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Effect of Poultry Litter Biochar on *Saccharomyces cerevisiae* Growth and Ethanol Production from Steam-Exploded Poplar and Corn Stover

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EFFECT OF POULTRY LITTER BIOCHAR ON *SACCHAROMYCES CEREVISIAE* GROWTH

AND ETHANOL PRODUCTION FROM STEAM-EXPLODED POPLAR AND CORN

STOVER

by

Oumou Diallo

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Biological Engineering

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UTAH STATE UNIVERSITY
Logan, Utah

2014

ABSTRACT

Effect of Poultry Litter Biochar on *Saccharomyces cerevisiae* Growth and Ethanol

Production from Steam-exploded Poplar and Corn Stover

by

Oumou Diallo, Master of Science

Utah State University, 2014

Major Professor: Dr. Foster A. Agblevor
Department: Biological Engineering

The following thesis is a three-part study, investigating the effect of poultry litter biochar on the growth of *Saccharomyces cerevisiae* and the ethanol production from steam-exploded poplar and corn stover. The first part of this study showed the effect of poultry litter biochar on the aerobic and anaerobic growth of *Saccharomyces cerevisiae* ATCC 204508/S288C. The second part focused on the effect of poultry litter biochar on the enzyme hydrolysis and fermentation of two different steam-exploded biomasses: poplar (0.25 M sodium hydroxide washed poplar, and unwashed poplar) and corn stover. The third part investigated optimal process parameters (biochar loading, biomass loading, and enzyme loading) on the reducing sugars production and ethanol yield from steam-exploded corn stover. The results obtained from the first part showed that *S. cerevisiae* can grow on the biochar medium under both aerobic and anaerobic growth conditions. The results in the second part showed that poultry litter biochar addition to steam-

exploded biomass improved the ethanol productivity of steam-exploded poplar up to a maximum of 3.20 g/l-h at 5% biochar loading, and the ethanol productivity of steam-exploded corn stover up to a maximum of 2.02 g/l-h at 1 % biochar loading. The results from the parametric study showed that biochar loadings had a significant effect on the ethanol yield (p-value = 0.0072), but the effect on the enzyme hydrolysis was not significant. At the optimal conditions of biochar loading (5%), biomass loading (15%), and enzyme loading (10 FPU/g^{-1}), the ethanol yield was 73.44%, which was 19.46% more than the non-optimized control at zero-level central point.

(145 pages)

PUBLIC ABSTRACT

Effect of Poultry Litter Biochar on *Saccharomyces cerevisiae* Growth and Ethanol

Production from Steam-exploded Poplar and Corn Stover

The use of ethanol produced from lignocellulosic biomass for transportation fuel offers solutions in reducing environmental emission and the use of non-renewable fuels. However, lignocellulosic ethanol production is still hampered by economic and technical obstacles. For instance, the inhibitory effect of toxic compounds produced during biomass pretreatment was reported to inhibit the fermenting microorganisms, hence there was a decrease in ethanol yield and productivity. Thus, there is a need to improve the bioconversion of lignocellulosic biomass to ethanol in order to promote its commercialization. The research reported here investigated the use of poultry litter biochar to improve the ethanol production from steam-exploded poplar and corn stover. The effect of poultry litter biochar was first studied on *Saccharomyces cerevisiae* ATCC 204508/S288C growth, and second on the enzyme hydrolysis and fermentation of two steam-exploded biomasses: (poplar and corn stover). The third part of the study investigated optimal process parameters (biochar loading, biomass loading, and enzyme loading) on the reducing sugars production, and ethanol yield from steam-exploded corn stover. In this study, it has been shown that poultry litter biochar improved the *S. cerevisiae* growth and ethanol productivity; therefore poultry litter biochar could potentially be used to improve the ethanol production from steam-exploded lignocellulosic biomass.

Oumou Diallo

ACKNOWLEDGMENTS

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CHAPTER 1

LITERATURE REVIEW

1.1. Ethanol for fuel

The use of ethanol as a transport fuel dates back to the origin of the automobile industry, for example, the early Henry Ford's Model T vehicle built in 1908 had a flexible carburetor that could be adjusted to run on either gasoline or ethanol (Rosillo-Calle and Walter, 2006; Zaldivar et al., 2001). Ford's vision was to build a vehicle powered by fuel ethanol that was not only affordable to the working family, but also as a means of boosting the rural farm economy (Bothast and Schlicher, 2005; Kovarik, 1998). This vision shared by Henry Ford, Charles F. Kettering, and many others in the automotive industry aimed at making ethanol from farm products and cellulosic materials as the fuel of the future (Kovarik, 1998) and as a result in the 1930s ethanol was utilized as a fuel source for cars in the United States. However, due to the abundance and low cost of petroleum and natural gas, the interest in ethanol as fuel remained low at that time (Bothast and Schlicher, 2005).

In the 1970s, there was renewed interest in ethanol because of the disruption of oil supply from the Middle East, increases in oil prices, concerns over fossil fuel depletion, and global climate change generated by the massive use of fossil fuels (Bothast and Schlicher, 2005). Ethanol was recognized as a potential sustainable alternative transportation fuel to fossil fuels mainly because it generates fewer emissions, no net CO_2, and is compatible with the current fuel distribution (González-García et al., 2010; Parawira and Tekere, 2011).

A number of studies have been conducted to investigate ethanol production from different substrates. The first generation ethanol was mainly from sugar cane in Brazil and corn starch in the USA (Bothast and Schlicher, 2005; Lennartsson et al., 2014; Solomon et al., 2007). However the use of human food such as corn and sugar cane as feedstock for fuels has led to considerable debates and is viewed as unethical (Lennartsson et al., 2014). Consequently, alternative substrates such as lignocellulosic biomass have been investigated for ethanol production. Research have shown that lignocellulosic biomass is an attractive potential renewable, non-food, and available feedstock for ethanol production (Alvira et al., 2010; González-García et al., 2010; Himmel et al., 2007; Zaldivar et al., 2001).

1.2. Lignocellulosic Biomass

Lignocellulosic biomass refers to plant biomass and usually classified as agricultural residues (e.g. corn stover, sugarcane bagasse, and rice straw) forestry residues and energy crops (e.g. switch grass, miscanthus, poplar, and willow). Lignocellulose biomass is composed of carbohydrate polymers: cellulose, hemicellulose, and lignin. Several decades of research has shown that lignocellulosic materials are a promising feedstock for the production of ethanol because they are low-cost, non-food material, renewable, and readily available raw material (Aden et al., 2002; González-García et al., 2010; Prasad et al., 2007; Sannigrahi et al., 2010). Zaldivar et al.(2001) reported that lignocellulose accounts for about 50% of the biomass in the world. In United States for example, the annual production of agricultural residue is estimated at 355 million metric dry tons including 200 million tons of corn stover and 70 million tons of cereal straw (Zambare and Christopher, 2012).

1.2.1. Poplar

Poplar is an energy crop cultivated in North America, and Europe. Poplar was reported as a promising feedstock for cellulosic ethanol due to its short rotation, fast and widespread growth area, and high productivity on the marginal lands (González-García et al., 2010; Kim et al., 2012; Luo et al., 2002; Sannigrahi et al., 2010). The yield of hybrid poplar species is estimated to be 14 Mg ha^{-1} year^{-1} in North America (Sannigrahi et al., 2010). Studies have shown that poplar is an attractive lignocellulosic biomass for the bioethanol production because it is readily available and has the following composition: cellulose (42-49%), hemicellulose (16-23%), and lignin (21-29%) (Sannigrahi et al., 2010).

1.2.2. Corn stover

According to the USDA (2002), corn is the most widely planted crop in United States (US). In 2009, US produced 41.9% of world corn (Ferguson, 2003; Zambare and Christopher, 2012). Corn stover is the residue after harvesting the corn grain; it is the non-grain part such as stalk, leaf, husk, and cob (Zambare and Christopher, 2012). Kadam and McMillan (2003) reported that approximately 60–80 million dry t/yr of corn Stover are potentially available for ethanol production. In addition to the availability, corn stover is a low cost agricultural residue and is composed of cellulose (32.4–37.4 %), hemicellulose (18.5–21.8%) and lignin (11.2–18 %) which makes it a potential feedstock for ethanol production (Aden et al., 2002; Weiss et al., 2010).

1.2.3. Cellulose

Cellulose is a major component of the plant cell wall, it was first discovered by

the French scientist Anselme Payen and its chemistry has been widely studied (Klemm et al., 2005). Cellulose has unique physical properties, it is a homogenous glucose polymer which has a linear structure consisting of 1000 to 1 million D-glucose units, linked by β-1, 4 glycosidic bonds, and composed of repeating unit of two glucose anhydride units called cellobiose (Robyt, 1998).

Mondragon et al.(2014) reported that cellulose is the most abundant organic material on earth and represents an important industrial polysaccharide due to its advantages and properties, such as biodegradability, recyclability, renewability, and biocompatibility. The cellulose present in lignocellulosic biomass is composed of crystalline and amorphous components, and is protected by the lignin which forms a barrier, and inhibits cellulose from degradation. Studies have shown that during the enzyme hydrolysis of lignocellulosic biomass, the amorphous component degrades more easily than the crystalline fraction, and the enzyme hydrolysis of cellulose with a high crystalline structure would result in lower enzyme accessibility, hence, lower sugar yield (Zhang et al., 2014).

1.2.4. Hemicellulose

Hemicellulose is a polymer also found in the plant cell wall. Unlike cellulose, hemicellulose is a more complex and heterogeneous polymer of pentose (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (Saha, 2003). Hemicellulose is linked to cellulose by covalent bonds (mainly α-benzyl ether linkages) and to lignin by ester linkage with acetyl units and hydroxycinamic acids, which restrict its liberation from the cell wall matrix (Gabrielii et al., 2000; Peng et al., 2010; Ren and Sun, 2010).

Studies have shown that the interactions of hemicellulose with cellulose affect many industrial processes, for example, production of cellulose, papermaking, and bioethanol production. The presence of hemicellulose on cellulose fiber surfaces has been shown to enhance the strength properties of the fiber network (Ren and Sun, 2010). Different methods such as alkaline extraction, alkaline peroxide extraction, and steam explosion extraction have been used to isolate hemicellulose from the plant cell wall (Alvira et al., 2010; Gáspár et al., 2007).

1.2.5. Lignin

Lignin is the third most abundant polymer found in all terrestrial plants. Lignin is made of monomers of phenyl propanoid building units. Unlike, cellulose and hemicellulose, lignin is a polymer arranged in a 3-dimensional network made of p-hydroxyphenyl propanoid units, and connected by C-C and C-O-C links (Morreel et al., 2010; Ralph et al., 2004). The structure and exact composition of lignin is still not fully determined due to its complexity (Fengel and Wegener, 1983; Ralph et al., 2004).

Lignin provides rigidity to vascular plants, and protects cellulose and hemicellulose from attacks from other organisms. For example lignin is extremely resistant to chemical and enzymatic degradation (Hammel, 1997). Lignin is not water soluble, and optically inactive which makes its degradation very difficult (Hendriks and Zeeman, 2009). However, lignin is soluble in aqueous alkali solutions due its alcohol precursors (Hammel, 1997). As a result, alkali hydrogen peroxide and sodium hydroxide have been recently used as a pretreatment process for the conversion of lignocellulosic biomass feedstock into biofuel (Alvira et al., 2010; Gupta and Lee, 2009 Zhang et al., 2010).

1.3. Pretreatment

Pretreatment of lignocellulosic biomass is the first step in bioethanol production. Studies show that pretreatment is an essential step for obtaining potentially fermentable sugars in the hydrolysis step (Alvira et al., 2010). The purpose of pretreatment is to increase accessibility to cellulose and the hemicellulose polymer, and disrupt the crystalline structure of cellulose (Alvira et al., 2010; Chiaramonti et al., 2012; Hendriks and Zeeman, 2009; Mosier et al., 2005; Zambare and Christopher, 2012). Lignocellulosic biomass pretreatment in general proceeds under high temperature, high pressure, acidity or alkality in order to alter its chemical composition and structure, and enhance the hydrolysis of the carbohydrate fractions into simple sugars (Chiaramonti et al., 2012; Mosier et al., 2005).

Several methods have been introduced for pretreatment of lignocellulosic materials prior to enzymatic hydrolysis. These methods are classified into biological (e.g. lignin degradation by white-rot fungi), physical (e.g. ball milling), chemical (e.g. dilute acid pretreatment), and physico-chemical (e.g. steam explosion, ammonia fiber explosion) (Alvira et al., 2010; Taherzadeh and Karimi, 2008; Zhang and Shahbazi, 2011). Pretreatment has been considered as the most expensive processing step in lignocellulosic ethanol processes, representing about 18% of the total cost (Yang and Wyman, 2008; Zhang and Shahbazi, 2011). Therefore, developing a cost-effective and efficient biomass pretreatment technology is necessary for efficient lignocellulosic biomass conversion to ethanol.

Taherzadeh and Karimi (2008) reported that the pretreatment should be efficient, minimize the carbohydrate degradation, and the formation of inhibiting byproducts, which can impede the progress of subsequent hydrolysis and fermentation processes.

Furthermore, the pretreatment should be economically feasible, such that the energy demand and the cost of pretreatment reactors construction are minimized.

1.3.1. Steam-explosion pretreatment

Steam-explosion is a physico-chemical process of pretreatment commonly used on lignocellulosic biomass. During the steam-explosion pretreatment, the biomass is subjected to a saturated steam at temperatures ranging from 160 to 260 ^{o}C and pressures of 0.69-4.83 MPa for a period of time, and then suddenly depressurized, which makes the materials undergo an explosive decompression (Alvira et al., 2010; Jeoh and Agblevor, 2001; Taherzadeh and Karimi, 2008). The most important factors affecting the steam-explosion are: the biomass particle size, operation temperature, and residence time (Alvira et al., 2010). Steam-explosion was reported as an effective method of pretreatment for lignocellulosic biomass compared to other pretreatment technologies. The advantages of steam explosion pretreatment include lower environmental impact, lower capital investment, more potential for energy efficiency, less hazardous process chemicals and conditions, and complete sugar recovery (Alvira et al., 2010; Avellar and Glasser, 1998).

However, steam-explosion pretreatment was also reported to lead to hemicellulose degradation, lignin transformation, and generation of some toxic compounds that affect the hydrolysis and fermentation steps (Alvira et al., 2010; Oliva et al., 2003). As a result of the formation of inhibitory compounds, some studies suggested that the steam-exploded biomass should be washed with water before fermentation in order to reduce the concentration of the inhibitory compounds. However, a loss of soluble reducing sugars was also observed after biomass washing (Cantarella et al., 2004b; Jeoh

and Agblevor, 2001; Öhgren et al., 2007). Cantarella et al., (2004b) and Lu et al., (2010) reported the main constituents of untreated and steam-exploded poplar and corn stover shown in Table 1.1. The glucan and lignin content increased in the steam-exploded biomass whereas, the xylan content slightly decreased or remained constant in the steam-exploded biomass.

1.3.2. Inhibitory compounds

Inhibitory compounds are reported as a major challenge to the commercial production of lignocellulosic bioethanol (Parawira and Tekere, 2011). The major lignocellulosic inhibitors are furan derivatives (Furfural and hydroxymethylfurfural (HMF)), weak acids, and phenolic compounds (Himmel et al., 2007; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000a; Parawira and Tekere, 2011; Taherzadeh and Karimi, 2011). Furfural and HMF are result of the dehydration of pentoses and hexoses respectively (Dunlop, 1948; Taherzadeh and Karimi, 2011; Ulbricht et al., 1984). Weak acids, such as acetic acid are produced by de-acetylation of hemicellulose, formic and levulinic acid are formed from HMF breakdown (Dunlop, 1948; Ulbricht et al., 1984). Phenolic compounds are generally generated due to the lignin breakdown (Parawira and Tekere, 2011).

Furfural was reported as a major fermentation inhibitor of many microorganisms used in fermentation. Its inhibitory effects varied depending on the concentration present in the medium and the microorganism used (Taherzadeh et al., 1999). Studies have shown that furfural at 1 g/l and above can affect yeast (*Saccharomyces cerevisiae)* metabolism by inhibiting its cells growth. In addition, furfural was reported to affect the glycolytic enzyme of alcohol dehydrogenase, which is responsible for converting

acetaldehyde to ethanol and as result decrease the rate of ethanol production (Palmqvist and Hahn-Hägerdal, 2000a; Taherzadeh and Karimi, 2011).

The effects of HMF on ethanol production by *S. cerevisiae* have also been reported (Almeida et al., 2008; Palmqvist and Hahn-Hägerdal, 2000b; Taherzadeh et al., 2000a). Taherzadeh et al., (2000a) and Mussatto and Roberto, (2004) observed that 1 g/l or higher of HMF can inhibit yeast fermentation and growth rate by prolonging its lag phase. Furthermore, a synergistic effect of furfural and HMF was also reported to inhibit the growth of yeast (Taherzadeh et al., 2000b).

High concentration of acetic acid, levulinic acid, and formic acid were also reported to inhibit the yeast and reduce the ethanol production (Larsson et al., 1999a). Acetic acid and levulinic acid were reported to lower the intracellular pH by diffusing into the cell cytoplasm. Formic acid was reported to be more toxic to yeast than both levulinic and acetic acid because it has a smaller size that facilitate its mass transport through the cell wall (Almeida et al., 2007; Parajó et al., 1998; Parawira and Tekere, 2011).

Phenolic compounds were also reported to be more toxic to microorganisms and enzymes than furfural and HMF (Mussatto and Roberto, 2004; Parajó et al., 1998). Palmqvist and Hahn-Hägerdal, (2000b) reported that phenolic compounds can decrease the rate of ethanol production, reduce cell growth, and sugar assimilation by affecting the biological membrane and inhibiting their ability to serve as a barrier and enzyme matrices. Parajó et al. (1998) also observed that xylose metabolism of *S. cerevisiae* was totally or partially inhibited when vanillin concentrations were 5 and 3.7 g/l, respectively in the wood hydrolysate. Cantarella et al. (2004b) reported the presence of several inhibitory compounds (weak acid, furan, and phenolic) in the steam-exploded poplar and

compared the concentration of the inhibitors of three samples including: unwashed slurry and two water washed samples. For the washed samples, the authors utilized two different volumes of water: 1.5 and 8 L and reported that the concentration of inhibitory compounds was higher in the unwashed slurry compared to the water washed biomass (Table 1.2). Additionally, washing the steam-exploded poplar with a larger volume of water (8 L) had reduced the concentration of inhibitory compounds by 10-1000 fold, however they also observed a removal of some soluble sugars.

Studies have shown the presence of weak acid, furan, and phenolic compounds in the steam-exploded corn stover (Huang et al., 2011 ; Öhgren et al., 2007) (Table 1.2). Öhgren et al. (2006; 2007) reported a lower concentration for acetic acid and furan because the biomass was washed with water after steam-explosion. While Huang et al., (2011) reported a higher concentration for acetic acid and formic acid, a lower concentration for HMF and furfural (below 1 g/L), and lower concentration for phenolic compounds in the unwashed pretreated corn stover (Table 1.2).

1.4. Enzyme Hydrolysis

Enzymatic hydrolysis of lignocellulosic biomass is a promising method for converting lignocellulosic biomass to fermentable sugars. Unlike acid hydrolysis, enzyme hydrolysis is carried out under mild conditions, is not toxic, and is environmentally friendly (Taherzadeh and Karimi, 2007b). Studies have shown that in order to increase the accessibility of the cellulose to enzymatic attack, lignocellulosic biomass should be pretreated prior to the enzymatic hydrolysis (Alvira et al., 2010; Taherzadeh and Karimi, 2007b). The enzyme hydrolysis of lignocellulosic biomass is carried out by highly specific cellulase enzymes which can break down the long chain of β-1$\rightarrow$4 glycosidic

Table 1.1. Composition of steam-exploded (SE) lignocellulosic biomass

Authors	(Cantarella et al., 2004b)		(Lu et al., 2010)	
Constituent	Poplar untreated (%)	SE poplar (%)	Corn stover Untreated (%)	SE corn stover (%)
Lignin	27.7	36.3	19.9	21.7
Glucan	48.9	52.2	36.9	38.6
Xylan	15.7	6.7	24.7	24.3
Arabinan	0.3	0.1	3.2	3.9
Galactan	0.3	0.3	1.7	2.4
Mannan	1	1.2	0.8	0.9
Ash	1.2	2.5	-	-

linkages of the cellulose polymer. Cellulase consists of three main groups of enzymes: endo-β-glucanase, exo-β-glucanase and β-glucosidase, collectively known as cellulases (Beguin and Aubert, 1994).

During the enzymatic hydrolysis process, cellulose is degraded by the cellulases to reducing sugars that can be further fermented by yeasts or bacteria to ethanol. Taherzadeh and Karimi (2007b) reported that the enzymatic hydrolysis of cellulose occurs in three steps: adsorption of cellulase enzymes onto the surface of the cellulose, biodegradation of cellulose to fermentable sugars, and desorption of cellulose. The important factors affecting the enzyme hydrolysis include: substrate concentration, cellulase activity, and reaction conditions (e.g. pH, temperature) The optimum temperature and pH of cellulase were reported to be in the range of 40°C to 50°C, and pH 4 to 5 respectively (Sun and Cheng, 2002; Taherzadeh and Karimi 2007b).

Table 1.2. Common inhibitory compounds and concentrations present in steam-exploded (SE) poplar and corn stover

Biomass	unit	acetic acid	formic acid	levulinic acid	Furfural	5-HMF	Vanillin	References
SE-poplar (unwashed slurry)	mg/g DW	27.8	11.2	0.79	5.9	2.6	0.35	(Cantarella et al., 2004b)
SE-poplar (washed with 1.5L di water)	mg/L	210.6	84.83	5.98	44.68	19.69	2.65	
SE-poplar (washed with 8L di-water)	mg/L	1.62	0.65	0.05	0.34	0.15	0.02	
SE. Corn Stover	g/L	2.6			0.6	0.7		(Öhgren et al., 2007)
SE. Corn Stover	g/L	7.81 ± 0.15	6.80 ± 0.1		0.71 ± 0.02	0.56 ± 0.02	4.5 ± 0.059	(Huang et al., 2011)
SE. Corn Stover Batch I	g/L	2.2	-	-	1.5	0.2		(Öhgren et al., 2006)
Batch II	g/L	2.1	-	-	1.3	0.2		

Substrate loading was reported as an important factor affecting the yield and rate of enzyme hydrolysis. Sun and Cheng (2002) reported that low substrate loading result in a low hydrolysis yield, and high substrate loading can cause substrate inhibition, and substantially lower the rate of the hydrolysis. Ioelovich and Morag (2012) also reported that high solid loading (15 to 30 wt. %) can cause cellulase deactivation and lower enzymatic conversion due to insufficient uniform mixing and mass transfer limitation. In addition, high biomass loading was also reported to reduce the degree of enzymatic conversion due to the enzyme inhibition by high concentration of sugars.

Furthermore, product inhibition was reported to affect enzyme activity by causing the irreversible adsorption of cellulase on cellulose and lead to cellulase deactivation (Sun and Cheng, 2002).

1.4.1. Effect of inhibitory compounds on enzyme hydrolysis

Inhibitory compounds formed during the pretreatment of lignocellulosic materials can have an effect on the enzyme hydrolysis. Tengborg et al. (2001) reported that furfural and HMF did not inhibit the enzyme hydrolysis of steam pretreated softwood. Similarly, Mes-Hartree and Saddler (1983) reported that the furan derivatives, furfural and HMF, were not inhibitory to enzyme hydrolysis at concentrations normally found in steam exploded wheat straw and aspen wood chips.

However, lignin and lignin derived product were reported as the major inhibitors of enzymatic hydrolysis. Alvira et al. (2010) reported that lignin limits the rate of enzyme hydrolysis by acting as a physical barrier preventing the digestible parts of the substrate from being hydrolyzed. Similarly, Ju et al. (2014) reported that lignin can reduce the activity of cellulase, and is a major recalcitrant factor to enzyme hydrolysis of

lignocellulosic substrates. Several studies have demonstrated that phenolic compounds appear to be the strongest inhibitors of enzyme hydrolysis (Ju et al., 2014; Tejirian and Xu, 2011; Ximenes et al., 2010; 2011). Tejirian and Xu (2011) reported that oligomeric phenolics were more inhibitory than simple phenolics, and Ximenes et al. (2011) reported that phenols are not only inhibitors but also cellulolytic enzymes deactivator.

1.4.2. CTec2 cellulase enzyme

The manufacturer (Novozymes) described Cellic Ctec2 as a blend of aggressive cellulase with high level of β-glucosidases and hemicellulase. They reported that Ctec2 has high conversion yield, compatible with multiple feedstocks, compatible with different pretreatment methods, and most important is tolerant to inhibitors. In addition Ctec2 was reported to be efficient at low dosage which contributes to lower cost of ethanol production from lignocellulosic biomass. The optimal temperature and pH of Ctec2 are 45 ^{o}C-50 ^{o}C and 5-5.5 respectively. Ju et al. (2014) reported the enzyme hydrolysis of modified poplar using Ctec2, *Accelerase* (ACC1500), and *Cytolase* cellulases. The authors reported that Ctec2 had a higher hydrolysability and showed a stronger capacity to overcome lignin inhibition compared to *Dupont Accelerase* (ACC1500) and *Cytolase* enzymes.

1.5. Fermentation

The fermentation of lignocellulose hydrolysate requires a metabolic process that converts the monomeric sugars to alcohol using microorganisms such as fungi or bacteria. *Saccharomyces cerevisiae*, known as Bakers' yeast, is the most commonly used

microorganism for industrial ethanol production (Margeot et al., 2009; Öhgren et al., 2006).

1.5.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a well-known yeast species and a preferred organism for ethanol production throughout recorded human history. *S. cerevisiae* was reported as a chosen species for industrial ethanol production because it is reasonably tolerant to ethanol, acid, and moderately high temperatures compared to bacteria (Almeida et al., 2007; Öhgren et al., 2006). Claassen et al. (1999) reported that *S. cerevisiae* has several distinct advantages over other yeasts, it has a high ethanol tolerance, amounting to 150 g/l ethanol. However several studies have reported that *S. cerevisiae* lacks ability to utilize the pentose sugars, xylose and arabinose, therefore is not very well equipped for the fermentation of lignocellulose (Claassen et al., 1999; Geddes et al., 2011; Margeot et al., 2009; Öhgren et al., 2006; Taherzadeh and Karimi, 2007a). Genetic engineering has been used to improve the conversion of pentose by *S. cerevisiae* (Sonderegger et al., 2004).

The effect of inhibitory compounds on *S. cerevisiae* has also been reported, Almeida et al. (2007) and Taherzadeh and Karimi (2007a) reported that furans (furfural and HMF) at high concentration can inhibit the growth of *S. cerevisiae*, and cause vacuole and mitochondrial membranes damage. Weak acid (acetic, levulinic, and formic acid) were also shown to inhibit the yeast growth by reducing the uptake of aromatic amino acids from the medium. Phenolic compounds were reported to reduce the volumetric ethanol productivity in *S. cerevisiae*. However, other studies reported that the effect of inhibitory compounds on *S. cerevisiae* depended on the strain used, some strains were reported to show tolerance to furan due to their ability to convert HMF and furfural

to less harmful compounds (Palmqvist and Hahn-Hägerdal, 2000b). The industrial *S. cerevisiae* strain TMB3000 was reported to have high tolerance to HMF (Nilsson et al., 2005). Some *S. cerevisiae* strains were also reported to have the natural ability to metabolize some phenolic compounds present in lignocellulose hydrolysates (Klinke et al., 2003).

1.5.2. Ethanol Fermentation

The fermentation of the pretreated lignocellulosic biomass is generally performed using two methods, simultaneous saccharification and fermentation (SSF), or separate hydrolysis and fermentation (SHF). The SSF method involves enzymatic hydrolysis of the pretreated lignocellulosic biomass and the fermentation of the resulting monomeric sugars simultaneously and in the same vessels. Whereas, in SHF method, the enzyme hydrolysis and the fermentation are preformed separately in different vessels. Several studies have reported that the overall ethanol yield for most lignocellulose biomass was higher when using SSF (Öhgren et al., 2006; Parawira and Tekere, 2011; Tomás-Pejó et al., 2008).

Cantarella et al. (2004a) and San Martín-Davison et al. (2014) reported the ethanol yield produced in SSF and SHF of detoxified steam-exploded poplar in Table 1.3. Cantarella et al. (2004a) used three different methods of detoxification (water rinsing, water-ethyl acetate, and overliming) while San Martín-Davison et al. (2014) used only simple water washing. Cantarella et al. (2004a) described that the steam-exploded poplar was washed with 1.5 L water in the sample A, and with 8.5 L water in the sample B. For the water-ethyl acetate two-phase contacting, 86 ml distilled water and 450 ml ethyl acetate were added to steam-exploded poplar and then rinsed with 8.5 L of water.

Finally for the $Ca(OH)_2$ overliming method, solution of $Ca(OH)_2$ was added to steam exploded poplar. Cantarella et al. (2004a) reported that the ethanol yield and productivity was higher in the overliming samples and water rinse samples but lower in ethyl acetate-water. The detoxification with overliming and water rinse improved the ethanol yield and productivity while the ethyl acetate-water method exhibited a longer lag phase which resulted in a low ethanol yield and productivity (Table 1.3). In addition, the authors reported that the undetoxified samples were not fermentable, and the most efficient detoxification method was overliming, and the least efficient was the ethyl acetate water system.

San Martín-Davison et al. (2014) reported that the ethanol yield in SSF of four poplar hybrids (H-29, H-32, H-34, and H-41) pretreated with steam-explosion at two different temperature (200 and 220 °C). The maximum ethanol yield obtained was 70% with 220 °C pretreated hybrid poplar H-29 (Table 1.3). The ethanol yield obtained in their study was lower compare to the study done by Cantarella et al. (2004a) who reported 80% ethanol yield when the steam exploded poplar was washed with water (Table 1.3).

Öhgren et al. (2007) and Chu et al. (2013) reported the ethanol yield of steam exploded corn stover in SSF and SHF shown in Table 1.3. The authors used two different methods of detoxification; Öhgren et al. (2007) washed the steam-exploded corn stover with water and further added sugar or xylanase, while Chu et al. (2013) used a lower substrate loading (10 % w/v) and vaccum evaporation to concentrate the hydrolysate after washing the biomass. Öhgren et al. (2007) stated that washing the material and then adding sugars was not a feasible process alternative, but was done in their study just to evaluate the inhibitory effect in SSF. They reported that SSF gave a 13% higher overall ethanol yield than SHF (Table 1.3). Chu et al. (2013) reported that the enzyme hydrolysis

yield was high (81.24%) and the ethanol yield was (94.50%) because low substrate loading was used, and the hydrolysate was concentrated by vaccum evaporation which removed volatile inhibitory compounds. The ethanol yield obtained in their study was higher compared to the study done by Öhgren et al. (2007); however both approaches are found economically feasible.

1.6. Detoxification Methods

Several detoxification methods have been reported to remove various inhibitory compounds from lignocellulosic hydrolysates. These methods are classified into physical (e.g. vacuum evaporation), chemical (e.g. overliming with calcium hydroxide, activated charcoal, ion exchange resins, solvent extraction), and biological (e.g. laccase enzymes, genes modification) (Chandel et al., 2007a; Larsson et al., 1999a; Mussatto and Roberto, 2004; Taherzadeh et al., 2000b). Table 1.4 summarizes the hydrolysate detoxification using various methods; each method was reported to remove a specific inhibitor from the hydrolysate.

1.6.1. Physical detoxification

Physical detoxification methods such as vaccum evaporation was reported to remove only volatile compounds such as acetic acid, furfural, and vanillin (Larsson et al., 1999b).Vacuum evaporation was shown to moderately increase the concentration of non-volatile toxic compounds (extractives and lignin derivatives), decrease the hydrolysate volume, and reduce the degree of fermentation (Larsson et al., 1999b).

Table 1.3. Fermentation of steam-exploded (SE) poplar and corn stover hydrolysates detoxified with different methods

Biomass	Detoxification	Bioprocess	Species	Ethanol yield (%)	Productivity (g/l-h)	References
SE-poplar	None	SSF	*S. cerevisiae*	Unfermentable	Unfermentable	
	Ethyl acetate-water system	SSF	*S. cerevisiae*	51	0.64	
	Ca(OH)$_2$	SSF	*S. cerevisiae*	86	1.08	(Cantarella et al., 2004a)
	water washed (sample A)	SSF	*S. cerevisiae*	80	0.83	
	(sample B)			82	0.74	
SE-poplar	None	SHF	*S. cerevisiae*	Unfermentable	Unfermentable	
	Ethyl acetate-water system	SHF	*S. cerevisiae*	37	0.11	
	Ca(OH)$_2$	SHF	*S. cerevisiae*	92	0.37	(Cantarella et al., 2004a)
	water washed (sample A)	SHF	*S. cerevisiae*	77	0.23	
	(sample B)			52.5	0.15	
SE-poplar H-29	water washed	SSF	*S. cerevisiae*	69.7± 1.16		
H-32		SSF	*S. cerevisiae*	69.2 ± 4.22		
H-34		SSF	*S. cerevisiae*	52 ± 0.83		(San Martín-Davison et al., 2014)
Control		SSF	*S. cerevisiae*	58.31 ± 0.59		
SE-corn stover	None (Whole slurry)	SSF	*S. cerevisiae*	78.2		
		SHF	*S. cerevisiae*	64.1		(Öhgren et al., 2007)
	water washed slurry/sugar	SSF	*S. cerevisiae*	69.3		
		SHF	*S. cerevisiae*	76.2		
	water washed slurry/xylanases	SSF	*S. cerevisiae*	81.5		
		SHF	*S. cerevisiae*	68.2		
SE-corn stover	Water washed (solid to liquid ratio 1:10) + vaccum evaporation	SSF	*S. cerevisiae*	94.5		(Chu et al., 2013)

1.6.2. Chemical detoxification

The chemical methods include precipitation of toxic compounds and ionization of some toxic compounds under certain pH values (Mussatto and Roberto, 2004). For example, ion exchange resins was reported as a detoxification method that can remove lignin-derived inhibitors, acetic acid and furfurals but it also lead to fermentable sugars loss. Chandel et al. (2007b) observed that ion exchange resins reduced furans by 63.4% and total phenolics by 75.8% from sugarcane bagasse acid hydrolysates but also led to a considerable loss of fermentable sugars.

Detoxification by the overliming procedure involves addition of a base, e.g. $Ca(OH)_2$ up to pH 10 or 11 at 25 or 60 ^oC, waiting for 30 to 60 min and then decreasing the pH to a level suitable for the fermentation (Millati et al., 2002). The effectiveness of overliming was reported to strongly depend on the treatment duration, pH and temperature. The process has been shown to remove volatile inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) from the hydrolysate but had no effect on acetic acid, and removed only a small percentage of phenolic compounds (Chandel et al., 2011b; Millati et al., 2002) (Table 1.4). Overliming method was also found uneconomical because it causes sugar loss and generates some waste by-products (Chandel et al., 2011b; Martinez et al., 2000; Millati et al., 2002).

Activated charcoal is another chemical detoxification method that was reported effective to adsorb toxic compounds (Chandel et al., 2011a). The effectiveness of the activated charcoal treatment was shown to depend on the pH, temperature, contact time, and the activated charcoal concentration (Lee et al., 2011). Converti et al. (1999) reported a 95% removal of phenolic compound when oak wood hydrolysate was treated with activated carbon. In addition, Lee et al. (2011) reported that activated carbon effectively

removed HMF and furfural, and partially formic acid and acetic acid but they also observed loss of fermentable sugars.

Solvent extraction was also reported as a chemical detoxification method. Grzenia et al. (2008) used membrane extraction and reported a removal of 60% acetic acid from corn stover hydrolysate. Later, Grzenia et al. (2010) reported a removal of acetic, formic and levulinic acid as well as 5-hydroxymethylfurfural and furfural when alamine 336, octanol and oelyl alcohol were used in the organic phase. However, the use of membrane extraction was not found to be economically feasible for lignocellulosic hydrolysate detoxification due to the high cost of operation, membrane, and solvent.

1.6.3. Biological detoxification

Biological methods were reported as an improvement on physical and chemical methods because it generated little waste, and had many advantages such as: mild reaction conditions, avoiding further use of toxic and corrosive chemicals, fewer side-reaction toxic products, and less energy demand (López et al., 2004). Fonseca et al. (2011); López et al. (2004); Parawira and Tekere (2011); and Taherzadeh and Karimi (2011) reported that the methods involved using microorganisms or enzymes that can act on the specific toxic compounds present in the hydrolysates and changed their composition or structure to less toxic ones. Fonseca et al. (2011) used *Issatchenkia occidentalis* CCTCC M 206097 yeast to detoxify hemicellulose hydrolysates and reported the reduction of syringaldehyde (66.67%), ferulic acid (73.33%), furfural (62%), and 5-HMF (85%). López et al. (2004) also reported that fungus *Coniochaeta ligniaria* C8 (NRRL30616) was effective in removing furfural and HMF from corn stover hydrolysate.

1.6.4. Effectiveness of detoxification methods

Mussatto and Roberto (2004) reported that an effective detoxification method should be inexpensive, easy to integrate into the process and able to remove the inhibitors from the lignocellulosic hydrolysate. However, studies show that many detoxification methods did not completely remove all the inhibitors from the hydrolysate and had some advantages and disadvantages. Larsson et al. (1999b) reported that the effectiveness of a detoxification method depend both on the type of hydrolysate and on the species of microorganism employed, because each type of hydrolysate has a different degree of toxicity, and each species of microorganism has a different degree of tolerance to inhibitor. Overall, some authors suggested that lignocellulosic hydrolysate detoxification should be avoided or considered only if the fermentation cannot succeed without it, because it could cause additional process cost, produce of additional wastes, and cause fermentable sugars loss (Almeida et al., 2007; Taherzadeh and Karimi, 2011).

1.7. Biochar

Biochar is a solid material obtained through a pyrolysis of biomass such as wood, manure or leaves (Hagner et al., 2013). The physical properties of biochar were shown to strongly depend on the starting organic material and the pyrolysis conditions. Temperature was shown as a very important factor that determines the physical and chemical properties of biochar. Gundale and DeLuca (2006) and Warnock et al. (2007) reported that coniferous wood biochars produced at high temperature (800 $^{\circ}$C) have higher sorptive capacity for cations than biochars generated at lower temperature (350 $^{\circ}$C). In addition the feedstock was also reported to affect the biochar physical properties. Keech et al. (2005) explained that plant species with large diameter cells in their stem

Table 1.4. Different detoxification strategies applied to lignocellulose hydrolysates for the removal of fermentation inhibitors

Lignocellulose hydrolysates	Detoxification methods	Changes in hydrolysate composition	References
Saccharum spontaneum	Overliming	Removal of furfural (41.75 %), total phenolics (33.21 %), no effect on acetic acid reduction of reducing sugars (7.61 %)	(Chandel et al., 2011b)
Wheat straw	Ethyl acetate + overliming	Removal of furfurals (59.76 %), phenolics (48.23 %), acetic acid (92.19 %)	(Zhuang et al., 2009)
Oak wood	Activated carbon	Removal of phenolics (95.40 %)	(Converti et al., 1999)
Corn stover	Membrane based organic phases alamine 336	60 % acetic acid	(Grzenia et al., 2008)
Sugarcane bagasse	*Issatchenkia Occidentalis CCTCC M 206097*	Reduction of syringaldehyde (66.67%) ferulic acid (73.33 %), furfural (62%), 5-HMF (85 %)	(Fonseca et al., 2011)
Corn stover	*Coniochaeta ligniaria*	80 % removal of furfural and 5-HMF	(López et al., 2004)

tissues produced biochar particles with greater quantities of macropores which can enhance the ability of biochar to adsorb larger molecules such as phenolic compounds. Biochar was reported to have three important physical properties: the aromatic structure, the porous structure, and the pores volumes (Biederman and Harpole, 2012). Lehmann et al. (2006) reported that biochar increased the recalcitrant carbon fraction of soil due to its aromatic structure, and explained that the resistance of biochar to decomposition was due to its aromatic structure. The study of Terra Preta soils in the Amazon showed that charcoal can remain in the soil for hundreds to thousands of years (Glaser et al., 2001; Lehmann et al., 2006). Keech et al. (2005) showed that pores represented more than 95% of the total wood composition and showed the presence of micro pores on the wood derived biochar.

Like the physical properties, the composition of biochar was also reported to vary according to the feedstock type and pyrolysis conditions. Biochars produced from wood materials were reported to have high carbon content (Lehmann et al., 2003; Lima and Marshall, 2005), whereas biochars produced from poultry litter had low carbon but high inorganic content. Table 1.5 shows the composition of oak wood, poultry litter, and giant reed biochars. The pyrolysis temperature was shown to affect the composition of biochar. Song and Guo (2012) and Zheng et al. (2013) studied the effect of pyrolysis temperature from 300 ^{o}C to 600 ^{o}C on the properties and nutrient values of biochars from giant reed and poultry litter. The authors reported that carbon and inorganic content of the biochar increased as the pyrolysis temperature increased, but the nitrogen content decreased with increasing pyrolysis temperatures (Table 1.5).

Zheng et al. (2013) reported that about 50% of nitrogen was lost from the giant reed biochar, and the remaining nitrogen was transformed to heterocyclic-N as the

temperature increased. In addition, the availability of nitrogen and phosphorous decreased as the pyrolysis temperature increased. The authors associated the decrease in nitrogen content to be due to the loss of TN (total nitrogen) and to the heterocyclization of nitrogen during the pyrolysis.

Poultry litter biochars produced at 350 ^{o}C and 600 ^{o}C had lower carbon content and higher nitrogen content compared to reed giant and oak wood biochars produced at the same temperatures (Table 1.5). Both Agblevor et al. (2010) and Song and Guo (2012) reported a high ash content for poultry litter biochar produced at 500°C compared to reed giant biochar produced at the same temperature (Table 1.5). In addition, the authors reported the presence of several inorganic elements in the poultry litter biochars such as: potassium, phosphorous, silicon, calcium, sodium, iron, and magnesium.

Zheng et al. (2013) reported that the specific surface areas (S_{BET}) of giant reed biochar produced at 500 ^{o}C and below were extremely low and varied between from 2.16 m^2/g to 3.04 m^2/g, however the biochar produced at 600 ^{o}C had a higher surface area of 50 m^2/g. In contrast, Song and Guo (2012) reported that poultry litter biochar had a low surface area of 5.79 m^2/g even when the biochar was produced at 600 ^{o}C. They reported that the specific surface area (S_{BET}) of the poultry litter biochar varied from 2.68 m^2/g to 5.79 m^2/g as the pyrolysis temperature increased from 300 ^{o}C to 600 ^{o}C.

1.7.1. Biochar application on agricultural soils

The application of biochar to soil has received much attention due to its high stability in soil and its potential to mitigate soil-derived greenhouse gas emissions. This fact was shown by the early study of the Terra Preta soils in the Amazonian rainforest by Glaser et al. (2001) and more recently by studies conducted by Beiderman and Harpole

Table 1.5. Composition of Biochars from different biomass

Biochar type	Components	300	350	400	450	500	550	600	References
Oak Wood: Quercus spp. (wt. %)	C		75.9					88.4	(Nguyen and Lehmann, 2009)
	H		4.27					2.13	
	N		0.1					0.10	
	O		19.6					9.0	
	K		0.11					0.22	
	Fe		0.003					0.13	
	Ca		0.07					0.09	
Poultry litter (wt. %)	C					23.40			(Agblevor et al., 2010)
	H					1.36			
	O					27.36			
	N					1.73			
	S					0.83			
	Cl					1.53			
	P					1.68			
Broiler-1	K					5.65			
	Fe					0.62			
	Ca					6.55			
	Mg					1.16			
	Ash					43.79			
Poultry litter (wt. %)	OC[a]	37.99	37.65	36.1	35.22	34.47	33.88	32.52	(Song and Guo, 2012)
	N	4.17	3.22	2.63	2.23	1.21	0.31	0.12	

[a] OC: organic carbon

Table 1.5 cont'd

Biochar type		Pyrolysis temperature (^{o}C)							References
	Components	300	350	400	450	500	550	600	
Poultry litter (wt. %)	P	2.27	2.4	2.63	2.66	2.79	2.98	4.05	(Song and Guo, 2012)
	K	6.93	7.46	8.12	8.57	8.79	8.97	9.15	
	Ca	7.18	7.64	8.34	8.79	9.06	9.3	9.4	
	Mg	1.86	1.97	2.15	2.28	2.33	2.38	2.42	
	S	2.7	2.88	3.12	3.32	3.4	3.5	3.53	
	Ash	47.87	51.29	56.62	58.66	60.58	60.65	60.78	
giant reed (wt. %)	C	65.26	66.97	72.25		73.12		78.61	(Zheng et al., 2013)
	H	4.51	4.46	4.09		3.01		2.22	
	O	21.03	21.67	18.72		11.54		11.24	
	N	0.65	0.64	0.69		0.63		0.55	
	P	0.12	0.12	0.13		0.16		0.17	
	K	3.7	3.8	4.18		4.77		5.02	
	Ash	7.69	7.73	8.45		10.7		11.27	
	S_{BET} (m^2/g)	2.72	2.16	3.04		2.58		50	

(2012); Chan et al. (2008); DeLuca et al. (2009); Farrell et al. (2013) Jien and Wang (2013); Lehmann et al. (2006); Lehmann and Joseph (2009); Lehmann et al. (2008); and Quilliam et al. (2013). These authors observed that biochar addition to soil, in general improved the physical and chemical properties of the soil.

Biochar addition to soil was reported to improve soil qualities by increasing its pH and nutrient availability (Glaser et al., 2001; Jien and Wang, 2013; Lehmann et al., 2006). Biederman and Harpole (2012) reported that the addition of biochar to soils resulted in a statistically significant increase in pH of acidic soils, aboveground productivity, soil phosphorous (P), soil potassium (K), total soil nitrogen (N), and total soil carbon (C) compared with the control conditions with no biochar addition.

In addition, Quilliam et al. (2013) observed other benefits of applying wood-derived biochar to the soils such as: sorption, stabilization of pesticides, nutrient ions, improve soil structure, and retention of soil moisture. They reported that these benefits were more related to the aromatic structure, surface density, and the pore size of the biochar. Lehmann et al. (2003) reported that biochar has the potential to increase plant nutrient availability by increasing cation exchange capacity, altering soil pH, or direct nutrient contributions from biochar.

Moreover, studies have suggested that biochar possesses the ability to increase plants productivity and reduce nutrients leaching in some agricultural systems (Jones et al., 2012). Chen et al. (2010) reported that bagasse biochar reduced soil dry density, enhanced available moisture of Shimajiri maji soil, and increased yields and sugar content of sugarcane. Similarly, Revell et al. (2012) reported that poultry litter biochar addition to soil increased lettuce germination due to its nutrients content. Quilliam et al. (2013) also observed that wood biochar application to agricultural soils improved soil

quality, and crop production due to the biochar large pores volume, which ameliorated soil aeration and water holding capacity. Zhang et al. (2012) reported that wheat straw biochar amendment showed a 20% to 30% increase of rice productivity.

1.7.2. Biochar effect on the environment

Biederman and Harpole (2012) reported that biochar application to soil can be solution for carbon sequestration and pollutants emission. The authors reported that biochar application to soil mitigated the anthropogenic CO_2 emission (12% CO_2 reduction), and reduced nitrous oxide (N_2O) emission. The CO_2 reduction was more associated with the physical properties of biochar such as porous structure, surface area, and affinity for charge particles. The reduction in N_2O emission was due to the increase in soil heavy metals caused by biochar addition to soil. Similarly, Zhang et al. (2012) reported that wheat straw biochar amendment can be used to decrease nitrous oxide (N_2O) and methane (CH_4) emission. Furthermore, Farrell et al. (2013) also mentioned that wood biochar has a potential to mitigate soil derived greenhouse gas emission and carbon sequestration due to its high carbon content.

1.7.3. Biochar effect on soil microbial growth

Studies have shown that biochar has a positive effect on the soil microbial population. This fact was illustrated by early research of Ishii and Kadoya (1994), which showed that charcoal application to the soil improved the growth of citrus trees, and vesicular arbuscular mycorrhizal (VAM) development. More recently, biochar impact on microbial communities structure and function has been reported by several studies (Biederman and Harpole, 2012; Jindo et al., 2012; Lehmann et al., 2011; Quilliam et al.,

2013; Van Zwieten et al., 2010; Warnock et al., 2007). Farrell et al. (2013) reported that biochar has many functions which could favor the microbial community such as: ability to increase water retention, soil aeration, soil sorption of toxics compounds, pH of acidic soils, and decreased leaching of nutrients.

Warnock et al. (2007) reported that biochar addition to soil increased mycorrhizal fungi abundance due to the increase in soil nutrients availability and the resistance to plant pathogen infections. The positive effect of biochar on microbial growth was attributed to the physical properties of the biochar. Jindo et al. (2012) and Warnock et al. (2007) suggested that the pores present in biochar served as a refuge for the microbes protecting them from predation and desiccation. In addition, Lehmann et al. (2011) explained that the impact of biochar on soil fauna is due to biochar sorption capacity, which presents high probability of altering native organic matter availability. Moreover, Rousk et al. (2010) reported that pH is a crucial factor and a key driver of microbial community structure and function, and therefore biochar effect on the microbial increase is associated with the pH increase of acidic soil.

Biederman and Harpole (2012) reported that soil microbial biomass, root nodulation by rhizobia increased with addition of biochar to soil. The authors explained that these increases were possibly due to the surface charge of biochar (which may have retained some nutrients and enhanced the microbial food resources), and the slight increase of soil nutrients alkalinity. Farrell et al. (2013) observed that gram positive bacteria dominated when wood biochar was added to soils and they also said that wood biochar application improved the soil pH, increased the microbial community, and provided habitat for microorganisms.

1.8. Concluding Remarks

In summary, the review of literature showed that lignocellulosic biomass is an attractive potential renewable feedstock for ethanol production. However, the conversion of lignocellulosic biomass into ethanol still encounters major technical challenges. Inhibitory compounds generated during the pretreatment step were shown to decrease the ethanol yield and productivity. Researchers are studying different methods to overcome the inhibitory compounds in order to improve the ethanol yield and productivity. Literature also shows that biochar produced from pyrolysis of lignocellulosic biomass is a valuable product, and has positive effect on agriculture soils. Success in incorporating biochar to improve the ethanol production from lignocellulosic biomass will help reduce the cost of detoxification.

1.9. Research Objectives

Although many detoxifications methods have been used to improve the ethanol production from the lignocellulosic biomass, the use of poultry litter biochar in ethanol production was not reported. The overall goal of this research was to improve the ethanol production from steam exploded poplar and corn stover using a low cost material poultry litter biochar, and develop a method that can be easily incorporated during the ethanol production process. The specific objectives include:

1. Determine the effect of poultry litter biochar on the aerobic and anaerobic growth of *Saccharomyces cerevisiae*

2. Determine the effect of poultry litter biochar on the enzyme hydrolysis of steam-exploded poplar and corn stover

3. Determine the effect of poultry litter biochar on ethanol production from steam-exploded poplar and corn stover

4. Compare the cellulosic conversion and ethanol yield of sodium hydroxide washed steam exploded poplar and unwashed steam exploded poplar

1.10. References

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CHAPTER 2

EFFECT OF POULTRY LITTER BIOCHAR ON THE GROWTH OF
SACCHAROMYCES CEREVISIAE AND ETHANOL PRODUCTION

2.1. Abstract

The effect of poultry litter biochar on the growth of yeast *Saccharomyces cerevisiae* was studied. Previous studies have reported that biochar contains high level of valuable nutrients and its application to agricultural soils increased plant productivity and soil microbial growth. In this study, we investigated the effect of poultry litter biochar on yeast growth under aerobic and anaerobic conditions. *S. cerevisiae* ATCC 204508/S288C was cultivated in the biochar, and the controls (YM and GYE) media, growth was measured by the optical density and standard plate count methods. Results show that addition of poultry litter biochar to the medium significantly improved the growth of *S. cerevisiae.* The doubling time in biochar medium was 1.5 ± 0.17 h compared to 2.2 ± 0.12 h for YM control, and 2.5 ± 0.28 h for the GYE control. The CFU count for the biochar medium was approximately 2 times that for the controls. The anaerobic growth data showed that glucose consumption and ethanol productivity were higher in the biochar medium compared to the control. For the same initial glucose concentration (50 g/l), the biochar medium glucose was consumed in 12 h compared to 24 h for control. Poultry litter biochar addition to the medium promoted the growth of yeast and ethanol production. When the glucose concentrations were 100 g/l and 150 g/l, the yeast growth and ethanol production were faster in the biochar media than the GYE control media.

2.2. Introduction

Poultry litter biomass is a mixture of bedding, manure, feathers, spilled feed, and a potential feedstock for biofuel production. Poultry litter biochar is the black solid residue obtained in addition to bio-oil and gases after pyrolysis of poultry litter biomass (Agblevor et al., 2010). Biochar has three important physical properties: the aromatic structure, the porous structure, and the pores volumes (Biederman and Harpole, 2012). The physical properties and the composition of biochar depend on the the feedstock and the pyrolysis conditions (Song and Guo, 2012).

Biochar have been the focus of several studies due to its potential uses and applications in different domain, and its impact on microbial growth, soil improvement, and plant growth (Farrell et al., 2013; Jindo et al., 2012). The application of biochar to soil has received much attention due to its high stability in soil and its potential to mitigate soil-derived greenhouse gas emissions. This fact was proved by the study of the Terra Preta soils in the Amazonian rainforest (Glaser et al., 2001) and more recently reported by the study conducted by (Lehmann et al., 2008).

Biochar impact on microbial communities structure and function have been reported as well by several works (Jindo et al., 2012; Lehmann et al., 2011; Quilliam et al., 2013; Van Zwieten et al., 2010; Warnock et al., 2007). Biochar was reported to increase soil microbial biomass, root nodulation by rhizobia, arbuscular mycorrhizal fungi (Biederman and Harpole, 2012; Warnock et al., 2007). The obvious positive attribute of biochar is more associated with its physical properties and nutrients value. Jindo et al. (2012) reported that biochar can affect soil microbial community through their high porosity which provides an adequate microhabitat for microorganisms in the soil. However, the exact effect of biochar on the microorganisms is still unclear

(Biederman and Harpole, 2012). Poultry litter biochar contains nutrients such as: Nitrogen, Phosphorous, Potassium, Calcium, Magnesium, Iron, and Sodium (Agblevor et al., 2010; Chan et al., 2008; Song and Guo, 2012). These minerals were reported favorable for biological uptake for microorganisms and also soil food web (Steiner et al., 2008).

S. cerevisiae is an important species used in the alcohol fermentation under anaerobic condition. The major constituents of culture media for yeast growth are: carbon source (e.g. sugars), nitrogen source such as organic (e.g. peptone, yeast extract) and inorganic (ammonium salts, nitrate nitrogen), minerals (e.g. K, Ca, Na, Mg, and trace elements). In this study, we hypothesize that poultry litter biochar can provide an additional nutrients to the growth medium and enhance the growth of *S. cerevisiae*. In this work we investigated the growth of *S. cerevisiae* ATCC 204508/S288C in biochar medium under aerobic and anaerobic conditions, and compared the growth curves to the controls (YM and GYE) media.

2.3. Materials and methods

2.3.1. Yeast strain

Saccharomyces cerevisiae ATCC 204508/S288C was used throughout this study.

2.3.2. Poultry litter Biochar

Poultry litter biochar (PLB) was obtained from USTAR Bioenergy Center, Utah State University; the sample was labelled (Buff + Berry). The initial poultry litter biomass was supplied by Virginia poultry farmer in Shenandoah Valley, Virginia (Agblevor et al., 2010). The moisture, ash content and elemental composition of the

biochar was determined. The previous study done by Agblevor et al. (2010) reported that poultry litter biochar ash contains some inorganic compounds shown in Table 2.1.

2.3.2.1. Moisture content

The moisture content of the PLB was determined using an infrared moisture analyzer (Denver Instrument IR-60, Bohemia, NY). 1.0 g of sample was weighed into a tared sample pan and its moisture was determined by heating it at 105 ^{o}C for 30 min. At the end of the 30 min, the moisture content was automatically calculated and displayed.

2.3.2.2. Ash content

The ash content of the PLB was determined using ASTM E1755-01 (Reapproved 2007) standard method: samples were prepared in triplicate, 1.0 g of each sample was weighed into a pre-weighed porcelain crucible and ashed at 575 ^{o}C using a muffler furnace for 8 h (Lindbergh Blue, Thermo Scientific, Asheville, NC), the ash content was calculated using the following equation.

$$\% \, ash = \frac{m_{ash} - m_{count}}{m_{od} - m_{count}} \, x \, 100$$

% ash = mass percent of ash, m_{ash} = mass of ash and container, m_{count} = tare mass of container, m_{od} = initial mass of 105 ^{o}C dried sample and container

2.3.2.3. Elemental Composition

The elemental composition (C, H, N, S, O) of the PLB were determined using a Flash 2000 organic elemental analyzer (CE Elantech Inc, Thermo Scientific, Lakewood, NJ).

2.3.2.4. Specific surface area

The Brunauer-Emmett-Teller (BET) specific surface area of the PLB was measured using a Quanta Chrome Monosorb instrument (model MS-16 Quanta chrome, Syosset, NY) through nitrogen adsorption. Before the measurement, the machine was calibrated by injection of 0.948 ml of air and the counter should display a reading of 2.84 $\pm$ 0.03. All samples before the BET surface area measurement were outgassed for 3 h at 220 °C. Three measurements were collected and averaged.

Table 2.1. Inorganic compounds of poultry litter biochar (Agblevor et al., 2010)

Element	Broiler-1	Broiler-2	Broiler-3
P (%)	1.68	2.59	1.73
K (%)	5.65	7.59	6.04
Ca (%)	6.55	8.64	5.73
Mg (%)	1.16	1.88	1.45
Na (%)	1.48	2.03	2
Al (%)	0.54	0.49	0.43
B (%)	bdl[a]	0.01	0.01
Fe (%)	0.62	0.57	0.38
Mn (%)	0.08	0.13	0.08
Cu (ppm)	0.08	0.11	0.16
Zn (ppm)	0.08	0.13	0.08
Cd (ppm)	4	3	1
Ni (ppm)	45	53	32

[a] bdl: below detection limit

2.3.3. Materials

Yeast *S. cerevisiae* was cultivated in biochar, YM, and GYE (glucose + yeast extract) media. For the liquid media preparation, yeast malt (YM) broth powder and glucose (D-(+) glucose) were purchased from Sigma Aldrich (Sigma Aldrich, St Louis MO), yeast extract (Bacto Yeast Extract) was purchased from Fischer Scientific (Pittsburgh, PA). Poultry litter biochar (PLB) was produced from the bench scale fluidized bed reactor, the poultry litter biomass was pyrolyzed at 500 ^{o}C. Yeast cells were grown in 250 ml Erlenmeyer flasks, duplicate flasks were prepared. Solid media were also prepared for colonies counting method.

2.3.3.1. YM media

According to Wickersham formulation, a 1 liter of YM broth contain: 3.0 g yeast extract, 3.0 g Malt extract, 5.0 g peptone, and 10.0 g dextrose. The YM media were prepared the same throughout the study, approximately 2.1 g of YM powder was weighed and added to 250 ml flask, 100 ml of deionized water (di- water) was added to dissolve the powder, and the pH was around 6.3. The mixture was autoclaved at 121 ^{o}C for 30 min, and left in the laminar hood to cool to room temperature.

2.3.3.2. Biochar media

Different types of biochar media were prepared, and each type was varied according to the glucose, yeast extract, and ammonium sulfate concentration; the PLB concentration was kept constant throughout the study (Table 2.2). In order to avoid reaction between the carbon source and the nitrogen source, the two were prepared separately. Two 250 ml Erlenmeyer flasks were used to prepare each biochar medium

type, glucose, biochar, and 50 ml of di-water were added to the first flask. Yeast extract, and 50 ml of di-water were added to the second flask. Flasks were autoclaved at 121 $^{\circ}$C for 30 min and left in the laminar hood to cool to room temperature. The yeast extract liquid was added to the glucose and biochar liquid to form the biochar medium. Before inoculation the pH was adjusted to 6.3 using 1 M HCl.

2.3.3.3. GYE media

Different types of GYE media were also prepared, and each type was varied according to the glucose, and yeast extract concentration, PLB was not added this time (Table 2.2). Glucose and yeast extract were prepared separately using two different flasks. Glucose and 50 ml of di-water were added to the first flask. Yeast extract, and 50 ml of di-water were added to the second flask. Flasks were autoclaved at 121 $^{\circ}$C for 30 min and left in the laminar hood to cool to room temperature. The yeast extract liquid was added to the glucose liquid to form the GYE medium. Before inoculation the pH was adjusted to 6.3 using 1 M HCl.

2.3.4. Inoculum

The inoculum was cultivated aerobically in YPD (Yeast Peptone Dextrose) medium (20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract) for 16 hours overnight before the experiment day, cultures were incubated in a shaker at 37°C and 220 rpm, and used to inoculate the YM, biochar, and GYE media. The optical density of the inoculum cultures varied between 5.1 and 6.8, and each time about 82.4 mg dry weight of yeast was aseptically added to each flask.

Table 2.2. Composition of Biochar media and GYE media

Aerobic growth								
Composition	Biochar media						Control (GYE media)	
Glucose (g/l)	10	20	20	20	20	20	10	20
PLB (g/l)	2	2	2	2	2	2	-	-
Yeast extract (g/l)	1	10	-	-	-	-	1	10
Ammonium sulfate (g/l)	-	-	-	1	5	10	-	-

Anaerobic growth								
Composition	Biochar media				Control (GYE media)			
Glucose (g/l)	20	50	100	150	20	50	100	150
PLB (g/l)	2	2	2	2	2	2	2	2
Yeast extract (g/l)	10	10	10	10	10	10	10	10

2.3.5. Growth and measurement methods

The cultures were incubated in an incubator-shaker (MaxQ 6000, Thermo Scientific, Asheville, NC) at 37 ^{o}C and under shaking at 220 rpm for 24 h. Batch cultivation was performed under aerobic and anaerobic conditions. For the aerobic growth foam stoppers and aluminum foils were used to plug the flasks to allow breathing, samples were taken every 3 h to measure the optical density and to count colonies. For the anaerobic growth, flasks were purged with nitrogen for 5 min and capped with screw cups to allow fermentation; samples were taken to measure the optical density, the glucose, and ethanol concentration.

2.3.5.1. Spectrophotometer

The spectrophotometer method is an indirect method used to measure the cell growth, each time a sample was taken to measure the optical density. The sample was diluted and 1 ml was added in a plastic cuvette, the optical density was read at 600 nm using a spectrophotometer (SpectraMax Plus 384, Molecular Device, Sunnyvale, CA). Before inoculating the cultures, samples were taken and used as blank for the spectrophotometer.

2.3.5.2. Standard Plate Count (SPC)

Yeast cultures were diluted with a series of sterile water blanks, the aliquots were then plated on agar nutrient plates using aseptic technique under a laminar hood. The number of colonies were counted after incubation for 48 h, and reported as colony forming units (CFUs). The dilution factors and the number colonies counted are shown in the Appendix.

2.3.6. Statistical analysis

The p-values of the growth curves at the exponential phase were calculated using QuickCalcs software (GraphPad Software, Inc. La Jolla, CA). The p-values of the yeast growth in the three different media (YM, biochar, GYE) were compared, and p-value < 0.05 was considered significant.

2.4. Results and Discussion

2.4.1. Biochar characterization

Table 2.3 shows the composition of the poultry litter biochar (PLB), the ash content was 55.3 ± 0.25 %, carbon content was 23.4 ± 1.34 % C, and nitrogen content was 2.3 ± 0.09 %. The values obtained are similar to the composition values of PLB analysis reported by Agblevor et al. (2010) and Revell et al. (2012). In general PLB had a low carbon, higher nitrogen and ash contents compared to wood biochar (Chan et al., 2008; Lehmann et al., 2003). Poultry litter biomass was reported to contain high inorganic elements and low carbon compared to woody biomass (Mante and Agblevor, 2011). The oxygen content determined by difference was 14.74 ± 1.37 %. In this study chlorine was not determined, and the oxygen content may also include chlorine. The BET specific surface area (SSA) was low (6.34 ± 0.19 m^2/g) because the biochar was produced at 500 ^{o}C. Biochar produced at 500 ^{o}C and below was reported to have extremely low surface area (S$_{BET}$) (Zheng et al., 2013).

2.4.2. Aerobic growth

2.4.2.1. Optical density

The optical density was measured at 600 nm and plotted against time to represent

Table 2.3. Composition of poultry litter biochar

Sample	Moisture (%)	Ash (%)	Elemental Analysis (wt. %)					SSA $(g/m^2)^b$
			C	H	N	S	O^a	
1	2.9	55.49	22.21	1.03	2.26	0.55	15.55	6.56
2	2.71	55.01	23.17	1.07	2.30	0.23	15.51	6.23
3	2.88	55.32	24.85	1.15	2.44	0.20	13.16	6.23
Average	**2.83 ± 0.10**	**55.27 ± 0.25**	**23.41 ± 1.34**	**1.09 ± 0.06**	**2.33 ± 0.09**	**0.33 ± 0.19**	**14.74 ± 1.37**	**6.34 ± 0.19**

[a] O by difference   [b] specific surface area

the growth curves. In Figure 2.1, the growth curves show that growth in the biochar medium was similar to the growth in YM medium (p-value = 0.9123 > 0.05). However, the growth in the biochar medium was higher than the growth in GYE medium (p-value = 0.0360 < 0.05) indicating that addition of poultry litter biochar (PLB) had a positive effect on the yeast growth. When the glucose and yeast extract concentrations were increased (Figure 2.2), the yeast growth in all media (biochar and GYE) increased and the increase with the biochar medium was statistically different from the growth in the YM medium (p-value = 0.0245 < 0.05) and GYE medium (p-value = 0.0283 < 0.05).

Chicken manure biochar amended soil was reported to increase microbial activity, and population growth capacity of the microbial community (Steiner et al., 2007). Also, biochar addition during composting of tomato stalk and chicken manure showed more influence on bacterial community (Xu et al., 2013). In this study, the growth of *S. cerevisiae* increased when PLB was added to the medium. When the glucose concentration was increased from 10 g/l to 20 g/l, the yeast growth rate increased (Figures 2.1 and 2.2). Steiner et al. (2008) reported a similar effect in their study (in a highly weathered Amazonian upland soil), when glucose was added to soil amended with biochar and water, soil microbial activity increased exponentially.

The data in Figure 2.1 appear to suggest that the lack of difference between the PLB medium and the YM broth medium was probably because the PLB medium rapidly consumed the glucose and stopped growing. However, at high glucose concentration the positive effect of PLB was clearly demonstrated as shown in Figure 2.2.

2.4.2.2. Microscopic pictures

The microscopic slides in (Figure 2.3) show *S. cerevisiae* in YM medium (21 g/l),

Figure 2.1. *S. cerevisiae* growth in YM medium (21 g/l), biochar medium (10 g/l glucose, 1g/l yeast extract, 2 g/l PLB), and GYE medium (10g/l glucose, 1g/l yeast extract)
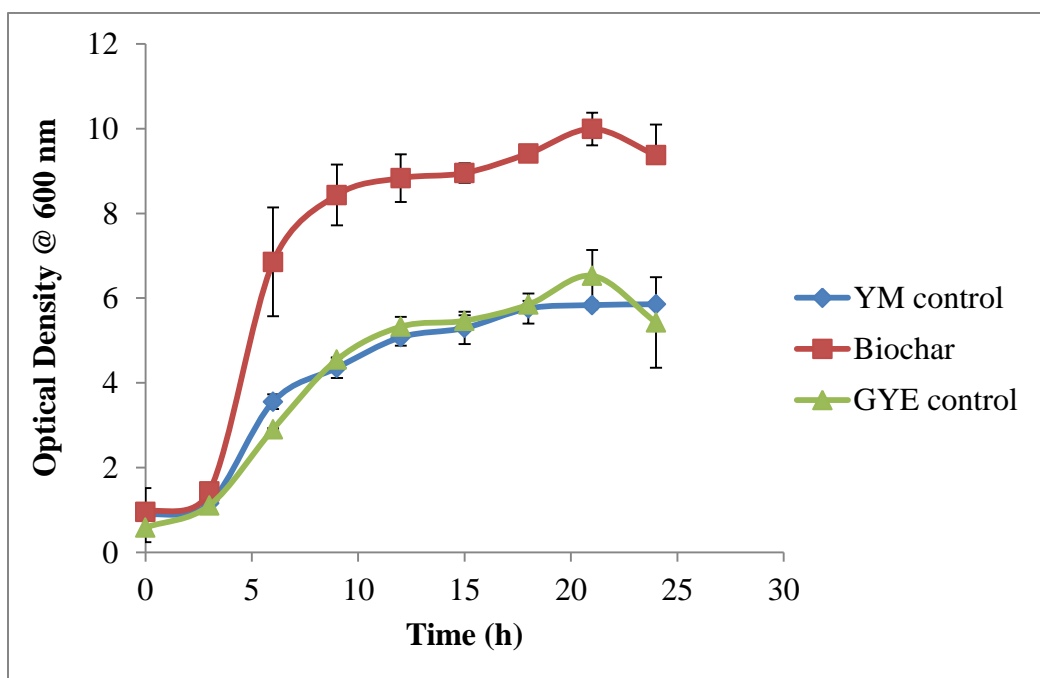
Figure 2.2. *S. cerevisiae* growth in YM medium (21 g/l), biochar medium (20 g/l glucose, 10 g/l yeast extract, 2 g/l PLB), and GYE medium (20 g/l glucose, 10 g/l yeast extract

Biochar medium (20 g/l glucose, 10 g/l yeast extract, 2 g/l PLB), and GYE medium (20g/l glucose, 10g/l yeast extract) cultivated aerobically for 9 h. The slides confirmed by visual observation that PLB stimulated the growth of *S. cerevisiae*; the cells were able to divide and multiply in the presence of biochar in the medium. In Figure 2.3a, the slide show that the yeast grown in the biochar medium after 9 h growth had more colonies than the yeast grown in the YM and GYE media (Figures 2.3b and 2.3c) for the same length of time.

2.4.2.3. Plate count

Yeast colonies were counted in order to confirm the results obtained from the spectrophotometer method. In Figure 2.4, the growth curves show that the biochar medium had more colonies than the YM and GYE media, which is similar to the trend observed in Figure 2.2. The number of CFU/ml at the exponential phase of the biochar growth curve was higher and significantly different from the YM growth curve (p-value = 0.0035 < 0.05) as well as the GYE growth curve (p-value = 0.0085 < 0.05). Figure 2.5; show a proportional relationship between the cell concentration and the optical density.

2.4.2.4. Growth rate and doubling time

Table 2.4 shows the maximum growth rate (μmax) and the yeast doubling time in the different media. Addition of poultry litter biochar to the medium increased the growth of yeast *S. cerevisiae* when compared to the growth in the control media (YM and GYE). The doubling time (Ʈd) of the yeast in the biochar medium was 1.5 h (Table 2.4). The improved doubling time of the *S. cerevisiae* is correlated by the CFU data that showed biochar CFU to be almost twice that of the control media. It appears some of the
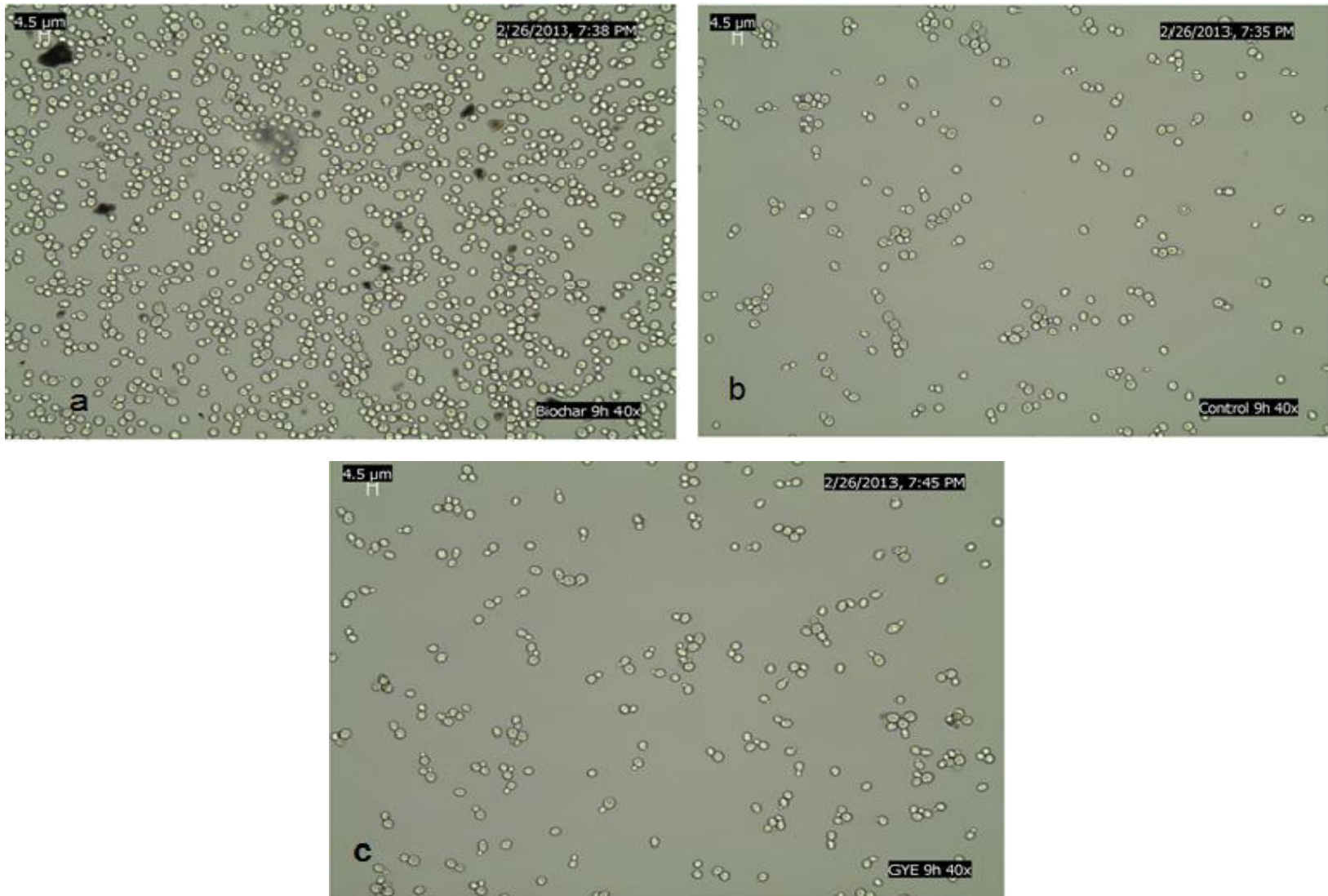
Figure 2.3. Microscopic slides of S. cerevisiae aerobic growth at 9 h in biochar and controls media (a) biochar, (b) YM control, and (c) GYE control

inorganic components such as calcium, potassium, iron, and magnesium of biochar contributed to the rapid growth of the cells.

2.4.2.5. Effect of yeast extract

Yeast extract is a source of trace elements, vitamins, and amino acids for the yeast growth medium. *S. cerevisiae* was cultivated in the biochar and glucose medium in the absence of yeast extract in order to investigate whether biochar can serve as a source of
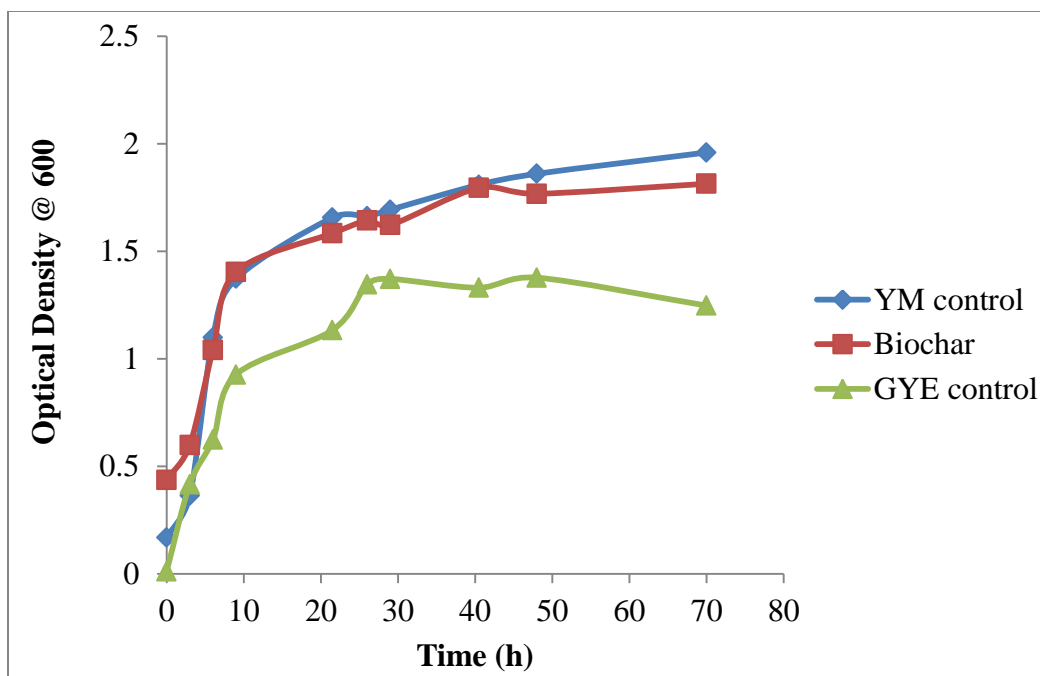

Figure 2.4. *S. cerevisiae* colonies in YM medium (21 g/l), biochar medium (20 g/l glucose, 10 g/l yeast extract, 2 g/l PLB), and GYE medium (20g/l glucose, 10g/l yeast extract)
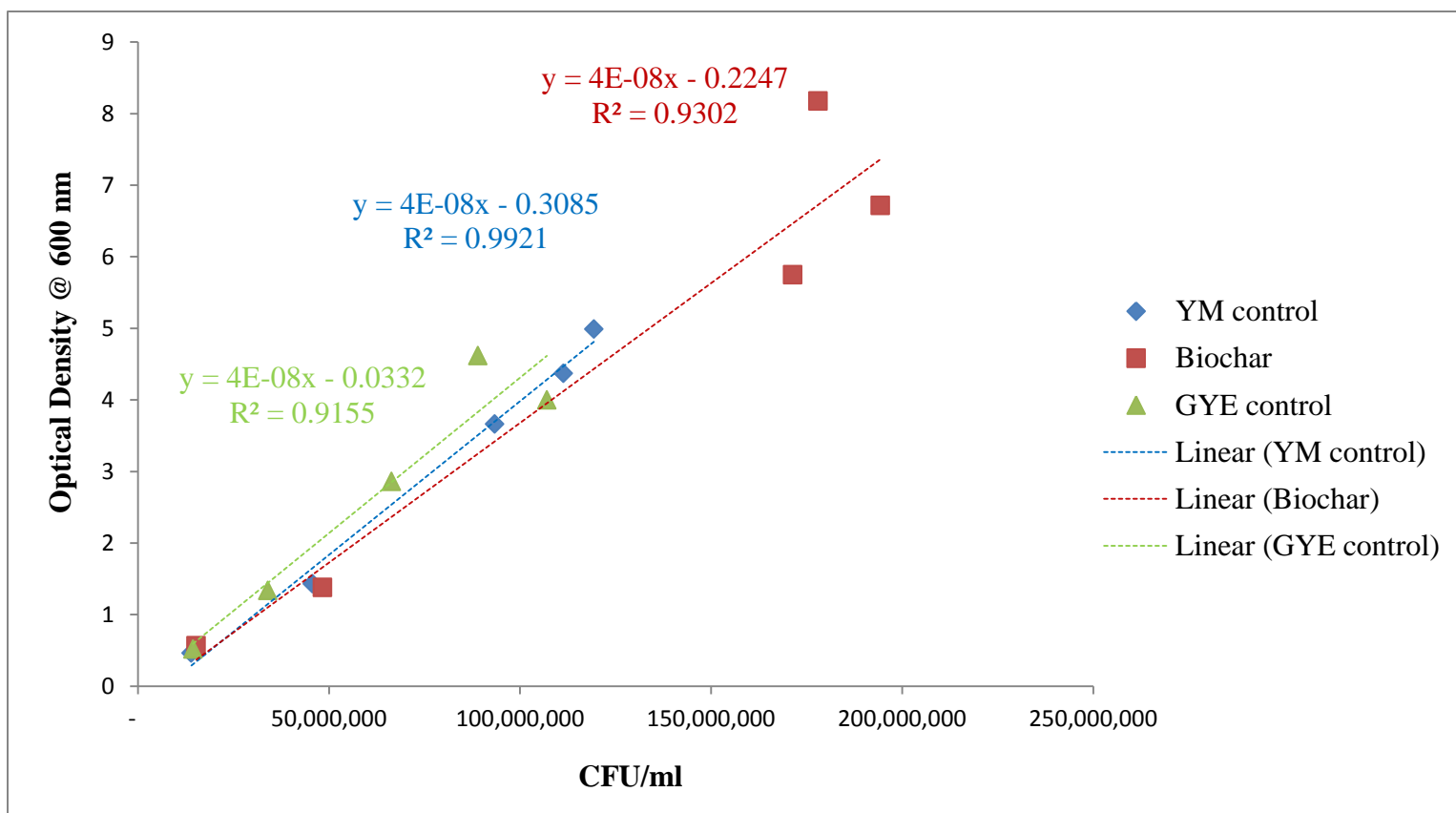
Figure 2.5. Linear correlation between the optical density and CFU/ml of *S. cerevisiae* growth in YM medium (21 g/l) biochar medium (20 g/l glucose, 10 g/l yeast extract, 2 g/l PLB), and GYE medium (20g/l glucose, 10g/l yeast extract)

Table 2.4. Maximum growth rate (μmax) and doubling time (Ʈd) at 37 ^{o}C

	YM medium	Biochar medium	GYE medium
μmax (h^{-1})	0.318 ± 0.017	0.468 ± 0.056	0.282 ± 0.033
Ʈd (h)	2.181 ± 0.122	1.496 ± 0.170	2.478 ± 0.279

nutrient. Results in Figure 2.6 show that the growth curve of the yeast in the YM medium was higher than for the yeast in the biochar (biochar only) medium, which indicates that the nitrogen in the biochar may not be bioavailable for the yeast uptake. Lang et al., (2005); Zheng et al., (2013) reported that the nitrogen (N) is lost during high temperature pyrolysis, and the decrease in available N in biochars begun at 400 ^{o}C during pyrolysis. DeLuca et al. (2009) also reported that N is associated with many organic molecules and it starts to volatilize at 200 ^{o}C.

Addition of an alternative source of nitrogen was found necessary in order for poultry litter biochar to stimulate the yeast growth. Revell et al. (2012) reported that addition of nitrogen fertilizer to biochar increased pepper yield in the sandy loam. The elemental composition of yeast extract was performed and result show that yeast extract contained approximately 10.29 ± 0.21 % N, the same amount of N was added to the biochar medium by substituting the yeast extract with ammonium sulfate. Ammonium sulfate ($(NH_4)_2SO_4$) contain 21 % of N, in order to supply the yeast 10.29 % N, 0.5 g $(NH_4)_2SO_4$ was added to the medium. Result in Figure 2.6 show that addition of $(NH_4)_2SO_4$ improved the yeast growth compared to the growth in (biochar only) medium

and the growth curves were statistically different (p-value = 0.0405< 0.05). However, the yeast growth in (biochar + $(NH_4)_2SO_4$) medium was less than the yeast growth in the YM medium. This suggests there are other components in yeast extract such as amino acids that contribute to the cell growth but are lacking in the poultry litter biochar.
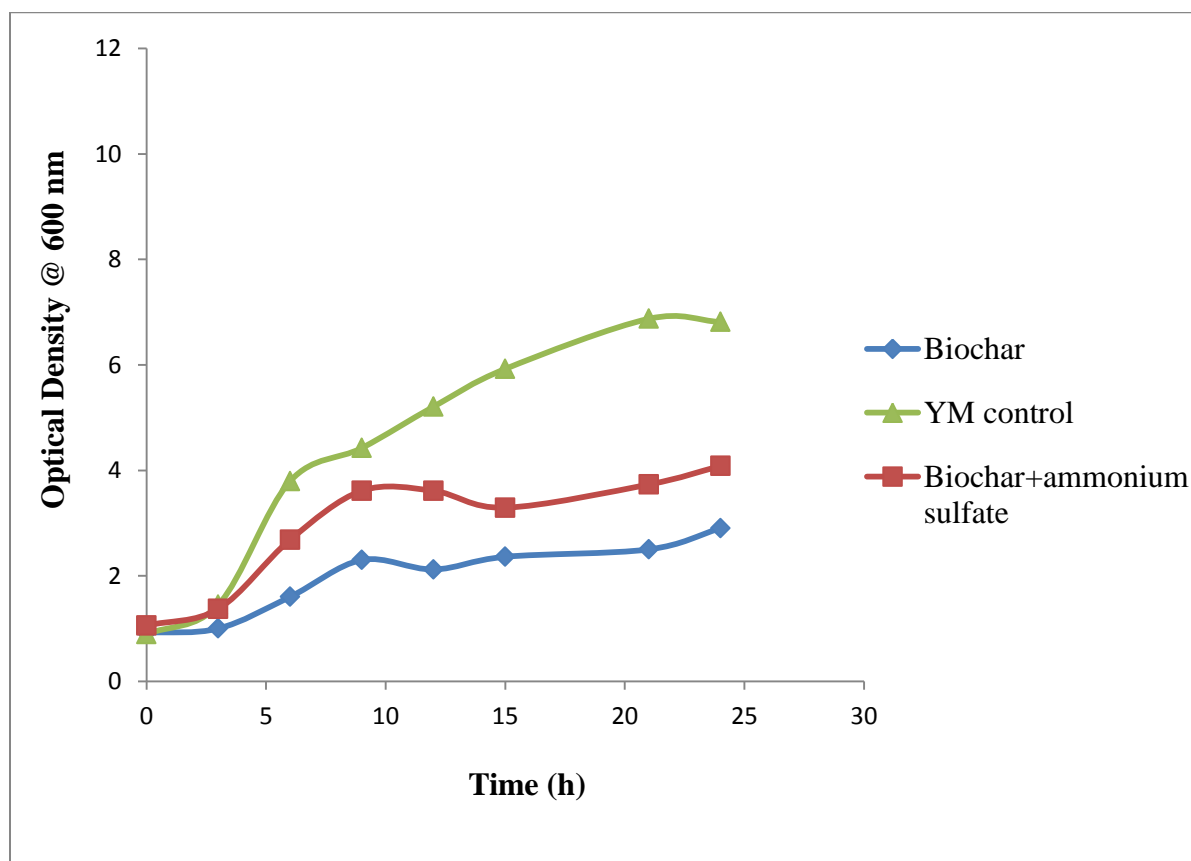
Figure 2.6. *S. cerevisiae* growth in YM medium (21 g/l), biochar (biochar only) medium (20 g/l glucose, and 2 g/l PLB), and biochar + ammonium sulfate medium (20 g/l glucose, 2 g/l PLB, 5 g/l $(NH_4)_2SO_4$)

2.4.3. Anaerobic growth

In Figure 2.7, the growth curves show the anaerobic growth of *S. cerevisiae* in the biochar, YM, and GYE media. The growth curve of the yeast in the biochar was higher and statistically significant from the yeast in the YM medium (p-value = 0.0003 < 0.05) and GYE media (p-value = 0.0382 < 0.05). Figures 2.8 present the optical density, the glucose and ethanol concentration of *S. cerevisiae* under anaerobic condition of the biochar and control GYE media; notice that the initial glucose and yeast extract concentrations were the same in both media.
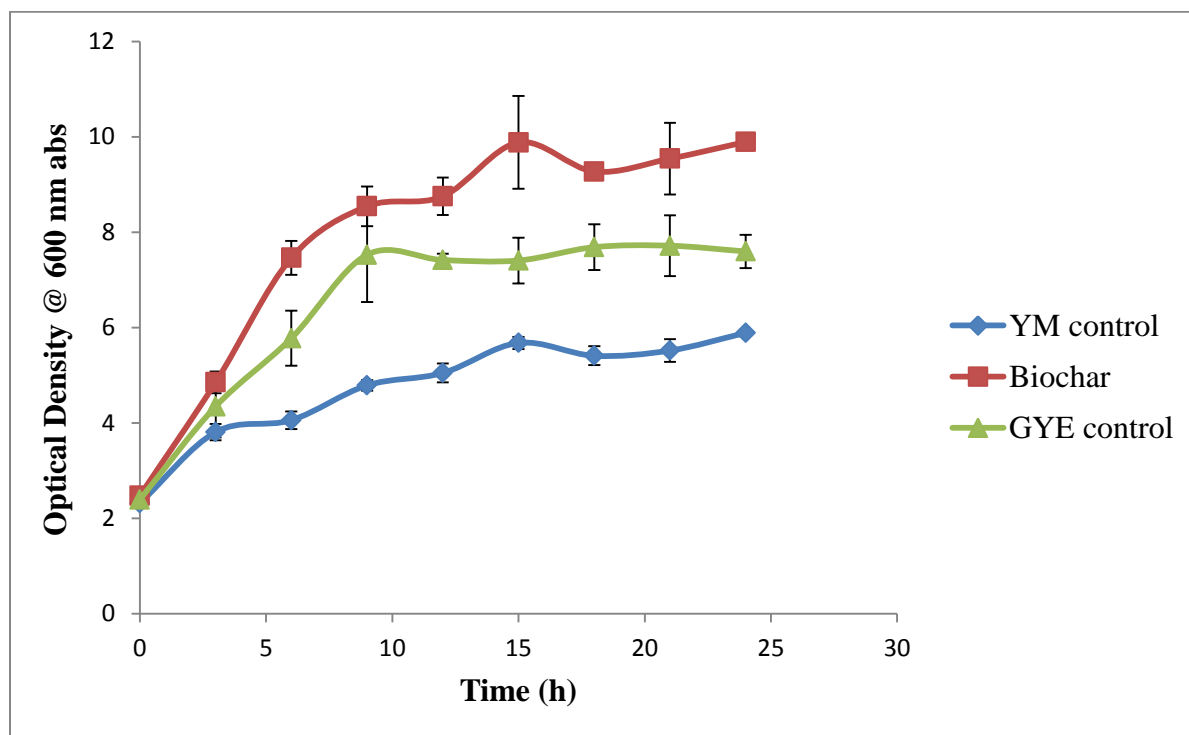
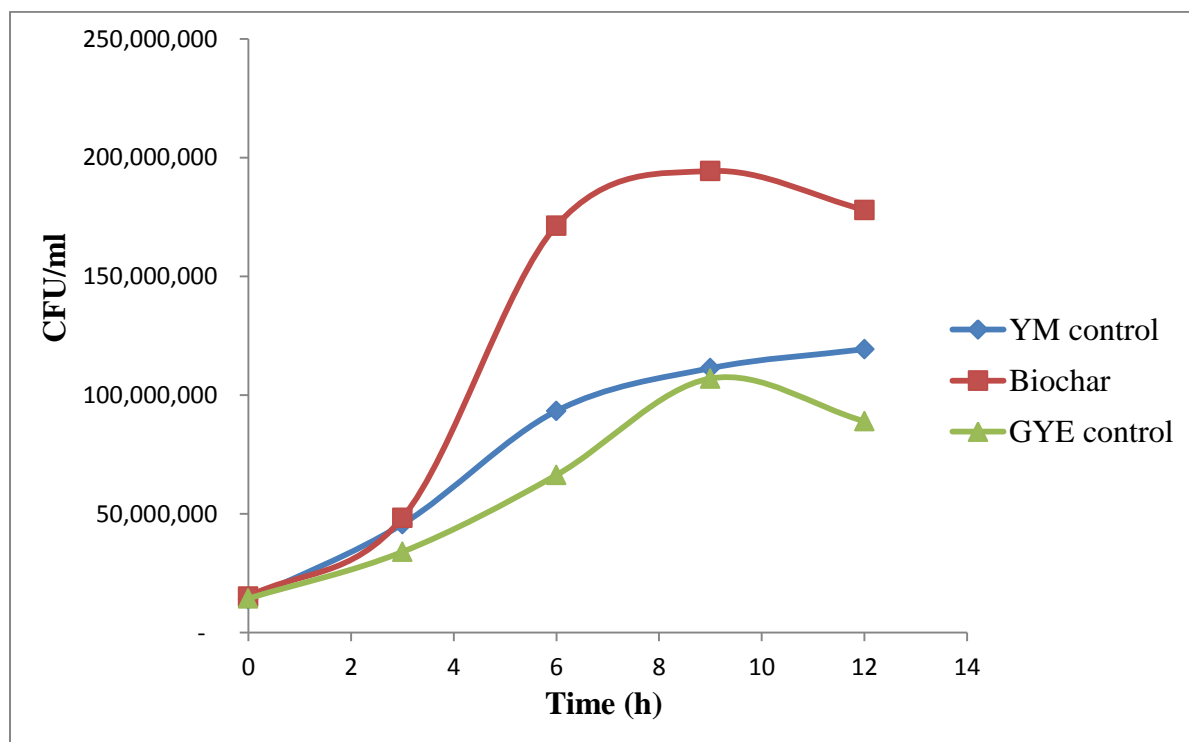Figure 2.7. *S. cerevisiae* growth under anaerobic conditions in YM medium (21 g/l), Biochar medium (20 g/l glucose, 10 g/l yeast extract, 2 g/l PLB), and GYE medium (20g/l glucose, 10g/l yeast extract)

Results confirmed the same growth pattern observed during the growth under aerobic conditions. Biochar medium had higher growth curve than the GYE medium (p-value = 0.0329 < 0.05). The glucose was completely consumed in 12 h in the biochar medium, while less than a half of the glucose in the GYE medium was consumed in 12 h, and it took at least 24 h for the glucose in the GYE medium to be completely consumed.

The ethanol production was faster in the biochar media compared to the control GYE media. The ethanol concentrations were 13.45 g/l in the biochar medium and 2.89 g/l in the GYE medium at 6 h when the initial glucose concentration was 50 g/l (Figure 2.8). In the biochar medium, the maximum ethanol was produced at 9 h, and was 15.46 g/l, then the ethanol started decreasing because the glucose was completely consumed. Whereas, in the GYE medium the maximum ethanol was 16.46 g/l at 9 h and it stabilized because more than half of the glucose still remained in the medium. Results show that glucose was consumed faster in the biochar medium than that in the GYE medium obviously because of the higher cell growth in the biochar medium (Figure 2.8).

When the initial glucose concentration was 100 g/l, the ethanol production in the biochar medium was rapid, the maximum ethanol was produced at 12 h and was 32.3 g/l and then stabilized, whereas the ethanol concentration in the control GYE medium was only 12.5 g/l at 12 h and slowly increased to a maximum of 37.8 g/l at 48 h (Figure 2.9). When the initial glucose concentration was increased to 150 g/l, the maximum ethanol concentration in the biochar medium was reached at 24 h and was 56.3 g/l compared to 30.1 g/l in the control at the same time (Figure 2.10). The slow ethanol production in the GYE control media for initial glucose 100 g/l and 150 g/l might be due the substrate inhibition, however addition of biochar to the high glucose concentration media 100 g/l and 150 g/l promoted the ethanol production at a faster rate than the control.

The optical density of the yeast growth in the biochar medium was higher and doubles the yeast growth in the control medium, and the same growth pattern was observed when the initial glucose concentrations were 100 g/l and 150 g/l (Figure 2.11).

Figures 2.12 and 2.13 are pictures for biochar and control fermentation media respectively at 9 h and 24 h. During the fermentation process it was observed that the flasks with the biochar media started to foam early but the flasks with the control GYE media did not foam during the early fermentation hours. At 9 h fermentation, flasks with the biochar media all had a lot of bubbles, whereas the flasks with the control GYE media did not had any bubbles at 9 h fermentation (Figure 2.12), indicating that the fermentation is faster in the biochar media compared to the control GYE media. This observation corroborates the high cell growth and ethanol production in the biochar media during the early fermentation hours. The fermentation was slower in the control GYE media and the presence of the bubbles was observed during the late fermentation hours. At 24 h fermentation, both flasks with the biochar and control GYE media had fermentation bubbles (Figure 2.13), indicating that the fermentation in the control GYE media preceded as well but it was just slower compared to the biochar media.

2.5. Conclusion

The poultry litter biochar used in the study had a high ash content of 55.27 ± 0.25, a moderate carbon 23.41 ± 1.34 and nitrogen content of 2.33 ± 0.09. Results obtained in this study show that *S. cerevisiae* can grow in the poultry litter biochar medium under both aerobic and anaerobic conditions. When glucose and yeast extract concentration were increased, the yeast growth in the biochar media was higher and statistically significant compared to the growth in the controls YM and GYE media. However,
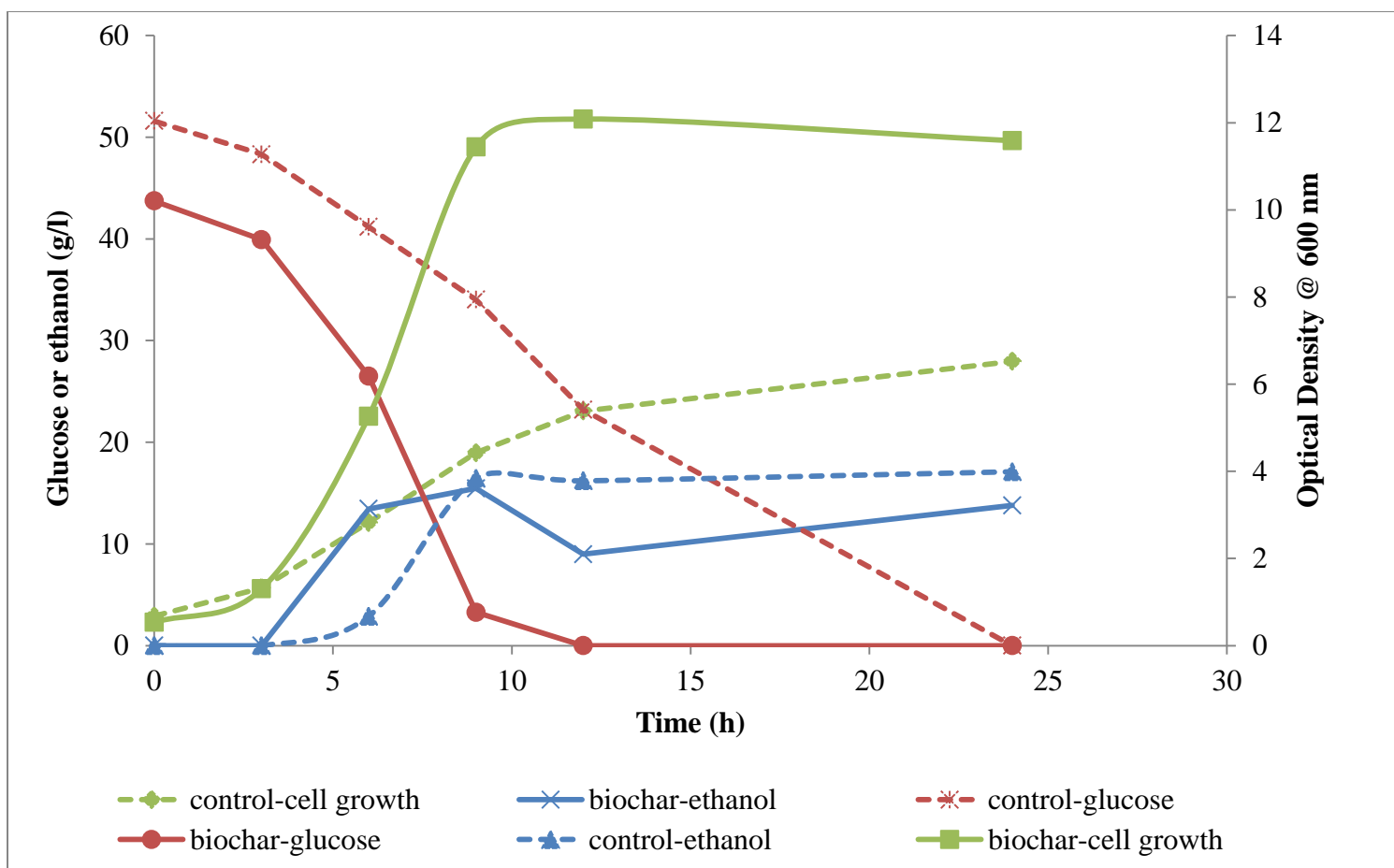
Figure 2.8. *S. cerevisiae* growth under anaerobic condition in the GYE control (50 g/l glucose, 10 g/l yeast extract) and biochar medium (50 g/l glucose, 10 g/l yeast extract, 2 g/l biochar)
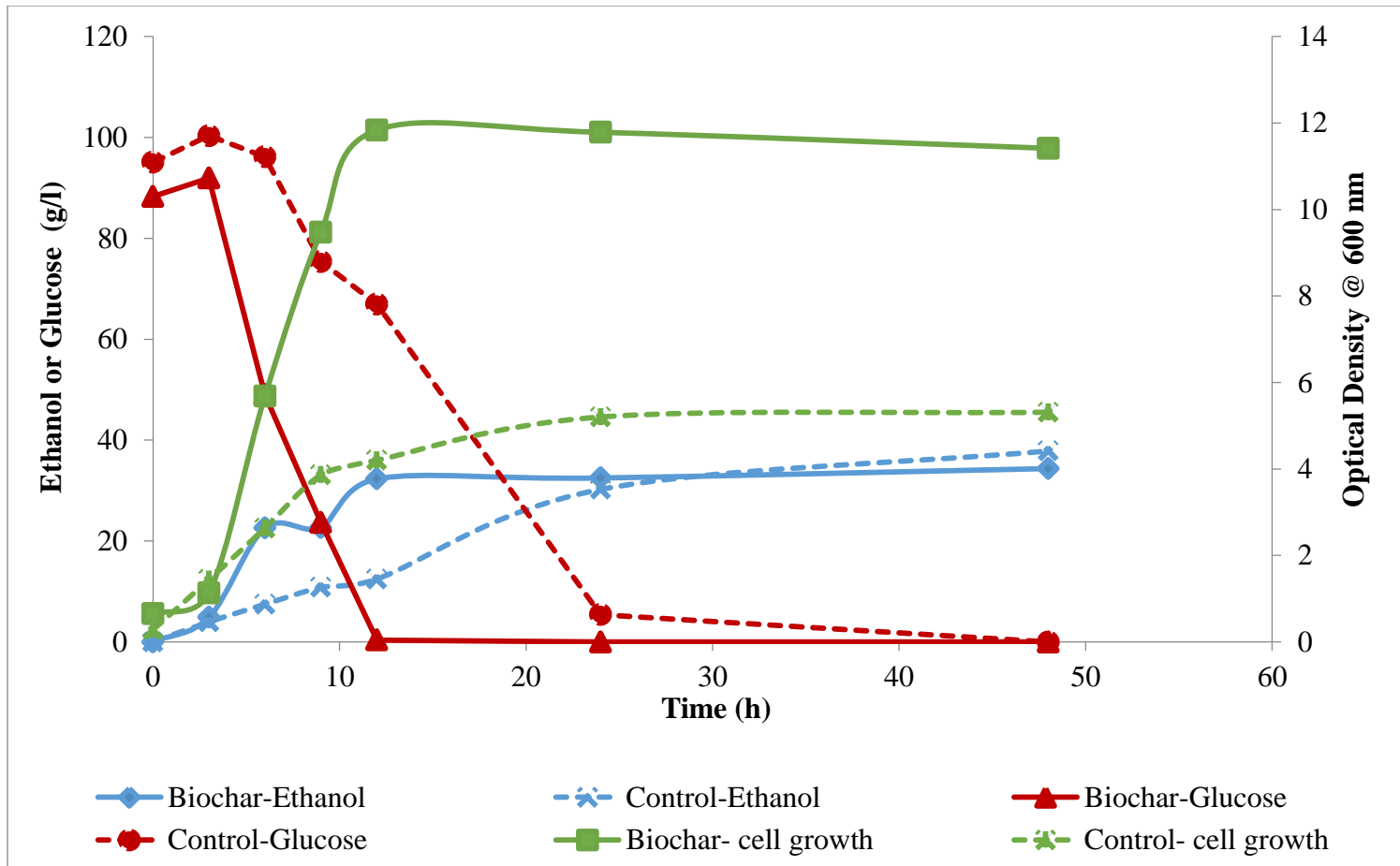
Figure 2.9. *S. cerevisiae* growth under anaerobic condition in the GYE control (100 g/l glucose, 10 g/l yeast extract) and biochar medium (100 g/l glucose, 10 g/l yeast extract, 2 g/l biochar)
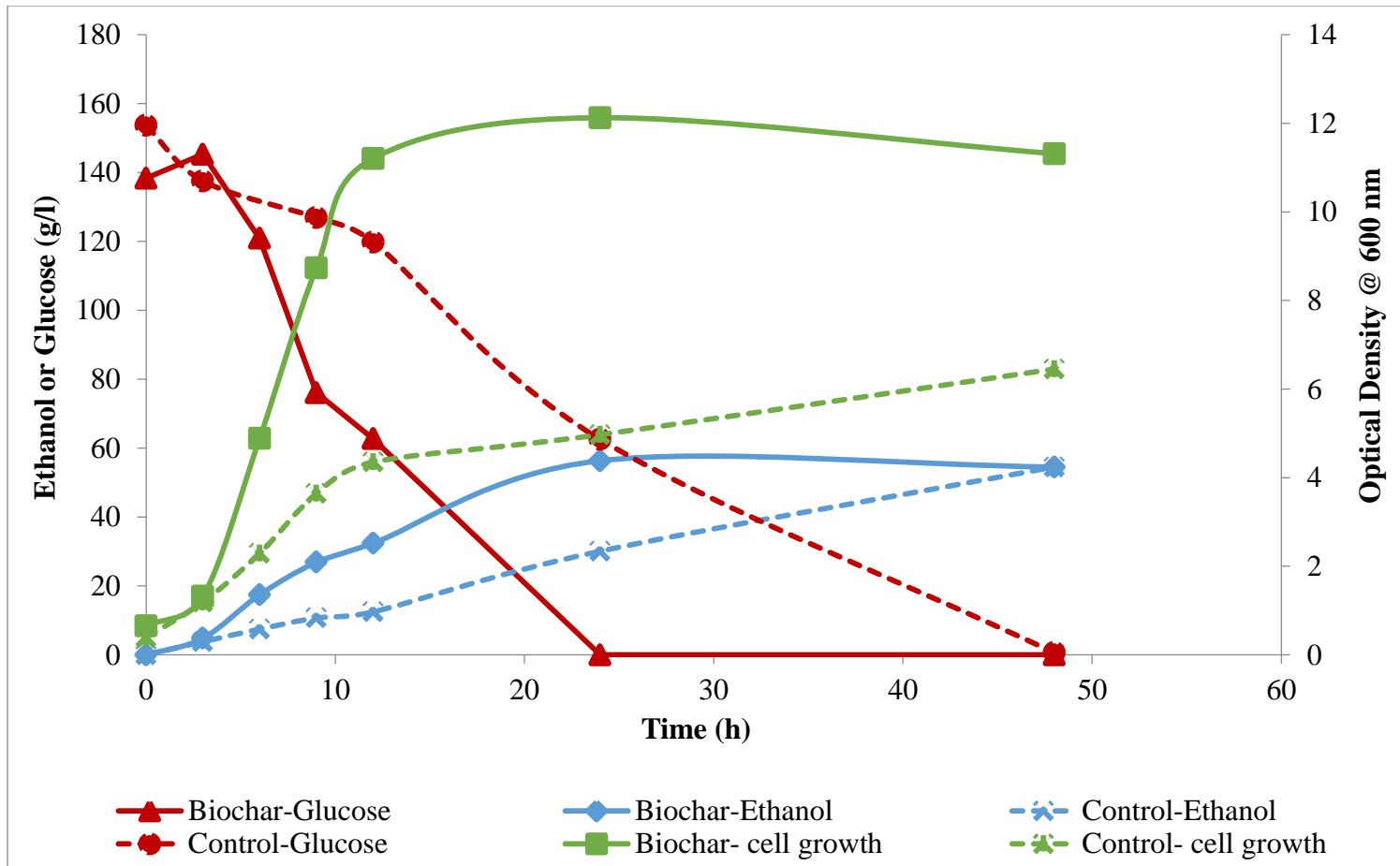
Figure 2.10. *S. cerevisiae* growth under anaerobic condition in the GYE control (150 g/l glucose, 10 g/l yeast extract) and biochar medium (150 g/l glucose, 10 g/l yeast extract, 2 g/l biochar)
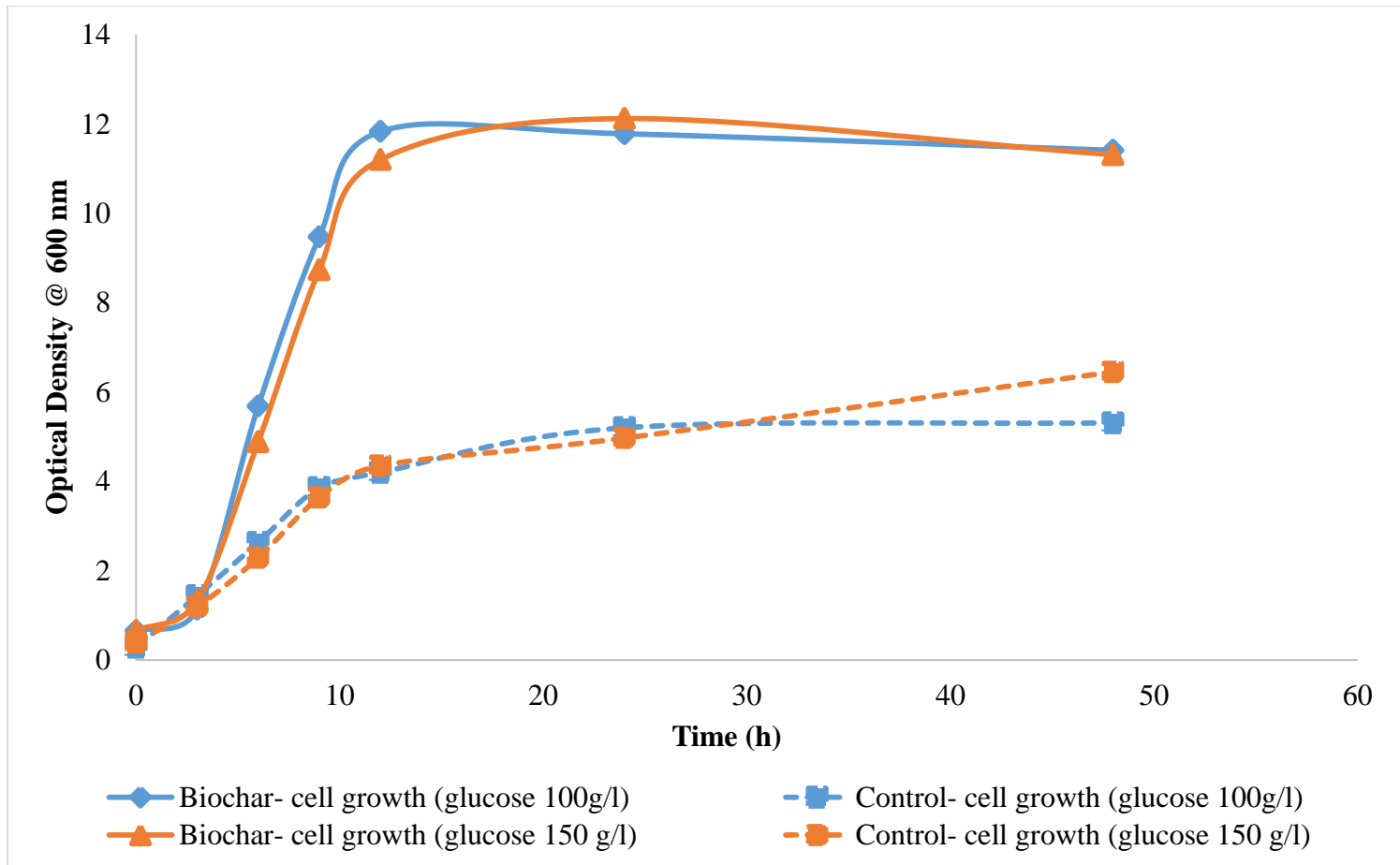
Figure 2.11. S. *cerevisiae* growth under anaerobic condition in the biochar (100 g/l or 150 g/l glucose, 10 g/l yeast extract, 2 g/l biochar) and in the GYE control media (100 g/l or 150 g/l glucose, 10 g/l yeast extract

Figure 2.12. Fermentation of biochar (flasks a and c had bubbles) and control (flasks b and d had no bubbles) media at 9 h

Figure 2.13. Fermentation of biochar (flasks c and d had bubbles) and control (flasks a and b had bubbles) media at 24 h

biochar was not able to supply the medium all the nutrient needed for the yeast growth, addition of a nitrogen source such as yeast extract or ammonium sulfate was found necessary for the biochar to be effective because biochar lacked some bioavailable nitrogen. Ethanol production and glucose consumption were faster in the biochar media compared to the control GYE media. Addition of poultry litter biochar in the high glucose concentration media 100 g/l and 150 g/l stimulated the ethanol production at higher rate compared to the control.

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CHAPTER 3

EFFECT OF POULTRY LITTER BIOCHAR ON ENZYME HYDROLYSIS AND

ETHANOL PRODUCTION FROM STEAM-EXPLODED BIOMASS

3.1. Abstract

The bioethanol yield and productivity obtained during fermentation of pretreated lignocellulosic biomass is normally low because of the presence of inhibiting compounds generated during the pretreatment. In the present study, we used two approaches to improve the ethanol production from steam exploded poplar and corn stover. First, the steam exploded poplar was washed with 0.25 M NaOH in order to remove lignin and potential inhibitors, and the remaining cellulosic biomass was hydrolyzed and fermented. Second, poultry litter biochar was added to the steam exploded poplar and corn stover, and its effect on ethanol production was studied. Results indicate that washing the steam exploded poplar with 0.25 M NaOH significantly improved the cellulose conversion and the ethanol yield. The glucose concentrations were 26.56 g/l for the untreated poplar and 39.02 g/l for the NaOH washed poplar, and the ethanol yield for the untreated poplar and NaOH washed steam exploded poplar were 67.33 % and 83.07 % respectively. Addition of poultry litter biochar to steam exploded poplar and corn stover improved the ethanol productivity up to a maximum of 3.2 g/l-h for poplar at 5% biochar loading, and 2.02 g/l-h for corn stover at 1 % biochar loading. .

3.2. Introduction

Lignocellulosic biomass is an attractive renewable feedstocks for bioethanol production due to their abundance and low-cost (Zaldivar et al., 2001). Bioethanol has received much attention as an alternative fuel to fossil fuels because it produces fewer emissions, no net CO_2, and is compatible with the current fuel distribution (Parawira and Tekere, 2011). Ethanol is currently used as a gasoline additive, for example, blend of 10% ethanol with gasoline is used as a transportation fuel in the US (González-García et al., 2010). Lignocellulosic biomass such as poplar and corn stover is used as feedstocks for bioethanol production. Poplar is a potential bioenergy crop because it has high cellulose content, and moderate lignin and hemicellulose which are desirable for ethanol production (Sannigrahi et al., 2010). In addition, poplar is a short rotation energy crop, and has ability to grow on marginal lands (González-García et al., 2010; Kim et al., 2012; Luo et al., 2002). Corn stover is also a potential feedstock for ethanol production, it is an abundant agricultural residue with low cost value, and contains cellulose and hemicellulose (Aden et al., 2002; Kadam and McMillan, 2003; Li et al., 2004; Zambare and Christopher, 2012).

The bioconversion of lignocellulosic biomass to ethanol requires many steps: pretreatment, enzyme hydrolysis, and fermentation. The purpose of the pretreatment is to break down the lignin structure and render the cellulose and hemicellulose polymers accessible to enzyme (Mosier et al., 2005). Cellulose and hemicellulose can be converted to monomeric sugars through enzyme hydrolysis and subsequently fermented to ethanol using *Saccharomyces cerevisiae.* In the last decade, thermochemical pretreatment such as steam explosion is considered one of the most effective pretreatments (Alvira et al., 2010;

Chiaramonti et al., 2012; Himmel et al., 2007; Mosier et al., 2005; Zhang and Shahbazi, 2011).

However, research has shown that when lignocellulose biomass is pretreated with steam-explosion, in addition to releasing the polymers (cellulose and hemicellulose), inhibitory compounds such as furfural, hydroxymethylfurfural (HMF), formic acid, levulinic acid, and acetic acid are also produced (Li et al., 2011). At high concentrations, these compounds are reported to inhibit the fermenting microorganisms and decrease the ethanol yield and productivity (Parawira and Tekere, 2011). Parawira and Tekere (2011) and Taherzadeh et al. (2000) reported that inhibitory compounds represent a major challenge for the commercial bioethanol production from lignocellulosic feedstocks. Several authors found necessary to incorporate a detoxification step before fermentation (Millati et al., 2002; Miyafuji et al., 2003; Mussatto and Roberto, 2004; Talebnia and Taherzadeh, 2006; Zhang et al., 2013).

Chemical and biological detoxification methods such as: overliming, membrane extraction, ion exchange, activated carbon, and enzyme treatments were used to improve ethanol production (Chen et al., 2013; Grzenia et al., 2012; Larsson et al., 1999; Millati et al., 2002; Parawira and Tekere, 2011). However, each detoxification method presented some advantages and disadvantages. For instance, overliming was shown to improve the ethanol production by 80%, however, the method is expensive, time consuming, and generates by-products which will add high ethanol production costs (Grzenia et al., 2012; Luo et al., 2002; Zhang and Shahbazi, 2011). But the use of poultry litter biochar to improve the production of bioethanol yield of steam exploded lignocellulosic biomass has not been reported. Poultry litter biochar is a by-product from the pyrolysis of

biomass, it is available with low cost value, and contain organic and inorganic materials and some valuables nutrients (Agblevor et al., 2010; Chan et al., 2008).

In this study, we hypothesize that poultry litter biochar can be used to increase the nutrient content of the hydrolysate, enhance microbial growth of yeast *Saccharomyces cerevisiae,* and reduce the toxicity of steam exploded hydrolysates. Poultry litter biochar was first added to steam exploded poplar and corn stover and its effect on enzyme hydrolysis and fermentation was monitored. Second, steam-exploded poplar was washed with 0.25 M NaOH in order to separate the lignin from the cellulosic biomass, and the cellulosic biomass was hydrolyzed and fermented with and without poultry litter biochar addition to produce ethanol.

3.3. Materials and Methods

3.3.1. Steam-exploded Poplar

The composition of the steam-exploded poplar (LSE Sol Res 9/02/05 and 9/05/05) was determined following the ASTM E 1721-01 (Reapproved 2009) procedure. Sulfuric acid (72% H_2SO_4) was used to hydrolyze 300 mg of dry biomass for 2 h at 30 ^{o}C in a water bath. At the end of the hydrolysis, samples were autoclaved for 1 h at 121 ^{o}C; the hydrolyzed substrates were filtered using medium porosity filtering glass crucibles. The filtrate was analyzed to determine the carbohydrate fraction using high performance liquid chromatography (HPLC analysis method was described in section 3.2.10). The solid residues were dried in the oven at 105 ^{o}C for 2 h and weighed. The dried residues were ashed in a muffler furnace (Lindbergh Blue, Thermo Scientific, Asheville, NC) for 3 h and weighed. The following equations were used to calculate percent acid insoluble residue and percent ash:

$$\% \ Acid \ Insoluble = \frac{insoluble \ residue - ash}{raw \ material} \ x \ 100 \qquad (3.2.1)$$

$$\% \ Ash = \frac{ash}{raw \ material} \ x \ 100 \qquad (3.2.2)$$

where,

% Acid Insoluble = percent acid insoluble residue on 105 ^{o}C oven-dried basis

% Ash = percent ash on 575 ^{o}C oven-dried basis

insoluble residue = oven-dried 105 ^{o}C weight of acid insoluble residue (g)

ash = weight of residue ashed at 575 ^{o}C (g)

raw material = initial weight of substrate

3.3.2. Steam-exploded poplar washing with sodium hydroxide

To separate the lignin fraction from the cellulosic biomass, 10 g of steam exploded poplar was dissolved in 1 liter 0.25 M sodium hydroxide (NaOH) solution, the mixture was stirred for 20 min. The mixture was then vacuum filtered to separate the black liquor and the solid biomass using 0.2 micron glass fiber filter paper. The black liquor was recovered and kept for the lignin recovery. The solid biomass was rinsed with approximately 600 ml of deionized-water and dried in the oven at 105 ^{o}C for 2 h and weighed. The lignin was recovered from the black liquor by adding a polymer (ZETAG 4125) solution, and concentrated acid. About 50 ml of the filtrate was poured in a first beaker (1) and another 50 ml of the filtrate in a second beaker (2), the filtrate was diluted by adding 50 ml of deionized water to each beaker, then, 2.5 ml of 0.25% g/ml polymer (ZETAG 4125) solution was added to both beakers, and stirred for 1 min.

Two different acids, phosphoric acid and citric acid were used to determine which produced the highest lignin recovery. Two and half milliliters of 50% phosphoric acid or

2.5 ml of 50% citric acid was diluted with 12.5 ml of deionized water; the phosphoric acid solution was added to the first beaker, and the citric acid solution to the second beaker containing the filtrate and the polymer. The mixtures were vacuum filtered using 0.2 micron glass fiber filter paper to recover the solid lignin from each solution, the lignin had a brownish color and the liquid was light yellow. The solid lignin was dried in the oven at 105 ^{o}C for 2 h and weighed and the liquid filtrate was analyzed by HPLC to determine the presence of sugar. Figure 3.2.1 show the different fractions obtained during the process. The following equations were used to calculate percent solid biomass and lignin recovered from the initial 10 g steam-exploded poplar:

$$\% \ carbohydrate \ = \frac{solid \ biomass}{initial \ biomass} \ x \ 100 \qquad\qquad (3.2.3)$$

$$\% \ lignin \ = \frac{solid \ lignin}{initial \ biomass} \ x \ 100 \qquad\qquad (3.2.4)$$

where,

% carbohydrate = percent of total biomass recovered after washing the steam exploded poplar with 0.25 M NaOH solution (%)

solid biomass = oven dried weight of the washed solid biomass (g)

% lignin = percent of total lignin recovered after washing the steam exploded poplar with 0.25 M NaOH solution (%)

solid lignin = oven-dried weight of the solid lignin (g)

initial biomass = initial weight of the steam exploded biomass (g)

3.3.3. Acid hydrolysis of substrates

Mircrocystalline cellulose, the carbohydrate portion of the washed poplar, and the unwashed poplar were acid hydrolyzed using 72% H_2SO_4 following the method described

above (section 3.2.1). The filrate were collected and analyzed by HPLC to determine the sugars. The cellulose conversion to glucose was calculated using the following equation:

$$\% \ carbohydrate \ conversion = \frac{glucose}{celluose} \ x \ 100 \qquad (3.2.5)$$

where,

carbohydrate conversion = percent of carbohydrate converted to glucose (%)

glucose = glucose concentration (mg/ml)

cellulose = concentration of available cellulose (mg/ ml)

3.3.4. Enzyme hydrolysis of substrates

Microcrystalline cellulose, the carbohydrate portion of the washed poplar, and the unwashed poplar were hydrolyzed using *CTec2*. 4 g of microcrystalline cellulose, 4 g of washed poplar, and 8 g of unwashed poplar were weighed and added to screw cups flasks, triplicate samples were prepared for each substrate, and 100 ml of citrate buffer (pH 4.8) was added to each flask. Flasks were autoclaved at 121°C for 30 min, *CTec2* was added at a dosage of 30% (g *CTec2*/ g cellulose), and flasks were incubated at 50°C and 130 rpm for 96 h. Samples were taken every 24 h to analyze the glucose concentration by HPLC. Cellulose conversion to glucose was calculated using equation (3.2.5).

3.3.5. Poultry litter biochar

The biochar used in this study was obtained from our lab, the initial poultry litter biomass was a mixture of buff and berry, the biomass was pyrolyzed at 500 ^{o}C in a fluidized bed reactor as described by (Agblevor et al., 2010). The elemental composition

and ash content of the biochar were determined by the method previously described in Chapter 2 (section 2.3.2).

3.3.6. Preparation of DNS reagent

The DNS reagent was prepared by adding 1 g of NaOH into 100 ml of deionized water, the mixture was stirred, and 18.2 g of potassium tartrate was added with continued stirring. When compounds dissolved, 1 g of 3, 5-dinitrosalicylic acid was added with continued stirring, 0.05 g of Na_2SO_3 and 0.2 g of phenol were then added with continued mixing until it all dissolved. The solution was stored in an amber bottle due to the light sensitivity of the DNS solution.

3.3.7. Enzyme activity

The enzyme used in this study was *CTec2* donated by Novozymes (Farmington, NC). The filter paper activity of the enzyme was measured using FPU by IUPAC procedure (Adney and Baker, 2008). The enzyme was first diluted with citrate buffer at a ratio of 1: 20, 50 mg of Whatman filter paper was added in a test tube, 1 ml of citrate buffer (pH 4.8) was added, 0.5 ml of the diluted enzyme was then added, and the mixture was incubated at 50 $^{\circ}$C for 1 h. Control test tubes were also prepared, the blank reagent contained only 1.5 ml citrate buffer, the enzyme control contained 1ml citrate buffer and 0.5 ml of diluted enzyme, and substrate control had 1.5 ml citrate buffer and 50 mg of filter paper strip. This was done in order to subtract any sugars that may have been dissolved in the enzyme solution or substrate samples before estimating the concentration of sugars hydrolyzed by the enzyme. After 1 h of incubation, the test tubes were removed and inserted into a boiling water bath for 5 min in order to deactivate the enzyme. The

amount of sugars released by the enzyme was estimated by the DNS method (as described below, section 3.2.10). The FPU was calculated using the following equation:

FPU / ml = ((concentration of sugars after 1 hour) x volume of solution x dilution factor)/ (0.18016 x 60 min)   (3. 2.6)

3.3.8. Enzyme hydrolysis

3.3.8.1. Steam-exploded poplar

Poultry litter biochar was added to the unwashed poplar and hydrolyzed using *CTec2*. Different concentrations of biochar were first tested on the unwashed biomass. Ten grams of the unwashed poplar was added to screw cup flasks, biochar (0.0 g, 0.2 g, 0.5 g, 1 g, 5 g, and 10 g) were added to each flask. One hundred milliliters of citrate buffer (pH 4.8) was added to each flask, and flasks were autoclaved at 121 ^{o}C for 30 min. The flasks were cooled to room temperature under laminar hood, and *CTec2* was added to each flask at a loading of 17 FPU/g (g of biomass dry matter). The flasks were then incubated at 50 ^{o}C and 130 rpm in a water bath shaker incubator (ALT, Precision, reciprocal shaking bath, East Lyme, CT) for 48 h.

3.3.8.2. Sodium hydroxide washed steam-exploded poplar

The washed biomass was hydrolyzed with and without biochar addition following the conditions described above (section 2.8.1). The biomass, biochar, and *CTec2* loadings were 10 wt. %, 1 wt. %, and 10 FPU/g respectively. Lower enzyme loading was used in this experiment in order to minimize the cost of hydrolysis.

3.3.8.3. Steam-exploded corn stover

The steam-exploded corn stover was hydrolyzed with and without biochar addition at the conditions mentioned above (section 2.8.1). The biomass loading was 10 wt. %, biochar loadings of 0.2 wt. % and 1 wt. % were used, and the *CTec2* loading was 10 FPU/g.

3.3.9. Fermentation

Hydrolysates fermentation was carried out using *S. cerevisiae* ATCC 204508/S288C. The inoculum was cultivated using YPD medium one day before the fermentation day; the cultures were incubated at 37 °C and 220 rpm. The cells were harvested after 16 h; the optical density at 600 nm was 6.16. The cells were centrifuged at 3000 x g for 10 min under sterile condition, the supernatant was discarded, and the cells were transferred to 250 ml screw-capped Erlenmeyer flasks containing the hydrolysates. Flasks were purged with nitrogen for 5 min then capped to allow fermentation but not completely tight in order to allow the CO_2 to escape. The cultures were incubated at 37 °C and 130 rpm in a water bath incubator shaker incubator. Fermentation samples were taken and analyzed for glucose and ethanol.

3.3.10. Methods of analysis

3.3.10.1. High performance liquid chromatography (HPLC) analysis of substrates

Hydrolysate samples were filtered through 0.2 µm nylon membranes and analyzed to determine the sugar concentration using high performance liquid chromatography (HPLC) method. The HPLC (LC-10AT Shimadzu) was equipped with evaporative light scattering detector (ELSD), an auto sampler (SIL-Shimdzu), and a

Prevail™ carbohydrate column (250 mm x 4.6 mm). The column temperature was 30 $^{\circ}$C, the detector temperature and pressure were 50 $^{\circ}$C and 350 kPa, respectively. The mobile phase composition was 20/80 water/acetonitrile, and the flow rate was 1 mL/min.

3.3.10.2. Ethanol extraction

All the chemicals used in this work were analytical grade, 99.5 % purchased from Sigma Aldrich (Sigma Aldrich, St Louis, MO). Steam-exploded poplar or corn stover biomass was dissolved in citrate buffer at 10 wt. % loading, the mixture was autoclaved, centrifuged, and the liquid biomass was used to prepare the calibration curve. Ethanol concentrations (1% v/v to 5% v/v) were prepared in the liquid biomass containing 1% n-propanol as internal standard. Methyl Isobutyl Ketone (MIBK) was added (0.5 ml x 3) to extract the ethanol and the propanol. The mixture was vortexed and centrifuged for 5 minutes at 3000 x g, and the upper layer was subjected to a gas chromatography (GC) analysis. Peak area ratios of the ethanol vs. n-propanol was calculated and plotted against ethanol concentrations (% v/v).

At the end of the fermentation, the broth was centrifuged in order to separate the biomass and the cells from the liquid broth. Before injecting the samples into the GC, extraction of the organic phase from the liquid broth was performed to avoid having the sugars into the GC column. Five hundred microliters of the fermentation broth was added to a centrifuge tube, and 5 μl of n-propanol was added as internal standard. The mixture was vortexed for 30 seconds, then (0.5 ml x 3) of MIBK was added; the mixture was again vortexed for 5 min. Finally, all tubes were centrifuged for 5 min at 3000 x g to facilitate phase separation. The organic phase (upper layer) was recovered and manually injected into GC for ethanol analysis.

3.3.10.3. Gas chromatographic

Analysis of ethanol and propanol was performed using HP 6890 Series GC, equipped with an Agilent column (30 m capillary column HP-5, 30m x 0.320 mm x 1.00 µm Id), and a flame ionization detector (FID). One microliter of the upper layer of the extract was injected at the following conditions: the detector and inlet temperatures were 300 ^{o}C, and 250 ^{o}C, respectively, the oven was programmed at initial temperature of 40 ^{o}C; this temperature was held for 1.7 min, the ramp was 15 ^{o}C/min, and the final temperature was 250 ^{o}C and held for 2.67 min. The ethanol was quantified using the calibration curve previously developed.

3.3.10.4. DNS method

Reducing sugars were determined using the DNS-assay and glucose was used for calibration curve (Miller, 1959). The DNS reagent was prepared as described above (section 3.2.6). Glucose concentrations (0.1 mg/ml to 1.0 mg/ml) were prepared in deionized water. Then 0.5 ml of each glucose solution or hydrolysate was added to a test tube and 0.5 ml of the DNS reagent was added, the mixture was boiled for 10 min at 90 ^{o}C in a water bath, and then the test tubes were inserted in iced water. The color formation was measured by a spectrophotometer (Spectra Max Plus 384, Molecular Device, Sunnyvale, CA) at 540 nm. The absorbance value and concentration of glucose solution were used to develop a calibration curve. The absorbance value of the hydrolysate was obtained and the glucose calibration curve was used to determine the reducing sugars concentration of the hydrolysate.

```
┌─────────────────────────┐        add        ┌─────────────────────────┐
│  Steam-exploded Biomass │ ◄──────────────►  │  Poultry litter biochar │
│   (Poplar/corn stover)  │                   │                         │
└─────────────────────────┘                   └─────────────────────────┘
             │                                              │
             └──────────────────────┬───────────────────────┘
                                     ▼
┌────────────┐            ┌─────────────────────────┐
│   CTec2    │ ─────────► │    Enzyme Hydrolysis    │
└────────────┘            └─────────────────────────┘
                                     │
                                     ▼
                          ┌─────────────────────────┐
                          │    Fermentable sugars   │
                          └─────────────────────────┘
                                     │
                                     ▼
┌────────────────┐        ┌─────────────────────────┐
│  S. cerevisiae │ ─────► │      Fermentation       │
└────────────────┘        └─────────────────────────┘
                                     │
                                     ▼
                          ┌─────────────────────────┐
                          │        Extraction       │
                          └─────────────────────────┘
                                     │
                                     ▼
                          ┌─────────────────────────┐
                          │         Ethanol         │
                          └─────────────────────────┘
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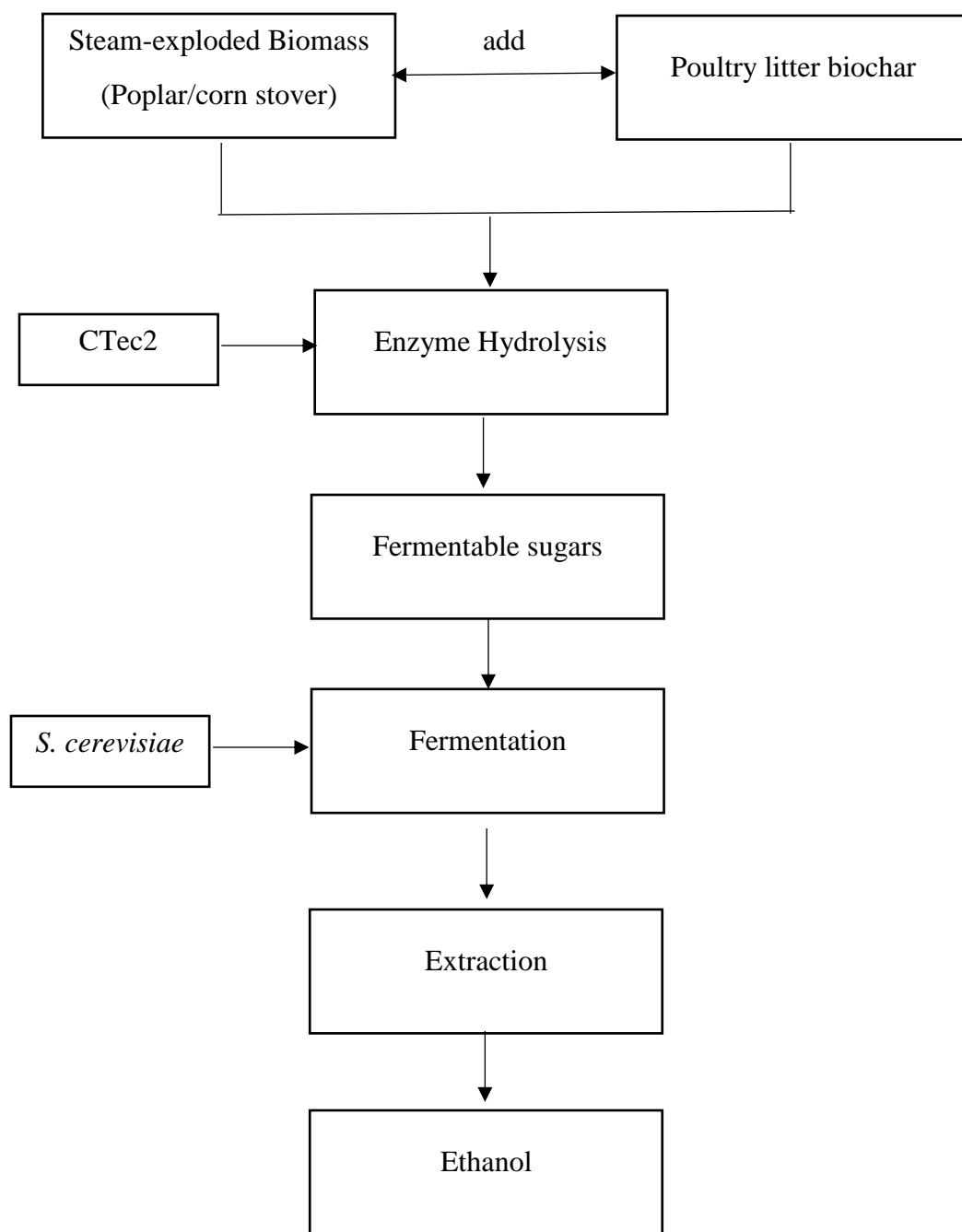
Figure 3.1. Flowchart outlining the general scheme employed in the hydrolysis and fermentation of steam-exploded poplar and corn stover

3.4. Results and Discussion

3.4.1. Composition of steam-exploded poplar

The steam-exploded poplar was composed of 28.5 ± 1.3 % acid insoluble, 0.5 ± 0.2 ash, and 57.4 ± 1.7 % glucose. The individual sugars such as xylose, arabinose, mannose, and galactose did not appear after the acid hydrolysis, this might be due to the severity factor used during the explosion. These results are in accordance with the literature where the xylan content of cotton gin waste, and yellow poplar were reported to decrease during steam explosion as the severity increased, and arabinan, galactan and mannan were completely degraded at higher severities (Jeoh and Agblevor, 2001) . Also, the results can be explained by the fact that the steam exploded poplar used in this study was washed with water after pretreatment, which probably removed some sugars during the washing.

3.4.2. Solid recovery

Traditionally, sodium hydroxide has been used to pretreat lignocellulosic biomass in order to remove lignin and improve cellulose digestibility (Li et al., 2004; Zhang and Shahbazi, 2011). In this study, when the steam-exploded poplar was added to 0.25 M NaOH solution, the lignin completely dissolved in the solution, and the carbohydrate fraction was separated from the solution by filtration. The filtrate was further coagulated to recover the solid lignin by adding Zetag 4125 polymer and phosphoric or citric acid. Figure 3.2.1 show the different fractions obtained during the process. Table 3.1 shows the solid recovered after washing. When phosphoric acid was used, the recovered carbohydrate and lignin fractions were 65.3%, and 30.4%, respectively. When citric acid was used, the recovered lignin fraction was 16%.

The recovered total solid (cellulose and lignin) percentages were 95.7% and 81.3% when phosphoric and citric acids were used respectively. To account for the missing 18.7% when citric acid was used, the filtrate (yellow liquid) was hydrolyzed with *CTec2* for 48 h, after lignin recovery in this case, however, noted that the sugar concentration was low.

3.4.3. Acid hydrolysis of washed poplar

After being washed with NaOH, the recovered biomass was hydrolyzed with 72% H_2SO_4 along with microcrystalline cellulose and the unwashed steam-exploded poplar. The conversion of cellulose to glucose was calculated using equation (2.5). Figure 3.3 shows that the glucose yields were 106.6%, for the microcrystalline cellulose, 99.2% for the washed biomass (cellulosic fraction), and 64.54% for the unwashed poplar. The high glucose yield of the washed biomass indicates that the biomass was almost pure cellulose.

3.4.4. Enzyme hydrolysis of washed poplar

The cellulose conversion by enzyme hydrolysis was 81.71% for the cellulosic fraction of the washed poplar, 60.91% for the microcrystalline cellulose, and 66.90% for the unwashed poplar (Figure 3.4). Microcrystalline cellulose (MC cellulose) had the lowest conversion due to its crystallinity, while the washed biomass had the highest (20.8% higher than the MC cellulose). This result can be explained by the high crystallinity index of the MC cellulose (ranging from 70% to 80%), which makes its degradation by enzyme difficult (Ji et al., 2012; Matthews et al., 2006). The crystallinity index is a factor used to determine the hydrolysis rate of cellulose and studies show that substrates with high crystallinity index are less accessible to enzyme and therefore more

Washed biomass

Steam exploded
poplar

0.25 M NaOH
solution

Polymer: Zetag 4125
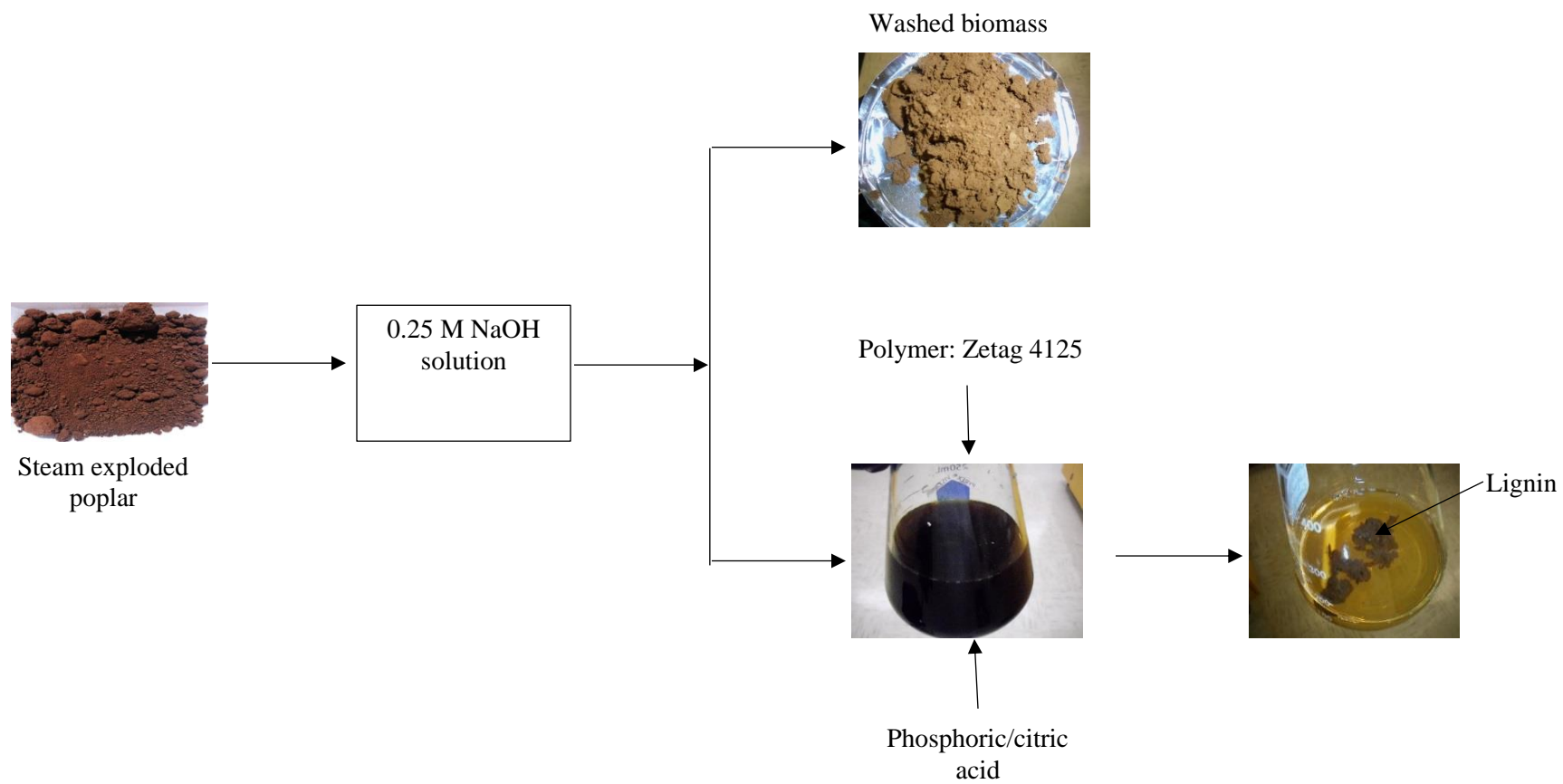
Lignin

Phosphoric/citric
acid

Figure 3.2. Flowchart outlining the general scheme employed in lignin recovery of steam-exploded NaOH washed poplar

Table 3.1. Solid recovery of NaOH washed steam-exploded poplar

Acid	Initial Biomass (g)	Carbohydrate fraction (g)	Lignin fraction (g)	Carbohydrate (% w/w)[a]	Lignin (% w/w)[b]	Total solid (% w/w)[c]
Phosphoric	10	6.53	3.04	65.26	30.40	95.70
Citric	10	6.53	1.60	65.26	16.0	81.30

[a] cellulosic (%) = cellulosic portion recovered (g) / initial biomass (g) x 100

[b] lignin (%) = lignin portion recovered (g) / initial biomass (g) x 100

[c] total solid (%) = (cellulosic portion + lignin portion) (g) / initial biomass (g) x 100

difficult to hydrolyze (Ji et al., 2012; Monschein et al., 2013). The presence of lignin in the lignocellulosic biomass was also reported to reduce the cellulose accessibility and slow down the hydrolysis rate during enzyme hydrolysis of lignocellulosic biomass (Berlin et al., 2006; Jørgensen et al., 2007; Monschein et al., 2013). In this study, the cellulose conversion of the steam-exploded poplar was improved by 22% when lignin was separated from the cellulosic biomass by washing with NaOH (Figure 3.4).
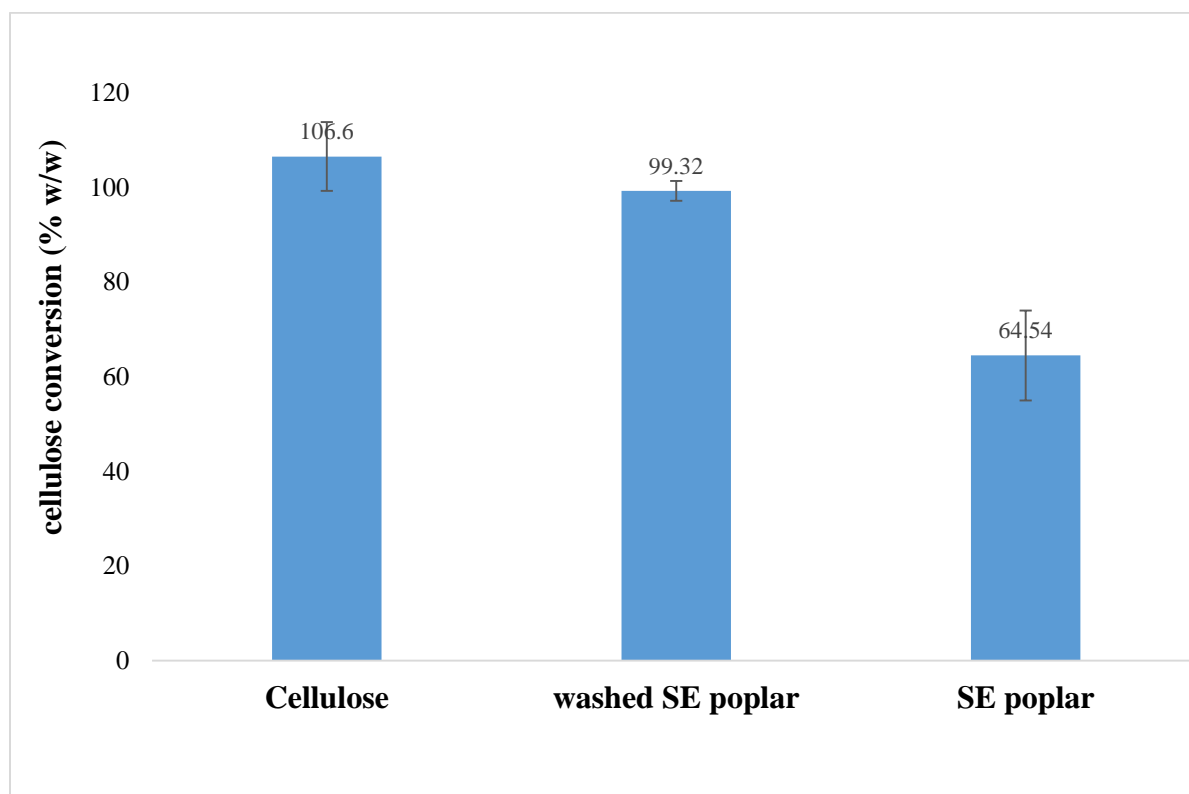

Figure 3.3. Acid hydrolysis of various feedstocks to glucose: cellulose (microcrystalline cellulose), washed SE poplar (NaOH washed steam-exploded poplar), and SE poplar (steam-exploded poplar)
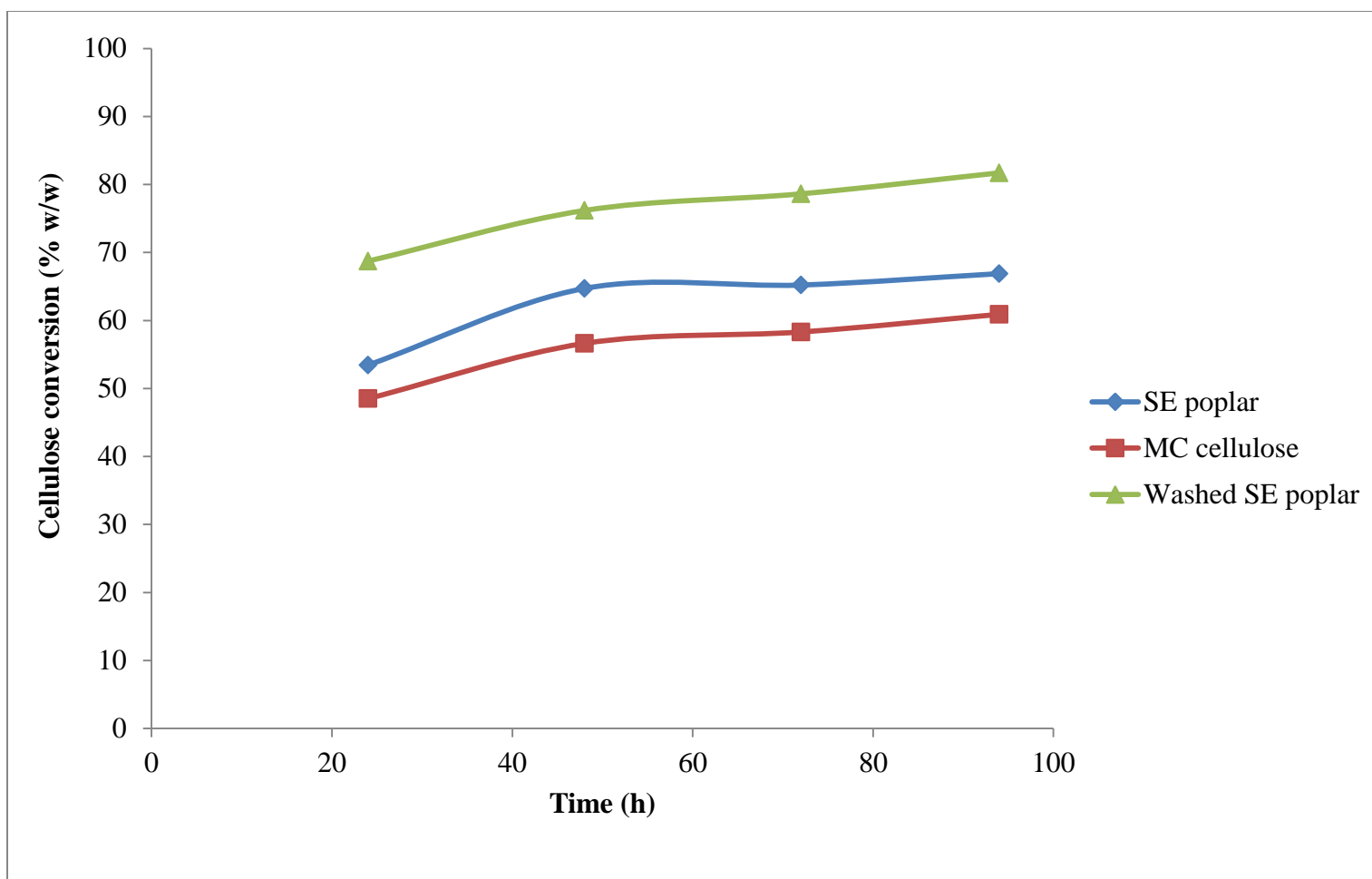
Figure 3.4. Eznyme hydrolysis of various feedstocks: MC cellulose (microcrystalline cellulose), washed SE poplar (NaOH washed steam-exploded poplar), and SE poplar (steam-exploded poplar)

3.4.5. Effect of poultry litter biochar on steam exploded poplar

The filter paper unit of the *CTec2* was 96 FPU/ml from the hydrolysis. The glucose concentrations after enzyme hydrolysis for samples' control (0 % biochar) and samples with biochar concentations of 0.2%, 0.5%, and 1% were 45.51 g/l, 43.61 g/l, 45.61 g/l, and 45.95 g/l, respectively (Figures 3.5, 3.6, and 3.7). Samples' Control and samples with biochar had a similar glucose concentration indicating that biochar at low concentration (0.2%, 0.5%, and 1%) did not have effect on the enzyme hydrolysis of the steam exploded poplar. However, when the biochar concentrations were 5% and 10%, the glucose concentration were lower than the control (38.41 g/l for 5% , and 31.45 g/l for 10%), indicating that biochar at high concentration can inhibit the enzyme hydrolysis (Figures 3.8 and 3.9). Studies show that poultry litter biochar contained some inorganic materials, such as Fe, Mn, Cu, Zn, Ni (Agblevor et al., 2010; Chan et al., 2008; Song and Guo, 2012), these materials at high concentration might inhibit the enzyme hydrolysis.

During the fermentation, the glucose was consumed faster by the yeast when biochar was added, as the biochar concentration increased, glucose consumption was even faster, this was also observed in the previous Chapter 2 (Effect of poultry litter biochar on the growth of *Saccharomyces cerevisiae* and ethanol production). The maxium ethanol was produced at 24 hours for the control sample (0% biochar), 12 h for the samples with lower biochar loadings (0.2%, 0.5%, and 1%), and at 6 h for the samples with higher biochar loadings (5%, and 10%). The maximum ethanol concentration for the control, 0.2%, 0.5%, 1%, 5%, and 10% biochar were 23.6 g/l, 22.3 g/l, 20.6, 20.7 g/l, 19.22 g/l, and 13.7 g/l, respectively (Figure 3.10).

The maximum ethanol produced decreased as the biochar concentration increased, this might be due to the susbstrate depletion in the biochar samples because we have

observed in Figures 3.8 and 3.9 that the enzyme hydrolysis of high biochar loading samples resulted a lower glucose concentration and the glucose was almost or completely consumed within 6 h.

The addition of biochar increased the ethanol porductitivity when compared to the control, as shown in Figure 3.11. The maximum ethanol productivity occurred around 6 h for the samples with biochar concentrations of 0.2%, 0.5%, 1%, 5%, and 10%; the ethanol concentrations were 1.94 g/l-h, 1.84 g/l-h, 2.26 g/l-h, 3.20 g/l-h, and 2.28 g/l-h respectively. The ethanol maximum concentration for the 10% biochar loading was 13.7 g/l versus 19.22 g/l for the 5% biochar loading. The decrease in the productivity from 3.20 g/l-h for the 5% biochar loading to 2.28 g/l-h for the 10% biochar loading was due to the decrease in ethanol production in case of the 10% biochar loading. The maximum ethanol productivity occurred at 12 h for the control sample and was 1.68 g/l-h. The sample with 5% biochar loading had the highest productivity (3.2 g/l-h) while the control had the lowest one (1.68 g/l-h).

3.4.6. Effect of poultry litter biochar on washed steam-exploded poplar

The glucose concentration increased significantly when the steam-exploded poplar was washed with 0.25 M NaOH and hydrolyzed with CTec2 enzyme. The glucose concentrations were 26.56 g/l for the unwashed biomass and 39.02 g/l for the washed biomass (Figure 3.12). The reducing sugars concentrations for the unwashed and washed biomass were 52.19 g/l and 58.88 g/l, respectively (Figure 3.13). Studies have shown that the pretreatment of lignocellulosic with NaOH can facilitate the removal of lignin and therefore increase the degree of enzymatic hydrolysis (Gupta and Lee, 2009; Zhang and Shahbazi, 2011). The addition of poultry litter biochar did not have a significant effect on

Figure 3.5. Ethanol production by *S. cerevisiae* from steam-exploded poplar with control and 0.2% biochar addition

Figure 3.6. Ethanol production by *S. cerevisiae* from steam-exploded poplar with control and 0.5% biochar addition

Figure 3.7. Ethanol production by *S. cerevisiae* from steam-exploded poplar with control and 1% biochar addition

Figure 3.8. Ethanol production by *S. cerevisiae* from steam-exploded poplar with control and 5% biochar addition
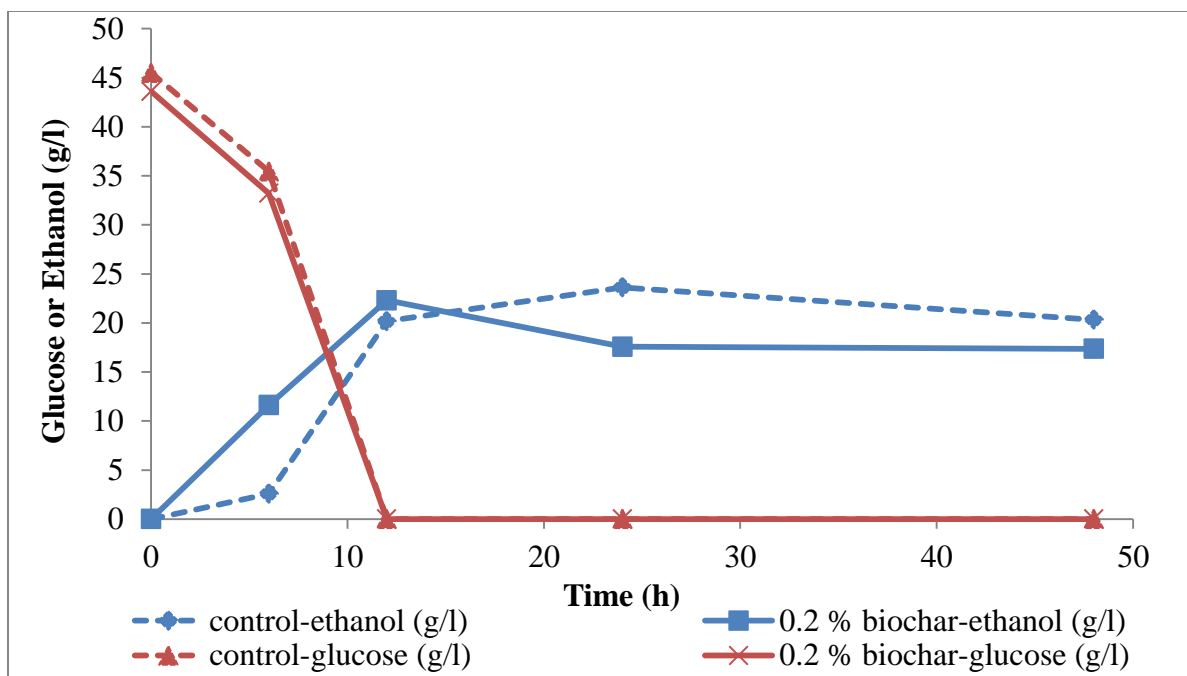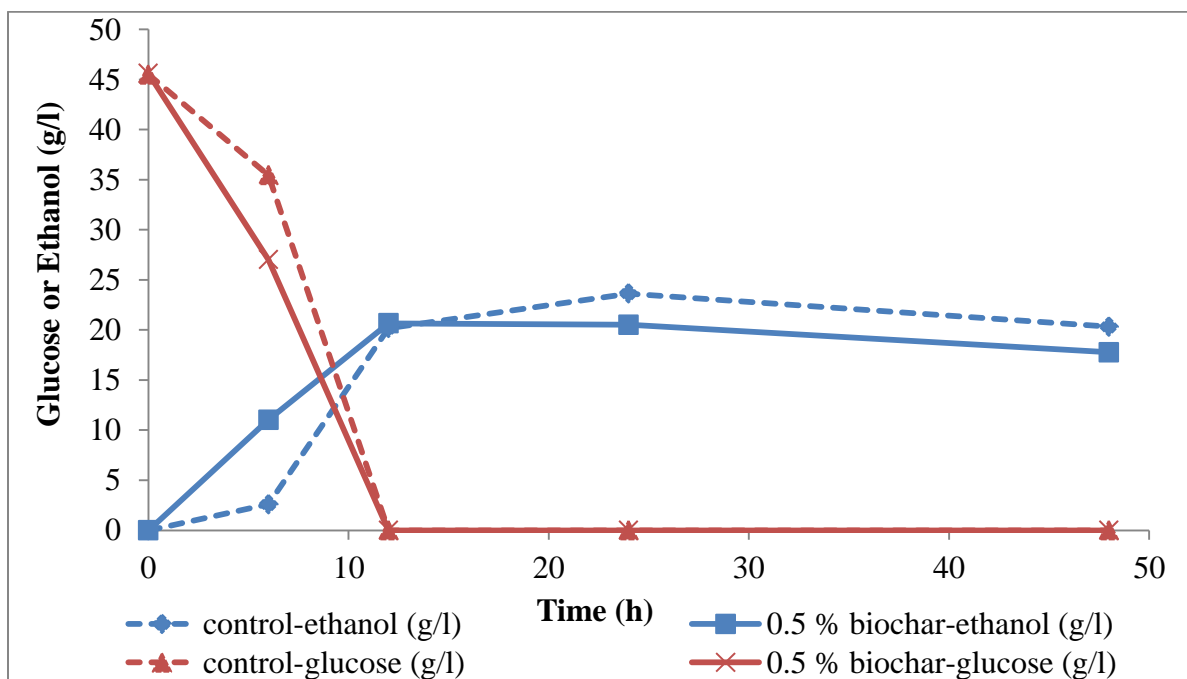
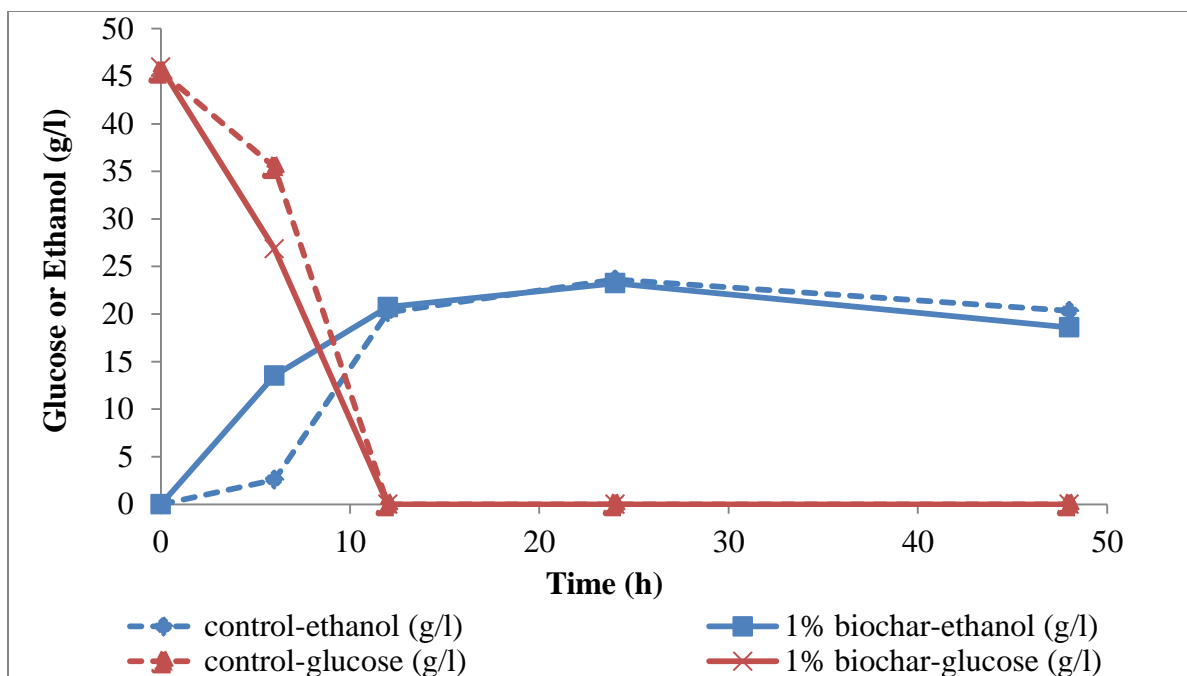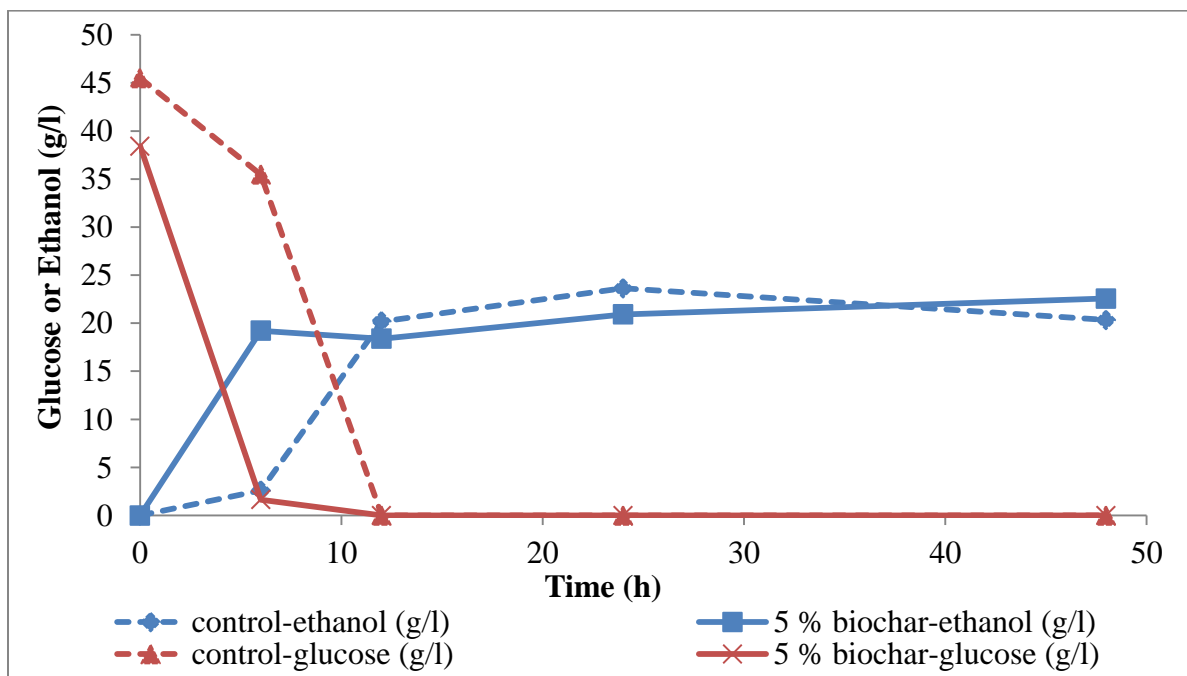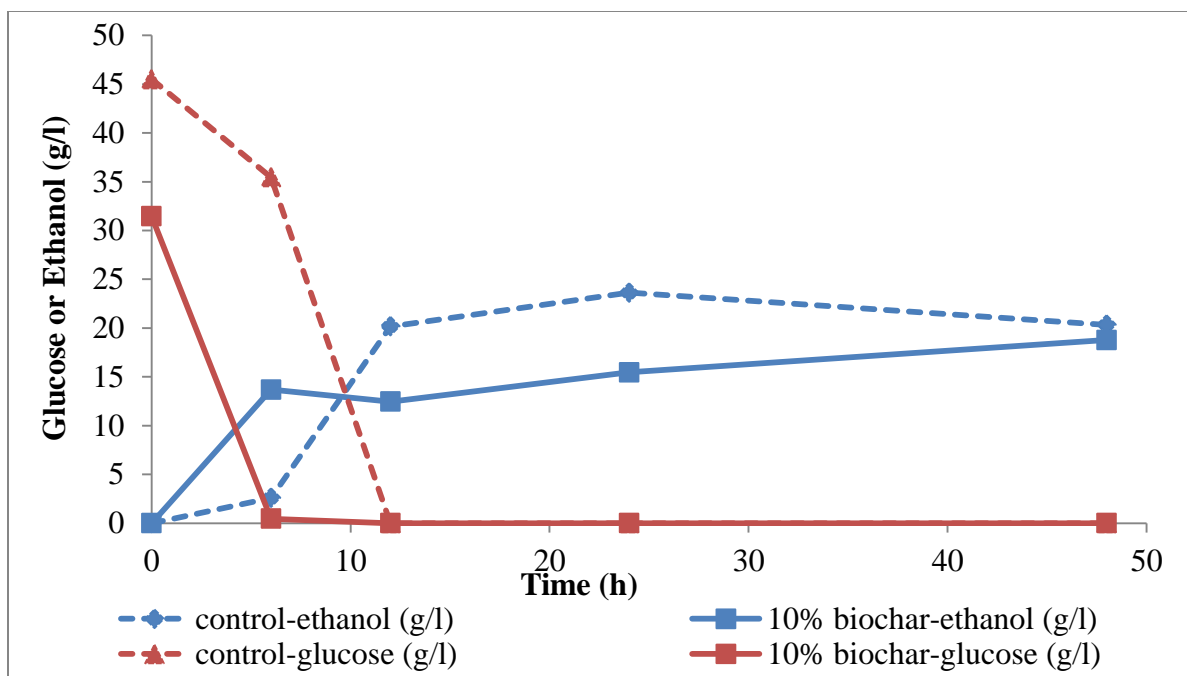Figure 3.9. Ethanol production by *S. cerevisiae* from steam-exploded poplar with control and 10% biochar addition
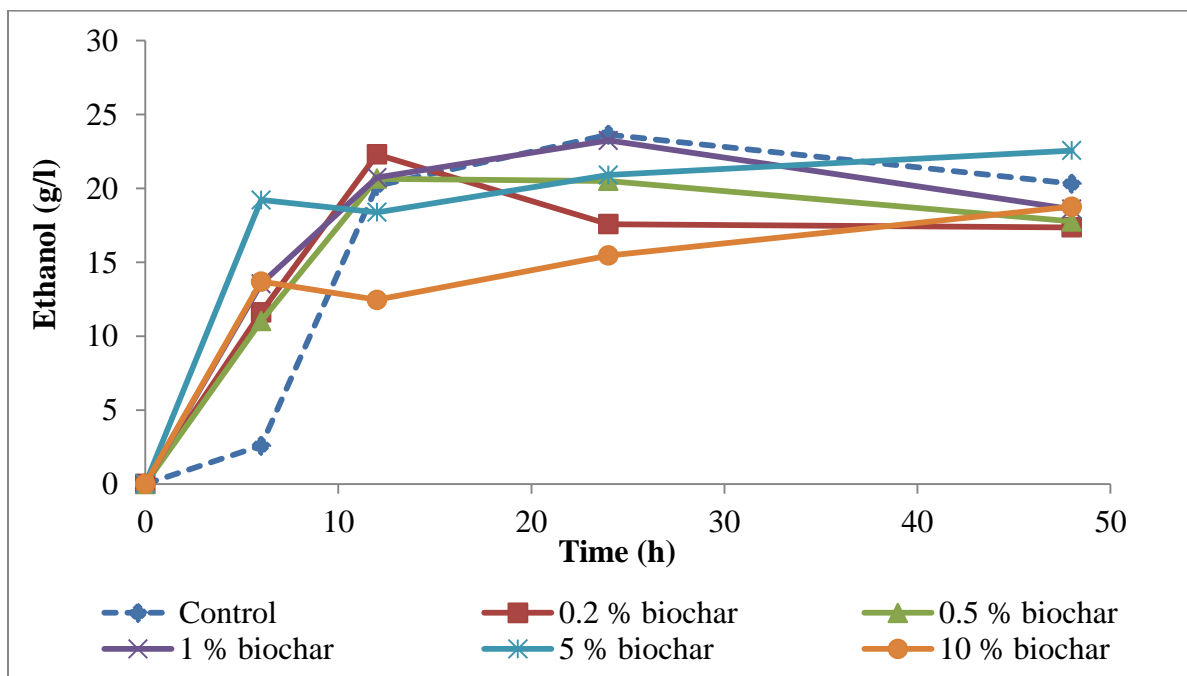

Figure 3.10. Ethanol production by *S. cerevisiae* from steam-exploded poplar for control (0% biochar), 0.2%, 0.5%, 1%, 5%, and 10% biochar addition
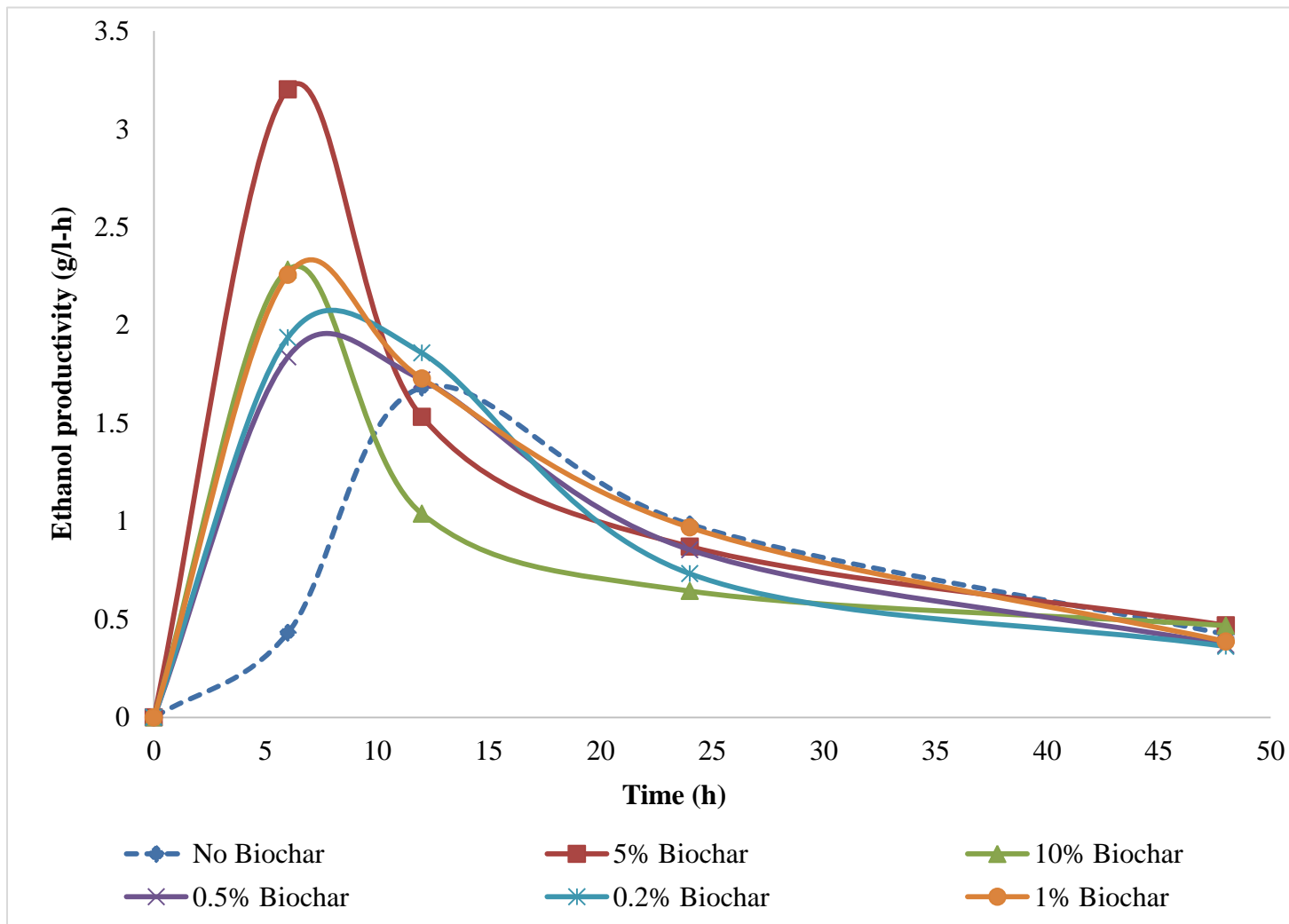
Figure 3.11. Ethanol productivity by *S. cerevisiae* from steam-exploded poplar for control (0% biochar), 0.2% , 0.5% , 1%, 5%, and 10% biochar addition

the enzyme hydrolysis. The glucose concentration increased from 39.02 g/l to 41.43 g/l when biochar was added to the NaOH, however the increase was not significant, for the unwashed poplar the glucose remained almost constant when biochar was added (Figure 3.12), similar effect was also observed for the total reducing sugars concentration (Figure 3.13).

The ethanol production was significantly improved when the biomass was washed with 0.25 M NaOH. The ethanol concentration was 17.19 g/l for the unwashed poplar, and 24.33 g/l for the washed poplar (Figure 3.14). The theoretical ethanol yields were 67.33% and 83.07% for the unwashed and the washed poplar, respectively (Figure 3.15). When biochar was added the ethanol concentrations were 19.25 g/l for the unwashed poplar, and 22.77 g/l for the washed poplar (Figure 3.14). The theoretical ethanol yields were 73.77% and 74.02% for the unwashed and for the washed poplar, respectively (Figure 3.15). The ethanol yield increased slightly for the unwashed poplar from 67.33% to 73.77% but the increase was not significant, and decreased for the washed biomass from 83.07% to 74.02% when biochar was added (Figure 3.15). The decrease in ethanol observed in the NaOH washed biomass might be due to substrate depletion in the biochar samples previously observed in section 3.3.5.

3.4.7. Effect of poultry litter biochar on steam-exploded corn stover

Poultry litter biochar was added to steam-exploded corn stover, the glucose and ethanol concentrations were measured during the fermentation and compared with the control sample (0 % biochar). The glucose concentrations after enzyme hydrolysis were 29.09 g/l for the control (0% biochar), 34.96 g/l for 0.2% biochar, and 33.64 g/l for 1% biochar (Figures 3.16, 3.17, and 3.18).

Figure 3.12. Glucose concentration of NaOH washed and unwashed steam-exploded poplar with no biochar and 1% biochar addition

Figure 3.13. Reducing sugars concentration of NaOH washed and unwashed steam-exploded poplar with no biochar and 1% biochar addition
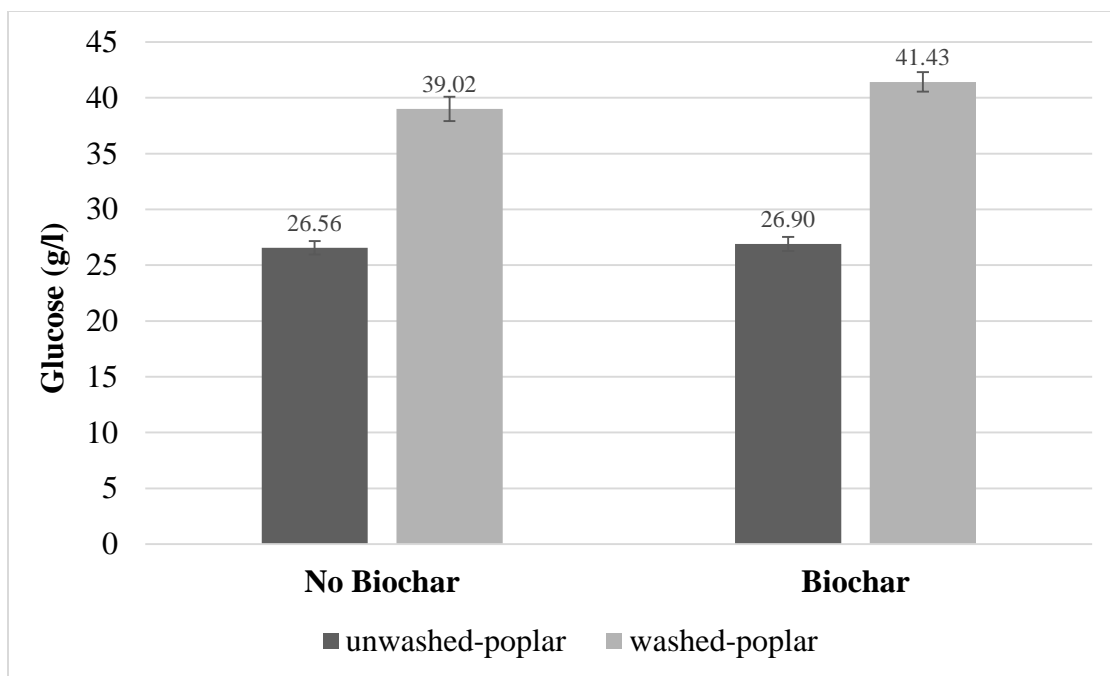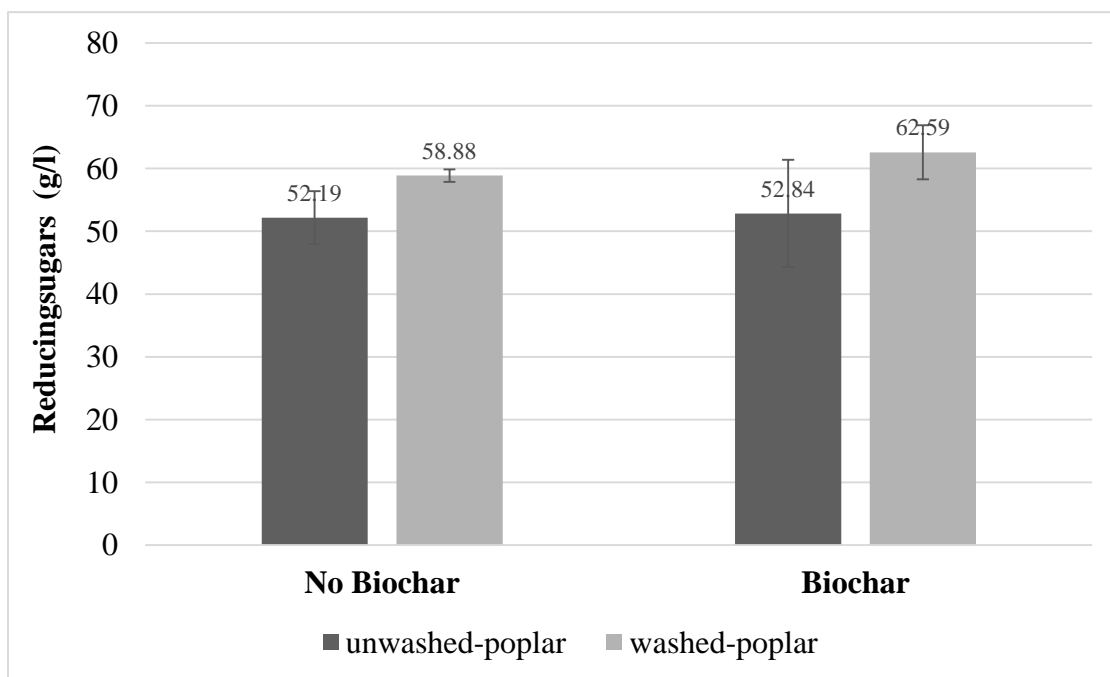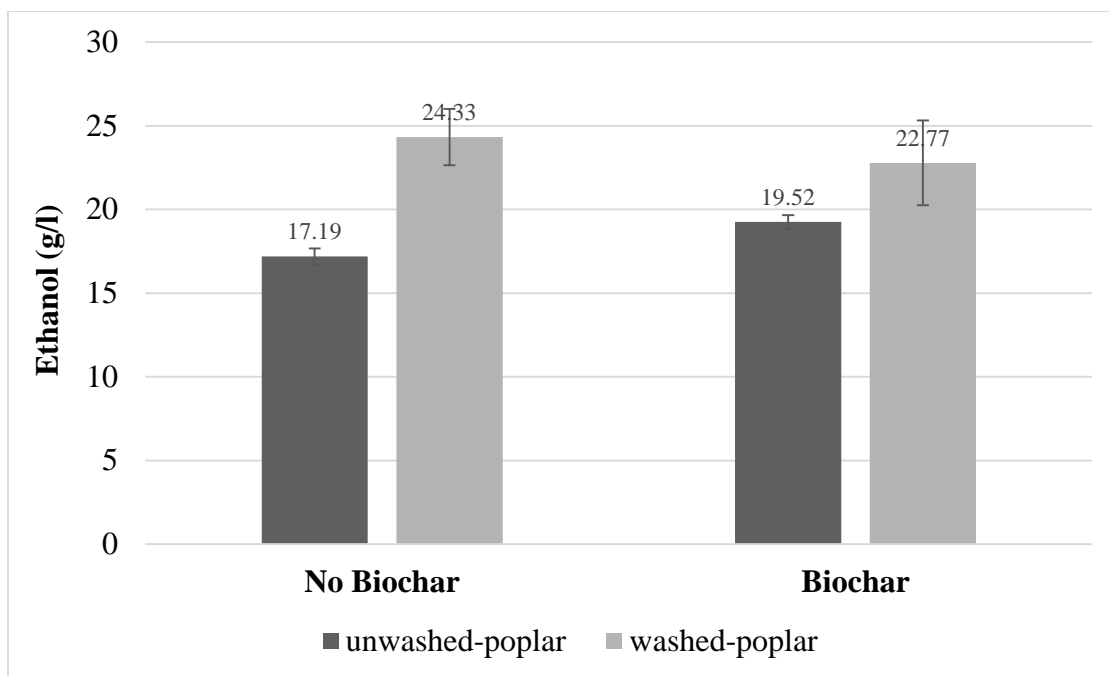
Figure 3.14. Ethanol concentration of NaOH washed and unwashed steam-exploded poplar with no biochar and 1% biochar addition
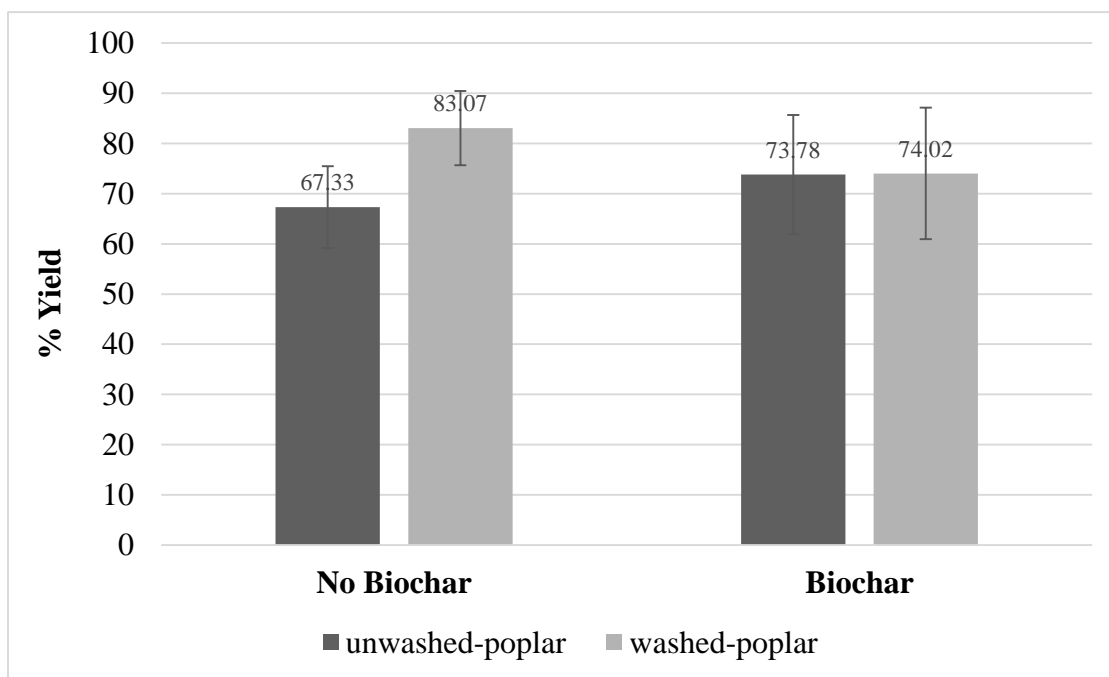

Figure 3.15. Ethanol yield of NaOH washed and unwashed steam-exploded poplar with no biochar and 1% biochar addition

In this case, glucose concentration increased sligthly when biochar was added, which was different from what was observed for poplar (section 3.3.5). During fermentation, the glucose consumption by the yeast was faster in the biochar samples than in the control sample, the same thing was also observed for poplar (section 3.3.5). Glucose was almost completely consumed within 9 h in 1% biochar sample, in 12 h in 0.2% biochar sample, and in 24 h in the control sample respectively (Figures 3.16, 3.17, and 3.18). The slower glucose consumption in the control sample might be due to the presence of some interfernces such as furan, acetic acid or formic acid in the untreated sample.

Poultry litter biochar addition to steam-exploded corn stover improved the ethanol production, the maxium ethanol concentrations were achieved within 12 h for the samples with 1% and 0.2% biochar loadings with the respective ethanol concentrations of 19 g/l and 16.7 g/l. For the control sample, the ethanol did not reach the maxium point until the glucose was completely consumed at 24 h (Figures 3.18, 3.19, and 3.20). Figure 3.19 summarize the fermentation results, the 1% biochar loading had the highest ethanol production, followed by the 0.2% one while the ethanol production in the control was much slower. As the biochar concentration increased from 0.2% to 1% the ethanol production increased and the glucose was consumed faster.

The maximum ethanol productivity was 2.02 g/l-h for the 1% biochar sample, and occurred at 6 h, it was 1.6 g/l-h for the 0.2% biochar sample and occurred at 9 h, and was 0.62 g/l-h for the control and occurred at 12 h (Figure 3.20). The maximum ethanol productivities obtained for the 1% biochar loading in the case of poplar and corn stover were 2.26 g/l-h and 2.02 g/l-h, respectively, they both occurred at the same time at 6 h. However, the maximum ethanol productivity for 0.2% biochar loading occurred at

different times and were 1.6 g/l-h at 9 h for corn stover versus 1.94 g/l-h at 6 h for poplar. The shorter ethanol production time observed in the case of steam-exploded poplar may be due to the fact that the biomass was washed with water after steam-explosion pretreatment unlike the steam-exploded corn stover that was not washed. Studies have shown that washing the biomass with water after steam-explosion pretreatment removed possible inhibitory compounds (Taherzadeh and Karimi, 2011), which can explain the overall increase in ethanol production from poplar compared to corn stover.

Figure 3.16. Ethanol production by *S. cerevisiae* from steam-exploded corn stover with no biochar addition

Figure 3.17. Ethanol production by *S. cerevisiae* from steam-exploded corn stover with 0.2% biochar addition
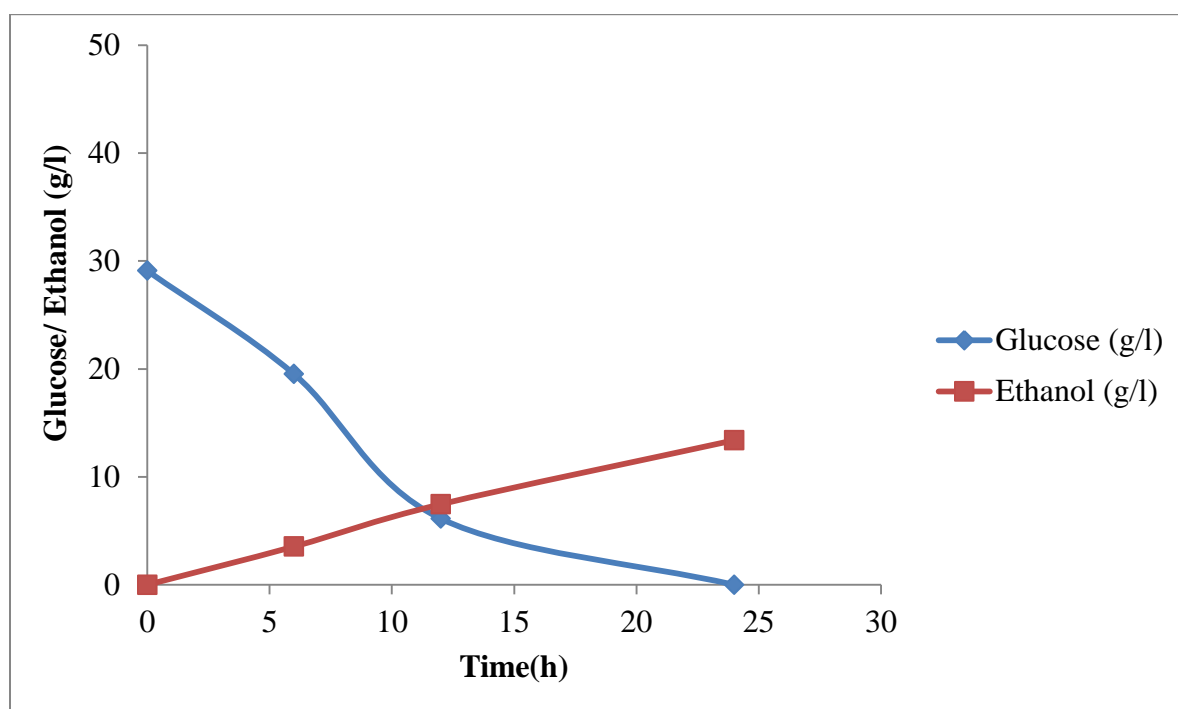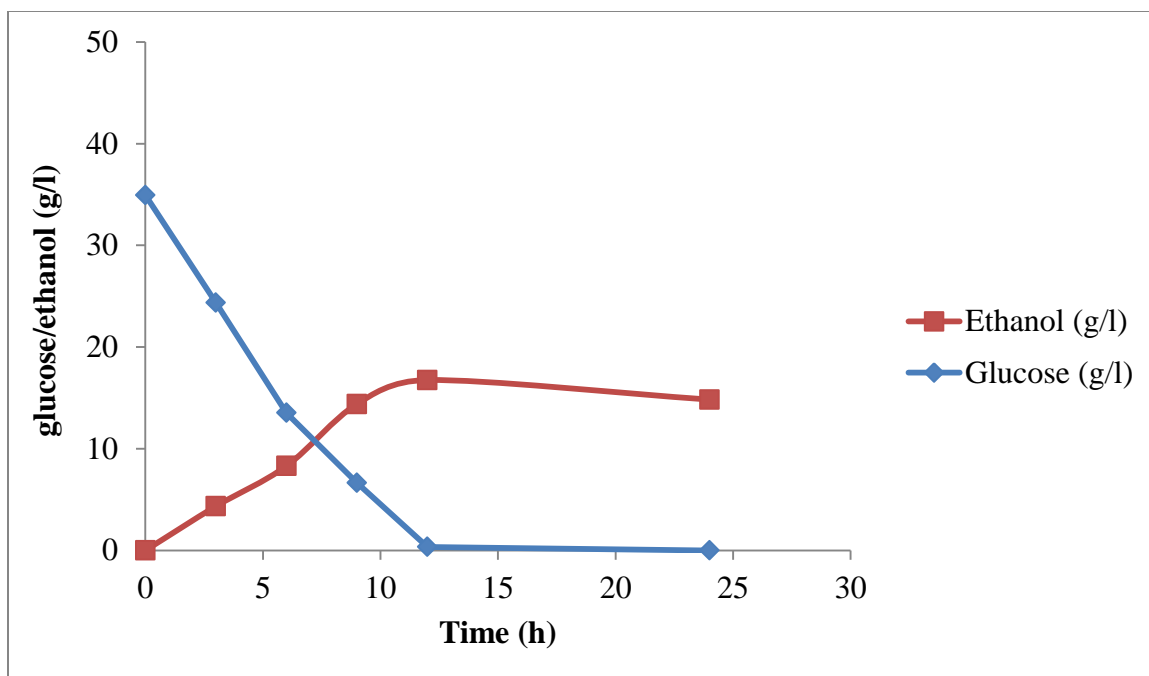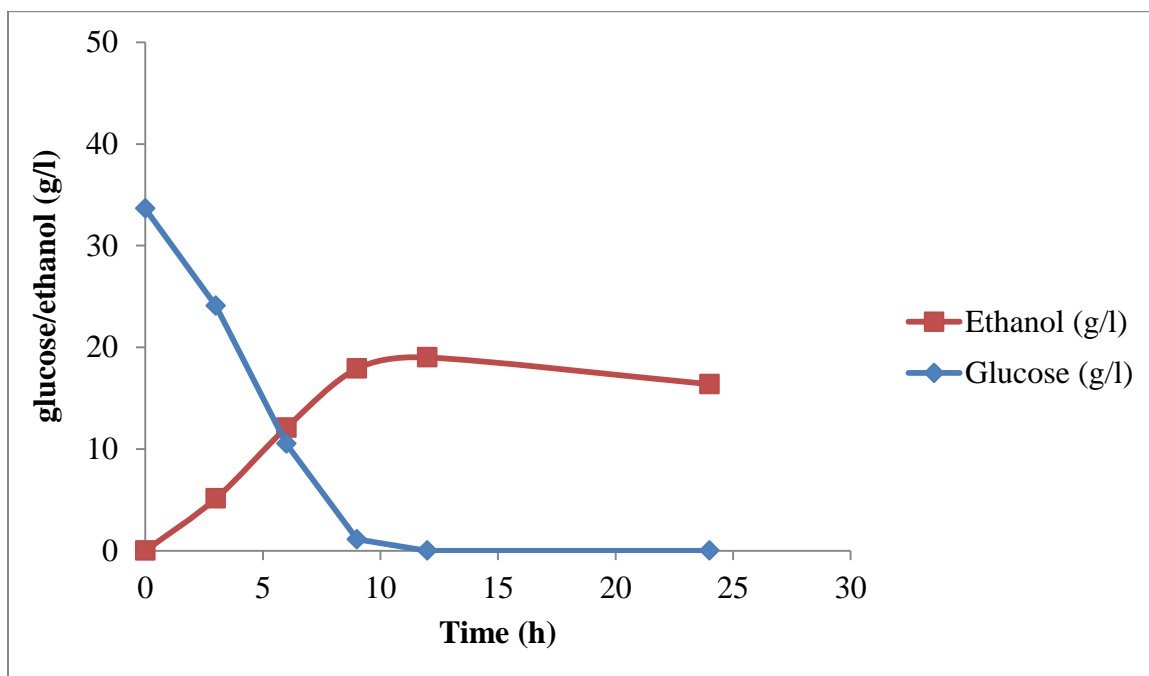

Figure 3.18. Ethanol production by *S. cerevisiae* from steam-exploded corn stover with 1% biochar addition
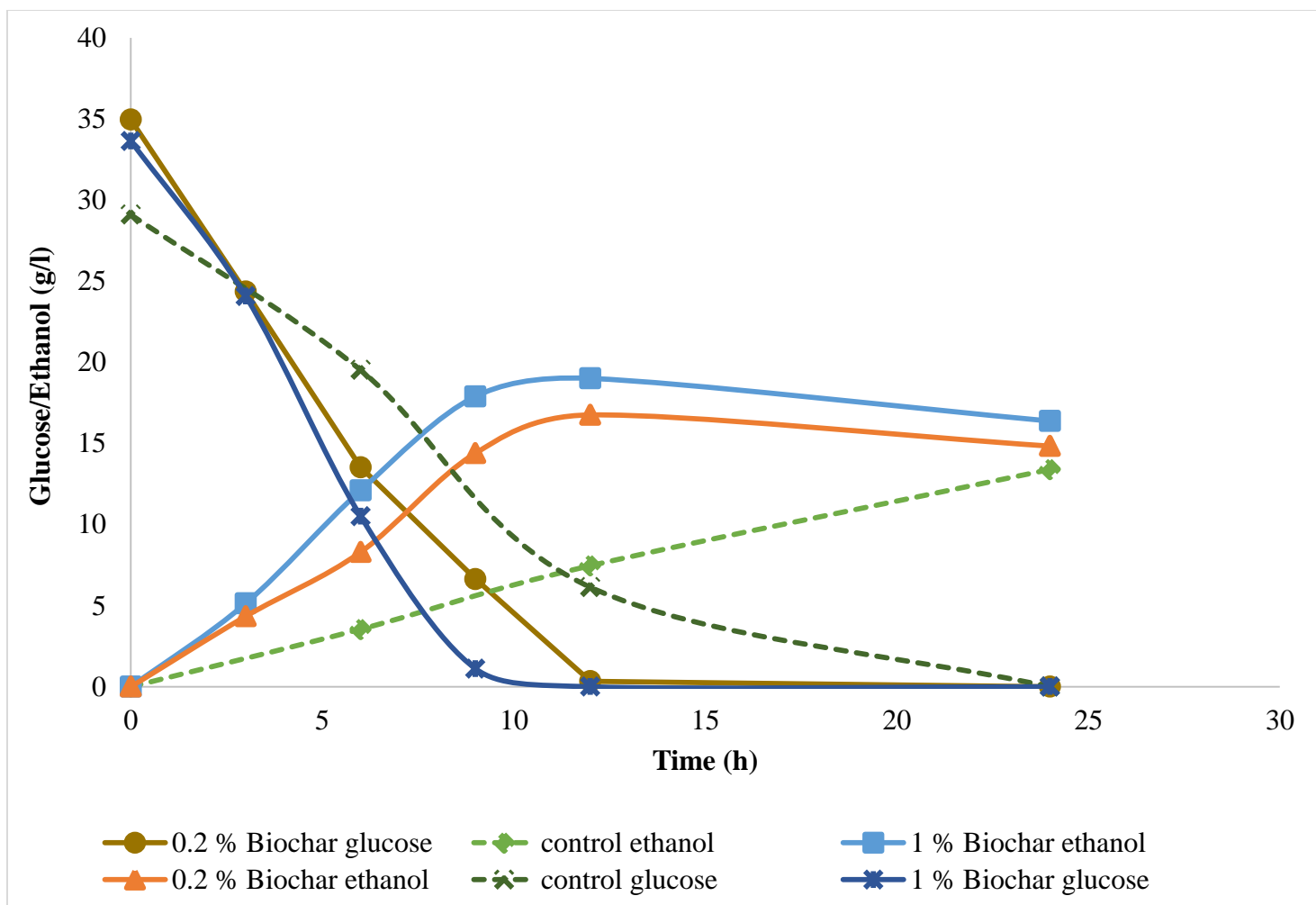
Figure 3.19. Ethanol production by *S. cerevisiae* from steam-exploded corn stover for control (0% biochar), 0.2%, and 1% biochar additions
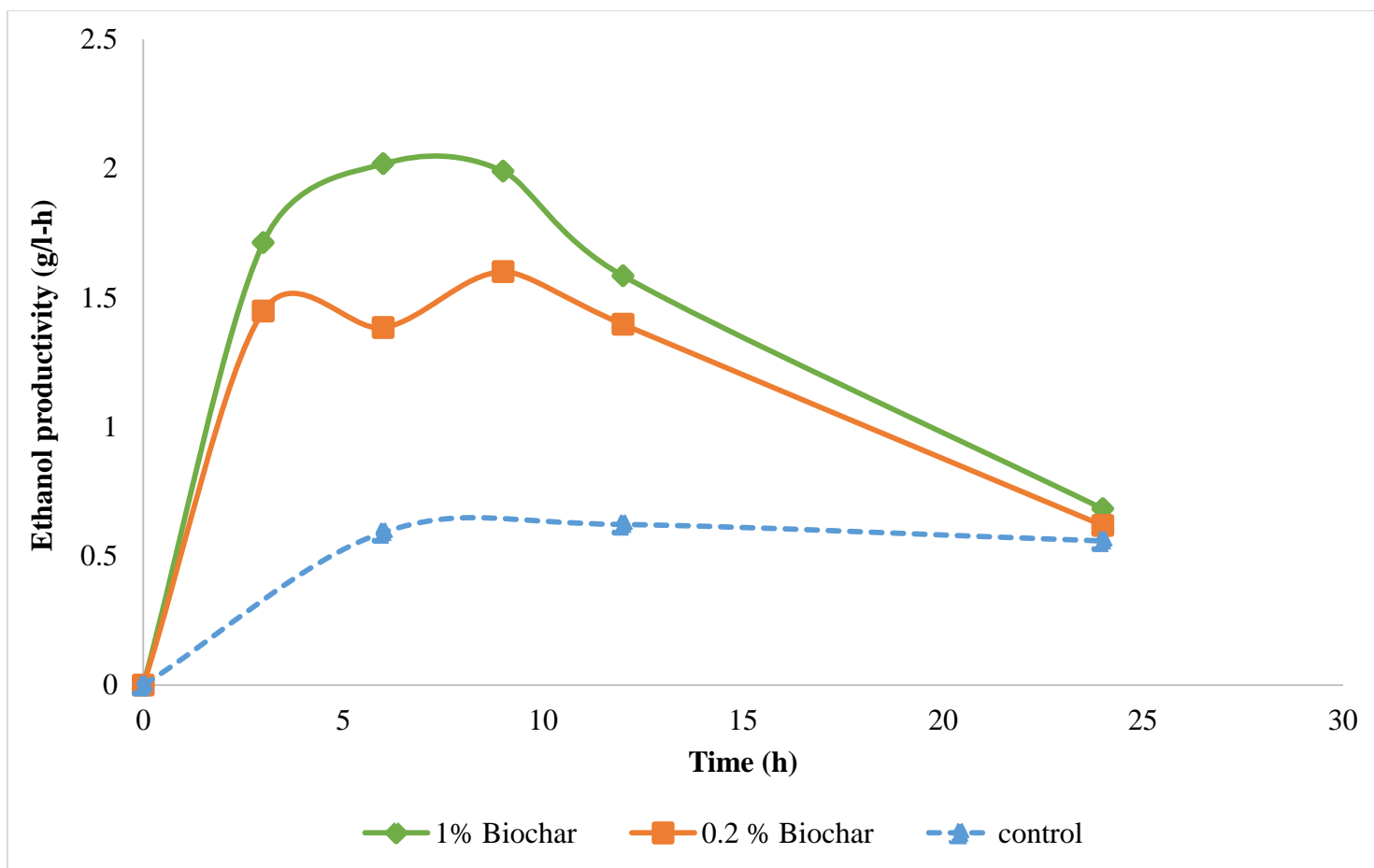
Figure 3.20. Ethanol productivity by *S. cerevisiae* from steam-exploded corn stover for control (0% biochar), 0.2%, and 1% biochar additions

3.5. Conclusion

Washing the steam-exploded poplar with 0.25 M NaOH had a significant effect on the enzyme hydrolysis and ethanol production from the steam-exploded poplar. The enzyme hydrolysis of NaOH washed poplar had a higher cellulose conversion than the microcrystalline cellulose and the unwashed poplar. Both the glucose and ethanol concentrations increased by washing the steam-exploded poplar with 0.25 M NaOH solution.

Addition of poultry litter biochar at low loadings (0.2%, 0.5%, and 1%) to the steam-exploded poplar did not have an effect on the glucose concentration; however, a decrease in the glucose concentration was observed at high biochar loadings (5%, and 10%). The ethanol production was faster in the biochar samples compared to the control, but the control had the highest final ethanol concentration. Biochar had a positive effect on the ethanol productivity; the highest value (3.2 g/l-h) was obtained in the case of 5% biochar loading and the lowest one (1.7 g/l-h) was obtained in the case of control sample.

Poultry litter biochar had also a positive effect on the steam-exploded corn stover, the glucose concentration was slightly higher in the biochar samples compared to the control. The addition of poultry litter biochar significantly improved the overall ethanol production from steam exploded corn stover. The ethanol productivities incresased with the increase of biochar concentrations: 0.62 g/l-hr for the control (no biochar), 1.60 g/l-h for (0.2 % biochar), and 2.018 g/l-hr for the (1% biochar).

3.6. References

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CHAPTER 4

PARAMETRIC STUDIES OF POULTRY LITTER BIOCHAR EFFECT ON

ETHANOL PRODUCTION FROM STEAM-EXPLODED CORN STOVER

4.1. Abstract

Steam-exploded biomass normally contains biomass degradation compounds that inhibit enzyme hydrolysis and efficient fermentation of the hydrolysate. In this study, poultry litter biochar was used to improve the ethanol production from steam-exploded corn stover, and response surface methodology was used to optimize the results. A three level, three variables central composite design was used in total of 17 experiments to evaluate the effects of poultry litter biochar loadings (1.27-5%), biomass loadings (5-15%), and enzyme loadings (10-30 FPU/g) on the reducing sugars and ethanol yield from the steam exploded corn stover. The steam-exploded corn stover was hydrolyzed with *CTec2* and then fermented with *Saccharomyces cerevisiae*. Results indicate that poultry litter biochar loadings had a significant effect on the ethanol yield (p-value = 0.0072), but the effect on the enzyme hydrolysis was not significant. At the optimal conditions of biochar loading (5%), biomass loading (15%), and enzyme loading (10 FPU/g^{-1}), the ethanol yield was 73.44% which was a 19.46% more than the non-optimized control at zero-level central point.

4.2. Introduction

Corn stover is an abundant agricultural by-product with a low commercial value, and is available for ethanol production (nearly 545 million dry tons produced in USA per year) (Aden et al., 2002; Zambare and Christopher, 2012). Studies showed that corn stover is an attractive feedstock for bioethanol production because it is composed of cellulose (32.4–37.4%), hemicellulose (18.5–21.8%) and lignin (11.2–18%) (Aden et al., 2002; Weiss et al., 2010). Corn stover (CS) biomass can be converted to bioethanol following three main steps: pretreatment, enzyme hydrolysis, and fermentation. Cellulose and hemicellulose polymers can be recovered using steam-explosion pretreatment and converted to monomeric sugars using enzymatic hydrolysis. The monomeric sugars can then be fermented to ethanol using traditional baker's yeast (*Saccharomyces cerevisiae*).

However, research showed that when lignocellulose biomass is pretreated with steam explosion, in addition to releasing the polymers (cellulose and hemicellulose), inhibitory compounds such as furfural, hydroxymethylfurfural (HMF), formic acid, levulinic acid, and acetic acid are also produced (Li et al., 2011). At high concentrations, these compounds are reported to inhibit the fermenting microorganisms and decrease the ethanol yield and productivity (Parawira and Tekere, 2011). Detoxification step was found necessary in order to improve the fermentability of lignocellulosic hydrolysates. Several methods of detoxification have been used and reported to improve the ethanol production such as ion exchange, overliming, activated carbon, and biological treatment (Chandel et al., 2011; In, 2001; López et al., 2004; Millati et al., 2002; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000). In Chapter 2 (effect of poultry litter biochar on *Saccharomyces cerevisiae* growth and ethanol production), we observed that poultry litter biochar had a positive effect on the yeast growth, the potential of using

poultry litter biochar to overcome the effect of inhibitory compounds in order to improve the ethanol production from the lignocellulosic biomass is an attractive opportunity.

In addition to hydrolysates detoxification, biomass loading and enzyme loading were shown as important factors that can affect the ethanol yield. Sun and Cheng (2002) reported that low biomass loading result in a low hydrolysis yield, and high biomass loading can cause substrate inhibition, and substantially lower the rate of the hydrolysis. The enzyme loading was also reported as an important factor that can affect the hydrolysis yield and the final ethanol production. Studies showed that the costs of cellulase account for up to 50% of the total ethanol production costs, therefore to lower the cost of lignocellulosic ethanol production, both substrates and enzyme loadings need to be optimized (Himmel et al., 2007; Ioelovich and Morag, 2012; Sun and Cheng, 2002; Wyman, 2007; Zambare and Christopher, 2012). The aims of this study is to estimate the effects of biomass loading, enzyme loading, and biochar loading to optimize the enzyme hydrolysis and ethanol yield from steam-exploded corn stover.

4.3. Materials and Methods

4.3.1. Steam-exploded corn stover

The steam-exploded corn stover (CS) used in this work was obtained from Virginia Tech, where corn stover treated with $Fe_2 (SO_4)_3$ was steam exploded at 220 ^{o}C for 5 min. The acid insoluble and ash fraction of the steam-exploded CS were determined following the ASTM E 1721-01 (Reapproved 2009) procedure as described in Chapter 3 (section 3.3.1).

4.3.2. Experimental Design

The effects of steam-exploded CS loading, PLB loading, and cellulase loading were investigated for the sugars released, ethanol concentration, and ethanol yield using a central composite design with three repetitions in the center. The design was generated with SAS 9.4 software (SAS Institute Inc. Cary, NC), the independent variables were chosen to be CS loadings (X_1, wt. %), PLB loadings (X_2, wt. %), and cellulase loadings (X_3, FPU/g). The dependent output variables were the reducing sugar concentration (g/l), the ethanol concentration (g/l), and the ethanol yield (%); star points were situated at ± 1.68 from the center to account for rotatability, the total runs was 17 treatments.

4.3.3. Enzymatic hydrolysis

The cellulase used to hydrolyze the biomass was *CTec2* donated by Novozyme, (Farmington, NC). Steam-exploded CS and PLB were weighed out and added to 250 ml screw cap Erlenmeyer flasks corresponding to CS loadings of 5.0, 10.0, and 15.0 wt. %, and PLB loadings of 1.27, 3.13, and 5.0 wt. %, 50 ml citrate buffer (0.05 M, pH 4.8) was added. Flasks containing biomass, biochar, and buffer were autoclaved for 30 min at 121 $^{\circ}$C. The enzyme was aseptically added to the flasks to obtain final enzyme loadings of 10.0, 20.0, 30.0 FPU/g (g of biomass dry matter). The reaction mixture was then incubated at 50 $^{\circ}$C in a water bath shaker incubator (ALT, Precision Reciprocal Shaking bath, East Lyme, CT) under shaking condition of 130 rpm. Aliquotes were withdrawn from each flask after 48 h, centrifuged at 3000 x g for 20 min, and supernatant was analyzed for reducing sugars concentration using the DNS method previously described in Chapter 3 (section 3.3.10.4).

4.3.4. Fermentation

Flasks were autoclaved at the end of hydrolysis before the fermentation to ensure the enzyme is not active. *Saccharomyces cerevisiae* ATCC 204508/S288C was used to inoculate the hydrolysates. The inoculum was prepared and cultivated under the same conditions described in Chapter 3 (section 3.3.9). The yeast cells were harvested after 16 h, the optical density of the culture at 600 nm was 7.36. The cells were centrifuged at 3000 x g for 10 min under sterile condition, the supernatant was discarded, and cells were transferred to 250 ml screw-capped Erlenmeyer flasks containing the hydrolysates. Flasks were purged with nitrogen for 5 min and tightly capped to allow fermentation to occur under anaerobic condition; the cultures were then incubated in a water bath shaker incubator (ALT, Precision Reciprocal Shaking bath, East Lyme, CT) at 37 °C under shaking at 130 rpm for 48 h. At the end of the fermentation, the broths were centrifuged to separate the biomass and the cells from the liquid broth, and ethanol was extracted from the liquid broth using the procedure described in Chapter 3 (section 3.3.10.2).

Ethanol was quantified using HP 6890 Series gas chromatograph equipped with an Agilent column (30 m capillary column HP-5, 30m x 0.320 mm x 1.00 μm id), and a flame ionization detector (FID). The GC method was described in Chapter 3 (section 3.3.10.3).

4.4. Results and Discussion

The steam exploded corn stover used in this study was composed of 26.7% acid insoluble residue, 6.5% ash, and 61.8% carbohydrates (Table 4.1).

4.4.1. Reducing Sugars

ANOVA (Table 4.3) show that both the CS loading and enzyme loading had effect on the reducing sugars p-values ($pX_1 < 0.0001$, $pX_3 = 0.0008$), however the PLB loading did not appear to have a direct effect on the reducing sugar production ($pX_2 = 0.3354 > 0.05$).

4.4.2. Ethanol fermentation

Table 4.2 shows the total reducing sugar concentrations, ethanol concentrations, and theoretical ethanol yield. In ANOVA (Table 4.4 and 4.5), the model indicates that both the CS loading and PLB loading had effect on the ethanol concentration ($pX_1 = 0.0004$, $pX_2 = 0.0338$), and yield ($pX_1 < 0.0001$, $pX_2 = 0.0072 < 0.05$). However, the enzyme loading did not appear to have a direct effect on the ethanol yield ($pX_3 = 0.9674 > 0.05$) because there was some reducing sugars that remained after fermentation.

For most of the time, the ethanol yield increased with increasing the PLB loading (run 1 and run 4, run 5 and run 7), and increasing biomass loadings (run 9 and run 10). The 0% yield at run 9 was due to the low biomass loading and the 0% yield at run 3 may be due to the high concentration of PLB in the hydrolysate. PLB contains numerous inorganic materials such as Fe, Cu, Ni; these substances at high concentration in the medium can inhibit the yeast growth and ethanol production. The predicted response (y) for ethanol yield (%) during fermentation is presented by the following equation:

$$y = 36.61411 + 18.78972X_1 + 7.215195X_2 - 0.08147X_3 + 10.12X_{12} - 7.1925X_{13} + 4.785X_{23}$$

where, X_1, X_2, and X_3 are the coded values for CS loading (wt. %), PLB loading (wt. %), and enzyme loading (FPU/g).

The goodness of model fit was $R^2 = 0.96$ which indicate only 4% of the variations could not be explained by the model. The response surface plot suggested that the ethanol concentration could be enhanced by increasing both the CS loadings and the PLB loadings (Figures 4.1, 4.2, and 4.3).

Results show poultry litter biochar (PLB) have a significant effect on the ethanol yield ($pX_2 = 0.0072 < 0.05$). The influence of PLB on the ethanol could be explained by the nutrients content in PLB that could enhance the yeast growth during fermentation. Many studies have already reported that biochar can increase soils microbial growth (Farrell et al., 2013; Jindo et al., 2012; Quilliam et al., 2013; Warnock et al., 2007), similarly in Chapter 2 (Effect of poultry litter biochar on the growth of *Saccharomyces cerevisiae*) we observed that the addition of PLB to the medium increased the growth of *S. cerevisiae*. Certainly, the increase in pH had also a significant effect, the presence of inhibitory compounds such as acetic acid and formic acid can make the steam-exploded corn stover hydrolysate acidic, addition of PLB increased the pH of the hydrolysate. Lee et al. (2011) reported in their study that the pH of wood autohydrolysate was increased when activated carbon was added and led to the removal of acetic acid and formic acid.

Another reason, might be due to the physical properties of PLB, Keech et al. (2005) showed the presence of macro pores on the wood derived charcoal and their ability to adsorb larger molecules such as phenolic compounds. In this study, the surface area of PLB was low (6.36 m^2/g) however the biochar might contribute to the neutralization of some inhibitory compounds. In order to prove this point, the inhibitory need to be identified before and after biochar addition to show the inhibitory compounds that were removed.

Table 4.1. Composition of steam-exploded corn stover

Composition	Average
Acid insoluble material (%)	26.68
Ash (%)	6.45
Carbohydrates (%)	61.77

Table 4.2. Central composite design of Reducing sugars ethanol concentration and ethanol yield

	X_1 Actual (Coded)	X_2 Actual (Coded)	X_3 Actual (Coded)	Responses			y
Runs	Biomass wt. %	Biochar wt. %	Enzyme FPU/g	Total sugars g/l	sugars consumed [a] g/l	Ethanol g/l	Yield [b] (%)
1	5(-1)	1.27(-1)	10(-1)	24.45	22.71	1.55	13.34
2	5(-1)	1.27(-1)	30(1)	31.17	28.03	1.70	11.92
3	5(-1)	5(1)	10(-1)	20.60	18.57	0.00	0.00
4	5(-1)	5(1)	30(1)	31.27	28.31	2.69	18.66
5	15(1)	1.27(-1)	10(-1)	50.30	29.61	6.85	45.36
6	15(1)	1.27(-1)	30(1)	78.58	17.95	1.48	16.11
7	15(1)	5(1)	10(-1)	54.41	38.11	14.27	73.44
8	15(1)	5(1)	30(1)	69.16	51.36	16.34	62.39
9	1.59(-1.68)	3.14(0)	20(0)	3.52	3.52	0.00	0.00
10	18.41(1.68)	3.14(0)	20(0)	64.30	55.81	17.47	61.38
11	10(0)	0.00(-1.68)	20(0)	44.98	35.74	6.32	34.69
12	10(0)	6.27(1.68)	20(0)	38.45	32.66	8.83	52.99
13	10(0)	3.14(0)	3.18(-1.68)	27.07	23.89	3.48	28.58
14	10(0)	3.14(0)	36.82(1.68)	56.37	48.81	10.36	41.63
15	10(0)	3.14(0)	20(0)	53.01	41.09	12.44	59.37
16	10(0)	3.14(0)	20(0)	54.97	43.43	12.29	55.48
17	10(0)	3.14(0)	20(0)	53.38	41.37	9.94	47.10

[a] sugar consumed = initial reducing sugars before fermentation − reducing sugars after fermentation

[b] Theoretical ethanol yield (%) = Ethanol produced/ (sugars consumed x 0.51) x100

Table 4.3. Analysis of variance (ANOVA) of reducing sugars (g/l) as function of biomass loadings (X_1), biochar loadings (X_2), and enzyme loadings (X_3)

Source	DF	Sum of Squares	Mean Square	F-value	p-value Pr> F
BIOMASS	1	4474.260	4474.260	162.309	<.0001
BIOCHAR	1	29.489	29.489	1.070	0.335
ENZYME	1	881.111	881.111	31.963	0.001
BIOMASS*BIOCHAR	1	0.303	0.303	0.011	0.919
BIOMASS*ENZYME	1	82.144	82.144	2.980	0.128
BIOCHAR*ENZYME	1	11.479	11.479	0.416	0.539

Table 4.4. Analysis of variance (ANOVA) of ethanol (g/l) as function of biomass loadings (X_1), biochar loadings (X_2), and enzyme loadings (X_3)

Source	DF	Sum of Squares	Mean Square	F-value	p-value Pr> F
BIOMASS	1	284.937	284.937	40.029	0.0004
BIOCHAR	1	49.284	49.284	6.923	0.034
ENZYME	1	9.055	9.055	1.272	0.297
BIOMASS*BIOCHAR	1	65.231	65.231	9.164	0.019
BIOMASS*ENZYME	1	4.743	4.743	0.666	0.441
BIOCHAR*ENZYME	1	12.450	12.450	1.749	0.228

Table 4.5. Analysis of variance (ANOVA) of ethanol yield (%) as function of biomass loadings (X_1), biochar loadings (X_2), and enzyme loadings (X_3)

Source	DF	Sum of Squares	Mean Square	F-value	p-value Pr> F
BIOMASS	1	4821.607	4821.607	95.192	<.0001
BIOCHAR	1	710.963	710.963	14.036	0.007
ENZYME	1	0.091	0.091	0.002	0.967
BIOMASS*BIOCHAR	1	819.315	819.315	16.176	0.005
BIOMASS*ENZYME	1	413.857	413.857	8.171	0.024
BIOCHAR*ENZYME	1	183.170	183.170	3.616	0.099
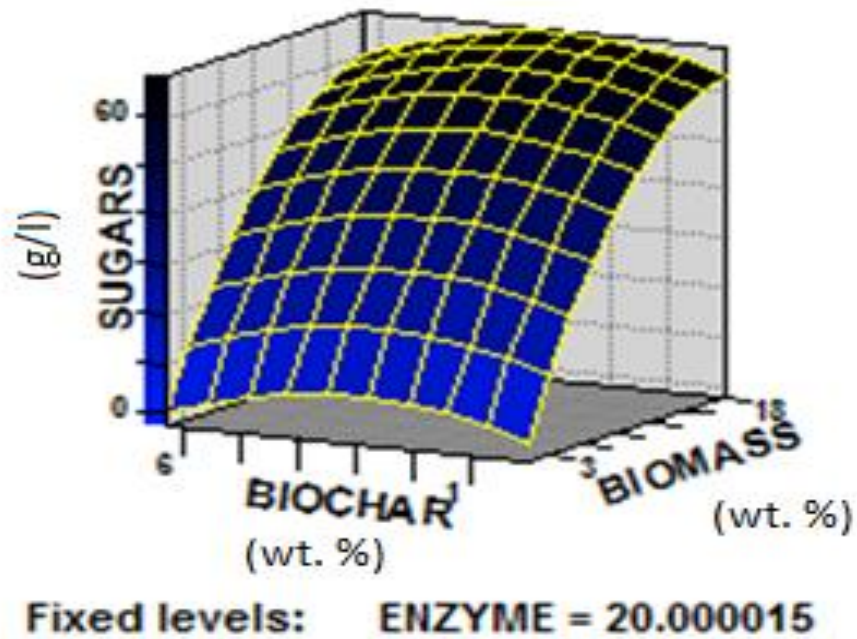
Figure 4.1. Reducing sugars concentration (g/l) with three independent variables biomass loadings X_1 (5%, 10%, and 15%), biochar loadings X_2 (1.27%, 3.14%, and 5%), and enzyme loadings X_3 (10 FPU/g, 20 FPU/g, and 30 FPU/g)

Figure 4.2. Ethanol concentration (g/l) with three independent biomass loadings X_1 (5%, 10%, and 15%), biochar loadings X_2 (1.27%, 3.14%, and 5%), and enzyme loadings X_3 (10 FPU/g, 20 FPU/g, and 30 FPU/g)
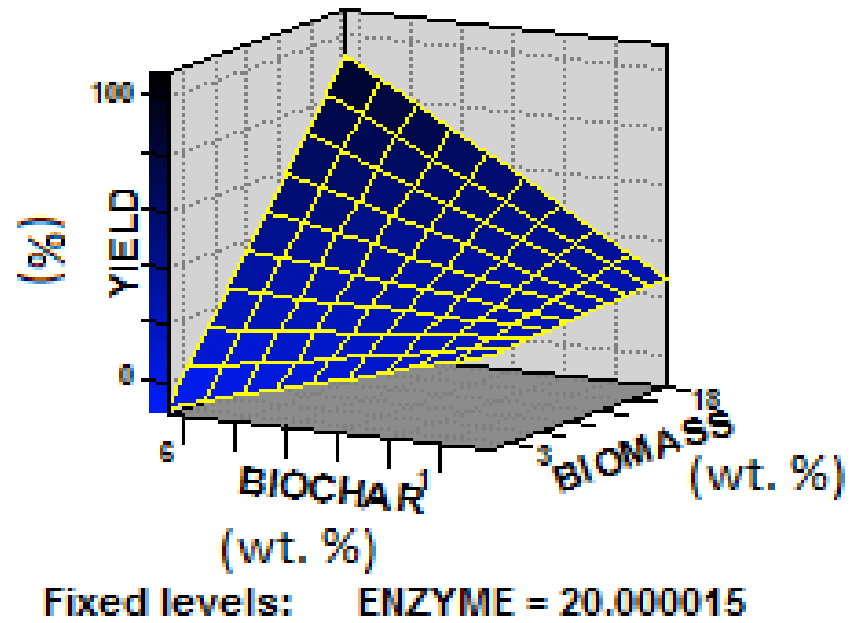
Figure 4.3. Theoretical ethanol yield (%) with three independent biomass loadings X_1 (5%, 10%, and 15%), biochar loadings X_2 (1.27%, 3.14%, and 5%), and enzyme loadings X_3 (10 FPU/g, 20 FPU/g, and 30 FPU/g)
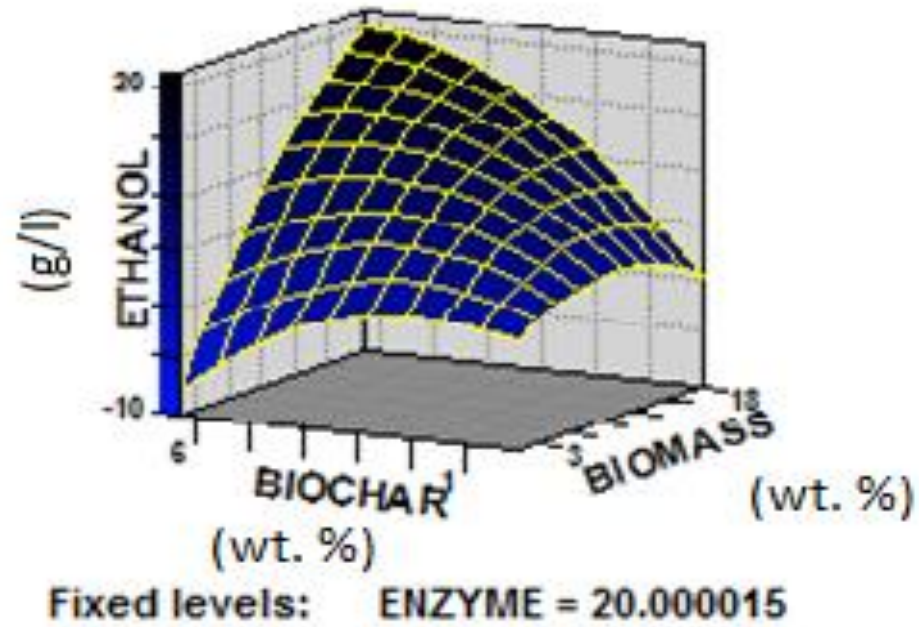
4.4.3. Effect of poultry litter biochar on the pH of steam-exploded corn stover

Addition of poultry litter biochar (PLB) to the steam-exploded corn stover increased hydrolysate pH: at 5% biomass loading, the pH increased from 4.6 to 5.9 when the PLB loading was increased from 1.27% to 5%. At 10% biomass loading, the pH increased from 3.8 to 4.5 and then to 5.2, with increasing PLB loading from 0 to 5%. At biomass loading 15%, the pH increased from 3.7 to 4.8 with increasing PLB loading from 1.27% to 5%.

Figure 4.4. Effect of poultry litter biochar loadings X_2 (1.27%, 3.14%, 5%) on the pH of steam-exploded corn stover hydrolysate at biomass loadings X_1 (5%, 10%, 15%)

4.5. Conclusion

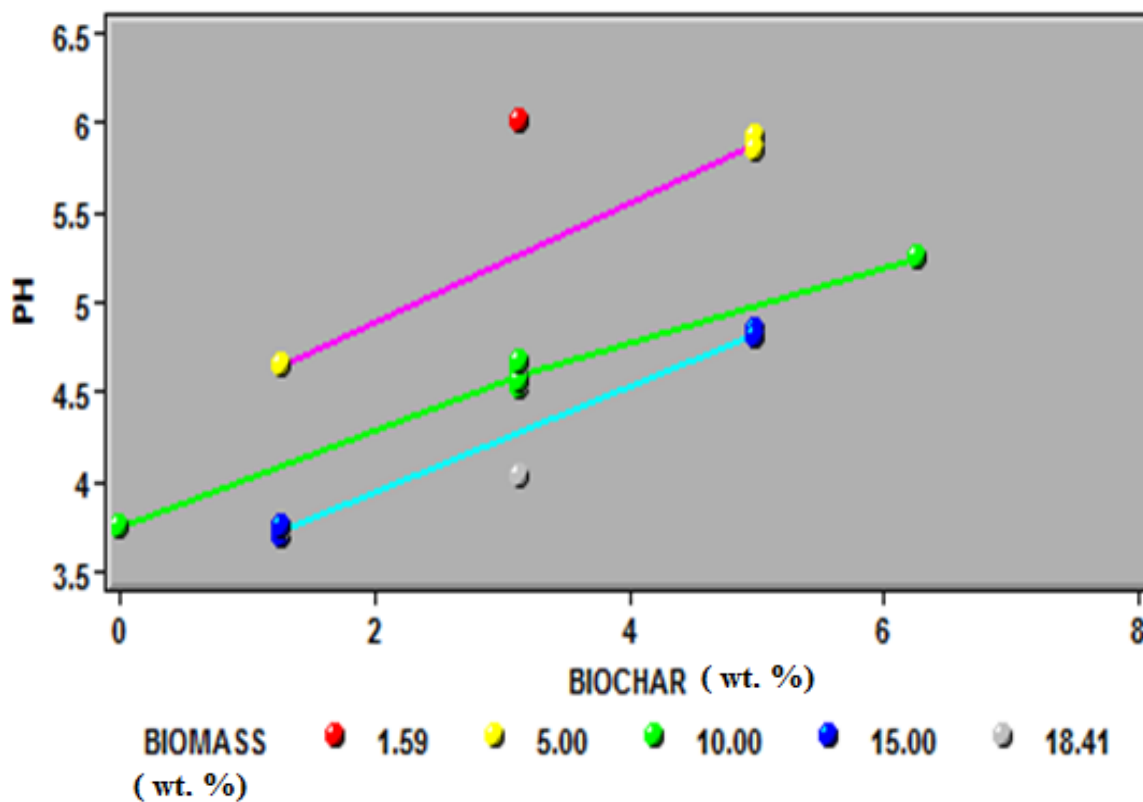The effect of biomass loadings, poultry litter biochar loadings, and enzyme loading as independent variables were investigated in a total of 17 experiments central composite design. The two variables, biomass and enzyme loadings showed a significant effect on the production of reducing sugars but biochar did not have any effect on the reducing sugars production. The two variables, biomass and biochar loadings showed a significant effect on the ethanol yield but enzyme loadings did not have any effect on ethanol production. The most important effect of poultry litter biochar addition to steam-exploded corn stover was the significant effect on the ethanol yield (p-value = 0.0072). Under the optimum conditions (biomass loading 15%, biochar loading 5% and enzyme loading 10 FPU/g) the ethanol yield was (73.44%) which represents a yield increase of 19.46% over the control at centers points (53.98%).

4.6. References

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CHAPTER 5

GENERAL CONCLUSIONS

The objectives of this research are to determine the effect of poultry litter biochar on *Saccharomyces cerevisiae* growth and on the ethanol production from steam-exploded poplar and corn stover. Our results showed the potential of using poultry litter biochar to improve yeast *S. cerevisiae* growth and ethanol production.

Based on our experimental results, it was observed that addition of 0.2% poultry litter biochar to the growth media had stimulated the growth of *S. cerevisiae* ATCC 204508/S288C under both aerobic and anaerobic conditions. However, addition of yeast extract was found necessary for biochar to enhance the growth. It was also observed that glucose was rapidly consumed by yeast in the presence of biochar in the medium, thus more ethanol is produced during the early fermentation hours and then stabilized when glucose was completely consumed. Based on these observations, we assumed that adding poultry litter biochar to continuous substrate feeding system would be more advantageous than to the batch system.

Addition of 0.2% poultry litter biochar to fermentation with high glucose concentrations 100 g/l and 150 g/l media was successful; the fermentation was faster when poultry litter biochar was added compare to control with no biochar. It seems that poultry litter biochar can be used to overcome the sugar inhibition, and it is interesting to try fermentation with higher glucose concentrations such as: 200 g/l, 300 g/l, and 500 g/l to determine the substrate inhibition point of the yeast in the biochar medium. Success in using poultry litter biochar to overcome the sugar inhibition would be a major finding for the ethanol production industry.

Addition of poultry litter biochar to steam-exploded poplar and corn stover showed a similar effect observed during the glucose fermentation. The glucose consumption and ethanol production were rapid when poultry litter biochar was added to biomass compare to the control with no biochar. The ethanol productivity from steam-exploded poplar and corn stover was improved at the beginning of the fermentation when biochar was added to the steam-exploded hydrolysates, meaning that poultry litter biochar could be used to detoxify the steam-exploded hydrolysates in order to improve the ethanol productivity.

However, the mechanism by which biochar promoted the ethanol production from the steam-exploded biomass is not clear. The biochar used in this study had a low surface area 6.34 g/m^2, which mean that the capacity of absorbing the toxic compounds by the biochar is low. Therefore, we associate the increase of the ethanol productivity to be due to the stimulation effect that biochar had on the *S. cerevisiae* growth observed during the glucose fermentation, and also to the pH increase of the acidic steam-exploded hydrolysates which provided the yeast a more favorable environment to grow. Identification of inhibitory compounds of steam-exploded biomass before and after biochar addition would help to better understand the effect of poultry litter biochar on the steam-exploded biomass.

APPENDIX

Section 2.3.5.2 Plate count

Tables A1, A2, and A3 show the yeast growth in the biochar and control (YM and GYE) media, the number colonies were counted and the CFU/ml (colony forming unit per ml) was calculated using the following equation:

$$\frac{CFU}{ml} = \frac{number\ of\ counted\ colonies\ x\ dilution\ factor}{plated\ volume\ (ml)}$$

Table A1. Growth in YM control media

Time (h)	Replicate plate	volume (ml)	dilution factor	Colonies (flask 1) [a]	CFU/ml	Colonies (flask 2) [b]	CFU/ml	average CFU/ml (flask 1&2)
0	1	0.1	10^4	134	13,400,000	119	11,900,000	
0	2	0.1	10^4	166	16,600,000	149	14,900,000	
0	3	0.1	10^4	136	13,600,000	n/a		
average				**145**	**14,533,333**	**134**	**13,400,000**	**13,966,667**
3	1	0.5	10^6	22	44,000,000	24	48,000,000	
3	2	0.5	10^6	20	40,000,000	27	54,000,000	
3	3	0.5	10^6	18	36,000,000	26	52,000,000	
average				**20**	**40,000,000**	**26**	**51,333,333**	**45,666,667**
6	1	0.5	10^6	43	86,000,000	48	96,000,000	
6	2	0.5	10^6	42	84,000,000	45	90,000,000	
6	3	0.5	10^6	54	108,000,000	48	96,000,000	
average				**46**	**92,666,667**	**47**	**94,000,000**	**93,333,333**
9	1	0.5	10^6	66	132,000,000	57	114,000,000	
9	2	0.5	10^6	44	88,000,000	52	104,000,000	
9	3	0.5	10^6	49	98,000,000	66	132,000,000	
average				**53**	**106,000,000**	**58**	**116,666,667**	**111,333,333**
12	1	0.5	10^6	58	116,000,000	67	134,000,000	
12	2	0.5	10^6	58	116,000,000	77	154,000,000	
12	3	0.5	10^6	55	110,000,000	43	86,000,000	
average				**57**	**114,000,000**	**62**	**124,666,667**	**119,333,333**

[a] number of colonies counted for flask 1

[b] number of colonies counted for flask 2

Table A2. Growth in Biochar media

Time (h)	Replicate plate	volume (ml)	dilution factor	Colonies (flask 1) [a]	CFU/ml	Colonies (flask 2) [b]	CFU/ml	average CFU/ml (flask 1&2)
0	1	0.1	10^4	135	13,500,000	148	14,800,000	
0	2	0.1	10^4	166	16,600,000	155	15,500,000	
0	3	0.1	10^4	159	15,900,000	120	12,000,000	
average				**153**	**15,333,333**	**141**	**15,150,000**	**15,241,667**
3	1	0.5	10^6	22	44,000,000	32	64,000,000	
3	2	0.5	10^6	22	44,000,000	22	44,000,000	
3	3	0.5	10^6	24	48,000,000	23	46,000,000	
average				**23**	**45,333,333**	**26**	**51,333,333**	**48,333,333**
6	1	0.5	10^6	76	152,000,000	88	176,000,000	
6	2	0.5	10^6	92	184,000,000	77	154,000,000	
6	3	0.5	10^6	101	202,000,000	80	160,000,000	
average				**90**	**179,333,333**	**82**	**163,333,333**	**171,333,333**
9	1	0.5	10^6	78	156,000,000	95	190,000,000	
9	2	0.5	10^6	91	182,000,000	116	232,000,000	
9	3	0.5	10^6	93	186,000,000	110	220,000,000	
average				**87**	**174,666,667**	**107**	**214,000,000**	**194,333,333**
12	1	0.5	10^6	92	184,000,000	81	162,000,000	
12	2	0.5	10^6	91	182,000,000	79	158,000,000	
12	3	0.5	10^6	105	210,000,000	86	172,000,000	
average				**96**	**192,000,000**	**82**	**164,000,000**	**178,000,000**

[a] number of colonies counted for flask 1

[b] number of colonies counted for flask 2

Table A3. Growth in GYE control media

Time (h)	Replicate plate	volume (ml)	dilution factor	Colonies (flask 1) [a]	CFU/ml	Colonies (flask 2) [b]	CFU/ml	average CFU/ml (flask 1&2)
0	1	0.1	10^4	141	14,100,000	154	15,400,000	
0	2	0.1	10^4	139	13,900,000	147	14,700,000	
0	3	0.1	10^4	132	13,200,000	154	15,400,000	
average				**137**	**13,733,333**	**152**	**15,050,000**	**14,391,667**
3	1	0.5	10^6	16	32,000,000	15	30,000,000	
3	2	0.5	10^6	15	30,000,000	16	32,000,000	
3	3	0.5	10^6	27	54,000,000	13	26,000,000	
average				**19**	**38,666,667**	**15**	**29,333,333**	**34,000,000**
6	1	0.5	10^6	43	86,000,000	32	64,000,000	
6	2	0.5	10^6	35	70,000,000	31	62,000,000	
6	3	0.5	10^6	32	64,000,000	26	52,000,000	
average				**37**	**73,333,333**	**30**	**59,333,333**	**66,333,333**
9	1	0.5	10^6	53	106,000,000	47	94,000,000	
9	2	0.5	10^6	60	120,000,000	44	88,000,000	
9	3	0.5	10^6	58	116,000,000	59	118,000,000	
average				**57**	**114,000,000**	**50**	**100,000,000**	**107,000,000**
12	1	0.5	10^6	48	96,000,000	44	88,000,000	
12	2	0.5	10^6	38	76,000,000	49	98,000,000	
12	3	0.5	10^6	36	72,000,000	52	104,000,000	
average				**41**	**81,333,333**	**48**	**96,666,667**	**89,000,000**

[a] number of colonies counted for flask 1

[b] number of colonies counted for flask 2