Wet Air Oxidation Pretreatment of Mixed Lignocellulosic Biomass to Enhance Enzymatic Convertibility

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Abstract – The present work explores the potential of wet air oxidation (WAO) for pretreatment of mixed lignocellulosic biomass to enhance enzymatic convertibility. Rice husk and wheat straw mixture (1:1 mass ratio) was used as a model mixed lignocellulosic biomass. Post-WAO treatment, cellulose recovery in the solid fraction was in the range of 86% to 99%, accompanied by a significant increase in enzymatic hydrolysis of cellulose present in the solid fraction. The highest enzymatic conversion efficiency, 63% (by weight), was achieved for the mixed biomass pretreated at 195 °C, 5 bar, 10 minutes compared to only 19% in the untreated biomass. The pretreatment under the aforesaid condition also facilitated 52% lignin removal and 67% hemicellulose solubilization. A statistical design of experiments on WAO process conditions was conducted to understand the effect of process parameters on pretreatment, and the predicted responses were found to be in close agreement with the experimental data. Enzymatic hydrolysis experiments with WAO liquid fraction as diluent showed favorable results with sugar enhancement up to 10.4 g L^{-1}.

Key words: Wet Air Oxidation, Pretreatment, Enzymatic Hydrolysis, Recycled Liquid Fraction, Lignocellulosic Biomass

1. Introduction

The depletion of fossil fuels and the increasing concern over greenhouse gas emissions (GHGs) has led to the growing interest in renewable form of energy sources. Bioethanol from renewable sources has been recognized as a potential alternative to petroleum based fossil fuel which reduces the net contribution of GHGs to the atmosphere [1]. Ethanol contains 35% oxygen, which results in cleaner fuel combustion, reducing particulate and NOx emissions [2]. India is currently following a 5% ethanol blending policy with gasoline and has proposed to move towards 20% blending by 2020. The currently available feedstocks in the form of sugarcane molasses are not sufficient to meet this demand and raise the food vs fuel debate [3]. Lignocellulosic waste is one of the most abundantly available renewable raw materials in India, and holds great potential as an alternative raw material for fuel ethanol production [4]. Lignocellulosic materials such as crop residues, grasses, sawdust, wood chips, and solid animal waste can serve as a source of low cost raw material for lignocellulosic ethanol production [5]. In India, the annual production of agricultural residues such as rice husk and wheat straw is estimated to be at 22.4 t and 109.9 t, respectively [6], which can serve as potential raw materials for biofuel generation. The bioconversion of lignocellulose to ethanol involves three steps: a pretreatment process to reduce substrate recalcitrance, enzymatic hydrolysis of the cellulose and hemicellulose components to simple sugars, and fermentation of

sugars to ethanol [7]. The crystalline structure of lignocellulose in association with hemicellulose and lignin complicates the task of hydrolyzing the lignocellulosic material into fermentable monosaccharide sugars. Owing to this structural and physico-chemical barrier, pretreatment is an important step to obtain potentially fermentable sugars during enzymatic hydrolysis [8]. Pretreatment, an upstream process increases the pore size of the biomass, solubilizes hemicellulose and removes lignin, thus making the cellulose present in lignocellulosic material more amenable for enzymatic hydrolysis [9]. An efficient, cost effective pretreatment strategy can considerably decrease the process cost of lignocellulosic bioethanol production. A number of pretreatment processes have been developed and reported so far, which include physical milling and grinding, alkaline or acid hydrolysis, gas treatment (ozone, sulfur dioxide), organo-solvent treatment, steam explosion, liquid hot water, Ammonia fiber explosion (AFEX), wet oxidation, and biological treatments [10-12]. Acid hydrolysis and steam explosion suffer from the major drawback of generation of inhibitory by-products like furfural and hydroxymethyl furfural, thereby entailing a separate detoxification step, resulting in increased process cost [13]. Gas treatment, organo-solvent treatment and AFEX require addition of extraneous chemicals. In this context, wet air oxidation at elevated temperatures (125-320 °C) and pressure (5-20 bar) using a gaseous source of oxygen [14] can be a potential pretreatment strategy [15,16]. WAO being an exothermic process minimizes the energy demand and can result in effective lignin removal and lower inhibitor generation [17]. The WAO process effectively fractionates the biomass into a cellulose rich solid fraction and a liquid fraction comprised of solubilized hemicellulose sugars which can be utilized for enzymatic hydrolysis and fermentation.

We used wet air oxidation as a pretreatment approach to enrich the

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cellulose content of the lignocellulosic biomass mixture (wheat straw and rice husk in 1:1 ratio) and facilitate subsequent enhanced enzymatic convertibility of cellulose. WAO reaction conditions (temperature, pressure and reaction time) were optimized using Minitab 16 statistical software. RSM (response surface methodology) was used to explore the effect of interaction between multiple WAO parameters so as to get an optimum response in terms of enhancement of cellulose, solubilization of hemicelluloses and removal of lignin to make the cellulose easily accessible for enzymatic hydrolysis. The utility of the WAO liquid fraction as an enrichment liquid medium during enzymatic hydrolysis was also investigated.

2. Methods

2-1. Raw Material

The raw material (rice husk and wheat straw) that was harvested in early May 2012 was obtained from local farmers of Kanhan (Taluka - Parshivni, District Nagpur, India, 21°14′39″N 79°15′15″E). Wheat straw is the dry stalk of plant and rice husk is the hard protecting coverings of grains of rice. The plant age was 3 months (post-harvest). The raw material was air dried at 45 °C for 48 hours in an oven (Bio-Technics, India) to a dry matter content of 95-96% and ground (Mixer Grinder, Philips, India) to pass through +20/-80 mesh sieves. The ground and sieved raw material was stored in glass bottles capped tightly and kept at room temperature. The materials were used shortly after storage.

2-2. Wet air oxidation pretreatment

The pretreatment process was in a Wet air oxidation reactor with a working volume of 1.8 L (Model-4578, Floor Stand HP/HT Reactor, Parr Instruments, IL, USA) with constant stirring at 100_{rpm}. 30 g of the pre-dried lignocellulosic material (rice husk and wheat straw in 1:1 ratio) was mixed thoroughly with 500 ml of water and 1 g Na_2CO_3. Air pressure in the range of 5-10 bar was applied before heating the suspension. After the pretreatment the reactor was cooled to room temperature and the biomass slurry was vacuum filtered to separate the solid cellulose-enriched fraction from the liquid hemicelluloses containing filtrate. The pH of the liquid fraction was measured and the solid fraction dried and weighed. The composition of both filtration cake and filtrate was analyzed.

2-3. Analysis of solid and liquid fraction

The filter cake was washed twice with distilled water and analyzed for its total solids and moisture content after drying at 105 °C. Extractives present in biomass were determined by a Soxhlet extractor with reagent grade ethanol as solvent during 24 h run period. Mineral components were determined by dry oxidation at 575± 5 °C for 3 h. Total cellulose content in the filter cake was estimated using the Monoethanolamine method [20]. A two stage acid hydrolysis process with 72% H_2SO_4 in the first stage and then consequently diluting it to 4% was used for lignin determination. The acid soluble lignin

fraction was quantified by UV spectrophotometric method measuring the absorbance of the acid hydrolysed samples at 320 nm. The acid insoluble lignin was quantified gravimetrically after accounting for ash by dry oxidation of the hydrolyzed samples at 575 °C. All the analyses were in accordance with NREL Laboratory Analytical Procedures [19].

The total organic carbon (TOC) in the WAO liquid fraction was analyzed by PC-controlled total organic carbon analyzer (Shimadzu, Japan, Model: TOC Vcph) with automatic internal acidification and sparging for IC pretreatment. The WAO filtrate was vacuum filtered through 0.2 μm millipore filter prior to analysis.

Monosaccharide sugar profile of the WAO pretreated liquid fraction was analyzed by using HPLC. The free monosaccharide sugars of the WAO liquid fraction were separated on Aminopropyl column using Shimadzu liquid chromatograph (Model: SCL-10AVP) and detected with an RI detector (Shimadzu RID-10A). Degassed acetonitrile and HPLC grade water in the ratio 80:20 were used as the mobile phase for chromatographic separation and the column temperature was kept at 30 °C.

The heavy metals were analyzed using ICP-OES (Perkin Elmer). The WAO liquid filtrate was digested with 2% nitric acid in a microwave digester and vacuum filtered through 0.2 μm millipore filter prior to analysis.

2-4. Enzymatic convertibility

Enzymatic hydrolysis of the pretreated solid fraction of WAO was performed to determine the cellulose convertibility and the improvement in enzymatic saccharification under different WAO conditions. The enzymatic conversion was performed at 1% dry matter loading in the presence of 0.1 M citrate buffer (pH=4.8) for a period of 72 hours at 50 °C with shaking at 150_{rpm}. The three sampling times were 24, 48 and 72 hours, respectively. The substrate concentration or the dry matter loading was kept at minimum of 1% to avoid problems of improper mixing and mass transfer. Hydrolysis was performed at a cellulase loading of 45 FPU g^{-1} cellulose and β-glucosidase loading of 90 CBU g^{-1}. After hydrolysis the glucose concentration in the hydrolyzate was measured by DNS method [20] taking the absorbance at 575 nm. The percentage of cellulose in the untreated and pretreated biomass enzymatically converted to glucose was calculated as:

Enzymatic convertibility of cellulose (% ECC) = grams glucose formed/grams cellulose added × 0.9 × 100

The factor 0.9 considers the molecular mass ratio between anhydro-glucose contained in cellulose and free glucose [21].

2-5. Statistical design of experiments

The effects of WAO process parameters were studied using 2^3 factorial design. Eight different experiments were conducted varying three factors: temperature, pressure and reaction time. The level of each factor is indicated in Table 1. Earlier reported work on rice husk and shea tree saw dust [15,16] was referred to select the levels of fac-

Table 1. Statistical 2^3 factorial design for Wet Air Oxidation experiments

Factors	High level	Low level
Temperature (°C)	195	170
Pressure (bar)	10	5
Reaction time (min)	20	10

tors. Optimum conditions were determined by choosing three variables designated as X_1, X_2 and X_3, corresponding to temperature, pressure, and reaction time. The model was constructed as a function of these variables and the predicted response was a second-order polynomial as follows:

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{1,2} X_1 X_2 + \alpha_{1,3} X_1 X_3 + \alpha_{2,3} X_2 X_3 \tag{1}$$

where, Y is the measured response associated with each factor level combination; α_0 to α_3 are the regression coefficients; X_1, X_2, X_3 are the factors. The optimal process parameters for WAO were estimated by MINITAB 16 software (PA, USA). The coefficients in the second-order polynomial (Eq. (1)) were calculated by regression analysis, based on the experimentally obtained data, and then the predicted responses using Eq. (1) were obtained [15,22]. The experimental order was randomized. All the experiments were performed in duplicate and the reported results indicate the average values of the duplicated experiments.

2-6. Scanning Electron Microscope

The scanning electron microscope (SEM) imaging of untreated, pretreated and enzymatically hydrolyzed biomass at various magnifications (250 to 1000X) was done using Analytical SEM (JSM-6380LA, JEOL, Japan) kindly made available at the centralized instrumentation facility at the National Institute of Technology, Karnataka. The biomass was dried at 105 °C prior to analysis. Before SEM imaging the non-conductive biomass samples were gold sputtered for 5 min for increasing the conductivity [18].

3. Results and Discussion

3-1. Solid biomass fraction post Wet Air Oxidation pretreatment

Wet air oxidation (WAO) experiments were statistically guided using 2^3 factorial design (Table 1). The experiments were accordingly conducted and the compositional analysis for the WAO pretreated solid and liquid fraction is detailed in Table 2. The WAO pretreatment facilitated the enrichment of the cellulose content of mixed biomass by solubilization of hemicellulose and lignin fractions. After WAO pretreatment, the cellulose content of the biomass mixture enhanced up to 63% under the pretreatment condition of 195 °C, 5 bar, 20 min compared to the initial cellulose content of 38.5%. The cause for cellulose enrichment was attributable to observed 70% solubilization of hemicellulose and 46.8% lignin removal from the biomass. The cellulose recovery in the solid fraction was found in the range of 87 to 99%. At higher temperature, pressure and reaction time, the cellulose recovery was lower, possibly due to undesirable cellulose degradation [13]. The 6-carbon monosaccharide sugars such as glucose and galactose detected via HPLC (Fig. 1) are found under higher WAO conditions (V-VIII), which correlates to cellulose degradation.

The hemicellulose solubilization was found to be higher under longer reaction time and higher temperature, pressure conditions. The WAO pretreatment at 195 °C, 10 bar, 20 min resulted up to 78% solubilization of hemicellulose. Higher temperature combined with high pressure and longer reaction time resulted in high biomass charring as evident from the dark brown color of the WAO pretreated solid biomass fraction under pretreatment conditions (VI), (VII), (VIII) as detailed in Table 2.

Model equations for cellulose enrichment, lignin removal and hemicelluloses solubilization were built from the experimentally obtained data to evaluate the effect of WAO conditions. Estimation of the regression coefficients was done by multiple regression analysis of the experimentally obtained data and the following second-order polynomials were obtained:

Table 2. Composition of the solid and liquid fractions of WAO pretreated mixed biomass

Reaction products		Oven dried untreated biomass	(I)	(II)	(III)	(IV)	(V)	(VI)	(VII)	(VIII)
Solid Fraction										
Dry matter	(g)	30	21.92	21.43	22.36	22.12	17.9	17.03	16.98	16.73
Cellulose	%	38.5	52.3	53.45	50.3	51.4	60.1	63.15	59.1	61.05
Lignin	%	22.8	27.28	27.23	26.46	24.28	18.4	21.33	21.7	21.8
Hemicellulose	%	24	16	15.35	18	16.6	13.1	12.47	11.65	9.25
Degree of charring		-	No charring	No charring	light	light	Severe	Severe	Severe	Severe
Liquid fraction										
TRS	g L^{-1}	-	1.87	2.32	1.86	1.92	5.62	5.74	4.32	5.29
pH		10.93	6.41	5.6	5.71	5.1	4.81	4.80	4.47	4.27
TOC	mg L^{-1}	3,304	4,162	3,316	3685	5,235	5,380	5,265	5,210	3,304
Heavy Met	mg L^{-1}	B(8) Ca (37) Mn(1.4) Zn(0.2)	B(11) Ca(43) Mn(1.9)Zn(0.5)	B(15) Ca(52) Mn(2.5)Zn(0.8)	B(15) Ca(68) Mn(2.9) Zn(0.6)	B(18) Ca(87) Mn(3.4)Zn(0.8)	B (22) Ca(103) Mn(4.9) Zn(0.6)	B(23.6) Ca(76) Mn(4, Zn(0.8)	B(19) Ca(39) Mn(3.9) Zn(0.5)	B(8) Ca (37) Mn(1.4) Zn(0.2)

%-w/w, (I)170 °C, 5 bar, 10 mins, (II) 170 °C, 5 bar, 20 mins (III) 170 °C, 10 bar, 10 mins, (IV) 170 °C,10 bar, 20 mins (V) 195 °C, 5 bar, 10 mins, (VI) 195 °C, 5 bar, 20 mins, (VII) 195 °C,10 bar, 10 mins, (VIII) 195 °C, 10 bar, 20 mins
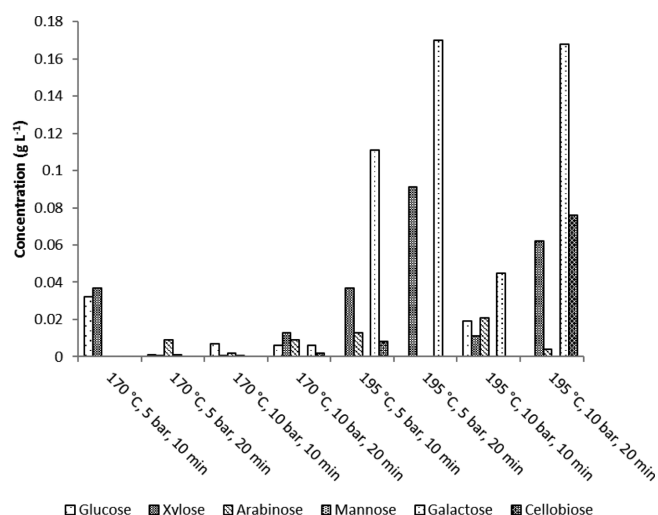
Fig. 1. HPLC sugar profile of free monosaccharide sugars obtained in WAO liquid fraction.

Cellulose enrichment % = Y_1 = $17.1437 + 0.2125 X_1 - 7.1105 X_2 + 1.65575 X_3 + 0.0394 X_1 X_2 - 0.0091 X_1 X_3 + 0.0005 X_2 X_3$, $R^2 = 1$

$$(2)$$

Lignin removal % = Y_2 = $-348.309 + 2.13714 X_1 + 8.33677 X_2 + 4.58031 X_3 - 0.0545415 X_1 X_2 - 0.0289921 X_1 X_3 + 0.113713 X_2 X_3$, $R^2 = 1$

$$(3)$$

Hemicellulose solubilization % = Y_3 = $41.3706 + 0.100739 X_1 - 19.3802 X_2 - 0.346627 X_3 + 0.10242 X_1 X_2 + 0.00225836 X_1 X_3 + 0.0478610 X_2 X_3$, $R^2 = 0.99$

$$(4)$$

When the values from X_1 to X_3 were substituted in the above equations, the predicted responses were obtained (Table 3). Note that the model equations take into consideration the linear effect of the individual parameters as well as the interaction effects of the various combinations of the process parameters. The predicted values were compared with the experimentally obtained values and the data were in close agreement.

Two-dimensional contour plots and three-dimensional response surface curves were plotted to study the interactions between the multiple parameters in WAO pretreatment of biomass mixture, and the optimum levels of each factor were determined so as to maximize the responses. Effects of individual factors on (% by weight) cellulose

content, lignin removal, and hemicellulose solubilization of the solid fraction are shown in Fig. 2. Fig. 2(a) shows the combined effect of temperature and reaction time on (%) cellulose content, where it can be concluded that higher temperatures and longer reaction times result in enhancement of cellulose content of WAO pretreated biomass. The effect of pressure is found to be insignificant in terms of cellulose enrichment (data not shown). The surface plot Fig. 2(b) emphasizes that at longer reaction times sustained at lower pressures result in more hemicellulose solubilization. An increase in temperature and decrease in pressure has a positive effect on lignin removal, as indicated in Fig. 2(c).

To gain insight into the structural changes taking place during pretreatment, scanning electron microscopy (SEM) was performed for untreated, pretreated and enzymatically hydrolysed pretreated biomass (Fig. 3) at various appropriate magnifications. The structure of the raw untreated biomass mixture at 300X can be seen to be compact (Fig. 3(a)). Fig 3(b) image represents pretreated biomass at 170 °C, 10 bar and 20 min at 270X magnification. Some disrupted structures (arrows in red) can be seen as a result of pretreatment. In Fig. 3(c) button-like structures or holes can be visible as indicated by red arrows under WAO condition of 195 °C, 5 bar, 10 min taken at 270X magnification. The biomass structure can be seen to be disrupted during the pretreatment. The holes can be the result of volatile constituents escaping from the disrupted biomass surface due to high temperature and pressure and also due to solubilization of hemicellulose and lignin. Fig. 3(d) SEM image at 250X shows the structural changes post WAO treatment (195 °C, 5 bar, 10 min) where cellulose microfibrils (arrows in red) were released due to disruption of biomass structure. Fig. 3(e) is an SEM image at 1000X post enzymatic hydrolysis of Lignocellulosic biomass mixture. Small holes (arrows in red) can be seen which may be the attachment points of the cellulose fibers to the silica sheath; the removal of cellulose fibers may have led to the formation of holes as reported by Banerjee et al. [21].

3-2. Liquid fraction post wet air pretreatment

The liquid fraction obtained post WAO pretreatment was analyzed for pH, TOC, heavy metals and total reducing sugars (TRS) (Table 2). The pH of the liquid fraction gradually decreased with the increase of temperature, pressure and reaction time. The decrease in pH can

Table 3. Experimental and Predicted results for cellulose content, lignin removal and hemicelluloses solubilisation (%w/w) in the solid fraction & pH and TRS in liquid fraction of WAO pretreated biomass mixture

| WAO Conditions	Cellulose Content		Lignin removal		Hemicellulose solubilisation		pH		TRS	
	E	P	E	P	E	P	E	P	E	P
I	52.3	52.319	12.576	12.531	51.289	51.418	1.87	2.025	6.41	6.361
II	53.45	53.431	14.687	14.733	54.312	54.184	2.32	2.165	5.6	5.649
III	50.3	50.281	13.494	13.54	44.095	43.996	1.86	1.705	5.71	5.759
IV	51.4	51.419	21.473	21.428	48.996	49.125	1.92	2.075	5.1	5.051
V	60.3	60.281	51.848	51.894	67.432	67.303	5.62	5.465	4.81	4.859
VI	59.1	59.119	46.893	46.848	70.505	70.634	5.74	5.895	4.8	4.751
VII	63.15	63.169	46.131	46.085	72.525	72.654	4.32	4.475	4.47	4.421
VIII	62.05	62.031	46.679	46.725	78.507	78.378	5.29	5.135	4.27	4.319

E: Experimental Values P: Predicted values using RSM
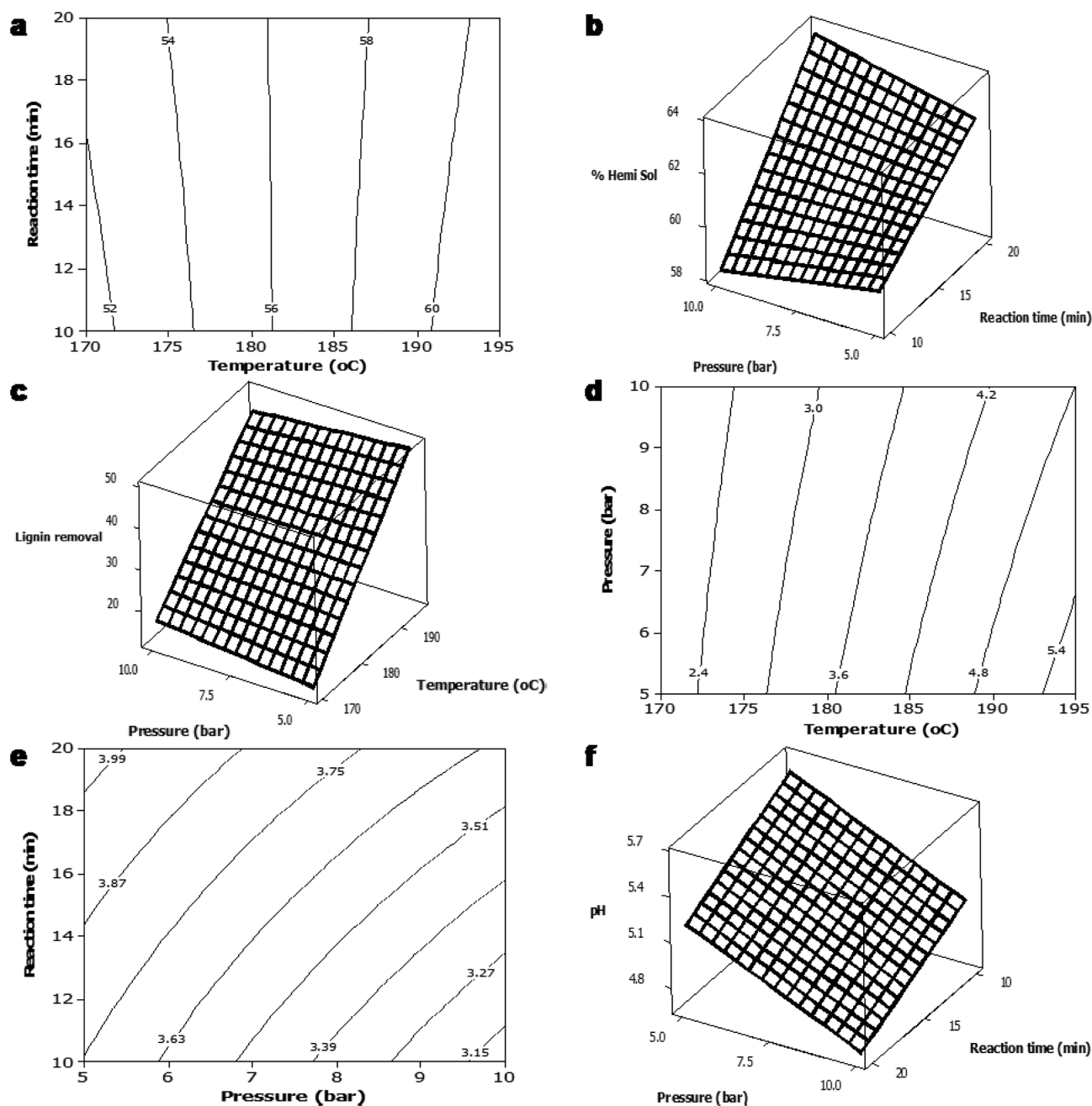
Fig. 2. Contour and surface plots of the various responses in WAO pretreated mixed biomass solid fraction and liquid fractions (a) Cellulose (%) vs Time& Temperature (b) Surface plot % Hemi. Sol vs Pressure & Time (c) Surface plot of % Lignin removal vs Pressure & Temperature (d) TRS in liquid fraction vs Pressure & Temperature (e) TRS in liquid fraction vs Time & Pressure (f) pH of liquid fraction vs Pressure and Time.

be attributed to the formation of carboxylic acids as degradation products under severe WAO conditions. The pH as low as 4.27 was found under the most severe WAO condition (195 °C, 10 bar, 20 min).The total organic carbon of the liquid fraction is indicated in Table 2. The solubilization of hemicelluloses, formation of carboxylic acids, phenols and furans accounted for the TOC. A high TOC of the liquid fraction signifies allow lignin content in the WAO pretreated biomass solid fraction [23].

A high TRS of 5.74 g L^{-1} was obtained under the WAO condition (VI). A low cellulose recovery in the WAO solid fraction of 87%,

high hemicelluloses solubilization 70.5% corresponding to high TOC of 5380 mg L^{-1} contributed to the high TRS yield. The monosaccharide sugar profile of the WAO pretreated liquid fraction was analyzed by HPLC (Fig. 1). The 6-carbon sugars such as glucose and galactose were found to be predominant at more severe WAO conditions, which indicates degradation of the cellulose in the solid biomass fraction at more severe conditions [13]. The hexose sugars were mostly preserved in the solid fraction as cellulose recovery was in the range of 87% to 99%. The 5-carbon sugars such as xylose and arabinose were found in liquid fraction at severe WAO conditions, suggesting effec-
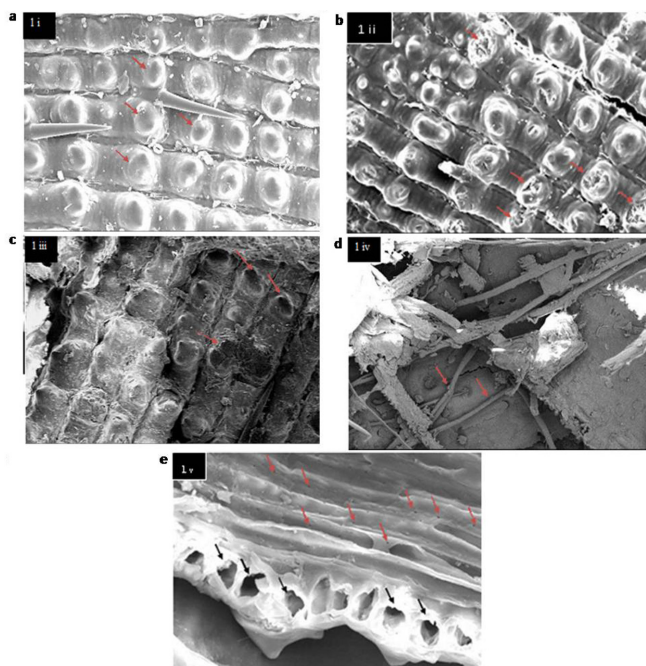
Fig. 3. SEM images at various magnifications for structural changes in biomass (a) untreated biomass (b) pretreated biomass at 170 °C, 10 bar and 20 min at 270X magnification (c-d) pretreated biomass at 195 °C, 5 bar, 10 min taken at 270X magnification (e) enzymatic hydrolysed biomass at 1000X magnification.

tive solubilization of the hemicellulose from the solid biomass fraction. Free monosaccharide sugars were found in higher concentrations corresponding to WAO runs (V-VII); this may be attributed to higher degree of hydrolysis of solubilized hemicellulose sugars in the liquid fraction under given WAO conditions. As reported by Palonin *et al*. [24], the WAO process oxidizes the monosaccharide sugars to carboxylic acids, while the oligosaccharides are more resistant to oxidation due to glycosidic linkages. Hence, the monosaccharide sugars were detected in trace amounts, suggesting that most of the sugars may be present in oligosaccharide form after WAO pretreatment. Hence, the WAO process catalyzed the high solubilization of the hemicellulose sugars from the solid fraction but was unable to result in cause a major hydrolysis of the solubilized hemicellulose molecules into monosaccharides [25]. The heavy metals were analyzed using ICP-OES indicated that Ag, Pb, Hg were not detected which could be inhibitory to the enzymes during enzymatic hydrolysis [26].

Model equations for TRS yield and pH were built from the experimentally obtained data to evaluate the effect of WAO conditions. Regression coefficients were estimated by multiple regression analysis of the experimentally obtained data and the following second order polynomials were obtained:

TRS yield (g L^{-1}) $= Y_4 = -23.5410 + 0.1528X_1 + 0.8012X_2$
$- 0.2062X_3 - 0.00536X_1X_2 + 0.00116X_1X_3 + 0.0046X_2X_3$, $R^2 = 0.991$

$$(5)$$

pH $= Y_5 = 23.1343 - 0.0909 X_1 - 0.345900 X_2 - 0.48315 X_3$
$+ 0.00132X_1X_2 + 0.00242X_1X_3 + 0.0001X_2X_3$, $R^2 = 0.994$ (6)

Two-dimensional contour plots and three-dimensional surface plots (Fig. 2) are plotted for TRS and pH to study the interactive effect of multiple factors during WAO pretreatment on the responses. The contour plot (Fig. 2) (d,e) emphasizes the positive effect of temperature and time on the TRS at relatively low oxygen pressure. The surface plot (Fig. 2(f)) illustrates that the pH gets lowered with longer reaction time and higher air pressure.

Concurrence of the experimentally determined TRS yields and pH values for different pretreatment conditions with the modelled output is given in Table 3.

3-3. Statistical analysis of results

The measured response values corresponding to the statistical design of experiments were determined (Table 4). P-values provide a valuable tool to assess the significance of each of the coefficients determined in the model, moreover, to help understand the presence of any underlying significant interactions among the variables. High T and low P values are indicative of the presence of significant coefficients in the developed model. Temperature was found significant on cellulose content (P = 0.003), while pressure is also significant on hemicellulose solubilization (P = 0.007).

All the main effects (except reaction time) and the interaction effects are significant on lignin removal (P < 0.05). This study points to dependency of lignin removal on multiple parameters; however, a more rigorous experimental design is required to adequately quantify the extent of interaction observed, The summary of analysis of variance (ANOVA) representing the results is discussed Table 5.

ANOVA is required to test the significance and adequacy of the models. The Fisher's variance ratio (F-value) is the measure of varia-

Table 4. Estimated t-values and p-values from response surface regression analysis

	t-value			p-value		
	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Lignin
Constant	3013.66	473.375	695.52	0.00	0.001	0.001
X_1	247.66	87.631	354.42	0.003	0.007	0.002
X_2	11.66	0.568	4.85	0.054	0.671	0.129
X_3	-0.33	16.482	15.57	0.795	0.039	0.041
X_1X_2	65.66	24.855	-37.37	0.01	0.026	0.017
X_1X_3	-30.33	1.096	-39.72	0.021	0.471	0.016
X_2X_3	0.33	4.646	31.16	0.795	0.135	0.020

X_1: Reaction Temperature (ºC) X_2: Pressure (bar) X_3: Reaction time (min)

Table 5. Analysis of Variance for polynomial model obtained from experimental design

Cellulose content

Source	DF	SS	MS	F	P
Main effect	3	187.6	31.2	11117.89	.007
2 - way interaction	3	14.7	4.9	1744.1	.018
Residual error	1	0.003	0.003		
Total	7	187.6			

Lignin removal

Source	DF	SS	MS	F	P
Main effect	3	2094.8	2090.4	125613.5	0.002
2 - way interaction	3	65.67	21.89	1315.3	0.02
Residual error	1	0.02	0.02		
Total	7	2160.57			

Hemicellulose solubilisation

Source	DF	SS	MS	F	P
Main effect	3	1054.8	351.6	2650.4	0.014
2 - way interaction	3	84.97	28.32	213.52	0.05
Residual error	1	0.13	0.13		
Total	7	1139.9			

DF=Degrees of Freedom. SS=Sum of squares. MS=Mean Square. F=F values. P=p values

tion in the data about the mean. Here the ANOVA of the multiple regression revealed that the quadratic models derived from the factorial design could adequately be used to predict the responses as evident from the high F values. In addition, the multiple correlation coefficients (R^2) of the regression equations obtained from ANOVA were 1 for cellulose content and lignin removal and 0.99 for hemicellulose solubilization. This was indicative of a good fit by the model with only a maximum of 1 % variation unexplained with respect to hemicellulose solubilization. Close agreement between the predicted and experimental values as indicated previously is shown in Table 3. The responses obtained at optimal conditions by both model and experimental obtained results are shown in Table 6, corresponding to WAO condition-V was in close agreement.

3-4. Enzymatic hydrolysis of WAO pretreated biomass

The WAO pretreated biomass mixture was enzymatically saccharified to determine the efficiency of pretreatment in terms of glucose yield and cellulose convertibility (Fig. 4-5).
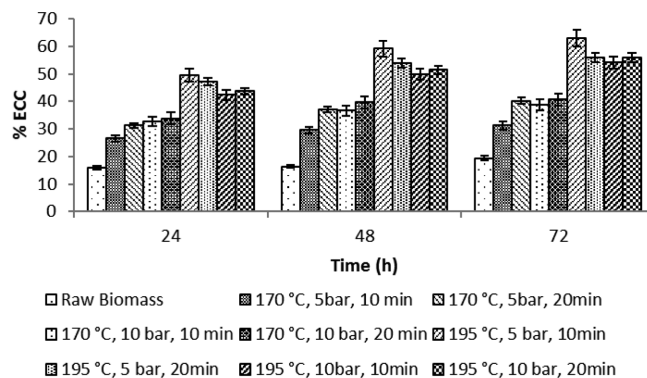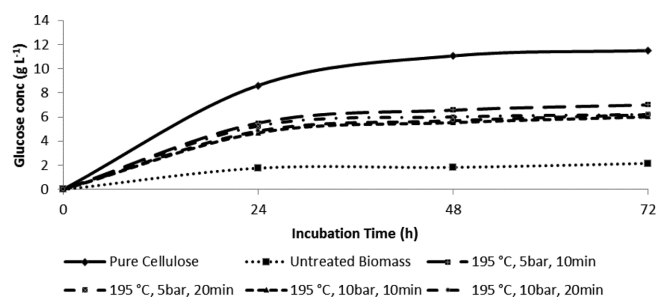
The glucose yield was in the range of 8-22.6 g per 100 g raw biomass and the cellulose convertibility was 31.2% to 63%. The optimal pretreatment condition was WAO-V because the cellulose convertibility of 63% as well as the glucose yield was 22.6 g per 100 g raw biomass. The highest glucose yield and cellulose convertibility resulted

Fig. 4. Enzymatic cellulose convertibility of raw and pretreated biomass at different WAO conditions.

Fig. 5. Glucose concentration after hydrolysis (45 FPU/g cellulase and 90 CBU/g β-glucosidase) of WAO pretreated solid fraction (in buffer) at 50 °C (1% DM loading).

due to the maximum lignin removal of 51.8% and hemicellulose solubilization of 67.4% during pretreatment. The enzymatic hydrolysis is limited by lignin content of biomass [5], as it is reported to irreversibly bind to the hydrolytic enzymes rendering them inaccessible for further enzymatic action [3]. Enzymatic convertibility of about 28% for WAO pretreated rice husk has been reported by Saumita *et al*. [21]; while Schmidt *et al*. [13] reported 66% enzymatic convertibility of wheat straw. Martin *et al*. [25] reported 57.4% and 48.9% convertibility of cellulose using wet oxidation and steam explosion, respectively, for sugarcane bagasse. Steam exploded wheat straw facilitated 51% cellulose conversion; while only 16% conversion has been achieved for acid pretreated wheat straw as reported by Kristensen *et al*. [27]. In our findings, wet air oxidation pretreatment facilitated up to 63% convertibility of the biomass mixture, which is similar or higher than that earlier reported for rice husk and wheat straw.

The utility of WAO pretreated liquid fraction as a diluent medium

Table 6. WAO optimised condition for biomass mixture

Factors	Optimum condition	Experimental condition	Response for solid fraction						Response for liquid fraction			
			% Cellulose content		%Hemicellulose solubilisation		% Lignin removal		pH		TRS (g L^{-1})	
			P	E	P	E	P	E	P	E	P	E
Temperature (°C)	195	195										
Pressure (bar)	5.5	5	60.3	60.3	68.3	67.4	50.6	51.8	4.7	4.8	5.4	5.6
Reaction time (min)	11.5	10										

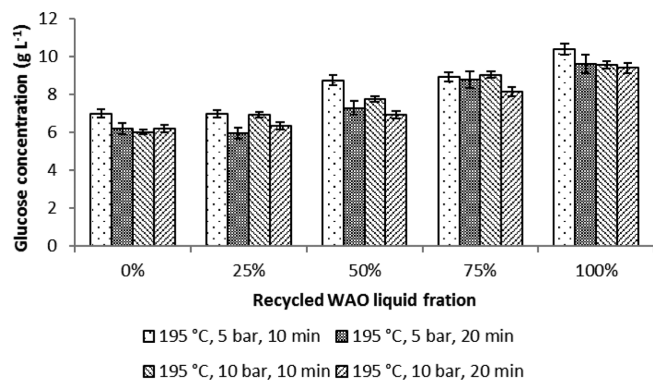E: Experimental Values P: Predicted values using RSM

Fig. 6. Recycled WAO liquid fraction as diluent for enzymatic hydrolysis of pretreated biomass.

for enzymatic hydrolysis was also evaluated (Fig. 6). Enzymatic hydrolysis experiments were performed using different proportions of WAO liquid fractions (0%, 25%, 50%, 75%, 100%) respectively in buffer medium. The glucose yield enhanced up to 10.4 g L^{-1} in WAO liquid fraction medium (100% recycle medium) compared to the yield of 7 g L^{-1} in buffer medium under WAO condition-V. The result indicates that inhibitors generated during the WAO in the liquid fraction were below the reported toxicity limits, and hence the WAO liquid fraction can be used for enhanced enzymatic hydrolysis without any prior detoxification step.

Conclusions

The wet air oxidation pretreatment of mixed lignocellulosic biomass of rice husk-wheat straw (1:1 mass ratio) was found to advantageous in disrupting the inherent structure of the biomass by removing lignin up to 52% and up to 67% solubilization of hemicellulose, resulting in enhanced enzymatic hydrolysis efficiencies. SEM analysis of the biomass revealed significant changes to the structural configuration due to pretreatment. A preliminary statistical design of experiments was performed, indicating significant effects of process parameters on the pretreatment efficiencies having a good fit with model parameters. Post WAO, enzymatic conversion efficiencies were observed to enhance up to 63% compared to only 19% in the untreated biomass. As inhibitors generated during the pretreatment process were at sub-inhibitory levels, the liquid fraction could be used directly without any prior detoxification step. An overall sugar enhancement of 10.4 g L^{-1} was observed. Further studies to capture the interactive effects on process parameters on the various steps outlined can be performed to arrive at better understanding of WAO as a potential pretreatment process.

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