Comparison of delignified coconuts waste and cactus for fuel-ethanol production by the simultaneous and semi-simultaneous saccharification and fermentation strategies

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HIGHLIGHTS

- Sequential Alk-H_2O_2/NaOH pretreatment was developed.
- FTIR, SEM, X-ray and crystallinity indexes have evidenced modifications in solids.
- Delignified MCF was more susceptible the enzymatic action.
- SSSF strategy allowed to obtain higher ethanol production than SSF.
- Step of presaccharification had a positive effect on the overall ethanol yield.

GRAPHICAL ABSTRACT
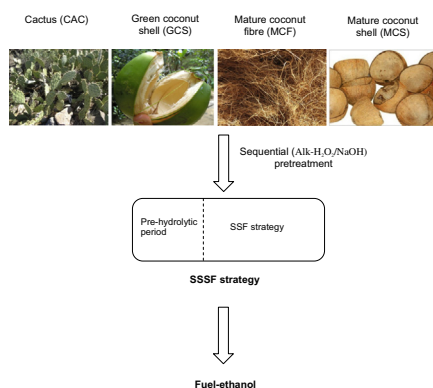
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ABSTRACT

It is of the highest importance to study different alternatives/strategies as simultaneous (SSF) and semi-simultaneous (SSSF) saccharification and fermentation process, as well as the prospects of the utilization of lignocellulosic residues as raw materials for fuel-ethanol production. In the first part of this work, different raw materials (cactus (CAC), green coconut shell (GCS), mature coconut fibre (MCF) and mature coconut shell (MCS)) were pretreated by sequential alkaline hydrogen peroxide (Alk-H_2O_2)–sodium hydroxide (NaOH) process. The characterization of the obtained solids by FTIR, SEM, X-ray and crystallinity indexes confirmed the higher susceptibility of these pretreated materials to enzymatic action. These results were further confirmed by the corresponding glucose conversion yields – 68.44%, 70.20%, 76.21% and 74.50% for CAC, GCS, MCF and MCS, respectively. Subsequently, the comparison between SSF and SSSF using *Saccharomyces cerevisiae*, *Pichia stipitis*, *Zymomonas mobilis* and pretreated MCF (selected in the enzymatic hydrolysis step) was done, being shown that a short presaccharification step at 50 °C for 8 h in the SSSF had a positive effect on the overall ethanol yield, with an increase from 79.27–84.64% to 85.04–89.15%. In all the cases, the SSSF strategy allowed the obtention of higher ethanol concentrations than SSF.

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1. Introduction

The use of biofuels, with emphasis on fuel-ethanol is an alternative to mitigate the pressure exerted by fossil fuels and their derivatives. However, fuel-ethanol production from corn, sugarcane and beet may be a problem in the near future due to the food competition in the use of these materials for bioenergy production [1]. One option is the production of cellulosic ethanol from coconut trees, as these crops are distributed in over 200 countries. According to FAO (http://www. www.faostat.org.br), the world production of coconut in 2009 was about 55 million tons, mainly in the Philippines (36%), Indonesia (28%) and India (20%). Brazil is the fourth largest producer of coconut, with a production of about 3 million tons (5.5%) (http://www.ibge.gov.br). Associated with the large volume coconut production, large amounts of not used agroindustrial waste, suitable to be applied in fuel-ethanol production, are also obtained. Just in Brazil, the production of CAC was 60,000 tons in 2009, mainly concentrated in the Northeast Region and was fully used in animal feed (http://www.ibge.gov.br).

Fuel-ethanol production from lignocellulosic materials (LCMs) is complicated due to the recalcitrant nature of the molecules present in these LCMs. In order to make cellulose and hemicellulose more accessible to the attack of cellulases and hemicellulases, a pretreatment is required [1,2]. Pretreatment processes can be physical, chemical, biological or a combination of these methods. The chemical pretreatments used in the delignification of LCMs provide a reduction of the degree of polymerization and crystallinity of cellulose, associated with the swelling of the sample and increase the internal area of LCMs [3]. The application of combined or sequential pretreatments strategies has been shown to be a good way to improve enzymatic hydrolysis and subsequently fuel-ethanol production [3].

The alkaline hydrogen peroxide (Alk-H_2O_2) process is based on the pretreatment of LCMs using hydrogen peroxide at alkaline conditions. This process is operated at low temperature and pressure and the peroxide decomposes into oxygen and water and so can be considered a process with a low environmental impact [4,5]. According to Gould [6], the use of hydrogen peroxide improves the subsequent delignification of LCMs, because hydrogen peroxide at alkaline conditions promotes the oxidative depolymerization of lignin, due to the break of carbon–carbon linkages in the lignin [7]. Xiang and Lee [8] reported two important factors in the oxidation process: pH of the reaction and decomposition of hydrogen peroxide. Additionally, the use of sodium hydroxide (NaOH) allows the delignification of LCMs by breaking the ester bonds crosslinking lignin and xylan, increasing the internal surface area [9].

On the other hand, there are different alternatives or strategies in the fermentation process for fuel-ethanol production [10]. During the last years, simultaneous saccharification and fermentation (SSF) has shown to have several advantages compared with separate hydrolysis and fermentation (SHF) in terms of overall ethanol yield and volumetric productivity of ethanol. Moreover, SSF reduces processing time as a consequence of the fast glucose conversion to ethanol by the fermenting microorganisms that reduce the enzyme inhibition due to the presence of sugars. Reduction in equipment costs is also obtained by carrying the hydrolysis and fermentation in a single reactor [11]. However, the difference between the optimal temperature for the enzyme action and microorganism growth is an issue that needs to be solved for an efficient SSF [12]. The operational strategy of semi-simultaneous saccharification and fermentation (SSSF) is a good alternative that includes a short presaccharification period before the SSF process and that has been shown to produce higher ethanol concentration, yield and productivity than SSF and SHF [10]. In this context, the objective of this work was to compare and evaluate the SSSF and SSF strategies for fuel-ethanol production by *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023 using a selected raw material as the MCF pretreated by the Alk-H_2O_2/NaOH process.

2. Materials and methods

2.1. Raw materials and chemical characterization

CAC, GCS, MCF and MCS were obtained from the agroindustries and urban locations in the Northeast of Brazil. The composition of the raw materials was obtained according to Gouveia et al. [13] and Sluiter et al. [14].

2.2. Pretreatment process

2.2.1. Preparation of raw materials before the pretreatment

The raw materials were washed five times with distilled water at 70 °C for removal of residual compounds. After this procedure, the LCMs were dried in an oven with air circulation at 40 °C for 24 h. The LCMs were milled to a particle size of 48 mesh (0.3 mm).

2.2.2. Alkaline hydrogen peroxide (Alk-H_2O_2) pretreatment

0.4 g of LCM were mixed with 31.75 mL of hydrogen peroxide in a flask with a concentration of 7.35% (v/v) at 25 °C for 1 h with agitation at 150 rpm. The pH of hydrogen peroxide solution was adjusted to 11.5 with NaOH. The LCM residual solid was separated via vacuum filtration and washed with distilled water [15].

2.2.3. Delignification process with sodium hydroxide (NaOH)

The Alk-H_2O_2 pretreated solids from each LCM were transferred to flasks with a 4% (w/v) solution of NaOH. The mixture remained at 100 °C under agitation at 100 rpm for 1 h. After delignification, the solids were separated from the liquor by filtration. The solids underwent seven washes with distilled water [16].

2.3. Characterization of delignified pretreated solids

2.3.1. Chemical composition after delignification

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

2.3.2. Fourier-transform infrared (FTIR)

The FTIR spectra of delignified pretreated solids and untreated LCMs were measured on an FTIR spectrometer (FTLA 2000 series, ABB Bomem Inc., Quebec, Canada). The conditions of analysis were: resolution of 4 cm^{-1} using 20 scans and frequency range of 400–4000 cm^{-1}. The samples were ground with spectroscopic grade potassium bromide (KBr).

FTIR analysis was conducted to examine the cellulose structure of delignified pretreated solids and untreated LCMs. Two infrared ratios related to cellulose structure were calculated: (1) 1426 cm^{-1}/896 cm^{-1}, the ratio of peak areas at 1426 and 896 cm^{-1}, which is referred to as crystallinity index [17] or lateral order index (LOI) [18]; (2) 1373 cm^{-1}/2917 cm^{-1}, the ratio of peak areas at 1373 and 2917 cm^{-1}, which is known as total crystallinity index (TCI) [19].

2.3.3. X-ray diffraction analysis and crystallinity

Cellulose crystallinity of delignified pretreated solids and untreated LCMs was analyzed in an X-ray diffractometer (Bruker D8 Discover, USA). The operating voltage and current were 40 kV and 40 mA, respectively. The crystallinity index (CI) was defined using the Eq. (1) [3].

$$CI = \frac{I_{002} - I_{am}}{I_{002}} \cdot 100 \qquad (1)$$

where, I_{002} = maximum intensity (2θ, 22.6°) of the (002) lattice diffraction; I_{am} = intensity of the amorphous diffraction (2θ, 18.7°).

2.3.4. Scanning electron microscopy

The surface of delignified pretreated solids and untreated LCMs was visualized by a scanning electron microscope (Nova NanoSEM 200, Netherlands).

2.4. Enzymes

Enzyme solutions, cellulases, β-glucosidase and hemicellulases (Cellic CTec2) and endoxylanase (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. [20]. In a tube were added 0.3 mL of the commercial enzyme diluted with 1.2 mL of sodium citrate buffer 0.5 μM 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. The β-glucosidase activity was determined for Cellic CTec2. The β-glucosidase activity was measured by incubating the enzyme solution with 15 μM of cellobiose and 50 mM sodium citrate buffer (pH 4.8) at 50 °C for 30 min. The reaction was stopped by immersing in boiling water for 5 min. Then, glucose concentration was determined using the GOD-POD method at 25 °C 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 μmol of glucose per min. The xylanase activity was determined for HTec2. Reaction mixtures contained 0.1 mL enzyme and 0.5% (w/v) of oat spelts 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 the DNS method [21]. One unit of xylanase activity (IU/mL) was defined as the amount of enzyme that released 1 μmol 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.

2.5. Enzymatic hydrolysis

2.5.1. Hydrolysis yield

The obtained delignified pretreated solids were used as substrate in the enzymatic hydrolysis. Enzymatic hydrolysis were performed with 4% (w/v) of delignified pretreated solids from each LCM, in an Erlenmeyer flask with a volume of 48 mL at 50 °C using Cellic CTec2 and HTec2 with an enzymatic load of 30 FPU, 75 CBU and 130 IU per gram of pretreated solid, 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 at 12 h intervals until a total time of 96 h [21,22]. All determinations were performed in duplicate. Sugars concentrations were determined by high performance liquid chromatography (HPLC) (see Section 2.7). The yield of enzymatic hydrolysis was calculated using Eq. (2) [22].

$$\text{Hydrolysis yield}(\%) = \frac{[\text{glucose}] + 1.053[\text{cellobiose}]}{(1.111)f\,[\text{biomass}]} \cdot 100 \qquad (2)$$

where, glucose = glucose concentration (g/L); cellobiose = cellobiose concentration (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 equivalent glucose; 1.053 = consists in the conversion factor of cellobiose to equivalent glucose.

2.5.2. Statistical analysis of enzymatic hydrolysis

The selection of the delignified pretreated solids was performed taking into account the susceptibility to enzymatic hydrolysis. The statistical analysis was carried out using single-factor analysis of variance (ANOVA), while multiple comparison tests were used to determine the statistical significance with a 95% confidence level. For the data analyses, Statistica software was used.

2.6. Fermentation process

2.6.1. Microorganisms

Microorganisms *P. stipitis*, *S. cerevisiae* and *Z. mobilis* were used in the fuel-ethanol production. *P. stipitis* Y7124, *Z. mobilis* B14023 and *S. cerevisiae* PE2 strains were obtained from microbiological collection of Institute Biotechnology and Bioengineering at the University of Minho. Microorganisms were maintained in Eppendorf at −80 °C (glycerol solution at 20% concentration) and subsequently lyophilized for use as working stock.

2.6.2. 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 [23]. For all cultures, the cell concentration in the inoculum was 2.0 (quantified by measuring the optical density at 600 nm in a UV–VIS spectrophotometer [12]. Subsequently, the cells were inoculated into 48 mL culture medium to start the SSF and SSSF processes.

2.6.3. Simultaneous saccharification and fermentation (SSF)

SSF experiments were conducted using delignified pretreated solid of MCF in accordance with the NREL standard procedure [22]. 4% (w/v) of delignified pretreated solids in 48 mL of sodium citrate buffer 50 mM (pH = 5.0) were added with Cellic CTec2 and HTec2 with an enzymatic load of 30 FPU, 75 CBU and 130 IU per gram of pretreated solid 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 [23]. SSF was started by adding enzymes and the microbial strains, incubated for 48 h 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, 8, 12, 24, 36 and 48 h. Concentrations of ethanol and sugars, glycerol and xylitol were determined by HPLC (see Section 2.7). All determinations were performed in duplicate.

2.6.4. Semi-simultaneous saccharification and fermentation (SSSF)

An 8 h pre-hydrolysis step followed by a 40 h SSF process was used for SSSF of MCF delignified pretreated solids. After 8 h of hydrolysis (50 °C), the medium temperature was adjusted to 30 °C and the SSF step was carried out as previously described (Section 2.6.3).

Ethanol yields from glucose fermentation (Eq. (3)) were calculated assuming that all the potential glucose in the pretreated delignified solids was available for fermentation and that 1 g of glucose yielded 0.511 g of ethanol and 1 g of cellulose gave 0.9 g of glucose [22]. Furthermore, ethanol yields from xylose fermentation by *P. stipitis* were calculated according to Dowe and McMillan

[22], but with the inclusion of xylose instead of glucose (Eq. (4)), and considering that 1.0 g of xylose yielded 0.51 g of ethanol.

$$\text{Ethanol yield } (\%) = \frac{[\text{ethanol}]}{[\text{glucose initial}] \cdot 0.51} \cdot 100 \qquad (3)$$

$$\text{Ethanol yield}(\%) = \frac{[\text{ethanol}]}{[\text{xylose initial} - \text{final xylose}] \cdot 0.51} \cdot 100 \qquad (4)$$

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

2.6.5. Statistical analyzes for SSF and SSSF

Statistical significance was evaluated by Fisher *F-test* for ANOVA and Student *t-test*, with a confidence level of 95%. Statistical analyzes were performed with the aid of Statistica software.

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

All the samples were centrifuged and filtered through a 0.2 μm sterile membrane filter for glucose, xylose, glycerol, ethanol and xylitol quantification. Chromatographic separation was performed using a Metacarb 87H 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).

Table 1
Chemical composition (% dry weight) of untreated, alkaline hydrogen peroxide (Alk-H_2O_2) pretreated and sequential alkaline hydrogen peroxide (Alk-H_2O_2)–sodium hydroxide (NaOH) pretreatment for MCF, GCS, MCS and CAC.

Mature coconut fibre			
Components	Untreated	Alk-H_2O_2	Alk-H_2O_2/NaOH
Cellulose	31.60 ± 0.51	41.53 ± 0.89	51.80 ± 0.78
Hemicellulose	26.33 ± 0.89	28.40 ± 0.74	25.81 ± 0.54
Insoluble lignin	25.02 ± 0.78	16.51 ± 0.26	8.83 ± 0.18
Soluble lignin	1.67 ± 0.19	0.83 ± 0.15	0.09 ± 0.01
Extractable	5.44 ± 0.24	0.36 ± 0.13	0.05 ± 0.04
Ash	3.31 ± 0.32	2.92 ± 0.22	2.98 ± 0.14
Green coconut shell			
Cellulose	32.88 ± 0.88	51.58 ± 0.87	54.14 ± 0.14
Hemicellulose	26.50 ± 0.45	27.94 ± 0.90	28.36 ± 0.28
Insoluble lignin	25.44 ± 0.75	9.07 ± 0.04	7.64 ± 0.43
Soluble lignin	1.44 ± 0.27	0.61 ± 0.01	0.25 ± 0.01
Extractable	3.27 ± 0.15	0.77 ± 0.28	0.26 ± 0.45
Ash	4.34 ± 0.20	1.95 ± 0.06	1.07 ± 0.07
Mature coconut shell			
Cellulose	30.47 ± 0.86	37.24 ± 0.69	53.88 ± 0.41
Hemicellulose	25.42 ± 0.29	29.29 ± 0.74	23.02 ± 0.59
Insoluble lignin	31.04 ± 0.18	18.11 ± 0.26	9.33 ± 0.21
Soluble lignin	2.11 ± 0.09	1.44 ± 0.14	0.89 ± 0.03
Extractable	2.71 ± 0.31	0.84 ± 0.04	0.53 ± 0.03
Ash	4.84 ± 0.09	4.34 ± 0.27	3.80 ± 0.07
Cactus			
Cellulose	38.33 ± 0.64	44.00 ± 0.79	54.91 ± 0.72
Hemicellulose	22.19 ± 0.59	21.39 ± 0.75	17.65 ± 0.29
Insoluble lignin	19.51 ± 0.29	13.66 ± 0.45	8.74 ± 0.11
Soluble lignin	1.39 ± 0.13	1.27 ± 0.24	0.71 ± 0.06
Extractable	5.82 ± 0.24	1.92 ± 0.05	0.48 ± 0.05
Ash	6.64 ± 0.21	8.80 ± 0.30	8.77 ± 0.12

3. Results and discussion

3.1. Compositions of raw materials

The composition of raw materials used (% dry weight) is presented in Table 1. The initial moisture content of CAC, GCS, MCF and MCS was 12.60%, 8.99%, 6.14% and 5.52%, respectively. The component present in higher amounts was cellulose with 38.33%, 32.88%, 31.6% and 30.47% for CAC, GCS, MCF and MCS, respectively, followed by hemicellulose and insoluble lignin, except for MCS, where lignin has a higher percentage than hemicellulose. Apart from the fact there are few reports regarding the composition of CAC, GCS, MCF and MCS. The composition of CAC depends on age, time of collecting and soil properties [24]. Vaithanomsat et al. [25] reported the chemical composition of coconut husk as: cellulose (39.31%), hemicellulose (16.15%) and lignin (29.79%). Overall, the chemical composition of these LCMs suggests their adequacy for the fuel-ethanol production.

3.2. Effect of sequential alkaline hydrogen peroxide (Alk-H_2O_2)–sodium hydroxide (NaOH) process

The purpose of using Alk-H_2O_2 and NaOH was to improve the efficacy of the delignification process in the LCMs. After Alk-H_2O_2 treatment, the obtained solid yields were 64.15%, 55.30%, 51.95% and 59.70% for CAC, GCS, MCF and MCS, respectively. In Table 1 the chemical composition of the different LCMs after Alk-H_2O_2 pretreatment is presented. The main effect of this process was the reduction of the lignin content in the pretreated LCMs, in comparison to the untreated LCMs. The observed reduction was 28.56%, 63.99%, 35.03% and 41.02% for CAC, GCS, MCF and MCS, respectively. In a recent work, Da Costa et al. [26] reported a 43.76% reduction in lignin for cashew apple bagasse pretreated with Alk-H_2O_2 at 35 °C for 24 h with hydrogen peroxide at a concentration of 4.3% (v/v) and Gould [6] reported that wheat straw was partially delignified using Alk-H_2O_2. Moreover, Gould [6] indicated that the delignification reaction is strongly dependent upon the pH of the reaction mixture with an optimum at pH 11.5–11.6. Ayeni et al. [27] used alkaline peroxide assisted wet air oxidation pretreatment of LCMs and obtained an up to 60% enrichment of cellulose with 80% and 17% reduction of hemicellulose and lignin, respectively. These results are in agreement with those reported in this work, evidencing the reduction of lignin in the solid after Alk-H_2O_2 pretreatment. However, a different result was reported by Brígida et al. [28] as a 4.31% pulp decrease and a 3.31% lignin increase in the coconut fiber pretreated with Alk-H_2O_2 (5.4% (v/v) hydrogen peroxide at 85 °C for 2 h).

The chemical composition of the different LCMs (% dry weight) after the application of the sequential Alk-H_2O_2/NaOH treatment is shown in the Table 1. The obtained solid yields are 50.90%, 38.40%, 46.35%, 49.76% for CAC, GCS, MCF and MCS, respectively, with corresponding reductions of 54.78%, 70.64%, 66.57% and 69.17% in the lignin content. The highest content of cellulose after this sequential process was 54.91% for CAC. This effect can be explained by the solubilization of lignin, revealing that the cellulose was almost not affected by the sequential process and consequently a pretreated solid with increased cellulose content was obtained. These results are in agreement with the ones obtained by Chen et al. [29] that reported that more than 95% of cellulose was conserved in alkaline pretreatment. In this work, for all the different materials considered, cellulose content increased and lignin content decreased while a reduction in hemicellulose was observed only for CAC, MCF and MCS. According to Siqueira et al. [30], removal of lignin increases the enzymatic hydrolysis of sugarcane bagasse, resulting in a larger conversion of cellulose to glucose. Presented results

suggest that the use of sequential Alk-H_2O_2/NaOH pretreatment increases the selectivity of lignin degradation, in comparison to the Alk-H_2O_2 pretreatment.

In what concerns cellulose and hemicellulose recovered, the obtained values were, respectively, 75.98% and 68.27% for MCF pretreated by Alk-H_2O_2 and 56.03% and 45.43% for the Alk-H_2O_2/NaOH pretreatment. In the case of GCS, the equivalent values were 81.50% and 76.32%, 54.77% and 49.60%; for MCS were 81.96% and 63.49%, 59.86% and 41.97%; and for CAC were 66.40% and 59.63%, 50.08% and 36.87%.

3.3. Characterization of delignified pretreated solids

3.3.1. X-ray diffraction analysis and crystallinity

The cellulose contained in the delignified pretreated solids has a crystalline region (highly ordered) and the applied pretreatment results in a region with different properties for each of the LCMs. The diffraction peaks around 15–16° and 21–22° (2θ) are characteristics of the cellulose from LCMs. The crystallinity indexes are presented in Fig. 1A–D.

The crystallinity indexes of untreated LCMs were 34.34%, 28.03%, 29.31% and 23.20% for MCF, MCS, GCS and CAC, respectively (Table 2). After the Alk-H_2O_2 pretreatment and sequential Alk-H_2O_2/NaOH pretreatment for MCF, GCS and CAC an increase in the crystallinity indexes was observed. For MCF, the increase was from 50.79% to 55.73%; for GCS from 49.18% to 49.89% and for CAC from 46.09% to 48.43%. For MCS a decrease from 55.98% to 53.77% was observed. This effect can be explained by the removal amorphous lignin and hemicellulose that causes an increase in the crystallinity while the swelling of cellulose in hydrogen peroxide solution softened the lignocellulosic structure and hence a decrease the crystallinity occurs [31].

3.3.2. Fourier-transform infrared (FTIR)

The infrared spectroscopy analysis was carried out with the purpose of obtaining information about the chemical groups present in the delignified pretreated solids, mainly the formation of free radicals in the polymer chains, indicating the existence of broken covalent bonds, a method commonly used by its simplicity and efficiency in biological analysis. The FTIR spectra in the region between 400 and 4000 cm^{-1} of LCMs are presented in Fig. 2.
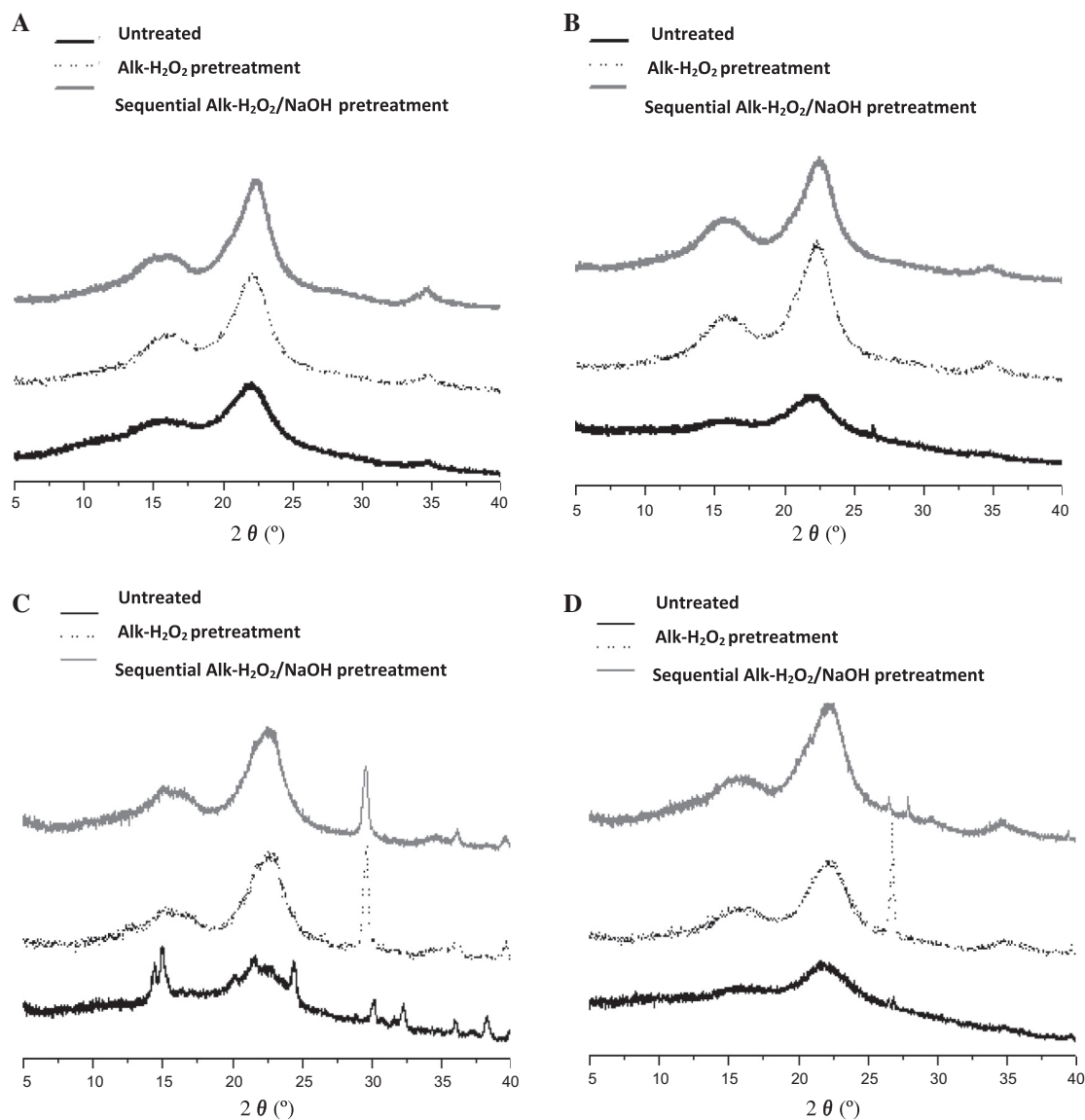

Fig. 1. X-ray diffraction curves of untreated, Alk-H_2O_2 pretreatment and sequential Alk-H_2O_2/NaOH pretreatment: (A) MCF, (B) GCS, (C) MCS and (D) CAC.

Table 2
Crystallinity index of untreated, alkaline hydrogen peroxide (Alk-H$_2$O$_2$) pretreated and sequential alkaline hydrogen peroxide (Alk-H$_2$O$_2$)–sodium hydroxide (NaOH) pretreatment for MCF, GCS, MCS and CAC.

Lignocellulosic material	LOI (FTIR)			TCI (FTIR)			CI (X-ray diffraction)		
	Untreated	Alk-H$_2$O$_2$	Alk-H$_2$O$_2$/NaOH	Untreated	Alk-H$_2$O$_2$	Alk-H$_2$O$_2$/NaOH	Untreated	Alk-H$_2$O$_2$	Alk-H$_2$O$_2$/NaOH
Cactus	0.679	0.908	0.924	0.816	0.944	1.014	0.232	0.461	0.484
Green coconut shell	0.852	0.910	0.913	0.861	1.039	1.056	0.293	0.492	0.499
Mature coconut fibre	0.888	0.934	0.957	0.919	1.014	1.073	0.343	0.508	0.557
Mature coconut shell	0.783	0.942	0.937	0.831	1.067	1.047	0.280	0.560	0.538

LOI: lateral order index based on FTIR (1426/896 cm^{-1}).
TCI: total crystallinity index based on FTIR (1373/2917 cm^{-1}).
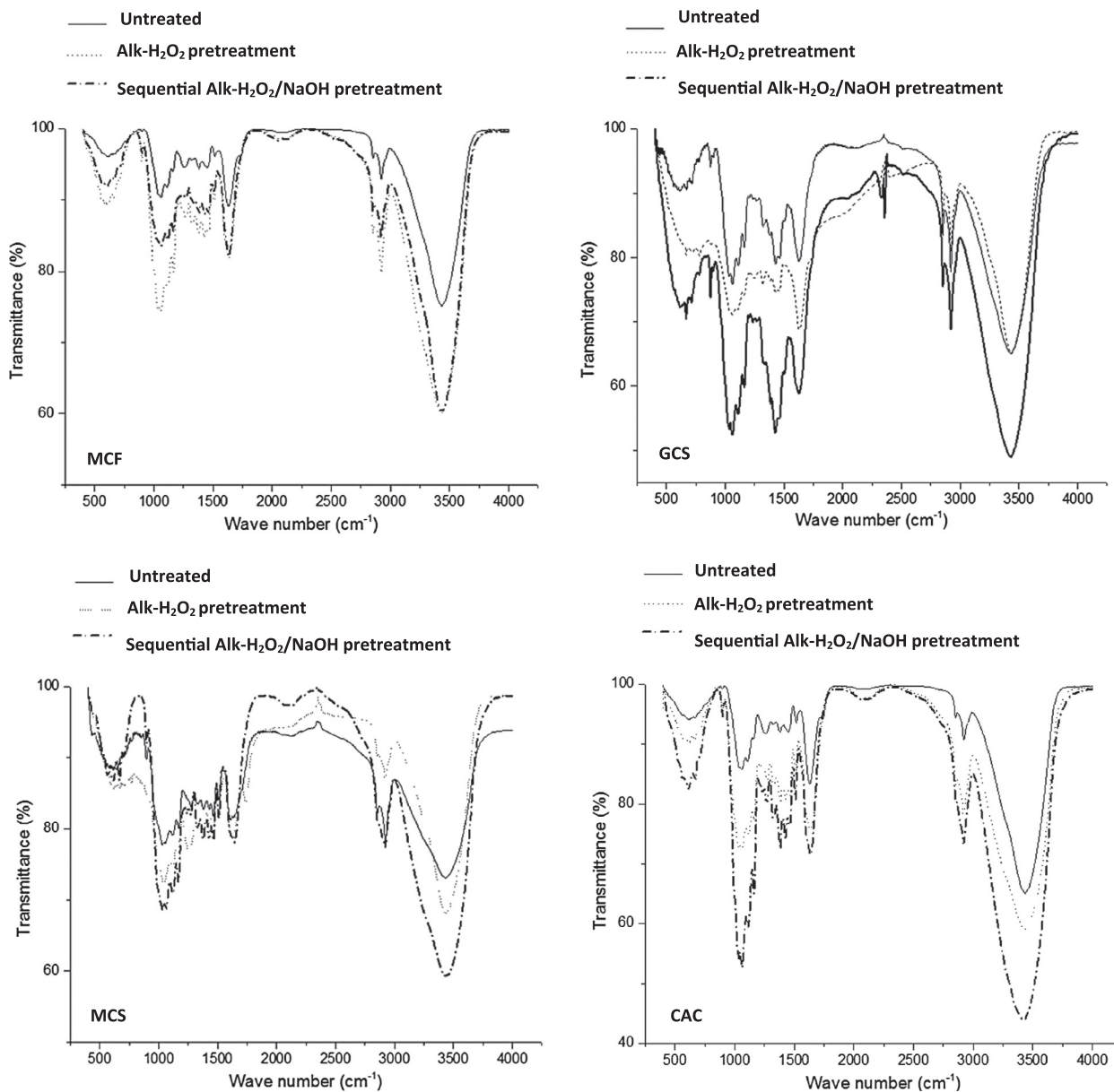CI: crystallinity index based on X-ray diffraction.

Fig. 2. FTIR spectra of untreated, Alk-H$_2$O$_2$ pretreatment and sequential Alk-H$_2$O$_2$/NaOH pretreatment for MCF, GCS, MCS and CAC.

Several relevant changes can be observed in the delignified pretreated solids compared to untreated LCMs. The presence of a peak at 1238 cm^{-1} relates to esters, ethers and phenolic groups and is attributed mainly to the presence of waxes in the epidermal tissue of the LCM, its disappearance after the pretreatment, representing the removal of these waxes fibers. Similar results were reported by Brígida et al. [28] in pretreated green coconut fiber. The carboxyl band (1636 cm^{-1}) in the spectrum of the untreated samples is related to the carboxyl groups of pectin associated with the cellulose fibers.

At 1254 cm^{-1} the absence of the peak corresponding to the double bond between carbon and oxygen lignin in the delignified pretreated solids, is an indication of the absence or reduction of lignin in the LCMs. The peaks at wavelengths 1030, 1241, 1360, 1405, 1430 and 1500 cm^{-1} are related to lignin [7]. The pretreated LCMs showed reductions in the intensity or absence of these peaks, indicating the rupture of lignin links [7]. These data that corroborates the reduction of lignin content in LCMs is supported by the presented SEM images.

The hemicellulose pattern shows characteristic peaks at wavelengths of 897, 1043, 1164, 1248 and 1728 cm^{-1}. These hemicellulose peaks were observed in untreated and pretreated LCMs, [29] and are in agreement with the chemical composition data presented in Table 1. The band ranging between 1370 and 1390 cm^{-1} where no peak is observed refers to a symmetrical structure and an asymmetrical deformation of cellulose and hemicellulose and may be indicative of a greater exposure of cellulose and hemicellulose on the fiber surface [28]. This is relevant information concerning the enzymatic digestibility of the LCMs.

The peak observed at a wavelength 3340 cm^{-1} is related to hydrogen bonding (OH) and indicates the stretching vibration of the structure of cellulose and lignin of the LCM. The band at 3400 cm^{-1} corresponds to the stretching of the hydroxyl and phenol groups at the LCMs and the observed peak increase after pretreatment indicates a reduction in the content of these groups in the obtained materials. These results may be related to the reduction of the degree of hydrogen bonding, resulting in the reduction of the superficial polarity of the fiber.

Overall, FTIR analysis corroborates the results on the chemical composition of delignified pretreated solids presented in Table 1, that demonstrate a reduced lignin content, while retaining hemicellulose and cellulose.

The crystallinity index (CI) was also obtained from values contained in the wavelengths of 1426/896 cm^{-1} and the total crystallinity index (TCI) from the values contained in the wavelengths of 1373/2917 cm^{-1} for both untreated and pretreated LCMs (Table 2). The 1426 cm^{-1} band represents CH$_2$ scissoring motion [32] and the 896 cm^{-1} band indicates the vibrational mode involving carbon and four atoms attached to it, which is characteristic of β-anomers or β-linked glucose polymers [32]. The 1373 cm^{-1} band is for CH bending mode [19] and the 2917 cm^{-1} band represents C–H and CH$_2$ stretching, which is unaffected by changes in crystallinity [19]. Therefore, higher values of LOI and TCI are indicative of biomass with a higher crystallinity and a more ordered structure of cellulose. The results obtained for LOI (FTIR) and TCI (FTIR) and presented in Table 2, show increased crystallinity indexes for the pretreated LCMs in comparison to the untreated ones. These results agree with the CI vales obtained by X-ray diffraction.

3.3.3. Scanning electron microscopy (SEM)

Differences in fiber structure between untreated, Alk-H$_2$O$_2$ and sequential Alk-H$_2$O$_2$/NaOH pretreated LCMs are presented in Fig. 3A–L. The untreated LCMs showed the fibers rigid surfaces intact and highly ordered (Fig. 3A, D, G and J). However, SEM images of Alk-H$_2$O$_2$ (Fig. 3B, E, H and K) and Alk-H$_2$O$_2$/NaOH pretreated (Fig. 3C, F, I and L) LCMs clearly show modified structures and a destruction of the fibers, fiber separation and the appearance of disordered fibers. These structural features may provide greater susceptibility of pretreated LCMs to enzymatic action.

3.4. Enzymatic hydrolysis for the selection of pretreated delignified material for further fermentation

The enzymatic hydrolysis of Alk-H$_2$O$_2$/NaOH pretreated LCMs will enable the selection of solid with the highest conversion yield and initial hydrolysis rate to be used in the fermentation stage. The

conversion yields (%) of CAC, GCS, MCF and MCS were 68.44% (0.50 g glucose/g LCM), 70.20% (0.56 g glucose/g LCM), 76.21% (0.59 g glucose/g LCM) and 74.50% (0.57 g glucose/g LCM), respectively, after 96 h (Fig. 4A). These results demonstrate the susceptibility of the LCMs pretreated by Alk-H$_2$O$_2$/NaOH to enzymatic attack. In a recent work, Rabelo et al. [15] reported a high glucose yield after enzymatic hydrolysis using sugarcane bagasse pretreated with Alk-H$_2$O$_2$ (7.35% (v/v) of hydrogen peroxide for 1 h at 25 °C). Gupta and Lee [7] also concluded that the use of hydrogen peroxide (5%) in alkaline solution (5%) on hybrid poplar at low temperature improved the delignification and the enzymatic hydrolysis, similar to the results presented in this work [7].

The results obtained in the enzymatic hydrolysis were evaluated by ANOVA and significant differences in the level of confidence of 95% were observed, being the highest sugar yields obtained for MCF.

Regarding the maximum initial hydrolysis rate (dG/dt), measured during the first 12 h (the slope of glucose concentration vs. time) (Fig. 4B) the highest, initial hydrolysis rate was obtained for MCF (1.49 g/(L h)) and MCS (1.43 g/(L h)), while lower values were reported for GCS (1.28 g/(L h)) and CAC (1.04 g/(L h)). In comparison, Ruiz et al. [2] reported initial hydrolysis rate of wheat straw pretreated by autohydrolysis of 0.47 g/(L h) using 30 FPU/g of cellulose. These results demonstrate the susceptibility of the pretreated LCMs to enzymatic attack.

The results obtained during enzymatic hydrolysis (see Fig. 4) emphasize that the highest conversion of pretreated LCMs into glucose occurred in the LCMs with higher crystallinity indexes (see Table 2). According to Kim et al. [33], the increased crystallinity can provide higher digestibility of LCM due to the higher exposure of the crystalline part on the surface of the LCM.

3.5. Fermentation process for fuel-ethanol production

SSF and SSSF strategies were evaluated using *P. stipitis* Y7124, *Z. mobilis* B14023, *S. cerevisiae* PE2 and MCF pretreated by the sequential Alk-H$_2$O$_2$/NaOH process. The pretreated MCF was selected based on the enzymatic hydrolysis yield and initial hydrolysis rate. The performance of the fermentation strategies was assessed by conversion yield (%) and volumetric productivity of ethanol (g/(L h)) [11].

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

The values obtained in the fuel-ethanol production by SSF using *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023 are shown in Fig. 5A–C, respectively. The obtained ethanol concentrations were 8.44 g/L, 9.12 g/L and 8.27 g/L for *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023, respectively and the process was completed after 48 h. Concerning ethanol yield, the results are presented in Table 3. The ethanol yield for *S. cerevisiae* PE2 was 84.64% (0.43 g ethanol/g sugar) and the volumetric productivity of ethanol 0.18 g/(L h), while for *P. stipitis* Y7124 and *Z. mobilis* B14023 the obtained values were 79.27% (0.40 g ethanol/g sugar) and 81.71% (0.42 g ethanol/g sugar) for ethanol yield and 0.19 g/(L h) and 0.17 g/(L h) for volumetric productivity of ethanol, respectively (Table 3). These results indicate that the glucose obtained from the enzymatic hydrolysis of pretreated MCF may be fermented to ethanol by *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023, the kinetic profiles having a similar pattern for glucose consumption, with a rapid glucose consumption during the initial 24 h (Fig. 5A–C). All microorganisms proved to suitable for the fermentation of sugars into ethanol.

In a recent work, Chaudhary et al. [34] produced fuel-ethanol through of a sequential alkaline and acid pretreatment using Kans Grass biomass as substrate and *P. stipitis* as microorganism,
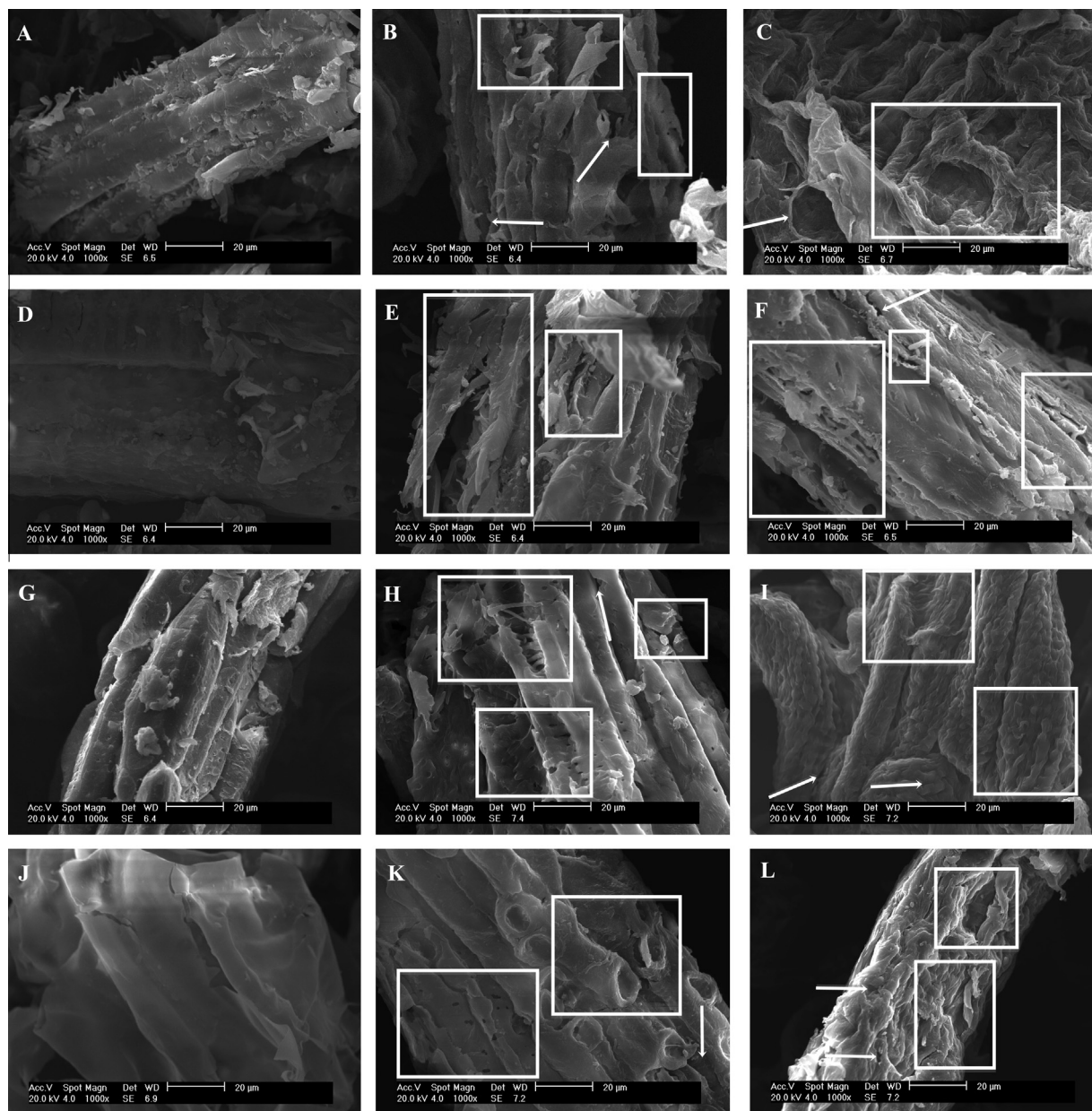
Fig. 3. Scanning electron microscopy images of MCF: (A) untreated, (B) Alk-H_2O_2 pretreatment, (C) sequential Alk-H_2O_2/NaOH pretreatment; GCS: (D) untreated, (E) Alk-H_2O_2 pretreatment, (F) sequential Alk-H_2O_2/NaOH pretreatment; MCS: (G) untreated, (H) Alk-H_2O_2 pretreatment, (I) sequential Alk-H_2O_2/NaOH pretreatment; CAC: (J) untreated, (K) Alk-H_2O_2 pretreatment, and (L) sequential Alk-H_2O_2/NaOH pretreatment. High porosity area, matrix separation and exposition fibers (white square).

reporting a volumetric productivity of ethanol of 0.22 g/(L h). In this work, using *P. stipitis* Y7124, the obtained volumetric productivity of ethanol was 0.19 g/(L h). Vaithanomsat et al. [25] studied the efficiency fuel-ethanol production using SSF and SHF processes with *S. cerevisiae* on coconut husk pretreated with NaOH as raw material and reported a conversion above 85% in both cases, a result similar to the one reported in this work where an ethanol yield of 84.64%) was obtained.

In the same Fig. (5A–C) are reported the equivalent values for ethanol fermentation using the SSSF strategy and the same LCM. Being 9.32 g/L and 89.15% (0.45 g ethanol/g sugar), 10.17 g/L and 85.04% (0.43 g ethanol/g sugar), 8.91 g/L and 85.65% (0.44 g ethanol/g sugar) for production and yield of ethanol by the *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023, respectively, after 48 h (Table 3). The highest volumetric ethanol productivity was 0.21 g/(L h) for *P. stipitis* Y7124, whereas the lowest was 0.19 g/(L h) for *S.*

cerevisiae PE2 and *Z. mobilis* B14023. All the experiments had a similar pattern for glucose concentration during the initial 8 h with a gradually decrease with increasing time (Fig. 5A–C). Results obtained for ethanol production and yield with the SSSF strategy are slightly higher compared with SSF (see Table 3). Moreover, higher volumetric productivities were obtained for SSSF. The higher fermentative efficiency for SSSF may be explained by the application of the short presaccharification period [35], which can enhance the conversion of cellulose to glucose and, in sequence, to ethanol.

In a recent work, Franco et al. [36] carried out the SSSF process (24 h of presaccharification and 24 h of SSF) using delignified *Pinus radiata* and *S. cerevisiae*, resulting in an ethanol yield and ethanol production of 90.0% and 15.5 g/L, respectively. These results are in agreement with the results obtained in this work for SSSF (ethanol yield between 85.65% and 89.15%). Martín et al. [37]
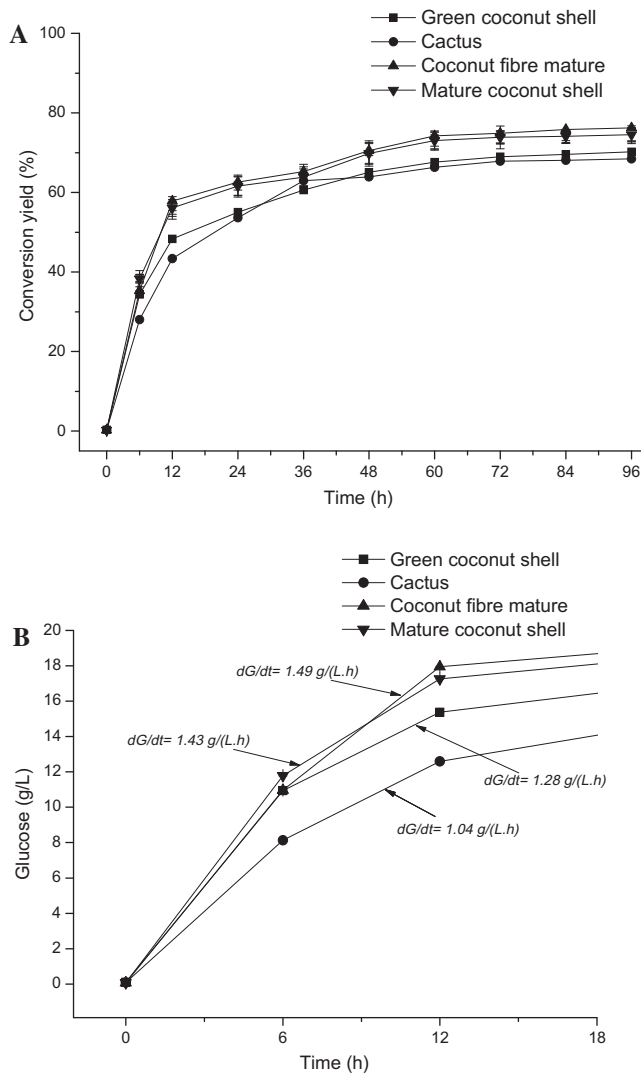
Fig. 4. Enzymatic hydrolysis of GCS, CAC, MCF and MCS pretreated with sequential Alk-H$_2$O$_2$/NaOH process. (A) Conversion yield%, and (B) Initial hydrolysis rate at 12 h.

reported that the presaccharification led to a rapid liquefaction and a good mixing was attained after 6 h, when the SSF process started. Santos et al. [38] carried out the SSSF process with 6 h of presaccharification period at 50 °C and subsequently the SSF at 37 °C using a delignified sugarcane bagasse as raw material and *S. cerevisiae* UFPEDA 1238, and the results were 27.71 g/L and 0.77 g/(L h) of ethanol production and volumetric productivity of ethanol, respectively. According to Souza et al. [12], the presaccharification has a possible positive effect, increasing the ethanol yield and volumetric productivity of ethanol. Additionally, they concluded that the use of a thermotolerant yeast and presaccharification stage are key points to increase yields in the SSF process for fuel-ethanol production.

SHF, SSF and SSSF strategies were compared by Mesa et al. [39] that reported that from one ton of sugarcane bagasse it is possible to obtain 192, 172 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 ethanol. Santos et al. [23] compared different process configurations for SSF and SSSF and reported that the highest cellulose to ethanol conversion and maximum ethanol productivities were completed with presaccharification prior to SSF. In this work, SSSF allowed to obtain a higher ethanol production than SSF.
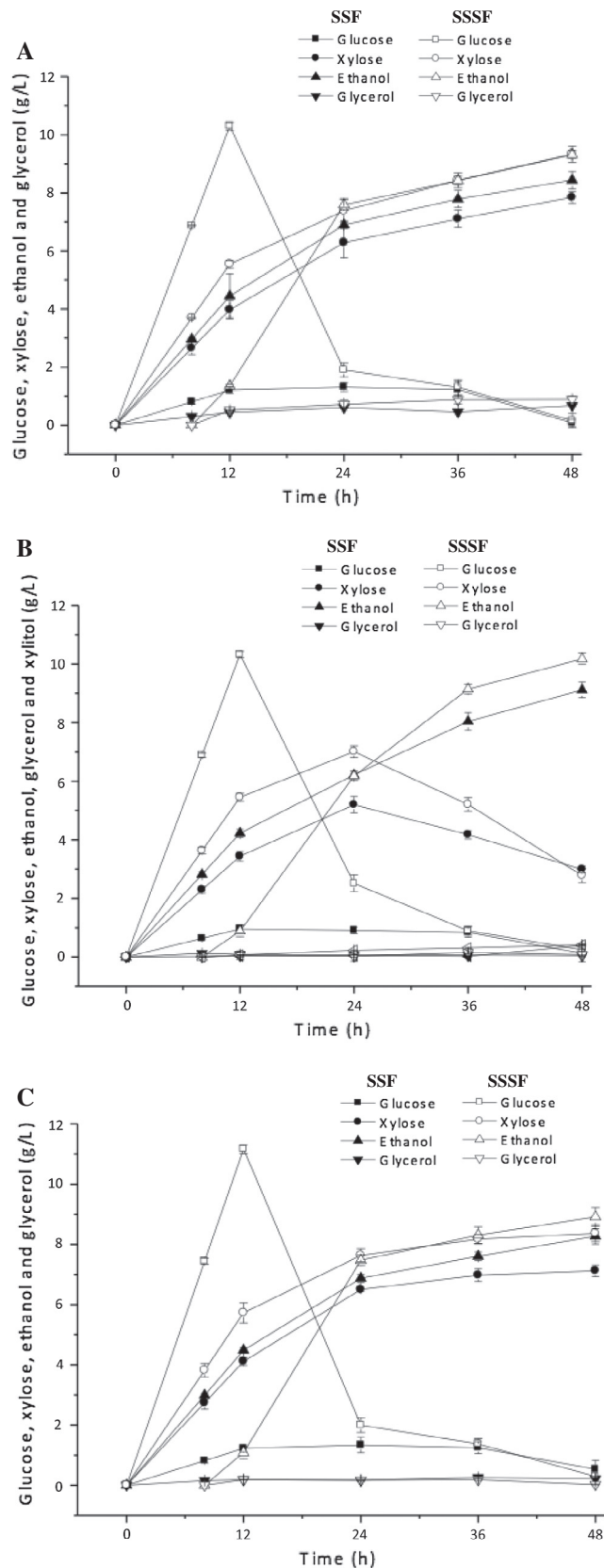

Fig. 5. Kinetics profiles of SSF and SSSF strategy using MCF pretreated with the sequential Alk-H$_2$O$_2$/NaOH process (A) *S. cerevisiae* PE2, (B) *P. stipitis* Y7124, and (C) *Z. mobilis* B14023.

The fuel-ethanol production by *S. cerevisiae*, *P. stipitis* and *Z. mobilis* using SSSF and SSF was evaluated statistically using *t-test*

Table 3
Kinetic parameters of fuel-ethanol production by *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023 using MCF pretreated with sequential alkaline hydrogen peroxide (Alk-H$_2$O$_2$)–sodium hydroxide (NaOH) as raw material in SSF and SSSF strategy.

Operational strategy	Microorganism	Ethanol yield (%)		Ethanol concentration (g/L)		Ethanol productivity (g/(L h))	
SSF	*S. cerevisiae*	84.64	±0.61	8.44	±0.06	0.18	±0.01
	P. stipitis	79.27	±1.56	9.12	±0.18	0.19	±0.00
	Z. mobilis	81.71	±0.60	8.27	±0.06	0.17	±0.00
SSSF	*S. cerevisiae*	89.15	±0.73	9.32	±0.08	0.19	±0.01
	P. stipitis	85.04	±0.54	10.17	±0.06	0.21	±0.00
	Z. mobilis	85.65	±1.02	8.91	±0.11	0.19	±0.00

and ANOVA (confidence level 95%). The fuel-ethanol production by the different microorganisms using SSF showed significant differences, when evaluated by the ANOVA. Similar results were also obtained when SSSF was applied. The comparison between results obtained by *S. cerevisiae* using SSF and *S. cerevisiae* using SSSF showed significant differences, when evaluated by the *t-test*. Similar results were also showed by the *P. stipitis* and *Z. mobilis* strains.

Glycerol is also formed as a byproduct in ethanol production during fermentation under anaerobic and aerobic growth conditions, and its can be influenced by microbial growth and several environmental factors as osmotic pressure [40]. In this work, the fermentations carried out by *S. cerevisiae* PE2, *P. stipitis* Y7124 and *Z. mobilis* B14023 in SSF and SSSF strategies presented minimal glycerol concentrations (Fig. 5A), in agreement with literature results for SSSF [10,38]. Moreover, no production of xylitol by *P. stipitis* Y7124 was observed for both SSF and SSSF strategies.

4. Conclusion

The present work was based on the evaluation of four raw materials (CAC, GCS, MCF and MCS) as promising materials for fuel-ethanol production. The effect of Alk-H$_2$O$_2$ and Alk-H$_2$O$_2$/NaOH pretreatments on solids properties was evaluated by FTIR, SEM, X-ray and crystallinity indexes determination. Moreover, sequential Alk-H$_2$O$_2$/NaOH pretreatment showed to be a suitable technology for generation of cellulose-enriched solids and MCF was selected for further fermentation taking into account its higher susceptibility to enzymatic hydrolysis. The short presaccharification at 50 °C for 8 h in SSSF had a positive effect on the overall ethanol yield, an increase from 79.27–84.64% to 85.04–89.15% being observed for the different microbial strains considered. The SSSF strategy allowed for the obtention of a higher ethanol production than SSF.

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