Comparison between Radio Frequency (RF) and Traditional Heating Assisted Alkaline Pretreatment on Lignocellulosic Biomass

by

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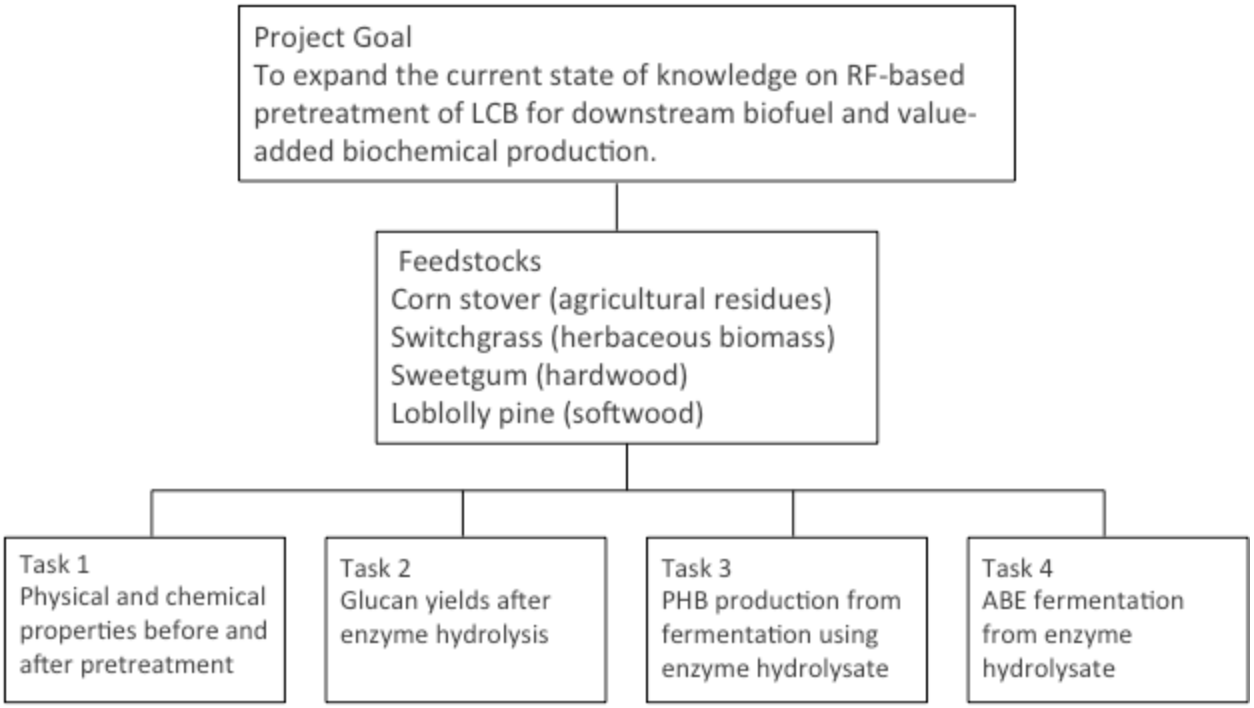
Abstract

Pretreatment plays an important role in making the cellulose accessible for enzyme hydrolysis and subsequent conversion because it more or less destroys resistance and recalcitrance of biomass. In this study, radio frequency (RF) assisted dielectric heating was utilized in the alkaline (NaOH) pretreatment. The substrates included agricultural residues (corn stover), herbaceous crops (switchgrass), and hardwood (sweetgum) to softwood (loblolly pine). Pretreatment was performed at 90℃ for both RF and traditional water bath (WB) heating for one hour after overnight soaking in NaOH solution (0.2g NaOH/g Biomass). Pretreated materials were characterized by chemical compositional analysis; enzyme hydrolysis, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The glucan yield from hydrolysis yield using RF heating method for these four feedstocks were 89.6%, 72.6%, 21.7% and 9.9%, while with conventional heating technique the yields were 89.4%, 51.8%, 19.6% and 9.8%. Interestingly, RF heating raised glucan yield on switchgrass and sweetgum but not much on corn stover or loblolly pine. The SEM images and FTIR spectra agreed with composition analysis and results of enzyme hydrolysis. Moreover, the acetic acid of switchgrass hydrolysate after radio frequency heating was 2.19g/L compared to that of traditional water bath heating at 1.58g/L. After pretreatment, the practicality of using switchgrass hydrolysate medium to grow recombinant *E-coli* utilizing pBHR68 plasmid for production of polyhydroxybutyrate (PHB), a biodegradable plastic, was explored in

this study. Switchgrass hydrolysates after alkaline pretreatment assisted by radio frequency heating and traditional water bath heating (original and added carbon source), as well as M9 medium (control group), were used as culture media. The RF media was shown to be optimal for PHB concentration produced, with final dry cell weight (DCW) 6.30 ± 0.11 g/L, PHB concentration 2.25 ± 0.13 g/L. Moreover, Switchgrass hydrolysates after alkaline pretreatment assisted by radio frequency heating and traditional water bath heating (original and added carbon source) were used as culture media for acetone-butanol-ethanol (ABE) fermentation by *Clostridium beijerinckii*. The hydrolysate was used after pH adjustment without sediment removal for ABE fermentation. ABE got to the maximum in the first medium with butanol concentration 3.9 g/L and total ABE concentration 5.91 g/L, corresponding to the maximum ABE yield (0.45) in the first medium, indicating that the enzymatic hydrolysates after alkaline pretreatment assisted by radio frequency was best for *Clostridium beijerinckii* growth. However, the difference of the switchgrass hydrolysate between radio frequency and traditional water bath heating was smaller with the addition of yeast extract, which verified that the radio frequency probably broke down into some nutrients in favor of ABE fermentation.

Visual Abstract



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List of Abbreviations

ABE	Acetone-butanol-ethanol fermentation
ADC	Acetoacetate decarboxylase
ADH	Acetaldehyde/ butyraldehyde dehydrogenase
AFEX	Ammonia fiber explosion
AK	Acetate kinase
BCD	Butyryl-CoA dehydrogenase
BDH	Ethanol/butanol dehydrogenase
BG	1,4-β-D- glucosidases
BK	Butyrate kinase
CBH	Exo-1, 4-β-D-glucanases
CFU	Colony forming unit
CO	Carbon monoxide
CS	Corn stover
CoAT	CoA transferase
CRT	Hydroxybutyryl-CoA dehydrolase
DCW	Dry cell weight
DI	Deionized
DW	Dry weight
EG	Endo-1, 4-β-D-glucanases

EMP	Embden-Meyerhoff pathway
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
H_2	Hydrogen
HBD	Hydroxybutyryl-CoA dehydrogenase
HMF	5-hydroxymethylfurfural
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
H_2SO_4	Sulfuric acid
LB	Luria-Bertani
LCB	Lignocellulosic biomass
LP	Loblolly pine
MW	Microwave
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
OD	Optical density
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PKA	Phosphate acetyltransferase
PLA	Polylactic acid
PTB	Phosphotransbutylase
RF	Radio frequency

rpm	revolutions per minute
SEM	Scanning electron microscope
SG	Sweet gum
SHF	Separate hydrolysis and fermentation process
SSF	Simultaneous saccharification and fermentation process
SW	Switchgrass
SWH	Switchgrass Hydrolysate
TGY	Tryptone–glucose–yeast extract medium
THL	Acetyl-CoA acetyltransferase
UV	Ultraviolet light
WB	Water bath
YE	Yeast extract

Chapter 1 Introduction and Research Objectives

1.1 Background

In order to reduce the dependence on foreign suppliers of petroleum, the United States is in need of alternative energy sources, which will also improve the cash flow balance for the country. The Energy Independence and Security Act of 2007 mandates a minimum of 36 billion gallons of renewable fuel production annually in the USA by 2022 (Mckendry, 2002). To meet the aforementioned goal, lignocellulosic biomass (LCB) needs to be produced on a cost competitive basis with fossil fuel sources (Jones and Mayfield, 2012; Maria et al., 2011). Compared with fossil fuels, biomass energy has a wide range of environmental and social benefits if produced in an efficient and sustainable manner. The advantages include reduction of CO_2 levels, waste control and other environmental issues. The southern U.S. is the primary wood-producing region of the country (Mitchell et al., 2008), with more than 200 million acres of commercial forestland and timber inventories that have increased each year for more than a decade (Sanderson et al., 1996). Therefore, the region's abundant timber resources become the most important feedstock for its ready availability and established markets.

The production of biofuel and value-added biochemicals involves an extensive process, including biomass pretreatment, enzyme hydrolysis, fermentation and product recovery (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008). The biomass pretreatment is one of the most crucial and expensive process steps because it is essential to reduce the crystallinity of the biomass and increase the surface area to enhance substrate digestibility, making it available to release fermentable sugars and then prepare

for the fermentation process (Galbe and Zacchi, 2012). Biomass can be pretreated in different ways, mainly physical, chemical and biological. In spite of the many different pretreatment methods extensively studied, none of these can be recognized as a "winner" by the scientific world (Chandra et al., 2007; Zheng et al., 2009; Zhu et al., 2006a). Most of the pretreatment methods involve a high temperature requirement, which is usually achieved through convection- or conduction- based heating. Dielectric heating is a promising and alternative method for conventional heating. Dielectric heating can be classified as microwave (MW) or radio frequency (RF) due to the wavelength used in heating devices (Baghurst and Mingos, 1992; Hu and Wen, 2008). Compared to MW, RF heating can penetrate dielectric materials more deeply, which is supposed to be easier to scale up (Guan et al., 2004; Ramaswamy and Tang, 2008; Wang et al., 2003). However, there has been no report on using RF heating for biomass pretreatment other than switchgrass as a substrate.

1.2 Research Objectives

The world population is increasing at an alarming rate, resulting in a large increase in primary energy consumption. Lignocellulosic biomass (LCB) can serve as a promising alternative energy source. Any type of fuel derived from biomass can be termed biofuel. The production of biofuel involves biomass pretreatment, enzyme hydrolysis, and fermentation. Pretreatment plays an important role in making the cellulose accessible for enzyme hydrolysis and subsequent conversion because it more or less destroyed resistance and recalcitrance of biomass. LCB can be pretreated in physical, chemical and biological ways. Radio frequency is one of the dielectric heating methods physically speaking.

The whole process of biofuel and value-added biochemical production can be divided into three stages: pretreatment, enzyme hydrolysis, and fermentation. There are mainly two objectives in this study. The first one is to compare the pretreatment of four different raw materials: corn stover (agricultural residues), switchgrass (herbaceous biomass), sweetgum (hardwood), and loblolly pine (softwood) using the alkaline pretreatment methods assisted by radio frequency and traditional water bath heating. The second one is to compare of switchgrass from different heating methods of pretreatment through enzymatic hydrolysis to fermentation, including chemical compositions before and after pretreatment, glucan yields after enzyme hydrolysis, PHB production after E-coli fermentation and ABE fermentation. Results from the pretreatment process will be utilized in biofuel and value-added biochemical production in both commercial and academic area.

1.3 Significance

The need for energy is constantly increasing because of increases in industrialization and population. The growth of the world's energy demand raises urgent problems. These real concerns force the search for alternative, sustainable, renewable, efficient and cost-effective energy sources (Tanger et al., 2013). In order to reduce the dependence on foreign suppliers of petroleum, the United States is in need of alternative energy sources, which will also improve the cash flow balance of the country (D. Humbird et al., 2011). LCB is a potential and competitive source for bioenergy production. There are two main reasons: biomass is one of the few energy sources that

can actually be utilized to produce several types of energy (motor fuel, electricity, heat); and cellulosic biomass is renewable and commonly found (Gellerstedt et al., 2009).

The production of biofuel and value-added products involves an extensive process, including biomass pretreatment, enzyme hydrolysis, and fermentation (Palmqvist and Hahn-Hägerdal, 2000a). The biomass pretreatment is one of the most crucial and expensive processes. Pretreatments of LCB are required to alter the structure of biomass in order to be converted to simple fermentable sugars. Biomass can normally be pretreated in conventional heating. Radio frequency (RF) heating is a promising kind of dielectric heating technology, which is an alternative method for conventional heating. Compared to MW, RF heating can penetrate dielectric materials more deeply, and it supposed to be easier to scale up to industrial scales (Cheng et al., 2011).

This research has a close relationship with the Energy for Sustainability program area in the National Science Foundation (NSF). This particular program supports fundamental research that will enable innovative processes for the sustainable production of electricity and transportation fuels (McKendry, 2002a; R.Tewfik et al., 2011; Ranjan and Moholkar, 2013). We propose here using environmental lignocellulosic biomass to compare different pretreatment methods for biofuel and value-added biochemicals production, which is alternative, sustainable, renewable, efficient and cost-effective. Since the need for energy is constantly increasing, this research will be of vital importance in energy for sustainability.

This research is systematic and unique and has never been discussed before for so many kinds of biomass feedstocks. First, it will be the first study to gain a comprehensive comparison of different types of lignocellulosic biomass using alkaline pretreatment assisted by radio frequency heating (Datta and Davidson, 2000; Keshwani and Cheng, 2010). To the best of our knowledge, there were only two papers regarding the alkaline pretreatment on biomass assisted by RF heating: Hu et al. (2008) collaborated with our lab handling switchgrass and Iroba et al. (2013) pretreated barley straw. But no research has been done on other types of lignocellulosic biomass. In the age of biofuel, we will continue with our long-term goal to produce renewable biofuels, value-added chemicals and biomaterials from biomass.

Results can contribute to two areas: commercial and engineering function. Through the comparison of chemical pretreatment assisted by dielectric and conventional heating, we will work out the best approach to get high biofuel and value-added bio-chemical yield, which gains an engineering insight into one of the most important biomass conversions. At the same time, we will gain an economical way to get biofuel, even value-added products, which would increase profits for companies.

Chapter 2 Literature review

2.1 Lignocellulosic Biomass

The need for energy is constantly increasing because of the finite supply of fossil fuel resources, as well as the industrialization and population (Valery B. Agbor, Nazim Cicek, Richard Sparling, Alex Berlin, 2011). Increased use of fossil fuels to meet global energy demand will significantly increase carbon dioxide emissions, which contributes to global climate change (McKendry, 2002a). These real concerns force the search for alternative, sustainable, renewable, efficient and cost-effective energy sources (Forest Products Laboratory, 2010; Larsson et al., 1999). In this regard, lignocellulosic biomass (LCB) has been considered a credible and viable alternative energy source.

LCB is a potential and competitive source for bioenergy production (Bull, 1990; Cantrell et al., 2008). There are two main reasons that LCB is superior to other feedstocks: biomass is one of the few energy sources that has no competition with food based biomass; and lignocellulosic biomass is renewable and commonly suitable for production, harvest, handling, storage and transportation because of the desired physical and chemical properties (Brethauer and Wyman, 2010; Chiaramonti et al., 2012; Chum and Overend, 2001; Gnansounou, 2010). LCB refers to plant biomass that is composed of cellulose, hemicelluloses, and lignin (Yamamoto et al., 2002). It is a complex matrix, comprising many different polysaccharides, phenolic polymers and proteins (Wyman et al., 2005a). LCB can be grouped into four main categories (P. Kumar et al., 2009): 1) agricultural residues (corn stover and straw, which can be used for production of second generation biofuels); 2) dedicated energy plants (switchgrass, eucalyptus etc.); 3) wood

biomass from hardwood and softwood trees; and 4) municipal solid waste mainly including municipal paper waste, packaging waste wood, household waste wood, market waste, food processing wastes, etc. (Chum and Overend, 2001; S. Kumar et al., 2009; Nanda et al., 2013; Zhang et al., 2013).

2.1.1 Physical structure of LCB

Lignocellulosic biomass has a complicated and hierarchical structure, which contributes to its special mechanical and physical properties (Zhu et al., 2010). Wood, depending on trunk, or root, is composed of different anatomical tissues, which are made of different wood cells with different functional roles. These cells are typically oriented in axial and radial directions (Brodeur et al., 2011). Plant cell walls have a wide array of disparate and sometimes opposing roles. For example, they have an important function to protect against pathogens, as well as resist the outside mechanical stress as well as the shape of the cell (Vanholme et al., 2010). However, they must be reasonably flexible to tolerate shear forces, and at the same time be permeable enough to allow the reaching into and out of cells for signaling molecules.

The cell wall is mainly composed of cellulose microfibrils (Yamamoto et al., 2002). In plant cell walls the cellulose microfibrils are encrusted in lignin and hemicellulose in a complete structure that, together with the crystallinity of cellulose shown in Figure 1, makes untreated cellulosic biomass recalcitrant to hydrolysis to fermentable sugars (Xiros et al., 2013).

Generally, wood is divided into two categories: softwoods (gymnosperms such as conifers) and hardwoods (angiosperms such as deciduous or broad-leaf trees) (Li, 2014; McKendry, 2002a; Tanger et al., 2013). In the southern forest area of the United States, approximately 52% is dominated by hardwoods-deciduous broadleaf trees such as oaks, while the remaining is dominated by softwoods-evergreen coniferous trees such as pines- or a mixture of hardwood and softwood species (Valery B. Agbor, Nazim Cicek, Richard Sparling, Alex Berlin, 2011).

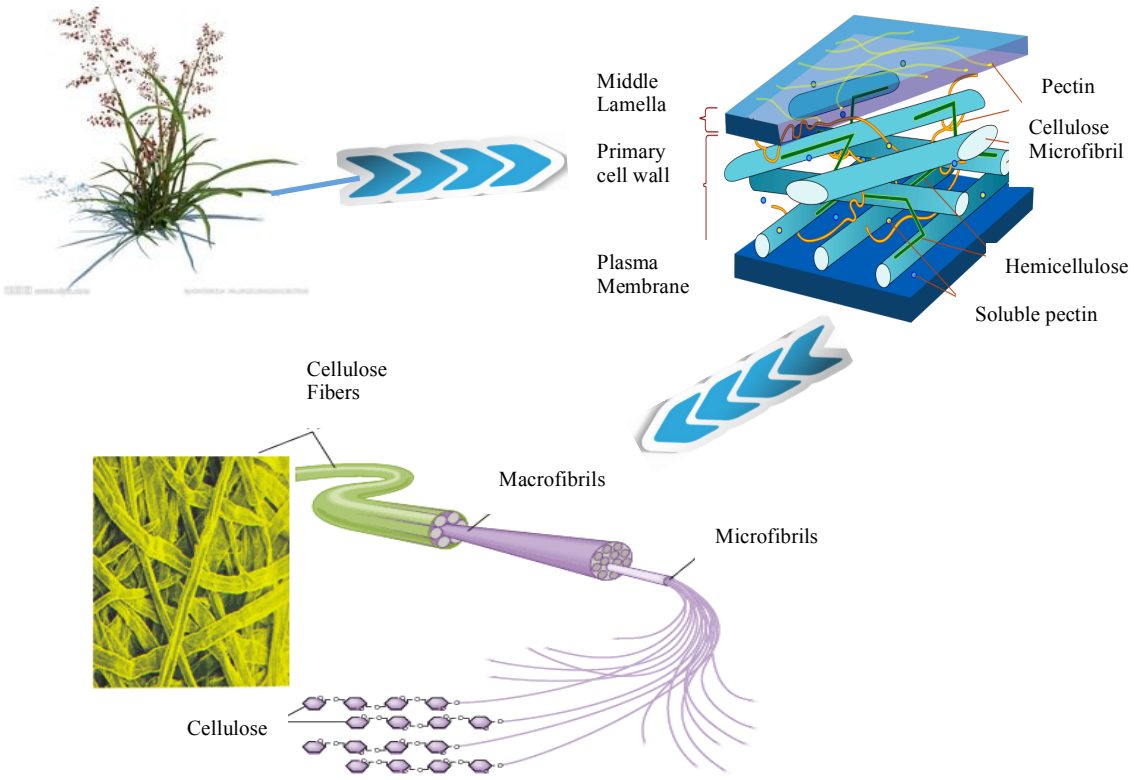


Figure 1 Structural organization of the plant cell wall

2.1.2 LCB chemistry

LCB is composed mainly of cellulose, hemicellulose and lignin that are closely associated with complicated components, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash (Alauddin et al., 2010; Park and Kim, 2012; Tomás-Pejó et al., 2011). The structure of LCB can be described as a skeleton of cellulose chains embedded in a cross-linked matrix of hemicellulose surrounded by a crust of lignin, shown in Figure 2. There are extensive interactions between cellulose, hemicellulose and lignin, and the natural barrier of lignin increases the difficulty of the access of hydrolytic enzymes to simple sugars. The amount of each component varies depending on the species of LCB (R. Kumar et al., 2009; Palmqvist and Hahn-Hägerdal, 2000a, 2000b). In addition, the percentage of different components within a single species of biomass differs with age, stage of growth, and other conditions. In general, hardwood has greater amount of cellulose, while wheat straw and leaves have more hemicellulose (Hendriks and Zeeman, 2009; Lloyd and Wyman, 2005), as shown in Table 1.

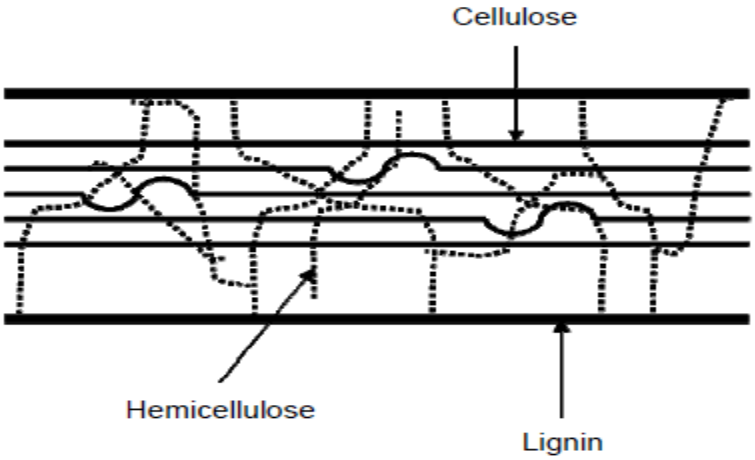


Figure 2 General Structure of Lignocellulosic Biomass

Table 1 Different lignocellulosic biomass chemical compositions (% dry basis)

Lignocellulosic biomass		***Cellulose***	***Hemicellulose***	***Lignin***
Softwood		40-50	15-20	23-33
	Loblolly pine	46.4	18.8	29.4
	Spruce	43.8	19.8	28.8
	Douglas fir	40	19.9	24.6
Hardwood		40-55	20-35	21-31
	Eucalyptus	45	19.2	31.3
	Polar	43.8	18.4	29.1
	Sweetgum	40.8	30.7	25.4
Herbaceous grasses		25-40	35-50	10-30
	Switchgrass	31.0	20.4	17.6
Agricultural residues		25-30	20-30	12-40
	Corn stover	37.5	22.4	17.6
	Rice straw	27.9	28.2	31.7
	Nut shells	31.1	22.3	13.3

Cellulose is the most abundant biopolymer in the world (Mussatto and Teixeira, 2010). From a structural point of view, cellulose is a linear polysaccharide of β-D-glucose units that are linked via β-1, 4 glucosidic bonds (Li and Huang, 1998; Singh et al., 2009), with repeating units of cellobiose shown in Figure 3. For the β-1, 4 glucosidic bonds, they form a linear chain of glucose molucules, which results in a highly ordered packing of cellulose chains. This chain interacts via inter-molecular and intra-molecular hyderogen bonds through a direct interaction of hydroxyl groups or the hydrogen atoms of neighboring glucose unit shown in Figure 4 (Arioli et al., 1998). Consequently, cellulose in biomass is present in both crystalline and amorphous forms (Chen et al., 2004; Harmsen and Huijgen, 2010; Qian, 2008). The crystalline regions are highly ordered and arranged, while amorphous-like regions are disordered. The degree of polymerization (DP) of cellulose is about 10,000 (Besombes and Mazeau, 2005; Jørgensen et al., 2007). Degrees of polymerization and cellulose crystallinity have been considered as important barriers for the subsequent enzymatic hydrolysis. Each cellulose chain owns one reducing end and one non-reducing end (Jørgensen et al., 2007; R. Kumar et al., 2009; Kurabi, 2004; Pinkert et al., 2009; Quiroz-Castañeda and Folch-Mallol, 2013), which need different kinds of enzymes to be hydrolyzed. From a functional point of view, cellulose is a major component of plant cell walls, and it provides mechanical strength and chemical stability.

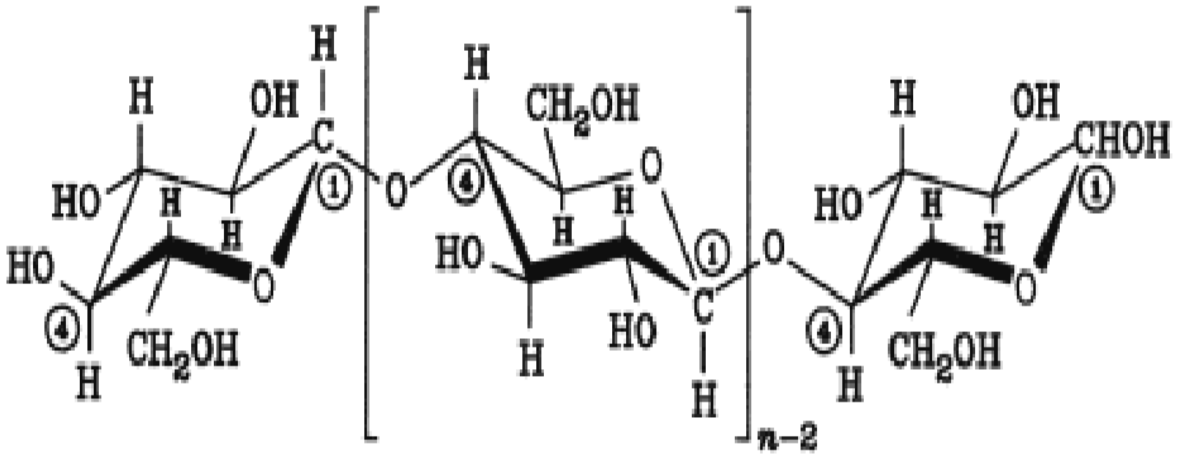


Figure 3 Structure of single cellulose molecule (Harmsen and Huijgen, 2010)

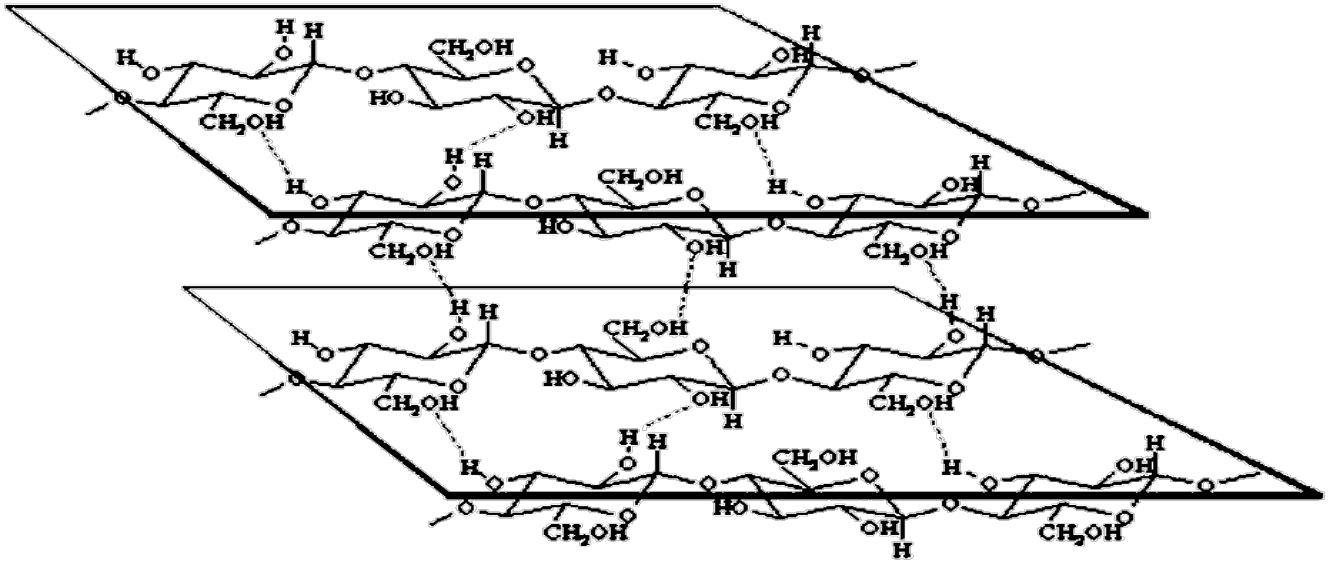


Figure 4 Demostration of the hydrogen bonding (Harmsen and Huijgen, 2010; Li, 2014)

In contrast to cellulose, which is a homopolysaccharide, hemicellulose is heteropolysaccharide that is made up of five carbon sugars (xylose and arabinose), six carbon sugars (mannose, glucose and galactose), and uronic acids (Chandra et al., 2007; Wyman et al., 2005b). These subunits are bonded together through linkages such as β-O-4, 1-2, 1-4, and 1-6 glycosidic bonds. From a structural point of view, cellulose remains almost the same for all lignocellulosic biomass. However, the structure and composition of hemicelluloses can vary. More specifically, the amount of hemicelluloses in wood typically varies between 20% and 30%. The structure and composition of the hemicelluloses in hardwoods differ from those in softwoods. For example, hemicelluloses in hardwood are rich in xylan polymers with small amounts of mannan, while softwood hemicelluloses are rich in mannan polymers (Chandra et al., 2007). The principal hemicellulose of softwoods is the galactoglucomannans (~ 20%), and the minor is arabinoglucuronoxylans (~5-10%). However, the primary hemicellulose of hardwoods is the glucuronoxylans (15-30%) while the minor is glucomannans (Mussatto and Teixeira, 2010). The structures of those four hemicelluloses are shown in Figure 5 in sequence. Although the backbone of the hemicellulose is similar to that of cellulose, the presence of side chains and occasional branching minimize hydrogen bonding, which further lower the crystallinity of hemicellulose. Most hemicelluloses have a DP of 150-200 (Kurabi, 2004) and it is relatively easy to be hydrolyzed to monomers. It has a lower molecular weight than cellulose, and its role is to connect cellulose fibers and lignin.

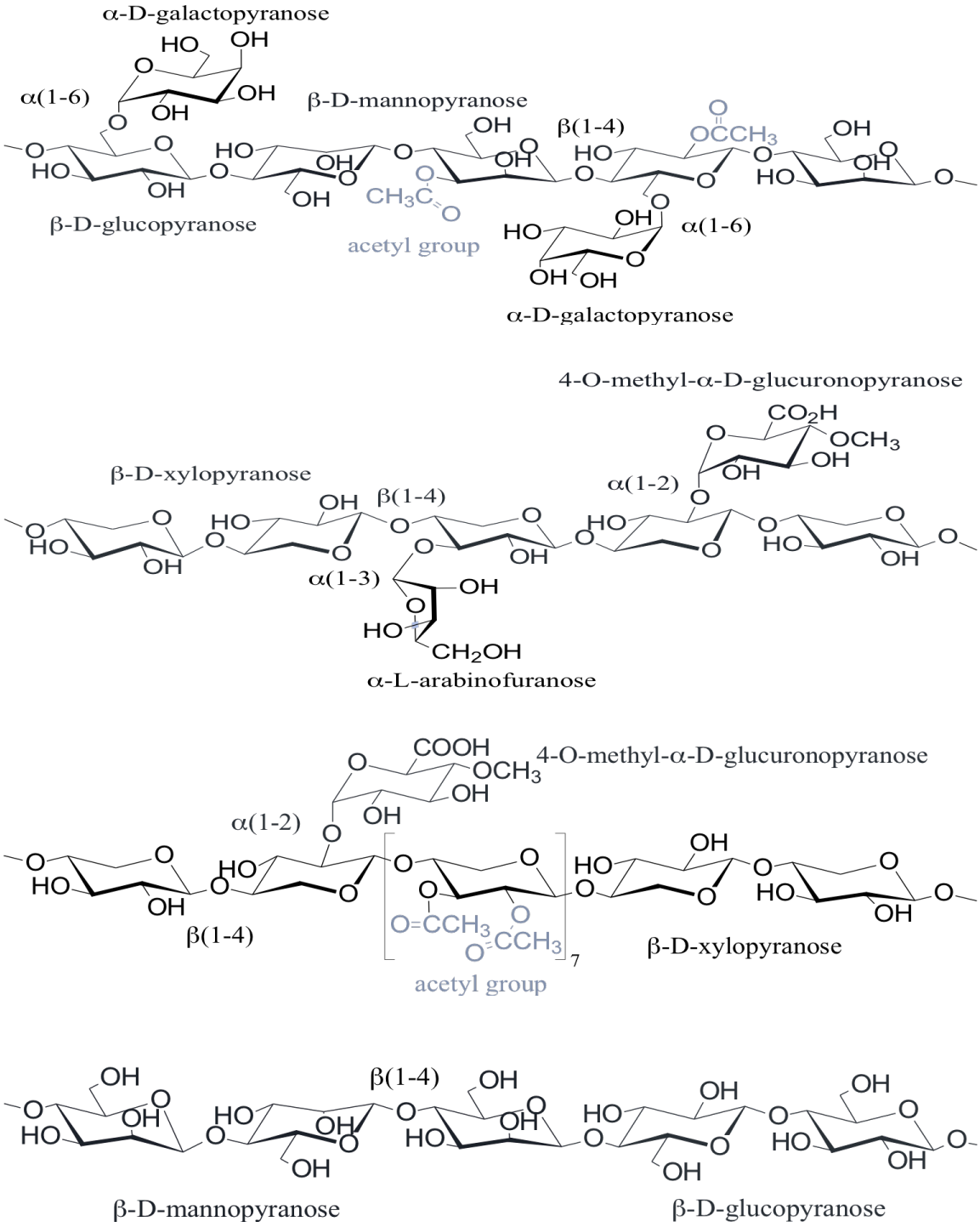


Figure 5 Structure of hemicelluloses in softwood and hardwood: galactoglucomannan; arabino-4-O- methylglucuronoxylan; glucuronoxylan; and glucomannan (Erik Hagglund, 1951)

Lignin is the second most abundant biopolymer on earth next to cellulose. It is the most complex natural polymer (Gellerstedt et al., 2009). From the structural point of view, it is a mixture of aromatic and aliphatic monomers. More specifically, it is made up of three types of basic units: β-dydroxyphenyl alcohol, coniferyl alcohol and sinapyl alcohol (Öhgren et al., 2007; Sjostrom, 1865) shown in Figure 6. There are also other monolignols (lignin monomers) including coniferal dehydes, acetylated coniferyl alcohol, and ferulic acid. The proportions of these three building blocks vary based on the type of the lignocellulosic biomass material. For example, softwood lignin can be described as "guaiacyl lignin" since it is composed mainly of coniferyl alcohol, that is guaiacyl (G) lignin sub-units, and may possess a small amount of coumaryl alcohol (Adler, 1977). Contrary to softwood, hardwoods contain mainly a "guaiacyl-syringyl" lignin that is made up of varying ratios of coniferyl and sinapyl alcohol type of units shown in Figure 7. For the hardwood and softwood, both the location and the type of lignin (G or S) probably have significant effect on the bio-recalcitrance of the substrate to cellulolytic enzymatic hydrolysis during the biomass utilization (Brebu and Vasile, 2010). In general, grasses such as switchgrass contain equal amounts of all three monolignols. These three basic units are connected together by C-O-C (ether) and C-C linkage. The β-O-4 arylether linkages dominate lignin structure. The other linkages include α-O-4, carbon 5-5, and 4-O-5 (Sjostrom, 1865). The C-C linkages are the strongest and are the main contributor to the barrier and recalcitrance nature of lignin. From the functional point of view, lignin acts as the "glue" that holds cellulose and hemicellulose together within the wood structure (Buranov and Mazza, 2008). In this regard, lignin plays a vital role in a plant's natural defense against degradation by microbial enzymes. For example, lignin works

well in cell's endurance and development, since it affects the transportation of water, nutrients and metabolites inside the plant wall.

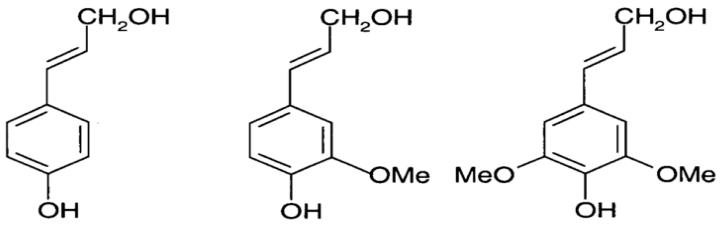


Figure 6 Basic building blocks of lignin

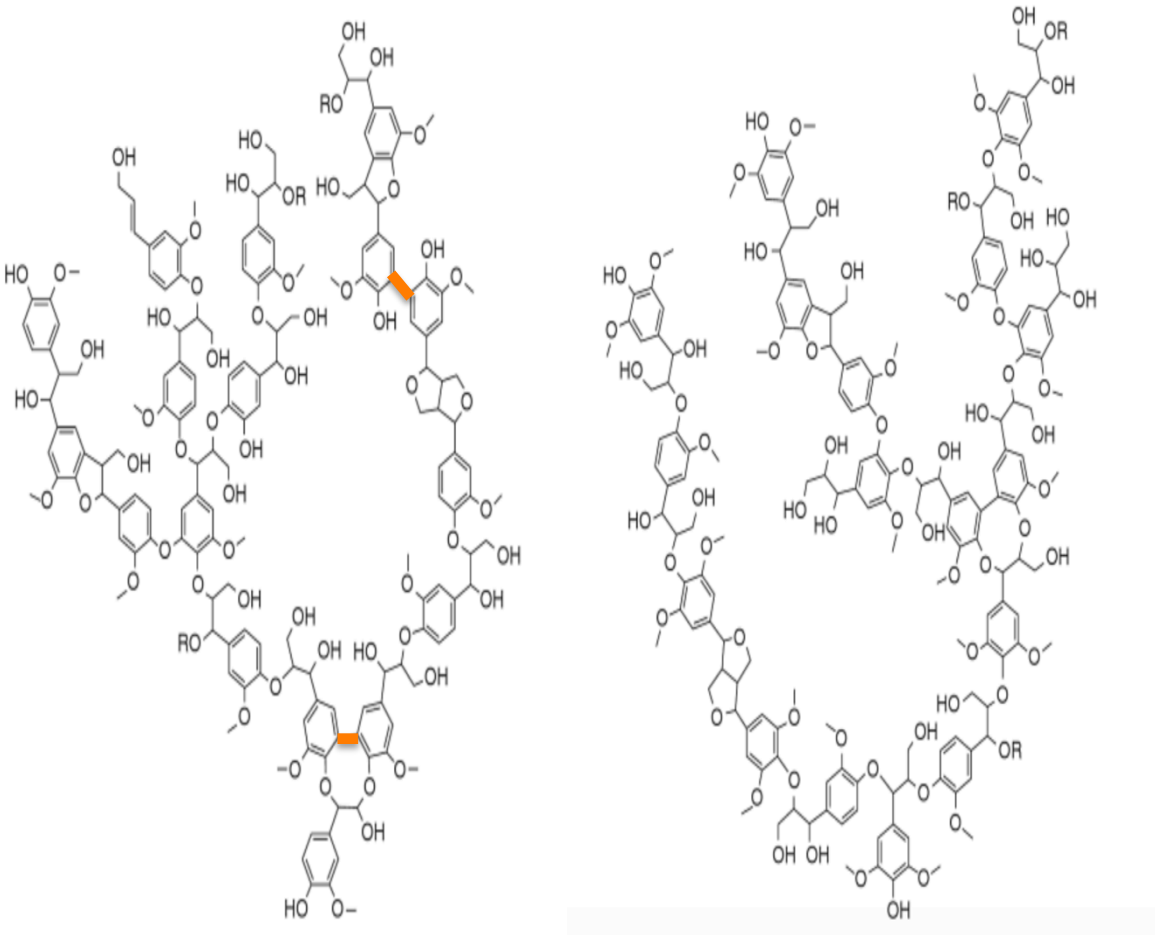


Figure 7 Lignin in softwood and hardwood, from left to right.

2.2 Biochemical conversion of LCB to fuels and chemicals

There are mainly two conversion pathways for LCB utilization shown in Figure 8. The first one is the thermochemical conversion. And the other one is biochemical conversion (Balan et al., 2012; Tanger et al., 2013; Wright and Brown, 2007).

For thermochemical conversion, lignocellulosic biomass is converted into intermediate products, which are further transformed into biofuels through chemical or biological routes (R. C. Brown, 2011; Tanger et al., 2013; Wright and Brown, 2007). Biomass can be broken down through heat energy and chemical catalysts and then be transformed into intermediate products (Bahng et al., 2009; Demirbaş, 2001). For example, gasification is to heat biomass with about one third of the oxygen necessary for complete combustion (McKendry, 2002a). It produces a mixture primarily composed of CO and H_2, known as syngas (Griffin and Schultz, 2012). Moreover, pyrolysis is to heat the biomass in the absence of oxygen at high temperatures. It decomposes into a liquid bio-oil, which can be used directly or can be converted to clean fuels and other valuable chemicals. Researchers are developing gasification and pyrolysis processes for the cost-effective thermochemical conversion of biomass to biofuels (Chundawat et al., 2010). For thermochemical conversion of biomass utilization, the major barriers and hurdles are the high cost associated with cleaning the product gas, undesirable contaminants, inefficiency due to the high temperatures utilized, and unproven use of syngas and bio-oil as transportation fuels (Boateng et al., 2008; R. Brown, 2011; Canabarro et al., 2013).

For biochemical conversion, multiple enzymes are used for the hydrolysis of pretreated biomass to obtain fermentable sugars (Foust et al., 2009; Kücük and Demirbas, 1997). There are mainly three basic steps in biochemical conversion of biomass, including converting biomass to sugar and other fermentation feedstocks through pretreatment and enzymatic hydrolysis; fermenting these biomass-derived feedstocks using microorganisms; and processing the fermentation product to produce fuel-grade ethanol and other fuels, chemicals, heat, and electricity (Balan et al., 2012; Balat, 2006; Ortegren, 1981; Saxena et al., 2009). Researchers are working to improve the efficiency and economics of the biochemical conversion process (Alvira et al., 2010; Ewanick and Bura, 2010; Szczodrak and Fiedurek, 1996). For this conversion, the major challenge is the pretreatment process and the utilization of expensive enzymes (Hendriks and Zeeman, 2009; McKendry, 2002b). In our experiment, we focus on the biochemical conversion process. It includes three steps: pretreatment, enzyme hydrolysis and fermentation.

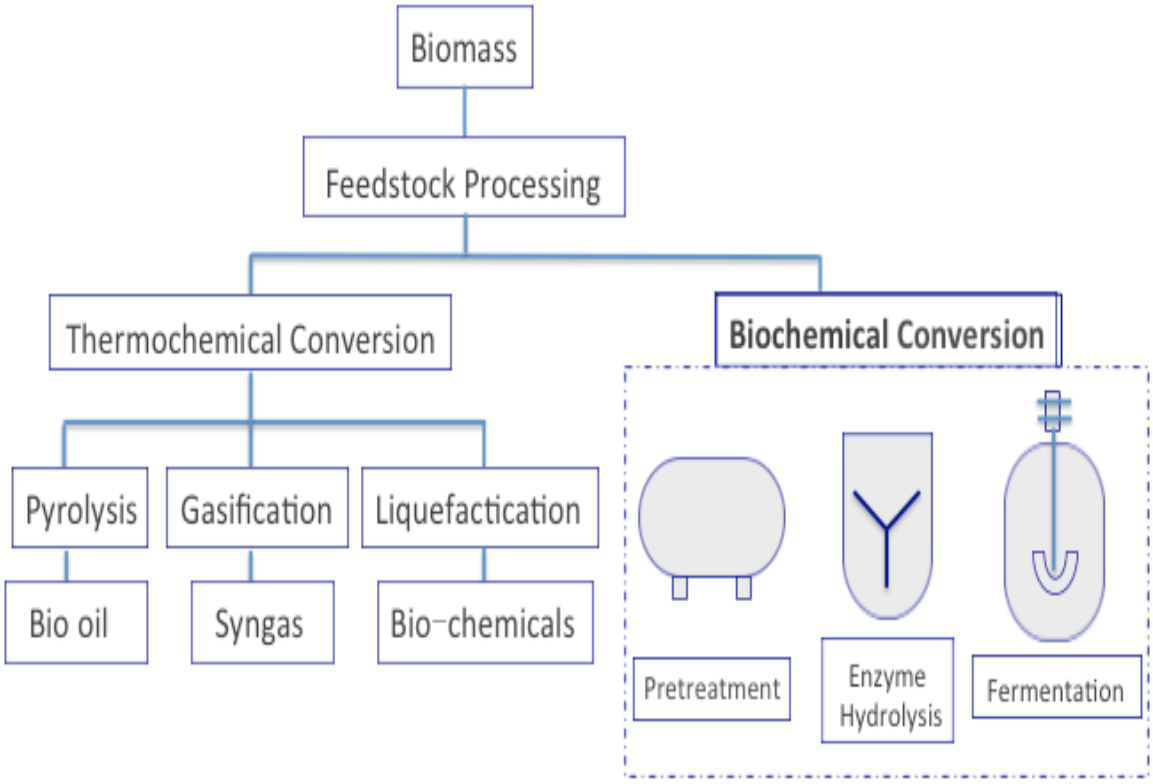


Figure 8 Major pathways for lignocellulosic biomass utilization

2.2.1 Pretreatment

In lignocellulosic biomass, cellulose is protected by a sheath of lignin and hemicellulose. Pretreatment technology is used to open up the structure of biomass to allow further enzyme hydrolysis of the cellulose to glucose (D. McMillan, 1994; Foston and Ragauskas, 2012; Harmsen and Huijgen, 2010). The basic objective of pretreatment is to reduce the recalcitrance of lignocellulose biomass in order to be converted to simple fermentable sugars. The bio-recalcitrance is caused by the physical structure of cellulose, hemicellulose and lignin, as well as the chemical linkages among the cellulose chain and the adjacent chains (Foston and Ragauskas, 2012; Himmel et al., 2007; Zhao et al., 2012a, 2012b; J Y Zhu and Pan, 2010).

The pretreatment process, shown in Figure 9, is required to remove lignin and hemicellulose, reduce the crystalline structure of cellulose, and increase the porosity of the materials (Behera et al., 2014; Galbe and Zacchi, 2012; Mosier et al., 2005a). An effective pretreatment is characterized by several criteria as follows (Lee and Dale, 2004; Mosier et al., 2005a; Susan Marie Hennessey et al., 2013; Zwart et al., 2006). From money point of view, it requires low substrate costs, capital costs and operation costs (Wyman et al., 2005a). From efficiency point of view, it needs to improve digestibility of cellulose in the subsequent enzyme hydrolysis (Zhu et al., 2010). From side effect of view, it should avoid degradation or loss of sugar and lignin, as well as lower the formation of degradation products that inhibit the growth of fermentative microorganism (Hendriks and Zeeman, 2009).

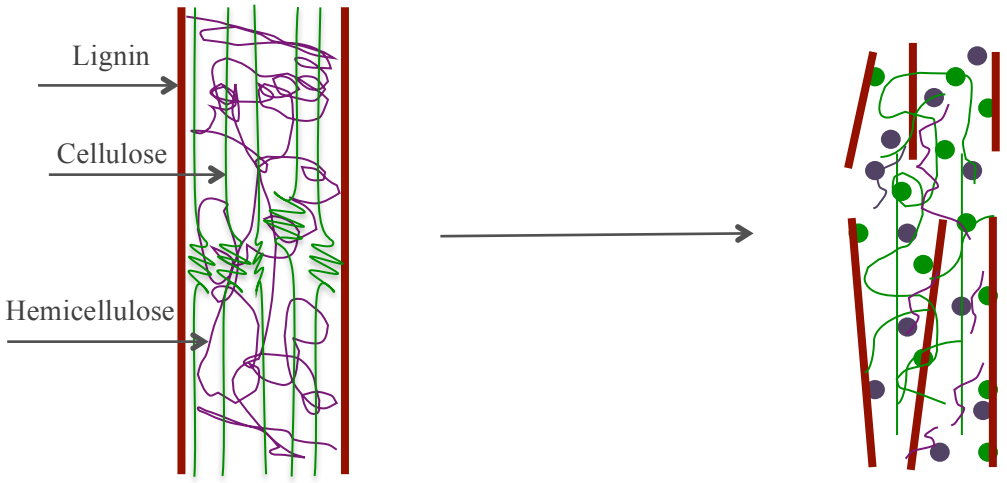


Figure 9 Schematic representation of pretreatment on lignocellulosic biomass

Feedstock pretreatment has been recognized as a necessary upstream process for enzymatic degradation (Hendriks and Zeeman, 2009). Since LCB materials are very complicated, their pretreatment is not easy either. There are several key factors to take into consideration for an effective pretreatment process (Galbe and Zacchi, 2012; J Y Zhu and Pan, 2010). For example, various pretreatmet methods have been shown to be better suited for specific type of lignocellulosic biomass. More specifically, the relationships between structural and compositional factors explain the complexity of LCB and further account for the varying digestibility between different sources of LCB (Hendriks and Zeeman, 2009; Sun and Cheng, 2002). Expect for the heterogeneous nature of LCB, the crystallinity and degree of polymerization of cellulose, the accessible surface area, crystallinity and degree of polymerization of cellulose, accessible surface area/porosity, protection of cellulose by lignin, and cellulose sheathing by hemicellulose all contribute to the bio-recalcitrance of LCB to hydrolysis (Chandra et al., 2007). In principle, an effective pretreatment successfully eliminates these barriers so that enzymes can cause hydrolysis in the following step.

Pretreatment technologies can be roughly divided into several different categories: physical (milling, irradiation, microwave, radio frequency, liquid hot water pretreatment, uncatalyzed steam-explosion, extrusion, pyrolysis, freeze pretreatment etc.), chemical (acid pretreatment, alkaline pretreatment, ionic liquid etc.), physicochemical (ammonia fiber explosion, steam explosion, alkaline/acid assisted by heating etc.), biological and others (Day, 2009; Haghighi Mood et al., 2013 and Zhu et al., 2010). Various pretreatment methods have been extensively studied. However, none

of these can be recognized as a "winner" (Ingram et al., 2011; Jäger and Büchs, 2012; Malherbe and Cloete, 2002; Menon and Rao, 2012) since each pretreatment has its intrinsic advantages and disadvantages shown in Table 2. These pretreatment methods result in different extent of lignin removal, enzyme accessibility, and fermentation efficiency.

Among the physico-chemical pretreatment methods, ammonia fiber explosion (AFEX) and alkaline/acid with heating are generally studied (Chundawat et al., 2007; Day, 2009; Taherzadeh and Karimi, 2008). Although these are all powerful methods for enzyme hydrolysis, they have their own drawbacks. AFEX is high-energy demanding and has unsafe factors.

For acid pretreatment, the main mechanism is the solubilization of hemicellulose, which is accompanied by the reduction of cellulose crystallinity, as well as the particial fracture of lignin, while the majority of lignin remains in solid phase (Chen et al., 2011; Eggeman and Elander, 2005; Guragain et al., 2011; Saha et al., 2005; Shekiro et al., 2012). However, the primary target of action for alkaline pretreatment is the removal of lignin (Zhao et al., 2008). At the same time, some hemicelluloses are removed by alkaline pretreatment since the structures are randomly amorphous especially at high temperature.

Table 2 Effect of chemical pretreatments on lignocellulosic biomass (Li, 2014)

Pretreatment	Chemical used	Reaction condition	Main effects	Pros	Cons
Comminution	Physical pretreatment	Mechanical operation	Reduce cellulose crystallinity	Structure little-changed	High energy needed; slow
Steaming or steam explosion (STEX)	Auto-hydrolysis (acetic acid)	180-210°C 1-10 min	Partial hydrolysis of hemicellulose; redistribution of lignin on fibers; fractionation of fibers	Surface area increased; structure little-changed; fast operation	Limited removal of lignin; little disrupt of crystallinity
Liquid hot water	Auto-hydrolysis (hydrothermolysis)	160-230°C 10-30 min	Removal of hemicelluloses and some lignin	No chemicals added; no neutralization needed; particle size insensitive	Limited disrupt of crystallinity; limited lignin removal; lignin structure altered
Acid hydrolysis	H_2SO_4 (0.3-3% w/w) SO_2/HCl/HNO_3/H_3PO_4	140-180°C 10-60 min	Removal of hemicelluloses	Enhanced susceptibility	Corrosiveness; degradation products; neutralization needed
Ammonia freeze/fiber explosion (AFEX)	NH_3 H_2O (5-15%)	90-100°C 5-10 min	Cleavage of lignin; partially depolymerization of cellulose and hemicellulose; less degradation products	Ammonia recyclable; little degradation products; high solid operation	Limited lignin removal; not well on softwood; Chemical cost
Lime	$CaCO_3$/NaOH	85-150°C; 1 h - days	Removal of lignin;	Low temperature; high delignification	Longer time; irrecoverable salts; biomass size sensitive
Wet oxidation	H_2O_2 (1-5%)	180-200°C 5-15 min	Removal of lignin; partial degradation of lignin; solubilization and oxidation of some hemicelluloses	Enhanced susceptibility	Much inhibitory
Ozonolysis	O_3	20-30°C 5-15 min	Degrade lignin and hemicelluloses	Effective delignification; less toxic products	Chemical cost
Organosolv	Alcohol (40-60%)/ organic acids (HCOOH, peracids)	100-250°C 30-60 min	Removal of lignin and some hemicelluloses (extracted out)	Isolate high quality lignin; removal of hemicellulose ; solvent recyclable	Some toxic; extra washing process; solvent cost
Biological	Fungi	20-30°C days	Degradation of lignin and hemicelluloses; partial degradation of cellulose	Low energy requirement; mild condition; enzymatic hydrolysis step partially skipped	Very slow

2.2.2 Enzyme hydrolysis

After pretreatment, lignocellulosic substrates are hydrolyzed by cellulases to yield sugars for subsequent fermentation (Alvira et al., 2010; Levine et al., 2010; Yang et al., 2011). In the pretreatment process, depending on the process employed, 80-100% of the hemicelluloses and 20-50% of the lignin are generally solubilized (Lynd et al., 2002). Enzymatic hydrolysis of cellulose is a complex reaction system (Yang et al., 2011; Zhang and Lynd, 2004), usually involving five steps. Firstly, transfer of the enzymes from bulk solution to the surface of the cellulose substrates; secondly, adsorb of the enzymes onto the cellulose substrate; thirdly, hydrolysis of cellulose by different cellulase components; fourthly, transfer of the glucose and cellobiose from the surface of substrate to the bulk solution; lastly, hydrolysis of cellobiose into glucose by β-glucosidase in the bulk solution.

The commonly used hydrolases include exo-1, 4-β-D-glucanases (CBH), endo-1, 4-β-D-glucanases (EG), 1,4-β-D- glucosidases (BG), endo-1,4-β-D-xylanases, 1,4-β-D-xylosidases, endo-1,4-β-D-mannanases, and 1,4-β-D-mannosidases. CBH I and CBH II hydrolyze the reducing and non-reducing ends of cellulose chains respectively (Jørgensen et al., 2007; Reese, 1955). EG randomly cut the cellulose chains at amorphous regions to produce new sites for CBH. Glucosidases hydrolyze cellobiose and cellotriose to glucose shown in Figure 10.

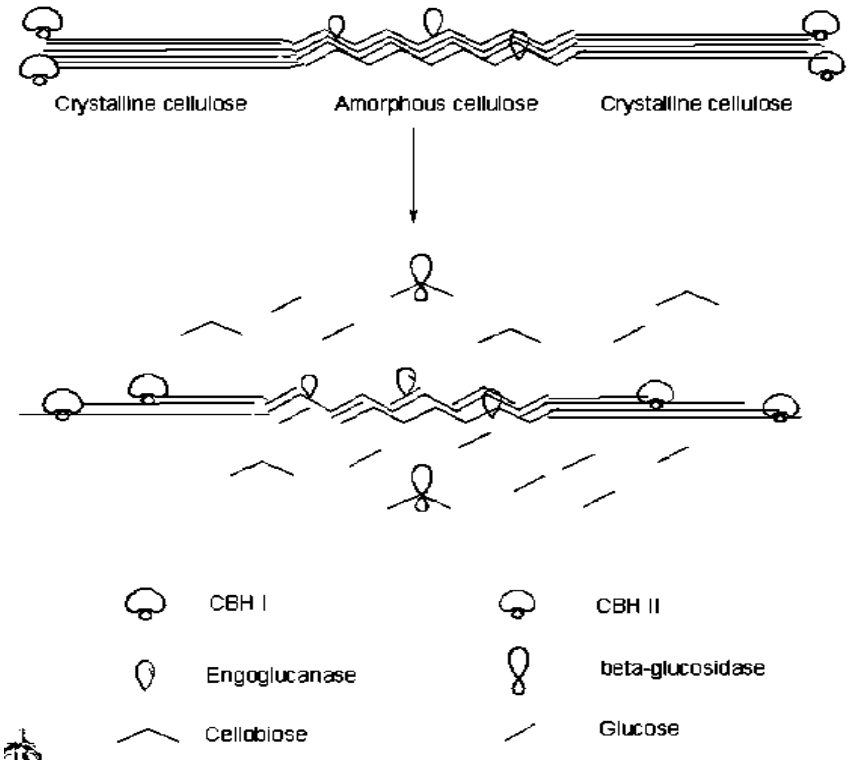


Figure 10 Cellulose hydrolysis by cellulases from Trichoderma reesei (Lynd et al. 2002)

There are mainly two categories of factors that limit enzymatic hydrolysis: enzymatic factors and substrate factors (Hendriks and Zeeman, 2009; Reese, 1955). Enzymatic factors that limit hydrolysis include lack of synergism, non-productive binding and end product inhibition. Bezerra and Dias (2005) showed that cellobiose is a strong inhibitor of both crude cellulases and exoglucanase (Cel7A). In addition to enzyme factors, the properties of the substrate are also known to play a major role in enzymatic hydrolysis. Due to their inherent structural characteristics, lignocellulosic substrates are naturally resistant to enzymatic hydrolysis. Substrate factors that limit enzymatic hydrolysis are summarized in Table 3.

Table 3 Substrate and enzyme factors limiting enzymatic hydrolysis (Lynd et al., 2002; Mosier et al., 2005b)

Impact factors	Characteristics
Degree of polymerization	DP affects the extent of hydrolysis ,but the correlation between hydrolysis and DP are low
Crystallinity	Amorphous cellulose hydrolyzes rapidly and crytalline hydrolysis slowly
Accessible surface area	Increasing the accessible surface area improves the lignocellulosic hydrolysis
Lignin	Lignin reduces the accessibility of cellulose to enzymes, and increases the non-productive binging with enzymes
Inhibition	Cellulases undergo strong end product inhibition thus requiring supplemental betaglucosidase
Synergism	Exoglucanases (CBHI and CBHII) and endoglucanases (EGI and EGII) act synergistically on lignocellulosic substract
Non-productive binding	Non-productive binding decreases the accessibility of cellulases to cellulose

2.2.3 Fermentation

Following enzymatic hydrolysis, fermentation is the phase of the bioconversion process where sugars from the hydrolysis step are fermented to ethanol, butanol and other biofuels and value-added biochemicals. Fermentation is a biological process in which enzymes produced by microorganisms catalyze chemical reactions that break simple sugars or amino acids into lower molecular weight materials such as organic acids and neutral solvents such as ethanol (Chisti, 2010; Humphrey, 1977; Rose, 1985). Although organisms exist to break down virtually any organic material, five- and six-carbon sugars are widely available in the plant and animal world (Munasinghe and Khanal, 2011; Panagiotou and Olsson, 2007). An enormous variety of bacteria, yeasts, and fungi exist to ferment these sugars. These microorganisms digest simple one and two molecule sugars to produce the energy and chemicals they need to live and reproduce, and give off byproducts such as carbon dioxide, organic acids, hydrogen, ethanol, and

other products (Awang et al., 1988; Jones and Woods, 1986; Lynd et al., 2002). Three approaches have been investigated for fermentation: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and direct microbial conversion (DMC) (Valery B. Agbor, Nazim Cicek, Richard Sparling, Alex Berlin, 2011). Separate hydrolysis and fermentation (SHF) is the most frequently applied process in bioconversion, and involves the fermentation of a pre-hydrolyzed substrate. Factors limiting the effectiveness of SHF include the end product (Saha et al., 2005). Fermentation processes using anaerobic microorganisms provide a promising path for converting biomass and agricultural wastes into chemicals and fuels (Ranjan and Moholkar, 2013). Acetone-butanol-ethanol fermentation (ABE fermentation) with the strict anaerobic bacterium, *Clostridium acetobutylicum*, was once (1917-1955) one of the largest fermentation processes ever developed in industry (Maddox, 1989; Patakova et al., 2011; Wu et al., 2012).

2.3 Fundamentals of radio frequency radiation-based process

Radio frequency (RF) heating is a promising kind of dielectric heating technology, which is an alternative method for conventional heating (Baghurst and Mingos, 1992). Convection (conduction) heating is based on superficial heat transfer, while dielectric heating utilizes the ability of some compounds to transform electromagnetic energy into heat that is volumetric and fast through a direct interaction between RF electromagnetic field and the object being heated (Wang, 2002). RF heating is also known as high frequency dielectric heating. During RF heating, the product to be heated forms a “dielectric” between two metal capacitor plates, which are alternatively

charged positively and negatively by a high frequency alternating electric field shown in Figure 11(Piyasena et al., 2003). In RF heating, there is a volumetric heat generation inside the product, which is a result of the interaction between the RF waves and the ions/molecules of the product (Hu et al., 2008; Liu et al., 2010; Ramaswamy and Tang, 2008; Tewari, 2007). Therefore, heat flows from inside the product to the outside, unlike conventional heating methods in which heat is transferred from the heating medium to the product via conduction or convection.

Depending on the wavelength used in the heating devices, dielectric heating can be divided into microwave and RF (Wang et al., 2003). Microwave (MW) heating has the same principle and mechanisms of heating as RF heating. Microwave-based heating has been studied in the pretreatment of various LCB (Zhu et al., 2006b). However, compared to microwave heating, RF heating systems have several advantages: 1) uniform electric field strength inside the application chambers, therefore preventing uncontrolled heating, overheating, local hot spots, and product degradation; 2) large penetration depth (10-30 m) of RF energy into a wide array of materials; and 3) higher electricity to electromagnetic power conversion efficiency (Al-Holy et al., 2005; Ramaswamy and Tang, 2008). In an RF chamber, the product is placed between two parallel electrodes (plates), which generate a uniform electric field around the product (Liu et al., 2010; Wang et al., 2003). Therefore, RF heating systems are more economically feasible, and they are more suitable for large commercial scale reactors. In RF and MW heating, heating is based on the product's ability to absorb electromagnetic radiation and convert it to heat. Applications of MW and RF improve product quality,

shorten the processing times, and reduce floor space when compared to conventional heating methods (Adair and Petersen, 2002; Guan et al., 2004; Koonen and Larrodé, 2008). RF heating is influenced by a number of system specific factors such as the frequency of heating, temperature, and product properties such as viscosity, moisture content, and structural and chemical composition (Ramaswamy and Tang, 2008). These influence the dielectric properties, and subsequently the RF heating of product. The RF band has a frequency range of 3 kHz to 300 MHz, but typically 13.56-27.12 MHz and 40.68 MHz are used (Roberts, 2006). The above-mentioned advantages associated with RF can enhance its commercialization and industrial application as a biomass pretreatment method as it can easily be scaled up (Al-Holy et al., 2005).

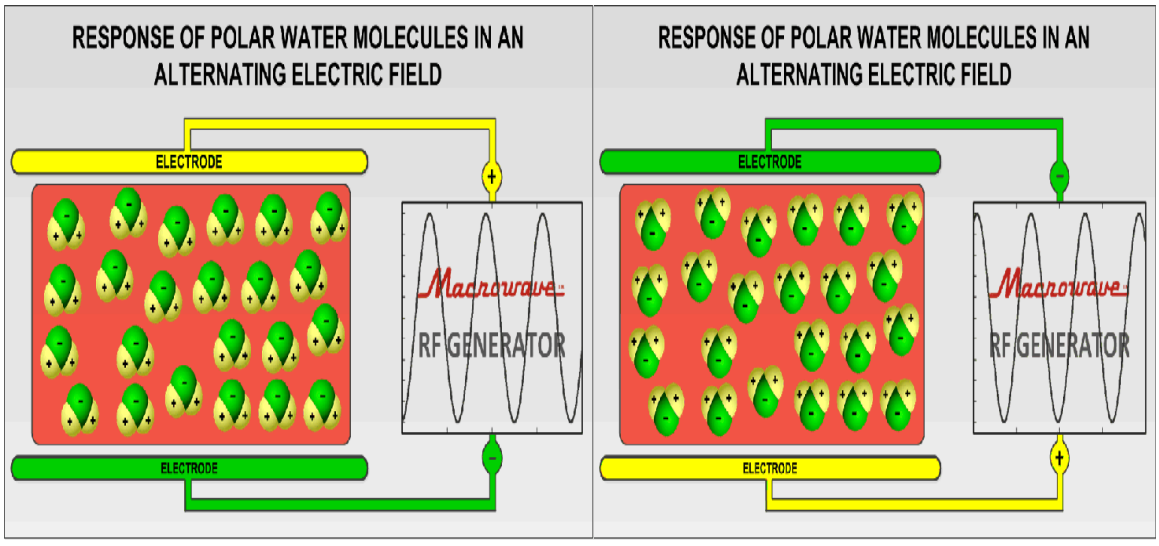


Figure 11 Schematic working mechanism of radio frequency heating (Radio Frequency Company, Millis, MA, United States)

Pre-hydrolysis treatment of lignocellulosic biomass, using RF with low concentration of NaOH solution as catalyst, can breakdown the cementing lignin matrix, and subsequently enhance the accessibility and digestibility of the energy potentials (cellulose and hemicellulose), and improve the enzymatic hydrolysis rates of lignocellulosic biomass for biofuel production (Hu et al., 2008; Iroba et al., 2013). If dielectric heating is applied to LCB, the more polar (lossy) part would absorb more energy, and thus, a "hot pot" would be generated within nonhomogeneous materials. It is reported that this special heating property results in an "explosion" effect among the particles and enhances the disruption of the crystallinity structures of LCB (Hu and Wen, 2008; Hu et al., 2008; Liu et al., 2010). At the same time, the electromagnetic field created in the dielectric field could generate nonthermal effects, which can also accelerate the destruction of the crystallinity structure (Shibata et al., 1996, Hu et al., 2008). In a RF heating system, the RF generator creates an alternating electric field between two electrodes. Thus the polar molecules being heated are kept reoriented to face opposite electrodes. This process offers the advantages of reduced processing times, as well as consistent quality, and simplified control. Moreover, RF heating used in this study utilized a temperature at less than 100°C in an open container which saves a lot of energy and provides a safer working circumstance compared to high temperature pretreatment methods. To the best of our knowledge, there were only two papers regarding the alkaline pretreatment on biomass assisted by RF heating. Hu et al. (2008) collaborated with our lab handling switchgrass and Iroba et al. (2013) pretreated barley straw.

2.4 Biodegradable plastics

In industrial society, polymeric materials such as rubber, resin etc. have been widely utilized since they provide a large variety of cost-effective products that improve the comfort and quality of our common life (Harding et al., 2007; Kolybaba et al., 2003). Today there are mainly two widely used types of plastics: petroleum-based plastics and biodegradable plastics (Amass et al., 1998; Flieger et al., 2003). In the research, we focus on the latter one.

Biodegradable plastic or polymer can be degraded through photo-degradation, oxidation, hydrolysis and/or biodegradation, and at the same time release no harmful residue to the environment (Bastioli, 2011; Chiellini and Solaro, 1996; Luzier, 1992). That is what can be environmentally accepted biodegradable. They are either partly or fully degraded by non-enzymatic hydrolysis, or by organized enzymes produced by some microorganisms. There are several types of biodegradable plastic, including polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and copolymers and blends of starch and polypropylene (Chanprateep, 2010; Khardenavis et al., 2007). There are also semi-degradable plastics made of starch and polypropylene compared to 100% biodegradable ones- PHAs (Kowalczyk and Piorkowska, 2012; Nonato et al., 2001).

2.4.1 A potential biodegradable plastic Polyhydroxybutyrate (PHB)

PHB (polyhydroxybutyrate) belongs to polyhydroxyalkanoates (PHAs), which are a kind of biodegradable plastic (B. Wang et al., 2013). The general polymer structure of PHAs is shown in Figure 12, and the R side chain gives the PHAs different chemical properties. A PHA molecule includes an ester group, which plays a significant role in making this type of polymer biodegradable through hydrolysis. For PHB, which is the most studied PHA, the R group assigns for a methyl (CH_3). Moreover, if the R group is an ethyl ($-C_2H_6$), this is known as polyhydrxyyalerate (Heinanen et al., 1999; Jacobson et al., 1999).

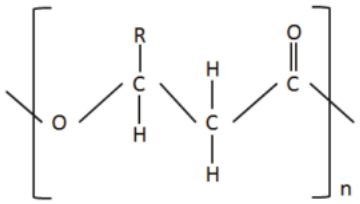


Figure 12 General structure of PHA

The melting temperature of PHB is about 179 °C. The crystallinity structure of PHB is between 30-70%. Pure PHB has similar physical and chemical properties compared to commonly used plastics, e.g. polypropylene (Reddy et al., 2003). From the mechanical properties, the melting temperature, Young's modulus and tensile strength are comparable to polypropylene and polystyrene and other bulk plastics. Moreover, PHB is unique because of other features such as thermoplastic process ability, resistant to water and moisture, and 100% biodegradability. In total, PHB can replace petroleum-based plastics. Hybrid polymers with PHB can be created, since the similarity of PHB and

commonly used bulk plastics as well as its unique characteristics (Rhu et al., 2003; Yamamoto et al., 2002).

2.4.2 Production of PHB

There are mainly two methods to produce PHB as biodegradable plastic: chemical synthesis and biosynthesis. For chemical synthesis, production of PHB can be realized utilizing β-butyrate or β- hydroxybutyric acid as monomers (Choi and Lee, 1997). However, the difficulty in controlling process conditions and high production cost make this approach unfeasible from an economic point of view. Compared to chemical synthesis, the process condition of biosynthesis is simpler and milder during production. Many kinds of microbes can generate PHB up to 30-80% of their dry cell weight, such as *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, and *Methylotroph* (Linton et al., 2012; Reusch and Sadoff, 1983; Spiekermann et al., 1999). As a consequence, the biosynthesis path, namely, fermentation, using specific microorganisms is a better approach for PHB production. For the biosynthesis process of the production of PHB, it can be done through either wild type bacteria or recombinant bacterial systems.

The wild type bacterial PHB production can be seen in (but not limited to) *Cupriavidus necator*, *Rhodopseudomonas palustris*, and *Methylobacterium organophilum* (Choi and Lee, 1997; De Gelder et al., 2008). The recombinant bacterial systems may include *E. coli* and *Cyanobacteria*. Because of relatively simple cultivation, easy growth and ease of downstream processing of bioproducts, *E. coli* is commonly regarded as an ideal candidate for large-scale production of PHB. There are three genes required for PHB production, including beta-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB),

and PHB polymerase (phbC) (or PHB synthase) (Reddy et al., 2003). These three genes from *R. eutrophus* (also known as *Alcaligenes eutrophus*) were successfully cloned into plasmid pBHR68 and subsequently expressed in *E. coli* (Antonio et al., 2000; Li et al., 2011 and Zhang et al., 2006). The pBHR68 plasmid is widely used in recombinant *E. coli* systems for the production of PHB.

The pathway for PHB production from acetyl-CoA is expressed in Figure 13 (Kusaka et al., 1997). Three genes encoding for beta-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB), and PHB polymerase (phbC) (or PHB synthase) required can be seen from this figure. Firstly, two molecules of acetyl-CoA made from carbohydrate can be converted to acetoacetyl-CoA by beta-ketothiolase (or acetyl-CoA acetyltransferase) encoded by PhaA gene. Secondly the acetoacetyl-CoA can be catalyzed by acetoacetyl-CoA reductase encoded by PhaB gene catalyzes through reduction to for a (R)-3-hydroxybutyryl-CoA (3HB-CoA). After that, PHB synthase encoded by PhaC gene can catalyze polymerization reaction of the monomer molecules ((R)-3-hydroxybutyryl-CoA (3HB-CoA)) to polymer (PHB) (Kang et al., 2008; Li et al., 2007).

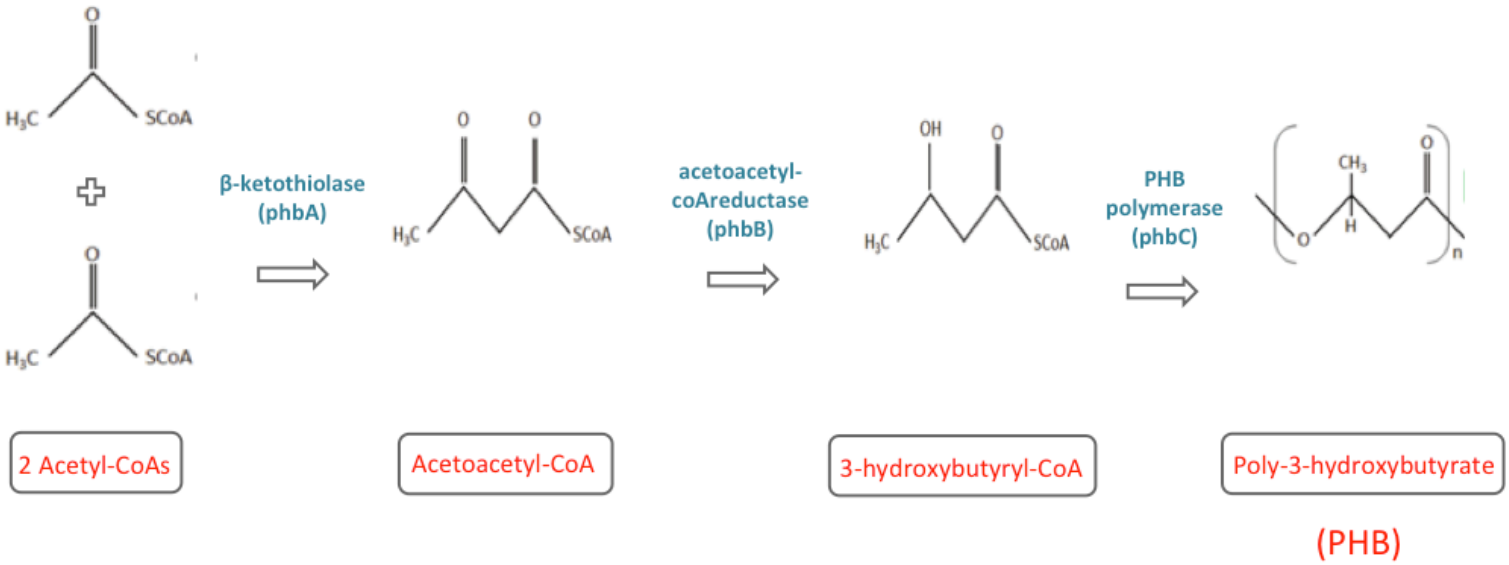


Figure 13 General pathway used to produce PHB from Acetyl CoA (Rahman et al., 2013)

2.4.3 PHB extraction

PHB is present as inclusion bodies inside the microorganism cell, as needs to be separated from cell material (Ojumu et al., 2004; Ramsay et al., 1994). It is hydrophobic and water insoluble as an intracellular product. Mechanical separation techniques, such as centrifugation and filtration, should be done to separate the cells from the culture medium (Jan et al., 1996a; Rahman et al., 2014). After that, cell lysis should be done to destroy the cell and isolate the PHB granules if highly purified PHB is expected. In other words, PHB extraction plays an important role in the whole process, which contributes to the production cost.

Many solvents have been studied for the extraction of PHB in order to find a relatively cheaper method, such as trichloroethylene (halogenated compound), methylene

chloride, 1,2-Dichloroethane, 1,1,2,2-etrachloroethane, dimethylformamide (nitrogen compound), ethyl acetate (esters), 2,2,2-trifluoro ethanol (alcohols), dimethylsulphoxide (sulfur compounds), dichloroethane, dichloromethane and chloroform (Deepak et al., 2009; Ramsay et al., 1994)ph. Among these solvents, chloroform is the most commonly and well known used one for PHB extraction. (Hahn and Chang, 1995) used chloroform and sodium hypochlorite solution to extract PHB from dry cell powder. The method used in our research was adapted from the gravimetric method of Kim et al. (1994). The mixture solution of sodium hypochlorite and chloroform were utilized for dry cell mass. After that, the solution was vortexed, incubated and centrifuged. Then the solution was layered and PHB was found in the organic layer.

2.4.4 PHB quantification and characterization

PHB dectection and quantification is typically done using the following methods including: High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), UV spectrophotometer and Nuclear Magnetic resonance (NMR). The first three methods used depend on characterization of its monomer, which was formed from PHB during acid digestion (Hahn and Chang, 1995; Jan et al., 1996a; Kacmar et al., 2006; Penloglou et al., 2008).

For the HPLC method, polymer samples of PHB-containing material were digested in concentrated sulfuric acid (De Gelder et al., 2008; Karr et al., 1983). The UV absorbance spectrum was measured at 210nm. Samples were eluted with dilute H_2SO_4 using an Aminex HPX-87H ion- exclusion organic acid analysis column (300 by 7.8 mm)

(Bio-Rad Laboratories, Richmond, Calif.) preceded by a guard column of Aminex HPX-85X. The amount of crotonic acid produced from PHB was calculated from the regression equation, which was derived from a set of crotonic acid standards. The concentration of crotonic acid was calculated from known standards or relative coefficient. For GC analysis, PHB polymers were hydrolyzed or methylated to produce volatile monomers, and these monomers are processed through GC and compared with standards (Jan et al., 1996a; Kacmar et al., 2006). For the NMR method, Jan et al. used NMR to detect, identify and quantify PHB. Quantitative NMR was combined with GC results in this study with standard curves generated (Jan et al., 1996b; Pan et al., 2012; Sindhu et al., 2011). The presence of PHB was shown in NMR spectra through 3 peaks shown in Figure 14, including: a doublet at 1.29ppm attributed to the methyl group coupled to one proton; a doublet of quadruplet at 2.57ppm attributed to the methylene group adjacent to an asymmetric carbon atom bearing a single atom and a multiplet at 5.27ppm which is characteristic of methylene group, corresponding to the different types of carbon atoms inside the PHB structure.

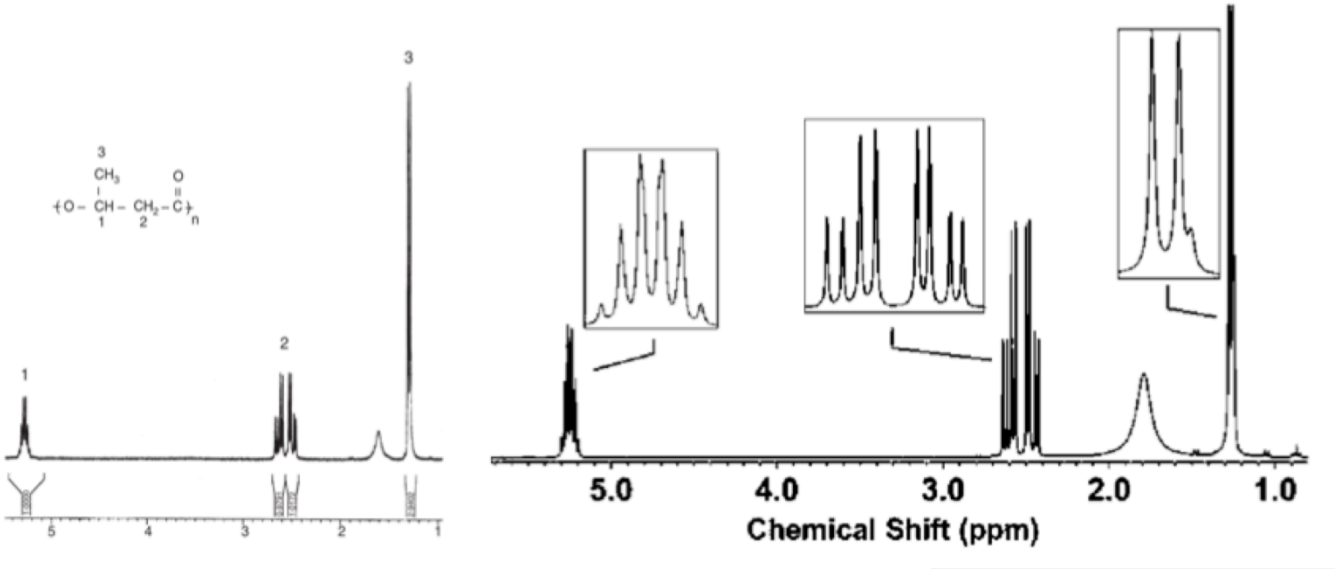


Figure 14 NMR spectra of PHB structure (Sindhu et al., 2011)

2.5 ABE fermentation

2.5.1 Biobutanol as a fuel and chemical feedstock

Butanol (n-butanol) is a kind of alcohol with four carbon atoms, and is colorless and flammable. It can be used as solvents, chemical intermediates, extract agents, industrial cleaners, or gasoline additives. Moreover, butanol has high heat value, high viscosity, less evaporative/explosive, high hydrophobicity, lower vapor pressure, less corrosive and other good properties (Maddox, 1989; Patakova et al., 2011). Therefore, butanol can be used as fuel, which has attracted people's attention recently.

2.5.2 Production methods of butanol

There are mainly two methods for production of butanol (Liu et al., 2009). The first one is using chemical technologies, such as oxo-synthesis and aldol condensation. The other one is to produce butanol as one of the products through

fermentation by microorganisms. The most commonly used bacteria species for fermentation are *Clostridium acetobutylicum* and *Clostridium beijerinkii.* The fermentation is also called acetone-butanol-ethanol (ABE) fermentation because of the main products including acetone, butanol and ethanol (Qureshi et al., 2008a; Raganati et al., 2012).

For the chemical production, oxo-synthesis is the primary industrial approach (Baral and Shah, 2014). It involves reaction of propylene with carbon monoxide and hydrogen using cobalt or rhodium as the catalyst. The condition is often at 10-20 MPa and 130-160℃ for cobalt, while for rhodium the condition is 0.7-3MPa and 80-120℃. The reactions are as follows:

$$CH_3CH - CH_2 + CO + H_2 \rightarrow CH_3CH_2CH_2CHO + (CH_3)_2CHCHO$$

$$CH_3CH_2CH_2CHO + H_2 \rightarrow CH_3CH_2CH_2CH_2OH$$

$$(CH_3)_2CHCHO + H_2 \rightarrow (CH_3)_2CHCH_2OH$$

The other chemical route is aldol condensation (Chheda and Dumesic, 2007). It involves the reaction of condensation and dehydration from two acetic aldehydes. After that, the product is hydrogenated into butanol at 180℃ and 0.2 MPa. The reactions are as follows:

$$2CH_3CHO \xrightarrow{condensation} CH_3CH(OH)CH_2CHO \xrightarrow{dehydration} CH_3CH = CHCHO$$

$$CH_3CH = CHCHO + 2H_2 \xrightarrow{hydrogenation} CH_3CH_2CH_2CH_2OH$$

In addition to the chemical routes, butanol can be obtained through biological ways by microorganisms through fermentation (Qureshi et al., 2008a). The *Clostridium* genus can utilize cellulose and hemicellulose to synthesize butanol under anaerobic

conditions, and the final products are generally the mixture of acetone, ethanol and butanol. There are several advantages over the chemical approaches. First, biological processes can utilize agricultural wastes, such as wheat straw, corncob etc., as well as other lignocellulose biomass (Mussatto and Teixeira, 2010; Thaddeus Ezeji, Nasib Qureshi, 2007). Furthermore, fermentation processes are simpler and milder than chemical processes (Qureshi et al., 2008b; Sukumaran et al., 2011). Last but not least, the products through fermentation process are high purity and easier to separate (Kraemer et al., 2011). The process of biobutanol production from lignocellulosic biomass can be shown in Figure 15.

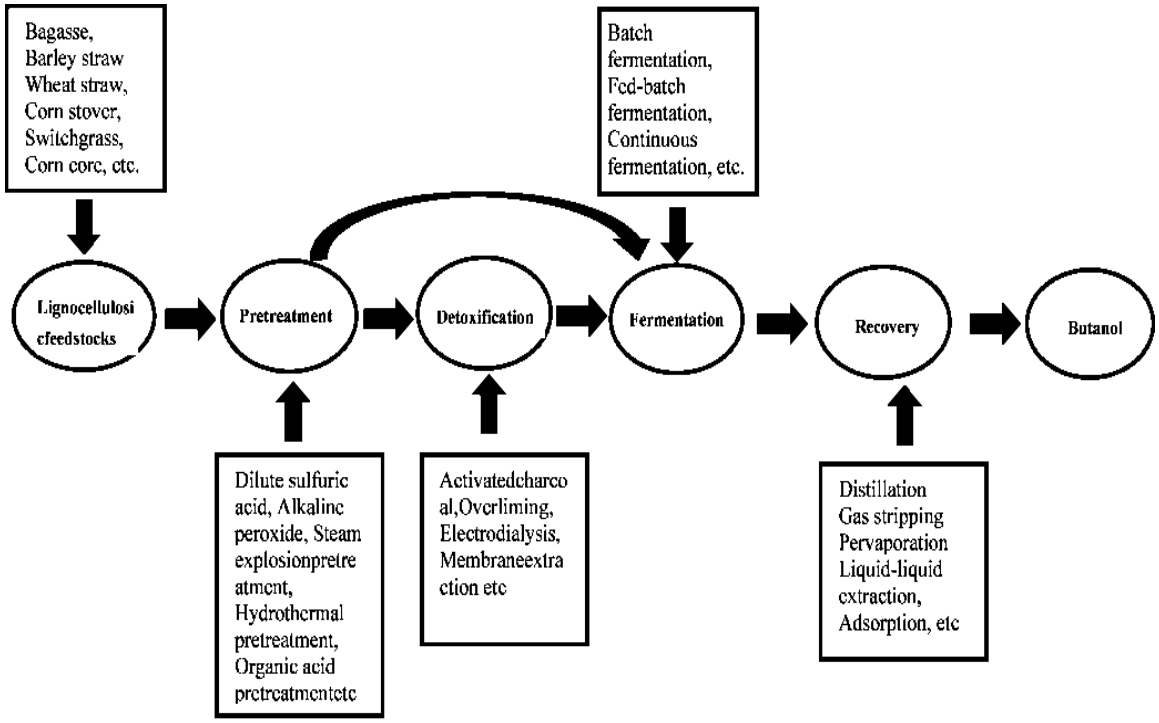


Figure 15 Butanol production from lignocellulosic biomass (Liu et al., 2009)

2.5.3 Metabolic pathways for butanol production

For the ABE feremtnation, the microorgansims can hydrolyze starch to glucose, and then glucose can be converted to pyruvate through Embden-Meyerhoff pathway (EMP, or glycolysis) (Liu et al., 2009). After that, it can be divided into two successive and distinct phases, namely the acidogenesis phase and the solvetogenesis phase (Y. Wang et al., 2013). The metabolic pathways of the ABE fermentation are illustrated in Figure 16. During the initial growth phase, the so-called the acidogenesis phase, the *Clostridia* may generate acetate, butyrate, hydrogen and carbon dioxide (Y. Wang et al., 2012). For the solvetogenesis phase, which was stationary after the culture steps, the formation of acids decreases while acetone and butanol become the dominant products (with a small fraction of ethanol) (Gheshlaghi et al., 2009; Mayank et al., 2012; Patakova et al., 2013). Throughout this whole process, the pH first decreases to lower levels due to the production of acids, and then increases after the cells enter the stationary state when the solvetogenesis phase begins with endospore forming (Y. Wang et al., 2013).

During the phase of acidogenesis, the products include acetate and butyrate. Acetate is created through acetyl-CoA, which is catalyzed by two kinds of enzymes, PTA (phosphotransacetylase, or phosphate acetyltransferase) and AK (acetate kinase) (Lin and Tanaka, 2006). Butyrate formation is relatively complicated through several steps (Ramey, 2004). Firstly, two molecules of acetyl-CoA is transformed into acetoacetyl-CoA through THL (acetyl-CoA acetyltransferase). Secondly, acetoacetyl-CoA is turned into butyryl-CoA through three enzymes: HBD (hydroxybutyryl-CoA dehydrogenase),

CRT (hydroxybutyryl-CoA dehydrolase), BCD (butyryl-CoA de-hydrogenase). Then butyryl-CoA is catalyzed by two enzymes: PTB (phosphotransbutylase or phosphate butyltransferase) and BK (butyrate kinase), to generate butyrate (Van-Thuoc et al., 2008). As the acid accumulates, pH drops to the lowest point during fermentation. This leads to the switch of acidogenesis phase to solvetogenesis phase (Taherzadeh et al., 1997). In the solvetogenesis phase, acetoactyl-CoA utilizes CoAT (CoA transferase) into acetyl-CoA and acetoacetate. Then acetone is obtained through ADC (acetoacetate decarboxylase). The former one has two pathways. In one route, it is converted into ethanol by ALD (aldehyde dehydrogenase) and ADH (alcohol dehydrogenase); in another, it is converted to butyryl-CoA. Then butyryl-CoA is converted to butyraldehyde by ADH (butyraldehyde dehydrogenase) and finally to butanol by BDH (butanol dehydrogenase) (Baral and Shah, 2014).

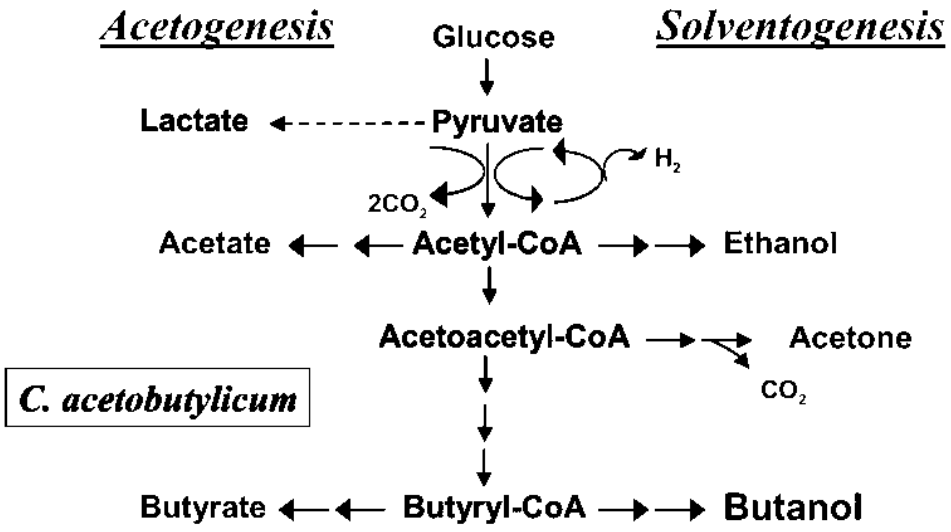


Figure 16 Metabolic pathway of ABE fermentation with acidogenesis phase and solvetogenesis phase (Ramey, 2004)

2.6 Economic analysis of biomass utilization

The utilization of lignocellulosic biomass, which is commonly accepted as a form of renewable source, plays an important role in helping reduce the environmental impact, as well as produce energy with main types of woody plants and herbaceous plants (McKendry, 2002a). The lignocellulosic biomasses store chemical energy in cellulose, hemicellulose and lignin, although the proportions vary with the species of plant. The conversion of biomass to bioenergy contains mainly two technologies: thermochemical and biochemical routes.

The selection of a decent conversion technology for biomass requires high sugar and value-added biochemical yields, low enzyme loading and cost, low capital costs etc. (Eggeman and Elander, 2005). Therefore, it is important to identify the economic impact of different conversion technologies, especially the pretreatment approach during biochemical route as related to capital and operating cost investment and glucose and xylose yields. However, it needs to be mentioned that it is not difficult to obtain good sugar yields, while it is very not easy to achieve good yields at low energy input (J. Y. Zhu and Pan, 2010). Therefore, the pretreatment energy input and the net energy output should be paid attention to in the biorefining community, which needs to be took into consideration in commercial scalability as a major technological barrier for future research and development.

Chapter 3 Comparison between radio frequency (RF) and traditional heating assisted alkaline pretreatment on four categories of lignocellulosic biomass

Abstract

Pretreatment plays an important role in making the cellulose accessible for enzyme hydrolysis and subsequent conversion because it more or less destroys resistance and recalcitrance of biomass. In this study, radio frequency (RF) assisted dielectric heating was utilized in the alkaline (NaOH) pretreatment. The substrates ranged from agricultural residues (corn stover), herbaceous crops (switchgrass), and hardwood (sweetgum) to softwood (loblolly pine). Pretreatment was performed at 90°C for both RF and traditional water bath (WB) heating for one hour after overnight soaking in NaOH solution (0.2g NaOH/g Biomass). Pretreated materials were characterized by chemical compositional analysis; enzyme hydrolysis, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The glucan yields from hydrolysis using the RF heating method for these four feedstocks were 89.6%, 72.6%, 21.7% and 9.9%, respectively, while with conventional heating technique the yields were 89.4%, 51.8%, 19.6% and 9.8%. Interestingly, RF heating raised glucan yield on switchgrass and sweetgum but not on corn stover or loblolly pine. The SEM images and FTIR spectra agreed with composition analysis and results of enzyme hydrolysis. GC-MS detected some compounds (including hydroxyl-acetaldehyde, propanoic acid, 2-methoxy-4-vinyl phenol, 2-methoxy phenol and 1,2-benzenediol (catechols)) that RF heated hydrolysate have, while no detection for WB pretreated ones. These compounds showed similarity for those pyrolysis processes.

3.1 Introduction

In order to reduce the dependence on foreign suppliers of petroleum, the United States is in need of alternative energy sources, which will also improve the cash flow balance for the country. The Energy Independence and Security Act of 2007 mandates a minimum of 36 billion gallons of renewable fuel production annually in the USA by 2022. To meet the aforementioned goal, lignocellulosic biomass (LCB) needs to be produced on a cost competitive basis with fossil fuel sources. Compared with fossil fuels, biomass energy has a wide range of environmental and social benefits if produced in an efficient and sustainable manner. The advantages include reduction of CO_2 levels, waste control etc. The southern U.S. is the primary wood-producing region of the country, with more than 200 million acres of commercial forestland and timber inventories that have increased each year for more than a decade. Therefore, the region's abundant timber resources become the most important feedstock for its ready availability and established markets.

LCB is a potential and competitive source for bioenergy production. There are two main reasons: biomass is one of the few energy sources that can actually be utilized to produce several types of energy (motor fuel, electricity, heat); and cellulosic biomass is renewable and commonly found (Sun and Cheng, 2002). LCB refers to plant biomass that is composed of cellulose, hemicelluloses, and lignin. It is a complex matrix, comprising many different polysaccharides, phenolic polymers and proteins (Wyman et al., 2005). LCB can be grouped into four main categories: 1) agricultural residues (corn stover and straw, which can be used for production of second generation biofuels); 2)

dedicated energy plants (switchgrass, eucalyptus etc.); 3) wood biomass from hardwood and softwood trees; and 4) municipal paper waste (mainly municipal solid waste, packaging waste wood, household waste wood, market waste, food processing wastes, etc.).

LCB is composed mainly of cellulose, hemicellulose and lignin that are closely associated with complicated components, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll, and waxes), and ash. Cellulose is a linear polysaccharide of β-D-glucose units that are linked via β-1, 4 glucosidic bonds. In contrast to cellulose, which is a homopolysaccharide, hemicellulose is heteropolysaccharide that is made up of five carbon sugars (xylose and arabinose), six carbon sugars (mannose, glucose and galactose), and uronic acids. It has a lower molecular weight than cellulose and its role is to connect cellulose fibers and lignin. Lignin is the most complex natural polymer. It is an amorphous three-dimensional polymer with phenylpropane units as the predominant building blocks. More specifically, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol are shown in Figure 6. The chemical constituents of lignin vary between softwoods and hardwoods. Lignin in softwoods can be described as "guaiacyl lignin" since it is composed mainly of guaiacyl (G) lignin sub-units “coniferyl alcohol”, while hardwoods contain mainly a "guaiacyl-syringyl" lignin that is composed of a higher amount of syringyl (S) units than G units. The structure of LCB can be described as a skeleton of cellulose chains embedded in a cross-linked matrix of hemicellulose surrounded by a crust of lignin, shown in Figure 2.

There are extensive interactions between cellulose, hemicellulose and lignin, as well as the natural barrier of lignin increase the difficulty of the access of hydrolytic enzymes to simple sugars. The amount of each component varies depending on the species of LCB. In addition, the percentage of different components within a single species of biomass differs with age, stage of growth, and other conditions. In general, hardwood has greater amount of cellulose, meanwhile wheat straw and leaves have more hemicellulose (Lloyd and Wyman, 2005), shown in Table 1.

Pretreatment of LCBs is required to alter the structure of biomass in order to be converted to simple fermentable sugars. The pretreatment process is required to remove lignin and hemicellulose, reduce the crystalline structure of cellulose, and increase the porosity of the materials (Galbe and Zacchi, 2012). An effective pretreatment is characterized by several criteria: 1) it results in high recovery of all carbohydrates; 2) it improves digestibility of cellulose in the subsequent enzyme hydrolysis; 3) it avoids degradation or loss of sugar and lignin; 4) it limits formation of degradation products that inhibit growth of fermentative microorganism; and 5) it requires low biomass costs, capital costs and operation costs.

Feedstock pretreatment has been recognized as a necessary upstream process for enzymatic degradation (Hendriks and Zeeman, 2009). Since LCB materials are very complicated, their pretreatment is not easy either. There are several key factors to take into consideration for effective pretreatment process: the structural and compositional features of LCB, the crystallinity and degree of polymerization of cellulose, the

accessible surface area/porosity, the protection of cellulose by lignin, and cellulose sheathing by hemicellulose. Therefore, the mechanism of pretreatment has always been a topic of concern for researchers. For example, alkaline treatment at high temperatures of the biomass samples significantly affected the amount of the lignin and carbohydrates contents by removal of the lignin and the increasing of glucan content (Q. Li et al., 2012). During the alkaline pretreatment, the first reactions taking place are solvation and saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components for example, lignin and other hemicellulose. This causes a swollen state of the biomass, making it more accessible for enzymes and bacteria, increasing the internal surface area of cellulose, decreasing the degree of polymerization and crystallinity, separating the structural linkages between lignin and carbohydrates, and provoking the lignin structure disruption (Mittal et al., 2011). Moreover, during the dissolution, the peeling of end-groups like acetyl and various uronic acid substitutions on hemicellulose that reduce the accessibility of hemicellulose and cellulose to enzymes, alkaline hydrolysis and degradation and decomposition of dissolved polysaccharides could take place (Chen et al., 2013). However, the effectiveness of alkaline pretreatment varies, depending on the lignin content of the materials. In general, alkaline pretreatment is more effective on agricultural residues, herbaceous crops and hardwood with low lignin content, and no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Alvira et al., 2010).

Pretreatment technologies can be roughly divided into several different categories: physical, chemical, physicochemical, biological and others (Haghighi Mood

et al., 2013). The methods of pretreatment of LCB are summarized as physical pretreatment (milling, irradiation, microwave, radio frequency, liquid hot water pretreatment, uncatalyzed steam-explosion, extrusion, pyrolysis, freeze pretreatment etc.), chemical pretreatment (acid pretreatment, alkaline pretreatment, ionic liquid etc.), physico-chemical pretreatment (ammonia fiber explosion, steam explosion, alkaline/acid assisted by heating etc.), and biological pretreatment (Zhu et al., 2010). Among the physico-chemical pretreatment methods, ammonia fiber explosion (AFEX) and alkaline/acid with heating are generally studied. Although these are all powerful methods for enzyme hydrolysis, they have their own drawbacks. AFEX is high-energy demanding and has unsafe factors. Alkaline/acid assisted by water bath (WB) heating is more energy consuming than alkaline/acid with RF heating. Various pretreatment methods have been extensively studied. However, none of these can be recognized as a "winner" since each pretreatment has its intrinsic advantages and disadvantages. Alkaline pretreatment was chosen in this study because the advantage of this pretreatment technology lies in the fact that it would create a washed clean substrate that is highly digestible and rich in cellulose and xylan. A relatively clean sugar stream could be obtained at reasonably high yield and economically relevant enzyme dose after enzymatic hydrolysis.

Radio frequency (RF) heating is a promising dielectric heating technology, and alternative method for conventional heating. Convection/conduction heating is based on superficial heat transfer, while dielectric heating utilizes the ability of some compounds to transform electromagnetic energy into heat that is volumetric and fast through a direct interaction between RF electromagnetic field and the object being

heated. If dielectric heating is applied to LCB, the more polar (lossy) part would absorb more energy, and thus, a "hot pot" would be generated within nonhomogeneous materials. It is reported that this special heating property results in an "explosion" effect among the particles and enhances the disruption of the crystallinity structures of LCB (Liu et al., 2010). At the same time, the electromagnetic field created in the dielectric field could generate nonthermal effects, which can also accelerate the destruction of the crystallinity structure (Hu et al., 2008). Depending on the wavelength used in the heating devices, dielectric heating can be divided into microwave and RF (Wang et al., 2003). Microwave-based heating has been studied in the pretreatment of various LCB (Zhu et al., 2006b). However, compared to microwave heating, RF heating systems have higher electricity to electromagnetic power conversion efficiency, and a much deeper penetration of RF energy into a wide array of materials. Therefore, RF heating systems are more economically feasible, and they are more suitable for large commercial scale reactors.

To the best of our knowledge, there were only two papers regarding the alkaline pretreatment on biomass assisted by RF heating. Hu et al. (2008) collaborated with our lab handling switchgrass and Iroba et al. (2013) pretreated barley straw.

The primary focus of this work was to carry out systematic study on RF heating technology and judge its feasibility for downstream biofuel production. We compared RF heating and conventional heating methods with chemical pretreatment on four different feedstocks including agricultural residues (corn stover), herbaceous crops

(switchgrass), hardwood (sweetgum) and softwood (loblolly pine). The resultant substrates were evaluated based on their chemical compositions and enzymatic hydrolysis yields and rates. We investigated the difference between RF heating and conventional WB heating on the residual xylan and lignin content, and on the substrates digestibility. In addition, the impact of different heating methods on the enzyme hydrolysis of four feedstocks was explored in this work. Physical characteristics were also measured and compared using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). Therefore, the objectives of this study were to explore the difference of physical and chemical characteristics of four different LCBs pretreated with alkaline assisted by RF heating and traditional heating.

3.2 Materials and Methods

3.2.1 Raw Materials

All samples were collected from recently harvested biomass. Briefly, corn stover, switchgrass (*Panicum virgatum*), sweetgum (*Liquidambar styraciflua*) and loblolly pine (*Pinus taeda*) were acquired from the Research Station at Auburn University. Corn stover was chosen as the most common agricultural residue in the southern United States, while switchgrass was chosen because its north–south range and ecological dominance. Sweetgum is one kind of hardwood, and it was chosen due to its fast growth rate in the same geographical region as loblolly pine. Finally, loblolly pine was chosen as the most likely conifer softwood since it is the most commercially important softwood in southern United States. The materials were air-dried and milled. All feedstocks were ground by Wiley mill (Thomas Scientific, Philadelphia, PA) for the same time period. The particles were reduced to an average size of $1.0 \times 2.0 \times 0.3$ cm^3 (L

× W × H) by a Waring commercial blender (Dynamics Corporation of America, New Hartford, CT) as raw materials. Then wood powder between 20 and 40 mesh was collected for chemical composition analysis. The air-dried materials were collected in sealed plastic bags, and stored at room temperature. The main components of raw material were analyzed before pretreatment.

Moisture, extractives, ash, cellulose (as glucan), hemicellulose (as xylan) and lignin (acid insoluble lignin and acid soluble lignin) in the raw substrates were analyzed according to NREL (National Renewable Energy Laboratory -USA) laboratory analytical procedures using the extractive-free samples. Chemical compositional analysis was determined on all of the four types of substrates using the NREL protocol. The extractive-free samples were hydrolyzed by 72% sulfuric acid for 1 h at 30°C after ethanol extraction of the raw biomass or pretreated substrates, and then followed by 4% sulfuric acid at 121 °C for 1 h.

3.2.2 NaOH Pretreatment with RF Heating

A RF heater (SO6B; Strayfield, Berkshire, England) was employed in this study, shown in Figure 17. This RF heating system worked at a frequency of 27.12 MHz and maximum power output of 6kW. Pretreatment was carried out inside the chamber, which had electrically insulated walls. Energy was created through a pair of parallel rectangular plate electrodes. The lower plate electrode was mounted at a fixed position, while the upper applicator was adjustable. The desired power of RF energy was achieved by adjusting the distance between the two plate electrodes. It was one free running

oscillator system and its power consumption was in proportion to the load. The distance between the two electrodes was fixed at 8.5 cm. Actually there are two ways to scale up the RF based pretreatment process by increasing the area of the plate electrodes to cover larger amount of switchgrass materials, or by adjusting the distance of the two plate electrodes to accommodate more materials.

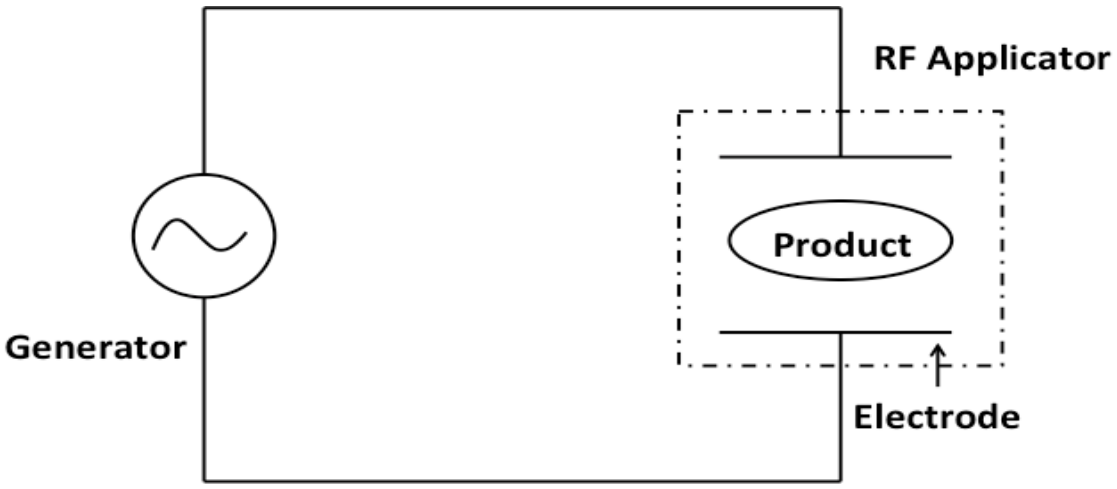


Figure 17 Schematic RF heating system

Before pretreatment, the four feedstocks were soaked in NaOH solution (0.2g NaOH/g Biomass) at a solid to liquid ratio of 1:10 in a 500ml plastic container at room temperature overnight. Then samples were treated by the RF system. During the RF heating process, four fiber-optic sensors (UMI, FISO Technologies, Quebec, Canada) were inserted into different locations of the container to monitor the sample temperature. The temperature was controlled at 90°C with ± 3°C fluctuation for 60 minutes. When the sample temperature reached 90°C, the RF heater was paused for 0.5 min followed by another 1-min RF heating in order to keep the sample at 90°C. This pause-heating pattern was repeated until the predetermined RF heating time was completed.

After pretreatment, the container was removed from the RF chamber and cooled down at room temperature. The pretreated substrate was collected by filtration through a Whatman No.4 filter paper in a Buchner funnel, washed with warm deionized water for at least three times to neutralize the pH to 7.0 and stored at 4°C. The wet pretreated substrate without drying was used for the chemical compositional analysis and following enzymatic hydrolysis.

3.2.3 NaOH Pretreatment with Conventional (WB) Heating

For NaOH pretreatment with conventional heating, in this study water bath (WB) heating was utilized in order to evaluate the efficiency of RF heating. Similar to RF heating, the four feedstock particles were soaked in NaOH solution (0.2g NaOH/g biomass) in a 500ml plastic container at a solid to liquid ratio of 1:10 at room temperature overnight. Before treating the biomass, the WB was prepared to 90°C. Then the mixture was put into the 90°C condition. Heating was maintained for 60 minutes. After pretreatment, the container was removed from the WB and cooled down at room temperature. Except for the heating method, the procedures for dealing with the pretreated samples were the same as those used in RF heating.

3.2.4 Enzymatic hydrolysis

Commercial cellulase, Novozym 22C, was obtained from Novozymes (Franklinton, NC). The filter paper activity of Novozym 22C was 100 FPU/ml, and its β-glucosidase activity was 343 IU/ml. One FPU is defined as the enzyme amount that

releases 1 μmol of glucose equivalents from Whatman no. 1 filter paper in 1 min. One unit of β-glucosidase activity is defined as the enzyme amount that converts 1 μmol of cellubiose to 2 μmol of glucose in 1 min. Novozym 22C is a cocktail of cellulase enzymes with sufficient β-glucosidase activity. The enzyme loading of Novozym 22C used in enzymatic hydrolysis was 2.5 or 5.0 FPU/g glucan.

Enzymatic hydrolysis was performed in 125 ml of 50 mM sodium citrate buffer (pH 4.8) at 2% glucan (w/v) with commercial enzyme (Novozym 22C) and pretreated biomass. The hydrolysis reaction was incubated at 50°C and 150 rpm for 72 h. During the hydrolysis, 1.5 ml of solution was sampled from the hydrolysate at various time intervals, and centrifuged at 10,000 rpm for 5 minutes. The supernatant was put through a 0.22 μm filter and stored for analysis of sugars. The concentration of sugars (glucose and xylose) was determined using an Agilent 1260 Infinity Quaternary LC VL HPLC (Agilent Technologies, Santa Clara, CA, United States) with refractive index detector (RID). The HPLC system consists of a solvent cabinet, quaternary pump, autosampler and thermostatted column compartment. It is equipped with a 300 mm × 7.8 mm i.d., 9μm, Aminex HPX-87P column and a 30 mm × 4.6 mm i.d. guard column of the same material (Bio- Rad, Hercules, CA) to separate and quantitate individual simple sugars. The column was used with nano-pure water as the mobile phase. The flow rate was set at 0.6 ml/min, and the column temperature was maintained at 80°C during the elution. Enzymatic hydrolysis was carried out in duplicate, and each data was presented as the average of two duplicates.

3.2.5 Scanning electron microscopy (SEM)

Scanning electron micrographs (SEMs) were obtained using a Zeiss DSM 940 scanning electron microscope. The images were taken at 20.00 kV. The samples were first dried under vacuum and gold-coated using a sputter coater (Pelco, model Sc-7) for 2 minutes and then imaged by SEM.

3.2.6 FTIR spectroscopy

FTIR spectra were collected using a PerkinElmer Spectrum model 100 (Perkin Elmer Co., Waltham, MA). This equipment utilized a single reflectance ATR diamond with a repeatable vertical pressure between samples to ensure repeatability in spectra acquisition between samples. All measurements were carried out at room temperature. Background spectra were measured before every sample to eliminate the noises contributed by carbon dioxide in air, moisture and oxygen. Each spectrum was recorded over 32 scans, in the range between 4000 and 650 cm^{-1} with a resolution of 4 cm^{-1}.

3.2.7 GC-MS of pretreated switchgrass

The alkaline pretreated switchgrass liquor assisted by radio frequency and water bath heating was tested for chemicals using GC-MS. Gas chromatography (GC) is a common method used to analyze gases produced during various chemical processes. GC/MS analysis of pretreated switchgrass was performed on an Agilent 6890N GC equipped with an Agilent 5973 mass-selective detector (MSD). The GC column used was a DB-1701 with a 60m × 0.25 mm, 0.25 μm film thickness. The oven was programmed to hold at 40 °C for 4 min, ramp at 3 °C/min to 250 °C, and hold there for 20 min. The

injector temperature was 250 °C, and the injector split ratio was set to 30:1. The flow rate was 1 mL/min of the He carrier gas. The samples were prepared as ~6% solutions in methanol that were filtered through a 0.45 μm PTFE filter prior to injection.

3.3 Results & Discussion

3.3.1 Characteristics of biomass before pretreatment

The biomass samples of corn stover, switchgrass, sweetgum and loblolly pine were used in this study to represent four types of biomass. Each type of the biomass was analyzed before pretreatment. The chemical composition of the four feedstocks is shown in Table 4. The untreated agricultural residue sample (corn stover) had 21.1% lignin and 70.4% carbohydrates while the raw dedicated energy crop sample (switchgrass) contained 21.4% lignin and 67.3% carbohydrates. For the untreated hardwood sample (sweetgum) there were 25.8% lignin and 68.1% carbohydrates while the untreated softwood sample (loblolly pine) had 28.7% lignin and 60.0% carbohydrates. Among all the biomass feedstocks, the major portion of the carbohydrates was glucan. Lignin, which contributes significantly to recalcitrance, was richest in the softwood (loblolly pine, 28.7%), followed by hardwood (sweetgum, 21.6%), and the least in herbaceous feedstocks (switchgrass, 21.4%) and agricultural residues (corn stover, 21.1%).

Table 4 Chemical compositions of corn stover, switchgrass, sweetgum and loblolly pine before and after using RF and WB heating (% dry basis)

Biomass	*Processes*	*Glucan*	*Xylan*	*Lignin*
Corn Stover	Untreated	44.9±0.05	25.5±0.67	21.1±0.72
	WB Pretreated	51.3±0.39	25.8±0.08	16.0±0.78
	RF Pretreated	52.1±0.49	24.6±0.12	15.8±0.44
Switchgrass	Untreated	43.7±0.23	23.6±0.24	21.4±0.79
	WB Pretreated	58.4±0.65	18.6±0.35	11.2±0.62
	RF Pretreated	60.7±0.55	16.6±0.19	10.4±0.14
Sweetgum	Untreated	47.8±0.48	20.3±0.28	25.8±0.57
	WB Pretreated	54.0±0.12	16.1±0.37	18.9±0.27
	RF Pretreated	56.6±0.33	14.0±0.54	18.2±0.38
Loblolly Pine	Untreated	52.1±0.37	7.9±0.38	28.7±0.49
	WB Pretreated	58.3±0.15	5.6±0.36	24.6±0.05
	RF Pretreated	57.3±0.24	3.8±0.29	24.8±0.10

3.3.2 Characteristics of biomass after pretreatment

Contents of glucan (as for cellulose), xylan (as for hemicellulose) and lignin in these four feedstocks were used as direct (first level) indicators to determine an extent of efficiency of biomass during pretreatment.

3.3.2.1 Chemical compositions of biomass after NaOH pretreatment with WB heating

The chemical compositions of the four feedstocks after NaOH pretreatment with traditional WB heating are also shown in the Table 4 for an easy comparison.

Results showed that softwood (loblolly pine) had the least change (suggesting no significant carbohydrate hydrolysis), especially in terms of lignin losses, followed by hardwood (sweetgum), while the other two types of LCB (corn stover and switchgrass) experienced more "damage". Alvira et al. (2010) reported that alkaline pretreatment is more effective on biomass with lignin content less than 26%. Since the lignin content in untreated sweetgum is about 25.8%, the glucan yield after enzyme hydrolysis was only around 20%. This was in accordance with what we found in our study.

It is reported that NaOH is capable of removing lignin from lignocellulose of agricultural residues, herbaceous biomass, and hardwood, but not much from softwood (Modenbach, 2013). The main mechanism of NaOH pretreatment on lignocellulosic biomass is to increase the porosity of biomass by means of delignification through breaking the ester bonds cross-linking lignin and xylan, and being accompanied with xylan solubilization. Among the three major components in LCB, hemicellulose was the most sensitive fractions to changes in pretreatment with its branched and somewhat irregular structure, while xylan was the most affected part among hemicellulose. In our experiment, lignin was partially solubilized, and degradation of the hemicellulose fraction occurred on switchgrass (herbaceous biomass) and corn stover (agricultural residues). And it was in accordance with the above-mentioned observation that NaOH works better in removing lignin from agricultural residues, herbaceous biomass and hardwood than from softwood.

Ninomiya (2012) reported that lignin structures vary with different sources of lignocellulose, which means that NaOH pretreatment may work more efficiently on some sources of biomass containing a higher proportion of syringyl units than others. Compared with softwood, the syringyl units in hardwood lignin are ~7-40 times higher, which makes hardwood more susceptible to alkaline pretreatment than softwood. Moreover, rice straw bagasse and some grasses have high levels of syringyl units (up to 65%) in the lignin fraction, which results in significant lignin removal following NaOH pretreatment (Cabrera et al., 2014). It explains why the NaOH is less capable of removing lignin from softwood. The relationship between syringyl unit and lignin removal further confirms the order of extent of "damage" in our study: corn stover, switchgrass, sweetgum and loblolly pine.

3.3.2.2 Chemical compositions of biomass after NaOH pretreatment with RF heating

The chemical compositions of the four biomass feedstock after NaOH pretreatment with RF heating are also shown in the Table 4, which indicated that agricultural residues and dedicated energy plants were more easily to be damaged by alkaline, followed by hardwood samples, while softwood had the least changes. Compared with WB heating assisted NaOH pretreatment, RF heating had the same order on the extent of "damage" among four types of biomass. According to Modenbach et al (2013), the final biomass composition was dependent on the type of biomass substrates. In our study, the results indicated that the intrinsic properties of the biomass played a vital role in pretreatment process.

3.3.3 Comparisons of chemical compositions between RF and WB heating

The order of the effectiveness of pretreatment assisted by RF heating results (Section 3.2.2) was exactly the same as that of WB heating in Section 3.2.1. However, RF heating showed advantage on delignification and xylan solubilization on almost every type of feedstock with remarkable difference on switchgrass and sweetgum versus WB heating. Similar results have been reported previously (Hu et al., 2008). They used RF based dielectric heating in the NaOH pretreatment of switchgrass to enhance its enzymatic digestibility. Results showed a higher xylose yield than the conventional heating method. Moreover, RF heating used in this study utilized a temperature at less than 100°C in an open container which saved a lot of energy and provided a safer working circumstance compared to high temperature pretreatment methods.

It appears that a major factor in the difference of "damage" among the pretreated biomass materials was the heating mechanism. RF heating has been considered as an alternative to conventional (convection and conduction) heating method, such as hot air and infrared heating. Compared with conventional heating, in which heat is transferred from the heating medium to the product, RF generates a volumetric heat inside the object as a result of the interaction between the RF waves and the ions or molecules of the object, and heat flows from inside to outside (Marra et al., 2007). Both of RF and MW heating methods, as dielectric heating techniques, produce rapid heat generation through direct interaction between an electromagnetic field and an object. If lignocellulose biomass is treated through dielectric heating, the more polar part would absorb more energy, and thus, a "hot spot" would be generated within nonhomogeneous

materials (Liu et al., 2010). A study by Hu and Wen (2008) indicated that this special heating property results in an "explosion" effect among the particles and enhances the disruption of the recalcitrant structures of LCB. At the same time, the electromagnetic field created in the dielectric field could generate athermal effects, which can also accelerate the destruction of the crystallinity structure. Thus, mechanism of heating method mentioned above explained why pretreatment assisted by RF worked better on biomass than WB.

3.3.4 Enzyme hydrolysis of biomass after pretreatment

Besides direct (first level) parameters (lignin, glucan and xylan) to determine the extent of "damage" of biomass during pretreatment, another indirect (second level) important parameter to judge an extent of "damage" is glucan yield produced by enzyme hydrolysis.

3.3.4.1 Enzyme hydrolysis of biomass after NaOH pretreatment with WB heating

Enzymatic digestibility of all the four different LCB pretreated by NaOH and WB was examined, and results are shown in Figure 18. The results showed that the different biomass had different enzymatic digestibility using the same pretreatment method. Among the four feedstocks, corn stover as a type of agricultural residue, had the highest enzymatic digestibility as the glucan yield could reach up to 89%, followed by the herbaceous biomass switchgrass with a 51.8% glucan yield, and then about 20% for hardwood sweetgum, while softwood- loblolly pine obtained less than 10% of glucan yield. Zheng et al. (2009) reported that structural and compositional features of lignocellulosic biomass form strong barriers to the biodegradation and the variability of

these characteristics explains the change of enzymatic digestibility among different sources of biomass. In our study, the glucan yield by enzyme hydrolysis of different biomass feedstocks was different even though the pretreatment was exactly the same (either NaOH with WB or NaOH with RF). It indicated that the enzymatic digestibility of biomass is substrate-specific.

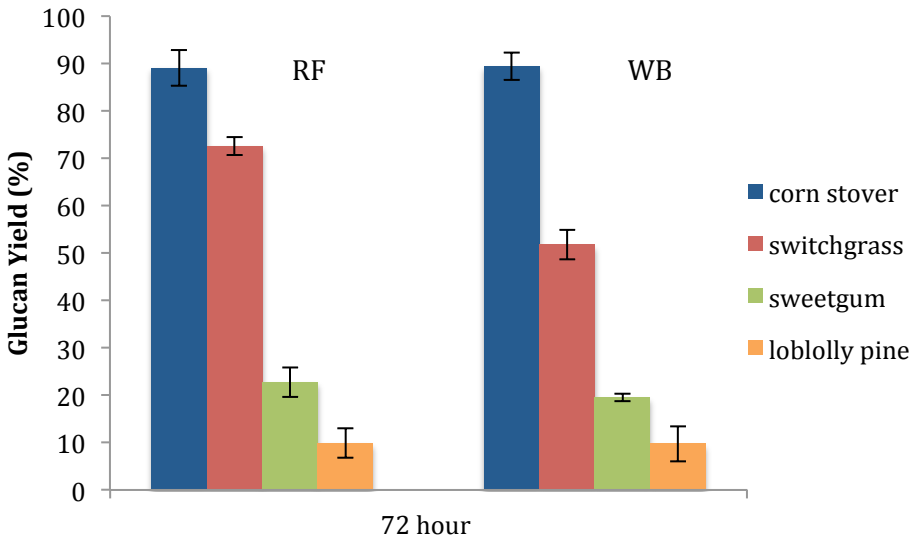


Figure 18 Effect of RF and WB heating pretreatment on enzymatic digestibility at 72 hour of sodium hydroxide pretreated corn stover, switchgrass, sweetgum and loblolly pine.

Sun and Cheng (2002) reported that lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. This explains the descending order of the glucan yield obtained from corn stove, switchgrass and sweetgum to loblolly pine, because the lignin content was exactly in the reversed order, from low to high, in these four untreated biomass, shown in Table 1.

3.3.4.2 Enzyme hydrolysis of biomass after NaOH pretreatment with RF heating

The results of enzymatic digestibility of all the four different LCB pretreated by NaOH and RF heating are shown in Figure 18. Among the four feedstocks, corn stover showed the highest enzymatic digestibility while a glucan yield could reach 89%, followed by switchgrass with a 72% glucan yield, and then about 20% for hardwood sample sweetgum, while softwood- loblolly pine showed only 10% of glucan yield. The order of glucan yield here was the same as that of the four biomass pretreated in section 3.3.1, no matter what kind of heating method was used. This happened to be the same as the results of chemical compositions in part 3.2, which provides additional evidence of the intrinsic properties of substrates.

3.3.4.3 Comparisons of enzyme hydrolysis between RF and WB heating

The order of the effectiveness of pretreatment assisted by RF heating results was the same as that of Section 3.3.1 using WB heating. However, RF showed superiority on the final glucan yield after enzyme hydrolysis on almost every type of feedstock with greatest difference on switchgrass (herbaceous grass) and sweetgum (hardwood).

During this research, both of the RF heating and conventional heating methods experienced a similar glucan yield of 89% from corn stover after enzyme hydrolysis shown in Figure 19. Chen et al. (2013) investigated the effect of pretreatment parameters on enzymatic hydrolysis of corn stover and concluded that the NaOH loading based on total solids (g NaOH/g biomass) is the most dominant variable for enzymatic digestibility since glucan conversion during hydrolysis was positively correlated with

NaOH loading. Here in our study the NaOH loading was 0.2g NaOH/g biomass for both RF-assisted heating and traditional heating methods. Corn stover is easy to be pretreated due to its structure and compositions. We believed that at this loading of NaOH, almost all the lignin could be removed at the temperature of 90°C no matter what heating method was adopted, which was in accordance with Chen's conclusion. Therefore, at this loading of NaOH pretreated on corn stover, both of these two heating methods are sufficient to breakdown the substrates.

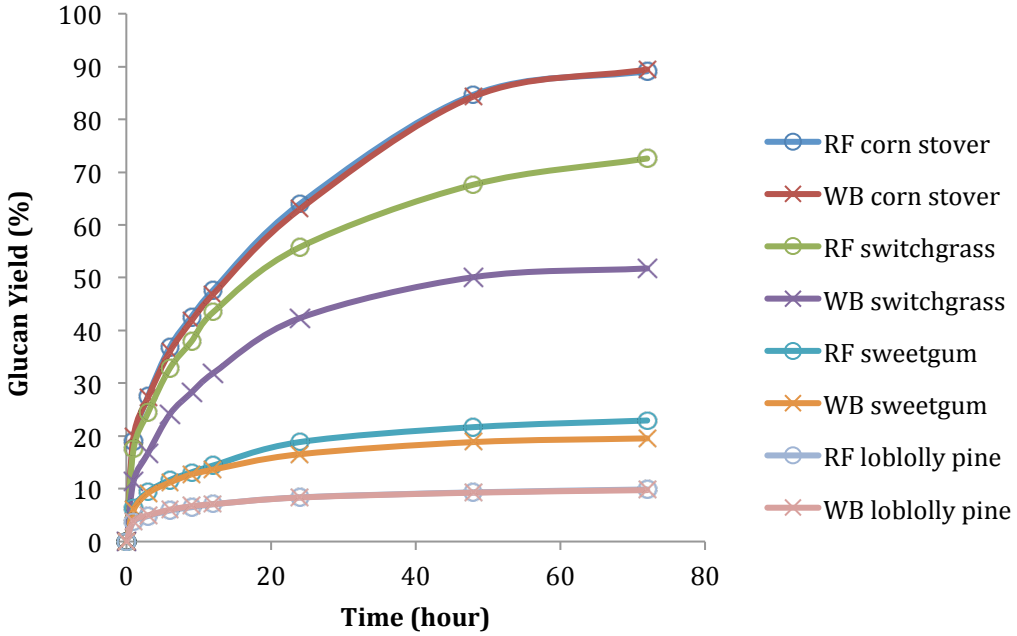


Figure 19 Effect of RF and WB heating on enzymatic hydrolysis sugar yield on corn stover, switchgrass, sweetgum, and loblolly pine

Figure 19 shows that in the hydrolysis stage, NaOH pretreated switchgrass with RF heating released 72.6% glucan, about 20% higher than that released from conventionally heated switchgrass (51.8%). According to Hu et al (2008), there was an improvement on alkaline pretreatment of switchgrass with RF heating method compared to traditional heating, which was in accordance with our results. Moreover, Hu et al. and

co-workers studied microwave-assisted alkaline pretreatment on switchgrass and found that the microwave heating method resulted in higher sugar yields than conventional heating. They pretreated the switchgrass by microwave or traditional heating after soaking samples in different concentrations of NaOH solutions. With alkaline loading of 0.1g/g biomass, they obtained the highest yield of 90% of maximum potential sugars. According to all above studies, we concluded that dielectric- assisted (both microwave and RF) heating on alkaline pretreatment is an efficient method to improve the enzymatic digestibility of switchgrass. Since the only difference between these two pretreatment methods was the heating method, RF versus conventional heating, we believe that there was a unique "strength" in the RF heating techniques compared to normal superficial heat transfer. Dielectric heating methods transform electromagnetic energy into heat between RF electromagnetic field and the object being heated. When switchgrass was being heated through RF, the more polar parts would absorb more energy and create a hot spot. This special heating resulted in an enhanced disruption of the recalcitrant structures of switchgrass, as well as accelerate in the destruction of the crystallinity structure. Moreover, there is an increasing attention paid to ionic liquid among the techniques of pretreatment of LCB. Recent researchers proposed that the ionic liquid such as [C4mim] Cl could effectively break the extensive network of intra- and intermolecular hydrogen bonds in cellulose, thus allowing cellulose dissolution in the ionic liquid (Cheng et al., 2011). Pinkert et al. (2009) suggested that cations in ionic liquids also play a role in solvation of cellulose. Knowing about these technologies and mechanisms of ionic liquid pretreatment of LCB, we believe that there may be similarities between RF heating techniques and ionic liquids reactions, but the reactions of ionic liquid were chemically

based while the RF utilized the physical “ionized” electromagnetic field. However, more researches about the mechanisms of RF heating should be conducted to explain this phenomenon.

For the sweetgum, the glucan yield after enzymatic hydrolysis through RF heating pretreatment was 21.7% while the glucan yield was 19.6% using traditional heating method, indicating that NaOH pretreatment assisted by RF heating was more effective than that of WB heating. The same argument on switchgrass could be applied here for sweetgum.

The enzymatic digestibility of the loblolly pine under different heating conditions is summarized in Figure 19, which showed no remarkable difference between RF and WB. In section 3.2, the major reasons why loblolly pine was very difficult to be pretreated using NaOH assisted by either RF or WB heating were explained, although RF heating has an advantage on switchgrass and sweetgum compared with WB heating. In addition, loblolly pine, has longer fibers than hardwood. Because of the long fiber, they have a compact structure that strongly resists on the pretreatment and biodegradation. Zheng et al. (2009) reported that alkaline pretreatment was more effective on hardwoods, herbaceous crops, and agricultural residues at the same pretreatment conditions because of generally higher lignin content of the softwood. Therefore, we believe that different types of LCB should be exposed to different pretreatment techniques to enhance their own chemistry and enzyme accessibility and digestibility for the subsequent processing.

The enzymatic digestibility of biomass is both substrate- and pretreatment method-specific.

3.3.5 SEM analysis on untreated and pretreated biomass

Since there was a portion of xylan and lignin removed by alkaline pretreatment, it was important to examine the physical changes in the biomass. For this purpose, we conducted SEM pictures on untreated and pretreated biomass samples. The surface morphology and microstructure of all four feedstocks before and after pretreatment were studied and are shown in Figure 20. In order to obtain more information about microstructure of these samples, the SEM micrographs were obtained at a magnification of 1000 X. For the untreated samples, the complete and compact lignocellulosic structure showed rigid and highly ordered fiber cells (Fig. 20 A B C D -1). After undergoing the pretreatment, a certain proportion of hemicelluloses and lignin has been removed, resulting in disruption of the biomass network structure.

For the corn stover pretreated with RF and WB heating, both of the structures have been damaged. The microfibrils were separated from the original connected structure (shown in untreated samples) and fully exposed to the air, even some fragments had flaked off from the biomass surface, shown in Fig. 20 A-2 and A-3. Moreover, similar results have been reported previously that there were significant morphological changes on corn stover by aqueous ammonia (Kim et al., 2003). The differences shown by these SEM images were in accordance with chemical analysis after enzymatic hydrolysis in section 3.3 previously.

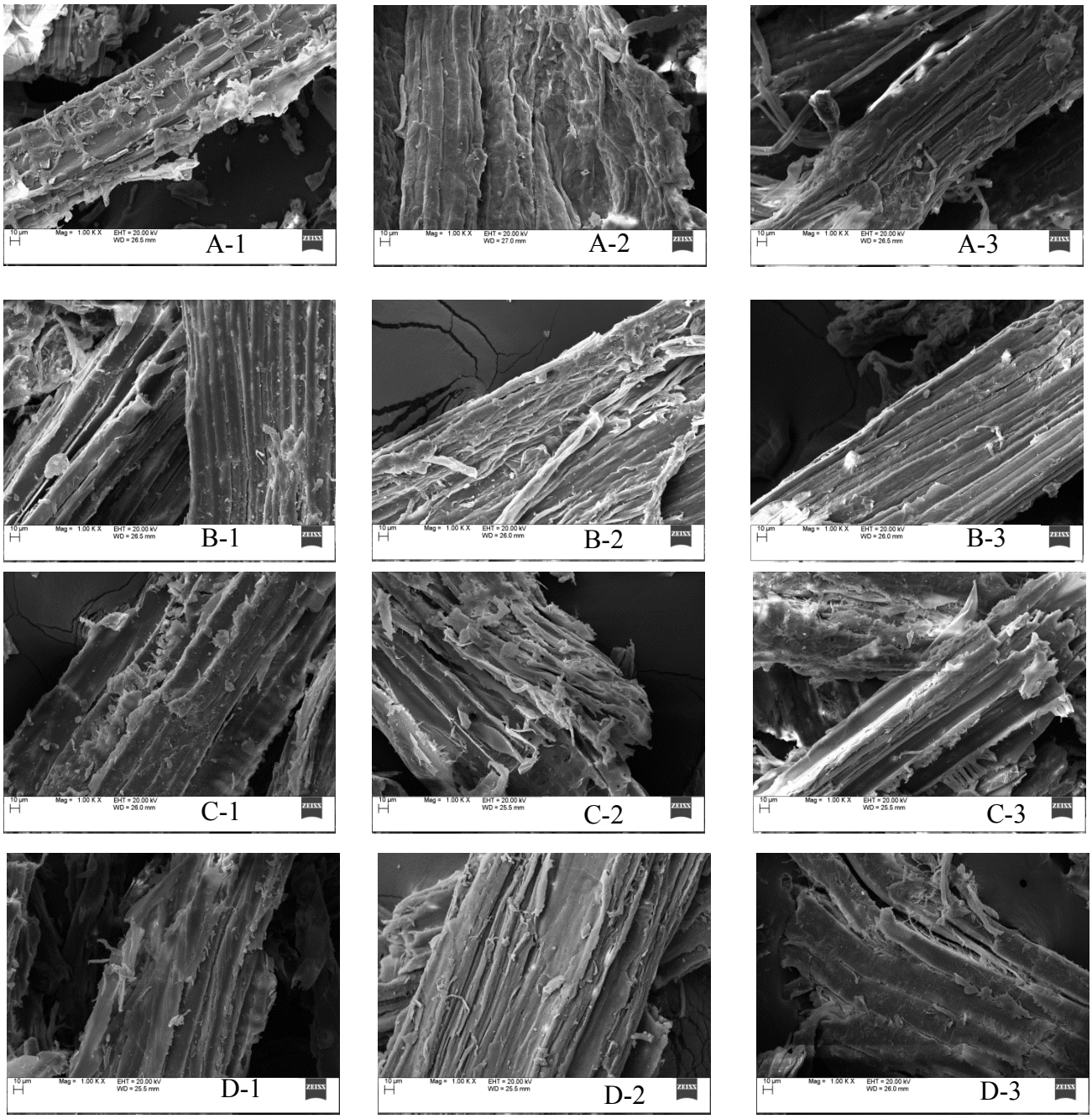


Figure 20 Scanning electron micrographs of untreated, RF and WB heating biomass samples. Note: A B C D stands for corn stover, switchgrass, sweetgum and loblolly pine. And 1, 2, and 3 means untreated, RF and WB heating pretreatment

For the WB pretreated switchgrass, the structure was damaged to some extent and some cracks were seen on the surface (Fig. 20 B-3). However, the RF pretreated switchgrass was affected in a significant way. As a result, much more debris was obtained and the disruption of the lignocellulosic structure became more pronounced (Fig.20 B-2). Results turned out the same as previous enzyme hydrolysis.

For the sweetgum as a kind of hardwood, things turned out the same as the chemical analysis and similar with the switchgrass. Some morphological changes occurred after pretreatment. The untreated sample exhibited highly ordered structure, while both pretreatment methods reduced fiber length and disrupted the structure.

For the loblolly pine samples, both of the pretreated samples show limited changes similar to the results of enzyme hydrolysis with light "damage". The ordered fibrils could still be seen (Fig.20 D-2 and 3). However, we found that the pretreated biomass appeared to be softer to touch than the untreated ones through hand-touch of the material.

3.3.6 FTIR analysis on untreated and pretreated biomass

Infrared spectroscopy is frequently used to investigate structure of materials and the chemical changes in lignocellulosic materials. FT-IR spectra for untreated and pretreated biomass samples are presented in Figure 21. For the FTIR spectrum, the vibrational frequencies of different peaks assign for different functional group in the biomass. The peaks around 3348cm^{-1} and 2900cm^{-1} are attributed to OH stretching and C-H stretching, respectively (Chundawat et al., 2007; Sun et al., 2005). The reductions in

the peaks around 1745cm^{-1} are attributed to hemicellulose acetyl and uronic ester groups or linkages in lignin and/or ester hemicellulose feurilic and p-coumaric acid carboxylic groups (P. Kumar et al., 2009). There is also a change in the intensity of the peak around 1610cm^{-1}, which are characteristic of amide linkages (Chundawat et al., 2007). The peaks around 1595, 1508 and 1458cm^{-1} are from aromatic skeletal vibrations in lignin (Sun et al., 2005). Peaks at 1428 and 1378cm^{-1} are the bands of cellulose and hemicellulose, respectively. Other important peaks correspond with different meanings shown in Table 5.

Figure 21 FTIR spectra of various biomass samples. Note: Black, blue, and red lines represent untreated, RF and WB heating treated methods, respectively

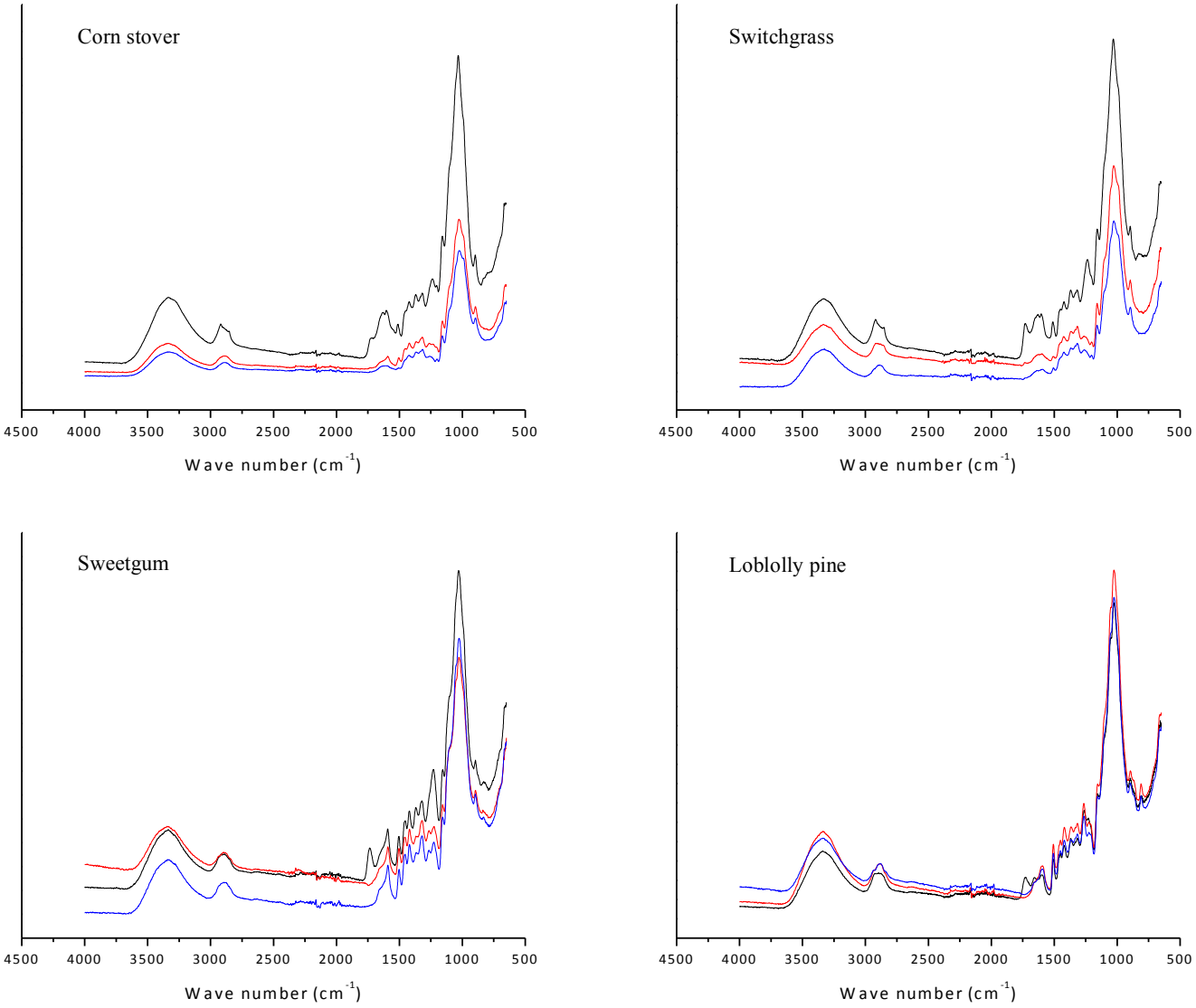


Table 5 Characterization of pretreated biomass by FTIR spectroscopy in terms of relative change in intensities

Band position (cm^{-1})	Assignment [e]	Corn stover		Switchgrass		Sweetgum		Loblolly pine	
		RF	WB	RF	WB	RF	WB	RF	WB
3348	O–H stretching (indicates rupture of cellulose hydrogen bonds)	63.6	53.5	54.6	28.1	36.1	-5.3	-15.4	-23.0
2900	C-H stretching (related to rupture of methyl/methylene group of cellulose)	63.7	51.6	61.5	29.1	47.0	-3.0	-13.2	-13.8
1745	Carbonyl bonds (associated with lignin side chain removal)	71.8	60.9	78.0	51.9	86.7	54.1	20.9	26.6
1720	Carboxylic acids or ester groups	75.6	66.7	79.2	56.5	84.7	47.9	25.8	31.0
1595	Aromatic ring stretch (associated to lignin removal)	75.8	62.6	70.3	49.9	44.0	22.2	-9.0	-14.2
1508	C=C stretching of aromatic ring in lignin	74.3	57.7	65.9	49.6	46.6	17.9	-6.9	-18.2
1458	C-H bending of methyl and methylene groups	67.8	51.9	59.6	41.2	35.9	17.2	-3.2	-15.0
1428	Band of cellulose	65.9	52.3	56.1	37.9	36.3	20.1	-4.2	-17.7
1378	Band of hemicellulose	66.1	54.7	58.2	41.6	43.5	26.7	1.0	-12.4
1260	Ester absorbance (related to removal of uronic acid)	72.0	58.5	64.1	52.5	47.7	31.3	5.4	-4.2
1245	C-O adsorption (resulting from acetyl groups cleavage)	74.6	62.3	68.8	58.0	53.0	39.4	12.6	3.9
1238	Hemicellulose-lignin linkage	75.8	62.8	70.5	60.1	52.3	40.1	13.8	5.5
1059	C-O stretch	61.5	51.6	52.2	36.6	21.0	25.4	-2.5	-10.9
1098	Heavy atom (CC and CO) stretching	66.0	55.2	56.3	42.4	23.1	23.9	-2.0	-11.0
900	Remove of amorphous cellulose	49.5	40.8	49.0	33.0	22.4	19.8	2.9	-4.8
1098/900 [d]	Amorphous to crystalline cellulose ratio	1.2	1.3	1.2	1.2	1.4	1.4	1.3	1.4

The effect of different pretreatment processes on four feedstocks was determined in terms of relative change in absorbance at specific band positions. The relative changes were calculated by the following equation:

$$\% \text{ Relative change} = 100 \times (\text{Intensity of untreated biomsss} - \text{Intensity of pretreated biomass}) / (\text{Intensity of untreated biomass})$$

Table 5 shows the relative percentage change in intensities of various bands between untreated biomass and differently pretreated biomass. Values of relative change for most of the absorption bands were positive for almost all situations, which demonstrated that there was efficient removal of the biomass components to different extent in various pretreatments. According to Singh (2014), the positive changes indicated a reduction of the particular component attributed to that band. For the corn stover in Table 5, both of the RF and the WB heating assisting alkaline pretreatment produced the greatest degree of change at almost every band positions presented, and these two methods showed no large difference. This indicated that they successfully disrupted the corn stover structure, which was in agreement with the chemical analysis after enzymatic hydrolysis in section 3.2.2. It also conformed to what Kumar (2009) did using lime to pretreat corn stover. However, for the switchgrass, RF heating assisting alkaline pretreatment showed better results for disrupting the methyl and methylene portions of cellulose (at 2,900cm^{-1}), as well as more cleavage of lignin side chains (1745cm^{-1}) in comparison with WB heating assisting pretreatment. This phenomenon was

also in accordance with the results from chemical analysis using HPLC in this study. As for the sweetgum, the RF heating had a better performance in disrupting the hydrogen bonds, methyl and methylene portions of cellulose, as well as linkages in lignin, compared to WB heating. Things happened to the same as in chemical hydrolysis. As for the loblolly pine, some of the relative change values were negative, which indicated that these two pretreatment methods did not efficiently remove biomass components (Sluiter et al., 2010). The loblolly pine was more recalcitrant than other types of LCB (Zhu and Pan, 2010). FTIR showed agreement with results from chemical analysis and SEM, and provided rapid indications of the effect of pretreatment method through the spectrum chart. Moreover, it is economical and more convenient to carry out.

3.3.7 Difference of GC-MS results of pretreated Switchgrass

For the pretreated switchgrass, compounds detected were different for heating by radio frequency versus traditional water bath heating. There were several compounds found in pretreated switchgrass with radio frequency heating, that were not detected in traditional water bath heating, including hydroxyl-acetaldehyde, propanoic acid, 2-methoxy-4-vinyl phenol, 2-methoxy phenol and 1,2-benzenediol (catechols). Mullen and Boateng (2008) conducted research on the chemical compositions of fast pyrolysis bio-oils from switchgrass and identified a total of 62 chemical species in the liquids including all of these five compounds detected in our experiment. Moreover, Wild et al. (2011) and Fivga (2011) presented a more detailed overview of important value-added chemicals that can be obtained from each of the main biomass fractions via pyrolysis. They found that acetic acid could be generated through hemicellulose degradation at the temperature

range of 150-300°C, and hydroxyl-acetaldehyde could be created through cellulose degradation at 200-400°C, while 2-methoxy phenols and catechols could be formed through lignin degradation around 150-600°C. Tumuluru et al. (2012) and Fang (2015) reported that lignin pyrolysis products include liquid (methanol, acetone, acetaldehyde, phenol, guaiacol, and eugenol and other multi-substituded phenols, etc), solid residues, gas and solid residues. (Fu et al., 2011) demonstrated that grass lignin produced p-vinylphenol as its major pylosis compound. Kelinke utilized alkaline wet oxidation pretreatment on wheat straw and found the main phenol monomers were 2-methoxy-4-vinyl phenol. However, we found the five compounds in pretreated Switchgrass assisted by radio frequency heating. Therefore, we could make a conclusion that radio frequency heating had a similar effect on pretreated switchgrass as that of pyrolysis and wet oxidation at high temperature. Futher, we could say that our conjectures about selectively overheating and an explosion inside the heated object happened during radio frequency heating, where the temperature could be high similar to a pyrolysis process.

3.3.8 Conjectures on the better performance of radio frequency

For the switchgrass and sweetgum, the radio frequency assisted pretreatment better than traditional heating methods. Up to now, there was no research published about why this phenomenon happened. However, research about the mechanism of microwave heating, another kind of dielectric heating, led to possible reasons for the improved pretreatment performance (Hu and Wen, 2008). Here are some of our conjures based on the mechanism of dielectric heating that made RF work better to conventional heating. One is the selectively overheating of ion and or polar liquids. Convection/conduction

heating is based on superficial heat transfer, while dielectric heating utilizes the ability of some compounds to transform electromagnetic energy into heat through a direct interaction between RF electromagnetic field and the object being heated. Energy transmission is produced by dielectric losses, and the magnitude of heating depends on the dielectric properties of the molecules (de la Hoz et al., 2005). These characteristics mean that absorption of the radiation and heating may occur selectively. Recently, ionic liquids have demonstrated great promise as effective solvents for biomass dissolution with easy recovery of cellulose upon anti-solvent addition (Li et al., 2010). Ionic liquids have been shown to be very effective in solubilizing crystalline cellulose and enhancing the rates of subsequent saccharification (Singh et al., 2009). For radio frequency heating, it may have some reaction mechanisms similar to ionic liquids because of selectively heating ion liquids. This ionic liquid topic needs further exploration before implementation. Moreover, Zhang and Zhao (2009) reported a variant of microwave-assisted pretreatment methods with ionic liquids. During the process, microwave irradiation at an appropriate power significantly reduced the reaction time and showed a better solubilization of the biomass (Zhu et al., 2006a). We hypothesized that there may be an "explosion" effect among the particles, which improves the disruption of the recalcitrant structures of lignocellulosic biomass.

The other reason could be non-thermal effects caused by the radio frequency heating. The agitation and mobility of molecules probably reduce the activation energy among the reaction (de la Hoz et al., 2005). It is reported that during the thermal decomposition of sodium bicarbonate, the activation energy of the reaction is reduced by

microwave radiation (Shibata et al., 1996). Even though the mechanism is not well understood, dielectric heating induces rapid rotation of the polarized dipoles and ionized ions in the molecules, which generates heat due to friction and increases the probability of contact between molecules and atoms simultaneously, thus enhancing the reaction rate and reducing the activation energy. However, these two conjectures are assumptions based on the mechanism of dielectric heating. In light of the differences in the physicochemical properties of LCB after radio frequency heating and traditional water bath heating, further research is needed to better understand the influence of biomass structure and composition during pretreatment process.

3.4 Conclusion

Pretreatment of biomass is an extremely significant step in a commercial biorefinery, and fundamental understanding and comparison of various pretreatment processes is essential. In this study RF and WB assisting alkaline pretreatments were compared on four different feedstocks: corn stover (agricultural residues), switchgrass (herbaceous biomass), sweetgum (hardwood), and loblolly pine (softwood). For corn stover, both of the RF and WB heating received similar level of disruption. For switchgrass and sweetgum, the RF heating method showed advantage over WB heating on both chemical compositions and glucan yields after enzyme hydrolysis. However, loblolly pine was not disrupted to a large extent using NaOH assisted by either RF or WB heating. Therefore, the chemical composition and enzymatic digestibility of biomass is both substrate- and pretreatment method-specific. Moreover, a critical assessment of the costs and benefits of using the radio frequency treatment on lignocellulosic biomass is

needed since the initial capital investment and operation cost are not inexpensive. Economic estimations, including the estimations of energy throughput, temperature rise and heat capacity, should be determined to assess the viability of using radio frequency for treating lignocellulosic materials.

Chapter 4 Production of polyhydroxybuyrate (PHB) by Recombinant *E-coli* using Switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency (RF) and traditional heating

Abstract

Pretreatment plays an important role in making the cellulose accessible for enzyme hydrolysis and subsequent fermentation conversion. In this study, radio frequency (RF), a promising kind of dielectric heating was utilized in the alkaline (NaOH) pretreatment. The practicality of using switchgrass hydrolysate as medium to grow recombinant *E-coli* utilizing pBHR68 plasmid for production of polyhydroxybutyrate (PHB), a biodegradable plastic, was explored in this study. Switchgrass hydrolysates after alkaline pretreatment assisted by radio frequency heating and traditional water bath heating (original and added carbon source), as well as M9 medium (control group), were used as culture media. The RF media was shown to be optimal for PHB production, with a higher final PHB concentration. Then 1g/L, 2g/L and 5g/L yeast extract were added into each of the medium. The differences between RF and WB heated hydrolysate diminished after the addition of yeast extract. More nitrogen content of RF heated switchgrass hydrolysate was detected through CHNS analysis. Moreover, the acetic acid of hydrolysate of Switchgrass after radio frequency heating was 2.19g/L compared to that of traditional water bath heating at 1.58g/L. Thus, RF heated hydrolysate showed better performance probably because of more nutrients (similar to yeast extract) as well as a higher content of acetic acid.

4.1 Introduction

Plastics are widely utilized in almost every manufacturing industry ranging from automotives to functional medicine. The primary raw material for current plastic production is petroleum. Petroleum is one kind of non-renewable energy source. Moreover, more and more attention has been paid to the harmful side effects of petrochemical-derived plastic materials in the environment recently (Mooney, 2009). For example, incinerating plastics is one of the options to deal with the non-degradable plastics (Lea, 1996). It is very expensive, as well as dangerous. Environmentally speaking, harmful chemicals like hydrogen chloride and hydrogen cyanide are generated and released during the incineration process (Goodship, 2007; Harding et al., 2007; Parish et al., 2000; Sahlin et al., 2007). Therefore, it becomes urgent and inevitable to replace non-biodegradable by biodegradable plastics. It interests both decision-makers and the plastic industry. Thus, more and more research has been conducted to improve the method of biodegradable plastic production, the selection of raw materials, as well as the conversions to suitable forms of biodegradable plastics using certain wastes (Mooney, 2009; Nonato et al., 2001; Ojumu et al., 2004).

There are three types of biodegradable plastics: photodegradable, semi-biodegradable and completely (100%) biodegradable (Matyjaszewski et al., 2004). For the first type, it has light sensitive groups, which work well on the backbone of polymers when exposed to extensive ultraviolet radiation. However, lack of sunlight makes them remain non-degradable (Simsek et al., 2013). For the second type, it is starch-linked plastic, where starch is incorporated with the soil bacteria to release the polymer

fragments that can be degraded. Bacteria would stop by the polymer fragments, and then plastics still remain non-degraded (Sanginario et al., 2006). For the last kind of biodegradable plastic, it is promising since it can be totally utilized by bacteria. This type includes polyhydryalkanoates (PHAs), polylactides (PLA), aliphatic polyesters, and polysaccharides (Kasirajan and Ngouajio, 2012).

Among the various biodegradable plastics being investigated, polyhydroxybutyrate (PHB) is attracting more and more attention because of its unique characteristics. Pure PHB has similar physical and chemical properties compared to commonly used plastics, such as polypropylene. In terms of mechanical properties, the melting temperature, Young's modulus and tensile strength are comparable to polypropylene and polystyrene and other bulk plastics. Moreover, PHB is resistant to water and moisture and 100% biodegradable (Kacmar et al., 2006; Reis et al., 2008). PHB has plenty of applications, including packaging materials, surgical materials and other medical purposes. However, the biggest obstacle of wide spread commercialization of PHB is the high production cost as discussed in 2.4.2. Therefore, looking for inexpensive and suitable raw feedstock for PHB production is of great importance to reduce production costs. It is reported that corn is commonly utilized by various biopolymer production companies like Cargill Dow Polymers, LLC for biopolymer production (Luzier, 1992; Rhu et al., 2003).

The pathway for PHB production from acetyl-CoA is expressed in Figure 13. Three genes encoding for beta-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB),

and PHB polymerase (phbC) (or PHB synthase) can be seen from this figure(Kang et al., 2008; Somleva et al., 2008). First, two molecules of acetyl-CoA made from carbohydrate can be converted to acetoacetyl-CoA by beta-ketothiolase (or acetyl-CoA acetyltransferase) encoded by the PhaA gene. Second, the acetoacetyl-CoA can be catalyzed by acetoacetyl-CoA reductase encoded by PhaB gene catalyzes through reduction to a (R)-3-hydroxybutyryl-CoA (3HB-CoA). After that, PHB synthase encoded by PhaC gene can catalyze the polymerization reaction of the monomer molecules ((R)-3-hydroxybutyryl-CoA (3HB-CoA)) to polymer (PHB). Different pathways for PHB production are shown in Figure 22 from different carbon sources through different metabolisms.

Switchgrass has been identified as a typical species of a herbaceous energy crop by the US Department of Energy (Balan et al., 2012; Hu et al., 2010). It shows promising utilization because of high productivity, suitability for marginal land quality, low water and nutritional requirements, environmental benefits, and flexibility for multipurpose uses (Schmer et al., 2008). Research has been reported that soy wastes, malt wastes, agro-industrial waste, hydrolyzed cornstarch and whey are resources for sustainable production of biodegradable plastics (Luzier, 1992). In our study switchgrass has been investigated as a sustainable lignocellulosic biomass. Pretreatments of switchgrass are required to alter the recalcitrant structure in order to be converted to simple fermentable sugars (Keshwani and Cheng, 2009). Also, the pretreatment is a necessary upstream process for enzymatic degradation. It has been published that pretreatment of switchgrass could obtain glucose yields ranging from 70-90% and xylose

yields ranging from 70-100% after hydrolysis. Moreover, it contains nuturients such as N, P, K, and Na that are suitable for microbial cultivation (McLaughlin and Kszos, 2005). Utilization of switchgrass hydrolysate as a medium for PHB production can potentially reduce the cost compared to the pure sugars.

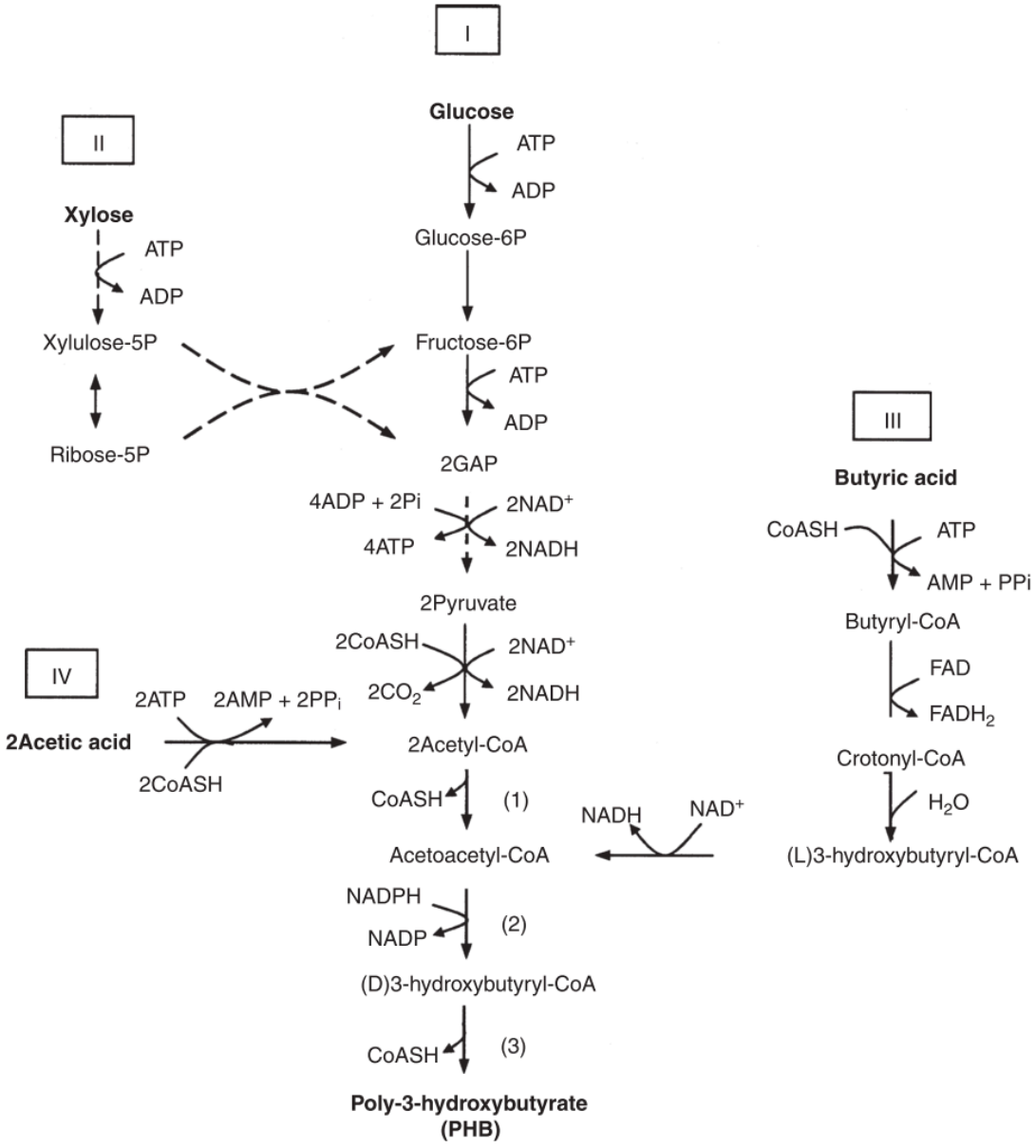


Figure 22 Pathways for the biosynthesis of poly(3-hydroxybutyrate) (PHB) from different carbon sources: (I) Glucose Embden–Meyerhof pathway; (II) Xylose via pentose-P pathway; (III) Butyric acid via acetoacetyl CoA and (IV) acetic acid. GAP refers to glyceraldehide-3-phosphate. Pathway for the biosynthesis of PHB from acetyl CoA involves three enzymes: (i) 3-keto-thiolase, (ii) acetoacetyl-CoA reductase and (iii) PHB synthase. The scheme was adapted from previous reports (Van-Thuoc et al., 2008).

Switchgrass was pretreated through alkaline solution assisted by radio frequency heating and traditional water bath heating. However, there is no literature on PHB production using switchgrass hydrolysate to make it suitable for industrial applications. For the microorganism, the pBHR68 plasmid is widely used in recombinant *E. coli* systems for the production of PHB (Anthony et al., 2013; Li et al., 2011; Rahman et al., 2014, 2013). These three genes from *R. eutrophus* (also known as *Alcaligenes eutrophus*) were successfully cloned into plasmid pBHR68 and subsequently expressed in *E. coli* (Asif Rahman, 2014). Therefore, this research focused on investigating the potential of Switchgrass hydrolysate based media for PHB production involving plasmid pBHR68. Moreover, this research focused on comparison of the media to culture *E-coli*, which were obtained after enzyme hydrolysis through alkaline pretreatment methods assisted by radio frequency heating and traditional water bath heating.

Generally, the pretreatment of lignocellulosic biomass (LCB) produces degradation products (Zha et al., 2014). Alkaline or acid pretreatment aims at disrupting the bio-recalcitrant structure of LCB and extracting fermentable sugars, which is essential

for production of biofuels and value-added bio-chemicals (Brodeur et al., 2011; X. J. Wang et al., 2012). This may result in inhibitors and byproducts that can inhibit subsequent fermentation process. Research has been conducted to identify compounds in pretreated biomass and biomass hydrolysates that cause inhibitory effects (Ibáñez and Bauer, 2014; Jönsson et al., 2013; Palmqvist and Hahn-Hägerdal, 2000a; Shuai et al., 2010). For these studies, it was found that inhibitors fall into four categories, 1) weak acids generated due to the degradation of hemicelluloses (i.e., acetic acid, formic acid, citric acid and levulinic acid) (Cho et al., 2012; Larsson et al., 1999; Palmqvist et al., 1999; Taherzadeh et al., 1997), 2) furan derivatives created from pentose and hexose sugars (i.e., hydroxymethylfurfural (HMF) and furfural) (Binder and Raines, 2009; Cantarella et al., 2004; Larsson et al., 1999; Palmqvist and Hahn-Hägerdal, 2000a), 3) phenolic compounds formed due to lignin degradation (i.e., ρ-coumaric acid, ferulic acid, hydroxybenzoic acid, vanillic acid, and syringaldehyde) (Cho et al., 2009; Klinke et al., 2002; Narayanaswamy et al., 2013), and 4) salts (i.e., sodium acetate, sodium chloride, and sodium sulfate) formed through acid-base reactions during pretreatment (Baral and Shah, 2014; Brethauer and Wyman, 2010; Harmsen and Huijgen, 2010; Van Niel et al., 2003).

Different pretreatment methods and mechanisms form different sugars and lignin degradation products. For example, for sulfuric acid pretreatment, which is one of the most widely used pretreatment processes for lignocellulosic biomass, furfural, acetic acid and phenolic compounds are the major byproducts formed (Qin et al., 2012). Steam explosion, which is an effective technique for cellulose conversion, also causes inhibitor

formation, mainly furfural, acetic acid, formic acid, and phenolic compounds (Cantarella et al., 2004). For alkaline pretreatment, such as aqueous ammonia and sodium hydroxide (NaOH), it has been reported that the low temperature for alkaline pretreatment decreases the formation of microbial inhibitors (Gao and Rehmann, 2014; Klinke et al., 2002).

4.2 Materials and methods

4.2.1 Chemicals

Standards of PHB (extracted from *Alcaligenes sp*) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The chloroform, stabilized with 0.6% w/w ethanol and the sodium hypochlorite (5% active chlorite) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The 99.8% atom-D chloroform containing 0.03% TMS was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). All other reactants were of analytical grade. Plasmid pBHR68 was a generous gift from Professor Charles Miller in Utah State University (Rahman et al., 2013).

4.2.2 Fermentation

E. coli harboring the pBHR68 plasmid was grown in Luria-Bertani medium (LB) the previous day. Overnight *E. coli* cultures with specific plasmids pBHR68 were inoculated from preculture into M9 medium with ampicillin (100 μg/ml), and grown in an orbital shaker operating at 220 rpm at 37℃ (Huang and Reusch, 1996; Li et al., 2007).The pBHR68 plasmid contains the three genes (phaA, phaB, and phaC) needed for PHB synthesis and confers ampicillin resistance (Rahman et al., 2014, 2013). Overnight cultures were then used to seed switchgrass hydrolysate after alkaline pretreatment

assisted by RF and WB heating at an initial optical density (OD600) of 0.05 at time 0 h. 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology, Inc. St. Louis, MO) was added to each flask at time 0 h. CFU/mL and PHB content were measured. Flasks were removed at 6, 12, 24, 48 h, 60h and 72h analyzed for PHB. CFU/mL was measured at time points 0, 4, 6, 8, 12, and 24 h. Experiments were carried out in triplicate.

4.2.3 Preparation and fermentation of swithcgrass hydrolysate for PHB production

E-coli containing the pBHR68 plasmid was cultured on four different media, namely, there were three experimental groups and one control group. A M9 medium was used as control. For the control group, M9 minimal medium has the same content of glucose as Switchgrass hydrolysate after alkaline pretreatment assisted by RF heating. M9 medium containing 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.02% $MgSO_4$ and 0.001% $CaCl_2$ was used as the growth media (Xie et al., 2012). Among the three experimental groups, two of them were Switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency and traditional water bath heating, respectively. The other one was switchgrass hydrolysate with WB heating added with glucose, xylose, arabinose, galactose, mannose, and acetic acid until equal content with the carbon source of RF heating. In the following results part, we use WBS as a symbol of this experimental group. For the detailed information of enzymatic hydrolysis of pretreated switchgrass and pretreatment method of raw materials, please refer to 3.4.2 to 3.4.4. Moreover, in order to find out the reasons for the difference in the PHB production, 1g/L, 2g/L and 5g/L yeast extract were added to M9 group, RF and WB heated Switchgrass hydrolysate separately.

4.2.4 Inhibitor analysis of pretreated swithcgrass before fermentation

The enzyme hydrolysate of alkaline ptrtreated Switchgrass assisted by radio frequency heating and traditional water bath heating were analyzed for inhibitors (including weak, furan derivatives, and phenolic compounds) before fermentation by high performance liquid chromatography (HPLC) using an Agilent 1260 liquid chromatography system (Agilent Technologies, Inc., CA, USA). The HPLC system was equipped with a strong cation-exchange column (Aminex HPX-87H, 300×7.8 mm) and a refractive index detector (RID-10A). The conditions are 45℃, 0.6 mL min, and 5.0 mM H_2SO_4 as mobile phase. A 60 min isocratic run was used for all samples.

4.2.5 PHB extraction and quantification

PHB extraction and quantification analysis was carried out using a direct polyhydroxyalkanoate analysis with 1H NMR as described previously Linton et al. (2012). After certain time points during fermentation, PHB producing *E. coli* were centrifuged at 4000 rpm for 20 min (Eppendorf, Eppendorf AG, Hamburg, Germany) at at -4°C. Then the precipitate of the cell samples was kept in -80°C for more than 12 hours until completely frozen. Frozen samples were dried using a freeze-dryer (Labconco lyophilizer; Labconco Corporation, Kansas City, MO, USA). Sample preparation in NMR was carried out in 2 mL vials. Approximately 5-10(±0.2) mg of lyophilized cells were dissolved in 0.7 mL of 5% sodium hypochlorite and 1mL of $CDCl_3$ (0.03% TMS). The samples from each time point were analyzed by NMR to allow for the maximum PHB production. 1H NMR sample preparation procedures were developed based on chloroform-sodium hypochlorite dispersion method for PHA extraction with modifications. These mixtures were vortexed for 10 min, and incubated on a shaker table

for 2 h at 30 °C. PHB standards were incubated at a higher temperature (50 °C) to facilitate dissolution. After the 2 h extraction, samples were centrifuged at 13,200 rpm for 10 min (Eppendorf, Eppendorf AG, Hamburg, Germany) to induce phase separation. This resulted in three different phases (as shown in Figure 24). The top layer was the aqueous hypochlorite solution, the middle layer included cells and other biological matter, and the bottom layer was rich in PHB content in chloroform. Because $CDCl_3$ was used for extraction, aliquots from the organic layer were subjected directly to 1H NMR analysis. The chloroform phase was needled out carefully, shown in Figure 25, after centrifugation. The organic phase was transferred to a 5mm NMR tube and was analyzed for PHB in a Bruker 400 MHz nuclear magnetic resonance NMR spectrometer. A standard NMR method was used to determine PHB concentrations as described in Jan et al. (1996). The TMS inside $CDCl_3$ was used as an internal standard to correct injection volume errors. Experiments were carried out in triplicate.

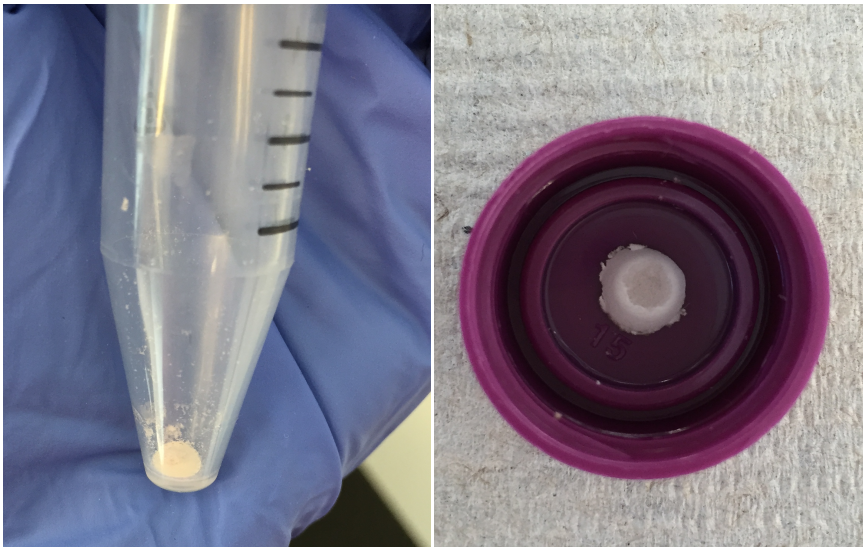


Figure 23 PHB obtained from Switchgrass hydrolysate fermentation

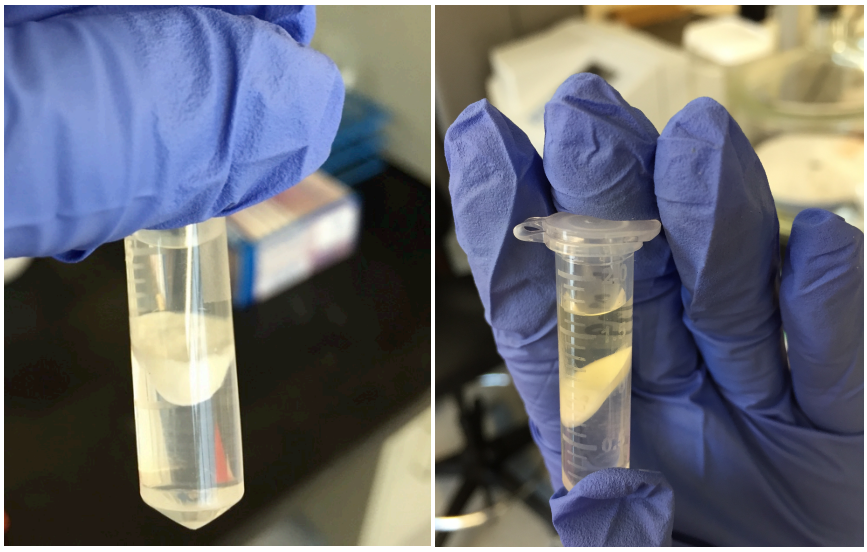


Figure 24 Three different phases appeared after centrifuging

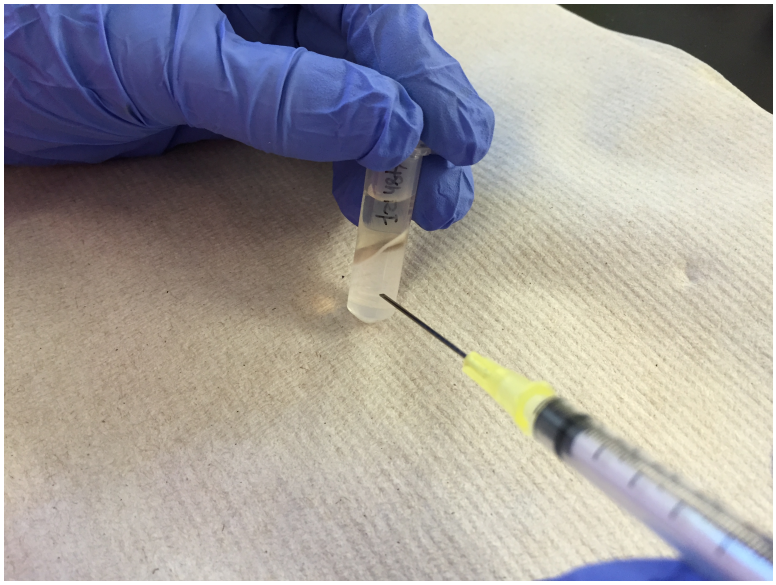


Figure 25 Separation of chloroform phase

4.2.6 Analytical procedures

4.2.6.1 Optical density (OD) and Dry cell weight (DCW)

During the fermentation process, optical Density (OD) of the suitably diluted cell suspension was measured at certain time points through a UV spectrophotometer at 600 nm against DI water as blank. Cell mass was harvested from 50 mL medium with centrifugation at 4,000 rpm for 20 min at 4 °C and freeze-dried. Samples were then kept in a desiccator and weighed to determine dry cell weight (DCW) for measurement of cell mass concentration and PHB content. Lyophilized biomass was used to determine dry cell weight and construct growth curves. Triplicates were determined in all experiments.

4.2.6.2 PHB identification analysis using NMR

The 1H nuclear magnetic resonance (NMR) spectrum was recorded at Burker 400 MHz (Bruker, Sikerstrifen, Germany) at room temperature. Data were averaged over 16 acquisitions on the internal standard. Values were calculated after examination of peak intensities. The spectrum was evaluated using standard Bruker uxnmr software. Chemical shifts were referenced to the internal reference TMS.

4.2.6.3 Statistical analysis

All experiments and analyses were performed in triplicate. For each run using different media, optical density (OD), dry cell weight (DCW), PHB yield coefficient relative to cell dry weight (Yp/x, g/g, defined as gram PHB produced per gram dry cell mass produced) (Grothe et al. 1999), PHB concentration (g/L, PHB produced per liter of culture), PHB content (g/g, defined as the ratio of to dry cell concentration) were measured and calculated accordingly for comparison after completion of the fermentation

process. Triplicates were tested in all experiments. ANOVA General Linear Model (GLM) analysis ($\alpha = 0.05$) and mixed analysis ($\alpha = 0.05$) using SAS® (version 9. 1. 3 SP4) were applied to compare the effect of different heating methods used in Switchgrass hydrolysates fermentation.

4.3 Results and Discussion

4.3.1 Growth kinetics and fermentation

E. coli harboring the pBHR68 plasmid was grown on four kinds of media, including SW hydrolysate after pretreated by RF heating (RF), SW hydrolysate after pretreated by WB heating (WB), equal glucose content of SW hydrolysate after pretreated by WB heating (WBS), and M9 medium as control (M9). The maximum optical density (OD_{600}) for RF media was around 1.15 where stationary phase was reached at approximately 20 h post induction (Figure 26). Both of the WB and WBS samples reached a maximum OD_{600} around 1.0. However, it should be noticed that at the beginning of cell growth, WB medium showed better performance before 12 hours. Cultures were allowed to continue growing until 72 h as this allowed time for finding maximum PHB accumulation.

Growth curves obtained for the PHB producing strains using four different media are presented in Figure 27. Surprisingly, SW hydrolysate after pretreatment by RF heating as medium obtained the best cell growth at 6.2 g/L, followed by that of WB heating around 3.0 g/L, and then the control group (M9 medium). However, the increasing rate of dry cell weight in WB was larger than that in RF before 12 hours in cell growth. For the SW hydrolysate after alkaline pretreatment through different heating

methods (RF and WB), there were distinct differences on the dry cell weight figure, which means that something in RF pretreated SW are more suitable for cell growth or inhibit the fermentation process less. Moreover, for SW hydrolysate after WB heating, there is no significant difference whether added carbon source until equal to that after RF heating. Thus it can be concluded that the glucose content was not the big problem in cell growth. Moreover, in order to find out what caused the difference between RF and WB pretreated SW hydrolysate, 1g/L, 2g/L and 5g/L yeast extract were added to the previous medium separately. The differences between RF and WB heated SW hydrolysate were becoming fewer, as the concentration of yeast extract increased. With the addition of yeast extract, the optical density of cells increased. However, the difference between RF and WB heated SW hydrolysate decreased with the increase of yeast extract. This demonstrated that the big difference between the two hydrolysates was nutrient to certain content.

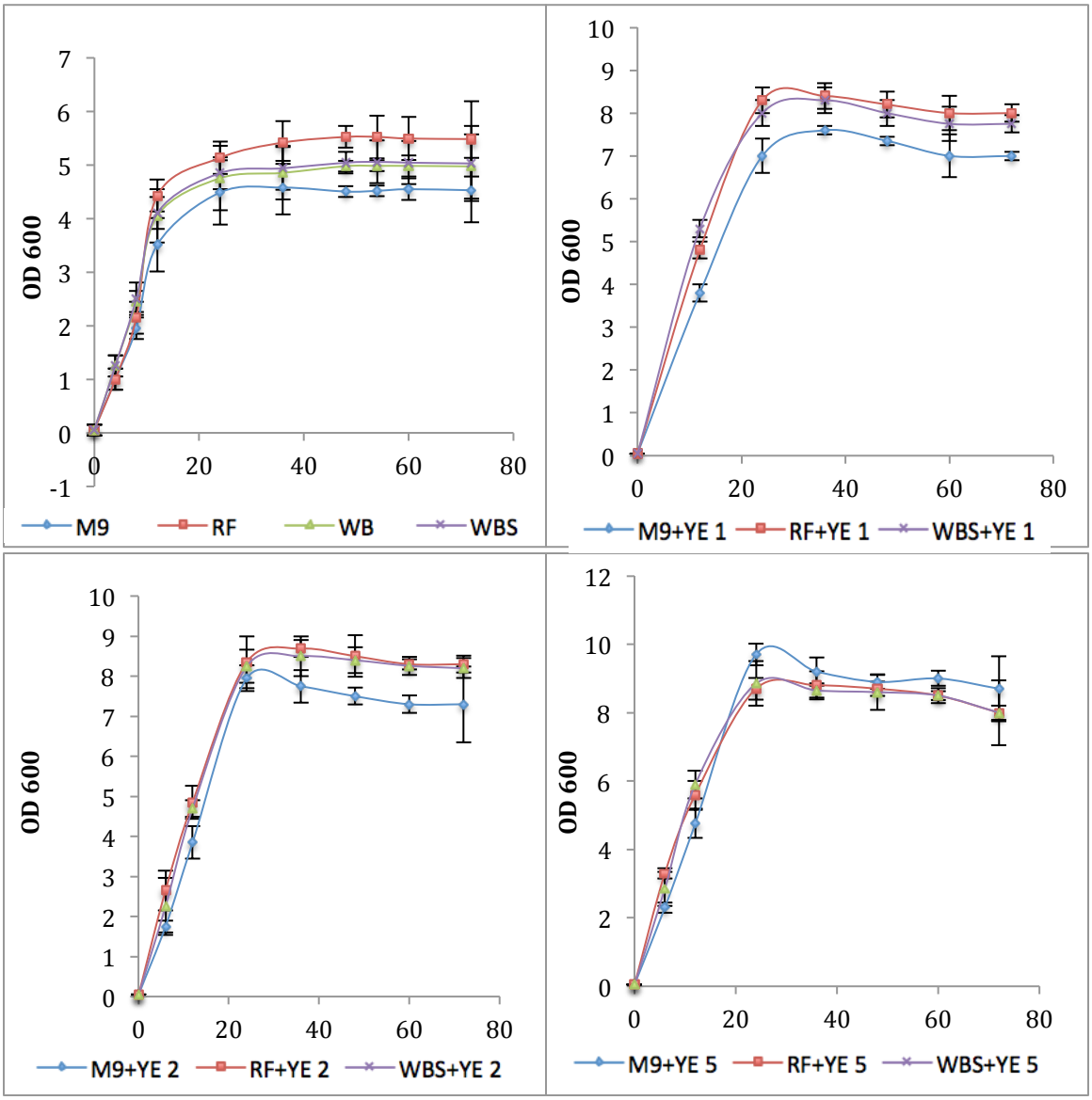


Figure 26 Optical density (OD_{600}) at certain time points during fermentation process

4.3.2 Identification analysis of PHB during fermentation

Switchgrass hydrolysate was used as C-source for PHB production. The PHB chemical structure was determined by NMR-spectroscopic analysis. During fermentation, cells were sampled to identify maximum PHB production at certain time points, namely

12h, 24h, 36h, 48h and 60h, in order to scale up PHB production through fermentation routes into industrial application. PHB concentration and PHB yield curves using four different media are presented in Figure 27 and Figure 28, respectively.

For the M9, RF and WB media without addition of yeast extract, PHB production reached the maximum concentration (around 2.2g/L) at 48h, and PHB yield reached the maximum yield (around 37%) at 48h. PHB production analysis was carried out 12, 24, 36, 48 and 60h. PHB production analysis was carried out 12, 24, 36, 48 and 60h after induction because after 20 h *E. coli* harboring the plasmid systems were in stationary phase. It is reported that PHB did not accumulate to significant levels during the exponential growth phase (Rahman et al., 2013). There is a delay between carbon source utilization and PHB accumulation since acetyl-CoA is required for cell synthesis during the exponential phase, as well as for PHB production in the stationary phase.

For PHB concentration, SW hydrolysate after pretreatment by RF heating achieved best results at 4.5 g/L, followed by that by WB heating around 3.0 g/L, and then the control group (M9 medium). However, for SW hydrolysate after WB heating, there was no significant statistical difference seen in whether added carbon source until equal to that after RF heating. Kang et al. (2008) reported that final PHB concentration was 3.52 g/l in *E. coli* DH5α harboring pBHR68 after 24-48 h. Moreover, it has been demonstrated from previous studies that PHB can accumulate in larger quantities in *E. coli* when using a bioreactor compared to a shaker flask (Choi et al. 1999; Lee SY et al. 1994). With the addition of yeast extract, the PHB concentration increased. Moreover, the

difference between RF and WB heated hydrolysates decreased with the increase of yeast extract.

For PHB yield, SW hydrolysate after pretreatment by RF heating achieved best results at around 36 %, followed by that by WB heating around 30%, and then the control group (M9 medium). For SW hydrolysate after WB heating, there was no significant statistical difference seen in whether added carbon source until equal to that after RF heating. After adding yeast extract, the PHB yield increased a little bit. At the same time, the difference lowered, until nearly the same. Rahman et al. (2013) reported that the non-secreting strain accumulated approximately 41.93 ± 13.5% and 47.24 ±6.0% PHB, respectively in the dry cell weight at 24 h and 48h after induction. Interestingly, the results of PHB concentration and PHB yield showed difference using Switchgrass hydrolysate after alkaline pretreatment by RF and WB heating.

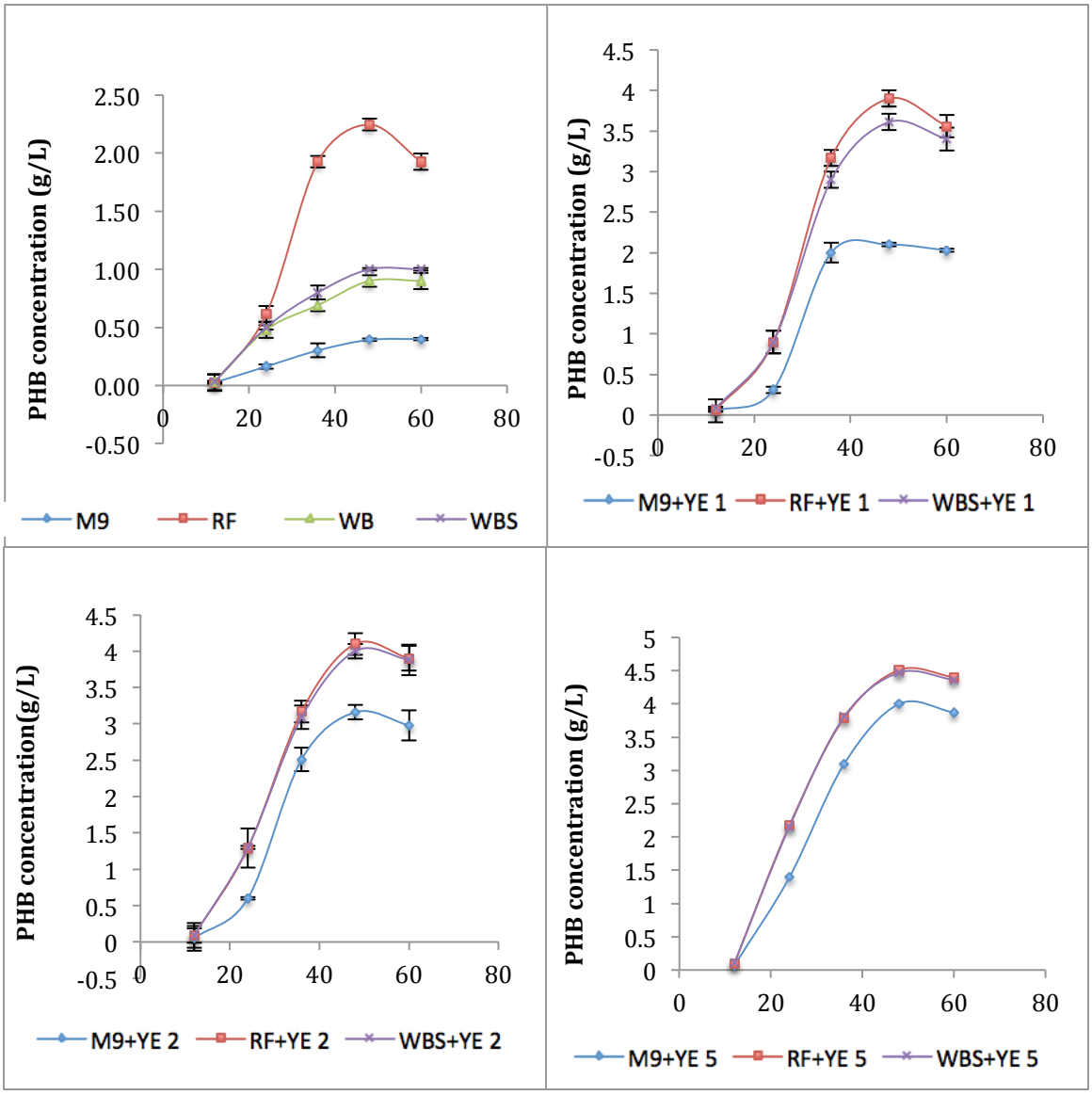


Figure 27 PHB concentration obtained at certain time points during fermentation process

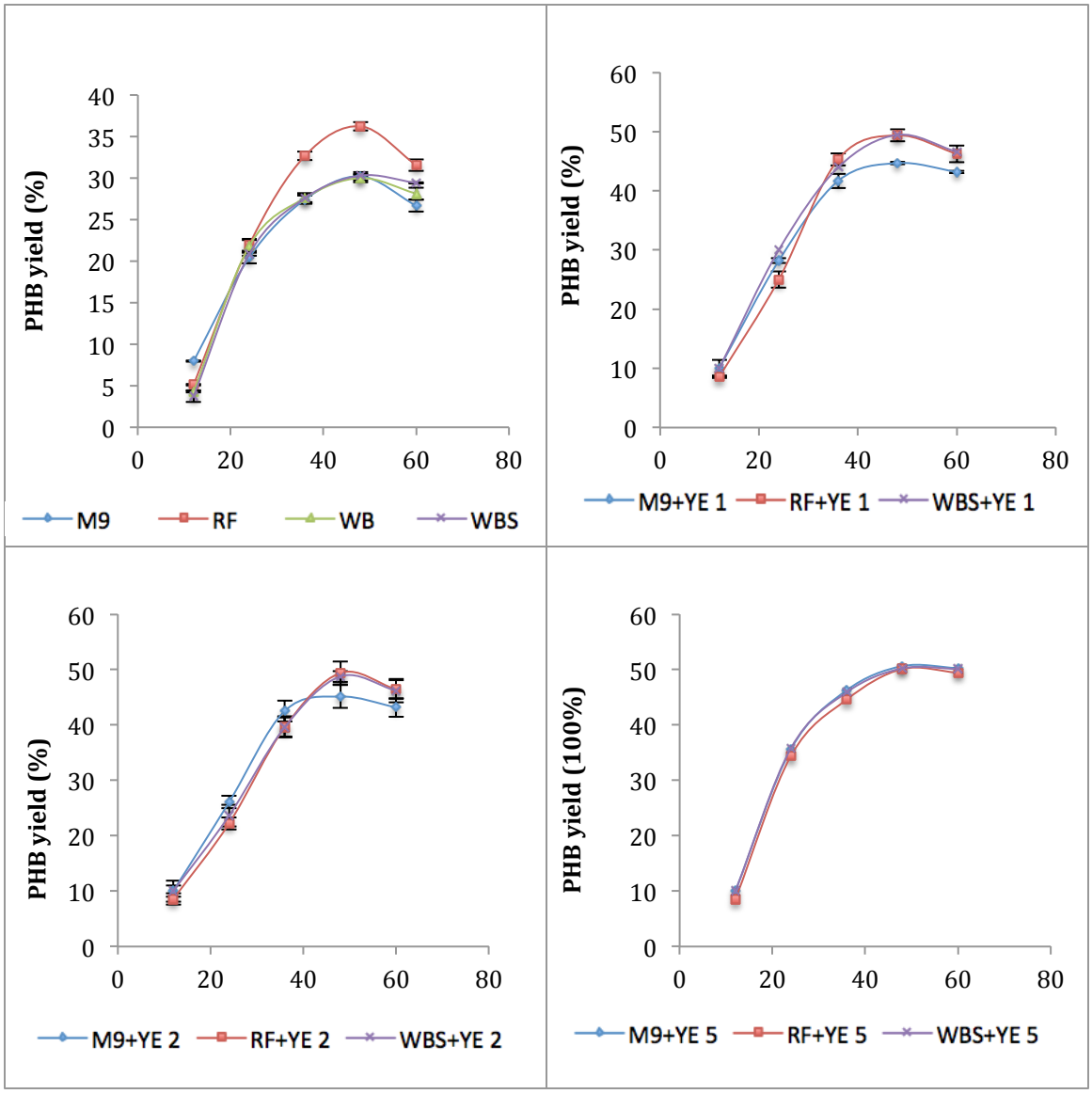


Figure 28 PHB yield obtained at certain time points during fermentation process

Table 6 Summarization of PHB concentration and PHB yield

Media	PHB concentration (g/L)	PHB yield (%)
M9	0.39±0.06	30.21±0.71
RF	2.25±0.01	36.23±0.12
WB	0.90±0.01	30.00±0.19
WB+ Glucose	1.00±0.06	30.30±0.39
M9+YE 1	2.10±0.07	44.68±1.41
RF+YE 1	3.90±0.01	49.37±0.20
WB+ Glu+YE 1	3.61±0.02	49.45±0.99
M9+YE 2	3.16±0.17	45.14±1.71
RF+YE 2	4.10±0.12	49.40±2.01
WB+ Glu+YE 2	4.00±0.10	48.78±1.96
M9+YE 5	4.00±0.07	50.63±0.17
RF+YE 5	4.51±0.01	50.11±0.19
WB+ Glu+YE 5	4.47±0.02	50.22±0.10

4.3.3 Comparison of fermentation and PHB production between RF and WB pretreated Switchgrass hydrolysate

For the inhibitor analysis of Switchgrass hydrolysate with GC-MS, results showed a big difference in acetic acid. Hydrolysate of Switchgrass by radio frequency heating obtained a 2.19g/L acetic acid content, while that of traditional water bath heating was 1.58g/L. Shuai et al. (2010) reported that acetic acid concentrations, as a potential fermentation inhibitor, that formed during the dilute acid and sulfite pretreatment were 5.3 and 2.7 g/L, respectively. This indicated that fewer inhibitors were formed from degradation of saccharides during the sulfite pretreatment than the dilute acid pretreatment. Compared to our study, alkaline produced the least acetic acid.

Acetic acid, which was found after pretreatment, is the most commonly used carboxylic acids. It is reported that its formation is caused by the hydrolysis of the acetyl

groups linked to the sugar or other linkages present in hemicellulose backbone (Shuai et al., 2010). Since the formation of acetic acid is inherent to hemicellulose hydrolysis, its formation cannot be prevented. And its generation mainly depends on the temperature and residence time of pretreatment until the acetyl groups are fully hydrolyzed (Taherzadeh et al., 1997). In this study, the acetic acid content in alkaline pretreated Switchgrass after RF heating is higher than that of WB heating. Then it can be concluded that RF heating hydrolyzed more acetyl groups linked to the hemicellulosic backbone. During the pretreatment process, it allows the hemicelluloses to hydrolyze in the lignocelluloses. Hemicelluloses can be degraded into xylose, mannose, acetic acid, galactose, glucose, etc. Chang and Holtzapple (2000) have reported that the alkaline pretreatment process removes acetyl and uronic acid groups present on hemicellulose and thus enhances the accessibility of the enzyme that degrades hemicellulose. In the alkali treatment process, ester linkages that join xylan and hemicelluloses residues are also hydrolyzed (Sun and Cheng 2002). RF irradiation showed a better solubilization of the biomass. We hypothesized that the selective heating probably caused an “explosion” effect among the particles, and improved the disruption of the recalcitrant structures of lignocellulosic biomass. Moreover, the dielectric heating induces rapid rotation of the polarized dipoles and ionized ions in the molecules (Hu et al., 2008; Piyasena et al., 2003), which generates heat due to the friction and increases the probability of contact between molecules and atoms simultaneously, thus hydrolyzing more acetyl groups lined to the hemicellulose. For Switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency heating, higher acetic acid content was analyzed, which subsequently gave a higher PHB concentration. From the pathway of PHB production, there are several

routes through different sources. One of them is from acetic acid, thus higher acetic acid should result in higher PHB production. Previous studies have demonstrated higher acetic acid content among certain range can increase ethanol yield. Cantarella et al. (2004) reported that in the presence of acetic acid, there was an increase of ethanol production during the fermentation of poplar wood hydrolysate using *Saccharomyces. cerevisiae*. Moreover, this is in agreement with the findings of Panagiotopoulos et al. (2009), an acetic acid concentration up to 1.0 g/L showed better fermentability utilizing biomass-derived sugars from wheat and barley straws by *Caldicellulosiruptor saccharolyticus*, while more than 3g/L acetic acid concentration inhibited the fermentation. However, the effect of acetic acid and the mechanism of that on growth of *E-coli* cultures should be investigated in the near future.

For the nitrogen content in RF and WB heated switchgrass hydrolysate, there was a significant difference, with a higher amount of the former one. In this condition, the RF pretreated hydrolysate contained a higher amount of nitrogen and could offer more cell growth and produce more PHB, and the results in turn verified the fermentation for PHB production. Borah et al. (2002) reported that the addition of an organic nitrogen source to media containing sucrose promoted PHB yield and productivity. The increase of PHB accumulation may be due to the presence of amino acids and peptides in the yeast extract. Song et al. (1999) reported that the PHB content of the cells was enhanced significantly by any supplement tested: nutrient broth, yease extract, peptone, or casein acid hydrolysate (each 0.5 g/l), respectively. Moreover, they thought that yeast extract as a supplement seemed to be the best candidate to obtain high amounts of PHB. Lee and

Chang (1995) reported that PHB synthesis was generally promoted by supplementing the medium with a small amount of complex nitrogen sources. Supplementation with 0.2% (w/v) tryptone, casamino acids, yeast extract or casein hydrolysates promoted PHB synthesis to a greater extent. It is well known that organic nitrogen sources such as yeast extract provides various amino acids, vitamins, minerals and growth factors that promote good growth of microorganisms. The complex nutrients provided precursors for the biosynthesis of amino acids, proteins and other cell constituents. Therefore, more acetyl-CoA was saved for conversion of PHB production, while PHB is synthesized from acetyl-CoA by three enzymatic conversion steps. The synthesis and accumulation of PHB are dependent on the amount of acetyl-CoA available. In our study, the more the yeast extract, the more amount of PHB production, as well as the less difference between RF and WB heated Switchgrass hydrolysate.

4.4 Conclusion

Pretreatment of biomass is an extremely significant step in a commercial biorefinery, and fundamental understanding and comparison of various pretreatment processes is essential. In this study RF and WB assisted alkaline pretreatments were carried out on Switchgrass. The acetic acid generated during pretreatment, which promotes cell growth at certain range of content, was also studied. The potential of Switchgrass hydrolysate as media for production of PHB by plasmid pBHR68 was highlighted in this study. Switchgrass hydrolysate, especially the one pretreated by radio frequency is better for achieving higher PHB concentration for more acetic acid, as well as more nutrients in RF hydrolysate. It is expected that results of this study can

subsequently be applied to enhance the conversion of lignocellulosic biomass for PHB as well as copolymer (such as PHBV and P (3HB-4HB)) production, thus increasing market potential and cost effectiveness.

Chapter 5 ABE fermentation using Switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency (RF) and traditional heating

Abstract

In this study, radio frequency (RF) assisted dielectric heating was utilized in the alkaline (NaOH) pretreatment of switchgrass. The acetic acid of hydrolysate of Switchgrass after radio frequency heating was 2.19g/L compared to that of traditional water bath heating at 1.58g/L. Moreover, the amount of nutrients in switchgrass hydrolysate after radio frequency heating was more than that of traditional water bath heating. Switchgrass hydrolysates after alkaline pretreatment assisted by radio frequency heating and traditional water bath heating (original and added carbon source) were used as culture media. Acetone butanol ethanol (ABE) was produced by *Clostridium beijerinckii*. The hydrolysate was used after pH adjustment without sediment removal for ABE fermentation. ABE reached the maximum in the first medium with a butanol concentration of 1.95 g/L and a total ABE concentration of 2.96 g/L, corresponding to the maximum ABE yield (0.20) in the first medium, indicating that the enzymatic hydrolysates after alkaline pretreatment assisted by radio frequency was best for *Clostridium beijerinckii* growth. However, the difference of the switchgrass hydrolysate between radio frequency and traditional water bath heating was smaller with the addition of yeast extract.

5.1 Introduction

Today the energy crisis was caused by the finite nature of fossil fuel resources. Moreover, the associated environmental effects including the release of green house gases and global warming have attracted public's eyes (McKendry, 2002a). Thus, the alternative bio-based fuels and chemicals from renewable sources develop fast to solve these problems. Solvents such as acetone, butanol, and ethanol (ABE) are important chemicals and potential fuels that can be produced by microbial fermentation of lignocellulosic biomass.

Biobutanol, which is a kind of alcohol with four carbon atoms, is colorless and flammable. Biobutanol can be used as solvents, chemical intermediates, extract agents, industrial cleaners, or gasoline additives. There are several advantages of butanol over ethanol, such as higher heat value, higher viscosity, less evaporative/explosive, higher hydrophobicity, lower vapor pressure, less corrosive (Maddox, 1989; Patakova et al., 2011). Therefore, butanol as a kind of promising fuel, has attracted people's attention recently. There are mainly two methods for production of butanol, including chemical technologies and fermentation by microorganisms (Liu et al., 2009). The chemical technologies are oxo-synthesis and aldol condensation. In additional to chemical routes, butanol can also be obtained through biological ways by microorganisms through fermentation (Qureshi et al., 2008a). For fermentation, the most commonly used bacteria species for fermentation is *Clostridium acetobutylicum.* The fermentation is also called acetone-butanol-ethanol (ABE) fermentation because of the main products including

acetone, butanol and ethanol (Qureshi et al., 2008a; Raganati et al., 2012). The process of biobutanol production from lignocellulosic biomass can be shown in Figure 29.

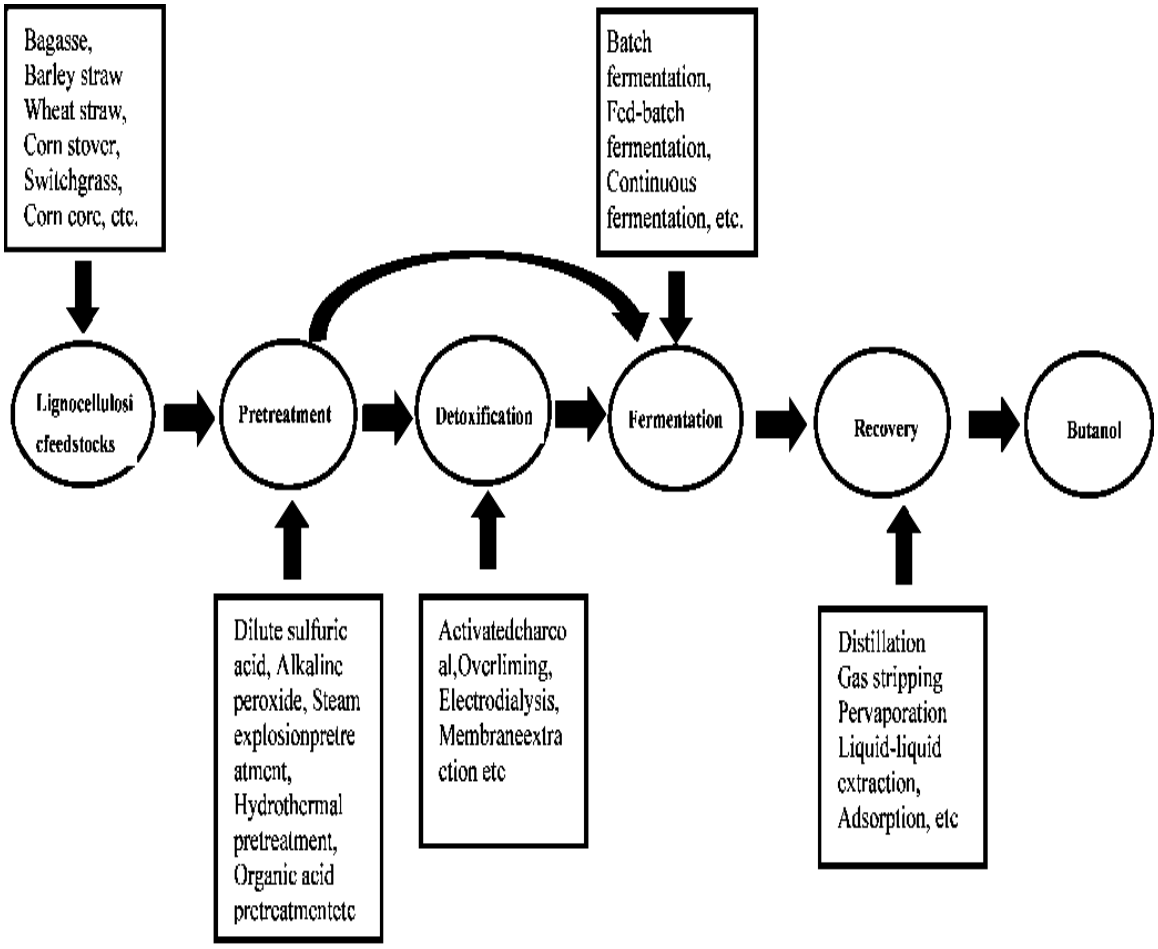


Figure 29 The process of biobutanol production from lignocellulosic biomass

Lignocellulosic biomass is the most abundant renewable resource on the planet. Some reports on the ABE fermentation of *Clostridia* strains utilizing the hydrolysate of lignocellulosic biomass are available in the scientific literature (Sukumaran et al., 2011). The lignocellulosic biomass used for the ABE fermentation includes agricultural residues (e.g., rice straw, wheat straw), wood (hardwood), and waste

paper. These substrates are composed mainly of polysaccharides that contain six and five carbon sugars. Due to the complexity and bio-recalcitrance of lignocellulosic biomass, enzymatic hydrolysis has to be carried out concurrently or prior to fermentation because the former process can degrade cellulose and hemicellulose into five- and six-carbon sugars. Moreover, prior to use of these substrates, these feedstocks must be pretreated to alter the physical structure of LCB. In this research, we utilized switchgrass as our raw materials and pretreated with alkaline solution assisted by radio frequency and traditional water bath heating. After that, pretreated feedstocks were hydrolyzed by enzymes to get five- and six-carbon sugars. Then the hydrolysates of Switchgrass were prepared to do fermentation tests. However, it should be noted that pretreatment of lignocellulosic biomass with acid/alkali forms inhibitors (Jönsson et al., 2013), which include weak acids (i.e., acetic, formic, and levulinic), furan derivatives (i.e., hydroxymethylfurfural (HMF) and furfural), and phenolic compounds (i.e., ρ-coumaric acid, ferulic acid, hydroxybenzoic acid, vanillic acid, and syringaldehyde) shown in Figure 31. For different pretreatment methods and process parameters, different kinds of inhibitors are produced. Weak acids are mainly generated because of the degradation of hemicellulose (Larsson et al., 1999). Furfuran and HMF are primarily produced from pentose and hexose sugars. Phenolic compounds are commonly created because of the degradation of lignin. Salts are normally formed by acid-base reactions during pretreatment (i.e., sodium acetate, sodium chloride, and sodium sulfate). The ABE fermentation is affected by the presence of these inhibitors.

It is reported that the most favorable *Clostridium* species, *Clostridium acetobutylicum* and *Clostridium berjerinckii*, share the same metabolic pathways. The main products include acids (acetic acid and butyric acid), solvents (butanol, acetone, and ethanol), and gases (CO_2 and H_2). Detailed mechanisms of metabolic pathways and their probable modifications are shown in Figure 16. For the ABE feremtnation, the microorgansims can hydrolyze starch to glucose, and then glucose can be converted to pyruvate through Embden-Meyerhoff pathway (EMP, or glycolysis). After that, it can be divided into two successive and distinct phases, namely the acidogenesis phase and solvetogenesis phase. In the initial growth phase, which is also called the acidogenesis phase, the *Clostridia* may generate acetate, butyrate, hydrogen and carbon dioxide. Acetate is created through acetyl-CoA, which is catalyzed by two kinds of enzymes, PTA (phosphotransacetylase, or phosphate acetyltransferase) and AK (acetate kinase). Butyrate formation is a relatively complicated process involving several steps (Y. Wang et al., 2013). First, two molecules of acetyl-CoA are transformed into acetoacetyl-CoA through THL (acetyl-CoA acetyltransferase). Second, acetoacetyl-CoA is transformed into butyryl-CoA through three enzymes: HBD (hydroxybutyryl-CoA dehydrogenase), CRT (hydroxybutyryl-CoA dehydrolase), and BCD (butyryl-CoA de-hydrogenase). Then butyryl-CoA is catalyzed by two enzymes: PTB (phosphotransbutylase or phosphate butyltransferase) and BK (butyrate kinase), to generate butyrate. As the acid accumulates, pH drops to the lowest point during fermentation. This leads to the switch of acidogenesis phase to solvetogenesis phase. During the solvetogenesis phase, which is stationary, the formation of acids decreases, and acetone and butanol become the dominant products (with a small fraction of ethanol).

In the solvetogenesis phase, acetoactyl-CoA is converted by CoAT (CoA transferase) into acetyl-CoA and acetoacetate. Then acetone is obtained through ADC (acetoacetate decarboxylase). The former one has two pathways. In one route, it is converted into ethanol by ADH (acetaldehyde dehydrogenase) and BDH (ethanol dehydrogenase); in another, it is converted to butyryl-CoA. Then butyryl-CoA is converted to butyraldehyde by ADH (butyraldehyde dehydrogenase) and finally to butanol by BDH (butanol dehydrogenase).

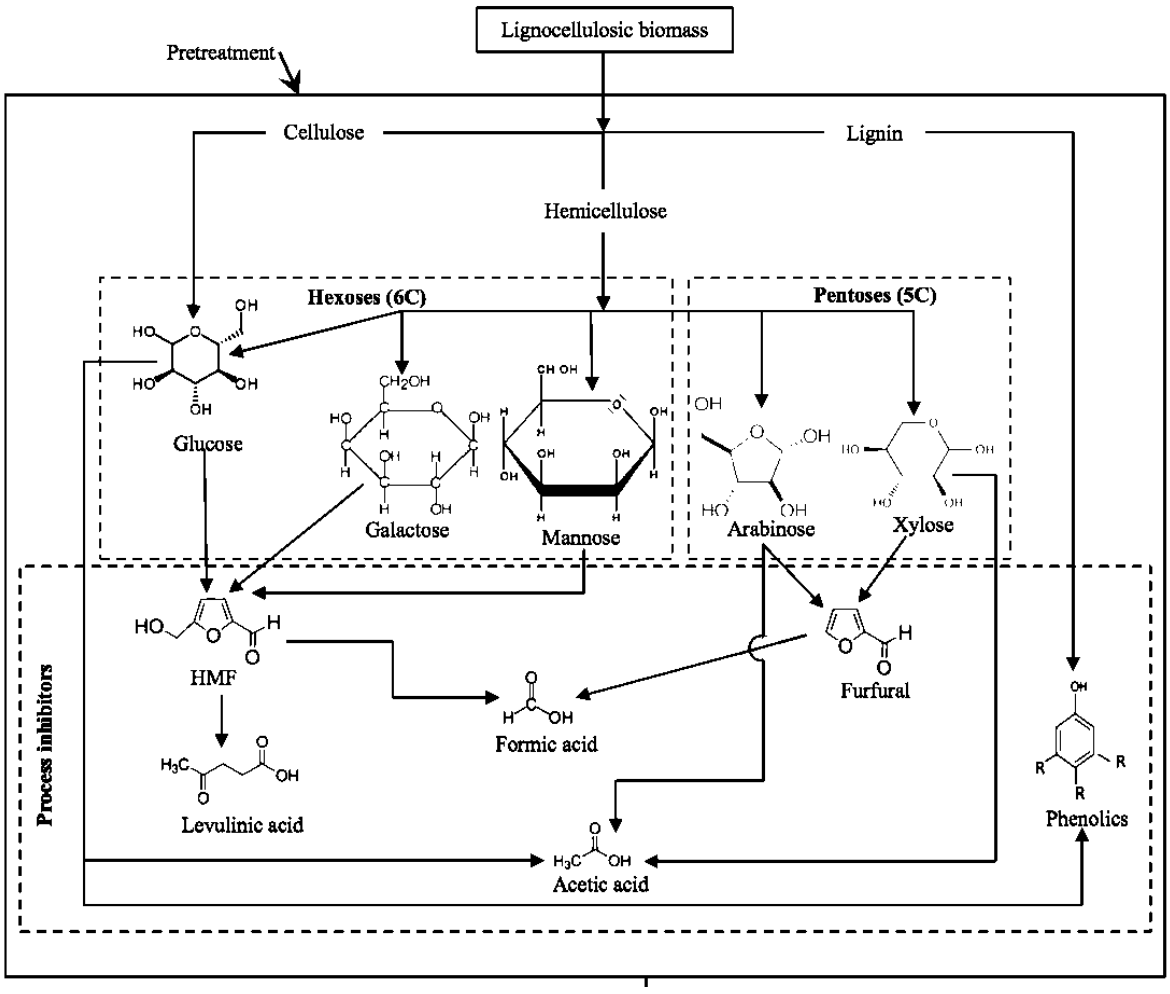


Figure 30 Microbial inhibitor formed during pretreatment and ABE fermentation processes. This figure was modified from Ibraheem and Ndimba (2013)

In the current study, we investigated utilization of switchgrass as a potential substrate for butanol fermentation employing *Clostridium. beijerinckii*. Switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency and traditional water bath heating was used to produce ABE. This research worked on investigating the potential of switchgrass hydrolysate based media for ABE fermentation and reducing substrate costs for ABE fermentation by using lignocellulosic biomass feedstocks. Moreover, this research focused on comparison of the media to culture *Clostridium. Beijerinckii*. These media were obtained after enzymatic hydrolysis through alkaline pretreatment methods assisted by radio frequency heating and traditional water bath heating to identify mechanisms for developing an efficient and sustainable conversion system for cellulosic biomass into biofuel based on integration of biomass pretreatment with fermentation.

5.2 Materials and methods

5.2.1 Bacterial culture

Clostridium. beijerinckii 8052 was used in these studies. A laboratory stock of *C. beijerinckii* 8052 spores were stored in sterile double distilled water at 4 ℃. It was routinely maintained as spore suspensions. The spores of *C. beijerinckii* 8052 were heat-shocked at 80 °C for 10 min, and then cooled on ice for 5 min. The heat-shocked spores were inoculated at a 1% inoculum level into 20mL tryptone–glucose–yeast extract (TGY) medium. The TGY medium contained 30 g/l of bactotryptone, 20 g/l of dextrose/glucose, 10 g/l of yeast extract, and 1 g/l of cysteine-HCl monohydrate. The TGY culture was

incubated at 35±1 ℃ for 12-14 h in an anaerobic chamber under N_2:CO_2:H_2 (volume ratio of 85:10:5) atmosphere.

5.2.2 Culture growth and fermentation experiment

After overnight TGY cultures grew to an optical density (OD600) of 0.8-1.0, the actively growing culture was inoculated with a 5% ratio into medium for fermentation at 35±1 ℃ in an anaerobic chamber. For all of these four media, 1% stock solutions (mineral, buffer, and vitamin) of P2 medium were added. The P2 medium contained the following compounds (in g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 0.01; p-Aminobenzoic Acid, 0.001; Thiamin-HCl, 0.001; Biotin, 0.00001. Before inoculation, the pH of the fermentation medium was adjusted to 6.8-7.0 in both media with filter-sterilized KOH. There were three experimental groups and one control group as medium that required keeping in the anaerobic chamber one night before innoculation. A medium including equal sugar content with that of hydrolysate after alkaline pretreatment assisted by RF heating was used as control. Among the three experimental groups, two of them were switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency and traditional water bath heating, respectively. The other one was switchgrass hydrolysate with WB heating added with glucose, xylose, arabinose, galactose, mannose, and acetic acid until equal content with the carbon source of RF heating. In the following results part, we use WBS as a symbol of this experimental group. Cell growth temperature was controlled at 35±1 ℃. The other whole groups were all the same as that of mentioned earlier, except that with addition of 1 g/L, 2g/L and 5g/L yeast extract, which means the medium was more nutrient for cell growth. Triplicates were carried out in experiment.

The cell density and product concentration were monitored through the course of fermentation. For the detailed information of enzymatic hydrolysis and alkaline pretreatment assisted by RF and WB heating of raw switchgrass as feesdtock, please refer to 3.4.2 to 3.4.4.

5.2.3 Fermentation products analysis

ABE, acetic acid, and butyric acid concentrations were quantified using high-performance liquid chromatography (HPLC) (Agilent Technologies 1260 series) equipped with an automatic sampler/injector and a refractive index (RI) detector. For HPLC analysis, the mixture was centrifuged at 13,200 rpm for 10 min. Before injection into the HPLC, the supernatant was diluted fivefold with distilled water. The HPLC column (HPX-87H; Aminex Resin- based) was obtained from BioRad (Hercules, CA, USA). The solvent (5mM H_2SO_4) flow rate was maintained at 0.6 mL/min at 45°C.

Butanol yield (g/g) was calculated as the total butanol produced divided by the total sugar utilized. ABE yield was defined as total grams of ABE produced divided by the per gram of glucose or sugar utilized. During the experiment, cell concentration was measured by optical density (OD) method. Culture growth was measured by following OD in the fermentation broth at 600nm using a UV-visible spectrophotometer. Triplicates samples were conducted during fermentation.

5.2.4 GC-MS study of enzyme hydrolysate of pretreated switchgrass

The alkaline pretreated switchgrass assisted by radio frequency and water bath heating was tested for chemicals using GC-MS. Gas chromatography (GC) is a common method used to analyze gases produced during various chemical processes. GC/MS

analysis of pretreated switchgrass was performed on an Agilent 6890N GC equipped with an Agilent 5973 mass-selective detector (MSD). The GC column used was a DB-1701 60m × 0.25 mm, 0.25 μm film thickness. The oven was programmed to hold at 40 °C for 4 min, ramp at 3 °C/min to 250 °C, and hold there for 20 min. The injector temperature was 250 °C, and the injector split ratio was set to 30:1. The flow rate was 1 mL/min of the He carrier gas. The samples were prepared as ~6% solutions in methanol that were filtered through a 0.45 μm PTFE filter prior to injection.

5.2.5 CHNS analysis

Elemental analyses (C, H, N, S) of the feedstocks and products were determined using a Perkin-Elmer CHNS/O analyzer. Ultimate analysis was carried out to determine the basic elemental composition of the biomass samples. The samples' ultimate analysis was done using a Perkin–Elmer model 2400, Series II CHNS/O analyzer to measure carbon, hydrogen, nitrogen and sulfur contents.

5.2.6 Statistical analysis

All experiments and analysis were performed in triplicate. For each run using different media, multiple one-way analyses of variance (ANOVA) were conducted to investigate the effect of different heating pretreatments on ABE production. ABE concentration, ABE productivity, ABE yield, cell concentration and cell yield were measured and calculated accordingly for comparison after completion of the fermentation process.

5.3 Results and Discussion

5.3.1 Growth kinetics

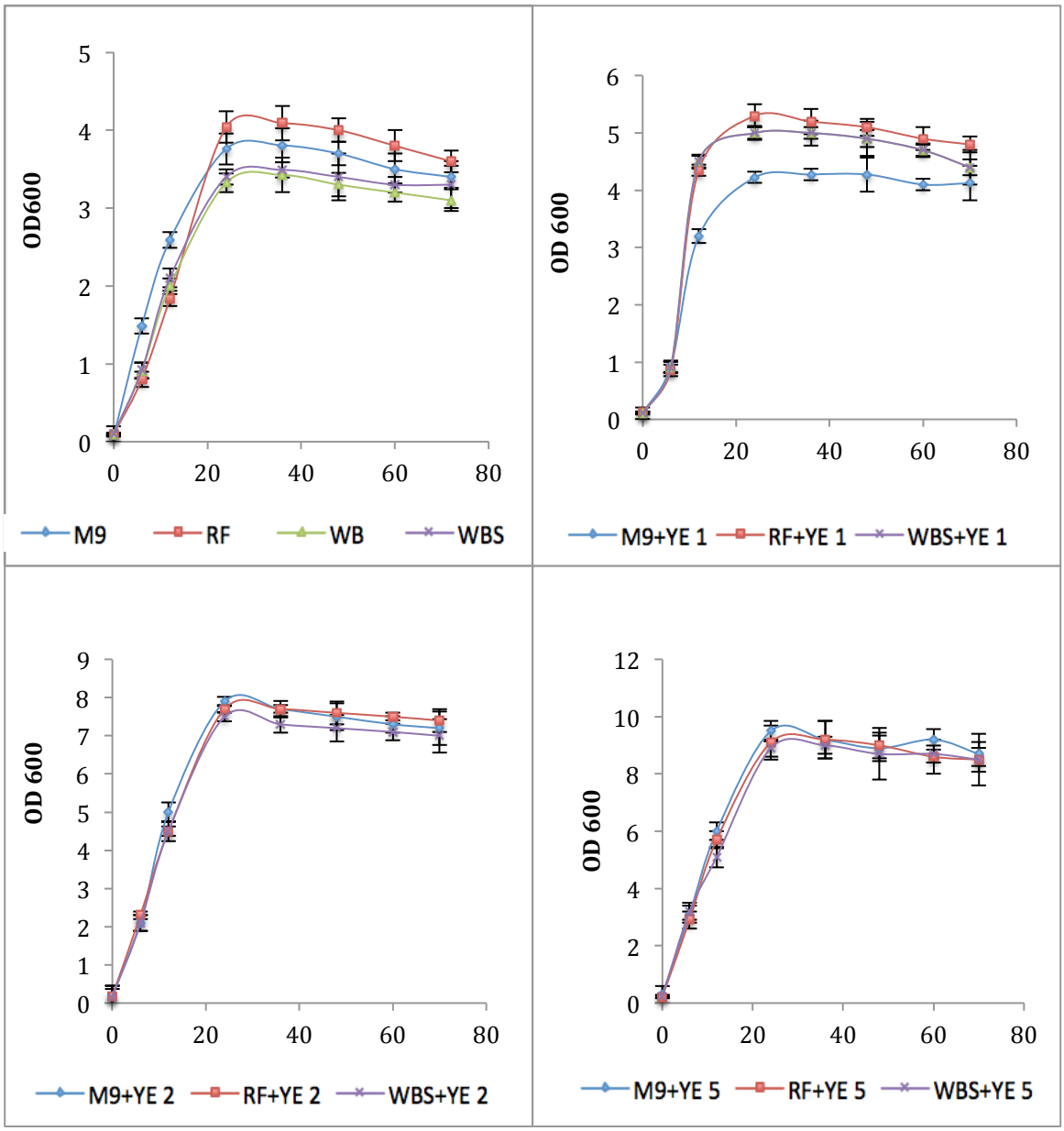


Figure 31 Optical density (OD_{600}) at certain time points during fermentation process

Clostridium. beijerinckii 8052 was grown on four kinds of medium, including SW hydrolysate after pretreated by RF heating (RF), SW hydrolysate after pretreated by WB heating (WB), equal glucose content of SW hydrolysate after pretreated by WB heating (WBS), and P2 medium as control (M9). The maximum optical density (OD_{600}) for RF medium was around 4.23 where the stationary phase was reached at approximately 24 h post induction (Figure 32). Both of the WB and WBS samples reached a maximum OD_{600} around 3.3. SW hydrolysate after pretreatment by RF heating as medium obtained best cell growth, followed by the control group, and then by the WB heating. However, the increasing rate of cell growth in the WB was larger than that in the RF before 12 hours. For SW hydrolysate after WB heating, there is no significant difference whether added carbon source until equal to that after RF heating. Cultures were allowed to continue growing until 72 h as this allowed time for ABE product accumulation.

Similar cell growth was observed on medium with the addition of yeast extract. The maximum optical density (OD_{600}) for RF+YE 1 medium was around 5.3, and the stationary phase was reached at approximately 24 h post induction. Both of the WB+YE and WBS+YE samples reached a maximum OD_{600} of almost 5. However, the difference between RF+YE and WB+YE was much smaller, compared to the medium without yeast extract. Moreover, with the larger amount of yeast extract addition, the OD_{600} increased, meanwhile the difference between RF and WB pretreated hydrolysate was becoming nearly zero.

5.3.2 ABE fermentation for pretreated Switchgrass hydrolysate

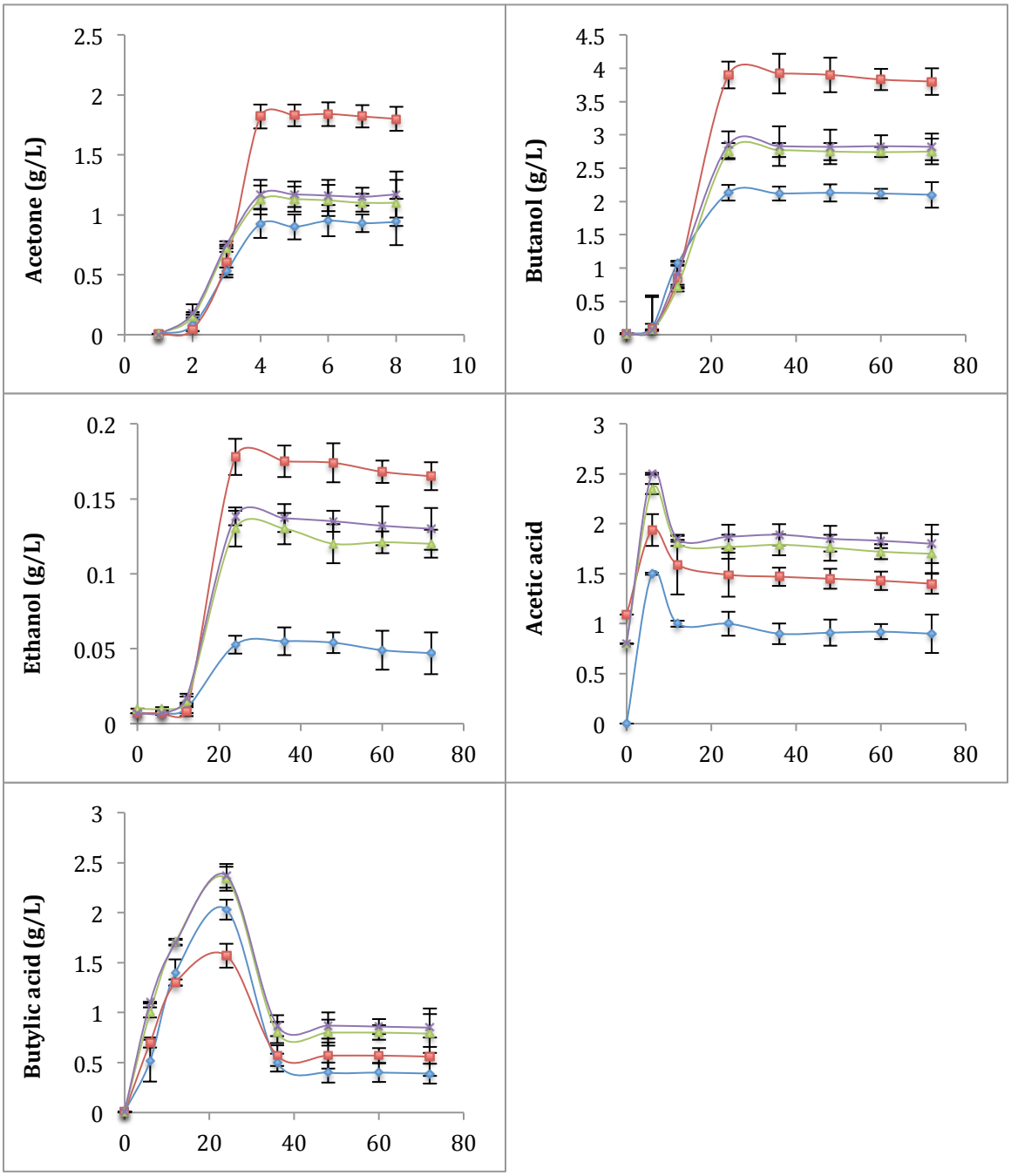


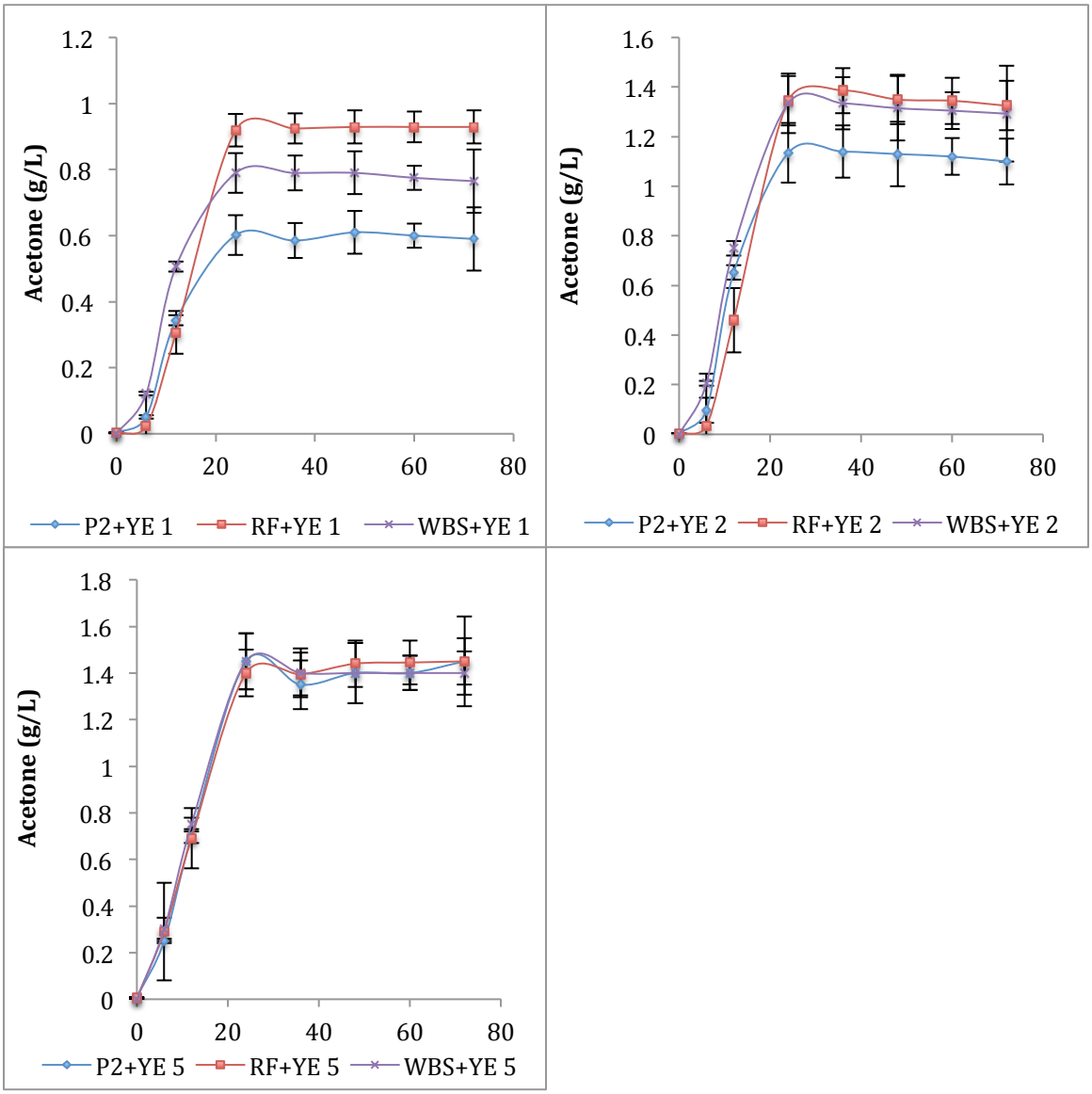
Figure 32 ABE fermentation profiles of the *C. beijerinckii* (Blue, red, green and purple stand for P2, RF, WB and WBS media, respectively; the horizontal axis represents for time)

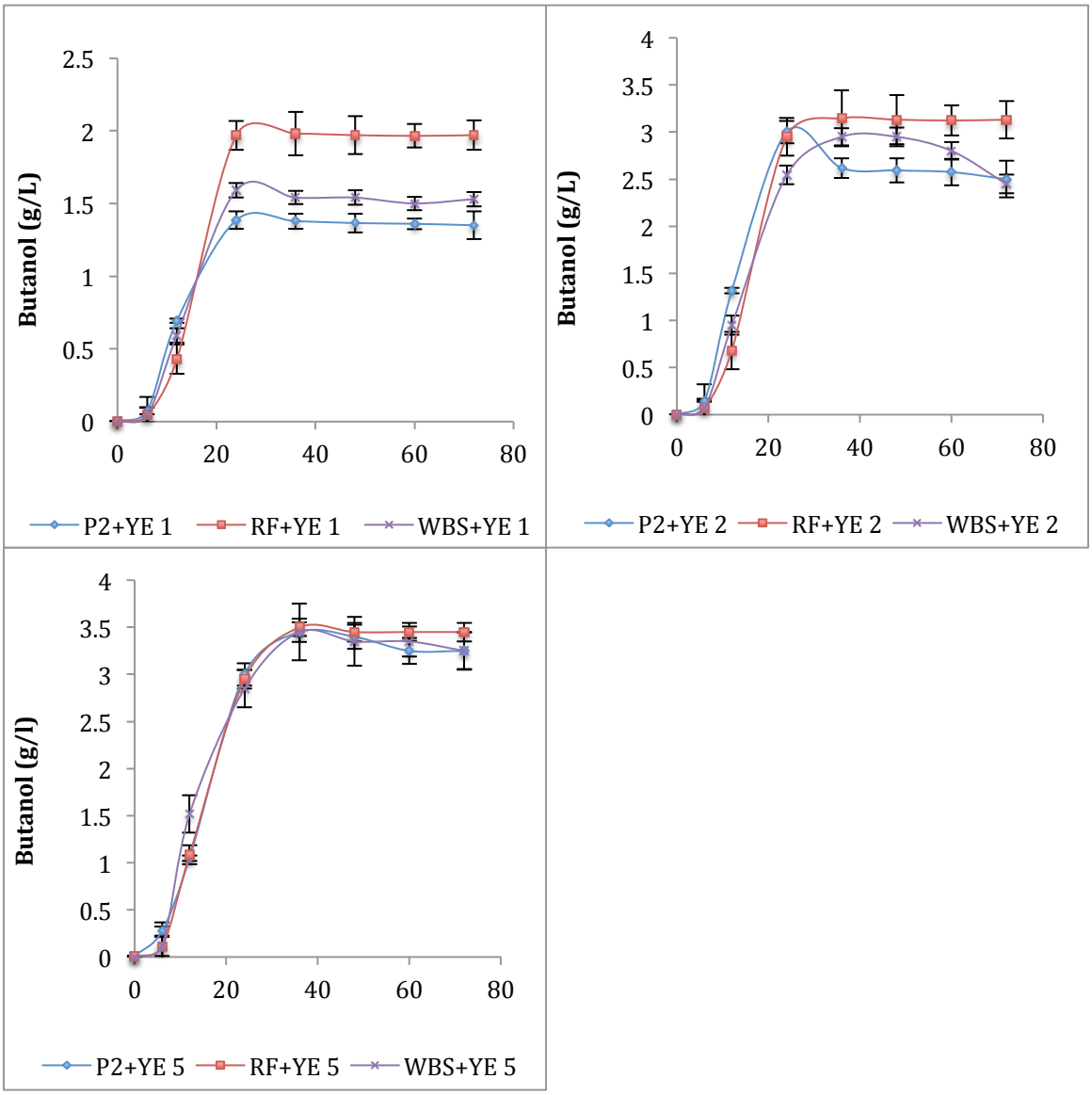
	Acetone (g/L)	*Butanol (g/L)*	*Butanol yield (g/gGlu)*	*Ethanol (g/L)*	*ABE (g/L)*	*ABE yield (g/g Glu)*
P2	0.47	1.06	0.07	0.03	1.56	0.10
RF	0.92	1.95	0.13	0.09	2.96	0.20
WB	0.56	1.38	0.09	0.06	2.00	0.13
WBS	0.59	1.41	0.09	0.07	2.07	0.14

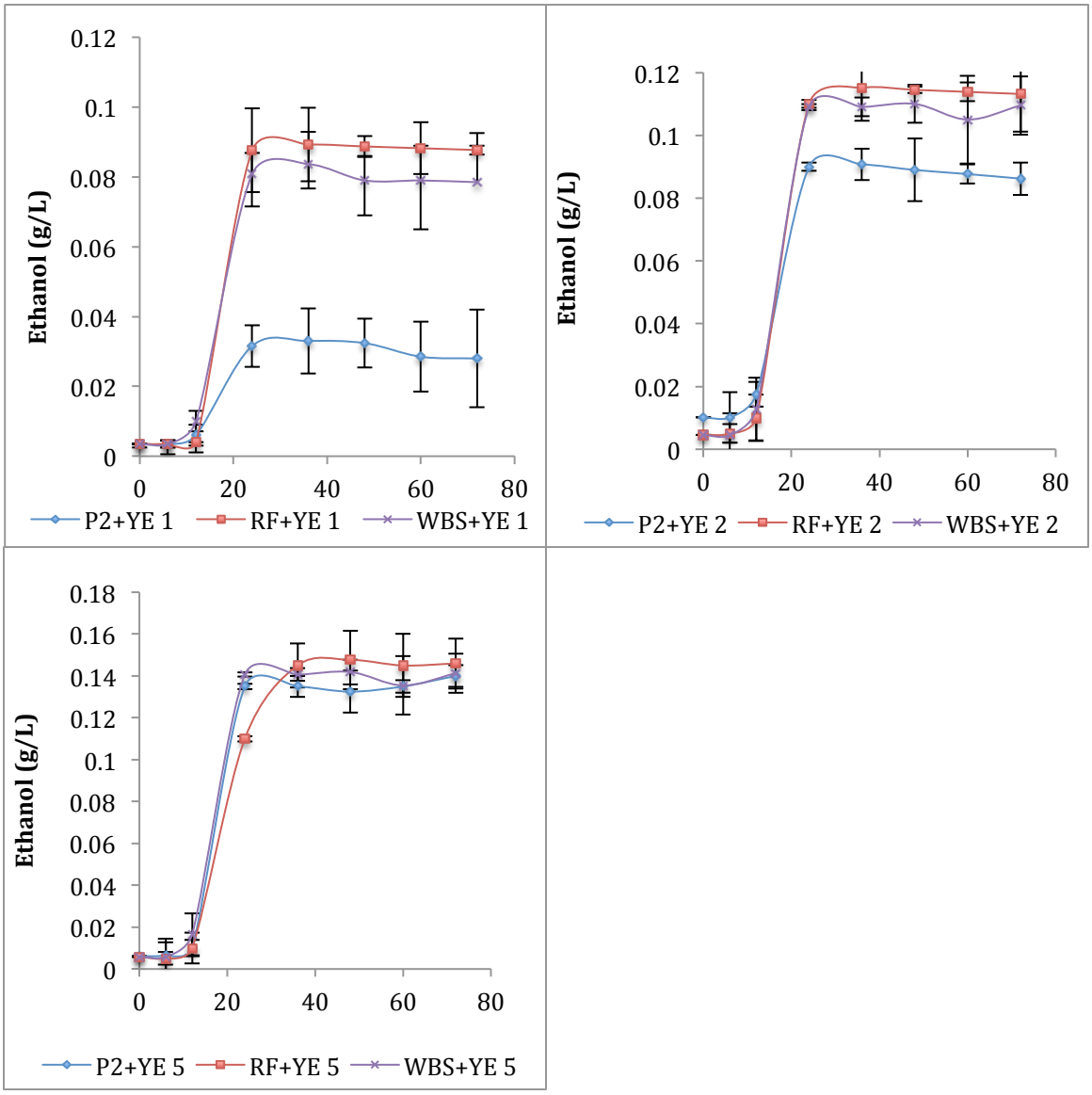
Table 7 Summary of fermentation results for the *C. beijerinckii* in different medium

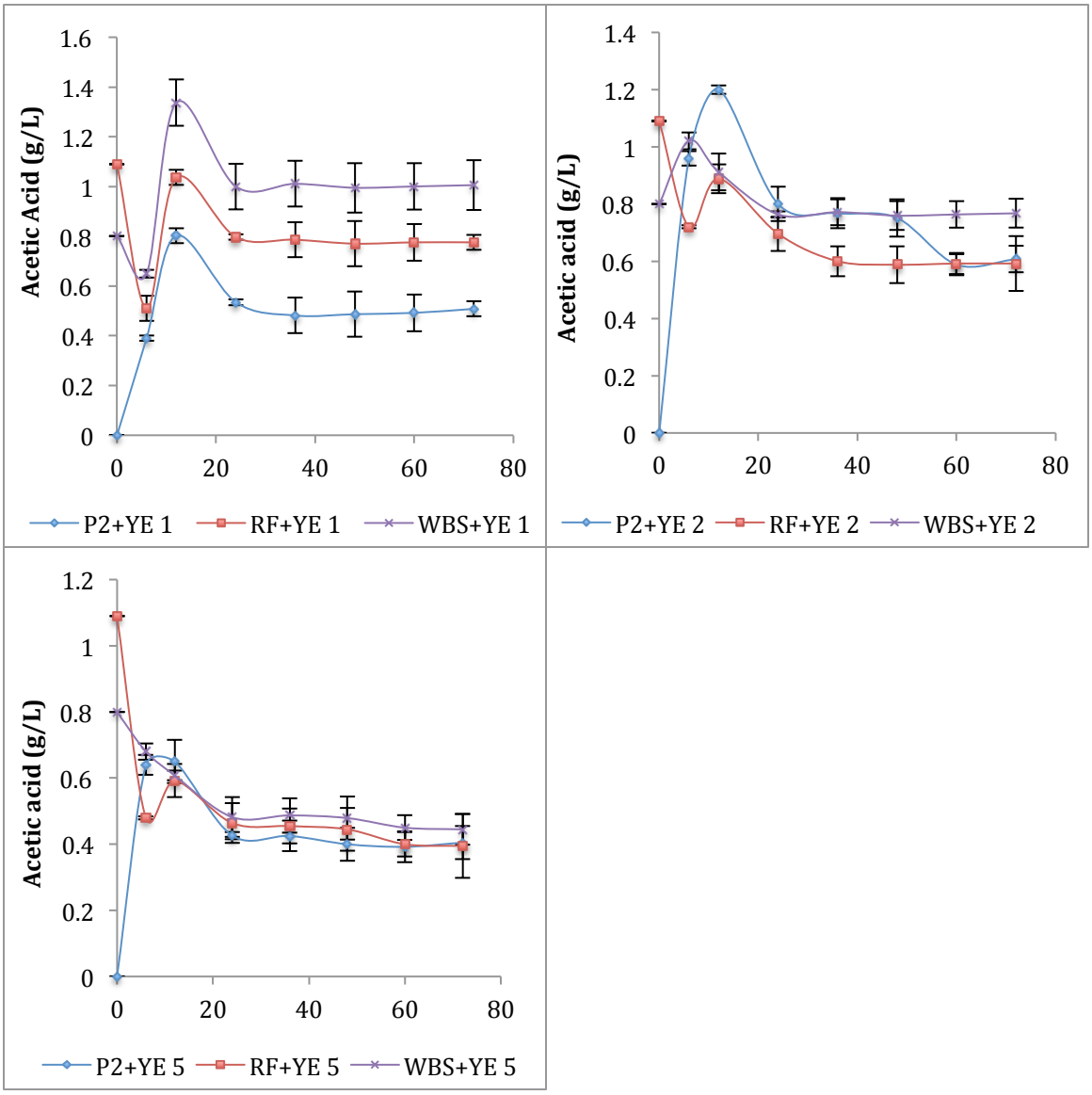
Switchgrass hydrolysate was used as the C-source for ABE fermentation. During fermentation, cells were sampled to analyze ABE products at certain time points in order to scale up into industrial application. For all of these four media, ABE products reached the maximum concentration (around 2.96g/L) in switchgrass hydrolysate after alkaline pretreatment assisted by radio frequency heating, followed by the switchgrass hydrolysate after traditional heating, and then the least in the control group. Moreover, for SW hydrolysate after WB heating, there is no significant difference whether added carbon source until equal to that after RF heating. For ABE yield, it has the similar trend for four media as that of ABE concentration: RF > (WB ≈ WBS) > Control.

5.3.3 ABE fermentation for pretreated Switchgrass hydrolysate with addition of yeast extract




Butanol (g/L)
2.5
2
1.5
1
0.5
0
0
20
40
60
80
P2+YE 1
RF+YE 1
WBS+YE 1
Butanol (g/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
P2+YE 2
RF+YE 2
WBS+YE 2
Butanol (g/l)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
P2+YE 5
RF+YE 5
WBS+YE 5


Ethanol (g/L)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
P2+YE 1
RF+YE 1
WBS+YE 1
Ethanol (g/L)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
P2+YE 2
RF+YE 2
WBS+YE 2
Ethanol (g/L)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
P2+YE 5
RF+YE 5
WBS+YE 5


Acetic Acid (g/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
P2+YE 1
RF+YE 1
WBS+YE 1
Acetic acid (g/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
P2+YE 2
RF+YE 2
WBS+YE 2
Acetic acid (g/L)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
P2+YE 5
RF+YE 5
WBS+YE 5

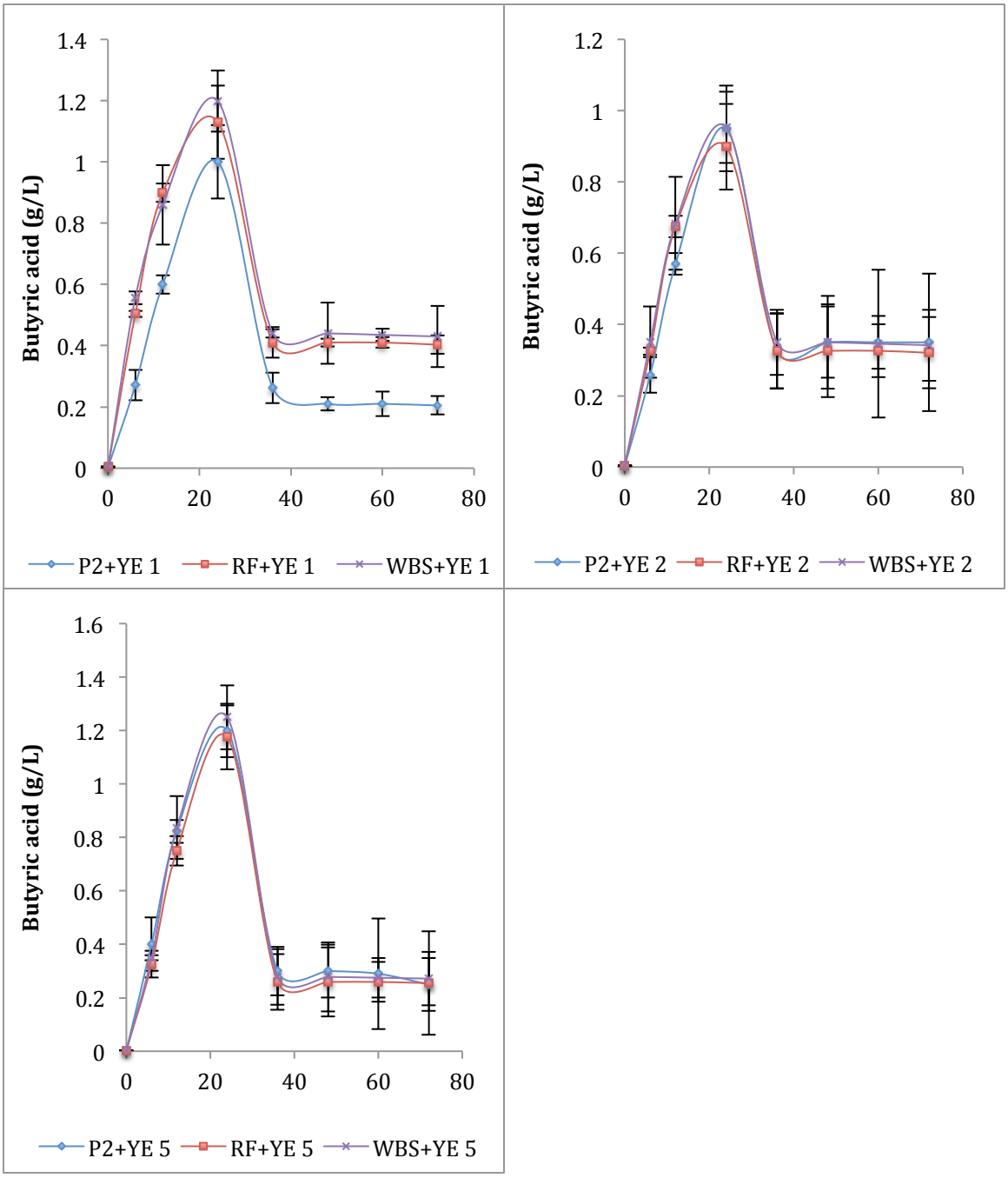


Figure 33 ABE fermentation profiles of the *C. beijerinckii* with addition of yeast extract (The horizontal axis represents for time)

	Acetone (g/L)	*Butanol (g/L)*	*Butanol yield (g/gGlu)*	*Ethanol (g/L)*	*ABE (g/L)*	*ABE yield (g/g Glu)*
P2+YE 1	0.59	1.35	0.09	0.03	1.97	0.13
RF+YE 1	0.93	1.97	0.13	0.09	2.99	0.20
WBS+YE 1	0.77	1.53	0.10	0.08	2.38	0.16
P2+YE 2	1.10	2.50	0.17	0.09	3.69	0.25
RF+YE 2	1.33	3.13	0.21	0.12	4.57	0.30
WBS+YE 2	1.30	2.88	0.19	0.11	4.28	0.29
P2+YE 5	1.45	3.25	0.22	0.14	4.84	0.32
RF+YE 5	1.45	3.45	0.23	0.15	5.05	0.34
WBS+YE 5	1.40	3.25	0.22	0.14	4.79	0.32

Table 8 Summary of fermentation results for the *C. beijerinckii* in different medium with addition of yeast extract

Switchgrass hydrolysate with addition of yeast extract was used as the carbon and nitrogen source for ABE fermentation. During the fermentation process, samples were taken to analyze ABE products. Among the four kinds of media with the addition of yeast extract, ABE products reached the maximum concentration at around 5.05 g/L in switchgrass hydrolysate with 5g/L yeast extract pretreated by alkaline assisted by radio frequency heating. Moreover, for SW hydrolysate after WB heating, there is no significant difference whether added carbon source and other carbon source until equal to that after RF heating. However, compared to the four media without yeast extract, the difference of the hydrolysate between radio frequency heating and water bath heating was smaller, probably because of the addition of yeast extract.

5.3.4 Comparison of ABE fermentation between RF and WB pretreated Switchgrass hydrolysate

Several methods have been extensively used to detect and quantify small carboxylic acids and furans in pretreated biomass and biomass hydrolysates, among which are High-Performance Liquid Chromatography (HPLC) (Zha et al., 2014). In our study, we test the pretreated Switchgrass using HPLC, results showed a big difference in acetic acid. Pretreated Switchgrass liquor by radio frequency heating achieved a 2.19g/L acetic acid content, while that of traditional water bath heating was 1.58g/L.

For acetic acid, it is the most commonly used carboxylic acid found after pretreatment. It is reported that acetic acid is formed by the hydrolysis of the acetyl groups linked to the sugar or other linkage present in hemicellulose backbone. The formation cannot be prevented due to its inherent to hemicellulose hydrolysis. And its formation mainly depends on the temperature and residence time of pretreatment until the acetyl groups are fully hydrolyzed. In this study, the acetic acid content in alkaline pretreated Switchgrass after RF heating is higher than that of WB heating. It can be hypothesized that RF heating hydrolyzed more acetyl groups linked to the hemicellulosic backbone. During alkaline pretreatment, the hemicellulose in Switchgrass is hydrolyzed. Hemicelluloses can be degraded into xylose, mannose, acetic acid, citric acid, formic acid, galactose, glucose, etc. Chang and Holtzapple (2000) have reported that the alkaline pretreatment process removes acetyl and uronic acid groups present on hemicellulose and thus enhances the accessibility of the enzyme that degrades hemicellulose. In the alkali treatment process, ester linkages that join xylan and hemicelluloses residues are also

hydrolyzed (Sun and Cheng 2002). In our experiment, the radio frequency heating method showed a better solubilization of the biomass. The similar explanation can be found in 4.3.3. Moreover, during the ABE fermentation process, the cell metabolism is affected by weak acids and other toxicities. For example, weak acids inhibit fermentation either by uncoupling of cell metabolism or intracellular anionic accumulation at certain range conetents. However, Ezeji et al. (2007) reported that a concentration of less than 1.98 g/L of acetates, furfural, and HMF did not inhibit growth or butanol production, particularly with *C. beijerinckii* BA101. As studied in this research, low concentrations of acetic acid benefit for the ABE fermentation. Therefore, ABE concentration was higher utilizing alkaline pretreated switchgrass assisted by radio frequency heating than that by traditional heating. However, the effect of acetic acid on growth and metabolism of *Clostridium berjerinckii* cultures should be investigated.

For the CHNS analysis, the nitrogen content showed significant difference between RF and WB heated switchgrass hydrolysate, in the amounts of 1.19 and 0.92, respectively. Therefore, there were more nutrients in RF pretreated hydrolysate, which could offer more cell growth and produce more ABE solvent, and the results in turn verified the fermentation for solvent production results. Li et al. (2012) reported that by adding yeast extract into cassava meal medium, a phase shift was triggered and fermentation performances were consequently improved. Total butanol concentrations/butanol productivities increased 15% in traditional fermentation compared to those with cassava substrate alone, and even reached the equivalent levels of those using corn substrate. Madihah et al. (2001) reported that the use of a mixture of

organic and inorganic nitrogen source (yeast extract +NH_4NO_3) enhanced the growth of *Clostridium acetobutylicum*, starch hydrolysis and solvent production compared to the use of yeast extract only. It is well known that organic nitrogen sources such as yeast extract provide various amino acids, vitamins, minerals and growth factors that promote good growth of microorganisms. Moreover, they drew a conclusion that the absolute concentrations of the distinct nutrients were more important factors for maximizing direct fermentation of starch to solvents. Roos et al. (1985) reported that mostly acids were produced under glucose limitation, but solvents were produced under nitrogen limitation. They believe that the nitrogen availability of organic nitrogen. In the switchgrass hydrolysate, it was not hard to say there were some kinds of unknown inorganic and organic nitrogen sources shown in CHNS analysis. In our study, there was a direct relationship between the yeast extract and the solvent production. The higher the yeast extract, the higher amount of solvent production, as well as the lower difference between RF and WB heated Switchgrass hydrolysate.

5.4 Conclusion

Pretreatment of biomass is an extremely significant step in a commercial biorefinery, and fundamental understanding and comparison of various pretreatment processes is essential. In this study RF and WB assisting alkaline pretreatments were carried out on switchgrass. The acetic acid generated during pretreatment was studied. The potential of switchgrass hydrolysate as media for ABE fermentation was highlighted in this study. Switchgrass hydrolysate, especially the one pretreated by radio frequency is optimal for achieving high ABE concentration. It can be concluded that at a certain range

of concentration, acetic acid is better for cell growth. Moreover, the larger amount of nitrogen source in RF pretreated switchgrass hydrolysate probably was better for *Clostridium beijerinckii* growth and solvent production. It is expected that the results of this study can subsequently be applied to enhance the conversion of lignocellulosic biomass for ABE fermentation.

Chapter 6 Conclusions and future work

6.1 Summary of results

During this study, radio frequency (RF) assisted dielectric heating was utilized in the alkaline (NaOH) pretreatment for lignocellulosic biomass. The substrates ranged from agricultural residues (corn stover), herbaceous crops (switchgrass), and hardwood (sweetgum) to softwood (loblolly pine). Pretreatment was performed at 90℃ for both RF and traditional water bath (WB) heating for one hour after overnight soaking in NaOH solution (0.2g NaOH/g biomass). The results obtained from this research are listed as follows:

1) Pretreated materials were characterized by chemical compositional analysis, enzyme hydrolysis, scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The glucan yield from hydrolysis yield using RF heating method for these four feedstocks were 89.6%, 72.6%, 21.7% and 9.9%, respectively. While the yields using conventional heating technique were 89.4%, 51.8%, 19.6% and 9.8%, respectively. Interestingly, RF heating raised glucan yield significantly on switchgrass and sweetgum, but less so on corn stover and loblolly pine. The SEM images and FTIR spectra confirmed results from composition analysis and enzyme hydrolysis.

2) The practicality of using switchgrass hydrolysate medium to grow recombinant *E-coli* utilizing pBHR68 plasmid for production of polyhydroxybutyrate (PHB), a biodegradable plastic, was explored in this study. RF, WB, WBS and M9 medium (control group) were used as culture media. The acetic acid concentration of pretreated switchgrass after radio frequency heating was 2.19g/L compared to that of traditional water bath heating at 1.58g/L. RF was shown to be optimal for PHB

concentration produced, with final dry cell weight (DCW) of 6.30 ± 0.11 g/L, and PHB concentration of 2.25 ± 0.13 g/L. However, with the addition of yeast extract, the differences between RF and WB heated hydrolysates diminished. Moreover, the larger the amount of yeast extract addition, the lower difference between RF and WB media.

3) RF, WB, WBS and M9 medium (control group) were used as culture media for acetone-butanol-ethanol (ABE) fermentation by *Clostridium beijerinckii*. The hydrolysate was used after pH adjustment without sediments removal for ABE fermentation. The acetic acid of pretreated switchgrass after radio frequency heating was 2.19g/L compared to that of traditional water bath heating at 1.58g/L. And the citric acid of pretreated Switchgrass after radio frequency heating was nearly zero, while that of traditional water bath heating at 0.59 g/L. After fermentation, ABE reached the maximum in RF with butanol concentration 3.9 g/L and total ABE concentration 5.91 g/L, corresponding to the maximum ABE yield (0.45) in the first medium, indicating that the enzymatic hydrolysates after alkaline pretreatment assisted by radio frequency was best for *Clostridium. beijerinckii* growth. However, the difference of the switchgrass hydrolysate between radio frequency and traditional water bath heating was smaller with the addition of yeast extract, which verified that the radio frequency probably broke down into some nutrients in favor of ABE fermentation.

6.2 Conclusion

Results showed differences between radio frequency and traditional water bath heating assisted alkaline pretreatment on different lignocellulose biomass. We drew several conclusions as follows:

1) Different biomass should be dealt with different pretreatment methods. Alkaline pretreatment works better on corn stover and swtichgrass, than on softwood species.

2) Radio frequency heating conditions are harsher compared to traditional water bath heating, causing a greater breakdown in functional groups and bonds. Because more small molecules were detected (e.g., acetic acid, hydroxyl-acetaldehyde, $-NH_2$ group etc.), RF resulted not only in better physical and chemical characteristics, but also in the next step of enzyme hydrolysis, which was further beneficial for fermentation.

6.3 Recommendations for future research

The mechanism of alkaline pretreatment on lignocellulosic biomass has not been fully understood. Further, radio frequency based pretreatment is still unclear. What is the difference in the heating mechanism between radio frequency heating and traditional water bath heating? How do these differences affect alkaline pretreatment of lignocellulosic biomass? What are the major functional groups and the primary chemical bonds being broken down through radio frequency heating assisted alkaline pretreatment? Can we quantify the interaction between radio frequency heating and these components and bonds? Why does the glucose yield show higher values after enzyme hydrolysis through radio frequency heating than that by traditional water bath heating? How do acetic acid and other "inhibitors" form during pretreatment? Do they inhibit enzymatic hydrolysis and fermentation? How do they effect PHB production and ABE fermentation? Do they have any effect on the PHB and ABE pathway inside cell body? How yeast extract affect the cell growth for *E-coli* and *C. beijerinkii*? Especially for

ABE fermentation, how organic and inorganic nitrogen sources affect the cell growth and solvent production?

These questions should be further investigated and elaborated in order to better understand the mechanism of alkaline pretreatment of LCB, as well as the principle of radio frequency heating technology. It will be important to further confirm the mechanism of acetic acid, as well as the mechanism of inorganic and organic nitrogen source for PHB production and ABE fermentation.

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