Chemical Engineering Journal 497 (2024) 154491

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Supercritical CO_2 as effective wheat straw pretreatment for subsequent mild fractionation strategies

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ARTICLE INFO

Keywords:
$scCO_2$
Enzymatic saccharification
Lignocellulose
Organosolv
Lignin

ABSTRACT

The efficient utilization of lignocellulosic biomass in biorefineries is pivotal for the transition to a carbon–neutral society, emphasizing the need for environmentally friendly fractionation techniques. While the extensive use of chemicals in biorefinery operations can yield favorable quantities of specific biomass components, adopting less chemically intense conditions is crucial for holistic methodologies that preserve the inherent potential of all biomass constituents. This study comprehensively investigates the use of supercritical carbon dioxide (sc-CO_2) as a green pretreatment to improve subsequent mild fractionation on wheat straw. Sc-CO_2 at 300 bars, 100 °C and 70% moisture was found to have minimal impact on the chemical composition and the lignin structure, while there were significant morphological changes as heightened surface area and reduced density. Apart from increasing enzymatic saccharification efficiency, the treatment notably enhanced subsequent mild delignification through alkaline and flow-through organosolv extractions. The combination of the pretreatments enhances the lignin solubilization yields from 49 to 79% for alkaline and 74 to 91% for organosolv extractions, while retaining a high β-O-4 conservation of 49 and 59 linkages per 100 aromatic units, respectively. Additionally, the combined use of sc-CO_2 with mild dilute acid pretreatment improved xylose solubilization from 59 to 76 % and enzymatic saccharification from 53 to 90%, albeit with increased lignin condensation. In summary, this study demonstrates the potential of sc-CO_2 pretreatment as a versatile tool for biomass valorization within the evolving bioeconomy, by combining enhanced extraction yields with minimal lignin structural impact. Our work thereby highlights the promise of the use of sc-CO_2 to contribute to the overall economic potential of improved biorefinery processes.

1. Introduction

The extensive reliance on a petroleum-based society has yielded profound consequences for the environment and society. Addressing this challenge by transitioning toward a more sustainable economic model necessitates focusing on a circular and bio-based economy [1,2]. Biomass, particularly lignocellulosic biomass, stands out for its potential to generate high-value products as alternatives to petroleum-based counterparts [3–5]. Lignocellulosic biomass, often a byproduct of agricultural or industrial processes, mitigates land competition for food production [6–8]. After rice, wheat is the most consumed and produced cereal in the world, generating huge amounts of residual side streams, of which wheat straw is the most prominent [7]. Therefore, it is important to develop dedicated strategies that can help valorize this biomass.

Despite the environmental benefits associated with the use of lignocellulosic biomass as feedstock, its inherent recalcitrance, stemming from the strong interaction between its primary components (cellulose, hemicellulose, and lignin), requires pretreatment to unlock its potential [9,10]. Fractionation strategies are in most cases based on chemical pretreatments. Of these, alkaline pulping is one of the most used in the paper industry to solubilize lignin and hemicellulose, obtaining a cellulose-rich residue [11,12]. Other commonly used pretreatments utilize acidic media, to solubilize hemicellulose and disrupt the biomass which can increase biomass enzymatic digestibility [13,14].

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https://doi.org/10.1016/j.cej.2024.154491
Received 16 May 2024; Received in revised form 5 July 2024; Accepted 1 August 2024

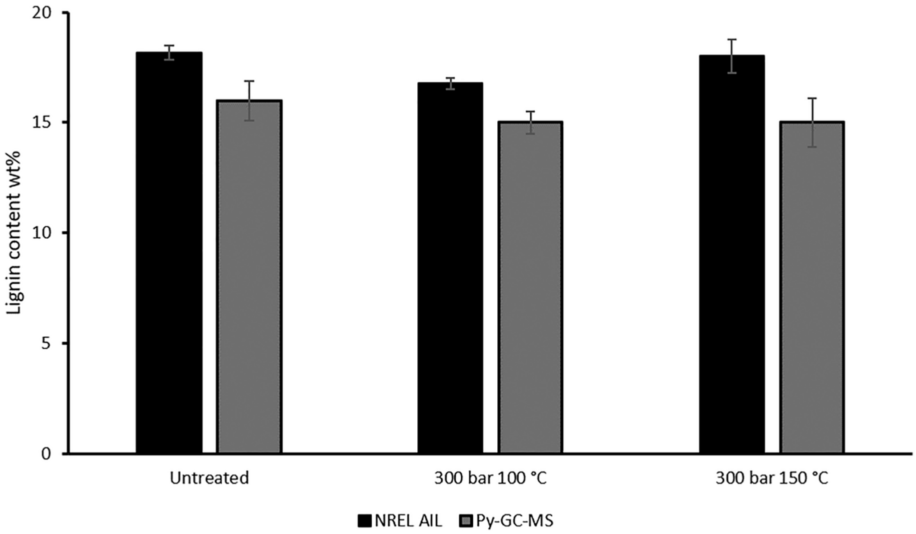

Fig. 1. Lignin content in the untreated and supercritical CO_2 pretreated wheat straw measured via NREL protocol (blue) and Pyrolysis-GC–MS with ^{13}C wheat straw lignin as internal standard. The pretreatment was done at 300 bars, 70% moisture, at 100 °C and 150 °C.

Table 1
^{13}C-IS pyrolysis-GC–MS relative abundance of lignin-derived compounds in untreated wheat straw REL and whole residues for untreated and pretreated wheat straw samples. Corrected for RRF and relative abundance of ^{13}C analogues. The average and standard deviation of analytical duplicates.

	Untreated REL	Untreated	sc-CO_2 100 °C	sc-CO_2 150 °C
Lignin subunits (%)				
H	15.0 ± 0.2	14.9 ± 0.2	15.5 ± 0.6	15.6 ± 0.1
G	53.1 ± 0.0	57.9 ± 0.0	57.9 ± 0.2	58.5 ± 0.0
S	31.8 ± 0.2	27.3 ± 0.0	26.6 ± 0.4	25.9 ± 0.1
S/G	0.60 ± 0.0	0.47 ± 0.0	0.46 ± 0.0	0.44 ± 0.0
***t*-CouA**[a]	3.0 ± 0.0	2.7 ± 0.0	3.0 ± 0.1	2.9 ± 0.1
***t*-ConA**[b]	55.4 ± 0.0	57.3 ± 0.0	57.1 ± 0.1	56.0 ± 0.3
***t*-SinA**[c]	41.7 ± 0.0	40.0 ± 0.0	39.9 ± 0.0	41.1 ± 0.2
***t*-SinA/*t*-ConA**	0.75 ± 0.0	0.70 ± 0.0	0.70 ± 0.0	0.73 ± 0.0
Structural moieties (%)				
Unsubstituted	6.4 ± 0.1	7.3 ± 0.1	8.0 ± 0.7	8.2 ± 0.2
Methyl	3.9 ± 0.1	3.7 ± 0.1	4.3 ± 0.2	4.3 ± 0.1
Vinyl	33.2 ± 0.2	41.0 ± 0.2	43.3 ± 1.1	44.3 ± 0.4
4-VP[d]	10.3 ± 0.2	10.1 ± 0.2	10.4 ± 0.3	10.5 ± 0.3
4-VG[e]	18.3 ± 0.1	26.3 ± 0.1	28.2 ± 0.6	29.5 ± 0.2
C_α-ox	3.5 ± 0.1	4.3 ± 0.1	4.5 ± 0.3	4.1 ± 0.2
diketones	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
C_β-ox[f]	1.0 ± 0.0	1.2 ± 0.0	1.4 ± 0.1	1.2 ± 0.1
C_γ-ox	47.4 ± 0.1	38.5 ± 0.1	33.9 ± 1.6	33.2 ± 0.2
Miscellaneous	4.6 ± 0.0	3.9 ± 0.0	4.5 ± 0.0	4.7 ± 0.0
PhCγ[g]	52.8 ± 0.1	43.3 ± 0.1	39.1 ± 1.7	38.4 ± 0.1
PhC_γ-corrected[h]	52.5 ± 0.1	42.9 ± 0.1	38.8 ± 1.7	38.1 ± 0.1

[a] *trans*-coumaryl alcohol.

[b] *trans*-coniferyl alcohol.

[c] *trans*-sinapyl alcohol.

[d] 4-vinylphenol.

[e] 4-vinylguaiacol.

[f] excluding diketones.

[g] phenols with intact α,β,γ carbon side chain.

[h] phenols with intact α,β,γ carbon side chain, excluding diketones.

However, the harsh conditions typically necessary for maximum efficiency compromise the applicability of solubilized compounds to high-end products, besides consuming substantial amounts of chemicals and energy [13,15].

In pursuit of minimizing environmental impact and preserving the value potential of all biomass components, ongoing research is focused on employing conditions with less chemical severity. This approach involves reducing the quantity of chemicals and temperatures used in biomass processing. Although milder conditions hold promise, they often entail a trade-off with reduced yield compared to their harsher counterparts [16–20]. Lignin, a potentially valuable component, undergoes severe condensation and concomitant value loss during harsh pretreatments. To overcome this, a wave of innovative approaches is emerging, referred to as 'lignin-first' concepts [3]. The primary objective is maintaining the crucial interunit linkage, more specifically the β-O-4 aryl ether linkage [21,22]. Preserving the primary linkage motif of lignin facilitates its subsequent valorization by for example catalytic cleavage to phenolic monomers. Additionally, the conservation of its native structure maintains the intrinsic properties and functionalities of the lignin, enhancing its overall utility [3,23,24]. Due to their high lignin yield and structure stabilization, mild alcohol organosolv extractions look promising [25]. This technique protects the benzylic cation via α-alkoxylation, [21,26] stabilizing the β-O-4 motif and increasing the solubility of the generated fragments in the solvent [21,27,28]. Alkaline extraction at harsh temperatures and high alkali concentrations also leads to condensed lignin. Still, it can solubilize high β-O-4 lignin with limited structural modifications when performed at mild temperatures and lower alkali concentrations. [18,29].

An additional alternative to mitigate the impact of emerging technologies is the adoption of greener solvents, such as supercritical CO_2 (sc-CO_2). This solvent proves advantageous as it is nontoxic, nonflammable, does not induce sugar dehydration at operational temperatures, and is proven cost-effective at large scale for various applications such as the synthesis of organic nitrogenous compounds via electrocatalytic C-N coupling [30–34].

Sc-CO_2 has been suggested as a green biomass pretreatment method to improve the enzymatic saccharification of biomass [32,35]. The distinctive properties of supercritical fluids involve a solvent with gas-like viscosity, allowing for higher diffusivity compared to liquid-state solvents while retaining liquid density [36]. When pressure is rapidly released, an explosive decompression occurs, resulting in enhanced biomass disruption. This disruption, as assessed by scanning electronic microscope (SEM) in various articles, is notably more pronounced when compared to biomass disruption achieved with non-supercritical steam explosion [37–41].

The second biomass disruption mechanism by the use of sc-CO_2 involves the in-situ formation of carbonic acid with the moisture present in the biomass. Numerous examples in the literature demonstrate a correlation between moisture content and increased enzymatic degradability in various biomasses, like guayale,[30] corn cobs,[42] corn

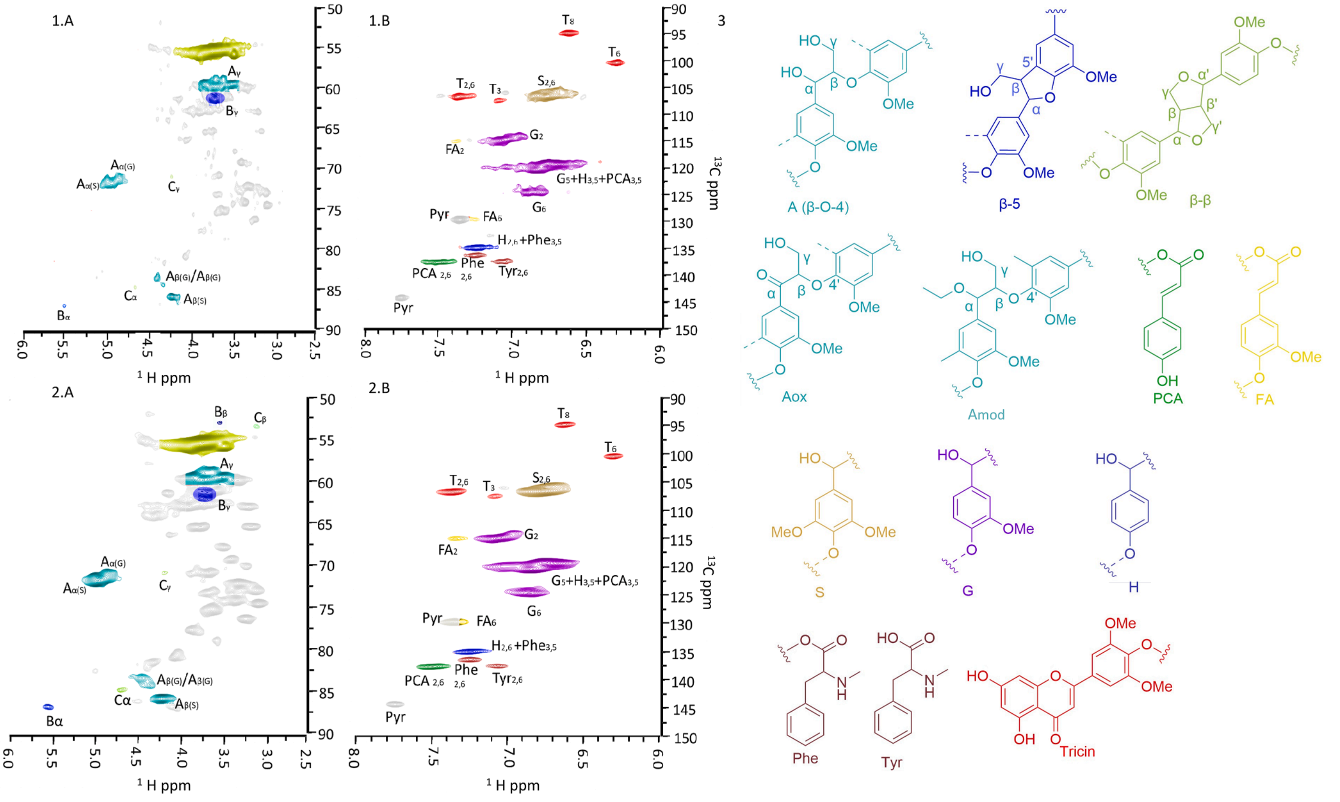

Fig. 2. Gel-state 2D HSQC NMR in pyridine-d_5/DMSO-d_6 (1:4) of REL from 1: untreated wheat straw, 2: sc-CO_2 pretreated wheat straw, (300 bars, 100 °C, 2 h, 70% moisture). A) Shows the aliphatic region and B) the aromatic region. The colours represent the different lignin structures that are represented in side panel 3.

Table 2
Main subunits and interlinkages of Residual Enzyme Lignin (REL) from non-pretreated and sc-CO2 pretreated wheat straw.

	β-O-4[a]	β-β	β-5	S[b]	G	H	FA	pCA	Tricin	$S_{condensed}$[c]	$S_{oxidized}$	S/G ratio
REL	63	4	3	39	56	5	7	9	9	0	0	0.7
SC300	61	5	6	45	51	4	5	11	10	0	0	0.8
SC400	63	3	4	39	57	4	7	8	10	0	0	0.7
SC150	60	3	3	41	56	3	9	8	11	0	6	0.7

[a] The linkages (β-O-4, β-β and β-5) are expressed per 100 aromatic units.

[b] Aromatic units and subgroups like FA, pCA, and tricin are expressed as a % ratio of total SGH units.

[c] $S_{condensed}$ and $S_{oxidized}$ are expressed as % of total aromatics. A representation of the structures presented in this table can be fine in Fig. 2.

stover,[43] oil palm wood,[37] and rice straw [39]. The acidic conditions generated by the added moisture have been occasionally linked to xylan dissolution [30,37]. However, investigations into the impact of sc-CO_2 on lignocellulose composition have yielded inconclusive results thus far. Some researchers contend that sc-CO_2 does not induce significant alterations in the primary biomass composition, as observed in cases like fruit bunches, flax, or guayule [30,39,44]. Conversely, other studies highlight substantial xylan solubilizations in rice straw and oil palm, reaching up to 34% of the initial xylan [37,45].

Despite the clear enhancement of enzymatic digestion after sc-CO_2 treatment, limited research has been conducted on the impact of sc-CO_2 pretreatment on lignin structure and retention of the valuable β-O-4 linkage. The available evidence is inconclusive and indirect; for instance, Santos et al. [38] inferred from photomicrographs that lignin undergoes breakdown during sc-CO_2 pretreatment. Importantly, most lignin related information is derived from the use of one pot reactions of alcohol-water mixtures with sc-CO_2. Previous studies,[46] demonstrated that 1-butanol under sc-CO_2 conditions (190 °C, 230 bar) can achieve delignification yields up to 95%. However, this process compromised the lignin structure and the carbohydrate fraction, resulting in low pulp yields of 8.7–30% [46]. More recent research indicates that ethanol–water systems combined with sc-CO_2 can also achieve high delignification yields, such as 90 % in corn stover (180 °C, 150 bar), albeit with significant alterations to the lignin structure [47]. Wang et al. confirmed that ethanol–water systems with sc-CO_2 (200 bar, 120 °C) lead to 79% delignification, with increased S and G unit condensation and a significant reduction in β-O-4 content from 69.4% to 25.8% [48]. Contrarily, some studies suggest that sc-CO_2 in ethanol–-water delignification of eucalyptus lignin results in minor modifications to β-O-4 content compared to processes without sc-CO_2, achieving yields of up to 33 % [49].

Thus, the impact of sc-CO_2 on the lignin structure and its potential application in obtaining high β-O-4 content lignin remains unclear. Therefore, this work assesses in detail the impact of sc-CO_2 pretreatment on the native structure retention of wheat straw lignin. The final goal is to evaluate if the lignin structure remains intact while the biomass becomes more accessible. The increased accessibility combined with structural retention of lignin can make sc-CO_2 pretreatment an interesting pretreatment to enhance further processing methods like the mild extraction of biomass components as studied in this study to potentially access higher value from all components.

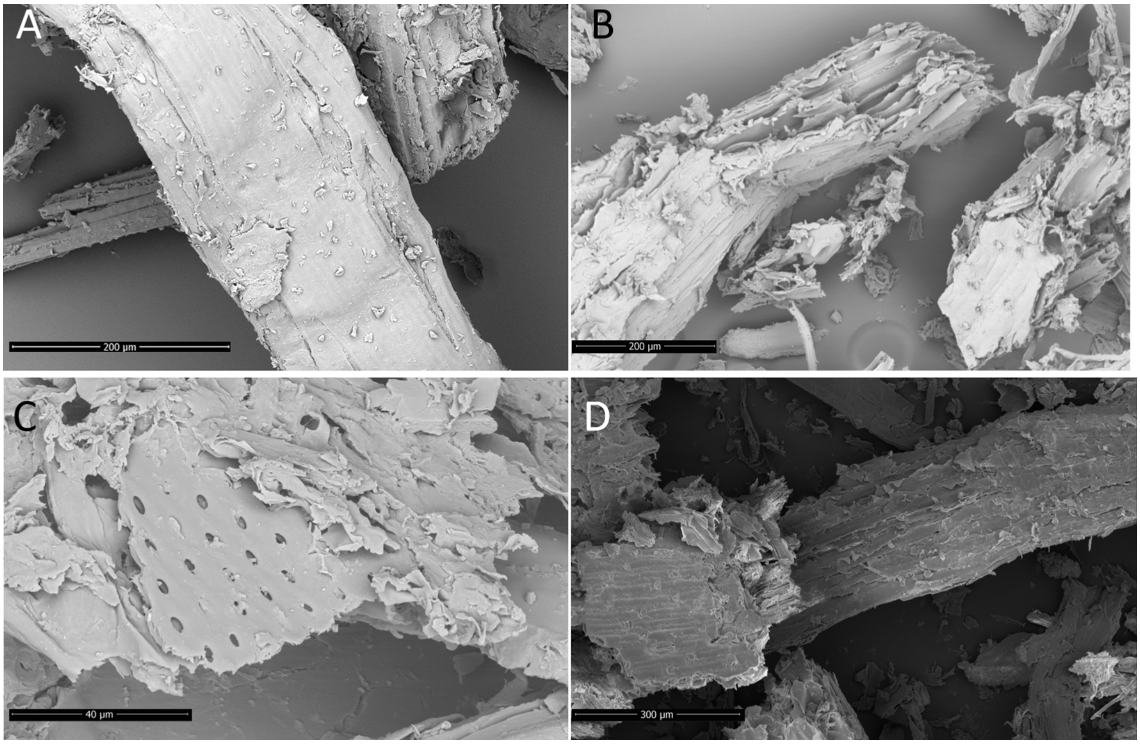

Fig. 3. SEM images of wheat straw pretreated at A) Untreated B) 300 bar 100 °C C) PFD D) 300 bar, 150 °C. Additional SEM images can be found in ESI Figure S7.

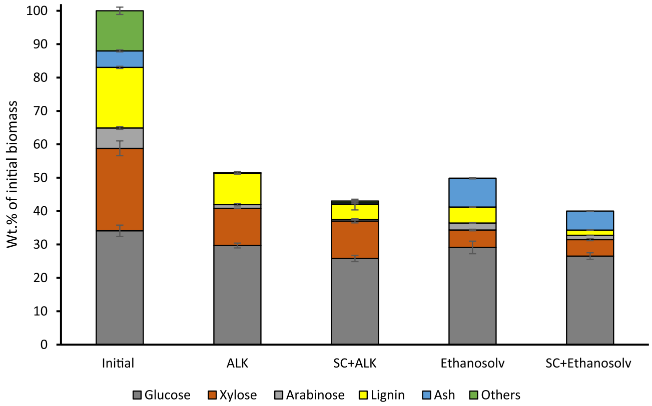

Fig. 4. The composition of the biomass before and after mild alkaline pretreatment (ALK), mild alkaline pretreatment after sc-CO_2 pretreatment (SC+ALK), mild ethanosolv pretreatment (ethanosolv) and mild ethanosolv pretreatment after sc-CO_2 pretreatment (SC+ethanosolv). For both the sc-CO_2 was conducted at 300 bars, 100 °C, 70% moisture for 2 h. The biomass was then directly applied to the next pretreatment, mild alkaline at 100 °C for 3 h and with 2 wt% of NaOH or mild ethanosolv with 8:2 ethanol/water with 0.18 M H_2SO_4 at 120 °C for 2 h. The results are expressed as the quantity present in the residue after the treatment as measured by NREL.

2. Materials and methods

2.1. Materials

Wheat straw was kindly provided by Idaho National Laboratory (Idaho, USA). The reagents were bought from Sigma-Aldrich unless noted otherwise. Sulfuric acid was purchased from Boom BV, with a specified purity of 95–97%. Acetone was purchased from Macron Fine Chemicals, with a specified purity of 99.5%. Acetone-d_6 was purchased from Acros Organics, with a specified purity of 99.5%.

2.2. Biomass preparation

All the biomasses were ground in a Perten lab mill 3303 and sieved to a particle size smaller than 0.6 mm, followed by a dewaxing step, as previously reported [50]. The extractives were discarded, and the biomass was dried under reduced pressure on a rotary evaporator and subsequently in a vacuum oven at 50 °C overnight.

2.3. Biomass composition

Biomass before and after pretreatments and all the residues were characterized following the National Renewable Energy Laboratory (NREL, USA) protocol [51]. Monosaccharides were quantified HPLC using an Agilent 1200 pump equipped with a Bio-Rad organic acid column (Aminex HPX-87H), a refractive index detector, and a UV detector (210 nm). The HPLC column was operated at 60 °C, and a 5 mM aqueous sulfuric acid solution was used as the mobile phase with a flow rate of 0.55 mL/min. The injection volume was set at 5 µL. The concentrations of individual compounds in the product mixture were determined using calibration curves obtained by analyzing standard solutions of known concentrations. The Acid Soluble Lignin (ASL) was measured via UV with a quartz cuvette in an Agilent Crosslab Cary 60 UV–Vis spectrophotometer. Acid insoluble lignin was measured as the weight difference in the filter crucible after drying the sample at 105 °C and corrected for the ash obtained after calcination at 575 °C. Moisture was measured with a PCE-MA 110 moisture meter.

2.4. Biomass characterization

The surface area was measured via N_2-physisorption isotherms and recorded on a Micromeritics ASAP 2420 apparatus at −196 °C. The samples were degassed at 110 °C for 12 h under reduced pressure before N_2 adsorption. The Brunauer-Emmett-Teller (BET) method was used to evaluate the surface area.

The determination of X-ray diffraction was conducted by a D8 ADVANCE Brochures instrument (Bruker, Germany) with Ni-filtered Cu

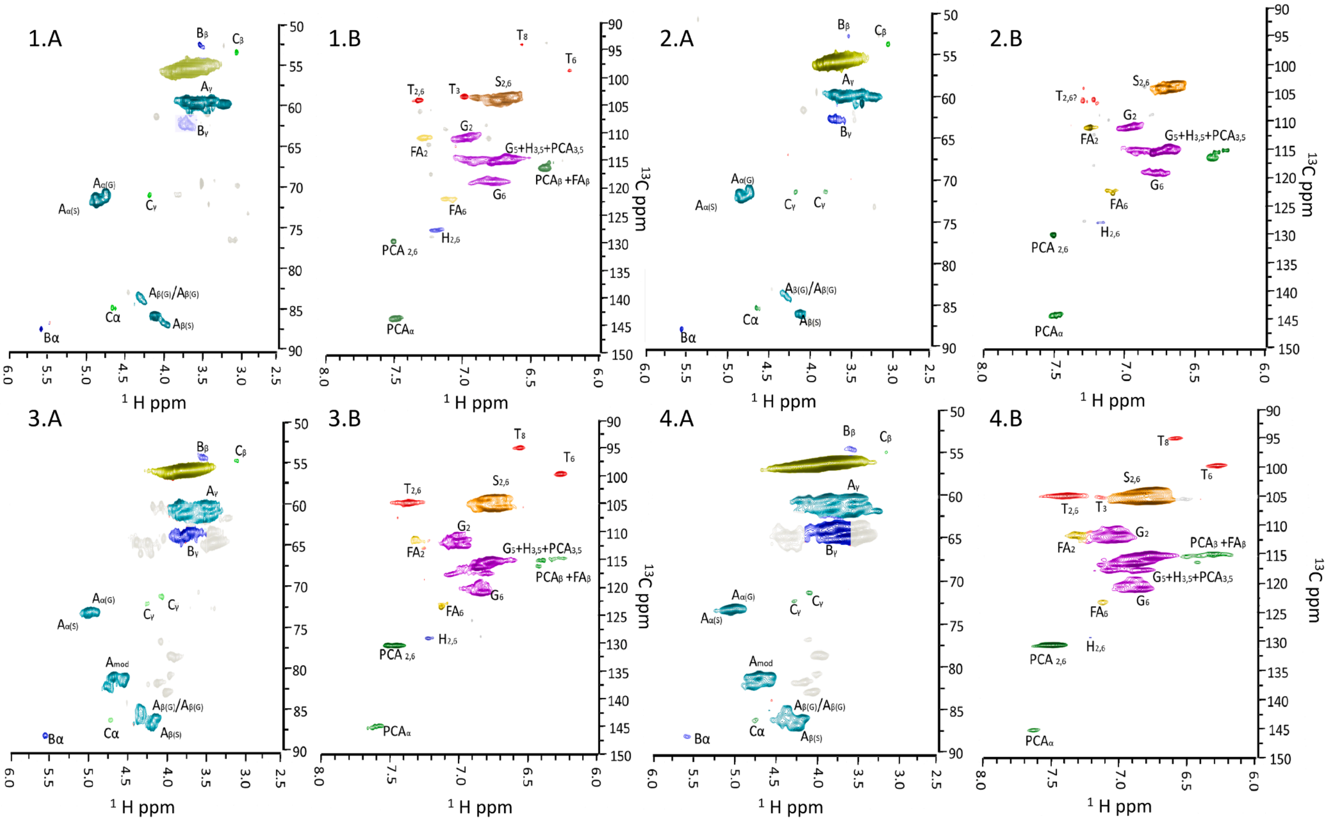

Fig. 5. 2D HSQC NMR with DMSO-d_6 (1,2) and acetone-d_6 (3,4) of 1) alkaline lignin 2) alkaline lignin on sc-CO_2 pretreated wheat straw 3) ethanosolv lignin 4) ethanosolv lignin on sc-CO_2 pretreated wheat straw. A) Shows the aliphatic region and B) the aromatic region. The colours represent the different lignin structures that can be seen in Fig. 2.

Table 3
Main units and interlinkages of lignin obtained from alkaline and ethanosolv treatments with and without a previous SC-CO_2 pretreatment. pretreated wheat straw.

	β-O-4 [a]	β-O-4′	Tβ-O-4 [b]	β-β	β-5	S[c]	G	H	FA	pCA	Tricin
Alkaline	48	0	48	2	3	48	50	2	10	2	0
SC+alkaline	49	0	49	2	3	55	44	1	14	4	0
Ethanosolv	25	35	60	4	2	47	52	1	9	7	8
SC+ethanosolv	27	32	59	4	2	47	51	2	10	8	8

[a] The linkages (β-O-4, β-O-4′, β-β, β-5 and Total β-O-4) are expressed per 100 aromatic units.
[b] The total β-O-4 is the sum of the non-modified β-O-4 linkage and the ethanol incorporated β-O-4′.
[c] aromatic units and subgroups like FA, pCA, and tricin are expressed as a % ratio of total SGH units. A representation of the structures presented in this table can be found in Fig. 2.

Kα radiation source (k = 0.15418 nm) generated at 40 kV and 40 mA. The sample holder material was PVC. The scattering angle (2θ) ranged from 5° to 50° using the reflection mode with fixed slit mode at a scanning speed of 2.58°/min with a step size of 0.043°. The crystallinity was calculated using two methods: Segal's method and deconvolution method. The former one is based on the intensity of the main crystalline domain of cellulose (200) and the amorphous intensity in an angle between the domains (200) and (110) described in Eq. (1). The deconvolution method to calculate the crystallinity of cellulose was performed using a code in python with a gaussian model for the deconvolution. Three peaks were associated to the crystalline contribution, one peak for amorphous contribution, and one for the background. The crystalline domains present were (1–10) and (110) were considered as one peak, (200) as the second crystalline peak, and (040). The centre of the crystalline domains were fixed and sigma and amplitude of the Gaussian were varying. All three parameters for the amorphous and background contribution were fixed and varying, respectively. The crystallinity was calculated as the ratio of the sum of areas for the crystalline peaks and the sum of crystalline and amorphous peaks, described in Eq. (2). The error of the fitting was calculated by the ratio of RMSE (root mean squared error) and the data points.

$$CrI = \frac{I_{200} - I_{am}}{I_{200}} \times 100\% \tag{1}$$

$$CrI = \frac{A_{cryst.}}{A_{cryst.} + A_{am}} \times 100\% \tag{2}$$

The samples after sc-CO_2 pretreatment were analyzed by scanning electron microscopy (SEM) with an accelerating voltage of 5.0 Kv at ZEISS EVO 18 (Carl Zeiss, Inc., Oberkochen, 46 Germany).

2.5. Sc-CO_2 pretreatment

Unless otherwise stated, 20 g of dewaxed wheat straw was mixed with 14 g of water (70 wt%) and introduced to the sc-CO_2 vessel. The vessel was then introduced in the preheated oven and pressure was built by pumping CO_2, once the internal temperature and pressure reached

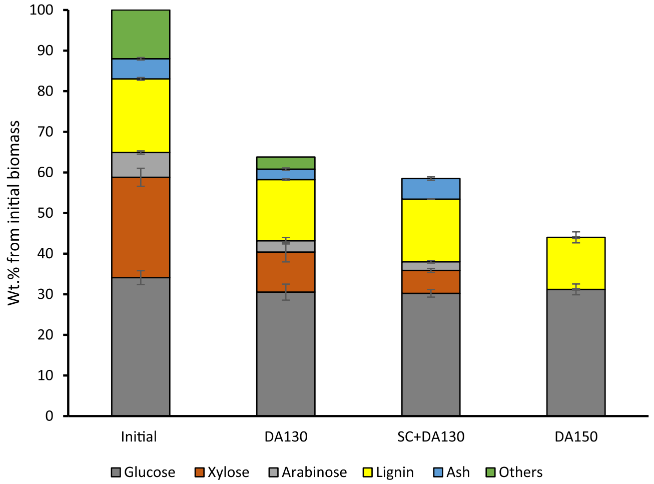

Fig. 6. Composition of the biomass before and after dilute acid (DA) pretreatments compared to the initial quantity in the biomass. Mild DA130 (130 °C, 0.75 wt% H_2SO_4, 30 min) or harsh D150 (150 °C, 1,5 wt% H_2SO_4,30 min). With or without sc-CO_2 pretreatment (SC) under optimal conditions (300 bar, 2 h, 70% moisture, 100 °C).

the desired level the reaction time was started. A constant flow rate of 50 g_{CO2}/min was used for 2 h while maintaining the working pressures (200–500 bar) and temperature (100–150 °C). At the end of the experiment, the pressure was released in a time of < 5 s. For the no moisture control sample, no moisture was added to the sample, while keeping the other parameters constant. For the pressurization-fast depressurization (PFD) sample, the biomass with 70% moisture was loaded in the reactor and after the 100 °C and pressure of 300 bars were reached the reactor was depressurized within <5 s. The parameters studied were selected following the optimized functional parameters described in [37].

The reactor used was an SFE 200 mL continuous extractor provided by Extratex (supercritical fluids innovation), and high-purity CO_2 (≥99.7% volume) was used in the experiments. The extractor comprises a liquid-only pump, necessitating the initial cooling of CO_2 from a cylinder in a refrigerator. Cooled CO_2 is then pumped into the reactor, which is itself contained within an oven (ESI Figure S1). This setup enables the regulation of the CO_2 temperature upon entry into the reactor. Subsequently, the CO_2 undergoes a separation process in which it can be effectively isolated from any extracted substances. Consequently, the CO_2 is consistently reintroduced into the system in a purified state while maintaining a continuous cycle.

2.6. *Alkaline lignin extraction*

Alkaline solubilization was performed based on a literature protocol [18]. Shortly, 15 g of sc-CO_2 pretreated or de-waxed biomass was introduced in a Parr reactor with 300 mL of 2% aqueous NaOH solution. The Parr reactor was a closed batch reactor (PREMEXreactor AG) with the overhead stirrer attached to the reactor stirring controlled by a frequency driver (Eurotherm Drivers) at 300 rpm, attached to a temperature controller to maintain the reaction at 100 °C for 3 h. After the reaction time, the reactor was cooled by flushing cold water around the cooling mantel.

After the reactor was cooled down, the slurry was centrifuged in a Thermo Scientific megafuge 40 centrifuge with a 75,003,607 rotor for 15 min at 4611 x *g* to separate the liquid containing the lignin from the residue. The liquid was neutralized using acetic acid to pH 6 and concentrated to 50 mL with a rotavapor, then was added dropwise to cold ethanol at a volume ratio of 1:3. The flocculent containing the hemicellulose was removed from the liquid via centrifugation for 15 min at 4611 x g and the liquid was concentrated to 40 mL. The concentrated liquid was added dropwise to an acidic water (pH 2) ratio of 1:7 and a precipitate appeared containing the lignin. Finally, the liquid was centrifugated at 4611 x *g* for 20 min and the lignin was dried in a freeze dryer.

2.7. *Ethanosolv lignin extraction*

16 g of the dewaxed biomass with or without sc-CO_2 pretreatment under optimal conditions were introduced to the flow-through reactor described in previous work [28,50]. Ethanol/water in a 4:1 ratio with 0.18 M of H_2SO_4 flowed through the stationary biomass bed until the generation of a back pressure of 7 bar. Once the pressure was stable, the heating started until it reached 120 °C and the eluent flowed through the biomass bed at a constant flow rate of 2 g/min for 2 h. The extraction liquor was neutralized with concentrated NaOH and dried in a rotatory evaporator. Once most of the solvent was evaporated the sample was resuspended in 30 mL of acetone and was added drop-wise to 400 mL of acidic water (pH 2). The flocculated lignin was separated by filtration,

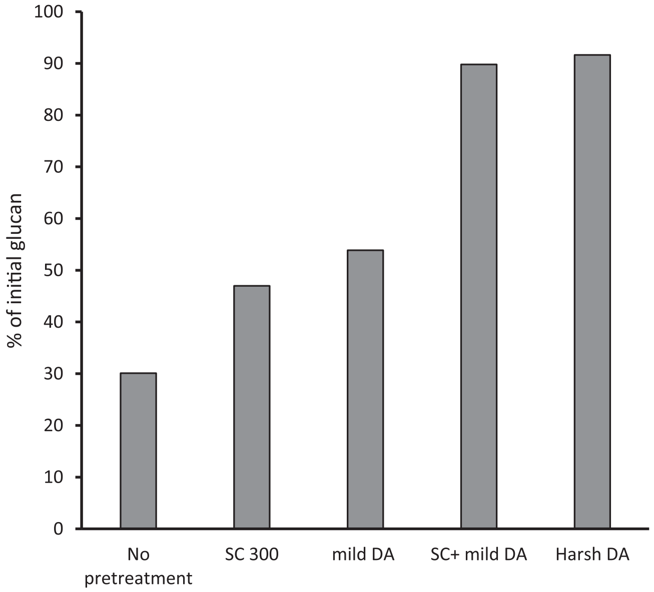

Fig. 7. Saccharification yield of dilute acid (DA) pretreated at mild conditions (130 °C, 0.75 wt% H_2SO_4,30 min) or harsh conditions (150 °C, 1.5 wt% H_2SO_4, 30 min). With or without sc-CO_2 pretreatment (SC) under optimal conditions (300 bar, 2 h, 70% moisture, 100 °C).

Table 4
Main units and interlinkages of Residual Enzyme Lignin (REL) from dilute acid pretreatment at different conditions with and without sc-CO_2 pretreatment.

	β-O-4 [a]	β-β	**β-5**	**S** [b]	**G**	**H**	FA	pCA	$S_{condensed}$	$S_{oxidized}$	**Tricin**
Mild DA	59	5	5	47	50	3	14	12	0	2	9
SC+mild DA	38	3	8	43	54	3	5	9	0	3	6
Harsh DA	11	8	0	66	34	1	7	13	36	4	0

[a] The linkages (β-O-4, β-β and β-5) are expressed per 100 aromatic units.

[b] aromatic units and subgroups like FA, pCA, and tricin are expressed as a % ratio of total SGH units. A representation of the structures presented in this table can be found in Fig. 2.

obtaining a carbohydrate-rich liquid and the precipitated lignin in the filter. The lignin component was air-dried, and its moisture content was measured as previously mentioned. The residual material remaining in the reactor was recovered and subjected to desiccation in a vacuum oven at 50 °C overnight for compositional characterization via the NREL protocol

2.8. *Dilute acid pretreatment*

For the mild diluted acid reaction, 15 g dewaxed or sc-CO_2 pretreated biomass under optimal conditions were introduced in the previously mentioned parr reactor together with 250 mL of 0.75 wt% H_2SO_4 and heated to 130 °C with agitation at 300 rpm for 30 min once the initial temperature was reached. The solid and liquid phases were separated with centrifugation at 4611 x g with the previously mentioned centrifuge and washed 3 times with water. The residue was then freeze-dried and characterized via NREL and the lignin was characterized after obtaining the residual enzyme lignin. The harsh diluted acid was done as the mild diluted acid but with 1.5 wt% H_2SO_4 and 150 °C.

2.9. *Enzymatic degradation*

2 g of never dried biomass samples were directly used after sc-CO_2 pretreatment or raw, and were mixed with 60 mL 50 mM acetate buffer at pH 5 supplemented with 0.8 mM tetracycline chloride to avoid bacterial growth. The enzymatic reaction took place in a shaking incubator at 150 rpm at 50 °C for 48 h. The enzyme dose was 3 $g_{protein}$/100 $g_{biomass}$ of cellulolytic enzymatic cocktail Cellic Ctec II (61.23 $mg_{protein/}$ mL_{enzyme} cocktail) and supplemented with a hemicellulolytic enzymatic cocktail ViscoStar 150L at an enzyme dosage of 0.5 $g_{protein}$/100 $g_{biomass}$ (40 $mg_{protein}$/mL_{enzyme}). After the incubation time, the enzymes were deactivated at 95 °C for 5 min. The solid was then separated from the liquid with centrifugation in a Thermo Scientific megafuge 40 centrifuge with a 75,003,607 rotor at 4611 x *g* for 15 min.

2.10. *Residual enzyme lignin (REL) isolation*

Residual enzyme lignin (REL) from initial biomass, different sc-CO_2 pretreated biomasses, and dilute acid biomass were obtained as described previously [52]. Briefly, 15 g biomass was milled for 24 h (effective milling time) in a planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) equipped with a 250 mL ZrO_2 jar and ZrO_2 balls (5 × 15 mm, 10 × 5 mm). A program with 450 rpm rotation, interchanging between 10 min milling followed by a 15 min pause was used. After milling, 5 g of the biomass was hydrolyzed using Cellic Ctec 2 in 50 mM acetate buffer at pH 5 and supplemented with 0.8 mM of tetracycline chloride to avoid bacterial growth for 72 h. The enzyme dosing was 3 $g_{protein}$/100 $g_{biomass}$ and the solid-to-liquid ratio was 30. After the enzymatic reaction, the lignin was separated from the carbohydrate-rich liquid and washed four times with water by centrifugation in a Thermo Scientific megafuge 40 centrifuges with a 75,003,607 rotor at 4611 x *g* for 15 min. The final solid was freeze-dried.

2.11. *2D HSQC NMR analysis*

2D HSQC NMR spectra of REL were recorded on a 600 MHz Bruker Biospin (BASIC PROBHD, Rheinstetten, Germany,) instrument. A 40 mg sample was swelled in a 0.6 mL mixture of DMSO-d_6 and pyridine-d_5 (4:1). Bruker standard pulse sequence 'hsqcetgpsisp.2′ was used for the ^{13}C–^{1}H correlation experiment. Reported parameters with minor modification were used for the analysis: spectra use 2048 data points from 11 to 0 ppm in F2 (^{1}H) (acquisition time 130 ms), 160 to 0 ppm in F1 (^{13}C) with 256 increments (acquisition time 6 ms) of 32 scans with 500 ms internal delay; the d1 delay was set to 86 ms. The total acquiring time is 3.54 h [52]. The signal of DMSO-d_6 was used as an internal reference (F2 δ 39.5 ppm, F1 δ 2.49 ppm). The data were processed by MestReNova x64 − 12.0.4–22023.

The structure of the soluble lignin obtained from the organosolv process and the alkaline process were analyzed as reported before [53]. Shortly, 60 mg of ehtanosolv lignin was dissolved in 0.7 mL of acetone-*d6*. A few drops of D_2O were added to ensure complete dissolution of the lignin. The NMR measurement was performed on a Bruker Ascend Neo 600 using the following parameters: (F2 = 11 to −1 ppm), (F1 = 160 to −10 ppm), nt = 4, ni = 512, d1 = 1.5, CNST [2] = 145 and the applied pulse sequence is hsqcetgpsi2. Analysis was performed with Mestrenova. The obtained values for the detectable linking motifs were divided by a factor of 1.3 as previous work showed that the 2D HSQC NMR measurement overestimated these values [53]. For alkaline lignin, the same procedure was done but using d6-DMSO as solvent.

The integration of the NMR spectra was done according to the used protocol, for NMR of soluble lignin with acetone-d_6 the integration was done according to a published procedure[53], with DMSO-d_6 according to literature,[54] and for gel-state NMR using pyridine-d_5/DMSO-d_6 this was done according to another published protocol[55].

2.12. *Pyrolysis-GC–MS with uniformly ^{13}C-Labeled lignin as internal standard*

Initial biomass, different sc-CO_2 pretreated biomasses, dilute acid biomass and REL of the initial, untreated biomass were analyzed for lignin content and structural features. Analytical pyrolysis coupled to GC with high-resolution (HR) mass spectrometric detection (Exactive Orbitrap, Thermo Scientific, Waltham, MA, USA) was performed as previously described, using an Agilent VF-1701 ms column (30 m × 0.25 i.d. 0.25 µm film) for chromatographic separation [56]. Uniformly ^{13}C-labeled lignin, isolated from ^{13}C wheat straw (*Triticum aestivum* L. cv." Baldus", 97 atom% ^{13}C) (IsoLife BV, Wageningen, The Netherlands) was used as an internal standard (^{13}C-IS) [57]. To each accurately weighed sample (80 µg) was added 10 µL of a ^{13}C-IS solution (1 mg/mL ethanol/chloroform 50:50 v/v). Samples were dried before analysis. All the samples were prepared and analyzed in duplicate. Lignin-derived pyrolysis products were monitored in full MS mode on the most abundant fragment per compound (both nonlabeled and uniformly ^{13}C labeled). Pyrograms were processed by TraceFinder version 5.1 software. Lignin contents and relative abundances of lignin-derived pyrolysis products were calculated as described previously [56].

3. Results and discussion

3.1. *Effect of the sc-CO_2 on the composition and morphology*

To find out more about the effect of the sc-CO_2, the biomass composition of the recovered samples was analyzed. Overall, the pretreated biomass exhibited only slight alterations in composition (ESI Figure S2). The marginal differences were mostly in the carbohydrate fraction through the partial removal of hemicellulosic sugars. Arabinose solubilization ranged from 32% to 22%, with xylose showing a relatively low solubilization of 10 % during the pretreatment at 300 bar (ESI Figure S2). Although discernible differences in composition were identified, a direct correlation between pretreatment conditions and the minor composition changes was not readily apparent. The glucan composition was not altered during the sc-CO_2 pretreatment, making the overall dry matter conservation exceed 90% across all tested conditions (ESI table S1), consistent with prior findings [30,37,39,44,58].

This high dry matter conservation was also achieved due to the conservation of lignin in the pretreated biomass, as assessed by two different methods; standard Klason lignin analysis (NREL) and quantitative pyrolysis-GC–MS with ^{13}C wheat straw lignin as internal standard [56]. Independently of the lignin quantification method used, no significant differences were observed between the untreated and sc-CO_2 pretreated samples, even at the harsher conditions tested (150 °C, 70% moisture, 300 bar) confirming the high retention of lignin (Fig. 1).

Concurrently with lignin content, also information on the structure of the lignin can be deduced from pyrolysis-GC–MS analysis. The structural integrity of lignin during the various sc-CO_2 pretreatments was evident in terms of subunit composition, both when considering general H:G:S pyrolysis product distributions and the more specific 4-hydroxyphenylpropanoid distributions (coumaryl alcohol (tCouA):coniferyl alcohol (tConA):sinapyl alcohol (tSinA)) (Table 1) [56]. Preservation of interunit linkages was also implied, depicted by the modest decrease in pyrolysis products with an intact three-carbon side chain (PhC_γ) as compared to the untreated sample [55]. A stable abundance of 4-vinylphenol further suggested the preservation of *p*-coumarate moieties upon sc-CO_2 pretreatment.

Other pretreatments with low compositional effects, such as microwave treatment, have reported changes in the lignin structure, presumably due to acid-catalyzed cleavage of interunit linkages, so sc-CO_2 pretreatment clearly stands out in that respect [59].

To assess the effect of sc-CO_2 on the lignin structure in more detail, residual enzyme lignin (REL) fractions were isolated [60,61]. As described by our group, REL provides high fidelity concerning lignin in the parent material and allows for obtaining gel-state 2D HSQC NMR spectra with good resolution (Fig. 2) [52,62]. Pyrolysis-GC–MS analysis confirmed that a representative lignin sample was obtained, showing similar structural features for the lignin analyzed in the (untreated) whole biomass and prepared REL sample (Table 1).

2D HSQC NMR analysis of the REL samples demonstrated that sc-CO_2 indeed had minimal effects on interunit linkage content nor subunit composition, maintaining β-O-4/100 aromatic units between 63 and 60, and S/G ratios between 0.7 and 0.8 for all pretreatments (Table 2) (ESI Figure S3-S4). The hydroxycinnamic acids ferulate and p-coumarate and flavonoid tricin were largely preserved as well. Condensed subunits were not observed (Fig. 2), and a small degree of Cα-oxidation of syringyl units was only prominent at higher pretreatment temperatures (150 °C) (Table 2). Indeed, 2D HSQC NMR and quantitative pyrolysis-GC–MS provided close-to-identical insight into the subunit composition, as demonstrated previously [63].

Finally, the minor effect of the sc-CO_2 pretreatment on the lignin was once more confirmed through the yield and distribution of phenolic products obtained after hydrogenolysis (ESI Figure S5). The hydrogenolysis resulted in similar bio-oil yields for the raw biomass with no pretreatment (21%) and the biomass pretreated (19%). Moreover, the distribution of the main generated products did not vary significantly.

To comprehend the effects of sc-CO_2 on biomass beyond chemical composition and lignin structure, XRD was conducted to assess potential changes in cellulose crystallinity. A minor decrease in crystallinity was observed only for the pretreatments performed at 500 bar pretreatments, but since this is within the experimental error margins it can be fairly considered that sc-CO_2 pretreatment did not significantly affect the crystallinity of cellulose (ESI Figure S6 and table S2). These findings are in agreement with the findings in studies performing sc-CO_2 pretreatments on diverse types of biomass like corn stover, corn cobs, corn stalks, and oil palm [37,38,43].

Despite minor crystallinity changes, SEM data revealed significant fibre disruption post-pretreatment, particularly in biomass treated with moisture and high pressure (Fig. 3). The disruption of the biomass was supported by the N_2-physisorption isotherm data, showing an increment of surface area. The pretreated biomass at 300 and 400 bar shows an increase of 53% and 27% in comparison with the untreated sample and the sample with no moisture (ESI table S3). While BET data requires careful evaluation due to the low biomass surface area (2.3 m^2/g), which is near the equipment detection limit, similar findings were reported by Moharreri et al. [58] for Guayule bagasse disruption after sc-CO_2 pretreatment.

Notably, sc-CO_2 also affected biomass density, decreasing from 0.37 g/cm^3 for untreated biomass to 0.17 g/cm^3 for samples pretreated under optimum conditions, indicating reduced biomass compression (ESI Figure S8).

Overall, the biomass disruption correlated with the increased enzymatic saccharification observed for the samples, where the biggest increment in enzymatic saccharification occurred under 300 bar, 100 °C and 70% moisture (Supplementary note 1).

Hence, the sc-CO_2 pretreatment demonstrated minimal alteration in the biomass composition and suggested a limited impact on the chemical structure of lignin, which is important since condensed lignin can have a negative impact on enzymatic saccharification [15,64,65].

3.2. Enhancement in lignin solubilization via sc-CO_2 pre-pretreatment

The increment of the surface area of biomass coupled with the minor compositional and structural effects on lignin set the stage for evaluating lignin extraction from sc-CO_2 disrupted biomass samples. Two extraction methods, mild alkaline extraction, [18] and mild flow-through ethanol extraction, [28] were tested. Typically, alkaline pretreatment has a more pronounced impact on lignin structure when applied at higher temperatures or alkali concentrations, whereas its effect is less significant at lower alkali concentrations and temperatures, allowing the fractionation of relatively uncondensed lignin [18,66].

The application of sc-CO_2 before the alkaline treatment demonstrated a notable increase in lignin solubilization from 49% to 76% (Fig. 4 and ESI Figure S9). Even with the increased solubilization, the sc-CO_2 had a limited effect on the structure of the isolated lignin, maintaining β-O-4 linkages at 48 and 49 per 100 aromatic units from samples without and with sc-CO_2 pretreatment, respectively (Fig. 5, Table 3). Despite this conservation of linkages, the pretreatment did appear to influence the S/G ratio, increasing the quantity of S to G units, which is often associated with higher lignin recovery yields [26].

The effect of sc-CO_2 on subsequent ethanosolv extraction showed a similar enhancement as for the alkali pretreatment. Upon application of sc-CO_2, the lignin solubilization increased from 74% to 91% (Fig. 4 and ESI Figure S10), while maintaining a high conservation indicated by the presence of 60 and 59 β-O-4_{total}/100 aromatic units found in the isolated ethanosolv lignin from wheat straw with and without sc-CO_2 pretreatment, respectively (Table 3 and Fig. 5). The consistently high structural conservation is further evidenced by the maintenance of similar S/G ratios and the tricin structure, typically situated at the ends of the lignin polymer (Fig. 5) [67,68]. This structure is often cleaved during lignocellulose pretreatments, showing the high conservation of this lignin [68]. Likewise, pCA and FA moieties were largely preserved in the ethanosolv extractions.

The combination of supercritical carbon dioxide (sc-CO_2) and the subsequent mild delignification strategy yields a remarkably high delignification while preserving the lignin structure, making it one of the most effective methods currently reported in the literature (ESI Table S4). This becomes especially clear when compared to similar expansion-disruption techniques such as steam explosion or hydrothermal pretreatments that are followed by lignin extraction, which typically achieve delignification rates of 19.5–48% with β-O-4 contents of only 12–18% [69–71]. Moreover, the high-quality lignin obtained through our method is comparable to, or even surpasses, that produced by leading mild lignin extraction processes developed within the lignin-first concept such as γ-valerolactone,[72] acid hydrotrope, [73] deep eutectic solvents,[74] acetone lignin extraction,[16] or cyrene extraction [75]. Additionally, the combination of sc-CO_2 followed by mild alkaline treatment yields one of the highest mild alkaline delignification rates reported in the literature (ESI Table S4). This includes comparisons with other pretreatments combined with alkaline treatment, such as ammonia pretreatment, ball milling, hot water pretreatment, sulfuric acid, and sonication combined with alkali treatments [18,76–78]. This highlights the exceptional efficacy of the proposed method in achieving high-quality lignin extraction.

The supercritical CO_2 pretreatment before lignin solubilization demonstrated potential for enhancing lignin removal without compromising its chemical structure, thus offering a valuable additional tool for biorefining approaches.

3.3. Improvement of carbohydrate saccharification via dilute acid

In order to assess the potential synergistic effects of sc-CO_2 in conjunction with other pretreatments that primarily target carbohydrate solubilization, we conducted a mild dilute acid (DA) (H_2SO_4) pretreatment aimed at extracting xylose from biomass. Fig. 6 illustrates that the introduction of sc-CO_2 pretreatment augmented the efficacy of a sequential mild DA treatment (0.75 wt% at 130 °C). The principal impact was observed in xylose solubilization, increasing from 59% to 76%, with a marginal effect on arabinose solubilization (from 55% to 59%, as depicted in ESI Figure S10). Notably, glucose solubilization remained unaffected by the previous addition of the sc-CO_2 pretreatment, and there was no discernible alteration in the quantity of lignin solubilized.

To establish a benchmark for a more intensive process, we also implemented a harsh DA pretreatment at 150 °C with 1.5 wt% acid concentration. This harsh pretreatment successfully removed nearly all xylose and arabinose while retaining 91 % of the initial glucose (ESI Figure S10). Despite the superior hemicellulose solubilization achieved under harsher conditions, such pretreatments compromise the quality of the residual lignin (Table 4 and ESI Table S5).

To evaluate the impact of the dilute acid pretreatments on lignin structure, including the addition of sc-CO_2, quantitative ^{13}C-IS pyrolysis-GC–MS was employed on the pretreated residues (Table S5). Additionally, REL samples were isolated and characterized by gel-state NMR (Table 4 and ESI Figure S11-S12). Despite differing in the absolute sense, in general, both analytical methodologies aligned on the relative structural changes observed, as already elaborated upon above and also recently demonstrated for various lignin fractions isolated from birch [66]. Both methods indicated that severe dilute acid treatment strongly impacted the interunit linkage abundance, showing that the aryl ether linkage content dropped from 59 to 11 per 100 aromatic rings. The effect of condensation at the harsh DA treatment was also confirmed by the increase of the condensed S units, accompanied by the complete disappearance of the tricin signals. Moreover, the S/G ratio also suffered a pronounced change from 0.69 to 1.94.

The total S/G composition with Pyrolysis-GC–MS showed also a very pronounced increase when t-ConA/t-SinA was considered, changing from 0.7 to 2.4, a consequence of the cleavage of interunit (Table S5). This was further confirmed by the decrease of PhCy products, especially excluding diketones.

Mild diluted acid treatment had a smaller effect on the lignin structure as confirmed by the higher aryl ether linkage content compared to the harsh DA treatment (Table 4). What is clear from the analysis with both techniques is that the combination of sc-CO_2 pretreatment and mild diluted acid has a more pronounced effect on lignin structure than the mild DA alone. This was evident in the reduction of β-O-4, tricin groups and the reduction of FA and pCA (Table 4). Moreover, pyrolysis-GC–MS showed depletion of PhCy products, while unsubstituted, methyl and diketone products increased (Table S5).

Finally, the enhanced performance of the sc-CO_2 DA pretreatment was also evident in the enzymatic saccharification of the glucan rich residue. The enzymatic saccharification is very high compared to what was achieved with dilute acid and sc-CO_2 pretreatments alone (Fig. 7). More specifically, we demonstrate that the introduction of sc-CO_2 prior to the mild dilute acid achieved an increment in glucose saccharification from 53% to 89.8% (Fig. 7), which is comparable to 91.6% obtained in the process with double the acid dose and higher temperature.

Hence, the biomass disruption applied caused by the sc-CO_2 can enhance the impact of mild dilute acid pretreatment, leading to improved solubilization of hemicellulose sugars and enhanced enzymatic saccharification of the glucan residue.

4. Conclusion

In conclusion, our investigation into the impact of supercritical CO_2 (sc-CO_2) pretreatment on wheat straw revealed that the increased enzymatic activity after sc-CO_2 was caused by morphological disruptions causing an increased surface area as indicated by SEM, BET and density data. This morphological disruption and improved accessibility do not come with a significant structural or compositional impact on the biomass, especially on lignin as evidenced by 2D HSQC NMR and pyrolysis-GC–MS. The morphological disruption generated by sc-CO_2 resulted in a clear enhancement of mild lignin solubilization strategies during subsequent extraction processes like ethanosolv and alkaline treatments while conserving the lignin structure. This presents a big potential to significantly increase the yields of extracting native-like lignin for the development of lignin-first biorefineries. The enhancing effect of sc-CO_2 pretreatment was not only limited to lignin solubilization pretreatments but also to dilute acid pretreatment. Xylose solubilization and enzymatic saccharification of the cellulose increased compared to mild DA without sc-CO_2. Overall, this comprehensive study highlights the possibility of using a green pretreatment like sc-CO_2 to enhance the performance of sequential milder treatments, helping to conserve the structure of the valuable biomass derived compounds.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the first author used Chat GPT in order to enhance the original text written by that author. After using this tool/service, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

CRediT authorship contribution statement

Salvador Bertran-Llorens: Writing – original draft, Methodology, Investigation, Conceptualization. **Federico Perondi:** Methodology, Investigation. **Ana Luiza Slama de Freitas:** Investigation. **Jiazhao Chen:** Investigation. **Gijs van Erven:** Writing – review & editing, Investigation, Conceptualization. **Peter J. Deuss:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data is included in the manuscript and SI

Acknowledgements

The contribution of COST Action LignoCOST (CA17128) in promoting interaction, exchange of knowledge, and collaboration in the field of lignin valorization is gratefully acknowledged. Salvador Bertran was financially supported by the Engineering and Technology Institute Groningen (ENTEG) and the Northern Netherlands Partnership (SNN), European Regional Development Fund (ERDF) for the province of Groningen. Ana Luiza Slama de Freitas was part of CellPro Chem together with Recell® group and was financially supported by the Province of Fryslân, the Northern Netherlands Partnership (SNN), European Regional Development Fund (ERDF), co-financed in the context of the Union's response to the COVID-19 pandemic, and the European Union (EU). Jiazhao Chen acknowledged the China Scholarship Council for funding (grant no. 202204910066).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2024.154491.

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