

## Article

# Single-Stage Fractionation of Vine Shoots Using Microwave Heating

Sandra Rivas and Juan Carlos Parajó \*

Department of Chemical Engineering, University of Vigo (Campus Ourense), 32004 As Lagoas, Spain; sandrarivas@uvigo.es

\* Correspondence: jcparajo@uvigo.es; Tel.: +34-988-387033

**Abstract:** Vine shoots are agricultural residues that can be used as a raw material in agro-biorefineries, in which their main constituents can be individually converted into valuable bioproducts. The treatment of vine shoots in uncatalyzed media containing water and 1-butanol enabled the single-stage separation of the major vine shoots constituents in different phases: the aqueous phase from treatments contained hemicellulose-derived products (mainly in the form of oligosaccharides), the organic phase accumulated the dissolved lignin, and the cellulosic fraction was recovered in solid phase. The aqueous phase from treatments was refined using membranes and processed with enzymes to obtain a refined product (RP) containing 92.2 g of oligosaccharides/100 g of non-volatile compounds. The oligosaccharides were mainly composed of anhydroxylose units substituted by acetyl and uronic groups. Enzymatic hydrolysis of RP with endo-xylanases reduced the average degree of polymerization to 2–3, which are preferred for application as healthy food ingredients. The solid phase from treatments was used as a substrate for enzymatic hydrolysis, enabling the production of solutions containing 34.9 g glucose /L and 4.2 g xylose /L.

**Keywords:** agro-residues; vine shoots; green solvents; enzymatic hydrolysis; membrane processing; cellulose; hemicelluloses; bioproducts; oligosaccharides; glucose

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## 1. Introduction

Agro-biorefineries represent a sustainable approach to manufacture bio-based products from lignocellulosic agricultural residues, operating in clean and efficient processes enabling the separation of their main constituents.

The worldwide area under vines was estimated at 7.4 million ha in 2018, with 13% of the world vineyard corresponding to Spain [1]. Vine shoots are agricultural wastes generated in vineyards at an estimated annual rate of 2–4 tons/ha [2], which are often abandoned in the field or burned, causing a number of ecological and environmental problems [3,4]. Owing to their large availability and compositional features, vine shoots are a potential resource for manufacturing a scope of bioproducts, such as lactic acid, xylitol [5], oligosaccharides (OS) [6], ethanol [7,8], biogas [9], glucose and lignin [10].

The major hindrance for using lignocellulosic biomass as an industrial raw material lies on its complex and heterogeneous structure, defined by the presence of interpenetrated polymers (hemicelluloses, cellulose and lignin, named “structural components”) with different chemical and physicochemical properties. For example, lignin is an aromatic polymer that can be dissolved by selected organosolvents, hemicelluloses are ramified polysaccharides of limited polymerization degree susceptible to hydrolysis reactions, and cellulose is a polysaccharide composed of anhydroglucose units that presents a limited susceptibility to hydrolysis. The vine shoots composition (referred to the structural components) is as follows: cellulose, 33–36 wt% [5,8]; hemicelluloses, 19–23 wt%; and lignin, 27.1–26.6 wt% [4,8].

The fractionation of lignocellulose involves the breakdown of the complex interlinks between the structural components, improving the accessibility of the fractionation agents to cellulose and hemicelluloses [11]. Usually, this goal is achieved by consecutive treatments, which allow an integral benefit of the feedstock with minimal or no waste generation [12,13]. A number of conversion techniques have been considered for this purpose [14–16], and several have been applied to vine shoots, including hydrothermal processing [6,8], acid hydrolysis [5], delignification with alkaline solutions or organosolvents [17] and enzymatic methods [4].

Organic solvents (pure or in mixtures with water, eventually in the presence of a catalyst), are suitable agents for lignocellulose fractionation: in typical treatments, lignin and hemicelluloses are partially dissolved and transferred to the liquid phase, whereas cellulose remains in solid phase. Although ethanol, glycerol and acetone are the most used solvents, 1-butanol represents an interesting alternative, owing to its partial water miscibility [18]. This characteristic allows the one-step fractionation of lignocellulose into three streams containing products derived from the structural components: lignin is dissolved in the organic phase, hemicellulose-derived products appear in the aqueous one, and cellulose is kept in solid phase. Recently, studies have been reported on biomass fractionation using partially immiscible solvents [19–21]. 1-butanol is considered as a green solvent that can be produced from biomass [22].

Efficient fractionation processes must provide an extensive lignin solubilization, yielding products that keep the structure and properties of the original polymer [23], and providing solids with enhanced cellulose contents. Additionally, the chemical treatment should be able to reduce the crystallinity of cellulose, increasing the available surface area, and enhancing the cellulose susceptibility to hydrolysis [24]. The enzymatic hydrolysis of cellulosic solids from fractionation leads to glucose solutions suitable as fermentation media for obtaining bioproducts such as ethanol or lactic acid [5,25].

The partial hydrolysis of hemicelluloses occurring in the aqueous phase leads to the formation of polysaccharide fragments (low molecular polysaccharides and/or OS) as main products [12]. OS find a number of applications in the food and pharmaceutical sectors, related to their bioactivity (for example, as prebiotics). The International Scientific Association for Probiotics and Prebiotics (ISAPP) defined “prebiotic” as “a substrate that is selectively utilized by host microorganisms, conferring a health benefit” [26]. The literature on the manufacture of OS with prebiotic properties from vine shoots indicates that OS are constituted by backbones of pentoses with different substitution patterns by hexoses and acetyl groups [6]. Besides OS, the aqueous phases contain undesired compounds for prebiotic manufacture, such as monosaccharides and non-saccharide compounds, which limit the purity of the final product [12,13].

Diverse methods have been reported on the manufacture and refining of hemicellulosic OS, including the enzymatic depolymerization into short chain OS, which are preferred for applications as food additives or nutraceuticals [27]. Additionally, liquid-liquid extraction, ion exchange or membrane filtration have been employed as purification treatments [28,29].

This work handles a single stage fractionation method based on water/1-butanol reaction media, which was able to separate the structural constituents of vine shoots (intact or as derived products) in separate streams, allowing their individual utilization. Specifically, the experimental duties considered in this study include: (i) compositional study of the substrate; (ii) fractionation under diverse experimental conditions and distribution of products among the process streams; (iii) selection of optimal reaction conditions based on the amount of oligosaccharides generated from hemicelluloses; (iv) in-depth evaluation of the composition hemicellulose-derived products; (v) DP tailoring of oligosaccharides by xylanases and purity assessment; and (vi) evaluation of the cellulose-enriched solid from processing as a substrate for glucose manufacture by enzymatic hydrolysis.

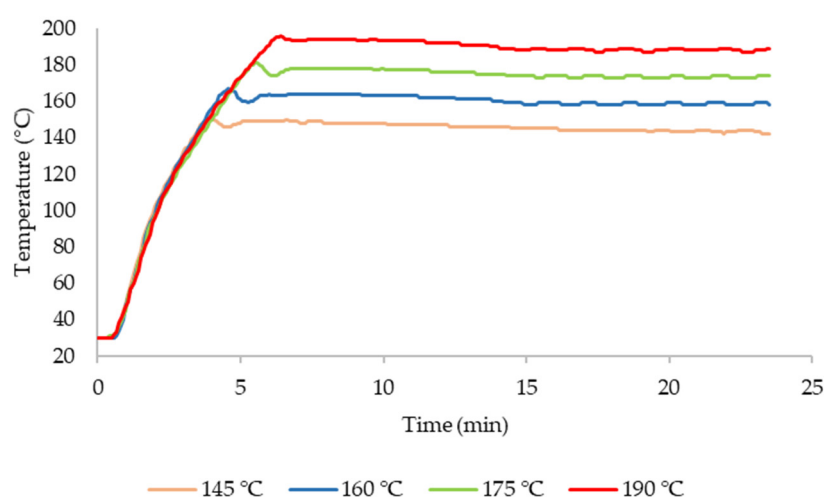
## 2. Materials and Methods

### 2.1. Preparation of Pretreated Vine Shoots

Vine shoots were kindly provided by a local producer from Ribeiro (Ourense, Spain), air dried, milled and screened to select the particles with size in the range of 0.5–2 mm. Samples were subjected to a mild aqueous extraction (130 °C) under non-isothermal conditions to remove water-soluble extractives while avoiding the dissolution of cellulose, hemicelluloses and lignin. The aqueous extraction was performed in a Parr reactor (Moline, IL, USA) at a liquid to solid ratio (LSR) of 10 g water/g of oven-dry vine shoots. After extraction, the media were filtered, and the extracted vine shoots were analyzed and used as fractionation substrates.

### 2.2. Fractionation Treatments

Water-extracted vine shoots were reacted with uncatalyzed water/1-butanol mixtures in a microwave accelerated reaction system (MARS 6, CEM Corporation, Matthews, NC, USA). The following operational variables were fixed: LSR, 12 mL/g; 1-butanol/water, 30/70 v/v; and reaction time, 20 min. The reaction temperature was employed as an operational variable within the range 145–190 °C. Figure 1 shows the temperature profiles followed in experiments.



**Figure 1.** Temperature profiles corresponding to fractionation treatments performed in the microwave reactor.

After treatments, the solid and liquid phases were separated by vacuum filtration. The processed solids, enriched in cellulose, were washed first with 1-butanol and then with distilled water, and assayed for composition and solid yield (SY). The aqueous phases, containing oligosaccharides, were analyzed (see below) and employed for DP tailoring and purification. The organic phases were separated by decantation and concentrated by evaporation. The distilled fraction, rich in 1-butanol, was condensed and reutilized in subsequent experiments. The liquid from distillation was mixed with water to induce lignin precipitation, centrifuged and dried in a vacuum oven at 40 °C. The aqueous phases were analyzed (see below) and employed for further processing for DP tailoring and purification.

The discussion of results is made in terms of the following variables: solid yield (%SY), delignification percentage (%D), percentage of hemicellulose removal (%H<sub>r</sub>), and cellulose content of processed solids (%C, expressed as wt%). These variables were defined as follows:

$$\%SY = 100 \times \frac{S_A}{S_B} \quad (1)$$

where  $S_A$  and  $S_B$  are the dry weights of the solids after and before fractionation treatment,

$$\%H_r = \frac{H_B - H_A \cdot \frac{SY}{100}}{H_B} \times 100 \quad (2)$$

where  $H_B$  and  $H_A$  are the weight percentages of hemicelluloses in the solids before and after fractionation treatment,

$$\%D = 100 \times \frac{L_B - L_A \cdot \frac{SY}{100}}{L_B} \quad (3)$$

where  $L_B$  and  $L_A$  are the weight percentages of total lignin (including Klason lignin and acid soluble lignin) in the solids before and after fractionation treatment,

$$\%C = 100 \times \frac{C_A}{S_A} \quad (4)$$

where  $C_A$  is the cellulose content of the solids from fractionation, expressed as wt%.

### 2.3. Refining of the Aqueous Phase from Fractionation Treatment

The aqueous phase from the fractionation treatment leading to the maximal concentration of OS (denoted as AqO) was refined with membranes operating in discontinuous diafiltration with volume reduction mode (DDVR), and to enzymatic processing. Figure 2 shows the scheme followed for AqO refining.

#### 2.3.1. Membrane Processing

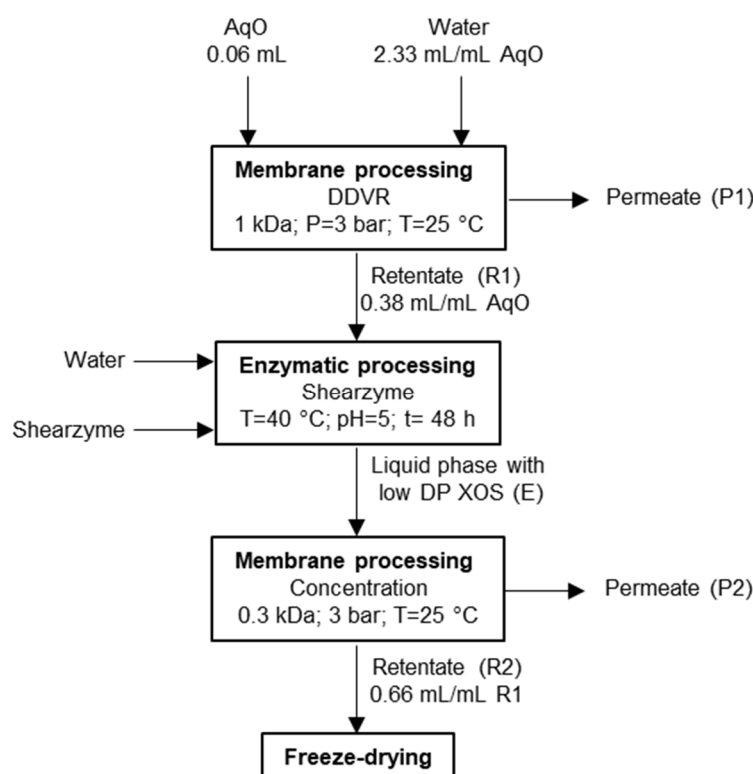
Membrane processing was performed in a 200 mL stirred Amicon Cell (Millipore) with a filtration area of 28.7 cm<sup>2</sup>, operating at a transmembrane pressure of 3 bar and room temperature. Operation was carried out as follows:

- DDVR of AqO was performed using a Millipore membrane of 1 kDa molecular weight cut-off (MWCO). Water was added at a volumetric ratio of 2.33 mL/mL AqO, and operation was continued until the volume of retentate 1 (denoted R1) corresponded to 38% of the feed volume;
- Concentration of the solution coming from the enzymatically processing of R1 (denoted E) was performed using a 0.3 kDa MWCO membrane (GE Osmonics Inc., Minnetonka, MN, USA). Operation continued until the volume of retentate (denoted R2) corresponded to 66% of R1.

#### 2.3.2. Enzymatic Processing

The treatments were performed using commercial endo-1,4-β-xylanases (Shearzyme from Novozymes, Spain). The enzymatic activity of the commercial solution (1000 U/mL of enzyme) was determined as per Vegas et al. [28]. R1 was diluted with water (1.10 kg of water/kg R1) and treated with enzymes under the following operational conditions: enzyme charge, 0.4 U/mL R1; temperature, 40 °C; pH, 5; and duration, 48 h. Experiments were performed in Erlenmeyer flasks with orbital agitation. The solution resulting from enzymatic processing, containing low DP OS, was named E.

The streams involved in the refining stage (AqO, R1 and R2) were assayed for composition and structure by HPLC, HPSEC, HPAEC-PAD and MALDI-TOF-MS as described below.



**Figure 2.** Scheme considered for refining the oligosaccharides present in the aqueous phase from fractionation (AqO).

#### 2.4. Enzymatic Hydrolysis of the Processed Solid from Fractionation Treatment

The solids (denoted PSO) obtained in the fractionation treatment with under the conditions leading to the maximum OS concentration were subjected to enzymatic hydrolysis using the commercial concentrate Cellic CTec2 (from Novozymes, Denmark). The activity of the Cellic CTec2 concentrate (determined as *per* Sun et al. [30]) was 137 Filter Paper Units (FPU)/g of the commercial preparation. Experiments were performed at 48.5 °C in Erlenmeyer flasks kept in an orbital incubator (150 rpm). The experiments were conducted for 0–96 h at a LSR of 15 g/g PSO and pH 4.85 (adjusted with citrate buffer). The enzyme-substrate ratios (ESR) were fixed at 10, 15 or 20 FPU/g PSO. The cellulose conversion into glucose was calculated as the percentage of glucose present in liquid phase respect to the potential glucose (resulting from stoichiometric cellulose hydrolysis). The conversion of xylan into xylose was calculated using the same method. Experiments were performed by triplicate.

#### 2.5. Analytical Procedures

The compositions of the feedstock and processed solid samples were assayed according to the following standard methods: extractive content, NREL/TP-510-42619; polysaccharides and Klason lignin, NREL-510-42618; and ash, NREL/TP-510-42622 [31–33]. The method NREL-510-42618 consisted of a two-step quantitative acid hydrolysis, performed with 72% and 4% H<sub>2</sub>SO<sub>4</sub>, separately, and led to an insoluble lignin residue (Klason lignin, KL) and to a liquid phase. KL was measured gravimetrically after oven drying at 105 °C. Acid soluble lignin (ASL) was determined spectrophotometrically at 205 nm. Samples from the liquid phase were analyzed by HPLC using an Agilent 1200 series instrument (Agilent Technologies, Santa Clara, CA, USA), fitted with a refractive index detector (RID). Aliquots were assayed for monosaccharides, acetic acid, and furans (furfural and hydroxymethylfurfural, HMF) using a 300 × 7.8 Aminex HPX-87H column (BioRad Life Science Group, Hercules, CA, USA) kept at 50 °C and eluted with 0.003 N H<sub>2</sub>SO<sub>4</sub> at 0.6

mL·min<sup>-1</sup>. Additional aliquots were neutralized with BaCO<sub>3</sub> and assayed for monosaccharides (glucose, xylose, galactose, arabinose and mannose) using a CARBOsep CHO-682 column (Transgenomic Inc. Omaha, NE, USA) kept at 80 °C, using water as a mobile phase (flow rate, 0.4 mL·min<sup>-1</sup>). Analyses were performed in triplicate.

Aqueous samples from the fractionation, membrane and enzymatic stages were subjected to quantitative post-hydrolysis (4% of H<sub>2</sub>SO<sub>4</sub> at 121 °C). The OS concentrations and their degree of substitution with acetyl groups (AG) were calculated from the increases in the amounts of monosaccharides and acetic acid upon post-hydrolysis. The content of total non-volatile compounds (NVC) was measured by oven-drying at 105 °C. The content of the fraction named “other non-volatile compounds” (ONVC) was calculated as the difference between NVC and the non-volatile compounds identified by HPLC. Analyses were performed in triplicate.

Hemicellulose-derived products in the aqueous phase from fractionation, membrane processing and enzymatic treatments were characterized using the following methods:

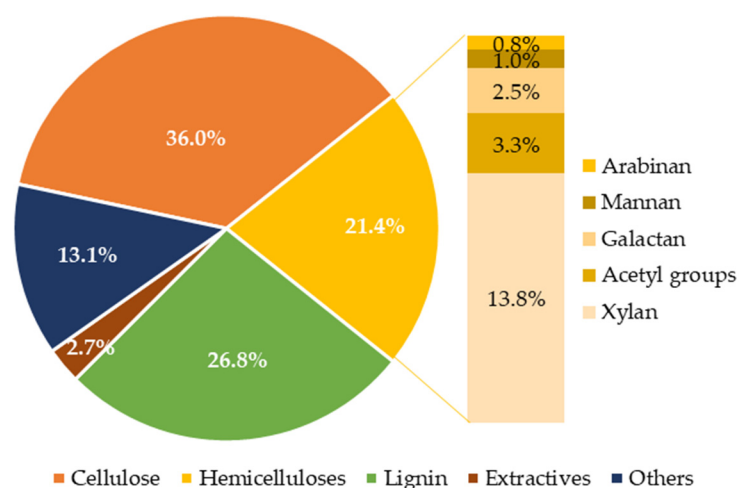
- HPLC, to assess the type and amount of OS structural units and substituents;
- High performance size exclusion chromatography (HPSEC), to assess the DP distribution of OS;
- High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), to provide a quantitative assessment on the small-DP OS derived from hemicelluloses;
- Matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF-MS) to elucidate the DP and substitution pattern of OS composed of backbones containing between 3 and 7 anhydroxylose units.

HPLC was performed using the method indicated above. HPSEC was conducted with two TSK Gel G3000PWXL and G2500PWXL columns in series combined with a PWX-guard column (Tosoh bioscience, Stuttgart, Germany) [34]. HPAEC-PAD was conducted using an ICS3000 chromatographic system (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac PA guard column and a CarboPac PA-1 column [35]. MALDI-TOF-MS analyses were performed using an Autoflex III Smartbeam Mass Spectrofotometer (Bruker Daltonics, Bremen, Germany). The spectra were acquired and treated using the Flex control 3.0 and Flex-Analysis 3.0 software (Bruker Daltonics) [36].

### 3. Results and Discussion

#### 3.1. Composition of Water-Extracted Vine Shoots

The average composition of extracted vine shoots is shown in Figure 3. Hemicelluloses were mainly composed of xylan (13.8%), acetyl groups (3.3%), galactan (2.5%) and minor amounts of mannan and arabinan. Although water extractives were mainly removed in the aqueous pretreatment, 2.7% extractives were still present in the pretreated vine shoots. Other components not quantified (protein, ashes, etc.) are not relevant for the purposes of this work and were accounted jointly by difference.



**Figure 3.** Average composition of water-extracted vine shoots, expressed as g of component per 100 g oven-dried material.

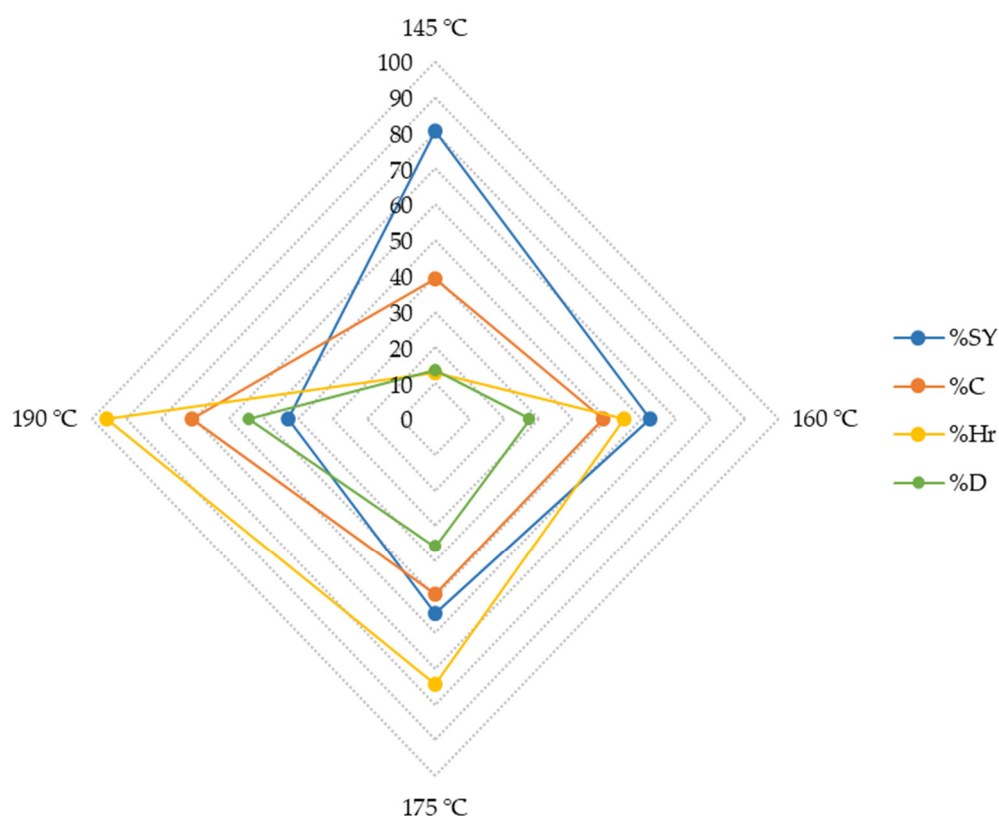
### 3.2. Fractionation

Treatments performed in media containing water/1-butanol mixtures enable the selective separation of hemicelluloses in the aqueous phase and lignin in the organic one [19–21,24]. Additionally, the organic phase can be processed to recover the organic solvent to separate the lignin as a solid material for further valorization [37].

Figure 4 shows the results achieved in fractionation treatments performed at different temperatures in terms of the variables %SY, %C, %Hr and %D defined above.

As a general trend, %SY decreased steadily with temperature, from more than 80% at 145 °C to 42.7% at the highest temperature assayed, mainly due to the solubilization of hemicelluloses and lignin. %Hr was near 100 at 190 °C, although significant hemicellulose solubilization (74.5%) was reached at 175 °C. At the mildest temperature assayed, %Hr hardly reached 13%. Similarly, %D increased with temperature, reaching values of 36% and 54.3% at 175 and 190 °C, respectively.

The cellulose content of the solids from fractionation (measured by %C) increased with temperature due to the lower susceptibility of this fraction to hydrolysis reactions. The highest value of %C (71%) was reached at the highest temperature assayed. The type of effects achieved by the treatments were in the range reported for vine shoot fractionation using other processing methods. Alves et al. [4] employed a two-stage method for vine shoot utilization: in the first step, the substrate was treated with dilute sulfuric acid at 120 °C for 30 min to dissolve 68.7% of the hemicellulose fraction, achieving a solid containing 75% cellulose with poor susceptibility to enzymatic hydrolysis. Obtaining a suitable hydrolysis substrate required a further treatment with 2% NaOH at 100 °C, conditions under which an almost complete conversion was obtained, no matter of the high lignin content (31.9%) of the material. Dávila et al. [17] also needed two chemical treatments for achieving a complete fractionation vine shoots: the first step, conducted to remove hemicelluloses, corresponded to a hydrothermal reaction conducted at 201 °C; whereas the second one consisted of a treatment with 8% NaOH at 124 °C, which provided a solid containing 55.4% cellulose and 33.8% lignin. Jiménez et al. [38] subjected vine shoots to one-stage processing in alkaline media (containing NaOH or mixtures of Na<sub>2</sub>S and NaOH) or organic solvents (ethanol or ethylene-glycol), looking for cellulose-enriched solids. In this study, the utilization of hemicelluloses was not considered. The cellulose and lignin contents of the treated solids were as follows (method/cellulose wt%/lignin wt%: NaOH/70.00/24.07; Na<sub>2</sub>S + NaOH/73.74/17.18; ethanol/53.70/31.92; ethylene glycol/60.22/35.26).



**Figure 4.** Solid yield (%SY), percentage of cellulose in the processed solid (%C), percentage of hemicellulose removal (%Hr) and percentage of delignification (%D) achieved in fractionation treatments performed at diverse temperatures.

Concerning the solubilization of hemicelluloses, Table 1 shows the effects of the fractionation treatment on the composition of the hemicellulose-derived compounds present in the aqueous phase. The NVC fraction, calculated as the joint contribution of OS, acetyl groups (AG), monosaccharides and ONVC, increased with temperature up to 175 °C, and then decreased, which is ascribed to the generation of volatile compounds (such as acetic acid and furans) from their respective non-volatile precursors. OS (including AG substituents) reached the highest concentration (18.67 g/L) at 175 °C. Xylooligosaccharides (XOS) were the most important OS components, reaching a maximum concentration (13.1 g/L) at the same temperature, and were partially decomposed into xylose and furfural (denoted F, originated from pentose dehydration) under harsher conditions. The presence of glucooligosaccharides (GOS) in the reaction media confirmed the solubilization of a small fraction of low-molecular weight hexosans. GOS were converted into glucose, which was dehydrated into hydroxymethylfurfural (HMF). Galactooligosaccharides (GaOS), arabinooligosaccharides (AOS) and mannoooligosaccharides (MOS) were found in minor amounts and showed a behavior similar to the one described for XOS, with maximal generation at 175 °C and further conversion into monosaccharides and dehydration products. The AG in OS reached concentrations up to 2.35 g/L (in the experiment performed at 175 °C). Harsher conditions caused the cleavage of AG, yielding acetic acid. The concentrations of the volatile compounds (HMF, F and acetic acid) increased with temperature, as a result of the reactions indicated above.



**Table 1.** Effects of fractionation treatments on the concentrations of the reaction products.

Composition (g/L)	145 °C	160 °C	175 °C	190 °C
<b>NVC</b>				
Glucose	0.07 ± 0.00	0.10 ± 0.01	0.23 ± 0.01	0.38 ± 0.02
Xylose	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.01	3.56 ± 0.21
Galactose	0.00 ± 0.00	0.00 ± 0.00	0.30 ± 0.02	0.00 ± 0.00
Arabinose	0.18 ± 0.02	0.25 ± 0.02	0.25 ± 0.01	0.00 ± 0.00
Mannose	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
GOS	4.32 ± 0.22	4.42 ± 0.25	1.44 ± 0.10	1.79 ± 0.08
XOS	0.89 ± 0.06	7.96 ± 0.09	13.1 ± 0.11	2.14 ± 0.20
GaOS	0.97 ± 0.10	1.46 ± 0.04	1.03 ± 0.04	0.00 ± 0.00
AOS	0.81 ± 0.05	0.59 ± 0.03	0.25 ± 0.02	0.00 ± 0.00
MOS	0.17 ± 0.02	0.10 ± 0.00	0.50 ± 0.03	0.00 ± 0.00
AG	0.40 ± 0.02	2.20 ± 0.12	2.35 ± 0.03	1.66 ± 0.08
ONVC	3.66 ± 0.23	5.53 ± 0.15	6.78 ± 0.22	9.57 ± 0.45
<b>Volatile compounds</b>				
Acetic acid	0.20 ± 0.02	0.71 ± 0.02	1.41 ± 0.04	2.21 ± 0.09
HMF	0.00 ± 0.00	0.03 ± 0.00	0.15 ± 0.02	0.28 ± 0.02
F	0.00 ± 0.00	0.04 ± 0.00	0.08 ± 0.01	0.75 ± 0.05

NVC: non-volatile compounds; GOS: glucose units in oligosaccharides (OS); XOS: xylose units in OS; GaOS: galactose units in OS; AOS: arabinose units in OS; MOS: mannose units in OS; AG, acetyl groups linked to OS; ONVC: other non-volatile compounds; HMF: hydroxymethylfurfural; F: furfural.

### 3.3. Refining of OS

Since the fractionation treatments performed at 175 °C provided the maximum OS concentration, this temperature was selected for further experimentation. The corresponding aqueous phase (AqO) contained both OS and undesired, volatile compounds and non-volatile components making part of the ONVC fraction. Additionally, AqO also contained 1-butanol because of its partial miscibility. The purification of hemicellulosic OS was conducted using a number of separation methods, including membranes. OS refining using membranes was applied to vine shoots [6] and to other types of biomass [12,13,34]. Membranes have been also employed for separating 1-butanol from the aqueous phase obtained in the organosolv treatment of sorghum bagasse [20].

Streams AqO, R1 and R2 in Figure 2 show the compositions indicated in Figure 5. The OS in AqO were mainly composed of anhydroxylose structural units. AG were present in XOS at a molar ratio 0.45:1 respect to xylose. In comparison, minor amounts of GOS, GaOS, AOS and MOS were also found in the aqueous media. The volumetric OS concentration (including substituents) reached 18.67 g/L. Monosaccharides (0.86 g/L) and ONVC (6.78 g/L) were contaminants, whose concentrations should be reduced. The volatile compounds (acid acetic, HMF and F) achieved a joint concentration of 1.64 g/L, whereas 1-butanol was found in the aqueous media at a high concentration (68.7 g/L).

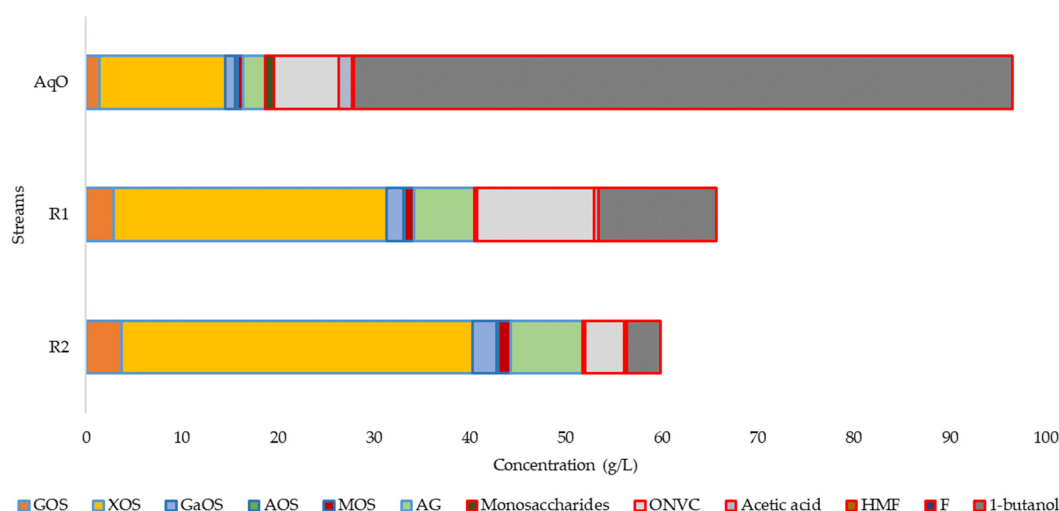
The first step of membrane processing (DDVR) allowed to remove 86.7 % of the 1-butanol present in the permeate P1 (see Figure 5), leaving a retentate (R1) containing 12.28 g 1-butanol/L together with monosaccharides and volatile compounds. However, the ONVC concentration after DDVR increased up to 12.23 g/L, almost proportionally to the increase observed for the concentration of total OS content (which reached 40.41 g/L). The XOS concentration increased remarkably (up to 28.46 g/L), with an almost proportional increase of the AG concentration. The DDVR stage performed using the 1kDa membrane enabled to recover in R1 of more than 90% of OS contained in AqO, increasing the OS content from 71 g OS/100 g NVC in AqO up to 76.3 g OS/100 g NVC.

Rivas et al. [34] reported on the enzymatic hydrolysis of OS from different sources for obtaining of low molecular weight oligomers. Following this idea, R1 was treated with

Shearzyme endo-xylanases (see Figure 2) for decreasing the average DP of XOS. This stage proceeded without xylose generation. In a related study, a similar behavior was reported for the enzymatic hydrolysis of rice husk xylooligosaccharides [28].

The solution resulting from the xylanase processing of R1 was processed in a 0.3kDa MWCO membrane to reduce the concentrations of ONVC and 1-butanol in the permeate (stream P2), whose concentrations in R2 dropped to 4.02 and 3.50 g/L, respectively. In the same stream, the OS concentration reached 51 g of substituted OS/L. Among OS, the XOS concentration accounted 36.5 g/L, keeping constant the degree of substitution by AG. The effluent R2 showed a final OS concentration of 92.23 g/100 g of NVC. Finally, R2 was freeze-dried to yield the refined product.

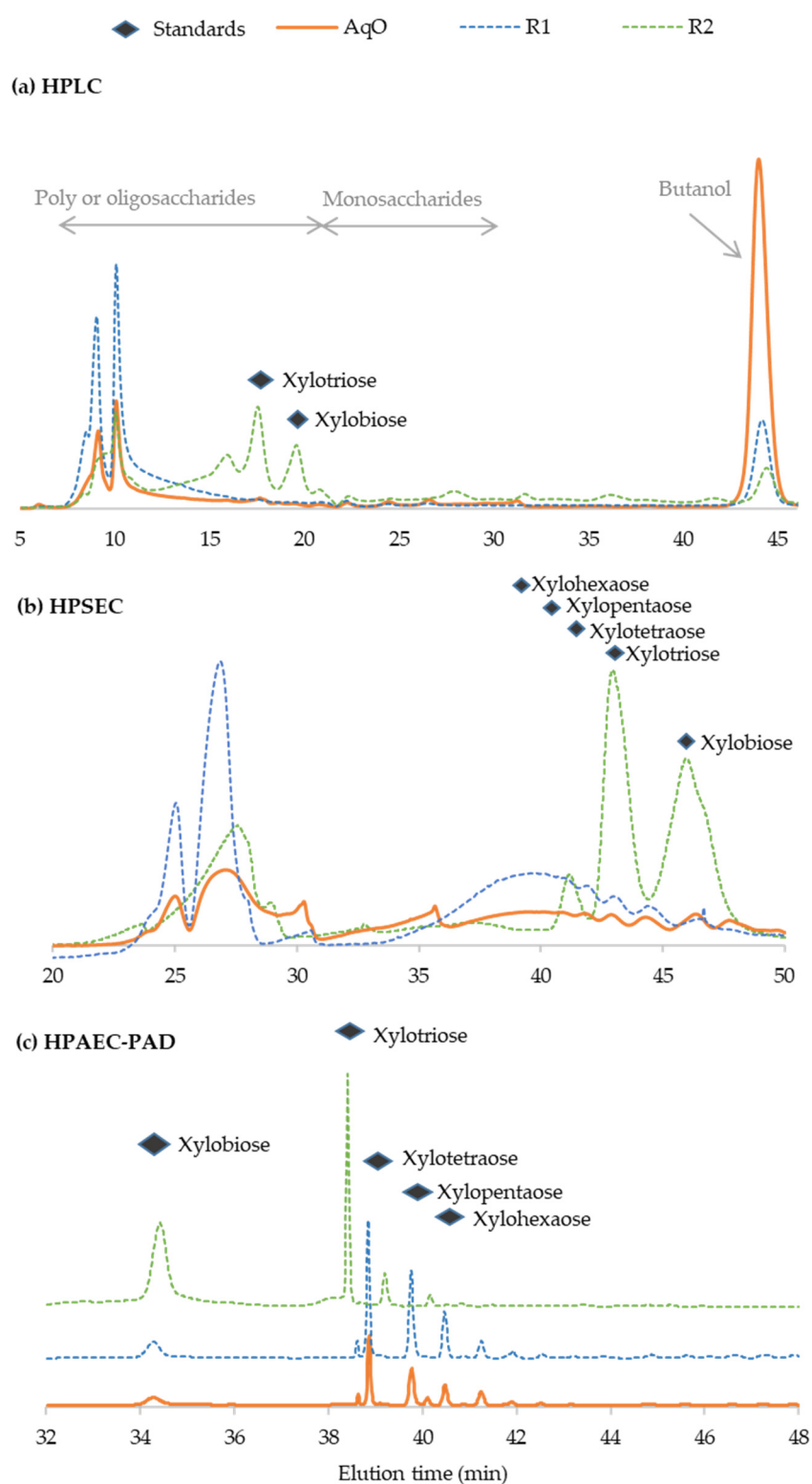
Diverse separation technologies have been employed in studies dealing with the refining of XOS from different sources [6,28,34,35,39]. For example, Vegas et al. [28] and Gullón et al. [35] reported that consecutive stages of membrane diafiltration, concentration and enzymatic hydrolysis with xylanases enabled the manufacture of low DP OS with purities above 90%.



**Figure 5.** Composition of the aqueous streams (AqO, R1 and R2) involved in the membrane processing. Nomenclature: GOS, glucose units in oligosaccharides (OS); XOS, xylose units in OS; GaOS, galactose units in OS; AOS, arabinose units in OS; MOS, mannose units in OS; AG, acetyl groups linked to OS; HMF, hydroxymethylfurfural; F, furfural; ONVC, other non-volatile compounds.

### 3.4. Characterization of Oligosaccharides from Vine Shoot Hemicelluloses

The type and relative proportions of structural units and substituents in streams AqO, R1 and R2 were studied by HPLC, HPSEC, and HPAED-PAD (see Figure 6).



**Figure 6.** Elution profiles obtained for streams AqO, R1 and R2 and standard XOS using: (a) HPLC (with the CARBOsep column); (b) HPSEC; and (c) HPAEC-PAD.

In the HPLC chromatograms (Figure 6a), the monosaccharides appeared at retention times between 20 and 28 min. The peaks eluted at shorter times corresponded to compounds included in the OS fraction, in order of decreasing DP. For comparison, the Figure

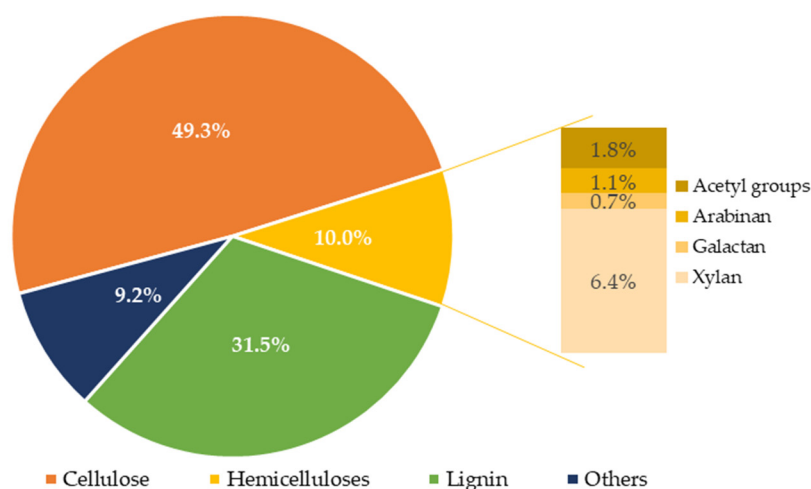
includes the peaks corresponding to XOS standards of DP 2-3. In the chromatograms obtained for AqO and R1, the concentration effects caused by DDVR can be observed. As a result of the xylanase stage, the product distribution determined for R2 is defined by the increased presence of low DP OS (in the range 2 and 3). The membrane step also caused the removal of 1-butanol (elution time, 44.5 min). The HPSEC chromatograms recorded for AqO, R1 and R2 (see Figure 6b) showed complex elution profiles, confirming diverse structural patterns for the substitution of the backbones composed of xylose units. The data revealed the presence of diverse compounds and substituents, including pentoses, hexoses and AG. The experimental data confirmed the major findings explained above concerning the concentration and DP distribution of the target compounds in streams AqO, R1 and R2. HPAEC-PAD provided additional information on the compounds contained in the OS fraction (see Figure 6c). The samples subjected to HPAEC-PAD analysis lacked AG substituents, which were saponified by the alkaline mobile phase [40]. The peaks corresponding to xylobiose (eluted at 34.2 min) and xylotriose (eluted at 38.8 min) in the chromatograms recorded for streams AqO, R1 and R2 clearly showed the generation of low-DP OS in the enzymatic step.

The experimental data showed that Shearzyme behaved as an excellent catalyst for reducing the DP of xylan-hydrolysis products without concomitant xylose generation. This finding is in agreement with data reported in related studies dealing with the processing of diverse xylan-containing materials (rice husks, wheat straw, corn cobs, and sunflower stalks) [28,41]. MALDI-TOF-MS spectra allowed the structural characterization of individual compounds making part of the OS fraction contained in AqO. The data are reported as sodium adducts of the respective compounds. OS below 500 m/z could not be identified because of the matrix interference (2,5-dihydroxybenzoic acid). Acetylated (A) backbones composed of pentose (P) chains, corresponding to the  $P_nA_m$  general structure, were identified. Their polymerization degree varied from  $P_3A_2$  to  $P_7A_5$ . Additionally, compounds bearing uronic substituents (U) were identified ( $P_6A_5U$ ). The spectra also showed the presence of saccharides composed of hexoses (H) with a wide DP range (from  $H_3$  to  $H_{13}$ ) in cases that were acetylated (such as  $H_{10}A$  and  $H_{10}A_2$ ). Based on the compositional data shown in Table 1 and Figure 5, the pentose chains type  $P_nA_m$  must be composed of xylose, whereas the hexoses correspond mainly to glucose, but also to galactose and mannose. The general structures deduced from the MALDI-TOF-MS data are in concordance with the ones reported for OS obtained from different lignocellulosic materials [6,12,13,35].

### 3.5. Enzymatic Hydrolysis of the Processed Solid from Fractionation Treatment

Biorefineries require the integral conversion of the feedstocks into a scope of biofuels, chemicals, and materials. For this purpose, the cellulose-enriched solids (denoted PSO) obtained under optimal fractionation conditions for OS manufacture were employed as substrates for enzymatic hydrolysis.

Figure 7 shows the compositional data obtained for PSO. Cellulose was the major component (49.3%), together with 6.4% xylan, which was not completely removed upon fractionation (%Hr = 74.5%). Considering all the hemicellulose components, this fraction accounted for 10% of PSO.



**Figure 7.** Average composition of the solids (PSO) from fractionation treatments performed at 175 °C and LSR = 12 for 20 min, in media containing 30% of organic solvent. Data expressed as g of component/100 g of oven dry PSO.

Table 2 lists the concentrations of xylose and glucose (and the respective conversions of xylan and cellulose) obtained in PSO hydrolysis performed at diverse reaction times and enzyme charges. The concentrations of glucose and xylose are expressed in g/L, whereas the conversions of cellulose and xylan into glucose and xylose were calculated based on their respective potential concentrations.

The highest glucose concentration (34.9 g/L, corresponding to 92.5% cellulose conversion) was reached, operating at ESR=20 for 98 h, although 29.3 g glucose/L (cellulose conversion, 77.5%) was achieved after 48 h. A comparison of data obtained in assays at ESR 10, 15 and 20 showed that lower glucose concentrations were obtained at ESR = 10, including if 83.1% of conversion was reached in this condition and at the longest enzymatic hydrolysis time considered.

The enzymatic complex caused not only the hydrolysis of cellulose, but also the conversion of xylan into xylose, owing to the xylanolytic activity of the Cellic CTec2 concentrate, a fact documented in literature [30,42]. Xylose concentrations in the range 4.2–4.3 g/L, corresponding to xylan conversions into xylose above 90%, were reached in all the experiments after 96 h.

**Table 2.** Results achieved in the enzymatic hydrolysis of solids from the fractionation stage (PSO). The concentrations of glucose and xylose are expressed as g/L  $\pm$  standard deviation. The conversions of cellulose and xylan into glucose and xylose are expressed as %  $\pm$  standard deviation.

Time (h)	Glucose concentration (g/L)			Conversion of cellulose into glucose (%)		
	ESR = 10	ESR = 15	ESR = 20	ESR = 10	ESR = 15	ESR = 20
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
24	16.7 $\pm$ 1.1	18.9 $\pm$ 0.2	22.8 $\pm$ 0.6	44.3 $\pm$ 2.0	49.9 $\pm$ 0.5	60.4 $\pm$ 1.2
48	22.8 $\pm$ 0.4	27.2 $\pm$ 0.1	29.3 $\pm$ 0.4	60.3 $\pm$ 0.8	71.9 $\pm$ 0.1	77.5 $\pm$ 0.7
72	28.4 $\pm$ 0.1	31.5 $\pm$ 0.8	32.9 $\pm$ 0.4	75.2 $\pm$ 0.2	83.5 $\pm$ 1.4	87.0 $\pm$ 0.7
96	31.4 $\pm$ 0.5	31.3 $\pm$ 0.4	34.9 $\pm$ 0.0	83.1 $\pm$ 0.9	82.8 $\pm$ 0.7	92.5 $\pm$ 0.1
Time (h)	Xylose concentration (g/L)			Conversion of xylan into xylose (%)		
	ESR = 10	ESR = 15	ESR = 20	ESR = 10	ESR = 15	ESR = 20
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
24	2.4 $\pm$ 0.1	2.6 $\pm$ 0.2	2.6 $\pm$ 0.1	53.1 $\pm$ 0.6	57.5 $\pm$ 2.3	56.5 $\pm$ 0.8

48	3.0 ± 0.1	3.6 ± 0.1	3.3 ± 0.3	66.1 ± 1.5	77.5 ± 1.5	71.6 ± 4.2
72	3.8 ± 0.1	4.2 ± 0.0	4.1 ± 0.3	82.0 ± 1.0	90.2 ± 0.4	89.2 ± 5.3
96	4.2 ± 0.0	4.2 ± 0.2	4.3 ± 0.2	91.0 ± 0.5	91.8 ± 2.4	93.6 ± 2.3

#### 4. Conclusions

Fractionation of vine shoots in 1-butanol/water media led to the partial separation of the main constituents of the feedstock in different phases: OS from hemicelluloses appeared in the aqueous phase, lignin in the organic one, and cellulose was kept in solid phase. OS were refined by membrane processing and subjected to xylanase hydrolysis for DP tailoring. After freeze-drying, a refined product containing low DP OS of high purity was obtained. Structural characterization of OS showed that the predominant structures corresponded to xylose backbones with different acetylation degrees, which were accompanied by minor amounts of OS composed of hexoses. The solids from the fractionation step were employed as substrates for enzymatic hydrolysis. The experiments were performed at diverse enzyme charges, and provided solutions containing up to 34.9 g glucose /L of and 4.3 g xylose /L.

The experimental data confirmed that the processing scheme proposed in this work is suitable for achieving the one-stage fractionation of vine shoots, enabling the utilization of the products deriving from hemicelluloses and cellulose for commercial purposes (OS with potential applications in food and nutraceutical sectors, and glucose-containing hydrolyzates suitable as fermentation media).

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