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Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis

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HIGHLIGHTS

- Enzymatic hydrolysis in fed-batch with pretreated sugarcane bagasse.
- Glucose concentration remained constant after 30 h due to higher lignin content.
- Fed-batch addition of both material led to higher glucose concentration with 12-h.
- The delignification and fed-batch hydrolysis led to an increase ethanol production.

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ABSTRACT

Enzymatic hydrolysis of pretreated sugarcane bagasse was performed to investigate the production of ethanol. The sugarcane bagasse was pretreated in a process combining steam explosion and alkaline delignification. The lignin content decreased to 83%. Fed-batch enzymatic hydrolyses was initiated with 8% (w/v) solids loading, and 10 FPU/g cellulose. Then, 1% solids were fed at 12, 24 or 48 h intervals. After 120 h, the hydrolysates were fermented with *Saccharomyces cerevisiae* UFPEDA 1238, and a fourfold increase in ethanol production was reached when fed-batch hydrolysis with a 12-h addition period was used for the steam pretreated and delignified bagasse.

1. Introduction

Lignocellulosic biomass, such as agricultural residues, potentially can be used for the biofuel production. Sugarcane bagasse, the major by-product of the sugarcane industry, is an economically viable and very promising raw bagasse for bioethanol and biomethane production (Rabelo et al., 2011; Badshah et al., 2012).

Bioethanol production from lignocellulosic biomass, by using *Saccharomyces cerevisiae*, comprises the hydrolysis of cellulose and sugar fermentation. In order to obtain fermentable sugars from sugarcane bagasse, the cellulose can be saccharified using either acids or enzymes. However, somewhat acid enzymes are preferred rather acid because enzymatic hydrolysis is free from fermentation inhibitory products (Rivera et al., 2010; Galbe and Zacchi, 2002; Sun and Cheng, 2002).

The task of hydrolyzing lignocellulose to fermentable monosaccharides is still technically problematic because the digestibility of cellulose is hindered by many physico-chemical, structural and compositional factors. Therefore, pretreatments, such as with steam explosion, alkaline, diluted acid, ammonia, organosolv water/ethanol, oxidation with Fenton's reagent and pelleting, among other methods, are required prior of enzymatic hydrolysis in order to make the cellulose more accessible to attack by enzymes (Galbe and Zacchi, 2002; Beukes and Pletschke, 2010; Michalska et al., 2012; Rijal et al., 2012).

Steam explosion is the most widely employed physico-chemical pretreatment for lignocellulosic biomass. In combination with the partial hemicellulose hydrolysis and solubilization, the lignin is redistributed and to some extent removed from the bagasse (Pan et al., 2005). On the other hand, alkaline pre-treatments solubilize

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lignin and a small percentage of the hemicelluloses (Beukes and Pletschke, 2010). The raw corn stover was pretreated in a process combining steam explosion and alkaline hydrogen-peroxide (Yang et al., 2010). Steam explosion was chosen as the first step to pretreat the corn stover. Lignin still was remained a protective physical barrier to against enzymatic attack, for it could not be effectively removed by steam explosion. After alkaline peroxide pretreatment, the sample contained 73.2% cellulose and less than 7% lignin.

Enzymatic hydrolysis of cellulose is also affected by the reaction conditions, the product inhibition, and substrate-related factors (Balat, 2011). Even if the efficiency of the enzymatic hydrolysis is high, the low solids concentration in the reaction mixture leads to low sugar production in the hydrolysates (Kuhad et al., 2010). Raising the substrate concentration in batch hydrolysis helps to increase sugar concentration, but it also changes the rheological properties of the fibrous suspensions, which difficult mixing and heat transfer (Rudolf et al., 2005). These problems can be avoided if the hydrolysis is carried out in fed-batch mode, i.e., by adding the substrate gradually to maintain the viscosity at low level (Chen et al., 2007).

In this work, the enzymatic hydrolysis of sugarcane bagasse was performed in fed-batch in order to evaluate the effects of steam explosion pretreatment coupled with alkaline delignification on ethanol production.

2. Methods

2.1. Steam-pretreated sugarcane bagasse and delignification

Sugarcane bagasse pretreated with steam explosion was kindly provided by Department of Biotechnology of Engineering College of Lorena (University of Sao Paulo, Brazil). The natural sugarcane bagasse was chopped to 5–6 mm size and then it was treated in a steam-exploded vessel at 200 °C for 7 min. The recovering of solids after steam explosion was 68% (Santos et al., 2012). The compositions of the raw and pretreated bagasse are shown in Table 1 (Gouveia et al., 2009).

The biomass was dried under room temperature and then it (containing 10% solids) was further pretreated with 1% (w/v) sodium hydroxide at 100 °C for 1 h in a 20 L rotary reactor fitted with mixing and heating systems (Regmed AUE/20, Regmed Indústria Técnica Ltda., Brazil). The pre-treated and delignified bagasse was filtered, and washed seven times by distilled water and dried at 50 °C. The pretreated biomass was used as the substrate in the entire work.

2.2. Enzymes and activities

A commercial preparation of *Trichoderma reesei* cellulases (Celluclast 1.5 L: 69.50 FPU/mL and 13.7 CBU/mL) and a β-glucosidase preparation (Novozym 188:1340 CBU/mL), both from Novozymes A/S (Bagsværd, Denmark), were used. The filter paper activity (FPU) and β-glucosidase activity (IU) were measured with improved method developed by Ghose (1987). The concentration of reducing sugar was determined using dinitrosalicylic acid reagent (Miller, 1959).

2.3. Enzymatic hydrolysis of sugarcane bagasse

Batch and fed-batch enzymatic hydrolysis of steam-exploded bagasse either delignified (D) or non-delignified (ND), were carried out at 8% (w/v) consistency in a sodium citrate buffer (pH 4.8; 50 mM). The cellulase load was 10 FPU/g cellulose in all the experiments, whereas the β-glucosidase load was 5% (v/v) of the added cellulase amount for the ND bagasse and 10% (v/v) for the D bagasse. These enzymatic loads were chosen according to previous results (Santos et al., 2012). The flasks with the reaction mixture were incubated in a rotary shaker at 50 °C and 150 rpm, and the hydrolysis carried out during 120 h. In fed-batch enzymatic hydrolysis, the experiments started with 8 g bagasse, and 1 g of fresh bagasse was added periodically after 12 h (ND12 and D12), 24 h (ND24 and D24) or 48 h (ND48 and D48) until reaching final solids loadings of 17%, 12% or 10%, respectively. Samples withdrawn at regular intervals were filtered (0.45 μm) and the supernatant was analyzed for glucose released. All the experiments were performed in duplicate.

2.4. Microorganism

An industrial strain (UFPEDA 1238) of *S. cerevisiae*, kindly provided by the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco, Brazil, was used. The strain was maintained in a solid medium containing (in g/L) glucose (20), yeast extract (4), peptone (3) and agar (15), at pH 7.0.

2.5. Inoculum preparation and fermentation

Inoculum was prepared by transferring of cells of *S. cerevisiae* UFPEDA 1238 into a 500 mL flask containing 100 mL of culture medium (20 g/L glucose, 3 g/L peptone, 4 g/L yeast extract; pH 7.0), and incubating at 30 °C for 12 h. Cells were harvested by filtration (0.45 μm filter), suspended in sterilized water and used to inoculate the fermentation medium. Cellulosic hydrolysate obtained from fed-batch hydrolysis, supplemented with 4 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4 and 0.75 g/L $MgSO_4{\cdot}7H_2O$, was utilized as a fermentation medium. The pH was adjusted to 5.5. Ethanol fermentations were carried out at 34 °C without agitation and in duplicate, with 4 g/L of initial concentration biomass into a 250 mL flask with a working volume of 100 mL. Samples were withdrawn with 24 h, filtered (0.45 μm filter), and the cell free supernatant was used for the determination of ethanol, glycerol, acetic acid and glucose by high performance liquid chromatography.

2.6. Analysis

The compositions of pretreated sugarcane bagasse were determined according to the analytical procedure validated by Gouveia et al. (2009). The microstructures were characterized by Scanning Electron Microscope (JSM 5900LV, JEOL, Japan). Superficial areas were evaluated in a Physisorption Analyzer (Micromeritics, ASAP 2020). The BET (Brunauer, Emmett e Teller) model was used for analysis of superficial area. This method is based in a multilayer adsorption model (Gelb and Gubbins, 1998). The crystallinity index (CI), measured by X-ray diffraction, was calculated from the height

Table 1
Chemical compositions of the sugarcane bagasses.

Component	Raw sugarcane bagasse, wt.%	Pretreated by steam explosion, wt.%	Pretreated by steam explosion, and NaOH, wt.%
Cellulose	42.8 ± 0.3	47.7 ± 0.5	87.3 ± 2.2
Hemicellulose	25.8 ± 0.3	8.9 ± 0.8	6.9 ± 0.7
Lignin	22.1 ± 0.3	34.3 ± 0.8	5.7 ± 0.1

ratio between the intensity of the crystalline peak with subtraction of amorphous and crystalline peaks (Driemeier et al., 2011).

Samples of enzymatic hydrolysis and fermentations were filtered through a 0.45 μm filter. Sugars, glycerol, acetic acid, ethanol and furan aldehydes were quantified by HPLC (Agilent HP 1100, Germany). Cellobiose, glucose, glycerol, acetic acid and ethanol were separated on an Aminex HPX-87H$^+$ column (Bio-Rad, Hercules, CA, USA). The mobile phase was 5 mmol/L H_2SO_4 at a flow rate of 0.6 mL/min, at 50 °C, and detected by index of refraction. Furfural and 5-hydroxymethylfurfural (HMF) were separated on a C-18 column (Beckman). The mobile phase was Acetonitrile/(1%) Acetic acid (12:88) at a flow rate of 0.8 mL/min at 25 °C and 274 nm. The total content of phenolic compounds was determined by colorimetrically Folin–Ciocalteu (Singleton et al., 1999) in a HP 8453 spectrophotometer (Agilent, Germany). Gallic acid was used as calibration standard.

3. Results and discussion

In this work, steam explosion was chosen as the first step to pretreat the sugarcane bagasse. The chemical compositions of sugarcane bagasse are shown in Table 1. After steam explosion, the percent hemicellulose content decreased from 25.8% to 8.9%. The percentage concentrations of lignin and cellulose increased from 22.1% and 42.8% to 34.3% and 47.7%, respectively. Lignin still was a protective physical barrier to enzymatic attack for it could not be effectively removed by steam explosion pretreatment. To improve the digestibility of the cellulosic residues, an alkaline delignification (NaOH) was used as the second step of pretreatment.

After delignification, the recovery of solids was 50%, and the obtained cellulosic pulp contained 87.29% cellulose, 6.88% hemicelluloses and 5.68% lignin. As result of the delignification, the content of lignin in the D bagasse was approximately 84% lower than in the ND bagasse. On the other hand, due to lignin solubilization, cellulose content increased in 83%. Lignin removal was higher than that obtained by Beukes and Pletschke (2010) for alkaline delignification (CaOH) of sugarcane bagasse (77%). NaOH has been reported to increase hardwood digestibility by reducing lignin content from 24–55% to 20% (Kumar et al., 2009).

Michalska et al. (2012) in the pretreatment from *Miscanthus giganteus*, *Sida hermaphrodita* and *Sorghum Moensch* with Fenton's reagent, obtained about 30–60% delignification. In hot water pretreatment coupled with organic solvent (organosolv) pretreatment of water/ethanol from almond (Prunus dulcis) shells, lignin removal reached 66% (Dong et al., 2011).

Superficial area of D bagasse was higher (1.34 ± 0.02 m^2/g) than the ND bagasse (1.24 ± 0.03 m^2/g), which is in agreement with the SEM observations. Crystallinity index of the D bagasse was around 67% lower than the ND bagasse. These results are consistent with the aims of pretreatments, which are to reduce crystallinity and thus improve the enzymatic conversion of cellulose (Hägerdal-Hahn et al., 2006). Enzyme accessibility should be affected by crystallinity, but it is also known to be affected by the lignin and hemicelluloses contents/distribution, particle size, and porosity of the native cell wall sample (Park et al., 2010). Sodium hydroxide causes swelling, decreasing the degree of polymerization and crystallinity, which provokes lignin structure disruption (Taherzadeh and Karimi, 2008).

In agreement with the above discussed phenomena, the results revealed a clear positive effect of delignification on the enzymatic hydrolysis. Maximal glucose concentration in the batch enzymatic hydrolysis of D bagasse (39 g/L) was 2.3 times higher than for the ND bagasse (17 g/L) (Fig. 1).

Maximal cellobiose concentrations were below 1 and 2 g/L in the hydrolysis of the ND and D bagasse, respectively. This indicates that the amount of β-glucosidase (5% of the added cellulase for the ND bagasse and 10% for the D bagasse) was sufficient to hydrolyze cellobiose to glucose. Glucose and cellobiose, are well-known inhibitors of cellulases (Mosier et al., 1999). Yang et al. (2010) observed slight inhibition of cellulose activity by 10 and 20 g/L cellobiose. On the other hand, 100 and 200 g/L glucose could decrease the enzymatic activity by 30% and 50%, respectively.

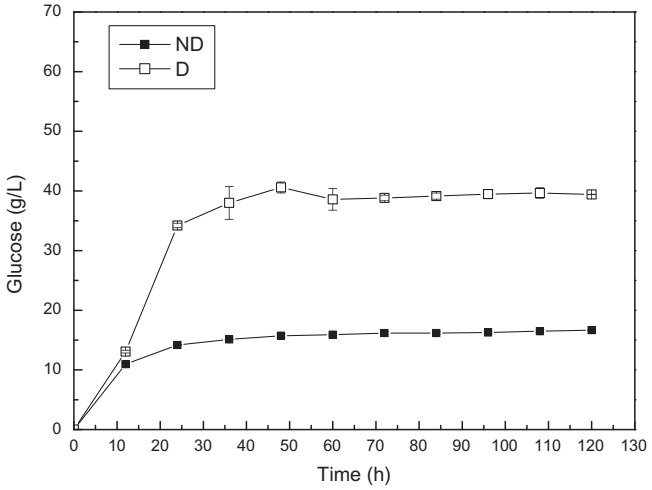

Fig. 1. Time course of batch enzymatic hydrolysis of non-delignified and delignified bagasses.

Fermentable sugars should be as high as possible in the practical bioethanol fermentation system to reduce the cost (Wingren et al., 2003). Raising the solids loading was considered as a direct way to enable high-concentration sugars. However, mixing and heat transfer problem became severe (Rudolf et al., 2005). Fed batch process was chose as an effective way to solve this problem.

In the fed-batch enzymatic hydrolysis, batch process was firstly taken in the beginning with 8% solids loading and 10 FPU/g cellulose. As the hydrolysis proceeded, the solid content of slurry decreased obviously. To increase the total solids loading, 1% fresh substrate was fed at intervals of 12, 24 or 48 h. Fig. 2 shows the effect of feeding time on the release of glucose from hydrolysis of sugarcane bagasse.

Fed-batch enzymatic hydrolysis improved the glucose concentration for both D and ND bagasse (Fig. 2) compared to the enzymatic hydrolysis carried out in batch (Fig. 1). The best results were achieved when the addition period was 12 h, where glucose

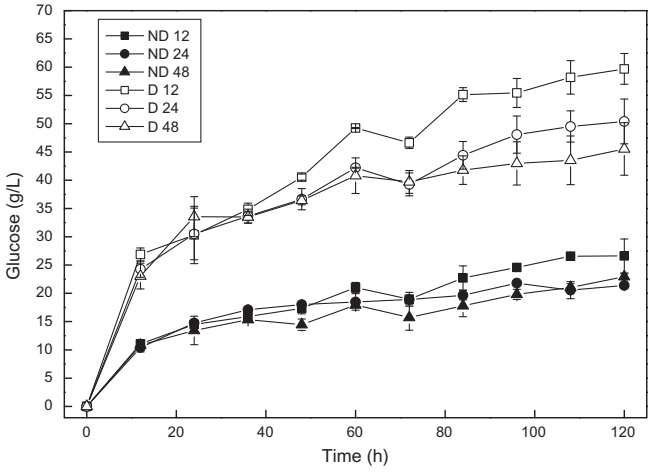

Fig. 2. Time course of fed-batch enzymatic hydrolysis of non-delignified (ND) and delignified (D) bagasses.

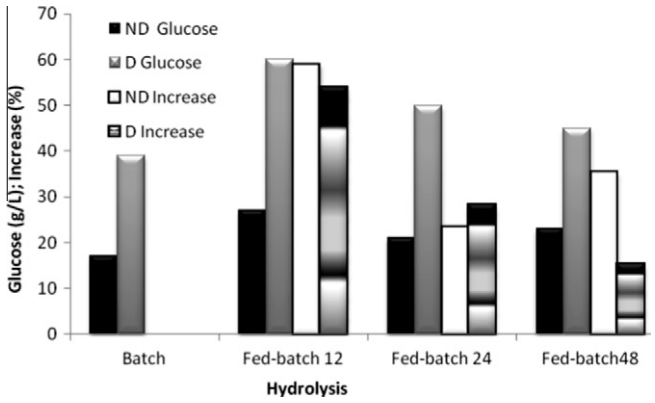

Fig. 3. Glucose concentration (g/L) after 120 h of fed batch enzymatic hydrolysis of non-delignified (ND) and delignified (D) bagasses, and increase compared to the batch mode (%).

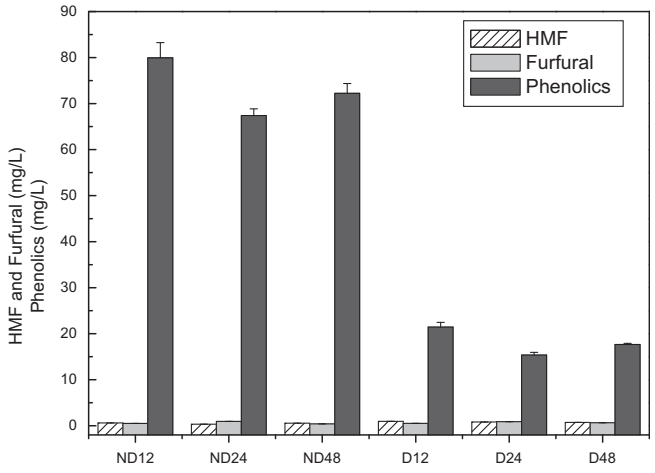

Fig. 4. Content of fermentation-inhibitory compounds in the hydrolysates obtained by fed-batch enzymatic hydrolysis of non-delignified (ND) and delignified bagasses (D).

concentration reached 53 g/L in the experiment with D bagasse (Fig. 2). Although in the hydrolysis of the ND bagasse, the maximum glucose concentration (23 g/L) was lower than that achieved for the D bagasse, it is evident that it was also positively influenced by the 12-h addition period (Fig. 2).

Besides fed-batch enzymatic hydrolysis, the pretreatment process was also a key factor for producing higher concentration glucose. Yang et al. (2010) in sugars production from corn stover, obtained also higher concentration glucose in process based on combined pretreatments and fed-batch enzymatic hydrolysis.

Regarding longer addition periods, no clear differences between the effect of 24-h and 48-h periods were observed up to 72 h for the D bagasse (Fig. 2). However, starting from 84 h, glucose concentration was considerably higher in the experiment with 24-h addition period than in the one with 48-h period. On the other hand, for the ND bagasse, glucose formation was generally higher for the 24-period than for the 48-h one.

Fig. 3 shows the effect of fed-batch hydrolysis on the increase of glucose concentration. In fed-batch process of 17% of solids loading, the increase was higher than 12% or 10% solids loading for both materials (ND or D bagasse). However, the analyses of variance showed that these results were significantly different for the hydrolysis of ND ($F = 3{,}916.40$; $\alpha = 0.05$) and D bagasse ($F = 4{,}388.78$; $\alpha = 0.05$).

The fermentation inhibitors furfural, HMF and phenolics compounds were low in all the enzymatic hydrolysates (Fig. 4). Furfural and HMF concentrations were comparable for all conditions. However, the total content of phenolics compounds was four times higher in the hydrolysates of ND bagasse. Enzymatic hydrolysis leads to higher yields of monosaccharide than dilute-acid hydrolysis, because cellulase enzymes catalyze only hydrolysis reactions and not sugar degradation reactions (Parisi, 1989).

Table 2 shows cell growth (ΔX), ethanol production (Et_f) and substrate consumption (ΔS) in the fermentations of fed-batch enzymatic hydrolysates of non-delignified (ND) and delignified (D) bagasses, with 12, 24, and 48 addition periods. The higher growth and ethanol production achieved in the fermentations of the hydrolysates of D bagasse can be explained by the higher initial glucose concentrations (45.5–59.7 g/L). Glucose was exhausted in all fermentations during 24 h.

The glucose consumption was utilized to calculate the fermentation efficiency, $Y_{E/S}/0.511 * 100$ (0.511 g/g is the theoretical

Table 2
Cell growth (ΔX), substrate consumption (ΔS) and ethanol production (Et_f) in the fermentations of fed-batch enzymatic hydrolysates of ND and D bagasses.

Hydrolysates	S_i (g/L)	S_f (g/L)	ΔS (g/L)	ΔX (g/L)	Et_f (g/L)
ND12	27.67 ± 1.49	0.34 ± 0.02	27.33 ± 1.47	0.88 ± 0.42	10.17 ± 0.56
ND24	21.40 ± 0.08	0.43 ± 0.01	20.97 ± 0.07	2.24 ± 0.15	6.18 ± 0.07
ND48	22.94 ± 0.52	0.47 ± 0.01	22.57 ± 0.52	2.05 ± 0.47	5.33 ± 0.48
D12	59.69 ± 2.72	1.34 ± 0.07	58.35 ± 2.80	3.19 ± 0.06	23.38 ± 0.46
D24	50.40 ± 3.96	0.38 ± 0.03	50.02 ± 3.99	3.13 ± 0.30	18.02 ± 1.55
D48	45.54 ± 4.65	0.43 ± 0.01	45.11 ± 4.65	4.02 ± 0.33	16.75 ± 0.49

X_f, final biomass concentration after 24 h; S_f, final glucose concentration after 24 h; S_i, initial glucose concentration; Et_f, final ethanol concentration.

Table 3
Yields and efficiencies of the fermentations of the hydrolysates of non-delignified (ND) and delignified (D) bagasse from fed-batch enzymatic hydrolyses.

Hydrolysates	$Y_{X/S}$ (g/g)	$Y_{E/S}$ (g/g)	$Y_{G/S}$ (g/g)	$Y_{AC/S}$ (g/g)	E_F (%)
ND12	0.03 ± 0.01	0.367 ± 0.02	0.024 ± 0.001	0.013 ± 0.003	73.00 ± 7.93
ND24	0.11 ± 0.01	0.289 ± 0.03	0.053 ± 0.001	0.019 ± 0.003	57.66 ± 0.49
ND48	0.09 ± 0.02	0.232 ± 0.20	0.061 ± 0.003	0.027 ± 0.001	46.47 ± 5.22
D12	0.05 ± 0.01	0.392 ± 0.007	0.048 ± 0.002	0.011 ± 0.001	78.47 ± 2.24
D24	0.06 ± 0.01	0.357 ± 0.30	0.047 ± 0.003	0.012 ± 0.001	70.51 ± 0.45
D48	0.09 ± 0.01	0.368 ± 0.010	0.057 ± 0.003	0.013 ± 0.002	73.18 ± 9.65

$Y_{X/S}$, biomass yield; $Y_{E/S}$, ethanol yield; $Y_{G/S}$, glycerol yield; $Y_{AC/S}$, acetic acid yield; E_F, efficiency.

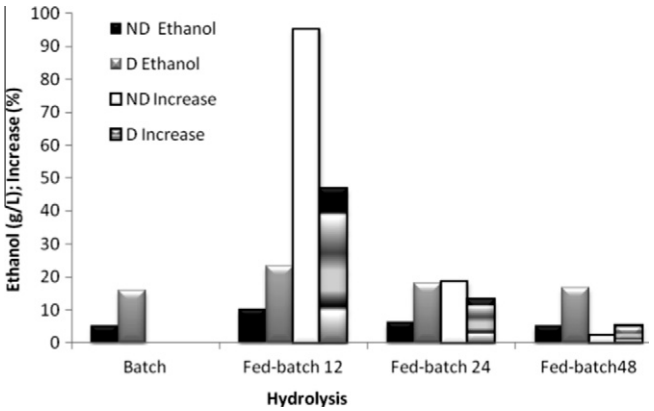

Fig. 5. Ethanol concentration (g/L) in the fermentation of hydrolysates from fed-batch enzymatic hydrolysis, and increase compared to the batch mode (%).

yield). The fermentation efficiencies of D12, D24, D48 and ND12 hydrolysates were above 70% (Table 3). Analyses of variance (ANOVA) were performed with all ethanol yields ($Y_{E/S}$). These results were significantly different ($F = 14.49$; $\alpha = 0.05$). However, the ANOVA between D12, D24, D48 and ND12 were not significantly different ($F = 0.62$, $\alpha = 0.05$).

The formation of by-products during the fermentation depends on the feedstock, process and strain. Often, the following by-products are considered fermentation inhibitors: glycerol, succinic acid, acetic acid, higher alcohols, esters, aldehydes, ketones, fatty acids, hydrogen sulfide and furfural (Martín et al., 2007).

Table 3 shows the glycerol ($Y_{G/S}$) and acetic acid ($Y_{AC/S}$) yields in fermentations of enzymatic hydrolysates. Acetic acid ($Y_{AC/S}$) yields were higher in the fermentation of ND24 and ND48 hydrolysates, which have shown lower ethanol yield. Lower ethanol yields were also correlated with higher glycerol yields.

Fig. 5 shows the effect of fed-batch hydrolysis on the increase in ethanol production. Analyses of variance were performed with these results, which were significantly different both in hydrolysis of ND ($F = 30{,}729.87$; $\alpha = 0.05$) and D bagasse ($F = 10{,}734.51$; $\alpha = 0.05$).

In the present study, were obtained an increase of about 450% in ethanol production, based on combined pretreatments and fed-batch enzymatic hydrolysis, i.e., comparing the fermentation of ND batch hydrolysate (5.21 g/L) and of D12 fed-batch hydrolysate (23.38 g/L). Kuhad et al. (2010), in study of bioconversion of the carbohydrate component of newspaper to sugars by enzymatic saccharification, and its fermentation to ethanol reported an increase of about 162% in ethanol production (from 5.64 to 14.77 g/L) when fed batch hydrolysis was performed.

The ethanol yields, when D bagasse was used, for any feeding intervals (0.39 g/g D12, 0.36 g/g D24 and 0.37 g/g D48), were quite close to those obtained by Chen et al. (2007), Kuhad et al. (2010). Comparing the productivities, experiments conducted in fed-batch using D bagasse also had similar results for 24 h (0.75 g/L h) and 48 h (0.70 g/h), but higher for 12 h (0.97 g/L h).

4. Conclusions

The alkaline delignification of steam-exploded bagasse increased surface area in about 8%, and reduced the lignin content and the crystallinity index in around 83% and 33%, respectively. The fed-batch addition of both ND and D bagasse within 12-h periods led to higher glucose formation, and, therefore, higher ethanol production, than 24-h or 48-h addition periods. The combination of alkaline delignification with fed-batch hydrolysis led to fourfold increase of ethanol production.

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