Dairy Manure as a Potential Feedstock for Cost-Effective Cellulosic Bioethanol

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This study investigated sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL) pretreatment and subsequent enzymatic digestibility of undigested dairy manure to preliminarily assess its potential use as an inexpensive feedstock for cellulosic bioethanol production. The sulfite pretreatment was carried out in a factorial analysis using 163 to 197 °C for 3 to 37 min with 0.8% to 4.2% sulfuric acid combined with 2.6% to 9.4% sodium sulfite. These treatments were compared with other standard pretreatments of dilute acid, and hot and cold alkali pretreatments. This comparative study showed that the sulfite pretreatment, through its combined effects of hemicellulose and lignin removal and lignin sulfonation, is more effective than the diluted acid and alkali pretreatments to improve the enzymatic digestibility of dairy manure.

Keywords: Dairy manure; Sulfite pretreatment; Bioethanol; Enzymatic hydrolysis

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INTRODUCTION

Cost-effective cellulosic ethanol can be produced from non-food and low-cost agricultural residues, such as wood residuals, corn stover, and perennial grasses (Zhang *et al.* 2013; Papa *et al.* 2015). Manure is an inexpensive lignocellulosic biomass because it is an animal feedlot waste and is concentrated at the farm, largely eliminating the need for transportation (Chen *et al.* 2005; Teater *et al.* 2011). More than 110 million tons of dairy manure is produced annually in the United States (Yue *et al.* 2010). Traditionally, manure is spread on agricultural fields as fertilizer. However if over-applied, it can cause environmental issues such as watershed pollution, high nitrogen and phosphorous soil loads, and generation of greenhouse gases (Gollehon *et al.* 2000).

Growing environmental concerns coupled with higher energy prices have recently led to a renewed interest in the utilization of manure to produce economically feasible bioenergy. Manure can be directly burned to produce heat, converted to bio-oil, or made into combustible syngas (Fernandez-Lopez *et al.* 2015). Alternatively, manure can be anaerobically digested to methane, which has a similar heating value to ethanol (Nasir *et al.* 2012). However, the remaining cellulose in manure is usually underutilized by anaerobic digestion. In fact, the anaerobically digested manure has more favorable compositional properties (less hemicellulose and similar or more cellulose) than other lignocellulosic biomasses, such as switchgrass (Yue *et al.* 2010). Technically, cellulosic bioethanol can be biologically produced from manure through fermentation of glucose derived from enzymatic hydrolysis of cellulose. Several studies have recently demonstrated the potential of manure as feedstock for cost-effective cellulosic bioethanol production (Yue *et al.* 2010; MacLellan *et al.* 2013; Zhang *et al.* 2013; Elumalai *et al.*

2014; Vancov *et al.* 2015). Like other lignocelluloses, an effective pretreatment is necessary for manure to remove recalcitrance to enzymatic hydrolysis of cellulose caused by hemicellulose and lignin. To this end, acid and alkali pretreatments have been applied to manure at elevated temperatures (Wen *et al.* 2004; Liao *et al.* 2006; Yue *et al.* 2010; MacLellan *et al.* 2013; Zhang *et al.* 2013; Elumalai *et al.* 2014; Vancov *et al.* 2015). However, several comparative studies have shown that sulfite pretreatment, usually referred to as the sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL), is generally more effective than acid and alkaline pretreatments to improve enzymatic digestibility of lignocellulosic biomass, such as switchgrass and spruce (Shuai *et al.* 2010; Li *et al.* 2012; Yang and Pan 2012; Zhang *et al.* 2013). The sulfite pretreatment also can offer other advantages, such as better carbohydrate recovery, less fermentation inhibitors, and great scalability over dilute acid and alkali pretreatments (Zhu *et al.* 2009).

To the best of our knowledge, there is no report on the sulfite pretreatment of manure for cellulosic bioethanol production. Therefore, this study focuses on the sulfite pretreatment of undigested dairy manure. Specifically, the objectives of this study were as follows: 1) to investigate the effects of pretreatment conditions (temperature, time, sulfuric acid, and sodium sulfite loadings) on the yield of cellulose and removal of lignin and hemicelluloses; 2) to compare dilute acid and alkali pretreatments; 3) to evaluate the enzymatic digestibility of pretreated dairy manure; and 4) to investigate the effects of pretreatment conditions on the enzymatic digestibility.

EXPERIMENTAL

Materials

Undigested dairy manure samples were collected from the Maple Leaf Dairy (Cleveland, WI), and were air-dried and ground to pass a 40-mesh screen using a Wiley mill. Sodium sulfite, sulfuric acid, urea, thiourea, and sodium hydroxide (50% w/w) were purchased from Thermo Fisher Inc. (Waltham, MA). Polyethylenimine (PEI), polyethylene glycol (PEG), tetracycline chloride, sodium acetate trihydrate, acetic acid, formic acid, levunilic acid, hydroxymethylfurfural (HMF), and furfural were purchased from Sigma-Aldrich (St. Louis, MO). Cellulase complex (Cellic CTec2) with activity of 120 filter paper units (FPU)/g was generously supplied by Novozymes (Franklinton, NC).

Pretreatments

Sulfite, dilute acid, and hot alkali pretreatments were conducted at temperatures ranging from 163 to 197 °C in 100-mL plastic vessels in batch modes using a microwave reactor (Mars, CEM Corp., Matthews, NC). Pretreatment liquors were prepared by mixing the required chemicals (sulfuric acid, sodium sulfite, and sodium hydroxide) with water. Chemical loading was based on the oven dry weight of raw manure. The pretreatment liquors containing 0.8% to 4.2% sulfuric acid and 2.6% to 9.4% sodium sulfite were used for the sulfite pretreatment, and the pretreatment liquors with 4.0% sulfuric acid and 8.0% sodium hydroxide were used for the dilute acid and hot alkali pretreatments, respectively. Briefly, 10 g of manure (oven dried) was mixed with 60 mL of pretreatment liquor. Pretreated manure was collected through filtration and thoroughly washed with deionized water. All pretreated manure samples were stored at 4 °C before enzymatic hydrolysis.

The sulfite pretreatment experiment was specially designed using Design-Expert (DX7, 2005) software with response surface methodology (RSM) combined with Hartley composite design (eight factorial points, eight star points, and five center points with a specified alpha value of 1.7 as an axial scaling). The obtained experimental data were further analyzed by JMP Pro 11 software (SAS Institute, Cary, NC) to establish fitted models, and then the three-dimensional surface plots were generated using MATLAB R2014 software (Math Works, Natick, MA), based on established models. The sulfite pretreatment conditions were chosen according to those reported for switchgrass and woody biomass in the literature with slight modifications to accommodate the manure biomass (Shuai *et al.* 2010; Yang and Pan 2012; Zhang *et al.* 2013). Specifically, the pretreatment experiments were composed of 21 runs, and the pretreatments were carried out at temperature ranges of 163 to 197 °C for 3 to 37 min using 0.8% to 4.2% sulfuric acid combined with 2.6% to 9.4% sodium sulfite, as summarized in Table 1. The alkali pretreatments at a cold temperature were carried out in 250-mL flasks at -20 °C. The alkali aqueous solutions were prepared by mixing 7.0 wt.% sodium hydroxide with 12.0% urea, 5.5% thiourea, 2.0% polyethylenimine, or 2.0% polyethylene glycol (Mohsenzadeh *et al.* 2012). Briefly, 10 g of manure (oven-dried) was mixed with 50 mL of alkali aqueous solution, and the resultant mixture was stirred for 30 min and then stored at – 20 °C for 12 h. Then, 100 mL of deionized water was added and stirred for 1 h. Pretreated manure was recovered through filtration and thoroughly washed with deionized water.

Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out in a 50-mL conical tube at 50 °C on a shaking incubator (New Brunswick Scientific Excella E24, Edison, NJ) at 180 rpm using 40 mL of sodium acetate buffer (50 mM, pH 4.8) at 2.0% consistency containing 2.0 mg of tetracycline chloride to deter bacterial action. The cellulase complex loading was 20 FPU/g of cellulose. Aliquots (0.4 mL) were taken periodically for glucose analysis, and sodium acetate buffers (0.4 mL, 50 mM, pH 4.8) were added to make up the volume.

Analytic Methods

Ash, extractives, and acid-insoluble (Klason) lignin were analyzed according to National Renewable Energy Laboratory (NREL) analytic procedures (Sluiter *et al.* 2010). Acid-soluble lignin was measured using a UV-vis spectrophotometer (Cary 50 Bio, Varian, Santa Clara, CA) at 205 nm with an extinction coefficient of 110 L $g^{-1}cm^{-1}$. Monosaccharides (glucose, galactose, arabinose, mannose, and xylose) were measured using high-performance ion chromatography (HPIC, ICS-3000, Dionex, Sunnyvale, CA, USA) equipped with an integrated amperometric detector and Carbopac guard (PA20, 3 × 30 mm) and analytic (PA20, 3 × 150 mm) columns. Fermentation inhibitors (acetic acid, formic acid, levunilic acid, hydroxymethylfurfural, and furfural) were measured using the Dionex ICS-3000 system (Sunnyvale, CA), equipped with a UV-vis detector and Superlcogel C-610H analytic (30 cm × 7.8 mm) and guard (5 cm × 4.6 mm) columns.

RESULTS AND DISCUSSION

Sulfite Pretreatment

The sulfite pretreatment was evaluated in terms of hemicellulose and lignin removal, concomitant changes in biomass components, substrate yield, and fermentation inhibitors produced. Cellulose and hemicellulose are expressed in terms of their monosaccharides rather than polysaccharides in this study, as saccharification was the final product. Component analysis showed that the dairy manure contained 23% cellulose, 23.5% lignin, 18% xylose, as well minor saccharides of 1.7% galactose, 2.3% arabinose, and 0.1% mannose.

The undigested dairy manure in this study had slightly more hemicellulose than reported digested manures, which was attributed primarily to the cows' diets (Yue *et al.* 2010; Teater *et al.* 2011; Vancov *et al.* 2015). Ash and extractives were 14.6% and 16.6%, respectively. It is well-established that hemicellulose and lignin can be removed through acid-catalyzed depolymerization reactions.

Effects of the pretreatment conditions on lignin and hemicellulose removal were determined and shown in Fig.1. As summarized in Table 1, the sulfite pretreatment under different conditions removed 11.6% to 38.3% lignin and 18.1% to 67.7% hemicellulose. Pretreatment temperature and time were set at their median values, when investigating effects of sulfuric acid and sodium sulfite loadings, and *vice-versa*.

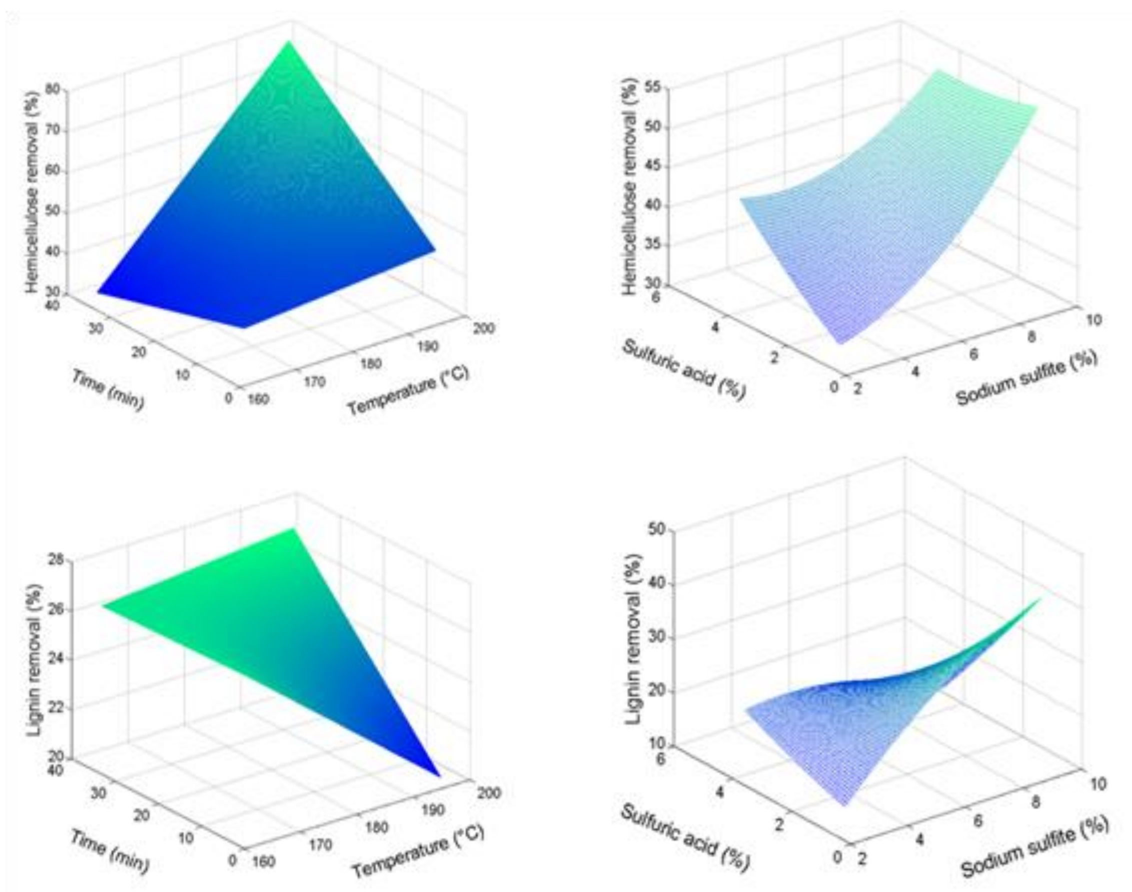

Fig. 1. Effects of pretreatment conditions on lignin and hemicellulose removals during sulfite pretreatment of manure

Table 1. Sulfite Pretreatment of Manure

Exp. No.	Pretreatment conditions[a]						Yield, %	Pretreated substrate[c]					CGC at 72 h, %	Pretreatment spent liquor[d]				
	Temp, °C	Time, min	SA, %	pH[a]	SS, %	pH[b]		Gla, %	Ara, %	Xyl, %	Glu, %	KL, %		Glu, %	SL, %	Xyl, %	AC, %	pH
1	180	20	2.5	1.86	6.0	5.43	56.7	2.4	2.4	20.2	36.5	23.4	57.7	0.2	6.6	6.2	1.0	6.44
2	197	20	2.5	1.86	6.0	5.43	48.8	1.9	1.4	11.5	36.7	26.4	62.3	0.5	5.3	12.1	1.1	6.52
3	163	20	2.5	1.86	6.0	5.43	69.6	3.1	2.9	20.7	31.6	24.3	36.6	0.1	5.9	3.9	0.6	6.88
4	190	10	3.5	1.78	8.0	2.93	8.2	2.1	1.8	20.9	38.8	26.4	56.8	0.1	6.3	4.9	0.9	6.61
5	170	10	3.5	1.78	4.0	2.09	72.0	2.1	2.4	20.1	33.4	24.6	36.8	0	5.5	3.3	0.8	6.91
6	180	37	2.5	1.86	6.0	5.43	45.4	1.9	2.1	19.2	35.1	24.7	57.6	0.7	6.0	8.9	1.0	6.65
7	170	30	1.5	1.91	8.0	6.63	65.0	2.3	2.1	19.5	36.5	22.6	34.9	0	5.9	6.8	0.8	6.23
8	180	20	2.5	1.86	6.0	5.43	57.9	1.7	1.6	15.9	39.1	25.8	52.1	0.1	5.7	4.1	1.1	6.66
9	180	20	2.5	1.86	6.0	5.43	56.2	2.5	2.2	19.3	35.8	24.6	38.5	0.3	4.7	9.2	0.8	6.92
10	180	3	2.5	1.86	6.0	5.43	71.3	2.1	1.9	19.1	32.0	25.6	46.8	0.1	5.2	9.6	0.8	6.78
11	180	20	2.5	1.86	6.0	5.43	55.7	1.5	1.5	14.9	38.9	25.4	48.5	0.1	6.3	7.1	0.9	6.88
12	190	30	1.5	1.91	4.0	6.69	55.4	1.4	1.4	14.6	37.4	28.3	55.4	0.2	4.4	10.1	3.6	6.44
13	180	20	2.5	1.86	2.6	2.12	61.9	2.0	2.0	17.0	30.5	27.7	34.6	0.4	4.2	6.1	0.7	6.43
14	180	20	2.5	1.86	9.4	6.26	42.3	2.2	2.0	18.3	36.1	22.3	48.3	0.8	6.4	9.2	0.7	6.60
15	170	30	3.5	1.78	8.0	2.83	62.2	2.1	1.8	18.4	35.5	25.1	36.3	0.1	2.7	6.0	0.6	6.58
16	180	20	4.2	1.71	6.0	2.15	52.6	1.8	1.9	16.3	33.7	28.1	48.8	0.5	5.1	9.2	0.7	6.71
17	180	20	2.5	1.86	6.0	5.43	56.4	2.3	1.9	21.2	37.7	26.5	42.2	0.2	7.5	6.3	0.7	6.98
18	180	20	0.8	1.99	6.0	6.62	61.0	2.0	1.7	18.3	36.9	23.9	51.4	0.1	7.2	6.5	6.0	6.57
19	190	10	1.5	1.91	8.0	6.63	63.4	1.3	1.2	15.9	35.7	23.7	54.9	0.1	9.0	7.6	5.9	6.69
20	190	30	3.5	1.78	4.0	2.09	52.5	0.7	0.7	12.2	38.5	34.6	49.6	0.3	4.5	10.3	0.9	6.34
21	170	10	1.5	1.91	4.0	6.69	77.2	2.0	1.8	17.2	30.1	26.7	38.5	0	5.6	4.3	0.4	6.60

Note: SS: sodium sulfite; SA: sulfuric acid; Gla: galactose; Ara: arabinose; Xyl: xylose; Glu: glucose; KL: Klason lignin; CGC: cellulose-to-glucose conversion; SL: soluble lignin; AC: acetic acid. Chemicals (SS and SA), % on oven-dry manure; [a]pH of sulfuric acid aqueous solution; [b]pH of sulfuric acid aqueous solution with sodium sulfite; [c]composition (xylose, glucose or lignin), % on dry pretreated manure; [d]composition (glucose, soluble lignin, xylose or acetic acid), % on dry untreated manure

As shown in Fig. 1, the hemicellulose and lignin removals were directly affected by the pretreatment conditions (temperature, time, sulfuric acid, and sodium sulfite loadings). The results showed that more lignin and hemicellulose can be removed under higher temperatures for longer time periods. In this study, both the pretreatment temperature and time were critical for hemicellulose removal. For instance, hemicellulose can be removed most when pretreated for 40 min at 200 °C. Lignin removal was more dependent upon the pretreatment time, because the least lignin removal can be obtained when the pretreatment is carried out at 195 °C for only 4 min. Also, more hemicellulose can be removed by increasing the dosage of sulfuric acid through accelerating acid-catalyzed hydrolysis of hemicellulose. However, sodium sulfite notably decreased the acidity of dilute acid aqueous solution, as evidenced by the fact that pH value greatly increased after addition of sodium sulfite. Therefore, the presence of sodium sulfite was not favorable to the acid-catalyzed hydrolysis of hemicellulose (Yang and Pan 2012; Zhang *et al.* 2013). Unexpectedly, it seems that more hemicellulose in this study can be removed with more sodium sulfite; however, minimal hemicellulose removal (18%) was observed even when the pretreated of sodium sulfite (with 2.5% sulfuric acid) was 6.0% at 163 °C for 20 min. This result indicates that the pretreatment temperature and time are more critical for the hemicellulose removal in this study. The acid-catalyzed lignin removal was accomplished mostly through the cleavage of *α*- and *β*-ether linkages (Cao *et al.* 2012). Therefore, the addition of more sulfuric acid resulted in more lignin removal. However, lignin depolymerization was accompanied through lignin condensation during the formation of intra-linkages, such as carbon-carbon bonding (Cao *et al.* 2012). Furthermore, the lignin condensation reaction tended to be more pronounced with increasing dosages of sulfuric acid. On one hand, sodium sulfite can facilitate lignin removal through sulfonation; increasing the dosage of sodium sulfite was generally beneficial for lignin removal in this study. On the other hand, a surplus sodium sulfite can hinder lignin removal by decreasing the acidity of the sulfuric acid aqueous solution. For example, when the dosage of sulfuric acid was in the range of 4.0% to 6.0%, further increasing the dosage of sodium sulfite from approximately 8.0% to 10.0% resulted in a decrease in lignin removal.

Removed lignin and hemicellulose found in the spent liquors respectively existed in the forms of soluble lignin and monosaccharides (xylose and glucose). Under severe acidic conditions at elevated temperature, xylose and glucose can be thermally degraded into furfural and HMF, respectively (Oefner *et al.* 1992; Woo *et al.* 2015). The acidity of the pretreatment liquor is decided by the dosages of sulfuric acid and sodium sulfite. As shown in Table 1, some pretreatment liquors with pH values of 2 were very acidic, while others are weakly acidic. However, when buffered by sodium sulfite and formed acetic acid by acidic hydrolysis of acetyl groups in hemicellulose, the pretreatment spent liquors exhibited similarity in pH value of 6.6 and were weakly acidic. Therefore, only small amounts of furfural (about 4.0%) and HMF (about 0.3%) were found in the pretreatment spent liquors. Although furfural may inhibit fermentation, the SPORL pretreatment may be low enough to not cause as much inhibition as dilute acid or alkali pretreatments.

Because of the removal of lignin and hemicellulose, the pretreated manures were enriched with cellulose (30.5 to 41.7%) relative to untreated manure (23%). Nevertheless, the pretreated manures still exhibited higher amounts of lignin (22.3% to 34.6%), primarily because of the removal of hemicellulose. In this study, the hemicellulose and lignin removals caused the manure substantial losses in mass during the sulfite pretreatment. The manure yield ranged from 42.3% to 77.2%. As discussed above, increasing the treatment temperature was favorable for the removal of lignin; however, the condensation of lignin can be likely pronounced as the pretreatment prolongs. Therefore, increasing temperature and extending pretreatment time can enhance dissolution of hemicellulose and even cellulose. Consequently, higher substrate yields can be achieved under either lower temperature or shorter treatment duration. For example, when the dosages of sodium sulfite and sulfuric acid were 6.0% and 2.5%, respectively, higher substrate yields (48.8% to 71.3%) were achieved under either shorter time (*e.g.*, 71.3% for run 10) or lower temperatures (*e.g.*, 69.6% for run 3). Increasing the loading of sulfuric acid or/and sodium sulfite can result in a decreased substrate yield because of the accelerated hemicellulose hydrolysis or/and delignification. Therefore, higher substrate yields are achieved under less sulfuric acid or/and sodium sulfite. For example, lower substrate yields (42.3% to 45.4%) were observed when the pretreatments (runs 6 and 14) were carried out at 180 °C for 20 to 37 min, using higher (2.5%) sulfuric acid addition, combined with greater (6.0% to 9.4%) sodium sulfite inclusion. The highest substrate yield (77.2%) in this study was achieved under mild pretreatment conditions (170 °C, 10 min, 4.0% sodium sulfite, and 1.5% sulfuric acid).

Enzymatic Hydrolysis

The sulfite-pretreated manures were then compared in enzymatic digestibility trials using untreated manure as a control. The time-dependent hydrolysis profiles, presented in Fig. 2, indicate that all pretreated manures showed higher initial hydrolysis rates (conversion at first hour) than untreated manure, and they exhibited much better performance as the hydrolysis progressed. To allow for facile comparison, cellulose-to-glucose conversions (CGC), after 72 h of hydrolysis, are summarized in Table 1. The data indicate that the untreated manure had a very poor enzymatic digestibility, with only approximately 3.0% of the cellulose enzymatically converted into glucose after 72 h of hydrolysis. By comparison, the sulfite-pretreated manures were much more digestible, with 36.6% to 62.3% CGCs after the same enzyme loadings. These results show that the sulfite pretreatment can noticeably improve the enzymatic digestibility of manure. The removal of lignin and hemicellulose was hypothesized to be responsible for the improvement in enzymatic digestibility by creating accessibility for enzymes to hydrolyze cellulose (Leu and Zhu 2013; Zhang *et al.* 2013; Meng and Ragauskas 2014).

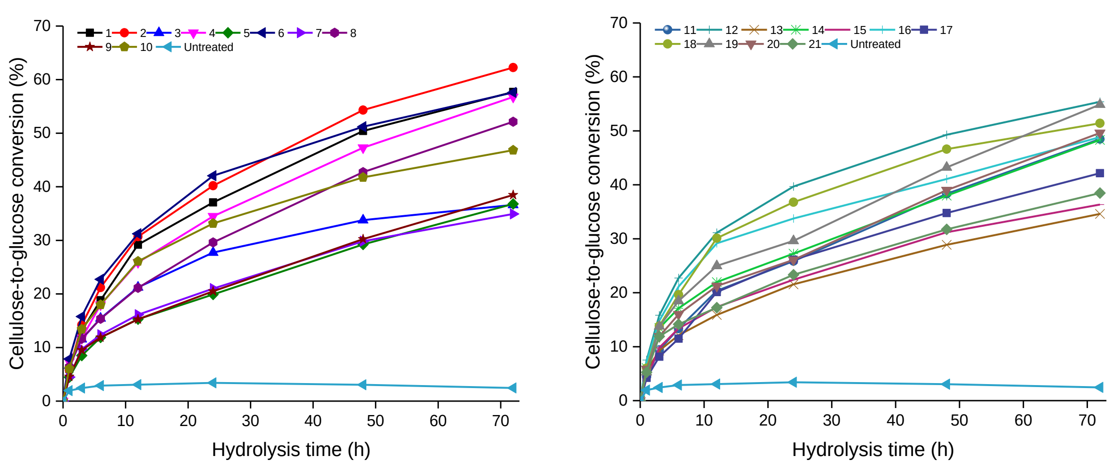

Fig. 2. Time-dependent enzymatic hydrolysis profiles of sulfite-pretreated manure experiments described in Table 1 (legends refer to experiment number).

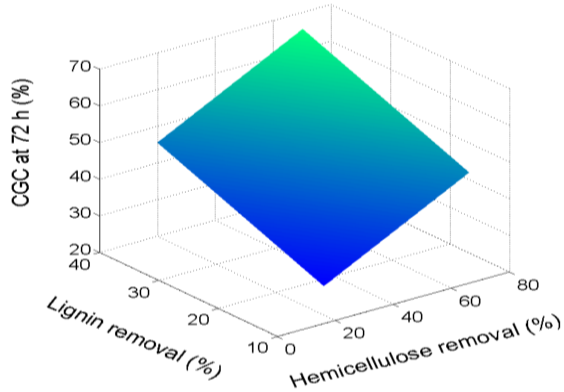

Fig. 3. Effects of lignin or hemicellulose removal on cellulose-to-glucose conversion (CGC) after 72 h in sulfite-pretreated manure

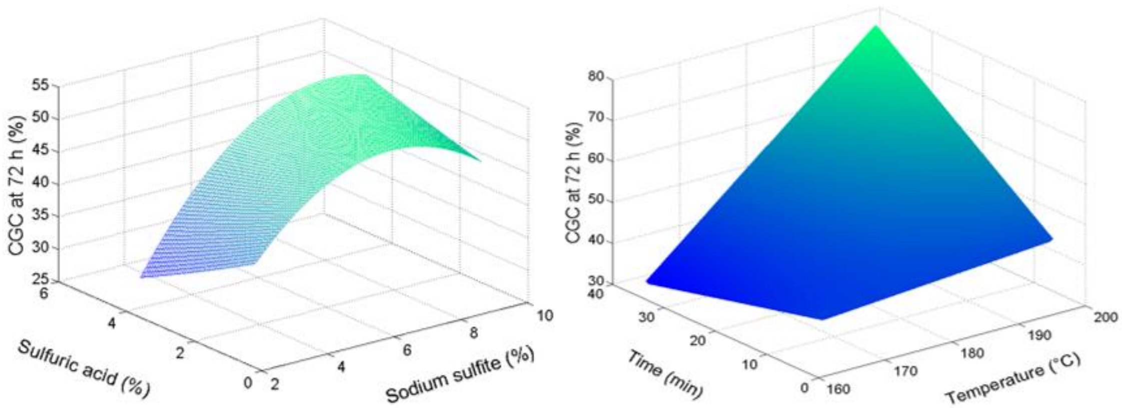

Fig. 4. Effects of pretreatment conditions on cellulose-to-glucose conversion (CGC) after 72 h in sulfite-pretreated manure

As shown in Fig. 3, the enzymatic digestibility (CGC at 72 h) of manure in this study was positively correlated with the removal of lignin and hemicellulose. The effects of the pretreatment conditions on the subsequent enzymatic digestibility (CGC at 72 h) are shown in Fig. 4. Increasing the pretreatment temperature or/and prolonging the pretreatment were beneficial to the enzymatic hydrolysis because of the enhanced removal of lignin and hemicellulose. For example, the manures pretreated at 180 to 197 °C generally achieved higher cellulose-to-glucose conversions (> 45%) than those pretreated at 163 to 170 °C (< 39%). Moreover, it seemed that increasing the pretreatment temperature was more effective than prolonging pretreatment. For example, the manure pretreated at 180 °C for only 3 min achieved a 46.8% CGC after 72 h of hydrolysis. This was likely because a higher temperature is necessary to initiate the acid-catalyzed delignification. The effect of sodium sulfite or sulfuric acid loading on the enzymatic digestibility is complicated, as shown in Fig. 4. In general, increasing the loading of sulfuric acid in the presence of sodium sulfite can improve the enzymatic digestibility by removing more hemicellulose and lignin. Similarly, increasing the dosage of sodium sulfite at a constant sulfuric acid loading rate can also improve the digestibility by enhancing the lignin removal through sulfonation. However, this was not always the case, as surplus sodium sulfite can also reduce the hemicellulose removal by decreasing the acidity of the pretreatment liquor. As discussed earlier, the hemicellulose removal in this study was more critical than the lignin removal, as shown in Fig. 3. Therefore, there should be a trade-off between the acid-catalyzed hemicellulose removal and sulfonation-caused lignin removal by increasing the loading of sodium sulfite to improve the enzymatic digestibility in this study.

Comparison of Sulfite, Diluted Acid, and Alkali Pretreatments

Diluted acid and alkali pretreatments are among the most common investigated routes for enhancement of cellulosic bioethanol production (Agbor *et al.* 2011). The dilute acid and alkali pretreatments were therefore investigated and compared with the sulfite pretreatment in this study. It has been well-recognized that sodium hydroxide, at high temperatures, can remove lignin through the cleavage of linkages, such as β-O-4. Sodium hydroxide hydrates at a high concentration can also penetrate the amorphous regions of cellulose, further altering the neighboring crystalline regions; it can even hydrolyze cellulose through a peeling reaction from the reducing end (Cai and Zhang 2005). At high concentrations and cold temperatures, some chemicals, such as urea, thiourea, polyethylene glycol (PEG), and polyethylenimine (PEI), can facilitate the dissolution of cellulose by interrupting or destroying the cellulose hydrogen bonds (Zhao *et al.* 2008; Mohsenzadeh *et al.* 2012). Therefore, in this study, sodium hydroxide alone was used for the hot alkali pretreatment, and sodium hydroxide combined with urea, thiourea, PEG, or PEI was used for the cold alkali pretreatment. The conditions for hot and cold alkali pretreatments were chosen according to the literature with modifications (Mohsenzadeh *et al.* 2012; Yang and Pan 2012; Zhang *et al.* 2013). Eight percent sodium hydroxide (based on oven-dry manure) was used for the hot alkali pretreatment, while 7.0% sodium hydroxide aqueous solutions containing one of the following: 12% urea, 5.5% thiourea, 2.0% PEG, or 2.0% PEI were used for the cold alkali pretreatments. The hot alkali pretreatment was carried out at 180 °C for 30 min, and the cold alkali pretreatments were conducted at -20 °C for 12 h. For comparison, the diluted acid pretreatment was also carried out at 180 °C for 30 min, and 4.0% sulfuric acid was used.

Table 2. Pretreatment Conditions of Manure using Sulfite, Diluted Acid, and Alkali

Pretreatment	Temp (°C)	Time (h)	Chemicals
Sulfite[a]	180	0.5	9.0% sodium sulfite + 4.0% sulfuric acid
Diluted acid[a]	180	0.5	4.0% sulfuric acid
Alkali-1[a]	180	0.5	8.0% sodium hydroxide
Alkali-2	-20	12	7.0% sodium hydroxide + 2.0% polyethylenimine
Alkali-3	-20	12	7.0% sodium hydroxide + 2.0% polyethylene glycol
Alkali-4	-20	12	7.0% sodium hydroxide + 5.5% thiourea
Alkali-5	-20	12	7.0% sodium hydroxide + 12% urea

[a]Chemical loading was based on the oven-dry weight of manure

As shown in Fig. 4, sodium sulfite improved the digestibility of sulfite-pretreated manure. Therefore, 9.0% sodium sulfite was chosen, and to compare with the diluted acid, the same amount of sulfuric acid (4.0%) was also used for the sulfite pretreatment, which was also conducted at 180 °C for 30 min. The presence of 9.0% sodium sulfite increased the pH value of the pretreatment liquor loaded with 4.0% sulfuric acid from 1.75 to 2.52. The sulfite, dilute acid, and alkali pretreatment conditions are summarized in Table 2, and the pretreatment results are shown in Fig. 5.

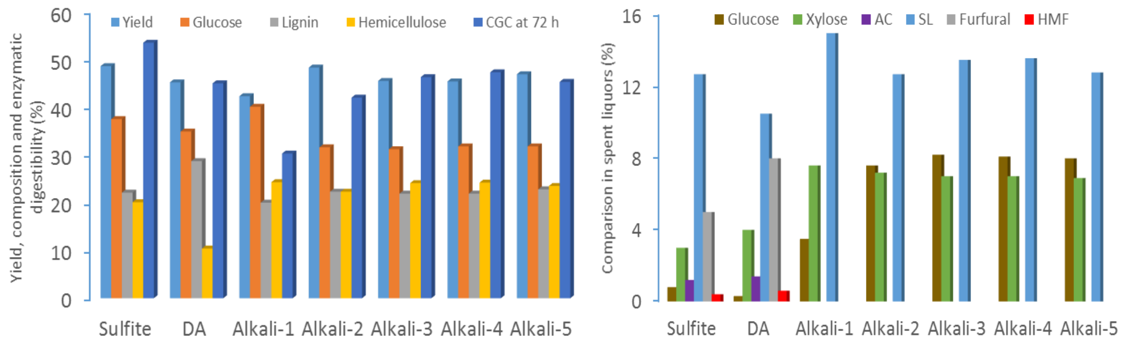

Fig. 5. Comparison of sulfite, dilute acid, and alkali pretreatments of manure

As expected, the dilute acid pretreatment removed more (74%) hemicellulose than the sulfite (45.7%), hot alkali (42.9%), or cold alkali (about 40%) pretreatments. As a result, the dilute acid-pretreated manure exhibited less hemicellulose (10.4%) than the sulfite (20.1%), hot alkali- (24.3%), or cold alkali- (21.9 to 22.3%) pretreated manures. The sulfite pretreatment spent liquor (pH=6.89) contained 5.0% and 0.4% furfural and HMF, respectively. Because of its stronger acidity (pH=4.92), more furfural and HMF (8.0% and 0.6%, respectively) were observed in the dilute acid pretreatment spent liquor. Therefore, in the pretreatment spent liquors, more xylose and glucose were found for the cold alkali (14.8% to 15.2%) and the hot alkali (11.1%) pretreatments than the dilute acid (4.3%) and the sulfite (3.8%) pretreatments. The hot alkali pretreatment removed more (63.8%) lignin than the cold alkali pretreatment (54% to 58%). Accordingly, more soluble lignin was observed in the hot alkali pretreatment spent liquor.

During the sulfite pretreatment, lignin can be removed through the acid-catalyzed cleavage of lignin linkages, as well as through sulfonation. Therefore, the sulfite pretreatment removed more (54% *vs*. 45%) lignin than the dilute acid pretreatment. The sulfite and cold alkali pretreated manures exhibited similar levels (about 22%) of lignin. However, the hot alkali-pretreated manure contained the least lignin (20%), while the dilute acid-pretreated manure had the highest lignin level (28.7%), which was attributed to greater removal of hemicellulose. Apparently, the removal of hemicellulose and lignin both influenced the substrate yield. These results showed that the sulfite (48.6%), dilute acid (45.2%), and cold alkali (45.4% to 48.3%) pretreatments resulted in relatively higher substrate yields than the hot alkali pretreatment (42.3%).

After lignin and hemicellulose are partially removed, manure is enriched in cellulose. The hot alkali pretreatment resulted in higher glucose conversion (40.1%) than the cold alkali one (about 31%), likely because more lignin was removed. Cellulose can be also hydrolyzed by sulfuric acid; however, sodium sulfite can alleviate the acid-catalyzed hydrolysis through decreasing the acidity (Yang and Pan 2012; Zhang *et al.* 2013). As a result, at the same sulfuric acid loading level, the sulfite-pretreated manure had more (37.5%) cellulose than the diluted acid-pretreated manure (34.9%). Acetic acid was observed in the diluted acid and sulfite pretreatment spent liquors, but it did not appear in the alkali pretreatment spent liquors, likely because it was neutralized by sodium hydroxide. The organic acids neutralizing the pH were observed with the pH value of hot alkali pretreatment liquor, which dropped from 11.7 to 9.1 (in the spent liquor) after pretreatment.

The results for enzymatic digestibility of manures pretreated by sulfite, diluted acid, and alkali are comparatively shown in Figs. 5 and 6. It was apparent that the sulfite-pretreated manure was hydrolyzed at the fastest rate and achieved the best enzymatic digestibility. A 53.5% cellulose-to-glucose conversion was obtained after 72 h of hydrolysis, which was close to the predicted value (54.4%) based on the fitted model (a function of temperature, time, sodium sulfite, and sulfuric acid) generated by JMP. Moreover, it can be concluded from Fig. 4 that the performance of sulfite pretreatment could be further improved under higher temperature for a longer time. For example, a 71% cellulose-to-glucose conversion after 72 h of hydrolysis was achieved when the pretreatment was carried out at 190 °C for 35 min by 9.0% sodium sulfite and 4.0% sulfuric acid. In contrast, the diluted acid-pretreated manure was less hydrolyzed, and therefore achieved a lower cellulose-to-glucose conversion (45%). However, the sulfite-pretreated manure had almost twice (20.1%) the hemicellulose than the dilute acid-pretreated manure (10.4%). The better digestibility for the sulfite-pretreated manure can be primarily attributed to its lower lignin content (22.1% *vs*. 28.7%). Looking at other biomass SPORL treatments, the residual lignin in the sulfite pretreatment was found to be less condensed than that of the diluted acid pretreatment, which should make the lignin more hydrophilic for the sulfite treatment (Rahikainen *et al.* 2013). Because of the weaker acidity and sulfonation, it can be reasonably hypothesized that the residual lignin in the sulfite-pretreated manure was less condensed and less hydrophobic than that of the dilute acid-pretreated manure.

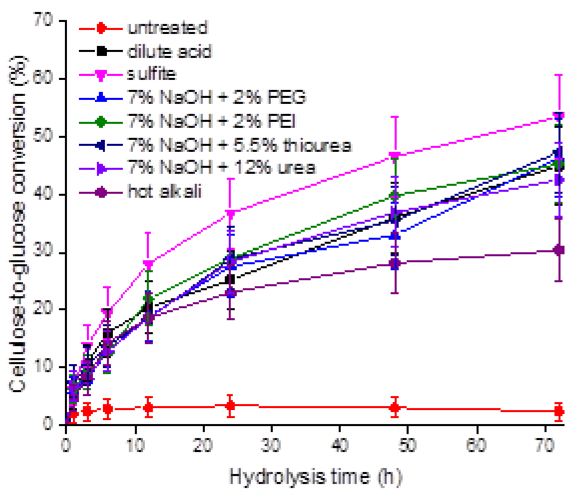

Fig. 6. Time-dependent enzymatic hydrolysis profiles of manure pretreated by sulfite, diluted acid, and alkali

Lignin adsorbs cellulolytic enzymes primarily through hydrophobic and ionic interactions (Eriksson *et al.* 2002). Therefore, with less content of lignin and less hydrophobic lignin, sulfite-pretreated lignocelluloses, such as switchgrass and agave substrates, can adsorb less cellulolytic enzymes compared with dilute acid-pretreated ones (Yang and Pan 2012; Zhang *et al.* 2013). The residual lignin with reduced hydrophobicity can adsorb less cellulolytic enzymes, leaving more enzymes for digesting cellulose. Therefore, the partial sulfonation of residual lignin might also contribute to the better digestibility of the sulfite-pretreated manure in this study. In comparison, the hot alkali-pretreated manure showed the poorest digestibility, with an almost 30.3% cellulose conversion after 72 h of hydrolysis, even though it had the lowest content (20%) of lignin. This result could have been caused by its higher content (24.3%) of hemicellulose. The hot alkali pretreatment also resulted in a poor enzymatic digestibility for switchgrass (Zhang *et al.* 2013). Conversely, the cold alkali-pretreated manures, with relatively higher contents of lignin (about 22%) and similar contents (about 24%) of hemicelluloses, exhibited favorable enzymatic digestibility, with over 40% cellulose conversion.

It has been reported that cold alkali pretreatment (sodium hydroxide combined with urea, thiourea, or PEG) may reduce the crystallinity of cellulose in both spruce and birch woods (Zhao *et al.* 2008). Glycosidic bonds in crystalline cellulose are protected by their strong inter- and intra-molecular hydrogen bonding. However, the disruption of hydrogen bonds (reduction in the crystallinity) can expose more β-1,4 glycosidic bonds for enzymatic hydrolysis. Therefore, it can be assumed that cellulose in the cold alkali-pretreated manure had a reduced crystallinity, and consequently showed favorable digestibility.

CONCLUSIONS

1. Sulfite pretreatment outperformed dilute acid (with same amount of sulfuric acid) and hot and cold (more chemicals and longer time) alkali pretreatments.
2. Sulfite pretreatment also exhibited other advantages, such as better sugar recovery and less fermentation inhibitors over the dilute acid pretreatment, and less chemicals required and shorter reaction times over the cold alkali pretreatment.
3. Sulfite pretreatment can notably improve the enzymatic digestibility of undigested dairy manure by reducing the recalcitrance through the removal of hemicellulose and lignin in the lignocellulosic matrix.
4. This study suggests that undigested dairy manure can be considered as a potential feedstock for cost-effective cellulosic bioethanol production. Further development and economic analysis will likely be required prior to commercialization.

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