1-1-2013

Ammonia Fiber Expansion (Afex) Treatment of Wheat Straw for Production of Bioethanol

Seyed Farshidreza Emam
Ryerson University

AMMONIA FIBER EXPANSION (AFEX) TREATMENT OF WHEAT STRAW FOR PRODUCTION OF BIOETHANOL

by

SEYED FARSHIDREZA EMAM

B.Sc., Tabriz University, Tabriz, Iran, 1999

A thesis

presented to Ryerson University

in partial fulfillment of the requirements for the degree of

MASTER OF APPLIED SCIENCE

in the program of

Environmental Applied Science and Management

Toronto, Ontario, Canada, 2013

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I authorize Ryerson University to lend this thesis to other institutions or individuals for the purpose of scholarly research.

I further authorize Ryerson University to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

Ammonia Fiber Expansion (AFEX) Treatment of Wheat Straw for Production of Bioethanol

Seyed Farshidreza Emam

Master of Applied Science, Environmental Applied Science and Management
Ryerson University
Toronto, Canada, 2013

Ammonia Fiber Expansion (AFEX) treatment is a technique that is able to enhance the enzymatic hydrolysis yield of lignocellulosic materials. In this technique, lignocellulosic materials are treated by liquid ammonia under pressure followed by rapid release of pressure that expands the fiber structure and increases enzyme access to lignocellulose polysaccharides. However, the AFEX treatment variables such as the mass ratio of ammonia to lignocellulosic biomass, moisture of lignocellulose (moisture content of biomass), temperature, and residence time need to be evaluated to find the maximum efficiency of this treatment.

The efficiency of the AFEX pretreatment was quantified by the yield of released sugars during enzymatic hydrolysis of the AFEX-treated wheat straw. The optimal treatment conditions for wheat straw were found to be: ammonia-to-wheat straw ratio, 1:1; temperature, 95°C; moisture content of wheat straw, 70% (dry weight basis); and residence time, 5 minutes. Under these conditions, almost 89% of the theoretical sugars were released by enzymatic hydrolysis of the AFEX-treated wheat straw. The enzymatic hydrolysis results showed the significance of AFEX pretreatment of wheat straw when compared to untreated wheat straw with released sugars yield of only 26 %.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation towards my supervisor, Dr. Ginette Turcotte, for accepting me as her graduate student and for her guidance and advice. She has been my mentor and has offered me her support at all times. I am very thankful to her for having given me the opportunity to work on my interesting topic and for her constant encouragement and faith in me.

I am also grateful to Dr. Michal Bardecki, Dr. Ronald Pushchak, and Dr. Noel George who have provided me with invaluable advice and guidance. Besides, I must thank the Program Administrator, Ms. Elias Chu for the tremendous amount of help she supplied.

I would also like to thank all Chemical Engineering faculty members at Ryerson University. Special thanks to Dr. Jiangning Wu for allowing me to work in her laboratory and use equipment. I would also like to extend my gratitude to Dr. Mehrab Mehrvar for all his help and valuable advice, and for letting me work in his laboratory when I needed it for performing AFEX experiments.

Furthermore, I must thank the Chemical Engineering office staff and technologists for all of the excellent work they do and help they supply. Special thanks to Alison Macleod, Isabella Fernandes, Louise Lichacz; and technologists, Ali Hemmati, Daniel Boothe, and Tondar Tajrobehkar.

DEDICATION

I would like to dedicate this thesis to my wonderful son Artin; it is he who gave me the strength and motivation, and lightened up my spirit to finish my thesis. And to my lovely wife, Shahrzad, who provided support and countless encouragements and personal sacrifices and helped me to succeed in my endeavors. And lastly, I would like to dedicate this thesis to my beloved parents who have always been incredibly supportive and encouraging. They instilled motivation, and self-confidence in me from an early age. *Without them I would truly not be where I am today.*

TABLE OF CONTENTS

Page

ABSTRACT iii

ACKNOWLEDGEMENTS iv

DEDICATION v

TABLE OF CONTENTS vi

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER 1 INTRODUCTION 1

Problem and objective of the research 3

CHAPTER 2 LITERATURE REVIEW 4

2.1 Bioethanol production from wheat straw 6

2.2 Lignocellulose structure 7

2.2.1 Structure of cellulose 8

2.2.2 Structure of hemicellulose 9

2.2.3 Structure of lignin 10

2.3 Pretreatment techniques 12

2.3.1 Physical pretreatment techniques 12

2.3.2 Chemical pretreatment technique 12

2.3.3 Physicochemical pretreatment technique 13

2.4 Hydrolysis of AFEX-treated wheat straw 15

CHAPTER 3 MATERIAL AND METHODS 18

3.1 Materials 18

3.2 AFEX pretreatment procedure 19

3.4 Design of experiments 29

3.5 Enzymatic hydrolysis of AFEX-treated wheat straw 32

CHAPTER 4 RESULTS AND DISCUSSION 34

4.1 Effect of AFEX pretreatment and enzymatic hydrolysis on appearance of wheat straw 34

4.2 Controlling the reactor temperature 36

4.3 Hydrolysis of wheat straw 38

4.4 Influence of AFEX conditions on released sugars yield enzymatic hydrolysis of wheat straw 42

4.4.1 Liquid ammonia to wheat straw ratio 42

4.4.2 Moisture content of wheat straw 45

4.4.3 Reactor temperature 46

4.4.4 Residence time 47

CHAPTER 5 CONCLUSION 51

FUTURE WORK AND RECOMMENDATIONS 53

APPENDICES 55

Appendix A: National Renewable Energy Laboratory protocol (LAP # 001) for total solids determination in wheat straw (NREL, 1995) 55

Appendix B: Enzymatic hydrolysis 58

Appendix C: Measurement of cellulase enzyme activity 93

Appendix D: Examples of calculations 103

REFERENCES 109

LIST OF TABLES

Page

Table 3-1. Recommended dosage and range of operating conditions for cellulases, according to Novozymes Bioenergy (2012). 18

Table 3-2. AFEX unit accessories and reactor parts..... 21

Table 3-3. The first set of AFEX pretreatment conditions for investigating the influence of ammonia to wheat straw ratio on released sugars yield of wheat straw..... 30

Table 3-4. The second set of AFEX pretreatment conditions for investigating the influence of moisture content on released sugars yield of wheat straw..... 30

Table 3-5. The third set of AFEX pretreatment conditions for investigating the influence of temperature on released sugars yield of wheat straw. 31

Table 3-6. The fourth set of AFEX pretreatment conditions for investigating the influence of residence time on released sugars yield of wheat straw. 32

LIST OF FIGURES

Page

Figure 2-1. Canadian bioethanol predicted production (Food and Agricultural Policy Research Institute, 2008). 5

Figure 2-2. Structure of cellulose with glucose subunits (Varga, 2003). 8

Figure 2-3. Subunits of hemicelluloses (Varga, 2003). 10

Figure 2-4. One of the lignin chemical structures and a phenylpropane subunit (Zimbardi et al., 2002). 11

Figure 2-5. Block diagram of AFEX pretreatment and enzymatic hydrolysis used in this research. 14

Figure 3-1. Schematic diagram of the AFEX unit used in this research..... 20

Figure 3-2. Reactor stainless steel tubing insert that was used inside the AFEX reactor. 22

Figure 3-3. Whole AFEX unit in the lab that was used for AFEX experiments in this research. 23

Figure 4-1. Wheat straw prior to enzymatic hydrolysis..... 35

Figure 4-2. Wheat straw after 164 hours of hydrolysis with enzymes NS22086 and NS2218 at 47.5 ºC..... 36

Figure 4-3. Overshoot of the Parr temperature controller at various set points (S.P.)..... 37

Figure 4-4. Reactor temperature against time using Parr temperature controller for target temperature of 95 ºC. 38

Figure 4-5. Glucose standard curve (using DNS method) for measurement of released sugars concentration..... 39

Figure 4-6. Released sugars concentrations from enzymatic hydrolysis of AFEX-treated and untreated wheat straw against enzymatic hydrolysis time at wheat straw moisture contents of 70 % and 50 % (dry weight basis), and AFEX reactor temperatures of 95 °C and 85 °C, and ammonia to wheat straw ratio of 1:1 after 164 h of hydrolysis. 41

Figure 4-7. Released sugars yield for AFEX treatment of wheat straw at 50% moisture content (dry weight basis) after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability. 43

Figure 4-8. Released sugars yield for AFEX treatment of wheat straw at 70 % moisture content (dry weight basis) after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability. 44

Figure 4-9. Released sugars yield for AFEX pretreatment of wheat straw at temperature of 95 °C and ammonia-to-wheat straw ratio of 1:1 after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability. 46

Figure 4-10. Released sugars yield for AFEX pretreatment of wheat straw at ammonia-to-wheat straw ratio of 1:1, and wheat straw moisture content of 70 % (dry weight basis) after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability. 47

Figure 4-11. Released sugars yield of AFEX-treated wheat straw at ammonia loading ratio of 1:1, wheat straw moisture content of 70 % and 80 % (dry weight basis) and reactor temperatures of 95 °C and 105 °C after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability..... 49

CHAPTER 1

INTRODUCTION

Today, many countries in the world are interested in replacing fossil fuels with clean and renewable energy sources. This interest emerges from the consequence of various economic and environmental problems such as uncertainty about the availability of fossil fuels, the energy crisis, and increasing the level of greenhouse gases in the atmosphere caused by the consumption of fossil fuels.

One source of clean and renewable energy that has been considered as an appropriate substitute for fossil fuels is bioethanol. Pure bioethanol can directly be used as a fuel in cars with a modified engine or blended with gasoline up to 30% and then used without any engine modifications.

Bioethanol can be produced from various types of biomass materials (materials produced by plants) such as sugar from sugarcane, starch from grains, or lignocellulosic waste materials such as plant residues. However, considering the world's human population growth and increasing demand for food, and consequently problems such as food security and food prices rising, lignocellulosic waste materials are the most viable alternatives for production of bioethanol in the near future.

Sources of lignocellulosic waste materials that could be available for bioethanol production are grasses (e.g., switchgrass, bermudagrass, chinesegrass), straws (e.g., rice straw, wheat straw, barley, and rye straw), corn stover, crop residues, sawdust, and waste wood chips. Among lignocellulosic waste materials, wheat straw is one of the cheapest and the most abundant agricultural residues from farming in countries which are the

major producers of wheat in the world such as Canada. Consequently, it could become one of the most attractive lignocellulosic materials for the production of bioethanol.

A major barrier to the commercialization of the production of bioethanol is the natural resistance of lignocellulosic materials to conversion into simple sugars which could be fermented to bioethanol by various microorganisms including yeasts, fungi, and bacteria. Two main techniques that are applied in the production of simple sugars from lignocellulosic materials are acid hydrolysis, and enzymatic hydrolysis.

Acid hydrolysis produces some inhibitors such as furan, which is a very toxic organic inhibitor for microorganisms in the downstream process. In addition, acid is a corrosive agent and may damage the equipment and increase the cost of acid hydrolysis. Enzymatic hydrolysis seems a better option because of the lack of corrosive compounds and formation of toxic substances. Despite the advantages of enzymatic hydrolysis, this method has a slow conversion rate, and is expensive due to the high price of enzymes. In order to improve the yield and the rate of enzymatic hydrolysis, a pretreatment step is required. Its goal is to make cellulose more accessible to enzymes through modifying and reducing the lignin fraction, prehydrolyzing the hemicellulose, and reducing the crystallinity of cellulose.

In Ammonia Fiber Expansion (AFEX) pretreatment, lignocellulosic materials are exposed to liquid ammonia at a mild temperature (60 °C -100 °C) and pressure for a short time (25-30 minutes) followed by a rapid release of pressure. The sudden release of pressure is an important factor for expanding the fiber structure of lignocellulose and increasing the accessible surface area for enzymes (Dale et al., 1983).

Problem and objective of the research

The AFEX pretreatment conditions have already been optimized for some lignocellulosic biomasses such as corn stover and switchgrass, and the data for the released sugars yield from enzymatic hydrolysis after AFEX pretreatment reported. However, no data for wheat straw seemed to be available.

The main purpose of this research was to determine the value of the major parameters (the ammonia-to-wheat straw ratio, the moisture content of wheat straw, temperature, and residence time) of an Ammonia Fiber Expansion (AFEX) pretreatment for wheat straw that result in its maximum enzymatic hydrolysis. The major parameters were selected based on previous work on agricultural residues such as corn stover by Teymouri et al. (2004). Since the main focus was on the pretreatment and enzymatic hydrolysis steps prior to the production of bioethanol, the subsequent steps of fermentation and distillation were not evaluated. The objectives of this research are outlined as follows:

- To investigate the effects of AFEX treatment conditions including liquid ammonia-to-wheat straw ratio, moisture content of wheat straw, temperature, and residence time for the enzymatic hydrolysis yield.
- To determine optimal AFEX conditions leading to maximum enzymatic hydrolysis of wheat straw.

CHAPTER 2

LITERATURE REVIEW

Significant environmental problems such as the increasing level of CO_2 in the atmosphere from the use of fossil fuels, the certain depletion of the world's fossil energy resources, and an unpredictable oil market, have stimulated societies around the world to search for renewable energy sources. However, among the various types of renewable sources of energy such as solar, wind, wave, tidal, biomass (organic materials produced by plants), and geothermal heat, only biomass is mostly used for production of liquid transportation fuels such as bioethanol.

Today, bioethanol is one of the most prevalent biofuels (fuel produced from biomass) in the world; and its production is continuously growing. In 2006, the global production of bioethanol mainly from sugar and starch was around 13 billion gallons (U.S.) per year, and total production capacity in 2015 was predicted to reach over 30 billion gallons (U.S.) per year (Licht, 2006).

In Canada, bioethanol production from various sources such as sugar, starch, and lignocellulosic materials has also shown a rising trend. Currently, however, only a small portion of produced bioethanol is from lignocelluosic materials. According to the Food and Agricultural Policy Research Institute (2008), bioethanol production capacity (from all sources) in 2007 was 0.147 billion gallons (U.S.) per year, and total output in 2017 is forecast to reach about 0.671 billion gallons (U.S.) per year (Figure 2-1). This increasing trend in Canada is due to various factors such as development of transportation industries, and government supports. The government supports include various incentives such as

tax incentives for biofuel producers, subsidies for the construction of new biofuel plants, and establishing bioethanol content policies such as that in the Canadian Environmental Protection Act (2010). According to this Act, fuel suppliers are committed to blend gasoline with at least 5 % vol. bioethanol. As a result of increasing bioethanol production in Canada, the demand for raw materials such as sugar and starch, and lignocellulosic materials such as agricultural residues that are used for production of bioethanol would be expected to increase.

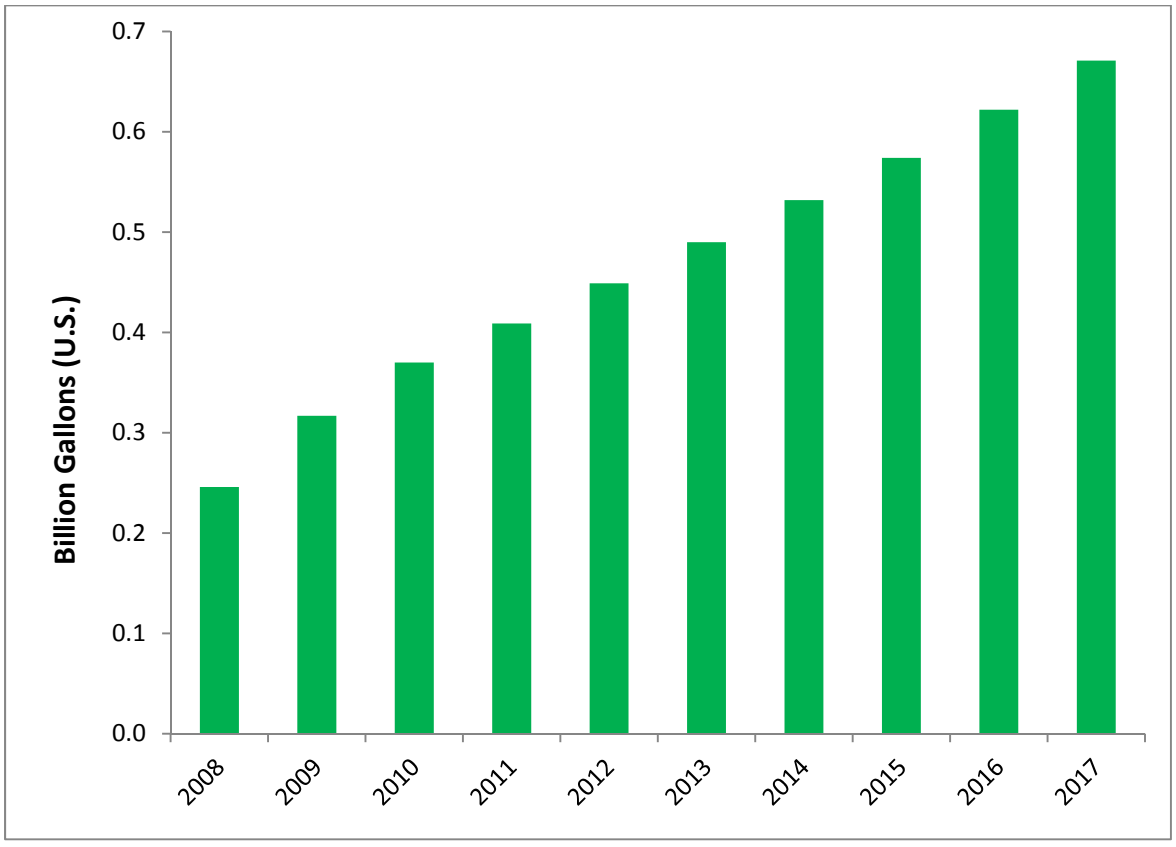

Figure 2-1. Canadian bioethanol predicted production (Food and Agricultural Policy Research Institute, 2008).

2.1 Bioethanol production from wheat straw

Currently, bioethanol is mainly produced by fermentation of fermentable sugars, which are derived from grain starch or are present in sugarcane juice. Fermentable sugars refer to simple sugars such as glucose that can be fermented to bioethanol by various microbes (i.e., *Saccharomyces cerevisiae*).

However, the cost of producing bioethanol from lignocellulosic materials is much higher than the cost of bioethanol production from sugarcane and grains. According to a Bloomberg New Energy Finance assessment (2013), the cost of bioethanol production from lignocellulosic materials was $ 3.6 per gallon (U.S.) in 2012, about 40% higher than the $ 2.5 per gallon (U.S.) cost of producing bioethanol from corn.

Today, production of bioethanol from food grains or sugarcane is been challenged due to human population growth and an increasing demand for food. As a result, the production of bioethanol from lignocellulosic waste materials is becoming more attractive. Agricultural residues (i.e., wheat straw, rice straw, and corn stover), energy crops (i.e., switchgrass, sweet sorghum), and wood are the most abundantly available sources of lignocellulosic biomass in the world. Among the agricultural residues, wheat straw is the second largest biomass feedstock in the world after rice straw.

Canada is the world's sixth-largest producer and one of the largest exporters of wheat, producing annually an average of 25 million tons of wheat grains or 4% of global wheat production (Agriculture and Agri-Food Canada, 2010; The Canadian Encyclopedia, 2012). With a mass ratio of wheat straw to grain equal to 1.6, about 40 million tons of wheat straw is annually produced in Canada (Agriculture and Agri-Food

Canada, 2003). Usually, a portion of the wheat straw biomass is intended to remain on the field for preventing soil erosion and maintaining soil fertility. In addition, a portion of the available wheat straw is used for livestock feeding and bedding. The remaining unused wheat straw is called surplus wheat straw. In the Canadian Prairie provinces alone (Alberta, Manitoba, Saskatchewan) the net surplus of wheat straw is about 14 Mt (Sokhansanj et al., 2006). Depending on the farmer's decision, the surplus wheat straw might be disposed of methods such as leaving it on the field, plowing it back into the land, burning it or even more simply, throwing it away. Obviously, these methods of disposal of surplus wheat straw do not generate any economic benefits for land owners. Additionally, the disposal of surplus wheat straw by burning releases huge amounts of air pollutants such as CO, NO_2, and particulate matter, which have negative impacts on human health. Therefore, finding an environmentally friendly method (for the disposal of surplus wheat straw) with economic benefits such as the production of bioethanol, is of high interest to society. However, in order to produce bioethanol from wheat straw or any lignocellulosic material, several barriers need to be removed. The major challenge is that the enzymes do not have easy access to the polysaccharides (cellulose and hemicellulose) in the lignocellulosic materials or biomass for enzymatic hydrolysis. This problem is mainly because polysaccharides are tightly bound to the lignin (Kim et al., 2004). In this regard, understanding the lignocellulose structure could be helpful for improving and developing new techniques in production of bioethanol from lignocellulosic materials.

2.2 Lignocellulose structure

The main components of lignocellulosic biomass include polysaccharides (cellulose and hemicellulose) with approximately 55-85% of the total weight of biomass,

and lignin (Demirbas, 2005). There are also smaller amounts of other compounds such as resins, salts, and minerals.

In lignocellulosic biomass, cellulose chains are bonded together to form cellulose fibers or cellulose microfibrils. Cellulose microfibrils are cross-linked to hemicellulose. Hemicellulose is also associated with lignin by ester cross-linkages between xylan subunits and uronic acids. Cellulose fibers and hemicellulose are wrapped by lignin.

2.2.1 Structure of cellulose

Cellulose is an insoluble organic compound consisting of a linear and unbranched polymer (polysaccharide) chain that is made up of thousands of D-glucose units (Figure 2-2). The cellulose formula is $(C_6H_{10}O_5)_n$ where n is the number of glucose subunits in the polymer.

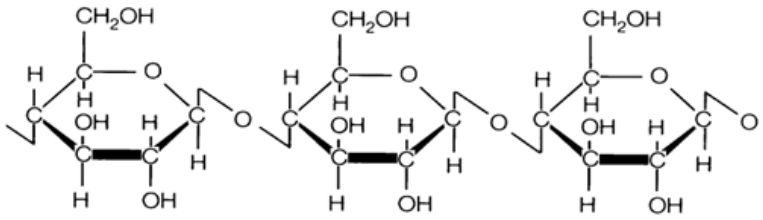

Figure 2-2. Structure of cellulose with glucose subunits (Varga, 2003).

In lignocellulosic materials, cellulose chains are bonded to each other by van der Waals forces and hydrogen bonding. These aggregations form primary fibrils, which can be further associated to form microfibrils (Campbell et al., 1999). The chemical bonds arrange polymer chains into various orders from a crystal lattice to a randomly ordered structure that are called crystalline regions and amorphous regions, respectively. The

degree of crystallinity of cellulose is very important in its chemical reactivity, because the enzymes are not able to have access to crystalline regions. An effective pretreatment technique would play a key role in the breakage of these crystalline regions to make cellulose more accessible to enzymes during the enzymatic hydrolysis step.

Crystallinity is one of the main reasons for the unreactivity of cellulose in lignocellulosic materials. However, another reason for the unreactivity of cellulose is the fact that cellulose microfibrils are wrapped in sheets of hemicellulose and lignin (Wyman, 1996).

2.2.2 Structure of hemicellulose

Hemicellulose is another significant polysaccharide found in the cell walls of lignocellulosic biomass. Based on the subunit (monosaccharide) composition, hemicelluloses are divided into eight groups: 1) xylans, which are hetero- and homopolysaccharides such as arabinoglucuronoxylans, arabinoxylans, and glucuronoxylans; 2) mannans, which are hetero- and homopolysaccharides such as glucogalactomannans, glucomannans, and galatomannans; 3) arabinans; 4) galacturonans; 5) glucofructosans and fructosans; 6) glucans, which are heteropolysaccharides including arabinoglucans, and homopolysaccharides such as β-(1-3)-D-glucan callose; 7) mannuronans; and 8) galactans, which are hetero- and homopolysaccharides such as arabinogalactans (Tarchevsky et al., 1991). Among all the different groups of hemicelluloses, xylans and mannans are the most dominant types of hemicelluloses in plant cell walls. Figure 2-3 shows some subunits of hemicellulose.

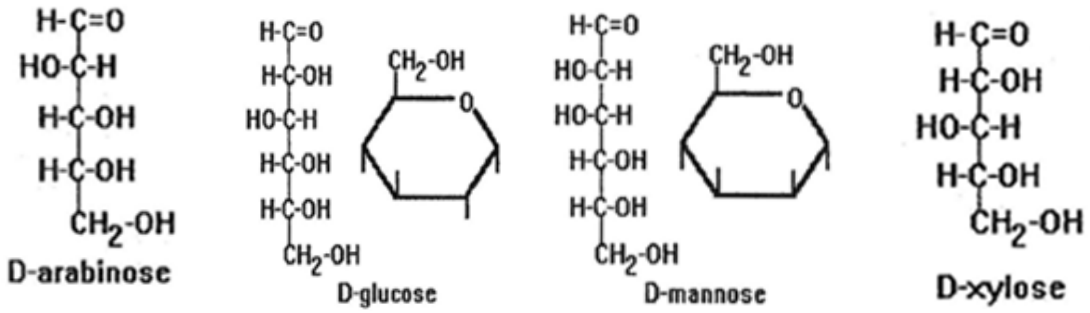

Figure 2-3. Subunits of hemicelluloses (Varga, 2003).

Unlike cellulose, which has a crystalline structure; and is resistant to reactant molecules, hemicellulose has little physical strength because of its having an amorphous structure. Therefore, hemicellulose can easily be hydrolyzed by enzymes to release simple sugars (Tarchevsky et al., 1991).

Since hemicellulose contains fermentable sugars such as glucose and fructose, its recovery, and its utilization during the fermentation process may increase the yield of ethanol produced from lignocellulosic materials. Xylose could also be fermented by some metabolically engineered microorganisms such as *Saccharomyces cerevisiae* to enhance the yield of ethanol production.

2.2.3 Structure of lignin

Lignin is an amorphous three-dimensional polymer with a high molecular weight (Figure 2-4). Lignin is composed of phenylpropane subunits that are held together by chemical bonds such as carbon-carbon and ether. Although there are many lignin structures, only few of them have been determined.

Lignin protects cellulose and hemicellulose from enzymatic attack through formation of a protective coating on them. Lignin physically binds with cellulase enzymes to prevent them having access to the cellulose (Mansfield et al., 1999).

Lignin, hemicellulose, and cellulose are bonded together by cross-linkages, which result in the recalcitrant structure of lignocellulose to hydrolysis (Tarchevsky et al., 1991). Thus, for bioethanol production, it is necessary to modify or disrupt the lignin that is behaves as a main obstacle to enzymatic hydrolysis (Grohmann et al., 1992).

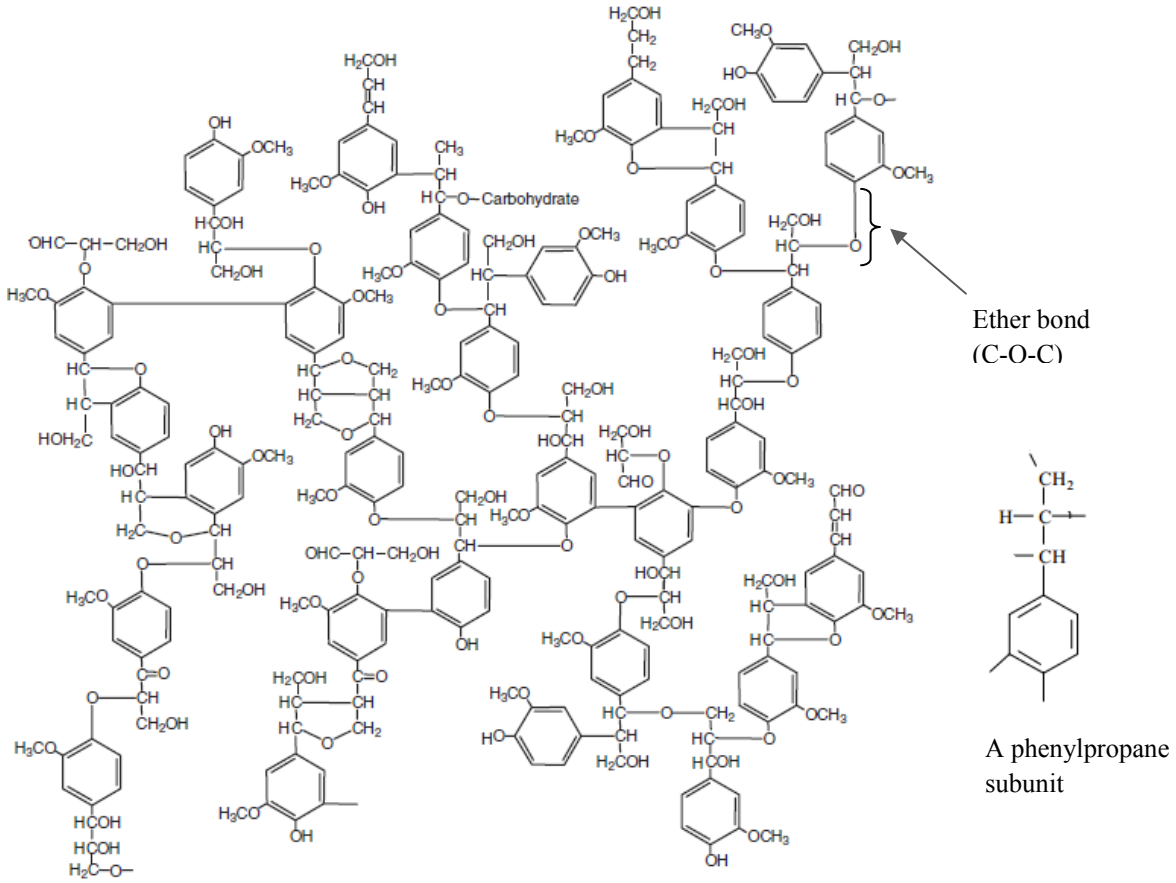

Figure 2-4. One of the lignin chemical structures and a phenylpropane subunit (Zimbardi et al., 2002).

2.3 Pretreatment techniques

In the bioethanol production process, a pretreatment step is required for reducing lignocellulose recalcitrance and making sugars available for fermentation into bioethanol. An effective pretreatment technique must preserve the fermentable sugars and prevent formation of inhibitor compounds.

There are three main categories of biomass pretreatment, including physical treatment, chemical treatment, and physicochemical treatment. Each pretreatment technique has some advantages and some drawbacks.

2.3.1 Physical pretreatment techniques

Physical pretreatment techniques, such as milling (e.g., ball milling) and grinding, enhance the enzymatic hydrolysis rate and yield through physical effects on lignocellulosic biomass. Compressive and shearing forces in milling increase the surface area accessible to enzymes through reducing the size of biomass (Abraham et al., 1997).

However, the major drawback for the physical pretreatment technique is that the consumption of electrical energy is higher than the inherent energy of lignocellulosic biomass (Kumar, 2009).

2.3.2 Chemical pretreatment technique

In this technique, lignocellulosic biomass can be treated with chemical compounds such as an acid or base. Strong acid or base treatment efficiently enhances the enzymatic hydrolysis of cellulose through removing lignin.

However, the major drawbacks in using chemicals in pretreatment are the high cost of chemicals, their corrosiveness, and their toxicity (formation of toxic compounds, such as furan, that may be inhibitory to microbes in the fermentation step). In addition, removing or neutralizing these chemicals is time consuming and expensive (Schell et al., 1996).

2.3.3 Physicochemical pretreatment technique

Physicochemical pretreatment technique is a combination of both physical and chemical processes. This technique has the advantages of both physical and chemical pretreatments. For instance, the consumption of energy is not as high as ball milling of physical pretreatment. Steam explosion (treatment of lignocellulosic materials with steam under temperature (215-260°C) and pressure (20-35 bar)) and Ammonia Fiber Expansion (AFEX) pretreatment are two examples of a physicochemical pretreatment technique.

The selection of an appropriate pretreatment technique is mainly based on the by-products generated by the pretreatment technique and the type of biomass used. One pretreatment technique that is effective for one type of biomass may not be appropriate for another type of biomass. For example, previous studies have demonstrated that Ammonia Fiber Expansion (AFEX) pretreatment has the potential to effectively increase the susceptibility of lignocellulose to enzymatic hydrolysis, especially for lignocellulosic materials with low amounts of lignin such as herbaceous and agricultural residues (Sun et al., 2002). AFEX does not generate inhibitory materials for subsequent steps, and the efficiency of this method is not dependent on the size of biomass (Mosier et al., 2005; Sun et al., 2002). In addition, on a large scale, the high volatility of ammonia makes it

easy to be recovered and recycled to reduce operating costs and prevent ammonia being released in the environment. Figure 2-5 shows the schematic diagram of AFEX pretreatment and the enzymatic hydrolysis process.

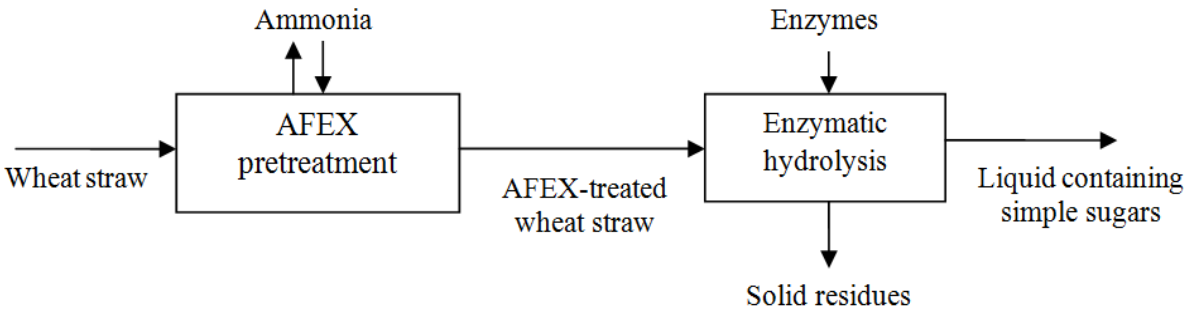

Figure 2-5. Block diagram of AFEX pretreatment and enzymatic hydrolysis used in this research.

The major variables that significantly affect the AFEX pretreatment are temperature, ammonia-to-biomass ratio, moisture content, and residence time. In this method, lignocellulosic materials are treated with liquid ammonia under pressure (e.g. 13.8-17 bar) and mild temperatures (e.g. 60°C -100°C) for a short period of time (e.g. 5-30 minutes) followed by the rapid release of pressure. The rapid release of pressure expands the fibers of lignocellulosic biomass materials; and as a result, the accessible surface area will be increased for enzymatic attack (Holtzapple et al., 1991; Dale and Moreira, 1983). By adjusting the pretreatment conditions, the ammonia fiber expansion (AFEX) method can effectively increase the yield of enzymatic hydrolysis (Holtzapple et al., 1991).

The mechanism for increasing the susceptibility of lignocellulose to enzymatic hydrolysis has not been established yet. However, it seems that there are some chemical and physical changes in the structure. One important change is that ammonia in liquid state can swell cellulose. Ammonia gets through cellulose and breaks the hydrogen bonds, and then reacts with the hydroxyl groups of cellulose (Barry et al., 1936). As a result of ammonia penetration into cellulose, the distances between the cellulose chains increase. Additionally, the structure of natural cellulose crystals (cellulose I) is transformed to ammonia treated cellulose crystals (cellulose III). Ammonia treated cellulose crystals (16-23 Å) are smaller than ordinary cellulose crystals (50-80 Å) (Lewin et al., 1971). These physical and chemical changes in the structure of cellulose provide more accessible surface area for enzymes to react with cellulose during enzymatic hydrolysis.

Another effect of ammonia on lignocellulose is the ammonolysis of the ester crosslinks of uronic acids with the xylan subunits that results in cleavage of the linkages between lignin and hemicellulose. In addition, ammonia can react with lignin and convert insoluble lignin macromolecules to smaller soluble molecules by cleavage of the C-C and C-O bonds (Wang et al., 1967). This partial degradation of hemicellulose and transformation of lignin enhances the susceptibility of lignocellulosic materials to enzymatic hydrolysis by making cellulose more accessible to enzymes.

2.4 Hydrolysis of AFEX-treated wheat straw

Ethanol can be produced by conversion of polysaccharides (hemicellulose and cellulose) in wheat straw into fermentable sugars and then, fermentation of these sugars

to bioethanol by selected microbes. Two techniques for hydrolyzing polysaccharides are acid hydrolysis and enzymatic hydrolysis. Acid hydrolysis with a dilute acid is a cheap method but the yield of sugars is low and the required high temperature for this method could degrade the sugars. In acid hydrolysis with a concentrated acid, the sugar yield is higher and the risk of sugar degradation is lower compared to a dilute acid, but the required subsequent acid neutralization and recovery processes make this technique costly. As an alternative to the acid hydrolysis technique and its problems, the enzymatic hydrolysis of polysaccharides with enzymes such as cellulases has a higher sugar yield without the degradation of sugars compared to acid hydrolysis.

Cellulase enzymes, mainly produced by microbes and fungi are a group of enzymes such as endo-glucanases, exo-glucanases, and glucosidases that act cooperatively in hydrolyzing cellulose to glucose (Wilke et al., 1983).

In enzymatic hydrolysis, the glycosidic linkages between glucose subunits are cleaved by adding one molecule of water to each linkage. One of the main obstacles to the commercialization of production of bioethanol from lignocellulosic materials is the high price of enzymes. Thus, one way to decrease the cost of bioethanol production is the consumption of smaller quantities of enzymes without reducing the yield of enzymatic hydrolysis. This target can be reached by evaluating the lignocellulose pretreatment parameters and finding the conditions, under which the enzymatic hydrolysis yield is higher despite using an equal amount of enzyme.

In conclusion, bioethanol as a clean and renewable source of energy is an alternative to fossil fuels; and its production is globally increasing. Currently, food-based

materials such as sugar and starch are used for bioethanol production. However, the use of these materials has been challenged today because of human population growth environmental impacts and food security issues. Thus, lignocellulosic materials such as agricultural residues are considered one of the most viable biomass sources for bioethanol production in the near future. Among agricultural residues, wheat straw, as a cheap and abundant agricultural residue, has the potential to be considered raw material for production of bioethanol instead of burning it or wasting it on the field. However, the costs of current technologies for producing bioethanol are high and the wheat straw to bioethanol production process cannot be commercialized. Today, more effective pretreatment processes have been developed that are able to enhance the yield of released sugars during enzymatic hydrolysis, and subsequently decrease the total cost of bioethanol production from lignocellulosic materials. The Ammonia Fiber Expansion (AFEX) method is a pretreatment technique that can effectively increase the yield of enzymatic hydrolysis if the pretreatment conditions (ammonia-to-wheat straw ratio, temperature, moisture content, and residence time) are properly adjusted.

CHAPTER 3

MATERIAL AND METHODS

The AFEX pretreatment conditions including the ammonia-to-biomass ratio, moisture content, temperature, and residence time were evaluated in this research.

3.1 Materials

Wheat straw

Wheat straw (*Triticum aestivum*) was generously provided by Mr. Terri Broadway (Newmarket, Ontario) in a bale in November 2011. Using a Retsch Cutting Mill SM 100, the wheat straw was milled and passed through a 6-mm sieve. Milled wheat straw was kept at room temperature in sealed plastic bags for subsequent use.

Enzymes

The enzymes NS22086 (Cellulase mixture comprising endo-glucanase, exo-glucanase and β-glucosidase enzymes) and NS22118 (β-glucosidase or cellobiase) were received from Novozymes A/S (Bagsvaerd, Denmark). Table 3-1 shows the range of operating conditions and recommended dosage.

Table 3-1. Recommended dosage and range of operating conditions for cellulases, according to Novozymes Bioenergy (2012).

Enzyme type	Density (g/ml)	Temperature (°C)	pH	Recommended dosage (% wt./wt. (total solids))
Cellulase mixture NS22086	1.15	45-50	5.0-5.5	1-5
β-glucosidase NS22118	1.2	45-70	2.5-6.5	0.2-0.6

Activity of the cellulase mixture NS22086 was determined by a modified NREL laboratory analytical procedure LAP- 006 (Appendix C), and was found to be 102 FPU/ ml. Filter paper unit (FPU) is the amount of enzyme releasing 1 μmol of glucose from filter paper per ml per minute.

Ammonia

Liquid anhydrous ammonia was purchased from Air Liquid Canada Inc. (Toronto, Ontario) in a 50-lb cylinder.

3.2 AFEX pretreatment procedure

The schematic diagram of the pressure vessel and the accessories used for the AFEX pretreatment of the wheat straw is shown in Figure 3-1, where all labeled accessories and reactor parts are found in Table 3-2. Figure 3-2 shows the whole AFEX unit assembly in the lab.

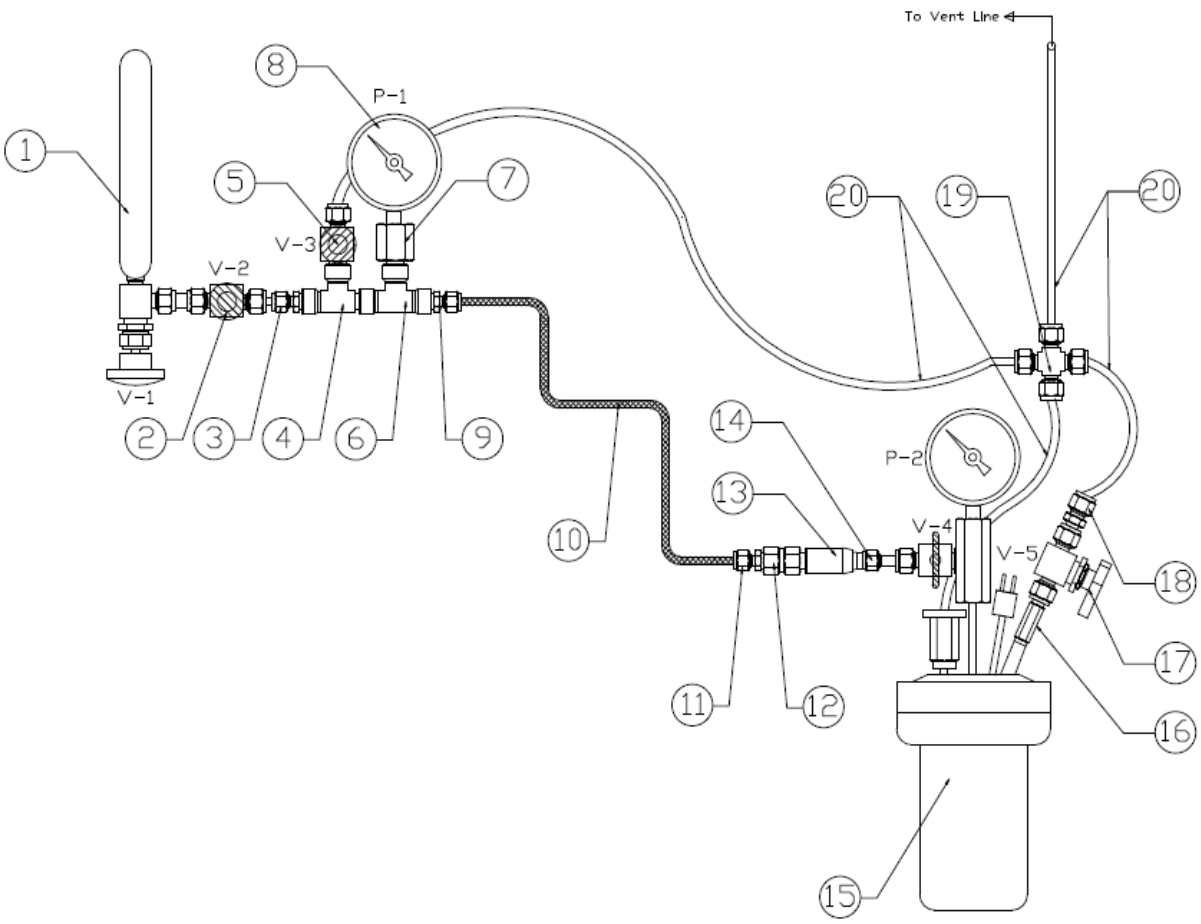

Figure 3-1. Schematic diagram of the AFEX unit used in this research.

Table 3-2. AFEX unit accessories and reactor parts

	Product Name and Description	Supplier	Symbol
1	Ammonia Cylinder and Ammonia Cylinder Valve (V-1)	Air Liquid	
2	Control Valve CGA 180 1/4" OD Tube	Matheson	V-2
3	Tube Fitting, Male Connector, 1/4 in.	Swagelok	
4	Stainless Steel Pipe Fitting, 1/4 in.	Swagelok	
5	Stainless Steel Integral Bonnet Needle Valve, 1/4 in.	Swagelok	V-3
6	Stainless Steel Pipe Fitting, 1/4 in.	Swagelok	
7	Stainless Steel Pipe Fitting, 1/4 in.	Swagelok	
8	Industrial Pressure Gauge 0 to 300 psi, 1/2 in.	Swagelok	P-1
9	Tube Fitting, Male Connector, 1/4 in.	Swagelok	
10	Parr 6ft Hose	Parr	
11	Tube Fitting, Male Connector, 1/4 in.	Swagelok	
12	Stainless Steel Pipe Fitting, 1/4 in.	Swagelok	
13	Stainless Steel PTFE-Sealed Quick-Connect, 1/4 in.	Swagelok	
14	Stainless Steel PTFE-Sealed Quick-Connect, 1/4 in.	Swagelok	
15	Parr reactor, 300ml, 3000 psi, Reactor Dimensions: 2.5 inch dia., 4.0 inch depth, 9lbs Parts list in Table 3-2	Parr	
16	Stainless Steel Pipe Fitting, 1/8 in.	Swagelok	
17	Stainless Steel Ball Valve, 1/8 in.	Swagelok	V-5
18	Stainless Steel Tube Fitting, Male Connector, 3/8 in.	Swagelok	
19	Stainless Steel Tube Fitting, 3/8 in.	Swagelok	
20	Vinyl Tubing, 1/4 in.	Swagelok	
21	Reactor Rupture Disk, Size: 0.25" ID Type: PST FS Burst Pressure: 3000psi@72ºF	Fike Corporation	
22	Integral Bonnet Needle Valve, Stainless Steel Needle Valve, Black Aluminum Bar Handle	Swagelok	V-4
23	Pressure Gauge, max-3000psi	Span	P-2
24	Parr Probe, Reactor Temperature Probe	Parr	
25	Reactor Stainless Steel Tubing Insert, two piece of in-house constructed tubing (one as a thermocouple well, and the other as an ammonia feeding port) which has distance of 3cm from the centers of the tube, Figure 3-2	Constructed in-house*	
26	Rubber Heater, Figure 3-3	Parr	
27	Omega Temperature Controller, Figure 3-3	Parr	
28	Parr Reactor Heater, Figure 3-3	Parr	
29	Parr Temperature Controller (Parr 4838), Figure 3-3	Parr	

*****This particular part was constructed by technologist in Chemical Engineering Department in order to be used inside the AFEX reactor.

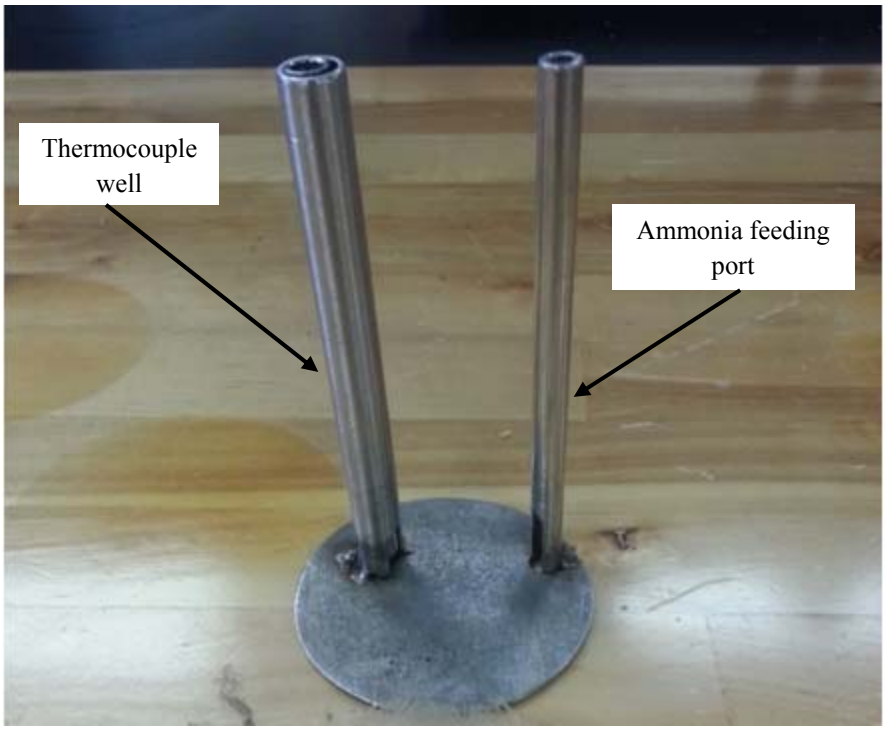

Figure 3-2. Reactor stainless steel tubing insert that was used inside the AFEX reactor.

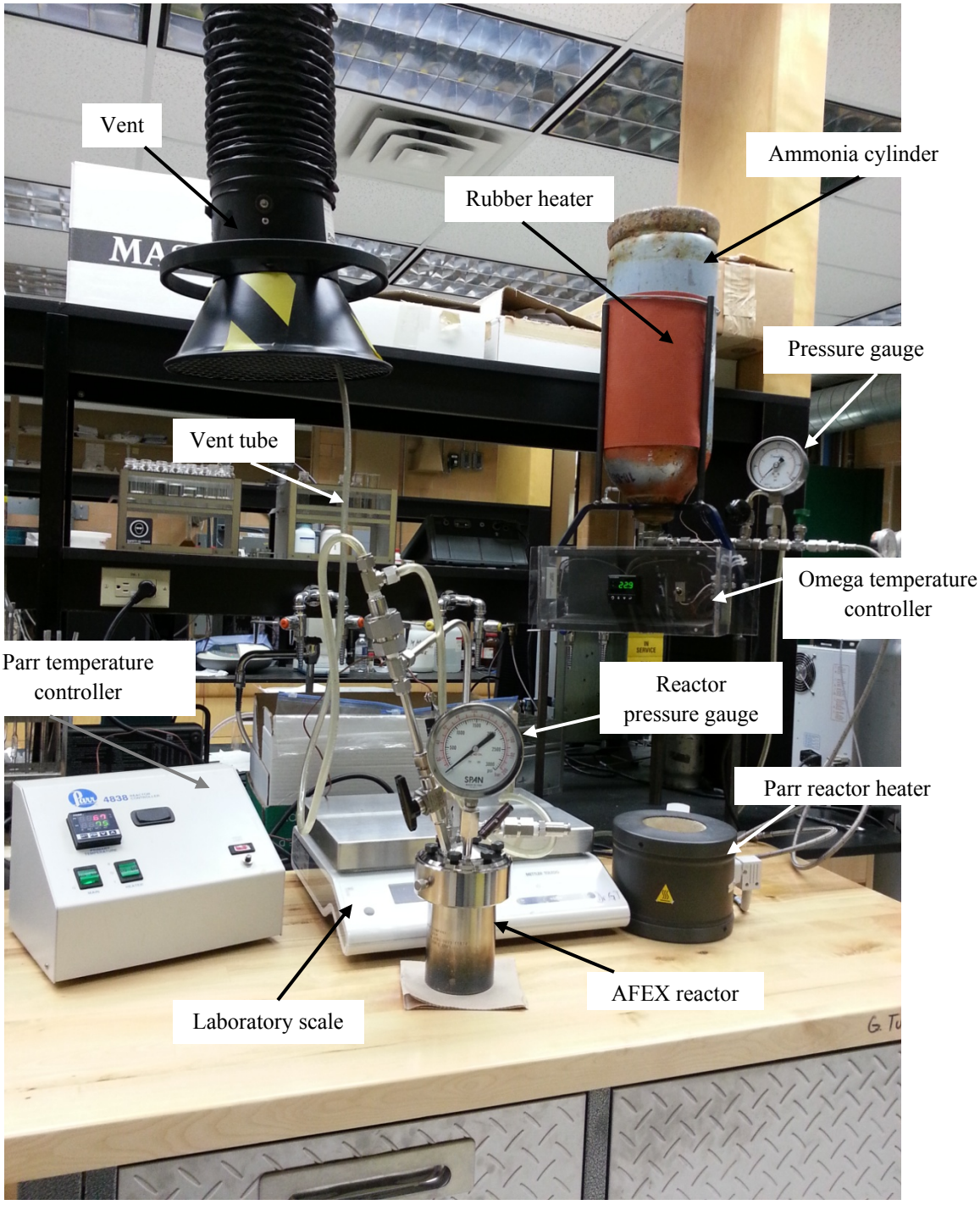

Figure 3-3. Whole AFEX unit in the lab that was used for AFEX experiments in this research.

The following procedure was used for each AFEX pretreatment run:

1. The AFEX unit was set up according to the Figures 3-1 and 3-3. The ammonia cylinder (50 lb) was placed inverted to ensure liquid ammonia delivery to the reactor.
2. The Omega temperature controller installed on the ammonia cylinder stand (Figure 3-3) was turned on for controlling the temperature of ammonia. Because of fluctuations in the lab temperature, the liquid ammonia in the cylinder was controlled to have a constant temperature, and to reduce the AFEX pretreatment heating time.
3. On the Omega temperature controller display, the set-point option was selected and adjusted the ammonia temperature set point at 30 °C using the arrow keys for option selection.
4. Left sign arrow key on the Omega temperature controller display was pressed to store the value.

   Note:

   - According to vendor recommendations, temperature of ammonia cylinder was not allowed to exceed 40 °C and also temperature of heating rubber pad attached to the ammonia cylinder (Figure 3-3) was not allowed to exceed 230 °C.
   - The "K" type thermocouple was always checked to see if it was properly attached by a tape to the ammonia cylinder opposite the rubber heater. The thermocouple was located at the back of the ammonia cylinder.
5. In thermocouple submenu option on Omega temperature controller display, the "K" type thermocouple was selected using the arrow keys.
6. 5 grams of wheat straw was mixed with the desired amount of water for desired moisture content in a beaker using a clean spatula. For example, for 70 % moisture

content, 2.82 g water was mixed with 5 g wheat straw containing 4.6 g total solids. Total solids were determined according to NREL laboratory analytical procedures LAP-001 (Appendix A) before each AFEX experiment. The wheat straw was mixed with water for few seconds until water entirely became absorbed by wheat straw, then the beaker was covered by a plastic foil.

7. Place the reactor stainless steel tubing insert into the 300 ml stainless steel reactor, PARR Instrument Co., IL (No. 15, Figure 3-1).
8. The wet wheat straw was immediately transferred into the reactor.
9. A plastic net was placed over the straw to prevent its dispersion during release of pressure at the end of the run.
10. To occupy the empty space (about half of the reactor) of the reactor, steel balls (diameter of 5 mm) were placed on top of the plastic net in order to decrease the empty volume and minimize the transformation of ammonia from liquid to gas before absorbing by wheat straw during ammonia loading.
11. The reactor lid was properly placed on the reactor in a direction that the "J" type thermocouple can be inserted in the thermocouple well. Then, the reactor lid was tightened using bolts and nuts.
12. The vent allocated for venting hazardous gas such as ammonia was turned on (see Figure 3-3).
13. The AFEX unit vent tube was connected to the vent (see Figure 3-3).
14. The ammonia cylinder valve (V-1) was opened. Before opening of the ammonia cylinder valve, and during the AFEX experiments, personal protective equipment such as a gas mask and gloves were worn. Ammonia (NH_3) in form of liquid or gas is

extremely hygroscopic (absorber of water). It easily dissolves in water and forms ammonium hydroxide (NH_4OH), which is a corrosive substance. Ammonia can readily be absorbed by the moist parts of human body such as eyes, respiratory tract, and moist skin that will result a chemical-type burn in them. Also, contact with liquid ammonia has the risk of skin freezing because of fast vaporization and quick absorption of skin heat (Davis, 1987; Kirk-Othmer, 1992).

15. Liquid ammonia was allowed to fill the ammonia line including tubes and Parr hose (6 ft, No. 10 in Figure 3-1) placed before reactor valve (V-4 in Figure 3-1). To ensure that the line was fully filled with liquid ammonia, the needle valve (V-3) was gently opened and let the ammonia gas be vented until observing the full flow of liquid ammonia in the vinyl tube (No. 20 in Figure 3-1). Then, the valve (V-3) was tightly closed.
16. The reactor was placed on the laboratory scale (Mettler Toledo, Model: MS32001L, Switzerland) and tare the scale after ending the weight fluctuations (see Figure 3-3). Patience was required for this step because vinyl tubes and thermocouple wire (referring to Figures 3-1 and 3-3) were connected to the reactor that could potentially be the sources of fluctuations, thus, at least 20 seconds was considered for stabilization.
17. The reactor needle valve (V-4) was opened very carefully and gently; and the desired weight of liquid ammonia (e.g. for 5 g wheat straw containing 4.6 g total solids, 4.6 g ammonia was required for an ammonia to wheat straw ratio of 1:1) was delivered to the reactor, then close the valve tightly.

Note: This step was a little challenging and required concentration, experience, and attention because rapidly opening the valve or moving the reactor on the scale during ammonia loading could lead to the delivery of extra ammonia to the reactor or weight fluctuations. In both cases, the experiment could be repeated from step 6 to 18.

18. The reactor was placed in the Parr reactor heater. Then, the Parr reactor heater and "J" type thermocouple was connected to the Parr temperature controller (see Figure 3-3).
19. The Parr temperature controller was turned on and adjusted the set-value at about half of the target temperature (e.g. set-value, 48 °C for target temperature, 95 °C) using "S.V" key on the temperature controller display. Then, state II for the heater key on the Parr temperature controller was selected to start heating with a fast rate.
20. The reactor parameters including time, temperature (reading from process value indicated as "P.V" on the temperature controller display), and pressure (reading from pressure gage (P-2) were recorded as soon as turning the heater on, and after every 2 minutes for 30 minutes (for residence time of 5 minutes) or 40 minutes (for residence time of 15 minutes).
21. Ammonia cylinder valve (V-1) was tightly closed.
22. After reaching half of the final temperature, the key state was changed from II to I to decrease the rate of heating. Also, the set-value was increased to a temperature about 10 °C lower than the final temperature.
23. After about 20 minutes, the heater was turned off. For residence time of 15 minutes, the heater needed to be turned on again after about 27 minutes for 2 minutes.

    Note:

- The Parr reactor heater was turned off few minutes prior to the release of pressure because the reactor and the heater stored the heat and released it when the Parr reactor heater was turned off. Also, the thermocouple well was near the center of the reactor, and consequently thermocouple had delay to receive heat from reactor walls.
- Sometimes the reactor needed to be taken out of the heater to maintain the target temperature and prevent overheating.

24. After 25 minutes, the initial temperature should reached its final temperature and became stable at its target temperature with a reasonable tolerance (e.g. ±1 °C) for desired residence time (e.g. 5 minutes or 15 minutes).
25. At the end of the residence time, the reactor ball valve (V-5) was rapidly opened to explosively release the pressure and vent the ammonia into the vent line.
26. The Parr temperature controller and Omega temperature controller were turned off.
27. The ammonia line was purged by opening the valve (V-3)
28. Approximately 5 minutes was needed before opening the reactor lid to vent the remaining ammonia gas in the reactor. The heat resistant gloves were worn and then, the reactor lid was opened and the steel balls and plastic net were transferred in a cold water bath to become cool, and then dry them using a clean paper towel.
29. The reactor stainless steel tubing insert along with AFEX-treated wheat straw were taken out. Then, AFEX-treated wheat straw was placed on an aluminum weighing dish and was put under a fume hood at room temperature to evaporate the remaining ammonia.
30. The reactor and reactor stainless steel tubing insert were placed in a cold water bath to become cool.

31. The steel balls, plastic net, reactor stainless steel tubing insert, and reactor were washed with distilled water.
32. The vent was turned off (Figure 3-3).
33. After one night, the AFEX-treated wheat straw was stored in sealed plastic bag in a refrigerator for subsequent enzymatic hydrolysis.

3.4 Design of experiments

The method of one-variable-at-a-time (OVAT) was used, in order to evaluate the interplay between AFEX pretreatment conditions (ammonia to wheat straw mass ratio, temperature, moisture content, and residence time) and to determine the pretreatment conditions that result in maximum enzymatic hydrolysis yield for wheat straw. In this regard, several sets of experiments were considered. All experiments were performed in duplicate. The residence time was set at 5 minutes for experiment sets 1 to 3.

The first set of AFEX pretreatment experiments was performed in four steps to investigate the influence of the ammonia-to-wheat straw ratio on the yield of released sugars from wheat straw (Table 3-3). The enzymatic hydrolysis was carried out after performing each step.

In the first step, the experiment was carried out at the ammonia-to-wheat straw ratio of 0.5:1, a moisture content of 50 %, and a reactor temperature of 85 °C. Then, the ammonia-to-wheat straw ratio was increased to 0.8:1, 1:1, 1.2:1, and 1.5:1, while the moisture content and temperature was fixed at 50 % and 85 °C respectively. The second step was performed similar to the first step, but the temperature was increased to 95 °C.

The third and the forth steps were carried out similar to the first and the second steps respectively, but only the moisture content was increased to 70 %. The experiments were performed in duplicate.

Table 3-3. The first set of AFEX pretreatment conditions for investigating the influence of ammonia to wheat straw ratio on released sugars yield of wheat straw.

Step	Ammonia to wheat straw ratio					Moisture	Residence time
	0.5:1	0.8:1	1:1	1.2:1	1.5:1		
1	T = 85 °C	T = 85 °C	T = 85 °C	T = 85 °C	T = 85 °C	50%	5 min
2	T = 95 °C	T = 95 °C	T = 95 °C	T = 95 °C	T = 95 °C		5 min
3	T = 85 °C	T = 85 °C	T = 85 °C	T = 85 °C	T = 85 °C	70%	5 min
4	T = 95 °C	T = 95 °C	T = 95 °C	T = 95 °C	T = 95 °C		5 min

The second set of experiments was carried out to investigate the effect of moisture content on the yield of released sugars from wheat straw (Table 3-4). In this regard, the first experiment was performed at a moisture content of 30 %, ammonia to wheat straw ratio of 1:1, and temperature of 95 °C. Then, the moisture content was increased to 50 %, 70 %, 80 %, and 90 %, while the temperature and ammonia-to-wheat straw ratio was fixed at 95 °C and 1:1 respectively. The experiments were performed in duplicate.

Table 3-4. The second set of AFEX pretreatment conditions for investigating the influence of moisture content on released sugars yield of wheat straw.

Moisture	Ammonia to wheat straw ratio	Temperature	Residence time
30%	1:1	95 °C	5 min
50%	1:1	95 °C	5 min
70%	1:1	95 °C	5 min
80%	1:1	95 °C	5 min
90%	1:1	95 °C	5 min

The third set of experiments was carried out to investigate the effect of temperature on the yield of released sugars from wheat straw (Table 3-5). The first run was performed at the temperature of 75 °C, moisture content of 70 %, and ammonia to wheat straw ratio of 1:1. Then, the temperature was increased to 85 °C, 95 °C, 105 °C, and 115 °C, while the moisture content and ammonia-to-wheat straw ratio was fixed at 70 % and 1:1 respectively. The experiments were performed in duplicate.

Table 3-5. The third set of AFEX pretreatment conditions for investigating the influence of temperature on released sugars yield of wheat straw.

Temperature	Ammonia to wheat straw ratio	Moisture	Residence time
75 °C	1:1	70%	5 min
85 °C	1:1	70%	5 min
95 °C	1:1	70%	5 min
105 °C	1:1	70%	5 min
115 °C	1:1	70%	5 min

The fourth set of experiments was carried out to investigate the effect of residence time on the yield of released sugars from wheat straw (Table 3-6). Some of the best AFEX conditions based on the enzymatic hydrolysis results were selected for this set. The residence time was set at 5 or 15 minutes for each experiment of this set. The first step of AFEX pretreatment of this set was performed at the ammonia-to-wheat straw ratio of 1:1, temperature of 95 °C, moisture content of 70 %, and residence time of 5 minutes. The second step was performed similar to the first step, but the residence time was increased to 15 minutes. In steps 3 and 4, the AFEX pretreatment experiments were carried out at the temperature of 95 °C, moisture content of 80 %, ammonia to wheat

straw ratio of 1:1, and residence times of 5 and 15 minutes respectively. In steps 5 and 6, the temperature was increased to 105 °C, the moisture content and ammonia to wheat straw ratio were fixed at 70 % and 1:1, and residence time was set at 5 and 15 minutes respectively. The experiments were performed in duplicate.

Table 3-6. The fourth set of AFEX pretreatment conditions for investigating the influence of residence time on released sugars yield of wheat straw.

Step	Residence time (minute)	Moisture	Ammonia to wheat straw ratio	Temperature
1	5	70%	1:1	95 °C
2	15	70%	1:1	95 °C
3	5	80%	1:1	95 °C
4	15	80%	1:1	95 °C
5	5	70%	1:1	105 °C
6	15	70%	1:1	105 °C

3.5 Enzymatic hydrolysis of AFEX-treated wheat straw

Enzymatic hydrolysis was carried out on AFEX-treated and untreated wheat straw to measure the effectiveness of the AFEX pretreatment. In this series of experiments, a modified form of the NREL protocol (LAP-009) was followed (Appendix B). The procedure (LAP-009) was modified using the recommended conditions (pH, temperature, and enzymes dosages) published by Novozymes Company for its new generation of enzymes. Recommended conditions can be found in Table 3-1. The new generation Novozymes enzymes used in enzymatic hydrolysis were enzymes NS22086 and NS22118.

Two grams of oven-dried wheat straw were placed in 250-ml Erlenmeyer flasks with 50 ml of sodium citrate (pH 5.25), 1 ml of sodium azide solution (2 wt. %) for sterilizing and distilled water to 100 ml. All flasks were sealed with stoppers and placed in a rotary incubator (New Brunswick, model INNOVA 40) at 47.5 ºC and 68 rpm (Appendix B). A 250-ml Erlenmeyer flask containing distilled water and a thermometer was also placed in the rotary incubator for reading the solution temperature. After raising the temperature of solutions to 47.5 ºC, 87 µl of cellulase mixture (NS 22086) equal to 5 % wt. /wt. (total solids) and 10 µl of β-glucosidase (NS 22118) equal to 0.6 % wt. /wt. (total solids) were added (Appendix B). All the Erlenmeyer flasks were again tightly closed; and the time was recorded as start point of enzymatic hydrolysis. Aliquots of 1.5 ml were taken using a 5.0 ml pipette with a cut plastic tip (in order to prevent clogging with wheat straw) at 2, 5, 20, 44, 68, 92, 116, 140 and 164 hours. These samples were placed in a 1.5 ml micro-centrifuge tube and centrifuged for about 2 minutes. A volume of 1.0 ml of the supernatant was analyzed for released sugars (glucose equivalents) with the DNS method (Appendix B). All enzymatic hydrolysis experiments were run in duplicate.

The yield of released sugars was calculated from:

$$Yield\ of\ released\ sugars\ \ (\%\ theoretical) = \frac{[Experimental\ released\ sugars\ \left(\frac{mg}{ml}\right)]}{[Theoretical\ released\ sugars\ \left(\frac{mg}{ml}\right)]} \times 100$$

The theoretical released sugars concentration is the amount of sugars that is expected to be released during the complete hydrolysis of hemicellulose and cellulose. The concentration of theoretical released sugars was calculated according to Appendix B.

CHAPTER 4

RESULTS AND DISCUSSION

The appearance of wheat straw, before and after AFEX pretreatment, and AFEX-treated and untreated after 164 hours of enzymatic hydrolysis, was evaluated to observe any possible physical changes in wheat straw appearance. Also, the AFEX pretreatment variables (ammonia-to-wheat straw ratio, moisture content, temperature, and residence time) were evaluated to determine the AFEX pretreatment conditions that result in maximum enzymatic hydrolysis. The effects of each parameter on the released sugars yield of wheat straw will be illustrated in this chapter.

4.1 Effect of AFEX pretreatment and enzymatic hydrolysis on appearance of wheat straw

Figure 4-1 shows the appearance of wheat straw before and after AFEX pretreatment. The only observable change in the macroscopic appearance of the AFEX-treated wheat straw was an increased darkness of wheat straw color with respect to untreated wheat straw. No information was found in the literature regarding the mechanism of this color change. However, it was observed that the darkening of color was increased by increasing the amount of ammonia and temperature.

(A) Untreated wheat straw (B) AFEX-treated wheat straw

Figure 4-1. Wheat straw prior to enzymatic hydrolysis.

Figure 4-2 shows untreated and AFEX-treated wheat straw after 164 hours of enzymatic hydrolysis. Enzymatic hydrolysis of AFEX-treated wheat straw resulted in a slurry-like mixture, while the structure of untreated wheat straw remained relatively immune to hydrolysis. The AFEX pretreatment is able to partially prehydrolyse hemicellulose and soluble lignin (Wang et al., 1967). Thus, lignin and hemicellulose could be transferred from wheat straw to the liquid portion in an enzymatic hydrolysis step. Additionally, cellulose was mostly hydrolysed by enzymes NS22086 and NS2218 and consequently, simple sugars were released into the liquid portion.

The slurry-like mixture that resulted after enzymatic hydrolysis of AFEX-treated wheat straw (Figure 4-2B) has some advantages such as easy materials handling compared to untreated wheat straw.

(A) Untreated wheat straw (B) AFEX-treated wheat straw

Figure 4-2. Wheat straw after 164 hours of hydrolysis with enzymes NS22086 and NS2218 at 47.5 ºC.

4.2 Controlling the reactor temperature

Figure 4-3 shows the overshoot of the Parr temperature controller at various set points. As this figure shows, controlling the reactor temperature by defining the final temperature as the set point for controller could be very difficult and inaccurate due to a wide range of overshoots from 3 ºC to 25 ºC for temperatures from 45 ºC to 125 ºC. Also, any effort performed by previous researchers in our lab for attaining an approximately constant temperature for reactor was useless. However, the proposed method in this research showed more accurate results compared to the previous works for controlling the reactor temperature using Parr temperature controller.

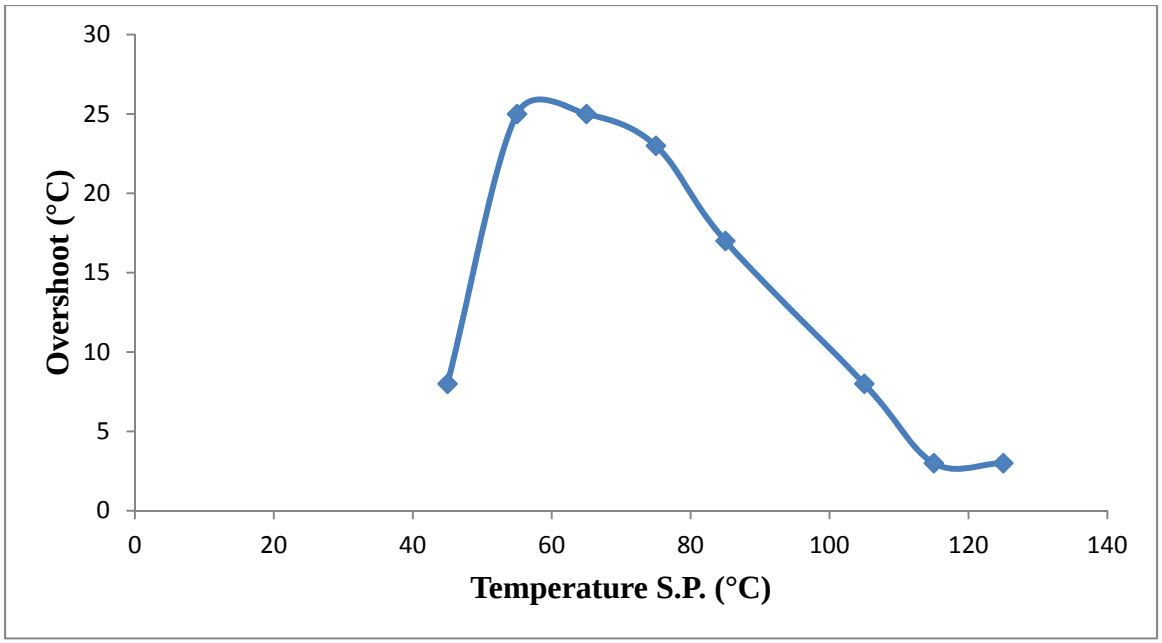

Figure 4-3. Overshoot of the Parr temperature controller at various set points (S.P.).

Figure 4-4 shows the reactor temperature against time for target temperature of 95 ºC using the proposed method in this research. As this figure shows, the target temperature of 95 ºC was obtained by an overshoot only about ±1 ºC. The temperature of 95 ºC was randomly selected for demonstrating the accuracy of the proposed method. However, other reactor temperatures including 75 ºC, 85 ºC, 105 ºC, and 115 ºC were also tested and resulted in a similar trend.

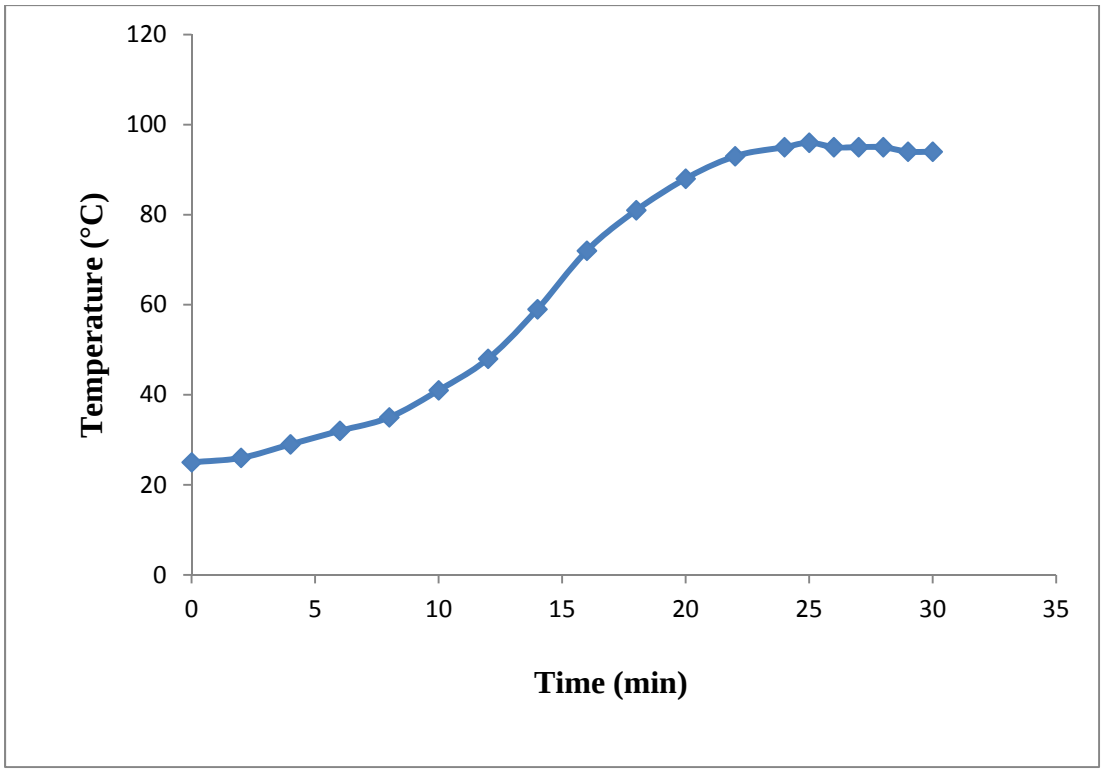

Figure 4-4. Reactor temperature against time using Parr temperature controller for target temperature of 95 ºC.

4.3 Hydrolysis of wheat straw

Figure 4-5 shows the glucose standard curve that was used to determine the amount of sugars released from the hydrolysis of wheat straw. The description of constructing this standard curve using the DNS method, the table of data, and statistics can be seen in Appendix B.

The absorbance for released sugars from enzymatic hydrolysis was read at wavelength 540 nm and assumed equivalent to the absorbance for glucose sugar in order to simplify the concentration measurement of released sugars using a glucose standard

curve. Then, the released sugars concentration (equivalent to glucose concentration) was calculated using equation (4-1).

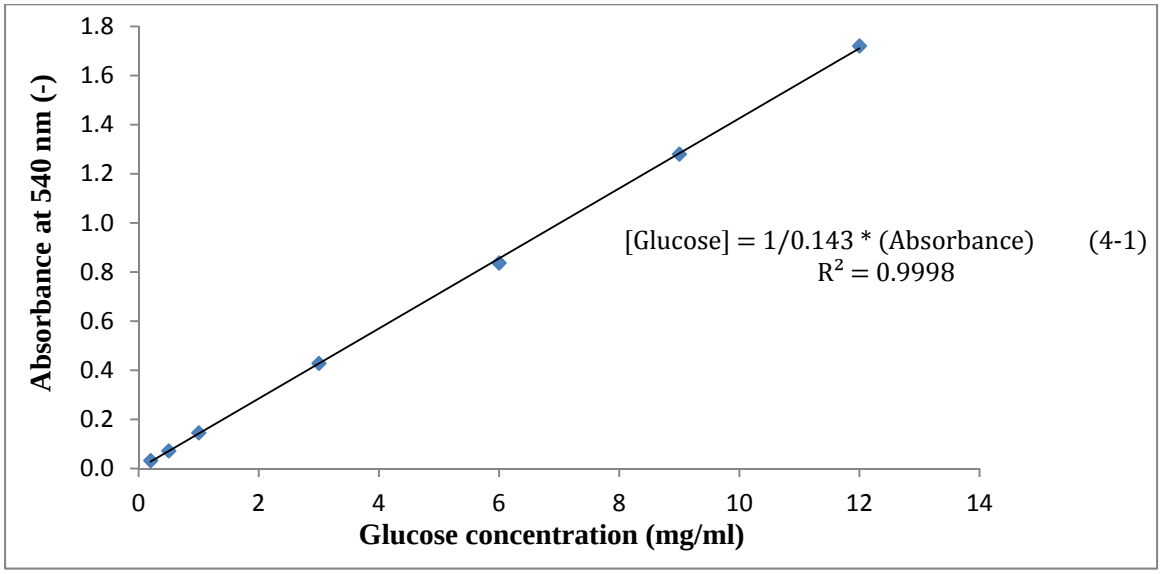

Figure 4-5. Glucose standard curve (using DNS method) for measurement of released sugars concentration.

The data for the duplicate runs of enzymatic hydrolysis of untreated and AFEX-treated wheat straw at a fixed ammonia-to-wheat straw ratio (1:1), moisture content (50 %, and 70 % dry weight basis), and temperature (85 °C, and 95 °C) are presented in Table B-2 (Appendix B). The method of obtaining released sugars (glucose equivalent) concentration and sample of calculation can be seen in Appendix B and Appendix D respectively. Figure 4-6 illustrates the release of sugars from AFEX-treated and untreated wheat straw during its enzymatic hydrolysis. Some of the AFEX pretreatment conditions were randomly selected from set 1 (Table 3-3) to investigate release of sugars across enzymatic hydrolysis time.

The curve for untreated wheat straw has a shape more or less similar to the other curves for AFEX-treated wheat straw. However, the maximum released sugars concentration for untreated wheat straw was only about 3 mg/ml, which was the lowest concentration of released sugars compared to AFEX-treated wheat straw. This low released sugars concentration was predictable for untreated wheat straw. Since cellulose is surrounded with layers of hemicellulose and lignin that are strongly cross-linked, enzymes do not have easy access to cellulose (Wyman et al., 1996). Cellulose crystallinity in untreated wheat straw is another barrier for enzymes (Lamptey et al., 1986).

All profiles show the higher rate of increasing released sugars concentration within the first 20 hours of enzymatic hydrolysis time. This occurs due to the fact that enzymes could have easier access to the cell wall polysaccharides (cellulose and hemicellulose) that are closer to the surface of the wheat straw. After 20 hours, the rate of released sugars concentration decreased because the enzymes had more difficulty reaching the internal polysaccharides of the wheat straw cell walls.

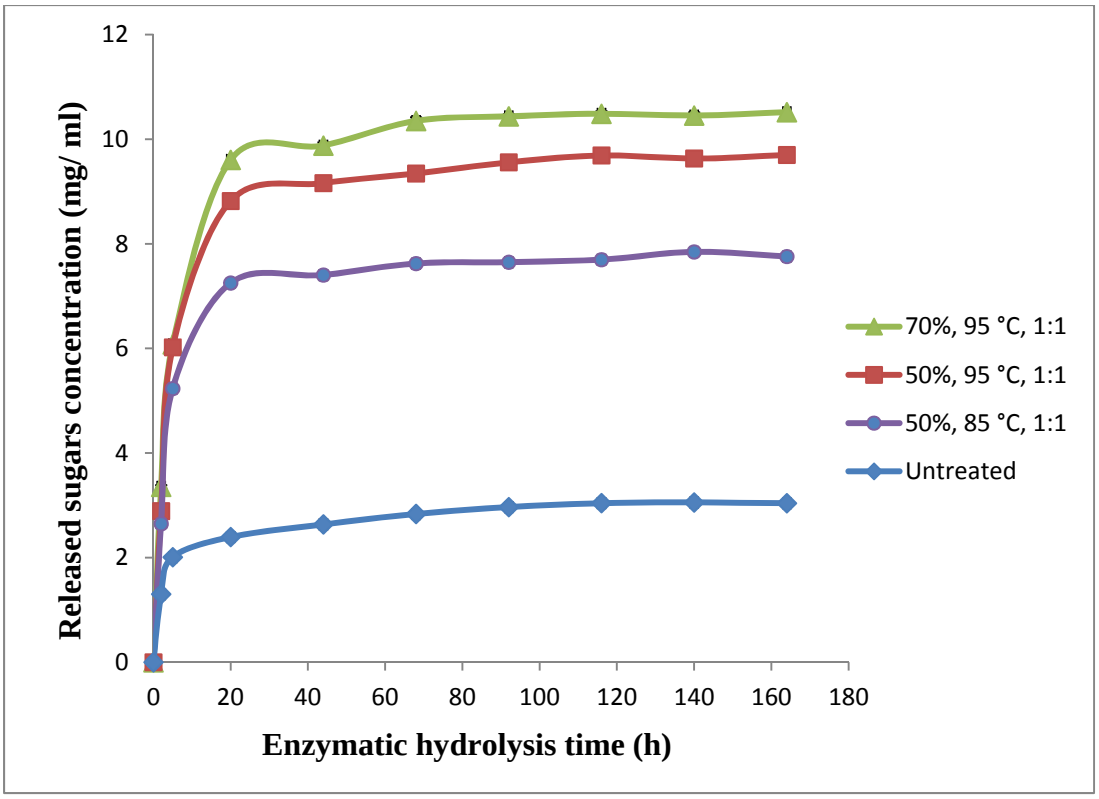

Figure 4-6. Released sugars concentrations from enzymatic hydrolysis of AFEX-treated and untreated wheat straw against enzymatic hydrolysis time at wheat straw moisture contents of 70 % and 50 % (dry weight basis), and AFEX reactor temperatures of 95 °C and 85 °C, and ammonia to wheat straw ratio of 1:1 after 164 h of hydrolysis.

There are various sources of error that might affect the values obtained for released sugars concentration. One source could be the assumption of having an equivalent absorbance for released sugars from enzymatic hydrolysis and glucose sugar at a wavelength of 540 nm. However, various sugars could have different absorbance than glucose absorbance and the assumption was considered to simplify the measurement of released sugars using the DNS method. The other source is the measurement of released sugars at high concentrations due to the chance of increased error at high glucose concentrations in the glucose standard curve. Another source of error could be the use of

wheat straw composition from Agblevor et al. (1993) to simplify the calculation of theoretical released sugars concentration for calculating the yield of released sugars.

Based on the Figure 4-6, the maximum sugar content was released after 164 hours of enzymatic hydrolysis. However, a large amount of sugars (more than 98 % of sugars released after 164 hours of enzymatic hydrolysis) was released after only 92 hours of hydrolysis. Thus, the yield of released sugars by AFEX treatment of wheat straw was reported in the next section after 92 hours of hydrolysis.

4.4 Influence of AFEX conditions on released sugars yield enzymatic hydrolysis of wheat straw

4.4.1 Liquid ammonia to wheat straw ratio

Figure 4-7 shows how the liquid ammonia to wheat straw ratio influences the yield of released sugars for an AFEX pretreatment at 85 °C and 95 °C with a 50 % moisture content (dry weight basis) of the straw, and after 92 hours of enzymatic hydrolysis. The AFEX pretreatment conditions can be seen in steps 1 and 2, Table 3-3.

Evaluating the effect of the ammonia-to-wheat straw ratio was the main focus of this section, thus, the temperatures and moisture contents were randomly selected. At 85 °C, increasing the ammonia-to-wheat straw ratio from 0.5:1 to 1:1 resulted in an increase of released sugars yield of 49 % to 65 %. Increasing the pretreatment temperature to 95 °C resulted in a similar trend, but the released sugars yield increased from 58 % to 82 % at the ratio of 1:1. Further increasing the ratio from 1:1 to 1.5:1 resulted in decreasing released sugars yields to 57 % and 73 % at temperatures 85 °C and 95 °C respectively.

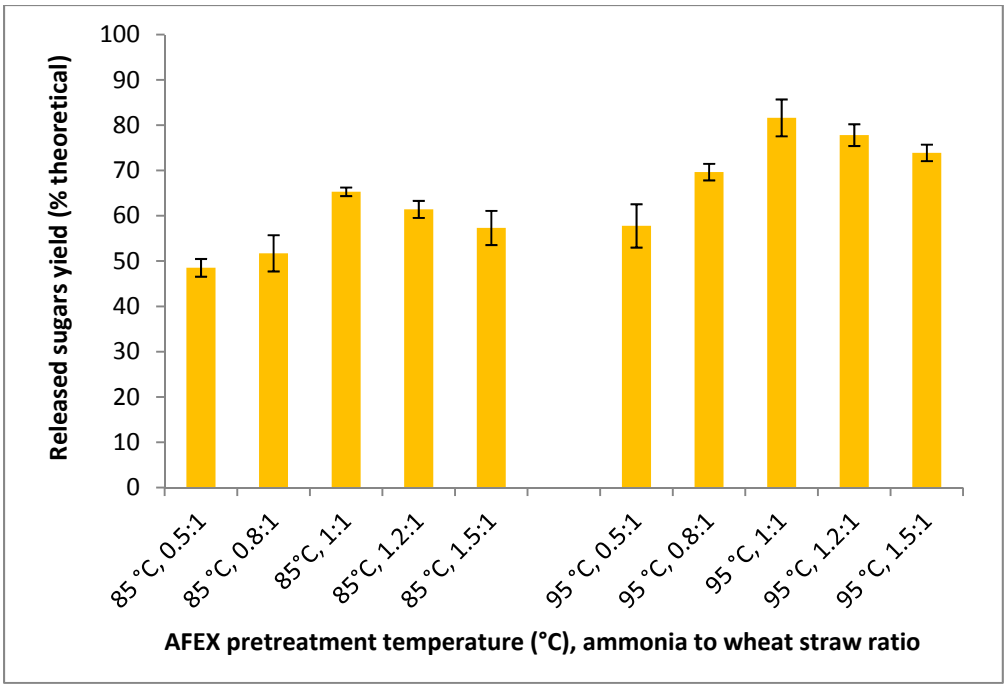

Figure 4-7. Released sugars yield for AFEX treatment of wheat straw at 50% moisture content (dry weight basis) after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability.

As Figure 4-8 shows, increasing the moisture content from 50 % to 70 % resulted in a similar trend for both temperatures 85 °C and 95 °C. However, the maximum yields of released sugars increased up to 67 % and 89 %. In addition, the released sugars yields decreased to 60 % and 77 % when the ratios increased from 1:1 to 1.5:1. The maximum yield of released sugars from untreated wheat straw was only 26 %. The AFEX pretreatment conditions can be seen in steps 3 and 4, Table 3-3.

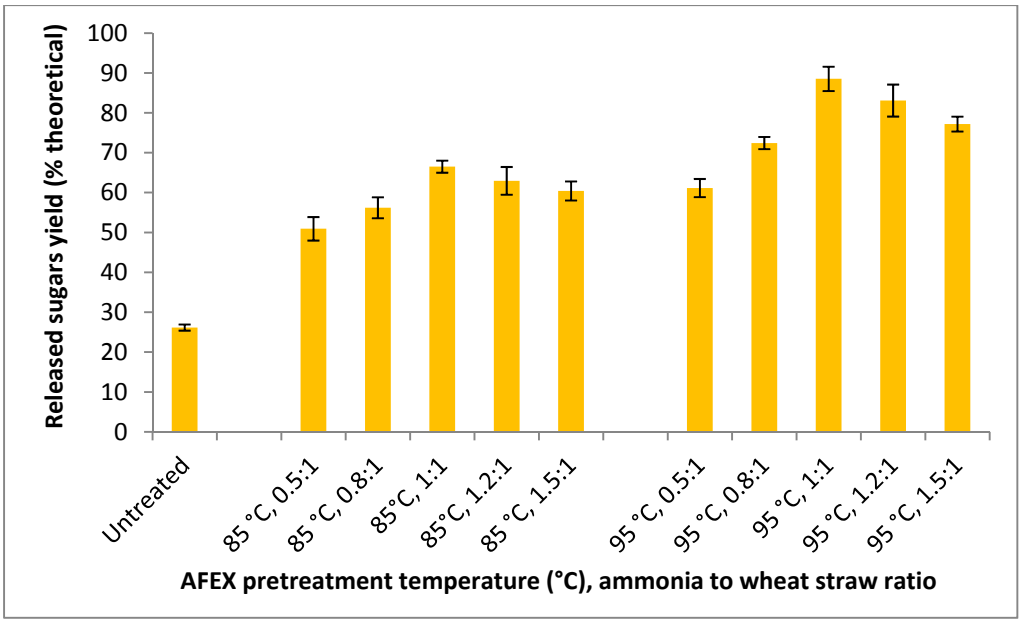

Figure 4-8. Released sugars yield for AFEX treatment of wheat straw at 70 % moisture content (dry weight basis) after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability.

It can be concluded that the released sugar yield is maximum at a ratio of 1:1. Other researchers (Alizadeh et al., 2005; Teymouri et al., 2004; Moniruzzaman et al., 1997) identified the same ratio of ammonia to biomass (1:1) as the optimum value for AFEX pretreatment of lignocellulosic materials such as switchgrass and corn stover.

A further increase of the ammonia-to-wheat straw ratio has an inverse influence and decreases the released sugars yield. It seems that an extra amount of ammonia could plasticize the cellulose fibers (O'Conner, 1972). That is, the cellulose fibers could soften and cellulose chains flow past one another when subjected to pressure (Schuerch, 1963). As a result, the disruptive effect of a rapid release of pressure decreases. Thus, it can be concluded that the ammonia-to-wheat straw ratio of 1:1 seems the best ratio of ammonia-to-wheat straw for AFEX pretreatment of wheat straw. Obviously, performing AFEX

pretreatments at this ratio could more effectively decrease ammonia consumption compared to the ratios of 1.2:1, and 1.5:1. The influence of various moisture contents on released sugar yield was also investigated at this ammonia-to-wheat straw ratio.

4.4.2 Moisture content of wheat straw

The effects of various moisture contents of wheat straw including 30 %, 50 %, 70 %, 80 %, 90 % (dry weight basis) on the released sugars yield for AFEX treatment of wheat straw at temperature of 95 °C and ammonia loading ratio of 1:1 after 92 h of hydrolysis are shown in Figure 4-9. The AFEX pretreatment conditions can be seen in Table 3-4. Increasing the moisture content from 30 % to 70 % resulted in an increased yield of released sugars from 62 % to 89 %. Ammonia reacts with the moisture of wheat straw and produces ammonium hydroxide (NH_4OH). Thus, alkaline hydrolysis of hemicellulose during the AFEX pretreatment could occur and influence the released sugar yield (Dale et al., 1985).

As these data show, at higher moisture contents from 70 % to 90 %, the released sugars yield tends to decrease (from 89 % to 85 %). It seems to indicate that ammonia is more diluted at higher moisture contents and the ability of ammonia to react with wheat straw is reduced. Thus, it can be concluded that the moisture content of 70 % seems the best value for moisture content for AFEX pretreatment of wheat straw. The influence of various temperatures on the released sugar yield was also investigated at this moisture content and ammonia to wheat straw ratio of 1:1.

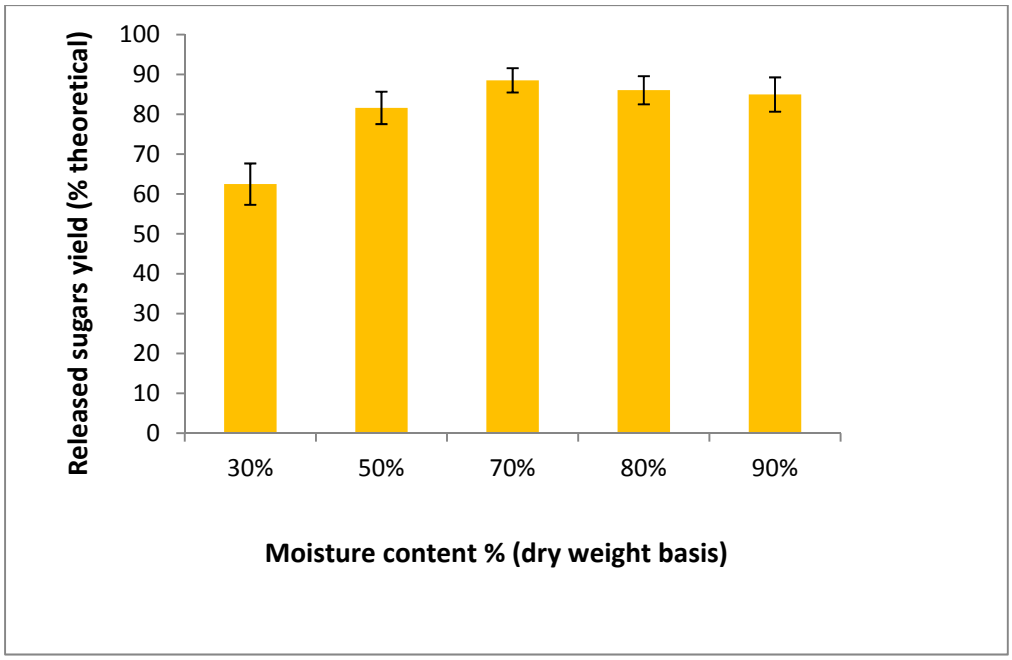

Figure 4-9. Released sugars yield for AFEX pretreatment of wheat straw at temperature of 95 °C and ammonia-to-wheat straw ratio of 1:1 after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability.

4.4.3 Reactor temperature

Figure 4-10 shows the effects of various reactor temperatures including 75 °C, 85 °C, 95 °C, 105 °C, and 115 °C on the released sugars yield for AFEX treatment of wheat straw at an ammonia-to-wheat straw ratio of 1:1 and wheat straw moisture content of 70% (dry weight basis) after 92 h of hydrolysis. The AFEX pretreatment conditions can be seen in Table 3-5. Increasing the temperature from 75 °C to 95 °C resulted in an increased yield of released sugars from 53 % to 89 %. Temperature can accelerate some chemical reactions such as hydrolysis of hemicellulose and increase the released sugars yield in a shorter pretreatment time (Teymouri et al., 2004). However, increasing the temperature from 95 °C to 115 °C resulted in a decline of released sugars yield from 89 % to 79 %. It seems that at temperatures higher than 95 °C, some polysaccharides may

degrade and consequently decrease the yield of released sugars. Thus, the temperature of 95 °C seems the best AFEX pretreatment temperature for wheat straw. Obviously, AFEX pretreatment at this temperature could save more electrical energy compared to higher temperatures (105 °C, and 115 °C). The influence of this temperature on released sugar yield was also investigated at residence time of 5 and 15 minutes.

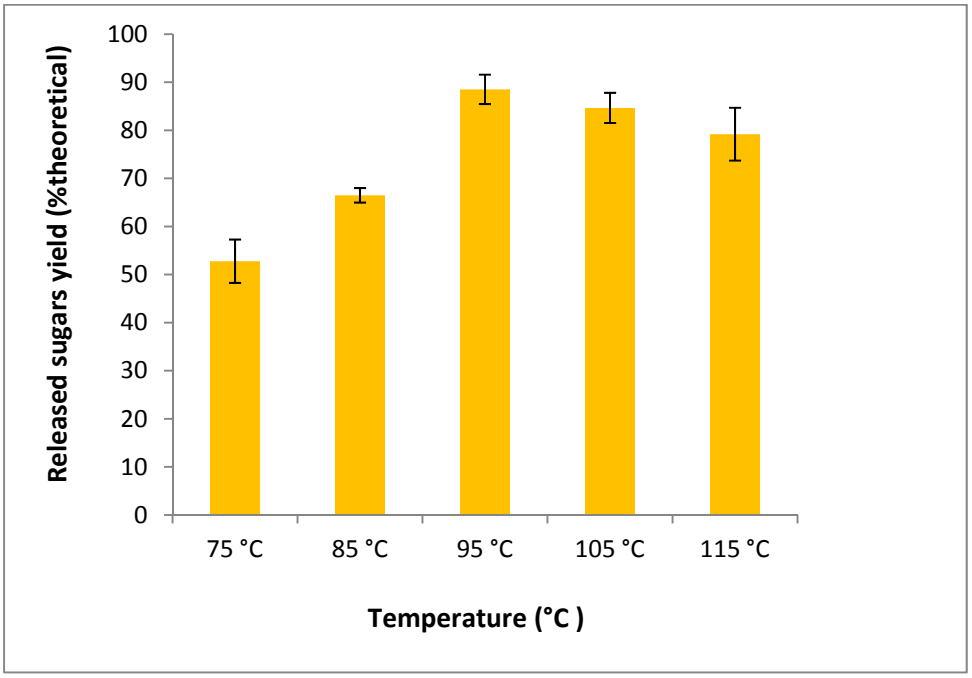

Figure 4-10. Released sugars yield for AFEX pretreatment of wheat straw at ammonia-to-wheat straw ratio of 1:1, and wheat straw moisture content of 70 % (dry weight basis) after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability.

4.4.4 Residence time

To investigate the effect of residence time, a series of experiments was performed under the best AFEX pretreatment conditions of: ammonia to wheat straw ratio of 1:1, temperature of 95 °C and 105 °C, and wheat straw moisture content of 70 % and 80 %

(dry weight basis). Both 5 and 15 minutes were studied. The AFEX pretreatment conditions can be seen in Table 3-6.

Figure 4-11 shows for each pair of residence times, the longer residence time of 15 minutes resulted in 9 % to 4 % less yield than its 5 minute counterpart. Data in this figure illustrate a decline in residence time longer than 5 minutes. A longer residence time may degrade more polysaccharides because of overheating of straw particles close to the reactor walls, or more cellulose fibres plasticizing because of longer contact of ammonia to wheat straw. Thus, the residence time of 5 minutes seems the best residence time for AFEX pretreatment of wheat straw. AFEX pretreatment at this residence time also could save more time and electrical energy compared to the 15 minute residence time.

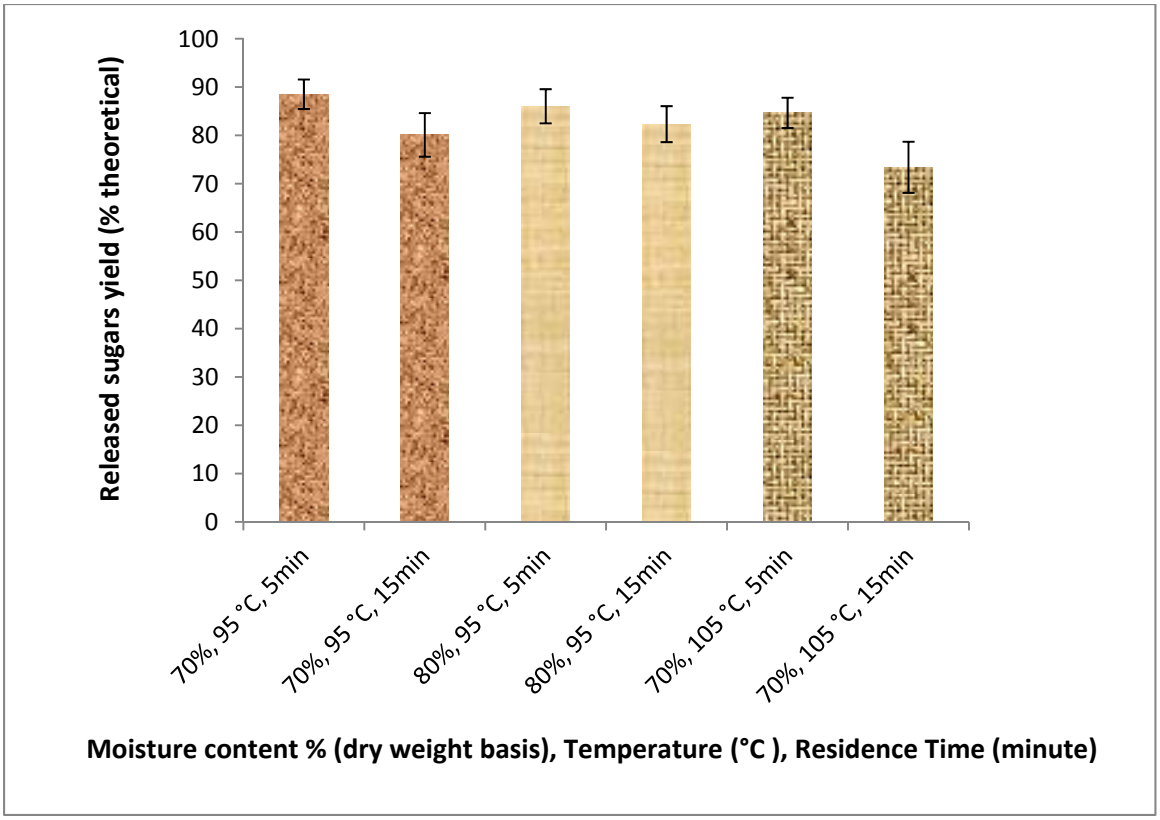

Figure 4-11. Released sugars yield of AFEX-treated wheat straw at ammonia loading ratio of 1:1, wheat straw moisture content of 70 % and 80 % (dry weight basis) and reactor temperatures of 95 °C and 105 °C after 92 h of hydrolysis. Vertical bars indicate the confidence intervals at 95 % probability.

It can be concluded that the released sugars concentration for AFEX-treated wheat straw under the best AFEX pretreatment conditions (ammonia-to-wheat straw ratio, 1:1; wheat straw moisture content, 70 %; temperature, 95 °C) reached 89 % after 92 hours of enzymatic hydrolysis, compared to only 26 % for untreated wheat straw. This shows the importance of AFEX pretreatment for increasing the released sugars yield. Additionally, despite using an equal amount of enzymes for all AFEX-treated wheat straw, AFEX pretreatment under pretreatment conditions other than the best AFEX pretreatment conditions (ammonia to wheat straw ratio, 1:1; wheat straw moisture content, 70 %; temperature, 95 °C) resulted in lower released sugars yield, compared to

the best AFEX pretreatment conditions. This indicates the importance of evaluating the AFEX variables for determining the AFEX pretreatment conditions that result in maximum released sugars yield in order to use enzymes efficiently.

CHAPTER 5

CONCLUSION

The most significant findings and conclusions of this research are:

1. An increased darkness of wheat straw color was the only observable change in AFEX-treated wheat straw appearance compared to untreated wheat straw.
2. After enzymatic hydrolysis of AFEX-treated and untreated wheat straw, the AFEX-treated wheat straw could be handled easier than untreated wheat straw due to formation of a slurry mixture.
3. The maximum yield of sugars released during enzymatic hydrolysis could be measured after 92 hours of hydrolysis.
4. All AFEX-treated wheat straw showed a consistently higher yield of released sugars than the untreated wheat straw.
5. The highest yield of released sugars was obtained with an ammonia-to-wheat straw ratio of 1:1, a temperature of 95°C, a wheat straw moisture content of 70% (dry weight basis), and a residence time at the target temperature for 5 minutes.
6. The enzymatic hydrolysis of AFEX-treated wheat straw under these conditions resulted in an 89% yield of released sugars versus only 26% released sugars yield for untreated wheat straw.
7. The set of conditions achieved for AFEX pretreatment of wheat straw are comparable with those for corn stover by Teymouri et al. (2004), and switchgrass by Alizadeh et al. (2005). Therefore, the designed AFEX treatment worked satisfactorily for wheat straw.

8. The data obtained for the best AFEX pretreatment conditions in this research could fill the current gap for production of biofuel from wheat straw using AFEX pretreatment process by making ammonia, enzyme, and energy consumption more efficient. This achievement could contribute to make production of bioethanol from agricultural residues and lignocellulosic waste materials technically and economically feasible.

FUTURE WORK AND RECOMMENDATIONS

In this research, wheat straw was considered as an appropriate lignocellulosic biomass material for production of bioethanol in Canada. In addition, AFEX treatment was recognized as a proper method for enhancing the enzymatic hydrolysis yield of wheat straw. However, this effort has the potential to be extended in the following ways:

- The best AFEX treatment parameters have been concluded based on the enzymatic hydrolysis yield, not the ethanol production yield; thus, more experimental work is needed to investigate the effect of these parameters on the yield of ethanol production from wheat straw.

- Bioethanol is a growing industry in Canada; thus, investigating the effect of AFEX pretreatment on the enzymatic hydrolysis yield from other types of cheap and abundant agricultural residues such as flax stalk, barley, and rye straw can enhance production of bioethanol in Canada.

- Due to some equipment limitations, the amount of released sugars from cellulose and hemicellulose were measured together. Therefore, more experiments are needed to measure the amount of released sugars from each polysaccharide separately to explore the effects of AFEX variables on each polysaccharide alone.

- So far, the AFEX treatment has not been applied to large scale bioethanol production in Canada; thus, the economic feasibility of this technique deserves to be investigated as well as the energy return on investment.

- The cost of enzymes used in the enzymatic hydrolysis step is high, and negatively affects the operating cost of bioethanol production from lignocellulosic materials. The dosage of enzymes used in this study was based on the recommended dosages by Novozymes Company for all types of pretreatments and lignocellulosic materials. Therefore, optimization of enzymatic hydrolysis conditions including enzyme loading, temperature, and pH can decrease the overall cost of process through reducing enzyme consumption.

APPENDICES

Appendix A: National Renewable Energy Laboratory protocol (LAP # 001) for total solids determination in wheat straw (NREL, 1995)

1) A predried aluminum foil weighing dish was precisely weighed, to the nearest 0.1 mg; and the weight is recorded.

2) Into the aluminium weighing dish 2 grams (to the nearest 0.1 mg) of wheat straw was weighed out; and the total weight (weight of aluminium dish and sample together) was recorded.

3) The sample was placed into a convection oven at 105 ºC ± 3 ºC and dried to constant weight (± 0.1 % change in the amount of moisture present upon one hour of reheating).

4) The sample was removed from the oven and placed in a desiccator to be cooled to room temperature.

5) The aluminium dish containing the oven-dried sample was weighed, to the nearest 0.1 mg, and the weight was recorded.

Calculations

The percent of total solids on a 105 ºC dry weight basis was calculated as follows:

$$\%Total\ solids\ = \frac{dried\ sample\ weight}{received\ sample\ wight} \times 100 \qquad (\mathrm{A}-1)$$

The percent moisture of wheat straw was also calculated as follows:

$$\%Moisture\ content\ of\ wheat\ straw\ = 100 - \%\ Total\ solids \qquad (A-2)$$

In appendix D, an example of the calculation of total solids and moisture content is presented. The calculated standard deviation, means, confidence functions, and the relative percent differences for total solids and moisture content of untreated and AFEX-treated samples are presented in Table A-1and A-2 respectively. The standard deviation (SD) for total solids and moisture content was calculated using equation (A-3).

$$SD = \sqrt{\frac{\sum(x - \bar{x}\,)^2}{n-1}} \qquad (A-3)$$

The confidence function (CF) for total solids and moisture content was calculated using the equation (A-4).

$$CF = 1.96 \times \left(\frac{SD}{\sqrt{n}}\right) \qquad (A-4)$$

In calculations, n (number of replicates) was considered equal to 2.

Relative Percent Difference (% RPD) was calculated using the equation (A-5).

$$\%\ RPD = \frac{|x_1 - x_2|}{\frac{(x_1 + x_2)}{2}} \times 100 \qquad (A-5)$$

The values x_1 and x_2 are the values of total solids percentage determined in replicate 1 and 2 respectively.

Note:

According to National Renewable Energy Laboratory protocol (LAP-001), if Relative Percent Difference (% RPD) for duplicate runs of determining total solids percentage was higher than 1.1 % then the experiment must be repeated.

Table A-1. Total solids and moisture content of untreated wheat straw

	Replicate 1	Replicate 2	Mean ± CF*	SD**	%RPD***
Weight of received wheat straw (g)	2.000	2.000	-	-	-
Weight of dry wheat straw (g)	1.844	1.848	-	-	-
Total solids (wt. %)	92.21	92.44	92.33 ± 0.22	0.16	0.25
Moisture content (wt. %)	7.79	7.56	7.68 ± 0.22	0.16	-

*CF: Confidence Function at 95 % probability

**SD: Standard Deviation

***RPD: Relative Percent Difference

Table A-2. Percent total solids and moisture content for AFEX-treated wheat straw produced under following AFEX conditions: temperature 95 °C, ammonia-to-wheat straw ratio 0.5:1, moisture content 70%

	Replicate 1	Replicate 2	Mean ± CF*	SD**	%RPD***
Weight of treated wheat straw (g)	2.000	2.000	-	-	-
Weight of dry wheat straw (g)	1.871	1.865	-	-	-
Total solids (wt. %)	93.55	93.25	93.40 ± 0.29	0.21	0.32
Moisture content (wt. %)	6.45	6.75	6.60 ± 0.29	0.21	-

*CF: Confidence Function at 95 % probability

**SD: Standard Deviation

***RPD: Relative Percent Difference

Appendix B: Enzymatic hydrolysis

B.1 A modified National Renewable Energy Laboratory protocol (LAP # 009) for enzymatic hydrolysis of wheat straw (NREL, 1995)

Materials

Citrate Buffer

In this research, enzymatic hydrolysis was performed at pH 5.20 using citrate buffer solution (0.1 M).

First, a stock solution of citrate buffer (1.0 M) was prepared. Citric acid monohydrate (210 g) was dissolved in 750 ml of distilled water. The pH of solution was adjusted to 4.3 by using 50-60 g of sodium hydroxide (NaOH). Then, 100 ml of this stock solution (1.0 M) was diluted to 1000 ml using distilled water. By using sodium hydroxide (NaOH), the pH of this solution was adjusted to 5.20.

Enzymatic hydrolysis procedure

For enzymatic hydrolysis, the total solids of all wheat straw samples were determined using LAP # 001 (described in Appendix A).

a) Two grams of AFEX-treated wheat straw was accurately weighed out and added to an Erlenmeyer flask (250 ml). Same amount of each AFEX-treated wheat straw sample was also added to a separate Erlenmeyer flask for using as a buffer blank in each enzymatic hydrolysis test. Each hydrolysis Erlenmeyer flask and relevant buffer blank Erlenmeyer flask was labeled. Each blank used as a reference when measuring the released sugars concentration. Each buffer blank solution used as a reference in

spectrophotometry step for measuring the absorbance and concentration of released sugars.

b) Another 250 ml Erlenmeyer flask considered as enzyme blank. For precise results, the amount of concentration measured for the enzyme blank need to be subtracted from released sugars concentration of each enzymatic hydrolysis test.

c) An amount of 50 ml of sodium citrate buffer solution (0.1 M) with pH 5.25 and 1 ml of sodium azide (2 wt. %) for sterilizing solution added to each Erlenmeyer flask.

d) The total volume of each Erlenmeyer flask was brought to 100 ml by adding 47 ml of distilled water.

Note:

In order to simplify the calculations, two assumptions considered.

1) Neglecting the volume of added enzymes because it was too small.

2) Density of 1.000 g/ml for all solutions and the wheat straw.

e) All the flacks were sealed with stoppers and placed in a rotary incubator and the temperature set at 47.5 ºC. After reaching the temperature of 47.5 ± 1 ºC, the recommended enzymes volumes including the cellulase enzyme (NS 22086) equal to 5 % wt. /wt. (total solids) and β-glucosidase enzyme (NS 22118) equal to 0.6 % wt. /wt. (total solids) were added to each enzymatic hydrolysis Erlenmeyer flask and to the enzyme blank flask.

According to Table 3-1, the density of cellulase enzyme (NS22086) and β-glucosidase (NS22118) is equal to 1.15 and 1.20 mg/µl respectively. Therefore, the required amount of enzymes was calculated as follows.

For cellulase enzyme (NS22086):

$$Cellulase = 0.05 \frac{mg\ of\ enzyme}{mg\ of\ total\ solid} \times 2000\ mg \times \frac{\mu l}{1.15\ mg} = 87\ \mu l$$

For β-glucosidase enzyme (NS22118):

$$\beta - glucosidase = 0.006 \frac{mg\ of\ enzyme}{mg\ of\ total\ solid} \times 2000\ mg \times \frac{\mu l}{1.20\ mg} = 10\ \mu l$$

Note:

1) Enzymes should not be added to the buffer blank Erlenmeyer flasks.
2) The enzymatic hydrolysis time is started as soon as adding enzymes to the flasks.

f) All the Erlenmeyer flasks were tightly closed and incubated with gentle rotation of 68 rpm and 47.5 ºC. The incubation continued until the released sugars concentration become nearly constant.

g) A 1.5 ml aliquot was taken (using a 5.0 ml pipette with a cut plastic tip in order to prevent clogging with wheat straw) at each predetermined time interval, for example 2, 5, 20, 44, 68, 92, 116, 140 and 164 hours. The sample was placed in a 1.5 ml micro-centrifuge and centrifuged for about 2 minutes. A volume of 1.0 ml of the supernatant was taken for analysis of released sugars concentration with DNS method.

B.2 Determination of released sugars by DNS (dinitrosalicylic acid) reagent (Ghose, 1987)

In order to determination of released sugars, the DNS reagent solution and glucose standard solutions were prepared according to Ghose (1987) procedure.

To prepare the DNS reagent solution, the amounts of 10.6 g of 3, 5 Dinitrosalicylic acid and 19.8 g of sodium hydroxide must be properly dissolved in distilled water. Then, 306 g of sodium potassium tartrate (Rochelle salts), 7.6 ml of phenol, and 8.3 g of sodium metabisulfite is respectively added to the solution. The final volume of solution should be reached to 1000 ml by using distilled water. An amount of 3 ml sample must be titrated by HCl (0.1 N) to the phenolphthalein endpoint. It must use 5-6 ml of HCl. sodium hydroxide (NaOH) will be add if required.

Glucose standard solutions

First, we prepared an anhydrous glucose stock solution (12 mg/ml). Then, we sealed and stored the aliquots of stock solution in the freezer. Before using standard solution, we thawed and vortex it to ensure proper mixing.

To prepare glucose dilutions, stock solution should be diluted in the following order:

1.0 ml + 0.0 ml citrate buffer = 1:1 (12.0 mg/ml)

1.0 ml + 0.33 ml citrate buffer = 1:1.33 (9.0 mg/ml)

1.0 ml + 1.0 ml citrate buffer = 1:2 (6.0 mg/ml)

1.0 ml + 3.0 ml citrate buffer = 1:4 (3.0 mg/ml)

1.0 ml + 11.0 ml citrate buffer = 1:12 (1.0 mg/ml)

1.0 ml + 23.0 ml citrate buffer = 1:24 (0.5 mg/ml)

1.0 ml + 59.0 ml citrate buffer = 1:60 (0.2 mg/ml)

After preparing glucose dilution, we take 1.0 ml of each dilution and transfer to a test tube (13 x 100 mm).

Development of color

a) Volumes of 1.0 ml of the supernatants from hydrolysis flasks, buffer blank flasks, and enzyme blank flasks were taken and put in test tubes (13 x 100 mm).

b) An amount of 3.0 ml of DNS reagent solution was added to each test tube containing hydrolysis sample, buffer blank, enzyme blank, and glucose standard.

c) In a water bath, the water was boiled first; and then all test tubes (assay, control, blank, and glucose standard) were placed in the boiling water for exact time of 5.0 minutes. The water in the water bath was enough to cover the portion of test tubes, which contain solution. After boiling for 5.0 minutes, test tubes were suddenly cooled down by transferring to a cold water bath containing ice.

d) All test tubes containing assay, control, blank, and glucose standard solutions were diluted using distilled water (0.200 ml of each solution with 2.5 ml of distilled water).

Calibration curve

The absorbance of glucose standard solutions was measured on a Biochrom UV spectrophotometer, model Ultraspec 50 (U.K.) at wavelength of 540 nm, and plotted against glucose concentration (as was shown in Figure 4-3). This standard curve used to determine the amount of released sugars from the hydrolysis of wheat straw.

The Method of Least Squares (Skoog et al., 2007) was used to calculate the error in the measurement of the equivalent glucose concentration of supernatants from enzymatic hydrolysis of AFEX-treated wheat straw. Table B-1 presents raw data for the calibration, and some statistics derived from it to evaluate the equation of the line passing through the data.

Table B-1. Calibration curve data for glucose standard solutions.

Glucose conc. (x_i)	Absorbance		Statistics for least square method			
			Absorbance mean (y_i)	(x_i^2)	(y_i^2)	(x_iy_i)
	Replicate 1	Replicate 2				
(mg/ml)	(-)	(-)	(-)	$(mg/ml)^2$	(-)	(mg/ml)
0.2	0.033	0.032	0.033	0.040	0.001	0.007
0.5	0.073	0.071	0.072	0.250	0.005	0.036
1.0	0.146	0.145	0.146	1.000	0.021	0.146
3.0	0.442	0.414	0.428	9.000	0.183	1.284
6.0	0.858	0.816	0.837	36.000	0.701	5.022
9.0	1.299	1.261	1.280	81.000	1.638	11.520
12.0	1.749	1.692	1.721	144.000	2.962	20.652
Sum of column above						
31.7			4.516	271.290	5.512	38.667

Three quantities, S_{xx}, S_{yy}, and S_{xy}, were used to define the slope and the intercept using the values of the sums reported in the last row of Table B-1 (N is the number of pairs of data used in preparing the calibration curve):

$$S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N} = 271.290 - \frac{(31.700)^2}{7} = 127.734\ (\frac{mg}{ml})^2$$

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} = 5.512 - \frac{(4.516)^2}{7} = 2.599\ (-)$$

$$S_{xy} = \sum x_i y_i - \frac{\sum x_i \sum y_i}{N} = 38.667 - \frac{31.700 \times 4.516}{7} = 18.216\ (\frac{mg}{ml})$$

The mean values of aqueous glucose standard concentration and absorbance of these solutions are:

$$\bar{x} = \frac{\sum x_i}{N} = \frac{31.700}{7} = 4.529\ (\frac{mg}{ml})$$

$$\bar{y} = \frac{\sum y_i}{N} = \frac{4.516}{7} = 0.645\ (-)$$

The slope of the straight line (m) passing through the calibration data is:

$$m = \frac{S_{xy}}{S_{xx}} = \frac{18.216}{127.734} = 0.143\ (\frac{ml}{mg})$$

The intercept (b) of the straight line is:

$$b = \bar{y} - m\bar{x} = 0.654 - 0.143 \times 4.529 = 0.0006 \approx 0.000\ (-)$$

So, the least squares straight line equation of the calibration curve is:

$$Absorbance = 0.143 \times Glucose\ concentration \qquad (B-1)$$

The standard deviation about the regression (S_r):

$$S_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}} = \sqrt{\frac{2.599 - (0.143)^2 \times 127.734}{7-2}} = 0.006689\ (-)$$

The standard deviation of the slope (S_m):

$$S_m = \sqrt{\frac{S_r^2}{S_{xx}}} = \sqrt{\frac{(0.007)^2}{127.734}} = 0.000619\ (\frac{ml}{mg})$$

The standard deviation of the intercept (S_b):

$$S_b = S_r \sqrt{\frac{1}{N - (\sum x_i)^2 / \sum x_i^2}} = 0.007 \times \sqrt{\frac{1}{7 - (31.700)^2 / 271.290}} = 0.003856\ (-)$$

The above statistics from the calibration curve are used to calculate the confidence limits (CL) associated with the imprecision of obtaining equivalent glucose concentration of supernatants, $\bar{x}_c$ (from enzymatic hydrolysis) from the calibration curve, equation B-1:

$$\bar{x}_c = {}^{\bar{y}_c}/_{0.143}$$

$$CL = \bar{x}_c \pm CF$$

The confidence function (CF) is:

$$CF = z \left(\frac{S_c}{\sqrt{M}}\right)$$

Thus, the confidence limits (CL) can be calculated using the following equation:

$$CL = \bar{x}_c \pm z \left(\frac{S_c}{\sqrt{M}}\right)$$

Where $\bar{x}_c$ is the mean of duplicate analysis of released sugars (values shown in Table B-2); z is the deviation from the mean of a population (for 95 % probability, $z = 1.96$); and M is now the number of replicate analysis of released sugars. So, for 164 h of hydrolysis, with an AFEX treatment done at 70 % moisture content in wheat straw, 95 °C and a 1:1 ratio,

$$\bar{x}_c = {}^{1.504}/_{0.143} = 10.517\ {}^{mg}/_{ml}$$

$$S_c = \frac{S_r}{m}\sqrt{\frac{1}{M}+\frac{1}{N}+\frac{(\bar{y}_c-\bar{y})^2}{m^2 s_{xx}}} \qquad (B-2)$$

With $\bar{y}_c$ being the mean absorbance value of the analyzed released sugars, while $\bar{y}$ is still the mean absorbance from the calibration curve. So,

$$S_c = \frac{0.006689}{0.143} \times \sqrt{\frac{1}{2}+\frac{1}{7}+\frac{(1.504-0.645)^2}{(0.143)^2 \times (127.734)}} \left(\frac{mg}{ml}\right)$$

$$S_c = 0.044996\ {}^{mg}/_{ml} \Rightarrow\ S_c = 0.045\ {}^{mg}/_{ml}$$

Consequently, the $CL = 10.517 \pm 1.96\left(\frac{0.045}{\sqrt{2}}\right) = 10.517 \pm 0.062\ {}^{mg}/_{ml}$

Individual values of S_c and CL for data from untreated wheat straw, and for all treatments were calculated similarly.

Table B-2 represents the absorbance data that were measured for duplicate runs of enzymatic hydrolysis at different enzymatic hydrolysis times. The mean (average) of absorbance for two replicates was calculated for calculating the concentration of released sugars by rearranging the least square technique (Equation B-1) for the line. The standard deviation (S_c) for the released sugars concentration (determined from the standard curve) was also calculated using equation B-2.

Table B-2. Enzymatic hydrolysis of untreated and AFEX-treated wheat straw at a fixed ammonia to wheat straw ratio (1:1 g of ammonia to g of wheat straw), moisture contents (50%, and 70% dry weight basis), and temperatures (85 °C, and 95 °C).

Hydrolysis time (h)	Untreated					50%, 95 °C, 1:1				
	Absorbance		Absorbance Mean ($\bar{y}_c$)	Mean released sugars concentration $\bar{x}_c \pm CF$	(S_c)	Absorbance		Absorbance Mean ($\bar{y}_c$)	Mean released sugars concentration $\bar{x}_c \pm CF$	(S_c)
	Replicate 1	Replicate 2				Replicate 1	Replicate 2			
	(-)	(-)	(-)	mg/ml	mg/ml	(-)	(-)	(-)	mg/ml	mg/ml
2	0.190	0.183	0.187	1.303 ± 0.007	0.005	0.373	0.453	0.413	2.889 ± 0.079	0.057
5	0.296	0.278	0.287	2.009 ± 0.018	0.013	0.878	0.844	0.861	6.021 ± 0.033	0.024
20	0.344	0.340	0.342	2.394 ± 0.004	0.003	1.272	1.250	1.261	8.819 ± 0.022	0.016
44	0.397	0.357	0.377	2.635 ± 0.039	0.028	1.279	1.341	1.310	9.161 ± 0.061	0.044
68	0.412	0.399	0.406	2.836 ± 0.012	0.009	1.307	1.365	1.336	9.345 ± 0.057	0.041
92	0.428	0.420	0.424	2.966 ± 0.008	0.006	1.333	1.401	1.367	9.559 ± 0.067	0.048
116	0.439	0.431	0.435	3.042 ± 0.008	0.006	1.348	1.424	1.386	9.690 ± 0.075	0.054
140	0.441	0.433	0.437	3.054 ± 0.008	0.006	1.342	1.413	1.378	9.632 ± 0.069	0.050
164	0.440	0.430	0.435	3.041 ± 0.010	0.007	1.352	1.422	1.387	9.700 ± 0.068	0.049
	70%, 95 °C, 1:1					50%, 85 °C, 1:1				
2	0.469	0.491	0.480	3.355 ± 0.022	0.016	0.353	0.404	0.379	2.647 ± 0.050	0.036
5	0.886	0.852	0.869	6.077 ± 0.033	0.024	0.715	0.782	0.749	5.233 ± 0.065	0.047
20	1.376	1.371	1.374	9.605 ± 0.006	0.004	1.039	1.034	1.037	7.251 ± 0.006	0.004
44	1.415	1.410	1.413	9.878 ± 0.006	0.004	1.058	1.059	1.059	7.403 ± 0.001	0.001
68	1.450	1.510	1.480	10.350 ± 0.058	0.042	1.100	1.080	1.090	7.622 ± 0.019	0.014
92	1.459	1.526	1.493	10.437 ± 0.065	0.047	1.098	1.090	1.094	7.648 ± 0.008	0.006
116	1.477	1.522	1.500	10.486 ± 0.044	0.032	1.107	1.094	1.101	7.696 ± 0.012	0.009
140	1.470	1.519	1.495	10.453 ± 0.049	0.035	1.131	1.113	1.122	7.845 ± 0.018	0.013
164	1.472	1.536	1.504	10.517 ± 0.062	0.045	1.115	1.104	1.110	7.757 ± 0.011	0.008

B.3 Wheat straw released sugars yield

The wheat straw released sugars yield is the amount of experimentally released sugars by enzymatic hydrolysis of AFEX-treated wheat straw to the theoretical amount of sugars that are expected to be released by complete conversion of wheat straw polysaccharides (cellulose and hemicellulose). The wheat straw released sugars yield can be calculated as follows (equation B-3):

$$Yield\ of\ released\ sugars\ \ (\%\ theoretical) = \frac{[Experimental\ released\ sugars\ \left(\frac{mg}{ml}\right)]}{[Theoretical\ released\ sugars\ \left(\frac{mg}{ml}\right)]} \times 100 \quad (\mathrm{B}-3)$$

According to Agblevor et al. (1993), wheat straw contains about 23 wt. % hemicellulose and 33 wt. % cellulose that can mainly be hydrolysed to sugars such as xylose and glucose. The enzymatic hydrolysis of hemicellulose and cellulose can be simplified as follows (equation B-4 and B-5):

$$[C_5(H_2O)_4]_n + nH_2O \rightarrow nC_5H_{10}O_5 \qquad \text{(B-4)}$$

hemicellulose n(xylose)

$$[C_6(H_2O)_5]_n + nH_2O \rightarrow nC_6H_{12}O_6 \qquad \text{(B-5)}$$

cellulose n (glucose)

According to Equations B-4 and B-5, 1 mol of hemicellulose (equivalent to 132 g) can release 1 mol of xylose (equivalent to 150 g); and 162 g of cellulose (equivalent to 1 mol glucose) can give 180 g of glucose (equivalent to 1 mol glucose).

Generally, if there is x amount of total solids in wheat straw sample, thus the amount of total released sugars can be calculated as:

$$Theoretical\ released\ xylose\ \ (g) = 0.23\ \times x\ \times \frac{150}{132} = 0.26\ x$$

$$Theoretical\ released\ glucose\ (g) = 0.33\ \times x \times \frac{180}{162} = 0.37\ x$$

Thus, the theoretical concentration of released sugars in 100 ml of solution can be obtained as follows:

$$Theoretical\ released\ sugars\ concentration\ (mg/ml) = \frac{[0.26\ x + 0.37x\ ]g}{100\ ml} \times \frac{1000\ mg}{g}$$

$$Theoretical\ released\ sugars\ concentration\ (mg/ml) = 6.3\ x$$

Or

$$Theoretical\ released\ sugars\ concentration\ (mg/ml) = 6.3\ (\frac{mg}{g\ ml}) \times [Total\ Solids\ (g)] \quad (\mathrm{B}-6)$$

As a result, the released sugars yield (% conversion) can be calculated as follow:

$$Yield\ of\ released\ sugars\ \ (\%\ theoretical) = \frac{[Experimental\ released\ sugars\ \left(\frac{mg}{ml}\right)]}{[6.3\ (\frac{mg}{g\ ml}) \times [Total\ Solids\ (g)]]} \times 100 \quad (\mathrm{B}-7)$$

Table B-3. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 50% moisture content (dry weight basis), and temperature of 95 °C (Replicate # 1).

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	% theoretical	mg/ml	% theoretical	mg/ml	% theoretical
0.5:1	1.905	11.965	2.729	22.81	4.148	34.67	6.216	51.95
0.8:1	1.896	11.909	3.520	29.56	5.639	47.35	7.862	66.02
1:1	1.892	11.884	2.607	21.94	6.143	51.69	8.893	74.83
1.2:1	1.908	11.984	3.311	27.63	6.021	50.24	8.679	72.42
1.5:1	1.897	11.915	2.594	21.77	5.818	48.83	8.257	69.30
			44		68		92	
0.5:1	1.905	11.965	6.319	52.81	6.400	53.49	6.249	55.23
0.8:1	1.896	11.909	7.923	66.53	8.226	69.07	8.322	69.88
1:1	1.892	11.884	8.944	75.26	9.142	76.93	9.319	78.42
1.2:1	1.908	11.984	9.304	77.64	9.413	78.55	9.481	79.11
1.5:1	1.897	11.915	8.447	70.89	8.541	71.68	8.898	74.68
			116		140		164	
0.5:1	1.905	11.965	6.696	55.96	6.745	56.37	6.619	55.32
0.8:1	1.896	11.909	8.354	70.15	8.484	71.24	8.405	70.58
1:1	1.892	11.884	9.424	79.30	9.382	78.95	9.453	79.54
1.2:1	1.908	11.984	9.567	79.83	9.399	78.43	9.472	79.04
1.5:1	1.897	11.915	8.993	75.48	8.964	75.23	8.915	74.82

Table B-4. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 50% moisture content (dry weight basis), and temperature of 85 °C. (Replicate # 1)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	% theoretical	mg/ml	% theoretical	mg/ml	% theoretical
0.5:1	1.897	11.915	2.196	18.43	3.787	31.78	5.389	45.23
0.8:1	1.903	11.953	2.103	17.59	3.422	28.63	5.332	44.61
1:1	1.887	11.852	2.472	20.86	4.998	42.17	7.268	61.32
1.2:1	1.895	11.902	3.068	25.78	3.950	33.19	6.593	55.39
1.5:1	1.892	11.884	2.296	19.32	4.216	35.48	6.294	52.96
			44		68		92	
0.5:1	1.897	11.915	5.551	46.59	5.757	48.32	5.856	49.15
0.8:1	1.903	11.953	5.779	48.35	5.814	48.64	5.920	49.53
1:1	1.887	11.852	7.399	62.43	7.694	64.92	7.677	64.77
1.2:1	1.895	11.902	6.831	57.39	6.990	58.73	7.122	59.84
1.5:1	1.892	11.884	6.319	53.17	6.432	54.12	6.499	54.69
			116		140		164	
0.5:1	1.897	11.915	5.940	49.85	5.863	49.21	5.900	49.52
0.8:1	1.903	11.953	5.973	49.97	6.004	50.23	5.938	49.68
1:1	1.887	11.852	7.743	65.33	7.910	66.74	7.796	65.78
1.2:1	1.895	11.902	7.053	59.26	7.153	60.10	7.195	60.45
1.5:1	1.892	11.884	6.611	55.63	6.531	54.96	6.583	55.39

Table B-5. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 50% moisture content (dry weight basis), and temperature of 95 °C. (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	% theoretical	mg/ml	% theoretical	mg/ml	% theoretical
0.5:1	1.899	11.928	2.259	18.94	4.491	37.65	6.544	54.86
0.8:1	1.908	11.984	3.22	26.87	5.502	45.91	7.708	64.32
1:1	1.892	11.884	3.171	26.68	5.899	49.64	8.744	73.58
1.2:1	1.902	11.946	2.811	23.53	5.660	47.38	8.478	70.97
1.5:1	1.887	11.852	2.348	19.81	5.048	42.59	7.620	64.29
			44		68		92	
0.5:1	1.899	11.928	6.913	57.96	3.372	58.27	7.138	59.84
0.8:1	1.908	11.984	7.848	65.49	7.973	66.53	8.054	67.21
1:1	1.892	11.884	9.378	78.91	9.548	80.34	9.798	82.45
1.2:1	1.902	11.946	8.557	71.63	8.658	72.48	8.963	75.03
1.5:1	1.887	11.852	7.986	67.38	8.272	69.79	8.513	71.83
			116		140		164	
0.5:1	1.899	11.928	7.224	60.56	7.288	61.10	7.182	60.21
0.8:1	1.908	11.984	8.140	67.92	8.249	68.83	8.234	68.71
1:1	1.892	11.884	9.956	83.78	9.882	83.15	9.946	83.69
1.2:1	1.902	11.946	9.030	75.59	9.066	75.89	9.149	76.59
1.5:1	1.887	11.852	8.552	72.16	7.609	72.64	8.647	72.96

Table B-6. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 50% moisture content (dry weight basis), and temperature of 85 °C. (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
0.5:1	1.909	11.990	2.432	20.28	3.448	28.76	5.123	42.73
0.8:1	1.891	11.877	2.584	21.76	3.619	30.47	5.612	47.25
1:1	1.896	11.909	2.822	23.70	5.467	45.91	7.234	60.74
1.2:1	1.885	11.840	3.129	26.43	4.607	38.91	6.917	58.42
1.5:1	1.906	11.972	2.270	18.96	4.803	40.12	6.594	55.08
			44		68		92	
0.5:1	1.909	11.990	5.313	44.31	5.378	44.85	5.627	46.93
0.8:1	1.891	11.877	6.101	51.37	6.231	52.46	6.320	53.21
1:1	1.896	11.909	7.406	62.19	7.550	63.40	7.619	63.98
1.2:1	1.885	11.840	7.100	59.97	7.169	60.55	7.278	61.47
1.5:1	1.906	11.972	6.721	56.14	6.922	57.82	6.981	58.31
			116		140		164	
0.5:1	1.909	11.990	5.670	47.29	5.867	48.93	5.698	47.52
0.8:1	1.891	11.877	6.469	54.47	6.493	54.67	6.385	53.76
1:1	1.896	11.909	7.649	64.23	7.780	65.33	7.718	64.81
1.2:1	1.885	11.840	7.400	62.50	7.315	61.78	7.385	62.37
1.5:1	1.906	11.972	7.037	58.78	7.057	58.95	7.092	59.24

Table B-7. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 50% moisture content (dry weight basis), and temperature of 95 °C.

Hydrolysis time (h)	0.5:1				0.8:1				1:1			
	Released sugars yield				Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2			# 1	# 2		
	% theoretical		% theoretical		% theoretical		% theoretical		% theoretical		% theoretical	
2	22.81	18.94	20.88 ± 3.79	2.74	29.56	26.87	28.22 ± 2.64	1.90	21.94	26.68	24.31 ± 4.65	3.35
5	34.67	37.65	36.16 ± 2.92	2.11	47.35	45.91	46.63 ± 1.41	1.02	51.69	49.64	50.67 ± 2.01	1.45
20	51.95	54.86	53.41 ± 2.85	2.06	66.02	64.32	65.17 ± 1.67	1.20	74.83	73.58	74.21 ± 1.22	0.88
44	52.81	57.96	55.39 ± 5.05	3.64	66.53	65.49	66.01 ± 1.02	0.74	75.26	78.91	77.09 ± 3.58	2.58
68	53.49	58.27	55.88 ± 4.68	3.38	69.07	66.53	67.80 ± 2.49	1.80	76.93	80.34	78.64 ± 3.34	2.41
92	55.23	59.84	57.54 ± 4.52	3.26	69.88	67.21	68.55 ± 2.62	1.89	78.42	82.45	80.44 ± 3.95	2.85
116	55.96	60.56	58.26 ± 4.51	3.25	70.15	67.92	69.04 ± 2.19	1.58	79.30	83.78	81.54 ± 4.39	3.17
140	56.37	61.10	57.77± 4.79	3.34	71.24	68.83	70.04 ± 2.36	1.70	78.95	83.15	81.05 ± 4.12	2.97
164	55.32	60.21	57.77 ± 4.79	3.46	70.58	68.71	69.65 ± 1.83	1.32	79.54	83.69	81.62 ± 4.07	2.93

	1.2:1				1.5:1			
2	27.63	23.53	25.58 ± 4.02	2.90	21.77	19.81	20.79 ± 1.92	1.39
5	50.24	47.38	48.81 ± 2.80	2.02	48.83	42.59	45.71± 6.12	4.41
20	72.42	70.97	71.70 ± 1.42	1.03	69.30	64.29	66.80 ± 4.91	3.54
44	77.64	71.63	74.64 ± 5.89	4.25	70.89	67.38	69.14 ± 3.44	2.48
68	78.55	72.48	75.52 ± 5.95	4.29	71.68	69.79	70.74 ± 1.85	1.34
92	79.11	75.03	77.07 ± 4.00	2.88	74.68	71.83	73.26 ± 2.79	2.02
116	79.83	75.59	77.71± 4.16	3.00	75.48	72.16	73.82 ± 3.25	2.35
140	78.43	75.89	77.16 ± 2.49	1.80	75.23	72.64	73.94 ± 2.54	1.83
164	79.04	76.59	77.82 ± 2.40	1.73	74.82	72.96	73.89 ± 1.82	1.32

Table B-8. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 50% moisture content (dry weight basis), and temperature of 85 °C.

Hydrolysis time (h)	0.5:1				0.8:1				1:1			
	Released sugars yield				Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2			# 1	# 2		
	% theoretical		% theoretical		% theoretical		% theoretical		% theoretical		% theoretical	
2	18.43	20.28	19.36 ± 1.81	1.31	17.59	21.76	19.68 ± 4.09	2.95	20.86	23.70	22.28 ± 2.78	2.01
5	31.78	28.76	30.27 ± 2.96	2.14	28.63	30.47	29.55 ± 1.80	1.30	42.17	45.91	44.04 ± 3.67	2.64
20	45.23	42.73	43.98 ± 2.45	1.77	44.61	47.25	45.93 ± 2.59	1.87	61.32	60.74	61.03 ± 0.57	0.41
44	46.59	44.31	45.45 ± 2.23	1.61	48.35	51.37	49.86 ± 2.96	2.14	62.43	62.19	62.31 ± 0.24	0.17
68	48.32	44.85	46.59 ± 3.40	2.45	48.64	52.46	50.55 ± 3.74	2.70	64.92	63.40	64.16 ± 1.49	1.07
92	49.15	46.93	48.04 ± 2.18	1.57	49.53	53.21	51.37 ± 3.61	2.60	64.77	63.98	64.38 ± 0.77	0.56
116	49.85	47.29	48.57 ± 2.51	1.81	49.97	54.47	52.22 ± 4.41	3.18	65.33	64.23	64.78 ± 1.08	0.78
140	49.21	48.93	49.07 ± 0.27	0.20	50.23	54.67	52.45 ± 4.35	3.14	66.74	65.33	66.04 ± 1.38	1.00
164	49.52	47.52	48.52 ± 1.96	1.41	49.68	53.76	51.72 ± 4.00	2.88	65.78	64.81	65.30 ± 0.95	0.69

	1.2:1				1.5:1			
2	25.78	26.43	26.11 ± 0.64	0.46	19.32	18.96	19.14 ± 0.35	0.25
5	33.19	38.91	36.05 ± 5.61	4.04	35.48	40.12	37.80 ± 4.55	3.28
20	55.39	58.42	56.91 ± 2.97	2.14	52.96	55.08	54.02 ± 2.08	1.50
44	57.39	59.97	58.68 ± 2.53	1.82	53.17	56.14	54.66 ± 2.91	2.10
68	58.73	60.55	59.64 ± 1.78	1.29	54.12	57.82	55.97 ± 3.63	2.62
92	59.84	61.47	60.66 ± 1.60	1.15	54.69	58.31	56.50 ± 3.55	2.56
116	59.26	62.50	60.88 ± 3.18	2.29	55.63	58.78	57.21 ± 3.09	2.23
140	60.10	61.78	60.94 ± 1.65	1.19	54.96	58.95	56.96 ± 3.91	2.82
164	60.45	62.37	61.41 ± 1.88	1.36	55.39	59.24	57.32 ± 3.77	2.72

Table B-9. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 70% moisture content (dry weight basis), and temperature of 95 °C. (Replicate # 1)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
0.5:1	1.886	11.846	2.092	17.66	3.976	33.56	6.264	52.88
0.8:1	1.890	11.871	2.924	24.63	3.935	33.15	7.285	61.37
1:1	1.885	11.839	3.277	27.68	6.196	52.35	9.619	81.25
1.2:1	1.892	11.884	3.426	28.83	5.987	50.38	9.012	75.83
1.5:1	1.897	11.915	3.782	31.74	5.795	48.64	8.418	70.65
			44		68		92	
0.5:1	1.886	11.846	6.629	55.96	6.910	58.33	6.898	58.23
0.8:1	1.890	11.871	7.650	64.44	7.867	66.27	8.024	67.59
1:1	1.885	11.839	9.896	83.59	10.142	85.67	10.204	86.19
1.2:1	1.892	11.884	9.467	79.66	9.853	82.91	10.035	84.44
1.5:1	1.897	11.915	8.539	71.67	8.831	74.12	8.973	75.31
			116		140		164	
0.5:1	1.886	11.846	7.103	59.96	7.131	60.2	7.105	59.98
0.8:1	1.890	11.871	8.294	69.87	8.475	71.39	8.506	71.65
1:1	1.885	11.839	10.327	87.23	10.282	86.85	10.296	86.97
1.2:1	1.892	11.884	10.114	85.11	10.126	85.20	10.117	85.13
1.5:1	1.897	11.915	9.073	76.15	9.060	76.04	9.084	76.24

Table B-10. Data for enzymatic hydrolysis of untreated and AFEX-treated wheat straw at various ammonia to wheat straw ratio, 70% moisture content (dry weight basis), and temperature of 85 °C. (Replicate # 1)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
0.5:1	1.871	11.752	1.933	16.45	3.707	31.54	5.405	45.99
0.8:1	1.885	11.840	2.223	18.78	3.389	28.62	5.845	49.37
1:1	1.878	11.796	3.325	28.19	5.651	47.91	7.552	64.02
1.2:1	1.887	11.852	3.277	27.65	5.372	45.32	6.993	59.00
1.5:1	1.883	11.827	1.767	14.94	3.663	30.97	6.348	53.67
Untreated	1.844	11.582	1.327	11.46	2.072	17.89	2.407	20.78
			44		68		92	
0.5:1	1.871	11.752	5.626	47.87	5.872	49.97	6.001	51.06
0.8:1	1.885	11.840	6.231	52.63	6.421	54.23	6.544	55.27
1:1	1.878	11.796	7.778	65.94	7.810	66.21	7.900	66.97
1.2:1	1.887	11.852	7.322	61.78	7.435	62.73	7.526	63.50
1.5:1	1.883	11.827	6.961	58.86	7.123	60.23	7.271	61.48
Untreated	1.844	11.582	2.774	23.95	2.881	24.88	2.994	25.85
			116		140		164	
0.5:1	1.871	11.752	6.21	52.84	6.120	52.08	6.162	52.43
0.8:1	1.885	11.840	6.917	58.42	7.158	60.46	6.813	57.54
1:1	1.878	11.796	7.926	67.19	7.949	67.39	7.935	67.27
1.2:1	1.887	11.852	7.666	64.68	7.624	64.33	7.671	64.72
1.5:1	1.883	11.827	7.301	61.73	7.282	61.57	7.290	61.64
Untreated	1.844	11.582	3.068	26.49	3.082	26.61	3.076	26.56

Table B-11. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 70% moisture content (dry weight basis), and temperature of 95 °C. (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
0.5:1	1.894	11.896	1.899	15.96	3.744	31.47	6.545	55.02
0.8:1	1.879	11.800	3.951	33.48	4.649	39.40	7.148	60.58
1:1	1.898	11.921	3.432	28.79	5.958	49.98	9.590	80.45
1.2:1	1.914	12.020	3.614	30.07	5.450	45.34	8.790	73.21
1.5:1	1.910	11.997	2.916	24.31	5.858	48.83	8.647	72.08
			44		68		92	
0.5:1	1.894	11.896	6.571	55.24	7.148	60.09	7.373	61.98
0.8:1	1.879	11.800	7.497	63.52	8.033	68.08	8.440	71.52
1:1	1.898	11.921	9.860	82.71	10.557	88.56	10.669	89.50
1.2:1	1.914	12.020	9.229	76.78	9.301	77.38	9.587	79.76
1.5:1	1.910	11.997	9.000	75.02	9.110	75.94	9.353	77.96
			116		140		164	
0.5:1	1.894	11.896	7.422	62.39	7.406	62.26	7.412	62.31
0.8:1	1.879	11.800	8.549	72.45	8.845	74.96	8.638	73.20
1:1	1.898	11.921	10.645	89.3	10.623	89.11	10.738	90.08
1.2:1	1.914	12.020	9.694	80.65	9.647	80.26	9.741	81.04
1.5:1	1.910	11.997	9.439	78.68	9.380	78.19	9.374	78.14

Table B-12. Data for enzymatic hydrolysis of untreated and AFEX-treated wheat straw at various ammonia to wheat straw ratio, 70% moisture content (dry weight basis), and temperature of 85 °C. (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
0.5:1	1.865	11.714	1.694	14.46	3.832	32.71	5.032	42.96
0.8:1	1.893	11.890	1.923	16.17	2.686	22.59	5.340	44.91
1:1	1.890	11.871	2.676	22.54	4.723	39.79	7.082	59.66
1.2:1	1.874	11.770	2.722	23.13	4.692	39.86	6.310	53.61
1.5:1	1.896	11.909	2.316	19.45	4.634	38.91	6.100	51.22
Untreated	1.857	11.664	1.278	10.96	1.946	16.68	2.381	20.41
			44		68		92	
0.5:1	1.865	11.714	5.120	43.71	5.617	47.95	5.733	48.94
0.8:1	1.893	11.890	5.685	47.81	5.978	50.28	6.171	51.90
1:1	1.890	11.871	7.189	60.56	7.724	65.07	7.726	65.08
1.2:1	1.874	11.770	6.588	55.97	6.821	57.95	6.990	59.39
1.5:1	1.896	11.909	6.176	51.86	7.000	58.78	7.160	60.12
Untreated	1.857	11.664	2.496	21.40	2.790	23.92	2.937	25.18
			116		140		164	
0.5:1	1.865	11.714	5.732	48.93	5.800	49.51	5.789	49.42
0.8:1	1.893	11.890	6.415	53.95	6.453	54.27	6.523	54.86
1:1	1.890	11.871	7.849	66.12	7.813	65.82	7.803	65.73
1.2:1	1.874	11.770	7.171	60.93	7.321	62.20	7.200	61.17
1.5:1	1.896	11.909	7.155	60.08	6.956	58.41	7.050	59.20
Untreated	1.857	11.664	3.016	25.86	3.026	25.94	3.005	25.76

Table B-13. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 70% moisture content (dry weight basis), and temperature of 95 °C.

Hydrolysis time (h)	0.5:1				0.8:1				1:1			
	Released sugars yield				Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2			# 1	# 2		
	% theoretical		% theoretical		% theoretical		% theoretical		% theoretical		% theoretical	
2	17.66	15.96	16.81 ± 1.67	1.20	24.63	33.48	29.06 ± 8.67	6.26	27.68	28.79	28.24 ± 0.77	0.78
5	33.56	31.47	32.52 ± 2.05	1.48	33.15	39.40	36.28 ± 6.12	4.42	52.35	49.98	51.17 ± 1.64	1.68
20	52.88	55.02	53.95 ± 2.10	1.51	61.37	60.58	60.98 ± 0.77	0.56	81.25	80.45	80.85 ± 0.78	0.57
44	55.96	55.24	55.60 ± 0.71	0.51	64.44	63.52	63.98 ± 0.90	0.65	83.59	82.71	83.15 ± 0.86	0.62
68	58.33	60.09	59.21 ± 1.72	1.24	66.27	68.08	67.18 ± 1.77	1.28	85.67	88.56	87.12 ± 2.83	2.04
92	58.23	61.98	60.11 ± 3.67	2.65	67.59	71.52	69.56 ± 3.85	2.78	86.19	89.50	87.85 ± 3.24	2.34
116	59.96	62.39	61.18 ± 2.38	1.72	69.87	72.45	71.16 ± 2.53	1.82	87.23	89.30	88.27 ± 2.03	1.46
140	60.20	62.26	61.23 ± 2.02	1.46	71.39	74.96	73.18 ± 3.50	2.52	86.85	89.11	87.98 ± 2.21	1.60
164	59.98	62.31	61.15 ± 2.28	1.65	71.65	73.20	72.43 ± 1.52	1.10	86.97	90.08	88.53 ± 3.05	2.20

	1.2:1				1.5:1			
2	28.83	30.07	29.45 ± 1.22	0.88	31.74	24.31	28.03 ± 7.28	5.25
5	50.38	45.34	47.86 ± 4.94	3.56	48.64	48.83	48.74 ± 0.19	0.13
20	75.83	73.21	74.52 ± 2.57	1.85	70.65	72.08	71.37 ± 1.40	1.01
44	79.66	76.78	78.22 ± 2.82	2.04	71.67	75.02	73.35 ± 3.28	2.37
68	82.91	77.38	80.15 ± 5.42	3.91	74.12	75.94	75.03 ± 1.78	1.29
92	84.44	79.76	82.10 ± 4.59	3.31	75.31	77.96	76.64 ± 2.60	1.87
116	85.11	80.65	82.88 ± 4.37	3.15	76.15	78.68	77.42 ± 2.48	1.79
140	85.20	80.26	82.73 ± 4.84	3.49	76.04	78.19	77.12 ± 2.11	1.52
164	85.13	81.04	83.09 ± 4.01	2.89	76.24	78.14	77.19 ± 1.86	1.34

Table B-14. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at various ammonia to wheat straw ratio, 70% moisture content (dry weight basis), and temperature of 85 °C.

Hydrolysis time (h)	0.5:1				0.8:1				1:1			
	Released sugars yield				Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2			# 1	# 2		
	% theoretical		% theoretical		% theoretical		% theoretical		% theoretical		% theoretical	
2	16.45	14.46	15.46 ± 1.95	1.41	18.78	16.17	17.48 ± 2.56	1.85	28.19	22.54	25.37 ± 5.54	4.00
5	31.54	32.71	32.13 ± 1.15	0.83	28.62	22.59	25.61 ± 5.91	4.26	47.91	39.79	43.85 ± 7.96	5.74
20	45.99	42.96	44.48 ± 2.97	2.14	49.37	44.91	47.14 ± 4.37	3.15	64.02	59.66	61.84 ± 4.27	3.08
44	47.87	43.71	45.79 ± 4.08	2.94	52.63	47.81	50.22 ± 4.72	3.41	65.94	60.56	63.25 ± 5.27	3.80
68	49.97	47.95	48.96 ± 1.98	1.43	54.23	50.28	52.26 ± 3.87	2.79	66.21	65.07	65.64 ± 1.12	0.81
92	51.06	48.94	50.00 ± 2.08	1.50	55.27	51.90	53.59 ± 3.30	2.38	66.97	65.08	66.03 ± 1.85	1.34
116	52.84	48.93	50.89 ± 3.83	2.76	58.42	53.95	56.19 ± 4.38	3.16	67.19	66.12	66.66 ± 1.05	0.76
140	52.08	49.51	50.80 ± 2.52	1.82	60.46	54.27	57.37 ± 6.07	4.38	67.39	65.82	66.61 ± 1.54	1.11
164	52.43	49.42	50.93 ± 2.95	2.13	57.54	54.86	56.20 ± 2.63	1.90	67.27	65.73	66.50 ± 1.51	1.09
	1.2:1				1.5:1							
2	27.65	23.13	25.39 ± 4.43	3.20	14.94	19.45	17.20 ± 4.42	3.19				
5	45.32	39.86	42.59 ± 5.35	3.86	30.97	38.91	34.94 ± 7.78	5.61				
20	59.00	53.61	56.31 ± 5.28	3.81	53.67	51.22	52.45 ± 2.40	1.73				
44	61.78	55.97	58.88 ± 5.69	4.11	58.86	51.86	55.36 ± 6.86	4.95				
68	62.73	57.95	60.34 ± 4.68	3.38	60.23	58.78	59.51 ± 1.42	1.03				
92	63.50	59.39	61.45 ± 4.03	2.91	61.48	60.12	60.80 ± 1.33	0.96				
116	64.68	60.93	62.81 ± 3.67	2.65	61.73	60.08	60.91 ± 1.62	1.17				
140	64.33	62.20	63.27 ± 2.09	1.51	61.57	58.41	59.99 ± 3.10	2.23				
164	64.72	61.17	62.95 ± 3.48	2.51	61.64	59.20	60.42 ± 2.39	1.73				

Table B-15. Summary of the duplicate runs of enzymatic hydrolysis of untreated wheat straw.

Hydrolysis time (h)	Untreated			
	Released sugars yield			
	Replicate		Mean ± CF	SD
	# 1	# 2		
	% theoretical	% theoretical	% theoretical	% theoretical
2	11.46	10.96	11.21 ± 0.49	0.35
5	17.89	16.68	17.29 ± 1.19	0.86
20	20.78	20.41	20.60 ± 0.36	0.26
44	23.95	21.40	22.68 ± 2.50	1.80
68	24.88	23.92	24.40 ± 0.94	0.68
92	25.85	25.18	25.52 ± 0.66	0.47
116	26.49	25.86	26.18 ± 0.62	0.45
140	26.61	25.94	26.28 ± 0.66	0.47
164	26.56	25.76	26.16 ± 0.78	0.57

Table B-16. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various moisture content (dry weight basis), ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw), and temperature of 95 °C. (Replicate # 1)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
30%	1.887	11.852	2.227	18.79	4.674	39.44	6.984	58.93
50%	1.892	11.884	2.607	21.94	6.143	51.69	8.893	74.83
70%	1.885	11.839	3.277	27.68	6.196	52.35	9.619	81.25
80%	1.896	11.909	2.955	24.81	5.964	50.08	9.474	79.55
90%	1.889	11.865	3.071	25.88	6.369	53.68	9.696	81.72
			44		68		92	
30%	1.887	11.852	7.167	60.47	7.569	63.86	7.632	64.39
50%	1.892	11.884	8.944	75.26	9.142	76.93	9.319	78.42
70%	1.885	11.839	9.896	83.59	10.142	85.67	10.204	86.19
80%	1.896	11.909	9.856	82.76	10.114	84.93	10.286	86.37
90%	1.889	11.865	9.959	83.94	10.057	84.76	10.238	86.29
			116		140		164	
30%	1.887	11.852	7.729	65.21	7.832	66.08	7.72	65.14
50%	1.892	11.884	9.424	79.30	9.382	78.95	9.453	79.54
70%	1.885	11.839	10.327	87.23	10.282	86.85	10.296	86.97
80%	1.896	11.909	10.431	87.59	10.385	87.20	10.462	87.85
90%	1.889	11.865	10.374	87.43	10.274	86.59	10.343	87.17

Table B-17. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various moisture content (dry weight basis), ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw), and temperature of 95 °C. (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
30%	1.906	11.972	2.452	20.48	4.408	36.82	6.696	55.93
50%	1.892	11.884	3.171	26.68	5.899	49.64	8.744	73.58
70%	1.898	11.921	3.432	28.79	5.958	49.98	9.590	80.45
80%	1.884	11.833	3.163	26.73	6.305	53.28	9.524	80.49
90%	1.879	11.802	2.68	22.71	6.085	51.56	9.246	78.34
			44		68		92	
30%	1.906	11.972	6.750	56.38	6.943	57.99	7.038	58.79
50%	1.892	11.884	9.378	78.91	9.548	80.34	9.798	82.45
70%	1.898	11.921	9.860	82.71	10.557	88.56	10.669	89.50
80%	1.884	11.833	9.620	81.30	9.826	83.04	10.007	84.57
90%	1.879	11.802	9.420	79.82	9.557	80.98	9.603	81.37
			116		140		164	
30%	1.906	11.972	7.165	59.85	7.140	59.64	7.164	59.84
50%	1.892	11.884	9.956	83.78	9.882	83.15	9.946	83.69
70%	1.898	11.921	10.645	89.3	10.623	89.11	10.738	90.08
80%	1.884	11.833	10.012	84.61	10.101	85.36	9.969	84.25
90%	1.879	11.802	9.684	82.05	9.752	82.63	9.769	82.77

Table B-18. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at various moisture content (dry weight basis), ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw), and temperature of 95 °C.

Hydrolysis time (h)	30%				50%				70%			
	Released sugars yield				Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2			# 1	# 2		
	% theoretical		% theoretical		% theoretical		% theoretical		% theoretical		% theoretical	
2	18.79	20.48	19.64 ± 1.66	1.20	21.94	26.68	24.31 ± 4.65	3.35	27.68	28.79	28.24 ± 0.77	0.78
5	39.44	36.82	38.13 ± 2.57	1.85	51.69	49.64	50.67 ± 2.01	1.45	52.35	49.98	51.17 ± 1.64	1.68
20	58.93	55.93	57.43 ± 2.94	2.12	74.83	73.58	74.21 ± 1.22	0.88	81.25	80.45	80.85 ± 0.78	0.57
44	60.47	56.38	58.43 ± 4.01	2.89	75.26	78.91	77.09 ± 3.58	2.58	83.59	82.71	83.15 ± 0.86	0.62
68	63.86	57.99	60.93 ± 5.75	4.15	76.93	80.34	78.64 ± 3.34	2.41	85.67	88.56	87.12 ± 2.83	2.04
92	64.39	58.79	61.59 ± 5.49	3.96	78.42	82.45	80.44 ± 3.95	2.85	86.19	89.50	87.85 ± 3.24	2.34
116	65.21	59.85	62.53 ± 5.25	3.79	79.30	83.78	81.54 ± 4.39	3.17	87.23	89.30	88.27 ± 2.03	1.46
140	66.08	59.64	62.86 ± 6.31	4.55	78.95	83.15	81.05 ± 4.12	2.97	86.85	89.11	87.98 ± 2.21	1.60
164	65.14	59.84	62.49 ± 5.19	3.75	79.54	83.69	81.62 ± 4.07	2.93	86.97	90.08	88.53 ± 3.05	2.20
	80%				90%							
2	24.81	26.73	25.77 ± 1.88	1.36	25.88	22.71	24.30 ± 3.11	2.24				
5	50.08	53.28	51.68 ± 3.14	2.26	53.68	51.56	52.62 ± 2.08	1.50				
20	79.55	80.49	80.02 ± 0.92	0.66	81.72	78.34	80.03 ± 3.31	2.39				
44	82.76	81.30	82.03 ± 1.43	1.03	83.94	79.82	81.88 ± 4.04	2.91				
68	84.93	83.04	83.99 ± 1.85	1.34	84.76	80.98	82.87 ± 3.70	2.67				
92	86.37	84.57	85.47 ± 1.76	1.27	86.29	81.37	83.83 ± 4.82	3.48				
116	87.59	84.61	86.10 ± 2.92	2.11	87.43	82.05	84.74 ± 5.27	3.80				
140	87.20	85.36	86.28 ± 1.80	1.30	86.59	82.63	84.61 ± 3.88	2.80				
164	87.85	84.25	86.05 ± 3.53	2.55	87.17	82.77	84.97 ± 4.31	3.11				

Table B-19. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various temperatures, 70% moisture content (dry weight basis), and ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw). (Replicate # 1)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
75 °C	1.901	11.940	2.104	17.62	3.519	29.47	5.562	46.58
85 °C	1.878	11.796	3.325	28.19	5.651	47.91	7.552	64.02
95 °C	1.885	11.839	3.277	27.68	6.196	52.35	9.619	81.25
105 °C	1.889	11.865	3.174	26.75	6.440	54.28	9.447	79.62
115 °C	1.903	11.953	3.068	25.67	6.091	50.96	9.065	75.84
			44		68		92	
75 °C	1.901	11.940	5.723	47.93	1.826	48.79	5.984	50.12
85 °C	1.878	11.796	7.778	65.94	7.810	66.21	7.900	66.97
95 °C	1.885	11.839	9.896	83.59	10.142	85.67	10.204	86.19
105 °C	1.889	11.865	9.570	80.66	9.735	82.05	9.837	82.91
115 °C	1.903	11.953	9.240	77.30	9.592	80.25	9.733	81.43
			116		140		164	
75 °C	1.901	11.940	6.057	50.73	6.124	51.29	6.027	50.48
85 °C	1.878	11.796	7.926	67.19	7.949	67.39	7.935	67.27
95 °C	1.885	11.839	10.327	87.23	10.282	86.85	10.296	86.97
105 °C	1.889	11.865	9.878	83.25	9.981	84.12	9.856	83.07
115 °C	1.903	11.953	9.764	81.69	9.87	82.57	9.803	82.01

Table B-20. Data for enzymatic hydrolysis of AFEX-treated wheat straw at various temperatures, 70% moisture content (dry weight basis), and ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw). (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
75 °C	1.876	11.783	2.758	23.41	3.676	31.20	5.672	48.14
85 °C	1.890	11.871	2.676	22.54	4.723	39.79	7.082	59.66
95 °C	1.898	11.921	3.432	28.79	5.958	49.98	9.590	80.45
105 °C	1.905	11.965	3.403	28.44	6.690	55.91	9.663	80.76
115 °C	1.909	11.990	3.254	27.14	5.972	49.81	8.698	72.54
			44		68		92	
75 °C	1.876	11.783	5.942	50.43	6.032	51.19	6.330	53.72
85 °C	1.890	11.871	7.189	60.56	7.724	65.07	7.726	65.08
95 °C	1.898	11.921	9.860	82.71	10.557	88.56	10.669	89.50
105 °C	1.905	11.965	9.893	82.68	10.085	84.29	10.125	84.62
115 °C	1.909	11.990	8.856	73.86	9.004	75.10	9.104	75.93
			116		140		164	
75 °C	1.876	11.783	6.467	54.88	6.442	54.67	6.490	55.08
85 °C	1.890	11.871	7.849	66.12	7.813	65.82	7.803	65.73
95 °C	1.898	11.921	10.645	89.3	10.623	89.11	10.738	90.08
105 °C	1.905	11.965	10.258	85.73	10.311	86.18	10.322	86.27
115 °C	1.909	11.990	9.133	76.17	9.267	77.29	9.162	76.41

Table B-21. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at various temperatures, 70% moisture content (dry weight basis), and ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw).

Hydrolysis time (h)	75 °C				85 °C				95 °C			
	Released sugars yield				Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2			# 1	# 2		
	% theoretical		% theoretical		% theoretical		% theoretical		% theoretical		% theoretical	
2	17.62	23.41	20.52 ± 5.67	4.09	28.19	22.54	25.37 ± 5.54	4.00	27.68	28.79	28.24 ± 0.77	0.78
5	29.47	31.20	30.34 ± 1.70	1.22	47.91	39.79	43.85 ± 7.96	5.74	52.35	49.98	51.17 ± 1.64	1.68
20	46.58	48.14	47.36 ± 1.53	1.10	64.02	59.66	61.84 ± 4.27	3.08	81.25	80.45	80.85 ± 0.78	0.57
44	47.93	50.43	49.18 ± 2.45	1.77	65.94	60.56	63.25 ± 5.27	3.80	83.59	82.71	83.15 ± 0.86	0.62
68	48.79	51.19	49.99 ± 2.35	1.70	66.21	65.07	65.64 ± 1.12	0.81	85.67	88.56	87.12 ± 2.83	2.04
92	50.12	53.72	51.92 ± 3.53	2.55	66.97	65.08	66.03 ± 1.85	1.34	86.19	89.50	87.85 ± 3.24	2.34
116	50.73	54.88	52.81 ± 4.07	2.93	67.19	66.12	66.66 ± 1.05	0.76	87.23	89.30	88.27 ± 2.03	1.46
140	51.29	54.67	52.98 ± 3.31	2.39	67.39	65.82	66.61 ± 1.54	1.11	86.85	89.11	87.98 ± 2.21	1.60
164	50.48	55.08	52.78 ± 4.51	3.25	67.27	65.73	66.50 ± 1.51	1.09	86.97	90.08	88.53 ± 3.05	2.20
	105 °C				115 °C							
2	26.75	28.44	27.60 ± 1.66	1.20	25.67	27.14	26.41 ± 1.44	1.04				
5	54.28	55.91	55.10 ± 1.60	1.15	50.96	49.81	50.39 ± 1.13	0.81				
20	79.62	80.76	80.19 ± 1.12	0.81	75.84	72.54	74.19 ± 3.23	2.33				
44	80.66	82.68	81.67 ± 1.98	1.43	77.30	73.86	75.58 ± 3.37	2.43				
68	82.05	84.29	83.17 ± 2.20	1.58	80.25	75.10	77.68 ± 5.05	3.64				
92	82.91	84.62	83.77 ± 1.68	1.21	81.43	75.93	78.68 ± 5.39	3.89				
116	83.25	85.73	84.49 ± 2.43	1.75	81.69	76.17	78.93 ± 5.41	3.90				
140	84.12	86.18	85.15 ± 2.02	1.46	82.57	77.29	79.93 ± 5.17	3.73				
164	83.07	86.27	84.67 ± 3.14	2.26	82.01	76.41	79.21 ± 5.49	3.96				

Table B-22. Data for enzymatic hydrolysis of AFEX-treated wheat straw at longer treatment time (15 minutes), temperatures (95 °C, and 105 °C), moisture content (70%, and 80% dry weight basis), and ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw). (Replicate # 1)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
70%, 95 °C	1.902	11.946	2.623	21.96	4.267	35.72	8.160	68.31
80%, 95 °C	1.897	11.915	3.472	29.14	5.810	48.76	9.053	75.98
70%, 105 °C	1.906	11.972	3.096	25.86	5.705	47.65	8.306	69.38
			44		68		92	
70%, 95 °C	1.902	11.946	8.512	71.25	8.773	73.44	11.946	77.18
80%, 95 °C	1.897	11.915	9.34	78.39	9.752	81.85	9.808	82.32
70%, 105 °C	1.906	11.972	8.681	72.51	8.965	74.88	9.030	75.43
			116		140		164	
70%, 95 °C	1.902	11.946	9.280	77.68	9.269	77.59	9.296	77.82
80%, 95 °C	1.897	11.915	9.956	83.56	9.974	83.71	10.038	84.25
70%, 105 °C	1.906	11.972	9.117	76.15	9.206	76.95	9.112	76.11

Table B-23. Data for enzymatic hydrolysis of AFEX-treated wheat straw at longer treatment time (15minutes), temperatures (95 °C, and 105 °C), moisture content (70%, and 80% dry weight basis), and ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw). (Replicate # 2)

Ammonia to wheat straw ratio	Total dried solids weight	Theoretical released sugars concentration	Enzymatic hydrolysis time (h)					
			2		5		20	
			Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield	Experimental released sugars concentration	Released sugars yield
	g	mg/ml	mg/ml	%theoretical	mg/ml	%theoretical	mg/ml	%theoretical
70%, 95 °C	1.895	11.902	2.793	23.47	4.382	36.82	8.440	70.91
80%, 95 °C	1.908	11.984	3.339	27.86	5.715	47.69	9.002	75.12
70%, 105 °C	1.903	11.953	2.609	21.83	2.277	44.15	7.812	65.36
			44		68		92	
70%, 95 °C	1.895	11.902	8.841	74.28	9.357	78.62	9.754	81.95
80%, 95 °C	1.908	11.984	9.306	77.65	9.501	79.28	9.610	80.19
70%, 105 °C	1.903	11.953	8.216	68.74	8.365	69.98	8.38	70.11
			116		140		164	
70%, 95 °C	1.895	11.902	9.800	82.34	9.893	83.12	9.810	82.42
80%, 95 °C	1.908	11.984	9.669	80.68	9.735	81.23	9.641	80.45
70%, 105 °C	1.903	11.953	8.434	70.56	7.407	70.33	8.452	70.71

Table B-24. Summary of the duplicate runs of enzymatic hydrolysis of AFEX-treated wheat straw at longer treatment time (15 minutes), temperatures (95 °C, and 105 °C), moisture content (70%, and 80% dry weight basis), and ammonia to wheat straw ratio 1:1 (g of ammonia: g of wheat straw).

Hydrolysis time (h)	70%, 95 °C				80%, 95 °C			
	Released sugars yield				Released sugars yield			
	Replicate		Mean ± CF	SD	Replicate		Mean ± CF	SD
	# 1	# 2			# 1	# 2		
	% theoretical		% theoretical	% theoretical	% theoretical		% theoretical	% theoretical
2	21.96	23.47	22.72 ± 1.48	1.07	29.14	27.86	28.50 ± 1.25	0.91
5	35.72	36.82	36.27 ± 1.08	0.78	48.76	47.69	48.23 ± 1.05	0.76
20	68.31	70.91	69.61 ± 2.55	1.84	75.98	75.12	75.55 ± 0.84	0.61
44	71.25	74.28	72.77 ± 2.97	2.14	78.39	77.65	78.02 ± 0.73	0.52
68	73.44	78.62	76.03 ± 5.08	3.66	81.85	79.28	80.57 ± 2.52	1.82
92	77.18	81.95	79.57 ± 4.67	3.37	82.32	80.19	81.26 ± 2.09	1.51
116	77.68	82.34	80.01 ± 4.57	3.30	83.56	80.68	82.12 ± 2.82	2.04
140	77.59	83.12	80.36 ± 5.42	3.91	83.71	81.23	82.47 ± 2.43	1.75
164	77.82	82.42	80.12 ± 4.51	3.25	84.25	80.45	82.35 ± 3.72	2.69
	70%, 105 °C							
2	25.86	21.83	23.85 ± 3.95	2.85				
5	47.65	44.15	45.90 ± 3.43	2.47				
20	69.38	65.36	67.37 ± 3.94	2.84				
44	72.51	68.74	70.63 ± 3.69	2.67				
68	74.88	69.98	72.43 ± 4.80	3.46				
92	75.43	70.11	72.77 ± 5.21	3.76				
116	76.15	70.56	73.36 ± 5.48	3.95				
140	76.95	70.33	73.64 ± 6.49	4.68				
164	76.11	70.71	73.41 ± 5.29	3.82				

Appendix C: Measurement of cellulase enzyme activity

Modified National Renewable Energy Laboratory protocol (LAP-006) (NREL, 1995)

Materials

Dinitrosalicylic acid (DNS) reagent

To prepare the DNS reagent solution, the amounts of 10.6 g of 3, 5 Dinitrosalicylic acid and 19.8 g of Sodium hydroxide must be properly dissolved in distilled water. Then, 306 g of Sodium potassium tartrate (Rochelle salts), 7.6 ml of Phenol, and 8.3 g of Sodium metabisulfite is respectively added to the solution. The final volume of solution should be reached to 1000 ml by using distilled water. An amount of 3 ml sample must be titrated by HCl (0.1 N) to the phenolphthalein endpoint. It must use 5-6 ml of HCl. Sodium hydroxide (NaOH) will be add if required.

Citrate Buffer

In this research, Novozymes Cellulase enzyme (NS 22086) assays were performed in pH 5.0 using citrate buffer solution (0.05 M).

First, a stock solution of citrate buffer (1.0 M) was prepared. The amount of 210 g citric acid monohydrate was dissolved in 750 ml of distilled water. The pH of solution was adjusted to 4.3 by using 50-60 g of sodium hydroxide (NaOH). Then, the volume of 50 ml of this stock solution (1.0 M) was diluted to 1000 ml using distilled water (950 ml). By using sodium hydroxide (NaOH), the pH of this solution was adjusted to 5.0.

Measurement of activity (Filter Paper Assay)

To detect the glycosidic bond dissociation by this technique, three sets of test tubes are considered including glucose standards, enzyme assay mixtures, and blanks and controls). Also, a 50 mg strip (about 1.0 x 6.0 cm) of Whatman filter paper No. 1 is considered as a substrate.

Preparing Blank and controls

a) Substrate control - 1.5 ml of citrate buffer (0.05 M) was added into a test tube containing a filter paper strip.

b) Reagent blank – an amount of 1.5 ml of citrate buffer (0.05 M) was added to a test tube.

c) Enzyme control: a volume of 1.0 ml of citrate buffer (0.05 M) was added to 0.5 ml enzyme dilutions. For each dilution, the control solution must separately be prepared.

Preparing assay mixtures tubes

a) A rolled Whatman filter paper strip was placed into each 13 x 100 mm test tube.

b) A volume of 1.0 ml citrate buffer (0.05 M) was added to each test tube. The filter paper strip should be saturated by citrate buffer.

c) Test tubes were incubated at 47.5 ºC to equilibrate buffer and substrate.

d) An amount of 0.5 ml diluted enzyme (diluted in citrate buffer) was added to test tubes. At least two dilutions were required to be prepared from each sample of

enzyme. One dilution must release slightly more than 2.0 mg of glucose (e.g., 2.1 mg glucose), and the other slightly less than 2.0 mg glucose (e.g., 1.9 mg glucose). However, depending on the enzymes these targets could be difficult to achieve, thus more dilutions need to be prepared.

e) Test tubes were incubated at 47.5 ºC for exact time of 60 minutes.

f) After incubation period, test tubes were removed and enzyme reaction was immediately terminated by adding 3.0 ml DNS reagent and mixing the solutions.

Glucose standards

First, we need to prepare an anhydrous glucose stock solution (10 mg/ml). Then, we seal and store the aliquots of stock solution in the freezer. Before using standard solution, we need to thaw and vortex it to ensure proper mixing.

To prepare glucose dilutions, stock solution was diluted in the following order:

1.0 ml + 0.5 ml citrate buffer (0.05M) = 1:1.5 (3.35 mg/0.5 ml).

1.0 ml + 1.0 ml citrate buffer (0.05M) = 1:2 (2.5 mg/0.5 ml).

1.0 ml + 2.0 ml citrate buffer (0.05M) = 1:3 (1.65 mg/0.5 ml).

1.0 ml + 4.0 ml citrate buffer (0.05M) = 1:5 (1.0 mg/0.5 ml).

To prepare glucose standard tubes, 0.5 ml of each of the above solutions (glucose dilutions) was added to 1.0 ml of citrate buffer solution (0.05 M) in a 13 x 100 test tube.

Glucose standards, controls and blanks, and enzyme assay mixture tubes were incubated all together at 47.5 ºC for 60 minutes, and then terminated the reactions by adding 3.0 ml of DNS reagent solution.

Development of color

a) In a water bath, the water was boiled first; and then all test tubes (glucose standards, controls, and blanks) were placed in the boiling water for exact time of 5.0 minutes. The water in the water bath should be enough to cover the portion of test tubes, which contain solution. After boiling for exactly 5.0 minutes, test tubes were suddenly cooled down by transferring to a cold water bath containing ice.

b) Enough time was considered for settlement of pulps in the test tubes. Also, the pulps were settle faster by centrifuging test tubes.

c) Test tubes were diluted by addition of water (2.5 ml of distilled water plus 0.2 ml of color developed solution). A pipette was used for mixing the solution by sucking the mixture solution into the pipette tip repeatedly.

d) The color was determined by measuring absorbance against the reagent blank solution at wavelength of 540 nm. Glucose standards explained above may give absorbance in the range of 0.1 to 1.0.

Calculations

a) A linear glucose standard curve was constructed first (absolute amounts of glucose plotted against A540.

b) This standard curve was used for determining the amount of released glucose for each sample test tube. The amount of enzyme blank was subtracted from the obtained amount for released glucose.

c) The concentration of enzyme which was able to release exact amount of 2.0 mg of glucose was estimated by using a plot of glucose standard curve. In this case, two data points that were very close to 2.0 mg were selected and a straight line was drawn between them. This line was used to interpolate between the two points for finding the enzyme dilution which would release exactly 2.0 mg glucose equivalents of released sugar.

   Note: Generally, in this plot and in the following equation for calculating FPU, the term "enzyme" was used for proportion of the original enzyme which was presented in each enzyme dilution. In another word, the mls of the original solution that were in each ml of the dilution.

$$Cellulase\ activity\left(FPU/_{ml}\right) = \frac{0.37}{[enzyme\ concentration\ for\ releasing\ 2.0\ mg\ glucose]} \quad (C-1)$$

Table C-1 shows the change of absorbance with change of glucose concentration. These data are also used for plotting glucose standard curve as seen in figure C-1.

In this research, the employed equipment for measurement of absorbance was Biochrom UV spectrophotometer with model number of Ultraspec 50, U.K. The plot of the glucose standard curve has been shown in Figure C-1.

Table C-1. Data for glucose standard curve (DNS method).

[Glucose] (x_i)	Absorbance		Absorbance Mean (y_i)	(x_i^2)	(y_i^2)	($x_i y_i$)
	Replicate 1	Replicate 2				
(mg/ 0.5ml)	(-)	(-)	(-)	(mg/ 0.5ml)^2	(-)	(mg/ 0.5ml)
1.00	0.216	0.212	0.214	1.000	0.046	0.214000
1.65	0.343	0.337	0.340	2.723	0.116	0.561000
2.50	0.524	0.512	0.518	6.250	0.268	1.295000
3.35	0.704	0.680	0.692	11.223	0.479	2.318200
8.50			1.764	21.1950	0.908584	4.388200

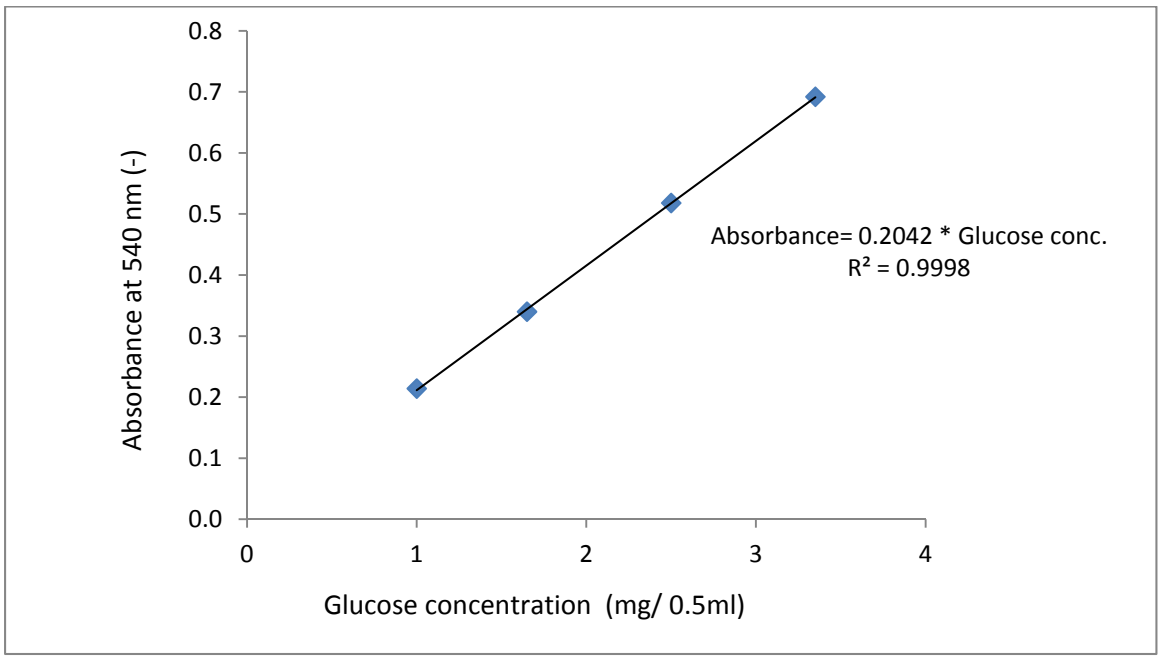

Figure C-1. Glucose standard curve (using DNS method) for measurement of cellulase enzyme activity.

In Table C-1, columns 5, 6, and 7 represent calculated values for x_i, y_i, and $x_i y_i$. The sums of values are shown at the last cell of each column. The data in this table are

used for analyzing the glucose measurements by the least square technique (Skoog et al., 2007).

Three quantities including S_{xx}, S_{yy}, and S_{xy} are used for defining the slope, and intercept as follows:

$$S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N} = 21.195 - \frac{(8.500)^2}{4} = 3.1325\ (\frac{mg}{0.5\ ml})^2$$

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} = 0.908584 - \frac{(1.764)^2}{4} = 0.13066\ (-)$$

$$S_{xy} = \sum x_i y_i - \frac{\sum x_i \sum y_i}{N} = 4.388200 - \frac{8.500 \times 1.764}{4} = 0.6397\ (\frac{mg}{0.5\ ml})$$

The slope of the line (m):

$$m = \frac{S_{xy}}{S_{xx}} = \frac{0.6397}{3.1325} = 0.20421\ (\frac{mg}{0.5\ ml})$$

The mean for x and y values:

$$\bar{x} = \frac{\sum x_i}{N} = \frac{8.500}{4} = 2.125\ (\frac{mg}{0.5\ ml})$$

$$\bar{y} = \frac{\sum y_i}{N} = \frac{1.764}{4} = 0.441\ (-)$$

The intercept (b):

$$b = \bar{y} - m\bar{x} = 0.441 - 0.20421 \times 2.125 = 0.007 \approx 0.000\ (-)$$

So, the least square line equation of the standard curve is:

$$Absorbance = 0.2042 \times [Glucose\ concentration] \qquad (C-2)$$

The standard deviation about regression (S_r):

$$S_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}} = \sqrt{\frac{0.13066 - (0.20421)^2 \times 3.1325}{4-2}} = 0.00383\ (-)$$

The standard deviation of the slope (S_m):

$$S_m = \sqrt{\frac{S_r^2}{S_{xx}}} = \sqrt{\frac{(0.00383)^2}{3.1325}} = 0.002164\ (\frac{0.5\ ml}{mg})$$

The standard deviation of the intercept (S_b):

$$S_b = S_r \sqrt{\frac{1}{N - (\sum x_i)^2 / \sum x_i^2}} = 0.00383 \times \sqrt{\frac{1}{4 - (8.500)^2 / 21.195}} = 0.00498\ (-)$$

The standard deviation of results (S_c) obtained from the standard curve:

$$S_c = \frac{S_r}{m} \sqrt{\frac{1}{M} + \frac{1}{N} + \frac{(\bar{y}_c - \bar{y})^2}{m^2 S_{xx}}}$$

$$S_c = \frac{0.00383}{0.20421} \times \sqrt{\frac{1}{2} + \frac{1}{4} + \frac{(\bar{y}_c - 0.441)^2}{0.20421^2 \times 3.1325}} \left(\frac{mg}{0.5\ ml}\right) \qquad (C-3)$$

In the above equation:
M = number of replicates
N = number of points used in the standard curve
$\bar{y}_c$= mean of the Absorbance measured in replicates 1 and 2
The confidence function (CF) was calculated at 95 % probability using the following equation:

$$CF = \pm 1.96 \times \left(\frac{S_c}{\sqrt{M}}\right)$$

Table C-2 represents the concentration of glucose measured using DNS test for dilutions of cellulase enzymes NS22086. The concentration data was used for finding a dilution that released 2.000 mg/ 0.5 ml of glucose sugar. This dilution was then applied for calculating the enzyme activity (Equation C-1). A sample of measurement of cellulase enzyme activity can be found in Appendix D (section D.4).

Table C-2. Results for dilution and glucose concentration of Cellulase enzyme stock (diluted in citrate buffer).

Run	Dilution factor	Replicate # 1		Replicate # 2		Glucose Conc. Mean ± CF	S_c
		Absorbance at 540 nm	Glucose conc.	Absorbance at 540 nm	Glucose conc.		
	(ml/ml)	(-)	(mg/ 0.5ml)	(-)	(mg/ 0.5ml)	(mg/ 0.5ml)	(mg/ 0.5ml)
1	0.0166	0.956	4.682	0.974	4.770	4.726 ± 0.18	0.13
2	0.0100	0.918	4.496	0.914	4.476	4.486 ± 0.05	0.03
3	0.0067	0.602	2.948	0.595	2.914	2.931 ± 0.07	0.05
4	0.0050	0.598	2.929	0.592	2.899	2.914 ± 0.05	0.04
5	0.0033	0.396	1.939	0.385	1.885	1.912 ± 0.03	0.08

Table C-3 represents enzyme activity of cellulase (NS22086) using filter paper unit technique. Also, the absorbance for duplicate runs with the mean, standard deviation (SD), and the confidence function (CF) at 95 % of probability are indicated in this table. The standard deviation (SD) was calculated using the equation C-4.

$$SD = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \qquad (C - 4)$$

The confidence function (CF) at 95% probability level was calculated using equation C-5:

$$CF = 1.96 \times \left(\frac{SD}{\sqrt{n}}\right) \qquad (C - 5)$$

"n" is equal to number of replicates.

Table C-3. Summary of duplicate runs of cellulase enzymes activity (FPU) measured by filter paper unit technique.

Cellulase enzyme	Replicate # 1		Replicate # 2		Cellulase activity Mean ± CF	SD
	[Enzyme]	Cellulase activity	[Enzyme]	Cellulase activity		
	(ml/ml)	FPU/ ml	(ml/ml)	FPU/ ml	FPU/ ml	FPU/ ml
NS22086	0.00363	102	0.00366	101	102 ± 0.98	0.71

Appendix D: Examples of calculations

D.1 Total solids and moisture content of wheat straw

An amount of 2.000 grams of wheat straw was weighed and dried at 105 °C in a convection oven, Thermolyne 9000, U.S.A. The percent of total solids and moisture content of replicate 1 (Table A-1) for untreated wheat straw was calculated as follow:

$$\%Total\ solids\ = \frac{1.844}{2.000} \times 100 = 92.21\ wt.\%$$

$$\%Moisture\ content\ = 100 - 92.21 = 7.79\ wt.\%$$

The mean of total solids percentage for Replicate 1 and 2 (Table A-1) was calculated as follow:

$$\%Total\ solids\ mean = \frac{92.21 + 92.44}{2} = 92.33\ wt.\%$$

The standard deviation (SD) of total solids percentage for Replicate 1 and 2 (Table A-1) was calculated as follow (using equation A-3):

$$SD = \sqrt{\frac{(92.21 - 92.33)^2 + (92.44 - 92.33)^2}{2 - 1}} = 0.16\ wt.\%$$

The confidence function (CF) of total solids percentage for Replicate 1 and 2 (Table A-1) at probability of 95 % was calculated as follows (using equation A-4):

$$CF = 1.96\ \times \left(\frac{0.16}{\sqrt{2}}\right) = 0.22\ wt.\%$$

The relative percent difference (RPD) of total solids percentage for Replicate 1 and 2 (Table A1) at probability of 95 % was calculated as follows (using equation A-5):

$$\% RPD = \frac{|92.21 - 92.44|}{\frac{(92.21 + 92.44)}{2}} \times 100 = 0.25\ wt.\%$$

D.2 Enzymatic hydrolysis

The yield of released sugars for AFEX-treated wheat straw under AFEX treatment conditions of temperature 95 °C, moisture 70%, and ammonia loading 1:1 after 164 hours of hydrolysis time in Figure 4-6, and Table B-9, replicate 1 was calculated as follow:

An amount of 2.000 grams of AFEX-treated wheat straw was used for determination of total solids according to the procedure described in Appendix A. The percent of total solids was found to be 94.25 wt. %. Another 2.000 grams of AFEX-treated wheat straw sample was considered for enzymatic hydrolysis. The total solids were calculated using the percent of total solids calculated earlier and rearranging Equation A-1.

$$Total\ solids = 2.000 \times \frac{94.25}{100} = 1.885\ g$$

The theoretical released sugars were calculated using Equation B-6:

$$Theoretical\ released\ sugars = 6.281\ {}^{mg}/_{gml} \times 1.885\ g = 11.840\ {}^{mg}/_{ml}$$

After 164 hours of enzymatic hydrolysis, three samples were taken from buffer blank, hydrolysis, and enzyme blank as explained in the Appendix B (enzymatic hydrolysis procedure). The absorbance for samples was measured using UV

spectrophotometer at 540 nm. Buffer blank was used as the reference. The absorbance of the enzyme blank was negligible in this experiment. Released sugars concentration for the enzymatic hydrolysis sample was calculated from relevant absorbance (1.472) using the glucose standard curve of Figure B-1.

$$Measured\ released\ sugars = 10.296\ {}^{mg}/_{ml}$$

Thus, the released sugars yield (% theoretical) was calculated using Equation B-7 as follow:

$$Yield\ of\ released\ sugars = \frac{10.296\ {}^{mg}/_{ml}}{12.562\ {}^{mg}/_{ml}} \times 100 = 81.96\ \%\ theoretical$$

D.3 The mean($\bar{x}$), standard deviation (SD), and confidence function (CF) for enzymatic hydrolysis

The mean($\bar{x}$), standard deviation (SD), and confidence function (CF) at 95 % probability For AFEX-treated wheat straw produced under AFEX conditions of temperature 95°C, ammonia loading 0.5:1, moisture content 70% after 2 hours enzymatic hydrolysis (Appendix B) were calculated as follow:

The mean ($\bar{x}$),

$$\bar{x} = \frac{x_1 + x_2}{2}$$

$$\bar{x} = \frac{17.66\ wt.\% + 15.96\ wt.\%}{2} = 16.81\ wt.\%$$

The standard deviation (SD),

$$SD = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

$$SD = \sqrt{\frac{(17.66 - 16.81)^2 + (15.96 - 16.81)^2}{2 - 1}} = 1.20$$

The confidence function (CF) at 95 % probability,

$$CF = 1.96 \times \left(\frac{SD}{\sqrt{n}}\right)$$

$$CF = 1.96 \times \left(\frac{1.20}{\sqrt{2}}\right)$$

$$CF = 1.67$$

D.4 Measurement of enzyme activity

The cellulase enzyme activity for enzyme NS22086 in the replicate 1 of Table C-2 (Appendix C) was calculated as follows:

All enzyme (NS22086) dilutions were prepared in citrate buffer (pH = 5.0), as shown in the Table D-1 from a working enzyme stock solution that was diluted 1:15 in citrate buffer solution.

Table D-1. Dilution of Cellulase enzyme NS22086 from enzyme stock that had been diluted 1:15 in citrate buffer.

Dilution	Citrate buffer	1:15 Enzyme	Dilution factor	Absorbance at 540 nm	[Glucose]
	(ml)	(ml)	(ml/ml)	(-)	(mg/ 0.5 ml)
1	1.65	0.35	0.0166	0.956	4.682
2	1.70	0.30	0.0100	0.918	4.496
3	1.80	0.20	0.0067	0.602	2.948
4	1.85	0.15	0.0050	0.598	2.929
5	1.90	0.10	0.0033	0.396	1.939

The term "dilution factor" represents the proportion of the original enzyme solution in the diluted solution added to the mixture of assay. For example, in dilution number 3 of Table D-1, a 1:10 dilution of the 1:15 working stock solution of enzyme has:

$$Dilution\ factor = \left(\frac{0.2}{\frac{0.2 + 1.80}{15}} \right) = 0.0067$$

After enzymatic hydrolysis (Appendix C), concentration of glucose of the cellulase enzyme assays were determined using glucose standard curve (Figure C-1). According to Table C-2, the cellulase enzyme dilution factor which releases 2.0 mg/ 0.5 ml glucose is between 0.0050 and 0.0033. Linear interpolation between these two

dilutions indicates that the enzyme dilution which releases 2.0 mg/ 0.5 ml of glucose is equal to 0.00363. Thus, the cellulase activity is calculated as follow (using Equation C-1):

$$Cellulase\ activity\left(^{FPU}/_{ml}\right) = \frac{0.37}{0.00363} = 102\ FPU/ml$$

REFERENCES

Abraham, M., Kurup G.M. (1997). Pretreatment studies of cellulose wastes for optimization of cellulase enzyme activity. Applied Biochemistry and Biotechnology, 62(2-3), 201-211.

Agblevor, F. A., Chum, H. L., Johnson, D. K. (1993). Compositional analysis of NIST Biomass Standards from the IEA Whole Feedstock Round Robin. Energy from Biomass and Wastes XVI: Proceedings of the Institute of Gas Technology Conference, 2-6 March 1992, Orlando, Florida. Chicago, IL: Institute of Gas Technology; 395-421.

Agriculture and Agri-Food Canada. (2003). Tillage Practices that Reduce Soil Erosion. Retrieved September 14, 2012, from: http://www.agr.gc.ca/pfra/soil/tillage_e.htm.

Agriculture and Agri-Food Canada. (2010). Market Outlook Report. Retrieved September 14, 2012, from: http://www.agr.gc.ca/pol/mad-dam/index_e.php?s1=pubs&s2=rmar&s3=php&page=rmar_02_06_2010-11-26.

Alizadeh, H., Teymouri, F., Gilbert, T. I., Bruce, E. D. (2005). Pretreatment of switchgrass by Ammonia Fiber Explosion (AFEX). Applied Biochemistry and Biotechnology, 121-124, 1133-1142.

Barry A.J., Peterson E., King A.J. (1936). X-ray study of the decomposition product of the cellulose complex. Journal of American Chemical Society, 58: 333

Bloomberg New Energy Finance (2013). Cellulosic ethanol heads for cost competitiveness by 2016. Retrieved July 02, 2013, from: http://about.bnef.com/press-releases/cellulosic-ethanol-heads-for-cost-competitiveness-by-2016/.

Campbell, N.A., Reece, J.B., Mitchell, L.G. (1999). Biology fifth edition, Benjamin. Cummings, 102-205.

Canadian Environmental Protection Act, 1999- Renewable Fuels Regulations-P.C. 2010-1080. (2010, August 23). Retrieved May 28, 2012, from Government of Canada: http://www.gazette.gc.ca/rp-pr/p2/2010/2010-09-01/html/sor-dors189-eng.html

Dale B.E., Moreira MJ. (1983). A freeze explosion technique for increasing cellulose hydrolysis. Biotechnology and Bioengineering Symposium, 12, 31-43.

Dale, B.E., Henk L., Shiang M. (1985). Fermentation of lignocellulosic materials treated by Ammonia Freeze Explosion. Development Industrial Microbiology, 26, 223-233.

Davis, D. (1987). Prevention Reference Manual: Chemical Specific, Volume 4: Control of Accidental Releases of Ammonia (SCAQMD), U.S. Environmental Protection Agency, Research Triangle Park, NC.

Demirbas, A. (2005). Bioethanol from cellulosic materials: A renewable motor fuel from biomass. Energy Sources, 27 (4), 327-337.

Food and Agricultural Policy Research Institute (2008). FAPRI 2008 U.S. and World Agricultural Outlook. Retrieved January 06, 2013, from: http://www.fapri.iastate.edu/outlook/2008/.

Ghose, T.K. (1987). Measurement of cellulase activities, Pure and Applied Chemistry, 59(2), 257-260.

Grohmann K, Wyman CE, Himmel ME. (1992). Potential for fuels from biomass and wastes, *In*: Emerging Technologies for Materials and Chemicals from Biomass. Eds. R.M. Rowell, T.P. Schultz, ACS Symposium Series 476, American Chemical Society, Washington, DC, 354-392.

Holtzapple, M.T., Jun, J.H., Ashok, G., Patibandla, S.L., Dale, B.E. (1991). The ammonia freeze explosion (AFEX) process: a practical lignocellulose pretreatment. Applied Biochemistry and Biotechnology (28/29), 59–74.

Kim, M., Aita, G., Day, D.F. (2004). Compositional changes in sugarcane bagasse on low temperature, long-term diluted ammonia treatment. Applied Biochemistry and Biotechnology, 161 (1-8), 34-40.

Kim, S., Dale, B.E. (2004). Global potential bioethanol production from wasted crops and crop residues. Biomass and Bioenergy, 26, 361–375.

Kirk-Othmer, D. (1992). Encyclopedia of Chemical Technology, 4th edition, John Wiley and Sons, New York.

Kumar, P., Barrett, D. M., Delwiche, M. J. and Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Research, 48, 3713–3729.

Lamptey J., Moo-Young M., and Robinson C.W., (1986). Pretreatment of lignocellulosic for bioconversion applications: process options. *In*: Biotechnology and Renewable Energy, Eds. Moo-Young M., Laptey J. Elsevier Applied Science Publishers, London and New York, 46-56.

Lewin M., Roldan L.G. (1971). The effect of liquid anhydrous ammonia in the structure and morphology of cotton cellulose. Journal of Polymer Science, Part C 36, 213-229.

Licht, F.O. (2006). World Ethanol Market: The Outlook to 2015, Tunbridge Wells, Agra Europe Special Report, UK.

Mansfield, S.D., Mooney, C., Saddler, J.N. (1999). Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnology Progress, 15, 804-816.

Meher, L.C., Vidya Sagar, D., Naik, S.N. (2006). Technical aspects of biodiesel production by transesterification - a review. Renewable and Sustainable Energy Reviews, 10, 248-268.

Moniruzzaman, M., Dale, B., Hespell, R., Bothast, R. (1997), Enzymatic hydrolysis of high-moisture com fiber pretreated by AFEX and recovery and recycling of the enzyme complex. Applied Biochemistry and Biotechnology, 67, 113-126.

Mosier, N., Wyman, C., Dale, B.E., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 96, 673-686.

National Renewable Energy Laboratory (NREL). (1995). Chemical Analysis and Testing Laboratory Analytical Procedures (LAP). Retrieved June 10, 2012 from http://infohouse.p2ric.org/ref/40/39182.pdf.

Novozymes Bioenergy. (2012). Enzymes for the hydroloysis of lignocellulose materials. Retrieved July 18, 2012 from http://www.bioenergy.novozymes.com/en/cellulosic-ethanol/samples/Documents/Application_Sheet_Cellulosic_ethanol_enzyme_kit_Final.pdf.

O'Conner, J. J. (1972). Ammonia Explosion Pulping, a new fiber separation process. Tappi 55(3), 353-358.

Schell, D.J. and Duff, B. (1996). Review of pilot plant programs for bioethanol conversion, *In*: Handbook on Bioethanol: Production and Utilization, Eds. C.E. Wyman, Taylor and Francis, Washington, DC, 381-394.

Schuerch, C. (1963). Plasticizing wood with liquid ammonia. Journal of Industrial and Engineering Chemistry, 55:39.

Skoog, D. A., Holler, F. J. and Crouch, S. R. (2007). Principles of Instrumental Analysis. Belmont, USA: Thomson Brooks/Cole.

Sokhansanj, S., Mani, S., Stumborg, M., Samson, R., Fenton, J. (2006). Production and distribution of cereal straw on the Canadian prairies. Canadian Biosystems Engineering, 6, 39-46.

Sun, Y., Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology 83, 1–11.

Tarchevsky, I., and Marchenko, G.N. (1991). Cellulose: Biosynthesis and Structure. Springer-Verlag New York, NY, 9-31, 335.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B.E. (2004). Ammonia fiber explosion treatment of corn stover. Applied Biochemistry and Biotechnology, 113, 951–963.

The Canadian Encyclopedia. (2012). Wheat. Retrieved September 25, 2012, from: http://www.thecanadianencyclopedia.com/articles/wheat.

Varga, E. (2003). Bioethanol production: pre-treatment and enzymatic hydrolysis of corn stover. Biochemistry, 90, 637-48.

Wang, P. Y., Bolker, H. I., and Purves, C. B. (1967). Uronic acid ester groups in some softwoods and hardwoods. Tappi 50, 123–124.

Wilke, C.R., Maiorella, B., Sciamanna, A., Tangnu, K., Wiley D., Wong, H. (1983). Enzymatic Hydrolysis of Cellulose: Theory and Application, Volume 218: Theory of Enzymatic Hydrolysis, Noyes Data Corporation, Park Ridge, NJ.

Wyman, C.E. (1996). Ethanol production from lignocellulosic biomass: overview *In*: Handbook on Bioethanol: Production and Utilization. Eds. Wyman, C.E., Taylor and Francis, Washington DC, 11-12.

Zimbardi, F., Viola, E., Gallifuoco, A., De Bari, I., Cantarella, M., Barisano, D., Braccio, G. (2002). Overview of the Bioethanol Production. Report ENEA and University of L'Aquila in the framework of the EU project "Production of clean hydrogen from fuel cells by reformation of bioethanol". Retrieved from www.cti2000.it.