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Stagewise Dilute-Acid Pretreatment and Enzyme Hydrolysis of Distillers' Grains and Corn Fiber

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Abstract Distillers' grains and corn fiber are the coproducts of the corn dry grind and wet milling industries, respectively. Availability of distillers' grains and corn fiber at the ethanol plant and their high levels of lignocellulosic material make these coproducts attractive feedstocks for conversion to ethanol. In this study, dilute sulfuric acid hydrolysis of these coproducts was investigated in a multistage scheme. After the completion of each pretreatment stage, the liquid substrate was separated and reused in the succeeding pretreatment stage with a fresh substrate. The substrate from each stage was also subjected to enzyme hydrolysis in a separate experiment. The sulfuric acid concentration and the substrate loading were maintained at 1.0 vol% and 15.0 wt.%, respectively, and the temperature was maintained at 120 °C in all the experiments. Experiments were also performed to study the effect of removing oil from the samples prior to the pretreatment. The highest concentration of monomeric sugars (MS) was observed when three stages of pretreatment were followed by the enzyme reaction. The enzyme hydrolysis of the three-stage pretreated dried distillers' grains and corn fiber yielded 122.6±5.8 and 184.5±4.1 mg/mL of MS, respectively. The formation of inhibitory products was also monitored.

Keywords Distillers' grains · Whole stillage · DDG · Corn fiber · Dilute sulfuric acid pretreatment · Enzyme hydrolysis · Monomeric sugars · Inhibitors · Ethanol

Introduction

Fuel ethanol production is one of the fastest growing industries in the USA. There are about 145 ethanol plants in the USA with annual production of 6.5 billion gallons in 2007. The current production of ethanol relies predominantly on starch and sugar-based agricultural material, such as corn and sugar canes. The production of starch and sugar-based ethanol is expected to reach 10–14 billion gallons per year by the year 2015 (Renewable Fuels

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Association). For ethanol industry to realize its anticipated production goals, it needs to rely on a more sustainable and inexpensive feedstock. Lignocellulosic biomass resources, such as agricultural residues, food-processing wastes, and wastes from the pulp and paper industry are plentiful and have the potential to be the feedstock for the production of ethanol.

Among the many potential lignocellulosic biomass resources, dry distillers' grains (DDG) and corn fiber are of particular interest as they are coproducts of the corn dry grind and wet milling industries, respectively. The availability of DDG and corn fiber at ethanol plants and their lower value compared with corn makes these products attractive feedstock for conversion to ethanol. What constitutes these products is a mixture of protein, oil, fiber, lignin, and residual starch not extracted during the milling and/or fermentation processes. On dry mass basis, DDG and corn fiber make up of about 30% and 14% of the corn kernel with about 50% and 70% lignocellulosic material and unutilized starch, respectively [1, 2]. Utilization of these products will result in a significant increase in ethanol production per bushel of corn.

Cellulose in lignocellulosic substrates is most resistance to biological degradation due to its crystalline structure. Moreover, it is embedded in a matrix consisting of lignin which forms a physical barrier limiting its availability for acid or enzyme hydrolysis. A variety of techniques have been studied for the conversion of lignocellulosic substrates to fermentable sugars; however, recent trends suggest a two-step scheme which includes an initial dilute-acid pretreatment of the biomass followed by enzyme hydrolysis of the pretreated substrate [3].

A number of studies have explored dilute-acid pretreatment followed by enzyme hydrolysis of the remaining solids for a variety of lignocellulosic substrates. Several studies by Bothast and coworkers examined the pretreatment and enzymatic saccharification of corn fiber under a variety of conditions [2, 4–6]. Pretreatment and enzyme hydrolysis of corn stover have been the topic of several other studies under dilute-acid or alkaline pretreatment conditions [7–11]. Other residual lignocellulosic materials such as quick fiber from a modified corn milling process [12], softwood [13], sorghum fiber [14], and poplar sawdust [15] have also been studied and shown potential as feedstock for ethanol production.

Recent research has also explored hot water pretreatment (LHW) and ammonia fiber expansion pretreatment (AFEX) methods prior to the enzyme hydrolysis of distillers' grains [16–19]. Both pretreatment methods were effective in enhancing the digestibility of the distillers' grains and minimizing the formation of monomeric sugars (MS) prior to the enzyme hydrolysis step. For example, Kim et al. [17] reported 100% of the theoretical yield for the conversion of sugars to ethanol for both LHW- and AFEX-pretreated wet distillers' grains. The reported concentration of sugars in the substrate after a single stage of AFEX pretreatment and enzyme hydrolysis of dry distillers' grain with solubles (DDGS) at 20% solid loading reached 68.0 mg/mL or 6.8% [18]. However, fermentation of a broth at this sugar concentration results in about half as much ethanol and, like previous such studies, are likely to result in less than 4% ethanol [12]. Low concentrations of ethanol in the beer product make this substrate economically unsuitable for distillation.

In the development of a cost-effective process for the conversion of lignocellulosic biomass to ethanol, the concentration of sugars in the fermentation broth is of great significance as it is an indicator of the achievable ethanol concentration in the beer. To achieve a higher concentration of sugars, studies have focused on stagewise pretreatment and enzyme hydrolysis schemes. Lee et al. [20] compared the pretreatment and the subsequent enzyme hydrolysis of hardwood material for a one-stage high-temperature pretreatment process (140–170 °C) versus a two-stage low-temperature process (100–120 °C). The

comparison was based on the suitability of the pretreated substrate for its simultaneous saccharification and fermentation. It was found that the one-stage process yields higher sugar concentrations; however, this was at the expense of a higher rate of the formation of inhibitory products such as 5-hydroxymethylfural and furfural. Nguyen and coworkers [21] studied a two-stage dilute sulfuric acid pretreatment followed by enzyme hydrolysis of softwood. The first stage was under relatively mild conditions where most of the hemicellulose was solubilized. The solid remains where then separated, washed, and was subjected to a second hydrolysis and under more severe conditions. The remaining solid was further hydrolyzed with cellulase enzymes. An overall sugar recovery of above 95% was achieved; however, use of excess water was noted as a concern due to its direct and significant effect on the overall ethanol production cost.

In this study, dilute sulfuric acid hydrolysis of the coproducts from the dry grind and wet milling ethanol production were investigated in a multistage scheme. After the completion of the first stage of pretreatment, the liquid substrate was separated and reused in the succeeding pretreatment stage with a fresh substrate. The substrate from each stage was also subjected to enzyme hydrolysis in a separate experiment. The sulfuric acid concentration and the substrate loading were maintained at 1.0% and 15.0% (db), respectively, and the temperature was maintained at 120 °C in all the experiments. Experiments were also performed to study the effect of removing oil from the samples prior to the pretreatment.

The substrates used in this study were DDG, corn fiber, and the whole stillage (WS) streams. WS is the residual stream from the first distillation column in a dry grind ethanol production where ethanol is separated from the beer stream. WS is centrifuged to yield wet distillers' grain (WDG) and thin stillage. Thin stillage is concentrated to form condensed solubles or syrup. Syrup and WDG are mixed to form wet distillers' grain with solubles (WDGS) and dried to form dry distillers' grain with solubles (DDGS). Hence, for all practical purposes, the composition of dry WS and DDGS is the same. On occasions, the processing facilities produce only dry WDG to make DDG without the addition of syrup.

Materials and Methods

Biomass and Chemicals

Distillers' grain (DDG, WS) and corn fiber were provided by Abengoa Bioenergy (dry grind corn ethanol facility, York, NE, USA) and Cargill (wet milling corn facility, Blair, NE, USA), respectively. All samples were stored in sealed containers at 4 °C before using. Carbohydrates (D(+)glucose; D(+)xylose; D(+)arabinose; D(+)cellobiose; D(+)galactose), acetic acid, glycerol, lactic acid, succinic acid, levulinic acid, 5-hydroxymethyl-2-furaldehyde (HMF), furfural, hexane, and enzymes (cellulase from *Trichoderma reesei* and β-glucosidase from *Aspergillus niger*) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sulfuric acid, sodium hydroxide, and sodium phosphate were purchased from Fisher Scientific (Pittsburg, PA, USA). Deionized water was purified for high-performance liquid chromatography (HPLC) mobile phase. Purification was performed with a Simplicity™ purification system (Millipore, Burlington, MA, USA).

Composition Determination Procedures

Analyses according to standard procedures were performed to determine the compositions of the distillers' grain and corn fiber samples. Analytical procedures were based on the

Laboratory Analytical Procedures documented by the National Renewable Energy Laboratory [22]. The procedures used were for moisture [23], oil [24], starch [25], carbohydrates and lignin [26], protein [27], and ash [28].

Equipment

The apparatus used for the pretreatment reactions was a 450 mL Parr 4562 bench-top high-pressure reactor with a detachable head (Parr Instrument, Moline, IL, USA). The reactor assembly was constructed of type 316 stainless steel. A 2.5-in. ID Parr 762HC2 glass liner was used to prevent corrosion in the reaction vessel. The reactor was equipped with a turbine impeller with a magnetic drive and a 1/12 hp variable speed motor, allowing for speeds up to 800 rpm. An electric heating mantle and an internal water-cooling loop maintained the desired temperature throughout each run via a proportional-integral-derivative (PID) controller. The reactor was also equipped with a thermocouple for temperature input into the controller. A Parr 4843 PID controller had the ability to ramp to reaction temperature, hold at the set point temperature, and ramp to the end temperature over selected time intervals. A cooling water solenoid valve, which was actuated by the controller, regulated temperatures in conjunction with the heating mantle. One of the reactor valve ports was connected to a 3-hp Pulsatron E plus series metering pump (Pulsafeeder-Standard Products Operation, Punta Gorda, FL), which provided acid to the reactor bomb. The pump settings were at 80% stroke rate and 100% stroke length which provided 30 mL of acid into the bomb in about 20 s.

Dilute-Acid Pretreatment Procedures

DDG and corn fiber were grinded in a coffee grinder (Mr. Coffee IDS55, Cleveland, OH) and passed through a 0.5-mm sieve (Ferrero Corp, Chicago, IL, USA). Prior to its use, the grinded biomass was dried in a convection oven at 65 °C. Defatted DDG was prepared by extraction of 100 g grinded DDG with 300 mL of hexane at room temperature for 30 min with a stirring rate of 300 rpm. After centrifugation at 11,000×*g* for 10 min and decantation of supernatant, the residue in the centrifuge tube was dried and used as defatted DDG. For the WS samples, the particle size of this solid mater was reduced by mixing in a blender (Warning Laboratory, Torrington, CT, USA) for 2 min at 22,000 rpm prior to use. WS so prepared was then used without further drying.

The reactor was charged with 30 g of the dried and grinded biomass and 170 mL of deionized water or 189.2 g of WS and 10.8 ml of deionized water. This resulted in a uniform solid loading in all cases. The acid injection metering pump was initially pressurized to about 60 psi. This was done to assure that sulfuric acid could be pumped into the reactor with ease when the reactor reached the desired temperature. The procedure was started by the stirring of the substrate at a constant speed of 500 rpm, while it was heated to the desired temperature (less than 5 min to reach 120 °C). As the desired temperature was reached, 30 mL of 6.67 vol% sulfuric acid solution was pumped into the reactor which resulted in 1.0 vol% acid concentration. The injection of acid to the reactor lasted about 20 s. The timing of the reaction started immediately after the complete charge of the acid solution into the reactor. Samples of about 15 mL were taken at 0, 5, 10, 15, 20, 30, 45, and 60 min for the analysis of the pretreated hydrolysate. The sampling port was flushed with compressed air prior to each sampling. To ensure the uniformity of samples compared with the remainder of the material inside of the reactor, a separate experiment was performed, and the solid content of the samples were measured. The highest deviation for the solid

content of the samples was less than 0.5 wt.% which confirmed the uniformity of the samples. Samples were immediately placed in an ice-water bath and then centrifuged at 10,000 rpm for 15 min. The liquid phase was then passed through a 0.2-μm cellulose acetate syringe filter (Toyo Roshi, Kaisha, Japan) and into a HPLC vial. For the stagewise pretreatment experiments, the reaction was carried out for 30 min without sampling. The reactor vessel was cooled down with ice-water immediately thereafter. The pretreated biomass was taken out of the reactor vessel, and the liquid phase was separated by vacuum filtration using grade 1 filter paper (Whatman, Maidstone, England). For the second stage of the reaction, the reactor was filled with the separated supernatant from the first stage of hydrolysis, and a specific amount of fresh biomass was added to maintain the solid loading equal to the first stage of the reaction (15 wt.%). The reaction was performed for 30 min with no additional sulfuric acid or deionized water. Three stages of dilute-acid hydrolysis pretreatments were performed in this manner. Parallel experiments were performed to the above procedure in which after each stage of pretreatment the whole pretreated biomass was subjected to the enzyme hydrolysis reaction as described in the next section and schematically shown in Fig. 1. Please note that the pretreatment and enzyme hydrolysis were not performed intermittently, as in such a scheme the level of glucose formed after the first enzyme hydrolysis step would be detrimental to the enzyme activity in the subsequent stages [29]. The unutilized solid after the pretreatment steps is rich in protein and cellulose and could be utilized further to make MS or be used in a variety of feed supplements.

Enzyme Hydrolysis Reaction Procedure

The enzyme activities were measured according to standard procedures [30]. The cellulase activities were 118.2 filter paper units (FPU/mL) and 49.6 cellobiase units/mL (CBU/mL). The β-glucosidase activities were 582.1 CBU/mL and 4.2 FPU/mL. The cellulase supplemented with β-glucosidase was used for their performance in the hydrolysis of

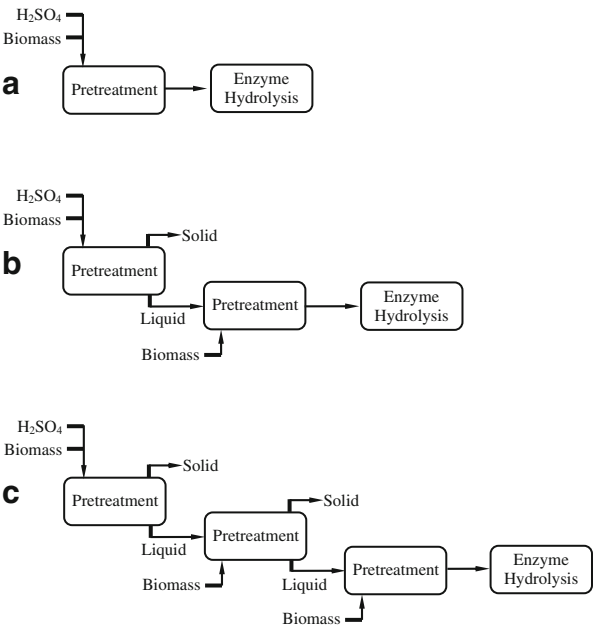

Fig. 1 Procedure schematic for stagewise dilute-acid pretreatment and enzyme hydrolysis of biomass; **a** one-stage pretreatment and enzyme hydrolysis, **b** two-stage pretreatment and enzyme hydrolysis, **c** three-stage pretreatment and enzyme hydrolysis

pretreated biomass without further purification. The weight ratio of the two enzymes used was 2:1 (cellulose/β-glucosidase). Based on the measured activity of the enzymes and at 15% biomass solid loading, the enzyme loading was at 22 FPU and 53 CBU/g carbohydrates for DDG and 15 FPU and 37 CBU/g carbohydrates for corn fiber samples.

Experiments were performed with dilute-acid pretreated as well as with fresh biomass. For the enzyme hydrolysis of the pretreated biomass, 57.5 g of the pretreated material was transferred into a 125-mL flask where its pH was adjusted to 5 with 10 N sodium hydroxide. For enzyme hydrolysis of fresh biomass, 7.5 g of dried and grinded biomass was placed in a 125-mL flask with 50 mL of pH 5 sodium phosphate (0.1 M) buffer. One gram of enzyme mixture was added. The reaction mixture was incubated in a temperature-controlled incubator (Imperial III incubator, Lab-Line Instruments, Melrose, IL, USA) in which a shaker (C2 classic platform shaker, New Brunswick Scientific, Edison, NJ, USA) was placed to mix the reaction flasks at 200 rpm. The timing of the reaction started immediately after addition of enzyme. About 5 mL aliquots were taken at 0, 1, 3, 6, 12, 24, 48, and 72 h for the analysis of the hydrolysate. After centrifugation and syringe filtration, samples were placed in a vial for HPLC analysis.

Analysis

The HPLC analysis was performed to quantify the concentration of MS (glucose, xylose, galactose, and arabinose), and inhibitory products (lactic acid, glycerol, acetic acid, succinic acid, levulinic acid, HMF, and furfural) in the hydrolysate. A waters 2695 HPLC Alliance system (Waters Corporation, Milford, MA, USA) was used for the chromatography work, and Waters Empower software was used for the analysis of data. For the analysis of MS, an ion-exchange Aminex® HPX-87P 300×8.7 mm column (Bio-Rad Laboratories, Hercules, CA, USA), a Bio-Rad micro-guard de-ashing 30×4.6 mm guard column, and a Waters 2414 refractive index (RI) detector were used. The column temperature was maintained at 85 °C inside a column heater module, and the RI detector was held at 30 °C. Purified deionized water was used as the mobile phase at an isocratic flow rate of 0.6 mL/min. The elution time with this method was about 7–9 min for hemicellulose oligomers, about 10 min for cellobiose, and 11–16 min for MS (glucose, xylose, galactose, and arabinose). For the analysis of inhibitors, an Aminex® HPX-87H 300×8.7 mm column with a micro-guard cation 30×4.6 mm guard column was used. The column temperature was at 65 °C. The mobile phase was 0.01 N H_2SO_4 at 0.6 mL/min of isocratic flow rate. Sample volumes of 20 μL were injected into the HPLC. The total running time for these methods was 50 min. Calibration of the HPLC methods was carried out by analyzing solutions of standard compounds for glucose, xylose, galactose, arabinose, lactic acid, glycerol, acetic acid, succinic acid, levulinic acid, HMF, and furfural. The calibration curves were adjusted on regular bases to ensure accuracy. All experiments were performed in replicates to determine the precision and repeatability of the analysis.

Result and Discussion

Biomass Composition

Analyses according to standard procedure were performed to quantify moisture, oil, carbohydrates (starch, cellulose, hemicellulose, lignin), crude protein, and ash in DDG, WS, and corn fiber samples. Experimental procedures outlined earlier in "Materials and Methods"

were followed. As the results presented in Table 1 show, the total carbohydrate content of DDG, WS, and corn fiber were 57.7±2.0, 59.3±1.9, and 77.0±1.0 wt.%, respectively. For the most part, the composition of DDG and WS were fairly similar with minor differences. The differences, mainly due to the absence of thin stillage in the DDG, were in a slightly larger fraction of oil and starch in WS and a larger fraction of protein in DDG. The thin stillage, which contains the solubles and finer particles of WS, is likely to contain unfermented glucose, a larger fraction of oil and residual starch, and a smaller fraction of protein. The overall carbohydrate content of the WS was slightly higher than DDG but was well within the standard deviations of the measurements. The experimental results were consistent with the previously reported compositions for these materials [6, 31].

Dilute-Acid Pretreatment

One-stage dilute sulfuric acid pretreatments of DDG, defatted DDG, WS, and corn fiber were performed as described earlier in "Materials and Methods." The results for the formation of MS as a function of time are presented in Fig. 2. Examination of the results for the pretreatment of biomass samples revealed an increasing trend in the formation of MS from the inception of the reaction to 30 min into the reaction. The formation of sugars started to slow down at this point, and further increases in the reaction time resulted in a slower but persistent increases in the total amount of the formed sugars. As this figure shows, the formation of sugars correlated well with the concentration of the carbohydrates in the samples with a general trend of corn fiber > defatted DDG > WS >DDG. For the DDG, defatted DDG, and WS samples, the differences in the concentration of the resulting MS were proportional to the differences in the initial concentration of carbohydrates in the substrates. For example, after 30 min of pretreatment (Fig. 2), WS resulted in 2.7 mg/mL more of total sugars than that of DDG which is attributed to 1.6% more carbohydrates

Table 1 Composition of DDG, WS, and corn fiber.

Components (%, dry basis)	DDG	WS	Corn fiber	DDGS[d]	Corn fiber[e]
Moisture	5.8±0.5	84.2±0.1	5.3±0.1	11.2±0.0	-
Crude oil	9.5±1.9	10.9±0.4	4.5±0.2	11.6±0.1	2.5±0.2
Carbohydrates	57.7±2.0	59.3±1.9	77.0±1.0	53.5	77.5±5.0
Starch[a]	6.2±0.4	13.2±0.3	17.7±0.2	27.3	19.7±0.9
Cellulose[b]	17.0±0.3	14.5±0.3	13.0±0.2	4.5	17.5±1.0
Hemicellulose[c]	25.8±0.5	23.9±0.9	38.8±0.4	3.1	32.5±0.4
xylose	11.7±0.3	10.7±0.3	20.3±0.4		17.6±1.8
galactose	2.7±0.1	2.6±0.2	4.4±0.1		3.6±0.3
arabinose	11.4±0.3	10.7±0.5	14.1±0.1		11.3±1.5
lignin	8.7±1.8	7.7±0.4	7.5±0.3		7.8±0.7
Crude Protein	30.3±0.8	24.0±0.6	9.9±0.5		11.0±0.5
Ash	1.0±0.1	4.5±0.2	0.6±0.1		0.6±0.1
Unknown	1.5	1.3	8.0		8.5[f]

[a] As a source of glucose from residual starch including residual glucose

[b] As a source of glucose from cellulosic part

[c] As a source of xylose, galactose, and arabinose

[d] Kim et al. [31]

[e] Grohmann and Bothast [6]

[f] Acetyl groups included

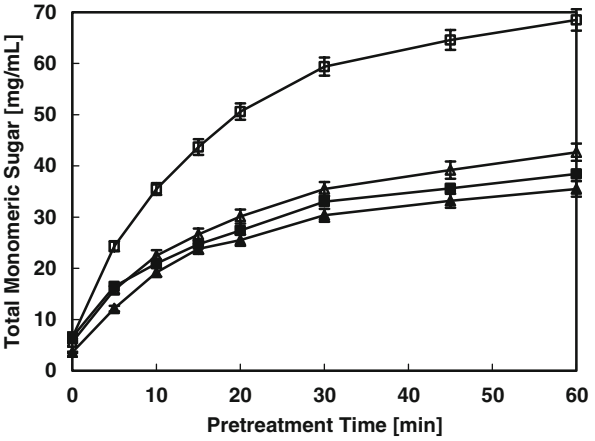

Fig. 2 Total MS production during the course of dilute-acid pretreatment of biomass at 120 °C, 15 wt.% biomass loading, and 1.0 vol% sulfuric acid concentration; *closed triangles* DDG, *open triangles* defatted DDG, *closed squares* WS, *open squares* corn fiber

(about 2.4 mg/mL at 15.0% biomass loading) in WS than DDG. The yield of MS for the DDG and WS, however, were within the standard deviations for the experiments. For the corn fiber samples, the formation of sugars out-performed the initial concentration of the carbohydrates when compared with the DDG and WS samples, and the yield for the formation of MS reached 50.7% compared to 36.8% for the DDG samples. This is mainly attributed to a much lower concentration of oil and protein in the corn fiber samples which appear to cause some mass transfer restrictions.

The pretreatment of the oil-free DDG was investigated due to an increasing interest in the conversion of the extracted oil to biodiesel and the increase in the total hydrocarbons of the resulting defatted substrate per unit mass of the material. For example, at 15% substrate loading, the removal of oil results in 5.2% increase in the total carbohydrates in the substrate (about 7.7 mg/mL at 15.0% biomass loading). The removal of oil may also minimize the transport limitations due to its presence in the reaction media which results in a higher viscosity of the substrate. As is shown in Table 2, the pretreatment of defatted DDG resulted in about 5.1 mg/mL more sugars than the DDG sample which was consistent with the increase in the availability of the carbohydrates in the defatted samples. As shown in Table 2, the overall pretreatment yield was slightly higher for the defatted DDG which may be attributed to the diverse mass transport effects caused by the presence of oil.

Stagewise Dilute-Acid Pretreatment

Stagewise dilute-acid pretreatments of DDG, defatted DDG, and corn fiber were carried out to increase the concentration of MS in the hydrolyzate which in turn is expected to increase the ethanol concentration in the beer. Stagewise pretreatment of WS was carried out for only one stage due to its high moisture content (about 84.2±0.1%) which prevented a multistage pretreatment at 15% solid loading. The results for the total amount of MS as a function of pretreatment stages are presented in Table 2. Examination of the stagewise pretreatment of DDG and defatted DDG confirms a nearly linear increase in the formation of MS as a function of pretreatment stages. For the DDG samples, the total amount of the sugars were 30.4±1.4, 59.4±2.1, and 91.3±2.9 mg/mL after one, two, and three pretreatment stages, respectively. This correlated linearly to the amount of pretreated biomass. The analysis of the MS also revealed for this increase to be mostly due to degradation of the hemicellulosic components. Based on the amount of fresh biomass

Table 2 Concentrations and yields of monomeric sugars in the stagewise dilute-acid pretreatment of biomass at 120 °C, 1.0% acid concentration, 15% biomass loading, 30 min reaction, and the enzyme hydrolysis of pretreated biomass at 45 °C, pH 5.0, and 24 h.

	DDG		Defatted DDG		Corn fiber	
	Concentration (mg/mL)	% Yield (g/100 g carbohydrates)	Concentration (mg/mL)	% Yield (g/100 g carbohydrates)	Concentration (mg/mL)	% Yield (g/100 g carbohydrates)
Pretreatment						
1 stage	30.4±1.4	36.8±1.7	35.5±1.7	38.9±1.8	59.4±1.9	50.7±1.6
2 stages	59.4±2.1	36.0±1.2	67.8±2.2	37.1±1.2	123.5±2.9	52.7±1.2
3 stages	91.3±2.9	35.8±1.2	98.5±2.9	36.0±1.1	160.5±3.2	45.7±0.9
Enzyme hydrolysis after						
0 stage pretreatment	24.3±1.1	29.4±1.3	22.9±1.1	25.1±1.2	26.3±0.5	22.4±0.5
1-stage pretreatment	62.5±2.8	75.7±3.4	59.8±2.7	65.5±3.0	92.0±2.3	78.5±2.0
2-stage pretreatment	98.6±2.8	59.7±1.7	94.8±4.5	52.0±1.6	147.9±3.7	63.1±1.6
3-stage pretreatment	122.6±3.2	49.5±1.3	125.6±3.0	45.9±1.1	184.5±4.1	52.5±1.2

which was added in each pretreatment stage, the average yield for the conversion of carbohydrates was about 36.5±0.5% after each stage of pretreatment (Table 2). The pretreatment of defatted DDG showed a similar trend with a slightly higher concentration of MS. The increase in the formation of sugars on the stagewise pretreatment of corn fiber showed a doubling-up from stage one to two or from 59.4±1.9 to 123.5±2.9 mg/mL. However, after the third pretreatment stage, the total amount of sugars was increased to 160.5±3.2 mg/mL. The average yield for the first two stages of pretreatment was about 51.7±1.4% and was at 45.7±0.9% for the third stage. The high concentration of MS along with the presence of other soluble material in the substrate (mainly proteins, lignin, and oil) may be responsible for imposing mass transfer limitation and as a result a lower trend in the formation of the MS in the substrate.

In the process of pretreatment of lignocellulosic material, the formation of other chemicals such as lactic acid, acetic acid, succinic acid, levulinic acid, glycerol, HMF (hydroxymethylfufural or 5-hydroxymethyl-2-furaldehyde), and furfural is of interest as these chemicals tend to inhibit the fermentation of MS to ethanol. These components are either directly released from the biomass itself, formed via sugar degradation during heat treatment of the biomass, or formed as by-products during the fermentation [32–34]. The effect of inhibitory compounds on the fermentation of various cellulosic biomasses has been studied and documented by researchers [35–37]. In this study, the formation of lactic acid, acetic acid, HMF, furfural, succinic acid, glycerol, and levulinic acid were monitored. The concentration of these chemicals at the end of each stage of pretreatment is presented in Table 3. The concentration range where the inhibitory effect of these chemicals on ethanol production becomes significant is termed inhibition concentration in Table 3 and is included in this table. At the inhibition concentration, a significant decrease in ethanol production is expected. As is shown in Table 3, for both DDG and corn fiber, no measurable quantities of levulinic acid and succinic acid were detected, and the concentration of glycerol, HMF, and furfural were well below the inhibition concentrations. For the hydrolyzate of corn fiber, the formation of lactic acid was also well below the inhibition concentration, while for the hydrolyzate of DDG, the concentrations of lactic acid were slightly higher but remained well below the inhibition concentration. A point of concern was the relatively high levels of acetic acid in the corn fiber hydrolyzate and some of the DDG samples. However, as shown by Graves and coworkers [38], an increase in pH is expected to increase the acetic acid tolerance level by the yeast during the fermentation of corn mesh. For example, at the acetic acid concentration of 0.8 wt.% (8.0 mg/mL) and the corn mash pH of 5.5, no significant loss in ethanol production was observed, whereas at the pH of 4.0 and otherwise identical

Table 3 Production of inhibitory compounds in the stagewise dilute-acid pretreatment of DDG and corn fiber at 120 °C, 1.0% acid concentration, 15% solid loading, 30 min reaction.

Substrates	DDG (mg/mL)			Corn fiber (mg/mL)			Inhibition concentration (mg/mL)
No. of pretreatments	1	2	3	1	2	3	
Lactic acid	1.30±0.06	2.37±0.10	3.81±0.16	0.28±0.01	0.73±0.02	1.22±0.04	10–40 [38]
Acetic acid	2.30±0.11	4.06±0.18	6.88±0.32	4.07±0.16	8.54±0.19	11.96±0.28	4–16 [38]
Glycerol	5.94±0.22	11.10±0.54	18.36±0.90	0.20±0.01	0.54±0.02	1.01±0.04	100–400 [39]
HMF	0.13±0.01	0.28±0.02	0.51±0.04	0.08±0.01	0.20±0.01	0.50±0.02	3–4 [40]
Furfural	0.06±0.01	0.31±0.01	0.92±0.03	0.15±0.01	0.51±0.02	1.20±0.04	2 [40]

The concentrations of succinic and levulinic acid were <0.01 mg/mL in all the cases studied

conditions, practically no ethanol was formed. The formation of inhibitory chemicals during the pretreatment of defatted DDG closely resembled the data for DDG, and the data are not presented here.

Enzyme Hydrolysis of Pretreated Biomass

Enzyme hydrolysis of fresh and pretreated biomass was performed as described earlier in the "Materials and Methods" section. The main purpose of these experiments was to increase the concentration of MS in the hydrolyzate which in turn is expected to increase the concentration of ethanol in the beer. The time courses of the pretreated DDG and defatted DDG at 15 wt.% initial solid loading using 22 FPU cellulase and 53 CBU β-glucosidase per gram biomass carbohydrate is presented in Fig. 3. As is shown in this figure, the enzyme hydrolysis of DDG and defatted DDG resulted in a significant increase in the total amount of sugars in the substrate. The rate of this increase was much faster at the onset of the reaction and gradually leveled off after 12 h. As the detail analysis of the MS revealed, this increase was mostly due to degradation of the residual starch and cellulosic components. The step increase in the amount of sugars after each enzyme hydrolysis stage correlated well with the carbohydrate loading, and the number of pretreatment stages did not appear to be a factor in this increase. For example, after 24 h reaction, the average incremental increase in the amount of sugars which was attributed to one enzyme hydrolysis stage was about 30.2±5.3 mg/mL for DDG and defatted DDG samples. For DDG samples, the individual yields for glucose and xylose were at 12.9± 1.3% and 59.0±4.2%, respectively, after one pretreatment and prior to the enzyme hydrolysis step. The yields for glucose and xylose reached 80.4±3.5% and 82.4±4.7% after 72 h enzyme hydrolysis (Fig. 4). Kim and coworkers reported about 68% and 20% yield for glucose and xylose, respectively, after enzyme hydrolysis of a single-stage hot-water pretreated DDGS and otherwise comparable conditions. For the DDG and defatted DDG samples, the highest concentration of sugars was observed when three stages of pretreatment was followed by a single enzyme-hydrolyzed step for 48 h (Fig. 3). This resulted in about 128±3.2 mg MS/mL which was about twice of what was formed when a single pretreatment stage was followed by one enzyme hydrolysis step. This level of sugar concentration (128 mg/mL) could potentially result in about 64 mg ethanol/mL substrate or about 6.4 wt.%. The overall yield of MS was about 78.0±3.7% after 48 h for the enzyme

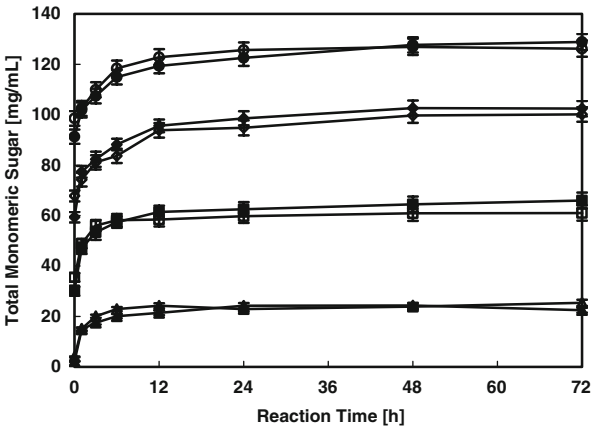

Fig. 3 Total MS production during the course of enzyme hydrolysis of pretreated DDG and defatted DDG at 45 °C and pH 5.0; *closed triangles* nonpretreated DDG, *closed squares* one-stage pretreated DDG, *closed diamonds* two-stage pretreated DDG, *closed circles* three-stage pretreated DDG, *open triangles* nonpretreated defatted DDG, *open squares* one-stage pretreated defatted DDG, *open diamonds* two-stage pretreated defatted DDG, *open circles* three-stage pretreated defatted DDG

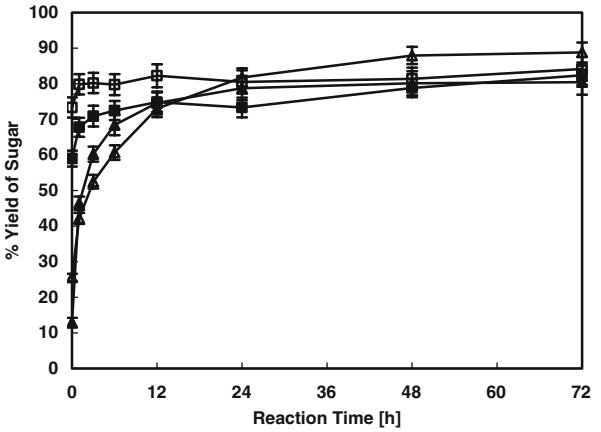

Fig. 4 Yields for glucose and xylose in the enzyme hydrolysis of pretreated DDG and corn fiber at 45 °C and pH 5.0; *closed triangles* glucose from DDG, *closed squares* xylose from DDG, *open triangles* glucose from corn fiber, *open squares* xylose from corn fiber

hydrolysis of DDG samples which were subjected to a single stage of pretreatment. The overall yield was considerably lower when two and three pretreatment stages were employed. This is primarily due to the processing scheme which excludes the solid fraction of the pretreated biomass after each pretreatment stage.

The time courses of the pretreated corn fiber at 15 wt.% initial solid loading using 15 FPU cellulase and 37 CBU β-glucosidase per gram carbohydrates is presented in Fig. 5. As is shown in this figure, enzyme hydrolysis of corn fiber resulted in a significant increase in the total amount of sugars in the substrate after each sequence of pretreatment stage(s). The effectiveness of the incremental increase in the total amount of MS due to enzyme hydrolysis appeared to diminish as the number of pretreatment stages, which enzyme hydrolysis superseded, was increased. For example, the increase in the amount of MS was 39.9±0.6, 31.8±0.6, and 29.0±1.1 mg/mL when the 72-h enzyme hydrolysis followed one, two, and three pretreatment stages, respectively. This decrease in the efficiency was similar to the decrease in the amount of MS after the pretreatment stages which showed a decreasing trend as the number of stages was increased. For the corn fiber samples, the highest concentration of sugars was at about 187.8±4.3 mg/mL when three stages of pretreatment was followed by a single enzyme-hydrolyzed step for 48 h (Fig. 5). This was about twice of the MS that was formed when a single pretreatment stage was followed by

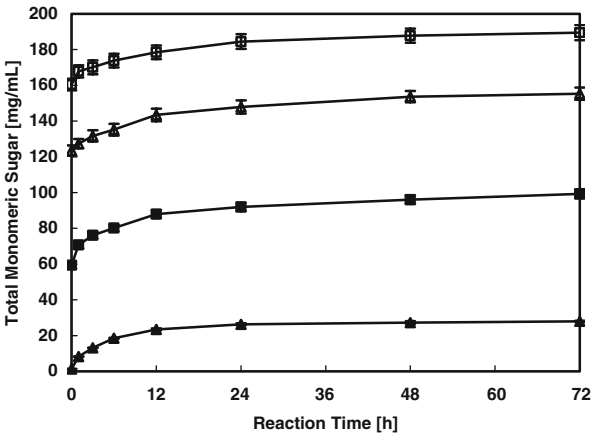

Fig. 5 Total MS production during the course of enzyme hydrolysis of pretreated corn fiber at 45 °C and pH 5.0; *closed triangles* nonpretreated corn fiber, *closed squares* one-stage pretreated corn fiber, *open triangles* two-stage pretreated corn fiber, *open squares* three-stage pretreated corn fiber

one enzyme hydrolysis step. This level of sugar concentration (187.8 mg/mL) could potentially result in about 94 mg ethanol/mL substrate or about 9.4 wt.%. The overall yield of MS was 84.7±2.9% when one stage of pretreatment was followed by enzyme hydrolysis for 72 h. Similar to the DDG samples, the overall yield was considerably lower when two and three pretreatment stages were employed which is attributed primarily to the processing scheme. Saha et al. reported about 87.4% yield for MS after a single-stage dilute-acid pretreatment and 72 h enzyme hydrolysis of corn fiber under otherwise comparable conditions [2].

The time course of the pretreatment for the formation of inhibitors was followed. The analysis of the results showed a slight but insignificant decrease in the concentration of lactic acid, acetic acid, glycerol, and HMF. However, there was a substantial decrease in the composition of furfural for all biomass samples. The concentration of furfural after 24 h enzyme hydrolysis of a three-stage pretreated DDG, defatted DDG, and corn fiber decreased from 0.92±0.04, 0.91±0.03, and 1.20±0.05 mg/mL to 0.57±0.03, 0.56±0.02, and 0.70±0.04 mg/mL, respectively. Furfural volatility has been blamed for the decrease in its concentration during the enzyme hydrolysis [41, 42]. The decrease in the furfural concentration was fairly insignificant when the enzyme hydrolysis followed a two-stage and a one-stage pretreated biomass. The concentrations of furfural in these cases (Table 3) were much lower than the hydrolyzate after the three-stage pretreatment which further supports its losses due to volatility in the case.

Conclusions

Lignocellulosic biomass resources such as agricultural residues and wastes from the corn milling facilities are plentiful and have the potential to be utilized in the production of ethanol. Availability of DDG and corn fiber at the ethanol plant and their high levels of lignocellulosic material make them attractive feedstock for conversion to ethanol. In this study, stagewise dilute sulfuric acid pretreatment and enzyme hydrolysis for the conversion of DDG, defatted DDG, WS, and corn fiber to MS was investigated. The main goal was to increase the concentration of MS in the hydrolyzate which in turn is expected to increase ethanol concentration in the fermentation products. The highest concentration of MS was reached when biomass was subjected to three pretreatment stages and followed by a single enzyme hydrolysis step. The enzyme hydrolysis of three-stage pretreated DDG and corn fiber resulted in 122.6±5.8 and 184.5±4.1 mg/mL, respectively. The formation of compounds which are known to inhibit the fermentation of MS to ethanol was monitored and was found to be below the inhibition concentration for these compounds.

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