



Bioethanol production by *Saccharomyces cerevisiae*, *Pichia stipitis* and *Zymomonas mobilis* from delignified coconut fibre mature and lignin extraction according to biorefinery concept



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ARTICLE INFO

Article history:

Received 11 September 2015

Received in revised form

1 February 2016

Accepted 9 March 2016

Keywords:

Hydrothermal pretreatment

Bioethanol

Lignin

Phenolics

Biorefinery

Fermentation

ABSTRACT

In search to increase the offer of liquid, clean, renewable and sustainable energy in the world energy matrix, the use of lignocellulosic materials (LCMs) for bioethanol production arises as a valuable alternative. The objective of this work was to analyze and compare the performance of *Saccharomyces cerevisiae*, *Pichia stipitis* and *Zymomonas mobilis* in the production of bioethanol from coconut fibre mature (CFM) using different strategies: simultaneous saccharification and fermentation (SSF) and semi-simultaneous saccharification and fermentation (SSSF). The CFM was pretreated by hydrothermal pretreatment catalyzed with sodium hydroxide (HPCSH). The pretreated CFM was characterized by X-ray diffractometry and SEM, and the lignin recovered in the liquid phase by FTIR and TGA. After the HPCSH pretreatment (2.5% (v/v) sodium hydroxide at 180 °C for 30 min), the cellulose content was 56.44%, while the hemicellulose and lignin were reduced 69.04% and 89.13%, respectively. Following pretreatment, the obtained cellulosic fraction was submitted to SSF and SSSF. *Pichia stipitis* allowed for the highest ethanol yield – 90.18% – in SSSF, 91.17% and 91.03% were obtained with *Saccharomyces cerevisiae* and *Zymomonas mobilis*, respectively. It may be concluded that the selection of the most efficient microorganism for the obtention of high bioethanol production yields from cellulose pretreated by HPCSH depends on the operational strategy used and this pretreatment is an interesting alternative for add value of coconut fibre mature compounds (lignin, phenolics) being in accordance with the biorefinery concept.

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1. Introduction

The emerging need to obtain clean, low cost and renewable energy in support of a sustainable energy matrix, demands for the development of biotechnology processes, as is the case of the production of biofuels, that will contribute to the energetic self-sufficiency worldwide. USA and Brazil have achieved a situation close to ideal as from the bioethanol production “food source” with innumerable economic gains. Being a step toward energetic sustainability, the matrix becomes more “green”, but does not fully

satisfy the socio-environmental issues and global geographic distribution. In this regard, an alternative solution is the bioethanol production from LCMs and other non-food source of carbohydrates, available according to the location, making possible a global production of this biofuel. However, some barriers are present in the production of cellulosic ethanol at commercial scale, especially the stages of pretreatment, enzymatic hydrolysis processing, fermentation (hexose and pentose) and the integral use of the raw material. There is also a need to select raw materials, according to the regions, that can be employed as substrate to produce bioethanol.

Brazil makes use of high amounts of sugarcane for the production of bioethanol and generates as waste straw and bagasse. However, in the current conjuncture of the Brazilian sugar and bioethanol production model, these residues are generally intended for electricity production [1], making uncertain its use for ethanol. As an alternative to mitigate this possible absence of

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residues from sugarcane, Brazil possesses raw materials from several other crops as it the case of the coconut residue (known as CFM). The cultivation of coconut is distributed in more than 200 countries and global production in 2009 was about 55 million tons [2]. Brazil is the fourth largest producer, with a production of about 3 million tonnes (5%), the Northeast Region accounting for 82.28% of the total cultivated area [3]. Gonçalves et al. [4] reported that the estimated content of bagasse (fibre) in the coconut in 50%.

For the production of second generation bioethanol within the biorefinery concept, a pretreatment stage for the fractionation of the material is necessary, with the finality of increasing the susceptibility of the material to enzymatic attack. Yang and Wyman [5] reported that the pretreatment stage is the most expensive step in bioethanol production. Pretreatment of LCMs are based on physical, chemical, biological, physicochemical and combined processes and are a key factor in the economic efficiency and one of the main challenges in large-scale production of ethanol [6].

According to Park et al. [7], the thermochemical pretreatment process is now regarded as the most effective one, being pH and temperature the main parameters to be considered. The alkaline pretreatment is used with the intention to remove mainly the lignin. Sodium hydroxide, calcium hydroxide and ammonia are highlighted in this pretreatment due to the low cost and the need of smaller enzymatic loads to convert cellulose into glucose [7]. The use of sodium hydroxide as catalyst pretreatment by steam explosion was carried out by Park et al. [7] and resulted in an elevated delignification and effective enzymatic hydrolysis on *Eucalyptus grandis*. Because a minimal reduction of the lignin on the solid phase in the LCM pretreated by steam explosion occurs [7], similar facts take place with the LCM pretreated by autohydrolysis [8]. Therefore, the use of sodium hydroxide in hydrothermal pretreatment consists an alternative to reduce the lignin content of the LCM, as used by Rawat et al. [9] in poplar (*Populus deltoides*) and Kim and Han [10] on rice straw.

During this pretreatment, the hemicellulose is degraded to low molecular weight oligomers, monomers and organic acids that form a black liquor [11]. Additionally, the black liquor contains a high lignin concentration and phenolic compounds, the recovery of lignin from the liquor being done by the acid precipitation method and converted into synthesis gas or as used as raw material for several aromatic substances of low molecular weight [11,12]. The phenolic compounds are natural sources of antioxidants and may be applied as food additives and cosmetic applications [8,13].

Several strategies for the production of cellulosic ethanol may be applied at the fermentation stage, for example, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). Attributes of SSF are less inhibition of enzyme and longer time of EH compared to SHF; SHF possesses a greater rate of hydrolysis [14]. As an alternative to merge the advantages provided by SHF and SSF, arises the use of semi-simultaneous saccharification and fermentation (SSSF) which includes a presaccharification stage before SSF [15]. SSSF is expected to have higher productivity and yield than SSF and SHF on their own, if the presaccharification time is appropriate [14,16].

Therefore, this work aims to evaluate a strategy for the application of CFM as a raw material for bioethanol production by carrying a HPCSH step followed by two alternative fermentation strategies – SSF and SSSF – using three fermenting microorganisms – *Saccharomyces cerevisiae*, *Pichia stipitis* and *Zymomonas mobilis* – and characterization of lignin obtained in the liquid phase.

2. Materials and methods

2.1. Raw material and chemical characterization

CFM was obtained from the agroindustries locations in the Northeast of Brazil. Chemical characterization was performed by quantitative acid hydrolysis with 5 mL of 72% (w/w) sulfuric acid for 1 h and quantitative post-hydrolysis with 4% sulfuric acid (adding water until 148.67 g) at 121 °C during 1 h. Before HPLC analysis, the solid residue from post-hydrolysis process was recovered by filtration and considered as Klason lignin. Monosaccharides sugars and acetic acid contained in the hydrolysates were determined by HPLC in order to estimate the contents of samples of cellulose and hemicellulose [15]. Composition of LCMs was determined according to the protocols of the a National Renewable Energy Laboratory [17]. Residual ash content was estimated from 2 g sample maintained at 550 °C for 5 h and weighed to measure the residual ash content [18]. The moisture was estimated from a 2 g sample maintained at 105 °C for 24 h and weighed to calculate the residual content [17].

2.2. Pretreatment process

2.2.1. Preparation of raw material before the pretreatment

The CFM was washed for the removal of non-lignocellulosic residues. After five washes with water at 70 °C, the material was dried in an oven with air circulation at 40 °C for 24 h. After this procedure, the CFM was milled into a particle size of 48 mesh (0.3 mm), with the purpose of standardizing the granulometry of the CFM [15].

2.2.2. Hydrothermal pretreatment catalyzed with sodium hydroxide (HPCSH)

The CFM, sodium hydroxide and water were mixed in order to obtain a 10:1 liquid/solid ratio, the conditions of HPCSH are shown in Table 1. These experiments were carried out in 50 mL total volume stainless steel cylinders reactors. The reactor was closed and mounted vertically and then submerged in a Julabo oil bath open heating circulator (Julabo, Germany) with PID temperature control, previously heated to the desired reaction temperature (Table 1). At

Table 1
Operational conditions of pretreatment.

Assay	Operational conditions					
	Normalized variables			Real value		
	T (°C)	t (min)	NaOH (%)	(°C)	(min)	(%)
X ₁	X ₂	X ₃	Temperature	Time	NaOH	
Lineal						
1	-1	-1	-1	160	10	1.0
2	-1	-1	1	160	10	4.0
3	-1	1	1	160	50	4.0
4	-1	1	-1	160	50	1.0
5	1	-1	-1	200	10	1.0
6	1	-1	1	200	10	4.0
7	1	1	1	200	50	4.0
8	1	1	-1	200	50	1.0
Central point						
9	0	0	0	180	30	2.5
10	0	0	0	180	30	2.5
Quadratics						
11	-1	0	0	160	30	2.5
12	1	0	0	200	30	2.5
13	0	-1	0	180	10	2.5
14	0	1	0	180	50	2.5
15	0	0	-1	180	30	1.0
16	0	0	1	180	30	4.0

the end of the desired reaction time (Table 1), the reactor was removed from the oil bath and cooled down by soaking in an ice-water bath for 5 min. The solid and liquid (liquor) were separated via vacuum filtration, both being characterized as mentioned in the Sections 2.3 and 2.4. The experiments were performed in duplicate.

2.2.3. Experimental design and statistical analysis

In order to relate the dependent variable cellulose (cellulose, %) and independent variables temperature (X_1 , °C), time (X_2 , min) and sodium hydroxide concentration (X_3 , %) on pretreated CFM, with the minimum possible number of experiments, a 2^3 central composite experimental design (CCD) for three factors that enabled the construction of a second-order polynomial in the independent variables and the identification of statistical significance in the variables was used.

The mathematical model (Equation (1)) corresponding to the experimental design is:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where:

Y_i = response function (cellulose %);

X_1, X_2, X_3 = values of the independent variables;

β_0 = coefficient relating the interception of the plane with the axis of response;

$\beta_1, \beta_2, \beta_3$ = linear coefficients estimated by the method of least squares;

$\beta_{11}, \beta_{22}, \beta_{33}$ = coefficient of the quadratic variables;

$\beta_{12}, \beta_{13}, \beta_{23}$ = coefficient of the interaction between the independent variables.

The quality of the fit of the polynomial model equation was evaluated by the coefficient of determination R^2 and the statistical significance was evaluated by the Fisher's F-test for analysis of variance (ANOVA) with a 95% confidence level. The effect of each independent variable and also their interaction effects were determined. ANOVA results generated the Pareto charts of interactions and effects. Experimental design package Statistica was the software used for data analysis.

2.3. Characterization of pretreated solids

2.3.1. Chemical composition after the pretreatment

The chemical composition was performed as described above (see Section 2.1.).

2.3.1.1. X-ray diffraction analysis. Cellulose crystallinity of pretreated and untreated CFM were analyzed using X-ray diffractometry (Bruker D8 Discover, USA). Determinations were made operating with radiation from copper $K\alpha$, voltage of 40 kV, electrical current of 40 mA and speed of 2° per minute using continuous scanning angle 2θ from 4 to 70. The crystallinity index (CI) was defined using the Equation (2) [19].

$$CI = \frac{I_{002} - I_{am}}{I_{002}} \cdot 100 \quad (2)$$

where,

I_{002} = maximum intensity (2θ , 22.6°) of the (002) lattice diffraction;

I_{am} = intensity of the amorphous diffraction (2θ , 18.7°).

2.3.1.2. Scanning electron microscopy. The scanning electron microscopy (SEM) surface of pretreated and untreated CFM were visualized by a scanning electron microscope (Nova NanoSEM 200, Netherlands) and photographed. Samples were initially coated with a gold layer by a cathodic sputtering process on voltage of 15 kV and afterwards visualized by SEM.

2.4. Characterization of liquid phase (liquor)

2.4.1. Liquor composition

The liquors were analyzed by HPLC (see Section 2.9.). The following terms correspond to the stoichiometric factor for the conversion of xylan into xylose or arabinan into arabinose (132/150), xylan into furfural (132/96), acetyl groups into acid acetic (43/60), cellulose into glucose (162/180) and cellulose into HMF (162/126) [20].

2.4.2. Total phenolic compounds

Total phenolic contents of liquor samples were determined by spectrophotometry using Folin-Ciocalteu reagent [21]. 100 μ L of liquor, 2 mL of sodium carbonate solution, 500 μ L of Folin-Ciocalteu reagent and 7.5 mL of distilled water were added in a tube. The tubes were placed in a water bath at 50°C for 5 min, cooled down at room temperature and agitated in the vortex. The absorbance of the samples was measured at 700 nm using gallic acid as a standard. A seven point standard curve (0–2000 mg/L) made possible to quantify the contents of total phenolics (in triplicate).

2.4.3. Acid precipitation of lignin in the liquor

Acid precipitation was used to recover the lignin present in liquors. This fraction could be precipitated together with hemicelluloses causing impurities. For that purpose the liquids were acidified to pH = 2 with sulfuric acid at 72% (w/w) and the acidic insoluble fraction was stored for 48 h to decant, washed and dried at 50°C until constant weight (modified from Egués et al. [11]).

Theoretical solubilized lignin (TSL) was calculated by Equation (3):

$$TSL(g) = ((SL \times 0.1) \times (IL \times 0.1)) + ((YL \times 0.1) \times (IL - PL) \times 0.1) \quad (3)$$

where,

SL: % solubilized LCM;

IL: % lignin of LCM untreated;

YL: % LCM unsolubilized;

PL: % lignin of LCM pretreated.

2.5. Characterization of lignin contained in the liquor after CFM pretreatment

2.5.1. Fourier-transform infrared spectra of lignin

Fourier-transform infrared (FTIR) spectra of lignin of pretreated CFM was obtained on an FTIR spectrophotometer (FTLA 2000 series, ABB Bomem Inc., Quebec, Canada). The conditions of analysis were as resolution 4 cm^{-1} , using 20 scans and frequency range of $500\text{--}4000\text{ cm}^{-1}$.

2.5.2. Thermogravimetric analysis of lignin

Thermogravimetric analysis (TGA) was performed using a Shimadzu TGA-50 equipment (Shimadzu Corporation, Japan), with thermal software TASYs. Samples were weighed (between 10 and 15 mg) in aluminium sample pans. The experiments were conducted under N_2 atmosphere, at a heating rate of $10^\circ\text{C}/\text{min}$ over a

temperature range between 20 and 600 °C.

2.6. Enzyme

Enzyme solutions, cellulases, β -glucosidase and hemicellulases (Cellic CTec2) and endoxylanase (Cellic HTec2) were kindly supplied by Novozymes A/S (Bagsvaerd, Denmark). The total cellulase activity from Cellic CTec2 was analyzed in accordance with the standard methodology established by Mandels et al. [22]. In a tube were added 0.3 mL of the commercial enzyme diluted and 1.2 mL of sodium citrate buffer 0.5 μ M at pH = 4.8 and 50 mg Whatman filter paper No. 1 as substrate. The medium was incubated in a water bath at 50 °C for 1 h, the glucose liberated was measured using the DNS method. For Cellic CTec2 enzyme, the β -glucosidase activity was also determined. The β -glucosidase activity was measured by incubating the enzyme solution with 15 μ M of cellobiose and 50 mM sodium citrate buffer (pH = 4.8) at 50 °C for 30 min. The reaction was stopped by immersing in water. Then, the concentration of glucose was determined using the GOD-POD method at room temperature for 10 min and the amount of glucose measured spectrophotometrically at 500 nm. One unit of enzyme activity (CBU/mL) was defined as the release of 1 μ mol of glucose per minute. The xylanase activity was determined for HTec2. Reaction mixtures contained 0.1 mL enzyme and 0.5% (w/v) of oat spelt xylan solution in acetate buffer, pH 5.0. The mixture was incubated at 50 °C for 10 min. After a predetermined period, the released reducing sugars were quantified by DNS method measured spectrophotometrically at 535 nm [23]. One unit of xylanase activity (IU/mL) was defined as the amount of enzyme that released 1 μ mol product per min under the assay conditions. The initial enzyme activities were 126 FPU/mL of cellulase, 269 CBU/mL of β -glucosidase for Cellic CTec2 kit and 1654 IU/mL of endoxylanase for Cellic HTec2 kit.

2.7. Enzymatic hydrolysis

The enzymatic hydrolysis (EH) of delignified pretreated solid of CFM was performed to measure the susceptibility of the pretreated material. EH was performed with 4% (w/v) of delignified pretreated solid of CFM, in an Erlenmeyer flask with a volume of 48 mL at 50 °C, using the enzymatic kit of Cellic CTec 2 and Cellic HTec 2 with an enzyme loading of 30 FPU, 75 CBU and 130 IU per gram of pretreated solid, respectively, in 50 mM sodium citrate buffer with 0.02% (w/v) sodium azide to prevent microbial growth. The agitation was maintained at 150 rpm for 96 h. The samples were taken at 6 h intervals for the first 12 h and 12 h intervals until a total time of 96 h [24,25]. All determinations were performed in duplicate. Concentrations of sugars were determined by HPLC (see Section 2.9). The yield of enzymatic hydrolysis was calculated using Equation (4) [24].

$$\text{Hydrolysis yield (\%)} = \frac{[\text{glucose}] + 1.053 [\text{cellobiose}]}{1.111f \cdot [\text{biomass}]} \cdot 100 \quad (4)$$

where:

- glucose = concentration of glucose (g/L);
- cellobiose = concentration of cellobiose (g/L);
- biomass = concentration of dry biomass initial of enzymatic hydrolysis (g/L);
- f = constitutes of the cellulose fraction of dry biomass (g/g);
- 1.111 = consists in the conversion factor of cellulose to glucose equivalent;
- 1.053 = consists in the conversion factor of cellobiose to glucose equivalent.

2.8. Fermentative process

2.8.1. Microorganisms

P. stipitis Y7124, *S. cerevisiae* PE2 and *Z. mobilis* B14023 strains were used in the production of bioethanol and obtained from the microbiological collection of the Centre of Biological Engineering at the University of Minho. Microorganisms were maintained in glycerol in Eppendorf tubes at –80 °C and subsequently lyophilized for use as a working stock.

2.8.2. Inoculum preparation

S. cerevisiae and *P. stipitis* were maintained in Petri dishes containing PDA (potato dextrose agar) culture medium and *Z. mobilis* was maintained in Petri dishes containing PCA (plate count agar) culture medium at 30 °C for 24 h. The strains for inoculation were grown in 250 mL Erlenmeyer flasks with 100 mL of sterile culture medium containing 50 g/L glucose, 1 g/L ammonium sulfate, 0.5 g/L potassium phosphate, 0.25 g/L magnesium sulfate, 10 g/L yeast extract and 10 g/L peptone at 30 °C and 200 rpm for *S. cerevisiae*, 250 rpm for *P. stipitis* and 150 rpm for *Z. mobilis* in an orbital shaker for 12 h and centrifuged [25]. The cell concentration was inoculated at an optical density of 2 for *S. cerevisiae*, *P. stipitis* and *Z. mobilis*, respectively, at 600 nm in a spectrophotometer UV–VIS [26]. Subsequently, the cells were inoculated into 48 mL culture medium to start the SSF and SSSF processes.

2.8.3. Simultaneous saccharification and fermentation (SSF)

The SSF experiments were conducted using delignified pretreated solids of CFM in accordance with the NREL standard procedure [24]. The SSF was performed with 4% (w/v) of delignified pretreated solids in 48 mL of sodium citrate buffer 50 mM (pH 5.0), using the enzymatic kit of Cellic CTec 2 and Cellic HTec 2 with an enzyme loading of 30 FPU, 75 CBU and 130 IU per gram of pretreated solid [15], supplemented with 1 g/L ammonium sulfate, 0.5 g/L potassium phosphate, 0.25 g/L magnesium sulfate, 2 g/L yeast extract and 1 g/L peptone [25]. The SSF was started by adding enzymes and the strains, incubated at 30 °C in an orbital shaker at 200 rpm for *S. cerevisiae*, 250 rpm for *P. stipitis* and 150 rpm for *Z. mobilis*. The samples were taken at 0, 6, 12, 24, 36 and 48 h. Ethanol and sugars concentrations were determined by means of HPLC (see Section 2.9). All determinations were performed in duplicate.

2.8.4. Semi-simultaneous saccharification and fermentation (SSSF)

The semi-simultaneous saccharification and fermentation was carried out for 12 h of presaccharification + 36 h of SSF process using delignified pretreated solids of CFM as substrate. The SSSF were performed with 4% (w/v) of delignified pretreated solids in 48 mL of sodium citrate buffer 50 mM (pH 5.0), using the enzymatic kit of Cellic CTec 2 and Cellic HTec 2 with an enzyme loading of 30 FPU, 75 CBU and 130 IU per gram of pretreated solid [15]; in this presaccharification period, the medium temperature was maintained at 50 °C. After 12 h of hydrolysis, the medium temperature was adjusted to 30 °C and supplemented with 1 g/L ammonium sulfate, 0.5 g/L potassium phosphate, 0.25 g/L magnesium sulfate, 2 g/L yeast extract and 1 g/L peptone. Then, the fermentative process was started by adding the strains in an orbital shaker at 200 rpm for *S. cerevisiae*, 250 rpm for *P. stipitis* and 150 rpm for *Z. mobilis*. The samples were taken at 0, 6, 12, 24, 36 and 48 h. Ethanol and sugars concentrations were determined by means of HPLC (see Section 2.9). All determinations were performed in duplicate. The ethanol yield (Equation (5)) was calculated assuming that all the potential glucose in the pretreated delignified solids was available for fermentation and that 1 g of glucose yielded 0.51 g of ethanol and 1 g of cellulose gave 0.9 g of glucose [24].

$$\text{Ethanol yield (\%)} = \frac{[\text{ethanol}]}{[\text{glucose or xylose initial}] \cdot 0.511} \cdot 100 \quad (5)$$

where:

ethanol = final ethanol concentration (g/L);
 glucose or xylose initial = initial concentration of glucose or xylose (g/L);
 0.511 = conversion factor of glucose or xylose to ethanol.

2.8.5. Statistical analyzes for SSF and SSSF

Statistical significance of ethanol production was evaluated by Fisher's F-test for analysis of variance (ANOVA) and Student *t*-test, with a confidence level of 95%. Statistical analyzes were performed with the aid of Statistica software.

2.9. Analysis of samples in high performance liquid chromatography

All the samples were centrifuged and filtered through a 0.2 μm sterile membrane filter and analyzed for glucose, xylose, ethanol, HMF, furfural and acetic acid. Chromatographic separation was performed using a Metacarb 87 H column (300 × 7.8 mm, Varian, USA) under the following conditions: mobile phase 0.005 mol/L sulfuric acid, flow rate 0.7 mL/min and column temperature 60 °C using a Jasco chromatograph 880-PU pump (Jasco, Tokyo, Japan) equipped with a Jasco 830-IR refraction-index detector (Jasco, Tokyo, Japan) and a Jasco AS-2057 Plus auto sampler (Jasco, Tokyo, Japan).

3. Results and discussion

3.1. Characterization of pretreated solids

3.1.1. Chemical composition after the pretreatment

The chemical composition of untreated and pretreated solid CFM is shown in Table 2. The moisture content of untreated CFM was 6.14%. A reduction between 39.48 and 78.36% in the amount of solids in pretreated CFM is observed and correlates to the increase of the operational conditions of HPCSH (Table 2).

Changes in the chemical composition of CFM in HPCSH in comparison to the untreated CFM were observed, corresponding to a cellulose increase of 32.18–59.71%, hemicellulose reduction of 27.81–8.61% and lignin reduction of 25.02–2.72% (Table 2). These modifications are strategic for the fractionation of CFM. Gonçalves et al. [15] carried out an autohydrolysis pretreatment (200 °C for 50 min) in CFM, that resulted in a solid yield of 76.89%, cellulose increase of 40.55%, hemicellulose reduction of 50.16% and lignin increase of 19.10%. Recently Kim and Han [10] carried out HPCSH on rice straw (5% (w/w)) with sodium hydroxide at 80 °C for 1 h, the result showed a composition of 50.6% cellulose, 21.7% hemicellulose and lignin 9.5%, with the reduction in lignin of 46.88%. Rawat et al. [9] carried out in poplar (*Populus deltoids*) the HPCSH (2.8% (w/w) with sodium hydroxide at 94 °C for 1 h) having as result an increase in cellulose of 26.15% and hemicellulose of 14.20%, with a reduction in lignin of 12.70%. Similar results were obtained in this work for cellulose and lignin, but a reduction of hemicellulose was obtained. According to Ballesteros et al. [27] increased hemicellulose degradation in agricultural waste occurs at high temperatures and high residence times, therefore, higher operating conditions will provide a higher reduction of hemicellulose. The reduction of lignin increases linearly with an increase in the conditions of the pretreatment, taking into account the alkali concentration, temperature and reaction time [10].

3.1.2. Scanning electron microscopy (SEM)

The use of SEM permits a detailed visualization of morphological and structural changes in CFM after the proposed thermochemical pretreatment. The SEM micrographs of CFM untreated and after HPCSH (2.5% (v/v) sodium hydroxide at 180 °C for 30 min) are shown in Fig. 3A–B.

The SEM images indicate that untreated CFM had intact epidermis, lumen and vascular bundles (Fig. 3A). The HPCSH developed cracks that were heterogeneous and scattered throughout the biomass. Development of these cracks can increase the surface area to facilitate the disruption of lignocellulose, a prerequisite for EH of cellulose and hemicellulose (Fig. 3B). Similar results were reported by Kim and Han [10] and Rawat et al. [9] on rice straw and poplar wood pretreated by HPCSH, respectively.

3.1.3. X-ray diffraction analysis

The analysis of X-ray diffraction for crystallinity index determination were carried out in untreated and pretreated CFM aiming

Table 2
Composition on the solid phase and yield of pretreated CFM.

Assay	Solid phase (%)				
	Solid yield	Cellulose	Hemicellulose	Klason lignin	Ash
Untreated	100 ± 0.00	32.18 ± 0.12	27.81 ± 0.74	25.02 ± 0.21	3.31 ± 0.32
1	60.52 ± 1.86	41.32 ± 0.15	15.79 ± 0.22	22.95 ± 0.10	4.36 ± 0.12
2	54.53 ± 0.53	46.25 ± 0.81	12.49 ± 0.85	20.20 ± 0.23	5.38 ± 0.82
3	48.36 ± 1.88	49.31 ± 0.84	12.88 ± 0.17	10.36 ± 0.21	7.90 ± 0.21
4	48.93 ± 0.42	51.61 ± 0.15	14.72 ± 0.43	14.14 ± 0.19	7.10 ± 0.39
5	54.36 ± 0.77	48.78 ± 0.36	14.79 ± 0.80	15.56 ± 0.13	6.96 ± 0.17
6	43.14 ± 0.92	58.72 ± 1.05	13.71 ± 0.43	11.76 ± 0.33	7.60 ± 0.31
7	21.64 ± 0.39	71.35 ± 1.08	8.61 ± 0.36	2.72 ± 0.35	9.20 ± 0.21
8	43.09 ± 1.06	59.71 ± 0.27	11.23 ± 0.43	5.06 ± 0.84	8.50 ± 0.16
9	43.14 ± 0.39	56.39 ± 0.02	12.26 ± 0.21	12.00 ± 0.11	8.30 ± 0.11
10	42.09 ± 0.28	56.44 ± 0.30	12.59 ± 0.88	11.72 ± 0.10	8.29 ± 0.37
11	48.67 ± 0.13	54.31 ± 0.31	12.85 ± 0.18	12.62 ± 0.11	8.88 ± 0.12
12	41.53 ± 0.60	58.76 ± 0.31	12.69 ± 0.16	10.34 ± 0.33	8.11 ± 0.04
13	45.58 ± 1.36	48.19 ± 0.17	13.02 ± 0.67	12.75 ± 0.21	7.35 ± 0.11
14	32.11 ± 0.15	56.70 ± 0.54	10.03 ± 0.23	7.84 ± 0.14	8.53 ± 0.17
15	45.87 ± 0.13	53.85 ± 0.58	12.30 ± 0.14	13.60 ± 0.28	7.07 ± 0.19
16	41.89 ± 0.63	57.02 ± 0.33	10.10 ± 0.32	9.44 ± 0.11	8.15 ± 0.26

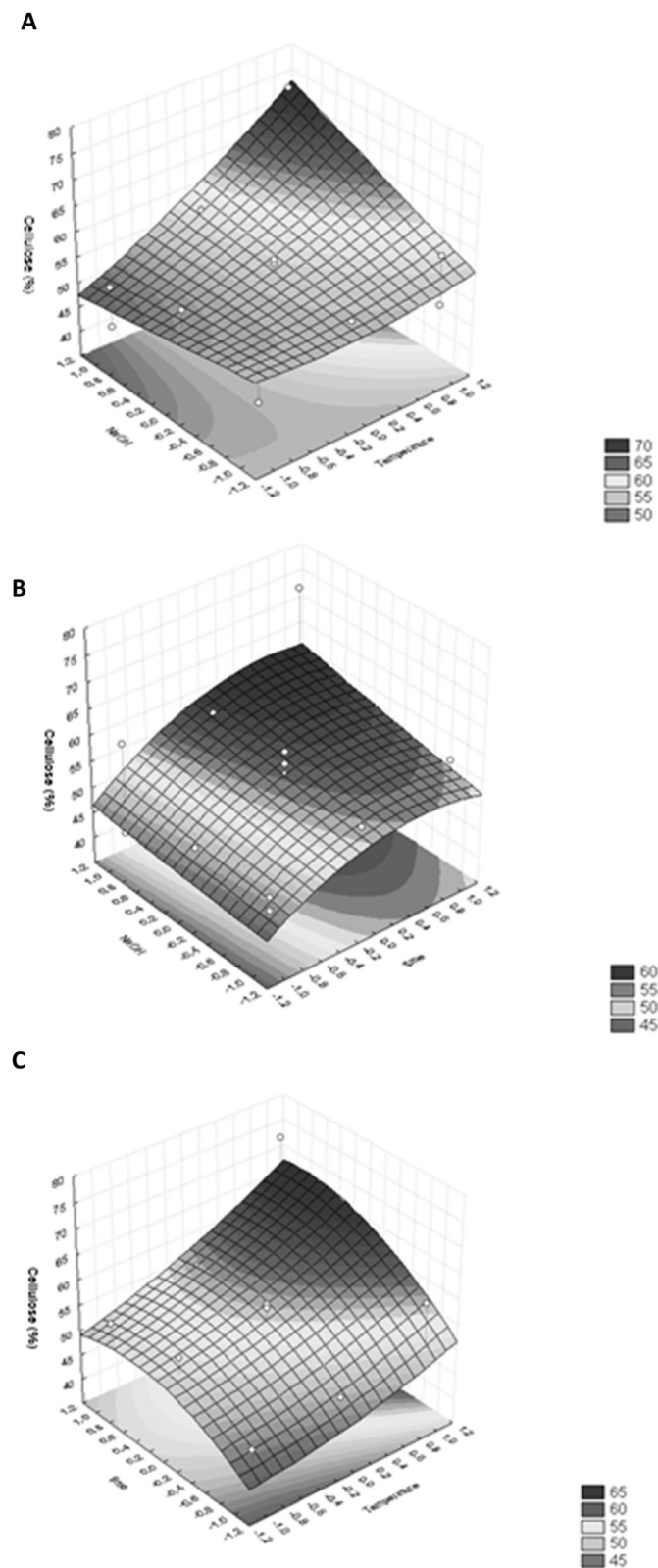


Fig. 1. Response surface and contour plot of pretreated CFM. A) Cellulose variation as a function of temperature and sodium hydroxide; B) Cellulose variation as a function of time and sodium hydroxide; C) Glucose variation as a function of time and temperature.

to analyze the crystalline structure.

Untreated CFM possesses a crystallinity index of 34.34%, while the CFM pretreated by HPCSH (2.5% (v/v) sodium hydroxide at 180 °C for 30 min) presents a value of 56.38% (Fig. 3C). The results showed an increase in the crystallinity index of pretreated CFM in relation to untreated CFM. According to Pu et al. [28], this effect can be explained by the removal of amorphous cellulose and hemicellulose that causes an increase in the crystallinity.

3.1.4. Statistical analysis of pretreatment processing

The experimental design was conducted to determine the variables that affect the pretreatment of CFM and influence in cellulose content of the pretreated material. The conditions used in the experimental design were temperature (160, 180 and 200 °C), time (10, 30 and 50 min) and the concentration of sodium hydroxide (1.0, 2.5 and 4.0%).

Multiple regression analysis and analysis of variance (ANOVA) of the experimental data were performed for the mathematical model fitting. The models in terms of normalized values (Table 1) expressed in Equation (6) represent the cellulose CFM content in HPCSH, as function of temperature (X_1), time (X_2) and concentration of sodium hydroxide (X_3).

$$\begin{aligned} \%Cellulose = & 55.70 + 5.65*Temperature + 4.64*time \\ & + 2.15*NaOH + 4.10Temperature*NaOH \end{aligned} \quad (6)$$

$$(R^2 = 0.9566; R_{adj} = 0.8916)$$

Response surfaces were drawn as three-dimensional plots of the second-order polynomial models (Equation (6)) as a function of the two most strongly influencing variables. Cellulose yield was plotted as a function of temperature, time and sodium hydroxide (Fig. 1) being demonstrated that the increase in cellulose yield is correlated with increases in temperature, time and concentration of sodium hydroxide during the HPCSH.

Results of ANOVA listed in Equation (6) revealed that the second-order polynomial models adequately represent the responses of cellulose yield with coefficients of determination R^2 , which indicates that 95.66% of the variability of response might be explained by the model. These values are in accordance with the adjusted coefficient of determination $R^2_{adj} = 0.8916$.

According to ANOVA results for cellulose yield in CFM pretreated by HPCSH (Table 4), the linear X_1 , X_2 and X_3 and interaction X_1X_3 terms have a significant effect on cellulose yield responses with p-value under a significance level of $\alpha = 0.05$. These effects can be visualized in the standardized Pareto charts (Fig. 2A). It is observed that the variables of temperature, time, concentration of sodium hydroxide and temperature-concentration of sodium hydroxide interaction are important in a confidence level of 95% on the cellulose yield and the effect of temperature, time and concentration of sodium hydroxide are positive, when increased from lower to higher values (Fig. 1). Furthermore, the predicted values versus observed values by the application of the model for multiple regression are shown in Fig. 2B and evidence the good quality of fit.

3.2. Characterization of the liquor

3.2.1. Liquor composition

The liquor separated by filtration of the solid phase from the solution of the HPCSH treated CFM, according to the conditions shown in Table 1, possesses pH variations during HPCSH between 9.3 and 13.6 (Table 3). This increase in pH value is correlated with increase in the operating conditions of the pretreatment (temperature, time and concentration of sodium hydroxide).

A linear correlation is observed between the increase in the pH

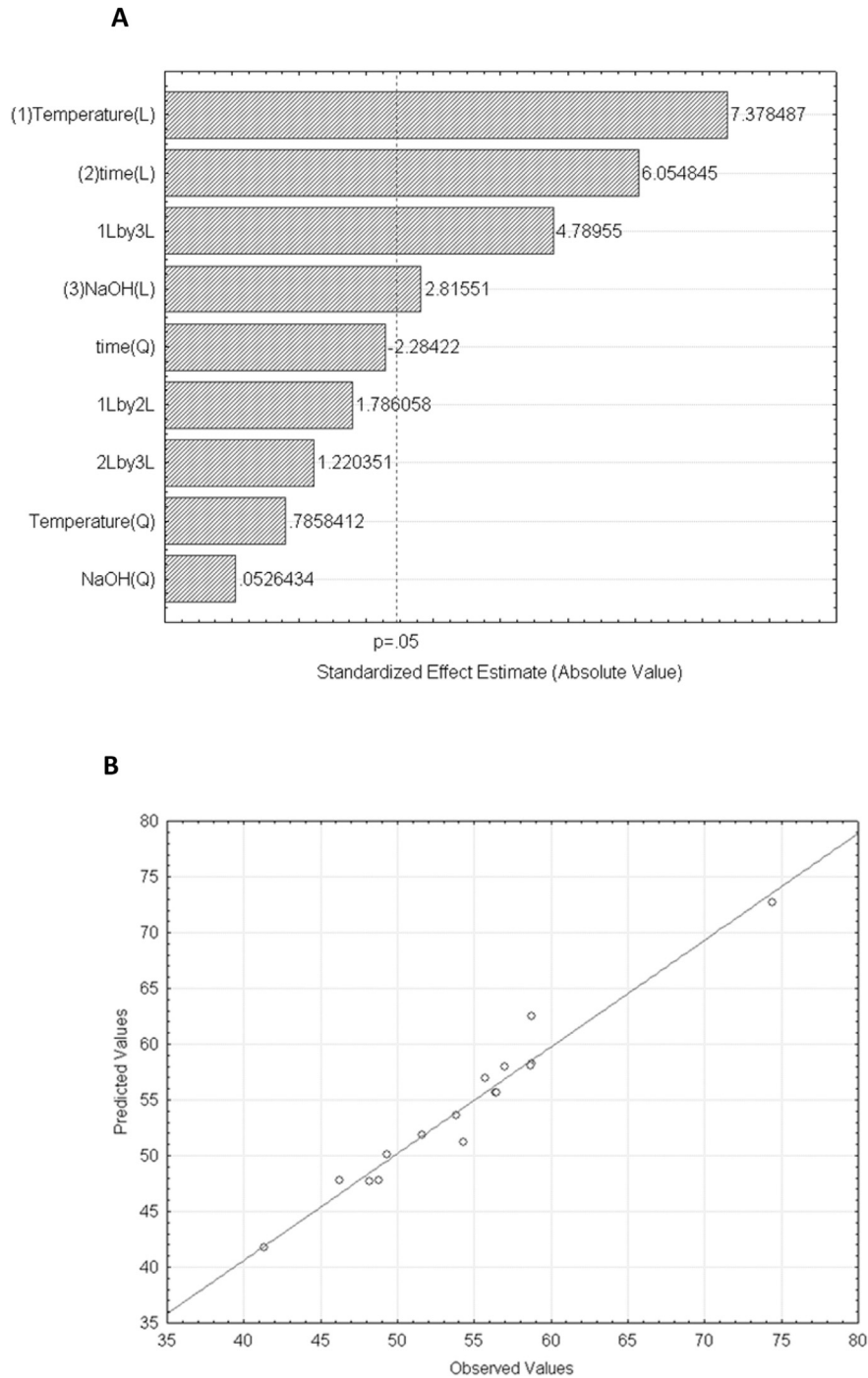


Fig. 2. Pretreated CFM. A) Pareto charts for standardized effects of temperature, time and sodium hydroxide; B) Charts of predicted values versus observed values of CFM pretreated by HPCSH related to cellulose (%).

and the increase in the concentrations of cellulose, hemicellulose, HMF, furfural and total phenolic compounds in the liquors. Furthermore, the pretreatment of CFM by PHCHS allowed for the obtention of concentrations of glucose, hemicellulose, HMF and furfural in the liquors as high as 4.09, 23.09, 0.21 and 0.88 g/L, respectively (Table 3). Monosaccharides, oligosaccharides, acetic acid, total phenolic compounds and lignin present in the liquors can be used in biotechnological and technological processes and in a biorefinery concept.

3.2.2. Total phenolic compounds in the liquid fraction

The liquors of CFM pretreated by HPCSH showed between 7.49 and 17.52 g/L of total phenolic compounds (total phenolic compounds between 74.9 and 175.2 mg/g of LCM dry) (Table 3). Faustino et al. [29] analyzed the black liquor of *E. globulus* in kraft pretreatment and the obtained phenolic compounds concentration was between 91.6 and 293.5 mg/g of LCM dry weight, values that are within the range of results reported in this work.

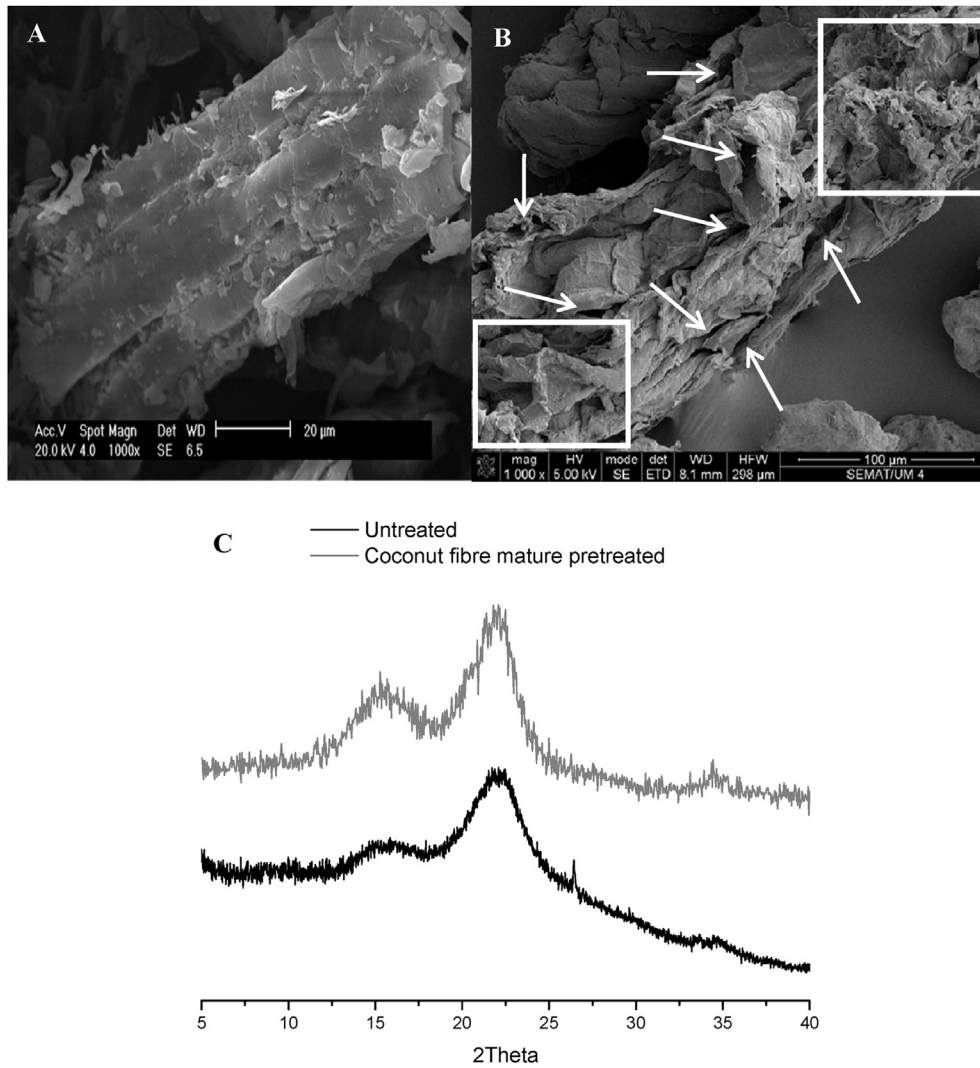


Fig. 3. CFM. A) SEM of CFM untreated; B) SEM of CFM pretreated by HPCSH (2.5% (v/v)) sodium hydroxide at 180 °C for 30 min). High porosity area, matrix separation and exposition fibres (white square); C) DRX of CFM untreated and pretreated.

Table 3

Composition of the liquid phase of pretreated CFM.

Assay	pH	Solubilized material (%)	Lignin recovered (%)	Liquid phase (g/L)					
				Lignin recovered	Phenolic compounds	Glucose	Hemicellulose	HMF	Furfural
1	11.6	39.48	85.27 ± 0.44	8.54 ± 0.09	7.49 ± 0.21	1.36	16.24	0.14	0.55
2	13.2	45.47	84.64 ± 0.50	9.84 ± 0.04	9.02 ± 0.07	0.89	18.69	0.17	0.70
3	13.3	51.64	88.35 ± 0.26	15.91 ± 0.13	11.18 ± 0.03	1.66	19.21	0.17	0.68
4	9.3	51.07	86.57 ± 0.81	16.01 ± 0.11	12.52 ± 0.01	1.88	18.34	0.15	0.60
5	9.9	45.65	88.92 ± 1.28	13.26 ± 0.01	11.86 ± 0.18	1.61	17.60	0.15	0.60
6	12.9	56.86	89.31 ± 0.16	15.69 ± 0.04	12.42 ± 0.45	2.57	19.49	0.16	0.65
7	13.1	78.37	87.88 ± 0.68	19.32 ± 0.14	17.52 ± 0.07	4.09	23.09	0.21	0.88
8	13.0	56.91	88.10 ± 0.14	18.11 ± 0.08	17.09 ± 0.20	2.57	20.44	0.18	0.76
9	13.0	56.86	88.79 ± 0.49	15.86 ± 0.13	14.63 ± 0.02	2.35	20.18	0.17	0.71
10	13.0	57.92	88.66 ± 0.41	16.03 ± 0.04	14.31 ± 0.02	2.35	20.04	0.17	0.70
11	13.0	51.34	88.14 ± 1.00	14.98 ± 0.13	12.17 ± 0.10	2.14	19.19	0.17	0.69
12	12.7	58.47	89.90 ± 0.50	16.43 ± 0.10	14.50 ± 0.27	2.57	20.06	0.17	0.69
13	13.3	54.42	88.40 ± 0.71	15.28 ± 0.13	14.39 ± 0.02	1.55	19.47	0.16	0.68
14	12.9	67.89	91.10 ± 1.45	18.45 ± 0.13	16.07 ± 0.03	2.28	21.88	0.19	0.82
15	9.6	54.13	85.81 ± 0.83	14.86 ± 0.11	11.55 ± 0.05	2.10	19.73	0.17	0.71
16	13.6	58.11	85.12 ± 0.51	15.50 ± 0.13	11.49 ± 0.06	2.41	20.99	0.19	0.81

Table 4

Analysis of variance (ANOVA) for glucose as a function of temperature (x_1), time (x_2) and sodium hydroxide (x_3).

Source	Sum of squares	d.f	Mean square	F-value	p-value
x_1	320.12	1	320.12	54.4420	0.00031*
x_1^2	3.63	1	3.63	0.61757	0.46185
x_2	215.57	1	215.57	36.6611	0.00092*
x_2^2	30.680	1	30.687	24544.6	0.06243
x_3	46.61	1	46.61	7.9270	0.03053*
x_3^2	0.0163	1	0.0163	0.0027	0.95972
x_1x_2	18.7578	1	18.7578	3.1900	0.12433
x_1x_3	134.890	1	134.8903	22.9397	0.00303*
x_2x_3	8.7571	1	8.7571	1.4892	0.26811
Pure error	35.2811	6	5.8801		
Total	814.2037	15			

d.f., degree of freedom, * significant, Fcalculated: 14.72, Ftubulated: 4.10.

3.2.3. Acid precipitation of lignin present in the liquors

Acid precipitation is the most common method for recovering lignin from black liquor [12]. In this context, liquors obtained from the pretreatment of CFM were precipitated by the addition of acid, resulting in values between 8.54 and 19.32 g/L of lignin, with a lignin recovery yield of 91.1% (in relation to the total lignin present in the liquor). In comparison, Egüés et al. [11] carried out the recovery of lignin of corn residues (contains 17.18% of Klason lignin) pretreated by HPCSH (160 °C and 75 min, solid/liquid ratio of 1:20 and shall the solubilization of LCM of 39.75%) resulting in 2.5 g/L of lignin. These results demonstrate the efficiency of the method for lignin recovery used in this work.

3.2.4. FTIR of lignin obtained by precipitation

The analyzes were carried out by infrared spectroscopy with the purpose of obtaining information from chemical groups present in the lignin [30]. The FTIR spectra in the region between 500 and 4000 cm^{-1} of standard lignin and recovered lignin of liquor from CFM pretreated by HPCSH (2.5% (v/v) sodium hydroxide at 180 °C for 30 min) are shown in Fig. 4.

The spectra are dominated by a wide band at 3396–3381 cm^{-1} due to aliphatic and phenolic OH-groups, followed by bands for CH stretching in methyl and methylene groups (2962–2874 cm^{-1}), the bands at 1604, 1516 and 1495 cm^{-1} assigned to aromatic skeletal vibrations, 1454 cm^{-1} assigned to CH deformations and aromatic ring vibrations, 1440 cm^{-1} assigned to aromatic skeletal vibrations, 1331 cm^{-1} assigned to syringyl ring breathing with CO stretching, 1284 cm^{-1} assigned to guaiacyl ring breathing with CO stretching and 1039 cm^{-1} assigned to aromatic CH guaiacyl type and CH deformation of primary alcohol. Besides these, there are bands observed at 1201, 1070, 753, 667 and 570 cm^{-1} . The absence of a peak at 1739 cm^{-1} are assigned to CO stretching of unconjugated ketone, carbonyl and ester groups, indicating the presence of hydroxycinnamates, such as *p*-coumarate and ferulate [31].

The peaks at wavelength of 1170–1164 cm^{-1} were not observed in the spectra of lignins. According to Tan et al. [32], the absence of this peak indicates the absence of sulfur in the lignin and becomes an important characteristic, because sulfur in the lignin is a contaminant and interferes with its use.

3.2.5. Thermogravimetric analysis (TGA) of lignin obtained by precipitation

The thermal stability of the recovered lignin of liquor from CFM pretreated by PHCHS (2.5% (v/v) sodium hydroxide at 180 °C for 30 min) was investigated by TGA and is shown in Fig. 4B. The lignin degradation profiles of the sample of standard lignin and the

sample of recovered lignin of liquor from CFM pretreated by PHCHS were similar. The residual weight at 600 °C of recovered lignin was 55.68%, whereas that of the standard lignin was 56.71%. Overall, the thermal decomposition behaviour of the lignin samples can be divided into three stages. The first stage (25–150 °C) involves the dehydration of lignin due to the absorbed water in lignin. The lignin sample contained a certain amount of bound water whose evaporation occurred at temperatures higher than 100 °C, indicating the strong interaction between water molecules and the hydroxyl groups in lignin samples. The next stage, occurred up to 350 °C, involves the fragmentations of internal linkages between lignin units. The major products in this stage were coke, organic acid and phenolic compounds together with gas products [33]. In the last stage (over 350 °C), pyrolytic degradation of lignin, decomposition and condensation of the aromatic rings occurred [34].

3.3. Susceptibility to the enzymatic hydrolysis

Conversions of CFM pretreated by HPCSH (2.5% (v/v) sodium hydroxide at 180 °C for 30 min) in glucose was 90.72% at 96 h of EH, being 88.90% at 72 h and 85.35% at 48 h of EH (Fig. 5A).

These results demonstrate the accessibility of CFM to the enzymatic attack. In comparison, for the CFM pretreated by autohydrolysis (200 °C for 50 min) the conversion in glucose by EH at 96 h was 84.10% [15]. The minor conversion of CFM pretreated by autohydrolysis in glucose may have been influenced by the higher content of lignin in the CFM (29.80% of lignin), when compared to the CFM pretreated by HPCSH (11.72% of lignin) [15]. The EH of poplar wood treated by HPCSH (2.8% (w/w) sodium hydroxide at 94 °C for 1 h) resulted in a conversion into glucose at 48 h of 41.5% [9]. The conversion to glucose during the EH of *Eucalyptus grandis* pretreated by steam explosion catalyzed by sodium hydroxide (7% (w/w) of sodium hydroxide at 210 °C for 9 min) was 65.55% in 72 h [7]. The EH of rice straw after HPCSH (5% (w/w) sodium hydroxide at 80 °C for 1 h) resulted in a conversion into glucose of 78.7% at 72 h [10].

Looking at the maximum initial hydrolysis rate (productivity of glucose) (dG/dt), that occurs in the first 12 h (the slope of glucose concentration vs time) shown in Fig. 5B, the initial hydrolysis rate of CFM was 1.27 g/(L h) (Fig. 5B). Gonçalves et al. [15] reported the initial hydrolysis rate of autohydrolysis pretreated CFM as 0.82 g/(L h) using 30 FPU/g of LCM. In comparison, Ruiz et al. [35] reported the initial hydrolysis rate of wheat straw pretreated by autohydrolysis as 0.47 g/(L h) using 30 FPU/g of cellulose. Rawat et al. [9] studied the initial hydrolysis rate of poplar wood using HPCSH and obtained 1.67 g/(L h) for 15 FPU/g of cellulose. These results demonstrate the susceptibility of CFM pretreated by PHCHS to the enzymatic attack.

3.4. Fermentative process for bioethanol production

SSF and SSSF strategies were carried out using *S. cerevisiae* PE2, *P. stipitis* Y7124, *Z. mobilis* B14023 on CFM pretreated by HPCSH (2.5% (v/v) sodium hydroxide at 180 °C for 30 min) as raw material. According to Shen and Agblevor [14], the performance of fermentative strategies can be assessed by two indicators: yield and volumetric productivity of ethanol.

3.4.1. Simultaneous saccharification and fermentation (SSF) and semi-simultaneous saccharification and fermentation (SSSF)

The SSF bioethanol production results by *S. cerevisiae*, *P. stipitis* and *Z. mobilis* are shown in Fig. 6A–C, respectively. The results of this study indicate that glucose from EH could be fermented to

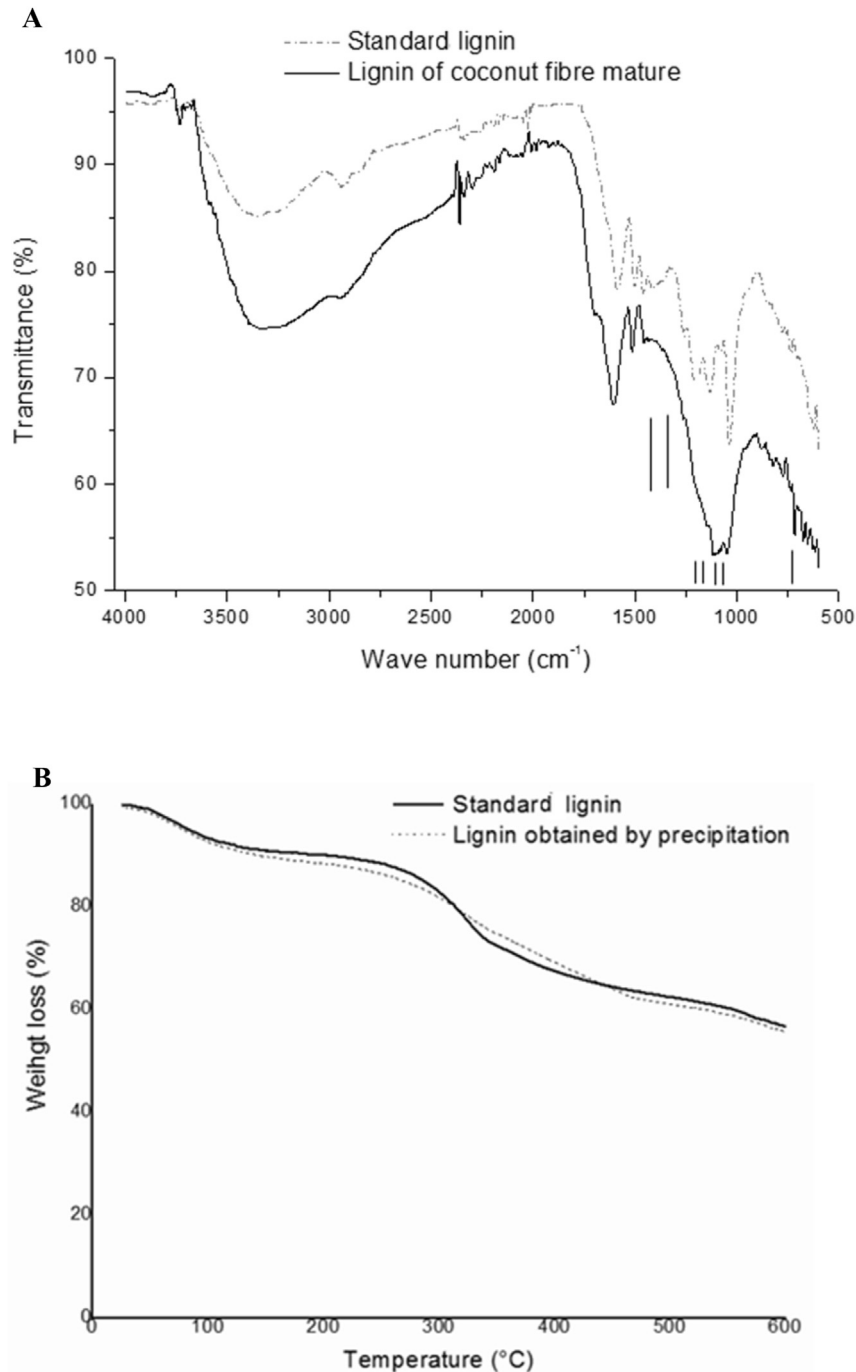


Fig. 4. CFM pretreated by HPCSH (2.5% (v/v)) sodium hydroxide at 180 °C for 30 min. A) FTIR of recovered lignin of liquor of pretreated CFM; B) TGA of recovered lignin of liquor of pretreated CFM.

ethanol by *S. cerevisiae*, *P. stipitis* and *Z. mobilis*, the kinetic profiles having a similar pattern for glucose, with rapid glucose consumption during the initial 24 h. The corresponding production of ethanol in SSF by *S. cerevisiae* and *Z. mobilis* were 10.91 g/L and 10.96 g/L, being the SSF process completed after 48 h (Fig. 6 and Table 5). The ethanol yield for *P. stipitis* was 87.44%, corresponding to a volumetric productivity of ethanol of 0.23 g/(L h) in 48 h, while for *S. cerevisiae* and *Z. mobilis* the ethanol yield were 85.31% and 84.56%, respectively. The *S. cerevisiae* and *Z. mobilis* presented

volumetric productivity of ethanol of 0.30 g/(L h) at 36 h (Table 5). The three microorganisms proved to be suitable for fermenting sugars to ethanol in SSF strategy.

In a recent work, Chaudhary et al. [36] produced bioethanol through of a sequential alkaline and acid pretreatment using Kans Grass biomass as substrate and *P. stipitis* as microorganism, reporting a productivity of 0.22 g/(L h). Vaithanomsat et al. [37] studied the efficiency in the bioethanol production using SSF process with *S. cerevisiae* and coconut husk pretreated with sodium

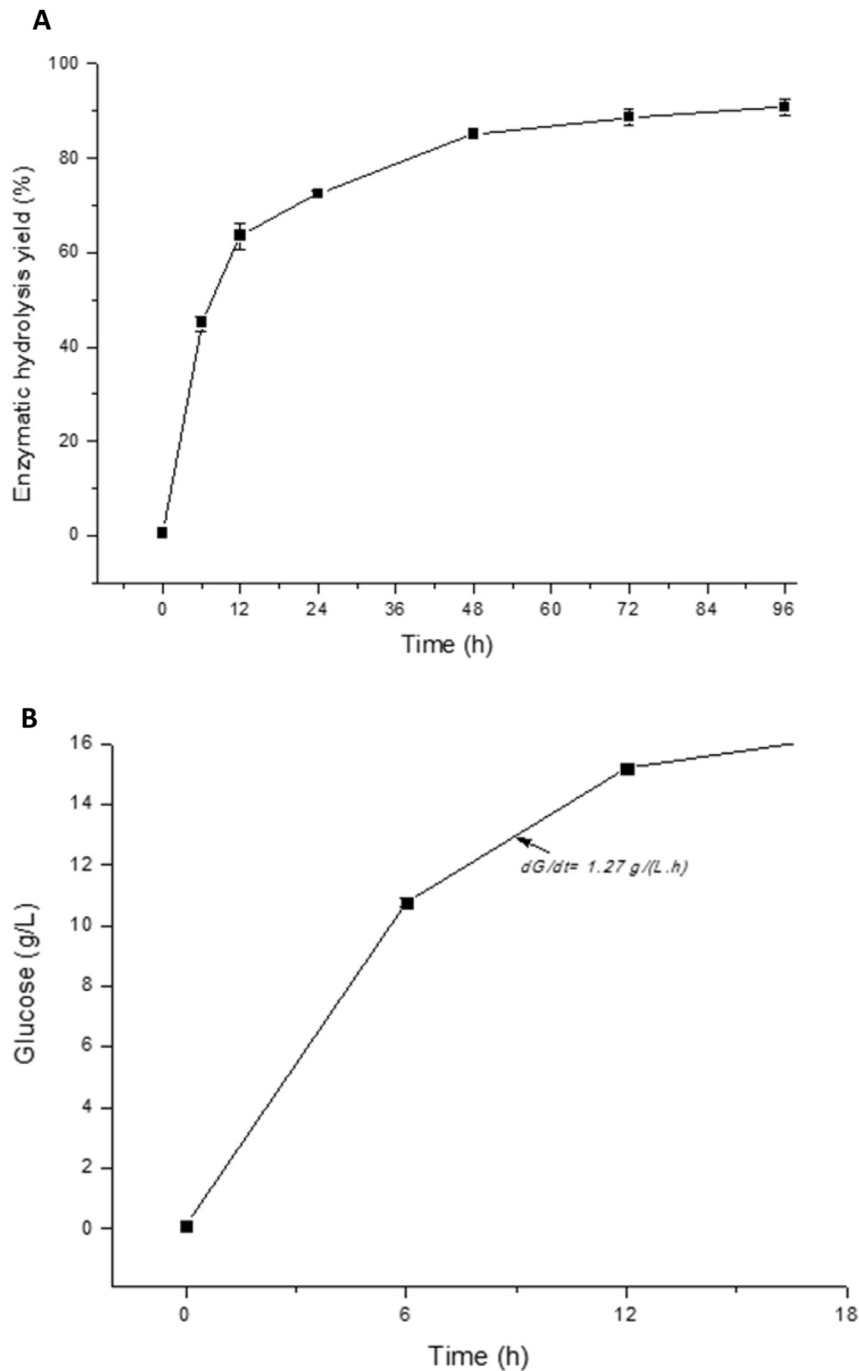
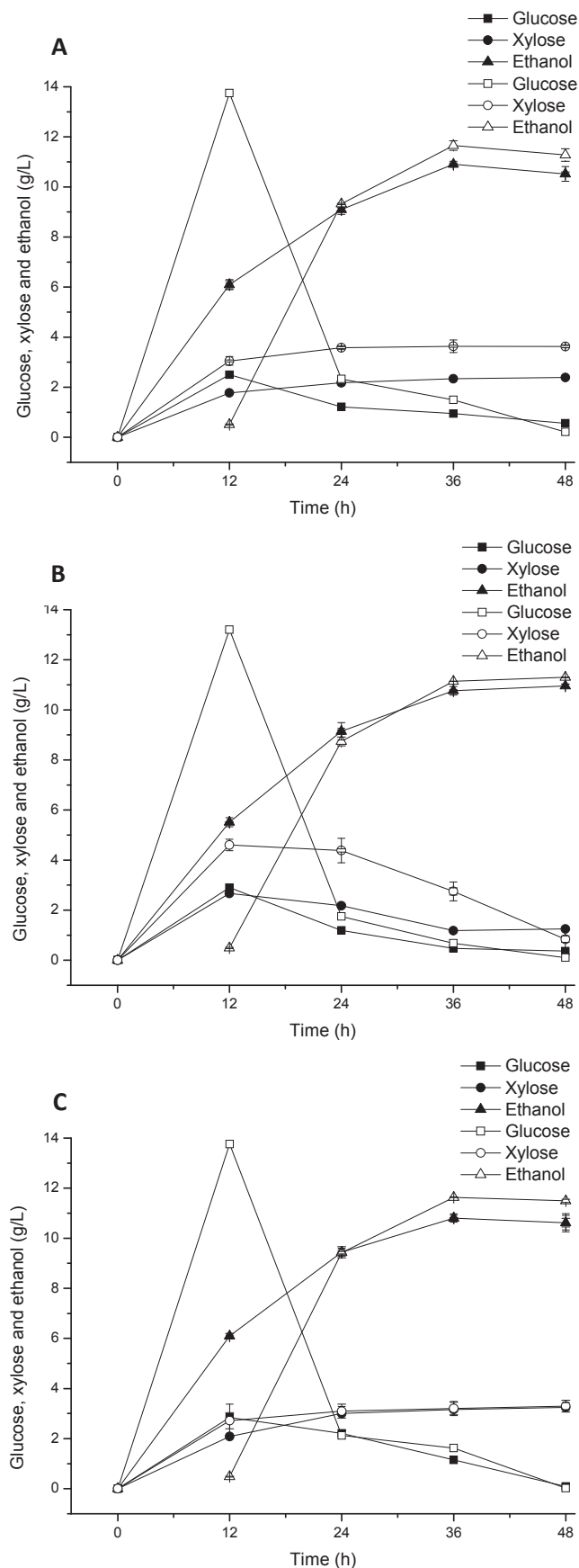


Fig. 5. EH of CFM pretreated by HPCSH (2.5% (v/v)) sodium hydroxide at 180 °C for 30 min. A) Conversion in glucose (%); B) Initial hydrolysis rate at 12 h.

hydroxide as raw material and reported a yield of ethanol of 85%. Gonçalves et al. [15] reported the ethanol production by *S. cerevisiae*, *P. stipitis* and *Z. mobilis* in SSF using autohydrolyzed CFM, producing ethanol with a concentration of 7.44 g/L, 8.47 g/L and 7.30 g/L and ethanol yield of 86.90%, 84.19% and 85.26%, respectively. These results are in agreement with those reported in this work.

The SSSF strategy had a similar pattern for glucose concentration during the initial 12 h and a gradual decrease with time (Fig. 6A–C). The corresponding production of ethanol in SSSF by

S. cerevisiae, *P. stipitis* and *Z. mobilis* were 11.65 g/L, 11.29 g/L and 11.64 g/L, being the SSSF process completed after 48 h, respectively (Fig. 6 and Table 5). The ethanol yield for *P. stipitis* was 90.18%, corresponding to a volumetric productivity of ethanol of 0.24 g/(L h), in 48 h, while for *S. cerevisiae* and *Z. mobilis* the obtained values were 91.17%, 91.03% and 0.32 g/(L h), 0.32 g/(L h) for ethanol yield and volumetric productivity, in 36 h, respectively (Table 5). The three microorganisms proved to be suitable for fermenting sugars to ethanol in SSSF strategy. In a recent work, Franco et al. [38] carried out the SSSF process (24 h of presaccharification and



24 h of SSF) using delignified *Pinus radiata* and *S. cerevisiae*, resulting in the production of 15.5 g/L of ethanol with a yield of 90.0%. Furthermore, Gonçalves et al. [15] reported ethanol production and ethanol yield values by *S. cerevisiae*, *P. stipitis* and *Z. mobilis* in SSSF using CFM pretreated by autohydrolysis of 7.71, 8.78 and 7.63 g/L, 90.09%, 87.25% and 89.16%, respectively. These results corroborate the results reported in this work for SSSF.

The ethanol production by *S. cerevisiae*, *P. stipitis* and *Z. mobilis* using fermentative strategies SSSF and SSF was evaluated statistically using ANOVA and *t*-test (confidence level 95%). The ethanol production by the microorganisms using SSF showed no significant differences, when evaluated by the ANOVA. A similar result was also obtained from the microorganisms using SSSF. The comparison between the results obtained by *S. cerevisiae* using SSF and *S. cerevisiae* using SSSF showed a significant difference, when evaluated by the *t*-test. A similar result was also shown for *Z. mobilis*.

Results obtained for ethanol production, ethanol yield and volumetric productivity with the SSSF strategy are slightly higher compared with SSF (see Table 5). The higher fermentative efficiency for SSSF may be explained by the application of the short presaccharification period that can enhance the conversion of cellulose to glucose and, in sequence to ethanol, thus, resulting in a decrease in the enzymatic inhibition. Furthermore, the short presaccharification process increases the solubility of the substrate, leading to the improved mass transfer of nutrients to the microorganism. Another important issue related to ethanol production is the need to increase cellulosic ethanol concentration to reduce costs in the distillation process. The use of a processing step, such as suggested in SSSF, that allows for high solids loading as a result of the reduction of fermentation medium viscosity may have a positive impact on the overall process [26,39].

In this sense, fermentative strategies SHF, SSF and SSSF were evaluated by some authors. For example, Santos et al. [25] carried out fermentation with *S. cerevisiae* in SHF (72 h), SSF (24 h), SSSF I (16 h of presaccharification and 24 h of SSF) and SSSF II (16 h of presaccharification and 24 h of SSF) with pretreated sugarcane bagasse by steam explosion and delignified with sodium hydroxide. The best results for the volumetric productivities of ethanol were obtained in SSSF I and SSSF II, 0.29 g/(L h) and 0.30 g/(L h), respectively. Mesa et al. [16] reported that from 1 ton of sugarcane bagasse it is possible to obtain 192 L, 172 L and 198 L of ethanol from SHF, SSF and SSSF, respectively. They concluded that SSSF is the best process strategy based on ethanol yield and volume of produced ethanol. These results demonstrate the importance of performing the stage of presaccharification before the fermentative process and corroborate with the results obtained in this work.

4. Conclusion

The CFM pretreated by HPCSH showed promising results for application as substrate in the bioethanol production with high conversions of CFM into glucose during the EH and ethanol by the fermentative strategies of SSF and SSSF. Moreover, the liquors of CFM pretreated by HPCSH contained a high content of lignin (high quality) and phenolics that could be recovered by precipitation for further utilization according to the biorefinery concept. It is also shown that the most efficient microorganism to be used in the production of bioethanol depends on the operational strategy used.

Fig. 6. Fermentation in SSF and SSSF of CFM pretreated by HPCSH (2.5% (v/v)) sodium hydroxide at 180 °C for 30 min). A) *S. cerevisiae*; B) *P. stipitis*; C) *Z. mobilis*. The SSF was represented by the black icon and SSSF was represented by the white icon.

Table 5

Kinetic parameters of ethanol fermentation of *S. cerevisiae*, *P. stipitis* and *Z. mobilis* in SSF and SSSF using CFM pretreated by PHCHS (2.5% (v/v)) sodium hydroxide at 180 °C for 30 min).

Bioprocess	Microorganism	Ethanol yield (%)	Ethanol production (g/L)	Ethanol productivity g/(L h)
SSF	<i>S. cerevisiae</i>	85.31 ± 1.17	10.91 ± 0.15	0.30 ± 0.15
	<i>P. stipitis</i>	87.44 ± 1.79	10.96 ± 0.22	0.23 ± 0.22
	<i>Z. mobilis</i>	84.56 ± 1.65	10.81 ± 0.21	0.30 ± 0.21
SSSF	<i>S. cerevisiae</i>	91.17 ± 1.46	11.65 ± 0.19	0.32 ± 0.19
	<i>P. stipitis</i>	90.18 ± 1.89	11.29 ± 0.24	0.24 ± 0.24
	<i>Z. mobilis</i>	91.03 ± 0.68	11.64 ± 0.09	0.32 ± 0.09

Acknowledgments

The authors gratefully acknowledge the Brazilian research funding agencies CNPq (Proc:470356/2011-1) and CAPES (Proc:BEX5951/11-9) for financial support.

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