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Cashew apple bagasse as new feedstock for the hydrogen production using dark fermentation process

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ABSTRACT

Cashew apple bagasse (CAB) has been studied as feedstock for the biohydrogen production using *Clostridium roseum* and the dark fermentation process. Pretreatment with alkaline hydrogen peroxide (CAB-AHP) on raw material and the acid and enzymatic hydrolysis have been taken into account to evaluate the H_2 yields. Results show that the acid hydrolysate obtained from CAB produced higher H_2 molar yield (HMY) (15 $mmol_{H2}/L_{hydrolysate}$) than the acid hydrolysate from CAB-AHP (4.99 $mmol_{H2}/L_{hydrolysate}$), These HMY were noticeably higher than values obtained from the enzymatic hydrolysate of CAB-AHP (1.05 $mmol_{H2}/L_{hydrolysa}$) and the enzymatic hydrolysate of CAB (0.59 $mmol_{H2}/L_{hydrolysa}$). The maximum biohydrogen productivity (12.57 mL_{H2}/L.h) was achieved using the acid hydrolysate from CAB, with a H_2 content of about 72% vol, that could be satisfactory in view of an energetic applications of the biogas. Results suggest that CAB could be considered for the hydrogen production process, providing an appropriate destination for this lignocellulosic biomass, and consequently, reducing the environmental impact it can exert.

1. Introduction

Over the last decades the international community has emphasized the importance of renewable energy sources, that includes solar and wind power, biogas, hydroelectricity and biomass. Much attention has been focused on identifying suitable biomass species, which can provide high-energy outputs, to replace conventional fossil fuel energy sources.

The conversion of biomass to energy (or bio-energy) includes a wide range of different types and sources of biomass, conversion options, end-use applications and infrastructure requirements. Biomass can be derived from the cultivation of dedicated energy crops, by harvesting forestry and other plant residues, and from biomass wastes such as sludge from organic industrial waste and organic domestic waste or the wastes themselves. In particular, the energy recovery of the waste biomasses derived from the agro industrial activities is an attractive way to recycle wastes and environmental conservation. In addition, the use of wastes biomasses does not affect the agricultural activities, and gives economic benefits deriving from the energy saving and the free availability of the residual farming activities materials (McKendry, 2002).

Among renewable energies, hydrogen is considered a promising fuel of strategic interest. It is commonly accepted by scientific and technical community that the global hydrogen economy would materialize in the next future, bringing a secure and sustainable energy system able to achieve the control of the greenhouse gases (GHG) emissions which have accelerated climate change (Suman, 2014). However, hydrogen is not an energy source but an energy carrier. Hydrogen has to be produced through various processes, such as the reforming of hydrocarbons and alcohols, photosynthesis, fermentation and electrolysis.

The biological production of hydrogen is the microbiological conversion of water and organic substrates into hydrogen, through the action of the enzyme hydrogenase (Hasi) or nitrogenase (Nasi) (Chong et al., 2009). Biological H_2 (or biohydrogen) production processes are important mainly because they use renewable energy resources and they are performed at ambient temperature and atmospheric pressure (Das and Veziroglu, 2016). The biological production of H_2 can be carried out in presence of light, using green algae, photosynthetic bacteria and cyanobacteria, or in the absence of light through the process named Dark Fermentation (DF), using heterotrophic bacteria (like as *Clostridium*) (Saratale et al., 2008).

Considering biological processes, the anaerobic fermentation comprises the most attractive pathway because the agroindustry wastes may be used as substrates. In these processes, the use of Gram-positive

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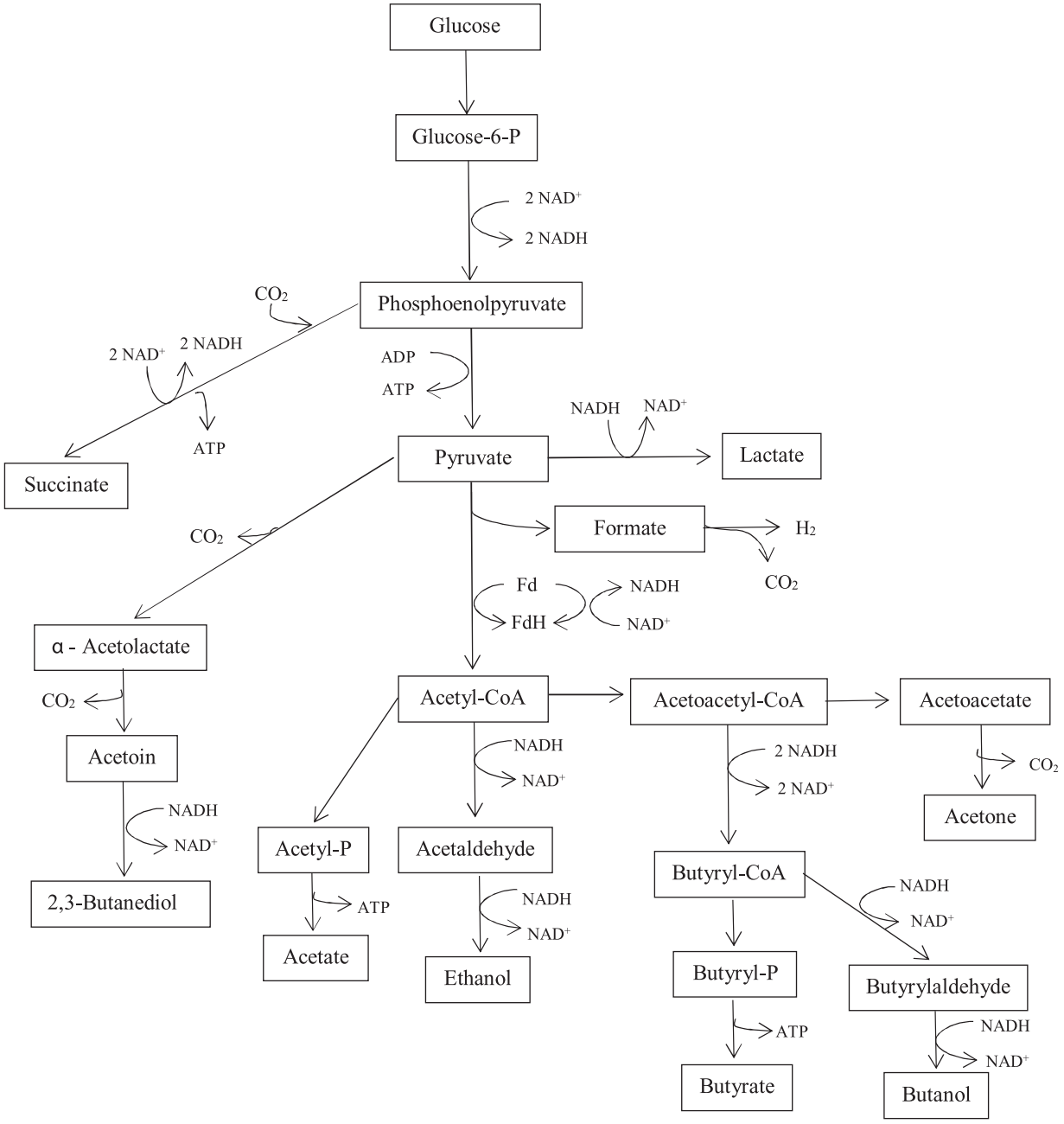

Fig. 1. Metabolic pathways of glucose metabolism in *Clostridium* species.
Source: Author's elaboration based on Wischral et al., 2016; Liu et al., 2012; Sund et al., 2013; Yu et al., 2015.

bacteria of the *Clostridium* genus has been preferred because it has a natural high hydrogen production rate. It is worthy of note that this microrganism can fellow different metabolic pathways, that may lead to the production of some other by-products of interest (i.e. ethanol) together with the hydrogen production, as an example, Fig. 1 shows the metabolic pathway of the *Clostridium* using glucose as substrate.

Different materials can be used as organic matter for hydrogen production, e.g., industrial waste like molasses and cheese whey (Mendes et al., 2012), *Arundo donax* (Ausiello et al., 2015), oat straw (Arreola-Vargas et al., 2015) and others material. One of the promising materials are lignocellulosic biomasses, because of its composition and low cost. In this context, cashew apple bagasse (CAB) could be considered a feasible feedstock for H_2 production.

CAB is a tropical pseudofruit and an abundant lignocellulosic waste in northeast Brazilian agro-industry and it appears to be a promising raw material for several potential applications. The industrial process of the cashew apple generates approximately 20–25% of residual fibre, that is discarded or used as animal feed supplement (Barros et al., 2017; Correia et al., 2013). But, there has been an increasing trend towards more efficient utilization of agro-industrial residues, which involves the production of biofuels, bioproducts and new materials. Some authors have already investigated the use of CAB as source for the production of biosurfactant (Franca et al., 2015; Rocha et al., 2009), enzymes (Rodrigues et al., 2008), ethanol (Barros et al., 2017; Rocha et al., 2014; Rodrigues et al., 2011; Rocha et al., 2011) and xylitol (Albuquerque et al., 2015; Rocha et al., 2014). Among the strategies to make this technological platform viable, the implementation of integrated biorefineries, which includes the production of biofuels, bioproducts and new materials, has been proposed as one of the most viable sustainable alternatives for the use of this raw material.

The aim of the present work is to study the CAB as feedstock for the biohydrogen production using the DF process. Pretreatments and

hydrolysis of the raw lignocellulosic material and the chosen of the proper microrganism are essential in a fermentation process. Therefore, according to pretreatments methods that have been found in recent literature data, such as the acid (Barros et al., 2017; Rodrigues et al., 2016) and the hydrogen peroxide pretreatments (Correia et al., 2013), this work takes into account four different hydrolysates of CAB as substrate for the production of biohydrogen using *Clostridium roseum* as inoculum for the DF.

2. Experimental

2.1. Materials

Raw cashew apple bagasse (*Anacardium occidentale* L) was kindly provided by Jandaia Industry of Juice (Ceará/Brazil). This material was washed three times with water, and then it was dried at 60 °C for 24 h, ground and sieved to 20–80 meshes (0.841–0.177 mm), after that the milled CAB was stored at 30 °C. Other reagents and chemicals used for pre-treatments and DF tests were of analytical grade and commercially available.

2.2. Pretreatment of cashew apple bagasse

CAB was pretreated with alkaline hydrogen peroxide according to the best conditions obtained by Correia et al. (2013). A solid loading of 5% (w/v) was slurred in 4.3% v/v alkaline hydrogen peroxide solution (AHP) at pH 11.5. The pretreatment was conducted in an orbital shaker at 35 °C and 250 rpm for 6 h. Afterwards, the solid and liquid fractions were separated by vacuum filtration. Then, the solid fraction was washed with distilled water and dried at 60 °C for 24 h. This solid fraction, named CAB-AHP, was used as substrate for the subsequent hydrolysis.

2.3. Obtention of hydrolysates

2.3.1. Diluted acid hydrolysis

CAB and CAB-AHP were subjected to acid hydrolysis to obtain the acid hydrolysates. The hydrolysis was conducted at 121 °C for 15 min in autoclave using a solution of 0.6 M H_2SO_4 and 20% w/v CAB. The liquid fraction was collected by vacuum filtration (Albuquerque et al., 2015). The pH of the solution was increased up to 5.5 adding $Ca(OH)_2$ under stirring condition, because this pH value performs the growth of *Clostridium roseum* (Bernal et al., 2013; Romero Aguilar et al., 2013), and it was filtrated and sterilized at 110 °C for 10 min. Then the sample was kept under stream of nitrogen before the inoculation. The hydrolysate obtained from CAB and CAB-AHP was named A and B, respectively, and they were used as a culture medium for the production of hydrogen. The yields of hydrolysis of cellulose and hemicellulose from untreated and treated CAB were calculated as proposed by Rodrigues et al. (2016).

2.3.2. Enzymatic hydrolysis

The enzymatic hydrolysis of CAB and CAB-AHP were performed using commercial enzymes complex of cellulase (Celluclast 1.5 L, 60 FPU/mL, Novozymes, Bagsvaerd, Denmark) and β-glucosidase (Novozymes 188, 360 CBU/mL, Bagsvaerd, Denmark) at enzyme loading per gram of cellulose of 15 FPU and 30 CBU, respectively. These hydrolysis were conducted in 100 mL of phosphate buffer (50 mM, pH 5) at 50 °C and 150 rpm for 72 h using 10% w/v solid (Rocha et al., 2009; Rodrigues et al., 2016; Correia et al., 2015; Ausiello et al., 2017). The enzymatic hydrolysates obtained from CAB was named C while the other obtained from CAB-AHP was named D, and they were used as a culture medium for fermentative hydrogen production. A schematic diagram summarizing the experimental setup is presented in Fig. 3.

The yields of hydrolysis of cellulose and hemicellulose from untreated and treated CAB were calculated as proposed by Rodrigues et al. (2016).

2.4. Dark fermentation tests

2.4.1. Microrganism and culture media

Inoculum for DF tests was the *Clostridium roseum* ATCC 17,797 supplied by Leibniz Institute DSMZ (Germany). Nutrient medium composition for microrganism growth was made by: NH_4Cl (1 g/L), K_2HPO_4 (0.3 g/L), KH_2PO_4 (0.3 g/L), $MgCl_2{\cdot}6\ H_2O$ (0.2 g/L), $CaCl_2{\cdot}2\ H_2O$ (0.1 g/L), NaCl (10 g/L), KCl (0.1 g/L), cysteine (0.5 g/L), CH_3COONa (0.5 g/L), yeast extract (2 g/L), tripeptone (2 g/L), NaOH solution at pH 11 (10 mL) and resazurin (0.1 g/L). The pH of the medium was corrected to 5.5 by the addition of a 10 M KOH solution and sterilized. Subsequently *C. roseum* was reactivated for 24 h of incubation at 37 °C ± 1 °C and 150 rpm in anaerobic conditions through nitrogen stream for 20 min, using inoculum/medium ratio of 10% v/v.

Each fermentation medium were prepared with working volume of 100 mL made by 80 mL of hydrolysate (A, B, C or D), 20 mL of inoculum and 0.025 wt% of resazurin. Then the vials were clamped with butyl rubber stopper pierced equipped with an aperture ring. Anaerobic conditions were obtained keeping the vial under stream of nitrogen for 20 min.

2.4.2. Experimental apparatus

DF tests were carried out at mesophilic condition (38 °C) using crimped Pyrex glass vials of 125 mL, Fig. 2. Samplings of liquid and gaseous phases from vials were performed according to standard anaerobic procedures (Strobel, 2009).

A volumetric method was used for biogas volume valuation by connecting with a capillary tube the headspace of the fermentation vial to an inverted vial filled with water (Toscano et al., 2014). The biogas volume was measured by weighing the water displaced through a second needle in the septum of collection vial. The collection vial was periodically replaced and the collected biogas analyzed by gas chromatography analysis, for the evaluation of the biohydrogen produced and other gas-phase products (mostly CO_2 and CH_4).

2.5. Analytical techniques

2.5.1. Characterization of raw material

Elemental composition of untreated and pretreated lignocellulosic biomass were examined with a PerkinElmer PE2400 Elemental CHNS/O analyser on samples of 1.0 g weight. Proximate analysis (moisture, volatile matter, fixed carbon and ash) were carried out with a LECO TGA701 according to ASTM 142–147 methods. Chemical composition in terms of cellulose, hemicellulose and lignin was determined

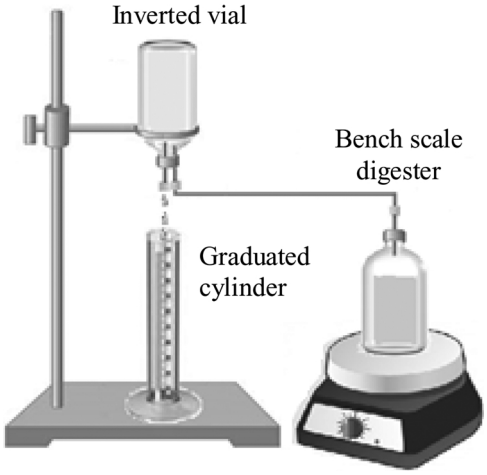

Fig. 2. Experimental apparatus used for hydrogen production by Dark Fermentation using *Clostridium roseum* ATCC 17797 and different hydrolysates from CAB.

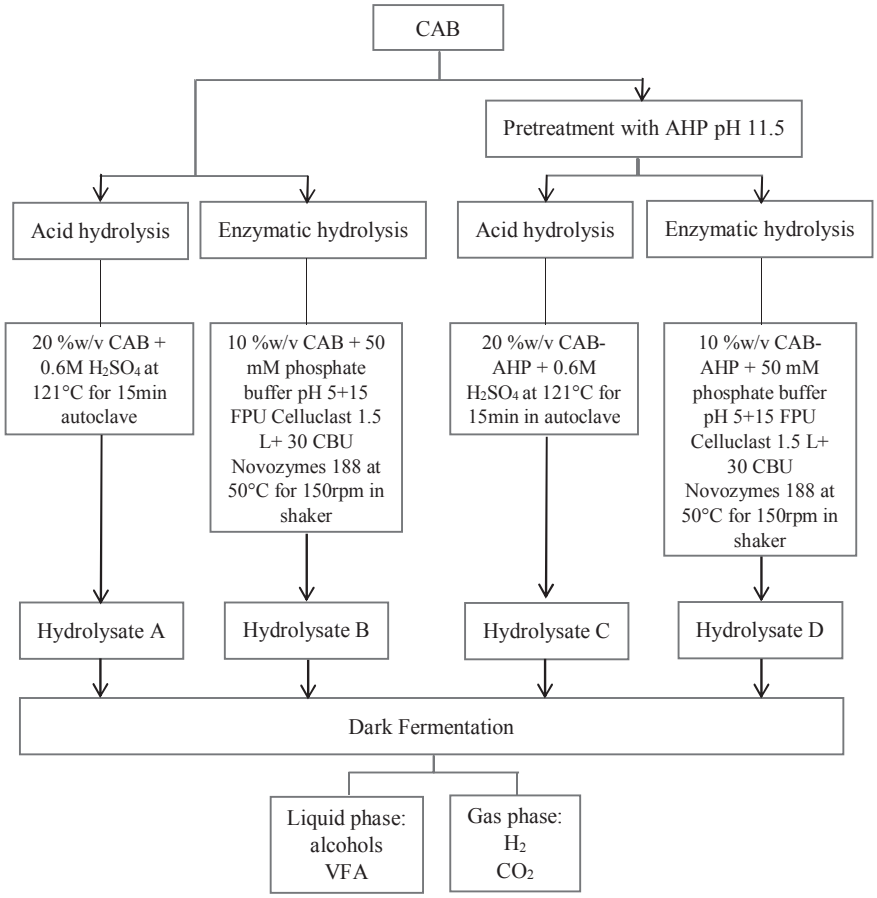

Fig. 3. Schematic diagram of the experimental stages.

Table 1
Composition of samples: CAB, cashew apple bagasse, and CAB-AHP, cashew apple bagasse pretreated with alkaline hydrogen peroxide, as received basis wt %.

Components	Composition (%w/w)	
	CAB	CAB-AHP
Moisture	8.40 ± 0.01	8.50 ± 0.01
Fixed carbon	24.88 ± 0.60	11.91 ± 0.08
Volatile	65.65 ± 0.65	74.53 ± 0.03
Ash	1.08 ± 0.06	5.07 ± 0.10
C	50.49 ± 0.49	44.12 ± 0.15
H	5.73 ± 0.03	5.86 ± 0.09
N	1.41	–
Cellulose	20.56 ± 2.19	44.16 ± 0.29
Hemicellulose	10.17 ± 0.89	18.27 ± 0.07
Lignin	35.26 ± 0.90	2.91 ± 0.07

according to the method proposed by Gouveia et al. (2009) and NREL Laboratory Analytical Procedures – LAP (Sluiter et al., 2008).

2.5.2. Liquid and gaseous phases analysis

Samples of the liquid phase were collected at 0, 24 and 48 h during the DF tests. The microbial biomass (MB) growth, carbohydrates, volatile acids and ethanol concentrations were monitored for each test.

MB growth was evaluated by measuring the optical absorbance at 600 nm (OD600) from a 1:10 diluted sample. Samples were centrifuged at 3000 rpm for 5 min and filtrated with 0.2 µm cut-off filters.

The activities of cellulase and β-glucosidase enzymes were determined according to a standard procedure (Ghose, 1987).

Carbohydrates were determined by HPLC using the procedure described by Barros et al. (2017).

Concentration of volatile acids (acetic acid, butyric acid, etc.) and alcohols (ethanol, butanol, etc.) were measured by gas chromatography, using a Shimadzu instrument GC-17A equipped with FID detector and a capillary column containing a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 µm film thickness, from SGE). These compounds were investigated according to the metabolic pathway of *C. Roseum* (Fig. 1). 1 µL samples were injected with a split-ratio of 1:10. Helium was fed as carrier gas with a flow rate of 6.5 mL/min. Injector and detector temperatures were set to 320 °C and 250 °C, respectively. Initial column temperature were set to 30 °C, kept for 3 min and followed by a ramp of 10 °C/min up to 140 °C.

Biogas (H_2, CO_2, CH_4, and others) composition was determined by GC analysis, using a HP 5890 series II equipped with a TCD detector and a double packed molecular sieves-porapack column. The injection volume was 0.2 mL and the temperature of column, injector and detector were kept at 60 °C. The gas carrier was Helium at pressure of 300 kPa.

3. Results and discussion

3.1. Compositional analysis of raw material and hydrolysates

Table 1 provides the results of the compositional analysis of CAB and CAB-AHP. CAB used in this investigation contained 20.56 ± 2.19%w/w cellulose, 10.17 ± 0.89%w/w hemicellulose and 35.26 ± 0.90%w/w lignin. Composition of CAB-AHP was 44.16 ± 0.29%w/w cellulose, 18.27 ± 0.07%w/w hemicellulose and 2.91 ± 0.07%w/w lignin. These values were in agreement with previous reported by literature (Rocha et al., 2011; Correia et al., 2013).

The lignocellulosic composition of the CAB and CAB-AHP on dry

Table 2
Sugars composition of the hydrolysates: (A) hydrolysate obtained from acid treatment of CAB, (B) hydrolysate obtained from acid treatment of CAB-AHP, (C) hydrolysate obtained from enzymatic treatment of CAB and (D) hydrolysate obtained from enzymatic treatment of CAB-AHP, and hydrolysis yield of cellulose and hemicellulose.

Hydrolysate	Sugar composition (g/L)				Yield of Hydrolysis (%)	
	Glucose	Xylose	Arabinose	Cellobiose	Cellulose	Hemicellulose
A	26.18 ± 0.37	18.90 ± 0.10	12.87 ± 0.60	7.56 ± 0.04	35.02 ± 0.43	13.09 ± 0.61
B	0.52 ± 0.01	0.27 ± 0.02	0.30 ± 0.02	1.19 ± 0.40	1.80 ± 0.04	0.30 ± 0.02
C	10.22 ± 0.25	6.08 ± 0.20	0.79 ± 0.03	0.34 ± 0.01	10.84 ± 0.27	0.85 ± 0.04
D	34.08 ± 0.25	10.23 ± 0.33	1.89 ± 0.25	3.23 ± 0.48	37.83 ± 0.26	1.97 ± 0.06

basis resulted in 55.82%w/w and 62.43%w/w respectively, it was made up by polysaccharides, lignin and other components.

The sequential procedures used were assessed to measure their capability to convert hemicellulose and cellulose from CAB to sugars. The acid hydrolysis affected predominantly the hemicellulose components while the alkaline pretreatment led to an effective removal of hemicellulose and lignin (Chen et al., 2008) due to the oxidizing radicals formed from H_2O_2 (Selig et al., 2009).

Table 2 reports the sugar composition of the hydrolysates and the yields of hydrolysis based on the initial composition of material (cellulose and hemicellulose). The glucose was the main sugar obtained in all hydrolysates, except for hydrolysate B, followed by xylose, arabinose, and cellobiose for hydrolysates A and C. CAB-AHP hydrolysates (B and D) presented more cellobiose than arabinose. These sugars were molecules that compose the structures of cellulose and hemicellulose. The highest concentrations and yields were achieved by acid hydrolysis from CAB (reported in Table 2 for A), and the main carbohydrates were pentoses (xylose and arabinose). The presence of these sugars can favour the fermentation process for the H_2 production: according to Arreola-Vargas et al. (2015), xylose produces similar amounts of H_2 as glucose, while arabinose produces about half of the H_2 produced by glucose and xylose.

The presence of some microbial inhibitors, i.e. furfural and hydroxymethylfurfural, were detected in hydrolysate A and C. However, the presence of these microbial inhibitors in the enzymatic hydrolysates does not affect the H_2 yield, as explained in Arreola-Vargas et al. (2015).

3.2. Dark fermentation tests

Results of DF tests carried out on the different hydrolysate obtained from CAB biomass were reported in the Fig. 4 and Tables 3 and 4.

Table 3 reports the volumes of biogas produced per volume of culture, the biogas compositions and H_2 yields obtained from the four hydrolysates (A–D), referring to cumulative volumes. Biogas production started from the first day but declined rapidly with no biogas produced at the end of 48 h. From the third day onwards there was no biogas production. This is in agreement with several literature data (Ghimire et al., 2015; Hosseini and Wahid, 2016; Ausiello et al., 2017), that showed a production of biohydrogen limited to the first 24–48 h of fermentation.

Biogas was made mainly by H_2 and CO_2 (dry condition), but the composition varied hugely with the samples, and significant amounts of other gases were not detect in the batch experiments. The highest biogas production were observed with A and C samples, that produced respectively about 420 and 240 $mL_{biogas}/L_{culture}$ and with a hydrogen concentration of 72 ± 2%vol and 43 ± 1%vol. Table 4 shows a comparison of dark fermentative H_2 production from different lignocellulosic substrates. In studies conducted by Sagnak et al. (2011) and Ausiello et al. (2017), the authors obtained 387 mL H_2 and 190.1 mL H_2, respectively, and they verified that the volume of the gas depended of operational conditions. The corresponding H_2 yields obtained from A, and C hydrolysates were 1.89 and 0.79 mL of H_2 per gram of lignocellulosic feedstock at 24 h of fermentation. As a general trend, it can be observed that using the hydrolysates B and D, obtained from CAB pretreated with AHP, resulted in a lower hydrogen yield, 0.08 and 0.33 mL_{H2}/g, and favoured the CO_2 production (90%vol in B and 74%vol in D) due to the presence of H_2O_2. Literature data reported the bactericidal activity of hydrogen peroxide, and the inhibition of *Clostridium* sp., due to the strong oxidizing effect of metabolite formed by the reaction between H_2O_2 and superoxides. Therefore, hydrogen peroxide inhibits bacterial growth, respiration, and viability (Bahl and Durre, 2001).

Table 3 reports also, the volumetric hydrogen production rate (VHPR) and hydrogen molar yield (HMY) at 24 h of dark fermentation. Thus resulting that the maximum hydrogen production rate calculated was 15 mmol $_{H2}/L_{hydrolysate}$ in 24 h for hydrolysate A. We considered these values in order to compare results of this work with CAB to other substrates present in literature data and reported in Table 4. A wide range of values of substrate conversion efficiency was reported by different authors. Cui et al. (2009) obtained yield values of hydrogen in the range of 0.34–52.33 mL H_2/g substrate, varying in function of the pH. At pH 4.0 and 5.0 the H_2 yields obtained were similar to that presented in this study (Table 4). These values are close to those found in this study, except for the substrates B and D. As with Wu et al. (2010) found maximum rate of hydrogen production of 10.3 mL/L h using *R. palustris* W004 with propionate and ethanol as substrate. On the other part, biohydrogen production by *Rhodopseudomonas* sp. from acetate and butyrate presented VHPR about 26.5 mL/L h, this value is higher than those obtained in the present study. Ozkan et al. (2011) investigated beet pulp through DF and obtained biohydrogen and carbon dioxide percentage in the total gas of 43.4–51.6 % vol and 48.4–56.6 % vol, respectively (see Table 4).

Analyses of liquid phase were used to monitoring the fermentation process and the metabolic pathway chosen by the microrganism. Trends of the biomass growth, reducing sugar concentration and pH were presented in Fig. 4.

The starting hydrolysates presented a concentration of glucose about 26.18 g/L and 10.22 g/L for hydrolysate obtained from CAB, A and C, whereas for hydrolysate obtained from CAB-AHP, B and D, was 0.52 g/L and 34.08 g/L respectively. This different in the initial glucose concentration is due to treatment conducted in the CAB. Glucose concentration slightly decreased in 48 h in spite of the biomass growth. It can be explained considering that the hydrolysed contains different monomers and reducing sugars and microrganism used at first the simplest sugars for a fast fermentation process and then reduced slower the complex sugars for the biomass growth. This also justified the not appreciable production of biogas and the slower growth of the biomass from 24 h to 48 h. Moreover, it is known that DF takes place optimally with a pH in the range 5.5–6.5, thus the acidification of the solution observed during the test, with a pH value that decreased from about 6 to approximately 4 in 48 h, influenced the fermentation process. In addition, some microrganisms of genus *Clostridium* show a biphasic behaviour in the metabolism, acidogenic and solventogenic phases. This behaviour is manifested as a change in the carbon uptake. In acidogenic conditions, the microrganisms grow exponentially, but

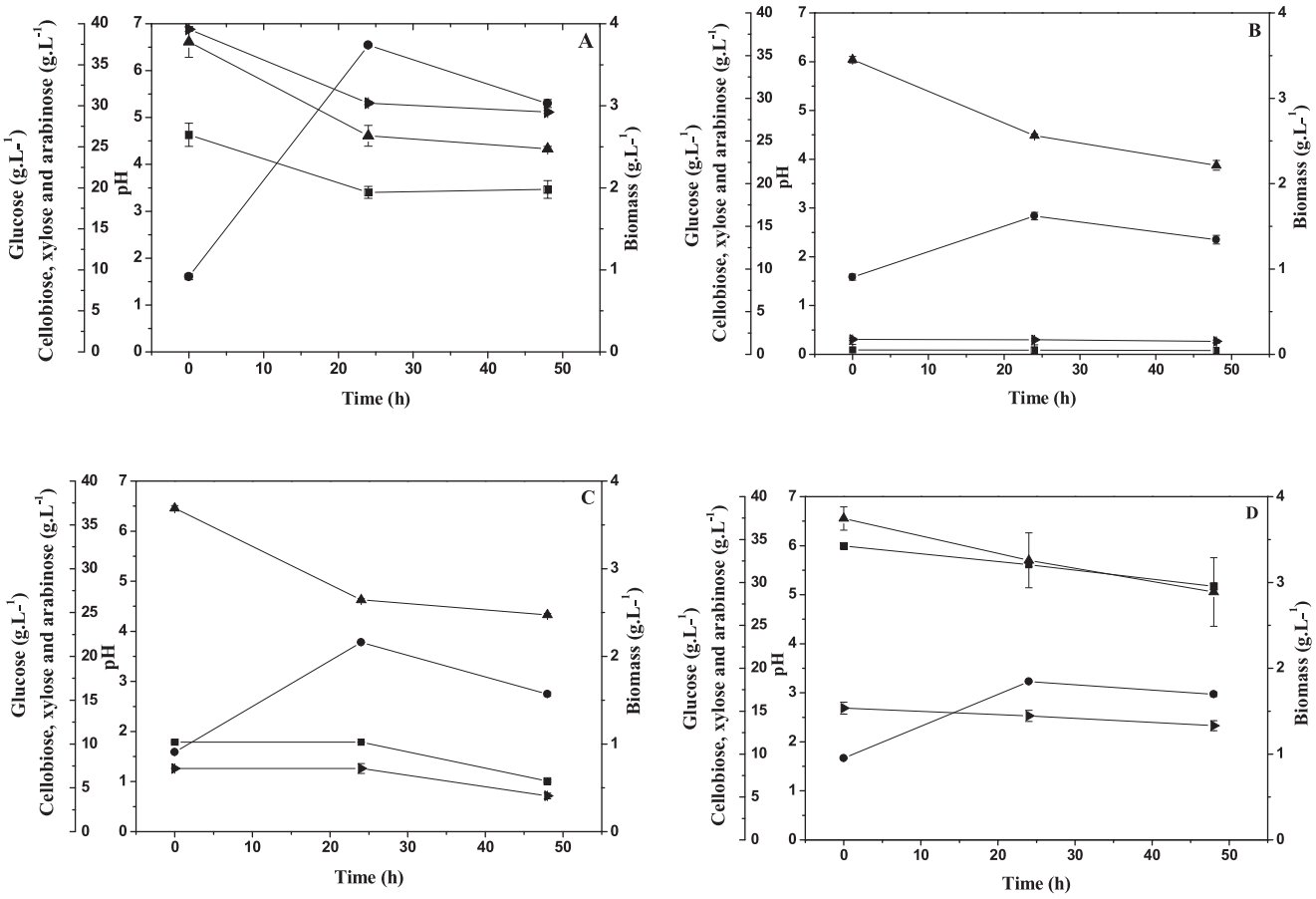

Fig. 4. Concentration of glucose (■), cellobiose, xylose and arabinose (►) biomass (●) and pH (▲), as function of time, of liquid phase produced during the Dark Fermentation by *C. roseum* at 38 °C using CAB: (A) hydrolysate obtained from acid treatment of CAB, (B) hydrolysate obtained from acid treatment of CAB-AHP, (C) hydrolysate obtained from enzymatic treatment of CAB and (D) hydrolysate obtained from enzymatic treatment of CAB-AHP.

under solventogenic conditions, they change to stationary phase (Milne et al., 2011; Dash et al., 2014, 2016).

The DF process transforms sugars, starches and other carbohydrates or fermentable organic substrates into biogas containing mainly H_2 and CO_2 together with organic acids, alcohols and other by-products (Liu and Whitman, 2008). Table 5 shows the concentration of volatile organic acids and alcohols produced during the DF process. These metabolites formed during the bioprocess were analysed because they can affect the hydrogen production.

Results evidenced that the acetic acid and butyric acid were the main metabolite produced during fermentation of the hydrolysates. The concentration of these organic acids had a maximum value at 24 h (data not shown), then the production decreased to values of 220, 1110, 152 and 1233 mg/L for the acetic acid and 119, 375, 400 and 1194 mg/L for butyric acid respectively for A, B, C and D hidrolysates (Table 5). However, it resulted that the APH treatment favoured the production of the acetic acid since the concentrations in B and D samples were about an order of magnitude higher than A and C, besides, the butyric acid contents were found about three times higher in the samples treated with the enzymatic hydrolysis, C and D, than the same samples treated with the acid hydrolysis, respectively A and B. The presence of these compounds led to the acidification of the solution (see Fig. 4) thus reducing the efficiency of DF process.

Analysis on the liquid phase detected ethanol and butanol, the presence of these compounds was also expected according to the metabolic pathway of *C. roseum* (Fig. 1). As mentioned before, the microrganism presented a biphasic behaviour of metabolism. During the acidogenic phase some acids are produced, such as acetate and butyrate, whereas into the solventogenic phase the acids are reassimilated back into the cell. The acid produced changes the pH of the media and forces the microrganism to change into the solventogenic phase producing solvents such as ethanol and butanol (Dash et al., 2016).

Table 3
Gas phase produced by *C. roseum* during the Dark Fermentation at 38 °C and 24 h of process using CAB: (A) hydrolysate obtained from acid treatment of CAB, (B) hydrolysate obtained from acid treatment of CAB-AHP, (C) hydrolysate obtained from enzymatic treatment of CAB and (D) hydrolysate obtained from enzymatic treatment of CAB-AHP.

Hydrolysate	$V_{biogas}/V_{culture}$ (L/L)	Composition (%vol)		VHPR (mL_{H2}/L h)	Yield ($mL_{H2}/g_{lignocellulosic\ feedstock}$)	HMY (mmol $_{H2}/L_{hydrolysate}$)
		H_2	CO_2			
A	0.42	72 ± 2	28 ± 1	12.57	1.89	15
B	0.12	10 ± 1	90 ± 2	0.50	0.08	0.59
C	0.24	43 ± 1	57 ± 1	4.23	0.79	4.99
D	0.08	26 ± 2	74 ± 1	0.89	0.33	1.05

Table 4
Comparison of dark fermentative H_2 yields obtained from different lignocellulosic substrates, pretreatments, microrganisms and conditions.

Substrate	Pretreatment/Obtaining of hydrolysate	Microrganism	Conditions	Hydrogen yield	Reference
Wheat straw	Enzymatic hydrolysis	*S. cerevisiae*	70 °C	178.0 mL H_2/g sugars	Kaparaju et al. (2009)
Beer lees	–	Mixed cultures	35 °C, 120 rpm,	0.34–52.33 mL H_2/g $_{substrate}$	Cui et al. (2009)
Corn stover	Enzymatic hydrolysis	*T. thermosaccharolyticum*	60 °C	108.5 mmol/L $_{hydrolysate}$	Ren et al. (2010)
Beet-pulp	Alkaline pretreatment	Mixed anaerobic culture	35 °C, 175 rpm	115.6 mL H_2/g COD	Ozkan et al. (2011)
Waste ground wheat	Acid hydrolysis	Anaerobic sludge	37 °C	1.52 mol H_2/mol glucose	Sagnak et al. (2011)
Food waste	Enzymatic hydrolysis	*A. awamori* and *A. oryzae*	37 °C, 300 rpm	39.14 mL H_2/g substrate	Han et al. (2015)
Wheat starch	Acid hydrolysis	*C. acetobutylicum*	30 °C, 100 rpm	2.37 mol H_2/ mol glucose	Hassan and Morsy (2015)
Corn stalk	–	*Clostridium sartagoforme* FZ11	35 °C, 120 rpm	87.2 mL H_2/g substrate	Zhang et al. (2015)
Arundo donax	Enzymatic hydrolysis	Mixed Microflora from sewage sludge	38 °C, 150 rpm	3.06 mol H_2/mol glucose	Ausiello et al. (2017)
Waste peach pulp	Partial hydrolysis	Anaerobic sludge	37 °C, 150 rpm	123.27 mL H_2/g TOC	Argun and Dao (2017)
CAB	Acid and enzymatic hydrolysis	*C. roseum*	38 °C, 150 rpm	0.08–1.89 mL_{H2}/g $_{substrate}$	This study

Table 5
Concentration of volatile acids and alcohols produced at 38 °C and 24 h by *C. roseum* during the Dark Fermentation process using CAB: (A) hydrolysate obtained from acid treatment of CAB, (B) hydrolysate obtained from acid treatment of CAB-AHP, (C) hydrolysate obtained from enzymatic treatment of CAB and (D) hydrolysate obtained from enzymatic treatment of CAB-AHP.

Hydrolysate	Composition (mg/L)			
	Butanol	Ethanol	Acetic Acid	Butyric Acid
A	35 ± 1	31 ± 1	220 ± 3	119 ± 3
B	–	40 ± 1	1110 ± 2	375 ± 3
C	52 ± 1	90 ± 1	152 ± 1	400 ± 3
D	–	299 ± 1	1233 ± 2	1194 ± 2

Ethanol reached concentration values of 31 mg/L, 40 mg/L, 90 mg/L and 299 mg/L respectively for A, B, C and D in 24 h and then remained approximately constant until the end of the test (data not shown). Butanol showed a maximum production in 24 h for A and C (35 mg/L and 52 mg/L) then the concentration decreased up to the value of 5 mg/L for A and C at 48 h of fermentation. Butanol may take part to the metabolic cycle of the microrganism for the survival, even though this mechanism is not well understand and explained in literature (Dash et al., 2016).

Other compounds were not found with appreciable amount during the tests, it can be hypothesized that the microrganism used the other organic acids that could have been produced as an energy source for the biomass growth.

The CAB is an alternative and inexpensive lignocellulosic material for the production of various biomolecules. However, CAB is highly recalcitrant to both microbial and enzymatic biotransformation, limiting its use and making its conversion into value-added products not economically feasible. A noticeable H_2 production was obtained using the hydrolysate A, that can be obtained easily and with a lower cost.

CAB has already been evaluated as an alternative raw material for the production of various products, such as ethanol and xylitol. Then, our research investigated for the first time the feasibility of CAB as energetic feedstock for H_2 production aiming in the future an integrated process applying the concept of biorefinery. Since it was a preliminary study, we were interested mainly about the H_2 yields using different reactional media (hydrolysates) obtained from the CAB and common experimental conditions. However, it is necessary to carry out a study to evaluate the optimal conditions for the hydrogen production using CAB.

4. Conclusion

This study investigated the effect of pretreatment and hydrolysis methods on biohydrogen production from CAB through dark fermentation process. The pretreatment with AHP affected microbial growth and this is interesting since delignification only slightly improved the overall sugar yield, suggesting that this step may be excluded from the sequential treatment in order to improve the hydrogen production.

Results obtained in this work showed that the dark fermentation of hydrolysed CAB with selected hydrogen producer bacteria (*C. roseum*) may be a promising process to obtain biogas with high H_2 content using hydrolysate A, which derived from the acid hydrolysis of the untreated CAB. Indeed, the produced biogas contained hydrogen at 72% and carbon monoxide at 28%, without any appreciable presences of others contaminants.

Lower H_2 yields were observed in B and D hydrolysates obtained from pretreated raw material indicating that H_2 production was not favourable for these feedstock.

These results represent a very promising starting point since the quality of the biogas obtained could be satisfactory in view of an energetic applications of biohydrogen (i.e. for fuell cell technology) and considering that it was produced in 24 h, a very short time especially if it is compared to other biogas producing process. Nevertheless the process of DF should be improved by controlling several parameters, such as the pH, since the production of organic acids caused the acidification of the liquid phase and reduced the efficiency of the process.

This is the first study that provides experimental evidence of using CAB as feedstock for the H_2 production.

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