Pretreatment of wheat straw with superheated steam and boiling water, its effect on cellulose structure, and fermentation by *Clostridium thermocellum*

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Abstract

The focus of this study was to determine the effects of pretreatment of wheat straw by superheated steam (SS) alone or in combination with boiling water (BW) on biomass structure and yields of fermentation products (cell mass and fermentation end-products) by *Clostridium thermocellum*. Different cultivars of wheat straw were ground to a particle size less than 355 µm, and exposed to the following methods of pretreatment: i) 15 min soaking in 119 °C boiling water under absolute pressure of 193 kPa, followed by processing with SS at atmospheric pressure at different temperatures and retention times; ii) 15 min processing with SS at atmospheric pressure; and iii) 15 min soaking in 119 °C boiling water under absolute pressure of 193 kPa. Processing with SS was conducted at a variety of temperatures in the range of 180-220 °C. The severity of pretreatment was expressed through a treatment severity factor as a measure of harshness of treatment. Pretreatment combinations of boiling water with superheated steam at different retention times inside the SS chamber were also investigated. Wheat straw samples were then used as substrates in fermentation reactions with *C. thermocellum*. The most noticeable effects on biomass structure and fermentation were observed at the highest severity factor of 6.5, corresponding to 15 min pretreatment with boiling water followed by 15 min treatment with SS at 220˚C. This pretreatment provided the maximum increase in percentage of contribution of amorphous cellulose (% CAC), and the highest fermentation yield in terms of hydrogen, carbon dioxide, and ethanol production.

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Nomenclature

ADF: Acid Detergent Fiber

AFEX: Ammonia Fiber Explosion

BW: Boiling Water

CAC: Contribution of Amorphous Cellulose

CBP: Consolidate Bioprocessing

DA: Diluted Acid

HMF: Hydroxymethyl Furfural

HPLC: High Performance Liquid Chromatography

GC: Gas Chromatography

LHW: Liquid Hot Water

MW: Molecular Weights

NDF: Neutral Detergent Fiber

SS: Superheated Steam

SE: Standard Error

TNO: The Netherlands-based Research Organization

WIS: Water Insoluble Solids

XRD: X-ray Diffraction

Chapter 1

1.1 Introduction

The use of renewable energies such as biomass is an important topic of discussion in today's society. There is a worldwide effort to expand the use of sustainable resources of energy, especially those systems which are based on biomass feed stocks. The term biomass describes the total mass of biological material that can be used for production of useful products, such as biofuels. Examples of biomass include forestry and agricultural residues. Cellulose, hemicellulose, and lignin are the main components of lignocellulosic biomass, and are arranged in a complicated structure. This prevents the sugars from being readily hydrolyzed and subsequently fermented. As a result, lignocellulosic biomass requires a suitable pretreatment process in order to liberate fermentable sugars. When these sugars are fermented, they can produce ethanol, which is a liquid biofuel (Agbor et al., 2011).

Characterization of biomass structure is difficult as it is affected by its composition. Crystallinity, porosity (available surface area), lignin protection, strength of fiber, and hemicellulose sheaths are parameters that influence biomass recalcitrance and the rate of hydrolysis of a given biomass. Lignin, which protects the cellulose of the cell wall, reduces biomass digestibility and limits enzymatic hydrolysis (Mosier a et al., 2005). Various kinds of biomass such as woody plants, herbaceous plants, grasses, and municipal solid waste, have varying amounts of cellulose with plants generally around 40-50 %, hemicellulose generally in the range of 20-40 % for plants, and lignin for plants in the range of 20-30 % (Chandra et al., 2007).

Sustainable energy systems based on renewable lignocellulosic biomass are in high demand because of global increase in energy demands, reduction of available fossil fuels, climate change. Biofuels are classified as either first or second generation. First generation biofuels are produced from sugars (bioethanol) or vegetable oils (biodiesel) derived from agricultural crops, while second generation biofuels are made from lignocellulosic biomass and agricultural waste materials.

The use of bioethanol in the transportation sector is an example of an energy system developed based on employing biomass (Agbor et al., 2011). Cellulose materials which are being used as feed stocks for biofuel production have several advantages such as being cost efficient, sustainable, and are the most abundant biomass in the world. Cellulose, hemicellulose, proteins, and lignin are the components of raw biomass. Cell walls of plants have a hetero-matrix which contains sugars fixed in cellulose fiber, making the biomass recalcitrant towards digestion by the bacteria. Pretreatment processes are methods that debilitate the lignin and hemicellulose fiber strength in the raw lignocellulosic biomass and increase the accessibility to cellulose in the conversion process (Lynd et al., 2002).

Consolidate Bioprocessing (CBP) is a strategy to decrease the production cost of end-products through conducting economical pretreatment methods and combination of production phases including cellulase production, substrate hydrolysis, and fermentation of sugars in one stage. This strategy reduces the biofuel production costs up to 41% (Lynd et al., 2008). CBP is conducted with soluble sugars and feed stocks such as cellulosic materials through processing with pretreatment and fermentation approaches. Consequently, CBP may be chosen as a desired method for hydrogen and ethanol production (Levin et al., 2006).

Lignocellulose biomass depolymerizing to simple sugars is a complex multi-step process consist of different steps such as loosening the structural complexity of lignocelluloses, hydrolysis of pectin, lignin degradation hemicellulose depolymerization and cellulose hydrolysis. Consolidated bioprocessing (CBP) has been known as the most promising fermentation approach for bioethanol production from lignocellulosic biomass. In CBP, all the processes, including the production of saccharolytic enzymes (cellulases and hemicellulases), polysaccharides hydrolysis which is the hydrolysis of carbohydrate components present in pretreated biomass to sugars, and the fermentation of hexose sugars (glucose, mannose and galactose) and the fermentation of pentose sugars (xylose and arabinose) to bioethanol or other valuable products proceed simultaneously (Parisutham et al., 2014; Kumagai et al., 2014; Olson et al., 2012; Lynd et al., 2005). On the other hand in the CBP conversion approach, all the stages of development including pretreatment, saccharification, and fermentation would be conducted within a single reactor to decrease operating cost, improve conversion efficiencies, and to decrease by-product inhibition (Favaro et al., 2015). This method can improve the operating procedures by increasing the efficiency of cellulose conversion and also causes the reduction of the costs for lignocellulosic biomass processing for biofuels production by improving the biomass solubilization effectiveness (Favaro et al., 2015). The main advantage of CBP technology is eliminating the need for added external exogenous hydrolytic enzymes and reducing the sugar inhibition of cellulases (Lynd et al., 2005; Olson et al., 2012).

The reason that CBP microorganisms do not need to exogenous saccharifying enzymes is that because they produce their own cellulolytic and hemicellulolytic enzymes for lignocellulose decomposition, which reduce the overall capital cost of the process (Favaro et al., 2015).

Furthermore, it simplifies the operating process by reducing the number of reactors and by minimizing the compatibility requirements of enzyme and fermentation systems.

For the purpose of generation of biofuels such as ethanol with using CBP and to have all the CBP benefits, cellulase generation, biomass saccharification, and fermentation of reducing sugars must happen in conjunction by a monoculture or coculture of microorganisms, such as bacteria like *C. thermocellum* and fungi including *Neurospora crassa*, *Fusarium oxysporum*, *S. cerevisiae*, and *Paecilomyces*. Acorrding to previous studies (Favaro et al., 2015), due to the native cellulolytic and ethanologenic capabilities of *Clostridium thermocellum*, this bacterium is a significant and strong biocatalyst candidate for CBP. This is due to *Clostridium thermocellum'*s natural ability to rapidly solubilize cellulose by way of its cellulosome, and also because of the multi-enzyme complexity structure as well as rapidly solubilize cellulose by way of its cellulosome, and eventually because of its ability to produce ethanol. Furthermore, *C. thermocellum* is an anaerobic thermophile which can significantly reduce operating expenditures and has inhibitory effects for enhanced biofuels production which eliminates the need for aeration, decreases contamination concerns, and improves temperature-dependent functionality of hydrolytic enzymes (Favaro et al., 2015).

The role of *C. thermocellum* in CBP approach is to hydrolyze cellulosic substrates into shorter chain sugars, primarily cellobiose for conversion to ethanol, acetate, and lactate as primary end products through several metabolic ways. *C. thermocellum* provides a collection of cellulolytic enzymes, which are stored into complex, large, multi-protein structures on the surface of cell, known as cellulosomes.

CBP technology with featuring cellulase production, cellulose hydrolysis and fermentation in one step has been reported as a promising approach with outstanding potential (Lynd et al., 2005).

The important aspect of CBF which distinguish it from other less highly integrated configurations in that it does not include a dedicated process step for production of cellulose. As it was reported by other researchers (Lynd et al., 2002), CBP offers potential the higher efficiency combining with lower cost and processes featuring dedicated cellulase production. In CBP strategy, the possibility of realizing higher hydrolysis rates could support with several factors including using the thermophilic organisms and/or complexed cellulase systems as well as enzyme-microbe synergy which cause the higher hydrolysis rates and therefore the reduced reactor volume and total capital investment.

The main objective of this work is to evaluate and characterize the fermentability of pretreated, local, wheat straw feedstocks (biomass) to be used for direct cellulose fermentation for production of biofuels and co-products by *Clostridium thermocellum* using superheated steam (SS) alone or in combination with boiling water (BW). As such pretreatments can improve substrate conversion rates, and end-product yields under consolidated bioprocessing conditions, the main objective is to identify and evaluate the changes in biomass properties resulting from pretreatment that can improve biofuel (ethanol and hydrogen) production via direct microbial conversion by fermentation. Studying the substrate characteristics of potential biorefining feedstocks as well as pretreatment methods to improve the synthesis of biofuels and consequently, co-products generated for a given substrate as we explore the fermentation options available to the integrated biorefining industry. Based on the main objective mentioned above, the following specific objectives are considered in this dissertation:

Objective 1:

to investigate the rate of conversion of pretreated different cultivars of wheat straw, each with different amounts of cellulose, hemicellulose, and lignin which are chosen as potential feedstocks for microbial conversion by *C. thermocellum* using CBP. The pretreatments will be conducted with superheated steam (SS), either alone, or in combination with boiling water (BW) at 119 °C. The effects of cellulose structure and the cellulose content on the conversion rate and yield of end-products of fermentation (ethanol, hydrogen, carbon dioxide) with *C. thermocellum* will be determined.

Objective 2:

To determine the effect of various pretreatments on biomass structure (related to crystalline and amorphous areas of cellulose) and microbial conversion of cellulose by *Clostridium thermocellum* to fermentation end-products. Therefore, different lignocellulosic feedstocks will be subjected to the same or similar type of physicochemical pretreatment, their rates of hydrolysis, and subsequent conversion as a function of the percentage of amorphous cellulose will be investigated and will be correlated with the efficiency of microbial conversion to fermentation end-products (i.e. yields of hydrogen and ethanol).

Objective 3:

To investigate the correlation between the severity factor, as a factor used to evaluate the effect of superheated steam (at different temperatures and retention processing times), with yield of fermentation end-products on biomass structure in order to find the optimized process condition (i.e. temperature and residence time) to reach the maximum production rate.

1.2 Literature Review

1.2.1 Lignocellulosic Biomass

Plant matter is the source of lignocellulosic biomass, which is composed of polymers such as cellulose, hemicellulose and lignin. Plants are considered to be a renewable resource because they convert solar energy to chemical energy stored in chemical bonds (McKendry et al., 2002). Depending on the type and sources of biomass, these polymers are arranged in different hetero-matrix structures and may have different composition of polymers (Carere et al., 2008).

Biomass obtained from grasses and other plants has a crucial role in renewable feed stock for industries. Lignocellulosic biomass has some advantages over starch and sucrose, such as being less expensive and is more accessible in large amounts. This makes it an attractive option for conversion to ethanol. Carbohydrates make up 50 to 80 % of lignocellulose by weight (Naik et al., 2010). These carbohydrates are polymer chains of both five and six carbon sugars. Biofuels such as ethanol can be produced by a series of either chemical or biological reactions involving carbohydrates such as sugar, cellulose, hemicellulose and starch. The conversion process of biomass can be divided into four main categories which are thermal, biological, chemical and physical conversion (Naik et al., 2010). This study investigates the effects of physical and thermal processes of size reduction, boiling water, and superheated steam pretreatments to wheat straw biomass.

In addition to plants, fungi and algae also have cell walls containing cellulose. Monomers of ß-D-glucopyranose are linked by ß-(1,4) glycosidic bonds to form polymers of cellulose. The forces holding cellulose together include covalent bonds, hydrogen bonds, and London dispersion forces (Perez et al., 2008). The more hydrogen bonds cellulose has, the straighter the

chain, making it harder to break. Cellulose structure (see Figure 1-1) is made of two regions: ordered (crystalline) regions and disordered (amorphous) regions (Perez et al., 2008).

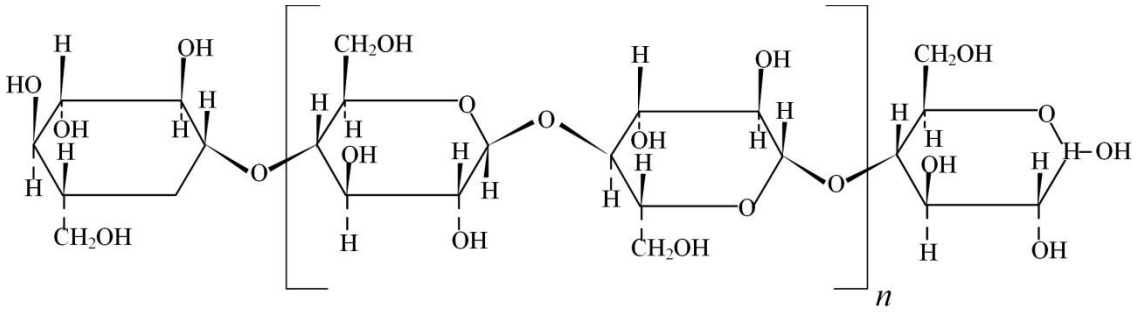

Figure 1-1. Cellulose unit (modified figure from Arvanitoyannis et al., 2009).

Hemicellulose contributes 20 to 50 % by weight of biomass. It is superseded in mass fraction of biomass only by cellulose. Hemicelluloses can join together to form branched polymers of pentoses, hexoses, and acetylated sugars. Their molecular weights (MW) are smaller than those of cellulose. Hemicellulose has short branches which are more easily hydrolyzed than cellulose. Hemicelluloses vary in composition depending on their source. For example, in agricultural biomass like straw and grasses, they mostly contain xylan. In contrast, softwood hemicelluloses include mostly glucomannan. Hemicellulose is the most thermally sensitive of the polymers of lignocellulose materials (Hendricks et al., 2009).

Cellulose fibrils are coated by hemicellulose (see Figure 1-2). To increase the digestibility of cellulose, a minimum of 50 percent of the hemicellulose should be eliminated. The hydrolysis and recovering rate of sugars derived from the hemicellulose can be increased after applying different types of pretreatment methods (Chandra et al., 2007).

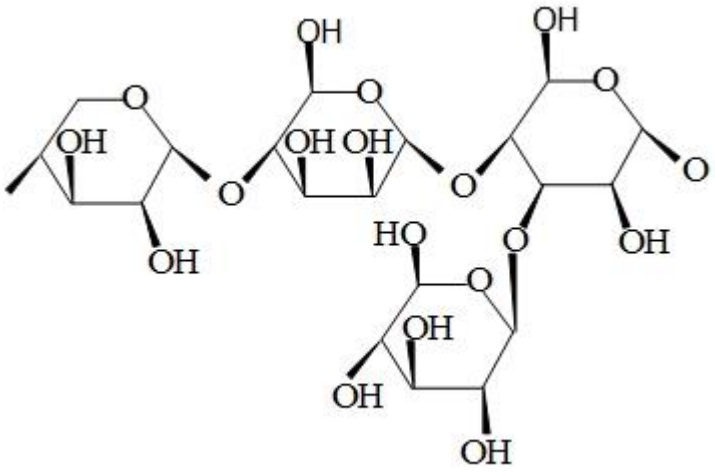

Figure 1-2. Hemicellulose unit (modified figure from Arvanitoyannis et al., 2009).

The third most abundant polymer in biomass is lignin. It is found in the cell wall of plant cells. Lignin has a shapeless hetero-polymer network of phenyl propane units and it is not easily degraded. Many microbes cannot penetrate lignin. It is resistant to oxidation and causes a reduction of the enzymatic effects on hydrolysis of biomass. The different components of lignocellulosic biomass are adhered together by lignin and this makes them insoluble in water. The content of lignin in the biomass varies with the biomass source and affects its digestibility. Therefore, depending on the lignin content of the biomass, different pretreatment methods are used (Hendricks et al., 2009).

Table 1-1 shows the cellulose, hemicellulose and lignin contents in different raw materials. Biomass with lower lignin content is preferable for utilization as a biofuel feedstock. Flax and hemp have the lowest lignin contents as well as the highest amounts of cellulose. This allows their pretreatment processes to be the most cost effective. In the case of this study, wheat straw was selected due to its abundance in Manitoba.

Table 1-1. Chemical composition of selected biomass (wt%) (modified from Le Digabel et al., 2006).

Substrate	Cellulose	Lignin	Hemicellulose
Bast fibers			
Flax	71	2	19
Hemp	75	4	18
Jute	72	13	13
Leaf fibers			
Abaca	70	6	22
Sisal	73	11	13
Seed-hair fibers			
Cotton	93	-	3
Wheat straw	51	16	26
Lignocellulose fillers	58	31	8

1.2.2 Second Generation Biofuels

Second generation biofuels refers to the use of lignocellulosic biomass, derived from forestry and crop residues and wastes, for biofuel production. Second generation biofuels have become of more interest because of the limited resources of first generation biofuels. The importance of biofuel is increasing due to the reduction of oil reserves. Overlay, yields of the first generation biofuels are lower than general energy needs. On the other hand, more energy is consumed for production than would be obtained from the final fuel. Second generation biofuels from lignocellulosic biomass is reported to be more promising owing to its high available quantity and productivity. Second generation biofuels, particularly from forest and crop residues and energy would significantly decrease net carbon emission, enhance energy efficiency and reduce energy dependency which are potentially overcoming the limitations of the first generation biofuels. Papini et al. showed that second generation biofuels from lignocellulosic biomass had a much higher productivity compared to traditional biofuel cultivation (Papini et al., 2010). The benefits of second generation biofuels compared with the first generation biofuels depend on the original location of feed stock and whether the biomass is a by-product or waste product.

There are two major methods for producing liquid biofuels from biomass: thermochemical and biochemical processing. Thermochemical processing describes the conversion of biomass into a variety of products by thermal decomposition, which results in chemical improvement. This is achieved by heating the biomass in the presence of different concentrations of oxygen. In thermochemical processing, almost all of the organic components of the biomass can be utilized. Biochemical processes such as anaerobic digestion emphasize using enzymes and chemicals to

break down the lignin shields of lignocellulosic biomass as well as the conversion of cellulosic materials to end-products (Naik et al., 2010).

Havlík et al. focused on three sources for second-generation biofuel production: 1) Biomass for second generation biofuels obtained from tree plantations, which are from croplands or pastures; 2) Biomass for second generation biofuels obtained from wood from forestry practices; and 3) Biomass for second generation biofuels obtained from tree plantations of non-agricultural land. The properties of the mentioned sources with respect to the greenhouse gas emissions were studied and it was figured out that in terms of GHG emissions, second generation biofuels had better performances than the first generation (Havlík et al., 2011).

1.2.3 Ethanol Production

Conversion of lignocellulosic to liquid biofuel such as ethanol has technical barriers because of structural and chemical resistance to conversion. Cellulose and hemicellulose can be hydrolyzed and fermented into ethanol. However, the long-chain sugars inside the cross-linking structure of the lignocellulose reduce the conversion process efficiency due to inaccessibility by the microorganisms (Wyman et al., 1999).

Conversion of lignocellulosics to ethanol consists of four major unit operations: pretreatment; hydrolysis; fermentation; and product separation or purification. Pretreatment is required to transform the biomass structure so that decomposition of the carbohydrate portions to monomeric sugars can occur more quickly and with higher efficiency. Hydrolysis is the process by which the carbohydrate polymers are converted to monomeric sugars. Enzymatic hydrolysis is a popular method at the industrial scale because of the high efficiency of biofuel production (Wyman et al., 1999).

Cellulose is hydrolyzed into sugars, specifically glucose, by enzymes such as cellulases or by chemicals such as sulfuric acid or other acids. Hemicellulose chains are hydrolysed by hemicellulases or acids. Glucose, galactose, mannose and other six carbon sugars, hexoses, are easily fermented to ethanol by organisms such as *C. thermocellum* (Gong et al., 1983).

Figure 1-3 illustrates the steps of ethanol production, starting with feed stock selection separated into solid and liquid phases, and continuing through pretreatment, separation, hydrolysis, fermentation, and purification processes. The fermentation process contains lignin, cellulose, hemicellulose and ash combined with enzymes and microorganisms, which are mixed in the bottom of the distillation column to be concentrated and separated into different end-products (Dwivedi et al., 2009).

Furan complexes, which are one of the most significant inhibitor groups in dilute-acid hydrolysates, are formed from numerous reactions of pentoses and hexoses during acid hydrolysis (see Figure 1-4). Furfural is produced from the degradation of pentoses such as xylose, while hydroxymethyl furfural (HMF) is made from hexoses such as glucose. As an inhibitor, furfural decreases the rate of ethanol production and growth of the microorganisms inside the media through suppression of enzymatic levels in the hydrolysis process (Banerjee et al., 1981).

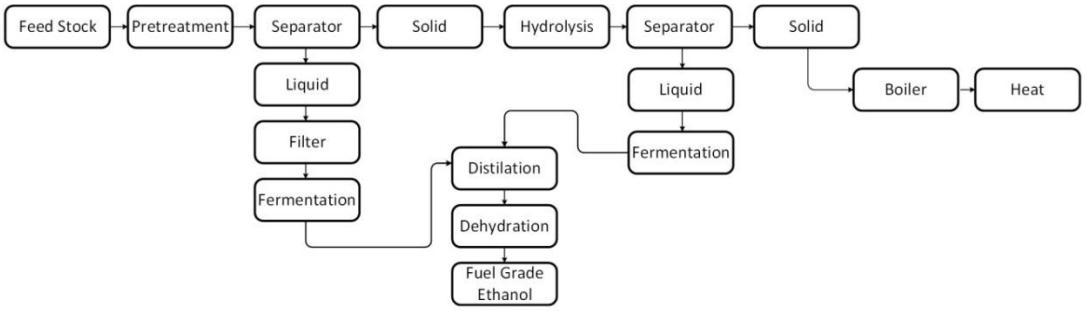

Figure 1-3. Schematic of ethanol production (modified from Dwivedi et al., 2009).

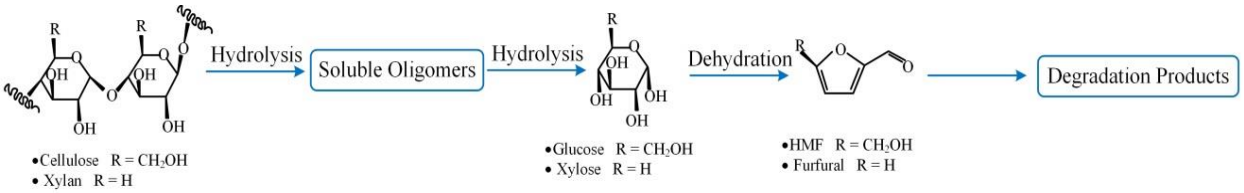

Figure 1-4. Schematic of furfural formation and degradation products (modified from Sánchez et al., 2011).

1.2.4 Biomass Pretreatment

Pretreatment processes aim to weaken the lignin and hemicellulose fiber strength in the raw lignocellulosic biomass and increase the accessibility of cellulose during the conversion process (see Figure 1-5). The purpose of pretreatment is to increase the digestibility of lignocellulose biomass, lower the crystallinity of cellulose, and to weaken the lignin shield and hemicellulose structure. Ideal attributes of the pretreatment process include increased yield of enzymatic hydrolysis, prevention of carbohydrate loss, prevention of byproduct formation, and reduction in costs. There are different categories of pretreatments: physical such as mechanical communication; chemical that includes the use of oxidizing agents, sulfuric or other acids; thermal such as steam explosion and ammonia fiber explosion; biological that is use of the microorganisms and enzymes; electrical including pulsed electric field pretreatment that is mostly in the forms of exponential decay or square waves; and any combination of the mentioned methods (Kumar et al., 2009). In order to enable fast hydrolysis of carbohydrates, pretreatment should change the submicroscopic structure and composition of biomass as well as its macroscopic size and structure (Chang et al., 2000).

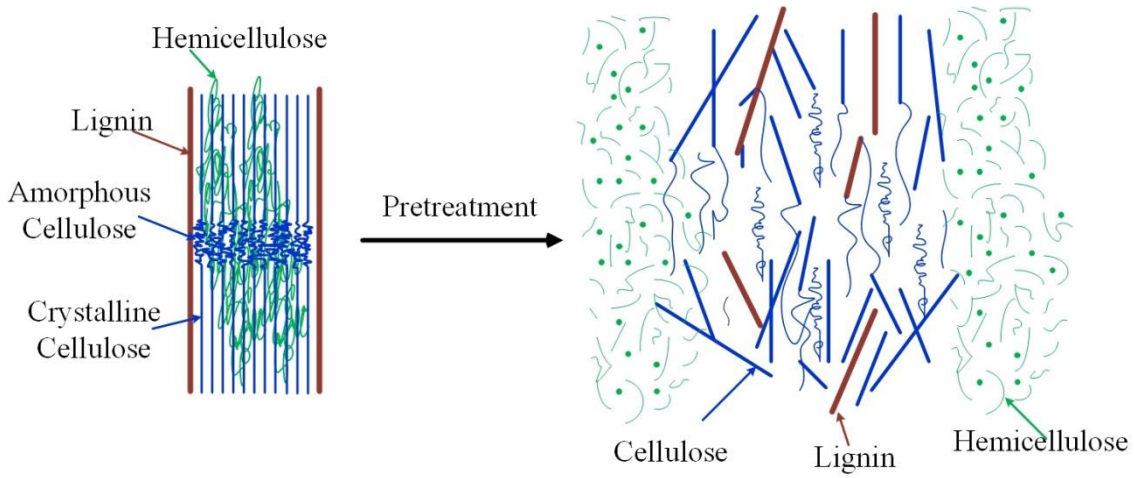

Figure 1-5. Effects of pretreatment on biomass structure (modified from Zheng et al., 2009).

When biomass is pretreated thermally, the effectiveness of the pretreatment is measured by the severity factor. The factor quantifies the effectiveness of the retention time, temperature, and acidity on the pretreatment. Pretreatments are assessed in several ways. One way is by analyzing unrestricted sugar monomers and oligomers in the liquid and the carbohydrate concentration in the water insoluble solids (WIS) after pretreatment. This determines the total number of recoverable carbohydrates. Another way is to assess the enzymatic hydrolysis of either the washed or unwashed WIS. Fermentation of the pretreated liquid to measure its fermentability directly or diluted to target concentration of fermenting microorganisms in terms of growth is another method of assessment. Another way to evaluate the pretreatment is by the fermentation of either the slurry or washed WIS for biofuels production. Pretreatments are also evaluated by the assessment of additional biotechnological potential of the pretreated fractions such as value added products (Galbe et al., 2007).

Hemicellulases or acids can be used to release the sugars in hemicellulose polymers. Glucose and galactose, sugars with six carbons are converted to ethanol through the fermentation process which is achieved using microorganisms such as bacteria. An effective pretreatment must keep the hemicellulose portions for fermentation to ethanol and hydrogen, limit the production of inhibitors, to be energy efficient and cost effective in terms of operation and equipment costs. Through the pretreatment of biomass, the complex matrix of lignin and hemicelluloses will be weakened prior to hydrolysis of cellulose (McKendry et al., 2002).

The type of biomass dictates the type of pretreatment method. There are several different pretreatment technologies available, each with their own advantages and disadvantages. The retrieval of valuable coproducts like proteins and lignin, salvaging of pretreatment catalyst, and

treatment of waste are also important parameters to consider when choosing the proper method of pretreatment (Palmqvist et al., 2000).

1.2.5 Physical Pretreatments

The advantages of mechanical pretreatment of cellulosic material such as chipping, crushing, and milling includes decreasing the crystallinity and the particle size in order to reduce the degree of polymerization, and increasing the digestibility of cellulose. Chipping can only reduce the size of cellulose to within a range of 20-30 mm. Milling with a vibratory ball reduces the size of particles to 0.2-2 mm and has the benefit of decreasing crystallinity more than routine ball milling (Sun et al., 2002). Various milling processes exist, including ball milling such as vibratory milling, squeeze milling, ball beating, and pan milling. The amount of energy used, the final size of particles, and the properties of the specific biomass determine the selection of the most suitable mechanical pretreatment. The amount of energy consumption required to reduce biomass particle size to 3-6 mm is below 30 kWh per ton of biomass (Cadoche et al., 1989).

1.2.6 Chemical Pretreatment

Chemical pretreatment is more efficient than mechanical pretreatment because it leads to reduction of lignin content and as a result, an increase in digestibility of cellulose by bacteria (Zheng et al., 2009).

1.2.6.1 Acid hydrolysis

The hydrolysis processing of biomass to fermentable sugars can be improved by acid hydrolysis pretreatment, which includes strong acids such as sulfuric acid and hydrochloric acid. However, the acid hydrolysis process is cost intensive due to the need for resilient reactors that can withstand the corrosive effects of the reagents. To make this process cost efficient, the

concentrated acids are recycled at the end of the process. Diluted acid such as sulfuric acid are used in this process as it is less corrosive and more efficient. This is because when the diluted sulfuric acid with a concentration lower than four percent by weight is added to the biomass, hydrolysis of hemicellulose to xylose and other dissolved sugars can be achieved that increases the digestibility of cellulose. In other words, pretreatment with diluted sulfuric acid considerably enhances hydrolysis of cellulose. In addition, raising the temperature during acid hydrolysis process can increase the effectiveness of this kind of pretreatment (Kumar et al., 2009).

1.2.6.2 Alkaline hydrolysis

Alkaline hydrolysis using basic solutions can be utilized for the pretreatment process. The efficiency of alkaline hydrolysis depends on the amount of lignin present in the biomass. Alkaline pretreatment conducted in a reactor needs less heat and pressure compared to thermal pretreatment methods. However, it requires more processing time than thermal pretreatments. Alkaline hydrolysis results in less degradation of sugar than acid hydrolysis. Typical alkaline chemicals utilized in pretreatment are sodium, calcium, potassium and ammonium hydroxides. Many of these corrosive salts can be recycled (Kumar et al., 2009).

Among alkaline pretreatments, calcium hydroxide is the most powerful agent as well as the most cost efficient. When an aqueous reaction which contains calcium is neutralized with carbon dioxide, calcium can be regained. Then, by using the lime kiln method, calcium hydroxide can be recovered. Lime kiln technology consists of three sections: first the lime is washed with water, the lime is then applied onto the biomass (particles of 10 mm or less) by spraying, and finally the sprayed material is stored for hours or weeks. By increasing the temperature, the contact time between the biomass and lime in this technology can be minimized. Crystallinity index is increased by the amount of lignin and hemicellulose present in the biomass, and lime

pretreatment can weaken and in some portions remove these substances. There are three fundamental parameters that affect the enzymatic digestibility: amount of lignin, amount of acetyl, and the crystallinity index of the substance. When there is significant delignification, digestibility of the biomass is increased, irrespective of acetyl concentrations. Enzymatic hydrolysis can be enhanced by delignification and deacetylation, and the hydrolysis rate can be influenced by the crystallinity. An efficient and powerful alkaline pretreatment of the lignocellulosic biomass will eliminate all of the acetyl groups and decrease the amount of lignin to ten percent by mass. Therefore, by rendering the cellulose susceptible to enzyme hydrolysis, alkaline pretreatment plays an important role in biomass processing (Kumar et al., 2009).

1.2.7 Biological pretreatment

In this process, microorganisms like brown-rot fungi, white-rot fungi or soft-rot fungi are responsible for degradation of hemicellulose and lignin. Brown rots attack the hemicellulose, while white and soft rots attack both hemicellulose and lignin. White-rot fungi cause lignin removal through the use of enzymes such as peroxidases and laccase. Biological treatments have some advantages including being safe and eco-friendly, lower costs and amounts of needed energy in order to remove lignin compare with physical and thermochemical processes (Kumar et al., 2009).

1.2.8 Thermal Biomass Pretreatment

1.2.8.1 Liquid hot water (LHW) pretreatment

In order to keep water in liquid phase at high temperatures, high pressure is required. Therefore, for liquid hot water pretreatment, biomass conversion is done under high temperature and pressure. Liquid hot water pretreatment has advantages such as increased cellulose digestibility and recovery of sugars (VanWalsum et al., 1996). When treating wheat straw with

liquid hot water pretreatment, 53 % recovery of sugar and a hydrolysis yield of 96 % can be expected (Perez et al., 2008). By optimizing LHW variables such as temperature and time, 80 percent xylose recovery and 91 percent hydrolysis yield can be obtained. LHW is a suitable method for a large scale implementation because in this process, biomass size reduction is not required and acid is not used (Perez et al., 2008).

The water in which the biomass was treated was analyzed by HPLC for the presence of glucose, xylose, and cellobiose. While no glucose was observed in the samples, trace amounts of cellobiose and significant amounts of xylose were detected from the hemicelluloses solubilized in the boiling water (BW) treatment. Hemicellulose is the least stable of three major plant biomass polymers and therefore most affected by the BW pretreament. Water at elevated temperatures has been previously reported to remove hemicellulose sugars while minimizing cellulose hydrolysis and the formation of sugar degradation products during liquid hot water pretreatments (Negro et al., 2003).

Over 80 % of hemicelluloses derived sugars were recovered in the liquid fraction of corn stover and sugar cane bagasse (Allen et al., 1996). This indicated that little or no amounts of glucose monomer units were solubilized and lost as a result of treatment in boiling water, and because cellulose chains were not substantially affected, hence almost all of the cellulose mass was potentially be available for bioconversion to end-products.

1.2.8.2 Ammonia Fiber Explosion (AFEX)

Another technology used for thermal pretreatment is ammonia fiber explosion. In this process, liquid ammonia at very high temperature and pressure is applied to lignocellulosic biomass. The pressure is then rapidly reduced. Standard parameters of the ammonia fiber explosion process are temperatures in the range of 60-90 °C, consumption of one to two kg of

ammonia per kg of biomass, and applied pressure of over 3 MPa with 10-60 minutes holding time (Alizadeh et al., 2005).

First, high pressure and temperature are applied to the biomass and ammonia for half an hour in a closed container. The biomass is maintained at the required temperature for 5 minutes, at which point the vent valve is unlocked in order to initiate an explosive decompression. The rapid opening of the valve results in rapid evaporation of ammonia. The cellulosic structure of the biomass is disrupted by ammonia and pressure, this causes destruction of crystalline regions of the cellulose through swelling of the biomass. This method of pretreatment is very effective for wheat straw, and it can dramatically increase the rate of biomass fermentation. By using ammonia fiber explosion pretreatment, hydrolysis yield of cellulose and hemicellulose of up to 90 percent can be obtained (Kumar et al., 2009). This is because this treatment causes the structure of the substances to change, which results in a higher capability for retaining water. This pretreatment method is less effective in cases where the biomass has lignin content over 15 percent, such as woods and nutshells (Kumar et al., 2009).

1.2.8.3 Steam pretreatment/steam explosion (ST/SE)

Steam pretreatment or steam explosion operates using a high temperature (as high as 240 °C) at a high pressure for short durations. The main purposes of this pretreatment method are to restrict inhibitor production and to increase the amount of available cellulose for hydrolysis. At the end of the process, due to high temperature, the lignin is converted and hemicellulose is solubilized in the liquid phase of steam that causes increasing the accessibility of the cellulose for digestion by the microorganism. Steam pretreatment and steam explosion pretreatment vary with respect to the rate of biomass cooling. In addition, in the steam pretreatment method, as moisture

content of the biomass increases, the time for steam pretreatment also increases (Sun et al., 2002).

Steam explosion is the most effective method of lignocellulosic pretreatment because it operates at very high-pressure steam and temperature of 160-260 °C. After applying steam with pressure of 0.69-4.83 MPa, rapid reduction of pressure to atmospheric pressure results in an explosive decompression of the biomass. Due to the extreme temperatures used in steam explosion pretreatment, lignin and hemicellulose, that are more thermo-sensitive polymers, are removed more easily. Removing these materials from biomass increases the surface area for enzymatic activity. Steam explosion has some disadvantages which include: the destruction of xylan structures, imperfect interruption of lignin structure, and allowing the formation of inhibitors of microorganism activities (Kumar et al., 2009).

1.2.8.4 Superheated steam

Since the middle of the twentieth century, superheated steam has been used as a method of pretreatment and drying of materials. Recycling steam through a heat exchanger can reduce processing costs. Superheated steam as a pretreatment method can increase the digestibility of biomass and production rate of fermentation end products (Sagehashi et al., 2006).

Using superheated steam (SS) as a method of pretreatment has some advantages and disadvantages. Some of the advantages of SS are: the high temperature of processing results in significant thermal diffusivity of the superheated steam inside the biomass to conduct the pretreatment process, and increased energy efficiency which can be achieved by recycling the heat of evaporation from condensed steam. However, there are some disadvantages as well: some substances with more hemicellulose contents are thermally sensitive and can be damaged by high temperature. Also, the initial condensation period around the sample especially on the wall of

sample container, causes a temporary increase in the moisture content of biomass (Beeby et al., 1985). Figure 1-6 shows a schematic of a simple superheated steam machine which includes a chamber with a hot air jacket to maintain the high temperature, a condenser to recycle the water inside the system, a boiler to produce the saturated steam, and steam superheater to increase the steam temperature which provides the superheated steam (Head et al., 2010).

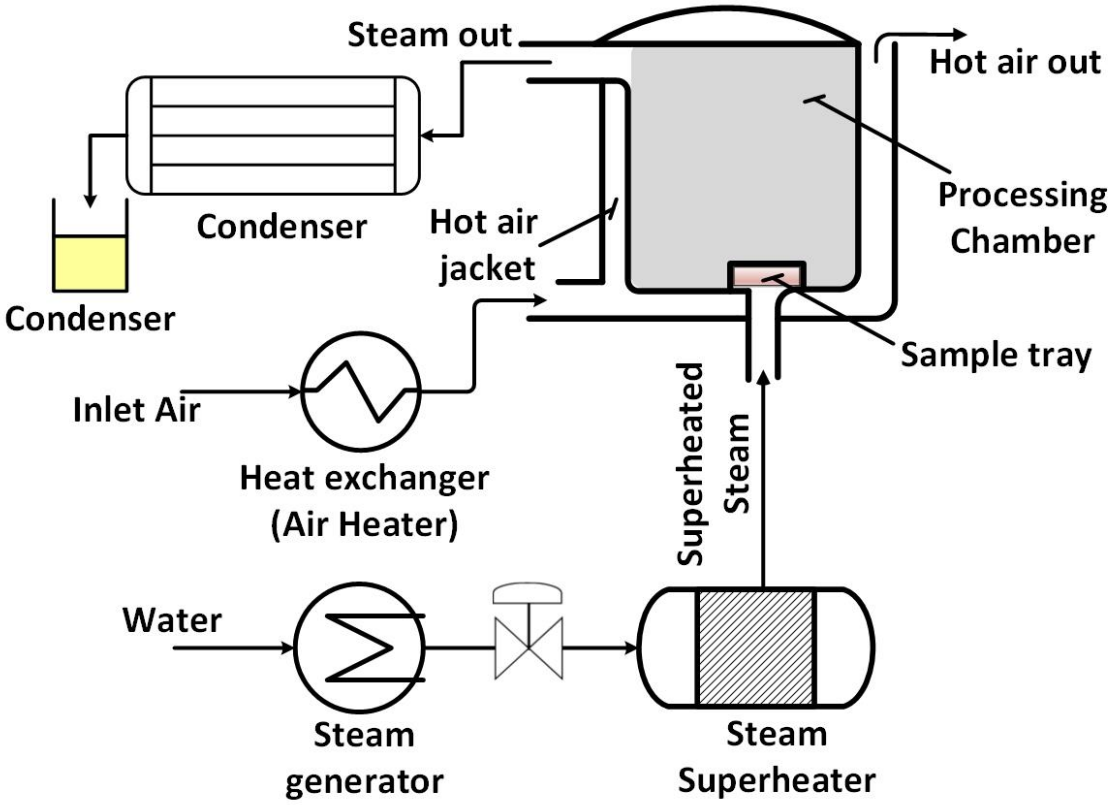

Figure 1-6. Schematic diagram of a superheated steam system (modified from Head et al., 2010).

1.2.8.5 Using Superheated Steam and Dilute Acid Pretreatment Process

Lower moisture content of the biomass reduces the heating and chemical costs in pretreatment methods by lowering the amount of acid required to reach high acid concentration. High dry mass concentration may also be beneficial in fermentation and downstream processing by producing higher substrate and product concentrations.

The Netherlands-based research organization, TNO, is exploring the possibility of pretreatment at higher dry matter concentrations, while minimizing reaction time and temperature. For that purpose, TNO uses diluted acid treatment in combination with passing a continuous stream of superheated steam (SS) through heaps of biomass (Groenestijn et al., 2012). When heating biomass using SS, heat transfer occurs by convection instead of conduction. During the SS pretreatment steam does not condense on the biomass surface and this eliminates the probability of diluting the acid (catalyst) in peripheral zones of the biomass particles. This is important when efficiencies near 100% must be reached. A continuous flow of superheated steam through the material is important as well, to keep the steam superheated and to evenly distribute the hot steam over the biomass material. The biomass should be porous enough to allow spaces between the biomass pieces. Pieces of straw and grass naturally act as spacers which allow steam to access every piece of biomass. Wheat straw, grass or corn stover) was used as the substrate, soaked in diluted sulfuric acid (2 percent by weight) and treated with SS at 120-190 °C. This method has some advantages including eliminating the need for cutting, fast heating and cooling, a short duration, low inhibitor concentrations, and any acid can be used as the catalyst (Groenestijn et al., 2012).

1.2.9 Evaluation of Biomass Pretreatment

A parameter labeled as "severity factor" has been used for the evaluation of the effectiveness of a thermal pretreatment method (Agbor et al., 2011). The factor is calculated based on temperature and duration of pretreatment. Several parameters which affect the assessment of pretreatment are:

(i) The content of sugars released after hydrolysis of cellulose and hemicelluloses;

(ii) Enzymatic hydrolysis rate of insoluble solids remaining in the water after pretreatment;

(iii) The growth of bacteria and time needed to reach the exponential phase during the fermentation process (Agbor et al., 2011).

The severity factor is expressed as (Galbe et al., 2007):

$$\log R_0 = \log(t \times \exp^{\left(\frac{T-100}{14.75}\right)}) \quad (1)$$

Where, log R_o: severity factor

t: processing time (min)

T: processing temperature ($°C$)

Table 1-2 summarizes some of the advantages and disadvantages of pretreatment methods for lignocellulosic biomass. Based on biomass composition and by-products of pretreatment, an appropriate pretreatment method can be selected. These important factors affect the costs associated with respective pretreatment methods. The pretreatments were examined for similarities and differences after enzyme treatment operating by cellulase enzyme system (Rosgaard et al., 2007).

Table 1-2. Summary of pretreatment methods.

Method	Process Description	Mode of action
Steam pretreatment (SP) or Steam explosion (SE)	Involves rapidly heating biomass with steam at elevated temperatures (190–240 °C) and pressures between 0.7 and 4.8 Mpa with duration times of 3–8 min followed by explosive decompression	Pressure is maintained from several seconds to a few minutes to promote hemicellulose hydrolysis and then released. Acetic acids from acetyl groups facilitate the breakdown of hemicellulose
Liquid hot water pretreatment (LHW)	In this process, lower temperatures (Ideal T is from 180 to 190 °C for corn stover) and low moisture content about (1-8 %) content are used leading to more poly and oligosaccharide production. In this process temperature can vary based on difficulty of the pretreatment. The temperature of 160-190 °C is used for pH controlled liquid hot water pretreatment	Hot water cleaves hemiacetal linkages thus liberating acids during biomass hydrolysis, which facilitates the breakage of ether linkages in biomass.
Dilute acid pretreatment (DA)	Diluted sulfuric acid and biomass are put together to solubilize hemicellulose thereby increasing the accessibility of the cellulose in the biomass. Steam can raise the temperature of this combination directly, while vessel walls of reactors heat the mixture by radiation.	The substrate dissolved in water is heated to the desired temperature and pretreated using pre-heated sulfuric acid (concentrations of < 4 wt %) in a stainless-steel reactor. The acid in DA pretreatment Influences polymers of carbohydrates which then results in liberation of oligomers and monomeric sugars.
Ammonia fibre / freeze explosion (AFEX), Ammonia recycle percolation (ARP) and Soaking aqueous ammonia (SAA)	Anhydrous liquid ammonia is loaded with biomass in a ratio of 1:1 to 1:2 (1 to 2 kg of ammonia to 1 kg of dry biomass) for 10-60 min at 60-90 °C and pressures above 3 MPa	The chemical effect of ammonia under pressure causes the cellulosic biomass to swell, thus increasing the accessible surface area while decrystalizing cellulose

Chapter 2 Materials and Methods

2.1 Biomass and Sample Preparation

Two sources of wheat straw were used in experiments. The sources were wheat straw harvested in September 2013 obtained from the Biovalco Inc., MB, including non-compacted (loose) wheat straw and compacted wheat straw and the Department of Plant Science at the University of Manitoba. Samples were chopped and then ground for five minutes to a fine powder using a coffee grinder. After grinding, the particles were separated into fractions using a Tyler shaker (Model RX-812, Mentor, Ohio) for fifteen minutes. Fractions passing the 355 μm sieve were used in the experiments. Superheated steam was generated by a unit designed in the Department of Biosystems Engineering, University of Manitoba. The unit consists of a boiler, super heater, processing chamber, connecting pipes and valves, and is monitored and controlled by a computer and data acquisition system. Wheat straw cultivars (McKenzie, Kane, AC Domain, AC Intrepid, Harvest, and 560 HR) were a gift from Dr. Belay Ayele, Department of Plant Science, at the University of Manitoba, and subjected to the same physical pretreatment as the samples obtained from Biovalco Inc.

2.2 Superheated Steam, System Design at University of Manitoba

Superheated steam is created when sensible heat is added to saturated steam. Therefore, superheated steam has the ability to lose heat without changing phase provided it remains above the saturated vapor temperature. Figure 3-1 shows the superheated steam system that consists of a boiler, heating elements in the superheater, transferring pipes, safety valves and chamber valves, the heat exchanger for condensation, vacuum breaker, pressure reducer, and data acquisition system. The steam generator functions to generate saturated steam for the system. The system uses an electric boiler with a steam capacity of 24.6 kg/h (54.2 lb/h) and requires a 3

Phase, 208 V, 50 A power supply. The boiler operates on regular tap water. The maximum working pressure for the boiler is 621 kPa (90 psi or 6.2 bar), which is well above the maximum system operating pressure. The system piping transports the superheated steam between the necessary instrumentation and equipment. Numerous valves function to control and direct the superheated steam in the system. All valves were selected to operate above 220 °C, which was chosen as the maximum operating temperature. The pressure reducing valve is required due to the higher pressure in the boiler. This helps to provide a uniform pressure and steam flow rate through the system. The vacuum breaker is between the 2 valves so that it can draw in significant amounts of air into the superheated steam system to create a suitable flow of steam. The superheated steam system is designed with a steam bypass that serves to divert the steam from the drying chamber during the warm-up and sample loading phases. As Figure 3-2 indicates, the process chamber has two sections. The lower one is smaller superheated steam passes from this section through the pipes to the second section where the sample is loaded. The exhaust steam is sent through a heat exchanger to preheat the process water before being pumped to the electric boiler (Gervais et al., 2003).

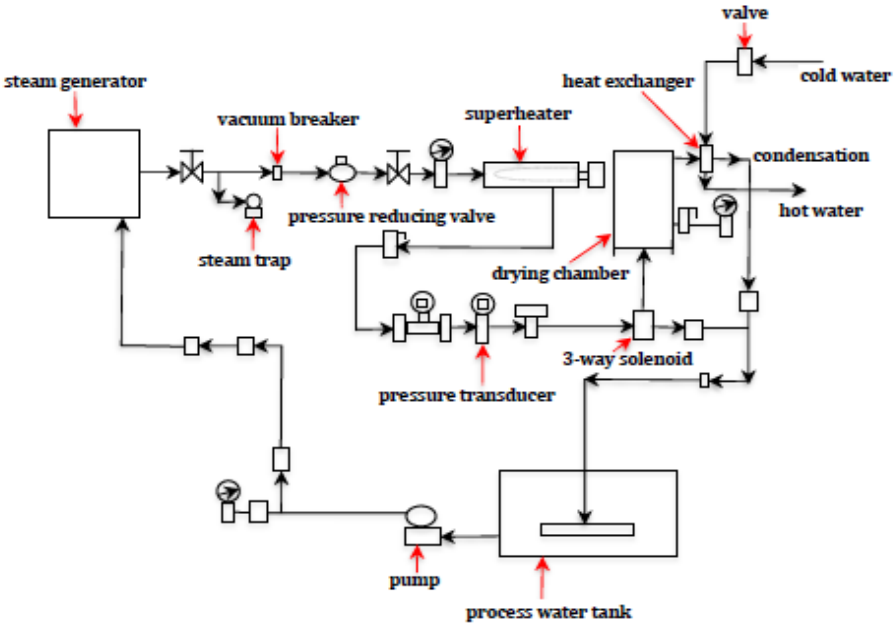

Figure 2-1. Superheated steam system at the University of Manitoba (modified from the superheated steam manual).

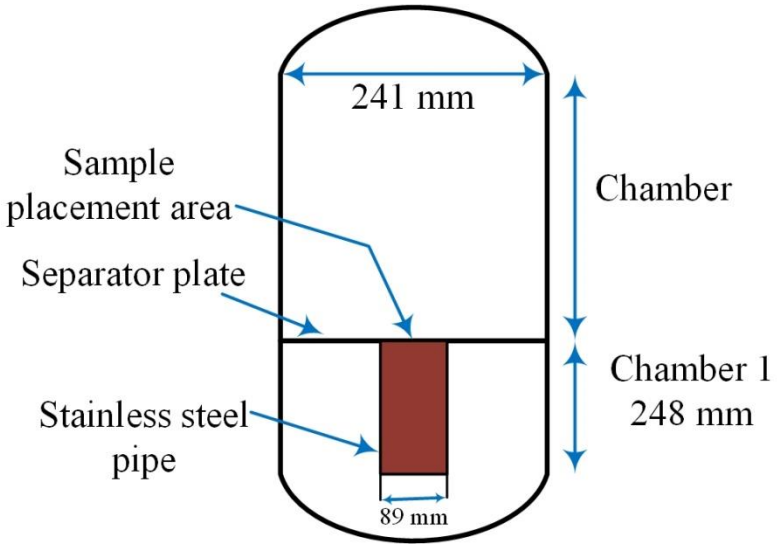

Figure 2-2. Superheated steam processing chamber.

2.3 Pretreatment Conditions

Three conditions were used to pretreat the samples: 1) 15 min of processing with SS at 180, 200, and 220 °C, under atmospheric pressure with a SS velocity of 1.93 m/s; 2) 15 min of soaking in pressurized boiling water (BW) in a pressure cooker at 119°C. A Lagostina 4.5 L, Italy pressure cooker was used; and 3) 15 min of soaking in pressurized boiling water (BW) in a pressure cooker (Lagostina 4.5 L, Italy) at 119°C, followed by the SS treatment at 180, 200 and 220 ºC. To reach the boiling point of 119°C, the pressure cooker was kept under an absolute pressure of 193 kPa. The pressure cooker was preheated to the saturation temperature, at which point a 2 g sample of ground wheat straw was inserted for 15 minutes.

Pretreatment with superheated steam was conducted under atmospheric pressure. For each experiment, the SS temperature at the inlet to the drying chamber was programmed to 180, 200, or 220 °C with an accuracy of ± 2°C. Due to the high SS velocity, spouted bed-like conditions were generated in the SS chamber. A 1g of ground wheat straw was placed in a 60 mm diameter glass-cylinder with wire mesh at the top and the bottom. The velocity of SS at the inlet was 1.93 ± 0.06 m/s, which corresponded to the average SS mass flow rate of 9.36 kg SS/h. The mass flow rate of SS was calculated based on the amount of condensate collected over a known period of time. To monitor changes in moisture content of the samples, mass measurements were taken at intervals of 30 s by a digital scale installed on top of the SS chamber.

In terms of energy consumption, the energy consumed for superheated steam production was calculated based on the saturated and superheated steam properties, power and working duration time of superheated steam equipment. It was estimated approximately 57 kJ/kg biomass, while the energy consumption for conducting steam explosion pretreatment was reported around 201 kJ/kg biomass (Adapa et al., 2011).

Figure 3-3 shows the monitor of control panel installed on the superheated steam machine that indicates the conditions of the processing inside the chamber such as set point temperature, mass varieties, step duration; and temperatures of different sections the chamber.

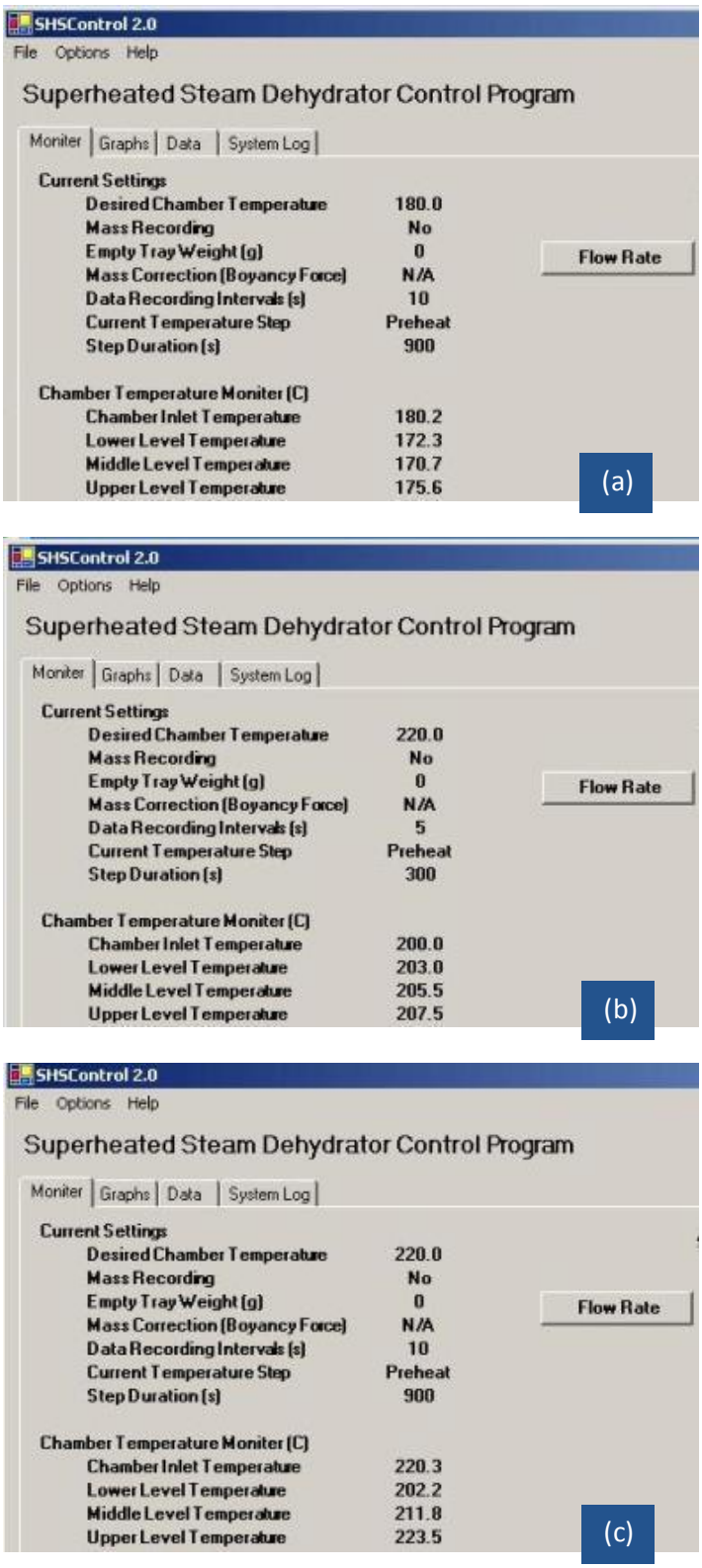

Figure 2-3. Screenshots of the superheated steam program indicating chamber inlet temperatures at (a) 180, (b) 200 and (c) 220ºC.

X-ray Diffraction

X-ray diffraction analyses (XRD) was conducted using a Siemens D5000 powder diffractometer (K710H, Siemens AXS, Inc., Madison, WI) using Cu radiation and operated at 40kV and 40mA, 2.7kW sealed-tube type X-Ray generator with a vertical goniometer housed in a fully-enclosed radiation cabinet (Figure 2-4). The goniometer is in Bragg-Brentano (θ-2θ) geometry. The system is equipped with computer-controlled divergence and receiving slits, a rotating sample holder, diffracted beam graphite monochromator and a scintillation detector. Data is collected using Bruker's DIFFRAC plus software and is processed with MDI Jade+ software.

A graph of the X-ray signal intensity (%) with respect to the angle of diffraction is known as two-theta degree angle. The analysis used the graph's highest and lowest peaks called the "XRD Peak height method". This method provides a comparison of contribution of amorphous cellulose (CAC) values among cellulosic samples exposed to different pretreatments. The anti-scatter and receiving slits were set to 1.0 mm. Samples were mounted in a depressed 'well' on a zero-background quartz plate and pressed into the well with a frosted glass slide. Scans were obtained from 8 to 42 degrees two-theta using a step-width of 0.05 degree two-theta and a dwell time of 1 s/step.

Figure 2-5 shows the schematic drawing of the principal parts of an x-ray diffractometer. X-rays from the tube's line focus are collimated by passage through a parallel slit assembly and a divergence slit before striking the powder on a glass slide or sample holder. While the slide (length exaggerated) rotates about the axis, as to be an angle θ to the direct beam, a detector scans through the angle 2θ. Step scan data for all samples were collected from 5 - 65° 2θ, step width of 0.02° 2θ, a dwell time of 1s/step, and divergence and anti-scatter slits of 1°. Figure 2-6

shows schematic of diffraction in a two-atom structure, which shows the angle of diffracted beams with molecules structure.

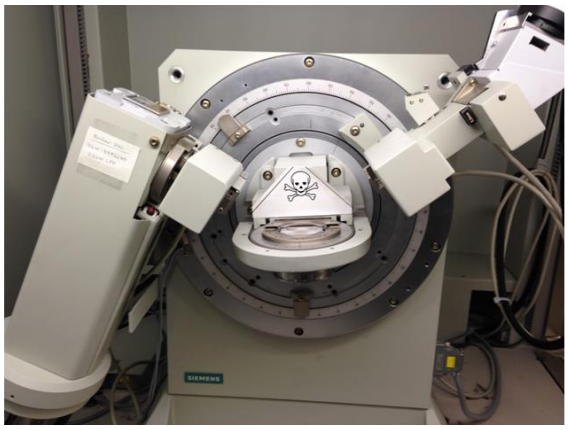

Figure 2-4. Sealed-tube type X-Ray generator used in this study (Department of Geology, University of Manitoba).

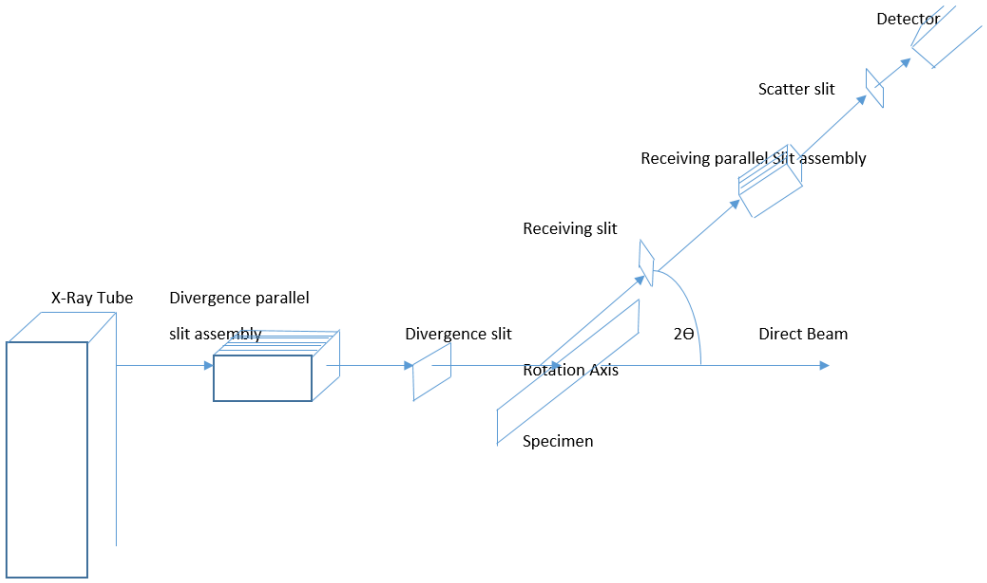

Figure 2-5. Schematic drawing of the principal parts of an X-ray diffractometer (modified from the Siemens manual).

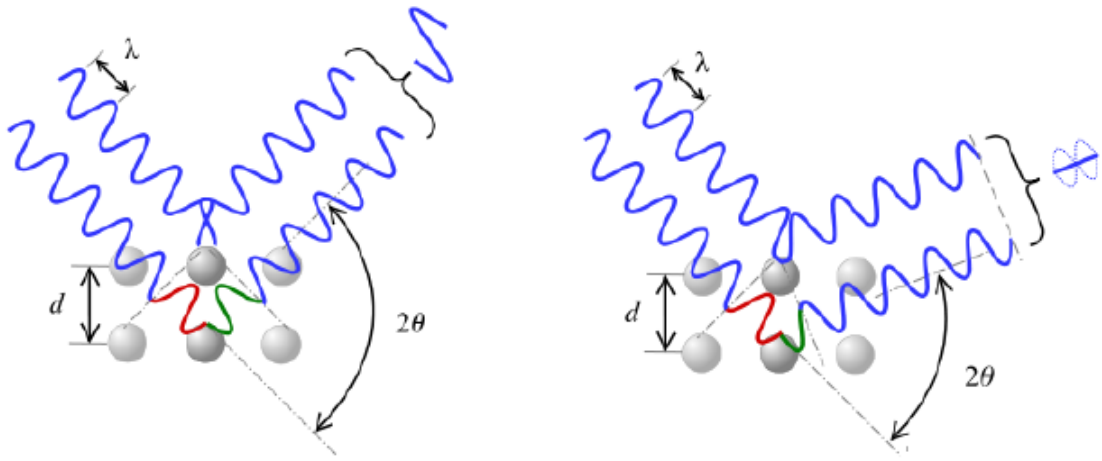

Figure 2-6. Schematic of reflected X-ray diffraction beams inside the structure of compound (modified form Cullity et al., 2001).

2.4 Fermentation

2.4.1 *Clostridium thermocellum*

Clostridium thermocellum is an anaerobic, thermophilic, spore-forming bacterium which produces Cellulosome complexes to hydrolyze cellulose to oligodextrins (cellobiose, cellotetrose, cellopentose, which are then metabolized to produce ethanol, organic acids (acetate, formate, ;acate), hydrogen and carbon dioxide (Maki et al., 2010). Cellulosome complexes are complicated multi-enzyme machines, consisting of hydrolytic enzymes attached to an anchoring protein called Scaffoldin. Degradative enzymes such as xylosidase, xylanases inside the Cellulosome hydrolyze the heterogeneous, insoluble hemicellulose inside the biomass (Maki et al., 2010).

Clostridium thermocellum DSM 1237 (synonymous collection numbers include ATCC 27405, JCM 12338, and NCIB 10682) obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) was used throughout these experiments. Figure 2-7 shows the enzymes produced by *C. thermocellum,* cellulases and hemicellulases for hydrolysis of lignocelluloses to sugars such as cellobiose and xylobiose. In addition, hexose sugars derived from celluloses can be used by *C. thermocellum* to produce ethanol.

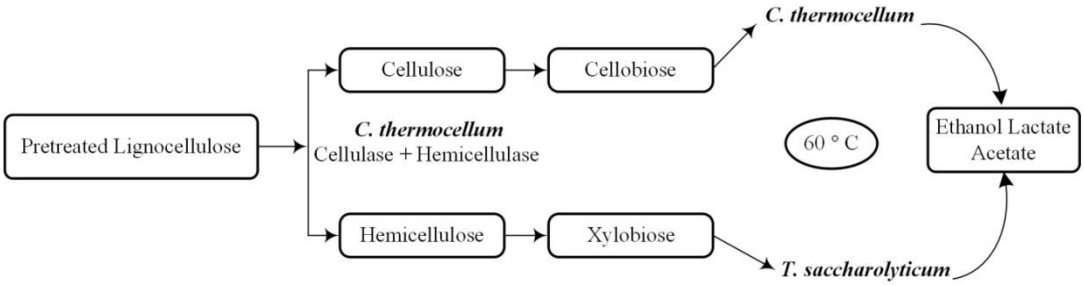

Figure 2-7. Using *C. thermocellum* (co-cultured with *Thermoanerobacterium saccharolyticum*) for ethanol production (modified from Maki et al., 2010).

2.4.2 Media

For the concentration of cellulose in the liquid phase of the media of approximately 2 g/L, wheat straw substrate was added to 45 mL of culture media (Islam et al., 2006). The media used for the experiments was 1191 cellulosic media, which contain the following ingredients:

- KH_2PO_4 Potassium phosphate
- Na_2HPO_4 Sodium phosphate
- NH_4Cl Ammonium chloride
- $MgCl_2.6H_2O$ Magnesium chloride
- Yeast extract
- Diluted Resazarin
- Cysteine hydrochloride

After mixing the media with ingredients in a 2 L beaker with a magnetic stirrer, the pH was, maintained at 7.2 and measured by a pH meter. As the pH of this specific media is around 6.8, NaOH pellets or liquid were added to increase the pH. The pH meter, was calibrated with pH 7 and 4 buffer solutions. Substrate and media were mixed in 100 mL serum bottles (Wheaton Science, Fisher Scientific). The bottles were air-sealed with butyl-rubber stoppers and crimped with aluminum seals. In order to maintain anaerobic conditions, oxygen was evacuated and nitrogen gas was added to each serum bottle. The bottles were gassed for 1 min and degassed for 4 min in four cycles with 100% nitrogen. Once anaerobic conditions were established, the bottles were autoclaved at a temperature of 122 °C and pressure of 213.7 kPa.

Media containing untreated wheat straw as a reference sample and pretreated wheat straw biomass were inoculated with *C. thermocellum*. Before inoculation of serum bottles containing test substrates, *C. thermocellum* was added to 500 mL serum bottles containing 1191 media and 2

g/L α-cellulose (Sigma Chemicals) then were incubated in a 60 °C water bath (Model 2332, Fisher Scientific MA) for 36 hours (Guo et al., 2010). Five (5) mL of log-phase (the period of cells' growth which numbers increase exponentially) *C. thermocellum* cells were removed from these cultures using a 5 mL syringe with a 20 gauge needle and transferred to the test bottles containing wheat straw samples. The test serum bottles were then inoculated at 60 °C for 53 hours.

Growth of *C. thermocellum* and fermentability of the substrates were inferred by measuring the concentrations of gaseous H_2 and CO_2 fermentation products, as a direct correlation between gas production and cell mass increase was previously identified by Islam et al., (2006; 2009). Hydrogen and CO_2 concentrations at the end of the fermentation reactions (53 hours) were measured by Gas Chromatography (GC) using an Agilent 7890 GC equipped with a thermal conductivity detector and flame ionization detector using a split inlet and two porous layer open tubular columns in series. Ethanol concentrations in the culture medium at the end of the fermentation reactions (53 hours), and the ethanol and sugar content in the samples and boiling water used for pretreatment were measured by High Performance Liquid Chromatography (HPLC) using a Breeze 2 HPLC system (Waters, Mississauga, ON) equipped with a HPX-87H column (BioRad, Mississauga, Ontario) with Micro-guard Cation H+ a guard column, and a 2414 refractive index detector. The mobile phase consisted of 5 mM of sulfuric acid at the flow rate of 0.6 mL/min with column temperature of 45 °C.

Chapter 3 Results and Discussions

3.1 Effect of Superheated Steam Processing on Moisture Content of Wheat Straw

Superheated steam can be successfully employed to remove moisture from pretreated biomass. Superheated steam decreases volume of the biomass. Representative graph of moisture content in percent wet basis versus time for straw processed at 220 °C and steam velocity of 1.93 m/s is reported in Figure 3-1. The drying rate follows an exponential curve, which is characteristic of many biological materials. After 15 min of processing with SS quilibrium was reached at 0.1 % w.b.

After placing the sample in the SS chamber an initial period of warming takes place during which the sample and tray transition from a temperature below the dew point to the operating temperature above the dew point. During this period, condensation on the surface of the tray and sample appeared resulting in an increase in the mass of sample from 79 g to 79.75 g, then it quickly evaporated (Figures 3-2).

Figure 3-3 shows the effect of pretreating straw by boiling it in water at 119 °C for 15 min before processing it with SS at 220 °C. The boiling step raises the moisture content of straw to approximately 36.9% w.b. Equilibrium moisture content was achieved under 0.2% w.b. The Figure 3-4 shows the initial stage of processing with steam in magnification. The initial warming period including the initial condensation on the surface of the tray that increased the mass of sample from 83.9 to 84.8 g and sample was followed by rapid drying. The amount of condensation in relation to the sample size depends on surface area and the thermal properties of the tray and sample as well as the prevailing convection currents of the steam inside the chamber.

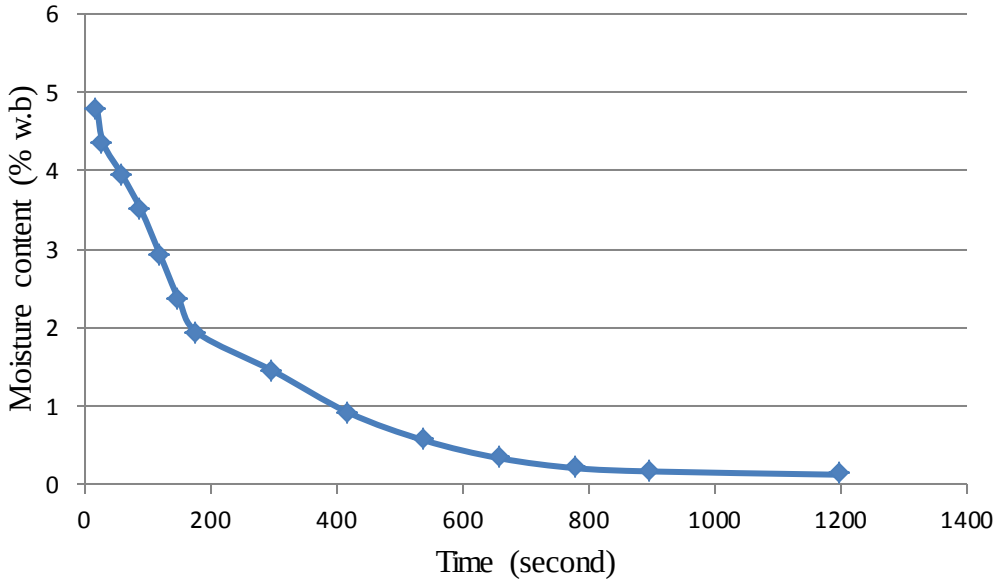

Figure 3-1. Moisture changes with time during processing of wheat straw with SS at 220 °C.

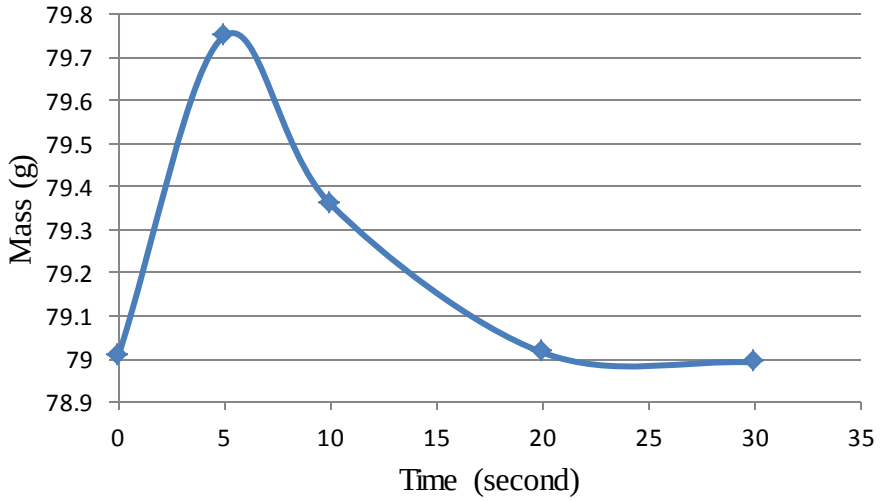

Figure 3-2. The mass changes of sample due to the initial condensation processed with SS at 220°C.

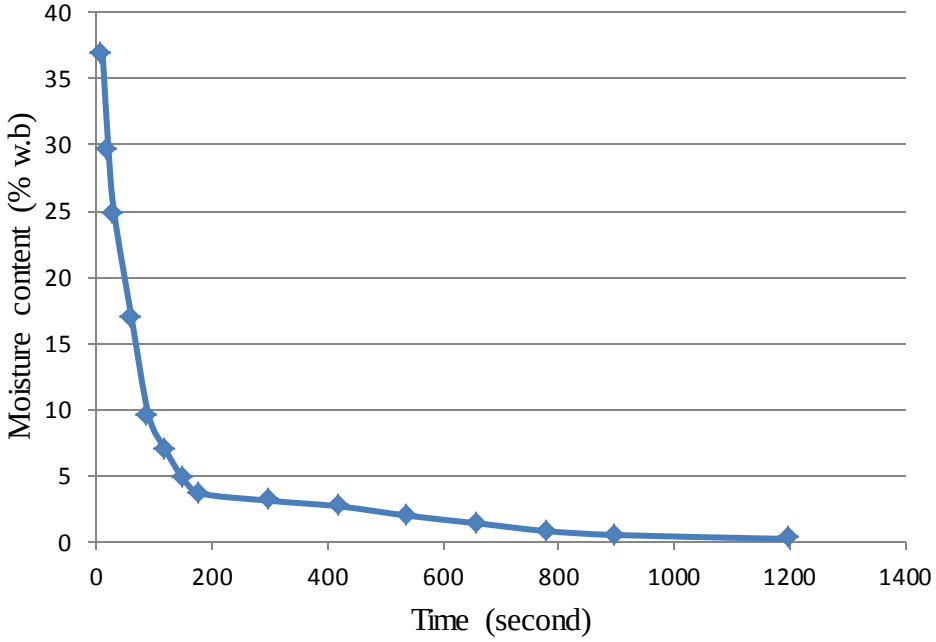

Figure 3-3. The effect of boiling straw at 119 °C on drying samples in SS at 220 °C.

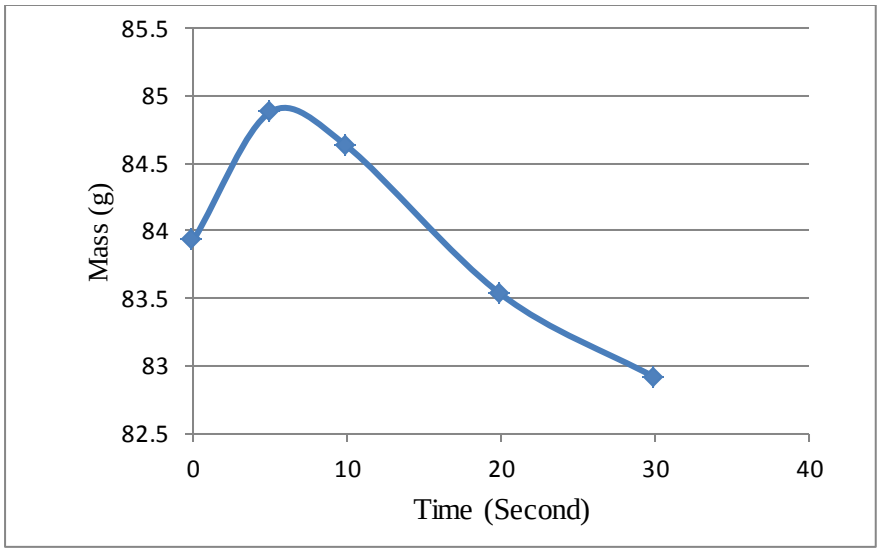

Figure 3-4. The mass changes of sample due to the initial condensation processed with the BW combination with SS at 220 °C

3.2 Compositional Analysis

Compositional analysis of wheat straw samples before and after pretreatment was conducted at the Feeds Innovation Institute, University of Saskatchewan. Samples were analyzed for lignin, hemicellulose, cellulose, NDF (Neutral Detergent Fiber) and ADF (Acid Detergent Fiber). The results were reported on a dry mass basis (see Table 3-1). The percentage of hemicellulose was determined by subtracting acid detergent fiber (ADF) from neutral detergent fiber (NDF), and the percentage of cellulose was determined by subtracting lignin from ADF. Each analysis was conducted in duplicate and was repeated when discrepancies of greater than three percent were found. Table 3-2 shows compositional analysis of straw samples that were exposed to BW at119 °C, SS at 180 and 220 °C and BW at 119 °C followed with SS at 180 and 220 °C. No significant differences in cellulose, hemicellulose and lignin contents were found between any of the treatment methods. Table 3-3 illustrates the compositional analysis for wheat straws obtained from the Plant Science (pilot plots) at the University of Manitoba. Based on the results, the AC Intrepid cultivar had the highest cellulose content followed by AC Domain, while 5602 HR had the lowest.

Table 3-1. Compositional analysis for loose versus compacted wheat straw samples obtained from industry (Biovalco Inc.). Analysis done by the Feeds Innovation Institute, University of Saskatchewan.

Sample ID	Method Used	Non-compacted Biovalco Inc.	Compacted Biovalco Inc.
Moisture (%)	AOAC 930.15	4.82	5.62
Dry Matter (%)	AOAC 930.15	95.18	94.38
Lignin (%)	AOAC 973.18	10.63	11.46
ADF (%)	AOAC 973.18	54.37	52.57
NDF (%)	AOAC 2002.04	78.4	75.47
Cellulose (%)	Cellulose = ADF - Lignin	43.74	41.12
Hemicellulose (%)	Hemicellulose = NDF - ADF	24.03	22.89

Table 3-2. Compositional analysis for raw and pretreated wheat straw obtained from industry (Biovalco Inc.). Analysis done by the Feeds Innovation Institute, University of Saskatchewan.

Component*	Raw	SS 180˚C	SS 220˚C	BW 119°C	BW+SS 180˚C	BW+SS 220˚C
Cellulose	54.45%	53.04%	55.83%	56.07%	55.66%	53.99%
Hemicellulose	33.10%	34.44%	30.70%	32.66%	33.05%	34.53%
Lignin	12.45%	12.53%	13.47%	11.24%	11.29%	11.48%

*values expressed as percentage of neutral detergent fiber (NDF) present in the sample

SS: Superheated steam, BW: Boiling water

Table 3-3. Compositional analysis of wheat straw cultivars grown by the Department of Plant Science, University of Manitoba

Cultivar	**Lignin (%)**		**Cellulose (%)**		**Hemicellulose (%)**	
Variety	Mean	SE*	Mean	SE	Mean	SE
5602 HR	5.3	0.04	31.4	0.76	20.0	0.68
AC Domain	6.7	0.79	40.1	1.46	25.4	0.58
AC Intrepid	6.5	0.09	41.2	0.53	26.7	0.37
Harvest	6.4	0.01	39.0	1.07	23.9	2.05
Kane	6.0	0.21	40.0	0.31	26.1	0.99
McKenzie	6.8	0.06	40.0	0.59	26.2	0.30

*SE = standard error

3.3 Severity Factor

Severity factor, log $R_{0,}$ is a term used in steam pretreatment as a quantitative measure for the severity of the pretreatment. Evaluation of biomass pretreatment depends on the severity factor, which is a function of the combined effects of temperature, acidity and duration of pretreatment. For any given acidity, the following equation (1) is used (Galbe et al., 2007):

$$\log R_0 = \log(t \times \exp\left(\frac{T-100}{14.75}\right))$$

Where t = time (min), and T = temperature ($°C$).

The results of calculations of the severity factor for various temperatures of SS are shown in Table 3-4. When a combined pretreatment of boiling water and SS was used, the severity factor was determined as the sum of the log R_0 of the individual pretreatments. Increasing the temperature of the superheated steam from 180 to 220°C, resulted in a linear rise in the severity factor from 3.53 to 4.70. Straw samples exposed to boiling water at 119 °C with pressure of 193 kPa with subsequent treatment using superheated steam at 180, 200 and 220 °C, resulted in linear rise in the severity factor from 5.26 to 6.44. Table 3-5 shows how severity factors vary based on retention times (5, 10, 15 min) in the SS chamber with a processing temperature of 220 °C with BW pretreatment and BW at 119 ºC treatment without subsequent SS treatment. Severity factor depends on the retention time of thermal pretreatment. The longer the retention time, the higher the severity factor was.

Table 3-4. Severity factors log R_0 for two types of pretreatments : 1) 15 min in SS and 2) 15 min in boiling water at 119˚C and 15 min in SS.

15 min boiling water + 15 min SS			**15 min SS**		**BW 15 min**
T (°C) of boiling water	*T* (°C) of SS	logR_0	*T* (°C) of SS	logR_0	log R_0
119	180	5.26	180	3.53	1.73
	200	5.85	200	4.12	
	220	6.44	220	4.70	

Table 3-5. Severity factors of different processing times for 220 ºC SS processing temperature.

Method of pretreatment	**Severity factor**
BW 119 ºC + 15 min	1.73
BW+ 5 min SS at 220ºC	5.96
BW+ 10 min SS at 220ºC	6.26
BW+ 15 min SS at 220ºC	6.44

SS: Superheated steam, BW: Boiling water

3.4 X-ray Diffraction

A typical X-ray diffraction graph for superheated steam pretreated straw is shown in Figure 3-5. The CAC was determined according to the method originally proposed by Segal et al. (Segal et al., 1962). This method utilizes the relative peak (I_{002}) and the amorphous hump (I_{AM}) values from the X-ray graph (Figure 3-5). The relative height is measured with respect to a minimum intensity of diffracted beams value in the tested range and can be considered as an approximation of the CAC only in the cellulose fraction of the biomass (Park et al., 2010):

$$CAC = \frac{I_{002} - I_{AM}}{I_{002}} \times 100 \quad (2)$$

Where, I_{002} is the total intensity of diffracted beams measured at $2\theta \cong 22°$ and I_{AM} is the height of the minimum intensity at $2\theta = 19°$ which represents the amorphous hump.

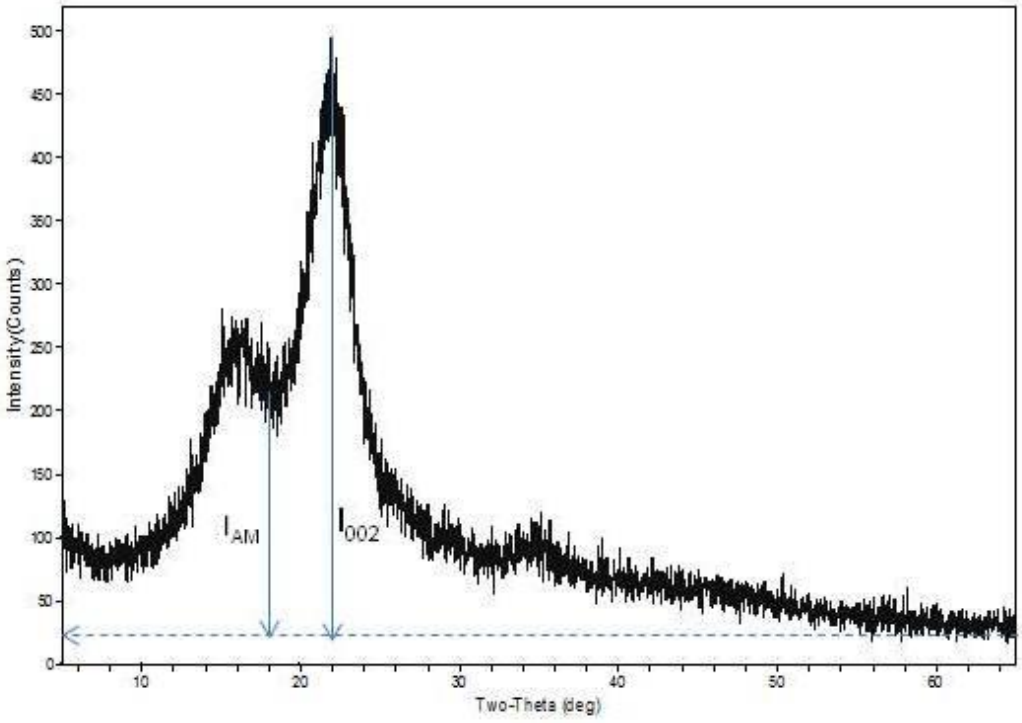

Figure 3-5. X-ray diffraction pattern for loose wheat straw processed with boiling water at 119°C followed by SS processing at 220 °C.

Figure 3-6 shows the pattern of X-ray diffractions for the pretreated samples at three different SS temperatures of 180, 200, and 220 ºC. Each graph has three patterns that illustrate three replications to obtain the standard error of the % CAC. The calculated values of the % CAC were used to compare effectiveness of different pretreatments. The amorphous region of biomass is more readily digested by bacteria in comparison to the crystalline region. Based on the results (Table 3-6), all pretreatments increased the amorphous region of the substrate with respect to the reference sample. The maximum increase was observed at a severity factor of 6.44, from 51.3 % for unprocessed straw to 62.6 %. Other factors that affect the fermentability of biomass by *C. thermocellum*, include the location of amorphous and crystalline regions in the cellulose, accessibility to these regions by bacteria and the density of cellulose (Peters et al., 1991).

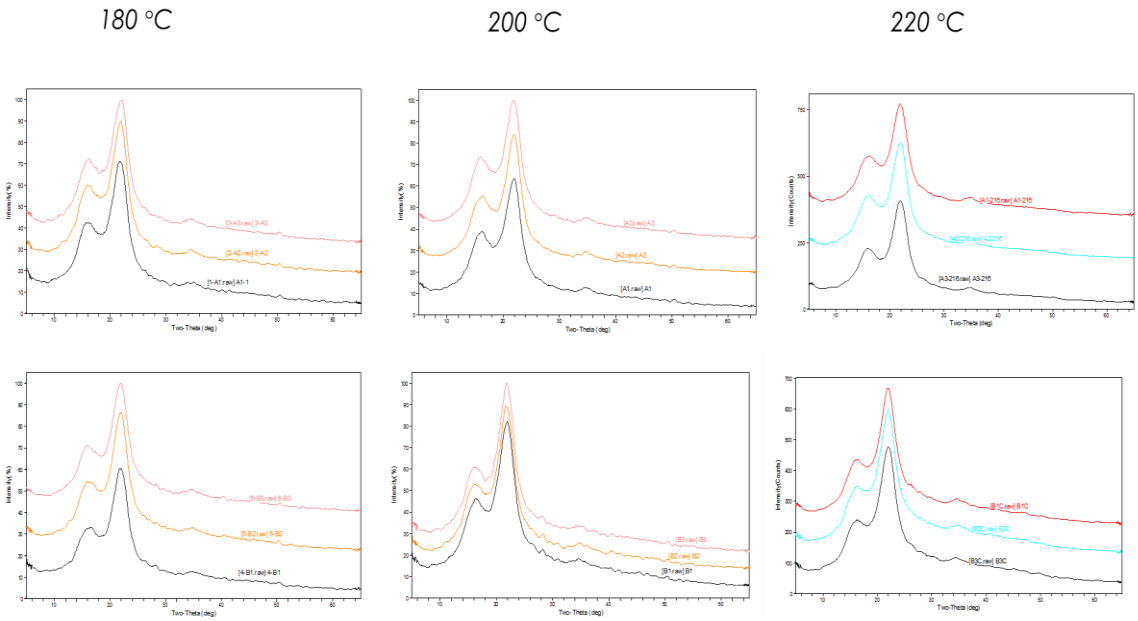

Figure 3-6. X-ray graphs for pretreated loose Biovalco Inc. wheat straw samples.

Table 3-6. Contribution of amorphous cellulose (% CAC) of loose wheat straw samples obtained from Biovalco Inc. pretreated with SS or boiling water and SS.

Method of pretreatment	Mean of % CAC	Standard Error (n=3)
15 min BW (119 °C) +15 min SS at 220 °C	62.6	0.58
15 min SS at 220 °C	56.1	0.15
15 min BW (119 °C) +15 min SS at 200 °C	60.4	0.36
15 min SS at 200 °C	54.8	0.1
15 min BW (119 °C) +15 min SS at 180 °C	58.3	0.18
15 min BW at 119 °C	57.36	0.349
15 min SS at 180 °C	52.9	0.43
Reference (Raw material)	51.3	0.46

SS, Superheated steam; BW, Boiling water.

Generally, boiling wheat straw followed by processing with superheated steam gave higher CAC values than processing with SS alone. Increasing the SS temperature from 180 to 220 °C resulted in an increase in % CAC values from 51.3% (reference sample) to 52.9, 54.8, and 56.1% for superheated steam pretreatments at 180, 200 and 220 °C, respectively. However, when boiling water was added as a pretreatment process, the CAC percentage increased from 51.3% (reference sample) to 57.36, 58.3, 60.4 and 62.6% for BW and BW followed by SS treatments at 180, 200 and 220 °C, respectively. Table 3-7 illustrates the % CAC for the compacted version of Biovalco Inc. wheat straw for the pretreated samples solely with SS at 180 °C and BW followed with SS (180 °C) for 15 min in comparison to the non-pretreated samples.

Table 3-7. Contribution of amorphous cellulose (% CAC) of compacted wheat straw obtained from Biovalco Inc.

Feedstock	Method of pretreatment	Mean of % CAC	Standard error (3 replications)
Compacted Biovalco wheat straw	15min soaking in BW (119°C)+15min SS at 180°C	57.61	0.223
	15min SS at 180°C	53.82	0.149
	Reference (Untreated material)	48.32	0.781

The % CAC was calculated for 6 different kinds of wheat straws from the Department of Plant Science at the University of Manitoba, for both treated samples with BW (119 °C) + SS at 220 °C and untreated wheat straw. The results reported in Table 3.8 illustrate that the % CAC of 560HR increased by at least 16.5 % after the pretreatment compared with the reference sample. McKenzie wheat straw had the highest % CAC between the treated samples followed by AC Intrepid and Kane. Consequently, the pretreatment method had the highest impact on the cellulose structure of McKenzie, AC Intrepid and Kane wheat straws.

The Biovalco wheat straw samples were exposed to 15 min boiling water at 119 ºC and then SS (220 ºC) was applied for 5, 10 and 15 min processing times inside the chamber including 5, 10 and 15 min. The % CAC was determined for all the samples. The results as indicated in Table 3-8 show that the retention time inside the SS chamber had a direct effect on the % CAC. Increasing the processing time of SS, raises the % CAC. There was seven percent increase in % CAC when retention time in SS increased from 5 to 15 min.

Table 3-8. Contribution of amorphous cellulose (% CAC) of wheat straw cultivars obtained from the Department of Plant Science, University of Manitoba, pretreated with combination of BW with SS at 220 ºC.

Substrate	Mean of %CAC	Standard error
AC Intrepid	59.26	0.04
Ref	50.23	0.32
Kane	58.92	0.41
Ref	49.99	0.19
McKenzie	60.51	0.47
Ref	49.49	0.58
AC Domain	58.73	0.18
Ref	49.29	0.39
Harvest	56.81	0.62
Ref	48.88	0.82
560HR	57.04	0.37
Ref	48.79	0.71

Ref, Reference (Raw).

Table 3-9. Contribution of amorphous cellulose (% CAC) of loose wheat straw obtained from the Biovalco Inc. pretreated at different processing retention times inside the SS (220 °C) chamber.

Pretreatment	Retention Time (min)	Mean of %CAC	Standard Error
Reference	-	51.3	0.46
BW 119 ºC	15	57.36	0.34
BW (119 ºC) + SS (220 ºC)	15+5	58.58	0.46
	15+10	60.85	0.63
	15+15	62.60	0.58

SS, Superheated steam; BW, Boiling water.

3.5 Fermentation End-Products: Hydrogen (H_2) and Carbon dioxide (CO_2)

3.5.1 Compacted Biovalco Inc. wheat straw substrate

Figure 3-7 illustrates the gas production (H_2 and CO_2) for compacted Biovalco Inc. wheat straw. The results indicated that 15 min soaking in boiling water at 119 °C with subsequent processing with superheated steam for 15 min, resulted in the highest H_2 and CO_2 production, 8.28 and 8.79 µmol/mL respectively, whereas the raw material (reference sample) had the lowest gas production, 4.83 and 4.2 µmol/mL for H_2 and CO_2 respectively.

The gas production using the compacted wheat straw substrates shows that there is no dramatic difference between 5 min and 15 min processing with superheated steam. In terms of energy consumption for superheated steam pretreatment calculated based on the superheated steam properties and retention time, 5 min pretreatment processing is more cost effective than 15 min.

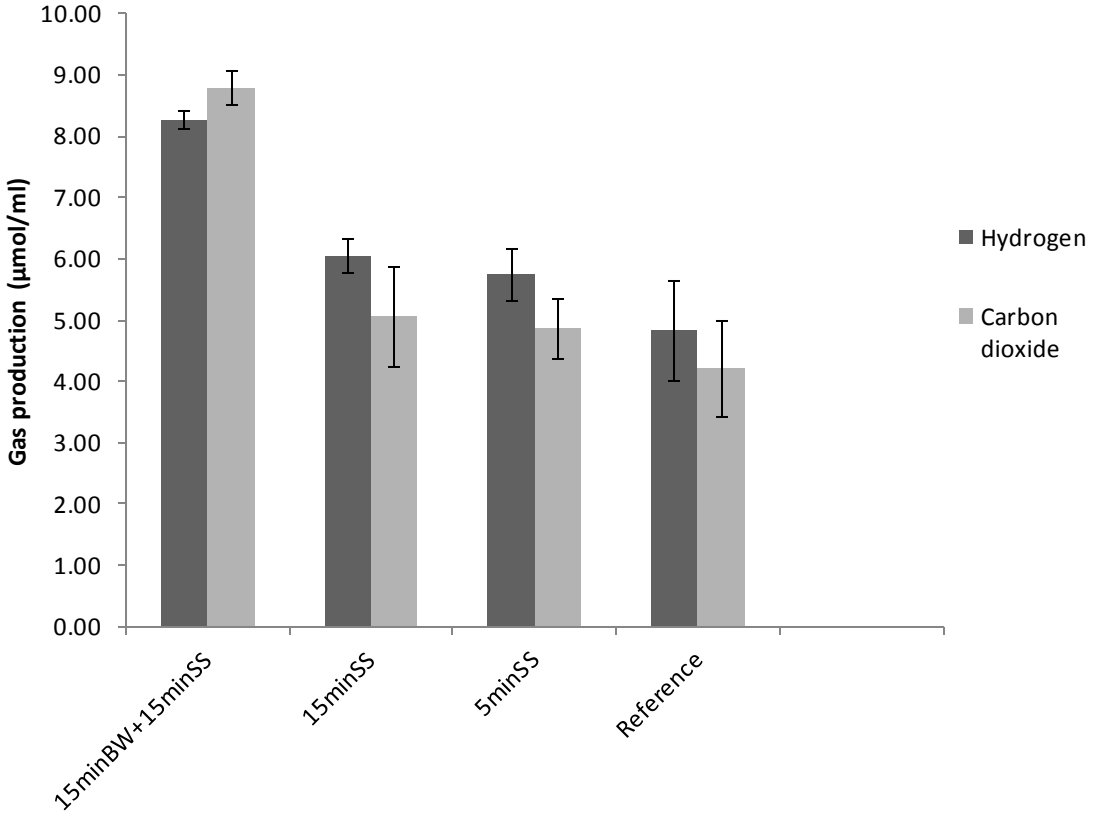

Figure 3-7. Hydrogen and carbon dioxide production for compacted wheat straw samples obtained from Biovalco Inc. pretreated with SS (180 ºC) and BW (119 ºC). SS, Superheated steam; BW, Boiling water.

3.5.2 Processing at different temperatures of SS

Figure 3-8 shows the concentrations of H_2 and CO_2 synthesized by *C. thermocellum* in cultures containing Biovalco wheat straw exposed to the two different pretreatment conditions: boiling water (119 ºC) plus superheated steam at different temperatures (180, 200 and 220 °C) versus solely employing superheat steam (180, 200 and 220 °C). Gas concentrations resulting from fermentation are shown in order of increasing SS temperatures (from right to left). The reference sample had the lowest H_2 and CO_2 concentrations (4.90 and 2.90 μmol/mL, respectively). Boiling the wheat straw in water at 119 °C for 15 minutes followed by pretreatment with superheated steam for another 15 minutes, resulted in higher concentrations of H_2 and CO_2 compared with the sole use of superheated steam treatment. Pre-processing in boiling water at 180°C + superheated steam treatment resulted in H_2 and CO_2 concentrations of 8.20 and 6.70 μmol/mL, respectively. When compared to the reference samples, this corresponds to a 67% and 132% increases in the production of H_2 and CO_2, respectively. When only superheated steam was used at180°C, H_2 and CO_2 concentrations were 5.11 and 3.57 μmol/ml, respectively, corresponding to increases of only 4 and 23%, respectively.

The same trends were observed for the pretreatments at 200 and 220°C. As the temperature of the superheated steam increased from 180 to 220 °C, the production of the two gases also increased, and at 220˚C the boiled and superheated steam pretreated samples showed a 95%, or a two fold increase in H_2 concentration and a 224%, or a threefold increase in CO_2 concentration with respect to the untreated sample. These results correlate with the %CAC outcome and confirm that increasing the amorphous region can increase the H_2 and CO_2 production by *C. thermocellum*.

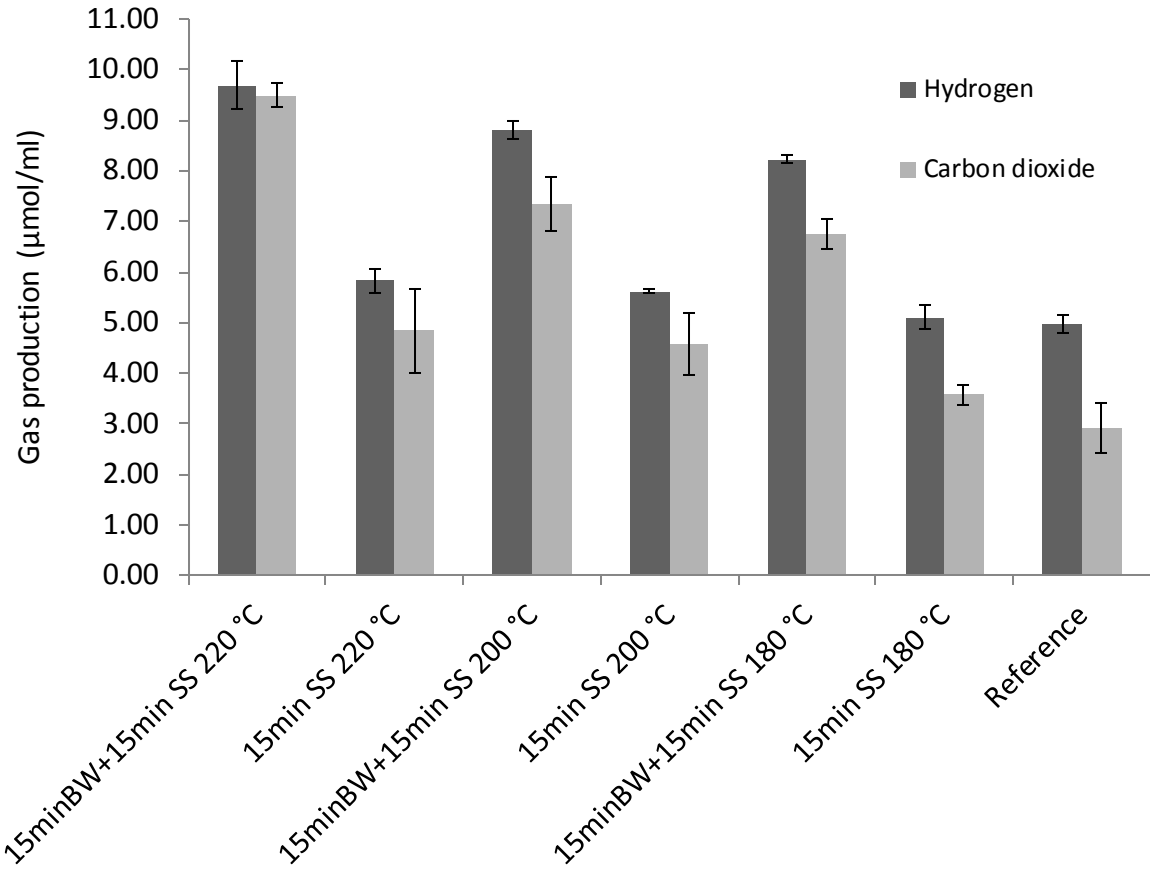

Figure 3-8. Hydrogen and carbon dioxide production by *C. thermocellum* cultured with untreated (reference) and pretreated loose wheat straw obtained from Biovalco Inc. SS, Superheated steam; BW, Boiling water.

3.5.3 Various wheat straw cultivars obtained from the Department of Plant Science, University of Manitoba

The Figure 3-9 shows the H_2 and CO_2 production for six different cultivars of wheat straws produced by the Department of Plant Science at the University of Manitoba. The pretreatments for all samples were done with BW at 119 °C followed by SS at 220 °C. The results indicate that the McKenzie and AC Intrepid had the highest gas production. There was close to a 55 and 53% increase in hydrogen and carbon dioxide production of treated McKenzie wheat straw compared with the raw material.

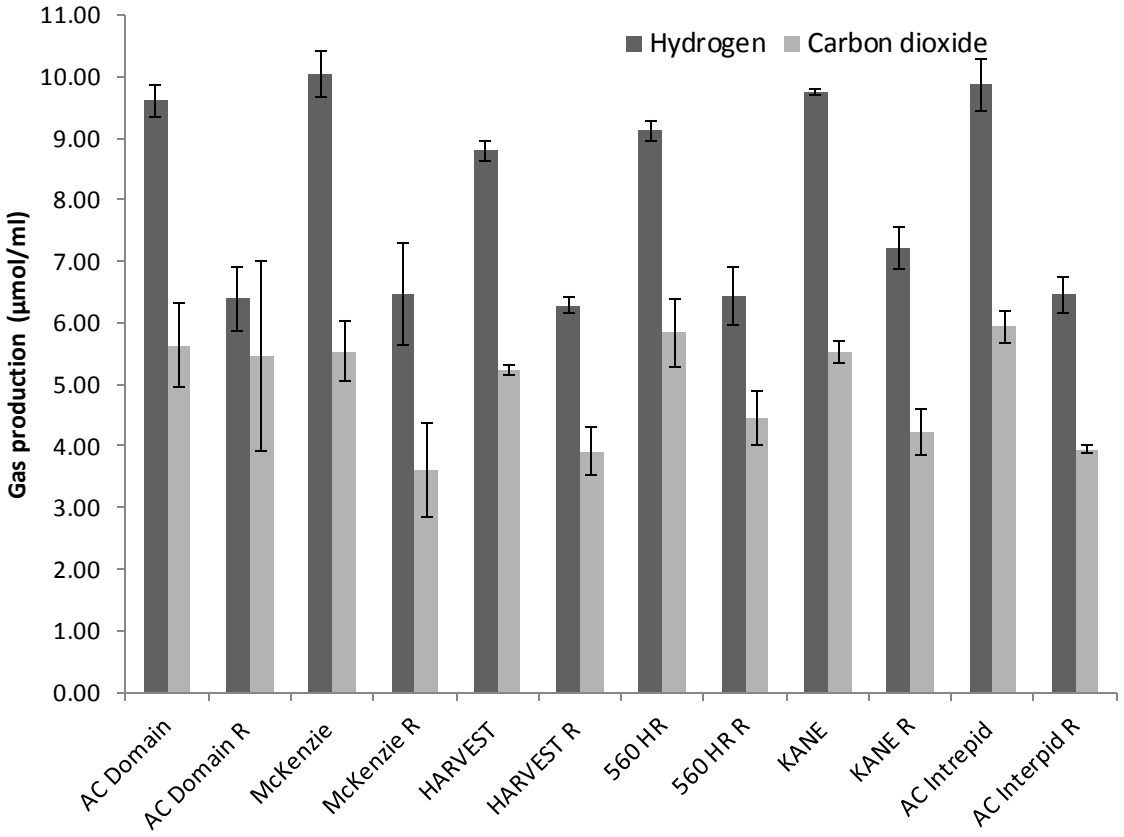

Figure 3-9. Hydrogen and carbon dioxide gas production from pretreated (boiling water at 119 °C plus superheated steam at 220 °C)and raw wheat straws derived from different cultivars provided by the Department of Plant Science (University of Manitoba) .R, Reference (Raw).

3.5.4 SS combination with BW pretreatment at different SS processing times

Figure 3-10 shows the gas production of treated Biovalco Inc. wheat straw using only BW at 119 °C (inside the pressure cooker for 15 min) vs BW+SS at three different retention times of SS treatment at 220 °C. The results indicate that using BW+SS for 15 min produced 24 and 22 % more hydrogen and carbon dioxide, respectively compared with just BW pretreatment. There was no significant difference in gas production between samples treated for 5 min with SS versus samples treated for 15 min with SS at 220 °C.

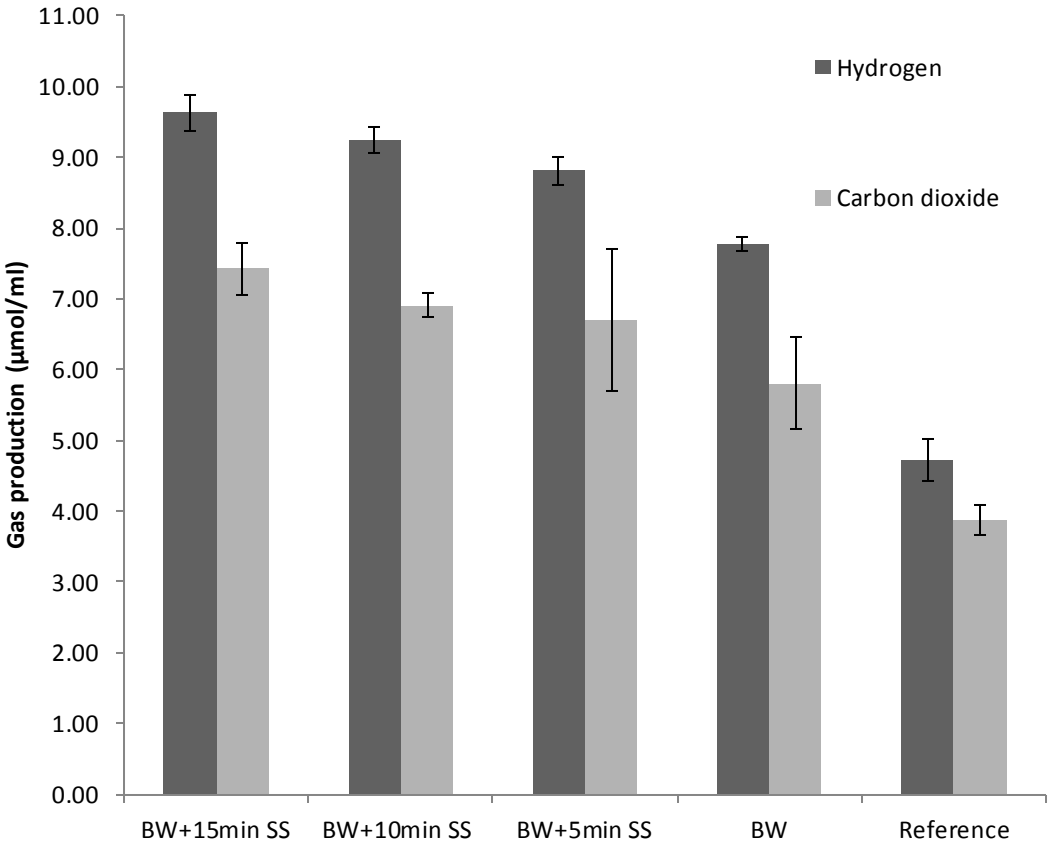

Figure 3-10. Gas production of processing at different retention times of SS. SS, Superheated steam; BW, Boiling water.

3.6 Fermentation End-Products: Ethanol

3.6.1 Compacted comparison with loose wheat straw obtained from industry

Figure 3-11 indicates the results of ethanol concentration after the fermentation process of compacted wheat straw obtained from Biovalco Inc. The HPLC results showed that 15 min soaking in boiling water plus 15 min processing with superheated steam resulted in the highest ethanol yield by *C. thermocellum*, followed by 15 min drying with superheated steam. The lowest ethanol production was observed in fermentation reactions with the reference sample for both loose and compacted wheat straw.

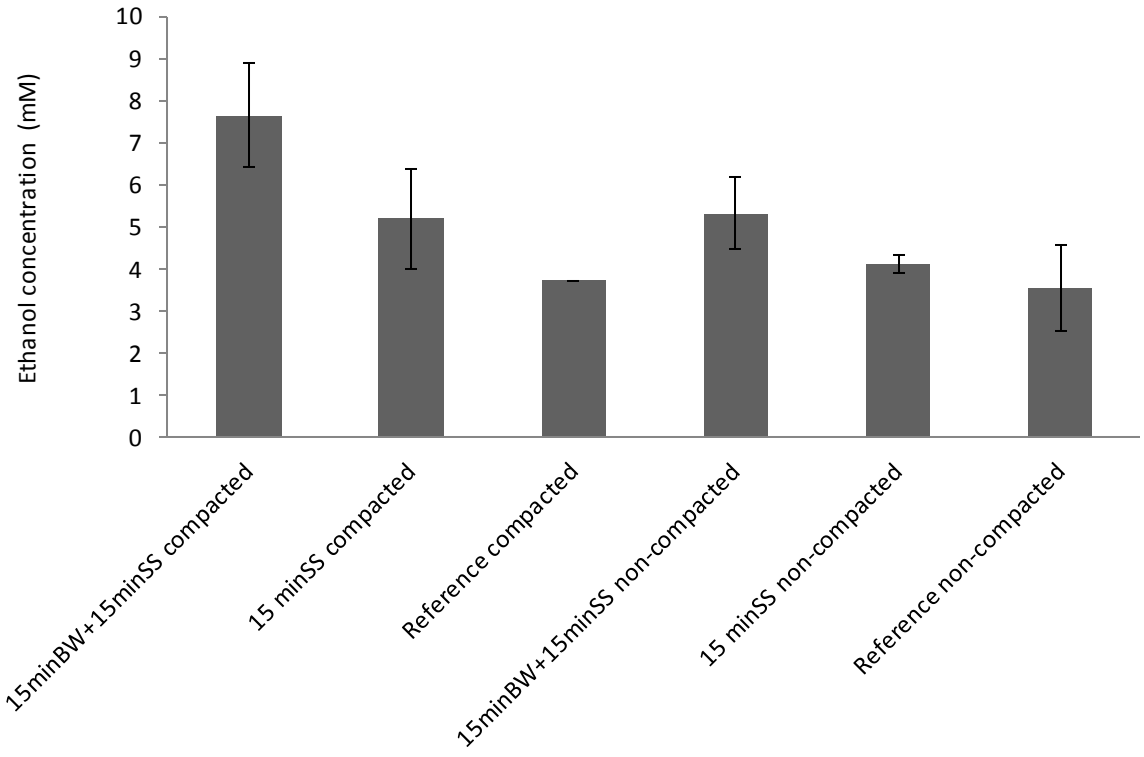

Figure 3-11. Ethanol concentrations from fermentation reactions containing compacted and non-compacted Biovalco Inc. wheat straw. SS, Superheated steam; BW, Boiling water.

3.6.2 Processing at Different Temperatures of SS Pretreatment

The trend in ethanol production by *C. thermocellum* cultured with untreated versus pretreated wheat straw followed a pattern that was very similar to that observed for gas production (see Figure 3-12). *C. thermocellum* fermentation reactions containing untreated wheat straw (reference) samples resulted in the lowest ethanol concentrations (1 mM). In contrast, the highest ethanol concentrations were obtained when wheat straw samples were boiled in water at 119°C followed by superheated steam treatment at 220 °C (4.8 mM). This corresponds to an approximate 5-fold increase in the ethanol production compared to the untreated samples. When the temperature of the superheated steam was lowered to 200 °C, the ethanol production decreased to 4.4 mM, which corresponds to a 3.5-fold increase in ethanol concentration. Processing with superheated steam alone gave 3-fold higher ethanol production in comparison to untreated samples.

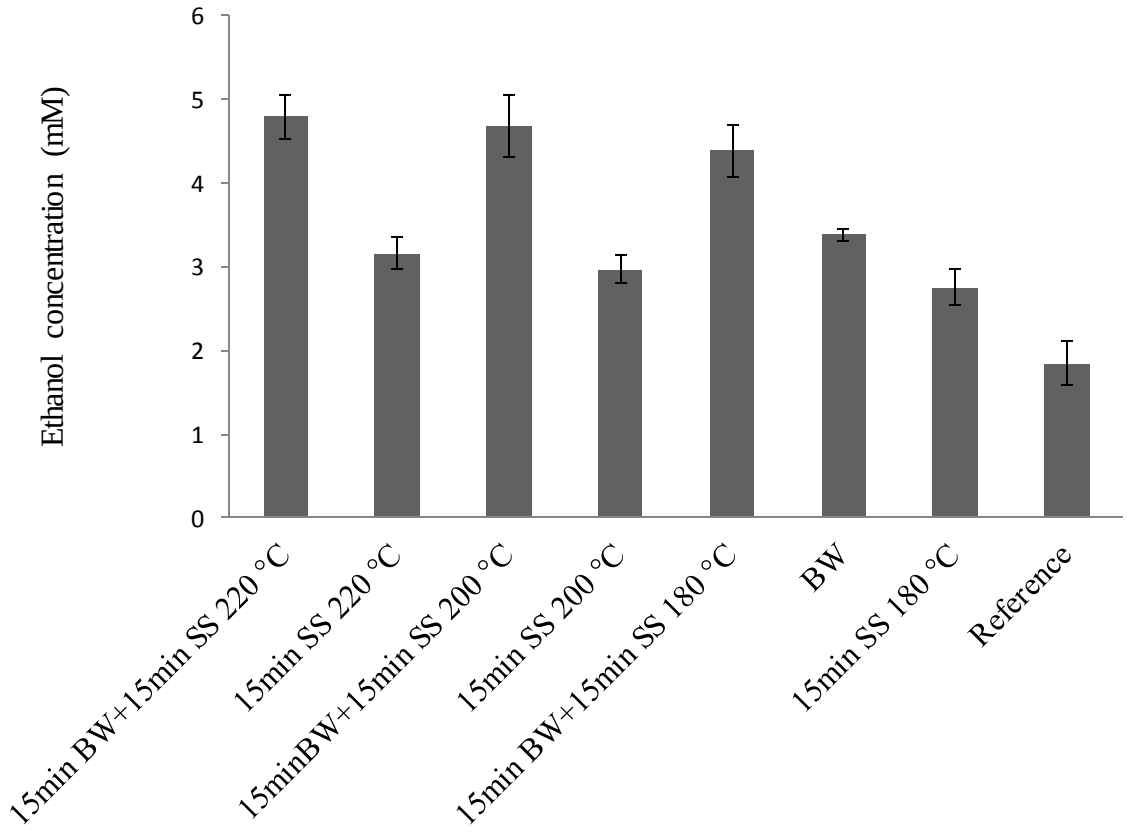

Figure 3-12. Ethanol concentrations resulting from fermentation of untreated (reference) and pretreated Biovalco Inc. wheat straw by *C. thermocellum*. SS, Superheated steam; BW, Boiling water.

3.6.3 Cultivars obtained from the Department of Plant Science (University of Manitoba)

Figure 3-13 shows the ethanol concentration of six different treated and untreated wheat straws cultivars grown at the University of Manitoba. Based on the results, the ethanol concentration showed a 1-fold increase after the pretreatment by BW + SS at 220 °C compared with the reference sample of AC Domain wheat straw. This was followed by a 70 and 45% increase of ethanol production after the pretreatment for AC Intrepid and McKenzie wheat straws, respectively.

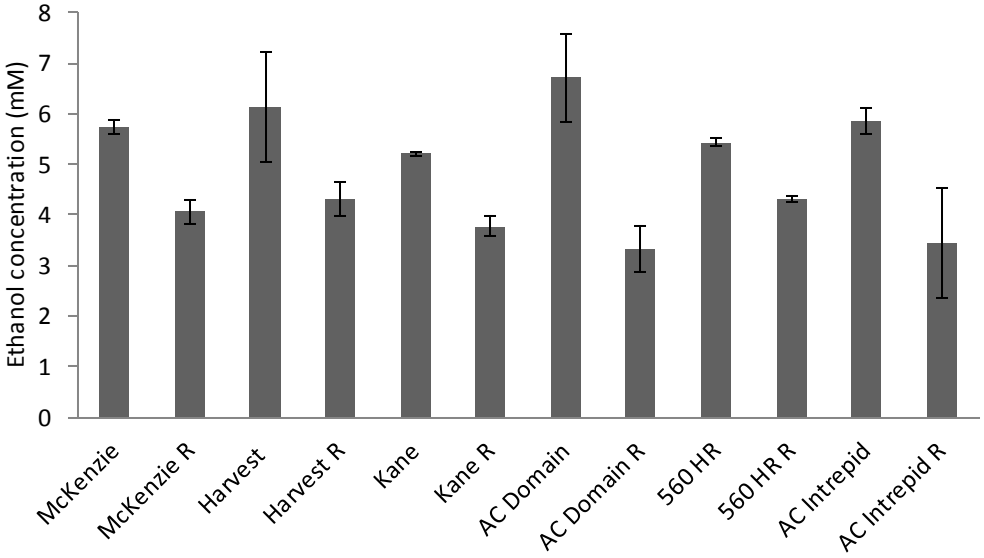

Figure 3-13. Ethanol concentrations resulting from fermentation of pretreated (boiling water at 119 °C plus superheated steam at 220 °C) wheat straw cultivars obtained from the Department of Plant Science, University of Manitoba. R, Reference (Raw material).

3.6.4 Pretreatment at Different Retention Times in SS Chamber

Figure 3-14 shows the ethanol concentrations for pretreatment methods using only BW versus BW + SS (220 ºC) at three different retention times (t = 5, 10, and 15 min) in the superheated steam chamber. There was a 75.5, 83.2, 99, and 104 % (two-fold) increase in ethanol concentrations obtained from the pretreated samples with BW, and BW+SS (220 ℃) at 5, 10, and 15 min (retention times inside the SS chamber), respectively in comparison to the reference wheat straw.

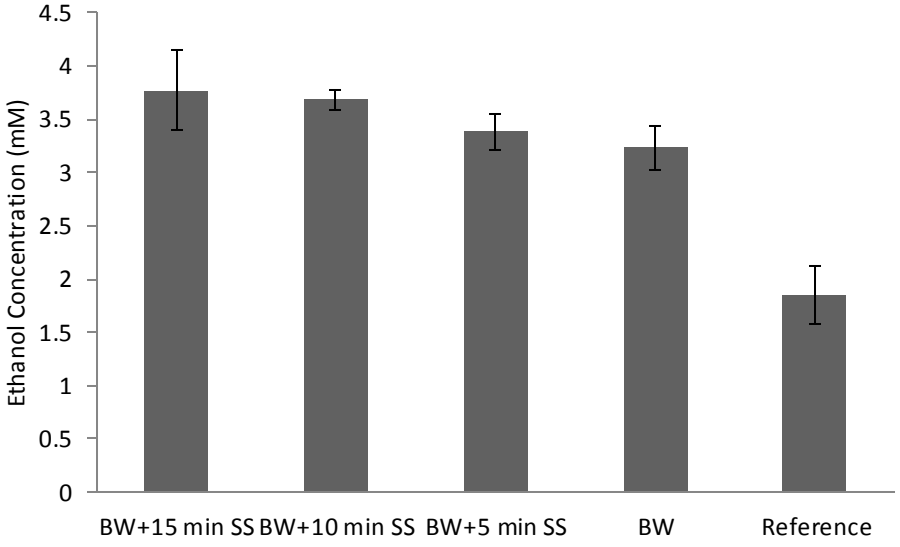

Figure 3-14. Ethanol concentration for different processing times inside the SS chamber. SS, Superheated steam; BW, Boiling water; Reference (Raw material).

3.6.5 Ethanol Yields

The water used during the BW phase of the pretreatment was analyzed by HPLC to detect glucose, xylose, and cellobiose. Trace amounts of cellobiose, but no glucose were detected in the samples; however, significant amounts of xylose were observed. This indicated that no glucose monomer units were solubilized and lost as a result of treatment in boiling water, and that all of the cellulose mass is available for conversion to ethanol. Ethanol yield calculations were based on the stoichiometric relationship between glucose monomer units present in the cellulose and ethyl alcohol produced in fermentation. The observed ethanol concentrations were compared to a theoretical maximum ethanol production of 0.004856 mol ethanol / g biomass. The ethanol yields are shown in Figure 3-15 expressed in a percentage with respect to production of ethanol from glucose. As indicated in Figure 3-16, AC Domain wheat straw pretreated by BW+SS at 220 °C has the highest ethanol yield of production followed by Harvest, AC Intrepid and McKenzie. Also the most significant difference between the reference sample and pretreated sample was observed in AC Domain, AC Intrepid and McKenzie wheat straws along with the same trend in gas production.

Figure 3-17 shows the ethanol yield for the Biovalco Inc. wheat straw pretreated with BW+SS at retention times (5, 10 and 15 min) inside the chamber compared with pretreated wheat straw samples with only BW. The results confirmed that the maximum processing time with SS resulted in the highest yield, followed by decreasing retention times. The lowest ethanol yield among the pretreated samples was for using only BW at 119 °C. The trends of ethanol yield were similar to the gas production trends.

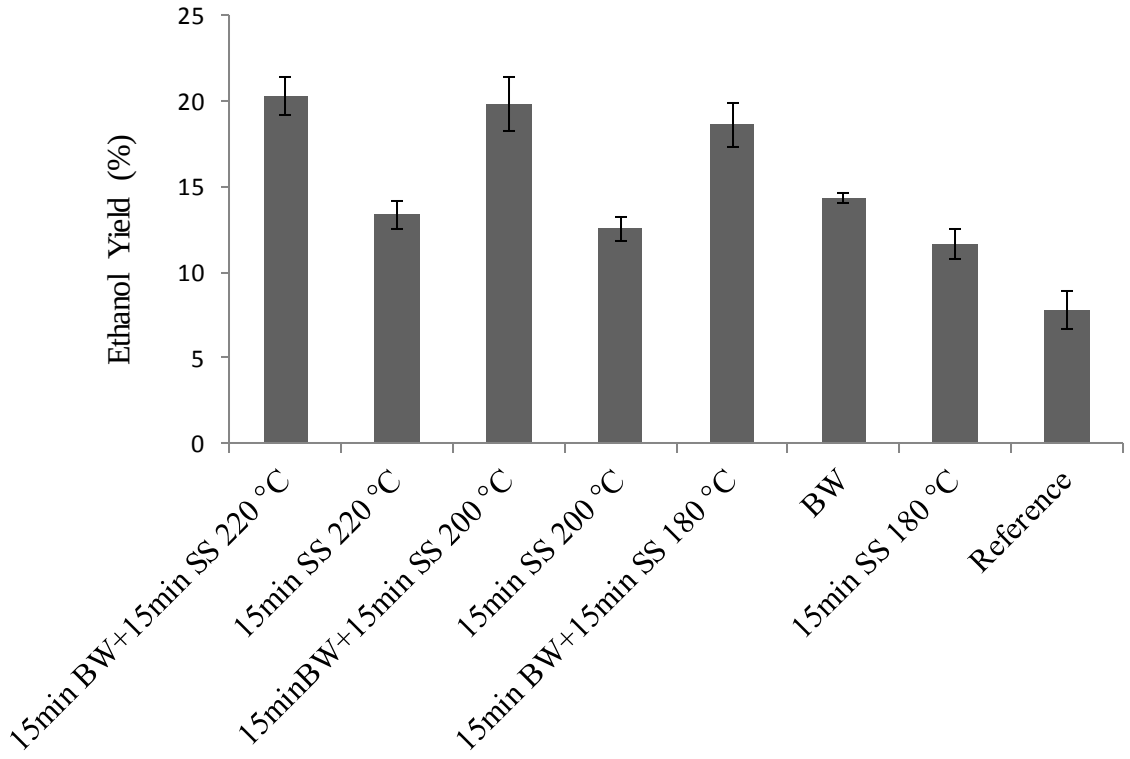

Figure 3-15. Ethanol yield with respect to glucose from fermentation of loose (non-compacted) Biovalco Inc. wheat straw as affected by various pretreatments. SS, Superheated steam; BW, Boiling water.

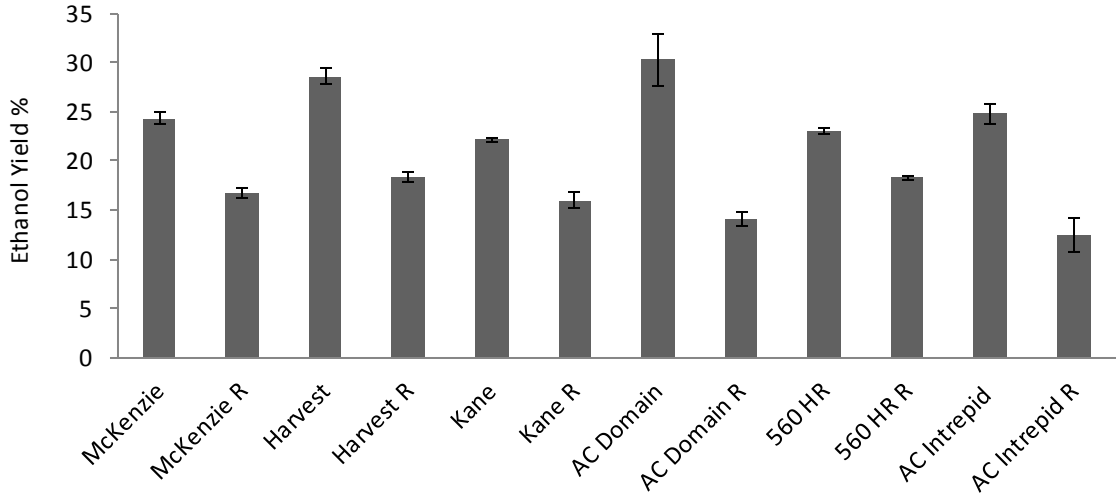

Figure 3-16. Ethanol yields for the Department of Plant Science (pretreated with boiling water at 119 °C plus superheated steam at 220 °C) samples. R, Reference (Raw material).

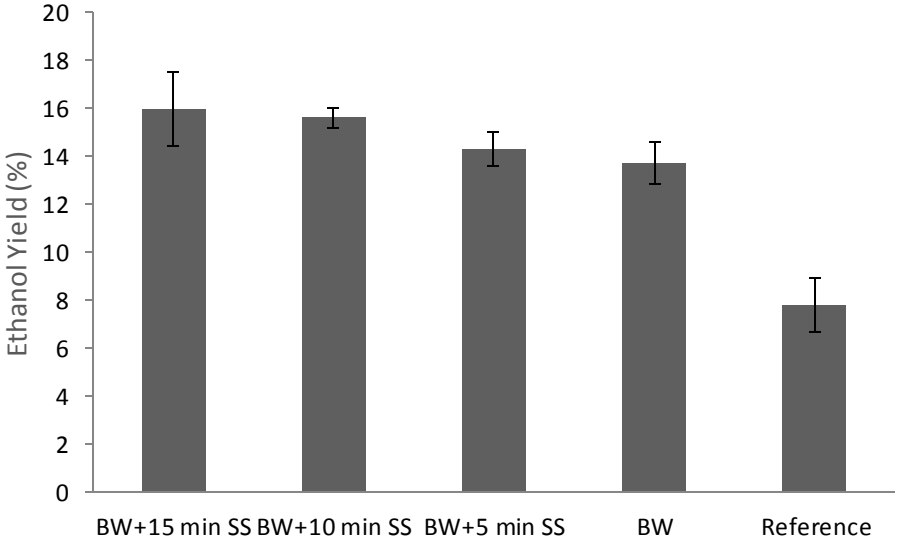

Figure 3-17. Ethanol yield of different times of process inside the SS chamber. SS, Superheated steam; BW, Boiling water; R, Reference (Raw material).

3.7 Effect of Severity Factor and contribution of amorphous cellulose (%CAC) of Pretreatment Methods

Figures 3-18 and 3-19 show the impact of severity factor on H_2 production and yields of ethanol resulting from the fermentation process. The pretreatment with boiling water followed by superheated steam was more severe than pretreatment with superheated steam alone. By increasing the severity factor from 0 (reference sample or untreated straw that had no pretreatment process) to 6.45 (boiling water at 119 °C followed by superheat steam at 220 °C), the production of H_2 increased 95% (Figure 3-18), and ethanol yield increased 175% (Figure 3-19).

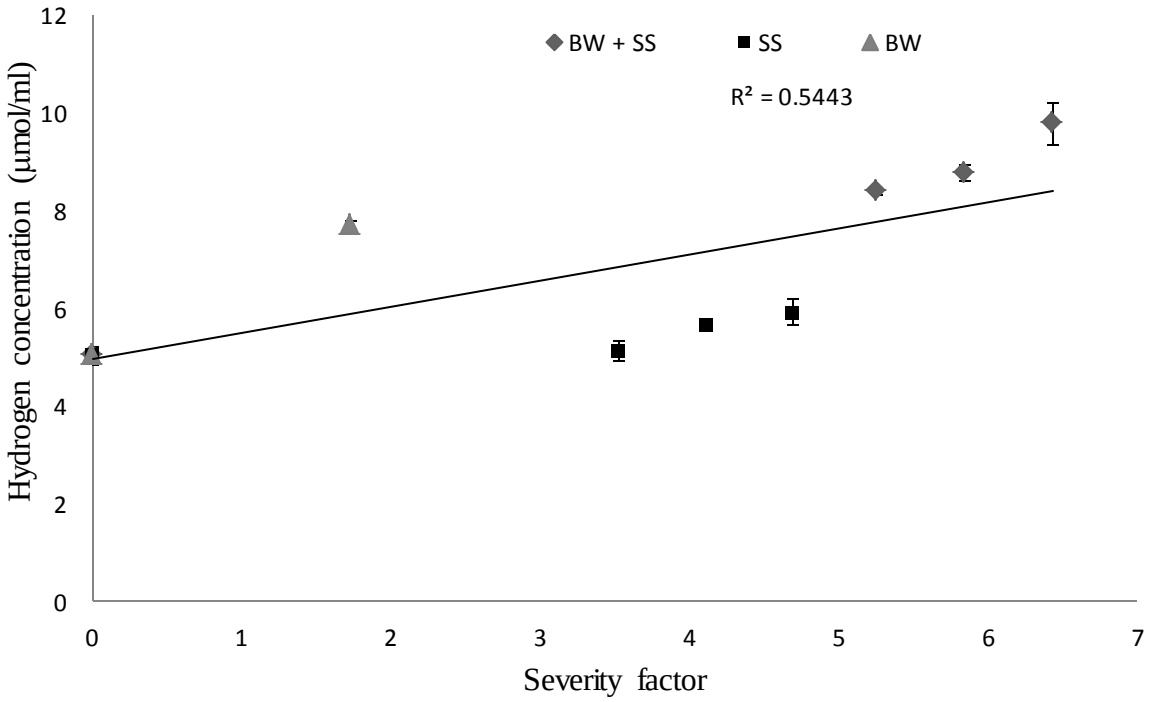

Figure 3-18. Preteatment severity factor versus hydrogen concentration.

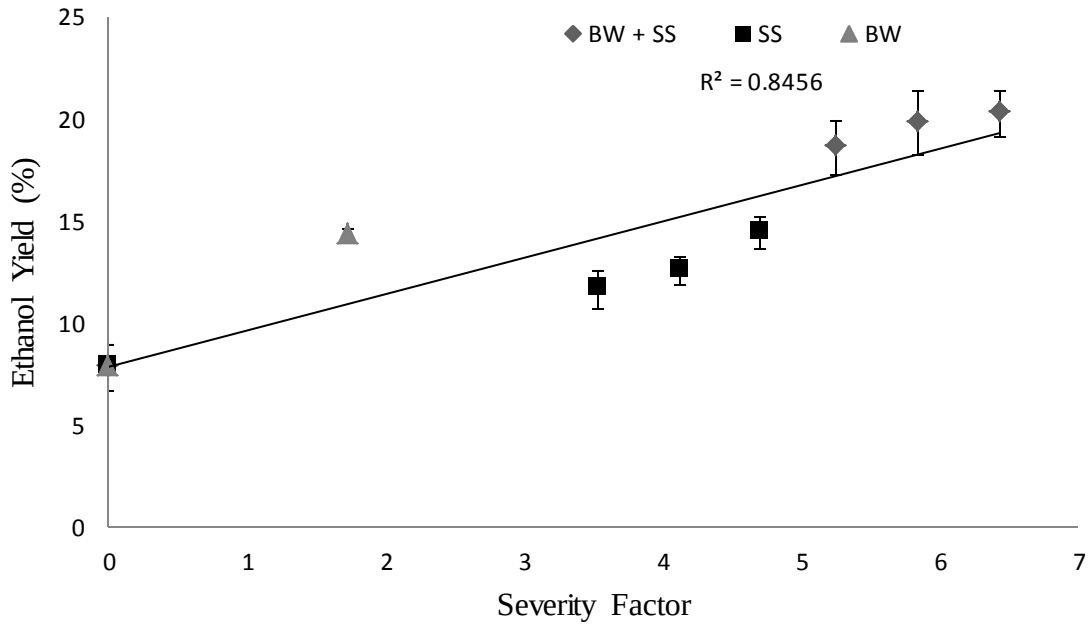

Figure 3-19. Preteatment severity factor versus ethanol yield.

Figures 3-20 and 3-21 indicate the effect of %CAC on H_2 production and yields of ethanol resulting from the fermentation process. The results confirm that there is a direct correlation between increasing percentage of amorphous area of the cellulose (CAC) and end-products concentrations. As %CAC increases, hydrogen and ethanol rate of production becomes greater. The samples treated with combination of BW and SS obtained higher %CAC values, causing higher concentration of fermented products following by samples treated with BW or SS alone.

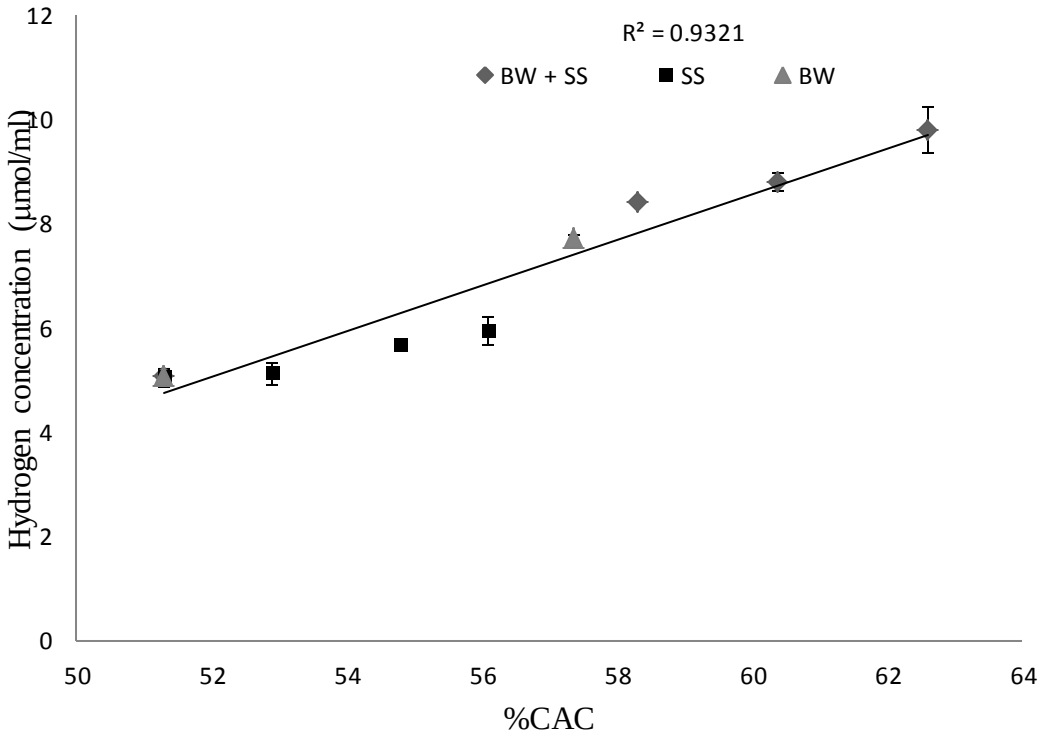

Figure 3-20. Preteatment % CAC versus Hydrogen production.

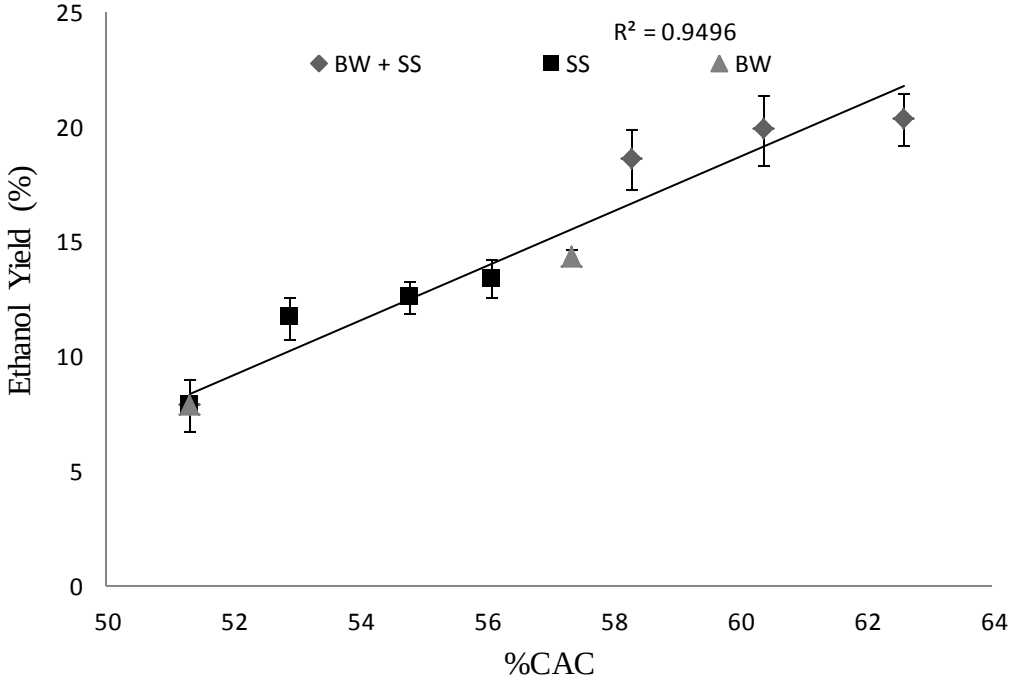

Figure 3-21. Preteatment % CAC versus Ethanol yield.

High severity factors resulting from the combined BW and SS treatment were directly correlated with the most significant structural changes in the wheat straw substrates, as estimated by changes in the % CAC, and with increased concentrations of both H_2 and ethanol. However, the BW treatment alone had a more pronounced effect than the SS treatment alone, contributing to a greater relative increase in % CAC, H_2 production, and ethanol yield at a comparatively lower severity. Figs 3-18 and 3-19 show the experimental results of the three different pretreatments with corresponding trend lines and R^2 values. The 15 minute pretreatment with BW and SS at 200 and 220 °C (corresponding to the 5.85 and 6.44 severity factor) resulted in maximum ethanol yield of approximately 12% higher than the ethanol yield obtained from the unprocessed sample (Figure 3-19).

The effects on % CAC, H_2 production, and ethanol yield from the combined BW and SS treatments at various severities can be approximated from the summation of the individual effects of the BW and SS treatments alone, with respect to the reference samples. For example, ethanol yield increased by 6.52% from the reference sample when exposed to the BW treatment, and 3.84% when exposed to SS at 180 °C alone. When these two values are added together, they give a predicted value of 10.36%, which is within the standard deviation of the measured increase in ethanol yield of 10.79% for the combined treatment (Figure 3-19). By increasing the severity factor from 0 (untreated straw) to 6.44 (boiling water followed by superheated steam), the production of H_2 increased 94%, ethanol yield increased 160% and % CAC increased 22%.

In the case of using BW (119ºC) + SS (at 220 ºC) with processing times (5, 10 and15 min), the maximum retention time of processing which also has the highest severity factor, obtained the maximum amorphous area of the cellulose (% CAC) which is more fermentable by the

bacteria. More severe pretreatment resulted in more H_2, CO_2 and ethanol production. The trend decreased by the lowering of the processing time inside the SS chamber from 15 to 10 and 5 min.

3.8 Energy Significance

Cellulose-adherent cellulolytic microorganisms could successfully increase the stability of industrial processes based on microbial cellulose utilization. Many efforts have been made to compare the costs of the production of ethanol from cellulosic biomass feedstocks to the private cost (the costs that the individual consumer pays the vendor. Also private cost can be explained as the interior costs of company's production purpose) and total social cost (private costs plus external costs) of producing ethanol using conventional methods from corn, and to analyze the sensitivity of the technology to the main parameters such as feedstock and energy prices, capital costs, and estimates of social costs (Woodson et al., 2015). Generated results show that existing ethanol technologies hold an economic advantage due to the larger capital costs and the higher complexity of the cellulosic ethanol conversion process. As addressed in more detail elsewhere (Greer, 2005), cellulose production costs have recently been reported in the range of 10 to 20 cents per gallon (¢/gal) of ethanol produced.

Lynd et al (Lynd et al., 2005) compared the projected costs for biological processing associated with ethanol production for a CBP process and for an advanced process featuring at-site dedicated cellulose production in combination with simultaneous saccharification with co-fermentation of hexose and pentose sugars (SSCF). Based on what they reported, the ethanol for dedicated cellulase production and for SSCF cost 9.90 ¢/gal and 9 ¢/gal respectively, which gives a total cost for biological processing of about 18.9 ¢/gal, which is not comparable with the 4.2 ¢/gal projected for CBP.

Woodson et al. (Woodson et al., 2015) applied the modeling approach to compare the economic and environmental performance of the two types of ethanol production technology; from biological feedstock that contains simple sugars (monosaccharides) to the traditional one. Based on the modeling approach of this research group, the traditional method of ethanol production from corn is the more economic from private and total social cost measures aspect view. They also reported that the private cost to produce one gallon of ethanol is $1.17 in the traditional ethanol plant and $1.56 in the cellulosic plant; total social costs are $1.29 and $1.59, respectively (Woodson et al., 2015). It has been also reported that the social costs of emissions from a traditional ethanol plant are $0.12 per gallon of ethanol produced, while for the cellulosic ethanol plant the value is only $0.03 per gallon which is a good proof of more environmentally benign of the production of ethanol from corn stover (Woodson et al., 2015).

In another study (Lynd et al., 2005) the cost for ethanol production can be highly affected by the price of the raw material. Generally, feedstock illustrates 60–75% of the total cost of bio-ethanol production. Bio-ethanol from sugar cane costs different from a place to place for instance in Brazil costs US$0.23–0.29 per liter, while in the United States and Europe the bio-ethanol production from sugar and corn would cost US$0.53 per liter and US$0.29 per liter, respectively (Balat et al. 2009). The comparison of the costs of bio-ethanol production from different feedstock is reported in Table 3-10 (Balat et al. 2009).

Table 3-10. Estimates of the costs of bio-ethanol production from different feedstock (exclusive of taxes), (US cents per liter) (Balat et al. 2009).

	2006	**Long-term about 2030**
Bio-ethanol from sugar cane	25–50	25–35
Bio-ethanol from corn	60–80	35–55
Bio-ethanol from beet	60–80	40–60
Bio-ethanol from wheat	70–95	45–65
Bio-ethanol from lignocellulose	80–110	25–65

3.8.1 Energy consumption and electricity cost

Superheated steam used in this study was produced using the following process;

- The tab water used and was boiled inside a reboiler.
- The saturated steam prepared from the steam generator using the pressure reducer and a superheated chamber to prepare the SS via dropping the pressure and enhancing the steam temperature to 220 ℃.

The energy consumption for the superheated steam production process has been calculated using the following method:

Specific Enthalpy of Saturated Water:

$h_f = C_W \times (t_f - t_0)$

h_f = enthalpy of water (kJ/kg)

C_W = specific heat of water = 4.19 (kJ/kg.°C)

t_f = saturation temperature (°C)

$h_f = 4.2 \times (100 - 20) = 336 kJ/kg$

Latent heat of water evaporation= $2257 kJ/kg$

Specific Enthalpy of Superheated Steam:

$h_s = h_g + C_{ps}(t_s - t_f)$

h_s = enthalpy of superheated steam (kJ/kg)

For saturated steam at standard atmosphere, the specific enthalpy, h_g is 2676 kJ/kg.

C_{ps} = specific heat of steam at constant pressure, 1.860 (kJ/kg.°C)

t_f = saturation temperature (°C)

t_s = superheated steam temperature (°C)

$h_s = 2676 + 1.86(220 - 100) = 2899.2 kJ / kg$

Total heat required for superheated steam generation from the tab water:

$2899.2 + 2257 + 336 = 5492.2 kJ / kg$

Based on the volume of tank inside the used reboiler, we could assume that 10 liters (10kg) of water was utilized in the superheated steam production for pretreatment of the biomass.

Energy consumed to produce superheated steam= $54922 kJ$

If we assume that the steam is being superheated up to 220 °C from saturated vapor excluding the energy consumption for boiling and evaporation of water inside the reboiler, considering the system works in a closed loop, the amount of energy will decrease substantially to approximately 29000kJ. Also energy from condensation could be used for heating water inside the system or heating other units. During the process of biomass with superheated steam, the optimum parameters were not established for the steam flow with respect to the quantity of the biomass. Also when steam was passing through the biomass loaded in the tray, sample balling happened due to several seconds of condensation on its surface and caused decreasing the penetration of steam right into the interior of the ball.

The second way of energy consumption calculation is based on the power of equipment used in the superheated steam machine. (Table 3-11).

Table 3-11. Energy consumption based on power of equipment

Equipment	Power (kw)	Time of working (second)	Energy (kJ)
Boiler	18	1800	32400
Heater	4	3600	14400
pump	1.4	600	840
actuator	0.085	120	10.2
control panel	2.5	3600	9000
flow meter	0.001	3600	3.6
pump control	0.23	3600	828
		Total energy (kJ) consumption	57481.8

If we assume that the calculated amount of energy is consumed in one hour operation of superheated steam machine, the following calculations show the operating electricity cost.

Utilized energy= 57481.8 kJ/h= 16 kWh

Current electricity rate (Manitoba hydro) = 0.0767 $/kWh

Estimate electricity cost per hour operation of SS= 1.22 $

Considering just superheating the steam from saturated vapor:

Utilized energy= 28992 kJ/h= 8 kWh

Electricity cost per hour= 0.61 $

3.9 Conclusions

Boiling water (BW), superheated steam (SS), and combinations of these methods were used to pretreat biomass samples, including loose and compacted Biovalco Inc. wheat straw, and wheat straw cultivars provided by the Department of Plant Science at University of Manitoba, to determine their effect on the contribution of amorphous cellulose (% CAC) of the cellulose substrates used and the concentrations of fermentation end-products (H_2 and ethanol) synthesized by *C. thermocellum*, Compositional analyses of the treated samples showed that there was no significant removal of lignin or hemicellulose from the substrates by any of the pretreatments. However, the % CAC of pretreated biomass, calculated from data generated by X-ray diffraction, confirmed that the pretreatment methods changed the structure of the cellulose of the biomass. As an example, the % CAC of pretreated biomass by BW followed by treatment with SS at the highest operational temperature (220 °C) was 22% higher than the corresponding untreated biomass sample.

Pretreatment of wheat straw with boiling water and/or superheated steam was found to alter the structure of cellulose by increasing the percent contribution of amorphous cellulose (%

CAC), which in turn enhanced its fermentability by the thermophilic cellulolytic bacterium, *Clostridium thermocellum*, as measured by both increased gas (H_2 and CO_2) and ethanol concentrations. As the temperature of the SS increased from 180 to 220 °C, production of the two gases increased, and at 220 ˚C the boiled and SS pretreated samples showed a two-fold, 94.2% increase in H_2 concentration and a threefold, or 221.5%, increase in CO_2 concentration with respect to the untreated sample. Increased ethanol yield was found to correlate with the severity of the treatment and increase in % CAC, resulting in a maximum increase in yield of 20.3% at the highest severity factor of 6.44, corresponding to treatment in boiling water for 15 minutes, followed by superheated steam at 220˚C.

Among the Department of Plant Science cultivars, AC Domain, McKenzie and Harvest showed more gas and yield of ethanol production after the pretreatment compared with other samples. The results obtained from those cultivars indicated that the greater severity factor caused more % CAC and concentration of end-products after fermentation. Pretreatment combination of BW with SS at 220 °C, for fifteen minutes resulted in the highest hydrogen and ethanol production. Increasing the retention time inside the SS chamber from five to fifteen minutes enhanced the hydrogen and ethanol production by 12and 5 %, respectively.

Chapter 4 Conclusion and Future Prospects

In this dissertation, Consolidated Bioprocessing (CBP) was considered as a potential strategy to reduce the costs of upstream pretreatment of lignocellulosic biomass for microbial production of biofuels and/or bioproducts. CBP combines unit operations of cellulase production, substrate hydrolysis, and fermentation of sugars into a single stage. However, although CBP is a less expensive method of producing biofuels from lignocellulosic biomass, the yields of fermentation end-products, like ethanol and hydrogen, are much lower than traditional processes of cellulosic biofuel production.

This thesis described experiments in which wheat straw biomass was subjected to pretreatments with superheated steam (SS), either alone, or in combination with boiling water (BW) at 119 °C. The effects of these pretreatments on both biomass structure (related to crystalline and amorphous areas of cellulose) and microbial conversion of cellulose by *Clostridium thermocellum* to fermentation end-products were investigated. Different cultivars of wheat straw, each with different amounts of cellulose, hemicellulose, and lignin were chosen as potential feedstocks for microbial conversion by *C. thermocellum* using CBP the biomass for this study and the cellulose structure was analyzed using X-ray diffraction technique.

Several conclusions may be drawn from this work. The severity factor parameter was used to correlate the effect of superheated steam (at different temperatures and retention processing times) on biomass structure with yields of fermentation end-products. The research revealed that higher operational temperatures of the superheated steam temperatures (which had higher severity values) had greater disruptive effects on the wheat straw biomass structure and resulted in greater yields of hydrogen and ethanol

X-ray diffraction analyses revealed that wheat straw samples that were exposed to pretreatment conditions with higher severity factors had higher percentages of amorphous cellulose (% CAC) compared to non-treated biomass. *C. thermocellum* was able to convert wheat straw samples with higher amorphous cellulose content more efficiently. Thus, the concentrations of end-products, including hydrogen, carbon dioxide, and ethanol, increased as a result of the pretreatment processing with BW and SS, compared with untreated feedstock. The amount of gases and ethanol enhancement rate highly depends on the selected pretreatment method. The higher applied temperature and retention processing time caused more end-product yields during the microbial conversion.

Pretreatment exclusively with SS or in combination with BW, provide considerable advantages over conventional pretreatment methods, such as chemical treatment, enzymatic treatment, and/or steam explosion. Of these advantages, use of SS alone or with BW eliminates the use of chemicals (acids or bases), which can have harmful environmental impacts, and is more cost effective compared to other methods, such as steam explosion and pyrolysis pretretaments, due to lower energy inputs:, approximately 57 kJ/kg biomass for the SS/BW method versus 201 kJ/kg biomass for steam explosion, which was reported in previous studies (Adapa et al., 2011).

Future Prospects

The previous section summarized what has been accomplished by the research reported in this thesis. With respect to continuation of this work and future research, several topics that were not investigated in this research could be pursued in order to develop industrial-scale consolidated bioprocesses of cellulosic feed stocks and increase biofuel production rate:

1) The maximum operational temperature and pressure used for SS in this were 220 °C and 1 bar (atmospheric pressure), respectively. The results reported in Chapter 3 of this thesis showed that

the higher SS temperature, the higher end-product yields. Therefore, it is suggested that higher operational temperatures, up to 250 ºC should be used for further investigations.

2) In this dissertation, the feedstocks tested were selected from different cultivars of wheat straw, which is plentiful in the province of Manitoba. Essentially the best choice for biomass feedstocks are substrates with higher amounts of cellulose, and lower amounts of lignin. Consequently, based on compositional analyses, biomass feedstocks with lower percentages of lignin should be utilized.

3) Exposure of wheat straw biomass to SS changed the structure of cellulose and increased end-product yields. In this study, BW was combined with SS as a complementary process to enhance yields of fermentation end-products. Other methods, such as catalyst addition to enhance the recovery of hemicellulose sugars, and soaking biomass in selected concentrations of dilute acid/base during the SS process could increase the solubilisation of lignin and hemicellulose, and thus enhance microbial conversion of the substrate to obtain higher fermentation end-product yields.

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