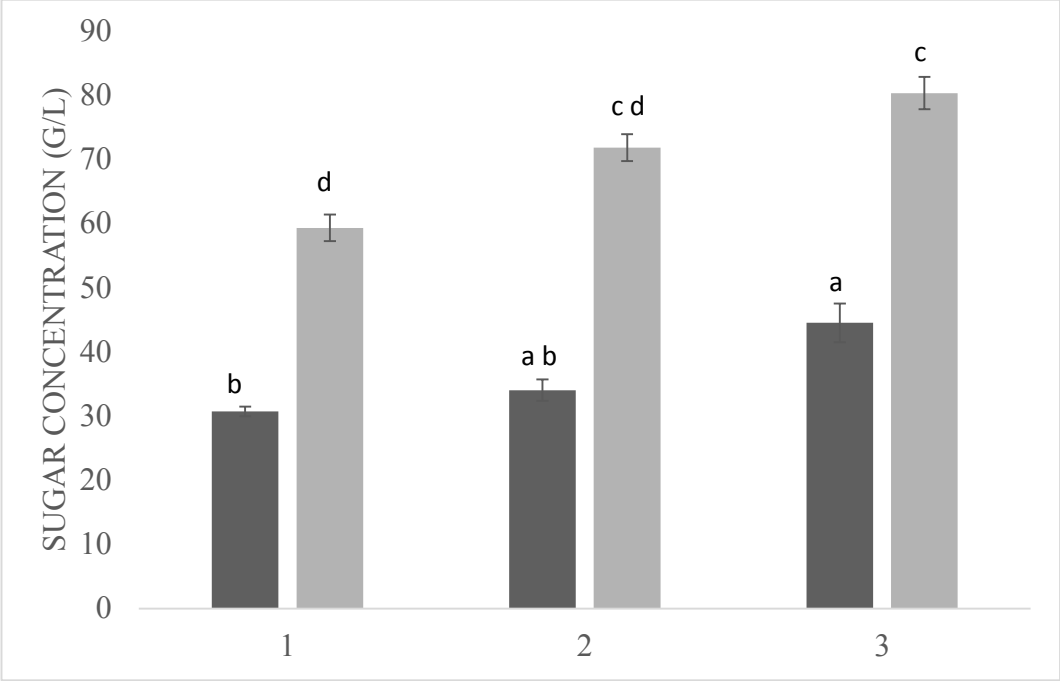

Figure 3.13: Glucose concentration/g/l) (■) and total reducing sugar concentration (g/l) (■) values of fresh orange peels hydrolyzed by 0.5 g cellulase (Biogazyme 2x) (BG). (1: 1st hydrolysis using only fresh orange peel and BG (10% solid load), 2:2nd hydrolysis by using hydrolysates obtained from 1st one and with the addition of new fresh orange peels (20% solid load), 3: 3rd hydrolysis by using glucose solution with the same concentration with 1st hydrolysates, newly added fresh orange peel and BG. Different letters represent significant difference ($p \leq 0.05$). (For the glucose and total reducing sugar concentration (g/l) values, one-way ANOVA was applied separately).

3.13 Effect of Pectinase and Cellulase Loading on Enzymatic Hydrolysis of Pomegranate Peels

In the literature, there is no study related with enzymatic hydrolysis of pomegranate peels. Pomegranate peels consists of 27.9% pectin, 10.8% hemicellulose, 26.2% cellulose and 5.7% lignin (Alaa I., 2013). Since it contains pectin and cellulose, it was decided to use of cellulolytic and pectic enzymes together as in the case of orange peels. Due its low lignin content, pretreatment methods aiming lignin removal was not

preferred before the enzymatic hydrolysis. The enzyme types and concentrations used in pomegranate hydrolysis are given in Table 3.4.

Table 3.3 Amount and Type of Enzymes Used in Hydrolysis of Pomegranate Peels

1	0.03 g BG
2	0.03 g BG+ 250 ul PC
3	0.06 g BG
4	0.06 g BG+250 ul PC
5	0.12 g BG
6	0.12 g BG +250 ul PC
7	0.25 g BG
8	0.25 g BG+ 250 ul PC
9	0.5 g BG
10	0.5 g BG+ 250 ul PC

According to the results shown in Fig. 3.14, if pectinase was not used and Biogazymex2 loading was increased step by step up to 0.5 gram, it was observed that change in glucose concentration was insignificant ($p>0.05$). However, when 0.5 gram BG was used, glucose concentration increased significantly ($p<0.05$). It was also seen that, if 250 ul pectinase was used with other enzyme loading of cellulase, in every step significant change was observed for the glucose concentration ($p<0.05$). Similar trend was also observed for the total reducing sugar concentration of the samples except for the first BG loading of 0.03 grams. Therefore, it could be stated that for the pomegranate peel hydrolysis, pectinase enzyme had an important role in addition to cellulolytic enzymes similar to orange peel hydrolysis.

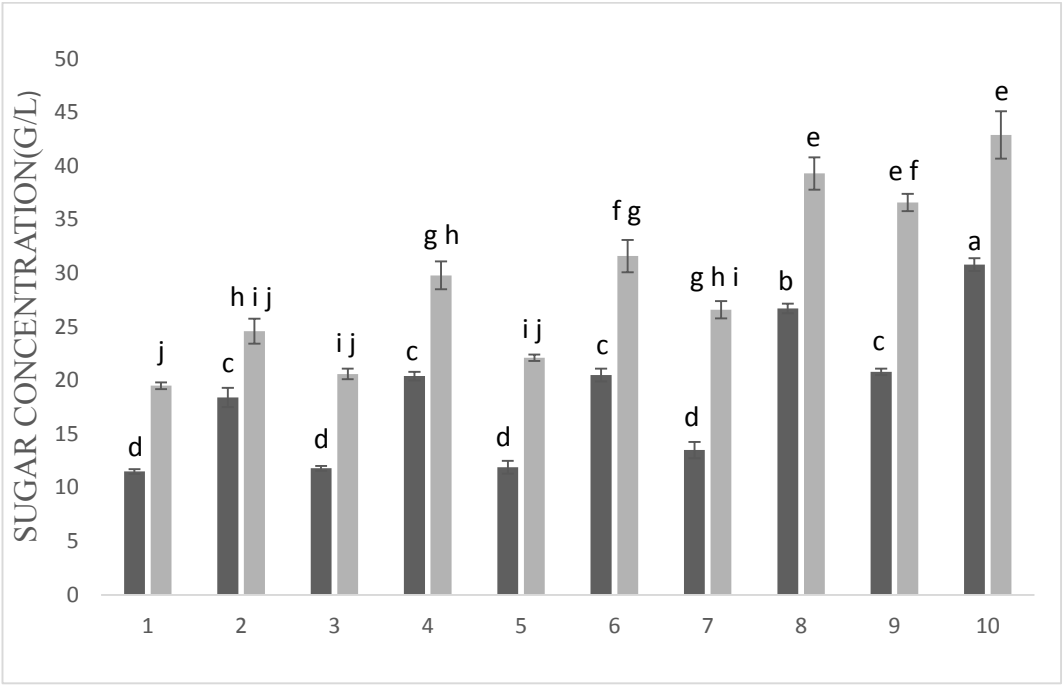

Figure 3.14 Glucose concentration (■) and total reducing sugar concentration values (■) of fresh pomegranate peels hydrolyzed by different load of pectinase (Pectinex Ultra SP-L) (PC) and cellulase(Biogazyme 2x) (BG)) Different letters represent significant difference ($p \leq 0.05$) . (For the Glucose concentration and total reducing sugar concentration values, two-way ANOVA was applied separately).

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

Due to their lignin composition, it is possible to divide lignocellulosic feed stocks into two main groups as lignin poor biomass and lignin rich biomass.

While corn cobs, saw dust are classified as lignin rich biomass because of their high lignin composition, fruit peels like orange and pomegranate peels are grouped in lignin poor biomass.

For the lignin rich biomass, alkaline pretreatment method was applied prior to enzymatic hydrolysis and it was found that it is suitable for the corn cobs rather than saw dust since significant increase was observed in glucose and total reducing sugar yield for the alkali pretreated corn cobs. ($p<0.05$).

For the corn cob hydrolysis addition of β-glucosidase significantly increased the glucose and total reducing sugar yield. On the other hand, if cellulase loading was increased between 150-600 ul, no significant difference was observed in glucose and total reducing sugar yields ($p>0.05$).

For the corn cob hydrolysis, addition of non-ionic surfactant (Tween 80) did not have a significant effect on sugar yield ($p>0.05$).

It was seen that, sugar yield (both glucose and total reducing sugar) reached a plateau at the end of 12 hours.

For the orange peel and pomegranate peel hydrolysis, pectolytic enzyme (pectinase) was required to increase sugar yield in addition to cellulolytic enzymes (cellulase and β-glucosidase).

Since glucose obtained from hydrolysis of these substrates is not sufficient for the lactic acid fermentation, addition of glucose which is obtained from other sources could be a solution for the economy of the process.

Moreover, in order to increase sugar yield obtained from hydrolysis, more effective pretreatment methods could be used prior to enzymatic hydrolysis.

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APPENDIX A

STANDARD CURVE FOR DINITROSALICYLIC ACID (DNS)

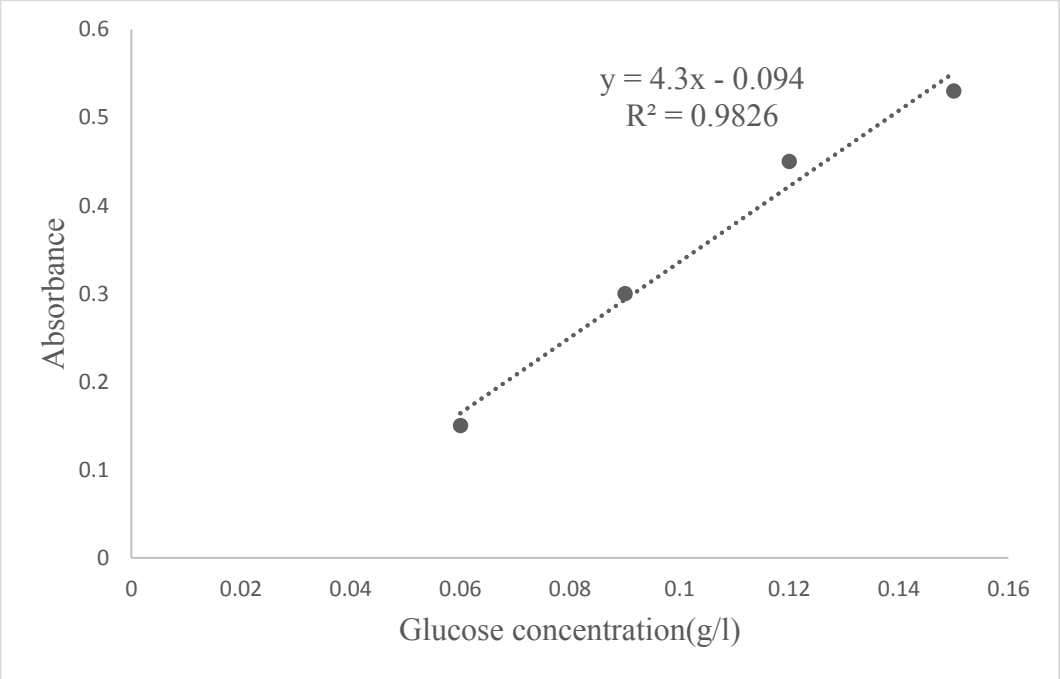

Figure A.1 The standard curve for DNS method

APPENDIX B

STANDARD CURVE FOR BLOOD GLUCOSE MONITOR (BGM)

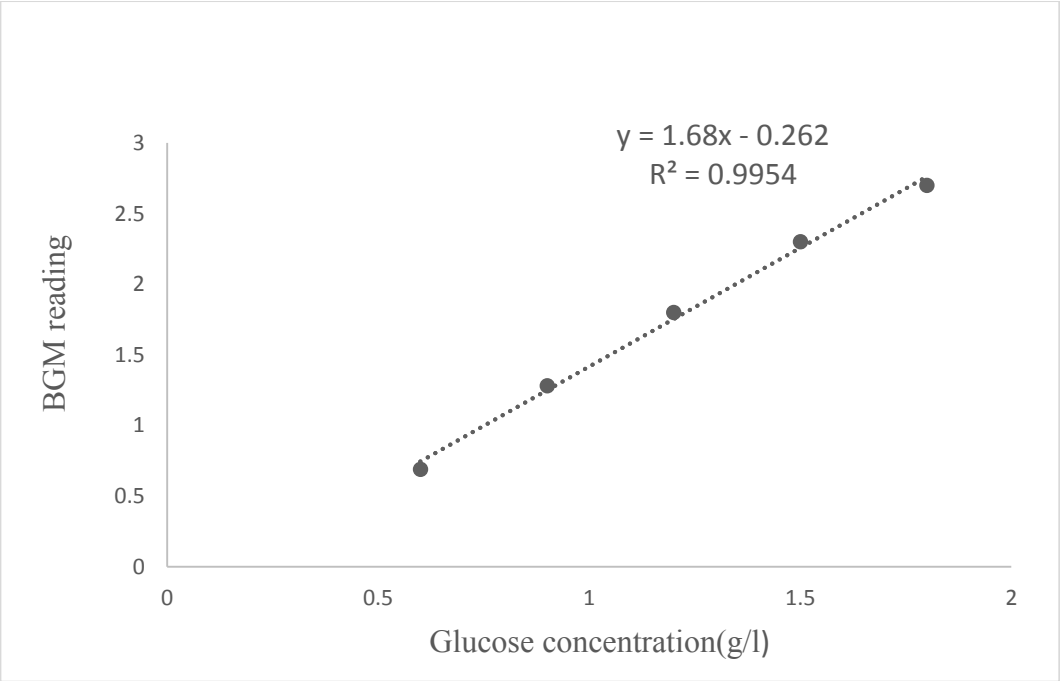

Figure B.1 The standard curve for BGM method

APPENDIX C

STATISTICAL ANALYSIS

Table C.1 Two way ANOVA for alkaline pretreatment effect on glucose yield of corn cobs and sawdust

Source	DF	Seq SS	Adj SS	Adj MS	F	P
subs	1	123.64	123.64	123.64	1204.9	0.000
pret	1	105.49	105.49	105.49	1028.02	0.000
subs* pret	1	105.49	105.49	105.49	1028.02	0.000
Error	4	0.41	0.41	0.10		
Total	7	335.02				

Table C.2 Two way ANOVA for alkaline pretreatment effect on total reducing sugar yield of corn cobs, sun flower seed husks and sawdust

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Subs	1	258.78	258.78	258.78	469.44	0.000
pret	1	148.78	148.78	148.78	269.9	0.000
Subs*pret	1	238.71	238.71	238.71	433.04	0.000
Error	4	2.2	2.2	2.2		
Total	7	648.48				

Table C.3 One way ANOVA for cellobiase effect on glucose yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Cellobiase	1	79.477	477	564.77	0.002
Error	2	0.281	0.141		
Total	3	79.759			

Table C.4 One way ANOVA for cellobiase effect on total reducing sugar yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Cellobiase	1	157.50	157.50	104.13	0.009
Error	2	3.02	1.51		
Total	3	160.53			

Table C.5 One way ANOVA for cellulase load effect on glucose yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
cell 1.5 L	3	18.62	6.21	4.36	0.095
Error	4	5.70	1.43		
Total	7	24.32			

Table C.6 One way ANOVA for cellulase load effect on total reducing sugar yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Cell 1.5 L	3	6.21	2.07	0.24	0.863
Error	4	34.08	8.52		
Total	7	40.30			

Table C.7 One way ANOVA for product inhibition effect on glucose yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Hydrolysis	2	676.60	338.30	111.90	0.002
Error	3	9.07	3.02		
Total	5	685.67			

Table C.8 One way ANOVA for product inhibition effect on total reducing sugar yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Hydrolysis	2	2570.09	1285.05	179.02	0.001
Error	3	21.53	7.18		
Total	5	2591.63			

Table C.9 One way ANOVA for CTec2 load effect on glucose yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Ctec2	5	397.99	79.60	60.84	0.000
Error	6	7.85	1.31		
Total	11	405.84			

Table C.10 One way ANOVA for CTec2 load effect on total reducing sugar yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
Ctec2	5	564.2	112.8	8.28	0.011
Error	6	81.8	13.6		
Total	11	646.0			

Table C.11 One way ANOVA for Tween 80 load effect on glucose yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
TW 80	2	3.430	1.715	3.71	0.154
Error	3	1.385	0.462		
Total	5	4.815			

Table C.12 One way ANOVA for Tween 80 load effect on total reducing sugar yield of alkaline pretreated corn cobs

Source	DF	SS	MS	F	P
TW 80	2	9.8	4.9	0.35	0.729
Error	3	41.8	13.9		
Total	5	51.6			

Table C.13 One way ANOVA for solid load effect on glucose yield of orange peels

Source	DF	SS	MS	F	P
SL (%)	2	199.023	99.512	370.85	0.000
Error	3	0.805	0.268		
Total	5	199.828			

Table C.14 One way ANOVA for solid load effect on total reducing sugar yield of orange peels

Source	DF	SS	MS	F	P
SL (%)	2	265.90	132.95	74.62	0.003
Error	3	5.34	1.78		
Total	5	271.25			

Table C.15 Two way ANOVA for low load PC&BG effect on glucose yield of orange peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
PC	1	63.368	63.368	63.368	44.10	0.000
BG	4	358.777	358.777	89.694	62.42	0.000
PC*BG	4	52.687	52.687	13.172	9.17	0.002
Error	10	14.370	14.370	1.437		
Total	19	489.202				

Table C.16 Two way ANOVA for low load PC&BG effect on total reducing sugar yield of orange peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
PC	1	461.76	461.76	461.76	66.01	0.000
BG	4	1122.39	1122.39	280.60	40.11	0.000
PC*BG	4	155.08	155.08	38.77	5.54	0.013
Error	10	69.95	69.95	7.00		
Total	19	1809.19				

Table C.17 Two way ANOVA for high load PC&BG effect on glucose yield of orange peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
BG	1	1306.82	1306.82	1306.82	77.22	0.000
PC	3	286.44	286.44	95.48	5.64	0.022
BG*PC	3	2.84	2.84	0.95	0.06	0.981
Error	8	135.39	135.39	16.92		
Total	15	1731.50				

Table C.18 Two way ANOVA for high load PC&BG effect on total reducing sugar yield of orange peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
BG	1	1185.08	1185.08	1185.08	49.90	0.000
PC	3	555.95	555.95	185.32	7.80	0.009
BG*PC	3	4.74	4.74	1.58	0.07	0.976
Error	8	190.01	190.01	23.75		
Total	15	1935.77				

Table C.19 Two way ANOVA for liquid boiling water effect on glucose and total reducing sugar yield of orange peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pretreatment	1	5.95	5.95	5.95	0.47	0.532
Type	1	2425.56	2425.56	2425.56	190.56	0.000
Pretreatment*Type	1	5.95	5.95	5.95	0.47	0.532
Error	4	50.91	50.91	12.73		
Total	7	2488.38				

Table C.20 One way ANOVA for product inhibition effect on glucose yield of orange peels

Source	DF	SS	MS	F	P
Hydrolysis	2	207.24	103.62	13.07	0.033
Error	3	23.79	7.93		
Total	5	231.03			

Table C.21 One way ANOVA for product inhibition effect on total reducing sugar yield of orange peels

Source	DF	SS	MS	F	P
Hydrolysis	2	444.64	222.32	22.44	0.016
Error	3	29.73	9.91		
Total	5	474.37			

Table C.22 Two way ANOVA for PG&BG effect on glucose yield of pomegranate peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
BG	4	317.135	317.135	79.284	136.34	0.000
PC	1	442.740	442.740	442.740	761.38	0.000
BG*PC	4	21.547	21.547	5.387	9.26	0.002
Error	10	5.815	5.815	0.582		
Total	19	787.237				

Table C.23 Two way ANOVA for PG&BG effect on reducing sugar yield of pomegranate peels

Source	DF	Seq SS	Adj SS	Adj MS	F	P
BG	4	796.41	796.41	199.10	69.97	0.000
PC	1	365.51	365.51	365.51	128.45	0.000
BG*PC	4	34.24	34.24	8.56	3.01	0.072
Error	10	28.46	28.46	2.85		
Total	19	1224.62				

APPENDIX D

IMAGES OF THE SUBSTRATES

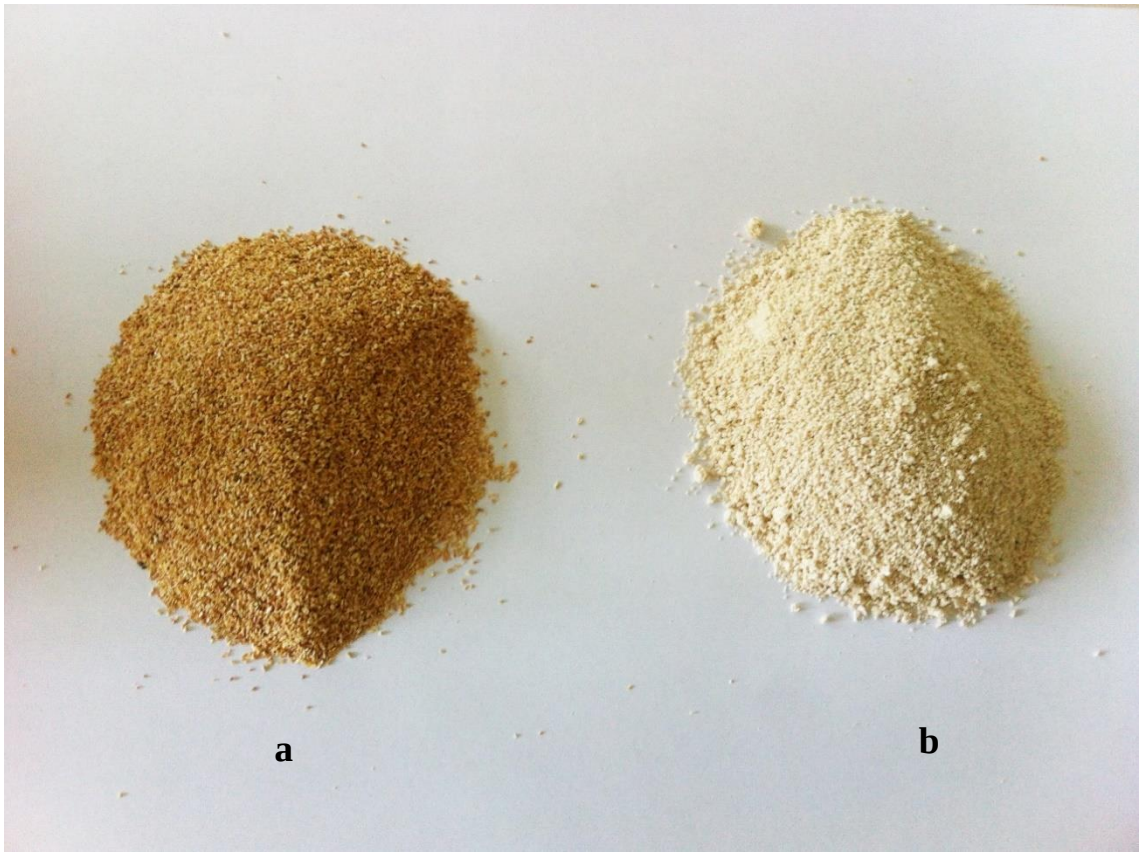

Figure D.1 Images of milled corn cobs **a)** Control **b)** Alkaline Pretreated

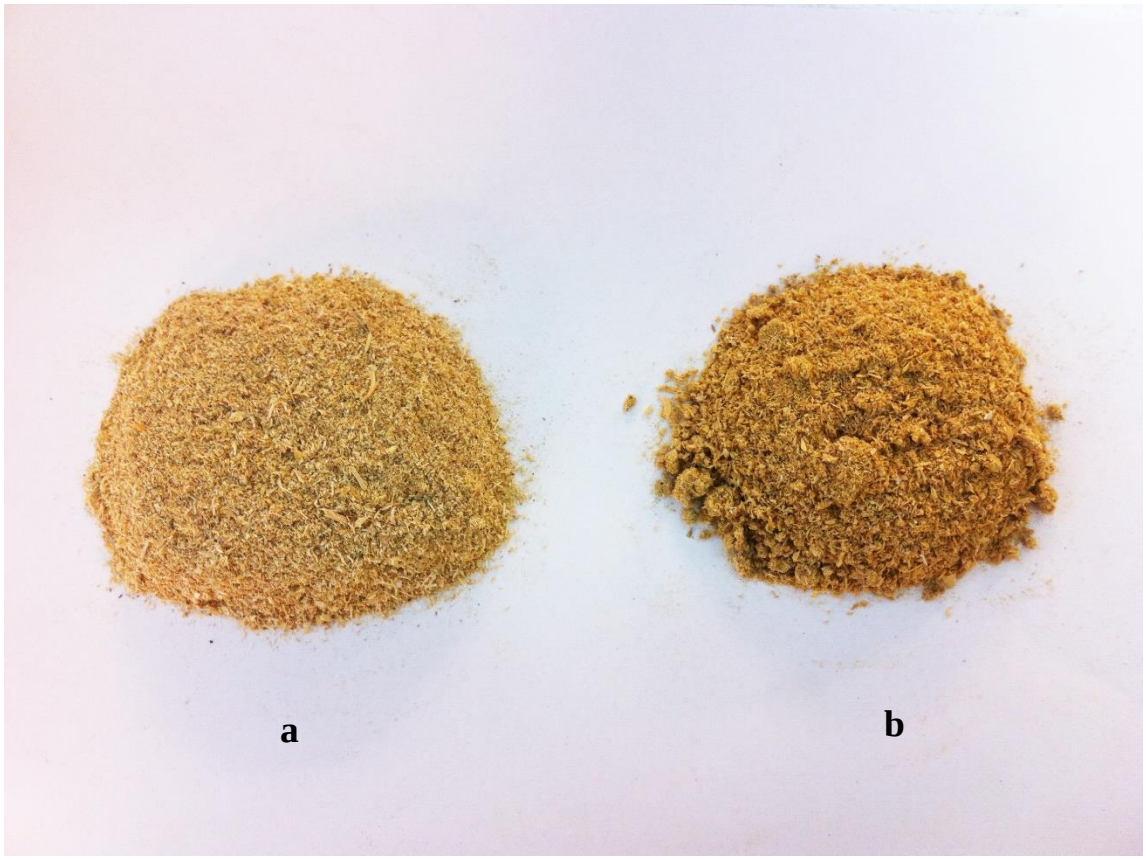

Figure D.2 Images of milled saw dusts **a)** Control **b)** Alkaline Pretreated

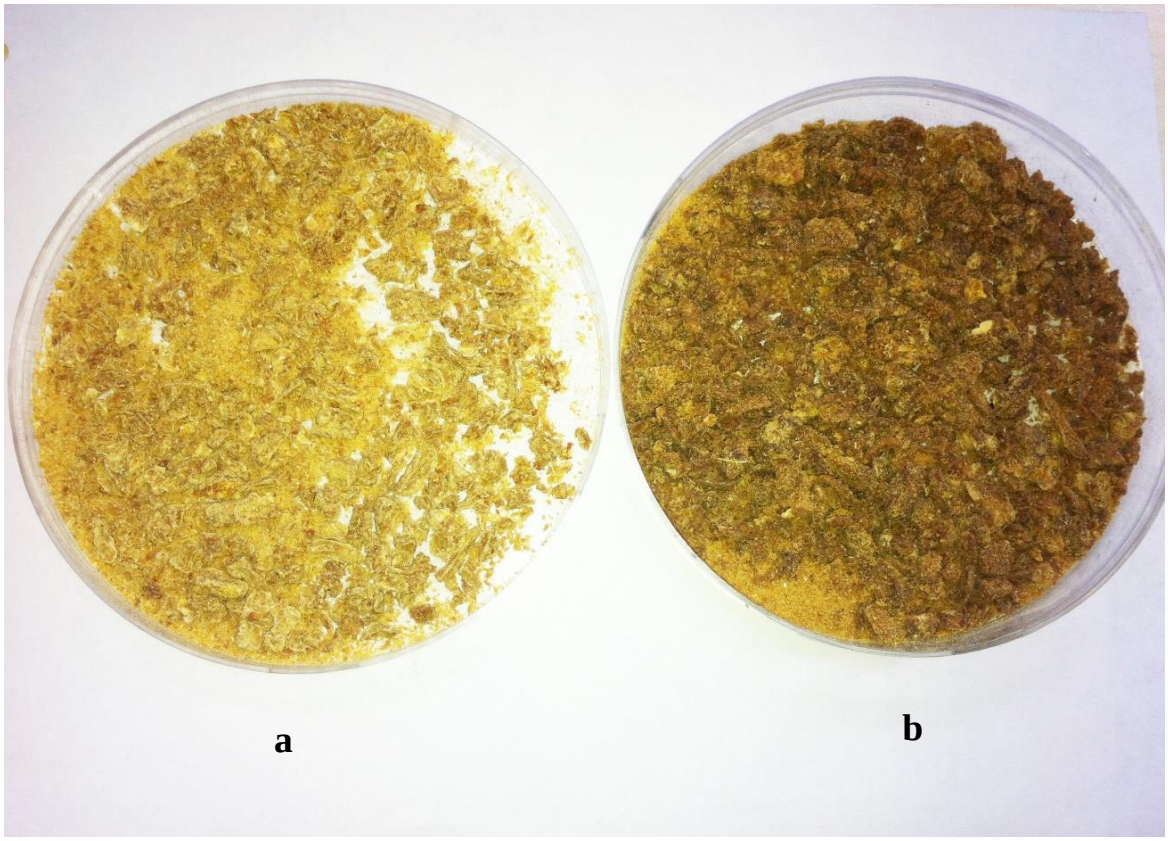

Figure D.3 Images of milled, dried orange peels **a)** Liquid boiling water pretreated **b)** Control