

Production of simple sugars from olive grove pruning using acid pretreatment and enzymatic hydrolysis

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Abstract. The purpose of this paper was to optimize the production of simple sugars from olive grove pruning (OGP) using acid pretreatment and enzymatic hydrolysis. This study was based on a model composition corresponding to a 34 orthogonal factorial design and employed the response surface methodology (RSM) to optimize the pretreatment and hydrolysis conditions, aiming to attain maximum glucose, xylose and arabinose extraction from cellulose and hemicellulose of biomass. The pretreatment operating conditions considered for optimization, were temperature (60–180 °C), residence time (30–120 min) and sulphuric acid concentration (0.5–5% w w⁻¹). Enzymatic hydrolysis experiments on solid fraction pretreated with diluted acid were performed at a solid concentration of 5% (w v⁻¹, based on dry weight), using 50 mM citrate buffer pH 4.8 with BSA at a concentration of 60 mg g⁻¹ dry biomass. The reaction mixture was incubated at 50 °C for 174 h on an orbital shaker at 150 rpm. Three commercial enzyme preparations (cellulase complex, b-glucosidase and xylanase) were used in enzymatic saccharification. Total carbohydrate content of the initial biomass was 51.25% (in dry mass), of which glucose was the major constituent with 33.59%. Contents of lignin and extractable found in biomass were 24.96% and 15.84%, respectively. In this work, it was possible to extract 93.1% of the sugars present in the olive grove pruning, with pretreatments carried out for 102 min at 156 °C with a sulfuric acid load of 4.09% (w w⁻¹), followed by enzymatic hydrolysis performed for 174 h, with an enzyme loading of 18 PFU, 36 p-NPGU and 36 IU per gram of substrate.

Key words: acid hydrolysis, enzymatic hydrolysis, olive pruning, pretreatment, RSM, sugars.

INTRODUCTION

The production of sugars from lignocellulosic materials comprises two steps: pretreatment and enzymatic hydrolysis (Láinez et al., 2018; Nashiruddin et al., 2020). The pretreatment is an important step to reduce the recalcitrance of the biomass for the subsequent steps of enzymatic hydrolysis and fermentation (Zhu & Pan, 2010). This step, although essential, because without it the efficiency of enzymatic hydrolysis rarely exceeds 20%, is one of the most expensive steps for production of sugars from cellulosic wood materials due to energy consumption, the use of chemical substances, and the need to treat and reuse process water (Sun et al., 2016; Zhu & Pan, 2022).

The main purpose of acid pretreatment is to solubilize the hemicellulose fraction of biomass in order to make cellulose more accessible to enzymatic attack. Such pretreatments are usually accomplished with dilute acid ($< 4\% \text{ w w}^{-1}$), being the sulfuric acid the most widely used reagent. The application of dilute acid pretreatments appears as the most advantageous method for industrial applications being considered cheap and effective (Huang et al., 2021; Jehadin et al., 2021; Mankar et al., 2021). In addition to sulfuric acid, other pretreatments have been tested, such as hydrothermal, alkaline and oxidative (Woiciechowski et al., 2020; Sarker et al., 2021; Scapini et al., 2021; Zhou et al., 2023).

The hydrolysis of cellulose in lignocellulosic materials requires the application of three enzymatic components: endoglucanases, exoglucanases and β -glucosidases. The hydrolysis of hemicelluloses, due to their heterogeneity, with different sugar backbones with different backbone linkages and side groups, requires complex enzymatic systems. Improving this step of the process can be approached by increasing the accessibility of the substrate, as already mentioned, modifying its chemical structure, thus promoting the performance of the enzymes (Luo et al., 2019). The application of surfactants in lignocellulosic materials have been shown to promote a substantial increase of enzymatic hydrolysis efficiency (Zheng et al., 2021; Sánchez-Muñoz et al., 2022). In recent years new types of surfactants have emerged to improve enzymatic hydrolysis, such as proteins like the bovine serum albumin (BSA), one of the most used (Brondi et al., 2019), or polymers such as polyethylene glycol (PEG) (Nogueira et al., 2022).

Olive grove pruning waste is an agricultural residue widely available in Portugal. It is estimated that the amount of material produced annually from olive grove pruning could amount to 290,000 t per year.

The purpose of this paper was to optimize the acid pretreatment and hydrolysis process and investigate the effects of acid concentration, temperature and residence time on the production of sugars (glucose, xylose and arabinose) as well as on the formation of degradation products (furfural, 5-hydroxymethylfurfural and acetic acid).

The work carried out aims to increase the profitability of olive farms in Portugal by using residues from olive grove pruning, which are usually burned on farms, as a raw material, to produce added value compounds, such as ethanol, xylitol, lactic acid, among others.

MATERIALS AND METHODS

Olive tree pruning's samples were collected and harvested on the farm of Higher School of Agriculture of Castelo Branco (GPS: 39.8197117, -7.4964662, Alt. 361 m). The raw material was collected after the olive grove pruning, consisting of small branches (diameter less than 4 cm) and fresh leaves.

Raw material analysis

The raw material was prepared for further analysis according with the standard NREL / TP-510-42620 (Hames et al., 2005), which defines a material particle size of 0.180 and 0.850 mm. For this purpose, the raw material was ground in a slide mill (Retch Mühle - West Germany) and subsequently sieved using 0.180 mm (80 mesh) and 0.500 (35 mesh) sieves in accordance with the specifications of the American Society for Testing and Materials (ASTM E1757-19).

The determination of the extractable content was carried out according to the Tappi 204 om-88 standard (Sithole et al., 1991).

The ash determination was performed in accordance with the standard NREL / TP-510-42622 (Sluiter et al., 2005) and the incineration was carried out in muffle furnace, using a temperature of 525 ± 25 °C.

The content of insoluble and soluble lignin was determined according to the standard NREL / TP-510-42618 (Sluiter et al., 2008a).

The concentration of total reducing sugars present in the solutions of the treated substrates was determined using the Miller method (Miller, 1959).

For the quantitative analysis of individual sugars and degradation products, a high-performance liquid chromatography apparatus (High-Performance Liquid Chromatography - HPLC) was used. For these analysis, an Aminex-87P column (Bio-Rad, Hercules, CA) was used to determine individual carbohydrates, namely glucose, xylose, galactose, arabinose and mannose, according to the standard NREL / TP-510-42618 (Sluiter et al., 2008a). The Aminex-87H column (Bio-Rad, Hercules, CA) was used for determining degradation products, namely acetic acid, formic acid, hydroxymethylfurfural (HMF) and furfural, following the standard NREL / TP-510-42623 (Sluiter et al., 2008b).

Pretreatment

Batch reactions were carried out under different operating conditions and a total of 34 runs, corresponding to the orthogonal factorial design, with different combinations of the variables were used, according to a central composite rotatable design (CCDR) generated using Design Expert 7 Trial Version (Stat-Ease inc. minneapolis) (Table 1). The reaction media was sulfuric acid, in concentrations ranging from 0.5 to 5.00% (w w⁻¹, dry biomass), using a solid-liquid ratio of 1.0 g dried biomass/10 mL liquid on a final volume of 100 mL. The temperature ranged from 60 to 180 °C and reaction time from 30 to 120 min. Sulfuric acid in this pretreatment was added from a solution with a concentration of 5 g L⁻¹ previously prepared. The dependent variable studied in the hydrolysate resulting from the pretreatment was the total reducing sugars content.

After this process, the material was washed with distilled water until the pH of the slurry became neutral (pH 7), to remove traces of acid and inhibitors, such as formic acid, which inactivates xylanase (Panagiotou, 2007). The solid residue resulting from pretreatment was used without drying in the subsequent phase of enzymatic hydrolysis, as drying can lead to the collapse of the pores of plant material, making enzymatic hydrolysis more difficult and consequently considerably reducing its yield. (Hendriks & Zeeman, 2009; Zhang et al., 2004).

Enzymatic hydrolysis

Enzymatic hydrolysis was performed at a solid concentration of 5% (w v⁻¹, based on dry weight), using 50 mM citrate buffer pH 4.8 with BSA at a concentration of 60 mg g⁻¹ dry biomass. The reaction mixture was incubated at 50 °C for 174 h on an orbital shaker at 150 rpm (Ferreira et al., 2010; Wei et al., 2012).

For enzyme digestion three commercial enzyme from Novozymes (Denmark) were used. A cellulase complex with 148 FPU mL⁻¹ (NS22086), a b-glucosidase with 426 p-NPGU mL⁻¹ (NS22118) and a xylanase with 7498 IU mL⁻¹ (NS22083).

In each hydrolysis assay, two enzymatic loads were tested, one with 18 FPU / 36 p-NPGU / 36 IU per gram of biomass and the other with 6 FPU / 12 p-NPGU / 12 IU per gram of biomass. The enzymatic load of b-glucosidase and xylanase was twice that of the cellulase complex to avoid inhibition caused by the accumulation of cellobiose. (Lloyd & Wyman, 2005; Wyman et al., 2005; Kumar & Wyman, 2009).

Samples were taken at incubation times of 0 (used as control), 2, 4, 6, 24, 48, 72 and 174 h. The enzymatic hydrolysis was stopped by immediate freezing on ice and centrifugation at 5,000 g, 4 °C for 10 min. The supernatants were separated for subsequent analytical characterization and stored at -20 °C until analysis. The analysis of sugars released during hydrolysis was carried out using the DNS method (Miller, 1959). All the assays were performed at least in triplicates.

RESULTS AND DISCUSSION

Characterization of the raw material

The chemical composition of the olive grove pruning biomass, is shown in Table 2. The HPLC analysis of the carbohydrates present in the biomass revealed a presence of 51.15% of sugars, these being constituted mostly by glucose (33.59%) and xylose (13.11%). Lignin is the second most significant component in the constitution of the material used, representing about 25% of its dry weight. The results obtained are similar to

Table 1. Test conditions tested in pre-treatment with sulfuric acid

Temperature (°C)	Residence time (min.)	Sulfuric acid (% w w ⁻¹)	Runs/Samples n°
60	75	2.75	2
84	48	1.41	2
84	102	1.41	2
84	48	4.09	2
84	102	4.09	2
120	75	0.50	2
120	30	2.75	2
120	75	2.75	6
120	120	2.75	2
120	75	5.00	2
156	48	1.41	2
156	102	1.41	2
156	48	4.09	2
156	102	4.09	2
180	75	2.75	2
Total			34

those obtained by other authors for olive pruning characterisation (Ballesteros et al., 2011; Requejo et al., 2012; Barbanera et al., 2015; Díaz et al., 2023).

Pretreatment

Fig. 1 presents the chemical constitution of the hydrolysates, resulting from pretreatments with sulfuric acid, based on determinations carried out with HPLC. These results show that xylose and glucose were the sugars with the highest concentration

in the hydrolysates. The highest sugar release rate for the hydrolysate, 29.9%, occurred in pretreatments carried out at a temperature of 156 °C, an acid concentration of 4.09% and a residence time of 48 min. As it can be seen, no large variations were recorded in the solubilization rates of glucose and xylose in pretreatments carried out at a temperature below 156 °C. Below this temperature value, glucose solubilization varied between 3.0% and 5.7%, and xylose solubilization between 3.8% and 7.1%. The greatest increase in glucose and xylose solubilization occurred, in pretreatments carried out at a temperature of 156 °C, when the acid load was increased from 1.41% to 4.09%. In this situation, glucose solubilization more than doubled, going from 5.7% to 12.2%, while there was a simultaneous increase in xylose solubilization from 7.1% to 13.1%. With an acid load of 4.09% it is possible to obtain solubilization rates of 100% of xylose and arabinose in pre-treatments carried out at 156 °C. Above this temperature, the release rates of these sugars decrease due to the formation of a greater quantity of degradation products.

These results are in line with those obtained by other authors. Wei and collaborators achieved an increase in glucose solubilization from 3% to 13% when the temperature increased from 140 °C to 170 °C, in pretreatments carried out with sulfuric acid using wheat straw as a substrate (Wei et al., 2012). Yat and collaborators also found, after carrying out pretreatments with sulfuric acid, at temperatures between 160 and 190 °C, maximum glucose solubilization of only 13%, concluding that acid pretreatments were not efficient in solubilizing this sugar. However, under the same conditions these authors achieved solubilizations of around 94% for xylose (Yat et al., 2008). Shen & Wyman also achieved similar xylose solubilization rates (93.1%) in pretreatments carried out with sulfuric acid on corn stover for 40 minutes, at 160 °C and with an acid load of 0.5% (w w⁻¹) (Yat et al., 2008; Shen & Wyman, 2011).

Fig. 1 also shows that the increase in concentrations of by-products (BP) and degradation products (DP) was directly related to the increase in temperature, acid concentration and time pretreatment.

The BP and DP are produced, in greater quantities, in pretreatments carried out at temperatures equal to or greater than 156 °C. The production of BP and DP increases from 5.6 to 6.2% when we increase the temperature from 156 °C to 180 °C even using lower acid concentrations and shorter pretreatment times.

Table 2. Chemical composition of raw material

Composition	Quantities (%) ± SD ^a
Glucose	33.59 ± 2.25
Xilose	13.11 ± 1.02
Arabinose	4.55 ± 0.20
Galactose	n.d.
Manose	n.d.
Acetic acid	3.53 ± 0.43
Insoluble acid lignin	18.07 ± 0.33
Soluble acid lignin	6.89 ± 0.06
Extractables	15.84 ± 0.44
Ashes	3.76 ± 0.01

^a Composition expressed on a dry basis; n.d. – not detect.

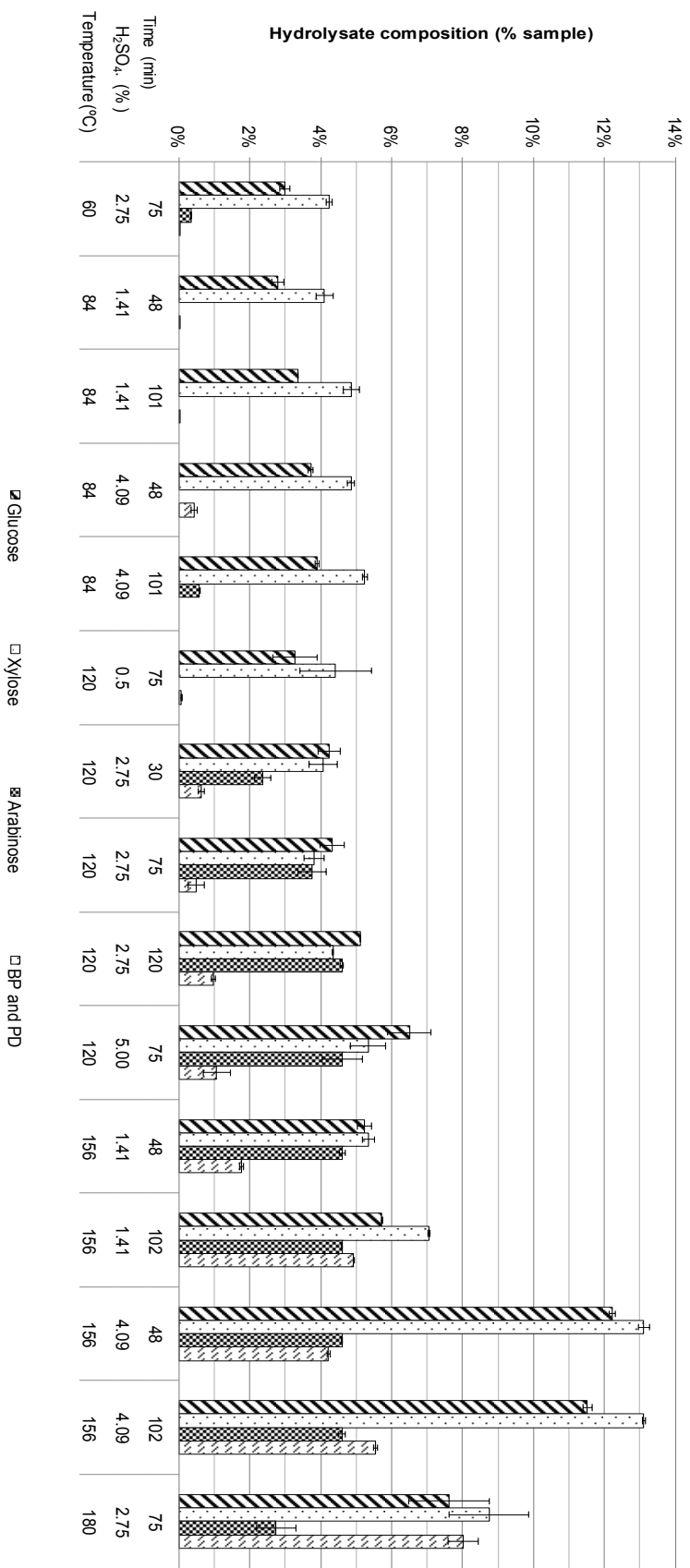


Figure 1. Chemical composition of hydrolysates resulting from pretreatment with sulfuric acid (in % of sample).
 * BP – By-products; DP – Degradation Products.

At 156 °C the production of BP and DP increases with the acid concentration and pretreatment time. In this temperature class, pretreatments carried out for 48 min with an acid concentration of 1.41% produced 1.8% BP and PD. However, when are increased the pretreatment time to 102 min and the acid concentration to 4.09%, the formation of BP and DP rise to 5.6%.

Cara et al., in 2008, with olive tree pruning, obtained a xylose solubilization rate of 97.3% in pretreatments with sulfuric acid at 210 °C, with an acid load of 1.4% (w w⁻¹) and for 10 min. However, under these conditions the production of BP and DP were 10.4% (Cara et al., 2008).

Kim et al., in 2011, obtained a xylose removal rate of 87.4% from yellow poplar wood in pretreatments carried out at 160 °C, with 3.7% (w w⁻¹) oxalic acid for 40 min, in which the production of 5-HMF and furfural was 0.10 g L⁻¹ and 0.66 g L⁻¹, respectively. However, when the pretreatment temperature was increased to 187 °C, even with a lower acid load of 2.5% (w w⁻¹) and for a shorter time, 30 min, the xylose removal rate increased to 96.3%, but the production of 5-HMF and furfural rise to 0.87 g L⁻¹ and 4.15 g L⁻¹, respectively (Kim et al., 2011).

The inhibitors, especially furfural and 5-hydroxymethylfurfural, have a severe effect on the microorganisms used for the fermentation process including a breakdown of DNA and reduced RNA synthesis, resulting in hampered enzymatic activity (Solarte-Toro et al., 2019; Woiciechowski et al., 2020).

From the results expressed above, it can be conclude that the conciliation between the solubilization of hemicelluloses and a minimum production of degradation products can be achieved with pretreatment temperatures around 150–160 °C, even with higher acid loads.

Enzymatic hydrolysis

As it can be observed in Table 3, for the same enzymatic concentration, increasing any of the factors used, temperature, concentration and pretreatment duration, has a positive effect on the enzymatic hydrolysis (EH) promoting the sugar release rate (SRR).

Table 3. Enzymatic hydrolysis yield (% sugars present in the pretreated solid residues)

Pre-treatment conditions			Enzymatic hydrolysis time (h)					
A	B	C	Enzymatic load ¹	12	24	48	72	174
156	48	1.41	6-12-12	11.7 ± 0.5	18.5 ± 1.1	19.5 ± 0.4	21.5 ± 0.6	25.3 ± 0.7
156	48	1.41	18-36-36	19.4 ± 0.1	25.0 ± 0.5	28.5 ± 0.4	32.6 ± 0.2	40.8 ± 1.6
156	102	1.41	6-12-12	11.6 ± 0.6	17.6 ± 1.1	20.3 ± 0.4	21.8 ± 1.0	26.4 ± 0.3
156	102	1.41	18-36-36	19.9 ± 0.9	29.8 ± 0.1	30.8 ± 0.7	36.2 ± 0.3	46.9 ± 1.4
156	48	4.09	6-12-12	9.9 ± 0.8	17.8 ± 0.5	22.4 ± 0.3	24.3 ± 0.3	34.8 ± 1.5
156	48	4.09	18-36-36	18.5 ± 0.5	31.5 ± 1	38.7 ± 0.6	51.6 ± 0.2	70.9 ± 1.0
156	102	4.09	6-12-12	9.8 ± 0.2	19.0 ± 0.8	25.0 ± 0.5	30.9 ± 0.9	44.8 ± 0.5
156	102	4.09	18-36-36	23.4 ± 0.4	33.1 ± 1.0	46.9 ± 0.2	64.3 ± 0.7	84.4 ± 3.0
180	75	2.75	6-12-12	12.8 ± 0.5	22.5 ± 0.2	30.4 ± 0.6	38.9 ± 0.7	57.1 ± 0.6
180	75	2.75	18-36-36	28.8 ± 0.5	42.2 ± 1.6	54.5 ± 1.0	71.4 ± 1.0	83.6 ± 2.3

A – Temperature (°C); B – Time (min); C – Concentration of H₂SO₄ (% w w⁻¹); ¹ – Enzymatic load: FPU g⁻¹ substrate – p-NPGU/g substrate – IU g⁻¹ substrate.

After 72 h of reaction, the highest EH yield were achieved with pretreatments carried out at the highest temperature (180 °C), reaching a SRR of 67% in tests performed with 18 FPU, 36 p-NPGU and 36 IU per gram of substrate. The results obtained after 72 h of enzymatic hydrolysis are lower than those obtained by other authors, possibly due to the use of lower pretreatment temperatures in this study. Cara and collaborators, using the same raw material and the same time duration of EH, achieved a SRR of 76.5% from pretreatments carried out at 210 °C, with a 1.4% sulfuric acid for 10 min with a enzyme loading of 15 FPU and 15 IU per gram of substrate (Cara et al., 2008).

It can also be observed that the release rates did not stabilize after 72 h of reaction, continuing to increase in all tests carried out. With material resulting from pretreatments carried out at 156 °C, for 102 min and with 4.09% H₂SO₄, it was observed an increase of 20.7%, from 64.3% to 84.0%, in tests carried out between 72 h and 174 h of enzymatic reaction and with higher enzyme concentration (18 PFU per gram of substrate, 36 p-NPGU per gram of substrate and 36 IU per gram of substrate).

Overall process yields

The greatest amount of sugars released, considering the two stages of the process, pretreatment and enzymatic hydrolysis, was obtained with pretreatments carried out at 156 °C, with a sulfuric acid load of 4.09% and for 102 min. Under these conditions, during the pretreatment was possible to solubilize 29.18 g of sugars per 100 g of raw material. The enzymatic hydrolysis process that follows, after 72 h, was able to release 13.46 g of sugar per 100 g of raw material. The sum of sugars released in pretreatment and EH corresponds to 83,2% of sugars content in the sample (42.64 g of sugars per 100 g of raw material). Using 174 h in EH, the release of sugars increased to 18.54% leading to a combined yield of 93.1%, considering the two stages, corresponding this value to a release of 47.72 g of sugars per 100 g of raw material.

Cara and collaborators, obtained a maximum of 36.3 g of sugars per 100 g of raw material from pretreatments carried out at 180 °C, with a 1.4% sulfuric acid during 10 min and an enzymatic hydrolysis performed for 72 h, with an enzyme loading of 15 FPU and 15 IU per gram of substrate (Cara et al., 2008). Also, in a study performed by Martínez-Patiño and collaborators, it was obtained an overall yield of 39.8 g total sugars/ 100 g extracted from olive tree biomass, with pretreatments carried out at 160 °C, with 4.9% sulfuric acid and a residence time of 10 min, using an enzyme loading of 15 FPU and 15 IU per gram of substrate (Martínez-Patiño et al., 2017).

CONCLUSIONS

This work shows that residues from olive grove pruning, with a sugar content of 51.15%, can play a relevant role in sugar production, transforming waste from olive grove maintenance into raw material likely to generate wealth and employment.

In this work, it was possible to extract 93.1% of the sugars present in the olive grove pruning, with pretreatments carried out for 102 min at 156 °C with a sulfuric acid load of 4.09% (w w⁻¹), followed by enzymatic hydrolysis performed for 174 h, with an enzyme loading of 18 PFU, 36 p-NPGU and 36 IU per gram of substrate.

The yield obtained in this process opens possibilities for the use of this agricultural residue, currently without any commercial use, as raw material for the extraction of sugars and subsequent conversion into value-added products for the food, pharmaceutical and

fuel production industries, through production of ethanol, xylitol, lactic acid, and furfural (Gírio et al., 2010; Seidl & Goulart, 2016; Attard et al., 2020; Gonçalves et al., 2022).

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