



## Integral process assessment of sugarcane agricultural crop residues conversion to ethanol

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### ABSTRACT

This work focuses a whole process assessment on post-harvesting sugarcane residues for 2G ethanol production by different saccharification-fermentation conditions at high solids loading, performed after steam explosion, alkaline and acidic pretreatments. Carbohydrate recoveries and enzymatic digestibility results showed that alkali and steam explosion pretreatments were effective for the biomass assayed. Due to a significant improvement (60%) of the glucose released by combining hemicellulases and cellulases only after the NaOH pretreatment, the most favorable process settled comprised an alkali-based pretreatment followed by a pre-saccharification and simultaneous saccharification and fermentation (PSSF). The produced ethanol reached 4.8% (w/w) as a result of an 80% conversion of the glucose from the pretreated biomass. Finally, an ethanol concentration of 3.2% (w/w) was obtained by means of a steam explosion followed by PSSF, representing a suitable start point to further develop a low environmental impact alternative for ethanol production.

### 1. Introduction

Towards a progressive substitution of fossil fuels for renewable energies, the use of non-food vegetable biomass for second generation (2G) ethanol production even now lacks enough economic feasibility. Accordingly, agricultural and forest lignocellulosic residues are targeted for extensive research and viability studies, as they are considered a large source of carbohydrate for chemical fuels (Sánchez and Cardona, 2008). Among them, sugarcane post-harvesting residues comprising part of the leaves, tops and trash are described as sugarcane straw or agricultural crop residues (ACR), excluding the sugarcane bagasse. This residue is an abundant lignocellulosic source in the North West of Argentina, where the sugarcane (*Saccharum* spp.) based industry is a primary driver of the economy. This activity reached 18,436,082 t of sugarcane cultivated in 2016 (Sugar harvest in Argentina, 2016), generating large amounts of residues that are partially left in the field to sustain soil quality, prevent erosion and to improve water retention. But most of them could provide an inexpensive and readily available source of lignocellulosic biomass (Sindhu et al., 2016).

For a suitable enzymatic mediated releasement of fermentable sugars from lignocellulose, the pretreatment represents a crucial step. The

use of different pretreatment methods has a remarkable impact on the global ethanol production process as they substantially affect enzymatic hydrolysis rates, enzyme loading, fermentation variables and even downstream procedures. Therefore, their conditions need to be carefully considered for each type of biomass (Tomás-Pejó et al., 2011; Bermúdez Alcántara et al., 2016). For sugarcane straw biomass, the pretreatments evaluated so far included milling (da Silva et al., 2010), diluted acid (Mesa et al., 2017), alkali (Carvalho et al., 2015), microwave (Moretti et al., 2016), un-catalyzed steam explosion (Oliveira et al., 2013), extrusion (Kuster Moro et al., 2017), and sequential pretreatment with glycerol assisted ferric chloride (Raghavi et al., 2016) and combination of wet disk milling and ozonolysis pretreatments (Barros et al., 2013). Between them, steam explosion is one of the most successful and widely used methods for fractionating and enhancing the enzymatic digestibility of lignocellulose (Duque et al., 2016). Also, the usefulness of diluted acid has been appointed because it allows a high recovery of pentoses (Alvira et al., 2010), whereas alkali-based pretreatments are advantageous given that they are carried out in relatively mild conditions producing high glucose yields, low inhibitor formation plus low capital costs (Kim et al., 2016).

Once the recalcitrant structure of lignocellulose is opened, 2G ethanol production can be accomplished by simultaneous

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saccharification and fermentation (SSF) or pre-saccharification and simultaneous saccharification and fermentation (PSSF) configurations. The SSF approach allows the sugar consumption by the yeast as it is produced by enzymatic hydrolysis, minimizing the glucose accumulation and the inhibition by cellobiose (Moreno et al., 2017). Alternatively, the utilization of a PSSF scheme involves the substrate incubation with hydrolytic enzymes during a relatively short period of time (8–24 h); then, the SSF proceeds when the microorganism is inoculated. This procedure permits to overcome enzymatic hydrolysis constraints due to the different temperature optima between the enzymes (50–55 °C) and most industrial yeasts (30 °C). Moreover, PSSF has shown to be suitable for elevated solids loads since it reduces the initial viscosity of the system thus facilitating the subsequent fermentation step (Jørgensen et al., 2007).

On post-harvesting residues from sugarcane, most of the available reports have separately focused on the individual stages of the processes, disregarding their integrated effect on the final product. Therefore, the aim of this study was to perform an overall assessment of 2G ethanol production from sugarcane agricultural crop residues (ACR). Our work encompassed the evaluation of different pretreatments on ACR by analyzing both, the chemical composition and the enzymatic digestibility of the solid fractions obtained. Then, saccharification conditions were adjusted by combining last generation cellulases and hemicellulases, and 2G ethanol production was carried out at high solids loading (20%, w/v) according to two strategies, SSF and PSSF (Fig. 1).

## 2. Materials and methods

### 2.1. Raw material and pretreatments

The agricultural crop residue (ACR) samples from sugarcane were kindly supplied by EEAOC (Estación Experimental Agroindustrial Obispo Colombres). The material was cut at particle size of 10–12 cm, water-washed, air-dried at 40 °C until moisture content near 10% and stored in dry place till used. For the biomass composition analysis, a portion of the ACR was milled at 1–2 mm particle size.

Un-catalyzed steam explosion of ACR was carried out at 204 °C and 20 min in a batch unit equipped with a 2 L reactor. The working

conditions were selected according to our previous optimization assays by means of response surface methodology, aimed to maximize the overall glucose yield from this substrate. Briefly: a water impregnation was carried out by soaking 125 g (dry matter) of ACR in 1.5 L of water overnight. The liquid in excess was then removed by filtration and the resulting moisture content of the impregnate raw material was ~60%. The pressure reactor was preheated at the set pretreatment temperature with saturated steam, thus less than 60 s were needed for the material to reach the working temperature. The exploded material (slurry) was recovered into a cyclone connected to the outlet of the reactor and cooled and filtered to recover both, liquid and solid fractions. Liquid fractions were analyzed for sugar content and water-insoluble solid (WIS) fractions were washed with deionized water until pH 7 and stored at 4 °C till further processing.

Acid and alkali pretreatments were carried out in 2 L bottles with 10% (w/v) of raw material as follows: aliquots of ACR involving 100 g (dry weight basis, dwb) were treated with 2% (w/v) of NaOH or H<sub>2</sub>SO<sub>4</sub> in autoclave at 121 °C during 60 min. The slurry was filtered and the liquid and WIS fractions were processed as is described above. These working conditions were selected based on bibliographic data (Li et al., 2014).

### 2.2. Characterization of the pretreatments

Following the pretreatments, the washed water insoluble fractions (WIS) were dried at 45 °C and milled at 1 mm particle size for composition analysis. The moisture content and chemical composition were analyzed according to the methods described below (Section 2.5). Solid recovery (SR) values were estimated as the dry weight of WIS remaining after pretreatment referred to 100 g of raw material (dwb). Cellulose, hemicellulose and lignin recovery on the WIS was calculated as follows:

$$\begin{aligned} \text{Component recovery (\%)} &= (\text{g compound in 100 g of WIS}) \\ &\times \text{SR} / (\text{g compound in 100 g of raw material}) \\ &\times 100 \end{aligned} \quad (1)$$

The sugars content (xylose, glucose, arabinose, mannose and galactose) of the liquid fractions was determined by HPLC before and after

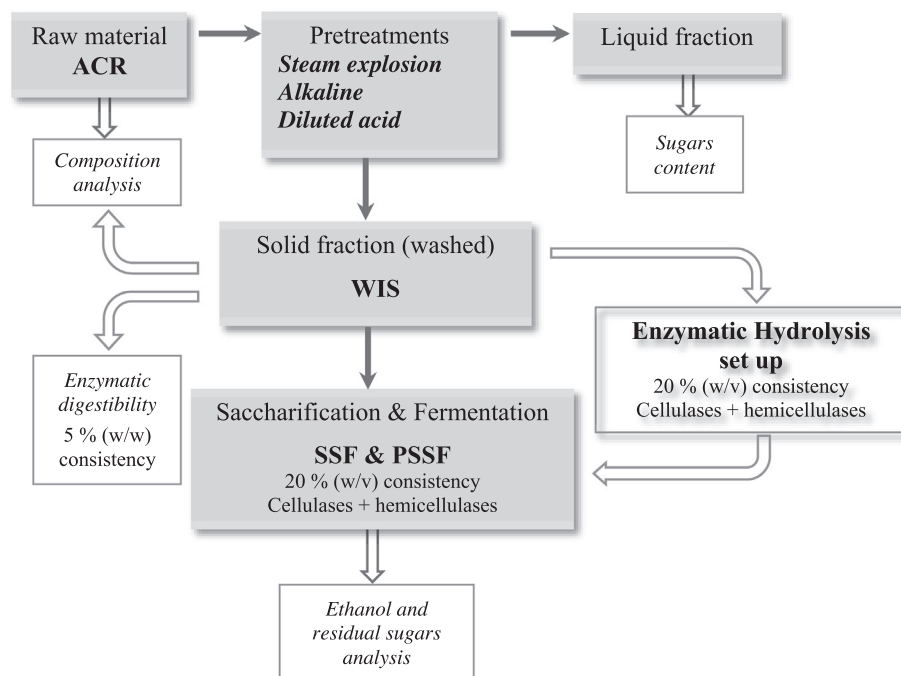


Fig. 1. Schematic flowsheet of the 2G ethanol production processes assayed and the complementary analyses performed.

an acid hydrolysis performed with sulphuric acid (3%, w/w) at 121 °C during 30 min. Sugars recovery into the liquid fractions was estimated according to:

$$\text{Sugar recovery in liquid (\%)} = \frac{(\text{g sugar in liquid fraction})}{(\text{g sugar in raw material})} \times 100 \quad (2)$$

In addition, the digestibility of the washed WIS fractions was assessed by standard enzymatic hydrolysis assays at 5% (w/w) of solids content, using a mixture of cellulases (Celluclast 1.5, Sigma-Aldrich) and  $\beta$ -glucosidase (Novo 188, Sigma-Aldrich), according to [Manzanares et al. \(2011\)](#).

### 2.3. Enzyme hydrolysis experiments

To determine a suitable combination of enzymes for an efficient release of sugars from the WIS to be used in SSF and PSSF assays, four saccharification experiments were carried out. These tests combined cellulases and hemicellulases, Cellic CTec2 (C) and Cellic HTec2 (H), which were kindly provided by Novozymes. The following proportions were tested: (i) 100% C (0.18 g/g glucan); (ii) 75% C (0.13 g/g glucan) plus 25% H (0.05 g/g glucan); (iii) 25% C (0.05 g/g glucan) plus 75% H (0.13 g/g glucan); and (iv) 100% H (0.18 g/g glucan). Enzymatic hydrolyses were performed at 20% (w/v) of washed pretreated solids (WIS) in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C during 72 h. The enzymes were then inactivated in a boiling water-bath for 5 min and the supernatants recovered after centrifugation (10,000g) were used to determine released sugars by HPLC. Blanks of the enzymatic mixtures were simultaneously processed and all experiments were run in duplicate. The enzymatic hydrolysis yield (EHY) was estimated according to the equation (3), where the potential glucose/xilose in the WIS was considered as total content glucose/xilose in pretreated solid.

$$\text{EHY (\%)} = \frac{[\text{g glucose (or xylose) released during enzymatic hydrolysis}]}{[\text{g of potential glucose (or xylose) in WIS}]} \times 100 \quad (3)$$

### 2.4. SSF and PSSF assays

*Saccharomyces cerevisiae* Ethanol Red® (Fermentis, France) was utilized for the fermentation assays. For inocula preparation, yeasts were grown at 30 °C and 150 rpm for 16 h in the medium containing, in g/L: yeast extract, 5; NH<sub>4</sub>Cl, 2; KH<sub>2</sub>PO<sub>4</sub>, 1; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.3 and glucose, 30.

SSF and PSSF assays were carried out at high solid loadings (20% w/v), in a final volume of 30 mL using 100 mL Erlenmeyer flasks. The medium was as described for inocula, replacing glucose with the pretreated substrate (WIS fraction). The SSF experiments were initiated with the yeast inoculation (1 g/L) and the selected enzyme mixtures (Section 2.3), in a rotary shaker at 150 rpm at 35 °C during 72 h. PSSF experiments involved a pre-hydrolysis step of the WIS during 24 h using the selected enzyme mixture at 50 °C and 150 rpm, and followed by SSF initiated by the yeast inoculation (1 g/L), without the addition of enzymes. Samples were taken each 24 h and ethanol concentration and residual sugars analyzed. All assays were performed in duplicate. The ethanol yield (%) was calculated as following:

$$\text{Ethanol yield (\%)} = \frac{(\text{g of ethanol produced})}{(\text{g of potential glucose in WIS})} \times 100 \quad (4)$$

The ethanol efficiency was estimated as the percentage of the theoretical yield from 0.51 g ethanol/g glucose.

### 2.5. Analytical methods

The composition of the raw material and pretreated biomass were determined according to [Sluiter et al. \(2010\)](#). The extractives were

determined as the solubilized material after an extraction with ethanol and water in a Dionex Accelerate Solvent Extractor System (ASE 200). Ashes were determined from material calcination at 575 °C for 8 h. Sugars were determined by high performance liquid chromatography (HPLC) using a Waters 2695 liquid chromatograph equipped with refractive index detector and a Carbosep CHO-782 (Transgenomic) carbohydrate analysis column, operating at 70 °C. Deionized water was utilized as mobile-phase under a flow rate of 0.6 mL/min. The ethanol produced was analyzed by gas chromatography using an Agilent 7693B Series injector equipped with flame ionization detector, and a column Carbowax 20 M operating at 85 °C as described earlier ([Tomás-Pejó et al., 2009](#)). The products furfural, 5-hydroxy-methyl-furfural (HMF), acetic and formic acid were determined by HPLC according to [Alvira et al. \(2011\)](#).

## 3. Results and discussion

### 3.1. Biomass characterization

The main components of the ACR from sugarcane showed average values of 29.7 ± 0.8% for cellulose; 22.8 ± 0.5% for hemicellulose and 26.8 ± 0.4% of lignin. The cellulose content was low compared with other reports on sugarcane straw ([Rueda-Ordóñez et al. 2015](#)), but the hemicellulose value was similar to that described by [Moretti et al. \(2016\)](#). The xylan fraction was of 19.1 ± 0.4% of the ACR, which showed a low degree of substitution for acetyl groups (0.3 ± 0.0%), accounted for 84% of the hemicellulose. Also, arabinan, 2.5 ± 0.1%; galactan 0.9 ± 0.0%, and mannan 0.3 ± 0.0% were determined. Regarding lignin content, acid soluble lignin (ASL) content was of 3.8 ± 0.1%, while acid insoluble lignin (AIL) was of 23.0 ± 0.3%, value that was similarly reported ([Szczerbowski et al., 2014](#), [Lachos Pérez et al., 2017](#)). By water extraction, 4.7% of the raw material was dissolved, whereas the subsequent ethanol extraction dissolved an additional fraction of 1.6% resulting extractives for a total of 6.3 ± 0.5%, so was similarly described for sugarcane straw samples ([Raghavi et al., 2016](#)). The high ashes content found (14.6 ± 0.1%) with respect to other data ([Barros et al., 2013](#), [Oliveira et al., 2014](#)), might be due to soil contamination during the ACR harvesting. Correspondingly, acid-insoluble ash near 7.7% (silica, etc.) was detected, which corresponded to ~53% of total ash.

It should be noticed that differences in raw material composition frequently relies on crop varieties, on storage conditions (temperature, period), on cultivation and harvesting approaches and even on the types of plant tissue analyzed ([Lachos-Pérez et al., 2017](#)). Such potential variations on crop dry matter performance and harvesting conditions may yield a suboptimal content of carbohydrates ([Carvalho et al., 2015](#), [Kuster-Moro et al., 2017](#)). By considering the cultivated varieties of sugarcane in Argentina, approximately 151 kg of ACR per ton of harvested sugarcane were estimated as potentially available for bio-refinery. This implied about 2.7 million tons of ACR vacant as a raw material for ethanol production in 2016. In this work, the ACR assayed presented a total carbohydrate content over 50%, thus representing a significant potential for fuels or chemicals production from this feedstock.

### 3.2. Evaluation of pretreatments for sugarcane agricultural crop residues

Pretreatment technologies are aimed to improve the lignocellulose digestibility and produce structural and compositional changes of feedstocks. Therefore, a thorough evaluation of pretreatments assayed involves not only the carbohydrate recovery but also the sugar yields by enzymatic hydrolysis as key indicators of effectiveness. The ACR biomass trimmed to an average piece size of 10–12 cm, was submitted to different pretreatments: (i) steam-explosion (SE; 204 °C; 20 min), (ii) diluted-acid (2% w/v H<sub>2</sub>SO<sub>4</sub>; 121 °C; 60 min) and (iii) alkaline (2% w/v NaOH; 121 °C; 60 min).

**Table 1**

Insoluble solid recovery (SR), WIS and liquid fraction compositions and enzymatic hydrolysis yields (EHY) from ACR-WIS after the different pretreatments assayed.

Pretreatment	SR (%)	WIS composition (% dwb)			Liquid fraction components (*) (g/100 g raw material)		EHY <sub>G</sub> (%)	EHY <sub>X</sub> (%) (**)
		Cellulose	Hemicellulose	Lignin	Glucose	Hemicellulosic-derived sugars		
SE-ACR	67.6	38.8 ± 0.2	4.3 ± 0.1	32.6 ± 5.3	1.7	7.1	77.6 ± 1.2	72.0 ± 0.4
NaOH-ACR	52.5	52.3 ± 0.1	26.3 ± 0.2	10.4 ± 0.8	0.9	10.3	80.0 ± 4.5	65.0 ± 3.4
H <sub>2</sub> SO <sub>4</sub> -ACR	64.8	41.0 ± 0.5	7.1 ± 0.3	37.2 ± 1.1	1.5	17.9	36.3 ± 0.4	36.9 ± 0.2

(\*) Liquid fractions were analyzed only for carbohydrates.

(\*\*) WIS enzymatic susceptibility assayed according to Section 2.2.

Solid recoveries (SR) of 67.6% and 64.8% were obtained for the steam explosion and acid-based pretreatments, respectively, whereas SR of 52.5% was determined for the alkali-based pretreatment (Table 1). Similar SR values of 56% and 51.1% were reported after an industrial-scale SE pretreatment of sugarcane straw performed under slightly less severe conditions (200 °C and 15 min, Oliveira et al., 2013) and from sugarcane bagasse pretreated with calcium hydroxide (Grimaldi et al., 2015), respectively. On the other hand, Carvalho et al. (2015) reported SR values of 37.3% and 55.8% for alkaline and acid pretreatments, respectively; the differences with the values founded here may be due to the higher temperature (175 °C) used by these authors.

In regard to the WIS fractions, considerable variations in composition were observed after the different pretreatments tested. Cellulose contents were of 38.8%, 52.3% and 41.0% following SE, alkali and diluted acid pretreatments, respectively. The main constituent removed from the WIS after pretreatments were hemicellulose for SE-ACR and H<sub>2</sub>SO<sub>4</sub>-ACR, as its content was of 4.3% and 7.1%, respectively, and lignin for NaOH-ACR (10.4%) (Table 1).

The analysis of the liquid fractions revealed that xylose was the most abundant sugar detected. Interestingly, ~76% and 100% of the hemicellulosic-derived sugars were present as oligosaccharides in the liquid fractions after the SE and alkaline pretreatments, respectively, while only monomeric sugars were identified in the samples retrieved from the diluted acid pretreatment of ACR (data not shown).

By referring the total carbohydrate recovery values to the raw material (in solid and liquid fractions), the cellulose recovery was > 93% with respect to the untreated ACR in all cases, as this compound is the least solubilized at the pretreatment conditions tested (Fig. 2). The hemicellulose was nearly completely recovered after the alkali-based pretreatment (99.8%), which concentrated this compound in the solid fraction. While the acid treatment produced a hemicellulose recovery of 88.3%, distributed 68.8% in the liquid fraction and 19.6% in the WIS, a different behavior takes place in the SE pretreatment where only 39.7% of hemicellulose was recovered, mostly in the liquid fraction (27.2%) (Fig. 2). In relation to the loss of hemicellulose, a partial hydrolysis of this component has been attributed to the acid conditions due to the sulphuric acid addition in the acid pretreatments, and to acetic acid and other organic acids released from the biomass during the steam explosion (Duque et al., 2016). Furthermore, the pronounced reduction (~60%) of hemicellulose that was verified after the SE assayed, conveyed the detection of the degradation compounds furfural (0.35 g/L), HMF (0.07 g/L), acetic acid (0.45 g/L) and formic acid (0.52 g/L) in the resultant liquid fraction. Since minimal loss of sugars was observed next to the alkali and acid based pretreatments, degradation compounds were not investigated in these cases.

Finally, lignin recovery in the WIS reached 82.2% and 89.8% for SE and diluted acid pretreatments, respectively. As expected, the alkali-based pretreatment on ACR produced the highest delignification observed, 79.7% (Fig. 2), value that was similar to those obtained from sugarcane tops residues at similar conditions (Shindu et al., 2014).

In summary, it was observed that the overall composition of the main lignocellulose components was modified as follows: SE produced a significant loss of hemicellulose while cellulose and lignin were

mostly recovered in the WIS fractions; the alkali-based pretreatment led to a nearly complete solubilization of lignin, and the diluted acid pretreatment allowed to an overall recovery of all the components, with recovery values of cellulose 94%, hemicellulose > 88% (mostly in the liquid fraction) and lignin ~90% (Fig. 2).

The susceptibility to the enzymatic attack to carbohydrates, a key feature of pretreated material, was further tested on all ACR-WIS fractions by standard enzymatic assays with Celluclast 1.5 and Novo 188. The resulting glucose conversion values EHY<sub>G</sub> were of 77.6 ± 1.2% and 80.0 ± 4.5% for SE-ACR and NaOH-ACR, respectively (Table 1). In the case of alkaline-based pretreatments, the effectiveness of the enzymatic hydrolysis has been primarily attributed to a high delignification (lignin content of 10.4%; Table 1) and to a swollen effect that increases the access to cellulose, diminishing its polymerization degree and crystallinity (Kim et al., 2016). Interestingly, a low enzymatic release of sugars was observed for H<sub>2</sub>SO<sub>4</sub>-ACR even when its cellulose content was close to that from SE-ACR, thus revealing a low enzymatic digestibility of the solid obtained.

### 3.3. Set up of saccharification conditions at 20% (w/v) consistency

An ethanol concentration of 4% (w/w) is considered to be the benchmark for an efficient distillation (Wingren et al., 2003). Such result entails the availability of sugar concentrations ≥8% (w/w) for fermentation, which implies the use of high solid loadings for most types of lignocellulosic material (Koppram et al., 2014). However, those substrate loadings involve significant drawbacks on saccharification, such as end-product inhibition of cellulolytic enzymes, mass transfer limitations and high concentration inhibitors in the system, among others (Jørgensen et al., 2007). With the aim of defining the suitable saccharifying mixtures to be further used for the ACR-WIS samples at 20% (w/v) consistency, the enzymes Cellic CTec2 (C) and Cellic HTec2 (H) were evaluated. These preparations were developed for biofuel production from lignocellulosic material in order to address some of the issues derived from high solids utilization. The enzymatic hydrolysis yields obtained were not significantly improved by the addition of different proportions of hemicellulase (H) to cellulase (C) when SE and acid pretreated biomasses were tested, although around 10% of sugars were additionally released from H<sub>2</sub>SO<sub>4</sub>-ACR with the aid of 25% (H) (Fig. 3). Otherwise, a significant improvement (60%) on glucose yield was observed from NaOH-ACR when cellulase (C) was combined with 25% or with 75% hemicellulase (H). This outcome could be explained by the high hemicellulosic content of NaOH-ACR biomass (26.3%) with respect to SE-ACR and H<sub>2</sub>SO<sub>4</sub>-ACR (Table 1). In this regard, the addition of xylanase and/or β-xylosidase to the saccharification step has shown to overcome the need for high enzyme dosages for an effective hydrolysis (Romaní et al., 2014). It is important to point out that the assays for the saccharification set up on SE-ACR and H<sub>2</sub>SO<sub>4</sub>-ACR, with a similar cellulose content, yielded 79.3% and 36.4% of glucose on average, respectively. This result further supports our observation about the strong influence that the structural changes arising from pretreatments present on its enzymatic digestibility.



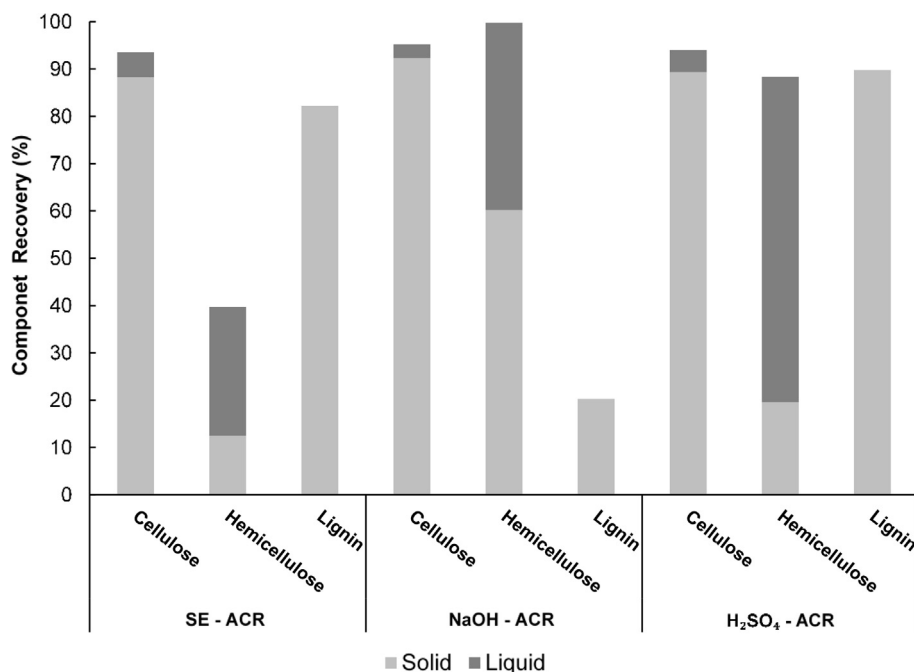


Fig. 2. Recovery of main structural compounds from sugarcane ACR in WIS and liquid fractions after pretreatments, with respect to raw material. Lignin content was only measured from the WIS fractions.

3.4. SSF and PSSF assays

For both configurations, the saccharification was settled on the basis of the results shown in Fig. 3: SE-ACR and H<sub>2</sub>SO<sub>4</sub>-ACR biomasses were treated with 100% Cellic CTec2 (C), whereas the combination consisting of 75% (C) + 25% (H) was applied for NaOH-ACR. From a general view, the SSF assays resulted in lower ethanol yields and efficiencies compared with the corresponding PSSF processes, mainly for [H<sub>2</sub>SO<sub>4</sub>-SSF] where ethanol was not detected (Table 2). These results are in good agreement with reports indicating that a hydrolysis preceding the SSF step improved the process using agricultural by-products at high solids loadings by facilitating the mass transference due to viscosity reduction (Rosgaard et al., 2007). Also, during SSF the saccharification proceeds at suboptimal enzymatic conditions (35 °C), fact that negatively affects the EH yields as it reduces the sugars release (Jørgensen et al., 2007).

The maximum ethanol concentrations obtained were 4.8% (w/w) for [NaOH-PSSF] and 4.5% (w/w) for [NaOH-SSF], implying ethanol

Table 2

Ethanol concentrations, yields and efficiencies obtained after 72 h of fermentation processes.

Process	Ethanol				
	% (w/w)	Yield (%)	L/ton ACR	Efficiency (%)	
PSSF	[SE-PSSF]	3.2 ± 0.1	36.8 ± 1.4	134.5	72.1 ± 2.7
	[NaOH-PSSF]	4.8 ± 0.2	40.8 ± 1.5	156.4	79.9 ± 2.9
	[H <sub>2</sub> SO <sub>4</sub> -PSSF]	1.4 ± 0.1	14.8 ± 0.6	54.6	28.9 ± 1.1
SSF	[SE-SSF]	2.2 ± 0.3	25.6 ± 3.0	93.4	50.1 ± 5.9
	[NaOH-SSF]	4.5 ± 0.1	38.4 ± 0.5	147.1	75.2 ± 1.0
	[H <sub>2</sub> SO <sub>4</sub> -SSF]	nd(*)	-	-	-

(\*) nd: not detected.

efficiencies over 75%, with no significant differences between both configurations (Table 2). Remarkably, these values successfully exceeded the minimum ethanol concentration reported as required for an

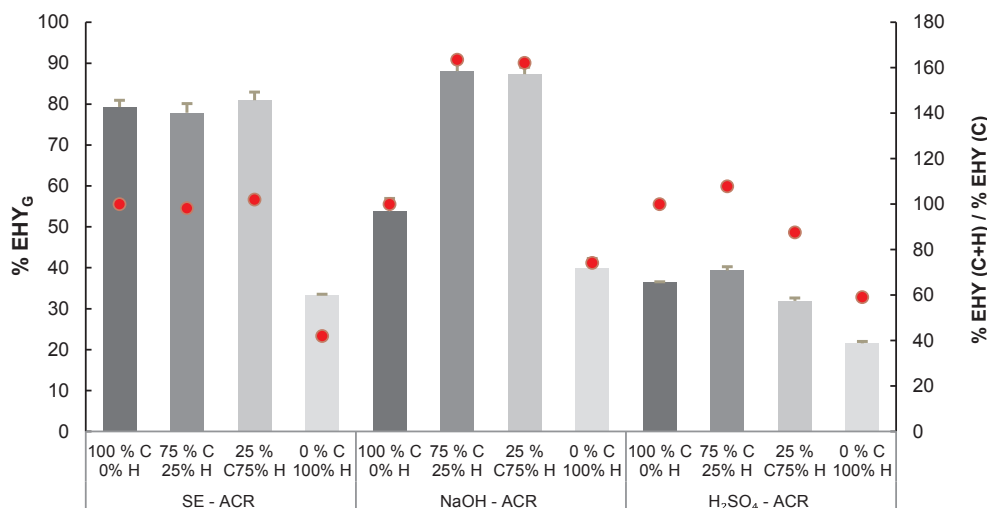
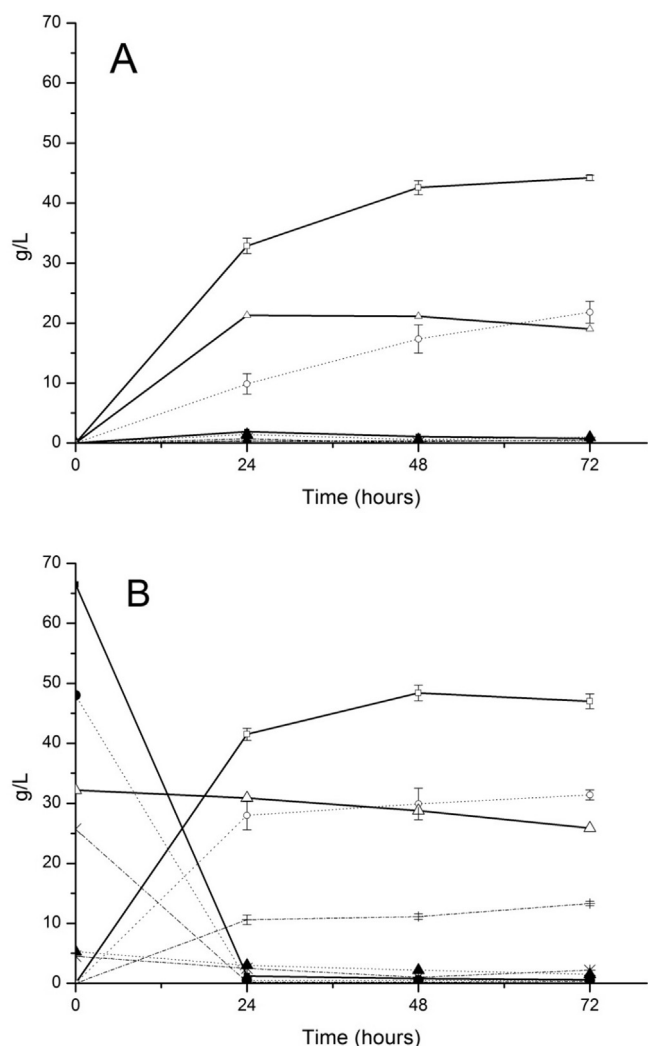


Fig. 3. Saccharification assays on 20% (w/v) WIS during 72 h using different combinations of Cellic CTec2 (C) and Cellic HTec2 (H). Bars represent the percentages of enzymatic hydrolysis yield (EHY<sub>G</sub>) of glucose obtained and red dots represent the ratio between the EHY<sub>G</sub> of each enzyme mixture (C + H) and the EHY<sub>G</sub> of the saccharification obtained with only (C), as percentage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Time course of ethanol production (g/L) and residual sugars of pretreated ACR submitted to A) SSF and B) PSSF at 20% (w/v) solid loading mixture. SE-ACR: --○-- ethanol, --●-- Glucose, --▲-- xylose; NaOH-ACR: —□— ethanol, —■— glucose, —△— xylose; H<sub>2</sub>SO<sub>4</sub>-ACR: --+-- ethanol, --x-- glucose, --x-- xylose.

economically feasible distillation (Koppram et al., 2014). Ethanol efficiencies found from the steam exploded material significantly declined in SSF, changing from 72.1% (PSSF) to 50.1% (SSF) (Table 2). It is important to bear in mind that such reductions could be meaningful for studies of viability and scale-up of the 2G ethanol production. Finally, [H<sub>2</sub>SO<sub>4</sub>-PSSF] was the less effective process assayed as it reached 1.4% (w/w) of ethanol production, whereas [H<sub>2</sub>SO<sub>4</sub>-SSF] produced negligible amounts of ethanol (Table 2).

Residual glucose was determined as an indicator of an efficient sugar consumption along ethanol production. Mostly, an efficient glucose consumption was observed. Residual concentrations  $\leq 0.7$  g/L of glucose were obtained by the SSF processes (Fig. 4A), whereas at 24 h of [SE-PSSF] and [NaOH-PSSF] processes, final values of 0.5 g/L to 1.2 g/L were reached from starting glucose concentrations of 48 g/L and 66 g/L, respectively. No meaningful variations of residual sugar were detected at 48 h and 72 h (Fig. 4 B). The [H<sub>2</sub>SO<sub>4</sub>-PSSF] process also showed similar residual glucose concentrations, although the sugar release was low due to the reduced enzyme susceptibility (*i.e.* low EH) observed for this pretreated biomass (Table 1).

It is worth to mention that the effective glucose consumption observed showed that the fermentative capacity of the microorganism used, *S. cerevisiae* Ethanol Red®, was not affected in the processes

performed. Interestingly, near 9 g/L of xylitol, a metabolic by-product from the yeast, were detected at 48 h of fermentation during [NaOH-PSSF] (data not shown). The significant amount of xylose released in this process (32.2 g/L, Fig. 4B) might sustain a further development of strategies towards a comprehensive exploitation of alkali pretreated sugarcane straw to produce a variety of bioproducts (Kwak and Jin, 2017).

The most favorable process assayed was [NaOH-PSSF], which produced 47.0 g/L of ethanol (Fig. 4B). This corresponded to an overall process yield of 12.3 g ethanol/100 g ACR, or 156.4 L ethanol/ton ACR, obtained from a glucose utilization of 79.9% of the raw material (Table 2). Mesa et al. (2017) reported the production of 14.8 g ethanol/100 g ACR after a PSSF process from an acid pretreated sugarcane straw using a solids consistency of 8% (w/v). However, the ethanol concentration obtained by these authors (14.47 g/L) represented the utilization 72.4% of the available glucose from the starting biomass. Therefore, we produced 3 times more ethanol concentration (47.0 g/L) at a 2.5 times higher solid loading (20%, w/v) by means of a [NaOH-PSSF] process.

It may perhaps be observed that the [SE-PSSF] process produced 134.5 L ethanol/ton ACR as a result of a 72.1% utilization of the glucose available (Table 2). Although the ethanol concentration reached of 3.2% (w/w) was below the 4% (w/w) considered as the target for a workable distillation product, the environmental and technical advantages of the steam explosion should be considered for a further improve the process efficiency towards a realistic ethanol production from ACR.

#### 4. Conclusions

An integrated process for 2G ethanol production was comprehensively evaluated by examining different pretreatments on post-harvesting sugarcane residues, and carrying out the saccharification-fermentation according to two process configurations, SSF and PSSF (Fig. 1). The most efficient process assayed was based on the use of alkali pretreated biomass, which released approximately 88% of its glucose content by adding last generation hemicellulases to cellulases. Such glucose availability for fermentation is considered as a reference for an economically viable distillation. As a result, by means of a [NaOH-PSSF] process at 20% (w/v) consistency, the overall process yielded 156.4 L ethanol/ton ACR.

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#### References

- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis, a review. *Bioresour. Technol.* 101, 4851–4861.
- Alvira, P., Negro, M.J., Ballesteros, M., 2011. Effect of endoxylanase and  $\alpha$ -L-arabinofuranosidase supplementation on the enzymatic hydrolysis of steam exploded wheat straw. *Bioresour. Technol.* 102, 4552–4558.
- Barros, R.R., Paredes, R.S., Endo, T., Bon, E.P., Lee, S.H., 2013. Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw. *Bioresour. Technol.* 136, 288–294.
- Bermúdez Alcántara, M.A., Dobruchowska, J., Azadi, P., Díez García, B., Molina-Heredia, F.P., et al., 2016. Recalcitrant carbohydrates after enzymatic hydrolysis of pretreated lignocellulosic biomass. *Biotechnol. Biofuels* 9, 207. <http://dx.doi.org/10.1186/s13068-016-0629-4>.
- Carvalho, D.M., Sevastyanova, O., Souza Penna, L., Pereira da Silva, B., Lindström, M., Colodette, J., 2015. Assessment of chemical transformations in eucalyptus, sugarcane bagasse and straw during hydrothermal, dilute acid and alkaline pretreatments. *Ind. Crop Prod.* 73, 118–126.
- da Silva, A.S.A., Inoue, H., Endo, T., Yano, S., Bon, E.P., S., 2010. Milling pretreatment of sugarcane bagasse and straw for enzymatic hydrolysis and ethanol fermentation.

- Bioresour. Technol. 101, 7402–7409.
- Duque, A., Manzanares, P., Ballesteros, I., Ballesteros, M., 2016. Steam explosion as lignocellulosic biomass pretreatment. In: Mussato, S.I. (Ed.), *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery*. Elsevier, pp. 349–368.
- Grimaldi, M., Marques, M., Lalue, C., Cilli, E.M., Sponchiado, S.R.P., 2015. Evaluation of lime and hydrothermal pretreatments for efficient enzymatic hydrolysis of raw sugarcane bagasse. *Biotechnol. Biofuels* 8, 205. <http://dx.doi.org/10.1186/s13068-015-0384-y>.
- Jørgensen, H., Vibe-Pedersen, J., Larsen, J., Felby, C., 2007. Liquefaction of lignocellulose at high-solids concentrations. *Biotechnol. Bioeng.* 96 (5), 862–870.
- Kim, J.S., Lee, Y.Y., Kim, T.H., 2016. A review on alkaline pretreatment technology for conversion of lignocellulosic biomass. *Bioresour. Technol.* 199, 42–48.
- Koppram, R., Tomás-Pejo, E., Xiros, C., Olsson, L., 2014. Lignocellulosic ethanol production at high-gravity, challenges and perspectives. *Trends Biotechnol.* 32, 46–53.
- Kuster-Moro, M., Teixeira, R.S.S., Sant'Ana da Silva, A., Duarte Fujimoto, M., Albuquerque Melo, P., Secchi, A.R., et al., 2017. Continuous pretreatment of sugarcane biomass using a twin-screw extruder. *Ind. Crop Prod.* 97, 509–517.
- Kwak, S., Jin, Y.S., 2017. Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*, a review and perspective. *Microb Cell Fact.* 16, 82.
- Lachos-Pérez, D., Tompsett, G.A., Guerra, P., Timko, M.T., Rostagno, M.A., Martínez, J., et al., 2017. Sugars and char formation on subcritical water hydrolysis of sugarcane straw. *Bioresour. Technol.* 243, 1069–1077.
- Li, J., Zhou, P., Liu, H., Xiong, C., Lin, J., et al., 2014. Synergism of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies. *Bioresour. Technol.* 155, 258–265.
- Manzanares, P., Negro, M.J., Oliva, J.M., Saéz, F., Ballesteros, I., et al., 2011. Different process configurations for bioethanol production from pretreated olive pruning biomass. *J. Chem. Technol. Biotechnol.* 86 (6), 881–887.
- Mesa, L., Martínez, Y., Barrio, E., González, E., 2017. Desirability function for optimization of diluted acid pretreatment of sugarcane straw for ethanol production and preliminary economic analysis based in three fermentation configurations. *Appl. Energy* 198, 299–311.
- Moreno, A.D., Alvira, P., Ibarra, D., Tomás-Pejó, E., 2017. Production of Ethanol from Lignocellulosic Biomass. In: Fang, Z., Smith, R., Qi, X. (Eds.), *Production of Platform Chemicals from Sustainable Resources*. Springer, Singapore, pp. 375–410.
- Moretti, M.M.S., Perrone, O.M., Nunes, C.C.C., Taboga, S., Boscolo, M., da Silva, R., et al., 2016. Effect of pretreatment and enzymatic hydrolysis on the physical-chemical composition and morphologic structure of sugarcane bagasse and sugarcane straw. *Bioresour. Technol.* 219, 773–777.
- Oliveira, F.M.V., Pinheiro, I.O., Soto-Maior, A.M., Nartin, C., Gonçalves, A.R., Rocha, G.M.J., 2013. Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added product. *Bioresour. Technol.* 130, 168–173.
- Oliveira, L.R.M., Nascimento, V.M., Gonçalves, R.A., Rocha, G.J.M., 2014. Combined process system for the production of bioethanol from sugarcane straw. *Ind. Crops Prod.* 58, 1–7.
- Raghavi, S., Sindhu, R., Binod, P., Gnansounou, E., Pandey, P., 2016. Development of a novel sequential pretreatment strategy for the production of bioethanol from sugarcane trash. *Bioresour. Technol.* 199, 202–210.
- Román, A., Ruíz, H.A., Pereira, J.A., Teixeira, J.A., Domingues, L., 2014. Integrated approach for effective bioethanol production using whole slurry from autohydrolyzed Eucalyptus globulus wood at high-solid loadings. *Fuel* 135, 282–291.
- Rosgaard, L., Andric, P., Dam-Johansen, K., Pedersen, S., Meyer, A.S., 2007. Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. *Appl. Biochem. Biotechnol.* 143, 27–40.
- Rueda-Ordóñez, Y.J., Tannous, K., 2015. Isoconversional kinetic study of the thermal decomposition of sugarcane straw for thermal conversion processes. *Bioresour. Technol.* 196, 136–144.
- Sánchez, O.J., Cardona, C., A., 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour. Technol.* 99, 5270–5295.
- Shindu, R., Kuttiraja, M., Binod, P., Sukumanan, R.K., Pandey, A., 2014. Physicochemical characterization of alkali pretreated sugarcane tops and optimization of enzymatic saccharification using response surface methodology. *Renew. Energy* 62, 362–368.
- Sindhu, R., Gnansounou, E., Binod, P., Pandey, A., 2016. Bioconversion of sugarcane crop residue for value added products. An overview. *Renew. Energy* 98, 203–215.
- Sluiter, J.B., Ruiz, R.O., Scarlata, C.J., Sluiter, A.D., Templeton, D.W., 2010. Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods. *J. Agr. Food Chem.* 58, 9043–9053.
- Sugar harvest in Argentina, 2016.** Sugar harvest in Argentina statistic reports for Argentina, <http://centroazucarero.com.ar/oldsite/zafas/zafra2016.html> [accessed 29 January 2018].
- Szczerbowski, D., Pitarello, A.P., Zandoná Filho, A., Ramos, L.P., 2014. Sugarcane biomass for biorefineries, comparative composition of carbohydrate and noncarbohydrate components of bagasse and straw. *Carbohydr. Polym.* 114, 95–101.
- Tomás-Pejó, E., García-Aparicio, M.P., Negro, M.J., Oliva, J.M., Ballesteros, M., 2009. Effect of different cellulase dosage on the cell viability and ethanol production by *Kluyveromyces marxianus* in SSF process. *Bioresour. Technol.* 100, 890–895.
- Tomás-Pejó, E., Alvira, P., Ballesteros, M., Negro, M.J., 2011. Pretreatment technologies for lignocellulose-bioethanol conversion. In: Pandey, A., Larroche, C., Ricke, S.C., Dussap, C.G., Gnansounou, E. (Eds.), *Biofuels Alternative Feedstocks and Conversion Processes*. Academic Press, pp. 149–176.
- Wingren, A., Galbe, M., Zacchi, G., 2003. Techno-economic evaluation of producing ethanol from softwood, comparison of SSF and SHF and identification of bottlenecks. *Biotechnol. Progr.* 19, 1109–1117.