ENZYMATIC AND PROCESS TECHNOLOGIES TO INCREASE CORN DRY GRIND SLURRY SOLIDS

BY

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THESIS

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Abstract

In conventional dry grind process, high glucose concentrations (>15% w/w) and liquefaction viscosities restrict slurry solids contents to 30 to 32% w/w. High slurry solids fermentations (above 33%) are important in reducing energy costs; decreased water input results in less evaporation, dehydration and distillation. There are numerous challenges associated with high solids fermentations: high slurry viscosities, high glucose concentrations that exert osmotic stress on yeast and high ethanol concentrations that result in loss of cell viability. The objective was to determine an economical and process efficient enzyme combination to be employed during simultaneous saccharification and fermentation (SSF) to reduce slurry viscosities and glucose concentrations at high solids. We also evaluated effects of nitrogen source and dose on high solids fermentation. The enzyme combination we considered for this study consisted of granular starch hydrolyzing enzymes (GSHE), alpha-amylase component of GSHE (GSHE AA) and glucoamylase (GA).

In this study, we showed that using low temperatures (55°C) and a combination of phytase and alpha-amylase during liquefaction reduced slurry viscosities at 35% solids by 81% compared to the conventional process. We compared eighteen SSF enzyme treatments to optimize each GSHE, GSHE AA and GA. These SSF enzyme treatments included two enzyme combinations: 1) GA and GSHE and 2) GA and GSHE AA, with different levels of enzyme concentrations. For all treatments except control, liquefaction (55°C for 90 min) was conducted at 35% solids using a formulation of alpha-amylase and phytase. SSF (32°C for 72 hr) was carried out using enzyme treatments, urea and yeast. The treatment containing 0.5 L glucoamylase and 1.25 L GSHE per g dry corn resulted in the highest fermentation efficiencies (92%) and ethanol yields (418 L/tonne). The control treatment resulted in the lowest fermentation efficiencies (84%) and ethanol yields (381 L/tonne). The above mentioned enzyme treatment also resulted in 34% lower peak glucose concentrations (9.87% w/v) compared to control treatment (13.49% w/v). Nitrogen source and dose effects were determined at 35 and 40% solids using modified process. Three nitrogen

sources (urea, ammonium sulfate, glutamine) and protease were compared for ethanol yields and other fermentation parameters. Urea and protease resulted in similar ethanol yields. However, fermentation rates were higher for protease during initial 12 hr of fermentation.

Effects of urea and protease levels were evaluated at 35 and 40% solids. At 35% solids, 2.16 mg urea and 0.71 mg protease resulted in highest fermentation efficiencies and ethanol yields. However at 40% solids, 4.32 and 2.16 mg urea and 0.71 mg protease gave highest fermentation efficiencies and ethanol yields. At 35 and 40% solids, increasing protease levels from 0.71 to 1.42 mg reduced fnal ethanol concentrations, ethanol yields and fermentation effciencies. Increasing solids content from 35 to 40% decreased fermentation effciencies and simultaneously reduced ethanol yields across all urea and protease levels.

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Chapter 1

Introduction

Production and use of renewable fuels in US has quadrupled since 2002. Declining petroleum reserves and combined with environmental concerns have stimulated the use of fuel ethanol. Ethanol is a renewable fuel which reduces America's dependence on foreign energy sources. US ethanol production has increased from 2.1 to 9.0 billion gal/yr from 2002 to 2009 (RFA 2009). Currently, there are 202 ethanol production facilities and 20 more under construction (RFA 2010). Ethanol is made primarily from corn or sorghum. Ethanol can be made from a variety of other agricultural grain crops such as wheat, barley, sugarcane, sugarbeets, cheese whey or potatoes. The two major processes by which corn is converted to ethanol are dry grind and wet mill. Wet mill facilities were more common in the industry's early days (before 1970), but dry grind ethanol plants account for more than 80% of US ethanol production. The domination of dry grind plants also relates to the fact that constructing wet mill plants is more complex and requires more capital per unit of capacity.

In the conventional dry grind process (Figure 1.1), grinding the corn kernel exposes starch granules for hydrolysis in subsequent processing. Ground corn is mixed with water to form slurry with 30 to 33% w/w dry solids. The slurry is liquefied at 85 to 105°C and pH 5.7 for 90 min in the presence of alpha-amylase and steam which degrades starch granule crystallinity. Alpha-amylases hydrolyze alpha-D-glucose alpha-1,4 bonds in the starch molecules releasing shorter, water soluble oligosaccharide chains called dextrins. Liquefied slurry is cooled to 32°C and pH is adjusted between 4.0 and 4.2. Slurry is saccharified and fermented simultaneously to produce ethanol. During simultaneous saccharification and fermentation (SSF), glucoamylase is added to convert oligosaccharides or short chain soluble sugars into mono-, di- and trisaccharides such as glucose, maltose and maltotriose. Yeast in the fermentation media convert these sugars into ethanol. Additional nutrients such as urea may be added as a nitrogen source for yeast.

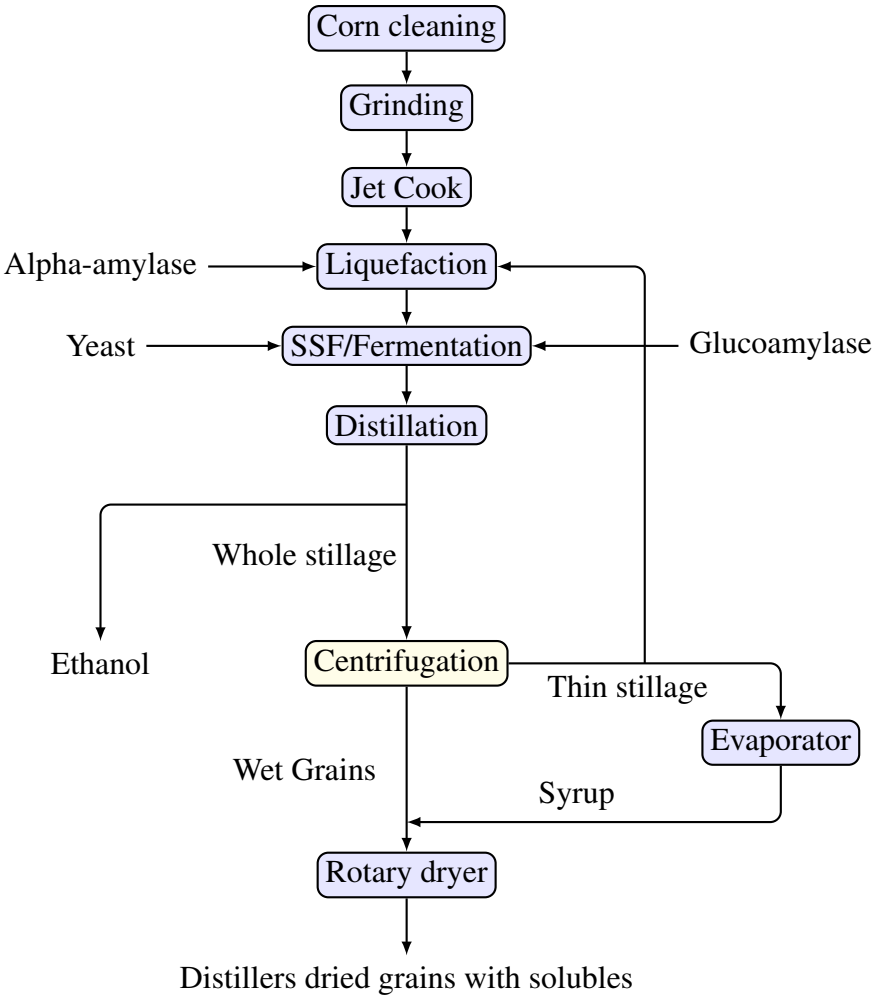

Figure 1.1. Schematic of dry grind ethanol production.

In the dry grind ethanol industry, corn makes up to 60% and steam makes up to 15% of total production costs (base case) estimated at US $1.61/gal (US $0.43/L), before credits for coproducts (Peschel et al 2006). Based on a 2002 United States department of agriculture (USDA) survey (Shapouri and Gallagher 2005), 50% of the total thermal energy used in the ethanol production can be assigned to three dry grind ethanol dewatering processes: evaporation, centrifugation and DDGS drying. The rest of the thermal energy used in dry grind plants can be attributed to fermentation and distillation steps. A major technology that could reduce production costs is high solids (>33% w/w) fermentation which would decrease water input and increase plant throughput, reducing energy costs (Devantier et al 2005).

High solids fermentation was defined as slurry fermentation having greater than 350 g/L dissolved solids. In the conventional dry grind process, typical solids concentrations range from 30 to 32%. High solids fermentation (>33% w/w) is important in increasing ethanol concentrations beyond 18% v/v, reducing process water requirements, increasing plant productivity and decreasing energy costs (Devantier et al 2005). High solids fermentation faces a number of challenges. First, high glucose concentrations ($\geq$15% w/w) produced during high solids fermentation exert osmotic stress on yeast, resulting in lower fermentation efficiencies and incomplete substrate utilization (Thatipamala et al 1992). High solids fermentation also increases slurry viscosities which lead to increased pumping power requirements and thus increased production costs. Finally, a consequence of high solids fermentation is higher ethanol stress on yeast which lowers yeast fermentative capacity (D'Amore and Stewart 1987). Ethanol causes the plasma membrane to be more permeable to protons and thus increases proton influx and ATP energy requirements to maintain intracellular pH (Piper 1995).

Process technologies are advancing; enzymes such as granular starch hydrolyzing enzyme (GSHE) and phytases can be used to overcome challenges in high solids fermentations. GSHE, a mixture of low temperature alpha-amylase and glucoamylase, produces gradual simultaneous liquefaction and saccharification (SSF) at $\leq$48°C resulting in lower glucose concentrations (<10% w/w). Phytases are enzymes that hydrolyze phytic acid into less phosphorylated inositol phosphate esters. Phytases help in reducing slurry viscosity and increasing alpha-amylase activity during liquefaction (Shetty et al 2008). Additional nutrients such as lipids and nitrogen sources have been used to improve yeast fermentation performance. With high solids fermentation, Wang et al (1999) and Ingledew (1999) achieved 23% v/v ethanol levels for wheat mashes by using high yeast inoculation levels, sufficient available nitrogen and adding yeast nutrients in the form of yeast extract to keep yeast growing under high stress environments.

In this work, we aim to develop a modified dry grind process that combined the use of conventional amylases, phytase and granular starch hydrolyzing enzyme to maximize fermentation efficiencies at high slurry solids concentrations (>33% w/w). We also evaluated

nitrogen source and dose to improve fermentation efficiencies in high solids fermentation. Specific objectives were to:

1. Evaluate effects of enzyme dose and liquefaction temperature on slurry viscosities and glucose concentrations at high solids (>33%) concentrations.

2. Determine effects of nitrogen source and dose to overcome nitrogen limitation and achieve higher fermentation efficiencies (>95%) at high solids (>33%) concentrations.

Chapter 2

Literature Review

2.1 Dry Grind Ethanol Production

The dry grind process involves the following steps to produce ethanol from corn: grinding, cooking, liquefaction, saccharification, fermentation and distillation. This process has a lower capital cost but suffers from production of low value coproducts, i. e., distillers dried grain with solubles (DDGS) compared to wet mill process. DDGS has a limited market because it is used only in ruminant animal diets. In the dry grind process, the whole kernel is ground using a hammer mill to facilitate water penetration in the cooking process. The milled corn is mixed with water to form slurry which is liquefied at 85 to 105°C for 1 to 2 hr using alpha-amylase to hydrolyze starch into dextrins. After cooking, slurry is cooled to 32°C and slurry pH is adjusted to 4.0. Glucoamylase, urea and yeast are added to undergo simultaneous saccharification and fermentation (SSF) at 32°C for 60 to 72 hr.

During saccharification, glucoamylase converts dextrins into monosacchrides, diasacchrides and trisacchrides of glucose, maltose and maltotriose, respectively. Yeast (*Sacchromyces cerevisiae*) ferments these sugars into ethanol anaerobically through the glycolytic pathway followed by conversion of pyruvate to ethanol (Embden-Myerhof-Parnas, or EMP, pathway) (Fiechter et al 1981). The EMP pathway operates in the presence or absence of oxygen to convert glucose into pyruvic acid, energy and reduced nicotinamide adenine dinucleotide ($NADH + H^+$) (Maiorella et al 1982; Petrik et al 1983). When oxygen levels are reduced to 5 to 20 ppm and glucose levels exceeds 0.1% w/v, pyruvic acid is converted to ethanol. Through the EMP pathway, glucose is converted into two molecules of carbon dioxide, ethanol and adenosine-triphosphate (ATP) molecules which serves as a stored energy source for the cell. This conversion results in production of 0.383 KJ of energy/g glucose. Part of this energy is used for

cell metabolism and a portion of it is lost as heat.

Batch or continuous fermentations can be used in the production of ethanol; batch fermentations are more common due to lower contamination risk (Ingledew 2003). Carbon dioxide (CO_2) released during fermentation can be captured and sold. CO_2 can be used in carbonated beverages, manufacturing dry ice and other industrial processes (Ronald 2001). Following fermentation, the beer stream, which contains ethanol, is distilled to produce 190 proof (95%) ethanol which is dewatered using molecular sieves to produce neat ethanol which is blended with 5% denaturant (gasoline) and stored for shipment. The solid and liquid fractions remaining after distillation are referred to as whole stillage which includes fiber, oil, protein and nonfermented starch.

Using centrifuges, thin stillage including the soluble solid fraction, is separated from whole stillage and sent to evaporators to remove water. After evaporation, the thick viscous syrup, known as thin stillage, is mixed with the insoluble solids, known as distillers grains, to create a product known as wet distillers grains with solubles (WDGS). WDGS, containing 65% moisture, has a short shelf life, and usually is dried to 10 to 12% moisture to produce distillers dried grains with solubles (DDGS). In 2008, dry grind ethanol plants produced more than 22 million tons of DDGS. This product is used domestically by livestock producers and exported to Europe, Mexico, Canada and Southeast Asia.

2.2 Benefits of High Solids Fermentation

In the conventional dry grind ethanol process, ethanol yields as high as 95% of the theoretical maximum have been observed at 30% solids (Lemuz et al 2009). Further process improvement can be achieved by reducing the fermentation time (increasing the rate of production) and increasing the concentration of ethanol by fermenting greater amounts of sugar using high solids fermentation. In high solids fermentation, slurry solids concentrations greater than 350 g/L dissolved solids are employed (Thomas and Ingledew 1992). There are numerous advantages of high solids fermentation owing to reduced water usage during slurry preparation.

In addition, lower energy requirements are required for heating ans cooling of the slurry and lower amounts of waste water. By increasing solids concentrations, the resulting sugar concentrations ($\geq$15% w/v) and consequently ethanol concentrations ($\geq$19% v/v) increase. Glucose concentrations greater than 15% w/v were obtained at 30% slurry solids and glucose concentrations as high as 19.3% w/v have been produced (Wang et al 2007). Thomas et al (1993) achieved 23% v/v final ethanol concentrations at 20°C using wheat mashes containing 38 to 39% w/v dissolved solids. Higher ethanol concentrations are expected to reduce distillation costs (Wingren et al 2003; Katzen et al 1999; Zacchi and Axelsson 1989).

2.3 Problems in High Solids Fermentation

2.3.1 Slurry Viscosity

Economical and process efficient high solids fermentation require a mash with low glucose concentrations and a mash consistency that is easy to handle and ferment. Unless specially treated, most grain mashes with high carbohydrate content are too viscous for normal handling. As the concentration of dissolved solids of a mash is raised, the viscosity and shear rate required for during mixing increases. This results in a greater demand for energy during mashing and fermentation. An abrupt increase in viscosity has been observed by increasing solids concentrations beyond 20% solids (Fan et al 2003; Mohagheghi et al 1992). Fan et al (2003) observed the necessary power requirements for mixing of paper sludges increased with solids concentration such that a 4% increase in solids content resulted in a five fold increase in power consumption. Moreover, increased slurry viscosity may lead to greater retention of CO_2 in the fermentation medium and this may inhibit yeast growth (Maiorella et al 1982). Therefore, successful implementation of high solids fermentation partially depends on preparation of low viscosity mashes.

In addition to solids content, another factor which affects slurry viscosity is cooking temperature. After slurry preparation, the slurry is cooked at 105°C. During starch

gelatinization, intermolecular bonds of starch molecules are broken down in the presence of water and heat, allowing the hydrogen bonding sites (the hydroxyl hydrogen and oxygen) to complex with water molecules. Penetration of water increases randomness in the general structure and decreases the number and size of crystalline regions. This process results in increased slurry viscosity before the slurry reaches starch gelatinization temperature. Bagley and Christianson (1982) measured viscosity of wheat starch granules at 60, 65, 70 and 75°C for 15, 30, 45, 60 and 75 min cooking; as cooking temperature was raised, viscosity at the end of 75 min cooking increased. They also showed that increasing cook time from 15 to 75 min at 60°C resulted in a corresponding increase in viscosity measured after cooking.

2.3.2 Glucose Inhibition

High solids fermentation results in glucose concentrations, ($\geq$13% w/v) that can exert osmotic stress on yeast, resulting in reduced cell growth and loss in cell viability (Thatipmala et al 1992). Yeast are unique microorganisms which can switch from respiration to fermentation depending on glucose and oxygen levels in the media. At concentrations greater than 3 to 30 g/L (depending on the yeast strain), catabolite repression of oxidative pathways (Crabtree effect) occurred even under fully aerobic conditions (Crabtree 1929). Under these conditions, yeast took a fermentative pathway to utilize glucose (Fiechter et al 1981). Glucose is converted to pyruvic acid which is converted through acetaldehyde into ethanol and CO_2. At higher glucose concentrations ($>$100 g/L), catabolite inactivation of enzymes in the glycolytic pathway takes place indicating onset of glucose inhibition as a result of high osmotic pressure and low water activity (Casey and Ingledew 1986; Stewart et al 1984).

Thatipamala et al (1992) showed that glucose inhibition resulted in reduced ethanol yields, $Y_{p/s}$ (g ethanol/g glucose) from 0.45 to 0.3. Cell viability decreased from 95 to 70% as initial glucose concentration increased from 150 to 280 g/L with a simultaneous increase in lag time. Substrate inhibition becomes important in the range of 15 to 25% (w/v) glucose and complete inhibition of growth occurs at 40% (w/v) glucose (Casey and Ingledew 1986; Holcberg and

Margalith 1981). Sugar concentrations greater than 20% (w/v) are not used under industrial conditions because increasing ethanol levels (>10 % w/v) in fermentation media delays yeast growth resulting in incomplete fermentation (Novak et al 1981; Strehaiano et al 1978; Kalmokoff and Ingledew 1985; Mota et al 1987).

2.3.3 Nutrient Limitation

Yeast requires nutrients such as nitrogen, oxygen, carbohydrates and inorganic ions. Assimilable nitrogen has been reported as the major limiting nutrient in high solids fermentations (Thomas and Ingledew 1990). Lack of nitrogen resulted in sluggish and incomplete fermentations (fermentation stopped before all the sugars are consumed) due to nutritionally induced yeast growth problems (Patel and Ingledew 1973). The rate of sugar consumption by yeast is faster (30 times or more) in growing conditions compared to the resting state. Thus, when cell growth ceases, sugar consumption slows down (Kirsop 1978; Searle and Kirsop 1979). Therefore, to ensure rapid fermentation during high solids concentrations, it is necessary to increase the duration and level of cell growth. This can be achieved by providing an abundant supply of growth limiting nutrients which must be increased proportionally to sugar concentrations.

Nutritional deficiencies were related mainly to nitrogen and dissolved oxygen levels in media. Oxygen was required by yeast for sterol and unsaturated fatty acid synthesis (Andreasen and Stier 1953) that enhanced yeast barrier forming capacity against ethanol. Lipids, such as sterols and fatty acids, were present in high gravity worts in suboptimal concentrations (David and Kirsop 1972). Moreover, at high solids concentrations, oxygen solubility decreased which led to reduced lipid synthesis (Baker and Morton 1977). Reduced lipid synthesis decreased ethanol tolerance capacity of yeast. Limiting sterol levels resulted in ceased reproductive cell growth (Aries and Kirsop 1977). Jones and Ingledew (1993) reported that fermenting wheat mashes contained sufficient dissolved oxygen in fermentation media for active dry yeast to allow fermentation completion. Nitrogen was found to be the major limiting nutrient resulting in

incomplete fermentations (Thomas and Ingledew 1990). Nitrogen limitation resulted in reduced synthesis of glycolytic enzymes (Salmon 1989), loss in biomass yield (Bisson 1991; Spayd et al 1995; Manginot et al 1998) and sugar transport catabolite inactivation (Lucero et al 2002). When adequate nitrogen was available in the media, yeast were capable of metabolizing high concentrations of sugar and fermentations were completed.

2.4 Overcoming Challenges in High Solids Fermentation

2.4.1 Control of Liquefaction Viscosities

Bagley and Christianson (1982) showed that increasing cooking temperatures from 60 to 75°C resulted in increase in viscosities for wheat starch solutions. Therefore, liquefying corn slurry at temperatures lower than starch gelatinization temperatures could lead to lower viscosities for liquefied slurry. However, liquefying corn slurry at lower temperatures would result in ungelatinized starch in the liquefied slurry. This problem can be overcome by using granular starch hydrolyzing enzyme (GSHE) during SSF that would hydrolyze ungelatinized starch. A combination of GSHE and low temperature liquefaction can be used to overcome slurry viscosities. GSHE, a mixture of glucoamylase and alpha-amylase, hydrolyzes starch to dextrins at temperatures less than 48°C and simultaneously converts dextrins to fermentable sugars during SSF at 32°C. Thus, use of GSHE after low temperature liquefaction would allow hydrolzying ungelatinized starch in the liquefied slurry.

Corn and other grain crops like wheat, barley and rye contain oil, protein, fiber and 0.89 to 1.14% phytic acid (Reddy 2002). Phytic acid binds essential minerals, such as zinc, calcium and magnesium, as well as reduces digestibility of proteins resulting in reduction of mineral availability for biochemical processes (Maenz 2001). Phytic acid has deleterious effects on alpha-amylase activity at high temperatures during liquefaction due to noncompetitive inhibition and calcium (an alpha-amylase cofactor) chelation (Knuckles and Betchart 1987). Therefore, phytic acid presence during liquefaction results in reduced starch hydrolysis. Phytases (inositol

hexaphosphate phosphohydolase, E.C. 3.1.3.8) are the enzymes which hydrolyzes phytic acid into less phophorylated inositol phosphate esters (Ins1, inositol with one phosphate group attached to Ins5, inositol with five phosphate groups). Plant phytases have optimum activity at pH 5.4 to 6.5 and a temperature optimum of 55 to 65°C. Some grains (wheat, rye, barley) contain considerable phytase activity; whereas, others (corn, oats, sorghum and oilseeds) have little or no phytase activity (Ravindran et al 1995).

Phytases applied in addition to alpha-amylase during liquefaction have been reported to reduce slurry viscosities (Shetty et al 2008). They used phytase amylase liquefaction system (PALS) process which consisted of pretreatment step to expose whole ground corn to phytase and alpha-amylase. Pretreatment was done at a pH 5.8 and temperature $65\,^{\circ}$C for 30 min prior to liquefaction at $>80\,^{\circ}$C. This resulted in lower slurry viscosities using half the conventional alpha-amylase dose during liquefaction at 36% dry solids. Phytase treatment also resulted in stabilization of alpha-amylase (SPEZYME^{TM} Xtra) between pH 5.8 and 5.2 at $85\,^{\circ}$C. This was due to reduction in phytic acid inhibition by phytase treatment which improved pH robustness of alpha-amylase. Improved alpha-amylase activity resulted in better starch hydrolysis.

Phytase may lead to release of inositol from phytic acid hydrolysis. Inositol has been shown to have positive effects on cell growth, ethanol production and ethanol tolerance of high ethanol producing *Saccharomyces sp.* (Chi et al 1999). Chi et al (1999) showed that phosphatidylinositol (PI) content decreased from 17.5 to 7.2% in cells grown in sucrose medium without inositol within 24 hr, while the PI content increased from 16.4 to 23.9% in cells grown with inositol within the same period. Fermentation media with inositol resulted in 16.3% (v/v) ethanol while only 15.5% (v/v) without added inositol. Moreover, yeast cells with high levels of PI content had higher cell viabilities (74.3%) when exposed to high ethanol concentrations (18.0% v/v). Keiji et al (2004) determined that yeast cellular pH was lower in inositol deficient yeast and observed that yeast cell membrane H+-ATPase activity maintained the membrane permeability barrier, thereby ensuring ion homeostasis in yeast that enhanced yeast ethanol tolerance. Therefore, loss in H+-ATPase activity resulted in reduced cell viability due to ethanol

inhibition and inositol limitation. Thus, inositol produced during phytic acid hydrolysis using phytases would improve yeast ethanol tolerance ability resulting in higher ethanol yields.

2.4.2 Overcoming Glucose Inhibition

Fermentation slurry at 30% solids produced mash glucose concentrations of 15% w/v which induced glucose inhibition of yeast as indicated by reduced biomass and product yields (Casey and Ingledew 1986; Thatipamala et al 1992). Glucose concentrations as high as 19.3% w/v have been produced at 25% solids (Wang et al 2007). High glucose levels inhibit cell growth; alternatively cause yeast to grow rapidly and then suddenly stop (Casey and Ingledew 1986). Wang et al (2007) compared GSHE treatment with two conventional treatments having different enzyme combinations. They found that peak glucose concentrations were 6.6% (w/v) for GSHE treatment while 19.3 and 18.7% w/v for the conventional treatments. GSHE resulted in lower glucose concentrations due to gradual simultaneous liquefaction and saccharification. As lower initial glucose concentrations were produced during SSF, the limits on solids contents imposed by glucose inhibition were eliminated permitting higher solids ($>$33%) in slurries.

2.4.3 Nitrogen Supplementation

Yeast requires nitrogen to be available during fermentation which can be provided from external sources of nitrogen, such as urea and salts of ammonium, because the complex nitrogen compounds (proteins) present in corn cannot be consumed by yeast directly. High solids fermentation requires nutrients for yeast growth in a stressed environment (Bafrncova et al 1999). Without adequate and available nitrogen, high sugar concentrations lead to sluggish and incomplete fermentations. This will result in yeast no longer converting sugars into ethanol and CO_2, thereby leaving high residual sugars in the fermentation media. Assimilable nitrogen was the only limiting nutrient in high solids fermentation (Thomas and Ingledew 1990). Low levels of nitrogen have been associated with lower biomass yield (Bisson 1991; Spayd et al 1995; Manginot et al 1998) and low cellular activity (Bely et al 1990).

Free amino nitrogen (FAN) helps in increasing fermentation rates (Thomas and Ingledew 1990). Low FAN levels reduce fermentation rates by slowing yeast cell growth and thus affect glycolysis rates. This phenomenon is a function of nitrogen levels and source; ammonia, glutamine and asparagine yielded higher cell growth rates than proline and urea (Schure et al 2000). During high solids fermentation of wheat mashes (350 g dissolved solids per liter), the following preference order was obtained in terms of nitrogen source: 1% w/v Yeastex-61 (yeast extract) $>$ 16 mM urea $>$ 12 mM diammonium phosphate and 12 mM ammonium sulfate (Jones and Ingledew 1993). They showed that urea functions equally well as a nitrogen source when compared to yeast extract and has been shown to allow yeast to produce final ethanol concentrations of 20% v/v. Mixtures of amino acids and ammonia delayed yeast growth when a high FAN content was present (Torija et al 2002).

All nitrogen sources are degraded into glutamate or ammonia, the former is converted into alpha-ketoglutarate and the latter by NAD dependent glutamate dehydrogenase (NAD-GDH) (Cooper 1982; Magasanik 1992). Yeast cells can synthesize all required proteins from inorganic nitrogen in the form of ammonium salts and inorganic carbon (Abramov et al 1994). Ammonium salts enhance glycolysis rate in two ways. First, it is a monovalent ion and activates glycolytic enzymes (6-phosphofructokinase and D-fructose-6-phosphate-1-phosphotransferase) *in vitro* and second, by supporting biosynthesis of nitrogenous constituents of the yeast cell (Saita and Slaughter 1984). The latter nitrogen utilization has been reported to be a major contribution of nitrogen supplementation in improving fermentation rates. Synthesis of enzymes, both in glycolytic and hexose monophosphate pathways, are controlled by nitrogen limitation and by glucose concentration in the medium (Thomas et al 1996). Relative flux of carbon through the glycolysis pathway is greater in a nitrogen limiting condition than in nitrogen excess due to consequent increase in phosphofructokinase (key regulatory enzyme for glycolysis) with a decrease in enzyme synthesis for hexose monophosphate pathway. Yeast fermentative capacity is higher under nitrogen limiting than nitrogen excess condition (Casey et al 1983; Thomas et al 1993).

Nitrogen requirements of *S. cerevisiae* depend on the strain (Jiranek et al 1995) and fermentation media conditions (glucose concentration, temperature, oxygen) (Valero et al 2003). Different nitrogen concentrations (0.38, 0.71 and 1.2 g/L) as ammonium sulfate were applied to four different yeast strains. Average sugar consumption rates improved with increase in nitrogen until 0.71 g/L. While at high nitrogen dose (1.2 g/L) residual sugars, average sugar consumption rates did not improve (Taillandier et al 2007). In another work in which different diammonium phosphate concentrations (16.5 to 805 mg N/L) were used, yeast strain was a poor nitrogen responder at high nitrogen concentration (267 to 805 mg/L) since maximum biomass concentration was found with 402 mg N/L. In excess nitrogen media (805 mg N/L), 30% of ammonia remained in the end regardless of the nitrogen source (Mendeis-Ferreira et al 2004). Although, biomass concentrations did not change with increased initial nitrogen levels, all yeast strains consumed more assimilable nitrogen (Taillandier et al 2007). Yeast stored excess nitrogen in intracellular vacoules (Torija et al 2002; Henschke and Jiranek 1993) which can be used during the stationary phase for new protein synthesis following protein turnover (Mendeis-Ferreira et al 2004).

Different studies reported different optimal nitrogen levels depending on the yeast strain and fermentation media. A nitrogen dose suggested by Saita and Slaughter (1984), (2.4 mg N/ds (3.1 mg N/g glucose)), resulted in excess nitrogen in fermentation media which did not improve fermentation performance. Also 0.48 to 0.71 mg N/g ds (Taillandier et al 2007) might result in nitrogen deficient media since yeast requires more nitrogen under high osmotic stress in high solids fermentation media (Thomas et al 1993).

Chapter 3

Granular Starch Hydrolyzing Enzyme and Phytase Application

3.1 Introduction

High solids fermentations, greater than 33% total solids, can improve dry grind ethanol process efficiency and throughput. To make high solids fermentations efficient, challenges related to high slurry viscosity and glucose inhibition need to be overcome. Problems associated with high slurry viscosity during high solids fermentation could be solved by using phytic acid hydrolyzing enzymes or phytases (Shetty et al 2008). They incubated corn slurry with alpha-amylase and phytase for 30 min at 62°C and pH 5.8 followed by raising the temperature to 82°C to continue liquefaction. This process resulted in a viscosity reduction at 36% solids. Incubating preliquefied slurry with phytase and alpha-amylase for 30 min also reduced alpha-amylase dose for liquefaction by 50%. Another strategy to reduce slurry viscosity was to keep liquefaction temperatures lower than starch gelatinization temperatures (62 to 65°C). Thus, a combination of lower liquefaction temperatures and phytase could be used to mitigate viscosity related problems during high solids fermentation. However, liquefying corn slurry at less than starch gelatinization temperature would result in large amounts of ungelatinized starch in the slurry which cannot be digested by conventional glucoamylase (GA) used during simultaneous saccharification and fermentation (SSF). This problem could be overcome by using a combination of granular starch hydrolyzing enzyme (GSHE) and conventional GA during SSF.

GSHE used in this process would help lower glucose concentrations during SSF, avoiding osmotic stress. GSHE is a mixture of alpha-amylase and GA. Alpha-amylase component converts starch into dextrins at <48°C and glucoamylase hydrolyzes dextrins into fermentable sugars during SSF. This simultaneous production of glucose, by alpha-amylase and GA, and

consumption of glucose by yeast helps maintain glucose concentrations much below inhibitory levels. Wang et al (2007) reported that at 25% solids, peak glucose concentrations remained below 6.0% w/v using GSHE compared to 19.3% w/v using conventional enzymes.

GSHE is two times more expensive than conventional enzymes and the level required is 5 times more than conventional enzymes. To make GSHE use economical, dosage levels need to be reduced. Improvements have been made to reduce GSHE dose via protein engineering of starch binding domains that would increase the ability of GSHE to adsorb onto the starch granule surface (Juge et al 2002). Also, synergism between endo and exoamylases was used to improve the rate of starch hydrolysis and thus reduce enzyme dose. Despite these improvements, high enzyme loadings are required to overcome mass transfer barriers typical in solid phase reactions. Using GSHE in combination with conventional GA would increase total GA dose in the fermentation medium. Devantier et al (2005) reported that increasing GA dose increased final ethanol concentrations from 106 to 126 g/kg (35% w/w) for dry milled corn mashes.

Wong et al (2007) reported that increasing alpha-amylase concentrations increased starch hydrolysis rate greater than achieved by increasing glucoamylase concentrations. We aimed to use high GSHE alpha-amylase (alpha-amylase component of GSHE) concentrations to eliminate any alpha-amylase limitation which might have been experienced if we used GSHE alone. GSHE alpha-amylase (GSHE AA) has a higher activity compared to alpha-amylase activity of GSHE. By using GSHE AA in conjunction with conventional glucoamylase, we can control the ratio of alpha-amylase to glucoamylase to achieve required process efficiencies.

For our study, we used modified and conventional processes. In the modified process, liquefaction was conducted at temperatures (55°C) lower than starch gelatinization temperatures (62 to 65°C) using a combination of conventional alpha-amylase and phytase. After liquefaction, SSF was conducted using a combination of GSHE and conventional glucoamylase. In the conventional process, liquefaction was conducted at 82°C using conventional alpha-amylase followed by SSF using conventional glucoamylase. Objectives were to:

1. Determine the effects of conventional and modified processes at 35 and 40% solids on slurry viscosities and final ethanol concentrations.

2. Assess effects of combination of GSHE and conventional glucoamylase for fermentation efficiencies and peak glucose concentrations during SSF at 35% solids.

3. Evaluate effects of conventional glucoamylase and GSHE AA addition during SSF at 35% solids to maximize fermentation efficiencies and minimize peak glucose concentrations.

3.2 Materials and Methods

3.2.1 Materials

Yellow dent corn was grown during the 2007 crop season at the Agricultural and Biological Engineering Research Farm, University of Illinois at Urbana-Champaign. Each corn sample was cleaned by sieving over a 12/64 h (4.8 mm) sieve to remove broken corn and foreign material. Cleaned corn was stored at 4°C. Corn (1 kg) was ground at 500 rpm with a laboratory hammer mill (1100 W, model MHM4, Glen Mills Inc., Clifton, NJ) to pass through 0.5 mm hole sieve size. Moisture content was determined using standard two stage convection oven method (Method 44-19, AACC 2002). Corn slurry pH was adjusted using 10*N* sulfuric acid (Ricca Chemical, Arlington, TX). Active dry yeast (*Saccharomyces cerevisiae*, Ethanol Red, Fermentis, Lesaffre Yeast, Milwaukee, WI) and urea (99.6% ACS grade, Fisher Scientific, Waltham, MA) were used during SSF. Yeast inoculate was prepared by mixing 5 g dry yeast and 25 mL distilled water and incubating at 32°C for 20 min at 120 rpm in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ).

3.2.1.1 Slurry Preparation

Each corn sample was cleaned by sieving over a 12/64 in (4.8 mm) round hole screen to remove broken corn and foreign material. Cleaned corn was stored at 4°C. Corn (1 kg) was ground at 500 rpm with a laboratory hammer mill (1100 W, model MHM4, Glen Mills Inc.,

Clifton, NJ) to pass through a 0.5 mm round hole sieve. Moisture content was determined using a standard two stage convection oven method (Method 44-19, AACC International 2002). Corn slurry was prepared by mixing 100 g (db) corn with deionized water to achieve required slurry solids.

3.2.1.2 Enzymes

Maxaliq^{TM} One (Genencor, Rochester, NY), a formulation of alpha-amylase derived from *Bacillus licheniformis* and phytase derived from *Trichoderma reesei*, was used for liquefaction in the modified process. The pH optimum of the enzyme was 5.7. Liquozyme SC (Novozymes, Franklinton, NC) was used for conventional liquefaction at 82°C. It was derived from mesophilic soil bacterium *Bacillus licheniformis* and had an activity of 120 to 138 KNU/g (KNU=kilo novo units).

Enzymes used during SSF were GSHE (Stargen 001; Genencor, Rochester, NY) and glucoamylase (1,4-alpha-D-glucan hydrolase; GC 147; Genencor, Rochester, NY). GSHE contained alpha-amylase from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger* and had an activity of 456 GSHU/g (GSHU is granular starch hydrolyzing units). GSHE hydrolyzes starch at low temperature (<48°C) and low pH (4.0 to 4.2). GC 147 is a glucoamylase that had an activity of 580 GTU/g (1 GTU is the amount of enzyme that will liberate 1 g of reducing sugars calculated as glucose/hr from soluble starch substrate). The enzyme had a pH range of 4.0 to 4.5 and optimal temperature range of 58 to 65°C.

For GSHE AA treatments, GC 626 (Genencor, Rochester, NY), the alpha-amylase component of GSHE, was used during SSF. GC 626 enzyme is a starch hydrolyzing alpha-amylase derived from a strain of *Aspergillus kawachi* expressed in *Trichoderma reesei*. The enzyme is an endoamylase that hydrolyzes alpha-1,4 glucosidic bonds of gelatinized and granular starch randomly, producing soluble dextrins and oligosaccharides. It had an activity of 10,000 SSU/g.

3.2.2 Treatments to Reduce Liquefaction Viscosities

3.2.2.1 Conventional Dry Grind Process

Corn slurry was prepared to achieve 35 and 40% slurry solids. Corn slurry pH was adjusted to 5.7 using 10*N* sulfuric acid. Alpha-amylase and GA levels used in the process were selected based on manufacturer's recommendations. Corn slurry was liquefied at 82°C for 90 min using 0.15 μL Liquozyme SC/g (db) corn. All runs were carried out in 500 mL flasks. The liquefied slurry was analyzed for final slurry viscosity at the end of 90 min liquefaction using a disk viscometer (RVT, Brookfield Engineering Laboratories, Middleboro, MA). The 500 mL flasks containing liquefied slurry were maintained at 32°C to measure viscosity using spindles no. 3 and 7 at 20 rpm and 32°C for 35 and 40% solids, respectively. Each dial reading was allowed to stabilize for 1 min. The dial reading was multiplied by the factor, 50 and 2000, corresponding to the RVT spindle no. 3 and 7, respectively, at 20 rpm.

Yeast inoculate levels were similar to levels used by Wang et al (2007) for corn slurry fermentations using GSHE at 25% solids. Glucoamylase levels were selected based on recommendations from the enzyme manufacturer. Urea level (48 mmol urea/g db solids) was chosen in excess to urea levels suggested by Jones and Ingledew (1993) for wheat mashes to avoid nitrogen limitation at 35% solids. Jones and Ingledew (1993) reported that 16 mmol urea/L wheat mash yielded 21.5% v/v ethanol at high inoculation levels for mashes at 30% solids.

A yeast inoculate was prepared by adding 5 g dry yeast to 25 mL deionized water and incubating at 32°C for 20 min. After adjusting the corn slurry pH to 4.0 using 10*N* sulfuric acid, yeast inoculate (0.02 mL/g db corn), glucoamylase (0.50 μL/g db corn) and urea (1.51 mg/g db corn) were added to the slurry. SSF was conducted at 32°C for 72 hr. All runs were carried out in 500 mL flasks in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ).

3.2.2.2 Modified Dry Grind Process

The modified process differed from the conventional process with respect to liquefaction temperatures and enzyme combinations used during liquefaction and SSF. In the modified

process, liquefaction was conducted at 55°C and pH 5.7 for 90 min using Maxaliq One at a rate of 0.30 μL/g db corn (dose based on manufacturer's recommendation). The liquefied slurry was analyzed for viscosity at the end of 90 min liquefaction using RVT viscometer as was done for the conventional process. Yeast inoculate was prepared as described in the conventional process. GSHE and glucoamylase were added based on the recommended doses from the manufacturer. Slurry pH was adjusted to 4.0 using 10*N* sulfuric acid. SSF was conducted using yeast inoculate (0.02 mL/g db corn), glucoamylase (0.50 μL/g db corn), GSHE (2.5 μL/g db corn) and urea (1.51 mg/g db corn). Fermentations were conducted at 32°C for 72 hr using 500 mL flasks in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ) constantly agitated at 120 rpm.

3.2.2.3 Metabolite Analyses

For metabolite analyses, slurry samples (1 mL) were taken at 0, 2, 4, 8, 12, 24, 48 and 72 hr during fermentation. Samples were centrifuged (Model 5415 D, Eppendorf, Westbury, NY) for 6 min at 11,000xg. The supernatant liquid was filtered through a 0.2 μm filter into 0.2 mL vial insert. Fermentation metabolite profiles were obtained by analyzing filtered supernatant liquid using an HPLC (high pressure liquid chromatography) equipped with a column (Aminex HPX-87H organic acid, BioRad, Hercules, CA; System: Breeze, Waters Corp, Milford, MA). Ethanol, glucose, maltose, maltotriose, glycerol, lactic acid and acetic acid concentrations were determined.

3.2.2.4 Fermentation Rates

Fermentation rates were defined as the overall percent ethanol change (% v/v/hr) during the initial 12 hr of fermentation. A linear regression fit, between ethanol concentration and fermentation time, was used to quantify initial fermentation rates as the slope of the regression line.

3.2.2.5 Ethanol Yields and Fermentation Efficiency

Starch and moisture contents of whole corn were analyzed in triplicate, using residual starch assay and convection oven method (AACC 2002), respectively. Corn mean moisture was 10.8% (wb) and mean starch was 69.8% (db). To determine theoretical ethanol yields, 100% starch conversion to glucose and 100% glucose conversion to ethanol was assumed. Starch in 100 g (db) corn was multiplied by 1.11 g glucose/g starch and 0.51 g ethanol/g glucose (based on molecular weight ratios and stoichiometric coefficients of substrate and product) to give theoretical ethanol (g). Total ethanol obtained based on 69.8% (db) starch in 100 g (db) corn was divided by ethanol density (0.789 g/mL) to obtain the ethanol volume. The ratio of ethanol volume produced and ground corn weight added to form slurry was reported as the theoretical ethanol yield (L/tonne). For calculating actual ethanol yield (L/tonne), ethanol volume produced was obtained by multiplying final ethanol concentrations by measured fermented slurry volume at 72 hr and dividing by ground corn weight added to make slurry. Fermentation efficiency was calculated by dividing actual ethanol yield by theoretical ethanol yield.

3.2.2.6 Experimental Design

Conventional and modified processes were conducted at 35 and 40% solids contents resulting in four treatments. Experiments were arranged as a 2x2 factorial in a complete randomized design with three replicates for each treatment. Metabolite profiles were analyzed to determine effects of low temperature liquefaction, phytase and GSHE addition on fermentation efficiencies and slurry viscosities in high solids fermentation. Nitrogen levels, yeast inoculate and pH were kept constant. For each treatment, liquefied slurry viscosities, final ethanol concentrations, fermentation efficiencies, ethanol yields and glucose concentrations were analyzed. Analysis of variance (ANOVA) and Fischer's least significant difference with a significance level of $p<0.05$ was used to compare means among treatments.

3.2.3 Effects of GSHE and Glucoamylase Loadings

Corn slurry at 35% solids was prepared by mixing 100 g ground corn with distilled water. After adjusting slurry pH to 5.7 using 10*N* sulfuric acid, Maxaliq One (0.30 μL/g db corn) was added and slurry was liquefied at 55°C for 90 min. Liquefied slurry pH was adjusted to 4.0 using 10*N* sulfuric acid. Yeast inoculate (0.02 mL/g db corn) and urea (50% w/v) (1.51 mg/g db corn) were added to the slurry. Enzymes were added according to the process treatment specifications in the experimental design. Enzyme level, 1X, was selected based on the enzyme dosage recommended by the manufacturer. Slurry was simultaneously saccharified and fermented at 32°C for 72 hr with constant agitation at 120 rpm in a shaking water bath. Fermented slurry samples (1 mL) were taken at 0, 2, 4, 8, 12, 24, 48 and 72 hr for metabolite analyses as in Section 3.2.2. Fermentation rates were calculated as described in Section 3.2.2. Similarly, ethanol yields and fermentation efficiencies were determined as described in Section 3.2.2.

3.2.3.1 Residual Starch Analysis

Residual starch was measured based on the acid hydrolysis method (Ebell 1969). Fermented slurry was dried overnight at 49°C, ground in a coffee mill (Black and Decker, Towson, MD) and analyzed for moisture content (AACC 2002). From ground samples, 1 g subsamples along with 1 g glucose and starch standards were weighed in triplicate. Samples were transferred to 100 mL autoclave safe glass bottles and 50 mL HCl (0.4M) added; bottles were autoclaved for 60 min at 126°C. After cooling to 100°C, bottles were placed in an ice water bath for 10 min. From bottled samples, 2 mL was transferred to a 25 mL centrifuge tube with a serological pipette. To centrifuge tubes, 220 μL Na_2CO_3 was added and mixed vigorously. All samples, including starch and glucose standards, were centrifuged for 5 min at 3000xg. Stillage samples were diluted 8:1 or 16:1 as required while glucose and starch standards were diluted to 40:1. A blank was prepared using 0.1 mL distilled water. Glucose standards (0.1 mL) at 0.25, 0.50, 0.75 and 1.00 mg/mL were prepared in glass tubes. From diluted samples, 0.1 mL was transferred to glass tubes with screw caps and 3 mL glucose oxidase and peroxidase (GOPOD)

reagent was added to each tube and vortexed. Tubes were incubated in a 50°C water bath for 20 min. After cooling to room temperature, sample absorbance was read at 510 nm against reagent blank (Helios spectrophotometer, Model 10 VIS, Thermo Scientific, Needham Heights, MA). An absorbance calibration curve was constructed from the glucose standards. Percent residual starch was determined by multiplying observed glucose concentration by the dilution and glucose recovery factors and dividing by a factor of 1.11.

3.2.3.2 GSHE Treatments

Combinations of GSHE and conventional glucoamylase (GA) were added during SSF to determine their effects on peak glucose concentrations, fermentation efficiencies and ethanol yields. A control treatment was conducted at 35% solids using the conventional dry grind process described in Section 3.2.2. Each process treatment was performed with three replications. For each treatment, fermentation profiles were plotted. Nine SSF enzyme treatments (Table 3.1) were arranged in a 3x3 factorial design. Enzyme levels of 1X corresponded to enzyme addition rates as recommended by the manufacturer. Temperature, yeast dose, nitrogen dose and solids content were held constant. Using SAS, a two factor ANOVA and Fisher's least significant difference (LSD) with $p<0.05$ were used to compare ethanol concentrations, glucose concentrations, ethanol yields and fermentation efficiencies among all treatments.

Table 3.1. Enzyme levels used in 3x3 factorial design to determine effects of glucoamylase and GSHE on high solids fermentation.

Glucoamylase	GSHE (μL/g dry corn)		
(μL/g dry corn)	0	1.25 (0.5X)	2.5 (1X)
0	x	x	x
0.25 (0.5X)	x	x	x
0.5 (1X)	x	x	x

Control treatment: 35% solids, conventional dry grind process.

3.2.4 Effects of GSHE Alpha-amylase and Glucoamylase Addition

3.2.4.1 Liquefaction and SSF

Corn slurry at 35% solids was prepared by mixing 100 g (db) corn with distilled water. Slurry pH was adjusted to 5.7 using 10*N* sulfuric acid. All GSHE AA treatments were conducted using modified process with liquefaction at 55°C for 90 min using Maxaliq One (0.30 μL/g db corn). After liquefaction, slurry pH was adjusted to 4.0 using 10*N* sulfuric acid. Yeast inoculate (0.02 mL/g db corn) and urea (1.51 mg/g db corn) were added to slurry. GSHE AA and GA were added according to the process treatment specifications in the experimental design (Table 3.2). Levels designated as 1X corresponded to the enzyme dosage recommended by the manufacturer. To avoid alpha-amylase limitation during SSF, 2X GSHE AA was selected as one of the enzyme levels for the experimental design. SSF was conducted at 32°C for 72 hr in 500 mL flasks with agitation at 120 rpm in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ). For metabolite analyses as described in Section 3.2.2, samples (1 mL) were taken at 0, 2, 4, 8, 12, 24, 48 and 72 hr. Fermentation rates, ethanol yields, residual starch and fermentation efficiencies were determined as described in Section 3.2.2 and 3.3.2.

3.2.4.2 Process Treatments

Combinations of GSHE AA and conventional GA were added during SSF to determine their effects on glucose concentrations, fermentation efficiencies and ethanol yields. Two variables, GSHE AA and GA, each with three levels were arranged in a 3x3 factorial completely randomized design resulting in nine treatments (Table 3.2). Each process treatment was performed with three replications. Temperature, nitrogen level, yeast inoculate, slurry pH were kept constant. Each process treatment was done in triplicate. For each treatment, fermentation profiles were obtained. A control treatment was conducted at 35% solids using the conventional dry grind process as described in Section 3.2.2. Analysis of variance (ANOVA) and Fischer's least significant difference with a significance level of $p<0.05$ were used to compare mean ethanol concentrations, ethanol yields, fermentation efficiencies and peak glucose concentrations

among treatments.

Table 3.2. Enzyme levels used in 3x3 factorial design to determine effects of GSHE AA and GA on high solids fermentation.

Glucoamylase	GSHE AA (μL/g dry corn)		
(μL/g dry corn)	0	0.625	1.25
		(1X)	(2X)
0	x	x	x
0.25 (0.5X)	x	x	x
0.5 (1X)	x	x	x

Control treatment: 35% solids, conventional dry grind process.

3.3 Results and Discussion

3.3.1 Treatments to Reduce Liquefaction Viscosities

The modified process resulted in 81 and 98% reduction in liquefied slurry viscosity at 35 and 40% solids, respectively (Table 3.3). Reduced slurry viscosity using the modified process would possibly result in lower pumping power requirements. Reduced slurry viscosities may have aided in removing CO_2 toxicity effects on yeast and thus higher yeast fermentation performance (Maiorella et al 1982). This was evident from higher ethanol concentrations observed for the modified process compared to conventional process at 35 and 40% solids (Table 3.3). The highest slurry viscosity (73,000 cP) was obtained at 40% solids using the conventional process. High slurry viscosities for conventional process was due to higher liquefaction temperature (82°C) and absence of phytase. Bagley and Christianson (1982) also observed an increase in viscosity for starch solutions with increasing liquefaction temperature (from 60 to 75°C).

Initial glucose concentrations were 0.86 and 0.00% w/v for the conventional process at 35 and 40% solids, respectively. For the modified process, initial glucose concentrations were 2.91

Table 3.3. Fermentation parameters for the modified and conventional processes at 35 and 40% solids (means of three observations).

Treatment		Process parameters			
Solids Content (% db)	Process	Slurry Viscosities (cP)	Ethanol Concentrations[a,b] (% v/v)	Peak Glucose[a,c] (% w/v)	Residual Glucose[a,d] (% w/v)
35	Conventional	2,700 b	18.1 c	13.5 c	1.99 c
	Modified	550 d	19.5 a	10.2 d	0.05 d
40	Conventional	73,000 a	18.7 b	16.7 a	2.89 a
	Modified	1,100 c	19.5 a	14.5 b	2.51 b

[a] Means corresponding to the same letter in the same column are similar ($p<0.05$).
[b] Fischer's LSD for final ethanol concentrations (72 hr) was 0.2% v/v.
[c] Fischer's LSD for peak glucose concentrations was 1.2% w/v.
[d] Fischer's LSD for residual glucose concentrations was 0.11% w/v.

and 2.78% w/v at 35 and 40% solids, respectively. Peak glucose concentrations were observed during initial 12 hr of fermentation (Figure 3.1). Peak glucose concentrations decreased from 13.5 to 10.2% w/v and from 16.7 to 14.5% w/v using the modified process at 35 and 40% solids, respectively (Table 3.3 and Figure 3.1). Increasing solids content from 35 to 40% solids resulted in 3.2 and 4.3% w/v increases in peak glucose concentrations using the conventional and modified processes, respectively (Table 3.3). Higher glucose concentrations in the conventional process indicated higher osmotic stress.

Glucose concentrations were less than 0.05% w/v for modified process treatments within 24 hr (Figure 3.1). However, for conventional process treatments glucose concentrations remained higher than 2% w/v throughout fermentation (Figure 3.1). This indicated reduced yeast fermentation performance resulting in higher residual glucose. Residual glucose concentrations ranged from 0.05 to 2.89% w/v for all the treatments, with maximum residual glucose concentrations obtained for the conventional process at 40% solids (Table 3.3). Differences were observed in residual glucose concentrations between 35 and 40% solids for the conventional and

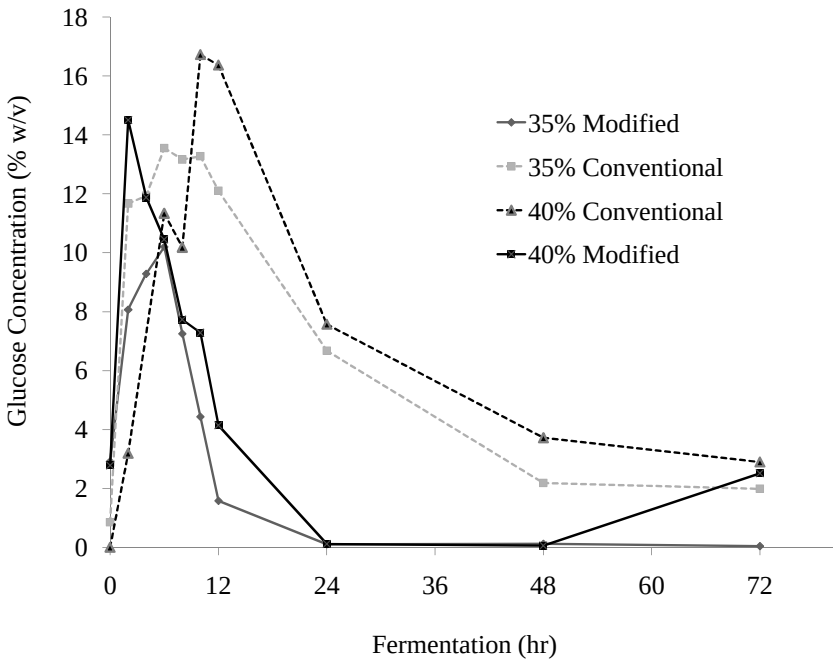

Figure 3.1. Glucose concentrations for conventional and modified processes at 35 and 40% solids (means of three observations).

modified processes. Higher residual glucose at 40% solids for conventional and modified processes (2.89 and 2.51% w/v, respectively) was attributed to a reduction in yeast fermentation performance, indicated by lower ethanol yields at 40% solids.

Ethanol concentrations remained highest for 40% solids using the modified process throughout fermentation (Figure 3.2). Although the conventional process at 40% solids resulted in lowest ethanol concentrations at 24 hr, final ethanol concentrations were 0.6% v/v higher compared to conventional treatment at 35% solids (Table 3.3 and Figure 3.2). For the modified process, final ethanol concentrations were 1.4 and 0.8% v/v higher compared to the conventional process at 35 and 40% solids, respectively. Final ethanol concentrations (19.5% v/v) obtained at 35% solids using the modified process were comparable to maximum ethanol concentrations (19.4% v/v) reported by Devantier et al (2005) for ground corn fermentations at 35% solids. Conventional process at 40% solids resulted in 18.7% v/v final ethanol concentrations which were similar to ethanol concentrations (18.8% v/v) reported by Shihadeh (2008) for ground corn fermentations at 40% solids using GSHE without separate liquefaction step.

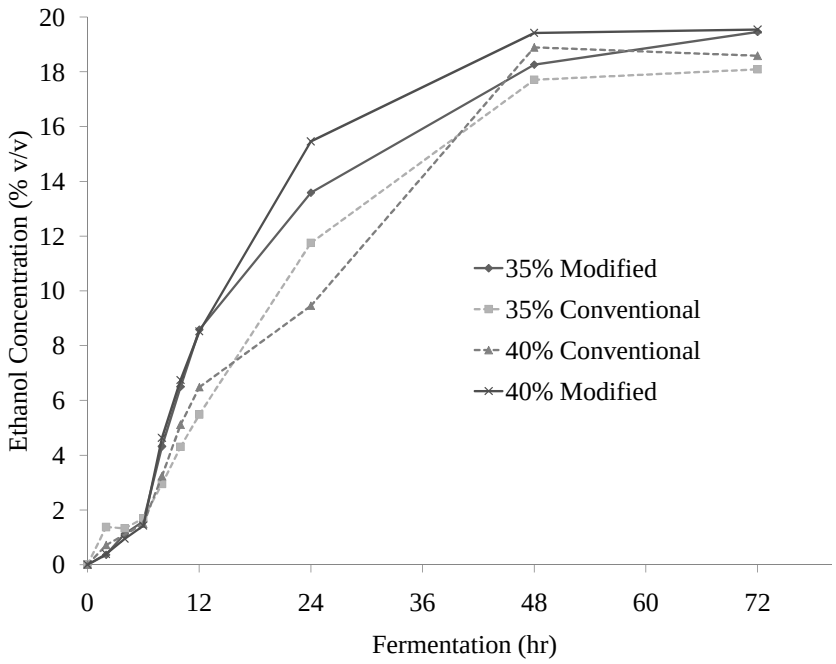

Figure 3.2. Ethanol concentrations for conventional and modified processes at 35 and 40% solids (means of three observations).

For the conventional process, increasing solids content from 35 to 40% resulted in 0.6% v/v increase in final ethanol concentrations (Table 3.3). However, for the modified process, treatments with 35 and 40% solids resulted in similar final ethanol concentrations (Table 3.3). High ethanol concentrations (>19% v/v) produced using the modified process may have exerted yeast stress leading to reduced yeast viability. This limited any further increase in final ethanol concentrations beyond 19.5% v/v. Shihadeh (2008) also observed reduction in yeast viability at high ethanol concentrations for ground corn fermentations at 40% solids.

Higher solids resulted in higher fermentation rates over initial 12 hr using the conventional process (Table 3.4). Using the modified process, fermentation rates increased by 0.23 and 0.15% v/v/hr at 35 and 40% solids, respectively. Fermentation rates were different between 35 and 40% solids for the conventional process (Table 3.4).

Glycerol concentrations were different for the conventional and the modified process at 35 and 40% solids (Table 3.4). However at 40% solids, glycerol concentrations were 0.20% w/v higher for the conventional process compared to the modified process, indicating higher yeast

Table 3.4. Fermentation efficiencies, ethanol yields, final glycerol and fermentation rates for modified and conventional processes at 35 and 40% solids content (means of three observations).

Treatment		Process parameters			
Solids Content (% db)	Process	Fermentation Efficiency[a,b,c] (%)	Ethanol Yields[a,b] (L/tonne)	Final Glycerol [a,b] (% w/v)	Fermentation Rates [a,b] (% v/v/hr)
35	Conventional	83 b	378 b	1.13 b	0.45 c
	Modified	91 a	416 a	0.99 c	0.68 a
40	Conventional	70 d	318 d	1.29 a	0.56 b
	Modified	73 c	330 c	1.09 b	0.71 a

[a] Means with the same letter in the same column are similar ($p<0.05$).
[b] Fischer's LSD for fermentation efficiency, ethanol yields, final glycerol and fermentation rates were 1%, 6 L/tonne, 0.04 % w/v and 0.03% v/v/hr, respectively.
[c] Fermentation efficiency was calculated as the ratio of experimental and theoretical ethanol yield.

stress in the conventional process. Lactic and acetic acid concentrations remained below inhibiting concentrations ($\geq$0.2% w/v for lactic acid and $\geq$0.05% w/v for acetic acid) throughout fermentation for all the treatments. This indicated that fermentations did not exhibit any stress by high organic acid (lactic and acetic) concentrations.

Fermentation efficiencies were 8 and 3% higher for the modified process compared to the conventional process at 35 and 40% solids, respectively (Table 3.4). Increasing solids content from 35 to 40% solids resulted in 28 and 18% lower fermentation efficiencies for the conventional and modified process (Table 3.4). At 35% solids, conventional and modified process resulted in 83 and 90% fermentation efficiencies, respectively. Wang et al (2007) also reported 88% fermentation efficiency for ground corn fermentations at 25% solids using GSHE and a different corn hybrid.

Ethanol yields increased by 38 and 12 L/tonne using the modified process at 35 and 40% solids, respectively (Table 3.4). Increasing slurry solids from 35 to 40% reduced ethanol yields by 60 and 86 L/tonne for the conventional and modified process, respectively (Table 3.4).

Ethanol yields obtained at 35 (416 L/tonne) and 40% (330 L/tonne) solids using the modified process were comparable to ethanol yields reported by Lemuz et al (2009) (419 and 353 L/tonne for 35 and 40% solids, respectively) for a conventional dry grind process.

3.3.2 Effects of GSHE and Glucoamylase Enzyme Loadings

3.3.2.1 Fermentation Profiles

During 72 hr fermentation, glucose concentrations peaked within 12 hr of fermentation (Figure 3.3). Control treatment resulted in highest peak glucose concentrations (13.49% v/v) (Figures 3.3 and Table 3.5). The rate of glucose consumption was the slowest for control treatment. It took 48 hr for the control treatment and 12 hr for all GSHE treatments to reduce glucose concentrations to less than 3.5% w/v. High glucose concentrations in the control treatment could have increased osmotic stress on yeast leading to reduced glucose consumption. This is evident from high residual glucose levels (72 hr) (1.95% w/w) obtained for control treatment (Figure 3.3). All other treatments resulted in less than 1.20% w/v residual glucose concentrations (Table 3.6 and Figure 3.3). Low residual glucose concentrations ($<$1% w/v) were indicative that fermentations for GSHE treatments were relatively complete.

Treatment with no enzyme resulted in lowest peak glucose concentrations (3.95% w/v) pertaining to insufficient hydrolyzed starch converted to glucose (Table 3.5). For 0 GSHE treatments, differences were observed in peak glucose concentrations between 0 and 0.5X GA levels, but no differences were observed between 0.5 and 1X GA levels. Increasing GA levels from 0 to 0.5X and 0.5 to 1X increased peak glucose concentrations by 5.26 and 0.35% w/v, respectively, for 0 GSHE treatments (Table 3.5). Differences were observed in peak glucose concentrations between 0, 0.5 and 1X GSHE levels for 0 GA level treatments (Table 3.5).

Final ethanol concentrations ranged from 8.2 to 19.4% v/v for all treatments (Table 3.5). The highest final ethanol concentrations (19.4% v/v) were obtained for 0.5X GSHE and 1X GA treatment (Table 3.5 and Figure 3.4). Treatment with no enzyme (0 GSHE and 0 GA) resulted in the lowest final ethanol concentrations (8.2% v/v). For all treatments except treatments with no

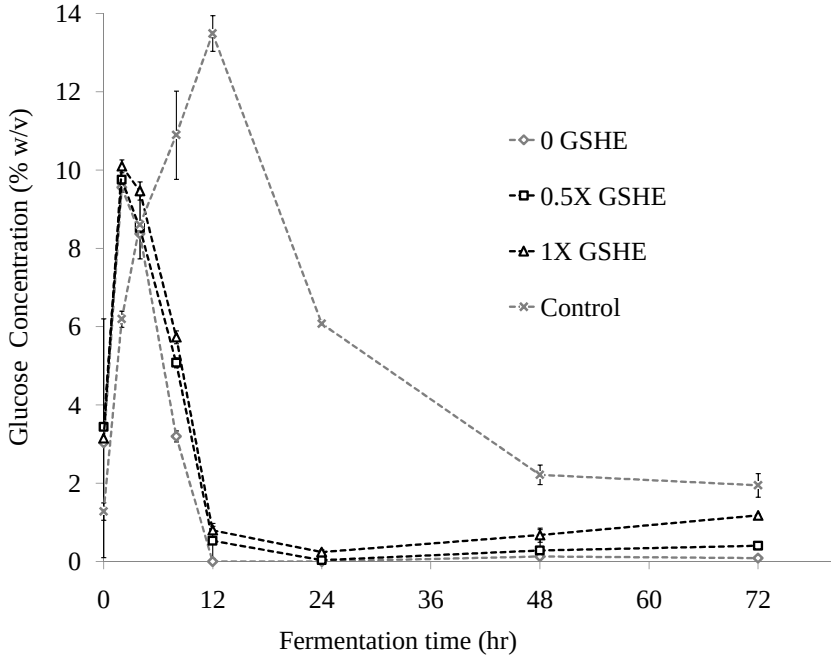

Figure 3.3. Glucose concentrations for GSHE process treatments at 1X GA level at 35% solids (means of three observations). Error bars are ±1 standard deviation.

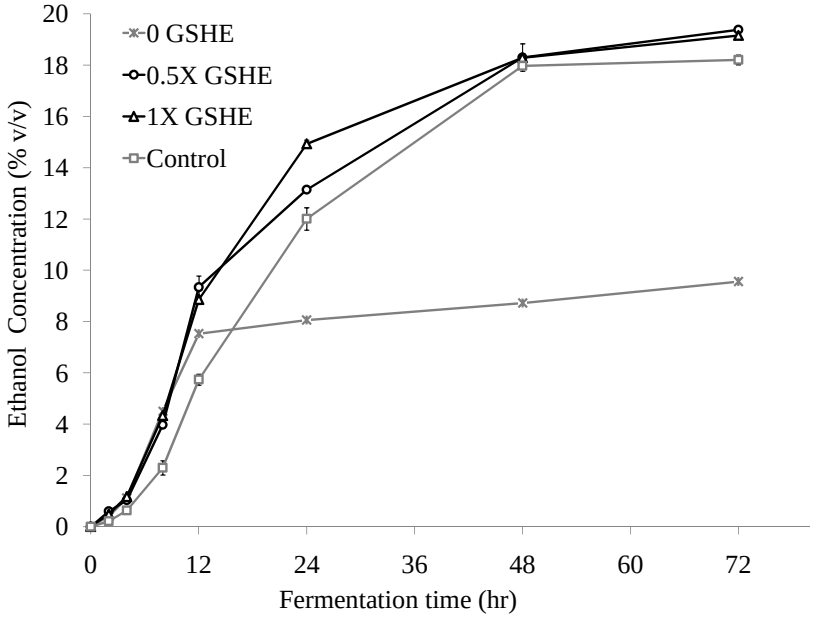

Figure 3.4. Ethanol concentrations for GSHE process treatments at 1X GA level at 35% solids (means of three observations). Error bars are ±1 standard deviation.

Table 3.5. Ethanol, glucose and glycerol concentrations with fermentation rates for GSHE and GA treatments at 35% solids (means of three observations ±1 standard deviation)

Treatment		Process parameters			
		Final Ethanol[a,b] (% v/v)	Peak Glucose[a,c] (% w/v)	Final Glycerol (% w/v)	Fermentation Rates (% v/v/hr)
0 GA	0 GSHE	8.2±0.02 e	3.95±0.09 e	0.51±0.02 f	0.58±0.01 def
	0.5X GSHE	18.5±0.50 c	9.10±0.53 d	0.94±0.06 cd	0.71±0.05 bc
	1X GSHE	19.0±0.07 ab	10.28±0.16 b	0.99±0.01 bc	0.72±0.01 bc
0.5X GA	0 GSHE	9.2±0.48 d	9.21±0.48 d	0.66±0.02 e	0.56±0.01 ef
	0.5X GSHE	18.9±0.06 b	9.81±0.09 bc	0.93±0.01 d	0.85±0.20 a
	1X GSHE	19.1±0.14 ab	10.38±0.13 b	1.00±0.04 b	0.68±0.01 bcd
1X GA	0 GSHE	9.1±0.22 d	9.56±0.56 cd	0.63±0.03 e	0.63±0.03 cde
	0.5X GSHE	19.4±0.04 a	9.87±0.09 bc	0.92±0.02 d	0.78±0.01 ab
	1X GSHE	19.2±0.08 ab	10.10±0.21 bc	0.96±0.02 bcd	0.74±0.01 bc
	Control*	18.2±0.04 c	13.49±0.45 a	1.19±0.02 a	0.48±0.00 f

[a] Means corresponding to the same letter in the same column are similar ($p<0.05$).

[b] Fischer's LSD for ethanol, peak glucose, final glycerol and fermentation rates were 0.4% v/v, 0.58% w/v, 0.05% w/v and 0.11% v/v/hr, respectively.

* Control treatment: 35% solids, conventional dry grind process.

Table 3.6. Fermentation efficiencies with ethanol yields, residual glucose and residual starch concentrations for GSHE and GA treatments at 35% solids (means ±1 standard deviation of three observations).

Treatment		Process parameters			
		Fermentation Efficiency[a,b] (%)	Ethanol Yields[a,b] (L/tonne)	Residual Starch[a,b] (% w/w)	Residual Glucose[a,b] (% w/v)
0 GA	0 GSHE	39±0.11 e	179±0.50 f	73.8±1.2 a	0.13±0.00 e
	0.5X GSHE	87±1.71 b	396±7.79 c	35.8±0.9 d	0.14±0.03 e
	1X GSHE	88±0.31 b	401±1.43 bc	27.1±0.4 g	0.76±0.03 c
0.5X GA	0 GSHE	44±2.29 e	198±10.40 e	68.8±1.5 b	0.129±0.02 e
	0.5X GSHE	90±1.08 ab	409±4.89 ab	30.2±1.5 f	0.41±0.03 d
	1X GSHE	91±0.40 a	413±1.84 a	29.8±0.6 f	0.82±0.19 c
1X GA	0 GSHE	43±1.48 d	194±6.70 e	64.9±2.5 c	0.09±0.08 e
	0.5X GSHE	92±0.34 a	418±1.55 a	28.0±0.2 g	0.40±0.01 d
	1X GSHE	90±0.37 ab	408±3.05 ab	27.2±0.5 g	1.17±0.24 b
	Control*	84±0.20 c	381±0.92 d	31.8±1.7 e	1.95±0.22 a

[a] Means corresponding to the same letter in the same column are similar ($p<0.05$).

[b] Fischer's LSD for fermentation efficiency, ethanol yield, residual starch and residual glucose were 2%, 9 L/tonne, 2% w/w and 0.21% w/v, respectively.

* Control treatment: 35% solids, conventional dry grind process.

GSHE, final ethanol concentrations were greater than 18% v/v (Table 3.5).

Increasing GSHE levels from 0 to 1X resulted in 1.17 to 6.33% w/v increase in peak glucose concentrations for 0, 0.5 and 1X GA treatments. Control treatment resulted in 0.3 to 1.2% v/v lower final ethanol concentrations (18.2% v/v) compared to treatments with only GSHE or a combination of GSHE and GA (Table 3.5). At fixed GA levels, differences were observed in final ethanol concentrations between 0 and 0.5X GSHE levels (Table 3.5 and Figure 3.5). Final ethanol concentrations increased by 10.3, 9.7 and 10.3% v/v when GSHE levels increased from 0 to 0.5X for 0, 0.5 and 1X GA treatments, respectively. No differences were observed in final ethanol concentrations between 0.5 and 1X GSHE levels for 0.5 and 1X GA treatments. However, final ethanol concentrations were different between 0.5 and 1X GSHE levels for 0 GA treatments; an increase of 0.5% v/v was obtained. These results implied that similar ethanol concentrations can be achieved using half GSHE dose (0.5X) when used in conjunction with conventional GA. Increasing GSHE levels from 0.5 to 1X did not improve final ethanol concentrations for 0.5 and 1X GA treatments when yeast was limited by high ethanol levels ($\cong$19% v/v). At these high ethanol concentrations, yeast growth gets inhibited leading to reduced yeast fermentative ability (Casey and Ingledew 1986).

For treatments with no GSHE, increase in GA levels from 0 to 0.5X increased final ethanol concentrations from 8.2 to 9.2% v/v. However, further increasing GA levels from 0.5 to 1X did not improve final ethanol concentrations (Table 3.5). For 0.5X GSHE treatments, differences were observed in final ethanol concentrations between 0, 0.5 and 1X GA treatments (Table 3.5 and Figure 3.5). For 1X GSHE treatments, final ethanol concentrations were similar at all GA levels (Table 3.5 and Figure 3.5).

Fermentation rates (initial 12 hr) were the lowest for control treatment (0.48% v/v/hr) (Table 3.5). GSHE treatments resulted in higher fermentation rates with maximum rate obtained for 0.5X GSHE and 0.5X GA treatment (0.85% v/v/hr). Fermentation rates increased by 0.13 to 0.29% v/v/hr with increasing GSHE levels from 0 to 0.5X for fixed GA treatments. However, increasing GSHE levels from 0.5 to 1X did not improve the rates (Table 3.5).

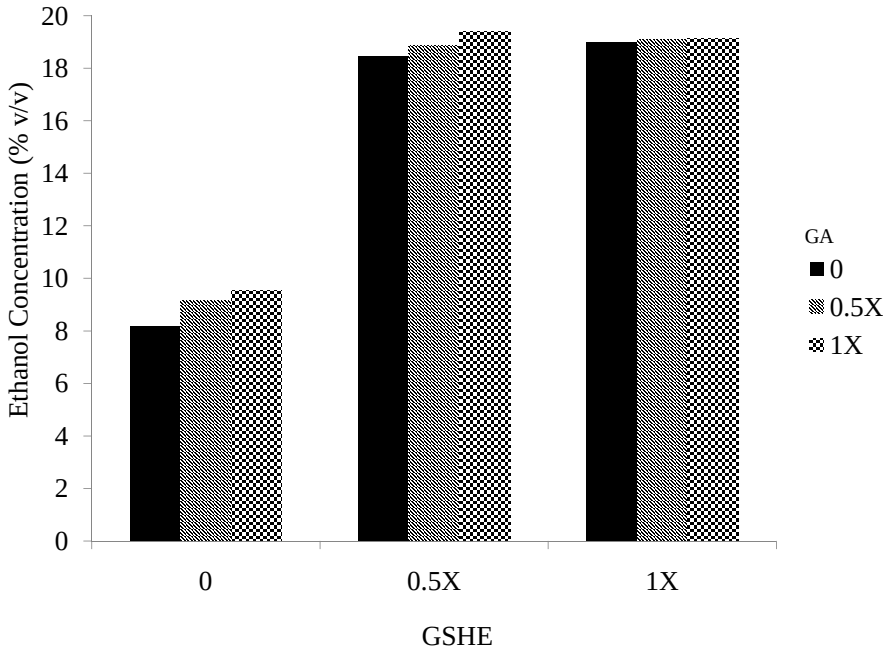

Figure 3.5. Ethanol concentrations for GSHE process treatments at 35% solids (means of three observations).

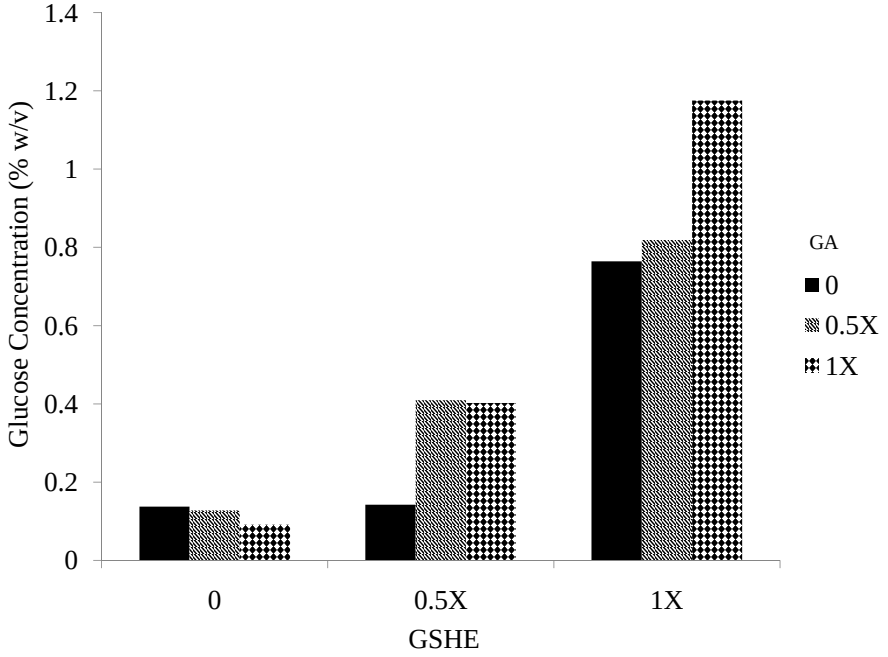

Figure 3.6. Residual glucose concentrations for GSHE process treatments (means of three observations).

3.3.2.2 Metabolite Profiles

Among treatments, final glycerol concentrations were the highest for the control treatment (1.19% w/v) (Table 3.5). This is in agreement with higher glucose concentrations observed for the control treatment and thus higher osmotic stress leading to production of more glycerol. For other treatments, glycerol concentrations were $\leq$1.00% w/v. The lowest glycerol concentrations (0.51% w/v) were obtained for the treatment with no enzyme. The treatment with no enzyme resulted in glucose ($<$3.95% w/v) and ethanol concentrations ($<$8.2% v/v) below inhibition levels throughout fermentation. Thus, fermentation exhibited no ethanol and glucose inhibition effects resulting in lower glycerol formation. Glycerol levels for all GSHE treatments were within the range typical for dry grind plants ($\leq$1.2 to 1.5% w/v, Russell 2003).

Final lactic acid concentrations remained below 0.07% w/v for all treatments. Narendranath et al (2001) reported that lactic and acetic acid stress yeast at $\geq$0.2 to 0.8 and $\geq$0.05 to 0.1% w/v, respectively. Therefore, fermentations for all enzyme treatments did not exhibit stress on yeast contributed by high organic acid concentrations.

3.3.2.3 Ethanol Yields and Fermentation Efficiency

Ethanol yields were the highest for 0.5X GSHE and 1X GA treatment (Table 3.6). The lowest ethanol yields (179 L/tonne) were obtained for treatment with no enzyme. Control treatment resulted in 4 to 9% lower ethanol yields compared to treatment containing only GSHE or a combination of GSHE and GA (Table 3.6). This can be attributed to osmotic stress exerted on yeast due to high glucose concentrations observed for control treatment during fermentation. Ethanol yields were different between 0 and 0.5X GSHE levels for 0, 0.5 and 1X GA treatments, but no differences were observed between 0.5 and 1X GSHE levels (Table 3.6). Ethanol yields increased by 217, 211 and 224 L/tonne when GSHE levels increased from 0 to 0.5X for 0, 0.5 and 1X GA treatments, respectively. For fixed GSHE treatments, ethanol yields were different for 0 and 0.5X GA levels, but no differences were observed between 0.5 and 1X GA levels. Differences of 19, 13 and 13 L/tonne were observed when GA increased from 0 to 0.5X for 0, 0.5

and 1X GSHE treatments, respectively.

Fermentation efficiency (92%) was the highest for 0.5X GSHE and 1X GA treatment. Control treatment resulted in 3 to 8% lower fermentation efficiency compared to treatments with GSHE or combination of GSHE and GA. Treatments with no enzyme, 0.5 or 1X GA only resulted in the lowest fermentation efficiencies, 39, 44 and 43%, respectively (Table 3.6). For 0, 0.5 and 1X GA treatments, increasing GSHE levels from 0 to 0.5X increased fermentation efficiencies by 46 to 49% while no differences were observed when GSHE levels further increased from 0.5 to 1X (Table 3.6). For treatments with fixed GSHE, differences were observed in fermentation efficiencies between 0 and 0.5X GA levels, but no differences were observed between 0.5 and 1X GA levels.

3.3.2.4 Residual Starch

Highest residual starch (>64% w/w) was obtained for treatment with no GSHE (Table 3.6). For 0, 0.5 and 1X GA treatments, increasing GSHE levels from 0 to 0.5X reduced residual starch by 36 to 38% w/w (Table 3.6). For 0 GA treatments, further increasing GSHE levels from 0.5 to 1X reduced residual starch by 8.7% w/w. However for 0.5 and 1X GA treatments, increasing GSHE from 0.5 to 1X did not reduce residual starch. Although increasing GSHE from 0.5 to 1X did not reduce residual starch levels, higher residual glucose levels were obtained for treatments with 1X GSHE compared to treatments with 0.5X GSHE (Table 3.6 and Figure 3.6). This indicated loss in yeast fermentation performance resulting in higher glucose remaining unconverted to ethanol at the end of fermentation. Similar residual starch contents were obtained for treatments with 1X GSHE, 0.5X GSHE and 1X GA, and 1X GSHE and 1X GA (Table 3.6). Control treatment resulted in 31.8% w/w residual starch which was similar to residual starch levels (31.3% w/w) reported by Shihadeh (2008) for ground corn fermentation at 30% solids using GSHE with no separate liquefaction. Our observations for residual starch levels were higher than residual starch levels suggested by Lewis (2007). They reported residual starch levels of above 9% w/w at 30% solids and 27% w/w at 40% solids.

3.3.3 Effects of GSHE Alpha-amylase and Glucoamylase Enzyme Loadings

3.3.3.1 Fermentation Profiles

Increasing GA levels from 0 to 0.5X increased peak glucose concentrations by 4.60, 3.90 and 3.68% w/v for 0, 1 and 2X GSHE AA treatments, respectively (Table 3.7). Further increasing GA levels from 0.5 to 1X led to smaller increase (1.10 to 1.95% w/v) in peak glucose concentrations; however, no differences were observed. Higher GSHE AA levels resulted in higher peak glucose concentrations for 0 and 1X GA treatments. Differences were observed in peak glucose concentrations between 0 and 1X GSHE AA levels for 1X GA treatments. While increasing GSHE AA from 0 to 2X, peak glucose concentrations increased by 0.66 and 1.04% w/v for 0 and 1X GA treatments, respectively.

Control treatment resulted in highest peak glucose (13.82% v/v) (Table 3.7 and Figure 3.7). Treatment with no enzyme resulted in the lowest peak glucose (3.64% w/v). Glucose concentrations were less than 10% w/v (below inhibitory levels, $\geq$ 12% w/v) for GSHE AA treatments (Table 3.7). Glucose concentrations reduced to less than 0.4% w/v within 24 hr for all treatments except control. The rate of glucose consumption was the lowest for control treatment with 1.95% w/v residual glucose (Figure 3.7). Glucose accumulation after 48 hr occurred due to reduced glucose consumption by yeast. For GSHE AA treatments, residual glucose concentrations were below 0.40% w/v (Table 3.8 and Figure 3.9). Treatment with 2X GSHE AA and 1X GA resulted in higher residual glucose (0.39% w/v) among GSHE AA treatments.

Final ethanol concentrations ranged from 8.3 to 18.7% v/v for all treatments (Table 3.7). The highest final ethanol concentrations (18.7% v/v) were obtained for 2X GSHE AA and 1X GA

Table 3.7. Ethanol, glucose and glycerol concentrations with fermentation rates for GSHE AA and GA treatments at 35% solids (means of three observations ±1 standard deviation)

Treatment		Process parameters			
		Final Ethanol[a,b] (% v/v)	Peak Glucose[a,b] (% w/v)	Final Glycerol[a,b] (% w/v)	Fermentation Rates[a,b] (% v/v/hr)
0 GA	0 GSHE AA	8.3±0.4 f	3.64±0.10 f	0.43±0.04 h	0.56±0.00 e
	1X GSHE AA	9.5±0.2 e	3.95±0.06 ef	0.50±0.02 g	0.58±0.00 cde
	2X GSHE AA	12.0±0.6 c	4.30±0.19 e	0.63±0.00 e	0.62±0.02 cd
0.5X GA	0 GSHE AA	10.0±0.6 de	8.24±0.02 d	0.70±0.01 d	0.57±0.01 de
	1X GSHE AA	17.0±0.3 b	7.85±0.12 d	0.90±0.02 c	0.70±0.02 b
	2X GSHE AA	18.5±0.4 a	7.98±0.39 d	0.89±0.03 c	0.80±0.07 a
1X GA	0 GSHE AA	10.5±0.1 d	7.91±0.08 d	0.59±0.02 f	0.62±0.02 c
	1X GSHE AA	18.4±0.3 a	9.80±0.51 b	0.95±0.05 b	0.74±0.01 b
	2X GSHE AA	18.7±0.3 a	8.95±0.20 c	0.90±0.00 c	0.74±0.01 b
	Control*	18.3±0.1 c	13.82±0.24 a	1.21±0.04 a	0.47±0.03 f

[a] Means corresponded to the same letter in the same column are similar ($p<0.05$).

[b] Fischer's LSD for ethanol, peak glucose, final glycerol and fermentation rates were 0.64% v/v, 0.39% w/v, 0.04% w/v and 0.05% v/v/hr, respectively.

* Control treatment: 35% solids, conventional dry grind process.

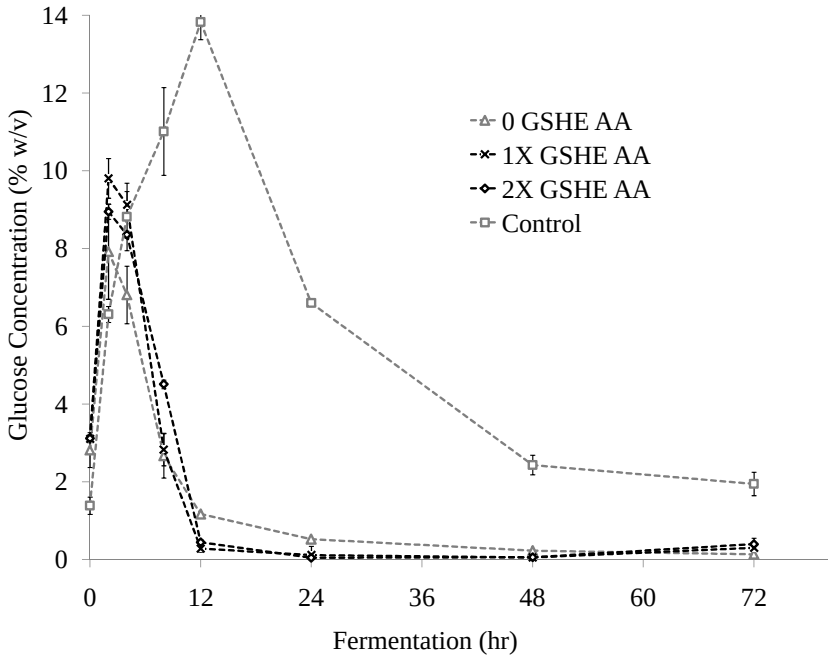

Figure 3.7. Glucose concentrations for GSHE AA process treatments at 1X GA level and 35% solids (means of three observations). Error bars are ±1 standard deviation.

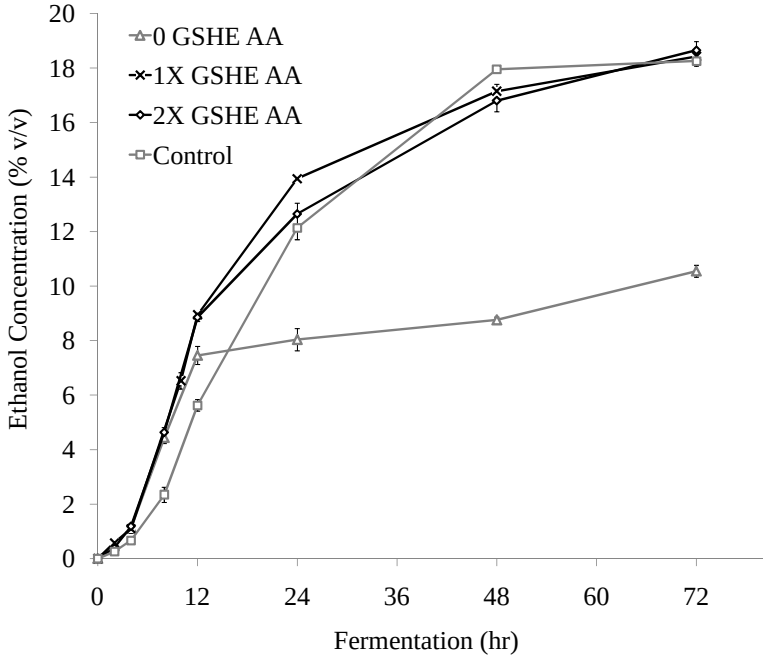

Figure 3.8. Ethanol concentrations for GSHE AA process treatments at 1X GA level and 35% solids (means of three observations). Error bars are ±1 standard deviation.

Table 3.8. Fermentation efficiencies, ethanol yields, residual glucose and residual starch for GSHE AA and GA treatments at 35% solids (means ±1 standard deviation of three observations)

Treatment		Process parameters			
		Fermentation[a,b] Efficiency[a,b] (%)	Ethanol[a,c] Yields[a,c] (L/tonne)	Residual[a,d] Starch[a,d] (% w/w)	Residual Glucose (% w/v)
0 GA	0 GSHE AA	40±1.93 g	182±9 g	73.8±1.0 a	0.07±0.03 cd
	1X GSHE AA	45±1.24 f	202±6 f	65.3±1.1 c	0.05±0.02 d
	2X GSHE AA	56±2.82 d	256±13 d	50.9±1.0 d	0.11±0.00 cd
0.5X GA	0 GSHE AA	48±2.38 e	217±11 ef	69.9±1.0 b	0.10±0.01 cd
	1X GSHE AA	79±1.81 c	360±8 c	36.6±2.3 e	0.30±0.42 bc
	2X GSHE AA	88±1.55 a	396±7 ab	27.4±1.0 h	0.12±0.06 cd
1X GA	0 GSHE AA	50±0.69 e	225±3 e	63.7±1.0 c	0.13±0.01 cd
	1X GSHE AA	88±1.47 a	402±7 a	33.7±3.5 f	0.30±0.02 bc
	2X GSHE AA	89±1.57 a	405±7 a	31.4±1.3 g	0.39±0.16 b
	Contol*	84±0.53 b	381±2 b	33.1±0.5 fg	1.95±0.22 a

[a] Means corresponded to the same letter in the same column are similar ($p<0.05$).
[b] Fischer's LSD for fermentation efficiency, ethanol yield, residual glucose and residual starch were 3%, 14 L/tonne, 0.25% w/v and 2% w/w, respectively.
* Control treatment: 35% solids, conventional dry grind process.

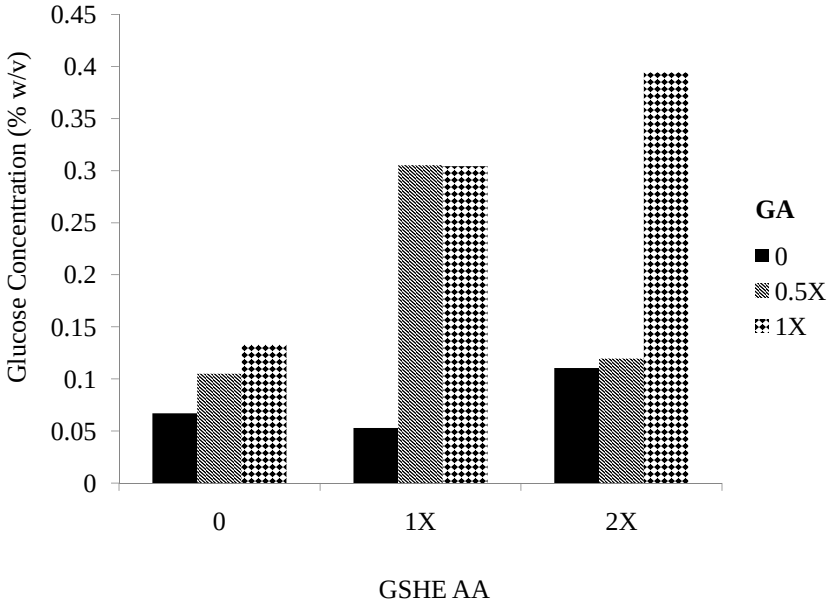

Figure 3.9. Residual glucose concentrations for GSHE AA process treatments (means of three observations).

treatment (Figure 3.8). Treatment with no enzyme resulted in the lowest final ethanol concentrations (8.3% v/v) (Table 3.7 and Figure 3.10). Among treatments, combinations of GSHE AA and GA resulted in 5.0 to 10.4% v/v higher ethanol concentrations than treatments with GSHE AA or GA only. Control treatment (18.3% v/v) resulted in 0.4% v/v lower final ethanol concentrations compared to treatments with 2X GSHE AA and 1X GA but no differences were detected (Table 3.7). For 0, 0.5 and 1X GA treatments, increasing GSHE AA levels from 0 to 1X increased final ethanol concentrations by 1.2, 7.0 and 7.9% v/v, respectively (Table 3.7). Similarly, increasing GSHE AA levels from 1 to 2X increased final ethanol concentrations by 2.5 and 1.5% v/v for 0 and 0.5X GA treatments, respectively. For 1X GA treatment, increasing GSHE AA from 1 to 2X did not affect final ethanol concentrations. For fixed GSHE AA treatments, differences were observed in final ethanol concentrations between 0 and 0.5X GA levels (Table 3.7); a difference of 1.7, 7.5 and 6.5% v/v was obtained in final ethanol concentrations for 0, 1 and 2X GSHE AA treatments, respectively. Further increasing GA levels from 0.5 to 1X did not affect final ethanol concentrations for 0 and 2X GSHE AA treatments, but

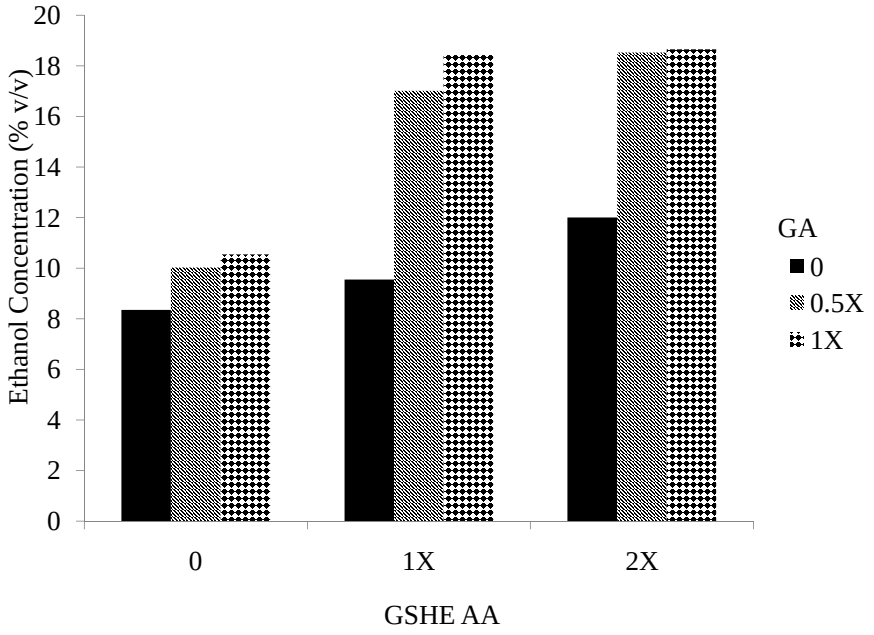

Figure 3.10. Final ethanol concentrations for GSHE AA process treatments (means of three observations).

for 1X GSHE AA treatments, differences were observed between 0.5 and 1X GA levels (Table 3.7). At high GSHE AA levels (2X), increasing GA levels beyond 0.5X did not improve final ethanol concentrations. High ethanol concentrations (>18% v/v) produced for 0.5X GA and 2X GSHE AA treatment, stressed yeast and affected the fermentation performance. This limited any further increase in ethanol concentrations although GA concentrations increased from 0.5 to 1X.

Compared to control, all GSHE AA treatments resulted in 0.08 to 0.32% v/v/hr higher fermentation rates (Table 3.7). Highest fermentation rates were obtained for 2X GSHE AA and 0.5X GA treatment. At fixed GA levels, increasing GSHE AA levels from 0 to 2X resulted in increased fermentation rates (Table 3.7). At fixed GSHE AA levels, increasing GA levels from 0 to 1X increased fermentation rates by 0.12 to 0.17% v/v/hr.

3.3.3.2 Metabolite Profiles

Final glycerol concentration was the highest for control treatment (1.21% w/v) (Table 3.7). For all treatments, glycerol levels were within or less than the range typical for dry grind ethanol

plants (1.2 to 1.5% w/v, Russell 2003).

Final lactic acid concentrations remained below 0.07% w/v for all treatments. All treatments resulted in lactic and acetic acid concentrations below inhibitory levels (>0.05 to 0.1% w/v for acetic acid and >0.2 to 0.8% w/v for lactic acid, Maiorella et al 1983; Narendranath et al 2001). Therefore, fermentations did not experience any yeast stress contributed by high organic acid concentrations.

3.3.3.3 Ethanol Yields and Fermentation Efficiency

Among all treatments, ethanol yields and fermentation efficiencies were the highest for 2X GSHE AA and 1X GA treatment (Table 3.8). Lowest ethanol yields (182 L/tonne) and fermentation efficiencies (40%) were obtained for treatment with no enzyme. Control treatment resulted in lower ethanol yields (a difference of 17 to 23 L/tonne) and fermentation efficiencies (a difference of 4 to 5%) compared to treatments with 2X GSHE AA and 1X GA, 0.5X GA and 2X GSHE AA, and, 1X GA and 1X GSHE AA (Table 3.8). Differences were observed in fermentation efficiencies and ethanol yields between 0 and 1X GSHE AA levels for 0, 0.5 and 1X GA treatments. Similarly, differences were observed between 1 and 2X GSHE AA levels for 0 and 0.5X GA treatments, but for 1X GA treatments, no differences were observed (Table 3.8). When GSHE AA levels increased from 0 to 1X, fermentation efficiencies and ethanol yields increased by 5 to 38% and by 20 to 177 L/tonne, respectively. When GSHE AA levels were further increased from 1 to 2X, fermentation efficiencies and ethanol yields increased by 9 to 11% and 36 to 54 L/tonne for 0 and 0.5X GA treatments, respectively. For fixed GSHE AA treatments, differences were observed in ethanol yields and fermentation efficiencies between 0 and 0.5X GA levels. No differences were observed in fermentation efficiencies and ethanol yields between 0.5 and 1X GA levels for 0 and 2X GSHE AA treatments, but differences were observed between 0.5 and 1X GA level for 1X GSHE AA treatments (Table 3.8). These results were in agreement with the trends observed for final ethanol concentrations.

3.3.3.4 Residual Starch

Among treatments, highest residual starch content was obtained for the no enzyme treatment (0 GA and 0 GSHE AA) (Table 3.8). Lowest residual starch was obtained for 2X GSHE AA and 0.5X GA (27.4% w/w). When accounted for residual glucose, 2X GSHE AA and 1X GA treatments resulted in lowest residual starch (18.93% w/w). Increasing GSHE AA levels resulted in reduced residual starch levels. Differences were observed in residual starch content between 0, 1 and 2X GSHE AA levels for fixed GA treatments. Increasing GSHE AA from 0 to 1X resulted in 8.5, 33.3 and 30.0% reduction in residual starch content for 0, 0.5 and 1X GA treatments, respectively. Similarly, further increasing GSHE AA levels from 1 to 2X reduced residual starch content by 14.4, 9.2 and 2.3% w/w for 0, 0.5 and 1X GA treatments, respectively. From these results, we can conclude that increased GSHE AA levels resulted in reduced residual starch for 0, 0,5 and 1X GA treatments.

3.4 Conclusions

Higher ethanol yields using the modified process permitted use of higher slurry solids (>33%). Use of low temperature liquefaction and phytase incubation in the modified process resulted in lower slurry viscosities. Higher final ethanol concentrations, fermentation rates and lower glucose concentrations were obtained using the modified process resulting in reduced glycerol levels and higher fermentation efficiencies. Employing lower liquefaction temperatures with GSHE and phytase addition would reduce overall energy cost in the process while maintaining high ethanol yields.

GSHE and GA concentrations had effects on fermentation including final ethanol concentrations and ethanol yields. Treatment with 0.5X GSHE and 1X GA resulted in the highest fermentation efficiencies and ethanol yields while reducing GSHE dose by 50%. Increasing GSHE levels from 0.5 to 1X at a fixed GA dose did not improve ethanol yields and fermentation efficiencies, rendering 0.5X as the optimum GSHE levels for fermentations at 35% solids.

Increasing GSHE AA and GA levels improved fermentation efficiencies and ethanol yields. Among all treatments, 2X GSHE AA and 1X GA treatment resulted in the highest fermentation efficiencies and ethanol yields. Liquefaction and saccharification produced by GSHE AA resulted in lower glucose concentrations (<10% w/v). Among GSHE and GSHE AA treatments, GSHE AA resulted in lower fermentation efficiencies and ethanol yields.

Chapter 4

Effects of Nitrogen Source and Dose During High Solids Fermentation

4.1 Introduction

Yeast (*Saccharomyces cerevisiae*) used in fuel ethanol plants require nutrients such as water, fermentable carbohydrates, lipids, oxygen, nitrogen and inorganic ions. In fermentation media, yeast cannot utilize nitrogen in the form of polypeptides larger than tripeptides (Russell 2003). Therefore, nitrogen sources such as organic nitrogen (glutamine, asparagine, tryptophan, urea), inorganic nitrogen (ammonium sulfate, diammonium phosphate, ammonia) or combinations of both need to be added to fermentation media. Not all nitrogen sources contribute to yeast growth equally. Some nitrogen sources such as glutamine, asparagine and ammonia are preferred by yeast more than proline and urea (Schure et al 2000). Yeast preferentially select a nitrogen source by a mechanism known as nitrogen catabolite repression (NCR) which is defined as the physiological response of inactivating gene expression in reaction to the nitrogen source present in the medium (Cooper 1982). When preferable nitrogen sources are present in growth medium, gene transcription involved in the utilization of poor nitrogen sources is repressed and their corresponding products are inactivated and degraded (Wiame et al 1985; Magasanik 1992).

High solids fermentations result in stuck and sluggish fermentations (fermentation that has stopped before all the sugars are consumed) due to lack of nutrients for yeast growth in a stressful environment (Bafrncova et al 1999). Free amino nitrogen (FAN) is the major limiting nutrient in high solids fermentation (Thomas and Ingledew 1990). FAN is a measure of individual amino acid or small peptides (di or tripeptides) that can be utilized by yeast. It plays an important role in controlling fermentation rates and alcohol and volatile compounds production (Bely et al 1990; Thomas and Ingledew 1990; Mendeis-Ferreira et al 2004) by enhancing sugar transport through

glycolysis pathway (Salmon 1989). Low levels of nitrogen were associated with lower biomass yield (Bisson 1991; Spayd et al 1995; Manginot et al 1998) and low cellular activity (Bely et al 1990). This was attributed to an arrest in protein synthesis (Salmon 1989). When protein synthesis is inhibited, hexose transport systems are irreversibly inactivated by a catabolite-inactivation process requiring the utilization of a fermentable substrate, glucose (Busturia and Lagunas 1986). This inactivation is responsible for the decrease in fermentation observed in ammonium-starved yeasts (Lagunas et al 1982).

In addition to nitrogen source, nitrogen dose plays an important role in fermentation performance. Taillandier et al (2007) showed that high nitrogen dose (0.71 g/L) improved average sugar consumption rates. While at higher nitrogen dose (1.2 g/L), average sugar consumption rates did not improve. Casey et al (1983) and Thomas et al (1993) reported that yeast fermentative capacity is higher under nitrogen limiting condition than in nitrogen excess condition. Nitrogen requirements of yeast not only depend on the strain (Jiranek et al 1995) but also on conditions of fermentation media (glucose and oxygen concentrations, temperature) (Valero et al 2003). Therefore, it is important to determine optimal nitrogen requirements in high solids fermentations to achieve maximum fermentation efficiencies. Effects of nitrogen source and dose have been studied at $<33\%$ solids but no studies have been published at $>35\%$ solids fermentations. Objectives of this work were to:

1. Evaluate the effects of nitrogen source during high solids fermentations.
2. Determine interaction effects of nitrogen source and nitrogen dose on high solids fermentations.

4.2 Materials and Methods

4.2.1 Materials

Yellow dent corn was grown during 2007 at the Agricultural and Biological Engineering Research Farm, University of Illinois at Urbana-Champaign. Moisture content was determined

using a standard two stage convection oven method (AACC 2002). Corn slurry was prepared using deionized water to achieve respective slurry solids. Slurry pH was adjusted using 10*N* sulfuric acid (Ricca Chemical, Arlington, TX). Slurry was liquefied using Maxaliq^{TM} One (1,4-alpha-D-glucan glucanohydrolase phosphoric monoester hydrolase, Genencor, Rochester, NY), a formulation of alpha-amylase derived from *Bacillus licheniformis* and phytase derived from *Trichoderma reesei*. The enzyme pH optimum was 5.7.

During SSF, active dry yeast (*Saccharomyces cerevisiae*) (Ethanol Red, Fermentis, Lesaffre Yeast, Milwaukee, WI) was used. Yeast inoculate was prepared by mixing 5 g dry yeast and 25 mL distilled water and incubated at 32°C for 20 min at 120 rpm in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ). Ammonium sulfate (ACS grade) and urea (99.6% ACS grade) were obtained from Fischer Scientific (Fair Lawn, NJ). L-Glutamine was obtained from Sigma (St. Louis, MO). A fungal endoprotease (GC 212, Genencor International, Rochester, NY) was used. The endoprotease was obtained by controlled fermentation of selected strain of *Aspergillus niger*. Its activity was 2000 SAPU/g (SAPU =spectrophotometric acid protease units). The endoprotease hydrolyzed corn protein maxtrix surrounding the starch granules into FAN. Peptide bonds randomly along the protein chain at low pH. Zein (corn protein bodies) were not affected by the endoprotease.

Enzymes used during SSF were GSHE (Stargen 001, Genencor, Rochester, NY) and glucoamylase (1,4-alpha-D-glucan hydrolase; GC 147, Genencor, Rochester, NY). GSHE, a mixture of alpha-amylase from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger* had an activity $\geq$456 GSHU/g (GSHU =granular starch hydrolyzing units).

For residual starch analysis, 0.4 M HCl (Fisher Scientific, Waltham, MA) prepared by adding 33.1 mL concentrated hydrochoric acid to 750 mL deionized (DI) water, Na_2CO_3 (Acros Organics, Geel, Belgium) (21.2% w/v, prepared by dissolving 21.2 g $Na_2CO_3H_20$ in 80 mL DI water), D-gluocse (D(+)-glucose anhydrous) (Acros Organics, Geel, Belgium), glucose determination reagent (glucose oxidase and peroxidase, GOPOD, Megazyme, Bray, Co. Wicklow, Ireland), standard glucose solutions: 0.25, 0.5, 0.75 and 1 mg/mL, and starch (Hylon V Corn

Starch, National Starch Food Innovation, Bridgewater, NJ) were used.

4.2.2 Effects of Nitrogen Source

4.2.2.1 Dry Grind Process

Each corn sample was cleaned by sieving over a standard 12/64 in (4.8 mm) sieve to remove broken corn and foreign material (BCFM). Cleaned corn was stored at 4°C. Corn (1 kg) was ground at 500 rpm to pass through 0.5 mm hole sieve with a laboratory hammer mill (1100 W, model MHM4, Glen Mills Inc., Clifton, NJ). Moisture content of ground corn was determined using a standard two stage oven method (AACC 2002). Milled corn (100 g db) was mixed with deionized water to obtain 35% slurry solids in 500 mL flasks. Slurry pH was adjusted to 5.7 using 10*N* sulfuric acid. Slurry was liquefied at 55°C using 0.3 μL Maxaliq One/g db corn for 90 min. Subsequent to liquefaction, corn slurry pH was set at 4.0.

Three nitrogen sources and an endoprotease were added respectively for the process treatments. GSHE (1.25 μL/g db corn), glucoamylase (0.5 μL/g db corn) and yeast inoculate (0.02 mL/g db corn) were added to corn slurry. SSF was conducted at 32°C for 72 hr with constant agitation at 120 rpm. Fermentations were carried out in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ). Fermentation samples (1 mL) were taken from the slurry at 2, 4, 8, 12, 24, 48 and 72 hr for metabolite analysis. After 72 hr, slurry volume was recorded using a 500 mL graduated cylinder. This slurry volume was multiplied by ethanol concentrations measured using HPLC (Column: Aminex HPX-87H organic acid, BioRad, Hercules, Waters Corp., Milford, MA) to obtain total ethanol volume. To evaporate ethanol, slurry was heated to 90°C and maintained for 2 hr . Solid material left after ethanol evaporation was dried in open aluminium cans for 24 hr in a convective oven at 49°C to form DDGS.

4.2.2.2 HPLC Analysis

Fermentation metabolite profiles were obtained by analyzing 1 mL slurry samples. Samples were centrifuged for 6 min at 11,000x*g* (Model 5425, Eppendorf, Westbury, NY).

Supernatant was passed through a 0.2 μm syringe filter into 0.2 mL vial insert. Filtered supernatant liquid was analyzed using HPLC (Column: Aminex HPX-87H organic acid, BioRad, Hercules, Waters Corp., Milford, MA) to determine ethanol, glucose, maltose, maltotriose, glycerol, lactic acid and acetic acid concentrations.

4.2.2.3 Fermentation Rates

Fermentation rates were defined as the overall percent ethanol change (% v/v/hr) during the initial 12 hr of fermentation. A regression line was fit to ethanol concentration data up to 12 hr fermentation time and the slope used to obtain initial fermentation rates.

4.2.2.4 Ethanol Yields and Fermentation Efficiency

Starch and moisture contents of whole corn were analyzed in triplicate using a residual starch assay (Section 3.2.3) and convection oven methods (AACC 2002), respectively. Mean moisture content was 10.8% (wb) and mean starch content was 69.8% (db). To determine theoretical ethanol yields, 100% starch conversion to glucose and 100% glucose conversion to ethanol was assumed. Starch in 100 g (db) corn was multiplied by 1.11 g glucose/g starch and 0.51 g ethanol/g glucose (obtained based on molecular weight ratios and stoichiometric coefficients of substrate and product) to give theoretical ethanol (g). Total ethanol obtained, based on 69.8% (db) starch in 100 g (db) corn, was divided by ethanol density (0.789 g/mL) to obtain ethanol volume. Ratio of total volume of ethanol produced and weight of ground corn added was reported as the theoretical ethanol yield (L/tonne). For calculating actual ethanol yield (L/tonne), total volume of ethanol produced was obtained by multiplying final ethanol concentrations with measured fermented slurry volume at 72 hr and dividing by weight of corn flour added. Fermentation efficiency was calculated by dividing the actual ethanol yield by the theoretical ethanol yield.

4.2.2.5 Experimental Design

Urea, ammonium sulfate ($(NH_4)_2SO_4$), glutamine and protease (GC 212, Genencor, Rochester, NY) (Table 4.1), were used to study the effects of nitrogen source and protease on yeast fermentation performance at 35% (db) slurry solids concentration. Urea was used as a control treatment. Nitrogen sources were added to fermentation media to achieve a fixed nitrogen dose of 1 mg N/g (db) corn suggested by Mendeis-Ferreira et al (2004). Amounts of urea, glutamine and $(NH_4)_2SO_4$ added were calculated based on their respective molecular weights and moles of nitrogen in a molecule. Resulting amounts of nitrogen sources added to fermentation media were 2.16 mg urea/g (db) corn, 4.77 mg $(NH_4)_2SO_4$/g (db) corn and 2.63 mg glutamine/g (db) corn. Protease (0.71 mg/g (db) corn) added for the fourth treatment was 1.5 times the dose recommended by the manufacturer. This dose (1.5X) also corresponded to one of the protease levels used by Wang et al (2009) for determining effects of exo and endoproteases using GSHE process. Wang et al (2009) used three levels of protease, 0.59, 1.18 and 2.36 μL/100 g (db) corn for their study. Amount of maximum FAN generated using 0.71 mg protease during first 24 hr was 0.52 mg FAN/g db corn.

Temperature, enzyme dose, yeast inoculate, slurry pH and solids content were kept constant. Each treatment was replicated three times. The experiment was arranged in a complete randomized design with a total of 12 observations. For each treatment, fermentation profiles were plotted; ethanol yields and fermentation efficiencies were determined. Analysis of variance (ANOVA) and Fischer's least significant difference with a significance level of $p<0.05$ was used to compare ethanol concentrations, fermentation rates, fermentation efficiencies, ethanol yields and peak glucose concentrations among treatments and control samples.

4.2.3 Effects of Nitrogen Source and Nitrogen Dose

We evaluated effects of urea and protease dose and slurry solids content (35 and 40% db) on fermentation efficiency and ethanol concentrations. Levels for urea were chosen to achieve optimum nitrogen doses recommended by Taillandier et al (2007) (0.71 mg N/g glucose),

Table 4.1. Three nitrogen sources addition rates to achieve 1 mg N/g db corn and protease addition at 0.71 mg/g db corn.

Treatment	Nitrogen source	Amount added (mg/g (db) corn)
N1	Urea (Control)	2.16
N2	$(NH_4)_2SO_4$	4.77
N3	Glutamine	2.63
N4	Protease	0.71

Mendeis-Ferreira et al (2004) (1.3 mg N/g glucose) and Saita and Slaughter (1984) (3.1 mg N/g glucose). These three levels used for urea were equivalent to 1.51, 2.16 and 4.32 mg urea/g (db) corn. Amounts of protease were chosen based on levels recommended by Wang et al (2009) and the enzyme manufacturer. Protease levels used in the experiment were 0.50, 0.71 and 1.42 mg/g (db) corn. The three variables, nitrogen source, nitrogen dose and solids content, were combined resulting in 12 treatments (Table 4.2).

For all treatments, liquefaction was conducted at 55°C, pH 5.7 for 90 min using 0.30 and 0.45 μL Maxaliq One/g (db) corn for 35 and 40% solids in 500 mL flasks, respectively. Urea and protease were added at three levels for the 12 treatments (Table 4.2). GSHE (1.25 μL/g (db) corn), glucoamylase (0.5 μL/g (db) corn) and yeast inoculate (0.02 mL/g (db) corn) were added to corn slurry. SSF was conducted at 32°C for 72 hr with constant agitation at 120 rpm in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ). Fermented slurry samples (1 mL) were taken at 2, 4, 8, 12, 24, 48 and 72 hr for metabolite analysis. Beer was analyzed for fermentation efficiency and metabolite (ethanol, glucose, acetic acid and lactic acid) concentrations as described in section 4.2.2. After 72 hr fermentation, ethanol was evaporated for 2 hr at 90°C. After oven drying for 24 hr, solid material left was analyzed for residual starch.

4.2.3.1 Residual Starch Analysis

Residual starch was measured based on an acid hydrolysis method (Ebell 1969). Fermented slurry solids were dried overnight at 49°C, ground in a coffee mill (Black and Decker,

Table 4.2. Urea and protease levels used at 35 and 40% solids to determine effects of nitrogen source and nitrogen dose on high solids fermentation.

Slurry solids (% db)	Nitrogen source	Levels (mg/g (db) corn)		
35	Protease	0.50	0.71	1.42
	Urea	1.51	2.16	4.32
40	Protease	0.50	0.71	1.42
	Urea	1.51	2.16	4.32

Towson, MD) and analyzed for moisture content (AACC 2002). From milled samples, 1 g subsamples along with 1 g glucose and starch standards were weighed in triplicate. Subsamples were transferred to 100 mL autoclave safe glass bottles and 50 mL HCl (0.4M) was added; bottles were autoclaved for 60 min at 126°C. After cooling to 100°C, bottles were placed in an ice water bath for 10 min. From a bottled sample, 2 mL was transferred to a 25 mL centrifuge tube with a serological pipette. To centrifuge tubes, 220 μL Na_2CO_3 was added and mixed vigorously. All samples, including starch and glucose standards, were centrifuged for 5 min at 3000xg. Stillage samples were diluted 8:1 or 16:1 as required while glucose and starch standards were diluted to 40:1. A blank was prepared using 0.1 mL distilled water. Glucose standards (0.1 mL) at 0.25, 0.50, 0.75 and 1.00 mg/mL were prepared in glass tubes. From each diluted sample, 0.1 mL was transferred to a glass tube with screw cap and 3 mL glucose oxidase and peroxidase (GOPOD) reagent was added and vortexed. Tubes were incubated in a 50°C water bath for 20 min. After cooling to room temperature, sample absorbance was read at 510 nm against reagent blank with a spectrophotometer (Helios Model 10 VIS, Thermo Scientific, Needham Heights, MA). An absorbance calibration curve was constructed from the glucose standards. Percent residual starch was determined by multiplying observed glucose concentration by the dilution and glucose recovery factors and dividing by a factor of 1.11.

4.2.3.2 Experimental Design

The experiment was arranged in a completely randomized design (Table 4.2) with a total of 36 observations. Temperature, enzyme dose, yeast inoculate and slurry pH were constant for all treatments. Each process treatment was replicated three times. For each treatment, fermentation profiles were obtained. Residual starch contents, final ethanol concentrations, ethanol yields, fermentation rates and fermentation efficiencies were analyzed. Analysis of variance (ANOVA) and Fischer's least significant difference with a significance level of $p<0.05$ were used to compare mean ethanol concentrations, fermentation rates, fermentation efficiencies and residual starch contents among treatments.

4.2.4 FAN Measurement

An experiment was conducted to determine maximum FAN production possible in protease treatments. Corn slurries at 35 and 40% solids were prepared by mixing 100 g (db) corn with distilled water in 500 mL flasks to achieve 35 and 40% solids. The slurry was liquefied using 0.30 and 0.45 μL Maxaliq One/g (db) corn at 5.7 pH and 55°C for 90 min. After liquefaction, a slurry sample (0 hr) was taken for FAN analysis. Liquefied slurry pH was adjusted to 4.0 using 10*N* sulfuric acid and 1.25 μL GSHE and glucoamylase 0.5 μL glucoamylase/g (db) corn were added. Three levels of protease, as shown in Table 4.2, were added at each 35 and 40% solids for 6 treatments. All treatments were performed in duplicate. Protease incubations were conducted at 32°C for 72 hr with constant agitation at 120 rpm in a shaking water bath (Model DHOD-182, Bellco Glass, Vineland, NJ). Slurry samples were taken at 6, 12, 24, 48 and 72 hr. Samples were centrifuged for 6 min at 11,000x*g*; the supernatant was added to 0.1*N* NaOH in 1:1 volume ratio. Samples were stored at -5°C. FAN was analyzed using the ninhydrin assay according to standard methods (AOAC 1980).

The experiment was arranged in a completely randomized design with 12 replicates. Variables such as temperature, slurry pH, GSHE and GA levels were constant for all 6 treatments. Each process treatment was replicated two times. For each treatment, FAN profiles were

obtained. Analysis of variance (ANOVA) and Fischer's least significant difference with a significance level of $p<0.05$ were used to compare maximum FAN levels produced among treatments.

4.3 Results and Discussion

4.3.1 Effects of Nitrogen Source

Final ethanol concentrations obtained for urea, protease and glutamine treatments were similar (Table 4.3). Ethanol concentrations obtained using protease (18.96% v/v) were higher than those reported by Wang et al (2006) for ground corn fermentation at 32% solids using GSHE (4 μL/g db corn) and an endoprotease (GC106) (2 μL/g db corn). Ammonium sulfate treatment (16.98% v/v) resulted in the lowest final ethanol concentration (Figure 4.1). Our results were in agreement with the findings of Jones and Ingledew (1993). For wheat mash fermentations, they reported that salts of ammonium resulted in lower final ethanol concentrations compared to other nitrogen sources such as urea and yeast extract.

Fermentation rates were highest for glutamine (0.77% v/v/hr) followed by protease treatment (Table 4.4). Ammonium sulfate had the lowest fermentation rate (0.59% v/v/hr) (Figure 4.1). For ammonium sulfate treatment, slurry pH decreased from 4.0 to 2.7 during fermentation which resulted in reduced rate of ethanol production. This result is supported by a study by Parsons et al (1984), which showed that yeast exhibited highest ethanol production rates between 3.2 to 4.0 pH.

There were no differences in peak glucose concentrations (Table 4.3 and Figure 4.2). Residual glucose concentrations (72 hr) were less than 1% w/v indicating complete fermentations (Table 4.3). For glutamine (0.84% w/v) and protease (0.77% w/v), relatively higher residual glucose concentrations (72 hr) were observed (Figure 4.2). Fermentations for ammonium sulfate and urea resulted in lower residual glucose concentrations ($\leq$0.37% w/v).

Glycerol concentrations (0.94 to 1.08% w/v) reached a maximum at 72 hr. No differences

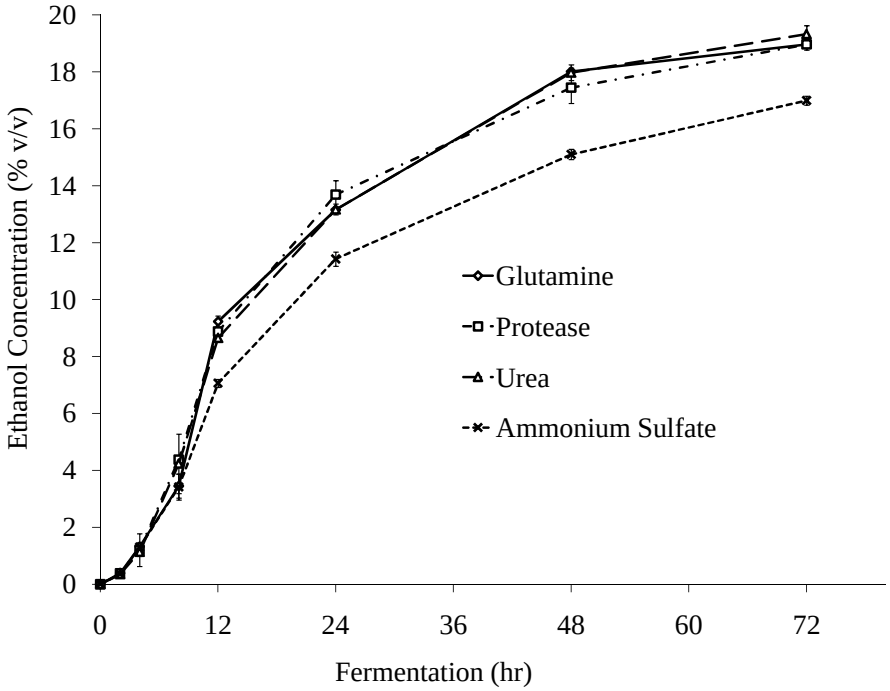

Figure 4.1. Ethanol concentrations for nitrogen treatments at 35% solids. Error bars are ±1 standard deviation.

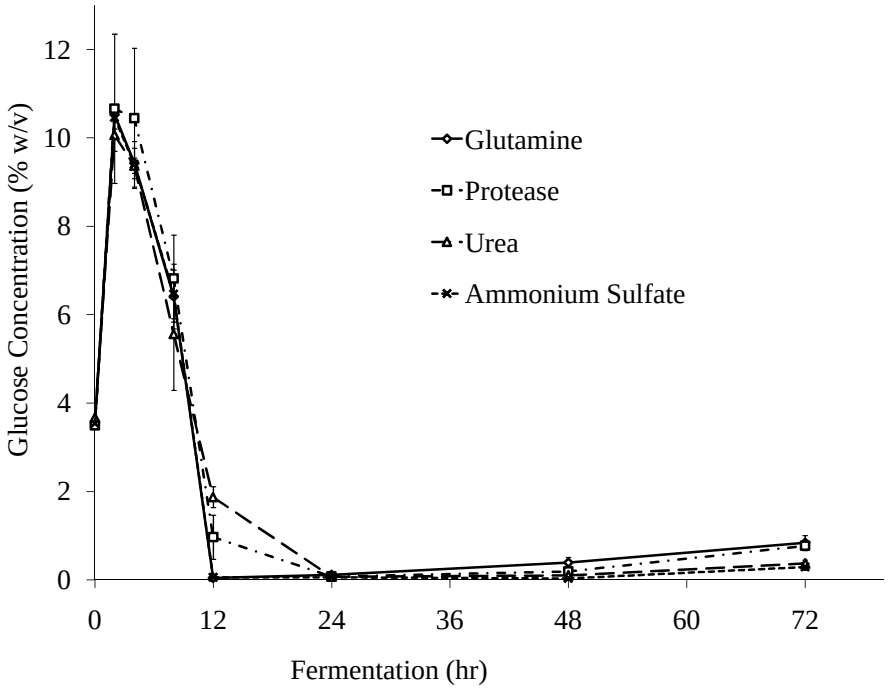

Figure 4.2. Glucose concentrations for nitrogen treatments at 35% solids. Error bars are ±1 standard deviation.

Table 4.3. Ethanol, glucose and glycerol concentrations for four nitrogen treatments at 35% solids (means of three observations).

Treatment	Process parameters			
	Final Ethanol[a,b] (% v/v)	Peak Glucose[a,b] (% w/v)	Residual Glucose[a,b] (% w/v)	Final Glycerol (% w/v)
Urea	19.32 a	10.06 a	0.37 b	1.04 a
Protease	18.96 a	10.65 a	0.77 a	0.96 a
Glutamine	18.96 a	10.52 a	0.84 a	0.94 a
Ammonium Sulfate	16.98 b	10.46 a	0.29 b	1.08 a

[a] Means followed by the same letter in the same column are similar ($p<0.05$).
[b] Fischer's LSD was 0.38% v/v, 1.64% w/v and 0.21% w/v for ethanol, peak glucose and residual glucose concentrations, respectively.

Table 4.4. Fermentation rates and efficiencies with ethanol yields for nitrogen treatments at 35% solids (means of three observations).

Treatment	Process parameters		
	Fermentation Efficiency[a,b](%)	Ethanol Yield[a,b] (L/tonne)	Fermentation Rate[a,b] (% v/v/hr)
Urea	91 a	414 a	0.72 c
Protease	90 a	410 a	0.74 b
Glutamine	88 b	399 b	0.77 a
Ammonium Sulfate	81 c	370 c	0.59 d

[a] Means followed by the same letter in the same column are similar ($p<0.05$).
[b] Fischer's LSD was 2%, 9 L/tonne and 0.02% v/v/hr for fermentation efficiencies, ethanol yields and fermentation rates, respectively.

were observed in final glycerol concentrations among treatments (Table 4.3). For all treatments, lactic and acetic acid concentrations remained below inhibiting concentrations (<0.2 to 0.8% w/v, lactic acid and <0.05 to 0.1% w/v, acetic acid, Narendranath et al 2001).

Fermentation efficiencies were the highest for urea and protease treatments (Table 4.4). Glutamine treatment resulted in lower fermentation efficiency (88%) compared to urea and

protease treatments. The lowest fermentation efficiency was obtained for ammonium sulfate. Ethanol yields followed similar trends as fermentation efficiencies with urea and protease resulting in the highest ethanol yields followed by glutamine (Table 4.4). Ammonium sulfate treatment resulted in the lowest ethanol yield. Ethanol yields (410 L/tonne) obtained using protease (GC 212) were higher than ethanol yields (360 L/tonne) reported by Wang et al (2006) for ground corn fermentations at 32% solids using GSHE (4 μL/g db corn) and endoprotease (GC106) (2 μL/g db corn).

4.3.2 Effect of Nitrogen Source and Nitrogen Dose

Mean final ethanol concentrations were similar for 35 and 40% solids across all urea and protease levels (Table 4.5). Differences were observed in final ethanol concentrations for urea and protease across all levels at 35 and 40% solids (Table 4.5). For protease treatments at 35% solids, increasing protease dose from 0.50 to 0.71 mg increased final ethanol concentrations from 18.44 to 19.01% v/v; however, no differences were observed at 40% solids (Table 4.5 and Figure 4.3). Further increasing protease levels from 0.71 to 1.42 mg resulted in lower (a difference of 1.92% v/v) final ethanol concentrations at 35% solids. Increasing urea levels from 1.51 to 2.16 mg at 35% solids resulted in similar final ethanol concentrations (Table 4.5). However, further increasing urea levels from 2.16 to 4.32 mg resulted in 0.68% v/v decrease in final ethanol concentrations. High urea (4.32 mg) and protease levels (1.42 mg) reduced final ethanol concentrations. This could be due to excess nitrogen present in fermentation media at high urea and protease levels. Under nitrogen excess condition, yeast fermentative capacity is reduced (Casey et al 1983; Thomas et al 1993). They suggested that there is a reduction in relative carbon flux through the glycolysis pathway in nitrogen excess condition due to consequent decrease in phosphofructokinase (a key regulatory enzyme for glycolysis) and a simultaneous increase in enzyme synthesis for hexose monophosphate pathway.

At 40% solids, similar ethanol concentrations were produced for 1.51 and 2.16 mg urea, and, 2.16 and 4.32 mg urea (Table 4.5). However, differences were observed between 1.51 and

4.32 mg urea treatments. Treatment with 4.32 mg urea resulted in higher final ethanol concentrations. At 40% solids, treatments with 0.50 and 0.71 mg protease produced similar ethanol concentrations (Table 4.5 and Figure 4.3). But differences were observed between 0.71 and 1.42 mg, and 0.50 and 1.42 mg protease levels. Increasing protease levels from 0.71 to 1.42 mg resulted in lower final ethanol concentrations. Urea resulted in higher mean final ethanol concentrations across all levels compared to protease at 35 and 40% solids (Table 4.5).

Initial fermentation rates at 35% solids (over first 12 hr) were higher for protease compared to urea across all levels (Table 4.5). These results were in agreement with results reported by Vidal et al (2010). They also observed that protease resulted in higher fermentation rates compared to urea for corn slurry fermentations at 25% solids. However, at 40% solids, urea resulted in higher fermentation rates compared to protease. Higher solids reduced enzyme performance resulting in lower fermentation rates (Shihadeh 2008). Among treatments with three urea levels at 35% solids, 2.16 mg urea treatment resulted in highest fermentation rates (0.741% v/v/hr). For protease treatments at 35% solids, 0.71 mg protease resulted in highest fermentation rates (0.771% v/v/hr). At 40% solids increasing urea and protease levels did not improve fermentation rates (Table 4.5).

Increasing urea and protease levels at 35 and 40% solids had an effect on peak glucose concentrations (Table 4.5). At 40% solids, increasing protease levels from 0.50 to 0.71 mg resulted in higher peak glucose concentrations (Table 4.5 and Figure 4.5). Treatment with 40% solids and 4.32 mg urea resulted in the highest peak glucose concentrations (16.25% w/v) (Table 4.5 and Figure 4.6). At 40% solids, increasing urea from 1.51 to 2.16 mg and protease levels from 0.50 to 0.71 mg resulted in 1.20 and 1.94% w/v increase in peak glucose concentrations, respectively. However, further increasing urea and protease levels at 40% solids from 2.16 to 4.32 mg and from 0.71 to 1.42 mg increased peak glucose concentrations; however no differences were observed. Peak glucose concentrations increased with increasing solids content from 35 to 40% (Table 4.5). At 35% solids, glucose concentrations ranged from 9.72 to 11.47% w/v and at 40% solids, glucose concentrations ranged from 12.65 to 16.25% w/v. Differences were observed

Table 4.5. Ethanol and peak glucose concentrations with fermentation rates for urea and protease treatments at 35 and 40% solids (means of three observations).

Treatment			Process parameters		
Slurry Solids (% db)	Nitrogen Source	Nitrogen Dose (mg/g (db) corn)	Ethanol[a,b] Concentration (%v/v)	Peak Glucose[a,c] (% w/v)	Fermentation Rates[a,d] (% v/v/hr)
35	Urea	1.51	19.09 ab	11.47 r	0.665 z
		2.16	19.39 a	10.88 s	0.741 x
		4.32	18.71 cd	11.40 rs	0.664 z
		Mean (Urea)[e]	19.06 A	11.25 A	0.690 B
35	Protease	0.50	18.44 de	11.38 rs	0.709 y
		0.71	19.01 bc	9.72 t	0.771 w
		1.42	18.09 e	9.97 t	0.693 y
		Mean (Protease)[e]	18.52 B	10.35 B	0.724 A
		Mean (Solids)[f]	18.79 X	10.79 Y	0.704 X
40	Urea	1.51	18.85 bc	13.92 t	0.686 wx
		2.16	19.01 ab	15.12 rs	0.675 wxy
		4.32	19.17 a	16.25 r	0.695 w
		Mean (Urea)[e]	19.01 A	15.09 A	0.685 A
40	Protease	0.50	18.67 c	12.65 u	0.671 xy
		0.71	18.67 c	14.59 st	0.654 y
		1.42	18.19 d	14.71 st	0.660 y
		Mean (Protease)[e]	18.51 B	13.98 B	0.662 B
		Mean (Solids)[f]	18.82 X	14.31 X	0.673 Y

[a] Means followed by the same letter within a solids content (35 or 40%) in the same column (abc) are similar ($p<0.05$).
[b] Fischer's LSD for ethanol concentrations were 0.36 and 0.29% v/v for 35 and 40% solids, respectively.
[c] Fischer's LSD for peak glucose were 0.54 and 1.20% w/v for 35 and 40% solids, respectively.
[d] Fischer's LSD for fermentation rates were 0.024 and 0.023% v/v/hr for 35 and 40% solids.
[e] Means followed by the same letter within a solids content for urea or protease (AB) in the same column are similar ($p<0.05$).
[f] Means followed by the same letter in the same column (XY) are similar ($p<0.05$).

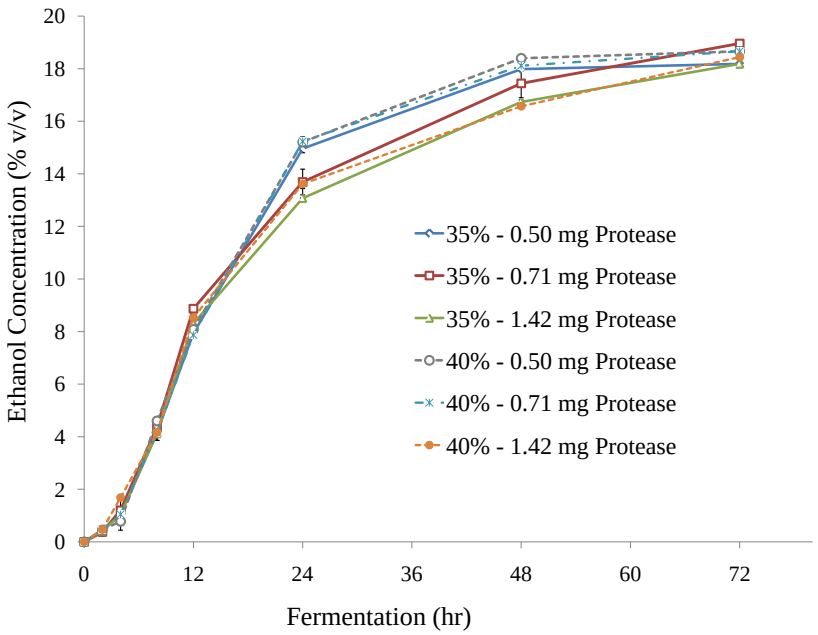

Figure 4.3. Ethanol concentrations for protease treatments at 35 and 40% solids. Error bars are ±1 standard deviation.

in peak glucose concentrations between urea and protease across all levels at 35 and 40% solids. Urea resulted in 0.90 and 1.11% w/v higher peak glucose compared to protease at 35 and 40% solids, respectively.

Highest fermentation efficiencies and ethanol yields were obtained at 35% solids across all urea and protease levels (Table 4.6). For treatments at 35 and 40% solids, differences were observed in fermentation efficiencies and ethanol yields between urea and protease across all levels (Table 4.6). Urea resulted in 2 and 3% higher fermentation efficiencies at 35 and 40% solids, respectively. Similarly, ethanol yields were 11 and 14 L/tonne higher for urea compared to protease across all levels at 35 and 40% solids, respectively. At 35% solids, increasing protease dose from 0.50 to 0.71 mg increased fermentation efficiency and ethanol yields by 3% and 10 L/tonne, respectively (Table 4.6). However, at 40% solids, no differences were observed between 0.50 and 0.71 mg protease loading. Increasing protease loadings from 0.71 to 1.42 mg reduced fermentation efficiencies (a difference of 6 and 3%) and ethanol yields (a difference of 24 and 15 L/tonne) at 35 and 40% solids, respectively (Table 4.6).

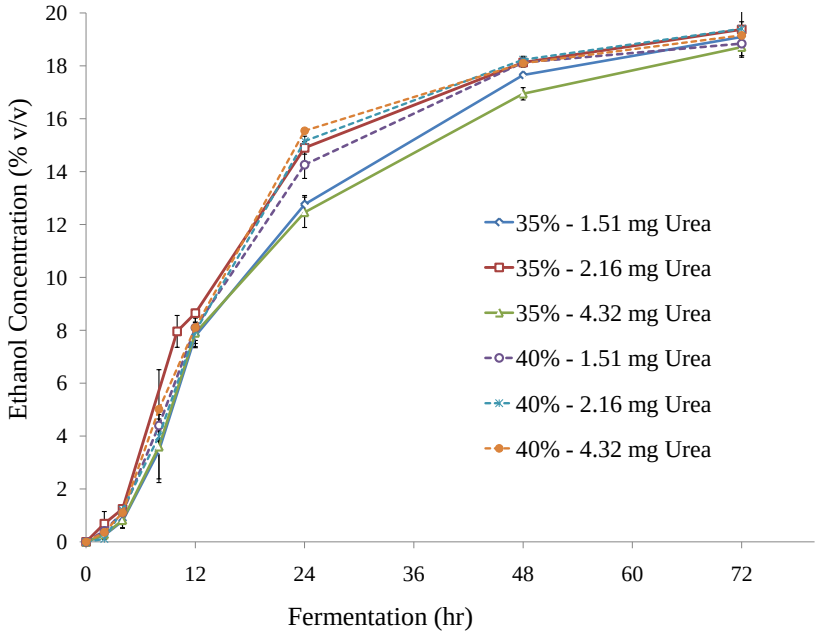

Figure 4.4. Ethanol concentrations for urea treatments at 35 and 40% solids. Error bars are ±1 standard deviation.

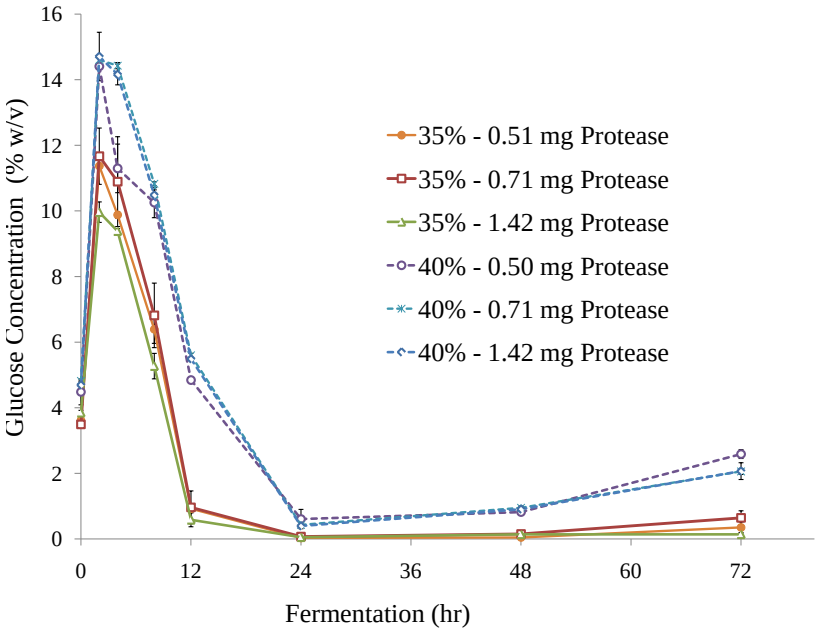

Figure 4.5. Glucose concentrations for protease treatments at 35 and 40% solids. Error bars are ±1 standard deviation.

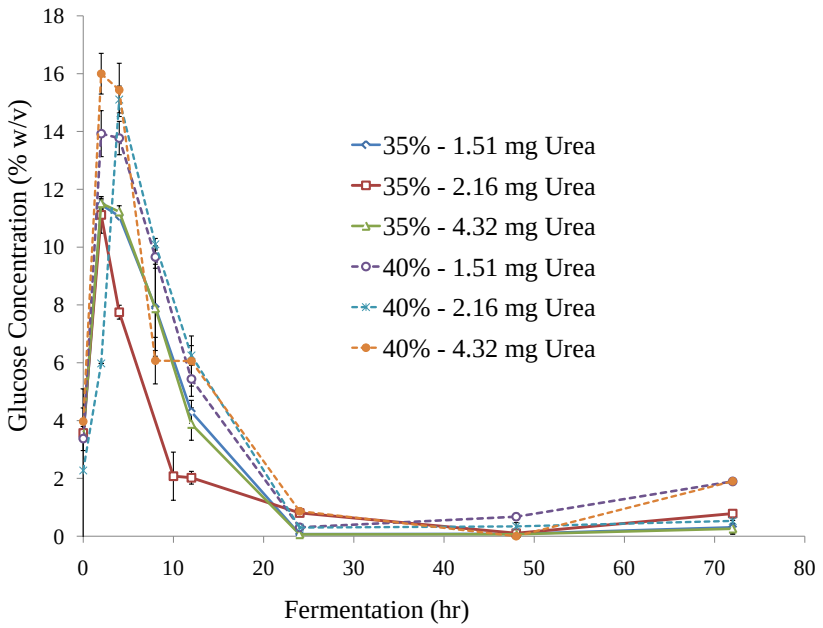

Figure 4.6. Glucose concentrations for urea treatments at 35 and 40% solids. Error bars are ±1 standard deviation.

At 35% solids, increasing urea levels from 1.51 to 2.16 mg increased fermentation efficiencies and ethanol yields by 2% and 12 L/tonne, respectively. However, further increasing urea dose from 2.16 to 4.32 mg at 35% solids reduced fermentation efficiencies and ethanol yields by 3% and 16 L/tonne, respectively (Table 4.6). At 40% solids, no differences were observed in fermentation efficiencies and ethanol yields between 2.16 an 4.32 mg urea levels.

For all urea and protease levels, treatments with 40% solids had 17.5% w/w higher residual starch content compared to treatments with 35% solids(Table 4.6). Higher residual starch at 40% solids might have resulted due to reduced yeast fermentation performance owing to high osmotic stress and ethanol inhibition. At 35% solids, 0.71 mg protease and 2.16 mg urea resulted in lower residual starch compared to other levels of urea and protease. Urea resulted in 2.6 and 9.0% w/w lower residual starch compared to protease at 35 and 40% solids, respectively.

Table 4.6. Fermentation efficiencies, ethanol yields and residual starch for urea and protease treatments at 35 and 40% solids.

Treatment			Process parameters		
Slurry Solids (% db)	Nitrogen Source	Nitrogen (mg/g (db) corn)	Fermentation Efficiency[a,b] (%)	Ethanol Yields[a,c] (L/tonne)	Residual Starch[a,d] (%w/w)
35	Urea	1.51	90 bc	408 s	30.9 yz
		2.16	92 a	420 r	30.3 z
		4.32	89 bc	404 s	32.1 y
		Mean (Urea)[e]	90 A	411 A	31.1 B
35	Protease	0.50	88 c	401 s	34.4 x
		0.71	91 ab	411 rs	31.3 yz
		1.42	85 d	387 t	35.7 w
		Mean (Protease)[e]	88 B	400 B	33.7 A
		Mean (Solids)[f]	89 X	410 X	32.4 Y
40	Urea	1.51	74 bc	338 st	46.1 y
		2.16	76 ab	346 rs	45.7 yz
		4.32	77 a	351 r	44.6 z
		Mean (Urea)[e]	76 A	345 A	45.4 B
40	Protease	0.50	73 c	334 t	53.1 x
		0.71	74 bc	337 st	54.9 w
		1.42	71 d	322 u	55.4 w
		Mean (Protease)[e]	73 B	331 B	54.4 A
		Mean (Solids)[f]	74 Y	338 Y	49.9 X

[a] Means followed by the same letter within a solids content (35 or 40%) in the same column are similar ($p<0.05$).
[b] Fischer's LSD for fermentation efficiency were 2% for 35 and 40% solids, respectively.
[c] Fischer's LSD for ethanol yield were 10 and 11 L/tonne for 35 and 40% solids, respectively.
[d] Fischer's LSD for residual starch was 1.3% w/w for 35 and 40% solids.
[e] Means followed by the same letter within a solids content for urea or protease (AB) in the same column are similar ($p<0.05$).
[f] Means followed by the same letter in the same column (XY) are similar ($p<0.05$).

Table 4.7. Maximum FAN generated during first 24 hr of protease incubation at 35 and 40% solids.

Solids Content	Protease Dose	Free Amino Nitrogen	
(% db)	(mg/g db corn)	(mg/L)	(mg/g db corn)
35	0.50	219 d	0.53
	0.71	218 d	0.52
	1.42	284 c	0.68
40	0.50	289 c	0.58
	0.71	326 b	0.65
	1.42	350 a	0.70

4.3.3 FAN Measurement

Differences in final ethanol concentrations between three protease levels can be explained based on FAN produced for protease treatments. FAN levels increase with increasing protease loadings (Table 4.7 and Figure 4.7). Highest FAN levels (350 mg/L) were obtained at 24 hr using 1.42 mg protease at 40% solids. Increasing protease dose from 0.50 to 0.71 mg did not have an affect on maximum FAN produced at 35% solids (Table 4.7). Increasing protease dose from 0.71 to 1.42 mg resulted in 66 mg/L increase in maximum FAN level at 35% solids. At 40% solids, increasing protease dose from 0.50 to 0.71 mg and 0.71 to 1.42 mg resulted in 37 and 24 mg/L increase in maximum FAN level (Table 4.7). At 72 hr, FAN levels reduced for all treatments which could be due to contamination. Wang (2008) also observed wild yeast contamination resulting in final ethanol concentration of 1% v/v. These high FAN levels up to 350 mg/L were associated with reduced final ethanol concentrations at high protease levels.

Vidal et al (2010) also observed that final ethanol concentrations decreased with high initial FAN levels (300 mg/L). This can be attributed to similar reasons (reduction in yeast fermentative capacity under excess nitrogen) suggested by Casey et al (1983) and Thomas et al (1993).

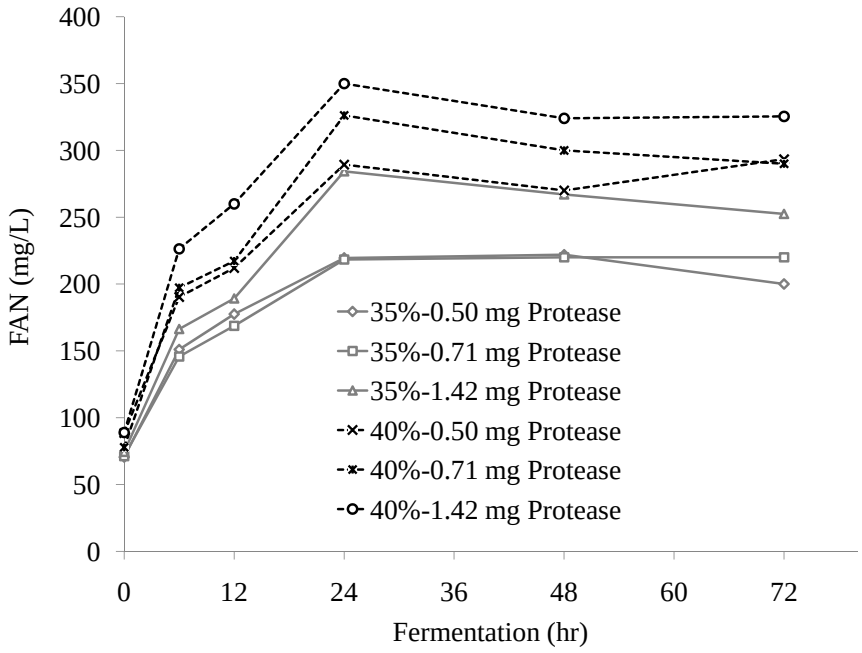

Figure 4.7. Free amino nitrogen (FAN) levels for protease treatments at 35 and 40% solids (means of duplicates).

4.4 Conclusions

Ethanol yields and fermentation efficiencies were highest using urea and protease as nitrogen sources. Similarly, higher final ethanol concentrations were observed for urea and protease. Although fermentation rates over first 12 hr fermentation were highest for glutamine, no differences were observed in final ethanol concentration among urea, glutamine and protease treatments.

At 35% solids, 2.16 mg urea and 0.71 mg protease resulted in highest fermentation efficiencies and final ethanol concentrations. However at 40% solids, 4.32 and 2.16 mg urea and 0.71 mg protease gave highest fermentation efficiencies and final ethanol concentrations. At 35 and 40% solids, increasing protease levels from 0.71 to 1.42 mg reduced final ethanol concentrations, ethanol yields and fermentation efficiencies. Similarly, at 35% solids, increasing urea levels from 2.16 to 4.32 mg decreased final ethanol concentrations and fermentation efficiencies. At all urea and protease levels, increasing solids content from 35 to 40% decreased

fermentation efficiencies and simultaneously reduced ethanol yields. Using 40% solids, higher urea levels resulted in higher ethanol yields and fermentation efficiency. At 35 and 40% solids, urea resulted in higher fermentation efficiencies and ethanol yields compared to protease.

Higher FAN levels were obtained at higher solids content (40%). At 40% solids, increasing protease dose resulted in an increase in FAN levels. At 35% solids, 1.42 mg protease dose produced highest FAN levels. Highest FAN levels at 35 and 40% solids corresponded to decreased final ethanol yields.

Chapter 5

Conclusions

The modified dry grind process resulted in lower viscosities, lower glucose concentrations, higher ethanol concentrations, higher fermentation rates and higher fermentation efficiencies for both 35 and 40% slurry solids content. Low viscosities and glucose concentration for the modified process were due to low temperature liquefaction, addition of phytase and GSH enzymes. At 35% solids content, the modified process had 80% lower slurry viscosities, lower peak glucose concentrations, higher (7.5%) final ethanol concentrations and 51% higher fermentation rates. At 40% solids content, the modified process had lower viscosities, lower peak and residual glucose concentration and higher ethanol concentrations than the conventional process; however, the results were in contrast to 35% solids content. At 40% solids content, SSF was not complete; more than 2.5% w/v residual glucose was left in the fermentation broth. Mean final ethanol concentration using the modified process at 40% solid content was 19.5% v/v and was similar to ethanol concentration achieved with modified process at 35% solids content. Enzyme level of 1.25 and 0.25 L/g db corn for GSH and GA enzymes, respectively, were selected as optimum enzyme doses for the modified process at 35% slurry solids content.

Among the nitrogen sources studied, urea and protease resulted in highest ethanol yields and fermentation effciencies. Highest fermentation rates over first 12 hr were observed for glutamine although final ethanol concentrations were similar for urea, glutamine and protease treatments. Further evaluation with urea and protease showed that at 35 as well as 40% solids, 2.16 mg urea and 0.71 mg protease can be used to achieve highest fermentation effciencies and final ethanol concentrations. We also observed that increasing urea and protease levels in fermentation media beyond 2.16 and 0.71 mg/g db corn, respectively, negatively impacted the ethanol yields. Optimal nitrogen levels were found to be closely dependent on the solids content, nitrogen source and FAN content in fermentation media. High FAN levels (350 mg/L) at 40%

solids corresponded to decreased ethanol yields. Moreover, increasing solids content from 35 to 40% resulted in reduced ethanol yields and fermentation efficiencies. By using the optimal urea or protease levels, the existing dry grind ethanol plants can improve ethanol production yields by 5% without altering the plant equipment or process flow.

Chapter 6

Recommendations for Future Work

Modified process involving use of GSHE, phytase and low temperature liquefaction resulted in improved ethanol yields and reduced slurry viscosities. We evaluated three enzyme concentrations for GSHE and GA and observed that 0.5X GSHE and 1X GA resulted in highest ethanol yields. Through a study on effects of nitrogen source and dose, we found that 2.16 mg urea and 0.71 mg protease resulted in highest fermentation performance for high solids fermentation at 35 and 40% solids.

Further process improvement could be achieved by undertaking following studies:

1. Evaluating effects of broader range of enzyme concentrations (GSHE and GA) on fermentation performance at high solids.

2. Determine effects of lipid and vitamin supplementation to overcome ethanol toxicity effects in high solids fermentation.

3. Studying effects of combination of nitrogen sources such as mixtures of amino acids in high solids fermentation. Effects of temperature staging can be evaluated to reduce ethanol toxicity effects on yeast performance.

4. Optimization of protease loadings to achieve higher fermentation efficiencies in high solids fermentation.

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