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Addition of cellulolytic enzymes and phytase for improving ethanol fermentation performance and oil recovery in corn dry grind process

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Keywords
corn dry-grinding, ethanol, oil recovery, fibers, hydrolytic enzymes

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Addition of cellulolytic enzymes and phytase for improving ethanol fermentation performance and oil recovery in corn dry grind process

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Abstract

Application of hydrolytic and other enzymes for improving fermentation performance and oil recovery in corn dry-grind process was optimized. Non-starch polysaccharide enzymes (BluZy-P XL; predominantly xylanase activity) were added at stages prior to fermentation at optimum conditions of 50°C and pH 5.2 and compared with conventional fermentation (30°C, pH 4.0). Enzyme applications resulted in faster ethanol production rates with a slight increase in yield compared to control. The thin stillage yield increased by 0.7-5% w/w wet basis with corresponding increase in solids content with enzymes treatment after liquefaction. The oil partitioned in thin stillage was at 67.7% dry basis after treatment with hydrolytic enzymes during fermentation. Further addition of protease and phytase during simultaneous saccharification and fermentation increased thin stillage oil partitioning to 77.8%. It also influenced other fermentation parameters, e.g., ethanol production rate increased to 1.16 g /g dry corn per h and thin stillage wet solids increased by 2% w/w. This study indicated that treatments with non-starch hydrolytic enzymes have potential to improve the performance of corn dry-grind process including oil partitioning into thin stillage. The novelty of this research is the addition of protease and phytase enzymes during simultaneous saccharification and fermentation stage of corn dry-grind process, which further improved ethanol yields and oil partitioning into thin stillage.

Keywords: Corn Dry-grinding, Ethanol, Oil Recovery, Fibers, Hydrolytic Enzymes

1. Introduction.

The US corn production in 2014 was approximately 14.2 billion bushels, with roughly 30% utilized for ethanol production (NCGA, 2015). Ethanol has been the most significant source of total biofuel usage in the US (94%), of which about 82% is produced using corn dry-grind

process (Wang, 2009a). In this process, ground corn is liquefied, saccharified, and fermented to convert monomeric glucose to ethanol. Non-fermentable residues result in a coproduct called distiller's dried grains with solubles (DDGS) after separation and drying with condensed solubles of thin stillage. On dry basis, DDGS usually contains 27.4, 11.7, 4.4, and 56.5% w/w of protein, oil, ash, and total carbohydrate, respectively (Liu, 2008). Approximately 40 million tons of DDGS were produced in 2012 and projected to reach 43 million tons in 2014 (Wisner, 2014). DDGS are also utilized as animal feed, with various incorporation levels for cattle and non-ruminant animals, higher fiber percentages limiting usage in the latter. Ethanol producers need to improve desirable characteristics in DDGS as animal feed to enhance its incorporation levels. Application of hydrolytic and other enzymes during processing could modify non-starch polysaccharides (NSP) more favorably for feed application and recover more oil upstream to make the process more profitable.

Corn oil is a higher-value coproduct of corn dry-grind process and is concentrated from 4% in corn kernel to about 14% in DDGS (Wang, 2008a, 2008b). Higher levels of oil in DDGS are sometimes undesirable and affect feed quality negatively; for example, higher amounts of oil could interfere with milk production in cattle and bacon texture in DDGS-fed swine (Wang, 2009b). Recovery of corn oil from the stillage will create a higher-value product stream than DDGS. Technologies for corn oil recovery from dry grind process are reported in the literature. Effect of physical treatments like grinding and flaking (Lamsal and Johnson, 2012), heating and solvent introduction before and after the corn dry-grind process (Majoni, 2011a; Wang 2008a, 2009a) were reported to enhance process performance. Use of hydrolytic enzymes is an environment-friendly and affordable method that can benefit corn dry-grind process (Johnston and McAloon, 2014), including recovery of corn oil (Majoni, 2011b).

Corn oil is mostly stored in germ cells as oil bodies or oleosomes and is secluded by phospholipids and layer of oleosin, an alkaline protein (Huang, 1996; Danso-boateng, 2011). During corn dry-grind process, oil bodies can be trapped between non-starch polysaccharide and protein matrix. Addition of protease and NSP hydrolyzing enzymes during the corn dry-grinding process can degrade such barriers and enhance oil recovery. Proteases are also suggested for free amino nitrogen production and utilization by yeast during fermentation (Vidal, 2010) that could result in higher ethanol production rates and yields.

This study compared the performance of corn dry-grind process upon addition of NSP hydrolytic enzyme cocktail (BluZy-P XL) and other enzymes. The application of BluZy-P XL cocktail, provided by Direvo Industrial Biotechnology GmbH (Cologne, Germany), during the simultaneous saccharification and fermentation (SSF) at 30°C for 60 h was compared with treatments at optimal enzyme conditions (pH, temperature, and process stages). Combination of the said enzyme cocktail with protease and phytase in corn dry-grinding process was also compared for enhanced performance indicators.

2. Materials and Methods

Yellow dent #2 corn used in the study was obtained from Iowa State University's research farm and stored at 15% moisture content in airtight bags placed inside an airtight plastic bin at 4°C. Corn contained 67% starch, 7% protein, and 3% lipid w/w on wet basis, the remainder being fiber, ash, and moisture. Corn was ground by using a hammer mill (Fitz Mill model DAS 06; Fitzpatrick Co., Elmhurst, IL) at 5,000 rpm with 3.18 mm screen opening (screen # 1531-0125). The ground corn meal had a particle size distribution of 4, 22, and 74 w/w % retained on mesh numbers 20, 12, and pan, respectively.

Liquid α-amylase Spezyme Xtra (13,642 R-amylase units/g) and protease GC 212 (2000 SAP units/g; SAP, spectrophotometer acid protease) were provided by Genecor international (Palo Alto, CA). Glucoamylase Spirizyme Excel XHS (Novozymes, Franklinton, NC), phytase Phytaflow (20,000 FYT/g, Novozymes, Bagsvaerd, Denmark), and dry yeast (*Saccharomyces Cerevisiae*) were provided by Lincoln way Energy (Neveda, IA). Lactrol (462 g virginiamycin /lb) was purchased from PhibroChem (Ridgefield Park, NJ). The BluZy-P XL enzymes cocktail, with mostly xylanase activity, was acquired from Direvo Industrial Biotechnology GmbH (Cologne, Germany). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

2.1 Optimal Temperature and pH conditions for addition of enzymes cocktail

The enzyme cocktail BluZy-P XL obtained from the company was experimental mix with a broad range of temperature and pH conditions, which needed narrowing down for best performance in corn dry-grind process being followed. Ground corn was mixed with distilled water at the ratio of 1:2 in 250-mL Erlenmeyer flaks for a total slurry weight of 200 g. For pH and temperature optimization experiments, the pH of slurry was adjusted to 3.8, 4.5, and 5.2 with 6.0 N sulfuric acid and incubated in incubator shaker at 150 rpm at 35, 42, and 50°C for 1 h. This range of temperature and pH was chosen following enzyme data sheet that showed a broader activity range. BluZy-P XL cocktail (400 ppm) was added and shaken steadily in incubator shaker at 150 rpm for 1.5 h (Innova 4300 incubator shaker, New Brunswick Scientific, NJ). The treated corn slurries were centrifuged to collect supernatant at 10,000 × *g* for 15 min (Sorvall Legend XTR Centrifuge, Fisher Scientific) and filtrated through 0.2μm filter for sugar analysis with HPLC. Triplicate experimental runs were carried out.

2.2 Application of hydrolytic enzymes cocktail BluZy-P XL and processing stages

Once the optimal working conditions for were arrived at, enzyme cocktail was then evaluated for its effectiveness in improving downstream fermentation by applying at three processing stages: post-grinding (Treatment A), post-liquefaction (Treatment B), and during simultaneous saccharification and fermentation (SSF) (Treatment C) (Fig 1). Enzyme cocktail application was at 400 ppm. While optimal enzyme temperature and pH conditions were maintained for Treatments A and B, SSF conditions prevailed for Treatment C.

The general procedure followed for corn dry-grind liquefaction and fermentation, along with enzymatic treatments, where indicated, was as following: 1:2 weight % ratio of ground corn: distilled water along with 0.67 mL of α-amylase Spezyme Xtra were mixed in a 2-L Erlenmeyer flask for a total weight of 1000 g. Liquefied occurred at 82°C first for 1 h with constant agitation followed by autoclaving (121°C, 103kPa, 20 min) and another 3 h liquefaction at 82°C with second application of α-amylase Spezyme Xtra (1 mL). The liquefied corn mash was cooled down to 30°C and pH-adjusted to 4.0 by using 6.0 N sulfuric acid. The evaporative weight loss during liquefaction was readjusted by adding sterile water to maintain the initial water: solid ratio.

SSF of pretreated slurry was carried with addition of glucoamylase Spiriyme at 0.04% w/w of corn, $(NH_4)_2SO_4$ at 150 ppm, antibiotic Lactrol at 0.004% w/w corn, and 0.67 g of dry yeast. The flask was capped with cotton and aluminum foil and incubated at 30°C for 72 h in incubator shaker (Innova 4300, New Brunswick Scientific, NJ) at 150 rpm. Two replications were carried for each treatment. Weight loss was recorded periodically during fermentation and was related to ethanol yields following the relationship proposed by Wang (2009a): ethanol yield (g per 100 g dry corn) =100 x (46 x total CO_2 production, g/44)/original dry corn mass, g. The initial ethanol production rates, g ethanol per 100 g dry corn per h, were calculated from the

slope of the linear portion of the ethanol yield versus time curves during initial periods, mostly 6 to 20 h.

2.3 Protease and phytase treatment (Treatment D)

Beside the study on BluZy-P XL treatment conditions, the effect of adding protease and phytase during SSF on process performance was also examined. Protease at 0.7 μL/g dry solid and 1.4 ppm of phytase was added during 72-h fermentation with the best performing BluZy-P XL treatment (previous section).

2.4 Post-fermentation separations and chemical analyses

Ethanol was distilled off by boiling and whole stillage (WS) was subjected to a simulated industrial decanting process called the multiple wash centrifugal filtrations (MWCF) for efficient partition of wet grains and thin stillage (Wang, 2009b). In short, 100-g WS in a permeable pouch was put in a cup-like assembly that was spun in a swing-bucket centrifuge at 3000x*g*. The supernatant was used to wash more fines from the wet solids by turning the device upside down and gently shaking without disassembling the device. This was repeated to wash the wet grains four times and finally to obtain wet cake (WC) and thin stillage (TS); wet yields and solid content of fractions were measured by drying at 105°C overnight. Total oil content was determined in WS and WC following acid hydrolysis method (AOAC 922.06). Representative wet cake samples from all enzymatic treatments were dried and analyzed in duplicate for extent of fiber modification using ANKOM procedure (ANKOM Technology Corporation, Macedon, NY). Cell solubles, cellulose and hemicellulose content in dry samples were calculated as:

Cellulose = % Acid Detergent Fiber (ADF) - % Acid Detergent Lignin (ADL)

Hemicellulose= %Neutral Detergent Fiber (NDF) - %Acid Detergent Fiber (ADF)

Cell solubles = 1- %Neutral Detergent Fiber (NDF)

The % values thus calculated reflected values based on dry cake samples, so they were recalculated on the basis of 100 g dry corn weight to account for weight loss due to hydrolytic and other enzyme treatments at various processing stages.

2.5 Calculations

At the end of fermentation, yield of wet grains and oil distribution in fractions were calculated as:

Wet yield of WC or TS (%) = [(Weight of WC or TS, g as is) × 100] / (wet weight of WS, g)

Dry solids (%) = [(Dry matter in WC or TS, g)×100] / (Dry matter in WS, g)

Oil in WC (%) = [(Oil in WC, g as is) × 100] / (Oil in WS, g)

Oil in TS (%) = [(1- Oil in WC, g) x 100] / (Oil in WS, g)

2.6 HPLC quantitation of xylose in enzyme hydrolysates and ethanol in stillage

Ethanol in whole stillage and xylose concentrations in hydrolysates were measured using an HPLC with HyperREZ XP Carbohydrate H+ 8 μm column (300×7.7 mm) and RI detector (Accela ultra high pressure; Fisher Scientific, USA), respectively. Injection was at 400 μL/min with 0.05 M sulfuric acid in water as a mobile phase at 70°C. Data was collected using ChromQuest system (EZChrom Elite, v 3.2.1, Scientific Software, Inc.).

3 Results and discussion

3.1 Best working conditions for BluZy-P XL enzyme cocktail:

The experimental enzyme cocktail BluZy-P XL was evaluated first for best working conditions in corn dry grind ethanol process by comparing the concentrations of xylose in the 200-mL corn slurry obtained at the end of 1.5 h incubation, since the enzyme had mainly xylanase activity (Fig 2). In general, there was significant trend in increase of xylose concentrations when treated between pH 3.8 and 5.2 and temperatures 35°C to 50°C. The xylose concentration at pH 5.2 and 50°C was the highest at 0.37 mg/mL; higher temperatures possibly help degrade cell matrix along with dissolution of hemicelluloses and provide more accessibility for enzymes to form a complex with substrate (Limayem, 2012; Poletto, 2013). It is known that higher temperatures enhance rates of enzymatic reactions for kinetics reasons and formation of enzyme-substrate complexes (Cornish-Bowden, 2012). In short, hydrolytic enzymes affected non-starch polysaccharides in corn matrix with temperature effect prominent than pH. The suitable condition for addition of enzymes BluZy-P XL was considered to be 50°C at corn slurry pH of ≈5.2-5.6.

3.2 Effect of enzyme systems on corn dry-grind process:

3.2.1 Fermentation performance

The effect of addition of enzymes cocktail BluZy-P XL and/or protease/phytase combination on corn dry-grind process was evaluated by treating corn mash at different process stages at 50°C without pH adjustment as the slurry had pH of pH≈5.5 (Fig. 1). The ethanol production profile for these treatments and final yields are shown in Figure 3 and Table 1, respectively. In general, treatment at any process stage by BluZy-P XL, which had predominantly xylose/ hemicellulose hydrolysis activity, improved ethanol production rates and

final concentrations compared to the control; however, a significant change in ethanol production rate was observed only for enzyme treatment after liquefaction (Treatment B; $p<0.05$). Treatment B resulted in production rate of 0.71±0.01 g of ethanol/100g of dry corn·h, which was higher than rate from pre-liquefaction stage enzyme treatment (0.63±0.00 g/g·h, Treatment A). Activity of NSP hydrolyzing enzymes (BluZy-P XL) in post-amylolytic liquefied mash may benefit from the easy substrate accessibility since complexity of cell wall network is reduced. Compared with treatment during fermentation at 30°C (Treatment C), enzyme cocktail was more effective at 50°C; however, the limitation of enzyme performance in Treatment A (post-grinding) was noticeable. The tight cellulosic/hemicellulosic structure in cell walls before liquefaction might have made it difficult to degrade by BluZy-P XL. In addition, the enzymatic treatments from liquefaction through fermentation (Treatment B) was more effective than Treatment C, due to possible greater accessibility by glucoamylase during saccharification.

In addition to hydrolytic enzyme mix, protease and phytase supplementation during SSF (Treatment D) was found to be beneficial for corn dry-grinding process for ethanol. The ethanol production rate with protease and phytase addition increased to 1.16g/ 100 g dry corn per•h, with final ethanol yield of 127.54±0.17 g/L (Table 1). Degradation of protein with enzyme in corn cell matrix can increase the accessibility of starch and other substrates to enzymes (Lamsal and Johnson 2012). In addition, mineral supplement from phytic acid degradation could improve efficiency of ethanol production by making more ion (Ca^{2+}) available for glucoamylase activity (Hruby, 2012). This suggests the need for these types of enzymes in ethanol process supplementing non-starch hydrolytic enzymes.

3.2.2 Solids distribution in coproduct fractions

Table 2 shows the effect of hydrolytic enzymes on wet solid recovery and solid contents, and oil partitioning in wet cake and thin stillage fractions as % of whole stillage. Treatments with enzyme cocktail BluZy-P XL and protease/phytase addition (Treatment D) had significantly increased solid partitioning in thin stillage compared to no-enzyme controls. The wet yield (total wet weight) of thin stillage ranged from 87.88-93.06%, with the highest from Treatment D, an increase of 1.6% compared with best BluZy-P XL treatments (Treatments B or C) and increase of 5.2% compared with no enzyme control. Consequently, the solids in wet cake reduced accordingly for the treatments, which explained the increase of dry matter in thin stillage. The degrading of fibers released more solubles into liquid fraction. The solid partitioned in thin stillage (dry matter) ranged from 59.25-69.70% with highest from protease and phytase addition (Treatment D).

3.2.3 Oil Partition

Table 2 also shows that addition of xylolytic enzyme cocktail BluZy-P XL significantly increased oil partitioned into thin stillage from 32.13%±1.50 (control) to 49.83%±2.44, 67.71%±3.64, and 54.57%±1.82 for Treatments A, B, and C, respectively. Treatment by enzyme cocktail after liquefaction (Treatment B) resulted in the highest oil content in thin stillage, and consequently, lower oil percentage in wet cake fractions (32.58±0.56%). Additional enzyme treatment with protease and phytase (Treatment D) resulted in significantly higher oil partitioning in thin stillage (77.8% w/w) compared with other enzyme treatments. Oil in different forms whole stillage can explain the oil partitioning after enzyme treatments. The oil in whole stillage can be in present in four different forms: oil-in-water emulsion, oil inside unbroken oil bodies (oleosomes), oil droplet attached to hydrophobic particle surfaces, and oil in unbroken (bigger) cells of germs and endosperm (Majoni et al, 2011a). Upon decanting of whole stillage,

emulsified oil and oil in oleosomes will partition into thin stillage, whereas, oil in unbroken matrices and that adhering to larger particles partition with wet cake. Non-starch carbohydrate hydrolysis enzymes, like BluZy-P XL, can hydrolyze cell walls of unbroken cells and release oil from them. Protease can hydrolyze protein particles and free the attached oil droplets as well as act on stabilizing proteins like oleosins in thin stillage emulsion. They all contribute to increase oil partitioning into thin stillage after decanting, and most of oil is recovered as free oil.

3.2.4 Distillers' wet grain after enzyme treatments

The wet distillers grains from enzyme treatments were dried after centrifugation and distribution of non-starch carbohydrates components were determined as weight % of dried samples. Since yields of wet distillers' grains at the end of enzyme treatments differed based on extent of hydrolysis, we expressed the constituent compositions (cellulose, hemicellulose, lignin) as % of starting 100 g dry corn. Hydrolysis of non-starch carbohydrate by BluZy-P XL resulted in lower amount of fiber content (both NDF and ADF), except for Treatment A, which had higher values than control group (Table 3). Control and Treatment A had significantly higher amount of fiber components than other treatments, whereas, protease/phytase treatment (Treatment D) had lowest. Treatments B and C were better stages than A for BluZy-P XL treatment because lower amount of non-digestible carbohydrates were found in wet grains. Additional protease/phytase addition during fermentation decreased that even further. It may be explained by the further structural decomposition leading to improvement in enzymes activity. Protease hydrolyzes protein and expose cell wall carbohydrates to other enzymes like BluZy-P XL. Moreover, phytase can hydrolyze phytic acid, which can chelate with metal and may reduce or inhibit enzyme activity, especially chelation of calcium, which could be detrimental to glycoamylase-type of enzymes. Decreasing phytic acid content in corn slurry can also improve activity of

carbohydrate hydrolysis enzymes (Mikulski et al, 2014). Cell solubles, which is the most digestible part in DDGS, also decreased with enzyme treatments. BluZy-P XL and protease/phytase treatments degraded larger molecules like polysaccharides, proteins; partitioning of these lower molecular weight molecules occurred in thin stillage fraction after decanting. Cell solubles, including protein, lipid, sugars and starch may also have been released from wet grains during enzyme hydrolysis and moved to thin stillage. The solid mass distribution in thin stillage and wet cake (Table 2) supports this explanation.

4 Conclusion:

This study demonstrated that incorporation of hydrolytic enzymes, including non-starch hydrolase, protease, and phytase, at their optimized conditions and process stages can promote fermentation performance for corn dry-grind process for ethanol. It can also enhance partitioning of solids and oil into liquid fraction (thin stillage) and produce DDGS with lower amounts of nondigestible carbohydrates. Best process performance was obtained with 1.5h incubation with BluZy-P XL after liquefaction (50°C) and protease/phytase addition during fermentation. This also resulted in favorably modified distillers grains that could be envisioned to have uses in monogastric feeding.

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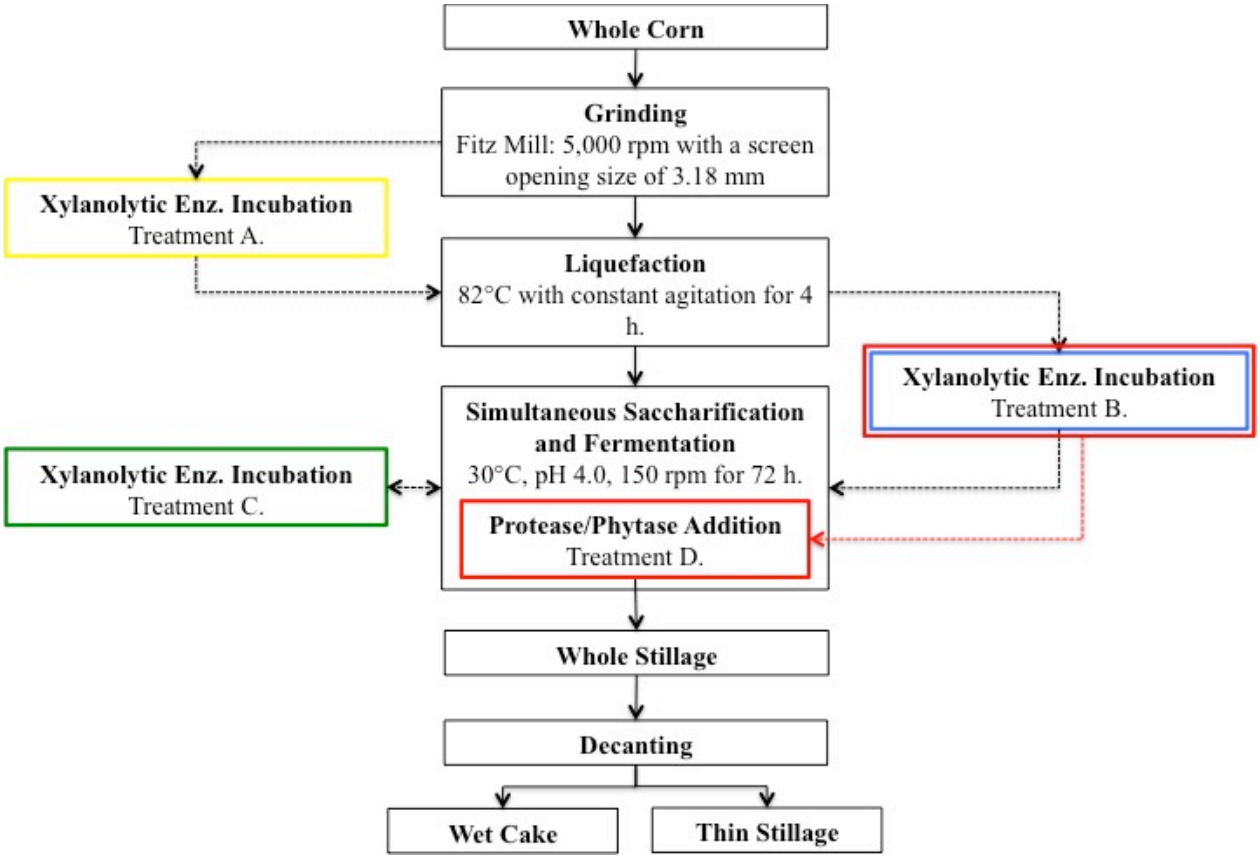

Figure 1. Flow chart for BluZy-P XL enzymatic treatment at different processing stages for corn dry-grind ethanol process. Treatments A, B, and C stand for xylolytic enzymes incubation at pre-liquefaction, post-liquefaction, and during-fermentation, respectively. Treatment D was a combination of Treatment B with protease and phytase supplementation during simultaneous saccharification and fermentation.

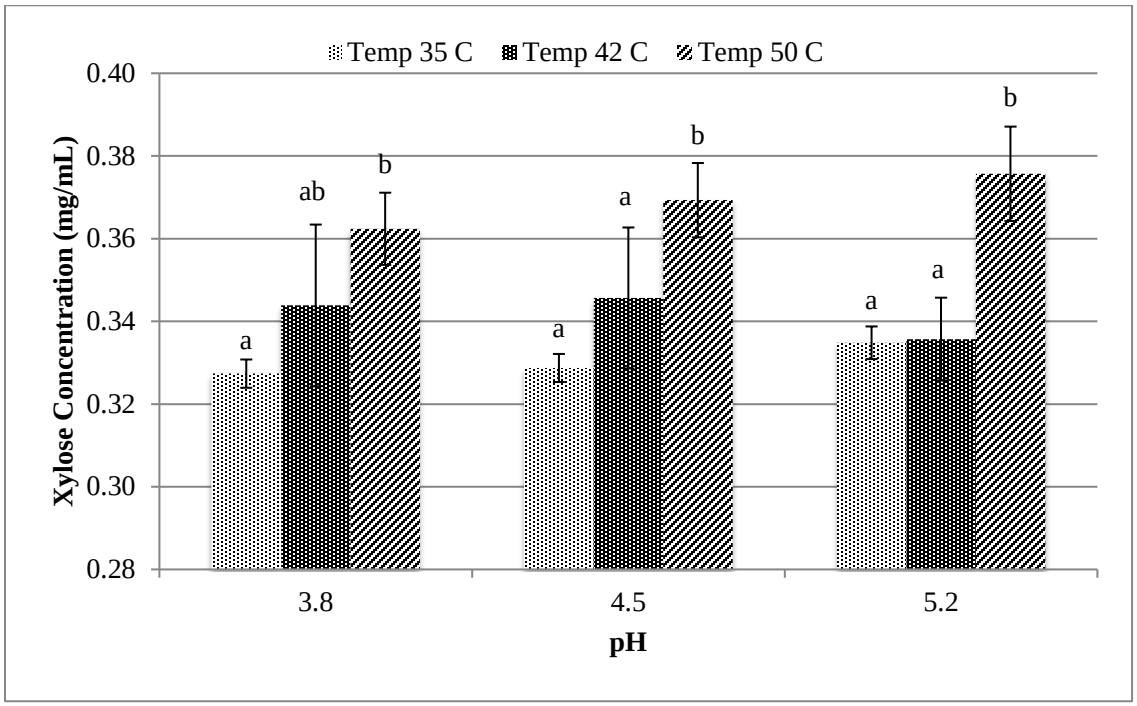

Figure 2. Xylose concentration from BluZy-P XL treatments at various pH and temperature conditions. The error bars represent the standard deviation of two replicates with three measurements each. Bars sharing same letter are not significantly different (p-value ≤ 0.05).

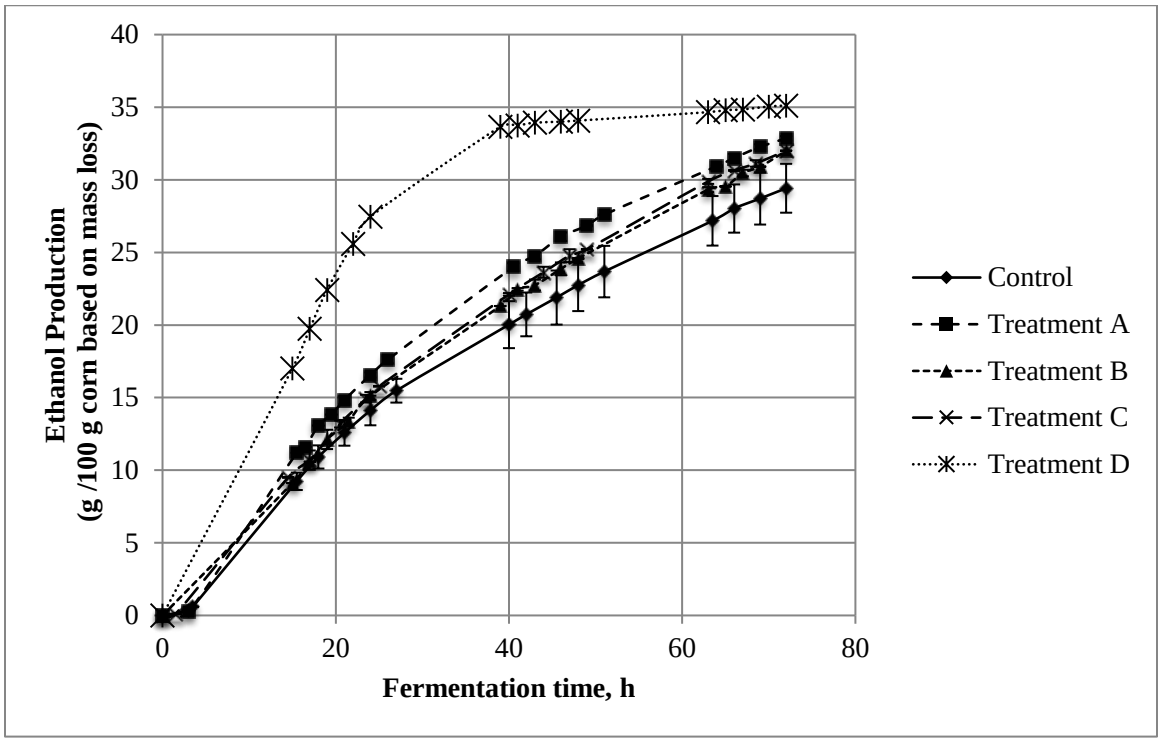

Figure 3. Ethanol production profiles for control, BluZy-P XL treatments (A, B, and C), and protease and phytase addition during fermentation (Treatment D). The error bars represent the standard deviation of two replicates with three measurements each.

Table 1. Fermentation performance as result of different enzymatic treatments

	Initial ethanol production rate (g /100g dry corn-h)	Final ethanol yields (g /100g dry corn)	Final ethanol yield by HPLC (g/L)
Control	0.54±0.03[a]	29.42±1.68[ab]	109.52±0.35[a]
Treatment A	0.63±0.00[a]	32.85±0.15[a]	121.20±0.05[b]
Treatment B	0.71±0.01[b]	31.99±0.00[b]	115.74±0.16[a]
Treatment C	0.63±0.02[a]	32.01±0.02[b]	117.00±0.25[a]
Treatment D	1.16±0.00[c]	35.11±0.02[c]	127.54±0.17[c]

* Treatment A, B, and C refer to BluZy-P XL treatments at post-grinding, liquefaction, and simultaneous saccharification and fermentation, respectively. Treatment D refers to BluZy-P XL treatment supplemented with protease/phytase addition during simultaneous saccharification and fermentation. Values sharing same letters are not significantly different (p-value ≤ 0.05)

Table 2. Weight distribution and oil partitioning in wet cake and thin stillage fractions relative to whole stillage:

	Wet Cake			
	Wet Yield (%wt.)	% Solid Content	Dry Matter Yield (%wt.)	Oil Partitioning (%wt.)
Control	11.88±0.27[a]	41.79±0.17[a]	43.99±1.01[a]	67.87±1.50[a]
Treatment A[1]	11.61±0.13[a]	41.79±0.50[ab]	42.26±2.20[a]	50.17±2.44[b]
Treatment B	8.53±0.09[c]	42.48±0.11[c]	32.58±0.56[b]	32.29±3.64[d]
Treatment C	8.37±0.09[b]	42.45±0.35[bc]	32.53±0.44[b]	45.43±1.82[c]
Treatment D[2]	6.71±0.27[c]	42.80±0.39[b]	30.46±1.18[c]	22.20±1.68[c]
	Thin Stillage			
Control	87.88±0.21[a]	8.07±0.17[ab]	62.93±2.54[a]	32.13±1.50[a]
Treatment A	88.56±0.37[b]	7.74±0.32[a]	59.25±3.26[a]	49.83±2.44[b]
Treatment B	91.23±0.19[d]	8.29±0.05[b]	67.93±1.14[b]	67.71±3.64[c]
Treatment C	91.50±0.08[c]	8.17±0.10[b]	68.48±0.74[b]	54.57±1.82[d]
Treatment D	93.06±0.34[c]	7.06±0.08[b]	69.70±1.11[c]	77.80±1.68[e]

Treatment A, B, and C refer to BluZy-P XL treatments at post-grinding, liquefaction, and simultaneous saccharification and fermentation, respectively. Treatment D refers to BluZy-P XL treatment supplemented with protease/phytase addition during simultaneous saccharification and fermentation. Values sharing same letters are not significantly different (p-value ≤ 0.05).

Table 3: Fiber distribution in wet grains (in dry basis) produced from 100 g dry corn after enzyme treatment at different stages

	Control	Treatment A	Treatment B	Treatment C	Treatment D
NDF (g)	3.81^{b}	4.48^{a}	2.98^{c}	2.76^{cd}	2.26^{d}
ADF (g)	0.96^{ab}	1.15^{a}	0.71^{bc}	0.63^{c}	0.65^{c}
ADL (g)	0.11^{ab}	0.13^{a}	0.09^{ab}	0.08^{b}	0.07^{b}
Cell solubles (g)	9.97^{a}	8.8^{b}	6.92^{c}	6.98^{c}	5.51^{d}
hemicellulose (g)	2.84^{b}	3.33^{a}	2.28^{c}	2.13^{c}	1.61^{d}
Cellulose (g)	0.85^{a}	1.02^{a}	0.62^{b}	0.55^{b}	0.58^{b}
Lignin (g)	0.12	0.13	0.09	0.08	0.07

NDF, ADF, and ADL referred to neutral detergent fiber, acid-detergent fiber, and acid-detergent lignin. Treatment A, B, and C refer to BluZy-P XL treatments at post-grinding, liquefaction, and simultaneous saccharification and fermentation, respectively. Treatment D refers to BluZy-P XL treatment supplemented with protease/phytase addition during simultaneous saccharification and fermentation. Values sharing same letters are not significantly different (p-value ≤ 0.05).