DILUTE ACID HYDROLYSIS OF SWEET SORGHUM BAGASSE AND FERMENTABILITY OF THE HEMICELLULOSIC HYDROLYSATE

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Abstract - This study aimed to determine the best dilute acid hydrolysis condition for the hemicellulosic fraction of sweet sorghum bagasse for ethanol production by *Scheffersomyces stipitis*. The experiment followed a 2^3 factorial design with four central points, and had as variables: sulfuric acid concentration, temperature and hydrolysis time. Sorghum bagasse presented the following chemical composition: 24.77% of lignin, 31.28% of hemicellulose and 34.80% of cellulose. The hydrolysis that resulted in the highest sugars concentration (14.22 g/L of xylose and 2.42 g/L glucose) was 1.75% H_2SO_4, 121 ºC and 40 minutes. This same condition provided low concentrations of toxic compounds (1.34 g/L of acetic acid, 0.90 g/L of phenol; 124.54 mg/L of hydroxymethylfurfural (HMF) and 978 mg/L of furfural). The fermentation of the hemicellulose-derived sugars by *S. stiptis* resulted in 22 g/L of ethanol, $Y_{P/S}$ 0.40 g/g and Qp 0.34 g/L.h.
Keywords: Fermentation; Hemicellulose; Bioethanol; *Scheffersomyces stipitis*.

INTRODUCTION

Most of the chemicals currently used in our society comes from fossil sources. However, the oscilation of prices of these resources, their uncertain availability, and environmental concerns of their exploration have created a demand for alternative chemicals based on renewable sources (Cherubini and Stroman, 2011). In this sense, the use of lignocellulosic biomass to obtain commodities tends to become increasingly important in the future society, as a complement to existing oil refineries (Jönsson et al., 2013).

The greatest potential for the long-term production of ethanol is the use of cellulosic materials, which comprise crop residues, energy crops, short rotation plants and forestry wastes (Wang et al., 2012). Several studies have focused on the use of agricultural waste as a low cost raw material for conversion into fuel ethanol, such as wheat straw (Singhania et al., 2014; Saha et al., 2015), rice straw (Belal, 2013; Silva et al., 2014), corn stover (Gladis et al., 2015; Buruiana et al., 2014) and bagasse (Rabelo et al., 2014; Gutiérrez-Rivera et al., 2014).

Lignocellulosic biomass is composed of three major structural biopolymers called cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer formed from glucose subunits linked together by glycosidic bonds of the β-1,4 type, whereas hemicellulose is a heteropolymer formed from hexose and pentose sugars such as glucose, mannose, galactose, xylose and arabinose (Pérez et al., 2002; Singh et al., 2011; Pu et al., 2013). Another difference between cellulose and hemicellulose is that the latter has ramifications made of short side chains and, in contrast to cellulose,

the polymers are more readily hydrolysable (Pérez et al., 2002). Lignin, in turn, is an aromatic polymer synthesized from phenylpropanoid precursors (guaiacyl, syringyl and p-hydroxyphenyl) and has high molecular weight, irregular structure and is highly insoluble and recalcitrant (Wong, 2009; Pu et al., 2013).

The process of ethanol production from lignocellulosic materials includes steps of pretreatment, hydrolysis of cellulose and hemicellulose into monomeric sugars, fermentation of sugars to ethanol, and finally, distillation and purification of ethanol (Jafari et al., 2011). During the pretreatment, the biomass is subjected to a combination of physical, thermal or chemical actions, which have the purpose of making the plant cell walls more susceptible to the following steps (Dien et al., 2009). The pre-treatment using dilute sulfuric acid has been the most widely used treatment for the separation of cell wall components with high efficiency and at low cost. This procedure results in hemicellulose hydrolysate and cellulignin (Dussán et al., 2014). This method can solubilize the hemicellulose into monomeric sugars (glucose, xylose, etc.) and soluble oligomers, thus improving the conversion of cellulose (Mehmood et al., 2009).

The cost efficiency in a biorefinery is dependent on the development of biomass pre-treatment technologies that are effective for obtaining high yields of fermentable sugars from biomass sources at a lower cost (Oh et al., 2015). In addition, the use of the different types of sugars present in the raw materials must be considered, including pentoses, such as arabinose and mainly xylose, derived from the hemicellulosic fraction of the biomass.

Some yeasts demonstrate great potential to ferment pentoses, such as yeast strains like *Scheffersomyces (Pichia) stipitis*, *Candida shehatae* and *Pachysolen tannophilus* (Agbogbo and Coward-Kelly, 2008). Among yeasts, *S. stipitis* has been shown to be industrially interesting for metabolizing xylose rapidly with high ethanol yields (Nigam, 2001) and low xylitol production (Toivola et al., 1984).

The generation of by-products such as aromatic compounds, aliphatic acids, aldehydes, furans, and inorganic ions, alcohols or other products that can act as microorganism inhibitors is strongly dependent on the feedstock and the method of pre-treatment used for the lignocellulosic biomass (Jönsson et al., 2013). These toxic compounds are produced during the hydrolysis process from the breakdown of lignocellulosic materials. Phenolic compounds are produced from the degradation of lignin (Barakat et al., 2012), while acetic acid is derived from acetyl groups present in the hemicellulose polymers that are released during the hydrolysis (Silva et al., 2013). Furfural and hydroxymethylfurfural (HMF) come from

the degradation of pentoses (xylose and arabinose) and hexoses (glucose, mannose and galactose), respectively (Dagnino et al., 2013).

Currently world ethanol production is basically derived from two crops, corn and sugarcane. However, with increasing demand for food production and increased consumption of fuel ethanol to unprecedented levels, raw materials for ethanol production must become more diversified. Accordingly, sweet sorghum (*Sorghum bicolor* L. Moench) is a raw material that contains high concentrations of sugars that can be directly converted to ethanol by fermentation (Wu et al., 2010). Hybrids developed from these cultures in genetic improvement programs for ethanol production have been tested with good production prospects (Barcelos et al., 2013). Therefore, sorghum is presented as an interesting complementary option to sugarcane to compose the energy matrix, especially in the sugarcane off season (May et al., 2012). Considering that the increase in the sweet sorghum crop areas for ethanol production will likely lead to a significant increase in the quantities of sorghum bagasse that can be used for second generation ethanol production, the aim of this study was to determine the best acid hydrolysis condition of the hemicellulosic fraction of sweet sorghum bagasse and evaluate the hydrolysate fermentability by the pentose fermenting yeast *S. stipitis*.

MATERIALS AND METHODS

Chemical characterization of sorghum bagasse

Sweet sorghum bagasse (cultivar CVSW 80007) was kindly provided by Canavialis/Monsanto, São Paulo, Brazil. The biomass was ground in a Wiley mill to a particle size of 50 mesh, packed and stored at -4 °C. The biomass was dried at 105 °C to constant weight and characterized for the lignocellulosic composition, ash, moisture and extractives according the method described by the National Renewable Energy Laboratory - NREL (Sluiter et al., 2008a; Sluiter et al., 2008b).

Evaluation of acid hydrolysis of sorghum bagasse

A central composite design (CCD) and response surface methodology was proposed to study the acid hydrolysis of the hemicellulosic fraction of sorghum bagasse. The tests were conducted according a 2^3 factorial design with four replications at the center point. The independent variables were the hydrolysis time in an autoclave (40, 50, and 60 min), sulfuric acid concentration (0.75, 1.25 and 1.75% w/v) and temperature (111, 115 and 121 ºC). The real and coded values are shown in Table 1. For each assay, 10 g of dry biomass were placed in 500 mL Erlenmeyer flasks, to which were added 200 mL of sulfuric acid. After

Table 1. Matrix of the 2^3 planning with the coded values, real values and the results of sugar concentration.

Assay	Variable							AA (g/L)	Phenolics (g/L)	HMF(g/L) (mg/L)	Fur (mg/L)
	Temp (ºC)	Time (minutes)	H₂SO₄ (%)	pH	Ara (g/L)	Xyl (g/L)	Glu (g/L)				
1	-1 (111)	-1 (40)	-1 (0.75)	1.15	2.61	2.2	1.08	0.78	0.58	40.36	403
2	1 (121)	-1 (40)	-1 (0.75)	1.11	2.08	5.12	1.31	0.85	0.79	91.23	772
3	-1 (111)	1 (60)	-1 (0.75)	1.13	1.92	1.75	0.69	0.93	0.42	52.87	481
4	1 (121)	1 (60)	-1 (0.75)	1.18	1.61	5.24	0.38	1.04	0.77	91.65	807
5	-1 (111)	-1 (40)	1 (1.75)	0.95	1.22	9.55	1.81	1.29	0.65	62.85	571
6	1 (121)	-1 (40)	1 (1.75)	1.02	0.95	14.22	2.42	1.34	0.90	124.54	978
7	-1 (111)	1 (60)	1 (1.75)	1.08	0.5	8.27	1.78	1.32	0.59	48.46	638
8	1 (121)	1 (60)	1 (1.75)	0.94	0.45	12.41	1.96	1.47	0.83	81.10	985
9	0 (115)	0 (50)	0 (1.25)	1.02	1.45	7.24	1.35	1.11	0.72	69.90	712
10	0 (115)	0 (50)	0 (1.25)	1.00	1.43	7.23	1.41	1.12	0.71	70.48	707
11	0 (115)	0 (50)	0 (1.25)	1.03	1.46	7.27	1.38	1.15	0.72	68.81	713
12	0 (115)	0 (50)	0 (1.25)	1.03	1.47	7.26	1.39	1.13	0.73	69.36	701

Temp: temperature; Ara: arabinose; Xyl: xylose; Glu: glucose; AA: Acetic acid; HMF: hydroxymethylfurfural; Fur: Furfural.

hydrolysis, the hydrolysate was filtered and analyzed for pH, concentration of xylose, glucose, arabinose, phenols, furfural, hydroxymethylfurfural (HMF) and acetic acid.

Validation of acid hydrolysis experiments

For validation of the results obtained during the CCD, experiments were carried out at 121 °C and 40 min (best conditions obtained in the acid hydrolysis study) and varying the sulfuric acid concentration (1.45, 1.60, 1.75, 2.15 and 2.30% w/v). The hydrolysates obtained were evaluated for the concentration of xylose, glucose, arabinose, phenols, furfural, HMF and acetic acid.

Bioethanol production from the hemicellulosic fraction of sweet sorghum bagasse

Preparation of sorghum bagasse hemicellulosic hydrolysate

The sorghum bagasse hemicellulosic hydrolysate obtained under the optimized condition was used for ethanol production. Before fermentation, hydrolysate was three-fold concentrated under vacuum at 65 °C. Then it was detoxified by pH change and adsorption with activated charcoal (Marton et al., 2006). The detoxified concentrated hydrolysate was analyzed for sugars and toxic compounds.

Microorganism and inoculum preparation

The yeast utilized was *Scheffersomyces stipitis* ATCC 58376, maintained in Malt Extract Agar medium at 4 °C. Inoculum preparation was carried out in 125 mL Erlenmeyer flasks containing 50 mL of YMP medium (20 g/L xylose, 3 g/L malt extract, 3 g/L yeast extract and 5 g/L peptone) in a rotating incubator at 200 rpm, 30 °C for 16 hours. Cells were recovered by centrifugation at 2000 x g for 20 min, rinsed twice with sterile distilled water, resuspended in sterile distilled water and used as inoculum at an initial cell concentration of 0.5 g/L.

Fermentations conditions

Fermentation was conducted in a bioreactor (Biotec 7.5 V, Tecnal) with working volume of 4.5 L. The detoxified concentrated hydrolysate was autoclaved at 121 °C and supplemented with the same nutrients of the medium YMP (except xylose) and pH was adjusted to 5.5 with NaOH (0.5 mol/L). The kLa (oxygen transfer coefficient) was 4.9 h⁻¹ (Silva et al., 2011), obtained with aeration of 0.25 vvm and agitation of 200 rpm. After inoculation, the fermentation was conducted at 30 °C for 78 hours.

Analytical methods

The concentrations of glucose, xylose, arabinose, acetic acid and ethanol in the hydrolysates were determined by high-performance liquid chromatography (HPLC) using a liquid chromatograph (Shimadzu 20A), Rezex column ROA Oganic Acids H + (8%) 150 x 7.8 mm; mobile phase H_2SO_4 0.005 mol/L, flow rate 0.6 mL/min, oven temperature 65 °C and a refractive index detector. Before injection samples were purified in Sep-Pak C18 cartridges (Waters).

For the determination of furfural and HMF, the following conditions were used: Column Kinetex Phenomenex, C18, 5 μm, 100 A, 150 x 4.6 mm; oven temperature 25 ºC; ultraviolet detector (SPD-20A) at 208 nm, mobile phase acetonitrile/water (1:8) with 1% acetic acid and flow rate 0.8 mL/min. Before injection samples were filtered in PTFE VertiPureTM (polytetrafluorethylene) syringe filters with pore size 0.45 μm and diameter 13 mm.

Total phenols were analyzed according to the Folin Ciocalteu method using a standard curve of vanillin (Singleton et al., 1999). The protein content was determined by the Kjeldahl method (AOAC, 1995). Cell concentration was determined by turbidimetry and calculated from a standard curve made by plotting dry weight of cells vs O.D.₆₀₀ₙₘ.

RESULTS AND DISCUSSION

Chemical characterization of lignocellulosic biomass

Sweet sorghum bagasse presented 10.41% (± 0.49) humidity; 3.31% (± 0.34) ash; 8.74% (± 1.33) extractives and 4.76% (± 0.58) protein. Lignocellulosic composition was 36.08% (± 1.13) cellulose, 31.18% (± 0.79) hemicellulose and 24.77% (± 1.99) lignin, values similar to those described by Liu et al. (2012) for sweet sorghum bagasse, which were 39.07% cellulose and 34.57% hemicellulose, except lignin, 13.29%. Kim and Day (2011) reported that the percentages of cellulose, hemicellulose and lignin in sweet sorghum bagasse were 45%, 27% and 21%, respectively. The variations in the chemical composition of sorghum biomass may be the result of the cultivar type or geographic and environmental factors in which the plant was grown. For sugarcane bagasse, a biomass widely studied as raw material for bio-processes including bioethanol, reported percentages are between 38-42% of cellulose, 27-24% of hemicellulose, and 22-18% of lignin (Kim and Day., 2011; Guilherme et al., 2015).

According to the results shown above, sorghum bagasse emerges as an alternative biomass for bioprocesses which utilize not only the cellulosic fraction but also hemicellulose as a source of sugars.

Statistical analysis of the acid hydrolysis

Table 1 presents the planning matrix for the acid hydrolysis of sweet sorghum bagasse with the variables in their coded and real values (in parentheses) and the results for sugars and inhibitory compounds.

Test 6 (sulfuric acid concentration 1.75% w/v, temperature 121 °C and hydrolysis time 40 minutes) resulted in the highest amounts of xylose (14.22 g/L) and glucose (2.42 g/L) (Table 1). After hydrolysis the glucose amounts were not superior to 2.42 g/L for all conditions tested, which may be explained by the fact that under the mild conditions cellulose was not hydrolyzed, the glucose possibly arising from the side chains of hemicellulose. According to Liu et al. (2012) the glucose release during hydrolysis of cellulose begins to occur when the reaction temperature increases. In this regard, the glucose yield increases significantly at 150 °C after 120 minutes. Low concentrations of glucose in the hemicellulosic hydrolysates have been considered acceptable because glucose contributes to microbial growth in the early hours of fermentation and assists in increasing product yields such as ethanol and xylitol in pentose-fermenting microorganisms (Agbogbo et al., 2006; Canilha et al., 2008).

Table 2 shows the main effects and interactions of the factors obtained in the study of the acid hydrolysis of sorghum bagasse. The main linear effects for the concentrations of sugars and toxic compounds were statistically significant at the significance level of 5% ($p < 0.05$).

The increase in the percentage of sulfuric acid from 0.75 to 1.75% promoted a positive effect on the glucose and xylose responses, resulting in 1.12 and 7.29-fold increases in the glucose and xylose concentrations in the hydrolysate, respectively. Regarding the temperature factor ($p = 0.000001$), the variation from 111 to 121 °C increased the xylose concentration by 3.59-fold. However, an increase of the hydrolysis time from 40 to 60 minutes contributed to reductions of 0.45- and 0.59-fold, respectively, for both of the sugars glucose and xylose ($p < 0.05$).

The results of this study are in agreement with those of Poonsrisawat et al. (2013) who affirm that, for sorghum bagasse, temperature and sulfuric acid concentration were the variables which had a significant effect on glucose and xylose yields; however, the residence time had no significant effect.

The highest arabinose concentration (2.61 g/L) was obtained with the lower levels (-1) of all variables (sulfuric acid 0.75%, 111 °C and 40 min), whereas under stronger conditions, arabinose suffered degradation (Table 1). Contrary to what was observed for glucose and xylose, the sulfuric acid factor ($p = 0.000002$) had a negative effect on arabinose. The increase in the percentage of acid used (0.75 to 1.75%) reduced the concentration of this sugar by 1.27-fold (Table 2). According to Liu et al. (2012), arabinose is released easily and is also susceptible to degradation into furfural at high temperatures.

With respect to inhibitor formation, the data presented in Table 1 show that the treatment 8, which consisted in the highest levels (+1) of the variables (sulfuric acid 1.75%, 121 °C and 60 minutes) resulted in the highest release of acetic acid (1.47 g/L). On the contrary, the lowest levels of the variables (-1) provided the lowest concentration of this inhibitor compound. Table 2 shows that acetic acid formation was favored by temperature ($p = 0.001754$), reaction time ($p = 0.000795$) and percentage of sulfuric acid ($p = 0.000018$), promoting increments of 0.09-, 0.12- and 0.45-fold, respectively, in the concentration of this acid in the hydrolyzate.

The strongest condition tested (sulfuric acid 1.75%, 121 °C and 60 minutes) also promoted an increase in the concentration of furfural (Table 1). This suggests that furfural formation was mainly related to the degradation of arabinose, as the concentrations of this sugar decreased upon increasing the acid concentration and temperature, as shown above.

From the results of Table 2 it is possible to verify statistically that the fator temperature exerted a more severe effect on the degradation of sugars into toxic compounds, resulting in increases of 44.99 and 362.25-fold for HMF and furfural, respectively. However, for the furfural response, besides the temperature

Table 2. Main effects of the acid hydrolysis planning with variation in temperature, time, acid concentration and their interactions for the responses concentration of sugars and toxic compounds.

Factor	Effect	Std.Err.	t(3)	p	-95%	+95%
			Arabinose			
Mean/Interc	1.43	0.004	289.88	0.000000	1.40	1.44
(1L) Temperature (ºC)	-0.29	0.012	-24.01	0.000158	-0.33	-0.25
(2L) Time (min)	-0.59	0.012	-49.27	0.000019	-0.63	-0.55
(3L) Acid (%)	-1.27	0.012	105.58	0.000002	-1.31	-1.23
1L by 2L	0.11	0.012	9.10	0.002796	0.07	0.14
1L by 3L	0.13	0.012	10.76	0.001714	0.09	0.16
2L by 3L	-0.01	0.012	-1.24	0.302445	-0.05	0.02
			Glucose (g/L)			
Mean/Interc	1.41	0.0072	195.83	0.000000	1.39	1.43
(1L) Temperature (ºC)	0.17	0.0176	10.04	0.002103	0.12	0.23
(2L) Time (min)	-0.45	0.0176	-25.59	0.000131	-0.50	-0.39
(3L) Acid (%)	1.12	0.0176	63.78	0.000008	1.07	1.18
1L by 2L	-0.24	0.0176	-13.71	0.000838	-0.29	-0.18
1L by 3L	0.21	0.0176	12.30	0.001156	0.16	0.27
2L by 3L	0.20	0.0176	11.73	0.001329	0.15	0.26
			Xylose			
Mean/Interc	7.22	0.0036	1989.26	0.000000	7.21	7.23
(1L) Temperature (ºC)	3.59	0.0088	403.48	0.000001	3.56	3.61
(2L) Time (min)	-0.59	0.0088	-66.87	0.000007	-0.62	-0.56
(3L) Acid (%)	7.29	0.0088	819.88	0.000003	7.26	7.32
1L by 2L	0.22	0.0088	25.28	0.000136	0.19	0.25
1L by 3L	0.31	0.0088	35.40	0.000050	0.28	0.34
2L by 3L	-0.45	0.0088	-50.57	0.000017	-0.47	-0.42
			Acetic acid			
Mean/Interc	1.12	0.0037	302.09	0.000000	1.11	1.13
(1L) Temperature (ºC)	0.09	0.0091	10.68	0.001754	0.06	0.12
(2L) Time (min)	0.12	0.0091	13.96	0.000795	0.09	0.15
(3L) Acid (%)	0.45	0.0091	49.56	0.000018	0.42	0.48
1L by 2L	0.03	0.0091	3.56	0.037815	0.003	0.06
1L by 3L	0.007	0.0091	0.82	0.471524	-0.02	0.03
2L by 3L	-0.04	0.0091	-4.65	0.018695	-0.07	-0.01
			Phenolics			
Mean/Interc	0.69	0.0027	252.66	0.000000	0.68	0.70
(1L) Temperature (ºC)	0.26	0.0067	38.77	0.000038	0.24	0.28
(2L) Time (min)	-0.07	0.0067	-11.44	0.001431	-0.09	-0.05
(3L) Acid (%)	0.10	0.0067	15.14	0.000626	0.08	0.12
1L by 2L	0.03	0.0067	4.80	0.017203	0,01	0.054
1L by 3L	-0.017	0.0067	-2.58	0.081432	-0.03	0.004
2L by 3L	0.012	0.0067	1.84	0.162008	-0.009	0.03
			HMF			
Mean/Interc	72.97	0.2091	348.91	0.000000	72.30	73.63
(1L) Temperature (ºC)	44.99	0.5122	87.83	0.000003	43.36	46.62
(2L) Time (min)	-12.22	0.5122	-23.86	0.000161	-13.85	-10.59
(3L) Acid (%)	11.21	0.5122	21.88	0.000209	9.57	12.84
1L by 2L	-9.28	0.5122	-18.12	0.000366	-10.91	-7.65
1L by 3L	0.17	0.5122	0.33	0.761841	-1.46	1.80
2L by 3L	-18.69	0.5122	-36.48	0.000045	-20.32	-17.05
			Furfural			
Mean/Interc	705.66	1.5877	444.45	0.000000	700.61	710.71
(1L) Temperature (ºC)	362.25	3.8890	93.14	0.000003	349.87	374.62
(2L) Time (min)	46.75	3.8890	12.02	0.001239	34.37	59.12
(3L) Acid (%)	177.25	3.8890	45.57	0.000023	164.87	189.62
1L by 2L	-25.75	3.8890	-6.62	0.007016	-38.12	-13.37
1L by 3L	14.75	3.8890	3.79	0.032165	2.37	27.12
2L by 3L	-9.7500	3.8890	-2.50	0.087166	-22.12	2.62

(p = 0.000003), the fator time (p = 0.001239) and percentage of sulfuric acid (p = 0.000023) also promoted increments of furfural concentration in the hydrolysate by 46.75 and 177.25-fold, respectively.

Jeevan et al. (2011) found that furfural and HMF levels increased when increasing the reaction time and the sulfuric acid concentration in optimization studies of the acid hydrolysis of corn cobs.

The interaction of the factors temperature x reaction time, as well as the interaction time x acid concentration had a negative effect (p < 0.05) on the formation of furfural and HMF, since they reduced the formation of these compounds in the hydrolysate. For HMF it was verified that the factors time and acid concentration together (p = 0.000045) reduced the formation of this compound by 18.69-fold, followed by the interaction of the factors temperature and reaction time (p = 0.000366), with a reduction in the order of 9.28-fold. For furfural, the interaction temperature x reaction time (p = 0.007016) led to a decrease of this inhibitor formation by 25.75-fold, while the factor reaction time x acid concentration (p = 0.087166) was not significant. The interaction temperature x acid concentration (p = 0.032165) favored the formation of fufural, increasing the concentration of this compound by 14.75-fold. Qi et al. (2008) found that the xylose degradation rate is faster than for glucose and the kinetic constant for degradation of xylose is mainly dependent on the temperature and hydrogen ion concentration.

With respect to the phenolic compounds, when the variables temperature, acid concentration and the interaction temperature x acid concentration were increased from the level (-1) to (+1) there was an increase in the concentration of phenolics. On the other hand, when time was increased from 40 to 60 minutes there was a decreased response for phenolics concentration. However, the interactions temperature x time and sulfuric acid concentration x time are below the significance limit.

According to the literature, the inhibitor concentrations vary for each type of biomass and hydrolysis condition employed. Zhang et al. (2015) obtained 0.99 g/L of acetic acid, 12.22 g/L of furfural, 0.73 g/L of HMF and 1.60 g/L of phenolics during hydrolysis of sorghum bagasse at 170 °C, acid concentration of 1.0% (w/w) and 10 min of reaction, a condition that resulted in the highest glucose concentration (70%). Concentrations values ranging from 0.25 g/L to 0.54 g/L furfural, 0.06 g/L to 1.56 g/L HMF, 1.87 to 2.80 g/L of acetic acid and 0.22 to 2.12 g/L phenolic were observed for corncob (Zhang et al., 2015; Ping et al., 2013), sorghum straw (Sene et al., 2011) and sugarcane bagasse (Martin et al., 2007). Thus, the wide range of the reported inhibitory concentrations depends on the type of biomass, hydrolysis conditions, severity of the treatment employed and the experimental conditions used in each experiment.

Analysis of variance

The analysis of variance (ANOVA) demonstrated that the proposed model is valid and that the equation

parameters fit the experimental data for arabinose, glucose and xylose, i.e., that the first order model (linear) was significant. Moreover, the coefficient of determination (R^2) was high, which indicates that the linear mathematical model for the production of sugars was valid for all the three cases.

The equations to predict the response variables are presented according to the coded values of the independent variables, indicating the statistically significant values at a confidence level of 95% for arabinose (Equation 1), glucose (Equation 2) and xylose (Equation 3). The statistically non-significant parameters (interaction time x acid concentration for arabinose) were eliminated from the model.

For the phenolic and HMF content, despite the lack of significant adjustment, the model can still be considered valid for predictive purposes, because the results showed high determination coefficient (R2> 0.96) at 95% confidence. For acetic acid (Equation 4) and furfural (Equation 5) the model exhibited no significant lack of fit, indicating that the predictive equation can be used for any combination of the variable values.

$$Arabinose(g/L) = 1.43 - 0.29X_1 - 0.59X_2 - 1.27X_3 + \\ + 0.11X_1X_2 + 0.13X_1X_3 \tag{1}$$

$$Glucose(g/L) = 1.41 + 0.17X_1 - 0.45X_2 + 1.12X_3 - \\ - 0.24X_1X_2 + 0.21X_1X_3 + 0.20X_2X_3 \tag{2}$$

$$Xylose(g/L) = 7.22 + 3.59X_1 - 0.59X_2 + 7.29X_3 + \\ + 0.22X_1X_2 + 0.31X_1X_3 - 0.45X_2X_3 \tag{3}$$

$$Acetic\ acid(g/L) = 1.12 + 0.097X_1 + 0.127X_2 + 0.452X_3 + \\ + 0.03X_1X_2 - 0.04X_1X_3 \tag{4}$$

$$Furfural(g/L) = 705.66 + 362.25X_1 + 46.75X_2 + 177.25X_3 - \\ - 25.75X_1X_2 + 14.75X_1X_3 \tag{5}$$

where X_1 is the encoded value for temperature, X_2 is the encoded value for time and X_3 the encoded value for acid concentration.

Surface response for the production of sugars and toxic compounds

The response surfaces were generated for arabinose, glucose and xylose (Figure 1) and inhibitors (Figure 2) on the basis of the significant factors of the independent variables.

The response surface of arabinose shows that the lowest temperature (111 ºC), the shortest time (40 minutes) and lowest acid concentration (0.75%) resulted in the highest amount of arabinose in the sorghum bagasse hemicellulosic hydrolysate.
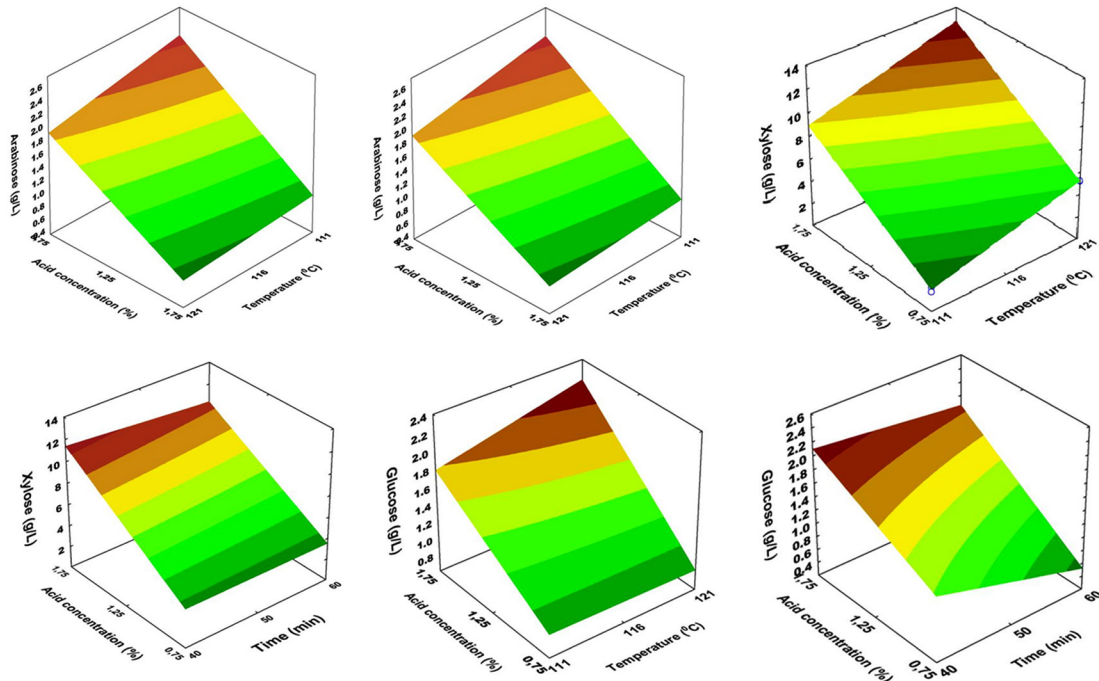
Figure 1. Response surfaces for the concentrations of arabinose, xylose and glucose obtained from the acid hydrolysis of sorghum bagasse.
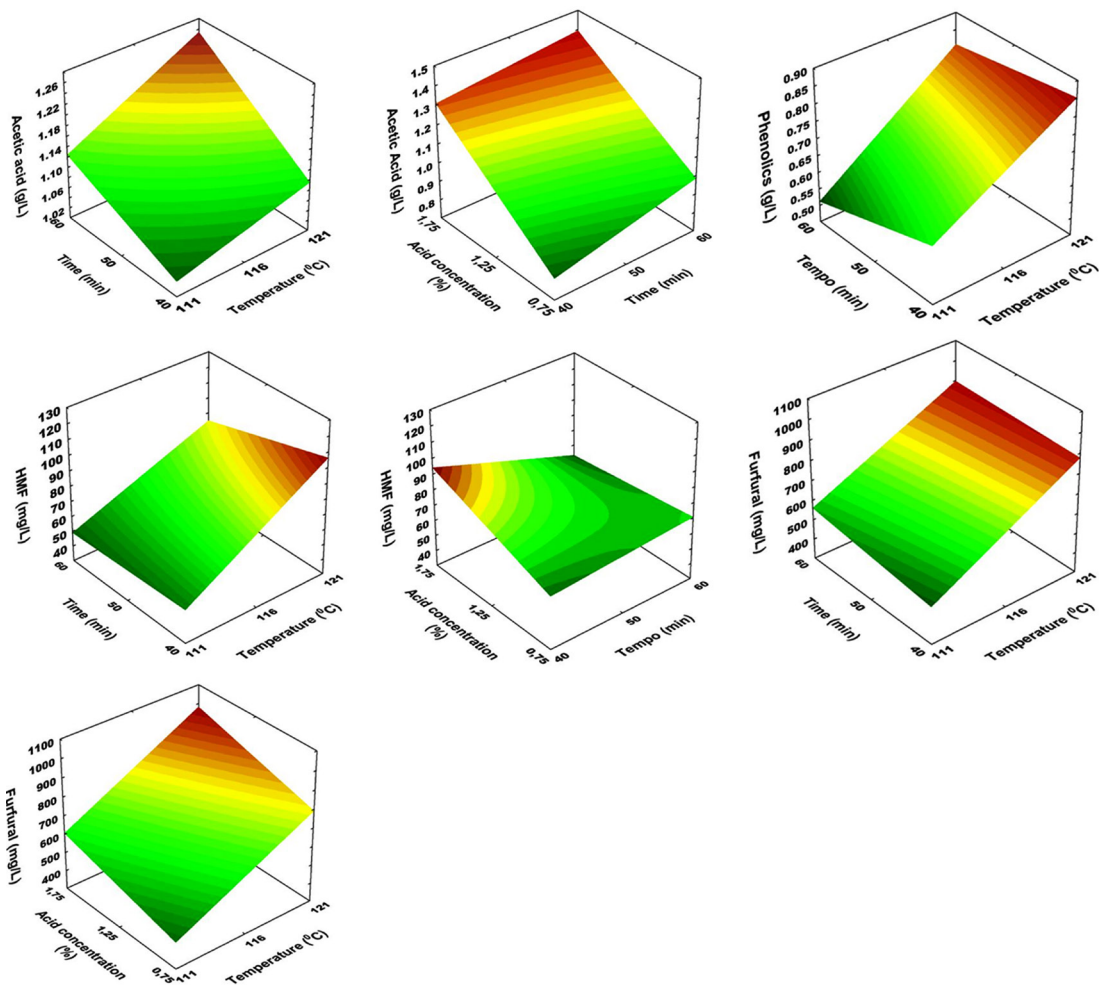

Figure 2. Response surfaces for the concentrations of acetic acid, phenolics, furfural and HMF obtained from acid hydrolysis of sorghum bagasse.

For glucose, the high level (+1) of temperature (121 ºC) and acid concentration (1.75%) resulted in the highest amount of glucose. In contrast, the independent variable time at the low level (-1) resulted in high glucose concentration in the hydrolysate.

The response surface plot for xylose, similar to that observed for glucose, shows that the acid concentration and temperature were the variables that most favored the hydrolysis of hemicellulose to xylose. The best xylose yields were obtained using an acid concentration of 1.75% and hydrolysis temperature of 121 ºC.

Camargo et al. (2015) described similar levels of xylose in the forage sorghum bagasse hemicellulosic hydrolysate (16.76 - 17.42 g/L) when hydrolysis was carried out with 2% sulfuric acid at 121 ºC for 70 minutes. With sorghum straw, Sene et al. (2011) obtained higher concentrations of xylose (17.69 g/L) and less glucose (2.1 g/L) and arabinose (1.81 g/L) than those found in the present work.

With regard to inhibitors, the response surface plot for acetic acid shows that the release of acetic acid was favored when the high levels of variables were employed. For the phenolic compounds, it is possible to observe that higher concentrations were obtained with the lowest hydrolysis time (40 minutes) and highest temperature (121 ºC).

For HMF the higher the temperature and concentration of the catalyst, the higher the amount of HMF. Similarly, the formation of furfural occured with the increase of temperature and acid concentration, indicating that to obtain lower concentrations of this toxic compound milder hydrolysis conditions should be used. The time, in turn, did not presented a significant effect on these results.

Experimental validation

For the model validation, a new hydrolysis assay was run at 121 ºC for 40 minutes, which were considered to be the best temperature and time during the CCD study for obtaining a hydrolysate rich in xylose. However, in these assays, the percent of sulfuric acid was varied (1.45, 1.60, 1.75, 2.15 and 2.30%). The results are shown in Figure 3.

The highest concentration of xylose (13.31 g/L) was obtained when the acid percentage was 1.75%, confirming that this was the best condition for obtaining this sugar with low occurrence of furfural degradation. Glucose concentration also increased when the acid percentage increased and the highest glucose concentration value (3.36 g/L) was obtained with 2.30% of sulfuric acid.

With respect to the inhibitors, an increase was observed in the concentrations of acetic acid, phenols, furfural and HMF when increasing the temperature to 121 °C, even at lower sulfuric acid concentrations. The highest acetic acid concentration (29.1 g/L), total
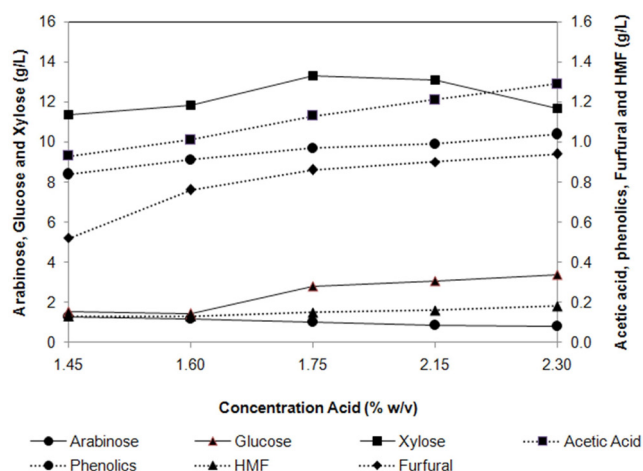

Figure 3. Variation of the concentration of sugars (arabinose, glucose, xylose) and toxic compounds (acetic acid, phenolics, furfural and HMF) obtained with the variation of sulfuric acid concentration during the hydrolysis of the hermicellulosic fraction of sweet sorghum bagasse.

phenolics (1.04 g/L), HMF (158.21 mg/L) and furfural (946.82 mg/L) were obtained with 2.30% H_2SO_4.

These results, therefore, confirm the validity of the model adopted, in which it is assumed that the variables time at the low level (-1) (40 minutes) and the variables temperature and acid concentration at the high level (+1) (121 º C; 1.75%), as observed previously (Table 1), compose the best condition for obtaining a hemicellulosic hydrolysate rich in sugars (mainly xylose) and low concentrations of inhibitory compounds.

Heredia-Olea et al. (2012), studying the total amounts of C6 sugars (cellobiose and glucose), C5 sugars (xylose and arabinose) and inhibitory compounds (acetic acid, HMF and furfural) generated by different acid treatments (sulfuric and hydrochloric), also found through surface response methodologies a high coefficient of determination (R^2) for the responses for treatment with sulfuric acid. In addition, they also observed that their equations predicted the real experimental values, although the quadratic effect was better adjusted to the model. The response surface behavior for sulfuric acid showed that higher amounts of sugars were released when higher acid concentrations and hydrolysis times were used

In the present work, the values for xylose were different from those predicted by the model (17.59 g/L) probably because the increase in acid concentration propitiated sugar degradation and inhibitor formation.

This situation in which the amount of xylose released is different from the value predicted by the model, even though a high mathematical correlation was obtained (90%), was also observed by Heredia-Olea et al. (2012) during acidic hydrolysis of sweet sorghum bagasse with sulfuric acid. According to the

authors, the prediction of the various acid treatments was underestimated compared to the expected and real values after the application of optimal treatments due to the high presence of inhibitory compounds, which indicated severe hydrolysis conditions for the raw material

Characterization of the original, concentrated and detoxified hydrolysates

The sorghum bagasse hemicellulosic hydrolysate obtained with 1.75% sulfuric acid at 121 ºC for 40 minutes presented in its composition: glucose 2.42 g/L, xylose 15.22 g/L, arabinose 0.95 g/L, phenols 0.9 g/L, acetic acid 1.34 g/L, furfural 950.01 mg/L and HMF 124.54 mg/L. After the concentration process, arabinose, glucose and xylose increased by 2.6; 3.6 and 2.5 fold, respectively, compared to the hydrolysate in natura, while the ratio of increase of the inhibitor compounds was 1.8 to 2.15 fold.

After hydrolysate detoxification, the following composition was found: glucose 2.89 g/L, xylose 42.05 g/L, arabinose 1.66 g/L, phenols 0.65 g/L, acetic acid 0.78 g/L, furfural 650.12 mg/L and HMF 88.07 mg/L. Therefore, the toxic compounds were partially removed in a ratio of approximately 70% compared to the non-detoxified hydrolysate. Sugar losses corresponded to 22%, 32% and 53% for xylose, arabinose and glucose, respectively. However, in sugarcane bagasse hemicellulosic hydrolysate detoxified with the same methodology used in this study, minor losses were found, representing 17; 15 and 20% for xylose, arabinose and glucose, respectively (Antunes et al., 2014).

Fermentability of sorghum bagasse hemicellulosic hydrolysate

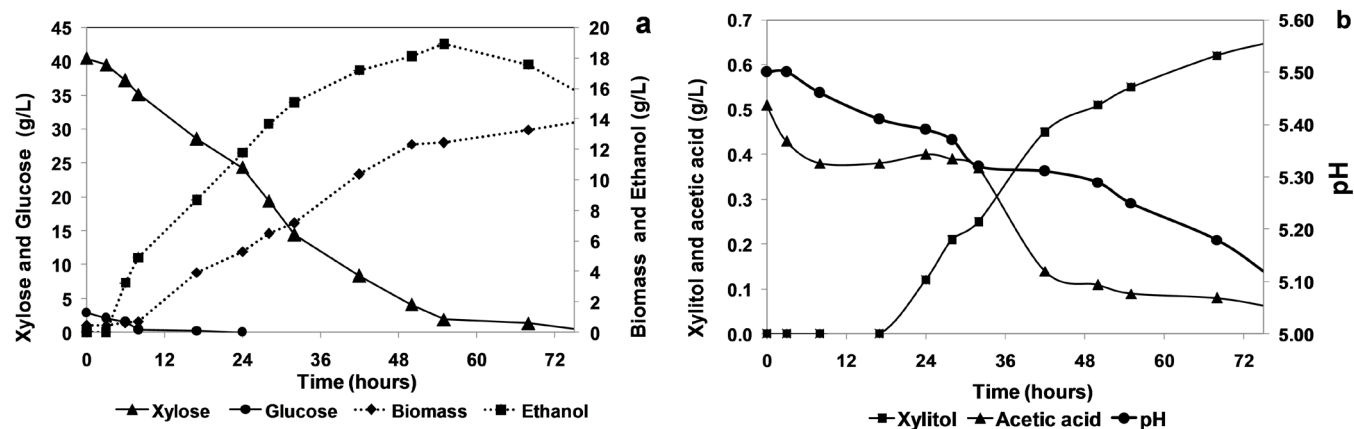Figure 4 exhibits the cell growth, glucose and xylose consumption and ethanol production by *S. stipitis* yeast in sweet sorghum bagasse hemicellulosic hydrolysate during 76 hours. The adaptation phase of the yeast lasted 8 hours, followed by intense cell division until 48 hours, when cell concentration reached 12 g/L. From that moment, there was no increase in microbial biomass. When Chaud et al. (2012) used this same yeast grown in oat hull hemicellulosic hydrolysate (40 g/L xylose) a final biomass concentration close to 6 g/L was observed in 72 hours fermentation.

According to Aloisio et al. (2014) *S. stipitis* was capable of converting glucose, xylose and mixtures of glucose/xylose to ethanol. Although this microorganism had no lag phase in a medium with glucose, in the presence of xylose a lag phase was verified that lasted 4-5 hours. In this work, xylose was consumed throughout the fermentation process, even concomitantly with glucose. It was observed that 99% of xylose had been used by the yeast at the end of 76 hours.

Arabinose assimilation (data not shown) was not observed during the fermentation process. This behavior is consistent with the results reported in other works during fermentation with *S. stipitis* (Chaud et al., 2012; Silva et al., 2014).

Figure 4a shows that ethanol production increased from the 10th hour of fermentation, reaching a maximum concentration of ethanol (22 g/L) at 55 hours. Slightly higher values (ethanol 26 g/L and cell concentration 15 g/L) were reported with *S. stipitis* yeast in synthetic medium (90 g/L xylose and 15 g/L glucose) in a bioreactor with a k_{LA} 4.9 h^{-1} after 96 hours of fermentation (Silva et al., 2012).

In this work, due to the sugars exhaustion, ethanol was used as carbon source and decreased to 18 g/L at the end of 76 hours. Ethanol consumption has also been observed by other authors with this same yeast (Stoutenburg et al., 2008; Fu et al., 2009; Ferreira et al., 2011). In a study to assess the volumetric oxygen transfer coefficient for ethanol production from xylose by *S. stipitis*, the maximum ethanol production (13.5

Figure 4. Cell growth, xylose and glucose consumption and ethanol production (a); acetic acid and pH variation and xylitol production (b) during fermentation of sweet sorghum bagasse hemicellulosic hydrolysate with *Scheffersomyces stipitis* ATCC 58376.

g/L) was verified with a kLa of 18.7 h^{-1} at 36 h of fermentation and then, part of the ethanol produced was assimilated when xylose was still available (40 g/L). In this case, ethanol assimilation by the yeast suggests that oxygen was supplied in excess in the medium (Silva et al., 2012). According to Liu et al. (2012) ethanol production by *S. stipitis* is strongly affected by the intracellular redox state. Therefore, ethanol production occurs under relatively low oxygen condition, while ethanol is reassimilated when the oxygen content is excessive.

The maximum ethanol yield Y$_{P/S}$ (12.40 g/g) and volumetric productivity Qp (0.34 g/L.h) obtained in this study after 55 hours of fermentation are comparable or even higher than those found in the literature for ethanol production in hemicellulosic hydrolysates of different materials. For example, when the same yeast strain used in this study was grown in sunflower meal hemicellulosic hydrolysate, the maximum Y$_{P/S}$ obtained was 0.23 g/g and Q$_P$ 0.12 g/L.h (Camargo and Sene, 2014). Ethanol production by *S. stipitis* DSM 3651 in sugarcane bagasse hemicellulosic hydrolysate resulted in Y$_{P/S}$ 0.30 g/g and Q$_P$ 0.16 g/L.h (Canilha et al., 2010). With *S. stipitis* NRRL 7154 grown in wheat straw hemicellulosic hydrolysate Y$_{P/S}$ 0.41 g/g and Q$_P$ 0.54 g/L.h were obtained (Nigam, 2001). Dussán et al. (2016) evaluated the influence of aeration, agitation rate and initial pH on ethanol production by *S. stipitis* NRRL Y-7124 in sugarcane bagasse hemicellulosic hydrolysate and obtained better results: Y$_{P/S}$ 0.42 g/g, Q$_P$ 0.25 g/L.h and 85% efficiency, using 100 rpm, initial pH 6.50 in limiting conditions of oxygen (0.7 vvm and. kLa 0.1 h^{-1}).

Values similar to those reported in this work were obtained in synthetic medium comprising 35 g/L glucose and 20 g/L xylose during the study of the effect of different aeration rates on ethanol production by *S. stipitis* DSM 3651 (Bellido et al., 2013). The authors obtained 22.33 g/L ethanol, Y$_{P/S}$ 0.40 g/g and Q$_P$ 0.30 g/L.h with kLa 3.3 h^{-1} in 72 hours of fermentation. In a synthetic medium with 60 g/L of sugars (30 g/L xylose and 30 g/L glucose) using *S. stipitis* CCUG18492, Rouhollah et al. (2007) obtained a maximum of 30 g/L of ethanol, with ethanol and cells yields of 0.40 g/g and 0.08 g/g, respectively, but a much higher productivity value (0.95 g/L.h).

In fermentations of the sorghum bagasse hydrolysates pretreated with 1.28% HCl, bagasse:liquid ratio 6% w/v, 121 °C and fibrolytic enzyme complex (Novozymes) with the strains *Saccharomyces cerevisiae* ATCC 20252™, *Pichia stipitis* ATCC 66278™, and *Issatchenkia orientalis* ATCC 20381™ all fermentations reached the maximum ethanol concentration after 24 hours. However, both yeasts *S. cerevisiae* and *I. orientalis* produced significant amounts of ethanol, 183.9 and 209.2 mg/g ethanol, respectively, while *P. stipitis* was unable to consume significant amounts of xylose and glucose during 72

h fermentation, producing minimal amounts of this alcohol (2.8 mg /g). According to the authors, *P. stipitis* was inhibited by the presence of toxic compounds such as acetic acid, HMF that were in sufficient concentrations (27.8, 4.8, 4.1 mg/g respectively), but did not significantly affect the other two strains of yeast. In addition, the fermentations were performed under anaerobic conditions and *P. stipitis* needs oxygen to generate ethanol (Heredia-Olea et al., 2013).

Figure 4b shows the variation of pH and the production of acetic acid and xylitol during the fermentation of sorghum bagasse hemicellulosic hydrolysate by *S. stipitis*. A decrease of acetic acid concentration was noted over time, which was more pronounced at the 30th hour, corresponding to an assimilation as carbon source of 88%.

Acetic acid consumption has been observed in other yeast species. During xylitol production by *C. guilliermondii* FTI 20037 in sorghum straw hemicellulosic hydrolysate containing 3.13 g/L of acetic acid a consumption was observed of about 28% after 72 hours (Sene et al., 2011). An assimilation of acetic acid higher than 70% was observed in *C. guilliermondii* FTI 20037 and *S. stipitis* NRRL Y-7124 during the fermentation of sugarcane bagasse hemicellulosic hydrolysate containing 2.5 g/L of acetic acid. According to the authors, it is possible that the acetic acid was used for cell growth while xylose could have been used for the formation of ethanol and xylitol by *C. guilliermondii* and *S. stipitis*, respectively (Silva et al., 2014). Despite the consumption of acetic acid, a slight pH reduction was observed during the process, probably due the excretion of other acidic metabolites not evaluated in this work.

At the end of the fermentation the xylitol concentration reached 0.6 g/L. A similar amount of xylitol (0.81 g/L) and 17.7 g/L ethanol were observed in the fermentation of *S. stipitis* Y-7124 with an initial xylose concentration of 50 g/L (Lee et al., 2000). Higher xylitol concentrations (1.48 g/L) were obtained during the fermentation of hemicellulose hydrolysate from sunflower meal (20.66 g/L glucose and 24.09 g/L xylose) by the same yeast strain used in this study (Camargo and Sene, 2014). Xylitol formation and its accumulation in the medium are attributed to a cellular response to environmental adversities such as redox imbalance caused under oxygen limitation, high substrate concentration, or the presence of inhibitors that may be generated during pre-treatment, especially under high severity conditions of hydrolysis (Betancur and Pereira, 2010).

CONCLUSION

Central composite design (CCD) and response surfaces allowed the evaluation of the acid hydrolysis of the hemicellulosic fraction of sweet sorghum

bagasse. The concentration of sulfuric acid was the factor that had the greatest influence on the hydrolysis of hemicellulose. Acid hydrolysis with 1.75% of sulfuric acid at 121 °C for 40 minutes was the condition that provided the highest sugar content and the lowest toxic compounds levels in the hemicellulosic hydrolysate. Furthermore, the model responded satisfactorily to the study and the concentration of sugar corresponded to the predicted values for the validation. *S. stipitis* was able to grow in the sorghum bagasse hemicellulosic hydrolysate, consuming efficiently sugars (xylose 99%) and producing 22 g/L ethanol, with ethanol yield similar to those found for this yeast in hydrolysates from other types of biomass. Therefore, sweet sorghum bagasse was demonstrated to be a promising raw material, mainly for fermentation processes that employ pentoses as carbon sources.

NOMENCLATURE

ANOVA	Analysis of variance
CCD	Central composite design
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
kLa	Oxygen transfer coefficient
NREL	National Renewable Energy Laboratory
O.D.	Optical density
pH	Potential of hydrogen ion
PTFE	Polytetrafluoroethylene
Q_p	Volumetric productivity
YMP	Yeast malt peptone
$Y_{P/S}$	Ethanol yield

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