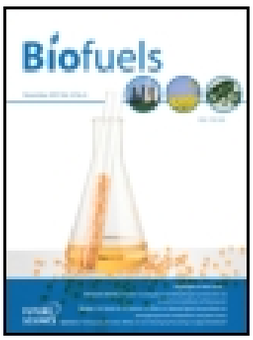

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# Second-generation ethanol production by separate hydrolysis and fermentation from sugarcane bagasse with cellulose hydrolysis using a customized enzyme cocktail

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**ABSTRACT**

Lignocellulosic materials are a valuable energy source that can be used to reduce dependence on fossil fuels. However, the high cost of enzymes and low efficiency of enzymatic hydrolysis are among the main bottlenecks regarding production of bioethanol. The purpose of the current work is to present the full use of sugarcane bagasse in the production of biofuels: as a carbon source for the production of competitive enzymes and as a platform for conversion of the cellulose and hemicellulose fractions into ethanol. Hydrolysis of pretreated sugarcane bagasse, using an enzymatic cocktail from naturally occurring microorganisms, was carried out, producing cellulose hydrolysate. This hydrolysate was further fermented to ethanol, achieving a concentration of 33 g/L, which corresponded to a fermentation efficiency of 89% and a high volumetric productivity of 11 g/(L·h). From the hemicellulose fraction, 18 g/L of ethanol was produced, which corresponds to a fermentation efficiency of 87%. Hence, from both cellulose and hemicellulose fractions, the overall ethanol concentration was 6.5% (v/v). These results indicate that production of ethanol through separate hydrolysis and fermentation (SHF) is a promising strategy, since it makes it possible to achieve high productivities during fermentation. Also, the possibility of converting xylose to ethanol can lead to an increase in production.

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**KEYWORDS**
Enzymatic hydrolysis; separate hydrolysis and fermentation; cellulase; ethanol

## Introduction

Biofuels could replace fossil fuels as a more sustainable energy source. Less harmful to the environment, their use presents some advantages, such as preservation of natural resources and reduced emissions of greenhouse gases (GHG). However, even though these advantages are well known, consumption of fossil resources has been increasing over the years: from the 1980s to now, an increase of over 84% with respect to carbon emissions from fossil fuels has been reported, and considering just the first few years of the current decade, this increase is about 7% [1].

Depending on its source, bioethanol is generally classified as first (from food-competing crops), second (from lignocellulosic material) or third generation (from algal biomass). Regarding the transportation sector, it is the most promising alternative to the more commonly used fossil fuels [2].

Brazil is the main producer of sugarcane and the second highest ethanol producer worldwide. In the 2015/2016 crop, approximately 666 million tons of sugarcane were produced, while ethanol production reached 30 million $m^3$, an increase of 185% in comparison with the 2000/2001 crop [3].

Ethanol production from sugarcane juice (first generation or 1G) comprises a well-established technological process. However, two thirds of the energy in sugarcane carbohydrates are stored in polysaccharides in the cell wall of the plant [4]. Polysaccharides correspond to nearly 75% of the components of plant cell wall and can be converted to fermentable sugars through the action of hydrolytic enzymes [5–7]. According to 'União da Indústria de Cana-de-Açúcar – Brazil', sugarcane cultivation generated about 166.5 million tons of bagasse (estimated) during the 2015/2016 period [3].

The quality of bioethanol is highly determined by its production route. Since production of 2G bioethanol consists of several stages, each stage (pretreatment, hydrolysis, fermentation, etc.) will have its consequences for both ethanol quality and cost [8].

Recent research has focused on developing more efficient technology. Despite advances, the hydrolysis step remains as the main bottleneck [2,9,10]. Limitations are associated with the high costs of hydrolytic enzymes, external dependence on enzyme production and low hydrolysis efficiencies. Zhuang [11] determined that costs of enzyme production represent 25–50% of the total production cost of lignocellulosic ethanol. It should be emphasized that the production cost of cellulolytic enzymes is not the only factor to influence the viability of 2G ethanol: enzyme loadings, hydrolysis and fermentation yields, as well as volumetric productivities, are parameters studied for the purpose of turning the production of 2G ethanol into a viable process.

In more than 40 countries, ethanol is used as a vehicular fuel, usually after being mixed with gasoline. In Brazil, for example, both this mixture and pure hydrated ethanol are

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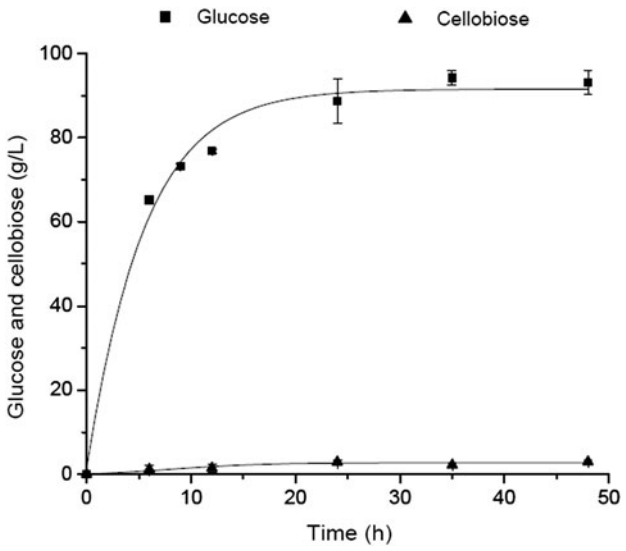


Figure 1. PDCL hydrolysis profile. Conditions: solid loading of 196 g/L, protein loading 4 of 24 mg/g cellulose (optimized mixture), 50 °C and pH 5.

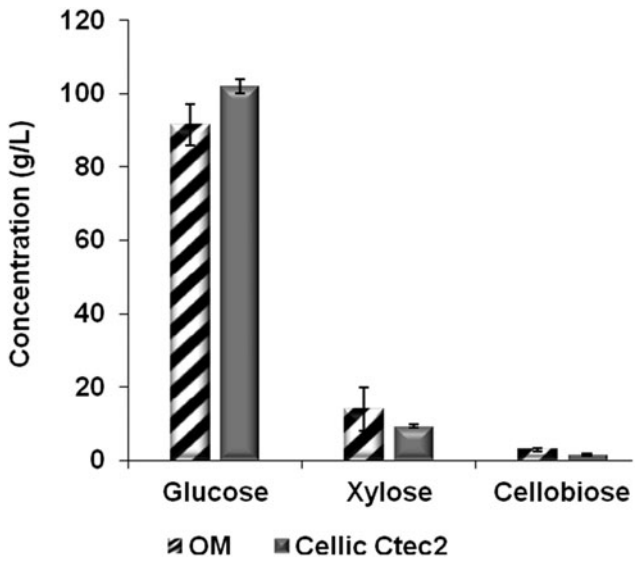


Figure 2. Sugars released, after 24 h, in partially delignified cellulignin (PDCL) hydrolysis using optimized mixture (OM) and Cellic Ctec2®. Conditions: solid loading of 196 g/L, protein loading of 24 mg/g cellulose, 50 °C, pH 5.

used. Due to its large consumption, however, ethanol production needs to be increased. One way of achieving this is making use of sugarcane bagasse, a natural resource which contains sugars that can be biotechnologically converted to ethanol [12]. Development of the current technology is pivotal for diffusion of 2G ethanol, and, consequently, a more sustainable future [8].

Therefore, this study aims at obtaining fermentable sugars from sugarcane bagasse, by making use of an enzymatic cocktail produced *in situ* and then converting these sugars into cellulosic ethanol. Furthermore, production of ethanol from hemicellulosic fraction was also investigated.

## Materials and methods

### Production of enzymes

Partially delignified cellulignin (PDCL) was used as carbon source for the production of enzymes. PDCL was obtained after acid and alkaline pretreatments according to Betancur et al. [13] and Vazquez et al. [14], respectively. Three different filamentous fungi, *Trichoderma harzianum*, *Penicilium funiculosum* and *Aspergillus niger*, were used for the production of three different enzymatic cocktails, which were combined in proportions of 15, 50 and 35%, respectively, with respect to proteic concentration, as described by Méndez et al. [15].

### Hydrolysis of pretreated sugarcane bagasse

Assays were performed in 1-L conical flasks containing 200 mL of reactional volume. Sodium citrate (50 mM) buffer solution, at pH 5.0, was used and temperature and stirring speed were 50 °C and 200 rpm, respectively. Solid loading was 196 g/L and protein loading was 24 mg/g cellulose, as described by Méndez et al. [15]. Glucose and cellobiose released through the process were quantified via high-performance liquid chromatography (HPLC), and the hydrolysis profile presents results for the first 48 h of hydrolysis.

### Fermentation of cellulosic hydrolysate

Fermentation of the PDCL hydrolysate was carried out using the yeast strain *Saccharomyces cerevisiae* JP1. The microorganism was maintained at 4 °C in a medium composed of 20 g/L glucose, 10 g/L yeast extract and 15 g/L agar-agar. Cells were activated and propagated in shake flasks at 200 rpm, 37 °C and pH 4.5, according to Pereira Jr. [16]. Activation and propagation steps lasted for 12 h and 6 h, respectively.

After sterilization at 0.5 atm for 20 min, cellulosic hydrolysate was supplemented with urea, yeast extract, $KH_2PO_4$ and mineral solution. All of these supplements were added to obtain the same concentrations of activation and propagation media. Approximately 15 g/L of cells (dry weight) were inoculated in the fermentation media. The pH was controlled and maintained at 6.0 through addition of 2 mol/L NaOH and/or 2 mol/L HCl. Temperature and stirring speed were kept at 30 °C and 200 rpm, respectively.

Kinetic profiles of substrate consumption and ethanol production were built based on samples that were withdrawn until depletion of substrate. These samples were centrifuged at 2500 × *g*, for 15 min at 10 °C, and the supernatant was separated and properly diluted for quantification of glucose and ethanol through HPLC.

### Fermentation of hemicellulose hydrolysate

In order to reduce concentrations of inhibitors such as hydroxymethylfurfural (HMF), furfural and acetic acid, prior to fermentation, the hemicellulose hydrolysate was detoxified in shake flasks for 2 h, at 200 rpm and 30 °C, using activated charcoal (5% m/v), according to Mussatto and Roberto (2004) [17]. Once detoxified, the hydrolysate was filtered twice with filter paper and then sterilized for further fermentation and conversion to ethanol. Xylose, glucose, HMF, furfural and acetic acid concentrations in the hemicellulose hydrolysate were quantified by HPLC before and after detoxification.

The yeast strain *Scheffersomyces stipitis* CBS5774 was used for fermentation. The microorganism was maintained

at 4 °C in medium composed of 5 g/L xylose, 2 g/L yeast extract and 3 g/L agar-agar, as described by Pereira Jr. [16]. Activation of cells was carried out using the medium previously described for *Saccharomyces cerevisiae* activation, with xylose instead of glucose. Temperature and stirring speed were kept at 30 °C and 200 rpm, respectively, for 22 h.

Fermentation was carried out in a Biostat B bioreactor, with nominal volume of 1.5 L and reaction volume of 1 L. Hydrolysate was supplemented with urea, yeast extract, $KH_2PO_4$ and mineral solution. All of these supplements were added to obtain the same concentrations as in the fermentation step of the cellulosic hydrolysate. Approximately 10 g/L of cells (dry weight) were inoculated. The pH was controlled and maintained at 6.0 through addition of 2 mol/L HCl and/or 2 mol/L NaOH, while temperature and stirring speed were kept at 30 °C and 200 rpm, respectively.

Kinetic profiles of substrate consumption and ethanol production were built based on samples that were withdrawn throughout the process. These samples were centrifuged at 2500 × *g*, for 15 min at 10 °C, and then the supernatant was separated and properly diluted for xylose, glucose, cellobiose and ethanol quantification through HPLC.

### Analytical methods

The HIPlex H column (Zorbax) was used for HPLC analyses. Glucose, xylose, cellobiose, mannose and ethanol were quantified with a refractive index detector (RID) , and HMF, furfural and acetic acid were quantified using a UV detector. Sulfuric acid at 0.0005 M in milli-Q water was used as the mobile phase at a flow rate of 0.6 mL/min, at oven and detector temperatures of 60 °C and 30 °C, respectively. Detection wavelengths for HMF and furfural were 235 nm, while for acetic acid it was 210 nm.

## Results

### Hydrolysis of pretreated sugarcane bagasse

The enzymatic cocktail, produced by *Trichoderma harzianum*, *Penicilium funiculosum* and *Aspergillus niger* from PDCL, presented cellulase activity of 13 ± 0.1 FPU/mL. Figure 1 displays the kinetic profile of the hydrolysis of pretreated sugarcane bagasse (PDCL), using the enzymatic cocktail (optimized mixture, OM) and optimized solid and protein loadings. The enzymatic cocktail composition, as well as solid and protein loadings, applied in this study were previously optimized by Méndez et al. [15]. In 24 h, the amount of glucose released reached 92 ± 6 g/L, corresponding to a hydrolysis efficiency of 63 ± 4%. After 48 h the glucose concentration obtained was 93 ± 3 g/L, with 64 ± 2% hydrolysis efficiency. There is no significant difference between results at 24 h and 48 h, since glucose levels were stabilized at 24 h, considering a 95% confidence level. Also, at 24 h volumetric productivity was 3.8 g/(L·h), while at 48 h it was 1.96 g/(L·h). The commercial cocktail Cellic Ctec2® was used to hydrolyze PDCL, under same conditions as in the current work, with the purpose to compare sugar released. Figure 2 presents the results for each cocktail (optimized mixture and Cellic Ctec2®).

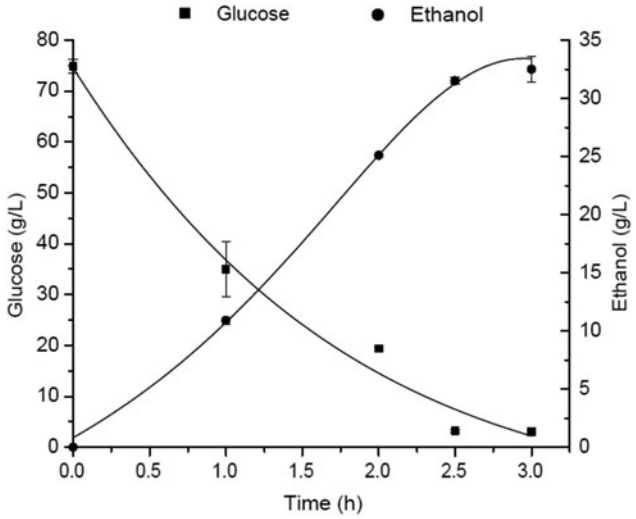


**Figure 3.** Alcoholic fermentation of cellulose hydrolysate, using industrial yeast *Saccharomyces cerevisiae* JP1 at 37 °C, and pH 4.5. Glucose was obtained through partially delignified cellulignin (PDCL) enzymatic hydrolysis using the optimized mixture.

### Fermentation of cellulose hydrolysate

Figure 3 presents the ethanol production profile, as well as glucose consumption, from the cellulose hydrolysate of sugarcane bagasse. At the end of the process, 33 g/L of ethanol were produced, corresponding to an efficiency of 89% and a volumetric productivity of 11 g/(L·h) during fermentation. Considering the saccharification step, which lasted for 24 h, volumetric productivity of ethanol in the separate hydrolysis and fermentation (SHF) process was 1.19 g/(L·h).

### Fermentation of hemicellulose hydrolysate

With the purpose of minimizing any inhibitory effect, detoxification of the hydrolysate was carried out (Table 1). A technique using activated charcoal produced the best results, mainly with respect to furfural and HMF removal – two substances that, according to Betancur [13], reduce the production and volumetric productivity of ethanol and

**Table 1.** Composition of hemicellulose hydrolysate before and after detoxification using activated charcoal.

| | Detoxification of hemicellulose hydrolysate | | |
|---|---|---|---|
| Component | Before (g/L) | After (g/L) | Removal efficiency (%) |
| Glucose | 2.32 ± 0.03 | 2.20 ± 0.02 | 5 ± 1.4 |
| Xylose and mannose | 46.48 ± 0.12 | 45.06 ± 0.11 | 3 ± 0.4 |
| Acetic acid | 19.28 ± 0.04 | 9.19 ± 0.017 | 52 ± 0.1 |
| Hydroxymethyl furfural | 0.07 ± 1.7E-4 | 0.00 ± 0.00 | 100 ± 0.0 |
| Furfural | 0.12 ± 2.7E-3 | 0.00 ± 0.00 | 100 ± 0.0 |

Table 2. Material balance for the production of ethanol from sugarcane bagasse.

| Step | Input | Output | Efficiency (%)* |
|---|---|---|---|
| Pretreatments | 1000 g SB + 2.8 L of 1.1% v/v $H_2SO_4$ + 1:20 4% m/v NaOH | 1.60 ± 0.01 L of hemicellulosic hydrolysate + 289 ± 1 g PDCL | 51 ± 1.5 |
| Hydrolysis | 289 ± 1 g PDCL | 135.6 ± 4.4 g glucose | 63 ± 4 |
| Glucose fermentation | 135.6 ± 4.4 g glucose | 59 ± 4 g ethanol | 89 ± 4 |
| Xylose fermentation | 1.60 ± 0.01 L of hemicellulosic hydrolysate containing 88.5 ± 4.8 g xylose | 38.5 ± 0.1 g ethanol | 87 ± 1 |

SB: Sugarcane bagasse. PDCL: Partially delignified cellulignin.
*Confidence interval of 95%.

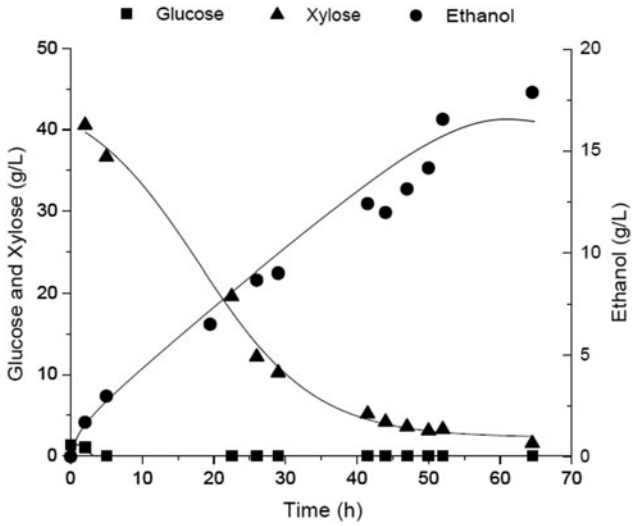


Figure 4. Fermentation of detoxified sugarcane bagasse hemicellulose hydrolysate using *Scheffersomyces stipitis*. Conditions: 30 °C and pH 6. Activated charcoal was used for detoxification.

the substrate consumption rate. Figure 4 displays ethanol production from the hemicellulose hydrolysate. It can be observed that *Scheffersomyces stipitis* CBS5774 was able to consume both xylose and glucose. By the end of the process (65 h) 18 g/L of ethanol had been produced, corresponding to a fermentation efficiency of 87% and a volumetric productivity of 0.3 g/(L·h).

The material balance was calculated for the process of glucose and xylose fermentation (with results of Figures 3 and 4). The purpose was to evaluate the amount of ethanol produced per dry weight of sugarcane bagasse. Table 2 shows the inputs and outputs for each step: pretreatments (acid and alkaline), hydrolysis and fermentations.

## Discussion

### Hydrolysis of pretreated sugarcane bagasse

The hydrolysis of pretreated sugarcane bagasse produced 92 ± 6 g/L of glucose, with a hydrolysis efficiency of 63 ± 4% at 24 h and a volumetric productivity of 3.8 g/(L·h), while at 48 h it was 1.96 g/(L·h). The kinetic profile presented in Figure 1 corroborates that of Arantes et al. [18], whose study indicated that enzymatic hydrolysis processes comprise three steps: liquefaction, a quick step during which enzyme adsorption occurs and the enzymes act on the most available substrate; an intermediate step, characterized by a moderate hydrolysis rate, at the end of which 50–70% of the substrate has been hydrolyzed; and the last stage, very slow, when the hydrolysis rate decreases and the substrate becomes more recalcitrant.

The higher substrate crystallinity could affect the action of enzymes, since the high ordering of the microfibrils hinders cellulase access. Besides, in the structure, chains might be linked with such a strength that the hydrolysis efficiency decreases [19]. Enzyme deactivation, synergism decrease and non-productive bindings are other factors that may be responsible for decreases in the hydrolysis rate [7,20].

A hydrolysis efficiency of 63% was achieved while using the optimized mixture along with a high solids loading. This result is similar to those reported in literature, as seen in Table 3. However, these previous studies comprise different lignocellulosic materials, and the hydrolysis efficiencies depend on the type of material used and the pretreatment carried out. Because of this fact, the commercial cocktail Cellic Ctec2® was used to hydrolyze PDCL under the same conditions as in the current research, with high solid loading (196 g/L) and a protein loading of 24 mg protein/g cellulose (Figure 2).

Under the same conditions, the commercial cocktail provided a hydrolysis efficiency in glucose of 70 ± 1%, a result 11% higher than that achieved with the optimized mixture. With respect to xylose release, there was no statistically significant difference between the two cocktails; both promoted a small accumulation of cellobiose, indicating that the optimized cocktail competes with the commercial cocktails.

### Fermentation of cellulose hydrolysate

Several strategies have been described for the production of 2G ethanol. In the present work, the SHF strategy was adopted. The optimized cocktail was added to the first stage, saccharification; this stage is characterized by high solids loading and moderate protein loading. This cocktail was produced from a mixture of enzymatic concentrates that had been produced *in situ* and induced with PDCL. In the second stage (fermentation), the system was inoculated with a high cell concentration and a high volumetric productivity was achieved (11 g/(L·h)). At the end of the process, 33 g/L of ethanol was produced, corresponding to an efficiency of 89% (Figure 3). In the saccharification step, the volumetric productivity of ethanol in the SHF process was 1.19 g/(L·h). These values are high when compared to those reported in the literature for different lignocellulosic biomasses [27–30]. These authors present volumetric productivity of ethanol in the SHF of 0.65 g/(L·h) by López-Linares et al. [27], 0.50 g/(L·h) by Gupta et al. [28], 0.58 g/(L·h) by Neves et al. [29], and 0.98 g/(L·h) by Mesa et al. [30]. In addition, Guilherme et al. [31] reached 31.5 g/L ethanol production from sugarcane bagasse by *S. cerevisiae* PE-2. You et al. [32] reported 16.2 g/L of ethanol from sugarcane bagasse by *S. cerevisiae*. Also, Huang et al. [33] produced

Table 3. Hydrolysis efficiency of pretreated biomass from this study compared with others reported in literature.

| Pretreated biomass | Solid loading (g/L) | Hydrolysis efficiency (%) | Ref. |
|---|---|---|---|
| Steam-exploded sugarcane bagasse | 200 | 69 | [21] |
| Slurries of pretreated corn stover | 200 | 35 | [22] |
| Steam-exploded or liquid hot water-pretreated olive tree biomass | 300 | 20 | [23] |
| Steam-exploded barley straw | 150 | 59 | [24] |
| Steam-pretreated wheat straw | 400 | 40 | [25] |
| Sweet sorghum bagasse pretreated with liquid hot water | 300 | 60 | [26] |
| Acid- and alkaline-pretreated sugarcane bagasse | 196 | 63 | This study |

18.8 g/L of ethanol from sugarcane bagasse by *S. cerevisiae* ZM1-5.

The SHF process presents some advantages, since it is possible for each stage (hydrolysis and fermentation) to be carried out in its optimum conditions. Also, cells can be recycled after fermentation, which contributes to lowering the global cost of the process. In comparison to the SSF (simultaneous saccharification and fermentation) process, studies have demonstrated that, in the latter, increases in the inoculum concentration do not promote higher ethanol production, indicating that hydrolysis is the limiting stage. In this case, enzymes do not act in their optimum conditions and, hence, the overall process is slower [34,35].

According to the relevant literature, inhibition of cellulase by the products is one of the main reasons for choosing the SSF process. It is known that glucose inhibits β-glucosidase action, leading to accumulation of cellobiose, and that this disaccharide has an inhibitory effect on cellobiohydrolases, which affects synergism in the hydrolysis process. In order to overcome these issues, many enzymatic cocktails are supplemented with β-glucosidases and, therefore, the SSF process has been identified as a strategy to minimize inhibition problems associated with cellulase.

Nevertheless, using the optimized mixture, no cellobiose accumulation ($<$ 0.5% w/v) was observed during the stage of enzymatic hydrolysis. This suggests that β-glucosidase was not inhibited by the product, indicating that the cocktail is well balanced with respect to this activity. As previously described, 35% of this mixture is composed by the *A. niger* cocktail, which presents high β-glucosidase activity, reinforcing the choice of the SHF process.

### *Fermentation of hemicellulose hydrolysate*

The first step of the sugarcane bagasse pretreatment comprised the addition of dilute acid, responsible for solubilizing and hydrolyzing the hemicellulose fraction, which is rich in xylose, a fermentable sugar that can be converted to a wide variety of bioproducts such as organic acids, solvents and others. In the current research, the hemicellulose hydrolysate was used for ethanol production. During the acid hydrolysis, despite the dilute concentration of the acid, some compounds that have an inhibitory effect on fermentation are produced, mainly furfural, HMF and acetic acid. Therefore, detoxification of the hydrolysate was carried out (Table 1). The activated charcoal technique was applied for detoxification, reaching 100% furfural and HMF removal, and 52% acetic acid removal.

Figure 4 displays ethanol production from the hemicellulose hydrolysate. It can be observed that *Scheffersomyces stipitis* CBS5774 was able to consume both xylose and glucose. By the end of the process (65 h) 18 g/L of ethanol had been produced, with a fermentation efficiency of 87% and a volumetric productivity of 0.3 g/(L·h). Betancur [13], who also carried out fermentation of sugarcane bagasse hydrolysate, achieved 19 g/L of ethanol after 40 h, which corresponds to a volumetric productivity of 0.47 g/(L.h). This difference might be associated with the high concentration of acetic acid (9 g/L) in the hydrolysate used in the present study, which is higher than that in Betancur's (2010) work and equivalent to 6 g/L.

Hilares et al. [36] obtained 17 g/L of ethanol from sugarcane bagasse by *Scheffersomyces stipitis* NRRL-Y7124. Bellido et al. [37] reported that ethanol production yields decrease with increasing acetic acid concentration. Scordia et al. [38] and Njoku et al. [39] observed that fermentation of hemicellulose hydrolysate by *P. stipitis* was inhibited by acetic acid and, to a lesser extent, by the presence of furfural. Studies carried out by Ferrari et al. [40], regarding the fermentation of eucalyptus wood by *P. stipitis* NRRL Y-7124, indicated that acetic acid was the main inhibitor of the process, lowering yields and production rates.

Acetic acid is usually generated from the acetyl groups that are present in the hemicellulose structure, at a pH that favors alcoholic fermentation (between 5 and 6). In its undissociated form, acetic acid is able to diffuse through the cell's cytoplasm, where, once dissociated, it promotes a reduction in the intracellular pH level. This situation increases the energetic demands of the cell, and might impair nutrient transportation through the membrane and inhibit the action of several enzymes in the glycolytic pathway [13,40,41].

Table 2 shows the inputs and outputs of material balance for each step: pretreatments (acid and alkaline), hydrolysis and fermentations. According to mass balance, the potential production of 97.5 g of ethanol was estimated from 1000 g of sugarcane bagasse. Efficiency of pretreatments was 51%, while the hydrolysis efficiency was 63% using the optimized mixture. In terms of fermentation, efficiency of this work reached 89% for glucose and 87% for xylose conversion to ethanol. It should be emphasized that in the current research, the concentration of ethanol obtained from the hemicellulose fraction corresponded to 56% of that obtained from the cellulosic fraction. This result suggests that the use of the hemicellulose portion could increase yields of ethanol produced from lignocellulosic biomass.

## Conclusions

Sugarcane bagasse generated during the production of 1G ethanol can be used for *in situ* production of efficient hydrolytic enzymes. These enzymes digest the cellulosic portion of the bagasse, allowing for an increase in ethanol production. The hemicellulose fraction, rich in xylose, can also be used for increasing 2G ethanol production. The

customized enzyme cocktail presented competitive hydrolysis efficiencies when compared to a commercial cocktail and to findings previously reported in the literature. The separate hydrolysis and fermentation (SHF) process provided high volumetric productivity (11 g/L·h) during the fermentation of glucose that arose from the cellulose enzymatic hydrolysis; this value is higher than those obtained in the industrial production of 1G ethanol (5–8 g/L·h). Besides, fermentation of the xylose obtained after acid pretreatment promoted an overall increase of 56% in the ethanol production, and by the end of the SHF process, using both cellulose and hemicellulose fractions, 6.5% (v/v) of ethanol was produced.

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## Disclosure statement

The authors declare that they have no conflicts of interest.

## Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors. The authors confirm that principles of ethical and professional conduct have been followed in this research and in the preparation of this article.

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