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Purification of oligosaccharides obtained from *Pinus pinaster* hemicelluloses by diafiltration

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ABSTRACT

The aim of this work was to assess the performance of diafiltration as a method for purification of oligosaccharides obtained by autohydrolysis of *Pinus pinaster* wood. *Pinus pinaster* wood hemicelluloses are mainly made up of galactoglucomannans, which were partially hydrolyzed in aqueous treatments (autohydrolysis) performed under suitable operational conditions (isothermal processing at 175°C for 24.5 min). The oligosaccharides present in autohydrolysis liquors obtained under optimal conditions were purified by diafiltration in a dead-end filtration device using a regenerated cellulose membrane (1 kDa MWCO). Continuous diafiltration resulted in increased weight percent of substituted oligosaccharides respect to the total non-volatile solutes (from 79.2% up to 94.7%), as well as in the selective removal of monosaccharides (the substituted oligosaccharides to monosaccharides ratio increased from 4.3 up to 17.8).

Keywords: Autohydrolysis; Biomass; Diafiltration; Galactomannans; *Pinus pinaster*

1. Introduction

Lignocellulosic biomass, the most abundant renewable resource in the Earth, is expected to play a key role in sustainable development. For this purpose, the "biomass refining" philosophy (based on the selective separation of the major biomass constituents) provides a valuable framework for process development.

Pinus pinaster is the most widespread conifer species in Spain [1], being distributed in about 1,000,000 ha [2]. Hemicelluloses of softwoods (including *Pinus pinaster*) are mainly made up of *o*-acetylgalactoglucomannans and galactoglucomannans, together with lower amounts of arabinoglucuronoxylans, arabinogalactans and xyloglucans [3–7]. Owing to their physicochemical properties as stiffeners and stabilizers of emulsions, and their lack of toxicity, galactoglucomannans are versatile chemicals that can be used for a variety of applications in the textile, pharmaceutical, biomedical, cosmetic and food industries. Acetylated galactoglucomannans can be used as bioactive polymers, hydrocolloids, papermaking chemicals or coating polymers [5,8]. Galactoglucomannans from various sources have been used as excipients for oral controlled drug release, as agents with in vitro immunostimulatory activity, or as substrates for the metabolism of human intestinal bacteria. Mannan-derived oligosaccharides have been proposed as ingredients for functional foods because of their potential prebiotic activity [9].

Fractionation of wood by hydrothermal treatments has been considered in the literature. The available methods suitable to dissolve hemicelluloses from lignocellulosic feedstocks have been summarized in a recent review [10]. One of the most promising biomass fractionation processes is autohydrolysis (treatments with hot, compressed water), in which no addition of chemicals to water is needed.

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In the autohydrolysis reaction, the hydronium ions from water auto-ionization and the *in situ* generated acids catalyze the depolymerization of hemicelluloses, by selective hydrolysis of glycosidic and ester linkages [10]. Depending on the operational conditions (temperature and time of reaction), the autohydrolysis products vary from oligosaccharides to monosaccharides and, under harsh conditions, to sugar degradation products. Liquors from autohydrolysis treatments contain a complex mixture of saccharides and non-saccharide products, together with a solid phase mainly made up of cellulose and lignin (which can be separated by further processing, according to the biomass refining philosophy). When the target products are oligosaccharides, the raw autohydrolysis liquors must be purified before utilization in a variety of applications.

Several studies have been carried out on the membrane processing of autohydrolysis liquors, intending to achieve purification and/or fractionation and/or concentration of the saccharide components [11–15].

This work deals with the effects of the autohydrolysis conditions on the solubilization of *Pinus pinaster* wood hemicelluloses, and with the subsequent purification of the oligomeric hemicellulose-derived saccharides present in reaction liquors obtained under optimal conditions by continuous diafiltration.

2. Experimental

2.1. Autohydrolysis

Pinus pinaster wood samples (kindly provided by Orember-Finsa, Ourense, Spain) were air-dried, milled to a particle size below 8 mm and homogenized in a single lot to avoid compositional differences. Milled wood was treated with water (at a liquor to solid ratio of 8:1 kg/kg) in a steel reactor (Parr Company) with temperature and agitation controls. Two sequential aqueous treatments were carried out: in the first one, the medium was heated up to reach 130°C (following the standard temperature profile) to remove extractives [16]; whereas in the second one (autohydrolysis) the extractive-free solid was heated up to reach temperatures in the range 160–240°C, in order to cause hemicellulose solubilization. The resulting liquors were analyzed for saccharides and total non-volatile solutes as described below.

2.2. Analytical methods

The amounts of extractives in the raw material and in the solid after aqueous treatment at 130°C were determined by Soxhlet extraction with ethanol.

Monosaccharides (glucose, xylose, mannose, galactose, and arabinose) in liquors were determined by HPLC–IR using a CARBOsep CHO 682 column (Transgenomic). The operational conditions were: mobile phase, distilled water; flow, 0.4 ml/min; temperature, 80°C.

Acetic acid was determined by HPLC–IR using an Aminex HPX-87H column (BioRad). The operational conditions were: mobile phase, 0.006 N H_2SO_4; flow, 0.6 ml/min; temperature, 60°C.

The concentrations of oligosaccharides (OS) and acetyl groups linked to them were determined from the concentrations of monosaccharides and acetic acid (measured by the above methods) present in liquors previously subjected to quantitative post-hydrolysis (treatment with 4% sulphuric acid at 121°C for 20 min). Before analysis in the CARBOsep CHO 682 column, post-hydrolysis samples were neutralized with $BaOH \cdot 8H_2O$. Concentrations were corrected for dilution.

Uronic acids were determined spectrophotometrically at 520 nm using the *m*-phenylphenol method [17].

The content of non-volatile compounds (NVC) of raw liquors, retentates and permeates was determined by oven-drying of aliquots at 105°C until constant weight.

2.3. Diafiltration

Continuous diafiltration of autohydrolysis liquors was carried out using a 400 ml dead-end filtration device (Amicon, Millipore) fitted with a reservoir for diafiltration water supply. Feed cell and reservoir were connected through a two position valve, which allowed either pressurization of both vessels with N_2 (at the beginning of the experiment), or supply of water from the reservoir to the feed tank to keep the volume constant. Operation was carried out using a regenerated cellulose membrane of 1 kDa cut-off (Millipore) with a membrane area of 41.8 cm^2.

Based on previous concentration experiments (data not shown), the operating pressure was fixed in 4 bar, as this pressure led to enhanced oligosaccharide retention. Two-diavolume diafiltration was carried out using autohydrolysis liquors as a feed. A diavolume is defined as the total diafiltration liquid volume introduced to the operation divided by the initial sample volume.

3. Results and discussion

3.1. Autohydrolysis

The first aqueous treatment (at 130°C) decreased the extractives content of wood by 28.5% of the initial value (resulting in samples containing 2.03 g extractives/100 g raw material), without causing significant hemicellulose degradation. This last point was confirmed by the negligible content of monosaccharides and oligosaccharides in water extractives.

In order to assess the selection of the optimum operating conditions in the autohydrolysis stage, extractive-free wood was processed under non-isothermal conditions at temperatures in the range 160–240°C. The different operational conditions were characterized by the maximum temperature of experiments, as well as by the severity (*S*), which also includes the effects of the heating profile. *S* is defined as:

$$S = \log \int_0^t \exp\left(\frac{T - T_r}{\omega}\right) dt \quad (1)$$

where T is the temperature (K), t is the time (min), T_r is the reference temperature (373 K) and ω is a paramenter related to the activation energy, with a value of 14.75 K [18]. Figs. 1a and b show the composition of the autohydrolysis liquors obtained under the operational conditions considered in this work.

Fig. 1a shows the concentrations of oligosaccharides (expressed as monosaccharide equivalents) in the liquors resulting from treatments carried out at different severities, as a function of *S* and the maximum temperature. The optimal oligosaccharide concentrations corresponded to *S* in the range 3.5–4.2, which led to media containing 12.2±0.8 g mannooligosaccharides/l, 2.7±1.4 g xylooligosaccharides/l, 3.0±0.3 g glucooligosaccharides/l, 2.0±0.4 g galactooligosaccharides/l and 0.3±0.3 g arabinooligosaccharides/l.

The optimum reaction conditions must be fixed considering additionally the monosaccharide concentrations (shown in Fig. 1b). For the purposes of this work, monosaccharides are undesired components, as they lack of prebiotic activity, and should be removed. On the other hand, the decrease in monosaccharide concentrations under harsh conditions is due to the participation of monosaccharide-degradation reactions leading to the generation of unwanted compounds (as hydroxymethylfurfural and furfural). Based on Fig. 1a and b, the optimal conditions were assumed to correspond to a severity of 3.6 (or to a maximal temperature of 200°C), as they led to both high concentrations of oligosaccharides and limited concentrations of monosaccharides. Under the optimal conditions, more than 90% of mannose, galactose, xylose and arabinose (as monosaccharides and oligosaccharides) were released from the raw material. The total glucose released from raw material was 6.1%. With the methods employed, no difference between glucose coming from cellulose or from hemicelluloses can be made. Considering that hemicelluloses are much more susceptible to solubilization than cellulose, it is expected that most of this percentage corresponds to hemicelluloses.

One of the advantages of the parameter *S* is that the optimal conditions determined for given operational conditions can be easily recalculated for other

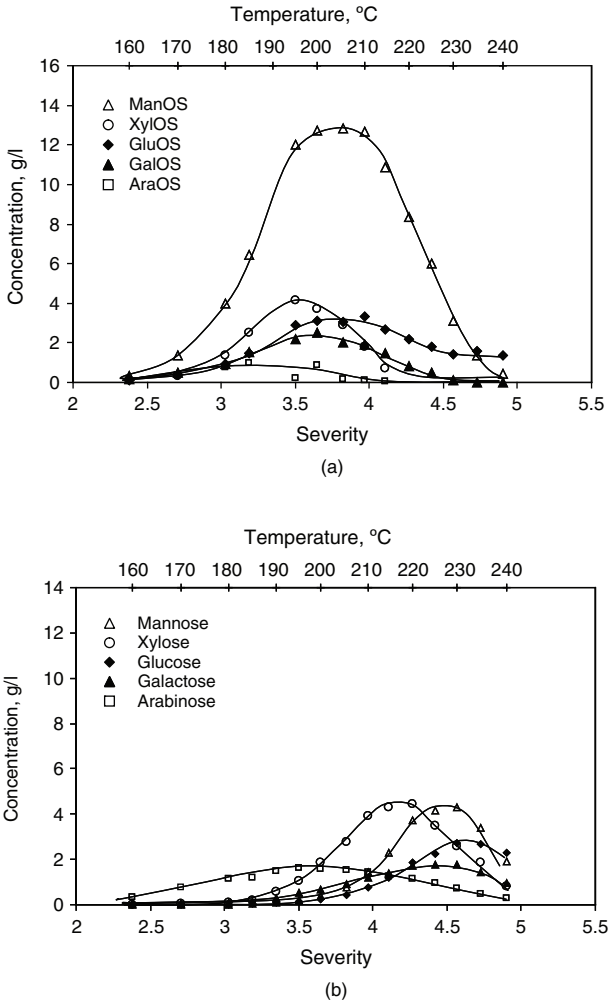

Fig. 1. Concentrations of (a) oligosaccharides and (b) monosaccharides in liquors obtained at different severities. ManOS: mannosyl units in oligosaccharides; XylOS: xylosyl units in oligosaccharides; GluOS: glucosyl units in oligosaccharides; GaOS: galactosyl units in oligosaccharides; AraOS: arabinosyl units in oligosaccharides.

temperature profiles. In our case, and in order to minimize experimental errors caused by small fluctuations in electric supply along heating, the optimal conditions were recalculated for a new temperature profile (heating up to 175°C and isothermal operation at this temperature for the required time). On the basis of the heating profile, the calculated duration of the isothermal reaction stage needed to achieve *S* = 3.6 was 24.5 min. The overall reaction time, including the heating time from T_r to 175°C, the isothermal period and the cooling time was 55 min.

Table 1 shows the concentrations and mass percentages respect to NVC in the autohydrolysis liquors obtained under the above conditions. Mannooligosaccharides were the major components of liquors, accounting for 40.3% of NVC. The total oligosaccharide fraction

Table 1
Composition of the autohydrolysis liquors obtained from *Pinus pinaster* wood at 175°C and S = 3.6 (oligosaccharides are expressed as monosaccharide equivalents)

Component	Concentration, g/l	Mass percentage, % of NVC
ManOS	10.45 ± 0.13	40.3 ± 0.7
XylOS	3.05 ± 0.29	11.8 ± 1.6
GluOS	2.16 ± 0.13	8.3 ± 0.7
GalOS	2.22 ± 0.04	8.6 ± 0.2
AraOS	0.16 ± 0.11	0.6 ± 0.6
AcOS	1.42 ± 0.02	5.5 ± 0.1
UrOS	1.10 ± 0.02	4.2 ± 0.1
Mannose	0.30 ± 0.13	1.2 ± 0.7
Xylose	1.74 ± 0.29	6.7 ± 1.6
Glucose	0.37 ± 0.13	1.4 ± 0.7
Galactose	0.65 ± 0.04	2.5 ± 0.2
Arabinose	1.75 ± 0.12	6.7 ± 0.7

Note: GluOS: glucosyl units in oligosaccharides, measured as glucose;
ManOS: mannosyl units in oligosaccharides, measured as mannose;
GaOS: galactosyl units in oligosaccharides, measured as galactose;
XylOS: xylosyl units in oligosaccharides, measured as xylose;
AraOS: arabinosyl units in oligosaccharides, measured as arabinose;
AcOS: acetyl groups linked to oligosaccharides, measured as acetic acid;
UrOS: uronic acids linked to oligosaccharides, measured as galacturonic acid.

(including the acetyl and uronic substituents), accounted for 79.2% of NVC; whereas monosaccharides accounted for 18.5%. The remaining 2.3%, calculated by difference among the overall NVC concentration (25.95 g/l liquor) and the concentrations of the various identified components, corresponded to non-saccharide, unwanted compounds (here denoted "other non-volatile compounds", ONVC). It can be noted that the ONVC fraction accumulates the experimental errors affecting the whole set of solutes identified, as well as that this variable was underestimated, as the oligosaccharide components are quantified as monosaccharides (without correction for hydration as a function of the average degree of polymerization). This fact comes from a limitation of the analytical methods employed, which are based on total hydrolysis (i.e., total conversion of saccharides into monosaccharides) as a necessary strategy for quantification.

Besides the non-volatile solutes shown in Table 1, the liquors contained acetic acid, at a concentration of 0.34 g/l.

3.2. Diafiltration

The autohydrolysis liquors were purified by continuous diafiltration in order to cause purification effects, derived from the selective removal of monosaccharides, which leads to increased mass fractions of oligosaccharides respect to NVC. Samples of permeate were taken at selected operation times and analyzed for composition using the methods listed above. The final retentate was also analyzed. The retentions of components were calculated using the following equation:

$$C_r = C_r^0 \cdot \exp\left(-\frac{Q_p}{V_r} \cdot (1 - R) \cdot t\right) \quad (2)$$

where C_r is the retentate concentration; C_r^0, the feed concentration; Q_p, the permeate volumetric flux; V_r, the retentate volume and R is the retention at time t.

R is defined as:

$$R = (1 - C_p/C_r) \quad (3)$$

where C_p is the permeate concentration.

Equation (2) is only valid for a continuous diafiltration, when both V_r and Q_p remain unchanged along processing. The relationship between the permeate volume with time gave a value of Q_p = 1.26 ± 0.01 ml/min with a correlation coefficient $R^2 > 0.999$. This Q_p corresponds to a permeate flux of 18 l/(h·m²). This behaviour showed that no significant fouling effects took place (a fact confirmed by water permeability measurement once the experiment was finished), suggesting that R can be considered constant.

Table 2 shows the composition of the retentate after two-diavolume filtration, expressed in terms of volumetric concentration and mass percentage respect to NVC, as well as the retentions calculated for the various liquor components.

Upon diafiltration, the ratio between oligosaccharides (including substituents) and monosaccharides increased from 4.0 to 17.8, evidencing the selectivity of the separation. The retention of each component was calculated taking into account the concentration of the retentate. Interestingly, the retentions of ManOS (0.881) and those of the rest of oligosaccharides (>0.76) were significantly higher than the ones of monosaccharides. Small imbalances in XylOS, which are not significant for the purposes of this study, were caused by limited precipitation in retentate.

On the other hand, the retention of monosaccharides was lower than 0.12, except for glucose and mannose. The low concentrations of glucose and mannose make the retention of this sugar scarcely significant for the purposes of this work.

It can be noted that the differences among the retentions of oligosaccharides and monosaccharides provides a practical method for purification of samples, which is

Table 2
Composition of retentate after two-diavolume filtration

Component	Concentration, g/l	Mass percentage, % of NVC	Retention
ManOS	8.16	54.8	0.876
XylOS	0.86	5.76	Not detected*
GluOS	1.86	12.47	0.925
GalOS	1.42	9.50	0.775
AraOS	0.18	1.18	1.000
AcOS	0.97	6.49	0.809
UrOS	0.68	4.54	0.760
Mannose	0.065	0.44	0.235
Xylose	0.29	1.91	0.095
Glucose	0.077	0.52	0.215
Galactose	0.11	0.73	0.112
Arabinose	0.25	1.69	0.027

*Theoretical value calculated from material balances: 0.789.

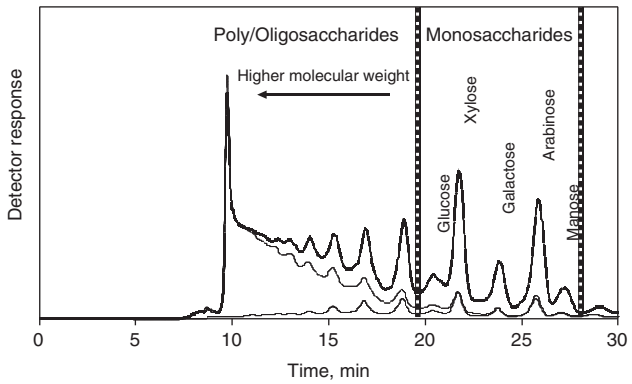

Fig. 2. Chromatographic elution pattern determined for (—) feed, (–) final retentate and (---) final permeate obtained with the CARBOsep CHO 682 column.

confirmed by the increase in the mass fractions of substituted oligosaccharides (measured respect to NVC) from 79.2% to 94.7%. This latter result is indicative of a final product directly suitable as a prebiotic. The evaluation of the prebiotic activity of diafiltered oligosaccharide samples is ongoing in our laboratory.

In order to provide additional insight on the effects caused by diafiltration, Fig. 2 shows the chromatograms obtained with the CARBOsep CHO 682 column for the raw liquors used as a feed and for the retentate and permeate obtained at the end of the experiment. The results confirm the above discussion, with significant removal of monosaccharides, partial loss of low molecular weight oligosaccharides and total retention of high molecular weight oligosaccharides. Depending on which polymerization degree is accepted as a limit between oligomers and polymers (not clearly established in literature), at least a part of the high molecular weight fraction of the retained saccharides could be ascribed to polymeric (instead to oligomeric) material.

4. Conclusions

Continuous dead-end diafiltration of autohydrolysis liquors obtained from water-extracted wood under optimal conditions (isothermal treatment at 175°C during 24.5 min) was carried out to cause purification effects (derived from the selective removal of monosaccharides and the increase in mass percentage of total oligosaccharides respect to non-volatile solutes). Two-diavolume diafiltration at 4 bar enabled the calculation of the retentions of the target compounds (oligosaccharides made up of mannose, galactose, glucose, arabinose and xylose units substituted by acetyl groups and uronic acids) and unwanted monosaccharides. The retentions of oligosaccharides (in the range 1.00–0.76) were significantly higher than the ones of monosaccharides, enabling the production of a retentate with a oligosaccharide/monosaccharide ratio of 17.8 (about four times higher than the value determined for the raw liquors used as a feed). The experimental results confirm that the studied reaction-diafiltration approach is suitable for obtaining a product with high oligosaccharide content (94.7% of non-volatile components remaining in the final retentate).

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Symbols

C_r — Concentration in retentate, g/l
C_r^0 — Initial concentration in retentate, g/l
Q_p — Volumetric permeate flux, ml/min
R — Retention, dimensionless
S — Severity factor
t — Time, min
T — Temperature, K

T_r — Reference temperature, K
V_r — Retentate volume, ml
ω — Parameter related to the reaction activation energy, K

Abbreviations

GluOS — Glucosyl units in oligosaccharides
ManOS — Mannosyl units in oligosaccharides
GaOS — Galactosyl units in oligosaccharides
XylOS — Xylosyl units in oligosaccharides
AraOS — Arabinosyl units in oligosaccharides
AcOS — Acetyl groups linked to oligosaccharides
UrOS — Uronic acids linked to oligosaccharides

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