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Combined sodium hydroxide and ammonium hydroxide pretreatment of post-biogas digestion dairy manure fiber for cost effective cellulosic bioethanol production

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Abstract

Background: The current higher manufacturing cost of biofuels production from lignocellulosics hinders the commercial process development. Although many approaches for reducing the manufacturing cost of cellulosic biofuels may be considered, the use of less expensive feedstocks may represent the largest impact. In the present study, we investigated the use of a low cost feedstock: post-biogas digestion dairy manure fiber. We used an innovative pretreatment procedure that combines dilute sodium hydroxide with supplementary aqueous ammonia, with the goal of releasing fermentable sugar for ethanol fermentation.

Results: Post-biogas digestion manure fiber were found to contain 41.1% total carbohydrates, 29.4% lignin, 13.7% ash, and 11.7% extractives on dry basis. Chemical treatment were applied using varying amounts of NaOH and NH₃ (2-10% loadings of each alkali on dry solids) at mild conditions of 100°C for 5 min, which led to a reduction in lignin of 16-40%. Increasing treatment severity conditions to 121°C for 60 min improved delignification to 17-67%, but also solubilized significant amounts of the carbohydrates. A modified severity parameter model was used to determine the delignification efficiency of manure fiber during alkaline pretreatment. The linear model well predicted the experimental values of fiber delignification for all pretreatment methods ($R^2 > 0.94$). Enzymatic digestion of the treated fibers attained 15-50% saccharification for the low severity treatment, whereas the high severity treatment achieved up to 2-fold higher saccharification. Pretreatment with NaOH alone at a variety of concentrations and temperatures provide low delignification levels of only 5 – 21% and low saccharification yields of 3 – 8%, whereas pretreatment with the combination of NaOH and NH₃ improved delignification levels and saccharification yields 2–3.5 higher than pretreatment with NH₃ alone. Additionally, the combined NaOH and NH₃ pretreatment led to noticeable changes in fiber morphology as determined by SEM and Crl measurements.

Conclusions: We show that combined alkaline treatment by NaOH and NH₃ improves the delignification and enzymatic digestibility of anaerobically digested manure fibers. Although pretreatment leads to acceptable saccharification for this low-cost feedstock, the high chemical consumption costs of the process likely will require recovery and reuse of the treatment chemicals, prior to this process being economically feasibility.

Keywords: Biogas, Digestion, Manure fiber, Sodium hydroxide, Ammonium hydroxide, Alkaline pretreatment, Enzyme saccharification

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Background

Global consumption of non-renewable fossil fuels in the transportation sector has increased vigorously during the last three decades with simultaneous increment in price of fuels [1,2]. For economic and environmental reasons, it is critical to find replacements for fossil fuels. Renewable, second-generation cellulosic biofuels offer the potential to improve energy security and reduce the deleterious environmental impact of first generation biofuels [3-6]. However, challenges remain in converting lignocellulosic biomass into sustainable biofuels in a cost- and energy-effective manner at large-scale [7-9]. The most commonly investigated lignocellulosic feedstocks for potential ethanol production are agricultural (crop residues) and forestry wastes (mill residues). Both of these feedstocks are natural composites consisting of three main biopolymers; cellulose, hemicellulose, and lignin [10-15]. Several studies [16-20] have demonstrated the potential of manure fibers (either pre- or post-biogas digestion) as a lignocellulose feedstock for the production of biofuels and value-added chemicals. The composition of manure fibers depends on the animal feed and the conditions of anaerobic digestion if carried out. The dry fibers typically have a high content of both carbohydrate (40 – 43%) and lignin (20 – 25%). Manure fiber is plentiful. In average, dairy cattle produce about 12.0 gal of manure per 1,000 lb live weight per day with 14.4 lb total solids. For example, the United States alone produces 110 million tonnes (d.b.) of manure annually. This manure supply could generate about 60 tonnes of biogas along with 60 million dry tonnes of undigested fibers that could produce an additional 7.6 billion liters (1.7 billion gallons) of ethanol [21,22]. Traditionally, most manure has been spread on fields, but digestion for biogas production is becoming more common in almost all countries [23-26]. The undigested manure byproduct of biogas production primarily is applied as a nutrient to farmland, but a small part is utilized for animal bedding [27], manure composts for organic fertilizer [28-30], and even the manufacture of particleboard [31]. Because manure fibers are known to be highly recalcitrant to enzymatic digestion, efforts on the conversion of manure fibers into biofuels have been limited [20]. Anaerobic digestion of manure for the production of biogas consumes hemicellulose and nearly all-available soluble sugars and leave cellulose and lignin untouched [20,32]. In addition, undesired components associated with nitrogenous extractives and ash increase the cost of biofuels production [33-35]. The recalcitrant nature of biomass is attributed to tight lignin wrapping, which prevents the accessibility of the biomass carbohydrate fractions (cellulose/hemicellulose) to enzymes, hemicellulose sheathing, cellulose crystallinity, and degree of polymerization [9,36]. Lignin not only hinders enzyme accessibility to cellulose but also provides the non-productive and/or irreversible binding of enzymes [37]. Therefore, a pretreatment step mechanical and/or chemical

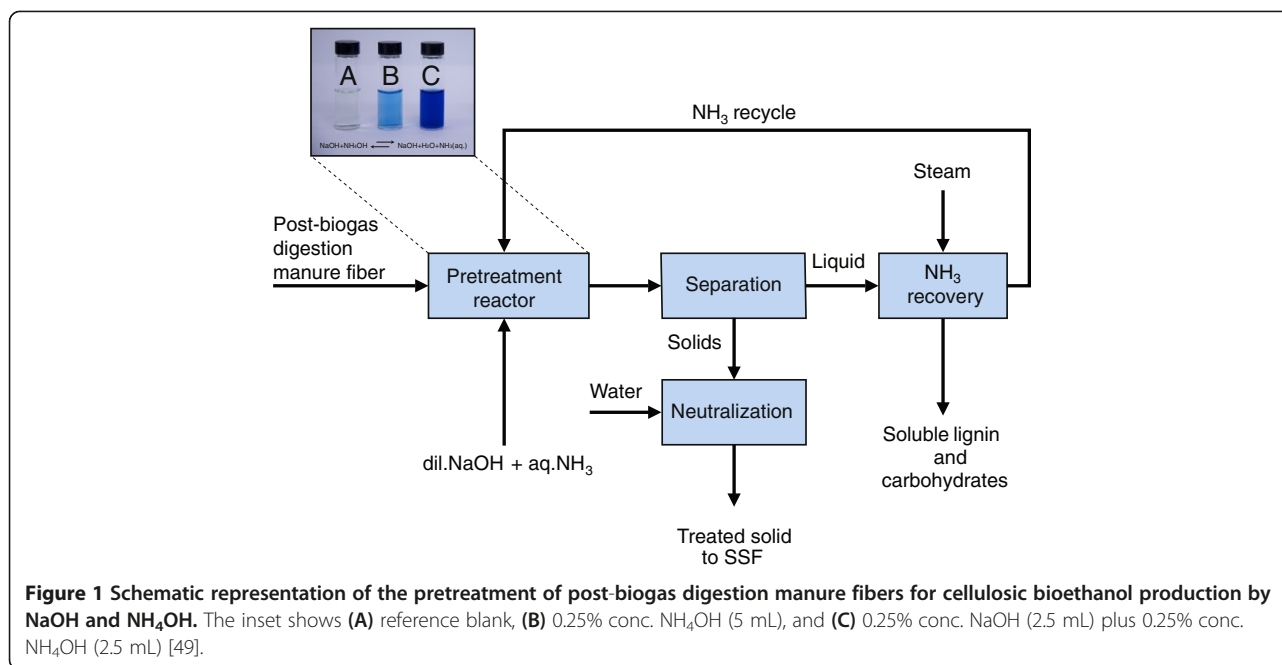
is necessary to modify the lignocellulose complex matrix structure in such a way as to disrupt lignin, dissolve hemicelluloses, and break down the cellulose crystallinity in order to enhance substrate accessibility to enzymes and in turn, release more fermentable sugars [38,39].

In general, nitrogenous matter in anaerobically digested fiber increases its alkalinity (to pH 8.5 – 9.0). Therefore, alkaline pretreatments are expected to require less chemical than acidic pretreatments [20]. Alkaline biomass pretreatment methods using either sodium hydroxide [40,41] or aqueous ammonia [42] have been studied in recent years and shown to have high efficiency and low cost [14,38]. Sodium hydroxide treatment effectively depolymerizes and removes the most labile biomass components, such as hemicelluloses and lignin, causes swelling that increases enzyme accessible surface area (for solvation and saponification reactions) and reduces the degree of polymerization and crystallinity of cellulose [40]. Aqueous ammonia reacts selectively with lignin by cleaving C-O-C bonds in lignin and ether and ester bonds in lignin-carbohydrate complexes, but carbohydrate removal and/or degradation is limited. In addition, these treatments cause significant morphological changes in the lignocellulose to improve enzyme accessibility [43,44]. However, aqueous ammonia may not be effective for the pretreatment of substrates having relatively higher lignin (wood feedstocks) [38]. According to reviews, maximum delignification (~64%) with enzymatic saccharification (~65%) could be achieved for anaerobically digested manure fiber by using dil. NaOH under elevated temperature [20,45]. Also, the addition of supplementary reagents to the alkali pretreatment chemicals, such as oxidizing agents [46] or lime [47], has been shown to further improve delignification and subsequent enzymatic digestion of lignocellulose substrates [48]. As pretreatment protocols for post-biogas digestion (PBD) manure fiber, which contain high residual lignin, aimed at improving enzymatic digestion for ethanol fermentation, we explored the use of dilute sodium hydroxide and/or aqueous ammonia. The addition of NaOH to NH_4OH shifts the equilibrium to form gaseous NH_3 , a reversible reaction that could be used to facilitate its recovery and reuse so as to improve the cost-effectiveness of this process (Figure 1) [49]. The fibers were then enzymatically saccharified to convert glucan to glucose to determine the effectiveness of the pretreatments. The pretreated fibers were also examined by scanning electron microscopy and x-ray diffraction measurements to determine cellulose crystallinity.

Results and discussion

Characterization of post-biogas digestion manure fiber

As determined by mechanical sieve analysis, the post-biogas digestion (PBD) manure fiber that had undergone alkaline pretreatment displayed a fiber size range from



2.4 mm to < 75 μ m with a calculated number average diameter (D_N) of 0.041 mm and volume surface mean diameter (D_S) of 0.415 mm [50]. Particles smaller than 18-mesh (1.0 mm) accounted for 93% of PDB fibers (dry basis), as compared to about 73% of pre-biogas digestion manure fibers. The effects anaerobic bacterial digestion lead to smaller particle sizes [20]. In general, particle size plays a significant role in the effectiveness of the pretreatment and fermentation steps [51,52], with smaller fiber size being advantageous for bioconversion. The PDB manure fiber contained 41.1% carbohydrate by weight, of which 23.6% was glucose and 17.5% other sugars (xylose, galactose, arabinose and mannose) (Table 1). The carbohydrate content of PDB manure fiber is 25–28% lower than those of other commonly used substrates for cellulosic ethanol production (corn stover, switch grass, sugarcane bagasse, and wheat straw) [15]. More accessible carbohydrate sugars are digested in the animal and during anaerobic digestion. The low carbohydrate content of PDB manure fibers leads to carbohydrate/lignin ratios 60–65% lower than in other agricultural biomass. PDB manure fibers contained 27.6% acid insoluble lignin (ash free and Klason) and 1.8% acid soluble lignin. Analysis of lignin monomers yielded 19:71:10 syringyl (S):guaiacyl (G):*p*-hydroxyphenyl (H) on dry basis, ratios consistent with corn stover lignin [42,53]. Fiber ash, a non-reactive and undesired component of manure fiber for biofuels production, which negatively affects ethanol yields particularly from thermochemical ethanol production [54], accounted for 13.7% (dry basis). It has been reported that manure fiber also contains ~12% solvent extractives, composed mainly of nitrogenous materials,

nonstructural sugars, inorganics, waxes, oils, and other compounds [55]. The predominant component, nitrogenous material, comes from indigestible forage proteins and ammonia and other nitrogen compounds in urine and manure. This nitrogen could be a potential nutrient source for microbial growth in ethanol fermentation [17,35]; however, biomass extractives interfere with analytical measurements [56,57] and thus were not considered in our study.

Alkaline pretreatment of PDB manure fiber

We investigated the pretreatment of PDB manure fiber by dilute sodium hydroxide and ammonium hydroxide (SHAH). We studied the effects of different pretreatment parameters, including alkali loading, temperature, and residence time, on the recovery and subsequent enzymatic digestion of PDB manure fiber (Table 2). We used low-severity protocols to evaluate pretreatment under conditions that minimized the cost of chemicals and the energy needed to heat the samples: 100°C for 5 min at concentrations of 2–10% (by dry fiber weight) of NaOH and NH₃ [41,58]. These pretreatment protocols led to substantial decreases in fiber residual lignin (16–40% delignification) and improved carbohydrate recovery (80–67%) and higher carbohydrate concentration (2–10%). The pretreatment yields were calculated based on a comparison between the weight of contents present in the sample before (initial) pretreatment and the weight of contents present in the solids remaining after pretreatment. These low severity pretreatment conditions achieved higher fiber delignification than higher severity conditions [20,47,59]. We also investigated the effects of higher

Table 1 Compositional analyses result of post-biogas digestion manure fiber (anaerobically bacterial digested)

Component	^a Post-biogas digestion manure fiber, dry basis
Carbohydrate	
Glucose	23.6 ± 0.3%
Xylose, Galactose, Arabinose, and Mannose	17.5 ± 0.3%
Lignin	
Acid insoluble lignin	27.6 ± 0.1%
Acid soluble lignin	1.8 ± 0.0%
Lignin monomer	
^b S/G ratio	0.3
S/H ratio	1.9
H/G ratio	0.1
Uronic acid	2.6 ± 0.1%
Ash	13.7 ± 0.1%
Extractives	11.7 ± 0.1%
Fiber elements	
C	39.1 ± 0.4%
N	3.3 ± 0.2%
P	1.1 ± 0.0%
K	1.4 ± 0.0%
Ca	4.8 ± 0.6%
Mg	0.8 ± 0.1%
S	1.0 ± 0.0%
Cl	0.4 ± 0.1%
Fe	0.3 ± 0.1%
Zn	0.1 ± 0.0%

^a Data are average of two replicates. Numbers with ± values represent standard errors; ^b Syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) lignin.

temperature (120°C at 15 psi) and longer residence time (60 min) at different concentrations of NaOH and ammonia (Table 2). The more stringent conditions led to modest increases in total sugars and reductions in total lignin. The results were consistent with the literature [20] and observed approximately 2-3-fold increase on pretreatment recovery.

We compared the effects of SHAH pretreatment protocols with those using NaOH and aqueous NH₃ singly under conditions of 121°C at 15 psi and 60 min (Table 2). In terms of solids recovered, total sugars, and total lignin, treatment with NaOH alone was equivalent to SHAH pretreatment; for both, increased alkali loading led to lower solids recovery, higher total sugars, and lower total lignin. Treatment with aqueous NH₃ alone led to higher solids recovery, slightly lower total sugars and higher total lignin. Increasing concentrations of aqueous ammonia had little effect on glucose, total sugars, or total lignin. A linear model relating modified severity parameter that

combines the effects of temperature, time, and alkali concentration to the percentage removal of lignin was used for the determination of fiber delignification during alkaline pretreatments. The model was developed by plotting $\log(M_0)$ vs percent delignification, as given in Figure 2. The modified severity parameter model was validated by plotting the experimental vs model predicted values of fiber delignification (Additional file 1: Figure S1) and observed $R^2 > 0.94$ for all pretreatment methods; indicating good predictive ability of the model.

Effects of alkaline pretreatment of PBD manure fiber on subsequent enzyme saccharification

We carried out enzymatic digestion of pretreated PBD manure fiber to determine the release of fermentable sugar for ethanol fermentation (Figure 3). The typical enzymatic hydrolysis profile showed rapid saccharification over 6 h followed by leveling off thereafter (Figure 3A). Enzymatic hydrolysis results of SHAH pretreatment at 100°C (Figure 3B) showed saccharification yields of 15-49% with 18-55% glucose conversion after 24 h with a corresponding increase in concentration of 5-3% points difference between each concentration increment. Approximately 1.5 – 2.0-fold increase in saccharification yield was achieved with 121°C at 15 psi and 60 min pretreatment (Figure 3B), indicating that removal of residual lignin (relatively 8-66% higher delignification) and other substrate features had significant impact on the improved enzyme accessibility for fiber digestion [60]. SHAH pretreatment achieved 3 – 8% and 1.5 – 2.5-fold higher saccharification than separate NaOH and NH₃ pretreatments, respectively (Figure 3C). Also, it was approximately 3-fold higher saccharification than Teater et al. [20].

Effects of pretreatment of PBD manure fiber on surface structure and cellulose crystallinity

We used scanning electron microscopy (SEM) to determine the effects of pretreatment on the surface features of the fibers. PBD manure fibers that were not pretreated (Figure 4A) or pretreatment with NaOH alone (Figure 4B) or aqueous NH₃ alone (Figure 4C) exhibited rigid and highly ordered surface structure. By contrast the SEM image of fibers that underwent SHAH pretreatment exhibited sponge-like structures and an apparent increase in fiber porosity (Figure 4D) [39,40].

We used X-ray powder diffraction pattern to determine the effects of pretreatment on cellulose crystallinity. The results (shown in Figure 5A) showed that the three treatment protocols at 121°C and 15 psi reduced crystallinity in the order: 10% NH₃ < 10% NaOH < 10% NaOH + NH₃. SHAH pretreatment greatly alters the crystalline structure by the competitive reaction of both alkalis resulting in the formation of different allomorphs that have different unit cell dimensions, chain packaging,

Table 2 Pretreatment recovery and solid residues composition of post-biogas digestion manure fiber after treatment using NaOH and NH₃ at different conditions

Alkali loading%, gm/gm dry solids		Temperature (°C)	Residence time (min)	^a Modified CS factor, log M ₀	Solids recovery%	Residue composition%, dry basis			
Dilute NaOH	Aqueous NH ₃					Glucose	^b Total sugars	^c Total lignin (AIL + ASL)	
2.0	2.0	100	5	0.09 (n = 0.89)	80.6 ± 0.1	24.2 ± 0.2	41.1 ± 0.6	31.1 ± 0.1	
4.0	4.0	100	5	0.35	76.6 ± 0.0	24.7 ± 0.4	42.0 ± 0.4	31.0 ± 0.4	
6.0	6.0	100	5	0.51	72.0 ± 0.5	25.0 ± 0.3	41.4 ± 0.5	29.0 ± 0.4	
8.0	8.0	100	5	0.62	68.9 ± 0.1	27.3 ± 0.7	44.4 ± 1.2	28.6 ± 0.2	
10.0	10.0	100	5	0.71	64.6 ± 0.3	28.2 ± 1.5	43.2 ± 1.2	27.6 ± 0.5	
2.0	2.0	121/15 psi	60	0.26 (n = 3.1)	78.1 ± 0.2	25.3 ± 0.2	42.1 ± 0.2	31.5 ± 0.1	
4.0	4.0	121/15 psi	60	1.20	71.2 ± 0.6	26.6 ± 0.8	42.9 ± 1.0	28.8 ± 0.3	
6.0	6.0	121/15 psi	60	1.74	63.0 ± 0.2	30.0 ± 0.7	46.9 ± 1.0	26.5 ± 0.1	
8.0	8.0	121/15 psi	60	2.14	57.0 ± 0.6	32.5 ± 0.0	52.3 ± 0.0	22.8 ± 0.0	
10.0	10.0	121/15 psi	60	2.43	51.2 ± 0.0	32.8 ± 0.0	53.5 ± 0.0	19.5 ± 0.1	
2.0	-	121/15 psi	60	0.21 (n = 2.2)	79.0 ± 0.1	25.5 ± 0.0	42.4 ± 0.0	33.2 ± 0.4	
4.0	-	121/15 psi	60	0.89	72.6 ± 0.8	27.0 ± 1.1	44.2 ± 1.2	30.5 ± 0.2	
6.0	-	121/15 psi	60	1.28	64.2 ± 0.1	31.1 ± 0.5	48.5 ± 1.3	27.3 ± 0.1	
8.0	-	121/15 psi	60	1.56	58.5 ± 0.1	32.2 ± 0.0	48.9 ± 0.0	23.9 ± 1.3	
10.0	-	121/15 psi	60	1.77	52.0 ± 0.3	31.8 ± 0.0	50.0 ± 0.0	20.1 ± 0.1	
-	2.0	121/15 psi	60	0.21 (n = 2.2)	83.1 ± 0.4	22.9 ± 1.2	41.2 ± 1.8	33.1 ± 0.3	
-	4.0	121/15 psi	60	0.89	79.5 ± 1.2	22.7 ± 0.1	42.2 ± 0.3	33.2 ± 0.2	
-	6.0	121/15 psi	60	1.28	79.3 ± 1.6	22.2 ± 0.3	42.0 ± 0.0	32.6 ± 0.7	
-	8.0	121/15 psi	60	1.56	77.2 ± 0.4	23.2 ± 0.4	43.1 ± 0.8	32.4 ± 0.2	
-	10.0	121/15 psi	60	1.77	76.8 ± 0.8	23.2 ± 0.4	43.1 ± 0.8	31.2 ± 0.8	
R ²					0.95	0.90	0.79	0.87	
RMSE					2.26	1.19	1.84	1.57	
Tukey's HSD (P < 0.05)					43.34	17.84	25.5	16.07	

^a Modified combined severity factor; ^b Total sugars including glucose, xylose, galactose, arabinose and mannose; ^c Total lignin including acid insoluble lignin (AIL) and acid soluble lignin (ASL) after ash correction. Data are average of two replicates. Numbers with ± values represent standard errors.

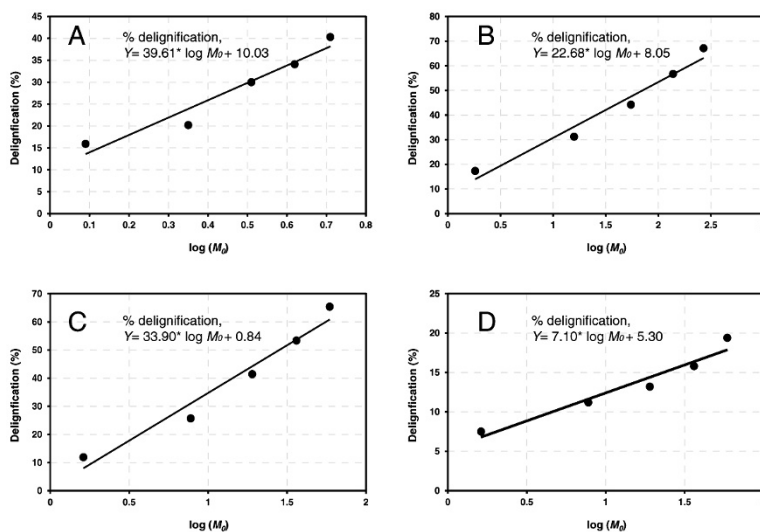
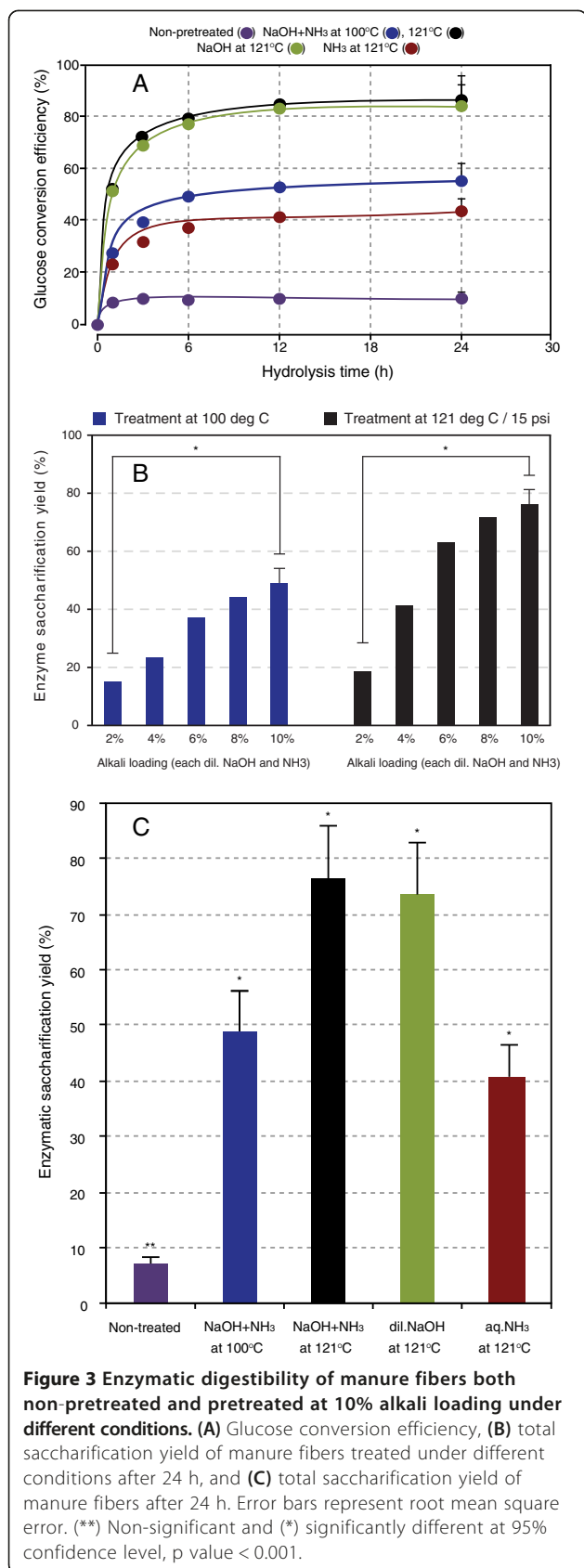


Figure 2 Plot of percent delignification of manure fibers vs modified severity parameter (log M₀) for alkaline pretreatment treated at (A) 100°C using combined NH₃ and NaOH, (B) 121°C/15 psi using combined NH₃ and NaOH, (C) 121°C/15 psi using NaOH, and (D) 121°C/15 psi using NH₃.



and hydrogen bonding relationships [61,62]. Studies recognized that alkaline pretreatment causes swelling of cellulose, leading to the decrease of degree of polymerization and crystallinity, and increases the surface area that facilitates more substrate exposed to cellulase attack [48]. However, the poor negative correlation between the cellulose crystallinity index and enzymatic digestion of fibers under different conditions ($R^2 < 0.1$) (Figure 5B – D), might be due to the dissolution of amorphous materials (xylan and lignin) and/or interference of other soluble materials [63,64].

Effect of pretreatment conditions on fiber delignification and enzymatic saccharification

We carried out a detailed study of the relationship between pretreatment parameters and fiber composition following enzymatic digestion by using a central composite design experiment with 3 dependent factors and 3 different levels (Table 3): 3 alkali loadings (x_1) of 2.0, 4.0 and 6.0% of each NaOH and NH₃, 3 treatment temperatures (x_2) of 80, 100, and 121°C, and three residence times (x_3) of 5, 30, and 60 min. The experimental parameters were selected on the basis of a previous SHAH pretreatment study on PBD manure fiber. The results showed a dependence on the pretreatment conditions of sugars released, mainly hemicellulose which is more vulnerable to chemical attack, and lignin (Table 4). The model identified that, within the studied range of experiments, chemical loading had the most significant effect on both sugar dissolution (regression coefficient, $\beta_3 = -5.4$) and delignification ($\beta_3 = 10.5$). Increasing alkali loadings from 2.0 to 6.0% led to a decrease in total sugar recovery by 20% and a decrease in residual lignin by 40%. The correlation coefficient values for the models ($R^2 \geq 0.95$) indicate that a large fraction of the variation in responses results from differences in the independent variables. Although enhanced removal of residual lignin is expected to improve subsequent enzymatic digestibility, the simultaneous loss of residual carbohydrate should decrease the yield of sugars through enzymatic hydrolysis [46].

Following enzymatic saccharification, chemical loading ($\beta_3 = 12.6$) had more significant effect than residence time ($\beta_2 = 11.0$) or treatment temperature ($\beta_1 = 5.9$). Three linear effects and one quadratic effect were observed with subsequent enzymatic digestibility of the treated fibers. An increase in chemical loading from 2.0 to 6.0% led to a 57% saccharification yield with 64% glucose conversion. This may be due to the enhanced removal of enzyme barriers, including residual lignin (~30%) and hemicelluloses (~22%), and surface modification during pretreatment which improves enzyme accessibility [9]. Linear terms of delignification and enzymatic saccharification correlated positively with the treatment parameters, indicating that these have the greatest effect on substrate deconstruction.

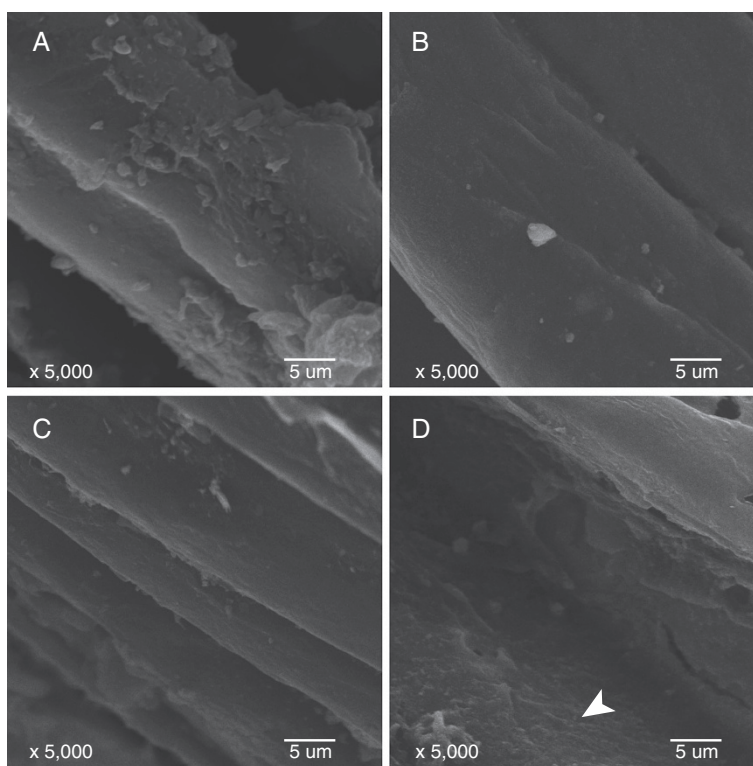


Figure 4 Scanning electron microscope images of manure fibers before and after treatment at 121°C and 10% alkali loading level. (A) non-pretreated (control), (B) pretreated by NH_3 alone, (C) pretreated by NaOH alone, and (D) pretreated by combined NH_3 and NaOH.

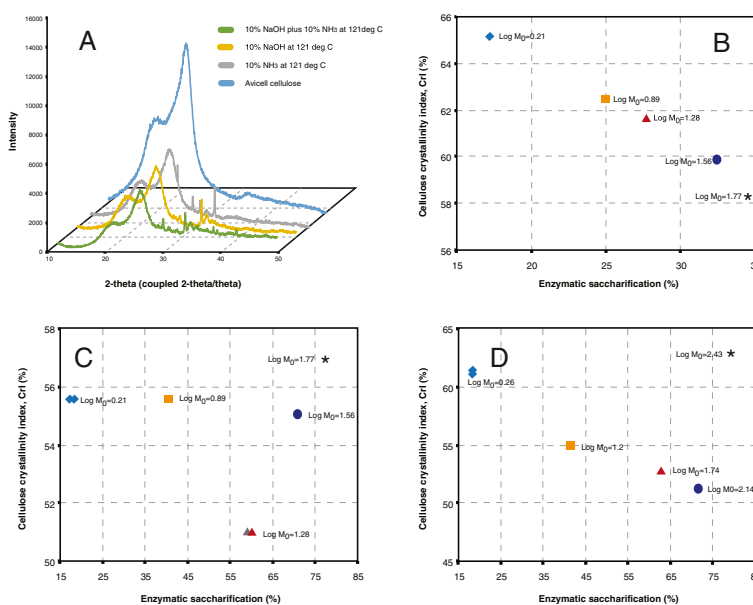


Figure 5 Cellulose crystallinity index (CrI) of alkaline-treated manure fibers and its relationship to the enzymatic digestibility (24 h). (A) powder X-ray diffraction spectrum of fibers after treatment at different conditions, (B) correlation between enzymatic digestibility and CrI of separate NH_3 treated fibers, (C) correlation between enzymatic digestibility and CrI of separate NaOH treated fibers, and (D) correlation between enzymatic digestibility and CrI of both NaOH and NH_3 treated fibers, under different conditions. Data are averages of two replicates.

Table 3 Central composite experimental design and the corresponding pretreatment responses with subsequent enzyme saccharification of post-biogas digestion manure fibers

Independent variables			Dependent variables, Y		
^a % NaOH and NH ₃ loading, gm/gm dry fiber, x ₁	Temperature (°C), x ₂	Residence time (min), x ₃	Total sugar recovery%	Delignification% dry basis	Enzyme saccharification%
8.0	100	30	87.3 ± 0.0	22.9 ± 0.3	24.5 ± 0.8
12.0	80	5	87.4 ± 0.0	25.7 ± 1.3	19.9 ± 4.3
8.0	100	30	87.3 ± 0.0	22.9 ± 0.3	24.5 ± 0.8
12.0	121	5	87.5 ± 0.4	21.6 ± 1.1	23.9 ± 1.2
8.0	100	60	86.0 ± 0.4	26.3 ± 0.8	34.0 ± 1.2
4.0	121	5	94.6 ± 0.1	5.9 ± 0.9	2.5 ± 0.4
12.0	80	60	82.7 ± 0.6	32.8 ± 0.5	44.5 ± 1.0
8.0	100	5	91.5 ± 0.1	17.1 ± 0.2	16.2 ± 0.1
8.0	100	30	87.3 ± 0.0	22.9 ± 0.3	24.5 ± 0.8
12.0	100	30	79.4 ± 0.1	37.1 ± 0.3	36.5 ± 3.8
4.0	80	60	93.4 ± 0.1	12.2 ± 0.1	10.9 ± 0.1
8.0	121	30	80.2 ± 0.8	30.3 ± 1.0	49.0 ± 2.8
12.0	121	60	73.3 ± 0.4	44.3 ± 0.8	59.0 ± 0.5
8.0	100	30	87.3 ± 0.0	22.9 ± 0.3	24.5 ± 0.8
4.0	100	30	93.1 ± 0.1	10.0 ± 1.3	13.7 ± 0.4
4.0	121	60	88.3 ± 0.1	17.5 ± 1.3	27.5 ± 0.2
4.0	80	5	95.2 ± 0.1	11.1 ± 1.1	3.1 ± 0.2
8.0	80	30	89.5 ± 0.1	21.1 ± 0.0	24.8 ± 0.5
8.0	100	30	87.3 ± 0.0	22.9 ± 0.3	24.5 ± 0.8

^a Percentage of each NaOH and NH₃ added to the dry manure solids. Data are average of two replicates. Numbers with ± values represent standard errors.

The second most important parameter affecting the overall process was the residence time [65]. This suggests that longer treatment times reduce fiber recalcitrance that limits sugar degradation [66] and improve energy utilization.

Conclusions

In the present study, enzyme recalcitrant post-biogas digestion (PBD) manure fibers were subjected to an innovative pretreatment method involving combined alkalis (dilute sodium hydroxide and aqueous ammonia). The effects of

Table 4 Statistical analysis of the effect of pretreatment parameters on manure fiber pretreatment recovery and following enzymatic saccharification

	Total sugar recovery		Delignification		Enzymatic saccharification	
^a R ²	0.96		0.97		0.95	
Prob. > F	< 0.0001*		< 0.0001*		< 0.0001*	
Terms	Estimate	^b Prob. > t	Estimate	^b Prob. > t	Estimate	^b Prob. > t
Temp (°C)	-2.43	0.0011*	1.66	0.061	5.88	0.0035*
Time (min)	-3.26	0.0001*	5.17	< 0.0001*	11.0	< 0.0001*
Chemical loading (% on dry solids)	-5.42	< 0.0001*	10.4	< 0.0001*	12.6	< 0.0001*
Temp x Temp	-1.50	0.1613	1.43	0.360	7.13	0.0345*
Time x Time	2.41	0.0372*	-2.61	0.113	-4.65	0.139
Chemical loading x Chemical loading	-0.079	0.938	-0.76	0.620	-4.64	0.140
Temp x Time	-1.73	0.015*	3.26	0.0046*	3.47	0.0681
Temp x Chemical loading	-0.44	0.463	0.91	0.322	0.33	0.848
Time x Chemical loading	-1.37	0.0414*	2.14	0.0363*	3.36	0.0755

^a Correlation coefficient; ^b p-value of the two-tailed test; * Numbers with asterisk indicate that the term has a significant effect at 95% confidence level.

pretreatment conditions were studied, including alkali loading on fibers, treatment temperature, and residence time. The results show that the dual alkali treatment improves fiber delignification (maximum 67.1%) and subsequent enzymatic digestion (maximum of 76.3%) of PBD manure fibers. Furthermore, the pretreatment alters the surface structural characteristics of the fiber apparently making them more prone to enzyme attack for enhanced sugar release. A positive factor in the economic viability of PBD manure fiber for cellulosic bioethanol production, is the high availability and relatively low cost of the feedstock. On the other hand, the costs of chemical consumption need to be taken into account, although these could be mitigated in part by recovery and reuse of the gas phase ammonia formed during the alkali reaction. In addition, it may be possible to improve the efficiency of the process by combined maceration (mechanical milling) and alkaline pretreatment with both NaOH and NH₃, and studies to evaluate this approach are ongoing.

Methods

Manure samples

Post-biogas digestion manure fibers were collected from Maple Leaf Dairy Farm, Cleveland, Wisconsin. The cattle feed was a mixture of alfalfa, corn silage and other proteins according to the National Research Council nutrient requirements of dairy cattle. The anaerobic digestion was running with a hydraulic retention time of 14–15 days at 35–40°C. The slurry containing undigested solids were separated by a 2.0 mm screen screw press. The collected fibers contained 60–65% moisture; they were air-dried and ground with a laboratory hammer mill (Christy & Norris Ltd., England, Model No. 1024XC) and then sieved. The fiber fraction within 40–50 mesh was used for the analysis.

Combined alkaline pretreatment

Sodium hydroxide (50% wt. Fisher Catalog No. SS254-4) and ammonium hydroxide (30% wt. Fisher Catalog No. 125) were used pretreatment. Weighed quantities of fiber in 50 ml Oak Ridge thermal resistant tubes (Fisher Catalog No. 05-563-10G) were treated with NaOH and/or NH₃ (at different loadings of each alkali 2–10% w/w) at room temperature for 2 h, followed by heating at 100 or 121°C for 1 h. The solid-to-liquid ratio was maintained at 1:7. After pretreatment, the supernatant was collected following centrifugation at 3,900 rpm (Eppendorf 510R) for 20 min, and the solid residues obtained were thoroughly washed with water until the pH reached neutrality. Finally, the solid residues were dried in a freeze-dryer (VirTis frezemobile 35ES) and stored at –80°C (New Brunswick U-700 freezer).

Enzymatic digestibility

Enzyme saccharification of fibers, both non-treated (control) and chemically treated, was carried out according to the standard NREL procedure (LAP 42629). Saccharification was conducted in 50 mL Falcon tubes at 2.0% (w/v) substrate consistency level using sodium acetate buffer pH 4.8. Tetracycline antibiotic was added at 0.02% (w/v) to prevent microbial contamination. Enzymes used in this study, Cellic CTec2 (cellulase complex containing cellulose and β-glucosidase) and Cellic HTec2 (hemicellulases including xylanase), were generously provided by Novozymes (Franklinton, NC). The Cellic CTec2 and Cellic HTec2 loadings on substrates were 5% and 2% w/w (gm enzyme/gm dry fiber), respectively. Substrates were pre-incubated at 50°C in sodium acetate buffer for 24 h prior to the addition of enzymes. Hydrolysis was conducted at 50°C in a shaker (New Brunswick Scientific Excella E24) at 200 rpm for 24 h. Samples were collected intermittently and analyzed for sugar concentration using High Performance Liquid Chromatography (HPLC) System (Agilent Technologies 1200 series). The HPLC was equipped with Bio-rad deashing micro-guard column (Cat No. 125–0118, Bio-Rad, CA) and Agilent Hi-Plex H (7.7 × 300 mm, 8 μm) analytical column operated at 60°C with 5 mM H₂SO₄ mobile phase at the flow rate of 0.7 mL/min. A refractive index detector (Agilent Technologies) was operated at 55°C. The mobile phase was filtered through a 0.22 μm nylon membrane (Millipore Corporation, MA) and degassed. The released glucose and other sugars (xylose, galactose, arabinose and mannose) at each time interval were used to calculate the glucose conversion and saccharification efficiency of the substrate as a percentage to the potential sugars available in the substrates. Each data point was the average of two replicates.

Analytical methods

Manure fiber moisture, extractives and ash contents were determined according to National Renewable Energy Laboratory (NREL) analytical procedures LAP 012, LAP 010 and LAP 005, respectively. Similarly, carbohydrate analysis of non-treated and chemically pretreated manure fiber was carried out according to NREL procedure LAP 009. Samples (0.3 g) were weighed (W_i) in a 5 ml centrifuge tube and hydrolyzed with 3 ml 72% H₂SO₄ (v/v) for 60 min. The hydrolyzate was diluted to 4% acid concentration (v/v) and autoclaved for 60 min at 121°C at 15 psi. The hydrolysis solution was vacuum filtered through the previously weighed filtering crucible. The filtrate was collected (F_i) and analyzed for carbohydrate and acid soluble lignin determination. Carbohydrate content, including glucose, xylose, galactose, arabinose, and mannose sugars, were analyzed on an HPLC System (Agilent Technologies 1200 series) equipped with

a Bio-Rad deashing micro-guard column (Cat No. 125–0118, Bio-Rad, CA) and an Agilent Hi-Plex H (7.7 × 300 mm, 8 μm) analytical column with a mobile phase of 5 mM H₂SO₄ operated at a flow rate of 0.7 mL/min at 60°C. The mobile phase was filtered through a 0.22 μm nylon membrane (Millipore Corporation, MA) and degassed, and peaks were detected by a refractive index detector (Agilent Technologies) operated at 55°C.

Acid insoluble lignin was calculated gravimetrically as acid-insoluble residue after correction for ash content. The lignin collected during filtration was washed with water and dried overnight in an oven at 105°C. The weight of the crucible with lignin was recorded (W_2), and the sample was ashed in muffle furnace for 4 h at 575°C. Finally, the weight of the crucible with ash content was recorded (W_3). Acid insoluble lignin (AIL) content of the manure fiber was calculated by the following equation:

$$AIL(\%) = \frac{(W_2 - W_3)}{W_1 \times m_i} \times 100$$

where m_i is the initial moisture content of the manure sample. The filtrate F_I was measured for the acid soluble lignin at 208 nm using UV/Vis spectroscopy (Agilent Cary 60) with 4% (v/v) sulfuric acid as reference blank.

Lignin monomer composition

Manure fiber lignin composition was determined by derivatization followed by reductive cleavage method [67].

Colorimetric assay of uronic acid

A *m*-hydroxydiphenyl colorimetric assay was followed for the determination of uronic acid content [39,68]. All chemicals were purchased from Sigma Aldrich (St. Louis, MO) and used as such. 200 μL of the acid hydrolyzate filtrate (F_I) was added to 1.2 mL H₂SO₄-tetraborate solution (476.8 mg sodium tetraborate dissolved in 500 mL 18 M H₂SO₄). Followed by, heating in a boiling water bath for 5 min and ice cooled. 20 μL of 0.15% *m*-hydroxydiphenyl reagent (22.5 mg 3-phenylphenol dissolved in 15 mL 0.5% w/v NaOH) was added to the reaction mixture and vortexed immediately until to get a dark pink color. Finally, the reagent mixture was read after the original color development at 520 nm using UV/Vis spectrophotometer (Agilent Cary 60). The uronic acid content was calculated from the OD value using the glucouronic acid/galacturonic acid calibration curve.

Scanning electron microscopy

The manure residues were collected after pretreatment at different conditions and washed with distilled water and

vacuum dried. The dry samples both non-treated and chemically treated were coated with gold in a SeeVac Auto conductavac IV sputter coater and scanned by scanning electron microscope (Hitachi S-570 LaB₆, Tokyo, Japan) at accelerating voltage of 10.0 kV (12.7 stub size).

Crystallinity index measurement

Cellulose crystallinity index (CrI) of both treated and non-treated manure fiber was measured by powder X-ray diffraction (PXRD) method using a Bruker D2 Phaser instrument (Bruker AXS Inc., Madison, WI). Dried samples (~0.5 g) were ground to a powder < 100 μm size and pressed into 40-mm diameter pellets. The pellets were measured in Bragg-Brentano geometry using a LynxEye detector with 4° opening. Ni-filtered copper radiation was generated at 30 kV/10 mA, and the pellets were scanned from 5° to 50° by 0.02° steps at 1 s each. The divergence slit was 0.6 mm, and the primary and secondary soller slits were 2.5° and 4°, respectively. TOPAS software version 4.2 was used to calculate the CrI of samples from the ratio of the area of all crystalline peaks to the total area by the deconvolution method [69].

Elemental analyses of manure fiber

Elemental analysis of the manure fiber was carried out by using a wavelength dispersive X-ray fluorescence (WDXRF) spectrophotometer S8 Tiger (Bruker AXS Inc., Madison, WI). About 10 g of an air-dried, non-pretreated PBD manure fiber sample was ground with inert binding material (amyl acetate, 5% collodion) (Bruker AXS GmbH, Germany) at a 5:1 ratio to assist grinding performance, increase pellet stability, and reduce material rewelding in vessel. This was followed by compression pressing of the powder (30 KN/m²) for 15 s in a 40-cm (dia) aluminum cup. The XRF spectrophotometer was equipped with 2 collimators (0.23° and 0.46°) and a set of 6 analyzer crystals (XS-GE-C, XS-CEM, XS-55, PET, LiF200 and LiF220). The measurement method used 27 kV/150 mA excitation for light elements and 60 kV/67 mA excitation for heavy elements using a Rhodium tube. The elemental composition was calculated by using QUANT-EXPRESS calibration (Bruker AXS GmbH, Germany).

Severity parameter and statistical data analysis

The severity parameter (R_0), a factor intended to quantify the energy intensity or severity of a pretreatment strategy, was initially defined by Overend and Chornet (1987) to relate temperature and time for steam explosion pretreated based on the assumption that the pretreatment effect follows first-order kinetics and obeys the Arrhenius equation [70]. Chum et al., (1990) later developed a modified severity parameter to use for sulfuric acid pretreatment that relates concentration with

an introduced arbitrary constant, temperature and residence time as follows [71]:

$$M_0 = t \times C^n \times \exp \left[\frac{(T_r - T_b)}{14.75} \right]$$

Where M_0 is the modified severity parameter; t is the residence time (min); C is the concentration of chemical (wt.%); T_r is the reaction temperature; T_b is the base temperature; n is the arbitrary constant. Silverstein et al. (2007) used the above equation for sodium hydroxide pretreatment by replacing the acid concentration with the alkali concentration [41]. Similarly, modified severity parameters of the alkaline pretreatment using NaOH and/or NH_3 were calculated from the above equation at different n -values obtained by data training while keeping positive log (M_0).

The more detailed relationship between the pretreatment parameters and pretreatment recovery following enzymatic digestion (dependent variables) of the PBD manure fiber was explored using less runs by using central composite design experiments [64,72]. Statistical software SAS JMP Pro version 10 was used for the design of experiments and to analyze the experimental data obtained. 3×3 central composite design experiments comprised a total of 19 runs with different combinations of pretreatment conditions derived by altering the three independent variables, alkali loading both NaOH and NH_3 (2–10% of each loading on dry manure fiber), treatment temperature (80, 100 and 120°C), and residence time (5, 30 and 60 min). The parameter levels were selected based on the previous study. The experimental data obtained were fitted to the following second order polynomial regression equation to identify the key variables:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_i^2 + \sum_{i=1}^n \sum_{j=1}^n \beta_{ij} x_i x_j$$

Where Y is the measured response (carbohydrate recovery, delignification efficiency and subsequent enzymatic digestibility); i, j are the linear and quadratic coefficients respectively; β_0 is the regression coefficient; x is the independent variable (alkali loading, temperature and residence time). The quality of model fit was expressed by the coefficient of determination, R^2 value. Model terms were evaluated based on the probability, p value with 95% confidence level.

Additional file

Additional file 1: Figure S1. Correlation plot of experimental and model predicted values of percent delignification of manure fibers pretreated at: (A) 100°C using combined NH_3 and NaOH, (B) 121°C/15 psi

using combined NH_3 and NaOH, (C) 121°C/15 psi using NaOH, and (D) 121°C/15 psi using NH_3 .

Abbreviations

PBD: Post-biogas digestion; NaOH: Sodium hydroxide; NH_3 : aqueous ammonia; SHAH: Sodium hydroxide and ammonium hydroxide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SE and TR designed the research with SE performing the experiments; SE, AR, TR, and JM analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

We gratefully acknowledge the United States Department of Agriculture-National Institute of Food and Agriculture for its financial support (USDA BRDI Grant number 2012-10006-19423). The authors thank GLBRC UW-Madison (Ruwan Ranatunga) for generously provided a Corn Stover-2010 sample for the comparison study. The authors thank Dharshana Padmakshan and Steven D. Karlen (GLBRC UW-Madison) for providing assistance with lignin monomer detection of manure fiber.

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Received: 22 March 2014 Accepted: 12 May 2014

Published: 20 May 2014

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doi:10.1186/2043-7129-2-12

Cite this article as: Elumalai *et al.*: Combined sodium hydroxide and ammonium hydroxide pretreatment of post-biogas digestion dairy manure fiber for cost effective cellulosic bioethanol production. *Sustainable Chemical Processes* 2014 **2**:12.

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