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A biorefinery approach for fractionation of *Miscanthus* lignocellulose using subcritical water extraction and a modified organosolv process.

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Abstract

Using a biorefinery approach, biomass polymers such as lignin and carbohydrates can be selectively purified from lignocellulosic feedstocks with the aim of generating not only lignocellulosic bioethanol but also high value bio-based compounds. Furthermore, the efficient use of the entire biomass can increase overall feedstock value and significantly contribute to process cost-effectiveness. Therefore, the aim of this work was to fractionate the main compounds of the energy crop *Miscanthus x giganteus* (MxG) using 'green' solvents in order to obtain cellulose-enriched fibres as well as non-

toxic streams rich in hemicellulose and lignin. Two processing routes were compared: a direct 1-step modified organosolv method for simultaneous lignin and hemicellulose removal; and a 3-step sequential process using subcritical water extraction for recovery of first extractives then hemicellulose, followed by modified organosolv lignin extraction. Both methods successfully generated cellulose-enriched fibres; from a complex mixture of compounds present in MxG, it was possible to obtain fibres comprising 78% cellulose without the use of commonly-applied toxic solvents that can potentially limit end uses for processed biomass and/or need additional neutralisation steps. Fibres generated by the direct and sequential processes were very similar in composition; however, physicochemical analysis of the fibres using scanning electron microscopy, Fourier-transform infrared spectroscopy and principal component analysis confirmed structural differences resulting from the two processing routes, which were demonstrated to have an impact on downstream processing.

Keywords: Subcritical water; Principal component analysis; Delignification; Biofuel; Biorefinery.

Abbreviations: MxG - *Miscanthus x giganteus;* SWE – Subcritical water extraction; PCA, Principle component analysis.

1. Introduction

The shift from a petroleum based economy towards one supported by renewable resources is not only environmentally beneficial, but it is also believed to be a way of achieving a sustainable economy and energy independence [1]. One potential renewable resource of current interest is lignocellulosic biomass, for example biomass comprising rapidly-growing plants or waste lignocellulosic biomass generated as a byproduct of agriculture and food processing [2-4]. In the former category, *Miscanthus x giganteus* (MxG) has been identified as an attractive source of biomass due to its

potential for high yields even with few inputs (nutrients, irrigation), high photosynthetic efficiency, low cost, and adaptability to low-quality land [5].

The biorefinery concept describes the utilisation of biomass to generate a range of products, for example fuels, platform chemicals and high-value chemicals, in a manner similar to the refinery of petrochemicals [2]. Interest in the biorefinery concept as part of a bio-based economy is increasing with technological advances in agriculture, biotechnology and chemistry, as well as societal drivers [2,6]. Moreover, it is believed that the successful implementation of an integrated biorefinery platform with the co-production of valuable products can make 2nd generation bioethanol cost-effective [7,8]. In this process, ethanol is generated from the fermentation of monosaccharides extracted and depolymerised from the cellulose and hemicellulose fractions of lignocellulosic biomass. However, due to the highly recalcitrant structure of lignocellulose, extraction and depolymerisation of monosaccharides is a difficult process, often with low monosaccharide yield due to decomposition of released monosaccharides under harsh reaction conditions. Moreover, available technologies for lignocellulosic fractionation are expensive, and frequently use toxic solvents to access biomass components, presenting an environmental concern [9].

In addition, it is widely reported that lignocellulose treatments to liberate monosaccharides result in the formation of fermentation inhibitors, which inhibit the production of ethanol from monosaccharides [10]. Thus, prevention of inhibitor formation during lignocellulosic processing to monosaccharides would potentially improve fermentative production of bioethanol.

An additional aim of biorefinery is similar in principle to chemical refineries: separation and purification of multiple commercially viable streams from a single feedstock. As well as hexose and pentose monosaccharides, useful for production of bioethanol via fermentation, potential streams from the biorefinery of lignocelluose include xylooligosaccharides (an emerging potential prebiotic [11]), and a variety of platform chemicals such as furan compounds, organic acids and phenolic compounds [12].

A major current issue with biorefineries using plant biomass as a feedstock is the use of harmful chemicals [13]. The use of ‘green’ solvents for lignocellulosic biomass processing is not only environmentally beneficial but it also holds the potential to generate non-toxic streams that could enhance the potential uses of biomass fractions for conversion into high-value products particularly for food and pharmaceutical applications [14]. Therefore, the use of subcritical water extraction (SWE) for hemicellulose extraction as a ‘green’ solvent is a potentially advantageous option that does not require additional catalysts, neutralization steps following processing or corrosion-resistant reactors [15,16]. SWE has previously been used for extraction of a wide range of different compounds in the biotechnology, food and pharmaceutical areas (reviewed by [17]). Lignin extraction can also be performed using ‘green’ solvents in a modified organosolv method using non-toxic solvents such as ethanol that can be recovered and re-used in the process [18] and alternative catalyst to replace bases (eg NaOH, KOH, ammonia) or mineral acids (H_2SO_4, HCl, H_3PO_4) used in delignification [13].

Previous work aiming to reduce MxG recalcitrance have been focused on lignin removal rather than biomass fractionation [19] and the use of mineral acids [19,20] and hydrogen peroxide [21] for extractions. Moreover, physicochemical evaluation of MxG fibres has been focused on visual evaluation of FTIR spectra rather than the use of a

statistical analysis such as PCA [20]. Therefore, the aim of this work was to evaluate two different routes to obtain purified cellulose fibres from MxG: a single-step modified organosolv approach; and a three-step SWE / modified organosolv approach designed to sequentially remove biomass extractives, hemicellulose and lignin from cellulose fibres (Fig. 1). Moreover, a physicochemical evaluation of the effect of these processing routes in the obtained fibre is presented using SEM, FTIR and PCA. Thus, this work proposes environmentally-friendly processes in a biorefinery approach as an attempt to fractionate lignocellulosic biomass and to obtain purified streams of hemicellulose and lignin and cellulose-enriched fibres that can be further processed into a variety of products including biochemicals and bioethanol.

2. Materials and methods

2.1. Materials

Air-dried *Miscanthus x giganteus* (MxG) was cultivated in Wales (UK), harvested in 2013, and kindly provided by Phytatec (Aberystwyth, UK). MxG used in this work contained (as percentage of dry weight): 11.5% of extractives, 22.6% of Klason lignin, and 18.3% of hemicellulose, all determined using NREL methods [22,23].

2.2. Extraction methods

2.2.1. Extractives SWE

0.01 kg (wet weight) of MxG was soaked in 0.2 L of distilled water at 50 °C for 20 min. The suspension was then ground in a domestic blender for 3 min and placed in a 0.5 L high-pressure reactor (Parr, alloy C276). The reactor was purged and pressurized to 5.0×10^6 Pa using N_2 and a heating jacket was set to 120 °C. The extraction lasted for 30 min (all residence times reported in this work starts when target temperature was achieved, i.e., heating time was not taken into consideration. Heating time varied

according to the target temperature and was from 12 to 27 min). At the end of the extraction, the reactor was cooled in an ice bath. Remaining fibres were filtered and dried completely at 65 °C. The fibres resulting from this procedure were called 120 °C fibres.

2.2.2. Hemicellulose SWE

0.01 kg of dried 120 °C fibres were placed in the same reactor as above and mixed with 0.2 L of distilled water. The reactor was purged and pressurized to 5.0×10^6 Pa with N_2 and a heating jacket was set to 180 °C for 30 min. After cooling the reactor in an ice bath, remaining fibres were filtered, dried completely at 65 °C and named 180 °C fibres. Temperatures for both extractives and hemicellulose SWE steps were chosen after preliminary tests.

2.2.3. Modified organosolv lignin extraction

The lignin extraction step was performed using a modified organosolv method adapted from Roque [24] in which mineral acids were replaced by pressurized CO_2 as catalyst. 0.25 L of 50 % (v/v) ethanol in distilled water (50 °C) was mixed with 0.005 kg of starting material (MxG, for direct delignification; 180 °C fibres for sequential extraction) and then allowed to soak for 20 min before being placed in the 0.5 L reactor. In the case of direct extraction, the suspension was ground in a domestic blender for 3 min before being placed in the reactor. The reactor was purged and pressurized to 5.0×10^6 Pa using CO_2 and set to 200 °C. The reaction lasted 60 min, and then the reactor was placed into an ice bath. Remaining fibres were filtered, air dried for 48 h and then dried completely at 65 ºC. Cellulose-enriched fibres obtained after lignin extraction were named DEL in the direct route and SEQ in the sequential extraction route (Fig. 1).

2.3. Quantitative/qualitative analysis

2.3.1. Extractives determination

The extractives content of the starting MxG material was determined using the National Renewable Energy Laboratory (NREL) protocol. This is a 2-step extraction procedure in a Soxhlet apparatus using first water (HPLC grade) as solvent for two consecutive days for 8h per day, and then ethanol as solvent for the same period of time [20]. Fibres were weighed before and after the extractions and the extractives compounds were calculated as the mass difference.

2.3.2. Lignin quantification

Lignin quantification was performed using the National Renewable Energy Laboratory (NREL) protocol [23] for Klason Lignin quantification using the Klason Lignin method.

2.3.3. High Performance Anion Exchange Chromatography (HPAEC)

Glucose (99.5%), arabinose (98%), xylose (99%), fructose (99%), cellobiose (98%), 5-hydroxymethyl-2-furaldehyde (HMF) (99%), erythrose (75%), and Avicel were purchased from Sigma Aldrich. Cellotetraose (95%) and cellohexaose (90%) were purchased from Megazyme, and galactose (99%) was purchased from Acros Organics).Sugar analysis in liquid samples were performed by High Performance Anion Exchange Chromatography coupled with Pulse-Amperometric Detection (HPAEC-PAD) from Dionex/Thermo (ICS-5000) using a guard CarboPac™ PA1 column (4x50mm) and an analytical CarboPac™ PA1 column (4x250mm). Oven and detector compartments were kept at 30 °C and 25 °C, respectively. Flow rate was 0.001 L/min and sample volume injected was 10×10^{-6} L, Milli-Q® water was used as solvent A and in the preparation of the other solvents. 0.2 M NaOH and 1 M NaOAc were used as solvent B and C respectively.

The method started with an isocratic step using 0.021 M of B during 20 min. At 20 min, B was increased to 0.080 M. Then, from 20 to 60 min, solvent C was introduced from 0-20 mM and B was kept at 0.080 M. A washing step was performed from 60 min in which B and C were increased to 0.120 M and 0.040 M, respectively, and kept constant for 10 min. At 70 min, C was set to 0 and B was set to 0.021 M for 20 min for column reconditioning. Total run time was 90 min per sample. Prior to HPAEC analysis, samples were acid-hydrolysed in 2 steps in order to break down polymers/oligomers into monomers to facilitate quantification. In the first step, 0.003 L of 72 % sulphuric acid (Fluka) was added to 0.0003 kg of sample and placed in a 30 °C water bath for 60 min. In the second step, distilled water was added to the sample in order to decrease acid concentration to 4 % and sample was placed into an oven at 121 °C for 60 min. Fibre compositional analysis was performed by two-stage acid hydrolysis as per Klason lignin determination [23] followed by HPAEC.

2.3.4. SEM imaging

Scanning electron microscopy (SEM) images were obtained using a Philips XL30 FEG Environmental scanning electron microscopy operating at 10 kV at several amplification magnitudes. Prior the analysis, samples were coated with platinum for 120 s using an Emscope Sc500 sputter coater.

2.3.5. Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR was performed in a Jasco FTIR 6300 spectrometer with a Specac Golden Gate ATR (Specac, Kent, UK). Samples were analysed with no prior preparation. Scans were obtained with resolution of 4 cm^{-1} and 32 scans between 4000-600 cm^{-1}, resulting in 1764 wavenumber data points for each spectrum.

2.3.6. Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed on the FTIR spectra data. PCA analysis was performed using the Unscrambler® X 10.3 software (CAMO). For the PCA data analysis, FTIR was performed on 5 independent samples of raw material (MxG) and each generated fibre (120 ^{o}C, 180 ^{o}C, DEL and SEQ) as well as on commercial cellulose (Avicel). Then, FTIR data was treated using smoothing followed by normalisation and 2nd-derivative, respectively, in order to decrease noise and increase spectral resolution [25-27]. Both smoothing and 2nd-derivative are tools available in the spectra software (Spectra Manager Version 2, Jasco®) and the software default parameters were used (smoothing – method, Means-Movement; convolution width, 25; and 2nd-derivative – algorithm, subtract; data points, 3). Normalisation was performed using the highest peak of each spectrum. This combination of data treatments was found to give the best clustering on scores plots.

2.3.7. Preliminary subcritical water hydrolysis

SEQ and DEL fibres were submitted to subcritical water (SW) hydrolysis and analysed for the production of glucose monomers. This was an exploratory evaluation in order to qualitatively compare the downstream processing of the fibres generated by direct and sequential routes.

SW hydrolysis was conducted in stainless steel tubes (thickness 0.0003 m) and caps (Swagelok, UK). Reactor dimensions were 0.0015 m of internal diameter and 0.0114 m length, 0.02 L total volume. Distilled water (0.015 L) was pre-heated to 50 °C prior to the reaction and mixed with 1.0% (w/v) of the biomass (SEQ or DEL) and placed into the reactor. The reactor was then placed into a pre-heated oven and the reaction lasted for a total of 20 min (heating time + residence time). Three temperatures were investigated: 220 °C; 250 °C; and 280 °C. After the residence time was completed,

reactors were placed in an ice bath to stop the reaction. The liquid fraction was then analysed for glucose concentration using HPAEC.

3. Results and discussion

Two different processing routes were evaluated in order to obtain cellulose-enriched fibres from MxG biomass: direct and sequential routes (Fig. 1). In the direct processing route, a modified organosolv treatment was used to solubilise and remove biomass extractives, hemicelluloses and lignin from cellulose fibres (remaining in the solid fraction) in a single step (Fig. 1A). In the sequential processing route, each biomass fraction (extractives, hemicellulose and lignin) was sequentially and selectively solubilised and separated from the solid fraction in a 3-step process comprising two subcritical water extractions (SWEs) of increasing severity and a final modified organosolv step identical to the direct route extraction (Fig. 1B). Removal of extractives and hemicellulose via the sequential route will first be considered, followed by lignin extraction from MxG (via the direct route) or from the fibres generated by the sequential route.

3.1. Extraction of extractives and hemicellulose via the sequential route

The sequential route was designed to first remove extractives such as non-structural polysaccharides (e.g. starch and pectin), as well as proteins and waxes, which are easily soluble in water and/or ethanol. The step to remove these extractives prior to hemicellulose extraction was intended to increase the purity of xylooligosaccharides (XOS) in the liquid phase in the second extraction step [28]. If the extractives are not removed prior to hemicellulose extraction, their removal from the hemicellulose fraction is very challenging.

MxG fibres were subjected to the first SWE step (H_2O, 5.0×10^6 Pa N_2, 120 °C, 30 min). 15 ± 2% of the MxG dry mass was removed as extractives in this step, leaving an extractives-free solid fraction (named here as “120 °C fibres”; Table 1). HPAEC analysis of the liquid extractives fraction revealed the presence of glucose, presumably derived from hydrolysis of starch, and very low concentrations (at the detection limit of the HPAEC used) of arabinose and xylose, indicating very limited hydrolysis of hemicellulose in this step (data not shown). The mass reduction by the first SWE extraction step was slightly higher than the extractives content of the MxG fibres as determined by the NREL method (11.5%, Table 2); this reflects differences in the methods used, the SWE extraction method likely extracting additional components of the MxG not solubilised by the NREL method.

The 120 °C fibres generated by the first SWE step were subjected to a second, more harsh, SWE step, hemicellulose extraction (H_2O, 5.0×10^6 Pa N_2, 180 °C, 30 minutes); analysis of the resultant fibres (named “180 °C fibres”) revealed substantial extraction of hemicellulose (from 20.6 % dry weight to 8.8 % dry weight; Table 2). Glucose could not be detected in the extracted liquid hemicellulose fraction, revealing that cellulose was not degraded in this step. For each 1 kg dry mass of 120 °C fibres subjected to hemicellulose extraction, 0.78 kg dry mass of 180 °C fibres were generated.

3.2. Comparison of delignification using direct and sequential routes

Direct delignification of MxG fibres using the modified organosolv process (50% EtOH, 5.0×10^6 Pa CO_2, 200 °C, 60 min) generates a liquid fraction rich in solubilised lignin, but which also contains: hemicellulose and its depolymerisation products (xylooligosaccharides and xylose); the decomposition products of hemicellulose such as acetic acid and furfural; and biomass extractives. Although lignin can be recovered

from this liquid fraction [29], separation of hemicellulose from the other components is challenging and its use in other processes can therefore be compromised. Comparison of the delignification step of the sequential extraction with the direct extraction route reveals that direct extraction was able to remove 73 % of the lignin from MxG fibres, whereas the organosolv delignification step of the sequential treatment removed 62 % of the lignin from the 180 °C fibres. It should also be noted that the hemicellulose extraction step (H_2O, 5.0 × 10^6 Pa N_2, 180 °C, 30 minutes) removed some lignin (equivalent to 12% of the lignin present) from 120 °C fibres. The resultant DEL fibres have a lower lignin content than SEQ fibres (Table 2).

Sequential extraction resulted in a lower percentage of lignin removal compared to direct delignification, most likely due to the severity of the organosolv process. As 180 °C fibres had already been exposed to two SWE treatments, it is likely that their lignin was more accessible than in the raw MxG. However, the severity of the organosolv process, especially under acidic conditions, has previously been associated with a decrease in lignin removal due to non-desirable reactions including condensation reactions, formation of pseudo-lignin, and/or re-precipitation of lignin into the remaining fibres [14, 30-32].

3.3. Analysis of final fibre composition

Comparison of fibre composition (Table 2) revealed that SEQ fibres generated by sequential extraction and DEL fibres generated by the one-step modified organosolv process had broadly similar compositions; SEQ fibres contained more lignin and less hemicellulose than DEL fibres. Assuming that the residual component (ie not lignin or hemicellulose) of the fibres was cellulose, then SEQ and DEL fibres were comparable in cellulose content.

The fibres and compositions obtained in this work are in agreement with other published studies using SBW and organosolv method catalysed by mineral acids extractions in MxG. Timilsena et al. [20] reported comparable results for MxG after a similar sequential extraction using SWE followed by acid-catalysed organosolv. In their study, the solid fibres after the sequential treatment presented mainly glucans (76.6%), lignin (17.3%) and residual xylan (5.8%) [20]. El Hage et al. [33] generated fibres containing 14.1% lignin after acid-catalysed organosolv treatment of MxG; the use of CO_2 as a catalyst in this work proved to be a potential replacement for mineral acids presenting comparable results for lignin extraction.

3.4. Effect of direct and sequential extraction on the physical and chemical properties of delignified cellulose fibres

The direct and sequential extractions aimed not only to recover and fractionate lignocellulosic components (extractives, hemicellulose, lignin), but also to obtain cellulose-enriched fibres and modify the structure of the cellulose in order to make it more accessible to hydrolysis so to act as a feedstock for second-generation bioethanol production by fermentation. Lignin extraction methods are thought to create pores in the lignocellulosic matrix, which might facilitate cellulose disruption [34].

Composition analysis of SEQ and DEL fibres did not show significant compositional differences between these fibres (Table 2). However, it remains unclear whether or not the SEQ and DEL fibres were physically different, which might impact on subsequent cellulose hydrolysis. Scanning electron microscopy (SEM) and Fourier Transfer Infra-Red (FTIR) spectroscopy analysis were used to compare the physical and chemical properties of SEQ and DEL fibres.

Visualising the fibres by SEM (Fig. 2), it is possible to see that the density of lignin droplets on the cellulose fibre surface is significantly higher for SEQ than for DEL fibres. This increase in density of droplets has been observed before and was attributed to increases in the severity of the pretreatment process [35]. Differences in lignin droplet size and shape, as shown in Fig. 2E and 2F, have also been previously observed [36] and smaller size and higher density of droplets present on SEQ fibres could indicate that the lignin has been more extensively fragmented when compared to the DEL fibres. Hence, in theory, cellulose from SEQ fibres is potentially more exposed than in DEL fibres.

FTIR is a rapid analysis method and can potentially indicate differences in chemical composition of lignocellulose fibres by differential analysis of generated spectra. However, the difficulty of using FTIR in analysis of lignocellulosic biomass is the overlapping of peaks due to the presence of large numbers of different chemical bonds found in lignocellulose [37]. Indeed, FTIR spectra of MxG, DEL and SEQ fibres were extremely complex (Fig. 3A). Therefore, in order to establish a better understanding of the chemical characteristics of MxG and the potential for change in chemical properties during delignification, the FTIR data were analysed by principal component analysis (PCA). FTIR data was manipulated prior to PCA using smoothing, normalisation and 2^{nd}-derivative functions.

Fig. 3B shows the PCA scores plots for MxG, 120 °C, 180 °C, SEQ and DEL fibres in which each plot is an independent sample. FTIR spectra data for commercial pure cellulose (Avicel PH101) was also used for comparison purposes. The scores plots of PCA present the samples grouped by their variability. The differences among the

samples presented in the scores plot are thought to be chemical (composition) and/or structural [38]; therefore, samples in the same cluster present similar features.

Samples were successfully separated into defined clusters; such clustering is impossible when visually analysing FTIR spectra (Fig. 3A). MxG and DEL fibres both present relatively broad clusters compared to the other fibres, possibly indicating their innate variability. According to Fig. 3B, it is also possible to suggest a trend in terms of changes in principle component 1 (PC1) and PC2 as fibres are processed. From the raw material (MxG), every treatment increased the PC1 value of the fibre, visualised in a move from left to right on the plot. It is known that each of the treatments performed resulted into an increase in cellulose percentage in the fibres, therefore PC1 could correlate to the cellulose contents and/or cellulose purity of the fibres. Moreover, the position of Avicel, comprising pure cellulose, in the positive region of PC1 supports this suggestion. However, SEQ and DEL have very similar cellulose contents (79.9 and 78.9%, respectively) and do not have the same PC1 value. Therefore, it is clear that cellulose content is not the only feature of the fibres that PC1 describes.

In addition, the sequential and direct processing routes generated fibres that had opposite values along PC2 (SEQ fibres being positive and DEL fibres being negative). However, the feature described by PC2 is not easily determined. PC2 might be related to hemicellulose contents, as the fibre that has the highest PC2 value (120 °C) is also the one that has the highest percentage of hemicellulose among all samples. Moreover, after losing a significant amount of hemicellulose, the PC2 value of 180 °C fibres was significantly lower than that of 120 °C fibres. Nevertheless, it is clear that hemicellulose is not the only feature described by PC2 as DEL fibres have higher hemicellulose contents than SEQ, but present lower PC2 values. These results indicate

that PC2 describe not one single characteristic, but a group of chemical and/or physical features of the fibres.

In conclusion, although DEL and SEQ fibres presented a similar composition in terms of cellulose, hemicellulose and lignin, physico-chemical analysis showed that they are rather different and, therefore, it is expected that they will behave differently during cellulose hydrolysis.

3.5. Effect of direct and sequential extraction on the hydrolysis of cellulose fibres

In order to evaluate if SEQ and DEL fibres would behave differently when subjected to further processing as hypothesised based on the PCA results, hydrolysis tests were conducted. The fibres were submitted to subcritical water (SW) hydrolysis at three different temperatures from 220-280 °C and generated glucose was quantified by HPAEC. This step aimed only to confirm that SEQ and DEL fibres had different hydrolysis properties; no optimization of hydrolysis conditions was conducted at this point.

Fig. 4 shows that glucose concentration after hydrolysis at different temperatures was indeed different for SEQ and DEL fibres. Under the same conditions, SEQ fibres generated higher glucose concentrations than DEL fibres at 250 °C and 280 °C, most likely due to the decrease in biomass recalcitrance resulting from the sequential extractions. Therefore, although the two proposed processing routes (direct and sequential extractions) led to fibres of similar composition, DEL and SEQ fibres are structurally different (as suggested by both PCA analysis and SEM images), reflected by different recalcitrance to hydrolysis. These results suggested that the sequential extraction could be a promising option for biomass fractionation in order to obtain both purified biomass fraction streams as well as less recalcitrant cellulose-enriched fibres.

A more detailed hydrolysis evaluation and optimization is required to further support this conclusion.

4. Conclusion

Using the biorefinery approach, cellulose-enriched fibres were successfully obtained from MxG using 'green' processes in two routes: 1) direct delignification; and 2) sequential extraction followed by delignification. Contrary to expectation, after the modified organosolv method step, DEL fibres had a lower percentage of lignin than SEQ fibres, most likely due to non-target reactions resulting from the accumulated severity of the SWE steps in the sequential route. Nevertheless, sequential extraction is still preferred because of the potential of using the liquid streams for high-value product generation, which has the potential of economically support production of 2nd-generation bioethanol. Although similar in composition, both SEM and FTIR-PCA analysis showed significant physicochemical and structural differences between SEQ and DEL fibres. Moreover, preliminary subcritical water hydrolysis suggested that these structural differences led to differences in monosaccharide production; SEQ fibres showed higher glucose production after hydrolysis. Therefore, the sequential extraction could be a promising option for biomass fractionation in a biorefinery approach.

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FIGURE CAPTIONS

Figure 1. Scheme of direct delignification and sequential extraction followed by delignification.

Figure 2. SEM images for cellulose-enriched fibres. Fibres visualised are DEL (A,C,E); and SEQ (B,D,F). Images magnification: A & B, 500x; C, 1 200x; D, 2 000x; E, 8 000x; and F, 12 000x.

Figure 3. FTIR analysis and PCA of fibres. A. FTIR spectra of Avicel (pure cellulose), DEL and SEQ fibres. B. PCA scores plots for FTIR data after smoothing+2nd-derivative+normalisation. Each data point is an independent sample.

Figure 4. Glucose production by subcritical water (SW) hydrolysis of SEQ and DEL fibres. Glucose concentrations (g/L) determined by HPAEC after SW hydrolysis of SEQ or DEL fibres at temperatures from 220 °C to 280 °C for 20 min and 1% biomass load. Mean data from three independent replicates are shown along with standard deviations as error bars.

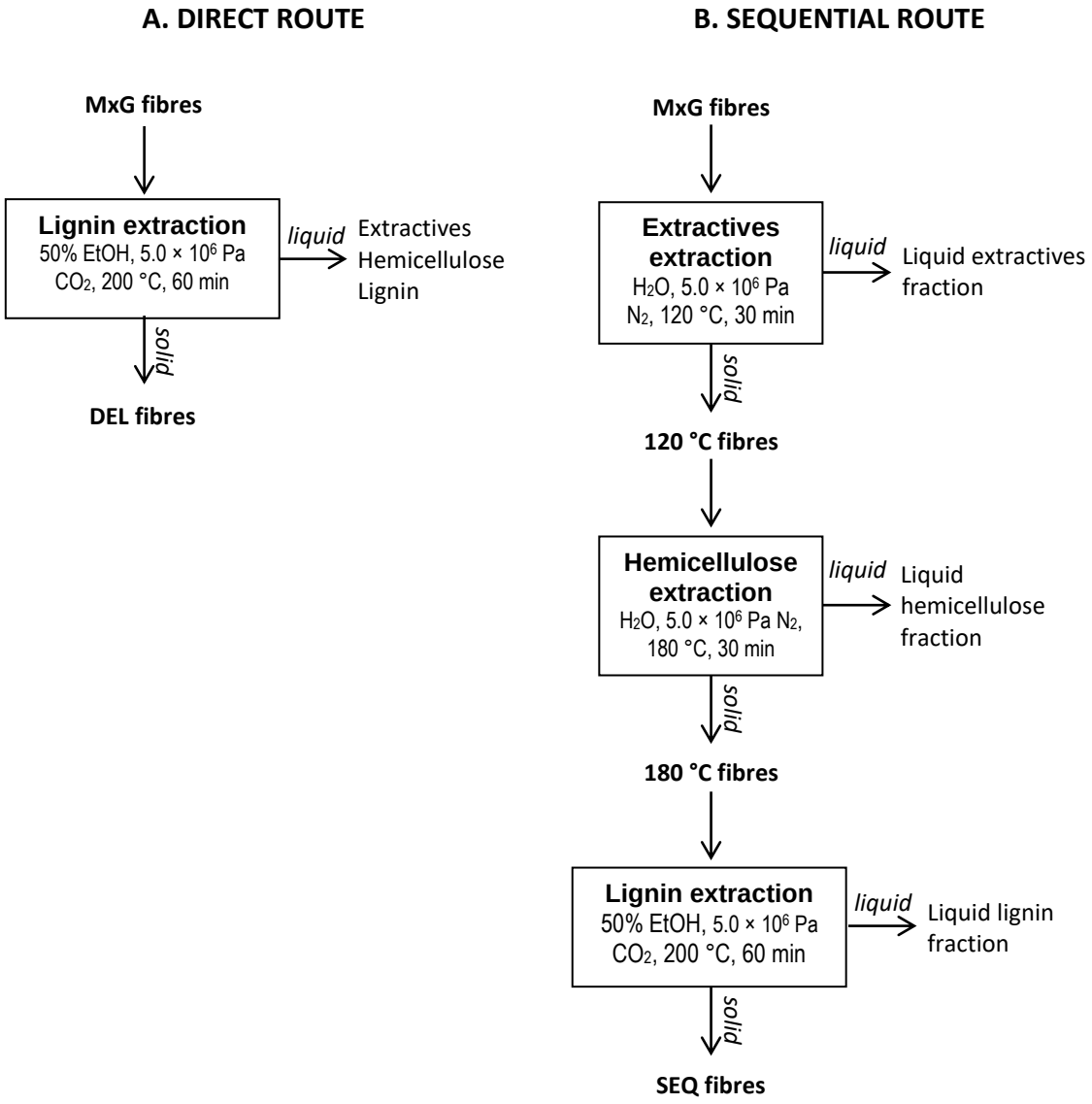
A. DIRECT ROUTE
B. SEQUENTIAL ROUTE
MxG fibres
Lignin extraction
50% EtOH, 5.0 × 10⁶ Pa
CO₂, 200 °C, 60 min
liquid
Extractives
Hemicellulose
Lignin
solid
DEL fibres
MxG fibres
Extractives extraction
H₂O, 5.0 × 10⁶ Pa
N₂, 120 °C, 30 min
liquid
Liquid extractives fraction
solid
120 °C fibres
Hemicellulose extraction
H₂O, 5.0 × 10⁶ Pa N₂,
180 °C, 30 min
liquid
Liquid hemicellulose fraction
solid
180 °C fibres
Lignin extraction
50% EtOH, 5.0 × 10⁶ Pa
CO₂, 200 °C, 60 min
liquid
Liquid lignin fraction
solid
SEQ fibres

Figure 1.

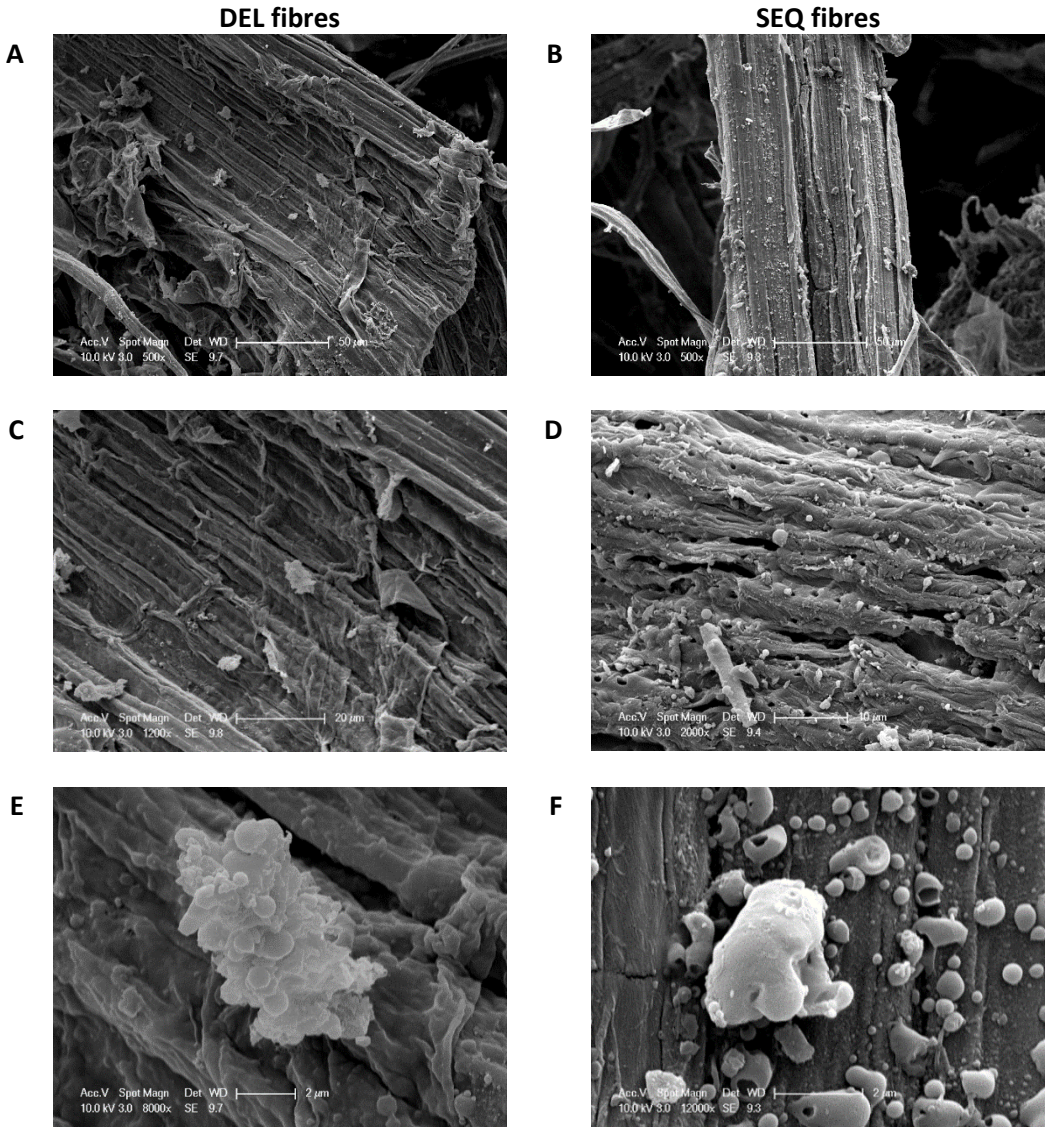

Figure 2.

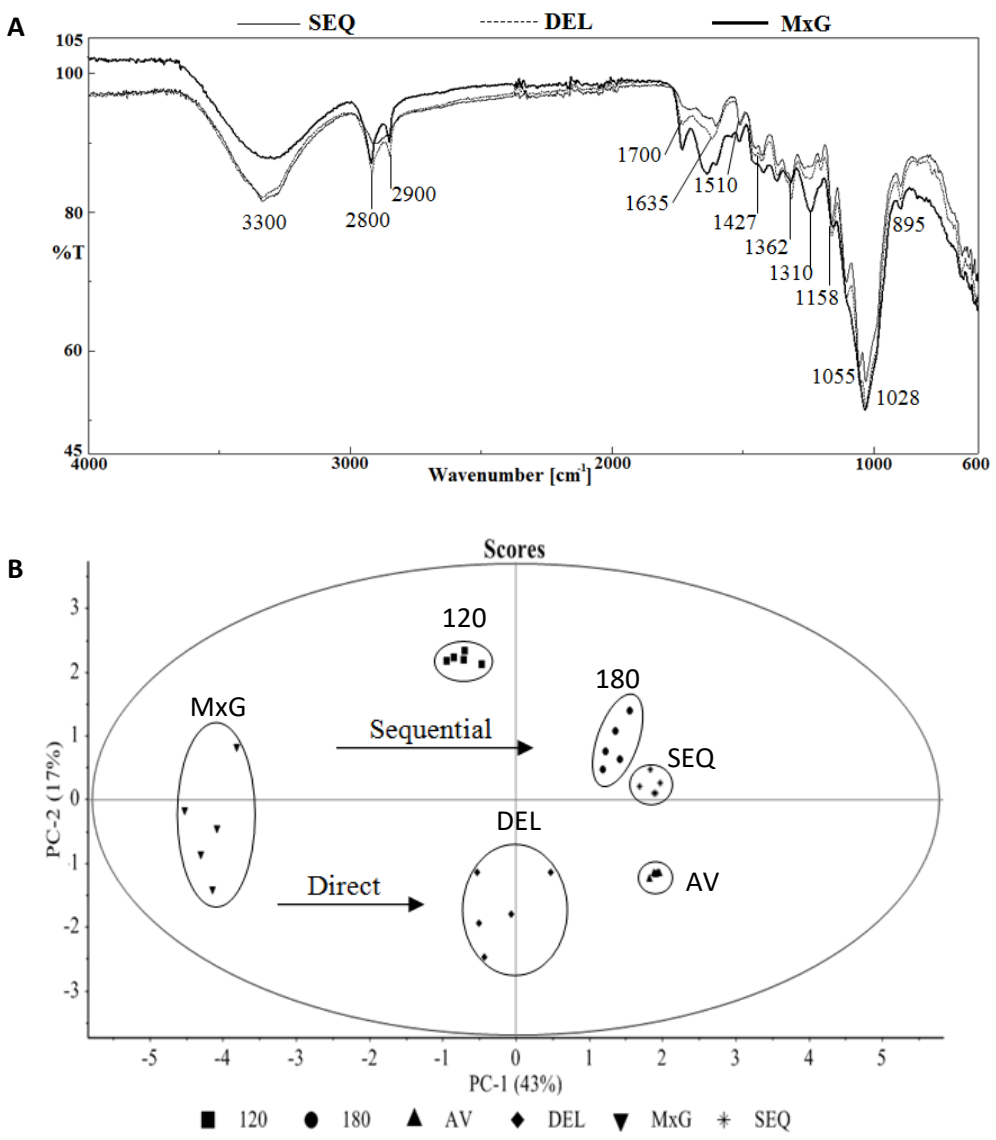
A
SEQ
DEL
MxG
105
100
80
%T
60
45
4000
3000
2000
1000
600
Wavenumber [cm-1]
3300
2800
2900
1700
1635
1510
1427
1362
1310
1158
1055
1028
895
B
Scores
120
180
MxG
Sequential
SEQ
DEL
Direct
AV
PC-2 (17%)
PC-1 (43%)
120
180
AV
DEL
MxG
SEQ

Figure 3.

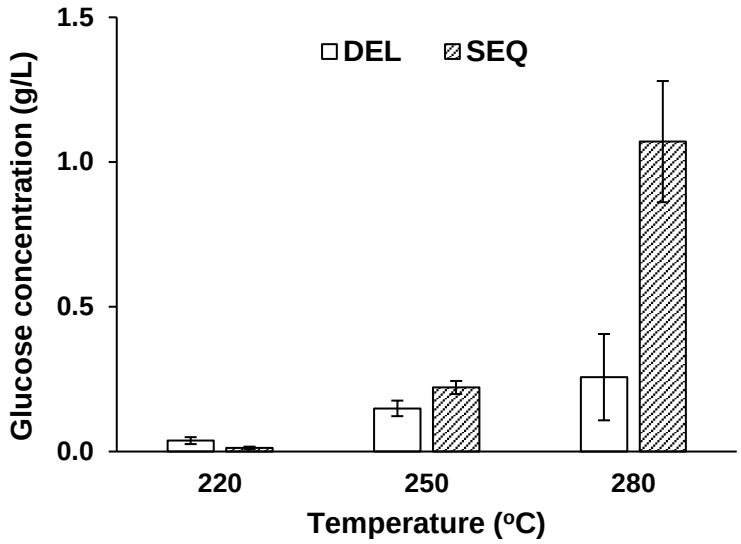
1.5
1.0
0.5
0.0
DEL
SEQ
Glucose concentration (g/L)
220
250
280
Temperature (ºC)

Figure 4.

Table 1. Mass balance for direct and sequential extraction routes.

Sequential route	**MxG (unprocessed)**	**120 °C fibres**	**180 °C fibres**	**SEQ fibres**
Dry mass	1 ± 0.10	0.85 ± 0.02	0.66 ± 0.03	0.44 ± 0.03
Direct route	**MxG (unprocessed)**	**DEL fibres**		
Dry mass	1 ± 0.10	0.48 ± 0.03		

Values are expressed in units of 1×10^{-3} kg; Masses are quoted in terms of dry mass; mean values ± standard deviations are given from multiple extraction experiments.

Table 2. Fibre compositions as percentage dry weight.

	MxG (unprocessed)	**120 °C fibres**	**180 °C fibres**	**SEQ fibres**	**DEL fibres**
Extractives†	**11.5 ± 0.05**	ND	ND	ND	ND
Hemicelluose:	**18.3**	**20.6**	**8.8**	**4.8**	**7.2**
Xylan	17.1 ± 1.1	19.2 ± 1.9	8.4 ± 0.8	4.8 ± 0.3	7.2± 0.6
Arabinan	1.0 ± 0.01	1.1 ± 0.01	0.3 ± 0.001	-	-
Galactan	0.2 ± 0.00	0.3 ± 0.00	0.1 ± 0.000	-	-
Klason lignin	**22.6 ± 0.6**	**25.4 ± 0.2**	**28.9 ± 0.7**	**16.4 ± 0.5**	**12.9 ± 0.5**
Cellulose*	**47.6**	**54.0**	**62.3**	**78.8**	**79.9**

Percentages are quoted as mean values ± standard deviations from multiple extraction experiments. Fibre composition was determined for a random sample of each type of fibres. † Quantity of extractives in MxG were determined using the NREL method. * Cellulose concentrations were not measured, but were assumed to constitute the balance of the mass of each fibre.