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Biomass Sorghum: Effect of Acid, Basic and Alkaline Peroxide Pretreatments on the Enzymatic Hydrolysis and Ethanol Production

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HIGHLIGHTS

- Alkaline, acid and alkaline peroxide pretreatments were evaluated in biomass sorghum hydrolysis.
- Acid and H_2O_2 pretreatments were the best in holocellulose protection and lignin removal.
- Highest monosaccharide yields were in hydrolysis of PTB1 (HCl 0.34%) and PTC1 (5% H_2O_2).
- Greatest ethanol yield was obtained in PTB1 hydrolysate (16.15%).

Abstract: This study evaluated the effects of three chemical pretreatments of biomass sorghum (BS): dilute alkaline (PTA1 and PTA2), dilute acid (PTB1 and PTB2) and alkaline hydrogen peroxide (PTC1 and PTC2) in the enzymatic hydrolysis and ethanol production. Among the six investigated conditions, the pretreatment with 7.36% H_2O_2 (PTC2) was the most efficient in the lignin removal and preservation of the polysaccharide fraction. After the enzymatic hydrolysis, increases in the glucose and xylose concentrations were observed in the pretreated BS hydrolysates, mainly in PTB1 and PTC1. All the hydrolysates obtained low concentrations of inhibitors. In the alcoholic fermentations with Pichia stiptis, the greatest ethanol yield was

obtained in PTB1 hydrolysate (3.84 g L^{-1}), corresponding to 16.15% of yield. The highest ethanol yield in PTB1 hydrolysate can be justified by the maximum concentration of xylose obtained in this hydrolysate, demonstrating the potential of P. stiptis in the fermentation of pentose to ethanol. The results indicated that biomass sorghum is an alternative lignocellulose source with potential for the production of second generation ethanol, opening up prospects for additional studies.

Keywords: biomass sorghum; chemical pretreatment; enzymatic hydrolysis; ethanol production.

INTRODUCTION

The increasing energy dependence, the depletion of oil reserves and the environmental problems have stimulated the search for biofuels from renewable sources in substitution to the fossil fuels [1]. In this scenario, lignocellulosic biomass can be a raw material of great potential and low cost for the production of biofuels and added-value bioproducts [2]. Among them, the second generation ethanol (2G) or cellulosic bioethanol is one of the most promising biomass-based fuels which can be produced from the released sugars of lignocellulose [3,4].

Lignocellulose is an attractive resource for the bioethanol production worldwide, since it is renewable and widely available, such as agricultural residues (sorghum stover, sugarcane bagasse, rice straw, wheat straw and maize straw) [5-7]. Energetic cultures of fast growth and high productivity can also be suitable for the production of bioethanol given its flexibility as source of starch, sucrose and lignocellulose [8]. For example, sorghum [*Sorghum bicolor* (L.) Moench] is a potential energy crop easily cultivable and adaptable to different temperature conditions throughout the world, being appropriate for the production of traditional first generation ethanol from its juice as well as cellulosic ethanol from the bagasse or stover[8]. Sorghum is a promising material due to its vigorous and rapid development, being able to grow up to five meters of height and produce more than 50 t ha^{-1} of dry matter per half-yearly average cycle [9]. In this context, researches focused on the generation of energetic varieties which contain elevated biomass composition have been conducted in Brazil, such as the program of genetic improvement from Brazilian Agricultural Research Corporation (EMBRAPA - Maize and Sorghum) in collaboration to Agricultural Research Company of Minas Gerais (EPAMIG), which recently developed a kind of sorghum with an increased biomass content, so-called *biomass sorghum* [10]. The composition of *biomass sorghum* (BS) resembles to other cultures conventionally employed for the production of cellulosic ethanol, such as sugarcane bagasse (SCB). Nevertheless, BS holds moderately low lignin content and elevated composition in polysaccharides (hemicellulose and cellulose), being considered a very promising energy crop for ethanol production [9,11]. Recent studies evaluated different genotypes of biomass sorghum with respect to agronomic potential and lignocellulose composition for bioethanol production and obtained average contents of 35-44% of cellulose, 25-30% of hemicellulose and 4.8-9% of lignin [12,13].

The carbohydrate polymer matrix of lignocellulose (cellulose and hemicelluloses) is strongly cross-linked and bound to lignin, limiting the use of this biomass for biotechnological applications [13,14]. As a result, effective methods of pretreatments are necessary to disrupt the heterogeneous matrix, increase the surface area and the porosity of the material in order to improve the enzymatic digestibility. Nevertheless, the effects of each pretreatment on the structure and the generation of possible inhibitory compounds can vary depending on the lignocellulosic material [1]. Among the chemical pretreatments, dilute acid pretreatment with sulfuric acid (H_2SO_4) or hydrochloric acid (HCl) have been successfully investigated to hydrolyze hemicelluloses and increase the accessibility to cellulose in different biomass, such as sugar beet pulp [15] and wheat straw [16]. Alkaline pretreatments with sodium or calcium hydroxides (NaOH or $Ca(OH)_2$) present delignificant effects, remove the xylan side chains, reduce the cellulose crystallinity and increase the porosity, enhancing the substrate susceptibility to the enzymatic attack [17]. Alkaline pretreatments under mild conditions have demonstrated high lignin reduction and remarkable sugar release in different lignocellulosic sources, such as wheat straw [3] and sweet sorghum bagasse [18].

The combination of alkaline and oxidizing reagents has also been highly efficient for the lignin and hemicelluloses reduction, increasing the yield of released sugars after enzymatic hydrolysis [19]. The pretreatment with alkaline peroxide (H_2O_2) has effectively been applied to different lignocellulose materials such as rapeseed straw [1], sweet sorghum bagasse [20] and rice hulls [21]. Toquero and Bolado [3] compared the effects of thermal, dilute acid, dilute basic and alkaline peroxide pretreatments of wheat straw on the enzymatic release of sugars and bioethanol biosynthesis and observed that the alkaline peroxide method was the most suitable to provide the highest sugar and ethanol yields. Bolado-Rodríguez and coauthors [22] compared the effects of pretreatments (thermal autoclaving, dilute HCl autoclaving, dilute

NaOH autoclaving and alkaline peroxide) of wheat straw and sugarcane bagasse on the methane potential and also observed that high rates of methane production were attained for solid fractions of both substrates.

Up to now, there are no studies comparing the effects of different pretreatments of biomass sorghum cultivars on the enzymatic hydrolysis and bioethanol production in literature. Only the prospection of other cultivars (sweet, forage and bagasse sorghum) or genotypes of biomass sorghum in terms of agronomic potential, biomass production and lignocellulosic composition have been described [13]. Based on prior studies with other kinds of sorghum and lignocelluloses, this work compared the effects of three pretreatments (dilute acid, dilute basic and alkaline peroxide) on the enzymatic hydrolysis of pretreated BS and ethanol production from the fermentable sugars released in hydrolysates.

MATERIAL AND METHODS

Materials

Biomass sorghum (BS), variety LE299, was kindly donated by the Agricultural Research Company of Minas Gerais (EPAMIG, Brazil). This variety of sorghum was genetically improved and is under testing process and patent certification (data not shown). BS samples were washed for particulate material removal, dried in a ventilated oven at 42 ºC, ground in an agricultural crusher to a size of 3-5 mm and stored in hermetic plastic bags until their use. The substrate was kept in an oven at 45 ºC until it reached a constant weight prior to compositional analysis and different pretreatments.

Chemical characterization of biomass sorghum

The chemical composition of raw and pretreated BS was determined in terms of total lignin (TL), acid soluble lignin (ASL), acid insoluble lignin (AIL) and structural carbohydrates (cellulose and hemicelluloses) contents [23]. All the assays were performed in triplicate.

Pretreatments

Three chemical pretreatments (dilute acid, dilute alkaline and alkaline hydrogen peroxide) of biomass sorghum were evaluated in this study according to Toquero and Bolado [3], with minor modifications. For each pretreatment, the experiments were performed at two different reagent percentages. For dilute alkaline and acid pretreatments, weighed amounts of raw BS were suspended in 1.0 and 2.0% NaOH solutions (PTA1 and PTA2) or in 0.34 and 1.5% w/w HCl solutions (PTB1 and PTB2), to obtain a solid:liquid ratio of 1:10 w/w. The assays were conducted in borosilicate flasks of 250 mL and the slurries were autoclaved during 1 h, at 120°C. For alkaline peroxide pretreatment, raw BS was suspended in 5.0 and 7.36% of H_2O_2 (PTC1 and PTC2) in a solid: liquid ratio of 1:20, and the pH was adjusted to 11.5 with 2 M NaOH. The mixture was then placed in a rotatory shaker at 50 ºC and 120 rpm for 1 h. After each pretreatment, the obtained pretreated slurry was recovered at room temperature and the residual solid was separated by vacuum filtration up to the maximum liquid removal. The liquid and solid fractions from each pretreatment were weighed and samples of both fraction were analyzed. Pretreated solid fractions were frozen until their subsequent enzymatic hydrolysis. All experiments were carried out in triplicate.

Enzymatic hydrolysis

The hydrolyses were conducted in Erlenmeyer flasks of 100 mL containing 10% w/w dry solid pretreated biomass sorghum obtained from each pretreatment and a mixture of commercial enzymes - 10 FPU of Celluclast 1.5 L and 30 CBU of Novozym 188 per gram of cellulose (dry basis), respectively [2]. Saccharifications were performed at 50 ºC, 150 rpm for 48 h, with sodium citrate buffer at pH 4.8 (0.05 M) in a final volume of 25 mL [2]. Samples of hydrolysates were filtered through 0.22 µm filters and stored under refrigeration for further analysis of sugars and inhibitory compounds. Experiments were conducted in triplicate.

Alcoholic fermentation

The fermentation of hydrolysates was carried out with the yeast *Sheffersomyces stipitis* (*Pichia stipitis*) DSM 3651 obtained from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Germany). *P. stipitis* DSM 3651 was maintained in YEPX agar plates (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} xylose, and 20 g L^{-1} agar) at 4 °C. For the inoculum preparation, it was grown aerobically in

Erlenmeyer flasks with YEPX liquid medium (10 g L^{-1} yeast extract and 20 g L^{-1} peptone previously autoclaved at 121 °C, 20 min; and 20 g L^{-1} of sterile filtered xylose in 0.22 **µ**m filters (Ministart Sartorius) and added later to avoid sugar degradation by temperature. The inoculum was grown aerobically on a rotatory shaker at 175 rpm and 30 °C during 24 h [3].

The fermentations were carried out in penicillin flasks with pretreated BS hydrolysates or sugar model solution (25 g L^{-1} glucose and 15 g L^{-1} xylose supplemented with salt solution containing 20 g L^{-1} peptone, 10 g L^{-1} yeast extract, 12.8 g L^{-1} KH_2PO_4, 0.51 g L^{-1} Na_2HPO_4, 0.47 g L^{-1} $(NH_4)_2SO_4$ and 0.47 g L^{-1} $MgSO_4 \cdot 7H_2O$). The growth medium was adjusted to pH 5.0 with a buffer comprising succinic acid 0.2 M (250 mL L^{-1}) and NaOH 0.2 M (267 mL L^{-1}). The model solution and hydrolysates whole slurries were autoclaved at 121 °C for 20 min, transferred to penicillin flasks and supplemented with 5 mL salt solution [3]. Afterwards, 3 mL of fresh 24 h *P. stipitis* pre-inoculum were added and the mixture was incubated semianaerobically at 150 rpm and 30 °C for 7 days. The fermentations were vacuum filtered and centrifuged at 10,000 g for 10 min. The supernatants were filtered through 0.22 µm filters (Ministart Sartorius) and analyzed by chromatography for ethanol quantification [24]. Experiments were conducted in triplicate.

Analytical methods

The chemical composition of raw BS and after each pretreatment was determined in terms of ash, acid insoluble lignin (AIL), acid soluble lignin (ASL), cellulose (as glucose) and xylan (as xylose), according to NREL procedures (National Renewable Energy Laboratory, USA) and Sluiter and coauthors [23]. Glucose, xylose, ethanol and organic acids (oxalic, formic and acetic acids) were measured by high performance liquid chromatography (HPLC) in a Waters Alliance e2695 separation module equipped with a Waters 2998 photodiode array detector and a Waters 2414 refractive index detector, all controlled and processed by Empower 3 (build 3471 from 2010) software (Waters). Sugars from compositional analysis procedures, hydrolysates and ethanol from fermentations were measured using the refraction index with a Phenomenex HPLC column RezexTM RPM-Monosaccharide Pb+2 (8%), 300 7.8 mm, at 80 °C, with MilliQ water as the eluent, at 0.6 mL min^{-1} according to Bolado-Rodríguez and coauthors[22]. Inhibitory compounds (oxalic, acetic and formic acids) in hydrolysates were measured by ultraviolet at 210 nm with a Bio-Rad Aminex HPLC HPX-87H column, 7.8 mm, at 50 °C, with 25 mM H_2SO_4 as the eluent, at 0.6 mL min^{-1}. Total phenolic compounds (TPC) in hydrolysates were determined by the Folin-Ciocalteu method at 765nm using gallic acid as calibration standard [3].

RESULTS

Chemical characterization of raw and pretreated biomass sorghum

After the compositional analysis, raw biomass sorghum presented relative percentages of 35.77% of cellulose, 12.52% of hemicellulose (xylan) and 28.47% of total lignin. Almeida and coauthors. [13] evaluated the lignocellulose composition of hybrids of biomass sorghum and obtained average contents of 35.81-39.07% of cellulose, 25.34-28.91% of hemicellulose and 4.79-7.31% of lignin, respectively, being the observed cellulose content close to that found in the present work. However, these authors observed lower lignin and higher hemicellulose percentages than in this study. Dos Santos and coauthors [12] characterized free of extractives biomass sorghum and observed close values of cellulose (30.72%) and lignin (24.71%) but higher content of hemicellulose (28.49%) than the present study. These differences in the chemical composition of BS among these studies can be due to the fact that the biomass can vary according to crop location, cultivars and harvesting time [13].

Pretreatments of BS resulted in the increase in the cellulose and hemicellulose compositions, with reduction in the lignin percentage (Table 1). After the alkaline pretreatment with NaOH (PTA), slight removals in the acid insoluble lignin (AIL) concentration were observed, with a reduction of 23.48% in PTA1 and 28.74% in PTA2. Increases of 65% in the concentration of acid soluble lignin (ASL) in PTA1 and of 30.32% in PTA2 were also observed (Table 1). The fractions of cellulose and xylan increased in 24 and 110% (PTA1) and 10 and 85% (PTA2) after the redistribution of the masses, respectively. This increase in the concentration of polysaccharides may be explained to the removal of part of the insoluble lignin, since the reduction of this component can cause changes in the distribution of the other components in the sorghum fibers [7]. These data indicated that the alkaline pretreatment was not very efficient for the lignin removal, but preserved the cellulose and hemicelluloses (xylan) fractions. Other studies reported more significant reductions in the lignin content after alkaline pretreatments in other kinds of lignocellulosic materials. Guilherme and coauthors [25]

obtained reduction of 75.77% in the lignin content in sugarcane bagasse pretreated with 4% NaOH and Maryana and coauthors [26] observed a removal of 57% in the lignin percentage after the pretreatment of sugarcane bagasse with NaOH 1N.

The dilute acid pretreatment with 0.34% HCl (PTB1) promoted low reduction of total lignin content (11.83%) and increase in the cellulose and xylan contents (Table 1). This increase in cellulose and hemicellulose fractions was even more pronounced in PTB2 pretreatment after the redistribution of the masses. Toquero and Bolado [3] observed an increase of 70.0% in the percentage of cellulose of sugarcane straw submitted to the same pretreatment, which was attributed to the drastic decrease of 76.8% in the hemicellulose content. In a study carried out with saccharin sorghum pretreated with HCl 1.39%, almost all of the hemicellulose content was removed, representing only 2.53% of the final composition in the acid cellulignin [27]. The cellulose and lignin contents increased from 40.42 and 19.79% in the initial composition to 45.78 and 27.79%, respectively, after dilute acid pretreatment, indicating a proportional increase of these fractions due to the great solubilization of hemicellulosic fraction [27].

Table 1. Main components of raw (BS) and pretreated biomass sorghum (PT). PTA1: alkali pretreatment with NaOH 1%; PTA2: alkali pretreatment with NaOH 2%; PTB1: acid pretreatment with HCl 0.34%; PTB2: acid pretreatment with HCl 1.5%; PTC1: alkaline peroxide pretreatment with H_2O_2 5% and PTC2: alkaline peroxide pretreatment with H_2O_2 7.36%.

Sample	[1]AIL	[2]ASL	[3]TL	Cellulose	Xylan	**Mass Balance
BS	23.48 ± 0.20	4.98 ± 0.16	28.47 ± 0.04	35.77 ± 1.04	12.52 ± 0.31	98.83 ± 0.10
PTA1	17.97 ± 0.53	8. 24 ± 0.82	26.21 ± 1.36	44.62 ± 2.75	26.40 ± 1.82	97.235 ± 0.15
PTA2	16. 73 ± 0.21	6.49 ± 0.41	23.23 ± 0.63	39.56 ± 0.16	23.17 ± 0.95	85.96 ± 0.24
PTB1	17.67 ± 0.38	7.42 ± 0.22	25.10 ± 0.61	46.35 ± 0.17	18.91 ± 0.12	90.36 ± 0.57
PTB2	18.85 ± 0.86	6.39 ± 0.02	25.24 ± 0.83	49.73 ± 2.29	15.00 ± 0.36	89.97 ± 0.78
PTC1	23.37 ± 0.01	5.95 ± 0.53	29.33 ± 0.54	44.08 ± 0.44	19.12 ± 0.17	92.53 ± 0.51
PTC2	4.29 ± 0.00	5.32 ± 0.31	9.62 ± 0.31	44.63 ± 0.11	18.79 ± 0.01	73.04 ± 0.81

[1]Acid insoluble lignin; [2]Acid soluble lignin; [3] Total lignin.
** The mass balance (dry basis) in pretreated biomass sorghum was calculated by the sum of TL, cellulose and xylan. For raw BS, ashes (4.46%) and extractives (17.61%) were also included in the mass balance.

The pretreatment with 7.36% of alkaline H_2O_2 (PTC2) triggered a drastic reduction of the total lignin content (66.2%). Increases of 24% of cellulose and 51% of xylan were also observed after PTC2. Rabelo and coauthors [28] obtained a reduction of 86% of total lignin and a reduction of 70.5% of hemicelluloses after alkaline H_2O_2 pretreatment (7.35%) of sugarcane bagasse. Toquero and Bolado [3] observed an increase of 38.8% in the cellulose composition and of 13.3% in the percentage of hemicelluloses after alkaline peroxide pretreatment of sugarcane bagasse. These authors also obtained a removal of 16.5% in AIL composition. This same method was applied to rapeseed straw by Karagöz and coauthors [1], which reported a slight increase of 14.7% in the cellulose composition with decreases of 18.0 and 21.1% in the hemicelluloses and lignin contents. In general, the percentage of cellulose increased after all the evaluated pretreatments due to the redistribution of the masses after the removal of lignin. Thus, these results indicated that, among the investigated pretreatments, the pretreatment with alkaline H_2O_2 at 7.36% (PTC2) was the most efficient for lignin removal and preservation of the polysaccharide fractions.

Regarding to the content of total phenolic compounds (TPC) which could have been generated during the pretreatments, the highest concentrations of these compounds were obtained in BS samples pretreated with HCl 0.34% (PTB1), with 0.492 g L^{-1} (Table 2). Close values of TPC were obtained after other pretreatments (Table 2). Highest levels of phenolic compounds were observed in previous studies. Alvira and coauthors [29] obtained 2.7 g L^{-1} of total phenols in hydrolysates of wheat straw pretreated by steam explosion. Toquero and Bolado [3] obtained 3.4 g L^{-1} of phenolics in wheat straw pretreated with NaOH. The determination of the total phenolic compounds (TPC) content in lignocellulosic biomass is of great importance since, during pretreatment, these compounds can be generated and can act as inhibitors of both enzymatic hydrolysis and fermentation of sugars [30]. Besides, during the enzymatic hydrolysis of pretreated lignocellulosic biomass, changes in the lignin structure may occur with consequent exposition of these compounds that may cause an inhibitory effect on the bioconversion of biomass [31]. Thus, a crucial point for the optimization of the hydrolysis can be the reduction of its inhibition by phenolic compounds, in order to increase the rate of bioconversion of sugars. Therefore, the obtained data indicated that the low amounts of phenolic inhibitors generated after the different pretreatments was a positive result on the composition of pretreated BS samples.

Concentration of sugars and inhibitors after enzymatic hydrolysis

The obtained data showed a great increase in the glucose concentration in the extracted biomass sorghum hydrolysate (ES) when compared to the raw biomass sorghum hydrolysate (BS). The increase in the glucose concentration was also very significant in the hydrolysates submitted to pretreatments, especially in the samples of biomass sorghum pretreated with HCl and H_2O_2 (Table 3). It was also observed that the maximum glucose concentrations were released after the hydrolysis of pretreated biomass sorghum with 1.5% HCl (PTB2) and 7.36% H_2O_2 (PTC2), with increase of 120 and 118% in glucose yields, respectively (Table 3). Among all the evaluated pretreated BS hydrolysates, PTC2 was the most efficient in the cellulose conversion in glucose (90.15%), followed by PTB2 (85.46%). Concerning the release of xylose, similar concentrations were observed in BS and ES hydrolysates (Table 3). However, in the pretreated BS hydrolysates, a great increase in its concentration was observed, especially in the hydrolysates of biomass sorghum pretreated with HCl 0.34% (PTB1) and with 5% H_2O_2 ((PTC2). Due to the lack of studies of hydrolysates of pretreated biomass sorghum, the obtained results were compared to studies carried out with other lignocellulosic biomasses. Toquero and Bolado [3] compared the effect of four types of pretreatments of wheat straw (thermal, 1% NaOH, 1.5% HCl and 5% H_2O_2) on the hydrolysis using commercial enzymatic complexes (10 FPU of NS50013 cellulase and 10 CBU of β-glycosidase NS50010, Novozymes). These authors also obtained maximum glucose concentrations in H_2O_2 pretreated wheat straw hydrolysates with 48.5% and 63.7% of glucose yield for washed samples and concluded that the alkaline peroxide pretreatment was advantageous for the retention of cellulose and to open the lignocellulosic structure, even with preserved lignin fraction. Karagöz and coauthors [1] reported 15.08 g L^{-1} of glucose production after the pretreatment of rapeseed straw with alkaline peroxide. Cao et al. [20] obtained 10.41 g L^{-1} of glucose in sweet sorghum bagasse hydrolysates submitted to alkaline peroxide pretreatment. In both cases, the glucose concentrations were lower than those ones obtained in the present study. With respect to the xylose release in the pretreated biomass sorghum hydrolysates from the present work, the obtained concentrations in hydrolysates of PTB and PTC (with HCl and H_2O_2) were higher than those obtained by Cao et al. [20] who obtained 4.98 g L^{-1} of xylose and slightly lower than that found by Karagöz et al. [1] which obtained 8.29 g L^{-1} of released xylose after the hydrolysis.

Table 2. Total content of phenolic compounds (TPC). PTA1: alkali pretreatment with NaOH 1%; PTA2: alkali pretreatment with NaOH 2%; PTB1: acid pretreatment with HCl 0.34%; PTB2: acid pretreatment with HCl 1.5%; PTC1: alkaline peroxide pretreatment with H_2O_2 5% and PTC2: alkaline peroxide pretreatment with H_2O_2 7.36%.

PT	TPC (g L^{-1})
PTA1	0.31
PTA2	0.34
PTB1	0.49
PTB2	0.44
PTC1	0.34
PTC2	0.31

Table 3. Glucose, xylose and inhibitors concentrations (g L^{-1}) in raw, extracted and pretreated hydrolysates of biomass sorghum. BS: raw biomass sorghum, ES: extracted biomass sorghum, Hydrolysates: PTA1: pretreated with NaOH 1%; PTA2: pretreated with NaOH 2%; PTB1: pretreated with HCl 0.34%; PTB2: pretreated with HCl 1.5%; PTC1: pretreated with H_2O_2 5% and PTC2: pretreated with H_2O_2 7.36%.

Hydrolysates	Glucose	Xylose	Glucose Yield (%)	Xylose Yield (%)	Oxalic acid	Formic acid	Acetic acid
BS	0.16	0.69	0.75 ± 0.19	8.69 ± 1.5	0.5365	_	0.2421
ES	8.49	0.7	32.60 ± 0.79	7.67 ± 0.01	0.5069	_	1.3084
PTA1	10.33	2.58	48.15 ± 3.10	34.28 ± 17.14	0.1170	_	0.5908
PTA2	7.96	2.11	37.09 ± 10.21	28.64 ± 2.90	0.2742	0.1089	0.5959
PTB1	15.75	**7.09**	73.40 ± 0.75	94.38 ± 1.46	0.4418	0.4677	0.8069
PTB2	**18.74**	6.35	85.46 ± 1.8	84.58 ± 1.7	0.5575	0.5741	0.8387
PTC1	17.73	**6.9**	82.60 ± 0.84	91.82 ± 4.24	0.5365	_	0.2421
PTC2	**19.35**	6.64	90.15 ± 1.27	88.48 ± 1.4	0.5069	_	1.3084

Among the evaluated hydrolysates, the hydrolysate of biomass sorghum pretreated with NaOH (PTA1 and PTA2) was the least efficient in the release of glucose and xylose, with the greatest yields obtained in PTA1 (10.33 g L^{-1} of glucose and 2.58 g L^{-1} xylose). These values were lower than those found by other authors in previous studies with other kinds of biomass. Nascimento et al. [32] studied the enzymatic

hydrolysis of sugarcane bagasse pretreated with NaOH using 20 FPU g^{-1} of Accelerase 1500 and obtained 38.8 g L^{-1} of glucose in the hydrolysate. Cao et al. [20] obtained 12.55 g L^{-1} of glucose and 4.98 g L^{-1} of xylose in sweet sorghum bagasse hydrolysates previously submitted to alkaline pretreatment. Therefore, the obtained results demonstrated that the pretreatment with alkaline H_2O_2 was the most efficient method in the release of glucose after the enzymatic hydrolysis of biomass sorghum. However, further studies might be carried in order to optimize the conditions and maximize the release of glucose and xylose from BS hydrolysates. In this work, 10% of dry matter was used in hydrolysis, according to previously established methodologies [3]. One of the alternatives would be to increase the percentage of dry mass during the saccharification. Some previous studies have demonstrated that simultaneous changes in the important parameters of enzymatic hydrolysis, such as time as well as the content of total solids can directly influence the final yield of released sugars from biomass hydrolysates [33].

Although the chemical pretreatments can favor the increase in the concentration of sugars in the pretreated hydrolysates, they also can produce inhibitor compounds of the fermentative process. This is a relevant limitation in the production of bioethanol from lignocellulosic materials not only due to the inhibition of the fermentation of hydrolysates but also due to the high cost and complexity of the detoxification stages [2]. The amounts and kinds of inhibitors produced during the lignocellulose pretreatment are dependent on the composition of the biomass, the type of pretreatment and the reaction conditions [34]. Regarding to the inhibitory compounds originated after each pretreatment, low concentrations of oxalic and acetic acids were obtained in biomass sorghum hydrolysates pretreated with NaOH 2% (PTA2). The lowest concentrations of oxalic, formic and acetic acid were obtained in BS hydrolysates pretreated with HCl (PTB1 and PTB2). In the hydrolysates of biomass sorghum pretreated with H_2O_2 (PTC1 and PTC2), low concentrations of these compounds were also observed (Table 3).

These obtained inhibitors concentrations in the present study were lower than those ones previously reported in other works. For example, Toquero and Bolado [3] obtained 3.59 g L^{-1} of acetic acid and 2.06 g L^{-1} of formic acid in hydrolysates of wheat straw pretreated with NaOH and Bolado-Rodrigues et al. [22] found 4.3 g L^{-1} of acetic acid in sugarcane bagasse pretreated with NaOH. The acetic acid is a product from the degradation of hemicelluloses typically found in hydrolysates which is produced by the hydrolysis of the acetyl groups. Its presence in the fermentative medium can cause an increase in the consumption of ATP by yeast. Thus, part of the energy that would be used for yeast growth or fermentation can be sidetracked to maintain its internal pH [35]. Therefore, the presence of high concentrations of acetic acid is undesirable for a good fermentative efficiency.

Comparing the concentrations of formic acid in BS hydrolysates pretreated with alkaline peroxide to the study of Toquero and Bolado [3], these authors found a concentration of formic acid slightly above (0.62 g L^{-1}) to that obtained in the present work. This acid is produced when 2-furfuraldehyde (FF) and 5-hydroxymethyl-2-furfuraldehyde (HMF) are decomposed after the pretreatment with alkaline peroxide [36]. Additionally, the formic acid eventually produced can be removed together with the extractives and contribute positively to the increase in the production of glucose and xylose in the extracted lignocellulose biomass. Based on the obtained results, it was concluded that the hydrolysates of the present work presented favorable characteristics for alcoholic fermentation, since low concentrations of formic acid and other inhibitors were observed in comparison with previous studies. Other inhibitory compounds commonly observed in sugarcane bagasse after pretreatments, such as FF and HMF, were not evaluated in this experiment, due to the low amounts obtained in previous studies with sugarcane bagasse submitted to the same kinds of pretreatments [3,22]. After the analysis of the results from the enzymatic hydrolysis, alcoholic fermentations were carried out only with the hydrolysates in which the highest concentrations of glucose and xylose were obtained (biomass sorghum hydrolysates pretreated with HCl and H_2O_2).

Ethanol production

Table 4 shows the ethanol production after 168 h of fermentation of pretreated biomass sorghum hydrolysates. The alcoholic fermentation with *P. stipitis* using the model solution obtained 12 g L^{-1} of ethanol with 30% yield. In the fermentations of BS pretreated hydrolysates, the highest concentrations of ethanol were observed after the fermentations of hydrolysates pretreated with HCl (PTB1 and PTB2) with 3.84 and 3.61 g L^{-1}, corresponding to 16.15 and 14.62% of yield, respectively. The superior ethanol yield of 30% in the fermentation using the in the model solution can be explained by the higher concentrations of monosaccharides (25 g L^{-1} glucose and 15 g L^{-1} xylose) in this solution than in the hydrolysates (highest concentrations of 19.35 and 7.09 g L^{-1}, respectively). Although the glucose concentration released in the

PTB1 hydrolysate was lower than PTB2, PTC1 and PTC2 (alkaline peroxide pretreatment at both concentrations), this hydrolysate obtained the highest xylose liberation, which may justify the highest production of ethanol from this fermentation (Table 4). In addition, the hydrolysate from PTB1 was the one that presented the lowest concentrations of inhibitors among the samples (oxalic and acetic acid) and formic acid was not detected (Table 3). Therefore, the hydrolysate of biomass sorghum pretreated with 0.34% HCl (PTB1) was selected as the best condition for the production of ethanol by *P. stipitis* as a function of the highest xylose concentration and lowest production of inhibitors.

Table 4. Production of bioethanol after alcoholic fermentation by *P. stiptis*. PTB1: dilute acid pretreatment with HCl 0.34%; PTB2: dilute acid pretreatment with HCl 1.5%; PTC1: alkaline peroxide pretreatment with H_2O_2 5% and PTC2: alkaline peroxide pretreatment with H_2O_2 7.36%.

Fermentation	Ethanol (g L^{-1})	Yield (%)
Model solution	12.02 ± 0.08	30.0± 0.00
PTB1	**3.84 ± 0.16**	16.15 ± 014
PTB2	3.61 ± 0.04	14.62 ± 0.49
PTC1	2.91 ± 0.49	11.87 ± 2.25
PTC2	2.36 ± 0.03	9.09 ± 0.15

Some earlier studies with other biomass sources showed similar yields in ethanol to that found in the present study. Pereira et al.[37] obtained 3.15 g L^{-1} of ethanol after the fermentation of hydrolysate of sugarcane bagasse pretreated by ozonolysis using the yeast *Saccharomyces cerevisiae*. Cao et al. [20] obtained 0.89 g L^{-1} of ethanol from fermentation of thermally pretreated sweet sorghum bagasse hydrolysates and 5.55 g L^{-1} of ethanol from sweet sorghum bagasse hydrolysates previously submitted to thermal and alkali pretreatments. In both cases, *S. cerevisiae* strains were used in fermentations. In the present work, the yeast *Pichia stipites,* which is able to ferment both pentose and hexose, was used in the alcoholic fermentation in despite of the most of studies which have described fermentations with *S. cerevisiae* strains [38]. Therefore, these results indicated that the ethanol yields from biomass sorghum were suitable. To date, few studies have been found in the literature demonstrating the use of biomass sorghum for the production of cellulosic ethanol, being this study, therefore, a pioneer in this research area.

CONCLUSION

The acid and alkaline peroxide pretreatments of biomass sorghum were the most favorable in the preservation of polysaccharides, with low formation of inhibitors and good release of glucose and xylose after enzymatic hydrolysis. The highest yields of glucose and xylose were obtained in the hydrolysates of BS pretreated with HCl 0.34% (PTB1) and 5% H_2O_2 ((PTC2). The PTB1 hydrolysate was selected as the most appropriate condition for the production of ethanol by *P. stipitis* as a function of the highest xylose content and lowest production of inhibitors. The percentages of the compounds in the pretreatments, the dry substrate charge in the hydrolysis and the conditions of fermentation were chief factors for the biosynthesis of ethanol. The results suggested biomass sorghum as a promising and alternative raw material for use in enzymatic saccharification and biofuel production.

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