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Research article

Microwave assisted acid and alkali pretreatment of *Miscanthus* biomass for biorefineries

Zongyuan Zhu [1], Rachael Simister [2], Susannah Bird [2], Simon J. McQueen-Mason [2], Leonardo D. Gomez [2], and Duncan J. Macquarrie [1,*]

[1] Green Chemistry Centre of Excellence, Department of Chemistry, University of York, Heslington, York, YO10 5DD, United Kingdom
[2] Center for Novel Agricultural Products, Department of Biology, University of York, York, Heslington YO10 5DD, United Kingdom

* **Correspondence:** Email: duncan.macquarrie@york.ac.uk; Tel: 0044-1904-324533.

Abstract: *Miscanthus* is a major bioenergy crop in Europe and a potential feedstock for second generation biofuels. Thermochemical pretreatment is a significant step in the process of converting lignocellulosic biomass into fermentable sugars. In this work, microwave energy was applied to facilitate NaOH and H_2SO_4 pretreatments of *Miscanthus*. This was carried out at 180 °C in a monomode microwave cavity at 300 W. Our results show that H_2SO_4 pretreatment contributes to the breakdown of hemicelluloses and cellulose, leading to a high glucose yield. The maximum sugar yield from available carbohydrates during pretreatment is 75.3% (0.2 M H_2SO_4 20 Min), and glucose yield is 46.7% under these conditions. NaOH and water pretreatments tend to break down only hemicellulose in preference to cellulose, contributing to high xylose yield. Compared to conventional heating NaOH/H_2SO_4 pretreatment, 12 times higher sugar yield was obtained by using microwave assisted pretreatment within half the time. NaOH pretreatments lead to a significantly enhanced digestibility of the residue, because the effective removal of lignin and hemicellulose makes cellulose fibres more accessible to cellulases. Morphological study of biomass shows that the tightly packed fibres in the *Miscanthus* were dismantled and exposed under NaOH condition. We studied sugar degradation under microwave assisted H_2SO_4 conditions. The results shows that 6–8% biomass was converted into levulinic acid (LA) during pretreatment, showing the possibility of using microwave technology to produce LA from biomass. The outcome of this work shows great potential for using microwave in the thermo-chemical pretreatment for biomass and also selective production of LA from biomass.

Keywords: microwave pretreatment; temperature dependence; *Miscanthus*; NaOH; H_2SO_4; digestibility; levulinic acid

1. Introduction

In recent years, there has been an upsurge of interest in the use of lignocellulosic material, such as corn stover [1], sugar cane bagasse [2], *Miscanthus* [3], rapeseed straw [4], wheat straw [5], and so forth, as feedstock for second generation bioethanol. *Miscanthus* is a C4 plant and a promising bioenergy crop in Europe [6]. A number of strategies have been put forward to convert polysaccharides contained in lignocellulosic material into bioethanol and other renewable fuels. Lignocellulosic biomass is a recalcitrant structure in which hemicellulose and cellulose are packed with layers of lignin, resulting in resistance towards enzymatic hydrolysis [7]. Therefore, various pretreatments have been studied to improve the yields of fermentable sugars from cellulose and hemicellulose, such as mechanical [8], steam explosion [9,10], ammonia fibre expansion[8,11], hot water, supercritical CO_2 [12], ozone pretreatment [8], biological [8], ultrasound [13], acid or alkali [1,14,15] and others. Pretreatments may alter the structure of cellulosic biomass to make it more accessible for enzymes, as well as decrease the degree of polymerization and cellulose crystallinity. Additionally, it can selectively remove hemicellulose and lignin from the lignocellulosic matrix [10,16].

There is growing interest in using microwaves in various biomass transformation processes. Compared with conduction/convection heating, microwave heating enables the heated object to directly interact with an electromagnetic field and generate heat. Therefore, it is more direct, rapid and uniform [17]. It can selectively heat the more polar parts in lignocelluloses and creates "hot spots" in heterogeneous materials. An 'explosion' effect could result from this unique heating mechanism, which further enhances the disruption of the recalcitrant structure of lignocelluloses [18]. Much research has been done in the field of microwave pretreatment, with different feedstocks, such as sugarcane bagasse [7], rapeseed straw [4], switchgrass [19], and wheat straw [20]. The efficiency of microwave processing strongly depends on the physical properties of the subject, such as structural arrangements, conductivity and dielectric properties [21]. In the present work, we monitored the effects of microwave assisted pretreatment in the presence of acid and alkali on *Miscanthus*. Our results show that microwave assisted pretreatments have a significant influence on the lignocellulosic biomass, specifically the type and quantity of sugar released during the pretreatment process and the digestibility of biomass.

2. Materials and Method

2.1. Biomass material and constituents

Miscanthus giganteus was grown in York, North Yorkshire, UK, under field conditions. The materials used represent the sixth year of harvest. After harvest and drying, it was milled using a hammer mill to 1 mm particles. The composition of raw *Miscanthus* is cellulose (34% ± 2.5%), hemicellulose (42 ± 2.8%), lignin (28 ± 2%) and ash (0.83 ± 0.03%).

2.2. Microwave pretreatment methods

The pretreatment was conducted in the CEM monomode microwave machine (CEM Discover SP-D, US). The CEM microwave reactor vessel (30 ml) was charged with 0.4 g of *Miscanthus* and 16 ml H_2SO_4 or NaOH solution (0.2M, 0.4 M and 1M). Pretreatment was carried out at 180 °C with a range of reaction times (5 min to 30 min). After pretreatment, the liquid was separated from solid residue by filtration. Liquid samples were neutralized by 150 mM $Ba(OH)_2$ or 1 M HCl. The solid fraction was rinsed with ethanol (3 × 10 ml) and dried at 50 °C overnight. Each pretreatment condition was done in triplicate.

2.3. Conventional heating pretreatment

Conventional heating pretreatment was conducted in an acid digestion vessel (Parr Instruments, Moline, IL). The acid digestion vessel was charged with 0.4 g biomass with 16 ml pretreatment media (0.2 M NaOH or H_2SO_4). The temperature was controlled at 180 °C, and hold time was 40 min. The same separation and samples preparation for analysis were carried out as mentioned above for microwave assisted pretreatment. Each pretreatment condition was done in triplicate.

2.4. Analysis of carbohydrates in liquid fraction

The monosaccharide analysis of the pretreatment liquid was carried out using High Performance Ion Exchange Chromatography, using a DionexICS-3000PC, Thermo Scientific, USA, equipped with an electrochemical detector to quantify the corresponding sugar content [22].

2.5. Hemicellulose quantification

Four mg biomass were hydrolyzed by adding 0.5 ml 2 M Trifluoroacetic acid (TFA). The vials were flushed with dry argon, then heated at 100 °C for 4 hours. Then vials were cooled to room temperature, and TFA was completely removed by evaporator with fume extraction overnight. Biomass was washed two times by adding 2 × 500 µl propan-2-ol. After evaporating propan-2-ol, the biomass sample was re-suspended in 200 µl ultra purified water. After centrifugation, the supernatant was transferred into a new tube and diluted 20 times to measure the monosaccharides in hemicellulose on DionexICS-3000PC.

2.6. Analysis of crystalline cellulose

To determine the percentage of crystalline cellulose in biomass, 10 mg untreated or pretreated biomass was hydrolysed using 500 µl 2M TFA at 100 °C for 4 h. The solids were subsequently hydrolysed using acetic acid: nitric acid: water (8: 1: 2 v/v) at 100 °C for 30 min. Finally, the resulting residue was crystalline cellulose, which was hydrolysed into glucose using 175 µl 72% H_2SO_4 at room temperature for 45 min and then diluting the H_2SO_4 to 3.2% and heating the samples at 120 °C for 2 h. Anthrone assay was used to quantify corresponding glucose [23].

2.7. Lignin quantification

Lignin was quantified as follows: 3.5mg of biomass (untreated or pretreated) was dissolved in 250 µl acetyl bromide solution (25% v/v acetyl bromide/glacial acetic acid), then 1 ml 2 M NaOH and 175 µl hydroxylamine HCl in a 5 ml volumetric flask were added. The solution was taken to 5 ml with acetic acid and diluted 10 times. The absorbance was read at 280 nm and the percentage of lignin was calculated using the following formula [24]:

ABSL%= {abs/(coeff × pathlength)} × {(total volume × 100%)/biomass weight}

Coefficient = 17.75; Pathlength =1 cm; Total volume= 5 ml; biomass= 3.5 mg.

2.8. Saccharification and sugar analysis

The saccharification of biomass was investigated by using a high throughput saccharification assay which is based on a robotic platform that can carry out the enzymatic digestion and quantification of the released sugars in a 96-well plate format. Enzymatic hydrolysis was carried out using an enzyme cocktail with a 4:1 (v/v) ratio of Celluclast and Novozyme 188 (both Novozymes, Bagsvaerd, Denmark). The enzymes were filtered using a Hi-Trap desalting column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) before use. 0.1 mg biomass was hydrolysed for 8 hours with 250 µl enzyme cocktail, in 250 ml of 25 mM sodium acetate buffer at pH 4.5, at 30 °C. Determination of sugars released after hydrolysis was performed using a modification of the method by Anton and Barrett using 3-methyl-2-benzothiazolinonehydrazone (MTBH) [25].

2.9. Chemical analysis of the solid residues

The chemical compositions of solid residues before and after the pretreatments were analysed by Fourier transformed infrared spectrometry (FT-IR) (VERTEX 70, Bruker).

Attenuated total reflection–Fourier transformed infrared spectroscopy (ATR-FTIR) was conducted using a Bruker Optics Vertex system (VERTEX 70, Bruker) with built-in diamond-germanium ATR single reflection crystal. Untreated and pretreated samples were pressed firmly against the diamond surface using a screw-loaded anvil. Sample spectra were obtained under 64 scans between 650 cm^{-1} to 2000 cm^{-1} with a spectral resolution of 4 cm^{-1}. Air was used as background for untreated and pretreated *Miscanthus*.

2.10.Morphological studies

Morphological characteristics of the raw materials and pretreated biomass residue were studied using a scanning electron microscope fitted with a tungsten filament cathode (JEOL, JSM-6490LV, Japan). Samples were sputter-coated with 7 nm Au/Pd to facilitate viewing by SEM. Images were obtained under vacuum, using a 5 kV accelerating voltage and a secondary electron detector.

2.11.Sugar degradation study

The liquid fraction from filtration was extracted with ethyl acetate (3 × 15 ml). The solvent was

removed by using a rotary evaporator. The organic product was a viscous dark brown substance, which was weighed and re-dissolved into ethyl acetate and $CDCl_3$ to conduct GC and NMR analysis respectively.

A HP 6890 GC equipped with a FID detector was used to quantify the levulinic acid and furfural. The flow rate for He was 1.3 ml/ min. A Stabilwax column (30 m × 0.25 mm × 0.25 μm) was used. Oven temperature was programmed to rise from 45 °C to 250 °C at 10 °C/min. The injection column volume was 0.4 μL.

Proton NMR experiments were carried out in a Jeol NMR 400 Spectrometer at Proton frequency of 399.78 MHz.

3. Results

We studied the effect of microwave assisted pretreatments of *Miscanthus* in the presence of diluted acid and alkali. The effect of the pretreatments was determined by monitoring the compounds released into the pretreatment liquor, monitoring the morphological changes and chemical composition changes in the resultant solid residue, and by determination of the saccharification potential in the solids after pretreatment.

3.1. Effect of catalyst and holding time on sugar release

Holding time and catalyst concentration can have strong effects on the release of sugars from *Miscanthus* during pretreatment. Figure 1 shows that, when H_2O is used as pretreatment medium, increasing the holding time increases the yield of reducing sugar from available carbohydrate released into pretreatment liquors up to a maximum value of 1.246 μmol of reducing sugars/mg of biomass after 20 min, corresponding to the yield of 28.7% from available carbohydrates. However, further holding time increase led to sugar yield decrease. High-temperature water can liberate acid components from biomass, acetic acid predominantly, and dissociate the acetal linkages between the rings in biomass during hydrolysis [26]. The pH of liquid hot water pretreated biomass is generally within the range of 4.0 to 5.0 [27]. The hydronium ions generated from both water and generated acid can promote hemicelluloses depolymerization to oligosaccharides and monosaccharides [28,29].

When H_2SO_4 or NaOH are used as pretreatment media, a similar pattern is observed. From 5 to 10 min, the reducing sugar yield rose sharply, and then more slightly until 20 min, followed by a reduction when the holding time is increased to 30 min. This could be explained by further degradation of produced sugars (major components, such as xylose or glucose) under severe pretreatment conditions. In our study, the biomass is partly carbonized, and produced levulinic acid and hydroxymethylfurfural (HMF) under the more severe acid conditions used (see the following SEM and sugar degradation discussions). H_2SO_4 produces higher sugar release than NaOH. When the acid concentration is increased from 0.2 M to 0.4 M, the total sugar production declined, due to stronger acid conditions facilitating further degradation of produced sugars [30]. The maximum reducing sugar yield is up to 3 μmol/mg biomass (yield from available carbohydrate: 75.3%) when *Miscanthus* was pretreated with 0.2 M H_2SO_4 for 20 min. Increasing the concentration of NaOH from 0.2 M to 0.4 M does not increase significantly the amount of sugar released. For holding times of 5 min, both concentrations of NaOH produced very low sugar yield. The sugar production reaches a maximum of 1.76 and 2.1 μmol/ mg biomass (yield from available carbohydrates: 43% and 50.7%)

when 0.2 M NaOH and 0.4 M NaOH are applied for 20 Min. Overall, H_2SO_4 is more efficient than NaOH in releasing reducing sugars from *Miscanthus* using microwave energy, using 0.2 M concentration.

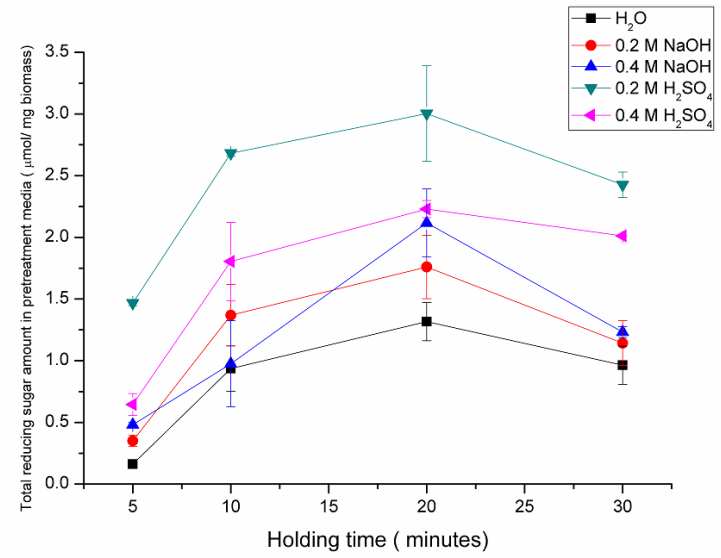


Figure 1. Total reducing sugar amounts present in the pretreatment liquors.

3.2. Monosaccharides analysis in pretreatment media

Holding time and catalysts have strong influence on the monosaccharide composition of the sugars released. In the cases of water and NaOH pretreatments, xylose is the major monosaccharide released, showing that hemicelluloses are broken down (Figure 2). These results indicate that the water and NaOH pretreatments extracted hemicelluloses fractions, which were composed mainly of glucuronoarabinoxylan or 1-arabino-D-xylans. Using a 5 min. holding time, a very small amount of sugar is released by water or low concentration of NaOH, and 1 M NaOH produces higher sugar amounts (sugar yield is 24% from available carbohydrate) (Figure 2a). However, under acid conditions, 0.2 M H_2SO_4 can give higher sugar release than 0.4 M and 1 M H_2SO_4, glucose being the major sugar component. When holding time was 10 min. (Figure 2b), the sugar production is greatly enhanced compared to that of 5 min. Almost equal amounts of glucose and xylose are produced by using 0.2 M H_2SO_4. However, when 0.4 M H_2SO_4 is applied for pretreatment, xylose yield is significantly reduced and glucose becomes the major product in the pretreatment media, indicating that cellulose starts to be broken down into glucose. The amount of xylose is decreased, suggesting that it is degraded into other chemicals under higher temperature and acid conditions. Xylose can be broken down into other chemicals such as furfural, formaldehyde, formic acid, crotonaldehyde, lactic acid, acetaldehyde, dihydroxyacetone [31]. In our study, furfural was the major product observed (see sugar degradation study section).

We have shown that 20 min. in combination with 0.2M H_2SO_4 gives maximum sugar yield (Figure 2c). When 0.2 M and 0.4 M H_2SO_4 are applied, glucose, derived from both cellulose and hemicellulose, is the major monosaccharide. The glucose productions from available carbohydrate are 1.8 and 1.9 μmol/ mg biomass respectively (yields: 47.6% and 50%) for 0.2 M and 0.4 M H_2SO_4.

Xylose yield decreases, due to its further degradation under acid conditions. In contrast, 0.4 M NaOH gives rise to the maximum yield of xylose (1.3 μmol/mg biomass). Similar results can be observed when the holding time is 30 min. (Figure 2d). In the case of water and NaOH, xylose production also declines, but it is still the major component.

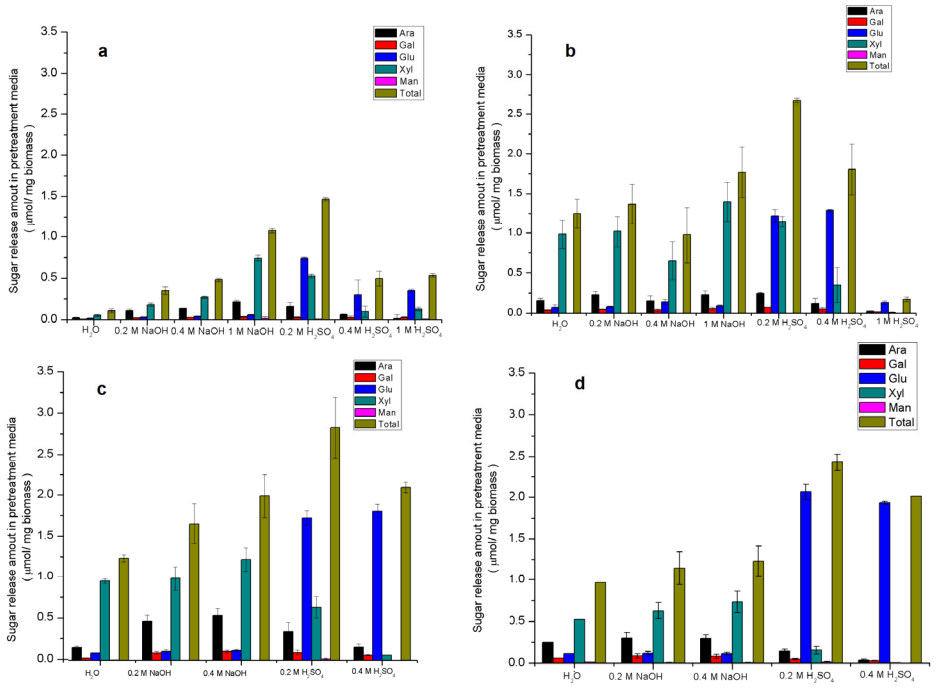


Figure 2. Monosaccharide amount after various pretreatments when holding time is varied. a) 5 min; b) 10 min; c) 20 min; d) 30 min.

Conventional H_2SO_4 and NaOH pretreatments are performed at 180 °C for 40 min. in order to compare. Supplementary Figure 1 shows the reducing sugar release during pretreatment. As can be seen, both water and NaOH have rather low production of reducing sugar. H_2SO_4 gives rise to relatively better reducing sugar production (0.25 μmol/mg biomass). In comparison, microwave assisted pretreatment led to 12 times higher sugar production within half the time. Brosse et al. used an H_2SO_4 assisted ethanol organosolv system to pretreat *Miscanthus*, and the results showed 0.14–9.08% glucan removal under similar temperature (170–180 °C) within longer holding time (60 min.) [32]. Yu et al. pretreated *Miscanthus* by using an aqueous ammonia/ hydrogen peroxide system under lower temperature (90–150 °C) with longer holding times (1–4 h), and the results showed lower cellulose removal during the pretreatment (2.4–19.1%) [33]. Haverty et al. studied peroxide/formic acid assisted pretreament for *Miscanthus* under autothermal condition, and the results showed 0.3–4.37% cellulose removal across conditions assayed [34]. Compared to other pretreament methods studied on *Miscanthus,* microwave assisted pretreatments in this study released sugars significantly more efficiently during the process. It is worth mentioning that the temperature of 180 °C has been identified as a crucial turning point in the microwave degradation of cellulose by Budarin et al. [35]. Below 180 °C with microwave, the polar groups in cellulose have less freedom to rotate easily, resulting in little effective interaction. Above 180 °C, the number of groups capable of rotating increases considerably, leading to a more effective interaction between cellulose and

microwave [35]. Therefore, under our microwave condition, the polar part of biomass is significantly involved in the alignment with the oscillating microwave field, resulting in a remarkable enhancement in sugar release.

3.3. Chemical compositions analysis

The lignin content in the 400 mg untreated *Miscanthus* is 112 mg. Figure 3a shows the amount of lignin remaining in the biomass after various pretreatments. After NaOH pretreatment the amount of lignin in the samples decreased sharply, in good agreement with previous studies showing that alkali conditions have a significant delignification effect [16,20,36]. 0.2 M NaOH can remove 84 mg lignin after 20 min. pretreatment. When the holding time increased to 30 Min, up to 93 mg lignin is removed. 0.4 M NaOH is more effective than 0.2 M NaOH, and 105.5 mg (94.2%) lignin is removed after 20 min. holding time. Lignin amount is also decreased in H_2SO_4 pretreatments, however, not as effectively as NaOH pretreatment. When 0.4 M H_2SO_4 is used for pretreatment, 10 Min removes more lignin than both 5 and 20 min. It could be explained by lignin extraction from the inner regions of the cell wall, and subsequent condensations and re-deposition on the surface. Li et al. reported that

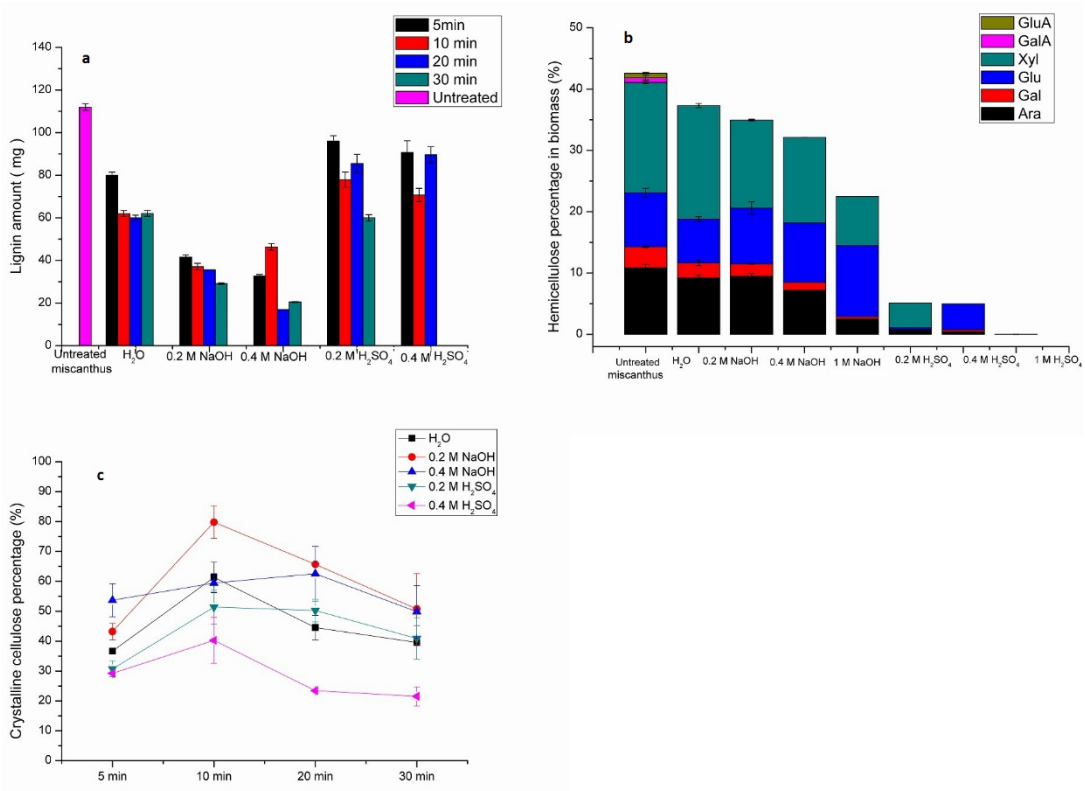


Figure 3. Chemical composition changes of biomass solid fraction after pretreatments. a) lignin content; b). hemicellulose percentage (holding time is 5 Min); c) crystalline cellulose percentage.

depolymerisation and subsequent re-polymerization of lignin occurs, with increasing severity of steam pretreatment of aspen wood[37]. Acetic acid assisted pretreatment of aspen wood also lead to similar results [37]. Under conventional heating conditions, NaOH effectively removed lignin, while

water and H_2SO_4 have little influence on lignin removal (see Table 1). Overall, more lignin is removed by using microwave heating pretreatment than conventional pretreatment. Because of the nature of its chemical structure, lignin is much less polar than polysaccharides and a significantly poorer microwave absorber than cellulose or hemicelluloses [17]. However, in our study, microwave assistance promotes lignin removal. The explanation could be the ester linkages between polysaccharides and lignin are influenced by microwave effect, leading to its more rapid cleavage and removal of lignin.

Table 1 Lignin amount in biomass solid fraction.

Conditions	H_2O	NaOH	H_2SO_4
Lignin amount (mg)	119.48 ± 6.76	50.61 ± 4.756	117.8 ± 9.06

The hemicellulose present in *Miscanthus* undergoes different degrees of removal depending on the pretreatment conditions. Untreated *Miscanthus* has 42% hemicellulose, comprising arabinose galactose, glucose, xylose, mannose, galacturonic acid and glucuronic acid, with xylose as the major component. Figure 3b shows hemicellulose percentages in the biomass material before and after pretreatment when the holding time was 5 min. Water pretreatment decreased the hemicellulose to 39%. Using NaOH pretreatment, the hemicellulose decreased further in a concentration dependent fashion. By using 1 M NaOH pretreatment, hemicellulose percentage in the biomass decreased to 22.5%. In contrast, H_2SO_4 pretreatment is significantly effective in removing hemicellulose from biomass. It sharply drops to 5% by using 0.2 M or 0.4 M H_2SO_4 pretreatment. Almost all of the hemicellulose is removed when 1 M H_2SO_4 is applied for pretreatment, albeit with significant decomposition of the monosaccharides released. Lower hemicellulose percentage is achieved by conventional pretreatments, which could have resulted from much longer pretreatment time (see Figure 4).

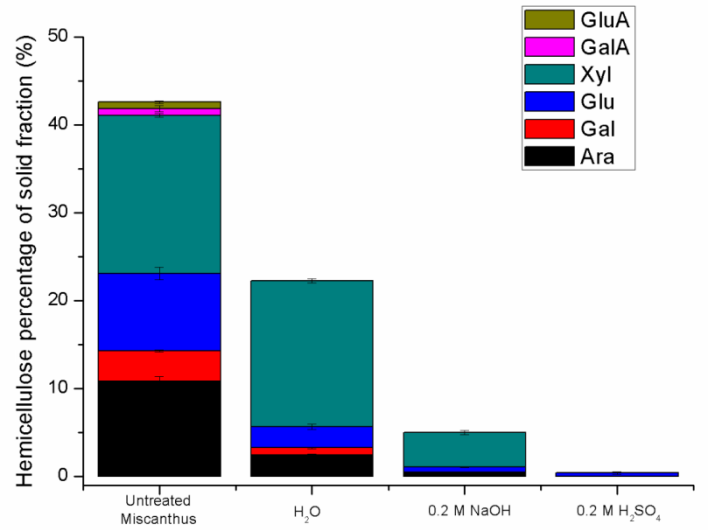


Figure 4. Hemicellulose percentage in biomass solid fraction after conventional heating pretreatment (180 ^{o}C, 40 min).

Crystalline cellulose percentage is a vital property influencing biomass digestibility. Figure 3c shows the crystalline cellulose percentages in biomass before and after various microwave assisted pretreatments. The crystalline cellulose percentage in untreated *Miscanthus* is 34%. Using short retention times in H_2SO_4 and water pretreatments, both hemicellulose and crystalline cellulose are partially broken down. When holding times are increased to 10 min., the crystalline cellulose percentage is relatively increased, because lignin and hemicellulose are being removed (Figure 3a and 3b). When holding times are further increased, crystalline cellulose percentages decrease, because crystalline cellulose undergoes hydrolysis under H_2SO_4 or water conditions, or changes into amorphous form under NaOH conditions [38]. Increasing the concentration of NaOH brings little difference to crystalline cellulose. However, stronger acid (0.4 M) can significantly decrease the percentage of crystalline cellulose (21%). In contrast, conventional pretreatments lead to lower crystalline percentage in the ptretreated biomass, see Table 2.

Table 2 Crystalline cellulose percentages of biomass residue.

Conditions	H_2O	H_2SO_4	NaOH
Crystalline cellulose percentage (%)	24.72 ± 0.32	19.12 ± 1.2	24.58 ± 0.86

The difference in crystalline cellulose between microwave and conventional pretreatments could be explained by different heating mechanism. Under conventional heating, biomass structure is disrupted by heat penetration from outside to inside and the cellulose changes from crystalline to amorphous structure, while under microwave condition the heat is generated by the interaction between polar part of biomass and oscillating microwave field. The cellulose fibres could be both ionic conducting (crystalline) and non-conducting (amorphous) [35]. A very ordered hydrogen bonded network is contained in the crystalline cellulose which could lead to a proton transport network under an electromagnetic field at the right conditions [35]. Therefore, the crystalline cellulose is able to act as an active microwave absorber, promoting the biomass decomposition. Therefore, along with the process of lignin/hemicellulose removal, crystalline cellulose percentage increases, followed by a decrease due to its self-promoting decomposition. In our study, when holding time is 10 min, high crystalline cellulose percentages are present in the biomass, which greatly enhance the microwave absorbing effect on biomass and lead to promising reducing sugar release at 20 min.

3.4. Enzymatic digestibility analysis

Biomass digestibility was measured. The digestibility of untreated biomass is 10 nmol sugar/ mg biomass × hour. It is increased after all the pretreatments, albeit to widely differing extents (Figure 5). It is remarkably enhanced by NaOH (up to 93 nmol sugar/ mg biomass × hour) and remains unaltered, irrespective of NaOH concentration and holding time. On the other hand, H_2SO_4 only improves *Miscanthus* digestibility slightly. For 0.2 M H_2SO_4, the holding time has negligible effect on digestibility, and they are all similarly increased (17–24 nmol sugar/mg biomass × hour). Nevertheless, when 0.4 M H_2SO_4 is applied, the digestibility is marginally increased using 5 min holding time, and thereafter it declines. The difference in saccharification after H_2SO_4 or NaOH pretreatments can be explained by the fact that the easily hydrolysed sugars were released into the

pretreatment liquor, reducing the amount of sugars available for enzymatic digestion. The higher saccharification after NaOH pretreatment is therefore consistent with the loss of significant amounts of lignin during pretreatment. Hemicellulose has a positive and dominant effect on biomass digestibility. In contrast, lignin is playing synergistic and negative roles in sugar production by the enzymatic hydrolysis after chemical pretreatment [14]. Several factors affecting the absolute enzymatic hydrolysis rate were outside the scope of the present study and were not optimised (solids loading; enzyme loading; the effect of various additives; the enzyme cocktail used; or the temperature and duration of digestion). Due to the delignification effect of NaOH, NaOH pretreated *Miscanthus* with low lignin percentage and higher crystalline cellulose percentage could generate more sugar in the hydrolysis process. 10 nmol glucose is obtained from 1 mg untreated *Miscanthus*, meaning only 0.53% cellulose-glucose conversion. With 0.4 M NaOH pretreament for 30 min., the cellulose-glucose conversion is increased to 3.34%. Under conventional heating conditions, NaOH leads to best digestibility (150 nmol sugar/mg biomass × hour, see Table 3), which is higher than that of microwave heating pretreatment. These results are expected, because fewer sugars were released during conventional heating pretreatment that during microwave heating pretreatment.

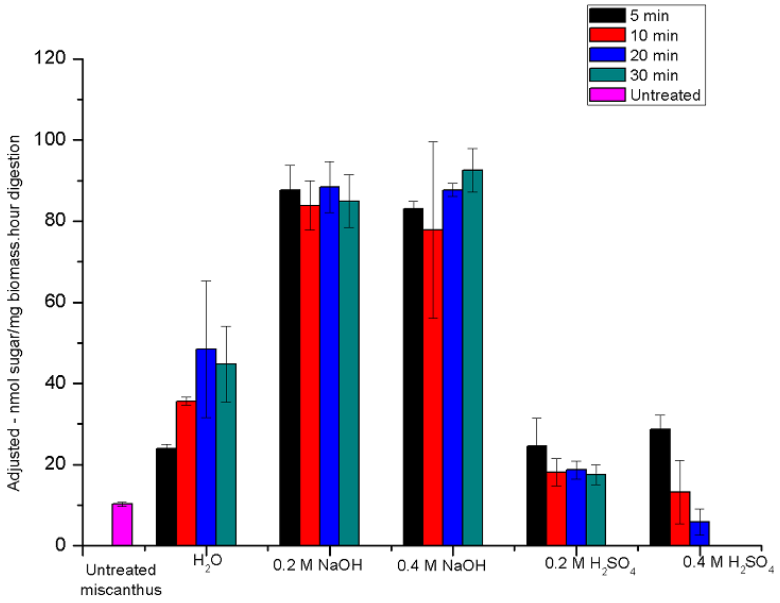


Figure 5. Digestibility analysis of un/pretreated *Miscanthus.*

Table 3. Digestibility of conventional heating pretreated biomass residue.

	H_2O	NaOH	H_2SO_4
Digestibility (nmol/ mg biomass.hour)	20.16766 ± 1.89	150.3739 ± 18.5	32.90467 ± 1.6

3.5. FT-IR analysis

Chemical changes in the surface of samples were qualitatively analysed by ATR-FTIR spectroscopy. Figure 6 shows sharp peaks at ν_{max}/cm^{-1} 897 and ν_{max}/cm^{-1} 1159 in the spectra, which

are attributed to C-O-C stretching at the β-glycosidic linkage between the sugar units (lit.,[39]897, 1161). The absorbance at ν_{max}/cm^{-1} 897, ν_{max}/cm^{-1} 1033, ν_{max}/cm^{-1} 1065 and ν_{max}/cm^{-1} 1108 can also be associated with cellulose [39,40]. Strong peaks at ν_{max}/cm^{-1} 1065 and ν_{max}/cm^{-1} 1033 relates to C-O stretching at C-3, C-C and C-O stretching at C-6 (lit., [39] 1064, 1030). When the concentration of NaOH was increased, a peak at ν_{max}/cm^{-1} 1065 became increasingly pronounced. This indicates that removing hemicellulose and lignin enhances cellulose-associated signals [40,41]. According to previous research, crystalline cellulose has a characteristic C-O vibration absorbance at ν_{max}/cm^{-1} 1098 [42,43,44]. However, this peak is not present here. The peak at ν_{max}/cm^{-1} 1108 appeared after NaOH pretreatments, and we infer it relates to the crystalline cellulose, whose appearance suggests that the biomass is more crystalline[42]. This result is in good correlation with the earlier discussion of crystalline cellulose percentage. Figure 5a shows that lignin has absorbance around ν_{max}/cm^{-1} 1424, ν_{max}/cm^{-1} 1512, and ν_{max}/cm^{-1} 1604 (lit., [40,45] 1422, 1512, 1600), and they have almost disappeared after NaOH pretreatment. The absorption at ν_{max}/cm^{-1} 1424 could be related to methyl groups present in lignin [46]. However, it could also be attributed to CH_2 symmetric bending of crystalline cellulose, and its disappearance during alkali treatment can also due to disruption of crystalline cellulose [47]. As discussed above, the percentage of crystalline cellulose in NaOH pretretreated *Miscanthus* is higher than that of untreated *Miscanthus* when holding time is 5 Min, and the peak at ν_{max}/cm^{-1} 1108 is stronger. Combining the results here, it can be confirmed that the peak at ν_{max}/cm^{-1} 1424 is related to lignin. The peak at ν_{max}/cm^{-1} 1239 (acetyl C-O stretching of hemicellulose) disappears after pretreatment (Figure 4a), implying that hemicellulose is deacetylated by pretreatment (lit., [48] 1235). The peak at ν_{max}/cm^{-1} 1731 represents the complex linkages between hemicellulose and lignin, such as ester-linked acetyl, feruloyl and *p*-coumaroyl groups (lit., [49] 1734). After pretreatment, the signal becomes very weak, indicating that the linkages were broken. The peak at ν_{max}/cm^{-1} 1634 is attributed to the bending mode of the absorbed water [50]. C-H deformation in cellulose and hemicellulose is at ν_{max}/cm^{-1} 1370 (lit., [48] 1375); C-H vibration in cellulose and C1-O vibration in syringyl ring derivatives are at ν_{max}/cm^{-1} 1320 (lit., [51]1320). The peak at ν_{max}/cm^{-1} 1033 (associated with cellulose) is less pronounced after 0.2 M H_2SO_4 pretreatment, while it becomes stronger after 0.4 M and 1 M H_2SO_4 (Figure 5b). Bands between

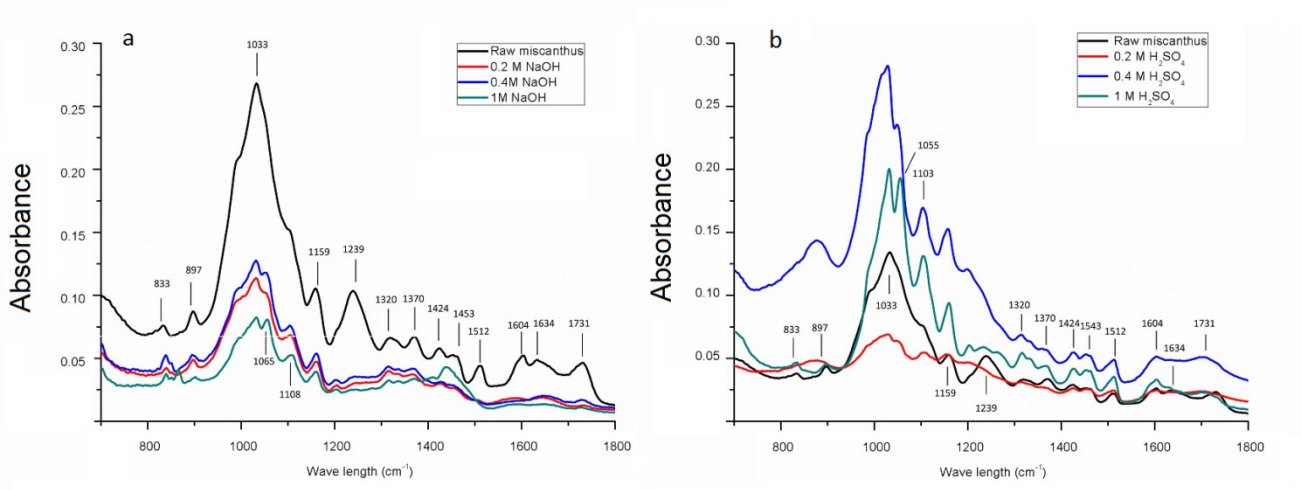


Figure 6. FT-IR analysis of Miscanthus after microwave pretreatments when holding time was 5 Min. a) NaOH; b) H_2SO_4.

ν_{max}/cm^{-1} 1170 and ν_{max}/cm^{-1} 1000 are typical of arabinoxylans [50]. Two peaks around ν_{max}/cm^{-1} 1055 and ν_{max}/cm^{-1} 1103 appeared after 0.4 M and 1 M H_2SO_4 pretreatments, which is contributed by polysaccharides content [49]. The hemicellulose peak around ν_{max}/cm^{-1} 1239 disappeared after 0.2 M and 0.4 M H_2SO_4 pretreatment. Lignin absorbances around ν_{max}/cm^{-1} 1424, ν_{max}/cm^{-1} 1512 and ν_{max}/cm^{-1} 1604 barely show any changes, confirming that H_2SO_4 has little influence on lignin. The peak around ν_{max}/cm^{-1} 1731 withered after pretreatment, suggesting that the ester linkages between hemicellulose and lignin are broken.

3.6. SEM analysis

Scanning electron microscopy was used to study the morphological characteristics produced by microwave assisted pretreatment of *Miscanthus* when the holding time was 20 min. Untreated *Miscanthus* presents vascular elements packed in bundles (Figure 7a and b) with relatively flat and clean surfaces (Figure 7c).

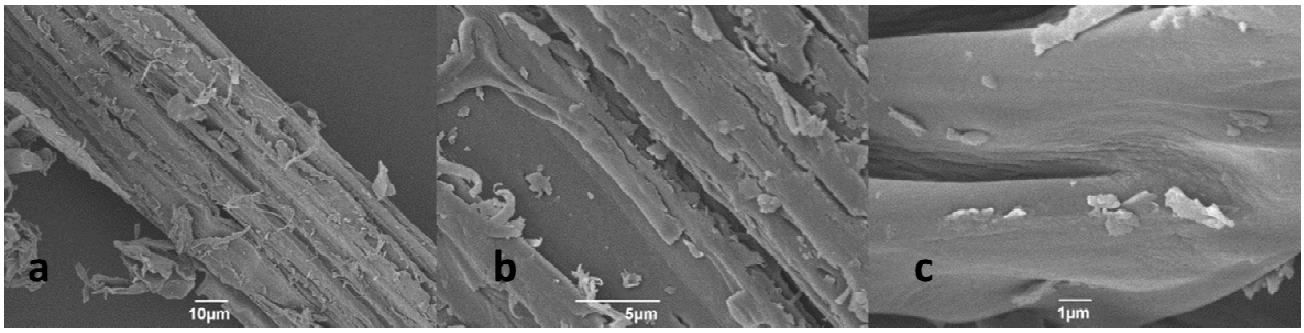


Figure 7. Surface images of the untreated *Miscanthus* obtained by SEM. (a) general view of a fibre surface, bar scale: 10 μm; (b) flat surface of a fibre showing, bar scale: 5 μm and (c) amplification of the surface, bar scale: 1 μm.

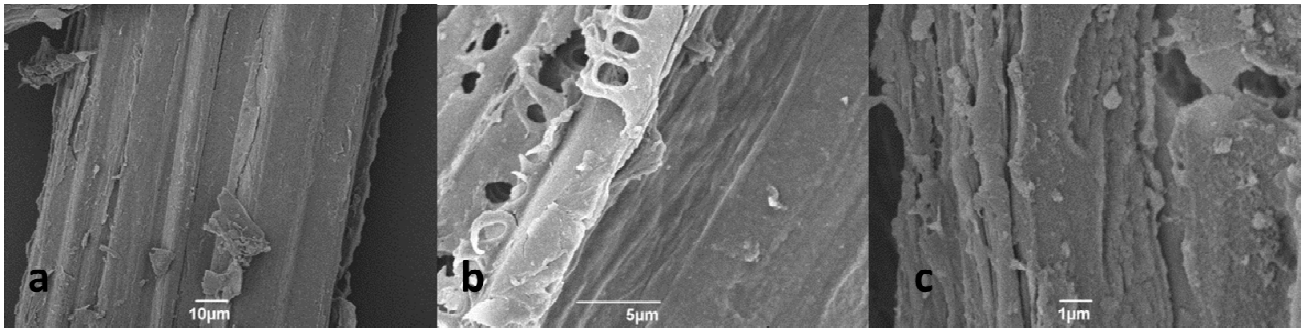


Figure 8. Surface images obtained by SEM on *Miscanthus* treated with water pretreatment, under a 300 W microwave power and three different magnifications with scale bars between 10 μm and 1 μm.

Miscanthus samples pretreated with water show a rough and striped surface only when observed at maximum magnification (Figure 8c), indicating that water treatment has a mild effect on the biomass surface. In contrast, NaOH has a pronounced effect on biomass. Figure 9 (a–c) presents the images from 0.2 M NaOH pretreatment. Firstly, the surface coating that can be observed in Figure 7

is damaged and the biomass surface becomes rough with the appearance of parallel strips. Additionally, some biomass fibres start to become exposed. With the application of 0.4 M NaOH, the effect of alkali is more obvious, and the biomass surface coating is totally removed and is covered with exposed biomass fibres (Figure 9 d–f). The exposed fibres' size is smaller after 0.4 M NaOH pretreatment, which could be due to higher NaOH concentration having a stronger influence on biomass surface. The tightly packed fibres in the raw *Miscanthus* start to dismantle and are exposed due to lignin removal from the interstices between the fibre bundles and is in agreement with the great decrease in lignin (Figure 3a).

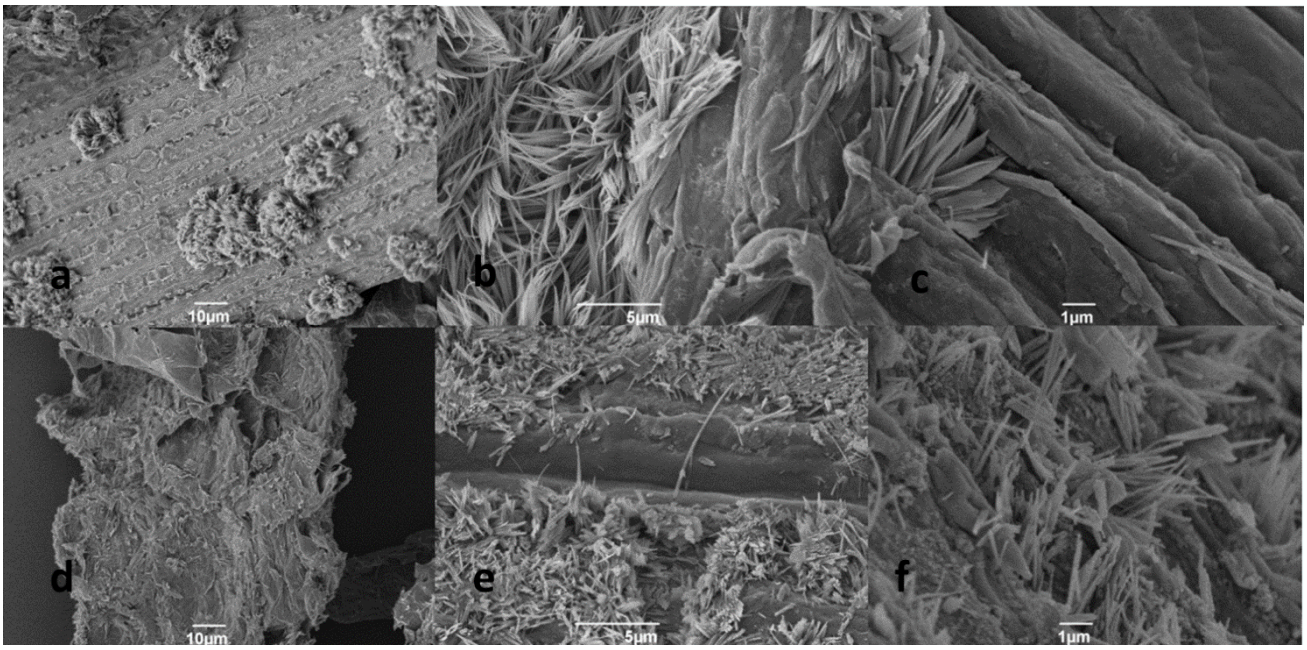


Figure 9. Surface images obtained by SEM on *Miscanthus* treated with 0.2 M and 0.4 M NaOH. Three different magnifications with scale bars between 10 µm and 1 µm. a–c: 0.2 M NaOH pretreatments; d–f: 0.4 M NaOH pretreatments.

The microwave assisted pretreatments under acid conditions using 0.2 and 0.4 M H_2SO_4 result in distinct morphological changes, when compared to the effects of the NaOH pretreatment. Figure 10 (a–c) and Figure 10 (e–f) shows the surface of *Miscanthus* treated with H_2SO_4 0.2 M and 0.4 M, respectively. The samples undergoing 0.2 M H_2SO_4 pretreatment show a very similar morphology to the untreated *Miscanthus*, indicating that the acid treatment is too mild under these conditions (Figure 10 (a–c) and 7 (a–c)). At higher magnification (Figure 10c), samples treated with acid present a tight and compact structure of cellulose microfibrils, similar to the raw bagasse and very different from the alkali effect on the ultrastructure of the cell wall. Samples treated with 0.4 M acid were carbonized. Macroscopically, the samples become a black powder and under microscope, present a degraded aspect, with spherical particles typical of carbonized samples (Figure 10 (d–f)) and showing a carbonized structure that keeps the general aspect of the fibre conducting bundles on the sample (Figure 10 d). Catalysed degradation of sugars to furans to hydrothermal carbon explains the very low amount of sugar released from the bagasse samples treated with higher concentration acid [52]. Under conventional heating conditions, water and H_2SO_4 pretreatments show mild influence on biomass, whereas NaOH has rather distinctive change on biomass surface (see

Supplementary Figure 2). Parallel strips and "lignin deposits" appeared on the biomass surface, which has been observed by others [53,54].

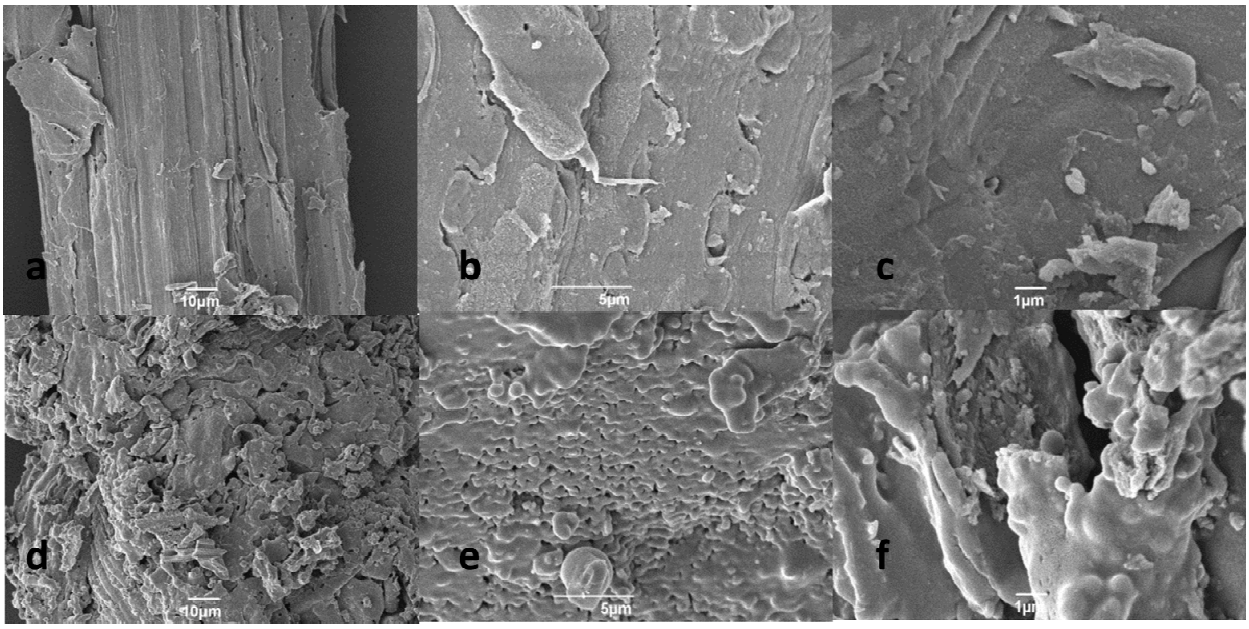


Figure 10. Surface images obtained by SEM on Miscanthus treated with 0.2 M and 0.4 M H2SO4. Three different magnifications with scale bars between 10 μm and 1 μm are shown. a–c: 0.2 M H_2SO_4 pretreatments; d–f: 0.4 M H_2SO_4.

3.7. Sugar degradation study

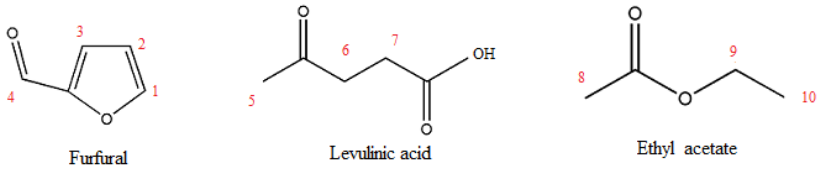
As it was shown in Figures 1 and 2, H_2SO_4 pretreatment led to promising yield of reducing sugars and selectively produced glucose, when holding time was 20 min. However, it is known that sugar dehydration also is facilitated by H_2SO_4, leading to the formation of furfural, formic acid, hydroxymethylfurfural (HMF) and levulinic acid, which are classified as inhibitors for fermentation [55]. Hence, the sugar dehydration products during microwave assisted pretreatment were analysed by GC and NMR (see supplementary Figure 3 and 4 for GC spectra). As can be seen, levulinic acid (LA) was identified as the predominant sugar degradation product during our microwave assisted acid pretreatment, and a very small amount of furfural was also obtained (see scheme 1). There are negligible amounts of other chemicals as well. The yield of LA was quantified by using anisole as an external standard. From 68–71 mg organic products were obtained from 400 mg biomass, of which LA was 25–31 mg (Table 4). The conversion of LA from biomass was between 6-8% under 0.2 M or 0.4 M H_2SO_4 conditions. The degradation products obtained from 0.4 M H_2SO_4 pretreatment were analysed by NMR (supplementary Figure 5). The chemical shifts from the spectrum are listed in Table 5. From NMR, we can confirm that levulinic acid was the predominant degradation product, with a small amount of furfural. Because ethyl acetate was used for extraction, it also is present in the NMR spectra. It was noticed that there was a gap in the mass balance, possibly because of the residue of ethyl acetate solvent. Secondly, during the step of re-dissolving organic products with ethyl acetate to make NMR samples, a black insoluble substance was present, which could be humins. Overall, there is a potential of using microwave technology to selectively produce LA from hexose or biomass under diluted H_2SO_4 conditions, which would be of

great interest in the biorefinery concept.

Table 4. Organic products during microwave assisted H_2SO_4 pretreatment.

Conditions (H_2SO_4, 20 Min)	Organic products (mg)	LA (mg)	Furfural (mg)	LA/ biomass (%)
0.2 M	71 ± 8	31 ± 10	5 ± 3	8 ± 3
0.4 M	68 ± 0.4	25 ± 5	1 ± 0.2	6 ± 1

*Each condition was done in triplicates and the results shown here were average with standard deviation.



Scheme 1. Chemical structure of Furfural, Levulinic acid.

Table 5. Assignments of ^{1}H NMR spectrum.

Proton positions	Chemical shifts (ppm)
Furfural	
1	8.00
2	6.58
3	7.67
4	9.61
Levulinic acid	
5	2.16
6	2.56, 2.58, 2.59
7	2.70, 2.72, 2.73
Ethyl acetate	
8	2.00
9	4.05, 4.07, 4.08, 4.10
10	1.19, 1.21, 1.23

4. Conclusion

Miscanthus is one of the most promising energy crops in Europe, and improvements in the processing of this species can contribute to realise second generation biofuels. In this work, microwave assisted pretreatment of *Miscanthus* with water, H_2SO_4 (0.2 M, 0.4 M or 1 M) and NaOH (0.2 M or 0.4 M or 1 M) was performed at various holding times (5 min. to 30 min.) under 180 °C. The total sugar yield firstly increases and then drops with the increase in holding time. 20 Min holding time removed the largest amount of sugars using various pretreatment media. H_2SO_4 has a

better sugar release production than NaOH during pretreatment, due to the efficient decomposition of both hemicellulose and cellulose, but it gives lower digestibility afterwards, because most of the digestible sugars are released during the pretreatment step. In comparison with conventional heating method, 12 times as much sugars were produced within half the time. Additionally, glucose is produced as the major component in the pretreatment media (maximal yield from available carbohydrate: 50%). Water and NaOH have a similar influence on sugar release during the pretreatment, giving rise to xylose as the major sugar component because of hemicellulose degradation. The xylose yield is as high as 27% from available carbohydrate when NaOH is used pretreatment media. In comparison with H_2SO_4, NaOH has a stronger performance on biomass surface, leading to exposed biomass fibres. The digestibility of *Miscanthus* pretreated with NaOH is 8 to 9 times higher than that of untreated *Miscanthus*, due to the efficient removal of hemicellulose and lignin and more opened biomass structure. Sugar degradation under microwave assisted H_2SO_4 condition was studied, and the results showed levulinic acid was selectively produced. About 8–9% biomass was converted into LA in this study. Overall, biomass compositions were more effectively broken down under microwave conditions, leading to higher sugar release during pretreatment than conventional heating pretreatment. Glucose and xylose were selectively produced by tuning pretreatment media. At the same time, microwave technology showed a great potential of producing LA from biomass.

Acknowledgments

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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