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Hydrogen peroxide-acetic acid pretreatment increases the saccharification and enzyme adsorption on lignocellulose

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ABSTRACT

Biomass delignification is a crucial condition for the effective production of fermentable sugars from lignocellulosic materials. Here, an effective method was used to pretreat lignocellulosic materials using hydrogen peroxide-acetic acid (HPAC) solution. The pretreatment of maize straw, sugarcane bagasse and eucalyptus bark with HPAC removed 45 to 75% of lignin and improved from 2.1 to 20.8-fold the saccharification process. Delignification caused by HPAC increased the enzyme adsorption capacities of pretreated substrates from 2.6 to 7.1-fold. The HPAC treatment clearly removes furfurals of the hydrolytic medium, contributing to more efficient ethanol fermentation. The applied method can be a useful alternative to improve biomass saccharification, reduce costs and increase the production of second-generation bioethanol.

1. Introduction

Two companies in Brazil that are using sugarcane bagasse residues (Raízen and GranBio) and one in the USA using maize residues (Poet-DSM consortium) are close to commercializing cellulosic ethanol with economic viability (Gírio et al., 2017). Although these industrial plants are already producing and selling cellulosic ethanol, they still require further reduction of the production cost. The main obstacles include the efficient removal of lignin from lignocellulosic biomass, the high cost to produce hydrolytic enzymes, and the low efficiency of yeasts to ferment pentoses (Marques, 2018). Pretreatment prior to enzymatic hydrolysis disrupts the recalcitrant structure of lignocellulosic biomass, enhancing the access of enzymes to the polysaccharides (Amorim et al., 2011; Oliveira et al., 2015; Mota et al., 2018). Several pretreatment procedures have been reported, including physical, biological and chemical methods, and their industrial applications can reduce the downstream operating costs for biofuel production (Alvira et al., 2010).

The hydrolysis rate is related to the number of enzymes adsorbed onto biomass (Kumar and Wyman, 2009; Lin et al., 2018). However, the relationship between cellulase adsorption kinetics and lignin removal in pretreated biomasses is not fully understood (Pareek et al., 2013). During enzymatic hydrolysis, enzymes tend to bind on the lignin-rich surfaces, inhibiting the enzymatic hydrolysis and harming the enzyme recycling (Pareek et al., 2013; Rahikainen et al., 2013; Lin et al., 2018), and finally, demanding higher enzyme loadings and increasing the costs of the process (Ko et al., 2015).

Ideally, a pretreatment method ought to present a low cost, efficient delignification of different lignocellulosic materials, minimum cellulose degradation and non-significant production of inhibitors for the subsequent enzymatic saccharification and fermentation (Gatt et al., 2018). Some strategies are being developed to decrease the content of inhibitory compounds, as furfural and 5-hydroxymethylfurfural (HMF) produced during the pretreatments. An efficient strategy is to use mild conditions like lower temperature and shorter times of pretreatment (Jönsson and Martin, 2016). By and large, hydrogen peroxide-acetic acid (HPAC) pretreatment meets these criteria because it efficiently removes lignin, using mild temperatures and weak acids (Wi et al., 2015). Previous studies revealed that HPAC pretreatment is quite effective for the delignification of pine and oak woods (Wi et al., 2015), sugarcane bagasse (Tan et al., 2010) and Jerusalem artichoke stalk (Song et al., 2016).

Together with sugarcane bagasse and maize straw, eucalyptus bark is a residue considered an interesting lignocellulosic material for cellulosic ethanol production (Lima et al., 2013; Reina et al., 2016). The large cultivated area of these plants generates a high amount of residues, with high potential for lignocellulosic biofuels. In addition,

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eucalyptus is widely used in building and paper industries. Based on the principle that HPAC efficiently removes lignin from different lignocellulosic sources, herein we evaluated the saccharification of HPAC-pretreated maize straw (MS, *Zea mays*), sugarcane bagasse (SCB, *Saccharum* sp.) and eucalyptus bark (EB, *Eucalyptus grandis*). Maize and sugarcane are important crops for food and ethanol. After HPAC pretreatment, the chemical modifications and structural features of lignocellulosic materials were characterized by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. The enzyme adsorption capacity on pretreated substrates and the degradation of furfurals were also evaluated.

2. Material and methods

2.1. Raw materials and chemicals

Maize straw, sugarcane bagasse and eucalyptus bark were air-dried, ball-milled to a fine powder and stored at room temperature. All chemicals used in this work were of analytical grade. Acetic acid and hydrogen peroxide were purchased from Nuclear (Brazil). Novozymes (Araucaria, Brazil) kindly donated cellulase complex NS22086, β-glycosidase complex NS22118 and Cellic® HTec2.

2.2. HPAC pretreatment

Each lignocellulosic material source was treated using the HPAC method described by Wi et al. (2015) with modifications. The HPAC solution was prepared by mixing hydrogen peroxide and acetic acid (1:1; *v/v*). One gram of lignocellulosic biomass was homogenized into a screw-capped plastic tube containing 10 mL of HPAC solution and incubated at 80 °C for 2 h. The HPAC-pretreated material was filtered to separate the liquor from the solid residue, and the solids were washed with distilled water and dried at 50 °C for 72 h.

2.3. Determination of furfurals

The liquor fraction obtained from HPAC pretreatment, with furfural (FURF) and 5-hydroxymethylfurfural (HMF) standards, were filtered through a 0.45-μm disposable syringe filter and analyzed by High-Performance Liquid Chromatography system (HPLC) (Moreira-Vilar et al., 2014).

2.4. Cell wall preparation and lignin determination

The dry matter of untreated or HPAC-pretreated biomasses was subjected to successive extractions with 80% ethanol (*v/v*) as described by Oliveira et al. (2016). The remaining solid material was defined as the alcohol insoluble residue (AIR). For lignin determination, AIR was subsequently washed with different solutions to obtain the protein-free cell wall fraction (Ferrarese et al., 2002) and quantified by the acetyl bromide method (Moreira-Vilar et al., 2014).

2.5. Monosaccharide composition

Five mg of AIR were hydrolyzed in 1 mL of 2 M trifluoroacetic acid (TFA) for 1 h at 100 °C, and the monosaccharides released were analyzed by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). The parameters used and the patterns obtained with standards for the monosaccharide separation are described by Pagliuso et al. (2018).

2.6. Enzymatic hydrolysis

The reaction mixtures were prepared with 15 mg of AIR, enzyme extract containing 5 U/mL cellulase and 30 U/mL xylanase, 0.02% sodium azide (*v/v*) and 50 mM sodium acetate buffer pH 5.0 at 50 °C (Oliveira et al., 2016). The reducing sugars were analyzed by DNS method (3,5-dinitrosalicylic acid) (Miller, 1959).

2.7. ATR-FTIR spectroscopy

ATR-FTIR spectroscopy was performed on a Bruker Vertex 70 FTIR Spectrometer equipped with an Attenuated Total Reflectance accessory and was carried out on AIR samples with 128 scans per sample at a 400 to 4000 cm^{-1} range with a resolution of 2 cm^{-1}.

2.8. Adsorption isotherms

Adsorption isotherms of proteins on lignocellulosic substrates were evaluated by varying the protein concentrations of the enzyme complex from 75 to 6000 μg/mL (5–400 mg protein/g AIR) according to the methods of Ko et al. (2015) with modifications. The concentration of non-adsorbed proteins in the supernatant was measured according to the Bradford method. Adsorbed protein data were fitted into the following Langmuir equation:

$$P_{ads} = (Pmax \times K_p \times P_{free})/ (1 + K_p + P_{free}) \quad (1)$$

where P_{ads} is the amount of adsorbed protein (mg protein/g AIR), P_{free} is the amount of non-adsorbed protein in the supernatant (mg protein/mL), $Pmax$ is the maximum protein adsorption capacity (mg protein/g AIR), K_p is the Langmuir constant (mL/mg protein) and the equation is a measurement for the adsorption affinity. Adsorption parameters were determined by non-linear regression of experimental data using GraphPad Prism (version 5.00 for Windows).

3. Results and discussion

3.1. HPAC pretreatment alters biomass composition

After 2 h of pretreatment, the total recovery of solids from maize straw (MS), sugarcane bagasse (SCB) and eucalyptus bark (EB) were 67%, 63% and 59%, respectively, and were similar to pinewood, oak wood and rice straw, which ranged from 59 to 75% (Wi et al., 2015). The decrease in HPAC-insoluble solids was mainly due to the solubilization of lignin-derived compounds and pectin-derived monosaccharides (Table 1). HPAC pretreatment removed 45%, 70% and 75% of lignin from MS, SCB and EB, respectively. In contrast, crystalline cellulose increased from 34% to 53% after pretreatment, and the hemicellulose content was barely reduced only in SCB (–8%), with no significant differences in MS and EB. The increased cellulose content together with the reduced lignin content resulted in an increased cellulose/lignin ratio in HPAC-pretreated materials. Due to the varied compositional features, different lignocellulosic materials can influence the pretreatment effectiveness and cell wall recalcitrance to hydrolysis in different ways (Alvira et al., 2010; Oliveira et al., 2019a).

The analysis of non-cellulosic monosaccharides by HPAEC-PAD revealed significant differences in the lignocellulosic materials pretreated with HPAC. Neutral monosaccharides released from hemicelluloses and pectin of MS, SCB and EB showed a high proportion of pentoses, before and after HPAC pretreatment. The higher content of xylose and arabinose in SCB and MS, when compared to EB, is related to the high content of arabinoxylan typical of grasses (de Souza et al., 2012d; Lima et al., 2014; Oliveira et al., 2019b). EB presented a higher amount of rhamnose (2.27 mg/g AIR) in comparison to MS (0.36 mg/g AIR) and SCB (0.16 mg/g AIR), indicating higher proportions of pectin in EB. Hexose contents of hemicelluloses were significantly changed (Table 1). HPAC pretreatment removed 68% and 55% of glucose from MS and SCB hemicelluloses, respectively. Differently from eucalyptus, sugarcane and maize cell walls contain significant quantities of mixed linkage (β–1,4 and β–1,3) glucans (Mota et al., 2018). Our results suggest that HPAC pretreatment partially removed the mixed linkage glucans,

Table 1
Chemical composition of untreated and HPAC-pretreated materials (expressed in mg/g AIR).

Samples	Lignin	Cellulose	Hemicellulose	Cellulose/Lignin ratio	Arabinose	Xylose	Fucose	Galactose	Glucose	Mannose	Rhamnose
MS	191.12 ± 4.28	283.53 ± 18.68	197.48 ± 15.55	1.48	32.36 ± 3.07	124.10 ± 5.98	0.53 ± 0.06	8.52 ± 0.75	30.22 ± 2.56	1.39 ± 0.09	0.36 ± 0.05
MS-HPAC	105.38 ± 1.89	380.27 ± 7.82	185.38 ± 6.57	3.61	31.37 ± 1.21	137.57 ± 5.74	0.21 ± 0.01	6.32 ± 0.30	9.55 ± 0.35	0.39 ± 0.01	0.17 ± 0.02
P-value	**< 0.001**	**0.022**	0.271		0.352	0.078	**0.001**	**0.039**	**0.006**	**0.001**	**0.022**
SCB	262.78 ± 8.84	395.34 ± 20.51	141.42 ± 0.45	1.50	13.79 ± 0.24	117.24 ± 1.45	0.55 ± 0.04	2.75 ± 0.07	5.66 ± 0.53	1.26 ± 0.02	0.16 ± 0.01
SCB-HPAC	79.64 ± 1.93	594.86 ± 40.50	130.36 ± 2.09	7.47	13.69 ± 0.22	112.27 ± 1.32	0.06 ± 0.01	1.59 ± 0.06	2.55 ± 0.20	0.14 ± 0.01	0.06 ± 0.01
P-value	**< 0.001**	**0.009**	**0.004**		0.387	**0.027**	**< 0.001**	**< 0.001**	**0.001**	**< 0.001**	**< 0.001**
EB	241.96 ± 7.04	307.22 ± 9.37	77.91 ± 2.51	1.27	14.58 ± 0.47	47.03 ± 1.58	1.14 ± 0.05	9.42 ± 0.40	2.60 ± 0.15	0.86 ± 0.05	2.27 ± 0.07
EB-HPAC	61.44 ± 3.56	468.89 ± 23.49	74.87 ± 1.62	7.63	2.58 ± 0.07	61.89 ± 1.30	1.20 ± 0.03	5.59 ± 0.15	2.74 ± 0.07	0.47 ± 0.01	0.39 ± 0.01
P-value	**< 0.001**	**0.001**	0.174		**< 0.001**	**< 0.001**	0.200	**< 0.001**	0.214	**< 0.001**	**< 0.001**

Mean values ± SEM (n = 3–4). P-values ≤ 0.05 that are statistically significant are showed in bold (unpaired two-sided t test).

modulating the hemicellulose composition. Although pentose fermentation is considerably less efficient than hexose, conversion of pentoses after enzymatic hydrolysis can be achieved using engineered or natural fermenting microorganisms (Almeida et al., 2011).

Changes in specific bands of ATR-FTIR spectroscopy were analyzed after HPAC pretreatment (Fig. 1A) and were based on previous studies (Bekiaris et al., 2015; Pereira et al., 2016). Except for bands at 1633 and 1660 cm^{-1} in EB, bands at 1465, 1510, 1600 and 1633 cm^{-1} assigned to lignin decreased considerably after pretreatment, in concordance with the lignin removal data determined by the acetyl bromide method (Table 1). The band assignment at 1735 cm^{-1}, usually attributed to the presence of acetyl groups of hemicellulose, did not change in any sample (Fig. 1B). Similar results were observed in pinewood, oak wood and rice straw (Wi et al., 2015). Bands at 1053, 1160, 1375, 2910 and 3450 cm^{-1} assigned to crystalline cellulose (Bekiaris et al., 2015), and the band at 898 cm^{-1} assigned to amorphous cellulose were similar in untreated and HPAC-pretreated materials, demonstrating that HPAC pretreatment did not degrade cellulose (Fig. 1B).

The ratio of the peaks between 1510 and 898 cm^{-1} was used to calculate the lignin/cellulose ratio after HPAC pretreatment. In MS, the ratio reduced from 1.17 (before pretreatment) to 0.62 (after HPAC pretreatment). In SCB, the pretreatment reduced from 1.09 to 0.51, and in EB, the ratio decreased from 1.51 to 0.87. These findings strongly suggest that HPAC reacts preferentially with the lignin fraction.

3.2. HPAC treatment improves the enzymatic hydrolysis

The HPAC pretreatment positively affected the saccharification in the different lignocellulosic materials (Fig. 2A). The amount of reducing sugars released from HPAC-pretreated MS increased 2.1-fold, from 6.03 to 12.58 g/L, at 50 °C for 96 h of hydrolysis. The enzymatic hydrolysis of SCB increased 7.1-fold the release of reducing sugars, raising it from 2.25 to 15.89 g/L, and in EB it increased 20.8-fold, from 0.59 to 12.24 g/L. In fact, these results indicate that HPAC pretreatment is a highly efficient process for improving biomass saccharification.

To better understand the relationships between cell wall components, enzymatic hydrolysis and protein adsorption, we constructed a heatmap of pairwise Pearson's correlations coefficients (r) comparing all lignocellulosic materials. The lignin contents of untreated and pretreated samples were negatively correlated with enzymatic hydrolysis at 96 h ($r = -0.95$; $P = 0.0032$). The high sugar yield after enzymatic hydrolysis obtained in HPAC-pretreated materials indicates that cellulose and hemicellulose became more accessible to enzymes. This probably occurs because delignification exposes the polysaccharides to the access of hydrolytic enzymes (Li et al., 2016). Compared to pretreatments with hydrogen peroxide, acetic acid, peracetic acid and sulphuric acid under the same conditions, HPAC pretreatment is more effective in improving the enzymatic saccharification of pine, oak woods, and rice straw (Wi et al., 2015). Our results indicate that HPAC is an efficient pretreatment for lignocellulosic materials of contrasting types of cell walls.

3.3. HPAC pretreatment enhances the enzyme adsorption

Due to the interference of lignin in the hydrolysis of polysaccharides by the impeding of enzyme accessibility to the substrates, we evaluated the effect of selective HPAC-delignification on enzyme adsorption. The adsorption isotherms were generated using untreated and HPAC-pretreated biomasses incubated with different enzyme loadings (75 to 6000 μg/mL). Representative predicted and experimental protein adsorption data are shown in Fig. 2B, and adsorption parameters were well fitted with the Langmuir isotherm, with $R^2 \geq 0.90$ (Table 2).

After pretreatment, the results revealed that adsorbed proteins were strongly increased (Fig. 2B). The maximum adsorption capacity (P_{max}) of MS increased 2.6-fold. In the same experimental condition, P_{max} of HPAC-pretreated SCB and EB were increased 3.0-fold and 7.0-fold,

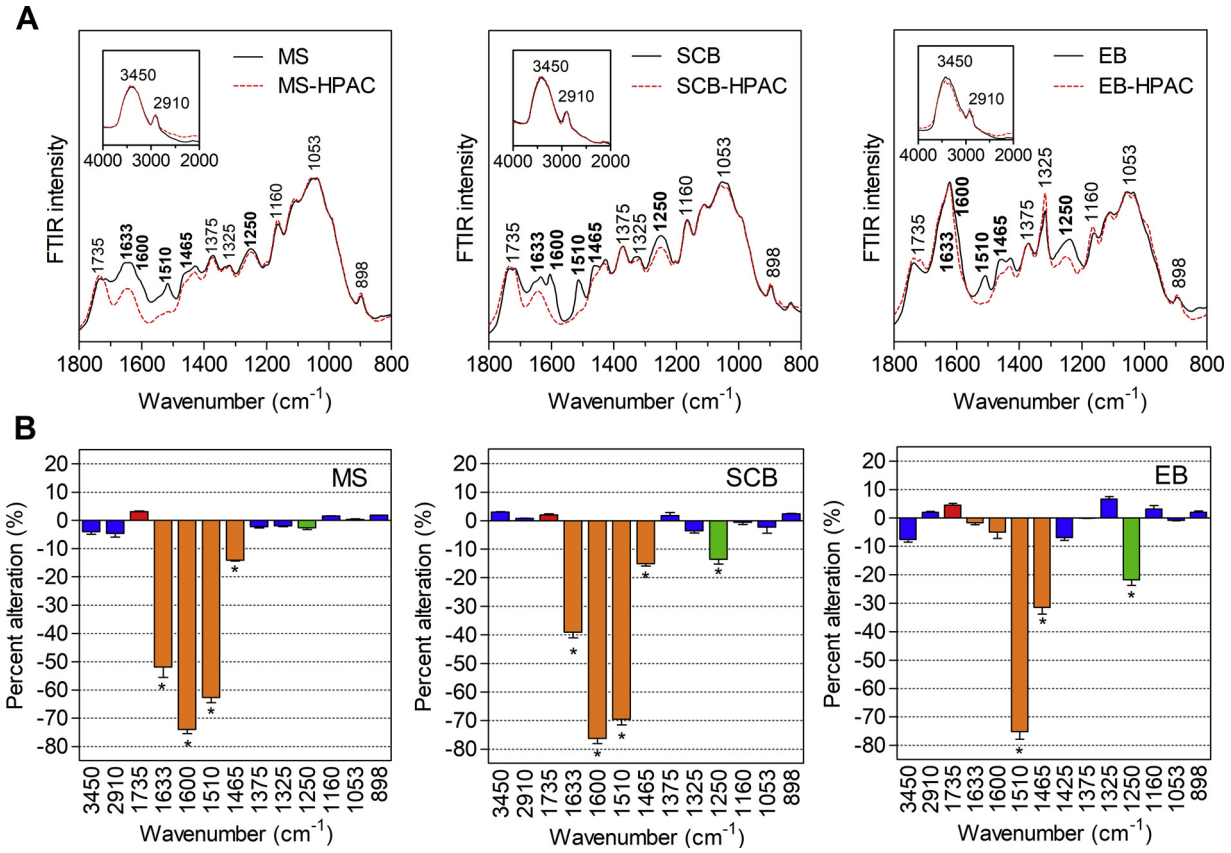

Fig. 1. ATR-FTIR spectra (A) and percent alterations in bands comparing the untreated and HPAC-pretreated substrates (B). Bands at 1465, 1510, 1600 and 1633 cm^{-1} correspond to lignin (orange bars), 1735 cm^{-1} corresponds to acetyl groups (red bar), 1250 cm^{-1} corresponds to xylan (green bar), bands at 898, 1058, 1160, 1325, 1375, 2910 and 3450 cm^{-1} correspond to cellulose (blue bars). Mean values ± SEM ($n = 3$). *$P < 0.05$, unpaired two-sided t test.

respectively. These findings show a nearly linear relationship between P_{max} *vs.* enzymatic hydrolysis (r = 0.90; $P < 0.05$) and P_{max} *vs.* lignin content (r = –0.90; $P < 0.05$). The high values of adsorption affinity (K_p) and adsorption strength (A) observed for untreated EB (3.61 mL/g protein and 57.05 mL/mg, respectively) may be related to the lower amount of reducing sugar released throughout the enzymatic hydrolysis (Fig. 2A). The HPAC pretreatment induced reductions in the K_p values of all the lignocellulosic materials (Table 2). As reported by Li et al. (2016), the lower K_p values suggest that delignified samples have more adsorption sites for proteins and, as expected, a possible relationship with the cell wall recalcitrance, as well as a higher efficiency for sugar yields. Instead, our analysis of pairwise correlations for K_p did not present clear relationships with enzymatic hydrolysis (r = -0.69; $P > 0.05$), lignin (r = 0.58; $P > 0.05$) or P_{max} (r = –0.69; $P > 0.05$), suggesting no evident correlation between K_p values and biomass recalcitrance.

The removal of lignin exposes more cellulose for protein adsorption (Kumar and Wyman, 2009). Delignification caused by HPAC had a strong positive effect on protein adsorption to the pretreated biomasses, suggesting relevant changes in the substrate accessibility. Pretreatments affect enzyme adsorption to the lignocellulose material altering its physicochemical properties (Pareek et al., 2013). After HPAC pretreatment, the reduction in lignin content led to a higher surface area of cell wall polysaccharides, which contributed to enhance productive enzyme adsorption on pretreated substrates and, consequently, to enzymatic hydrolysis rate.

3.4. HPAC pretreatment avoids the production of furfurals

Taking into account the pretreatment conditions (acidic solution at 80 °C for 2 h), the production of furfurals was expected (Jönsson and Martin, 2016). However, after HPAC pretreatment, no furfural (FURF) or 5-hydroxymethylfurfural (HMF) accumulated in the liquor (Fig. 3A). Therefore, we hypothesized that HPAC solution degrades furfurals in these pretreatment conditions. To strengthen this hypothesis, FURF and HMF standards (at 0.25 mM) were incubated with HPAC solution, deionized water, acetic acid and hydrogen peroxide in the same conditions used for the pretreatment (Fig. 3B). After 20 min, only 2.6% of initial HMF and 3.7% of FURF concentrations were detected, and after 40 min, both furfurals were completely degraded (Fig. 3C). In brief, this property of HPAC is an advantage for pretreatment of lignocellulose, since it avoids the accumulation of furfurals in the reaction medium.

4. Conclusions

Our results showed the potential of maize straw, sugarcane bagasse and eucalyptus bark as sources of fermentable sugars for bioethanol production after HPAC pretreatment. The HPAC pretreatment efficiently removed lignin from lignocellulosic materials with different cell wall types (types I and II). The delignification exposed cellulose and hemicellulose leading to more efficient saccharification of lignocellulose materials, without accumulation of furfurals, inhibitors of ethanol fermentation by yeasts. Furthermore, HPAC increased the adsorption of hydrolytic enzymes onto lignocellulose with potential to maximize sugar yields. Based on our results, HPAC pretreatment may be applied in feedstocks with different cell wall types focusing on cellulosic ethanol production. Altogether, these findings suggest that HPAC treatment is a valuable strategy to decrease the costs of second-generation bioethanol processing in the industry.

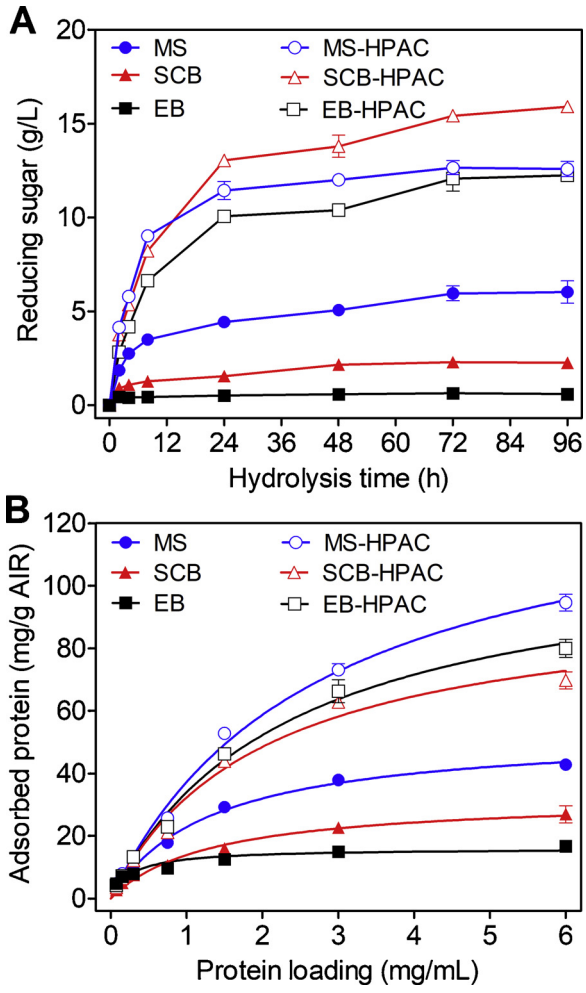

Fig. 2. Reducing sugar yields of enzymatic hydrolysis (A) and adsorption isotherms of proteins on untreated and HPAC-pretreated biomasses (B). Lines indicate the predicted amounts of adsorbed proteins well fitted with the Langmuir isotherm. Mean values ± SEM (n = 4).

Table 2
Maximum enzyme adsorption capacity (*Pmax*), adsorption affinity (K_p) and adsorption strength (A) constants for different lignocellulosic biomass. Mean values ± SEM (n = 4).

Samples	P_{max} (mg protein/ g biomass)	K_p (mL/mg protein)	$A = P_{max} \times K$ (mL/mg)	R^2
MS	53.33 ± 0.80	0.75 ± 0.03	39.90	0.98
MS-HPAC	138.80 ± 2.65	0.37 ± 0.02	50.85	0.99
SCB	32.34 ± 1.04	0.75 ± 0.07	24.30	0.93
SCB-HPAC	97.13 ± 2.26	0.50 ± 0.03	48.36	0.98
EB	15.79 ± 0.56	3.61 ± 0.28	57.05	0.90
EB-HPAC	111.50 ± 4.75	0.44 ± 0.04	49.61	0.98

Declaration of Competing Interest

The authors declare no conflicts of interest.

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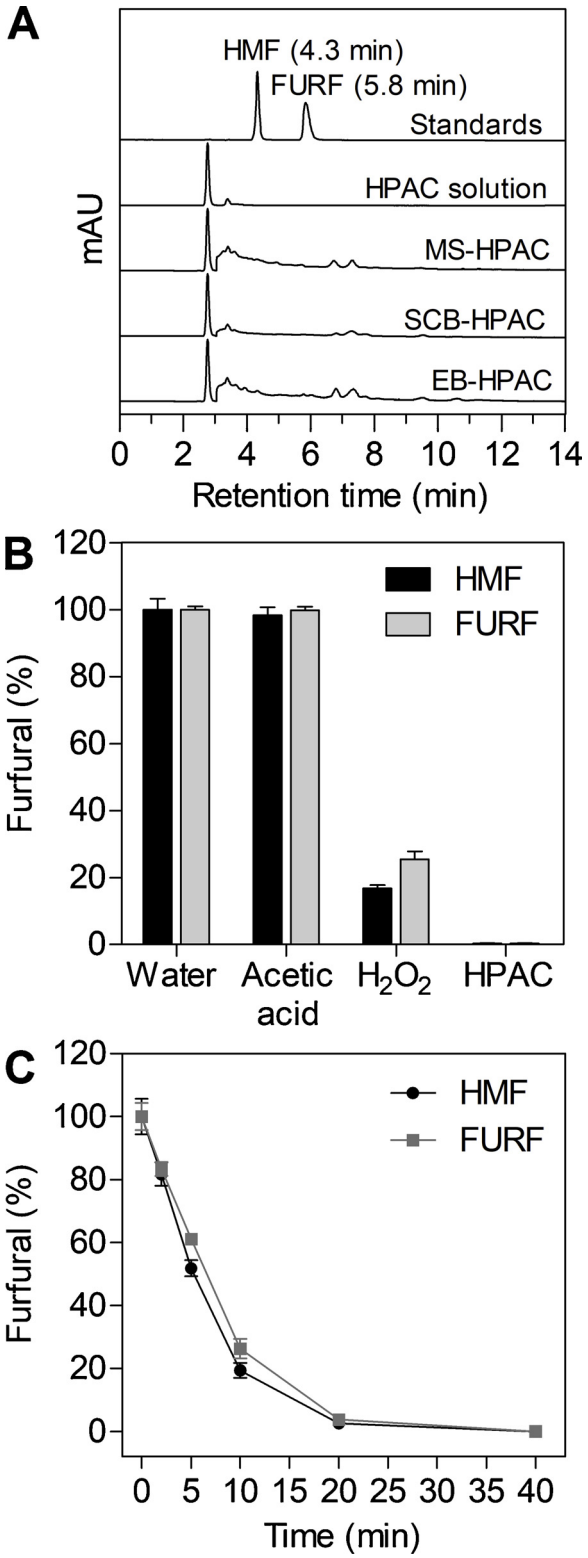

Fig. 3. Furfural analysis. (A) Chromatogram profile of liquor from different HPAC-pretreated biomass, standards and HPAC solution; (B) incubation of furfurals (at 0.25 mM) in water, acetic acid, hydrogen peroxide and HPAC solution, and (C) degradation of furfurals (at 0.25 mM) by HPAC after incubation in different times. HMF, 5-hydroxymethylfurfural; FURF, furfural. Mean values ± SEM (n = 3).

and 2014/50884-5) and (CNPq 574002/2008-1 and 465319/2014-9).

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