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Optimizing deep eutectic solvent pretreatment for enhanced glucan recovery from miscanthus

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This work focuses on the use of the deep eutectic solvent composed of choline chloride and acetic acid (1 : 2) in the pretreatment of the perennial energy crop Miscanthus, commonly called silvergrass. The pretreatment time and temperature were optimized to achieve a maximum of the glucan and minimum of the lignin contents in the pretreated biomass. In the optimization approach, the maximal glucan recovery in the pretreated solid was also considered. The performed optimization resulted in conditions (2 h 52 min and 150 °C), at which a pretreated biomass contained 74.1 wt% and 9.5 wt% of glucan and lignin respectively, and a glucan recovery was as high as 87.0 wt%. Furthermore, the biocompatibility of deep eutectic solvent was evaluated by using enzymatic hydrolysis washed and unwashed pretreated biomass produced at optimal conditions. The enzymatic hydrolysis of washed biomass resulted in higher glucan and xylan conversion than those achieved from unwashed biomass, deeming the step of biomass washing necessary. This was confirmed by the fractal kinetics modelling that confirmed higher accessibility of glucan for washed biomass than for unwashed Miscanthus sample.

1. Introduction

The exponential growth of the global population and the expansion of energy-intensive industries have resulted in the unsustainable overexploitation of our limited resources. While fossil fuels have propelled remarkable industrial advancements, their utilization comes at a considerable environmental cost. The combustion of these non-renewable resources has led to the emission of substantial amounts of greenhouse gases into the atmosphere, deepening global warming and climate change.[1] Therefore, it is necessary for industries to move towards sustainable alternatives. In this context, lignocellulosic biomass emerges as a promising solution.[2] Derived from non-edible organic sources such as crop residues, forestry waste, and dedicated energy crops, biomass offers a renewable and carbon-neutral resource that can be effectively exploited to generate a variety of commodities including, *e.g.*, biomaterials and biofuels.[3]

Utilizing agricultural and forestry biomass residues is important to limit potential greenhouse gas emissions as well as landfill waste. Hence, an integration of energy crops is necessary to address the drawbacks of first-generation biomass valorization.[4] Energy crops can be a controlled and reliable source of feedstock that can counter the seasonality issues typical for agricultural residues. The combination of these feedstocks in the context of biorefinery allows for more controlled and continuous production of renewable resources. Additionally, the use of underutilized degraded or marginal lands can ensure a sustainable and efficient approach for sustainable alternative to fossil resources.[5]

Miscanthus has been widely studied for its potential as an energy crop due to its high biomass productivity, low need for pesticides, fertilizers, and low water footprint.[6] However, the conversion of Miscanthus, like any lignocellulosic biomass, presents significant challenges, mainly due to its resistant nature.[7] Lignocellulosic biomass requires pretreatment before conversion, particularly in biological conversion processes, to make the cellulose more accessible to enzymatic hydrolysis.[8] This pretreatment step is one of the most costly and energy-intensive stages of the overall biomass conversion process.[9,10] Furthermore, conventional volatile organic solvents used in pretreatment, although highly efficient, present concerns regarding their toxicity and harmful environmental impact.[11,12]

Deep eutectic solvents (DES) have emerged as a promising alternative to traditional solvents, offering various potential economic and environmental benefits.[12–14] DES are solvents with low volatility and toxicity, and are easily prepared by mixing a hydrogen bond acceptor (HBA), usually choline

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chloride (ChCl), and a hydrogen bond donor (HBD), which can vary from carboxylic acids and carbohydrates to amino acids.[14] Such compounds are usually not only biodegradable and biocompatible but also widely available.[12] The process of biomass pretreatment can be simplified if the solvent used has a negligible effect on the efficiency of the enzymatic hydrolysis and fermentation process needed for biomass conversion.[15] This feature makes the biomass washing step after pretreatment and before conversion either unnecessary or less extensive compared to other solvents such as ionic liquids and organic solvents.[16] In addition, the selectivity of DES for hemicellulose and lignin extraction from biomass also offers the possibility of lignin valorization, which can bring economic advantages to the biorefinery.[17]

Literature reports have shown that processing Miscanthus with DES has a significant potential. For example, pretreatment with ChCl/glycerol for 6 h at 100 °C led to the production of an amorphous cellulose-rich extract that, after enzymatic hydrolysis with 200 FBU per $g_{cellulose}$ of Celluclast 1.5 L and β-glucosidase for 72 h at 50 °C, resulted in a glucose hydrolysis efficiency of 96 wt%.[18] Similarly, Miscanthus pretreated with ChCl/glycerol, using the heteropoly acid silicotungstic as a catalyst, for 3 h at 120 °C yielded a glucose hydrolysis efficiency of 97.3 wt% when processed under the following conditions of 50 °C for 72 h using 15 FPU per $g_{cellulose}$ of Celluclast 1.5 L.[19]

This work focuses on the pretreatment of Miscanthus using ChCl/acetic acid in 1 : 2 ratio. The parameters of time and temperature were evaluated in terms of their influence on glucan and lignin content in the pretreated biomass, as well as on the yield of recovered glucan. Furthermore, the pretreated biomass produced under optimal conditions was subjected to enzymatic hydrolysis, in which both glucan and xylan hydrolysis yields were assessed for washed and unwashed pretreated biomass, thus evaluating the biocompatibility of the DES, and its effect on such process. The approach used in this work is presented in a schematic way in Fig. 1.

2. Materials and methods

2.1 Raw materials and chemicals

Miscanthus biomass was originally from Comgoed (NL), delivered by TU Delft, and distributed by TNO as part of the cooperation in BRISK2 project. Before use, the biomass was milled to 1.5 mm using a knife mill (IKA WERKE GmbH & Co, Staufen, Germany), and was stored in a closed glass container at room temperature. The biomass size distribution was assessed using a Retsch (Haan, Germany) vibratory sieve shaker (EVS1, Endecotts, England) for 30 minutes, with sieves of pore diameters of 1.00, 0.71, 0.50, 0.25 and 0.15 mm. The mass fractions of each granulometry range were assessed by weighing the remaining material in each sieve.

The DES used in the pretreatment was composed of choline chloride (>98 wt% Sigma Aldrich, St. Louis, USA) and acetic acid (>99.8 wt% Fluka-Honeywell, Charlotte, USA). The pretreatment medium was prepared as described elsewhere,[20] by mixing ChCl and acetic acid in a 1 : 2 molar ratio in an 80 °C water bath (Memmert W200) resulting in a DES with a pH of 1.7.

Basylone M-350 oil purchased from Bayer (Leverkursen, Germany), was used in all biomass pretreatment assays as the heating medium. The PURELAB Classic Elga system was employed to produce distilled water (15 MΩ cm^{-1}) for all needed procedures. After the biomass pretreatment, lignin was solubilized using ethanol 96% v/v purchased from AGA-Álcool e Géneros Alimentares, S.A. (Prior Velho, Portugal). The solids, namely the cellulose-rich fraction and the lignin-rich fraction, obtained after each processing stage, were separated using filtration membranes made of cellulose fibers and very pure cotton linters whose content in alpha cellulose is virtually 100% (∅ = 150 mm, no. 1235) from Filter-Lab, Filtros Anoia, S.A. (Barcelona, Spain). Sulfuric acid (72 wt%) purchased from Carlo Erba (Milan, Italy) was used for hydrolyzing biomass in the procedure of quantitative acid hydrolysis. The resulting hydrolysates both from quantitative acid hydrolysis and enzymatic hydrolysis were analyzed with HPLC with glucose, xylose, arabinose, and acetic acid standards of analytical grade purchased from Sigma Aldrich (St. Louis, USA). All samples for HPLC analyzes were filtered using 0.22 μm nylon membranes (Agilent Captiva, Agilent Technologies, USA).

Enzymatic hydrolysis was conducted with the commercial enzyme Cellic CTec2, generously provided by Novozymes (Denmark). Enzymatic hydrolysis samples were prepared using a 50 mM sodium citrate buffer (pH = 4.8), prepared using tris-sodium citrate (>99 wt% purity) and citric acid monohydrate (99.7 wt% purity), both purchased from VWR International Ltd.

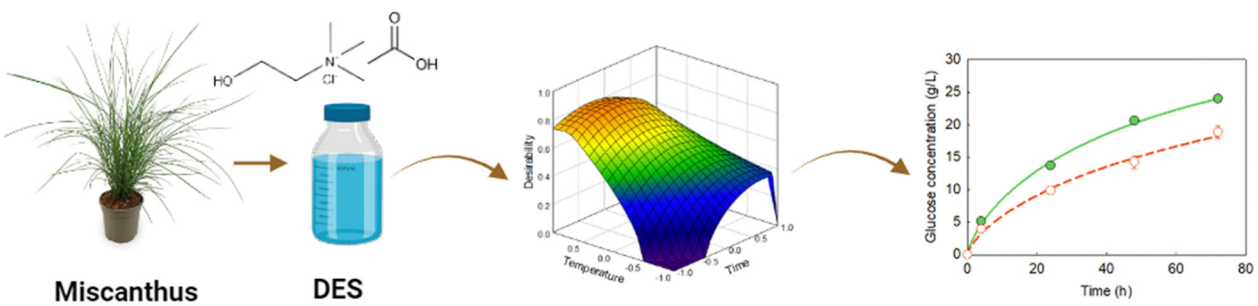

Fig. 1 Schematic of DES pretreatment of Miscanthus.

(Leicester, England). Aseptic conditions during these assays were maintained using sodium azide 99 wt% acquired from Merck (Darmstadt, Germany).

2.2 Biomass pretreatment

The biomass pretreatment reaction was conducted as described in a previous work.[21] The process involved a solid-to-liquid ratio of 1 : 10, with 5 g of dry biomass to 50 g of DES, and 20 g of dry biomass to 200 g of DES. The mixture was placed in Schott flasks of twice the mixture's volume (100 mL for 5 g and 500 mL for 20 g) and heated in an oil bath using an IKA heating plate and thermometer. After the reaction, the pretreated biomass was cooled, washed, and centrifuged twice with 20 mL and 10 mL of ethanol per gram of biomass at 4000 rpm for 15 minutes. The solids were vacuum filtered and dried at 45 °C for 24 hours. The liquid fraction was distilled to separate ethanol, and the resulting liquid lignin was precipitated by adding distilled water. The lignin-rich fraction was centrifuged, washed with a 1 : 9 ethanol-to-water solution, and vacuum filtered. The cellulose-rich fraction was washed with 40 mL of distilled water per 0.5 g of biomass, stirred for 20 minutes, vacuum filtered, and dried for 24 hours at 45 °C. For enzymatic hydrolysis with unwashed biomass, this stage was omitted.

2.3 Compositional analysis of solids

The moisture and ash content of the biomass were determined following the National Renewable Energy Laboratory (NREL) protocols TP-510-42621[22] and TP-510-42619,[23] respectively. Biomass composition, *i.e.*, its cellulose (as glucan), xylan and arabinan contents were determined using the acid hydrolysis procedure described in the NREL/TP-510-42618 method.[24] The hydrolysates resulted from this procedure were also used to quantify acid-soluble lignin using UV spectrophotometry at 320 nm.[25]

Hydrolysates from quantitative acid hydrolysis and enzymatic hydrolysis were analyzed with an Agilent 1100 series HPLC system (Santa Clara, CA, USA) with a Bio-Rad Aminex HPX-87H column (Hercules, CA, USA) and a refractive index (RI) detector, for the detection of glucose, xylose, acetic acid, and arabinose. For such analysis column was operated at 50 °C, applying a 0.6 mL min^{-1} flow rate using 5 mM H_2SO_4 as the mobile phase.

2.4 Enzymatic hydrolysis

Enzymatic hydrolysis assays were conducted in 50 mL Erlenmeyer flasks placed in an incubator, equipped with an orbital shaker, at 50 °C with 125 rpm of agitation. Each assay had a total volume of 10 mL, and was composed of biomass, 50 Mm acetate buffer (pH 4.8), distilled water sodium azide (2% w/v), and Cellic CTec2 enzyme, added immediately before incubation. Biomass quantities in each experiment were calculated to maintain a constant level of enzymatic activity, of 15.45 FPU per g_{glucan}. The enzymatic activity was determined as described by Ghose (1987),[26] and according to the NREL/TP-510-42628 method[27] and amounted to a total of 154.17 FPU mL^{-1}. To analyze the reaction kinetics, a 0.5 mL sample was taken at the reaction time intervals of 4, 24, 48, and 72 h. The sugars concentration and conversion yields were determined by HPLC analysis described in Section 2.3. For this purpose, the enzymes still present in solution were inactivated by boiling each sample for 10 min, and the solids were separated by centrifugation. Sugars conversion yields were determined as follows:

$$\text{Glucan to glucose yield (wt\%)} = \frac{\left(\frac{162}{180}\right) \times [\text{Glucose}] \times V}{\text{glucan content}} \times 100\%,$$

$$\text{Xylan to xylose yield (wt\%)} = \frac{\left(\frac{132}{150}\right) \times [\text{Xylose}] \times V}{\text{xylan content}} \times 100\%,$$

where [Glucose] and [Xylose] are glucose and xylose concentrations in g L^{-1}. V is the volume of the enzymatic solution in L. Glucan and xylan content refers to their amount in the pretreated biomass before the enzymatic hydrolysis experiment.

2.5 Fractal kinetics modeling of enzymatic hydrolysis

For the evaluation of the enzymatic hydrolysis reaction kinetics the following fractal kinetics equation was used: $P(t) = S_0\left[1 - e^{-kt^{1-h}}\right]$, where $P(t)$ is the product concentration, S_0 is the initial concentration of substrate and t is time in h. These parameters are obtained experimentally. The factors from the equation, which are calculated are k, the rate coefficient in h^{-1}, and h ($0 \leq h < 1$), the fractal exponent. These values were calculated by simulating $P(t)$ values using random k and h values and minimizing the sum of squared errors between the simulated values of $P(t)$ and the experimental data, using the excel solver tool with variables of h and k.[28]

2.6 Experimental design

Pretreatment reaction conditions were defined using the Doehlert matrix chemometric tool for time and temperature. In a first iteration the variables intervals were set as: time from 1 to 5 h, and temperature from 100 °C to 150 °C. Such conditions were chosen based on similar pretreatment reactions assessed elsewhere.[18,19] The result of such design is presented as X_1^* and X_2^* in Table 1. A second iteration was necessary, as explained in

Table 1 Doehlert matrix experimental domains for time and temperature and their respective codifications for the first iteration (X_1^* and X_2^*), and for the final optimization (X_1 and X_2)

		First iteration			
Absolute values		Codified values		Final optimization	
t/h	T/°C	X_1^*	X_2^*	X_1	X_2
3.0	125.0	0.00	0.00	−0.20	−0.17
5.0	125.0	1.00	0.00	0.60	−0.17
1.0	125.0	−1.00	0.00	−1.00	−0.17
4.0	146.7	0.50	0.87	0.20	0.56
2.0	103.4	−0.50	−0.87	−0.60	−0.89
4.0	103.4	0.50	−0.87	0.20	−0.89
2.0	146.7	−0.50	0.87	−0.60	0.56
6.0	155.0	—	—	1.00	0.83
6.0	160.0	—	—	1.00	1.00

the further sections, and conditions were extended for a time interval of 1 to 6 h, and for a temperature range from 100 °C to 160 °C. The experimental conditions that resulted from the new design are also presented in Table 1, as X_1 and X_2.

2.7 Statistical analysis

The results of glucan and lignin content, as well as glucan recovery were statistically analyzed using multiple regression analysis tool in the Design-Experiments 7.0 software (Stat-Ease, Inc., Minneapolis, MN, USA). For this purpose, a quadratic surface model was used: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} {X_1}^2 + \beta_{12} {X_2}^2$ (4), where Y is the performance category analyzed, β_i are the regression coefficients, X_1 represents time, and X_2 temperature. β_0 describes the response at the center of the experimental domain, β_1 and β_2 describe the effect of the reaction time and temperature, respectively. β_{12} describes the influence of both time and temperature and can also be defined as the interaction parameter. Finally, β_{11} and β_{22} demonstrate how the response surface folds quadratically: downward (negative values) or upward (positive values). The effect and statistical significance of each regression coefficient was determined using the ANOVA method.

The Design-Experiments 7.0 software also allowed the optimization of the reaction conditions. For this, specific goals were defined as the maximization of glucan content, the minimization of lignin content and maximization of glucan recovery in pretreated solid. To perform such multidimensional optimization the following desirability function was used:[29] $D(x) = (d_1 \times d_2 \times d_3)^{\frac{1}{n}}$, where d_i are the desirable values for the purposes mentioned above, which vary between 0 and 1, from least to most desirable, and n is the number of responses in the measurement, *i.e.*, $n = 3$.

2.8 Experimental uncertainty

All measurements in the experiment demonstrate a certain standard uncertainty (u). The temperature in the pretreatment reactions was measured with the temperature uncertainty, $u(T)$, of 0.1 °C, while in all other measurements $u(T)$ was of 1 °C. Each measured weight uncertainty, $u(m)$, was 0.1 mg. All measured time uncertainty, $u(t)$ was 1 s.

Pretreatment and enzymatic hydrolysis experiments were performed in duplicates, the results presented in the Results and discussion section are the average values of such results. For all analyses, it was estimated that there was a consistent error of maximal 10% of the measured value, which helped to assess the extent to which other experimental errors related to measurements occurred in the calibration technique employed to quantify product concentrations.

3. Results and discussion

3.1 Miscanthus composition and particle size distribution

The moisture content was 7.63 ± 0.12 wt%. The macromolecular composition of Miscanthus raw biomass is presented in Table 2. As noted, the high total polysaccharide content of 64.34 wt%, and the high-lignin content of 24.79 wt%, demonstrate the biomass potential for valorization. These values are in agreement with those found in literature.[30]

Table 2 Macromolecular composition of Miscanthus obtained in this study and in literature

	This work	
Component	Wt% (dry weight)	Ref. 30
Cellulose	45.86 ± 0.89	44.5
Hemicellulose	18.48	26.6
Xylan	13.88 ± 0.17	—
Arabinosyl group	2.87 ± 0.04	—
Acetyl group	1.73 ± 0.01	—
Lignin	24.79	26.5
Acid-insoluble	23.69 ± 0.03	—
Acid-soluble	1.10 ± 0.02	—
Protein	1.71 ± 0.01	—
Ash	2.02 ± 0.02	3.0
Extractives	6.09	—
Water	4.01 ± 0.06	—
Ethanol	2.08 ± 0.14	—

The biomass used in this work exhibited different particle sizes. The Miscanthus size distribution and their respective mass fractions, with a maximum size of 1.5 mm, are presented in Table 3. As can be noted, the majority of the biomass, 67.75 wt%, has a granulometry between 1.00 and 0.25 mm.

3.2 Optimization of reaction conditions

The primary aim of this study was to achieve pretreated Miscanthus with a high glucan and low lignin content. This approach was intended to enhance the enzymatic susceptibility of the biomass, enabling the production of elevated glucose concentrations. To achieve these goals, the optimization of reaction conditions was performed based on the Doehlert design of experiments, with the experimental domains represented in Table 4 as X_1^* and X_2^*. However, the optimization led to inconclusive results, as a wide range of maximum desirability function was obtained, as shown in Fig. 2.

To address this issue, both experimental domains, *i.e.*, temperature and time, were extended and two additional experimental conditions (6 h, 155 °C and 6 h, 160 °C) were assessed. The new experimental domains are presented in Table 1 as X_1 and X_2. Furthermore, the desirability function was calculated using a new set of outputs, which included two previously mentioned outputs (maximization of glucan content and minimization of lignin content in the pretreated biomass) and, additionally, the glucan recovery in the pretreated biomass. This approach allowed maximization of the valorization

Table 3 Miscanthus particle size distribution

Particle size range (mm)	Miscanthus fraction (wt%)
1.50–1.00	17.69
1.00–0.71	20.72
0.71–0.50	21.19
0.50–0.25	25.84
0.25–0.15	8.27
<0.15	6.28

Table 4 Glucan and lignin contents, as well as glucan recovery in the pretreated biomass used for a first iteration attempt and for the final optimization

First iteration				Final optimization				
X_1^*	X_2^*	Glucan content (wt%)	Lignin content (wt%)	X_1	X_2	Glucan content (wt%)	Lignin content (wt%)	Glucan recovery (wt%)
0.00	0.00	60.8 ± 3.0	18.3 ± 0.3	−0.20	−0.17	60.8 ± 3.0	18.3 ± 0.3	91.2 ± 0.5
1.00	0.00	63.5 ± 2.1	16.0 ± 1.6	0.60	−0.17	63.5 ± 2.1	16.0 ± 1.6	91.7 ± 0.8
−1.00	0.00	53.1 ± 1.1	21.5 ± 0.3	−1.00	−0.17	53.1 ± 1.1	21.5 ± 0.3	91.9 ± 0.7
0.50	0.87	73.8 ± 1.8	10.2 ± 1.1	0.20	0.56	73.8 ± 1.8	10.2 ± 1.1	93.3 ± 1.7
−0.50	−0.87	46.2 ± 0.9	24.2 ± 0.5	−0.60	−0.89	46.2 ± 0.9	24.2 ± 0.5	95.4 ± 1.6
0.50	−0.87	51.6 ± 0.9	22.1 ± 0.3	0.20	−0.89	51.6 ± 0.9	22.1 ± 0.3	95.9 ± 1.9
−0.50	0.87	74.1 ± 0.0	9.0 ± 0.0	−0.60	0.56	74.1 ± 0.0	9.0 ± 0.0	90.1 ± 2.5
—	—	—	—	1.00	0.83	76.8 ± 0.1	12.4 ± 0.0	84.1 ± 0.1
—	—	—	—	1.00	1.00	75.4 ± 0.0	10.6 ± 0.1	83.2 ± 0.1

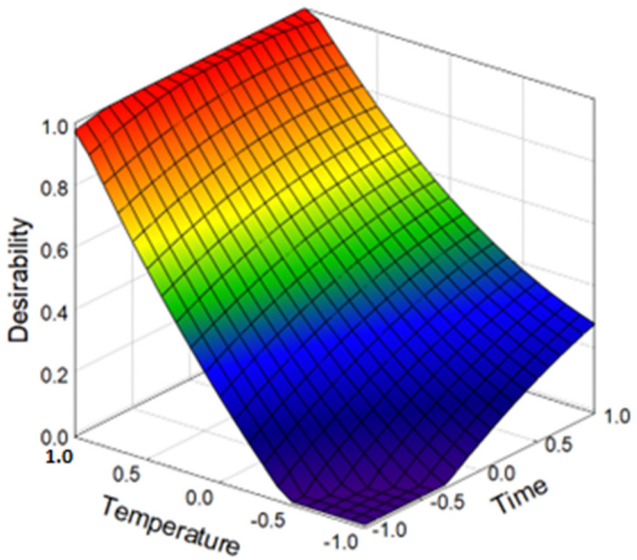

Fig. 2 Desirability function for maximization of X_1^* (glucan content in pretreated biomass) and minimization of X_2^* (lignin content in pretreated biomass) under the range of temperature and time evaluated.

potential and helped to design a new maximum for the desirability function. All conditions used for this optimization and respective results in terms of biomass composition and glucan recovery are summarized in Table 4.

In terms of biomass composition, the glucan content in the pretreated biomass ranged from 76.8 ± 0.1 wt% at 6 h and 155 °C, to 46.2 ± 0.9 wt% at 2 h and 103.35 °C. In the range of studied conditions, the lignin content varied from 9.0 ± 0.0 wt% at 2 h and 146.65 °C, to 24.2 ± 0.5 wt% at 2 h and 103.35 °C. For the glucan recovery, all yields were high, ranging from 95.9 ± 1.9 wt% at 4 h and 103.35 °C, to 83.2 ± 0.1 at 6 h and 160 °C.

The obtained data was analyzed using the ANOVA statistical method to acquire deeper insights into the impact of studied variables on the glucan and lignin contents, as well as on the glucan recovery in the pretreated biomass. The ANOVA results are presented in Table 5.

The second-order polynomial response for the three studied outputs (glucan and lignin contents as well as the glucan recovery), in terms of time (t) and temperature (T) in coded factors, can be represented as by following equations:

$$\text{Glucan content (wt\%)} = 64.66 + 3.00t + 16.91T - 5.24tT - 3.87tt + 1.80TT,$$

$$\text{Lignin content (wt\%)} = 15.51 - 0.52t - 9.16T + 4.16tT - 2.71tt - 2.32TT,$$

$$\text{Glucan recovery (wt\%)} = 92.90 - 1.45t - 3.16T + 0.85tT - 3.78tt - 0.55TT.$$

The proposed model for the maximizing glucan content is statistically significant, with a p-value lower than 0.0001, an insignificant lack of fit with a value of 0.1794, and a high R^2 of 0.98. Analyzing the statistical significance of the different terms of the second-order polynomial response regarding glucan content in the pretreated biomass, only the linear coefficients, for both time, t and temperature, T, and the interactive coefficient, tT, were statistically significant, with p-values of 0.009, <0.0001, and 0.026, respectively. Regarding the effect of the parameters on the final glucan content, temperature, with a coefficient value of 16.91, had approximately six times greater impact than time, with a coefficient value of 3.00. The greater impact of temperature might be explained by the reduction in viscosity of DES, leading to weaker hydrogen bonds between its components, which in turn facilitates the interaction between the DES and Miscanthus.[31]

The model designed for minimizing lignin content in the pretreated biomass is also significant, with a p-value lower than 0.0001 and a high R^2 of 0.97. However, the lack of fit is significant, with a p-value of 0.023. Regarding the model terms, only T, tT and the quadratic factor for time, tt are statistically significant, with p-values of <0.0001, 0.010 and 0.046, respectively. Both increasing time and temperature negatively impact the lignin content of pretreated biomass. However, temperature, with a coefficient of −9.16, has a greater impact on the lignin value, being almost eighteen times higher than that of time, which has a coefficient value of only −0.52.

In previous work on the use of ChCl–acetic acid for wheat straw pretreatment, time had a greater influence on the value of both glucan and lignin contents. In contrast, in the present

Table 5 ANOVA statistical analysis for response surface quadratic model of the glucan and lignin contents, as well as the glucan recovery in the pretreated biomass. All model coefficients are presented in coded terms

			Linear		Interactive	Quadratic				
		Model	t	T	tT	tt	TT	Lack of fit	R^2	Adjusted R^2
Glucan Content	Coefficients		3.00	16.91	−5.24	−3.87	1.80		0.98	0.97
	F value	100.61	10.35	327.68	6.87	4.60	1.01	2.17		
	p-value[a]	<0.0001	0.009	<0.0001	0.026	0.058	0.339	0.179		
Lignin Content	Coefficients		−0.52	−9.16	4.16	2.71	−2.32			
	F value	59.42	0.70	221.12	9.96	5.17	3.83	6.13	0.97	0.95
	p-value[a]	<0.0001	0.422	<0.0001	0.010	0.046	0.079	0.023		
Glucan recovery	Coefficients		−1.42	−3.16	−0.85	−3.78	0.51			
	F value	7.43	1.86	8.91	0.14	3.40	0.06	4.06	0.79	0.68
	p-value[a]	0.004	0.202	0.014	0.715	0.095	0.807	0.058		

[a] Statistically significant when p-value < 0.05, not statistically significant when p-value > 0.05.

work, temperature has a greater impact on the response. It is also interesting to note that in this previous work, the lignin content of wheat straw increased with an increase of time and temperature, while in this work, the opposite effect is observed, *i.e.*, t and T are negative. Although both wheat straw and Miscanthus are grass biomasses, pretreatment of Miscanthus resulted in considerable higher glucan and lower lignin contents than wheat straw. The discrepancy in ash content between wheat straw and Miscanthus significantly affects their pretreatment with ChCl–acetic acid 1 : 2 DES (deep eutectic solvent). Ash, primarily composed of inorganic minerals, influences the pretreatment process by modifying the pH and buffering capacity of the solution. Wheat straw contains 9.11 wt% ash compared to 2.02 wt% in Miscanthus, which contributes to variations in pretreatment efficiency.

Recent studies have demonstrated that DES like ChCl–acetic acid, which is highly acidic with a pH of 1.7, is critical for effective biomass deconstruction. However, the high ash content in wheat straw can partially neutralize this acidity. Minerals in the ash dissolve in the acidic medium and act as buffers, raising the pH and reducing the solvent's effectiveness.[32] This neutralization decreases the hydrogen bonding capability between the solvent and biomass, which is essential for breaking down lignocellulosic structures.[33]

Moreover, ash can interfere with the dissolution of lignin and hemicellulose during pretreatment. Recent research has shown that higher mineral content can hinder the solubilization process, thus reducing the overall efficiency of biomass conversion. This is particularly problematic for pretreatments that rely on acidic conditions, as the buffering action of the ash diminishes the DES's capacity to disrupt biomass components.[34] Regarding glucan recovery yield, the proposed model is significant, with a p-value of 0.004, and the lack of fit insignificant, with a p-value of 0.058. However, the R^2 was only 0.79. Among model terms, only the linear factor of temperature is significant. Both time, with a coefficient of −1.42, and temperature, with a coefficient of −3.16, negatively impact the yield of recovered glucan, with temperature having almost twice the influence on the expected output.

A desirability function was calculated considering three outputs, *i.e.*, maximization of both glucan content and glucan recovery and minimization of lignin content in the pretreated biomass. This optimization, shown in Fig. 3, defined the optimal conditions as 150 °C and 2 h and 52 min of reaction time. According to the previously detailed models, these conditions should achieve a glucan content of 76.84 wt%, a lignin content of 7.83 wt%, and a glucan recovery of 91.26 wt% in the pretreated biomass. These values were confirmed experimentally, with the obtained results being 74.1 wt%, 9.5 wt% and 87.0 wt% for glucan and lignin contents and glucan recovery, respectively.

Studies addressing Miscanthus pretreatment with ChCl-based DES are still limited, with the majority focusing on microwave-assisted procedures, which significantly shortens reaction time.[19,35,36] The ultrafast (45 s) microwave-assisted pretreatment of Miscanthus using ChCl and lactic acid (1 : 2) led to a pretreated biomass with a lower glucan content, of 67.70 wt%, and a higher lignin content of 18.41 wt%.[36] Another study evaluated the pretreatment capacity of ChCl–ethylene

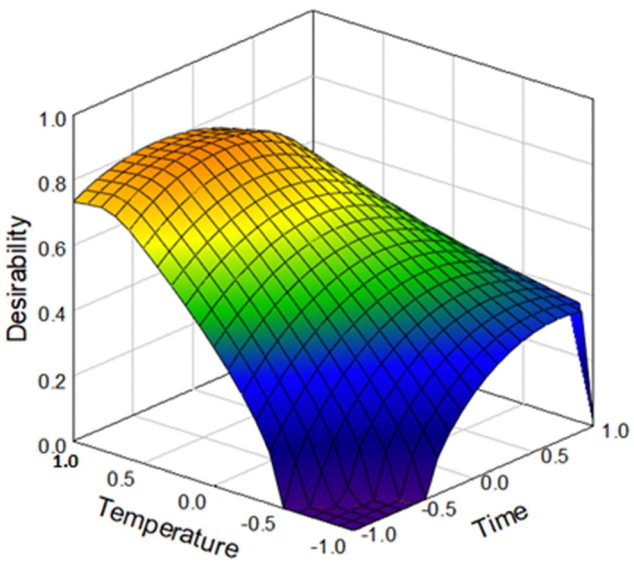

Fig. 3 Desirability function for maximal glucan and minimal lignin content in the pretreated biomass, as well as maximal glucan recovery yield for studied range of time and temperature.

glycol, urea, or glycerol with Miscanthus.[35] ChCl–glycerol (1 : 2) produced the best results with a Miscanthus composition of 12.8 wt% of lignin content, 16.8 wt% of hemicellulose and 67.5 wt% of glucan.[35] The authors concluded that ChCl–glycerol, despite being a neutral solvent that facilitates mild reaction conditions, offers a higher hydrogen bond donor (HBD) capacity due to the presence of glycerol, surpassing other chemicals like acetic acid in this regard. This, in turn, allows the production of biomass with a high cellulose content.[35] Another study on the effect of ChCl and glycolic acid pretreatment of Miscanthus, enhanced with heteropoly acids, allowed achieving glucan contents between 64.7–75.3 wt% and lignin removal yields as high as 89.5 wt%. Heteropoly acids enhanced the neutral deep eutectic solvent pretreatment capacity.[19] Therefore the results obtained in that study cannot be directly compared to those obtained in this work. When comparing the obtained results to the results of various biomass pretreatment methods, traditional approaches often fall short in efficiently separating hemicellulose, cellulose, and lignin fractions. The only promising candidates capable of achieving this are imidazole and certain ionic liquids.[37–39] For instance, the pretreatment of wheat straw and Eucalyptus residues with imidazole resulted in an increase in cellulose content from 35.9 wt% to 55.1 wt% for wheat straw pretreated at 160 °C for 2 hours. Under the same conditions, lignin content decreased from 16.7 wt% to 5.3 wt%, achieving over 68% lignin removal. More severe conditions, such as 160 °C for 4 hours, resulted in delignification as high as 85.9%.[40]

Comparing these results to those obtained with ionic liquids, especially cholinium-based ones, shows similar efficiency. For example, Ren *et al.*[37] reported a maximum delignification of 68.8 wt% using cholinium taurate at 90 °C for 6 hours. In another study, da Costa Lopes reported that using 1-butyl-3-methylimidazolium thiocyanate at 120 °C for 6 hours produced a solid fraction containing up to 83.4 wt% cellulose and only 2.8 wt% lignin. These findings highlight the superior efficiency of imidazole and certain ionic liquids in biomass pretreatment compared to classical methods.[41]

For Eucalyptus residues, the authors demonstrated that the increase in cellulose content was less remarkable compared to wheat straw, as it rose from 44.1 wt% to 54.3 wt% after treatment at 160 °C for 4 hours.[40] Additionally, Bernardo *et al.*[42] demonstrated that an acidic environment is not ideal for achieving sufficient delignification of Eucalyptus residues. However, when an acetate-based ionic liquid was used, the lignin content in the solid fraction pretreated at 120 °C for 2 hours was reduced to as low as 0.7 wt%.

In case of pretreatment of Miscanthus × giganteus with the protic ionic liquid triethylammonium hydrogen sulfate with 20% water as a co-solvent, the authors solubilized hemicellulose and lignin simultaneously, while enriching the cellulose content in the solid fraction. After 24 hours of treatment at 120 °C, the composition of the pretreated solid fraction was 75 wt% cellulose, 5 wt% hemicellulose, and 20 wt% lignin,[43] which means less efficient fractionation of lignin and major polysaccharides than in the presented study.

For the alkaline pretreatment of Miscanthus, Den *et al.*[44] obtained also less efficient fractionation because after the pretreatment performed using sodium hydroxide (2% NaOH) at 80 °C for 2 hours, the lignin content was reduced by 60%, increasing the cellulose content in the solid fraction from 45% to 70%. The overall efficiency of fractionation indicated that approximately 85% of lignin was solubilized, while 90% of the cellulose remained in the solid fraction.

3.3 Enzymatic hydrolysis

Miscanthus pretreated under optimal conditions was enzymatically hydrolyzed with both washed and unwashed samples from the same pretreatment batch. Comparison of the results of the two reactions for glucose and xylose hydrolysis yields helped to clarify the need for a biomass washing step prior to enzymatic hydrolysis.

Before the enzymatic hydrolysis tests, a scaling-up of the pretreatment reaction was performed to produce enough biomass for all the necessary assays. The results of the scaled-up pretreatment reaction, performed at optimal time and

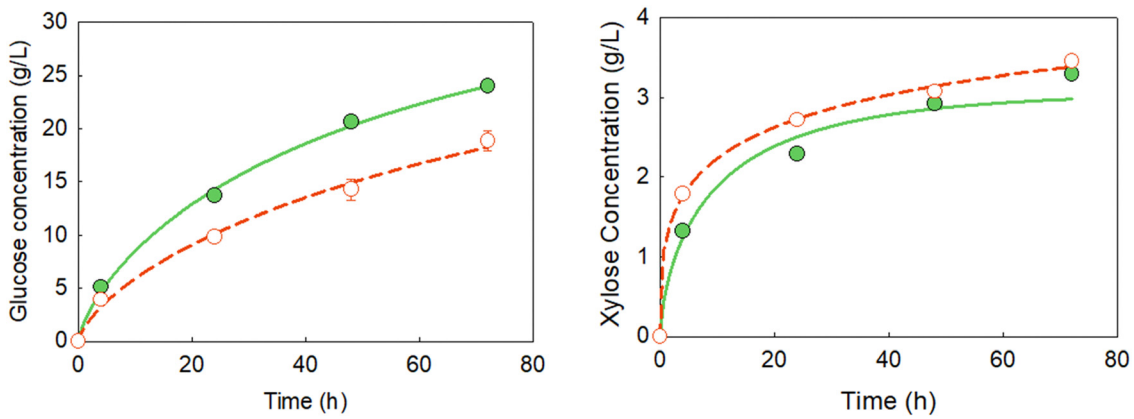

Fig. 4 Glucose (left) and xylose (right) concentration (g L^{-1}) for washed (closed symbol), and unwashed (open symbols) pretreated Miscanthus subjected to the enzymatic hydrolysis assays. Solid and dashed lines, for washed and unwashed biomass, respectively were calculated using fractal kinetics model.

temperature of 2 h 52 min and 150 °C, were as follows: a glucan recovery yield of 88.7 wt%, a glucan content of 72.8 wt% and a lignin content of 16.4 wt%. The most significant difference was observed for the lignin content, which showed a considerable increase. The origin of this significant difference might be due to rheological issue related to the particle sizes of the Miscanthus sample and challenges related to the efficient agitation of the reaction mixture. Nevertheless, since the absolute amounts the lignin content was still low, especially when compared to glucan this issue was not further studied in this work.

The biomass produced in this way was hydrolyzed enzymatically. The evolution of glucose and xylose concentration during the enzymatic hydrolysis is shown in Fig. 4, and the values of glucan to glucose and xylan to xylose yields are presented in Table 6. After 72 hours of reaction, the final glucose concentration was higher for washed biomass, at 24.0 g L^{-1}, compared to unwashed biomass, at 18.9 g L^{-1}. The final glucan to glucose yield was also higher for washed biomass, at 64.1 wt%, compared to 49.4 wt% for unwashed biomass. Regarding xylose, the final concentration achieved was statistically similar for both washed and unwashed biomass, with values of 3.3 g L^{-1} and 3.5 g L^{-1}, respectively.

In a previously mentioned study,[34] Miscanthus was pretreated with ChCl–lactic acid using an ultrafast microwave-assisted protocol taking only 45 s. Although the resulting pretreated biomass was similar in terms of glucan and lignin content, at 67.7 wt% and 18.4 wt%, respectively, enzymatic hydrolysis after 48 h resulted in lower glucan and xylan conversion yields of 35 wt% and 27 wt%, respectively. However, it is worth noting that the enzyme loading in the study was approximately half of that used in the present work. Song *et al.* reported that in the case of willow and corn stover pretreated by DES based on choline chloride and lactic acid, non-productive cellulase binding on lignin was observed. Hence, the enzymatic hydrolysis of biomass pretreated with DES is often affected by a greater amount of phenolic hydroxyl groups of the lignin.[45]

Another previously mentioned study of Miscanthus pretreatment with ChCl–glycerol obtained a pretreated biomass with a similar composition of 67.5 wt% of glucan and 12.8 wt% of lignin.[18] Similarly, enzymatic hydrolysis achieved much higher conversion values, with a glucan conversion yield of 96.2 wt% and a glucose concentration of 35.9 g L^{-1}. This may suggest that the level of HBD, *i.e.*, its ability to form hydrogen bonds, may be more relevant than the acidity of the solvent in the pretreatment reaction to produce cellulose more susceptible to enzymatic attack. Wang *et al.* reported that the presence of DES improves the saccharification efficiency of lignocellulosic biomass. Through molecular dynamic simulations they found that cholinium chloride primarily interacts with the cellulose substrate rather than the enzyme itself. This interaction alters the structural configuration of cellulose, making it more accessible to enzymatic attack. The study demonstrated that the presence of choline chloride leads to a reduction in cellulose crystallinity and an increase in the amorphous regions of cellulose, which are more easily hydrolyzed by cellulases. Furthermore, Wang *et al.* stated that choline chloride helps in the stabilization of the lignin component, preventing its recondensation during pretreatment. This stabilization effect further facilitates the accessibility of cellulose for enzymatic hydrolysis. The results showed a substantial increase in glucose yield, confirming that choline chloride-based pretreatment improves the overall efficiency of enzymatic saccharification by modifying the cellulose structure and enhancing enzyme accessibility.[46] Another important aspect of using DES is their biodegradability, which is only partially true for ionic liquids.[47] The use of biodegradable solvents is particularly important because it helps avoid the need for neutralization to achieve low environmental impact, or any negative influence on process efficiency.[48] The enzymatic hydrolysis results of the present work suggest that the DES still present in the unwashed pretreated biomass inhibits the enzymatic hydrolysis reaction. This inhibition was not observed in the previously obtained enzymatic hydrolysis results for wheat straw, which showed comparable results for both washed and unwashed biomass. This difference may be due to the smaller particle size of Miscanthus, with 67.75 wt% of its granulometry between 1.00 and 0.25 mm, compared to the wheat straw used, with 68.65 wt% of its granulometry between 2.36 and 1.00 mm. In other words, the smaller particle size of Miscanthus may retain more DES compared to wheat straw, leading to a higher concentration of DES in solution during Miscanthus enzymatic hydrolysis, negatively affecting the efficiency of the reaction. The post-washing process is a subject of various literature studies as it would help to avoid or at least partially mitigate the need for one of the critical steps in terms of biomass processing. The post-washing aspect is more relevant at high-solid loading processes,[49,50] as it helps remove some inhibiting agents, *e.g.* humic acids[51] or means used to pretreat biomass.[52]

The results of the enzymatic hydrolysis were used to determine the kinetics of the reactions. Table 7 presents the kinetic parameters of k, h, and the R^2 values that resulted from fitting the

Table 6 Glucan and xylan hydrolysis yields obtained along the enzymatic hydrolysis reaction time

	Glucan to glucose yield (wt%)		Xylan to xylose yield (wt%)	
Time (h)	Washed	Unwashed	Washed	Unwashed
0	0	0	0	0
4	10.5 ± 0.0	7.1 ± 0.3	40.3 ± 0.0	33.7 ± 1.1
24	35.1 ± 0.0	23.9 ± 0.0	71.6 ± 0.0	53.2 ± 0.9
48	54.5 ± 0.0	36.3 ± 2.8	91.6 ± 0.0	60.9 ± 1.7
72	64.1 ± 0.0	49.4 ± 2.7	100.0 ± 0.0	70.0 ± 0.7

Table 7 Fractal kinetic parameters fitted to washed and unwashed enzymatic hydrolysis data

	Washed		Unwashed	
	Glucose	Xylose	Glucose	xylose
k (h^{-1})	0.053	0.209	0.037	0.290
h	0.279	0.343	0.301	0.660
R^2	0.998	0.981	0.994	0.999

data for glucose and xylose concentrations in the enzymatic hydrolysis to the fractal modelling. For glucose, the reaction with washed Miscanthus had a higher rate, with a k, of 0.053 h^{-1}, compared to unwashed Miscanthus, which had a k value of 0.037 h^{-1}. The rate of xylose reaction was similar for both washed and unwashed biomass; however, it was almost 40% higher for unwashed biomass, with a k value of 0.290 h^{-1}, compared to washed biomass, with a k value of 0.209 h^{-1}.

The h values indicate the accessibility of the substrate and range from 0 to 1, the lower the value, the better the substrate availability. The h-values for glucan in the washed biomass were lower than for the unwashed biomass, with values of 0.279 and 0.301, respectively. Regarding the xylan reaction, the unwashed biomass showed greater substrate inaccessibility, with h-values significantly higher than for washed biomass, 0.660 and 0.343, respectively.

The literature is scarce in terms of fractal kinetics modelling especially when considered DES and Miscanthus. However Wojtusik *et al.* on the basis of results for different biomasses (wheat straw, corn stover and cardoon steam) and pretreatment types (diluted sulfuric acid and acid ethanol–water extraction) concluded that the best k and h values could be ascribed to a more efficient removal of lignin and xylans, which indeed is also observed in this work.[53]

4. Conclusion

The DES composed of choline chloride–acetic acid (1 : 2), was shown to be effective for the pretreatment of the lignocellulosic biomass Miscanthus, increasing the enzymatic susceptibility of cellulose. The optimized DES pretreatment resulted in a biomass with a glucan content of 74.1 wt%, a lignin content of 9.5 wt%, with a glucan recovery yield of 87.0 wt%. Enzymatic hydrolysis of the pretreated biomass reached glucan yield of 64.1 wt% while xylan conversion was quantitative. The apparent presence of DES in the pretreated biomass showed inhibiting the enzymatic reaction, as the sugar yields obtained from the reaction with washed was much higher than for unwashed biomass. The fractal kinetics modelling showed that the enzymatic hydrolysis reaction with washed Miscanthus had a higher rate, with k, of 0.053 h^{-1}, than unwashed Miscanthus, with a k value of 0.037 h^{-1}. This can also be confirmed by higher accessibility because for the washed biomass glucan accessibility was higher than for unwashed.

Author contributions

Conceptualization and methodology – R. M. Ł.; writing – original draft preparation, P. J. P., M. M. N., writing – review & editing, R. M. Ł. and G. D.; funding acquisition, R. M. Ł.

Data availability

The data sets can be created directly from the manuscript as all data supporting this article have been presented herein. In case of difficulties, direct assistance can be obtained from the authors.

Conflicts of interest

There are no conflicts to declare.

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