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High-yield selective conversion of pretreated milled softwood mixture to glucose by oxalic acid impregnation and microwave-assisted hydrolysis – Application to bio-ethanol production

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A B S T R A C T

In this study, a deep eutectic solvent pretreated milled softwood mixture was impregnated with oxalic acid before performing a water hydrolysis to obtain glucose using a microwave. Analysis of variance of a central composite design (CCD) showed that both temperature and time were significant parameters to consider when studying the cellulose conversion, glucose yield and glucose selectivity. The optimal conditions were 174 °C and 2 h, which resulted in 27.7% cellulose conversion, 22.4% glucose yield and 81.1% glucose selectivity. Oxalic acid affected the amorphous domains of cellulose at low hydrolysis temperature, while both the amorphous and the crystalline regions were affected at high temperature. Successive treatments of the solid hydrolysis residues resulted in 98.4% cellulose conversion, 79.0% glucose yield and 80.3% selectivity. Bioethanol was produced from the recovered glucose within 24 h with a fermentation efficiency of 42.9%.

1. Introduction

The high consumption of fossil energy and the associated negative environmental impact have led to the search for sustainable energy sources. Lignocellulosic biomass (LCB), derived from agricultural, industrial, and forestry wastes, has become the sustainable energy source of choice for most research studies due to its abundant and renewable nature. LCB has therefore become a widely accepted material to produce bioethanol, an alternative fuel with minimal environmental impact (Ching et al., 2017).

The main constituents of lignocellulosic biomass (LCB) are cellulose (40 – 60%), hemicellulose (20 – 40%) and lignin (10 – 25%), which are intimately interconnected forming the structure of the wall of the vegetal cells (Manmai et al., 2020). Cellulose, the main polysaccharide in LCB, is tightly bound forming crystalline structures through intramolecular hydrogen bonds that are protected against chemical and biological attack by the presence of lignin. To separate the cellulose from the rest of the LCB, a suitable pretreatment technique is required (Liu et al., 2021). Pretreatment with a deep eutectic solvent (DES) has been shown to be effective in removing both lignin and hemicellulose with minimal effect on the cellulose, resulting in a high purity cellulose for further processing (Ceaser et al., 2023).

The production of cellulosic bioethanol involves the pretreatment or isolation of cellulose from biomass, its hydrolysis to glucose, and the subsequent fermentation to bioethanol. Cellulose hydrolysis can be accomplished by either enzymatic or chemical treatment. The low specific activity of enzymes on pretreated cellulose in enzymatic hydrolysis and the relatively slower hydrolysis rate make the chemical treatment method more competitive (Sasaki et al., 2020). Chemically-catalyzed cellulose hydrolysis is faster in the amorphous regions of cellulose than in the crystalline regions. This means that an efficient amorphization of cellulose can lead to faster hydrolysis, high glucose yield, and high selectivity with limited degradation byproducts, while keeping catalyst and equipment costs low.

Conventional chemical hydrolysis uses concentrated inorganic acids such as sulfuric, phosphoric or hydrochloric acids, which generate large amounts of cellulose degradation products like hydroxymethyl furfural and humic substances. The hydrolysate requires purification, neutralization, and removal of the acid salts to reduce their negative effects on the fermentation of glucose (Ceaser and Chimphango, 2021; Hirayama et al., 2020; Sasaki et al., 2019). In addition, the use of concentrated inorganic acids increases investment and operation cost of the hydrolysis unit due to corrosion-related issues. Organic acids such as oxalic acid, maleic acid, and citric acid, are a suitable alternative to

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concentrated mineral acids for cellulose hydrolysis since they are biodegradable, safer to use, and easy to store (Yang et al., 2021).

The efficient hydrolysis of cellulose is a major concern in its application. Recently, a two-step process of first impregnating the cellulosic material with a concentrated acid at a low temperature to enhance cellulose depolymerization while minimizing further hydration. The impregnated cellulose is then hydrolyzed to glucose with water as the solvent at an elevated temperature. To date, few studies have been conducted using organic acids as catalysts in cellulose impregnation before performing a microwave-assisted water hydrolysis into glucose, although they have been proven to be more effective than inorganic acids when used as pretreatment solvents (Li et al., 2018). In general, studies conducted using inorganic acids such as sulfuric acid for impregnation prior to water hydrolysis have resulted in a 53 – 78% cellulose conversion, a 27 – 30% glucose yield, and a low glucose selectivity of 39 – 51% due to the formation of large amounts of degradation products (Ching et al., 2017; Gavilà et al., 2017, 2015). A study conducted by our research group found that commercial microcrystalline cellulose impregnated with oxalic acid yielded more glucose after hydrolysis than cellulose impregnated with sulfuric acid (Chimentão et al., 2014). Moreover, microwave-assisted hydrolysis of the cellulose impregnated with oxalic acid resulted in a 53% cellulose conversion, a 85% glucose selectivity and a 45% glucose yield. Furthermore, the microwave-assisted hydrolysis resulted in a shorter hydrolysis time than conventional heating (6 h v 20 h) and improved cellulose conversion, glucose selectivity and yield. The improved selectivity was primarily due to oxalic acid's dicarboxylic nature, enhancing selectivity to glucose by suppressing the formation of degradation products. This makes the hydrolysate more favourable for fermentation compared to that obtained with inorganic acids (Liu et al., 2021). Although Chimentão et al. (2014) achieved high glucose selectivity and yield with the oxalic acid impregnation, the microwave-assisted hydrolysis was conducted at a low solid concentration (1.5 w/v%) at 120 °C for 6 h. The low solids loading, and the extended hydrolysis time impeded the industrial feasibility of the process. On the other hand, Ching et al. (2017) optimized the hydrolysis of microcrystalline cellulose impregnated with sulfuric acid to obtain a 53% cellulose conversion, 50% glucose selectivity and 27% glucose yield at 10 w/v% solids loading, 180 °C and for 10 min. This implies that optimization of the conditions of cellulose hydrolysis with oxalic acid could lead to a shorter hydrolysis time and a better glucose yield and selectivity at higher solids loading, which would be beneficial for ethanol production.

So far, the studies reported in the literature on cellulose hydrolysis with organic acid-impregnated biomass have mainly used commercial microcrystalline cellulose as feedstock, while cellulose from LCB has not been investigated. In this study, we focused on the use of a commercial softwood mixture pretreated with a DES delignification process (PB) optimized in a previous work (Ceaser et al., 2023). The PB was impregnated with oxalic acid before the microwave-assisted hydrolysis was performed at a 5 w/v% solid concentration which is higher than the initial 1.5 w/v% used for oxalic acid (Chimentão et al., 2014). The effects of the impregnation and the hydrolysis conditions on the conversion of the PB cellulose, the glucose selectivity and yield were determined and optimized. At the optimal conditions, the unconverted solid retentate was consecutively hydrolysed to determine the treatment efficiency. The glucose obtained at the optimal condition was further converted to bioethanol using the yeast *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Materials

The cellulose material used in this study was obtained by following the procedure described by Ceaser et al. (2023). A pre-milled homogeneous batch of a softwood mixture (MSM), sieved to a maximum size of 100 μm, was pretreated using a DES of choline chloride (ChCl)/formic acid (FA) at a molar ratio of 1:4. The MSM was pretreated at 140 °C for 14 min in a microwave reactor with a MSM/DES mass ratio of 1/10. The pretreated biomass (PB) was washed with 50% ethanol/water, recovered by vacuum filtration and dried in an oven at 50 °C overnight. The resulting dry PB which was 51% of the initial starting material had a composition of 89.2% cellulose, 4.2% hemicellulose and 6.5% lignin, and was used in the hydrolysis study without further modification. This corresponds to a cellulose loss below 6% during pretreatment (Ceaser et al., 2023).

Commercial microcrystalline cellulose (CC) was obtained from Sigma-Aldrich and it was used as reference material in the hydrolysis experiments. Oxalic acid (98%, Acros Organics), formic acid (98%, Labkem), choline chloride (98%, Merck), cellobiose (99%, Sigma-Aldrich), glucose (99%, Sigma-Aldrich), furfural (99%, Sigma-Aldrich), hydroxymethylfurfural (99%, Sigma-Aldrich), and levulinic acid (98%, Sigma-Aldrich) were used as received without further purification.

2.2. Optimisation of the organic acid-assisted hydrolysis of PB

The hydrolysis with organic acid consisted of two steps, impregnation with the organic acid, followed by the hydrolysis reaction (Chimentão et al., 2014). Firstly, the PB was impregnated with a 6 wt% of the oxalic acid at 1:5 solid to liquid ratio and dried at 100 °C for 20 h. After drying, 6 mL of water was added to 0.3 g of the acid-impregnated PB and placed in a Milestone Synthwave Single Reaction Chamber microwave reactor. The reactor was pressurized to 20 bar with N_2 and rapidly heated (<15 min) to the desired temperature at 800 W and held constant for the reaction time at a stirring rate of 200 rpm throughout the experiment. Reaction temperature (140 – 180 °C) and time (1 – 3 h) were varied to determine the optimum conditions using a central composite design (CCD). The optimum was defined based on the conversion of the cellulose in the impregnated PB, the yield of glucose, and the selectivity to glucose. At the end of the hydrolysis, the samples were cooled to 40 °C and the hydrolysate was separated by centrifugation at 6000 rpm for 10 min before it was analyzed by high performance liquid chromatography (HPLC) as described below. The solids were washed with 30 mL of distilled water and dried at 100 °C. Cellulose conversion (X_C), glucose yield (Y_G) and glucose selectivity (S_G) were calculated as follows, where CE denotes cellobiose, G denotes glucose, HMF is hydroxymethyl furfural, LA is levulinic acid and FA is formic acid:

$$X_C(\%) = \left(\frac{0.474 \times \mathrm{CE(g)} + 0.9 \times \mathrm{G(g)} + 1.286 \times \mathrm{HMF(g)} + 1.396 \times \mathrm{LA(g)} + 3.522 \times \mathrm{FA(g)}}{\mathrm{Cellulose\ in\ PB(g)}}\right) \times 100 \quad (1)$$

$$Y_G(\%) = \left(\frac{0.9 \times \mathrm{G(g)}}{\mathrm{Cellulose\ in\ PB(g)}}\right) \times 100 \quad (2)$$

$$S_G(\%) = \left(\frac{Y_G}{X_G}\right) \times 100 \quad (3)$$

2.3. Consecutive hydrolysis of the unconverted solid retentate

The dried solid residue (HPB) from the initial hydrolysis was then used in the consecutive recycle experiments. The dried solid residues were again impregnated with 6 wt% oxalic acid and dried in an oven overnight at 100 °C. Distilled water was added to the dried samples at a 1:20 w/v ratio and placed in the microwave at the optimum conditions. After the hydrolysis the samples were cooled, and the hydrolysate separated for analysis while the solid retentate was used in the next cycle. Three cycles (RHPB-1, RHPB-2 and RHPB-3) were conducted using this method to determine the process efficiency.

2.4. Fermentation of the hydrolysate to bioethanol

The bioethanol fermentation was conducted using yeast from *S. cerevisiae* (Lalvin QA23 obtained from Lallemand Brewing Portugal). A medium containing 5 g/L yeast extract, 10 g peptone and 10 g glucose was topped up to 500 mL with distilled water and sterilized at 121 °C for 20 min. A 10% w/v% of activated *S. cerevisiae* was prepared by using sterilized distilled water as the solvent and placed in an incubator at 37 °C for 20 min. A 0.5 mL solution of the activated yeast was transferred into 30 mL of the prepared fermentation medium aseptically.

The produced hydrolysate (25 mL) was fermented by adding 2% (w/v) of yeast obtained from the fermentation medium (Manmai et al., 2020). The solution mixture was fermented for 96 h. An aliquot of the fermented solution was taken at 0, 24, 48 and 96 h to measure the concentration of the glucose in the hydrolysate (H_{GC}) and the ethanol produced (F_{EC}) by HPLC. The kinetic parameters for the ethanol yield coefficient (EYC), the ethanol productivity (EYP), and the fermentation efficiency (FE) as described (Manmai et al., 2021; Tong et al., 2023) were calculated as follows:

$$\mathrm{EYC}(g/g) = \frac{\mathrm{F}_{EC}(g/L)}{\mathrm{H}_{GC}(g/L)} \tag{4}$$

$$\mathrm{EYP}(g/L \bullet h) = \frac{\mathrm{F}_{EC}(g/L)}{\text{Fermentation time(h)}} \tag{5}$$

$$\mathrm{FE}(\%) = \frac{\mathrm{F}_{EC}(g/L)}{0.51 \times \mathrm{H}_{GC}(g/L)} \times 100 \tag{6}$$

2.5. Product analysis by HPLC

The fermentation produced ethanol and hydrolysates containing glucose, cellobiose, levulinic acid (LA), furfural, formic acid and 5-hydroxymethylfurfural (HMF) were analyzed using an Agilent 1100 series HPLC chromatograph system equipped with a refractive index detector (RID) and a diode array detector (UV-DAD). The samples were filtered through a 0.22 µm syringe filter before analysis. The analysis was conducted using an Aminex HPX-87 H column at 50 °C with 0.6 mL/min of 0.005 M H_2SO_4 as the solvent. The DAD was operated at a wavelength of 254 nm to determine the concentration of HMF, levulinic acid and furfural, and the remaining compounds were determined with the RI detector. The concentration of each product was measured from a calibration performed with standards prepared with analytical grade compounds.

2.6. X-ray diffraction (XRD)

The effect of the organic acid impregnation on the crystallinity of the cellulose contained in the samples were determined by XRD on a Bruker D2 Phaser X-ray diffractometer. The samples were analyzed within a range of 5–50°, at a voltage of 40 kV using a CuKα radiation source (λ = 1.54184 Å) at a current of 40 mA. The sample scan speed was 1°/min with a 0.02° step size (Ceaser and Chimphango, 2021). The crystallinity (CrI) of the samples was calculated as follows:

$$\mathrm{CrI}(\%) = \frac{\mathrm{I}_{200} - \mathrm{I}_{\mathrm{am}}}{\mathrm{I}_{200}} \times 100\% \tag{7}$$

2.7. Fourier transform infrared (FTIR)

The functional groups present within the acid-impregnated residue after hydrolysis were analyzed on a Jasco FT/IR-600 Plus equipped with ATR Specac Golden Gate. The residues were analyzed at a resolution of 4 cm^{-1} within a range of 400–4000 cm^{-1}. The signals were assigned to specific functional groups according to literature (Ceaser et al., 2023).

2.8. Statistical analysis and model fitting

The optimisation of the hydrolysis process parameters was conducted through a central composite design by using Statistica 10.0 software. A two factor, three level statistical design with a total of 12 experiments was conducted using 4 central points. The experimental results for cellulose conversion (Z_1), glucose yield (Z_2), and glucose selectivity (Z_3), were fitted to a second order polynomial function (Eq. 8) of temperature *T*, in degrees Celsius, and time *t*, in minutes. The significance of the model was determined based on the F-test and the P-value (< 0.05). The model fitness was evaluated based on the coefficient of determination (R^2), the adjusted coefficient of determination (adj. R^2) and the lack of fit test showing no significance. At the optimum condition for the CCD, the experimental results were expressed as the mean ± the standard deviation of 3 replicates (Ceaser et al., 2023).

$$Z_j = a_{j,0} + a_{j,1}T + a_{j,2}T^2 + a_{j,3}t + a_{j,4}t^2 + a_{j,5}Tt \tag{8}$$

3. Results and discussion

3.1. Analysis of variance (ANOVA) of response models

The ANOVA for the cellulose conversion, glucose yield and glucose selectivity in Table 1 showed that the R^2 and adjusted R^2 values for all the responses were greater than 0.97 and 0.95 respectively. The marginal difference in the values indicates a good correlation between the values predicted by the model and the experimental values. In addition, the lack of fit for the various responses was not significant representing the ability of the model to explain the experimental results (Ceaser et al., 2023). The effect of temperature and time on the responses was also evaluated depending on their significance ($p < 0.05$) and the corresponding experimental plots given in Fig. 1.

3.2. Effect of temperature and time on organic acid impregnated PB conversion

The effect of temperature, time and their interactions on PB conversion was shown in Table 1. The factors (reaction temperature and time) considered were both observed to affect the cellulose conversion with reaction temperature exhibiting a stronger effect than time. The presence of an effect based on the interaction of temperature and time implied that the cellulose conversion was dependent on both factors. The best-fit values of the parameters of the cellulose conversion mathematical model (Z_1) with respect to temperature (T) and time (t) are given in Table 2.

Fig. 1(a) shows that increasing temperature from 130 °C to c.a. 170 °C produces an increase in cellulose conversion, while a further increase in temperature reduces the cellulose conversion. Interestingly, the results show that at ≤ 140 °C more cellobiose was produced than glucose (glucose yield < 13%) although the cellulose conversion was high (22.4%). This implies that to convert cellulose to glucose using an oxalic acid impregnation process it is necessary to operate at temperatures above 140 °C. Observing the trend for the reaction time shows an increase of cellulose conversion from 22.2% to 28.7% as time increased

Table 1
Analysis of variance (ANOVA) summarised for cellulose conversion, glucose yield and glucose selectivity based on a central composite design.

	Cellulose conversion (Z_1)		Glucose yield (Z_2)		Glucose selectivity (Z_3)	
Factor	F-value	p-value	F-value	p-value	F-value	p-value
T	90.91	0.002447*	318.34	0.000384*	17113.75	0.000001*
T^2	26.78	0.014004*	301.97	0.000415*	21068.18	0.000001*
t	39.80	0.008048*	12.59	0.038123*	181.10	0.000887*
t^2	47.19	0.006317*	42.24	0.007396*	45.65	0.006623*
Tt	11.95	0.040753*	2.69	0.199233	2662.56	0.000016*
Lack of fit	0.79	0.574116	1.13	0.461760	7.13	0.070505
Pure error		0.5025		0.4092		0.0620
R-squared		0.9744		0.9902		0.9994
Adjusted R-squared		0.9531		0.9820		0.9989

[*] Significant at 95% confidence level with p value less than 0.05

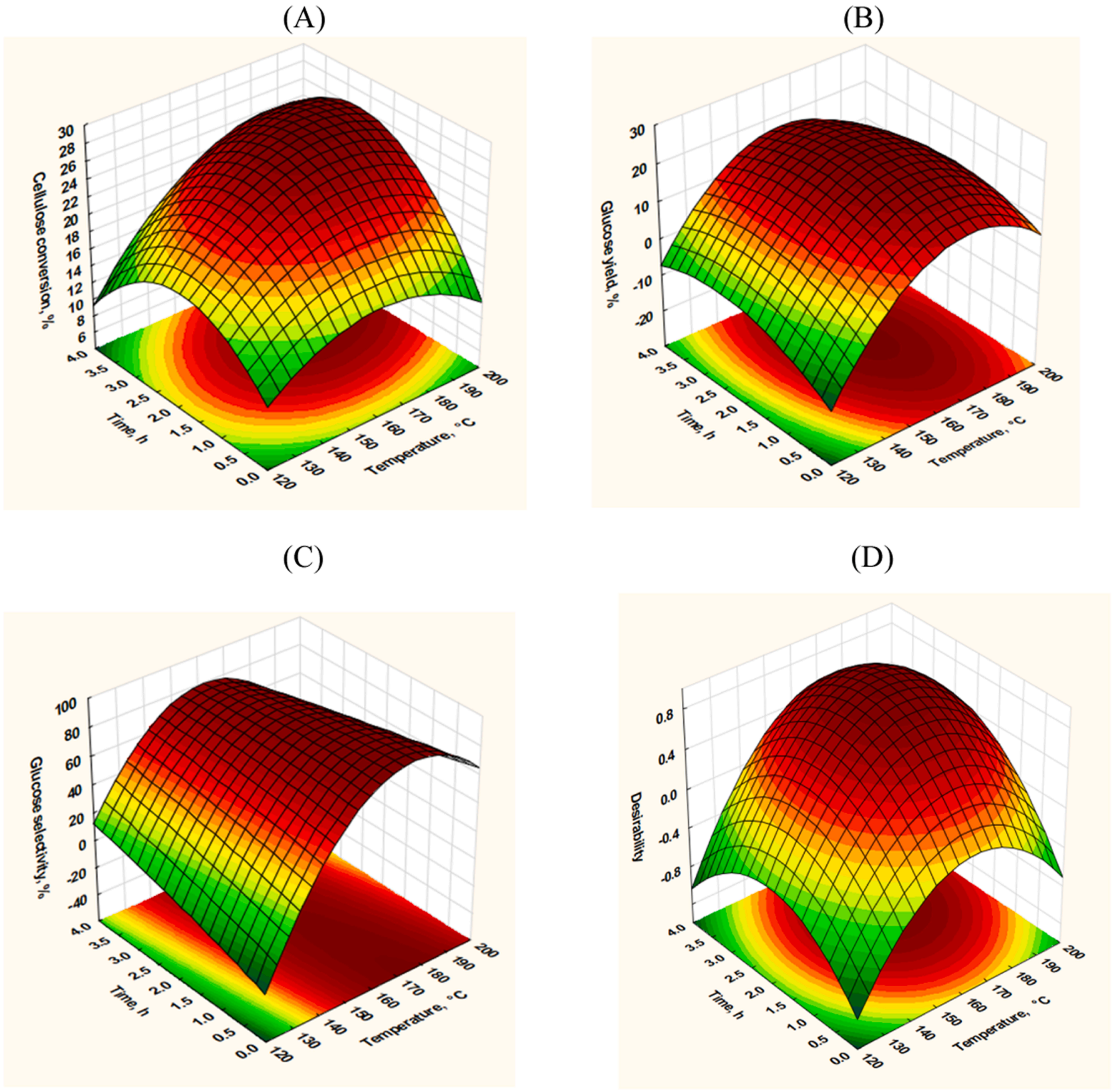

Fig. 1. Response surface for a) Cellulose conversion, b) Glucose yield, c) Glucose selectivity, d) Desirability, with hydrolysis temperature and time.

Table 2
Mathematical models for cellulose conversion (Z_1), glucose yield (Z_2) and glucose selectivity (Z_3) as represented by Eq. 8.

Z_j	$a_{j,0}$	$a_{j,1}$	$a_{j,2}$	$a_{j,3}$	$a_{j,4}$	$a_{j,5}$
Z_1	-76.1794	1.1573	-0.0036	-0.5198	-1.9248	0.0061
Z_2	-308.9053	3.7701	-0.0110	11.5759	-0.6433	0.0263
Z_3	-1035.6891	12.7008	-0.0359	53.0812	-0.6677	0.3225

from 0.59 h to 2 h. A gradual decrease in cellulose conversion was observed with a further increase in reaction time. The reduction in cellulose conversion with a further increase in temperature and time could be attributed to a generation of humin in the solution resulting in a decrease in the total concentration of cellulose products observed in the filtrate. This is supported by the observed reduction in the concentrations of formic and levulinic acid concentrations observed with samples 12 and 7 (Table 3).

3.3. *Effect of temperature and time on organic acid impregnated PB glucose yield*

The glucose yield was similarly affected by both the reaction temperature and time with no significant effect presented by the interaction of both temperature and time (Table 1). This showed that the effect of the factors on the glucose yield were independent of each other. In addition, reaction temperature was also observed to present the highest effect on the glucose yield when considering both the linear and quadratic terms. The model for the glucose yield (Z_2) is represented in Table 2.

In Fig. 1(b) a sharp increase in glucose yield from 7.3% to 22.6% was observed as the temperature increased from 132 °C to 160 °C while reducing to 18.9% with a further increase in temperature to 188 °C. The reduction in the glucose yield could be attributed to the conversion of glucose to HMF which was observed as more than a double of the HMF concentration when sample 9 is compared to sample 6. As reaction time increased from 0.59 h to 2 h the glucose yield gradually increased from 17.9% to 22.6% while a further increase in time to 3.41 h resulted in the reduction of the glucose yield to 19.5%. Given the reduction in the concentration of the degradation products (Table 3), it can be surmised that the extended reaction time led to the constant degradation of both glucose and the generated degradation products to humin resulting in a decrease in the glucose yield.

3.4. *Effect of temperature and time on organic acid impregnated PB glucose selectivity*

Temperature had the highest effect on the glucose selectivity followed by the interaction of both temperature and time (Table 1). Additionally, the reaction time had the least effect on the glucose selectivity. The mathematical model representing the glucose selectivity is given in Table 2.

The glucose selectivity increased sharply from 33.9% to 78.8% as temperature increased from 132 °C to 160 °C before reducing to 66.5% as temperature reached 188 °C (Fig. 1c). Ching et al. (2017) similarly observed a reduction in the selectivity of the desired products as temperature increased to 180 °C due to the formation of humins. On the other hand, at 160 °C, the glucose selectivity remained almost constant (c.a. 78.7%) as time increased from 0.59 h to 2 h with a slight reduction to 75.6% when reaction time was extended to 3.41 h. Furthermore, as temperature increased, the highest selectivity to glucose (82.2%) was reached within a short time, while at low temperatures took a longer time to reach the highest obtainable glucose selectivity. However, at high temperature and time (Fig. 1c), the glucose selectivity decreased due to an increase in the formation of degradation products such as HMF and furfural (Sample 4, Table 3) which led to the formation of humins. The latter is consistent with observations from previous studies (Ching et al., 2017; Patil and Lund, 2011).

3.5. *Validation of optimum multiple response conditions and determination of degradation products*

The optimum oxalic acid impregnation hydrolysis conditions according to the model were 174 °C and 2 h, with predicted values of cellulose conversion, glucose yield and glucose selectivity of 28.0 ± 1.1%, 21.9 ± 0.9%, and 79.5 ± 0.8%, respectively. The respective experimental values at those conditions were 27.7 ± 0.2%, 22.4 ± 0.2% and 81.1 ± 0.9%, which fully agree with the model predictions within the uncertainty limits (Fig. 2a).

To assess the influence of the type of biomass, additional experiments were conducted at the optimum conditions for the pretreated softwood biomass (174 °C and 2 h), using commercial cellulose as substrate. In Fig. 2a, when commercial cellulose was used as feedstock, cellulose conversion, glucose yield and selectivity were 26.2%, 20.1% and 76.7%, respectively. This can be explained by the lower crystallinity of the pretreated softwood in comparison with the commercial cellulose. Chimentão et al. (2014) reported that oxalic acid acts on the amorphous regions of cellulose during acid impregnation to produce glucose. Furthermore, the high treatment temperature could also improve the conversion of the cellulose into glucose. The concentration of the degradation products produced from both commercial cellulose and pretreated biomass was comparable (Fig. 2b), indicating that the used conditions did not result in a severe degradation of either feedstock.

The effect of oxalic acid was observed by substituting the acid with water during impregnation step for both pretreated biomass and commercial cellulose (Fig. 2a). Cellulose conversion, glucose yield and glucose selectivity were 2.1%, 1.2% and 56%, respectively, without significant differences between both substrates. Concerning the cellulose degradation products, the results were similar except formic acid. The initial formic acid content was 8.5 g/ 100 g PB, most likely due to

Tablee 3
Degradation products obtained at various conditions during central composite design optimisation experiment.

	Hydrolysis conditions		Degradation products, g/L				
Sample	Temperature, °C	Time, h	Formic acid	Acetic acid	Levulinic acid	HMF	Furfural
1	140	1	1.22	0.03	0.03	0.06	0.28
2	140	3	2.31	0.03	0.16	0.13	0.41
3	180	1	3.54	0.04	0.26	0.47	1.10
4	180	3	4.43	0.05	0.91	1.25	0.99
5	132	2	1.00	0.03	0.08	0.35	0.22
6	188	2	4.07	0.06	0.44	2.08	2.42
7	160	0.59	3.65	0.03	0.18	0.29	1.08
8	160	3.41	3.94	0.05	0.47	0.88	1.42
9	160	2	4.20	0.04	0.41	0.92	1.33
10	160	2	4.15	0.04	0.45	0.81	1.19
11	160	2	4.18	0.05	0.40	0.88	1.29
12	160	2	4.24	0.04	0.43	0.88	1.20

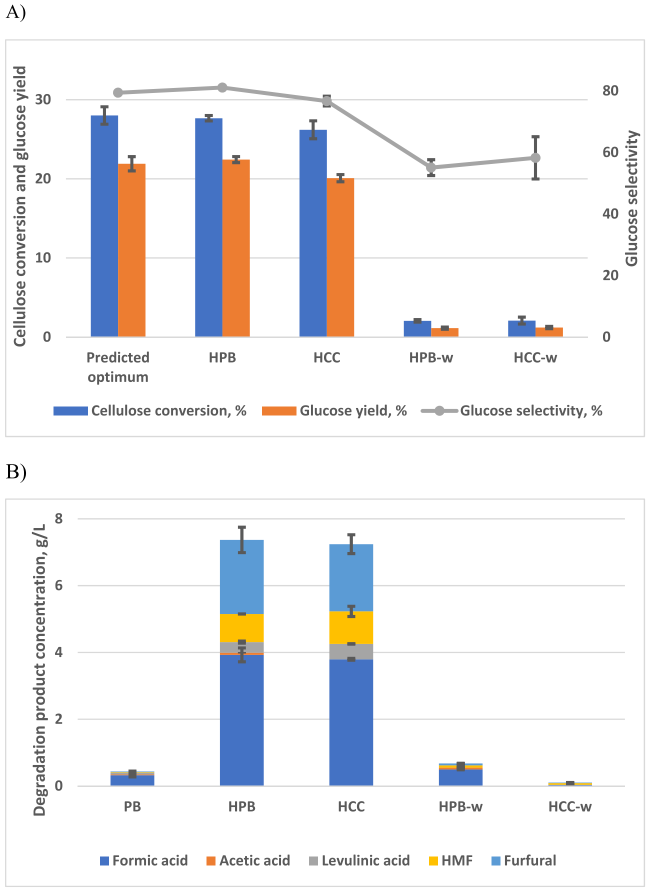

Fig. 2. A) Cellulose conversion, glucose yield and glucose selectivity B) Degradation product concentrations for pretreated biomass (PB), hydrolysis of oxalic acid impregnated pretreated biomass (HPB) and commercial cellulose (HCC) and water impregnated pretreated biomass (HPB-w) and commercial cellulose (HCC-w).

residual formic acid from the DES pretreatment whiles the other degradation products (acetic acid, levulinic acid, HMF and furfural) were almost non-existent. There was more formic acid released from the pretreated biomass as compared to the commercial cellulose (0.5 g/L vs. 0.1 g/L) (Fig. 2b). The excess formic acid was likely due to residual formic acid that existed within the DES ChCl/FA used during the pretreatment. Furthermore, the amount of degradation products was larger when oxalic acid was used instead of water (Fig. 2b). The concentration of HMF and levulinic acid were found to be below 1 g/L for both commercial cellulose and pretreated biomass, even when treated with oxalic acid. In the case of formic acid, the concentration was c.a. 3.8 g/L for both cases. The rise in the formic acid concentration in comparison to the other degradation products could be due to decomposition of oxalic acid during the hydrolytic reaction. Chen et al. (2020) studied the reactivity of oxalic acid at high pressures and noticed that even at a high pressure of 600 bar, it was possible for oxalic acid to decompose between 130 and 180 °C, producing formic acid and CO_2. Therefore, considering that the hydrolysis was conducted at 20 bars and 174 °C, it is likely that the oxalic acid partially decomposed to produce formic acid leading to an increase in the formic acid concentration. Additionally, the lowest formic acid concentration during the CCD, 1.0 g/L, was obtained at 132 °C (Table 2), indicating a partial decomposition of oxalic acid with increasing temperature. However, operating at such low temperature implies a 4.5% glucose yield and a 25.4% selectivity, even with an extended reaction time of 4 h.

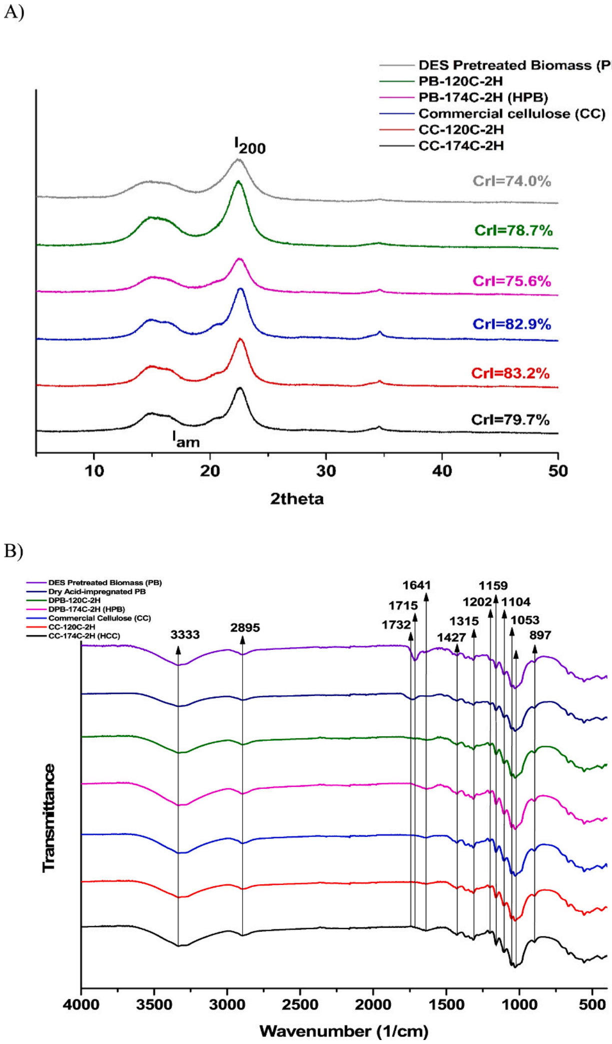

Fig. 3. A) XRD patterns and B) FTIR of untreated and hydrolysis residues from commercial cellulose and DES pretreated biomass.

3.6. *Characterization of the hydrolyzed solid residues*

3.6.1. *Crystallinity*

To better understand the effect of treatment conditions on cellulose, the crystallinity index was determined for the raw biomass, the biomass pretreated by DES delignification (PB), and the hydrolyzed solid residues (Fig. 3a). The crystallinity of PB (74%) was similar to the crystallinity obtained in our previous work (72%) after performing DES pretreatment albeit slightly higher (Ceaser et al., 2023). Since temperature was noticed to be the factor that had the larger influence on the hydrolysis response variables, the crystallinity was measured at samples treated for 2 h both at 120 °C and 174 °C. From Fig. 3a), at 120 °C the crystallinity index of the cellulose remaining in the hydrolyzed solids increases (78.7%), indicating that the oxalic acid impregnation mainly affected the amorphous regions as reported by Chimentão et al. (2014). Similarly, a slight increase in crystallinity was observed when commercial cellulose was used as the substrate during hydrolysis at 120 °C (83.2% vs. 82.9%). However, at 174 °C the crystallinity decreased to 75.6%. This shows that oxalic acid was able to cause amorphization to the cellulose by partially reducing the crystalline regions as the temperature increased. This particular phenomenon has mainly being observed in inorganic acids such as sulfuric and phosphoric acid, since they mainly attack the crystalline regions thereby increasing the amount of glucose (Hirayama et al., 2020; Sasaki et al., 2020). Commercial cellulose also showed a similar reduction in crystallinity when treated at 174 °C (79.7%) as compared to the crystallinity at 120 °C (83.2%). The high glucose yield and selectivity obtained at the optimum conditions could therefore be contributed to the combined effects of both oxalic acid and high temperature (174 °C).

3.6.2. Residual hydrolyzed cellulose functional groups

The FTIR spectra for the DES pretreated biomass, commercial cellulose, and the hydrolyzed solid residues at 120 °C and 174 °C were analyzed and presented in Fig. 3b. The peaks characteristic of cellulose were observed in all samples. After drying of the DES oxalic acid-impregnated pretreated biomass, a peak was observed at 1731 cm^{-1}. This peak represented the presence of C=O which resulted from partial degradation of amorphous cellulose thereby exposing hemicetals groups (Liu et al., 2017). The signal attributed to cellulose hydrogen bonds O-H stretching vibration at 3334 cm^{-1} became sharper with a lower intensity at 120 °C for both the pretreated biomass and the commercial cellulose as compared to the original cellulose while an increase in the peak intensity was observed at 174 °C (Ciolacu et al., 2011). This could imply that operating at 120 °C the oxalic acid affected the amorphous regions of cellulose thereby resulting in a reduction of the intensity of this peak while the partial conversion of oxalic acid into formic acid at 174 °C caused a reduction in the crystalline regions of the samples. This agrees with the results obtained from the XRD analysis where crystallinity increased at 120 °C and reduced at 174 °C.

The peak at 1427 cm^{-1} and 897 cm^{-1} attributed to the crystalline and amorphous regions of cellulose were noticed in all the samples (Ceaser et al., 2023). Furthermore, the cellulose characteristic peaks at 1029, 1053, 1104, 1159, 1202 and 1315 cm^{-1} assigned to C-O stretch of C-6, C-O stretch of C-3, in-plane ring stretching, asymmetric stretch, C-O-H in-plane bending at C-6 and CH_2 wagging were found in all samples (Hassan and Mutelet, 2022; Holilah et al., 2022). At 120 °C, the peaks at 897, 1020, 1104 and 1159 cm^{-1} increased weakly whiles at 1202, 1315 and 1427 cm^{-1} remained almost constant indicating that the oxalic acid first affected the surface and amorphous regions breaking the hydrogen bonds and exposing C-OH, C-O-C and C-C bonds. At 174 °C, the peaks at 897, 1020, 1104 and 1159 cm^{-1} decreased weakly whiles the peaks at 1202, 1315 and 1427 cm^{-1} increased weakly indicating that the organic acids were able to finally attack the crystalline regions of the cellulose (Sun et al., 2008). This explains the increase in the crystallinity of the residues obtained at 174 °C in comparison to the pretreated biomass at 120 °C.

The peak at 1630 – 1640 cm^{-1} was attributed to the absorbed water in all samples (Holilah et al., 2022). The presence of residual formic acid in the DES pretreated biomass can be observed by the presence of a peak at 1718 cm^{-1} (Sun et al., 2008). This peak disappeared after the microwave-assisted hydrolysis indicating that the residual formic acid was deposited in the hydrolysate resulting in the observed presence of formic acid even at low temperatures. These results agree with the HPLC analysis performed on the hydrolysate.

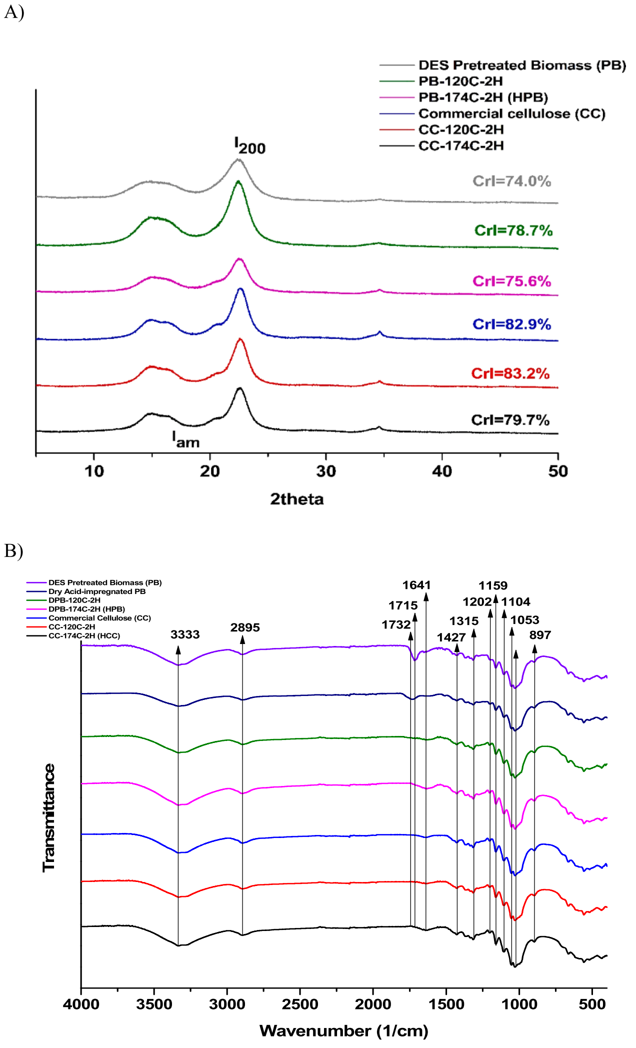

Fig. 4. A) Cellulose conversion, glucose yield and glucose selectivity B) Degradation product concentrations for hydrolysis of oxalic acid impregnated pretreated biomass (HPB), first recycle without acid impregnation (RHPB-1 w/o acid), first recycle with acid impregnation (RHPB-1), second recycle with acid impregnation (RHPB-2) and third recycle with acid impregnation (RHPB-3).

3.7. Consecutive hydrolysis treatments

As discussed above, conversion during the hydrolytic process was incomplete, and the hydrolyzed solids still contained a significant amount of cellulose. A series of experiments was conducted in which the unhydrolyzed solid was further processed with consecutive treatments performed at the optimum hydrolysis conditions, both with and without additional acid impregnation steps (Fig. 4). Performing a second reaction step without impregnation of the solid resulted in a low cellulose conversion of 4.6%, whereas when the solid was reimpregnated with oxalic acid, a 22.6% cellulose conversion was obtained (Fig. 4a). The glucose yield (18.2%) and the glucose selectivity (80.4%) approached those obtained in the first hydrolysis step (22.4% and 81.1%, respectively). This represents an 81% increase in the total yield of glucose in comparison to that of the single hydrolysis step. In the second hydrolysis step, the concentration of degradation products was lower than that of the first hydrolysis step (Fig. 4b). On the other hand, without acid impregnation only a 3.8% of the glucose yield of the step could be obtained on the second step, with a cellulose conversion and glucose selectivity of 4.6% and 80.2%, respectively (Fig. 4a).

When the hydrolyzed solid was processed through three consecutive reimpregnation/hydrolysis steps (RHPB-1 to RHPB-3 in Fig. 4), the total combined cellulose conversion and glucose yield were 98.4% and 79.0%, respectively, with a glucose selectivity of 80.3%. The glucose yield in each step was similar, but cellulose conversion and glucose selectivity were only comparable in RHPB-1 and RHPB-2 (Fig. 4a). In the RHPB-3 cellulose conversion was higher (25.6%), comparable to that attained during the first hydrolysis step, and glucose selectivity was lower due to an increase in the production of HMF (2.9 g/L, Fig. 4b). The results indicated that the first and second additional cycles made it easier for the organic acid to act on the cellulose by exposing the fibers, promoting the increase in cellulose conversion and yield. In addition, this also caused a faster conversion of the produced glucose into HMF, thereby resulting in the higher HMF concentration obtained in RHPB-3.

The crystallinity of the solids after the consecutive hydrolysis treatments was determined (Fig. 5a). Crystallinity diminished after each cycle from 75.6% to 55.6%, indicating that each hydrolysis stage

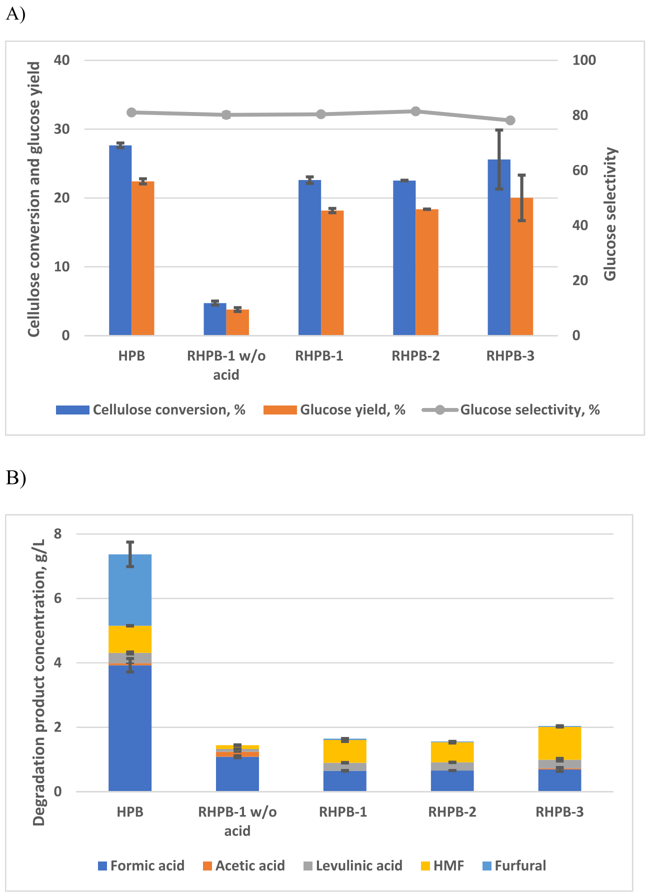

Fig. 5. A) XRD patterns and B) FTIR of untreated and hydrolysis residues from commercial cellulose and DES pretreated biomass.

converted more crystalline regions into amorphous cellulose that subsequently depolymerized to glucose. The magnitude of the variation in crystallinity doubled after every successive hydrolysis treatment, with RHPB-3 having the highest (2.2-fold increase). The slight increase in the crystallinity change form 2-fold to 2.2-fold in the third cycle supports the observation of an increase in cellulose conversion and production of HMF observed in the third cycle.

The changes in functional groups were analysed by FTIR spectroscopy (Fig. 5b). Very similar functional groups were observed for the solids, regardless of the number of impregnation/hydrolysis treatments. The main differences were a reduction in the peak intensities of the characteristic cellulose peaks after every successive cycle. Furthermore, the peak associated with the adsorbed water at 1640 cm^{-1} shifted to 1600 cm^{-1} during the cycles (Holilah et al., 2022).

3.8. Bioethanol production from the hydrolysate solutions

Saccharomyces cerevisiae was used in the fermentation of the hydrolysate obtained after the microwave-assisted hydrolysis of the oxalic acid impregnated pretreated biomass. The yeast strain is known for its ability to mainly ferment hexose sugars such as glucose into ethanol. Since the glucose yield was found to be comparable after each run (10–12 g/L) with the initial hydrolysis having the highest degradation product concentrations, the initial hydrolysate was used without any further purification to test the bioethanol production efficacy using *S. cerevisiae* for 96 h.

Bioethanol production was observed to increase during the first 24 h (Fig. 6). After 24 h, ethanol production rate declined until 96 h probably due to the yeast entering a death phase (Table 4). This phenomenon has been noticed and described by several researchers (Aranda et al., 2019).

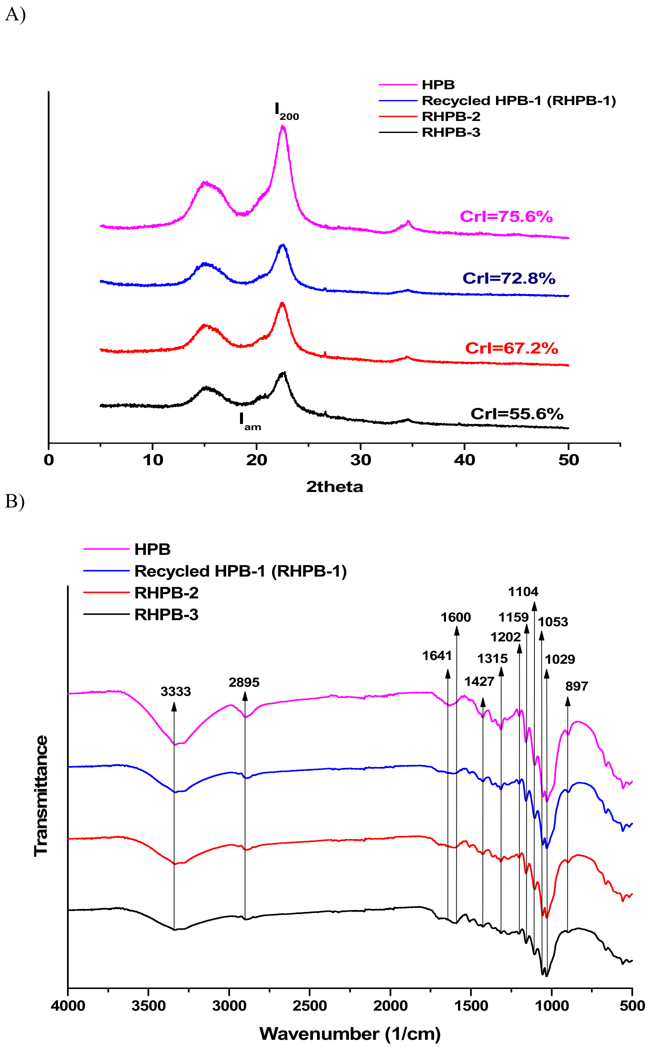

Fig. 6. Glucose, ethanol concentration and optical density (OD) profiles during fermentation process of glucose containing hydrolysate.

Table 4
Kinetic parameters of ethanol production with time (average values ± standard deviation).

Time (h)	Ethanol, × 10^2 (g/L)	Ethanol yield coefficient, × 10^2 (g/g)	Ethanol productivity, × 10^2 (g/L·h)	Fermentation efficiency, (%)
0	0	0	0	0
24	226 ± 8	21.9 ± 1	9.42 ± 0	42.9 ± 0.02
48	24.5 ± 7	2.37 ± 1	0.510 ± 0	4.65 ± 0.01
72	1.84 ± 7	0.178 ± 0	0.0255 ± 0	0.329 ± 0.01
96	0	0	0	0

Zheng et al. (2013) noticed that the maximum ethanol concentration during fermentation processes were generally obtained between 24 – 48 h and remained almost constant with further increase in fermentation time. In our study, at 24 h, the optimum fermentation time to produce bioethanol, the ethanol yield, the ethanol productivity, and the fermentation efficiency were 0.22 g/g, 0.09 g/L h and 42.9%, respectively (Table 4). These values were higher than the 0.16 g/g ethanol yield, 0.17 g/L h ethanol productivity and 30.33% fermentation efficiency obtained at 48 h by fermenting glucose obtained from a sequential chemical pretreatment and enzymatic hydrolysis of sorghum stalk (Manmai et al., 2020). The results indicated that despite the presence of inhibitors such furfural, HMF, formic acid, acetic acid and levulinic acid in the hydrolysate (Jung and Kim, 2017), it can be used for bioethanol production without any purification step.

4. Conclusion

The hydrolysis of softwood delignified by a DES pretreatment was investigated by a two-step process consisting of impregnation with concentrated oxalic acid at low temperature followed by aqueous-phase hydrolysis in a microwave reactor. The effect of temperature and time on the microwave-assisted hydrolysis step was investigated, and the conditions optimized. The hydrolysis temperature had the strongest effect on cellulose conversion, glucose yield, and glucose selectivity. At the optimum treatment conditions, 174 °C and 2 h, 27.7% cellulose conversion, 22.4% glucose yield and 81.1% glucose selectivity were obtained. At mild temperature (120 °C), the hydrolysis only attacked the amorphous regions of cellulose, thus resulting in an increase in crystallinity. At 174 °C, both the crystalline and the amorphous regions of cellulose were attacked, resulting in a decrease in the crystallinity of the unconverted solid. A sequence of four consecutive treatments of impregnation/hydrolysis led to a total cellulose conversion of 98.4%, with a 79.0% glucose yield and a 80.3% selectivity to glucose. Finally, the resulting glucose-rich hydrolysate could be directly fermented to bioethanol without any purification step.

CRediT authorship contribution statement

Regan Ceaser: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Daniel Montané:** Methodology, Formal analysis, Writing – review & editing, Supervision. **Magda Constantí:** Methodology, Formal analysis, Writing – review & editing, Supervision. **Francesc Medina:** Formal analysis, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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