INVESTIGATING THE EFFECT OF DIFFERENT FACTORS ON ENZYMATIC HYDROLYSIS OF SUGAR BEET PULP & CORN COB

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BERNA LEYLUHAN YURTSEVEN

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INVESTIGATING THE EFFECT OF DIFFERENT FACTORS ON ENZYMATIC HYDROLYSIS OF SUGAR BEET PULP & CORN COB

submitted by **BERNA LEYLUHAN YURTSEVEN** in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Assoc. Prof. Dr. Can Özen
Head of Department, **Biotechnology** _____

Assoc. Prof. Dr. Mecit Halil Öztop
Supervisor, **Biotechnology, METU** _____

Assoc. Prof. Dr. Çağdaş Devrim Son
Co-Supervisor, **Biology, METU** _____

Examining Committee Members:

Prof. Dr. Alev Bayındırlı
Food Engineering, METU _____

Assoc. Prof. Dr. Mecit Halil Öztop
Biotechnology, METU _____

Prof. Dr. Hami Alpas
Food Engineering, METU _____

Assoc. Prof. Dr. Yeşim Soyer
Food Engineering, METU _____

Assist. Prof. Dr. Emin Burçin Özvural
Food Engineering, Çankırı Karatekin University _____

Date: 26.11.2019

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname: Berna Leyluhan Yurtseven

Signature:

ABSTRACT

INVESTIGATING THE EFFECT OF DIFFERENT FACTORS ON ENZYMATIC HYDROLYSIS OF SUGAR BEET PULP & CORN COB

Leyluhan Yurtseven, Berna
Master of Science, Biotechnology
Supervisor: Assoc. Prof. Dr. Mecit Halil Öztop
Co-Supervisor: Assoc. Prof. Dr. Çağdaş Devrim Son

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Hydrolysis of biomass obtained as the waste of industrial or agricultural production has gained significant interest in recent years. In this study, 2 different biomasses were studied.

In the 1st part of the study, sugar beet pulp (SBP) hydrolysis was optimized using *Response Surface Methodology (RSM)*. Sugar beet pulp is an important by product of sugar manufacturing and its hydrolysis could yield valuable sugars that can be used in fermentation processes. As the parameters of SBP hydrolysis by RSM, effect of enzyme concentration, hydrolysis time and pulp amount were examined. A central composite design with 5 levels was constructed using; substrate loadings, two different enzyme- Pectinex Ultra SP-L and Cellic Ctec3 concentrations and hydrolysis time. It was found that at 20% SBP subtrate loadings, as the volume of Pectinex Ultra SP-L was increased above 250 µl, yield reached its maximum- above 90 g/L. As the SBP loading increased, even while using lower volumes of Cellic Ctec3 -around 150 µl, yield reached its maximum. Analysis showed that combining Pectinex Ultra SP-L with Cellic Ctec3 resulted in a synergetic response. RSM estimates indicated that the optimal point for maximum reduced sugar yield was beyond the experimental range used in this thesis. In addition to RSM, classification and regression tree (CART)

method was used to investigate the effects of substrate amount, enzyme loadings and hydrolysis time. Regression tree analysis results confirmed the estimations from RSM. Moreover, classification tree analysis results form a basis for future optimization studies.

In the 2nd part of the study, corn cob was used as the substrate for hydrolysis. Corn cob is a high lignin product and its hydrolysis requires the use of different pretreatment methods. In this study, rather than using a pretreatment method, surfactants were utilized to increase the accessibility of the enzymes to the cellulosic network through lignin. Tween 20 and 80 were used as the surfactants. Hydrolysis was performed in 0.05 M sodium citrate buffer at a pH of 4.8. Celluclast 1.5L and Novozyme 188 were used at a fixed concentration of 150 μL as the enzymes and the hydrolysis was monitored for 24 hours. Results showed that effect of surfactant addition was not significant on hydrolysis ($p<0.05$) as long as lignin was not removed by a pretreatment method.

Keywords: Enzymatic hydrolysis, Surfactant, Tween 20, Tween 80, Corn cob, Sugar beet pulp (SBP), Pectinex Ultra SP-L, Cellic Ctec3, Celluclast 1.5L, Novozyme 188

ÖZ

FARKLI FAKTÖRLERİN ŞEKER PANCARI KÜSPESİ ve MISIR KOÇANININ ENZİMATİK HİDROLİZİNE ETKİSİNİN ARAŞTIRILMASI

Leyluhan Yurtseven, Berna
Yüksek Lisans, Biyoteknoloji
Tez Danışmanı: Doç. Dr. Mecit Halil Öztop
Ortak Tez Danışmanı: Doç. Dr. Çağdaş Devrim Son

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Endüstriyel veya tarımsal üretim atıkları olarak elde edilen biyokütlenin hidrolizi son yıllarda önemli ilgi görmektedir. Bu çalışmada 2 farklı biyokütle çalışılmıştır.

Çalışmanın 1. bölümünde, şeker pancarı küspesi (SBP) hidrolizi, Yanıt Yüzey Metodolojisi (RSM) kullanılarak optimize edilmiştir. Şeker pancarı küspesi, şeker üretiminin bir ürünüdür ve hidrolizi, fermantasyon işlemlerinde kullanılabilecek değerli şekeri üretmeye olanak sağlar. SBP'nin RSM ile hidroliz parametreleri olarak enzim konsantrasyonunun etkisi, hidroliz süresi ve küspe miktarı incelenmiştir. Deney tasarımı, 5 seviyeli merkezi kompozit tasarım kullanılarak yapılmış vesubstrat miktarı, iki farklı enzim-Pectinex Ultra SP-L ve Cellic Ctec3 konsantrasyonu ve hidroliz süresi etkisi incelenen parametreler olmuştur. %20 SBP kullanıldığında, Pectinex Ultra SP-L'nin miktarının 250 µl'nin üzerine çıkarılması sonucunda verimin 90 g / L'nin üstüne çıktığı bulunmuştur. SBP yüzdesi arttıkça, 150 µl civarında düşük hacimli Cellic Ctec3 kullanırken bile, verimin maksimuma ulaştığı tespit edilmiştir. Analiz sonuçları, Pectinex Ultra SP-L'nin Cellic Ctec3 ile beraber kullanımının sinerjik bir etki oluşturduğunu göstermektedir. RSM modeli sonucunda elde edilen tahminler, maksimum indirgenmiş şeker verimi için optimal noktanın bu tezde kullanılan deneysel aralığın ötesinde olduğunu göstermiştir. RSM'ye ek olarak,

substrat miktarı, enzim konsantrasyonları ve hidroliz süresinin etkilerini araştırmak için sınıflandırma ve Regresyon Ağacı (CART) yöntemi kullanılmıştır. Regresyon Ağacı analizi sonuçları, RSM'deki tahminleri doğrulamıştır. Ayrıca, analizi sonuçları ilerideki optimizasyon çalışmaları için bir temel oluşturabilir niteliktedir.

Çalışmanın 2. bölümünde, mısır koçanı hidroliz için substrat olarak kullanılmıştır. Mısır koçanı yüksek lignin içerikli bir ürünüdür ve hidrolizi, farklı ön işlem yöntemlerinin kullanılmasını gerektirmektedir. Bu çalışmada, ön-işlem yöntemi kullanmak yerine, enzimlerin selülozik ağa lignin yoluyla erişebilirliğini arttırmak için yüzey aktif maddeler kullanılmıştır. Yüzey aktif madde olarak Tween 20 ve 80 kullanılmıştır. Hidroliz, pH'ı 4.8 olan 0.05 M sodyum sitrat tamponunda gerçekleştirilmiş, Celluclast 1.5L ve Novozyme 188, enzim olarak 150 µL sabit konsantrasyonda kullanılmış ve hidroliz, 24 saat süresince izlenmiştir. Sonuçlar, yüzey aktif madde ilavesinin etkisinin, lignin bir ön muamele yöntemi ile ortamdan uzaklaştırılmadığı sürece hidroliz üzerinde etkili olmadığını göstermiştir ($p<0.05$).

Anahtar Kelimeler: Enzimatik hidroliz, Sürfektan, Tween 20, Tween 80, Mısır koçanı, Şeker pancarı küspesi, Pectinex Ultra SP-L, Cellic Ctec3, Celluclast 1.5L, Novozyme 188

To my beloved son, Kerem

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INTRODUCTION

1.1. Why do we need a better biomass conversion process?

Today, to replace fossil fuels by biofuel is one of the primary goals of nations not only to decrease cost of the energy but also to save the world by decreasing or eliminating the bad effects of fossil fuels on environment. Since the emission of carbon dioxide is almost at zero level when cellulose biomass-based biofuel is used, it is the better replacement of fossil fuels (Ullah et al. 2018). Bioethanol is the most used environmental biofuel; it is not toxic and does not contaminate water sources (Sánchez and Cardona 2008). In addition, it is processed from the crops like sugar cane, sugar beet, maize etc. which can lead to food crisis if not consumed responsibly. Waste has increased dramatically in recent years as a result of increased demand for processed foods, lignocellulosic biomass is widely available in the Earth (Radenkovs et al. 2018). So, the proposed generation of biofuels from agro-wastes are gaining worldwide attention (Ullah et al. 2018).

As can be seen in Figure 1.1, bioethanol is generally produced by fermenting monomeric sugars obtained from the enzymatic treatment of cellulosic and hemi cellulosic polymeric chains, of which exposure to enzymes is often enhanced by pretreatment methods. Particle size is reduced generally reduced by milling. Smaller particle size is associated with reduction of crystallinity of lignocellulosic biomass (Paulova et al. 2015).

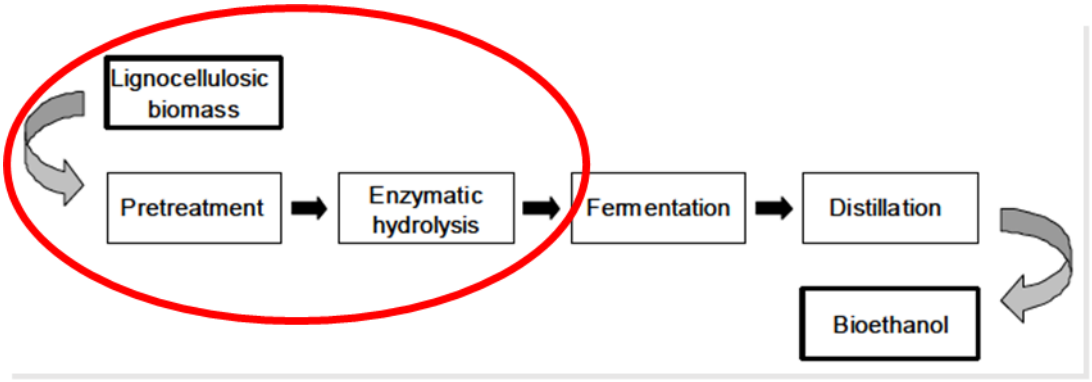

Figure 1.1. Bioethanol production process

The process steps can be carried out consecutively (separate hydrolysis and fermentation) or simultaneously. Main drawback of consecutive process is the end-product (glucose) inhibition, especially high at high substrate loadings. On the other hand, since optimum temperature of cellulolytic enzymes (usually 45 – 50°C) and that of fermenting microorganisms (mostly 28 – 37°C) are different, simultaneous production does not always give the expected results, generally results in decrease of ethanol yield. There are many studies result in new approaches to overcome these kinds of drawbacks. The economical bioethanol production from lignocellulosic biomass technology can be influenced by many factors; when carried out consecutively, during the hydrolysis, these are the type of lignocellulosic feedstock, pretreatment methods and the type & amount of cellulolytic enzymes (Paulova et al. 2015).

Due to the advances in enzyme technology the cost of biomass-based bioethanol production has decreased dramatically in recent years. On the other hand, the conversion of cellulosic components into fermentable sugars efficiently are still the major challenge and the enzymatic hydrolysis still forms a major cost factor, mainly due to the high cost of enzymes in the conversion (Maitan-Alfenas, Visser, and Guimarães 2015; Viikari, Vehmaanperä, and Koivula 2012).

1.2. Lignocellulosic biomass practices in agro-industry

1.2.1. Lignocellulosic biomass and its characteristics

Lignocellulosic biomass consists of cellulose, hemicellulose, lignin and small amount of pectin, protein and ash. Composition and structure differ according to the source of biomass which makes the conversion difficult. Cellulose, hemicellulose, and lignin are formed a complex cell wall structure, so biodegradation becomes a major challenge. Organic materials, generally treated as waste such as straw, corn stover, grass bagasse, rice straw, olive tree branches in addition to soft or hard wood are abundantly available and their acquisition is highly cost effective. So, it is possible that lignocellulosic biomass could solve or contribute to solve some problems currently facing with; energy crises, food shortages and climate change are some examples (Chen 2014).

1.2.1.1. Structure and Properties of Cellulose

Cellulose is a biodegradable and a water insoluble polymer with the molecular formula $(C_6H_{10}O_5)_n$ (*n*, called the degree of polymerization (DP)) and made up of glucose units liked by β-(l-4)-glycosidic bonds which form a dimer, cellobiose. It is widely available on earth and found mainly in higher plants as the main component of the plant cell. Cellulose has both crystalline and amorphous regions. Cellulose molecules are arranged regularly at its crystalline region and the crystallinity of cellulose makes it recalcitrant, so the chemical processing of cellulose is difficult. On the other hand, amorphous part is arranged irregularly which leads to more relaxed structure (Chen 2014). Interactions between and within the cellulose chains specify the properties of cellulosic materials (Suhas et al. 2016). Cellulose is usually covered by hemicellulose and lignin.

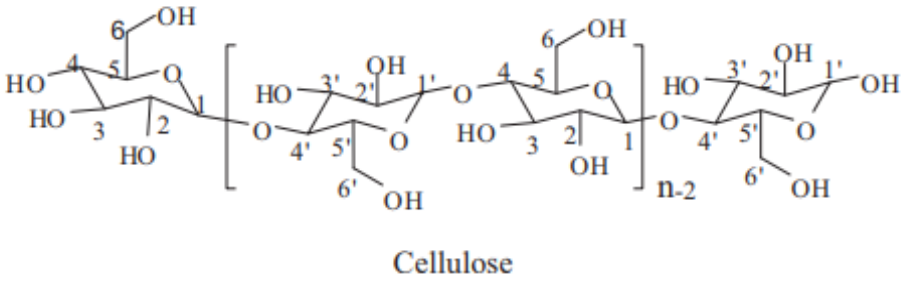

Figure 1.2. Structure of cellulose (Suhas et al. 2016)

1.2.1.2. Structure and Properties of Hemicellulose

Hemicellulose is an amorphous material with a low polymerization degree. Its structure makes hemicellulose a complex molecule. Hemicellulose composition is different in various plants (Chen 2014). Hemicelluloses are polymers composed of pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids. Xylan and glucomannan are the main hemicellulosic components of hardwoods and softwoods, respectively (Kumar, Singh, and Singh 2008).

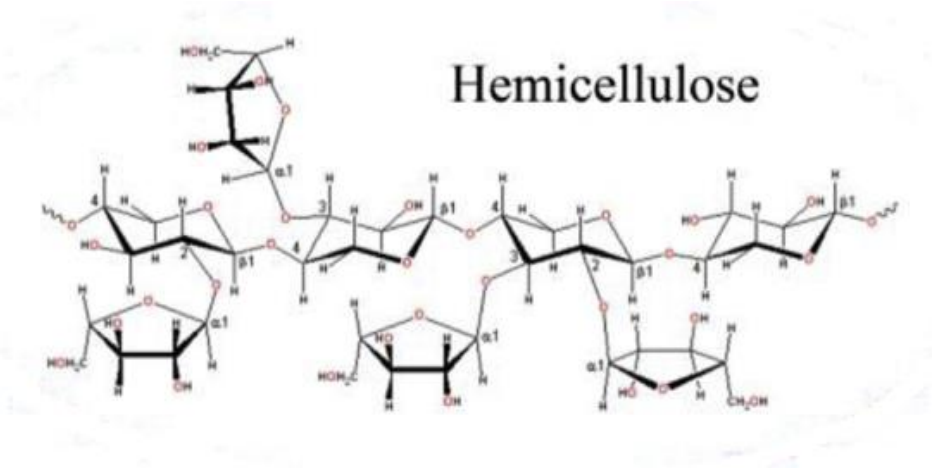

Figure 1.3. Structure of hemicellulose (Radenkovs et al. 2018)

1.2.1.3. Structure and Properties of Lignin

Lignin is the most abundant non-carbohydrate organic compound and it is the second most abundant biopolymer after cellulose. Lignin is a three-dimensional amorphous polymer mainly linked by ether bonds between monomeric phenylpropane units most of which are not readily hydrolysable, is a constituent of the plant cell wall. There are differences in lignin composition between various plants and within the different tissues of the same plant which makes lignin unusual. Since their exact composition is not known, it is hard to decompose lignin by using enzymes and microorganisms. Lignin covers the cellulose and hemicellulose and prevents enzymes to reach them for the biochemical conversion. Lignin produces polyphenols which adversely affect the quality of the fermentation process (Elbersen et al. 2017).

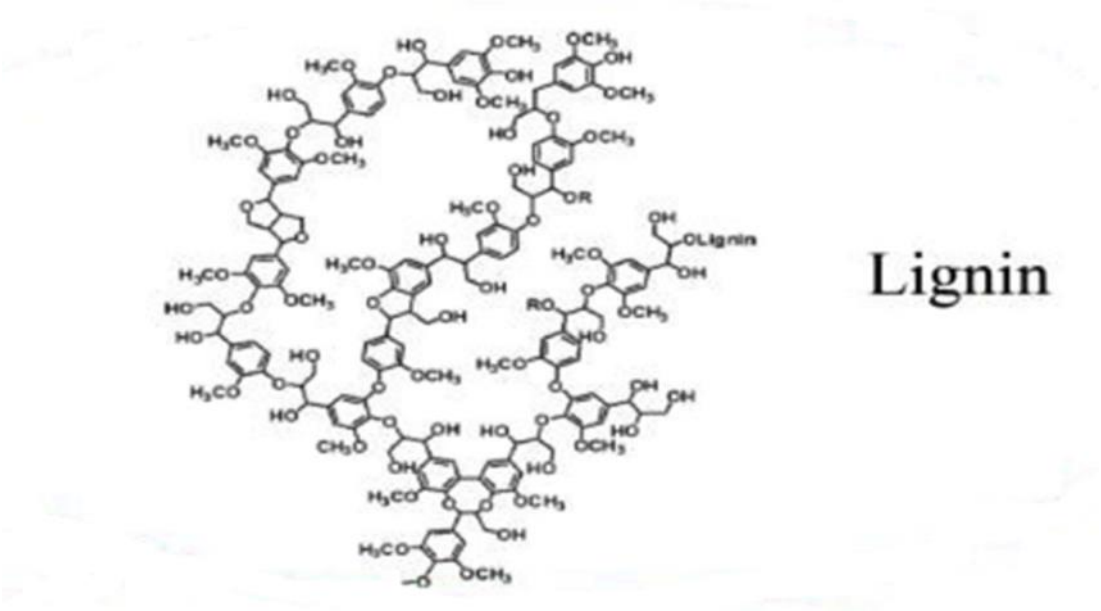

Figure 1.4. Structure of lignin (Radenkovs et al. 2018)

1.2.1.4. Pectin

Pectin, the major component of the cell wall, is composed of homo-galacturonic acid regions with neutral sugar side chains (L-rhamnose, arabinose, galactose and xylose)

(Kumar, Singh, and Singh 2008). Galacturonic acid is composed of carboxyl groups which are esterified by methyl groups and neutralized by sodium, potassium or ammonium ions. Pectic substances are grouped as protopectin, pectic acid, pectinic acid and pectin (Kashyap et al., n.d.). On the other hand, sugar beet pectin is composed of homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) regions. Since acetyl groups are present in both HG and RG-I, the degree of acetylation is higher when compared with other pectin sources. Side chains of RG-I are linear β-(1,4)-linked galactan and highly branched arabinan, composed of α-(1,5)-linked backbones with α-(1,2) and/or α-(1,3) arabinofuranosyl substitutions (Leijdekkers et al. 2013). Degradation of pectin makes way for the enzymes to reach cellulosic materials for hydrolysis.

1.2.2. Lignocellulosic feedstocks used in enzymatic hydrolysis

Lignocellulose is a heterogenous polymeric material that forms a complex structure with a 3D network (Gupta et al. 2016). Although composition of lignocellulosic feedstock varies in nature, it is mainly composed of cellulose (40-50%), hemicellulose (25-30%) and lignin (15-20%). Pectin, proteins, ash, salt and minerals are other components found in lignocellulose. Lignin is a complex aromatic alcohol polymer. On the other hand, cellulose consists of glucose chains linked by β-1,4 linkages. The major component of hemicellulose is mannan and it is composed of sugars like mannose, galactose and glucose. Pectin is widely available in nature and its major component is the galacturonic acid (Van Dyk and Pletschke 2012).

Polysaccharide cellulose and hemicellulose can be converted into its monomers, thus biofuels. Today, the methods are developed, or studies are carried on keeping maximum polysaccharide fraction within the lignocellulosic biomass to obtain higher amount of total sugars (Van Dyk and Pletschke 2012). Future trends include the production of genetically modified plant materials used as a biomass to produce bioethanol (Sánchez and Cardona 2008).

Substrate loading is determined according to type of sugars utilized in fermentation and the loading level varies depending on the carbohydrate and lignin composition. As a general approach, high solid loadings are preferred due to its advantages; such as increased sugar and ethanol concentrations and decreased operating costs, over low or moderate solid loadings.

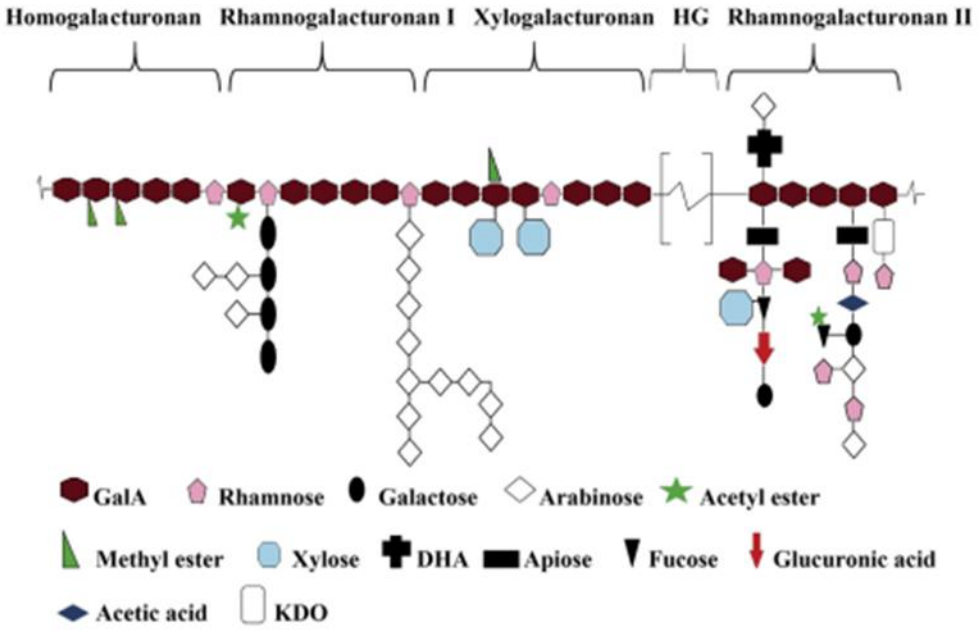

Figure 1.5. Structure of pectin (Radenkovs et al. 2018)

1.2.2.1. Sugar Beet Pulp (SBP)

Sugar beet is the raw material of table sugar -sucrose - and globally 35% of sucrose is extracted from sugar beets. Carbohydrate content is high in SBP, which is a byproduct of the sugar beet industry. After sucrose extraction, drying the pulp and selling as animal feed is a common practice. On the other hand, processing SBP for animal feed does not have any remarkable return; so, it could be more useful to use it as a feedstock for bioethanol production (Donkoh et al. 2012). Its lignin content is low, and pectin and hemicellulose content are high. Average composition of dried SBP consists of 20-24% cellulose, 25-36% hemicellulose (essentially arabinan), 20-25% pectin, 1-2%

lignin and 7-8% protein (Foster, Dale, and Doran-Peterson 2001). The SBP polysaccharide composition comprised of glucan (25.2%), arabinan (18.1%), galacturonic acid (15.4%), galactan (6.6%), and xylan (2.3%) (Nahar, Rorick, and Pryor 2014).

To achieve the conversion of carbohydrates to soluble sugars, without formation of inhibitory products, pretreatment methods must be efficiently applied and optimized (Foster, Dale, and Doran-Peterson 2001). Acid hydrolysis pretreatment produces acids, furan derivatives and phenolic compounds as inhibitors; on the other hand, it is a very effective method (Guo, Chang, and Lee 2018). Technical (high specificity, mild reaction conditions) and economic (cost of processes) advantages of enzymatic hydrolysis over chemical is high (Spagnuolo et al. 1997). Because of the low lignin content of SBP, the costly pretreatment step was eliminated in this study.

Galactose, arabinose and galacturonic acid are the sugars hydrolyzed from hemicellulose and pectin in SBP. Studies showed that highest sugar yields were obtained when pectinases combined with cellulases or hemicellulases (Nahar, Rorick, and Pryor 2014). In other words, hydrolysis of lignocellulosic material components is efficient if various enzymes are combined synergistically to degrade both crystalline and amorphous parts of the fiber and to make the structure more permeable (Spagnuolo et al. 1997). This is the reason of choosing pectinase and cellulase & hemicellulase combination in this study.

Table 1.1. *Sugar beet pulp composition (*(Foster, Dale, and Doran-Peterson 2001)

Constituents	*Content (% w/w)*
Cellulose	20-24
Hemicellulose	26-36
Pectin	20-25
Lignin	1-2

1.2.2.2. Corn Cob

Corn cob is a potential feedstock to produce bioethanol. 700 kg of mono and oligosaccharides can be extracted from the saccharification of a ton of corn cob. Average composition of dried corn cob consists of 36.3 - 41.3 % cellulose, 39.2 – 49.6 % hemicellulose, 9.6 - 14.2 % lignin. In addition, it involves 0.28 – 0.32 % fat, 3.3 – 5.22 % protein and 2.77 – 2.99 % ash (Pointner et al. 2014). Depending on the type of the corn cob or harvesting conditions, the composition could be different.

1.2.3. Enzymatic Hydrolysis of Lignocellulosic Biomass

Lignocellulosic materials are highly recalcitrant due to the structural integrity of cellulose; the strong intra- and intermolecular hydrogen bonds which are the constituents of crystalline cellulose contrary to hemicellulose and amorphous cellulose. In addition, the highly organized cell wall structures in plants and the presence of lignin also contribute to biomass recalcitrance (Viikari, Vehmaanperä, and Koivula 2012). So, pretreatment methods, the most expensive parts of the transformation, are developed to increase the accessibility of enzymes to lignocellulosic biomass (Manisha and Yadav 2017). Acid reaction, steam explosion, ammonia fiber growth, organosolv, sulfite pretreatment to beat the recalcitrance of lignocellulose, alkalescent wet reaction and gas pretreatment are some of the used pretreatment techniques (Ullah et al. 2018). Physical methods; chipping, grinding and milling, are not preferred due to their high energy and capital costs, but they reduce the biomass crystallinity, and increase the access of cellulases (Sánchez and Cardona 2008). Biological methods; such as, direct enzymatic hydrolysis or fungal hydrolysis have low capital cost with respect to other methods and environmentally friendly but has long reaction times (Guo, Chang, and Lee 2018). Pretreatment methods are selected by considering the composition of lignin and hemicellulose of biomass which is highly dependent on the type of plant which the biomass is obtained, age of crop and harvesting method etc. (Sánchez and Cardona 2008).

Combination of enzymes selected from a broad spectrum are expected to create a synergetic effect on the enzymatic hydrolysis of lignocellulosic biomass after pretreatment (Viikari, Vehmaanperä, and Koivula 2012). Besides, lower amount of enzyme is needed depending on the used substrate and the selection of suitable pretreatment method (Van Dyk and Pletschke 2012). Endoglucanases, cellobiohydrolases, and β-glucosidases are the enzymes used for cellulose degradation which hydrolyze the β-1,4 covalent bonds within the glucose units. The enzymatic breakdown of hemicellulose requires many enzymes; non-arabinose- and arabinose liberating endo-xylanases, endo-xylanases, β-xylosidases, xyloglucanases, acetyl xylan esterases and ferulic esterases, β-mannanases, /-galactosidases, /-arabinofuranosidases and /-glucuronidases (Gupta et al. 2016).

Lignin also binds both cellulases and hemicellulases unproductively during the enzymatic hydrolysis. Adding various surface-active compounds, such as Tween 20, Tween 80, or polyethylene glycol (PEG) is also known as promising way to prevent unproductive binding of enzymes to lignin (Viikari, Vehmaanperä, and Koivula 2012). On the other hand, the economic feasibility is doubtful. The efficiency highly depends on the type of biomass used and the pretreatment methods (Paulova et al. 2015).

Exoglucanases and β-glucosidases are inhibited by their reaction products; cellobiose and glucose (Gupta et al. 2016). Detoxification of lignocellulosic hydrolysates is needed to eliminate the inhibitors produced before fermentation. And some of the methods have stimulatory effects (Sánchez and Cardona 2008).

Since the presence of both pentose and hexose sugars in the fermentation broth is one of the major issues of bioethanol process, efficient production of ethanol is highly dependent on efficient hydrolysis of lignocellulosic biomass (Paulova et al. 2015).

Other types of products; such as chemicals like toluene, benzene, xylene, lignin monomers like propylphenol eugenol, syringols, aryl ethers, alkylated methyl aryl ethers, syringaldehyde, vanillin, vanillic acid etc. aromatic, aliphatic acids and

quinones, which are used as food supplements or prebiotics, can also be obtained by the enzymatic hydrolysis of biomass (Manisha and Yadav 2017).

Recently, researches focus on to seek new pretreatment methods, simultaneous or continuous saccharification and fermentation processes, technological developments in enzymes and novel techniques on enzyme-substrate interactions (Guo, Chang, and Lee 2018).

1.2.4. Surfactant Effect in Enzymatic Hydrolysis of Lignocellulosic Biomass

As already mentioned, to obtain high amount of reducing sugars from lignocellulosic biomass, one way is to use high concentrations of enzymes. Using high volume of expensive enzymes makes process economically less feasible. So, a common approach to decrease the cost of hydrolysis by decreasing enzyme loadings has been evolved. On the other hand, surfactants are known as their ability of increasing the conversion to reducing sugars during the enzymatic hydrolysis of lignocellulosic biomass. It was shown that in steam-pretreated spruce, conversion of cellulose, when non-ionic surfactants are added, was found in between 43% - 48%; while conversion of cellulose without the addition of surfactants is 33%. Tween (non-ionic) surfactants (Tween 20 and Tween 80) was demonstrated as the most effective surfactants during the conversion of cellulose and the reason can be elucidated as its only stabilizing effect – since no effect is present on the catalytic mechanism of the enzymes (Eriksson, Börjesson, and Tjerneld 2002). Steam-exploded wood was hydrolyzed, and it was observed that Tween 80 increased the rate of hydrolysis. Adding some amount of non-ionic surfactant to the hydrolysis medium eliminated the need of extra enzyme addition (almost 2-fold higher) to obtain the same hydrolysis rate (Helle, Duff,', and Coopes, n.d.). Tween 20 treatment increased the cellulose conversions at the end of 72 hours for substrates with various lignin compositions (Seo, Fujita, and Sakoda 2011). Both Tween 20 and Tween 80 ensured cellulase to be stable and effective while disrupting the lignocellulosic material of pretreated corn stover which led to increase rates and conversion of the hydrolysis (Kaar and Holtzapple 1998).

It was shown that if crystallinity of the substrate was higher and the cellulase concentration was lower, then the effectiveness of the surfactant was higher on the pretreated biomass (Eriksson, Börjesson, and Tjerneld 2002). Surfactants caused to decrease the adsorption of enzymes to cellulose, to increase the available surface area of cellulose or to remove the lignin part during the hydrolysis (Helle, Duff,', and Coopes, n.d.). When Tween 80 was added before the pretreatment of corn stover, it was observed that pretreatment efficiency increased; lignin removal became higher, as the time was prolonged (Qing, Yang, and Wyman 2010). Surfactants were found to alter the interfacial properties between different phases within the system (Seo, Fujita, and Sakoda 2011).

1.2.5. Enzymes Used in Hydrolysis of Lignocellulosic Biomass

Hydrolytic enzymes (hydrolases) play an important role to obtain value added products from lignocellulosic biomass, are used in various industrial processes; such as, production of food, consumables etc. In general, the microbes, *Pseudomonas, Clostridium, Bacillus, Aspergillus, Trichoderma and Penicillium*, are utilized to produce this vast group of enzymes. Cellulase, β-glucosidase, laccase, xylanases, and mannanases are some well-known hydrolases (Manisha and Yadav 2017).

Ligninases (manganese peroxidase, lignin peroxidase, versatile peroxidase, and laccase), endoglucanases and exoglucanases (acting on reducing and non-reducing ends of cellulose), with or without a carbohydrate-binding module (CBM), as well as various hemicellulases are required for the total degradation of lignocellulosic feedstock to fermentable sugars (Gupta et al. 2016). It was shown that pectinolytic enzymes are more efficient when compared with the results of acid hydrolysis; total reducing sugar values were in between 16.4 to 29.7% of dry weight pulp (Spagnuolo et al. 1997). Minimum enzyme combination which lead to maximum yield of total reducing sugar was the goal.

The main effect on hydrolysability is the removal of lignin. But for hemicellulose saccharification, accessory enzymes can also be used. Although the function is still

not known very well, there are some findings that proteins secreted by cellulolytic microorganisms when grown on cellulosic substrates, have a role during the hydrolysis of lignocellulose (Van Dyk and Pletschke 2012; Gupta et al. 2016).

1.2.5.1. Cellulase

Cellulase enzymes constitute a large part of the world enzyme market. *Clostridium, Cellulomonas*, *Thermomonospora*, *Trichoderma*, and *Aspergillus* are the main microbial sources of cellulases (Manisha and Yadav 2017). Cellulase secreted by the fungus *Trichoderma reesei* is widely used to destroy the crystalline structure of cellulose, and *Trichoderma spp*. generally lacks β-glucosidase activity. This leads to incomplete conversion of cellobiose, which inhibits the cellulose conversion. Thus, blending *Trichoderma spp*. with *Aspergillus spp.* for the maximization of conversion is a common way as reported by many studies (Maitan-Alfenas, Visser, and Guimarães 2015; Zhang et al. 2010).

Endoglucanases, exoglucanases (or cellobiohydrolases) and β-glucosidases are the components of cellulase and depending on the source of the enzyme their part in enzyme varies. Endoglucanase and exoglucanase are the bounded fractions, β-glucosidase is the unbounded part. Endoglucanases have a role in the conversion of cellulose to cello oligomers by binding the reducing and non-reducing ends of cellulose, likewise exoglucanases convert cello oligomers to cellobiose and finally β-glucosidase acts on cellobiose to degrade it into its monomers (F. and Shastri 2016).

Typically, the optimum temperature and pH of cellulose hydrolysis is between 40 & 55 °C and 4.5 to 5.5, respectively (F. and Shastri 2016). High amount of cellulase increases the yield of hydrolysis and conversion rate of cellulose and at some point, it starts to negatively affect the yield and conversion (Sun and Cheng, n.d.).

1.2.5.2. Hemicellulase

Hemicellulase hydrolyses the hemicellulose fraction of lignocellulosic biomass. Xylan is the major polymer of hemicellulose and it requires xylanase for the

conversion of xylo-oligosaccharides. Besides, β-xylosidase releases xylose and some accessory enzymes; such as, α-L-arabinofuranosidase, α-glucuronidase, α-galactosidase, acetylxylan esterase and ferulic acid esterase are needed for the complete conversion of hemicellulose to its monomers (Maitan-Alfenas, Visser, and Guimarães 2015).

1.2.5.3. Pectinase

Pectinases, which are commercially produced from the filamentous fungi *Aspergillus sp.* in general, are used for the degradation of pectin. Pectinases are classified as pectin esterases, depolymerizing enzymes and protopectinase. Depolymerizing enzymes are grouped as the enzymes hydrolyzing glycosidic linkages and cleaving. The enzymes hydrolyzing glycosidic linkages include polymethylgalacturonases (PMG) and polygalacturonases (PG); PMG catalyze the hydrolytic cleavage of a-1,4-glycosidic bonds and PG catalyze hydrolysis of α-1,4-glycosidic linkages in pectic acid (polygalacturonic acid). The enzymes grouped as cleaving catalyze the cleavage of α-1,4-glycosidic linkages by trans-elimination (Kashyap et al., n.d.).

It is apparent that pectinases play an important role during the hydrolysis of sugar beet pulp. As a result of enzymatic hydrolysis of SBP, galacturonic acid and arabinose are released (Zheng et al. 2012).

1.2.6. Commercial Enzymes Used in Hydrolysis of Lignocellulosic Biomass

Total effects of single enzymes are lower than the total effects of combined enzymes. In addition, the synergistic effects of combined enzymes – at least two of them - are related with the kind and the characteristics of the enzyme. According to a study, sugar beet pulp hydrolysis was increased by 69.2% when Celluclast 1.5 L *(a cellulase mixture that will be explained later*) and Pectinex were used in combination, this number was higher than sum of the separate Celluclast 1.5 L and Pectinex activities. In that study, the amount of glucose released was increased by 1.8 – 5.5 times when the pulp was hydrolyzed by the combination of cellulases and pectinases (Spagnuolo et al. 1997). It was suggested that Celluclast 1.5 L was the most efficient enzyme for

sugar beet pulp hydrolysis (Spagnuolo et al. 1997; Zheng et al. 2012). In another study, arabinan and galactan solubilization increased with the increase in cellulase loading, so it was concluded that commercial cellulase might have some arabinose and/or pectinase activities (Zheng et al. 2012). It was observed that enzyme mixture of Viscozyme L and an experimental pectinase preparation from *Aspergillus niger* gave rise to 79% galacturonic acid and 82% arabinose after 48 h of incubation of SBP; on the other hand, galacturonic acid and arabinose content decreased to 58% and 29% respectively if only Viscozyme L was used (Leijdekkers et al. 2013).

Enzymes may be reused or recycled if unproductive binding of lignin to enzymes can be prevented. High carbohydrate and low lignin content of SBP make enzyme recycling and reuse much more feasible (Zheng et al. 2012).

Still, our knowledge is limited regarding the complete use of hydrolases and appropriate enzyme combinations to maximize the saccharification has not been achieved yet. Many factors are considered while optimizing the hydrolysis; including substrate loadings, enzyme concentrations, inhibitors, and surfactants. Nowadays, because of the increasing usage of the enzymes, engineered enzymes manipulated and overexpressed in a different host have become very popular (Manisha and Yadav 2017).

1.2.6.1. Pectinex Ultra SP-L

Pectinex Ultra SP-L is a pectinase from *Aspergillus aculeatus*.

1.2.6.2. Cellic CTec 3

Cellic CTec3 is a highly efficient cellulase and hemicellulase complex produced by Novozyme. Optimal performance of Cellic CTec3 occurs at a temperature range of 50-55 °C and at pH 4.75-5.25.

1.2.6.3. Celluclast 1.5L

Celluclast 1.5L is a cellulase produced from the fungus *Trichoderma reesei* and catalyzes the breakdown of cellulose into glucose, cellobiose, and higher glucose

polymer. Optimal pH range is 4.5 - 6.0, and the optimal temperature range is 50 - 60°C (Sigma Aldrich).

1.2.6.4. Novozyme 188

Novozyme 188 is mainly composed of cellobiase obtained by submerged fermentation of an *Aspergillus niger*. It hydrolyzes cellobiose to glucose.

1.2.7. Surfactants Used in Enzymatic Hydrolysis of Lignocellulosic Biomass

Surfactants can be used during pretreatment, during enzymatic hydrolysis and for recycling of enzymes after batch hydrolysis (Van Dyk and Pletschke 2012). When used in enzymatic hydrolysis, lower enzyme concentrations are required.

While non-ionic surfactants (Tween 80 and Tween 20) caused an increase in reducing sugar concentration during the hydrolysis of steam-exploded wood, the effect of anionic surfactants on hydrolysis rate was not as high as non-ionic ones, and cationic surfactant had no effect on the hydrolysis rate (Helle, Duff,', and Coopes, n.d.).

1.2.7.1. Tween 80

Tween 80 is a polysorbate type non-ionic surfactant and $C_{32}H_{60}O_{10}$ is its molecular formula. It is frequently used as an emulsifier in foods and cosmetics.

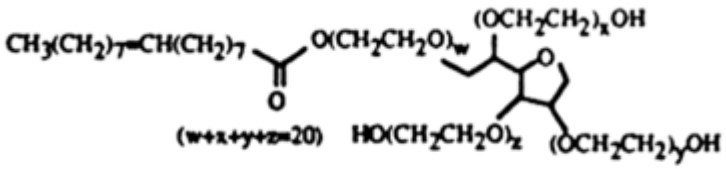

Figure 1.6. Tween 80

1.2.7.2. Tween 20

Tween 20 is a polysorbate type non-ionic surfactant and $C_{26}H_{50}O_{10}$ is its molecular formula. They are non-toxic and widely used in biochemical applications (Eriksson, Börjesson, and Tjerneld 2002).

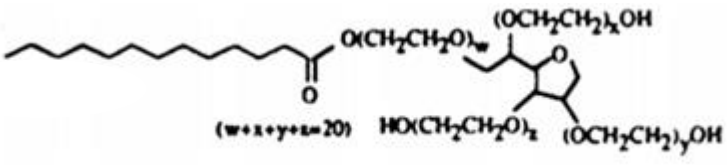

Figure 1.7. Tween 20

1.3. Objective of the Study

The study was carried in two different parts. The objective of the 1st part was to optimize the SBP hydrolysis wrt to proper enzyme substrate loadings and hydrolysis time. For that purpose, effects of different enzymes, enzyme and substrate loadings on the hydrolysis properties of sugar beet pulp were investigated. A screening analysis was also carried out using a half – fractional 2^4 design. Following the screening analysis, a response surface model was formed using the data from a 5-point central composite design to study the effects the aforementioned factors. Data were examined and results were verified by using classification and regression analysis.

Long hydrolysis times and large quantity of enzymes set a limit to the commercial utilization of biomass. And especially the pretreatment methods could bring additional costs. In that regard, as an alternative to pretreatment methods, use of surfactants has been shown to be promising. Thus, the objective of second part of the study was to examine the enzymatic hydrolysis of corn cob by using different nonionic surfactants (Tween 20 and Tween 80) and see the effects of different factors (surfactant loadings, enzyme loadings, pretreatment, substrate loading) on the sugar yields.

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1 Materials

Fresh sugar beet pulp obtained from Kayseri Sugar Plant in Kayseri, Turkey, was dried at 105°C. Dried sugar beet pulp was milled to particle sizes between 10 μm-2mm. A kitchen type food processor was used for this purpose. Corn cobs, which were acquired from local markets in Ankara, Turkey were ground to particle sizes between 10 μm and 2mm using a laboratory type mill. Corn cobs had been dried at 100°C before they were milled. Dried and ground corn cob samples were then treated with surfactant. Tri-sodium citrate dihydrate and citric acid monohydrate were purchased from Merck (Darmstadt, Germany). 3-5 Dinitrosalicylic acid, sodium sulfate and phenol were purchased from Sigma-Aldrich (St. Lois, MO, USA).

Enzymes Pectinex Ultra SP-L (pectinase) & Cellic Ctec3 (cellulase and hemicellulose complex) for sugar beet pulp and Celluclast 1.5L & Novozyme 188 were kindly provided by Novozymes (Bagsvaerd, Denmark). They were stored at 4°C when not in use.

2.2 Methodology

2.2.1 Enzymatic Hydrolysis of Sugar Beet Pulp

No pretreatment was applied prior to enzymatic hydrolysis due to the low lignin content of sugar beet pulp. Fresh sugar beet pulp was dried up at 105°C for 72 hours. Before enzymatic hydrolysis, reducing sugar content of the sugar beet pulp was found to be around 1.2 g/l. Before designing the experiment, four parameters; substrate loadings, two different enzyme loadings and time, were selected. The feasible substrate content for an appropriate experimental setup was determined to change

between 4 – 20 % solid/liquid ratio on dry basis. Pectinex Ultra SP-L and Cellic Ctec3 enzymes were used. Pectinex Ultra SP-L is a pectinase obtained from *Aspergillus aculeatus* and Cellic Ctec3 is a cellulase and hemicellulase complex. Pectinase hydrolyzes pectin, which is a component of the cell wall, and allows access of cellulase to cellulose. Pectinex Ultra SP-L and Cellic Ctec3 were combined at varying volumes ranging from 100-500 µl. Min and max, 6 and 30-hours hydrolysis time was particularly chosen. A shaking incubator was used for the hydrolysis experiments. Working conditions of the incubator were set at 50°C, 150 rpm for 6 to 30 hours. 0.05 M sodium citrate buffer solution with a pH of 4.8 pH was used. Samples were immersed in boiling water for 5 minutes in to terminate the hydrolysis. Following this, samples were centrifuged at 13,000 rpm for 3 minutes. Following the centrifugation, DNS method (Miller, 1959) was used to determine the reducing sugar content of the supernatant of the samples. Enzymatic hydrolysis was conducted in triplicates.

2.2.2 Enzymatic Hydrolysis of Avicel and Corn Cob

Since corn cob involves significant amount of lignin, pretreatment is needed to obtain high yields of reducing sugar. In our study, costly pretreatment methods were not applied, instead, to see the effect of surfactants when pretreatment step is eliminated, Tween 20 and Tween 80 were used. In order to see the effects of surfactants on the structure of cellulose, avicel – pure cellulose - was used and selected as a control. Preliminary experiments were conducted both by simultaneous addition of surfactant & enzyme to the mixture and by sequential addition of surfactant & enzyme. Sequential addition comprises stirring the solution for 24 hours at 450 rpm before incubation. Temperature of the stirrer was adjusted as 0°C, 50°C and 90°C. A shaking incubator was utilized for the enzymatic hydrolysis. Working conditions of the incubator were set at 50°C, 150 rpm for 24 hours. 0.05 M sodium citrate buffer solution with a pH of 4.8 was used. Celluclast 1.5L and Novozyme 188 were the enzymes used. The volume of each enzyme was kept constant as 150 µl, since this was the optimum volume found in a previous study for the same substrate and the

enzymes (Pocan et al. 2018). To see the surfactant effect, mixture of corn cob and avicel; 40% corn cob & 60 % avicel and 20% corn cob & 80% avicel, in addition to the substrates of only avicel and only corn cob, were used. Experiments were conducted with the surfactant volumes of 135 µl, 250 µl, 400 µl, 500 µl, 600 µl, 1000 µl, 3000 µl and 5000 µl. Hydrolysis lasted for 24 hours. Samples were immersed in boiling water for 5 minutes to terminate the hydrolysis process. Following hydrolysis, samples were centrifuged at 13,000 rpm for 3 minutes. Following the centrifugation, DNS method (Miller, 1959) was used to determine the reducing sugar content of the supernatant of the samples. Enzymatic hydrolysis was conducted in triplicates.

2.2.3 Determination of Reducing Sugar Content

D - glucose was used as a standard for the DNS analysis. Before the addition of the DNS reagent, supernatant part of the medium from the enzymatic hydrolysis was diluted with distilled water. Ratio of the DNS agent was set 1:1.5 on a volume basis. After the addition of the DNS reagent, obtained solution was maintained in a 100°C water bath for 5 minutes; then the color change in the solution was observed. Optizen Pop Nano Bio spectrophotometer was used to measure absorbance of the samples at 540 nm. Calibration curves were prepared to calculate the concentrations of reducing sugar in the samples.

2.2.3.1 Statistical Analysis of Sugar Beet Pulp Hydrolysis

It is necessary to select the variables with major effects since many variables may affect the system studied. To identify and control the small contributions of variables, screening designs should be carried out to determine which of the several experimental variables and their interactions present more significant effects. Since it is economical and effective, full fractional two-level factorial design was preferred for screening (Bezerra et al. 2008). The results from screening activities were analyzed and experimental plan was prepared by Response Surface Method (RSM) for optimization of reducing sugar amount of sugar beet pulp. Independent variables are selected as % substrate (w/v), amount of Pectinex Ultra SP-L (µl), amount of Cellic Ctec3 (µl) and

hydrolysis time (hours). Among the more known second order symmetrical designs are the three-level factorial design, Box–Behnken design, central composite design, and Doehlert design (Bezerra et al. 2008). Central composite design was selected for the further studies. The variable % substrate (w/v) was investigated at five levels; 4, 8, 12, 16 and 20. Similarly both enzymes (µl) and time (hours) were investigated at five levels; 100, 200, 300, 400, 500 and 6, 12, 18, 24, 30 respectively. Response was determined as the difference between the initial and final amount (g/l) reducing sugar of sugar beet pulp. After acquiring data related to each experimental point, since it is necessary to fit a mathematical equation to describe the behavior of the response according to the levels of values studied, Minitab was run (Version No 16).

2.2.3.2 ANOVA

Analysis of variance - is generally used as a more reliable way to evaluate the quality of the model fitted. Thus, it is possible to evaluate the significance of the regression used to foresee responses considering the sources of experimental variance. ANOVA was applied and the significance of regression was evaluated by the lack of fit test. No lack of fit was detected in the model.

2.2.3.3 Analysis with Classification and Regression Tree (CART)

Decision trees are represented by a series of questions. Therefore, the initial dataset is divided into smaller pieces through a series of questions. When the tree is set up, the tree is created with the initial data set, i.e. training data. This data is randomly selected from the dataset. With this randomly selected data, a classification model is created by creating a classification rule. The remainder of the data set is called test data and estimates the accuracy of the classification rule created during the tree formation process. If the predicted accuracy is acceptable, this rule also applies to new data. Thus, a classification model is created with decision trees.

A regression tree model was formed to investigate the effects of substrate content, enzyme amount and hydrolysis time on the reducing sugar amount of sugar beet pulp. '*rpart*', a recursive partitioning tool developed by Therneau and Atkinson (2000) for

R! statistical package, was used for the classification tree analysis. Moreover, reduced sugar amount was divided into quartiles and a classification tree model was estimated to predict the quartile class based on independent variables described above.

2.2.3.4 Statistical Analysis of Avicel and Corn Cob Hydrolysis

Student ‘t’ test was conducted to verify the statistical significance of the mean differences between the control group and samples in which surfactants were used.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Enzymatic Hydrolysis of Sugar Beet Pulp

It was shown that pretreated SBP - with dilute sulfuric acid - loadings ranging from 0.66% and 2.34% did not have any significant effect on hydrolysis yield (Donkoh et al. 2012). SBP solid loadings, ranging from 2% to 10%, led to the increase in the concentration of reducing sugars as expected. Hydrolysis yield decreased from 45% - at a solid loading 2%- to 41.5% - at a solid loading 10%- after 72 hours of incubation (Zheng et al. 2012). In another study, SBP solid loadings from 10% to 16% increased the hydrolysate sugar concentrations, on the other hand, yields decreased at solid loadings above 10% (Nahar, Rorick, and Pryor 2014). To obtain high fermentable sugars, it is obvious that high solid loadings are necessary. Whereas high solid content may adversely affect the process; mainly end-product inhibition in addition to mixing (Zheng et al. 2012).

Arabinose, galacturonic acid, and galactose are the sugars obtained after the hemicellulose and pectin hydrolysis. In addition, glucose is produced at the end of cellulose hydrolysis. Multiple interactions occur between enzymes on complex substrates and this still requires investigation (Van Dyk and Pletschke 2012). To increase enzyme productivity, additional knowledge is needed on the enzyme efficiency and enzyme recycling techniques (Maitan-Alfenas, Visser, and Guimarães 2015).

It was found that both cellulases and pectinases are important enzymes for the hydrolysis of sugar beet pulp. Although β-glucosidase can be used additionally, it was shown that hemicellulase was not needed to improve the effectiveness of hydrolysis

(Zheng et al. 2012). Substrate loading, and reaction time were important factors while maximizing the reducing sugars.

It was shown that the loss of enzyme activity during the enzymatic hydrolysis of SBP was essentially due to the hydrolysis products inhibition (Zheng et al. 2012). Rapid removal of the end products was important for the efficiency of the process (Guo, Chang, and Lee 2018).

Protein content increase during SBP hydrolysis was observed in a study which indicated the protease side activity of the enzymes. This might increase the access of the enzymes to pectin. To obtain high yields of pectin monomers, some degree of cellulose degradation w required (Leijdekkers et al. 2013).

The relationships between the enzyme loadings and sugar conversion produced are shown in the two-dimensional contour in Figure 3(1)-(6). In correspondence with the regression models, an increase in cellulase and pectinase loadings resulted in an increase in sugar release. The maximum yields within the design space were approximately 87 g/l after 18 h of hydrolysis, using 300 µl Cellic Ctec3 and 300 µl Pectinex Ultra SP-L at %20 substrate loading. As the regression models demonstrated a larger range of enzyme concentrations might be needed to be further investigated to observe optimum concentration.

3.1.1 Response Surface Model for Yield

Data used in the response surface model is presented in Appendix B; whereas coding of the predictors used in the response surface model is given in Table 3.1.

Table 3.1. *Coding of independent variables*

Coded value	X1	X2	X3	X4
-2	4	100	100	6
-1	8	200	200	12
0	12	300	300	18
1	16	400	400	24
2	20	500	500	30

A full quadratic model, i.e. a model consisting of first and second order polynomials of the predictors in addition to their interaction terms, was estimated. Unusual observations may influence the estimation of regression coefficients and their standard errors. Therefore, an iterative approach was adopted to identify such observations. After the initial model estimation, observations with absolute standardized residual greater than 2 were removed from the data set and the full quadratic model was estimated again. This process was continued until there were no unusual observations in the data set.

Following this process, statistical significance of the regression coefficients was scrutinized. Similar to the previous phase, predictors with the lowest absolute t statistics were discarded from the model one by one. Enhancement of the model fit due to the aforementioned iterative processes is summarized in Table 3.2, whereas the final model is given in Table 3.3.

Table 3.2. *Goodness of fit parameters for iterative estimations*

Iteration	R squared	F (lack of fit)	p (lack of fit)	Unusual observations	Omitted predictor
1	95.48	5.11	0.062	5, 15, 28	-
2	97.61	2.68	0.175	43, 45	-
3	98.56	1.61	0.346	17	
4	98.86	2.18	0.286	3	
5	99.09	1.77	0.354	42	
6	99.33	1.30	0.472	34	
7	99.45	1.01	0.575	-	-
8	99.45	0.96	0.599	-	X2 * X2
9	99.41	0.97	0.599	-	X4 * X4

Table 3.3. *Response surface model estimation results*

Variable	Coefficient	Standard Error	t	P
Constant	66.329	0.619	107.027	0.000
X1	16.026	0.341	47.047	0.000
X2	1.843	0.347	5.309	0.000
X3	1.369	0.354	3.866	0.001
X4	4.945	0.357	13.837	0.000
X1 * X1	-1.658	0.383	-4.335	0.000
X3 * X3	-1.047	0.383	-2.737	0.013
X1 * X2	0.869	0.384	2.265	0.035
X1 * X3	2.317	0.406	5.709	0.000
X1 * X4	1.475	0.432	3.416	0.003
X2 * X3	2.201	0.396	5.309	0.000
X2 * X4	1.063	0.443	2.400	0.026
X3 * X4	-1.512	0.443	-.3411	0.003
R2	99.41%			
Adjusted R2	98.95%			

Regression results indicated that all the main effects were positive. As expected, incorporating a higher enzyme content with more concentrated substrate over a longer period increased the yield. On the other hand, second order effect coefficients for substrate amount and Ctec 3 content were negative, suggesting optimal operation points might have existed for these variables. Moreover, the interaction term between Ctec 3 and time also had a negative coefficient. Therefore, it was hypothesized that the optimal values of the substrate amount, Ctec3 concentration and hydrolysis time could be found to optimize the process yield. In addition, the interaction term for Ctec3 and Pectinex Ultra SP-L had a positive coefficient, indicating that these enzymes displayed a synergetic response. Analysis of variance for the final model is given in Table 3.4.

Table 3.4. *ANOVA for yield*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	4	1929.8	2089.56	522.39	168.72	0.000
Regression	12	8575.4	8575.41	714.62	230.81	0.000
Linear	4	8155.3	7980.17	1995.04	644.37	0.000
X1	1	7540.5	6853.04	6853.04	2213.42	0.000
X2	1	76.2	87.27	87.27	28.19	0.000
X3	1	65.7	46.29	46.29	14.95	0.001
X4	1	472.9	592.77	592.77	191.45	0.000
Square	2	65.7	63.80	31.90	10.30	0.001
X1*X1	1	42.0	58.17	58.17	18.79	0.000
X3*X3	1	23.7	23.19	23.19	7.49	0.013
Interaction	6	354.3	354.33	59.06	19.07	0.000
X1*X2	1	0.7	15.89	15.89	5.13	0.035
X1*X3	1	81.5	100.90	100.90	32.59	0.000
X1*X4	1	89.9	36.13	36.13	11.67	0.003
X2*X3	1	108.5	87.26	87.26	28.18	0.000
X2*X4	1	37.8	17.83	17.83	5.76	0.026
X3*X4	1	36.0	36.03	36.03	11.64	0.003
Residual Error	20	61.9	61.92	3.10		
Lack-of-Fit	17	52.4	52.36	3.08	0.97	0.598
Pure Error	3	9.6	9.56	3.19		
Total	36	10567.1				

Contour plots of predictor variable couples are shown in Figures 3.1 to 3.6. As can be seen in Figure 3.1 and Figure 3.2, yield increased with higher amounts of substrate and enzyme concentration. Negative second order regression coefficient for substrate amount suggested that yield should decline after a certain point, i.e. an optimal substrate amount should exist. However, estimation results also indicated that such an optimal substrate amount was well beyond the experimental range used in this study.

Moreover, feasibility of the optimality of a higher substrate amount is equivocal. Difficulties were encountered during the trials while taking 1 ml of supernatant from the samples containing 20% substrate in order to conduct DNS assay. Therefore, it is almost impossible to find any supernatant in the sample above this percentage of substrate.

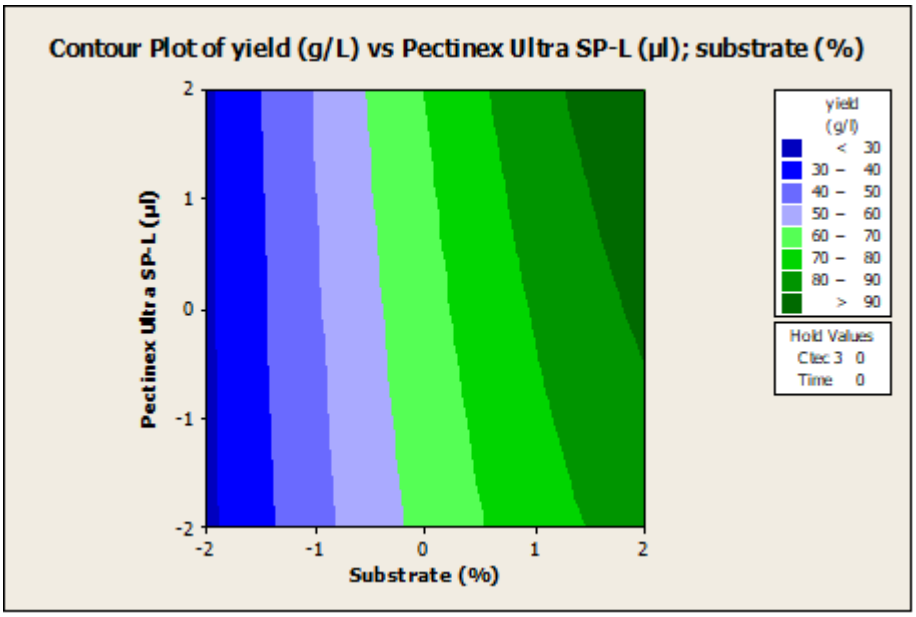

Figure 3.1. Contour plot of yield vs Pectinex Ultra SP-L; substrate

It is seen from Figure 3.1 that as the percent of substrate and enzyme volume increases, yield increased. When 20% substrate was used, as the volume of Pectinex Ultra SP-L was increased above 250 µl, yield reached its maximum - above 90 g/L. It was inferred that the variation in substrate % is relatively important than the variation in amount of Pectinex Ultra SP-L, since former affected the yield more. In addition, yield was almost constant at constant substrates but increasing enzyme volumes.

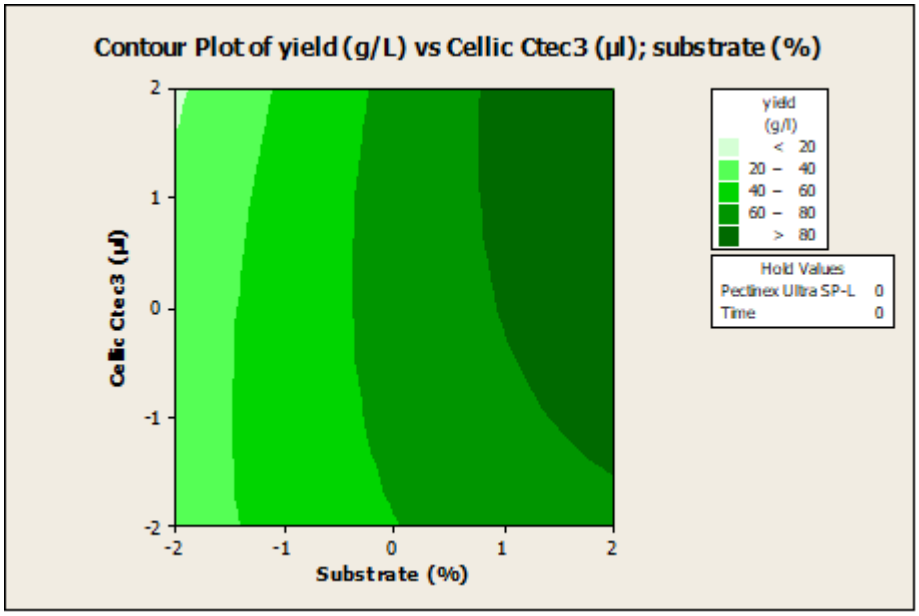

Figure 3.2. Contour plot of yield vs Cellic Ctec3; substrate

It is seen from Figure 3.2 that as the percent of substrate increased, even while using lower volumes of Cellic Ctec3 -around 150 µl, yield reached its maximum. If Figure 3.2 is compared with Figure 3.1, in Figure 3.2 lower substrate % and Cellic Ctec3 volume led to slightly higher yields. This result was expected since cellulose content was higher in SBP with respect to pectin.

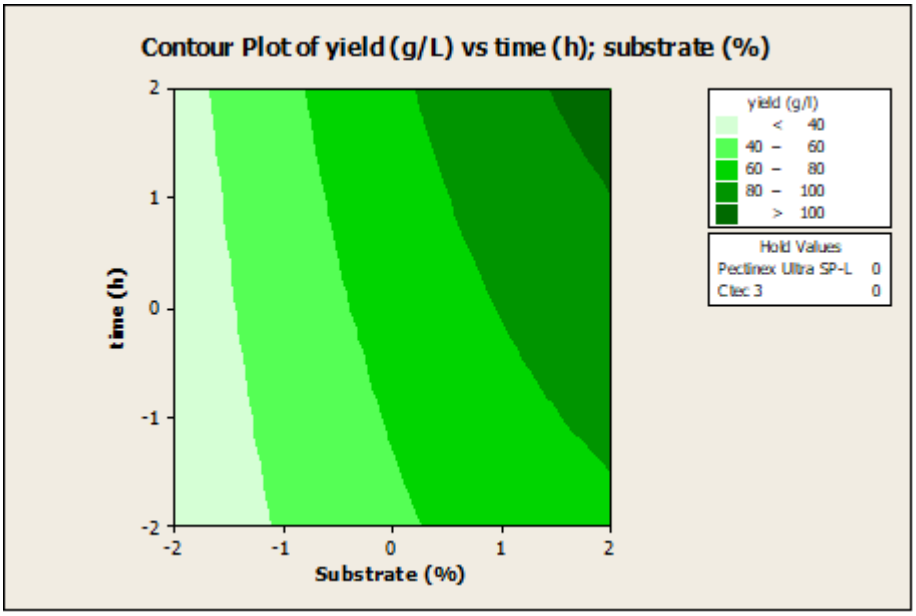

Figure 3.3. Contour plot of yield vs time; substrate

As can be seen in Figure 3.3, yield increased with both higher substrate amount and longer reaction time as expected. It was shown that during the hydrolysis of sugar beet pulp 50% of hydrolysate was composed of galacturonic acid and arabinose after 48 h of incubation. Sampling was done at the end of 12 h and 24 h incubation and it was observed that 50% and 80% of these monomers have been released at the end of 12 h and 24 h respectively (Leijdekkers et al. 2013). Another study indicated that 53% arabinose, 57% galactose and 44% rhamnose were released after 8 h of hydrolysis of SBP which were half of the monomers observed 48 h after hydrolysis (Micard, Renard, and Thibault, n.d.).

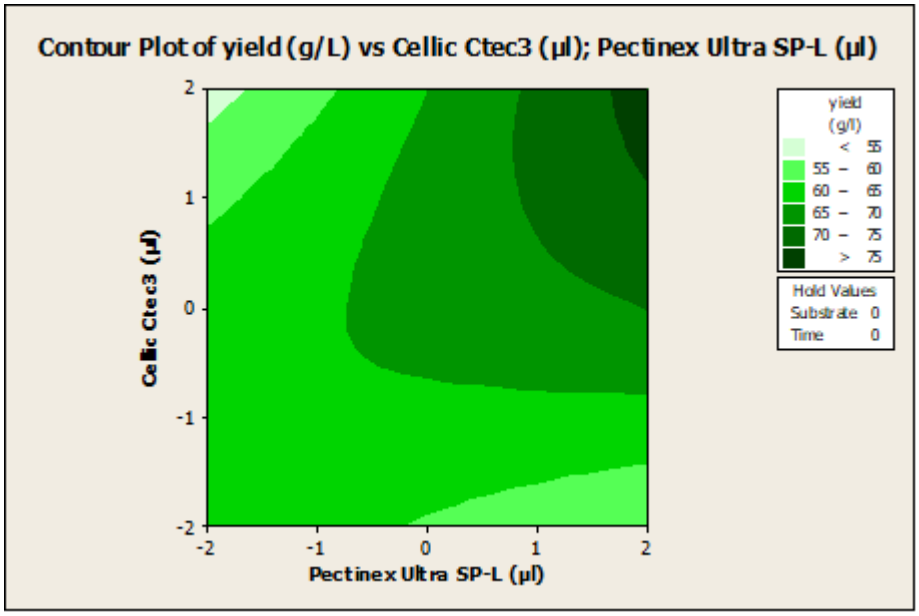

Figure 3.4. Contour plot of yield vs Cellic Ctec3; Pectinex Ultra SP-L

Analysis results showed that combining Pectinex Ultra SP-L with Cellic Ctec3 created a synergetic response. As presented in Figure 3.4, [1,1] combination (i.e. 400 µl Pectinex Ultra SP-L and 400 µl Ctec 3) gave a higher yield than [2,0] or [0,2] combinations. Similarly [0,0] combination produced a higher yield than [1, -1], [-1,1], [2, -2] and [-2,2] combinations.

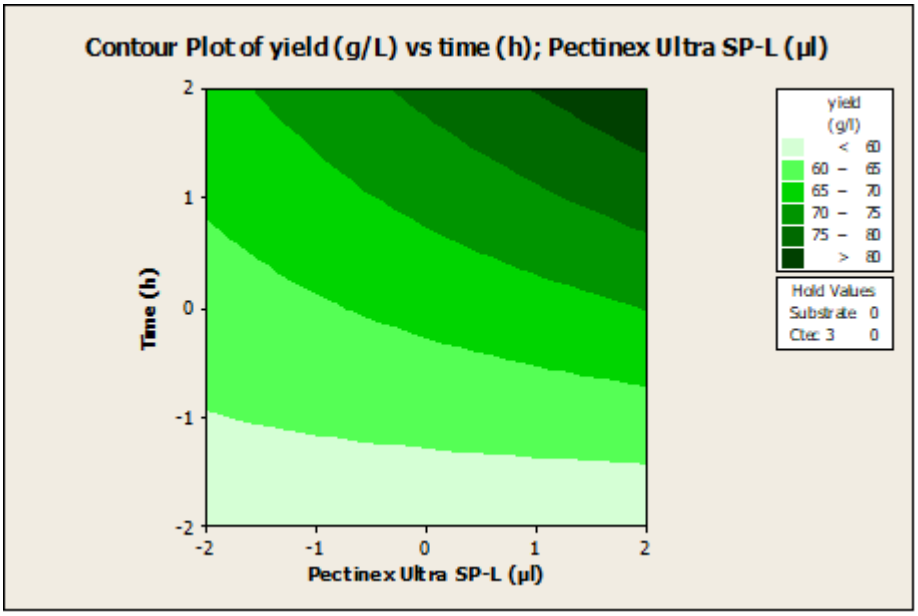

Figure 3.5. Contour plot of yield vs time; Pectinex Ultra SP-L

As shown in Figure 3.5, obtained findings for the interaction of Pectinex Ultra SP-L and time conformed with the intuitively expected outcome. Time had more effect on the extent of saccharification.

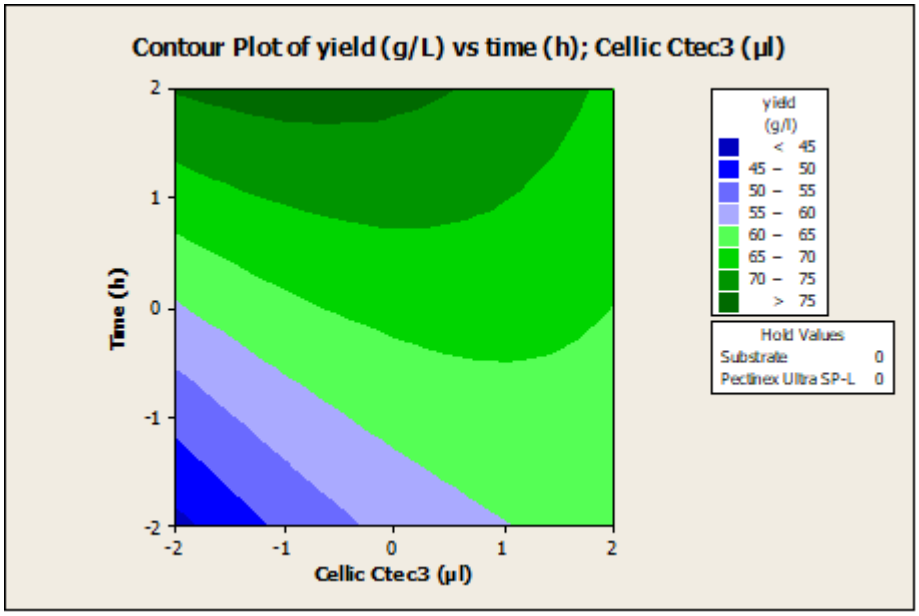

Figure 3.6. Contour plot of yield vs time; Cellic Ctec3

Negative interaction between reaction time and Cellic Ctec3 content is presented in Figure 3.6. Inhibition was observed at higher volumes of Cellic Ctec3 as expected since cellobiose or glucose formation slowed the rate of hydrolysis – end product inhibition. At higher substrate loadings, since end-product inhibition can be observed, it was estimated that yield will drop beyond the experiment range.

3.1.2 Classification and Regression Tree Analysis

Regression tree, which was used to model the effects of substrate content, enzyme amount and hydrolysis time on the reducing sugar amount of sugar beet pulp is shown in Figure 3.7. As can be seen in Figure 3.7, the resulting decision tree displays the interaction of substrate amount and reaction time, as well as the two different types of enzymes used in the experiments.

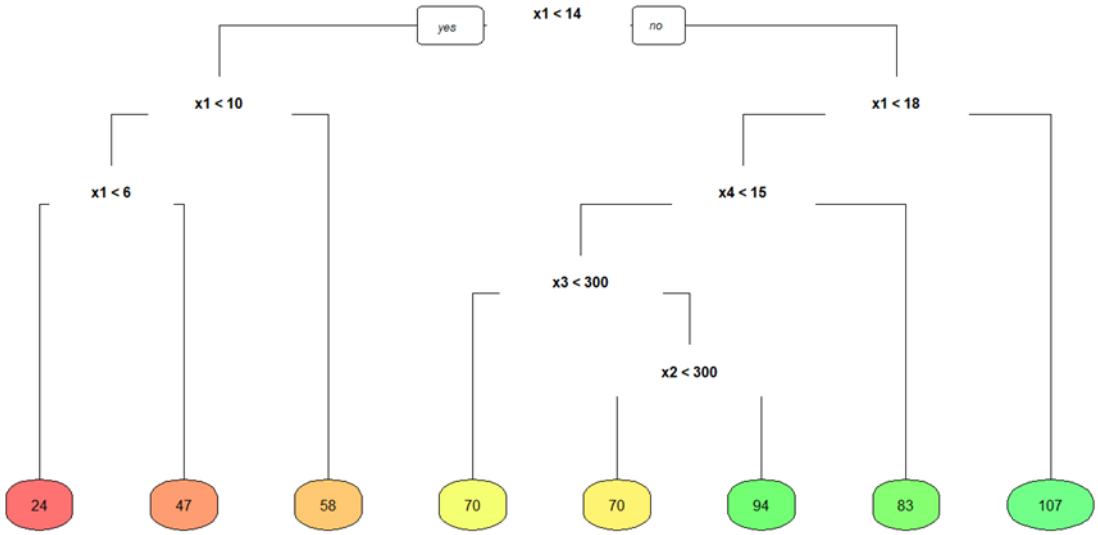

Figure 3.7. Regression tree model for reducing sugar yield

Regression trees can be used to deduce rules from the resulting decision tree. In this sense, rules regarding the reduced sugar yield can be summarized as follows:

Table 3.5. *Rules derived from the regression tree*

Rule	*Reducing Sugar Yield*
%substrate is smaller than 6%	24 g/l
% substrate is between 6% and 10%	47 g/l
% substrate is between 10% and 14%	58 g/l
% substrate is between 14% and 18% and reaction time is smaller than 15 hours and Cellic CTec3 is larger than 300 μl and Pectinex Ultra SP-L is smaller than 300 μl	70 g/l
% substrate is between 14% and 18% and reaction time is smaller than 15 hours and Cellic CTec3 is smaller than 300 μl	70 g/l
% substrate is between 14% and 18% and reaction time is larger than 15 hours	83 g/l
% substrate is between 14% and 18% and reaction time is smaller than 15 hours and Cellic CTec3 is larger than 300 μl and Pectinex Ultra SP-L is larger than 300 μl	94 g/l
%substrate is greater than 18%	107 g/l

Classification tree, which was formed to predict the quartiles of reduced sugar yield is presented in Figure 3.8. Substrate amount and reaction time dominated the classification results, hence obtained results confirmed the findings from other models.

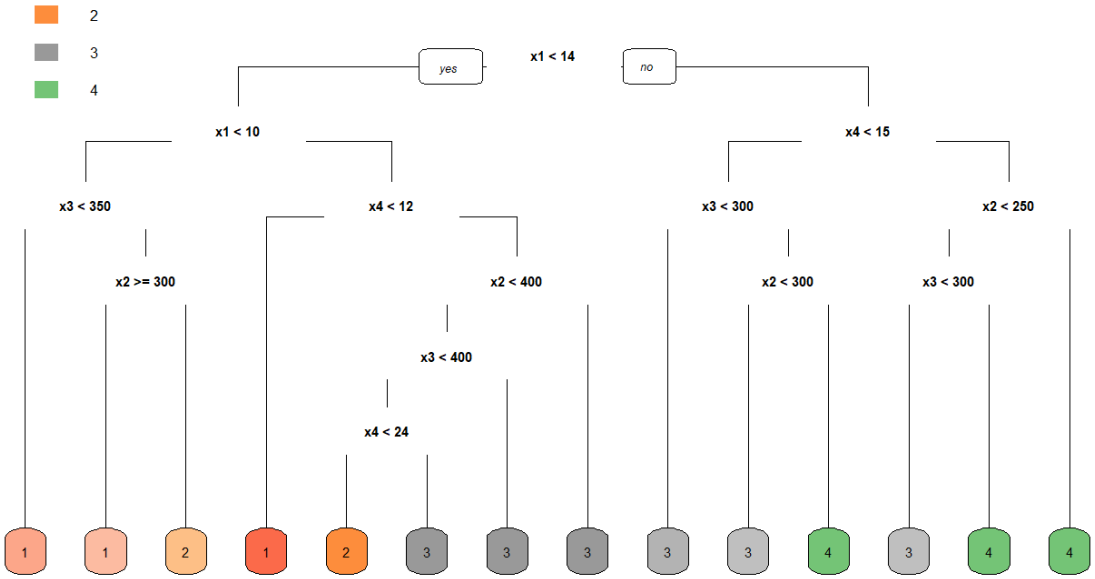

Figure 3.8. Classification tree for reducing sugar yield quartiles

Quartile predictions from the classification tree are given in Table 3.6.

Table 3.6. *Classification tree predictions*

		Predicted			
		<25th Percentile	>25th Percentile < 50th Percentile	>50th Percentile < 75th Percentile	>75th Percentile
Actual	<25th Percentile	12	2	0	0
	>25th Percentile < 50th Percentile	3	10	0	0
	>50th Percentile < 75th Percentile	2	1	11	0
	>75th Percentile	0	0	3	11

The diagonal in Table 3.6 shows the correct predictions by the classification tree. As can be seen in Table 3.6, the classification tree model had an accuracy of 80%, which indicated that 44 of the 55 cases were correctly classified.

Classification tree analysis provides a different perspective, by predicting group memberships instead of point estimates for reduced sugar yield. Rules derived from this classification tree can be used to determine experimental range of substrate amount, enzyme loadings and hydrolysis time in future optimization studies. Classification tree analysis results show that substrate content should be greater than 14%, reaction time should be at least 15 hours, and enzyme loadings should be 250 to 300 μl in order to obtain high reducing sugar yield. These values can form a basis for future optimization studies.

3.1.3 Corn Cob

Surfactant usage does not eliminate the pretreatment step but increases the yield of conversion of cellulose. It was shown in a study that, samples without pretreatment and with Tween 80 demonstrated an increase, from 50 to 80 mg equiv glucose/g dry stover, on sugar yield. It was found that Tween 20 was more effective when compared with Tween 80, during the hydrolysis of pretreated corn stover. In addition, at high substrate concentrations, it was seen that Tween was more effective during the saccharification of pretreated corn stover (Kaar and Holtzapple 1998).

In another study although, the adsorption of cellulase decreased with the addition of Tween 20 in steam-pretreated spruce (SPS) hydrolysis medium, there was no significant decrease in enzyme adsorption when delignified SPS and avicel used (Eriksson, Börjesson, and Tjerneld 2002).

During the pretreatment process, the structure of lignin surfaces changes so enzymes are easily adsorbed by the lignin surfaces (Eriksson, Börjesson, and Tjerneld 2002).When substrates with various lignin composition were hydrolyzed, it was observed that presence of lignin highly affected the adsorption capacity of Tween 20 and it was higher than pure cellulose. On the other hand, since there was no linear relationship between the lignin amount and the adsorption capacity, it was concluded that acidic groups within the substrate or pH of the medium might be effective on adsorption behavior of Tween 20. Structural changes in avicel and the substrate with highest amounts of lignin were not observed. Although structural changes were observed within the other samples having various amount of lignin composition, it was inferred that Tween 20 effect on crystalline structure was not significant (Seo, Fujita, and Sakoda 2011).

The effects of surfactant use on reducing sugar yield of avicel were investigated by incorporating two different types of surfactants, namely Tween 20 and Tween 80. Enzyme amount, pulp content, and hydrolysis time were kept constant at 300 μ L (Cellulast 150 μ L, Novozyme 150 μ L), pulp amount, and 24 hours respectively.

Obtained results are presented in Table 3.7. All data acquired in the study is also presented in Appendix D. It is important to mention that in all corn cob hydrolysis experiments to check the activity of the enzymes, avicel was also hydrolyzed for each run. By this way possible errors due to instruments or enzymes were controlled.

Table 3.7. *Reducing sugar yield of avicel treated with Tween 20 and Tween 80*

Group	Observations	Minimum (g/l)	Maximum (g/l)	Mean (g/l)	Standard Deviation	Coefficient of Variation
Control	22	11.36	26.68	21.19	4.36	20.57%
Tween 20	15	17.49	32.06	22.55	3.61	16.00%
Tween 80	15	16.43	28.56	23.32	3.00	12.86%

Mean yield of samples containing surfactants was found to be higher than that of the control group. t test was conducted to verify the statistical significance of the mean differences between the control group and samples in which surfactants were used. Significance level α was set as 5%. t-test results are given in Appendix C.

Although the previous studies showed that the inclusion of surfactants Tween 20 and Tween 80 could increase the reducing sugar yield, this effect could not be verified statistically in this study. t-test results showed that mean difference between treated and control groups was not different ($p>0.05$). Therefore, it was concluded that surfactant use while keeping the other parameters constant (i.e. enzyme content, substrate amount and hydrolysis time) did not necessarily increase the reducing sugar yield from avicel.

Even trials with lower and higher amounts of surfactants (T20, 135 µl through 500 µl), higher and lower amounts of enzymes (300 µl +300 µl, 75 µ + 75 µl), various substrate compositions (40% corn cob + 60% avicel, 20% corn cob + 80% avicel, 100% corn cob) did not give statistically different results. Data can be found in Appendix D.

Crystallinity of cellulose in corn cob can be higher; so, the conversion in structure-to amorphous- can be negligible. Thus, enzymes are not able to reach cellulose easily. On the other hand, surfactant might behave as an inhibitory product since enzyme amount could be more that it expected to be. It can be inferred that pretreatment of corn cob is needed even if it is costly, to remove lignin and to make enzymes reach cellulose.

CHAPTER 4

CONCLUSION

Elimination of the costly pretreatment methods is the focus of researchers to study the lignocellulosic biomass utilization. Lignin composition varies on different the sources of biomass. While corn cob has higher amount of lignin when compared with most of the biomass sources, sugar beet pulp has lower amount of lignin. Surfactants have been using recently in the pretreatment step of lignocellulosic biomass; and some studies showed promising results of the surfactants increasing the yield.

In this study, two biomasses were enzymatically hydrolyzed. In the first part, sugar beet pulp hydrolysis was optimized using Response Surface Methodology (RSM) and Classification and Regression Tree (CART). No pretreatment was applied to sugar beet pulp since its lignin amount is low. Findings from RSM indicated that the variation in substrate % was relatively important than the variation in amount of Pectinex Ultra SP-L, since former affected the yield more. Since cellulose content was higher in SBP with respect to pectin, lower substrate % and Cellic Ctec3 volume led to slightly higher yields. Yield increased with both higher substrate amount and longer hydrolysis time as expected. Results showed that combining cellulase, hemicellulase and pectinase created a synergetic response and increased the yield of reducing sugar. Negative interaction between the reaction time and Cellic Ctec3 content was also presented. Inhibition was observed at higher volumes of Cellic Ctec3 as expected since cellobiose or glucose formation slowed the rate of hydrolysis – end product inhibition. Regression tree results confirmed the findings from RSM; when %substrate was greater than 18%, maximum reducing sugar yield was obtained. Reducing sugar yield was higher when % substrate was between 14% and 18%, reaction time was shorter than 15 hours, Cellic CTec3 was larger than 300 μl and Pectinex Ultra SP-L was larger than 300 μl. Classification tree analysis results showed that substrate content should

be greater than 14%, reaction time should be at least 15 hours, and enzyme loadings should be 250 to 300 μl in order to obtain high reducing sugar yield. Substrate amount and reaction time dominated the classification results as well.

To see the effects of surfactant on lignin containing biomass, enzymatic hydrolysis experiments of both avicel and avicel-corn cob combinations were conducted. It was concluded that Tween 20 and 80 had no significant effect on the hydrolysis if a pretreatment method was not used. Presence of lignin was still a barrier and could not be overcome by surfactant only.

Since sugar beet pulp has very low level of lignin, costly pretreatment step can be eliminated easily. Yield was reasonably higher below 24 hours reaction times. So, the main cost comes into prominence as the cost of enzymes. To decrease enzyme amount during the hydrolysis of SBP should be another goal. To study on enzyme mixtures which includes pectinase, cellulase and hemicellulose together could give remarkable results as well. For lignin rich biomass, surfactant addition while eliminating the pretreatment step does not necessarily give meaningful results. It is recommended for further studies to observe the surfactant effect during the pretreatment of lignocellulosic biomass.

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APPENDICES

A. STANDARD CURVE FOR DINITROSALICYLIC ACID (DNS)

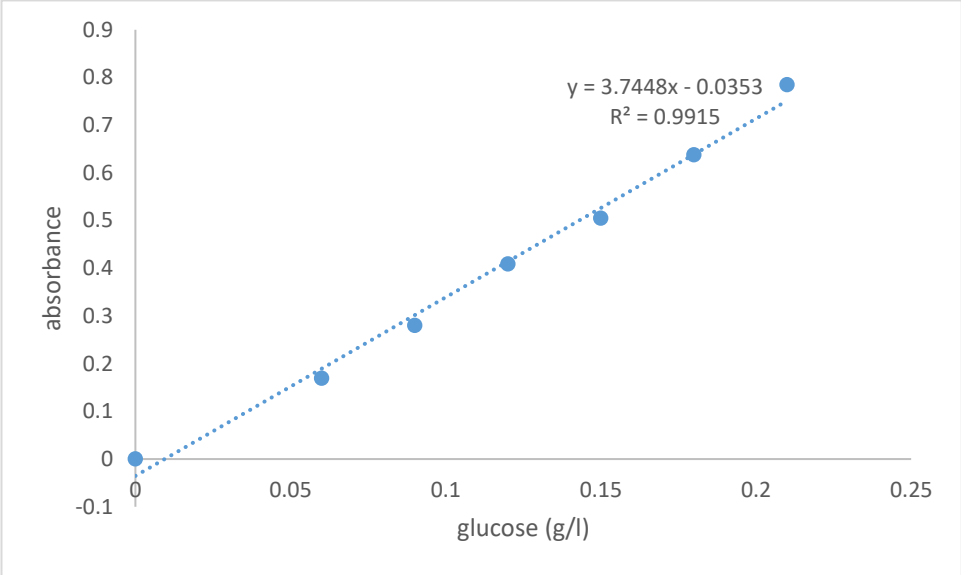

Figure A.1 DNS method calibration curve for avicel & corn cob

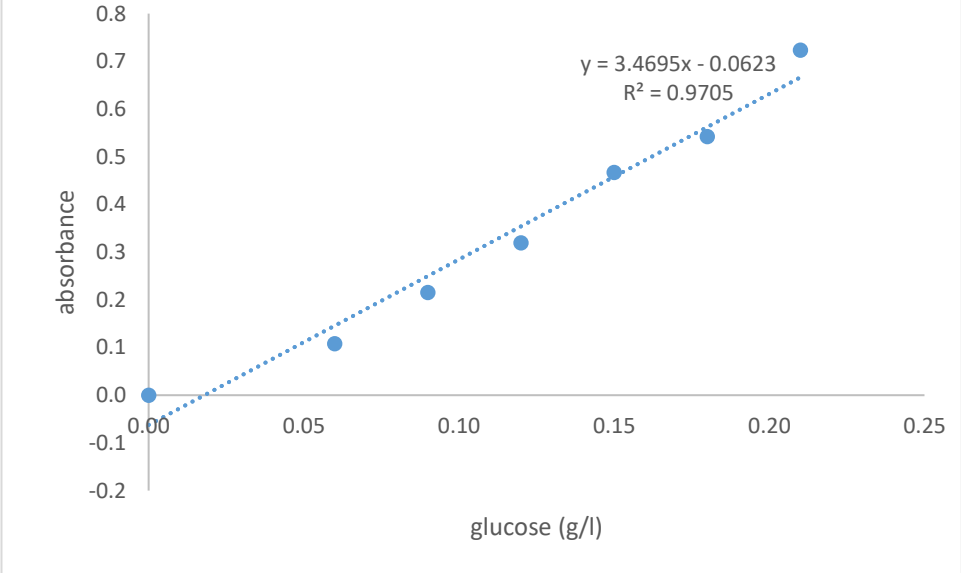

Figure A.2 DNS method calibration curve for avicel, corn cob & sugar beet pulp

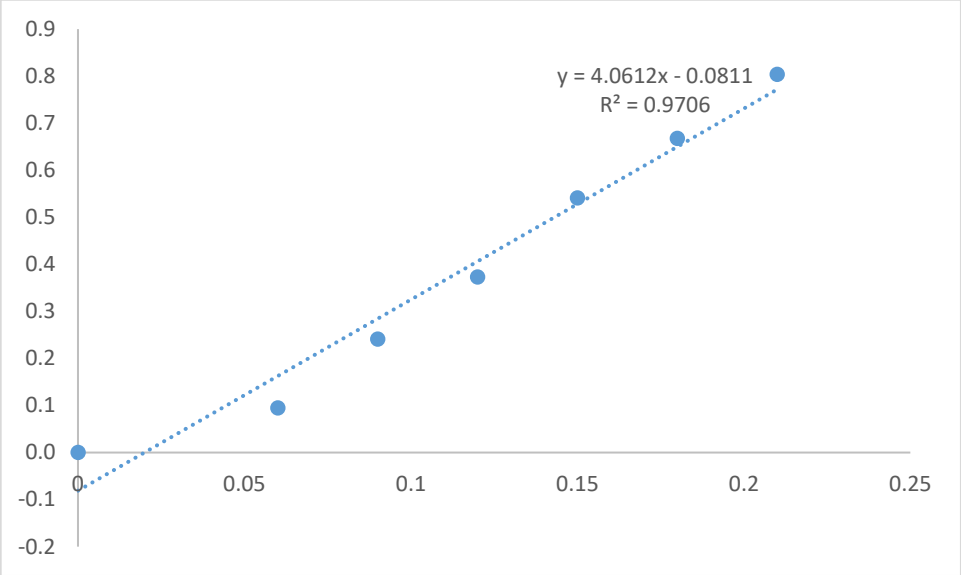

Figure A.3 DNS method calibration curve for sugar beet pulp

B. RESPONSE SURFACE METHOD DATA

Table B.1 *Response surface method data*

Observation	% substrate (w/v) X1	Pectinex Ultra SP-L (µl) X2	Cellic Ctec3 (µl) X3	Time (hours) X4	Block	Yield (g/l)
1	-1	1	1	-1	1	53.56
2	1	1	-1	-1	1	70.78
3	1	-1	1	-1	1	72.52
4	-1	-1	-1	-1	1	50.08
5	1	1	1	1	1	86.87
6	1	-1	-1	1	1	89.64
7	-1	-1	1	1	1	50.83
8	-1	1	-1	1	1	58.42
9	-1	1	-1	-1	2	53.63
10	-1	-1	1	-1	2	56.59
11	1	-1	-1	-1	2	82.29
12	1	1	1	-1	2	94.41
13	-1	1	1	1	2	64.26
14	1	-1	1	1	2	95.41
15	-1	-1	-1	1	2	45.71
16	1	1	-1	1	2	99.77
17	0	0	0	0	3	56.98
18	1	1	1	0	3	85.70
19	-1	1	-1	0	3	45.73
20	0	0	2	0	3	61.45
21	2	0	0	0	3	87.39
22	0	2	0	0	3	66.98
23	0	0	-2	0	3	54.82
24	0	-2	0	0	3	56.82
25	-2	0	0	0	3	23.98
26	0	0	0	0	3	61.68

27	0	0	0	0	4	56.22
28	1	1	1	0	4	84.91
29	0	0	0	0	4	53.17
30	0	0	0	0	4	56.73
31	0	0	0	0	4	57.06
32	0	0	0	-2	4	46.91
33	0	0	0	2	4	67.74
34	-1	1	1	1	4	35.93
35	1	-1	1	1	4	71.68
36	1	-1	-1	1	4	71.24
37	-1	1	1	-1	5	40.33
38	1	1	-1	-1	5	65.34
39	-1	-1	1	1	5	39.77
40	1	-1	1	-1	5	70.30
41	-1	-1	-1	-1	5	42.40
42	-1	1	-1	-1	5	44.13
43	-1	-1	-1	1	5	39.69
44	1	1	-1	1	5	78.80
45	-1	-1	1	-1	5	48.66
46	1	-1	-1	-1	5	64.51
47	-1	1	1	-1	6	43.60
48	1	1	-1	-1	6	69.58
49	1	-1	1	-1	6	66.89
50	-1	-1	-1	-1	6	40.29
51	1	1	1	1	6	76.95
52	1	-1	-1	1	6	68.70
53	-1	-1	1	1	6	51.47
54	-1	1	-1	1	6	37.21
55	2	1	1	1	6	127.04

C. T-TEST RESULTS

Table C.1 *Group mean comparison between Tween 20 and Control*

Group	Mean Difference	95% Confidence Interval		t value	Degrees of Freedom
Control vs Tween 20	1.35	-1.42	4.12	0.99	35
H_0: Difference=0	P(|T|>|t|) = 0.33			Difference in means is not statistically significantly different from zero	
H_a: Difference>0	P(T>t) = 0.16			Difference in means is not statistically significantly different from zero	

Table C.2. *Group mean comparison between Tween 80 and Control*

Group	Mean Difference	95% Confidence Interval		t value	Degrees of Freedom
Control vs Tween 80	2.12	-0.51	4.76	1.64	35
H_0: Difference=0	P(|T|>|t|) = 0.11			Difference in means is not statistically significantly different from zero	
H_a: Difference>0	P(T>t) = 0.06			Difference in means is not statistically significantly different from zero	

D. AVICEL AND CORN-COB DATA

Avicel	Reducing sugar_ average (g/l)	Standart Deviation
Control	21.19	4.26
T20 (250 µl)	18.85	1.98
T20 (400 µl)	17.58	1.66
T20 (500 µl)	22.87	3.49
T20 (600 µl)	18.21	1.53
T20 (1000 µl)	23.23	0.83
T20 (3000 µl)	18.96	0.08
T20 (5000 µl)	17.95	0.56
T80 (250 µl)	19.58	1.66
T80 (400 µl)	17.89	0.72
T80 (500 µl)	23.32	2.90
T80 (600 µl)	17.36	0.00
T80 (1000 µl)	23.22	0.64
T80 (3000 µl)	29.13	2.63
T80 (5000 µl)	20.00	0.00

Corn Cob	Reducing sugar_ average (g/l)	Standart Deviation
Control	2.96	0.24
T20 (500 µl)	2.93	0.00
T20 (500 µl)	3.15	0.06

80% avicel & 20% corn cob	Reducing sugar_ average (g/l)	Standart Deviation
Control	23.01	0.74
T20 (500 µl)	22.45	0.66
T20 (500 µl)	22.65	0.95

60% avicel & 40% corn cob	Reducing sugar_ average (g/l)	Standart Deviation
Control	16.46	0.56
T20 (500 µl)	16.25	0.86
T20 (1000 µl)	16.45	0.04
T20 (3000 µl)	14.68	0.74
T20 (5000 µl)	17.75	4.69

T80 (500 µl)	15.93	0.30
T80 (1000 µl)	17.35	0.75
T80 (3000 µl)	13.81	1.20
T80 (5000 µl)	16.00	3.88

T20=Tween 20; T80= Tween 80

() represents the amount of T20 or T80