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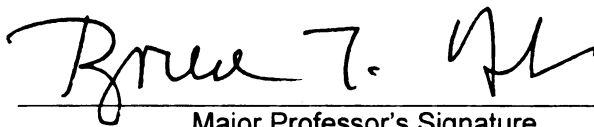
ENZYMATIC HYDROLYSIS OF AMMONIA FIBER
EXPLOSION (AFEX) PRETREATED REED CANARYGRASS
AND SWITCHGRASS

presented by

Tamika Calicia Bradshaw

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Materials Science Engineering



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**ENZYMATIC HYDROLYSIS OF AMMONIA FIBER EXPLOSION (AFEX)
PRETREATED REED CANARYGRASS AND SWITCHGRASS**

By

Tamika Calicia Bradshaw

A THESIS

Submitted to
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In partial fulfillment of the requirements
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ABSTRACT

ENZYMATIC HYDROLYSIS OF AMMONIA FIBER EXPLOSION (AFEX) PRETREATED REED CANARYGRASS AND SWITCHGRASS

By

Tamika Calicia Bradshaw

Enzymatic hydrolysis is a chemical process which extracts sugar polymers from lignocellulose by the addition of a water molecule. However, lignocellulosic biomass is resistant to enzymes as a result of the unreactive nature of cellulose to chemicals. The strong bonding between cellulose, hemicellulose, and lignin within the crystalline regions of lignocellulose makes it rather difficult to access the sugar polymers in the cell wall. An effective pretreatment is essential to permit cellulose to become susceptible to enzymatic hydrolysis thereby allowing the retrieval of these sugars. Ammonia Fiber Explosion (AFEX), a physiochemical pretreatment, has shown to decrease cellulose crystallinity and particle size, while increasing the surface area exposed to enzymatic attack. AFEX weakens the association among lignin, hemicellulose, and cellulose facilitating more hydrolysis.

In this study, reed canarygrass (RCG) and switchgrass (SWG) were pretreated using AFEX, enzymatically hydrolyzed, and analyzed for glucose and xylose. The pretreatment conditions tested were temperature and ammonia loading at constant moisture content (60%). The objectives of this study were to evaluate suitable AFEX pretreatment conditions and determine glucose and xylose yields possible at different stages of RCG and SWG growth.

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DEDICATION

*In memory of Mary J. Robertson,
1923-1990*

To my heavenly father, who has given me this incredible gift of learning and intelligence, thank you so very much for blessing me with the mind and ability to achieve higher goals in life. To my mother, Gwendolyn G. Bradshaw: I thank you for all of your support throughout the years. You have dedicated so much of yourself to give me the chance at a better life. Your strength, your will, your love has helped me to become the woman that I am. To my father, Calvin R. Bradshaw: I thank you for your support and for always believing in me. You always told me that if I worked hard, I would accomplish great things. Thank you for all instilling that in me. To my brother, CJ, who has always stood by my side: I thank you for all of your encouraging words and guidance. You are truly the best! To my fiancé, DJ: Thank you for being a friend when I needed it most. I can't wait to begin the next chapter of my life with you. To my family and friends: Thank you for your continued support and prayers. Whenever I needed you, you were always there. I love you all!

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ABBREVIATIONS

AFEX-	Ammonia Fiber Explosion
DM-	Dry Material
FPU-	Filter Paper Unit
FSWG-	Frost of Switchgrass
GLU-	Glucose
HPLC-	High-Performance Liquid Chromatography
LAP-	Laboratory Analytical Procedure
M.C.-	Moisture Content
MW-	Molecular Weight
NREL-	National Renewable Energy Laboratory
NSC-	Non-Structural Carbohydrates
pp-	Percentage Points
RCG-	Reed Canarygrass
S.C.-	Solid Content
SC-	Structural Carbohydrates
SRCG-	Seed of Reed Canarygrass
SWG-	Switchgrass
VRCG-	Vegetative Reed Canarygrass
WM-	Wet Biomass
XYL-	Xylose

Chapter 1

Introduction

The U.S. fuel ethanol industry is currently producing over 3 billion gallons per year of ethanol from starch and sugar sources, primarily corn grain. However, starches and sugars are only a small fraction of total biomass materials. Biomass is the only potentially renewable source of organic chemicals, organic materials and liquid transportation fuels [1]. Biomass is also relatively inexpensive and compares favorably with petroleum on a cost per pound basis and, frequently, on a cost per unit of energy [1]. However, the cellulosic portion of biomass represents an immense source of sugars which awaits development of the technology necessary for its economical utilization [2]. Cellulose and hemicellulose form the bulk of most plant materials (biomass), and effective ethanol production from these components can expand the types and amount of available feedstock [3]. Cellulosic biomass as an alternate feedstock could provide very large quantities of ethanol with considerable environmental benefits. Bioethanol is an admirable alternative fuel currently used as a gasoline additive to reduce carbon monoxide and other toxic air emissions, ground level ozone formation, and to boost octane. However, there is a key problem with the ability to break cellulose and hemicellulose down into their sugar components.

The chemically unreactive nature of cellulose, in particular its resistance to hydrolysis and the close association among lignin, hemicellulose, and cellulose makes it rather difficult to convert to sugars [3, 4]. Enzymatic hydrolysis of this

material is possible but requires treatment of the lignocellulosic material before the enzymes can access the sugar polymers [4]. Pretreatment processes produce a pretreated biomass that is more amenable to enzymatic hydrolysis by cellulases and related enzymes than native biomass.

The ammonia fiber explosion (AFEX) process may offer both an effective and economically attractive means of increasing yields of fermentable sugars from lignocellulosic biomass [5]. AFEX is a process in which liquid ammonia is used to treat biomass in a pressure vessel at a desired residence time (approximately 5 minutes) for a given temperature, moisture content and ammonia loading. The pressure is released causing the biomass to explode. After the ammonia has evaporated, the biomass is prepared for hydrolysis. AFEX has been shown to decrease cellulose crystallinity and particle size, while increasing the surface area exposed to enzymatic attack [6]. AFEX weakens the association among lignin, hemicellulose, and cellulose facilitating more hydrolysis.

In this study, the biomass materials used were: reed canarygrass (RCG) and switchgrass (SWG). SWG is a sod-forming, warm-season grass, which was an important component of the native, highly productive North American tallgrass prairie [7]. SWG is a productive herbaceous crop valuable for its flexibility both as a forage species and as a biofuel raw material, its positive environmental attributes, including low nutrient use, low pesticide requirements, and its perennial growth habit [8]. RCG is also a potential biofuel raw material,

and is a cool season grass alternative to SWG [8]. RCG's rhizomatous growth habit also makes it appealing, particularly on soils which SWG, a bunchgrass, does not form thick stands and where erosion is a problem [8]. AFEX pretreatment and enzymatic hydrolysis was performed to determine the glucose and xylose yields possible at different stages of RCG and SWG growth. The conversion yields were accessed by a High Performance Liquid Chromatography (HPLC) unit. The objectives of this research were to evaluate suitable AFEX treatment conditions for SWG and RCG, estimate glucose and xylose yields obtainable, and to determine the most effective AFEX condition as measured by glucose and xylose hydrolysis profiles. The most effective condition implies to the AFEX treatment condition that provided the highest % conversions achieved for both glucose and xylose observed for the selected materials.

Chapter 2

Background

2.1 Biomass

Biomass is a very heterogeneous and chemically complex renewable resource [1]. The term *biomass* means any plant-derived organic matter [1]. Biomass feedstocks include herbaceous and woody energy crops, agricultural food and feed crops, agricultural crop wastes and residues, wood wastes and residues, aquatic plants, and other waste materials including some municipal wastes [9]. The use of lignocellulose (biomass) has the potential to solve or mitigate problems, such as global warming, balance of trade, Third World debt, air pollution, trash disposal, soil erosion, herbicide/pesticide accumulation, deforestation, and fuel shortages [10]. Biomass conversion to ethanol would provide both an environmentally and economically attractive alternative means for gasoline. Figure 2.1 illustrates the process of converting biomass to ethanol.

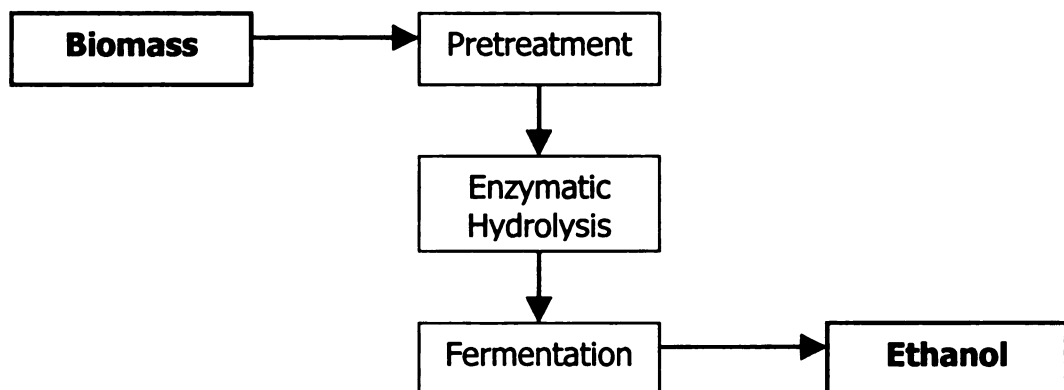


Figure 2.1: Process for biomass conversion to ethanol.

Biomass materials consist of three primary constituents within the plant cell wall: cellulose, hemicellulose, and lignin. The lignin, hemicellulose, and cellulose (lignocellulose) have a special three-dimensional relationship and through extensive cross-linking they comprise the very rigid cell wall matrix of the plant [11]. The proportion of lignin, hemicellulose, and cellulose varies among plant species, plant parts, and the stage of growth [11]. These materials prevent cellulolytic enzymes (enzymes containing cellulase) from contacting a sufficient number of glucosidic links in the cellulose to permit significant hydrolysis [12].

2.2 Cell Wall

Plant cell walls have a number of functions: they provide rigidity to the cell for structural and mechanical support, maintaining cell shape, the direction of cell growth as well as preventing expansion when water enters the cell [13]. The cell wall, as defined by Oxford, is “the rigid outer layer that surrounds the plasma membrane of plant, fungal, algal, and bacterial (but not animals) cells [14].” Complex carbohydrates (sugars) such as cellulose, hemicellulose, and lignin form the cell wall of the plant. These carbohydrates are known as the structural (SC) or non-soluble carbohydrates. Non-structural (NSC) or soluble carbohydrates are simple carbohydrates (i.e. starches and sugars) stored inside the cell and used as an energy source for plants. Figure 2.2 illustrates where the structural and non-structural carbohydrates are found within the cell.

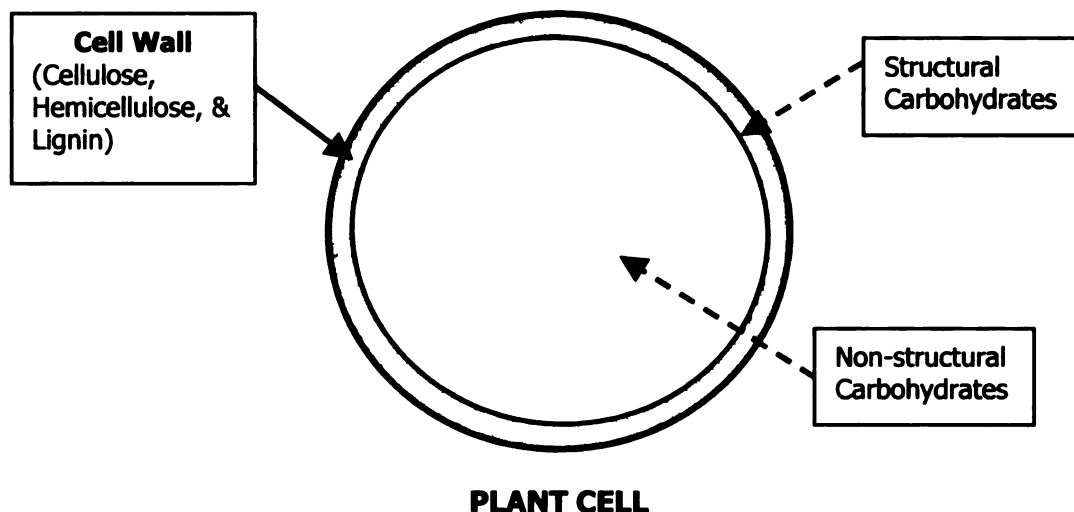


Figure 2.2: Structural and non-structural carbohydrates within the plant cell. (The dark gray region represents the cell wall of the plant, where cellulose, hemicellulose and lignin reside.)

2.2.1 Cellulose

Cellulose, by far the most abundant organic material on earth, forms the bulk of the cell wall material of all higher plants [12]. Cellulose is a polysaccharide which consists of a long unsplit chain of glucose units. It is the main constituent of the cell walls of all plants and is responsible for providing the rigidity of the cell wall [14]. Cellulose is one of man's least expensive and most useful natural resources [12]. It is unique among major industrial raw materials in being available from renewable and therefore potentially inexhaustible sources of supply [12].

In a plant, cellulose chains are surrounded by hemicelluloses and lignin. The hemicellulose, and lignin are closely packed within the crystalline regions of the cell wall causing a strong attraction between them. These compounds shield

the cellulose from cellulase enzymes and may even irreversibly absorb some of these enzymes [15]. Therefore, the crystalline cellulose makes it difficult to access the monosaccharides due to its resistance to enzymatic hydrolysis. If the lignin and hemicellulose sheath can be removed or reduced, effective microbial degradation can occur to obtain glucose, xylose, and other simple sugars [16].

2.2.2 Hemicellulose

Hemicellulose is a polysaccharide found in the cell walls of plants and it binds to cellulose microfibrils to form a network of cross-linked fibres [14]. Unlike cellulose which is composed completely of polymerized glucose, hemicellulose is a complex linkage of a mixture of sugars containing primarily xylose [15].

2.2.3 Lignin

Lignin is a virtually indigestible portion of plant material which gives mechanical strength and is known to inhibit utilization of cellulose and hemicellulose [16]. Oxford describes lignin as “a complex organic polymer that is deposited within the cellulose of plant cell walls during secondary thickening; Lignification makes the walls woody and therefore rigid [14].” However, lignin does not contain carbohydrates. It consists of a highly condensed phenylpropanoid polymer of high molecular weight. Lignin has also been shown to vary widely according to the maturity of the plant due to the varying

proportions of different monomers [17]. However, the presence of lignin in the cell wall impedes enzymatic hydrolysis of carbohydrates [18].

2.3 Pretreatment

Pretreatment is an essential process for enhancing the reactivity of enzymatic hydrolysis. It alters the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrates into fermentable sugars [18]. Pretreatment processes produce a solid pretreated biomass residue that is more amenable to enzymatic hydrolysis by cellulases and related enzymes than native (untreated) biomass.

Pretreatments are classified as physical, chemical, or physiochemical. Physical pretreatments alter the appearance and structure of the biomass by physical means to reduce the particle size of the biomass. Examples of physical treatments include ball milling to very small mesh sizes, two-roll milling, attrition milling and sieving [19]. Such milling reduces the crystallinity of the cellulose, increases the surface area and bulk density, and decreases the lignin sheath [2]. Chemical pretreatments cause the cellulose to swell and decrystallize. Chemicals penetrate through the crystalline regions of the cellulose causes the structure to disrupt [2]. Most chemical treatments involve hydrolytic and oxidative agents [11]. Physiochemical pretreatments involve both a chemical reaction and physical change of the structure [11]. AFEX is an example of a physiochemical pretreatment.

2.4 Ammonia Fiber Explosion (AFEX)

The AFEX process treats lignocellulose with high-pressure liquid ammonia and then explosively releases the pressure [20]. AFEX is a physiochemical pretreatment whose combined chemical effect (cellulose decrystallization) and physical effect (increased accessible surface area) dramatically increases lignocellulose susceptibility to enzymatic attack [20]. There are several parameters that can be altered when using AFEX such as ammonia loading, moisture content, temperature, residence time, and pressure to improve hydrolysis. Ammonia treated material is a suitable substrate for enzymatic hydrolysis, single-cell protein production, cellulase enzyme production and ethanol production.

2.5 Enzymatic Hydrolysis

Hydrolysis is a chemical process in which a molecule is cleaved into two parts by the addition of a water molecule. During enzymatic hydrolysis, the biomass samples consist of a glucan and xylan content which converts to glucose and xylose, respectively, by the addition of water. Glucan is glucose in the anhydrous form (meaning loss of water molecule). The molecular weight of glucan ($C_6H_{10}O_5$) is 162 g/mol and 180 g/mol for glucose ($C_6H_{12}O_6$). The difference between the two forms of glucose is the loss of a water molecule (H_2O : 18 g/mol). In addition, xylan is an anhydrous xylose ($C_5H_8O_4$: 132 g

/mol), and differs from xylose ($C_5H_{10}O_5$: 150 g/mol) by a loss of water molecule.

The conversion factors used to convert the anhydrous sugar forms to its corresponding sugar components are presented in section 3.2.3.2.

Enzymatic hydrolysis of cellulose is attractive because of its specificity and absence of the competitive degradation which normally accompanies acid hydrolysis [2]. Enzymatic hydrolysis of lignocellulosics is a heterogeneous reaction and is therefore, influenced by the structural features of the biomass such as crystallinity of cellulose, lignin content and surface area [17, 21]. Crystalline cellulose refers to aggregates of cellulose polymers held tightly together by extensive hydrogen bonding and therefore water molecules are excluded from the crystalline inner structure [11]. The inability of water to infiltrate the micro fibril prevents hydration of the internal cellulose polymers of the micro fibril, which in turn prevents cellulose hydrolysis by cellulolytic enzymes [22]. Cellulase is a carbohydrate-digesting enzyme (a carbohydrase) that hydrolyses cellulose to sugars, including cellobiose (a disaccharide consisting of two β - (1, 4) linked molecules of glucose) and glucose [14]. Cellulase breaks the β -glycosidic links that join the constituent sugar units of cellulose [14].

Chapter 3

Enzymatic Hydrolysis of AFEX Pretreated Reed Canarygrass and Switchgrass

3.1 Materials

Materials utilized were: the vegetative stage of RCG (VRCG), the seed stage of RCG (SRCG), and the frost stage of SWG (FSWG). The vegetative stage (the period when leaves begin to grow) is the earliest stage of grass maturity. It is followed by the jointing, boot, heading, blooming, and finally, the seed development. The frost (for SWG) used in this study was the “killing frost” or “post frost” seedling that occurs after harvest (October to November). RCG was dried and ground through a 1-mm screen in a Wiley mill at Dairy Forage Research Center (Madison, WI). SWG was dried at 50°C and ground through a 2-mm screen in a Wiley mill. Samples of RCG and SWG are shown in figure 3.1.

Images in this thesis are presented in color.

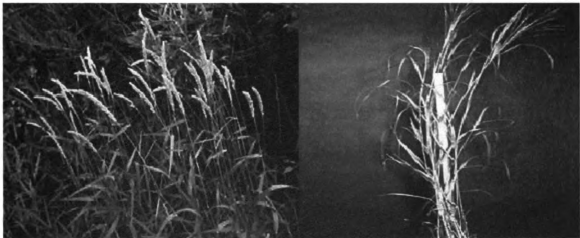


Figure 3.1: Samples of RCG (left) and SWG (right) [23].

The cell wall carbohydrates, glucan and xylan, and the carbohydrate composition for each of these materials are summarized in Table 3.1 for RCG and SWG. Furthermore, table 3.2 provides an analysis of the glucose composition for the materials. Refer to Appendix A for complete data on the carbohydrate composition for these materials and for a detailed explanation for glucose percentages of these soluble carbohydrates (provided for table 3.2).

Table 3.1: Carbohydrate composition of biomass samples (g/kg DM) [24].

<u>Species†</u> Stage	<u>Cell Wall Carbohydrates</u>							
	Glc	Xyl	Ara	Gal	Man	Rha	Fuc	UA
<u>Reed Canarygrass</u>								
Veg (VRCG)	209	117	30	16	6	1	1	22
Seed (SRCG)	265	163	28	13	6	1	1	21
<u>Switchgrass</u>								
Frost (FSWG)	322	223	30	12	5	2	1	7
	<u>Soluble Carbohydrates</u>						<u>Total Carbohydrates</u>	
	Glc	Fru	Suc	Raf	Sta	Storage [‡]	NSC	SC
<u>Reed Canarygrass</u>								
Veg (VRCG)	4	5	69	3	0	35	116	402
Seed (SRCG)	2	12	30	0	1	54	99	498
<u>Switchgrass</u>								
Frost (FSWG)	7	7	13	0	0	7	34	602

NSC- Non- structural Carbohydrates SC- Structural Carbohydrates

†Data for reed canarygrass and switchgrass are for whole herbage (g/kg DM).

Glc, glucose; Xyl, xylose; Ara, arabinose; Gal, galactose; Man, mannose; Rha, rhamnose; Fuc, fucose; UA, uronic acids; Fru, fructose; Suc, sucrose; Raf, raffinose; Sta, stachyose

‡Storage carbohydrate for switchgrass was starch. VRCG had fructans and SRCG had both fructans and starch (37 and 17 g/kg DM, respectively).

Table 3.2: Glucose composition of biomass samples (g/kg DM).

Soluble (NSC) Carbohydrates	Glu Content (g/kg DM)		
	VRCG	SRCG	FSWG
Glu	4	2	7
Sucrose	35	15	13
Raffinose	1	0	0
Stachyose	0	0.25	0
Storage	12	29	7
Total Glu in NSC	52	46.25	27
Total Glu in SC	209	265	322
Total Glu	261	311	349

Note that the for storage carbohydrates of SRCG, 17 g/kg DM is of starch and 12 g/kg DM is from fructans.

3.2 Experimental Methods

3.2.1 AFEX Pretreatment

Materials were AFEX treated with anhydrous liquid ammonia in a laboratory-scale batch ammonia reactor unit. See figure 3.2 for the AFEX equipment used. The AFEX pretreatment conditions varied were temperature (80°C - 120°C) and ammonia loading (0.8, 1.0, and 1.2 kg per 1 kg DM) at 60% moisture content (dwb). Following ammonia addition and heating to the desired temperature, the temperature was held for a residence time of 5 minutes and then the pressure was explosively released. After AFEX pretreatment, the materials were allowed to air dry overnight under a fume hood to evaporate residual ammonia. The AFEX pretreated material was collected in plastic bags and refrigerated for later use. See Appendix C for complete AFEX procedure. AFEX treated and untreated each of materials are displayed in figures 3.3, 3.4, and 3.5, respectively.

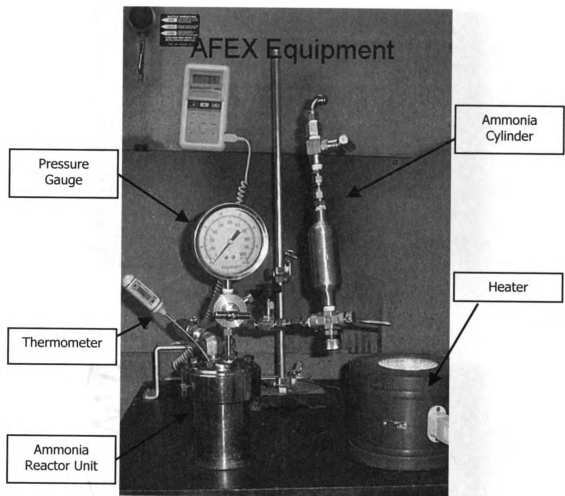


Figure 3.2: AFEX Equipment.

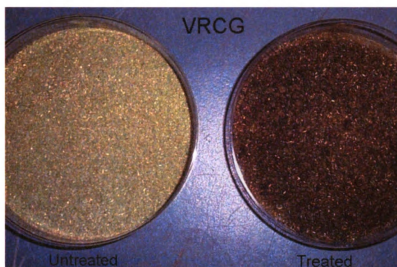


Figure 3.3: Untreated and AFEX treated VRCG.

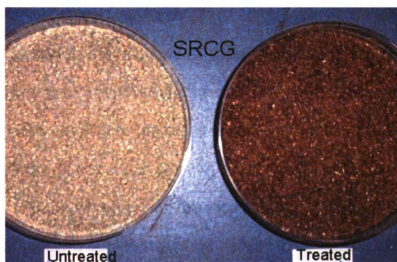


Figure 3.4: Untreated and AFEX treated SRCG.

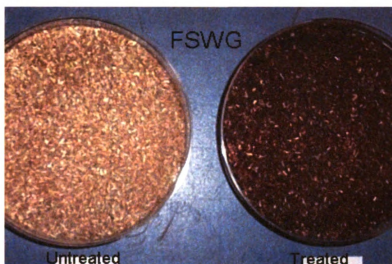


Figure 3.5: Untreated and AFEX treated FSWG.

3.2.1.1 Pretreatment Conditions

The objectives of this study were to test the effects of different AFEX conditions on enzymatic hydrolysis for the different growth stages of RCG and SWG. The initial pretreatment conditions tested were: 80°C, 90°C, 100°C for temperature; 0.8, 1.0, and 1.2 kg per 1 kg dry material (DM) for ammonia loading; 60% M.C. However, during experimentation, it was concluded that pretreatment conditions outside this range needed to be tested to fulfill the objective for this study. Testing outside the range also provided an insight on the effect that temperature and ammonia loading had on each of the materials tested. Elevated temperatures (110°C and 120°C) and ammonia loadings (1.4 and 1.6 kg per 1kg DM) were tested to determine the most effective pretreatment condition for each material. Table 3.3 provides all treatment conditions tested for VRCG, SRCG, and FSWG.

Table 3.3: AFEX Treatment Conditions tested for VRCG, SRCG, and FSWG.

Material	Temperature (°C)	MC (%)	Ammonia Loading (kg DM: kg ammonia)	Residence Time (min)
VRCG	80	60	0.8:1	5
VRCG	80	60	1:1	5
VRCG	80	60	1.2:1	5
VRCG	90	60	0.8:1	5
VRCG	90	60	1:1	5
VRCG	90	60	1.2:1	5
VRCG	100	60	0.8:1	5
VRCG	100	60	1:1	5
VRCG	100	60	1.2:1	5
VRCG	100	60	1.6:1	5
VRCG	110	60	1:1	5
VRCG	110	60	1.6:1	5
SRCG	80	60	0.8:1	5
SRCG	80	60	1:1	5
SRCG	80	60	1.2:1	5
SRCG	90	60	0.8:1	5
SRCG	90	60	1:1	5
SRCG	90	60	1.2:1	5
SRCG	100	60	0.8:1	5
SRCG	100	60	1:1	5
SRCG	100	60	1.2:1	5
SRCG	110	60	0.8:1	5
SRCG	110	60	1.6:1	5
FSWG	80	60	0.8:1	5
FSWG	80	60	1:1	5
FSWG	80	60	1.2:1	5
FSWG	90	60	0.8:1	5
FSWG	90	60	1:1	5
FSWG	90	60	1.2:1	5
FSWG	90	60	1.4:1	5
FSWG	100	60	0.8:1	5
FSWG	100	60	1:1	5
FSWG	100	60	1.2:1	5
FSWG	100	60	1.4:1	5
FSWG	110	60	0.8:1	5
FSWG	110	60	1:1	5
FSWG	110	60	1.2:1	5
FSWG	120	60	0.8:1	5
FSWG	120	60	1:1	5
FSWG	120	60	1.2:1	5
FSWG	120	60	1.4:1	5

3.2.2 Enzymatic Hydrolysis with Spezyme CP Cellulase

AFEX treated and untreated materials were enzymatically hydrolyzed the using National Renewable Energy Laboratory (NREL) procedure (LAP 009) at 15 FPU cellulase/ gram glucan. This procedure can be obtained at the following website: <http://www.eere.energy.gov/biomass/progs/searchdb2.cgi?4692>.

Hydrolysis was performed simultaneously for each AFEX treated and untreated sample. Each sample contained 0.25 gram glucan hydrolyzed using Spezyme CP cellulase enzyme (59 FPU/mL; Genecor) and Novozyme 188 β -glucosidase (64 pNPGU; cellobiase). The total volume of hydrolysis mixture for each sample was 25mL. Later the hydrolysis was scaled down to 0.15 gram glucan and 15 mL for the total volume for each sample. The cellulase and β -glucosidase enzyme loading are determined using equations 3.1 and 3.2, respectively.

$$\text{Cellulase (mL)} = \frac{15 \text{ FPU}}{1 \text{ g glucan}} \times \frac{1 \text{ mL enzyme}}{59 \text{ FPU}} \times \text{g glucan of hydrolysis} \quad (3.1)$$

$$\beta\text{-glucosidase (mL)} = \frac{64 \text{ pNPGU}}{1 \text{ g glucan}} \times \frac{1 \text{ mL enzyme}}{250 \text{ pNPGU}} \times \text{g glucan of hydrolysis} \quad (3.2)$$

Hydrolysis was done in capped vials which were placed in a 75 rpm incubator at 50°C for 168 hrs. Samples of 1400 μ L were taken at 24 hr, 72 hr, and 168 hr. The collected samples were centrifuged and filtered through a syringe filter containing a nylon membrane into a vial. The vials were placed in the freezer until further analysis.

3.2.3 Analytical Methods

3.2.3.1 High-Performance Liquid Chromatography

HPLC is a sensitive technique for separating or analyzing mixtures, in which the sample is forced through the chromatography column under pressure [14]. Samples are injected into the HPLC via an injection port. Then the sample is dissolved in water, and forced to flow through a column under a high pressure. In the column, the mixture is resolved into its sugar components. The small molecules found within the sample quickly pass through the column, while larger molecules get tangled within the column. This process helps to separate the molecules of the sample. Once the molecules are separated, the sample passes through the Refractive Index detector that measures the light refracted off of the molecules. The detector then signals a peak on the chromatogram, which is then read to determine sugar concentrations. For analysis, the samples from enzymatic hydrolysis were thawed and placed in a tray with degassed water and standard sugars. The standard sugars of glucose and xylose from Sigma-Aldrich (USA) were prepared for various concentrations (i.e. 1, 2, 3, 6, and 12 g/L) and used to calibrate the HPLC. The operating conditions for the HPLC are listed below in table 3.4.

Table 3.4: HPLC operating conditions.

	Operating Conditions
Temperature	85°C
Pressure	700-900 psi
Injection Volume	10µL
System Flow Rate	0.6 mL/min
Run Time	20 min/vial

Once the HPLC is calibrated and operating conditions are met, the samples are loaded into the HPLC for sugar analysis of glucose and xylose. All data was processed with Peak Simple analytical software.

3.2.3.2 Conversion Calculations

The following calculations were performed in determining % conversions for glucose and xylose. Percent (%) conversion is defined in equation 3.3.

$$\% \text{ Conversion} = [\text{Actual (g)} / \text{Theoretical (g)}] \times 100\% \quad (3.3)$$

The sugar analysis obtained from HPLC provided concentrations (g/L) for glucose and xylose of each sample. The concentrations are multiplied by the hydrolysis volume (L) to establish the actual (g) amount of glucose and xylose found in each sample [equation 3.4].

$$\text{Actual (g)} = \text{concentration (g/L)} \times \text{hydrolysis volume (L)} \quad (3.4)$$

The theoretical value is the expected amount that is believed to achieve 100% conversion. But before the theoretical values are determined, the conversion factors must first be defined. The conversion factors are ratios of the sugar to its anhydrous sugar form (i.e. glucose to glucan) as described in section 3.1.2. These factors convert the anhydrous sugar to its corresponding monomeric sugar by dividing the molecular weight (MW) of each sugar form. The MW for the sugars was presented in section 3.1.2. The conversion factors for glucose and xylose are provided as the following:

$$\frac{\text{MW glucose}}{\text{MW glucan}} = \frac{(180 \text{ g/mol glucose})}{(162 \text{ g/mol glucan})} = \frac{1.111 \text{ g glucose}}{\text{g glucan}}$$

$$\frac{MW \text{ xylose}}{MW \text{ xylan}} = \frac{(150 \text{ g/mol xylose})}{(132 \text{ g/mol xylan})} = \frac{1.136 \text{ g xylose}}{\text{g xylan}}$$

The theoretical (g) values for glucose and xylose are calculated using equations 3.5 and 3.6. Glucan and xylan contents for each material are given in table 3.1.

Theoretical (g) glucose

$$= \text{g glucan of hydrolysis} \times \frac{1.111 \text{ g glucose}}{\text{g glucan}} \quad (3.5)$$

Theoretical (g) xylose

$$= \text{g glucan of hydrolysis} \times \frac{\text{g xylan content}}{\text{g glucan content}} \times \frac{1.136 \text{ g xylose}}{\text{g xylan}} \quad (3.6)$$

The actual (g) and theoretical (g) values are applied to equation 3.3 to determine % conversion yields for glucose and xylose. An average % conversion is then estimated for each set of AFEX treated and untreated samples. The data and results in this research are shown for average % conversions (as the average of two samples).

3.3 Results and Discussion

3.3.1 Vegetative Stage of Reed Canarygrass

Several different VRCG samples were studied, including the untreated control sample. These samples varied in AFEX parameters of temperature and ammonia loading and were observed for glucose and xylose generated over 168 hrs of hydrolysis. Figure 3.6 shows glucose and xylose conversions for VRCG after 168 hrs of hydrolysis. Percent conversions ranged from 74% to 131% for glucose and 55% to 77% for xylose. AFEX treatments that provided at least 100% theoretical glucose yield show correlations between temperature and ammonia loading. Temperature determines the amount of ammonia vaporized during the explosive flash (for AFEX) [25]. It appears that at the lowest temperature (80°C) tested, more ammonia must be used (1.2 kg ammonia: 1 kg DM) to achieve 100% of theoretical glucose conversion, while at a higher temperature (100°C), less ammonia (1 kg ammonia: 1 kg DM) achieved 100% of theoretical glucose. More ammonia vapors flash at higher reactor temperatures, causing greater disruption of the fibrous structure [25]. It could be that at a lower temperature more ammonia is needed to produce as much disruption by ammonia vapor as with a higher temperature requiring less ammonia. Then again, a temperature of 90°C successfully reached 100% theoretical glucose yield at all ammonia levels tested. Elevating the temperature beyond 100°C reduced conversions yields for both glucose and xylose. Experimental work to better understand the deleterious effects of high temperature is ongoing. The most

effective AFEX condition for VRCG was found to be 100°C, 60% moisture, 1.2 kg: 1 kg (ammonia to DM), which provided a theoretical yield of 131% glucose based on total carbohydrates and 77% xylose conversion. It appears that glucose conversions observed over 100% were obtained from the soluble (non-structural) carbohydrates found in VCRG (i.e. sucrose and storage carbohydrates). The total glucose that can be obtained from both NSC and SC for VRCG (261 g per kg DM) accounts for 125% theoretical glucose conversion, near the observed yield of 131% of theoretical based on structural carbohydrates only. Thus the NSC can yield an additional 25% glucose for VRCG. With this said, the most effective AFEX condition for VRCG, 100°C, 60% moisture, 1.2 kg: 1 kg (ammonia to DM), which yielded 131% theoretical glucose, produced “only” 106% theoretical glucose from SC. Equation 3.7 shows the calculation used to determine the theoretical glucose yielded from both NSC and SC (DM).

$$DM \% Glu = 100 \% SC Glu \times (DM Total Glu / SC Total Glu) \quad (3.7)$$

The theoretical glucose % conversions from NSC and SC of the AFEX treated VRCG are illustrated in figure 3.7. On the other hand, figure 3.6 also revealed that 100% theoretical xylose was not attained for VRCG with the AFEX pretreatment conditions studied. Although temperature and ammonia loading was increased beyond standard AFEX pretreatment conditions to explore the possibility of achieving higher theoretical yields for both glucose and xylose,

VRCG did not behave well under such conditions. Nonetheless, AFEX treated VRCG increased glucose conversions by 72 percentage points (pp) and 67 pp for xylose over those observed for untreated VRCG. By the end of 168 hrs of hydrolysis, AFEX treated VRCG enhanced both glucose and xylose conversion yields compared to its untreated counterpart.

Figure 3.8 shows the hydrolysis profile for AFEX treated and untreated VRCG at an enzyme level of 15 FPU cellulase/g glucan. The degree of digestibility was analyzed for the most effective and least effective AFEX treatment conditions for VRCG and untreated VRCG. It is interesting to note that the xylose content of untreated VRCG is completely resistant to enzymatic hydrolysis, whereas it gives about 30% glucose conversion at 168 hrs hydrolysis. With AFEX pretreatment, the surface area is exposed allowing the enzymes to better digest VRCG, increasing both glucose and xylose conversions. For example, after only 24 hrs, the most effective AFEX condition observed for VRCG (100°C, 60%, 1.2 kg ammonia: 1kg DM) generated approximately 6 times more glucose and 32 pp more xylose theoretical yield than that of untreated VRCG. By 72 hrs, the treated VRCG produced 100 pp more glucose and 70 pp more xylose than the yield from untreated material. At the end of 168 hrs, treated VRCG showed no change in glucose and a 4 pp increase in xylose yields compared to 72hrs. The data shows that by the end of hydrolysis (for the most effective AFEX condition), glucose and xylose yields increased by 100 pp and 77 pp, respectively, with AFEX pretreatment.

The hydrolysis profile also summarizes the amount of time needed to completely digest the enzymes. The figure shows that the most effective AFEX condition for VRCG reached a peak for glucose after 72 hrs and 120 hrs of hydrolysis for xylose. It took approximately 100 hrs and 168 hrs of hydrolysis to achieve the peak glucose and xylose yields, respectively, for the least effective condition (80°C, 60%, 1 kg ammonia: 1kg DM). Peak glucose and xylose yields for the untreated sample were attained after 168 hrs of hydrolysis. Increased temperature and ammonia loading seems to enhance the rate of digestion for VRCG compared to the untreated sample.

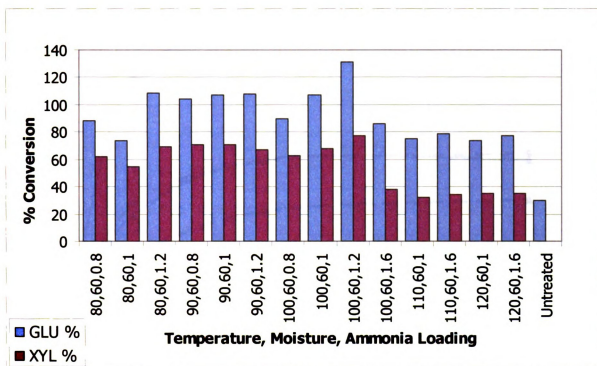


Figure 3.6: % Conversion for Glucose and Xylose after 168 Hr Hydrolysis for VRCG (15 FPU cellulase/ g glucan).

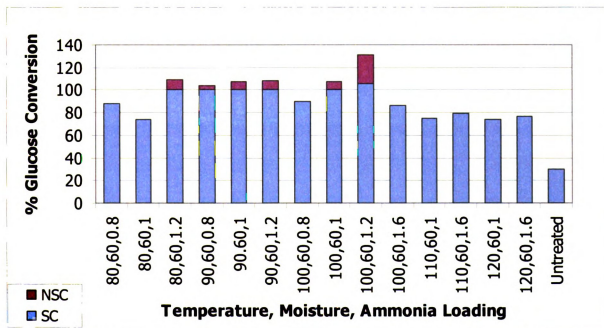


Figure 3.7: % Glucose Conversion contributed from NSC and SC after 168 Hr Hydrolysis for VRCG (15 FPU cellulase/ g glucan).

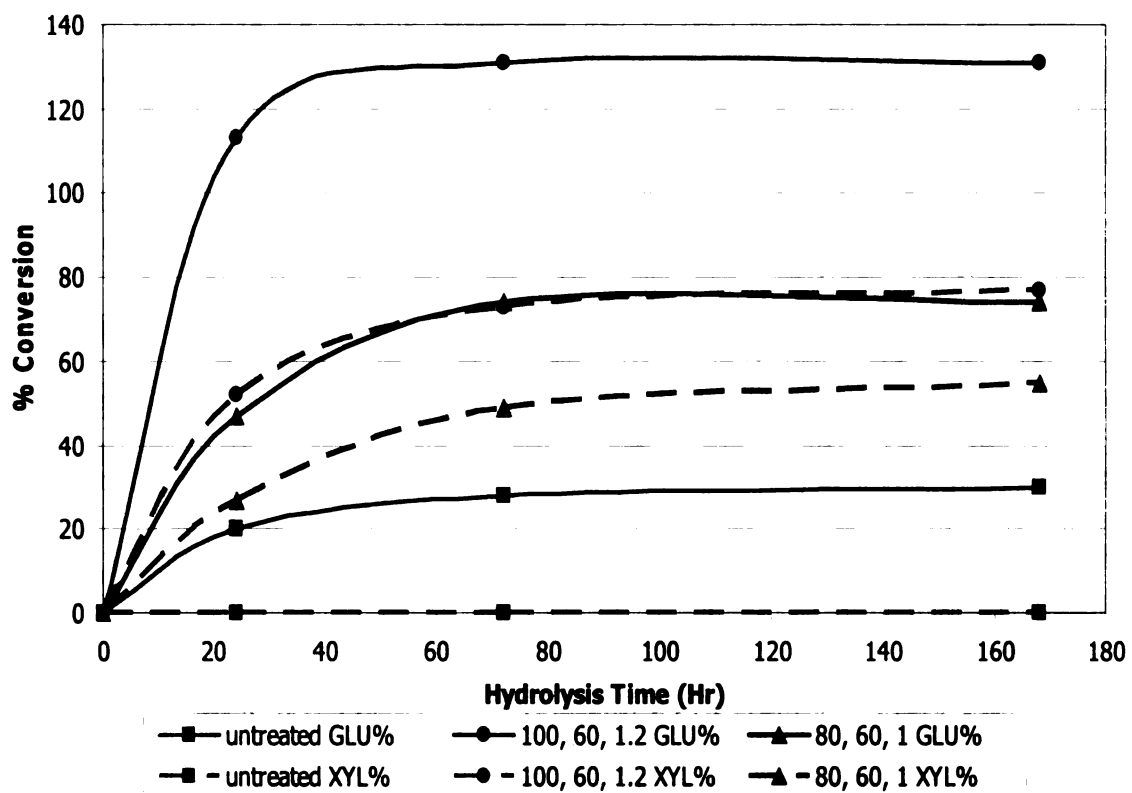


Figure 3.8: Glucose and xylose conversion vs. hydrolysis time at enzyme loading 15 FPU cellulase/g glucan for VRCG.

3.3.2 Seed Stage of Reed Canarygrass

SRCG (including untreated SRCG) were observed for glucose and xylose conversions at various treatment parameters. Figure 3.9 displays the 168 hr conversions for SRCG. The treatment effectiveness improved at each temperature with increasing ammonia loading. However at 100°C, the conversion levels decrease with increasing ammonia loading. Ammonia can react with lignocellulosics by ammonolysis of the ester crosslinks of some uronic acids with the xylan units [10], and cleaving the bond linkages between hemicellulose and lignin [15, 26]. It is possible that extra liquid ammonia plasticizes the cellulose and thereby reduces the disruptive effect of sudden pressure release [27]. In Figure 3.9, SRCG treated at (90°C, 60%, 1.2), (100°C, 60%, 0.8) and (100°C, 60%, 1) is shown to provide 120% conversion for glucose and 80% for xylose. The most effective AFEX condition for SRCG (100°C, 60%, 0.8) produced 121% theoretical glucose and 81% theoretical xylose conversion. As observed with VRCG, these conversions greater than 100% might be due to the glucose found in the soluble carbohydrates (NSC) of SRCG. As mentioned for VRCG, 100% theoretical glucose conversion would require that all of the glucose from SC to be extracted. Table 3.2 presented the SRCG glucose content as 46 (g/kg DM) for the NSC, 265 (g/kg DM) for the SC, and the total glucose content for SRCG (both NSC and SC) as 311 (g/kg DM). Using equation 3.7, the maximum theoretical glucose that can yield from the total glucose content (both NSC and SC) of SRCG is 117%. Accordingly, 17% additional glucose is from the NSC of

SRCG. The most effective AFEX condition for SRCG, as a result, contributed 104% glucose conversion from the SC. The theoretical glucose % conversions from NSC and SC of the AFEX treated SRCG are illustrated in figure 3.10.

Figure 3.11 illustrates the hydrolysis profile for the most and least effective AFEX conditions for SRCG and untreated SRCG at 15 FPU cellulase/g glucan enzyme loadings. It is apparent from figure 3.11 that the structure of SRCG is disrupted by AFEX allowing for nearly 4.4 times more glucose and a 45 pp increase in xylose with the most effective AFEX condition compared to untreated SRCG at the end of 24 hrs. At 72 hrs of hydrolysis, glucose and xylose conversions were consistent with 24 hr results for the most effective AFEX condition for SRCG. However, the glucose conversion for the untreated sample increased by 13 pp. By the end of 168 hrs of hydrolysis, AFEX treated SRCG increased 30 pp for both glucose and xylose compared with the 72 hr results. No change was noticed in the untreated sample. Glucose and xylose yields show 70 pp and 75 pp, respectively, enhancement for AFEX treated SRCG over the untreated sample. Again, as observed for VRCG, the xylose content for the untreated SRCG is completely resistant to enzymatic hydrolysis, xylose conversions improve with AFEX pretreatment, and 100% theoretical xylose conversion is not observed for the AFEX treatments tested. For the most effective AFEX condition for SRCG (100°C, 60%, 0.8), 100% theoretical glucose conversion was achieved near 100 hrs; however, glucose conversion increases further with digestion (up to 168 hrs). The peak theoretical xylose conversion

for the most effective AFEX condition is attained at 168 hrs producing only 80% of theoretical conversion. The least effective AFEX condition for SRCG as well as the untreated sample reached peaks for both glucose and xylose conversions after 168 hrs of hydrolysis. It appears that if the AFEX treated material was allowed to digest longer than 168 hrs, the percent conversions might slightly increase.

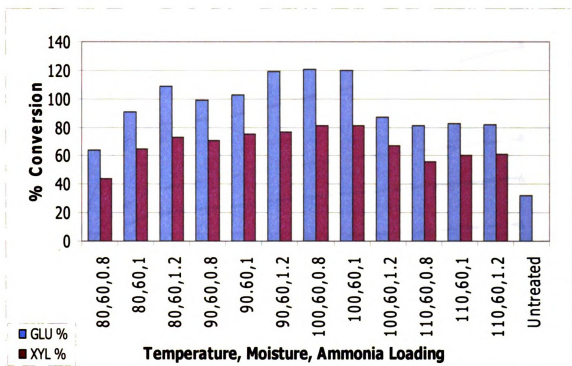


Figure 3.9: % Conversion for Glucose and Xylose after 168 Hr Hydrolysis for SRCG (15 FPU cellulase/ g glucan).

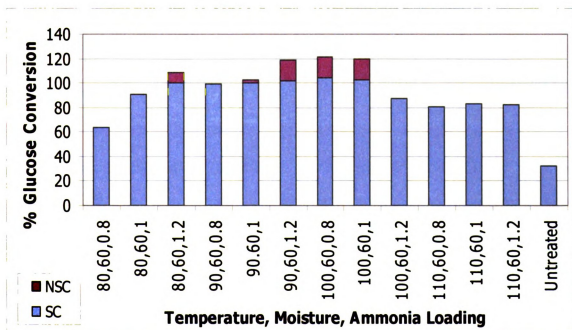


Figure 3.10: % Glucose Conversion contributed from NSC and SC after 168 Hr Hydrolysis for SRCG (15 FPU cellulase/ g glucan).

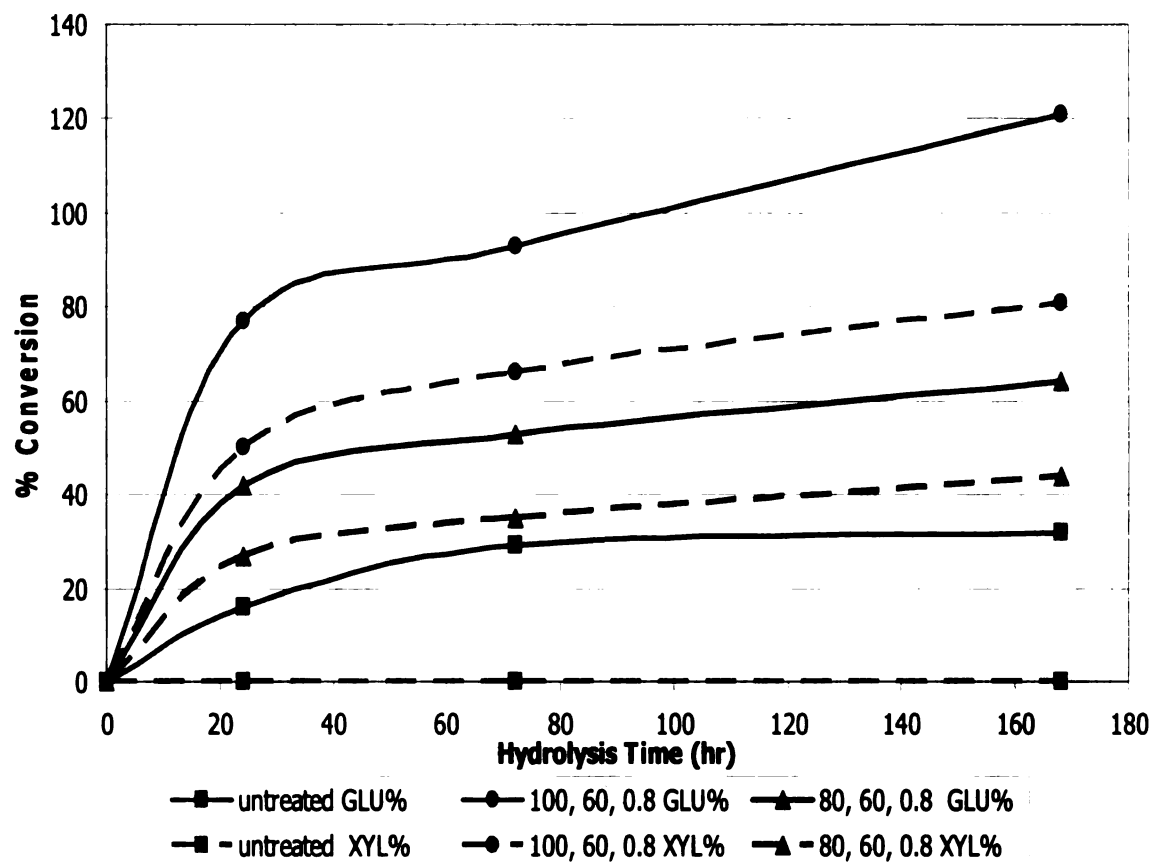


Figure 3.11: Glucose and xylose conversion vs. hydrolysis time at enzyme loading 15 FPU cellulase/g glucan for SRCG.

3.3.3 Frost Stage of Switchgrass

Several AFEX pretreatment conditions were tested to improve theoretical glucose and xylose conversions for FSWG. An AFEX pretreatment condition that would give 100% of theoretical conversion for either glucose or xylose has yet to be found. From table 3.1, a yield of 322 g glucose/ kg DM and 233 g xylose/ kg DM from FSWG would represent 100% theoretical conversion for glucose and xylose, respectively. Hence, the AFEX pretreatment conditions tested did not effectively remove all of the glucose and xylose present within the SC for FSWG. After 168 hrs, Figure 3.12 shows that glucose and xylose conversion yields for FSWG improved with increasing temperature and ammonia. Then, as with VRCG and SRCG, as the ammonia loading is further increased, the conversions are shown to drop slightly. The extra ammonia may have inhibited the disruption of the cellulosic structure of FSWG. Figure 3.12 also displays the most effective AFEX condition for FSWG as 120°C, 60%, 1.2 kg ammonia: 1kg DM, which provided the peaks of 85% theoretical glucose and 70% theoretical xylose conversion.

Figure 3.13 illustrates the effect of AFEX pretreatment for FSWG during a hydrolysis period of 168 hrs at a 15 FPU cellulase/ g glucan enzyme loading. AFEX treated FSWG is shown to enhance the enzyme digestibility and increase conversions compared with the untreated sample. As noticed with untreated VRCG and SRCG, the xylose content for the untreated FSWG is also completely resistant to enzymatic hydrolysis, whereas 23% theoretical glucose was

obtained. After 24 hrs of hydrolysis, the most effective AFEX condition is shown to increase theoretical glucose conversion yield by 24 pp (nearly 3 times more) and xylose by 36 pp compared to the conversions achieved without AFEX pretreatment. Conversions continue to improve after 72 hrs of hydrolysis providing nearly 50 pp more glucose and xylose; and at the end of 168 hrs, 62 pp more theoretical glucose and 70 pp theoretical xylose is achieved. The hydrolysis profile for FSWG also reveals that all peak conversions for the most and least effective AFEX condition, as well as the untreated sample, were accomplished after 168 hrs. This suggests that the enzymes were less able to access FSWG than they were for both VRCG and SRCG.

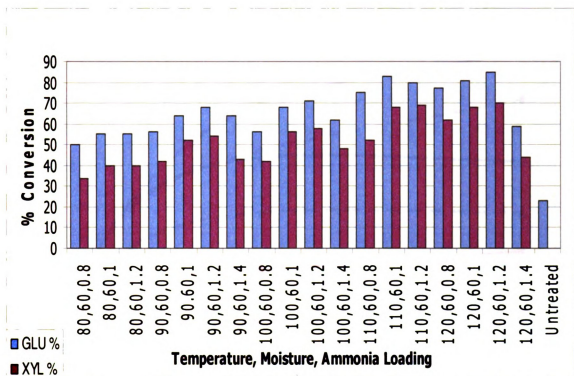


Figure 3.12: % Conversion for Glucose and Xylose after 168 Hr Hydrolysis for FSWG (15 FPU cellulase/ g glucan).

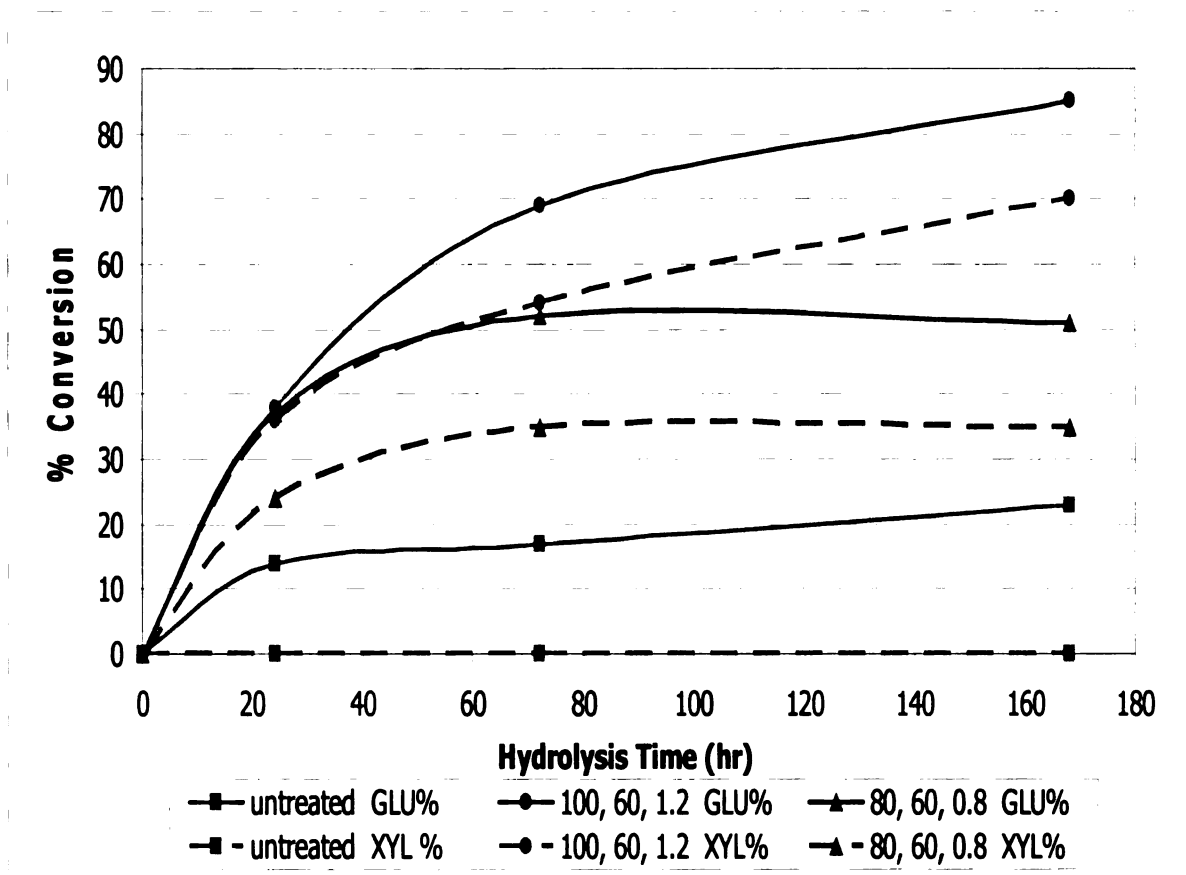


Figure 3.13: Glucose and xylose conversion vs. hydrolysis time at enzyme loading 15 FPU cellulase/g glucan for FSWG.

3.3.4 Discussion

RCG and SWG both had shown to enhance theoretical glucose and xylose conversions with AFEX pretreatment. However, it seems that the maturity of the materials probably influenced % conversions of the sugars. In a previous study, it was shown that the maturity level for RCG and SWG affected the lignin concentrations which could explain the rapid digestion observed for some of the materials studied. Dien et al. stated that "harvesting more mature forage (biomass material) resulted in higher concentrations of cell wall glucose and non-glucose sugars. Lignin concentrations also increased for the more mature samples [24]." Therefore, more mature stages of RCG and SWG shown higher lignin content as well as higher concentrations in structural carbohydrates. And if the lignin content is higher for more mature material, accessing the sugars from these materials would probably require more digestion to achieve higher % conversions. Plant material in its earlier stage growth with lower lignin content would provide higher % conversions with less hydrolysis time (or digestion). In table A.1, the lignin content is expressed as 109 (g/kg DM) for VRCG, 148 (g/kg DM) for SRCG, and 173 (g/kg DM) for FSWG. Consequently, the lignin content is lower for VRCG (which is an earlier stage of RCG) compared to those of SRCG and FSWG. This could provide evidence as to why higher % conversions were achieved with VRCG than with SRCG and FSWG.

Chapter 4

Enzymatic Hydrolysis with Cellulase and Xylanase Combination

4.1 Xylanase

Theoretical xylose conversions for RCG and SWG did not yield near 100% as was observed for glucose conversions. Cellulase hydrolyzes cellulose, which is comprised of glucose and therefore it is expected to see higher glucose yields than xylose yields. Existing commercial cellulases have very little xylanase activity. The xylan content of the material needs to be hydrolyzed with a xylanase that would allow greater conversion than those observed with the use of cellulase alone. Several xylanase enzymes were sent to Michigan State University in May 2005 from Genecor, Inc. See Appendix D for a description of the xylanases. These xylanases were assayed for protein content using a BCA (Bicinchoninic Acid) Protein Assay. The protein contents of the xylanases are provided in table 4.1.

4.2 Xylanase Screening

The xylanase enzymes were tested on AFEX treated (90°C, 60%, 1) VRCG, SCRG, and FSWG to determine an effective xylanase that most influenced glucose and xylose yields. Each xylanase was loaded at 10% (by weight) of the cellulase loading. Equation 4.1 displays the calculation used in determining

xylanase volume of a percentage of cellulase loading. Note that the protein content for Spezyme CP cellulase is 123 mg/mL.

$$\text{Xylanase (mL)} = \frac{\text{Cellulase (mL)} \times 123 \text{ mg/mL}}{\text{xylanase protein content (mg/mL)}} \times \text{percentage} \quad (4.1)$$

The samples were hydrolyzed for 168 hrs and analyzed for glucose and xylose conversions using HPLC. Multifect 720 was found to be the most effective xylanase for all of the materials tested.

Table 4.1: Protein content of xylanases used for screening.

Xylanase	Lot #	Total Protein Content (mg/mL)
Optimash BG	301-04168-115	136
Optimash XL	301-04232-162	180
Multifect Xylanase	301-04296-205	42
Multifect XL	301-03228-134	150
GC 260	2503098	20.5
Multifect A40	301-04247-171	138
Multifect 720	501-04285-193	93.5

4.3 Results and Discussion

The most effective AFEX condition found for each of the materials were enzymatically hydrolyzed for 168 hrs with a combination of Spezyme CP cellulase and Multifect 720 xylanase at 15 FPU cellulase/ g glucan. The AFEX treated samples were tested at various xylanase loadings (10%, 25%, and 50%).

Figure 4.1 shows the increase in glucose and xylose conversions attained for the most effective AFEX conditions for VRCG, SRCG, and FSWG for this specific enzyme combination. The relative pp conversion is given as the percentage increase shown over the AFEX treated material hydrolyzed with cellulase alone. Multifect 720 xylanase was shown to enhance both glucose and xylose conversions for RCG and SWG. As the xylanase loading increased, higher glucose and xylose conversions were generated for each material. But unlike the glucose conversion shown in figure 4.1, the xylose conversions steadily increased with increasing loading of xylanase. At a 10% xylanase loading, both maturity stages for RCG produced about 6 pp glucose and 5 pp xylose conversions, while FSWG gave 4 pp glucose and 3 pp xylose. At 25% xylanase loading, the glucose conversion for RCG nearly doubled while the xylose conversion gradually increased by 1 pp and 2pp for VRCG and SRCG, respectively. But both glucose and xylose conversions doubled for FSWG. As observed at 50% xylanase loading, VRCG continued to progress, while the more mature RCG, SRCG, showed no change with the increase of xylanase. FSWG provided 13 pp glucose

conversions, although the xylose conversion did not show improvement with the increase of xylanase loading.

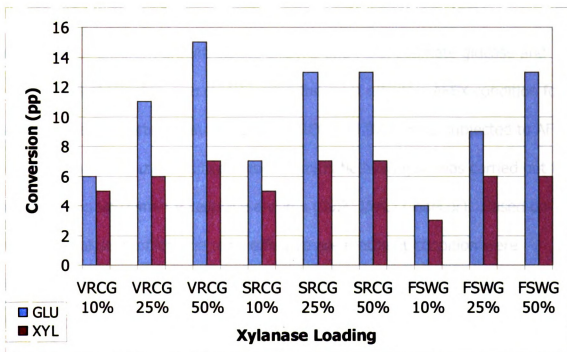


Figure 4.1: Percentage point conversion increases for glucose and xylose at 168 Hrs with Spezyme CP cellulase and Multifect 720 xylanase (on a relative scale based on results obtained with Spezyme CP cellulase alone).

Chapter 5

Conclusions and Recommendations

The objectives of this research were to evaluate suitable AFEX treatment conditions for different growth stages of SWG and RCG, estimate glucose and xylose yields obtainable, and to determine the most effective AFEX condition for each material. Selected maturity levels of RCG and SWG were subjected to AFEX pretreatment and enzymatic hydrolysis. Enzymatic hydrolysis was carried out for 168 hrs using Spezyme CP cellulase enzyme (59FPU/mL; Genecor). Based on 168 hr hydrolysis profiles, the most effective pretreatment condition were determined for each of the materials. The research shows AFEX to be an effective pretreatment for the materials tested. AFEX pretreated RCG and SWG produced greater amounts of glucose and xylose than the untreated samples. All pretreatment conditions tested were shown to enhance enzyme digestibility and susceptibility as well as improve sugar conversions for both RCG and SWG compared to its untreated counterparts. The standard AFEX treatment conditions (80°C, 90°C, 100°C for temperature; 0.8, 1.0, and 1.2 kg per 1 kg dry biomass for ammonia loading; 60% MC) were suitable for VRCG and SRCG in providing the most effective conditions for each. However, these treatment conditions did not provide theoretical sugar conversion yields for FSWG. It could be concluded that both the maturity and lignin content of the materials may have had a direct effect on the conversion yields that were achieved.

In a previous study, Dien et al. states that "the more mature the material was, the higher the lignin content [24]." Higher lignin content would imply that the cell wall of the material is very rigid and much more resistant to enzymatic hydrolysis than a material that is lower in lignin content. As observed in table A.1, FSWG has the highest lignin content of the materials studied and is in the latter stages of growth for SWG. Due to its extremely resistant behavior to cellulase, more treatment conditions (outside of the standard AFEX treatment range) were tested and evaluated to determine the most effective treatment condition for FSWG. After 168 hr hydrolysis using 15 FPU/g glucan and Spezyme CP cellulase, the most effective treatment conditions were determined as:

VRCG- 100°C, 60% MC, 1.2:1 kg DM per kg ammonia (106% glu, 77% xyl);

SRCG- 100°C, 60% MC, 0.8:1 kg DM per kg ammonia (103% glu, 81% xyl);

FSWG- 120°C, 60% MC, 1.2:1 kg DM per kg ammonia (85% glu, 70% xyl).

Enzymatic hydrolysis was then performed using a combination of Spezyme CP cellulase and Multifect 720 xylanase at various xylanase loadings to study the effect xylanase would have on the xylan content of each material. Multifect 720 was shown to enhance pp conversions for glucose and xylose for VRCG, SRCG, and FSWG.

With continuing research, AFEX pretreated RCG and SWG could prove to be beneficial to the bioethanol industry. It is recommended that further AFEX testing (varying temperature, moisture content, and ammonia loading) be

conducted to determine if xylose conversions for the growth stages of RCG and both glucose and xylose conversions for SWG could improve.

Recommendations:

- Test cellulase enzyme loading levels below 15 FPU/ g glucan using Spezyme CP cellulase.
- Test SWG in its earlier stages of maturity with AFEX pretreatment. These samples may provide higher % conversions because the lignin content is less than that of SFWG.

Appendices

Appendix A

Carbohydrate Composition Data

Table A.1: Protein, lipid, ash, organic acids, lignin, carbohydrate, and gross energy content of biomass forage samples (g/kg DM) [24].

Species† Stage	Crude Protein	Ether Extract	Ash	Organic Acids	Klason Lignin	Carbohydrates	Total of Components	Gross Energy
<u>Reed</u>								
<u>Canarygrass</u>								
Veg (VRCG)	88	22	128	24	109	518	889	4230
Seed (SRCG)	45	13	95	10	148	597	908	4216
<u>Switchgrass</u>								
Frost (FSWG)	30	16	57	3	178	650	915	4465

†Data for reed canarygrass and switchgrass are for whole herbage (g/kg DM). Crude protein, ether extract, ash, organic acids, lignin, carbohydrates, and total of components are given as g/kg DM. The gross energy is given as kcal/kg

Description of Carbohydrates:

Sucrose is a disaccharide consisting of glucose and fructose. Sucrose is 50% glucose and 50% fructose by weight.

Raffinose (melitose) is a trisaccharide which yields galactose, fructose, and dextrose on hydrolysis. Dextrose is an isomer of glucose. For that reason, raffinose is a polysaccharide of galactose and sucrose. A third (33%) of raffinose is glucose.

Stachyose is a tetrasaccharide that yields glucose, fructose, and galactose on hydrolysis. A fourth (25%) of stachyose is glucose.

Storage carbohydrates are starch and fructans. Starch is a polysaccharide consisting of various proportions of two glucose polymers, amylose and amylopectin and ultimately yields glucose when digested [28]. The starch in the storage is 100% glucose. Fructan is a polysaccharide composed of D-fructose units linked with glucose. Fructan yields sucrose and fructose. Hence, a third (33%) of fructan is glucose.

Note: Fructose and galactose are both stereoisomeric with glucose (having the same MW).

Appendix B

AFEX Procedure

1. Determine the solid and moisture content for the wet material (WM) sample. This can be obtained either by using a moisture analyzer or drying the sample in an oven for 24 hrs.
- 1b. If drying is to be used to determine the solid and moisture content:
 - Weigh an aluminum boat and tare scale.
 - Weigh approximately 1 g of sample. Record this weight as *sample weight*.
 - Remove boat with sample and re-tare scale.
 - Re-weigh boat with sample. Record this weight as *before*.
 - Place boat with sample into oven and allow drying for 24 hrs.
 - After 24 hrs, weigh boat with sample. Record weight as *after*.
 - Determine solid and moisture content:

$$\text{solid content (S.C.)} = 1 - [(before - after) / sample weight]$$

$$\text{moisture content (M.C.)} = 1 - \text{solid content}$$

2. Load ammonia cylinder and record weight of ammonia.
3. Determine the amount of dry material (DM) and WM:

$$\text{DM (g)} = \text{weight of ammonia} \times \text{ratio of DM to ammonia}$$

i.e. 1 kg DM: 0.8 kg ammonia

$$\text{WM (g)} = \text{DM (g)} / \text{S.C.}$$

4. Calculate amount of water to add to wbm to achieve the desired M.C. for the AFEX treatment. (*i.e.* the desired M.C. = 60%)

Amount of H₂O to add (g)

$$= \text{Amount of H}_2\text{O (g)} - \text{Amount of H}_2\text{O present}$$

$$= [\text{DM (g)} \times (\text{desired M.C.} / 100)] - [\text{WM (g)} - \text{DM (g)}]$$

5. Mix the wbm and water together. Carefully pour the sample into the reactor. Pack the space within the reactor with ball bearings and attach the lid to the reactor.
6. Vacuum the reactor for approximately 5 minutes to remove all air that may be contained within.
7. Weigh the reactor and tare scale.
8. Attach the ammonia cylinder to the reactor and load ammonia.
9. Re-weigh the reactor to determine if all of the ammonia is loaded. If not successful, re-attach ammonia cylinder to capture remaining ammonia that may be trapped inside. Re-weigh.
10. Once ammonia is loaded, record the initial temperature and pressure. Place the reactor into the heater.
11. Record the temperature and pressure at 2 minute increments. When temperature reaches 10 degrees below the treatment temperature, remove reactor from the heater and allow the temperature to reach ± 1 of the treatment temperature.
12. Hold for residence time and then release the pressure.

13. Remove sample from reactor and dry under a hooded vent for approximately 24 hrs. This allows for excess ammonia to evaporate from the sample.
14. Collect sample into a zip-lock bag and record treatment date and conditions onto the bag. Immediately place into refrigerator until further analysis.

Appendix C

Enzymes

GC 260 is a purified xylanase produced by submerged fermentation of a genetically modified strain of *Bacillus licheniformis* transformed by introduction of a xylanase gene from *Bacillus pumilis*. The enzyme is stable in the acidic pH range (pH 4.5-7.0) at moderately high temperature (55°C). The enzyme is an endo-1, 4-β-xylanase and exhibits high specificity towards the soluble pentosan fraction in wheat.

Multifect® 720 enzyme is a purified alkaline xylanase produced by submerged fermentation of genetically modified strain of *Bacillus licheniformis* transformed by introduction of a xylanase gene from a selected strain of *Bacillus alcalophilus*. Multifect® 720 enzyme causes a random hydrolysis of xylan polymers in the alkaline pH range (pH 6.5-8.5) at an elevated temperature (50-70°C) and can be used as a processing aid to reduce the amount of bleaching chemicals used during processing of Kraft pulps.

Multifect® A40 enzyme preparation contains cellulase and hemicellulases as main enzyme actives together with low level of xylanase and mannanase. Multifect® A40 enzyme is produced using a submerged fermentation of a selected strain of *Trichoderma reesei*. Multifect® A40 enzyme acts mainly on the

surface of the fiber in the acidic to neutral pH range (pH 4.0-7.0) and between temperatures of 40-65°C. The fiber modification by Multifect® A40 enzyme results in the enhancement of water removal from paper pulp processing. If refining follows the treatment, increased fibrillation can be observed due to enhanced beatability/refining of the fibers.

Multifect® XL enzyme is an endoxylanase derived from a selected strain of *Trichoderma reesei*. Typical application areas for the Multifect® XL product include baking, waste treatment and agricultural silage.

Multifect® Xylanase is derived from a genetically modified strain of *Trichoderma reesei*. A typical application area for Multifect® Xylanase is animal feed.

Optimash™ BG is an enzyme preparation intended from the fuel alcohol industry. This product is capable of reducing viscosity of barley and wheat mashes. Optimash™ BG enzyme contains a combination of enzymes which effectively modify and digest non-starch carbohydrates, the structural material of plant cells. Optimash™ BG is produced by submerged fermentation of genetically modified strain of *Trichoderma reesei*.

Optimash™ XL cellulase/xylanase is an enzyme preparation intended for the fermentation alcohol industry. This product is capable of reducing viscosity and improving separation of different grain fractions of wheat and rye mashes. Optimash™ XL cellulase/xylanase contains a combination of enzymes which effectively modify and digest non-starch carbohydrates, the structural material of plant cells. The plant material is composed mainly of cellulose, arabinoxylans and β -glucans which are cross-linked with each other and also with lignin, pectins, proteins, starch and lipids. Optimash™ XL enzyme is produced by controlled fermentation of *Trichoderma reesei*.

Spezyme® CP cellulase is an enzyme preparation intended for the starch and alcohol industries. This product is capable of reducing viscosity and improving separation of different grain fractions. Spezyme® CP cellulase contains a combination of enzymes which effectively modify and digest non-starch carbohydrates, the structural material of plant cells. The plant material is composed mainly of cellulose, hemicellulose, and β -glucans which are cross-linked with each other and also with lignin, pectins, proteins, starch, and lipids. Spezyme® CP enzyme is produced by controlled fermentation of *Trichoderma reesei* (formerly *longibrachiatum*).

All descriptions for enzymes are provided by:

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