EXTRACTION AND CHARACTERIZATION OF HEMICELLULOSES FROM *Pinus radiata* AND ITS FEASIBILITY FOR BIOETHANOL PRODUCTION[1]

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ABSTRACT – Galactoglucomannans are the major hemicellulosic fraction present in softwoods, and is mainly composed by the hexoses: galactose, glucose and mannose. The efficient and selective isolation of these hemicelluloses is regarded as one of the most critical obstacles to overcome for their utilization. The objective of the current work was to extract and characterize acidic and neutral aqueous solutions of hemicelluloses from *Pinus radiata* wood chips and investigate their feasibility for bioethanol production. Hemicelluloses in *P. radiata* represented 26 g/100 g wood (o.d.w.) and hexoses are responsible for approximately 64% of this amount. According to the different extraction conditions, approximately 50% of the hemicellulosic fraction was solubilized and recovered after precipitation with ethanol. The recovered hemicellulosic fractions were in the form of oligomers with weight-average molecular weigth (M_w) varying from $4x10^3$ to $4x10^5$ g/mol. Hemicellulosic oligomers were hydrolyzed with dilute sulfuric acid and the hydrolysates concentrated until approximately 70 g/L of hexoses and fermented by *Saccharomyces cerevisiae* yeast. Fermentation results showed that sugar obtained from acid and neutral extractions were fermented to ethanol with maximum yields of 63% and 54% (22 g/L and 19 g/L), respectively. The conversion of wood hemicellulosic substrates to ethanol is feasible but the low ethanol yields obtained make the process not economically attractive and optimization of the process or alternatives uses for hemicelluloses should be evaluated.

Keywords: *Pinus radiata*, Hemicelluloses, Bioethanol.

EXTRAÇÃO E CARACTERIZAÇÃO DE HEMICELULOSES DE Pinus radiata E SUA VIABILIDADE PARA A PRODUÇÃO DE BIOETANOL

RESUMO – As galactoglucomananas são as principais frações de hemiceluloses presentes nas madeiras moles e contêm, principalmente, as hexoses galactose, glicose e manose. O isolamento eficiente e seletivo dessas hemiceluloses é um obstáculo crítico a superar para sua utilização. Os objetivos deste trabalho foram extrair e caracterizar soluções aquosas ácidas e neutras de hemiceluloses de cavacos de madeira de ***Pinus radiata****, bem como avaliar sua viabilidade para a produção de bioetanol. As hemiceluloses em* ***P. radiata*** *representam 26 g/100 g de madeira (base seca), e as hexoses são responsáveis por aproximadamente 64% dessa quantidade. De acordo com as diferentes condições de extração, cerca de 50% da fração hemicelulósica foi solubilizada e recuperada depois de uma precipitação com etanol. As frações recuperadas de hemiceluloses estavam na forma de oligômeros com peso molecular médio (M_w) variando entre $4x10^3$ e $4x10^5$ g/mol. Os oligômeros hemicelulósicos foram hidrolisados com ácido sulfúrico diluído e os hidrolisados concentrados até aproximadamente 70 g/L hexosas e fermentados pela levedura* ***Saccharomyces cerevisiae****. Os resultados de fermentação indicaram que os açúcares obtidos dos extratos ácidos e neutros foram fermentados com rendimentos máximos de etanol de 63% e 54% (22 g/L e 19 g/L), respectivamente. A conversão de hemiceluloses da madeira em etanol é viável, porém seu baixo rendimento faz que o processo não seja economicamente atrativo, razão por que melhorias no processo ou usos alternativos das hemiceluloses devem ser avaliados.*

Palavras-Chaves: ***Pinus radiata****, Hemicelulosas, Bioetanol.*

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1. INTRODUCTION

The need for alternative and sustainable fuels is a worldwide concern and a subject of several studies whilst global sources of oil continue to decline and the energy requirements to increase. Bioethanol is one of the most promising biofuels with potential as a sustainable transportation fuel to replace gasoline. It can be produced from any feedstock containing appreciable amounts of sugars or polysaccharides that can be converted into fermentable sugars (WYMAN, 1999; PETROU et al., 2009). Also, an alternative of readily available raw material is the hemicellulosic fraction from wood. Hemicelluloses can be used in several processes aimed to produce fuels and chemicals for different industry sectors (VIIKARI et al., 1993; SAHA, 2003; GIRIO et al., 2010). One of the alternatives to develop a wood-based biorefinery is extracting hemicelluloses previous to kraft or mechanical pulping for use in the production of ethanol, xylitol or other bioproducts (Van HENNING, 2006; CARVALHEIRO et al., 2008; MAMMAN et al., 2008; HOUTMAN et al., 2011).

The hemicellulosic fraction comprises between 20-30% of wood dry weight and presents a varied composition and structure depending on the wood species (TIMELL, 1967; LAWTHER et al., 1995). Galactoglucomannans (GGM) is the main hemicellulose in softwoods, with approximately 15-30% of the wood composition (WILFÖR et al., 2005, MORAIS et al., 2005). Galactoglucomannans could be a potential source of fermentable sugars for ethanol production inasmuch as, according to Gottschalk (1947), the six carbon monosaccharides, D-galactose, D-glucose and D-mannose, can be directly fermented by yeasts to ethanol. The efficient use of the hemicellulosic fraction requires its extraction from wood matrix and characterization to determine the its possible use and products (SONG et al., 2008; CARVALHEIRO et al., 2008; CHAA et al., 2008; AMIDON, 2009; ROOS et al., 2009).

Although the extraction and characterization of hemicelluloses has been an important focus of study using hardwood species (*Betula pendula*, *Eucalyptus grandis* among others) (WILLFÖR et al., 2005, GOUVÊA et al. 2009), the extraction of hemicelluloses from softwoods, especially pine species, has been less studied. Based on these antecedents, the objectives of this work were: (1) to characterize the chemical composition and molar mass distribution of the hemicellulosic fraction extracted under acid and neutral conditions from *P. radiata* wood chips (2) to evaluate the biochemical conversion of the hemicellulosic liquors in bioethanol using the yeast *Saccharomyces cerevisiae*.

2. MATERIAL AND METHODS

2.1 Wood

Pinus radiata wood chips from trees at around 11 years of age were obtained from a pulp mill located in the Bío-Bío Province (Chile). Wood chips with average size of 2.0 x 1.5 x 0.2 cm were air-dried until 10% moisture and stored in plastic bags under low humidity conditions until use.

2.2. Hemicelluloses extraction

The extraction of hemicelluloses from *P. radiata* was carried out with aqueous solutions at different pH, time and temperature (Table 1). The extraction was carried out in a 1.2-L high-pressure digester with recirculation liquor (HAATO) loaded with 200 g of wood chips and 1000 mL liquor, using a temperature ramp of 5°C/min. After each reaction, the material was cooled to room temperature, filtered and washed with 1000 mL of water. Solids were air-dried, the exact moisture was determined and the residual wood chips were weighed. To 100 mL of the recovered liquor fraction were added 400 mL of 95% ethanol to precipitate the hemicelluloses. The precipitated was filtered in a 0.25 ìm membrane filter, washed with ethanol and dried in vacuum oven at 40°C. Solids were weighed to determine the yield of hemicellulose extracted and further used for chemical, and molar mass distribution analysis.

Table 1 – Experimental conditions used for hemicelluloses extraction from *P. radiata* wood chips.

Tabela 1 – *Condições experimentais usadas para extração de hemiceluloses de cavacos de madeira de* ***P. radiata****.*

Experiment	Time (min)	Temperature (°C)	Initial pH
P-1	75	120	2
P-2	30	145	2
P-3	120	145	2
P-4	75	170	2
P-5	30	120	7
P-6	120	120	7
P-7	75	145	7
P-8	30	170	7
P-9	120	170	7

2.3 Wood and hemicelluloses characterization

Approximately 50 g of wood chips were milled in knife mill and sieved to 40/60 mesh. Three grams of milled wood was extracted with acetone 90% for 16 h. Extractive-free wood (300 mg) was hydrolyzed with 72% sulfuric acid. Acid-insoluble and soluble lignins were determined by gravimetry and UV-Vis spectrophotometry, respectively. Glucose was determined by HPLC and further converted to glucans using a 0.9 hydrolysis factor (FERRAZ et al., 2000). The monomeric composition of the hemicelluloses in wood and in the ethanol-precipitates was determined by acid methanolysis followed by gas chromatography according to the methodology described by Sunderg et al. (1996). All experiments were performed in triplicate.

Weight-average molecular weight (M_w) and number-average molecular weight (M_n) of the hemicelluloses obtained from the different extraction conditions were determined by size-exclusion chromatography (SEC). Measurements were performed by using a JASCO SEC system (degasser DG980-50, pump PU-980, refractive index detector RI-930, Suprema precolumn, columns Suprema 1000 and Suprema 30, from Kromatek, Essex, UK), in aqueous 0.1 M $NaNO_3$ containing 0.05% NaN_3 as eluent (1.0 mL/min). Pullulans were used as standards to determine M_w and M_n of hemicelluloses.

2.4 Fermentation of hemicellulosic extracts to ethanol

The hemicellulosic extracts with the highest amount of sugars from one of the each neutral or acidic pH conditions were concentrated in a rotaevaporator to obtain a concentration of approximately 70 g/L hexose. The concentrated hydrolysate was adjusted with $Ca(OH)_2$ to pH 7. Then, the pH was adjusted to 5 with H_2SO_4. The treated hydrolysate was centrifuged at 1500 rpm for 15 min to remove the precipitate resulting from the pH adjustment. Fifty millimeters of hydrolysate was supplemented with 5 g/L yeast extract, 5 g/L peptone, 1 g/L NH_4Cl, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, and 3 g/L of the yeast *Saccharomyces cerevisiae* IR2-9a. Assays were carried out at 37°C, 150 rpm for 72 h. A parallel assay of fermentation was performed using a synthetic medium prepared with 70 g/L mannose and the same amount of nutrients and fermentation conditions as used previously. From each assay, aliquots of 1 mL were periodically withdrawn for ethanol quantification in the time-course of fermentation. Ethanol concentration was determined by gas chromatography on a Perkin-Elmer autosystem XL Headspace using a FID detector and a HPSMS 30 m column. The theoretical ethanol yield was calculated assuming that the hexoses in the medium were available for fermentation with an ethanol yield of 0.51 g ethanol/g hexose. Furfural and hydroxymethylfurfural (HMF) were determined by HPLC (Merck Hitachi) in a Biorrad HPX-87H column at 65°C, eluted with 0.3 mL/min 30% acetonitrile and 70% H_2SO_4 5 mM, and using an UV detector at 270 nm. All experiments described in this section were performed in triplicate.

3. RESULTS

3.1 Hemicelluloses extraction and characterization

The chemical composition of *P. radiata* wood chips showed that hemicelluloses represented approximately 26% of wood dry weight and, out of this, hexoses (glucose, mannose and galactose) were responsible for 64% of its composition (Table 2). Hemicelluloses from *P. radiata* wood chips were extracted by applying aqueous solutions with different initial pH and cooking conditions (Table 1). When the extraction was performed at pH 2, the ethanol precipitated hemicelluloses amount varied from 4 to 13 mg hemicellulose/g wood and in neutral pH. Those values ranged from 2 to 15 mg hemicellulose/g wood (Table 3). For fermentation, it is desired a high amount of mannose, glucose and galactose. The amount of hexoses and their contend in the extracted liquors varied according to the liquor composition and extraction conditions.

Table 2 – Chemical composition of wood and hemicelluloses from *P. radiata**.

Tabela 2 – *Composição química da madeira e hemiceluloses de* ***P. radiata*****.

Component	g/100 g wood	g/100 g hemicellulose
Cellulose	41.2	
Hemicellulose	26.0	
Anhydroarabinanose	1.3	5.0
Anhydrogalactose	3.1	11.9
Anhydroglucose	3.9	15.0
Anhydromannose	9.7	37.3
Anhydrorhamnose	0.7	2.7
Anhydroxylose	5.0	19.2
Uronic groups	2.3	8.9
Lignin	27.8	
Acetone-soluble extractives	1.9	

* Standard deviation was lower than 3% of the average value from triplicate analysis.

Table 3 – Chemical composition and average molecular weight of ethanol-precipitated hemicelluloses.
Tabela 3 – *Composição química e peso molecular médio das hemiceluloses precipitadas com etanol.*

Recovered Hemicellulose recovered (g/100 g wood)*	Composition (mg/g hemicellulose)**							Molecular weight (x10^3 g/mol)	
Assay	Ara	Gal	Glc	Man	Rha	Xyl	OC	M_w	M_n
P-1	4.4	3.4	21.3	12.5	37.1	1.4	7.1	17.2	1.9 e 4	5.1
P-2	3.8	1.7	18.1	12.4	46.0	0.3	9.6	11.9	7.9 e 4	8.3
P-3	10.5	2.3	17.4	15.4	39.2	0.5	12.5	12.7	9.7 e 3	2.7
P-4	13.3	1.8	15.7	13.5	44.5	0.4	5.6	18.5	1.0 e 4	2.8
P-5	2.2	4.3	16.2	14.7	24.7	5.0	5.0	30.2	5.4 e 5	14
P-6	6.4	3.9	19.7	15.0	35.8	2.5	6.7	16.3	1.1e 4	4.0
P-7	5.78	2.9	11.2	7.7	30.8	1.1	18.6	27.6	2.5 e 4	6.6
P-8	10.8	1.1	10.5	7.7	29.4	1.1	18.6	17.7	1.7 e 5	10
P-9	15.2	2.1	13.9	19.0	58.7	0.4	4.9	1.1	3.7 e 3	1.5

* Amount of hemicellulose recovered from the liquid fraction by precipitation with ethanol.
** Abbreviations: Ara: arabinose; Gal: galactose; Glc: glucose; Man: mannose; Rha: rhamnose; OC: other or unidentified components; Xyl: xylose. Mw: weight-average molecular weight; Mn: number-average molecular weight.

3.2 Bioethanol from the hemicellulosic hydrolysates

Hydrolysates with high content of hemicelluloses were prepared for testing fermentation to bioethanol. Extraction conditions selected for these assays were: pH 2, 170°C, 75 min and pH 7, 170°C, 120 min. Before fermentation, a mild hydrolysis with diluted sulfuric acid was performed in order to hydrolyze the oligomeric fraction and the hydrolysates were concentrated until approximately 70 g/L of hexoses.

Ethanol concentration obtained after 72 h of fermentation was 30 g/L (84% yield) for the synthetic medium prepared with mannose indicating that the yeast strain use was able to ferment adequately this monosaccharide to ethanol. Fermentation results from the hemicellulosic hydrolysates showed that sugars obtained from acid and neutral extractions were fermented rather similar to ethanol with maximum yields of 22 and 19 g/L (63% and 54% yield), respectively (Table 4). Volumetric productivity values (Qp) are 0.31 for extract obtained at pH 2 and 0.27 for extract obtained at pH 7.

Table 4 – Ethanol yield obtained from the synthetic medium with mannose and hemicellulosic hydrolysates.
Tabela 4 – *Rendimento de etanol obtido deste meio sintético com manose e hidrolisados hemicelulósicos.*

Sugar source	Ethanol (g/L)	Ethanolyield (%)*	Qp (g ethanol/h)
Synthetic medium (70 g/L mannose)	30 ± 2	84 ± 2	0.42 ± 0.02
Acid extract (70 g/L hexoses)	22 ± 2	63 ± 2	0.31± 0.03
Neutral extract (70 g/L hexoses)	19 ± 2	54 ± 2	0.27 ± 0.01

Qp: Volumetric productivity, * as % of the maximal theorethical.

4. DISCUSSION

4.1 Hemicelluloses extraction and characterization

There are a number of methods to extract these hemicellulose sugars. Previous results showed that acid and neutral conditions are capable of extracting the hemicellulose sugars from *P. radiata*. In general, high extraction time and temperature were needed to obtain high hemicellulose yields regardless to the initial pH. Ban et al. (2008), when extracting hemicellulose from birch (*Betula pendula*), also reported that the temperature is the most important factor for extraction which does not dependent on the initial pH of the liquor, followed by the cooking time, especially when working at low temperatures. Authors obtained approximately 8.7 mg hemicelluloses/g wood and the acidic environment provided a slightly higher extract yield compared to other conditions evaluated.

Composition of the recovered hemicellulose also varied for the different monosaccharides present in its structure (Table 3). For fermentation, it is desired a high amount of mannose, glucose and galactose. The amount of hexoses and their proportion in the extracted liquors varied according to the liquor

composition and extraction conditions. Low M_w was found in the hemicelluloses recovered by ethanol precipitation indicating its fragmentation during acid and neutral extraction procedures (Figure 1 and Table 3). However, the fermentation yeast is not able to convert oligomers to simple sugars and a mild acid or even an enzymatic hydrolysis is needed to obtain the monosaccharides from the hemicellulosic fraction for further bioconversion to ethanol.

4.2 Bioethanol from the hemicellulosic hydrolysates

Hemicellulose fermentation involves the fermentation of multiple sugars, sometimes in the presence of inhibitors. Based on the results obtained for the fermentation of mannose in synthetic medium, the yeast was able to ferment this hexose with yields over 80%. During the fermentation of liquors from hemicellulose extractions, which were previously hydrolyzed and then buffered to pH 4.8, the ethanol yield was not so high, with yields up to 63% (Table 4). One hypothesis for the low ethanol yield obtained could be the presence of some inhibitors in the medium that were not removed in the detoxification step or the presence of residual oligomers that were not fully hydrolyzed. Concentration of inhibitory compounds in the acid hydrolysate was 0.03 g/L and 0.21 g/L furfural and 5-hidroximetl furfural (5-HMF), respectively, while in neutral hydrolysates it was 0.5 g/L and 0.3 g/ furfural and 5-HMF, respectively. Some losses of sugars during the neutralization with $Ca(OH)_2$ could also have occurred and contributed to the decrease in ethanol yields. Treatment with $Ca(OH)_2$ was used to detoxify the hydrolysates and increase fermentability (VAN ZYL et al., 1988). The effect of these factors in ethanol production from hemicelluloses is under evaluation in the ongoing work of our group.

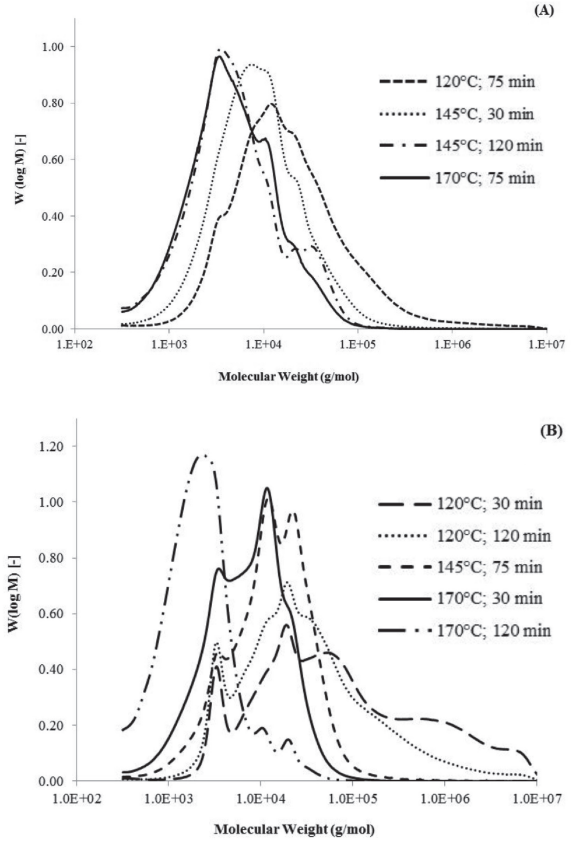

Figure 1 – Size-exclusion chromatograms of (a) acid and (b) neutral hemicellulosic extracts.

Figura 1 *– Cromatogramas de exclusão por tamanho de extratos hemicelulósicos (a) ácido e (b) neutro.*

5. CONCLUSIONS

Hemicelluloses from *P. radiata* wood chips could be extracted up to 60% by means of neutral or acid aqueous solutions. The conversion of wood hemicellulosic substrates to fermentable sugars is feasible but the low concentration of sugars in the extracts and the low ethanol yields obtained make the process not economically attractive and optimization of the process or alternatives uses for hemicelluloses should be evaluated in future studies.

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