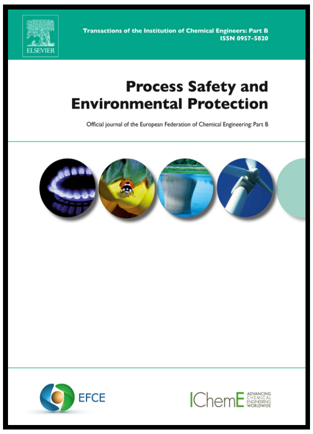

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Sustainable vine shoots-to-ethanol valorisation by a sequential acid/organosolv pretreatment

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Abstract

In this study, a fractionation and valorisation scheme for vine shoots is proposed for biofuel and lignin production. This agricultural waste was fractionated by acid/organosolv sequential pretreatment. In the first step, acid pretreatment was optimised at 150ºC and 1.2% H_2SO_4 to release hemicellulosic sugars, of which 76% could be recovered. This sugar stream was co-fermented by *E. coli* with an ethanol yield higher than 98% after detoxification with resins or NH_4OH. The solid obtained under optimal acid pretreatment conditions was delignified by organosolv treatment, and a delignification rate of 43% was reached at 180ºC. This substrate with 83% enzymatic digestibility was bio-converted into ethanol by simultaneous saccharification and fermentation, with a yield of 76%. Additionally, lignin was recovered from the organosolv liquor, aiming for the full valorisation of the biomass, which showed a syringyl/guaiacyl ratio of 0.92 by nuclear magnetic resonance, complying with the data provided for Fourier transform infrared spectroscopy and confirming the aromaticity of this fraction for further valorisation.

Keywords

vine shoots; organosolv pretreatment; co-fermentation; sugars; ethanol; lignin

1. Introduction

Agricultural residues are some of the main and most promising sources of energy and, after a suitable conversion process, of renewable chemical compounds. They are produced on a yearly basis and must be removed from the fields, have low costs and alternative applications (Tye et al., 2016). If these materials are processed to obtain products of interest, an additional environmental advantage is achieved since an alternative disposal method is provided as opposed to the usual practice of direct burning in the fields, which is linked to fire risks and uncontrolled CO_2 emissions, in addition to economic costs (Ginni et al., 2021).

One of the difficulties regarding the practical use of agricultural residues is that they are generally scattered in the fields, resulting in considerable costs of accumulation and transportation. Therefore, for taking full advantage, their integral use is necessary. Since, as lignocellulosic materials, they basically consist of three different basic polymers, i.e., cellulose, hemicelluloses and lignin, a common strategy is to separate these fractions and process them in the most appropriate way.

Vine shoots (VS) constitute a prominent example of agricultural residues. They are generated yearly and, so far, have no practical applications and are therefore burned on the fields. Spain is one of the top three wine producers, with an estimated yearly generation of VS per hectare between 1 and 3 tonnes (Biovino, 2021). This makes vine shoots some of the most relevant agricultural residues in quantitative terms, also considering that in 2021, the global wine cultivation area was 6.7 million ha (FAOSTAT, 2023).

Fractionation or separation of the main fractions in which the lignocellulosic residues are constituted imply the transformation of each fraction (or of its components) into other products of interest, generally related to a biorefinery approach (Vaez et al., 2021). Cellulose, composed of glucose, is usually enzymatically hydrolysed, and the released glucose is transformed to a variety of products, among which ethanol has been widely proposed because of its direct use as biofuel for the total or partial substitution of gasoline (Moreno et al., 2021).

Hemicelluloses, heterogeneous polymers of several sugars, mainly xylose, can also be hydrolysed, and the sugars are converted into ethanol (Romero-García et al., 2022). Since hemicellulosic sugars, e.g., xylose, cannot be converted into ethanol by conventional yeasts, but may play a role in an economically feasible process, the use of modified microorganisms has been the focus of different studies. For example, the ethanologenic *Escherichia coli* can efficiently transform both xylose and glucose (a process referred to as co-fermentation) into ethanol (López-Linares et al., 2020). The third main polymer of lignocellulosic materials, lignin, is also applied in different processes after being fully characterised (Dávila et al., 2019).

Regarding VS, several studies investigating the production of renewable compounds from the different fractions have recently been reviewed (Contreras et al., 2021; Filippi et al., 2022). In the case of ethanol production, Senila et al. (2020) reported an ethanol concentration of 20 g/L obtained from pretreated and delignified VS by *Saccharomyces cerevisiae*, a microorganism unable, in its native form, to convert pentoses. A similar ethanol concentration (20.8 g/L) was reported using diluted sulphuric acid pretreatment and *Debaryomyces nepalensis* (Egües et al., 2013).

Considering that the sum of the three fundamental polymers of cellulose-based materials in general (and vine shoots in particular) exceeds 80% (Contreras et al., 2021), the objective of this work is to propose an efficient scheme for obtaining sugars (from the cellulose and hemicellulose fractions) and their subsequent transformation into ethanol, as well as the recovery and characterization of the solubilised lignin from the treatment. As new features, the present work proposes VS fractionation by sequential acid-organosolv pretreatment, the optimisation of hemicellulosic fraction solubilisation by acid pretreatment and further co-fermentation by xylose-fermenting microorganisms of the resulting hemicellulosic sugars to ethanol.

2. Materials and methods

2.1 Raw material

Vine shoots were collected after pruning following the harvesting of the grapes. In the laboratory, the raw material was air-dried at room temperature and to a final moisture content of 10.33% ± 0.12%. Subsequently, the material was milled using a laboratory hammer mill (Retsch) to a particle size smaller than 1 cm, homogenised in a single lot and stored until used. The chemical composition of VS was as follows (% w/w, dry basis): cellulose (as glucose), 37.3; hemicellulose, 18.5 (xylose 18.0, galactose 1.5, arabinose 0.8, mannose 0.5); lignin, 23.9; ash, 3.0; acetyl groups, 3.4; extractives, 9.0 (Silva Rabelo et al., 2023).

2.2 Acid pretreatment

The VS were subjected to acid pretreatment to separate the hemicellulose from the remaining lignocellulose matrix. Acid pretreatment was carried out according to a central composite experimental design using the response surface methodology. The influence of temperature and H_2SO_4 concentration was evaluated and 13 different experimental conditions were assayed (Table 1). The VS were pretreated in a laboratory-scale stirred Parr reactor with a total volume of 1 L. The solid loading used was 15% (w/v) in all experiments (90 g of dry biomass and 600 mL of acid solution). When the pretreatment temperature was reached, cooling started. The reactor was kept sealed, and the suspension was agitated until it was cooled down to approximately 40ºC. After pretreatment, the resulting slurry was vacuum-filtered to separate the acid liquor (liquid fraction) from the acid-pretreated solid (solid fraction). The acid-pretreated solids were washed with distilled water to neutral pH, dried at 40°C for 24 h and chemically characterised to determine their cellulose, hemicellulose and lignin contents. The liquid fractions were analysed for sugars and inhibitory compounds as described below and stored at 4°C.

Once all experiments were completed, the experimental data were analysed using the statistical software Design Expert 8.0.2, Stat-Ease Inc., Minneapolis, USA. The optimum conditions were determined by analysis of variance (ANOVA). Acid pretreatment of VS under optimum conditions was carried out, and both liquor and pretreated solid were characterised and stored at room temperature for later use.

2.3 Organosolv pretreatment

In a second step, the acid-pretreated solid obtained under optimum conditions was subjected to organosolv pretreatment. This pretreatment was carried out at 150°C and 180°C with ethanol 50% (v/v) and sulphuric acid 1% (w/v) as catalysts, using a solid loading of 15%. The reactor used for organosolv pretreatment was the same as that applied in the acid stage. The suspensions resulting from this pretreatment were vacuum-filtered to separate organosolv liquors from organosolv-pretreated solids. The organosolv liquors were chemically characterised according to Section 2.10 and stored at 4°C. The organosolv-pretreated solids were washed with distilled water to neutral pH, dried at 40°C for 24 h and stored at room temperature. Biomass delignification attained by organosolv treatment was calculated by measuring the amount of acid insoluble lignin in the biomass before and after organosolv pretreatment.

2.4 Enzymatic hydrolysis

Raw VS, acid-pretreated solid obtained under optimised conditions and organosolv-pretreated solids attained at 150°C and 180°C were enzymatically hydrolysed to determine the effect of pretreatment on their enzymatic digestibility. In 100-mL Erlenmeyer flasks, 1.25 g of dry substrate was placed with 25 mL total volume which was composed of a buffer solution (50 mM sodium citrate) at pH 4.8 and 15 FPU/g dry substrate of Cellic® CTec2 (Novozymes A/S, Bagsværd, Dinamarca) enzyme supplemented with β-glucosidase (Novozymes 50010) at 10% of the volume of Cellic® CTec2 enzyme. A solid/liquid ratio of 5% (w/v) was used in all cases. Erlenmeyer flasks were placed in an orbital shaker at 50°C and 150 rpm for 72 h. All experiments were performed in triplicate, and a 1-mL aliquot of liquid samples was taken every 24 h and analysed by HPLC to measure the glucose content. Enzymatic digestibility was calculated as grams of glucose attained by enzymatic hydrolysis per 100 grams of glucose in substrate.

2.5 Microorganisms and inocula

Saccharomyces cerevisiae (Fermentis ethanol red, France) was used in the simultaneous saccharification and fermentation of the 180ºC organosolv-pretreated solid. The inoculum was prepared by growing lyophilised yeast in an orbital shaker at 30ºC and 150 rpm for 24 h. Inoculation was performed in 100-mL Erlenmeyer flasks with 25 mL of culture medium. A needle was attached to allow the gases formed by the metabolic activity of the yeast to escape. The culture medium contained 30 g/L glucose, 5 g/L yeast extract, 2 g/L NH_4Cl, 1 g/L KH_2PO_4 and 0.3 g/L $MgSO_4{\cdot}7H_2O$. After 24 h, the yeast was reinoculated into a new Erlenmeyer flask with culture medium on an orbital shaker at 35°C and 150 rpm for 16 h, and the inoculum was ready to be used in the simultaneous saccharification and fermentation (SSF) experiments.

Escherichia coli SL100 was used to co-ferment pentoses and hexoses in acid liquors. The microorganism previously inoculated, and AM1 medium was used for growing the inoculum, with the following composition (in g/L): xylose 16 g/L, glucose 10 g/L, $(NH_4)_2HPO_4$ 2.63 g/L, $NH_4H_2PO_4$ 0.87 g/L, $MgSO_4{\cdot}7H_2O$ 0.246 g/L, KCl 0.149 g/L, betaine 0.117 g/L, citric acid 0.1 g/L (Fernandes-Klajn et al., 2018). Cells were incubated in 250-mL Erlenmeyer flasks using 75 mL of AM1 medium. The volume of inoculum required was determined by spectrophotometry at 620 nm, calculating an initial concentration in the liquid of 0.8 g/L (dry basis). The flasks were placed on an orbital shaker at 37°C and 200 rpm for 20 h. After incubation, *E. coli* cells were separated by centrifugation from the AM1 medium, washed and added to the culture medium (acid liquor).

Prior to use, all instruments were autoclaved at 120°C for 30 min. Inoculation and handling of microorganisms were performed in a laminar flow hood previously sterilised using UV radiation for 10 min.

2.6 SSF of delignified vine shoots

The SSF experiments were performed under microaerobic conditions in 100-mL Erlenmeyer flasks with a total volume of 25 mL in citrate buffer (50 mM, pH 5.0), using 180-organosolv pretreated solid as substrate at 10% and 20% (w/v). The SSF tests started by simultaneously adding Cellic® CTec2 enzymes (15 FPU/g dry substrate) and *S. cerevisiae* inoculum (4% v/v), corresponding to a cell addition of 0.25 g/L. These experiments were carried out in an orbital shaker at 40°C, 180 rpm, for 72 h. Every 24 h, a 1-mL aliquot of the sample was taken and centrifuged at 6,000 rpm for 10 min, filtered through a 0.45-µm nylon filter and analysed by HPLC as described in Section 2.10.

2.7 Detoxification of acid liquor

The acid liquor obtained at optimised conditions was subjected to a detoxification stage to reduce its concentration of inhibitory compounds, using two methods. Ion exchange resins were used to detoxify the acid liquor at two consecutive contacts. First, the resins and the liquor were mixed (0.1 g resin/mL liquor) and placed in an orbital shaker at 50°C and 150 rpm for 30 min. Then, the suspension was vacuum-filtered, and the filtered acid liquor was mixed with new ion exchange resins under the same conditions used for the first contact.

In the second detoxification method, the acid liquor was adjusted to pH 9 with NH_4OH. Once this pH was reached, the acid liquor was taken to an orbital shaker at 150 rpm and 50°C for 30 min. Subsequently, it was filtered under vacuum to separate the material that could have precipitated from the acid liquor.

2.8 Fermentation of acid liquors

Fermentation tests were carried out in 300-mL glass fermenters. Undetoxified acid liquor and detoxified liquors were adjusted to pH 6.7 with KOH and H_2SO_4 as needed. A volume of 150 mL of acid liquor was added to each fermenter, and the fermentation experiments were carried out in duplicate. The *E. coli* cells centrifuged as described in Section 2.3 were resuspended with a small portion of liquor and then added to the total volume inside the glass fermenter. Yeast extract (5 g/L), tryptone (10 g/L) and NaCl (10 g/L) were added to each fermenter. Sodium metabisulfite (5 mM) was added to enhance the xylose metabolism by *E. coli* and to reduce the toxicity of the fermentative medium (Fernandes-Klajn et al., 2018).

The glass fermenters were transferred to a water bath at 37°C provided with an individual agitation system at 350 rpm. The pH was maintained at 6.7 throughout the experiment, adjusted by an automatic controller using KOH 2M. Fermentation lasted for 72 h, and 2-mL medium samples were taken at different time points (5, 24, 48 and 72 h). The samples were centrifuged at 3,500 rpm, and the liquid fraction was filtered (0.45 µm) and analysed by HPLC to measure sugars and ethanol, as described in Section 2.10.

2.9 Lignin characterisation and recovery

The lignin solubilised during organosolv pretreatment at 180ºC was recovered from the organosolv liquor by precipitation. The liquor was diluted in a 4:1 water/liquor (w/w) ratio and subsequently agitated in an orbital shaker at 30°C and 150 rpm for 2 h (Padilla-Rascón et al., 2022). The precipitated lignin was then separated from the liquid by centrifugation at 6,000 rpm and dried at 40°C for 48 h.

After this, the ultimate analysis of the raw material and the precipitated lignin was performed. The carbon, hydrogen, nitrogen and sulphur contents were determined in a Leco TruSpec Micro (St. Joseph, MI, USA). The attenuated total reflectance (ATR)-transform infrared spectroscopy (FTIR) analysis of the lignin sample was carried out in a Bruker Vertex 70 Fourier Transform Infrared Spectrometer (Bruker corporation, Bremen, Germany), equipped with a platinum ATR unit and a DLaTGS/KBr detector. The spectra were obtained from 400 to 4,000 cm^{-1}, at a resolution of 4 cm^{-1}, and 32 scans were accumulated. The analyses were performed in triplicate.

The ^1H and the 2D-HSQC spectra of the organosolv lignin were obtained by nuclear magnetic resonance (NMR) using a Bruker Advance equipment at 500 MHz, referenced to dimethyl sulfoxide (DMSO)-d6 at 2.50 ppm. Data was processed on MestReNova 15.0 and automatic baseline optimization was applied after automatic phasing on all 2D data.

2.10 Analytical methods

The chemical composition of acid-pretreated solids and organosolv-pretreated solids regarding cellulose, hemicellulose and lignin was analysed using the National Renewable Energy Laboratory (NREL) methodology (Sluiter et al., 2008). Acid liquors were analysed by high-performance liquid chromatography (HPLC) for the measurement of monomeric sugars using a Waters Prostar liquid chromatograph with a refractive index detector (Waters 2414), equipped with a CARBOSep CHO-782 Pb column and operating at 70°C with ultrapure water as mobile phase (0.6 mL/min). The samples were previously neutralised with $CaCO_3$ and filtered through a 45-µm nylon filter. The concentrations of inhibitory compounds (acetic acid, furfural, hydroxymethilfurfural (HMF) and formic acid) and sugars (sum of xylose, galactose and mannose (XGM), glucose and arabinose) in the liquors as well as the ethanol concentrations in liquids from SSF and fermentation media were measured by HPLC, using an Agilent

Technologies liquid chromatograph (1260 model) with the refractive index detector mentioned above but with an ICSep ICE-COREGEL 87H3 column operating at 65ºC and with 5 mM H_2SO_4 as mobile phase (0.6 mL/min). All analytical determinations were performed in triplicate, and the average results are shown. The relative standard deviations were below 3%.

3. Results and discussion

3.1 Acid pretreatment of vine shoots

Acid pretreatment has been reported as an efficient treatment to hydrolyse amorphous cellulose and mainly hemicellulose from lignocellulosic biomass. Among the most used acids, sulphuric acid stands out for its low cost and high efficacy (Shukla et al., 2023). In this work, VS were pretreated at a solid loading of 15% (w/v), and the remaining pretreatment variables were selected according to a central rotatable experimental design ($\alpha = \pm 1.41$): temperature (144 ºC–186ºC) and sulphuric acid concentration (0.2%–2.3% w/v) (Table 1).

Biomass recoveries ranged from 45% (runs 9, 11) to 82% (run 1) depending on the harshness of the treatment (Table 1). Acid treatment mainly resulted in the hydrolysis of hemicellulose and this fraction was completely removed from almost all solids except for pretreated solids resulting from runs 1 and 2, which remained with more than 10% hemicellulose because these experiments were carried out under milder conditions (Table 1). The solubilisation of hemicellulose resulted in solids with a cellulose content higher than that of the raw VS (37%) when the pretreatment was carried out under central point conditions, 165ºC and 1.25% H_2SO_4 (runs 6, 7, 8, 10, 13).

Table 2 shows the composition of acid liquors in sugars and inhibitory compounds. The solubilised sugar concentrations in liquors ranged from 17.5 g/L (run 1) up to 35.1 g/L (run 4). These sugars were present mainly in monomeric form and only in the case of runs 1 and 2, with 89% and 41% oligomers, respectively, making a post-hydrolysis step necessary to determine their total sugar contents. Xylose was the main sugar in most liquors, and a maximum xylose concentration, 19.3 g/L, corresponding to an HSR of 78.2%, was determined when the pretreatment was carried out at 150ºC with 2% H_2SO_4 (run 4). Nevertheless, the presence of glucose in these acid liquors was also noticeable, reaching a concentration of 18.4 g/L at the highest temperature, 186ºC (run 9), indicating that 33% of glucose in raw VS was released during acid pretreatment. This glucose could derive partly from the starch content in raw VS (4.06% ± 0.07%), from a more labile cellulose fraction, known as amorphous cellulose, or even from hemicellulose. Dávila et al. (2021) reported the presence of glucose associated to VS hemicelluloses, which was solubilised during the autohydrolysis pretreatment of VS. Castro et al. (2023) reported a glucose recovery of 18% in the prehydrolysate after steam explosion pretreatment of VS impregnated with H_2SO_4 1.63% at 190ºC. Moldes et al. (2007) obtained a liquor containing 18 g/L xylose, 11 g/L glucose and 4 g/L arabinose after prehydrolysis of VS at 130ºC, 3% H_2SO_4 for 15 min, using a liquid/solid ratio of 8/1.

As a consequence of the hydrolysis of hemicellulose, the acetic acid concentration was approximately 7 g/L in most liquors. Additionally, the presence of furfural was noteworthy, especially in runs 9 and 11 (higher than 6 g/L), due to xylose degradation, as indicated by the low xylose concentrations measured in these liquors, with 5.5 and 5.9 g/L, respectively. These experiments yielded the maximum values of total inhibitory compound concentration (TIC), over 16 g/L (Table 2). Using VS pretreated by steam explosion at 170 ºC –190ºC, furfural concentrations below 1 g/L were measured in the prehydrolysate, which indicates a lower xylose degradation, although acetic acid concentrations of 7 g/L were reached (Castro et al., 2023).

3.2 Optimisation of acid pretreatment

In this pretreatment stage, the influence of both temperature and acid concentration on the biomass was studied according to the response surface methodology. The aim of this stage was to hydrolyse hemicellulose and recover as much hemicellulosic sugars as possible in the liquid fraction. For this purpose, high solubilisation is required, although it is important to simultaneously maintain a low formation of inhibitory compounds, which can be translated into a low sugar degradation. Thus, the diluted acid pretreatment was focused on maximising the hemicellulosic sugar recovery (HSR) and minimising the formation of inhibitory compounds (TIC). Second-order polynomial equations were employed to express these responses as functions of the coded independent variables. The models were highly predictive for the responses, and both factors, temperature and acid concentration, were significant in all cases (Table 3). Temperature was the most significant factor impacting HSR, with a clear negative influence, whilst the acid concentration showed a positive influence on this response, according to the equation corresponding to HSR. Fig. 1a shows the response surface and contour plots obtained from the HSR model. The highest HSR level was achieved at the lowest temperature and the highest acid concentration. According to the models for acetic acid and furfural concentrations (Table 3), both temperature and acid concentration exerted a positive effect on the formation of these inhibitory compounds. Nevertheless, in the case of acetic acid, the effect of the sulphuric acid concentration in the pretreatment was more significant than that of temperature, and there was a synergistic effect between both factors. The model corresponding to the total inhibitory compound concentration indicated a similar positive influence of both factors (Table 3).

The influence of both factors on acetic acid formation is shown in Fig. 1b. When the sulphuric acid concentration used in the pretreatment increased, the acetic acid concentration also increased, regardless of the temperature. However, the temperature increase meant a slight increase in the acetic acid concentration only at low levels of sulphuric acid concentration (Fig. 1b). Regarding the furfural concentration in the liquors, the highest level, above 7 g/L, was determined when the pretreatment was performed at the highest levels of both factors (Fig. 1c).

This can be attributed to the degradation of xylose under harsh conditions. Likewise, the highest levels of both factors yielded maximum values of TIC, close to 18 g/L (Fig. 1d).

Acid pretreatment of VS was optimised by maximising the hemicellulosic sugar recovery in the liquor (HSR) and minimising the formation of total inhibitory compounds (TIC). According to the central composite design, the optimal conditions were 150°C and 1.2% H_2SO_4, resulting in an HSR of 75% and 7.6 g/L of TIC. These conditions were validated in the laboratory to obtain the actual values. The experimental values for the responses HSR and TIC were 76 ± 1.70% and 7.91 ± 0.40 g/L, respectively. Both values are slightly higher than those predicted by the model, although within the limits of variability at the confidence level of 95%. Castro et al. (2023) reported a 68% hemicellulosic sugar recovery in the prehydrolysate from VS pretreated by steam explosion at 190°C and 1.6% H_2SO_4. Other authors removed hemicellulose from the same feedstock by autohydrolysis, but the sugars were recovered in the oligomeric form. Thus, using the same feedstock, a hemicellulose removal of 88% was reached by autohydrolysis at 200ºC for 20 min (Dávila et al., 2021) and 33% at 180ºC for 10 min (Senila et al., 2020).

3.3 Co-fermentation of the acid liquor

Advanced bioethanol production based on lignocellulose feedstock is required to meet Sustainable Development Goals (Shukla et al., 2023). Nevertheless, the integral use of cellulosic and hemicellulosic sugars in these raw materials is required to obtain a global process viability (Duque-Acevedo et al., 2021; Fernandes-Klajn et al., 2018). It is worth noting that hemicellulosic sugars in VS represent 36% of the total sugar content, making their valorisation crucial to the viability of a biorefinery product based on this raw material. Nevertheless, the co-fermentation of pentoses and hexoses is one of the main barriers to ethanol production by the biochemical conversion of lignocellulose biomass to renewable fuels (Young et al., 2010). In this work, the acid liquor obtained via an optimised VS acid pretreatment was co-fermented by *E. coli* to convert its C5 and C6 sugars into ethanol. The composition of this liquor was as follows (in g/L): glucose 11.89 ± 0.59, xylose 20.14 ± 0.63, galactose 3.83 ± 0.06, mannose 0.10 ± 0.02, arabinose 2.58 ± 0.13, acetic acid 6.27 ± 0.25, formic acid 0.63 ± 0.02, furfural 0.71 ± 0.04, HMF 0.30 ± 0.01 and phenolic compounds 1.08 ± 0.02 (Table 2). This raw liquor was subjected to fermentation, but *E. coli* could not grow, and a detoxification step was therefore necessary to remove inhibitory compounds from the medium. Two different methods were applied to reduce the concentrations of these compounds: (i) exchange ionic resins and (ii) NH_4OH treatment.

Fig. 2 shows the removal percentage of inhibitor compounds attained with the two detoxification methods assayed. Resin treatment was more effective in the removal of acetic acid and phenols, reducing their concentrations by 21% and 65%, respectively, after two 30 min of contact of the liquor with the resins. However, ammonia detoxification achieved a higher removal of formic acid, 56% *vs* 13%. Likewise, NH_4OH treatment removed more furans than the resins (70% *vs* 54% for furfural and 80% *vs* 10% for HMF). In addition to inhibitory compounds, detoxification methods affected the sugar concentrations. However, detoxification using resins resulted in a sugar loss lower than 1%, while the use of NH_4OH meant a sugar loss of 10%.

Raw and detoxified liquors from both treatments were subjected to fermentation by *E. coli* to evaluate their fermentability and their capacity to produce ethanol. When the raw liquor was used as fermentation medium, *E. coli* was not able to assimilate glucose after 72 h, indicating an inhibitory effect exerted by the toxic compounds present in this liquor. This behaviour could be attributed to the presence of phenols (1.08 ± 0.05 g/L) in this medium. Nevertheless, it is difficult to stablish a clear relation between a given inhibitor concentration and its influence on microbial metabolism due to possible synergistic effects (Mussatto and Roberto, 2004). However, detoxification treatment with resins or NH_4OH yielded liquors containing approximately 5 g/L of acetic acid and 0.25 g/L of furfural, which could be fermented by *E.*

coli. In both cases, a preferential glucose consumption by *E. coli* could be observed, although the initial glucose concentration in both media was approximately half that of XGM. After 24 h, *E. coli* had assimilated all sugars in the liquor detoxified by resins (Fig. 3a) and by NH_4OH (Fig. 3b). According to Palmqvist and Hahn-Hägerdal (2000), the simultaneous presence of a high acetic acid concentration and a low furfural concentration in the medium can exert a positive effect on the fermentation. Maximum ethanol concentrations of 19 and 17 g/L were reached after treatment with resins and NH_4OH, respectively, corresponding to ethanol yields higher than 98% (referred to the theoretical ethanol yield) and a productivity of 0.70 g/L/h in both cases. These results compare favourably with those reported for a hemicellulosic liquor obtained from the autohydrolysis of VS after post-hydrolysis and detoxification with resins (9 g/L of ethanol after 72 h fermentation with *Scheffersomyces stipitis* and 70% ethanol conversion) (Dávila et al., 2019).

3.4 Organosolv pretreatment and cellulose conversion

Organosolvent treatment is an eco-friendly chemical process to remove lignin from lignocellulose biomass (Robak and Balcerek, 2020). After the solubilisation and recovery of hemicellulosic sugars, VS fractionation includes an ethanol-organosolv pretreatment to remove lignin and enhance the enzymatic digestibility of the biomass. The ability of organic solvents, such as ethanol, to solubilise lignin and enhance cellulose hydrolysis has been widely reported (Xie et al., 2023). According to Gómez-Cruz et al. (2021), organosolv treatment is considered the most environmentally friendly and economical process, since the solvent can be recycled. In addition, this process generates high-purity lignin with good antioxidant properties.

In this work, the solid resulting from the acid pretreatment of VS at optimal conditions (acid-pretreated VS) with a cellulose content higher than 45% (Table 1), was subjected to an organosolv pretreatment at 150ºC and 180ºC using ethanol 50% (v/v) and sulphuric acid 1% (w/v) as catalysts. During the organosolv pretreatment, the biomass was partly solubilised, and solid recoveries of 75.7% and 53.8% were determined at 150ºC and 180ºC, respectively. The biomass solubilised in this step corresponds mainly to the lignin fraction, whilst high glucose recoveries were determined in the pretreated solids, with 74% and 83% at 150ºC and 180ºC, respectively (Fig. 4). Based on these results, temperature plays an essential role in biomass delignification. When the organosolv pretreatment was performed at 150ºC, 25.2% of lignin was removed and when the temperature was increased up to 180ºC, a delignification rate of 43.4% was obtained (Fig. 4). Senila et al. (2020) removed 90% of lignin by the treatment of VS with acid chlorite at 80ºC for 2 h. Bu et al. (2021) reported 59% of lignin removal from sugarcane bagasse pretreated by peroxyformic acid for 10 min.

This process step yielded a liquid stream containing solubilised lignin and a cellulose-enriched solid, which was enzymatically hydrolysed to evaluate the cellulose conversion increase obtained via the organosolv treatment. For comparative purposes, acid-pretreated VS was also enzymatically saccharified, and an enzymatic digestibility of only 19.7% was reached after 72 h. The raw material, i.e., untreated VS, was submitted to enzymatic hydrolysis with no glucose production. However, taking into account that lignin reduces the accessibility and susceptibility of cellulose to microbial enzymatic degradation (Banerjee et al., 2023), the enzymatic digestibility of VS was noticeably enhanced after organosolv pretreatment since this step meant the partial removal of lignin, especially when the pretreament was carried out at 180ºC. At this pretreatment temperature, an enzymatic digestibility of 83% was determined after 72 h, whereas the enzymatic digestibility reached after pretreatment at 150ºC was only 47.3%. These results show the positive influence of the organosolv pretreatment on the cellulose conversion of VS since the lignin binds to cellulose chains and hinders the enzymatic hydrolysis by acting as a physical barrier that limits the productive enzyme-cellulose bonds (Shukla et al., 2023). These results are comparable with those reported using the same feedstock, either after autohydrolysis and delignification with NaOH 12% at 124ºC and 105 min (54–79 g glucose/100 g substrate)

(Dávila et al., 2017) or after applying a sequential acid/alkaline pretreatment at 130ºC using 2% H_2SO_4 and 12% NaOH, respectively (Bustos et al., 2005).

3.5. *Ethanol production from 180ºC-organosolv-pretreated solid*

The solid resulting from organosolv pretreatment at 180ºC, containing 46% cellulose (Table 1), was selected to produce ethanol by simultaneous saccharification and fermentation (SSF) at 10% and 20% (w/v) solid loading. This organosolv-pretreated solid (180ºC-organosolv-pretreated solid) was subjected to an SSF process using Cellic® CTec2 and *S. cerevisiae* at 40ºC to assess its ethanol production potential. Fig. 5 shows the evolution of glucose consumption and ethanol production during SSF. The level of glucose in the medium remained below 0.7 g/L in both experiments. Maximum ethanol concentrations of 17.6 and 34 g/L were obtained after 72 h SSF at 10% and 20% substrate loading, respectively. These concentrations correspond to ethanol yields of 78.3% and 75.7% (referring to the theoretical ethanol yield), respectively, which means that the increase in substrate loading did not result in a significant drop in yield. High ethanol concentrations are desirable in order not to increase the cost of the distillation step and improve the feasibility of the overall process (Yan et al., 2020). Chen et al. (2022) reported an ethanol concentration of 85 g/L from sugarcane bagasse by simultaneous saccharification co-fermentation with SHY07-1 yeast after ultrasonic/dilute acid sequential pretreatment. Saini et al. (2023) reported an ethanol production yield of 73–85% using also *S. cerevisiae* to ferment enzymatic hydrolysates from rice straw pretreated by laccase-assisted sodium chlorite in a sequential saccharification and fermentation process.

As expected, the increase in substrate loading resulted in a more concentrated ethanolic solution. By doubling the solid loading, the ethanol concentration almost doubled after 72 h of SSF (34 *vs* 17.6 g/L). The use of high solid loadings in these processes is interesting to obtain concentrated ethanolic solutions. Nevertheless, it has been widely reported that the increase in substrate loading results in a noticeable decrease in SSF yield (Modenbach and Nokes, 2013;

Wang et al., 2011). Baptista et al. (2023) pretreated VS by autohydrolysis and obtained 50 g/L ethanol by SSF when the medium was supplemented with grape must, although the corresponding ethanol yield was lower than 65% due to glucose accumulation in the medium after 24 h. Yan et al. (2020) achieved an ethanol concentration of 100 g/L from grass waste pretreated by NaOH supplemented with H_2O_2 under mild conditions using a solid loading of 30% in a simultaneous saccharification co-fermentation with *Saccharomyces ce*revisiae and *Pichia stipitis.*

3.6 Chemical characteristics of lignin

During the organosolv pretreatment, lignin was partly solubilised and recovered from the organosolv liquor by precipitation. The lignin recovered from the organosolv liquor obtained at 180ºC consisted of 58.7% carbon, 5.7% hydrogen, 0.6% nitrogen, and 0.05% sulphur. The carbon content was enhanced in the lignin fraction compared to the raw biomass, which presented 45.1% carbon, 5.7% hydrogen, 0.6% nitrogen, and 0.05% sulphur. This carbon enrichment was also observed for other organosolv lignins when compared with the raw biomasses, e.g., in exhausted olive pomace (Gómez-Cruz et al., 2021), and pine wood (Wang et al., 2015). It is expected that the recovered lignin has a higher calorific value than the raw biomass, according to the higher carbon content (Bychkov et al., 2017). The purity of the organosolv lignin was determined by acid hydrolysis to release potential carbohydrate impurities bound to the lignin according to NREL methodology (Section 2.10). The high purity determined for this lignin, 91%, is clearly advantageous because the value-added products that can be obtained from the lignin depends of the quality of the lignin (Pan and Saddler, 2013).

In the present study, ATR-FTIR spectroscopy was applied to determine the molecular features of the organosolv lignin. In the absorption spectrum (Fig. 6), a wide band was observed at approximately 3,376 cm^{-1}, which is commonly attributed to the O–H stretching vibrations, which can be associated with the presence of aromatic and aliphatic hydroxyl groups, along with those in cellulose (Amine et al., 2021). The peak at approximately 2,934 cm^1 indicates the C–H stretching vibration of methyl or methylene groups (Amine et al., 2021; García et al., 2010). The CH vibration of the methyl group of the aromatic methoxyl groups could be associated with the wavelength of 2,845 cm^{-1} (García et al., 2010). The region between 1,600 and 1420 cm^{-1} is highly characteristic of lignin and related to the aromatic skeletal vibration of this polymer, with absorption at around 1,602, 1,512 and 1,425 cm^{-1} (Amine et al., 2021; García et al., 2010). The presence of syringyl (S) units is associated with the wavelengths of 1,318 and at 1,112 cm^{-1}, related to the skeletal vibrations of the ring breathing with C–O stretching (Amine et al., 2021; García et al., 2010) and the aromatic C–H deformation of S units, respectively (Gómez-Cruz et al., 2021). The guaiacyl (G) unit is evidenced at 1,266 cm^{-1} (G ring breathing with C–O stretching), which is characteristic of this phenol moiety (Lancefield et al., 2019). The band at approximately 1,030 cm^{-1} has been associated with C–H in-plane deformation of the G ring plus C–O deformation, symmetric stretching vibration of C–O–C of ethers in lignin and OH stretching of primary alcohols (García et al., 2010; Pereira et al., 2023; Yang et al., 2016). The peak at 834 cm^{-1} could be related to C–H out-of-plane deformation vibrations of the lignin units but is also related to the presence of p-hydroxyphenyl (H) units (García et al., 2010; Pereira et al., 2023). The band at 579 cm^{-1} could be attributed to the C=O bend of aldehyde moieties of aromatic structures having C=C–C=O, such as in coniferyl aldehyde (Bock and Gierlinger, 2019). Overall, these absorption bands agreed well with those described in lignins obtained from extracted VS delignified with dioxane/water (9:1, v/v) containing 0.2 M HCl (Pereira et al., 2023). In general, the ATR-FTIR absorption spectrum of the recovered lignin resembled the spectra of SG and SGH-type lignins, according to the study by Lancefield et al. (2019). This agreed with the results obtained by nuclear magnetic resonance.

The organosolv lignin was also evaluated by NMR, and Fig. 7a shows the 1H spectrum of the recovered lignin. It exhibited striking similarities to the spectrum of commercial alkaline lignin previously evaluated (Lara-Serrano et al., 2019). Fig. 7a reveals peaks corresponding to aliphatic protons. In the range of 2.0 to 1.0 ppm, signals attributed to methyl or methylene groups can be observed, whereas the region between 1.0 and 0.8 ppm shows peaks associated with saturated aliphatic chains of lignin. Notably, the presence of water contamination is evident due to the incomplete drying of lignin and the impurities present in the DMSO-d6 solvent. Within the range of 4.0 to 3.4 ppm, the protons of methoxy groups are observed in proximity to water protons. Additionally, there are weak peaks indicating the presence of aliphatic protons between 4.2 and 5.6 ppm. The signals between 6.0 and 4.0 ppm correspond to the protons of α, β and γ carbons in the β-O-4, β-5 and β-β linkages, respectively. In the range of 8.0 to 6.0 ppm, the spectrum exhibits characteristic signals of the aromatic region and the vinyl protons of S and G. Peaks between 8.5 and 8.0 ppm can be attributed to phenolic groups within the lignin molecule, whereas signals above 9.0 ppm indicate the shielding effect between G groups (Korbag and Saleh, 2016; Toledano et al., 2010; Lara-Serrano et al., 2019).

Lignin is a macromolecule composed of common monomer units with a phenolic base structure, aromatic rings and a propyl group attached to it. It can exist in linear form or with branching. Therefore, one expectation is that if it consisted only of S units, the polymers would be mainly linear, whereas in the case of predominantly G units, it would be mostly linear with some branching. If it consisted of only H units, it would be linear with significant branching. Hence, a higher proportion of S units in lignin indicates a more linear structure, whereas a higher proportion of G monomers indicates greater branching. If the S/G ratio is below 1, the sample is branched and has a high molecular weight. If the S/G ratio is greater than 1, the lignin sample is linear and has a medium molecular weight. In this work, the percentages of S, G and H units determined for VS organosolv lignin were 47.3%, 51.6% and 1.1%, respectively. Therefore, this lignin with an S/G ratio of approximately 0.92 is mainly non-linear, which can be explained by the selective degradation of S units, which occurs when the delignification process is carried out in an acid medium (Ovejero-Pérez et al., 2022). Nevertheless, G-rich substrates promote the removal of derived-lignin compounds that act as inhibitors in fermentation processes (Li et al, 2014), and substrates rich in G and S units favour sugar solubilisation (Wang et al., 2019). The 2D-HSQC spectra of the sample showed the percentages of the different linkages (Fig. 7b): 1.9% β-O-4, 6.2% β-β and 2.1% β-5. A low value of β-O-4 bonds was also observed in organosolv lignin obtained from poplar wood, which was associated with its hydrolysis when the pretreatment was acid-catalysed (Meng et al., 2020). When poplar wood was organosolv pretreated without acid catalyst, the percentage of these linkages was approximately 37% aromatic units (Ovejero-Pérez et al., 2022). The presence of β-O-4 ether linkages is interesting to promote the depolymerisation of the lignin to aromatic monomers (Meng et al., 2020). Alternatively, lignins with higher C-C interunit bonds (e.g., β-β and β-5) are more thermally stable and can be potentially applied as reinforcement materials and fire retardants (Ovejero-Pérez et al., 2022).

3.7 Overall process mass balance

Fig. 8 shows the overall mass balance of the process scheme proposed in this work for VS fractionation and valorisation. The flow diagram includes an acid treatment to hydrolyse hemicelluloses followed by an organosolv pretreatmet to solubilise lignin and to improve the enzymatic digestibility of biomass. This fractionation scheme generated (i) a liquid stream containing mainly hemicellulosic sugars that were bioconverted into ethanol after a detoxification step with resins, (ii) a cellulose-enriched solid, which was used as a substrate to produce ethanol and, (iii) a liquor containing high-purity organosolv lignin.

As shown, acid pretreatment at optimal conditions recovered 81% of total sugars in raw material, 33% of them in the liquid fraction. Moreover, this stream also contains a relevant amount of glucose, 21% of glucose in raw VS, which was solubilised during acid treatment.

Therefore, the valorisation of glucose in this stream proposed in this scheme is important for an integral use of VS.

Subsequently, organosolv pretreatment at 180ºC solubilised 46% of biomass, mainly lignin. Considering the lignin content measured in the resulting solid, 43% of lignin was solubilised and, although only 20% of this organosolv lignin could be recovered from the liquor, its purity was as high as 91%. In addition to lignin, organosolv pretreatment yielded a cellulose-enriched solid with high enzymatic digestibility (83 g glucose/100 g acid-pretreated solid), which was used as substrate to produce ethanol by SSF. Thus, the bioconversion scheme proposed resulted in an overall ethanol yield of 15 g ethanol/100 g raw VS. This result is comparable with those reported for other lignocellulosic residues such as olive tree biomass (Martínez-Patiño et al., 2018), rapeseed straw (López-Linares et al. 2016) or brewery spent grain (Rojas-Chamorro et al., 2018).

Conclusions

Vine shoots can be used as a sustainable raw material to produce sugars, ethanol and lignin. The process strategy developed in this work can effectively fractionate this biomass by a sequential acid-organosolv pretreatment and recover more than 80% of sugars in raw VS to be converted into ethanol. The liquor containing hemicellulosic sugars is toxic for *E. coli*, resulting in metabolism inhibition. Therefore, a previous detoxification step is required using resins or NH_4OH, which probed highly efficient. The second fractionation step, organosolv pretreatment, is favoured by the temperature, and when carried out at 180ºC, results in a high-purity organosolv lignin and a glucose-rich substrate to produce ethanol by SSF. Future works will be focused on improving the recovery of this high-purity organosolv lignin to find valorisation alternatives as well as recovering and characterising the lignin contained in the solid issued from SSF.

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Figure captions

Fig. 1 Response surface curves representing the interactive effects of pretreatment temperature and concentration of sulphuric acid on hemicellulosic sugar recovery (a), concentration of acetic acid (b), furfural (c), and total inhibitory compounds (d) in the acid liquors.

Fig. 2 Removal of inhibitory compounds from hemicellulosic liquor attained by detoxification with resins and NH_4OH.

Fig. 3 Evolution of sugar consumption and ethanol production during fermentation by *E. coli* of the acid liquor detoxified with (a) resins and (b) NH_4OH. XGM: Sum of xylose, galactose and mannose.

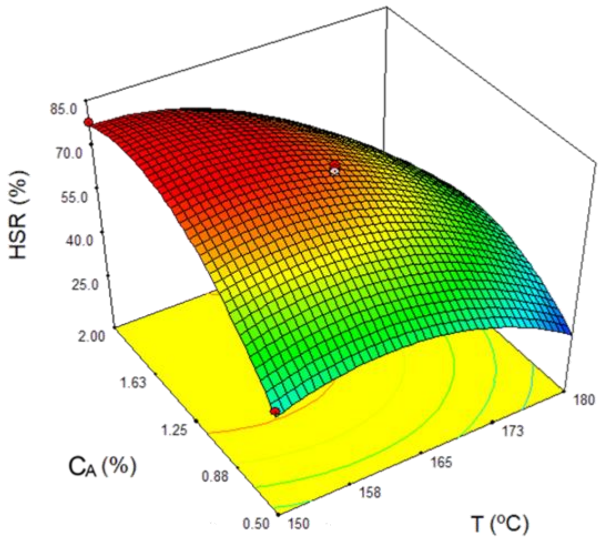
Fig. 4 Organosolv pretreatment of vine shoots at 150ºC and 180ºC with ethanol 50% (v/v) and sulphuric acid 1% (w/v) as catalysts using a solid loading of 15%.

Fig. 5 Glucose and ethanol concentrations during the simultaneous saccharification and fermentation (SSF) of 180ºC-organosolv-pretreated solid at 10% and 20% solid loadings.

Fig. 6 Absorption spectrum of organosolv lignin from vine shoots by attenuated total reflectance-transform infrared spectroscopy

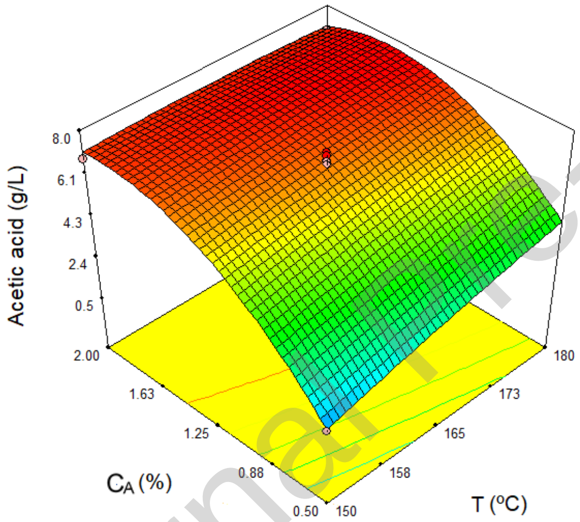
Fig.7 (a) 1H NMR spectrum of organosolv lignin and (b) 2D-NMR HSQC spectra of the aromatic region and aliphatic side-chain region of organosolv lignin

Fig. 8 Mass balance of the fractionation and valorisation process proposed for vine shoots.

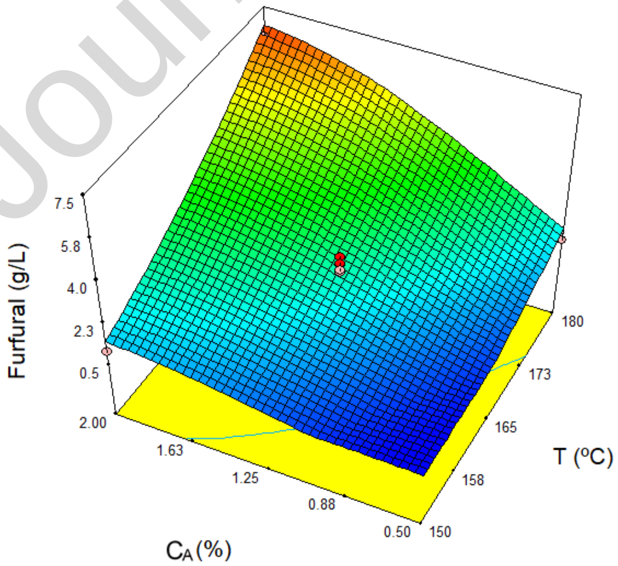

85.0
70.0
55.0
40.0
25.0
HSR (%)
2.00
1.63
1.25
0.88
0.50
CA (%)
150
158
165
173
180
T (ºC)

(a)

(


8.0
6.1
4.3
2.4
0.5
Acetic acid (g/L)
2.00
1.63
1.25
0.88
0.50
CA (%)
150
158
165
173
180
T (ºC)

b)


7.5
5.8
4.0
2.3
0.5
Furfural (g/L)
2.00
1.63
1.25
0.88
0.50
CA (%)
150
158
165
173
180
T (ºC)

(c)

(d)

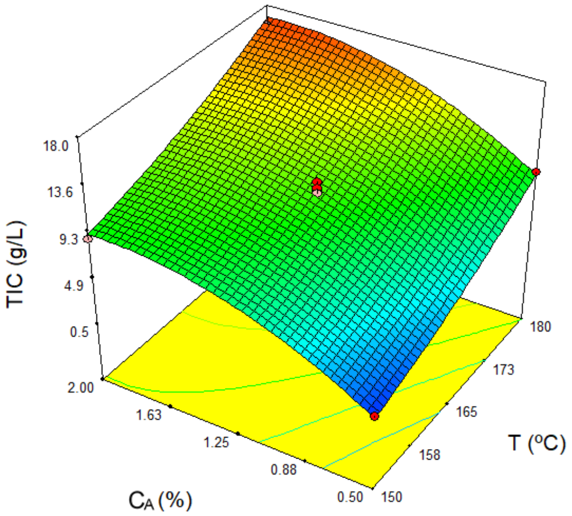

TIC (g/L)
18.0
13.6
9.3
4.9
0.5
2.00
1.63
1.25
0.88
0.50
C_A (%)
150
158
165
173
180
T (ºC)

Fig. 1

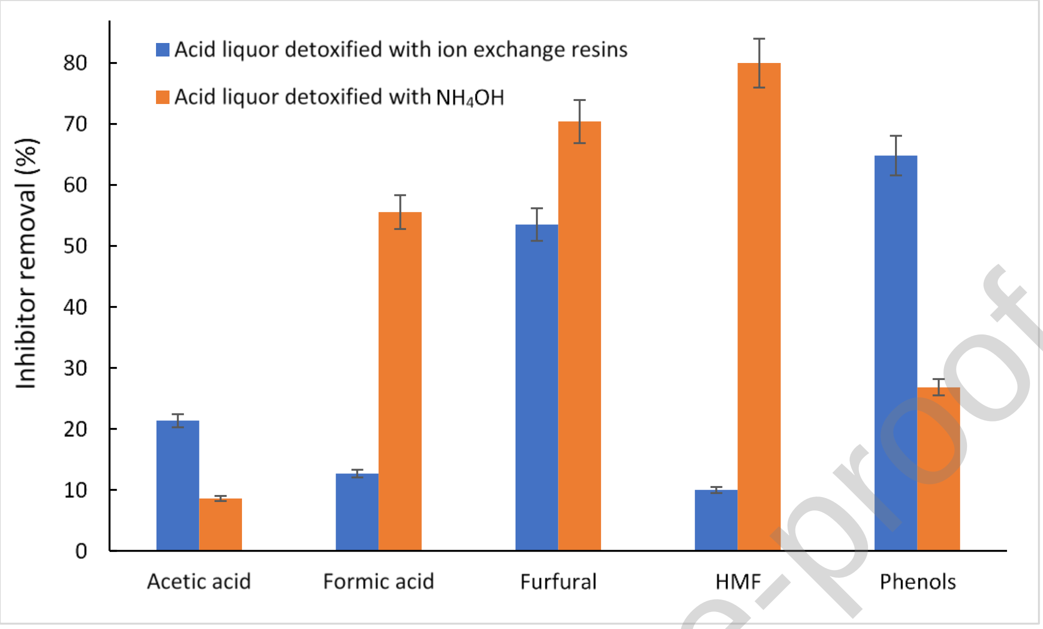
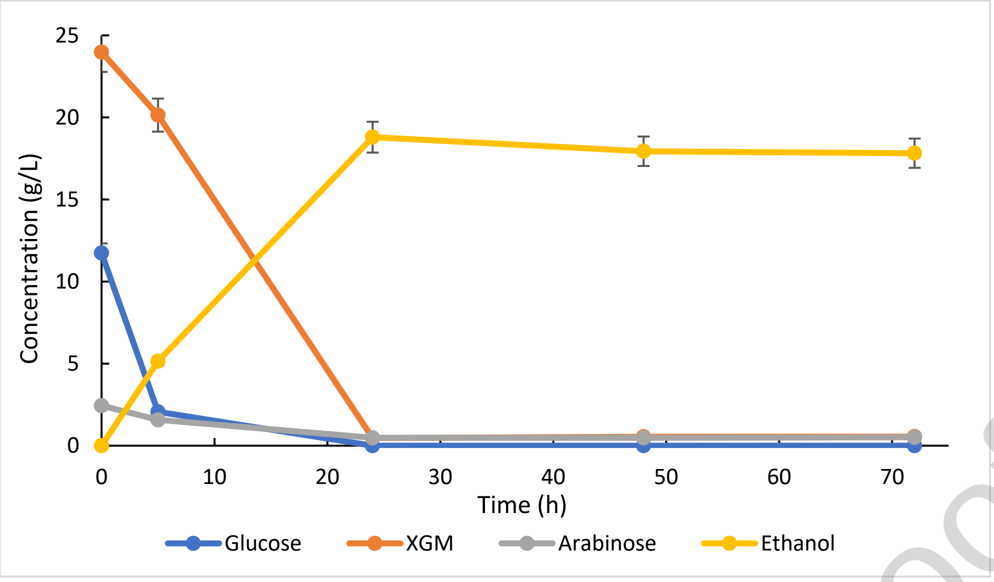
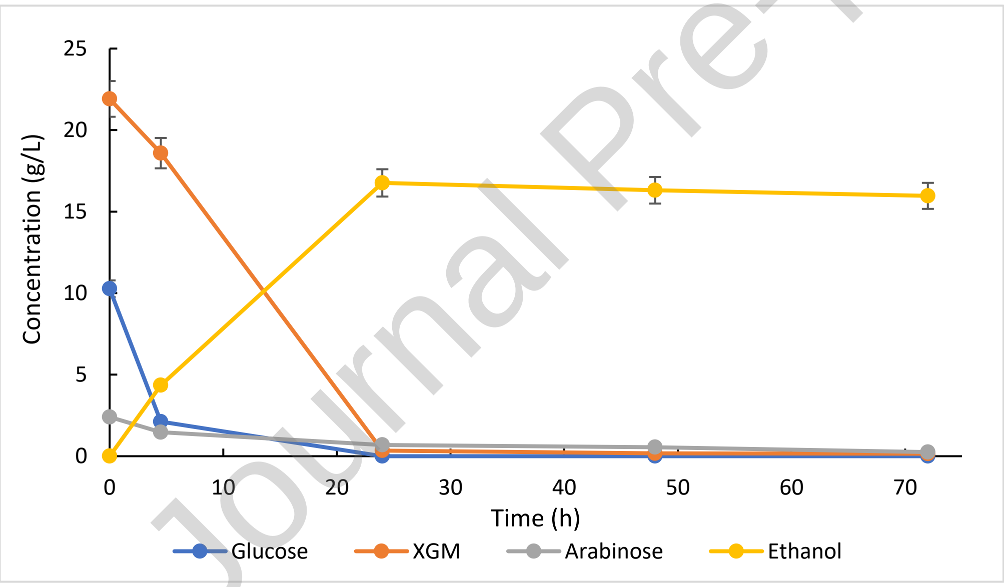

Inhibitor removal (%)
0
10
20
30
40
50
60
70
80
Acid liquor detoxified with ion exchange resins
Acid liquor detoxified with NH₄OH
Acetic acid
Formic acid
Furfural
HMF
Phenols

Fig. 2


Concentration (g/L)
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Time (h)
Glucose
XGM
Arabinose
Ethanol

(a)


Concentration (g/L)
25
20
15
10
5
0
0
10
20
30
40
50
60
70
Time (h)
Glucose
XGM
Arabinose
Ethanol

(b)

Fig. 3

Fig. 5

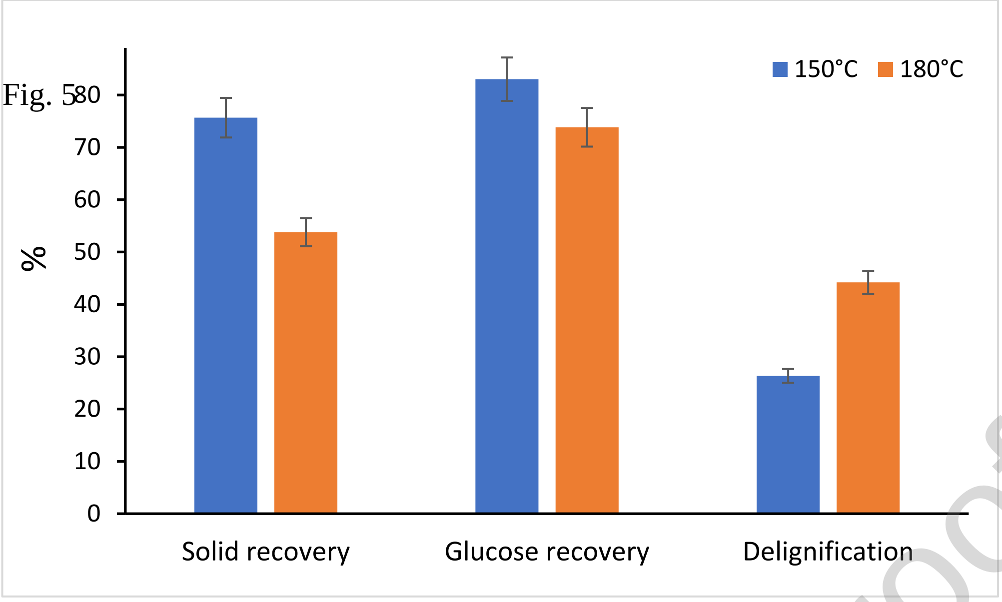

■ 150°C
■ 180°C
%
0
10
20
30
40
50
60
70
80
Solid recovery
Glucose recovery
Delignification

Fig. 4

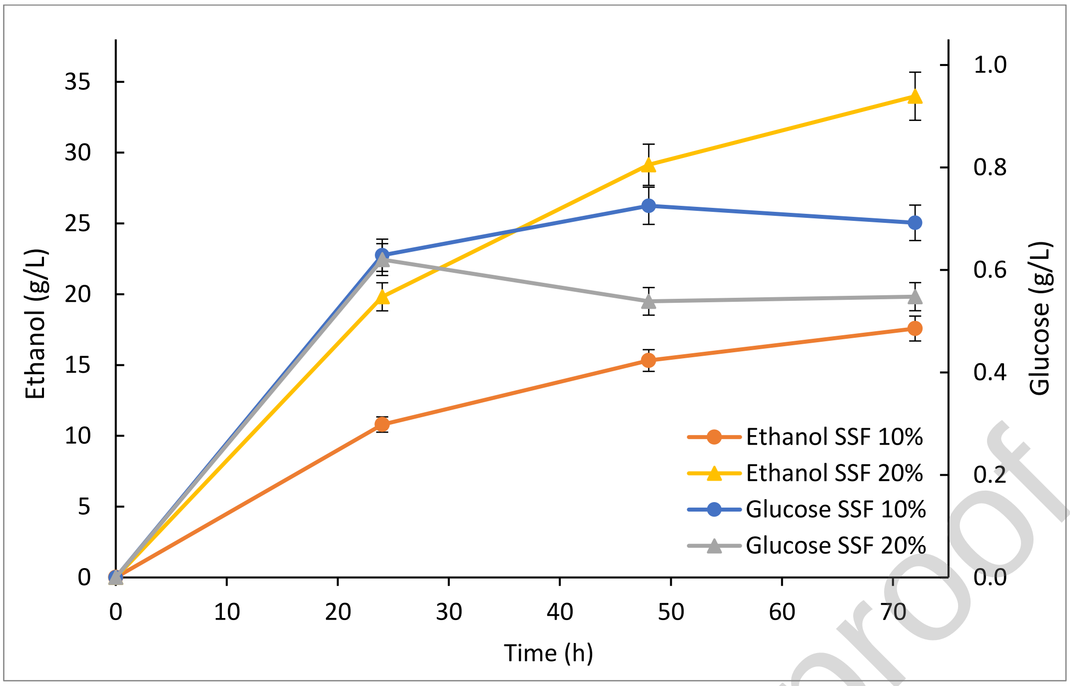

Ethanol (g/L)
Glucose (g/L)
Time (h)
0
5
10
15
20
25
30
35
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
70
Ethanol SSF 10%
Ethanol SSF 20%
Glucose SSF 10%
Glucose SSF 20%

Fig. 5

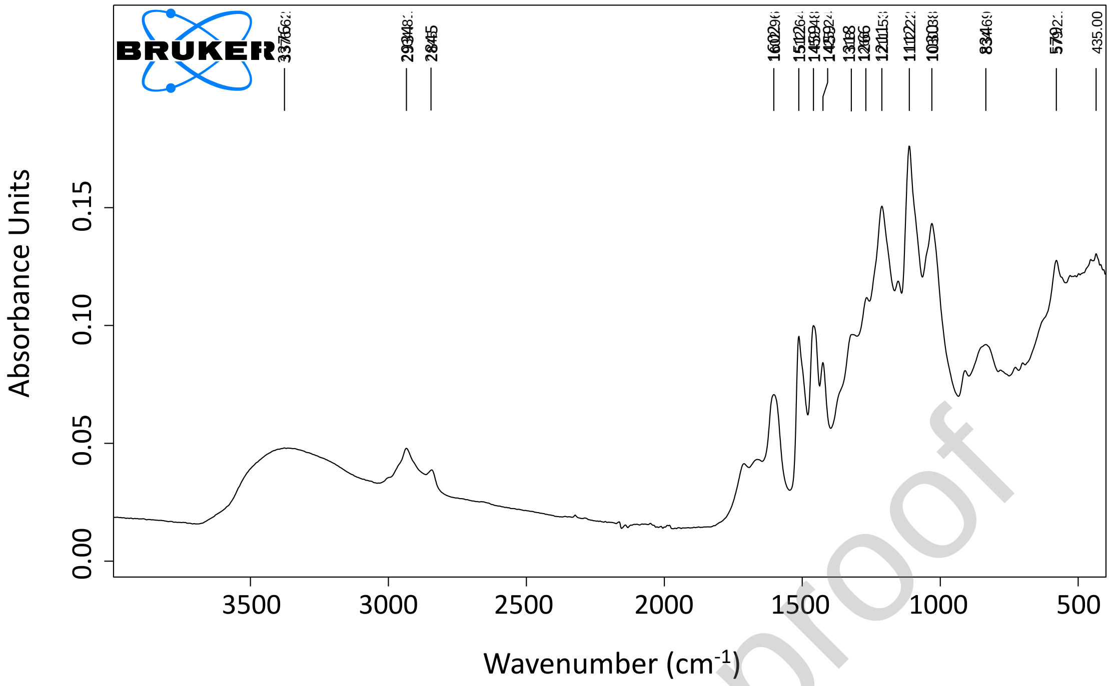
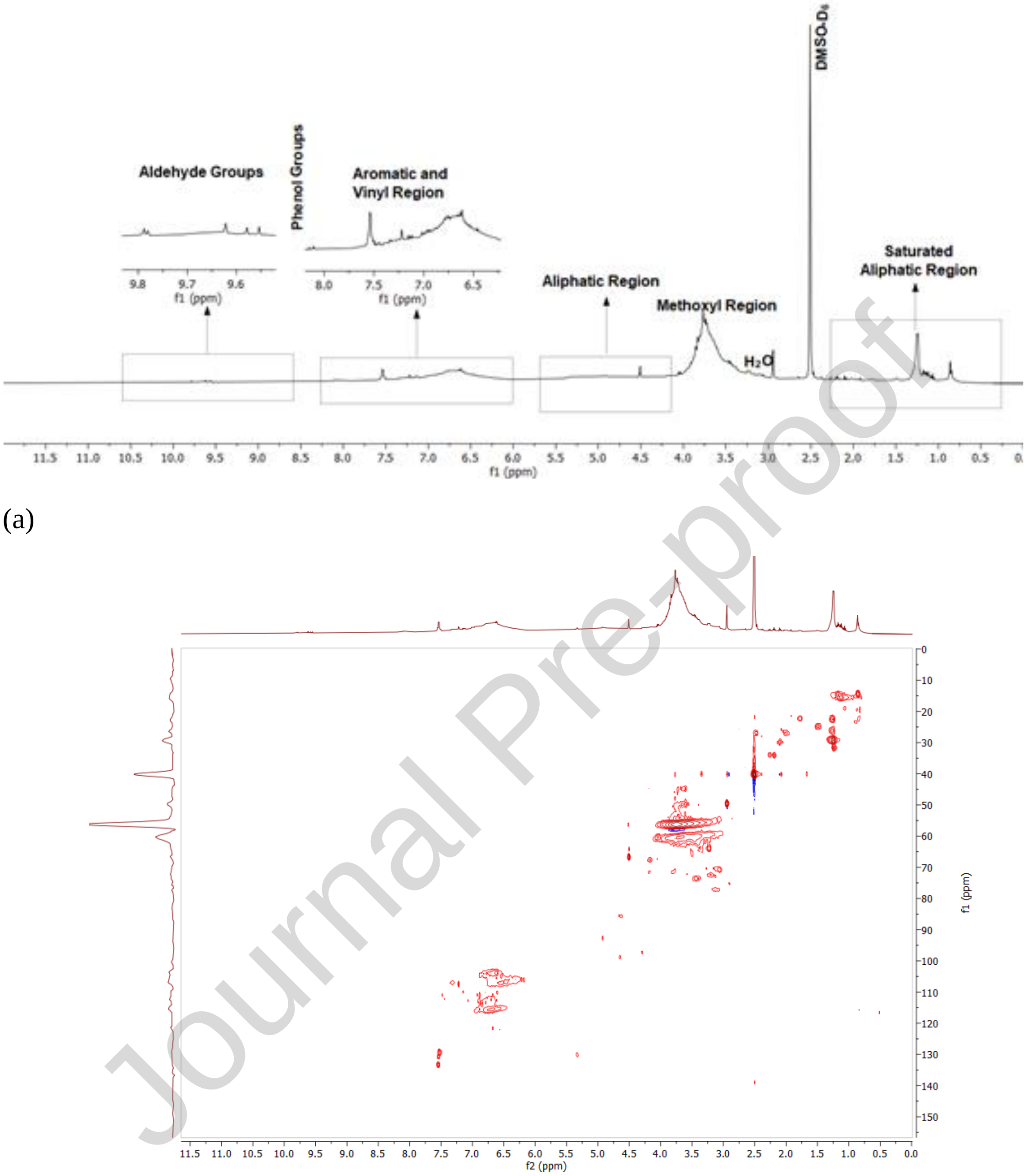

3376
2934
2845
1602
1512
1459
1432
1318
1266
1211
1112
1030
834
579
435.00
Absorbance Units
0.00
0.05
0.10
0.15
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm^{-1})

Fig. 6


Aldehyde Groups
9.8
9.7
9.6
f1 (ppm)
Phenol Groups
Aromatic and
Vinyl Region
8.0
7.5
7.0
6.5
f1 (ppm)
Aliphatic Region
Methoxyl Region
H₂O
DMSO-D₆
Saturated
Aliphatic Region
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
f1 (ppm)
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f2 (ppm)

(a)

(b)

Fig. 7

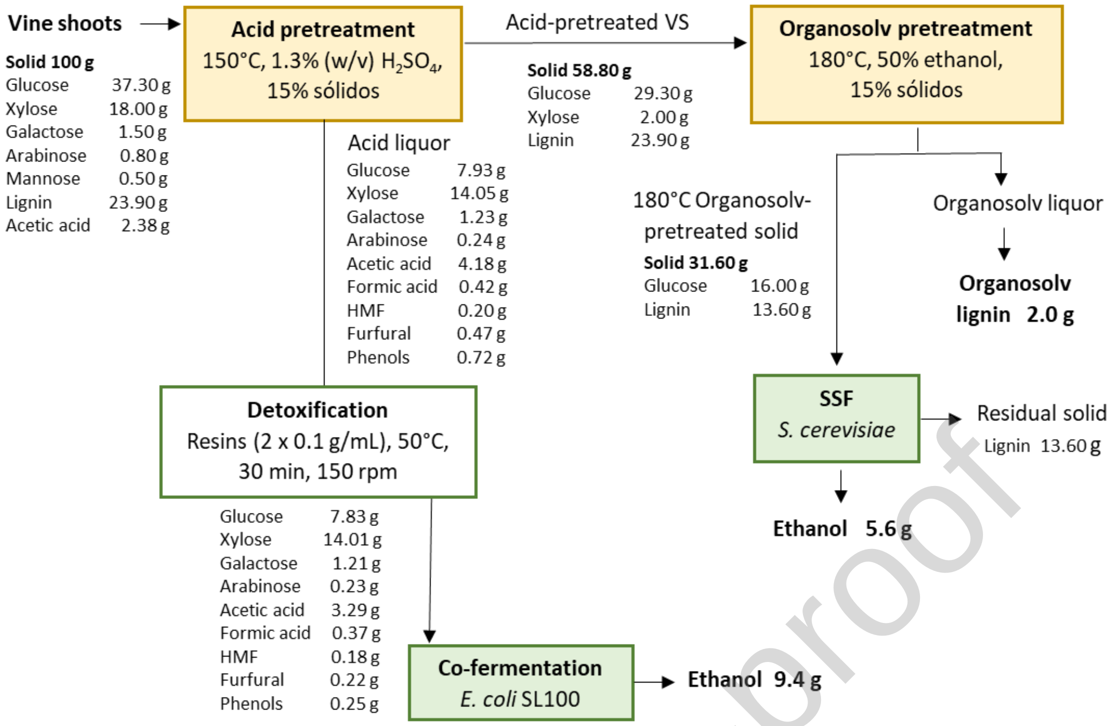

Vine shoots
Solid 100 g
Glucose 37.30 g
Xylose 18.00 g
Galactose 1.50 g
Arabinose 0.80 g
Mannose 0.50 g
Lignin 23.90 g
Acetic acid 2.38 g
Acid pretreatment
150°C, 1.3% (w/v) H_2SO_4,
15% sólidos
Acid-pretreated VS
Solid 58.80 g
Glucose 29.30 g
Xylose 2.00 g
Lignin 23.90 g
Organosolv pretreatment
180°C, 50% ethanol,
15% sólidos
Acid liquor
Glucose 7.93 g
Xylose 14.05 g
Galactose 1.23 g
Arabinose 0.24 g
Acetic acid 4.18 g
Formic acid 0.42 g
HMF 0.20 g
Furfural 0.47 g
Phenols 0.72 g
180°C Organosolv-pretreated solid
Solid 31.60 g
Glucose 16.00 g
Lignin 13.60 g
Organosolv liquor
Organosolv lignin 2.0 g
Detoxification
Resins (2 x 0.1 g/mL), 50°C,
30 min, 150 rpm
SSF
S. cerevisiae
Residual solid
Lignin 13.60 g
Ethanol 5.6 g
Glucose 7.83 g
Xylose 14.01 g
Galactose 1.21 g
Arabinose 0.23 g
Acetic acid 3.29 g
Formic acid 0.37 g
HMF 0.18 g
Furfural 0.22 g
Phenols 0.25 g
Co-fermentation
E. coli SL100
Ethanol 9.4 g

Fig. 8

Table 1. Solid recovery and composition of VS after acid and organosolv pretreatment (%)

Run	**T (ºC)**	**C_A (%)**	**Solid recovery**	**Cellulose**	**Hemicellulose**	**Lignin**
			Acid pretreatment			
1	165	0.19	82.1	33.8	17.9	26.7
2	150	0.50	75.1	33.2	11.5	26.6
3	180	0.50	57.2	34.5	0.0	19.9
4	150	2.00	58.0	34.3	0.0	24.1
5	144	1.25	63.2	36.5	0.0	24.7
6	165	1.25	55.8	46.5	0.0	44.1
7	165	1.25	56.3	46.8	0.0	42.9
8	165	1.25	55.1	47.7	0.0	45.7
9	186	1.25	45.3	18.6	0.0	26.3
10	165	1.25	55.1	48.4	0.0	44.0
11	180	2.00	45.3	17.5	0.0	27.7
12	165	2.31	51.7	24.9	0.0	26.4
13	165	1.25	54.6	45.6	0.0	44.4
Optimised conditions	150	1.2	58.8	45.3	3.0	42.6
			Organosolv pretreatment			
	150	--	75.7	37.0	0.6	41.2
	180	--	53.8	46.0	0.0	43.0

Table 2. Composition of liquid fractions in sugars and inhibitors (g/L) and hemicellulosic sugar recovery (%).

Run	Sugar concentration (g/L)*				HSR (%)	Inhibitory compounds concentration (g/L)				
	Glucose	Xylose	Galactose	Arabinose		Acetic acid	Formic acid	Furfural	HMF	TIC
1	9.49	4.67	1.84	1.44	25.77	0.45	0.37	0.07	0.08	0.96
2	9.88	9.53	2.29	1.65	43.53	1.61	0.27	0.05	0.10	2.02
3	9.14	18.76	3.27	1.62	76.50	6.52	1.12	1.55	0.52	9.70
4	10.71	19.30	3.17	1.76	78.23	6.81	0.59	1.07	0.32	8.80
5	9.89	16.55	2.64	1.88	68.00	5.99	0.41	0.33	0.25	6.98
6	10.56	18.59	2.43	1.64	73.15	6.95	0.88	2.30	0.44	10.58
7	10.66	18.95	2.24	1.88	74.50	7.11	0.89	2.95	0.45	11.41

8	10.39	18.00	2.66	1.68	72.25	6.58	0.92	2.38	0.44	10.32
9	18.37	5.54	1.49	0.60	25.13	7.54	1.84	7.15	1.31	17.83
10	10.68	18.89	2.28	1.38	72.90	6.76	0.93	2.60	0.45	10.74
11	17.71	5.89	1.68	0.73	26.98	6.93	1.86	6.35	0.98	16.12
12	12.66	13.37	1.66	1.20	52.50	6.68	1.08	4.37	0.48	12.61
13	10.54	18.23	2.37	1.42	71.19	6.72	0.95	2.67	0.45	10.79
Optimised conditions	11.89	20.14	3.83	2.58	76.00	6.27	0.63	0.71	0.30	7.91

*Mannose concentrations lower than 0.2 g/L (data not shown). HSR: hemicellulosic sugars recovery; HMF: hydroxymethylfurfural; TIC: total inhibitory compounds concentration.

Table 3. Model equations and ANOVA parameters

Equations in terms of coded factors*	**CV (%)**	**SD**	**R^2**	**Adjusted R^2**	**F-value**	**p-value**	**Lack of fit p-value**
HSR (%) = $+ 72.80 - 16.06 \cdot T + 9.07 \cdot C_A - 8.66 \cdot T \cdot C_A - 12.85 \cdot T^2 - 16.57 \cdot C_A^2$	2.86	1.63	0.9968	0.9941	370.15	<0.0001	0.1394
Acetic acid (g/L) = $+ 6.82 + 0.60 \cdot T + 2.11 \cdot C_A - 0.59 \cdot T \cdot C_A - 0.11 \cdot T^2 - 1.71 \cdot C_A^2$	4.71	0.28	0.9921	0.9856	151.19	<0.0001	0.1443
Furfural (g/L) = $+ 1.59 + 0.69 \cdot T + 0.58 \cdot C_A - 0.25 \cdot C_A^2$	9.86	0.14	0.9746	0.9662	115.33	<0.0001	0.0781
TIC (g/L) = $+ 10.77 + 3.79 \cdot T + 3.71 \cdot C_A + 0.71 \cdot T^2 - 2.10 \cdot C_A^2$	5.55	0.55	0.9909	0.9863	216.73	<0.0001	0.1764

HSR: hemicellulosic sugar recovery in liquor (%); TIC: total inhibitory compounds concentration (g/L); T: temperature (°C); C_A: sulfuric acid concentration (%).

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.

Graphical abstract