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Ethanol production using hemicellulosic hydrolyzate and sugarcane juice with yeasts that converts pentoses and hexoses

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The use of vegetable biomass as substrate for ethanol production could reduce the existing usage of fossil fuels, thereby minimizing negative environmental impacts. Due to mechanical harvesting of sugarcane, the amount of pointer and straw has increased in sugarcane fields, becoming inputs of great energy potential. This study aimed to analyze the use of hemicellulosic hydrolyzate produced by sugarcane pointers and leaves compared with that of sugarcane juice fermented by yeasts that unfold hexoses and pentoses in the production of second generation biofuel, ethanol. The substrates used for ethanol production composed of either sugarcane juice (hexoses) or hemicellulosic hydrolyzate from sugarcane leaves and pointers (pentoses and hexoses), and the mixture of these two musts. Fermentation was performed in a laboratory scale using the J10 and FT858 yeast strains using 500 ml Erlenmeyer flasks with 180 ml of must prepared by adjusting the Brix to 16 ± 0.3°; pH 4.5 ± 0.5; 30°C; 10^7 CFU/ml with constant stirring for 72 h, with four replications. Cell viability, budding, buds viability, and ethanol production were evaluated. Among the yeasts, the cell viability was greater for J10. The use of FT858 + J10 was effective in producing ethanol. The hemicellulosic hydrolyzate had low efficiency in ethanol production compared with sugarcane juice.

Key words: Hydrolysis of sugarcane straw and pointers, sugarcane juice, xylose, cell viability, ethanol.

INTRODUCTION

With the decline in world oil reserves, along with price instability and the appeal for the sustainable use of natural resources, the search for alternative such as biofuel production has intensified (Oderich and Filippi, 2013). Brazil stands out as the world's largest producer of sugarcane, with an estimated production of 25.77 billion

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gallons of ethanol in the 2013/2014 harvest (Conab, 2013). In order to increase ethanol production, the technology of converting lignocellulosic biomass into fermentable sugars for ethanol production is an alternative to meet the global demand for fuels (Santos et al., 2012).

Due to the expansion of energy crops in conjunction with environmental responsibility measures, an agro-environmental protocol on the cooperation between the government and the sugar-energy sector was established with the purpose of ending sugarcane burning and expanding mechanized harvesting. Without previous burning of sugarcane trash, mechanized harvesting results in large amounts of straw and pointers in the field, reaching 5-20 tons per hectare (Foloni et al., 2010).

The dry bagasse (which has now been used in cogeneration) and sugarcane trash account for two-thirds of the planted area, that is, only one third of the biomass in plants is used in the production of ethanol or sugar, but they have great potential to produce second generation ethanol (Fugita, 2010). For straw and sugarcane bagasse cellulose content with an average of 39 and 43% respectively, there is a potential ethanol production of about 88/101 billion liters (Nunes et al., 2013).

The ethanol production from lignocellulosic hydrolysates in an economically feasible process, requires micro-organisms that produce ethanol with a high yield from all sugars present (hexoses and pentoses), have high ethanol productivity and can withstand potential inhibitors; furthermore, an integration of fermentation with the rest of the process should be investigated (Olsson and Hahn-Hägerdal, 1996).

Several studies have been conducted focusing on viable and low cost alternatives for the production of biofuel from biomass (Canilha et al. 2012; Cheng et al., 2008). In order to allow the release of sugars present in the hemicellulosic fraction of the mechanized harvesting residues and to make it available for fermentation using microorganisms, prior hydrolysis of biomass is required.

Amongst the available processes, acid hydrolysis provides recovery of up to 90% of fermentable sugars present in the hemicellulosic fraction (Rodrigues, 2007). However, this process may generate inhibitors, such as the phenolic compounds which are mainly formed during partial degradation of lignin (Martin et al., 2007), thereby inhibiting the fermentation process and resulting in low efficiency and low industrial production (Ravaneli et al., 2006).

The objective of this study was to evaluate the production of second generation biofuel, the ethanol from a hemicellulosic hydrolyzate obtained from sugarcane leaves and pointers, sugarcane juice and their mixture, fermented by two different yeasts.

MATERIALS AND METHODS

Raw material

The raw material obtained from sugarcane variety RB867515 (straw, pointers and juice) was collected from a production unit in the region of Jaboticabal, SP. The straw and pointers were subjected to hydrolysis process. Before and after this process, these fractions were characterized as cellulose, hemicellulose and lignin (Van Soest and Robertson, 1985). The sugarcane juice was adjusted and available to fermentative process.

Hydrolysis

In order to obtain the hemicellulosic hydrolyzate, 2 kg of leaves and pointers previously dried in an aerated-oven at 60°C to constant weight were used. Acid hydrolysis of the hemicellulosic fraction was performed in a 40 L reactor under the following conditions: temperature of 121°C, residence time of 20 min, and 105 ml of sulfuric acid in 20 L of water.

Musts

To obtain the hemicellulosic hydrolyzate must (HHM), the hydrolysis fraction were initially detoxified for the removal of the fermentation inhibitors. The solution pH was adjusted to 7.0 by the addition of calcium oxide (CaO), followed by an adjustment to pH 4.0 using phosphoric acid (H_3PO_4). Furthermore, the hydrolyzate underwent adsorption using activated carbon (1%) in an incubator at 50°C (B.O.D) for 30 min. At the end of each pH adjustment step, the hydrolyzate was centrifuged and filtered (Marton, 2002), resulting in the must to be fermented.

To obtain the sugarcane juice must (SJM), the original juice was subjected to clarification process for the removal of impurities. This process consisted of 300 mg/L of phosphoric acid and pH adjusted to (6.0 ± 0.1) with calcium hydroxide (0.76 mol/L) of analytical reagent grade (a.r.). The lime juice was then heated to 100 to 105°C and was transferred to beakers, and allowed to rest for 20 min for all impurities settling. To promote high settling rate, the beakers contained a polymer (Flomex 9074 – 2 mg/L) that group the small amount of impurities in high molecular weight flocs. After that, the juice was filtered through a 14 µm filter paper in order to separate the precipitated impurities, thereby resulting in a clarified juice. The clarified juice was standardized with distilled water to 16° Brix (soluble solids), and its pH was adjusted to 4.5 with sulfuric acid (± 0.3) at a temperature of 30°C, resulting in the must.

The third must (HSJM) was obtained by mixing the sugarcane juice must and hemicellulosic hydrolysate must in the ratio 1:1 (v/v).

Yeast strains

The following yeasts were isolated and mixed at the ratio of (1:1) (four replications): 1. J10 (*Rhodotorula glutinis* -xylose metabolizing) obtained from a stock-culture maintained at 4°C provided by the yeast bank of the Laboratory of Sugar and Ethanol Technology of the Department of Technology - School of Agrarian and Veterinary Sciences, UNESP, SP, Jaboticabal, (Guidi, 2000); 2. FT858 (*Saccharomyces cerevisiae* - used for industrial ethanol production) with the following characteristics: high-yield fermentation; resistant at low pH; tolerance to higher levels of alcohol; high viability during cell recycling fermentation; low foam formation; non-flocculent yeast strain; good fermentation speed (8 h when used in sugarcane industry), and low residual sugar levels in the must (Amorim, 2011).

The initial cell viability was determined for 72 h using a Neubauer cell-counter chamber (Lee et al., 1981), and a cell mass of both strains, containing a sufficient amount of cells to start fermentation (10^7 CFU/ml), was used.

Fermentation and ethanol production

Fermentation was performed in laboratory scale using 500 ml

Table 1. Cellulose, hemicellulose and lignin from the straw and sugarcane tip before and after hydrolysis of the hemicellulose fraction.

Percentage	Cellulose	Hemicellulose	Lignin
Composition before acid hydrolysis	37.32	35.98	5.52
Composition after acid hydrolysis	22.44	28.71	11.80

Values are represented as means.

Table 2. Analytical of pretreated substrates used for ethanol production.

Evaluated parameters	SJM	HHM	HSJM
Brix	16.1	16	16.3
pH	4.51	4.24	4.31
Sulfuric Acid Concentration (g/L)	0.73	5.72	2.69
Phenolic Compounds (g/L)	0.17	2.85	1.82
Total Monosaccharides (g/L)	111.7	81	97.5

Values are represented as means. SJM, Sugarcane juice; HHM, hemicellulosic hydrolyzate sugarcane leaves and pointers; HSJM, the mixture of these two substrates.

Erlenmeyer flasks containing the substrate used for ethanol production (180 mL): SJM, HHM and HSJM. A total cell concentration of 10^7 CFU/mL of the following strains J10, FT858, and J10 + FT858 was used. The flasks after inoculation with respective cultures at desired cell concentration were incubated at 30± 1°C with continuous stirring for 72 h. Cell viability, budding and buds viability were determined at 0, 6, 12, 24, 36, 48, and 72 hof fermentation (Lee et al., 1981).

The concentrations of sugars and ethanol were determined by HPLC (Waters, Milford, MA) with a Bio Rad Aminex HPX-87H column under the following conditions: column temperature 45°C, eluent: H_2SO_4, 0.005 mol/L, flow rate of 0.6 ml/min, and an injection volume of 20 µL.

The aliquots collected at 0, 6, 12, 24, 36, 48, and 72 h of fermentation, for analysis of sugar consumption and ethanol production, were properly diluted and filtered through a “Sep Pack” C18 filter (Millipore). The eluent was prepared by subjecting it to vacuum filtration using Millipore membrane filter (0.45 pm, Hawp) and was degassed in an ultrasound bath (Microsonic SX-50) for 15 min which was subsequently analyzed by HPLC.

Statistical analysis

The results of cell viability and ethanol production were subjected to analysis of variance by the F test, and the comparison of the means was performed by the Tukey test (Barbosa and Maldonado, 2011).

RESULTS AND DISCUSSION

The composition of the cellulose and hemicellulose was reduced when considering the results reported by Santos et al. (2012) (Table 1). After hydrolysis there was a reduction in the percentage of cellulose and hemicellulose, and an increase in lignin concentration.

The cellulose and hemicellulose have a low calorific value and after the hydrolysis process, the sugar were released and used as a substrate for ethanol production. Lignin has a high calorific value and can be used in cogeneration. The values obtained from cellulose, hemicellulose and lignin, for the sugar cane bagasse are around 48, 7.8 and 34.5%, respectively. These differences are explained by the straw characteristics and tips of sugarcane used in the study, which are structurally less rigid than the bagasse from sugarcane stalks.

The average values of Brix, pH, sulfuric acid concentration, total monosaccharides and phenolic compounds of the musts are shown in Table 2. It can be seen that the three musts (SJM, HHM and HSJM) had similar characteristics in terms of pH and Brix. Regarding to total acidity, highest values were found in the hemicellulosic hydrolyzate probably because sulfuric acid (0.5%) was added in the hydrolysis process.

The concentration process means an increase in the content of sugar and phenolic compounds, which were higher than the values reported in the literature. Phenolic compounds and other compounds that remain after detoxification can inhibit fermentation (Polakovic et al., 1992) directly affecting cell viability and ethanol production (Ravaneli et al., 2006; Garcia et al., 2010).

The presence of toxic compounds may influence fermentative organisms to an inefficient use of reducing sugar and formation of the product (Mussatto and Roberto, 2004). Martinez et al. (2000) observed a synergistic effect when inhibitors compounds combined; including a variety of phenolic, aromatic compounds and several types of acids, derived from lignin degradation, that ethanol production by *E. coli* was affected.

On the other hand, the results of yeast cell viability in the three substrates are given in Table 3**,** which clearly show that the yeast cell viability in the sugarcane juice

Table 3. Variance analysis and comparison of means by the Tukey test (5% probability) of the microbiological analysis results using the musts composed of sugarcane juice (SJM), hemicellulosic hydrolyzate (HHM), and sugarcane juice + hemicellulosic hydrolyzate (HSJM), with the strains J10, FT858, and J10 + FT858.

Musts and yeats	Cell viability (%)	Budding (%)	Buds viability (%)
Musts			
SJM	88.69^{A}*	7.57^{A}	87.46^{A}
HHM	68.89^{C}	3.67^{B}	63.54^{C}
HSJM	78.59^{B}	5.77^{A}	77.71^{B}
Yeasts			
J10	83.77^{A}	6.14^{A}	80.27^{A}
FT858	73.84^{C}	5.45^{A}	69.85^{B}
J10 + TF858	78.55^{B}	5.42^{A}	78.58^{A}
Must X Yeasts	4.66**	15.04**	2.80^{ns}

**Significant at 1% ($P<0.01$); ns, non-significant ($P\geq0.05$); *Means followed by the same uppercase in letters in a column are not significantly different according to the Tukey Test.

must was 22.33% higher than that in the hemicellulosic hydrolyzate must. It was found that J10 had the best cell viability, while the worst viability was found for FT858; the mixture of these two yeasts showed intermediate viability values.

There was a continuous decrease in cell viability after 72 h of fermentation for all strains. This behavior is due to the natural metabolism of yeast strains since they transform sugar into fermentation products such as ethanol, acids, glycerol and other compounds that accumulate in the culture medium inhibiting their metabolic process, negatively affecting cell viability (Amorim et al., 1996).

The fermentation process was evaluated for 72 h, which in an industrial scale is considered a process too long for ethanol production. In the present study, the fermentation process occurred within the first 10 h, with cell viability of approximately 90, 86, and 78% for the sugarcane juice must, mixture (broth and hydrolyzate), and for the hydrolyzate must , respectively. Very low values around 40% were found for the hydrolyzate must at the end of the process due to the combination of inhibitory compounds which accumulate over time.

During sugarcane juice must fermentation, cell viability was statistically the highest, followed by the mixture of the fermented hemicellulosic hydrolyzate and sugarcane juice. Among the yeasts, the best performance was found for J10 and the mixture of J10 and FT858. The strain FT858 had shown lowest cell viability.

The bud was the highest in sugarcane juice broth and was found to be lowest in the hemicellulosic hydrolyzate. When strains used in the present study was compared, no statistical significant differences was obtained in terms of budding. The optimum budding index of a fermentation process should generally range between 5 and 15% (Amorim et al., 1996); while, in the present report, the hemicellulosic hydrolyzate was the one with values lower than the optimal ones reported in literature (3.67%. on average), probably affected by the presence of inhibitor compounds.

The buds viability yeast cells in the fermentation of the sugarcane juice was also statistically higher, followed by that in the fermentation of the must composed of the mixture of hemicellulosic hydrolyzate and sugarcane juice. Among the yeasts, the best performance was found for J10; FT858 produced the lowest performance, and the mixture of J10 and FT858 produced intermediate performance.

Literature reports suggest that hexoses and pentoses were completely consumed in the first few of fermentation as glucose is the universal carbon source (Schirmer-Michel et al. 2008). Similar results has been reported by Cheng and coworkers (2008) in sugarcane bagasse hydrolyzates. Xylose consumption in this study (the main sugar in the hemicellulosic hydrolyzate), however, was not complete (Table 4).

Our results are in accordance with the report of Toivari et al. (2001) wherein a higher concentration of phenolic compounds and acids could be responsible for lower production of ethanol. Evaluating the effect of the fermentation time (Figure 1) on the musts, it was observed that in 24 h of fermentation, the highest concentration of ethanol with the clarified broth of sugarcane juice yield was 70% higher than that of the hemicellulosic hydrolyzate (around 9 g/L) in same time period. The sugarcane juice must produced the highest level of ethanol (33 g/L), followed by the must composed of the mixture of hemicellulosic hydrolyzate and sugarcane juice (22 g/L).

The variation in the ethanol production, cell viability, budding and buds viability was mainly attributed to the

Table 4. Variance analysis and comparison of means by the Tukey test (5% probability) of the use of xylose by yeasts J10, FT858, and J10 + FT858.

Time of fermentation (h)	Use of xylose by yeasts		
	J10	FT858	J10 + FT858
0	49.80^{A}	56.55^{A}	52.51^{A}
6	37.51^{B}	45.91^{B}	42.96^{B}
12	34.49^{B}	35.76^{C}	35.67^{C}
24	24.15^{C}	25.80^{D}	27.03^{D}
36	23.40^{C}	21.49^{DE}	22.24^{DE}
48	22.55^{C}	20.45^{DE}	19.03^{EF}
72	18.09^{C}	18.59^{E}	14.74^{F}
Teste F	53.92**	92.26**	81.69**

**Significant at 1% (P<0.01); *Means followed by the same uppercase in letters in a column are not significantly different according to the Tukey Test.

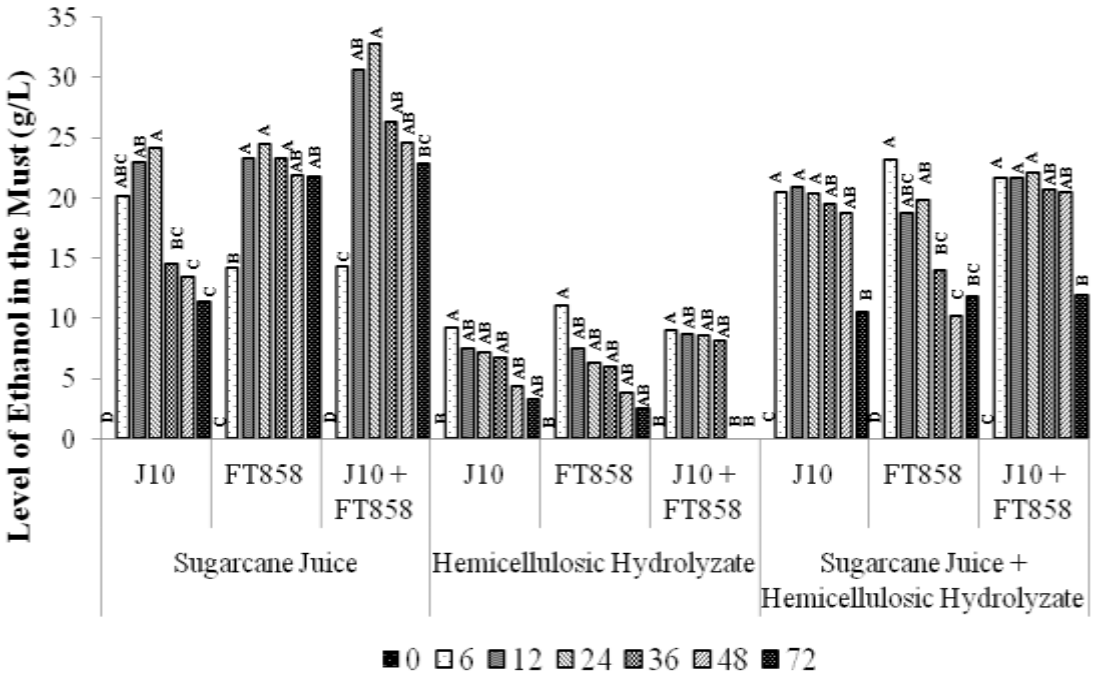

Figure 1. Graphical representation of the unfolding of the musts and yeasts (J10. FT858 and J10 + FT858) over a 72 h period for ethanol production.

composition of the pretreated substrates, which contain large concentration of inhibitory compounds; which were not efficiently removed during the detoxification process and this might have negatively influenced the final result. Some toxic compounds can stress fermentative organisms to an inefficient utilization of sugar resulting in product formation decreases (Silva, 2004).

The final ethanol concentration varies according to the concentration of sugar, nutrients, contaminants, and inhibitors presents in the substrate. Accordingly, it was found that only one single detoxification process was not sufficient for the removal of acids and phenolic compounds, which negatively influenced the production of ethanol from the hemicellulosic hydrolyzate. The detoxification method has to be based on concentrations and the degree of microbial inhibition caused by the compounds. To a certain types of compounds, better results can be obtained by combining two or more different detoxification method (Silva, 2004).

In the present investigation, the level of ethanol produced using clarified broth of sugarcane juice, although lower than most of the literature reports using sugarcane juice, in the present investigation, the level of ethanol produced

using clarified broth of sugarcane juice was the higher (about 9 g/L) compared with 1.5 g/L reported by Fugita (2010) that used sugarcane bagasse as raw material and J10 yeasts.

In conclusion, we observed highest cell viability and ethanol production in the clarified broth of sugarcane juice using the strain J10. The detoxification process used promoted a partial removal of acids and phenolic compounds. The use of a yeast co-culture produced the best performance in ethanol production. The pointer and straw cane are an important raw material to be considered for the ethanol production.

Conflict of interests

The authors did not declare any conflict of interest.

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