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Aqueous Ammonia Soaking of Switchgrass Followed by Simultaneous Saccharification and Fermentation

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Abstract Simultaneous saccharification and fermentation (SSF) of switchgrass was performed following aqueous ammonia pretreatment. Switchgrass was soaked in aqueous ammonium hydroxide (30%) with different liquid–solid ratios (5 and 10 ml/g) for either 5 or 10 days. The pretreatment was carried out at atmospheric conditions without agitation. A 40–50% delignification (Klason lignin basis) was achieved, whereas cellulose content remained unchanged and hemicellulose content decreased by approximately 50%. The *Sacccharomyces cerevisiae* (D_5A)-mediated SSF of ammonia-treated switchgrass was investigated at two glucan loadings (3 and 6%) and three enzyme loadings (26, 38.5, and 77 FPU/g cellulose), using Spezyme CP. The percentage of maximum theoretical ethanol yield achieved was 72. Liquid–solid ratio and steeping time affected lignin removal slightly, but did not cause a significant change in overall ethanol conversion yields at sufficiently high enzyme loadings. These results suggest that ammonia steeping may be an effective method of pretreatment for lignocellulosic feedstocks.

Keywords Switchgrass · Pretreatment · Aqueous ammonia soaking · Ethanol production · Simultaneous saccharification and fermentation (SSF)

Introduction

Switchgrass (*Panicum virgatum*) is a warm season, perennial grass that is resistant to harsh conditions, pests, and diseases [1]. It is also capable of producing high biomass yields at low fertilizer application rates [1]. These attributes, along with the environmental benefits

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associated with perennial vegetation, make switchgrass a good candidate for a dedicated energy crop [2]. However, lignin in lignocellulosic feedstocks is known to create obstacles such as inhibition of enzymatic hydrolysis and microbial activity in the ethanol fermentation. Besides the goal of reducing compounds that may inhibit fermentation of sugars to ethanol, pretreatment is also required to either partially remove or break up the lignin structure, so that enzymes can diffuse into the cellulose polymer and degrade it into monomeric fermentable sugars. While a variety of pretreatment methods have been developed and tested at lab-scale [3], pretreatment of biomass remains one of the most costly steps in lignocellulosic biofuels production and affects subsequent operations [4]. For example, improvements in pretreatment can reduce the amount of enzymes (e.g., cellulases) used [4]. Teymouri et al. [5] indicated effective enzymatic hydrolysis of ammonia fiber explosion (AFEX)-treated biomass at enzyme loadings as low as 7 FPU/g of glucan could be achieved by adjusting the pretreatment parameters. Kim et al. [6] reported the enzymatic digestibility of corn stover treated by the ammonia recycled percolation to be 90% with an enzyme loading of 10 FPU/g–glucan. Although many biological, chemical, and physical methods have been attempted over the years, further development of pretreatment methods is needed to reduce overall costs of lignocellulosic bioconversion [7]. Dilute acid treatment, water pretreatment with pH control, AFEX, ammonia recycle percolation (ARP), and lime pretreatment are among the most promising and most studied technologies [3]. Many of these pretreatment methods, however, require high temperature and/or high pressure. The extreme conditions used to increase the digestibility of the biomass decrease the reaction time required for the pretreatment, but they increase capital and operating costs. Extreme conditions may also cause the formation of compounds that are inhibitory to the fermentative organisms, and they may cause degradation of some fraction of the fermentation substrate. For these reasons, ambient temperature and pressure pretreatments are of interest.

Removing lignin with alkaline chemicals to improve cellulose digestibility and ammonia steeping/soaking at room temperature has been previously studied on several types of biomass [8–10]. The steeping method is a simple method that does not require high pressures and high temperatures. Ammonia soaking of corn stover at room temperature can remove as much as 74% of the lignin, but retain nearly 100% of the glucan and 85% of the xylan [8].

Currently, simultaneous saccharification and fermentation (SSF) is one of the most commonly used processes for ethanol production [5, 6, 8, 11–15]. This process combines two steps in the same vessel to generate ethanol: enzymatic break down of the complex sugars into glucose and fermentation of the glucose into ethanol by yeast. This process has been widely adopted because of the reduction of glucose inhibition during enzymatic hydrolysis. In addition, the risk of bacterial contamination and capital investments are lower, as both the hydrolysis and the fermentation steps take place in the same reactor. Although SSF of switchgrass has been studied after numerous types of pretreatment [11–13, 16], the effect of aqueous ammonia soaking at room temperature and atmospheric pressure on ethanol yield of switchgrass has not been reported. The objectives of this study were to determine the effect of soaking time and liquid–solid ratios on the composition of ammonia-steeped switchgrass, and on ethanol production from SSF of ammonia-steeped switchgrass.

Materials and Methods

Switchgrass samples were collected from mature stands of the Cave-in-Rock cultivar while dormant (early spring) in Chariton, IA. Dry switchgrass was ground to a size of 5–6 mm by

the Biomass Energy Conversion Center (BECON), Nevada, IA. The composition of the switchgrass except for Klason lignin was determined by Iowa State University, Department of Agronomy, using the Ankom method (Ankom Technology, Fairport, NY) as described by Vogel et al. [17]. Untreated switchgrass contained 42% cellulose, 31% hemicellulose, 6% acid detergent lignin (ADL), 22% Klason lignin and 0.7% ash.

Cellulase enzyme (Spezyme CP, lot no. 301-05021-011) was provided by Genencor International (Palo Alto, CA) and had an activity of 77 filter paper units (FPU)/ml, measured using standard procedures [18]. The yeast (*Sacccharomyces cerevisiae* D_5A) was supplied by the National Renewable Energy Laboratory and preserved at 4°C after freeze drying with 20% skim milk.

Forty grams of dry switchgrass was soaked in reagent—grade 29.5 wt% aqueous ammonium hydroxide (Fisher Scientific) in 1 l high density polyethylene bottles at room temperature without any agitation. Two different aqueous ammonia loading rates (5 and 10 ml/g) were applied for both 5 and 10 days. Each treatment was performed in duplicates. At the end of the pretreatment, the biomass was washed in the same bottles with 20 l DI water using a customized fluidized bed-biomass washing system (Fig. 1). For treated fiber washing, deionized water was supplied from the bottom of the bottle that was placed on top of a magnetic stir plate, and the rinsate was collected from the top of the bottle. A metal screen and glass wool was used to keep the biomass inside the bottle during washing. This system allowed homogeneous continuous rinsing of the biomass in situ to minimize reactor handling.

The pretreated samples were then analyzed for cellulose, hemicellulose (using Ankom method), and for Klason lignin. The biomass was dried completely for Klason lignin analysis, which was performed following Crawford and Pometto [19] with a slight modification, namely that glass fiber filters (Fisherbrand, G6, 1.6 μm) were used for capturing lignin residues instead of Whatman #1 filter papers. Employing the glass fiber

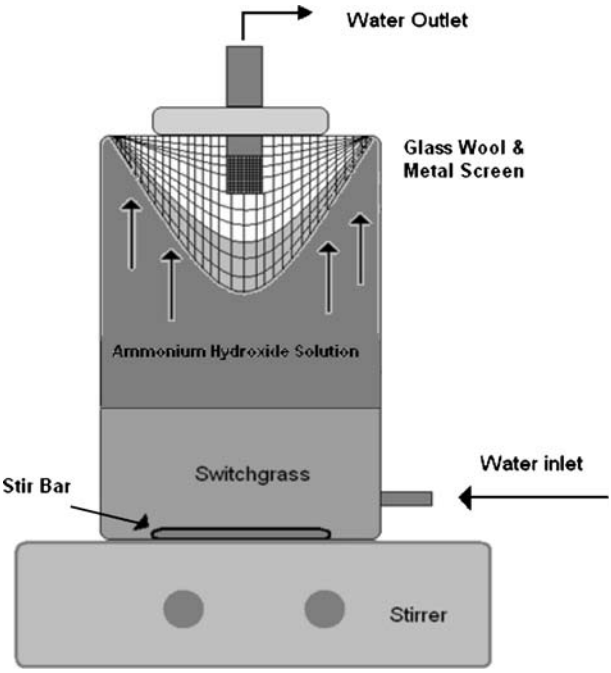

Fig. 1 Fluidized bed biomass washing set-up

filters avoided errors because of the rapid adsorption of atmospheric humidity onto the dry filter papers.

The wet biomass obtained from the washing system was used for SSF experiments following established procedures [20]. Specifically, 250-ml Erlenmeyer flasks were used for fermentation with 100-ml working volume. Two cellulose loadings (3 and 6%) and three enzyme loadings (26, 38, and 77 FPU/ml) were evaluated on ammonia-treated switchgrass. The switchgrass after the pretreatment contained 56.6% cellulose with 80% wet basis moisture content. Example cellulose loading calculation for one flask [20]:

$$\begin{aligned}&26.6\text{ g wet pretreated biomass} \times 20\ \%\text{ solid content} \times\ 56.6\ \%\text{ cellulose content}\\&= 3.01\text{ g cellulose in 100 ml working volume fermentation flask}\end{aligned}$$

The fermentation media contained 1% *w*/*v* yeast extract, 2% *w*/*v* peptone and 0.05 M citrate buffer (pH 4.8). Yeast-free saccharification flasks were run alongside each fermentation flasks to monitor sugar production in the absence of fermentative organisms. Samples were analyzed for sugars (cellobiose, glucose, and xylose) and ethanol by high-performance liquid chromatography (HPLC; Varian ProStar 210, MetaCarb 87P column with mobile phase of water, flow rate of 0.4 ml/min, column temperature of 80°C, and injection volume of 20 μl) with a refractive index detector. Total sugars and reducing sugars in both saccharification and fermentation flasks were determined by using phenol-sulfuric [21] and dinitrosalicylic acid (DNS) methods [22], respectively. Water-soaked switchgrass and α-cellulose were fermented using the same procedure and reported as control and reference, respectively. All of the experiments are performed in duplicate (n=2).

Theoretical ethanol yields were calculated as follows considering the maximum (51%) conversion of glucose into ethanol by yeast [8].

$$\text{Theoretical ethanol yield }(\%) = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial sugar (cellulose) (g) in reactor} \times 0.511} \times 100$$

Results and Discussion

Ammonia steeping proved to be an effective method for removing Klason lignin, preserving the cellulose fraction and enhancing the subsequent SSF of switchgrass. Figure 2 illustrates the effect of different treatments on the recovery of sugars and total dry biomass. Almost all the cellulose was retained, whereas nearly half of the hemicellulose was removed with ammonia treatment. More than 75% of the original dry biomass was collected after soaking. Removing hemicellulose has sometimes been considered a desired characteristic of a biomass pretreatment because this reduces inhibitory compounds such as furfural generated from hemicellulose degradation via dilute acid treatment at high temperature and pressures [23] and some process designs have included fermentation of hexose and pentose sugars in separate reactors. However, the development of genetically modified microorganism capable of fermenting both pentose and hexose sugars offers the advantage of greater ethanol yields [24, 25] and lower capital cost. Therefore, the feasibility of capturing the rinsate pentoses should be determined in future studies. In contrast to the 50% removal of hemicellulose that we observed, Kim and Lee [8] observed around 15% xylan reduction after 10 days of aqueous ammonia soaking of corn stover with 12 ml/g loading (they did not report hemicellulose reductions specifically). The higher reduction in

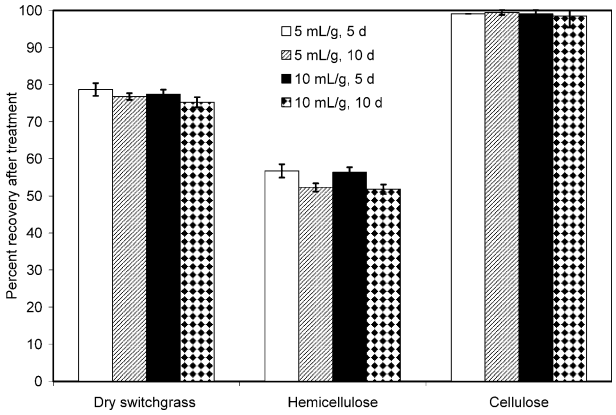

Fig. 2 Effect of different aqueous ammonium hydroxide loadings (ml/g of ground switchgrass) and soaking time on the recovery of dry switchgrass, hemicellulose, and cellulose (n=2)

pentose polymers in our study could be due to the thorough washing of treated biomass employed to ensure neutral pH before fermentation; the degree of washing performed by Kim and Lee was not reported. However, the difference might also reflect fundamental cell wall differences between corn stover and switchgrass.

Estimates of lignin content can vary greatly between different procedures [26]. For example, although ADL and Klason lignin are both common for determination of forage lignin content, Klason lignin values are generally two to four times greater than ADL estimates for grasses [26]. This study used Klason lignin values to be consistent with previous studies [8, 10, 14, 15].

The influence of different treatment conditions on Klason lignin is presented in Fig. 3. As anticipated, more lignin was removed with higher aqueous ammonia loadings and longer treatment. The highest delignification (47%) was achieved with ammonium hydroxide soaking for 10 days at 10 ml/g biomass. These results are consistent with Kim and Lee [8] who report approximately 50% lignin removal from corn stover in 4 days with loading of 12 ml/g ground corn-stover. Cao et al. [10] report that between 80–90% lignin can be removed from corn cobs in 24 h with an ammonium steeping ratio of 5 ml/g at 26°C. Higher delignification with lower pretreatment duration in that case could be caused by structural differences between corn cob and corn stover. Chang et al. [14] reported approximately 30% lignin solubilization after lime treatment of switchgrass. Kim and Lee

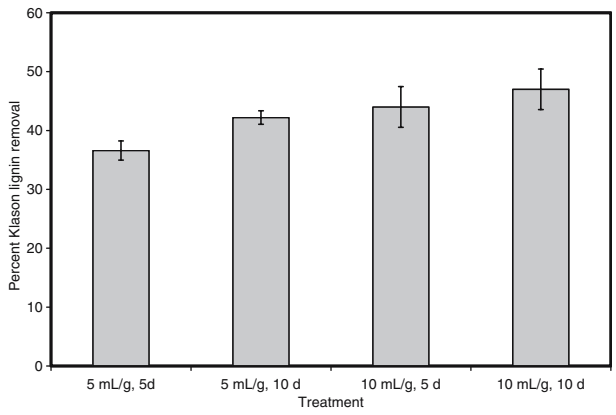

Fig. 3 Percent klason lignin removal with different aqueous ammonium hydroxide loadings (ml/g ground switchgrass) and soaking time (n=2)

[15] have also reported that the ARP process can remove up to 85% of lignin from corn stover.

The SSF of aqueous ammonia pretreated switchgrass followed a classical SSF production curve in which ethanol concentration increased with time, whereas glucose and cellobiose concentrations increased initially then decreased once the yeast began consuming sugars at higher rates (Fig. 4). The accumulation of xylose indicated the presence of hemicellulases in our enzyme complex and could potentially inhibit ethanol fermentation [8]. The glucose concentrations were generally higher than cellobiose concentrations throughout the fermentations, indicating the effective conversion of cellobiose into glucose by β-glucosidase in the enzyme solution. The ethanol concentration remained relatively constant after 24 h, but to ensure fermentation was completed, the 96-h data are presented in subsequent figures.

Two extreme ammonium pretreatment conditions were selected (5 days with 5 ml/g and 10 days with 10 ml/g switchgrass) to explore the effect of steeping time and loading rate on sugar release and ethanol production (Fig. 5). The lower residual sugar concentrations observed in the fermentation flasks (compared to saccharification flasks) suggested that the enzymes and yeast were metabolizing sugars. As expected, higher aqueous ammonium hydroxide loadings and longer soaking times led to greater sugar release in subsequent saccharification tests. This implied that higher ethanol concentrations could be achieved by these more aggressive conditions, and this is borne out in the data presented in Fig. 6, which illustrates the final ethanol concentrations achieved through SSF of pretreated switchgrass. The highest ethanol concentration (22 g/l) was observed in flasks fermenting switchgrass treated for 10 days at 10 ml/g ground switchgrass, using a glucan loading rate of 6% and an enzyme loading rate of 38.5 FPU/g cellulose. This also corresponded to the treatment showing the highest sugar release (Fig. 5, T4). Pretreatment loading rates and durations had no significant effect on ethanol production at high enzyme loadings (Fig. 6, T1 and T2). However, at medium and low enzyme loadings (38.5 and 26 FPU/g cellulose, respectively), final ethanol concentrations were sensitive to pretreatment conditions in the range studied. Specifically, final ethanol concentrations increased approximately up to 40% when the pretreatment went from 5 days with 5 ml/g loading to 10 days with 10 ml/g aqueous ammonia loading (Fig. 6, T3 vs T4, and T5 vs T6). Doubling the cellulose loading while halving the enzyme loadings resulted in a doubling of ethanol concentrations at 96 h (Fig. 6, T1 vs T3, and T2 vs T4). This suggests effective conversions at higher cellulose concentrations with medium enzyme loadings. The trade-offs among pretreatment intensity,

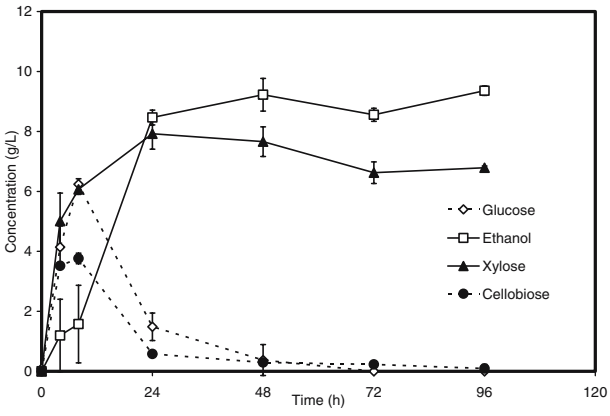

Fig. 4 Time courses of sugars and ethanol concentrations for SSF of aqueous ammonium hydroxide steeped switchgrass (5 ml/g, 5 days, 3% cellulose, 77 FPU/g cellulose; n=2)

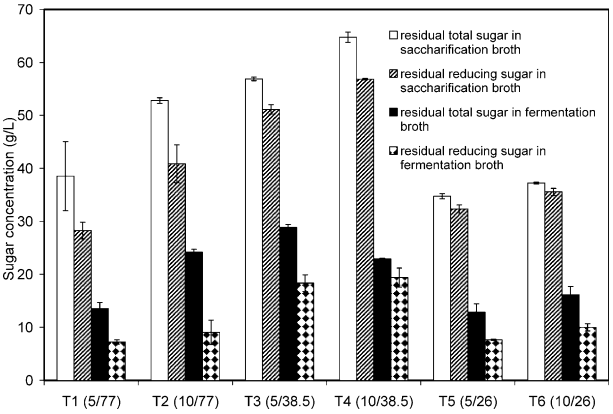

Fig. 5 Effect of aqueous ammonium hydroxide loading and soaking time on sugar release at 96 h. Saccharification and fermentation flasks had two cellulose loadings (3 and 6%) and three enzyme loadings (26, 38.5, and 77 FPU/g cellulose). *T1 (5/77)* Treatment 1, 5 ml/g, 5 days, 3% cellulose, 77 FPU/g cellulose; *T2 (10/77)* treatment 2, 10 ml/g, 10 days, 3% cellulose, 77 FPU/g cellulose; *T3 (5/38.5)* treatment 3, 5 ml/g, 5 days, 6% cellulose, 38.5 FPU/g cellulose; *T4 (10/38.5)* treatment 4, 10 ml/g, 10 days, 6% cellulose, 38.5 FPU/g cellulose; *T5 (5/26)* treatment 5, 5 ml/g, 5 days, 3% cellulose, 26 FPU/g cellulose; *T6 (10/26)* treatment 6, 10 ml/g, 10 days, 3% cellulose, 26 FPU/g cellulose

enzyme loadings, and cellulose loadings need to be addressed in detail before scale-up of the aqueous ammonia steeping procedure.

Three percent α-cellulose fermentation with high enzyme loading (77 FPU/g cellulose) was performed as a reference. At equal glucan loadings, α-cellulose yielded approximately 25% more ethanol compared to aqueous ammonia pretreated switchgrass (Fig. 6, REF vs T1 and T2). The lower ethanol productions from switchgrass fermentation were likely

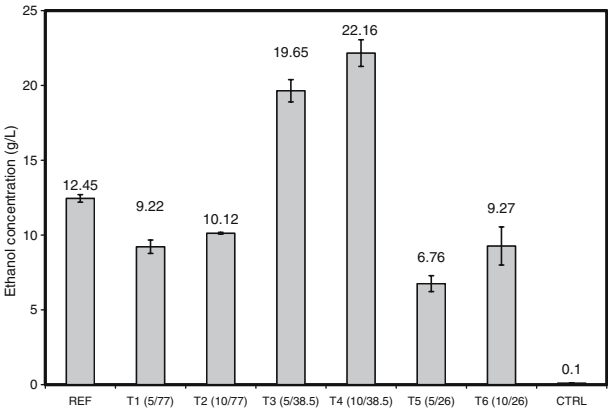

Fig. 6 Ethanol concentrations in different fermentation flasks at 96 h. *REF* Reference, 3% α-cellulose fermentation, 77 FPU/g cellulose; *T1 (5/77)* treatment 1, 5 ml/g, 5 d, 3% cellulose, 77 FPU/g cellulose; *T2 (10/77)* treatment 2, 10 ml/g, 10 days, 3% cellulose, 77 FPU/g cellulose; *T3 (5/38.5)* treatment 3, 5 ml/g, 5 days, 6% cellulose, 38.5 FPU/g cellulose; *T4 (10/38.5)* treatment 4, 10 ml/g, 10 days, 6% cellulose, 38.5 FPU/g cellulose; *T5 (5/26)* treatment 5, 5 ml/g, 5 days, 3% cellulose, 26 FPU/g cellulose; *T6 (10/26)* treatment 6, 10 ml/g, 10 days, 3% cellulose, 26 FPU/g cellulose; *CTRL* control, water soaked switchgrass fermentation, 5 ml/g, 5 days, 3% cellulose, 77 FPU/g cellulose

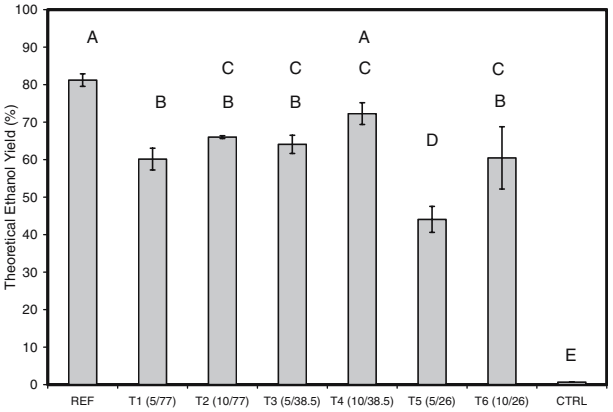

Fig. 7 Theoretical ethanol yields of different fermentations. *Letters on top of the columns* indicate significant differences (Tukey test, α=0.05). *REF* Reference, 3% α-cellulose fermentation, 77 FPU/g cellulose; *T1 (5/77)* treatment 1, 5 ml/g, 5 days, 3% cellulose, 77 FPU/g cellulose; *T2 (10/77)* treatment 2, 10 ml/g, 10 days, 3% cellulose, 77 FPU/g cellulose; *T3 (5/38.5)* treatment 3, 5 ml/g, 5 days, 6% cellulose, 38.5 FPU/g cellulose; *T4 (10/38.5)* treatment 4, 10 ml/g, 10 days, 6% cellulose, 38.5 FPU/g cellulose; *T5 (5/26)* treatment 5, 5 ml/g, 5 days, 3% cellulose, 26 FPU/g cellulose; *T6 (10/26)* treatment 6, 10 ml/g, 10 days, 3% cellulose, 26 FPU/g cellulose; *CTRL* control, water soaked switchgrass fermentation, 5 ml/g, 5 days, 3% cellulose, 77 FPU/g cellulose

because of the accumulation of inhibitory compounds such as xylose [8]. Fermentation of water-soaked switchgrass was attempted as a negative control, and no ethanol production was observed, illustrating the critical role of aqueous ammonia soaking in overcoming the recalcitrance of switchgrass.

The percent of maximum theoretical ethanol yield achieved was computed for each of the SSF treatments using the conversion rate of 51 g ethanol per 100 g cellulose. Results ranged from 72% for 10 days with the 10 ml/g treatments (Fig. 7, T4) to 44% for 5 days with 5 ml/g and low enzyme case (Fig. 7, T5). The average percent of maximum theoretical ethanol yield achieved was 60. Kim and Lee [8] reported 73% of maximum theoretical ethanol yield achieved after fermenting corn stover pretreated in aqueous ammonium hydroxide at 8 ml/g for 10 days. No statistically significant differences in percent of maximum theoretical ethanol yield achieved were observed between low, medium, and high enzyme loadings at high liquid–solid ratios and soaking times (Fig. 7, T2, T4, and T6). In contrast, 5 days with 5 ml/g with the lowest enzyme loading had a statistically significantly lower ethanol yield (Fig. 7, T5). This reinforces the interrelationship between enzyme requirements and pretreatment intensity discussed earlier.

Conclusion

Aqueous ammonia soaking at room temperature and atmospheric pressure is an effective pretreatment method for switchgrass before SSF for ethanol production. The percent of maximum theoretical ethanol yield achieved by this method was as high as 72%, and this result reflected minimal optimization of the process. At high enzyme loadings, ethanol production was not greatly influenced by soaking time and liquid–solid ratio. However, at low enzyme loadings, significant increases in ethanol production were observed for the

samples pretreated with higher intensity. The interrelationship between pretreatment conditions and enzyme requirements should be an area of further study and optimization.

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