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Bioethanol production from coconuts and cactus pretreated by autohydrolysis

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ABSTRACT

The use of coconut fiber mature, green coconut shell, mature coconut shell and cactus is an important alternative as substrates for bioethanol production, since these lignocellulosic materials (LCMs) are abundant in Brazil, mainly in the Northeast Region. The first objective of this work was to evaluate the autohydrolysis pretreatment (AP) on these LCMs and the susceptibility of the treated materials to enzymatic hydrolysis (EH). The second part of the work deals with the application of semi-simultaneous saccharification and fermentation (SSSF) and simultaneous saccharification and fermentation (SSF) using *Zymomonas mobilis*, *Pichia* stipitis, *Saccharomyces cerevisiae* and as substrate the green coconut shell (selected according to the results obtained in the first part of the work). The LCMs after AP using the highest severity factor (4.64) showed changes in the chemical composition in comparison to the untreated LCMs: between the LCMs the cellulose increase was 48.55%, the hemicellulose decrease was 76.77% and an increase of 62.26% was observed for lignin. The green coconut shell was characterized by SEM, X-ray and FTIR after AP and its EH conversion into glucose was 92.52%. The best results on ethanol yield (90.09%) and ethanol productivity (0.21 g/(L h)) from green coconut shell were obtained by *S. cerevisiae* using SSSF. Overall, an efficient process for the bioethanol production from green coconut shell was developed.

1. Introduction

In search to mitigate climate change and fossil fuel dependence of some countries, arise as an alternative biofuel production, especially ethanol produced from sugarcane sucrose and cornstarch. However, both processes have economical and environmental limitations for its productive expansion. Thus, a promising option to increase the ethanol supply is the use of lignocellulosic materials (LCMs) as substrate, which are initially converted in fermentable sugars from pretreatment and EH processes and subsequent fermentation. Attempts to produce ethanol from LCMs are old and originated in Germany and Russia over 80 years ago from the saccharification of LCM by acid hydrolysis (Bastos, 2007).

The LCMs used for ethanol production should be from forests (extracted from vegetable or cultivated), agroindustrial and urban wastes, vegetables grown in inhospitable environments and photosynthetic aquatic biomass (Gonçalves et al., 2014). In this context, this work uses agroindustrial waste (coconut fiber mature, green coconut shell and mature coconut shell), urban waste (green coconut shell) and vegetable cultivated in inhospitable environments (cactus) aiming at the bioethanol production. According to FAO (2012), the global production of coconut in 2009 was approximately 55 million tons, mainly in the Philippines, Indonesia and India. The fourth largest producer of coconut is Brazil (IBGE, 2012). Besides, in 2009, the production of cactus in Brazil was 60,000 tons (IBGE, 2012).

The recalcitrance of these LCMs demands a pretreatment to facilitate enzymatic action (Romaní et al., 2010). Several methods for vegetable biomass pretreatment have been studied, e.g., biological, physical, chemical or its combination. The autohydrolysis pretreatment (also called liquid hot water or hydrothermal processing – AP) was initially used by Bobleter et al. (1976) to increase susceptibility to EH of LCMs. Normally, the advantages in the use of AP are the simple operation, absence of corrosion in the equipment, unnecessary use of chemical solvent, except water, addition to low operating costs (Cybulska et al., 2010; Romaní

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et al., 2010). In this way, the AP has important features in the preservation of the environment (Garrote et al., 2003).

The AP is carried out by the heating the aqueous suspension in the presence of the LCMs and resulting in the depolymerization and solubilization of the hemicellulose in the liquid phase (oligosaccharides and monosaccharides) (Romaní et al., 2012; Ruiz et al., 2013), as well as products of sugar degradation (furfural and hydroxymethylfurfural – HMF) and acetic acid (Ruiz et al., 2013). Surface area and porosity of LCM increases (Cybulska et al., 2010) and re-location of the lignin on the surface of the LCMs (Ruiz et al., 2013) occurs that, together with an increase in the fraction of the cellulose in the pretreated LCMs, contributes to the improvement of the susceptibility of these solids to EH (Ruiz et al., 2013). The solubilization of hemicellulose by AP may be considered as the first stage in the implementation of the biorefinery concept (Romaní et al., 2011) that makes possible the selective separation of the most important components contained in the LCMs (hemicellulose, cellulose and lignin) including the recovery of lignin after distillation of the fermented broth.

This biorefinery concept consists of an industrial installation that unites equipment and conversion processes to produce power, fuels and other chemicals derived of LCMs (NREL, 2012), use the resources in a sustainable way, without producing waste and other environmental pollutants, based on the association of cleaner processes (Luo et al., 2010). The biorefinery based on lignocellulose is a promising strategy to the implementation of this concept (Uihlein and Schebek, 2009).

Moreover, there are different fermentative strategies for bioethanol production (Shen and Agblevor, 2011). Lately, the separate hydrolysis and fermentation (SHF) have shown to have several disadvantages relative the yield and volumetric productivity of ethanol compared with SSF. Furthermore, the SSF has less processing time, enzymatic inhibition and equipment costs (Shen and Agblevor, 2010). However, for an efficient SSF is necessary approaches the optimal temperature of enzymatic action and the growth of microorganism (De Souza et al., 2012). The fermentative strategy of SSSF consists an interesting alternative that has a small presaccharification period prior to the SSF and shows higher ethanol productivity, yield and concentration compared to SHF and SSF strategies (Shen and Agblevor, 2011).

The objective of this work was to study the AP on cactus, coconut fiber mature, green coconut shell and mature coconut shell followed by EH as a suitable LCMs material for bioethanol production. Moreover, SSF and SSSF strategies for bioethanol production utilizing *Pichia stipitis*, *Saccharomyces cerevisiae* and *Zymomonas mobilis* were developed.

2. Materials and methods

2.1. Raw material, chemical and physical agents in the chemical characterization

The LCMs used in this work were the cactus, coconut fiber mature, green coconut shell and mature coconut shell derivatives of the urban locations and agroindustries in Brazil (Northeast Region).

The chemical characterization was carried out with 0.3 g from LCMs and 5 mL of sulphuric acid (concentration of 72% (w/w)) during 1 h. The post-hydrolysis using 4% sulphuric acid and adding water (until 148.67 g) at 121 °C for 1 h. Before analyzing in high performance liquid chromatography (HPLC), the LCMs solids residues derivatives of post-hydrolysis process were recovered by the filtration and regarded as Klason lignin, based on standard test methods (T-249 and T- 264) of the Technical Association of the Pulp and Paper Industry (TAPPI) (www.tappi.org). Monosaccharides, acetic acid, furfural and HMF contained in the hydrolysates were determined by the HPLC, with the purpose of content estimate of the acetyl groups, arabinan, cellulose and xylan of samples. Compositions of LCMs were based in the protocol analysis of National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008) and its subsequent amendments. Residual ash content was estimated from 2 g sample maintained at 550 °C by 5 h and weighed to measure the residual ash content (Sluiter et al., 2008). Moisture was determined from 2 g sample maintained at 105 °C by 24 h and weighed to calculate the dry weight (Sluiter et al., 2008).

Table 1
Autohydrolysis pretreatment of LCMs. (A) Operational conditions; (B) severity factor.

A

Assay	Operational conditions			
	Real value		Normalized variables[a]	
	T (°C)	t (min)	T (°C)	t (min)
	X_1	X_2	X_1	X_2
1	160	10	−1	−1
2	160	50	−1	1
3	160	30	−1	0
4	200	10	1	−1
5	200	50	1	1
6	200	30	1	0
7	180	10	0	−1
8	180	50	0	1
9	180	30	0	0
10	180	30	0	0

B

Severity factor (R_0)			
T (°C)/t (min)	10	30	50
160	2.76	3.24	3.46
180	3.35	3.83	4.05
200	3.94	4.42	4.64

Note: the mathematical model (Eq. (1)) corresponding to the severity parameter $\log R_0$ is $R_0 = \int_0^t \exp\left(\frac{T-100}{14.75}\right)\ dt$.

[a] X_1: temperature, X_2: time.

2.2. Pretreatment stage

2.2.1. Preparation of lignocellulosic materials before of the pretreatment

The LCMs were initially washed with the purpose of withdrawing the residual compounds existing. Afterwards of five washes utilizing water (70 °C), the LCMs were dried in an oven with air circulation during 24 h at 40 °C. After this procedure, the LCMs were milled to standardize a particle size of 0.3 mm (48 mesh) (Gonçalves et al., 2014).

2.2.2. Autohydrolysis pretreatment (AP)

The LCMs and water were mixed to obtain a ratio 1:10 solid/liquid (v/w), the conditions and severity of pretreatment are shown in Table 1A and B. The stainless steel cylinders reactors with total volume of 50 mL were utilized in the experiments. These reactors were closed and then immersed in oil bath (Julabo, Germany) equipped with a PID temperature control, pre-heated to the desired reaction temperature, based on Table 1. After the end of the desired reaction time established in Table 1, the reactors were removed from the oil bath and submerged in an ice-water bath during 5 min. The solid phase and liquid (liquor) were separated by vacuum filtration (Ruiz et al., 2012), both being characterized according to the methods presented in Sections 2.3 and 2.4. The effects of the temperature and time on pretreated LCMs were analyzed according to severity parameter of the $\log R_0$ (Overend and

Chornet, 1987) (Eq. (1)). The experiments were performed in duplicate (Table 1A).

The mathematical model (Eq. (1)) corresponding to the severity parameter log R_0 is:

$$R_0 = \int_0^t \exp\left(\frac{T-100}{14.75}\right)\ dt \quad (1)$$

where t: reaction time (min); T: temperature (°C); 100: reference temperature; 14.74: empirical parameter related to the activation energy (pseudo first order kinetics).

The residence times during heating at constant temperature and the constant set temperatures allowed the calculate of log R_0 values. Both the heating and cooling were not considerate.

2.2.3. The experimental design and the statistical analysis

For determining the dependent variable: cellulose (cellulose, %); independent variables: temperature (X_1, °C) and time (X_2, min) in LCMs pretreated by AP, a 2^2 with central composite experimental design using 3 factors, enabling the production of second order polynomials in the independent variables. Besides using the identification of the variables related to statistical significance.

The mathematical model related to the experimental design is shown in Eq. (2) as:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (2)$$

where Y_i: response function; X_1, X_2: values in relation to the independent variables; β_0: coefficient relating to the interception of the plane with the axis of response; β_1, β_2: linear coefficients estimated by the method of least squares; β_{11}, β_{22}: coefficient of the quadratic variables; β_{12}: coefficient of the relationship among the independent variables.

The coefficient of determination R^2 allowed to evaluate the quality of the fit in the polynomial model equation. The Fisher's F-test allowed to evaluate the statistical significance from analysis of variance (ANOVA) using confidence level of 95%. Also, the effects of the independent variables and the interaction effects correspondent were determined. The Pareto charts regarding the effects and interactions were created from the ANOVA results. The software utilized in the data analysis was Statistica 7.0.

2.3. Characterization of the solids after autohydrolysis pretreatment

2.3.1. Chemical composition of LCMs

The LCMs were characterized as described above (see Section 2.1).

2.3.2. Crystallinity and X-ray diffraction analysis

The LCMs untreated and pretreated were analyzed in the X-ray diffractometry (Bruker D8 Discover, USA) to determine the crystalline of the cellulose. The operational conditions were the radiation from Cu Kα, 40 kV of voltage, 40 mA of electrical current and a rate of 2 degrees per min using continuous scanning angle 2θ from 4 to 70. Eq. (3) was used for defining the crystallinity index (CI) (Segal et al., 1959).

$$CI = \frac{I_{0\,0\,2} - I_{am}}{I_{0\,0\,2}}.100 \quad (3)$$

where I_{002}: maximum intensity (2θ, 22.6°) for lattice diffraction (0 0 2); I_{am}: amorphous diffraction intensity (2θ, 18.7°).

2.3.3. Scanning electron microscopy (SEM) of LCMs

The surfaces of LCMs untreated and pretreated were analyzed in a Nova NanoSEM 200. The samples were initially covered by a gold layer using a cathodic sputtering process, a voltage of 15 kV and afterwards visualized by SEM.

2.4. Characterization of the liquid phase after autohydrolysis pretreatment

The liquor phase was separated by filtration after the autohydrolysis pretreatment. The liquors were analyzed by HPLC (see Section 2.8). A second sample with 20 mL of this liquor was utilized for quantitative the pos-thydrolysis. Thus, the sample was subjected to a solution of 4% sulphuric acid at 121 °C for 1 h, and then the HPLC analysis (Garrote et al., 2002). This posthydrolysis step of the liquor resulting of autohydrolysis pretreatment consists in quantify the oligosaccharides from acid hydrolysis (sulphuric acid), because autohydrolysis pretreatment possesses low hydrolysis these oligosaccharides.

Eqs. (4)–(13) were used to determine the following components: (1) the proportion of furfural (F_C), xylooligosaccharides (XO_C) and xylose (Xyl_C) from of xylan; (2) the proportion of arabinose (Ara_C) and arabino-oligosaccharides (ArO_C) from of arabinan; (3) the proportion of acetic acid (AcI_C) and acetyl groups linked to oligosaccharides (AcO_C) from of acetyl groups; (4) the proportion of HMF (HMF_C), glucose (Glu_C) and glucooligosaccharides (GO_C) from of cellulose (Garrote et al., 2002).

$$XO_C = \frac{132}{150} \cdot \frac{XO}{Xyl_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (4)$$

$$Xyl_C = \frac{132}{150} \cdot \frac{Xyl}{Xyl_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (5)$$

$$ArO_C = \frac{132}{150} \cdot \frac{ArO}{Ara_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (6)$$

$$Ara_C = \frac{132}{150} \cdot \frac{Ara}{Ara_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (7)$$

$$F_C = \frac{132}{96} \cdot \frac{F}{Xyl_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (8)$$

$$A_C O_C = \frac{43}{60} \cdot \frac{A_C OS}{A_C l_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (9)$$

$$A_C l_C = \frac{43}{60} \cdot \frac{A_C l}{A_C l_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (10)$$

$$GO_C = \frac{162}{180} \cdot \frac{GO}{Glu_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (11)$$

$$Glu_C = \frac{162}{180} \cdot \frac{Glu}{Glu_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (12)$$

$$HMF_C = \frac{162}{126} \cdot \frac{HMF}{Glu_{FS}} \cdot \frac{W_L}{W_{FS}} \cdot 10 \quad (13)$$

where XO_C: xylooligosaccharides; Xyl_C: xylose; F_C: furfural; ArO_C: arabino-oligosaccharides; Ara_C: arabinose; AcO_C: acetyl groups linked to oligosaccharides; $A_C L_C$: acetic acid; GO_C: glucooligosaccharides; Glu_C: glucose; HMF_C: hydroxymethylfurfural; W_L: initial weight of liquor; W_{FS}: final weight of the liquor submitted to post-hydrolysis step; $_C$: converted; $_L$: initial; $_{FS}$: final.

The following ratios are related to the stoichiometric factors of the conversion: cellulose into HMF is 162/126; cellulose into glucose is 162/180; acetyl groups into acid acetic is 43/60; arabinan into arabinose is 132/150; xylan into furfural is 132/96 and xylan into xylose is 132/150 (Garrote et al., 2002).

2.5. Enzymes

Enzyme solutions: Cellic CTec 2 containing cellulases, hemicellulases and β-glucosidase; HTec 2 containing hemicellulases (endoxylanase) from Novozymes A/S (Bagsvaerd, Denmark).

In the Cellic CTec 2, the total cellulase activity and β-glucosidase activity were analyzed using the standard methodology of Mandels et al. (1976) and Ghose (1987), respectively. In the HTec 2, the xylanase activity was determined using the standard methodology of Ghose (1987). The Cellic CTec 2 kit shown the initial enzyme activities from β-glucosidase and cellulase of 269 CBU/mL and 126 FPU/mL, respectively, and Cellic HTec 2 kit shown 1654 IU/mL of endoxylase.

2.6. Enzymatic hydrolysis (EH)

2.6.1. Hydrolysis yield

The pretreated LCMs were utilized as substrate in the EH step. The EH were carried out using 4% (w/v) solutions of pretreated LCM in Erlenmeyer flasks with a volume of 48 mL at 50 °C, using the enzymatic kit of Cellic CTec 2 and HTec 2, an enzyme loading of 130 IU, 75 CBU and 30 FPU per gram of pretreated LCM, respectively, and 0.02% (w/v) of sodium azide inserted into 50 mM sodium citrate buffer. Submitted the agitation of 150 rpm and the samples were withdrawals at the 0 h, 6 h and 12 h, after in intervals of 12 h until reach 96 h of process (Dowe and McMillan, 2001; Santos et al., 2010). The samples were analyzed by HPLC (see Section 2.8). All procedures were performed in duplicate. The Eq. (14) was used for calculate the yield EH (Dowe and McMillan, 2001).

$$\text{Hydrolysis yield}\,(\%) = \frac{[\text{glucose}] + 1.053\,[\text{cellobiose}]}{1.111f * [\text{biomass}]} \times 100 \qquad (14)$$

where glucose: glucose concentration (g/L); cellobiose: cellobiose concentration (g/L); biomass: dry concentration of the biomass initial in the EH (g/L); *f*: cellulose fraction in the dry biomass (g/g); 1.111: conversion factor of the cellulose into glucose equivalent; 1.053: conversion factor of the cellobiose into glucose equivalent.

2.6.2. Statistical analysis of the enzymatic hydrolysis

The single-factor from ANOVA was used in the statistical analysis of the EH and the statistical significance was with a confidence level of 95%, using multiple comparison tests. The Statistica 7.0 software was used for the data analyses.

2.7. Fermentative stage

2.7.1. Microorganisms

The microorganisms' *P. stipitis*, *S. cerevisiae* and *Z mobilis* were utilized for ethanol production. *P. stipitis* Y7124, *S. cerevisiae* PE2 and *Z. mobilis* B14023 strains were obtained from the microbiological collection of the Centre of Biological Engineering (CEB) at the University of Minho (UMINHO). The microorganisms were stored in Eppendorf with glycerol at −80 °C and lyophilized, used as a material stock.

2.7.2. Preparation of inoculum

The *S. cerevisiae* and *P. stipitis* were kept in the Petri dishes containing as culture medium the potato dextrose agar (PDA) and *Z. mobilis* was kept in ghe Petri dishes containing as culture medium the plate count agar (PCA) at 30 °C during 24 h.

The microorganisms were cultivated in 250 mL Erlenmeyer flasks containing: 0.25 g/L magnesium sulphate; 0.5 g/L potassium phosphate; 10 g/L peptone; 1 g/L ammonium sulphate; 10 g/L yeast extract and 50 g/L glucose diluted in 100 mL of sterile culture medium at 30 °C and the agitation for *S. cerevisiae* was 200 rpm, *Z. mobilis* was 150 rpm and *P. stipitis* was 250 rpm into an orbital shaker during 12 h (Santos et al., 2010). The cells in the inoculum were grown till an optical density of 2 for all strains (*S. cerevisiae*, *P. stipitis* and *Z. mobilis*) measured in a UV–vis spectrophotometer in the wavelength at 600 nm (De Souza et al., 2012).

2.7.3. Simultaneous saccharification and fermentation process (SSF)

The SSF experiments were conducted using the NREL standard procedure (Dowe and McMillan, 2001; Gonçalves et al., 2014). The culture medium was supplemented in accordance with Santos et al. (2010), using *P. stipitis*, *S. cerevisiae* and *Z. mobilis*. The samples were withdrawals at 0 h, 6 h and 12 h, after an interval of 12 h until it reached 48 h of process. The samples were analyzed in the HPLC (see Section 2.8). All procedures were performed in duplicate.

2.7.4. Semi-simultaneous saccharification and fermentation process (SSSF)

The SSSF included at 12 h of pre-saccharification + 36 h of SSF process using pretreated green coconut shell as substrate established by Gonçalves et al. (2014), and *P. stipitis*, *S. cerevisiae* and *Z. mobilis* as microorganism. The culture medium was supplemented in accordance with Santos et al. (2010). The samples were withdrawals at 0 h, 6 h and 12 h, after an interval of 12 h until reach 48 h of process. The samples were analyzed in the HPLC (see Section 2.8). All procedures were performed in duplicate.

The ethanol yield (Eq. (15)) was calculated for SSF and SSSF. The fermentative processes were estimated with all glucose contained in the pretreated green coconut shell. Furthermore, establishing that 0.51 g of ethanol from 1 g of glucose and 0.9 g of glucose from 1 g of cellulose (Dowe and McMillan, 2001).

$$\text{Ethanol yield}(\%) = \frac{[\text{ethanol}]}{[\text{initial glucose or xylose}] \times 0.511} \times 100 \qquad (15)$$

where ethanol: final concentration of ethanol (g/L); initial glucose or xylose: initial concentration of the glucose or xylose (g/L); 0.511: conversion factor of the glucose or xylose into ethanol.

2.7.5. Statistical analyzes from SSF and SSSF

The statistical significance was with a confidence level of 95% for Fisher *F*-test (ANOVA) and Student's *t*-test. The Statistica 7.0 software was used for the data analyses.

2.8. Analysis of the samples in the high performance liquid chromatography (HPLC)

The samples of chemical characterization, enzymatic hydrolysis and fermentations were processed (centrifuged, filtered and analyzed by HPLC) to verify the concentration of organic compounds contained. Using Metacarb 87H column (300 mm × 7.8 mm) (Varian, USA) in a Jasco chromatograph 880-PU pump containing a Jasco 830-IR refraction-index detector and a Jasco AS-2057 Plus auto sampler (Jasco, Japan). The analysis conditions: column at 60 °C; mobile phase of sulphuric acid (0.005 mol/L) and flow rate 0.7 mL/min.

3. Results and discussion

3.1. Composition of the LCMs

The chemical compositions of the LCMs (untreated and pretreated) are shown in Table 2. The moisture content of the untreated LCMs was 12.60%, 5.52%, 6.14% and 8.99% for cactus, mature coconut shell, coconut fiber mature and green coconut shell, respectively.

The LCMs after AP using the highest severity factor (4.64) showed changes in chemical composition in comparison to the untreated LCMs: for cactus the cellulose content increased 35.39%, hemicellulose was reduced 76.77% and lignin increased 37.72%; for green coconut shell, the cellulose increased 23.38%, hemicellulose was reduced 72.27% and lignin increased 62.26%; for coconut fiber

Table 2
Composition and yield of pretreated LCMs. (A) Coconut fiber mature; (B) mature coconut shell; (c) green coconut shell; (d) cactus.

Ro	Assay	Solid phase (%)				
		Solid yield	Cellulose	Hemicellulose	Klason lignin	Ash
A						
0	Untreated	100 ± 0.00	32.18 ± 0.12	27.81 ± 0.74	25.02 ± 0.01	3.31 ± 0.02
2.76	1	92.74 ± 0.50	32.54 ± 0.51	29.15 ± 0.46	29.29 ± 0.15	1.59 ± 0.07
3.24	2	87.76 ± 0.74	33.86 ± 0.37	19.48 ± 0.05	28.20 ± 0.25	1.60 ± 0.16
3.46	3	82.96 ± 1.28	34.59 ± 0.21	19.46 ± 0.21	26.66 ± 0.41	2.60 ± 0.16
3.94	4	79.86 ± 0.42	38.35 ± 0.12	17.41 ± 0.03	28.12 ± 0.16	1.98 ± 0.04
4.42	5	75.47 ± 0.33	42.55 ± 1.04	16.67 ± 0.12	28.68 ± 0.32	1.67 ± 0.19
4.64	6	73.94 ± 0.87	45.23 ± 1.23	13.86 ± 0.06	29.80 ± 0.25	1.35 ± 0.02
3.35	7	83.76 ± 1.81	34.52 ± 0.56	17.80 ± 0.14	27.01 ± 0.20	1.18 ± 0.03
4.05	8	77.44 ± 1.12	39.98 ± 0.24	18.73 ± 0.15	28.37 ± 0.40	2.53 ± 0.07
3.83	9	81.89 ± 0.40	39.53 ± 0.15	19.46 ± 0.01	27.44 ± 0.08	2.02 ± 0.02
3.83	10	81.78 ± 0.33	39.85 ± 0.15	19.82 ± 0.01	27.48 ± 0.08	2.46 ± 0.03
B						
0	Untreated	100 ± 0.00	29.58 ± 0.50	27.77 ± 0.79	31.04 ± 0.10	3.84 ± 0.08
2.76	1	90.85 ± 0.09	30.35 ± 0.17	30.31 ± 0.02	29.13 ± 0.47	1.55 ± 0.11
3.24	2	87.58 ± 0.19	32.50 ± 0.52	25.98 ± 0.88	27.53 ± 0.49	0.82 ± 0.20
3.46	3	85.44 ± 0.44	32.53 ± 0.27	22.07 ± 0.24	29.23 ± 0.40	0.89 ± 0.05
3.94	4	85.69 ± 0.59	32.70 ± 0.59	18.67 ± 0.49	31.48 ± 0.22	0.68 ± 0.06
4.42	5	66.87 ± 0.20	41.21 ± 0.11	12.48 ± 0.15	34.39 ± 0.09	0.73 ± 0.03
4.64	6	62.08 ± 0.02	43.94 ± 0.53	7.02 ± 0.05	36.14 ± 0.05	0.71 ± 0.07
3.35	7	86.62 ± 0.31	32.19 ± 0.52	23.66 ± 0.17	24.70 ± 0.20	0.90 ± 0.10
4.05	8	76.98 ± 0.47	32.26 ± 0.41	16.50 ± 0.53	31.28 ± 0.03	0.69 ± 0.10
3.83	9	84.13 ± 0.15	36.52 ± 0.18	20.06 ± 0.13	28.93 ± 0.13	0.68 ± 0.11
3.83	10	83.62 ± 0.84	34.74 ± 0.90	19.88 ± 0.09	28.64 ± 0.41	0.63 ± 0.13
C						
0	Untreated	100 ± 0.00	33.23 ± 0.24	29.14 ± 0.22	25.44 ± 0.12	2.34 ± 0.11
2.76	1	89.43 ± 1.40	30.65 ± 0.03	23.25 ± 0.04	32.15 ± 0.47	1.71 ± 0.05
3.24	2	86.83 ± 0.53	32.07 ± 0.08	21.10 ± 0.07	30.93 ± 0.38	1.52 ± 0.29
3.46	3	84.44 ± 0.36	33.11 ± 0.12	19.87 ± 0.04	34.00 ± 0.43	1.73 ± 0.02
3.94	4	80.46 ± 0.72	34.14 ± 0.17	18.11 ± 0.01	31.05 ± 0.32	0.70 ± 0.42
4.42	5	74.31 ± 0.20	38.57 ± 0.10	14.55 ± 0.06	37.58 ± 0.10	1.06 ± 0.23
4.64	6	68.36 ± 0.44	41.96 ± 1.10	8.08 ± 0.10	41.28 ± 0.87	1.22 ± 0.04
3.35	7	88.77 ± 1.60	30.64 ± 0.09	20.07 ± 0.04	30.36 ± 0.40	1.88 ± 0.31
4.05	8	77.45 ± 1.13	35.92 ± 0.07	16.83 ± 0.11	33.92 ± 0.03	0.77 ± 0.01
3.83	9	82.56 ± 0.54	33.52 ± 0.21	18.27 ± 0.12	34.80 ± 0.26	1.41 ± 0.05
3.83	10	82.38 ± 1.60	34.01 ± 0.14	18.34 ± 0.22	34.22 ± 0.31	1.52 ± 0.30
D						
0	Untreated	100 ± 0.00	38.12 ± 0.75	23.50 ± 0.42	19.51 ± 0.13	5.64 ± 0.21
2.76	1	62.34 ± 0.87	36.67 ± 0.17	13.22 ± 0.05	26.73 ± 0.23	9.37 ± 0.22
3.24	2	56.69 ± 0.14	38.94 ± 0.18	12.89 ± 0.31	23.87 ± 0.31	10.62 ± 0.26
3.46	3	53.19 ± 0.78	39.95 ± 0.50	11.87 ± 0.05	26.90 ± 0.31	11.47 ± 0.41
3.94	4	45.17 ± 0.85	43.14 ± 0.25	11.01 ± 0.11	22.12 ± 0.21	10.45 ± 0.34
4.42	5	42.95 ± 1.28	49.82 ± 0.15	9.88 ± 0.33	27.11 ± 0.08	11.37 ± 0.31
4.64	6	40.36 ± 0.47	51.61 ± 0.04	5.46 ± 0.17	26.87 ± 0.06	12.25 ± 0.72
3.35	7	56.18 ± 0.09	39.59 ± 0.96	12.00 ± 0.03	21.34 ± 0.26	11.30 ± 0.29
4.05	8	44.74 ± 0.54	43.65 ± 0.52	10.92 ± 0.10	21.85 ± 0.11	10.34 ± 0.16
3.83	9	47.15 ± 0.73	42.65 ± 1.05	11.74 ± 0.08	21.89 ± 0.35	11.56 ± 0.38
3.83	10	46.85 ± 0.65	41.83 ± 0.04	11.45 ± 0.13	22.22 ± 0.24	10.89 ± 0.76

mature the cellulose increase was 40.55%, the hemicellulose reduction was 50.16% and lignin increase 19.10%; and for mature coconut shell the cellulose increase was 48.55%, the hemicellulose decrease was 74.71% and an increase of 16.43% was observed for lignin. For all pretreated LCMs, a reduction in the hemicellulose content, and increase of cellulose and lignin was observed, these aspects being strategic for the fractionation of LCM, as it allows the hydrolysis of hemicellulose and enriches the material in cellulose and lignin (Ruiz et al., 2013). According to Ballesteros et al. (2002) the hemicellulose degradation in agricultural wastes increases at high temperatures and high residence times, therefore, higher severities factors in operating conditions will provide a higher reduction of hemicellulose, similar to the results obtained in this work. Ruiz et al. (2013) emphasized that the AP causes the re-location of lignin on LCM surface. Thus, the accessibility of enzymes to pretreated LCMs is favored, increasing the hydrolysis of cellulose. Increased porosity and surface area improve EH efficiency (Cybulska et al., 2010).

Pretreated LCMs presented a reduction of solid between 7.26% and 59.64%, this being correlated with an increase in the severity factor (Table 2). Minimum reductions were observed for cellulose in pretreated mature coconut shell, green coconut shell and coconut fiber mature. Romaní et al. (2010) reported reductions of cellulose mass after the pretreatment in the range of 5.29% and 19.55% at 240 °C and 250 °C, respectively. In addition to Sakaki et al. (2002), they evidence the appearance of the hexoses from cellulose and degradation of oligosaccharides after 230 °C and the 295 °C practically all the cellulose was degraded.

3.2. Liquid phase composition after autohydrolysis pretreatment

The liquid phase composition after AP carried out at several severities is shown in Table 1B. In all situations, a reduction in pH is observed, the lowest one occurring for cactus liquor (Table 3). However, the pH values of the cactus liquors were close to the minimum pH values encountered for other LCMs, indicating an

Table 3
Composition of the liquid phase of pretreated LCMs. (A) Coconut fiber mature; (b) mature coconut shell; (c) green coconut shell; (d) cactus.

Ro	Assay	pH	Liquid phase (g/L)										Total sugar	[XO] %
			Glucose	Xylose	Arabinose	Acetic acid	HMF	Furfural	[GO]	[XO]	[ArO]	[AcO]		
A														
2.76	1	5.85	0.13	1.16	0.11	0.25	0.01	0.10	0.39	1.80	1.22	2.79	4.81	37.42
3.24	2	5.00	0.12	2.17	0.30	0.12	0.02	0.16	0.44	2.44	1.51	3.47	6.98	34.96
3.46	3	4.70	0.13	2.22	0.67	0.37	0.04	0.18	0.43	2.68	1.99	2.84	8.12	33.00
3.94	4	4.80	0.12	2.52	0.39	0.32	0.03	0.18	0.45	3.79	1.42	2.84	8.69	43.61
4.42	5	4.30	0.13	2.46	1.09	0.60	0.09	0.32	0.58	4.89	1.53	3.00	10.68	45.79
4.64	6	3.95	0.12	2.77	0.86	0.86	0.06	0.76	0.56	5.01	1.21	3.10	10.53	47.58
3.35	7	5.30	0.13	3.17	0.36	0.27	0.01	0.18	0.43	2.70	1.31	2.78	8.10	33.33
4.05	8	4.40	0.13	2.35	0.11	0.50	0.07	0.24	0.55	4.26	1.85	2.98	9.25	46.05
3.83	9	4.55	0.12	2.25	0.69	0.37	0.04	0.18	0.49	2.65	1.85	2.86	8.05	32.92
3.83	10	4.60	0.13	2.28	0.97	0.43	0.06	0.20	0.51	3.18	2.17	2.94	9.24	34.42
B														
2.76	1	5.45	0.13	0.88	0.11	0.27	0.02	0.18	0.42	2.04	1.18	1.79	4.76	42.86
3.24	2	4.65	0.13	1.06	0.18	0.30	0.02	0.18	0.43	2.12	1.21	1.75	5.13	41.33
3.46	3	4.40	0.14	1.20	0.26	0.33	0.02	0.18	0.49	3.37	1.28	2.93	6.74	50.00
3.94	4	4.70	0.14	1.77	0.19	0.30	0.03	0.18	0.40	2.63	1.18	3.00	6.31	41.68
4.42	5	3.70	0.15	1.41	0.41	0.69	0.05	0.52	0.54	13.55	1.20	4.02	17.26	78.51
4.64	6	3.40	0.16	1.90	0.36	1.29	0.11	0.83	0.61	16.52	1.01	4.31	20.56	80.35
3.35	7	5.10	0.13	1.16	0.13	0.27	0.02	0.16	0.39	2.07	1.16	2.72	5.04	41.07
4.05	8	3.80	0.16	1.47	0.47	0.53	0.03	0.30	0.48	11.35	1.15	3.83	15.08	75.27
3.83	9	4.20	0.14	1.23	0.32	0.37	0.02	0.19	0.45	6.85	1.24	3.08	10.23	66.96
3.83	10	4.15	0.14	1.26	0.34	0.37	0.02	0.21	0.50	5.52	1.27	3.12	9.03	61.13
C														
2.76	1	5.20	0.13	1.15	0.16	0.33	0.01	0.16	0.38	1.85	1.24	2.78	4.91	37.68
3.24	2	4.45	0.13	1.18	0.40	0.29	0.02	0.16	0.46	2.92	1.64	2.88	6.73	43.39
3.46	3	4.15	0.13	1.21	0.56	0.32	0.02	0.18	0.51	4.15	2.23	3.05	8.79	47.21
3.94	4	4.25	0.13	1.20	0.39	0.30	0.02	0.18	0.49	5.32	1.60	2.89	9.13	58.27
4.42	5	3.80	0.14	1.79	0.93	0.62	0.05	0.38	0.61	11.56	1.20	3.93	16.23	71.23
4.64	6	3.60	0.17	3.03	0.53	1.18	0.08	0.86	0.65	12.15	1.12	3.76	17.65	68.84
3.35	7	4.65	0.14	1.16	0.18	0.26	0.04	0.18	0.42	3.10	1.33	2.82	6.33	48.97
4.05	8	3.90	0.13	1.50	0.94	0.48	0.03	0.32	0.61	8.16	1.59	3.64	12.93	63.11
3.83	9	4.10	0.13	1.26	0.66	0.36	0.04	0.18	0.51	5.39	1.54	3.09	9.49	56.80
3.83	10	4.00	0.13	1.28	0.70	0.38	0.04	0.20	0.55	5.68	1.67	3.15	10.01	56.74
D														
2.76	1	4.10	1.73	1.72	0.16	0.27	0.06	0.22	0.22	4.52	1.96	0.28	10.31	43.84
3.24	2	4.00	2.67	2.84	0.53	0.35	0.08	0.24	2.49	7.56	5.12	0.94	21.21	35.64
3.46	3	3.90	2.52	2.77	0.87	0.58	0.23	0.22	3.30	6.89	4.51	0.90	20.86	33.03
3.94	4	4.10	1.74	3.01	0.45	0.34	0.21	0.20	3.52	5.14	3.33	0.62	17.19	29.90
4.42	5	4.00	2.43	3.75	1.09	0.78	0.44	0.70	6.57	5.06	4.15	1.30	23.05	21.95
4.64	6	3.80	2.72	3.71	1.29	1.06	0.54	0.94	7.23	5.47	4.32	1.20	24.74	22.11
3.35	7	4.10	2.79	2.83	1.10	0.31	0.21	0.38	5.07	6.20	3.62	0.50	21.61	28.69
4.05	8	3.90	2.87	2.87	1.05	0.67	0.41	0.38	6.34	7.15	2.82	1.53	23.10	30.95
3.83	9	4.00	2.36	2.78	1.07	0.51	0.24	0.24	5.84	7.26	4.01	0.96	23.32	31.13
3.83	10	3.90	2.16	3.05	1.11	0.52	0.27	0.26	5.92	7.75	3.60	0.87	23.59	32.85

Note: GO: glucooligosaccharides; XO: xylooligosaccharides; ArO: arabino-oligosaccharides; AcO: acetyl groups linked to oligosaccharides.

increased sensitivity of cactus to the different severities used in autohydrolysis pretreatment. Pretreated LCMs present a correlation between the reduction of the pH and the increase in the concentration of acetic acid, furfural, HMF, arabinose, xylose, GO, XO, AcO and ArO in the liquor. Cybulska et al. (2010) studied the importance of monitoring and controlling the pH to maximize the solubilization of hemicellulosic fraction. Cara et al. (2007) reported a decreased pH for AP materials correlated with an increased concentration of the degradation product of hemicellulose (furfural); similar results were obtained in this work (Table 3).

The highest concentrations of acetic acid, furfural, HMF, arabinose, xylose, XO and AcO were observed for green coconut shell, coconut fiber mature and mature coconut shell. Furthermore, oligosaccharides were obtained at higher concentrations than monosaccharides, these results also are being correlated with pH reduction and with the severity factor. Low amounts of cellulose or hemicellulose were degraded into GO in coconut fiber mature, mature coconut shell and green coconut shell. Similar results were obtained for AP rye straw (Gullón et al., 2010). Cactus presented higher concentrations of monomeric sugars, acetic acid, HMF, furfural, GO and ArO. These values are related to an increase in the severity factor and the highest concentrations obtained in the cactus liquors indicate a higher sensitivity of cactus to AP.

Monosaccharides and oligosaccharides present in the liquor can be used in others biotechnological processes (Ruiz et al., 2013). The Moure et al. (2006) reported the possibility of using these carbohydrates in food and pharmaceutical industries, and on the production of cellulosic ethanol and xylitol.

3.3. Experimental design and statistical analysis of pretreated LCMs

The experimental design was conducted to determine variables that affect the pretreatment of LCMs and influence the cellulose content contained in the pretreated material. The conditions used in the experimental design were temperature (160, 180 and 200 °C) and time (10, 30 and 50 min).

The ANOVA and multiple regression analysis of the experimental data were carrie out from mathematical model fitting. Models describing the AP materials cellulosic content the temperature (X_1) and time (X_2) as a function and using normalized values (Table 1A) are described by Eqs. (16)–(19) for coconut fiber mature, mature

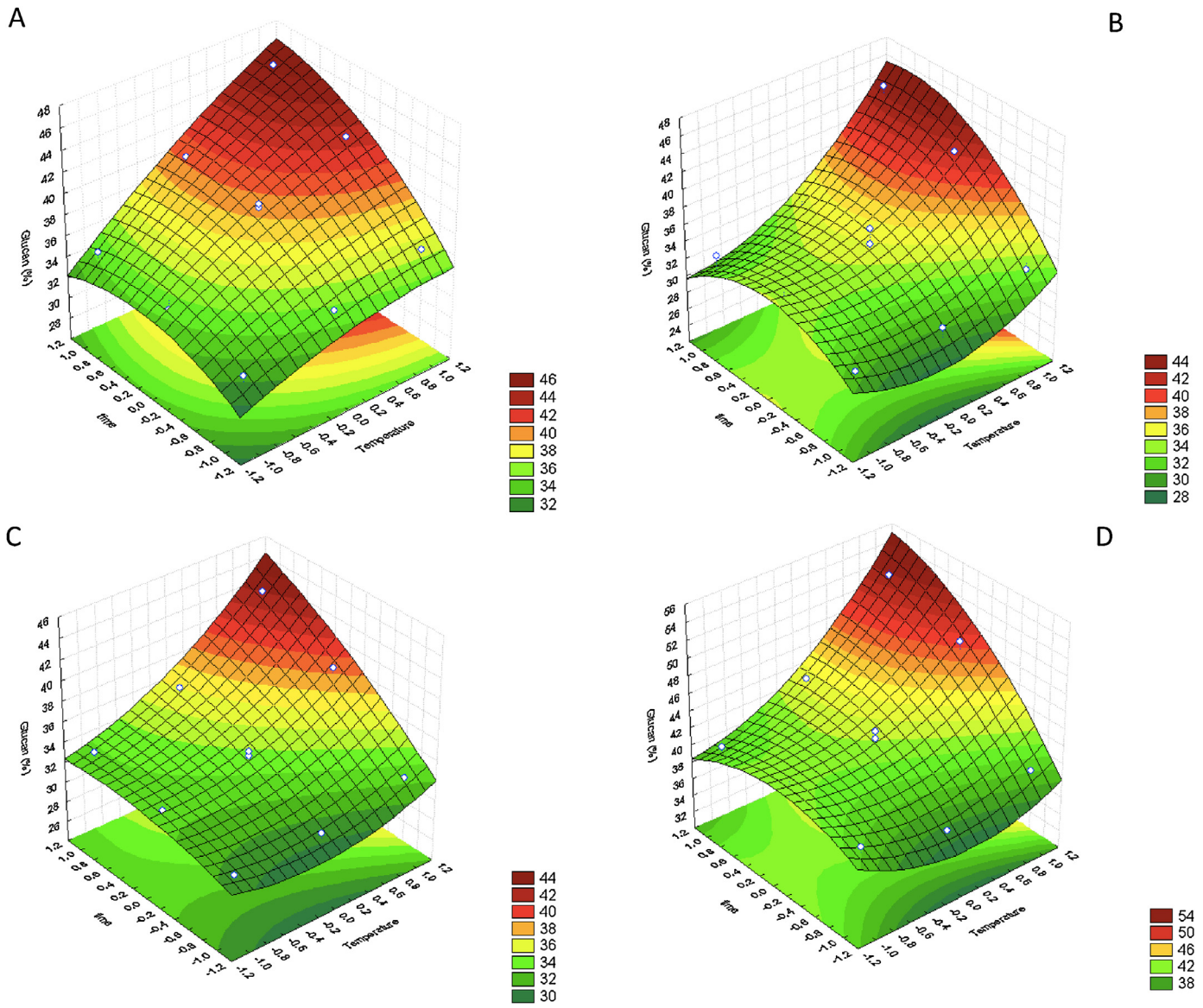

Fig. 1. Response surface and contour plot of LCMs pretreated by autohydrolysis. Cellulose variation as a function of temperature and time. (A) Coconut fiber mature; (B) mature coconut shell; (C) green coconut shell; (D) cactus.

coconut shell, green coconut shell and cactus, respectively,

$$\text{Coconut fibre mature}: \%\,\text{Cellulose} = 39.28 + 3.85 * \text{Temperature} - 0.67 * \text{Temperature}^2 + 2.73 * \text{time} - 1.63 * \text{time}^2 + 1.70 * \text{Temperature} * \text{time} \quad (R^2 = 0.9878;\ R^2_{adj} = 0.9725) \tag{16}$$

$$\text{Mature coconut shell}: \%\,\text{Cellulose} = 34.99 + 3.74 * \text{Temperature} + 2.5 * \text{Temperature}^2 + 2.51 * \text{time} - 2.93 * \text{time}^2 + 2.26 * \text{Temperature} * \text{time} \quad (R^2 = 0.9251;\ R^2_{adj} = 0.9315) \tag{17}$$

$$\text{Green coconut shell}: \%\,\text{Cellulose} = 33.85 + 2.80 * \text{Temperature} + 1.35 * \text{Temperature}^2 + 2.92 * \text{time} - 0.68 * \text{time}^2 + 1.83 * \text{Temperature} * \text{time} \quad (R^2 = 0.9914;\ R^2_{adj} = 0.9808) \tag{18}$$

$$\text{Cactus}: \%\,\text{Cellulose} = 42.24 + 3.23 * \text{Temperature} + 2.37 * \text{Temperature}^2 + 3.33 * \text{time} - 2.09 * \text{time}^2 + 3.08 * \text{Temperature} * \text{time} \quad (R^2 = 0.9834;\ R^2_{adj} = 0.9627) \tag{19}$$

The response surfaces were designed as three dimensional plots of the second order polynomial models (Eqs. (16)–(19), as a function of the two most strongly influencing variables. Cellulose variation was represented in function of the temperature and time (Fig. 1) and it was demonstrated that the increase in cellulose is correlated with the increase in the AP of the temperature and time.

The ANOVA results (see Eqs. (16)–(19)) showed that the second order polynomial models represents properly the responses in the cellulose variation. Obtained coefficients of determination R^2 show that 98.8%, 92.5%, 99.4% and 98.3% of the variability of response may be described by the model. The values are in conform to adjusted coefficients of determination – $R_{adj}{}^2$ = 0.973, 0.932, 0.980 and 0.963, respectively.

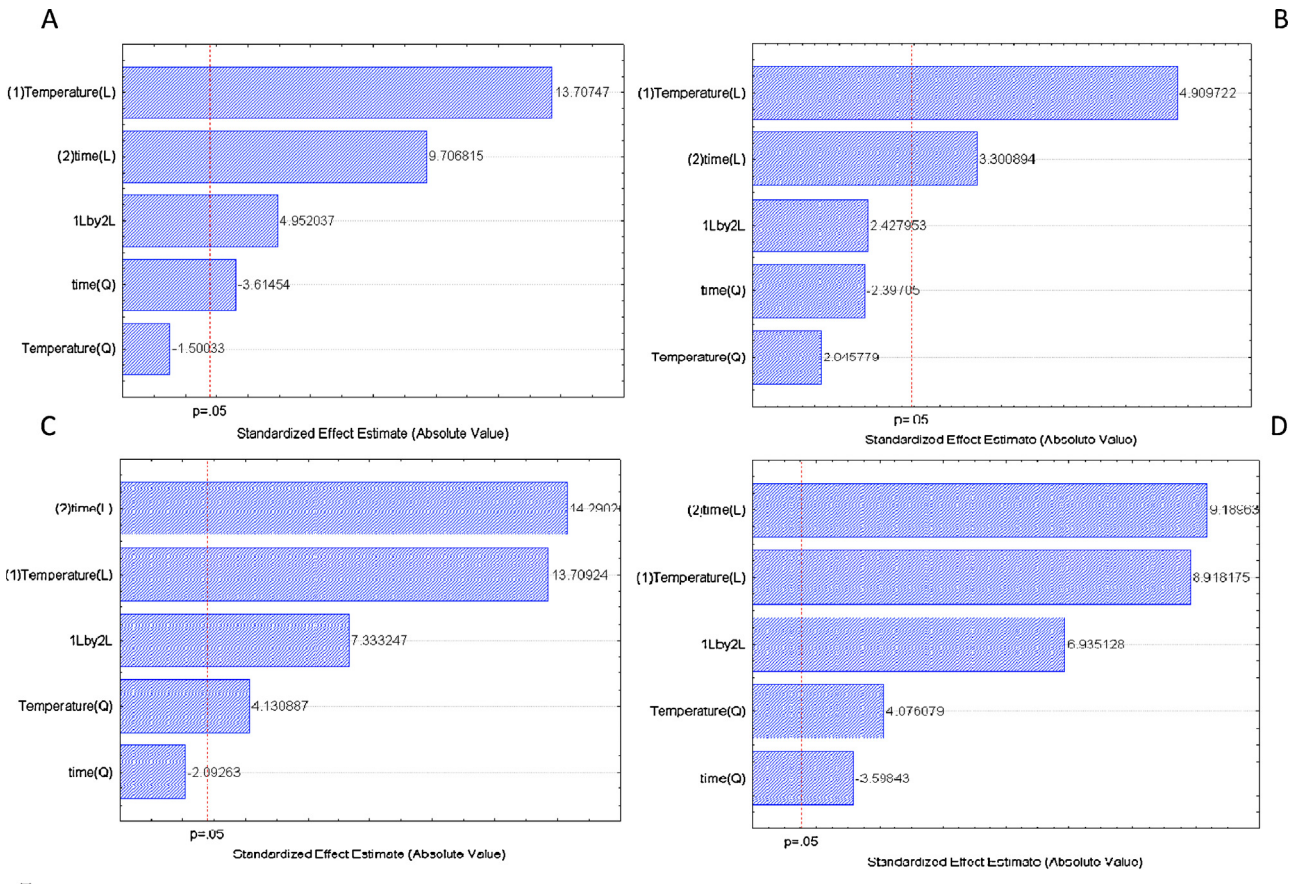

Fig. 2. Pareto charts for standardized effects of temperature and time of LCMS pretreated by autohydrolysis. (A) Coconut fiber mature; (B) mature coconut shell; (C) green coconut shell; (D) cactus.

According to ANOVA results for cellulose variation in cactus pretreated by AP, the linear X_1, X_2, square ${X_1}^2$, ${X_2}^2$ and interaction X_1X_2 have a significant effect on cellulose variation responses and p-value with a significance level of $\alpha = 0.05$. The effects can be visualized in Fig. 2D (standardized Pareto charts) and if observes the temperature, time, and temperature-time interaction are important with a confidence level of 95% on the cellulose variation and that the effect of temperature and time are positive, when increased from lower to higher values (Fig. 1D). The ANOVA results for cellulose variation in AP coconut fiber mature evidentiate that the linear X_1, X_2, square ${X_2}^2$ and interaction X_1X_2 have a significant effect on cellulose variation responses with p-value under a significance level of $\alpha = 0.05$. The effects can be visualized in Fig. 2A (standardized Pareto charts)and it can be seen that temperature, time, and temperature–time interaction are important with a confidence level of 95% on the cellulose variation, and the effect of the temperature and time are positive, when increased from lower values to higher values (Fig. 1A). The ANOVA results for cellulose variation in mature coconut shell in AP show that the linear X_1 and X_2 have a significant effect on cellulose variation responses and p-value with a significance level of $\alpha = 0.05$. The effects can be observed in Fig. 2B (standardized Pareto charts). As in previous situations, the variables temperature and time are important in the confidence level of 95% of the cellulose variation and the effect of temperature and time are positive, when increased from lower values to higher values (Fig. 1B). The ANOVA results for cellulose variation in mature coconut shell after AP demonstrate that the linear X_1, X_2, square ${X_1}^2$ and interaction X_1X_2 have a significant effect on cellulose variation responses and p-value with a significance level of $\alpha = 0.05$. The effects can be observed in Fig. 2C (standardized Pareto charts) Similar conclusions to those observed for other materials were obtained on the effects of the temperature and time on cellulose variation in Fig. 1C. Furthermore, Fig. 3 shows predicted and observed values of the LCMs.

Table 4
Kinetic parameters of ethanol fermentation of *S. cerevisiae*, *P. stipitis* and *Z. mobilis* in SSF and SSSF using green coconut shell pretreated by autohydrolysis (200 °C and 50 min).

Bioprocess	Microorganism	$Y_{P/S}$	Ethanol yield (%)	Ethanol production (g/L)	Ethanol productivity g/(L h)
SSF	*S. cerevisiae*	0.44 ± 0.00	86.90 ± 0.59	7.44 ± 0.17	0.15 ± 0.00*
	P. stipitis	0.43 ± 0.01	84.19 ± 2.60	8.47 ± 0.26	0.18 ± 0.01
	Z. mobilis	0.43 ± 0.01	85.26 ± 1.45	7.30 ± 0.12	0.15 ± 0.00*
SSSF	*S. cerevisiae*	0.45 ± 0.01	90.09 ± 1.60	7.71 ± 0.14	0.16 ± 0.00*
	P. stipitis	0.44 ± 0.01	87.25 ± 2.10	8.78 ± 0.21	0.18 ± 0.00
	Z. mobilis	0.45 ± 0.00	89.16 ± 0.58	7.63 ± 0.05	0.16 ± 0.00*

Note: * values are significant at a confidence level of 95%.
$Y_{P/S}$: ethanol yield (g ethanol/g sugar).

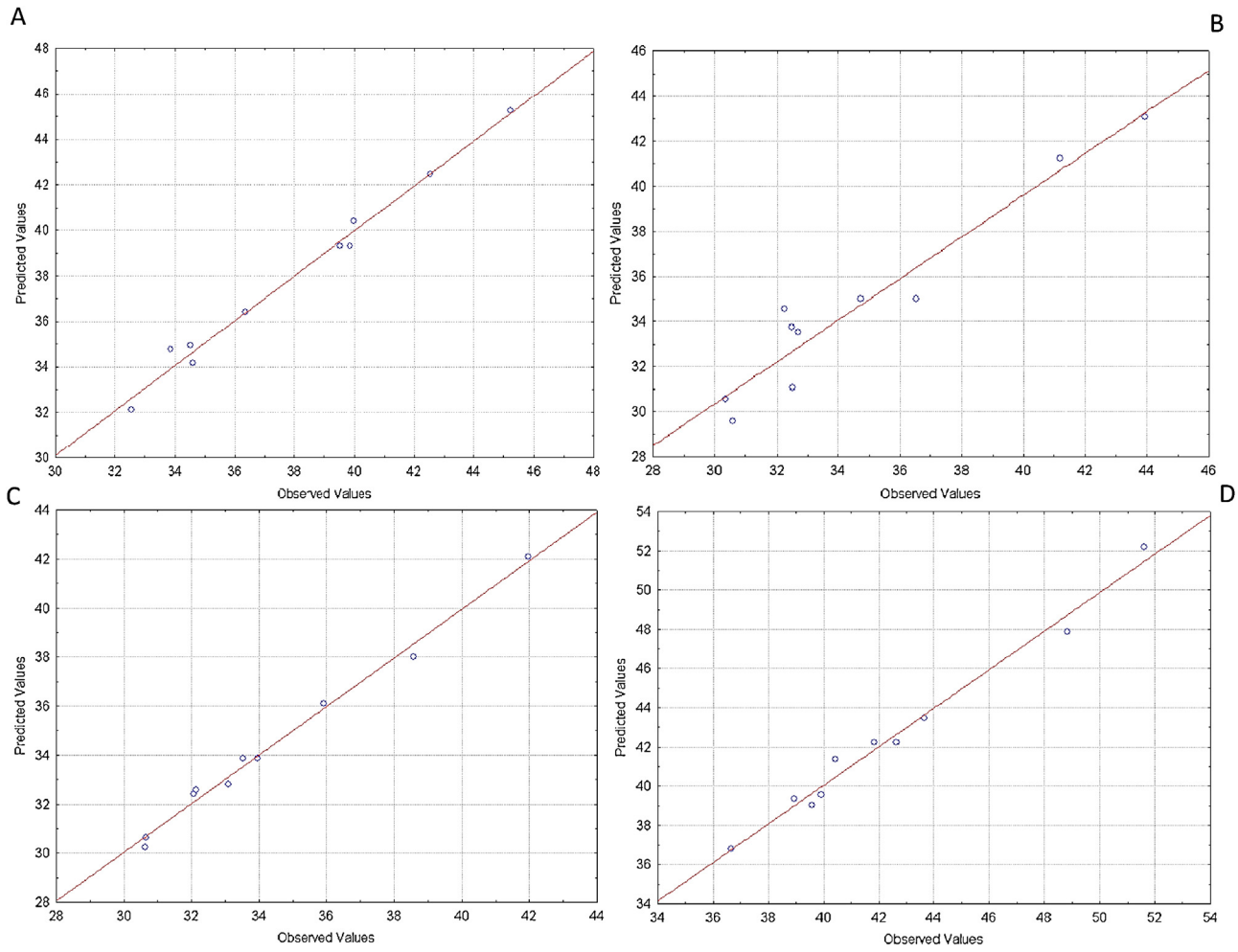

Fig. 3. Graphs of predicted values versus observed values of LCMs pretreated by autohydrolysis related to cellulose (%). (A) Coconut fiber mature; (B) mature coconut shell; (C) green coconut shell; (D) cactus.

3.4. Scanning electron microscopy

The SEM images shown in Fig. 4 allowed to observe the effects caused by AP in the green coconut shell structure (200 °C and 50 min). The untreated sample shows the fibers highly ordered and surfaces intact (Fig. 4A). However, the LCM after AP (Fig. 4B) show modified structures, with increased surface area and porosity, fiber separation and disordered fibers. Structural features can give greater susceptibility to the pretreated LCM during the EH.

3.5. Crystallinity and X-ray diffraction analysis

The X-ray diffraction analysis and the crystallinity index determination were carried out in the untreated and AP green coconut shell aiming to analyze their crystalline structure.

Untreated green coconut shell crystallinity index was 29.31%, while for AP pretreated material (200 °C and 50 min) the obtained value was 43.47% (Fig. 4C). The results showed an increase in the crystallinity index for the pretreated green coconut shell. When processing biomass by AP, Xu et al. (2013) observed the occurrence of increased crystallinity and specific surface area, mass loss and removal of the amorphous phase. Thompson et al. (1992) carried out AP in native mixed hardwood (with a crystallinity index of 71.6%), the treatment resulting in crystallinity index values of 78.1%, 85.2% and 85.8%, for increasing values – 240 °C, 260 °C and 280 °C – of the processing temperature. The results corroborate with the results obtained in this work, that green coconut shell submitted to AP also provides increased crystallinity index. According to Wei and Cheng (1985), the removal of amorphous cellulose and hemicellulose causes an increase in the crystallinity.

3.6. Enzymatic hydrolysis

The LCMs used in the EH stage were selected according to the cellulose content present in the solid phase after AP (Table 2 and Fig. 1A). Therefore, selected LCMs were those processed at a severity factor $R_0 = 4.64$ (200 °C and 50 min); for these conditions, the composition of cellulose in cactus, coconut fiber mature, mature coconut shell and green coconut shell was 51.61%, 45.23%, 43.94% and 41.96%, respectively.

Observed values for the conversion of cactus, coconut fiber mature, mature coconut shell and green coconut shell into glucose were 90.91% (0.43 g/g), 84.10% (0.42 g/g), 89.20% (0.43 g/g) and 92.52% (0.43 g/g) in 96 h of EH (Fig. 5A). The results evidence the accessibility of the pretreated LCMs in the EH and are according to the results shown by others authors, as Cybulska et al. (2010) that using AP (210 °C for 10 min), prairie cord grass obtained a glucose conversion of 97.96%, after 72 h. Romaní et al. (2010) used *Eucalyptus globulus* in AP (200 °C) and the obtained conversion in glucose by EH was 65%, after 96 h. Ruiz et al. (2012) used wheat straw in AP (180 °C for 30 min), obtaining an enzymatic conversion in glucose of 90.88%, after 96 h. Manzanares et al. (2011) studied olive tree

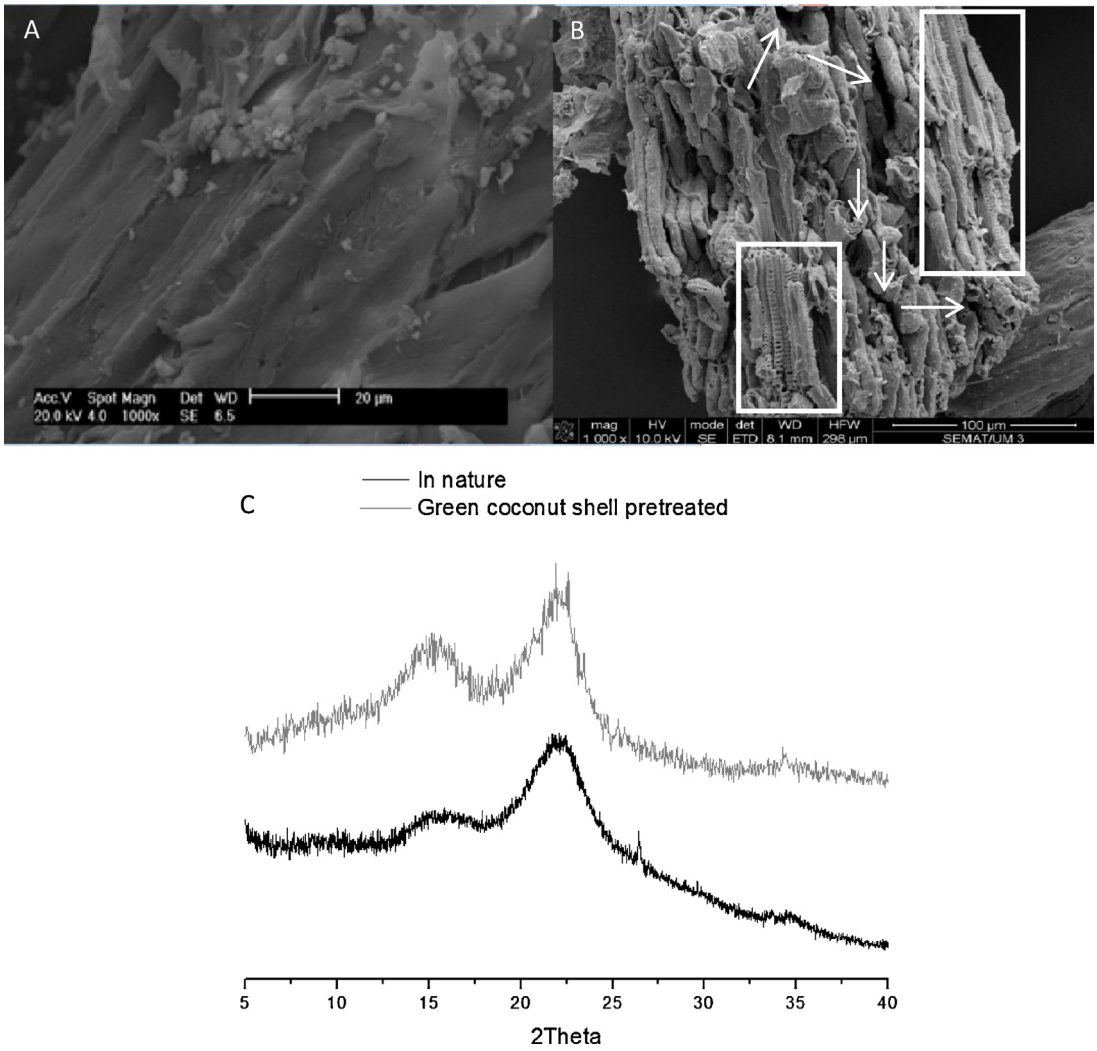

Fig. 4. SEM of green coconut shell. (A) Untreated; (B) pretreated by autohydrolysis (200 °C and 50 min). X-ray of green coconut shell. (C) Untreated and pretreated by autohydrolysis (200 °C and 50 min). High porosity area, matrix separation and exposition fibers (white square).

pruning in AP (210 °C for 10 min) with an enzymatic conversion into glucose of 65%, in 72 h. The results obtained in the EH were analyzed by ANOVA and showed in the level of confidence at 95% a significant differences, being the green coconut shell the LCM that allowed for the obtention of the highest yields of sugars.

Fig. 5B shows the maximum rate of initial hydrolysis (dG/dt), corresponding to the 12 h initial (glucose concentration vs time). The maximum rate of initial hydrolysis for coconut fiber mature and mature coconut shell were 0.82 g/(L h) and 0.91 g/(L h), respectively. Green coconut shell and cactus had higher initial hydrolysis rates – 0.95 g/(L h) and 1.03 g/(L h), respectively. In comparison, Ruiz et al. (2012) reported the maximum rate of initial hydrolysis of 0.47 g/(L h) using AP wheat straw and 30 FPU/g of cellulose. The results obtained in this work evidence the susceptibility of the LCMs to the enzymatic attack.

3.7. Fermentative process for bioethanol production

The SSF and SSSF were performed the *S. cerevisiae* PE2, *P. stipitis* Y7124, *Z. mobilis* B14023 and 4% (w/v) of pretreated green coconut shell (200 °C and 50 min) as substrate, selected based on the EH yield (Fig. 5). The efficiency of the SSF and SSSF was examined by two indicators: yield and volumetric productivity of ethanol (Shen and Agblevor, 2010).

3.7.1. Simultaneous saccharification and fermentation and semi-simultaneous saccharification and fermentation

The SSF ethanol production results by *P. stipitis*, *Z. mobilis* and *S. cerevisiae* are shown in Fig. 6A–C, respectively, obtaining results indicate that glucose from EH could be fermented to ethanol by the microorganisms. The glucose presented the standard kinetic profiles with a rapid consumption in the first 24 h. Final ethanol concentration and ethanol yield in SSF by *P. stipitis*, *Z. mobilis* and *S. cerevisiae* were 8.47 g/L and 0.43 g/g (84.19%), 7.30 g/L and 0.43 g/g (85.26%), 7.44 g/L and 0.44 g/g (86.90%), being the SSF process completed after 48 h, respectively (Fig. 6 and Table 4). For *P. stipitis* the volumetric productivity of ethanol was 0.18 g/(L h), while for *Z. mobilis* and *S. cerevisiae* the obtained values were 0.15 g/(L h), for both (Table 4). The three microorganisms proved to be suitable for fermenting sugars to ethanol by SSF. The results reported in this work are in conformity with the results presented by Romaní et al. (2010) for ethanol production by SSF of AP *E. globulus* (10% w/v of pretreated solids) using *S. cerevisiae*, that obtained production, conversion and productivity of ethanol values of 15.1 g/L, 86.4% and 0.21 g/(L h), respectively. Additionally, Romaní et al. (2012) carried out ethanol production by SSF of AP *E. globulus* (10% w/v of pretreated solids) by *S. cerevisiae*, reporting production, conversion and productivity of ethanol values of 67.4 g/L, 91.1% and 0.93 g/(L h), respectively.

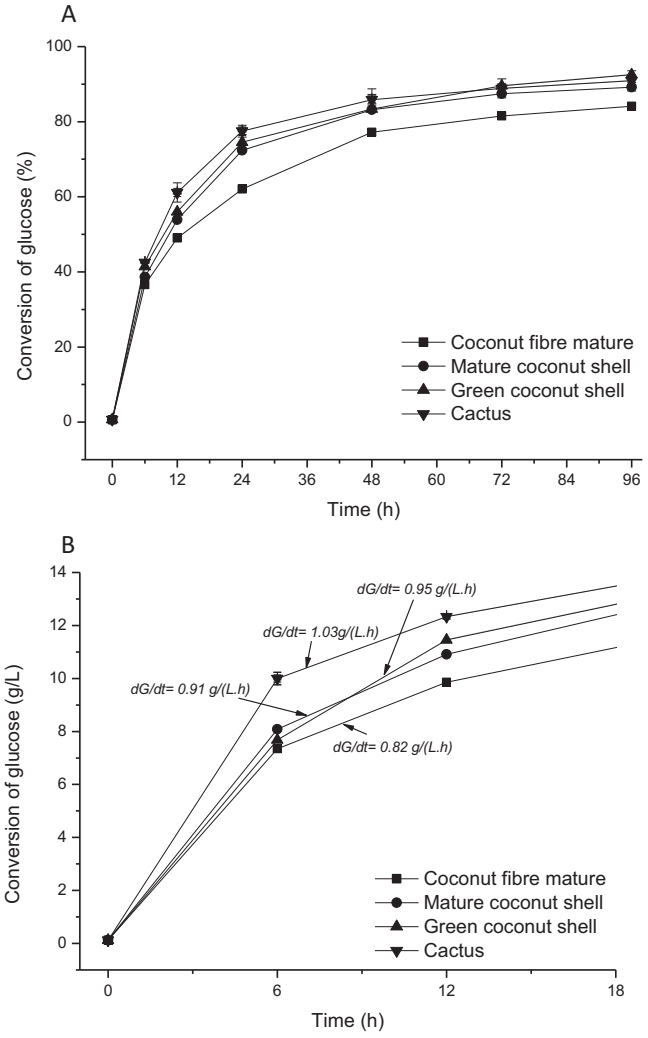

Fig. 5. Enzymatic hydrolysis of LCMs pretreated by autohydrolysis (200 °C and 50 min). (A) Conversion yield (%); (B) initial hydrolysis rate at 12 h. Enzyme loading of 30 FPU, 75 CBU and 130 IU per gram of pretreated solid.

In the experiments, SSSF had similar results concerning glucose availability during the initial phase of 12 h, with a gradual reduction of glucose with time (Fig. 6A–C). The values obtained of the ethanol concentration and yield with the SSSF strategy using *P. stipitis*, *Z. mobilis* and *S. cerevisiae* are presented in Fig. 6A–C and Table 4, being 8.78 g/L and 0.44 g/g (87.25%), 7.63 g/L and 0.45 g/g (89.16%), 7.71 g/L and 0.45 g/g (90.09%), respectively for each microbial strain after 48 h. For *P. stipitis* the volumetric productivity of ethanol was 0.18 g/(L h) in 36 h, while for *Z. mobilis* and *S. cerevisiae* the achieved values were 0.16 g/(L h) and 0.21 g/(L h), respectively (Table 4). The results achieved in this work corroborated with the results reported by Manzanares et al. (2011) that carried out the SSSF, being 24 h of presaccharification and 72 h of SSF, using residue pruning olive as raw material (17% w/v of pretreated solids). The application of SSSF resulted in the ethanol production at a concentration of 50% of the theoretical value. Mesa et al. (2011) used pretreated sugarcane bagasse, producing 15.7 g ethanol/100 g of raw material (68.4% of ethanol).

The ethanol production by the *P. stipitis*, *Z. mobilis* and *S. cerevisiae* utilizing the fermentative strategies SSF and SSSF was assessed statistically using ANOVA and *t*-test, both with 95% confidence level. The SSF using microorganisms demonstrated no significant differences in the ethanol production, when assessed by the ANOVA. The fermentation performance of the microorganisms

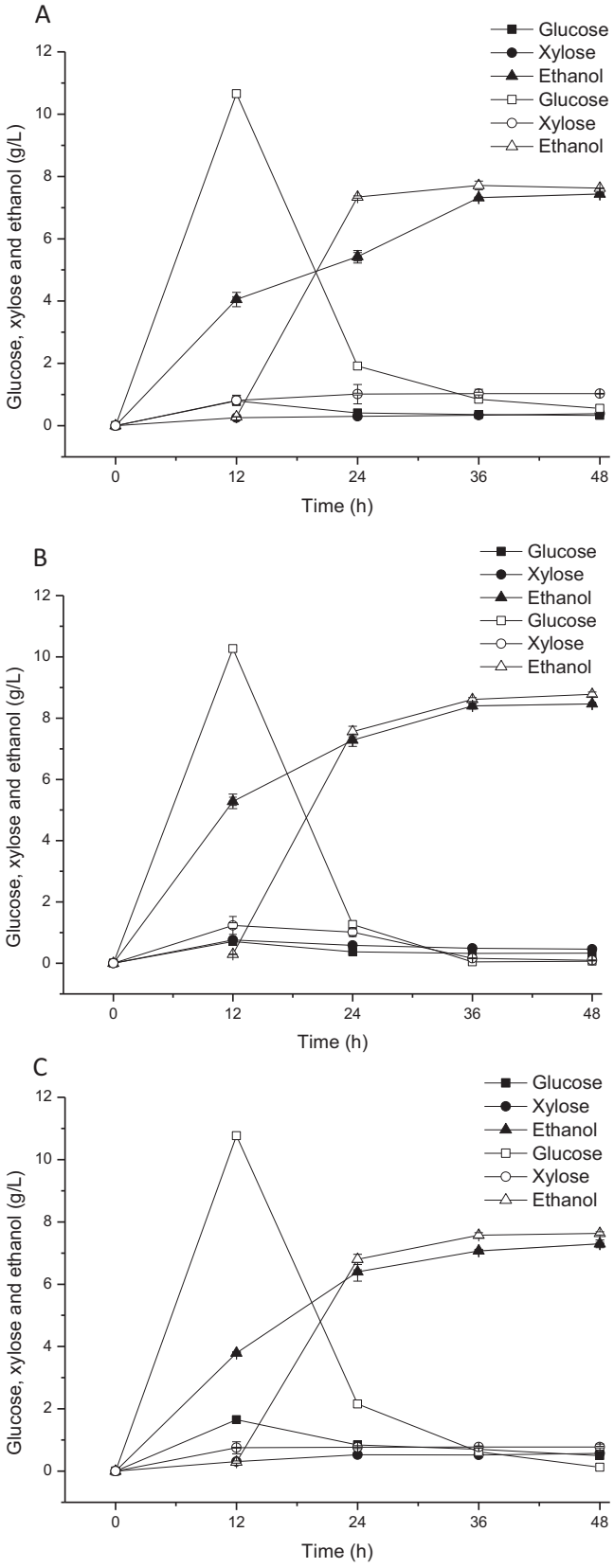

Fig. 6. Fermentation in SSF and SSSF of green coconut shell pretreated by autohydrolysis (200 °C and 50 min). (A) *S. cerevisiae*; (B) *P. stipitis*; (C) *Z. mobilis*. The SSF was represented by the black icon and SSSF was represented by the white icon.

using SSSF showed a similar result the SSF. However, the comparison of the achieved results by the *Z. mobilis* utilizing the SSSF and SSF showed a significant difference, when assessed by the *t*-test. A similar result was also shown by *S. cerevisiae*.

The achieved results in the production and the yield of ethanol in SSF are minors, when compared the SSSF (Table 4). De Souza et al. (2012) reported that in relation to SHF and SSF the presaccharification has a possible positive effect, increasing the yield and volumetric productivity of ethanol. Öhgren et al. (2007) showed the influences on the global ethanol yield of pretreated corn stover utilizing the presaccharification before of the SSF. They concluded that the fast decrease in the viscosity provided by the presaccharification can have a relevant impact on the unit operations before SSF. Santos et al. (2010) utilized the SSSF and SSF processes obtained the higher production and productivity of ethanol with the use of the pre-saccharification before to SSF. Mesa et al. (2011) also evaluated the application of SHF, SSF and SSSF for ethanol production using 1 ton of sugarcane bagasse, obtaining 192, 172 and 198 L of ethanol, respectively. These results demonstrate the importance of performing the presaccharification step before the fermentative process and corroborate the results achieved in this work.

4. Conclusion

Cactus, mature coconut shell, coconut fiber mature and green coconut shell submitted to autohydrolysis pretreatment were shown to be promising raw materials for application in the context of biorefinery, as from the elevated versatility and practicality in the fractionation of the LCMs (a reduction in the hemicellulose content, and increase of the cellulose and lignin) with high conversions into glucose by EH. As the best results on EH were obtained from green coconut shell (92.52%), this LCM was selected for ethanol production being shown that the best results on ethanol yield (90.09%) and ethanol productivity (0.21 g/(L h)) from green coconut shell were obtained by *S. cerevisiae* using SSSF. Therefore, an efficient process was developed for the bioethanol production from green coconut shell.

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