INCREASING DRY GRIND ETHANOL YIELD THROUGH FIBER PRETREATMENT AFTER LIQUEFACTION

BY

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THESIS

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Abstract

Concentrations of starch and fiber in corn grain are 72 and 10% on a dry basis (db), respectively. In the conventional dry grind process, starch is converted to ethanol; whereas, corn fiber remains unconverted. Corn fiber contains 12 to 18% cellulose and 11 to 23% starch which can be hydrolyzed and fermented into ethanol. Conversion of corn fiber into ethanol would lead to an increase in ethanol yield, decrease in downstream processing costs, improvement in DDGS quality and D3 RIN generation for dry grind plants. Recalcitrance towards enzyme hydrolysis is a major challenge associated with the conversion of corn fiber into ethanol. Objectives of this study were to study the effects of hot water pretreatment, disk milling and cellulase dose on conversion of corn fiber into ethanol and to modify the conventional dry grind process for converting corn fiber into ethanol.

Mash obtained after liquefaction was filtered and retentate (referred to as fiber) was used to study effects of pretreatments on ethanol yield. Fiber contained 23.5% db structural carbohydrates and 62.1% db extractives which were mostly soluble sugars. Fiber was pretreated using hot water at 20% solids at 160°C for 5, 10 and 20 min residence times and 3 cycles of disk milling at 20 and 45% solids. SSF was performed at a 10% db fiber concentration with the addition of commercially available glucoamylase, cellulase (30 FPU/g fiber) and hemicellulase enzymes and ethanol red yeast. SSF of untreated biomass was performed with 120 FPU/g fiber cellulase dose to understand effects of excess cellulase addition on ethanol yields. Conversion for hot water pretreated fiber was higher than that of untreated biomass (69.4%), with highest increase using a 5 min residence time at 160°C (76.6%). Disk milling had no effect on conversion. Use of excess cellulase (120 FPU/g fiber) resulted in higher conversion (92.5%) than that at normal cellulase (30 FPU/g fiber) (69.8%). Corn fiber could be converted to ethanol in the conventional dry grind process through the addition of cellulolytic enzymes during SSF.

Conventional dry grind process was modified for converting corn fiber into ethanol. These modifications included addition of 30 or 120 FPU/g fiber cellulase during SSF, disk milling (3 cycles) slurry and combination of disk milling and cellulase (30 FPU/g fiber) addition during SSF. Cellulase addition (30 FPU/g fiber) during SSF resulted in higher conversion (85.3%) compared to the conventional dry grind process (81.1%). Disk milling corn slurry had no effect on ethanol yield compared to the conventional process. Cellulase addition (120 FPU/g fiber) at

the SSF stage and combination of disk milling with cellulase addition (30 FPU/g fiber) achieved lower ethanol yields compared to conventional dry grind process. Residual glucose was observed at the end of fermentation in all experiments with cellulase addition. Cellulase addition in the conventional dry grind process might lead to yeast inhibition. Cellulase addition at the SSF stage can increase ethanol yields in the conventional dry grind process by 0.14 gal/bushel corn.

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Chapter 1

Introduction

Use of fossil fuels to meet the increasing demand for energy has resulted in increased risk of extinction of these resources and global climate changes due to increased greenhouse gas (GHG) emissions. Bioethanol is one of the most promising alternatives to replace fossil fuels and to reduce fossil fuel utilization and GHG emissions. Presently, starch and glucose rich crops such as corn, wheat, sugarcane and sugar beet are used as feedstocks for bioethanol production. Ethanol production in the US was 15 billion gallons in 2016 (ERS 2016, RFA 2017).

Traditionally corn has been dedicated for food production. Starch, fiber, protein and fat are major components of the corn kernel. Proportions of starch, protein, fiber and fat on dry basis are 65 to 72%, 9 to 10%, 9 to 10% and 4%, respectively (Kelsall and Pigott 2009). Currently a major proportion of corn production in the US is utilized for producing ethanol with 40% gross share of corn production and net acreage of 25% dedicated to ethanol production in 2011 (Mumm et al 2014).

Wet milling and dry grinding are two techniques used primarily for the production of ethanol. High capital investment, water requirements and operating costs associated with wet milling have favored construction of dry grind ethanol plants (Rausch and Beylea 2006, Bothast and Schlicher 2005). Consequently, in the US approximately 90% of ethanol is produced by the dry grind industry (RFA 2017).

In the dry grind process, corn is milled and mixed with water to form slurry. The slurry goes through jet cooking, liquefaction and SSF (simultaneous saccharification and fermentation) steps during which starch is converted to ethanol and carbon dioxide. The ethanol-water mixture is distilled and further separated by molecular sieves to obtain 200 proof ethanol. The solids remaining after distillation (whole stillage) go through several operations of dewatering and dehydration to produce a coproduct known as distillers dried grains with solubles (DDGS). In a typical dry grind plant, 1 bu (25.4 kg) corn produces 2.7 gal (10.4 L) ethanol, 0.7 lb (0.3 kg) corn oil and 15 lb (7.5 kg) DDGS (Rausch and Beylea 2006, Bothast and Schlicher 2005).

DDGS and corn oil are coproducts from the dry grind ethanol process and play an important role in reducing ethanol production costs. DDGS has a low market value due to its high fiber content which restricts animal feed use to ruminants. Inconsistent nutrient composition of DDGS interferes with animal diet formulation, further reducing its market value (Beylea et al 2004, Corrigan and Mass 2009, Rausch and Beylea 2006, Singh et al 2005). Higher ethanol production, low fiber and high protein contents in DDGS and consistency in DDGS composition contribute to the profitability of a dry grind ethanol plant.

Storage carbohydrates are broken down into fermentable sugars during cooking and hydrolysis steps and are fermented into ethanol in the conventional dry grind processes. Corn fiber contains 12 to 18% cellulose, 30 to 50% arabinoxylan and 11 to 23% starch, which can be hydrolyzed and fermented into ethanol. However due to its recalcitrant nature, corn fiber is difficult to be broken down into sugars; therefore, it remains underutilized. Fiber accumulates in DDGS, decreasing its market value (Dien and Bothast 2009).

Benefits of converting corn fiber into ethanol include:

1. Potential increase in ethanol production of 0.2 gal/bu corn (Grohmann and Bothast 1997) and decrease in downstream processing costs (Madson 2009).
2. Lower fiber and higher protein contents in DDGS compared to conventional DDGS.
3. D3 RIN generation for cellulosic ethanol production.

Hot water and wet disk milling are chemical free and environmentally friendly pretreatment methods that can reduce biomass recalcitrance to improve sugar release during hydrolysis. These pretreatment methods also have low capital and operating cost requirements compared to other pretreatments (Kim et al 2016a, 2016b, Hideno et al 2009) and can be incorporated in dry grind ethanol plants. Sugar release in the hydrolysis step also is highly dependent on cellulase and hemicellulase loadings.

Many technologies are being developed for integrating cellulosic ethanol into conventional dry grind production. Technologies developed by ICM and Edeniq have increased cellulosic ethanol yield in dry grind plants by 7 and 2.5%, respectively (Warner et al 2017). Implementation of technologies developed by ICM and Edeniq throughout the dry grind ethanol industry could result in 300 to 1500 million gal cellulosic ethanol produced annually (Warner et

al 2017). Although these technologies are promising, there is a lack of published data to support the claims. Evaluating effects of hot water pretreatment, disk milling and enzyme loading on ethanol yield from corn fiber and the entire corn kernel would contribute to understanding these technologies.

Thus, study objectives were to compare:

1. The effects of hot water pretreatment conditions (time and temperature) on conversion of corn fiber into ethanol.
2. The effects of cellulase enzyme doses and disk milling conditions on ethanol conversion for corn fiber and in the conventional dry grind process.

Chapter 2

Literature review

2.1. Carbohydrates in the corn kernel

Corn is composed of starch, fiber, protein and fat at concentrations of 65 to 72% w/w, 9 to 10% w/w, 9 to 12% w/w and 4% db, respectively (Kelsall and Pigott 2009). Carbohydrates account for 74 to 82% db of a corn kernel and can be classified broadly into two categories: storage carbohydrates and structural carbohydrates. Storage carbohydrates (starch) are present in the endosperm and provide energy to the embryo of nascent plant (germ); whereas, structural carbohydrates (corn fiber) are present in the pericarp which presents a physical barrier to seed damage (Haefele and Ross 2009).

Starch is composed primarily of two polymers: amylose and amylopectin. Amylose is a linear polymer with α-1,4-glucosidic bonds. Amylopectin is a branched polymer with α-1,4-glucosidic and α-1,6-glucosidic bonds. The amylose chain consists of approximately 1000 glucose units whereas amylopectin consists of approximately 10,000 glucose units; corn starch contains 10 to 25% amylose and 75 to 90% amylopectin (Kelsall and Pigott 2009).

Corn fiber is composed of cellulose, hemicellulose and lignin. Cellulose is a polymer of glucose with β-1,4-glucosidic bonds with a degree of polymerization of 10 to 1000 glucose molecules. Strong interchain and intrachain hydrogen bonds render cellulose highly crystalline and insoluble in aqueous solutions. Hemicellulose is an amorphous short chain molecule that is composed mainly of pentoses such as xylose and arabinose, galactose and other sugar acids. Lignin is a polymer composed of phenyl propane units (p-coumaryl, sinapyl and coniferyl alcohols) linked by ether and carbon-carbon bonds (Dien and Bothast 2009, Kumar and Murthy 2016, Kozliac et al 2016). Corn fiber is composed of cellulose (15 to 20%), arabinoxylan (30 to 50%) and adherent starch (11 to 23%) (Gáspár et al 2007, Kim et al 2016c).

2.2. Dry grind ethanol process

The dry grind process is designed for the conversion of corn kernel into ethanol. The process can be divided into 4 parts: grinding and slurrying, cooking and hydrolysis, simultaneous saccharification and fermentation (SSF) and downstream processing. Corn is ground using a

hammer mill through a 30 mesh screen and mixed with water to form a slurry with 32% solids. Objective of the cooking and hydrolysis steps is to produce fermentable sugars by breaking down corn starch. A thermostable α-amylase is added to the slurry which is heated above 100ºC in a jet cooker. Starch loses its crystallinity during the jet cooking step (known as gelatinization) which increases solubility and accessibility of enzymes, facilitating further breakdown of starch. Alpha-amylase added during the cooking and hydrolysis step hydrolyzes α-1,4-glucosidic bonds in starch and converts starch into short chain glucose polymers called dextrins. The slurry is cooled to 85ºC in a flash tank and an additional dose of α-amylase is added in the liquefaction tank to complete starch hydrolysis. The mash obtained after liquefaction is cooled to 32ºC and the pH of the mash is adjusted to 4.5. During the SSF step, glucoamylase, yeasts and nitrogen source are added to the fermenter. Glucoamylase hydrolyzes dextrins into glucose molecules, yeasts ferment glucose into ethanol and carbon dioxide and the nitrogen source (usually urea or ammonium sulfate) provides nutrition to yeasts. Proteases also are added during fermentation to convert corn proteins into amino acids to provide additional nitrogen source. SSF maintains a low glucose concentration in fermentation beer, reducing osmotic stress on yeast. SSF is carried out at 32ºC for 48 to 72 h for complete starch conversion. Ethanol concentration of approximately 17% v/v is achieved during the SSF process. The fermentation beer undergoes distillation where 95% v/v ethanol is produced. The ethanol-water mixture obtained after distillation is passed through molecular sieves that separate ethanol from water to produce pure ethanol. The remainder of post distillation beer is known as whole stillage which includes corn protein, oil, fiber and unfermented starch. Whole stillage is centrifuged to produce wet grains and thin stillage of which 15 to 30% thin stillage is recycled to the slurrying process and the remainder is concentrated into syrup using evaporators. Corn oil is separated from syrup using decanter centrifuge and the remainder of the syrup is mixed with wet grains and dried to produce dried distillers grains with solubles (DDGS) which is composed of 33% fiber and 34% crude protein (db) (Kim et al 2008). Revenue obtained from DDGS and corn oil is important for the sustainability of dry grind plants. Corn fiber is not converted into ethanol during the conventional dry grind process and accumulates in DDGS increasing the fiber content in DDGS (Bothast and Schlicher 2005, Kelsall and Pigott 2009, Rausch and Beylea 2006). High fiber content of DDGS restricts use of DDGS to ruminant animal diets (Beylea et al 2004, Corrigan and Mass 2009, Rausch and Beylea 2006, Singh et al 2005).

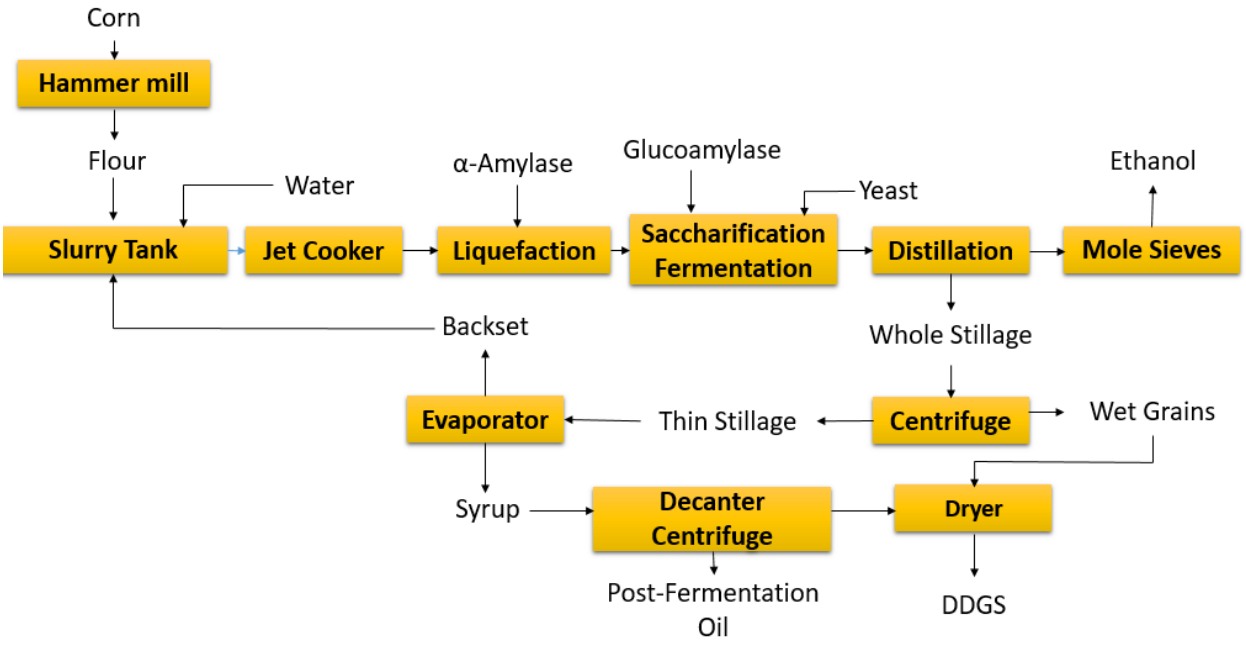

Fig 2.1. Schematic of dry grind ethanol process.

2.3. Benefits of converting structural carbohydrates into ethanol

2.3.1. Increase in ethanol yield and reduction in downstream processing costs

Corn fiber is composed of cellulose (15 to 20%), arabinoxylan (30 to 50%) and adherent starch (11 to 23%) (Gáspár et al 2007, Kim et al 2016c) which can be converted into ethanol. Grohmann and Brothast (1997) reported that complete utilization of corn fiber can yield 13% additional ethanol which would equal 0.22 gal/bu. Moreover, an increase in ethanol yield would decrease the energy requirement for the energy intensive distillation process (Madson 2009).

2.3.2. Improvement in DDGS quality

Conversion of corn fiber into ethanol would lead to DDGS with low fiber and high protein contents. Lower fiber content presents an opportunity for DDGS in the nonruminant market. Secondly, DDGS with increased protein content can be sold at a higher market price (Rausch and Beylea 2006, Martinez-Amezcua et al 2007).

2.3.3. Renewable fuel standard compliance

Because ethanol generated from corn fiber will be regarded as cellulosic biofuel, a plant can generate D3 Renewable Identification Numbers (RIN) for every gallon of bioethanol generated

from corn fiber (Gibson 2017, EPA 2017). Along with an increase in ethanol production, D3 RINs have a value of $2 per gallon of cellulosic ethanol and can contribute to the profitability of a dry grind plant (Gibson 2017).

2.4. Challenges associated with incorporation of cellulosic ethanol production in conventional dry grind ethanol plants

2.4.1. Biomass recalcitrance

Corn fiber is more recalcitrant towards hydrolysis than corn starch due to low enzyme accessibility and enzyme effectiveness, differences in composition (lignin, hemicellulose and cellulose), high crystallinity and insolubility of cellulose and structural complexity (Dien and Bothast 2009, Kim et al 2016a). Furthermore, lignin and hemicellulose components reduce enzyme accessibility and inhibit enzyme activity (Dien and Bothast 2009, Kumar and Murthy 2011, Kumar et al 2016, Kim et al 2016a).

2.4.2. Inability of yeast (*Saccharomyces cerevisiae*) to metabolize pentose sugars

Hemicelluloses account for 30 to 40% of biomass which can be broken down into pentoses such as xylose and arabinose. Due to the inability of yeasts (*Saccharomyces cerevisiae*) to metabolize pentoses, a large portion of biomass is not converted to ethanol. In addition, the pentose utilizing strains grow slowly and produce low ethanol yields (Bothast and Schlicher 2005, Bothast et al 1999). The use of genetically modified organisms during fermentation can be an approach to utilize biomass to its full potential (Kumar and Murthy 2011, Kumar et al 2016, Kim et al 2016a, Dien and Bothast 2009).

2.4.3. High pretreatment and enzyme cost

Biomass recalcitrance is responsible for requirement of an energy intensive pretreatment step in the cellulosic ethanol production that is not required in the conventional dry grind process (Bothast and Schlicher 2005). High costs associated with cellulolytic enzymes and high enzyme loading requirements due to biomass recalcitrance, low enzyme accessibility and enzyme inhibition in cellulosic ethanol production present barriers for incorporating fiber conversion in the dry grind process (Bothast and Schlicher 2005, Dien and Bothast 2009, Kumar et al 2016).

2.5. Ethanol production from lignocellulosic biomass

Biomass is reduced to an average size of 0.41 to 0.58 cm for further processing (Kim et al 2016a). Biomass pretreatment, hydrolysis and fermentation are three major steps in biomass processing following size reduction.

2.5.1. Biomass pretreatment

After a size reduction step, a pretreatment step is required for disrupting the biomass structure to reduce biomass recalcitrance and enhance enzyme accessibility. The pretreatment can be classified as physical pretreatment (mechanical refining, extrusion), chemical pretreatment (dilute acid pretreatment, dilute alkali pretreatment, liquid hot water pretreatments) and biological pretreatment (white rot fungi) and physiochemical pretreatment (AFEX (ammonia fiber expansion), steam explosion). Pretreatment partially hydrolyzes hemicellulose and cellulose, reduces cellulose crystallinity and enhances hydrolysis efficiency. Harsh pretreatment conditions can produce compounds (furfural, hydroxymethyl furfural, acetic and formic acids, phenolic compounds) inhibitory to enzymes and yeasts which significantly can reduce process efficiency. An ideal pretreatment should be cost effective, have high sugar yields and result in low inhibitor concentrations (Dien and Bothast 2009, Kumar and Murthy 2011, Kumar et al 2016, Kim et al 2016a). Pretreatments used in this study included hot water pretreatment and disk milling.

2.5.1.1. Hot water pretreatment

Hot water pretreatment is performed at temperatures of 160 to 230ºC with liquid water. At these temperatures and pressures water ionizes into protons and hydroxyl ions which makes the solution weakly acidic and catalyze the biomass breakdown. Acetic and uronic acids released from the hemicellulose fraction under high temperatures and pressures further catalyze the reaction. Hemicellulose-lignin bonds are hydrolyzed and the hemicellulose fraction is hydrolyzed to oligomers. Use of higher pressures and temperatures and high enzyme requirement in hot water pretreatment compared to other chemical pretreatment methods are the major drawbacks of the process. However, this method is chemical free and environmentally friendly and requires lower capital and operating costs compared to other pretreatments (Kim et al 2016a, Kim et al 2016b, Kumar and Murthy 2011, Mosier 2013).

2.5.1.2. Wet disk milling

Mechanical refining pretreatment of biomass includes different milling techniques such as hammer milling, knife milling, ball milling and disk milling. The major effects of mechanical refining are as follows (Kim et al 2016a, Park et al 2016):

Shortening: Reduction in particle size.

External fibrillation: Removal of primary cell walls of biomass due to shearing action, which includes cellulose, hemicellulose and lignin.

Internal fibrillation: Disruption of intra fiber hydrogen bonds and formation of hydrogen bonds with water molecules.

Milling techniques such as hammer milling and knife milling reduce biomass particle size and increase surface area and flowability of solids but cause minimal fibrillation (Kim et al 2016a). Thus, these techniques do little to reduce biomass recalcitrance and cannot be used alone as a pretreatment methods. Techniques such as ball milling and disk milling can be for biomass pretreatment as they lead to fibrillation of biomass.

A disk mill consists of two grooved disks: one disk remains stationary while the other rotates or both the disks counter rotate to provide shearing action for defiberizing biomass. Disk milling is more economical and scalable than other milling techniques and can be used in a continuous mode. Water and high energy requirements are drawbacks of wet disk milling (Kim et al 2016a, Kim et al 2016b, Hideno et al 2009, Hideno et al 2012, Park et al 2016).

2.5.2. Biomass hydrolysis

Pretreatment is followed by hydrolysis and fermentation which produce sugars from the pretreated biomass and ferment sugars to ethanol, respectively. Cellulases, hemicellulases and ligninases are three enzymes used for hydrolyzing cellulosic biomass. Cellulases are composed of endocellulases, exocellulases and β-glucosidases. Exocellulases hydrolyze cellulose from reducing (CBH I) and nonreducing (CBH II) ends; whereas, endoglucanases hydrolyze internal cellulose bonds to produce cellobiose which is hydrolyzed to glucose by β-glucosidases. Hemicellulases are responsible for the saccharification of xylose oligomers and increasing cellulose digestibility. Hemicellulases include endoxylanases, β-xylosidases and accessory

enzymes for the hydrolysis of complex hemicellulose structure. Ligninases (lignin peroxidases, manganese peroxidases and laccases) are not used extensively for biomass pretreatment or hydrolysis (Dien and Bothast 2009, Kumar and Murthy 2016).

2.5.3. Biomass fermentation

A limitation of *Saccharomyces cerevisiae* in biomass fermentation is inability to convert pentoses into ethanol. Thus, genetically modified strains capable of cofermenting glucose and xylose such as *Zymomonas mobilis* (Kumar and Murthy 2011), *Escherichia coli* strains K011, SL40 (Dien et al 1997, Lau et al 2008) and FBR5 (Dien et al 2004, Dien et al 2005) and *Saccharomyces cerevisiae* 424A (LNH-ST) (Mosier et al 2005) have been used in biomass fermentation studies. Similarly, genetically engineered *Saccharomyces cerevisiae* capable of cofermenting glucose and xylose along with inhibitory compounds such as cellobiose and acetate (Ha et al 2010, Fox et al 2011, Zhang et al 2016) have been developed.

2.6. Utilization of corn fiber for ethanol production

Pretreatments such as hot water and dilute acid pretreatment (sulfuric acid) have achieved up to 85% conversion of polysaccharides to fermentable sugars after hydrolysis (Weil et al 1998, Grohmann and Bothast 1997). Low pH used in dilute acid pretreatment aids in the hydrolysis of hemicellulose to xylose and arabinose and of cellulose fractions to glucose oligomers and monomers (Kim et al 2016a, Kumar and Murthy 2011, Trajano and Wyman 2013).

Kim et al (2017) studied effects of pericarp size on enzymatic hydrolysis of pericarp in the dry grind process using cellulases and concluded that a smaller pericarp size released higher quantities of phenolic compounds which inhibited cellulases. Phenol tolerant *Aspergillus niger* pectinase enzyme achieved a higher conversion of pericarp than cellulases derived from *Trichoderma ressei*. Combining phenol tolerant cellulases and intact pericarp could be a solution for the conversion of pericarp to ethanol in the dry grind process. Incorporating xylanolytic enzymes and proteases increased ethanol yield and oil recovery in the dry grind process (Luangthongkam et al 2015). Extruding flaked, ground and cracked corn prior to fermentation achieved a higher ethanol yield compared to autoclaving flaked corn. Addition of cellulases and proteases to flaked and extruded corn increased ethanol yield by 4% (Wang 2009).

Modified dry grind processes such as QGQF (quick germ quick fiber) and E-milling (enzymatic milling) allowed separation of fiber prior to fermentation in dry grind ethanol processes. The QGQF process separates pericarp (quick fiber) and germ prior to fermentation whereas E-milling separates pericarp fiber and endosperm fiber prior to fermentation (Singh et al 2005). Quick fiber was pretreated using dilute acid, hydrolyzed using cellulases, β-glucosidases and glucoamylases and fermented using *Saccharomyces cerevisiae* and *Escherichia coli* FBR5 with yeast fermentation giving 32% and bacterial fermentation achieving 85% theoretical yield based on total sugars released (Dien et al 2004). A similar study performed using endosperm and pericarp fiber obtained from E-milling achieved a total ethanol yield of 0.37 L/kg with endosperm fiber giving a 20.5% higher ethanol yield than pericarp fiber (Dien et al 2005).

Fiber separation has been attempted in the downstream processing stages for incorporating cellulosic ethanol in the dry grind process. Sieving whole stillage after grinding separated fiber with 45 to 100% NDF yielded 90.8% glucose and 92.9% xylose after dilute acid pretreatment and hydrolysis (Kim et al 2016c). Another approach for converting fiber in the dry grind processing is utilizing DDGS as a fiber source. DDGS has been treated with different pretreatment techniques such as liquid hot water (LHW), dilute acid and AFEX and hydrolysis and fermentation of pretreated DDGS has been studied. AFEX treatment involves heating biomass to moderate temperatures with liquid concentrated ammonia at high pressures. AFEX is known to decrystallize cellulose, partially hydrolyze hemicellulose and remove surface lignin (Bals et al 2006). Virtually complete conversion of cellulose to glucose was observed after AFEX pretreatment and hydrolysis for 72 h for DDGS (Bals et al 2006). Hydrolysis and fermentation of AFEX and LHW pretreated wet grains (13 to 15% moisture) by glucose fermenting *Saccharomyces cerevisiae* ATCC 4124 resulted in 100% conversion of glucose to ethanol (Kim et al 2008). Similarly, the effect of xylose fermenting strains *Escherichia coli* strains K011 and M011 (strain adapted due to selective evolution) was studied where M011 resulted in a better fermentation performance than K011 strain (Lau et al 2008). Similarly, the effect of compositional variability of DDGS on ethanol production was studied by Kim et al (2010).

Table 2.1. Studies performed for incorporation of corn fiber in the dry grind process.

Biomass	Pretreatment	Hydrolysis Enzymes	Fermentation strain	Source
Corn fiber obtained from wet milling	Dilute acid	Cellulase β-glucosidase Amyloglucosidase	*E. coli* K011	Grohmann and Bothast (1997)
	Hot water	Cellulase	-	Weil et al (1998)
	Hot water	Novozyme 188 Cellucast 1.5L	*S. cerevisiae* 424A (LNH-ST)	Mosier et al (2005)
Corn hulls Germ meal	-	Dilute Sulfuric Acid	*E. coli* K011 *E. coli* SL40	Dien et al (1997)
'Quick' fiber	Dilute acid	Cellulase β-glucosidase Amyloglucosidase	*E. coli* FBR5 *S. cerevisiae*	Dien et al (2004)
Endosperm pericarp fiber	Dilute acid	Cellulase β-glucosidase Amyloglucosidase	*E. coli* FBR5 *S. cerevisiae*	Dien et al (2005)
Corn pericarp	-	Cellulase Pectinase	-	Kim et al (2017)
Wet distillers grains	Dilute acid	Cellulase	-	Kim et al (2016c)
	Hot water AFEX	Cellulase β-glucosidase	*S. cerevisiae* ATCC 4124	Kim et al (2008)
DDGS	Hot water AFEX	Cellulase β-glucosidase	*S. cerevisiae* ATCC 4124	Kim et al (2008)
	AFEX	Spezyme CP β-glucosidase	*E. coli* K011 *E. coli* M011	Lau et al (2007)
	AFEX	Spezyme CP β-glucosidase	-	Bals et al (2006)
	-	Dilute Acid	-	Xu et al (2010)
	Hot water AFEX	Spezyme CP β-glucosidase	*S. cerevisiae* 424A (LNH-ST) *S. cerevisiae* D5A	Kim et al (2010)
	Hot water AFEX	Cellulase Pectinase Feruloyl Esterase	-	Dien et al (2008)

Commercially available enzymes are not efficient in degrading hemicellulose in corn fiber (Bothast and Schlicher, 2005, Hespell et al 1997). A low xylose concentration was observed in AFEX pretreated corn fiber (1.8 g/L at 5% solid concentration) and corn fiber xylan (25%

conversion) hydrolyzed by a combination of commercial glucoamylase, xylanase and cellulase enzyme preparation (Hespell et al 1997). Dien et al (2008) obtained a low xylose yield (< 40%) using a combination of cellulase (15 FPU/g), β glucosidase (40 U/g cellulose) and xylanase (40 U/g cellulose) in AFEX and LHW pretreated DDGS. A combination of cellulase (15 FPU/g), β -glucosidase (40 U/g cellulose), multifect pectinase (50 U xylanase/g DDGS) (for debranching and feruroyl esterase activities) and feruloyl esterase (3 FAE IU/g DDGS) could produce 82% xylose in AFEX and LHW pretreated DDGS. The complex nature of corn xylan with approximately 70% of the xylan backbone substituted with arabinose, glucoronic acid esters and uronic acid is responsible for ineffective hydrolysis (Hespell et al 1997). Corn fiber xylan strands are connected to phenolics (diferulates and triferulates) forming crosslinks between them (Bunzel et al 2005, Grabber et al 1995). Ferulate ester crosslinks reduce efficiency of the enzymatic hydrolysis of xylan (Grabber et al 1998a, 1998b).

2.7. Current status of cellulosic ethanol production in dry grind plants

National Renewable Energy Laboratory (NREL) collected data from 98 US demonstration, pilot and commercial projects and 13 international projects producing nonstarch fuels; of these 39 US and 13 international projects produced alcohol through biochemical and thermochemical processes. The majority of US (89%) and international (85%) projects used acid or enzymatic treatment with fermentation for ethanol production. The assignment of D3 renewable identification numbers was a result of increased cellulosic ethanol production with approximately 3.3 million gallons of cellulosic ethanol produced in 2016 (Warner et al 2017).

Many technologies are being developed with the goal of becoming integrated directly in existing dry grind ethanol plants and producing cellulosic ethanol from corn pericarp. ICM's Added Cellulosic Ethanol (ACE) + Enogen and Edeniq's Cellunator + enzyme technologies are examples of such technologies. ICM's technology separates corn fiber for pretreatment, fermentation and distillation parallel to corn ethanol production and can increase cellulosic yields up to 10% (ICM 2016, Gibson 2017). Edeniq's technology involves cellulosic fiber mixing, fine milling and fermentation with slurry using their licensed enzymes which can increase cellulosic ethanol yields up to 2.5% and total ethanol yields up to 7% of total ethanol production (Edeniq 2016). Implementation of technologies developed by ICM and Edeniq can increase plant capacity 2 to 10%, contributing to plants profit (Warner et al 2017).

Chapter 3

Increasing ethanol yield in corn dry grind processing through fiber pretreatment after liquefaction

3.1. Introduction

Conversion of fiber to ethanol potentially would increase the yield of the conventional dry grind process up to 13% (Grohmann and Bothast 1997). Subsequently, a decrease in downstream processing costs and improved quality of DDGS would contribute to the profitability of the process (Bothast and Schlicher 2005, Madson 2009, Rausch and Beylea 2006). Due to the recalcitrant nature of corn fiber, conversion to ethanol would require further processing (Bothast and Schlicher 2005). Furthermore, separation of corn fiber in the dry grind process would require capital intensive processing (Gibson 2017).

Hot water pretreatment and disk milling can reduce corn fiber recalcitrance to improve sugar release during hydrolysis and are less capital intensive than other pretreatment techniques (Kim et al 2016, Hideno et al 2009). Biomass hydrolysis also is dependent on cellulase loading.

Liquefied mash was filtered and the retentate (referred to as fiber) was used in SSF. Fiber was pretreated using hot water and wet disk milling techniques; SSF was performed using commercially available glucoamylase, cellulase, hemicellulase and yeast at 30 FPU/g fiber cellulase loading. Effects of cellulase loadings were investigated by performing SSF on untreated fiber with 120 FPU/g fiber cellulase loading.

Specific objectives were to compare:

1) Effects of hot water pretreatment and disk milling conditions on the fermentation characteristics of fiber (ethanol concentration and conversion efficiency).
2) Effects of cellulase loadings on the fermentation characteristics of untreated fiber (ethanol concentration and conversion efficiency).

3.2. Materials

3.2.1. Experimental material

Yellow corn (P1197AMXT) harvested in year 2016 was obtained from DuPont Pioneer (Champaign, IL). Corn was sieved over 12/64 (4.8 mm) sieve, manually cleaned to remove broken corn and foreign material and stored in Ziploc bags at 4ºC.

3.2.2. Enzymes, yeasts and other chemicals

SPEZYME®CL (DuPont Industrial Biosciences, Palo Alto, CA) was a thermostable α-amylase obtained from genetically modified strain of *Bacillus licheniformis.* SPEZYME®CL, had a pH of 5.5 to 6.5 and a density of 1.17g/mL. DISTILLASE® SSF (DuPont Industrial Biosciences, Palo Alto, CA) was an enzyme cocktail containing glucoamylase (1,4-α-D-glucan hydrolase E.C. 3.2.1.3), amylase (1,4-α-D-glucan glucanohydrolase - EC 3.2.1.1) and aspergillopepsin I (EC 3.4.23.18). DISTILLASE® SSF was derived from genetically modified *Trichoderma ressei*. DISTILLASE® SSF, had a density of 1.1 to 1.14 g/mL and an optimum pH of 4.0 to 4.5. Cellic®Ctec2 (Novozymes, Franklinton, NC) was a commercial blend of cellulases, β-glucosidases and hemicellulases used for hydrolyzing cellulose to glucose. Cellic®Htec2 (Novozymes, Franklinton, NC) was a commercial blend of endoxylanases capable of hydrolyzing hemicellulose. Yeast culture for SSF was prepared by mixing 5 g yeast (Ethanol Red, Lesaffre Yeast Corp., Milwaukee, WI) with 25 mL deionized (DI) water at 32ºC at 95 rpm for 20 min. Citrate buffer (0.1 M, pH 5) was prepared by mixing 0.1 M citric acid monohydrate (Sigma Aldrich, St. Louis, MO) and trisodium citrate dihydrate (Sigma Aldrich St. Louis, MO) in a 7:13 ratio and adjusting the pH to 5. pH adjustment for slurry, buffer and mash was performed using 10*N* sulfuric acid (Ricca Chemical, Arlington, TX) and 5 M sodium hydroxide (Sigma Aldrich, Aldrich St. Louis, MO).

3.3. Methods

3.3.1. Fiber preparation

Corn was ground using a hammer mill (1100W, model MHM4, Glen Mills Inc., Clifton, NJ) at 500 rpm using a 0.5 mm sieve. Moisture content was measured by drying at 105ºC for 16 h (AACCI Method 44-15.02). Ground corn (100 g db) was diluted with DI water to a 32% solids content and slurry pH was adjusted to 5.1. Alpha-amylase (SPEZYME®CL), 25.7 µL, was

added to the slurry and slurry was liquefied at 85ºC for 90 minutes. Mash was vacuum filtered in a 4 L flask through Whatman No.4 filter paper. Filtered liquid analyzed by HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA) and retentate was weighed and dried for 72 h at 49ºC. The dried retentate was ground using a hammer mill (1100W, model MHM4, Glen Mills Inc., Clifton, NJ) at 500 rpm through a 2 mm sieve. Moisture content of the ground retentate was measured by drying a sample at 105ºC for 16 h (AACCI Method 44-15.02). Ground retentate was stored in Ziploc bags at -18ºC. The ground mash retentate was defined as fiber.

3.3.2. Composition analysis of fiber

Composition analysis of fiber was performed using procedures developed and published by NREL (National Renewable Energy Laboratory). Extractives were determined by the procedure adapted from Sluiter et al (2008a). Samples were wrapped in filter bags (XT4, Ankom Technology, Macedon, NY) and extraction was performed using DI water and 95% ethanol for 16 h each. Extracted samples were dried for 24 h in a 49ºC oven and extractives content was calculated from difference between weights of initial and final samples. The extractive free samples were analyzed for carbohydrate and lignin contents using a procedure adapted from Sluiter et al (2008b). Samples were digested in 72% w/w sulfuric acid at 30ºC for 60 min and in 4% w/w sulfuric acid at 121ºC for 60 min. Digested samples were filtered through filtering crucibles and filtrate obtained was analyzed for structural carbohydrates and acid soluble lignin. To determine structural carbohydrates, 10 mL of sample was neutralized to pH 5 to 6 by adding 4.6 g barium carbonate (Sigma Aldrich, St. Louis, MO). The neutralized sample was analyzed for sugars in HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA). The remaining solution was used for analysis of acid soluble lignin. Liquid was diluted 1000 times using deionized (DI) water and absorbance was measured at 205 nm using a UV-Vis Spectrophotometer (Evolution array, Thermo Scientific, Waltham, MA) to determine acid soluble lignin. Crucibles with retentate were dried at 100ºC to determine dry weight and ashed in the oven at 575ºC for 4 h to eliminate carbon. Acid insoluble lignin was determined as the difference between the dried crucible weight and the weight of ashed crucible. Ash content (Sluiter et al 2008c) in the biomass was determined by ashing a specified weight of sample in a muffle furnace at 575ºC for 4 h and measuring the final sample weight. Soluble sugar determination was performed by diluting fiber to 10% w/w solids and agitating in a vortex mixer (Fisher Scientific Mini Vortexer, Cat #

128101, Hampton, NH) for 30 min. The solution obtained was analyzed in HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA). The filtrate obtained after filtering mash was analyzed for sugars in HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA).

3.3.3. Starch content determination

Total starch content of corn was determined by a method adapted from Vidal et al (2009). Fiber was milled to pass through a 0.5 mm screen. Alpha-amylase solution was prepared by diluting 1 mL thermostable α-amylase (Megazymes, Wicklow, Ireland) in a 1:30 ratio with 0.1 M sodium acetate buffer of pH 5. Ethanol (80% v/v), 0.2 mL, was added to 0.1 g ground fiber and stirred in vortex mixer to aid dispersion. Alpha-amylase solution, 3 mL, was added to the mixture and the mixture was placed in a boiling water bath for 6 min. The mixture was incubated with 0.1 mL thermostable glucoamylase (Megazymes, Wicklow, Ireland) at 50ºC for 30 min. Mixture was diluted to 10 mL with distilled water and mixed thoroughly. The diluted mixture was centrifuged (1500 x g) for 10 min and the filtrate obtained was analyzed in HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA). Blank determination was performed with 0.1 mL water to account for glucose present in enzyme solutions and subtracted from the starch content. The glucose concentration was determined for pure starch by similar method using 0.1 g starch to account for the recovery factor.

3.3.4. Hot water pretreatment

Hot water pretreatment was adapted from Mosier et al (2005). Fiber (10 g db) was diluted using DI water to 20% w/w solids. The mixture was filled in batch tubular 316 stainless steel reactors (Swagelok SS-T12-S-065-20, Chicago Fluid System Technologies, Chicago, IL) with 19.1 mm O.D. × 1.7 mm wall thickness and 104.8 mm length and capped on both sides with 19.1 mm screwed 316 stainless steel caps (Swagelok SS-T12-S-065-20, Chicago Fluid System Technologies, Chicago, IL). Hot water pretreatment was performed by heating the reactors in a fluidized sand bath (Model 01187-00 bath and 01190-72 temperature controller; Cole-Parmer, Vernon Hills, IL). To monitor internal temperature, one tubular reactor had 19.1 to 6.4 mm reducing union to accommodate a thermocouple (39105K212, Penetration/Immersion Thermocouple Probe Mini Conn (Pointed-Tip, Type K, -250 to 900ºC), McMaster-Carr, Robbinsville, NJ). Temperature data were recorded using a data logger (HH306/306A, Data Logger Thermometer, Omega, Stamford, CT). Hot water pretreatment was performed by heating

reactors to 160ºC and maintaining the temperature for 5, 10 or 20 min. The reactors were cooled by immersion in cold water.

3.3.5. Wet disk milling

Disk milling was performed according to procedure outlined by Kim et al (2016b). Disk milling (3 cycles) was performed on fiber with 20% w/w and 45% w/w dry solids. Disk milling (Quaker City Grinding Mill model 4E, Straub Co., Philadelphia, PA) was operated at a disk speed of 89 rpm with zero clearance between stationary and rotating disks. A sample from the disk milled slurry was used for moisture determination. Disk milled slurry was diluted to 10% w/w solids to perform SSF.

3.3.6. Simultaneous saccharification and fermentation (SSF) of fiber using *Saccharomyces cerevisiae*

The procedure outlined by Dien et al (2004) was adapted for carrying out SSF. For control treatment, 10 g db fiber was diluted to 10% w/w solids with 0.1 M citrate buffer (pH 5). Cellulase enzyme (Cellic CTec2, 135 FPU/mL), 2.2 mL, 0.55 mL hemicellulase enzyme (Cellic HTec2), 7 µL glucoamylase (DISTILLASE® SSF), 0.25 mL yeast culture and 0.05 mL 50% w/v urea solution was added at the start of SSF. For hot water and disk milling pretreatments, pH of 10 g db pretreated fiber was adjusted to 5.0 and the fiber was diluted to 10% w/w solids with 0.1 M citrate buffer (pH 5). SSF was performed similar to the control treatment. For excess cellulase treatment, 10 g db fiber was diluted to 10% w/w solids with 0.1 M citrate buffer (pH 5). Cellulase enzyme (Cellic CTec2, 135 FPU/mL), 8.8 mL, 0.55 mL hemicellulase enzyme (Cellic HTec2), 7 µL glucoamylase (DISTILLASE® SSF), 0.25 mL yeast culture and 0.05 mL 50% w/v urea solution was added at the start of SSF. For all treatments, SSF was performed for 72 h at 32ºC with continuous agitation at 95 rpm in a water bath. SSF was monitored by taking 1 mL samples at 0, 6, 12, 24, 48 and 72 h. Samples were analyzed in duplicate to determine sugar and ethanol content in an HPLC (Model 2414, Waters Corporation, Milford, MA; Aminex HPX-87H column, Bio–Rad Laboratories, Hercules, CA). Samples were prepared according to the procedure outlined by Singh et al (2005). Each treatment was performed with three replicates. To account for ethanol production from sugars present in enzymes, SSF was performed with all constituents except biomass. Ethanol concentrations in blank flasks were subtracted from treatment flasks. Figure 3.1 shows the experiment design for the study.

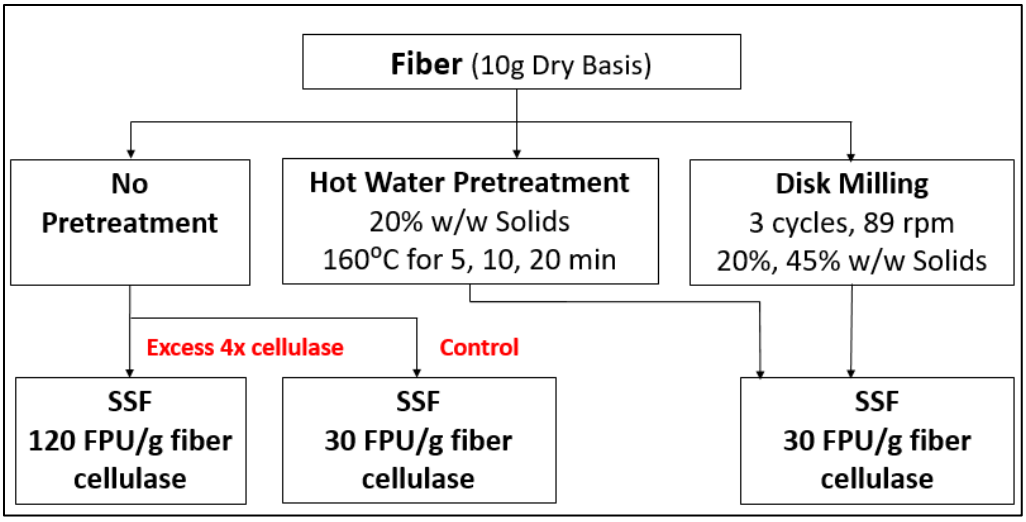

Fig. 3.1. Experiment design for the study

3.3.7. Data analysis

Every experiment was performed in three replicates and the results were analyzed in duplicates. To compare mean differences, analysis of variance (ANOVA) and Tukey's HSD tests (R core team, Vienna, Austria) were performed at 95% level of significance. The procedure outlined by Kumar et al (2017) was used to calculate conversion efficiency.

$$V_{EtOH} = \frac{(V_{H2O}\rho_{H2O}) + (W_{Corn}MC_{Corn}) - (NV_n) - (W_{Corn}(1 - MC_{Corn})S(0.11))}{\frac{\rho_{EtOH/H2O}}{C_{EtOH}} - \rho_{EtOH}}$$

$$V_{EtOH\,max} = \frac{W_{Corn}(1 - MC_{Corn})S(1.11)(0.511) + G(0.511)}{\rho_{EtOH}}$$

$$E_{Conversion} = \frac{V_{EtOH}(100)}{V_{EtOH\,max}}$$

where V_{EtOH} is the volume of ethanol in beer, $V_{H2O}\rho_{H2O}$ is the mass of water added in beer, W_{Corn} is the weight of corn in beer, MC_{Corn} is the moisture content of corn, N is the number of samples taken during process, V_n is the volume of liquid in each sample, S is the starch content of corn on dry basis, G is glucose concentration on dry basis, 1.11 is the hydrolytic gain of starch during hydrolysis, 0.511 is the stoichiometric ratio of ethanol to glucose during fermentation, $\rho_{EtOH/H2O}$ is the density of water-ethanol mixture, C_{EtOH} is the concentration of sample in % v/v, ρ_{EtOH} is the density of ethanol (0.789 g/mL), $V_{EtOH\,max}$is the maximum concentration of ethanol

achieved by conversion of starch into sugars and $E_{Conversion}$ is the percentage conversion efficiency.

3.4. Results and discussion

3.4.1. Fiber and mash filtrate composition

Weights of fiber and filtrate obtained after filtration were 29.5% and 70.5% of mash weight, respectively. Fiber had a moisture content of 56.1% wb (wet basis) and filtrate had 72.1% wb moisture. Dried and ground fiber had a moisture content of 4.8% wb.

Fiber derived after liquefaction and filtration on a dry basis was composed of 10.8% glucan, 7.0% xylan, 5.5% arabinan, 5.9% acid soluble lignin, 3.9% acid insoluble lignin, 1.2% ash and 62.1% db extractives (Table 3.1). Fiber contained 21.5% structural carbohydrates and 49.3% db starch on a dry basis that implied the hydrolysis and saccharification of fiber could yield a large amount concentration of fermentable sugars. Soluble sugars present in the fiber were glucose (1.8%) and maltose (7.8%); no pentoses were detected in the analysis. Mash filtrate was composed of 1.3% w/v glucose, 5.6% w/v maltose, 0.03% w/v arabinose and 0.05% w/v galactose. There was no xylose detected in the filtrate.

A low concentration of monomeric sugars in fiber and filtrate was expected as α-amylase cannot hydrolyze starch to monomeric sugars. A high extractive yield (62.1%) and moisture content (56.1% before drying) of fiber was suggestive that a large amount of filtrate (with soluble sugars and dextrins) was retained in the fiber.

Table 3.1. Composition of fiber (mash retentate).

Component	Amount (% w/w db)*
Extractives	62.1 ± 0.5
Glucan[a]	10.8 ± 0.6
Galactan[a]	0.2 ± 0.3
Xylan[a]	5.7 ± 0.3
Arabinan[a]	4.8 ± 0.5
Acid Soluble Lignin	5.9 ± 0.1
Acid Insoluble Lignin	3.2 ± 0.1
Ash	1.2 ± 0.1
Glucose	1.8 ± 0.1
Maltose	7.8 ± 0.2
Starch	49.3 ± 0.2

*Mean ± standard deviations from three replicates. [a] Anhydrous monosaccharides.

Table 3.2. Composition of mash filtrate.

Component	Amount (% w/v)*
Glucose	1.30 ± 0.06
Galactose	0.04
Arabinose	0.03
Maltose	5.6 ± 0.2

*Mean ± standard deviations from three replicates.

3.4.2. Effect of hot water pretreatment on ethanol yield and glucose release

Ethanol yields of hot water pretreated fiber except for the 160ºC for 20 min pretreatment were higher than control (Table 3.3). There were no differences among the ethanol yields of fiber pretreated with hot water at 160ºC except among 5 and 20 min hot water pretreatments (Table 3.3).

Glucose profiles of 160ºC for 5, 10 and 20 min and control were similar during SSF (Fig. 3.3). The glucose concentration decreased with time and nearly all glucose was fermented after

24 h of fermentation indicating completion of fermentation (Fig. 3.3). There was no inhibitor (furfural or HMF) formation after hot water pretreatment. Absence of monomeric sugar formation during pretreatment has been correlated positively with absence of inhibitors (Mosier et al 2005).

Loosening of the cellulosic structure of corn fiber and the release of cellulose and hemicellulose oligomers during pretreatment were responsible for the higher ethanol concentration in hot water pretreated fiber. An increase in sugar yield by hot water pretreatment was observed for sugarcane bagasse (Gao et al 2013, Yu et al 2013), oil palm biomass (Zakaria et al 2015), corn stover (Kim et al 2016, Mosier et al 2005), palm kernel cake (Goh et al 2010, 2012) and miscanthus (Khullar et al 2016, Boakye-Boaten et al 2015).

3.4.3. Effect of disk milling on ethanol yield and glucose release

The final ethanol concentrations of fiber disk milled at 20 and 45% w/w solids and control were not different (Table 3.3). Nearly all glucose was fermented after 24 h for control and disk milling treatments (Fig 3.3).

Wet disk milling has been an effective pretreatment for increasing sugar and ethanol yields in different feedstocks (Chen et al 2013, da Silva et al 2010, Jones et al 2013, Kim et al 2016b, 2017, Zakaria et al 2015, Kumagai et al 2015, Hideno et al 2009, 2012). In this study, the feedstock contained high quantities of extractives (mostly soluble sugars and dextrins) compared to other biomass. Extractives lost during disk milling is one of the factors responsible for low ethanol yield during the SSF of disk milled fiber compared to the control. The low initial glucose concentration in the disk-milled fiber compared to the control is an indicator of loss of extractives. Solid loss in disk milling was observed by Kim et al (2017) due to solids being stuck to the mill. Thus, the gain obtained after disk milling treatment was offset by the loss of extractives in the milling step.

Fine grinding or enhanced milling technologies coupled with cellulosic enzymes can increase ethanol yields in commercial dry grind plants by releasing starch adhered to fiber and converting cellulose fiber into fermentable sugars (Gibson 2017). In the laboratory, corn was ground to a particle size of 0.5 mm which released most of the bound starch, thus additional grinding did not improve ethanol yield.

Table 3.3. Ethanol concentration achieved with different pretreatments after 72 h SSF.

Pretreatment	Ethanol Concentration (% v/v)*
Control	3.35 ± 0.11^{ba}
Hot Water, 160ºC, 5 min	3.65 ± 0.06^{c}
Hot Water, 160ºC, 10 min	3.57 ± 0.19^{cd}
Hot Water, 160ºC, 20 min	3.46 ± 0.06^{abd}
Disk Milling, 3 cycles, 20% solids	3.29 ± 0.15^{a}
Disk Milling, 3 cycles, 45% solids	3.44 ± 0.05^{bd}

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance (p>0.05).

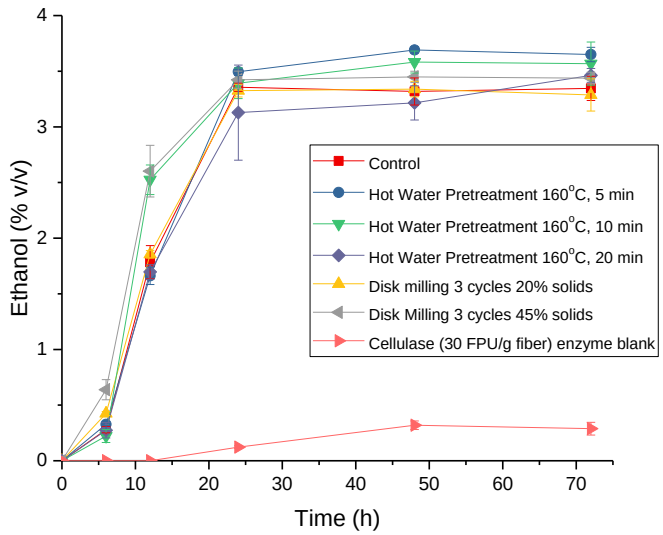

Fig 3.2. Ethanol profile of fiber pretreated using hot water and disk milling.

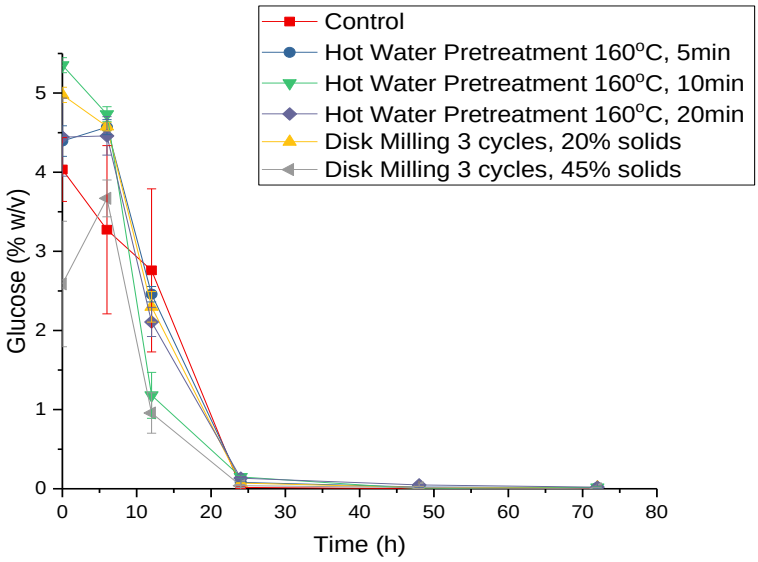

Fig 3.3. Glucose profiles of fiber pretreated using hot water and disk milling.

3.4.4. Effect of excess cellulase (120 FPU/g fiber) addition on ethanol yield and glucose release

SSF performed with excess cellulase dose achieved a higher ethanol concentration compared to the control (Table 3.4). Glucose present in the added enzyme solution and cellulose hydrolysis were factors responsible for higher ethanol concentration in the excess cellulase treatment compared to control. Enzyme blanks were prepared to account for differences in ethanol yields due to glucose present in enzymes. After accounting for ethanol yield due to glucose present in the enzymes, excess cellulase dose achieved a 31% increase in net ethanol yield compared to the control (Table 3.6). Nearly all glucose was consumed at 24 h for the control treatment whereas the excess cellulase treatment required 48 h to complete fermentation (Fig. 3.5).

Higher fermentation efficiency achieved by excess cellulase dose was indicative of incomplete biomass hydrolysis with commercially recommended dose of cellulase. As the biomass was not pretreated, biomass recalcitrance can be a reason for incomplete hydrolysis of biomass. Firm binding of cellulose and hemicellulose polymers by lignin, cellulose crystallinity, low accessible surface area and protection by lignin fraction are reasons contributing to biomass recalcitrance. Pretreatment is required for loosening biomass structure and reducing recalcitrance for efficient hydrolysis (Kumar and Wyman 2009).

Release of phenols from ground pericarp can be responsible for cellulase inhibition in corn fiber hydrolysis (Kim et al 2017). The concentration and type of phenolic compound influence cellulase inhibition (Kim et al 2011, 2017, Tejirian and Xu 2011, Ximenes et al 2010, 2011, Oliva-Taravilla et al 2015). Ximenes et al (2010) identified inhibitory effects of individual phenolic compounds on cellulase and β-glucosidase activities. The time of enzyme incubation with phenolic compounds is an important factor affecting hydrolysis (Ximenes et al 2011); therefore, the long hydrolysis time was responsible for enzyme deactivation. The removal of phenolic compounds improved hydrolysis efficiency in pretreated maple hydrolysate (Kim et al 2011). Cellulases derived from *Trichoderma ressei* are more sensitive to phenol inhibition compared to *Aspergillus niger* cellulases (Kim et al 2011, 2017, Tejirian and Xu 2011, Ximenes et al 2010, 2011). A high protein to phenol ratio improves hydrolysis efficiency (Kim et al 2011, 2017, Tejirian and Xu 2011, Ximenes et al 2010, 2011).

Cellobiose inhibits both exocellulases and endoglucanases. Although β-glucosidase converts cellobiose to glucose, both glucose and cellobiose are inhibitors of β-glucosidase (Saha et al 1998). Cellobiose concentrations of 3 and 8.5 g/L were observed in control and excess cellulase treatments after 12 h SSF which were hydrolyzed to glucose by β-glucosidases. Unhydrolyzed starch polysaccharides inhibit cellulases; whereas, hydrolyzed starch polymers inhibit β-glucosidases (Ximenes et al 2010). Xylose and xylooligosaccharides are also cellulase inhibitors (Kim et al 2011, Kumar and Wyman 2009b, Qing et al 2010, Ximenes et al 2010) but a low xylose concentration (< 0.3% w/v) was observed in the study.

Table 3.4. Ethanol concentration achieved with different pretreatments after 72 h SSF.

Treatment	Ethanol Concentration (% v/v)*
Control	3.17 ± 0.12^{a}
Excess Cellulase dose	5.01 ± 0.05^{b}

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance ($p>0.05$).

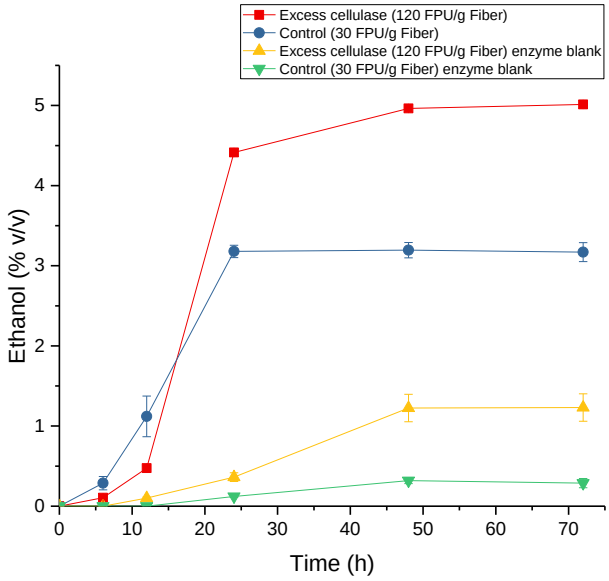

Fig 3.4. Ethanol profile during SSF of fiber using excess cellulase dose.

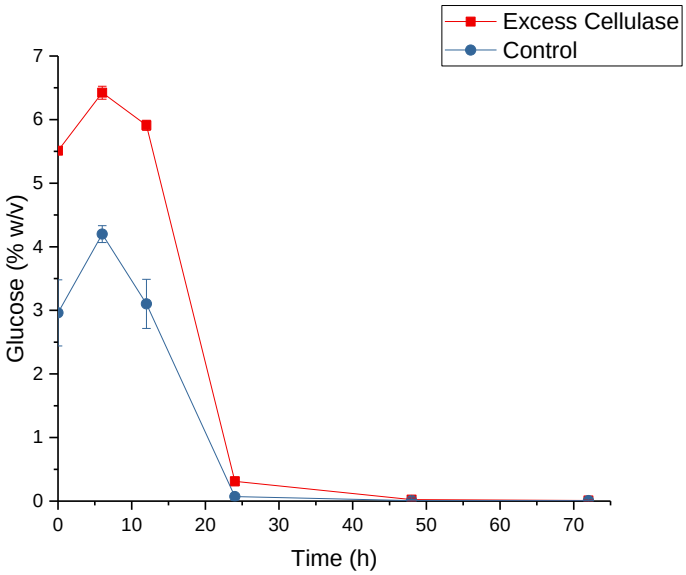

Fig 3.5. Ethanol profile during SSF of fiber using excess cellulase dose.

3.4.5. Comparison of ethanol yields achieved from different pretreatment methods

Cellulases used in the SSF process had a comparable amount of sugars which affected ethanol yields. Enzyme blanks were fermented to account for the effect of glucose present in enzymes on ethanol yields and calculate ethanol yield due to fiber hydrolysis. The enzyme blanks for control

and excess cellulases resulted in ethanol concentrations of 0.29 and 1.23% v/v respectively. Conversion efficiencies achieved ranged from 68.0% to 92.5% (Table 3.5, Table 3.6). Hot water pretreatments at 160ºC for 5 min and 10 min had higher ethanol concentrations and conversions compared to the control. Conversion efficiencies for all hot water pretreatment conditions were similar. Disk milled and control treatments achieved similar ethanol concentrations and conversion efficiencies after 72 h fermentation. Corn ground using a screen size of 0.5 mm released most of the starch adhered to fiber. Thus, disk milling post slurry making did not affect the ethanol yield. In a commercial dry grind process, hammer milling does not release all the starch adhered to fiber. Fine grinding in a commercial dry grind process would release more starch increasing ethanol yield. Disk milling treatment had a final ethanol concentration similar to that of fiber pretreated at 160ºC for 10 and 20 min whereas fiber pretreated at 160ºC for 5 min had a higher ethanol concentration compared to the disk milled fiber. Excess cellulase addition yielded the highest ethanol concentration and conversion efficiencies among the treatments. Hot water pretreatment, disk milling and excess cellulase addition resulted in 10, 3 and 31% increases in ethanol concentrations relative to control, respectively (Table 3.5, Table 3.6). Corn fiber could be converted into ethanol without pretreatment. Corn fiber could be converted into ethanol in a conventional dry grind process by addition of cellulolytic enzymes during SSF as fiber pretreatment was not required for conversion of corn fiber into ethanol.

Table 3.5. Ethanol concentrations, conversion efficiencies and ethanol yield gains achieved with different pretreatments after accounting for enzyme blanks.

Pretreatment	Ethanol Concentration (% v/v)*	Conversion Efficiency (%)*	Gain in ethanol concentration (%)
Control	3.06 ± 0.11^{b}	69.4 ± 2.6^{b}	NA
Hot Water, 160ºC, 5 min	3.36 ± 0.06^{c}	76.6 ± 1.5^{c}	10
Hot Water, 160ºC, 10 min	3.29 ± 0.19^{cd}	74.6 ± 4.6^{cd}	7
Hot Water, 160ºC, 20 min	3.17 ± 0.06^{abd}	72.1 ± 1.5^{abcd}	4
Disk Milling 3 cycles, 20% solids	3.00 ± 0.15^{a}	68.0 ± 3.4^{a}	-2
Disk Milling 3 cycles, 45% solids	3.15 ± 0.05^{bd}	71.5 ± 1.2^{bd}	3

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance ($p>0.05$).

Table 3.6. Ethanol concentrations, conversion efficiencies and ethanol yield gains achieved with different pretreatments after accounting for enyme blanks.

Pretreatment	Ethanol Concentration (% v/v)*	Conversion Efficiency (%)*	Gain in ethanol concentration (%)
Control	2.88 ± 0.11^{a}	69.8 ± 3.0^{a}	NA
Excess Cellulase	3.78 ± 0.05^{b}	92.5 ± 1.2^{b}	31

*Mean ± standard deviations from three replicates. Means followed by same letter are not significantly different at 95% level of significance ($p>0.05$).

3.4.6. Xylose release

Xylose and arabinose concentrations were low (< 0.3% w/v of xylose and negligible arabinose) which corresponded to less than 50% of xylose release and negligible arabinose release during SSF. Low efficiency of commercially available hemicellulase enzymes in hydrolyzing corn pericarp hemicellulose is responsible for low xylose concentration in all the experiments (Bothast and Schilier, 2005, Hespell et al 1997, Dien et al 2008).

3.5. Conclusions

Hot water pretreatment performed at 160ºC for 5 and 10 increased conversion efficiencies compared to the control treatment. Fiber pretreated at 160ºC for different times had no differences in conversion efficiencies. Ethanol concentrations achieved after 3 cycles of disk milling at 20% and 45% w/w solids were not different from the control. SSF performed with excess cellulase dose (120 FPU/g fiber cellulase) achieved a higher final ethanol concentration compared to the control (30 FPU/g fiber cellulase). SSF performed with excess cellulase achieved a higher conversion efficiency compared to the other pretreatments.

Hot water pretreatment, disk milling and excess cellulase addition could achieve 10, 3 and 31% increase in ethanol concentrations, respectively. Similarly, excess cellulase addition could achieve higher conversion efficiency compared to other treatments tested. Separation and pretreatment of corn fiber in the dry grind process might not be necessary for converting corn fiber into ethanol. Conversion of corn fiber to ethanol can be achieved by adding cellulolytic enzymes during SSF in the conventional dry grind process.

Chapter 4

Use of cellulases in the dry grind ethanol process

4.1. Introduction

Fermentable sugars can be produced through hydrolysis of corn fiber (Grohmann 1997, Hespell 1997) present in DDGS (Dien et al 2008), whole stillage (Kim et al 2016c), wet grains (Ximenes et al 2010) and the dry grind process (Kim et al 2017). Corn fiber obtained from wet milling (Mosier et al 2005, Saha et al 1998), the quick fiber dry grind process (Dien et al 2004) and the enzymatic milling dry grind process (Dien et al 2005) can be converted into ethanol. Similarly, fermentation of the corn fiber present in DDGS (Kim et al 2008, Kim et al 2010, Lau et al 2008, Xu et al 2008) has been studied.

Luangthongkam et al (2015) observed that addition of cellulase cocktail post grinding improved ethanol yields by 10.7%; whereas, addition of cellulase cocktail, phytase and protease during SSF improved ethanol yields by 16.5% in the conventional dry grind process. Addition of protease and cellulase improved ethanol yields by 9.2% when SSF of flaked and extruded corn was performed with the addition of liquefaction and saccharification enzymes and yeasts (Wang et al 2009).

Corn fiber is composed of 15 to 20% adherent starch (Gáspár et al 2007) which can be converted to ethanol. Fine grinding corn releases the starch adhered to fiber, increases enzyme accessibility and pretreats corn fiber for hydrolysis (Edeniq 2016, Gibson 2017). Wet disk milling pretreatment (which involves the principle of fine grinding) increased sugar and ethanol yields in different biomass materials (Chen et al 2013, da Silva et al 2010, Jones et al 2013, Kim et al 2016b, 2017, Zakaria et al 2015, Kumagai et al 2015, Hideno et al 2009, 2012).

It is claimed that recent technologies based on fine grinding and the cellulolytic enzyme addition can improve overall ethanol yields (Edeniq 2016, Gibson 2017). These technologies require lower capital cost and implementation time compared to technologies involving fiber separation and fermentation (Gibson 2017).

The specific objectives of this chapter were to evaluate:

1. Fermentation performance in the conventional dry grind process as affected by cellulase dose.
2. Effects of fine grinding and combined fine grinding and cellulase addition on fermentation performance in the conventional dry grind process.

4.2. Materials and methods

4.2.1. Dry grind procedure

Yellow corn (P1197AMXT) harvested in 2016 was obtained from DuPont Pioneer (Champaign, IL). Corn was sieved over 12/64 (4.8 mm) sieve, manually cleaned to remove broken corn and foreign material (BCFM) and stored in Ziploc bags at 4ºC. Corn was ground using a hammer mill (1100W, model MHM4, Glen Mills Inc., Clifton, NJ) at 500 rpm using a 0.5 mm sieve. The ground corn moisture content was measured by drying at 105ºC for 16 h (AACCI Method 44-15.02).

In the conventional dry grind process (control), ground corn (50 g db) was suspended in DI water to 32% solids and slurry pH was adjusted to 5.1. Alpha-amylase (SPEZYME®CL), 11 µL, was added to the slurry which was liquefied at 85ºC for 90 min. Liquefied mash pH was adjusted to 4.8, and SSF was performed by adding 35 µL glucoamylase (DISTILLASE® SSF), 1 mL yeast and 0.2 mL 50% w/v urea solution. Modifications to the conventional process included: addition of 30 FPU/g fiber cellulase during SSF, addition of 120 FPU/g fiber (excess) cellulase during SSF, disk milling (3 cycles), disk milling slurry and cellulase addition (30 FPU/g fiber). In 30 FPU/g fiber cellulase treatment, liquefaction was performed with conditions similar to conventional dry grind process. SSF was performed by adding 1.65 mL cellulase enzyme (135 FPU/mL) (Cellic CTec2), 0.41 mL hemicellulase enzyme (Cellic HTec2), 35 µL glucoamylase (DISTILLASE® SSF), 1 mL yeast and 0.2 mL 50% w/v urea solution. In disk milling treatment, ground corn (50 g db) was suspended in deionized water to 32% solids and pretreated with 3 cycles of disk milling. Liquefaction and SSF was performed similar to control. In another treatment, based on the results in the previous chapter, SSF was performed with (excess cellulase) 4 times the recommended cellulase dose (30 FPU/g fiber). In excess cellulase treatment, liquefaction was performed similar to control. SSF was performed by adding 6.7 mL

cellulase enzyme (135 FPU/mL) (Cellic CTec2), 0.41 mL hemicellulase enzyme (Cellic HTec2), 35 µL glucoamylase (DISTILLASE® SSF), 1 mL yeast and 0.2 mL 50% w/v urea solution. In disk milling slurry and cellulase addition (30 FPU/g fiber) treatment, ground corn (50 g db) was suspended in DI water to 32% solids and pretreated with 3 cycles of disk milling. Liquefaction and SSF were performed similar to cellulase addition treatment. SSF was performed for 72 h at 32ºC with continuous agitation at 150 rpm in an incubator shaker (New Brunswick™ Innova®42, Eppendorf, Hamburg, Germany). SSF was monitored by taking 1 mL samples at 0, 4, 8, 24, 48 and 72 h. To determine sugar and ethanol contents, samples were analyzed in duplicate in an HPLC (Model 2414, Waters Corporation, Milford, MA; Aminex HPX-87H column, Bio–Rad Laboratories, Hercules, CA). Samples were prepared according to the procedure outlined by Singh et al (2005). All treatments were replicated three times. To account for differences in ethanol due to the addition of enzymes, a blank reactor was prepared consisting of all reactant constituents except for milled corn. The ethanol concentrations in blank reactors were subtracted from those in the treatment flasks. Figure 4.1 shows the experimental design for the study.

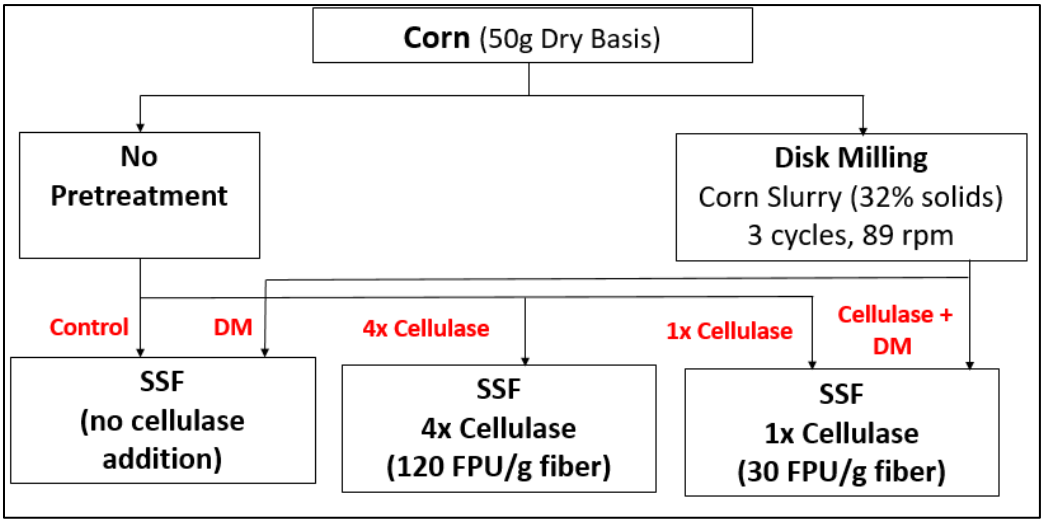

Fig. 4.1. Experiment design for the study

4.2.2. Wet disk milling

It was hypothesized that disk milling corn slurry would release more starch and increase ethanol yield in the dry grind process. Wet disk milling was performed according to the procedure used by Kim et al (2016). Three cycles of disk milling were performed on slurry with 32% w/w dry solids. Disk milling (Quaker City grinding mill model 4E, Straub Co.,

Philadelphia, PA) was operated at an output speed of 89 rpm with zero clearance between stationary and rotating disks. A sample from the disk milled slurry was used for moisture determination.

4.2.3. Starch content determination

Total starch content of corn was determined by method adapted from Vidal et al (2009). Corn was milled to pass through a 0.5 mm screen. Alpha-amylase solution was prepared by diluting 1 mL thermostable α-amylase (Megazymes, Wicklow, Ireland) in a 1:30 ratio with 0.1 M sodium acetate buffer of pH 5. Ethanol (80% v/v), 0.2 mL, was added to 0.1 g ground fiber and stirred in a vortex mixer to aid dispersion. Alpha-amylase solution (3 mL) was added to the mixture and the mixture was placed in boiling water bath for 6 min. The mixture was incubated with 0.1 mL thermostable glucoamylase (Megazymes, Wicklow, Ireland) at 50ºC for 30 min. Mixture was diluted to 10 mL with distilled water and mixed. The diluted mixture was centrifuged (1500xg) for 10 min, and filtrate obtained was analyzed in HPLC (Aminex HPX-87P, Bio-Rad, Hercules, CA). To account for glucose present in the enzyme solutions and subtracted from the starch content, blank determination was performed with 0.1 mL water. Glucose concentration was determined for pure starch by similar method using 0.1 g starch to account for the recovery factor.

4.2.4. Glucose fermentation by *Saccharomyces cerevisiae* in the presence of cellulases

Based on observations in modified dry grind processes, it was hypothesized that cellulase addition during SSF might lead to yeast inhibition. We performed an experiment to compare glucose fermentation in the presence or absence of cellulase. Glucose (Sigma Aldrich, St. Louis, MO) was dissolved in 0.1 M citric acid buffer (pH 4.8) to a make a 20% w/v glucose solution. Fermentation was performed by adding 4.45 mL cellulase enzyme (135 FPU/mL) (Cellic CTec2), 0.2 mL yeast culture, 40 µL 50%w/v urea solution as a nitrogen source and 1 g yeast extract for additional nutrients. Fermentation was performed for 72 h at 32ºC with continuous agitation at 150 rpm in an incubator shaker (New Brunswick™ Innova®42, Eppendorf, Hamburg, Germany). Fermentation was monitored by taking 1 mL samples at 0, 24, 48 and 72 h. Samples were analyzed in duplicate to determine sugar and ethanol contents in an HPLC (Model 2414, Waters Corporation, Milford, MA; Aminex HPX-87H column, Bio–Rad Laboratories, Hercules, CA). Samples were prepared according to the procedure outlined by Singh et al (2005). The

control treatment was performed with the addition of 4.45 mL citrate buffer instead of cellulase keeping other parameters similar. To account for differences in ethanol due to the addition of enzymes, a blank reactor was prepared consisting of all reactant constituents except glucose. The ethanol concentrations in blank reactors were subtracted from those of treatment flasks.

4.2.5. Data Analysis

Every experiment was performed in three replicates and the results were analyzed in duplicates. To compare mean differences, analysis of variance (ANOVA) (R core team, Vienna, Austria, 2013) was performed at a 95% level of significance. The procedure outlined by Kumar et al (2017) was used to calculate the conversion efficiency and ethanol yield.

$$V_{EtOH} = \frac{(V_{H2O}\rho_{H2O}) + (W_{Corn}MC_{Corn}) - (NV_n) - (W_{Corn}(1 - MC_{Corn})S(0.11))}{\frac{\rho_{EtOH/H2O}}{C_{EtOH}} - \rho_{EtOH}}$$

$$V_{EtOH\ max.} = \frac{W_{Corn}(1 - MC_{Corn})S(1.11)(0.511) + G(0.511)}{\rho_{EtOH}}$$

$$E_{Conversion} = \frac{V_{EtOH}(100)}{V_{EtOH\ max.}}$$

$$Y_{\frac{L}{kg}dry\ corn} = \frac{V_{EtOH}}{W_{Corn}(1 - MC_{Corn})}$$

$$Y_{\frac{gal}{bu}} = \frac{V_{EtOH}(25.4)}{W_{Corn}(3.785)}$$

where V_{EtOH} is the volume of ethanol in beer, $V_{H2O}\rho_{H2O}$ is the mass of water added in beer, W_{Corn} is the weight of corn in beer, MC_{Corn} is moisture content of the corn, N is the number of samples taken during process, V_n is the volume of liquid in each sample, S is the starch content of corn on wet basis, $\rho_{EtOH/H2O}$ is the density of water-ethanol mixture, C_{EtOH} is the concentration of sample in % v/v, ρ_{EtOH} is the density of ethanol (0.789 g/mL), $V_{EtOH\ max.}$ is the maximum concentration of ethanol achieved by conversion of starch into sugars ,$E_{Conversion}$ is the conversion efficiency in percentage, $Y_{\frac{L}{kg}dry\ corn}$ is the ethanol yield in L/ kg dry corn and $Y_{\frac{gal}{bu}}$ is the ethanol yield in gal/bu

4.3. Results and discussion

4.3.1. Effect of (1x) cellulase (30 FPU/g fiber) addition on fermentation performance

The addition of cellulase at 30 FPU/g fiber increased beer ethanol concentrations. Ethanol concentrations for cellulase and control treatments were 19.2 and 18.3% v/v, respectively, after 72 h of fermentation (Fig. 4.2). Cellulase treatment achieved higher ethanol concentration (15.3% v/v) relative to control (13.1% v/v) treatment after 24 h of fermentation. Ethanol yields for the 1x cellulase and control treatments were 0.46 (2.74 gal/bu) and 0.43 L/kg dry corn (2.6 gal/bu), respectively. Conversion efficiencies for 1x cellulase treatment and control were 85.3% and 81.1%, respectively (Table 4.1). Addition of cellulases hydrolyzed corn fiber cellulose to glucose and released starch adhered to fiber similar to observations by Kim et al (2017). Cellulose hydrolysis and increase in starch release were factors responsible for increase in ethanol yield (Li et al 2018, Luangthongkam et al 2015).

Peak glucose concentrations achieved for cellulase and control treatments were 14.8 and 18.1% w/v, respectively (Fig 4.3). Lower peak glucose concentrations obtained through cellulase addition explained a higher initial fermentation rate for cellulase treatments. Higher glucose concentrations in control treatment led to osmotic stress in yeast which affected fermentation rate and efficiency (Thatipamala et al 1992). Glucose concentrations after 72 h of fermentation for cellulase and control treatments were 1 and 0.4% w/v, respectively (Table 4.1). Higher fermentation efficiencies were achieved for cellulase treatment.

Table 4.1. Effect of 1x cellulase addition in conventional dry grind process.

Treatment	Ethanol (% v/v)*	Residual Glucose (% w/v)*	Conversion Efficiency (%)*	Ethanol Yield (L/kg dry corn)*	Ethanol yield (gal/bu)*	Glycerol (% w/v)*
1x cellulase**	19.17 ± 0.32[a]	1.01 ± 0.38[a]	85.6 ± 1.7[a]	0.46	2.74 ± 0.06[a]	0.73
Control	18.33 ± 0.06[b]	0.4 ± 0.10[b]	81.1 ± 0.3[b]	0.43	2.60 ± 0.01[b]	1.16

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance (p>0.05). ** Cellulase addition of 30 FPU/g fiber.

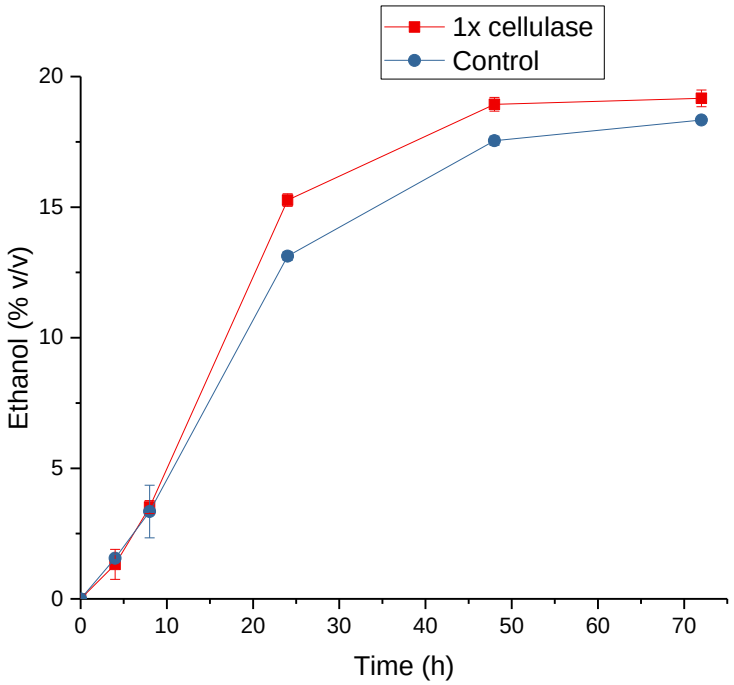

Fig 4.2. Ethanol profile for SSF performed with cellulase (30 FPU/g fiber) addition in the conventional dry grind process.

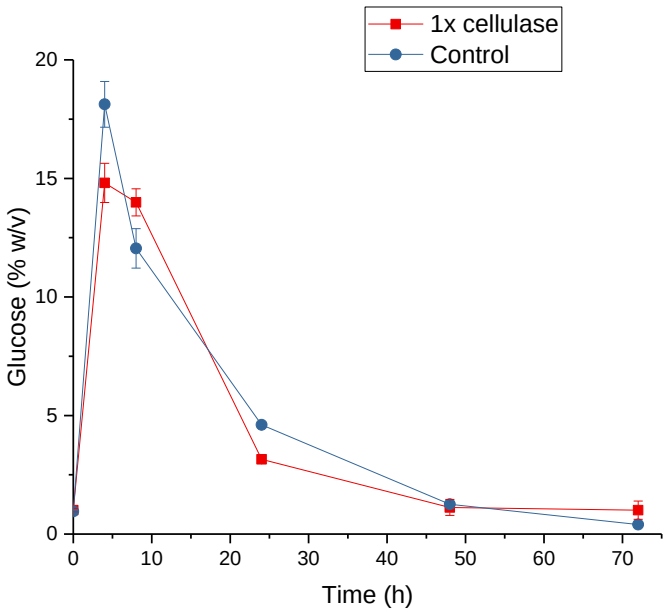

Fig 4.3. Glucose profile for SSF performed with cellulase (30 FPU/g fiber) addition in the conventional dry grind process.

4.3.2. Effect of excess (4x) cellulase (120 FPU/g fiber) dose on fermentation performance

SSF performed with excess cellulase dose (120 FPU/g fiber) achieved lower ethanol concentration (16.5% v/v) than the control (17.5% v/v) after 72 h (Fig. 4.4). The ethanol profile was similar for the control and excess cellulase treatments for 48 h of fermentation. Differences in ethanol concentrations in the control and excess cellulase treatments were observed after 72 h of fermentation. Ethanol yields for the 4x cellulase and the control treatments were 0.39 and 0.42 L/kg dry corn, respectively. The conversion efficiencies for 4x cellulase and the control treatments were 73.8 and 79.0%, respectively (Table 4.2). This observation was contrary to the expected result. After 72 h, 2.5% w/v residual glucose was observed in the excess cellulase treatment; whereas, no glucose was observed in the control treatment (Table 4.2). Residual glucose with excess cellulase treatment was indicative of incomplete fermentation. Residual glucose with excess cellulase was the reason that a low ethanol yield was observed.

Table 4.2. Effect of 4x cellulase addition in the conventional dry grind process.

Treatment	Ethanol (% v/v)*	Residual Glucose (% w/v)*	Conversion Efficiency (%)*	Ethanol Yield (L/kg dry corn)*	Ethanol Yield (gal/bu)*	Glycerol (% w/v)*
4x cellulase**	16.51 ± 0.06[a]	2.5	73.8 ± 0.3[a]	0.39	2.39 ± 0.01[a]	0.06 ± 0.01[a]
Control	17.49± 0.03[b]	0	79.0 ± 0.2[b]	0.42	2.55 ± 0.01[b]	1.33 ± 0.01[b]

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance (p>0.05).** Cellulase addition of 120 FPU/g fiber.

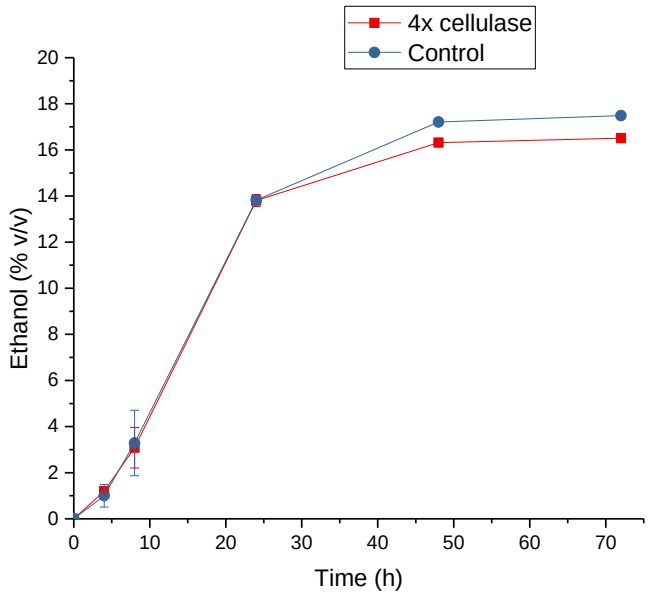

Fig 4.4. Ethanol profile for SSF performed with cellulase (120 FPU/g fiber) addition in the conventional dry grind process.

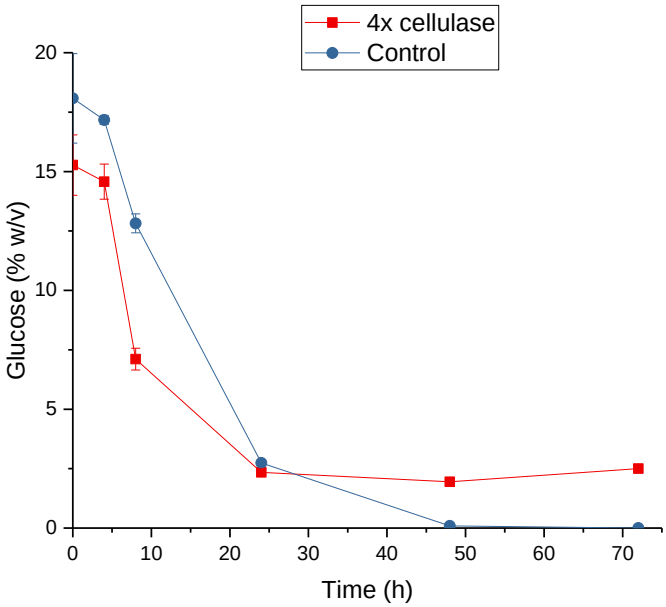

Fig 4.5. Glucose profile for SSF performed with cellulase (120 FPU/g fiber) addition in the conventional dry grind process.

4.3.3. Effect of disk milling slurry on fermentation performance

Ethanol concentrations for disk milling and control treatments were 18.5% and 18.2% v/v, respectively. Ethanol profiles of the treatment and control treatments were similar (Fig 4.6). Fermentation required 72 h for completion for both the treatments. Ethanol yields for the disk milling and control treatments were 0.42 and 0.41 L/kg dry corn, respectively. Conversion efficiencies for the disk milled and control treatments were 78.2 and 77.1%, respectively. Ethanol concentrations, ethanol yields and conversion efficiencies for disk milled and control treatments were similar (Table 4.3).

Fine grinding at the laboratory scale did not improve ethanol yields, as the particle size of less than 0.5 mm achieved during hammer milling released all bound starch. In the commercial dry grind process, hammer milling does not release all bound starch. Wet disk milling corn slurry would release bound starch and improve the ethanol yield of the conventional dry grind process at a commercial scale.

Table 4.3. Effect of disk milling prior to liquefaction in the conventional dry grind process.

Treatment	Ethanol (% v/v)*	Residual Glucose (% w/v)*	Conversion Efficiency (%)*	Ethanol Yield (L/kg dry corn)*	Ethanol yield (gal/bu)*	Glycerol (% w/v)*
Disk Milling	18.42 ± 0.08[a]	0.00	78.2 ± 0.4[a]	0.42	2.53 ± 0.01[a]	1.24 ± 0.01[a]
Control	18.22 ± 0.29[a]	0.03	77.2 ± 1.5[a]	0.41	2.50 ± 0.04[a]	1.33 ± 0.01[a]

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance ($p>0.05$).

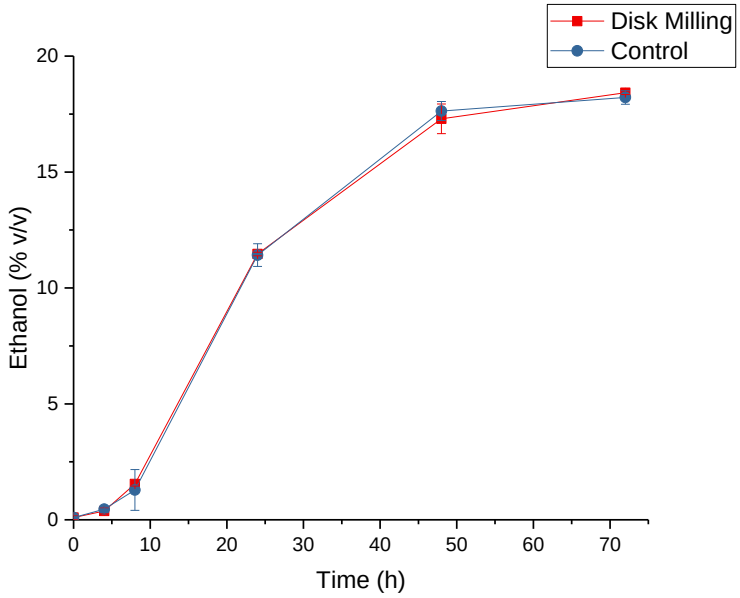

Fig 4.6. Ethanol profile for SSF performed with disk milled slurry in conventional dry grind process.

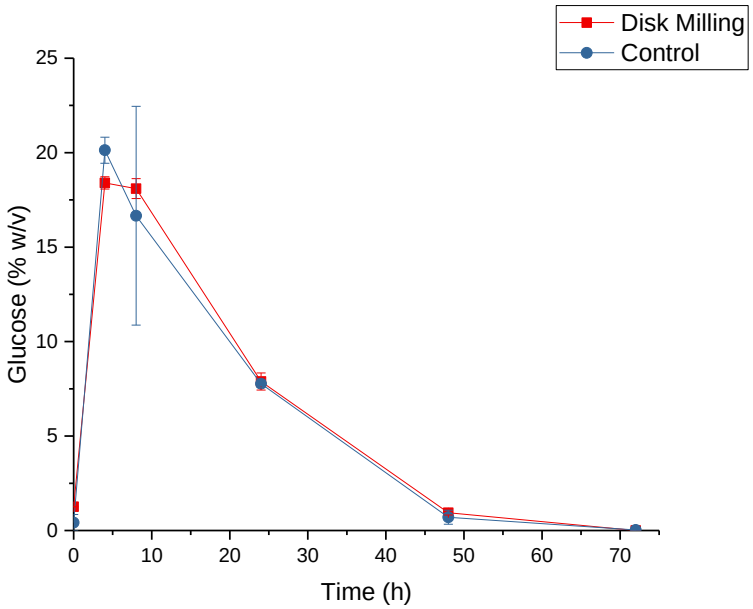

Fig 4.7. Glucose profile for SSF performed with disk milled slurry in conventional dry grind process.

4.3.4. Effect of disk milling slurry and cellulase addition (30 FPU/g fiber) on fermentation performance

Ethanol concentrations between disk milling with cellulase (30 FPU/g fiber) treatment, disk milling treatment and control treatment were compared. In the first experiment, disk milling with cellulase (30 FPU/g fiber) treatment was compared to the control treatment; in the second experiment, disk milling with cellulase treatment (30 FPU/g fiber) was compared to disk milling treatment.

A lower final ethanol concentration for disk milling with cellulase treatment (17.6% v/v) compared to the control (18.2% v/v) (Table 4.4) was observed. Differences in ethanol concentrations in control and treatment were observed between 48 and 72 h of fermentation. The ethanol profiles were similar for control and treatment in the first 48 h (Fig 4.8). After 72 h fermentation, 1.66% w/v residual glucose was observed in disk milling with cellulase treatment; whereas, the glucose concentration for control treatment was negligible (Fig 4.9). The ethanol yields for disk milling with cellulase and control treatments were 0.41 and 0.43 L/kg dry corn, respectively. Conversion efficiencies for disk milling with cellulase and control treatments were 77.3 and 80.3% (Table 4.4), respectively.

Table 4.4. Effect of disk milling slurry with 1x cellulase addition in SSF in conventional dry grind process.

Treatment	Ethanol (% v/v)*	Residual Glucose (% w/v)*	Conversion Efficiency (%)*	Ethanol Yield (L/kg dry corn)*	Ethanol yield (gal/bu)*	Glycerol (% w/v)*
Disk milling with 1x cellulase**	17.62 ± 0.11[a]	1.66	77.3 ± 0.6[a]	0.41	2.50 ± 0.02[a]	0.91 ± 0.03[a]
Control	17.95 ± 0.22[b]	0.01	80.3 ± 0.8[b]	0.43	2.60 ± 0.02[b]	1.31 ± 0.01[b]

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance ($p>0.05$). ** Cellulase addition of 30 FPU/g fiber.

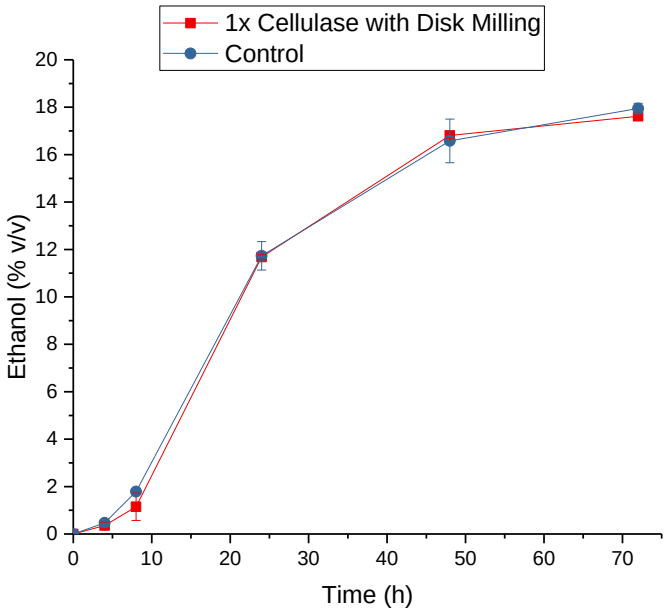

Fig 4.8. Ethanol profile for SSF performed using disk milled slurry with cellulase (30 FPU/g fiber) addition in conventional dry grind process.

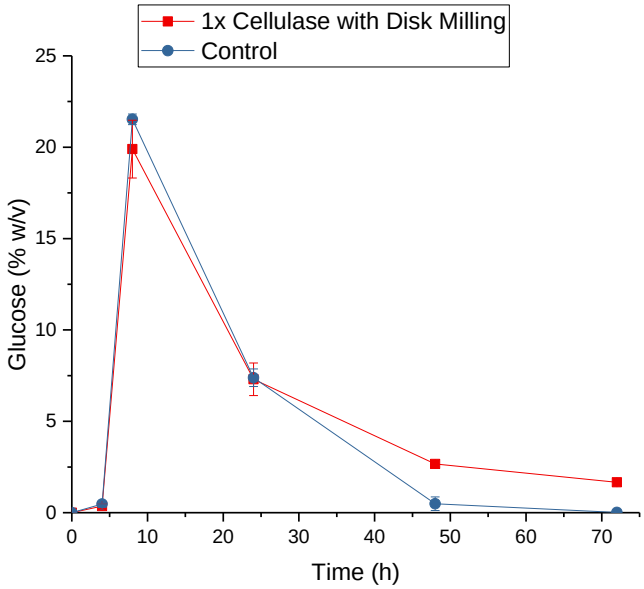

Fig 4.9. Glucose profile for SSF performed using disk milled slurry with cellulase (30 FPU/g fiber) addition in the conventional dry grind process.

The ethanol profile was different for the first 24 h with cellulase treatment having a higher ethanol concentration (11.9% v/v) than the control treatment (10.2% v/v). At 48 h of fermentation, ethanol concentrations in disk milling with cellulase treatment and only disk milling treatment were similar. At the end of fermentation, ethanol concentrations in disk milling with cellulase treatment were lower than disk milling treatment (Fig 4.10). Glucose profiles for the disk milling with cellulase treatment and only disk milling treatment were similar for first 48 h (Fig 4.11). After 72 h SSF, 1.9% w/v residual glucose was observed for disk milling with cellulase treatment; whereas, no glucose was observed in only disk milling treatment. Ethanol yields for disk milling with cellulase treatment and only disk milling treatment were 0.43 and 0.44 L/kg dry corn, respectively. Conversion efficiencies for disk milling with cellulase treatment and only disk milling treatment were 78.7 and 81.9%, respectively (Table 4.5).

Table 4.5. Effect of disk milling slurry with 1x cellulase addition in SSF in conventional dry grind process.

Treatment	Ethanol (% v/v)*	Residual Glucose (% w/v)*	Conversion Efficiency (%)*	Ethanol Yield (L/kg dry corn)*	Ethanol yield (gal/bu)*	Glycerol (% w/v)*
Disk Milling with 1xCellulase**	17.9 ± 0.27[a]	1.90	78.7 ± 1.4[a]	0.43	2.55 ± 0.05[a]	0.93 ± 0.03[a]
Disk Milling	18.48 ± 0.12[b]	0.01	81.9 ± 1.3[b]	0.44	2.65 ± 0.04[b]	1.38 ± 0.04[b]

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance ($p>0.05$). ** Cellulase addition of 30 FPU/g fiber.

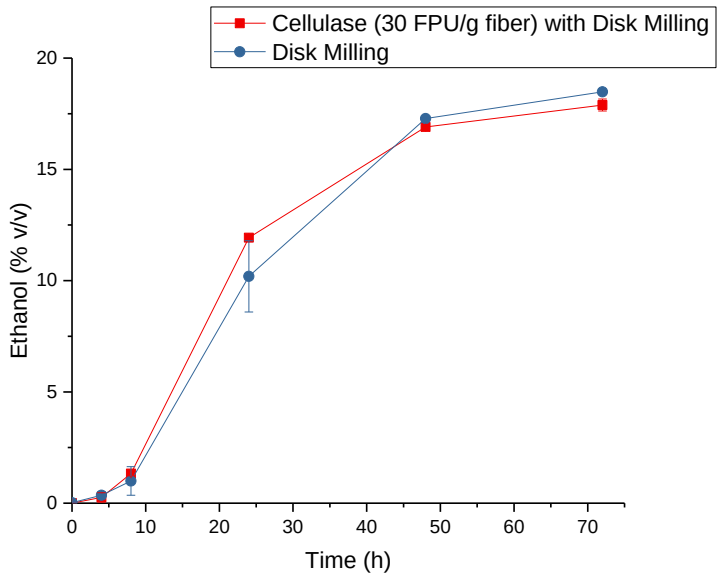

Fig 4.10. Ethanol profile for SSF performed using disk milled slurry with cellulase (30 FPU/g fiber) addition in conventional dry grind process.

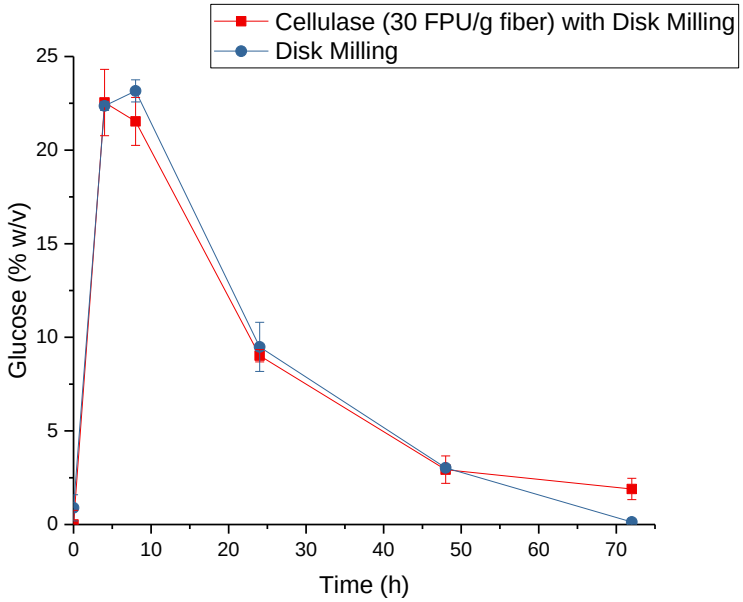

Fig 4.11. Glucose profile for SSF performed using disk milled slurry with cellulase (30 FPU/g fiber) addition in conventional dry grind process.

Ethanol and residual glucose concentrations at 72 h in disk milling with cellulase treatment for both the experiments were not different. Similarly, final ethanol concentrations for disk milling treatment in this set of experiments were not different from the final ethanol concentrations in previous set of disk milling treatment.

4.3.5. Yeast Inhibition

Yeast inhibition by cellulase addition in dry grind process was responsible for residual glucose at the end of 72 h fermentation (Table 4.6). The ethanol concentration achieved in all processes except disk milling exceeded the ethanol concentrations achieved from the control after the conversion of residual glucose to ethanol. Ethanol concentration estimated after conversion of residual glucose was highest for 1x cellulase treatment. Lower ethanol yields in other treatments relative to 1x cellulase treatment were a result of lower hydrolysis efficiency in these treatments. Enzyme inhibition was responsible for lower hydrolysis efficiency.

Table 4.6. Final ethanol concentrations for conventional and modified dry grind processes when residual glucose is converted into ethanol.

Treatment	Residual Glucose (% w/v)	Ethanol (% v/v)*
1x cellulase**	1.01	19.8 ± 0.3^{a}
Control	0.4	18.6 ± 0.1^{b}
4x cellulase***	2.5	18.1 ± 0.1^{a}
Control	0	17.5 ± 0.3^{b}
Disk Milling	0.00	18.4 ± 0.8^{a}
Control	0.03	18.2 ± 0.3^{a}
Disk Milling with 1x cellulase**	1.66	18.7 ± 0.1^{a}
Control	0.01	18.0 ± 0.2^{b}
Disk Milling with 1x cellulase**	1.90	19.1 ± 0.3^{a}
Disk Milling	0.01	18.5 ± 0.1^{b}

*Mean ± standard deviations from three replicates. Means followed by same letter are not different at 95% level of significance ($p>0.05$). ** Cellulase addition of 30 FPU/g fiber. *** Cellulase addition of 120 FPU/g fiber.

Addition of cellulolytic enzymes improved biomass hydrolysis and fermentation efficiency (Boakye-Boaten et al 2015, da Silva et al 2010, Dien et al 2008, Lau et al 2008, Mosier et al 2005, Saha et al 1998). Similar results were observed in SSF performed with mash retentate at a 10% w/w dry solids loading where all glucose in beer was consumed. The dry grind process involves slurry of 30 to 33% solids (Bothast and Schlicher 2005). Higher inhibitor concentration,

mass transfer limitations and substrate and product inhibition at 32% w/w dry solids compared to 10% w/w dry solids lead to yeast stress (Dien et al 2008, Kim et al 2008, Lau et al 2008).

Cell walls represent 26 to 32% cell dry weight in *Saccharomyces cerevisiae* (Nguyen et al 1998). Polysaccharides account for 85 to 90% of the cell wall dry weight (Nguyen et al 1998) with the proportion of mannoproteins, β-1,6 glucans, β-1,3 glucans and chitin in cell walls being 35 to 40, 5 to 10, 50 to 55% and 1% db, respectively (Klis et al 2002). Cell wall proteins can be released by β-glucanase digestion (Kaptyen et al 1999). We hypothesized that cellulolytic enzymes were responsible for glucan hydrolysis resulting in cell wall damage and yeast inhibition due to cell wall damage led to the incomplete conversion of glucose.

We studied the effect of cellulase addition on the fermentation performance of yeasts. Cellulase (60 FPU/g glucose) addition increased ethanol concentration and conversion efficiency compared to the control (Figure 4.12). Ethanol concentrations after 72 h fermentation in the cellulase and control treatments were 10.7 and 9.9 % v/v respectively (Table 4.7). Conversion efficiencies in the cellulase and control treatments were 98.3 and 88.7%, respectively (Table 4.7). Lower glycerol production in the cellulase treatment compared to the control was responsible for higher ethanol yields in the cellulase treatment. Cellulase addition did not lead to yeast inhibition.

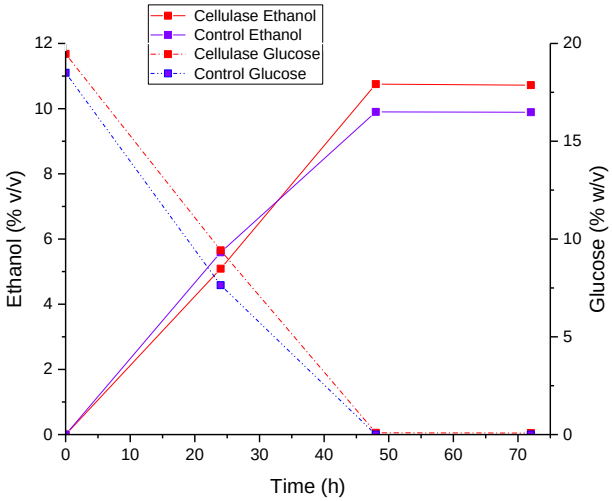

Fig 4.12. Ethanol and glucose profiles of glucose fermented with and without cellulase (60 FPU/g glucose) addition

Table 4.7. Final ethanol concentrations for glucose fermentation in the presence of cellulase.

Treatment	Ethanol (% v/v)	Conversion Efficiency (%)	Glycerol (% w/v)
Cellulase**	10.72	98.27 ± 0.03[a]	0.53 [a]
Control	9.89	88.66 ± 0.07[b]	1.12 [b]

[*]Mean ± standard deviations from triplicates. Means followed by same letter are not different at 95% level of significance (p>0.05). ** Cellulase addition of 60 FPU/g glucose.

4.3.6. Comparison with other studies

Luangthongkam et al (2015) found that addition of 1.2 FPU/g fiber cellulase cocktail (assuming enzyme activity of 135 FPU/mL) post grinding increased ethanol concentration in beer to 121 g/L; whereas, addition of cellulase cocktail, phytase and protease during SSF increased ethanol concentrations to 128 g/L compared to the control (110 g/L) in the conventional dry grind process. The addition of cellulase cocktail and combination of cellulase cocktail, phytase and protease achieved 10.6 and 16.5% increase in ethanol yield, respectively. Li et al. (2018) found that addition of cellulase (6.7 FPU/g fiber) during SSF improved ethanol concentrations to 129 g/L compared to the control (127 g/L) in the conventional dry grind process with 30% dry solids loading, respectively. In the same study, 1.8 and 1.2% increases in ethanol yields compared to the control were observed for 30 and 33% corn dry solids loading, respectively. A residual glucose concentration of 3.7 g/L was observed for 33% corn dry solids loading by Li et al 2018. In our study, the addition of 30 FPU/g fiber cellulase dose at the SSF stage achieved a 4.6% increase in ethanol yield. A residual glucose concentration of 10 g/L was observed at 32% w/w corn solids loading and 30 FPU/g fiber cellulase enzyme addition. Ethanol concentrations achieved by Luangthongkam et al (2015) and Li et al (2018) in control and cellulase treatment were lower than the ethanol concentrations achieved for control (145 g/L) and treatment (151 g/L) in this study, respectively. Luangthongkam et al (2015) used a two stage liquefaction procedure and used BluZy-PXL cocktail with high xylanolytic activity for fiber hydrolysis in the process. Luangthongkam et al (2015) performed dry grind process at 30% w/v corn solids and a 400 ppm cellulase enzyme (1.2 FPU/g fiber) dose. Li et al (2018) did not provide specific information on the cellulolytic enzyme and corn hybrid used in the study. Liquefaction was performed at 85ºC for 4 h; whereas, SSF was performed for 5 days in the study

by Li et al (2018). Li et al (2018) performed most of the experiments at 30% w/w solid loadings and lower cellulase dose (6.7 FPU/g fiber) compared to those in our study (30 FPU/g fiber).

4.4. Conclusions

Corn was processed using conventional and modified dry grind processes. Modifications to the conventional process included addition of 30 and 120 FPU/g fiber cellulase during SSF, disk milling (3 cycles) of slurry and disk milling (3 cycles) of slurry with the addition of 30 FPU/g fiber cellulase during SSF. The 30 and 120 FPU/g cellulase doses corresponded to one (1x cellulase) and four times (4x cellulase) the recommended cellulase dose, respectively.

Addition of cellulase at 30 FPU/g fiber dose resulted in higher ethanol concentration compared to conventional dry grind process. SSF performed with disk milled slurry had ethanol yields similar to the conventional process. Addition of 120 FPU/g fiber cellulase and disk milling with cellulase (30 FPU/g fiber) addition achieved lower ethanol yield compared to the control. Yeast inhibition was responsible for residual glucose observed at the end of fermentation in all modified process involving cellulase addition. Addition of 30 FPU/g fiber cellulase dose during SSF stage improved yield of the dry grind ethanol process by 0.14 gal/bu.

Chapter 5

Recommendations for future work

Hot water pretreatment increased the conversion efficiency of mash retentate to ethanol; whereas, disk milling had no effect on the conversion efficiency of mash retentate to ethanol. The increased cellulase dose increased the conversion efficiency of mash retentate (10% w/w solid loading). Increased dose of cellulolytic enzymes in conventional and modified dry grind processes (32% w/w solid loading) resulted in incomplete fermentation. The following are suggestions for the further analysis:

1. Hot water pretreatment increased the conversion efficiency of mash retentate to ethanol which was suggestive that fiber separation and pretreatment can increase ethanol yields in conventional dry grind process. For a complete understanding of subject a techno economic analysis of the process should be performed.
2. SSF for mash retentate was performed at 10% w/w solid loading. As there was no inhibitor formation in the process, SSF at a higher solid content can be performed.
3. The addition of cellulolytic enzymes resulted in residual sugars in conventional dry grind process which was suggestive of yeast inhibition. The investigation of factors responsible for the low ethanol yield seem to be the next steps. Similarly, alleviating yeast inhibition at 32% solids content would need further research.
4. The conversion of corn fiber to ethanol due to cellulolytic enzymes would decrease the proportion of fiber in DDGS which can improve DDGS quality. Thus, composition analysis of DDGS obtained after evaporating ethanol and drying stillage should be performed.
5. The concentration of arabinoxylans in corn fiber is 30 to 50% db and the conversion of arabinoxylan fraction can further improve ethanol yields in dry grind process. A study using a yeast strain cofermenting pentose and hexose sugars can be performed.

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