RESEARCHREPORT

ANIMAL SCIENCE — Nutritional Efficiency Consortium

Title: Fate of Lysine and Phytate During the Bioprocess of Making DDGS–**NPB #09-119**

Investigator: Y. Zhang

Institution: National Corn-to-Ethanol Research Center (NCERC), Southern Illinois University Edwardsville

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Table of Contents

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Part A

Project Title: Fate of Lysine During the Bioprocess of Making DDGS

National Pork Board Project Identification Number: #09-119

Principal Investigator: Yanhong Zhang, Ph.D, Assistant Director, Analytical Chemistry

Institution: National Corn-to-Ethanol Research Center (NCERC)

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I. Industrial Summary

The objective of this study is to monitor the changes in lysine concentration and reactivity through the unit operations and processes that are used to make fuel ethanol. The concentrations of lysine and heat related compound, i.e. furosine, will be measured in the corn used as the feedstock, the distiller's dried grains with solubles (DDGS) that is produced as the final coproduct, and at important intermediate steps in the process in order to identify processes and steps which result in heat damage of lysine during the bioprocess of making DDGS.

Among all the indispensable amino acids in DDGS, lysine is the most variable amino acid and has the lowest digestibility in pig feeding trials. The culprit was suggested to be heat damage that occurs during DDGS production. In this study, we conducted a pilot-plant trial to investigate the effects of corn-to-ethanol unit operation conditions on lysine quality in intermediates and DDGS. We also focused on three operational parameters for DDGS production: fermentation completeness (reducing sugar level), syrup addition rate (ratio of syrup to wet cake), and dryer outlet temperature. In order to better estimate the heat damage to lysine, we tested furosine, a compound produced when lysine is damaged during a heat induced reaction called the Maillard reaction. A total of eighty samples were analyzed and they include corn flour, slurry, mash, fermentation broth, whole stillage, thin stillage, syrup, wet cake, DDG and DDGS. What we learned from this study is summarized in the following:

1. High temperatures, long residence times and high glucose levels are the major factors causing heat damage to lysine during the bioprocess of making DDGS. Among all the unit operations, liquefaction, evaporation and drying are the main processes contributing to the heat damage to lysine.
2. With the lysine level in corn around 0.27% and L/CP (percentage of lysine in crude protein) of corn around 3.0%, the DDGS produced in this study contained lysine levels ranging from 0.52% to 1.1% and L/CP from 1.9% to 3.2%, due to the various extent of heat damage to lysine. The DDGS generated under high temperature and incomplete fermentation had the lowest lysine level and L/CP.
3. Furosine is not a naturally occurring compound in corn, but the furosine levels in DDGS in this study ranged from 0.03% to 0.22% due to the various extent of heat damage to lysine. The high end of the furosine levels in DDGS suggests that heat damage could lead to lysine digestibility as low as 70%.

4. The furosine to lysine ratio of DDGS can be a good indicator for heat damage to lysine. Based on this study, the DDGS with good quality lysine had F/L (percentage of furosine in lysine) lower than 10%, while the DDGS with heat damaged lysine contained F/L as high as 40%.
5. Besides F/L, the L/CP can be a good estimate for lysine quality if the L/CP of the corn (as the feedstock) is known. The lower the L/CP of DDGS compared with that of corn, the more damage to lysine caused by heat.
6. Color of DDGS is not a very good indicator for heat damage to lysine.

It is strongly recommended that the dry grind ethanol plants monitor and control processing parameters during corn to DDGS production. The total sugar level in the drop fermentation broth sample should be lower than 1% to ensure the completeness of fermentation, and the dryer temperature should be kept as low as possible to meet the moisture level requirement and protect lysine quality.

III. Keywords DDGS, heat damage to lysine, furosine, lysine variability, lysine digestibility

IV. Scientific abstract

Among all the indispensable amino acids in dried distiller's grains with solubles (DDGS), lysine is the most variable amino acid and has the lowest digestibility in swine feeds. The culprit was suggested to be heat damage that occurs during DDGS production. The sensitivity of lysine to heat is known in food due to a temperature dependent reaction between reducing sugars and lysine, known as the Maillard reaction, however, the fate of lysine through unit operations and processes used to make DDGS is unknown. In this study, we measured the changes in the levels of lysine and furosine, a known indicator for heat damage of lysine in food, in intermediate products and DDGS, and found out that the three unit operations, liquefaction, evaporation and drying, are the main processes contributing to the heat damage to lysine in DDGS.

We learned that the heat damage to lysine in DDGS can be caused by the combination of high temperatures, long reaction times and high glucose levels during the corn to DDGS production. With the lysine level in corn about 0.27%, the lysine levels in the DDGS generated from this study varied from 0.52% to 1.1%, responding to different processing conditions, and the estimate of lysine bioavailability varied from 70% to 98%.

We have confirmed that the furosine to lysine ratio of DDGS can be a good indicator for heat damage to lysine. The DDGS with good quality lysine produced from this study contained F/L (percentage of furosine in lysine) lower than 10%, while the DDGS with damaged lysine contained F/L as high as 40%. Besides F/L, the L/CP can be a good estimate for lysine quality if the L/CP of the corn (as the feedstock) is known. The lower the L/CP of DDGS compared with that of corn, the more damage to lysine caused by heat. Color of DDGS is not a very good indicator for heat damage to lysine.

V. Introduction

The tremendous growth in the fuel-ethanol industry has been accompanied by concomitant growth in the production of DDGS, and the potential for increased use of DDGS as animal feed is great. DDGS can be a valuable ingredient in animal feeds because it contains higher concentrations of essential nutrients than corn (e.g., amino acids, phosphorus) (Stein, 2007). Unfortunately, the relatively large variation of important nutritional characteristics in DDGS from different sources has made it difficult to increase its inclusion level in swine diets

(Thaler, 2002). Among all the indispensable amino acids, the concentration of lysine is most variable in DDGS (Stein *et al.*, 2006). This variability is suggested to be partly due to heat damage that occurs during DDGS production (Fastinger and Mahan, 2006; Pahm *et al.*, 2008; Amezcua *et al.*, 2004). In addition, lysine has the lowest digestibility in pig feeding trials around 60% (Stein *et al.*, 2006; Pahm *et al.*, 2008). Both of these factors make it difficult to determine appropriate inclusion rates for DDGS in swine diets, and low lysine digestibility leads to the use of synthetic lysine as feed supplement.

High temperatures encountered in processing of DDGS can promote the Maillard reaction to occur. The Maillard reaction is a chemical reaction between protein and reducing sugars at elevated temperature. This reaction has been detected and studied in a wide array of food products (Acquistucci *et al.*, 1996; Hidalgo *et al.*, 1995; Sanz *et al.*, 2000; Ledl, 1990). The first step of the Maillard reaction consists of a condensation reaction between an amino acid and a reducing sugar, for DDGS production, most likely lysine and glucose. The formed compound from the condensation undergoes an Amadori rearrangement and becomes ε-deoxy-L-fructosyl-lysine. When testing lysine in DDGS, the protein in DDGS is hydrolyzed using 6 N HCl (AOAC 994.18), under which condition the formed ε-deoxy-L-fructosyl-lysine is decomposed and the break down products including 32% lysine and 40% furosine (Bujard and Finot, 1978; Finot *et al.*, 1981). The part of lysine formed from the decomposition is considered unreactive lysine, and the total lysine minus unreactive lysine gives reactive lysine, which might be directly related to the bioavailable lysine of DDGS in swine feed (Stein *et al.*, 2006). While the early steps of the Maillard reaction are relatively simple and well studied, the later stages are extremely complex due to the decomposition of the Amadori rearrangement product, as a result, some heat damaged lysine may never be retrieved during acid hydrolysis of DDGS. Overall, the Maillard reaction can lead to high variability of lysine in DDGS and low lysine digestibility of DDGS as swine feed. In order to better characterize the heat damage to lysine caused by the Maillard reaction, we tested furosine content in DDGS and other important intermediates.

Drying of DDGS is the process that has been most consistently blamed for the observed variations in ileal lysine digestibility in growing pigs, but other processes and operations involved in converting corn to ethanol and DDGS, such as liquefaction, distillation, and evaporation, also expose the material to high temperature and could cause similar damage to lysine. It is well known that reaction kinetics and the product yield of the Maillard reaction depend on the reaction conditions, such as temperature, pH, reactant concentration, reaction time, etc. (Martins *et al.*, 2000; Wijewickreme and Kitts, 1997; Lingnert, 1990). In this study, we conducted several pilot-scale runs to investigate the effects of corn-to-ethanol unit operation conditions on the concentrations of lysine and furosine in process intermediates and DDGS. Also, we studied the impact of plant processing variables, including the dryer outlet temperature, the residual sugar concentrations at the end of fermentation, and the relative amounts of syrup and wet cake that were combined to make DDGS, on the concentrations of lysine and furosine in DDGS. Since the absolute lysine level can change with crude protein depending on the partition of other major components in the sample, we determined crude protein content in most samples to better characterize lysine change due to heat damage. Also included was color measurement of DDGS since the color of DDGS could be a potential indicator for heat damage to lysine.

VI. Stated Objectives from the Original Proposal

The objective of this study is to monitor the changes in lysine concentration and reactivity through the unit operations and processes that are used to make fuel ethanol. The concentrations of lysine and related compound, *i.e.*, furosine, will be measured in the corn used as the feedstock in a pilot-plant trial at NCERC, the DDGS that is produced as the final coproduct, and at important intermediate steps in the process, including liquefaction, fermentation, distillation, and coproduct processing (*e.g.*, the concentrations will be measured in wet cake, thin stillage, and syrup). All the samples for the study will come from a pilot plant trial conducted at NCERC in cooperation with USDA scientists.

VII. Materials and Methods

Chemical standards and reagents: Furosine dihydrochloride was purchased from Polypeptides Laboratories (San Diego, CA). LC/MS grade methanol and trifluoroacetic acid (TFA) and amino acid standards were purchased from Sigma. Chemical and supplies related to the lysine test were purchased from Pickering Laboratories.

Sample preparation: Except for the corn flour, fermentation broth and DDGS samples, all the other samples were freeze dried using a Millrock MD53 freeze dryer to get rid of the high moisture level in the original samples. The residual moisture level in the dried samples was measured using a moisture balance (Mettler Toledo HR83) in order to calculate the lysine, furosine and crude protein levels in each sample on a dry matter basis.

All the samples were hydrolyzed according to the method of AOAC 994.12. After hydrolysis, an aliquot of the hydrolysate was saved for the furosine test, and the rest of the hydrolysate was processed for the lysine test.

Lysine test: The measurement of lysine in hydrolysate was based on the method of AOAC 994.12, a method for the determination of regular amino acids in feeds. After adding L-norleucine as an internal standard, the hydrolysate was evaporated to dryness, conditioned with sodium citrate buffer, and run on an Agilent HPLC system with OPA post column derivatization. The detection of amino acids was performed on an Agilent fluorescence detector (330 nm for excitation and 465 nm for emission). The method was validated, and the repeatability of the entire method was lower than 2%, the recoveries for spiked samples were between 98% and 102%, and the detection limit for lysine in DDGS was 0.01%.

Furosine test: Furosine content was determined with a Shimadzu SPD 20 HPLC system consisting of an auto-sampler, a degasser, a dual head pump, and a thermostated column oven set at 40º C. A binary solvent system was used: solvent A was 0.1% w/v TFA in water and solvent B was 0.1% w/v TFA in methanol. An Insertsil ODS-4 C18 column and guard column were used for the chromatographic separation. The chromatographic separation was performed at a flow rate of 1 mL min^{-1} under a gradient elution program. Identification and detection of the analytes were performed by a triple quadrupole mass spectrometer (3200 QTrap, AB Sciex) equipped with an ESI turbo ion source operated at positive mode. Furosine was detected using the 255 m/z ion, corresponding to [M+H]+ ion and the 130 m/z ion generated by fragmentation the former ion. Quantitation of furosine was based on external calibration using furosine dihydrochloride. The method was validated, and the repeatability of the entire method was lower than 8%, the

recoveries for spiked samples were between 99.0-107.0%, and the detection limit for furosine in DDGS was 0.01%.

Crude protein test: As a major component in DDGS and other intermediates, crude protein level in a sample can change due to the variation of other major components, such as residual sugars. When the crude protein level changes, the lysine level changes accordingly, which is not due to the Maillard reaction. To better characterize the variation of lysine due to the Maillard reaction, we determined the crude protein content in most samples. Crude protein was analyzed by combustion (AOAC 990.03). The method was validated, and the repeatability of the entire method was lower than 1%, the recovery for QC standard was about 99%, and the detection limit for crude protein in DDGS was 0.01%.

Color test: Color was measured using a HunterLab Colorflex colorimeter. The colorimeter was calibrated using black and white tiles before measuring samples. Color was recorded as L (brightness, where pure black is 0 and pure white is 100), a (positive values indicate the degree of redness and negative values indicate the degree of greenness), and b (positive values indicate the degree of yellowness and negative values indicate the degree of blueness). The color a and b values were always positive, indicating that DDGS color is dominated by red and yellow hues, rather than greens and blues.

Pilot plant trial: Samples were generated in a multiple fermentor pilot plant trial which was supported by USDA. The study focused on three operational parameters for DDGS production: fermentation completeness (reducing sugar level), syrup addition rate (ratio of syrup to wet cake), and dryer outlet temperature. Each combination of the factors constituted a single condition, and each condition was run in independent duplicates. Independence of the replicates was insured by using separate fermentors for each. For this study, we focused on four fermentor runs (Table 1), and samples representing unit operations from those runs were collected. Besides these four runs (Table 1), DDGS from other fermentor runs were collected in order to obtain a large data base to examine the impact of processing conditions on heat damage to lysine.

For front end operation, slurry (32% dry solids, w/w) was prepared by milling No. 2 yellow-dent corn using a hammer mill equipped with a 7/64-inch (2.78 mm) screen, and mixing the flour with hot water (85°C) and alpha-amalyse for 30 min. The slurry was pumped through a jet cooker, where it was heated to 106°C for 7 min, then into a liquefaction tank (mash tank) where additional α-amalyse added and the temperature was maintained at 85°C for 90 min. Since the front end operation was the same for all fermentor runs, we collected triplicate samples of corn flour, slurry and mash for this study.

For fermentation, about 30,000 lbs of mash was transferred into each fermentor over a 24-hr period. The fermentors were inoculated with yeast about 1 hour after filling began. Fermentation lasted approximately 50 hours, and the temperature was maintained at 32^{o}C throughout. The average final concentration of total sugars (sum of the concentrations of DP4+, DP3, DP2, and glucose, where DP*x* represents oligosaccharides consisting of *x* glucosyl units) was 1.0 ± 0.3% (w/v) for complete fermentors and 3.7 ± 1.7% (w/v) for incomplete fermentors. Incomplete fermentation was induced by limiting the nitrogen available to support yeast growth: nitrogen was supplied as urea to a final concentration of 500 mg nitrogen /kg mash for the complete fermentors and 250 mg nitrogen /kg mash for incomplete fermentors. The fermentation broth samples were collected at three fermentation time points (about 10 hr., 30 hr. and end of

fermentation) to represent early, active and late stage of fermentation from each fermentor (Table 1).

For back end operation, the fermentation broth was pumped through a continuous distillation system to separate ethanol from the nonvolatile residue, which is called whole stillage. The distillation system operated at a pressure of about 6 psig (140 kPa), and the whole stillage was kept at 113°C for about 38 minutes before being separated into solid (wet cake) and liquid (thin stillage) fractions by continuous centrifugation in a decanter centrifuge operating at 3500 rpm. Syrup was produced from the thin stillage by evaporation under vacuum at 68°C. The total solids were concentrated from 5% to 40% over about a 15 hour period. DDGS was produced by mixing syrup, wet cake, and recycled DDGS, and the mixture was introduced into a direct-fired Davenport rotary drum dryer. Approximately 20% of the material that exited the dryer was pneumatically conveyed to DDGS storage, while the remaining 80% was recycled back to the mixer where syrup and wet cake were added. The temperature at the outlet of the dryer was used as a measure of the temperature of DDGS production, and it varied from 93^{o}C to 149^{o}C. The residence time of DDGS inside the dryer was about 60 min. From each fermentor (Table 1), one sample of whole stillage, wet cake, thin stillage and syrup, and two samples of DDGS with different dryer temperatures were collected. In addition, 9 DDG and 26 DDGS samples were collected from the USDA trial representing more running conditions.

In total, 80 samples were collected and tested for the concentrations of lysine, furosine and crude protein.

VIII. Results

The lysine, furosine and crude protein concentrations reported in this study are on a dry weight basis. We introduced the percentage of lysine in crude protein (L/CP) to better evaluate the fate of lysine, and the percentage of furosine in lysine (F/L) to better evaluate the heat damage to lysine.

Table 2 lists the concentrations of lysine, furosine and crude protein in samples collected from the front end operation. The results for the three replicates of corn flour and slurry were consistent: the level of lysine was around 0.27% in corn flour and slurry, and the level of furosine went from not detected in corn to just above the detection limit in slurry. The lysine level in mash stayed about 0.27%, two out of three mash samples showed higher levels of furosine, about seven times higher than that in the third replicate mash samples and in slurry samples. The crude protein in slurry was consistently around 8.8% and the L/CP was about 3.0%.

Table 3a through 3d list the concentrations of lysine, furosine and crude protein in samples collected from fermentation and the following back end unit operations. Each table represents one fermentor run (F2, 4, 8, 9, Table 1). The data from each fermentor showed similar trends in the change of lysine and furosine levels in fermentation broth samples: both the lysine and furosine levels in fermentation broth increased with fermentation time. Within the analytical uncertainty of the furosine data (we tested furosine in the original fermentation broth samples not in freeze dried sample and the furosine level was just above detection limit), no obvious change was observed in the F/L of the fermentation broth during fermentation, which was about 16%.

For whole stillage, the lysine level was about 0.9%, the furosine level was about 0.15% and the F/L was about 15%. For wet cake from complete fermentation, the lysine level increased to be above 1.10% and the L/CP was about 3.0% (Tables 3a, 3b); for wet cake from incomplete fermentation, the lysine level was about 1.00% and the L/CP was about 2.9%; the furosine level in wet cake from the four runs was slightly above detection limit which gave a F/L of lower than 5%. For thin stillage, the lysine level dropped to about 0.5% but the L/CP increased to be above 3.0%, and the furosine level increased significantly ranging from 0.2% to 0.3% which led to the F/L higher than 40%. For syrup, the lysine level dropped to around 0.5% but the L/CP increased to above 3.5, and the furosine level ranged from 0.25% to 0.33% which led to the F/L to be above 50% (Table 3).

The lysine level and L/CP of DDGS varied. For DDGS from complete fermentation, the lysine level was above 0.85% with the L/CP ranging from 2.8 to 3.1%, and the furosine level was below 0.1%, which led to the F/L lower than 10% (Table 3a, 3b). For DDGS from incomplete fermentation, the lysine level was relatively low of below 0.80% with L/CP ranging from 1.9% to 2.9%, and the furosine level was higher than 0.05%, which gave the range of F/L between 6% and 39% (Table 3c, 3d). For DDGS from the same fermentor run, the ones produced under higher dryer temperature always had higher furosine levels, lower lysine levels and lower L/CP (Tables 3a – 3d).

When comparing data from similar fermentation runs with different syrup addition rates, such as F2 vs. F4 and F8 vs. F9, no obvious trend was observed in any of the ingredients tested between the corresponding samples, i.e., wet cake from F2 vs. wet cake from F4.

When examining the large database for DDG and DDGS (Table 4a, 4b, 4c), the DDG from complete fermentation had the highest lysine levels ranging from 1.1 to 1.3% and L/CP ranging from 2.2 to 3.2%, while the ones from incomplete fermentation had lysine levels ranging from 1.0 to 1.1% and L/CP ranging from 2.5 to 2.9%, and the furosine levels in all DDG were below 0.1% which gave the F/L lower than 8%. The DDGS from complete fermentation had the lysine levels ranging from 0.79 to 1.1%, L/P ranging from 2.5 to 3.2% and furosine levels ranging from 0.03% to 0.11%, which gave the range of F/L from 3% to 13%. The DDGS from incomplete fermentation had the lysine levels ranging from 0.52 to 0.82%, L/CP ranging from 1.9 to 2.9%, and the furosine levels ranging from 0.05% to 0.22%, which gave the range of F/L from 6% to 40%.

IX. Discussion

Unit Operations of Corn to DDGS Bioprocess

The lysine level in the feedstock of No. 2 yellow-dent corn was 0.27% (Table 2), which is close to the lysine content in corn (0.28%) from a published study (Batal and Dale, 2010), and the L/CP of slurry is also close to the reported value (3.2%) (Batal and Dale, 2010). No furosine was found in corn, which indicates that furosine is not a naturally occurring compound in corn. The furosine level increased from slurry samples to mash samples (Table 2), which corresponds to the liquefaction condition of high temperature (85^{o}C) and decent residence time (90 min.), a favorable condition for the Maillard reaction**.** The fact that one out of the three mash samples showed low level of furosine may indicate that not all the mash material underwent the Maillard reaction and the mash sample was not very homogeneous.

During fermentation, both the lysine and furosine levels in the broth increased because the total dry matter in the fermentation broth decreased due to the production of carbon dioxide and ethanol from starch. The reason that the lysine levels in the drop samples from complete fermentation were slightly higher than that in the drop samples from incomplete fermentation, was due to the higher residual sugars in the drop samples from incomplete fermentation, not because of heat damage to lysine. The fermentation temperature of 32^{o}C was probably not high enough to induce the Maillard reaction, and that is why the F/L of the fermentation broth stayed around 16%.

The lysine levels in whole stillage was around 0.9%, almost three times the level in corn, but the L/CP was around 3.0% which was close to that of slurry. The F/L of whole stillage was very close to that of fermentation end sample indicating that the distillation process, which was under high temperature (113^{o}C) and with a period of 30 min. had no obvious impact on lysine quality. According to our previous study (Wrenn and Caupert, 2010), after centrifuging, 80% of the protein in whole stillage partition in wet cake, while 20% of the more water soluble or lipophillic protein partition in thin stillage. This explains the higher lysine level in wet cake than in whole stillage but similar L/CP of wet cake to that of whole stillage. The F/L of the wet cake samples was the lowest among all the back end products.

The protein in thin stillage was very different from that in wet cake and whole stillage. The L/CP increased significantly from that in whole stillage, but the lysine level dropped to around 0.5%, and the furosine level in thin stillage was significantly higher than that in whole stillage and fermentation broth, which led to the F/L of thin stillage over 40%. The F/L of syrup was mostly higher than that of thin stillage, indicating that the evaporation process under a temperature of 68^{o}C for a period of 15 hours had impact on lysine quality.

Since DDGS was produced by mixing wet cake and syrup, the lysine and furosine levels in DDGS were expected to be somewhere between those in syrup and wet cake (Table 3). Compared with whole stillage, the L/P of DDGS from complete fermentation was similar (Table 3a, 3b), but the L/P of DDGS from incomplete fermentation was markedly lower, indicating that incomplete fermentation can lead to stronger heat damage to lysine during drying. It is as expected that within the same fermentor, the DDGS produced with a higher dryer temperature had higher furosine, lower lysine and lower L/CP (Table 3a – 3d).

F/L, an Indicator for Heat Damage to Lysine during the Bioprocess of Making DDGS

The data from above have suggested that the level of furosine in various intermediates and DDGS can be a good indicator for how the processing conditions impact lysine quality. Since the variation of lysine and furosine during the bioprocess of making DDGS was not always caused by heat damage, we used F/L, instead of the absolute furosine level, to evaluate the whole bioprocess of the four fermentation runs (Table 1, Fig. 1). Data from these runs showed similar trends that while the thin stillage and syrup had the highest F/L due to protein partition after centrifuging, the unit operations such as liquefaction, evaporation and drying, produced high F/L. When looking at the processing temperature and residence time for each unit operation (Fig. 2), the three unit operations identified above have relatively higher reaction temperature and decent reaction time. It is not clear why the distillation process did not increase the F/L significantly, maybe because the reaction time was not long enough. Finally, the drying process provides the best evidence on how heating can damage lysine quality: the DDGS generated under higher

dryer temperature always had higher F/L than those generated from lower temperature regardless of fermentation condition. In summary, during the bioprocess of making DDGS, higher temperature and longer reaction time are the most favorable conditions for the Maillard reaction to occur, and the unit operations of liquefaction, evaporation and drying are the main culprits for heat damage to lysine.

When the reaction temperature and residence time are similar for the same unit operation, the concentration of reducing sugars becomes the dominant factor for the reactivity of the Maillard reaction. This is well demonstrated when comparing the F/L of DDGS generated from incomplete fermentation versus that of DDGS generated from complete fermentation (Fig. 1). The glucose level in the samples from incomplete fermentation was probably four times higher than that in the samples from complete fermentation, as a result, the F/L of DDGS from incomplete fermentation was always higher, although not four times higher. When increasing the syrup addition rate about 50% (F4 vs. F2, F9 vs. F8), which means the incoming furosine level and glucose level were increased 50%, the impact on F/L of DDGS was not obvious.

Fate of Lysine during the Bioprocess of Making DDGS

Regarding the fate of lysine during the bioprocessing of making DDGS, the lysine level stayed at 0.27% during the front end of operation (Fig. 3), increased to around 0.9% in whole stillage due to the mass loss of starch, and after centrifuging, the protein partition led to slightly increased level of lysine in wet cake to above 1.0%, and lower level of lysine in thin stillage of around 0.5%. The lysine level varied greatly in DDGS ranging from 0.52% to 1.1% (Table 3).

To truly examine the lysine change caused by heat damage, we looked at the change of L/CP during the bioprocessing of making DDGS (Fig. 4): the L/CP stayed at 3.0% during the front end of operation (Fig. 4), stayed about 3.0% in whole stillage, and after centrifuging, the protein partition led to lower L/CP of 2.9% in wet cake, and higher L/CP of 3.5% to 4.0% in thin stillage and syrup. While the L/CP of DDGS from complete fermentation was close to that of slurry, the L/CP of DDGS from incomplete fermentation decreased to as low as 1.9%.

In Summary, the quality of lysine in thin stillage and syrup was different from that in other intermediates and DDGS because the F/L and L/CP were higher. The quality of lysine in DDGS was definitely influenced by the processing parameters under which the DDGS was produced: higher temperature and glucose level will lead to more damage to lysine, therefore, higher F/L and lower L/CP.

The Large Database of DDGS

The USDA trial that this study piggy-backed on was a sixteen fermentor trial and generated DDG and DDGS under forty-eight conditions, with each condition representing a combination of processing treatment factors, such as fermentation completeness (reducing sugar level), syrup addition rate (ratio of glucose to lysine), and dryer outlet temperature. Among all the DDG and DDGS generated during the USDA trial, we selected 9 DDG and 34 DDGS samples and measured the lysine, furosine and crude protein levels in them (Table 4a – 4c).

Figure 5 shows a plot of F/L against dryer temperature under which the DDG and DDGS were produced. The DDG has the lowest F/L (lower than 5%) regardless of dryer temperature and fermentation completeness because there was almost no source of glucose to contribute to

the Mailllard reaction. For DDGS, the F/L increased with dryer temperature when other processing factors, such as fermentation completeness and syrup addition rate, were kept constant. For the same dryer temperature, the DDGS produced from incomplete fermentation had higher F/L than the one produced from complete fermentation. No obvious change was observed with increasing syrup addition rate. The L/CP plot against dryer temperature has shown similar trend (Fig. 6): the L/CP decreased with dryer temperature when other processing factors, such as fermentation completeness and syrup addition rate, were kept constant. For the same dryer temperature, the DDGS produced from incomplete fermentation had lower L/CP than the one produced from complete fermentation. No obvious change was observed with increasing syrup addition rate. In conclusion, the DDGS data provided the same suggestion as the data from the unit operations that temperature and fermentation completeness are the major processing parameters contributing to the Maillard reaction, which can lead to heat damage to lysine and enrichment of furosine.

When plotting the L/CP versus F/L of DDGS (Fig. 7), it shows that the higher the F/L of DDGS, the lower the L/CP and the coefficient of determination between L/CP and F/L is 0.776. This suggests that the F/L of DDGS can be a very good indicator for heat damage to lysine, and the higher L/CP, the better quality of lysine. Also, this suggests that when the L/CP of the feedstock is known, the lower L/CP the DDGS has, the more heat damage to lysine in DDGS. We also plotted F/L and L/CP versus the color measurement of DDGS, the brightness (L) and yellowness (b) (Fig. 8, 9), the coefficients of determination for F/L or L/CP and color measurement were generally about 0.3, and therefore no strong association was observed.

This study has provided an in-depth investigation of the fate of lysine during corn to ethanol production. The lysine level in corn started about 0.27% for each of the sixteen fermentors, and as the end product of the bioprocessing, the DDGS contained lysine levels ranging from 0.50% to 1.1%, almost 2 times difference from the lowest to the highest level; and the L/CP of corn started about 3.0%, the L/CP of DDGS varied from 1.9 to 3.2%. The furosine level in DDGS ranged from 0.03% to 0.22%, which equals to the range of unreactive lysine level in DDGS from 0.02% to 0.18%. The F/L of DDGS ranged from 3% to 40% which suggests the percentage of reactive lysine in total lysine ranging from 68% to 98%. It is well known that lysine digestibility of DDGS in swine could be as low as 70% (XXX). Our pilot plant study has demonstrated that heat damage to lysine can cause the variation of lysine in DDGS as large as 200%, and lysine digestibility as low as 70%.

Implications for the Ethanol Industry and the Animal Feed Industry

To monitor and control processing parameters for DDGS production is the key to produce high quality co-products. Fermentation is an important process to monitor for glucose and other reducing sugar levels. It is helpful to keep the total sugar level in the final fermentation sample below 1.0%. The dryer temperature is another important parameter to monitor and it is challenging to keep the dryer temperature low while maintain the moisture level around 10% in DDGS. Wet cake has better quality of lysine because the early Maillard products tend to be enriched with the thin stillage.

Furosine is confirmed to be a good indicator for heat damage of lysine, which is supported by a swine trial study (Stein, 2010). It is recommended for animal nutritionists to

monitor furosine level in DDGS to estimate the potential lysine digestibility of DDGS as swine feed. When furosine data is not available, the lysine to crude protein ratio can be another indicator for lysine quality if the lysine to crude protein ratio of corn as the feedstock is known.

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Table 1: The Selection of Samples from the USDA Plant Trial

Fermentor No.	Fermentation samples	Syrup addition rate	Samples of whole stillage, wet cake, thin stillage and syrup	DDGS samples at dryer outlet temperature of 110^{o}C	DDGS samples at dryer outlet temperature of 149^{o}C
Complete Fermentation					
F2	3	1.2 lbs/min	4	1	1
F4	3	2.0 lbs/min	4	1	1
In addition, 6 DDG and 14 DDGS samples were collected from other fermentor runs					
Incomplete Fermentation					
F8	3	1.6 lbs/min	4	1	1
F9	3	2.2 lbs/min	4	1	1
In addition, 3 DDGS and 12 DDGS samples were collected from other fermentor runs					
Corn Samples	Slurry Samples	Mash Samples			
3	3	3		Total samples for this study	80

Table 2. Lysine, Furosine and Crude Protein (CP) Contents in Samples from Front End Operation (%, wt/wt, on dry basis)

	Lysine	Furosine	F/L (%)[@]	CP	L/CP (%)[#]
Corn 1	0.27	n.d.[*]	0	N.A.[**]	N.A.
Corn 2	0.27	n.d.	0	N.A.	N.A.
Corn 3	0.27	n.d.	0	N.A.	N.A.
Corn mean	**0.27**	**n.d.**	**0**	**N.A.**	**N.A.**
Slurry 1	0.27	0.01	2	8.7	3.1
Slurry 2	0.27	0.01	2	8.8	3.0
Slurry 3	0.26	0.01	3	8.8	3.0
Slurry mean	**0.27**	**0.01**	**2**	**8.8**	**3.0**
Mash 1	0.26	0.08	32	N.A.	N.A.
Mash 2	0.27	0.07	27	N.A.	N.A.
Mash 3	0.27	0.01	3	N.A.	N.A.
Mash mean	**0.27**	**0.05**	**20**	**N.A.**	**N.A.**

[*] n.d., not detected

[**] N.A., data not available

[@] F/L (%): the ratio of furosine to lysine times 100

[#] L/CP (%): the ratio of lysine to crude protein times 100

Table 3a. Lysine, Furosine and Crude Protein (CP) in Samples from Run F2 (%, wt/wt, on dry basis)

Sample Source	Lysine	Furosine	F/L (%)[@]	CP	L/CP (%)[#]
Early Ferm.[*]	0.29	0.07	24	N.A.[**]	N.A.
Active Ferm.[*]	0.52	0.07	13	N.A.	N.A.
End Ferm.[*]	1.12	0.18	16	N.A.	N.A.
Whole stillage	1.10	0.14	13	31.8	3.5
Wet cake	1.14	0.01	1	39.3	2.9
Thin stillage	0.61	0.29	48	19.6	3.1
Syrup	0.60	0.33	56	15.2	3.9
DDGS (149^{o}C)	1.03	0.08	8	33.5	3.1
DDGS (110^{o}C)	1.02	0.04	4	35.9	2.8

[*] Early Ferm., Active Ferm., End Ferm.: with fermentation time points of about 10 hr., 30 hr. and end of fermentation

[**] N.A., data not available

[@] F/L (%): the ratio of furosine to lysine times 100

[#] L/CP (%): the ratio of lysine to crude protein times 100

Table 3b. . Lysine, Furosine and Crude Protein (CP) in Samples from Run F4 (%, wt/wt, on dry basis)

Sample Source	Lysine	Furosine	F/L (%)[@]	**CP**	**L/CP (%)[#]**
Early Ferm.[*]	0.31	0.05	16	N.A.[**]	N.A.
Active Ferm. [*]	N.A.	0.13	N.A.	N.A.	N.A.
End Ferm. [*]	1.07	0.18	17	N.A.	N.A.
Whole stillage	0.92	0.15	17	30.6	3.0
Wet cake	1.24	0.01	1	39.9	3.1
Thin stillage	0.51	0.19	37	14.6	3.5
Syrup	0.53	0.28	53	14.9	3.5
DDGS (149^{o}C)	0.85	0.09	11	29.9	2.9
DDGS (110^{o}C)	0.96	0.03	3	31.3	3.1

[*] Early Ferm., Active Ferm., End Ferm., with fermentation time points of about 10 hr., 30 hr. and end of fermentation

[**] N.A., data not available

[@] F/L (%): the ratio of furosine to lysine times 100

[#] L/CP (%): the ratio of lysine to crude protein times 100

Table 3c. Lysine, Furosine and Crude Protein (CP) in Samples from Run F8 (%, wt/wt, on dry basis)

Sample Source	Lysine	Furosine	F/L (%)[@]	CP	L/CP (%)[#]
Early Ferm.[*]	0.35	0.05	14	N.A.[**]	N.A.
Active Ferm.[*]	0.59	0.12	20	N.A.	N.A.
End Ferm.[*]	0.74	0.12	16	N.A.	N.A.
Whole stillage	0.79	0.15	19	33.7	2.3
Wet cake	1.01	0.03	3	34.4	2.9
Thin stillage	0.51	0.21	40	16.3	3.2
Syrup	0.53	0.26	50	13.2	4.0
DDGS (149^{o}C)	0.58	0.21	36	30.1	1.9
DDGS (110^{o}C)	0.80	0.07	9	27.8	2.9

[*] Early Ferm., Active Ferm., End Ferm., with fermentation time points of about 10 hr., 30 hr. and end of fermentation

[**] N.A., data not available

[@] F/L (%): the ratio of furosine to lysine times 100

[#] L/CP (%): the ratio of lysine to crude protein times 100

Table 3d. Lysine, Furosine and Crude Protein (CP) in Samples from Run F9 (%, wt/wt, on dry basis)

Sample Source	Lysine	Furosine	F/L (%)[@]	CP	L/CP (%)[#]
Early Ferm.[*]	0.45	0.04	9	N.A.[**]	N.A.
Active Ferm.[*]	0.62	0.07	11	N.A.	N.A.
End Ferm.[*]	0.90	0.16	18	N.A.	N.A.
Whole stillage	0.93	0.16	17	28.0	3.3
Wet cake	1.01	0.02	2	34.4	2.9
Thin stillage	0.51	0.28	53	11.8	4.3
Syrup	0.53	0.25	47	12.2	4.3
DDGS (149^{o}C)	0.54	0.21	39	29.2	2.0
DDGS (110^{o}C)	0.82	0.05	6	29.6	2.5

[*] Early Ferm., Active Ferm., End Ferm., with fermentation time points of about 10 hr., 30 hr. and end of fermentation

[**] N.A., data not available

[@] F/L (%): the ratio of furosine to lysine times 100

[#] L/CP (%): the ratio of lysine to crude protein times 100

Table 4a. Lysine, Furosine and Crude Protein (CP) in DDG (%, wt/wt, on dry basis)

	Lysine	Furosine	F/L (%)[@]	CP	L/CP[#] (%)	Syrup rate (lbs/min.)	Dryer T (^{o}C)
Complete fermentation							
DDG 1	1.27	0.02	2	40.2	3.2	0	110
DDG 2	1.29	0.02	2	40.3	3.2	0	110
DDG 3	0.98	0.04	4	44.8	2.2	0	110
DDG 4	1.24	0.03	2	38.7	3.2	0	129
DDG 5	1.06	0.05	5	40.2	2.6	0	129
DDG 6	1.16	0.09	8	38.8	3.0	0	149
Incomplete fermentation							
DDG 1	1.00	0.05	5	39.4	2.5	0	110
DDG 2	1.03	0.03	3	38.2	2.7	0	110
DDG 3	1.10	0.03	3	38.4	2.9	0	129

[@] F/L (%): the ratio of furosine to lysine times 100

[#] L/CP (%): the ratio of lysine to crude protein times 100

Table 4b. Lysine, Furosine and Crude Protein (CP) in DDGS from Complete Fermentation Runs (%, wt/wt, on dry basis)

	Lysine	Furosine	F/L (%)[@]	CP	L/CP[#] (%)	Syrup rate (lbs / min.)	Dryer T (°C)
DDGS 1	1.02	0.04	4	35.9	2.8	1.2	110
DDGS 2	0.95	0.03	3	33.7	2.8	1.2	110
DDGS 3	0.98	0.05	5	34.9	2.8	1.2	129
DDGS 4	1.03	0.08	8	34.3	3.0	1.2	129
DDGS 5	1.03	0.08	8	34.3	3.1	1.2	149
DDGS 6	0.90	0.09	10	35.7	2.5	1.2	149
DDGS 7	1.08	0.03	3	34.0	3.2	1.6	110
DDGS 8	1.07	0.03	3	35.0	3.0	1.6	110
DDGS 9	0.96	0.08	8	31.8	3.0	1.6	129
DDGS 10	0.97	0.07	7	31.1	3.1	1.6	129
DDGS 11	0.81	0.08	10	29.5	2.7	1.6	149
DDGS 12	0.79	0.10	13	29.4	2.7	1.6	149
DDGS 13	0.96	0.03	3	31.3	3.1	2.0	110
DDGS 14	0.97	0.03	3	32.9	3.0	2.0	110
DDGS 15	0.95	0.06	6	30.4	3.1	2.0	129
DDGS 16	0.98	0.07	7	33.0	3.0	2.0	129
DDGS 17	0.85	0.09	11	29.9	2.9	2.0	149
DDGS 18	0.82	0.11	13	31.9	2.6	2.0	149

@ F/L (%): the ratio of furosine to lysine times 100

L/CP (%): the ratio of lysine to crude protein times 100

Table 4c. Lysine, Furosine and Crude Protein (CP) in DDGS Samples from Incomplete Fermentation Runs (%, wt/wt, on dry basis)

	Lysine	**Furosine**	**F/L (%)[@]**	**CP**	**L/CP (%)[#]**	**Syrup rate (lbs / min.)**	**Dryer T (^{o}C)**
DDGS 1	0.80	0.07	9	27.8	2.9	1.6	110
DDGS 2	0.58	0.20	34	28.4	2.0	1.6	129
DDGS 3	0.77	0.10	13	29.0	2.6	1.6	129
DDGS 4	0.58	0.21	36	29.9	1.9	1.6	149
DDGS 5	0.78	0.05	6	31.5	2.5	1.9	110
DDGS 6	0.76	0.07	9	28.6	2.7	1.9	110
DDGS 7	0.73	0.05	7	28.3	2.6	1.9	129
DDGS 8	0.68	0.12	18	25.7	2.7	1.9	129
DDGS 9	0.55	0.22	40	28.5	1.9	1.9	149
DDGS 10	0.52	0.21	40	26.5	2.0	1.9	149
DDGS 11	0.54	0.21	39	26.6	2.0	1.9	149
DDGS 12	0.82	0.05	6	32.1	2.5	2.2	110
DDGS 13	0.71	0.05	7	27.6	2.6	2.2	110
DDGS 14	0.70	0.15	21	26.7	2.6	2.2	129
DDGS 15	0.67	0.11	16	26.7	2.5	2.2	129
DDGS 16	0.54	0.21	39	25.6	2.1	2.2	149

@ F/L (%): the ratio of furosine to lysine times 100

L/CP (%):the ratio of lysine to crude protein times 100

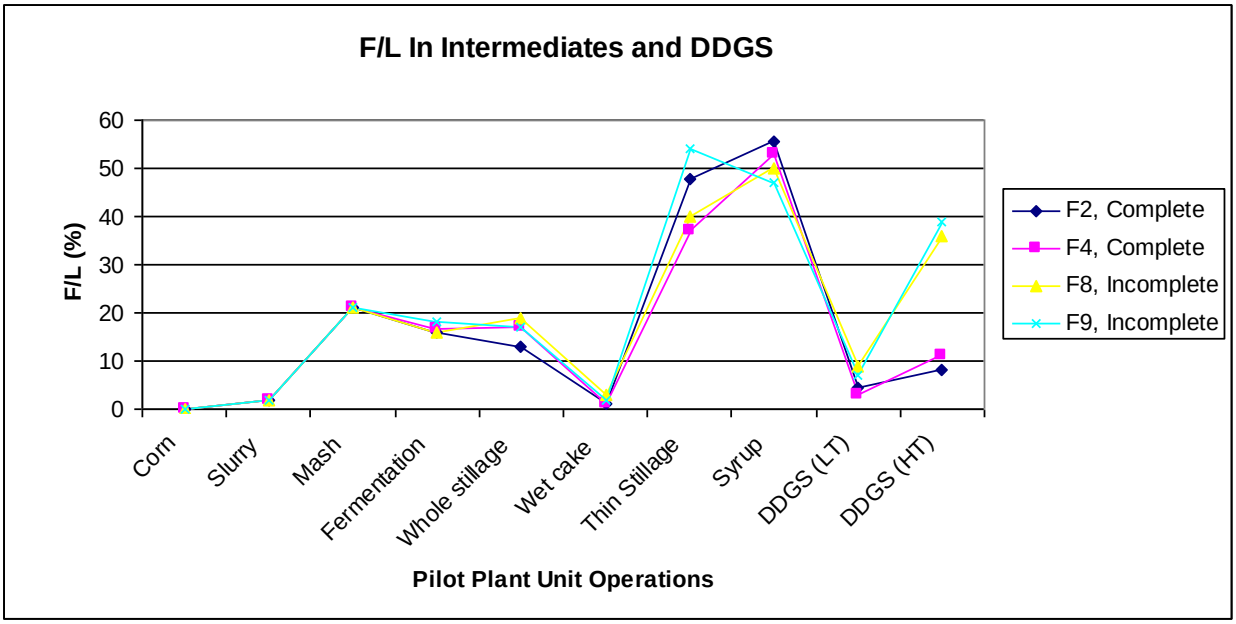

Fig. 1 The change of the percentage of furosine in lysine (F/L) during the bioprocess of making DDGS.

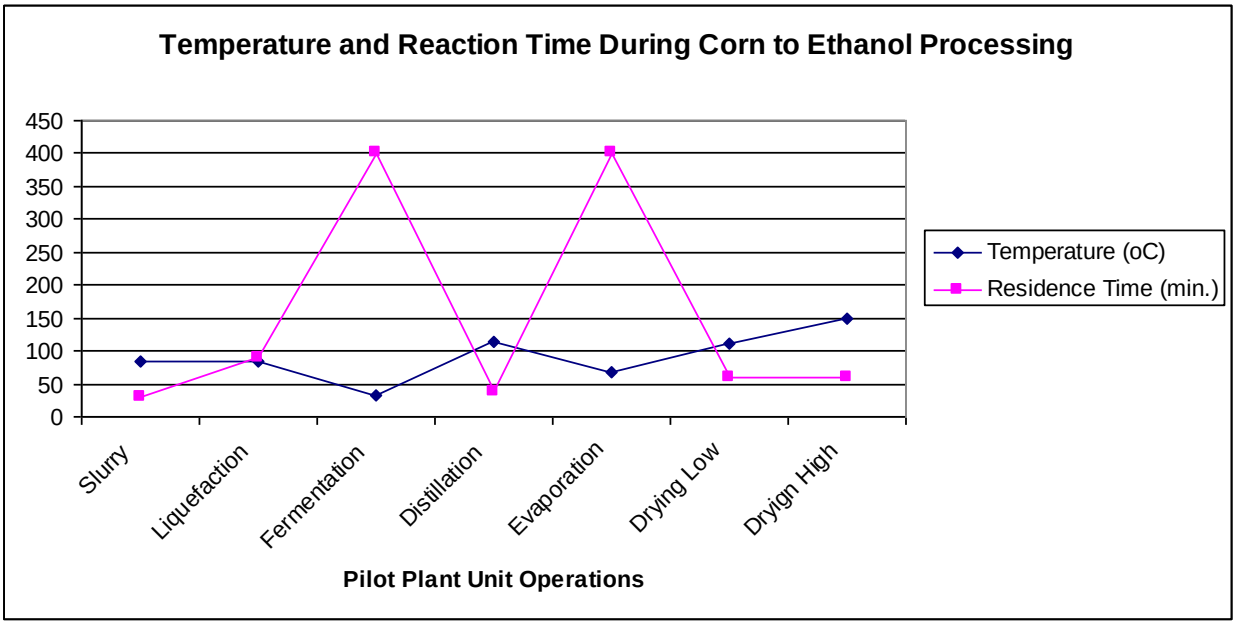

Fig. 2 The temperature and residence time of plant unit operations.

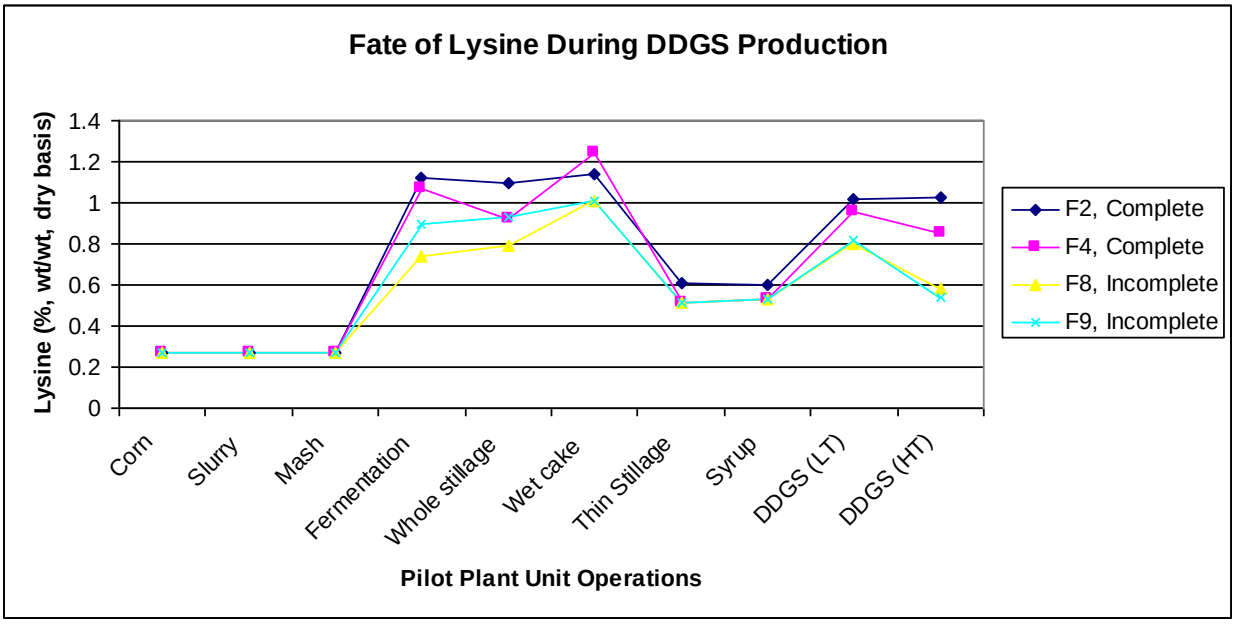

Fig. 3 The change of lysine content during the bioprocess of making DDGS.

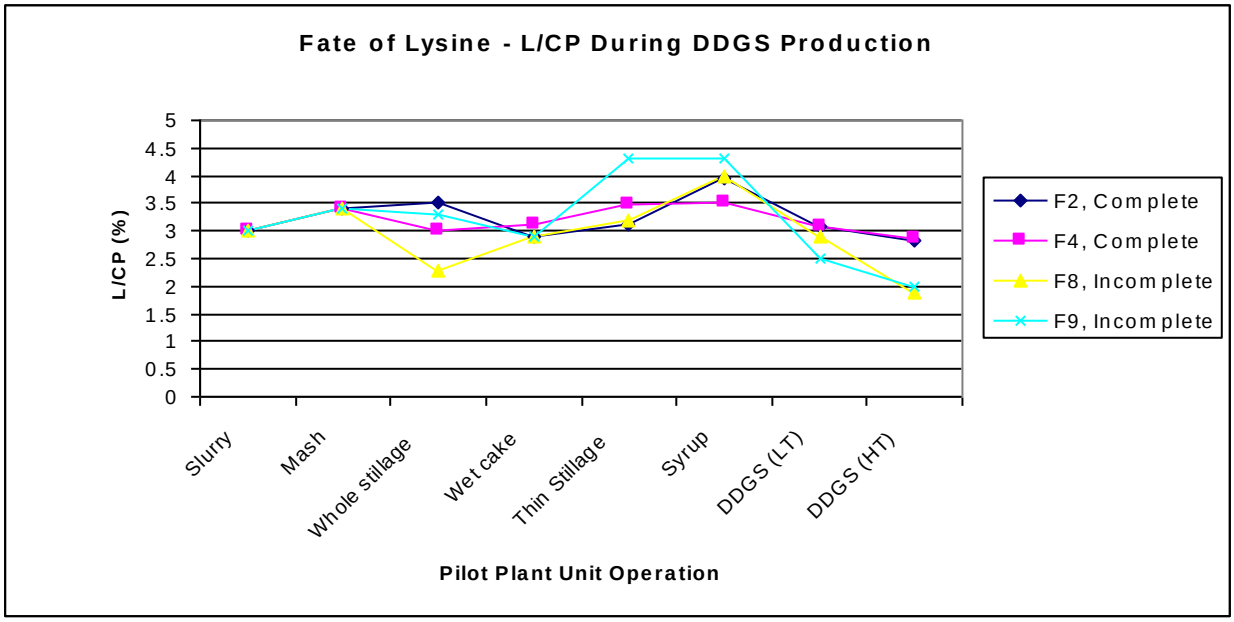

Fig. 4 The change of the percentage of lysine in crude protein (L/CP) during the bioprocess of making DDGS.

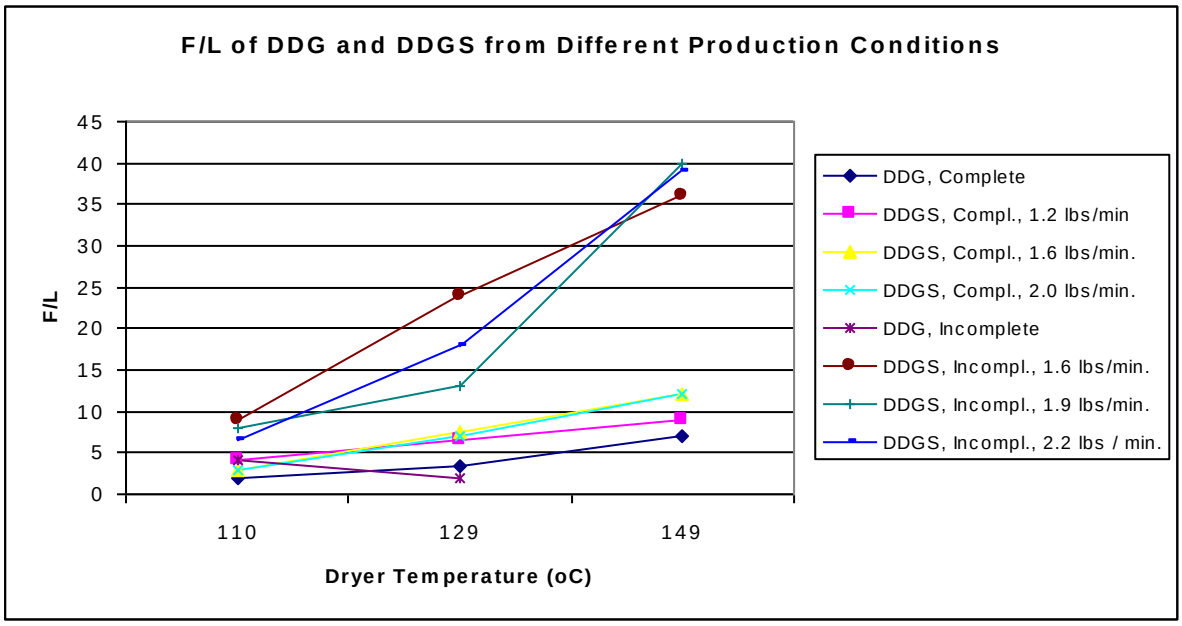

Fig. 5 The percentage of furosine in lysine (F/L) of DDG and DDGS generated from various processing conditions

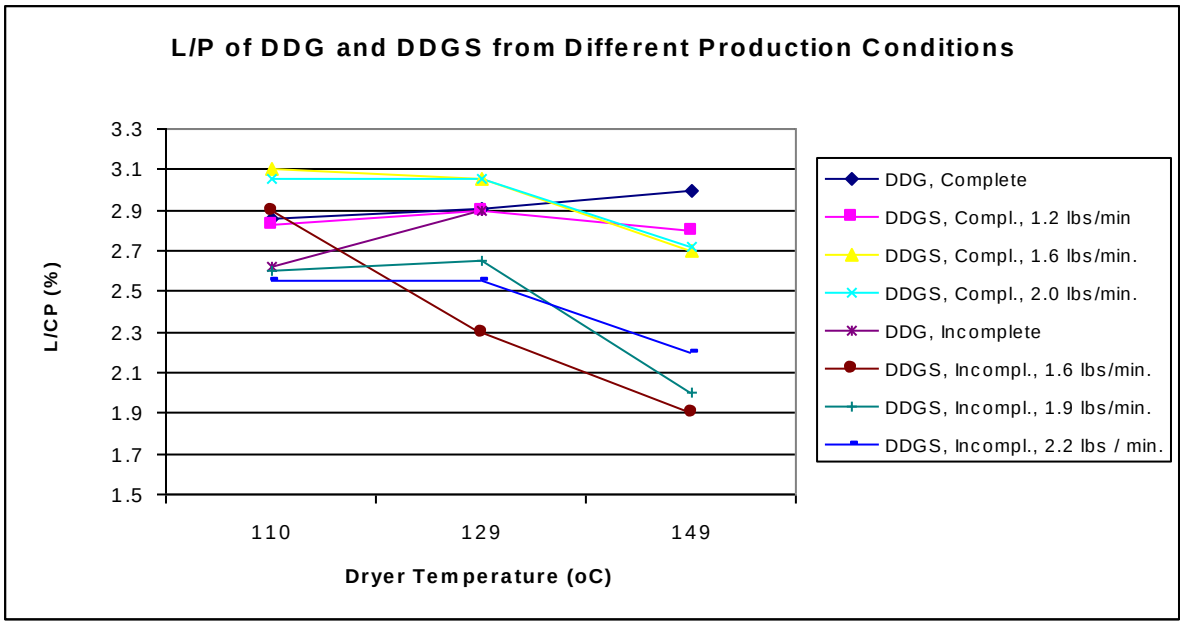

Fig. 6 The percentage of lysine in crude protein (L/CP) of DDG and DDGS generated from various processing conditions

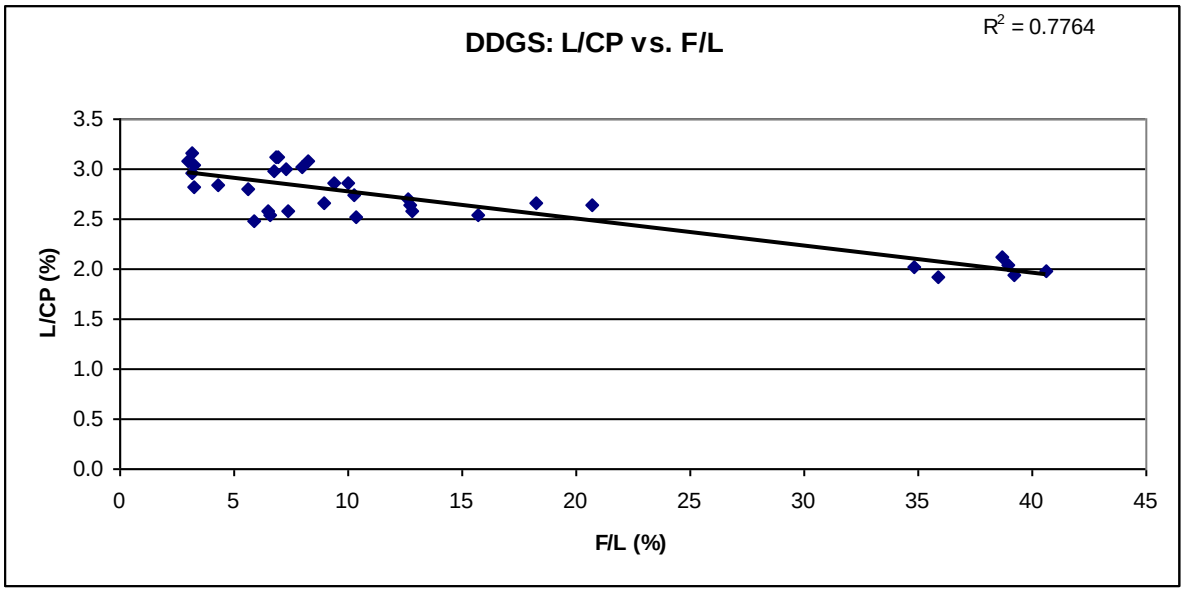

Fig. 7 The percentage of lysine in crude protein (L/CP) versus the percentage of furosine in lysine (F/L) in DDGS generated from various processing conditions.

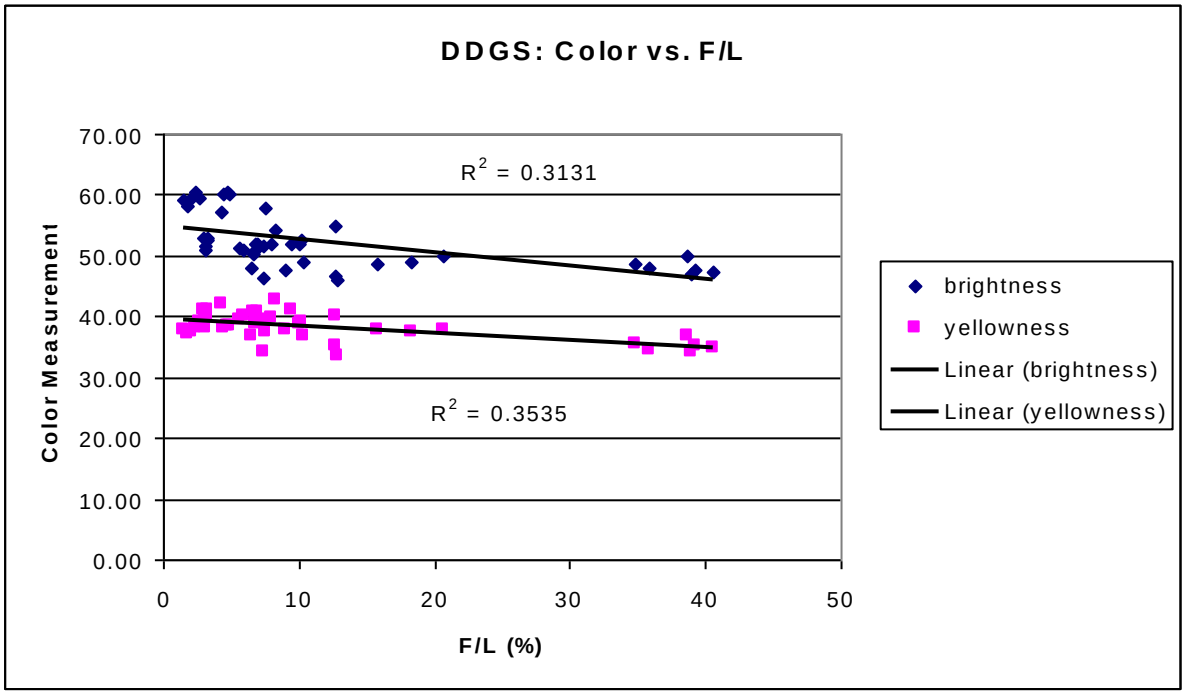

Fig. 8 The color measurement from Hunter Lab, brightness and yellowness versus the percentage of furosine in lysine (F/L).

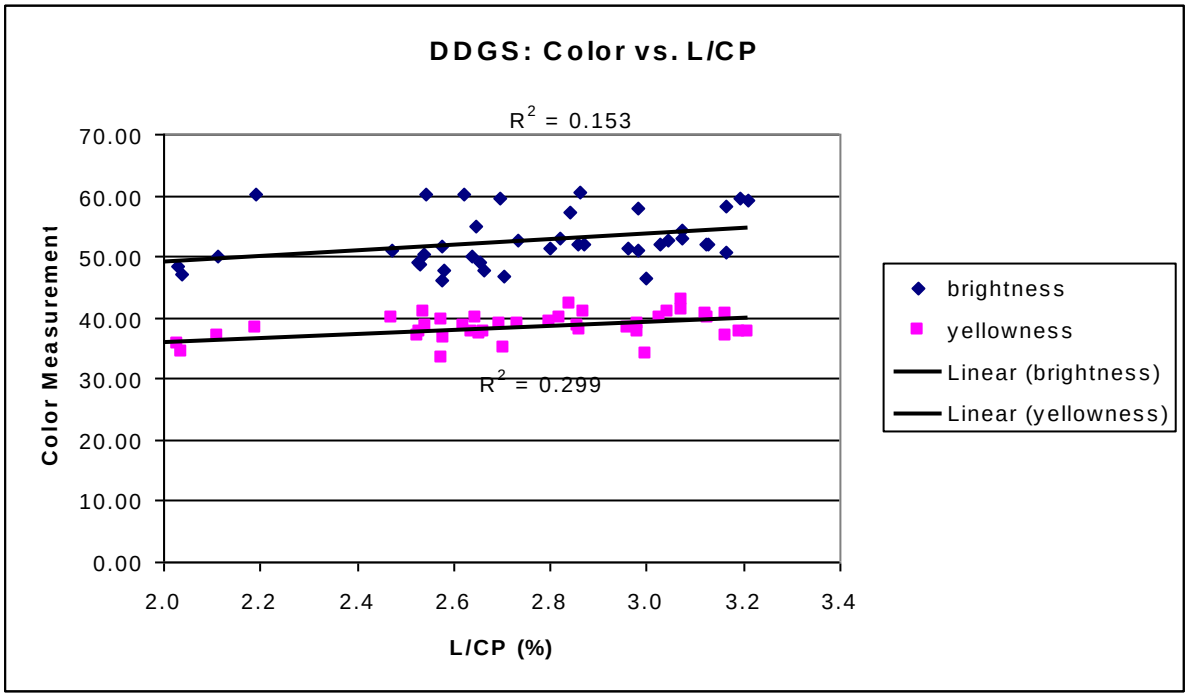

Fig. 9 The color measurement from Hunter Lab, brightness and yellowness versus the percentage of lysine in crude protein (L/CP).

Final Report for National Pork Board, Project #09-119

Y. Zhang

National Corn-to-Ethanol Research Center (NCERC),

Southern Illinois University Edwardsville;

Part B

(Fate of **Phytate** During the Bioprocess of Making DDGS)

Table of Contents

II. Project Title: Fate of Phytate During the Bioprocess of Making DDGS

National Pork Board Project Identification Number: #09-119

Principal Investigator: Yanhong Zhang, Ph.D, Assistant Director, Analytical Chemistry

Institution: National Corn-to-Ethanol Research Center (NCERC)

Date final report submitted: November 15, 2010.

III. Industrial Summary

This objective of this study is to monitor the changes in phytate concentration through the unit operations and processes that are used to make fuel ethanol. The concentrations of phytate and total phosphorus will be measured in the corn used as the feedstock in a pilot-plant trial at NCERC, the distiller's dried grains with solubles (DDGS) that is produced as the final coproduct, and at important intermediate steps in the process, including liquefaction, fermentation, distillation, and coproduct processing (*e.g.*, the concentrations will be measured in wet cake, thin stillage, and syrup).

Phosphorus (P) is an important nutrient in swine feed. More than 80% of the P in corn is in the form of phytate P which is not bioavailable, but the data on various P forms in DDGS are rare and the P bioavailability of DDGS is not well understood. In this study, we conducted a pilot-plant trial to investigate the effects of corn-to-ethanol unit operation conditions on the conversion of phytate P to inorganic P which is bioavailable in intermediates and DDGS. The operational parameters of the bioprocess we focused on included fermentation completeness (reducing sugar level), syrup addition rate (ratio of syrup to wet cake), and dryer outlet temperature. A total of forty-five samples were analyzed and they included corn flour, slurry, mash, fermentation broth, whole stillage, thin stillage, syrup, wet cake and DDGS. What we learned from this study is summarized in the following:

1. Among all the unit operations of the bioprocess of making DDGS, the fermentation process was the only unit operation which had a strong impact on the conversion of phytate P to inorganic P, with complete fermentation causing stronger conversion of phytate P to inorganic P than incomplete fermentation.

2. While the percentage of phytate P in total P was about 80% in corn as feedstock, that percentage decreased to 40% in DDGS generated from complete fermentation, and to 50% in DDGS generated from incomplete fermentation.

3. The unit operation of centrifuging changed the absolute level of phytate P and total P in wet cake and thin stillage due to mass partition, but the ratio of phytate P to total P stayed close to that of fermentation end product and DDGS.

4. It is confirmed that the yeast strain used for the corn to ethanol production was able to produce phytase to convert phytate P to bioavailable P. The more vital the yeast, the stronger the conversion of phytate P to bioavailable P.

It is highly recommended that dry grind ethanol plants monitor and control fermentation in order to produce more bioavailable P in DDGS. Growing healthy yeast is the key to produce more bioavailable P in DDGS. Including DDGS in swine diets can certainly help to reduce the amount of inorganic phosphate and phytase needed.

III. Keywords DDGS, phosphorus bioavailability, phytate, total phosphorus

IV. Scientific Abstract

More than 80% of phosphorus (P) in corn is in phytate P form, which is not bioavailable. As a result, it is common to include inorganic phosphate and phytase in swine diets to help to improve phosphorus digestibility and reduce environmental impact caused by high phosphate concentrations in manure. The yeast used in fermentation during the bioprocess of making distiller's dried grains with solubles (DDGS) is likely to be able to produce phytase, but how much of the phytate P can be converted to bioavailable P during fermentation is not clear, and whether any other unit operations of the bioprocess of making DDGS have any impact on the change of P speciation is unknown. In this study, we measured the changes in the levels of total P, phytate P and inorganic P in intermediate products and DDGS produced from four different pilot plant runs with various combinations of processing parameters. We learned that the fermentation process was the only unit operation contributing to the phytate P degradation to inorganic P. Our study showed that the percentage of phytate P in total P went from 80% in corn to 40% in DDGS generated from complete fermentation, and to 50% in DDGS generated from incomplete fermentation. The intermediates generated in the back end, such as wet cake and thin stillage, contained different levels of total P and phytate P from DDGS, but the percentage of phytate P in total P was similar to that of DDGS. The key to generate DDGS with high bioavailable P is to grow healthy yeast during fermentation process.

V. Introduction

The tremendous growth in the fuel-ethanol industry has been accompanied by concomitant growth in the production of distiller's dried grains with solubles (DDGS), and the potential for increased use of DDGS as animal feed is great. DDGS can be a valuable ingredient in animal feeds because it contains higher concentrations of essential nutrients than corn (e.g., amino acids, phosphorus) (Stein, 2007). More than 80% of the P in corn is in the form of phytate P which is not bioavailable, but the data on various P forms in DDGS are rare and the P bioavailability of DDGS is not well understood.

The concentration of phosphorus, like other nonfermentable components, increases by a factor of about three in DDGS produced from corn, because starch represents more than two-thirds of the dry mass of corn and it will be converted to ethanol and carbon dioxide during the production of DDGS. Phytate, myo-inositol hexaphosphate (IP6), is a major phosphorus storage form in corn. A confirmed antinutrient, phyate P is not nutritionally available to growing pigs due to its ability to bind minerals and proteins to alter their solubility, functionality, digestibility and absorption (Pointillart *et al*., 1984, 1985, Rickard and Thompson, 1997). In addition to its lack of nutritional benefit, phytate P in swine feed can cause environmental problems due to the resulting high phosphate concentrations in manure. Besides phytate, there are other inositol

phosphates occurring in corn and they are myo-mono, bis-, tris, tetrakis- and pentakisphosphates, or simply IP1, 2, 3, 4 and 5.

It was reported that the bioavailability of phosphorus in DDGS is higher than that in the grain that is used as the feedstock for fermentation (Widyaratne and Zijlstra, 2007), and the available fraction of total phosphorus in DDGS can vary from about 50% to 100% (Pedersen *et al*., 2007; Widmer *et al*., 2007; Amezcua and Parson, 2007). Microorganism like yeast is known to be able to produce phytase enzyme to convert phytate P via penta- to monophosphates and orthophosphate through a stepwise IP6 hydrolysis, therefore helping to increase phosphorus bioavialability (Walz and Pallauf, 2002; Minihane and Rimbach, 2002). Some researchers suggest that fermentation increases phosphorus availability (Mahajan and Chauhan, 1988; Reale *et al*., 2004), whereas others suggest that heating is important (Amezcua and Parson, 2007). Understanding the changes that occur in phytate concentration through the ethanol production process will not only improve the ability of swine nutritionists to evaluate the contribution of DDGS to available phosphorus in swine diets, but it will also help to predict, and thus minimize the environmental impacts of swine excreta.

In this study, we conducted several pilot-scale plant runs to investigate the effects of corn-to-ethanol unit operation conditions on the concentrations of total phosphorus (P), phytate P and inorganic P (P from orthophosphate and pyrophosphate) in process intermediates and DDGS. We used the ratio of phytate P to total P to monitor the degradation of phytate P during the bioprocess of making DDGS, and the ratio of inorganic P to total P to verify the conversion of phytate P to the end product.

VI. Stated Objectives from Original Proposal

This objective of this study is to monitor the changes in phytate concentration through the unit operations and processes that are used to make fuel ethanol. The concentrations of phytate and total phosphorus will be measured in the corn used as the feedstock in a pilot-plant trial at NCERC, the DDGS that is produced as the final coproduct, and at important intermediate steps in the process, including liquefaction, fermentation, distillation, and coproduct processing (*e.g.*, the concentrations will be measured in wet cake, thin stillage, and syrup). All the samples for the study will come from a pilot plant trial conducted at NCERC in cooperation with USDA scientists.

VII. Materials and Methods

Sample preparation: Except for the corn flour, fermentation broth and DDGS samples, all the other samples were freeze dried using a Millrock MD53 freeze dryer to get rid of the high moisture level in the original samples. The residual moisture level in the dried samples was measured using a moisture balance (Mettler Toledo HR83) in order to calculate total P, phytate P and inorganic P in each sample on a dry matter basis. All the tests related to P forms were performed at a commercial testing laboratory.

Total P test: The sample was ashed in a muffle furnace. The ash residue was dissolved in dilute acid, and the solution was analyzed using a Technicon Auto-Analyzer system, where the solution was treated with molybdic-sulfuric acid reagent, forming phosphomolybdate. Stannous chloride-hydrazine sulfate was added to reduce the phosphomolybdate to produce a blue color. The concentration of P was determined by comparing the absorbance of the sample solution to

the absorbance of standard solutions using colorimetric spectroscopy. The method was validated, and the repeatability of the entire method was about 3.5%, the recovery for QC standard was around 95%, and the detection limit in DDGS was 0.01%. This test was based on the method of AOAC 965.17.

Phytate test: Phytic acid was extracted with diluted hydrochloric acid solution and separated from inorganic phosphate on an anion exchange column. Phytate was eluted with a sodium chloride solution. The eluate was digested with sulfuric /nitric acid, freeing phosphorus, which was reacted with ammonium molybdate and sulfonic acid solutions, forming a blue color complex which was measured spectrophotometrically. Phosphorus concentration was quantitated from a set of standards of known concentration that were taken through the color reaction. The phytate P obtained from this test came from the inositol phosphates of IP6, 5 and 4, and the P of IP1, 2 and 3 was not accounted for by this method. The method was validated and the repeatability of the entire method was about 5%, the recovery for QC standard was about 95% and the detection limit for phytate in DDGS was 0.01%. This test was based the method of AOAC 998.16.

Orthophosphate test: The sample was defatted using petroleum ether. Orthophosphate and pyrophosphate were extracted with dilute hydrochloric acid solution. Sample extracts were injected on ion chromatograph with a conductivity detector and quantitated from a set of standards of known concentration that were injected on the IC. The phosphorus from orthophosphate and pyrophosphate was reported as Inorganic P. The method was validated, and the repeatability of the entire method was lower than 5%, the recoveries for QC standard was about 95%, and the detection limit for lysine in DDGS was 0.01%. This test was adapted from a method provided by Dionex.

Pilot plant trial: Samples were generated in a multiple fermentor pilot plant trial which was supported by USDA. The study focused on three operational parameters for DDGS production: fermentation completeness (reducing sugar level), syrup addition rate (ratio of syrup to wet cake), and dryer outlet temperature. Each combination of the factors constituted a single condition, and each condition was run in independent duplicates. Independence of the replicates was insured by using separate fermenters for each. For this study, we focused on four fermentor runs (Table 1), and samples representing unit operations from those runs were collected.

For front end operation, slurry (32% dry solids, w/w) was prepared by milling No. 2 yellow-dent corn using a hammer mill equipped with a 7/64-inch (2.78 mm) screen, and mixing the flour with hot water (85°C) and alpha-amalyse for 30 min. The slurry was pumped through a jet cooker, where it was heated to 106°C for 7 min, then into a liquefaction tank (mash tank) where additional α-amalyse was added and the temperature was maintained at 85°C for 90 min. Since the front end operation was the same for all fermentor runs, we collected triplicate samples of corn flour, slurry and mash for this study.

For fermentation, about 30,000 lbs of mash was transferred into each fermentor over a 24-hr period. The fermenters were inoculated with yeast about 1 hour after filling began. Fermentation lasted approximately 50 hours, and the temperature was maintained at 32^{o}C throughout. The average final concentration of total sugars (sum of the concentrations of DP4+, DP3, DP2, and glucose, where DP*x* represents oligosaccharides consisting of *x* glucosyl units) was 1.0 ± 0.3% (w/v) for complete fermentors and 3.7 ± 1.7% (w/v) for incomplete fermentors. Incomplete fermentation was induced by limiting the nitrogen available to support yeast growth:

nitrogen was supplied as urea to a final concentration of 500 mg nitrogen/kg mash for the complete fermentors and 250 mg nitrogen/kg mash for incomplete fermentors. The fermentation broth samples were collected at three fermentation time points (about 10 hr., 30 hr. and end of fermentation) to represent early, active and late stage of fermentation from each fermentor (Table 1).

For back end operation, the fermentation broth was pumped through a continuous distillation system to separate ethanol from the nonvolatile residue, which is called whole stillage. The distillation system operated at a pressure of about 6 psig (140 kPa), and the whole stillage was kept at 113°C for about 38 minutes before being separated into solid (wet cake) and liquid (thin stillage) fractions by continuous centrifugation in a decanter centrifuge operating at 3500 rpm. Syrup was produced from the thin stillage by evaporation under vacuum at 68°C. The total solids were concentrated from 5% to 40% over about a 15 hour period. DDGS was produced by mixing syrup, wet cake, and recycled DDGS, and the mixture was introduced into a direct-fired Davenport rotary drum dryer. Approximately 20% of the material that exited the dryer was pneumatically conveyed to DDGS storage, while the remaining 80% was recycled back to the mixer where syrup and wet cake were added. The temperature at the outlet of the dryer was used as a measure of the temperature of DDGS production, and it varied from 93^{o}C to 149^{o}C. The residence time of DDGS inside the dryer was about 60 min. From each fermentor (Table 1), one sample of whole stillage, wet cake, thin stillage and syrup, and two samples of DDGS with different dryer temperatures were collected.

In total, 45 samples were collected and tested for the concentrations of total P and phytate P and among them, 15 were tested for inorganic P.

VIII. Results

All of the concentrations reported in this study are on a dry weight basis and calculated based on elemental P in each P forms. To better evaluate the fate of phytate, we calculated the percentage of phytate P in total P as PP/TP, and the percentage of inorganic P in total P as IP/TP.

Table 2 lists the concentrations of total P, phytate P and inorganic P in samples collected from the front end operation. The results showed that within analytical uncertainty, the levels of total P, phytate P and inorganic P were similar in corn flour, slurry and mash: total P was about 0.32%, phytate P about 0.25% and inorganic P about 0.03%, which led to the PP/TP about 80% and IP/PP around 10%.

Table 3a through 3d list the concentrations of total P and phytate P in samples collected from fermentation and the following back end unit operations, and inorganic P in some samples from F2 and F4. Each table represents one fermentor run (Fermentor 2, 4, 8, 9, Table 1). The data from each fermentor showed similar trends in the change of total P and phytate P in fermentation broth samples. Both total P and phytate P in fermentation broth increased with fermentation time, and the PP/TP decreased from 60% to 40% for complete fermentation, and from 55% to 50% for incomplete fermentation. For F2 and F4, the IP/PP started high around 95%, but ended between 30% and 50% in the fermentation end sample.

For whole stillage, the total P was about 1.0%, the phytate P was about 0.4% which gave the PP/TP close to 40% (Table 3). For wet cake, the total P was about 0.50%, phytate P was

about 0.25% and PP/TP was around 50%. For thin stillage and syrup from complete fermentation, the total P was around 1.90%, phytate P was around 0.7% and PP/TP around 35%, while for thin stillage and syrup from incomplete fermentation, the total P was around 1.60%, phytate P was around 0.7% and PP/TP around 40% (Table 3). For DDGS from complete fermentation, the total P was around 0.9%, phytate P around 0.35% and PP/TP around 40%; and for DDGS from incomplete fermentation, the total P was around 0.9%, phytate P was around 0.45% with PP/TP around 50% (Table 3a – d).

For F2, the IP/PP was about 30% for whole stillage, 19% for wet cake, 40% for thin stillage and syrup and 30% for DDGS.

IX. Discussion

Unit Operations of Corn to DDGS Bioprocess

The total P in the feedstock of No. 2 yellow-dent corn was 0.30% (Table 2), which is slightly higher than the reported value (Batal and Dale, 2010). Over 80% of the total P in corn and slurry was phytate P, which agrees with the reported value (Punna and Roland, 2001). The front end operations does not seem to have any impact on the total P or phytate P, even though the distillation process involves high temperature and decent residence time. When comparing total P with the sum of phytate P and inorganic P for each sample, there is about 10% of total P unaccounted for, which is likely to be related to the P in IP1, 2 and 3.

During fermentation, both the total P and phytate P in the broth increased because the total dry matter in the fermentation broth decreased due to the production of carbon dioxide and ethanol from starch. The reason that the total P in the drop samples from complete fermentation was slightly higher than that in the drop samples from incomplete fermentation, is due to the higher residual sugars in the drop samples from incomplete fermentation. The obvious change in PP/TP occurred during fermentation: the PP/TP of the fermentation broth from complete fermentation decreased from 65% to 40%, and the PP/TP of the fermentation broth from incomplete fermentation decreased from 55% to 50%. It is clear that the fermentation process had led to the degradation of phytate P to inorganic P, because the IP/PP of fermentation broth increased from 10% in slurry and mash to between 30% and 50% in fermentation end sample (Table 3a, 3b). In addition, between 10% and 20% of total P in fermentation end samples were related to IP1, 2 and 3. It is not clear why the early fermentation sample contained extremely high level of inorganic P. It could be an analytical error caused by interference from other ions in the early fermentation samples.

The total P in whole stillage was around 0.9%, which is almost three times the level in corn, and within analytical uncertainty, the total P and phytate P in whole stillage were very close to those in the fermentation drop sample (Table 3), indicating that the distillation process did not impact on the change of phytate P. According to our previous study (Wrenn and Caupert, 2010), after centrifuging, mass partition would result in different compositions of major ingredients in wet cake and thin stillage. This explains why wet cake contained lower total P and phytate P but higher PP/TP than whole stillage, and thin stillage and syrup contained higher total P and phytate P and similar PP/TP to whole stillage. It seems that the thin stillage and syrup from complete fermentation contained relatively lower PP/TP than those from incomplete fermentation, which agrees with the data from the fermentation broth that complete fermentation

caused higher conversion of phytate P. The fact that there was not obvious difference in the total P, phytate P and PP/TP between thin stillage and syrup, indicates that evaporation process did not impact on the change of phytate P.

The total P, phytate P and PP/TP in DDGS was very similar to those of the fermentation end sample, indicating that the back end unit operations did not impact on P speciation.

In conclusion, during the bioprocess of making DDGS, the fermentation process was the only unit operation which had a strong impact on the conversion of phytate P to inorganic P, with complete fermentation leading to higher conversion of phytate P to inorganic P than incomplete fermentation. The mass partition of centrifuging changed the absolute levels of total P and phytate P in wet cake, thin stillage and syrup, but the PP/TP of wet cake and thin stillage was close to that of fermentation end samples. In corn, more than 80% of P is phytate P, the phytate in total P dropped to 40% in DDGS generated from complete fermentation and to 50% in DDGS generated from incomplete fermentation.

Fate of Phytate P during the Bioprocess of Making DDGS

Regarding the fate of phytate during the bioprocessing of making DDGS, the phytate P stayed at 0.25% during the front end operation, increased to around 0.4% in whole stillage, and after centrifuging, was around 0.25% in wet cake and 0.6% in thin stillage and syrup. The phytate P ended with around 0.40% in DDGS (Table 3). To better evaluate the phytate P change, we plotted PP/TP against unit operations during the corn to DDGS bioprocess (Fig. 1). It shows that the PP/TP stayed at 80% during the front end operation, decreased drastically during fermentation to between 40% and 50%, and stayed close to the PP/TP of the fermentation drop sample among the back end products with slight change in wet cake, thin stillage and syrup due to mass partition.

In order to better understand how the phytate P degraded, we compared the IP/TP, PP/TP, and (TP-PP-IP)/TP of samples from F2 and F4 (Fig. 2), with the (TP-PP-IP) representing P from the IP1, 2 and 3 which are likely the intermediate products of phytate P degradation to inorganic P and more bioavailable than IP4, 5 and 6. The IP/TP of slurry and mash stayed at 10% during the front end of operation, increased drastically during fermentation and ended between 30% and 50% in fermentation end sample, whole stillage and DDGS. The (TP-PP-IP)/TP started around 10% in the front end operation, stayed around 10% for F2 fermentation and increased to 25% for F4 fermentation, and stayed close to 10% in DDGS from F2. The inorganic P is the direct indicator for P bioavailability and the DDGS produced from complete fermentation contained between 30% and 50% bioavailable P, and the additional 20% P from the inositol phosphates of IP1, 2 and 3 in DDGS could be easily converted to inorganic P.

Conversion of Phytate P by Yeast Fermentation

The data from the four fermentation runs confirmed that the yeast strain used in this study was able to produce phytase to convert phytate P in corn to inorganic P. When the yeast growth was prohibited by limited supply of nutrients during incomplete fermentation, the conversion of phytate P to inorganic P was markedly slowed down comparing with what happened during complete fermentation, and as a result, the PP/TP of fermentation end sample from incomplete fermentation was about 50% versus the PP/TP of fermentation end sample from complete

fermentation of about 40%. The difference of PP/TP between different fermentation end samples carried through to the DDGS produced later.

Even with the same fermentation condition, such as F2 vs. F4, the fermentation of F4 led to higher conversion of phytate P to inorganic P comparing with that of F2, because the IP/PP of fermentation end sample from F4 is higher and the (TP-PP-IP)/PP of fermentation end sample from F4 is lower (Fig. 3). It is not clear why the same yeast performed differently with the two complete fermentation runs.

Overall, the phytate P degradation during fermentation is controlled by the yeast performance. It seemed that when yeast was active and healthy, the conversion of phytate P to inorganic P was easier.

Implications for Ethanol Industry and Animal Feed Industry

Over 80% of phosphorus in corn was phytate P, after complete fermentation, about half of the phytate in fermentation mash was converted into inorganic P, which led to the bioavailability of P in DDGS between 30% and 50%. Comparing with corn, the high bioavailability of P in DDGS has made it possible to reduce the amount of inorganic phosphate and phytase that are included in swine diets.

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Table 1: The Selection of Samples from the USDA Plant Trial

Fermentor No.	Fermentation samples	Syrup addition rate	Samples of whole stillage, wet cake, thin stillage and syrup	DDGS samples at dryer outlet temperature of 110^{o}C	DDGS samples at dryer outlet temperature of 149^{o}C
Complete Fermentation					
F2	3	1.2 lbs/min	4	1	1
F4	3	2.0 lbs/min	4	1	1
Incomplete Fermentation					
F8	3	1.6 lbs/min	4	1	1
F9	3	2.2 lbs/min	4	1	1
Corn Samples	Slurry Samples	Mash Samples			
3	3	3		Total samples for this study	45

Table 2. Total P, Phytate P and Inorganic P in Samples from Front End Operation (%, wt/wt, on dry basis)

	Total P	Phytate P	PP/TP (%)*	Inorganic P	IP/TP** (%)
Corn 1	0.31	0.28	89	N.A.***	N.A.
Corn 2	0.32	0.23	72	0.03	10
Corn 3	0.27	0.23	84	N.A.	N.A.
Corn mean	**0.30**	**0.24**	**82**	N.A.	N.A.
Slurry 1	0.33	0.26	78	N.A.	N.A.
Slurry 2	0.32	0.25	77	0.02	6
Slurry 3	0.30	0.26	86	N.A.	N.A.
Slurry mean	**0.32**	**0.26**	**80**	N.A.	N.A.
Mash 1	0.36	0.24	67	N.A.	N.A.
Mash 2	0.32	0.26	80	0.03	9
Mash 3	0.32	0.25	79	N.A.	N.A.
Mash mean	**0.33**	**0.25**	**76**	N.A.	N.A.

* PP/TP (%): the ratio of phytate P to total P times 100

** IP/TP (%): the ratio of inorganic P to total P times 100

*** N.A., data not available

Table 3a. Total P, Phytate P and Inorganic P in Samples from Run F2 (%, wt/wt, on dry basis)

Sample Source	Total P	Phytate P	PP/TP (%)*	Inorganic P	IP/TP** (%)
Early Ferm.@	0.33	0.21	64	0.32	95
Active Ferm. @	0.52	0.27	52	0.13	24
End Ferm. @	1.07	0.48	45	0.33	31
Whole stillage	0.96	0.41	43	0.31	32
Wet cake	0.44	0.22	49	0.08	19
Thin stillage	2.20	0.87	40	0.82	37
Syrup	1.89	0.73	38	0.84	44
DDGS (149^{o}C)	0.85	0.37	44	0.25	31
DDGS (110^{o}C)	0.77	0.35	45	0.26	33

* PP/TP (%): the ratio of phytate P to total P times 100

** IP/TP (%): the ratio of inorganic P to total P times 100

@ Early Ferm., Active Ferm., End Ferm.: with fermentation time points of about 10 hr., 30 hr. and end of fermentation

Table 3b. Total P, Phytate P and Inorganic P in Samples from Run F4 (%, wt/wt, on dry basis)

Sample Source	Total P	Phytate P	PP/TP (%)*	Inorganic P	IP/TP** (%)
Early Ferm.@	0.33	0.21	63	0.30	91
Active Ferm. @	0.48	0.29	60	0.11	23
End Ferm. @	0.99	0.40	41	0.50	50
Whole stillage	0.95	0.35	37	N.A.***	N.A.
Wet cake	0.52	0.26	51	N.A.	N.A.
Thin stillage	1.95	0.65	33	N.A.	N.A.
Syrup	1.88	0.64	34	N.A.	N.A.
DDGS (149^{o}C)	1.05	0.44	42	N.A.	N.A.
DDGS (110^{o}C)	1.02	0.39	38	N.A.	N.A.

* PP/TP (%): the ratio of phytate P to total P times 100

** IP/TP (%): the ratio of inorganic P to total P times 100

*** N.A., data not available

@ Early Ferm., Active Ferm., End Ferm.: with fermentation time points of about 10 hr., 30 hr. and end of fermentation

Table 3c. Total P and Phytate P in Samples from Run F8 (%, wt/wt, on dry basis)

Sample Source	Total P	Phytate P	PP/TP (%)*
Early Ferm.@	0.36	0.20	57
Active Ferm. @	0.60	0.32	53
End Ferm. @	0.80	0.42	53
Whole stillage	N.A.**	N.A.	N.A.
Wet cake	0.57	0.34	60
Thin stillage	1.41	0.60	43
Syrup	1.75	0.73	42
DDGS (149^{o}C)	0.95	0.48	50
DDGS (110^{o}C)	0.93	0.46	49

* PP/TP (%): the ratio of phytate P to total P times 100

** N.A., data not available

@ Early Ferm., Active Ferm., End Ferm.: with fermentation time points of about 10 hr., 30 hr. and end of fermentation

Table 3d. Total P and Phytate P in Samples from Run F9 (%, wt/wt, on dry basis)

Sample Source	Total P	Phytate P	PP/TP (%)*
Early Ferm.@	0.43	0.24	55
Active Ferm. @	0.66	0.32	48
End Ferm. @	0.91	0.45	49
Whole stillage	1.08	0.46	43
Wet cake	0.56	0.29	52
Thin stillage	1.73	0.69	40
Syrup	1.56	0.66	42
DDGS (149^{o}C)	0.98	0.49	50
DDGS (110^{o}C)	0.98	0.48	49

* PP/TP (%): the ratio of phytate P to total P times 100

@ Early Ferm., Active Ferm., End Ferm.: with fermentation time points of about 10 hr., 30 hr. and end of fermentation

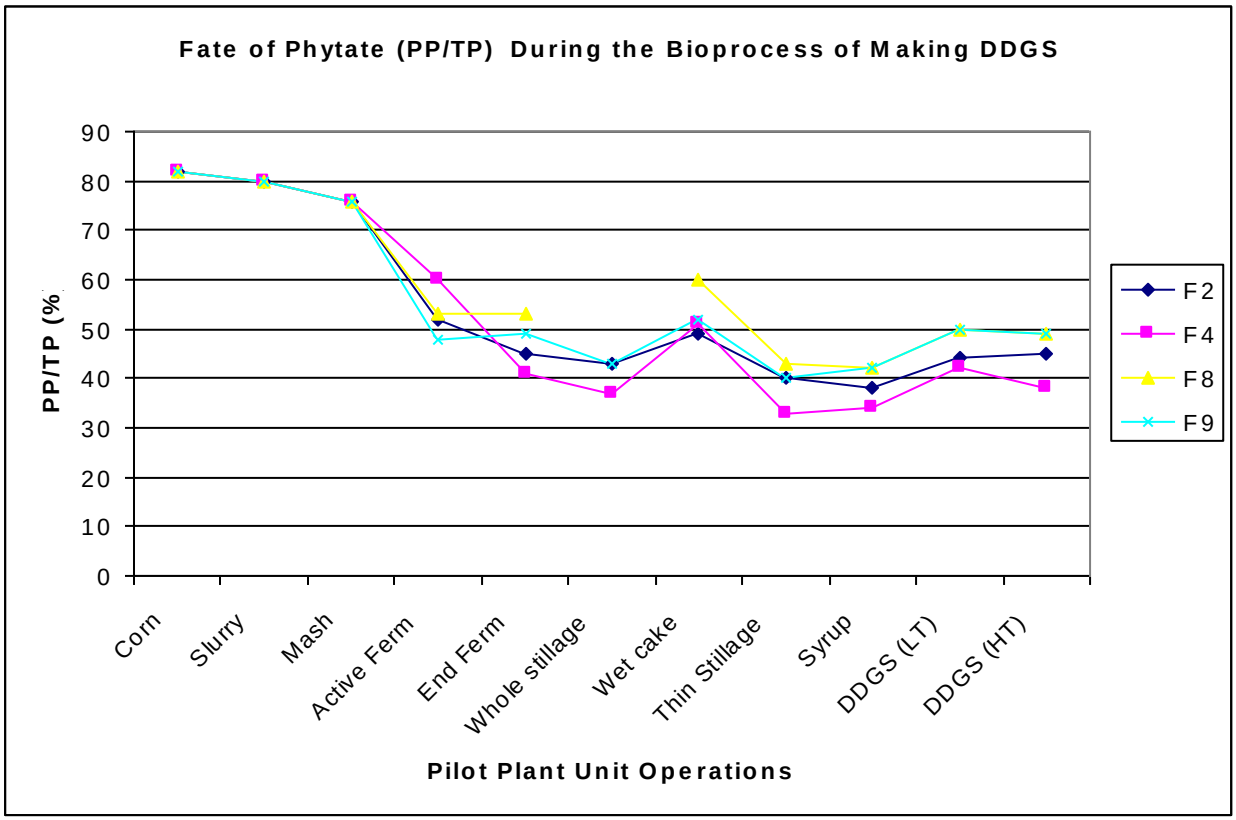

Fig. 1. Change of the percentage of phytate P in total P (PP/TP) during the bioprocess of making DDGS

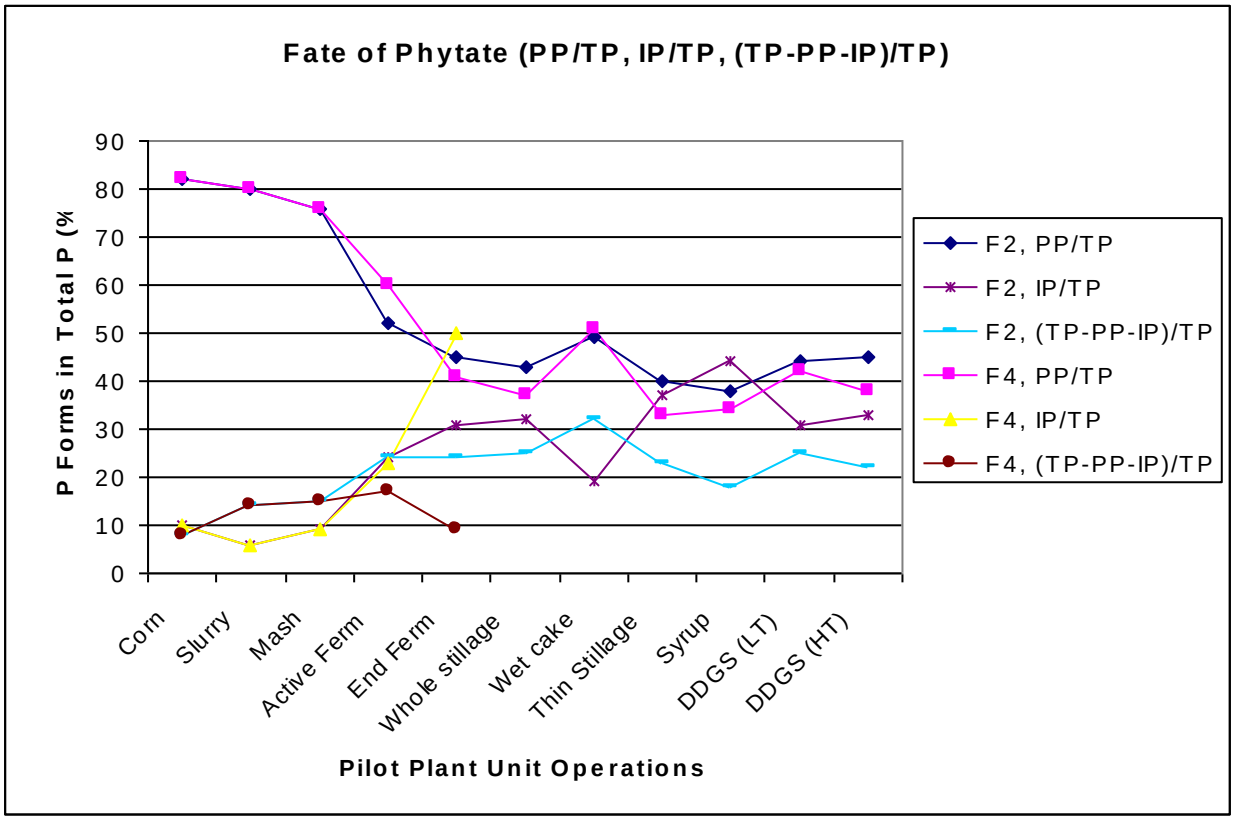

Fig. 2. The change of the percentages of phytate P in total P (PP/TP), inorganic P in total P (IP/TP), and P from IP1, 2, 3 in total P ((TP-PP-IP)/TP) during the bioprocess of making DDGS from Fermentor 2 and 4.

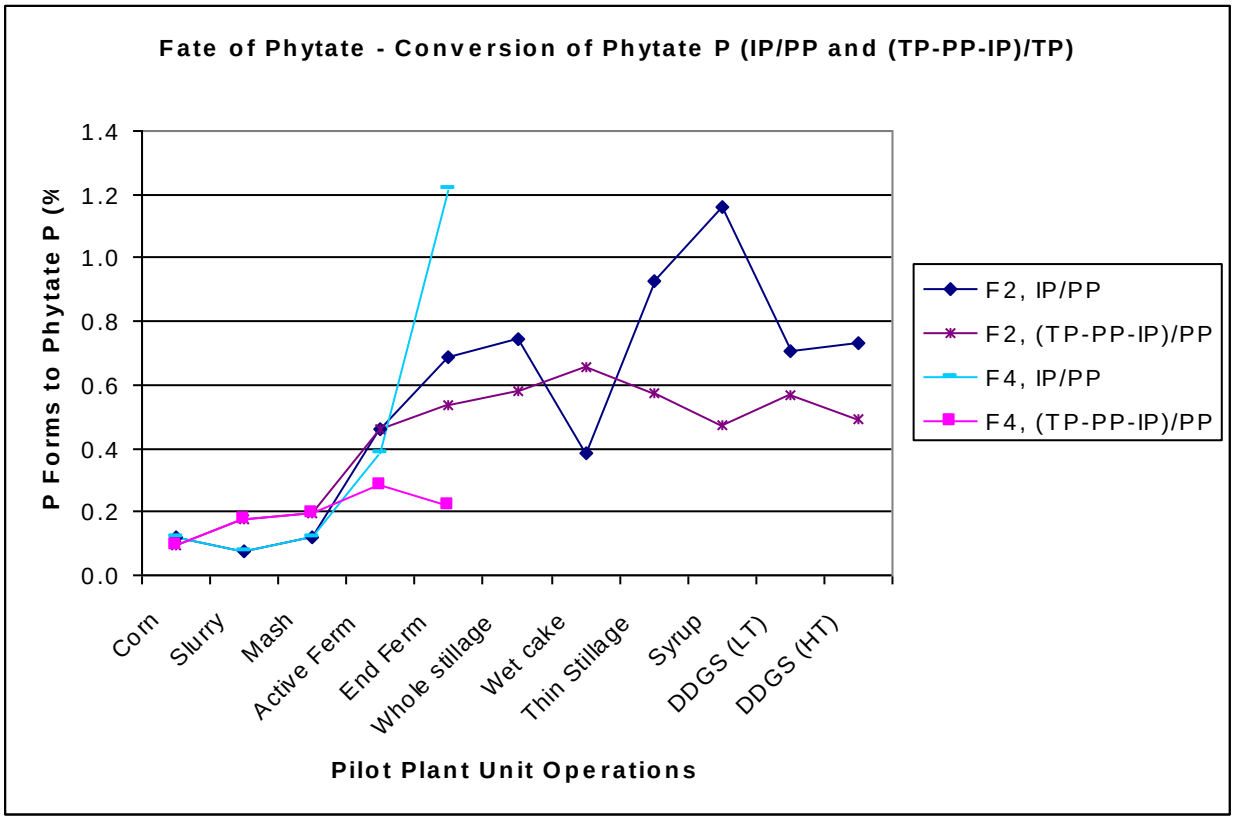

Fig. 3. Conversion of phytate P to inorganic P (IP/PP) and P from IP1, 2, 3 ((TP-PP-IP)/PP) during the bioprocess of making DDGS fro Fermentor 2 and 4.