

Effect of chemical input during wet air oxidation pretreatment of rice straw in reducing biomass recalcitrance and enhancing cellulose accessibility

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Abstract—The present study was aimed at evaluating the effect of variable sodium carbonate (Na_2CO_3) loading during wet air oxidation (WAO) pretreatment of rice straw in reducing biomass recalcitrance. The research study was intended to increase the cellulose recovery, hemicellulose solubilization, lignin removal in the solid fraction and limiting the generation of inhibitors in the liquid fraction while reducing the chemical input. The operating condition of 169 °C, 4 bar, 18 min and 6.5 g/L Na_2CO_3 loading resulted in maximum cellulose recovery of 82.07% and hemicellulose solubilization and lignin removal of 85.43% and 65.42%, respectively, with a total phenolic content of 0.36 g/L in the liquid fraction. The crystallinity index increased from 47.69 to 51.25 along with enzymatic digestibility with an increase in Na_2CO_3 loading from 0 to 6.5 g/L as a result of removal of barriers for saccharification *via* effective cleavage of ether and ester bonds cross-linking the carbohydrates and lignin as indicated by FT-IR spectroscopy. A further increase in the Na_2CO_3 loading to 9.5 g/L did not significantly increase the sugar release. Thus, it was concluded that 6.5 g/L Na_2CO_3 during WAO is sufficient to increase the delignification and deacetylation, leading to significant changes in apparent cellulose crystallinity *inter alia* improvement in cellulose accessibility and digestibility of rice straw.

Keywords: Alkaline Wet Air Oxidation Pretreatment, Delignification, FT-IR, Rice Straw, Saccharification, Sodium Carbonate, XRD

INTRODUCTION

The inevitable depletion of fossil fuel reserves and issues related to energy security has led to a hunt for alternative renewable and sustainable resources for the production of platform chemicals and liquid transportation fuels. Lignocellulosic biomass (LCB), especially rice straw, provides a sustainable solution owing to its abundant availability at the regional and global level, renewability and limited competitive uses [1]. Moreover, it has high cellulose content (35-40%) with 15 kJ/g combustion energy [2]. Nonetheless, biomass conversion to sugars requires the following steps: (1) overcoming biomass recalcitrance through a pretreatment by partial breakdown of plant cell wall with a concomitant increase in accessibility to embedded cellulose, and (2) enzymatically hydrolyzing the insoluble cellulose to soluble sugars. Thus, pretreatment and enzymatic hydrolysis play a crucial role during lignocellulosic bioconversion. Although various pretreatments have been reported in literature [3,4], alkaline wet air oxidation (AWAO) for rice straw has received less attention.

WAO involves oxidation of organic matter in aqueous condition at a higher temperature and pressure using air as oxidant, while AWAO is carried out in aqueous alkaline conditions. It is known that the acidic milieu during WAO leads to degradation of cellulose to a certain extent, thereby reducing the carbohydrate recovery

[5]. However, it is reported that alkali pretreatment aids in removing lignin along with uronic acid from hemicellulose with negligible cellulose degradation [6,7]. Thus, it is beneficial to combine these two strategies, i.e., WAO and alkaline pretreatment for an effective pretreatment of rice straw.

Sodium carbonate (Na_2CO_3) has been reported for conventional alkaline pretreatment and AWAO of LCB since it is a relatively strong alkali [8]. Moreover, sodium carbonate is economical, has no or slight lignocellulosic degradation and has no environmental concerns, thereby making it a potential choice for AWAO at industrial scale [6]. Further, alkali loading is one of the key factors that dictate the degree of delignification, which makes it imperative to examine its effect during the pretreatment. There are only a few reports that examine the role of sodium carbonate in reducing biomass recalcitrance; however, they are limited to a few feedstocks and are mostly assessed for conventional alkaline pretreatments and seldom investigated thoroughly with respect to WAO of rice straw. Additionally, the information available from different LCB does not provide the hydrolysis behavior of the biomass with same provenance, which necessitates studying their individual behavior. Therefore, although AWAO was studied with respect to a few LCB, the effect of alkali loading was not studied and the experimental trials were at a fixed alkali concentration, which indicated that the alkali concentration might not be a significant parameter [9]. However, some studies indicated the use of very high concentration of alkali for effective fractionation [10]; nonetheless, the effect of alkali loading at varying concentrations during AWAO was still not studied exclusively. Therefore, to minimize the chemical input and elucidate the effect

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of alkali loading at varying concentrations on AWAO, the present study was carried out exclusively on the optimized condition for AWAO of rice straw. The optimized condition of 169 °C, 4 bar, 18 min was obtained from our extensive previous work on optimization of operating parameters for AWAO of rice straw [11]. Further, the chemical costs that are dictated by the chemical loading and chemical recovery costs significantly affect the total pretreatment cost. This collectively necessitates study of alkali effect during WAO pretreatment on this underutilized renewable feedstock in order to evaluate the pretreatment efficacy while minimizing the chemical input and cost involved in pretreatment processes.

In the present study, the effect of varying Na₂CO₃ loading during WAO of rice straw was evaluated. The changes in composition and characteristics of pretreated rice straw, the degradation of cell wall constituents, chemical changes in the functional groups, cellulose crystallinity and degree of cellulose digestibility were examined to unveil the effect of Na₂CO₃ loading and its role in making the embedded cellulose amenable for subsequent hydrolysis by overcoming the recalcitrance.

MATERIALS AND METHODS

1. Feedstock

Rice straw was collected from the fields near Kanhan, Maharashtra, India (21.2300 °N, 79.2370 °E). It was dried at 45 °C for 48 h followed by its milling and sieving through +20/–80 mesh (ASTM specification). The straw particles ranged from 0.85–0.18 mm. They were further analyzed for composition. All the chemicals were procured from Fischer Scientific, Mumbai, India and were of analytical grade.

2. Pretreatment

30 g rice straw was mixed with 500 mL water containing varying concentration of Na₂CO₃ (0–9.5 g/L) in a stainless steel Parr high pressure-high temperature reactor (1.8 L capacity). The schematic representation of the reactor design is shown in Fig. S1 of the supplementary material. The reactor was sealed properly to ascertain a leak-proof reactor and the contents were mixed thoroughly with the agitation speed of 220 rpm. Agitation was continued till the end of the reaction. 4 bar air pressure was applied before heating the suspension and the temperature was maintained at 169 °C for 18 minutes. The temperature and pressure limits were maintained and the reactor was again tested for air leakage. The effect of alkali loading was studied by varying the concentration of Na₂CO₃ from 0 g/L to 9.5 g/L and the WAO was carried out at 169 °C, 4 bar, 18 minutes. The optimum reaction condition (169 °C, 4 bar, 18 min) was selected based on the prior extensive study, which was carried out for optimization of operating conditions for AWAO of rice straw [5,11]. The previous study only dealt with the optimization of operating parameters; however, the effect of alkali loading was not studied in the previous work. The reactor vessel was cooled after the desired reaction time and it was ensured that the air pressure was released before opening the reactor in order to ensure safety working conditions. The slurry was vacuum filtered to obtain a cellulose-rich solid fraction and a hemicellulose-rich liquid fraction. The solid fraction was washed twice with deionized water, dried, weighed and analyzed for composition. The solid fraction was fur-

ther subjected to enzymatic hydrolysis. The liquid fraction was analyzed for pH, sugar and inhibitor profiling and total phenolic content.

3. Statistical Analysis

All the experiments were replicated and the reported values are the mean of all observations. The pre- and post-pretreatment compositional analysis was analyzed statistically through one-way ANOVA using MINITAB 16.0 software (PA, U.S.A.). The distribution of data was examined through normality test, while Levene's test was used for examining the equality of variances. The post-hoc test was performed after ANOVA using Tukey method to determine the different statistically significant groups.

4. Enzymatic Hydrolysis

To examine the effect of Na₂CO₃ charge during pretreatment on digestibility of biomass, the untreated and pretreated rice straw were subjected to enzymatic hydrolysis for 48 h at 50 °C in the presence of 10 FPU/g dry matter (DM) cellulase and 5 IU/g DM β-glucosidase in citrate buffer (0.1 M, pH-4.8) at 100 rpm. Cellulase obtained from *Trichoderma reesei* and β-glucosidase from almonds which had an activity of 108 FPU/mL and 9.6 CBU/mg, respectively, were purchased from Sigma Aldrich, U.S.A. The saccharification was carried out at 6% solid loading. The samples were drawn at specific time intervals, boiled at 100 °C to deactivate the enzymes and centrifuged at 7,500 rpm for 10 min to remove the residual fibers. Glucose, xylose and cellobiose released were estimated using high performance liquid chromatography (HPLC), and total reducing sugar (TRS) was determined using dinitro salicylic acid assay. The enzymatic digestibility (%) was calculated as follows:

$$\begin{aligned} \text{Enzymatic digestibility (\%)} \\ &= \frac{\text{Amount of cellulose digested (g)}}{\text{Amount of cellulose added (g)}} \times 100 \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Amount of cellulose digested} \\ &= \text{Glucose (g/mL)} \times 0.9 \times \text{Reaction volume (mL)} \end{aligned} \quad (2)$$

5. Analytical Methods

5-1. Compositional Analysis

The solid fraction was analyzed for total solids, moisture, ash, extractives and lignin according to NREL protocols [12]. Cellulose content was estimated using monoethanolamine method, while the holocellulose content was determined gravimetrically [13,14]. The carbohydrate content in the liquid fraction was determined using HPLC (Waters) equipped with BioRad Aminex HPX-87P column (300×7.8 mm) and Waters 2414 refractive index (RI) detector. The analysis was performed at 80 °C using HPLC-grade water as mobile phase at a flow rate of 0.6 mL/min. The organic acid and other degradation products in the liquor were analyzed by HPLC using BioRad Aminex HPX-87H Organic acid column (300×7.8 mm) at 65 °C using 0.005 M H₂SO₄ as mobile phase at a flow rate of 0.6 mL/min and detected with RI Detector. Folin-Ciocalteu assay was used to estimate the total phenolic content (TPC). The estimation of TPC by Folin-Ciocalteu assay is based on the electron transfer from phenolic compounds to phosphomolybdic and phosphotungstic acid complexes in alkaline medium, which are measured thereafter by determining the absorbance at 765 nm [15].

5-2. FT-IR

The solid fraction was further analyzed qualitatively for the chemi-

Table 1. Effect of alkali loading during AWAO on chemical composition of rice straw

Components	Untreated rice straw	Alkali loading (g/L) during AWAO pretreatment			
		0 g/L	3.5 g/L	6.5 g/L	9.5 g/L
Moisture (%)	1.44±0.03	4.28±0.29	3.88±0.20	3.63±0.03	3.59±0.46
Extractives (%)	2.5±0.05	2.29±0.92	2.19±0.65	1.36±0.07	0.977±0.85
Ash (%)	22.15±0.52	19.74±0.71	18.71±0.45	13.62±0.25	16.46±0.37
Cellulose (%)	40.66±0.37	42.96±0.45	44.35±0.66	56.27±0.55	64.10±0.80
Hemicellulose (%)	20.6±0.78	21.01±0.43	24.48±2.75	5.06±0.8	5.00±3.22
Total Lignin (%)	12.3±0.84	13.78±0.16	10.92±1.56	7.18±0.29	7.69±0.48
Acid soluble lignin (%)	1.33±0.94	1.74±0.08	0.62±0.09	0.39±0.15	1.03±0.21
Acid insoluble lignin (%)	11.47±0.04	12.37±0.07	10.54±1.0	6.90±0.12	6.74±0.19
Solid recovery (%)	NA	73.44±2.14	70.28±2.23	59.36±1.97	50.29±2.31
Total volume of liquid lost (%)	NA	26±1.7	24±1.41	13.5±0.99	5±1.13
Initial pH	NA	6.19±0.05	11.9±0.02	11.9±0.02	11.9±0.03
Final pH	NA	4.51±0.03	6.63±0.04	6.56±0.01	6.55±0.04
TRS (g/kg untreated rice straw) in liquid fraction	NA	34.29±0.37	11.91±1.14	22.06±1.3	25.49±0.16
TRS (g/kg untreated rice straw) in liquid fraction after acid hydrolysis	NA	78.81±0.25	14.44±2.3	34.6±3.4	44.18±1.47

Values given in Table represent the average values for replicate trials

cal changes occurring at variable Na_2CO_3 loading through Fourier transform-infrared spectroscopy (FT-IR) (Bruker ATI Matson Infinity 60 AR). The samples were ground with spectroscopic grade potassium bromide (KBr) and scanned 16 times to obtain a spectrum from $4,000\text{ cm}^{-1}$ to 400 cm^{-1} .

5-3. XRD

Crystallinity of the untreated and pretreated rice straw was analyzed by X-ray diffractometer (Rigaku MiniFlex II, Japan). The samples were placed on the glass sample holder; Ni-filtered $\text{CuK}\alpha$ radiation ($\lambda=1.54\text{ \AA}$) generated at a voltage of 30 kV and current of 15 mA was used. The scanning data was collected from 10° to 40° with a step size of 0.02° and scan speed of 5 s. The crystallinity index (CI) for untreated and pretreated samples was calculated as:

$$\text{Crystallinity index (\%)} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (3)$$

where I_{002} is the intensity of the crystalline region at $2\theta=22.1$ and I_{am} is the intensity of the amorphous region at $2\theta=18.1$ (in this study, the peak at 22.1 represented the crystalline region, while the peak at 18.1 represented the amorphous region).

RESULTS AND DISCUSSION

1. Effect of Alkali Loading During AWAO on Chemical Composition

The effectiveness of a pretreatment is usually gauged on the basis of effective LCB fractionation into its components--cellulose, hemicellulose and lignin--while generating limited sugar and lignin degradation products while using minimum alkali. To evaluate the role of alkali loading during AWAO, compositional analysis was carried out pre- and post- pretreatment.

1-1. Analysis of Solid Fraction

The solid fraction was analyzed for various parameters as given

in Table 1. It was observed that the post-pretreatment solid recovery reduced with increasing alkali concentration during WAO. The solid recovery decreased from 73.44% to 50.29% based on the original weight of rice straw, when the Na_2CO_3 concentration was increased from 0 to 9.5 g/L, thereby, indicating the significance of alkali loading in the solid recovery. This decrease in solid yield might be a result of removal of carbohydrates and lignin in the rice straw, however, to different extents. Moreover, the lower solid yield at higher alkali loading is also indicative of presence of high amount of alkali soluble components; the dissolution of which resulted in a reduced solid yield [6]. Note also that the total volume lost during WAO decreased with an increase in alkali loading. This might be due to the loss of water holding capacity of the pretreated solid fraction, which could be observed from the physical changes occurring during the pretreatment. At lower alkali concentration, the water lost was retained in the biomass as indicated by the moisture content of biomass, whereas at higher concentration of alkali, the biomass could not hold water, which was reflected from the lower moisture content. This effect was evident from the physical changes occurring in the biomass (Fig. 1). The untreated rice straw was pale yellow and straw particles could be seen as single entities. However, noticeable color change and clumping was clearly visible at higher alkali loading as a consequence of a pronounced bleaching effect at higher alkali loading.

Further, the extractives and ash content reduced with an increase in alkali loading indicating their removal during the pretreatment (Table 1). The decline in the ash content was attributed to the dissolution of inorganic salts. Additionally, an enhancement in cellulose content (%) was observed from the initial 40.66% to a maximum of 64.10% at a higher alkali loading of 9.5 g/L. This enhancement in cellulose content can be ascribed to removal of hemicellulose and lignin. A significant decrease in hemicellulose (%) and lignin (%) from 20.6% and 12.3% in untreated to 5.00% and 7.18%

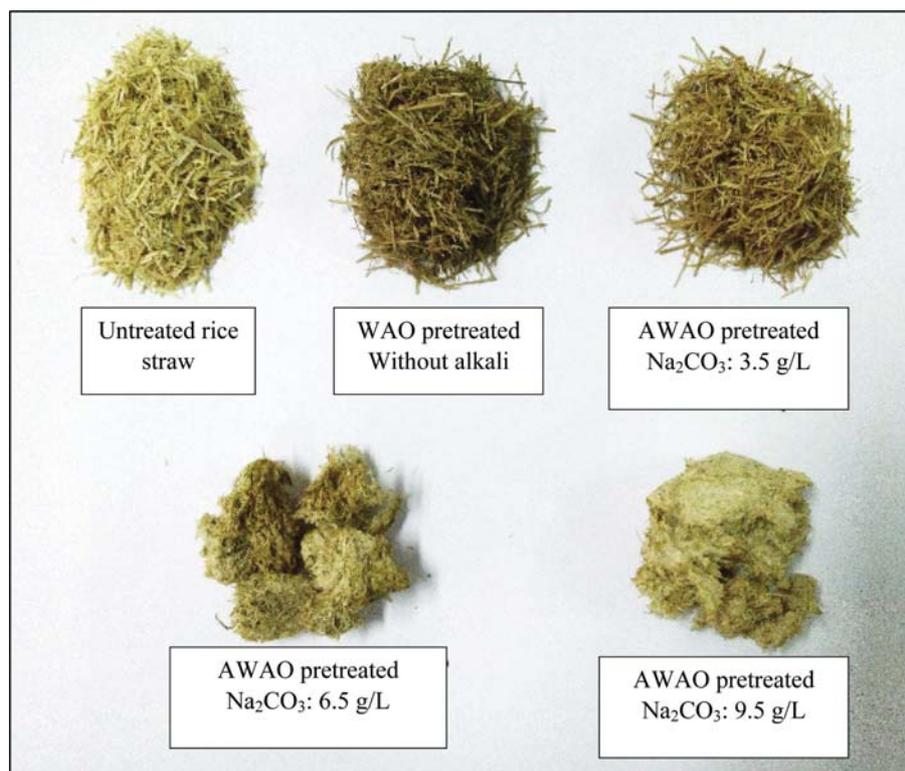


Fig. 1. Effect of alkali loading during WAO on the physical changes occurring in rice straw (a) untreated rice straw, (b) without alkali, (c) 3.5 g/L Na_2CO_3 , (d) 6.5 g/L Na_2CO_3 , and (e) 9.5 g/L Na_2CO_3 .

Table 2. ANOVA for % cellulose recovery, % hemicellulose solubilization, % lignin removal and total phenolic content

Alkali loading (g/L)	% Cellulose recovery $R^2=90.72\%$ F=13.03 P=0.016	% Hemicellulose solubilization $R^2=97.6\%$ F=67.64 P=0.000	% Lignin removal $R^2=91.14\%$ F=24.02 P=0.000	Total phenolic content (g/L) $R^2=93.39\%$ F=56.47 P=0.000
0	77.523±0.801 B	25.07±1.58 B	17.927±1.720 B	0.345±0.0023 B
3.5	76.586±1.131 B	16.49±9.38 B	37.763±0.371 B	0.323±0.0017 C
6.5	82.065±0.799 A	85.43±2.31 A	65.422±14.172 A	0.356±0.0074 B
9.5	79.214±0.996 B A	87.78±7.86 A	68.613±6.649 A	0.373±0.0079 A

Means that do not share a letter are significantly different using Tukey method

in AWAO pretreated rice straw at 9.5 g/L and 6.5 g/L alkali loading, respectively, was observed as a result of hemicellulose solubilization and lignin removal. Further it can be noted that a slight increase in % total lignin was observed for rice straw pretreated with WAO without alkali; however, when calculated based on solid recovery, it indicates a slight reduction in total lignin of WAO pretreated rice straw. This is explicitly due to the stability of acid insoluble lignin at WAO treatment condition without alkali. The slight reduction in total lignin content was due to the solubilization of acid soluble lignin. This dissolution of acid soluble lignin is an outcome of partial cleavage of α -ether bonds in lignin [16]. However, a further increase in the alkali loading resulted only in gentle removal of acid soluble lignin (Table 1). Additionally, at higher alkali loading, lesser removal of acid soluble lignin was observed, while a higher removal of acid insoluble lignin was observed owing to increased

delignification at severe alkaline conditions.

The values for cellulose recovery, hemicellulose solubilization and lignin removal at different Na_2CO_3 loading are presented in Table 2. The results of the compositional analysis from Table 2 entail an effective biomass fractionation along with preservation of cellulose which can be indicated from a maximum cellulose recovery of 82.06±0.79% and removal of hemicellulose and lignin by a maximum of 87.78±7.86% and 68.61±6.64%, respectively. The slightly lower cellulose recovery in non-alkaline conditions might be a result of cellulose degradation due to the acidic environment, while the higher cellulose recovery observed at 6.5 g/L alkali loading was a result of low reactivity of cellulose with alkali. It is noteworthy that although cellulose recovery enhanced till alkali loading of 6.5 g/L, it declined when further alkali concentration was increased to 9.5 g/L. The higher hemicellulose solubilization and lignin removal

might have resulted in loosening of the complex lignocellulosic structure, which might have resulted in minor degradation of cellulose, leading to slight reduction in cellulose recovery at alkali loading of 9.5 g/L. We further observed that hemicellulose solubilization and lignin removal increased with an increasing alkali loading. It may be ascribed to the higher alkaline degradation of rice straw at higher alkali loading as a result of cleavage of ester linkages between the carbohydrates and lignin, cleavage of α -benzyl ether bonds cross-linking hemicellulose and lignin along with uronic acid substitutions and deacetylation of acetyl groups in hemicellulose [16]. Moreover, the results obtained also suggest employing a moderate concentration of Na_2CO_3 during WAO as a replacement for other alkali--NaOH, $\text{Ca}(\text{OH})_2$ --owing to selective and effective lignin removal without cellulose degradation. Moreover, the cost of NaOH (\$300-660/ton) and $\text{Ca}(\text{OH})_2$ (\$190-450/ton) is higher than that of Na_2CO_3 (\$180-235/ton), which further makes it more preferable alkali to be used during AWAO [17].

1-2. Analysis of Liquid Fraction

The liquid fraction obtained during WAO was analyzed for pH, TRS in acid hydrolyzed and non-acid hydrolyzed liquor, presence of monosaccharide sugars and inhibitors. It can be seen from Table 1 that the pH of the non-alkali WAO treated rice straw had an initial pH of 6.19, which decreased to 4.51 post-pretreatment. This indicated a certain degree of acidic degradation of carbohydrates. On the contrary, the presence of alkali led to hemicellulose deacetylation and oxidation of its acidic components along with fragmentation of lignin and its oxidation to phenolic compounds and aliphatic carboxylic acids [18]. This is indicated by the presence of carboxylic acids, acetic acid and formic acid in liquor (Fig. 2) [19]. The sugar and degradation products' profiling of the liquor is shown in Fig. 2. The near neutral pH of the liquor is indicative of minimum degradation products under alkaline conditions of WAO. A slightly higher concentration of acetic acid under alkaline conditions is a result of enhanced hemicellulose solubilization

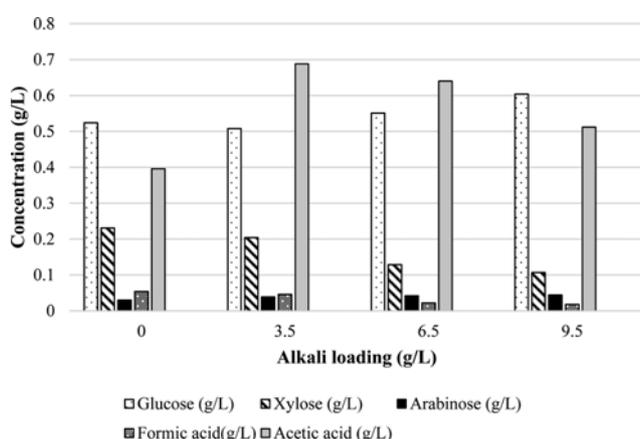


Fig. 2. Effect of alkali loading during WAO on compositional analysis of liquid fraction of pretreated rice straw indicating sugar and inhibitor profiling. The potent inhibitors furfural and 5-hydroxymethyl furfural were absent in the liquid fraction and therefore not detected during HPLC and hence not shown. Cellobiose content was found to be less than 0.01 g/L and hence was not included in the graph.

under these conditions. A minor difference in hemicellulose solubilization can be seen at non-alkaline and 3.5 g/L Na_2CO_3 loading conditions; however, the difference is statistically insignificant compared to higher alkali loadings (discussed in details in section 3.1.3). Nonetheless, higher deacetylation is observed at Na_2CO_3 loading of 3.5 g/L than at non-alkali condition, while the amount of xylose is comparatively lesser at these conditions. This can be explicitly due to preservation of xylose in xylooligosaccharide form under alkaline conditions unlike its release under acidic conditions [20, 21]. On the other hand, a slight variation in acetic acid concentration at varied alkaline conditions might be a result of a reversible reaction between the generated acetic acid and sodium carbonate to form sodium acetate and carbonic acid. This might have resulted in the presence of lower acetic acid at alkali loadings of 6.5 g/L and 9.5 g/L, even after undergoing higher hemicellulose solubilization. We observed that higher amount of xylose was released during WAO without alkali, while maximum glucose release was observed at a higher alkali loading of 9.5 g/L. Fig. 2 indicates a collectively higher concentration of total sugars at the pretreatment conditions with no or low alkali loading, which is comparatively lower at higher alkali loading. This indicates that the latter pretreatment conditions are better at preserving the carbohydrates owing to its low reactivity with alkali [16,22]. To further validate the preservation of carbohydrates in oligosaccharide form, the liquor was further examined for the total reducing sugar content pre- and post-acid hydrolysis to release monosaccharides. It can be seen from Table 1 that the TRS increased after acid-hydrolysis indicating that the sugars are preserved in the oligosaccharide form due to incomplete hydrolysis during the pretreatment. HPLC analysis of the liquor further indicated absence of potent inhibitors: furfural and 5-hydroxymethylfurfural (Fig. 2). Further, it can be observed from Table 2 that the total phenolic content (TPC) ranged from 0.32-0.37 g/L. TPC increased when the alkali concentration increased from 3.5 to 9.5 g/L. However, a slight decrease in TPC was observed at 3.5 g/L Na_2CO_3 loading as compared to that without alkali. It is known that lignin is a mixture of phenolic compounds and flavonoids present in bound form including esters and glycosides. TPC is related to release of phenolic compounds, which can be correlated from the lignin removal to an extent; however, lignin removal also involves removal of flavonoids along with phenolic compounds [23]. The slightly lower TPC at 3.5 g/L Na_2CO_3 loading might be the result of release of comparatively less phenolic compounds compared to that without alkali; nonetheless, the same condition might have resulted in higher flavonoid release, which contributed to higher lignin removal compared to that without alkali. Further, TPC increased with increase in alkali from 3.5 to 9.5 g/L as a result of enhanced delignification owing to breakdown of bonds between the polysaccharides leading to release of lignin at higher alkali concentrations. The TPC >2 g/L is reported to be inhibitory to the fermenting microorganisms [24]. Therefore, the reuse of liquid fraction as a diluent during the fermentation step can be suggested owing to the lower concentration of phenolic content.

1-3. Statistical Analysis

The effect of Na_2CO_3 loading during WAO of rice straw was envisaged through the cellulose recovery, hemicellulose solubilization and lignin removal in the solid fraction and total phenolic con-

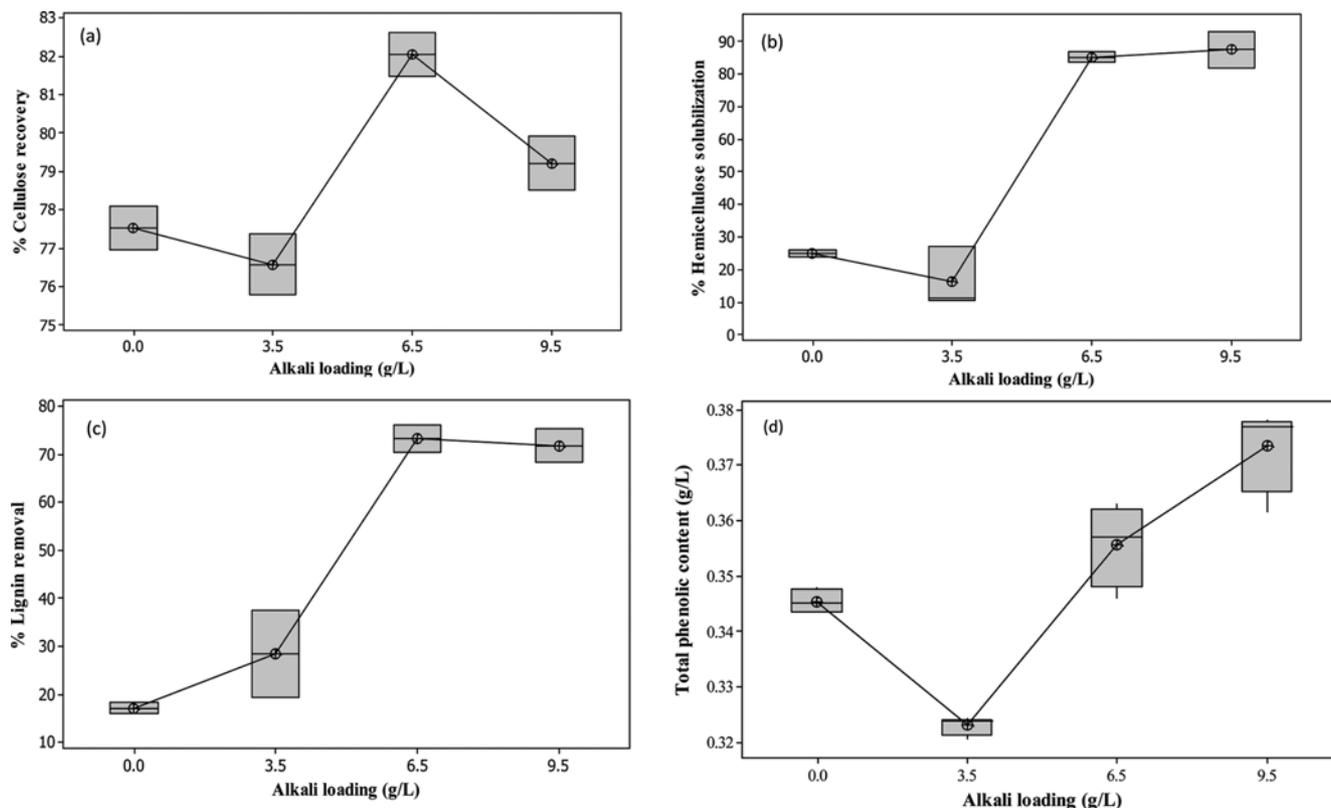


Fig. 3. Box plots indicating the effect of alkali loading during AWAO on compositional analysis of rice straw (a) % cellulose recovery, (b) % hemicellulose solubilization, (c) % lignin removal and (d) total phenolic content.

tent in liquid fraction since an effective pretreatment usually balances delignification and preservation of carbohydrates. One-way analysis of variance (ANOVA) was performed for the experimental data obtained and the average values of the above-mentioned responses along with standard deviation are represented in Table 2. The examination of the data did not indicate presence of any outliers as assessed by the inspection of the boxplots for different responses: cellulose recovery, hemicellulose solubilization, lignin removal and total phenolic content. The boxplot for cellulose recovery (Fig. 3(a)) indicates that the alkali loading of 6.5 g/L had the highest % cellulose recovery values, while alkali loading of 3.5 g/L had lowest % cellulose recovery values. Fig. 3(b) represents the boxplot for % hemicellulose solubilization, which indicates that higher concentration of alkali led to higher hemicellulose solubilization. It further indicates that the hemicellulose solubilization values for alkali loading of 3.5 g/L were very spread out, while that for non-alkali were least spread out. The boxplot for lignin removal (Fig. 3(c)) indicates that highest lignin removal values were obtained for alkali loading of 6.5 g/L, and the increase in alkali loading did not show a significant increase in lignin removal. However, a significant increase in TPC was observed when the alkali loading was increased from 6.5 g/L to 9.5 g/L (Fig. 3(d)). One-way ANOVA was performed for the experimentally obtained responses based on the assumptions that the dependent variables, i.e. the responses, were normally distributed and showed homogeneity of variances. To substantiate these assumptions, normality test and Levene's test for investigating the homogeneity of variance were performed. The higher P-

values (>0.05) in Levene's test for all the responses indicated the homogeneity of variances. The results of ANOVA are represented in Table 2. The P-value (<0.05) indicates that the Na_2CO_3 loading had a significant effect on all the responses and the means in different groups of alkali loading were significantly different statistically. Moreover, the higher F-values and the higher determination coefficients ($R^2 > 90\%$) for all the responses indicate the competence of the model. Further, although the results of one way ANOVA indicated a statistically significant difference among the different alkali loading groups, it did not reveal which specific groups differed. However, this was confirmed by performing the post-hoc test by Tukey's method. Table 2 further provides the grouping of means indicated by a letter; the means that do not share a letter are significantly different.

2. Chemical Changes in the Pretreated Rice Straw

2-1. FT-IR Analysis

FT-IR analysis was carried out to scrutinize the chemical changes occurring during rice straw pretreatment as a result of change in Na_2CO_3 concentration, and the FT-IR spectra for the same are presented in Fig. S2 and Table S1 (Supplementary material). The untreated rice straw exhibited some fingerprint peaks for cellulose, hemicellulose and lignin which have been either reduced, shifted or diminished in the pretreated samples at varying alkali loading. It was observed that the peak at 665.62 cm^{-1} in the untreated rice straw which is attributed to COH out of plane bending vibrations of cellulose is shifted to higher wavenumber that might be attributed to the cellulose transformation [25]. The peak at 884.53 cm^{-1} in the

untreated rice straw represented the C-O-C stretching at β -glucosidase linkage, which is attributed to the amorphous cellulose (Fig. S2(a)) [26]. However, its absence from the pretreated samples indicated its easy removal during AWAO. The peaks at $1,040.01\text{ cm}^{-1}$ and $1,090.88\text{ cm}^{-1}$ in the untreated sample were ascribed to the >CH-O-CH stretching vibrations and the skeletal C-C vibrations of hemicellulose which showed a peak shift in the pretreated samples [26,27]. The noticeable peaks observed at $1,348.73\text{ cm}^{-1}$ and $1,423.4\text{ cm}^{-1}$ were attributed to the C-H ring in-plane bending vibrations of hemicellulose and the vinyl C-H in-plane bend of the crystalline region of cellulose, respectively [27]. These peaks were shifted to a lower wavenumber in rice straw pretreated with 0 g/L and 3.5 g/L alkali (Fig. S2(b), S2(c)), while they were absent at 6.5 g/L alkali loading (Fig. S2(d)) and were shifted to higher wavenumber at 9.5 g/L alkali (Fig. S2(e)), which is indicative of changes in the hemicellulose-cellulose linkages and the cellulose crystallinity owing to the variable alkali loading during the pretreatment. Additionally, the fingerprint peak of lignin at $1,596.2\text{ cm}^{-1}$ in the untreated rice straw, which is representative of the C=C-C aromatic ring stretch, aromatic C-O stretch in the phenylpropanoid polymer of lignin, showed a slight shift to lower wavenumber which signified the rearrangement in the lignin structure due to pretreatment [27]. The intensity of this peak varied with varying pretreatment conditions. The shoulder peak at $1,653.76\text{ cm}^{-1}$ in the untreated rice straw, which represented the lignin-hemicellulose-cellulose crosslinks, was eliminated in all the pretreated samples except at higher alkali loading where it showed a peak shift to lower wavenumber. This suggested breakdown of the linkages between these polymers in the former pretreatment conditions and their rearrangement in the latter.

The FT-IR spectrum of untreated rice straw indicated peaks at $3,233.41\text{ cm}^{-1}$ and $3,269.07\text{ cm}^{-1}$ representing the intermolecular hydrogen bonding for 6-OH---O-3' in cellulose I; $3,328.55\text{ cm}^{-1}$ and $3,269.07\text{ cm}^{-1}$ denoting the stretching of OH group along with the peaks at $3,522.22\text{ cm}^{-1}$ and $3,577.09\text{ cm}^{-1}$ that exemplify the valence vibration of hydrogen bonded OH-group [25]. It was noted that the FT-IR spectrum of non-alkaline WAO pretreated straw showed the presence of intramolecular hydrogen bonding of cellulose and absence of intermolecular hydrogen bonding of cellulose indicating their breakdown. Furthermore, all the alkali pretreated rice straw showed intra- and intermolecular hydrogen bonding with a slight peak shift indicating transformation associated with the change in intra- and intermolecular bonds in cellulose.

Thus, collectively, FT-IR spectra indicated hemicellulose solubilization, lignin removal and cellulose transformation, as indicated by the cleavage of ester and ether linkages and changes in inter- and

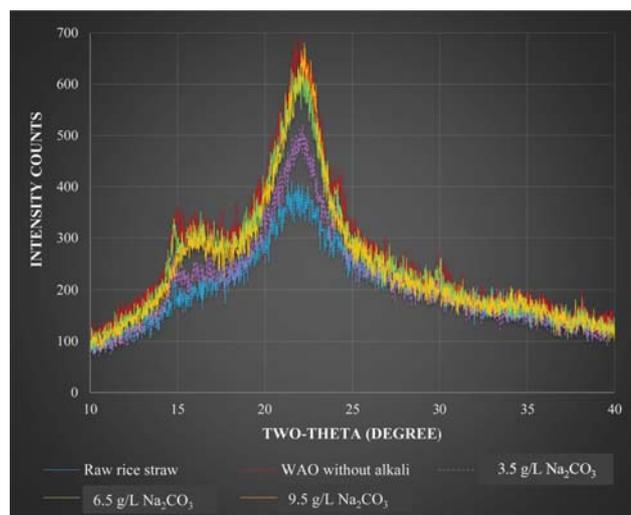


Fig. 4. X-ray diffraction pattern of untreated rice straw, WAO pretreatment without alkali, AWAO with 3.5 g/L Na_2CO_3 , 6.5 g/L Na_2CO_3 and 9.5 g/L Na_2CO_3 .

intramolecular hydrogen bonding of cellulose, respectively, with a slight degree of variability at different Na_2CO_3 loading.

2-2. XRD Analysis

Another governing factor indicating the changes occurring at variable Na_2CO_3 during WAO and the subsequent enzymatic hydrolysis step is the cellulose crystallinity, which is expressed in terms of crystallinity index (CI) which was calculated from Eq. (3). However, measuring true cellulose crystallinity *via* XRD is difficult owing to the presence of amorphous impurities, hemicellulose and lignin; thus, cellulose crystallinity measured *via* XRD is apparent. The XRD patterns for untreated and pretreated rice straw are shown in Fig. 4, while the calculated CI values are given in Table 3. It can be observed from Table 3 that WAO with and without Na_2CO_3 resulted in changes in the CI as well as the crystal structure system of cellulose. It was noted that the CI increased as a result of WAO pretreatment. This might be ascribed to the removal of the amorphous components: hemicellulose and lignin in the pretreated rice straw. Further, AWAO pretreated rice straw had a comparatively higher CI against that pretreated without alkali, which might be attributed to higher lignin removal under alkaline conditions, which can be corroborated from Table 2. Furthermore, CI increased with an increase in Na_2CO_3 loading as a consequence of removal of hemicellulose, lignin and amorphous portion of cellulose leaving behind the crystalline portion of cellulose intact, as compared to non-alkaline WAO. This may further be attributed to the peeling reaction

Table 3. Crystallinity index of untreated and pretreated rice straw

Sr. No.	Pretreatment condition	Crystallinity index
1	Untreated rice straw	46.55
2	Wet air oxidation without alkali pretreated rice straw	47.69
3	Alkaline wet air oxidation with 3.5 g/L Na_2CO_3	48.46
4	Alkaline wet air oxidation with 6.5 g/L Na_2CO_3	51.25
5	Alkaline wet air oxidation with 9.5 g/L Na_2CO_3	54.04

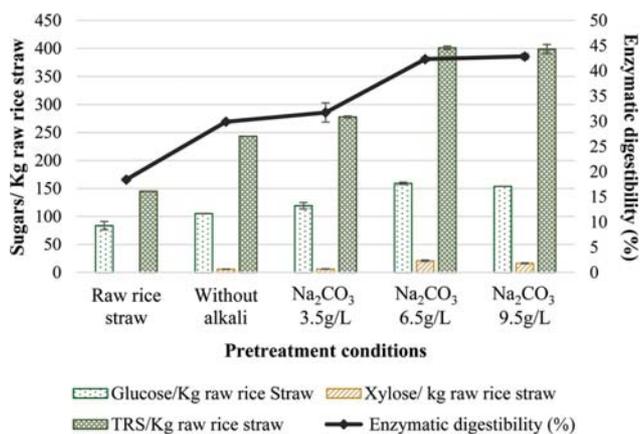


Fig. 5. Effect of alkali loading during pretreatment on sugar release and % saccharification during enzymatic hydrolysis of WAO pretreated rice straw.

due to Na_2CO_3 in amorphous region of biomass [26,28]. Similar results have been reported by other researchers for WAO of other substrate [29]. Although these results indicate changes in crystallinity due to variability in alkali loading, its effect on the subsequent step of saccharification is of utmost importance as it is deemed that crystallinity affects the accessibility and digestibility of LCB. Therefore, these effects were investigated and are reflected in successive section 3.3.

3. Effect of Alkali Loading on Enzymatic Hydrolysis

To make the lignocellulosic bioconversion process economically feasible, it is suggested to employ lower enzyme loadings to reduce the cost contribution from enzymes. However, it has always been a shortcoming while examining the efficacy of any pretreatment. Therefore, in the present study, the enzymatic saccharification was performed using lower enzyme loading, and glucose, xylose and TRS released during hydrolysis were estimated based on which enzymatic digestibility was calculated.

The glucose and TRS yields obtained during the pretreatment at different Na_2CO_3 loading during enzymatic hydrolysis are shown in Fig. 5. It was observed that the glucose and TRS concentration increased significantly with an increasing Na_2CO_3 loading. Although the sugar yields at Na_2CO_3 loading of 6.5 g/L and 9.5 g/L did not differ significantly; however, a difference in enzymatic digestibility was observed at these alkali loadings.

The glucose, xylose, TRS released were calculated on basis of per kg untreated rice straw along with % saccharification and are represented in Fig. 5. The experimental results indicated that glucose released per kg of untreated rice straw increased with Na_2CO_3 loading from 105.19 ± 0.5 g glucose/kg untreated rice straw in samples pretreated without Na_2CO_3 to 159.11 ± 2.87 g glucose/kg untreated rice straw in samples pretreated with 6.5 g/L Na_2CO_3 . Additionally, xylose yield was 5.83 ± 0.38 g per kg of untreated rice straw after WAO pretreatment without alkali, which increased to 21.03 ± 1.88 g at alkali loading of 6.5 g/L. However, xylose was released in very minor amounts as compared to glucose, which is apparent from the fact that the saccharification was not supplemented by hemicellulose degrading enzymes such as xylanase in the present study. Further, the TRS yield increased from 243.35 g/

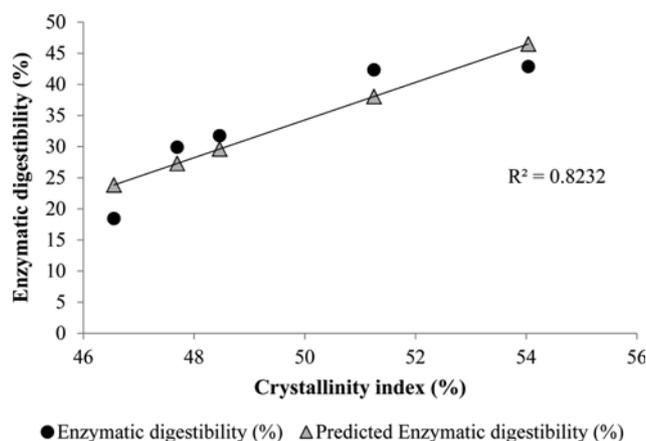


Fig. 6. Relation between crystallinity index (%) and enzymatic digestibility (%).

kg untreated rice straw in non-alkali pretreated rice straw to 401.21 ± 2.97 g TRS/kg untreated rice straw in WAO with 6.5 g/L Na_2CO_3 . Also, further increasing the Na_2CO_3 concentration from 6.5 g/L to 9.5 g/L did not result in significant increase in sugar concentration. Nonetheless, enzymatic digestibility increased from $29.90 \pm 0.15\%$ to $42.85 \pm 0.54\%$ with increase in Na_2CO_3 from nil to 9.5 g/L. A sharp increase in enzymatic digestibility was observed when Na_2CO_3 increased from 3.5 g/L to 6.5 g/L.

The saccharification yields obtained at moderate Na_2CO_3 loading were a result of higher lignin removal in those samples as compared to those pretreated without Na_2CO_3 or at lower Na_2CO_3 loading. Moreover, although it is reported that lignin results in unproductive binding of cellulases, complete delignification is not a prerequisite to attain maximum saccharification. Generally, partial delignification of 20-65% is adequate to improve the accessibility of cellulase to cellulose and for the subsequent improvement in digestibility [30]. Additionally, the relation between CI and enzymatic digestibility was evaluated using linear regression analysis and the results are depicted in Fig. 6. A linear regression established that the CI could statistically significantly predict the % saccharification (F-value=13.97 and p-value=0.03; $R^2=0.82$). This reflected that the increased CI for every increase in Na_2CO_3 during AWAO was associated with removal of barriers (i.e., hemicellulose and lignin), which subsequently increased the cellulose accessibility during enzymatic hydrolysis leaving the cellulose exposed. However, increase in Na_2CO_3 above 6.5 g/L led to enhancement in crystallinity but no significant difference in sugar yield, which indicates that increasing the alkali loading during AWAO above 6.5 g/L is futile. The saccharification results obtained in the present study were compared with that reported for rice straw and other LCB such as corn stover, wheat straw, switchgrass, etc. by conventional alkali pretreatments under different conditions as can be seen from Table 4 [6,16,28,31]. However, the other pretreatments employed high amount of alkali during pretreatment as well as higher loadings of enzyme. Thus, alkali loading of 6.5 g/L resulted in maximum saccharification yield.

4. AWAO Technology Analysis

An effective pretreatment is alleged to enhance cellulose recov-

Table 4. Comparative saccharification results for different biomass and pretreatments

Sr. No.	Feedstock	Pretreatment	Enzyme loading	% Glucan conversion	Reference
1	Rice straw	8% Sodium carbonate	20 FPU/g dry matter	71.7%	[6]
2	Corn stover	0.08 g NaOH/g solid	4 mg protein/g glucan	70%	[16]
3	Rice straw	Aqueous ammonia	30 FPU/g dry matter	78.04%	[28]
4	Corn stover	1 g Na ₂ CO ₃ /g solid	30 mg protein/g glucan	95.1%	[31]
	Miscanthus			62.3%	
	Switchgrass			81.3%	
5	Wheat straw	8% Sodium carbonate	20 FPU/g dry matter	74.5%	[33]

ery, hemicellulose solubilization, lignin removal while utilizing least amount of chemicals and utilities and generating limited amount of inhibitors and waste. The only chemical used during AWAO is Na₂CO₃, which was used in the range of 0-0.16 g/g rice straw (0-9.5 g/L), which is far less than that reported in literature for the same substrate pretreated using similar strategy (52.99 g/L sodium carbonate) [10]. Since the results from WAO pretreatment with 6.5 g/L and 9.5 g/L Na₂CO₃ did not vary significantly, addition of another 3 g/L Na₂CO₃ would only lead to wastage of chemical without any significant difference in outcome. Thus, 6.5 g/L Na₂CO₃ charge could be considered optimum from this study. So, the chemical requirement for AWAO calculated on this basis and 6% solid loading would result in 108 kg Na₂CO₃/ton untreated rice straw, which costs \$19.4-25.4 at the rate of \$180-235/ton. Further, the energy required in AWAO pretreatment is less as compared to other commercially available pretreatments like steam explosion. This is because the energy required to heat water (718-898 kJ/kg) is less than that required to vaporize it as necessary in steam explosion (2,755-2,794 kJ/kg) [32]. Additionally, AWAO is an exothermic process, which makes it a self-supporting process in terms of heat once the reaction is initiated, thereby, decreasing energy requirements. The near neutral pH of the liquid fraction (6.55) and absence of potent inhibitors make it a suitable diluent for fermentation, which would reduce the freshwater requirements along with wastewater generation and, in turn, the wastewater treatment cost. However, this recommended usage of liquor needs to be validated for its effect on the biofuel yield during fermentation. Since the regeneration and recovery of Na₂CO₃ is deemed to be less economic owing to the approximate recovery costs of \$121.7-242.1 [16], the reuse of liquor seems to be beneficial. A further detailed insight into the techno-economic analysis is restricted since the main objective of the present research was to study the effect of alkali loading on AWAO pretreatment of rice straw in order to employ optimal chemical input, thereby reflecting a reduction in the cost contribution from chemical usage in the pretreatment process. Collectively, the comparatively lesser usage of chemical, absence of potent inhibitors and waste, increased cellulose accessibility and digestibility, as indicated in the present study might reinforce its prospects in a biorefinery. A proper integration of different stages through usage of lesser utilities, recycle and reuse of water, integration into biohydrogen and/or biomethane plant would lead to proper process intensification that would further aid in reducing the process costs. Furthermore, the outcome of present study might be constructive in designing new scale-up experiments for further studies.

CONCLUSION

The effect of alkali loading during WAO of rice straw was evaluated to minimize the chemical input. It was found to considerably affect the cellulose recovery, hemicellulose solubilization, lignin removal and TPC. This was statistically concluded through ANOVA, which exhibited a statistically significant difference in all responses with variable Na₂CO₃ loading. An effective fractionation of biomass was evident from the FT-IR spectra and XRD patterns, which was further substantiated through the increased cellulose accessibility and % saccharification at moderate alkali loading. The alkali loading of 6.5 g/L yielded favorable outcomes, which is far less than that reported in literature for AWAO of rice straw, thereby, reducing the chemical input and its cost in the pretreatment process.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at <http://www.springer.com/chemistry/journal/11814>.

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Supporting Information

Effect of chemical input during wet air oxidation pretreatment of rice straw in reducing biomass recalcitrance and enhancing cellulose accessibility

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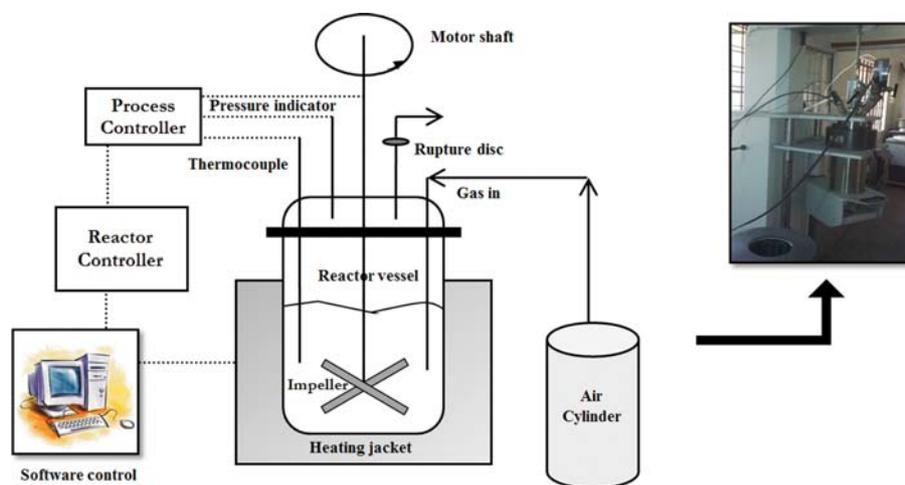


Fig. S1. Schematic representation of the high pressure-high temperature reactor used for AWAO.

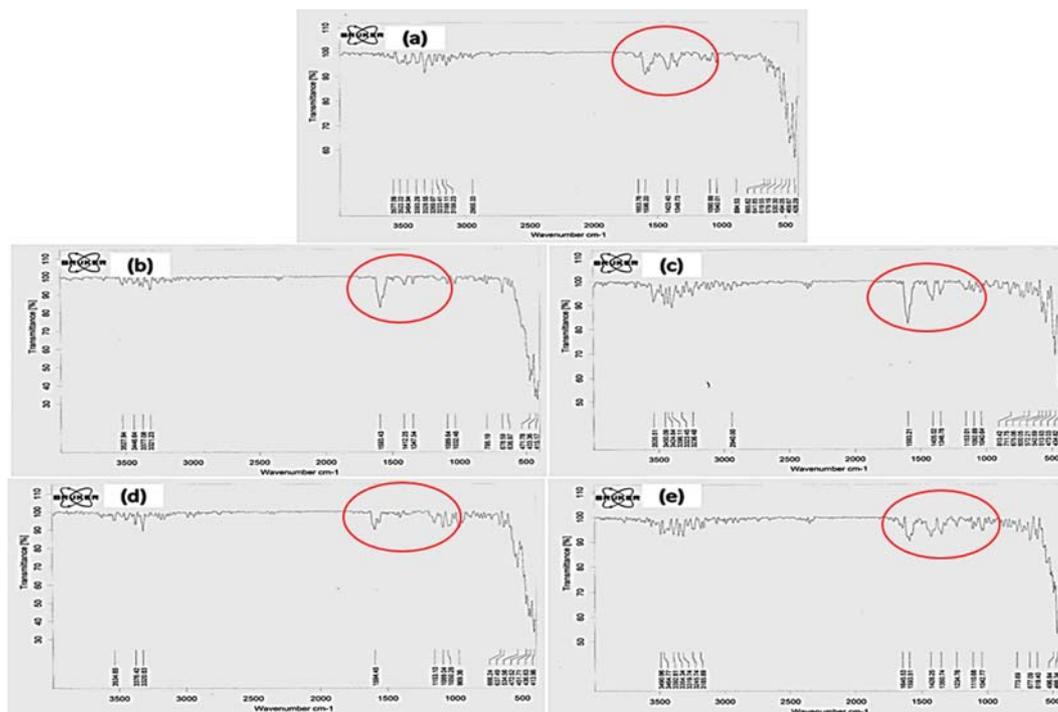


Fig. S2. FT-IR analysis showing effect of alkali loading during WAO of rice straw (a) untreated rice straw (b) without alkali, (c) 3.5 g/L Na₂CO₃, (d) 6.5 g/L Na₂CO₃ and (e) 9.5 g/L Na₂CO₃.

Table S1. FT-IR analysis showing effect of alkali loading during WAO of rice straw

Wavenumber (cm ⁻¹)	Representative functional group	Intensity of the band/Variation in the band				
		Untreated rice straw	0 g/L Na ₂ CO ₃	3.5 g/L Na ₂ CO ₃	6.5 g/L Na ₂ CO ₃	9.5 g/L Na ₂ CO ₃
884	C-O-C vibration of β -1,4 glycosidic linkage in sugar monomers i.e. glucose ring of cellulose and hemicellulose and out-of-plane bending of C-O-H [27,34]	Weak	Shift to 795 cm ⁻¹	Shift to 813 cm ⁻¹	Shift to 969 cm ⁻¹	Shift to 872 cm ⁻¹
1040	C-O, C=C, C-C-O vibration stretching and deformation bands in cellulose, hemicellulose and lignin; Pyranose ring vibration of carbohydrate [35]	Medium	Weak Shifted to 1032 cm ⁻¹	Absent	Weak Shifted to 1050 cm ⁻¹	Medium Shifted to 1042 cm ⁻¹
1090	C-O-C-O-C bonds in cellulose	Medium	Weak Shifted to 1089 cm ⁻¹	Weak Shifted to 1092 cm ⁻¹	Weak Shifted to 1089 cm ⁻¹	Weak Shifted to 1110 cm ⁻¹
1348	Symmetric CH ₂ bending and wagging in cellulose and hemicellulose [34]	Strong	Medium Shifted to 1347 cm ⁻¹	Medium Shifted to 1346 cm ⁻¹	Absent	Weak Shifted to 1350 cm ⁻¹
1423	CH ₂ bending vibration of crystalline cellulose [35,36]	Strong	Medium Shifted to 1412 cm ⁻¹	Medium Shifted to 1405 cm ⁻¹	Absent	Medium Shifted to 1428 cm ⁻¹
1596	C=C aromatic ring vibrations of lignin; Aromatic C-O stretch [14]	Strong	Strong Shifted to 1593 cm ⁻¹	Medium Shifted to 1593 cm ⁻¹	Medium Strong Shifted to 1594 cm ⁻¹	Weak Shifted to 1593 cm ⁻¹
1653	Carbonyl stretching conjugate with aromatic ring	Medium	Absent	Absent	Absent	Shifted to 1645 cm ⁻¹
3233	Intermolecular hydrogen bonding for 6-OH...O-3' in cellulose I [25]	Medium	Absent	Weak Shifted to 3236 cm ⁻¹	Absent	Absent Shifted to 3245 cm ⁻¹
3269		Medium	Absent	Absent	Absent	Absent
3464	Valence vibration of H-bonded OH group	Weak	Weak Shifted to 3446 cm ⁻¹	Weak Shifted to 3236 cm ⁻¹	Absent	Weak Shifted to 3484 cm ⁻¹
3522		Weak	Weak Shifted to 3527 cm ⁻¹	Weak Shifted to 3535 cm ⁻¹	Weak Shifted to 3534 cm ⁻¹	Absent
3577		Weak	Absent	Absent	Absent	Absent