Journal of Industrial and Engineering Chemistry 145 (2025) 693–704

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

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

Enhancing the biorefinery of brewery spent grain by deep eutectic solvent pretreatment: Optimisation of polysaccharide enrichment through a response surface methodology

M.G. Morán-Aguilar [a], I. Costa-Trigo [a], M. Calderón-Santoyo [b], M.G. Aguilar-Uscanga [c], R. Pinheiro de Souza Oliveira [d], J.M. Domínguez [a,*]

[a] Industrial Biotechnology and Environmental Engineering Group "BiotecnIA", Chemical Engineering Department, University of Vigo (Campus Ourense), 32004 Ourense, Spain
[b] National Technological Institute of Mexico/I. T. of Tepic, Integral Food Research Laboratory, C.P. 63175 Tepic, Nayarit, Mexico
[c] National Technological Institute of Mexico /I. T. Veracruz, Food Research and Development Unit, C.P. 91860, Veracruz, Mexico
[d] Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, São Paulo University, Av. Prof Lineu Prestes, 580, Bl 16, São Paulo 05508-900, Brazil

ARTICLE INFO

Keywords:
Biorefinery
Brewery spent grain
Pretreatment
Acid-based deep eutectic solvent
Enzymatic digestibility
Value-added product

ABSTRACT

One of the main challenges in biorefinery is the efficient fractionation and use of lignocellulosic biomass. In this sense, pretreatment with deep eutectic solvents (DES) is highlighted as a clean and effective separation method, due to its selective solubilisation of hemicellulose and lignin fractions while preserving cellulose. This study presents a process of the enrichment of polysaccharide content and the improvement of enzymatic digestibility using choline chloride (ChCl) and lactic acid (LA) or glycerol (Gly) in brewery spent grain (BSG) pretreatment. Additionally, it describes how improvements can be made in obtaining polysaccharide-rich material through a response surface methodology, this by means of analysing the operational conditions (temperature, reaction time, and molar ratio) using ChCl:LA. The optimised operational conditions (130 °C, 90 min, and 1:8 mol/mol) generate a 75 % enrichment of polysaccharide fraction and the removal of 77.13 %, 50.70 %, and 100 % of acid-soluble lignin, xylan, and arabinan, respectively. Moreover, the process enhances the saccharification of glucan and xylan to almost 70 % using Cellic CTec2, and to 80 % of glucan and 40 % of xylan using *A. niger* CECT 2700 enzymatic extract. This constitutes a promising approach to the fractioning of cellulose and lignin from BSG through DES pretreatment, which is unfeasible using traditional pretreatment methods.

Introduction

Biorefinery involves applying a variety of techniques to fully use and transform biomass resources into different biofuels and chemical products of industrial interest [1]. Biomass is considered the most abundant renewable resource on the earth and has received a great deal of attention; in that it is seen as a sustainable alternative to fossil fuels. Biomass generally comes from forestry, agricultural, and agro-industrial wastes, including spent grain from breweries (BSG) [2].

BSG is the insoluble and solid fraction of the malted barley grain generated during the production of beer [3]. BSG represents 85 % of brewery by-products and 30 % of the malted grain [4], with an annual production estimated at ~3.4 Mt in the EU [5]. Its composition is notable in comparison to other agro-industrial residues, in that it is rich in protein, fibres, cellulose, arabinoxylan, and lignin, compounds which are considered to be precursors to a variety of products of industrial interest [6]. Hence, the use of BSG represents a significant ecological and economic approach as a feedstock for obtaining a range of value-added products, since it is surplus waste, involves low levels of handling, and has a low market value [3].

However, the recalcitrance of biomass caused by the interlocking network of cellulose, lignin, and hemicellulose components represents a considerable challenge for any successful biorefinery process [7]. An efficient pretreatment to fractionate lignocellulose into its three usable forms has thus been recognised as the key for promoting an integrated multi-product manufacturing biorefinery concept that can contribute to

* Corresponding author.
E-mail address: jmanuel@uvigo.es (J.M. Domínguez).

https://doi.org/10.1016/j.jiec.2024.10.066
Received 20 June 2024; Received in revised form 21 October 2024; Accepted 28 October 2024
Available online 13 November 2024

sustainability and a circular economy [8]. Previous studies using acidic, alkaline, organosolv, and ionic liquids as pretreatments have shown various degrees of success in biomass separation. However, the degradation of some compounds into non-recoverable molecules (5-hydroxymethylfurfural and furfural from hexose and pentose sugars) leads to a waste of resources, hence limiting the application of economically competitive biorefineries [7]. The discovery and application of deep eutectic solvents (DESs) has led to new perspectives in the selective fractionation of biomass through the solubilisation of lignin and hemicellulose without breaking the cellulose, improving its hydrolysis during the saccharification stage due to the swell/deconstruction of biomass [6]. Besides, compared to conventional pretreatments, DESs are recognised as promising, economical, non-toxic, easy to prepare, biocompatible and biodegradable, making them as the solvents of choice [9].

DESs are composed of a hydrogen bond acceptor (HBA, e.g., choline chloride) and a hydrogen bond donor (HBD, e.g., polyols, carboxyl acids, alcohols, amine, or amides) at fixed ratios, forming liquid phase eutectic mixtures at moderate temperature (between 50 and 100 °C) and ambient pressure conditions (1 atm) [10]. The HBD in DES may consist of polyols (such as glycerol), which generally present lower freezing temperatures and are liquid at room temperature, as well as being positively correlated with the decrease in saccharification time after pretreatment [11]. Furthermore, polyols are used extensively in an array of industrial applications, especially in the food and pharmaceutical industries [12]. On the other hand, recent studies have evaluated the influence of carboxylic acids (e.g. lactic acid, acetic acid, citric acid, levulinic acid, among others) as HBD on biomass fractionation, with a notable potential for application in the effective deconstruction of biomass [13]. For example, Chen et al., [14] report the effective application of betaine:lactic acid (LA) pretreatment in sugarcane bagasse, demonstrating a lignin and xylan removal of 47.1 % and 44.6 %, respectively, and an increase in cellulose digestibility 4.2 times higher than the raw biomass. Likewise, Raj et al., [15] show that [ChCl]:LA pretreatment at 10 % (w/w) solids, 140 °C and 2 h of reaction time, solubilises 78.8 % of lignin and 80.4 % of hemicellulose, allowing 82.7 % enzymatic conversion of glucans to glucose. Moreover, Morán-Aguilar et al., [16] studied the sugarcane pretreatment using [ChCl]:acetic acid (1:4 mol/mol), reporting an enrichment in glucan (>60 %) and xylan (>20 %) and an improvement in cellulose and xylan digestibility of 89 % and 73 %, respectively.

In this regard, the recent literature has highlighted the potential of acid-based DES (ADES) as a biomass pretreatment due to the good correlation between the polarity and acidic nature of the ADES. In addition, DES is an effective pretreatment for hemicellulose and lignin fractioning; it improves the release of sugars during enzymatic hydrolysis and allows generally closed cycle biorefinery processes for more cost-effective and environmentally sustainable processes [15,17].

However, studies here generally use DES as a pretreatment in lignocellulosic biomass, focusing on the examination of one variable or parameter (pretreatment temperature, reaction time, biomass-to-solvent ratio, or biomass particle size) and assessing efficiency in terms of the lignocellulosic fraction or the subsequent saccharification step [11].

Only a small number of papers have involved the study of a set of parameters using experimental design tools and maximising or minimising a response variable (e.g., polysaccharide content or saccharification maximisation or lignin content after pretreatment minimisation) [7,18,19].

The optimisation of variables allows for cost reductions in the pretreatment process, through a better understanding of the chemical mechanisms in DES reaction [5]. Among these, the use of the Box-Behnken design, a second-order multivariate method based on reduced factorial designs at three levels, has been highlighted. This method facilitates the optimisation analysis through the Response Surface Methodology (RSM), a statistical tool which is considered to provide reliable and adequate response studies through a mathematical model which is adjustable to experimental data [18].

Therefore, the present study, proposes the analysis of operating conditions (reaction time, molar ratio, and temperature) during DES pretreatment using the RSM method with the aim of improving polysaccharide enrichment following BSG pretreatment, leading to a more effective biomass deconstruction and enhanced enzymatic hydrolysis. Such an approach aims to establish a theoretical basis for the efficient and environmentally friendly conversion in a sustainable BSG biorefinery process.

Materials and methods

Materials and reagents

BSG was supplied by the brewery company UNICER located in Portugal. The biomass was dried at 50 °C in an oven (Celsius 2007, Memmert, Schwabach, Germany) for 24 h, and then crushed with a stainless-steel mill (SOGO, SS-111 5430 models, Sanysan Appliances SL, Valencia, Spain). The dry biomass was then sieved using a sieve with a mesh size of 1 mm, following the methodology described by Hames et al., [20]. Finally, it was stored in propylene bags at 25 °C until further use.

Choline chloride (ChCl) (purity > 99 %) was purchased from Sigma Aldrich (Spain) and used as HBA. In order to avoid moisture absorption, [ChCl] was kept in an oven for 24 h at 100 °C. Two HBD were assayed: glycerol (99.5 %) from Fisher Scientific (Hampton, NH, USA) and lactic acid (purity > 90 %) from the CARLO ERBA company (France).

The multienzyme complex Cellic CTec2 (Cellic CTec2-SAE0020) was purchased from Sigma-Aldrich and its enzymatic activity was evaluated as 254.50 ± 4.53 FPU/mL (cellulase activity) and 12084.88 ± 169.33 U/mL (xylanase activity) [21].

Methods

DES synthesis

DES preparation was performed by mixing HBA and HBD in a molar ratio (mol/mol) of 1:2 for choline chloride-glycerol [ChCl]:Gly according to the methodology describe by Morán-Aguilar et al., [21] and at ratios of 1:4, 1:8 and 1:12 for [ChCl]:LA [22,23]. The mixtures were subjected to heating (60 °C) and constant stirring until a homogeneous transparent liquid was obtained [13]. Finally, the generated DES were stored in glass bottles until use.

DES pretreatment

Initially, an evaluation was made of the influence of a neutral or acid HBD (glycerol and lactic acid, respectively) in the DES mechanism through efficiencies in enhancing the polysaccharides-rich material (PRM). Consequently, one gram of BSG was pretreated with [ChCl]:Gly and [ChCl]:LA with molar ratios of 1:2 and 1:4 (mol/mol), respectively, using a liquid–solid ratio (LSR) of 15:1 (v/w) in a sand bath at 130 °C for 90 min, following the methodology described in our previous studies [13]. Once the reaction had finished, DES was recovered by adding an antisolvent, in this case acetone-distilled water (1:1 v/v), in a LSR of 25:1 (mL:g). The solution was stirred at 250 rpm for 30 min in orbital shakers (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) and centrifuged to obtain a solid fraction. The PRM (solid fraction) was cleaned with distilled water (LSR of 25:1 mL:g), dried at 50 °C for 24 h in an oven (Celsius 2007, Memmert, Schwabach, Germany), and stored until use. On the other hand, lignin-rich material (LRM) was obtained by adding distilled water at 1:1 (v/v) to the liquid fraction (DES, lignin, and acetone/water). After 24 h, the precipitated LRM was obtained by centrifugation at 2755 x g for 30 min and dried at 50 °C for 24 h in an oven before analysis.

Experimental design

A Box-Behnken design was used to investigate the effects of the independent variables during [ChCl]:LA pretreatment in BSG. The

pretreatment optimisation process involved three independent variables: temperature (X_1), reaction time (X_2), and molar ratio (X_3) with three levels each (−1, 0, and 1), and the response variable was the maximum enrichment of total polysaccharides (%) after DES pretreatment (Table 1).

The behaviour of the DES system is explained by the quadratic polynomial equation (**Eq. (1)**) as a function of independent variables involving the quadratic interactions and the square terms.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{i<j}^{k} \beta_{ij} X_i X_i + \varepsilon \quad (1)$$

where Y is the predicted response of the process, k is the number of independent variables, X_i and X_j are the independent variables (i and j are the index numbers for the range of the pattern from 1 to k), β_0 is the intersection coefficient of the model, and β_j, β_{jj} and β_{ij} are the interaction coefficients of the linear, quadratic and second order terms, respectively. Finally, ϵ is the random error of the discrepancies or uncertainties between the predicted and measured values [24,25].

In addition, an ANOVA analysis was performed based on the proposed model to determine the relationship between the factors and the response variable. The quality of the fit of the regression model was expressed by the coefficient of determination R^2 and R^2 adj. Likewise, factors based on the probability value *p* with a confidence value of 95 % were selected.

Using the results obtained, response surface plots and contour plots based on the Box-Behnken design were generated to visualise the individual and interactive effects of the independent variables on the response variable enrichment of polysaccharides in PRM recovery after DES pretreatment on BSG.

Enzymatic hydrolysis of PRM

To understand the degree of enzyme digestibility after each run of the experimental design, the PRMs obtained were subjected to enzymatic hydrolysis. These experiments were performed in 50 mL sealed bottles with 1 g of PRM and 30 mL of citrate butter solution (50 mM at pH 4.8) with an enzymatic charge of 40 FPU/g of PRM using the multienzyme complex Cellic CTec2. The operational working conditions were 50 °C and 150 rpm for 72 h [13]. Control experiments were also conducted with untreated BSG substrate.

Since the costs associated with the step of biomass pretreatment and enzymatic hydrolysis during biorefinery processes can hinder its application in practical scenarios, we also applied an enzymatic cocktail produced from the same residue under simple and economical process conditions. The multi-enzyme complex obtained, following our previous studies [26] using *Aspergillus niger* CECT 2700 and solid-state fermentation (SSF), was then tested using two enzyme extract-solid ratios, 20:1 and 40:1 (v/w), in the untreated and [ChCl]:LA pretreated BSG obtained under the optimal operational conditions through the Box–Behnken design. Finally, the sugars in the aliquots were determined and quantified by HPLC to calculate glucan and xylan digestibility.

Table 1
Box-Behnken design for optimising polysaccharide content in BSG.

Variable	Coded variables	Levels		
		−1	0	1
Temperature (°C)	X_1	90	110	130
Reaction time (min)	X_2	90	135	180
Molar ratio (mol/mol)	X_3	1:4	1:8	1:12

$$Glucan\ digestibility\ (\%) = \left[\frac{Glucose\ amount\ in\ enzymatic\ hydrolysate * 0.9}{Glucan\ amount\ in\ substrate}\right] * 100 \quad (2)$$

$$Xylan\ digestibility\ (\%) = \left[\frac{Xylose\ amount\ in\ enzymatic\ hydrolysate * 0.88}{Xylan\ amount\ in\ substrate}\right] * 100 \quad (3)$$

Multi-enzyme complex production from A. niger CECT 2700 in SSF

The enzymatic extract from *Aspergillus niger* CECT 2700 was obtained following the methodology described by Morán-Aguilar et al., [27], using BSG after autoclave pretreatment as a substrate and the SSF methodology. The enzyme extract was characterised for cellulase and xylanase activity using the methodology described in section 2.6. The analysis of enzymatic extract attained 0.36 ± 0.02 FPU/mL (cellulase activity) and 228.69 ± 5.89 U/mL (xylanase activity).

Analytical procedures

Polysaccharide and lignin content. The composition of untreated BSG, PRMs, and LRMs obtained after DES pretreatment was determined by quantitative acid hydrolysis in two stages, following the process described in the National Renewable Energy Laboratory (NREL) Technical Report [28]. The analysis of polysaccharides was carried out by means of a HPLC system (Agilent model 1200, Palo Alto, CA, USA) equipped with a refractive index detector and an Aminex HPX-87H ion exclusion column (Bio Rad 300 × 7.8 mm, 9μ particles) with a guard column.

All the samples were eluted with 0.3 g/L of sulfuric acid at 0.6 mL/min and 50 °C. The lignin was measured including acid-soluble lignin (ASL) and Klason lignin (KL). The removal rate (%) from ASL, xylan, and arabinan loss after DES pretreatment were calculated according to Han et al., [29]:

$$ASL(\%) = \left[1 - \left[\frac{m_{ASLr}}{m_{ASLo}}\right]\right] * 100\% \quad (4)$$

$$Xylan\ loss(\%) = \left[1 - \left[\frac{m_{Xr}}{m_{Xo}}\right]\right] * 100\% \quad (5)$$

$$Arabinan\ loss(\%) = \left[1 - \left[\frac{m_{Ar}}{m_{Ao}}\right]\right] * 100\% \quad (6)$$

where m_{ASLo}, m_{Xo} and m_{Ao} are the quantities of lignin, xylose, and arabinose in the original BSG feedstock, and m_{ASLr}, m_{Xr}, and m_{Ar} are the quantities of lignin, xylose, and arabinose in PRM residue pretreated with DES, respectively.

Physicochemical composition analysis. The structure of native and pretreated BSG under the optimal operational condition with [ChCl]:LA pretreatment, as well as the lignin recovery in the pretreatment, were assayed through SEM analysis to observe morphological changes, using a JEOL JSM6010LA Scanning Electron Microscope (SEM).

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was conducted with a Thermo Nicolet 6700 FTIR Spectrometer (Thermo Fisher Scientific Inc., Madison, WI, USA), and an ATR accessory equipped with a diamond crystal (Smart Orbit Diamond ATR,

Thermo Fisher, USA). Native BSG, pretreated BSG and recovered solid lignin were recorded without preparation in the range 4000 to 400 cm^{-1} at 4 cm^{-1} resolution and 20 scans using a deuterated triglycine sulphate (DTGS) KBr detector. Also, cellulose crystallinity modifications were evaluated through the Lateral Order Index (LOI) expression (**Eq. (7)**) through the absorbance obtained in each sample [30].

$$LOI = \frac{A_{1437cm^{-1}}}{A_{898cm^{-1}}} \quad (7)$$

Finally, the crystalline index (*CrI*) was analysed by means of X-ray spectroscopy (Siemens D500) using diffraction angles ranging from 2θ = 2–45°, with a step size of 0.02° and a step time of 0.5 s. The *CrI* was calculated using the following expression [31]:

$$CrI = \left[\frac{I_{cry} - I_{am}}{I_{cry}}\right] * 100 \quad (8)$$

where I_{cry} is the intensity of the crystalline region at 2θ = 22.35 and I_{am} is the intensity in the amorphous region at 2θ = 16.17.

Enzymatic activities assay

Cellulase activity (FPU/mL) was determined using the methodology described by Ghose [32], based on the hydrolysis of 1 × 5.5 cm (50 ± 0.1 mg) Whatman No. 1 filter paper (Healthcare, Buckinghamshire, UK). The assay was conducted at pH 4.8 using 1 mL of 50 mM sodium citrate buffer and 0.5 mL of the enzyme solution, followed by incubation at 50 °C for 60 min.

Xylanase activity was assayed by the quantification of reducing sugars released from birch xylan solution (1 % w/v) diluted in 50 mM sodium phosphate buffer at pH 6 [32]. The reaction was carried out using 0.45 mL of birch xylan solution and 0.05 mL of enzyme solution at 50 °C for 10 min.

The quantification of reducing sugars was performed using the dinitro salicylic acid (DNS) method at 540 nm. For this study, one unit (U) of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars (glucose or xylose) per min under the conditions of the assay [26].

Statistical analysis

The data obtained by the experimental design were analysed using the statistical package Statgraphics (Statgraphics Centurion XVI version 16.1.11; 32 bit) to obtain the ANOVA analysis of variance and the optimum values for higher polysaccharide content (%) in the PRM obtained. Additionally, responses were adjusted to the second-order polynomial model. The comparison of means was established by means of the Tukey test at 95 % confidence.

Results and discussion

Effect of HBD in DES pretreatment

Chemical composition analysis of PRM

Table 2 sets out the results obtained from the characterisation of untreated and pretreated BSG, and as can be seen, the untreated BSG composition coincides with that reported in the extensive literature, as reviewed by Mussatto [33]. In this sense, it is well known that BSG is a lignocellulosic residue rich in sugars, proteins and minerals. However, the composition of this residue may suffer significant chemical variations due to the barley variety used in the process, the harvest season, cultural practices, malting and mashing conditions, and the amount and type of the adjuncts added to the barley malt for making the wort [33].

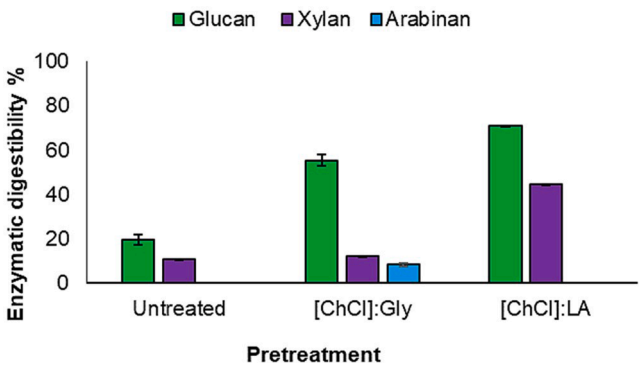

Fig. 1. Enzymatic digestibility of untreated and DES-pretreated BSG using Cellic CTec2 at 72 h of reaction.

Table 2 also shows the effect of [ChCl]:Gly pretreatment on untreated BSG. In this case, the polysaccharide fraction was reduced to 15.4 % glucan, 18.06 % xylan, and 8.18 % arabinan. This could be linked to the physicochemical properties of the eutectic mixture used, since neutral solvents (polyols, glucose, and amino acids) as HBD contain the main functional groups C═O, NH, and OH [13]. These functional groups have available lone pair electrons in the outermost orbitals which form strong hydrogen bonds with the dominated lignin structures in the biomass. Likewise, these hydrogen bonds are oriented and saturated, holding the active sites of the lignin to improve the main components fraction of the lignocellulose under certain conditions [34].

In addition, Chen et al., [35] indicated that the type of biomass (lignocellulose composition) and the operating conditions (temperature and reaction time) can change the yield in [ChCl]:Gly pretreatment system. Therefore, the total polysaccharides content can differ when applying conditions from 15 h and 150 °C for corncob to 1 h and 110 °C for switchgrass [36].

By contrast, the use of [ChCl]:LA promoted the enrichment of glucan composition to 33.22 % and a reduction to 13.52 % (xylan), 3.33 % (ASL), and all the arabinan content regarding untreated BSG. This could be related to the polarity value of DES mixtures using the Kamlet-Taft parameters α, β and π* (which represent the hydrogen-bond providing capacity, hydrogen-bond accepting capacity, and polarity/polarisability, respectively), indicating that the [ChCl]:LA mixture was able to release more acidic protons (H^+) compared to [ChCl]:Gly. Therefore, the presence of H^+ provided by lactic acid promotes the incision of ester bonds and allows selective removal of lignin and hemicellulose [37,38].

Moreover, these results are consistent with those obtained by Morán-Aguilar et al., [13] using [ChCl]:LA in sugarcane bagasse, where a total lignin removal close to 55 % was obtained. Likewise, these authors reported that the improvements in efficiency of pretreatment using ADES could be due to its ionic properties. This means that, ADES generates

Table 2
Polysaccharide and lignin composition in untreated BSG and PRMs obtained after DES pretreatment.

	Chemical composition (%)				
Pretreatment	**Glucan**	**Xylan**	**Arabinan**	**ASL**	**KL**
Untreated	26.59 ± 1.11	22.03 ± 0.79	9.83 ± 1.04	7.88 ± 0.17	11.54 ± 0.11
[ChCl] :LA	33.22 ± 3.84	13.52 ± 0.08	0.00 ± 0.00	3.33 ± 0.02	13.18 ± 0.51
[ChCl] :Gly	15.40 ± 2.43	18.06 ± 1.61	8.18 ± 0.89	5.46 ± 0.11	16.54 ± 1.51

ASL: Acid soluble lignin; KL: Klason lignin; [ChCl]:LA: choline chloride-lactic acid; [ChCl]:Gly: choline chloride-glycerol.

additional hydroxyl groups in its alkyl chain, which are slightly esterified with the OH hydroxyl groups in lignin, promoting ionic interactions with aromatic groups (bond β-O-4) and generating their incision and condensation [8].

For this reason, the delignification rate using ADES could be different from that in other eutectic mixtures. Similarly, recent reports have shown the high efficiency of [ChCl]:LA as pretreatment of grapevines due to the high conductivity (1448 μS/cm a 25 °C), low viscosity, and pH (0.04) of various DES mixtures [39].

Enzymatic saccharification of PRM

Fig. 1 represents the percentage of digestibility obtained after enzymatic hydrolysis using untreated and pretreated BSG following pretreatments with different DESs mixtures. The percentages of enzymatic digestibility of 55.40 ± 2.51 %, 12.19 ± 0.12 %, and 8.31 ± 0.67 % were achieved for glucan, xylan, and arabinan, respectively, using [ChCl]:Gly. Meanwhile, the application of [ChCl]:LA increased the percentage of digestibility to 71.09 ± 0.13 % for glucan and 44.62 ± 0.04 % for xylan, which represents a 4-fold increase over the performance obtained using the untreated BSG.

The improvement in enzymatic digestibility with [ChCl]:LA over [ChCl]:Gly can be attributed, first, to the physicochemical characteristics of the DES mixture, because [ChCl]:LA presents a lower viscosity, which may contribute to a better mass transfer between BSG and DES during pretreatment [40]. Second, the carboxylic acid group of lactic acid enhances the cleavage of glycoside, ester, and ether bonds with respect to the OH group of glycerol, improving the accessibility of the enzyme during hydrolysis [38].

Additionally, ADES is able to generate stronger ionic interactions with biomass, producing lignin dissolution and the generation of amorphous zones with larger contact areas, as well as catalysing cleavage in the β-(1–4)-glycoside bonds of cellulose, reducing its degree of polymerisation, which can facilitate enzyme accessibility by the substrate [8,41–43].

Consequently, the importance of the proper selection and combination of HBA and HBD in eutectic mixtures has been underlined, due to previous studies have suggested that the functional groups in the HBD substantially affect the ability to deconstruct lignocellulosic structure and dissolve lignin in biomass [44]. In this sense, the [ChCl]:LA was selected to evaluate various factors that influence modifications in the lignocellulose structure and functional groups, with the aim of generating a straightforward and effective process to support viable BSG biorefinery.

Analysis of Box-Behnken experimental design and model fit

Polysaccharide and lignin content in PRM

Table 3 presents the design of experiments with the non-coded variables, as well as the experimental values obtained for each experiment, quantifying glucan, xylan, arabinan, KL, and ASL composition and the removal rate according to **Eqs. (4), (5), and (6)**. Experiments 13–15 also show the design centre points as a means of quantifying experimental error.

The results (Table 3) demonstrate the effect on the total polysaccharide content and lignin removal rate after DES pretreatment. In this sense, experiment 12 (110 °C for 180 min at a molar ratio of 1:12) generated a minimum polysaccharide content (45.91 %), resulting in a removal rate of 63.47 %, 100 %, and ~74 % of xylan and arabinan, and ASL, respectively. It seems that the severity of the pretreatment processes is closely related to the stoichiometric ratio and the type of HBA and HBD in the DES system, because this affects the properties of the DES solvent and, consequently, the efficient removal of lignin or enrichment of the polysaccharide fraction [45].

The amount of HBD (lactic acid) in ADES, such as [ChCl]:LA, has a dominant role in biomass pretreatment. For instance, Zhang et al., [46] reported that the increased stoichiometric ratios (molar ratio) of formic acid as HBD in the DES pretreatment of poplar wood without bark could release more free H^+ (acidic protons) and reduce the pH of the medium, which is conducive to an acidic environment for the selective cleavage of the ether bonds of the lignin-polysaccharide complex. Also, according to Shishov et al., [47] the displacement of hydrogen bonding forces by the different proportions of the HBA and HBD can affect or enhance the deconstruction and separation of lignin, hemicellulose, and cellulose in lignocellulose.

On the other hand, it is important to note that the application of a high HBD-HBA molar ratio and prolonged pretreatment times can also negatively affect the enrichment of polysaccharides. Thus, Li et al., [48] reported that the maximum lignin removal from wood occurs with the application of high temperatures (120 °C) and longer reaction times (12 h) in combination with an elevated HBD-HBA molar ratio ([ChCl]:LA 1:10) during DES pretreatment. A greater fraction of hemicelluloses (mainly xylan and arabinan) is converted into soluble products of low molecular weight, which cannot be recovered in the PRM after pretreatment through the application of more severe conditions.

Apart from the effect generated by the molar ratio in the DES solvent, pretreatment temperature and reaction time are also crucial factors to be controlled during the pretreatment to enhance the polysaccharide composition of PRM and avoid the severity of the biomass deconstruction [49].

Table 3
The lignocellulosic composition of BSG obtained by the Box Behnken design.

No.	Factors			Composition (%)					Total polysaccharides	Removal rate (%)				
	X_1	X_2	X_3	Glucan	Xylan	Arabinan	ASL	KL	(%)	Glucan	Xylan	Arabinan	ASL	KL
1	90	90	1:8	40.23	19.15	8.02	9.11	23.29	67.40	–	28.03	49.42	14.08	–
2	130	90	1:8	56.42	13.12	0.00	1.84	25.58	69.54	–	50.70	100.00	77.13	–
3	90	180	1:8	42.23	17.93	7.46	8.65	21.00	67.61	–	32.64	52.95	18.43	–
4	130	180	1:8	52.52	10.56	0.00	2.42	29.24	63.09	–	60.31	100.00	77.18	–
5	90	135	1:4	38.50	16.68	6.88	9.93	17.88	62.06	–	37.31	56.61	6.35	–
6	130	135	1:4	54.44	14.27	0.00	4.10	26.94	68.71	–	46.38	100.00	61.35	–
7	90	135	1:12	37.24	17.81	4.51	9.26	19.13	59.56	–	33.07	71.52	12.62	–
8	130	135	1:12	43.18	12.00	6.64	5.24	25.12	61.82	–	54.92	58.12	50.60	–
9	110	90	1:4	42.50	14.50	0.00	8.84	19.64	57.01	–	45.49	100.00	16.64	–
10	110	180	1:4	43.56	11.66	0.00	6.21	21.10	55.22	–	56.18	100.00	41.40	–
11	110	90	1:12	44.66	13.26	0.00	5.38	22.06	57.92	–	50.17	100.00	49.22	–
12	110	180	1:12	36.19	9.72	0.00	2.76	47.82	45.91	–	63.47	100.00	73.97	–
13	110	135	1:8	47.99	12.59	0.00	5.50	24.45	60.58	–	52.70	100.00	48.08	–
14	110	135	1:8	49.41	12.71	0.00	5.31	24.76	62.12	–	52.24	100.00	49.95	–
15	110	135	1:8	52.78	13.91	0.00	4.29	25.05	66.69	–	47.73	100.00	59.57	–

X_1: Temperature (°C); X_2: Reaction time (min); X_3: Molar ratio (mol/mol); Total polysaccharides are considered as the sum of glucan, xylan and arabinan for each experiment; –: unquantified.

Experiment 2 achieved the most enriched polysaccharide fraction with ~70 % (glucan and xylan included) and an ASL reduction of ~77 %. This might indicate that the application of short pretreatment times (90 min) and moderate pretreatment conditions such as temperatures below 130 °C and an HBA-HBD molar ratio of 1:8 mol/mol enhanced BSG polysaccharide enrichment.

The stable formation of hydrogen bonds between the eutectic solvent and the lignocellulose in the system pretreatment is crucial. Therefore, the analysis and control of pretreatment factors are important issues to be addressed, because the efficiencies of the pretreatment could vary greatly depending on the conditions established [50]. For instance, the increment in temperature could generate a reduction in viscosity and consequently an improved extraction performance. However, this increment in temperature should be reasonable, since it can harm the thermolabile compounds [16,51]. The influence of temperature on chemical reactions and intermolecular forces in solvent systems can be verified through the Gibbs free energy equation [50].

Hence, the perfect configuration of factors (temperature, reaction time and molar ratio HBA-HBD) during DES pretreatment can improve the lignin removal yield or an enrichment in polysaccharides, which in turn can generate efficient, scalable and low-cost processes due to the reduction of energy used during the process, as well as the excess of reagents implement in DES solvent synthesis [52].

The polynomial equation of the quadratic model (**Eq. (8)**) describes the relationship between the three factors (X_1, X_2, and X_3) and the response variable (total polysaccharides %) obtained through multiple regression analysis and the coefficients of the equation. It is observed that the molar ratio (X_3) with a coefficient of 7.879 had the largest positive linear effect among the factors, followed by the reaction time (X_2) with a coefficient of 0.836.

$$\textit{Total polysaccharides}\ (\%) = 200.916 - 3.978{*}X_1 + 0.836{*}X_2 + 7.879{*}X_3 + 0.021{*}X_1^2 - 0.004{*}X_1{*}X_2 - 0.009{*}X_1{*}X_3 - 0.001{*}X_2^2 - 0.012{*}X_2{*}X_3 - 0.356{*}X_3^2 \quad (8)$$

The model in **Eq. (8)** presented high R^2 and R^2 adjusted (0.9733 and 0.9253 respectively), showing that the regression models are adjusted to the experimental data. Additionally, the model indicates that 97 % of the content of polysaccharides obtained during BSG pretreatment were attributed to the analysed variables, while the model cannot explain around 3 % of the data process.

Figure S2 represents a contour and main effect plots in BSG pretreated with DES to evaluate the effect of the factors on the response. As can be observed in **Fig. S2a**, higher values of total polysaccharides (%) in the PRM can be achieved by increasing the temperature (from 90 °C to 130 °C) in shorter periods (<120 min), which can reduce the cost of the energy required to perform the pretreatment process. The molar ratio 1:8 mol/mol (centre point) positively affects the enrichment of polysaccharides after DES pretreatment using short periods (<100 min).

Figure S2b shows the main effect plots, where, through the slope, the significance and effect of each variable on the responses for positive (+) and negative (−) values can be evaluated. Thus, **Fig. S2b** demonstrates a more significant effect for the factors reaction time (X_2) and molar ratio (X_3), which coincides with the data presented in **Table S1**.

Table S1 also presents the ANOVA analysis of the Box Behnken design, where the *p*-value and F-value were used in the hypothesis testing. A higher F-value indicated greater reliability of the model, and a *p*-value (≤0.05) was used to determine whether an operating variable significantly affected the selected responses [53]. Hence, these results show that reaction time (X_2) and molar ratio (X_3) are the main factors that exert a significant effect ($p < 0.05$) on the response variable. This in in line with the report by Sharma et al., [54] who indicate that the success in the application of DES as biomass pretreatment depends on the type of eutectic mixture, its molar ratio, the pretreatment conditions, as well as the nature of the biomass.

Similarly, it is observed that the quadratic effect of temperature (X_1X_1), molar ratio (X_3X_3), and the interaction effect between temperature and reaction time (X_1X_2) have a significant effect on the enrichment of total polysaccharides (%) during the pretreatment in BSG with [ChCl]:LA.

The molar ratio is an important factor in DES pretreatment because it can affect the mechanism of biomass deconstruction (lignin-polysaccharide complex fractionation), as well as the degradation of the polysaccharides into low molecular weight molecules (such as glucose, xylose, or arabinose) dissolved in the eutectic solvent that cannot be recovered in the solid fraction (PRM) [55]. Therefore, a balance between the amounts of stoichiometric composition (moles of HBD) is necessary because an increase in the amount of lactic acid could generate an adverse.

In this regard, pretreatment conditions such as temperature and reaction time influence the removal and degradation of polysaccharides in

Table 4
Comparative analysis on acid-based DES and operational conditions on chemical composition in different biomass.

Substrate	DES pretreatment condition	Biomass composition (%)		Reference
		Raw	After pretreatment	
Poplar sawdust	[ChCl]:LA (1:6 mol/mol), 130 °C, 1.5 h	• 46.9 Glucan • 18.6 Xylan • 28.3 Lignin	• 78.7 Glucan • 8.2 Xylan • 7.5 Lignin	[59]
	[ChCl]:LA (1:10 mol/mol), 110 °C, 1.5 h		• 54.5 Glucan • 13.5 Xylan • 17.5 Lignin	
Rice straw	[ChCl]:LA (1:3 mol/mol), 120 °C, 3 h and 15 % of biomass loading	• 34.0 Glucan • 20.7 Xylan • 20.7 Lignin	• 51.9 Glucan • 11.7 Xylan • 11.11 Lignin	[49]
Wheat straw	TEBAC:LA (1:9 mol/mol), 100 °C, 10 h, LSR:15:1	• 35.0 Glucan • 19.0 Xylan • 18.0 Lignin	• 55 Glucan • 9 Xylan • 9 Lignin	[23]
Corncob	BTMAC:LA (1:2 mol/mol), 140 °C, 2 h	• 37.1 Glucan • 31.7 Xylan • 16.1 Lignin	• 72.2 Glucan • 6.1 Xylan • 5.9 Lignin	[58]
Brewery spent grain	[ChCl]:LA (1:8 mol/mol), 130 °C, 1.5 h, LSR: 15:1 (v/w)	• 26.6 Glucan • 22.0 Xylan • 10.0 Arabinan • 19.5 Lignin	• 56.4 Glucan • 13.12 Xylan • 0 Arabinan • 27.44 Lignin	Present study

LSR: liquid–solid ratio; TEBAC: triethylbenzyl ammonium chloride; BTMAC: benzyltrimethylammonium chloride; LA: lactic acid.

ADES pretreatment. For instance, Lee et al., [56] showed that increasing the period from 1 to 2 h can double the production of furfural in the pretreatment of oil palm leaves using [ChCl]:oxalic acid. Likewise, Isci and Kaltschmitt [57] reported that sequential pretreatments, microwave-assisted with [ChCl]:formic acid in wheat straw, increased furfural concentration by prolonging the pretreatment time from 50 s to 120 s. Therefore, it is critical to balance pretreatment severity by optimising pretreatment conditions.

The analysis of the Box-Behnken design by STATGRAPHICS Centurion program showed, as optimal conditions, a temperature of 130 °C, 90 min of reaction, and a molar ratio of 1:8 (mol/mol) with a predicted value of the maximum polysaccharides content of 76 %, comprised mainly of glucan and xylan. These operational conditions were validated in triplicate, obtaining 68.50 ± 1.22 % of total polysaccharide, which represents 90 % replicability.

The current study presents more moderate time and temperature conditions than those reported in Table 4 below. In particular, the study reported by Liu et al., [23] using TEBAC as HBA at 100 °C for 10 h of reaction, obtained a 1.6-fold increase in glucan yield after pretreatment. Likewise, Guo et al., [58] present a 1.94-fold increase in glucan enrichment after pretreatment in corncobs. This is also consistent with the findings reported by Huang et al., [49] and Su et al., [59] who present temperature conditions between 110 °C to 130 °C and reaction times between 1.5 and 3 h, obtaining glucan enrichment between 1–1.68-fold compared to rice straw and untreated poplar sawdust, respectively.

Therefore, our study highlights the fact that we have been able to obtain more than twice the glucan enrichment after the optimal analysis in terms of temperature, reaction time, and DES molar ratio (130 °C, 1.5 h, and 1:8 mol/mol) in the BSG. This underlines the need to study different variables synergistically in DES pretreatments to reduce energy consumption, as well as other special requirements (e.g. reagents, additional pretreatment steps, equipment, etc.) that contribute to the generation of simple and applicable processes in the industry.

To our knowledge, only a limited number of studies have been based on the optimisation of different factors in DES pretreatment related to the efficiency of polysaccharide content, sugar release, or enzymatic digestibility, using experimental designs based on the RSM. For instance, Panakkal et al., [18] studied the solid-DES-biomass ratio, reaction time, and temperature in a DES pretreatment system, to improve the release efficiency of fermentable sugars in mg/g of *Napier grass*, and reported an optimal operating condition using relatively low temperatures (80 °C) and long operating times (5 h) with a total polysaccharide content similar to that obtained in our study (~70 %). Likewise, Ceaser et al., [19] analysed the effect of a DES molar ratio, temperature, reaction time, and solid loading in *Pinus insignis* based on a central composite rotatable design in combination with microwave treatment to improve high-quality glucan and lignin solid recovery. That study reported that

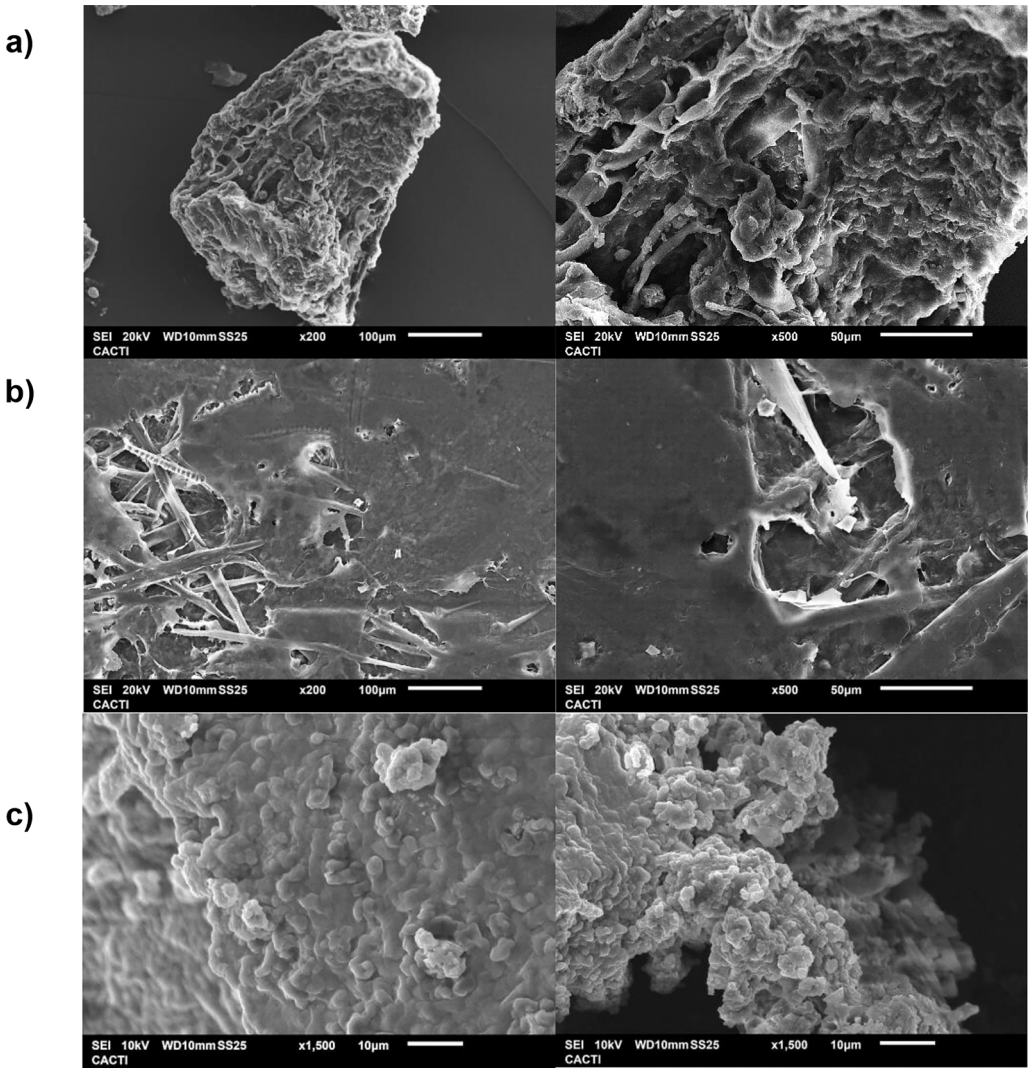

Fig. 2. Morphological characterisation by SEM of BSG a) native; b) pretreated with [ChCl]:LA under optimal operational condition; and c) LRM recovered after DES pretreatment.

the application of [ChCl]:formic acid (1:4 mol/mol) at 140 °C, 14 min, 800 W, and 15 % (w/v) generated a 96.2 % hemicellulose removal, 90.1 % delignification and 93.5 % glucan retention. Elsewhere, Sunar et al., [7] analysed the reaction time, temperature, and DES concentration in terms of the effect of two types of DES [ChCl]:oxalic acid (1:2 mol/mol) and [ChCl]:trifluoroacetic acid (1:1.5 mol/mol) in sugarcane bagasse. They reported optimal pretreatment conditions of 108.98 °C, 41.27 min, and 39.66 % (v/v) for [ChCl]:oxalic acid and 111.02 °C, 42.80 min and 37.62 % (v/v) for [ChCl]: trifluoroacetic acid achieved 33.49 % and 42.62 %, respectively for reducing sugars.

In this sense, the aforementioned works report operating conditions with prolonged times (5 h), the application of combined pretreatments with DES (microwave or ultrasound treatment), or large amounts of eutectic solvent (~40 % v/v) to perform the reaction compared to the optimal conditions reported in our present study.

Thus, although there is support in the literature for the effect of DES pretreatments on different lignocellulosic residues, scant information is available on evaluating the synergistic effect of different variables during biomass pretreatment towards explaining the behaviour of DES pretreatment more deeply and creating scalable and economical

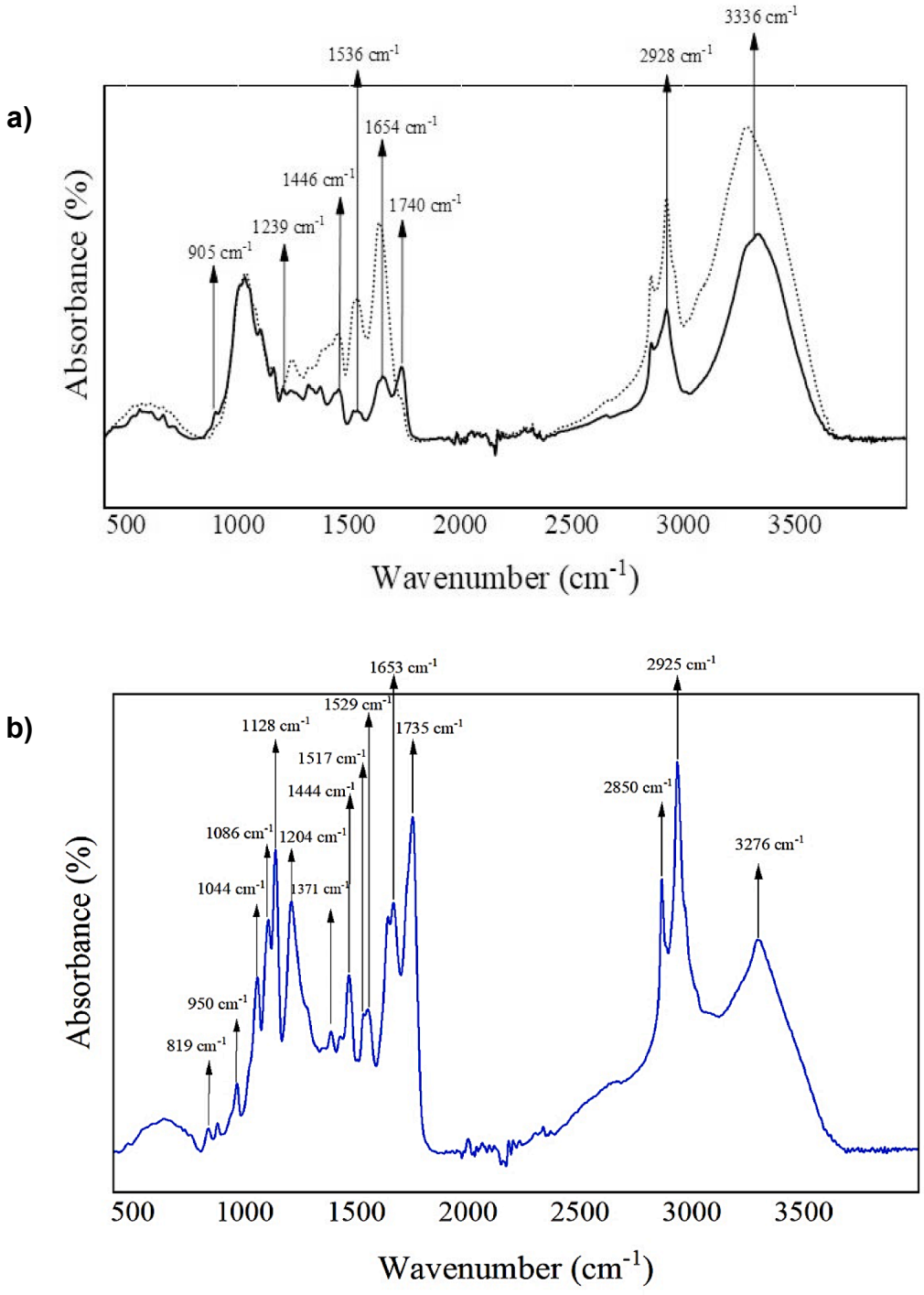

Fig. 3. ATR-FTIR of a) BSG native (dashed line) and PRM obtained at 130 °C, 90 min and molar ratio of 1:8 (black line), b) Spectra of LRM solid recovered after [ChCl]:LA pretreatment on BSG.

processes that can improve sustainable economics in biorefinery processes. It is worth bearing in mind in this context that the pretreatment process represents about 40 % of total production costs [19].

Physicochemical composition analysis

The morphological analysis presented in Fig. 2**a** indicates a recalcitrant structure denoting porous, flats and heterogeneous surfaces formed by various rigid and ordered fibril debris. By contrast, Fig. 2**b** shows the micrographs of pretreated BSG which exhibited the appearance of a smooth and consistent surface, which could indicate the presence of a more ordered cellulose structure than the native BSG [13]. In addition, the micrography exposes a morphological change due to an improved deformation with loss of fibres. According to Lin et al., [60], ADES pretreatment enhances cellulose reactivity through a deconstruction/swelling process, by removing lignin and hemicellulose to expose the innermost cellulosic component of biomass.

Finally, the morphology of LRM recovered after [ChCl]:LA pretreatment of BSG was investigated using the SEM technique. Fig. 2**c** shows a homogeneous material formed by spherical nanoparticles on the surface of the recovered material, and this could be linked in that lignin is often present as tightly packed because of the intense electron interactions within it [61].

Moreover, LRM obtained after DES pretreatment had 78.50 ± 0.02 % KL and 2.88 ± 0.03 % of ASL with a purity higher than 80 %. Minor sugar impurities were also detected and expressed as glucan (2.60 ± 1.00 %). In this regard, similar results were achieved by Zhu et al, [39] with 81.10 % purity using [ChCl]:LA 1:4 (mol:mol) at 130 °C during 360 min in grapevine. Likewise, Raj et al., [15] affirmed that [ChCl]:LA was a good candidate to extract a high-quality lignin since their report showed better lignin yields using [ChCl]:LA 1:2 (mol:mol) than [Betaine]:LA 1:2(mol:mol) in oilcane bagasse after 140 °C during 120 min, obtaining purities above 95 % from oilcane bagasse.

Therefore, the effective recovery of high purity lignin from BSG using [ChCl]:LA under mild operational conditions (130 °C, 90 min) has been demonstrated. Additionally, these results indicate that the [ChCl]:LA might extract lignin with low molecular weight which could be suitable for the preparation of bioactive materials [15].

In light of the fact that ATR-FTIR spectroscopy analysis provides information related to the presence or absence of specific functional groups, as well as the chemical structure of polymer materials. This study evaluated the spectrum of native BSG and pretreated BSG with [ChCl]:LA (Fig. 3**a**). Thus, the band at 3336 cm^{-1} to 2928 cm^{-1} corresponding to OH bonds and stretching in C–H are mostly represented in the native BSG spectrum due to the cellulose, hemicellulose, and lignin compounds in the biomass without pretreatment. By contrast, after DES pretreatment a reduction in this peak is observed (black line).

This could be due to the fact that during ADES pretreatment, the hemicellulose can undergo partial acetylation. Additionally, the ester bond is broken to generate acetic acid during the pretreatment process, while xylose, arabinose, rhamnose (pentoses), mannose, and galactose (hexoses) are solubilised in the DES solvent [62].

In addition, the peaks at 1740 cm^{-1} and 1654 cm^{-1} assigned to the stretching of C=O hemicellulose and lignin [63] were reduced in pretreated BSG. According to Hou et al., [64], the free protons (H^+) in HBD might catalyse the breaking of ether bonds between lignin and hemicellulose, facilitating the separation and extraction during DES pretreatment.

On the other hand, the bands in 1536, 1446, 1239 cm^{-1} assigned to vibration C=C (guaiacyl aromatic), C–H (methyl and methylene) and C–O (guaiacyl unit of lignin) were reduced in pretreated BSG spectra, since [ChCl]:LA was able to fracture the ether bonds between phenylpropane structural units of lignin, thereby accelerating the depolymerisation of lignin, similar to the effect after lignin hydrochloric acid-mediated hydrolysis [65].

LOI can be used to interpret qualitative modifications in cellulose crystallinity and is based on the ratio of absorbance bands at specific wavenumbers (1437 cm^{-1} correlated with crystalline cellulose and 898 cm^{-1} corresponding to amorphous cellulose) [30]. Therefore, a decrease in LOI value indicates the reduction of crystallinity.

The LOI values obtained for native BSG was 8.01 and for pretreated BSG with [ChCl]:LA (130 °C; 90 min and 1:8 mol/mol) was 1.71, which indicates the reduction of cellulose crystallinity after DES pretreatment. This might also be understood as the generation of increased amounts of amorphous cellulose susceptible to the enzymatic attack.

On the other hand, the results obtained from the crystallinity analysis by XRD showed a *CrI* of 44.34 in PRM after pretreatment with [ChCl]:LA at 130 °C, 90 min and 1:8 mol/mol, whereas the native BSG achieved 14.28. This increase of crystallinity was more than three times that of native BSG, which indicates the effectiveness of [ChCl]:LA under the optimum conditions.

Cellulose reactivity was enhanced by removal of the lignin and hemicellulose (amorphous compounds) from the PRM through a deconstruction/swelling of the biomass due to DES pretreatment. This might be explained by the ADES action as a mild acid-base catalytic solution, breaking the β-O-4 aryl ester bonds between the lignin-polysaccharide complex, as well as ester linkages between lignin and 4-O-methylglucuronic acid xylan chains.

The current study has also analysed the relevant absorbance bands of LRM obtained after DES pretreatment on BSG (Fig. 3**b**). In this sense, the band at 3276 cm^{-1} associated with the $^{-}$OH stretching of the phenolic and aliphatic moiety of the lignin skeleton, the peaks at 2925 and 2850 cm^{-1} linked to stretching vibration of symmetric and asymmetric C–H of methyl and methylene [63], the characteristic peak at 1735 cm^{-1} corresponding to the carbonyl stretching of unconjugated ketones, and the peak at 1653 cm^{-1} for C–C stretching in the side chain of lignin subunits [7].

Moreover, the band which describes the C=C and C–C stretching vibration of the aromatic ring and phenolic ring of lignin were observed at 1517 and 1529 cm^{-1}. Likewise, aromatic skeletal vibrations in the lignin structure were observed in the corresponding band at 1444 cm^{-1} [19].

The high purity of the recovery LRM was also confirmed by the guaiacyl and syringyl unit peaks [19], which correspond to the peak at 1204 cm^{-1} indicating the stretching vibration of the C-O (guaiacyl units), the band at 1128 cm^{-1} related to aromatic C–H in-plane deformation of the syringyl ring, and the band at 1086 cm^{-1} C–O bond in (aromatic guaiacyl units) [63]. In this sense, the extracted LRM could be applied as a potential feedstock to produce chemicals or aromatic derivatives as a co-product under a closed loop process due to the amount of guaiacyl units [66].

Table 5
Enzymatic digestibility of PMRs obtained from the Box-Behnken design using [ChC]:LA as eutectic solvent.

No.	Factors			Enzymatic digestibility (%)		
	X_1	X_2	X_3	Glucan	Xylan	Arabinan
1	90	90	1:8	43.32	42.71	18.68
2	130	90	1:8	68.00	67.56	0.00
3	90	180	1:8	39.05	38.03	4.26
4	130	180	1:8	55.41	64.67	0.00
5	90	135	1:4	41.61	46.12	11.57
6	130	135	1:4	56.86	56.72	0.00
7	90	135	1:12	40.36	47.95	14.53
8	130	135	1:12	43.27	59.02	11.11
9	110	90	1:4	44.28	44.08	0.00
10	110	180	1:4	63.08	69.95	0.00
11	110	90	1:12	53.46	61.15	0.00
12	110	180	1:12	44.42	43.14	0.00
13	110	135	1:8	60.49	69.38	0.00
14	110	135	1:8	58.91	68.36	0.00
15	110	135	1:8	57.42	64.84	0.00

X_1: Temperature; X_2: Reaction time; X_3: Molar ratio.

Additionally, the band at 1044 cm^{-1} related to the stretching and vibrations in $–CH_2OH$ and CO groups of lignin structure and the peak observed in the wavenumber at 819 cm^{-1} is attributed to the C–H out-of-plane bending in positions C_2, C_5, and C_6 of guaiacyl units of lignin [67].

However, the peak at 950 cm^{-1} could be linked to polysaccharide impurities, since this band is attributed to the stretching C-O-C of β-(1,4) glycosidic linkage [63]. Besides, this is consistent with the composition reported in section 3.2.2. for the LRM recovered after [ChCl]:LA pretreatment.

The band at 905 cm^{-1} assigned to stretching of C–O–C of β-(1,4) glycosidic linkage in cellulose polysaccharide was mainly observed for pretreated BSG, which indicates the efficiency of LA as HBD in the deconstruction of cellulose through the formation of a greater number of amorphous zones [13].

Enzymatic saccharification

Table 5. shows the efficiency of biomass deconstruction by enzymatic digestibility in each experiment from the Box-Behnken design by the Cellic CTec2 enzyme complex. Hence, experiment 2 (130 °C; 90 min and 1:8 mol/mol) corresponds to the saccharification percentage of ~ 70 % for glucan and xylan, demonstrating the best efficiency in biomass pretreatment.

This could be related to efficient delignification during biomass pretreatment. Yu et al., [65] established a linear correlation between enzymatic digestibility and lignin removal, as lignin restricts cellulase accessibility, leading to ineffective cellulase adsorption during enzymatic hydrolysis [68]. Furthermore, a PRM with reduced lignin content can provide more contact points between cellulose and cellulase, which enhance the sugar released [38].

By contrast, it has been shown that experiments performed with molar ratio values above 1:8 mol/mol or long-time reactions >90 min result in a decreased efficiency of saccharification. This could be due to the collapse of pores in lignocellulose caused by the increased presence of organic acids in the DESs molar ratio, which impacts the adsorption of cellulase onto the cellulose surface [65]. Moreover, during the pretreatment, the hydroxyl groups and reducing end groups of cellulose can also be oxidised [69], and thus chemical oxidation of the reducing ends of cellulose could harm the interaction of cellobiohydrolase (1,4-β-D-glucan-cellobiohydrolase or exoglucanase), which targets the reducing end of cellulose, which in turn could reduce the efficiency during enzymatic hydrolysis.

On the other hand, Fig. 4 shows that higher LSR (40 mL:1 g) improved the enzymatic digestibility of PRM obtained with [ChCl]:LA, attaining 81.40 ± 0.08 % and 41.60 ± 0.04 % for glucan and xylan, respectively. This might be explained by considering that fungi generate a great diversity of enzymes with complementary or synergistic activities during their growth on complex materials such as lignocellulose [70], among which feruloyl esterases produced by *A. niger* CECT 2700 have been studied [27,71]. These enzymes perform an important role in the synergistic degradation of biomass where feruloyl esterase is involved in breaking ester or ether bonds between lignin and polysaccharides.

Table 6 also describes the biomass pretreatment through enzymatic digestibility using DES or ionic liquids compared with this study. Thus, similar results of glucan and xylan digestibility (~70 %) to those reported by [23,49,72] can be observed. However, we might note that the present study applies operating conditions with shorter times (≤ 1.5 h). This is beneficial as it reduces the amount of energy needed to carry out the process. In this regard, Ceaser et al., [19] indicate that the use of extended times could have a negative impact on product quality and process cost without guaranteeing any improvement in the yield and quality of the pretreated biomass.

On the other hand, higher cellulose digestibility yields (>50 %) have been achieved in shorter periods (<108 h) and less enzymatic load than those reported by Su et al., [59] using operational conditions similar to those in the present study.

Therefore, it can be affirmed that the DES pretreatment conditions applied in this study improved the digestibility of BSG. Additionally, the application of an enzymatic extract obtained from the same residue could offer a closed loop process with a positive impact on the circular economy, bearing in mind that Patel et al., [73] estimated the cost of enzymes to be 30–40 % of the total expense in biorefinery processes, as well as the pretreatment.

The value-added products derived through biorefinery processes using lignocellulosic biomass as feedstock require the development of more cost-effective and environmentally friendly catalytic pathways that overcome the challenges of the high production costs and low competitiveness of traditional processes. Hence, this study presents the efficient application of the pretreatment stage by analysing the factors involved in the optimal performance for polysaccharide enrichment and lignin recovery as value-added products through the application of green and renewable processes such as DES valorising brewing industry wastes.

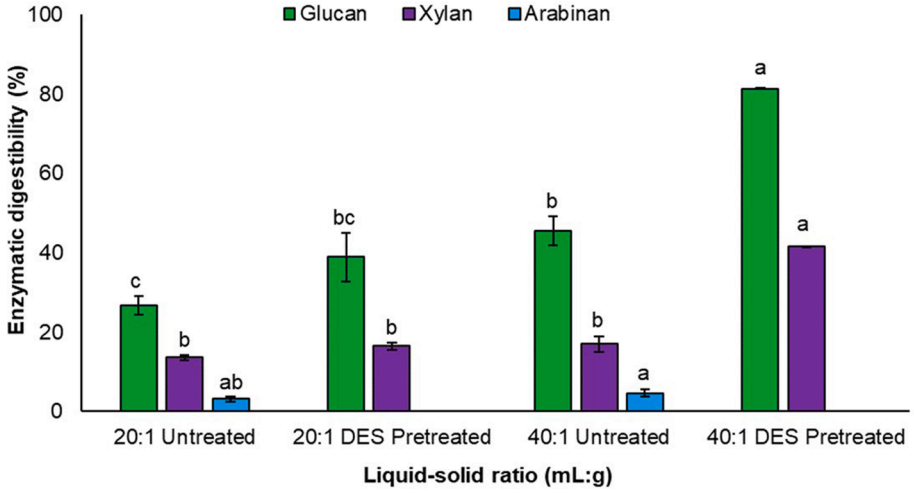

Fig. 4. Enzymatic hydrolysis of pretreated BSG with [ChCl]:LA under optimal operational conditions using different liquid–solid- ratios of enzyme extract from *A. niger* CECT 2700. Different letters represent statistically significant differences (one-way ANOVA, Tukey's test; $p < 0.05$).

Table 6
Comparative study of the different enzymatic digestibility obtained of different pretreated lignocellulosic residues.

Substrate	DES pretreatment condition	Enzymatic load	Hydrolysis condition	Enzymatic digestibility*	Reference
Poplar sawdust	[ChCl]:LA (1:6 mol/mol), 130 °C, 1.5 h	30 FPU of Cellic CTec 2 per g of glucan.	50 °C, 108 h, 150 rpm.	~50 % of cellulose	[59]
	[ChC]l:LA (1:10 mol/mol), 110 °C, 1.5 h			37.7 % of cellulose	
Rice straw	[ChCl]:LA (1:3 mol/mol), 120 °C, 3 h and 15 % of biomass loading	20 FPU of Celluclast 1.5L per g of biomass and 0.1 % Viscoyme L.	50 °C; 150 rpm, 192 h.	78.7 % of cellulose 71.6 % of xylan	[49]
Wheat straw	TEBAC:LA (1:9 mol/mol), 100 °C, 10 h, LSR:15:1	35 FPU of Celluclast 1.5L per g of biomass and 82 CBU of β-glucosidase/ g of biomass.	50 °C, 200 rpm, 60 h.	89 % of cellulose 71 % of xylan	[23]
Brewery spent grain	[N1112OH] [Gly] 90 °C,16 h, 5 wt% of solid loading.	Enzymatic extract produced by *Aspergillus niger* CECT 2700 and *Trichoderma reesei* CECT 2414.Solid-liquid ratio 1:60 (w/v).	50 °C, 150 rpm, 72 h.	80.68 %, 54.29 % and 19.58 % of glucan, xylan and arabinan, respectively.	[72]
Brewery spent grain	[ChCl]:LA (1:8 mol/mol), 130 °C, 1.5 h, LSR: 15:1 (v/w)	40 FPU of Cellic CTec 2 per g of biomass.	50 °C, 150 rpm, 72 h	~70 % of glucan and xylan	Present study
		Enzymatic extract produced by *Aspergillus niger* CECT 2700. Solid-liquid ratio 1:40 (w/v).	50 °C, 150 rpm, 72 h.	~80 % of glucan ~40 % of xylan	

* Enzymatic digestibility in biomass pretreated with DES; LSR: liquid–solid ratio; TEBAC: triethylbenzyl ammonium chloride; BTMAC: benzyltrimethylammonium chloride; LA: lactic acid.

Conclusion

The cost of biomass pretreatment and enzymatic hydrolysis efficiency are closely associated with the issue of economically viable bioconversion in biorefinery processes. The present study has examined the effects of [ChCl]:LA pretreatment on polysaccharide enrichment and the enzymatic digestibility in PRM recovery using promising waste from the brewing industry. The results showed that molar ratio was the most influential parameter, followed by reaction time, for the improvement of polysaccharide content after DES pretreatment. This was particularly evident in the recovery of cellulose in the PRM, attributed to the effective removal of lignin and hemicellulose (xylan and arabinan) from BSG. Additionally, according to the ANOVA analysis, the determination of coefficient R^2 (97.33 %) and adjusted R^2 (92.53 %) values towards responses are sufficiently high, confirming the desired suitability of the predicted experimental results. In conclusion, the optimised process parameters via RSM analysis have been shown to improve polysaccharide content in the BSG residue and enhance the enzymatic digestibility process. Thus, these results can form the basis of a simple and workable pretreatment process, with positive effects on the feasibility of DES pretreatments of agro-industrial waste.

CRediT authorship contribution statement

M.G. Morán-Aguilar: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **I. Costa-Trigo:** Supervision, Methodology. **M. Calderón-Santoyo:** Supervision. **M.G. Aguilar-Uscanga:** Writing – review & editing, Supervision. **Ricardo Pinheiro de Souza Oliveira:** Writing – review & editing, Supervision, Investigation, Funding acquisition. **J.M. Domínguez:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, 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.

Acknowledgments

The authors are grateful for Grant PID2020-115879RB-I00 funded by MICIU/AEI/10.13039/501100011033, to Xunta de Galicia of Spain (GRC-ED431C 2024/24), São Paulo Research Foundation FAPESP (processes n. 2018/25511-1 and n. 2023/09256-0) and National Council for Scientific and Technological Development (CNPq, grants 408783/2021-4 and 312923/2020-1). Funding for open access charge: Universidade de Vigo/CRUE-CISUG is also acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jiec.2024.10.066.

References

[1] Y. Wang, K.H. Kim, K. Jeong, N.K. Kim, C.G. Yoo, Curr. Opin. Green Sustain. Chem. (2021), https://doi.org/10.1016/j.cogsc.2020.100396.
[2] R. Amoriello, Tiziana, Ciccoritti, Sustain. 13 (4) (2021) 2355, https://doi.org/10.3390/su13042355.
[3] Z. Qazanfarzadeh, A.R. Ganesan, L. Mariniello, L. Conterno, V. Kumaravel, J. Clean. Prod. 385 (2023) 135726, https://doi.org/10.1016/j.jclepro.2022.135726.
[4] A. Estévez, L. Padrell, B. Iñarra, M. Orive, D.S. Martin, Aquaculture 543 (2021), https://doi.org/10.1016/j.aquaculture.2021.736921.
[5] W. Li, H. Yang, T.E. Coldea, H. Zhao, LWT 139 (2021) 110582, https://doi.org/10.1016/j.lwt.2020.110582.
[6] A.C. Cassoni, P. Costa, I. Mota, M.W. Vasconcelos, M. Pintado, Chem. Eng. Res. Des. 192 (2023) 34–43, https://doi.org/10.1016/j.cherd.2023.01.053.
[7] S.L. Sunar, R.K. Oruganti, D. Bhattacharyya, D. Shee, T.K. Panda, J. Ind. Eng. Chem. (2024), https://doi.org/10.1016/j.jiec.2024.05.030.
[8] D. Tian, Y. Guo, J. Hu, G. Yang, J. Zhang, L. Luo, Y. Xiao, Int. J. Biol. Macromol. 142 (2020) 288–297, https://doi.org/10.1016/j.ijbiomac.2019.09.100.
[9] V. Ashokkumar, R. Venkatkarthick, S. Jayashree, S. Chuetor, S. Dharmaraj, G. Kumar, W.H. Chen, C. Ngamcharussrivichai, Bioresour. Technol. 344 (2022) 126195, https://doi.org/10.1016/j.biortech.2021.126195.
[10] T. El Achkar, H. Greige-Gerges, S. Fourmentin, Environ. Chem. Lett. (2021) 3397–3408, https://doi.org/10.1007/s10311-021-01225-8.
[11] S. Massayev, K.M. Lee, J. Clean. Prod. 360 (2022) 132239, https://doi.org/10.1016/j.jclepro.2022.132239.
[12] H. Shekaari, M.T. Zafarani-Moattar, A. Shayanfar, M. Mokhtarpour, J. Mol. Liq. 249 (2018) 1222–1235, https://doi.org/10.1016/j.molliq.2017.11.057.
[13] M.G. Morán-Aguilar, M. Calderón-Santoyo, R.P. de Souza Oliveira, M.G. Aguilar-Uscanga, J.M. Domínguez, Carbohydr. Polym. 298 (2022), https://doi.org/10.1016/j.carbpol.2022.120097.
[14] Y. Chen, C. Ma, W. Tang, Y.C. He, Bioresour. Technol. 386 (2023), https://doi.org/10.1016/j.biortech.2023.129485.

[15] T. Raj, B.S. Dien, V. Singh, Chem. Eng. J. 493 (2024), https://doi.org/10.1016/j.cej.2024.152657.
[16] M.G. Morán-Aguilar, I. Costa-Trigo, M.G. Aguilar-Uscanga, A. Paz, J. M. Domínguez, Biomass Bioener. 186 (2024) 107256, https://doi.org/10.1016/j.biombioe.2024.107256.
[17] X. Li, W. Tang, Y.C. He, Ind. Crops Prod. 206 (2023), https://doi.org/10.1016/j.indcrop.2023.117691.
[18] E.J. Panakkal, K. Cheenkachorn, S. Chuetor, P. Tantayotai, N. Raina, Y.S. Cheng, M. Sriariyanun, Sustain. Energy Technol. Assess. 54 (2022), https://doi.org/10.1016/j.seta.2022.102856.
[19] R. Ceaser, S. Rosa, D. Montané, M. Constantí, F. Medina, Bioresour. Technol. 369 (2023), https://doi.org/10.1016/j.biortech.2022.128470.
[20] B. Hames, R. Ruiz, C. Scarlata, A. Sluiter, J. Sluiter, D. Templeton, Preparation of Samples for Compositional Analysis: Laboratory Analytical Procedure (LAP); Issue Date 08/08/2008, 2008.
[21] M.G. Morán-Aguilar, I. Costa-Trigo, A.M. Ramírez-Pérez, E. de Blas, M. Calderón-Santoyo, M.G. Aguilar-Uscanga, J.M. Domínguez, Energies 15 (11) (2022) 4101, https://doi.org/10.3390/en15114101.
[22] Z. Chen, A. Ragauskas, C. Wan, Ind. Crops Prod. (2020), https://doi.org/10.1016/j.indcrop.2020.112241.
[23] Y. Liu, J. Zheng, J. Xiao, X. He, K. Zhang, S. Yuan, Z. Peng, Z. Chen, X. Lin, ACS Omega 4 (22) (2019) 19829–19839, https://doi.org/10.1021/acsomega.9b02709.
[24] D.R. Pinheiro, R. de F. Neves, S.P.A. Paz, Micropor. Mesopor. Mat. 323 (2021) 111250, https://doi.org/10.1016/j.micromeso.2021.111250.
[25] V. Hosseinpour, M. Kazemeini, A. Mohammadrezaee, Appl. Catal. A. Gen. 394 (1–2) (2011) 166–175, https://doi.org/10.1016/j.apcata.2010.12.036.
[26] M.G. Morán-Aguilar, M. Calderón-Santoyo, J.M. Domínguez, M.G. Aguilar-Uscanga, Biomass Convers. Biorefin. 13 (9) (2021) 7983–7991, https://doi.org/10.1007/s13399-021-01808-z.
[27] M.G. Moran-Aguilar, I. Costa-Trigo, M. Calderón-Santoyo, J.M. Domínguez, M. G. Aguilar-Uscanga, Biochem. Eng. J. 172 (2021), https://doi.org/10.1016/j.bej.2021.108060.
[28] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Biomass Anal. Technol. Team Laboratory Anal. Procedure (2011) 1–14.
[29] S. Han, R. Wang, K. Wang, J. Jiang, J. Xu, Bioresour. Technol. 363 (2022), https://doi.org/10.1016/j.biortech.2022.127905.
[30] A. Kljun, T.A.S. Benians, F. Goubet, F. Meulewaeter, J.P. Knox, R.S. Blackburn, Biomacromolecules 12 (11) (2011) 4121–4126, https://doi.org/10.1021/bm201176m.
[31] D. Outeiriño, I. Costa-Trigo, A. Rodríguez, N. Pérez, J. Manuel, Sep. Purif. Technol. 254 (2021) 117651, https://doi.org/10.1016/j.seppur.2020.117651.
[32] T.K. Ghose, Int. J. Sel. Assess. 59 (2) (1987) 257–268, https://doi.org/10.1111/j.1468-2389.1995.tb00038.x.
[33] S.I. Mussatto, J. Sci. Food Agric. 94 (7) (2014) 1264–1275, https://doi.org/10.1002/jsfa.6486.
[34] X. Chen, Q. Liu, B. Li, N. Wang, C. Liu, J. Shi, L. Liu, Int. J. Biol. Macromol. 259 (2024), https://doi.org/10.1016/j.ijbiomac.2024.129354.
[35] Z. Chen, X. Bai, A. Lusi, C. Wan, A.C.S. Sustain, Chem. Eng. 6 (9) (2018) 12205–12216, https://doi.org/10.1021/acssuschemeng.8b02541.
[36] A. Procentese, E. Johnson, V. Orr, A. Garruto Campanile, J.A. Wood, A. Marzocchella, L. Rehmann, Bioresour. Technol. 192 (2015) 31–36, https://doi.org/10.1016/j.biortech.2015.05.053.
[37] E.S. Morais, A.M. Da Costa Lopes, M.G. Freire, C.S.R. Freire, J.A.P. Coutinho, A.J. D. Silvestre, Molecules 25 (16) (2020), https://doi.org/10.3390/molecules25163652.
[38] D. Huo, Y. Sun, Q. Yang, F. Zhang, G. Fang, H. Zhu, Y. Liu, Bioresour. Technol. 376 (2023) 128937, https://doi.org/10.1016/j.biortech.2023.128937.
[39] Y. Zhu, T.X. Yang, B.K. Qi, H. Li, Q.S. Zhao, B. Zhao, Int. J. Biol. Macromol. 236 (2023), https://doi.org/10.1016/j.ijbiomac.2023.123977.
[40] Y.-L. Loow, T.Y. Wu, G.H. Yang, L.Y. Ang, E.K. New, L.F. Siow, J.M. Jahim, A. W. Mohammad, W.H. Teoh, Bioresour. Technol. 249 (2018) 818–825, https://doi.org/10.1016/j.biortech.2017.07.165.
[41] J.A. Sirviö, M. Visanko, H. Liimatainen, Green Chem. 17 (6) (2015) 3401–3406, https://doi.org/10.1039/c5gc00398a.
[42] V.R. Chourasia, A. Pandey, K. Kishore, R.J. Henry, Bioresour. Technol. 337 (2021) 125480, https://doi.org/10.1016/j.biortech.2021.125480.
[43] Q. Ji, X. Yu, A.E.A. Yagoub, L. Chen, A.T. Mustapha, C. Zhou, Renew. Energy 172 (2021) 304–316, https://doi.org/10.1016/j.renene.2021.03.050.
[44] C. Alvarez-Vasco, R. Ma, M. Quintero, M. Guo, S. Geleynse, K.K. Ramasamy, M. Wolcott, X. Zhang, Green Chem. 18 (19) (2016) 5133–5141, https://doi.org/10.1039/c6gc01007e.
[45] A. Alhadid, L. Mokrushina, M. Minceva, Molecules 25 (2020) 5, https://doi.org/10.3390/molecules25051077.
[46] Z. Zhang, J. Xu, J. Xie, S. Zhu, B. Wang, J. Li, K. Chen, Carbohydr. Polym. 290 (2022) 119472, https://doi.org/10.1016/j.carbpol.2022.119472.
[47] A. Shishov, A. Bulatov, M. Locatelli, S. Carradori, V. Andruch, Microchem. J. (2017) 33–38, https://doi.org/10.1016/j.microc.2017.07.015.
[48] L. Li, L. Yu, Z. Wu, Wood Research 64 (3) (2019) 499–514.
[49] Z.J. Huang, G.J. Feng, K.P. Lin, F.L. Pu, Y.M. Tan, W.C. Tu, Y.L. Han, X.D. Hou, H. M. Zhang, Y. Zhang, Ind. Crops Prod. 152 (2020), https://doi.org/10.1016/j.indcrop.2020.112515.
[50] T. Xiao, M. Hou, X. Guo, X. Cao, C. Li, Q. Zhang, W. Jia, Y. Sun, Y. Guo, H. Shi, Renew. Sust. Energ. Rev. (2024), https://doi.org/10.1016/j.rser.2023.114243.
[51] A. Saini, A. Kumar, P.S. Panesar, A. Thakur, Appl. Food Res. (2022), https://doi.org/10.1016/j.afres.2022.100211.
[52] Z. Chen, Y. Wang, H. Cheng, H. Zhou, Ind. Crops Prod. 187 (2022) 115335, https://doi.org/10.1016/j.indcrop.2022.115335.
[53] K. Manatura, B. Chalermsinsuwan, N. Kaewtrakulchai, Y.C. Chao, Y.H. Li, Results Eng. 18 (2023) 101139, https://doi.org/10.1016/j.rineng.2023.101139.
[54] V. Sharma, M.L. Tsai, C.W. Chen, P.P. Sun, A.K. Patel, R.R. Singhania, P. Nargotra, C. Di Dong, Bioresour. Technol. (2022), https://doi.org/10.1016/j.biortech.2022.127631.
[55] W. Wang, D.J. Lee, Bioresour. Technol. 339 (2021) 125587, https://doi.org/10.1016/j.biortech.2021.125587.
[56] C.B.T.L. Lee, T.Y. Wu, C.H. Ting, J.K. Tan, L.F. Siow, C.K. Cheng, J.M. Jahim, A. W. Mohammad, Bioresour. Technol. 278 (2019) 486–489, https://doi.org/10.1016/j.biortech.2018.12.034.
[57] A. Isci, M. Kaltschmitt, Biomass Convers. Biorefin. 12 (2022) 197–226, https://doi.org/10.1007/s13399-021-01860-9.
[58] Z. Guo, Q. Zhang, T. You, X. Zhang, F. Xu, Y. Wu, Green Chem. 21 (11) (2019) 3099–3108, https://doi.org/10.1039/c9gc00704k.
[59] Y. Su, C. Huang, C. Lai, Q. Yong, Bioresour. Technol. 321 (2021), https://doi.org/10.1016/j.biortech.2020.124471.
[60] W. Lin, S. Xing, Y. Jin, X. Lu, C. Huang, Q. Yong, Bioresour. Technol. 306 (2020), https://doi.org/10.1016/j.biortech.2020.123163.
[61] V. Singh, N. Mittal, S. Dhukia, A.K. Atri, V. Singh, Sustain. Chem. Pharm. 39 (2024), https://doi.org/10.1016/j.scp.2024.101518.
[62] Z.K. Wang, H. Li, X.C. Lin, L. Tang, J.J. Chen, J.W. Mo, R.S. Yu, X.J. Shen, Bioresour. Technol. 307 (2020), https://doi.org/10.1016/j.biortech.2020.123237.
[63] A. Azizan, N.A.A. Jusri, I.S. Azmi, M.F. Abd Rahman, N. Ibrahim, R. Jalil, Mater Today Proc. 63 (2022) S359–S367, https://doi.org/10.1016/j.matpr.2022.03.548.
[64] X.D. Hou, A.L. Li, K.P. Lin, Y.Y. Wang, Z.Y. Kuang, S.L. Cao, Bioresour. Technol. 249 (2018) 261–267, https://doi.org/10.1016/j.biortech.2017.10.019.
[65] Y. Yu, D. Wang, L. Chen, H. Qi, A. Liu, M. Deng, X. Wu, K. Wang, Ind. Crops Prod. 187 (2022), https://doi.org/10.1016/j.indcrop.2022.115542.
[66] Y.T. Tan, G.C. Ngoh, A.S.M. Chua, Bioresour. Technol. 281 (2019) 359–366, https://doi.org/10.1016/j.biortech.2019.02.010.
[67] V. Hemmilä, R. Hosseinpourpia, S. Adamopoulos, A. Eceiza, Waste Biomass Valori. 11 (11) (2020) 5835–5845, https://doi.org/10.1007/s12649-019-00878-5.
[68] C. Huang, X. Jiang, X. Shen, J. Hu, W. Tang, X. Wu, A. Ragauskas, H. Jameel, X. Meng, Q. Yong, Renew. Sust. Energ. Rev. 154 (2022) 111822, https://doi.org/10.1016/j.rser.2021.111822.
[69] C.I. Ishizawa, T. Jeoh, W.S. Adney, M.E. Himmel, D.K. Johnson, M.F. Davis, Cellulose 16 (4) (2009) 677–686, https://doi.org/10.1007/s10570-009-9313-1.
[70] C. Sánchez, Biotechnol. Adv. 27 (2) (2009) 185–194, https://doi.org/10.1016/j.biotechadv.2008.11.001.
[71] N. Pérez-Rodríguez, C.D. Moreira, A. Torrado Agrasar, J.M. Domínguez, Enzyme Microb. Technol. 91 (2016) 52–58, https://doi.org/10.1016/j.enzmictec.2016.05.011.
[72] D. Outeiriño, I. Costa-Trigo, A. Ochogavias, R. Pinheiro de Souza Oliveira, N. Pérez Guerra, J.M. Salgado, J.M. Domínguez, N. Biotechnol. 79 (2024), https://doi.org/10.1016/j.nbt.2023.12.010.
[73] A. Patel, A.R. Shah, J. Bioresour. Bioprod. 6 (2) (2021) 108–128, https://doi.org/10.1016/j.jobab.2021.02.001.