Effect of Cellulase, Substrate Concentrations, and Configuration Processes on Cellulosic Ethanol Production from Pretreated *Arundo donax*

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Arundo donax was used to investigate the effect of the enzyme and substrate concentrations on hydrolysis, pre-hydrolysis and simultaneous saccharification and fermentation (PSSF), in comparison to simultaneous saccharification and fermentation (SSF). Hydrolysis was performed at 37 and 50 °C. At the highest biomass (10%) and enzyme (69.6 FPU/g cellulose) loadings, the highest glucose concentration (32.4 g/L) was obtained (at 50 °C). SSF resulted in a cellulose conversion (91.9%) and an ethanol concentration (19.8 g/L) higher than what was obtained using PSSF at 37 °C (86.9% and 18.8 g/L, respectively) and PSSF at 50 °C (81.6% and 17.7 g/L, respectively). A positive correlation between the cellulase concentration, cellulose conversion, and ethanol content was observed. In PSSF, the increase in the solids loadings caused a reduction in the % cellulose conversion, but the ethanol concentration in PSSF and SSF increased. SSF appeared to be the most advantageous process for bioethanol production from *A. donax*.

Keywords: Cellulosic ethanol; Pre-hydrolysis and simultaneous saccharification and fermentation (PSSF); Simultaneous saccharification and fermentation (SSF); Cellulase loading; Water-insoluble solids (WIS) concentration

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INTRODUCTION

Organic chemicals, fuels, and polymers are currently derived mainly from petroleum. Environmentally friendly energy resources for alternative fuels have increased in the last decade, with the aim of progressively replacing fossil fuels and reducing the amount of greenhouse gases released into the atmosphere (Hoyer *et al.* 2009; Menegol *et al.* 2016; Ventorino *et al.* 2017). Bioethanol, produced from lignocellulosic biomass, is a possible alternative to fossil fuels used in transportation (Saini *et al.* 2015). Bioethanol can be produced from several agro-industrial residues, municipal solid waste, sawdust, and dedicated energy crops, such as miscanthus, switchgrass, black poplar (*Populus nigra* L.), and giant reed (*Arundo donax* L.) (Liguori *et al.* 2016; Neves *et al.* 2016; Ventorino *et al.* 2016a). Lignocellulosic ethanol offers several advantages compared to first-generation bioethanol, which is generated from the fermentation of sugar and starch. Lignocellulosic ethanol avoids competition for food and feed production and takes advantage of lignocellulosic biomass, which is a resource available in huge quantities (García-Aparicio *et al.* 2011).

A. donax is a perennial, herbaceous, nonfood crop that may be a promising candidate for bioethanol production because of its high energy, high biomass yield, and ability to grow quickly on marginal, contaminated, and degraded lands that are unusable for food crops (Fiorentino *et al.* 2013; Mutturi and Lidén 2013; Lemons e Silva *et al.* 2015; Ventorino *et al.* 2015). Moreover, *A. donax* can improve soil fertility and reduce soil erosion, and the bioethanol production from giant reed has been reported to be higher than for other energy crops (Fagnano *et al.* 2015; Castiglia *et al.* 2016; Fiorentino *et al.* 2017).

The efficiency of cellulosic ethanol production may be affected by several factors, such as the type of lignocellulosic raw materials, concentration of cellulolytic enzymes, solids concentration (water-insoluble solids, WIS), microorganisms used, operational parameters (*e.g.*, temperature), and configuration process (Mesa *et al.* 2011; López-Linares *et al.* 2014a; Paulova *et al.* 2015). In particular, considerable effort has been aimed at reducing the enzyme concentration required and lowering the associated process costs. In fact, enzymes contribute to the high cost of cellulosic ethanol, as they account for approximately 20% to 30% of the total cost (Koppram *et al.* 2014; Chen and Fu 2016). However, any reduction in the enzyme loading must be consistent with the target yields and process length to maintain the overall efficiency of bioethanol production (Olofsson *et al.* 2008). To a point, increases in the enzyme load are sometimes necessary for an efficient process (Koppram *et al.* 2014).

The WIS concentration is another important parameter to consider. A high concentration of WIS is fundamental to increase the ethanol concentration in the fermentation batch (Öhgren *et al.* 2007). Higher solids loadings lead to higher amounts of hydrolyzed sugars, which improves the fermentation step (García-Aparicio *et al.* 2011). Moreover, higher solids loadings are associated with a reduction in the global production costs, and decreases the overall water consumption and downstream processing costs (Manzanares *et al.* 2011).

The production of ethanol may be carried out by one of the following three main process strategies: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and pre-hydrolysis and simultaneous saccharification and fermentation (PSSF) (López-Linares *et al.* 2014b). In SHF, hydrolysis and fermentation are performed in two separate, consecutive steps using their respective optimal conditions.

However, end-product inhibition of the cellulolytic enzymes may reduce the ethanol yield (Chen and Fu 2016). In the SSF process, both steps are carried out in one vessel, reducing the cost of the industrial process (López-Linares *et al.* 2014b). Moreover, hydrolysis and fermentation are simultaneous, which minimizes the end-product inhibition (López-Linares *et al.* 2014b). The main disadvantage of the SSF process is the difference in the two temperature optima that may exist for the enzymes and yeast (Paulova *et al.* 2015). The PSSF strategy consists of a hydrolysis step followed by SSF in a single batch, which partially overcomes the SSF drawbacks due to the discrepancy in the temperature optima for enzyme and microorganism and reduces the viscosity of the slurry (Paulova *et al.* 2015).

In this study, the effect of cellulase and WIS concentration on the hydrolysis, PSSF, and SSF processes was evaluated for bioethanol production using pretreated *A. donax* as the substrate. The effect of the temperature on the glucose and ethanol production in the hydrolysis and PSSF processes was also investigated. Moreover, the PSSF and SSF processes for bioethanol production were compared for the first time.

EXPERIMENTAL

Effect of Temperature on the Selected Yeast Strain

The *Saccharomyces cerevisiae* NA227 strain used in this study was previously isolated from traditional "Passito wine Moscato di Saracena" produced in the Calabria region of Southern Italy. This strain was selected on the basis of its high resistance to osmotic stress and high tolerance to ethanol stress (Aponte and Blaiotta 2016). To evaluate the effect of the temperature on the growth of *S. cerevisiae* NA227, the yeast strain was inoculated in triplicate in 0.2 mL of YPD broth (10 g/L yeast extract, OXOID, Milan, Italy; 20 g/L peptone, OXOID; 20 g/L dextrose, OXOID; pH = 6.5) at approximately 1 x 10^6 CFU/mL. The yeast kinetic growth was analyzed at 30, 37, 40, and 45 °C using a 96-well BioTek Elx808 microtiter plate reader (BioTek Instruments Inc., Winooski, VT) at 600 nm with readings performed every 30 min for 12 h. The spectrophotometric data were processed using the Gompertz equation and Curve Expert 1.4 software (Daniel Hyams, Madison, AL, USA) to calculate the maximum growth rate (μ_{max}).

Pretreated *A. donax* Composition and Enzymes

A. donax biomass, pretreated according to Garbero *et al.* (2010) and De Bari *et al.* (2013), was utilized as the substrate in the enzymatic hydrolysis, PSSF, and SSF experiments. The macromolecular composition of the pretreated giant reed was 38.2% glucans, 5.7% xylans, and 36.1% Klason lignin.

Commercial cellulase (Cellic CTec2, Novozymes, Bagsværd, Denmark) was used to hydrolyze the pretreated *A. donax*. The enzymatic activity for Cellic CTec2 was measured according to the methods reported by Ghose (1987). The enzymatic activity for cellulase is expressed in filter paper units (FPU)/mL. The enzymatic activity for Cellic CTec2 was measured as 162 FPU/mL.

Enzymatic Hydrolysis Test

Enzymatic hydrolysis was carried out with 5.0%, 7.5%, and 10.0% (w/v) pretreated *A. donax* biomass loadings (*A. donax* broth) and seven different cellulase concentrations (1.09, 2.18, 4.35, 8.07, 17.41, 34.81, and 69.63 FPU/g cellulose). Saccharification tests with each loading (enzyme and pretreated biomass) were performed in 250-mL Erlenmeyer flasks sealed with rubber stoppers fitted with one-way air valves (Check valve, Fisher Scientific, Pittsburgh, PA) to maintain an anaerobic environment. Each flask contained 100 mL of sodium acetate buffer (0.05 M, pH = 5) (Huang *et al.* 2014). The *A. donax* broth was autoclaved at 121 °C for 15 min and cooled to room temperature before adding the enzymes. All of the flasks were incubated at 37 °C for 72 h in an orbital shaker (ES-80 Shaker-Incubator, Grant Bio, Cambridge, UK) at 120 rpm. The hydrolysis tests at 50 °C (optimum enzyme temperature) were performed using two Cellic Ctec2 concentrations (34.81 and 69.63 FPU/g cellulose) at various WIS concentrations (5.0%, 7.5%, and 10.0%, w/v). The experiments were performed using the same operational conditions reported above. Samples were taken at 24, 48, and 72 h for the glucose concentration measurements. All the saccharification experiments were performed in triplicate.

Inoculum Preparation

To standardize the *S. cerevisiae* NA227 concentrations, 0.3 mL of culture (3 x 10^8 CFU/mL) was inoculated in 30 mL of YPD broth. After incubation for 24 h at 30 °C, the cells were centrifuged (5289 x g) for 10 min, and washed three times to remove any sugar and ethanol residues. The cells were harvested and inoculated to achieve viable counts of approximately 10^8 CFU/mL in the fermentation media (pretreated biomass for SSF or biomass hydrolyzate for PSSF).

PSSF

The *A. donax* hydrolyzate obtained after 72 h of enzymatic hydrolysis at 37 °C or 50 °C was used in the PSSF experiments. The SSF step was performed by inoculating *S. cerevisiae* NA227 in 100 mL of biomass hydrolyzate, and was incubated at 37 °C for 96 h in an orbital shaker (120 rpm). Samples were collected after 24 and 96 h for ethanol determination. All of the PSSF experiments were performed in triplicate.

SSF

The SSF of the pretreated *A. donax* was carried out in 250-mL Erlenmeyer flasks sealed with rubber stoppers fitted with one-way air valves with a total volume of 100 mL of sodium acetate buffer (0.05 M, pH = 5) (Huang *et al.* 2014). The tests were performed at 37 °C for 168 h under shaking at 120 rpm (Faga *et al.* 2010). Three concentrations of pretreated biomass (5%, 7.5%, and 10%, w/v) and two different concentrations of cellulase (34.81 and 69.63 FPU/g cellulose) were tested. The SSF was begun by simultaneously adding the enzymes and *S. cerevisiae* NA227. Samples were collected at 72, 96, and 168 h of SSF for ethanol analysis. All the SSF experiments were performed in triplicate.

Analytical Method and Calculations

The ethanol and glucose concentrations in the samples were analyzed by high-performance liquid chromatography (HPLC, Refractive Index Detector 133, Pump 307, Gilson, Middleton, WI, USA) using a Varian METACARB 67H column (Varian, Palo Alto, CA, USA). The column was used at 65 °C with 0.01 N of H_2SO_4 as the eluent with a flow rate of 0.4 mL/min for 30 min (Ventorino *et al.* 2016b).

The glucose yield was calculated as the percent of the theoretical yield (% hydrolysis) using the following formula:

$$\%\, Hydrolysis = \frac{[Glu]}{1.11\, f\, [BIOMASS]} * 100 \quad (1)$$

where [*Glu*] is the glucose concentration (g/L) at time *t*, *f* is the fraction of glucan in dry solids (g/g), [*Biomass*] is the initial concentration of solids (g/L), and 1.11 is the mass conversion factor of glucan hydrolysis to glucose (g/g).

The percent of the theoretical ethanol yield (% cellulose conversion) was calculated using the following formula:

$$\%\, Cellulose\ Conversion = \frac{[Ethanol]}{0.51\ (1.11\, f\, [BIOMASS])} * 100 \quad (2)$$

where [*Ethanol*] is the ethanol concentration (g/L) produced at time *t*, 0.51 is the mass conversion factor of glucose to ethanol (g/g), *f* is the fraction of glucan in dry solids (g/g),

[*Biomass*] is the initial concentration of solids (g/L), and 1.11 is the mass conversion factor of glucan hydrolysis to glucose (g/g).

Statistical Analyses

One-way analysis of variance (ANOVA), followed by Tukey's post-hoc tests for the pairwise comparison of mean values ($P < 0.05$), was used to assess the differences in glucose and ethanol production in the hydrolysis, PSSF, and SSF processes. The statistical analyses were performed using the SPSS 21.0 statistical software package (SAS Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Temperature Tolerance of *S. cerevisiae* NA227

S. cerevisiae NA227 was determined to be able to grow at temperatures above 30 °C, which is its optimal growth temperature. An increase in the temperature was necessary to favor enzymatic hydrolysis in the SSF process and SSF step of the PSSF process. The cell density profile showed a reduction in the growth rate (μ_{max}) with increasing temperature ($P < 0.05$, Fig. 1). The growth rate (μ_{max}) at 30, 37, and 40 °C was 0.62, 0.40, and 0.27 h^{-1}, respectively; moreover, *S. cerevisiae* NA227 was not able to grow at 45 °C. Woo *et al.* (2014) and Mendes *et al.* (2016) tested the *S. cerevisiae* growth rate by increasing the temperature from 30 to 42 °C, and they observed a decrease from 0.49 to 0.12 h^{-1} and from 0.44 to 0.29 h^{-1}, respectively. On the basis of the results of this study and taking into account the literature data (Olofsson *et al.* 2008; Paulova *et al.* 2015), 37 °C was recognized as the best temperature that compromised between the enzymatic hydrolysis and fermentation processes, and enabled both a high yeast growth rate and cellulase activity; therefore, the SSF process and SSF step of the PSSF process were performed at 37 °C in this work.

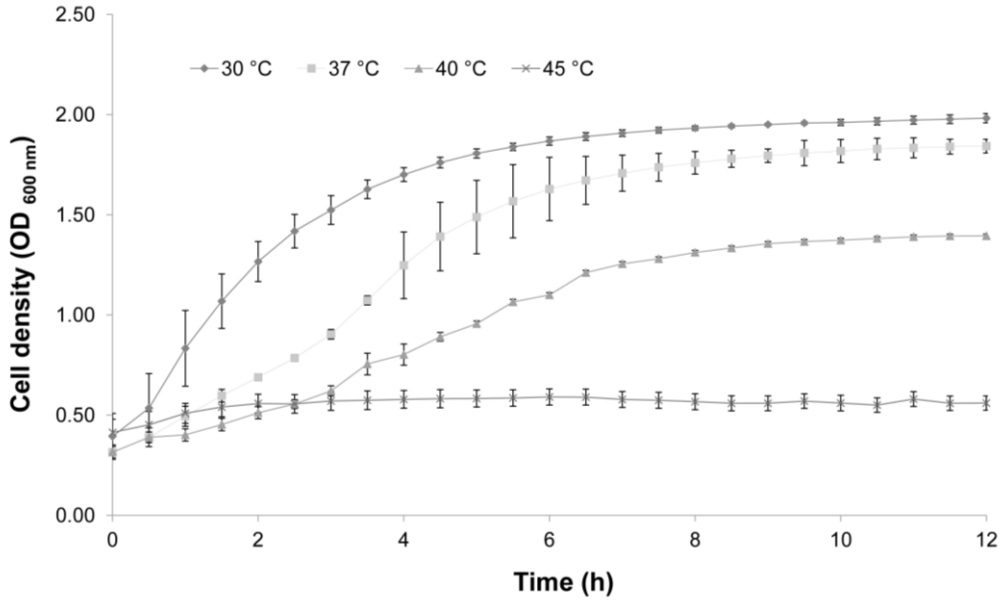

Fig. 1. Growth profiles of *S. cerevisiae* NA227 in YPD broth medium over 12 h of incubation at 30, 37, 40, and 45 °C

Effect of Enzyme Loading and Solids Amount on Enzymatic Hydrolysis

Enzymatic hydrolysis is a fundamental step for cellulosic ethanol production. For this reason, in the present work, the effect of different enzyme concentrations, biomass loadings, temperature, and time on the percent hydrolysis and glucose concentration from the pretreated lignocellulosic biomass of *A. donax* were evaluated (Tables 1 to 3). A positive correlation between the enzyme concentration and percent hydrolysis was found at 37 °C. In fact, a significant increase, from 6-fold up to 9-fold depending on reaction time and biomass loading, on the glucan hydrolysis was shown when the enzyme concentration was increased from 1.09 to 69.63 FPU/g cellulose (Table 1). Similar results were also reported by Van Dyk and Pletschke (2012) and Wang *et al.* (2012). The increase of reaction time was positively correlated with hydrolysis yield also. In fact, respect to 24 h of reaction time, an increase of 12 to 13% and 17 to 18 % of hydrolysis was recorded at 48 h and 72 h, respectively (Table 1).

By contrast, the increase of biomass loading was negatively correlated with hydrolysis yield: reductions of 8 to 9% and 13 to 14% were shown ranging from 5% to 7.5% and from 5% to 10% of biomass loading, respectively. This result could have been due to several factors, such as the higher viscosity that reduced enzyme mobility, lower enzyme binding, and end-product inhibition (Van Dyk and Pletschke 2012). In fact, the maximum hydrolysis yield (94%) was shown at the highest enzyme concentration and lowest biomass loading. However, the highest glucose concentration (30 g/L) in the hydrolyzate was shown at highest biomass loading at each incubation time (Table 2). These findings were consistent with previous works in which an increase in the amount of elephant grass (from 4% to 12%) or steam-pretreated *A. donax* (from 10% to 20%) resulted in a decrease in the percent hydrolysis and an increase in the glucose concentration (Palmqvist and Lidén 2012; Menegol *et al.* 2016).

The hydrolysis at 50 °C, instead of 37 °C, low increased both hydrolysis yield (<15 %) (Table 1 and 3) and glucose concentration in the hydrolyzate (<3%) (Tables 2 and 3). The best performance in the enzymatic hydrolysis is usually obtained at approximately 50 °C (Paulova *et al.* 2015). Mutturi and Lidén (2013) and Linde *et al.* (2007) obtained an increase of 8 to 12 % in the percent hydrolysis by increasing the temperature from 32 to 45 °C.

De Bari *et al.* (2013) achieved 80% hydrolysis using a 10% solids loading (steam-explosion pretreated *A. donax*) and approximately 39 FPU/g cellulose after more than 150 h of incubation at 50 °C (enzyme optimal temperature). In this study, at 10% of loading biomass, the same hydrolysis yield was shown using about 70 FPU/g cellulose in the half time (72 h) at 37 °C (Table 1). Moreover, under the conditions tested in this study (69.63 FPU/g cellulose, 10% biomass loading, and pH = 5.0), a hydrolysis yield of 63% was obtained after 24 h of incubation at 37 °C. This value was approximately 22% higher than that reported by Lemons e Silva *et al.* (2015), who reported a maximum (52%) after 30 h of saccharification at 50 °C and pH of 5.0 using approximately 35 FPU/g cellulose and a 10% loading of acid- and alkaline-pretreated *A. donax*. Moreover, at the optimal enzyme temperature (50 °C), after 24 h of saccharification with 69.63 FPU/g cellulose and a 10% pretreated *A. donax* loading, the percent of hydrolysis shown, in this study (71.4%), was approximately 36% higher than that reported by Lemons e Silva *et al.* (2015) after 30 h of hydrolysis (35 FPU/g cellulose and 10% pretreated giant reed loading), which confirmed that the increase in the enzyme loading led to a significant increase in the percent hydrolysis.

The high enzyme concentration needed to obtain high hydrolysis yield on lignocellulosic biomass of *A. donax*, as shown in this study, may be due to its high lignin content (36%). The enzyme loading required to hydrolyze biomass depends on the specific lignocellulosic material and its composition, as well as the type of pretreatment used (Van Dyk and Pletschke 2012).

Although 7 to 33 FPU/g substrate are generally used to hydrolyze glucan, higher enzyme loadings are necessary for the efficient conversion of the substrate in the presence of a high lignin content (Van Dyk and Pletschke 2012). In fact, kraft pulp with a 28% lignin content was not totally hydrolyzed when using 750 FPU/g cellulose (Van Dyk and Pletschke 2012). Moreover, Olsen *et al*. (2011) demonstrated that enzyme concentrations of approximately 70 FPU/g cellulose are necessary to increase the percent hydrolysis of corn stover biomass.

Table 1. Percent Hydrolysis (%) Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 72 h of Hydrolysis at 37 °C

Enzyme loading (FPU/g cellulose)	Time (h)								
	24			48			72		
	Biomass loading (%, w/v)								
	5	7.5	10	5	7.5	10	5	7.5	10
69.63	76.4±1.7^{aE}	68.1±0.4^{aF}	63.2±1.0^{aG}	89.9±0.2^{aB}	81.0±0.4^{aD}	75.8±1.2^{aE}	94.1±0.5^{aA}	85.7±1.3^{aC}	81.0±0.6^{aD}
34.81	54.5±2.9^{bD}	46.0±2.9^{bE}	45.1±2.2^{bE}	66.7±4.7^{bBC}	63.9±1.6^{bC}	58.4±0.1^{bD}	79.5±3.5^{bA}	71.5±1.6^{bB}	65.2±1.4^{bC}
17.41	40.0±1.8^{cF}	35.4±1.4^{cG}	34.0±0.7^{cG}	51.9±1.5^{cC}	49.0±1.0^{cD}	45.1±0.1^{cE}	60.6±1.9^{cA}	55.9±0.4^{cB}	51.4±1.1^{cCD}
8.70	25.5±0.6^{dF}	24.4±1.0^{dFG}	22.9±0.5^{dG}	37.0±1.6^{dD}	33.7±0.7^{dE}	31.6±0.5^{dE}	41.8±0.3^{dA}	40.0±1.0^{dBC}	37.9±0.7^{dCD}
4.35	16.1±0.1^{eC}	15.9±0.2^{eC}	15.9±0.9^{eC}	25.5±2.1^{eA}	21.2±1.6^{eB}	20.6±0.3^{eB}	27.6±0.2^{eA}	27.6±1.9^{eA}	25.1±0.4^{eA}
2.18	11.2±0.1^{fD}	11.4±0.4^{fD}	11.1±0.2^{fD}	16.3±0.4^{fBC}	15.1±0.8^{fC}	14.4±0.4^{fC}	19.2±2.1^{fA}	18.0±1.1^{fAB}	17.7±0.2^{fAB}
1.09	7.9±0.1^{gD}	8.5±0.1^{gD}	8.2±0.1^{gD}	11.1±1.0^{gBC}	10.8±0.3^{gC}	10.7±0.4^{gC}	12.4±0.3^{gA}	12.6±0.7^{gA}	12.0±0.2^{gAB}

The values are the means ± the standard deviation (SD) of three replicates from independent experiments.
Different letters after the values indicate significant differences ($P < 0.05$) in the same column (lowercase letters) or the same row (uppercase letters).

Table 2. Glucose Concentration (g/L) Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 72 h of Hydrolysis at 37 °C. The glucose was undetectable (<0.25 g/L), in all cases, at beginning of hydrolysis.

Enzyme loading (FPU/g cellulose)	Time (h)								
	24			48			72		
	Biomass loading (%, w/v)								
	5	7.5	10	5	7.5	10	5	7.5	10
69.63	14.6±0.3^{aH}	19.5±0.1^{aE}	24.1±0.4^{aC}	17.1±0.0^{aG}	23.2±0.1^{aD}	28.9±0.5^{aB}	17.9±0.1^{aF}	24.5±0.4^{aC}	30.9±0.2^{aA}
34.81	10.4±0.6^{bG}	13.2±0.9^{bF}	17.2±0.9^{bD}	12.7±0.9^{bF}	18.3±0.5^{bD}	22.3±0.0^{bB}	15.1±0.7^{bE}	20.4±0.5^{bC}	24.8±0.5^{bA}
17.41	7.6±0.3^{cG}	10.1±0.4^{cF}	13.0±0.3^{cE}	9.9±0.2^{cF}	14.0±0.3^{cD}	17.1±0.1^{cB}	11.5±0.4^{cF}	16.2±0.1^{cC}	19.6±0.4^{cA}
8.70	4.9±0.1^{dG}	7.0±0.3^{dF}	8.9±0.3^{dD}	7.0±0.3^{dF}	9.6±0.2^{dC}	12.0±0.2^{dB}	8.0±0.1^{dE}	11.4±0.3^{dB}	14.4±0.3^{dA}
4.35	3.2±0.0^{eF}	4.6±0.1^{eE}	6.0±0.3^{eC}	4.9±0.4^{eE}	6.0±0.4^{eCD}	7.9±0.1^{eB}	5.3±0.0^{eDE}	7.9±0.5^{eB}	9.6±0.1^{eA}
2.18	2.1±0.00^{fF}	3.2±0.1^{fDE}	4.2±0.1^{fC}	3.1±0.1^{fE}	4.3±0.2^{fC}	5.5±0.1^{fB}	3,7±0.4^{fD}	5.2±0.3^{fB}	6.7±0.1^{fA}
1.09	1.5±0.0^{gG}	2.4±0.0^{gE}	3.1±0.0^{gD}	2.1±0.2^{gF}	3.1±0.1^{gD}	4.1±0.2^{gB}	2.4±0.1^{gEF}	3.6±0.2^{gC}	4.6±0.1^{gA}

The values are the means ± the SD of three replicates from independent experiments.
Different letters after the values indicate significant differences ($P < 0.05$) in the same column (lowercase letters) or the same row (uppercase letters)

Table 3. Percent Hydrolysis (%) and Glucose Concentration (g/L) Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 72 h of Hydrolysis at 50 °C. The glucose was undetectable (<0.25 g/L), in all cases, at beginning of hydrolysis.

Enzyme loading (FPU/g cellulose)		Time (h)								
		24			48			72		
		Biomass loading (%, w/v)								
		5	7.5	10	5	7.5	10	5	7.5	10
Hydrolysis (%)	69.63	82.4±1.2^{aE}	75.5±1.1^{aF}	71.4±0.2^{aG}	92.0±2.8^{aB}	82.5±0.6^{aDE}	80.7±0.3^{aE}	95.8±1.5^{aA}	89.3±0.6^{aC}	84.9±0.4^{aD}
	34.81	66.7±0.9^{bE}	61.5±1.1^{bF}	46.2±0.6^{bG}	75.1±0.3^{bB}	70.8±2.3^{bC}	63.4±1.3^{bF}	81.8±2.0^{bA}	75.6±0.5^{bB}	68.7±0.7^{bDE}
Glucose concentration (g/L)	69.63	15.7±0.2^{al}	21.6±0.3^{aF}	27.2±0.1^{aC}	17.5±0.5^{aH}	23.5±0.4^{aE}	30.8±0.1^{aB}	18.3±0.3^{aG}	25.5±0.2^{aD}	32.4±0.2^{aA}
	34.81	12.7±0.2^{bH}	17.6±0.5^{bE}	20.4±0.2^{bC}	14.3±0.1^{bG}	20.3±0.7^{bD}	24.2±0.5^{bB}	15.6±0.4^{bF}	21.6±0.2^{bC}	26.2±0.3^{bA}

The values are the means ± the SD of three replicates from independent experiments.
Different letters after the values indicate significant differences (P < 0.05) in the same column (lowercase letters) or the same row (uppercase letters).

Pre-Hydrolysis and Simultaneous Saccharification and Fermentation (PSSF)

PSSF is a process commonly used to overcome several problems that limit other conversion processes by combining the advantages of SHF and SSF (Paulova *et al.* 2015). In order to investigate on the effect of enzyme concentration and solids loading during PSSF using giant reed biomass for bioethanol production, flasks containing *A. donax* hydrolyzate for 72 h at 37 °C, as previously described, were inoculated with *S. cerevisiae* NA227 and ethanol production was monitored for 96 h.

After 24 h of fermentation, part of the sugars remained unfermented; in fact, the maximum cellulose conversion and ethanol concentration were observed only at 96 h (Tables 4 and 5). The cellulose conversion and ethanol concentration were positively correlated with enzyme concentration. These results are in agreement with those obtained Linde *et al.* (2007) who, using a 7.5% steam-pretreated barley straw loading, observed a 1.3-fold increase in the cellulose conversion and ethanol content after increasing the enzyme concentration from 5 to 10 FPU/g cellulose. Enzyme loading is one of the main factors that influences PSSF, and in fact, an increase in the enzyme level leads to a higher ethanol concentration (Mesa *et al.* 2011).

After 96 h of fermentation, as expected, the highest cellulose conversion (about 92%) was detected employing the highest enzymatic concentration and lowest substrate amount (Table 4). By contrast, the higher ethanol concentration (about 19 g/L) at the end of fermentation was produced when both enzyme and biomass were at higher levels (Table 5). A similar trend was shown when analyzing data obtained from fermentations of hydrolyzates obtained at 50 °C for 72 h (Table 6). Even if a little lower cellulose conversion and ethanol concentration in the fermented mixture were recorded, which was probably due to a partial deactivation of the cellulolytic enzymes exposed to a high temperature (50 °C) for extended periods (Manzanares *et al.* 2011; Saini *et al.* 2015). Furthermore, when using a 10% pretreated biomass loading, the fermentation was slower than with a 5% or 7.5% solids loading (Table 6, Fig. 2). These results could have been due to the release of inhibitory substances in the substrate during the hydrolysis performed at a high temperature (50 °C) over prolonged time periods (3 d) in the presence of the highest WIS concentration. These substances could have led to an inhibitory effect on the fermentative metabolism of the yeast strain.

During PSSF, several factors can lead to a decrease in the ethanol content that increases the lignocellulosic biomass concentration, including end-product inhibition (glucose and cellobiose), mass transfer efficiency, substrate composition, and deactivation and adsorption of the enzyme (Manzanares *et al.* 2011; López-Linares *et al.* 2014b). However, the inhibition of enzyme adsorption by the end-products present in the solution has been suggested to be the main cause of decreased cellulose conversion with an increasing solids content (Manzanares *et al.* 2011).

Table 4. Cellulose Conversion (%) in Ethanol Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 96 h of Fermentation at 37 °C After Pre-Hydrolysis at 37 °C for 72 h.

Enzyme loading (FPU/g cellulose)	Time (h)					
	24			96		
	Biomass loading (%, w/v)					
	5	7.5	10	5	7.5	10
69.63	85.3±0.8^{aD}	81.6±0.0^{aE}	73.8±0.6^{aF}	91.9±0.9^{aA}	89.4±0.5^{aB}	86.9±0.2^{aC}
34.81	74.4±4.0^{bC}	69.6±0.6^{bD}	63.7±0.9^{bE}	83.9±4.9^{bA}	82.9±0.1^{bA}	78.3±0.1^{bC}
17.41	56.8±2.9^{cB}	55.4±1.1^{cCD}	53.2±2.7^{cD}	66.4±1.2^{cA}	68.6±0.8^{cA}	65.4±1.9^{cA}
8.70	39.3±0.8^{dD}	41.3±1.7^{dD}	37.7±0.8^{dD}	48.8±2.6^{dC}	54.5±1.1^{dA}	51.9±3.0^{dBC}
4.35	26.9±0.8^{eBC}	28.8±1.4^{eB}	23.3±2.8^{eC}	33.9±2.3^{eA}	37.6±1.8^{eA}	36.0±1.5^{eA}
2.18	17.9±0.0^{fD}	19.6±2.1^{fCD}	17.9±0.1^{fD}	21.4±0.8^{fBC}	25.3±1.4^{fA}	24.4±2.2^{fAB}
1.09	12.4±1.3^{gC}	14.2±1.6^{gBC}	13.3±0.3^{gC}	13.9±0.2^{gBC}	15.8±0.3^{gAB}	16.9±1.5^{gA}

The values are the means ± the SD of three replicates from independent experiments.
Different letters after the values indicate significant differences ($P < 0.05$) in the same column (lowercase letters) or the same row (uppercase letters).

Table 5. Ethanol Concentration (g/L) Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 96 h of Fermentation at 37 °C After Pre-Hydrolysis at 37 °C

Enzyme loading (FPU/g cellulose)	Time (h)					
	24			96		
	Biomass loading (%, w/v)					
	5	7.5	10	5	7.5	10
69.63	9.2±0.1^{aF}	13.2±0.0^{aD}	16.0±0.1^{aB}	9.9±0.1^{aE}	14.5±0.1^{aC}	18.8±0.1^{aA}
34.81	8.1±0.4^{bE}	11.3±0.1^{bC}	13.8±0.2^{bB}	9.1±0.5^{bD}	13.5±0.0^{bB}	16.9±0.0^{bA}
17.41	6.2±0.3^{cE}	9.0±0.2^{cC}	11.0±0.2^{cB}	7.2±0.1^{cD}	11.1±0.1^{cB}	14.2±0.4^{cA}
8.70	4.3±0.1^{dE}	6.7±0.3^{dC}	8.2±0.2^{dB}	5.3±0.3^{dD}	8.9±0.2^{dB}	11.2±0.7^{dA}
4.35	2.9±0.1^{eE}	4.7±0.2^{eC}	5.0±0.6^{eC}	3.7±0.3^{eD}	6.1±0.3^{eB}	7.8±0.3^{eA}
2.18	1.9±0.0^{fD}	3.2±0.3^{fC}	3.9±0.0^{fB}	2.3±0.1^{fD}	4.1±0.2^{fB}	5.3±0.5^{fA}
1.09	1.3±0.1^{gD}	2.3±0.3^{gC}	2.9±0.1^{gB}	1.5±0.0^{gD}	2.6±0.1^{gBC}	3.7±0.6^{gA}

The values are the means ± the SD of three replicates from independent experiments.
Different letters after the values indicate significant differences ($P < 0.05$) in the same column (lowercase letters) or the same row (uppercase letters).

Table 6. Cellulose Conversion (%) in Ethanol and Ethanol Concentration (g/L) Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 96 h of Fermentation at 37 °C After Pre-Hydrolysis at 50 °C for 72 h.

Enzyme loading (FPU/g cellulose)		Time (h)					
		24			96		
		Biomass loading (%, w/v)					
		5	7.5	10	5	7.5	10
Cellulose conversion (%)	69.63	81.2±0.6^{aC}	71.5±4.7^{aD}	25.4±5.3^{aE}	87.4±0.2^{aA}	84.5±0.0^{aB}	81.6±1.0^{aC}
	34.81	70.6±2.4^{bC}	56.0±2.7^{bE}	24.5±1.6^{aF}	75.4±0.9^{bA}	72.9±1.0^{bBC}	67.5±0.4^{bD}
Ethanol concentration (g/L)	69.63	7.8±0.3^{aE}	11.6±0.7^{aC}	5.5±1.2^{aF}	9.5±0.0^{aD}	13.7±0.0^{aB}	17.7±0.2^{aA}
	34.81	7.2±0.1^{bE}	9.1±0.4^{bC}	5.3±0.3^{aF}	8.1±0.1^{bD}	11.8±0.2^{bB}	14.6±0.1^{bA}

The values are the means ± the SD of three replicates from independent experiments.
Different letters after the values indicate significant differences ($P < 0.05$) in the same column (lowercase letters) or the same row (uppercase letters).

Furthermore, osmotic pressure and other inhibitors (weak acids, furan derivatives, and phenol compounds) lead to lower ethanol yields at high solids loadings, which influences the yeast performance (Huang *et al.* 2011; Chen and Fu 2016). Huang *et al.* (2014) reported a decrease in the fermentation yield (from 94.2% to 75.7%) and an increase in the ethanol concentration (from 9.3 to 20.8 g/L) after increasing the hydrothermally pretreated pomelo peel loading from 5% to 14%. Öhgren *et al.* (2007) observed the same trend when using steam-pretreated corn stover, where an increase in the biomass loading from 10% to 11.5% led to a reduction in the % cellulose conversion from 75% to 67.6% and an increase in the ethanol concentration from 26.4 to 28.5 g/L.

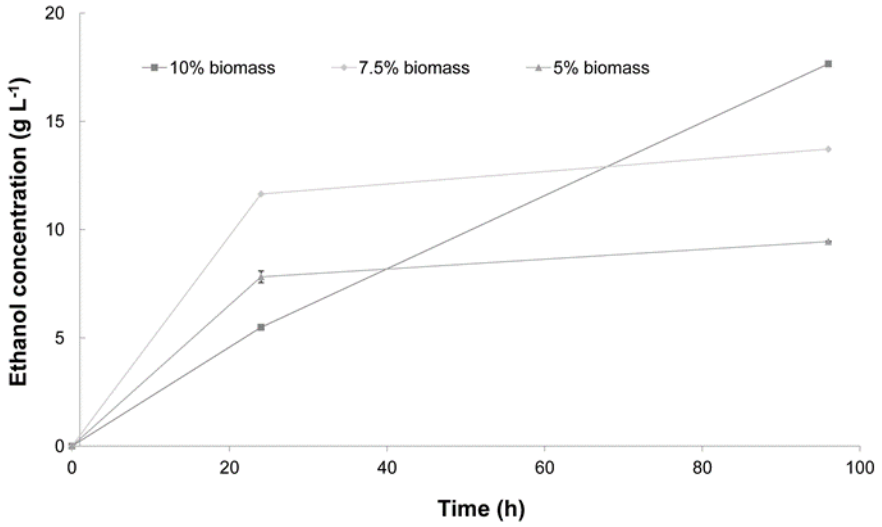

Fig. 2. Ethanol concentration (g/L) in the PSSF (pre-hydrolysis temperature: 50 °C) with 5%, 7.5%, and 10% pretreated *A. donax* biomass loadings using 69.63 FPU/g cellulose.

Interestingly, in both conditions (37 and 50 °C), the final ethanol content was higher than that achievable from the glucose concentration obtained by pre-hydrolysis. This result could have been due to the reactivation of the cellulase enzymes after the conversion of glucose by the yeast during the fermentation step (Vásquez *et al.* 2007).

Simultaneous Saccharification and Fermentation (SSF)

The SSF process is one of the main processes used for cellulosic ethanol production because it is performed in a single vessel and the end-product inhibition is very limited (López-Linares *et al.* 2014b). Thus, in this work, SSF experiments were also performed to evaluate the effect of enzyme concentration and solids loading on the bioethanol production using *A. donax*.

At the end of the process (168 h), the highest cellulose conversion and ethanol concentration were obtained by increasing the enzyme concentration observing an increase of 7 to 12% depending on biomass loading (Table 7). These results are in agreement with Mendes *et al.* (2016) and Faga *et al.* (2010) that observed an increase of both cellulose conversion and ethanol content increasing the enzyme loading from 5 to 15 FPU/g cellulose in SSF experiments. Indeed, Mesa *et al*. (2011) demonstrated that the cellulase loading was the only factor that significantly increased the ethanol concentration in the SSF process.

Table 7. Cellulose Conversion (%) in Ethanol and Ethanol Concentration (g/L) Using 5%, 7.5%, and 10% Pretreated *A. donax* Biomass Loadings and Various Enzyme Concentrations Over 168 h of Simultaneous Saccharification and Fermentation (SSF) at 37 °C for 168 h.

Enzyme loading (FPU/g cellulose)		Time (h)*								
		72			96			168		
		Biomass loading (%, w/v)								
		5	7.5	10	5	7.5	10	5	7.5	10
Cellulose conversion (%)	69.63	85.6±1.0^{aE}	86.2±0.7^{aE}	83.2±1.0^{aF}	89.1±0.8^{aCD}	90.3±0.2^{aBC}	87.8±0.9^{aDE}	93.5±0.5^{aA}	93.6±0.5^{aA}	91.9±1.0^{aAB}
	34.81	75.4±2.4^{bC}	76.0±0.1^{bC}	69.8±0.5^{bD}	76.3±2.9^{bC}	78.1±0.3^{bBC}	75.1±2.9^{bC}	86.5±0.0^{bA}	85.0±0.1^{bA}	80.4±1.9^{bB}
Ethanol concentration (g/L)	69.63	8.4±0.3^{al}	13.7±0.3^{aF}	18.0±0.2^{aC}	9.0±0.0^{aH}	14.6±0.0^{aE}	19.0±0.2^{aB}	10.0±0.2^{aG}	15.7±0.2^{aD}	19.8±0.1^{aA}
	34.81	7.1±0.0^{bH}	12.3±0.0^{bE}	15.1±0.1^{bC}	8.2±0.3^{bG}	12.7±0.0^{bE}	16.2±0.6^{bB}	9.3±0.0^{bF}	13.8±0.0^{bD}	17.4±0.4^{bA}

*The values of the cellulose conversion (%) and ethanol yield (g/L) recorded at 24 h and 48 h were not shown in this table.
The values are the means ± the SD of three replicates from independent experiments.
Different letters after the values indicate significant differences ($P < 0.05$) in the same column (lowercase letters) or the same row (uppercase letters).

Interestingly, the results of the current study indicated that the highest enzyme concentration used (69.63 FPU/g cellulose) was necessary to significantly increase the cellulose conversion of pretreated *A. donax* biomass. Pessani *et al.* (2011) reported that the highest cellulose conversion was obtained by increasing the enzyme loading from 0.1 to 0.7 mL/g cellulose; whereas no increase in the % cellulose conversion was observed for an enzyme concentration above 0.7 mL/g cellulose. Therefore, higher cellulase concentrations do not necessarily lead to an increase in the cellulose conversion because of the competition for the same binding sites (Van Dyk and Pletschke 2012).

Moreover, according to Hoyer *et al*. (2009) and López-Linares *et al.* (2014a), the use of higher pretreated biomass loadings (up to 10%) resulted in a higher ethanol concentration and, at same time, no significant decrease in the cellulose conversion occurred. These results confirmed that there was no end-product accumulation during the SSF process when using a less than 10% biomass concentration, which was the limit to obtain good cellulose conversion (Olofsson *et al.* 2008). Interestingly, the cellulose conversions obtained at all of the substrate concentrations with 69.63 FPU/g cellulose were similar to the maximal yield assumed by Olofsson *et al.* (2008).

PSSF *vs*. SSF

The results obtained in the PSSF and SSF processes were compared because of the fact that the process configuration is recognized as an important factor that affects the ethanol results (Mesa *et al.* 2011).

In this work, the SSF process achieved a higher ethanol content than what was obtained in the PSSF ($P < 0.05$) using the 7.5% and 10% pretreated biomass loadings (69.63 FPU/g cellulose). These findings were in agreement with Romaní *et al.* (2014) and Hoyer *et al*. (2009), who reported better performance for SSF than PSSF. The PSSF process significantly increased the % cellulose conversion only when the % cellulose conversion and ethanol concentration were low in the SSF process, but otherwise, it did not have a significant effect, or even had a negative effect when a high cellulose conversion and ethanol content were obtained without a pre-hydrolysis step (Hoyer *et al.* 2013).

Interestingly, at the 7.5% and 10% pretreated giant reed loadings using 69.63 FPU/g cellulose, the SSF configuration just after 96 h (Table 7) achieved the same ethanol concentration obtained after 168 h in the PSSF process (Table 5). In the PSSF configuration, when the yeast was inoculated into the pre-hydrolyzate substrate with excess glucose, some monosaccharides were transformed into byproducts, which reduced the final ethanol content (Öhgren *et al.* 2007). Moreover, the lower ethanol production in the PSSF could also have been explained by end-product inhibition (García-Aparicio *et al.* 2011).

Using a 5% pretreated *A. donax* loading, no significant differences in the ethanol concentration were observed between the PSSF (Table 5) and SSF (Table 7) processes. This result are in agreement with those of García-Aparicio *et al.* (2011), who suggested that at low pretreated biomass concentrations, the final ethanol content was not affected by the end-product inhibition for the lower glucose concentration.

CONCLUSIONS

1. The results demonstrated that an increase in the enzyme loading significantly affected the process, which led to an increase in the cellulose conversion and ethanol concentration during PSSF and SSF of pretreated *A. donax*.
2. In PSSF, the lowest pre-hydrolysis temperature determined the highest ethanol yield and concentration.
3. The increase in the solids loadings induced a reduction of cellulose conversion, more significant in PSSF respect SSF.
4. The overall results showed that SSF is an advantageous strategy respect PSSF for bioethanol production from *A. donax*.
5. However, in all case, a high concentration of enzyme is needed to obtain a high cellulose conversion drastically increasing the production costs.

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