Improvement of the Hydrolysis and Fermentation of Rice Straw by *Saccharomyces cerevisiae* by Ammonia-Based Pretreatments

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This work aimed at improving the hydrolysis and fermentation processes of rice straw through different ammonia-based pretreatments to aid in bioethanol production. For this purpose, pretreatment was performed at 70 °C for 12 h, followed by enzymatic hydrolysis at 50 °C for 24 h and 72 h using 15 FPU cellulase and 30 CBU cellobiase. The best hydrolysis yield, based on the production yield and rate, for the 24-h digestion period was samples that had been soaked in methanolic aqueous ammonia (SMAA), with 72% of the theoretical maximum. However, for the 72-h digestion period, soaking in ethanolic aqueous ammonia (SEAA) was the best method, with 88% yield. In the case of ethanol production after 24 h, the SMAA pretreatment and SSF resulted in the highest yield at 72%. However, after 72 h of simultaneous saccharification and fermentation (SSF), SMAA-pretreated rice straw showed a yield of 85%, while the SEAA-pretreated sample resulted in a noteworthy yield of 89% of the theoretical maximum. However, with regard to the production yield and rate and pretreatment cost, the best method for ethanol production was judged to be the SMAA with 5% methanol, particularly after 24 h of SSF.

Keywords: Rice straw; Ethanolic ammonia pretreatment; Methanolic ammonia pretreatment; Ethanol; SSF; Saccharomyces cerevisiae

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INTRODUCTION

The progressive depletion, increasing demand and price, and prevalent environmental issues of petroleum have caused many to consider ethanol as an efficacious alternative to gasoline as a fuel, as well as an additive to petro-fuels, due to the fact that it can be produced from the bioconversion of sustainable resources (Campbell and Laherrère 1998; Dewulf and Van Langenhove 2006; Drapcho *et al*. 2008; Demirbas 2009).

Biomass, particularly agricultural waste, is a promising feedstock for biofuel production, and rice straw is considered the most abundant agricultural waste in the world. Moreover, it has mostly been considered unusable and is usually burned in agricultural fields (Ko *et al*. 2009; Binod *et al*. 2010; Poornejad *et al*. 2013).This substance contains a significant amount of cellulose and hemicellulose that, through the use of appropriate enzymes, can be converted to fermentable sugars. However, when only cellulolytic enzymes are used, even in high amounts, the recalcitrance of the native rice straw structure acts as a hindrance to achieving a high productivity in hydrolysis processes. Hence, pretreating in order to lessen this hindrance and gain a higher yield seems indispensable (Karimi *et al.* 2006; Ko *et al.* 2009; Binod *et al*. 2010).

Ammonia pretreatments, such as soaking in aqueous ammonia (SAA) or ethanolic ammonia (SEAA), are alkaline treatments that result in the delignification of biomass. They are highly effective for substrates with low lignin content, such as bagasse and rice straw (Kim *et al.* 2009a; Hedayatkhah *et al.* 2013). Methanol and ethanol could also bring about delignification, but a higher temperature is needed, which requires higher energy input. Aqueous ammonia pretreatment might give lower enzymatic hydrolysis efficiency, so this method was investigated in this study. SAA is an encouraging method for lignocellulosic pretreatment, yet, in comparison with other methods such as dilute acid treatment, it has been less investigated and developed.

It is possible to modify SAA pretreatment by its supplementation with other chemicals, such as ethanol or methanol (Kim *et al*. 2009a; Hedayatkhah*et al.* 2013). Methanol is a delignifying agent by itself, but, except for the previous work on bagasse by Hedayatkhah *et al*. (2013), there are few studies on the supplementation of methanol in ammonia pretreatment. Moreover, ethanolic ammonia has only been applied to corn stover (Kim *et al*. 2009), and there have been no studies conducted regarding its effect on rice straw. This article followed two previous studies conducted on the degradation and bioconversion of cellulose (Pourramezan *et al*. 2012; Hedayatkhah *et al.* 2013) and aimed to improve the enzymatic hydrolysis, simultaneous saccharification, and fermentation of rice straw using an aqueous ammonia pretreatment and compare the given method with enhanced SAA methods, including ethanolic SAA (SEAA) and methanolic SAA (SMAA) pretreatments. The reason behind these improvements was also investigated by analyzing the composition and structure of the treated and untreated substrates.

EXPERIMENTAL

Rice Straw

Rice straw was obtained from Yasuj farm lands (Yasuj, Iran). The air-dried straw was ground and subsequently screened by sieves of 10 to 35 mesh size (Hames *et al.* 2008). In order to estimate the cellulose, hemicellulose, and lignin content of the substrates, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were measured by a standard fiber analysis procedure described in previous literature (Banchorndhevakul 2002; Sulbarán-De-Ferrer *et al.* 2003). Briefly, NDF, ADF, and ADL analyses represent cellulose, hemicellulose, and acid soluble lignin levels of rice straw. The cellulose, hemicellulose, and lignin of each sample were related to the NDF, ADF, and ADL analyses based on summation relations:

- ADL = lignin (Only the acid soluble part of total lignin)
- ADF = lignin + cellulose
- NDF = lignin+ cellulose + hemicellulose.

The analysis showed that the rice straw contained 41.4% cellulose, 30.6% hemicellulose, and 3.8% acid-soluble lignin.

Pretreatment

Ten grams of rice straw (dry weight) were transferred to 500 mL pretreatment bottles. All pretreatment solutions were provided in a solid-to-liquid (S: L) ratio of 1:6. An aqueous ammonia solution was prepared at 15% (v/v) ammonia (Ko *et al.* 2009). Methanolic pretreatment was performed using only absolute methanol. The ethanolic ammonia (SEAA) and methanolic ammonia solutions (SMAA) were prepared according to the proportions given in Table 1. Pretreatment conditions were adjusted to 70 °C, without any agitation, for 12 h (Kim *et al*. 2009a,b; Ko *et al*. 2009; Hedayatkhah *et al.* 2013). After incubation, the slurry was separated, and the solid portion was washed with 2 L of distilled water and stored at 4 °C for further use.

Enzyme Digestibility Test

Pretreated and untreated substrates (1.75 g dry weight at 105 °C) were transferred to 250 mL Erlenmeyer flasks. Cellulase at 15 filter paper unit (FPU) (Sigma-C2730) and cellobiase at 30 cellobiase unit (CBU) (Sigma-C6105), supplemented with 42 mgL^{-1} of tetracycline and 30 mgL^{-1} of cyclohexamide (to avoid contamination), were loaded and subsequently adjusted to a volume of 100 mL by citrate buffer (50 mM, pH 4.8). All flasks were incubated at 50 °C and 150 rpm for 24 h and 72 h. The released glucose was measured by way of a glucose oxidase reagent (GOD) (Selig *et al*. 2008).

Samples of 1 g of Avicel and 3 g of untreated rice straw were also tested as references. Using the enzymatic hydrolysis results, the best pretreatment cases, based on the productivity yield, were chosen, and their compositional analyses (NDF, ADF and ADL) were investigated.

Inoculum Preparation

Saccharomyces cerevisiae (ATCC 96581) was cultivated on yeast extract, peptone, dextrose (YPD) agar plates (1% yeast extract, 2% peptone, and 2% dextrose and agar). This cultivar was incubated at 37 °C for 24 h, and single colonies were selected to inoculate YPD broth flasks (100 mL, 1% yeast extract, 2% peptone, and 5% dextrose). After an incubation of 13 ±1 h, the broth was centrifuged at 4500 rpm for 5 min, and the supernatant was removed. The pellets were then re-suspended in 5 mL of a 50-mM sterile citrate buffer (pH 4.8) under aseptic conditions. The harvested cells were used as inoculum in SSF tests (Dowe and McMillan 2001).

Simultaneous Saccharification and Fermentation

Avicel and treated and untreated substrates were simultaneously saccharified and fermented by cellulase, cellobiase, and *S. cerevisiae* (ATCC 96581), according to the standardized procedure, under anaerobic conditions at 37 °C for 24 h and 72 h at 150 rpm (Dowe and McMillan 2001). For each previously selected pretreated substrate, 5 g of the sample was transferred to 500-mL bubble traps and sterilized at 121°C for 15 min. Afterwards, 15 FPU of cellulase, 30 CBU of cellobiose, and *S. cerevisiae* inoculation were added to each bottle, and the flasks were adjusted to a volume of 100 mL by citrate buffer (50 mM, pH 4.8). The obtained samples after 72 h of the SSF process were centrifuged at 13000 rpm for 20 min (Dowe and McMillan 2001). Samples of 5 g of untreated rice straw and 3 g of Avicel were also tested as references.

Analytical Methods

The glucose quantities in the hydrolysates were estimated using a glucose oxidase reagent (Selig *et al*. 2008). A Fourier transform infrared (FTIR) spectrometer equipped with a universal attenuated total reflection (ATR) accessory and a deuterated triglycine sulfate (DTGS) detector was used to determine the crystallinity index (CI) and the alterations in the substrate structure before and after pretreatment. Based on the enzymatic hydrolysis results, the best modes of pretreatment were selected for the FTIR analysis, and their spectra were obtained in a range of 600 to 4000 cm^{-1} (Alemdar and Sain 2008; Gupta 2008; Nieves *et al*. 2011; Poornejad *et al*. 2013).

To estimate the cellulose, hemicellulose, and lignin contents of the untreated and pretreated rice straw, all samples were subjected to a standard method of fiber analysis to measure neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) (Goering and Van Soest 1970; Banchorndhevakul 2002; Sulbarán-De-Ferrer *et al.* 2003).

The ethanol levels after the SSF process were determined by HPLC using an Aminex HPX-87H column (Bio-Rad, USA) at 60 °C with 0.6 mL min^{-1} of 5 mM sulfuric acid as an eluent (Dowe and McMillan 2001; Li *et al*. 2010).

The ethanol yields were calculated according to the following equation (Eq. 1).

$$Ethanol\ yield\ \% = \frac{\text{Ethanol produced (g)}}{\text{Initial glucan in the sample (g)}\times 0.511} \times 100\ (\%) \tag{1}$$

RESULTS AND DISCUSSION

Effect of Pretreatment on Enzymatic Hydrolysis

To determine the effect of pretreatment on rice straw, liquid samples were taken periodically. All the tests were conducted in triplicate, and the mean of the three samples was reported (Table 1). In addition, Avicel and untreated rice straw were tested as references. Table 1 shows that when 1 g of Avicel was hydrolyzed, 7.4 g L^{-1} and 8.9 g L^{-1} of glucose were released after 24 h and 72 h of digestion, which represented 67% and 80% of the maximum theoretical yield, respectively.

Table 1. Digestion Test Results for SAA and SEAA Pretreatments

Substrate	Pretreatment Type	Amount of Released Glucose(g/L)		Glucose Production Yield (%)	
		24 h	72 h	after 24 h	after 72 h
Rice straw	-	1.2	2.0	15	25
Rice straw	SAA	7.6	9.4	68	84
Rice straw	SEAA(20%E*+15%A**)	8.2	10.3	70	88
Rice straw	5%M*+15%A**	8.7	9.5	72	79
Rice straw	15%M+15%A	8.6	9.2	-	-
Rice straw	25%M+15%A	8.0	9.1	-	-
Rice straw	50%M+12.5%A	7.9	8.7	-	-
Rice straw	75%M+6.25%A	5.8	7.2	-	-
Rice straw	85%M+3.75%A	4.4	7.0	-	-
Rice straw	95%M+1.25%A	2.9	4.1	-	-
Rice straw	100%M+0%A	2.0	2.9	-	-
Avicel	-	7.4	8.9	67	80

*x%E/M= The percentage of methanol or ethanol in solution
**y%A= The percentage of ammonia in solution

After 24 h and 72 h of digestion of the untreated rice straw, only 1.2 g L^{-1} and 2.0 g L^{-1} of glucose were released, respectively, equal to 15% and 25% of the maximum theoretical yield. This result implied that untreated rice straw is not an adequate feedstock for bioethanol production.

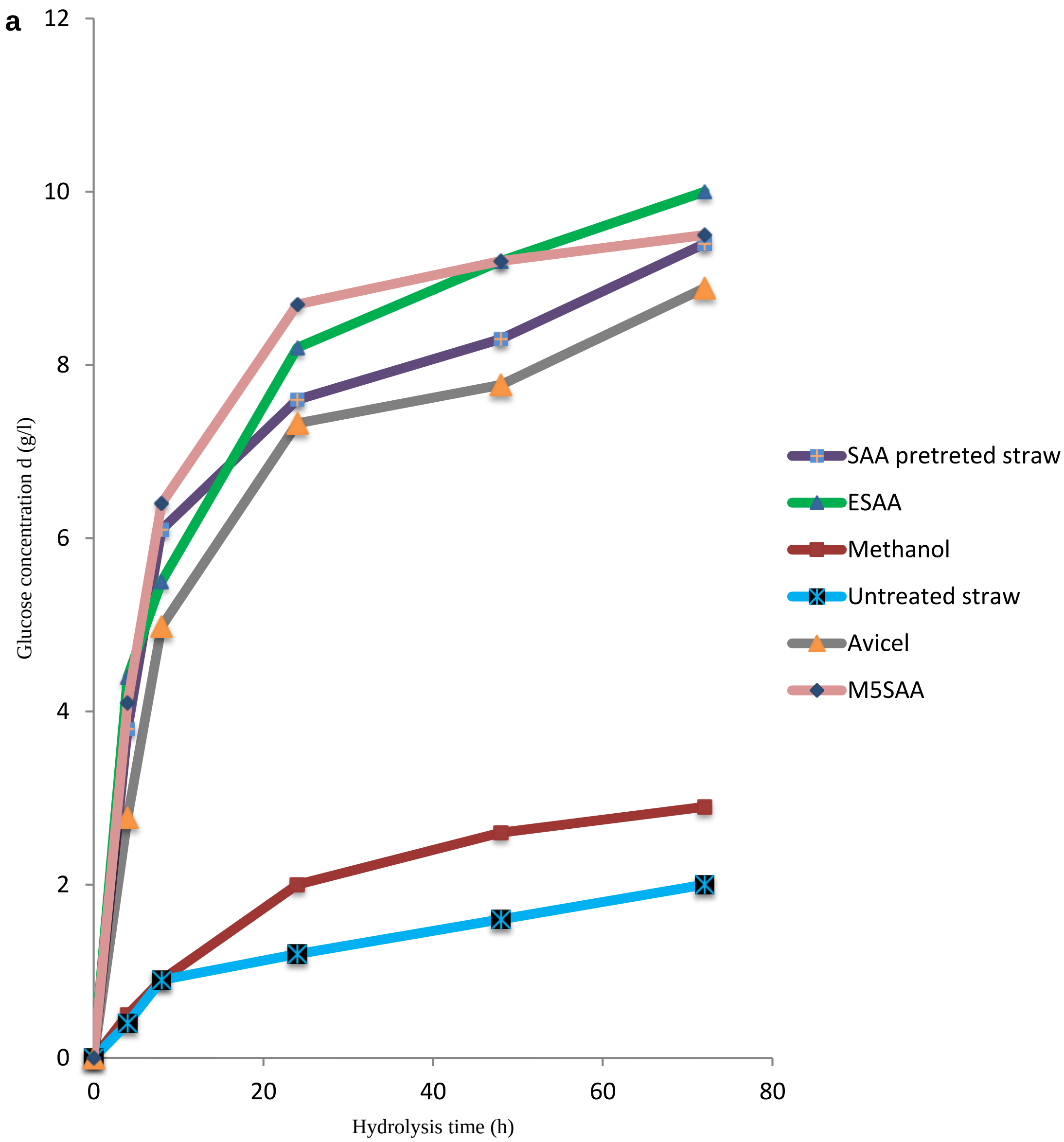

Fig. 1. A: Enzymatic hydrolysis of untreated rice straw, Avicel, SAA-pretreated rice straw, SEAA-pretreated rice straw, SMAA-pretreated (at 5% methanol) rice straw and methanol pretreated rice straw.
M*SAA: soaking in methanolic aqueous ammonia (SMAA) with different shares of methanol*; e.g.,* M25SAA= SMAA pretreatment with 25% methanol.

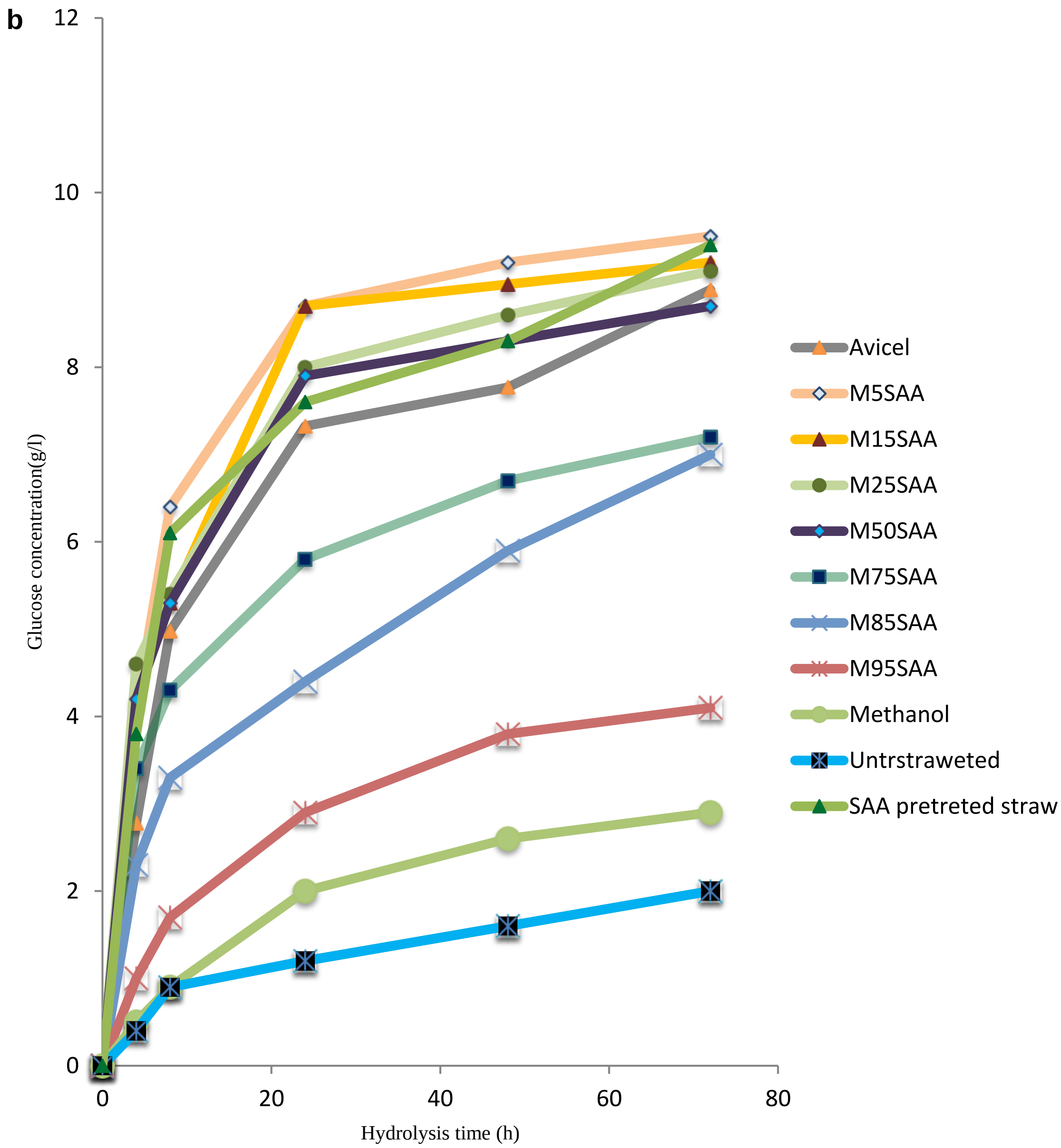

Fig. 1. B: Glucose yield following pretreatment of rice straw with different concentrations of methanol (from 5% to 95%), untreated rice straw, Avicel, and methanol-pretreated rice straw. M*SAA: soaking in methanolic aqueous ammonia (SMAA) with different shares of methanol; *e.g.,* M25SAA= SMAA pretreatment with 25% methanol.

With methanolic pretreatment, the hydrolysis yields after 24 h and 72 h of digestion were 2.0 g L^{-1} and 2.9 g L^{-1} of glucose, respectively. Methanol is a delignification agent, but this test revealed that it did not have any significant effects on the digestibility yield at moderate temperature and only resulted in a 0.9 gL^{-1} increase in hydrolysis yield compared to untreated straw.

Using SAA pretreatment, the released glucose was increased to 7.6 g L^{-1} and 9.4 gL^{-1} after 24 h and 72 h of hydrolysis, respectively, equal to 68% and 84% of the maximum theoretical yield. Compared to untreated rice straw, SAA-pretreated rice straw resulted in close to 4 and 3.5 times the growth in enzymatic digestibility yield, respectively, after 24 h and 72 h of digestion. Furthermore, in the case of untreated rice straw, the production rate was the slowest, and Avicel showed a desirable production rate; applying the SAA pretreatment to rice straw led to a higher production rate at all sampling points compared to the untreated rice straw and even the Avicel (Fig.1 a).

When ammonia solution was blended with methanol (SMAA), different results were observed. Table 1 indicates that in the case of SMAA, when 5% methanol and 15% ammonia were mixed, (M5SAA) 8.7 g $L^{-1}$and 9.5 g L^{-1} of glucose were produced after 24 h and 72 h of hydrolysis, respectively, equal to 72% and 79% of the maximum theoretical yield. The production yield of M5SAA after 24 h of hydrolysis was slightly higher than that of SAA, though it became negligibly lower after 72 h of digestion. However, supplementation of SAA with 5% methanol resulted in a higher digestion rate that was obvious at all sampling points, particularly after 24 h of digestion, an observation that may warrant further analysis.

When 15% methanol was blended with ammonia (M15SAA), the amount of released glucose was decreased to 9.2 g L^{-1} compared to M5SAA. Although the production yields after 72h was lower than with SAA, in all previous sampling points the hydrolysis rate of M15SAA was higher than that of SAA. This trend was also observed with some other SMAA solutions (25% and 50% methanol) (Fig. 1 b). The final values of released glucose after 72 h of digestion were 9.2 g L^{-1} and 9.1 g L^{-1} for M25SAA and M50SAA, respectively.

From the obtained results, two important facts were observed about SMAA pretreatment. It can be concluded that as the methanol portion of the pretreatment solution increased, the production yield slightly decreased. When, in addition to an increase in the methanol portion of the pretreatment solution, the ammonia portion was reduced, the production yield decreased at a higher rate. However, a previous study showed that, in the case of bagasse, the production yield from the M15SAA pretreatment (S: L ratio of 1:6) increased compared to the M5SAA pretreatment, indicating that higher concentrations of methanol led to higher yield. However, higher methanol concentrations (25 and 35% methanol) led to a reduction in the production yield. In another case (S:L ratio of 1:10), the maximum production yield was obtained using M25SAA pretreatment, followed by M15SAA, then M5SAA; however, with M35SAA (35% methanol), the glucose production yield fell sharply to the lowest value. This phenomenon was attributed to the fact that methanol, at low concentrations, led to the facile accessibility of ammonia to the lignin portion of the substrate, while at higher values of methanol the desirable effects of ammonia were obstructed by the competitive inhibitory effect of methanol on ammonia (Hedayatkhah *et al*. 2013). In other words, a low concentration of methanol had a synergistic effect on ammonia, but methanol competes with ammonia in delignification. Thus, at higher concentrations of methanol, the competitive inhibitory effect surpassed the synergistic effect and resulted in a decrease in the delignification effect of ammonia.

Table 1 displays the results using the other SMAA solutions; as the proportion of ammonia decreased in the blend, the hydrolysis yield and rate were reduced, though they were still greater than those of untreated rice straw. The minimum production yield was observed for M95SAA, which had the lowest aliquot of ammonia (1.25%), producing 2.9 g L^{-1} and 4.1 g L^{-1} of glucose after 24 h and 72 h of hydrolysis, respectively. However, this was almost 2 times higher than the production yield of untreated rice straw.

When a blend of 20% ethanol and 15% ammonia was used (Kim *et al*. 2009b), the glucose production yield was 8.2 g L^{-1} and 10.3 g L^{-1} after 24 h and 72 h of hydrolysis, respectively, the highest achieved hydrolysis yield among all performed methods, achieving 70% and 88% of the maximum theoretical yield. The supplementation of ethanol with ammonia led to a greater preservation of hydrocarbons in the substrate during the pretreatment process (Kim *et al*. 2009b).

Compositional Analysis and Crystallinity Index (CI)

Based on the results of the hydrolysis tests, the untreated, SAA-pretreated, SEAA-pretreated, and best SMAA-pretreated (with 5% methanol) rice straw were selected for compositional analysis and FTIR testing. Table 2 shows NDF, ADF, and ADL analyses that, respectively, represent cellulose, hemicellulose, and acid-soluble lignin levels of rice straw before and after subjecting samples to the treatment methods. The analysis revealed that applying the SAA pretreatment resulted in a 23.7% reduction in ADL compared to the untreated rice straw, while the ADL was reduced by 21.1% and 28.9% using SEAA and SMAA, respectively.

The residual glucan and xylan content after applying a pretreatment method is an important factor in the bioconversion process. For glucan, the best method in this regard was SMAA with 62.1% glucan remaining (and 19.7% xylan), while the best results for xylan were obtained with SEAA with 23.2% (and 60.0% glucan). Kim *et al.* (2009b) reported that the supplementation of ammonia with ethanol led to a decreased reduction in xylan during pretreatment processes.

Table 3 shows the FTIR results; after applying pretreatments to rice straw, crystallinity index (CI) decreased and most of the lignin was removed, which was indicated by reduced wave numbers after almost all treatment methods compared to the untreated rice straw. The greater reduction of these factors indicated more desirable effects of pretreatment on the delignification and fractionation of substrates. The eminent peak at 1741 cm^{-1} in the spectra could have been ascribed either to the acetyl and uronic ester groups of the hemicelluloses or to the ester linkage of the carboxylic group of lignin and/or hemicelluloses (Alemdar and Sain 2008). The absorbance at this wave number decreased from 0.00375 for untreated rice straw to 0.00241 for SAA-pretreated rice straw and to 0.00275 for SMAA-pretreated rice straw. The same pattern was observed for 1610 cm^{-1}, 1598 cm^{-1}, and all other lignin-representing wave numbers, which indicated that the values of these bonds decreased for the pretreated samples compared to untreated rice straw as a result of partial delignification.

Table 2. Composition Analysis and Glucose Production Yield of Rice Straw for Selected Methods

Pretreatment Type	Pretreatment Yield (%)	NDF (%)	ADF (%)	ADL (%)	Cellulose (%)	Hemicellulose (%)	Glucose Yield After24 h of Hydrolysis(%)	Glucose Yield After 72 h of Hydrolysis(%)
Untreated	100	76.1	45.2	3.8	41.4	30.6	15	25
SAA	64.8	81.6	60.4	2.9	57.5	21.2	68	84
SEAA*	65.5	86.2	63.0	3.0	60.0	23.2	70	88
M5SAA**	66.0	84.5	64.8	2.7	62.1	19.7	72	79

Table 3. FTIR Spectra of Pretreated and Untreated Rice Straw

Wave-number (cm^{-1})	Functional Group	Assign-ment	Un-treated	SAA Pre-treated	SEAA Pre-treated	SMAA Pre-treated
3175	-OH stretching intra-molecular hydrogen bonds	Cellulose II	0.00503	0.00548	0.00488	0.00525
2900	C–H stretching	Cellulose	0.00463	0.00528	0.00464	0.00518
1741	C=O stretching of acetyl or carboxylic acid	Hemi-cellulose and lignin	0.00357	0.00241	0.00266	0.00275
1610	C=C stretching of the aromatic ring	Lignin	0.00676	0.00471	0.00504	0.00504
1598	C=C	Lignin	0.00646	0.00463	0.00477	0.00477
1510	C=C stretching of the aromatic ring	Lignin	0.00415	0.00313	0.00346	0.00346
1465	Asymmetric bending in C–H3	Lignin	0.00593	0.00528	0.00482	0.00482
1430	C–H2 bending	Cellulose	0.0067	0.00653	0.00569	0.00693
1420	C–H2 symmetric bending	Cellulose	0.0068	0.00651	0.00575	0.007
1375	C–H bending	Cellulose	0.00755	0.00725	0.00611	0.00742
1335	–OH (in plane bending)	Cellulose	0.00743	0.00759	0.00627	0.00769
1315	C–H2 wagging	Cellulose	0.00821	0.00819	0.00673	0.00837
1158	C–O–C asymmetric stretching	Cellulose	0.0165	0.015	0.01604	0.0151
898	Asym., out of phase ring stretching (cellulose)	Cellulose	0.01378	0.01935	0.01789	0.01722
Crystallinity Index (CI)	A1430/A898	CI	0.49	0.34	0.32	0.4
	A1375/A2900	CI	1.63	1.37	1.32	1.43

Furthermore, the A1430/A898 absorbance ratio, which is defined as the CI, was 0.49, 0.4, 0.32, and 0.34 for untreated, SMAA-pretreated, SEAA-pretreated, and SAA-pretreated rice straw, respectively. These results revealed that treatment with these methods, except SMAA, decreased the absorption band at 1430 cm^{-1} (cellulose I) and increased the band at 898 cm^{-1} (cellulose II). Total crystallinity could also be correlated with the ratio of A1375 to A2900 (Nieves *et al*. 2011). This CI factor decreased from 1.63 for untreated rice straw to 1.43, 1.37, and 1.32 for SMAA-, SAA-, and SEAA-pretreated rice straw, respectively.

The M5SAA method led to a higher preservation of cellulose during pretreatment, but it also led to a higher removal of hemicellulose compared to the SEAA and SAA methods. This study also showed that SEAA will not only lead to a greater preservation of hemicellulose in the substrate during pretreatment but will also result in lower delignification. Kim *et al*. (2009b) reported the same findings.

Effect of SAA Treatment on Simultaneous Saccharification and Fermentation

Table 4 shows that only 8% and 14% of the maximum theoretical yield of ethanol production was achieved after 24 h and 72 h of SSF, respectively, when using 5 g of untreated rice straw per 100 mL of working volume as the reference. When 3 g of Avicel was hydrolyzed and fermented in the SSF process, 65% and 77% of the maximum theoretical yield was achieved after 24 and 72 h, respectively.

Table 4. Amount of Produced Ethanol per 50 gL^{-1} of Substrate and 30 gL^{-1} of Avicel

Substrate	Loading Value ($g L^{-1}$)	Yield After 24 h of SSF (%)	Yield After 72 h of SSF (%)
Untreated straw	50	8	14
SAA-Pretreated straw	50	68	84
SEAA-Pretreated straw	50	70	89
SMAA-Pretreated straw	50	72	85
Avicel	30	65	77

In the case of SAA pretreatment, the ethanol production yield from 5 g of pretreated rice straw was 68% and 84%, respectively, after 24 h and 72 h; these values were almost 8.5 and 6 times higher than those of untreated rice straw (Fig. 2). When SEAA-pretreated rice straw was subjected to the SSF process, 70% and 89% of the maximum production yield was achieved after 24 h and 72 h, respectively, and the production yield showed significant growth between these two time periods. Moreover, achieving 89% of the maximum production yield after 72 h was the highest yield among all of the performed methods. In comparison with the untreated rice straw, SEAA pretreatment led to 8.7 and 6.4 times the increase in ethanol production yield, respectively, at the 24 h and 72 h check points.

In the case of the SMAA-pretreated substrate, 72% of the maximum theoretical yield was achieved, which was the highest production yield after 24 h of SSF and was, remarkably, 9 times higher than the production yield of untreated rice straw. However, after 72 h of SSF, the production yield only reached 85% of the maximum theoretical yield, which was lower than the SEAA production yield. Similar to the hydrolysis test, the reduction in ethanol production yield might have been due to the higher crystallinity index of the SMAA-pretreated substrate compared to the SEAA-pretreated substrate.

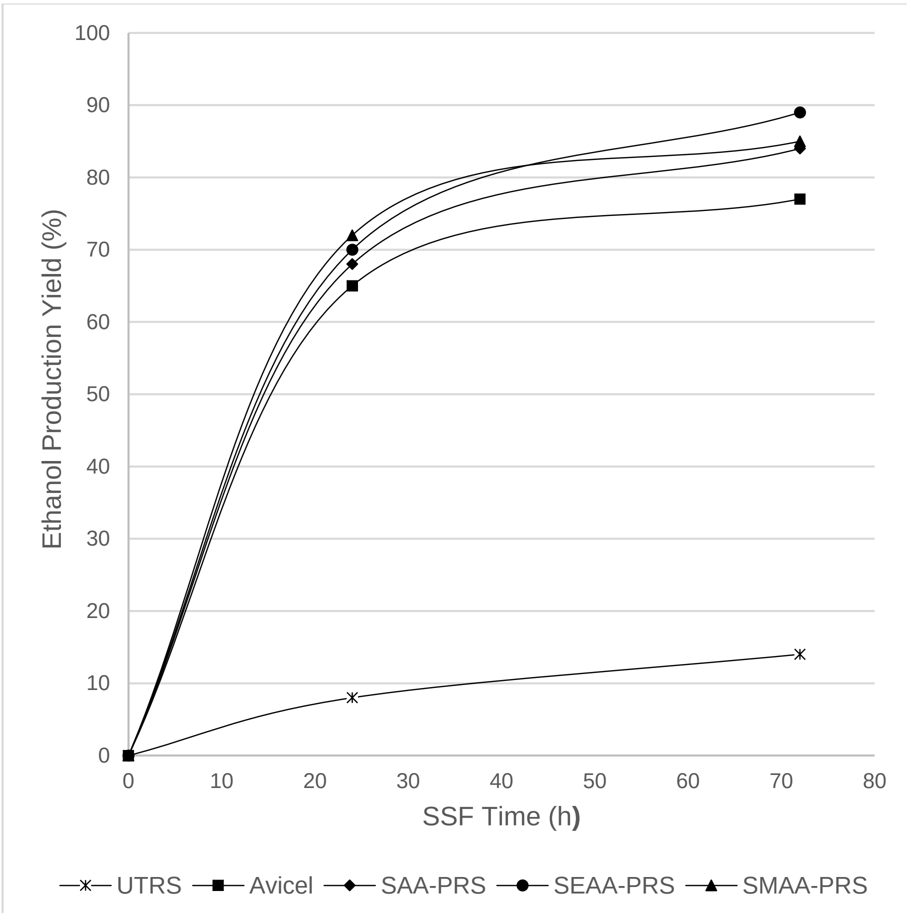

Fig. 2. SSF results for untreated rice straw (UTRS), Avicel, SAA-pretreated rice straw (SAA-PRS), SEAA-pretreated rice straw (SEAA-PRS) and SMAA-pretreated rice straw (SMAA-PRS).

From the obtained results, two important facts were observed about SMAA pretreatment. It can be concluded that as the methanol portion of the pretreatment solution increased, the production yield slightly decreased. When, in addition to an increase in the methanol portion of the pretreatment solution, the ammonia portion was reduced, the production yield decreased at a higher rate.

The results showed that the best method of pretreatment based on the glucose production rate and production yield after 24 h was M5SAA, followed by SEAA, then SAA. However, the best method of pretreatment just based on the glucose production yield after 72 h was SEAA, followed by SAA, then M5SAA (Fig. 1).

Ko *et al.* (2009) studied the effect of SAA pretreatment on rice straw, reporting a digestibility yield of 71.1% for SAA-pretreated rice straw after 12 h at 69 °C and a S:L ratio of 1:6. Compared to Ko *et al.* (2009), this study modified the SAA pretreatment time and temperature (Hedayatkhah *et al.* 2013), achieving a yield equal to 84% of the theoretical maximum. Enhancing the SAA pretreatment by supplementation with ethanol and methanol achieved 88% and 79%, respectively, of the maximum theoretical yield. To sum up, SAA yield in this study was 84% and SMAA actually decreased the yield by 5% while SEAA increased it by 4%.

Previous studies showed that the enzymatic digestibility of corn stover was 85% when SAA pretreatment was conducted at 60 °C for 12 h (Kim and Lee 2007). A previous study on bagasse showed a 88.6% production yield using M15SAA pretreatment at a S:L ratio of 1:6 (Hedayatkhah *et al*. 2013). Kim *et al.* (2009a) reported 88.5% yield using an ammonia-recycled percolation (ARP) and hot water pretreatment process and 99% and 92.5% yield using an ARP process with 60 FPU and 10 FPU cellulase, respectively. They also reported that SEAA pretreatment of corn stover led to approximately 90% digestibility yield (Kim *et al*. 2003; Kim and Lee 2005; Kim *et al*. 2009b).

The results from hydrolysis, considering both the compositional and FTIR analyses of the samples, showed that the delignification and fractionation effects of ammonia and methanol pretreatments led to an increase in the carbohydrates' accessibility to enzymes. These results demonstrated that, although delignification resulted in higher digestibility, it was not necessary to remove a great amount of lignin in order to achieve a high degree of hydrolysis (Kim and Lee 2005; Kim *et al*. 2009a; Ko *et al*. 2009). Table 2 indicates that the SMAA method led to the highest reduction of lignin content. It can be concluded from this reduction that the SMAA-pretreated rice straw showed the highest glucose production yield and rate after 24 h of hydrolysis. However, the production rate and yield for SMAA-pretreated samples did not show any significant increase between 24 h and 72 h of hydrolysis. Another important factor in hydrolysis yield is the cellulose crystallinity index (CI); the highest CI was observed using the SMAA pretreatment, among that method and SAA-or SEAA-pretreated rice straw. Thus, a greater reduction in CI was also responsible for the higher production yield.

In brief, the cellulose CI and lignin content in a sample were the two important factors influencing the hydrolysis yield. Hence, the hydrolysis yields of all pretreated rice straw samples were significantly higher than those of the untreated rice straw samples due to the reduction in CI and lignin content. Moreover, the SEAA and SMAA pretreatments showed higher production rates than the SAA pretreatment. By considering the production rate and treatment cost, it seems that the SMAA pretreatment with 5% methanol would be better than the SEAA and SAA pretreatments, particularly if the goal is to perform the process in a more practical and cost-effective time period (24 h).

The increase in ethanol production yield during the SSF process between the untreated rice straw and the pretreated samples was due to delignification, the reduction in cellulose crystallinity in the substrates, and the fractionation of the substrates through the treatment processes. These effects led to higher glucose liberation during enzymatic digestion and a subsequent increase in ethanol production yield. In other words, the enzymatic digestibility test was an appropriate method for estimating ethanol production and assessing the quality of a pretreatment method.

Li *et al*. (2010) reported a yield of 84% of the theoretical maximum, based on the total carbohydrates (glucan and xylan), using SAA-pretreated corn stover in a two-phase SSF process. Kim and Lee (2007) reported an SSF yield of 84% of the theoretical maximum using low-liquid, ARP-treated corn stover. Poornejad *et al*. (2013) reported a production yield of 93.3% for N-methylmorpholine N-oxide-pretreated rice straw.

CONCLUSIONS

1. All the selected pretreatment methods were efficacious and desirable methods for the delignification of rice straw. The given procedures were also highly effective in enhancing the yield of enzymatic digestion and simultaneous saccharification and fermentation processes.
2. The best hydrolysis yield, based on production yield and production rate after 24 h of digestion, was obtained from the SMAA-pretreated sample, with 72% of the maximum theoretical yield. However, SAA yield in this study was 84% and SMAA actually decreased the yield by 5% while SEAA increased it by 4%.
3. The best pretreatment method, based on ethanol production yield, was SEAA, with 89% yield. However, if, in addition to the production yield, the production rate and pretreatment cost were considered, the best method for ethanol production was SMAA with 5% methanol, particularly after 24 h of SSF. In other words, for rapid fermentation processes, SMAA would be more desirable and cost effective than SEAA.

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