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Bioethanol production from agro-industrial *Saccharum* spp. residues: using *Trametes versicolor* in simple fermentation and saccharification processes

Figueroa-Rosales, Edna X.[1]; Aguilar-Huerta, Eréndira[1]; Granados-Baeza, Manuel J.[1]; Quinto-Hernández, Alfredo[1*]

[1]Tecnológico Nacional de México - Instituto Tecnológico de Zacatepec, Calzada Tecnológico 27, Zacatepec, Morelos, México, 62780.

***Corresponding author**: alfredo.qh@zacatepec.tecnm.mx

ABSTRACT

Objective: To quantify the production of total and reducing sugars, as well as bioethanol from sugarcane bagasse (*Saccharum* spp.) pretreated with *Trametes versicolor* and 20 %(W/V) NaOH solution.

Design/methodology/approach: An experimental 2^K design was used to determine the effect of the volume of inoculant solution added to wild sugarcane bagasse samples (Factor A; 8 and 10 mL), its incubation period (Factor B; 18 and 24 d), and the bagasse weight/volume of 20 %(W/V) NaOH solution (Factor C; 5 and 20 %(W/V), on the production of total and reducing sugars during simple saccharification. ANOVA analysis was used to evaluate the significance of these factors. The production of bioethanol was achieved via simple fermentation using a commercial strain of *Saccharomyces cerevisiae*.

Results: It was found that the highest yields of reducing and total sugars were obtained with the use of 10 mL of inoculant solution and with an 18 d incubation period during biological pretreatments. The ANOVA analysis suggests that Factors A and B influence the release of sugars, while Factor C was irrelevant. The bioethanol production registered concentrations of up to 7.3 mg mL^{-1} for every 5 g of sugarcane bagasse.

Limitations/implications: This study focused on how treatments using *T. versicolor* and NaOH affected the release of sugars. Optimization of bioethanol production needs to be considered subsequently in another study.

Findings/conclusions: The results have implications for the use of pretreatments for the production of bioethanol from agro-industrial residues.

Keywords: biofuel, cellulose, residues, fermentation.

INTRODUCTION

Biomass is the fundamental resource for the production of biofuels. Biofuels are considered an alternative energy source to petroleum, with the characteristic of being renewable and low environmental pollution indices. Similar to other biofuels such as biodiesel and biogas, bioethanol is easily produced, although it can compete with food production for human consumption when it is processed directly from crops. Using agro-industrial residues as a source of bioethanol aims to make its production more appealing by not competing with arable lands, contributing to decrease production costs since it is a low cost material, and by generating a lower carbon footprint. In this sense, current bioethanol production from agro-industrial residues does not solve the problem of providing energetic security, since there are still many challenges such as excessive water consumption

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during pretreatment of agro-industrial residues, fostering the production high-added value products within secondary biorefining of agro-industrial residues, and finding strategies to strengthen the energetic density in biofuels which is naturally low.

Lignocellulosic residues, such as sugarcane bagasse (*Saccharum* spp.) aside from cellulose, primarily contain the biopolymers hemicellulose and lignin in their matrix. The presence of these components depends on the species, age, and growth phase of the plant (Cardona and Sanchez, 2007). In order to produce bioethanol, it is necessary to remove the complex matrix of hemicellulose and lignin which surrounds cellulose, by applying pretreatments. Any pretreatment applied to agro-industrial residue should take into account increasing to the maximum the enzymatic conversion of polymeric scaffolds, minimizing the loss of sugars, and avoiding the production of inhibitors which could affect subsequent fermentation processes. There are a great number of physicochemical pretreatments (based on trituration, vapor, acids, strong bases, or a combination of these) and biological pretreatments reported, with well-established advantages and disadvantages (Alvira *et al*., 2010; Cardona *et al*., 2010). The purpose of pretreatments is the removal of lignin and hemicellulose, or rather to break the crystalline structure of cellulose in order to make subsequent saccharification and fermentation processes more efficient. In other words, pretreatments increase the accessibility of enzymes during the processes of hydrolysis, allowing the transformation of cellulose and hemicellulose into fermentable sugars (Sun and Cheng, 2002). Biological and physico-biological pretreatments are based on microorganisms, such as fungi, bacteria or algae, and they are generally characterized as being environmentally friendly, and not generating enzymatic inhibitors of any kind; however, they are slow. Nevertheless, the manipulation of a variety of microorganisms, including those genetically modified, can be attractive in order to make subsequent saccharification and fermentation pretreatment processes of lignocellulosic material more efficient (Alvira *et al*., 2010). Some examples of these systems include strains of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* (thermotolerant strain), which have been used for simple saccharification and fermentation (SHF), simultaneous saccharification and fermentation (SSF), or pre-saccharification followed by SSF (Kádár *et al*., 2010; Mesa *et al*., 2011; Cardona *et al*., 2010). These types of systems have achieved satisfactory results in accordance to those expected from glucose production from residues. *Trametes versicolor* is a white-rot fungus which degrades lignin. These characteristics permit easier saccharification of exposed cellulose in order to transform it to glucose, which in turn can be transformed into bioethanol via fermentation. The release of reducing and total sugars from pretreated fibers is achieved by using a mixture of commercial cellulases during simple saccharification. The production of bioethanol was developed in conditions of simple fermentation using *Saccharomices cerevisiae*. Taking this into consideration, this study evaluated the effects of biological pretreatments using *Trametes versicolor* on the production of total and reducing sugars from sugarcane bagasse.

MATERIALS AND METHODS

Sugarcane bagasse (*Saccharum* spp.) was collected from the sugar refinery "Emiliano Zapata", in Zacatepec, Morelos, Mexico. For its use, we proceeded to dry the bagasse by solar radiation for 72 hours, and subsequently sieved until 0.5 × 0.5 mm fibers were obtained.

The bagasse cane fiber was pretreated in two stages. First pretreatments were applied using *Trametes versicolor*, while in the second stage, a 20 %(W/V) NaOH solution was added under thermal conditions. The experimental design consisted in alkaline-biological pretreatments applied to sugarcane bagasse for the removal of lignin and to decrystallize the resulting cellulose structure, taking into account eight different conditions with two replicas, thus resulting in a 2^k experimental design with two levels of response. This allowed the data analysis through an ANOVA approximation.

A. Obtaining *Trametes versicolor*. *Trametes versicolor* was cultivated in a PDA medium for 6 d at 25 °C (Felisa incubator). An inoculum from this microorganism was prepared by placing a sample (1 cm^2 of the fungus) in another liquid medium through puncturing, forcing it to grow in spores. The composition of the second liquid medium was 30 g L^{-1} malt extract, 10 g L^{-1} yeast extract, and 10 g L^{-1} glucose anhydride. Afterwards the spores were incubated for another 96 hours at 25 °C at a speed of 100 rpm (LabTech incubator, Model LSI-3016A), then subsequently washed, liquefied, and finally centrifuged (Hermle Centrifuge, Model Z323K) with Czapek Dox solution.

B. *Trametes versicolor* inoculation. 5 g of sugarcane bagasse were placed in glass bottles (413 mL) with a cotton plug, to which a certain amount of inoculum solution was added (Factor A: Inoculum volume, 4 and 10 mL). See Table 1. Before the inoculation, 30 mL of Czapek Dox solution was added and the bagasse was sterilized for 15 min at 121 °C. The bagasse was incubated for a certain time (Factor B: Incubation time; 18 and 24 d), and was subsequently washed with ~2L of distilled water in order to eliminate any microorganism residues. The samples were dried and stored for their later use.

C. Alkaline pretreatments. The biologically pretreated fibers had a second conditioning when alkaline treatments were applied. For this process, a 20 %(W/V) NaOH solution was incorporated in accordance with the proportion of weight of the bagasse treated biologically vs volume of alkaline solution (Factor C: 5 and 20 %(W/V), as indicated in Table 1. These samples were again thermally treated at 121 °C for 30 minutes, and subsequently washed until a neutral pH was obtained, and then dried. Figure 1 depicts the sequence of processes carried out during alkaline pretreatments.

Table 1. Description of factors used during pretreatment based on solutions with *Trametes versicolor* and 20 %(W/V) NaOH.

Pretreatment	Factor A	Factor B	Factor C
	Inoculum volume (mL)	Incubation time (Days)	Weight of pretreated bagasse/ Volume of NaOH solution (%W/V)
1	4	18	5
2	10	18	5
3	4	24	5
4	10	24	5
5	4	18	20
6	10	18	20
7	4	24	20
8	10	24	20

Simple Saccharification

In order to saccharify pretreated bagasse, 1.5 g of sample was initially put in contact with an enrichment medium and 0.2 mL of Tween 80, at pH=4.8 (Mesa *et al.*, 2010). The enrichment medium was composed of 0.2 g L^{-1} $CaCl_2$, 1 g L^{-1} NaCl, 1.7 g L^{-1} $MgSO_4$, 2 g L^{-1} K_2HPO_4 and 3 g L^{-1} of yeast extract. The pH was conditioned with a 50 mM citric acid solution. Once these conditions were obtained, the mixture was supplemented with 1.8 mL of cellulases 1,4-(1,3:1,4)-β-D-Glucan, 4-glucano-hydrolase (Celluclast 1.5 L, ≥700 units/g, *Trichoderma reesei* ATCC 26921, Sigma Aldrich) and incubated (180 rpm, 5 °C) for 72 hours (Giese *et al.*, 2012). Once the saccharification process was concluded, the supernatant was separated from the bagasse by centrifugation at 3500 rpm for 20 minutes.

Simple Fermentation

An inoculum of *Saccharomices cerevisiae* was prepared for simple fermentation of the saccharified samples. For this, it was necessary to seed fresh commercial yeast in an agar medium of yeast extract (10 g L^{-1} yeast extract,

Figure 1. Pretreatment process applied to sugarcane bagasse (*Saccharum* spp.).

15 g L^{-1} bacteriological agar, 20 g L^{-1} dextrose and 20 g L^{-1} peptone), during an incubation period of 3 days at 25 °C. After incubation, the mixture was stored at 4 °C. At the same time, a nutritive solution (20 g L^{-1} dextrose, 20 g L^{-1} peptone and 10 g L^{-1} yeast extract) was prepared and added for the inoculum to grow, which required another incubation period at 30 °C and 100 rpm for 24 h. Our simple fermentation was carried out in batches. In order to achieve this, ~24 mL of the centrifuged liquid fraction of the saccharified samples was transferred to 50 mL test tubes and conditioned to a pH=6.5. A 0.1 M NaOH solution was added by dosage. The inoculum produced from the yeast was re-suspended and incorporated to this same saccharified portion, and subsequently incubated at 30 °C for 8 h. The fermented sample was centrifuged for 20 min at 3500 rpm in order to analyze the supernatant and discard the precipitate (de Souza *et al.*, 2012). Figure 2 illustrates the sequence of procedures applied during simple saccharification and simple fermentation.

Quantification of total sugars, reducing sugars and ethanol concentration

The quantification of total sugars was achieved with the phenol-sulfuric acid method with glucose calibration (Dubois *et al.*, 1956), in both saccharification and fermentation stages under each of the proposed conditions. Similarly, the DNS colorimetric technique was used to determine reducing sugar concentrations (Harisha, 2007), which is based on the capacity of glucose to reduce 3, 5-dinitrosalicylic acid. All of the colorimetric determinations were conducted at 540 nm in a UV-Visible Jenway spectrophotometer, Genova Model. Finally, the quantification of bioethanol was also performed through colorimetry using the enzyme assay equipment Megazyme (Megazyme International Ireland, Wicklow, Ireland) to detect ethanol. In this stage, the assay consisted of two subsequent enzymatic reactions. During the first reaction (Equation (1)), the bioethanol is catalyzed by alcohol dehydrogenase (ADH) to acetaldehyde, in the presence of nicotinamide adenine dinucleotide (NAD^+),

$$CH_3CH_2OH + NAD^+ (ADH) \rightarrow CH_3CHO + NADH + H^+ \quad (1)$$

Afterwards, the same acetaldehyde is oxidized to acetic acid in the presence of aldehyde dehydrogenase (Al-DH) and NAD^+ (Equation (2)).

$$CH_3CHO + NAD^+ + H_2O(Al-DH) \rightarrow CH_3COOH + NADH + H^+ \quad (2)$$

The quantification of bioethanol was achieved by determining the amount of NADH produced, which presents a significant absorption at 340 nm.

RESULTS AND DISCUSSION

Table 2 shows the quantification of total sugars after saccharification and fermentation processes, applied to samples pretreated sequentially with *T. versicolor* and NaOH. At a glance, these results reveal two tendencies when 10 mL of the inoculum was added to the wild samples (Factor A): higher production of total sugars after saccharification, and lower concentration

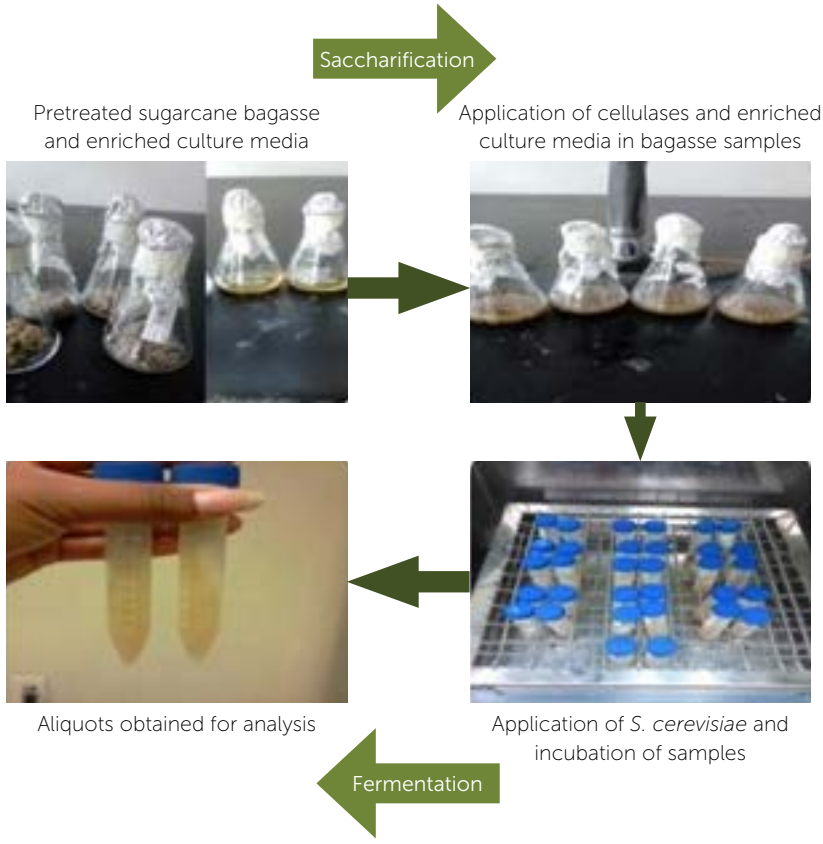

Figure 2. Sequence of processes for saccharification of pretreated sugarcane bagasse samples (*Saccharum* spp.), and of fermentation in their respective liquid extracts, both in simple conditions.

Table 2. Quantification of total sugars after simple saccharification and fermentation processes of samples pretreated with *Trametes versicolor*/NaOH.

No.	Factor A	Factor B	Factor C	Total sugars (mg/mL)			
	Inoculum volume (mL)	Incubation Time (days)	% W/V Bagasse/ NaOH Solution	After Saccharification		After Fermentation	
				Rep. 1	Rep. 2	Rep. 1	Rep. 2
1	4	18	5	13,35	25,28	13,52	16,25
2	10	18	5	30,61	35,98	10,6	9,4
3	4	24	5	37,52	32,32	16,62	13,85
4	10	24	5	25,8	39,05	14,79	12,21
5	4	18	20	16,46	17,5	8,39	8,35
6	10	18	20	36,19	34,38	14,75	12,62
7	4	24	20	24,62	22,21	17,78	18,68
8	10	24	20	46,69	25,35	16,25	15,91

of these sugars after fermentation. These tendencies occur independently of the bagasse/NaOH %(W/V) ratio (Factor C), which allows one to assume that this factor was irrelevant during saccharification and fermentation.

A simple analysis can also be applied to the results of reducing sugars, as shown in Table 3, highlighting that analogous trends are present in the concentration of reducing sugars as with total sugars; namely, that the addition of 10 mL of *Trametes versicolor* inoculum influences the production of sugars, and that Factor C is irrelevant. This should be taken into account given that the bioethanol yield directly depends on the amount of reducing sugars present. The results shown in Table 3 also suggest that in both saccharification and fermentation processes, a big portion of the total sugars actually corresponds to reducing sugars, within the margins of experimental error.

Elevated sugar concentrations are to be expected in our saccharified samples due to the high cellulose content in the sugarcane bagasse (~50%) (Sun and Cheng, 2002). In order to determine which factor or source had the highest attributable effect on production of total and reduced sugars, an ANOVA analysis was conducted considering the factorial design described above. Table 4 shows the ANOVA analysis with a significance of α=0.05 for both types of sugars. The value of α indicates from which probability ANOVA will not detect a significant difference. Thus, at lower probability there will be higher certainty that there is a significant difference.

The ANOVA analysis confirms the initial observations. Factor C % (bagasse weight/volume of 20 %(W/V) NaOH solution) is not significant in the release of reducing or total sugars during the saccharification process. On the contrary, the volume of solution incorporated containing *Trametes versicolor* (Factor A) has an important effect,

Table 3. Quantification of reducing sugars and ethanol after simple saccharification and fermentation processes of samples pretreated with *Trametes versicolor*/NaOH.

No.	Factor A	Factor B	Factor C	Total sugars (mg/mL)				Ethanol (mg/mL)	
	Inoculum volume (mL)	Incubation Time (days)	% W/V Bagasse/ NaOH Solution	After Saccharification		After Fermentation			
				Rep. 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2
1	4	18	5	8.29	18.64	8.67	15.15	4.03	5.42
2	10	18	5	32.68	24.99		4.02	4.63	5.69
3	4	24	5	25.3	25.53	9.41	10.82	6.13	6.33
4	10	24	5	34.31	24.85	9.22	7.34	5.52	5.49
5	4	18	20	14.54	16.93	1.83	2.34	5.05	5.47
6	10	18	20	36.65	38.58	11.64	6.95	7.31	6.81
7	4	24	20	18.56	17.33	14.37	12.38	5.08	4.81
8	10	24	20	28.41	25.17	12.3	12.77	5.64	5.64

as well as its interference on the number of incubation days (Factor AB). In other words, statistically speaking the volume of inoculum that was added to the wild samples affects the production of both types of sugars. On the other hand, the ANOVA analysis also suggests that the days of incubation in this study (18 and 24 d) are not relevant for the release of reducing sugars, but is important for total sugars. It is important to point this out, since one of the disadvantages of biological pretreatments is the time required for the biomass to be exposed to microorganisms. We suggest a separate study to explore this finding further.

Table 4. ANOVA analysis for the quantification of total and reducing sugars after saccharification of wild samples.

Origin of variation	Total sugars		Reducing sugars	
	F	F (0.05)	F	F (0.05)
A	8,57	0,019	36,44	0
B	2,29	0,169	0,24	0,637
AB	2,34	0,164	8,48	0,02
C	0,32	0,584	0,01	0,927
AC	1,8	0,217	1,81	0,216
BC	0,27	0,616	6,55	0,034
ABC	0,55	0,478	0,05	0,832

The impact of fermentation on sugars is also taken into account. This is analyzed in Table 5. Regardless of the type of pretreatment applied to the sugarcane bagasse, it stands out that simple fermentation induced a significant decrease in reducing sugars. Once more the effect of adding 10 mL of inoculum solution was noted, since the increased concentration of reducing sugars permitted higher consumption and better yield when producing bioethanol, compared to adding 4 mL.

It is difficult to correlate the direct effect of the of the biological-alkaline pretreatment conditions with bioethanol efficiency, excluding the intuitive idea of having an elevated concentration of reducing sugars. The fermentation process has its own variables that need to be controlled, including fermentation temperature, yeast concentration, time of exposure and presence of inhibitors, among others. Therefore, a separate study is needed to analyze optimization of fermentation using samples pretreated with *Trametes versicolor*. The importance of this study is valued when we consider that sugarcane is one of the principal crops in the country, and especially in the state of Morelos. Thus, finding adequate methods for producing bioethanol from its residues is of great importance.

CONCLUSIONS

This study established conditions for production of reducing and total sugars, as well as bioethanol, from sugarcane bagasse that was pretreated with *Trametes versicolor*. A volume of 10 mL of this inoculum had a significant influence on the liberation of reducing and total sugars, and in the subsequent production of bioethanol. Under these same conditions, the incubation time intervals for the fungus acting on the fiber (18 and 24 d) allowed obtaining a concentration of reducing sugars in a range of 25.17 to 38.58 mg mL^{-1}. Slightly higher concentrations were found in the samples incubated for 18 d. Our ANOVA analysis sugests that during the alkaline pretreatment, the proportion of pretreated sugarcane bagasse mass *vs* the volume of 20 %(W/V NaOH solution used were not significant enough to influence the production of sugar.

Table 5. Concentration of reducing sugars consumed and bioethanol production.

Pretreatment	Factor A	Factor B	Factor C	Reducing Sugars				Ethanol (mg/mL)	
	Inoculum volume (mL)	Incubation time (days)	% W/V Bagasse/NaOH Solution	Consumption (m/mL)		% Consumption			
				Rep. 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2
1	4	18	5	−0,38	3,49	−4,58	18,72	4,03	5,42
2	10	18	5	22,8	20,97	69,76	83,91	4,63	5,69
3	4	24	5	15,89	14,71	62,81	57,62	6,13	6,33
4	10	24	5	25,09	17,51	73,13	70,46	5,52	5,49
5	4	18	20	12,71	14,59	87,41	86,18	5,05	5,47
6	10	18	20	25,01	31,63	68,24	81,99	7,31	6,81
7	4	24	20	4,19	4,95	22,58	28,56	5,08	4,81
8	10	24	20	16,11	12,4	56,71	49,27	5,64	5,64

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