PROTEASE USE IN ETHANOL PRODUCTION FROM DRY FRACTIONATED CORN

BY

BERNARDO-JR C. VIDAL

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agricultural and Biological Engineering in the Graduate College of the University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Doctoral Committee:

Associate Professor Vijay Singh, Chair and Director of Research
Associate Professor Kent D. Rausch
Emeritus Professor M. E. Tumbleson
Associate Professor Elvira G. de Mejia
Dr. Jim Liu, Novozymes
ABSTRACT

Fractionating the corn kernel to separate endosperm from germ and pericarp improves corn ethanol processing by increasing fermentation throughput and generating salable coproducts. One such technology, dry fractionation, suffers from loss of germ derived nutrients and amino acids, resulting in poor fermentation kinetics. In the fuel ethanol industry, such deficiencies may be addressed by increasing inorganic nitrogen and other nutritional supplements. We investigated the addition of a commercial protease as an alternative to exogenous nitrogen supplementation. Our goal was to understand how protease can be used more effectively in corn dry fractionation for ethanol production.

We compared and evaluated the relative impacts of protease use among wet fractionation (E-Mill) and dry fractionation processes (using both conventional and granular starch hydrolyzing enzyme processes, referred to as dry conv and dry RS, respectively). With protease treatment, residual starch in the endosperm fiber was reduced by 1.9% w/w (22% relative reduction) in dry conv and 1.7% w/w (8% relative reduction) in dry RS, while no reduction was observed in E-Mill. Protease treatment increased ethanol production rates early in fermentation (0 to 36 hr), especially in dry conv and dry RS (0.3 and 0.6 g/L/hr higher than no protease controls, respectively). We therefore observed a greater benefit from using protease in dry fractionation process than in wet fractionation process.

To understand protease efficacy in dry fractionation ethanol process, we studied protease’s effects on each unit operation step. We found no evidence to suggest that protease pretreatment of dry fractionated endosperm increased glucose production rates during conventional liquefaction and saccharification. Instead, protease affected fermentation performance via free amino nitrogen (FAN) generated for yeast consumption. Protease generated FAN resulted in fermentation being 99% complete in 48 hr, compared to 93% with a urea supplemented control. Yeast growth and FAN consumption rates were not different between fermentations that were supplemented with both urea and FAN and with FAN alone, indicative of FAN being utilized preferentially. Only when urea was limiting (<2.5 mg N/g glucose) did FAN supplementation increased ethanol yields. With high protease loading
(generating 1.6 mg FAN/g glucose), ethanol yields were 2 g/L lower than a urea control. This reduced ethanol yield was attributed to poorer utilization of maltose, evident from the increase in maltose concentrations after fermentations with increasing initial FAN. Using glucose and maltose solutions, we observed high residual maltose during fermentations with high FAN supplementation. However, in contrast to conventional process employing separate high temperature liquefaction, a granular starch hydrolyzing enzyme (GSHE) process did not result in unutilized maltose or reduced ethanol yields, including at high initial FAN concentrations (435 mg/L in the mash supernatant). At intermediate concentrations of protease generated FAN (244 mg/L), ethanol yields from GSHE process were higher by 3 g/L compared to a urea control.

Finally, we studied protease use to generate FAN from germ as a supplement for endosperm fermentation. Ethanol yields were dependent on mash FAN concentrations, increasing to a maximum when FAN level was 80 to 90 mg FAN/100 g ds. At half the optimal FAN level (40 mg FAN/100 g ds), nitrogen limitation occurred. As was observed with endosperm derived FAN, maltose concentrations at the end of fermentation increased with increasing initial germ derived FAN. The magnitude of the residual maltose concentrations resulting from these two FAN sources differed; germ derived FAN resulted in residual maltose concentrations <50% of those resulting from endosperm derived FAN (for the same FAN levels). Ethanol production rates at 0 to 24 hr fermentation period were higher with germ FAN supplementation than with a urea control. Protease use to generate optimal FAN levels (80 to 100 mg FAN/100 g ds) in mash could improve economics of dry fractionated corn ethanol production by increasing fermentation rates and, consequently, reducing fermentation time.
ACKNOWLEDGEMENT

A wise person once said it takes a village to raise a child. It is likewise with a PhD thesis. I wish to thank numerous people who have contributed to this work and my scientific training. To my main adviser, Dr. Vijay Singh, and coadvisers Drs. Kent Rausch and Mike Tumbleson; they have shone a path towards my goal. To Drs. Elvira de Mejia (Food Science and Human Nutrition) and Jim Liu (Novozymes) for their time and dedication in serving in my committee. To Larry Pruiett, Li Xu, Jingwei Su (Dr. Yuanhui Zhang’s laboratory), Vermont Dia (Dr. de Mejia’s laboratory) and Dr. David Johnston (USDA-ARS) for their technical help. To the administrative staff of Agricultural and Biological Engineering, headed by Dr. K. C. Ting, for their material and moral support. To Novozymes, especially the Research and Development group headed by Dr. Anne Glud Hjulmand, for financial and scientific assistance given to this project. To my present and former colleagues, Wei Liu, Esha Khullar, Prabhjot Kaur, Jameel Shihadeh, Dr. Vivek Sharma and Dr. Ping Wang; all shared their labors, ideas and friendships that sustained me. To all whom I have come across within and outside the university, who in one way or another inspired me to become better in what I do. Lastly, to my family, especially Sharon, who had to be Penelope in my graduate school odyssey, I humbly dedicate my efforts.
# TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION ................................................................. 1

CHAPTER 2: LITERATURE REVIEW ......................................................... 4
  2.1 Dry Grind and Modified Dry Grind Processes for Ethanol Production............ 4
  2.2 Corn Protein and Starch Matrix: Implications on Starch Digestion, Separation 
      and Other Processes ........................................................................ 7
  2.3 Yeast Nitrogen Metabolism: Repression Mechanisms and Effects on Fermentation......10

CHAPTER 3: EFFECTS OF PROTEASE USE IN MODIFIED DRY 
GRAIND PROCESSES ........................................................................ 14
  3.1 Materials and Methods........................................................................ 14
  3.2 Results ................................................................................................. 19
  3.3 Discussion ............................................................................................. 23
  3.4 Conclusions .......................................................................................... 26

CHAPTER 4: PROTEASE TREATMENT OF CORN ENDOSPERM: 
EFFECTS ON LIQUEFACTION, SACCHARIFICATION 
AND FERMENTATION ......................................................................... 27
  4.1 Materials and Methods........................................................................ 27
  4.2 Results ................................................................................................. 32
  4.3 Discussion ............................................................................................. 41
  4.4 Conclusions .......................................................................................... 43

CHAPTER 5: GERMDERIVED FREE AMINO NITROGEN AS 
SUPPLEMENT FOR CORN ENDOSPERM 
FERMENTATION .................................................................................. 45
  5.1 Materials and Methods........................................................................ 45
  5.2 Results ................................................................................................. 48
  5.3 Discussion ............................................................................................. 52
  5.4 Conclusions .......................................................................................... 54

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE 
STUDIES .............................................................................................. 56
REFERENCES ............................................................................................................................ 59

APPENDIX A: STARCH HYDROLYZING ACTIVITY IN PROTEASES AND ITS EFFECT ON GRANULAR STARCH HYDROLYZING ENZYME (GSHE) PERFORMANCE ........................................... 69

APPENDIX B: ENDOSPERM FERMENTATION RESULTS USING REDUCED PROTEASE LOADINGS ....................................................................................................................... 72

APPENDIX C: EFFECT OF PROTEASE TREATMENTS ON ENDOSPERM FERMENTATION USING GRANULAR STARCH HYDROLYZING ENZYMES (GSHE) ......................................................... 74

APPENDIX D: POLYPEPTIDE AND AMINO ACID PROFILES GENERATED BY PROTEASE TREATMENT OF ENDOSPERM AND GERM ........................................................................... 76

APPENDIX E: AMINO ACID CONSUMPTION PROFILES DURING 24 HR FERMENTATION ............................................................................................................................... 79

APPENDIX F: PARTICLE SIZE DISTRIBUTIONS AMONG SOLIDS IN GERM HYDROLYZATES ......................................................................................................................... 81

APPENDIX G: ETHANOL YIELDS AND SUBSTRATE CONSUMPTION CALCULATIONS ................................................................................................................................. 85
CHAPTER 1

INTRODUCTION

Fuel ethanol production in the US in 2009 reached 10.6 billion gal/yr ($4.0 \times 10^{10}$ L/yr), the highest production of any country (RFA 2010). Corn ($Zea\ mays$) is the primary feedstock; 3.8 billion bushels (97 million metric tons) were processed in 2009, mainly by the dry grind industry. The capacity is expanding, projected to reach $>14$ billion gallons per year once all ethanol producing facilities under construction are completed and in full capacity (RFA 2010). The main driver for this expansion is the signing into law in 2007 of the Energy Independence and Security Act (EISA) which increased the target of the Renewable Fuels Standard (RFS) to 36 billion gallons annual ethanol production by 2022.

Recognizing constraints imposed by agricultural land use, the RFS schedule caps corn derived ethanol at 15 billion gal/yr by 2015. This implies that other biofuels (eg, cellulosic ethanol) would have to bridge the gap between this capped capacity and the RFS target. Indeed, authors have warned of the unintended consequence grain based biofuel production has on land use changes that can exacerbate greenhouse gas (GHG) emissions (Searchinger et al 2008; Fargione et al 2008). Concerns also have been raised on its impact on world food prices (World Bank 2008). While these are debatable issues, the seeming consensus is that of grain based biofuel (ie, corn ethanol in the US) acting as a partial and bridging solution to the renewable energy challenge.

Opportunities exist for improving yield and efficiency in dry grind corn ethanol process, tipping the balance towards more positive net energy gain and GHG reduction. One of these opportunities is fractionation of corn kernel. The conventional dry grind process consists of grinding the entire corn and cooking it into mash for fermentation. The result is production of large quantities of distillers dried grains with solubles (DDGS), marketed as ingredients for animal food. In 2009, 31 million metric tons of this coproduct was generated by the ethanol industry (RFA 2010); finding a market large enough to absorb additional production could pose a challenge. On the other hand, coproducts such as corn germ and pericarp fractions have
untapped market potentials (Rausch and Belyea 2006). Germ oil, fiber oil, fiber gum and
cellulosic ethanol are some of the derivative products from the coproduct streams. In addition,
separation of these fractions concentrates the starch in the fermenter, thereby increasing process
throughput for higher ethanol production (Singh et al 2005). At the abstract level, the
fractionation process moves the concept of biorefinery (Vertès et al 2008) closer to realization as
an ideal alternative to the current petroleum based refinery.

Dry fractionation (DF) is based on dry milling (tempering, degerming systems), which
has been used in cereal food production for decades (Alexander 1987). An advantage is its use
of sieving and aerodynamic separation to achieve fractionation, thus minimizing water usage.
Fermentation of DF endosperm has been reported to reduce fermentation rates and ethanol
yields, attributed to the absence of germ components that are beneficial for yeast (S. cerevisiae)
growth (Murthy et al 2006a). These components may include lipids and amino acids that would
be released from germ during corn grinding and slurrying in the conventional dry grind ethanol
process or during soaking in wet fractionation. Supplementation with B vitamins (B1, B2, B6,
niacinamide, pantothenic acid, para- amino benzoic acid, inositol, folic acid and B12) and germ
soak water or with exogenous lipids was found to increase fermentation rates of DF endosperm
(Murthy et al 2006a,b).

To avoid nitrogen deficiency during fermentation, corn mash is supplemented with
inorganic nitrogen. Urea, produced industrially for fertilizer, commonly is used. Production of
ammonia (a precursor to urea) via Haber-Bosch process is energy intensive (consumes about 1% of
the world energy supply) and utilizes fossil fuel to generate hydrogen as a starting material
(Smith 2002). In addition, urea is a nonpreferred nitrogen substrate of yeast, and requires
carboxylases (dur1, dur2) for catabolism and a specific permease (dur3) for active transport,
which are under nitrogen catabolite repression (Eiberry et al 1993). On the other hand, peptides
and amino acids have been characterized as excellent nitrogen sources for yeast fermentation of
wheat mashes (Thomas and Ingledew 1990). One way to generate these nitrogen sources may be
to hydrolyze the corn endosperm protein matrix surrounding the starch granules. Addition of a
protease in dry grind ethanol fermentation using a granular starch hydrolyzing enzyme was
effective in increasing ethanol yields (Wang et al 2009).
Dry fractionated germ contains >15% w/w protein (Johnston et al 2005). Some of these proteins are germ proteins (albumins and globulins with physiological functions) as well as endosperm proteins such as prolamins (zein) and glutelins in the adhering endosperm (Parris et al 2006). Proteins also are found in the cementing layer at the boundary of the endosperm and the germ epithelium (Wolf et al 1958). Protein content of wet fractionated germ remained high (18 to 20% w/w) even after prolonged water soaking, indicative that most proteins were not leached out easily into solution (Johnston et al 2005). Proteases could hydrolyze germ proteins and release amino acids, thus also making these available to yeast. Due to high starch content (20% w/w) in the dry fractionated germ (Johnston et al 2005), recovery of starch for fermentation is another potential benefit that can be derived from protease use. Reducing residual starch could increase germ’s value by increasing oil content on a per weight basis.

The goal of this dissertation is to understand how protease can be used more effectively in corn dry fractionation for ethanol production. The goal consists of four specific objectives:

1. Evaluate and compare the impacts of protease use in three modified dry grind processes, including wet and dry fractionation, on fermentation performance.

2. Assess the effects of protease incubation on the liquefaction and saccharification of dry fractionated endosperm.

3. Investigate the fermentation performance of dry fractionated endosperm under different levels of urea and free amino nitrogen (FAN) generated by protease incubation.

4. Determine optimal FAN levels derived from dry fractionated germ by protease incubation for supplementing endosperm fermentation.
CHAPTER 2

LITERATURE REVIEW

2.1 Dry Grind and Modified Dry Grind Processes For Ethanol Production

Corn is the main feedstock for ethanol production in the US. Processing of corn to ethanol mainly is by the dry grind process (Figure 2.1). In the dry grind process, whole corn kernel is ground by hammer mill to particle sizes of typically ≤ 4 mm diameter (sieve opening), then cooked at temperatures exceeding gelatinization point (>80°C) in the presence of α-amylase, an enzyme which rapidly reduces viscosity during cooking. The resulting mash, as the cooked or liquefied ground corn is referred to, is then fermented by brewer’s yeast (Saccharomyces cerevisiae) with the addition of glucoamylase, an enzyme which releases the sugars from the starch in the mash, a process referred to as simultaneous saccharification and fermentation (SSF) (Inlow et al 1988). After 48 to 72 hr fermentation, the resulting beer of 15% or higher ethanol content is concentrated and purified by distillation and molecular sieve separation to a final 100% (neat) ethanol. The heavy stream (bottoms during distillation) goes through evaporation and drying to yield what is the sole coproduct of the dry grind process, an animal food ingredient called distillers dried grains with solubles (DDGS).
Kernel fractionation, one of the proposed modifications to the conventional dry grind process, separates the different components of the corn kernel structure (Figure 2.2). This technology of applying fractionation in corn ethanol production also has been called modified dry grind processes. By separating germ and pericarp, the endosperm fraction, wherein starch is concentrated, is fermented primarily, rather than the entire kernel. By such a process, additional coproducts (namely, germ and pericarp fiber) are obtained, while increasing the mass throughput and starch concentration in the fermenter (Singh et al 2005). One such process is E-mill or enzymatic dry grind (Figure 2.3). The process consists of first water soaking the corn kernels (6 to 12 hr), followed by coarse grinding typically in a high speed blender (>2500 rpm) so that kernels are broken to expose the endosperm and release the germ. The ground corn slurry is incubated with starch hydrolyzing enzymes and protease for another 2 to 4 hr. Due to the increase in the slurry’s specific gravity (due to increase in sugar concentrations), it is possible to separate the lighter germ and pericarp fiber from the starch rich slurry by gravity separation. The starch rich slurry is screened (0.074 mm opening) to separate the endosperm fiber prior to SSF.
or, in one modification, plate milled to a finer grind size, followed by SSF, ethanol stripping and screening of endosperm fiber (through 0.15 mm opening) (Wang et al 2005). E-Mill has been reported to increase both ethanol titers (concentrations at the end of fermentation) and increase fermentation rates compared to conventional dry grind or nonenzymatic wet fractionation methods such as Quick Germ and Quick Germ/Quick Fiber (Singh et al 2005).

Another fractionation method is based on corn dry milling, primarily for the production of corn flakes and grits (Alexander 1987). It is referred to as dry fractionation to emphasize its dependence on particle size screening and aerodynamic separation, rather than on water as a separation medium as used in wet fractionation methods (eg, E-mill). This method was adopted for laboratory scale fermentation, in a process called dry degerm defiber or 3D (Murthy et al 2006a) (Figure 2.3). In this method, corn is first tempered (ie, contacted with water) to raise the moisture to >20%. This allows the germ to resist fracturing under mechanical pressure, when the tempered corn is passed through a degerminator mill. The milled corn is sieved to separate size fractions, after which larger size fractions are roller milled. By a series of screening operations, endosperm rich and germ rich fractions can be obtained. Fiber can be separated from both germ and endosperm fractions by aspiration.

Figure 2.2. The corn kernel structure.
Dry fractionated endosperm resulted in slow fermentation kinetics, likely due to the loss of germ derived nutrients beneficial for yeast growth. Supplementation with germ soak water and B vitamins (B1, B2, B6, niacinamide, panthothenic acid, para-amino benzoic acid, inositol, folic acid and B12) were shown to improve fermentation kinetics (Murthy et al 2006a). Lipid supplementation, in particular addition of fatty acid ester, alkylphenol and ethoxylated sorbitan ester, also increased final ethanol yields from dry fractionated endosperm (Murthy et al 2006b).

2.2 Corn Protein and Starch Matrix: Implications on Starch Digestion, Separation and Other Processes

A review of the protein fractions in the corn kernel can be found in Wilson (1987). According to Osborne (1924), the main proteins in the endosperm are the prolamins and glutelins. Prolamins are storage proteins that are aggregated into protein bodies, soluble in alcohol and consist mostly of zein (Duvick 1961). Glutelins are residual proteins left after alcohol solubilization, which can be extracted by the action of alkali or reducing agents such as
sulfite used in wet milling (Christianson 1969). These proteins are mostly amorphous in character and constitute the bulk of what is called the protein matrix, believed to hold together the starch granules in the endosperm (Christianson 1969; Wolf et al 1969). More recently, proteins have been shown to associate more tightly with starch granules, and to occur within granule interiors as well (Mu-Forster and Wasserman 1998). Internal granular proteins have been detected within starch granule channels, where they can be accessed and degraded by the protease thermolysin (Han et al 2005). In contrast to aggregating proteins on the outer surface of starch granules (ie, zein rich protein bodies), these internal proteins likely are single polymers which can undergo covalent modifications but do not crosslink readily (Mu-Forster and Wasserman 1998).

The specific role of the protein matrix in ruminal digestibility of starch granules has been studied (McAllister et al 1993). It is believed that the protein matrix, along with structural carbohydrates, is the main barrier to microbial enzyme attack on cereal grains fed to ruminants (McAllister et al 1993). Rooney and Pflugfelder (1986) found that waxy grains have higher ruminal digestibility as a result of higher protein digestibility. Using Opaque 2, a high lysine recombinant corn variety that is deficient or completely lacking in protein bodies, Redd et al (1975) observed increased microbial degradation in the rumen. Higher glutelin content in the protein matrix of different types of maize grain was linked to a lower fraction of potentially degradable starch in the rumen (Philippeau et al 1998).

It is not surprising that processes affecting the protein matrix would significantly alter the in vitro digestibility of cereal starch. Ezeogu et al (2005) showed that with cooking, more extensive polymerization of proteins occurred, possibly by disulfide bond crosslinking, causing lower starch degradability. This appeared to be the case in both maize and sorghum, wherein the addition of a reducing agent (2-mercaptoethanol) during cooking increased starch in vitro digestion by α-amylase. The same effect was attained by pressure cooking, possibly because of physical rupture of the disulfide bonds (Ezeogu et al 2005). Surprisingly, protein digestibility did not decrease with cooking in maize, although it did in sorghum (Duodo et al 2002). Nevertheless, predigestion of the maize and sorghum flour with α-amylase slightly increased protein digestibility in both, indicative of protein-starch interaction (Duodo et al 2002). Using microscopy, Ezeogu et al (2008) confirmed weblike structures that emerged from extensive
crosslinking of the protein matrix during cooking. Especially in the case of vitreous endosperm, cooking caused the collapse and matting of the protein matrix. In contrast, the addition of mercaptoethanol resulted to an expansion of the matrix putatively due to the breaking of disulfide bonds (Ezeogu et al. 2008). In a microscopic study done on pasta, Fardet et al (1999) revealed that higher protein content resulted in formation of protein networks that encapsulated starch, which likely have retarded α-amylase degradation in the protein enriched pasta.

Because proteases are enzymes that hydrolyze peptide bonds, breaking down proteins into smaller molecules of peptides, even down to amino acid units, their use has found application in starch separation processes. Johnston and Singh (2001) reported use of commercial protease preparations reduced SO₂ steeping time in corn wet milling when kernels were soaked and slightly ground prior to protease incubation. They subsequently demonstrated that reduced amounts of SO₂ can be used with the protease bromelain to produce better starch yields than with SO₂ alone (Johnston and Singh 2005). Use of protease in itself or with a cell wall degrading enzyme also resulted in higher starch recovery (>90%) from yellow dent corn when added and incubated for 4 hr after 20 hr of SO₂ soaking (Mezo-Villanueva and Serna-Saldivar 2004; Perez-Carillo and Serna-Saldivar 2006). An interesting use of protease in corn wet milling was in conjunction with high intensity sonication, wherein the best results were achieved by neutral protease incubation followed by sonication (Cameron and Wang 2006). As it was shown that sonication by itself increased starch yield and purity in corn wet milling (Zhang et al. 2005), the protease might have facilitated the physical rupture of starch-protein interactions during subsequent sonication.

Reflecting the importance of grain derived starch as fermentation feedstock, the impact of high temperature mashing was studied on sorghum proteins and was found to be similar to the effect of cooking (Zhao et al. 2008). Conversion efficiency in ethanol production was correlated with protein solubility and negatively with formation of weblike microstructures during mashing. As well as starch granules, oligosaccharides and polysaccharides were found to be trapped inside these weblike protein structures (Zhao et al. 2008). Although the study did not include maize, based on the studies cited on the effect of cooking (see previous paragraph), conclusions derived from the mashing study could be extrapolated to maize, albeit the effect may be of lesser magnitude. Perez-Carillo and Serna-Saldivar (2007) observed higher starch hydrolysis rates
with addition of protease to dry grind corn slurry prior to mashing (lesser than was observed for sorghum), supporting the same mechanism operated in corn, although to a lesser degree than sorghum because of differences in protein compositions between these two cereal grains.

2.3 Yeast Nitrogen Metabolism: Repression Mechanisms and Effects on Fermentation

Yeast (*S. cerevisiae*) utilizes a broad range of nitrogen sources, including all naturally occurring amino acids. Like other eukaryotic microorganisms, yeast possesses two general classes of amino acid transporters: specific systems for one amino acid or a family of structurally related amino acids and general systems shared by a large number of amino acids (Horak 1986). Regardless of source, these amino acids and other nitrogen sources (eg, urea) are converted ultimately within the cell to only two forms, glutamate and glutamine, which together with ammonium form the central nitrogen metabolism (ter Schure et al 2000) (Figure 2.4). However, yeast grows differently on different amino acids and this preferential utilization of nitrogen source is what is termed nitrogen catabolite repression (NCR) (ter Schure et al 2000). As a first regulation step, NCR relies on the expression of specific permeases depending on the predominant nitrogen source in the media. In particular, the general amino acid permease (Gap1p) of *S. cerevisiae* is responsible for sensing amino acids in the growth media and prepares the cell for their assimilation (Garret 2008).

The GATA family transcriptional activators, Gln3p and Gat1p, are implicated in NCR control (Cooper 2002) (Figure 2.5). When the media is nitrogen rich, Gln3p and Gat1p bind Ure2p, their complexes localize in the cytoplasm and NCR sensitive gene expression decreases. When nitrogen is low, Gln3p and Gat1p are dephosphorylated and released by Ure2p, causing the activators to enter the nucleus and increase NCR sensitive gene expression. Another pair of genes, Dal80p and Deh1p, act negatively on the Gln3p and Gat1p dependent transcription, and altogether these four genes not only regulate the expression of amino acid permeases as cited above but also expression of proteases that degrade intracellular proteins for nitrogen metabolism (Hofman-Bang 1999). More recent findings point to Ure2p as a sensor for a decrease in intracellular glutamine, which then triggers the entry of Gln3p into the nucleus to induce NCR sensitive gene expression (Magasanik 2005).
Figure 2.4. Pathways through which various nitrogen sources ultimately arrive at the yeast’s central nitrogen metabolism, wherein glutamine, glutamate and NH$_4^+$ are interconverted. Reprinted from Godard et al (2007).

Figure 2.5. Nitrogen catabolite repression regulates nitrogen utilization under limiting and excess nitrogen conditions. Adopted from Cooper (2002).
With the sequencing of the yeast genome (Goffeau et al. 1997), genomic analysis revealed the nature of nitrogen source supporting yeast growth affects transcription of large number of genes and the general response corresponded with the classification of the nitrogen source as being good (higher growth and catabolite production rate) or poor (Boer et al. 2007; Godard et al. 2007).

Numerous studies have been done on the effect of nitrogen deficiency in the oenological and brewing field because of the common problem of sluggish or stuck fermentation (Alexandre and Charpentier 1998; O’Connor-Cox and Ingledew 1989). Deficiency of nitrogen has been implicated in decreasing the sugar transport activity in yeast, mainly because synthesis of the sugar transporter protein is arrested (Busturia and Lagunas 1986). About 50 hr after ammonium depletion, glucose transport ceases (Schulze et al. 1996). The minimum nitrogen required is 120 to 140 mg N/L (Alexandre and Charpentier 1998), although higher amounts (>200 mg N/L) have been reported (Mendes-Ferreira et al. 2004). Minimum nitrogen requirements are difficult to generalize since many factors interact with nitrogen availability in affecting fermentation performance. Among these factors are yeast strain (Gardner et al. 2002; Taillandier et al. 2007; Manginot et al. 1998), temperature (Coleman et al. 2007) and biotin availability (Bohlscheid et al. 2007). By mutational disruption of two genes in S. cerevisiae, Gardner et al. (2005) managed to increase sugar catabolism under nitrogen limitation, thus producing mutant yeasts that utilize nitrogen sources more efficiently during fermentation.

Fermentation outcomes are affected not only by the concentration but also the form of nitrogen source. Thomas and Ingledew (1990) observed shorter fermentation time when amino acids (casamino acids or glutamic acid) were used in fuel ethanol production from wheat mash; however, the effect was reversed when glycine was used. Fermentation time also was reduced when protease was used to produce amino nitrogen in situ as substitute for exogenous nitrogen source (Thomas and Ingledew 1990). Complexity of amino acids can affect fermentation, as was demonstrated in a study of wheat mash fermentation where lysine inhibited yeast growth when used as a single nitrogen source but increased fermentation rates when supplemented by another amino acid (Thomas and Ingledew 1992). Utilization of different carbohydrate substrates during fermentation is affected differently depending on the nitrogen source. Batistote et al. (2006) observed in different brewing and wine yeast strains that free amino acid consistently performed...
better for maltose utilization, peptone (short peptides) induced higher fermentation rates when glucose was the substrate and ammonium salt performed the least irrespective of strain or substrate. The same general trend of better performance with peptone and poorer performance with ammonium salt was observed with baking and brewing ale and lager yeast strains (da Cruz et al 2002).

Global gene transcription analysis has been used to study the differential response of yeast to addition of either amino acids or ammonia when under nitrogen depletion (Jimenez-Marti et al 2007). In alcoholic fermentation, ammonia addition has been shown to result to higher expressions of genes involved in amino acid synthesis; whereas, amino acid addition to the growth media resulted in higher expression of genes responsible for preparing the cell for protein biosynthesis (Jimenez-Marti and del Olmo 2008). The latter may be linked to higher metabolic flux due to the synthesis of critical enzymes in the metabolic pathway. Comparing L-alanine with ammonium, global transcriptional analysis showed altered transcript levels of some 1400 genes mostly focused on metabolic functions, supporting the observed phenotypic response of higher growth and anabolic activity with L-alanine compared to ammonium (Usaite et al 2006).
CHAPTER 3
EFFECTS OF PROTEASE USE IN MODIFIED DRY GRIND PROCESSES

In this chapter, effects of protease treatment on fermentation performance and on starch separation from endosperm fiber in modified dry grind processes are discussed. Endosperm fractions were obtained by two processes: a wet fractionation process known as E-Mill (Singh et al 2005) and a dry fractionation process similar to dry milling. In the dry fractionation process, both conventional liquefaction and granular starch hydrolysis as routes to fermentation were investigated. Within these different schemes, protease treatments at some stage of the process were compared against no protease controls. Specific objectives were to (1) assess the effectiveness of protease treatment in hydrolyzing endosperm proteins by measuring the kinetics of free amino nitrogen (FAN) production, (2) determine fermentation performance with protease treatment under nonlimiting nitrogen condition and (3) evaluate the effectiveness of protease treatment in releasing starch from endosperm fiber.

3.1 Materials and Methods

3.1.1 Enzymes

All enzymes were from Novozymes, Franklinton, NC. Protease was NS50045, containing both endoprotease and exoprotease, having a declared activity of 500 LAPU/g (LAPU is the unit of enzyme activity to hydrolyze 1 μmol L-leucine-p-nitroanilide /min). The granular starch hydrolyzing enzyme (GSHE) was NS50086, a mixture of glucoamylase and α-amylase with native starch degrading capability. Liquozyme® SC (α-amylase from Bacillus licheniformis, with a declared activity of 120 KNU-S/g) and Spirizyme® Fuel (glucoamylase with acid fungal activity derived from Aspergillus niger and having a declared activity of 750 AGU/g) were used in conventional liquefaction and saccharification. These activity units were reproduced from manufacturer data sheets and were based on proprietary assays (reported
without standard International Units). Enzyme loading was reported in % v/w slurry, or equivalently, in mL/100 g slurry.

### 3.1.2 Endosperm Fractionation

Endosperm fractions were obtained from standard yellow dent corn grown in 2007 in University of Illinois at Urbana-Champaign, Agricultural and Biological Engineering Research Farm. After hand cleaning corn kernels free of fragments and foreign materials, moisture content was determined to be 13.2% (Method 44-19, AACC International 2000). Starch content (Method 76-13, AACC International 2000) and protein content (Method 4.2.08, AOAC 1995) were 62.86±0.03% and 8.87±0.01% w/w, respectively. Processes used to obtain endosperm fractions and endosperm fiber after fermentation are illustrated in Figure 3.1.

Figure 3.1. Flow diagrams of three schemes used to recover endosperm fiber at the end of fermentation.
**Dry Fractionation**

Dry fractionation was based on a modified procedure for dry milling as previously reported (Murthy et al 2006a). One kilogram corn was tempered for 20 min by gently mixing it with water to bring the moisture content to 22.5%. Tempered corn was passed through a degermenator mill (model SPL56CC17 F20 51EP, Marathon Electric, Wausau, WI), returning unbroken kernels for a second pass. Milled corn was placed in a 49°C oven for 1 hr to remove excess moisture; then passed three times through a roller mill. The resulting flakes and grits were sifted on US No. 10 (2.06 mm opening) and No. 18 (1.0 mm opening) screens. Those retained on the No. 10 were germ and bran. Those on the No. 18 were endosperm grits mixed with smaller fiber. The +18 grits were separated from fiber by aspiration and combined with fines collected at the bottom; this shall be referred to as dry mill grits in subsequent discussions. To obtain particle size suitable for fermentation, dry mill grits were ground in a crossbeater mill (Type SK100, Retsch GmbH & Co., Haan, Germany) to pass through a 0.5 mm sieve. Endosperm had starch and protein contents of 73.6% and 7.9±0.1% w/w, respectively.

**Enzymatic Milling**

A modified E-Mill procedure following Wang et al (2005) was used. Corn weighing 140 g was soaked in 280 mL water for 4 hr in a water bath heated at 55°C and shaken at 100 rpm. Soaked corn was ground using a tachometer controlled blender (Waring Products, Torrington, CT) for 1 min at 3500 rpm followed by 5 min at 2500 rpm. The ground corn slurry was transferred to a 2 L beaker using 30 mL rinse water. Slurry was adjusted to pH 4.2 by adding 10N H₂SO₄. Slurry was incubated in 48°C water bath with continuous stirring at 40 rpm. The first 2 hr incubation was done with or without 0.2% v/w protease, after which 0.05% v/w GSHE was added for another 2 hr incubation. At the end of 4 hr incubation, slurry was cooled to 25°C; germ and pericarp were skimmed. Skimming was done for 30 min or until no germ was left in the slurry. Remaining endosperm fraction was ground using a Quaker City plate mill (model 4-E, The Straub Co., Hatboro, PA). Solids adhering to the mill were rinsed with the 100 mL washings used for the germ and pericarp fraction. The finely ground slurry, with a solids content of 22.7±0.3%, was used for fermentation.
3.1.3 Proteolysis Kinetics

The progress of proteolysis was measured by the amount of FAN production in the course of 6 hr. Ground corn slurry was prepared according to the E-Mill procedure (up to just before enzyme incubation). The slurry was adjusted to pH 4.0 by addition of 10N H₂SO₄ or left at pH 5.9, which was closer to the enzyme’s 6.5 pH optimum as declared by the manufacturer. Slurry was incubated in 48°C as above with 0.2% v/w protease. Samples were taken at 0.5, 1, 2, 4 and 6 hr and frozen prior to FAN analysis using the ninhydrin colorimetric method (Official Method 945.30L, AOAC 1980).

An analogous kinetics study was conducted on dry mill grits obtained by dry fractionation (before hammer milling). Slurry was prepared by mixing 100 g grits in 300 mL water. Prior to protease addition, slurry was incubated in 55°C water bath for 2 hr, to simulate the soak treatment of corn during E-Mill, or used as was. The kinetics of FAN production were determined following the same procedure as the ground (soaked) corn slurry.

3.1.4 Fermentation and Endosperm Fiber Recovery

Granular Starch Hydrolysis and Fermentation

Fermentations were conducted in 1 L flasks (Bellco Biotechnology, Vineland, NJ) with pressure release caps, shaken at 100 rpm in a 30°C water bath. Slurry was prepared from the ground dry mill grits by adding 300 mL water to 100 g ground grits. The prepared slurry had a dry solids content of 22.3%. Slurry was adjusted to pH 4.0 by addition of 10N H₂SO₄; 0.1% v/w protease was added for the treated samples. The following were added: 1 mL of 45% urea as nitrogen supplement, 0.05% v/w GSHE and 2 mL of yeast inoculum prepared by dispersing 1 g activated dry yeast (Ethanol Red, Lesaffre Yeast Corp., Milwaukee, WI) in 5 mL water and incubating at 30°C for 20 min. E-Mill slurry (22.7% dry solids) was obtained after grinding of the endosperm fraction; 420 to 430 g of slurry was fermented following the same procedure above but omitting the protease step.
**Conventional Liquefaction and Fermentation**

Slurry was prepared from endosperm flour as above but without pH adjustment; 0.2 % v/w protease was added. Slurry was heated to 48°C for 2 hr in a programmable incubator (Labomat BFA-12, Werner Mathis AG, Switzerland), after which 0.025% v/w α-amylase was added. The temperature was increased to 85°C (3°C/min) and held for 90 min for liquefaction. Resulting mash was adjusted to pH 4.0 and transferred to the fermentation flask. Urea, glucoamylase (0.05% v/w) and yeast inoculum were added at similar amounts used in the no cook procedure. Fermentation was conducted under similar conditions (30°C, 100 rpm shaking).

**Fermentation Monitoring**

Fermentation was monitored by HPLC (Waters Corp., Milford, MA) using an ion exchange column (Aminex HPX-87H, Bio-Rad, Hercules, CA). Samples were collected at 7 time points over the course of 60 hr fermentation and analyzed for fermentation products and fermentable sugars (Singh et al 2005).

**Endosperm Fiber Recovery**

At the end of 60 hr fermentation, beer was filtered through a US No. 100 screen (150 μm opening). The retained fiber was washed with water (initial 200 mL stage followed by two 500 mL stages). After pressing out most of the water, fiber was dried in a 49°C oven for 6 hr and stored in sealed bags at 4°C prior to residual starch analysis.

**3.1.5 Residual Starch Assay**

Starch analysis was carried out using an acid hydrolysis method based on the procedure first reported in Ebell (1969) (Vidal et al 2009). One gram samples were autoclaved in 50 mL HCl (0.4 M) for 1 hr at 127°C. After cooling to room temperature, a 10 mL aliquot was added with 1.1 mL Na₂CO₃ (2 M) to bring the pH to 7. Neutralized samples were centrifuged for 10 min at 1000×g; sufficient volumes of supernatant were diluted to within the linear range of the glucose assay standard (< 1 g/L glucose). Glucose was measured by glucose oxidase-peroxidase colorimetric method using the Megazyme assay kit (Megazyme, Bray, Ireland). Starch content
was calculated from the determined glucose concentration and corrected for glucose lost during acid reaction. Starch content was reported on a % w/w dry basis.

3.1.6 Statistical Analysis

Experiments were conducted in pairs of treatment and control for each scheme presented in Figure 3.1. Experiments were repeated three times. Ethanol concentrations at sampled time points were reported as means of difference (between treatment and control); significance was determined using paired difference t test ($\alpha=0.05$). Analysis of variance (ANOVA, $\alpha=0.05$) was conducted in a randomized block design, with each repeat experiment representing a block. SAS 9.1 was used for statistical analysis.

3.2 Results

3.2.1 Proteolysis Kinetics

The kinetics of FAN production in coarsely ground soaked corn (referred to as ground corn) and endosperm grits obtained by dry milling (referred to as dry mill grits) are depicted in Figure 3.2. Difference between the two samples when no protease was added was about 50 mg/L FAN throughout 6 hr. This difference, referred to as baseline difference between controls, could be attributed to higher solids content in ground corn slurry (0.37% w/w) than in dry mill grits slurry (0.27% w/w), and to amino acid contributions from germ in the case of ground corn. The small change in FAN concentration (26 mg/L in dry mill grits; 29 mg/L in ground corn) during 6 hr without protease addition indicated that endogenous protease activity was minimal. This was the case even in ground corn slurry wherein soaking and grinding in the presence of germ could have released endogenous enzymes.

Taking into account the baseline difference between controls, the protease added samples (ie, ground corn slurry and dry mill grits slurry) exhibited similar FAN kinetics. Differences between the final (6 hr) and initial FAN concentrations in the two samples were comparable: 312±61 mg/L and 304±31 mg/L in ground corn and dry mill grits, respectively. This similarity was suggestive that FAN production by proteolysis was mainly from the endosperm fraction,
with germ and pericarp having minimal contribution. Conversely, the presence of germ and pericarp did not appear to inhibit protease activity, as again suggested by FAN kinetics.

To examine whether heat treatment during soaking prior to grinding had any effect on the proteolysis kinetics of the ground corn slurry, dry mill grits were subjected to similar treatments of 55°C for 2 hr. There was no change in the kinetics of presoaked dry mill grits, nor was there a difference in the baseline FAN level (at 0 hr) indicating that soaking did not contribute to the degradation of the protein matrix. Since both samples were not pH adjusted during the kinetics experiment, FAN production at pH 4.0 was investigated. The results (data not shown) were not different from results obtained at the initial pH of 5.9 to 6.1.

![Figure 3.2](image-url)

Figure 3.2. Kinetics of free amino nitrogen (FAN) production with and without addition of 0.2% v/w protease. Upper panel: dry mill grits (25% solids slurry). Lower panel: ground soaked corn. Error bars are ±1 SD of means (two replicates).
3.2.2 Fermentation Performance

The effect of protease treatment on fermentation performance was evaluated for each of the processes tested (E-Mill, dry RS and dry conv; Figure 3.1) by monitoring ethanol production during fermentation. In all three schemes, the difference occurred during the first half of fermentation (≤ 24 hr), coinciding with high ethanol production rates (Table 3.1). Protease treated samples had higher concentrations during this stage, indicating a higher rate of ethanol production with protease treatment. Among the three schemes, dry RS exhibited the highest increment of 1.62% v/v difference after 24 hr.

In dry RS and E-Mill, final ethanol concentrations at 60 hr were not different between protease treated and control. In E-Mill, which experienced the highest maximum average rate (0.63% /hr) among all treated samples during the 0 to 18 hr period, the concentration difference was not different from zero beginning at 24 hr. A different trend was observed in dry conv, wherein the difference in concentrations became negative late in the fermentation (≥ 36 hr). Protease treated samples of dry conv had about 0.3% v/v lower concentrations beginning at 36 hr until the end of fermentation. The sugar profile of dry conv after protease treatment was indicative of uneven liquefaction (Table 3.2). Whereas E-Mill had equivalent glucose concentrations between protease treated and control at time zero of fermentation, glucose concentration in protease treated dry conv was lower than in the control. In addition, glucose concentration in protease treated dry conv had a larger standard deviation (Table 3.2). The occurrence of high concentrations (up to 5% w/v) of maltose, which was observed only in protease treated dry conv, was supportive of the hypothesis that protease treatment altered starch degradation by α-amylase. No similar effect on starch degradation products using GSHE was observed in E-Mill (Table 3.2).
Table 3.1. Ethanol concentration differences among protease treatments and no protease treatments (controls) during fermentation. \(^{a,b}\)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>E-Mill Control (% v/v)</th>
<th>Δ (% v/v)</th>
<th>Dry Raw Starch Control (% v/v)</th>
<th>Δ (% v/v)</th>
<th>Dry Conventional Control (% v/v)</th>
<th>Δ (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>5.67</td>
<td>1.15 **</td>
<td>5.49</td>
<td>0.86 ***</td>
<td>4.89</td>
<td>0.75 **</td>
</tr>
<tr>
<td>18</td>
<td>10.48</td>
<td>0.85 *</td>
<td>8.51</td>
<td>1.40 *</td>
<td>9.03</td>
<td>0.67 **</td>
</tr>
<tr>
<td>24</td>
<td>12.91</td>
<td>-0.05</td>
<td>10.26</td>
<td>1.62 **</td>
<td>11.52</td>
<td>0.88 **</td>
</tr>
<tr>
<td>36</td>
<td>14.06</td>
<td>-0.21</td>
<td>12.87</td>
<td>0.30 *</td>
<td>13.14</td>
<td>-0.30 *</td>
</tr>
<tr>
<td>48</td>
<td>14.18</td>
<td>-0.08</td>
<td>13.32</td>
<td>0.03 **</td>
<td>13.21</td>
<td>-0.31 *</td>
</tr>
<tr>
<td>60</td>
<td>14.32</td>
<td>-0.12</td>
<td>13.45</td>
<td>0.01</td>
<td>13.19</td>
<td>-0.26 *</td>
</tr>
</tbody>
</table>

\(^a\) Ethanol concentration difference (Δ) are difference of means (treatment – control) from three repeated experiments.

\(^b\) \(p\) values obtained from the pairwise difference \(T\) test are indicated as follows: *** for ≤ 0.001, ** for ≤ 0.01, * for ≤ 0.05, and unmarked for values greater than \(\alpha=0.05\).

Table 3.2. Glucose and maltose concentrations after protease treatment prior to fermentation in E-Mill and conventional dry fractionation (\(dry\ conv\)). \(^a\)

<table>
<thead>
<tr>
<th>Process</th>
<th>Protease Treatment</th>
<th>Glucose (% w/v)</th>
<th>Maltose (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>E-Mill</td>
<td>+</td>
<td>6.63</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>6.52</td>
<td>0.26</td>
</tr>
<tr>
<td>(dry\ conv)</td>
<td>+</td>
<td>16.53</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>19.67</td>
<td>0.97</td>
</tr>
</tbody>
</table>

\(^a\) Means and standard deviations computed from three observations.
3.2.3 Endosperm Fiber Residual Starch

Dry RS and dry conv produced endosperm fibers that had lower starch contents when treated with protease (Figure 3.3). Dry conv protease treatment resulted in the largest reduction in residual starch of 1.9% w/w (22% reduction from the control). Protease treatment reduced starch in dry RS by 1.7% w/w (8% reduction from the control).

Protease treatment did not reduce residual starch in E-Mill endosperm fiber. However, E-Mill had the lowest residual starch in the control (without protease treatment); 2.6% w/w compared to 8.5% for dry conv and 21% for dry RS. It also had the lowest endosperm fiber yield of 5.6% w/w in the unfermented slurry compared to 8.5% w/w for dry conv and 8.7% w/w for dry RS (no protease controls). One difference E-Mill had from dry RS and dry conv was the second grinding of the endosperm slurry after protease incubation. The effects of second grinding (e.g., mechanical shearing) might have overwhelmed the effects due to protease. The same reason may be cited to explain the smaller endosperm fiber yield in E-Mill, as finer grinding of the fiber would have caused it to pass more easily through the No. 100 screen used for separation.

3.3 Discussion

Protease effectiveness was demonstrated in facilitating the release of starch from endosperm fiber in two of the three process schemes (all except E-Mill). Protease treatment reduced residual starch in the endosperm fiber by 22% in dry conv and 8% in dry RS relative to controls without protease addition. FAN production kinetics in dry mill grits and ground corn (E-Mill process) were similar, with the source being degradation of the endosperm protein matrix. This similarity in their kinetics was indicative of proteolysis being equally effective in all three schemes. However, no reduction of starch in the endosperm fiber was observed in E-Mill due to the dominant effect of second grinding as mentioned above.
Fermentation rates in all three schemes increased during the early stage of fermentation (Table 3.1). The amount of glucose and other carbon substrates available to the yeast could not account for the difference in rate during this growth phase, since from the onset these were equivalent in both protease treated and control, while in the case of dry conv the control was even higher (Table 3.2). The difference in rate could be attributed only to the amount and characteristic of nitrogen sources. In particular, amino acid nitrogen generated by proteolysis provided additional nitrogen source to that originally in the slurry and subsequently added in the form of urea. While urea was added in sufficient amount (525 mg/L of nitrogen) to ensure that nitrogen limitation would not be a confounding factor, the presence of additional amino acids nevertheless influenced the fermentation rates. As opposed to amino acids such as glutamine and asparagine, urea is a known poor nitrogen source because it needs to be degraded to
ammonia in two steps, a reason why urea utilization is subject to NCR (ter Schure et al 2000). Yeast gene expression patterns during alcoholic fermentation have shown that addition of amino acids (as opposed to ammonia) primes the yeast for protein biosynthesis (Jimenez-Marti and del Olmo 2008). The additional amino acids generated from protease activity would have the effect of increasing metabolic enzyme production by yeast cells, resulting to higher fermentation rates (Thomas and Ingledew 1990).

Final ethanol concentrations were similar between treatment and control for dry RS and E-Mill. This was despite the residual starch results indicating that protease treatment facilitated starch release from endosperm fiber. Starch utilization during fermentation was enzyme (glucoamylase) limited (as indicated by steady state glucose concentrations close to zero) and accessibility to substrates was only one factor limiting starch degradation, albeit an important one in the case of ruminal digestion (McAllister et al 1993). Physical release of starch from the endosperm matrix did not imply consequent enzymatic degradation, if for instance resistance to enzymatic attack was due to carbohydrate structure (eg, crystallinity or amylose to amylopectin ratio). Conversely, starch degradation could take place even when the protein matrix was left intact as Elkhalifa et al (2006) revealed in a microscopic study of sorghum flour fermentation.

Dry conv produced the highest reduction in endosperm fiber starch as a result of protease treatment. However, ethanol production in dry conv was affected adversely in the latter half of fermentation, with the result that protease treated samples had 0.3% v/v lower final ethanol concentrations. As mentioned, liquefaction was affected by protease treatment, resulting in either lower availability or altered assimilation of carbon substrates. Perez-Carillo and Serna-Saldivar (2007) observed no similar effect on liquefaction of dry grind corn with protease treatment, although no fermentation was conducted with their samples after liquefaction. In their study, FAN concentration increased concurrently with reducing sugar formation and attained less than 200 mg/L final FAN concentration (approximately half of what was attained in this study; Figure 3.2). Considering the lack of deleterious effect on granular starch hydrolysis (ie, no effect on GSHE activity based on our unpublished data), the observed effect on liquefaction (presumably through an altered α-amylase activity) needs further elucidation. This could be important since cooking and mashing have been shown to produce crosslinking in both corn and sorghum endosperm protein matrix, resulting to lower protein solubility and formation of
weblike structures that adhere more tightly to starch granules (Ezeogu et al 2008; Zhao et al 2008). Thus, carrying out proteolysis prior to liquefaction, rather than after it, may have benefits for the conventional ethanol process.

3.4 Conclusions

We demonstrated the use of protease treatment as a method to improve modified dry grind corn processes. Up to 22% reduction in residual starch in endosperm fiber was achieved with protease treatment. Lower residual starch content in the endosperm fiber fraction would be desirable for producing purer fiber components such as corn fiber gum and for more efficient cellulose conversion. Although protease treatment did not result in an increase in ethanol yields, fermentation rates increased when using protease treatment, helping to reduce fermentation time and avoid sluggish fermentations. One issue that needs further elucidation is the adverse effect on conventional liquefaction observed with protease treatment. Further understanding of how protease acts in the context of the modified dry grind processes would allow its more effective use.
CHAPTER 4

PROTEASE TREATMENT OF CORN ENDOSPERM: EFFECTS ON LIQUEFACTION, SACCHARIFICATION AND FERMENTATION

Protease incubation as a treatment for dry fractionated (DF) endosperm was investigated. Protease treatment may assist dry grind ethanol fermentation via two mechanisms. The first is protein matrix proteolysis, potentially increasing the accessible surface area of starch granules to amylolytic enzymes. This can affect starch liquefaction and saccharification, during which α-amylase and glucoamylase hydrolyze starch to produce sugars needed for yeast growth. Second is production of amino nitrogen for subsequent utilization by yeast during fermentation. Both mechanisms were evaluated as to their relative contributions to the overall effect on fermentation performance. In particular, amino nitrogen requirements during fermentation were studied under varying levels of urea supplementation, both to understand whether interactions exist in the presence of these two nitrogen sources and evaluate whether protease treatment could displace urea in the dry grind industry.

4.1 Materials and Methods

4.1.1 Substrates and Reagents

Endosperm was obtained from a single yellow dent corn hybrid grown in 2008 at the University of Illinois at Urbana-Champaign, Agricultural and Biological Engineering Research Farm. The corn had a starch content of 69.3% w/w (Method 76-13, AACC International 2000) and crude protein content of 8.9% w/w (AOAC 1995). Endosperm grits and fines were fractionated by a laboratory procedure for dry milling one kilogram corn (referred to as kg DF) (Murthy et al 2006a). Product yields were 77.2% endosperm, 4.8% pericarp fiber and 6.4% germ by weight, with the remainder consisting of fine endosperm and fiber components. The collected endosperm fractions were hammer milled to pass through a 1.0 mm opening sieve.
Endosperm samples obtained by kg DF were used in the liquefaction and saccharification experiments (Sections 4.1.3 and 4.1.4).

To generate sufficient endosperm samples for the fermentation experiments (Section 4.1.5), dry fractionation was conducted (on corn from the same sample lot) in a 25 kg batch pilot plant scale (referred to as pilot DF) (Gupta et al 2001). Starch and crude protein (db) contents of endosperm samples obtained from these two dry fractionation procedures (kg and pilot DF) were: 74.2±0.5 and 75.8±0.8% starch, and 8.7±0.1 and 8.3±0.1% protein, respectively. For model solution fermentations, we used laboratory grade reagents: glucose (Acros Organics, Morris Plains, NJ), maltose monohydrate (Acros Organics, Morris Plains, NJ) and purified casein (MP Biochemicals, Solon, OH).

4.1.2 Enzymes and Yeast

Protease NS50045 (Novozymes, Franklinton, NC) contained both endoprotease and exoprotease activities, with a total protein content of 230 mg/mL (Pierce Protein BCA Assay, Thermo Scientific, Rockford, IL) and specific gravity (sg) of 1.26. The pH optimum of the enzyme was 6.5, with an application range of pH 5 to 7; the optimal temperature was 50°C. The enzyme had a declared activity of 500 LAPU/g (where LAPU was the enzyme activity to hydrolyze L-leucine-p-nitroanilide at 1 μmol/min, based on manufacturer’s assay protocol). To further characterize this protease, we compared the relative activity (as measured by the amount of generated FAN) of NS50045 on 5 g/L casein (pH 5.2, 37°C, 10 min) with a protease of known activity (Subtilisin A, Type VIII, Sigma-Aldrich, St. Louis, MO). We found 0.5% v/v NS50045 had three times higher activity on casein compared to 5 Units/ml subtilisin. Protease loading was reported as volume in mL/100 g slurry (wb). With 25% (db) endosperm slurry, a protease loading of 0.1 mL/100 g slurry corresponded to 0.005 g/g dry solids.

The α-amylase was from Bacillus licheniformis (Liquozyme® SC, Novozymes, Franklinton, NC), with a declared activity of 120 KNU-S/g (sg 1.25), where KNU-S stands for Kilo Novo Units (Stearothermophilus), which was determined by a Novozymes proprietary procedure. Liquozyme® SC DS was a similar α-amylase as above but concentrated to two times activity of Liquozyme® SC. Spirizyme® Ultra (Novozymes, Franklinton, NC) was
glucoamylase with a declared activity of 900 AGU/g (sg 1.15), where AGU was the amount of enzyme able to hydrolyze maltose at 1 μmol/min at 37°C and pH 4.3.

Active dry yeast (ADY) was Ethanol Red (Lesaffre Yeast Corp., Milwaukee, WI). Yeast inoculants were prepared by dispersing 1 g ADY in 5 mL distilled water and incubating in a 30°C water bath for 20 min (80 rpm shaking).

4.1.3 Liquefaction Experiment

To study the effects of protease treatment on endosperm liquefaction, 25 g (22 g db) endosperm samples obtained by kg DF were dispersed in 75 mL acetate buffer (50mM, pH 5.2), in 250 mL flasks (Pyrex® Vista™, Corning Inc., Corning, NY). A final pH of 5.5 was attained after complete mixing of the slurry. NS50045 was added at loadings of 0, 0.1 and 0.2 mL/100 g slurry. Flasks were incubated in a shaking water bath (MAX Q, Barnstead International, Melrose Park, IL) for 2 hr at 45°C and 120 rpm shaking. Subsamples (1 mL) were taken after incubation, centrifuged for 3 min at 11,000×g, and the supernatant added to 0.10 N NaOH at 1:1 volume ratio. Samples were frozen by submerging in ethanol at -5°C and stored frozen. FAN was analyzed using the ninhydrin assay (Friedman 2004) according to standard methods (AOAC 1980). Sugars and oligosaccharides were analyzed by HPLC using an ion exchange column (Aminex HPX-87H; Bio-Rad, Hercules, CA) equipped with Waters 2414 refractive index detector (Waters, Milford, MA) and 5 mM H₂SO₄ mobile phase with 0.6 mL/min elution rate (Singh et al 2005).

After protease incubation, Liquozyme SC (0.025 mL/ 100 g slurry) was added. Samples were transferred to a reciprocating water bath (Gyromax™ 939XL, Amerex Instruments, Lafayette, CA) operating at 85°C and 100 rpm. Subsamples (1 mL) were taken after 1 and 2 hr, and processed as described above (including NaOH addition and immediate freezing to stop the reaction) prior to sugar analyses using HPLC and FAN analysis (AOAC 1980). The treatments were replicated three times; results were analyzed by 1-way ANOVA with means comparison using Tukey’s test (α=0.05). All statistical analyses, including in subsequent sections, were conducted using OriginPro 8 built in statistics (v8.0891, OriginLab Corporation, Northampton, MA).
4.1.4 Saccharification Experiment

To study the effects of protease treatment on saccharification of liquefied endosperm, 50 g (44 g db) endosperm samples obtained by kg DF were dispersed in 150 mL acetate buffer (50 mM, pH 5.2). Treatment samples were prepared by adding protease NS50045 (0.2 mL/100 g slurry), while control samples were prepared without protease addition. Samples were incubated in a Labomat incubator (BFA-12, Werner Mathis AG, Switzerland), ramping at 3°C/min to 45°C, and holding at 45°C for 1 hr. Without removing from the incubator, samples were treated with Liquozyme SC (0.025 mL/100 g slurry); the temperature was ramped (3°C/min) to 85°C and held at 85°C for 1 hr. After cooling to room temperature (25°C), samples were adjusted to pH 4.3 by addition of glacial acetic acid (1 mL). Samples were transferred to 500 mL glass flasks (Bellco Biotechnology, Vineland, NJ); Spirizyme Ultra was added at a loading of 0.025 mL/100 g slurry, which was equivalent to 1 AGU/g substrate. Samples were incubated in a shaking water bath (40°C, 60 rpm), and subsamples (1 mL) were taken at various time points (0, 0.5, 1, 2, 3, 4, 6, 8 and 20 hr). Sampling procedure was as described in Section 2.3 above; subsample supernatants were treated with stopping reagent (0.10N NaOH, 1:1 volume ratio) and frozen until analyzed for sugars and oligosaccharides using HPLC. Treatments were done in two replicates (duplicate samples), and repeated twice (repeat experiments are referred to as sets A and B). To account for differences in liquefaction results between the two sets, glucose concentrations were reported as percentage of theoretical glucose (TG). TG was obtained by summing glucose concentrations and equivalent glucose concentrations of maltose, maltotriose and DP4+ (oligosaccharides of four or more glucose units) measured by HPLC in the supernatant at the beginning of saccharification.

Results for 20 hr glucose concentration were analyzed by 2-way ANOVA, in which each of the two repeated sets of treatments was treated as a block. This was done to account for variance due to liquefaction, which influenced glucose production kinetics during saccharification. Means comparisons were done using Tukey’s test (α=0.05). Rates were determined for treatment and control samples by taking the slopes of regression lines through mean glucose concentrations (as percentage of TG) sampled over the periods 0 to 2 hr (4 data points) and 3 to 8 hr (4 data points). The initial 2 hr corresponded to high DP4+ consumption.
rates, consequently high glucose production rates; whereas, the subsequent 3 to 8 hr
corresponded to nearly constant DP4+ concentrations and lower glucose production rates.

4.1.5 Endosperm Fermentation Experiment

To investigate the effect of protease pretreatment (with or without additional urea) on
fermentation performance, slurry with 25% solids (db) was prepared by dispersing endosperm
samples (obtained by pilot DF) in water to a final weight of 400 g. Protease incubation and
liquefaction steps were carried out in a Labomat incubator (BFA-12, Werner Mathis AG,
Switzerland). Protease was added at loadings specified in the next paragraph and incubated for
120 min at 48°C. Without removing slurry samples from the incubator, Liquozyme SC DS was
added at 0.015 mL/100 g slurry and incubated for 60 min at 85°C. After cooling to room
temperature, samples were adjusted to pH 4.0 by addition of H2SO4 (10N). Urea (levels
specified below), glucoamylase (0.025 mL/100 g slurry) and yeast inoculants (2 mL as prepared,
or 0.4 g cell mass/100 g ds) were added to the fermentation slurry. Fermentation (72 hr) was
conducted in a shaking water bath (Gyromax™ 939XL, Amerex Instruments, Lafayette, CA)
maintained at 30°C and 100 rpm shaking. Subsamples (1 mL) were collected at 0, 12, 24, 48 and
72 hr and analyzed for fermentation products and sugars using HPLC. Additional subsamples
(1 mL) were taken from selected fermentation runs for FAN measurement (AOAC 1980) and
viable yeast count (CFU, colony forming units) using Petrifilm™ (3M, St. Paul, MN).

Protease loading and urea addition levels for the above procedure were chosen such that
interaction effects between urea and protease generated FAN during fermentation could be
evaluated. A 3^2 factorial experiment was conducted with varying levels of protease loading (0.4,
0.2 or 0 mL/100 g slurry) and urea addition (112, 34 or 0 mg/100 g slurry). The highest urea
level was 3 mg N/g glucose, while the midlevel was 1 mg N/g glucose, in the range typically
used during laboratory fermentation (Saita and Slaughter 1984; Taillandier et al 2007). Each
experimental treatment was replicated three times. Results for final ethanol concentrations
(72 hr) and extent of completion at 48 hr (ethanol concentrations at 48 hr relative to final
concentrations) were analyzed by 2-way ANOVA with interactions, and means comparison
using Tukey’s test (α=0.05).
4.1.6 Fermentations Using Model Solutions

Fermentations were conducted using model solutions to confirm the effect of FAN concentrations on maltose uptake. FAN was generated by adding NS50045 (0.2% v/v) to 10 g/L solution of casein in phosphate buffer (50 mM, pH 7.6) and incubating for 16 hr in a 37°C water bath. Casein was used since it was obtainable in high purity. In addition, casamino acids (acid hydrolyzates of casein) have been used by studies involving amino acid mixtures in fermentation (eg, Thomas and Ingledew 1990). Using SDS-PAGE, we confirmed digestion of casein by NS50045 from the complete disappearance of its characteristic bands upon protease treatment (data not shown). The hydrolyzate was filtered through Whatman filter paper #4, and sterilized (121°C, 25 min). Hydrolysate solutions were prepared (by dilution with phosphate buffer) with FAN concentrations of 420 or 105 mg/L (high or low, respectively). A phosphate buffer solution with no FAN was prepared as a control. After adjusting hydrolysate solutions to pH 4.3 with H₂SO₄ (10N), urea (1.4 g/L) and biotin (4 μg/L) were added to ensure excess available N.

Glucose and maltose were each added to the solutions at an average concentration of 56 g/L (as measured by HPLC). Yeast inoculants (prepared as in Section 4.1.2) were added at 0.5 mL/100 mL solution. Fermentations were conducted using 10 mL solutions in centrifuge tubes (50 mL) equipped with pressure releasing caps and submerged in a reciprocating water bath (Gyromax™ 939XL, Amerex Instruments, Lafayette, CA) set at 30°C and 100 rpm. Subsamples (1 mL) were taken at 24 and 48 hr time points for HPLC analyses of sugars and ethanol. Treatments were replicated three times. Results for ethanol concentration and remaining glucose and maltose (as percentages of initial concentrations) were analyzed using 1-way ANOVA and Tukey’s test (α=0.05).

4.2 Results

4.2.1 Effect of Protease Treatment on Liquefaction

Protease treatment resulted in lower DP4+ (oligosaccharides of four or more glucose units) concentrations after 1 or 2 hr liquefaction (difference of 10 to 20 g/L) (Figure 4.1). No differences were observed in DP4+ concentrations between protease loadings (0.1 or 0.2 ml/100 g slurry). Protease treated samples had higher sugar (sum of glucose, maltose and maltotriose)
concentrations than untreated controls (difference of 10 g/L), including at time zero (ie, before start of liquefaction). No differences were observed in sugar concentrations between protease loadings (0.1 or 0.2 ml/100 g slurry) (Figure 4.1).

![Figure 4.1](image_url)

Figure 4.1. Sugar (DP1 to 3) and DP4+ product profiles with time of liquefaction of dry fractionated (DF) corn endosperm. Error bars denote ±1 SD; letters (a,b) above bars compare means (α=0.05, Tukey’s test) among protease treatments. Protease treatments P-0.2, P-0.1 and P-0.0 are 0.2, 0.1 and 0.0 mL protease/100 g slurry, respectively.

### 4.2.2 Effect of Protease Treatment on Saccharification

Glucose production rates during the initial 2 hr were two times faster in experiment set B, resulting from higher initial DP4+, relative to experiment set A; whereas, subsequent rates (3 to 8 hr) of the two sets were similar as DP4+ concentrations equalized between them (Table 4.1, Figure 4.2). Comparing between protease treated samples and untreated controls in both experiment sets, glucose production rates were similar (within 95% CI) (Table 4.1). Although initial glucose concentrations were different between treated and untreated samples, their final (20 hr) glucose concentrations were not different (Table 4.1). Maltose and maltotriose
concentrations (20 hr) likewise were not different between protease treated and untreated samples (data not shown).

![Graph showing glucose production]

Figure 4.2. Glucose production with time as percent of theoretical glucose (%w/w TG) during saccharification of dry fractionated (DF) endosperm. A and B denote experimental sets (repeated experiments with protease treated samples and untreated controls in duplicates). Error bars denote ±1 SD (of duplicates). Inset: DP4+ oligosaccaride trend during the first 4 hr of saccharification (for the same experimental sets).

4.2.3 Effect of Protease Treatment on Fermentation Yields and Rates

Interaction effects on final ethanol concentrations and fermentation rates were found between urea and protease treatments (Table 4.2). Increase in urea levels resulted in increased final ethanol concentrations only when protease was absent (Table 4.2). In this case, the addition of <112 mg urea/100 g slurry led to incomplete fermentation, as indicated by lower ethanol concentrations (Table 4.2) and glucose detection at 72 hr (54±3 and 11±4 g/L for urea added at 0 and 34 mg/100 g slurry, respectively). In all other treatments, no residual glucose was detected after 72 hr fermentation.
Table 4.1. Glucose production rates and concentrations during saccharification of dry fractionated (DF) corn endosperm with and without protease treatment.\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Experiment Set</th>
<th>Protease Loading (ml/100 g slurry)</th>
<th>Theoretical Glucose (TG) Concentration (g/L)</th>
<th>Initial Glucose Concentration (0 hr) (g/L)</th>
<th>Final Glucose Concentration (20 hr) (g/L)</th>
<th>Glucose Production Rate (0 to 2 hr) (% w/w TG/hr)</th>
<th>Glucose Production Rate (3 to 8 hr) (% w/w TG/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.2</td>
<td>226±3</td>
<td>22</td>
<td>177</td>
<td>6.9±0.5</td>
<td>4.4±0.5</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>217±9</td>
<td>16±1</td>
<td>177±1</td>
<td>7.5±0.3</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>B</td>
<td>0.2</td>
<td>263±11</td>
<td>20±1</td>
<td>211±2</td>
<td>13.9±3.5</td>
<td>4.7±0.5</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>264±4</td>
<td>14</td>
<td>209±1</td>
<td>15.1±4.3</td>
<td>5.3±0.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Theoretical glucose (TG) was the glucose equivalent concentration of the summed sugar concentrations of glucose, maltose, maltotriose and DP4+ at the beginning of saccharification (after liquefaction) measured by HPLC.

\textsuperscript{b} Glucose production rates were derived from the slopes of the mean glucose produced (% w/w TG) as a function of time. Values of the slopes are shown as linear regression estimates±1 standard error.
Table 4.2. Mean±1 SD ethanol concentrations, fermentation extents (48 hr), and free amino nitrogen (FAN) concentrations generated from dry fractionated (DF) corn endosperm for different levels of protease pretreatment and urea addition.\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th>Protease Loading (mL/100 g slurry)</th>
<th>FAN Generated (mg/L)</th>
<th>Fermentation Extents (48 hr) (%)</th>
<th>72 hr Ethanol Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No urea</td>
<td>Low urea</td>
</tr>
<tr>
<td>0.0</td>
<td>62±2</td>
<td>77±3</td>
<td>76±2</td>
</tr>
<tr>
<td>0.2</td>
<td>343±6</td>
<td>99±1 b</td>
<td>99±1 b</td>
</tr>
<tr>
<td>0.4</td>
<td>480±16</td>
<td>100±1 b</td>
<td>99 b</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Fermentation extent was obtained by dividing ethanol concentration at 48 hr by ethanol concentration at 72 hr; expressed in percentage.

\textsuperscript{b} Urea levels (mg/100 g slurry) were as follows: no urea = 0, low urea = 34, high urea = 112.

\textsuperscript{c} Superscript lowercase letters (a,b) are used to compare means (level of significance $\alpha = 0.05$) of protease levels within a urea level (columns), while superscript uppercase letters (A,B,C) are used to compare means of urea levels within a protease level (rows). Comparisons are based on Tukey’s test for a factorial design (2-way ANOVA with interactions).
In the absence of protease, addition of increasing amounts of urea resulted in the following fermentation extents (48 hr): 77±3, 76±2 and 93±2% respectively (Table 4.2). With protease addition, there were no differences in fermentation extents regardless of urea amount (Table 4.2). The average fermentation extent (48 hr) among protease treated samples was 99±1%, indicating nearly complete fermentation.

Protease treatment had an effect on the final ethanol concentrations relative to an untreated control, but no differences were observed between loading levels (0.2 and 0.4 mL/100 g slurry) (Table 4.2). Protease treatment increased final ethanol concentrations in the case of low (34 mg/100 g slurry) and no urea addition (Table 4.2). However, with urea addition of 112 mg/100 g slurry, protease treatment (0.4 mL/100 g slurry) resulted in 2 g/L reduction in final ethanol concentrations relative to an untreated control.

4.2.4 FAN Consumption and Cell Growth

Initial FAN concentrations attained during protease pretreatment at different loadings are shown in Table 4.2. To understand the observed reduction in ethanol yield with protease treatment relative to urea added control (112 mg/100 g slurry), we investigated how initial FAN was consumed under different treatments: urea only, urea+protease and protease only. In addition, we monitored cell growth during 48 hr fermentation. Viable yeast cell numbers were not different among the three treatments (Figure 4.3 top panel). With respect to FAN consumption, no difference in rate was observed between protease treatment with and without urea in the first 24 hr (Figure 4.3 bottom panel). This was consistent with FAN being preferred over urea for cell assimilation. There was minimal change in FAN after 24 hr, with remaining FAN possibly from less preferred amino acids (eg, proline and glycine) (Appendix E) and inassimilable peptides. In the presence of another nonpreferred N source such as urea, FAN (from nonpreferred amino acids) likely would not be consumed as rapidly. This could explain the small difference in the remaining FAN between treatments (protease only and urea+protease) at 48 hr.
Figure 4.3. Upper panel: viable cell numbers as colony forming units (CFU) during fermentation of dry fractionated (DF) corn endosperm, treated with urea only, protease only or combination of both (all at nonlimiting N levels). Lower panel: free amino nitrogen (FAN) concentrations during the same fermentations. Error bars denote ±1 SD.

4.2.5 FAN and Sugar Uptake

In determining nitrogen sufficiency, we based fermentation completeness on glucose utilization (ie, no glucose detected by HPLC after 72 hr fermentation). To investigate if FAN levels affect ethanol yields through yeast utilization of other fermentable sugars, we plotted maltose and maltotriose concentrations at 72 hr with respect to initial FAN (Figure 4.4). Correlations were observed between sugar (maltose and maltotriose) concentrations and initial FAN (Pearson’s r >0.97). As protease treatment did not affect enzyme performance during saccharification, the correlation was indicative that utilization of sugar substrates other than glucose was affected by FAN. With a difference in remaining (72 hr) maltose concentrations of
3.9 g/L between protease (0.4 mL/100 g slurry) and urea only treatments, the predicted loss in ethanol yield was 2.1 g/L, similar to our findings (Table 4.2).

We tested whether our observations held for nonstarch fermentations, using glucose and maltose solutions and supplementing with FAN from casein hydrolysis. Ethanol produced and glucose and maltose remaining at 24 and 48 hr are presented in Table 4.3. Ethanol concentration and % glucose remaining at 24 hr were indicative that fermentation rates increased with higher FAN concentrations, in agreement with observations for endosperm fermentations. There were no differences among maltose consumptions at 24 hr. Low FAN supplementation had higher ethanol concentration at 48 hr than urea only, but was not different from high FAN supplementation. Glucose was no longer detected at 48 hr in all three treatments, indicating complete glucose consumption. Smaller remaining maltose (48 hr) was observed with low FAN supplementation than with high FAN supplementation (Table 4.3), in agreement with our findings (Figure 4.4).

![Figure 4.4](image)

Figure 4.4. Remaining (72 hr) maltose and maltotriose concentrations after fermentation of dry fractionated (DF) corn endosperm, plotted as functions of initial FAN (time=0 hr). Pearson’s correlation \( r \) for maltose and maltotriose are 0.97 and 0.98, respectively. Error bars represent ±1 SD (3 replicates).
Table 4.3. Product and substrate profiles during fermentation of model solutions containing 56 g/L of glucose and maltose and supplemented with free amino nitrogen (FAN) from casein hydrolysis.\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Nitrogen Supplement</th>
<th>24 hr</th>
<th>48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol ((\text{g/L}))</td>
<td>Remaining Glucose (%)</td>
</tr>
<tr>
<td>Urea</td>
<td>8.5±0.6 \textsuperscript{a}</td>
<td>54.3±3.6 \textsuperscript{a}</td>
</tr>
<tr>
<td>Urea+FAN (low)</td>
<td>12.2±0.4 \textsuperscript{b}</td>
<td>39.1±1.6 \textsuperscript{b}</td>
</tr>
<tr>
<td>Urea+FAN (high)</td>
<td>17.4 \textsuperscript{c}</td>
<td>19.9±0.5 \textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Urea added was 1.5 g urea/L in all treatments. FAN (low) = 105 mg FAN/L from casein hydrolysis. FAN (high) = 420 mg FAN/L from casein hydrolysis.

\textsuperscript{b} bdl: below detection limit or <0.2 g/L; superscript letters (a,b,c) are used to compare means (\(\alpha = 0.05\), Tukey’s test) among different nitrogen source treatments.
4.3 Discussion

In Chapter 3, we found that protease use had a larger impact on fermentation performance of endosperm obtained by dry fractionation compared to wet fractionation. This motivated our investigation on the effects of protease activity on each step of the dry fractionated corn ethanol process. We hypothesized the addition of protease would result in more efficient starch hydrolysis by amylolytic enzymes (α-amylase and glucoamylase) during the liquefaction and saccharification steps of the DF corn dry grind ethanol process. The hypothesis stemmed from analyzing previous works wherein protease treatment resulted in better starch separation in wet milling (Johnston and Singh 2001; Mezo-Villanueva and Serna-Saldívar 2004) and lesser residual starch in endosperm fibers recovered at the end of fermentation (Chapter 3). During liquefaction, we observed differences in DP4+ and sugar concentrations (lower and higher, respectively) with protease pretreatment (Figure 4.1). Having noted that lower DP products (e.g., glucose and maltose) have higher reducing equivalence than higher DP products (e.g., oligosaccharides), our observations were in agreement with a study on ground corn liquefaction (Perez-Carillo and Serna-Saldívar 2007), in which they reported a small increase in reducing sugar concentration with protease treatment. The product profile was what would be expected with higher exoamylase activity during liquefaction. The protease (NS50045) itself had amylolytic activity (Appendix A). Differences in sugar concentrations existed even before liquefaction was conducted (time=0 hr, Figure 4.1), indicative that differences in liquefaction product profile resulted from the protease’s amylolytic activity. During saccharification (addition of glucoamylase to the liquefied mash), protease treatments and untreated controls resulted in similar glucose production rates and 20 hr glucose yields (Figure 4.2 and Table 4.1). This was indicative that protease pretreatment, notwithstanding its effect on liquefaction product profiles, did not alter the hydrolysis efficiency of the glucoamylase. Based on the foregoing observations, there was no evidence to suggest that proteolysis could exert a controlling factor in fermentation rates by increasing starch hydrolysis rates during fermentation.

Without protease treatment, nitrogen limitation was encountered up to a urea addition of 34 mg/100 g slurry (Table 4.2). This level was equivalent to 0.75 mg N/g glucose, excluding the 0.2 mg FAN/g glucose present in the mash. This value was within the optimal N requirement reported for different strains of S. cerevisiae used in wine making and growing in high sugar
synthetic media (Taillandier et al 2007). Complete fermentation was achieved by increasing the urea amount to 2.5 mg N/g glucose (uncorrected for intrinsic FAN), which was closer to the N requirement reported by Saita and Slaughter (1984). With protease treatment, 343±6 mg FAN/L, equivalent to 1.2 mg FAN/g glucose, was sufficient for complete fermentation. Adding more protease to increase the FAN level to 480±6 mg/L, or 1.6 mg FAN/g glucose, did not result in higher ethanol yields, likely because N requirement already had been attained.

As 343 mg FAN/L was in excess of the required nitrogen (Figure 4.3 lower panel), a lower protease loading of 0.05 mL/100 g slurry (generating 162±12 mg FAN/L) was investigated (Appendix B). In this case, fermentation was complete (no detected glucose at 72 hr) with final ethanol concentrations (127±1 g/L) similar to urea control, albeit with lower extent of completion at 48 hr (88% as opposed to 93% in urea control). As reported by others (Arrizon and Gschaedler 2007; da Cruz et al 2002; Thomas and Ingledew 1980), structural complexity of nitrogen source (ie, diversity of nitrogenous compounds available to yeast) had as much effect on fermentations as amount of total N content in the media. Yeast selects among available nitrogen sources, preferring amino acids such as asparagine and glutamine, via NCR (ter Schure et al 2000).

Utilization of protease generated FAN resulted in more rapid fermentations as evidenced by fermentation extents at 48 hr (Table 4.2). At a protease loading of 0.2 mL/100 g slurry, fermentation was complete in 48 hr, while urea only supplemented fermentation was 93% complete. At this protease loading, further urea addition neither affected rates (Table 4.2), nor FAN consumption profile (Figure 4.3 lower panel), indicative of FAN being assimilated preferentially by yeast. When comparing cell growth rates over this fermentation period (0 to 48 hr), there was no difference between protease and urea control treatments (Figure 4.3); the increase in ethanol production rate could be interpreted as an increase in yeast ethanol productivity (ie, efficiency of yeast in converting carbon substrate to ethanol). In their study of yeast global gene transcription in response to amino acids or ammonia addition during alcoholic fermentation (Jiménez-Martí and del Olmo 2008), the authors found that ammonia addition resulted in higher expression of genes involved in amino acid synthesis; whereas, amino acid addition resulted in higher expression of genes responsible for protein biosynthesis. The latter
could imply higher metabolic fluxes due to faster syntheses of rate limiting enzymes, possibly explaining the higher ethanol productivity observed in FAN supplemented fermentations.

Reduction in final ethanol concentrations with high FAN levels was correlated with a higher concentration of maltose (and to a lesser degree, maltotriose) remaining after fermentation. The observation that higher FAN concentrations led to higher unutilized maltose remaining in the media was confirmed in a model fermentation experiment (Table 4.3). Utilization of sugars, such as maltose, less preferred than glucose is regulated by a catabolite repression mechanism: when glucose is depleted, expression of maltose (and other less preferred sugar) transporters in cell membranes are upregulated (Rautio and Londesborough 2003). With granular starch hydrolyzing enzymes (GSHE), wherein glucose concentrations were kept low during fermentation, unutilized maltose was not observed, including at high FAN levels. Consequently, no ethanol yield reduction occurred in GSHE process (Appendix C).

The diminished ability to utilize maltose has not been reported for nitrogen rich media. On the contrary, nitrogen starvation has been implicated in inactivating sugar transporters, which would suggest that higher nitrogen availability would enhance, not hinder, maltose uptake and utilization (Lucero et al 2002). However, others (Batistote et al 2006; da Cruz et al 2002) indicated that nitrogen source structural complexity influenced the sugar utilization characteristics of yeasts in a strain specific manner, pointing to a more complex interaction between carbon and nitrogen metabolism.

4.4 Conclusions

The effect of protease treatment on fermentation rates was attributed mainly to FAN production during proteolysis. With protease generated FAN >300 mg/L, fermentations completed at a faster rate than with urea alone (99 vs 93% completed in 48 hr). A reduction in ethanol concentration (2 g/L) was observed at the highest protease loading (480 mg FAN/L), correlated with higher concentrations of maltose (and to a lesser degree, maltotriose) at the end of fermentation. We found no evidence to suggest that protease pretreatment of dry fractionated endosperm increased glucose production rates during conventional liquefaction and saccharification, wherein starch undergoes gelatinization and solubilization to be accessible to
amylases. Because protease treatment resulted in faster fermentation, it has the potential to increase fermenter throughput and reduce capital investment.
We investigated the use of a protease in generating free amino nitrogen (FAN) from germ as an alternative to exogenous nitrogen supplementation in endosperm fermentation. Dry fractionated germ has high protein content (>15% w/w) and high residual starch (>20% w/w), making it attractive to recover these substrates and nutrients for ethanol fermentation. To achieve this goal, we wanted to study the (1) effectiveness of a protease in generating free amino nitrogen (FAN) from germ, (2) effects on ethanol yields and fermentation rates of germ hydrolyzates compared to urea supplementation and (3) optimal free amino nitrogen (FAN) concentrations in the fermentation mash supplemented with germ hydrolyzates.

5.1 Materials and Methods

5.1.1 Corn Endosperm and Germ

Corn was a yellow dent hybrid grown in 2008 at the University of Illinois at Urbana-Champaign, Agricultural and Biological Engineering Research Farm. Endosperm and germ were obtained by dry fractionation using a 25 kg batch pilot plant scale procedure (Gupta et al 2001). Endosperm fractions were pooled and ground in a hammer mill to pass through a 1.0 mm opening sieve. Ground endosperm contained 10.9% moisture, 85.9% starch, 8.6% crude protein and 1.8% fat. Germ contained 11.4% moisture, 22.2% starch, 18.4% crude protein and 23.1% fat.

5.1.2 Enzymes and Yeast

Protease NS50045 (Novozymes, Franklinton, NC) contained both endoprotease and exoprotease activities, with a total protein content of 230 mg/mL (Pierce Protein BCA Assay, Thermo Scientific, Rockford, IL) and specific gravity (sg) of 1.26. The enzyme pH optimum was 6.5, with an application range of pH 5 to 7; the optimal temperature was 50°C. The enzyme
had a declared activity of 500 LAPU/g (where LAPU was the enzyme activity to hydrolyze L-leucine-p-nitroanilide at 1 μmol/min, based on manufacturer’s assay protocol). Its activity on casein, using subtilisin as reference, was reported in Chapter 4. Liquozyme® SC DS was α-amylase from *Bacillus licheniformis* having a declared activity of 240 KNU-S/g (sg 1.25), where KNU-S stands for Kilo Novo Units (*Stearothermophilus*), which was determined by a Novozymes proprietary procedure. Spirizyme® Ultra (Novozymes, Franklinton, NC) was glucoamylase with a declared activity of 900 AGU/g (sg 1.15), where AGU was the amount of enzyme able to hydrolyze maltose at 1 μmol/min at 37°C and pH 4.3. Active dry yeast (ADY) was Ethanol Red (Lesaffre Yeast, Milwaukee, WI). Yeast inoculants were prepared by dispersing 1 g ADY in 5 mL distilled water and incubating in a 30°C water bath for 30 min (80 rpm shaking).

### 5.1.3 Kinetics of FAN Production From Germ

A time course experiment was conducted to investigate the production of FAN from germ at different protease loadings. Germ slurry was prepared in 250 mL Erlenmeyer flasks by mixing 10 g (8.9 g ds) germ (as fractionated) in 40 g distilled water. Because the slurry had a pH of 6.5, no further adjustment was made. Protease was added at loadings of 0, 0.05, 0.10 and 0.20 mL/100 g slurry. Samples in two replicates were incubated for 6 hr at 50°C (120 rpm) in a reciprocating water bath (Gyromax™ 939XL, Amerex Instruments, Lafayette, CA). To avoid issues of microbial contamination, longer incubation times were not investigated. Subsamples (0.5 mL) were collected at 0, 1, 2, 3, 4, 5 and 6 hr. After subsamples were centrifuged at 11000×g for 3 min, supernatant was pipetted and mixed (1:1 volume ratio) with TCA buffer (0.1 M trichloroacetic acid, 0.2 M sodium acetate, 0.3 M acetic acid) to terminate the reaction. Subsamples were frozen by submerging in ethanol (-5°C) until measured for FAN by the ninhydrin method (AOAC 1980).

### 5.1.4 Germ Hydrolyzate Production

Hydrolyzates were produced from germ to supplement endosperm fermentations. To generate sufficient quantities of hydrolyzates, each treatment was carried out in two sample batches (375 g slurry each). For each batch, germ (75 g wet basis, as fractionated) was added to distilled water (300 g) in 500 mL glass flasks (Bellco Biotechnology, Vineland, NJ). Protease
was added at loadings of 0, 0.05 and 0.2 mL/100 g slurry referred to as no protease (NP), low protease (LP) and high protease (HP), respectively. Samples were incubated for 6 hr at 50°C (120 rpm) in a gyrating water bath (Lab-line® Max Q 7000, Barnstead International, Melrose Park, IL). After incubation, hydrolyzates were separated from intact germ by filtering the slurry through a Buchner funnel (CoorsTek Chemical and Scientific Labware, Golden, CO) attached to a vacuum. Hydrolyzates from each of two batches were pooled and frozen. Subsamples of pooled hydrolyzates were collected for FAN analysis (AOAC 1980). Additional subsamples were collected for dry solids determination (Method 44-15A, AACC International 2000) and total fermentable substrates using HCl hydrolysis method (Vidal et al 2009). Total fermentable substrates (referred to as fermentable substrates in subsequent discussions) includes glucose, \( \alpha \)-1,4-glucans and starch, aggregately expressed in mass units as glucose. The hydrolyzate production procedure was repeated three times.

5.1.5 Endosperm Fermentation Supplemented With Germ Hydrolyzates

To investigate the effect of germ hydrolyzates on endosperm fermentation, mash samples (25% db) were prepared by mixing endosperm with hydrolyzates (NP, LP and HP). Hydrolyzates were added to endosperm resulting in a dry solids content ratio of 2:23 (hydrolyzate:endosperm). For urea control, endosperm was mixed with water to 25% ds. Dry solids content was the variable controlled because it was determined before slurry preparation and because enzyme (ie, \( \alpha \)-amylase and glucoamylase) loadings were normalized on dry solids basis as is typical in dry grind fermentations.

After adjusting to pH 5.7 with \( \text{H}_2\text{SO}_4 \) (10N), slurry (400 g) was liquefied by adding 0.6 mL \( \alpha \)-amylase in a Labomat incubator (BFA-12, Werner Mathis AG, Switzerland) set at 85°C (60 min holding time, 3°C/min ramping ending in 48°C) and 120 rpm. Liquefied samples were cooled to room temperature (20°C) and adjusted to pH 4.0 with \( \text{H}_2\text{SO}_4 \) (10N). Urea (112 mg/100 g slurry) was added to the control (containing endosperm only). Subsamples (0.5 mL) were taken from samples for FAN analysis following the procedure outlined in 5.1.3. After adding glucoamylase (0.1 mL) and yeast inoculants (2 mL), samples were immersed in a gyrating water bath (Lab-line® Max Q 7000, Barnstead International, Melrose Park, IL) set to 30°C and 130 rpm for 60 hr. Subsamples (1 mL) were taken at 0, 12, 24, 36, 48 and 60 hr for analyses of
sugars, maltodextrins (DP4+) and fermentation products by HPLC (Singh et al 2005). The HPLC used was an ion exchange column (Aminex HPX-87H; BioRad, Hercules, CA) equipped with Waters 2414 refractive index detector (Waters Corp., Milford, MA). To evaporate the ethanol at the end of fermentation, samples were submerged for 2 hr in a stationery water bath (Precision, Jouan, Winchester, VA) set at 90°C. Stillage was collected in aluminum pans and dried overnight in a 49°C oven. Dried stillage was stored inside polyurethane bags at 4°C until analyzed for fermentable substrates using HCl hydrolysis method. Experimental treatments were replicated three times.

5.1.6 Data and Statistical Analysis

Because we controlled for mash dry solids content, variability in fermentable substrates in hydrolyzates added to endosperm could result in differences in fermentable substrates content among mash treatments. To account for these differences, final ethanol concentrations were normalized to initial fermentable substrates as %Ys, or ethanol yield as percentage of theoretical yield based on initial fermentable substrates. In addition, the percentage of fermentable substrates consumed, %Xs, was determined from the fermentable substrates remaining in the stillage. With these values, it was possible to determine %Yxs, the ethanol yield as percentage of theoretical yield based on fermentable substrates consumed. %Yxs can be used to deduce the efficiency with which yeast converts sugar to ethanol, in contrast to %Ys which measures the overall efficiency of the conversion process, including enzymatic starch hydrolysis. Calculations for these parameters are given in Appendix G.

Regression analysis and analysis of variance (ANOVA) to test for significance (α=0.05) were conducted using OriginPro 8 built in statistics (v8.0891, OriginLab Corporation, Northampton, MA). Means were compared using Tukey’s test.

5.2 Results

5.2.1 Kinetics of FAN Production From Germ

Without protease addition, 60% of total FAN was produced in the first hr of incubation (Figure 5.1). Likely sources of FAN were amino acids that diffuse out of germ or hydrolysis of
soluble proteins by endogenous enzymes. With protease, 40 to 50% of total FAN was produced in the first hr, followed by linear trends ($r^2=0.97$ to 0.99) in the 2 to 6 hr period. Slopes during this period were not different among protease treatments (within the 95% C.I.), with a mean value of 55 mg FAN/L-hr. Between 200 to 300% more FAN were produced with protease addition compared to no addition during the 6 hr incubation.

![Graph showing FAN generation with protease loadings](image)

Figure 5.1. Free amino nitrogen (FAN) generated from germ (18 g ds/100 g slurry) with time of incubation and different protease (NS50045) loadings. Error bars: ±1 SD (3 replicates).

5.2.2 Germ Hydrolyzate Production

Germ hydrolyzates obtained from germ incubation with protease have higher FAN concentrations than the no protease control (Table 5.1). Dry solids and fermentable substrates were not different among treatments. Fermentable substrates in hydrolyzates were correlated with dry solids contents ($R^2=0.88$).
Table 5.1. Dry solids, FAN and fermentable substrates concentrations of hydrolyzates obtained from germ incubations (Mean ± 1 SD of 3 repeated experiments).\(^{a,b,c}\)

<table>
<thead>
<tr>
<th>Incubation Treatments</th>
<th>Dry Solids (g/100 g hydrolyzate)</th>
<th>FAN (mg/100 g hydrolyzate)</th>
<th>Fermentable Substrates (g/100 g hydrolyzate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Protease (NP)</td>
<td>6.7±0.5 (^a)</td>
<td>30±2 (^a)</td>
<td>2.1±0.6 (^a)</td>
</tr>
<tr>
<td>Low Protease (LP)</td>
<td>7.1±0.5 (^a)</td>
<td>63±3 (^b)</td>
<td>2.6±0.7 (^a)</td>
</tr>
<tr>
<td>High Protease (HP)</td>
<td>7.3±0.4 (^a)</td>
<td>85±7 (^c)</td>
<td>3.0±0.2 (^a)</td>
</tr>
</tbody>
</table>

\(^a\) Protease loading for no, low and high protease treatments: 0, 0.05 and 0.2 mL/100 g germ slurry, respectively.

\(^b\) Fermentable substrates is the sum of glucose, \(\alpha\)-1,4-glucans and starch (expressed in aggregate as glucose mass) in the hydrolyzate as measured by HCl hydrolysis assay.

\(^c\) Superscript letters (a, b, c) are used to compare means (\(\alpha = 0.05\), Tukey’s test) among different protease treatments.

5.2.3 Endosperm Fermentation Supplemented With Germ Hydrolyzates

Mash (25% ds) prepared by supplementing endosperm with germ hydrolyzates had lower initial fermentable substrates than the urea control (containing endosperm only) (Table 5.2). This was expected as germ hydrolyzates contained lower fermentable substrates than endosperm (on dry basis). Fermentable substrate consumed (as percent of initial fermentable substrate), or \(\%Xs\), was higher with urea control than with germ hydrolyzate added samples (Table 5.2). However, there were no differences in ethanol yields, as percentage of initial or consumed fermentable substrate theoretical yields (\(\%Ys\) and \(\%Yxs\), respectively), among germ hydrolyzate samples and urea control (Table 5.2).
Table 5.2. Initial mash compositions (dry solids, free amino nitrogen (FAN) and fermentable substrates) and fermentation results (ethanol yields and substrate consumption).\(^{a,b,c}\)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dry Solids (g/100 g mash)</th>
<th>FAN (mg/100 g ds)</th>
<th>Fermentable Substrates (g/100 g ds)</th>
<th>%Xs</th>
<th>%Ys</th>
<th>%Yxs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosperm + urea</td>
<td>25</td>
<td>14±1</td>
<td>94</td>
<td>99±0 (^a)</td>
<td>89±1 (^a)</td>
<td>90±1 (^a)</td>
</tr>
<tr>
<td>Endosperm + NP</td>
<td>25</td>
<td>47±6</td>
<td>89±1</td>
<td>97±0 (^b)</td>
<td>88±3 (^a)</td>
<td>91±2 (^a)</td>
</tr>
<tr>
<td>Endosperm + LP</td>
<td>25</td>
<td>80±10</td>
<td>89±1</td>
<td>98±0 (^{bc})</td>
<td>91±0 (^a)</td>
<td>93±0 (^a)</td>
</tr>
<tr>
<td>Endosperm + HP</td>
<td>25</td>
<td>100±14</td>
<td>90±0</td>
<td>98±0 (^c)</td>
<td>90±0 (^a)</td>
<td>92±0 (^a)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ±1 SD of samples (replicated three times). Fermentable substrates: sum of glucose, \(\alpha\)-1,4-glucans and starch (expressed in aggregate as glucose mass) determined by HCl hydrolysis assay in the endosperm and hydrolyzate samples.

\(^b\) NP (no protease), LP (low protease) and HP (high protease): germ hydrolyzates generated by 0, 0.05 and 0.2 mL protease/100 g germ slurry treatments, respectively. Urea added was 112 mg urea (34 mg N)/100 g mash.

\(^c\) %Xs: percent fermentable substrate consumed during fermentation; %Ys: percent of theoretical ethanol yield based on initial fermentable substrate; %Yxs: percent of theoretical ethanol yield based on fermentable substrate consumed. Calculations in Appendix G.

\(^d\) Superscript letters (a,b,c) are used to compare means (\(\alpha = 0.05\), Tukey’s test) among different treatments.
FAN concentrations were variable (COV>10%) within treatments among germ hydrolyzate added samples (Table 5.2). To account for FAN variability within treatments, ethanol yields were plotted as a function of FAN concentrations for all germ hydrolyzate treatments (Figure 5.2, upper and middle panels). Ethanol yields (both %Ys and %Yxs) followed a parabolic curve ($p<0.05$), with maximum values at 80 to 90 mg FAN/100 g ds. At half the optimal FAN level (ie, 42 mg FAN/100 g ds), ethanol yields were below the predicted value, reflective of incomplete fermentation (2.2 g glucose/L remaining at 60 hr) due to nitrogen limitation.

Rate of ethanol production based on 0 to 24 hr ethanol concentration profiles (Figure 5.3) were higher with HP hydrolyzate addition than with urea control. With NP hydrolyzate addition, the rate was lower than with urea control, suggestive of sluggish fermentation (Figure 5.3).

5.3 Discussion

During fermentation with germ derived FAN, ethanol yields were dependent on FAN concentrations, increasing to a maximum at 80 to 90 mg FAN/100 g ds (Figure 5.3). This was true for yields on substrate consumed (ie, %Yxs), indicative that FAN affects the efficiency with which yeast converts sugars to ethanol. Based on a study of yeast global gene transcription in response to amino acids or ammonia addition during alcoholic fermentation (Jiménez-Martí and del Olmo 2008), amino acid addition resulted in higher expression of genes responsible for protein biosynthesis. This would mean higher metabolic fluxes due to faster syntheses of rate limiting enzymes, thus possibly higher ethanol productivity in FAN supplemented fermentations.

Despite the observed relationship between FAN levels and ethanol yields, germ derived FAN did not increase ethanol yields compared to urea control (Table 5.2). Part of the reason could be that germ FAN added fermentations exhibited lower substrate consumption (Table 5.2). With endosperm that had been protease incubated, we observed high maltose concentrations remaining after fermentation with high initial FAN (Chapter 4). In the case of germ derived FAN, we found a similar trend (Figure 5.2, bottom panel). With urea control, residual maltose was 0.50 g/100 g substrate, below what was observed with germ FAN addition.
Figure 5.2. Ethanol yields as percent theoretical yield on initial substrate (Ys) and substrate consumed (Yxs), and residual maltose (60 hr) plotted as functions of initial free amino nitrogen (FAN) concentrations in mash after germ hydrolyzate or urea supplementation. Regression models were fitted only to observations from germ hydrolyzate supplementation.
Figure 5.3. Ethanol concentrations with time for endosperm fermentations supplemented with germ hydrolyzates (GH). No protease, low protease and high protease: 0, 0.05 and 0.2 mL protease/100 g germ slurry, respectively. Urea control was 112 mg urea (34 mg N)/100 g mash.

Residual maltose (on initial fermentable substrate basis) resulting from germ hydrolyzate addition was half that from endosperm incubation, at the same initial FAN levels. This could explain ethanol yield reductions with endosperm derived FAN supplementations (Chapter 4) but not with germ derived FAN supplementations. Differences could have stemmed from differences in amino acid contents between germ and endosperm (Wilson 1987). Likewise, this difference was observed in the hydrolyzates obtained from protease incubation of germ and endosperm (Appendix D).

5.4 Conclusions

FAN generated from germ is a viable source of nitrogen for ethanol fermentation of dry fractionated corn endosperm. Protease increased the amount of FAN in the hydrolyzates produced during germ incubation. Optimal FAN concentration in mash was 80 to 90 mg
FAN/100 g dry solids. Nitrogen limitation was observed at half optimal FAN concentration. Although ethanol yields on an initial substrate basis were not different between FAN and urea added fermentations, germ derived FAN (at optimal concentrations) increased ethanol production rates, thus helping avoid sluggish fermentation.
CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

The goal of this dissertation was to understand how protease can be used more effectively in corn dry fractionation for ethanol production. In line with the four specific objectives stated in the Introduction, the following are our major conclusions.

1. Protease had a greater impact on dry fractionation than on wet fractionation, in terms of increasing fermentation rates and reducing residual starch in endosperm fiber recovered at the end of fermentation.

2. As far as conventional liquefaction is concerned, protease incubation neither affected liquefaction outcomes nor subsequent saccharification of liquefied mash. Two qualifications regarding this finding need to be stated: (1) starch hydrolyzing enzymes (i.e., amylases) were used at recommended loadings, and therefore, our conclusion cannot extend to lower enzyme loadings and (2) saccharification extended only to 20 hr, at which point product inhibition (by glucose and maltose) likely had taken effect, and therefore, our conclusion cannot be extended to longer saccharification periods (>20 hr) expected during fermentation.

3. Under any level of urea supplementation, the addition of protease to generate free amino nitrogen (FAN) from endosperm increased fermentation rates. When urea was limiting (<2.5 mg N/g glucose), FAN supplementation increased ethanol yields, but not when urea was sufficient. With FAN supplementation at the highest level (480 mg FAN/L in the mash or 140 mg FAN/100 g ds), ethanol yield was less (by 2 g/L) compared to a urea control during fermentation using conventional liquefaction. This yield reduction was correlated with a higher amount of unutilized maltose at the end of fermentation. No such ethanol yield reduction occurred during fermentation using a granular starch hydrolyzing enzyme (GSHE) process.
4. In the case of germ derived FAN, the optimal level for improving ethanol yields and production rates was 80 to 90 mg FAN/100 g ds. Germ FAN resulted in lower unutilized maltose, around half the amount resulting from endosperm derived FAN (on similar FAN level basis).

From this study, we identified four potential issues that would benefit from further investigation.

1. On the issue of using a protease to facilitate starch hydrolysis (ie, liquefaction and saccharification), study should be made whether lower enzyme loadings (of amylases) would favor protease treatment over control. Expressed in question form: would optimal amylase loadings be lower with protease treatment? In addition, how would such a response to lower amylase loading with protease treatment be different between conventional and GSHE process?

2. How is maltose utilization by yeast during fermentation tied to free amino nitrogen levels? Maltose consumption occurs late in fermentation when glucose is depleted because of catabolite repression in yeast (ie, high glucose concentrations inhibit maltose uptake and utilization). On the other hand, preferred amino acids are consumed quickly (first 24 hr) during fermentation, while leaving less preferred amino acids (especially proline and glycine) in the media. Could the timing suggest more than coincidence and could the accumulation of nonpreferred amino acids actually influence the efficiency of maltose utilization?

3. Related to issue (2) above is the observation that fermentation using GSHE process did not result in unutilized maltose even with high FAN supplementation. It would be interesting to test the hypothesis that lower steady state glucose concentrations during GSHE process prevents catabolite repression of maltose in yeast, thereby negating the effect of high FAN on maltose utilization.

4. For the same FAN level, supplementing with germ derived FAN resulted in smaller amounts of unutilized maltose remaining after fermentation than with
endosperm derived FAN. This points to differences between amino acid compositions of the two sources of protease generated FAN. It would be interesting to investigate whether such a composition can be optimized, and whether we could select for proteases based on this optimal amino acid composition.
REFERENCES


Cooper, T. G. 2002. Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiol. Rev. 26:223-238.


We tested amylolytic side activities of various commercial protease preparations by incubating (in 45°C, pH 4.2) proteases in 5% w/v starch solution (Fisher Chemical, Fairlawn, NJ) and then measuring the reducing sugar liberated after 30 min using DNS assay (Miller 1959). We observed starch hydrolyzing activities in these proteases (Table A.1).

Table A.1. Reducing sugar liberated from soluble starch after incubation with various proteases. \(^{a,b,c}\)

<table>
<thead>
<tr>
<th>Protease</th>
<th>Reducing Sugar (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS5000P</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>NS50045</td>
<td>0.68 ± 0.10</td>
</tr>
<tr>
<td>GC106</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>No Enzyme Control</td>
<td>bdl</td>
</tr>
</tbody>
</table>

\(^a\) Protease loading was 0.0001% v/v.
\(^b\) Values are mean±1 average deviation (2 replicates); bdl: below detection limit.
\(^c\) NS5000P and NS50045 (Novozymes, Franklinton, NC); GC106 (Genencor, Palo Alto, CA)

We assessed the significance of proteases’ intrinsic amylolytic activities when applied with granular starch hydrolyzing enzymes (GSHE) to dry grind corn. GSHE NS50040 (0.2 mL/100 g slurry) was added to 25% solids slurry made up of ground corn (hammer milled through 1-mm opening). Incubation was conducted for 3 hr at 45°C, pH 4.2, and samples collected for reducing sugar measurements. Contributions from protease side activities were negligible (Figure A.1 and A.2).
Figure A.1. Reducing sugar liberated from 25% w/w dry grind corn with addition of GSHE NS500040 (0.2% v/w) and protease NS5000P (0, 0.1 and 0.2% v/w). $P_t - P_0$: reducing sugar at time t (0 to 3 hr) minus reducing sugar at time 0 hr.
Figure A.2. Reducing sugar liberated from 25% w/w dry grind corn with addition of GSHE NS50040 (0.2% v/w) and proteases NS50045 (0.1% v/w) and GC106 (0.1% v/w). $P_t - P_0$: reducing sugar at time t (0 to 3 hr) minus reducing sugar at time 0 hr.
In Chapter 4, the minimum FAN supplementation level used to assess effectiveness as a nitrogen source for endosperm fermentation was 340 mg FAN/L. We found from the FAN consumption profile (Figure 4.3) the average FAN consumed during fermentation was close to 200 mg FAN/L. We examined whether a much lower protease (NS50045) loading of 0.05 mL/100 g slurry (generating 162±12 mg FAN/L) could support complete fermentation without urea supplement. We also examined whether the protease could be added at the start of fermentation (or simultaneous proteolysis (SP), rather than pretreating the samples (PP)). Except for the lower protease loading and the latter modification (SP), the procedure followed exactly Section 4.1.5 (Chapter 4).

Ethanol production trends (SP and PP) are shown in Figure B.1, along with 0.2 mL protease/100 g slurry treatment and urea control (112 mg urea/100 g slurry) for comparison. Fermentations were complete in 72 hr (no remaining glucose detected by HPLC) and final ethanol yields for the lower protease loading (SP and PP) were comparable to that of urea only (127±1, 128±1 and 127±1 g/L for PP, SP and urea only, respectively). Therefore, the use of a lower protease loading (0.05 mL/100 g slurry) had not resulted in a similar reduction in ethanol yields as had higher protease loadings (0.40 mL/100 g slurry). However, the values for fermentation extent at 48 hr (88±1 and 84±2% for PP and SP, respectively) were lower than those obtained with higher protease loading or with urea control.
Figure B.1. Ethanol production during fermentation of DF corn endosperm treated as follows: SP 0.05, simultaneous proteolysis at protease loading of 0.05 mL/100 g slurry (ie, added right before fermentation); PP 0.05 and 0.20, pretreatment at protease loadings of 0.05 and 0.20 mL/100 g slurry, respectively; and urea supplemented control (112 mg urea/100 g slurry).
APPENDIX C

EFFECT OF PROTEASE TREATMENTS ON ENDOSPERM FERMENTATION USING GRANULAR STARCH HYDROLYZING ENZYMES (GSHE)

We examined the effect of protease incubation on endosperm fermentation using a granular starch hydrolyzing process. A similar range of FAN (≤430 mg/L, Table C.1) used during conventional fermentations in Chapter 4 was obtained by protease incubation. Fermentation conditions were similar to those in Section 4.1.5 (Chapter 4), but instead of going through a liquefaction step after protease incubation, slurry was adjusted directly to pH 4 and fermented after adding 0.05 mL GSHE/100 g slurry (NS50086 from Novozymes, Franklinton, NC). Ethanol product profiles were indicative of faster fermentation rates with protease treatment compared to urea control (Figure C.1). Final maltose concentrations were below detection, including at the highest FAN level (Table C.1). At the highest FAN level, ethanol yield was not different from the urea control; whereas, at the intermediate FAN level, ethanol yield was higher than the control (Table C.1).
Figure C.1. Ethanol production profile of endosperm fermentation using granular starch hydrolyzing enzyme (GSHE) NS50086.

Table C.1. Initial free amino nitrogen (FAN) and final maltose and ethanol concentrations during endosperm fermentation using granular starch hydrolyzing enzyme (GSHE) NS50086. a,b,c

<table>
<thead>
<tr>
<th>Protease (mL/100 g slurry)</th>
<th>FAN (0 hr) (mg/L)</th>
<th>Maltose (72 hr) (g/L)</th>
<th>Ethanol (72 hr) (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (urea control)</td>
<td>71±1</td>
<td>bdl</td>
<td>126.7±0.9 a</td>
</tr>
<tr>
<td>0.1</td>
<td>244±14</td>
<td>bdl</td>
<td>130.2±0.3 b</td>
</tr>
<tr>
<td>0.4</td>
<td>435±30</td>
<td>bdl</td>
<td>127.4±0.8 ab</td>
</tr>
</tbody>
</table>

a bdl: below detection limit
b Values reported as Mean±1 SD (2 replicates)
c Letters (a,b) denote difference in means (2 replicates) using Tukey’s test (α=0.05).
APPENDIX D

POLYPEPTIDE AND AMINO ACID PROFILES GENERATED BY PROTEASE TREATMENT OF ENDOSPERM AND GERM

Product profiles from protease incubation of both dry fractionated endosperm and germ were characterized using SDS-PAGE (Figure D.1) and amino acid analyzer (Figure D.2). A brief description of the procedure is as follows. Slurry was prepared from ground endosperm and intact germ (25 and 18% ds, respectively) in acetate buffer (pH 5, 50 mM). Protease NS50045 and subtilisin (Subtilisin A, Type VIII, Sigma-Aldrich, St. Louis, MO) were loaded at 0.1 mL/100 g slurry and 50 U/100 g slurry, respectively. Slurry was incubated in water bath (50°C) for 3 hr. For amino acid analysis, hydrolyzate supernatant samples were filtered through 0.2 µm filters (Waters, Milford, MA) into HPLC vials and stored frozen. Amino acid analysis was accomplished using Zorbax Eclipse-AAA column in Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA). For SDS-PAGE, supernatant samples were 6.25 times diluted before mixing with SDS denaturing buffer (1:1 volume ratio). Samples were boiled for 10 min before loading into 4 to 20% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA).
Figure D.1. Polypeptide molecular weight profiles of hydrolyzates obtained after protease incubation of endosperm and germ. –C: no protease control; +N: NS50045 added; +S: subtilisin added.
Figure D.2. Molar change in individual amino acids as a result of protease treatment with NS50045. Values were obtained by subtracting individual amino acid contents of protease treated samples by untreated controls and normalizing to 100 moles change in total amino acids. Normalization was necessary to account for the greater change in total amino acids resulting from germ proteolysis (32 µmol/mL) compared to endosperm proteolysis (8 µmol/mL). Amino acids are arranged left to right from most to least preferred (Appendix E).
APPENDIX E

AMINO ACID CONSUMPTION PROFILES DURING 24 HR FERMENTATION

The consumption of individual amino acids during fermentation was determined from amino acid profiles taken at 0 and 24 hr periods. Endosperm incubation, liquefaction and fermentations all followed the procedures outlined in Section 4.1.5 (Chapter 4). Two treatments were conducted: one with urea added (112 mg/100 g slurry) and one without. In both treatments, protease NS50045 was added at 0.2 mL/100 g slurry. Samples were collected at 0 and 24 hr using a similar procedure for HPLC sugar analysis (Singh 2005). Amino acid analysis was accomplished using Zorbax Eclipse-AAA column in Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA). Amino acid consumption profiles (Table E.1) are expressed in molar percentage (ie, moles amino acid consumed/100 moles amino acid initially in mash).
Table E.1  Amount of amino acids consumed from the mash during 24 hr fermentation. a

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>% Moles Consumed (24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Urea</td>
</tr>
<tr>
<td>Lysine</td>
<td>100</td>
</tr>
<tr>
<td>Threonine</td>
<td>97±1</td>
</tr>
<tr>
<td>Methionine</td>
<td>93±2</td>
</tr>
<tr>
<td>Serine</td>
<td>93±2</td>
</tr>
<tr>
<td>Asparagine</td>
<td>90±5</td>
</tr>
<tr>
<td>Glutamine</td>
<td>88±5</td>
</tr>
<tr>
<td>Leucine</td>
<td>83±1</td>
</tr>
<tr>
<td>Aspartate</td>
<td>82±7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>81±2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>79</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>76</td>
</tr>
<tr>
<td>Histidine</td>
<td>63±6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>55±3</td>
</tr>
<tr>
<td>Valine</td>
<td>53±1</td>
</tr>
<tr>
<td>Glutamate</td>
<td>49±11</td>
</tr>
<tr>
<td>Arginine</td>
<td>47±2</td>
</tr>
<tr>
<td>Cystine</td>
<td>35±5</td>
</tr>
<tr>
<td>Alanine</td>
<td>21±3</td>
</tr>
<tr>
<td>Proline</td>
<td>11±12</td>
</tr>
<tr>
<td>Glycine</td>
<td>7±2</td>
</tr>
</tbody>
</table>

a Values presented are means±1 average deviation (2 replicates).
APPENDIX F

PARTICLE SIZE DISTRIBUTIONS AMONG SOLIDS IN GERM HYDROLYZATES

We characterized particle size distributions among solids in germ hydrolyzates as functions of protease loading. This information provides indirect evidence of starch release from the protein matrix, as well as potential polypeptide aggregation that might occur during incubation. Spherical form of polypeptide aggregates have been shown to be within the size range 0.1 to 1 micron (Masuda et al 1978). Size distributions were obtained for germ hydrolyzates that had been treated with 0, 0.05 and 0.2 mL protease/100 g slurry (Section 5.1.4, Chapter 5). Particle size analysis was using a particle analyzer (Horiba LA-300, Horiba Ltd., Kyoto, Japan). Distributions based on % mass undersize and calculations for geometric mean diameters were done using Horiba LA-300’s built-in software (Figures F.1 to F.3). Samples were analyzed in duplicates. Particle geometric mean diameters decreased from 29 µm without protease treatment to 19 and 16 µm with 0.05 and 0.4 mL protease/100 g slurry added, respectively. The distribution also became closer to normal distribution with protease addition. These trends were indicative of starch granules (5 to 30 µm diameter) being released from the protein matrix, as was suggested by an increase in dry solids content of hydrolyzates with protease treatment (Chapter 5, Table 5.1). No particle sizes below 1 µm, suggestive of polypeptide aggregates, were observed in all treatments.
Figure F.1. Particle size distribution among solids in hydrolyzates obtained from germ incubation without protease treatment. Geometric mean diameters were 29.7 µm and 27.7 µm for measurements 1 and 2, respectively.
Figure F.2. Particle size distribution among solids in hydrolyzates obtained from germ incubation with 0.05 mL protease/100 g slurry. Geometric mean diameters were 18.8 µm and 19.4 µm for measurements 1 and 2, respectively.
Figure F.3. Particle size distribution among solids in hydrolyzates obtained from germ incubation with 0.2 mL protease/100 g slurry. Geometric mean diameters were 15.6 µm and 15.8 µm for measurements 1 and 2, respectively.
APPENDIX G

ETHANOL YIELDS AND SUBSTRATE CONSUMPTION CALCULATIONS

CALCULATION OF FRACTION SUBSTRATE CONSUMED DURING FERMENTATION:

Data input:

\[ S_i \] substrate content (including glucose, \( \alpha \)-1,4-glucans and starch) before fermentation (mass fraction of dry solids)

\[ S_f \] starch content after fermentation (mass fraction of dry solids)

Data output:

\[ \alpha_S \] fractional substrate conversion

Calculation variables:

\[ X_S \] mass substrate converted during fermentation

\[ T_i \] dry solids mass before fermentation

\[ T_S \] substrate mass before fermentation

Assumption:

Loss in dry solids mass is mainly due to substrate consumption (starch and its hydrolysis products), thus essentially, \( T_f = T_i - X_S \).

Calculation:

\[
\frac{T_s}{T_i} = S_i
\]  
(1)
\[
\frac{T_s - X_s}{T_i - X_s} = S_f
\]  
(2)

Dividing with \(T_i\) both numerator and denominator of LHS of (2)

\[
\frac{S_i - X_s}{1 - \frac{X_s}{T_i}} = S_f
\]

Solving for \(X_s/T_i\)

\[
X_s = \frac{S_i - S_f}{1 - S_f} \cdot T_i
\]

Solving for \(\alpha_s\)

\[
\alpha_s = \frac{X_s}{T_s}
\]

\[
\alpha_s = \frac{X_s}{S_i} \cdot \frac{T_i}{T_s}
\]

CALCULATION OF ETHANOL YIELD ON SUBSTRATE INPUT \((Y_S)\) AND ETHANOL YIELD ON SUBSTRATE CONSUMED \((Y_{XS})\):

Data input:

- \(C_{pf}\) final ethanol concentration (mL ethanol/mL aqueous sample)
- \(T_D\) dry solids content in slurry (g dry solids /g slurry)
- \(\alpha_s\) fractional substrate conversion
$S_i$ substrate content (includes glucose, $\alpha$-1,4-glucan and starch) before fermentation (mass fraction of dry solids)

$\rho_{Et}$ Density of pure ethanol

$\rho_{PW}$ Density of aqueous ethanol (at $C_{PW}$)

Data output:

$Y_P$ ethanol yield on dry solids input (g ethanol / g dry solids in slurry)

$Y_S$ ethanol yield on substrate input (g ethanol/g substrate)

$Y_{XS}$ ethanol yield on substrate consumed (g ethanol / g starch consumed)

Calculation variables:

$C_{PW}$ final ethanol concentration in mass units (g ethanol/ g aqueous sample)

$C_{P/W}$ final ethanol concentration (g ethanol/ g water)

$M_W$ water content in the slurry (g water/ g dry solids in slurry)

Assumptions:

1. Volume of aqueous sample can be taken as volume of ethanol-water mixture (other solute contributions to volume are negligible).

2. Loss of water from chemical reaction (eg, starch hydrolysis) and evaporation is negligible compared with total water mass in the system.

Calculation:

Converting $C_{Pf}$ to $C_{P/W}$

$$C_{PW} = C_{Pf} \left( \frac{\rho_{Et}}{\rho_{PW}} \right)$$
\[ C_{P/W} = \frac{C_{PW}}{1-C_{PW}} \]

Solving for \( M_W \)

\[ M_W = \frac{1-T_D}{T_D} \]

Solving for \( Y_P \)

\[ Y_P = C_{P/W}M_W \]

Solving for \( Y_S \)

\[ Y_S = \frac{Y_P}{S_i} \]

Solving for \( Y_{P/S} \)

\[ Y_{XS} = \frac{Y_P}{\alpha_S(S_i)} \]

**EXPRESSION OF SUBSTRATE CONSUMPTION AND ETHANOL YIELDS AS PERCENTAGES:**

\[ \%X_S = 100\alpha_S \]

Let \( Y_T \)=0.51, theoretical mass yield of ethanol from glucose.

\[ \%Y_S = 100Y_S/Y_T \]

\[ \%Y_{XS} = 100Y_{XS}/Y_T \]