Increased Production of Cellulosic Sugars from Lignocellulosic Materials

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To my life-coaches, my parents Hussein & Zahra: because I owe it all to you. Many Thanks!
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Abstract

Renewable energy is energy that is regenerated naturally for infinite time. Renewable energy can be categorized into solar, hydro, wind, geothermal, and biomass. Biomass is an organic material such as agricultural, forest residues, energy crops (Jatropha, miscanthus, switch grass and etc) and algae. The current study focusing on biomass based renewable and sustainable energy production from lignocelluloses biomass. As the name suggest lignocellulosic biomass is made up of lignin, cellulose and hemicellulose and the cellulose, hemicellulose are made up of polymerized C5 and C6 sugars such as glucose, xylose, arabinose, mannose, and galactose. Due to the complex nature of lignocellulosic biomass, the sugars present in plant biomass are not readily accessible for production of biofuels and biochemicals. Hence, a pretreatment of lignocellulosic biomass to loosen up the lignocellulosic matrix, followed by a hydrolysis process with enzymes or acids is necessary to obtain a fermentable stream of monomeric sugars.

The objectives of this study were to: 1) investigate the enzymatic production of fermentable sugars (glucose) from underutilized, low-cost lignocellulosic biomass such as poplar wood, and 2) examine factors that can enhance the efficiency of enzymatic hydrolysis leading to higher glucose yields and lower production costs. A two-stage steam-exploded poplar wood biomass was used as substrate in this work. Initially the effect of inhibitors that generally form during high temperature pretreatment was evaluated by washing the biomass with distilled water. There were no significant improvement in hydrolysis yield was obtained, perhaps significant reduction in sugar yield was noticed after 96h of hydrolysis. Therefore unwashed biomass was used in all our studies. There after the effect of original pH (3.0) of pretreated poplar pulp and optimum pH (5.0) of cellulase on hydrolysis yield was studied. Interestingly the commercial cellulase

preparation was active at pH 3.0, however pH 5.0 was chosen for further studies due to slightly higher sugar yield.

To overcome the low hydrolysis efficiency, critical parameters such as enzyme loading, substrate consistency, hydrolysis time, and substrate recycling were evaluated. Based on the results, 5% enzyme loading and 5% substrate consistency were found to be optimal. Substrate recycling could possibly reduce the enzyme usage in successive hydrolysis cycling. A 50% reduction in sugar yield was observed after 2 successive recycling of substrate, hence adding fresh enzyme at low concentration was recommended. Further, the effect of different surfactants on hydrolysis was studied. Compared to the control (without surfactant), 1% PEG4000 produced highest sugar yield of 58.5% at 5% substrate consistency and 5% enzyme loading. Compared to PEG4000, other surfactants studied (PEG8000, Tween 20, and TritonX100) improved hydrolysis yield to a lower extent. Therefore, addition of surfactant can enhance the hydrolysis efficiency of enzymes. The most likely mechanism for this effect is believed to be by a surfactant-facilitated blocking of the non-productive sites on lignin, which results in increase in the concentration of free enzymes available for enzymatic hydrolysis.

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Abbreviations

AFEX	Ammonium fiber explosion
ANOVA	Analysis of variance
DW	Cell dry weight
FPU	Filter paper unit
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
L	Liter
LCB	Lignocellulosic biomass
M	Molarity
mL	Milliliters
mM	Millimolar
N	Normal
NREL	National renewable energy laboratory

Chapter 1

1. INTRODUCTION

Biomass is an important source of energy and biomaterials. Therefore understanding the nature of biomass (lignocellulosic biomass) and its successive conversion in to fuel and bio products is essential to develop a viable bioprocess. Hence this chapter attempts to critically review biomass conversion technologies such as types of biomass, different types of pretreatment, hydrolysis processes and fermentation techniques. Production of energy using biomass is expected to address challenges associated with the use of environmentally non-friendly and unsustainable fossil fuels [42]. Bioethanol is one amongst the biofuels produced from lignocellulosic biomass through biomass pretreatment, hydrolysis and fermentation [43]. However, the production of monosaccharides from lignocellulosic biomass is an energy intensive, tedious and laborious process. Thus, various technical and economic challenges have to be addressed to make such conversion route efficient and cost effective [4]. One among them is enzymatic hydrolysis of lignocellulosic biomass. Enzymatic hydrolysis is a green, specific and efficient method to convert biomass based cellulose into its monomeric form. There was ample research has been taken up in past decades. However, a cost effective hydrolysis method for biofuel production is not yet developed. Therefore this review also provides a quick insight into the lignocellulosic biomass, its structure, pretreatment, chemical/enzymatic hydrolysis, potential application and the different approaches to valorize it.

1.1 Biomass Production

Biomass is one of the abundant feedstock's available in nature. With the increase in demand for biomass based products, the global biomass production has increased. Between 2000

and 2011, biomass production has increased by 49 million tons compared to oil equivalent [42, 72]. Biomass is also regarded as the 4th leading source of energy and contributes to around 10% of global energy supply. Such enormous amount of biomass can potentially be converted into fuels to meet the world's energy demand. Bioenergy from biomass is carbon neutral process due to the re growing of plants recaptures the CO_2 from the atmosphere for producing energy through photosynthesis. Hence the net CO_2 released from biofuel is nearly equivalent to the CO_2 captured. Considering the above facts a high importance has been given among the researchers across the globe to focus on developing an efficient method to valorize biomass feedstock generated energy [31].

Production of energy from biomass existed before the industrial revolution. Biomass was the primary source of energy (fire wood and plant oil) in the early days. Indeed, the first motorized vehicle (Ford model T) was run by plant-based oil [81]. With the discovery of fossil fuel, the use of biomass as a source of energy was phased out. However, with the increased energy demands across the world, fluctuating prices of fuels and environmental issues attached with the use of fossil fuels, needs of biomass-based energy re-emerged in 1970. Since 1975, more attention was drawn towards the second-generation biofuels (i.e. Lignocellulose and nonfood crops and algae) than first generation (food crops).Between 1975 and 1980 countries such as the United States formulated policies to improve the research on biomass based biofuel technologies [29]. The utilization of biomass for energy generation gained more attention in 1983 as forest thinning's, wood waste and household wastes were being converted to energy. Due to the strict environmental policies, mandatory biofuel blending (two to five percent) with fossil fuels introduced to reduce the pollution [47]. The development of advanced bioreactors has also revolutionized the biofuel production from biomass [1].

The global biomass production was roughly estimated to be 13 billion metric ton dry matter per annum. Out of which 82% was used for food and fodder applications. An 11% of global biomass production was used for biofuel production along with 7% of biomaterials. However, the other renewable and sustainable biomass resources such as forest residues, food industry waste were underutilized [73].

Canada has a highly diversified source of biomass attributed to its large landmass, forest, and vibrant agricultural industry. Biomass production and its application in energy sector contribute 4.4% of total energy demand, which makes it the second source of renewable energy after wind energy. The other renewable energy contributors are solar and tidal power [54]. It is also reported by Kumarappan et,al that the high water demand associated with the production of biomass puts pressure on the environment [34].

The disposal of agricultural waste such as wheat straws and corn stalks is cumbersome process considering their high volume of production. As a result, a large portion of the biomass was left as unutilized or used for mulching and composite production [80]. The form of disposal increases the likelihood of buildup of pest and diseases. Another option, which involves the incineration of the wastes, increases carbon footprint while disrupting the soil structure and causing potential loss of nutrients. Burning also does not offer any benefit other than heat energy, however, alternative use of agricultural waste in to liquid fuel can lead to increased revenue generation [25].

1.2 Lignocellulosic biomass

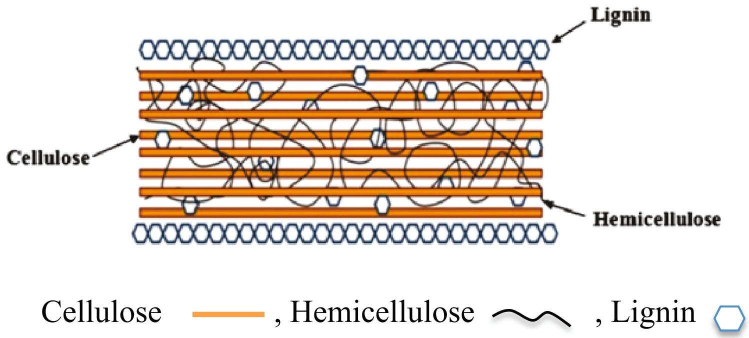
Lignocellulosic biomass (LCB) is regarded as a promising, renewable and sustainable substrate for biofuel production. Some of the reasons for the preference of lignocellulosic biomass include its low cost, availability and sustainable supply [36]. Examples of the

lignocellulosic biomass include the plant and plant-derived materials such as agricultural wastes including wheat straws and bagasse from sugar cane, corn stover, rice straw, reed and switch grass [43]. Energy crops such as willow and temperate grasses are the other examples of lignocellulosic biomass. Organic wastes obtained from municipal solid waste, pulp and paper industries and agricultural residues have also been considered as lignocellulosic biomass [5].

The recalcitrant nature of lignocellulosic biomass reduces the access of available sugar polymers (i.e. cellulose). Hence, understanding the composition of the lignocellulosic biomass is important in developing a pretreatment approach to breaking down the biomass into its monomeric components followed by the production of biofuels [13].

1.2.1 Structure and Composition of lignocellulosic biomass

Structurally, the cellulosic fibrils of lignocellulosic biomass is embedded in lignin and a hemicellulosic portion (Figure 3), which gives the strength to the plant. The different components of lignocellulosic material are held together by various bonds such as covalent, intramolecular bridges and loose forces of attraction known as Vander Waals forces [79]. These forces make the lignocellulosic material hard to hydrolyze. The cellulose is tightly linked to hemicellulose through hydrogen bonds while the hemicellulose is bonded to lignin by covalent linkages [77]. The image below (Figure 3) shows the general structure of lignocellulose material [43].



Cellulose ——, Hemicellulose, Lignin

Figure 1: The arrangement of the various components of a lignocellulosic biomass [43]

In its native form, LCB is unsuitable for biofuel/biochemical production. The recalcitrant nature of lignocellulosic biomass affects the enzymatic digestibility significantly. Thereby it increases enzyme requirement and reduces the monomeric sugar yield, which directly affect the biomass conversion process [79]. Moreover, the crystallinity of cellulose and the vast cross-linkages between lignin and the hemicellulose render the biomass resistant to hydrolysis by microbial action [30].

Therefore, a need exists for the disruption of the native structure of lignocellulosic biomass to facilitate breakdown of lignocellulose to yield fermentable monomeric sugars (i.e. C5 or C6 sugars). The disruption of the lignocellulosic biomass can be achieved through pre-treatment steps that remove hemicellulose/lignin and expose the cellulose molecule for easier hydrolysis.

1.2.1.1 Cellulose

The cellulose makes up 40-45% of lignocellulosic biomass. Cellulose is a carbohydrate polymer made up of several glucose units linked together by β-1,4 glycosidic bonds [14]. On the other hand, plants synthesize glucose as monomers, which are polymerized into cellulose, starch,

amylose (one of the polymeric compound in starch). The number of glucose molecules found in the cellulose chain depends on the source material. Cellulose found in native wood containing up to 10,000 glucose monomers compared to kraft pulped wood which contains an average of 1000 glucose per fibril [36]. Cellulose in a lignocellulosic biomass forms an association called cellulose microfibrils that aggregate to form fibrils. The cellulose fibrils are linked by intra and inter-molecular hydrogen bonds that make the molecule to form a stiff complex structure that is 70% crystalline in shape and 30% amorphous [14]. Depending on rigidity and polymerization, plant cellulose are classified in to crystalline and amorphous cellulose. Amorphous cellulose is non-orderly arranged glucose polymers. They do not have sharp melting point and it undergoes irregular breakage. On the other hand crystalline cellulose is orderly arranged long range cellulosic polymer, which melts at sharp temperature and can be cleaved along definite planes. The complex structure has high density and is more resistant to the enzymatic hydrolysis. The complexity of the cellulose fibrils makes the cellulose molecule insoluble in water or organic solvents. Figure 1 shows the aggregation of the cellulose units to semi-crystalline fibril structure [8].

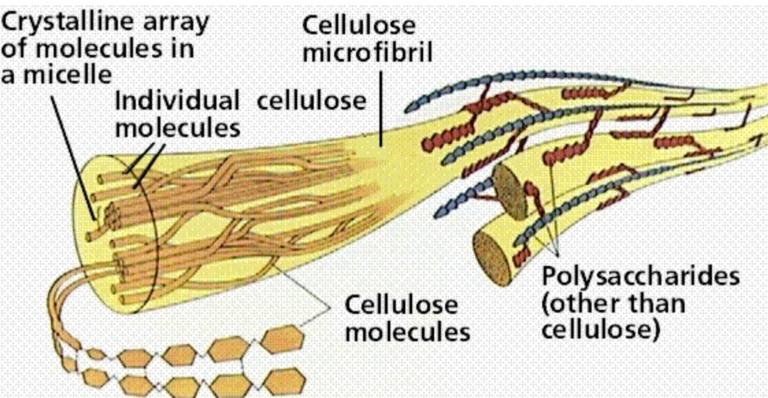


Figure 2: The image showing cellulose aggregation to form the complex fibril structure (82)

1.2.1.2 Hemicelluloses

The second largest component that makes up the lignocellulosic biomass is hemicellulose that constitutes between 24-40% of the total biomass. Hemicelluloses are mainly found in the secondary cell walls and they often cover the cellulose fibrils. Unlike the cellulose, hemicelluloses are highly branched in nature. The molecule is made up of pentose and hexose sugars. The main pentose sugars found in hemicellulose include xylose and arabinose, while glucose, mannose and galactose are the major hexose sugars. An uronic acid such as α-D-galacturonic acids also constitutes the hemicellulose structure. Hemicelluloses have low molecular weight (Average MW 132) compared to cellulose (162.1), lack the crystalline structure and are easy to depolymerize. Despite, the fact that hemicellulose is easy to hydrolyze, its presence adds complexity in break down of the biomass [14].

The composition of the hemicellulose varies with the type of lignocellulose biomass. For plant material such as wheat straw, xylan is the main hemicellulose component while glucomannan is largely present in softwood. Based on the composition of hemicellulose, the pretreatment methods also vary. For instance, presence of high concentration of xylan requires acid or alkaline treatment while glucomannan extraction is performed in strictly alkaline conditions [18].

1.2.1.3 Lignin

Lignin is one of the most abundant non-carbohydrate aromatic organic polymer after cellulose. The lignin constitutes 25-36% of the lignocellulose biomass. Lignin is amorphous, highly branched polyphenolic material copolymerized from coniferyl, synapyl and p-coumaryl alcohols [38]. Lignin forms a bond "gluing" together the other constituents of the lignocellulosic biomass [79]. Lignin molecule is resistant to hydrolysis and together with hemicellulose, it acts as a protective sheath to the cellulose chains. Figure 2 shows the structure of lignin molecule.

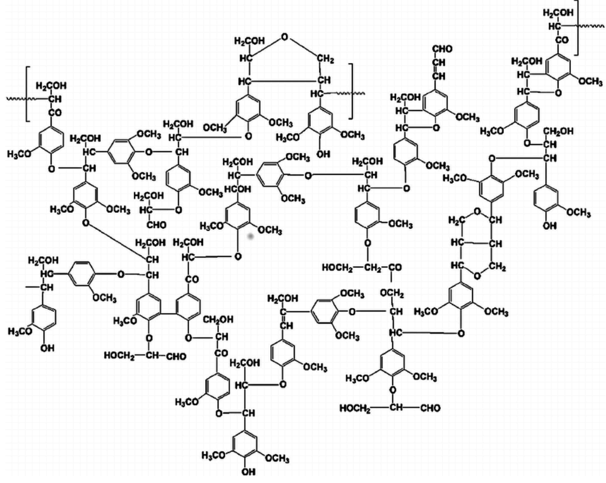


Figure 3: The structure of lignin molecule obtained from a hardwood [38]

1.3 Conversion of biomass to energy and value-added products

In general, biomass is used to generate heat energy by burning or combustion. For example, the sawdust along with lignin are used to generate heat in pulp and paper industry.. Hence, to convert the LCB to high energy fuels, it requires a set of processes that modify the LCB to liquids such as ethanol, propanol, and acetone or bio oil. This can be achieved by employing thermo-chemical and/or biochemical conversion processes [23].

Thermochemical conversion of biomass to energy is based on the heat treatment of biomass under controlled conditions of pressure and oxygen supply. The end product of biomass thermochemical process can be heat, syngas (a mixture of carbon monooxide and hydrogen), or liquid (eg. bio oil) [50, 23]. Thermochemical conversion of biomass can be carried out using various approaches. A commonly used thermal conversion method includes gasification, combustion and pyrolysis [66]. Gasification involves the conversion of biomass to combustible gas (also known as syngas) using air gasifiers. Pyrolysis involves burning of biomass under controlled temperature and oxygen to convert biomass feedstocks into gas, oil or forms of

charcoal [76]. The production of biofuel using pyrolysis occurs at temperature ranging from 500-800 °C and results in rapid conversion of biomass to biofuels [19]. However, the use of pyrolysis is impeded by high production cost, which is associated with energy input [11]. On the whole, most of these thermochemical processes are not sustainable, energy intensive and often associated with the irreversible destruction of organic matter [23].

Biochemical conversion of biomass is regarded as more sustainable and environmentally friendly. The biochemical conversion of biomass can be carried out in two ways (i) anaerobic digestion (ii) fermentation. Anaerobic digestion of biomass can be carried out in two ways. First is primary anaerobic digestion that involves the use of anaerobic microbes to convert the lignocellulose biomass in to biogas and organic acids [23]. Second involves the anaerobic digestion of biomass, which involves the enzymatic break down of solid biomass to soluble compounds. The hydrolyzed products of the enzymatic process then undergo acidosis step to produce short chain organic acids. Finally, the short chain acids are converted to methane by strict anaerobes through methanogenesis [55]. The methanogenesis occurs at moderate pH (pH 6.5-8.0) and at temperature ranging from 30-60°C. Although anaerobic digestion requires smaller production units, the method is associated with inflated cost and production of large volume of sludge [55].

Fermentation is the alternative to the anaerobic digestion. Fermentation requires simple sugars. In order to make the lignocellulose biomass in to fermentable sugars, the biomass must be pretreated and saccharified using hydrolyzing enzymes. The fermentation process involves the microbial conversion of simple sugar into biofuels. The fermentation results in the production of alcohol such as bioethanol, biobutanol, acetone, propanol etc., which can be used as liquid fuels. The biomass used in this process includes the crops waste such as sorghum, cassava [2, 32].

Apart from waste lignocellulosic materials, cellulose and hemicellulose from pulp and paper industries can also be used as substrates for ethanol fermentation process. Wayman et al.(1992) reported that a ton of waste paper can yield up to 400 liters of ethanol[70].

1.4 Pretreatment methods

Pretreatment of lignocellulose biomass helps in the disruption of the structure of lignocellulosic constituents and facilitates efficient hydrolysis of cellulose to simple sugars by making the substrate more accessible to the hydrolytic enzyme(s). There are four main types of lignocellulose pretreatment methods. This includes (i) physical (ii) physicochemical (iii) chemical and (iv) biological pretreatment method.

1.4.1 Physical/Mechanical

Physical pretreatment methods can disrupt the crystalline structure of cellulose through activities such as grinding and milling. This approach is termed a "comminution" [12]. The physical pretreatment of biomass using UV light is indicated by structural variation by interfering with the cell wall and tissue structure. The UV rays are also effective in bond cleavage and the creation of free radicals that stimulate degradation of lignin structure [15]. Physical pretreatment of lignocellulosic biomass by use of ultrasonication disrupts the biomass structure by breaking the bonds in lignin thereby exposing cellulose for hydrolysis [75]. The use of ultrasound can be enhanced if it is used at high frequency and in combination with oxidizing agents such as hydrogen peroxide and peracetic acid [12].

1.4.3 Chemical Pretreatment method

Chemicals such as acids, alkalis and organic/ionic solvents have the property to change the structural integrity of the biomass. Alkali is known to interfere with the structure of the

lignocellulosic biomass by causing them to swell, and change in their internal surface area and reduction in cellulose crystallinity [12]. The uronic acids and acetyl molecules in the hemicellulose are also removed by alkalis. The treatment of lignocellulose with alkalis also leads to an alteration in the structure of lignin through the development of point breakages [9]. Examples of the alkalis used in the pretreatment of lignocellulosic biomass includes NaOH, $Ca(OH)_2$ and anhydrous ammonia. The challenge with the use of alkalis is the high concentration required for pretreatment.

Dilute acids such as H_2SO_4, HCl hydrolyze the hemicelluloses to their simplest sugars, hence exposing the cellulose for degradation. The acids are, however, not preferred due to their corrosive nature. When acids are used, alkalis should also be used to neutralize the hydrolysate. This adds to overall cost of production of value added compounds from biomass. Additionally, the hydrolysis of hemicelluloses mainly results in a pentose sugars such as xylose, which are not readily utilized by microorganisms as a carbon source. The variation in the temperature and the pretreatment holding time are associated with the generation of undesirable chemicals that include furfurals, hydroxymethyl furfurals (HMF) all of which inhibit the growth of fermentation microbes and therefore productivity [13]. Use of ionic liquids expose the cellulose by dissolving lignin and hemicellulose from lignocellulosic biomass [56]. However, use of such solvents can permanently inactivate the activities of cellulase enzyme used during the bioconversion process. Thus, use of alternative solvents such as N-methyl morpholine N-oxide for pretreatment of biomass has also been explored [21].

Chemical treatment can also be accomplished through wet oxidation, which involves the oxidization of the compounds dissolved in water using oxygen. Wet oxidation results in the decomposition of about 70% of the lignin. Wet oxidation can be combined with alkali treatment

to reduce the formation of inhibitors such as organic acids [12]. Treatment of lignocellulose with hydrogen peroxide disrupts the biomass through delignification. Hydrogen peroxide solubilizes lignin and disrupts the crystalline structure of cellulose by rupturing the intra chain hydrogen bonds [22].

1.4.2 Physicochemical

1.4.2.1 Steam explosion

This approach uses the physical and chemical methods to disrupt the lignocellulose structure. Steam explosion disrupts the cellulosic fibrils through the use of high pressure and saturated steam followed a drastic depressurization. Fibril disruption leads to the exposure of the cellulose to degradation. Typical steam explosion conditions are 160-260°C with 0.69 to 4.83 MPa pressure. Acid molecules formed during stem explosion solubilize hemicellulose [61]. On the other hand, use of acid catalyst under above-mentioned specified conditions improves hemicellulose disruption [51]. The catalyst reduces the formation of inhibitors while enhancing the disruption of hemicellulose. Lignin also undergoes structural changes when it is exposed to high temperature and pressure [62]. Chemical disruption of lignocellulosic material is achieved through the glycosidic bond cleavage and delignification.

1.4.2.2 Ammonia fiber explosion (AFEX)

AFEX is similar to steam explosion however the pretreatment process involves liquid ammonia at high pressure and temperature. The biomass is treated at a controlled temperature and pressure for a period of time and the pressure is released rapidly. The drastic drop in pressure decomposes the biomass, which improves the enzyme digestibility and water holding capacity of pretreated biomass. In other words, expansion of ammonia causes the biomass to swell altering the linkage of the different components of the lignocellulosic material. Ammonia fiber explosion

does not remove lignin but it disrupts the lignin-carbohydrate complexes [24].

1.4.2.3 Carbon dioxide explosion

Pretreatment of lignocellulosic biomass by use of carbon dioxide explosion occurs through the weakening of the cell wall structure as the carbon dioxide diffuse into the cellulose structure and explode [12]. Increased acidity due to the interaction between carbon dioxide and water in the biomass also disrupts the lignocellulosic structure. It is important to note that the use of carbon dioxide explosion pretreatment helps to reduce the carbon dioxide released into the atmosphere by utilizing the carbon dioxide produced during fermentation of sugars to bioethanol [60]. Other physicochemical pretreatment techniques include the liquid hot water, ammonia recycle percolation and supercritical fluid pretreatment.

1.4.4 Biological

Biological pretreatment of lignocellulosic biomass is regarded to be a mild and inexpensive, but time-consuming approach. Biological pretreatment is also eco-friendly and a sustainable. Biological pretreatment proceeds through the breakdown of lignin material by the enzymes secreted by microorganisms. The microbial breakdown of lignin and hemicellulose is achieved through the use of two main of groups of enzymes, which include the phenol oxidase and peroxidases [9]. Phenol oxidase, also is known as laccase, uses redox mediators in the breakdown of the non-phenolic lignin compounds and therefore, aid in the breakdown of lignin molecule. The laccase enzyme is found in both fungi such as *Phanerochaete chrysosporium* and in bacteria such as *Bacillus subtilis* [18]. Similarly, there are several groups of microbial peroxidases that help in the breakdown of lignin. Examples of the peroxidases include the lignin peroxidase, which is involved in the degradation of the lignin polymer to smaller chains. The degradation occurs through the catalytic breakdown of the β-o-4 ether bonds and of Cα–Cβ

bonds in the lignin structure. The disruption of the lignin structure by lignin peroxidase is also achieved through a catalytic cleavage of the aromatic ring and the formation of quinine. Another group of microbial peroxidases that breakdown the lignin structure is the manganese peroxidase. Lignin degradation by manganese peroxidase is influenced by the presence of hydrogen peroxide and manganese ions [69]. For efficient pretreatment of the lignocellulosic biomass, the laccase in combination with one of the microbial peroxidases should be used. Hence, it is important to identify microbes with the ability to produce laccase and peroxidases to attain effective biological pretreatment. fungi (*Phanerochaete chrysosporium* and *Jungua separabilima*) possess the capability to produce laccase enzyme and an array of peroxidase enzymes including the lignin peroxidase and manganese peroxidase [69]. *Trichoderma reesei* has the ability to degrade hemicellulose, meantime it produces high titers of cellulase. However, the organism is not able to breakdown lignin material. Furthermore, the incorporation of the biological agents in a lignocellulosic pretreatment process helps to reduce the build-up of undesirable products and inhibitors.

One of the biggest challenges with the use of biological pretreatment method is its slow rate of reaction during the lignin degradation. It is indicated that the biological pretreatment can take more than 14 days. Meanwhile, biological pretreatment requires strict sample /media preparation and operation conditions. Hence, biological pretreatment is preferred in biomass with low levels of lignin. The approach can also be used as the first step when used in combination with other pretreatment methods [9]. The combination of the biological option with other forms of pretreatment helps to cut down on the operation cost mainly by reducing the initial energy requirement. Based on the literature review, physicochemical method has been found more

effective on lignocellulosic biomass since it removes lignin and hemicellulose effectively depending upon the chemical and physical condition.

1.4.5 Biomass pretreatment for enzymatic hydrolysis

The degradation action of hydrolytic enzymes is affected by various factors. One of the factors is the surface area of the substrate. The pretreatment should improve the surface are of cellulosic fibres on which the enzymes can attach and hydrolyze. Hence a good pretreatment condition substantially removes the hemicellulose and lignin from the biomass and improves the surface area of cellulosic fibres [59]. It is, therefore, important that the hemicellulose/lignin surface area should be reduced to enhance the activity of the hydrolytic enzymes [59]. Fermentation inhibition occurs when inhibitors such as aliphatic carboxylic acids and sorbic acid exist above the concentration of 200 to 450mMl^{-1} respectively [46].

The consistency of the substrate (also known as biomass to enzyme loading ratio) and reaction kinetics determines the amount of sugars produced from the hydrolytic process. The consistency also influences the production costs. In the hydrolytic degradation of lignocellulose biomass, a consistency of less than 5% of the solid material is usually used. At this consistency, the enzymatic hydrolysis yields 5% sugar solution. Increased sugar output can be achieved by increasing the consistency. Increasing the consistency helps in reducing the operation cost and the capital requirement [37]. The increase of consistency from 5% to 8% is indicated to result in 20% reduction in the cost associated with enzymatic hydrolysis of biomass. However, caution should be exercised when increasing the consistency to avoid inefficient mixing caused by high concentration of solid materials. It is indicated that a consistency of more than 10% results in poor enzymatic degradation due to inefficient mass transfer.

1.5 Types of biomass hydrolysis

The conversion of biomass to sugars occurs through a process termed hydrolysis. The hydrolytic degradation of biomass involves the cleavage of glycosidic bonds that exist within carbohydrate polymers. There are two types of biomass hydrolytic processes - acid hydrolysis and enzymatic hydrolysis [23].

Acid hydrolysis of biomass involves the use of dilute acid or concentrated acid. The dilute acid process involves two reactions with first reaction involving the hydrolysis of hemicellulose while the second reaction involves the hydrolysis of cellulose [68]. The reactions occur at same conditions leading to sugar generation and generation of unwanted material. The formation of byproduct leads to the inhibition of the fermentation process. The concentrated acid process is associated with enhanced glucose yield from cellulose. However, concentrated acid hydrolysis is associated with the formation of large amount of fermentation inhibitors [46, 14, 68].

Enzymatic hydrolysis involves the use of cellulolytic enzymes obtained from various microorganisms. In this regard, use of *T. reesei* for the production of cellulase enzyme has gained more attention due to the high enzyme productivity and effectiveness of enzyme produced. Cellulase is a multi-enzyme complex that works together (synergistic action) to break down cellulose to simple sugars (Serrano-Ruiz, & Dumesic, 2011). The endoglucanases hydrolyze the cellulose by cleaving the chain at random sites using their open active sites. Thus, resulting in the production of short chains that are then hydrolyzed to cellobiose by exoglucanases. The short chains of cellobiose and cellodextrins that are produced by the exoglucanases and cellodextrinases are then converted to simple sugars by β-glucosidases.

1.6 Enzymatic hydrolysis

1.6.1 Approaches for enhanced enzymatic hydrolysis of lignocellulosic biomass

1.6.1.1 Enzyme and substrate recycling

During the hydrolysis of lignocellulose, the accumulation of glucose can cause end-product inhibition [64]. One of the approaches used to overcome the inhibition is through the use of high concentration of enzymes [65]. However, the cost associated with the use of high concentration of enzyme is discouraging [71, 77]. Enzyme recycling, however, provides a means of sustaining high enzyme concentration to ensure sustained production of sugars [71]. One of the enzyme recycling approaches is the readsorption of the free enzyme contained in the liquid phase of the hydrolysis process through the use of fresh substrates [64]. The addition of fresh substrates into the liquid phase can facilitate the recycling of up to 82% of the free enzymes in the liquid phase [64]. Another approach is through the recycling of the substrate [71]. The recycling of the insoluble biomass is based on the fact that close to 30% of the enzyme remained bound to the insoluble biomass during the hydrolysis process [64]. The recycling of solid biomass helps to sustain high production of sugars while facilitating the reduction of the required dosage of enzyme [71].

1.6.1.2 Addition of surfactants

One of the challenges in the lignocellulose hydrolysis is the adsorption of cellulase to lignin, which results in the unavailability/inactivity of the enzymes [39]. The addition of anionic and non-ionic surfactants reduces the adsorption of the enzyme by 10% [16]. The addition of surfactant-polyethylene glycol has the potential to reduce the concentration of the adsorbed enzyme by 11.25% while increasing enzyme activity by 51.06%, hence increasing the production of sugars [78]. Tween 80 also increases desorption of the enzymes from lignin through

competitive adsorption [39]. Enhanced hydrolysis of the lignocellulose substrate is also achieved through the increase in the carboxylic content of lignin, which results in the reduction in the amount of the cellulase that is bound to lignin [44].

1.6.1.3 High (substrate) solids processing

Increased biofuel titer is achieved through high (substrate) solid processing [35]. However the economic importance of high solids processing can be realized by addressing the challenge of enzyme loading and energy costs [41]. The adoption of fed-batch enzymatic saccharification with simultaneous fermentation helps to solve some of the challenges encountered in the use of high solids [35]. Fed-batching of solids through sustained solid loadings leads to high ethanol titer at considerably low enzyme loading [35].

1.6.1.4 High temperature

Increased production of sugars from lignocellulose biomass can be achieved through optimization of process temperature [53]. High temperature enhances enzymatic hydrolysis by reducing viscosity, therefore, increasing the mixing of biomass slurry. High temperatures also facilitate the high mass transfer and increased solid loadings. However, the advantages of high temperature can only be realized when using thermophilic enzymes that have the capability to withstand the high temperature of about 70-90 °C [53].

1.6.1.5 Enzyme dose

Increasing the enzyme dose leads to enhanced yield and rate of enzyme hydrolysis. However, the rate of increase varies based on the existing cellulase concentration. Below enzyme concentration of 10 FPU/g increase in enzyme dose leads to rapid increase in enzyme hydrolysis but the rate of increase in hydrolysis drops above the enzyme concentration of 10 FPU/g [60]. Effective hydrolysis of lignocellulose is also influenced by the time of enzyme dosing. To

achieve the required motion in a biomass slurry enzyme dosing should be done one hour before substrate dosing [60].

1.6.1.6 Hydrolysis time

Hydrolysis time influence the degree of cellulase hydrolysis. The amount of sugars produced increases with increase in the hydrolysis time given that other factors are not limiting [71]. However, it should be noted that without controlling for other limiting factors such as enzyme dose, the degree of hydrolysis is less time dependent [71].

1.6.2 Types of cellulases

Cellulase is a multienzyme complex. Each subunit of cellulase performs different function. Based on the microbe used for its production, the degree of each subunit varies [58]. One of the subunit known as endoglucanases hydrolyzes the cellulose polymers by randomly attaching to a site in the polymer and breaking it into shorter chains [53]. Examples of endoglucanases include the ROI 89-05: NREL's endoglucanases obtained from *Acidothermus cellulolyticus.* These endoglucanases have high thermostability and can be used at high temperature (80°C). Another type of cellulases are the exoglucanases which are also termed as cellobiohydrolases. The exoglucanases, unlike the endoglucanases, hydrolyze the cellulose polymer by progressive removal of the sugar molecules from one end of the chain. The exoglucanases remove between two to four sugars residues from the end of the sugar polymer chain [58]. The exoglucanases can be further grouped based on the point from which they begin their progressive removal of oligosaccharides. The type 1 exoglucanases are those that remove the oligosaccharides from the reducing end of the polymer, while the type II exoglucanases cleave the short chains from the non-reducing end of the cellulose chain [53]. Another group of cellulase enzymes are the cellobiases whose function is to hydrolyze the cellobiose produced

from the degradation of cellulose by exoglucanases to glucose. The cellobiases are also termed as β-glucosidase. Similarly, certain groups of cellulase enzymes termed as oxidative cellulase hydrolyzes and cellulose phosphorylase hydrolyzes can hydrolyze the cellulose through radical reaction and phosphorylation reaction respectively [58].

1.6.3 Cellulase structure

The structure of cellulase enzymes (Figure 4) varies based on the type of source organism. For aerobic organism, the enzyme structure is characterized by a catalytic domain that is joined to cellulose binding domains while the catalytic domain is joined to a dockerin domain in aerobic organisms [10]. The cellulose-binding domain is important in enabling the binding of the cellulase to crystalline or the amorphous structure of cellulose. Other features that make up the structure of cellulase enzyme include the fibronectin-type III domain and NodB-like domain.

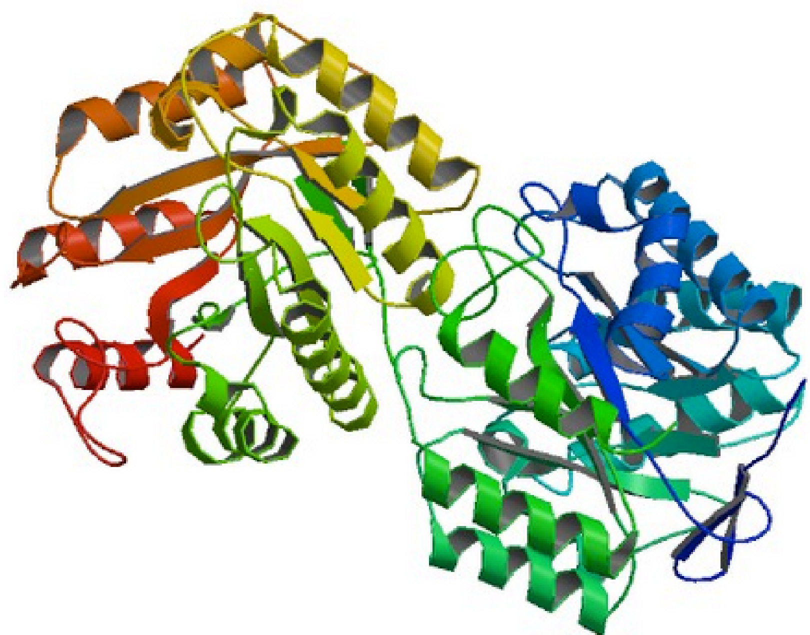


Figure 4: Cellulase cel5G [49]

Cellulose is as multienzyme complex generally made up of three types of activities includes, endo and exo-glucanase and β-glucosidase. Cellulose is produced by bacteria, fungi,

actenomycetes and algae. Based on the source the structure and function varies. The 3-D structure of the cellulase enzyme give information on their function. The active site of the enzyme is contained in an acidic cleft that is located at the carboxyl end of the barrel. Adjacent to the active site is a highly folded subdomain consisting of amino acid chain made up of 4 α-helixes and 2 β-structure strands. The subdomain extends to the top of the barrel at one end to form a substrate-binding cleft. The proton donor (Glu-140) responsible for mediating the process of catalysis is also located in the active site [48].

The 3-D structure of exoglucanases such as *T. reesei* cellobiohydrolase CbhII consists of a α/β barrel of 7 strands connected in α-helices except for the 6th and 7th that are connected by irregular strands. The carboxyl end of the barrel contains two extensive loops with side chains that form an enclosed tunnel with other side chains from the barrel. The tunnel provides a site where the non-reducing end of the carbohydrate polymer threads. The binding sites, a proton donor, and nucleophile are located in the tunnel [63].

1.6. 4 Biosynthesis of cellulases

Cellulases are naturally produced by certain microbes. New methods for obtaining the cellulase from microbes such as the use of genetically engineered microbes are being optimized. In this approach, bio prospecting is first done to identify the genetic make up the organism that can produce a higher titer of cellulase enzymes within a short period. Once the genetic combination has been identified and constructed, it is then inserted into a selected microbe through a process called transformation. The transformed organisms with the capability to express the inserted gene are then used as recombinant cellulase enzyme producers [74].

Generation of the cellulase enzyme can also be carried out by providing conducive conditions to the enzyme-producing organism. It is indicated that the variation in the substrate on

which the microbes of interest are cultured influences the cellulose production. Cellulase activity was observed to increase 2 to 10 fold when *Termitomyces clypeatus* was cultured in soluble carbohydrate obtained from wheat bran [45]. The researchers added wheat bran to media in with mustard straw with high cellulose and hemicellulose was used as the only carbon source. The use of wheat bran was observed to increase enzyme production by 10 fold compared to the general media used in cellulose production.

1.6.5 Mechanism of cellulase hydrolysis of cellulose

Catalytic breakdown of cellulose by cellulase proceeds through two main mechanisms that include the retaining mechanisms and the inverting mechanism (Fig 5)[33]. The cleavage of the β-glycosidic bond between the residues occurs in a stereo selective manner. The bond cleavage through the retaining mechanism occurs in two steps in which the displacement reaction occurs twice leading to the retention of the configuration at anomeric carbon level. The first step of the retaining mechanism is mediated by an acid group that donates a proton to the glycosidic oxygen [33]. This result in the cleavage of the bond resulting 2 fragments, one with a non-reducing end and the other is glycosyl-enzyme intermediate that undergoes the second step of reaction resulting in the retention of the configuration. The retaining mechanism is usually observed in exoglucanases [33]. The inverting mechanism, however, consists of only one step and results in the change of configuration at anomeric carbon level [41]. In this mechanism of action, the proton donor such as the Glu-55 protonates the glycosidic oxygen. As protonation takes place a negatively charged aspartate-201 residue ionizes a water molecule resulting in the formation of hydroxyl ions, which target the anomeric carbon resulting in the inversion of the configuration and bond cleavage. The cleavage of the bond in the inverting mechanism do not result in the formation of glycosyl-enzyme intermediate but the reaction proceeds through an

oxocarbonium ion-like transition state as is the case for the retaining mechanism [41].

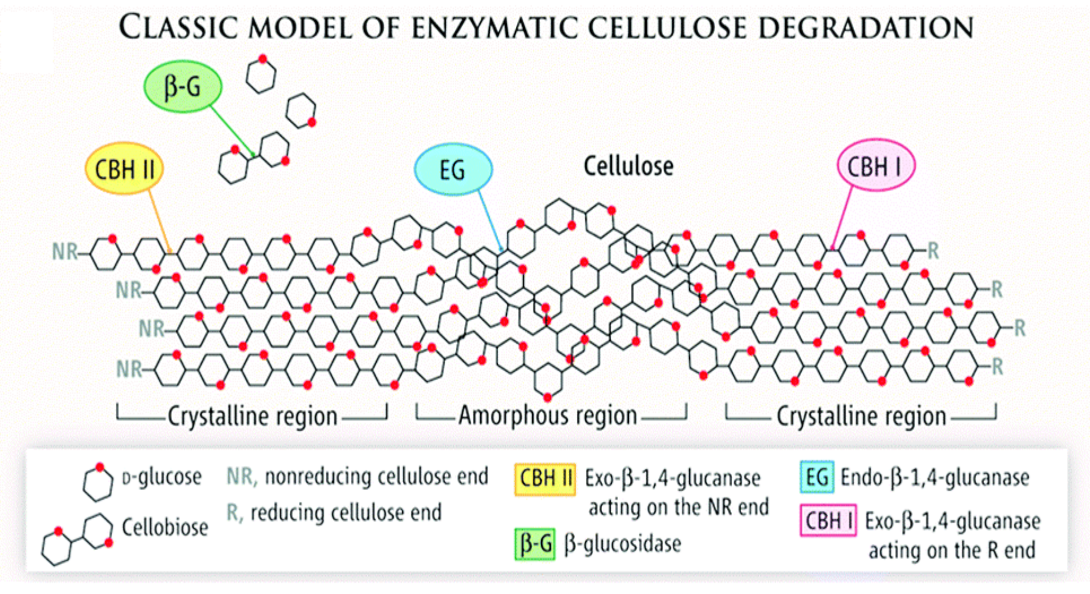


Figure 5: general mechanism of cellulose hydrolysis by cellulase; CBH I- cleaves the reducing end of cellulose polymer and CBH II acts on non reducing end. Endo glucanase cleaves the open chains that created by CBH I &II and releases cellobiose units. β-glucosidases acts on cellobiose units and cleaves the β-1,4 glucoside link and releases glucose monomers.

1.6.6 Inhibitors of enzymatic hydrolysis

The main inhibitors of enzymatic hydrolysis are the end products or undesired compounds released during biomass pretreatment process. Beyond certain levels of concentration in the media, products formed during the fermentation can potentially inhibit the activity of enzymes used. Products which show such inhibition include the organic alcohol such as ethanol and butanol, the organic acids (butyric acid) and acetone. The inhibitors such as butanol work by suppressing the cellulolytic activities of the enzyme and saccharification of the pretreated substrate [6]. These inhibitors can work singly or can work in combination leading to increased inhibition.

If chemical method such as acid pretreatment is used in combination with biological pretreatment methods, various compounds such as furfurals, hydroxymethylfurfural (HMF),

formic acids and acetic acids formed during the former process may inhibit the activity of latter process (i.e. biological) and during the fermentation [57].The other groups of inhibitors are the aromatic compounds produced from lignin degradation during the pretreatment process. Aromatic compounds can also be formed from sugars.]. Aliphatic acids such as acetic acids produced during the degradation of hemicellulose inhibit the growth of microbes and sometimes kill the organism by causing a decline in the intracellular pH. Accumulation of the products of enzyme hydrolysis such as cellobiose also results in enzyme inhibition [28].

1.7 Summary

The above literature review evaluates the use of LCB as a potential source of bioenergy. However, the conversion of LCB into biofuels or value added biomaterials will require a set of unique processes. Pretreatment of LCB removes hemicellulose or lignin from the biomass, helps to reduce the complex nature of biomass, and increases the surface area of cellulose. Hydrolytic enzymes can access and convert the pretreated biomass into fermentable sugars. Even though the research into biofuel production from LCB has a long tradition, a complete industrial process is not yet developed. Based on the literature review it is evident that the enzyme cost is one of the major bottlenecks in LCB to biofuels conversion. Hence, more research needs to address the issues related to substrate and enzyme recycling or recovery to improve the process economy.

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Chapter 2

Impact of enzymatic hydrolysis conditions on glucose production from poplar pulp

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ABSTRACT

The current study aimed to evaluate the impact of critical parameters of enzymatic hydrolysis on enzymatic saccharification of poplar pulp to glucose using a commercial enzyme product (CTec2, Novozymes). It was found that pre-washing of biomass had no effect on enzymatic hydrolysis. However, adjusting the pH of the substrate to pH 5, as compared to the initial substrate pH of 3, improved hydrolysis efficiency. The optimum biomass and enzyme loadings, based on the maximum sugar yield obtained from poplar pulp (41.9% w/w), were both 5% w/w. However, hydrolysis efficiency was reduced by 19.3% at 15% substrate consistency. Increasing the enzyme loading from 1% to 10% resulted in a 2.1-fold increase in sugar yields. The substrate recycling was found to produce additional amounts of glucose, which further increased hydrolysis efficiency. After three consecutive repeated hydrolysis of substrate, 92.1% cellulose was converted in to glucose. Hence, substrate recycling can be used as a means to 1) extract additional amounts of sugar from the substrate, and 2) recover and reuse residual substrate-bound enzyme. Therefore, substrate recycling could reduce the overall cost of enzymatic hydrolysis.

Keywords: Enzymatic hydrolysis, CTec2, Poplar pulp, Substrate recycling, Glucose yield

1. INTRODUCTION

The ever growing concerns of the negative effect of fossil fuels on environment and fuel demand across the globe lead to renewable and sustainable fuels research [13]. An increasing demand for fossil fuels and petroleum based non-biodegradable products has assured a viable future for the development of alternative fuels/chemicals obtained from renewable sources such as lignocellulose biomass. Lignocellulose is the plant biomass that consists three polymers namely, lignin, hemicellulose, and cellulose. It is an essential feedstock for the production of renewable fuels including bio-ethanol [4]. Lignocellulosic biomass can broadly be categorized into energy crops, waste biomass, and virgin biomass [5; 6]. With its large resources, the forests in Canada represent an abundant and rich source of biomass such as biomass harvest residues and biomass plantations such willow and poplar species [7; 8]. Derbowka *et al.* [9] describe willows and poplars as shrubs and trees that belong to the members of "*Salicaceae*" family. Being among the fastest growing tree species as reported by Mamashita, Larocque *et al.* [10], hybrid poplars, are considered by both the provincial and federal governments as suitable for the production of bio-energy to increase biomass-derived energy availability in Canada. Several efforts have been made to primarily boost the breeding as well as selection and assortment of poplar plants in Canada to enhance their potential as a sustainable and renewable biofuel resource. [8]. For efficient utilization of lignocellulose biomass for biofuel and biochemical synthesis, a primary removal of non-cellulosic materials is a pre requisite [11; 12]. Bensah and Mensah [13] reported the importance of chemical pretreatment of lignocellulosic biomass is a significant factor that plays an influential role in the cellulose conversion processes. In the study of Amin *et al.* [14] on the pretreatment methods of lignocellulosic biomass for anaerobic digestion, the observed that pretreatment was an influential factor in changing the cellulosic biomass structure, making

cellulose readily accessible to the enzymes responsible for converting carbohydrate polymers into soluble and fermentable sugars. Pretreatment of the lignocellulosic material is performed to overcome recalcitrance through the amalgamation of structural and chemical changes to carbohydrates and lignin [14]. In this regard substrate washing and non-washing have been widely studied for different feedstock especially waste biomass enzymatic hydrolysis [15]. Toquero and Bolado [16] investigated four pretreatments techniques followed by a washing step to remove the inhibitors formed during the pretreatment step. The authors reported that washed alkaline peroxide pretreated biomass provided the highest sugar concentrations (31.8 g/L glucose, and 13.8 g/L xylose). Further, the above study revealed that the washing of pretreated biomass could attribute a higher yield of ethanol (17.4g/L) upon fermentation. Also, Zheng *et al.* [17] reported that washing helped in the neutralization and deashing of the substrate before saccharification. Frederick, Zhang *et al.* [15] investigated the effect of washing of pretreated biomass on enzymatic hydrolysis and fermentation to ethanol from poplar wood pulp. The authors reported that the biomass washed with 3 volumes of water produced the highest ethanol yields (up to 0.4 $g\ g^{-1}$ glucose) and was significantly greater than those from the non-washed sample ($\leq$0.3 $g\ g^{-1}$ glucose).

Cellulosic ethanol is one of the viable products from lignocellulosic biomass synthesized by three major steps, namely, pretreatment, hydrolysis, and fermentation [18; 19]. In hydrolysis, hemicelluloses and cellulose are broken down into monomeric sugars through the addition of enzymes or acids [20; 21]. Enzymatic hydrolysis of lignocellulosic biomass is affected by substrate consistency [22; 23], enzyme dosage [24; 25], reaction temperature [26; 27], addition of surfactant [28; 29], pH [30], and substrate pretreatment [16; 31].

Elsewhere, Weiss *et al.* [32] studied insoluble solids recycling during enzyme-catalyzed lignocellulose hydrolysis to improve cellulase productivity. The authors demonstrated that recycling the insoluble biomass fraction could lead to: 1) increased glucose production and 2) recovery of a substantial amount of cellulase activity that resulted in 30% reduction in enzyme dosage while achieving the same yields of glucose under the most conducive conditions.

With the above background in mind, this study posed to study the factors such as effect of pretreatment inhibitors, pH, enzyme and substrate loading in biomass hydrolysis. Further the study is aimed to recover the substrate bound enzyme (substrate recycling) to reduce the use of enzyme in hydrolysis.

2. MATERIALS AND METHODS

2.1. Substrate

Steam exploded poplar was used as a substrate for enzymatic hydrolysis in the present study. Poplar wood chips (8 kg) were soaked in water at 1:4 ratio (w/v) overnight and then drained through a sieve of mesh size 4 (pore/opening size of 4750 μm). The soaked chips were then loaded into a custom-made pressurized percolation reactor and pre-steamed at 100°C for 60 min, followed by cooking with saturated steam at 170°C for 120 min. To adjust temperature, the purging steam was frequently discharged from the reactor, condensed and collected (liquid purge). At end of the cooking, the pressure of the reactor was instantly released to atmosphere through a discharge valve, which caused fibrillation of the wood chips. The fiber-like biomass was then pressed in a custom-made hydraulic press cylinder at 3,000 psi through a sieve of mesh size 80 (pore/opening size of 180 μm) for 25 min until the liquid (prehydrolysis liquor) in biomass was completely drained. The steam-pretreated PP was stored at 4°C until use.

Initial moisture and total solid contents were analysed by an automated moisture analyzer (Sartorius 44-1, Germany). Further, the samples was stored at -80□C until further use.

2.2. Impact of washing on production of cellulosic sugars

A sample of 15 g of pretreated poplar pulp was washed using distilled water while new sample 15g of the substrate was left unwashed. The pH of the washed and unwashed substrate was adjusted to 5 using 2M sodium hydroxide and then placed into the fridge for overnight [33]. The substrate pH was tested after 24 h storage in the refrigerator and readjusted to 5. Initially the moisture and total solid content of the washed and unwashed biomass was determined using moisture analyzer (ML-50, A&D, Limited, Tokyo, Japan). Based on the total solid content the biomass consitancy was adjusted to 5% and the total reaction volume was 30mL in both experiments. Glass beads (10g) were added to each substrate in a flask to improve substrates mixing. The volume of enzymes added to the flask was determined based on substrate dry weight using the following equation (EQ. 1):

$$Enzyme\ \ volume = \frac{Substrate\ dry\ weight \times Enzyme\ \%}{Enzyme\ density\left(1.2\frac{g}{ml}for\ CTec2\right)} \qquad (1)$$

The enzyme dossage was clculated based on the substrate (total solids in the reaction mixture) content. Percentage of enzyme dosage substituted in the above equation was 1% which corresponded to 125 µL of enzyme volume (total solid 7.5g, 1% enzyme and the reaction volume is 150mL). The substrate was pre-warmed in the incubator for 30 minutes after which 125 µL enzymes (CTec 2, Novozymes) was added. The substrate was incubated at 50 °C, agitation speed of 200 rpm for 96 hours. Sampling was carried out at a time interval of 24, 48, 72, and 96 hours. HPLC (1200 Infinity, Agilent Technologies, Santa Clara, CA, USA) technique was employed to determine the concentration of sugars in hydrolyzate. The HPLC was equipped with

a column (Aminex HPX-87H, Bio-Rad, Hercules, California, USA) and a refractive index detector (RID) [34]. The mobile phase (5mM H_2SO_4) flow rate was 0.5 mL/min. The column and detector temperatures were maintained at 60°C and 35°C, respectively. The analysis was performed by adding 1 ml of the aliquot to 2 ml centrifuge tubes and further diluted by 1 ml of distilled water in the tubes. The samples were centrifuged at 13000 × g for 3 min, followed by filtration using a 0.2 μm syringe (manufacturer) filter before HPLC injection. Samples were prepared and run in triplicate.

2.3. Impact of pH

For this experiment, 15 g of unwashed poplar pulp with the of the actual substrate pH (3) and the pH of a fresh sample (15 g) of unwashed poplar pulp was adjusted to 5.0 using 2 M sodium hydroxide. In order to stabilize the pH of substrates, after adjusting the pH the samples were placed in the refrigerator for 24 h. After 24h the samples were retrieved and brought to room temperature and the pH was readjusted to 5.0. The substrate consistency was equally determined using moisture analyzer after which 137.82 mL distilled water was added to each of the substrates [33]. Glass beads (10 g) were added to flask to improve sample homogenization. The enzymes volume added to the flask was calculated as described in Eq.1.

2.4. Impact of enzyme dosage

Four samples of 15 g of unwashed poplar pulp adjusted to pH 5 using 2 M sodium hydroxide were used for the analysis. All four samples were placed in the refrigerator for 24 h. The samples pH level was adjusted and determined after the refrigeration period to conform with the initial pH of the substrates. The substrate consistency was adjusted to 5% by the addition of 137.82mL

of distilled water to all the four samples. Glass beads (10 g) was added to the flask for better mixing.

The samples were incubated for thirty minutes (pre-warmed) before the addition of enzyme. Four levels of enzyme dosages (CTec 2, Novozymes) were studied as follows: 1% (125 μL), 5% (625 μL), 8% (1000 μL), 10% (1250 μL) to appropriately labelled sample of 15g unwashed substrate containing 51%w/w total solid.The total reaction volume was 150ml in all experiments[33]. The volume of enzyme added to the flask was determined based on substrate dry weight using Eq.1. The substrate was incubated at 50 °C, agitation speed of 200 rpm for 96 hours. During the incubation period, aliquot sampling was carried out at a time interval of 24, 48, 72, and 96 hours.

2.5. Impact of substrate consistency

The effect of substrate consistency on the production of soluble sugars was carried out according to Zhao, Song *et al.* [35]. The pH of three samples of 15g each of unwashed poplar pulp was adjusted to 5 using 2M Sodium hydroxide. All the three samples were refrigerated for 24 h. The pH level of the substrates was adjusted after the refrigeration period once the samples were reached room temperature. The substrate consistencies of three samples were adjusted to 5%, 10% and 15% and the total volume of the reaction was maintained at 150ml [36]. The substrate consistency was calculated based on Equation 2. Homogeneous mixing of the substrates was achieved by adding 10 g of glass beads. The volume of enzymes required for the experiment was determined using Eq.1 based on substrate dry weight. A constant amount of enzyme (5%) quantified as 625 μL was used for all the samples.

$$Substrate\ consiste`ncy = \frac{Total\ solids(\frac{g}{l})}{Totalreaction\ volume\ (mL)} * 100\ (Eq.2)$$

2.6. Impact of substrate recycling

This research aimed to evaluate the sugar produced by recycled poplar pulp. The pH of two samples of unwashed poplar pulp (15 g) was adjusted to 5.0 with 2M sodium hydroxide. The pH adjusted samples were refrigerated for 24 h. After 24h the samples were thawed to room 25□C and the pH was determined and readjusted to 5. The substrate consistency (5%) was calculated based on the total solid content of the substrate. The volume of enzymes added to the flask was calculated based on substrate dry weight according to Eq. 1. The substrates were pre-warmed in an incubator for 30 min before enzymes were added as discussed in Section 2.1. The two samples were incubated at 50 °C, agitation speed of 200 rpm for 24 hours, and 5 % (625 µL) of the enzyme. The soluble sugars synthesis was analyzed using the HPLC technique as discussed in Section 2.1.

At the end of 96 h of hydrolysis, the separated residue from the supernatant was transferred to aluminium containers and placed in an oven heated at 105 °C overnight. Then the containers were transferred to a desiccator for 30 min to cool down to room temperature. The dry mass was estimated following hydrolysis and the liquefied rate determined. The solid dried pulp was prepared to conform with 5% consistency by adding distilled water after which fresh enzyme dosage was added and incubated for 96 h. After every 24 h, a sample was withdrawn from the incubated mixture and analyzed using HPLC. After 96 h, the above procedure was repeated twice. The percentage of glucose yield was calculated based on the equation 3. The glucose yield was calculated by differentiating the initial glucose content of the substrate and the glucose content of the hydrolyzate received after each time intervals of hydrolysis.

$$Glucose\ yield\ (\%) = \frac{Glucose\ concentration\ in\ hydrolyzate\left(\frac{g}{g}\right)X100}{Glocose\ content\ of\ initial\ substrate(\frac{g}{g})} \quad (Eq.3)$$

3. RESULTS AND DISCUSSION

3.1. Impact of substrate washing and pH of enzymatic hydrolysis

Thermochemical pretreatment such as steam explosion is known to create potential enzyme and fermentation inhibitors derived from sugar and lignin degradation of lignocellulose. Washing is one of the widely accepted methods of inhibitor removal. Therefore hydrolysis of biomass was investigated with and without washing the biomass. From Fig. 1 it is understandable that the washing of biomass was not effective in improving the glucose yields. At 96 h of hydrolysis time, a sugar yield of 22.27% w/w was obtained from washed pulp and 20.69% w/w in the case of unwashed biomass. However, Fedrick *et al.* [37] reported a seven-fold increase in reducing sugar yield from washed acid-pretreated poplar wood. The increase in hydrolysis was attributed to reduction of inhibitors such as gallic, vanillic, syringic, p-coumaric, ferrulic, trans-cinnamic, and salicylic acids at concentrations below 0.07 mg/ml in pretreated pulp.

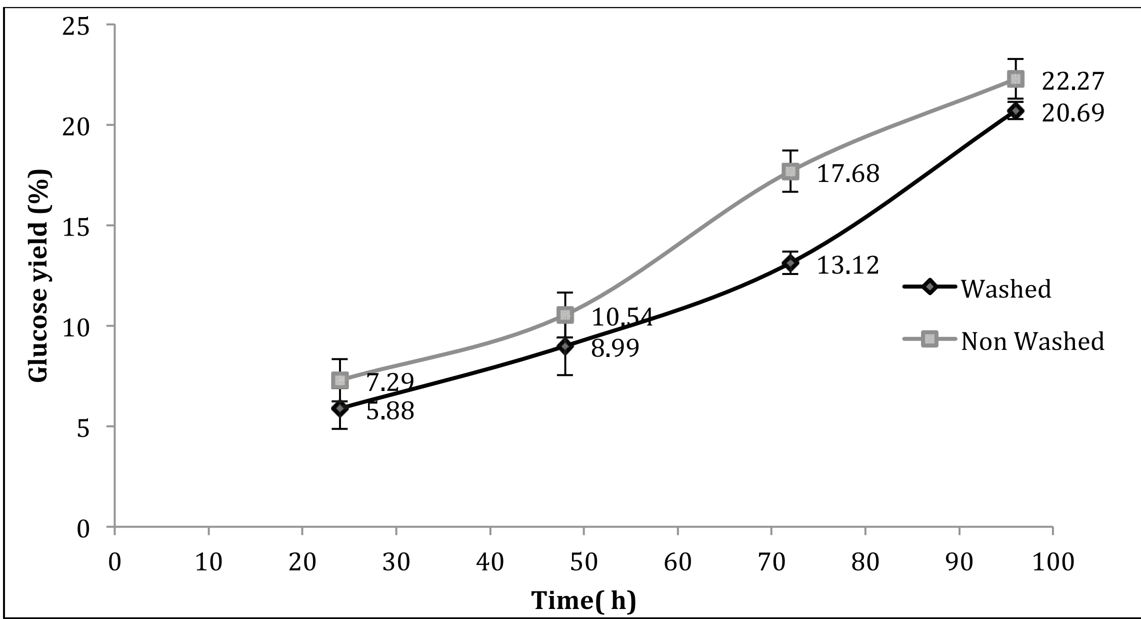

Fig. 1: Effects of substrate washing on glucose production following enzymatic hydrolysis of poplar pulp with CTec2 cellulase

Contradicting that, Soares et al. [25] showed no effect of washing on enzymatic hydrolysis of steam-exploded sugarcane bagasse. Moreover, significant amounts of sugars were lost as a result of washing [25], which agrees well with our results. Further work in this study was therefore carried out with unwashed substrate.

pH is one of the major factors in enzymatic hydrolysis since it provides the optimum buffering condition for the enzyme. Moreover, CTec2 cellulase used here is known to be more active at a pH range from 4.5 to 5.5 (Fig. 2). In the current investigation, two different pHs (3 and 5) were evaluated. A slightly higher sugar yield was noticed in the case of pH 5 (23%). In the case of pH 3, the sugar yield reached 20.6%w/w at 96 h of incubation. Moreover, the results obtained for both pH 5 and 3 are not statistically significant. Based on the above results, it can be concluded that pH 5 was the parameter of choice for achieving optimal glucose yields.

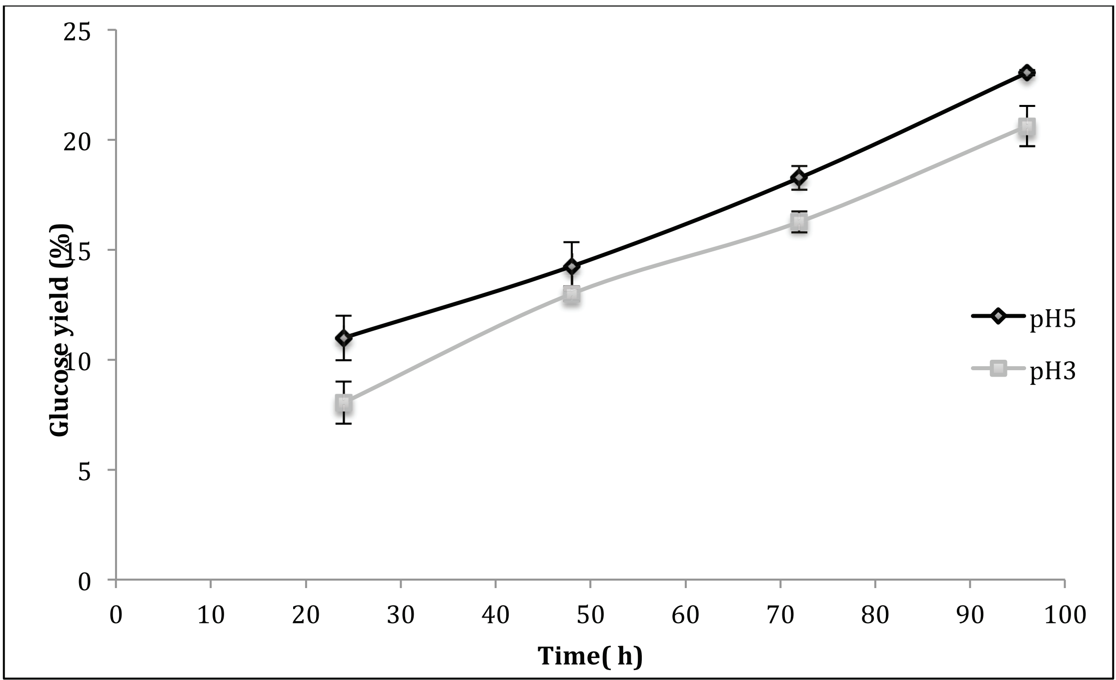

Fig. 2: Effect of pH on glucose production from poplar pulp following enzymatic hydrolysis of poplar pulp with CTec2 cellulase

3.2. Impact of enzyme dosage

The influence of enzyme dosages was examined at four levels (1, 5, 8, and 10% w/w). The lowest sugar conversion efficiency (23.1% glucose yield) was noticed at 1% w/w enzyme (Fig. 3). As expected, increasing the enzyme dosage resulted in high cellulose to glucose conversion. A high sugar yield of 48.9 % was obtained with 10% w/w enzyme charge, followed by 8% w/w (46.9%) and 5% w/w (40.6%). The above results were comparable with the work reported by Yang et al [38], where hydrolysis of poplar pulp resulted in 48.2% sugar yield. Comparing 1% with 5, 8 and 10% enzyme dosages, a significant improvement in hydrolysis was attained. It is obvious that the free enzyme concentration or substrate to enzyme ratio is higher at higher enzyme loading. However, statistical analysis showed a less significant effect on enzyme concentration on hydrolysis when 5, 8, and 10 wt.% was used ($p<0.05$) (Fig. 3). Increasing enzyme load 5% w/w enzyme to 10% w/w resulted in only 8.39% increase in sugar yield, which is statistically insignificant. Similarly, the statistical differences in sugar yields between 5 and 8%w/w enzyme and 8 to 10%w/w enzyme were not significant. Mussatto et al [39] reported that high enzyme concentration is one of the highly significant principal factors that affect the hydrolysis in lignocellulosic biomass. In the present study 5%w/w (enzyme/g of biomass) (6.7FPU/g) enzyme loading resulted in 40.6% hydrolysis efficiency and increasing the enzyme loading resulted in slightly higher hydrolysis efficiency. However in the study conducted by Mussatto et, al.(2008) reported an enzyme loading of 45FPU/g of substrate resulted in 99.4% conversion. Compared to the above study the enzyme loading was 6.7 fold lesser in the present case. However the figure 3 reveals an increasing hydrolysis trend upon hydrolysis time. Hence increasing the hydrolysis time with high enzyme loading can result better cellulose conversion.

[39].

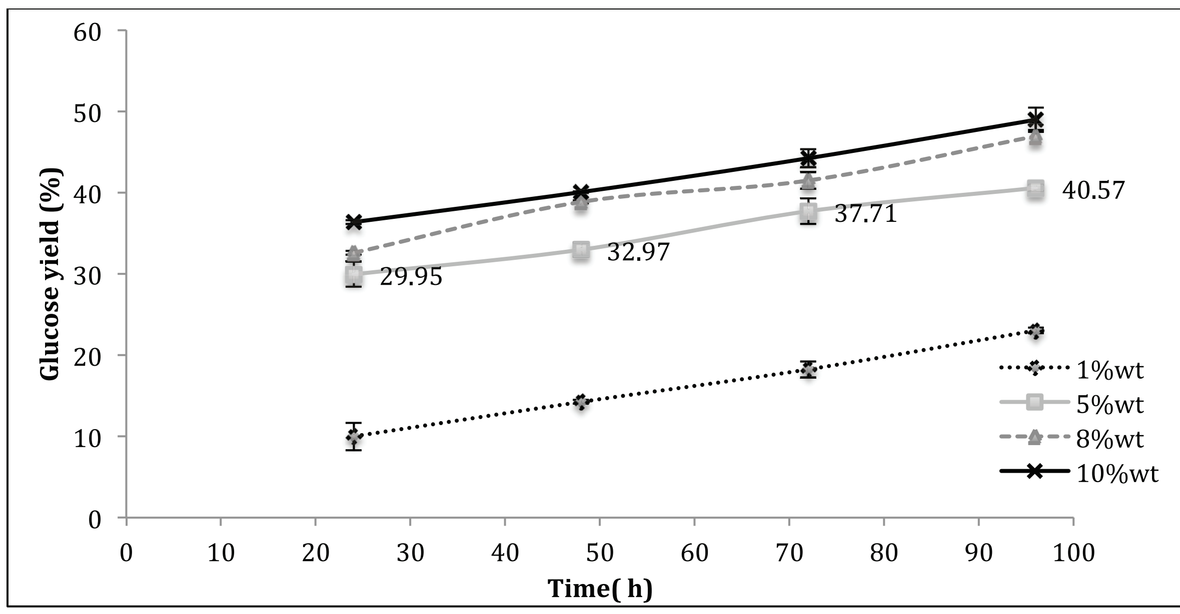

Fig. 3: Impact of enzyme dosage on glucose production following enzymatic hydrolysis of poplar pulp with CTec2 cellulase

It is evident from our results that increasing the enzyme concentration beyond an optimum did not improve hydrolysis. However, the use of more enzyme certainly increases the cost of sugar conversion in a significant manner. The current experiment showed an enzyme dosage of 5% w/w at 15% w/w substrate consistency as the optimum for hydrolysis of poplar pulp. However, further research is required to optimize the enzyme dosage for glucose production from poplar pulp.

3.3. Effects of substrate consistency

Substrate consistency is a crucial factor that affects the yield and initial rate of enzymatic hydrolysis of lignocellulose biomass [22; 23]. Three levels of substrate consistencies (5%, 10%, and 15% wt. of the substrate) at 5% enzyme dosage were investigated in this study. Fig. 4 shows the effects of substrate consistency on the sugar yield from the enzymatic hydrolysis of poplar

pulp. Lowest yield of sugar was obtained at highest substrate consistency. There was no significant difference ($p<0.05$) between 10 and 15% substrate consistencies. At low substrate concentration, an increase in substrate concentration resulted in increase of the sugar concentration and possible improvement in hydrolysis reaction rate [40; 41]. From Fig. 4 it is evident that increasing the substrate consistency negatively affected the total sugar yield. The current experiment shows a 41.9% w/w sugar yield at 5% substrate loading and 5% w/w enzyme loading. A linear decrease in sugar yield is evident indicating that high substrate concentrations can hinder the enzyme hydrolysis. Similar results were obtained by Jørgensen, Kristensen *et al.* [42], Rosgaard, Andric *et al.* [22], and Shen, Hu *et al.* [41]. Cara *et al.* [43] reported that enzymatic hydrolysis at high substrate concentration (≥20%) is possible, yielding a concentrated glucose solution (>50 g/L). This could be due to the free enzyme availability in the reaction mixture. On the other hand, feedback inhibition of cellulolytic enzymes was reported to reduce the hydrolysis efficiency at high substrate concentrations. At high substrate concentration, cellobiose inhibits the β-glucosidase activity considerably [44]. Moreover, in the current study, no surfactants were used to facilitate the enzyme adsorption and desorption from the substrate or to reduce the non-productive binding of the enzyme on lignin. Increasing the hydrolysis time may lead to enzyme deactivation, which in turns results in poor hydrolysis. Eventually, a high substrate concentration during hydrolysis might cause substrate inhibition, which substantially lowers the rate of hydrolysis while the extent of substrate inhibition depends on the ratio of the total substrate and enzyme dosage [45].

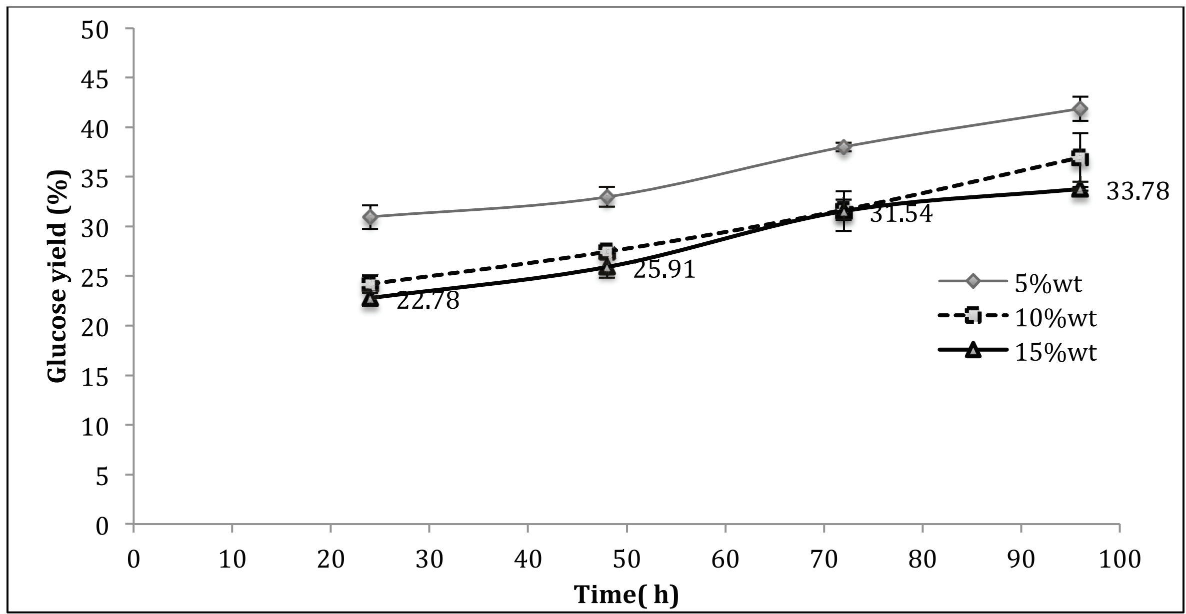

Fig. 4: Effect of substrate consistency on glucose production following enzymatic hydrolysis of poplar pulp with CTec2 cellulase

3.4. Impact of substrate recycling

Fig. 5 shows two consecutive runs of substrate residue recycling each for up to 96 h hydrolysis time. A decrease in glucose yield was observed as the recycling number increased. Usually, cellulases are found free in the liquid fraction (supernatant) at the end of hydrolysis, however, binding of cellulose to the residual substrate also occurs. Most of the enzyme bound to the substrate residue is still active and retains its capacity to efficiently adsorb onto fresh substrates for additional enzymatic hydrolysis [32]. In the current study, 41.5% glucose was released after 96h of hydrolysis. When the same substrate was reused for the second cycle, the glucose yield was dropped to 29.7% and which is 11.8% less than the initial cycle. Further recycling the substrate (3rd cycle) resulted in 20.6% reduction in glucose yield compared to initial hydrolysis yield. While compiling the data obtained after three cycles of hydrolysis an overall glucose yield of 92.1% (41.5+29.7+20.9) was obtained at 270h (90h*3cycles) of hydrolysis (Fig. 5). A significant buildup of lignin-rich residues and cellulose avilability would eventually have an

adverse effect on the hydrolytic ability of the bound and fresh enzymes might be responsible for these results [46]. Another possible reason might be due to the limited availability of easily hydrolyzable substrate (cellulose) in the pulp residue [47].

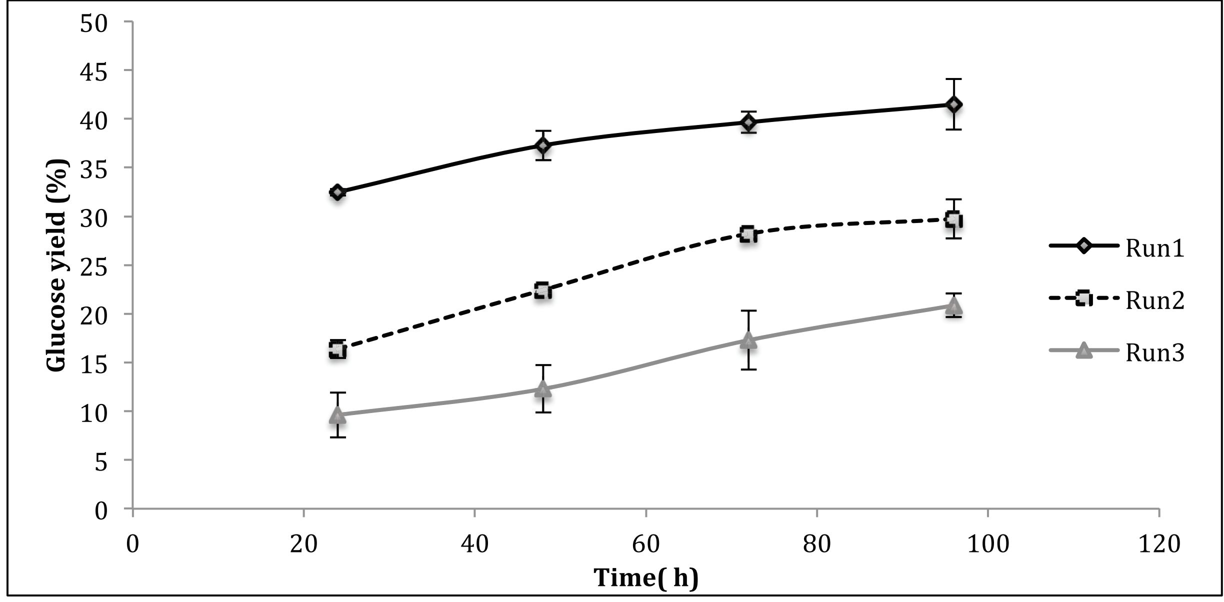

Fig. 5: Impact of substrate recycling on glucose production following enzymatic hydrolysis of poplar pulp with CTec2 cellulase

4. CONCLUSIONS

The effects of enzymatic hydrolysis factors on the production of glucose from pretreated poplar pulp were investigated. The substrate and enzyme consistency and hydrolysis time were identified as significant factors in hydrolysis. The higher the substrate consistency the lower the enzymatic hydrolysis yield of glucose from poplar pulp. On other hand, increasing the enzyme loading resulted in increased pulp to sugar conversion. Substrate recycling produced additional amounts of glucose from poplar pulp. At initial hydrolysis 41.5% cellulose conversion was attained. After two repeated hydrolysis cycling, 92.1% cellulose conversion efficiency was attained and which add 50% additional sugar to the hydrolysate. The reduction in the glucose yield was most likely due to reduced availability of easily hydrolysable substrate in poplar pulp,

and accumulation of lignin, which is a known inhibitor of enzymatic and fermentative processes. However, recycling of the substrate provides the opportunity to more efficiently utilize the substrate for glucose release, and reuse some of the substrate-bound enzyme, which may lead to the use of lower enzyme dosages. This approach has the potential to enhance the cost-efficiency of enzymatic hydrolysis and will be further optimized for developing an economically-viable process of sugar production from renewable, low-cost biomass sources.

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Chapter 3

Enhancing enzyme-aided production of fermentable sugars from poplar pulp in presence of non-ionic surfactants

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Abstract Addition of surfactants to enzymatic hydrolysis has been reported to enhance the hydrolytic potential of enzymes in bioconversion of lignocellulosic biomass to fermentable sugars. The objective of this investigation was to evaluate the effects of four non-ionic surfactants (PEG4000, PEG8000, TritonX-100, and Tween 20) on the efficiency of enzymatic hydrolysis of steam-pretreated poplar using a commercial cellulase preparation (Cellic® CTec2). Statistical discriminant analysis at four variable factors (surfactant type, surfactant concentration, hydrolysis time, and substrate consistency) revealed that enzymatic hydrolysis was significantly enhanced in presence of PEG4000, with 19.2% increase in glucose yield over control without surfactant, whereas ANOVA test indicated substrate consistency and hydrolysis time as the most significant factors ($P<0.05$). Hydrolysis of poplar pulp at 5% w/w pulp consistency with CTec2 in presence of 1 % w/w PEG4000 produced the highest glucose yield of 58.5% after 96 h reaction time.

Keywords Enzymatic hydrolysis, HTec2 cellulase, Poplar pulp, Non-ionic surfactants, Fermentable sugars, Discriminant analysis

1. Introduction

Lignocellulosic biomass is the only renewable resource on Earth that holds the key to a sustainable production of fuels and chemicals without compromising human food security. Furthermore, plant biomass has the potential to significantly decrease and eventually substitute the use of oil-derived products of environmental concern, increase energy security and independence, and enhance rural economy [1].

Production of high-yield fermentable sugars from plant biomass is a prerequisite for the establishment of economically feasible bioconversion process to value-added products such as bioethanol, organic acids, enzymes, solvents, etc. For this reason, enzymatic hydrolysis of lignocellulosic biomass into soluble sugars has been extensively investigated [2-4]. The efficient production of sugars from cellulosic biomass is impeded by several factors [5]. The rapid decrease in hydrolysis rate with hydrolysis time leads to low yields and long processing times [6]. High cellulose conversion rates are normally attained at high enzyme dosages, which increases production costs [6-10]. In addition, enzyme recovery and reuse is problematic due to enzyme adsorption to residual lignocellulose and non-specific and non-productive binding to lignin, which results in enzyme activity loss [6,7]. Furthermore, the presence of sugar and lignin degradation products formed during biomass pretreatment may inhibit enzyme activities and hydrolysis efficiency [8, 9].

Several researchers have attempted to elucidate the factors responsible for the decreasing rate of cellulose hydrolysis [6,8, 9-11]. In the investigation of Pihlajaniemi et al. [9] on the rate-constraining changes in the enzymatic saccharification of wheat straw, the authors concluded that partial permanent activity loss due to irreversible non-productive enzyme binding may be

responsible for the declining hydrolysis rates. Irreversible binding of cellulases by lignin and steric hindrance of enzymes were pointed as the most likely causes for the gradual slowing down of the reaction rate during enzymatic hydrolysis of pretreated poplar and switchgrass. The other factors included substrate availability in a hydrolysis. Cellulose surface area decreased linearly with hydrolysis, in correlation with total cellulose content, hence the hydrolysis efficiency decreases corresponding to increasing hydrolysis time and cellulose content [12]. The presence of lignin in complex lignocellulosic substrates has been reported to exert a negative effect on enzymatic hydrolysis in several studies [12-14]

The use of additives, such as surfactants and polymers, has shown promise in improving the enzymatic digestibility of cellulose and hydrolysis yields [17-22]. Although the exact mechanism of the surfactant-substrate-enzyme interaction is not fully understood, it is believed that surfactants prevent the non-productive enzyme adsorption onto lignin, which in turn increases the amount of free enzyme available for cellulose saccharification [15,16]. The effects of different types of surfactants have been examined on cellulosic substrate hydrolysis by several researchers. Cao, Aita [17] studied the enzymatic hydrolysis and ethanol production of combined surfactant and diluted ammonia-treated sugarcane bagasse. The authors showed that PEG 4000 and Tween 80 gave the highest cellulose digestibility (62%, 66%) and ethanol yields (73%, 69%) as compared to the use of only dilute ammonia (38%, 42%) or water (27%, 26%) as catalysts, respectively. Similarly, an enhanced enzymatic hydrolysis of sugarcane bagasse with ferric chloride pretreatment and surfactant was developed by Zhang et al. [18]. A synergistic surfactant-assisted ionic liquid pretreatment of lignocellulosic waste improved the enzymatic hydrolysis by 21% upon surfactant addition [19]. In another study, applying a surfactant-mediated ionic liquid, the rate of enzymatic hydrolysis was significantly increased and 12.5%

more lignin was removed from sugarcane bagasse using surfactants as compared with ionic liquid alone Nasirpour et al. [20].

The type of surfactant, substrate and enzyme all influence the enzymatic hydrolysis of lignocellulose biomass, and improved hydrolysis rates and yields in presence of surfactants may be attributed to one or most likely a combination of synergistic effects such as: a) surfactant effect is higher at low cellulase concentration [10]; b) surfactants adsorb at the air–liquid interface and increase enzyme stability thus protecting enzymes from possible denaturation during hydrolysis [21-23], c) surfactants prevented enzyme inactivation and facilitate enzyme desorption from substrate [24,25], d) surfactants promote availability of reaction sites which leads to increased hydrolysis rates [19,15], e) adsorption of enzymes to cellulose during hydrolysis decreases in presence of surfactants [24], f) surfactants assists in increase of the available cellulose surface and/or removal of inhibitory lignin [26].

Henceforth, the aim of this study was to evaluate the effectiveness of different types of non-ionic surfactant in enzymatic hydrolysis of poplar pulp (PP). We attempted to (i) enhance glucose yields from PP by addition of surfactants to enzymatic hydrolysis; (ii) to better understand the relationship between significant factors that influence the efficiency of enzymatic hydrolysis.

2. Materials and methods

2.1. Poplar pulp

Steam-pretreated PP was used as a substrate in the hydrolysis experiments. Poplar wood chips (8 kg) were soaked in water at 1:4 ratio (w/v) overnight and then drained through a sieve of mesh size 4 (pore/opening size of 4750 μm). The soaked chips were then loaded into a custom-made

pressurized percolation reactor and pre-steamed at 100°C for 60 min, followed by cooking with saturated steam at 170°C for 120 min. To adjust temperature, the purging steam was frequently discharged from the reactor, condensed and collected (liquid purge). At end of the cooking, the pressure of the reactor was instantly released to the atmosphere through a discharge valve, which caused fibrillation of the wood chips. The fibre-like biomass was then pressed in a custom-made hydraulic press cylinder at 3,000 psi through a sieve of mesh size 80 (pore/opening size of 180 µm) for 25 min until the liquid (prehydrolysis liquor) in biomass was completely drained. The steam-pretreated PP was stored at 4°C until use.

2.2. Chemical composition

The carbohydrate composition of PP was determined according to procedures described by Sluiter et al [27]. An automated moisture analyzer (Sartorius MA37-1, Goettingen, Germany) was used to measure the total solid content of PP. In order to determine the monomeric sugar composition, 0.3±1g of moisture corrected sample was weighed into a clean screw capped test tube, and to that, 3 ml of 72% w/v H_2SO_4 was added. The well-mixed sample was hydrolyzed at 30°C for 2 h with intermediate mixing. After 2 h of initial hydrolysis, 84 mL of deionized water was added and the sample was autoclaved at 121°C for 1 h. Thereafter the sample was cooled down to room temperature and filtered through a Whatman 0.45 µm filter paper. The solid residue collected on the filter paper was used for ash and acid insoluble (Klason) lignin determination. The supernatant was neutralised to pH 5.5-6.0 with $CaCO_3$ and filtered through a Whatman 0.2µm filter paper prior to sugar analysis. The cellulose content of PP was calculated as % of total biomass dry weight according to Eq. 1:

$$Cellulose\ (\%) = \frac{glucose\ content}{poplar\ pulp\ dry\ weight} \times 0.9 \times 100 \quad (1)$$

where *glucose content* is the amount of glucose (g) released from poplar pulp upon complete acid hydrolysis of PP, and *poplar pulp dry weight* is the amount of dry (water-free) poplar pulp (g) used for the determination. 0.9 is the conversion factor for glucose to cellulose.

2.3. Surfactants

Four non-ionic surfactants were used in this study: polyethylene glycol PEG8000 (Fisher Scientific, NJ, USA), polyethylene glycol PEG4000 (Alfa Aesar, MA, USA), polyethylene glycol octyl phenol ether, TritonX-100 (Sigma Aldrich, MO, USA), and poly(oxyethylene)20 sorbitan monolaurate, Tween20 (Sigma Aldrich, MO, USA).

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of PP was carried out with a commercial cellulase preparation Cellic(®) CTec2 (Novozymes A/S, Bagsvaerd, Denmark). Cellic(®) CTec2 was used according to the manufacturer recommendations under optimum pH of 5.0 and temperature of 50°C. The enzyme loading applied on PP was 5% (w/w). The total reaction volume was 150mL with 7.5g of total solid. The enzyme loading and the surfactant concentrations were calculated based on the equitation's 2 and 3. PP was enzymatically treated at a pulp consistency of 5, 10, and 15 % (w/w) for up 96 h. pH was adjusted to 5.0 using 2 M sodium hydroxide. The impact of surfactants on enzymatic hydrolysis was studied as described by Börjesson et al. [23]. Following pH and consistency adjustment, surfactants were mixed with PP at three concentrations of 1, 5 and 8 % (w/w) 24 h before addition of CTec2 cellulase. To facilitate mixing during enzymatic hydrolysis, glass beads were added to each flask (250 ml) and samples (each containing 15 g dry

weight of PP) were placed in a shaking incubator (Innova 44, Maine, USA) at 200 rpm. All samples were analyzed in triplicate.

$$Enzyme\ \ volume = \frac{Substrate\ dry\ weight \times Enzyme\ \%}{Enzyme\ density\ \left(1.2\frac{g}{ml} for\ CTec2\right)} \tag{2}$$

$$Surfactant\ percentage = \frac{Substrate\ dry\ weight \times Surfactant\ \%}{surfactant\ density} \tag{3}$$

Where substrate dry weights is the total biomass added and the enzyme density is the weight of 1ml of Novozymes CTech 2 cellulose preparation. In the case of enzyme and surfactant addition total, dry biomass was considered as 100%

2.4.1. Sugar analysis

Following enzymatic treatment of PP, enzymatic hydrolyzates were analyzed for glucose in a 1200 Series High-Performance Liquid Chromatography (Agilent Technology, Toronto, Canada) using a refractive index detector (RID) and an Aminex HPX-87H column (300 x 7.7). For elution, 5 mM sulfuric acid was used as an isocratic eluent at a flow rate of 0.5 mL/min. The column and RID temperature were maintained at 60°C and 35°C, respectively.

2.4.2. Glucose yield

Glucose yield (%) was calculated as follows:

$$Glucose\ yield\ (\%) = \frac{glucose\ released}{initial\ glucose} \times 100 \tag{4}$$

where *glucose released* is the amount of glucose (g) produced by enzymatic hydrolysis of PP, and *initial glucose* is the amount of glucose (g) contained in PP prior to enzymatic hydrolysis.

2.4.3. Statistical analysis

A full factorial design was used to verify the effects of the surfactants on the conversion of PP to glucose. Statistical analysis was carried out to investigate the effect of individual factors, leverage and desirability using JMP® (Statistical Analysis Systems, Version 13.0.0, SAS Institute

Inc., Cary, NC, USA). Discriminant analysis was used for the classification of the surfactant types using canonical variate analysis (CVA) [28]. To predict a suitable surfactant that could best enhance enzymatic hydrolysis of PP, a discriminant analysis of all four surfactants was carried out using surfactant concentration, reaction time, and substrate consistency. Fischer test (F-value) and probability (P-value) were applied to determine the significance of the model and individual parameter. F-value was determined to compare the variance of the corresponding term with the residual variance, and was estimated as mean square of the term to the mean square of variance. P-value of any term is the probability of getting F-value of a size in which a P-value of less than 0.05 ($P<0.05$) is considered to have a significant effect, whereas $P>0.05$ is deemed insignificant.

3. Results and discussion

3.1. Chemical composition of poplar pulp

The chemical composition of pretreated PP is presented as % of the total dry weight of substrate in Table 1. The PP biomass constituted 81.6% polysaccharides (cellulose and hemicellulose). Assuming all glucose was derived from hydrolysis of the cellulose fraction, the cellulose content of poplar pulp was 66.1%. According to the literature [29], untreated poplar wood contains from 42.2 to 47.5% w/w cellulose, which suggests that the steam pre-treatment applied in this work was successful in enriching the carbohydrate, in particular, cellulose content of biomass. The high sugar content, of which nearly 90.5% was glucose (comparing the total reducing sugars), suggests that PP is a suitable feedstock for biorefinery applications [30].

Table 1 Carbohydrate composition of poplar pulp

Component	% w/w
Glucose	73.9±2.8
Xylose	5.6±0.7
Arabinose	2.1±0.2

3.2. Effect of hydrolysis time

Four non-ionic surfactants were evaluated for their ability to enhance enzymatic hydrolysis of PP The surfactants are described to have several advantages; 1) it can extract and block lignin and hemicellulose degradation products, 2) it protects the enzyme from denaturation at high temperature, 3) improved electrostatic interaction between surfactant monomer or micelles and enzyme causes an enhanced enzyme activity, and 4) reducing the surface tension and viscosity of liquid that in turn diminishes the contact of enzyme with air-liquid interface. In the present investigation, four different surfactants based on their ethylene oxide (EO) group was used to evaluate the hydrolysis (PEG4000/8000 10 -180 EO units and Tween80 and TritonX100 10 -19 EO units)[37]. The efficiency of the enzymatic hydrolysis was evaluated based on the glucose yield obtained after 24, 48, 72, and 96 h of hydrolysis time (Fig. 1). As evident from Fig. 1, a nearly linear increase in glucose yield with time was observed with or without surfactant and irrespective of the surfactant type. For example, the glucose yield obtained after enzymatic hydrolysis of PP for 96 h in absence of surfactant (control) was 39.3% w/w. In presence of 1% of PEG4000, the glucose yield increased from 39.6% (at 24 h) to 58.5% (after 96 h). Similarly, in presence of 1% Tween80, the glucose yield increased 17.1% when hydrolysis time was prolonged from 24 h to 96 h. Furthermore, all surfactants improved the glucose yield over the

control to a different extent depending on the surfactant type. Best results were obtained with PEG4000, PEG8000 and Tween80, which improved the actual glucose yield by 19.2%, 14.1% and 12.8%, respectively, over control after 96 h. In contrast, the sugar yield improvement with TritonX-100 was only marginal compared to control (Fig. 1). The positive effect of surfactant addition could be due to the prevention of the non-specific binding of cellulose to lignin and other cell wall structures [31,32]. An early study on PEG4000 reported a 59% improvement in cellulose conversion [33]. The addition of PEG4000 increased the concentration of free enzyme in solution up to 96% and lowered the extent of protein binding on lignin. Elsewhere, the addition of Tween80 was reported to improve the sugar (hexose) of steam-exploded reed by 91%, with 1.7-fold improvement in the overall hydrolysis [26].

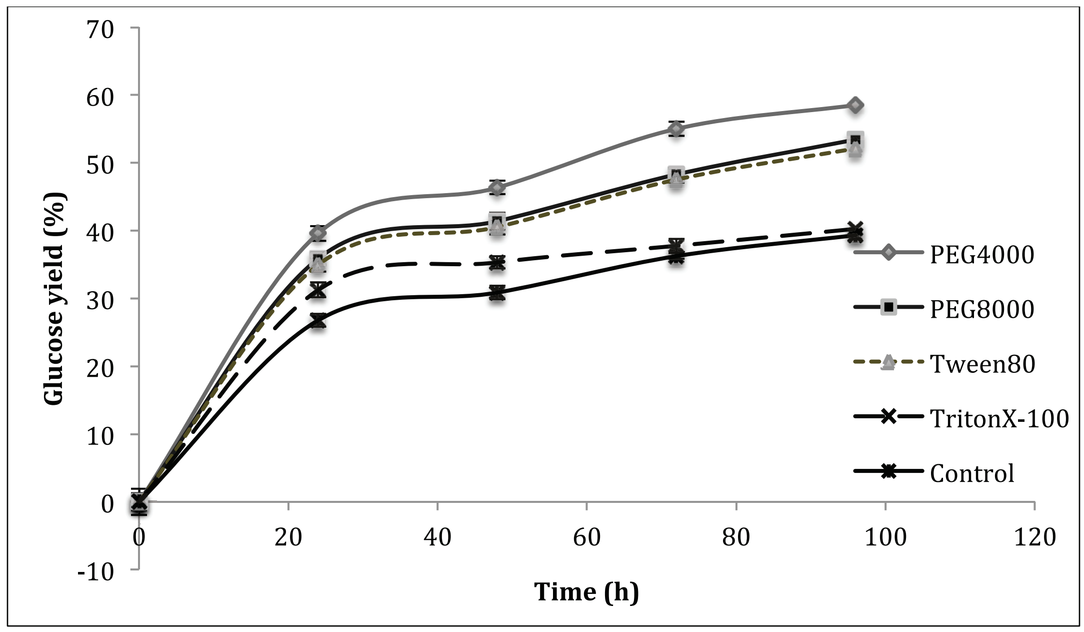


Fig. 1 Impact of hydrolysis time on glucose yield following enzymatic hydrolysis of poplar pulp at 5% w/w substrate consistency and 1% w/w surfactant concentration

As demonstrated here, higher sugar concentrations could be attained at longer hydrolysis times. However, it is known that processing times directly impact the overall process cost. Alongside, under prolonged residence times, high glucose concentrations may reversibly affect

the effectiveness of enzymatic hydrolysis by feedback inhibition [33]. Moreover, the residence time of enzymatic hydrolysis could be dependent upon the biomass composition and structure, reaction conditions and availability of free enzyme [34]. Hence, a proper optimization of resident time is an essential factor for enhancing the yield of fermentable sugars from lignocellulosic biomass.

3.3. Effect of surfactant concentration

The effect of surfactant concentration on hydrolysis efficiency was evaluated and compared with the control conditions (Fig. 2). As stated above, at 1% w/w surfactant concentration, enzymatic hydrolysis in presence of PEG4000 produced the greatest glucose yield of 58.5% whereas the lowest sugar yield of 40.3% was observed in presence of 1% w/w TritonX-100. However, with the exception of TritonX-100, the increase in the surfactant concentration to 5% and 8% did not lead to any further improvements in the glucose yield, and in fact, a decrease in the sugar release from PP was observed (Fig. 2). This decrease was more evident at 8% w/w surfactant concentration than 5% w/w, suggesting that the surfactant effect on enzymatic hydrolysis is both surfactant type and concentration dependent. Our results suggest that increased surfactant concentrations can lead to enzyme inhibition by denaturation and loss of enzyme activity, which negatively affects the cellulose conversion efficiency.

Literature reports on surfactant impact on hydrolysis are controversial. Zhang et al. [35] reported a positive correlation in reducing sugar concentration with surfactant (PEG4000) concentration up to an equilibrium point at a surfactant concentration at which the non-productive sites in biomass had been surfactant-counteracted. Further increase in surfactant concentration did not improve sugar yields but caused a decline in the hydrolysis efficiency

instead. Another study [36] reports a significant increase in sugar yield (by 57.5%) in presence of TritonX-100 at higher concentrations (2-5%), which is in agreement with our results. This suggests that the complex biomass-enzyme-surfactant interaction is influenced by the surfactant type and concentration, among others. More specifically, non-ionic surfactants containing ethylene oxide groups such as PEG may be more advantageous in lignocellulose hydrolysis as both the hydrophobic and hydrophilic groups of PEG can interact with the phenyl and methyl groups of lignin to create a hydrated layer on lignin which prohibits protein (enzyme) binding on lignin [37]. It is demonstrated that surfactants can significantly increase the concentration of free (non-bound) enzyme available for a hydrolytic reaction [24,20]. For example, Eriksson et al. [32] reported that surfactants enabled a two-fold increase in the free enzyme compared to control.

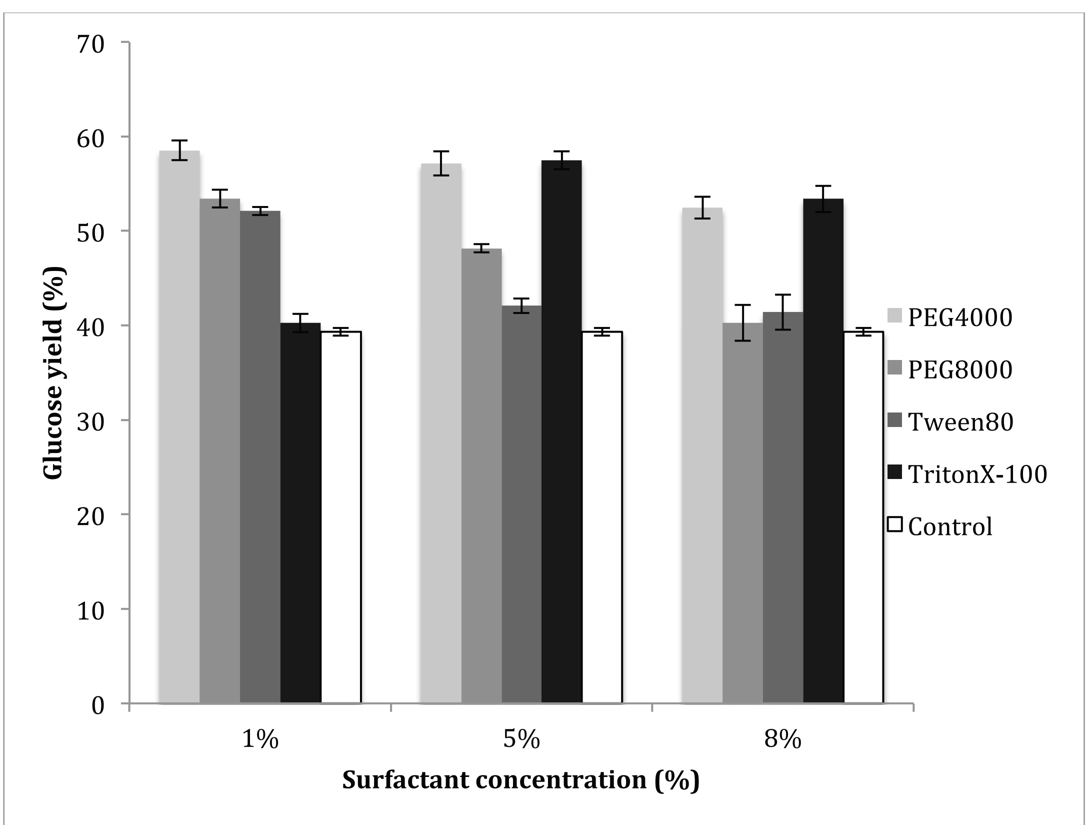


Fig. 2 Impact of surfactant concentration on glucose yield following enzymatic hydrolysis of poplar pulp at 5% w/w substrate consistency for 96 h

3.4. Effect of substrate consistency

Substrate consistency is another factor in enzymatic hydrolysis of lignocellulosic biomass which directly affects the sugar yields increasing the substrate/solids concentration improves the overall sugar concentrations/titers. However, the substrate consistency is a dependent parameter, which could be influenced by the amount of free enzyme and surfactant in the hydrolysis reaction.

In the current study, the effect of substrate (PP) consistency was evaluated (Fig. 3) at three different levels (5, 10 and 15% w/w) with varying surfactant concentrations (1, 5 and 8% w/w). As noted, the highest hydrolysis yield was obtained with PEG4000 at 5% w/w PP consistency. The increase in substrate consistency from 5 to 10 and 15% w/w significantly affected the sugar yield. For example, at 10% w/w PP consistency and 1% w/w PEG4000 concentration, an 18.4% drop in sugar yield over control was detected (Fig. 3b). The cellulose to glucose conversion efficiency at 15% w/w solids was significantly lower (2.2-fold) than 5% w/w substrate consistency. With PEG4000, over 50% loss in glucose yield was noticed at 15% w/w compared to 5% w/w solids (Fig. 3c). However, increasing the PEG8000 concentration from 1 to 5% w/w with 15% substrate loading resulted in 13.8% improvement in sugar yield, compared to 1% w/w surfactant dose. Similarly, with all other surfactants, a slight improvement in glucose yield was attained when the surfactant concentration was proportionally increased to the substrate solids (Fig 3a,b,c). Interestingly, compared to 1% w/w surfactant concentration and 5% w/w solids (Fig 3a), a significant increase in the glucose yield of 57.5% was recorded with TritonX-100 at 5% w/w surfactant concentration (Fig. 3b). This could be explained by the effect of formation of hydrated "micelles " in presence of phenolic compounds (such as TritonX-100) that have the affinity to bind to lignin as a highly branched phenolic polymer which results in the release of

enzyme non-specifically bound to the lignin surface [38]. Accordingly, earlier studies on substrate concentration revealed a similar result [33,39,40]. The factors associated with substrate concentration that affects the sugar yield include product inhibition, pre-treatment inhibitors, lignin, mass transfer and non-productive adsorption of enzymes. Increase in substrate consistency negatively impacts mass transfer (mixing) and leads to increased sugar and lignin concentrations that can cause inhibition and prohibit efficient cellulose conversion.

Our results agree well with the fact that increasing the surfactant concentration with the substrate loading reduces the non-productive enzyme binding, which leads to improved sugar yield [37]. In other words, surfactant binds hydrophobically with lignin, which facilitates enzyme desorption from non-productive sites.

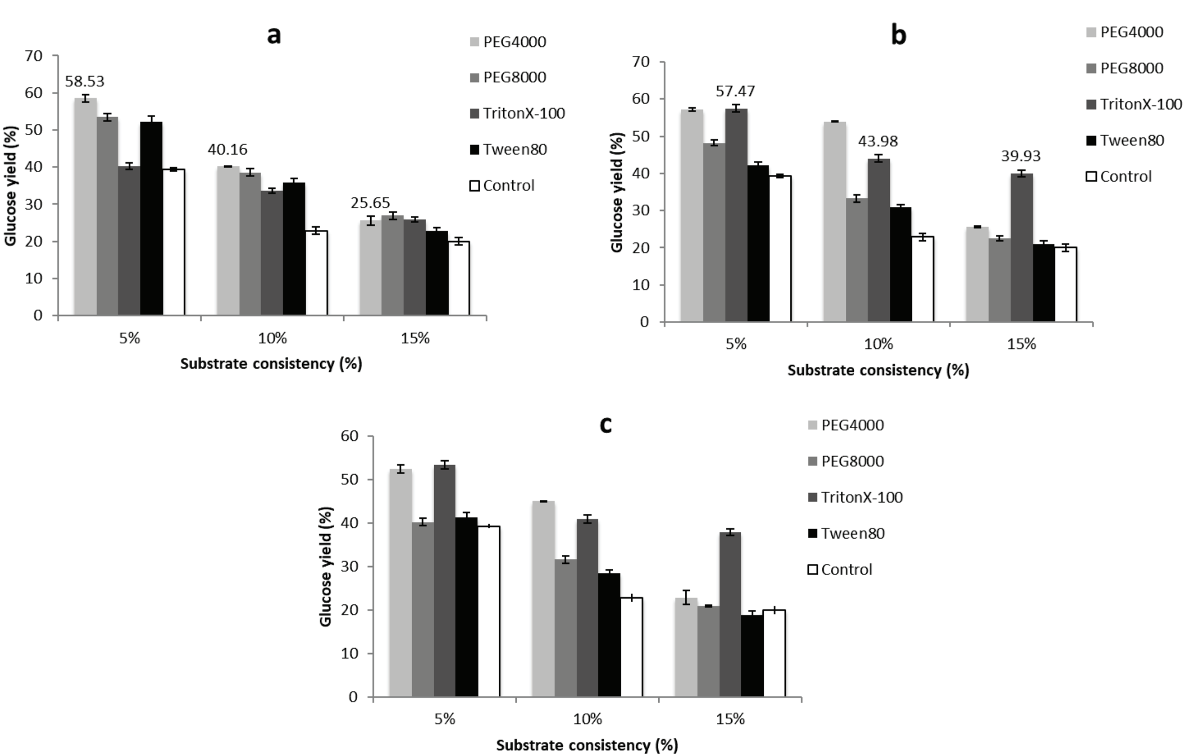
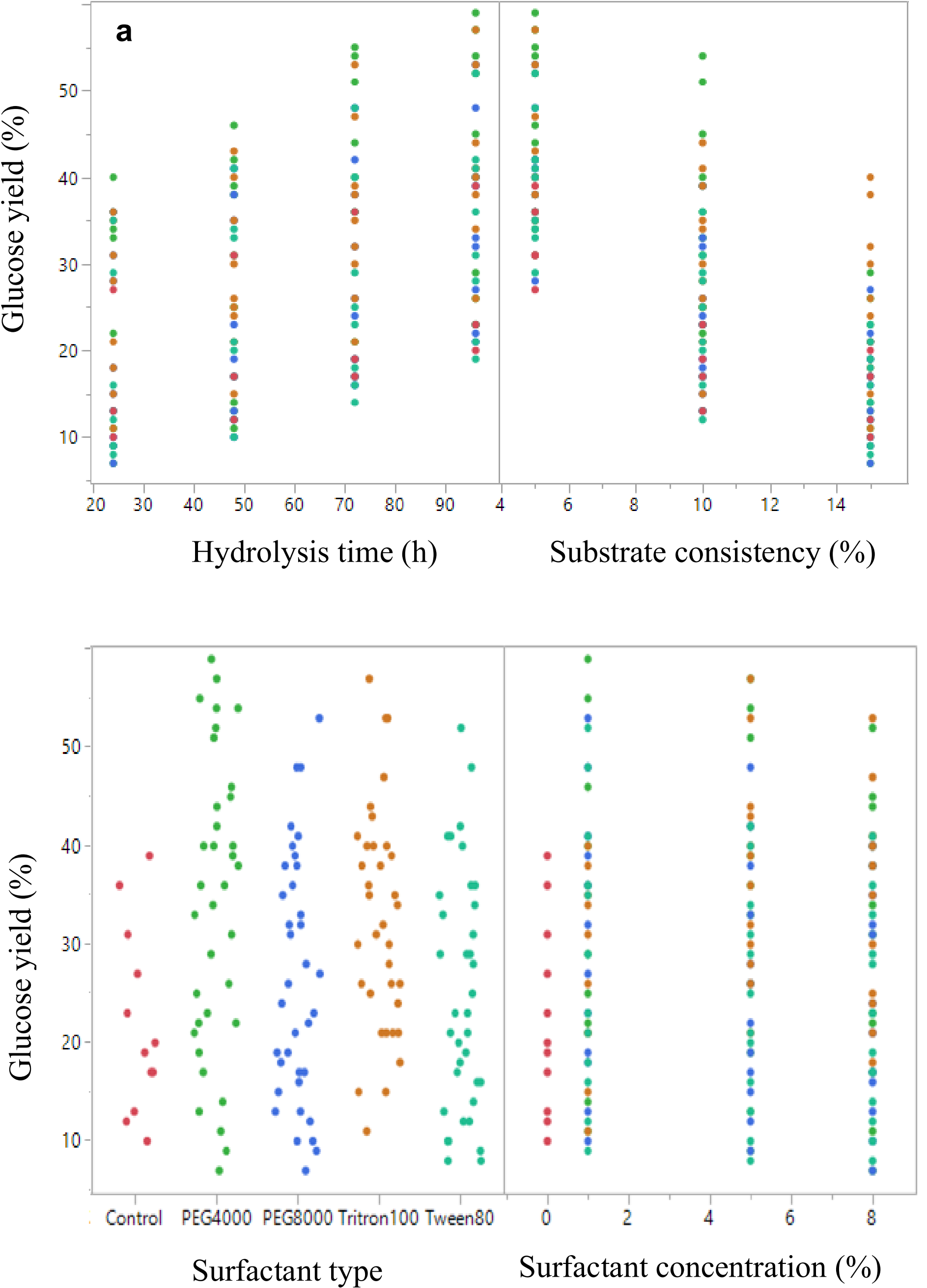


Fig. 3 Impact of substrate consistency on glucose yield following enzymatic hydrolysis of poplar pulp for 96h at surfactant concentration of **a** 1 % w/w, **b** 5 % w/w, **c** 8 % w/w

3.5. Discriminant analysis of surfactant impact

The substrate consistency, reaction time, surfactant type, and surfactant concentration were used as variable factors to statistically classify the best surfactant suitable for high glucose yield from PP hydrolysis. This analysis equally provided information on the hierarchy of level of significance of each of the parameters on glucose production from PP. A total of 156 lines of data that comprised all the selected parameters were used for the discriminant analysis. Stepwise variable selection model was employed from JMP 13.0, a statistical analysis software for the data classification and analysis. Fig. 4 shows the scatterplot matrix and discriminant analysis results used to determine the best surfactant suitable for the high conversion of PP to fermentable sugars. From Fig. 4a, the scatterplot of the surfactants type revealed that PEG4000 showed the highest influence on the conversion of PP to soluble sugar. It was also confirmed that the glucose yield increased with reaction time and decreased with substrate consistency. The scatterplot equally revealed a decrease in the conversion of PP with an increase in the surfactant concentrations. CVA has the capacity to classify the product effect in the two-way (product and subject) multivariate ANOVA model, which is the natural extension of the classical univariate approach consisting of ANOVA of every attribute [41,28].


a
Glucose yield (%)
Hydrolysis time (h)
Substrate consistency (%)
Glucose yield (%)
Control
PEG4000
PEG8000
Tritron100
Tween80
Surfactant type
Surfactant concentration (%)

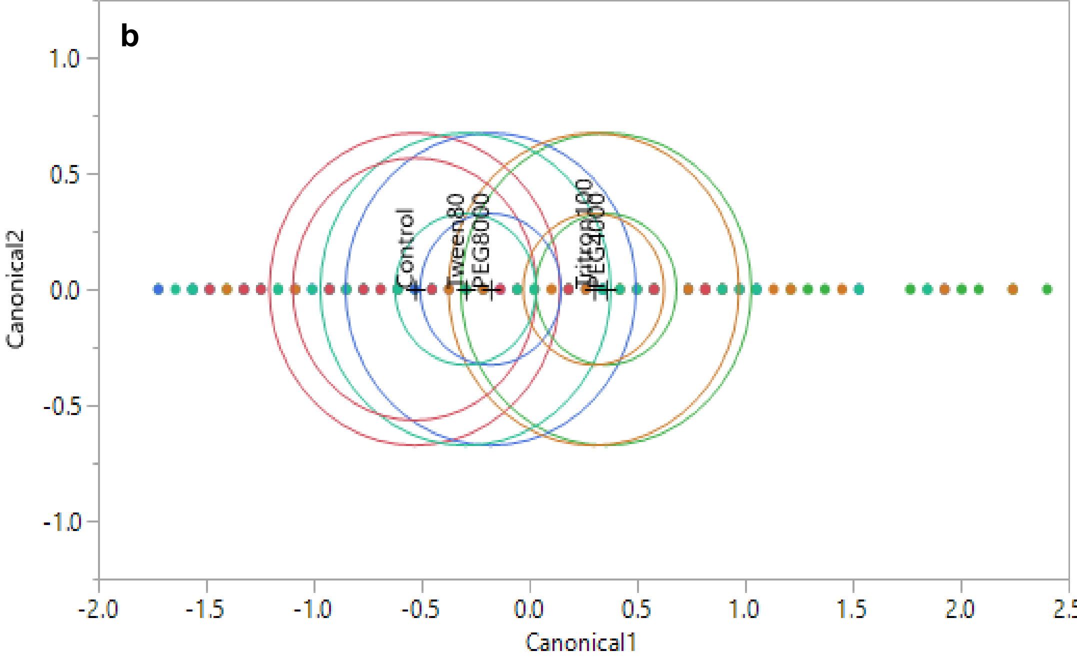


Fig. 4 Discriminant analysis of surfactant effect on enzymatic hydrolysis of poplar pulp under different conditions of hydrolysis time, substrate consistency, surfactant type and concentration **a** scatterplot matrix, **b** discriminant analysis of surfactant effect

Fig. 4b displays the discriminant results of the effect of surfactants on the enzymatic hydrolysis of PP. PEG4000 was found to have the highest positive impact on the hydrolysis of PP to glucose, followed by TritonX-100. This finding was based on the combined effects of all the selected parameters. TritonX-100 produced better results at 5% surfactant concentration and 10% substrate consistency (Fig.4b). The control had the highest negative effect on the conversion of PP to glucose, which means that the addition of surfactant significantly improved the hydrolysis process. Similar results were reported by Jin et al. [26] in their investigation of the effects of Tween80 to enhance enzymatic saccharification of steam-exploded biomass and ethanol production by lessening cellulase absorption with lignin in common reed. The authors reported that Tween80 specifically blocks lignin absorbing with cellulase for high biomass

digestion. A ranking screening of the parameters involved in the discriminant analysis of the surfactant effects was presented in Table 2. Surfactant type was ranked third out of four while surfactant concentration was the least significant parameter in the ranking. The substrate consistency was ranked as the most important parameter for enhancing the glucose yield from PP biomass. High substrate consistency requires the use of high surfactant concentrations to limit the negative impact of non-specific enzyme bonding to lignin and possible enzyme inhibition. Further analysis of multivariate pairwise comparison of the three discrete variables with the soluble sugar yield from PP was performed (Table 3). Pairwise comparison between sample consistency and reaction time with the glucose yield was found to significantly ($P<0.05$) influence the conversion of PP to glucose. From the analysis of variance (ANOVA), surfactants significantly ($P<0.05$) influenced the conversion of lignocellulosic biomass to soluble sugars. The contribution of each surfactant to the conversion of lignocellulosic biomass (PP) declined in the following descending order: PEG4000 > Tween80 > PEG8000 > TritonX-100.

Table 2 Effects of predictor screening of parameters on glucose yield

Predictor	Contribution	Portion	Rank
Substrate consistency	2321.35	0.4844	1
Time	1637.47	0.3417	2
Surfactant type	760.22	0.1586	3
Surfactant concentration	72.86	0.0152	4

Table 3 Multivariate pairwise comparison of variables

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
Surfactant concentration	Glucose yield	0.0189	156	-0.1387	0.1755	0.8153
Pulp consistency	Glucose yield	-0.7374	156	-0.8017	-0.6564	**<.0001***
Pulp consistency	Surfactant concentration	0.0000	156	-0.1571	0.1571	1.0000
Time	Glucose yield	0.4802	156	0.3495	0.5927	**<.0001***
Time	Surfactant concentration	0.0000	156	-0.1571	0.1571	1.0000
Time	Sample	0.0000	156	-0.1571	0.1571	1.0000

4. Conclusion

The current investigation highlighted the importance of surfactants as additives to enzymatic hydrolysis that can enhance the efficiency of cellulose conversion and yields of fermentable sugars for biorefinery applications such as the production of biofuels and biochemicals. Addition of surfactants (PEG4000) proved efficient at concentrations as low as 1% w/w, and their effects on glucose yield were both surfactant type and concentration dependent. Enzymatic saccharification of poplar pulp for 96 h was significantly enhanced in presence of PEG4000, yielding 58.5% glucose which translates into 19.2% increase in glucose yield over control without surfactant. The discriminant analysis employed in this study appears as a useful tool for classifying, identifying, and discriminating the impact of different surfactants on glucose production from biomass.The most significant factors ($P<0.05$) that can positively influence the outcome of enzymatic hydrolysis were a substrate (poplar pulp) consistency and reaction time. Overall, with exception of TritonX-100, the increase in the surfactant concentration and substrate consistency reduced the glucose yield from poplar pulp. At a given glucose yield, the use of surfactants in enzymatic hydrolysis of lignocellulosic biomass would allow reduction of the

enzyme (cellulase) dosage and potentially improve the economics of any biorefinery process that requires second-generation fermentable sugars as feedstock. Work is underway to optimize the amounts of PEG4000 and cellulase for the establishment of a cost-effective process for sugar production from currently underutilized biomass sources such as poplar.

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Chapter 4

CONCULSIONS AND FUTURE WORK

The objective of this work was to examine enzymatic hydrolysis as a tool to utilize a currently underutilized biomass source (poplar) for sustainable production of sugars (glucose) as the feedstock for numerous biorefinery applications. Providing an inexpensive source of glucose is the key to unlocking the enormous potential of lignocellulosic biomass for the establishment of Integrated Biorefineries as a major pillar for the emerging Bioeconomy. Lignocellulosic biomass is the only renewable resource on Earth that can be utilized for sustainable production of fuels and chemicals without compromising human food security. Furthermore, plant biomass has the potential to significantly decrease and eventually substitute the use of oil-derived products of environmental concern, increase energy security and independence, and enhance rural economy.

Chapter 1 provided an extensive and critical review of recent literature, with 80 most recent references on the topic, emphasizing sugar production from plant biomass by enzymatic hydrolysis, composition of biomass, pretreatment methods, drawbacks and opportunities for enhancing sugar yields from biomass, and ways to make enzymatic hydrolysis more cost-efficient. The review points out to the fact that more research is needed to address and overcome challenges related to process economics that currently prevent large-scale biorefinery applications. In this context, enzyme cost is one of the major bottlenecks in the biomass valorization to value-added products.

In Chapter 2, the impact of critical parameters of enzymatic hydrolysis on enzymatic saccharification of poplar pulp to glucose using a commercial enzyme product (CTec2, Novozymes) was investigated. It was found that prewashing of biomass had no effect on enzymatic hydrolysis. However, adjusting the pH of the substrate to pH 5, as compared to the

initial substrate pH of 3, improved hydrolysis efficiency. The optimum biomass and enzyme loadings, based on the maximum sugar yield obtained from poplar pulp (41.87% w/w), were both 5% % w/w. However, hydrolysis efficiency was reduced by 19.3% at 15% substrate consistency. The substrate and enzyme consistency and hydrolysis time were identified as significant factors in hydrolysis. The higher the substrate consistency the lower the enzymatic hydrolysis yield of glucose from poplar pulp. On other hand, increasing the enzyme loading resulted in increased pulp to sugar conversion. For example, increasing the enzyme loading from 1% to 10% resulted in a 2.1-fold increase in sugar yields. The substrate recycling was found to produce additional amounts of glucose which further increased hydrolysis efficiency, although sugar yields declined two-fold after two consecutive recycling runs. The reduction in the glucose yield was most likely due to reduced availability of easily hydrolysable substrate in poplar pulp, and accumulation of lignin, which is a known inhibitor of enzymatic and fermentative processes. However, recycling of the substrate provides the opportunity to more efficiently utilize the substrate for glucose release, and reuse some of the substrate-bound enzyme, which may lead to use of lower enzyme dosages. This approach has the potential to enhance the cost-efficiency of enzymatic hydrolysis and will be further optimized for developing an economically-viable process of sugar production from renewable, low-cost biomass sources.

In Chapter 3, the effect of surfactants addition to enzymatic hydrolysis to enhance the hydrolytic potential of enzymes in bioconversion of lignocellulosic biomass to fermentable sugars was investigated. Addition of surfactants to enzymatic hydrolysis has been reported to enhance the hydrolytic potential of enzymes in bioconversion of lignocellulosic biomass to fermentable sugars. The objective of the work was to evaluate the effects of four non-ionic surfactants (PEG4000, PEG8000, TitronX-100, and Tween 20) on the efficiency of enzymatic

hydrolysis of steam-pretreated poplar using a commercial cellulase preparation (Cellic® CTec2). Statistical discriminant analysis at four variable factors (surfactant type, surfactant concentration, hydrolysis time, and substrate consistency) revealed that enzymatic hydrolysis was significantly enhanced in presence of PEG4000, with 19.2% increase in glucose yield over control without surfactant, whereas ANOVA test indicated substrate consistency and hydrolysis time as the most significant factors ($P<0.05$). Hydrolysis of poplar pulp at 5% w/w pulp consistency with CTec2 in presence of 1 % w/w PEG4000 produced the highest glucose yield of 58.5% after 96 h reaction time. This chapter highlighted the importance of surfactants as additives to enzymatic hydrolysis that can enhance the efficiency of cellulose conversion and yields of fermentable sugars for biorefinery applications such as production of biofuels and biochemicals. Addition of surfactants proved efficient at concentrations as low as 1% w/w, and their effects on glucose yield were both surfactant type and concentration dependent. The discriminant analysis employed in this study appears as a useful tool for classifying, identifying, and discriminating the impact of different surfactants on glucose production from biomass. The most significant factors ($P<0.05$) that can positively influence the outcome of enzymatic hydrolysis were substrate (poplar pulp) consistency and reaction time. At a given glucose yield, the use of surfactants in enzymatic hydrolysis of lignocellulosic biomass would allow reduction of the enzyme (cellulase) dosage and potentially improve the economics of any biorefinery process that requires second-generation fermentable sugars as feedstock.

Finally, this study also paves the way for further related research on the topic of cost-effective production of fermentable sugars from lignocellulosic biomass: 1) better understanding of the mechanisms of surfactant action and interaction with biomass during enzymatic hydrolysis; 2) optimization of surfactant and enzyme doses; 3) optimization of substrate

recycling; 4) investigation into enzyme recycling and optimization of enzyme recycling; 5) optimization of the combined effects of surfactant, substrate and enzyme recycling for maximal sugar production at minimal production costs; 6) techno-economic analysis of the developed enzymatic hydrolysis process; 7) investigation of pathways to utilize the fermentable sugars generated through the developed enzymatic process for cost-effective production of biofuels and value-added biochemicals.