SURFACTANT-ASSISTED MICROWAVE-ACID PRETREATMENT OF LEAF LITTER BIOMASS FOR ENHANCED ENZYMATIC RELEASE OF SUGARS

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Leaf litter biomass from *Eucalyptus globulus* was pretreated using a novel surfactant assisted microwave-acid pretreatment (SAMAP) technique for enhanced enzymatic release of sugars. Screening of the parameters for the SAMAP technique was carried out using the Taguchi experimental design, followed by enzymatic saccharification. The critical parameters identified were time, acid concentration and surfactant concentration. To optimize the saccharification of SAMAP biomass, the combined effects of the significant parameters were further investigated using response surface methodology (RSM). As compared to the reducing sugar yield of 0.05 g/g in native biomass, enzymatic hydrolysis of the recovered solid fraction of SAMAP biomass (0.5 mm) showed a 93% increase in reducing sugar (0.69 g/g), when treated with 1.25% H_2SO_4 and 0.2% Tween-80 followed by 80 FPU/g of enzymatic hydrolysis for 42 h. The composition of native biomass was 31% cellulose, 18% hemicellulose and 12% total lignin. Delignified SAMAP biomass after recovery showed a 33% increase in cellulose content, whereas hemicellulose and lignin content was reduced by 39 and 66%, respectively. Native and pretreated biomass was characterized by scanning electron microscopy (SEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), Fourier transform infrared (FTIR) and solid state ^{13}C cross polarizing magic angle spinning (CP/MAS) NMR spectroscopy.

Keywords: hydrolysis, leaf litter biomass, microwave, pretreatment, surfactant

INTRODUCTION

Lignocellulosic biomass is the most abundant, incredible, renewable, low-cost and promising substrate for bioethanol production. Agricultural residues serve as a low-cost feed stock for the production of biofuels.[1] Utilization of these feed stocks for biofuel production requires the development of pretreatment techniques to break up lignin structures and to enhance enzymatic saccharification of cellulose.[2] Due to the deep encapsulation of cellulose by lignin and hemicelluloses, the highly crystalline structure of native lignocelluloses resists enzymatic hydrolysis for bioconversion to sugars and further to ethanol. Pretreatment is an essential step towards the development and industrialization of efficient second generation lignocellulosic ethanol processes.[3] The major challenge involved in the pretreatment of lignocelluloses is to develop novel strategies to improve the enzymatic release of sugars for bioethanol production. Pretreatment processes separate lignin from polysaccharides and reduce cellulose crystallinity making the glycoside bonds more accessible to the hydrolytic enzymes.[4] The pretreatment of lignocellulosic biomass using acid, alkali, steam explosion, hot water and ammonia fibre explosion (AFEX) techniques has been tested for different types of biomass, however it cannot be used for all feedstocks, because of the large variation in the composition of biomass from different sources.[5]

Several reports are available on the pretreatment of lignocellulosic biomass using acid,[1] alkali,[6] ionic liquids,[7] organosolvent,[8] organic acids,[9] microwave,[10] ultrasound,[11] biological[12] and combined pretreatment.[13]

Eucalyptus species managed as short-rotation crops for bioenergy are of increasing interest in many parts of the world.[14] Out of 300 species of *Eucalyptus* all over the world, *Eucalyptus globulus*, an evergreen tree, frequently found growing in India in a cool, moist, equitable climate and deep soil.[15] The tree bears a lot of

leaves, which are simple, entire, gland-dotted, and pendulousite. It plays an important role in cellulose production,[16] and may be used as potential feedstock for sugars.

The use of microwave radiation is a promising pretreatment process, which utilizes thermal and nonthermal effects generated by microwaves in aqueous environments,[17] as it breaks down the lignin-hemicellulose complex and exposes more of the accessible surface area of cellulose to cellulase.[18] Surfactant-assisted ultrasound pretreatment of sugarcane tops,[19] microwave-assisted alkali pretreatment of corn cob[20] and microwave-assisted alkali pretreatment of switch grass[17] have also been reported for improved enzymatic release of sugars. However, surfactant-assisted microwave-acid pretreatment (SAMAP) of lignocellulosic feedstock has not been investigated so far to enhance the enzymatic hydrolysis to the best of our knowledge.

The objective of the present study was to develop a novel process for enhancing the reducing sugar yield from *Eucalyptus* leaf litter biomass by adopting the SAMAP technique. The optimization of process parameters for pretreatment and hydrolysis was done using the response surface methodology (RSM) and the characteristics of native and pretreated samples were investigated by scanning electron microscopy (SEM), x-ray diffraction (XRD), thermal gravimetric analysis (TGA), Fourier transform infrared (FTIR) spectroscopy and solid state cross polarizing magic angle spinning (CP/MAS) ^{13}C NMR spectroscopy.

EXPERIMENTAL

Leaf litter biomass

Eucalyptus globulus leaf litter was collected from Thapar University campus, Patiala, Punjab (India), located at 30°19'48"N, 76º24'0"E and 310 m above the sea level. Leaf litter was washed to remove adhered debris, dried, ground and sieved to a particle size of 0.5 mm. The sieved sample was stored in an air tight container at room temperature for further use. The compositional analysis of native and pretreated leaf litter was carried out by two-step acid hydrolysis as per standard protocol from National Renewable Energy Laboratory.[21]

Pretreatment of leaf litter biomass

Primary screening

Pretreatment was performed in 150 ml stoppered conical flasks with biomass loading of 10% (w/v). The samples were soaked with different surfactants (1% w/v), such as Tween-80, Tween-40, Tween-20, Triton X-100, PEG 6000, SDS and CTAB, for 60 min. Soaked biomass samples (10% w/v) were placed in a proprietary express vessel and subjected to microwave pretreatment using a Microwave Accelerated Reaction System (MARS6®, CEM, Buckingham, United Kingdom) with an operating frequency of 2450 MHz and power range of 100-1800 W. The pretreatment was initially carried out for 60 s at 200 W and 150 °C and then the samples were autoclaved (121 °C, 15 lb) for 60 min. The samples were neutralized using tap water devoid of sugar in two consecutive wash runs and then dried at room temperature (32 ± 2 °C). Reducing sugar was analysed in the native and recovered solid fraction of biomass using the 2,5-dinitrosalicylic acid method.[22]

Optimization of process parameters for pretreatment by the Taguchi experimental design

The Taguchi statistical design was used to find out the best pretreatment conditions for SAMAP of *Eucalyptus* leaf litter biomass. According to the orthogonal array, five variables in 16 experiments were used to evaluate the pretreatment efficiency. The design and execution of the experiments were carried out to generate experimental data considering all possible interactions between different process variables (Table 1). Design-Expert statistical software (Version 8.0.7.1, Stat-Ease, Minneapolis, Minn. USA) was used for experimental design and validation. The pretreated samples were washed with tap water, dried at room temperature (32 ± 2 °C) and reducing sugar was estimated in recovered biomass as per the protocol discussed earlier.[22]

Characterization of native and pretreated leaf litter biomass

Scanning electron microscopy (SEM)

Physical changes in native and SAMAP biomass were observed by SEM. Images of the native and treated biomass were taken using SEM (Model: JEOL JSM-6510 LV, USA) at a magnification of 1,000X. The samples were coated with gold using gold sputter at a voltage of 10-15 kV.

X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) analysis revealed the crystallinity of native and SAMAP biomass. The samples were analyzed at room temperature in the scanning angle of 5-50° at the scan speed of 5° min^{-1} using a PANalytical X'Pert PRO diffractometer (Netherlands) with Ni-filter, operated at 45 KV and 40 mA with λ (Cu Kα) = 1.5406 Å. The crystallinity index (*CrI*) of the leaf litter biomass was calculated and determined as described by Segal *et al.*[23] as follows:

$$CrI = [(I_{002}-I_{am})/I_{002}] \times 100$$

where I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ of 22.5°, and I_{am} is the peak for the amorphous portion (i.e., cellulose,

hemicellulose, and lignin) at about 2θ of 18° in most literatures.[24-25]

Thermal gravimetric analysis (TGA)

To understand the devolatilization characteristics, the TGA of native and SAMAP biomass was performed using a Mettler Toledo instrument (Model: TGA/SDTA 851e). The devolatilization of biomass was studied from 20 to 700 °C at a heating rate of 10 °C min^{-1} with purge gas (Nitrogen) flow rate of 60 ml min^{-1}.

Fourier transform infrared (FTIR) spectroscopy analysis

Fourier transform infrared (FTIR) spectroscopy analysis was carried out to detect changes in the functional groups of native and SAMAP biomass. The spectra were obtained using a Nicolet instrument (Model: MAGNA 550, USA). The biomass (10 mg) was mixed well with 200 mg of KBr and the mixture was compressed for preparing the pellets. Each spectrum was the average of 64 scans with a total scan time of 15 s in the IR range of 400-4000 cm^{-1} at 1 cm^{-1}.

Solid state CP/MAS ^{13}C NMR spectroscopy

Solid state ^{13}C CP/MAS NMR spectroscopy is an important tool to analyze the structural features of lignocellulosic biomass. Solid state ^{13}C CP/MAS NMR spectra of native and SAMAP biomass were obtained at 75.475 MHz using a Bruker AV-300 FT NMR spectrometer (Germany). Dry samples were spun throughout at a rate of 10 kHz using 4 mm zirconia (ZnO_2) rotor with CP pulse programme. Typically 18,000 transients were accumulated in the time domain at a contact time of 1 ms and a recycle delay of 3 s. Tetramethylsilane (TMS) was used as an external standard for the calibration of the ^{13}C chemical shift.

Enzymatic hydrolysis

Enzymatic saccharification of SAMAP leaf litter biomass was carried out in 150 ml stoppered conical flasks by incubating 10% (w/v) of biomass with commercial cellulase (Zytex India Private Limited, Mumbai, India). The enzyme loading was 50 filter paper units (FPU)/g of pretreated biomass, 20 µl of 1x antibiotic solution (Penicillin-Streptomycin cocktail), 0.2% w/v surfactant (Tween-80) and 0.5% v/v acid (H_2SO_4) concentration. Total reaction volume was made up to 20 ml with citrate buffer (100 mM, pH 4.8). Samples were incubated at 50 °C for 42 h under shaking conditions (120 rpm). Samples were centrifuged (10,000 rpm, 10 min) after incubation to remove unhydrolyzed biomass and reducing sugar was analyzed as discussed earlier.[22] The reducing sugar yield was expressed in g/g pretreated biomass, taking into consideration the material loss during the pretreatment stage.

Optimization of enzymatic saccharification by Box-Behnken design

Box-Behnken (BB) design was applied to study the effect of independent variables on the response and interaction of factors with different combination of variables. The critical parameters that affected enzymatic hydrolysis were enzyme loading, acid concentration, surfactant concentration and incubation time.

Table 1
Taguchi design for optimization of SAMAP leaf litter

Runs		Factors					Reducing sugar (g/g)	
Nr	Order	A: Time (min.)	B: Temperature (°C)	C: Power (watt.)	D: Acid (% v/v)	E: Surfactant (% w/v)	Experimental	Predicted
7	1	10	50	200	0.5	0.05	0.211	0.208
12	2	10	100	400	1	0.1	0.254	0.269
5	3	10	150	600	1.5	0.15	0.261	0.266
14	4	10	200	800	2	0.2	0.301	0.283
6	5	20	50	400	1.5	0.2	0.398	0.413
4	6	20	100	200	2	0.15	0.443	0.44
2	7	20	150	800	0.5	0.1	0.465	0.447
9	8	20	200	600	1	0.05	0.487	0.492
16	9	30	50	600	2	0.1	0.311	0.316
13	10	30	100	800	1.5	0.05	0.421	0.403
15	11	30	150	200	1	0.2	0.502	0.521
11	12	30	200	400	0.5	0.15	0.478	0.484
8	13	40	50	800	1	0.15	0.377	0.359
3	14	40	100	600	0.5	0.2	0.412	0.417
1	15	40	150	400	2	0.05	0.198	0.214
10	16	40	200	200	1.5	0.1	0.168	0.165

Table 2
Box-Behnken design for enzymatic hydrolysis

Runs		Factors				Reducing sugar (g/g)	
Nr	Order	A: Acid (% v/v)	B: Surfactant (% w/v)	C: Enzyme (FPU/g)	D: Time (h)	Experimental	Predicted
5	1	1.25	0.125	20	24	0.164	0.149
7	2	1.25	0.125	20	60	0.256	0.216
14	3	1.25	0.2	20	42	0.321	0.351
27	4	1.25	0.125	50	42	0.562	0.565
3	5	0.5	0.2	50	42	0.612	0.618
26	6	1.25	0.125	50	42	0.576	0.565
20	7	2	0.125	80	42	0.583	0.594
21	8	1.25	0.05	50	24	0.317	0.359
28	9	1.25	0.125	50	42	0.581	0.565
16	10	1.25	0.2	80	42	0.689	0.664
6	11	1.25	0.125	80	24	0.354	0.375
12	12	2	0.125	50	60	0.446	0.484
4	13	2	0.2	50	42	0.563	0.570
24	14	1.25	0.2	50	60	0.563	0.534
13	15	1.25	0.05	20	42	0.301	0.330
22	16	1.25	0.2	50	24	0.376	0.385
15	17	1.25	0.05	80	42	0.587	0.561
9	18	0.5	0.125	50	24	0.384	0.349
11	19	0.5	0.125	50	60	0.41	0.439
8	20	1.25	0.125	80	60	0.539	0.534
29	21	1.25	0.125	50	42	0.566	0.565
1	22	0.5	0.05	50	42	0.512	0.486
2	23	2	0.05	50	42	0.602	0.577
17	24	0.5	0.125	20	42	0.298	0.300
10	25	2	0.125	50	24	0.374	0.348
19	26	0.5	0.125	80	42	0.563	0.584
23	27	1.25	0.05	50	60	0.432	0.436
18	28	2	0.125	20	42	0.342	0.334

In the BB experimental design, the total number of experimental combinations is $N=K^2+K+C_P$, where K is the number of independent variables and C_p is the rotatable central point number. Table 2 presents 29 experimental runs, designed for optimizing enzymatic saccharification, in which each variable was tested at three different levels coded as -1, 0 and +1, corresponding to lower, middle and higher values, respectively.

In the developing regression equation, factors were coded according to the equation:

$$X_I = (x_i - x_{oi})/x_i$$

where X_I is the coded value of i^{th} independent variable, x_i is the natural value of i^{th} independent variable; x_{oi} is the natural value of the i^{th} independent variable at the central point. Reducing sugar yield was determined for each trial and a second order polynomial was fitted to the response data obtained from the design. The general polynomial equation is as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2$$

where Y is the predicted response (reducing sugar in g/g of biomass), β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient for the interaction effect, and X_I is a dimensionless coded value of x_i (independent variable).

RESULTS AND DISCUSSION

The composition analysis of native and SAMAP biomass was carried out to determine cellulose, hemicellulose and total lignin content. Native biomass contained 31.25% cellulose, 17.58% hemicellulose, 16.34% total lignin and reducing sugar of 0.05 g/g, which achieved values of 46.56, 10.76, 4.18% and 0.50 g/g, respectively, for SAMAP biomass (Table 3). The recovery of the SAMAP biomass was of 64% after pretreatment, which was mainly due to the removal of hemicellulose and lignin. SAMAP biomass showed an increase in cellulose and reducing sugar content by 33 and 90%, respectively, and a decrease in hemicellulose and lignin content by 39 and 74%, respectively. Identical observations were earlier reported with

surfactant-assisted ultrasound pretreatment of sugarcane tops[19] and alkali-assisted microwave pretreatment of cotton plant residue.[13]

Primary screening

Among the surfactants screened, Tween-80 was found to be the most effective agent in terms of reducing sugar yield (0.426 g/g). Other surfactants, such as Tween-40 and Tween-20, were also found to be potent, but the reducing sugar yields were of 0.378 g/g and 0.393 g/g, respectively, i.e. lower than that obtained by the Tween-80 pretreatment. PEG 6000, Triton X-100 and CTAB gave almost the same reducing sugar yield (0.349, 0.346 and 0.353 g/g). The positive effect of Tween on the pretreatment of corn stover and sugarcane tops was also reported by Qing *et al.*[26] and Sindhu *et al.*[19] The non-ionic surfactant improves cellulose conversion by increasing the available cellulose surface area and preventing nonspecific binding of cellulase enzyme.[26]

Effect of different process parameters for pretreatment by Taguchi experimental design

The Taguchi design was adopted for optimizing selected parameters, such as time, temperature, power, acid concentration (v/v) and surfactant concentration (w/v). Run order 2 and 3 gave nearly identical reducing sugar yields (0.254 and 0.261 g/g). The maximum reducing sugar was observed to be 0.502 g/g achieved under the conditions of run order 11 (Table 1), which were selected for further enzymatic hydrolysis studies.

Table 3
Cellulose, hemicellulose, total lignin, reducing sugar and *CrI* of native and SAMAP biomass

Biomass	Cellulose (%)	Hemicellulose (%)	Total lignin (%)	Reducing sugar (g/g)	*CrI* (%)
Native	31.25	17.58	12.34	0.05	32.23
SAMAP	46.56	10.76	4.18	0.50	45.5

Table 4
Analysis of variance (ANOVA) for selected factorial mode of Taguchi design

Source	Sum of squares	df	Mean square	F value	Probability > F	
Model	0.299882	12	0.02499	30.47267	0.0084	Significant
A: Time	0.167349	3	0.055783	68.02103	0.0029	
B: Temperature	0.0135	3	0.0045	5.487349	0.0979	
D: Acid	0.060295	3	0.020098	24.50777	0.013	
E: Surfactant	0.058737	3	0.019579	23.8754	0.0135	
Residual	0.00246	13	0.00082			
Cor. Total	0.302342	15				

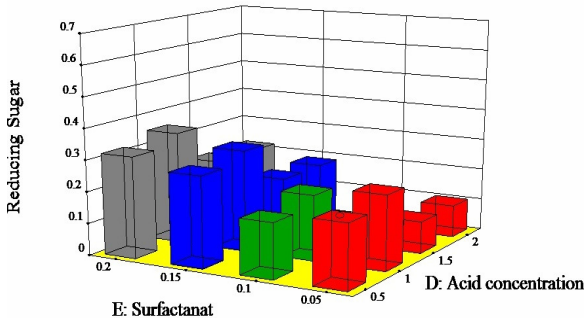

Figure 1: 3D-contour plot showing the interaction between acid and surfactant concentration

Among all the variables studied, the analysis of variance (ANOVA) results predicted three statistically significant factors such as time, acid concentration and surfactant concentration. The F-value computed for the Taguchi model was 30.47, which reflected the strength of the model (Table 4). The analysis of sugar yield from SAMAP biomass using the Taguchi design revealed that

time, acid concentration and surfactant concentration contributed to the maximum impact with P (probability) > F less than 0.05 among all selected factors. Predicted R^2 (0.76) and adjusted R^2 (0.95) were in a reasonable agreement with each other.

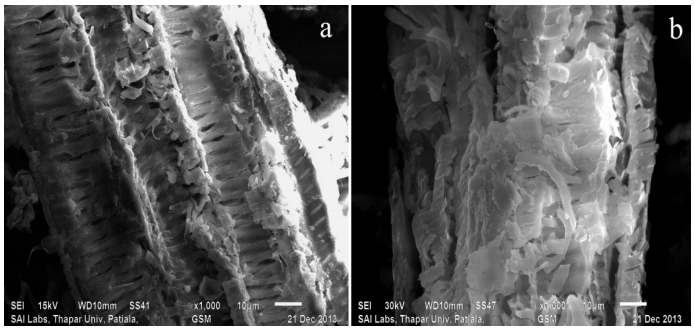

Figure 2: Scanning electron micrographs of (a) native and (b) SAMAP biomass (magnification: 1,000X)

The model has an adequate precision of 19.874, this suggests that the model can be used to navigate the design space. The adequate precision value is an index of the signal to noise and a value >4 is an essential prerequisite for a model to be a good fit. The effect of interaction between surfactant concentration and acid concentration on the reducing sugar yield of SAMAP biomass is depicted in Figure 1.

Non-ionic surfactants modify the biomass surface and interact hydrophobically with lignin to remove it and increase the accessible area of cellulose.[26] An identical observation was reported earlier for the surfactant-assisted ultrasound pretreatment of sugarcane tops.[19] Dilute acid pretreatment was reported to break down the lignin shield surrounding lignocellulose and cause hemicellulose solubilization to open the structure of biomass, which improved enzyme accessibility to cellulosic fraction.[27] A decrease in sugar yield was observed with an increase in acid concentration beyond 1%, as it imposed a negative effect, which may be due to the formation of inhibitors, such as furfural, acetic acid and hydroxyl-methyl furfural.[28]

The effect of non-ionic surfactant assisted dilute acid pretreatment of wheat straw on enzymatic hydrolysis and simultaneous saccharification and fermentation for ethanol production was reported by Qi *et al*.[29] A comparison of three types of microwave pretreatment using sugarcane bagasse as lignocellulosic biomass has been made to evaluate the enzymatic saccharification efficiency and lignin removal.[10] A comparative study on alkali-assisted microwave pretreatment of cotton plant residue and high pressure reactor pretreatment, as well as modelling of the alkali-assisted microwave pretreatment followed by the evaluation of enzymatic saccharification, was also carried out by Vani *et al*.[13]

Characterization of native and SAMAP biomass

SEM and XRD analyses

SEM observation of untreated (Fig. 2a) and SAMAP biomass (Fig. 2b) showed that the pretreatment induced structural changes in the biomass. SEM revealed an ordered and compact structure, which was destroyed in the pretreated biomass. Similar structural changes were observed for sugarcane bagasse pretreated with microwave alkali pretreatment[10] and sugarcane tops pretreated by ultrasound assisted with a surfactant.[19]

The XRD profile of native and SAMAP biomass is presented in Figure 3. *CrI* in native and SAMAP biomass was 32.23 and 45.5%, respectively (Table 3). A significant increase (13.27%) in *CrI* was recorded in SAMAP biomass as compared to native biomass. The result was in close proximity with the earlier report of surfactant-assisted ultrasound pretreatment of sugarcane tops.[19] Treatments by microwave-acid, microwave-alkali and microwave-alkali followed by acid pretreatment of sugarcane bagasse also showed 5.35, 11.85 and 12.11% increases in *CrI*.[10] An increase in *CrI* by

6% was also reported for enzyme pretreated *Lantana camara* biomass.[25] The crystallinity is mainly related to the molecular mass of hydrocarbons and fatty components of biomass.[30] Probably, crystallinity has been damaged owing to the synergistic effect of the surfactant, microwave and acid treatment, leading to enhanced saccharification of the leaf litter biomass.

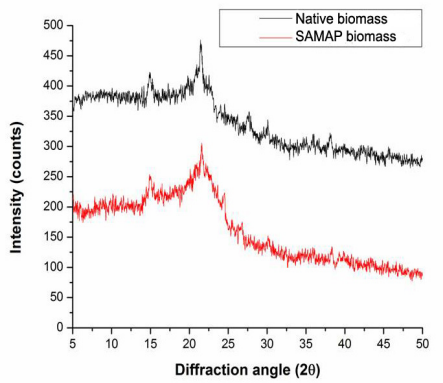

Figure 3: XRD profile of native and SAMAP biomass

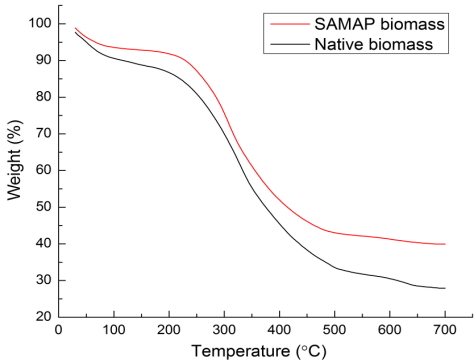

Figure 4: TGA of native and SAMAP biomass

Thermal gravimetric analysis (TGA)

The TGA of leaf litter biomass indicated the onset temperature of devolatilization in the range of 200-250 °C, which corresponded to 10% weight loss with respect to the final weight loss (Fig. 4). The behaviour of biomass materials during devolatilization was related to the presence of chemical constituents such as cellulose, hemicellulose and lignin.[31] The main weight loss ended at 350-390 °C, followed by a slow and continuous weight loss. The former step was due to primary devolatilization, whereas the latter was attributed to the degradation of heavier chemical structures in the solid matrix.[32] Similar devolatilization properties were also found with hot water pretreated biomass of *Tamarix ramosissima*[33] and heat pretreated sugarcane bagasse.[34] A tail flattening beyond 500 °C was observed mainly due to the removal of lignin in SAMAP biomass, as the long tail was due to the high percentage of mannose or lignin content.[35]

Fourier transform infrared (FTIR) spectroscopy analysis

Changes in the functional groups of native and SAMAP biomass are presented by FTIR spectra (Fig. 5) and peak assignments have been described based on literature.[36-39] The most prominent bands in the native sample were located at 3416, 2911, 1714, 1458, 731 cm^{-1}. The broad band at 3416 cm^{-1} is related to O-H stretching vibrations caused by the presence of alcoholic and phenolic hydroxyl group involved in the hydrogen bond. The O-H stretching band was changed to a higher wave number and somewhat broadened as a result of the pretreatment, which is an indication of weaker intra and intermolecular hydrogen bonding and thereby lower crystallinity.[40] The band position at 2911 cm^{-1} attributed to C-H stretching vibration showed a slight enhancement in peak intensity, indicating that the methyl and methylene portions of cellulose were ruptured. The highest reduction was observed in the 1714 cm^{-1} band, attributed to hemicellulose acetyl and uronic ester groups or linkages in lignin and/or from the ester linkage of the carboxylic group of the ferulic and p-coumaric acids of lignin and/or hemicellulose. This signal was almost absent in SAMAP, which indicated that the pretreatment cleaved the ester bond from the hemicellulose and/or lignin. The absorption peak at 1458 cm^{-1} represents absorption due to C-H deformation within the methoxyl group of lignin and hemicellulose. Since the adsorption of the band greatly reduced in SAMAP, this suggested the release of acid-soluble lignin. The bands in the range of 1000-1200 cm^{-1} were related to structural features of cellulose and hemicelluloses. The enhancement of the absorption peak at 1076 cm^{-1} after pretreatment

indicated an increase in cellulose content in the solid residue.[10]

Solid state CP/MAS ^{13}C NMR spectroscopy

Solid state CP/MAS ^{13}C NMR spectra of the native and SAMAP biomass are presented in Figure 6. The peaks have been assigned based on the literature.[33,41-43] The peak at 22.4 ppm can be attributed to the $-CH_3$ in the acetyl group of hemicellulose and is more prominent in native biomass. The peaks at 31.6 and 55.9 assigned to the aryl methoxyl carbons of lignin have been greatly reduced after pretreatment. The prominent peaks in the SAMAP biomass, as compared to the native one, in the region between 60 and 110 ppm are predominantly due to the different carbons of cellulose. The signals from the lignin phenolic groups arising from syringyl (S) and guaiacyl (G) type lignin are present between 110-160 ppm. In this region, the peaks from 110 to 120 ppm can be assigned to carbons (C) of G lignin and the peaks between 120-140 ppm correspond to quaternary carbon in S and G lignin. The higher cellulose contents of the SAMAP biomass was seen as a distinct peak (72 ppm) assigned to the C2, C3 and C5 of cellulose. A less prominent peak at 172.2 ppm in SAMAP biomass corresponds to acetyl groups of hemicellulose, ascribed to hemicellulose degradation. The results were in good agreement with those reported for hot water pretreated biomass of *Tamarix ramosissima*[33] and biologically pretreated lime wood.[42]

Optimization of enzymatic saccharification by Box-Behnken design

The Box-Behnken design was used for optimization of the enzymatic hydrolysis of SAMAP biomass. The polynomial equation was derived to represent the reducing sugar yield as a function of the independent variables tested.

Reducing sugar (g/g) = 0.5652 + 0.010917*A + 0.031083*B + 0.136083*C + 0.056417*D- 0.03475*A*B - 0.006*A*C + 0.0115*A*D + 0.0205*B*C+ 0.018*B*D+ 0.0232*C*D - 0.01264*A^2 + 0.010608*B^2 - 0.09914*C^2 - 0.14689*D^2

where A, B, C, D are coded values for acid concentration, surfactant concentration, enzyme loading, incubation time, respectively. Testing of the model was performed by the Fisher's statistical test for ANOVA using Design Expert software (Table 5).

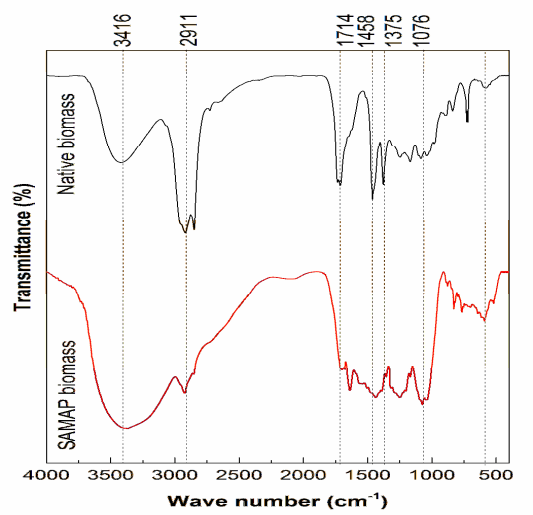

Figure 5: FTIR spectra of native and SAMAP biomass

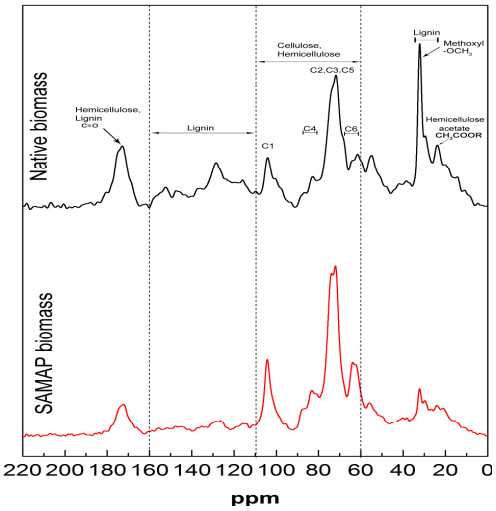

Figure 6: Solid state CP/MAS ^{13}C NMR spectra of native and SAMAP biomass

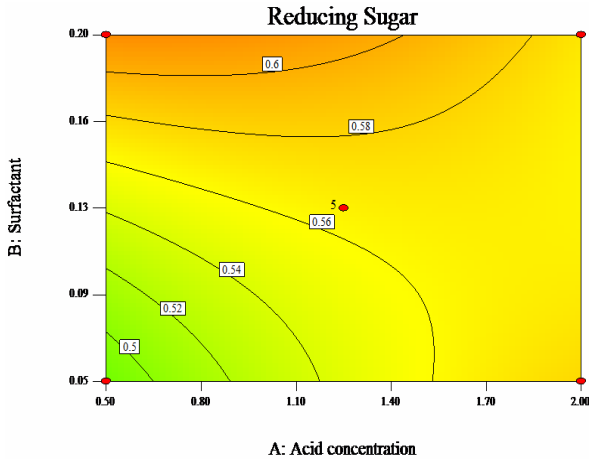

Figure 7: 2D-contour plot showing the interaction between acid and surfactant concentration

ANOVA of the quadratic regression model suggests that the model is significant with a computed *F* value of 31.56. The values of $P > F$ (<0.0001) imply that the model terms (enzyme loading and incubation time) are significant. The equation obtained from ANOVA indicates a R^2 value of 0.96, which is in a reasonable agreement with the predicted R^2 (0.83) and adjusted R^2 (0.93). A lower value for the coefficient of variation (CV) suggests higher reliability of the experiment and the obtained CV value of 7.11% demonstrates a greater reliability of the trials.

The effect of interaction between acid and surfactant concentration is shown in a contour plot (Fig. 7). It was observed that reducing sugar yield decreased after 42 h of incubation with enzyme loading of 20-80 FPU/g. As compared to the reducing sugar yield of 0.05 g/g in native biomass, enzymatic hydrolysis of the recovered solid fraction of SAMAP biomass (0.5 mm) showed a 93% increase in reducing sugar (0.69 g/g), when treated with 1.25% H_2SO_4 and 0.2% Tween-80 followed by 80 FPU/g of enzymatic hydrolysis for 42 h (Table 2).

Table 5
Analysis of variance (ANOVA) for selected factorial of Box-Behnken model

Source	Sum of squares	df	Mean square	F value	Probability > F	
Model	0.478098	14	0.03415	31.56848	< 0.0001	Significant
A: Acid	0.00143	1	0.00143	1.321983	0.2695	
B: Surfactant	0.011594	1	0.011594	10.71768	0.0055	
C: Enzyme	0.222224	1	0.222224	205.4261	< 0.0001	
D: Incubation time	0.038194	1	0.038194	35.30698	< 0.0001	
AB	0.00483	1	0.00483	4.46513	0.053	
AC	0.000144	1	0.000144	0.133115	0.7207	
AD	0.000529	1	0.000529	0.489013	0.4958	
BC	0.001681	1	0.001681	1.553933	0.233	
BD	0.001296	1	0.001296	1.198035	0.2922	
CD	0.002162	1	0.002162	1.998805	0.1793	
A^2	0.001037	1	0.001037	0.958259	0.3442	
B^2	0.00073	1	0.00073	0.67479	0.4252	
C^2	0.063756	1	0.063756	58.93679	< 0.0001	
D^2	0.13996	1	0.13996	129.3804	< 0.0001	
Residual	0.015145	14	0.001082			
Lack of fit	0.014182	10	0.001418	5.891982	0.0511	Not significant
Pure error	0.000963	4	0.000241			
Cor total	0.493243	28				

An initial increase in the reducing sugars with the increase in enzyme dose from 20 to 50 FPU/g and a slow increase in reducing sugars afterwards were observed, which might be due to the lower adsorption efficiency for higher enzyme concentrations than for diluted ones,[44] probably due to the enzyme saturation of the substrate surface. The low reducing sugar yield with increased hydrolysis time after 42 h may be due to the inhibition of the enzyme action by the accumulated hydrolysis products.[45] The addition of non-ionic surfactant (Tween-80) improved enzymatic hydrolysis effectively, as it reduced non-productive adsorption of the enzyme to the lignin component of the lignocellulose biomass by removing lignin. An enhanced yield of sugar was also reported for the hydrolysis of sugarcane tops using Tween-40 by Sindhu *et al*.[19]

CONCLUSION

Surfactant (Tween-80) assisted microwave-acid pretreatment of *Eucalyptus globulus* leaf litter biomass was effective for deriving sugar after enzymatic saccharification. The maximum yield of reducing sugar (0.69 g/g) was obtained with an optimum surfactant concentration of 0.2% (w/v), 80 FPU/g of enzyme loading after 42 h of incubation. SEM, XRD, TGA, FTIR, solid state CP/MAS ^{13}C NMR spectroscopy and composition analysis revealed differences between native and pretreated biomass. To the best of our knowledge, this is the first report on surfactant-assisted microwave acid pretreatment of leaf litter biomass. The technique was found to be highly effective in removing hemicellulose and lignin to provide accessible area for enzymatic hydrolysis. Further studies need to be carried out to determine the overall process efficiencies and the feasibility of the adopted strategy using different kinds of lignocellulosic biomass.

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