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Preprocessing of Biomass Using Mechanical, Chemical and Microbial Techniques

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PREPROCESSING OF BIOMASS USING MECHANICAL, CHEMICAL AND MICROBIAL TECHNIQUES

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ABSTRACT

Biomass-derived fuels have acquired a lot of attention recently due to increasing emphasis on energy independence, efforts to utilize abundance of green resources and mitigation of greenhouse gas emissions. Grasses, agricultural residues, animal residues and waste, used oils, etc., can be used as starting materials in the production of biofuels. Various preprocessing techniques used in the preprocessing of biomass, such as microbial preprocessing, mechanical preprocessing and chemical pretreatment, are used for enhancing the digestibility of biomass to sugars for ethanol production. In this work, studies were conducted to improve the microbial, chemical and mechanical preprocessing of switch grass by decreasing the treatment time, optimizing the pretreatment temperature and enzyme requirements, and by developing a mechanical method to identify the heterogeneous fractions of switch grass.

Switch grass was preprocessed with the fungus *Phanerochaete chrysosporium* and enzyme profiles were determined for various cellulase and lignin related enzymes. The enzyme profiles peaked at the 7^{th} day during the 28 day treatment. Following the enzyme profile results, a seven day enzyme hydrolysis of switch grass resulted in a 5 % w/w increase in total sugar yields and 5 % increase in glucan % w/w by composition, and decreased the treatment time fourfold when compared to previous literature.

A mechanical size separation method was developed for switch grass to identify the heterogeneous fractions in bulk and the pretreatment and enzyme requirements were estimated

for individual fractions using design of experiments. This study demonstrated that each fraction had different composition in terms of glucan, xylan and lignin, and had different pretreatment and enzyme requirements for hydrolysis. The recalcitrant fraction, <1 mm, was identified based on greater pretreatment and enzyme requirements, lower glucose yields and higher crystallinity, suggesting biomass enrichment by about 10 % through its elimination from unpartitioned switch grass.

Microbial preprocessing on size separated fractions of switch grass showed higher enzyme activity for >1 mm size fraction. The activity profiles varied by enzyme and by peak times during a 12 day preprocessing period for each of the fractions. Size separated fractions had lower glucose yields compared to the unpartitioned switch grass after microbial preprocessing. However, preprocessed samples had higher glucose yield compared to the raw samples for all fractions.

The studies, improved the glucose yield of switch grass through various preprocessing techniques, decreased the microbial preprocessing time, and identified the recalcitrant fraction of switch grass.

DEDICATION

This thesis is dedicated to my father, for his dream to see me as a doctorate and to my better half for his unending encouragement and support in achieving this dream.

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1. BACKGROUND AND LITERATURE REVIEW

1.1 Components of a Plant

Plants have a high potential for supplementing fuel production as they are an important source of cellulosic ethanol. Mean percentages of total glycans and lignin for corn stover, switch grass and poplar are 60 % and 20 %; 57 % and 23 %; 58 % and 29 % respectively [1]. Cellulose and hemicelluloses are the polymeric hexoses and pentoses respectively. The major sugars present in the polymeric carbohydrates are glucose, fructose, xylose, mannose, arabinose, galactose. These sugars in the polymeric form are associated with each other through H-bonds forming multiple layers in the plant cell wall. In plants like cotton, these fibres are extended to great distances with high strength and flexibility. Hemicellulose, forms multiple cross-linked network fibres with the cellulose using pectin as the binder.

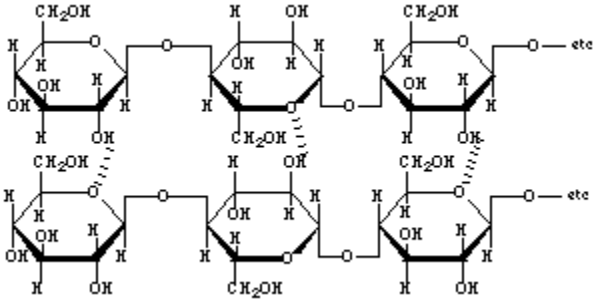

Figure 1. 1: Polymerized cellulose layers through H-Bonding

http://www.bio.indiana.edu/~hangarterlab/courses/b373/lecturenotes/cellwall/fig5.gif

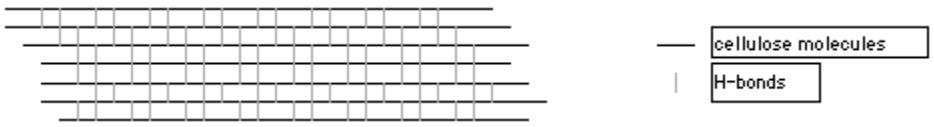

Figure 1. 2: Polymerized cellulose layers through H-Bonding

http://www.bio.indiana.edu/~hangarterlab/courses/b373/lecturenotes/cellwall/fig6.gif

Lignin, the most recalcitrant part of the plant, impedes conversion of sugars by fixing them physically in the structure. The sugars which are entangled in the structure are called structural sugars, while those not in the structural skeleton are called nonstructural sugars. Nonstructural sugars can be removed by extraction with water. Structural sugars are difficult to be removed by normal procedures as they have a shield called lignin. Lignin, in a way, protects these non-structural sugars of a plant from microbial and chemical attack. Lignin formation, the last step in the formation of plant cell wall after initial accumulation of carbohydrates, is the formation through dehydrogenative polymerization of p-coumaryl, coniferyl-CA- and sinaply alcohol [2]. Lignin as a biopolymer has several unusual properties as it is a heterogeneous compound lacking in a defined structure. Lignin in plants is present in different forms. Polyphenols which can be removed by organic chemical extraction procedures are a form of lignin present in plants. Its composition varies significantly from different plant types. The amount of lignin present contributes to the strength of the plant. Lignin content is developed with the growth of plant, thus imparting greater strength physically and against any microbial attack. Several parts of the plant have different compositions of lignin, for example, leaves of a tree have lesser lignin than its bark [3]. Therefore, plants with varying compositions of cellulose, hemicellulose and lignin exhibit varying properties, those having the higher cellulose,

hemicellulose or starch are used as feed stocks and those having higher lignin could be used for building purposes. However, the higher the content of lignin in forage plants the lesser digestible it is for the animals[4].

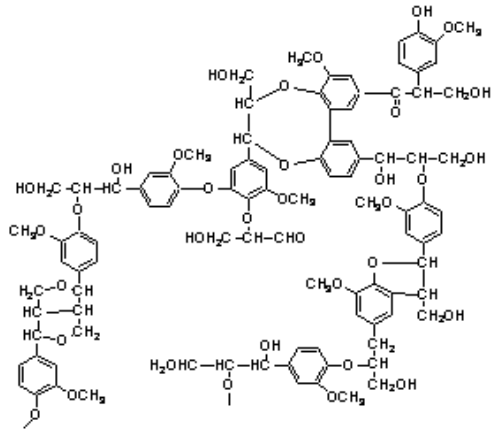

Figure 1. 3: A small section of a lignin polymer illustrating typical chemical linkages

http://www.lignin.org/01augdialogue.html

1.2 Energy Requirements

From a recent report by DOE, the official energy statistics from the US government, for the transportation fuel consumption, there was a 41 % increase of total fuel consumption, including natural gas and petroleum, from 1985 to 2005, which is a 2 % increase on average per year. There was an increase of 0.26 % from the 2005 to 2006(total consumption calculated for both the years until December). The demand for biofuels has escalated from 1985(statistics available from 1985) at a rate of 27.88 % increase of consumption per year until 2005, and an increase of 34.21 % from 2005-2006[5]. These figures depict a prominent development of the consciousness for the conservation of fossil fuels attributing it to an increase in demand for the renewable sources of energy for transportation.

With increasing demands for transportation fuel, renewable sources of energy have gained importance in the past years. Important fuel parameters are energy contents, combustion quality such as octane or cetane number, volatility, freezing point, toxicity and its adaptability to current combustion engines [6]. Biofuels such as hydrogen, methane, ethanol, butanol and biodiesel are of current interest in replacing (in partial or complete) gasoline to mitigate greenhouse gas emissions.

Table 1.1: Properties of various biofuels (adapted from sources [6] and http://en.wikipedia.org/wiki/Energy_density). *not required for lower blending.

	Hydrogen	Methane	Ethanol	Butanol	Biodiesel	Gasoline
Heat of Vaporization, KJ/Kg	451.9	760	920	430	2639.9	360
Energy Density, MJ/L	10.1 (liq)	0.0378	19.6	29.2	37.3	32.0
Research Octane Number	>130	135	129	96	>25	97-98
Air to Fuel Ratio	34	17.2	9.0	11.2	13.5	14.6
Freezing Point, °F	-435	-296.5	-173.2	-128.7	26-66	-40
Flash Point, Closed Cup, °F	-423	-306.4	55	84	212-338	-45
Solubility in Water, Volume %	-	-	100	9	Negligible	Negligible
Technology	Microbial	Microbial	Microbial	Microbial Chemical	Chemical Enzymatic	Chemical Physical
Status	Laboratory	Industrial	Industrial	Laboratory	Industrial Laboratory	Industrial
Engine Application	Blend Pure	Blend Pure	Blend Pure	Blend Pure	Blend Pure	N/A
Current Engine Modification	Required	Required	Required for Higher Blends	Not Required	Not Required*	N/A

Table 1.1 presents a comparative data for various fuels against gasoline and can be produced from biochemical conversion of biomass. Current working status of these fuels is also mentioned in the table 1.1. Among the fuels mentioned in the table, butanol and biodiesel (biodiesel from pure vegetable oils) can be used in existing gasoline and diesel engines respectively with little modification. For others, engine modification is required. For ethanol, lower blends in gasoline do not require engine modification. Use in higher blends requires engine modification. Engine modification is required for some non-gasoline fuels due to difference in their air-fuel ratio, latent heat of evaporation and corrosiveness. Air-fuel ratio of gasoline is 14.6 kg air for 1 kg of fuel. However, 10 % v/v ethanol blend of gasoline has 3.5 % w/w oxygen in the fuel which influences the air-fuel ratio at which the engine performs. Engine management systems in modern vehicles adjust the air-fuel ratio to maintain the stoichiometric oxygen in the fuel. Absence of engine management system or use of higher blend gasoline/biodiesel alters the air-fuel ratio, therefore requiring engine modification. Ethanol and biodiesel have higher latent heat of evaporation compared to gasoline, which may present difficulties with starting in cold conditions. To avoid cold start difficulties, vehicles require a small tank fitted to accommodate gasoline to initiate combustion. Moreover, viscosity of biodiesel increases during cold conditions requiring alternative starting methods for vehicles using higher blends of biodiesel. Higher blends of ethanol are known to be corrosive on fuel lines and tank therefore vehicles using 20 % v/v ethanol blend gasoline, require to have nickel plated steel fuel lines and tank.

1.3 Biomass

Until the 1970s, the idea of agricultural residues such as corn stover and grasses such as switch grass, giant miscanthus, sorghum sudan grass being potential sources of lignocellulosic

ethanol was not well recognized. The crises during the 1970s and 1980s have been one of the major reasons for such a breakthrough, where potential alternatives for fossil fuels and engines were investigated. These include Hybrid Electric Vehicles (HEV) and compressed natural gas, hydrogen fuel cell and biomass fuels [7]. Biomass fuels are the most cost effective alternatives to date in spite of facing criticism, often erroneously, for an unfavorable net energy balance, and significant arable land and water requirements[8]. But biofuels have proved to significantly reduce the CO_2 emissions[9].

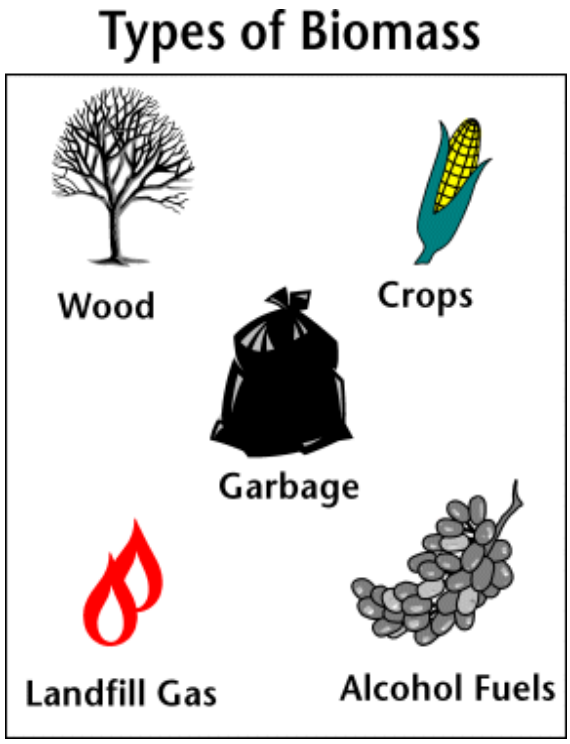

Figure 1. 4: Types of biomass

Biomass represents all materials derived from plant, animal and microbial origins. Classification of biomass used in conversion to biofuels, may be based on the origin (plant/animal), carbon source (woody/herbaceous) and physical and chemical characteristics. However, biomass from plant origin is considered highly desirable for its abundance and

potential to mitigate emission of greenhouse gases. Carbohydrate monomers in plants are formed through photosynthesis, in which the atmospheric carbon dioxide is converted by sunlight to chemical energy. Moreover, the same amount of carbon dioxide is released, when biomass-derived fuels for energy are used, as taken up during the plant growth using sustainable means, therefore, production of more biomass, consequently mitigates and does not add up to the atmospheric carbon dioxide [10].

Biomass can be majorly divided into woody plants, herbaceous plants or grasses, aquatic plants and manures. Among these, some herbaceous plants, aquatic plants and manures contain high moisture content and are suitable for wet processing or biochemical processing. Aqueous processing or wet processing is generally initiated through enzyme action. This method is suitable for high moisture content biomass because of challenged efficiency of overall energy retrieval, compared to the energy required for drying involved in dry processing. Moisture content, carbon source and cellulose to lignin ratio are the most important factors affecting the wet process. Biomass with low moisture content is subjected to dry process or thermal treatment such as gasification, pyrolysis and combustion. Factors that influence the dry processes are ash content, alkali and trace components as they adversely affect the thermal conversion processes [10].

The products of wet processes are ethanol, butanol and biogas. Ethanol and butanol products majorly depend on the plant composition-cellulose, hemicellulose and lignin. Cellulose, hemicellulose and lignin are the three main components of any plant material. Cellulose is a polymer of glucose with linear chains of (1,4)-D-glucopyranose units in β-configuration with an average molecular weight of around 100,000. Another polymer of glucose with linear chains of (1,4)-D-glucopyranose units in α-configuration, called amylose constitute about 20 % of starch.

Starch also includes amylopectin, a branched polymer chain of D-glucose molecules called α-1,6 glycosidic linkage [11]. Starch can be more easily digested to sugars compared to cellulose due to the high crystallinity offered by cellulose linear structure. Starch can be obtained from any of the food storage units of plants, while cellulose constitutes all the other parts of the plant.

Hemicellulose is a heterogeneous polymer of pentoses (xylose and arabinose) primarily xylose, hexoses (mannose, glucose and galactose) and sugar acids. Although it is not covalently bonded, it is tightly bonded to the surface of each cellulose microfibril. Cellulose digestibility to sugars partially depends on the hemicellulose content.

After cellulose and hemicellulose, lignin is the third most abundant biopolymer, consisting primarily of phenyl propane units most commonly linked by ether bonds. It provides structural support and, through its hydrophobic nature impermeability and resistance to microbial and oxidative attack [12, 13]. Additionally, woody plants have higher lignin than herbaceous plants, thus imparting lesser strength in the latter due to loosely bound fibers [14]. Lignin also inhibits the conversion of carbohydrates to ethanol making it imperative to maximize the elimination of lignin in biomass. However, woody plants having higher lignin proportions resist moderately severe treatments, unlike herbaceous biomass. Some herbaceous plants like switch grass and miscanthus require less severe treatments for lignin removal. Since lignin alone causes inhibition to conversion of sugars and to ethanol, cellulose to lignin ratio is an important factor effecting conversion. Removed lignin can be used for combustion in boilers for energy generation.

For dedicated energy crops, cultivation of herbaceous plants is greatly encouraged compared to the woody biomass for several reasons such as, shorter harvest time, ease of harvesting, usage of surplus land, less intensive agricultural practices, less lignin content and less

severe treatment for conversion. Selection of plants for energy production depends on the climatic conditions, geographical location, availability and type of treatment employed (either thermal or biochemical).

In the UK, a perennial crop, miscanthus, has attained a lot of attention for energy production through biochemical conversion due to the ease in growing, harvesting and good annual yield. This thin-stemmed crop has been considered a good energy crop due to its annual harvest and low mineral content, and is grown in ten countries in Europe. In the USA, another thin-stemmed crop, switch grass, is a model crop for the Oak Ridge National Laboratory, as it yields high ethanol from fermentation with the existing technologies. Its low ash and alkali content allows it to be used for combustion. Brazil, one of the pioneers for the production of ethanol for fuel uses sugarcane as the source [10]. Sources of biomass other than herbaceous plants include agricultural residues such as wheat straw, rice straw, corn fiber, corn stover, bagasse etc., Animal residues such as pig slurry [15], cattle dung, horse dung[16] etc. are used for biogas production, which upon upgrading to >97 % methane, can be used as transport fuel. Marine algae have gained importance as potential sources for biofuel production, both as substrates for fermentation to hydrogen, ethanol and butanol, and as oil rich sources for biodiesel production. Due to their less energy and water requirement, higher carbon dioxide capture and negligible lignin, they are considered as superior to terrestrial biomass [17, 18]. However, several factors including availability, moisture content and cellulose/lignin ratio impact the biochemical production of biofuels.

1.4 Process overview

Major processes involved in the biochemical production of biofuels are biomass handling, biomass pretreatment, hydrolysis and fermentation. However, depending on the source

of biomass, the route of conversion to biofuel and the type of biofuel, the series of processes can alter. Figure 1.5 shows a schematic representation of some common unit operations and processes for the biofuels mentioned in this section.

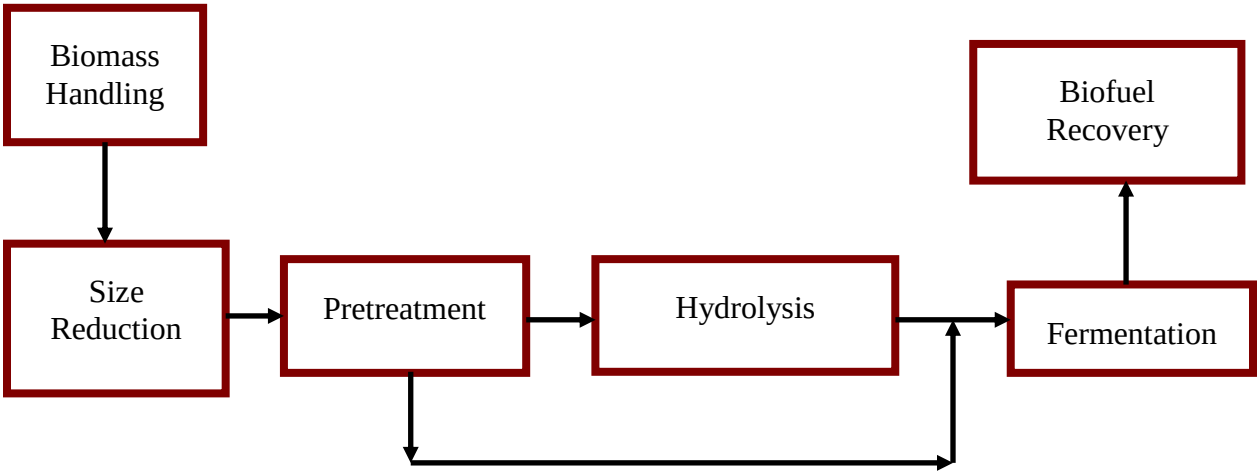

Figure 1. 5: Schematic representation of processes in biochemical conversion of biomass to fuels.

1.4.1 Handling

Biomass, either grown or obtained from various sources, needs to be transported to the production sites for biochemical conversion to fuels. Post harvest it is prepared as bales, pellets and briquettes for which, the biomass has to be size reduced. Size reduction is an important mechanical preprocessing step to increase the bulk density and flowability of particles for transportation. Biomass is generally ground to 3-8 mm particles to compact it into pellets or briquettes of higher density. Important parameters in evaluating the efficiency of size reduction are particle size, particle size distribution, shape, surface area, density and energy efficiency of mill used [19]. Due to the unavailability of a continuous supply of biomass feedstocks, storage of biomass becomes important to ensure uninterrupted supply for continuous production of biofuels. Although outdoor storing of wood chunks is a commonly practiced method, studies

show that terpenes are emitted from wood due to exposure of direct heat from sunlight [20]. Large silos and specially constructed facilities are used for biomass storage to protect feedstock from the effects of weather, rodents and microbial growth. Microbial growth during storage causes loss of substrate and also has the potential to result in self-ignition due to exothermic reactions. Therefore, maintenance of dry conditions is required to allow little microbial activity in the biomass during storage. Field drying post harvest is a common method for drying in sunny regions. However, thermal or mechanical drying techniques using drum driers are available for drying biomass after harvest and before storage in colder regions[21].

1.4.2 Pretreatment

Pretreatment plays an important role in the biochemical conversion yields of biofuels. Complex structures in biomass are broken down into oligomeric sub units through pretreatment. These oligomers are further broken down into monomeric units during hydrolysis and fermentation. Pretreatment enhances the product yields by disrupting and solubilizing the hemicelluloses and lignin structures in biomass. Key properties affecting the conversion of lignocellulose are the crystallinity of cellulose, degree of polymerization, moisture content, available surface area, and lignin content [12]. The aim of pretreatment is to disrupt the lignocellulosic structure by: (1) removing hemicellulose, increasing mean pore size, and facilitating the entrance of enzymes and hydrolysis, (2) removing or redistributing lignin to reduce its "shielding" effect [22].

Pretreatment processes will ideally achieve the following [23]:

- High yields for multiple crops, sites ages, harvesting times
- Highly digestible pretreated solid

- Minimum amount of toxic compounds
- Biomass size reduction not required
- Operation in reasonable size and moderate cost reactors
- Non-production of solid-waste residues
- Effective at low moisture content
- Obtains high sugar concentration (from hydrolysis)
- Fermentation compatibility (minimal production of inhibitors)
- Lignin recovery
- Minimum heat and power requirements

The main classes of pretreatment are mechanical, chemical/physiochemical, and microbial.

1.4.2.1 Mechanical

Milling uses grinding to reduce particle size and crystallinity. Specific surface area is increased and degree of polymerization decreased. Numerous milling systems can be employed: ball, hammer, roller, colloid, and vibro energy milling [22, 24]. Coupled with other pretreatment, milling can increase hydrolysis yield for lignocellulose by 5-25 % and reduces digestion time by 23-59 % [25, 26]. There are limits to effectiveness. Size reduction below #40 mesh does not improve hydrolysis yield or rate [12]. Power requirements are large, which will limit economic feasibility [27].

1.4.2.2 Chemical/ Physiochemical

Pretreatments for bioethanol production may be performed using chemicals such as sulfuric acid, sodium hydroxide, ammonium hydroxide, supercritical ammonia and supercritical

carbon dioxide at both high and low temperature and pressure conditions to separate undesirable components such as lignin from biomass. Pretreatment disrupts the biomass structure and increases the surface area to enhance enzyme access during the hydrolysis stage. Several pretreatment methods such as hot water treatment, steam explosion, dilute sulfuric acid treatment and ammonia fiber expansion can be employed to remove lignin and/or depolymerize lignocelluloses structure in biomass.

Thermal processes include liquid hot water (LHW) and steam pretreatment. At temperatures above 150-180 ^{o}C, hemicellulose and then lignin begins to dissolve [28, 29]. Hot water pretreatment primarily dissolves hemicellulose to increase access for enzyme hydrolysis and to limit formation of inhibitors [30]. Liquid hot water has removed up to 80 % of the hemicellulose to improve enzymatic hydrolysis by increasing the accessible surface area of the cellulose [30, 31]. pH should be kept between 4 and 7 to maintain hemicellulosic sugars in oligomeric state, reducing formation of degradation products and thus inhibitors [30]. Hemicellulose can be hydrolyzed to form acids which further hydrolyze the hemicelluloses [32]. The main advantages for LHW are recovery of pentoses and minimization of inhibitors, compared to steam explosions, and minimal need for chemical and neutralization as compared to dilute acid pretreatment [24]. Hot water pretreatment of lignocellulosic biomass has three types of reactor configurations, co-current, counter current and flow through. In co-current pretreatment, biomass and water are heated to a desired temperature and held in the reactor for a controlled residence time before cooling. In counter current flow system, biomass slurry and water are allowed to flow in opposite directions into the reactor. In flow through configuration, hot water is allowed to flow through a stationary bed of biomass [33]. Therefore, pretreatment

technologies have been developed to be carried out in both batch and continuous flow reactor configurations.

Steam explosion has been widely tested in lab and pilot-scale systems. Biomass is pressurized with steam at 160-260 ^{o}C for several seconds to minutes and pressure is rapidly released. Mechanical forces separate fibers and the high temperature promotes conversion of acetyl groups to acetic acid [22, 24]. The main action of the acetic acid is probably to catalyze the hydrolysis of soluble hemicellulose oligomers [28]. Lignin is redistributed and some times removed [34]. Removing hemicellulose increases accessibility of enzymes to the cellulose [22]. The advantages of steam explosion include use of larger chip size, reduced need for acid catalyst, high sugar recovery, and feasibility for industrial-scale use [22]. The primary disadvantages include partial hemicellulose degradation and generation of inhibitory compounds [34]. Steam explosion can be combined with addition of sulfur dioxide and sulfuric acid to enhance recovery of cellulose and hemicellulose. It improves the solubilization of hemicelluloses, lowers optimal treatment temperatures, and partially hydrolyzes cellulose [34, 35]. Acid addition is particularly effective with softwoods, which have a low content of acetyl groups [36].

Acid pretreatment removes hemicellulose to make cellulose more accessible. It can also hydrolyze fermentable sugars. Acid pretreatment can be practiced using high concentrations of acid (generally sulfuric) at low temperatures or low concentrations at high temperatures [24]. Use of concentrated acid requires corrosion resistant process equipment. Recovery of the acid is energy intensive, and produces degradation products inhibitory to fermentation [22, 24, 37]. Use of dilute acid is more promising, for example at 0.1 to 1 % sulfuric acid at 140-190 ^{o}C. This achieves almost total hemicellulose removal and high cellulose conversion [24]. Production of inhibitory compounds is lessened [27]. Addition of nitric acid greatly improves solubilization of

lignin in newspaper [38]. The use of acid pretreatment for methane production is more forgiving because, methanogens can tolerate the inhibitory compounds [38, 39].

Alkali pretreatment uses NaOH, $Ca(OH)_2$, or NH_4OH. Lime is very effective [27]. It removes acetyl groups, has lower cost and less safety concerns. Solvation and saponification reactions [27] lead to swelling. The swelling increases internal surface area of cellulose, decreases polymerization and crystallinity, and disrupts lignin structure and removes some lignin and hemicellulose [24], increasing accessibility to enzymes enhancing saccharification [40]. Processing can be done at low (ambient) temperature [41] for long time periods (24 hours), or at elevated (120-130 ^{o}C) levels for minutes to hours [42]. Production of inhibitory compounds is significantly less [24]. But, solubilization and redistribution of lignin and modifications in crystalline state of cellulose can counteract the benefits of the method [32]. Addition of hydrogen peroxide to alkaline pretreatment enhances lignin removal and improves enzymatic hydrolysis [43]. Alkaline pretreatment, as with acid, is more forgiving for production of methane, versus ethanol [44].

Ammonia fiber explosion or "expansion" (AFEX) is analogous to the steam expansion method. Anhydrous ammonia is added to biomass at approximately 1 kg NH_3: 1 kg dry biomass, and held at temperatures of approximately 100-120 ^{o}C for several minutes. Pressure is rapidly released, swelling and disrupting the lignocellulose structure [22, 24]. Only a solid residue is produced, and little hemicellulose and lignin are removed [45]. Enzyme hydrolysis yields and ethanol production are increased [46]. AFEX does not produce inhibitors, although some lignin may remain on the biomass surface [22]. It is more effective on lower-lignin crop residues and herbaceous crops than woody material [45].

CO_2 explosion uses CO_2 at high pressure to penetrate the pores of lignocellulose. Explosive depressurization disrupts the cellulose and hemicellulose structure and improves enzymatic hydrolysis. Supercritical conditions at 35 ^oC and 73 bar more effectively remove lignin and increase digestibility [22]. However, in general pretreatment with appropriate conditions is a highly desirable step for lignocellulosic biomass to improve its digestibility.

Other physiochemical methods include organosolv and wet oxidation. Organosolv uses organic solvents to dissolve lignin. Solvent recovery is essential, and inexpensive, low molecular weight alcohols are favored. The recovery of low molecular weight lignin as a co-product is potentially a significant advantage [34]. Wet oxidation uses water and oxygen under elevated pressure and temperature [24]. Hydrogen peroxide can be used at ambient temperature can also be used to enhance enzymatic hydrolysis [47]. Batch treatment of corn stover using $FeCl_3$ in tubular reactors resulted in the hydrolysis yield of 98 % compared to 22.8 % yield for the untreated corn stover [48].

Alvira et al. conclude that chemical and thermo chemical methods are the most effective and promising technologies for industrial applications [22]. They suggest combination of different pretreatments should be considered for optimal fractionation of components and high yields. They also stress the need for additional fundamental research of plant cells to better understand the reactions induced by pretreatment.

Taherzadeh and Karimi [24] concluded that concentrated acids, wet oxidation, solvents and metal complexes are effective, but too expensive [30, 49]. They concluded that steam pretreatment, lime pretreatment, LHW systems and ammonia-based pretreatments have a high potential. Eggeman and Elander [50] presented an economic evaluation showing only small

differences in cost for five different pretreatment technologies (dilute acid, hot water, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), and lime). This analysis appeared in the special issue 'Coordinated development of leading biomass pretreatment technologies'[45]. Optimizing enzyme blends and hydrolyzate conditioning may better differentiate process economics.

1.4.2.3 Microbial

With research and development with respect to the pretreatment methods, potential in microbes to convert some dangerous industrial pollutions has been discovered. Various fungi are involved in lignin degradation, three main types of wood degrading fungi are white rot fungi, brown rot fungi and soft rot fungi [48], among which, white rot fungi are capable of degrading all major components of wood (cellulose, hemicelluloses, lignin). Lignocellulosics treated with *Ceriporiopsis subvermispora* and *Cyathus stercoreus* showed a significant increase in the biological delignification [51]. *Phanerochaete chrysosporium* is known to degrade different synthetic chemicals, most of which are recalcitrant to biodegradation [52]. Mushroom compost, obtained from growing *Agaricus bisporus* on straw and hay, can be divided into three major components, cellulose, lignin, organic and inorganic nitrogen sources [53]. Modifications in lignin were observed by atomic force microscopy and high performance liquid chromatography in lignin samples treated with bacterial consortium [54]. Extracellular enzymes like lignin peroxidases (LiP) and manganese peroxidases (MnP) are the most important components of lignin degrading enzymes systems [55] and the enzymes produced during the process are critical in determining the path of microbes. *P. chrysosporium* demonstrates capabilities of degrading two structurally different dyes by the extracellular enzymes produced in a fixed bed bioreactor, the fixed bed reactor proved to be a suitable reactor configuration for MnP and LiP showing

activities of 1293 and 225 U/l [56]. A bacterium capable of degrading peanut hull lignin was isolated using growth techniques and C^{14} labelled techniques [57]. Enzymatic digestibility of corn stover after pretreatment with *Cyathus stercoreus* and reduction in shear force suggesting the effectiveness in improving the forage digestibility [58]. Investigations regarding co-culturing of two white-rot fungi on aspen wood chips suggest that a combination of *Pleurotus ostreatus* with *Ceriporiopsis subvermispora* or with *Physisporinus rivulosus* yielded higher MnP activities than the other combinations considered, and a combination of *Pleurotus ostreatus* with *Ceriporiopsis subvermispora* yielded high laccase activity than other combinations worked [59]. Thus a suitable microbial agent can be employed to improve the enzymatic digestibility of the agricultural substrate.

1.4.3 Hydrolysis and Fermentation

1.4.3.1 Hydrolysis

During hydrolysis, breaking down of polymeric and oligomeric cellulosic structure, to simpler molecules such as glucose, cellobiose, xylose, galactose, arabinose and mannose, takes place. It is done by the action of either chemical or enzymatic agents. Enzymatic hydrolysis is a complicated process that takes place at the solid/liquid interphase. Several processes such as, chemical and physical changes in the solid biomass, primary hydrolysis of soluble intermediates from the surface, and secondary hydrolysis to ultimately simpler molecules such as glucose, take place simultaneously [60].

Hydrolysis of the pretreated biomass can be performed both chemically and biochemically. Chemical hydrolysis uses a continuous two-step dilute sulfuric acid process. The first step involves low temperature treatment and the second step, a high temperature treatment, as hemicellulose depolymerizes at lower temperature than the cellulose polymer. In the first step,

the hemicellulose fraction is removed, followed by the second step in which hexose release occurs. A batch process, using concentrated sulfuric acid, is also used for biomass hydrolysis; however, use of concentrated acid requires high capital investment due to the requirement of corrosive resistant process equipment. Additionally, it requires acid recycling and recovery for economic viability of the process [60].

Biochemical hydrolysis is the most developed process in recent years and is commonly called as saccharification. It is initiated by enzymes that cleave the cellulose-lignin matrix into various monomeric, dimeric and oligomeric sugars. Most of the common enzymes that act synergistically for cellulose hydrolysis, called cellulases, are endoglucanases or endo-1,4-*β*-glucanases (EG), exoglucanases or cellobiohydrolases (CBH) and *β*-glucosidases (BGL). While endoglucanases cleave the intra-molecular bonds of the cellulose polymer, CBH and BGL catalyze the release of cellobiose and glucose from oligomeric ends, and glucose from cellobiose respectively as shown in the Figure 1.6. A synergistic effect of an enzyme component system consisting of at least endo-*β*-glucanases, exo-*β*-glucanases and *β*-glucosidases results in hydrolytic efficiency [61, 62].

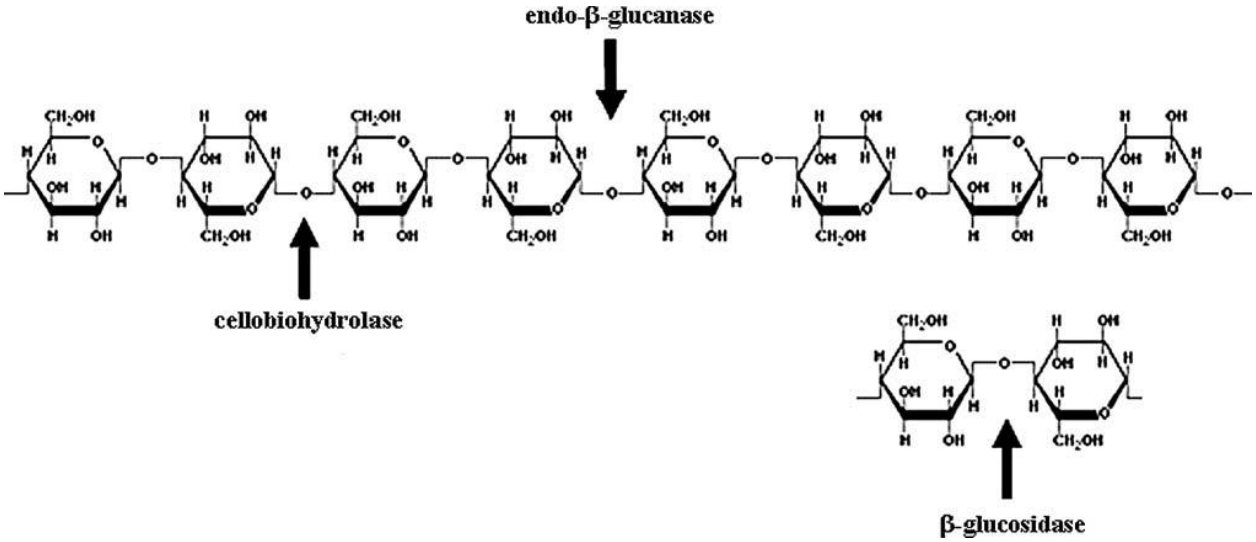

Figure 1. 6: Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and *β*-glucosidase [63].

Enzymes related to hemicellulose hydrolysis, hemicellulases, are mainly endo-1,4- *β*-xylanase, *β*-xylosidase, *α*-glucuronidase, α-L-arabinofuranosidase and acetylylan esterase as shown in Figure 1.7. Therefore, the hydrolyzate contains both hexoses, pentoses and their oligomeric forms depending on the treatment [64].

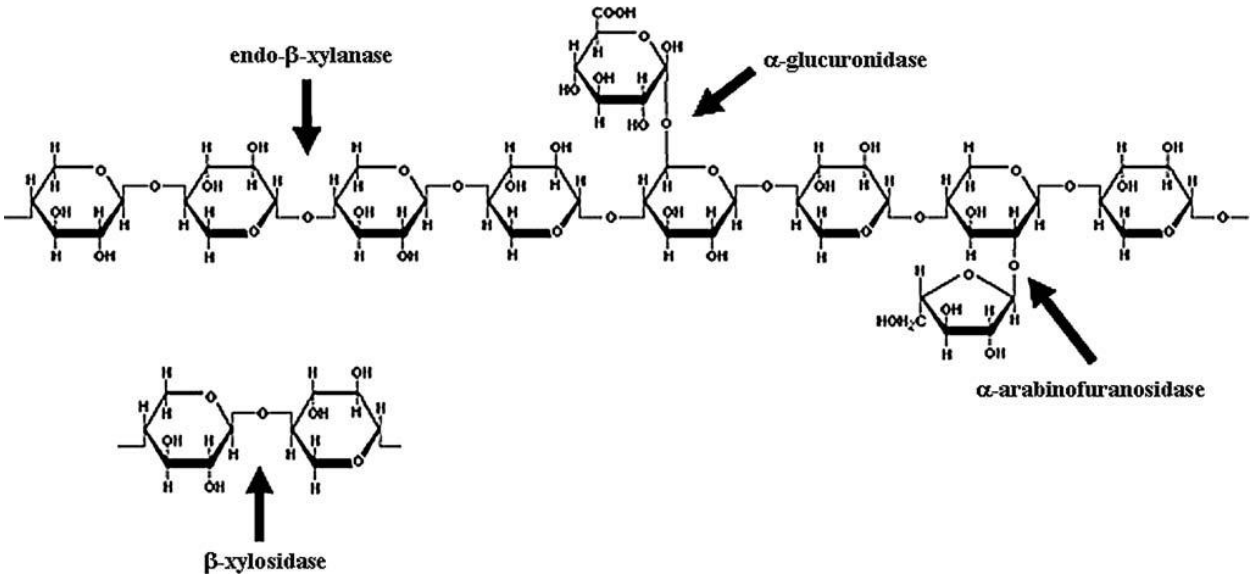

Figure 1. 7: Polymeric chemical structure of hemicellulose and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation [63].

Various genera of bacteria such as *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora* and *Streptomyces* produce these enzymes to hydrolyze lignocelluloses. Fungal genera such as *Trichoderma*, *Ceriporiopsis*, *Aspergillus* and *Sporotrichum* also include species that possess the cellulolytic abilities to hydrolyze lignocellulosic biomass. Therefore, enzyme extracts from these cultures are used for hydrolyzing biomass and recent developments in enzyme technology have reduced their price of production significantly.

The factors that influence the enzymatic hydrolysis include temperature, pH and substrate concentration. At low substrate concentration, an increase in substrate concentration increases

the yield and reaction rate of hydrolysis. However, at high substrate concentration, yield and reaction rate decreases due to substrate inhibition of enzymes [37, 62]. Temperature and pH of enzyme varies by the microbe source from which it is derived. However, most commonly used industrial cellulases are derived from wild and modified strains of *Trichoderma reesei* and have an optimum temperature between 45-50 ^{o}C. Hydrolysis yields are also increased by addition of surfactants such as Tween-20. It is reported that addition of Tween-20 resulted in 8 % increase in ethanol and 50 % reduction in cellulases dosage, increase in enzyme activity and the hydrolysis rate [65].

Consolidated microbial treatment of biomass is another method of saccharification of biomass. Loss of sugars during the process is inevitable, due to its consumption by microbes, which makes the use of enzyme extracts advantageous for hydrolysis. Enzyme hydrolysis is limited by product inhibition, which requires continuous removal of hydrolysis products apart from use of BGL for subsequent conversion of the generated cellobiose to glucose. Therefore, simultaneous saccharification and fermentation (SSF) is a potential solution for product inhibition, where release of glucose using enzyme hydrolysis and its subsequent fermentation to ethanol by yeast takes place in the same system [60].

1.4.3.2 Fermentation

Conversion of simpler carbohydrates to alcohol through action of microbes is called as fermentation. Fermentation of biomass to ethanol is commonly carried out using yeast such as *Saccharomyces* and *Pichia*, bacteria such as *Zymomonas* and *Escherichia*, and non yeast fungi such as *Aspergillus.* Products of hydrolysis, sugars, are converted to ethanol producing carbon dioxide as byproduct and energy for cell growth. The most commonly used microbe, *Saccharomyces cerevisiae*, ferments sugars to ethanol at almost anaerobic conditions, although it

requires a certain amount of oxygen for essential poly-unsaturated fats and lipids. Figure 1.8 depicts the ethanol fermentation pathway of *Saccharomyces* from glucose. It briefly describes the conversion of glucose to ethanol through intermediate biochemical reactions involving NAD^+ and NADH (Nicotinamide adenine dinucleotide - oxidized and reduced forms respectively). Since, lignocellulosic biomass consists of several components such as pentoses, hexoses, acids (acetic acid), degradation products derived from the pretreatment stage could inhibit the fermentation process. Chemical, physical and biological methods have been developed to overcome the inhibition effect of these compounds by detoxification. *Trichoderma reesei* has been reported to degrade the inhibitors present in willow hydrolyzate after steam pretreatment. Overnight extraction of spruce hydrolyzate with diethyl ether at pH 2 showed detoxification effects with ethanol yields comparable to the reference fermentation. Detoxification by alkali treatment at pH 9 using $Ca(OH)_2$ and readjustment of pH to 5.5 allowed better fermentability due to precipitation of toxic compounds [66].

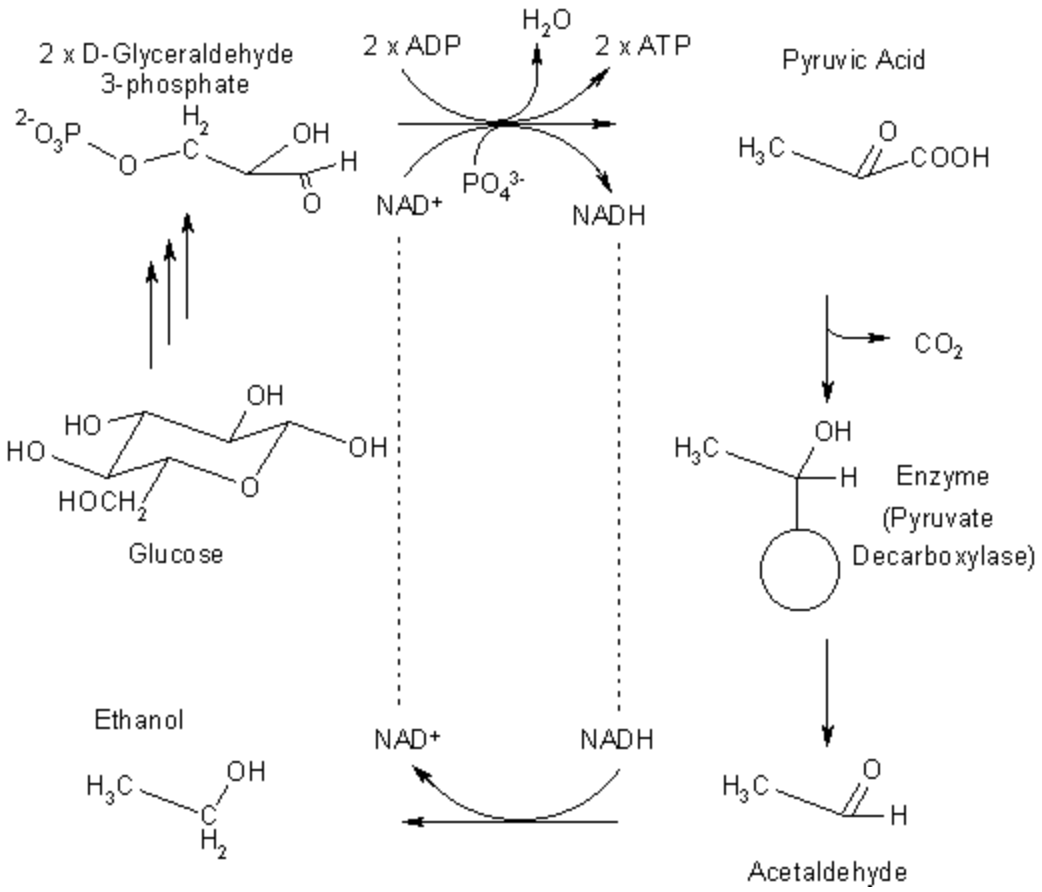

Figure 1. 8: Ethanol fermentation pathway of *Saccharomyces.*

Usually, the temperature of operation is in the mesophilic range (15-40°C) for most of the species mentioned above. Increases in temperature beyond the optimum condition result in a decrease in ethanol yield and eventually in cell death. Another important factor in maintaining good cell growth is pH, generally a pH range of 6.5-7.5 [67] is suitable for ethanol fermentation for most of the strains, although, yeast and fungal strains can tolerate down to 3.5-5.0. pH below 4.0 reduces the potential of bacterial contamination thus alleviating the requirement of aseptic techniques [60].

Fermentation of biomass is affected by several other factors such as ethanol tolerance, substrate concentration and by product inhibition. Ethanol tolerance is one of the factors which determine the maximum ethanol concentration that can be reached during fermentation, as most

of the microbes responsible for fermentation cannot tolerate high concentrations of ethanol, eventually leading to cell death. *Zymomonas* has higher ethanol tolerance, and achieves 5 % higher ethanol yields, as compared to yeast strains [68]. Increase in substrate concentration decreased the ethanol yield. However, batch-wise charging of substrate reduces this kind of inhibition. Therefore, fed-batch reactors are more suitable for industrial applications. Byproduct inhibition is overcome by chemical, mechanical or biological detoxification [60].

A combination of hydrolysis and fermentation is another process where simultaneous break down of complex carbohydrates to simpler ones, and conversion to alcohol takes place. This process is commonly called as simultaneous saccharification and fermentation (SSF). Product yields from SSF are higher than separate hydrolysis and fermentation (SHF), as the end product inhibition during hydrolysis of higher carbohydrates to glucose and cellobiose, is relieved by simultaneous fermentation of glucose to ethanol [60].

Hydrolysis and fermentation are carried out in both batch and continuous modes. Batch reactors require higher reactor volume compared to the continuous reactors to achieve similar product yields. Two basic types of continuous reactors used in biochemical reactions are continuously stirred tank reactor (CSTR) and plug flow reactor (PFR). Most commonly, CSTR is used for hydrolysis and fermentation during the biochemical production of biofuels. Studies show usage of a packed bed reactor (PBR) in comparison with upflow anaerobic sludge bed (UASB) for the production of hydrogen from organic fraction of municipal solid waste, where the PBR was packed with municipal solid waste. The retention times of 50 and 24 hours with maximum hydrogen yields of 23 % v/v and 30 % v/v (based on volume of waste) for PBR and UASB respectively [69]. Another study investigated combined or sequential two-stage processes involving co-production of hydrogen and methane since hydrogen is an intermediate byproduct

of methane production [70-72]. Dissolved oxygen and heat transfer are known to be limited by reactor volume. Fermentation for hydrogen, methane, ethanol and butanol production is anaerobic, and the reactor volume is not limited by the dissolved oxygen and heat transfer when run in continuous mode. Therefore, CSTR fermentation systems with recycling of cell mass are sufficient to overcome solvent toxicity and limited cell growth [73].

This chapter is adapted from Master's Thesis by Swetha Mahalaxmi "Microbial conditioning of Biomass" and "Handbook of Climate Change Mitigation" book chapter: "Biochemical conversion of biomass to fuels", Springer Publications, authors: Swetha Mahalaxmi, Clint Williford.

2. OVERVIEW

The overall aim of this dissertation is to study various techniques applied to preprocessing of biomass (microbial preprocessing, mechanical preprocessing and chemical pretreatment) that are used for enhancing the digestibility of biomass to sugars for ethanol production. In this work switch grass was used as the model biomass substrate for all the preprocessing and pretreatment methods. For microbial preprocessing, *Phanerochaete chrysosporium* was used as a model microorganism for all the studies.

Chapter 3, "Estimation of treatment time for microbial preprocessing of biomass", studies the use of enzyme profiling as a method to estimate an appropriate treatment time for preprocessing switch grass with *P. chrysosporium*. Determining appropriate preprocessing time using enzyme profiles gives a good estimate to obtain higher glucose and total sugar yield. This work was published in Applied Biochemistry and Biotechnology 162: 1414-1422.

Chapter 4, "Pretreatment and enzyme requirements of size separated fractions of switch grass", investigates biomass size partitioning, a mechanical process, as a potential process to separate the heterogeneous fractions present in switch grass. The study determines size partitioning as a method to separate heterogeneous fractions of biomass, for optimal usage of chemical pretreatment conditions and enzymes during enzyme hydrolysis, and to identify fractions with higher lignin, lower glucose yields and recalcitrance.

Chapter 5, "Fungal preprocessing of size separated fractions of switch grass", studies the estimation of treatment times for a microbial preprocessing using *P. chrysosporium* (based on chapter 3) for various size separated fractions of switch grass (based on chapter 4). This study

shows that the microbial preprocessing time and effect varies with different size fractions in biomass due to compositional differences. Efforts to utilize agricultural substrates for enzyme production can be optimized based on the findings in this work in identifying and utilizing the most desirable components in agricultural residues for enzyme production.

Chapter 6 provides bibliography of this dissertation. Chapter 7, “Appendix”, elaborates some of the procedures used in this work.

3. ESTIMATION OF TREATMENT TIME FOR MICROBIAL PREPROCESSING OF BIOMASS

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Abstract

Biochemical conversion of lignocellulosic biomass to ethanol involves size reduction, preprocessing, pretreatment, enzyme hydrolysis and fermentation. In recent years, microbial preprocessing has been gaining attention as a means to produce labile biomass for lessening the requirement of pretreatment severity. However, loss of sugars due to microbial consumption is a major consequence, suggesting its minimization through optimization of nutrients, temperature and preprocessing time. In this work we emphasized estimation of fungal preprocessing time, at which higher sugar yields can be achieved after preprocessing and enzyme hydrolysis. The estimation is based on the enzymatic activity profile obtained by treating switch grass with *Phanerochaete chrysosporium* for 28 days. Enzyme assays were conducted once in every 7 days for 28 days, for activities of phenol oxidase, peroxidase, β-glucosidase, β-xylosidase and cellobiohydrolase. We found no activity for phenol oxidase and peroxidase, but the greatest activities for cellulases on the seventh day. We then treated switch grass for 7 days with *P.chrysosporium* and observed that the preprocessed switch grass had higher glucan (39 %),

xylan (17.5 %) and total sugar yields (25.5 %) than the unpreprocessed switch grass(34 %, 37.5 % and 20.5 %, respectively, $p < 0.05$). This verifies the utility of using enzyme assays for initial estimation of preprocessing time to enhance sugar yields.

3.1 Introduction

With increasing demands for fuel and environmental concerns, biofuels have gained new importance [7]. In spite of advances in fermentation technology, commercialization of ethanol from lignocellulosic biomass is still hindered by the recalcitrance of biomass. Various pretreatment processes can reduce the recalcitrance of biomass, however obstacles to hydrolysis and fermentation remain due to the production of degradation products during pretreatment, as a result of greater pretreatment severity [74]. Although washing and other chemical methods of treating the degradation products can alleviate inhibition, significant water and chemical usage combined with capital equipment and energy contribute to higher costs [75]. Microbial preprocessing of biomass before pretreatment may potentially lessen the requirements of pretreatment severity. Solid state fungal treatment is one such technique involving less aggressive treatment with simpler processing parameters and equipment. It also offers a simpler reactor system with minimum downstream processing [76] and brings the preprocessing technology closer to the farms that are the source of agricultural residues. A solid state fungal system producing cellulases may potentially deconstruct the biomass, improving digestibility and thus ethanol production.

Phanerochaete chrysosporium is a white rot fungus known to degrade different synthetic chemicals, most of which are recalcitrant to biodegradation [52]. *P. chrysosporium* is a potential lignin degrading fungus with ability to partially breakdown lignin carbohydrate complexes [58]. Although it produces lignin peroxidase and managanese-dependent peroxidase [56] and laccase

[77] to break lignin, it also produces multiple endoglucanases which exhibit endo- exo synergism with cellobiohydrolases. Beta glucosidase obtained from *P.chrysosporium* can also cleave hemicellulose to produce xylose, mannose and arabinose due to its non-specificity [78]. Besides the lignin degrading capability of *P.chrysosporium*, its potential for partial-cellulose degradation can be explored to recover sugars during the prolonged storage of biomass.

Although most of the work related to using *P.chrysosporium* for producing higher cellulosic materials from biomass has shown significant lignin degradation, considerable cellulose losses have been reported. Corn stover treated for 29 days using *P. chrysosporium* showed reduced viscosity, but showed no improvement in enzyme digestibility [58]. *P. chrysosporium*-treated cotton stalks also showed similar results of reduced digestibility over a 14 day solid state treatment in spite of lignin degradation [79]. It is apparent from these previous works that, although prolonged treatment time resulted in lignin degradation, loss in sugars was observed due to microbial consumption. Thus a method to determine an appropriate treatment time is critical, for exploiting the capacity of the fungus to partially degrade cellulose and hemicellulose complexes, to minimize the sugar consumption and produce higher sugar yields.

In the present study, we used enzyme profiling as a method to estimate an appropriate treatment time for preprocessing switch grass. We treated switch grass under solid state conditions with *P. chrysosporium* (in triplicate) for 28 days and assayed for activities of phenol oxidase (laccase), peroxidase, β-glucosidase, β-xylosidase and cellobiohydrolase. Assays were conducted for every 7 days of incubation starting with the initial day, to obtain a profile of activities against time, for the 28 day period. A time point on the plot of enzyme activity against time with highest activities on the plot was chosen to be an appropriate treatment time. In a subsequent experiment, we treated switch grass for the time period obtained from the previous

step. The treated samples were analyzed for glucose and total sugar yields, showing an increase in total glucose yield and total sugar yield, thus validating the method of using enzyme profiling for estimation of treatment time.

3.2 Materials and Methods

3.2.1 Propagation of inoculum

P. chrysosporium (strain BKM-F-1767) was obtained from USDA Forest Products Laboratory (Madison, WI) and was propagated onto potato dextrose agar (PDA) plates of 90mm × 12mm size and allowed to grow at 37 °C for 7 days [58]. Stock cultures were stored for a week at 4 °C, and the culture was maintained by periodically transferring to fresh PDA plates. Prior to treatments, *P. chrysosporium* was grown for 7 days on PDA plates, and a spore suspension was prepared by scraping the spores aseptically from 3 plates into 60 ml sterile water, ensuring uniform spore distribution in the liquid by vortexing. A 5 ml sample of this suspension was used as inoculum for each treatment flask.

3.2.2 Preparation of Switch grass and Solid state treatment with *P.Chrysosporium*

Switch grass was obtained from Waller labs, University of Mississippi, where it was grown, harvested, air dried and ground to 3mm mesh size. The ground samples were stored under dry conditions. These were further dried at 35 °C for 2 days prior to experimentation in a convection incubator. Ten grams of switch grass was weighed, placed in a 250 ml flask, and autoclaved (121 °C, 30 min). Fifty milliliters of sterilized water was added to maintain approximately 80 % moisture and 5 mL of supplemental growth media ($NaNO_3$ – 3 g, KCl – 0.5 g, $MgSO_4.7H_2O$ – 0.5 g, $FeSO_4.7H_2O$ – 0.5 g, KH_2PO_4 – 1.0 g, Glucose – 20 g in 1 liter solution) [80], sterilized separately, was added in addition to 20 µL of tetracycline (20mg/mL in

ethanol) to minimize bacterial contamination in the flasks. Treatment flasks received 5 mL of *P. chrysosporium* suspension while other flasks, supplemented with 5 mL of additional sterilized water and no *P. chrysosporium*, were used as controls. Flasks (3 treated (with *P. chrysosporium*) and 3 controls (without *P. chrysosporium*), were allowed to incubate at 37 °C under solid state conditions for 7 days.

3.2.3 Enzyme activity assays

Flasks (3 treated and 3 controls) were incubated at 37 °C under solid state conditions for 28 days and were sampled for phenol oxidase, peroxidase, β-glucosidase, β-xylosidase and cellobiohydrolase activities for every 7 days during the 28 day period of treatment. The substrate used for phenoloxidase and peroxidase tests is 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA), and those for β-glucosidase, β-xylosidase and cellobiohydrolase tests are 5 mM pNP-β-glucopyronoside, 5 mM pNP-β-xylopyranoside and 5 mM pNP-cellobioside respectively.

A known amount (approximately a gram) of sample was taken in a test tube and diluted to 5 mL by addition of water and mixed well, 150 µL of supernatant was incubated with 150 µL of substrate solution (and 15 µL of 0.3 % H_2O_2, only for peroxidase assay) for a noted time, and the mixture was analyzed spectrophotometrically at 460 µm and 410 µm for L-DOPA assays and cellulose assays respectively. The units of activity are defined as µmoles of the substrate reacted with the enzyme in 1ml of sample per hour of incubation (U/ml) [81].

3.2.4 Composition Analysis and Enzyme hydrolysis

Samples, before and after treatment, were analyzed for the glucan and xylan compositions. Commercial enzymes, Novozyme 188 (10 FPU/ 0.5g glucan) and celluclast (15

FPU/ 0.5 g glucan) were used for 72 hour enzyme hydrolysis of samples using laboratory analytical procedure (LAP) from National Renewable Energy Laboratory (NREL).

3.2.5 Analysis

Activities of phenol oxidase, peroxidase, β-glucosidase, β-xylosidase and cellobiohydrolase were determined at day 0, 7, 14, 21 and 28 of the 28 day solid state microbial treatment. Flasks at the end of the treatment were washed with 25 mL of water heated up to 50 °C, and the switch grass was filtered and stored in plastic bags. To estimate overall changes, yields in washate (for free sugars) and solid phases of the treated substrate were accounted for. Samples, before and after treatment, were analyzed for the glucan and xylan compositions. Commercial enzymes, Novozyme 188 (10 FPU/ 0.5g glucan) and celluclast (15 FPU/ 0.5 g glucan) were used for 72 hour enzyme hydrolysis of samples using laboratory analytical procedure (LAP) from National Renewable Energy Laboratory (NREL).

The samples from compositional analysis were analyzed using HPLC with an Aminex HPX-87P column at 85 ^{0}C, using deionized water as mobile phase and refractive index detector at 50 ^{0}C. Analysis of enzyme hydrolysis samples was done using an Aminex HPX-87H column at 65 ^{0}C, 0.05N H_2SO_4 as mobile phase and a refractive index detector at 50 ^{0}C. Calculations were performed as follows:

$$\text{Glucose Yield} = \frac{\text{Concentration of glucose}}{\text{Amount of sample}} \times \text{volume of liquid} \times 100$$

$$\text{Total Glucose Yield} = \text{Glucose Yield}_{\text{enzyme hydrolysis}} + \text{Glucose Yield}_{\text{washate}}$$

$$\text{Sugar Yield} = \frac{\text{Total sugar concentration}}{\text{Amount of sample}} \times \text{volume of liquid} \times 100$$

$$\text{Total Sugar Yield} = \text{Sugar Yield}_{\text{enzyme hydrolysis}} + \text{Sugar Yield}_{\text{washate}}$$

3.3 Results and Discussion

3.3.1 Enzyme activity profiles and estimation of treatment period

Cellobiohydrolase, β-glucosidase, and β-xylosidase activities increased from the initial day until the 7th day and then decreased over the 28-day period of incubation (Figure 3.1). Beta glucosidase activity decreased gradually until 21 days, but increased again on the 28th day. Beta xylosidase and cellobiohydrolase activities decreased steeply until the 21st day and increased slightly on the 28th day. Beta glucosidase activity was significant throughout the 28-day period compared to β-xylosidase and cellobiohydrolase activities, with the highest activity of 5.5 U/ml on the 7th day of incubation. Deconstruction of cellulose network results from production of enzymes for release of free sugars necessary for metabolic growth of *P. chrysosporium* [82]. However, evidence from earlier works [58, 82] suggests that with long preprocessing time, degradation of lignin was accompanied by considerable loss in cellulose and glucose yield due to the sugar consumption by the fungi. Thus a time point (7 days from figure 3.1) with highest activity from the enzyme profiles can be an appropriate treatment time due to greater rate of sugar release compared to the rate of sugar consumed.

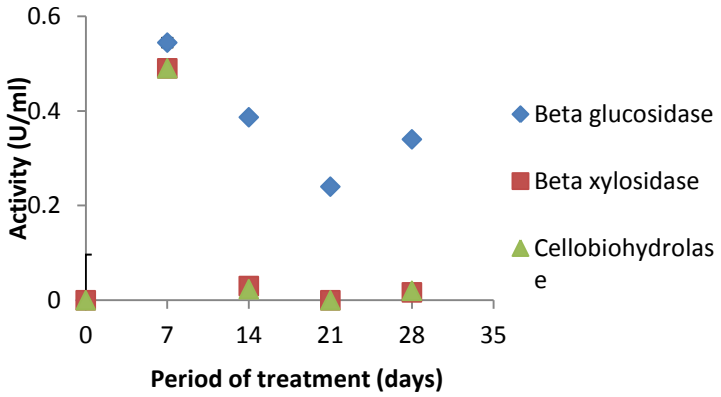

Figure 3. 1: Profile of beta glucosidase (diamond), beta xylosidase (square) and cellobiohydrolase (triangle) activities during the 28 days treatment of switch grass with *P. chrysosporium*.

Activities of phenol oxidase and peroxidase were not observed during this period which could be because of their very low concentrations. Besides, white rot fungi do not use lignin as growth substrate [83] leading to utilization of the initial glucose and a partial degradation of cellulose without the release of lignin degrading enzymes.

3.3.2 Composition Analysis

Glucan % and xylan % were determined for the samples before and after fungal treatment. The samples include treated samples (with *P. chrysosporium*), control samples (without *P. chrysosporium*) and raw switch grass sample. Glucan and xylan composition of the preprocessed (with *P. chrysosporium*) and control samples (without *P. chrysosporium*) are higher ($p < 0.005$) than the unprocessed (raw) switch grass (Figure 3.2). This supports that a higher proportion of non-cellulosic part of the switch grass underwent degradation [82]. An increase in % glucan composition for the treated samples resulted from the shorter treatment time (7 days) derived from the enzyme activity profiles. Thus activity profiles seem to give a good estimation of treatment time for obtaining increased glucan % and xylan %, in contrast with the

samples having decreased glucan % with 14 day treatment work by Shi et al [79]. Higher glucan composition also results in lesser biomass loading in the enzyme hydrolysis stage than untreated substrate for an equivalent glucan weight, thus reducing the operational costs of hydrolysis reactors.

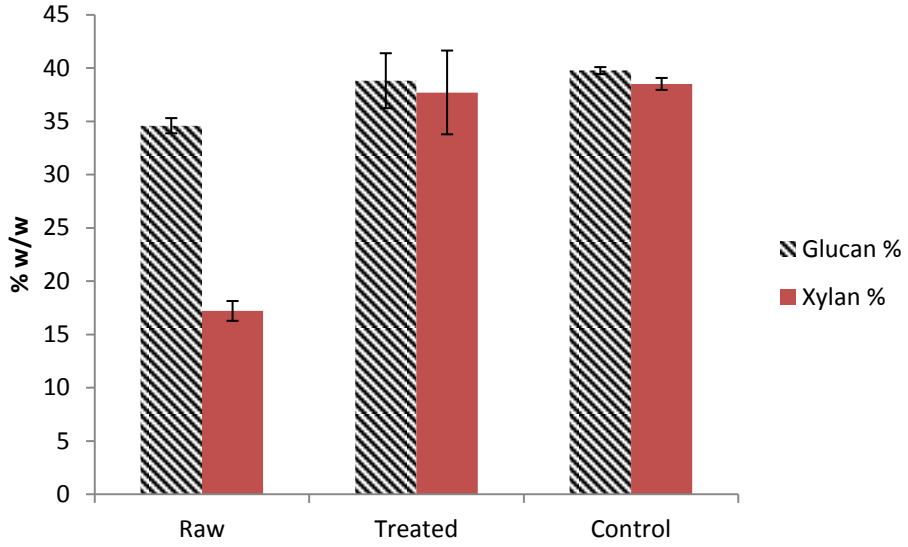

Figure 3. 2: Composition (in terms of glucan % and xylan %) of raw, treated (preprocessed with *P. chrysosporium*) and control (preprocessed without *P. chrysosporium*) samples. The error bars represent 95 % confidence interval.

3.3.3 Free Sugar Concentration

Figure 3. 3 displays the concentration of free sugars present in the liquid phase from the day-0 to day-7 of the fungal treatment. Cellobiose, glucose and mannose were the three free sugars present in significant amounts in all the samples. Glucose and mannose concentrations in the treated samples decreased with respect to both the 0 day samples and controls indicating the utilization of glucose and mannose by *P. chrysosporium*. Controls had higher free sugar concentrations than the treated samples, confirming the consumption of sugars by the fungus.

However, cellobiose concentration increased during the 7-day treatment (Figure 3.3(a)) which is a reflection of the cellobiohydrolase activity that releases cellobiose from cellulose (Figure 3.1). Moreover, cellobiose concentration in the treated samples could be lower than that in the controls, indicating its conversion to glucose due to beta glucosidase activity. Total free sugar concentration (in washate) of the treated sample was lower than the initial day and the control samples (Figure 3.3(d)) further confirming the monomeric sugar consumption by *P. chrysosporium* for metabolic growth.

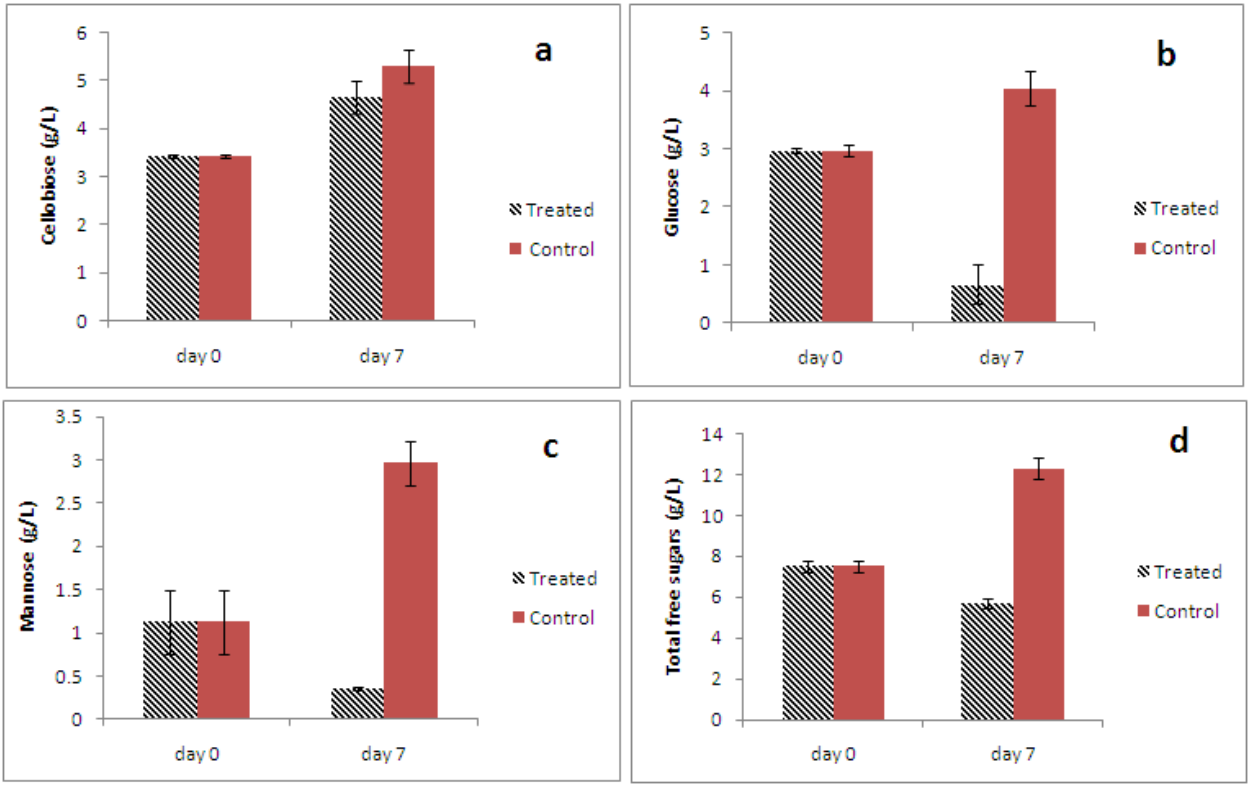

Figure 3. 3: Concentration of free sugars from the washate: (a) cellobiose (b) glucose (c) mannose and (d) concentration of total free sugars released after 7 day fungal conditioning of switch grass. The error bars represent 95 % confidence interval.

3.3.4 Glucose and Total sugar yield

Glucose yield from enzyme hydrolysis for treated samples was lower than the raw and controls samples (Figure 3. 4(a)). Enzyme hydrolysis glucose yield of the control was similar to

that of the raw sample and higher than that of the treated sample. However the total glucose yield of treated samples is significantly higher ($p < 0.05$) than the raw samples (Figure 3.4(b)) considering the glucose equivalent of cellobiose in the free sugars. Also, the controls had higher total glucose yield than the treated and raw samples, indicating the contribution of free glucose and the glucose equivalent cellobiose released during the treatment, in increasing the total glucose yields.

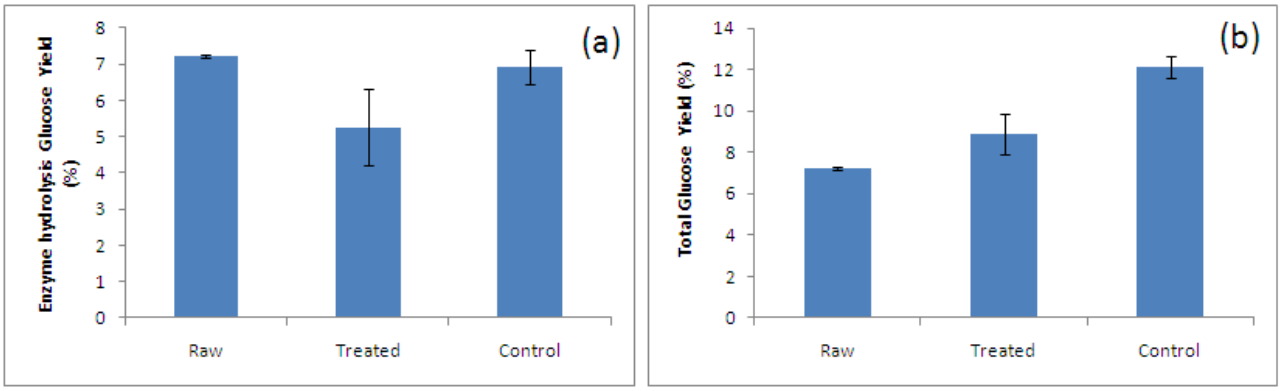

Figure 3. 4: (a) Glucose yield (%) of raw, treated and control samples of switch grass (b) Total Glucose yield (%) of raw, treated and control samples of switch grass after 72 hr Enzyme hydrolysis. The error bars represent 95 % confidence interval.

Total sugar yield per gram of switch grass evaluated for samples with and without treatment shows that, the treated samples showed a 24 % higher total sugar yield than the raw switch grass (Figure 3.5(a)). However, treated switch grass also showed lesser total sugar yield than the control. Sugar yields after 72 hr enzyme hydrolysis for raw, treated and control samples were not significantly different from each other (Figure 3.5(b)). Thus the increase in glucose and free sugar yields was majorly contributed by the free sugar release during fungal treatment, a highly desirable effect if optimized.

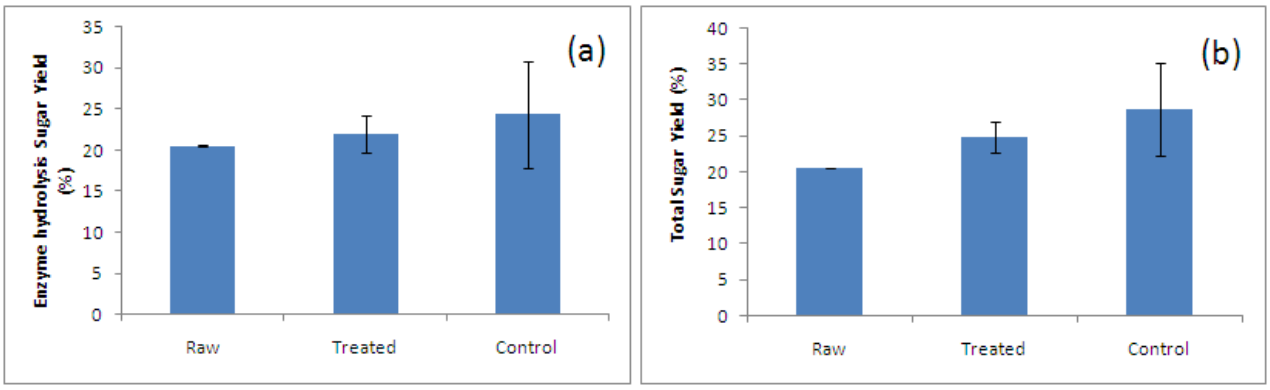

Figure 3. 5: (a) Sugar yield of raw, treated and control samples (b) Total sugar yield (%) of switch grass for raw, treated and control samples obtained after 72 hr enzyme hydrolysis. The error bars represent 95 % confidence interval.

3.4 Conclusions

A profile of β-glucosidase, β-xylosidase and cellobiohydrolase activities during fungal preprocessing of switch grass was helpful in estimating an appropriate treatment time of 7 days based on the time of highest activity. Fungal preprocessing of switch grass for 7 days with *P. chrysosporium* resulted in higher glucose yields and monomeric sugar yields. Glucan and xylan compositions were also higher for the treated sample than the raw sample. Higher sugar yields and glucose yields are attributed to the free sugars released during preprocessing of switch grass with *P. chrysosporium.* Although improvements in sugar yields and glucose yields due to the fungal treatment were not extremely high, observed improvement supports the concept of using enzyme activity profiles for initial estimation of treatment time. Further work should be pursued in the direction of decreasing treatment time during fungal preprocessing and optimizing it for higher sugar yields.

Acknowledgements

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4. PRETREATMENT AND ENZYME REQUIREMENTS OF SIZE SEPARATED FRACTIONS OF SWITCH GRASS

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Abstract

Separation based on anatomical differences such as cobs, leaves and stems for biomass such as corn and wheat straw is known to affect sugar yields during pretreatment and enzyme hydrolysis. However, separation of biomass such as switch grass into heterogeneous portions is challenging due to its undifferentiated anatomy. Size separation is a potential process for obtaining heterogeneous fractions of switch grass based on Glucan/(Lignin+Xylan) ratio and crystallinity. Pre-milled switch grass (1'' grind size) was separated into three fractions, with mesh sizes of >2 mm, 1-2 mm and <1 mm. Among the three fractions, >2 mm and 1-2 mm had similar Glucan/(Lignin+Xylan) ratios (1.3-1.5), while <1 mm size fraction had significantly (p < 0.05) different Glucan/(Lignin+Xylan) ratio (0.9-1.1). Effect of dilute sulfuric acid pretreatment conditions and enzyme loading conditions, 235 F - 260 F and 0 FPU/g - 20 FPU/g of biomass respectively, was investigated using response surface method on the three fractions. Response surface study demonstrated that <1 mm size fraction was the most recalcitrant among the other fractions (p < 0.05) and required higher pretreatment and enzyme loading conditions. Therefore, separate processing or elimination of <1 mm size fraction enriches biomass for better sugar yield during hydrolysis of biomass.

4.1 Introduction

Various biomasses such as corn stover, wheat stover and switch grass are used in the production of ethanol. It has been shown that different anatomical components in biomass vary in hydrolysis and fermentation of sugars. Rumen digestibility of stem and leaf fractions of rice straw were 36 % and 46 % respectively [84]. Hand separated fractions of wheat stover showed varied glucan, xylan, lignin and ash contents, internodes had higher glucan content (38 %) (dry basis) compared to leaves (25 %). However, leaves had higher glucan conversion (80 %) compared to internodes (77 %) and higher ethanol yield compared to other fractions [85]. Similarly, cobs, leaves, and husks from corn stover showed varying glucose concentrations after hydrolysis [86]. Dilute sulfuric acid pretreatment and NaOH pretreatment of different anatomical fractions of corn stover showed that cobs, husks and leaves responded best to the pretreatment, top of stalks slightly less and bottom of stalks the least [87]. Duguid et al. [87] suggested that integrating biomass collection with the process of removal of low yielding fraction minimizes ethanol production costs. Moreover, the required pretreatment conditions for each of the anatomical fractions could differ from each other. In an another study, hybrid poplar mixed with sparse wood chips was pretreated to obtain fractions with varying lignin content, acetyl content and crystallinity to study their effects on the enzymatic digestibility. Lignin removal and reduction in crystallinity greatly enhanced hydrolysis yield and the initial hydrolysis rate respectively [88].

It is evident from these previous studies that, separation based on anatomical differences allows effective use of enzymes for hydrolysis. However, hand separation of anatomical fractions, is a time consuming and a labor intensive process. Additionally, hand separation is not suitable for biomass such as switch grass, due to its undifferentiated anatomy unlike corn stover,

which can be separated into cobs, husks, leaves and stalks. Therefore, a mechanized process which can separate the heterogeneous fractions of biomass is desirable.

Papatheofanous et al. showed that wheat straw milled and sieved into two major fractions, chip fraction consisting of stem internodes and meal fraction consisting of leaves, nodes and husks, varied in composition [89]. Therefore, separation of heterogeneous components in biomass can be achieved by sieving milled biomass into fractions based on varying particle size. Particle size distribution is a potential indicator of compositional and crystallinity differences, and is a valuable tool to identify the heterogeneous fractions in biomass such as switch grass. Although investigations on biomass size separation were conducted earlier [89, 90], pretreatment and enzyme requirements during hydrolysis, for size separated fractions were not extensively studied. Additionally, release of inhibitory by-products during pretreatment may vary among fractions.

The objective of this research was to investigate biomass size separation as a potential process to separate the heterogeneous fractions present in switch grass. Pre-milled switch grass (1'' grind) was separated into three fractions >2 mm, 1-2 mm and <1 mm size and their composition, in terms of glucan, xylan, acid insoluble lignin and ash, was determined. The three fractions and un-partitioned switch grass (UP) were pretreated with dilute sulfuric acid (0.69 % w/v) at different temperatures, 235 °F, 239 °F, 248 °F, 257 °F and 260 °F for 30 minutes. Sulfuric acid pretreatment with varying acid concentration (0.69 % to 10 %) at 260 °F for 30 minutes was conducted on the three fractions and UP, to investigate the release of degradation products such as 5-(Hydroxymethyl) furfural (HMF), furfural and phenolic compounds which are fermentation inhibitors. Response surface method was employed to investigate and compare the pretreatment temperature and enzyme loading requirements for the three fractions and UP.

4.2 Materials and Methods

4.2.1 Size separation and preparation of biomass

Switch grass of 1'' grind size is obtained from BioDimensions, Memphis, TN. A stacked sieve system, comprising U.S.A Standard Testing Sieves (arranged in a top to bottom sequence) #10 (2 mm), #18 (1 mm) and a collection pan, was used for separating the 1'' grind switch grass into three fractions, >2 mm (material remained above the #10 pan), 1-2 mm (material remained below the #10 sieve and above the #18 sieve) and <1 mm (material remaining in the collection pan). A known amount of un-partitioned switch grass (UP) is taken in the pan #10, of the staked sieve system, and subjected to manual shaking for a minute. This procedure was repeated five times and the fractions obtained were weighed and collected separately for further experiments. The weight fraction of each size fraction in the UP is represented the Figure 4.1 for all the size fractions. The UP and the three fractions were subjected to milling in an IKA MF 10.1 impact mill with an internal 1 mm circular screen, in order to eliminate the effect of the particle size for further experiments.

4.2.2 Design and analysis of experiments

A rotatable central composite design (CCD) is chosen to determine the effect of pretreatment temperature and enzyme loading on glucose yield during enzyme hydrolysis. Table 4.1, represents the set of experiments for various conditions of enzyme loading (B) on biomass pretreated with dilute sulfuric acid at various temperatures (A) in duplicate, and is generated by the statistical software Design-Expert 8.0.3. The UP and the three fractions obtained from sieving are subjected to dilute sulfuric acid pretreatment at temperatures 235 °F, 239 °F, 248 °F, 257 °F and 260 °F as mentioned in section 4.2.3.1. The enzyme hydrolysis of the pretreated samples is described in section 4.2.5. Analysis of variance and significance test of the empirical

equations are presented in the Table 4.2. Estimated coefficients of empirical equations are presented in Table 4.3.

Table 4. 1: Experimental design conditions (temperature and enzyme loading) and the responses (glucose yield (%)) for UP (R1), >2 mm (R2), 1-2 mm (R3), <1 mm (R4).

Run	Temperature (F), A		Enzyme (FPU/g), B		UP Glucose yield (%), R1	>2 mm Glucose yield (%), R2	1-2 mm Glucose yield (%), R3	<1 mm Glucose yield (%), R4
	Coded	Uncoded	Coded	Uncoded				
1	0.00	248	0.00	10.00	17.25	15.65	17.87	16.20
2	0.00	248	1.41	20.00	20.14	18.87	20.14	18.91
3	0.00	248	0.00	10.00	17.34	15.66	18.38	16.29
4	-1.00	239	-1.00	2.93	12.45	12.38	15.20	11.14
5	1.00	257	1.00	17.07	22.17	21.97	26.34	19.79
6	-1.00	239	1.00	17.07	17.05	17.09	17.73	15.22
7	-1.41	235	0.00	10.00	15.07	15.93	16.75	13.50
8	1.00	257	1.00	17.07	21.74	23.87	26.54	19.41
9	-1.00	239	-1.00	2.93	11.65	12.07	14.14	10.42
10	0.00	248	0.00	10.00	17.40	15.73	19.65	16.34
11	0.00	248	0.00	10.00	17.18	15.31	18.30	16.14
12	0.00	248	0.00	10.00	17.70	15.86	19.04	16.62
13	1.41	260	0.00	10.00	21.85	20.44	21.25	20.76
14	1.41	260	0.00	10.00	22.83	21.42	23.74	21.70
15	0.00	248	0.00	10.00	16.98	15.06	18.00	15.95
16	1.00	257	-1.00	2.93	17.11	15.98	19.81	15.32
17	0.00	248	0.00	10.00	16.25	15.32	18.93	15.26
18	0.00	248	0.00	10.00	17.07	14.72	18.79	16.03
19	0.00	248	0.00	10.00	16.93	15.25	18.17	15.90
20	0.00	248	0.00	10.00	15.72	14.13	18.27	14.77
21	-1.41	235	0.00	10.00	15.07	15.51	16.51	13.50
22	0.00	248	1.41	20.00	20.04	18.70	21.35	18.81
23	-1.00	239	1.00	17.07	16.46	17.30	19.07	14.70
24	0.00	248	-1.41	0.00	0.64	1.24	0.90	0.64
25	0.00	248.00	-1.41	0.00	0.64	1.24	0.90	0.64
26	1.00	257.00	-1.00	2.93	16.74	16.86	19.13	14.99

4.2.3 Pretreatment

4.2.3.1 Dilute sulfuric acid pretreatment

Dilute sulfuric acid pretreatment was carried out on >2 mm, 1-2 mm, <1 mm and UP samples obtained in section 4.2.1. The reaction mixture, comprising 5 g of biomass, 95 mL of water and 959 µL of 72 % H_2SO_4, was subjected to autoclaving at 235 °F, 239 °F, 248 °F, 257 °F and 260 °F for 30 minutes in tightly capped 250 mL flasks. After pretreatment, the flasks were allowed to cool at room temperature, the samples were vacuum filtered and the residue was washed with 100 mL of water. The residue was dried at 40 °C in a convection oven for 24 hours and stored at -4 ºC for further experiments. The collected filtrate was measured for concentration of sugars.

4.2.3.2 Sulfuric acid pretreatment

In another set of experiments, >2 mm, 1-2 mm, <1 mm and UP samples were subjected to 0.69 %, 2 %, 5 % and 10 % concentrations of sulfuric acid pretreatment for 30 minutes in 20 mL tightly capped hungate tubes at 260 °F. The reaction mixture comprised 1 g biomass and 10 mL of H_2SO_4 (0.097 mL, 0.28 mL, 0.69 mL and 1.39 mL of 72 % H_2SO_4 made up to 10 mL with water, to make 0.69 %, 2 %, 5 % and 10 % H_2SO_4, respectively). The tubes were allowed to cool to room temperature, and the reaction mixture was filtered and washed to obtain filtrate and residue. The filtrate was analyzed for sugars, furfural, hydroxyl methyl furfural (HMF) and polyphenols using HPLC.

4.2.4 Composition analysis and Crystallinity Index measurement

Composition analysis of samples was performed using NREL's standard operating procedure with some modifications. A 0.1 g sample of biomass was taken in a pressure tube and 1 ml of 72 % H_2SO_4 was added. The sample was mixed and incubated at 30 °C for 1 hour. Periodic mixing was done for every 10 minutes using a glass rod, to allow uniform particle to acid contact. Upon completion of an hour of incubation with 72 % H_2SO_4, 25 mL of water was added to the sample to dilute the acid to 4 %. The pressure tubes were tightly capped and autoclaved for 1 hour at 121 °C. The samples obtained after autoclaving were allowed to cool to room temperature and filtered into 125 mL flasks using glass crucibles. All samples are tested in duplicate.

The glass crucibles were previously dried at 100 °C, cooled in a desiccator and recorded for weight before using for filtration. The acid insoluble lignin remaining in the crucible after filtration was subjected to heat in an oven at 100 °C for 24 hours. The difference in weight after the 24 hour heating at 100 °C was recorded as acid soluble lignin in the sample. To the clear hydrolyzate obtained from filtration, $CaCO_3$ was added slowly to neutralize the acid to a pH between 5 and 6. The neutralized solution was decanted, and the supernatant was filtered using 0.2 µm syringe filter into a glass vial for High Performance Liquid Chromatography (HPLC) analysis. The samples were stored at -4 °C for a week before analysis.

Ash analysis of samples was done by taking about 0.1 g of dry biomass sample in a dry crucible (previously dried in a heat oven at 100 °C and stored in a desiccator). The crucible with the sample was weighed before subjecting it to 575 °C in a furnace for 24 hour. The crucibles were cooled in a desiccator and weighed to record the difference as the amount of ash in the biomass sample.

Biomass crystallinity was measured by powder X-ray diffraction method using X'Pert PRO (PANalytical). Biomass samples obtained from section 4.2.1 were further reduced to # 45 sieve size and used for analysis. The samples were scanned at 1°/min from 2θ = 10° to 40° with a step size of 0.05°. Crystallinity Index (*CrI*), the percentage of crystalline material in biomass is defined in equation (1)

$$CrI = (I_{002} - I_{am})/I_{002} \times 100 \quad (1)$$

I_{002} is the intensity of the peak at 2θ = 25^0 and I_{am} is the intensity of the background scatter at 2θ = 21.5^0. The values of 2θ measured are different from the other studies [88, 91] as cobalt was the X-ray source in the present study.

4.2.5 Enzyme hydrolysis

The biomass samples obtained from section 4.2.3.1 were enzyme hydrolyzed using Celluclast and Novozyme 188. A 0.5 g glucan equivalent of biomass was taken in a 125 mL flask, 25 mL of citric acid buffer pH 4.8, 0.4 mL of tetracycline, appropriate amount of Celluclast and Novozyme 188 (β-glucosidase) (2.8 CBU of Novozyme 188 for every 1FPU of Celluclast) as per the experimental design mentioned in Table 1, were added and made up to a final volume of 50 mL with water and the flasks were incubated at 50 °C at 130 rpm for 72 hours. After the 72 hour enzyme hydrolysis, the supernatant was filtered using 0.2 µm syringe filters in to a vial and analyzed using HPLC. The samples were stored at -4 °C for a week before analysis.

4.2.6 HPLC Analysis

Samples from section 4.2.4 are analyzed for sugars using Aminex HPX 87-P column, with injection volume of 20 µL, at column temperature 85 °C, refractive index detector at 50 °C, with de-ionized water as mobile phase at 0.6 mL/ min and run time of 30 minutes for each sample.

Samples from section 4.2.3 and 4.2.5 are analyzed for sugars, HMF and furfural using Aminex HPX 87-H column, with injection volume of 20 µL, at column temperature 65 °C, refractive index detector at 50 °C, with 0.005 N H_2SO_4 as mobile phase at 0.6 mL/min. Run time for samples in section 2.5 was 50 minutes for each sample.

4.2.7 Polyphenols analysis

Samples from section 4.2.3.2 are analyzed for polyphenols concentration using the method developed by Graham [92]. The reaction mixture comprised 3 mL hydrolyzate sample from section 2.3.2, 1 mL 0.016 M $K_3Fe(CN)_6$ and 1 mL 0.02 M $FeCl_3$ in 0.1 M HCl, the contents were mixed well and allowed to stand at 24 °C for 15 minutes. A blue unstable precipitate was obtained, to which, 3 mL of 6.03 M H_3PO_4 was added, mixed well and allowed to stand for 2 minutes. After 2 minutes, the reaction was stopped by adding 2 mL of 1 % gum acacia solution and mixed well. The final solution was measured for color density at 700 nm using a UV-Vis spectrophotometer. Tannic acid was used as the calibration standard with concentrations between 5-40 µg in 3 mL.

4.3 Results and Discussion

4.3.1 Size separation

The weights of the fractions >2 mm, 1-2 mm and <1 mm obtained after sieving using the stacked sieving system as mentioned in section 4.2.1 are recorded and represented as weight of the fraction in the UP in Figure 4.1. From figure 4.1, >2 mm is 68 %, 1-2 mm is 22 % and <1 mm is 9 % of the UP, where >2 mm fraction constitutes the greatest and the <1 mm fraction constitutes the lowest weight in the UP. Therefore, any major chemical or physical differences that are observed in these fractions will be potential sources of heterogeneity in the un-partitioned material due to the observed weight distributions.

4.3.2 Composition analysis of unpretreated and pretreated fractions

The three fractions and the UP before pretreatment were subjected to composition analysis as mentioned in section 4.2.4 and the results are shown in figure 4.1. Glucan, xylan, lignin and ash content of the four materials UP, >2 mm, 1-2 mm and <1 mm were determined as mentioned in section 4.2.4. The UP had significantly higher glucan % than <1 mm and similar glucan % as that of >2 mm and 1-2 mm samples. Xylan % of the UP was significantly lower than that of the three fractions. The <1 mm sample had the highest lignin % among the samples and had significantly higher lignin % than the UP. Ash content of <1 mm sample was significantly higher than the other samples. Therefore, <1 mm sample had lower glucan %, higher lignin % and higher ash % compared to other samples. Alternately, the UP sample had higher glucan %, lower lignin % and lower ash % compared to other samples. Cellulose imparts strength and flexibility to biomass [93] and it is known that, lower cellulose content leads to increased fragility in biomass samples [87], therefore, <1 mm sample having lower glucan % is more fragile and less resistant to mechanical disruption, while >2 mm sample having higher

glucan % is less fragile and more resistant to size reduction. Figure 4.1 also shows the crystallinity indices (*CrI*) of the four samples, *CrI* of <1 mm and UP are similar and higher than that of the other samples, and *CrI* of 1-2 is lower than other samples. Therefore, with decreasing size of the fraction, the *CrI* increased. It is known that the enzymes attack the finest particles first [94], which in the present study is <1 mm fraction, which contains higher lignin %, higher ash %, lower glucan % and higher *CrI*, leading to irreversible binding of lignin to the active sites of cellulase, reducing their activity for cellulose conversion during enzyme hydrolysis and producing inhibitory compounds during acid pretreatment. Therefore, composition analysis of the three fractions suggests separation of <1 mm sample from the UP due to its contribution towards heterogeneity in the UP, moreover, separation of <1 mm sample from UP results in reduction of ash by approximately 17 %.

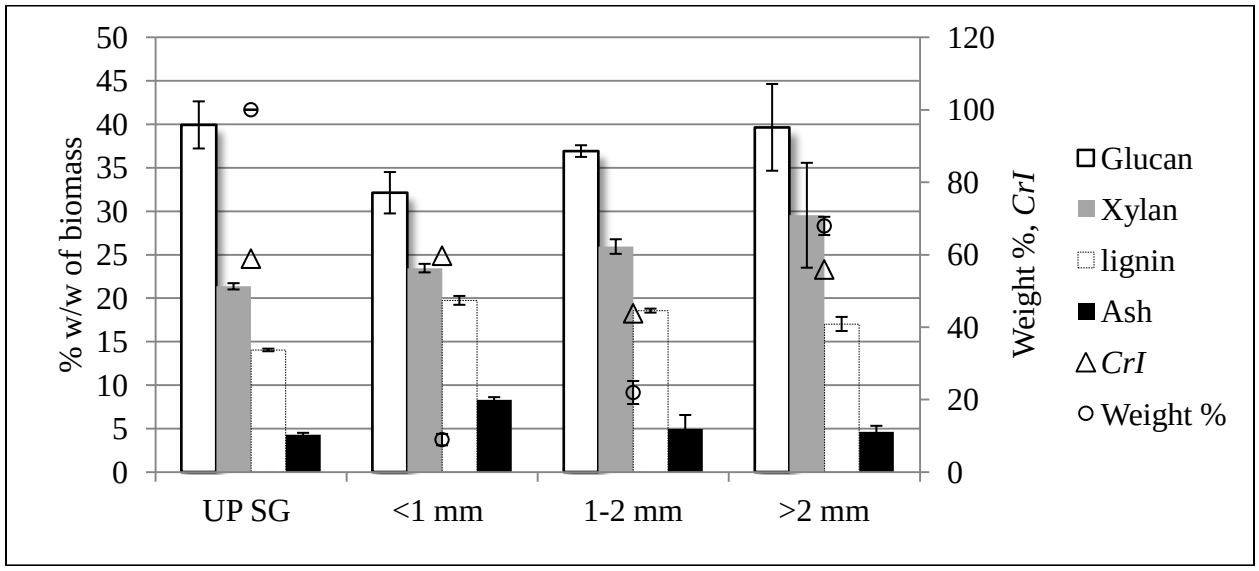


Figure 4. 1: Composition of un-pretreated UP, <1 mm, 1-2 mm and >2 mm samples in terms of Glucan, Xylan, Lignin, Ash (% w/w of biomass). Triangles (Δ) represent the crystallinity index (*CrI*) of the samples and (O) represent the weight % in UP (secondary axis).

The UP and the three fractions after dilute acid pretreatment at 235 °F, 248 °F and 260 °F for 30 minutes, as mentioned in section 4.2.3.1, were subjected to composition analysis as

mentioned in section 4.2.4 and the results are shown in figure 4.2. Figure 4.2 presents a comparison of (a) glucan %, (b) xylan % and (c) lignin % for the UP and the three fractions at various pretreatment temperatures. Figure 4.2(a) shows that >2 mm has higher glucan % and <1 mm has lower glucan % than other samples, from figure 4.2(b), xylan % of the samples are not significantly different from each other. However, xylan % of all the samples decreased with increasing pretreatment temperature due to hemicellulose solubilization during pretreatment. Figure 4.2(c), shows that lignin % of <1 mm is significantly higher than that of the UP and other fractions. Increase in pretreatment temperature has an increasing effect on glucan % and lignin % for all samples. The trend of xylan % among samples after pretreatment is similar to that of before pretreatment. However, the trend of glucan % and lignin % for UP and fractions after pretreatment is different from that of before pretreatment, that is, the maximum improvement in glucan % after pretreatment for UP is by 20 % and that for >2 mm sample is 25 %. Similarly, the maximum improvement in lignin % after pretreatment for <1 mm is by 10 % and that of >2 mm is by 5.5 %. As mentioned earlier, the compositional differences in the fractions are enhanced due to the effect of pretreatment consequently affecting the heterogeneity of pretreated UP. Therefore, separate pretreatment of the fractions will enrich the glucan content of >2 mm sample which constitutes 68 % by weight in UP and the lignin enriched <1 mm fraction, could be employed for other applications.

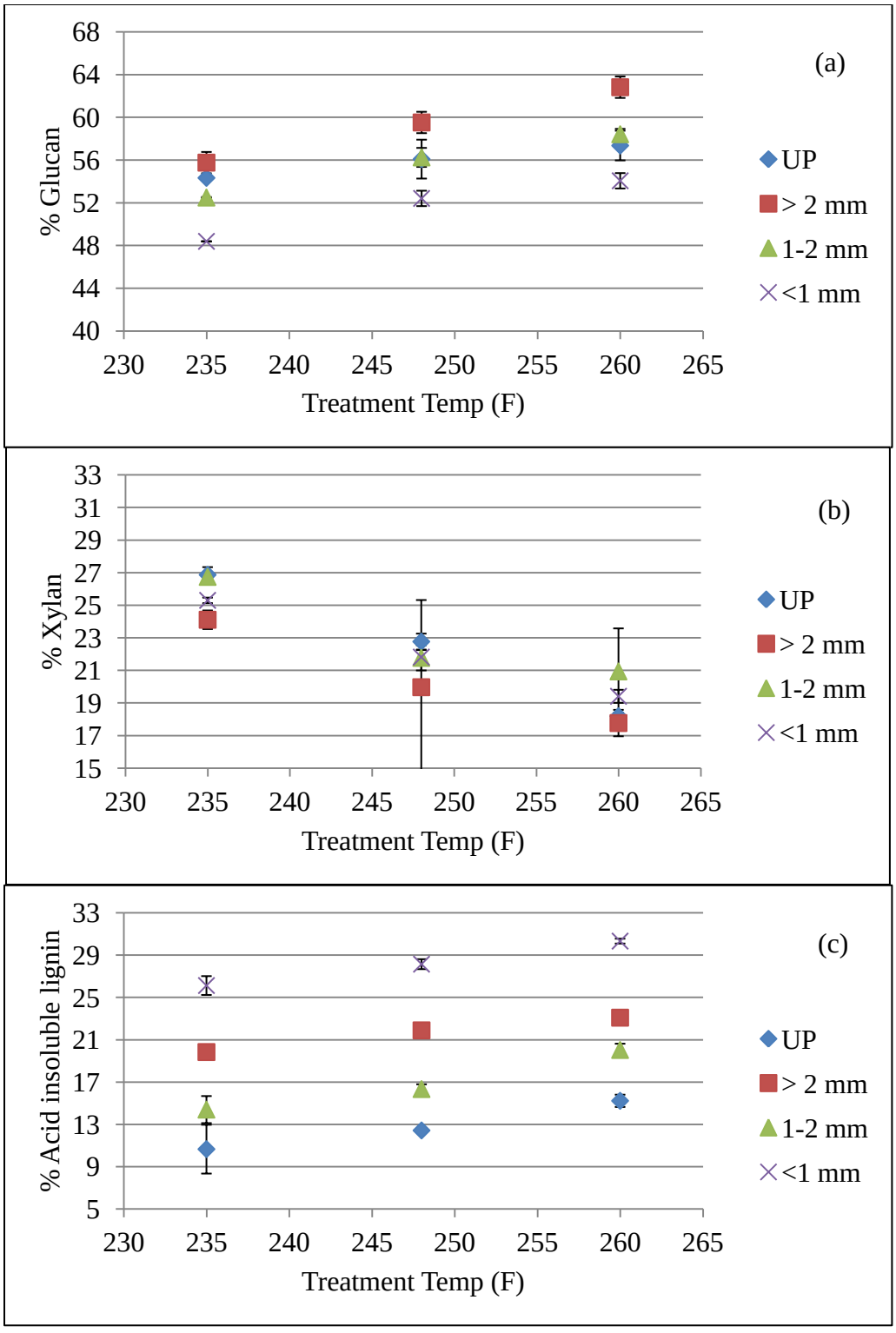


Figure 4. 2: Composition, (a) Glucan, (b) Xylan, and (c) Lignin (% w/w) of dilute sulfuric acid pretreated UP SG, <1 mm, 1-2 mm and >2 mm samples at 235 °F, 239 °F, 248 °F, 257 °F and 260 °F for 30 minutes.

Figure 4.3 shows a comparison of Glucan/(Lignin+Xylan) (GLX) ratio for UP and the three fractions before and after dilute sulfuric acid pretreatment at different temperatures (section 2.3.1). From figure 4.3, the three fractions and UP showed an increase in GLX ratio with increasing pretreatment temperatures and the ratio decreased with decreasing size fraction. The smallest size fraction, <1 mm has the least GLX ratio and UP has the highest GLX ratio both before and after pretreatment at different temperatures. The GLX ratios of >2mm and 1-2 mm are similar to each other before and after pretreatment at all temperatures. It is known that cellulose/lignin ratio is an important factor that dictates biochemical conversion of biomass [95]. Moreover, from the above discussion, decreasing size of the fraction with decreasing GLX ratio concurs with Lee [93] that cellulose imparts strength to biomass, therefore separation of pre-milled biomass into heterogeneous fractions by sieving, can be based on significant differences in GLX ratios.

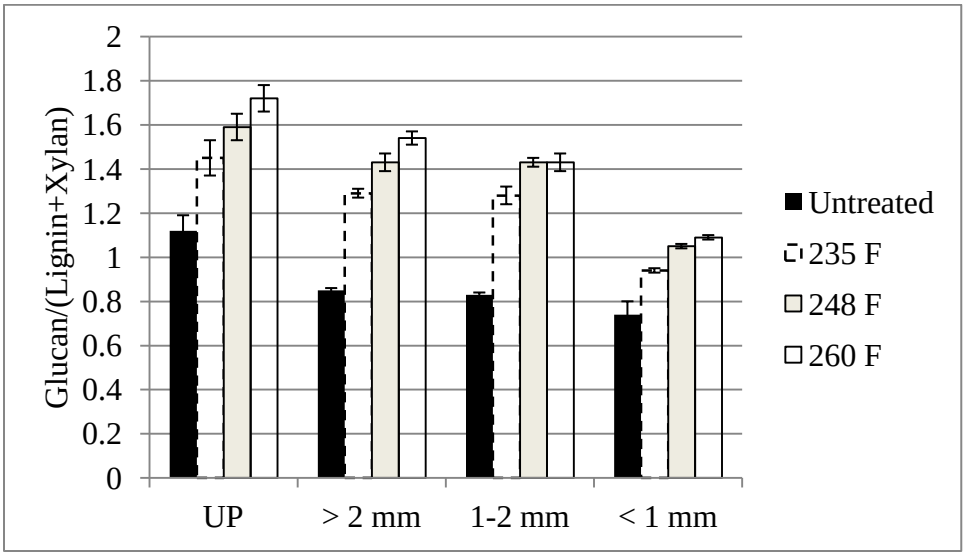
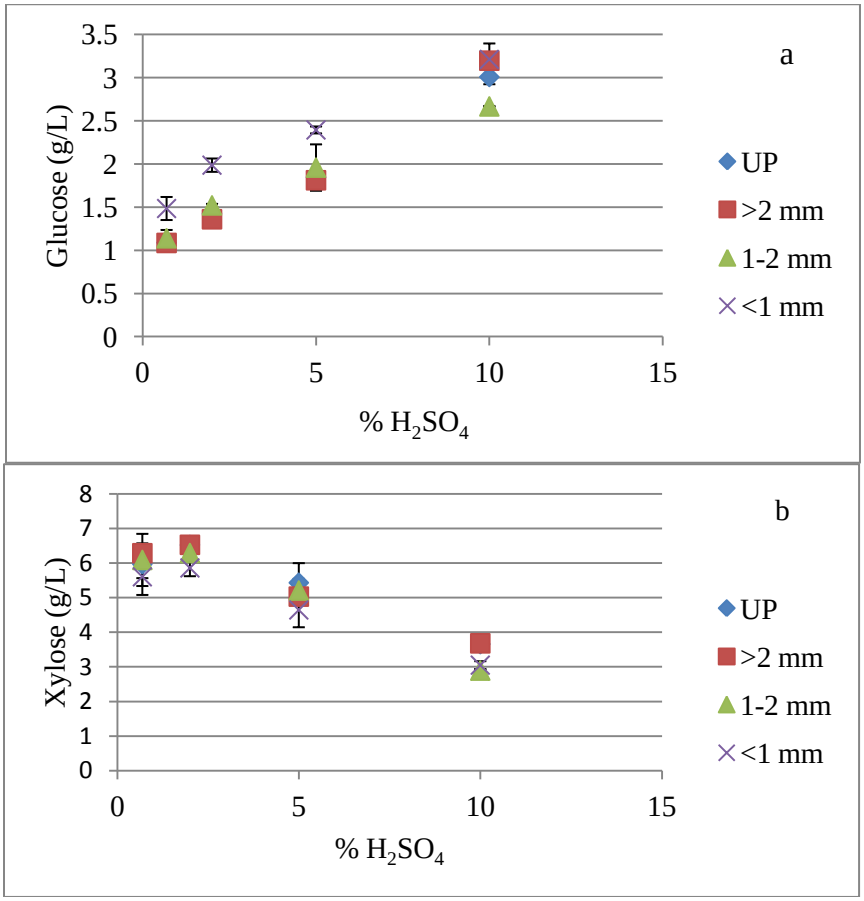


Figure 4. 3: Ratio of Glucan and (Lignin+ Xylan) for untreated and dilute sulfuric acid pretreated (at 235°F, 248°F and 260 °F) UP SG, <1 mm, 1-2 mm and >2 mm samples.

4.3.3 Byproducts of sulfuric acid pretreatment of different size fractions of switch grass

Pretreatment of samples with various concentrations of sulfuric acid (0.69 %, 2 %, 5 % and 10 %) at 260 °F for 30 minutes was conducted on UP and the three fractions (section 4.2.3.2), to observe the amount of sugars and inhibitory compounds released during the process. The results are presented in the figure 4.4. Figures 4.4(a), 4.4(b) and 4.4(c) represent the concentrations of glucose, xylose and arabinose respectively in the pretreatment hydrolyzate and figures 4.4(e) and 4.4(f) represent the concentration of furfural and polyphenols respectively for UP, and the three fractions. From figure 4.4(a), glucose concentration in the hydrolyzate increased with the increasing sulfuric acid concentration and the glucose concentration for <1 mm sample was higher compared to the other samples for pretreatment at 0.69 %, 2 % and 5 % sulfuric acid concentration. At 10 % H_2SO_4 pretreatment, glucose concentrations of UP, >2 mm and <1 mm samples in the hydrolyzate were similar, and that of 1-2 mm was lesser than that of the other fractions. Higher glucose concentration in the hydrolyzate for <1 mm sample until 5 % H_2SO_4 could be due to greater H_2SO_4 availability to cellulose in the sample. Figure 4.4(b) shows that the xylose concentration in the hydrolyzate for all samples is decreasing with increasing H_2SO_4 concentration and there is no significant difference among the fractions. Arabinose concentration (figure 4.4(c)) of <1 mm is higher than that of other samples at 2 % and 10 % H_2SO_4 pretreatment. From figure 4.4(d), furfural concentration of all samples increased with increasing H_2SO_4 concentration, and at 10 % H_2SO_4 pretreatment, <1 mm sample showed lesser furfural concentration compared to that of other samples. Polyphenols concentration decreased with increasing H_2SO_4 concentration during pretreatment (figure 4.4 (e)) however, <1 mm showed higher concentration than the other samples at 10 % H_2SO_4 pretreatment. HMF concentrations in hydrolyzate for all samples were similar at all pretreatment concentrations and

showed no effect of increasing pretreatment conditions (not shown in the figure 4.4). Therefore, at low pretreatment severities, <1 mm sample showed higher glucose release and at high severity it showed higher polyphenols release in the hydrolyzate compared to other fractions and UP. Hence, it is important to note that, <1 mm sample produced degradation products both at low and high pretreatment severities which can be otherwise minimized by treating <1 mm sample separately at different conditions.



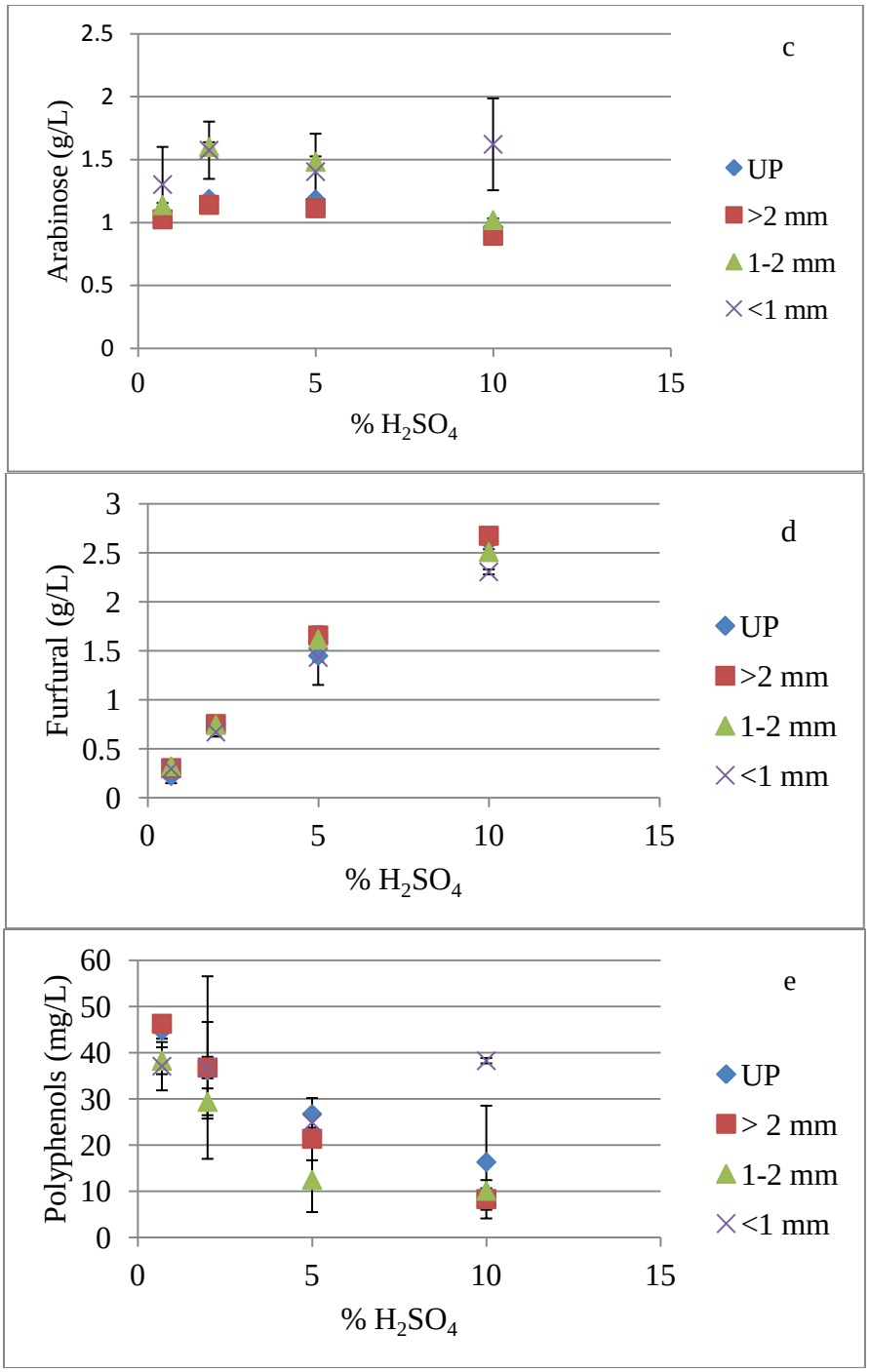


Figure 4. 4: Concentration of sugars (a) Glucose, (b) Xylose and (c) Arabinose in g/L, and inhibitory compounds (d) Furfural and (e) Polyphenols in the hydrolyzate after pretreatment of UP, >2mm, 1-2 mm and <1 mm samples with 0.69 %, 2 %, 5 % and 10 % sulfuric acid at 260 °F for 30 minutes.

4.3.4 Analysis of response surface

Statistical significance of the respective model was checked using *F*-test analysis of variance (table 4.2) for all the fractions and UP. Glucose yield is the response variable for which the CCD was analyzed, A- pretreatment temperature (ºF) and B- enzyme loading (FPU/g biomass). Table 4.2 represent ANOVA of glucose yield of (a) UP (R1), (b) >2 mm (R2), (c) 1-2 mm (R3) and (d) <1 mm (R4) respectively. Since the ratio of maximum to minimum glucose yield in the experimental sets is observed to be >10, the response variables R1, R2, R3 and R4 were power transformed. The probability (*p*-value < 0.0001) for empirical equations indicate that the fits are highly significant and insignificant lack-of-fits (*p*-value > 0.05) for equations indicate that the experimental data is in good agreement with the empirical equations [96]. The equations for all the samples are reduced cubic equations, since insignificant terms from the complete cubic equation are eliminated. Estimated coefficients and the empirical equations in coded terms of A and B are presented in table 4.3 for (a) UP, (b) >2 mm, (c) 1-2 mm and (d) <1 mm samples respectively. The fitness of the equations (R^2) for UP, >2 mm, 1-2 mm and <1 mm are 0.984, 0.973, 0.973 and 0.988 respectively, indicate their respective response variability. Reasonable agreement between the Pred R^2 and Adj R^2 for all the samples in tables 4.3 (a), (b), (c) and (d) indicate that the reduced cubic empirical equations fit the experimental data adequately. The equations in coded factors for UP and >2 mm are similar, equations for 1-2 mm and <1 mm have higher and lower values of coefficients respectively than others, therefore indicating that 1-2 mm and <1 mm has higher and lower glucose yield respectively, than other fractions. Therefore from empirical equations, <1 mm is the most recalcitrant fraction and 1-2 mm the most desirable fraction among the fractions.

Table 4. 2: ANOVA of power transformed glucose yield for (a) UP, (b) >2 mm, (c) 1-2 mm and (d) <1 mm, where A is the pretreatment temperature (ºF) and B is the enzyme loading (FPU/ g of biomass)

(a) Analysis of variance for power transformed glucose yield of UP ($R_1^{2.03}$)					
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	488277	6	81379	197	< 0.0001
A	162060	1	162060	392	< 0.0001
B	194697	1	194697	471	< 0.0001
AB	2339	1	2339	5	0.0279
A^2	28983	1	28983	70	< 0.0001
B^2	25122	1	25122	61	< 0.0001
A^2B	16643	1	16643	40	< 0.0001
Residual	7840	19	412		
Lack of Fit	1674	2	837	2	0.1298
Pure Error	6166	17	362		
Total	496118	25			
(b) Analysis of variance for power transformed glucose yield of >2 mm ($R_2^{2.11}$)					
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	782863	6	130477	112	< 0.0001
A	203406	1	203406	175	< 0.0001
B	403543	1	403543	348	< 0.0001
AB	14211	1	14211	12	0.0024
A^2	71913	1	71913	62	< 0.0001
B^2	14197	1	14197	12	0.0024
A^2B^2	18856	1	18856	16	0.0007
Residual	22019	19	1158		
Lack of Fit	6053	2	3026	3	0.0651
Pure Error	15966	17	939		
Total	804883	25			

(c) Analysis of variance for power transformed glucose yield of 1-2 mm ($R_3^{2.32}$)					
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4980441	7	711491	91	< 0.0001
A	485250	1	485250	62	< 0.0001
B	2209692	1	2209692	284	< 0.0001
AB	218977	1	218977	28	< 0.0001
A^2	66310	1	66310	8	0.0091
B^2	269179	1	269179	34	< 0.0001
AB^2	95680	1	95680	12	0.0025
A^2B^2	331280	1	331280	42	< 0.0001
Residual	139928	18	7773		
Lack of Fit	14607	1	14607	2	0.1773
Pure Error	125321	17	7371		
Total	5120370	25			

(d) Analysis of variance for power transformed glucose yield of <1 mm ($R_4^{1.57}$)					
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	21436.67	6	3572.78	264	< 0.0001
A	3798.27	1	3798.27	280	< 0.0001
B	10024.37	1	10024.37	740	< 0.0001
A^2	683.27	1	683.27	50	< 0.0001
B^2	2312.37	1	2312.37	170	< 0.0001
A^2B	1519.25	1	1519.25	112	< 0.0001
AB^2	107.17	1	107.17	8	0.0111
Residual	257.13	19	13.53		
Lack of Fit	43.77	2	21.88	2	0.2047
Pure Error	213.36	17	12.55		
Total	21693.80	25			

Table 4. 3: Estimated coefficients for power transformed glucose yield and the final model equation for (a) UP, (b) >2 mm, (c) 1-2 mm and (d) <1 mm

(a) Estimated coefficients for power transformed glucose yield of UP ($R_1^{2.03}$)

Factor	Coefficient Estimate	df	Standard Error	95 % CI Low	95 % CI High
Intercept	314.30	1	6.42	300.85	327.74
A	100.64	1	5.08	90.01	111.27
B	156.00	1	7.18	140.97	171.04
AB	17.10	1	7.18	2.07	32.13
A^2	45.64	1	5.45	34.24	57.04
B^2	-42.49	1	5.45	-53.89	-31.09
A^2B	-64.50	1	10.16	-85.76	-43.25

Final Equation in Terms of Coded Factors:

$R_1^{2.03} = 314.30 + 100.64A + 156.00B + 17.10AB + 45.64A^2 - 42.49B^2 - 64.50A^2B$

$R^2 = 0.984$; Adj $R^2 = 0.979$; Pred $R^2 = 0.972$

(b) Estimated coefficients for power transformed glucose yield of >2 mm ($R_2^{2.11}$)

Factor	Coefficient Estimate	df	Standard Error	95 % CI Low	95 % CI High
Intercept	315.02	1	10.77	292.49	337.55
A	112.75	1	8.51	94.94	130.56
B	158.81	1	8.51	141.00	176.63
AB	42.15	1	12.04	16.96	67.34
A^2	79.32	1	10.07	58.25	100.40
B^2	-35.25	1	10.07	-56.32	-14.17
A^2B^2	68.66	1	17.02	33.03	104.29

Final Equation in Terms of Coded Factors:

$R_2^{2.11} = 315.01 + 112.75A + 158.81B + 42.15AB + 79.32A^2 - 35.25B^2 + 68.66A^2B^2$

$R^2 = 0.973$; Adj $R^2 = 0.964$; Pred $R^2 = 0.938$

(c) Estimated coefficients for power transformed glucose yield of 1-2 mm ($R_3^{2.32}$)

Factor	Coefficient Estimate	df	Standard Error	95 % CI Low	95 % CI High
Intercept	876.01	1	27.88	817.43	934.58
A	246.29	1	31.17	180.79	311.78
B	371.63	1	22.04	325.32	417.94
AB	165.45	1	31.17	99.95	230.94
A^2	76.17	1	26.08	21.38	130.97
B^2	-153.47	1	26.08	-208.26	-98.68
AB^2	154.66	1	44.08	62.04	247.28
A^2B^2	287.79	1	44.08	195.17	380.40

Final Equation in Terms of Coded Factors:

$R_3^{2.32} = 876.01 + 246.29\ A + 371.63\ B + 165.45\ AB + 76.17\ A^2 - 153.47\ B^2 + 154.66\ AB^2 + 287.7$

A^2B^2

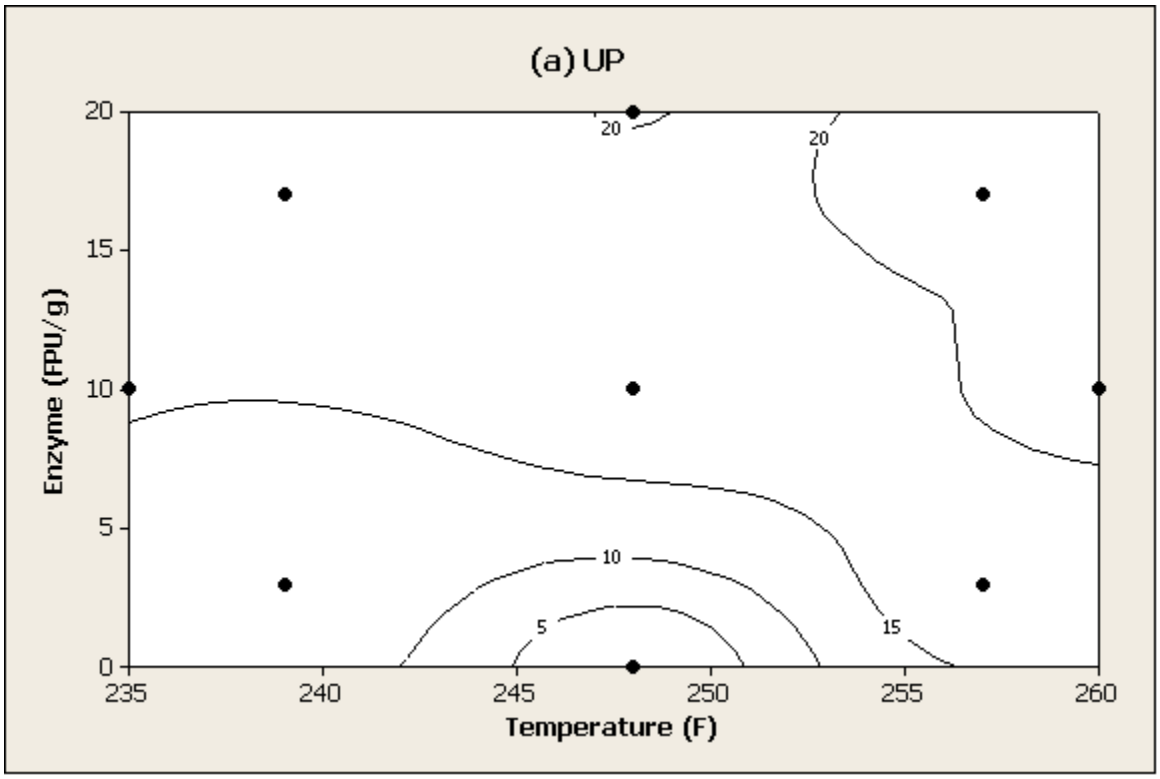
$R^2 = 0.973$; Adj $R^2 = 0.962$; Pred $R^2 = 0.918$

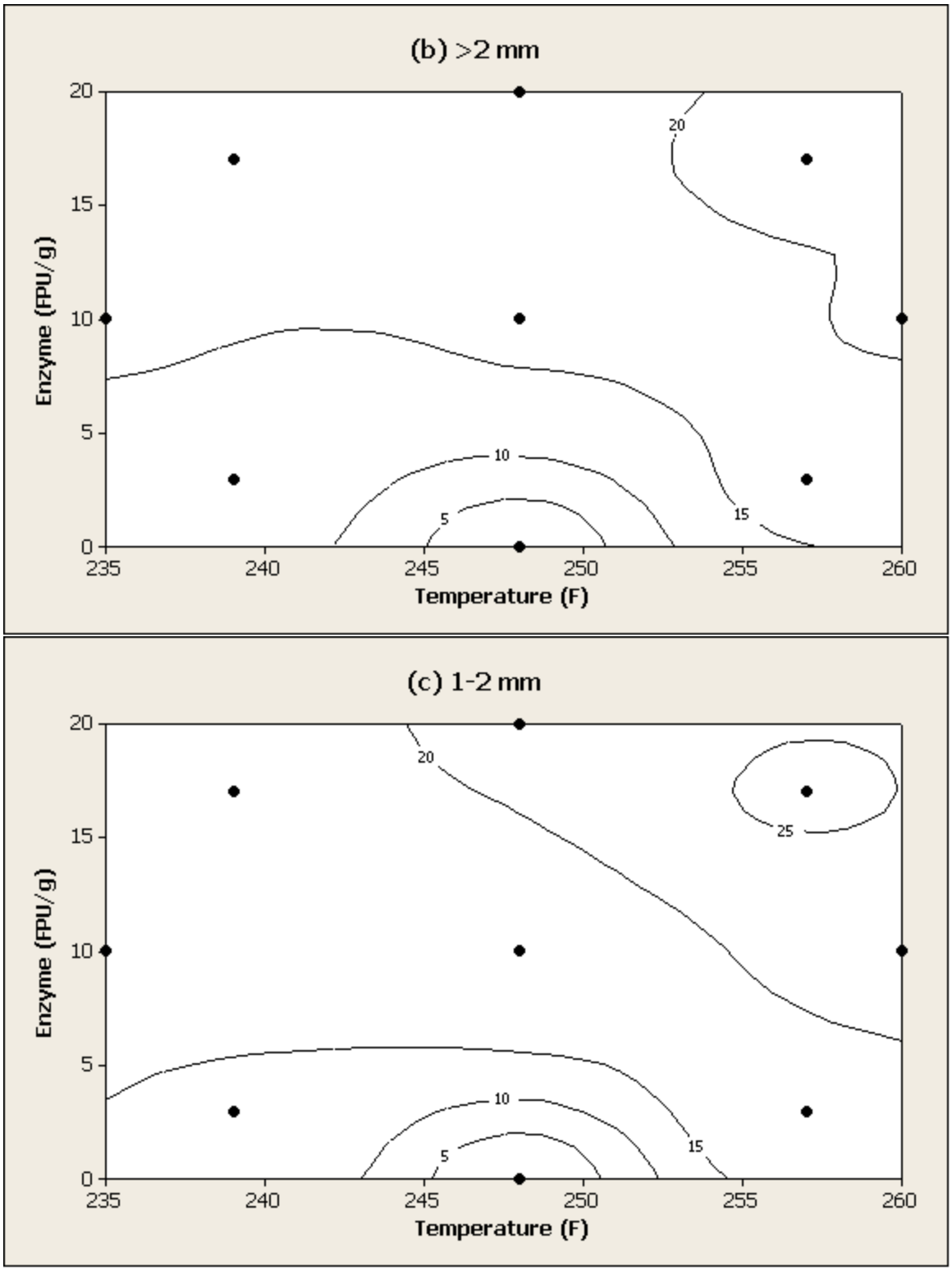
(d) Estimated coefficients for power transformed glucose yield of <1 mm ($R_4^{1.57}$)					
Factor	**Coefficient Estimate**	**df**	**Standard Error**	**95 % CI Low**	**95 % CI High**
Intercept	77.36	1	1.16	74.93	79.80
A	21.79	1	1.30	19.07	24.51
B	35.40	1	1.30	32.68	38.12
A^2	7.01	1	0.99	4.94	9.07
B^2	-12.89	1	0.99	-14.96	-10.83
A^2B	-19.49	1	1.84	-23.34	-15.64
AB^2	-5.18	1	1.84	-9.03	-1.33
Final Equation in Terms of Coded Factors: $R_4^{1.57} = 77.36 + 21.79\ A + 35.40\ B + 7.01\ A^2 - 12.89\ B^2 - 19.49\ A^2B - 5.18\ AB^2$ $R^2 = 0.988$; Adj $R^2 = 0.984$; Pred $R^2 = 0.979$					

4.3.5 Response surfaces of glucose yield versus pretreatment temperature and enzyme loading

Figure 4.5 shows the contour plots of glucose yield versus pretreatment temperature and enzyme loading for (a) UP, (b) >2 mm , (c) 1-2 mm and (d) <1 mm. The contour plots show that the glucose yield increased with increasing pretreatment temperature and enzyme loading. Figures 4.5(a), 4.5(b), 4.5(c) and 4.5(d) show similar areas of <5 % glucose yield, 4.5(a), 4.5(b) and 4.5(c) show similar areas of 5-10 % glucose yield, while 4.5(d) shows greater area of 5-10 % yield than others indicating, greater pretreatment and enzyme loading requirements of <1 mm sample. For 10-15 % glucose yield, figures 4.5(a) and 4.5(b) have similar areas, pretreatment and enzyme conditions, 4.5(c) has lower pretreatment and enzyme loading conditions and 4.5(d) has the highest area of 10-15 % yield, higher pretreatment and enzyme loading conditions. Figures 4.5(a) and 4.5(b) show similar areas, pretreatment and enzyme loading conditions for 15-20 % and >20 % yield. Figure 4.5(c) shows lower and 4.5(d) shows higher pretreatment and enzyme loading requirements compared to the other fractions. Therefore, 1-2 mm sample (figure 4.5(c))

is the least recalcitrant and <1 mm sample (figure 4.5(d)) the most recalcitrant fraction among the others which can as well be explained from the *CrI* values of the samples in Figure 1. The 1-2 mm sample having lower *CrI,* showed reduced pretreatment and enzyme loading conditions and <1 mm sample having higher *CrI,* showed higher pretreatment and enzyme loading conditions. It is also evident that <1 mm has the least GLX ratio and it produced inhibitory products both at lower and higher pretreatment severities. It is known that lower hemicellulose % (xylan) and lignin % in the sample, increases enzymatic hydrolysis efficiency [97, 98], therefore, removal of <1 mm fraction from UP reduces the undesirable components such as lignin and ash from UP even without pretreatment.




(b) >2 mm
Enzyme (FPU/g)
Temperature (F)
20
15
10
5
0
235
240
245
250
255
260
20
15
10
5
(c) 1-2 mm
Enzyme (FPU/g)
Temperature (F)
20
15
10
5
0
235
240
245
250
255
260
25
20
15
10
5

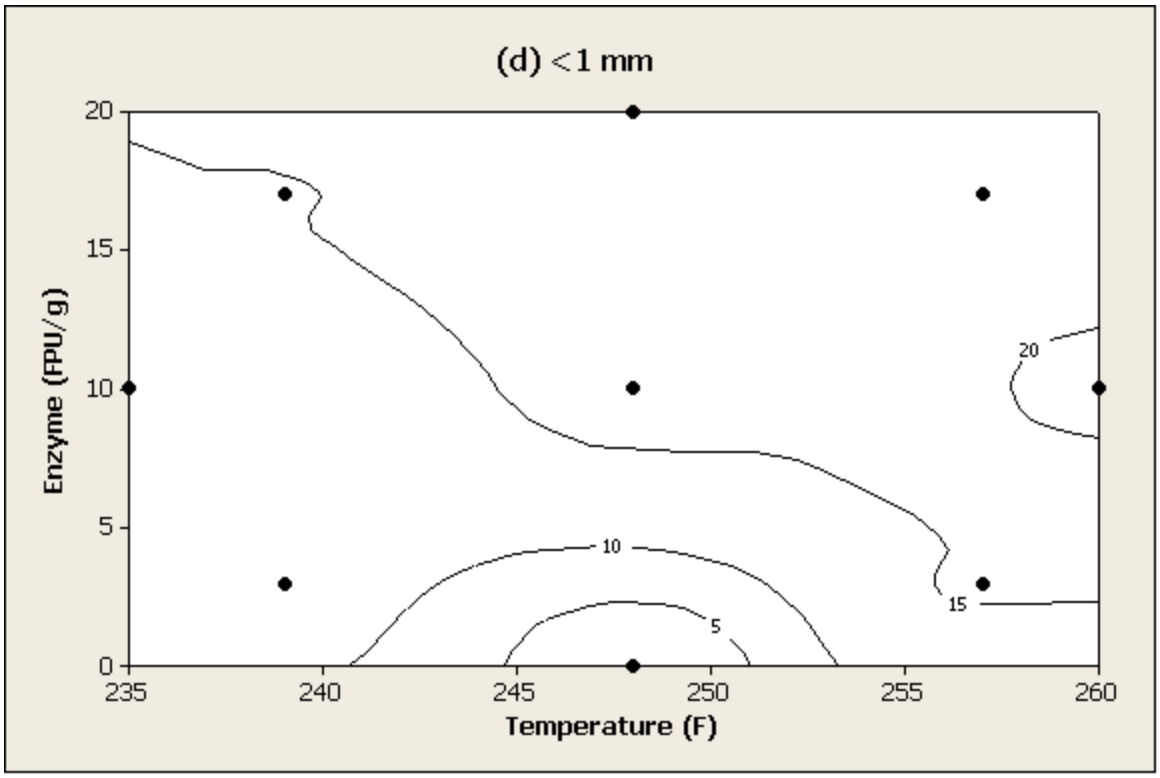


Figure 4. 5: Contour plots showing enzyme hydrolysis glucose yield (% w/w) versus pretreatment temperatures (°F) and enzyme loadings (FPU/g) for samples (a) UP SG, (b) >2 mm, (c) 1-2 mm and (d) <1 mm.

4.4 Conclusions

Separation of biomass into different size fractions can be based on significant differences in GLX ratio, as the fraction with significantly lower GLX ratio is rich in lignin. The fraction, < 1 mm, has the lowest GLX ratio, higher crystallinity and higher lignin % compared to other fractions both with and without pretreatment. During pretreatment with varying acid concentrations, <1 mm produced greater glucose and polyphenols in the hydrolyzate at lower and higher pretreatment severities respectively. Response surface method indicates that the pretreatment and enzyme loading requirements for <1 mm sample are higher than the other fractions. The empirical equations generated for glucose yield based on pretreatment temperature and enzyme loading, also clearly indicate this difference. Therefore, <1 mm fraction can be

eliminated from the un-partitioned switch grass to increase the glucose yield, to decrease the pretreatment and enzyme loading conditions, and to reduce the production of inhibitory by-products during pretreatment. Moreover, since milling and sieving are already a part of mechanical treatment of biomass, separation or sieving of biomass into size fractions based on significant differences in GLX ratio and crystallinity, can be incorporated into the existing technology to eliminating the undesirable fractions through a mechanized process.

Acknowledgement

The author, Swetha Mahalaxmi ,would like to thank Dr. Stephen Threlkeld, Professor, Department of Biology, University of Mississippi, for his guidance in the concepts of DOE and Response Surface Method.

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5. FUNGAL PREPROCESSING OF SIZE SEPARATED FRACTIONS OF SWITCH GRASS

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Abstract

Separation based on anatomical differences such as cobs, leaves and stems for biomass such as corn and wheat straw is known to affect sugar yields during pretreatment and enzyme hydrolysis. Separation of biomass such as switch grass into several heterogeneous fractions based on size was shown to have different requirements for pretreatment temperature and enzyme loading. In the current study, effect of fungal preprocessing on heterogeneous fractions of switch grass was studied. Heterogeneous fractions identified from the previous study, >1 mm and <1 mm, were subjected to microbial preprocessing using *Phanerochaete chrysosporium* along with un partitioned (UP) switch grass and times of preprocessing for each fraction was evaluated based on the enzyme profiles obtained for 12 days. Maximum enzyme activity for >1 mm and <1 mm was observed on 6^{th} and 3^{rd} day respectively. The fractions were subjected to preprocessing using *P. chrysosporium* for respective treatment times and followed with 144 hr enzyme hydrolysis. *P. chrysosporium* preprocessing of switch grass size fractions showed up to 23 % increase in glucose yields and up to 15 % in total sugar yields compared to that of raw samples. Preprocessed UP sample showed about 15- 23 % higher glucose and total sugar yields

compared to >1 mm and <1 mm samples. Preprocessed UP sample also showed about 20 % higher glucose and total sugar yields compared to that of raw UP sample. Therefore, UP is the most desirable material for sugar recovery, and >1 mm samples is the most desirable for enzyme production. <1 mm sample, which is rich in lignin can be considered for energy generation in boilers.

5.1 Introduction

Use of agricultural residues for biofuels, chemicals and bioproducts is gaining attention in order to employ renewable sources for their potential to surplus availability and to reduce greenhouse emissions [99, 100]. Switch grass, a potential feedstock with short harvest time and high yielding capacity [10], is most suitable for production of enzymes, biofuels, chemicals and other byproducts.

Various parts of corn plant had different enzyme hydrolysis yields, leaves had higher cellulose compared to stalks and cobs [85]. Also from chapter 4, it is evident that switch grass is heterogeneous and can be separated into various size fractions based on compositional differences. Based on previous literature [90, 101] and work from chapter 4, heterogeneous fractions of biomass had varying glucan and lignin content, pretreatment and enzyme requirements, and glucose yields. However, work related to microbial preprocessing on various size fractions of switch grass to understand the effect on glucose yield and enzymes released during the treatment is not yet studied. In the current study, the effect of fungal preprocessing on different size fractions of switch grass was studied to compliment the previous work in chapter 4. Switch grass size fractions, >1 mm and <1 mm were subjected to microbial preprocessing using *Phanerochaete chrysosporium*, and enzyme activity profiles were determined for a period of 12 days. Based on the earlier work by Mahalaxmi et al. [101], each size fraction is subjected to

preprocessing for respective amount of time obtained from the peak times of enzyme profiles. At the end of the treatment, the fractions are determined for composition and were further hydrolyzed to obtain glucose and total sugar yields.

5.2 Materials and Methods

5.2.1 Size separation and preparation of biomass

Switch grass of 1'' grind size is obtained from BioDimensions, Memphis, TN. A stacked sieve system, comprising U.S.A Standard Testing Sieves (arranged in a top to bottom sequence) #18 (1 mm) and a collection pan, was used for separating the 1'' grind switch grass into two fractions, >1 mm (material remained above the #18) and <1 mm (material remained in the collection pan). Fractions collected by this procedure were used in further experiments. The un-partitioned switch grass is abbreviated as UP. All the samples were further ground in an IKA MF 10.1 impact mill with an internal 1 mm circular screen, in order to eliminate the effect of the particle size for further experiments.

5.2.2 Propagation of inoculum

P. chrysosporium (strain BKM-F-1767) was obtained from USDA Forest Products Laboratory (Madison, WI) and was propagated onto potato dextrose agar (PDA) plates of 90mm × 12mm size and allowed to grow at 37 ˚C for 7 days [58]. Stock cultures were stored for a week at 4 ˚C, and the culture was maintained by periodically transferring to fresh PDA plates. Prior to inoculation, *P. chrysosporium* was grown for 7 days on PDA plates, and a spore suspension was prepared by scraping the spores aseptically from 3 plates into 60 ml sterile water, ensuring uniform spore distribution in the liquid by vortexing. A 5 ml sample of this suspension was used as inoculum for each treatment flask [101].

5.2.3 Preprocessing using *Phanerochaete chrysosporium*

Switch grass samples, UP, >1 mm and <1 mm, were subjected to preprocessing using *P. chrysosporium* inoculums prepared from section 5.2.2. Moisture content for each of the samples was determined using the moisture oven. It was recorded that the moisture content of switch grass samples was between 7 % and 8 %. Ten grams of switch grass (dry basis) was placed in a 250 ml flask, and autoclaved (121 °C, 30 min). Fifty milliliters of sterilized water was added to maintain approximately 80 % moisture and 5 mL of supplemental growth media ($NaNO_3$ – 3 g, KCl – 0.5 g, $MgSO_4.7H_2O$ – 0.5 g, $FeSO_4.7H_2O$ – 0.5 g, KH_2PO_4 – 1.0 g, Glucose – 20 g in 1 liter solution, sterilized separately) [80, 101], was added in addition to 20 µL of tetracycline (20mg/mL in ethanol) to minimize bacterial contamination in the flasks. Treatment flasks received 5 mL of *P. chrysosporium* suspension while other flasks, supplemented with 5 mL of additional sterilized water and no *P. chrysosporium*, were used as controls. Flasks (3 treated (with *P. chrysosporium*) and 3 controls (without *P. chrysosporium*) for each of UP, >1 mm and <1 mm, were allowed to incubate at 37 °C under solid state conditions. For enzyme activity profile experiments, the samples were incubated for 12 days and for other experiments, the samples were incubated for respective peak times obtained from enzyme activity profile results. After treatment, 100 mL of deionized water was added to the samples and they were subjected to autoclave at 121 °C for 20 minutes to minimize viable microbial presence. The autoclaved samples were vacuum filtered using coffee filters and dried at 40 °C for 48 hrs, for further composition analysis and enzyme hydrolysis.

5.2.4 Enzyme activity assays

Flasks (3 treated and 3 controls) were incubated at 37 °C under solid state conditions for 12 days and were sampled for phenol oxidase, peroxidase, β-glucosidase, β-xylosidase and cellobiohydrolase activities for every 3 days during the 12 day treatment. The substrate used for phenoloxidase and peroxidase tests is 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA), and those for β-glucosidase, β-xylosidase and cellobiohydrolase tests are 5 mM pNP-β-glucopyronoside, 5 mM pNP-β-xylopyranoside and 5 mM pNP-cellobioside respectively, all prepared in 50 mM (pH 5.0) acetate buffer [101].

A known amount (precisely a gram) of biomass sample was taken from the flask in to a test tube and diluted to 5 mL by addition of water and vortexed, 150 µL of supernatant was incubated with 150 µL of substrate solution, in a 96 well plate (and 15 µL of 0.3 % H_2O_2, only for peroxidase assay), for a noted time, and later subjected to centrifugation at 2000-5000 g for 5 minutes. The clear supernatant obtained after centrifugation was analyzed spectrophotometrically at 460 µm and 410 µm for L-DOPA assays and cellulase assays respectively. The units of activity are defined as µmoles of the substrate reacted with the enzyme in 1ml of sample per hour of incubation (U/(mL)) [81, 101].

5.2.5 Composition analysis

Composition analysis of samples was performed using NREL's standard operating procedure with some modifications. A 0.1 g sample of biomass was taken in a pressure tube and 1 ml of 72 % H_2SO_4 was added. The sample was mixed and incubated at 30 °C for 1 hour. Periodic mixing was done every 10 minutes using a glass rod, to allow uniform particle to acid

contact. Upon completion of an hour of incubation with 72 % H_2SO_4, 25 mL of water was added to the sample to dilute the acid to 4%. The pressure tubes were tightly capped and autoclaved for 1 hour at 121 °C. The samples obtained after autoclaving were allowed to cool to room temperature and filtered into 125 mL flasks using glass crucibles. All samples are tested in duplicate.

Glass crucibles were previously dried at 100 °C, cooled in desiccators and recorded for weight before using for filtration. The acid insoluble lignin remaining in the crucible after filtration was subjected to heat in an oven at 100 °C for 24 hours. The difference in weight after the 24 hour heating at 100 °C was recorded as acid insoluble lignin in the sample. To the clear hydrolyzate obtained from filtration, $CaCO_3$ was added slowly to neutralize the acid to a pH between 5 and 6. The neutralized solution was decanted, and the supernatant was filtered using 0.2 µm syringe filter into a glass vial for High Performance Liquid Chromatography (HPLC) analysis. The samples could be stored at -4 °C for a week before analysis.

Biomass crystallinity was measured by powder X-ray diffraction method using X'Pert PRO (PANalytical). Biomass samples obtained from section 2.1 were further reduced to - 45 mesh size and used for analysis. The samples were scanned at 1°/min from 2θ = 10° to 40° with a step size of 0.05°. Crystallinity Index (*CrI*), the percentage of crystalline material in biomass is defined in equation (1)

$$CrI = (I_{002} - I_{am})/I_{002} \times 100 \quad (1)$$

I_{002} is the intensity of the peak at 2θ = 25^0 and I_{am} is the intensity of the background scatter at 2θ = 21.5^0. The values of 2θ measured are different from the other studies [88, 91] as cobalt was the X-ray source in the present study.

5.2.6 Enzyme hydrolysis

The biomass samples obtained from section 5.2.3 were enzyme hydrolyzed using Celluclast and Novozyme 188. A 0.5 g glucan equivalent of biomass was taken in a 125 mL flask, 25 mL of citric acid buffer pH 4.8, 0.4 mL of tetracycline, 10 mL aliquot of enzyme cocktail, containing 10 FPU of Celluclast and 28 CBU Novo 188, were added and made up to a final volume of 50 mL with water and the flasks were incubated at 50 °C at 130 rpm for 144 hours. For every 12 hours of 144 hour enzyme hydrolysis, the supernatant was filtered using 0.2 µm syringe filters in to a vial and analyzed using HPLC. The samples could be stored at -4 °C for a week before analysis. The samples analyzed are used for calculating the glucose and total sugar yield using the following equations:

$$\text{Glucose Yield} = \frac{\text{Concentration of glucose}}{\text{Amount of biomass sample}} \times \text{volume of liquid} \times 100$$

Total Sugar Yield

$$= \frac{\text{Glucose concentration} + \text{Xylose Concentration}}{\text{Amount of biomass sample}} \times \text{volume of liquid} \times 100$$

5.2.7 HPLC Analysis

Samples from section 5.2.5 are analyzed for sugars using Aminex HPX 87-P column, with injection volume of 20 µL, at column temperature 85 °C, refractive index detector at 50 °C, with de-ionized water as mobile phase at 0.6 mL/ min and run time of 30 minutes for each sample.

Samples from section 5.2.6 are analyzed for sugars, HMF and furfural using Aminex HPX 87-H column, with injection volume of 20 µL, at column temperature 65 °C, refractive index detector at 50 °C, with 0.005 N H_2SO_4 as mobile phase at 0.6 mL/min and run time for 50 minutes for each sample.

5.3 Results and Discussion

5.3.1 Enzyme Activity Profiles

Cellobiohydrolase, β-glucosidase, and β-xylosidase activities for >1 mm, <1 mm and UP, for 12 days are shown in figure 5.1. β-xylosidase (figure 5.1(a)), cellobiohydrolase (figure 5.1(b)) and β-glucosidase (figure 5.1(c)) activities increased gradually for 6 days and then decreased steeply until 12^{th} day of incubation for >1 mm sample. For <1 mm sample, the activities increased for 3 days and then decreased steeply from the 6^{th} day. Significant profile patter was not observed for UP, cellobiohydrolase, β-xylosidase and β-glucosidase activities for UP remain relatively low compared to >1 mm and <1 mm samples. A difference in activities for β-xylosidase, cellobiohydrolase and β-glucosidase is observed among the samples, >1 mm, <1 mm and UP, which can be explained from the differences in the glucan composition of the samples. Higher cellulase activities in >1 mm sample compared to other samples is due to higher glucan % of the sample. Among enzymes, highest activity was observed for β-glucosidase which concurs with previous work [101].

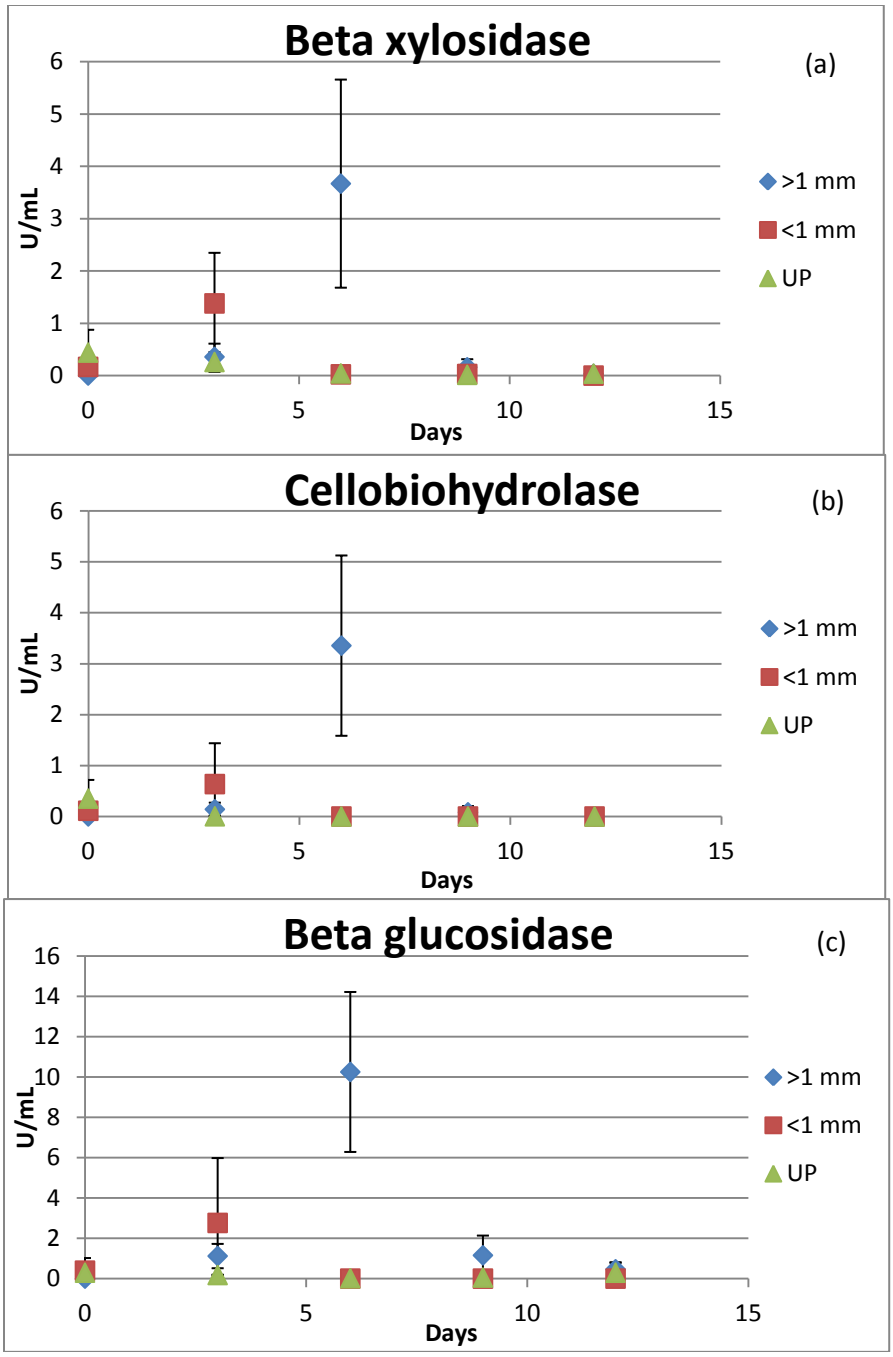

Figure 5. 1: Enzyme activity profiles, (a) Beta xylosidase, (b) Cellobiohydrolase and (c) Beta Glucosidase for a period of 12 days for UP, >1 mm and <1 mm samples (error bars represent 95 % confidence interval for triplicates)

5.3.2 Free sugar analysis in washate

Figure 5.2 shows the concentration of glucose (g/L) in washate for UP, >1 mm and <1 mm at preprocessed and unpreprocessed conditions. Glucose was not present in <1 mm sample at both preprocessed and unpreprocessed conditions. For UP, glucose was higher in unpreprocessed condition than at preprocessed condition, for >1 mm sample it is undeterminable. However, glucose concentration in washate attributes to the cellulase activities observed in the samples.

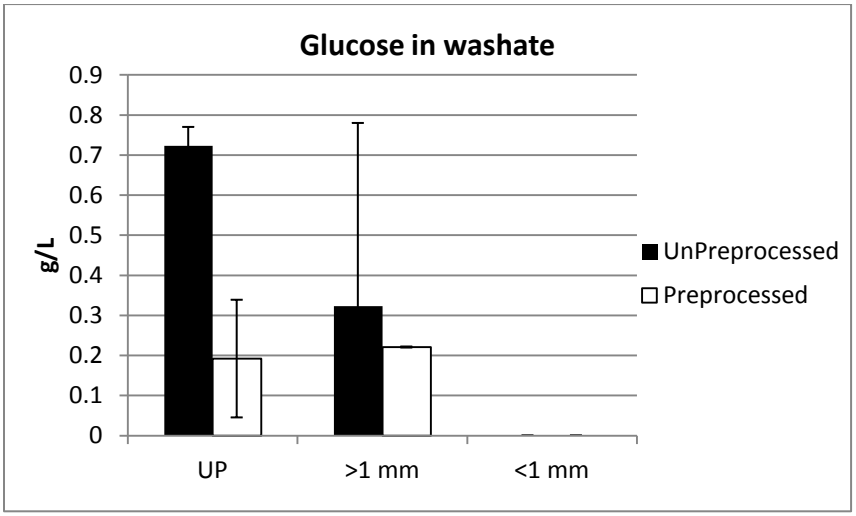

Figure 5. 2: Glucose (g/L) in washate for samples UP, >1 mm and <1 mm at preprocessed and unpreprocessed conditions.

Figure 5.3 shows cellobiose, glucose and xylose concentrations (g/L) in washate for preprocessed >1 mm sample. Glucose concentration was higher than cellobiose and xylose concentrations. High glucose and low cellobiose could be attributed to the high β-glucosidase activity during fungal preprocessing and high initial glucan present in >1 mm sample. UP and <1

mm samples showed absence of cellobiose and xylose in washate at both preprocessed and unpreprocessed conditions (not shown in figures 5.2 and 5.3) which could be due to the lower glucan % of samples.

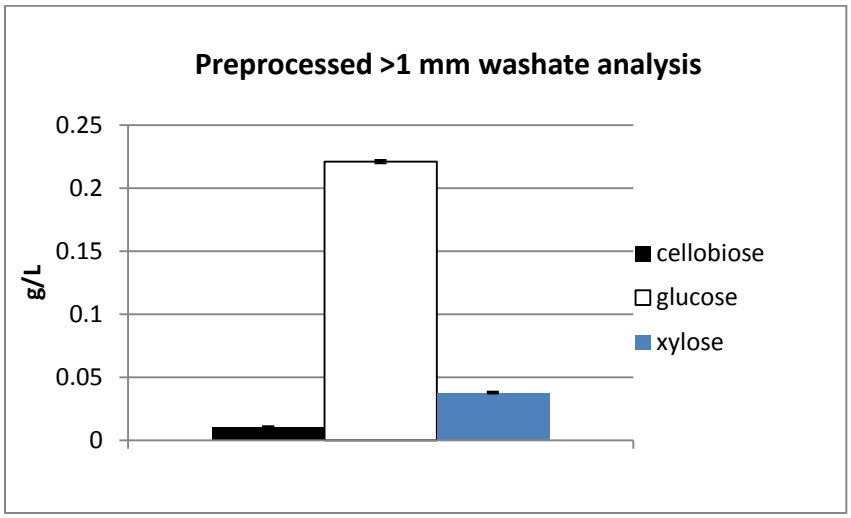

Figure 5. 3: Cellubiose, glucose and xylose concentration (g/L) in the washate for preprocessed >1 mm sample.

5.3.3 Composition analysis of unpreprocessed and preprocessed fractions

The UP, >1 mm and <1 mm samples with and without preprocessing were subjected to composition analysis as mentioned in section 5.2.5 and the results are shown in Figure 5.4. Figure 5.4 (a) Glucan, (b) xylan and (c) lignin of the three materials UP, >1 mm and <1 mm were determined as mentioned in section 5.2.5. The UP and >1 mm have higher glucan % than <1 mm. Raw samples have lower glucan % than preprocessed and unpreprocessed (control) samples. Glucan % decreased due to preprocessing due to the glucose consumption during fungal metabolism as observed from the enzyme activities of the samples in figure 5.1. However, higher glucan % in unprocessed samples can be attributed to hydrolysis of sugars due to wetting and absence of *P. chrysosporium*.

Xylan % of UP and >1 mm samples are higher than <1 mm sample. However, xylan % decreased with preprocessing which can again be explained due to fungal metabolism. <1 mm

sample has the highest lignin % among the samples at all conditions such as raw, preprocessed and unpreprocessed (control). Lignin % decreased with preprocessing for all samples showing the effect of *P.chrysosporium*.

Therefore, <1 mm sample has lower glucan %, lower xylan % and higher lignin % compared to other samples. Alternatively, the UP and >1 mm has similar glucan %, xylan % and lignin %.

Cellulose imparts strength and flexibility to biomass[93] and it is known that, lower cellulose content leads to increased fragility in biomass samples [102], therefore, <1 mm sample having lower glucan % is more fragile and less resistant to mechanical disruption, while >1 mm sample having higher glucan % is less fragile and more resistant to size reduction. Table 5.1 also shows the crystallinity indices (*CrI*) of the samples, the increasing order of the values for crystallinity indices is >1 mm, UP and <1 mm. Enzyme activity profiles from figure 5.1 also indicate greater activity for >1 mm which is less crystalline compared to other samples. Washate analysis from figures 5.2 and 5.3 also indicate that >1 mm sample had higher free sugar concentration compared with UP and <1 mm, confirming that its higher glucan % renders digestibility to free sugars present in washate. Apparently, <1 mm did not have any free sugars present in washate inferring the possible effect of lower glucan % on cellulose degradation of switch grass during preprocessing.

Table 5. 1: Crystallinity indices of >1 mm, UP and <1 mm samples.

Sample	*CrI*
>1 mm	57.35253
UP	58.96481
<1 mm	59.7479

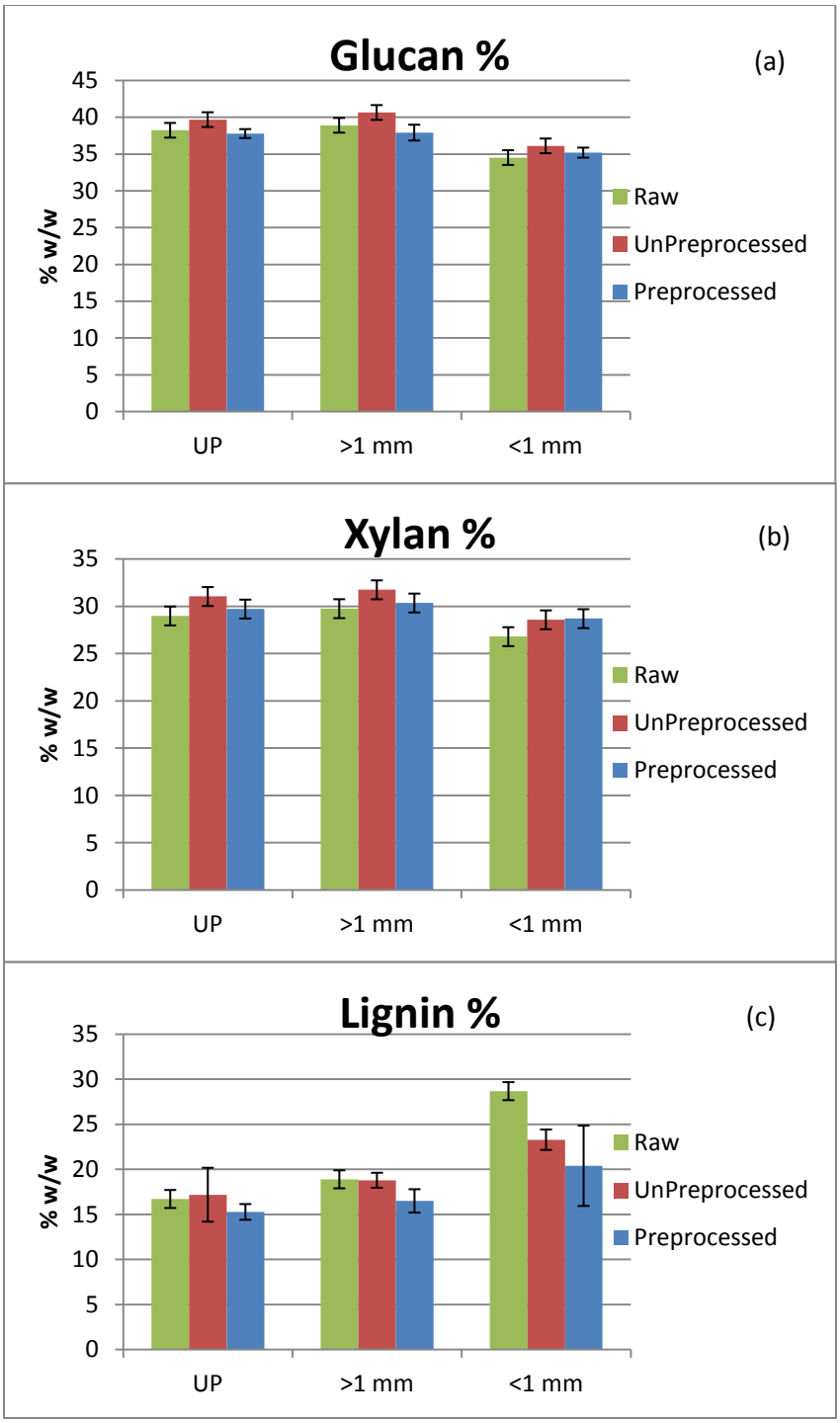

Figure 5. 4: Composition analysis in terms of (a) glucan %, (b) Xylan % and (c) Lignin % for UP, >1 mm and <1 mm samples at raw, control and preprocessed conditions. Error bars are 95 % confidence internvals.

5.3.4 Enzyme Hydrolysis

Enzyme hydrolysis results are shown in figures 5.5 and 5.6. Figure 5.5 shows the glucose yield (% w/w) on dry biomass basis, of raw (figure 5.5 (a)), unpreprocessed (figure 5.5 (b)) and preprocessed (figure 5.5 (c)) samples of UP, >1 mm and <1 mm for a period of 144 hours. Glucose yield of raw samples (figure 5.5 (a)) of UP, >1 mm and <1 mm are not significantly different from each other. From figure 5.5 (b), it is observed that the glucose yields of unpreprocessed samples of UP and >1 mm are higher than that of unpreprocessed <1 mm sample and a decreasing order in glucose yield is observed from UP to >1 mm to <1 mm sample. From figure 5.5 (c), the glucose yields of preprocessed samples of UP are higher than that of >1 mm and <1 mm samples. There is no significant difference in the glucose yields between preprocessed >1 mm and <1 mm samples. At all conditions, the glucose yield increased with increasing time of hydrolysis. The highest glucose yield for raw samples is about 3.25 %, and that for preprocessed and unpreprocessed samples is about 4 % and 4.25 % respectively. Preprocessed and unpreprocessed samples have very slight different glucose yields, however, they have higher glucose yield compared to raw samples. Increasing order for initial rate of hydrolysis (12 hours) is UP, >1 mm and <1 mm at unpreprocessed and preprocessed conditions, however, no difference for initial hydrolysis is observed among raw samples in the first 12 hours.

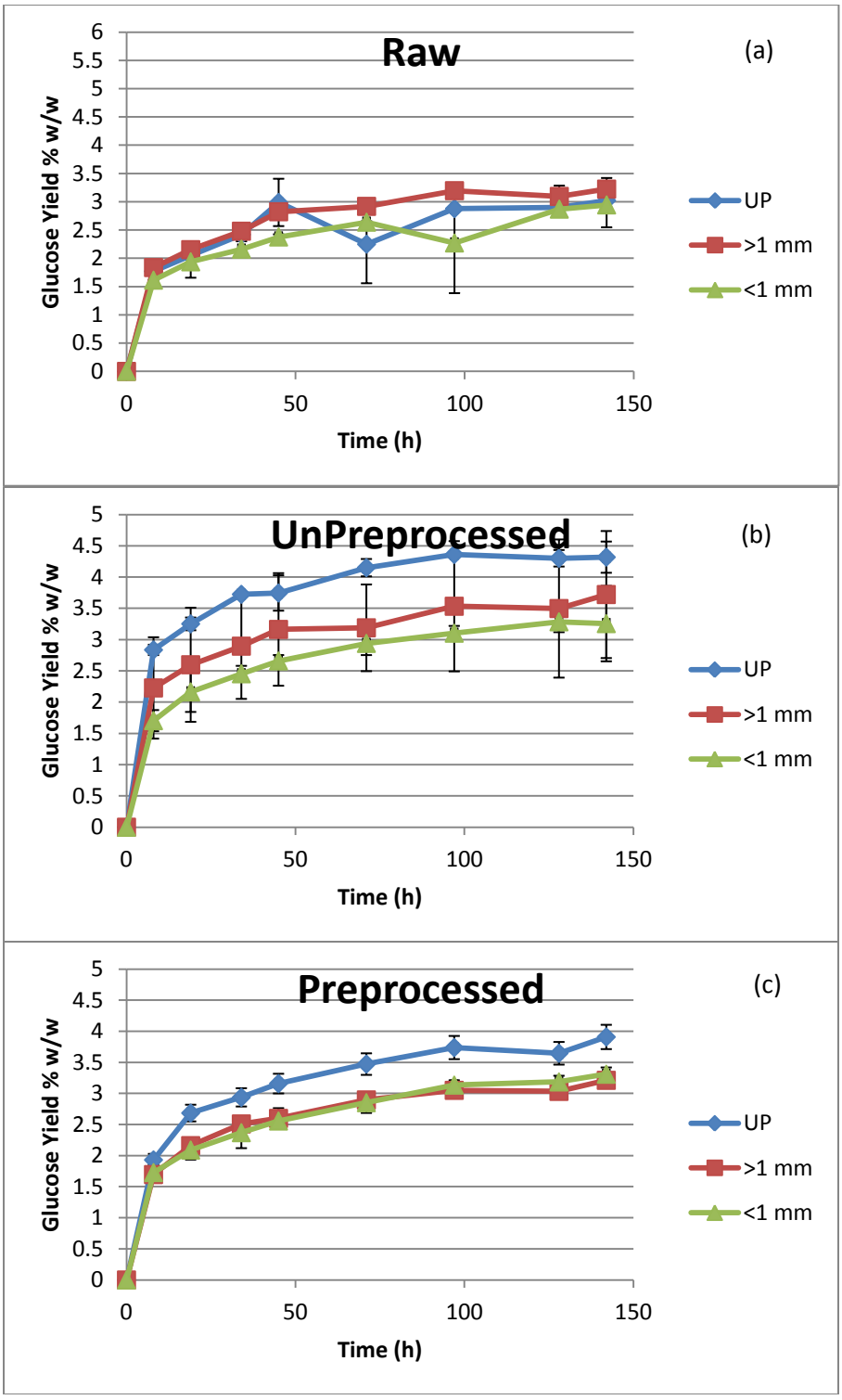

Figure 5. 5: Glucose Yield (% w/w of dry switch grass) of UP, >1 mm and <1 mm samples for 150 h of enzyme hydrolysis with error bars representing 95 % confidence intervals.

Figure 5.6 depicts the total sugar yield (glucose and xylose) of UP, >1 mm and <1 mm samples at (a) raw, (b) unpreprocessed and (c) preprocessed conditions. At all the processing conditions, total sugar yield increased with increasing time of hydrolysis. Similar to the glucose yield results, initial rate of hydrolysis in the first 24 hours of hydrolysis for total sugars is highest for UP at unpreprocessed and preprocessed conditions, which could be due to its higher *CrI* . However, no difference for initial hydrolysis is observed among fractions at raw conditions, therefore showing the effect of preprocessing. Total sugar yield (figure 5.6 (a)) of UP, >1 mm and <1 mm at raw conditions are not significantly different from each other. It is observed that the total sugar yields of unpreprocessed samples (figure 5.6 (b)) of UP and >1 mm are higher than that of <1 mm sample. A decreasing order in total sugar yield is observed from UP to >1 mm to <1 mm sample at unpreprocessed and preprocessed conditions. From figure 5.6 (c), the total sugar yields of preprocessed samples of UP are higher than that of >1 mm and <1 mm. The highest total sugar yield for raw samples is about 3.5- 4 %, and that for unpreprocessed and preprocessed samples is about 5 % and 4.5 % respectively.

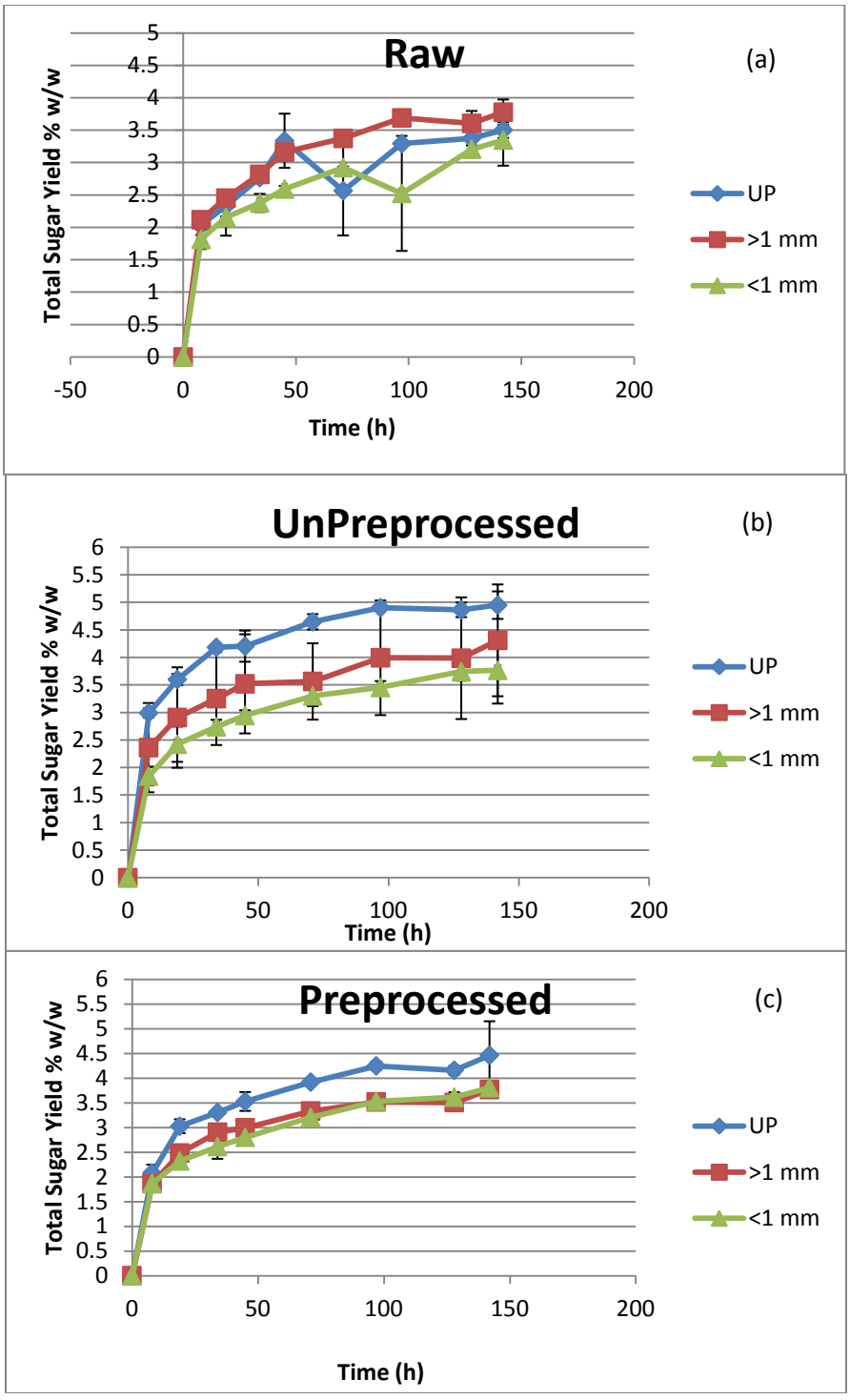

Figure 5. 6: Total Sugar yields (Glucose +Xylose) (% w/w of dry biomass) of UP, >1 mm and <1 mm samples for 150 h of enzyme hydrolysis with error bars representing 95 % confidence intervals.

From figures 5.5 and 5.6, glucose yields, total sugar yields and initial hydrolysis rates increased after preprocessing switch grass with *P. chrysosporium*. Although >1 mm sample did not show higher glucose or total sugar yields as expected based on the findings from Chapter 4, results still show that size partitioning had an effect on enzyme activities of cellobiohydrolase, β-glucosidase, and β-xylosidase. If fungal preprocessing on switch grass is used for enzyme production, this work suggests that under the experimental conditions, >1 mm sample has higher capacity to produce cellulase enzymes, which could be recovered and later used for cellulose degradation. Efforts to utilize agricultural substrates for enzyme production [99, 100, 103, 104] [99, 100, 103, 104] can be optimized based on the findings in this work by identifying the most desirable components in agricultural residues for enzyme production.

5.4 Conclusions

Preprocessing of switch grass size fractions, UP, >1 mm and <1 mm samples, with *P.chrysosporium* studied for 15 days showed enzyme profiles for cellobiohydrolase, β-glucosidase, β-xylosidase, phenol oxidase and peroxidase, with peak times of 3 days and 6 days for >1 mm and <1 mm samples respectively. The profile for UP was not relatively significant. Enzyme activities for phenol oxidase and peroxidase were not observed for all the samples. Among the size fractions, highest cellulase activity was observed for >1 mm sample. Therefore, >1 mm sample has higher capacity to produce cellulase enzymes compared to the other fractions. Preprocessing of switch grass size fractions showed 12-14 % increase in glucose and total sugar yields compared to that of raw samples. After preprocessing, UP sample showed about 20 % higher glucose and total sugar yields compared to >1 mm and <1 mm samples. Preprocessed UP sample also showed about 28 % higher glucose and total sugar yields compared to that of raw UP sample. Therefore, for sugar recovery from switch grass, UP material is most desirable.

6. FUTURE WORK

In the past work, preprocessing of biomass was performed using mechanical, chemical and microbial techniques, to modify the biomass structure to yield higher sugar yields during enzyme hydrolysis.

The concept of microbial preprocessing is known to enrich biomass through lignin degradation. During microbial preprocessing, lignin present in the biomass is degraded, however cellulose is consumed by the microbes due to the prolonged treatment times. This problem is addressed in chapter 3 suggesting shortening the treatment time, using enzyme profiles as the basis for treatment time estimation. Optimization of microbial preprocessing is a promising area of research which involves studying the process parameters, reaction volume, reactor design, temperature, media and amount of inoculum.

In chapter 4, a mechanical separation method was developed to identify and separate the heterogeneous fractions of biomass such as switch grass to eliminate or separately process the recalcitrant fractions present in it. This method can be easily incorporated in the present biomass handling system, it decreases the pretreatment and enzyme requirements, and reduces the amount of inhibitors in the pretreatment hydrolyzate of biomass. Further work in the direction of process design for separating biomass using sieving method is desirable to investigate the economic feasibility of the process.

Future research interests would be in engineering biochemical methods using various microbes, scale-up and scale-down of fermentation processes. Conversion of biomass to

chemicals using biochemical means is another area of interest. Generating chemicals from renewable sources is not only an interesting concept but also a solution to rising need of industrial chemicals. Chemicals such as acetone, ethanol are widely used in the industry and can be produced by biochemical methods that require, less severe processing methods, thereby reducing the severity to treat the downstream water.

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List of Appendices

Appendix: A

A. PROCEDURES

A.1 Composition Analysis

Sample of one to two grams is ground in IKA impact mill using a 1 mm circular mesh. Each sample is weighed accurately to 0.1±0.01 grams and placed into a thick walled test tube/pressure tube. This is equilibriated to 30 ^{0}C in a shaker-incubator, and 1±0.1 ml of 72 % H_2SO_4 is added. The bottle is set in the shaker-incubator with a glass stir rod in it for 60±5 minutes, stirring the contents for every 5 to 10 minutes. Mixing at regular time periods is important to ensure uniform liquid to solid contact. All samples are tested in at least duplicate. Upon completion of the 60 minute hydrolysis 25 ml of water is added to the tube, bringing the concentration of H_2SO_4 to 4 %, and then mixed thoroughly to avoid phase separation between high and low acid concentrations. The test tube is tightly capped and autoclaved at 121^{0}C for 1 hour. A set of sugar recovery standards (SRS) which include D-(+)glucose, D-(+)xylose, D-(+)galactose, L-(+)arabinose and D-(+)mannose. SRS sugar concentrations should closely resemble the concentrations in the biomass. In another tube, 1 ml of 72 % H_2SO_4 is added, diluted to 4 % by adding 25 ml water. The mixture of sugar recovery standards is added and capped/sealed and autoclaved along with the test samples. The tubes are then cooled to room temperature. After cooling, the tubes are mixed well by shaking. Hydrolysis liquid is taken in a 50 ml Erlynmeyer flask and slowly neutralized with $CaCO_3$ till the pH is between 5 and 6. The supernatant is decanted and filtered into HPLC vials using 0.2 µm Nylon syringe filters. The samples can be stored for a maximum of two weeks before they are analyzed. Calculations for

glucan, xylan, galactan, mannan, arabinan are shown in the equation B.1.

A.2 Enzyme hydrolysis

Sample of 0.5 gram glucan equivalent weight (calculations in section 5.2.2) is weighed into a 250 ml Erlynmeyer flask. Stock solutions of tetracycline and cycloheximide are prepared and stored in the freezer. Required amount of 0.1 M Citric acid buffer is prepared and adjusted to a pH of 4.8±0.3 with NaOH. Enzyme cocktail is prepared using 2.8 CBU of Novo-188 for every 1 FPU of Celluclast in 1 mL 10 mL of aliquot goes in to each flask containing the 0.5 g glucan equivalent of biomass sample.

To each flask containing 0.5 g glucan equivalent of biomass sample, 25 ml of 0.1 M citric acid buffer, 400 µL tetracycline, 300µL of cycloheximide are added and total volume is made up to 40 ml by adding the required amount of water. The amount of water to be added is calculated by subtracting the volumes of citric acid, sample weight, tetracycline and cycloheximide. The loaded flasks are equilibrated in a shaker-incubator for 30 minutes at 50 ^{0}C with shaker off and during equilibration, enzyme cocktail can be prepared. The flasks are taken out of the incubator-shaker; 10ml of enzyme cocktail is added to each flask and placed back. The flasks are sealed with the aluminum foil and are allowed to incubate for 72 hours at 50 ^{0}C and 200 RPM. At the end of 72 hour hydrolysis, the samples are taken in a 1.5 ml centrifuge tubes and centrifuged at 2000 RPM for 20 minutes. The supernatant is again filtered through a 0.2 µm nylon filter using a syringe into HPLC vials. The samples can be stored in a freezer for 2 weeks before analysis. The enzyme hydrolysis yield of the sample is calculated based on the equation B.2.

A.3 HPLC Analysis

Each sample to be analyzed using HPLC is initially filtered into HPLC vials using a 0.2 µm syringe filter and samples are stored at -4 ˚C for future analysis.

Mobile phase is vacuum filtered through a 0.45 µm membrane filter using a vacuum filtration set up, in to a liter flask. The filtered mobile phase is transferred to a clean mobile phase tank placed over the HPLC pump. It is ascertained that there are no particulate matter present in the tank, also the level of the mobile phase in the tank is always above the diffuser connected to the end of the tubing through which the mobile phase is pumped into the pump system. Bottles named 30 % methanol in the pump and water in the auto sampler are ensured to be up to the desired level. Switch on the pump, the auto sampler and RI detector, and wait until the initialization process is complete for all of them.

In the computer connected to the HPLC system, double click on the Chromeleon (software) icon to initialize the software, press the start button and wait until the icon turns grey in the bottom right corner of the task bar. After the Chromeloeon software is initialized, double click on the Chromeloeon icon on the desktop to open the panel displaying the pump, auto sampler and the RI Detectot tabs. On the pump tab, press the ‘Connect’ button to have remote access to the pump controls. Similarly, on the auto sampler and RI detector tab, press the ‘Connect’ button to have remote access to the auto sampler and RI detector controls respectively. For every change of mobile phase and a new start of the system, purge the pump for about 3-4 minutes as set in the system. Purging is performed by turning the purge valve in the pump anti-clockwise by about 4 threads and pressing the ‘purge on’ button in the pump tab. After the pump is purged, the purge valve is closed.

Samples are placed in the auto sampler and a new sequence is created based on the positions in the auto sampler, using an existing sequence and saving it with a new file name.

Based on the method of analysis, the column (Aminex HPX 87P for composition analysis and Aminex HPX 87H for enzyme hydrolysis) is fitted in the column and an appropriate guard cartridge is fixed in the guard cartridge holder in line, before the column. It is ensured that the directions of the guard cartridge and the column are coherent with the direction of flow of the mobile phase. Column heater is turned on and the temperature is set at 65 ˚C for Aminex HPX 87H and at 85 ˚C for Aminex HPX 87P columns. On the RI detector panel, 'Acquisition on' button is pressed to monitor the base line during equilibration of the system.

On the pump panel in the computer, flow of mobile phase is slowly started with 0.2 mL/.min. After the temperature of the column reaches the set temperature, the flow rate of mobile phase is ramped up by 0.1 mL/min for every 10 minutes until 0.6 mL/min. The system is equilibrated if the baseline monitored in the RI panel is stable as a straight line. After the system equilibration, the acquisition is turned off on the RI panel and the sequence is started by choosing the sequence tab and then opting for 'Batch Start'. Once all the samples are analyzed, the flow rate of the system is ramped down to 0.2 mL/min at 0.1 mL/min for every 10 minutes. The column is turned off and the mobile phase is allowed to flow through the system until the column temperature if returned to room temperature.

A.4 Enzyme Activity Assays

Table A. 1: Substrates and respective incubation times for the enzyme activity assays.

Enzyme	Substrate	Typical Incubation time
β-glucosidase	5 mM pNP-β-glucopyranoside	0.5-2 hrs
cellobiohydrolase (CBH)	2 mM pNP-cellobioside	3-4 hrs
β-xylosidase	5 mM pNP-β-xylopyranoside	2-3 hrs
Phenol oxidase/Peroxidase	5 mM L-3,4,-dihydroxyphenylalanine (L-DOPA)	1-2 hrs

Substrate solutions are made in 50 mM, pH 5.0, acetate buffer. L-DOPA must be made freshly every time, and other substrates can be used for 2-3 weeks. Biomass samples to be analyzed for enzyme assays are aseptically taken from the experimental flasks using sterile glass rod or spatula for every flask. For fungal preprocessing experiements a noted amount of solid sample is taken from every flask and is made up to 5 mL using 50 mM, pH 5.0, acetate buffer in a test tube. From each test tube, 150 µL of sample + 150 µL of the enzyme substrate solution (+10 µLof H_2O_2 solution only for peroxidase activity assay) are transferred to a 96 well micro centrifuge plates and incubated as mentioned in the above the table. Enzyme substrate control (150 µL of sample + 150 µL of the buffer) and sample control (150 µL of enzyme substrate + 150 µL of the buffer) are the reference samples whose absorbance is used as reference for the experimental samples. After appropriate incubation time, the micro well plates are subjected to centrifugation at 2000-5000 g for 5 minutes and 100 µL of supernatant is transferred to 96 well

micro titer plates with 200 µL deionized water and the absorbance is measured at 410 µm and 460 µm for cellulase and phenol oxidase (peroxidase) enzymes respectively. The enzyme activity of the sample is calculated based on the equation B.3.

Appendix: B

B. Equations

B.1 Composition Analysis

$$\%\ R\ (for\ sugar\ recovery\ standards)\ = \frac{conc.\ detected\ by\ HPLC, g/L}{known\ concentration\ of\ sugar\ before\ hydrolysis, g/L} * 100$$

$$C\ = \frac{Chplc \times dilution\ factor}{\%R} * 100$$

$$C, anhydrous\ = C \times anhydro\ correction$$

Anhydro correction is 0.88 for C-5 sugars and 0.9 for C-6 sugars.

$$\%\ Sugar\ = \frac{C, anhydrous \times V, filtrate \times {}^{1g}/_{1000mg}}{weight\ of\ sample} * 100$$

$$V, filtrate = \ 87.00\ ml$$

B.2 Enzyme Hydrolysis

$$weight\ of\ sample\ equivalent\ to\ 0.5\ g\ glucan\ = \frac{0.5 \times 100 \times 100}{glucan\ \%\ \times (100 - \%\ moisture)}$$

$$\%\ Sugar(extractive\ free)\ = \frac{C, anhydrous \times V, filtrate \times {}^{1g}/_{1000mg}}{weight\ of\ sample} \times 100$$

B.3 Enzyme Activity

$$Final\ Abs\ = sample\ Abs - sample\ control\ Abs - substrate\ control\ Abs$$

$$Activity\ \left(\frac{\mu moles}{hr \times ml}\right) = \ {Final\ Abs}/{(C \times incubation\ time \times Vol\ of\ sample)}$$

Where C =14.71 for cellulase assays and C = 2.387 for L-DOPA based assays, volume of sample= 100 µL

VITA

Swetha Mahalaxmi, was born in Hyderabad, Andhra Pradeesh, India on December 6 1983. She is the eldest daughter of Moola Papi Reddy and Moola Janaki Devi. She pursued her B. E in Chemical Engineering at University College of Technology, Osmania University and excelled with distinction in 2005. She started her graduate studies in January 2006, obtained MS Chemical Engineering from University of Mississippi in 2008 and continued with Ph.D in Chemical Engineering at University of Mississippi. During her graduate study, Swetha obtained several prestigious awards such as Summer Research Fellowship, Graduate Student Council Research Grant Award and Dissertation Fellowship. She presented her work at AIChE Annual meetings and Society for Industrial Microbiology symposia. She complimented her academics with industrial internships at Michigan Biotechnology Institute and Mascoma Corporation and gained recognition. She also participated in cultural activities at India Student Association at University of Mississippi. Swetha is currently working as Post doctoral researcher at Audubon Sugar Institute and attributes her success to her hard work, intelligence, family and nicest people she got to work with.