Recovery and Characterization of Cellulosic Ethanol from Fermentation of Sugarcane Bagasse

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Abstract. Ethanol from the fermentation using renewable sugarcane feedstock has been demonstrated a sustainable fuel chain production in Brazil. This work was focused on the production of cellulosic ethanol from sugarcane bagasse applying the current bioprocessing strategies with the aim to recover and characterize the product. The feedstock was pretreated at 190 °C and residence time of 10 min. The enzymatic hydrolysis was performed with commercial cellulolytic enzymes. Fermentation substrate were formulated with hydrolysate and supplemented with sugarcane molasses. The fermentations were set up to mimic the industrial conventional fermentation in Brazil ethanol distilleries with cell recycling. The fermentation resulted in a reproducible performance by yield of 0.49 g/g, productivity of 6.96 g/(L·h), and cell viability of 95.3 %. The fermented media was distilled in a lab-scale batch system. The distilled product showed compatible values compared to the fuel ethanol standard regulation. The bottom product of distillation presented characteristics suitable for fertilizer or biogas application.

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Keywords: sugarcane bagasse, cellulosic ethanol, fermentation, cell recycling, distillation, vinasse, ethanol fuel standard

Introduction

The production of renewable fuels plays a key role in the development of the non-petroleum-dependent society contributing to climate change mitigation by reducing greenhouse gas emissions. Ethanol produced by biological route has been used broadly as additive, fuel or blended with conventional gasoline[1]. Ethanol uses beyond fuels comprehend the production of several industrial interest products such as ethylene, propylene, and butadiene[2]. Moreover, ethanol is widely used in food, pharmaceutical, cosmetics, detergents, and medical care areas. Brazil and USA are the largest producers that are responsible for 75 % of the ethanol production in the world[3]. Corn and sugarcane are the most abundant and common renewable sources of sugars used for the anaerobic conversion by *Saccharomyces cerevisiae* into ethanol. Nowadays, lignocellulosic feedstock such as sugarcane bagasse, wheat straw, and cultivated wood have been exploited for the production of second generation ethanol[4–6].Integration of conventional and second-generation processes have been adopted as a strategy principle to minimize the capital investment, optimize the feedstock logistic, and promote the advancement of biorefineries by producing added-value chemicals[7,8]. The process integration for ethanol conversion occurs after obtaining the cellulosic hydrolysate (C6 fractions). This alternative source of carbon is directly integrated in the fermentation using the conventional process configuration. The conventional ethanol fermentation configuration in Brazil comprises high cell density and its recycling[9,10]. The substrate is obtained from sugarcane juice and molasses. Most of the installations use fed-batch mode with 4 h of feeding followed by 2 h or more of batch to complete sugars exhaustion. At the end of fermentation, the cells were recovered by centrifugation to reuse for the next new fermentation by previous cell washing with sulfuric acid at pH 2-3. In addition, the amount of inoculum corresponds to 1/3 of the final volume, and the rest is dedicated for substrate feeding. The current ethanol titer at the end of fermentation is between 8 and 12 % (v/v) and reaching efficiency of 92 %. The ethanol is recovered and concentrated by distillation. The conventional distillation consists of a set of 3 distillation columns and 2 rectification columns[11,12]. In the distillation columns is obtained ethanol-rich stream containing approximately 40 % ethanol, and the bottom product stream called vinasse. Vinasse is practically free of ethanol. The ethanol-rich stream in a vapor phase goes to the rectification columns to

concentrate near the binary ethanol-water azeotrope. Either a heater exchange or direct steam injection is used in the distillation column. Moreover, during the distillation impurities are removed. The distillation columns are also designed to remove separately secondary alcohol and incondensable components (CO_2 and SO_2). The rectification columns are provided of condensers to remove fusel oil and higher oil streams[12]. Fusel oil is composed mainly by 3-methyl-1-butanol (amyl alcohol) and 2-methyl-1-propanol (isobutyl alcohol)[13]. Higher oil is composed by n-propanol, isobutanol, and n-butanol. The current distillation yield is 99.5 % and the generation of bottom product is between 10 and 15 L of vinasse/L of ethanol[3]. Therefore, the production of ethanol from sugarcane has an overall high efficiency and well-established method to obtain a fuel ethanol grade. This work was focused on the production and recovery of cellulosic ethanol from sugarcane bagasse applying the current bioprocessing strategies, and characterization of ethanol for comparison to the fuel standard regulation.

Material and Methods

The feedstock sugarcane bagasse was pretreated in a 350-L stirred reactor (Pope Scientific Inc., USA) at 190 °C and 10 min of residence time using 10 % (w/v) of solids. The pretreated bagasse was recovered by Nutsche filter (Pope Scientific Inc., USA), then followed by intense washing with water until reach stable value of pH. The enzymatic hydrolysis was performed in the same stirred reactor at 50 °C, pH of 4.8 during 72 h, 10 % (w/v) of solids, and 10 FPU/mL of cellulolytic enzymes Cellic Ctec2 (Novozymes, DK). The cellulosic hydrolysate was recovered and concentrated in a wiped film evaporator (Pope Scientific Inc., USA) at 80 °C and 475 mbar. Afterwards, the concentrated hydrolysate was conditioned as substrate for fermentation. The conditioning steps and the formulation of the substrate to meet the nutrients requirement of the yeast were done at the same manner as previous work[14]. The equivalent of total sugars in term of reducing sugars was adjusted to obtain a final ethanol concentration of 7.5 °GL (59.2 g/L), which corresponds to the current industrial overall value of the first generation ethanol distilleries[15]. Moreover, the fermentations were set up to mimic the current industrial conventional Brazilian ethanol fermentation. The cell concentration of the inoculum adopted was 70 g/L (dry basis). Ten repeated fermentations were performed in 2-L Bioflo 115 bioreactor (New Brunswick, USA) at 33 °C, 200 rpm, without air sparging. An isolated industrial strain of *Saccharomyces cerevisiae* was used as culture starter. After each fermentation, yeast cells were recovered by centrifugation in a centrifuge Avanti J-26 XP (Beckman Coulter, EUA) at 13,225 x g, 15 min, and 20 °C. After that, the cells pellet was suspended in a sterile tap water and transferred back to the bioreactor's vessel. Then, the acidic yeast treatment was performed by adjusting the operating conditions at 33 °C, 800 rpm, and 0.2 L/min of air, and the pH was adjusted to 2.5 with 2 M sulfuric acid. After 30 min of acidic treatment, the bioreactor conditions were setup for fermentation (33 °C, 200 rpm) by immediately substrate feeding. The centrifuged fermented media of all fermentation runs were recovered and combined for ethanol recovery, which was performed in a batch distillation system Autodest® 800AC (iFISCHER, DE) supervisioned by Indusoft Web studioTM v6.2[16]. The distillation column system comprehended a column packed with Pro-Pak® of 6 mm in 316 SS. The column is equipped with a silvered vacuum mantle and a thermometer for temperature control, and the set-point was adjusted at 78 °C. The reboiler is equipped with an external mantle and two thermometers. The temperature set-points were adjusted at 100 °C (top jacket) and 170 °C (bottom jacket). A cryostat with a capacity of 360 Kcal at 0 °C was used in the condenser. The reflux ratio of 3 was adopted. This batch distillation system is equivalent a column of 15 theoretical plates (ASTM D 2892). The distillate streams were collected at 5 time-points between 38 °C to 80 °C.

The feedstock was characterized according to NREL's standard procedure[17–19]. The composition of hydrolysate and the fermentation metabolites were analyzed by high-performance liquid chromatography (HPLC). Sucrose, glucose, fructose, and xylose were determined in an Agilent Infinity 1260 with IR detector at 50 °C, Aminex column HPX-87P 300 mm x 7.8 mm at 60 °C and 0. 5 mL/min of ultrapure Milli-Q water as eluent phase. Acetic acid, ethanol, formic acid and glycerol were determined in a Dionex Ultimate 3000 with IR detector Shodex RI-101, Aminex column HPX-87H 300 mm x 7.8 mm at 50 °C and 0.5 mL/min of 5 mM sulfuric acid as eluent phase. Furfural and 5-HMF were determined in an Agilent Infinity 1260 with UV detector at 274 nm, Acclaim 120 - C18 150 x 4.8 mm at 25 °C and 0.8 mL/ min of acetonitrile with water (1:8) with 8 % of acetic acid as eluent phase. The composition of distillate products were quantified by gas chromatography (GC) in a GC-2010 Shimadzu equipment and following the European Standard EN 15721. The characterization of distilled fermented media were characterized by: color (ASTM D 1209); acidic (ABNT NBR 16047) using a potentiometric titration KEM (Kyoto Electronics); electrical conductivity at 25 °C (ABNT NBR 10547) using Orion Star conductivity meter; sodium content (ABNT NBR 10422); permanganate test (ASTM D 1363); sulfur content (ASTM D 5453). The bottom product (vinasse), was characterized according to Standard Method for the Examination of Water and Wastewater (SMEWW) 22nd edition. Analytics of the fermentation were done according to the previous work[20]. Substrate (sum of glucose and equivalent

reducing sugars (glucose and fructose) of sucrose) to product conversion yield ($Y_{P/S}$) was calculated as the ratio between the product formed and substrate consumed. The volumetric productivity (Q_p) corresponded to the product formation divided by the fermentation time.

Results and Discussion

The composition of the feedstock sugarcane bagasse corresponded to in wt%: cellulose, 38.57 ± 6.23; hemicellulose, 25.10 ± 4.09; lignin, 28.30 ± 0.97; acetic acid, 2.84 ± 0.36; extractives, 3.13 ± 0.08; ashes, 5.42 ± 0.11. This composition is in agreement with the reported values[21,22]. The moisture corresponded to 19.42 ± 0.46 %. The composition of the concentrated hydrolysate corresponded to in g/L: glucose, 212.29; xylose, 11.69; glycerol, 0.10; acetic acid, 0.72; HMF, 0.092; furfural; 0.002. The composition of the fermentation substrate that comprehended the combination of sugarcane bagasse hydrolysate and 8% (w/w) of sugarcane molasses, applied in all fermentation experiments contained in g/L: sucrose, 27.80; glucose, 121.19; fructose, 10.85; xylose, 8.46; acetic acid, 0.53; lactic acid, 4.76; glycerol, 0.15, HMF, 0.10; furfural, 0.05. Table 1 shows the results of the performance of all 10 fermentations in terms of productivity, ethanol yield, and cells viability. The ethanol titer achieved 59.09 ± 2.39 g/L that corresponds to approximately 7.5 °GL as expected. The value of ethanol conversion, productivity, and cells viability were similar along all fermentations reaching conversion of 0.49 ± 0.02 g ethanol/g sugars, productivity of 6.96 ± 0.21 g/(L·h), and 95.33 ± 3.02% of cells viability. The ethanol conversion met the typical industrial value (higher than 0.46 g/g), but the values of productivity and cells viability were higher than the industrial values[23,24]. This is, probably, because the bioreactor vessel in a bench-scale conditions has an impeller system that provides a good mixture of cells and substrate homogenization. In addition, the end of the fermentation can be determined precisely using MIR instrument, which gives on time results of sugars and ethanol[20,25]. Thus, both contributed to achieve higher values of productivity and cells viability than the industrial values. Therefore, the scaling down processing of the Brazilian ethanol fermentation using sugarcane bagasse hydrolysate supplemented with molasses was well succeed in bench scale (Figure 1). Additionally, only xylose (data not shown) remained as residual sugar at the end of the fermentations.

Table 1. Performance of cellulosic fermentation of sugarcane bagasse.

Sequence	Ethanol titer (g/L)	$Y_{P/S}$ (g/g)	Q_p (g/(L·h))	Cells viability (%)
1	53.55	0.47	6.74	96.61
2	56.74	0.43	6.63	98.84
3	59.37	0.48	6.88	98.16
4	60.33	0.51	6.97	98.82
5	60.04	0.51	7.03	95.30
6	61.93	0.51	7.36	90.58
7	60.00	0.50	7.10	96.00
8	60.89	0.49	7.15	90.62
9	58.65	0.49	6.89	94.17
10	59.37	0.47	6.91	94.20
Mean	59.09	0.49	6.96	95.33
STD	2.38	0.02	0.21	3.02



Fig. 1 Cellulosic ethanol fermentation in 2-L bioreactor.

The volume of fermented media of 10 fermentations totalized 15.3 L. During the batch distillation, 5 samples were recovered as shown by Table 2. The total amount of hydroalcoholic mixture corresponded to 1.7 L. Figure 2 shows the samples collected during the distillation. Sample 1 exhibited a whitish appearance while Sample 2 presented a yellowish appearance. These colors can be attributed to the presence of impurities. Samples 3, 4, and 5 were colorless. Apart from Sample 1, the superior alcohols were quantified as depicted in Table 3. Sample 2 presented highest amount of all volatiles analyzed (butanol, isoamyl alcohol, propanol, isobutyl alcohol, acetaldehyde, acetal, acetone, and ethyl acetate). Such by-products mixture (isoamyl alcohol (3-Methyl-1-butanol), propanol, and isobutyl alcohol (2-Methyl-1-propanol)) form the called higher alcohols or fusel oils that can be converted into valuable compound such as organic carbonates[3]. The excess of amino acids during fermentation leads to amino acid deamination and decarboxylation (Ehrlich Pathway) forming these higher alcohols[26]. Molasses is a source of amino acids[27]; also the cellulolytic enzymes broth could contribute to the total amino acids presented in the fermentation. Moreover, Table 3 shows the correspondent content of these volatiles in the fermented media. This composition was

compared with current values of ethanol produced from sugarcane juice fermentation[13]. Among those volatiles, butanol and isobutyl alcohol were higher than the reported values of ethanol from sugarcane juice fermentation[13].

Table 2. Samples of recovering ethanol by distillation.

Samples	Column temperature (°C)	Volume (mL)	Ethanol titer (% v/v)
1	25 - 38	110	33.45
2	38 - 72	524	81.78
3	72 - 80	440	79.50
4	72 - 80	500	43.90
5	72 - 80	120	13.06



Fig. 2 Samples of distillation, left to right, samples 1 to 5.

Table 3. Content of volatiles compounds in the hydro alcoholic samples in comparison to ethanol from sugarcane juice.

Compound	Sample 2 (mg/kg)	Sample 3 (mg/kg)	Sample 4 (mg/kg)	Sample 5 (mg/kg)	Value in the fermented media (wt %)	Ethanol from sugarcane juice (wt%) [13]
Butanol	65	34.4	3.8	< 10	3.40×10^{-4}	$(0 – 2.4) \times 10^{-4}$
3-Methyl-1-butanol	1086	41.3	5.0	21	3.94×10^{-3}	$(2.7 – 18.8) \times 10^{-3}$
Propanol	772	500	95	28	4.50×10^{-3}	$(2.1 – 6.8) \times 10^{-3}$
2-Methyl-1-propanol	1960	602	36.6	38	8.75×10^{-3}	$(1.3 – 4.9) \times 10^{-3}$
Acetaldehyde	425	60	31	< 50	1.76×10^{-3}	$(1.0 – 8.3) \times 10^{-3}$
Acetal	42	< 10	12.2	< 16	1.87×10^{-4}	-
Acetone	26	< 10	< 10	< 10	9.07×10^{-5}	-
Ethyl acetate	207	10.5	< 10	< 10	7.53×10^{-4}	$(5.5 – 11.9) \times 10^{-4}$

Characterization of distilled samples was done for the Sample 3 and Sample 4 as shown by Table 4, following the standard specification of ANP (National Agency of Petroleum - Brazilian Agency) for ethanol fuel. Sulfur and ethanol content were not comparable parameters due to the applied distillation method that gave a lower values than expected for ethanol fuel. Apart from it, Sample 3 presented most the desired characteristics such as total acidic, electrical conductivity, and pH. The high color index can be attributed to the hydrolysate itself that contained furfural, 5-HMF, and phenolic compounds, molasses, and byproducts formed during the autoclave processing in the pre-treatment step. Even after the application of the clarification process, the color still remained on the product. Thus, clarification has an important role for ethanol fuel quality by removing colloids particles, soluble proteins, and some colorful compounds of hydrolysate and molasses. Moreover, the composition of the hydroalcoholic fraction obtained in this work was slightly different from previous work that processed fermented media from sugarcane juice fermentation using the same distillation system. The reported values[28] were: ethanol, 80 % (w/w); 3-Methyl-1-butanol, 1.5 % (w/w); acetaldehyde, 22.5 mg/L; acetone,7 mg/L; ethyl acetate, 30 mg/L; propanol, 70 mg/L; 2-Methyl-1-propanol, 325 mg/L; butanol, 6 mg/L. The content of butanol and propanol of Sample 3 and Sample 4 were pronounced higher compared to these reported values. Thus, the ethanol process of cellulosic hydrolysate of sugarcane bagasse produced more volatile compounds than the conventional process. The distillation bottom product, vinasse, correspondent to approximately 13.5 L was characterized. Vinasse is an important side stream product that is current used as fertilizer[12], but it can be applied for the production of biogas[29].Table 5 presents the vinasse characterization in comparison to reported values[30] for distillation of fermented sugarcane juice. The vinasse originated from cellulosic ethanol can be applied as fertilizer, because the content of phosphorous (128 ± 4 mg/L) and magnesium (211 ± 1 g/L) were similar to the current values. Also, it can be applied for biogas production, since the quantity of biochemical oxygen demand was high (25,049 ± 4 mg/L), also higher compared to the current values (6,000 - 16,500 mg/L). However, sodium and sulfate contents were high more than expected. Both salts came from de pH adjustment during the enzymatic hydrolysis. The difficulties of mixing solids at the beginning of saccharification impacted the pH measurement and its control.

Table 4. Characterization of distilled samples and comparison with ANP standard specification.

Parameter	Sample 3	Sample 4	ANP standard
Color	< 20	< 20	5
Total acidic (mg/L)	29.3	51.1	30 (max.)
Electrical conductivity (µS/m)	312	980	300 (max.)
pH	5.6	4.9	6.0 - 8.0
Permanganate test (min)	< 1	< 1	Not detected
Sodium content (mg/kg)	2.7	0.9	2.0 (max.)
Sulfur content (mg/kg)	< 3.5	< 3.5	Not detected
Ethanol (% v/v)	79.5	43.9	92.5 – 94.6
Methanol (%v/v)	< 0.03	< 0.03	0.5 (max.)

Table 5. Vinasse characterization.

Compound/ parameter	Value (this work)	Value[30]
Calcium (mg/L)	404 ± 2	130 – 1,540
Cupper (mg/L)	0.024 ± 0.001	-
Iron (mg/L)	26 ± 1	-
Magnesium (mg/L)	211 ± 1	200 – 490
Manganese (mg/L)	7.315 ± 0.004	-
Molybdenum (mg/L)	0.230 ± 0.001	-
Nickel (mg/L)	6.11 ± 0.08	-
Potassium (mg/L)	1,194 ± 1	1,200 – 2,100
Selenium (mg/L)	< 0.005	-
Sodium (mg/L)	10,752 ± 18	-
Sulfate (mg/L)	24,870 ± 100	600 - 760
Zinc (mg/L)	0.500 ± 0.003	-
Total nitrogen (mg NH_3/L)	28.1 ± 0.4	
Total nitrogen (mg N/L)	108.60 ± 0.35	150 - 700
Total phenolic (mg/L)	< 0.010	-
Total phosphorous (mg/L)	128 ± 4	10 – 210
Total solids (mg/L)	62,490 ± 86	23,700
Biochemical oxygen demand (mg/L)	25,049 ± 4	6,000 – 16,500
Chemical oxygen demand (mg O_2/L)	54,062 ± 26	15,000 – 33,000

Conclusion

The fermentation of cellulosic hydrolysate of sugarcane bagasse using the current configuration was well-succeed by reaching similar industrial performance. The characterization of the distilled product showed discrepancies compared to the standard values required for fuel, which can be solved by adjustment of operating parameter to remove all impurities. The bottom product presented similar characteristics of current vinasse from conventional process.

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