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ScienceDirect
Bioresource Technology 99 (2008) 5206–5215
BIORESOURCE TECHNOLOGY

Enzyme hydrolysis and ethanol fermentation of liquid hot water and AFEX pretreated distillers' grains at high-solids loadings

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Available online 19 November 2007

Abstract

The dry milling ethanol industry produces distiller's grains as major co-products, which are composed of unhydrolyzed and unfermented polymeric sugars. Utilization of the distiller's grains as an additional source of fermentable sugars has the potential to increase overall ethanol yields in current dry grind processes. In this study, controlled pH liquid hot water pretreatment (LHW) and ammonia fiber expansion (AFEX) treatment have been applied to enhance enzymatic digestibility of the distiller's grains. Both pretreatment methods significantly increased the hydrolysis rate of distiller's dried grains with solubles (DDGS) over unpretreated material, resulting in 90% cellulose conversion to glucose within 24 h of hydrolysis at an enzyme loading of 15 FPU cellulase and 40 IU β-glucosidase per gram of glucan and a solids loading of 5% DDGS. Hydrolysis of the pretreated wet distiller's grains at 13–15% (wt of dry distiller's grains per wt of total mixture) solids loading at the same enzyme reduced cellulose conversion to 70% and increased conversion time to 72 h for both LHW and AFEX pretreatments. However, when the cellulase was supplemented with xylanase and feruloyl esterase, the pretreated wet distiller's grains at 15% or 20% solids (w/w) gave 80% glucose and 50% xylose yields. The rationale for supplementation of cellulases with non-cellulolytic enzymes is given by Dien et al., later in this journal volume. Fermentation of the hydrolyzed wet distiller's grains by glucose fermenting *Saccharomyces cerevisiae* ATCC 4124 strain resulted in 100% theoretical ethanol yields for both LHW and AFEX pretreated wet distiller's grains. The solids remaining after fermentation had significantly higher protein content and are representative of a protein-enhanced wet DG that would result in enhanced DDGS. Enhanced DDGS refers to the solid product of a modified dry grind process in which the distiller's grains are recycled and processed further to extract the unutilized polymeric sugars. Compositional changes of the laboratory generated enhanced DDGS are also presented and discussed.

Keywords: Pretreatment; Distiller's grains; DDGS; Ethanol; Dry milling

1. Introduction

The recent increase in ethanol demand as an alternative fuel has boosted ethanol production in the US. Currently, about 80% of ethanol is produced via dry milling process (RFA Annual Ethanol Industry Outlook, 2007). Distiller's grains and distiller's dried grains with solubles are the major co-products of the dry milling process. The production of these co-products is expected to increase significantly over the next few years as a result of the escalating demand for bioethanol. Distiller's grains recently have been drawing much attention as an additional feedstock to increase overall ethanol yield in the current dry grind ethanol facilities. Wet distiller's grains (wet cake) contain about 20% total glucan which can be hydrolyzed to glucose monomers (Kim et al., 2008a). The total glucan includes cellulose and residual starch. The compositional analysis showed that about 15–16% of the distiller's grains is

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doi:10.1016/j.biortech.2007.09.031

cellulose with the remaining 5% being starch (Kim et al., 2008a). Due to the recalcitrant nature of the cellulose, it requires a pretreatment step to enhance hydrolysis efficiency by disrupting cellulose crystallinity and increasing accessibility of cellulolytic enzymes to the feedstock.

Among various pretreatment methods, controlled pH, liquid hot water pretreatment (LHW) has been shown to be effective in removing and solubilizing the hemicellulose fraction of the recalcitrant cellulosic biomass, and disrupting cellulose and cell wall structure thus, improving the subsequent hydrolysis efficiency (Walch et al., 1992; Ladisch et al., 1998; Mosier et al., 2005a; Zeng et al., 2007; Weil et al., 1997, 1998). The formation of monomeric sugars and further degradation to toxic compounds is restricted by pH control of the liquid phase during the hot water pretreatment (Mosier et al., 2005a; Weil et al., 1998; Ladisch et al., 1998; Bobleter, 1994). Previous study by Weil et al. (1998) and Mosier et al. (2005a,b) has shown that the liquid hot water pretreatment of corn fiber at 160 °C for 20 min resulted in 50% dissolution of the initial material. Loss of the dissolved carbohydrates to degradation products was less than 1%.

Another pretreatment that has been used to improve hydrolysis yields is ammonia fiber expansion (AFEX). Highly concentrated aqueous ammonia is added to biomass and heated to moderate temperatures (70–100 °C) and high pressure (150–400 psi). After a residence time of 5 to as much as 30 min, the pressure is explosively released. The ammonia can be recycled during this process. This pretreatment decrystallizes cellulose, partially hydrolyzes and solubilizes hemicellulose, increases pore size, and removes lignin to the surface of the biomass (Bals et al., 2006; Mosier et al., 2005b). AFEX has been shown to be effective in increasing the rate and extent of cellulose hydrolysis in several types of grasses and agricultural residues. Xylose yields after enzymatic hydrolysis also tend to be fairly high with little or no extra xylanase addition (Teymouri et al., 2005; Alizadeh et al., 2005; Murnen et al., 2007). Previous research with DDGS shows high glucose yields at the relatively low temperature of AFEX pretreatment conditions (Bals et al., 2006).

Distillers' grains, either wet or dried, are considered a good source of supplemental protein for ruminant and poultry diet due to their high protein content. In addition, high energy value and digestible fiber content of the distiller's grains make these by-products attractive as an energy source. One of the critical issues of utilizing DDGS or WDG as an additional source of fermentable sugar is associated with the changes in composition of the final product. The co-product of a modified dry grind process where distiller's grains are recycled in the process to extract the unutilized sugars is termed "enhanced DDGS (eDDGS)." Because DDGS is mainly sold as animal feed, its nutritional value is of particular interest.

The purpose of this paper is to present results for the application of two different pretreatment methods, controlled pH liquid hot water (LHW) pretreatment and AFEX treatment, to distillers' grains, to enhance its digestibility by cellulolytic enzymes. We present hydrolysis yields of the pretreated distiller's grains as well as results for subsequent fermentability of the hydrolyzate. The solids that remain after the fermentation is referred to as "enhanced distiller's grains" throughout this special issue. Changes in the composition of enhanced DDGS (eDDGS) relative to conventional distiller's grains are also discussed in this paper. The enhanced DDGS prepared via laboratory scale hydrolysis and fermentation of the pretreated WDG (wet cake) was analyzed for its feed and nutritional changes and is compared to conventional DDGS.

2. Methods

2.1. Materials

DDGS, wet distiller's grains (WDG or wet cake), and thin stillage were obtained from Big River Resources, LLC (West Burlington, IA). Spezyme CP (cellulase), GC220 (cellulase), and Multifect Pectinase FE (xylanase) were provided by Genencor International, Inc. (Rochester, NY) and Novo 188 (beta glucosidase, Novo Nordisk, Denmark) was purchased from Sigma (Cat. No. C6150). Depol 740 L (feruloyl esterase) was provided by Biocatalysts Enzymes (Wales, UK). All other reagents and chemicals, unless otherwise noted, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Controlled pH liquid hot water (LHW) pretreatment

The aqueous pretreatment of DDGS (moisture = 11%) consisted of mixing DDGS with distilled, de-ionized water at 15.7% solids loading (w/v, g dry solids per 100 mL liquid) and heating at 160 °C for 20 min under pressure in order to keep the water in a liquid state. Pretreatment of wet distiller's grains (WDG, 64% moisture) was done at the same conditions except that it was mixed with thin stillage instead of de-ionized water at different solids loadings (13–30% wt of dry DG per total wt). For the pretreatment, 1 in. OD × 0.083 in. (2.54 cm × 2.1 mm) wall thickness, 316 stainless steel tubing and a pair of 1 in. (2.54 cm) Swagelok tube end fittings (Swagelok, Indianapolis, IN), were used. Each tube was 4.5 in. (11.4 cm) in length and 45 mL in total volume. The sample volume was only 33.7 mL to give about 25% of free space for liquid expansion during heating to 160 °C. The reactor tube containing the DDGS, slurried in water, was placed in a Tecam® SBL-1 fluidized sand bath (Cole–Parmer, Vernon Hills, IL) at 160 °C for 24 min consisting of a 4 min heat up time and 20 min pretreatment time. After pretreatment, each tube was quenched in water before transfer to ice-water slurry for cooling. Pretreated material was stored frozen until analysis and hydrolysis experiments.

2.3. Ammonia fiber expansion (AFEX) treatment

The AFEX pretreatment process was performed in a 300 mL stainless steel pressure vessel. For each batch, 25 g

dry weight of DDGS at 11% moisture content (total weight basis) was added to the vessel. Glass spheres were added to minimize void space, thereby reducing the amount of ammonia in the vapor phase within the reactor. The lid was bolted shut, and a sample cylinder containing 21.0 g of liquid anhydrous ammonia was connected to allow the ammonia to be charged into the vessel. Approximately 1 g of ammonia remained in the sample cylinder after charging, so that the total ammonia loading was 0.80:1 g/g dry biomass. The reactor was heated to 70 °C using a 400 W PARR heating mantle, requiring between 14 and 18 min, and allowed to stand at a constant temperature (±2 °C) for 5 min. At these conditions, the final pressure of the reactor was measured ranging from 350 to 430 psi. The pressure was explosively released by rapidly turning the exhaust valve. The treated biomass was removed and placed in a fume hood overnight to remove any residual ammonia. Multiple batches were combined and thoroughly mixed before being used for future experiments.

AFEX pretreatment for wet cake was performed in a similar manner. For wet cake at approximately 60% moisture (total weight basis), 15 g of anhydrous ammonia was used, and the temperature was raised to 90 °C. Due to the greater moisture content and higher temperatures, longer heating times were required for wet cake compared to DDGS. Recent AFEX process designs, carried out since the preparation of this manuscript both for laboratory and commercial scale equipment, feature nearly instantaneous heat up to final reaction temperatures. Rapid heating should reduce damage to biomass carbohydrates and proteins due to the pretreatment (note, for example, the effects of pretreatment on amino acid composition of DDGS given later in this article).

2.4. Enzyme digestibility test

2.4.1. Low-solids digestion

Enzymatic saccharification of native or pretreated distiller's dried grains with solubles (DDGS) was done by following a modified LAP 009 procedure. This procedure is modified from the standard NREL laboratory analytical procedure (LAP) 009 by scaling-up the masses and volumes by a factor of 10. The material was used, as is, without grinding. Enzyme loading for the hydrolysis was 15 FPU/g glucan of cellulase (Spezyme CP) and 40 U/g glucan of β-glucosidase (Novo 188). The 250 mL Nalgene bottles containing the pretreated DDGS solids at about 5% dry solids loading (w/w) and enzymes were placed in a New Brunswick Scientific model G24 Environmental Incubator Shaker (Edison, NJ) set at 50 °C and an agitation rate of 200 rpm. The pretreated slurry was allowed to digest at 50 °C for up to 72 h. A 1.0 mL aliquot was removed at regular intervals for the analysis.

$$\%\ \text{digestion} = \frac{\text{g of glucan(total)digested}}{\text{g of initial glucan(total)}} \times 100$$

2.4.2. High-solids digestion

The high-solids hydrolysis was done using wet distiller's grains as substrate. The wet distiller's grains (WDG) was pretreated at 13–30% (w/w) solids loadings by liquid hot water pretreatment as described above and the entire contents of the pretreated material was hydrolyzed at 50 °C, 200 rpm for 48 or 72 h. Unlike the standard enzyme digestibility test following the LAP 009 procedure, no additional buffer or water was added to the pretreated materials and the starting material was not milled. For the hydrolysis the whole slurry of the pretreated wet distiller's grains from three reactor tubes was emptied out to a 250 mL Nalgene bottle. Spezyme CP or GC220 was pipetted into the flasks to achieve a cellulase loading of 15 FPU/g of total glucan (as calculated from the amount of glucan initially loaded into the pretreatment reactor tubes). In addition, Novo 188, obtained from Sigma–Aldrich (St. Louis, MO), was pipetted into the flasks to achieve a β-glucosidase loading of 40 IU/g total glucan. In some cases, 50 U xylanase (Multifect Pectinase FE) and 2 U feruloyl esterase (Depol 740 L) enzymes per g dry solids were supplemented to increase the overall sugar yields as described by Dien et al., 2008. Sugar yields after the hydrolysis were measured by HPLC analysis. Glucose and xylose yields of the hydrolysis runs were calculated based on the initial total glucan (cellulose and starch) and xylan, respectively. The initial total glucan or xylan includes glucan or xylan from the WDG plus soluble glucan or xylan from the thin stillage.

AFEX treated WDG was provided by Biomass Conversion Research Laboratory at Michigan State University. The pretreatment procedure is described above. For the hydrolysis, the AFEX treated WDG was mixed with pH 4.8 buffer to give 15 w/w solids loading (wt of dry WDG per total wt). Enzymatic hydrolysis of the AFEX treated WDG was carried out at the same conditions as for the LHW treated WDG. The resulting hydrolyzate was analyzed by HPLC for amount of released sugars. As the AFEX treated WDG did not involve thin stillage, the glucose and xylose yields were calculated based on the initial total glucan and xylan from WDG only.

It is important to note that this approach in enzymatic hydrolysis was taken to put AFEX and LHW on the same footing for subsequent analysis. The AFEX process is dry to dry, while the LHW process is a wet process. Any stirrable concentration of AFEX treated solids can be hydrolyzed or hydrolyzed and fermented via fed-batch addition of solids to the hydrolysis/fermentation vessel.

2.5. Fermentation of pretreated WDG and generation of enhanced DDGS

Enhanced DDGS was prepared from both liquid hot water pretreated WDG and AFEX treated WDG. Pretreatment of the distiller's grains is as described previously. Three pretreatment tubes of LHW treated distillers

grains at 13% (w/w) solids loading were emptied into a 250 mL Nalgene bottle and hydrolyzed by cellulase enzymes at a loading of 15 FPU Spezyme CP/g glucan and 40 U Novo 188/g glucan. A total of 8 bottles of the LHW treated wet cake were prepared for enzymatic hydrolysis to give enough material for sampling and subsequent fermentation. Another 8 bottles of the AFEX treated WDG at 15% (w/w) solids loading were prepared for the hydrolysis and the subsequent fermentation. The hydrolysis time course was obtained in a way that each data point represents a single sample from one of the 8 hydrolysis runs. This approach in sampling was taken to prevent excessive loss of solids caused by multiple samplings from a single hydrolysis run, which adds variability to the overall sugar yields measured at the end of each run.

The pretreated mixtures were placed in a shaking incubator at 50 °C, 200 rpm. From each bottle, aliquots of 5–7 mL of sample were taken at different times for analysis. After 72 h the hydrolyzate was pooled together and centrifuged at 10,000 rpm for 25 min. The liquid supernatant was collected and concentrated via lyophilization with a target of 100 mL final volume. The solids recovered from the centrifugation were placed into a drying oven at 40 °C for 24 h. The 100 mL of the concentrated liquid hydrolyzate was transferred into side-arm flask, pH adjusted to 5.5 with ammonium hydroxide, then inoculated with *Saccharomyces cerevisiae* strain ATCC 4124 (non-recombinant).

For inoculum generation, 8 mL of seed culture were used to inoculate 100 mL YEPD (YEP plus 2% glucose) in a 300 mL baffled Erlenmeyer flask equipped with a side-arm. The inoculation cultures were incubated in a shaker at 28 °C and 200 rpm and grown aerobically for 24 h (final OD 500–550 KU). The yeast was harvested by centrifugation at 3000*g* for 5 min at room temperature. The supernatant was discarded and the cells were transferred into a 300 mL baffled Erlenmeyer flask containing the concentrated WDG hydrolyzate. The initial cell mass concentration prior to the fermentation in each experiment was 8.5–9 g dry weight/L. The flasks were then sealed with plastic wrap to allow fermentation to be carried out under largely anaerobic conditions. The cultures were placed in a shaker and incubated at 28 °C for 48 h. At regular intervals 1 mL samples of the fermentation mixture were removed for monitoring the fermentation. Fermentation broth from the final 48 h of fermentation was poured into centrifuge bottles that were centrifuged for 20 min at 3100*g* and 4 °C. The liquid was decanted and the corresponding solids recovered from the centrifugation were dried for 24 h.

Ethanol in the fermented mash was allowed to evaporate for 24 h in a fume hood. The solid samples were then further dried at around boiling point (96–98 °C) overnight in order to make the drying process comparable to a dry milling operation. The final enhanced DDGS samples ground to 1/4 inch mesh in a mill were sent to the Experiment Station Chemical Laboratories in University of Missouri for feed analysis.

2.6. HPLC analysis

HPLC analysis of liquid samples was performed on a system consisting of a Varian 9010 Solvent Delivery System, Waters 717plus Auto sampler, Aminex HPX–87 H column (Biorad, Hercules, CA), Waters 2414 Refractive Index Detector, Waters 2487 Dual λ Absorbance Detector, and a Hewlett Packard HP3396G Integrator. The mobile phase was 5 mM H_2SO_4 filtered through 0.2 μm nylon filter (Millipore) and degassed. The mobile phase flow rate was 0.6 mL/min and the column temperature was maintained at 60 °C by an Eppendorf CH–30 Column Heater controlled by an Eppendorf TC-50.

3. Results

3.1. Effect of pretreatment of distiller's grains

Compositions of the distiller's grains (both DDGS and wet cake) and thin stillage are presented in a separate paper (Kim et al., 2008a) in this special issue. The wet distiller's grains (WDG) contain 18.5% total glucan, 14.9% xylan, and 5.5% arabinan, by dry mass basis. The thin stillage obtained from Big River Resources, LLC for this research contained an average of 13 g/L soluble glucan and 3.8 g/L soluble xylan. DDGS contains slightly higher glucan (21%) and 8.2% xylan.

First, the distiller's dried grains with solubles (DDGS) were evaluated for its digestibility by cellulase (15 FPU/g glucan Spezyme CP) and β-glucosidase (40 IU/g glucan Novozyme 188). Hydrolysis was carried out by following the modified LAP 009 procedure as described in Materials and Methods. The percent dry solids concentration for the modified LAP 009 procedure was about 5% (w/w). The results shown in Fig. 1 are an average of multiple runs by several of the members of the Midwest Consortium (Purdue, USDA NCAUR, University of Illinois and Michigan State University). Cellulose digestibility of the pretreated and untreated DDGS is compared.

The results show that the untreated DDGS digests rapidly compared with some other biomass feedstocks, such as corn stover, even without any pretreatment prior to addition of enzymes. For example, enzymatic hydrolysis of unpretreated corn stover particles (53–75 μm) at the same enzyme loading (15 FPU/g glucan) resulted in 25% glucan conversion after 7 days of hydrolysis (Zeng et al., 2007).

Digestion of the untreated DDGS leveled off after 3 days (72 h) resulting in a glucose yield of 76% (measured at 168 h). The conversions were higher for the pretreated forms of the DDGS, either by aqueous pretreatment or AFEX, than the untreated DDGS. Pretreatment dramatically increases rates of hydrolysis. Pretreatment for 20 min using the LHW system increased the ultimate yield of glucose to 98% at 72 h of hydrolysis and increased the initial hydrolysis rate by 10 ×, with hydrolysis leveling off at 5 h. AFEX pretreatment also resulted in a significantly increased hydrolysis rate and an enhanced glucose yield.

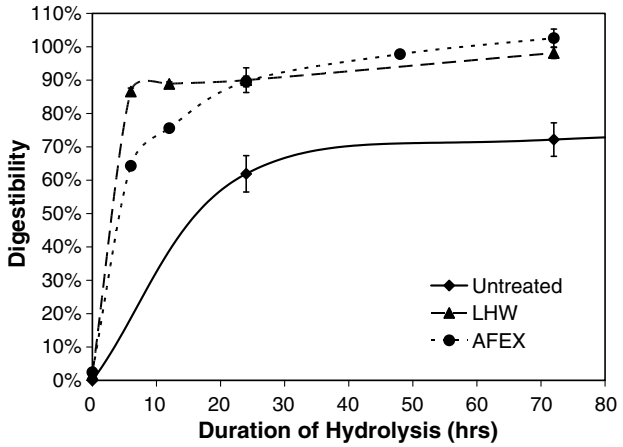

Fig. 1. Glucan digestibility of untreated DDGS versus DDGS pretreated by liquid hot water pretreatment (LHW) at 15.7% solids, 160 °C, 20 min and DDGS pretreated by AFEX at an ammonia loading of 0.8 g NH_3/g dry DDGS and at 70 °C. Enzyme loading: 15 FPU/g glucan Cellulase (Spezyme CP), 40 IU/g glucan β-glucosidase (Novozyme 188). Hydrolysis conditions: < 5% (w/w) solids loading, pH 4.8, 50 °C, 200 rpm, following modified LAP 009 procedure. Error bars represent 95% CI.

Glucose yield for the AFEX treated DDGS was 102.6% after 72 h. The yield higher than 100% is likely due to variability associated with measuring the total initial glucan of the DDGS. For the AFEX pretreated DDGS the digestibility again occurred at an enhanced rate that started to level off after 24 h of hydrolysis. These results clearly demonstrated that the pretreatment of the distiller's grains significantly enhanced both the rate and extent of hydrolysis. This is of significance when the large throughput of DDGS in the dry mill plants is considered.

The liquid hot water pretreatment solubilized only 2.9% of the total glucan as glucose in the solution upon pretreatment. Also, there was no detectable amount of sugar degradation products in the liquid fraction of the liquid pretreated DDGS. During the enzymatic hydrolysis, yields of xylose and arabinose from the hemicellulose fraction were much lower than glucose yields. Xylose yields were in a range of 20–40% with a maximum yield of 44% (data not shown). Xylose yields for AFEX treated DDGS were also low, obtaining less than 20% yield after 72 h of hydrolysis. The low yields of xylose illustrate the potential impact of hemicellulases on the hydrolysis yields. The study of enzyme formulation to enhance overall sugar yields is discussed in the separate paper by Dien et al. (2008) in this special issue.

3.2. Digestibility of wet distiller's grains at high-solids loadings

Pretreatment, hydrolysis, and fermentation at high-solids loadings is an important parameter in enhancing the economic attractiveness of processing wet distiller's grains (WDG) or DDGS into value-added products. Higher loadings (i.e., higher initial concentrations of WDG or DDGS) result in higher sugar concentrations and greater ethanol titers, which in turn require less energy and in some areas, smaller equipment for a given throughput.

Fig. 2 shows hydrolysis time courses of the LHW treated WDG at 13% (w/w) solids loading and the AFEX treated WDG at 15% (w/w) solids loading using 15 FPU cellulase (Spezyme CP) and 40 IU β-glucosidase (Novo 188). For each time course in Fig. 2, a total of 8 separate hydrolysis runs were made. Unlike the modified LAP 009 procedure the hydrolysis of the LHW treated WDG was carried out without diluting the pretreated material by buffer or DI water. After 3 days of hydrolysis the glucose yield was 68% for both LHW and AFEX treated WDGs. Xylose yield was 20% for the liquid hot water pretreatment and 12.2% for the AFEX treatment. The final sugar concentrations at these conditions were 27.1 g/L glucose and 5.3 g/L xylose for the LHW treated WDG and 24 g/L glucose and 3.5 g/L xylose for the AFEX treated WDG. The final glucose concentration for the LHW treated WDG was slightly higher than AFEX pretreated WDG despite the same glucose yield. This is because, as described in Materials and Methods, LHW pretreatment uses thin stillage as its pretreatment media, which contains additional soluble glucan.

The maximum glucose yields from the hydrolysis of WDG at these solids loadings were lower by approximately 30% compared to the glucose yields in Fig. 1 where glucose conversions were achieved by following the modified LAP 009 procedure. The standard LAP 009 procedure is done at an optimal initial pH using pH 4.8 buffer and at a 5% w/w solids loading. This difference suggests that there may be inhibition of cellulase enzymes by end-products or other inhibitors present in the distiller's grains which become noticeable at a high-solids loading. This effect was studied at 15%, 20%, and 30% loading for hydrolysis as well as pretreatment steps.

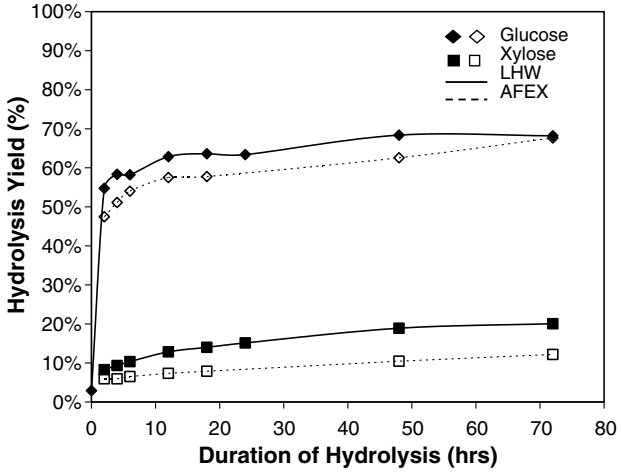

Fig. 2. Glucan and xylan digestibility of LHW treated and AFEX treated WDG. Enzyme loading: 15 FPU cellulase (Spezyme CP) and 40 IU β-glucosidase (Novozyme 188) per gram total glucan. Hydrolysis conditions: 15% (w/v) (equivalent to 13% w/w) solids loading for LHW treated WDG and 15% (w/w) for AFEX treated WDG. Hydrolysis at 50 °C, 200 rpm.

Enzyme digestibility of the pretreated WDG at three different solids loadings of hydrolysis (15%, 20%, and 30% w/w) utililzed a modified enzyme in which the formulation based on the work of Dien et al. (2008), included not only cellulase (GC220) and β-glucosidase (Novo 188), but also is supplemented with xylanase (Multifect Pectinase FE) and feruloyl esterase (Depol 740L). The two auxiliary enzymes are both commercially available. The activities and effects of these supplementary enzymes on the digestibility of DDGS are reported by Dien et al. (2008) in another paper in this special volume. The enzyme loading for the high-solids hydrolysis of WDG in this paper was 15 FPU cellulase and 40 IU β-glucosidase per gram glucan plus 50 U xylanase and 2 U feruloyl esterase per g dry solids.

The final sugar concentrations and their corresponding sugar yields after 48 h of hydrolysis are given in Table 1. Theoretical maximum sugar concentrations from hydrolysis of LHW treated WDG at 15% (w/w) solids loading are 45 g/L glucose and 31 g/L xylose. At the same solids loading the AFEX treated WDG gives 34 g/L glucose and 27 g/L xylose as the maximum theoretical concentrations. The difference in the maximum theoretical concentrations between LHW and AFEX treated WDGs derives from the method of pretreatment. Liquid hot water pretreatment utilizes thin stillage as pretreatment media, which also contains soluble polymeric sugars. The additional soluble glucan and xylan contributes to the higher maximum theoretical sugar concentrations of the aqueous pretreated WDG.

In the absence of supplementary enzymes, hydrolysis of LHW treated WDG at 15% and 20% (w/w) using 15 FPU GC220 and 40 IU Novozyme 188 per g glucan gave 65% glucose yields (data not shown). The 65% yield is similar to the yield (68%) from Fig. 2 which was obtained by using Spezyme CP instead of GC220 at the same enzyme loading. There was no visible liquefaction of the mixture at 30% dry solids loading in the absence of the auxiliary enzymes, which is an indication of poor hydrolysis efficiency. When the same materials are hydrolyzed at 5% solids complete conversions result.

Hydrolysis of LHW treated WDG at 15% and 20% solids loadings with the addition of auxiliary enzymes resulted enhanced glucose yield to 80%. Xylose yields were between 40% and 50%, which are at least 2 times higher than the case without the addition of xylanase and feruloyl esterase, although further development of xylanase activity in a cellulolytic enzyme formulation is needed. Both glucose and xylose yields were higher than the yields shown in Fig. 2 obtained without the auxiliary enzymes. However, the overall sugar yields for the case of 30% (wt/wt) solids loading was less (67% glucose yield, 48% xylose yield), which, at first examination, suggests that the concentration of end-products or other inhibitors may interfere with the enzyme activity even though the ratio of enzyme to substrate is constant.

Enzyme digestion of the AFEX treated WDG at 15% solids loading with the addition of the auxiliary enzymes showed a slight increase (from 68% to 72%) in the glucose yield and approximately 4 times increase in the xylose yield (from 12% to 45%) as compared to the case without addition the xylanase and feruloyl esterase enzymes. The rise in glucan conversion as well as xylan is likely due to synergistic effects between the different enzymes. As more hemicellulose is hydrolyzed, this likely increases the glucan susceptibility for attack, thereby slightly improving glucose yield as well.

As noted previously, AFEX is a dry to dry process. Dry biomass enters the process and after AFEX treatment and ammonia recovery, the treated material is substantially dry. Thus AFEX treated material can be batch fed to any stirrable concentration in the hydrolysis vessel; over 30% solids (300 g solids per liter) has been achieved in early work. Future research work planned within the Midwest Consortium will consider this aspect of the utilization of AFEX treated DDGS and WDG. This paper compares LHW and AFEX treated materials under comparable conditions and hence dry to dry runs are not reported here.

3.3. Fermentation of pretreated wet distiller's grains

To generate the enhanced DDGS (eDDGS), hydrolyzates of LHW treated WDG or AFEX treated WDG were pooled together, concentrated, and fermented by non-recombinant *Saccharomyces* yeast strain ATCC 4124. Hydrolysis time courses of the LHW treated and AFEX treated WDGs for generation of the eDDGS are shown in Fig. 2. The final hydrolyzates were concentrated via lyophilization prior to the yeast fermentation to reduce

Table 1
Glucose and xylose yields from hydrolysis of pretreated WDG at high-solids loadings

Type of pretreatment	Loading as % solids wt/wt	Glucose			Xylose		
		Yield %	Concentration, g/L		Yield %	Concentration, g/L	
			Observed	Theoretical		Observed	Theoretical
LHW	15	77	34.8	45.1	41	12.8	30.9
	20	83	47.8	56.3	50	20.2	40.9
	30	67	56.6	85.0	38	25.3	66.1
AFEX	15	72	24.4	34.2	45	12.3	27.4

Enzyme loading: 15 FPU/g glucan Cellulase (GC220), 40 IU/g glucan β-glucosidase (Novozyme 188), 50 U/g dry solids Xylanase (Multifect Pectinase FE), and 2 U/g dry solids feruloyl esterase (Depol 740). Hydrolyzed at 50 °C, 200 rpm, 48 h.

post-fermentation drying time of the final fermented mash. By-products such as glycerol, lactic acid and acetic acid were present at higher levels than they usually are in a conventional fermented broth. In an industrial process, recirculation of process liquid streams will cause accumulation of nonmetabolizable compounds in the hydrolyzate as well as in the fermentation, thereby potentially inhibiting the yeast.

Accumulation of the toxic compounds in process streams of a modified dry grind process is discussed in another paper in this special volume (Kim et al., 2008b). Simulation of a modified dry grind process has shown that the recycle of WDG in the process can result in 2–5 times higher concentrations of the inhibitory substances in fermentation mash as compared to the conventional dry grind process at the same level of backset. The laboratory hydrolyzate we obtain from the pretreated WDG does not involve accumulation of these inhibitory compounds, as it is a one-cycle operation. The concentration of hydrolyzate via lyophilization enabled us to test fermentability of a hydrolyzate that contains increased levels of yeast inhibitors. Although the concentrated hydrolyzate is not representative of an industrial process, it is a useful material to test impact of unidentified inhibitors.

Inhibition of the enzyme by unspecified inhibitors in the hydrolysate is insufficient to explain the different results. If inhibition were the only factor, the decrease in activity should be a constant at constant enzyme/solids loading ratio. Other explanations may include diffusional or mass transfer resistances that result in higher, localized ratios of products or other inhibitors, or agglomeration of small particles into larger ones with reduced accessible surface areas. Hence, lack of mixing could also be a factor. Particle size effects for corn stover (ground corn stalks) have recently been shown to result in a $2\times$ difference in the extent of hydrolysis (Zeng et al., 2007), and hence, an induced increase in size at high-solids loading, while not proven, should be considered a possible explanation.

Lyophilization of the hydrolyzate of the LHW treated WDG concentrated the sugars and other components by 5 times, resulting in 120 g/L glucose, 21 g/L xylose, 47 g/L glycerol, 4.0 g/L lactic acid, and 2.5 g/L acetic acid. The hydrolyzate of AFEX treated WDG was concentrated by 3.4 times, giving 75 g/L glucose, 9 g/L xylose, 12 g/L glycerol, 1.1 g/L lactic acid, and 1.4 g/L acetic acid. Sugar degradation products such as HMF and furfural were at non-detectable levels even after the lyophilization.

Fermentation time courses are given in Fig. 3. Over 95% of the glucose was consumed within 24 h for the LHW treated WDG hydrolyzate. Glucose was completely consumed within 6 h for the hydrolyzate of AFEX treated WDG. The slower fermentation rate for the hydrolyzate of LHW treated WDG is due to the high concentrations of initially present sugars that may affect the yeast metabolism. The metabolic yield after 48 h of fermentation was 0.53 g ethanol/g consumed sugar or 104% of the theoretical yield for the LHW treated WDG. It was 0.61 g ethanol/g consumed sugar for the AFEX treated WDG hydrolyzate, which is equivalent to 120% of the theoretical yield. The yields higher than 100% of the theoretical ethanol yields imply that co-current hydrolysis and fermentation of oligosaccharides or colloidal cellulose in the WDG hydrolyzates is occurring in the fermentor.

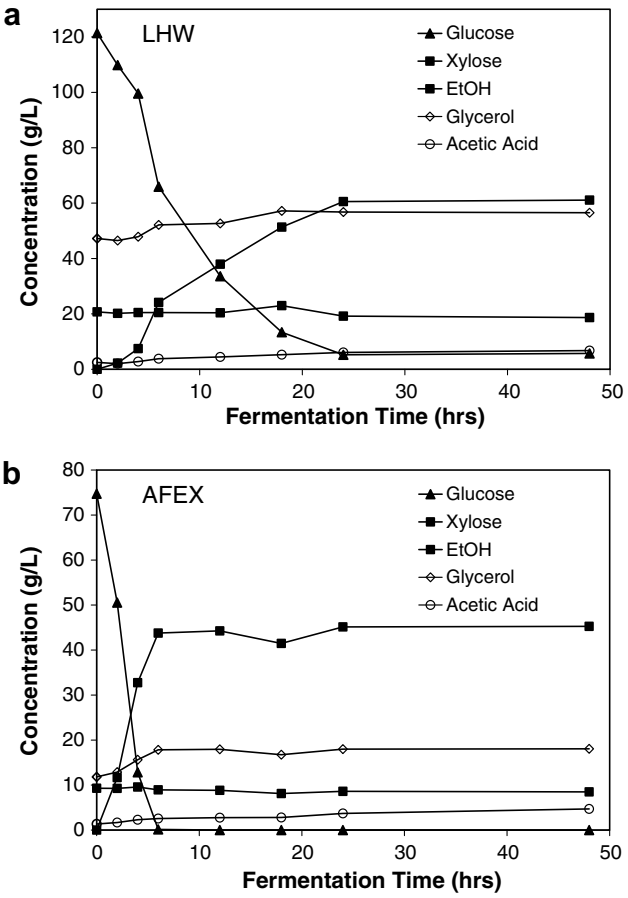

Fig. 3. Fermentation of (a) hydrolyzate of LHW treated WDG at 13% solids (w/w), and (b) hydrolyzate of AFEX treated WDG at 15% solids (w/w). Enzyme loading: 15 FPU/g glucan cellulase (Spezyme CP) and 40 IU/g glucan β-glucosidase (Novozyme 188). Hydrolysis at 50 °C, 200 rpm for 72 h. Final hydrolyzates were concentrated by 5 times for LHW treated WDG and 3.4 times for AFEX treated WDG via lyophilization, prior to the fermentation.

Final ethanol concentration was 61 g/L for the LHW treated WDG and 45 g/L for the AFEX hydrolyzate. In both cases the glucose was almost totally consumed, with the difference in ethanol being proportional to the initial sugar yields. Results suggest that, although the sugar consumption rate was slower for the LHW treated WDG, the extent of the fermentation was not significantly affected at the levels of sugars and inhibitory substances found in the concentrated hydrolyzate.

3.4. Feed analysis of enhanced DDGS

The fermented slurry mixed with the previously recovered solids fraction of the hydrolyzate was dried and

analyzed for compositional changes (Table 2). In this paper, we only report contents of the major components, such as crude protein, crude fat, crude fiber and ash, to show a proximate change in these compounds upon processing of the WDG. Amino acid profiles of the enhanced DDGS (eDDGS) are also given and compared to that of the conventional DDGS in Fig. 4. Although there is considerable variation in its composition, typically DDGS contains about 30% of crude protein, 11% of crude fat, 9% of crude fiber and 6% of ash (Spiehs et al., 2002). As shown in Table 2, the DDGS used in this study contains slightly higher fat and lower fiber and ash than average DDGS. Differences in composition of feed corn, processing methods, fermentation efficiency, and extents of process liquid recycle cause the variability in the composition of DDGS.

Table 2
Feed analysis results of DDGS and enhanced DDGS

% Compositions	DDGS Average value[d]	DDGS, (this work)	Enhanced DDGS (from LHW WDG)	Enhanced DDGS (from AFEX WDG)
Moisture	11.1	10.4	6.6	11.5
Crude protein[a]	30.2	28.3	41.2	50.8
Crude fat	10.9	14.5	14.7	7.2
Crude fiber	8.8	6.5	2.9	0.5
Ash	5.8	4.8	5.3	6.0
Pepsin digestibility[b]	–	86.7	86.7	92.2
Carbohydrates[c]	–	52.5	38.8	36.0

Results are expressed on a dry matter basis (wt/wt%).
[a] Crude protein by Kjeldahl.
[b] Percentage of crude protein digested by pepsin.
[c] Carbohydrates calculated by difference from proximate data.
[d] Spiehs et al., 2002.

The enhanced DDGS from LHW treated WDG was found to contain about 50% more protein, 60% less fiber, and 13% more ash than the DDGS. There was no significant change in the crude fat content. For the enhanced DDGS generated from AFEX treated WDG, the changes were 80% more protein, 50% less fat, 90% less fiber, and 25% more ash. The enhanced DDGS, either from LHW treated or AFEX treated WDG, exhibited increased protein and decreased fiber contents as a result of fiber and residual starch removal and their subsequent conversion to ethanol. Due to the decrease in fiber content, it may be possible that the enhanced DDGS market could be suitable for the swine and poultry markets. Although the increased protein content alone may enhance the value of the DDGS as a supplementary protein source in livestock diet, a more detailed analysis on the quality of the resulting proteins is required for an accurate evaluation of its value as feed for different livestock animals. For purposes of economic analysis, and based on the amino acid profile of the eDDGS, Perkis et al. (2008) in another paper of this special issue, assume the value of eDDGS to be the same as the starting DDGS.

Possible heat damage to the proteins was suggested by the amino acid profiles of the enhanced DDGSs (Fig. 4). Among the amino acids, lysine, an essential amino acid in animal nutrition, is of particular interest as it is known to be deficient in corn-based products and to be heat

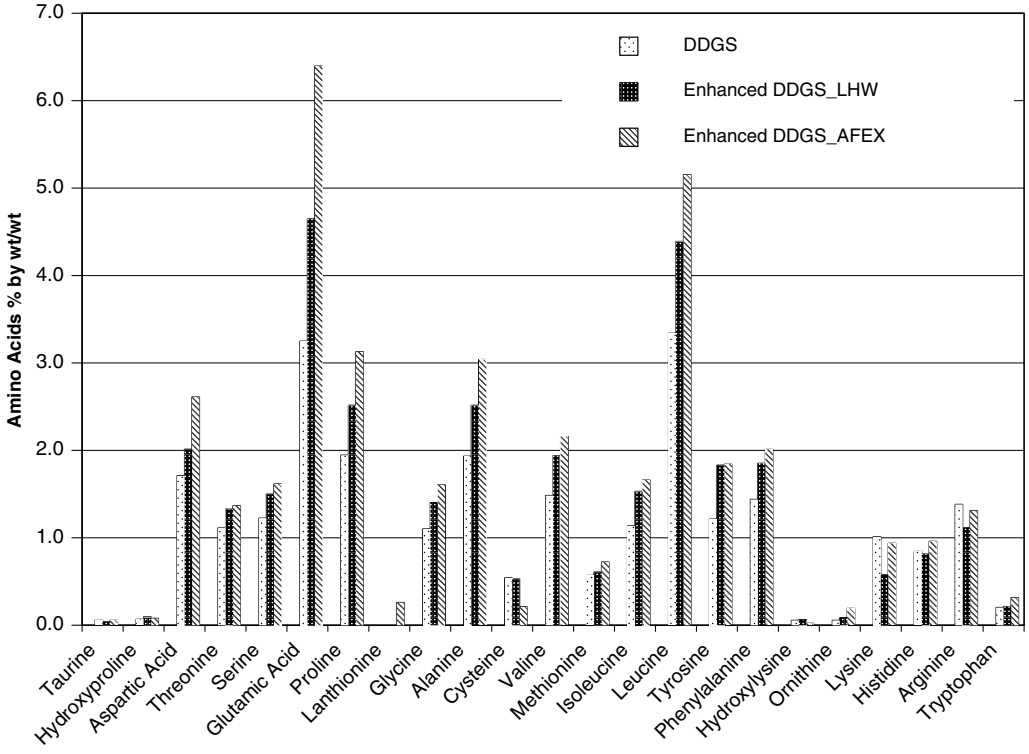

Fig. 4. Amino acid profiles of DDGS and enhanced DDGSs. Results are expressed on a dry matter basis (w/w%).

sensitive (Adrian, 1974; Carpenter and Booth, 1973). Other heat labile amino acids include methionine, tryptophan and cystine (Van Soest, 1983). While most of the amino acid contents were increased in the enhanced DDGS, the heat sensitive amino acids were at the same levels or decreased. Especially, lysine was found to significantly decrease in the enhanced DDGS from LHW treated WDG as compared to the other heat sensitive amino acids. Lysine content of the AFEX enhanced DDGS was also slightly lower than that of the DDGS. For the most of the other amino acids, there were significant increases in their content.

The combined total amount of amino acids in DDGS was 25.6% (by dry weight) as calculated from the amino acid profile. The number is close to the protein content (28.3%) as determined from the total Kjeldahl nitrogen number given in Table 2. However, for the enhanced DDGS, the total combined amino acids content was only 31.6% for the LHW eDDGS and 37.6% for the AFEX eDDGS, which is about 30% lower than the protein value estimated from the total nitrogen content (41.2% for LHW eDDGS and 50.8% for AFEX eDDGS in Table 2). Considering that there was no addition of nitrogen containing nutrients in the process of generating the laboratory enhanced DDGS from the LHW treated WDG, the difference in the measured protein contents of the enhanced DDGS suggests possible heat damage of the proteins resulting in the same nitrogen level but less intact amino acids. On the other hand, AFEX process involves ammonia which can contribute to an increased Kjeldahl nitrogen number. Therefore, the main causes of the difference in the protein contents of the AFEX eDDGS are less clear. However, for both LHW and AFEX eDDGSs, the protein contents measured as sum of the amino acids were still higher than for the DDGS. The protein content determined from the amino acid profile was 23% higher for the LHW eDDGS and 47% higher for the AFEX eDDGS than the conventional DDGS. The results indicate that an accurate protein content measurement for the eDDGS should be based on measuring the constituent amino acids, rather than by measuring the total nitrogen. Pepsin digestibility of the protein in the enhanced DDGS was unaffected by the possible heat damage. The pepsin digestibility was the same for both DDGS and LHW eDDGS (86.7%) and was slightly higher for the AFEX eDDGS (92%).

Changes in color and smell of the feed are critical issues that may affect the feed's quality and market value. The enhanced DDGSs were darker than the conventional DDGS. Color darkness is also an indicator of heat damage via Maillard reactions. Maillard reactions during the heat treatment of grains are known to cause partial indigestibility of lysine and formation of non-nutritive compounds (Barrier-Guillot et al., 1993; Carpenter and Booth, 1973; Mauron, 1981). Although the results suggest a possible heat damage of the enhanced DDGS, a more detailed nutritional analysis still needs to be conducted to examine its quality as an animal food. An alternate approach to avoid heat damage to the material would be to fractionate proteins before milling and fermentation steps to produce a protein-rich feed that has a more value as livestock feed than the conventional DDGS. In addition, refined pretreatment process designs, such as those mentioned earlier for a rapid heat up AFEX system, should also reduce damage to sensitive components.

Other than as a supplementary protein source in livestock diet, the enhanced DDGS could be used efficiently as an alternative energy source to generate electricity and heat for the process. Comprehensive analysis by Morey et al. (2006a,b) on characteristics of DDGS as a fuel source for the ethanol plant provides insights into alternate strategies for using DDGS and obtaining possible energy saving in a modified dry grind processes. According to their study, dry distiller's grains have a lower heating value of 8819 Btu/lb, and dry distillers grains with solubles (DDGS) is 8703 Btu/lb (lower heating value). These values are greater than that of corn stover (7192 Btu/lb, dried), which is also another potential biomass energy source, due to the oil fraction of DG or DDGS (see crude fat content in Table 2). The enhanced DDGS still contains the high fat content (approximately 7–15% depending on the pretreatment method applied). Therefore, it is expected that its heating value per dry mass does not change significantly as compared to the conventional DDGS. Energy required for the additional unit operations to extract sugars from distiller's grains could be supplemented by the heat energy stored in the co-products (enhanced DDGS or enhanced DG) of a modified dry grind process.

4. Summary and conclusions

In this study, liquid hot water pretreatment (LHW) and ammonia fiber expansion (AFEX) treatment were applied to increase the enzymatic digestibility of distiller's grains. For the aqueous pretreatment, pretreatment conditions of 160 °C and 20 min appeared to be sufficient to extract sugars from distiller's grains with minimal sugar degradation. AFEX pretreatment conditions ranged from 70 to 90 °C and 20–30 min. Over 90% of glucose yield was achieved from the hydrolysis of both LHW and AFEX treated DDGSs (dried distiller's grains with solubles) with 15 FPU cellulase and 40 IU β-glucosidase per gram of total glucan within 24 h. Reduced sugar yields of distiller's grains at high-solids loadings between a range of 13% to 30% (wt of dry solids/total wt) as compared to the low solids hydrolysis indicated that reduced activity of cellulolytic enzymes, due to either inhibition or mass transfer limitation, may become an issue for the high-solids hydrolysis. Further study is needed to confirm the major source and mechanism of the possible inhibition at high-solids loadings of the distiller's grains. Such work is required to enhance the overall sugar yields for high-solids hydrolysis of distiller's grains, and subsequently, to avoid possible costs associated with dealing of dilute sugar streams in the process.

Addition of supplemental xylanase and feruloyl esterase enzymes along with cellulase and β-glucosidase to the

LHW pretreated wet distiller's grains at 15–20% (wt dry solids/wt total) dry solids increased glucose yields from 65% to 80%. Xylose yields were also enhanced by at least 2 times for the LHW treated distiller's grains and by 4 times for the AFEX treated wet distiller's grains when the cellulases were supplemented with xylanase and feruloyl esterase. These results suggest that the digestibility of distiller's grains can be greatly improved with an optimal mixture of enzymes for the high-solids hydrolysis. Concentrated hydrolyzates of both LHW and AFEX pretreated wet distiller's grains were successfully fermented by *Saccharomyces* yeast with 100% of the theoretical (metabolic) ethanol yield being achieved. The laboratory prepared enhanced DDGSs were found to contain 20–50% higher proteins than conventional DDGS. Feed analysis and amino acid profiles of the enhanced DDGS indicated that several heat sensitive amino acids were damaged during heat treatment of the distiller's grains. This could be caused by Maillard reaction during pretreatment of distiller's grains, which also explains the color change of the processed distiller's grains. These preliminary observations necessitate a study of utilization of the enhanced DDGS as animal feed, in much greater depth, to evaluate its value over that of DDGS accurately.

Acknowledgements

We thank Genencor International, Inc. for a gift of enzymes and Big River Resources, LLC for supplying distiller's grains and thin stillage. The authors also thank Bruce S. Dien for his valuable advice regarding the enzyme recipe. This work was supported by US Department of Energy (Contract: DE-FG36-04GO14220) in cooperation with Midwest Consortium for Biobased Products and Bioenergy.

References

Adrian, J., 1974. Nutritional and physiological consequences of the maillard reaction. World Rev. Nutr. Diet. 19, 72–120.

Alizadeh, H., Teymouri, F., Gilbert, T.I., Dale, B.E., 2005. Pretreatment of switchgrass by ammonia fiber explosion (AFEX). Appl. Biochem. Biotechnol. 121–124, 1133–1141.

Bals, B., Dale, B., Balan, V., 2006. Enzymatic hydrolysis of distiller's grain and solubles (DDGS) using ammonia fiber expansion pretreatment. Energy Fuels 20, 2732–2736.

Barrier-Guillot, B., Zuprizal, Jondreville, C., Chagneau, A.M., Larbier, M., Leuillet, M., 1993. Effect of heat drying temperature on the nutritive value of corn in chickens and pigs. Anim. Feed Sci. Technol. 41, 149–159.

Bobleter, O., 1994. Hydrothermal degradation of polymers derived from plants. Prog. Polym. Sci. 19, 797–841.

Carpenter, K.J., Booth, V.H., 1973. Damage to lysine in food processing: its measurement and its significance. Nutr. Abstr. Rev. 43, 424–445.

Dien, B., Ximenes, E.A., O'Bryan, P.J., Moniruzzaman, M., Li, X., Balan, V., Dale, B., Cotta, M.A., 2008. Enzymatic characterization for hydrolysis of AFEX and liquid hot-water pretreated distiller's grains and their conversion to ethanol. Bioresour. Technol. 99, 5216–5225.

Kim, Y., Mosier, N.S., Hendrickson, R., Ezeji, T., Blaschek, H., Dien, B., Cotta, M., Dale, B., Ladisch, M.R., 2008a. Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. Bioresour. Technol. 99, 5165–5176.

Kim, Y., Mosier, N.S., Ladisch, M.R., 2008b. Process simulation of modified dry grind ethanol plant with recycle of pretreated and enzymatically hydrolyzed distiller's grains. Bioresour. Technol. 99, 5177–5192.

Ladisch, M.R., Kohlmann, K., Westgate, P., Weil, J., Yang, Y., 1998. Processes for treating cellulosic material. US Patent 5,846,787.

Mauron, J., 1981. The maillard reaction in food: A critical review from the nutritional stand-point. Prog. Food Nutr. Sci. 5, 5–35.

Morey, R.V., Tiffany, D.G., Hatfield, D.L., 2006a. Biomass for electricity and process heat at ethanol plants. Appl. Eng. Agri. 22, 1–6.

Morey, R.V., Hatfield, D.L., Sears, R., Tiffany, D.G., 2006. Characterization of feed streams and emissions from biomass gasification/combustion at fuel ethanol plants. ASABE Annual International Meeting, Portland, Oregon.

Mosier, N.S., Hendrickson, R., Brewer, M., Ho, N., Sedlak, M., Dreshel, R., Welch, G., Dien, B.S., Aden, A., Ladisch, M.R., 2005a. Industrial scale-up of pH-controlled liquid hot water pretreatment of corn fiber for fuel ethanol production. Appl. Biochem. Biotech. 125, 77–97.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M.R., 2005b. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technol. 96, 673–686.

Murnen, H., Balan, V., Chundawat, S., Bals, B., Sousa, L., Dale, B., 2007. Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of Miscanthus x giganteus to fermentable sugars. Biotechnol. Progr. 23, 846–850.

Perkis, D., Tyner, W., Dale, R., 2008. Economic analysis of a modified dry grind ethanol process with recycle of pretreated and enzymatically hydrolyzed distillers' grains. Bioresour. Technol. 99, 5243–5249.

Renewable Fuels Association, 2007. Ethanol industry outlook 2007: Building new horizons.

Spiehs, M.J., Whitney, M.H., Shurson, G.C., 2002. Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota. Journal of Animal Science 80 (10), 2639–2645.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B., 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. Bioresource Technol. 96, 2014–2018.

Van Soest, P.J., 1983. Nutritional ecology of the ruminant. O and B Books Inc. USA.

Walch, E., Xemann, A., Schinner, F., Bonn, G., Bobleter, O., 1992. Enzymatic saccharification of hemicellulose obtained from hydrothermally pretreated sugar-cane bagasse and bark. Bioresource Technol. 39, 173–177.

Weil, J., Sarikaya, A., Rau, S.L., Goetz, J., Ladisch, C., Brewer, M., Hendrickson, R., Ladisch, M.R., 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. Appl. Biochem. Biotechnol. 68, 21–40.

Weil, J.R., Sarikaya, A., Rau, S.L., Goetz, J., Ladisch, C.M., Brewer, M., Hendrickson, R., Ladisch, M.R., 1998. Pretreatment of corn fiber by pressure cooking in water. Appl. Biochem. Biotechnol. 73, 1–17.

Zeng, M., Mosier, N.S., Huang, C., Sherman, D.M., Ladisch, M.R., 2007. Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. Biotech. Bioeng. 97, 265–278.