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Alkaline deacetylation as a strategy to improve sugars recovery and ethanol production from rice straw hemicellulose and cellulose

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ABSTRACT

A mild alkaline pretreatment (deacetylation) prior to the dilute acid pretreatment was evaluated as a strategy to improve the sugars recovery and ethanol production from both hemicellulose and cellulose fractions of rice straw. This pretreatment was carried out using different conditions of temperature (50–70 °C) and NaOH loading (20–80 mg NaOH/g biomass), which were combined according to a 2^2 central composite design. In this step the removal of acetyl groups as well as the impact of this step on biomass composition were evaluated. In order to assess the impact of the deacetylation on hemicellulosic hydrolysate composition, the influence of the reaction time (30–90 min) and sulfuric acid concentration (0.5–1.5% w/v) was also studied, using the alkaline-pretreated solid (deacetylated) and rice straw *in natura*. The best sequential pretreatment conditions were scaled-up to 50-L reactor, being obtained a cellulose-rich pretreated solid (cellulignin) and a hemicellulosic hydrolysate, which was concentrated to 70 g/L xylose to be used as fermentation medium. A significant improvement on ethanol production from xylose by *Scheffersomyces stipitis* NRRL Y-7124 was observed when the biomass was submitted to deacetylation (about 4-fold). The enzymatic conversion of cellulose was also improved (from 73 to 89%) when the deacetylated cellulignin was used, resulting in an enhancement of the ethanol production (from 12.7 to 20.4 g/L) during the simultaneous saccharification and fermentation with *Kluyveromyces marxianus* NRRL Y-6860. In brief, biomass deacetylation prior to dilute acid pretreatment was an efficient strategy for rice straw processing, substantially improving the ethanol production from both pentose and hexose sugars.

1. Introduction

Lignocellulosic materials are abundant and renewable resources that can be converted into valuable products, such as biofuels and chemicals, by biotechnological processes. These materials are mainly composed of sugars in the form of cellulose and hemicellulose polysaccharides, interspersed with a polyphenolic macromolecule named lignin. The utilization of these three main components is of fundamental importance for the development and implementation of competitive and sustainable biorefinery platforms (Mussatto and Dragone, 2016). Nevertheless, the conversion of polysaccharides from biomass into monomeric sugars is a challenge due to the natural recalcitrance of the lignocellulosic matrix. An adequate pretreatment step is therefore crucial for a successful recovery of cellulose and hemicellulose sugars from the lignocellulosic structure. A variety of pretreatment techniques has been reported in the literature, including physical, chemical and biological methods, and their combinations (Mussatto, 2016a). Each method has a different impact in the structure of the lignocellulosic material, and has a large impact on all the other steps of the biomass conversion process, in terms of sugar recovery, toxicity of hydrolysates, enzymatic hydrolysis and fermentation, as well as energy and waste water treatment demands.

Alkaline pretreatments at severe conditions (high temperature and alkali loadings) have been widely studied to promote biomass delignification. However, these operational conditions are not feasible to match the biorefinery concept since hemicellulose is wasted due to the high amount of lignin degradation compounds that are also solubilized in the alkaline black liquor, making the pentose use unviable (Guo et al., 2013). The loss of hemicellulosic sugars, mainly xylose, must be avoided because these sugars can be converted into ethanol or other higher value compounds, including xylitol and

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2,3-butanediol among others (Zhao et al., 2011; Gurpilhares et al., 2009), contributing to the economic success of the biorefineries.

Dilute acid pretreatment is a widely studied method that matches the requirement of selective and non-destructive separation of the polysaccharide fractions from lignocellulosic biomass, and, when compared to other pretreatment technologies, is pointed as the best choice in economic terms (Mussatto, 2016b). Besides to efficiently recover hemicellulosic sugars in the liquid fraction, this method also improves the cellulose digestibility of the resulting solid (Castro and Roberto, 2015). However, one of the major drawbacks is that several undesirable toxic compounds are also generated in the produced hemicellulosic hydrolysate, including sugar degradation products (5-hydroxymethylfurfural (HMF) and furfural), lignin degradation products (ferulic acid, *p*-coumaric acid, vanillin, vanillyl alcohol) and acetic acid (Mussatto and Roberto, 2004). Acetic acid, in particular, is one of the most important inhibitors for pentose-fermenting yeasts such as *Scheffersomyces stipitis* (Bellido et al., 2011), *Pachysolen tannophilus* (Harner et al., 2014) and the recombinant xylose-fermenting *Saccharomyces cerevisiae* (Martín et al., 2002). Since the ethanol production cost is sensitive to the pentose fermentation efficiency (Kumar and Murthy, 2011), the development of techniques able to reduce the concentration of inhibitor compounds in the hemicellulosic hydrolysate is strongly required to become the ethanol production from biomass more economically competitive.

In order to decrease the toxicity of hemicellulosic hydrolysates, physical, chemical, and/or biological detoxification methods can be used (Mussatto and Roberto, 2004). Nevertheless, not only inhibitor compounds but also part of the sugars can be removed during this stage. In addition, usually these methods do not provide reasonable results when applied alone, being necessary a combination of them, which increases the operational costs (Jönsson et al., 2013). In the present study, a strategy was developed to produce a sugar rich hydrolysate suitable for use as fermentation medium without necessity of applying a detoxification step. This strategy was based on performing a pretreatment step previous to the dilute acid pretreatment, with the purpose of selectively removing the toxic compounds without affecting the polysaccharide's structure. More specifically, a mild alkaline pretreatment was applied prior to the use of dilute acid in order to remove the acetyl groups (as acetate) with partial solubilisation of the lignin in the alkaline liquor, thus reducing the toxicity of the hydrolysate produced in the following step using dilute acid. There are some few reports in the literature on biomass deacetylation using other types of biomass such as yellow poplar (Cho et al., 2010), Kans grass (*Saccharum spontaneum*) (Chaudhary et al., 2012), and corn stover (Chen et al., 2014, 2012a; Shekiro et al., 2014). However, these studies are focused on the effect of the deacetylation in only one of the polysaccharide fractions (cellulose or hemicellulose), and not in both fractions as the present study. Additionally, to the best of our knowledge, this is the first study on the evaluation of the effect of deacetylation also in the fermentation of pentoses and hexoses sugars to produce ethanol.

2. Materials and methods

2.1. Feedstock

Rice straw was collected from fields in the region of Canas (São Paulo, Brazil). The material was dried until 10% moisture content, hammer-milled to obtain particles of about 1 cm in length and 1 mm in thickness, and stored until processing. The composition of this raw material, determined according to Sluiter et al. (2012), was (% w/w): 35.3 ± 0.2 cellulose, 23.8 ± 0.4 hemicellulose (19.9 xylan; 3.9 arabinan), 13.1 ± 0.7 acid insoluble lignin, 4.4 ± 0.2 acid soluble lignin, 2.6 ± 0.4 acetyl groups, 11.3 ± 0.1 ash, and 14.0 ± 0.2 extractives.

2.2. Mild alkaline pretreatment (deacetylation)

The mild alkaline pretreatment was carried out using different conditions of temperature (50–70 °C) and NaOH loading (20–80 mg NaOH/g biomass), which were combined according to a 2^2 face-centered central composite design. The experiments were performed in 250-mL Erlenmeyer flasks with 4.3 g rice straw (dry weight). The NaOH loading was obtained as a combination of different NaOH solutions (0.2, 0.6 or 0.8% w/v) and solid-to-liquid ratio (1:6; 1:8 or 1:10). The NaOH solutions were previously placed at the same temperature required for each assay. After homogenization of the mixture with a glass rod, the flasks were incubated in a water bath (Dubnoff Bath, Nova Ética, Brazil) under agitation of 100 cycles/min, during 45 min. Afterwards, the flasks were cooled in an ice bath and the solids were separated by filtration using 120 mesh sieves. The liquid fractions (alkaline black liquors) were stored and analyzed to determine the contents of monomeric sugars and acetate. The recovered solids (deacetylated rice straw) were washed with tap water until pH 6.5 and sun dried to 10% moisture, being subsequently characterized to determine the biomass compositional changes (Sluiter et al., 2012).

To obtain the necessary amount of deacetylated rice straw for subsequent dilute acid pretreatment, the alkaline pretreatment under the optimized conditions was scaled-up to a 50-L stainless steel reactor heated by electric resistance and stirred by rotation on its own axis (2 rpm). At the end of reaction, the reactor was cooled to room temperature, the alkaline liquor was removed and the deacetylated rice straw was washed with water until pH 6.5 and sun dried to 10% moisture content.

2.3. Dilute acid pretreatment

For the dilute acid pretreatment, 4.0 g of the sample (deacetylated or *in natura* rice straw) were placed in 125-mL Erlenmeyer flasks and impregnated with 40 mL H_2SO_4 solution. The reactions were carried out at 121 °C using different concentrations of H_2SO_4 solution (0.5–1.5% w/v) and residence times (30–90 min) according to a 2^2 face-centered central composite design. At the end of reactions, the residual solid material (cellulignin) was separated by filtration using 120 mesh sieves. The hemicellulosic hydrolysates were analyzed to determine the concentration of monomeric sugars (glucose, xylose and arabinose) and by-products (acetic acid, furfural, 5-HMF, furoic acid and low molecular weight phenolic compounds) present. The results were expressed in terms of hemicellulose hydrolysis efficiency (HHE), which was calculated using Eq. (1), where C is the concentration of xylose and arabinose in the liquid phase (g/L), M is the amount of deacetylated or *in natura* rice straw (dry matter) employed in the experiment (g), V is the volume of liquid solution employed (L) and Y_{max} is the maximum yield of recovered sugars that can be attained (g per 100 g dry matter). In addition, the concentration of inhibitory compounds was also assessed.

$$\mathrm{HHE}(\%) = \frac{C \times V}{M \times Y_{max}} \times 100 \quad (1)$$

To obtain enough amount of hemicellulosic hydrolysate and cellulignin for the subsequent experiments, the dilute acid pretreatment under the optimized conditions was also scaled-up to the same 50-L reactor described in the previous section. The reactions were carried out at 121 °C using 3.6 kg of solid material (deacetylated or *in natura* rice straw) and 36 L of acid solution. After reaction, the reactor was cooled until room temperature and the hemicellulosic hydrolysate was separated from the solid fraction by filtration in 120 mesh sieves. Both hemicellulosic hydrolysates (deacetylated and *in natura*) were analyzed for solubilized sugars and inhibitor

compounds, while the remaining solids were washed until pH 6.5, sun dried until 10% moisture content and stored for further use.

To be used as fermentation media, the hemicellulosic hydrolysates were submitted to a vacuum concentration process at 70 ± 5 °C in order to increase the xylose content to 70 g/L, and had the pH adjusted to 5.5 by addition of NaOH pellets. The concentration of sugars and inhibitors compounds in the concentrated hydrolysates were also determined.

2.4. Microorganisms and inoculum

Scheffersomyces stipitis NRRL Y-7124 was the yeast strain used for fermentation of the hemicellulosic hydrolysates, whereas for experiments of simultaneous saccharification and fermentation (SSF) of cellulignin was employed the yeast *Kluyveromyces marxianus* NRRL Y-6860. Both cultures were maintained on malt extract agar slants at 4 °C.

The inoculum of *S. stipitis* was prepared by transferring cells from the maintenance medium to test tubes containing sterilized water. Aliquots of cell suspension were transferred to 250-mL Erlenmeyer flasks containing 50 mL of *in natura* hemicellulosic hydrolysate (xylose content adjusted to 30 g/L and pH adjusted to 5.5 with 10 M NaOH) supplemented with 3.0 g/L yeast extract. The flasks were incubated in a rotary shaker at 200 rpm, 30 °C for 24 h. Then, the cells were recovered by centrifugation (2500 rpm, 20 min) and resuspended in the hydrolysates to obtain a concentration of 1.0 g/L at the beginning of the fermentation.

For preparation of the inoculum of *K. marxianus*, the cells from agar slants were transferred to 125-mL Erlenmeyer flasks containing 25 mL of medium composed of (g/L): glucose (30.0), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (1.5), $MgSO_4{\cdot}7H_2O$ (0.1) and yeast extract (3.0), prepared in 50 mM sodium citrate buffer (pH 4.8). The flasks were maintained in a rotatory shaker at 200 rpm, 40 °C, for 16 h. The cells were then recovered by centrifugation (2500 rpm, 15 min), washed twice in sterile distilled water, and resuspended in sterile distilled water to obtain a concentrated cell suspension that was used as inoculum for the SSF experiments.

2.5. Hemicellulosic hydrolysate fermentation

Fermentation assays using the hemicellulosic hydrolysates were carried out in 125-mL Erlenmeyer flasks containing 50 mL of concentrated hemicellulosic hydrolysate (pH = 5.5) supplemented with yeast extract (3.0 g/L) and inoculated with 1 g/L of *S. stipitis*. The flasks were incubated in a rotatory shaker at 30 °C, 200 rpm for 96 h. Samples were withdrawn periodically to determine the concentration of sugars and ethanol.

2.6. Enzymatic hydrolysis and SSF

Both solid fractions obtained after dilute acid hydrolysis (deacetylated cellulignin and reference cellulignin – pretreated only by dilute acid), were submitted to enzymatic hydrolysis and also to SSF experiments. Cellic CTec2 (Novozymes) was the enzyme cocktail used in both cases.

The enzymatic hydrolysis assays were performed in 125-mL Erlenmeyer flasks (50 mL reaction volume) using the following conditions: enzyme loading of 20 FPU/g cellulose, 8% (w/v) cellulignin content, pH 4.8 (sodium citrate buffer 50 mM), at 100 rpm and 43 °C. Samples were withdrawn periodically for sugar analysis being immediately heated for 5 min on a boiling water bath to inactivate the enzymes and stop the reactions. The mixture was then centrifuged (3000 rpm, 10 min) and the supernatant was analyzed. Cellulose conversion (CC, %) was estimated by the ratio between the amount of glucose produced and the total amount of glucose available in the substrate (considering the cellulose content of each sample).

SSF experiments were carried out using the same conditions applied for enzymatic hydrolysis (20 FPU/g cellulose, 8% w/v solids content, 100 rpm, 43 °C), but with additional supplementation of the medium with (g/L): $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (1.5), $MgSO_4{\cdot}7H_2O$ (0.1) and yeast extract (3.0), and addition of 1 g/L of *K. marxianus* at the beginning of the runs. Samples were withdrawn periodically for sugar and ethanol analyses being immediately heated for 5 min on a boiling water bath to stop the reactions.

2.7. Analytical methods and severity factor calculation

Glucose, xylose, arabinose, acetic acid and ethanol concentrations were determined by high performance liquid chromatography (HPLC) using an Agilent Technologies 1260 Infinity (Taunton, MA) chromatograph equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Hercules, CA) at 45 °C. Sulfuric acid (0.005 M) was used as eluent in a flow rate of 0.6 mL/min. Monomeric phenolic and furan compounds were also determined by HPLC but using an UV detector (at 276 nm) and a Waters Spherisorb C18 5 μm ODS2 column (4.6 × 100 mm) at room temperature, acetonitrile/water/acetic acid a ratio of 88:11:1 as eluent in a flow rate of 0.8 mL/min.

The combined severity factor (CSF), which integrates the effects of hydrolysis temperature, time and acid concentration into a single variable, was calculated according to Eq. (2), where t is the hydrolysis time (min), T_H is the hydrolysis temperature (°C), T_R is the reference temperature (most often 100 °C), and pH is the acidity of the aqueous solution in terms of acid concentration (Lloyd and Wyman, 2005).

$$\mathrm{CSF} = \log\{t \cdot \exp[(T_H - T_R/14.75)]\} - \mathrm{pH} \quad (2)$$

3. Results and discussion

3.1. Effect of mild alkaline pretreatment (deacetylation) on rice straw composition

Different temperatures and NaOH loadings were evaluated for deacetylation of rice straw. Table 1 shows the chemical composition of rice straw *in natura* and after each alkaline pretreatment condition. As can be seen, acetyl groups, ash and lignin were the main fractions affected by this pretreatment, being their removal favored when increasing the NaOH loading, independently of the temperature. The highest acetyl removal (98.8%) was achieved when using the conditions of the assay 4 (70 °C, 80 mg NaOH/g biomass), in which were also removed 42.7% of lignin and 59.4% of ash, with small losses of glucan (1.2%) and hemicellulose (7.7%). Chen et al. (2012b) found lower values of acetyl removal (75%) during the alkaline pretreatment of corn stover at 80 °C for 3 h, using 48 mg NaOH/g biomass. According to these authors, the impact of deacetylation on chemical composition of corn stover was highly dependent on the vegetal variety.

Under the evaluated conditions, the polysaccharide fractions (glucan and hemicellulose) were only slightly affected by the deacetylation, the highest removals being correspondent to 6.9 and 10.6%, respectively (assay 10). Despite cellulose is reported to be unreactive under mild alkaline conditions (Chen et al., 2013), grasses like rice straw also contain mixed-linkage glucan, a hemicellulosic cell wall polysaccharide with an unbranched β(1-3)(1-4)-glucan backbone (Vega-Sanchez et al., 2013), which explains the partial glucose solubilization in the alkaline liquor.

From these results it can be concluded that the objective proposed for the mild alkaline pretreatment was successfully achieved since acetyl group was the main fraction removed from biomass

Table 1
Effect of different conditions of alkaline deacetylation on chemical composition of rice straw.

Assay	Independent variables[a]		Rice straw composition (g/100 g)					MR[b] (%)	Removal after deacetylation pretreatment (%)				
	X_1	X_2	Glucan	Hemi	Lignin	Ash	Acetyl		Glucan	Hemi	Lignin	Ash	Acetyl
1	50	20	37.9	24.2	16.3	9.7	2.0	91.0	2.3	7.5	15.2	21.9	30.0
2	70	20	37.6	24.3	15.6	9.9	1.8	93.5	<0.5	4.5	16.6	18.1	35.3
3	50	80	42.4	27.5	13.3	5.9	0.2	82.8	<0.5	4.3	37.1	56.8	93.6
4	70	80	43.3	27.3	12.5	5.7	0.04	80.5	1.2	7.7	42.5	59.4	98.8
5	60	60	41.5	26.8	14.5	6.2	0.3	83.9	1.4	5.5	30.5	53.9	90.3
6	60	60	42.1	26.7	15.2	6.9	0.2	86.9	<0.5	2.5	24.5	46.9	93.3
7	60	60	40.7	26.4	15.7	6.8	0.3	85.2	1.8	5.5	23.6	48.7	90.2
8	50	60	41.8	26.9	14.9	7.1	0.4	82.1	2.8	7.2	30.1	48.4	87.4
9	70	60	42.2	28.1	14.3	6.2	0.3	84.6	<0.5	<0.5	30.8	53.6	90.2
10	60	20	38.1	24.7	15.8	9.6	1.7	86.2	6.9	10.5	22.2	26.8	43.6
11	60	80	42.1	26.7	13.7	4.6	0.2	81.9	2.3	8.1	35.9	66.7	93.7
RS[c]	–	–	**35.3**	**23.8**	**17.5**	**11.3**	**2.6**	–	–	–	–	–	–

[a] X_1 = temperature (°C), X_2 = (mg NaOH/g biomass).
[b] % mass recovery after the treatment.
[c] Rice straw *in natura*; Hemi = hemicellulose.

structure. Additionally, the alkaline pretreatment removed significant amount of lignin (15.26–42.31%) and ash (18.10–66.67%), which also contribute for the obtainment of a sugar rich hydrolysate with minimum concentration of toxic compounds during the subsequent biomass processing steps.

A statistical analysis of the results was then performed in order to select the process conditions that maximize the removal of acetyl groups with minimum effects on glucan and hemicellulose fractions. Mathematical models describing the variations of these responses as a function of the temperature and NaOH loading variations were established (Eqs. (3)–(5)). All the models presented high coefficient of determinations ($R^2 > 0.92$), which means a close agreement between the experimental results and those predicted by the models. According to the model equations, the linear effect of NaOH loading (X_2) was the only variable statistically significant ($p < 0.10$) for all the responses. For the acetyl removal, in particular, the linear effect of NaOH loading (X_2) was about thirteen times higher than the linear effect of the temperature (X_1), confirming the direct analysis of the data, which suggested greater influence of NaOH loading on the removal of acetyl groups.

$$\text{Acetylremoval}\ (\%) = 83.58 + 2.22 \cdot X_1 - 3.39 \cdot X_1^2 + 29.53 \cdot X_2 - 15.48 \cdot X_2^2 (R^2 = 0.99) \quad (3)$$

$$\text{Hemicellulosecontent}\ (\%\ \text{w/w}) = 26.44 + 0.18 \cdot X_1 + 0.48 \cdot X_1^2 + 1.38 \cdot X_2 - 0.97 \cdot X_2^2 (R^2 = 0.92) \quad (4)$$

$$\text{Glucancontent}\ (\%\ \text{w/w}) = 40.51 + 2.46 \cdot X_2 (R^2 = 0.92) \quad (5)$$

In order to select the best pretreatment conditions, a graphical optimization based on overlaying the curves of these three responses was performed and a condition was assigned as optimum point, which corresponded to the use of 70 °C and 80 mg NaOH/g rice straw. Under these conditions, the model predicts an acetyl removal of 96.5%, along with hemicellulose and glucan contents of 27.5% and 43.0%, respectively, in the pretreated biomass. The predicted responses were validated by performing additional experiments (in quadruplicate) at the selected conditions. The results obtained in these experiments (acetyl removal of 98.1%, with hemicellulose and glucan contents of 27.8% and 43.5% in the pretreated biomass), were very close to the predicted values. Under these conditions, the removal of lignin and ash were 52.8% and 80.4%, respectively, which will also positively impact in the subsequent processing steps of rice straw.

Finally, the alkaline pretreatment under the optimized conditions (70 °C and 80 mg NaOH/g rice straw) was scaled-up to 50-L reactor to obtain a higher amount of deacetylated rice straw for the subsequent assays. The chemical composition of rice straw before and after the scale-up experiments, as well as the mass recovery and removal percentage of each fraction, are shown in Table 2. As expected, acetyl group was the main fraction removed, followed by ash and lignin. However, a lower total mass recovery was attained when compared to the shake flask experiments (Table 1), probably due to the differences between the experimental apparatus, such as the agitation system, that might have affected the mass transfer during the reactions.

The liquid fraction obtained after this pretreatment stage (alkaline black liquor) was mainly composed of acetate (2.1 g/L) and phenolic compounds, mostly hydroxycinnamic acids (0.46 g/L ferulic and 0.26 g/L *p*-coumaric). The presence of hydroxycinnamic acids in the alkaline black liquor was expected since rice straw contains approx. 5–15% hydroxycinnamic acids in the composition, based on the total lignin content (Sun et al., 2002), and such phenolic acids are easily released during alkaline treatments (Buranov and Mazza, 2008; Mussatto et al., 2007). Phenolic acids present properties with important benefits for the health including antioxidant, anti-mutagenic, anti-allergenic, anti-inflammatory and anti-microbial properties, which are of great interest for food, cosmetic and pharmaceutical industries (Martins et al., 2011; Meneses et al., 2013; Mussatto et al., 2007). Such a fact opens up new possibilities for obtaining other high added-value products from rice straw, being of interest for the development of biorefineries.

3.2. Effect of deacetylation on hemicellulose sugars recovery by dilute acid pretreatment

In order to assess the impact of the deacetylation on hemicellulosic hydrolysate composition, dilute acid pretreatment was performed using the pretreated solid obtained under the optimized alkaline conditions and rice straw *in natura* as a reference, and the influence of the reaction time (30–90 min) and sulfuric acid concentration (0.5–1.5% w/v) was also studied. Table 3 shows the chemical composition of the hydrolysates obtained under the different process conditions.

Regarding the sugars solubilisation, glucose concentration was not above 1.8 g/L in all the hydrolysates indicating low cellulose degradation independent of the process condition and substrate (deacetylated or *in natura* rice straw). Arabinose concentration was also low and not significantly different for the different hydrolysates, with an average value of 4.5 g/L for the deacetylated hydrolysates and 2.2 g/L for the *in natura* hydrolysates.

Table 2
Chemical composition, mass recovery and removal of each fraction from rice straw after alkaline deacetylation pretreatment, under the optimized process conditions, in a 50-L reactor.

Components	Composition (g/100 g)		Mass recovery (g)[a]	Removal (%)
	In natura	Deacetylated		
Glucan	**35.3**	**42.8**	**30.0**	**15.1**
Hemicellulose	**23.8**	**28.3**	**19.8**	**16.8**
Xylan	*19.9*	*23.9*	*16.7*	15.9
Arabinan	*3.9*	*4.4*	*3.1*	21.0
Acetyl groups	**2.6**	**0.5**	**0.4**	**86.5**
Lignin	**17.5**	**16.5**	**11.6**	**34.0**
Acid insoluble lignin	*13.0*	*11.5*	*8.1*	37.7
Acid soluble lignin	*4.5*	*3.7*	*2.6*	42.2
Ash	**11.3**	**6.4**	**4.5**	**60.4**
Others	**9.5**	**5.5**	**3.9**	**59.5**
Total	**100**	**100**	**70.0**	**30.0**

[a] Calculated considering 70% total solids recovery.

On the other hand, the xylose concentration strongly varied according to the conditions used for dilute acid pretreatment (2.8–20.4 g/L in deacetylated hydrolysates and 1.6–18.2 g/L in *in natura* hydrolysates), revealing a significant influence of the studied variables on hemicellulose hydrolysis efficiency (HHE). As can be seen in Table 3, xylose and arabinose concentrations were higher in the deacetylated hydrolysates. This result indicates that previous deacetylation might have weakened hemicellulose structure, since lignin and structural proteins have been suggested to participate in intermolecular interactions with arabinoxylan (Agger et al., 2010), and therefore its removal would make hemicellulose linkages more exposed to degradation. However, this does not mean that HHE from deacetylated rice straw was always greater since xylan and arabinan contents of this material were also higher than those found in rice straw *in natura*, as shown in Table 2.

With respect to the inhibitor compounds, all deacetylated hydrolysates presented acetic acid concentrations below 0.1 g/L, which was expected due to the effective removal of acetyl groups during the first step of pretreatment. On the other hand, the hydrolysates obtained from rice straw *in natura* presented acetic acid concentration varying from 0.4 to 1.9 g/L, being the highest concentrations (above 1.7 g/L) related to the increase of combined severity factor (CSF) from 1.88 to 2.06. According to Fengel and Wegener (1989), the acetic acid release depends on the biomass type and process conditions. In fact, Lee et al. (2015) reported higher concentrations of acetic acid (0.95–3.79 g/L) during the dilute acid pretreatment of corn stover at CSF varying from 0.8 to 2.4.

Furans (furfural and HMF) were found at relatively small amounts in all the hydrolysates (<0.33 g/L). However, their concentration significantly varied according to the acid pretreatment condition employed, being the highest levels obtained when using the highest CSF (assay 4) (Table 3). This result reveals that the sugars degradation was sensitive to the process severity. Similar behavior was reported during the dilute acid pretreatment of other raw materials, such as corn stover (Lee et al., 2015) and cotton stalk (Gaur et al., 2016).

Phenolic acids (ferulic and *p*-coumaric) were also found in all the hydrolysates, but the highest concentrations were observed in the hydrolysates produced from rice straw *in natura.* The presence of lower concentrations of these compounds in deacetylated hydrolysates is justifiable since such acids had been partially removed during the alkaline pretreatment. It is worth mentioning that the concentration of *p*-coumaric acid in the hemicellulosic hydrolysate was always higher than the concentration of ferulic acid (ferulic/*p*-coumaric ratio ranging from 0.2 to 0.66), whereas an inverse behavior was observed in the alkaline black liquor (ferulic/*p*-coumaric ratio of 1.78). Such difference can be explained by the molecular bonds of hydroxycinnamic acids in the lignin-carbohydrate complex, since phenolic acids (in particular *p*-coumaric and ferulic) are directly involved in the lignin association with hemicellulose in the plant cell wall. Alkaline treatment promotes the cleavage of ester linkages with carbohydrates, releasing ferulic acid in the alkaline solution, whereas the acid treatment mainly break the ether linkages with condensed units of lignin, releasing *p*-coumaric acid in the acid solution (Buranov and Mazza, 2008).

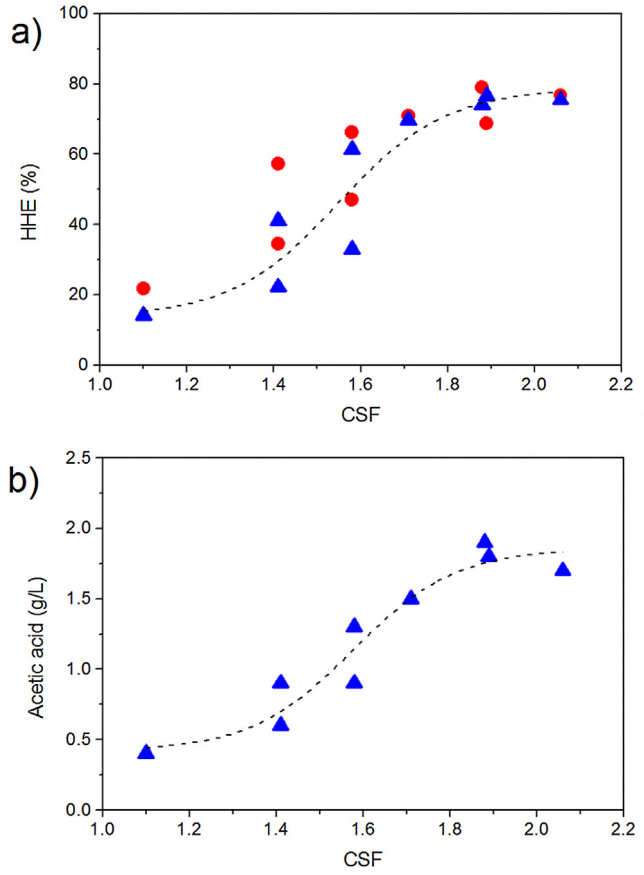

Fig. 1. Effect of combined severity factor (CSF) on a) hemicellulose hydrolysis efficiency (HHE) and b) acetic acid formation in the hydrolysates obtained from deacetylated (circles) and rice straw *in natura* (triangles).

The correlation of hemicellulose hydrolysis efficiency (HHE) with the combined severity factor (CSF) used in each experiment is shown in Fig. 1a. As can be seen in this figure, the HHE increased until CSF = 1.88 and no more improvements were observed at

Table 3
Chemical composition of rice straw hemicellulosic hydrolysates and hemicellulose hydrolysis efficiency (HHE) obtained under different conditions of dilute acid pretreatment.

Assay	Variables		CSF	Rice straw substrate	Sugars			Furans		Acids			HHE (%)
	Time (min)	H_2SO_4 (% w/v)			Glucose (g/L)	Xylose (g/L)	Arab. (g/L)	Furfural (mg/L)	HMF (mg/L)	Acetic (g/L)	Ferulic (mg/L)	*p*-Coumaric (mg/L)	
1	30	0.5	1.10	Deacetylated	0.1	2.8	4.2	13.0	11.3	nd	24.8	80.8	21.8
				In natura	0.1	1.6	2.2	10.9	35.9	0.4	27.2	105.0	14.1
2	90	0.5	1.58	Deacetylated	0.2	10.6	4.5	81.9	13.9	nd	31.1	94.9	47.0
				In natura	0.3	6.5	2.4	53.6	55.2	0.9	53.0	192.5	32.9
3	30	1.5	1.58	Deacetylated	0.6	16.6	4.7	70.6	8.5	nd	45.7	108.1	66.2
				In natura	0.6	13.9	2.7	52.0	53.1	1.3	56.7	184.5	61.4
4	90	1.5	2.06	Deacetylated	1.8	20.3	4.4	328.1	27.5	0.1	34.4	56.8	76.8
				In natura	1.8	17.5	2.9	293.8	100.5	1.7	79.4	120.0	75.4
5	30	1.0	1.41	Deacetylated	0.3	13.6	4.8	39.2	3.9	nd	34.1	112.9	57.2
				In natura	0.3	8.8	2.3	35.1	55.8	0.9	52.0	198.7	41.0
6	90	1.0	1.88	Deacetylated	1.4	20.4	5.0	222.8	23.4	nd	48.9	91.4	79.0
				In natura	1.5	17.4	2.6	198.4	91.1	1.9	78.3	163.0	74.0
7	60	0.5	1.41	Deacetylated	0.1	6.6	4.5	44.1	6.2	nd	23.2	94.3	34.5
				In natura	0.3	3.6	2.4	25.1	48.4	0.6	32.5	153.6	22.2
8	60	1.5	1.89	Deacetylated	1.4	17.8	4.3	208.3	13.5	nd	34.1	66.9	68.7
				In natura	1.6	18.2	2.5	179.5	69.8	1.8	73.6	110.2	76.5
9[a]	60	1.0	1.71	Deacetylated	0.7	17.9	4.8	98.3	8.7	nd	49.2	116.6	70.9
				In natura	0.8	15.9	3.0	81.9	75.0	1.5	69.1	212.1	69.7

[a] Central point value correspond to the average of four replicates; CSF = combined severity factor; HMF = hydroxymethylfurfural; HHE = hemicellulose hydrolysis efficiency; nd = not detected (<0.01 g/L).

higher severities. It is interesting to notice that for both substrates (deacetylated and *in natura*), HHE values exceeding 70% were achieved only when the CSF was greater than 1.70. Similar behavior was observed for the acetic acid release from rice straw *in natura* (Fig. 1b), as the highest concentrations of acetic acid (>1.7 g/L) were also obtained under the conditions that resulted in the highest HHE (>70%). This can be explained by the fact that acetyl groups are structurally linked to hemicellulose. Hsu et al. (2010) and Guo et al. (2008) have also obtained the maximum solubility of acetic acid in rice straw hemicellulosic hydrolysate at the same conditions that resulted in the highest HHE.

In the present study, the highest HHE values (79 and 76.8%) were obtained when using deacetylated rice straw under the conditions of the assays 6 (90 min, 1.0% w/v sulfuric acid – CSF = 1.88) and 4 (90 min, 1.5% w/v sulfuric acid – CSF = 2.06), respectively. Under these conditions, the HHE of rice straw *in natura* was only slightly lower (Table 3), suggesting that under more severe process conditions the hemicellulose solubilisation is not influenced by the deacetylation step. This behavior was also observed by Chen et al. (2012b), who found that increasing the severity reduced the xylose yield difference between the untreated and the deacetylated corn stover. According to these authors, under conditions of increased severity, the esterified fraction of xylan, which can reach up to 40% total xylan, is easily hydrolyzed and hence the effect of the alkaline pretreatment is lessened.

Statistical analysis of the data was carried out in order to select the conditions of dilute acid pretreatment that maximize the HHE from deacetylated rice straw, with low formation of furfural. Mathematical models describing the responses variations as a function of the variations in reaction time (X_1) and H_2SO_4 concentration (X_2) were established (Eqs. (6) and (7)). The models presented high coefficient of determination R^2 (0.99 for HHE and 0.92 for furfural concentration), revealing a close agreement between the experimental results and those predicted by the models.

$$\text{HHE (\%)} = 70.21 + 9.59 \cdot X_1 - 0.74 \cdot X_1^2 + 18.09 \cdot X_2 - 17.22 \cdot X_2^2 - 3.65 \cdot X_1 \cdot X_2 \quad (6)$$

$$\text{Furfural (g/L)} = 104.2 + 85.0 \cdot X_1 + 14.97 \cdot X_1^2 + 78.0 \cdot X_2 + 10.16 \cdot X_2^2 + 47.15 \cdot X_1 \cdot X_2 \quad (7)$$

As can be seen through the model equations, both variables, reaction time (X_1) and H_2SO_4 concentration (X_2), were significant at 95% confidence level for both the responses. Regarding to HHE, the linear effect of acid concentration was almost double the linear effect of reaction time, confirming the direct analysis of the data, which suggested greater influence of acid concentration on HHE. It is also noteworthy that the interaction between time and acid concentration showed a negative effect for this response, indicating that the HHE can be enhanced when higher acid concentration is employed during lower reaction time.

For the furfural concentration, the effect of reaction time (X_1) was higher than the effect of the acid concentration (X_2), indicating a greater influence of reaction time on pentose degradation reactions. For this response, the interaction between time and temperature showed a positive effect, indicating that the furfural concentration is increased when higher acid concentration is employed during longer reaction time.

In order to maximize the HHE, a graphical optimization based on overlaying the curves of these two responses was performed and a condition was set as optimum point, which corresponded to the use of 1.0% w/v sulfuric acid during 85 min. Under these conditions, the model predicts HHE of 78.2% and 197.9 mg/L furfural in the hydrolysate (at $p < 0.05$). The predicted responses were validated by performing extra experiments (in triplicate) at the selected pretreatment conditions. The results obtained in these

Table 4
Composition of rice straw hemicellulosic hydrolysates (original and after concentration) obtained by dilute acid pretreatment in a 50-L reactor under the optimized conditions.

Compounds	Original hydrolysates		Concentrated hydrolysates	
	in natura	Deacetylated	*in natura*	Deacetylated
Glucose (g/L)	2.0 ± 0.1	2.1 ± 0.1	8.9 ± 0.1	8.6 ± 0.1
Xylose (g/L)	18.1 ± 0.5	19.2 ± 0.5	69.0 ± 0.1	70.9 ± 0.9
Arabinose (g/L)	2.9 ± 0.1	5.2 ± 0.2	11.1 ± 0.1	16.6 ± 0.2
Acetic acid (g/L)	1.4 ± 0.1	<0.01	1.8 ± 0.1	<0.01
5-HMF (mg/L)	100.7 ± 1.4	27.2 ± 0.4	379.6 ± 5.2	126.1 ± 5.5
Furfural (mg/L)	282.6 ± 8.9	368.9 ± 2.4	252.6 ± 2.7	119.8 ± 3.7
Furoic acid (mg/L)	49.6 ± 5.9	126.6 ± 2.8	650.9 ± 7.4	595.8 ± 23.4
Vanillic acid (mg/L)	24.7 ± 0.2	8.2 ± 0.5	88.8 ± 1.0	33.4 ± 2.9
Vanillin (mg/L)	72.7 ± 1.3	12.7 ± 0.4	88.2 ± 2.2	36.5 ± 3.8
p-Coumaric acid (mg/L)	135.3 ± 1.0	17.9 ± 0.4	91.0 ± 1.7	22.3 ± 2.3
Ferulic acid (mg/L)	27.0 ± 2.2	13.4 ± 0.1	112.0 ± 1.1	20.9 ± 0.1

assays (HHE of 74.9 ± 2.2% and 191 ± 9.1 mg/L furfural) were in close agreement with the predicted values. In addition, low concentrations of hydroxycinnamic acids (66.1 ± 0.7 mg/L *p*-coumaric and 29.2 ± 4.7 mg/L ferulic acid) were found in the hydrolysate produced under these process conditions.

The conditions optimized for dilute acid pretreatment (1.0% w/v H_2SO_4, 85 min, 121 °C) were then scaled up to 50-L reactor using the rice straw deacetylated under the optimized alkaline conditions. The HHE for the scale-up experiments reached 75.9 ± 3.0%, which is in accordance with the value predicted by the model optimized for dilute acid pretreatment. However, the furfural concentration obtained in these experiments was higher than the value obtained in shake flasks, probably due to the longer time required for cooling the reactor, which might have favored the xylose degradation to furfural.

For comparison, assays using rice straw *in natura* were also carried out in the 50-L reactor. As can be seen in Table 4, similar contents of glucose and xylose were found in both hydrolysates. However, a higher concentration of arabinose was observed in deacetylated rice straw hydrolysate, and the concentration of inhibitory compounds was also quite different for both, deacetylated and *in natura* hydrolysates. Both hydrolysates were later concentrated to increase the xylose content for fermentation. After concentration, the sugars contents were increased proportionally to the concentration factor employed. The same did not occur for the contents of by-products, specially furfural that had its concentration decreased, suggesting partial degradation or volatilization of these compounds during the concentration step, that would be beneficial for the subsequent fermentation process.

Besides do not contain acetic acid, the concentrated hydrolysate from deacetylated rice straw contained lower concentration of furans and phenolic compounds than the concentrated hydrolysate produced from rice straw *in natura*. It should be highlighted that the identified low molecular weight phenolic compounds (vanillic acid, vanillin, *p*-coumaric acid and ferulic acid) are only a small fraction of the total phenolics present in rice straw hemicellulosic hydrolysate, which also contains high amounts of polyphenolic structures (Silva et al., 2013). Nevertheless, the identified phenolic compounds would be an indicative of the hydrolysate toxicity, since they have been suggested to be inhibitory to the microbial metabolism (Mussatto and Roberto, 2004). Silva et al. (2013) reported that the removal of total phenols (above 40%), low molecular phenolic compounds (above 95%) and furans (above 52%) improved approximately twice the ethanol volumetric productivity by *S. stipitis* NRRL Y-7124 in rice straw hemicellulosic hydrolysate. Therefore, the mild alkaline pretreatment proposed in the present study, which reduced the concentration of potential fermentation inhibitors in the hydrolysate, should also improve the rice straw hydrolysate fermentability.

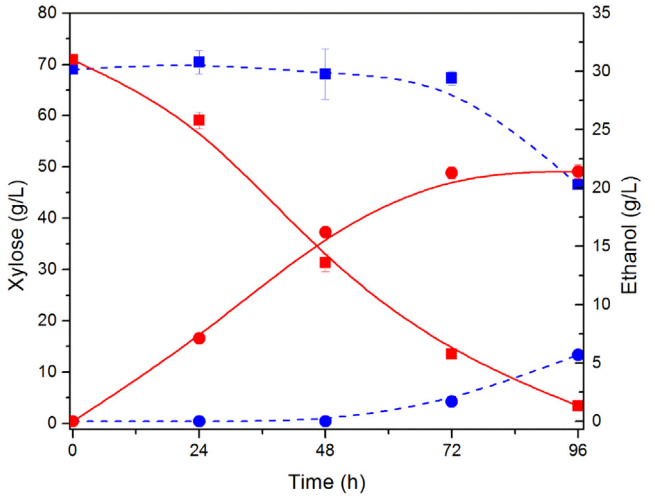

Fig. 2. Xylose consumption (squares) and ethanol production (circles) by *S. stipitis* NRRL Y-7124 from both deacetylated (solid lines) and *in natura* (dashed lines) rice straw hemicellulose hydrolysates.

3.3. Effect of deacetylation on ethanol production from hemicellulose and cellulose

In order to investigate the effects of deacetylation on ethanol production from hemicellulosic hydrolysate, fermentation experiments employing the yeast *S. stipitis* NRRL Y-7124 were carried out using the hydrolysates of both deacetylated and *in natura* rice straw. As can be seen in Fig. 2, the yeast was able to consume only 30% of the total xylose content and produced about 6 g/L ethanol in the *in natura* hydrolysate. The results were greatly improved when using the deacetylated rice straw hydrolysate (almost 70 g/L xylose were consumed and about 20 g/L ethanol were produced), resulting in an increase on the ethanol volumetric productivity (Q_P) from 0.06 to 0.30 g/L h. These results can be attributed to the absence of acetic acid and lower concentrations of phenolic compounds and furfural in deacetylated hydrolysate. Recently, some authors reported a positive effect of the deacetylation step on ethanol production from hemicellulosic hydrolysates of different raw materials, such as yellow poplar (Cho et al., 2010; Kundu et al., 2015) and corn stover (Chen et al., 2012b, 2012c). The present study is the first report on the effects of a deacetylation pretreatment on ethanol production from rice straw hemicellulosic hydrolysate.

It is also important to highlight that it was not necessary to submit the deacetylated rice straw hydrolysate to any previous detoxification method prior the yeast inoculation for fermentation. In general, the fermentation of non-detoxified hemicellulosic hydrolysates is characterized by slow kinetics, with limited yield and productivity when compared with the fermentation of com-

Table 5
Fermentative parameters of ethanol production from C5 and C6 streams obtained from the evaluated pretreatments of rice straw.

C5 fermentation (hemicellulose hydrolysate)	Deacetylated	*in natura*
Xylose consumption (%)	95.2	32.6
Ethanol (g/L)	21.3	5.7
$Y_{P/S}$ (g/g)	0.37	0.25
Q_P (g/L h)	0.30	0.06
C6 fermentation (cellulignin by SSF)	**Deacetylated**	**Reference**
Cellulose conversion (%)[a]	88.9	70.1
Ethanol (g/L)	20.4	12.7
$Y_{P/S}$ (g/g)	0.45	0.40
Q_P (g/L.h)	0.57	0.35

[a] Results obtained from enzymatic hydrolysis of cellulignin only, without yeast inoculation.

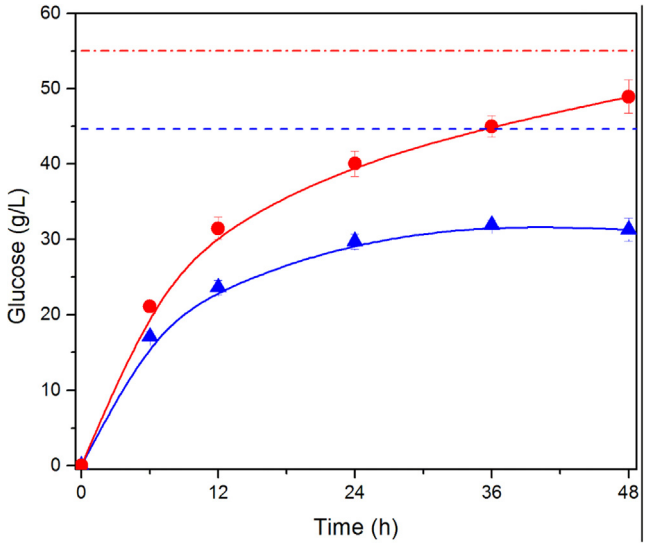

Fig. 3. Glucose concentration from enzymatic hydrolysis of deacetylated cellulignin (circles) and reference cellulignin (triangles) at 8% solids and enzyme loading of 20 FPU/g cellulose (Cellic CTec2). The horizontal lines represent maximum glucose concentrations (100% cellulose conversion yield) for deacetylated (dash dot) and reference (dash) cellulignin.

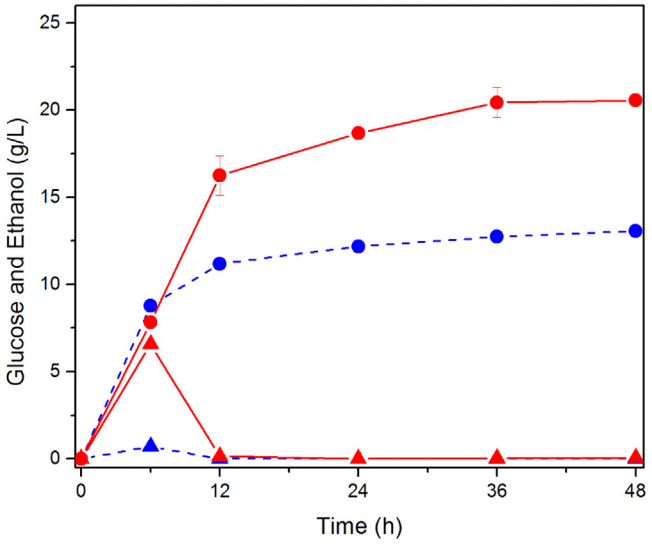

Fig. 4. Ethanol production (circle) and glucose concentration (g/L) during simultaneous saccharification and fermentation (SSF) at 8% solids, from deacetylated (solid line) and reference rice straw cellulignin (dotted line), at enzyme loading of 20 FPU/g cellulose (Cellic CTec2) and 1 g/L of *K. marxianus* NRRL Y-6860 initial cell concentration.

mercial sugars or detoxified hydrolysates (Mussatto and Roberto, 2004). Therefore, the process costs for ethanol production from pentoses using lignocellulosic materials could be decreased by employing the deacetylation prior to the dilute acid pretreatment and, even more, if adding value to the alkaline liquor generated during this process for the recovery of acetic acid and/or antioxidant phenolic compounds, for example.

In order to investigate the effects of deacetylation on ethanol production from cellulose, enzymatic hydrolysis assays were carried out using the solid fractions obtained after the dilute acid pretreatment (cellulignins). Additionally, assays for ethanol production by SSF employing the yeast *K. marxianus* NRRL Y-6860 were also performed. The results revealed that the deacetylated solid was more susceptible to enzymatic hydrolysis than the reference cellulignin (Fig. 3) since about 50 g/L glucose (89% cellulose conversion) were obtained from the deacetylated rice straw while only 30 g/L glucose (73% cellulose conversion) were produced from the reference material. Such improvement in the material digestibility can be attributed to the structural modifications caused by the alkaline pretreatment, which produced a solid with higher cellulose (61.8%) and lower lignin (17.1%) and ash (6.0%) contents than the reference material (50.2%, 25.5% and 13.8% cellulose, lignin and ash contents, respectively). The positive effect of a deacetylation step prior to the dilute acid pretreatment on enzymatic conversion of cellulose was also described by Chen et al. (2012b). These authors evaluated four hybrids varieties of corn stover and reported an average increase of 15% when using deacetylated substrates instead of those submitted only to dilute acid pretreatment.

As a consequence of the lower recalcitrance to enzymatic hydrolysis, the ethanol production by SSF process was also improved (about 60%) by using the deacetylated cellulignin, as shown in Fig. 4. It is worth mentioning that no pre-saccharification step was used in the present work, since cellulase enzymes and yeast were added at the same starting point, and the temperature was kept at 43 °C during all the process.

Finally, the impact of the mild alkaline pretreatment (deacetylation) on ethanol production from both hemicellulose and cellulose fractions of rice straw is summarized in Table 5. As can be seen in this table, all the fermentative parameters were substantially improved when the biomass was pretreated by sequential deacetylation and dilute acid pretreatment, especially those regarding to ethanol production from hemicellulose (ethanol concentration and Q_P were increased 3.7 and 5-fold, respectively).

4. Conclusions

Mild alkaline pretreatment under the optimized conditions (70 °C and 80 mg NaOH/g biomass) was an effective method for the removal of acetyl groups from rice straw (deacetylation) provid-

ing also partial removal of lignin and ash. This deacetylation step improved the quality of the hemicellulosic hydrolysate obtained in the subsequent step of pretreatment using dilute acid by lowering the concentration of inhibitory compounds generated. Ethanol production from xylose was also strongly improved when compared to the production from non-deacetylated material. Furthermore, the deacetylated cellulignin was more susceptible to enzymatic hydrolysis than the reference cellulignin, also providing improved results of ethanol production by SSF process. In brief, the deacetylation pretreatment prior to polysaccharides hydrolysis by dilute acid process enhanced the ethanol production from both xylose and glucose, thus providing an efficient route for rice straw processing.

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