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Second generation bioethanol production from *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.

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ABSTRACT

Saccharum (*Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.), is a rapidly growing, wide ranging high-yield perennial, suitable for second generation bioethanol production. This study evaluated oxalic acid as a pretreatment for bioconversion. Overall sugar yields, sugar degradation products, enzymatic glucan hydrolysis and ethanol production were studied as effects of temperature (150–190 °C), reaction time (10–40 min) and oxalic acid concentration 2–8% (w/w). Time and temperature were combined into a single parameter, Severity Factor (SF) [Log (R_0)], and related to oxalic acid using a response surface methodology. Maximum total sugar yield was attained at a SF of 2.93 and 6.79% (w/w) oxalic acid, while maximum formation of sugar degradation products was observed at the highest SF (4.05) and 5% (w/w) oxalic acid. These were also the conditions for maximum simultaneous saccharification and fermentation (SSF) of the residual solids. Commercial cellulases and *Saccharomyces cerevisiae* attained 89.9% glucan conversion and 17.8 g/l ethanol. *Pichia stipitis* CBS 6054 fermented hemicellulosic hydrolysates from less severe conditions to ethanol with a yield of 0.35 (g/g). Maximal product yields were 69% of theoretical value and 90% of the SSF conversion efficiency for hydrolysate fermentation and SSF, respectively.

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1. Introduction

Rising concerns over the cost of petroleum and the prospect of global warming are driving a major effort to develop alternative feedstocks for ethanol production from crop residues, forest by-products, perennial grasses and other forms of lignocellulosic plant biomass (Jeffries, 2006; Ohlrogge et al., 2009). Concerns have been expressed over the competition for land use between agriculture and biofuel production, but when bioconversion of lignocellulosics can be conducted without displacing natural forests or encroaching on productive agricultural lands, it has the potential to reduce greenhouse gases while providing an alternative to fossil fuels (Tilman et al., 2009).

Perennial herbaceous energy crops, well adapted to the climatic and soil conditions of a specific area, could reduce the raw material cost for this technology. Once established, they do not require annual reseeding. They require lower energy inputs of fertilizer and pesticide than annual crops (Vecchiet and Jodice, 1996). They have a high production of biomass and they can often be grown on mar-

ginal cropland (Mckendry, 2002; McLaughlin et al., 2002). They also have environmental benefits including enhanced carbon sequestration and the potential to reduce erosion in sloping so (Cosentino et al., 2004). Most herbaceous perennial crops have not been cultivated for biomass production, so their growth yield, compositions, fiber and bioconversion characteristics are not as well known as traditional agricultural residues.

Understanding the complexity of plant cell walls and ways to efficiently release the sugars are priorities in this technology. Lignocellulosic cell walls have natural resistance, often called “recalcitrance”, to microbial and enzymatic deconstruction (Zhu et al., 2009). Therefore, biomass pretreatment is necessary to catalyze the hydrolysis of hemicellulose and make the cellulose fraction more accessible for enzymatic digestion prior to the ethanol fermentation.

Although cellulose can be efficiently hydrolyzed to glucose and fermented to ethanol, xylose and other C5 sugars, contained in hemicelluloses are much more difficult to ferment efficiently (Jeffries, 2006). Among dilute acid pretreatments, organic acids are thought to be more selective for the hydrolysis of β -(1,4)-glycolic bonds than sulfuric acid, due to the decreased ability to cause glucose degradation (Lu and Mosier, 2007). This research tested the hypothesis whether oxalic acid with its dual pKa could provide more efficient and specific hydrolysis of hemicellulose than could sulfuric acid.

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Oxalic acid is one of the strongest organic acids known. Due to its pKa's of 1.27 and 4.28, respectively, it can catalyze the hydrolysis of hemicelluloses while sparing cellulose. At the same time it is less toxic to yeasts and other microorganism than acetic or sulphurous acids, because its lower pKa restricts diffusion or transport of the ionized form across the cellular membrane (Lee et al., 2009). As a result, the hemicellulosic derived sugars can be fermented to ethanol by xylose-fermenting yeasts such as *Pichia stipitis* and *Candida shehate* (Jeffries and Kurtzman, 1994). After mild pretreatment, the cellulose-enriched fraction can be used for papermaking (Kenealy et al., 2007), or after more extensive pretreatment, the residual solids can be hydrolyzed by cellulolytic enzymes to fermentable sugars for ethanol production.

In the Mediterranean semi-arid environment, perennial energy crops such as *Miscanthus*, which is native to subtropical areas, encounters difficulty because of the dry summer period (Cosentino et al., 2007). In contrast *Saccharum* (*Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.), a perennial, herbaceous plant belonging to the *Poaceae* family is wild in the Mediterranean area. It has adapted to live over a wide range of grassland climatic habitats: from oriental Asia to the southern region, in the warm-temperate areas of Africa and in Mediterranean regions (Australian Government, 2004).

The *Saccharum* genus contains thirteen species (US Department of Agriculture) and it is similar to the *Miscanthus* genus; it differs from the latter by the different disposition of the spikelets in the bloom and the rachis fragility (Scully et al., 1997). Moreover, the crop shows high aboveground biomass yield starting after the second year of establishment, whereas this occurs only after the third year in the *Miscanthus* genus (Cosentino et al., 2006).

Pretreatment studies have been conducted on various cultivars of commercial sugar cane (*Saccharum* hybrids), but almost no such research has been directed to wild species that can grow in more arid habitats. The objective of our present work was to evaluate the effect of temperature, reaction time and dilute oxalic acid (OA) concentration during steam-pretreatment of *Saccharum* using a response surface methodology.

We examined the hemicellulosic derived sugars and sugar degradation products released in the hydrolysate fraction (HF) for its fermentation to ethanol, the susceptibility of residual cellulose to enzymatic hydrolysis (EH), and the simultaneous saccharification and fermentation (SSF) of the solid fraction after pretreatment.

2. Methods

2.1. Raw material

Saccharum biomass (stems and leaves) was harvested in southern Italy, from a well established plantation located in the Experimental Fields of the Facoltà di Agraria, University of Catania, (Primosole area, 10 m at sea level, 37°25' N latitude; 15°30' E longitude), milled to a particle size smaller than 2 cm (Wiley Mill model No. 2, Philadelphia, USA), homogenized and stored at room temperature until used. Biomass was harvested in February 2008, after one year growing season, according to the perennial cultivation system.

Moisture content ($5.1 \pm 0.84\%$) of leaves and stems was determined from a representative sub-sample, according to the NREL LAP-001 (Ehrman, 1994).

2.2. Pretreatment

Pretreatment was performed at the USDA Forest Service, Forest Product Laboratory, Madison, WI, USA, in a custom 23-L tumbling steam digester. Three stainless steel reactor vessels were placed in-

side the digester to be heated, which was then rotated to keep the liquor in contact with the material during cooking (James, 1999).

Each vessel, (64 mm dia $\times$ 360 mm, approximately 1100 cc volume), was loaded with 100 g of material (dry weight basis) and sufficient acid/water mixture to reach a total 4:1 (w/w) solvent/solid ratio. Oxalic acid (OA) was used for hydrolysis.

Factors governing the effectiveness of pretreatment are temperature, residence time and catalyst/acid concentration. The reaction temperature and time can be combined in a single severity term, defined as Severity Factor (Overend and Chornet, 1987) (hereinafter referred as SF):

$$\text{Log}(R_0) = \text{Log}[t \cdot \exp(T - T_{\text{ref}})/14.75]$$

where, t is the time (min), T the pretreatment temperature ($^{\circ}\text{C}$) and T_{ref} the reference temperature, usually set to 100 $^{\circ}\text{C}$.

Heat treatment was performed at temperatures ranging from 150 to 190 $^{\circ}\text{C}$, from 10 to 40 min of residence time and from 2 to 8 (% w/w) of diluted-OA as catalyst/acid concentration.

The residual solid after pretreatment was vacuum filtered in order to separate the HF, washed with deionized hot water at a liquid to solid ratio of 4:1, weighed and used as substrate for both EH and SSF experiments.

2.3. Experimental design

Dilute-OA-pretreatment of *Saccharum* biomass was optimized and evaluated using a full factorial 2ⁿ central composite design (CCD) with three input parameters. The CCD is a design widely used for estimating second-order response surfaces. The parameters studied were temperature and reaction time (combined as SF) and OA concentration in the formation of monomeric sugars and sugar degradation products released in the HF, glucan conversion and ethanol production after EH and SSF, respectively. Quadratic models were fitted to obtain predict values at any points within the experimental design

$$z = a_0 + a_x \cdot x + a_y \cdot y + a_{xy} \cdot x \cdot y + a_{xx} \cdot x^2 + a_{yy} \cdot y^2$$

where, z is the response, x is the [(SF - its mean)/SD], y is the [(OA concentration - its mean)/SD], and a 's are coefficients that are estimated by fitting the z 's to the predictor variables via least squares. The significance of the coefficients and two factors interaction was estimated by ANOVA. The experimental conditions, run in full randomized order, are shown in Table 1.

2.4. Enzymatic hydrolysis (EH)

Dilute-OA-pretreated *Saccharum* was subjected to EH in 125 ml Erlenmeyer flasks, each containing 2% (w/v) dry substrate in 50 ml (5 mM) sodium citrate buffer solution (pH 4.8). Hydrolysis was conducted at 50 $^{\circ}\text{C}$ using an orbital shaking incubator (New Brunswick Scientific, Innova 4400) at 200 rpm. A commercial preparation of Accellerase 1000 enzymes (Genencor Inc., A Danisco division), with a loading of 0.50 ml/g cellulose (corresponding to an endoglucanase activity of 1000 carboxymethylcellulose (CMC) U/g cellulose and β -glucosidase activity of 160 para-nitrophenyl- β -D-glucopyranoside (pNPG) U/g cellulose, respectively), was used. One CMC unit of activity liberates 1 $\mu\text{mol}/\text{min}$ of reducing sugars (expressed as glucose equivalents) under specific assay conditions of 50 $^{\circ}\text{C}$ and pH 4.8. One pNPG unit denotes 1 μmol of nitrophenol liberated from para-nitrophenyl- β -D-glucopyranoside in 10 min at 50 $^{\circ}\text{C}$ and pH 4.8 (Genencor Product Information).

The manufacturer states that some hemicellulase activities are present in the commercial preparation, but they do not provide quantities and these activities were not assessed in the present study.

Table 1Experimental conditions and relative Severity Factor for dilute-OA-pretreatment of *Saccharum spontaneum* ssp. *aegyptiacum*, used in the central composite design.

Experiment	Coder			Factors			Severity Factor
Run	X_1	X_2	X_3	Temperature (°C)	OA (% w/w)	Time (min)	Log (R_0)
1	−1	−1	−1	158.1	3.21	16.07	2.93
2	1	−1	−1	181.9	3.21	16.07	3.59
3	−1	1	−1	158.1	6.79	16.07	2.93
4	1	1	−1	181.9	6.79	16.07	3.59
5	−1	−1	1	158.1	3.21	34.33	3.24
6	1	−1	1	181.9	3.21	34.33	3.93
7	−1	1	1	158.1	6.79	34.33	3.24
8	1	1	1	181.9	6.79	34.33	3.93
9	−1.68	0	0	150.0	5.00	25.00	2.87
10	1.68	0	0	190.0	5.00	25.00	4.05
11	0	−1.68	0	170.0	2.00	25.00	3.46
12	0	1.68	0	170.0	8.00	25.00	3.46
13	0	0	−1.68	170.0	5.00	10.00	3.06
14	0	0	1.68	170.0	5.00	40.00	3.66
15	0	0	0	170.0	5.00	25.00	3.46
16	0	0	0	170.0	5.00	25.00	3.46
17	0	0	0	170.0	5.00	25.00	3.46

2.5. Microorganisms and growth conditions

P. stipitk CBS 6054, a native xylose-fermenting yeast, was used for fermentation of the hemicellulose hydrolysate, while *Saccharomyces cerevisiae* FPL 450 was used for SSF of the solid fraction. Strains were maintained on YPD plates and stored at 4 °C. Cells were grown in 1000 ml Erlenmeyer flasks containing 400 ml of YPD (10 g/l yeast extract, 10 g/l peptone, 20 g/l glucose) in an orbital shaker incubator at 30 °C, shaken at 200 rpm. Following 24 h growth, cells were harvested by centrifugation (6708 g, 10 min, 4 °C), washed with sterile DI water and adjusted to a calculated concentration of 30 g/l dry cell weight (DCW) via standard curves relating 600 nm absorbance to DCW/l concentration (Agilent 8453, UV-Visible Spectroscopy system).

2.6. Preparation of hemicellulose hydrolysate fraction (HF) and inoculation

Saccharum hemicellulose hydrolysate was obtained after dilute-OA-pretreatment under the following condition: 158 °C, 16 min (2.93 SF), 3.21% (w/w) OA concentration (hereinafter referred as [OA]). The pH was increased to 5.0 with Ca(OH)₂, adjusted with NaOH to 5.5, 6.0 and 6.5, filter sterilized, and then transferred to sterilized 125 ml Erlenmeyer flask containing: 45 ml of HF supplemented with 3 ml of nutrients (5 g/l urea, 0.5 g/l MgSO₄·7H₂O and 1 g/l KH₂PO₄) and 2 g/l initial cell density of *P. stipitis* CBS 6054. Flasks were stoppered with cotton plugs and incubated at 30 °C, 150 rpm for 96 h.

2.7. Simultaneous saccharification and fermentation (SSF)

SSF experiments were carried out in 125 ml Erlenmeyer flasks, each containing 10% (w/v) dry dilute-OA-pretreated Saccharum substrate, 50 ml (5 mM) sodium citrate buffer solution (pH 5.5), 0.50 ml/g cellulose of Accellerase 1000 enzymes and 5 ml of nutrients containing: 0.44 g/l (NH₄)₂SO₄, 0.5 g/l MgSO₄·7H₂O and 1 g/l KH₂PO₄. The initial cells density used as inoculum was 2 g/l of *S. cerevisiae* FPL 450. Flasks were stoppered with cotton plug and incubated at 30 °C, 150 rpm for 96 h.

2.8. Analytical methods

Carbohydrate compositions of the raw materials and dilute-OA-pretreated solid samples were measured by the Analytical Chemistry and Microscopy Laboratory (USDA Forest Products Laboratory)

using an improved high-performance anion exchange chromatography (ICs-3000, Dionex, Sunnyvale, California) with pulsed amperometric detection (HPAEC-PAD), according to the method of Davis (1998). The solid material was milled using a Wiley mill (Thomas Scientific, Swedesboro, New Jersey) before acid hydrolysis. The supernatant solution of the acid hydrolysate was directly used for ion chromatography (IC) analysis. The measurement relative standard deviation (RSD) was reported based on internal regular quality assurance (QA) and quality control (QC).

Monomeric sugar concentrations in the HF were determined by HPLC equipped with a refraction index detector (Hitachi High Technologies Corporation model L-2490, Japan), using an organic acid column (Bio-rad Laboratories Inc., Hercules, CA) HPX-87H (300 × 7.8 mm) operating at 55 °C, 5 mM H₂SO₄ as mobile phase and 0.3 ml/min as flow rate. Acetic acid and ethanol concentrations were measured by the same method. A mild post-acid hydrolysis (4% H₂SO₄, 121 °C, 1 h) was performed to estimate the monomeric forms in the HF, and the resultant hydrolysate analyzed as above.

Hydroxymethylfurfural (HMF) and furfural in the HF were measured by HPLC (HP, 1090 Series II, Hewlett-Packard, Now Agilent Technologies, Palo Alto, CA) with a Phenomenex C18 (2) column (250 × 4.6 mm) and acetonitrile (ACN), water and acetic acid 1% as mobile phase at a flow rate of 0.8 ml/min. All samples were properly diluted and filtered through 0.22 µm spin-filter before analysis to remove the particle size.

Total phenol compounds were estimated colorimetrically by the Folin-Ciocalteu method via standard curve relating 760 nm absorbance (Scalbert et al., 1989).

3. Results and discussion

Our results showed that use of oxalic acid enabled better control of hydrolysis during pretreatment, which led to fewer degradation products and a selective hydrolysis of hemicellulose over cellulose in the pretreated solids subjected to mild severity. However, more severe treatments were necessary in order to maximize enzymatic saccharification. Under these more severe conditions, which were similar to those obtained by others during dilute-sulfuric acid pretreatment, sugar degradation rates were higher.

3.1. Raw material

Saccharum raw material composition is shown in Table 2. Carbohydrates account for 61.5% of the total dry weight (tdw) while

Table 2
Raw material composition of *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack

Composition	Dry matter ^a (%)
Glucan	36.81 ± 0.13
Xylan	21.53 ± 0.04
Galactan	0.72 ± 0.01
Arabinan	2.16 ± 0.01
Mannan	0.16 ± 0.04
Rhamnan	0.14 ± 0.01
Acetyl groups	3.68 ± 0.00
Proteins	2.35 ± 0.18
K. lignin	20.03 ± 0.12
Ash	5.40 ± 0.21
AL ash	1.21 ± 0.09

^a Mean values and standard deviation of two determinations.

cellulose constitutes the main glucan fraction (36.8%). The main sugars in the hemicelluloses fraction are arabino-xylans with 2.1 and 21.5% (tdw), respectively. Galactan, mannan and rhamnan make up only a small fraction in *Saccharum* hemicelluloses; acetyl groups are 3.7%. These results confirm the intrinsic chemical composition of this monocot crop, since arabino-xylan have been identified as the main hemicelluloses in other monocots as corn stover, wheat, rye, barley, oat, rice and sorghum (Ebringerova and Heinze, 2000; Polizeli et al., 2005).

Acid-insoluble lignin (20.0%) is at the high end of the range reported for other grasses (15–20%) but is relatively low when compared to hardwood or softwood (20–35%) (Chang, 2007). Protein content is 2.3%, while the whole ash and acid-insoluble lignin (AL) ash are 5.4 and 1.2% (tdw), respectively. *Saccharum* biomass is comparable in its carbohydrates composition to other lignocellulosic substrates used with this pretreatment technology such as wood and herbaceous agricultural residues, which makes this crop an adequate substrate for second generation bioethanol production.

Saccharum biomass was subjected to dilute-OA-pretreatment in order to depolymerize the hemicelluloses contained in the raw material and collect the hemicellulosic derived sugars in the HF. The residual solid fraction, mainly composed of enriched-glucan, lignin and part of hemicelluloses, was used for EH and SSF after minimal washing with DI hot water.

Previous studies have shown that oxalic acid removes hemicellulosic sugars selectively from wood and other lignocellulosic res-

idues (Kenealy et al., 2007; Lee et al., 2009; Scordia et al., 2009). One apparent advantage of pretreating wood with oxalic acid is that it is possible to remove a portion of the hemicelluloses without significantly degrading the cellulosic fibers (Lee et al., 2009).

3.2. Solid fraction

Our approach processed the residual solid and hemicellulosic fractions separately since the conditions required for fermentation of hemicellulosic hydrolysate and for the saccharification and fermentation of residual cellulose are quite different.

Composition of the residual solid following dilute-OA-pretreatment is shown in Table 3. The xylan content, which constitutes the largest fraction of hemicelluloses in *Saccharum* biomass, is the most important indicator of effectiveness for dilute acid pretreatment (Ballesteros et al., 2008). About 50% of xylan content, relative to the original dry weight (odw) was removed under mild SF (2.93) and low [OA] (3.21% w/w), while 96% was removed under the most severe treatments. Both glucan and lignin contents ranged from 51 to 59% and 25.4 to 34.5% (odw) following mild and severe treatments of SF, respectively.

Galactans, mannans and rhamnans were almost completely removed from the raw material, ranging from 0.12% to not detected in the residual, at low and high combined severity (Table 3).

3.3. Sugar yield and inhibitor compounds in the HF

Hemicellulosic derived sugars released in the HF after dilute-OA-pretreatment are shown in Table 3, and reported as monosaccharide yield (g/l) and monomeric forms (%). Xylose is the major sugar detected in the HF accounting for 85% of the total sugars, in the average of all conditions tested. Glucose was found in significant amounts at higher SF or [OA], accounting for 13% of the average total sugars released. Arabinose accounts for only 1.8%, while galactose and mannose were detected in negligible amounts. Table 3 also shows the fraction of total sugars present as monomers in the HF following dilute-OA-pretreatment. This was determined by subsequent mild post-acid hydrolysis (4% H₂SO₄, 121 °C, 60 min) of the HF and a second round of sugar analysis. This fraction was calculated for both xylose and glucose, the two main sugars present in the HF. It ranges from 71 to 100% for xylose (with 100% monomers in 10 of the conditions) and from 38 to 100% for

Table 3

Pretreatment conditions with respective severity factor [Log (*R_s*)], composition of the residual solid, hemicellulosic derived sugars and sugar degradation products released in the hydrolysate fraction following dilute-OA-pretreatment of *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.

Factors		Solid fraction composition				Hydrolysate fraction composition				Inhibitory compounds				
severity	Factor	[OA]	Glucan	Xylan	Lignin	Glucose ^a	Xylose ^a		Arabinose	Acetic acid	Furfural	HMF	Phenolic	
Log (<i>R_s</i>)		(%w/w)	(%w/w)	(%w/w)	(%w/w)	(g/l) [%]	(g/l)	[%]	(g/l)	(g/l)	(g/l)	(g/l)	compounds (g/l)	
2.87		5.00	52.51	8.74	26.00	3.40 [62.96]	36.30	[90.30]	2.80	5.00	0.69	0.11	6.17	
2.93		3.21	51.13	10.78	25.40	2.60 [54.17]	30.70	[93.73]	1.40	3.60	0.68	0.10	6.58	
2.93		6.79	55.15	5.24	27.70	5.00 [89.29]	43.00	[100.0]	0.40	6.80	1.57	0.22	6.64	
3.06		5.00	54.77	6.74	26.20	5.20 [86.67]	38.50	[100.0]	0.40	6.20	2.03	0.28	7.26	
3.24		3.21	50.73	7.21	28.00	2.70 [51.92]	31.80	[84.35]	0.40	4.00	1.17	0.12	6.65	
3.24		6.79	59.21	4.42	26.70	5.20 [89.89]	40.90	[100.0]	0.40	7.00	2.15	0.23	6.72	
3.46		2.00	53.72	7.68	27.00	1.90 [38.00]	24.10	[71.09]	1.80	4.00	1.94	0.24	7.23	
3.46 ^b		5.00	56.18	3.86	29.33	5.80 [96.24]	38.80	[100.0]	0.40	7.60	3.43	0.40	6.57	
3.46		8.00	57.28	1.26	27.00	5.60 [100.0]	31.50	[100.0]	0.40	7.60	4.20	0.43	7.05	
3.59		3.21	55.41	3.37	29.60	3.90 [72.22]	25.30	[92.67]	0.60	6.20	4.46	0.42	6.77	
3.59		6.79	56.74	1.29	32.29	6.30 [90.00]	26.50	[100.0]	0.60	7.30	5.32	0.62	7.08	
3.66		5.00	59.06	2.42	30.00	5.70 [100.0]	32.10	[100.0]	0.40	7.40	4.50	0.50	6.84	
3.93		3.21	55.87	1.64	31.20	2.80 [58.33]	15.70	[100.0]	0.60	7.70	6.08	0.78	7.21	
3.93		6.79	58.77	0.75	34.50	6.50 [96.86]	15.50	[100.0]	0.60	8.40	6.90	0.93	7.21	
4.05		5.00	59.33	0.80	33.70	4.50 [97.59]	10.80	[100.0]	0.60	8.40	7.04	1.02	7.77	

^a Values in brackets are the % monomeric sugars in the hydrolysate fraction (HF) as determined by subsequent mild post-acid-hydrolysis (4% H₂SO₄, 121 °C, 1 h).

^b Average value of three center point determinations.

glucose (with 100% monomers in only two conditions). The lowest concentration of monomers for both xylose and glucose was detected at the lowest concentration of OA (2% w/w). The low pH found in all conditions tested (-2.0) can aid the depolymerisation of oligomeric to monomeric forms, as it has been reported for dilute-sulfuric acid pretreated cardoon biomass (Ballesteros et al., 2007). By contrast, Balan et al. (2009) found very low concentrations of monomeric forms prior to mild post-acid hydrolysis compared to our work, when corn stover and poplar biomasses were pretreated with AFEX. After post-acid hydrolysis, they observed a small increase in glucose and about 54-fold increase in xylose for poplar biomass, while in corn stover, they observed 2.9 and 300-fold increase in glucose and xylose, respectively. The alkaline environment during AFEX-pretreatment does not depolymerize oligomers to monomers as in an acid environment. Thus, dilute-OA-pretreatment can hydrolyze hemicelluloses to monosaccharides in a single stage pretreatment. This might be a disadvantage under severe conditions since monomer accumulation can lead to furfural or HMF production.

To optimize the dilute-OA-pretreatment process for HF fermentation, a multiple variant response surface analysis for total sugars was carried out. Fig. 1 shows the response of total sugars (xylose, glucose and arabinose) at different SF conditions versus the [OA].

As expected, different responses were observed when SF or [OA] varied. According to the model, at low SF (2.87–3.24), xylose yield increased linearly with increasing [OA]. After that point, the greater the SF or the [OA], the lower the xylose recovery; glucose followed the opposite trend, as it was found in significant amount at higher SF or [OA]. Optimal total sugar production was attained at SF 2.93 (158 °C, 16 min) and 6.79% (w/w) [OA] with 43.0 g/l of xylose, 5.0 g/l of glucose and 0.40 g/l of arabinose. At this condition, xylose and glucose represent 89 and 10%, respectively of the total sugars released at a yield (%) of 70.3, 5.0 and 6.5, respectively for maximum potential xylose, glucose and arabinose based on original untreated *Saccharum* substrate (g of xylose, glucose and arabinose/100 g dry untreated material). The remaining fraction of xylose, glucose and arabinose was recovered in the pretreated solid, and as furfural and HMF for pentoses and hexoses degradation, respectively (see Table 3). The lowest value was attained at the highest SF (4.05), where the total monomeric sugar concentration was only 15.9 g/l, and in accordance with the predicted model value (16.0 g/l). Response of total sugars for both main effects, SF and

[OA], resulted to be statistically significant at a confidence level of 99%, as well as its interaction and both quadratic effects ($p \geq 0.01$). At the central point of the design (3.46 SF and 5% (w/w) [OA]), which was replicated three times, the model predicted a total sugars production (for xylose and glucose) of 42.8 g/l. We obtained values of overall xylose and glucose yield close to the predicted one, attaining 44.6 ± 0.60 g/l at the same condition of SF and [OA]. *Saccharum* hydrolysate fractions contain inhibitory compounds such as acetic acid from the acetyl group in hemicelluloses, furfural from pentose degradation, HMF from hexose degradation, and phenolic compounds from lignin degradation (Palmqvist and Hahn-Hagerdal, 2000). These compounds are known to inhibit the growth of yeasts (Liu et al., 2005).

Inhibitory compounds detected in the HF following dilute-OA-pretreatment of *Saccharum* are shown in Table 3. Acetic acid, furfural, HMF, and total phenolic compounds, increased as the SF of the pretreatment rose, and increased with [OA] when SF was held constant. The lowest values were found at 2.93 SF and 3.21% (w/w) [OA] with (g/l) 3.60, 0.68, 0.10 and 6.58 against the highest values at 4.05 SF and 5.0% (w/w) [OA] with (g/l) 8.40, 7.04, 1.02 and 7.77 for acetic acid, furfural, HMF and total phenolic compounds, respectively. Acetic acid, furfural as well as HMF are strongly influenced of the SF, as they increased 2.3, 10.4 and 10-fold, respectively when the SF rose from 2.93 to 4.05, which is consistent with the hydrolysis of acetyl groups and both pentoses and hexoses degradation at elevated temperatures (Palmqvist and Hahn-Hagerdal, 2000). However, HMF concentration is substantially low, even at the highest SF or [OA], implying that cellulose is virtually unaffected by dilute-OA-pretreatment under the range of conditions employed and in accordance with kinetics studies on glucose degradation performed with organic acid pretreatments (Mosier et al., 2002). Total phenolic compounds followed a different pattern, showing a small fluctuation at different SF and [OA] tested.

The fermentation of xylose by *P. stipitis* is inhibited by acetic acid (Jeffries, 2008) and to a lesser extent by the presence of furfural (Roberto et al., 1991). We therefore examined a response surface for both inhibitory compounds (Fig. 2a and b).

The release of acetic acid is influenced by the [OA]. Its concentration increased 1.9-fold as the [OA] rose from 2 to 8% (w/w). By comparison, the SF has a more pronounced effect on production of furfural than does [OA], since its concentration increased only 2.1-fold from the lowest to the highest [OA] compared to 10-fold when the SF increased from the lowest to the highest value. Kinetics studies on furfural formation from xylose and arabinose degradation have been reported (Lu and Mosier, 2007; Kootstra et al., 2009). In the previous studies, performed with mineral and organic acid pretreatments, the authors observed larger degradation rate constants, for both xylose and arabinose, with sulfuric than with organic acids. Moreover, their results indicate that (1) the stronger the acid and the higher the temperature the larger the resulting degradation constant and that (2) arabinose is more stable than xylose under the same conditions. The response of acetic acid formation to both main effects indicates statistical significance, as well as its interaction and the quadratic effect of [OA] ($p \geq 0.01$), but no significance was observed for the SF quadratic effect. The furfural response was statistically significant only for the main effects ($p \geq 0.01$), so its behaviour could be explained by a first-order equation. In the central point of the design the model predicted values of 7.30 and 3.52 g/l for acetic acid and furfural, respectively, compared to our results of 7.60 ± 0.9 and 3.43 ± 0.1 g/l, respectively. Our results support the idea of using moderate severity and low acid concentration during the dilute-OA-pretreatment, which will prevent loss of fermentable sugars and at the same time avoid formation of inhibitory products in the HF that could compromise its fermentability.

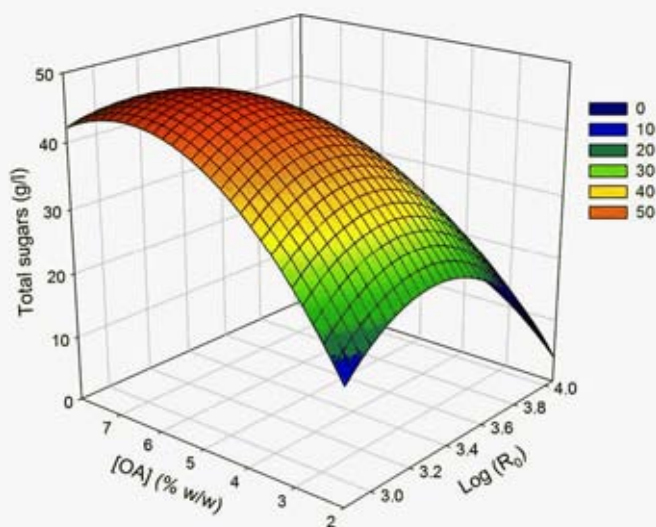


Fig. 1. Perspective plot of the fitted total sugars (g/l) response surface of severity factor [Log (R_0)] versus oxalic acid concentration after dilute-OA-pretreatment of *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.

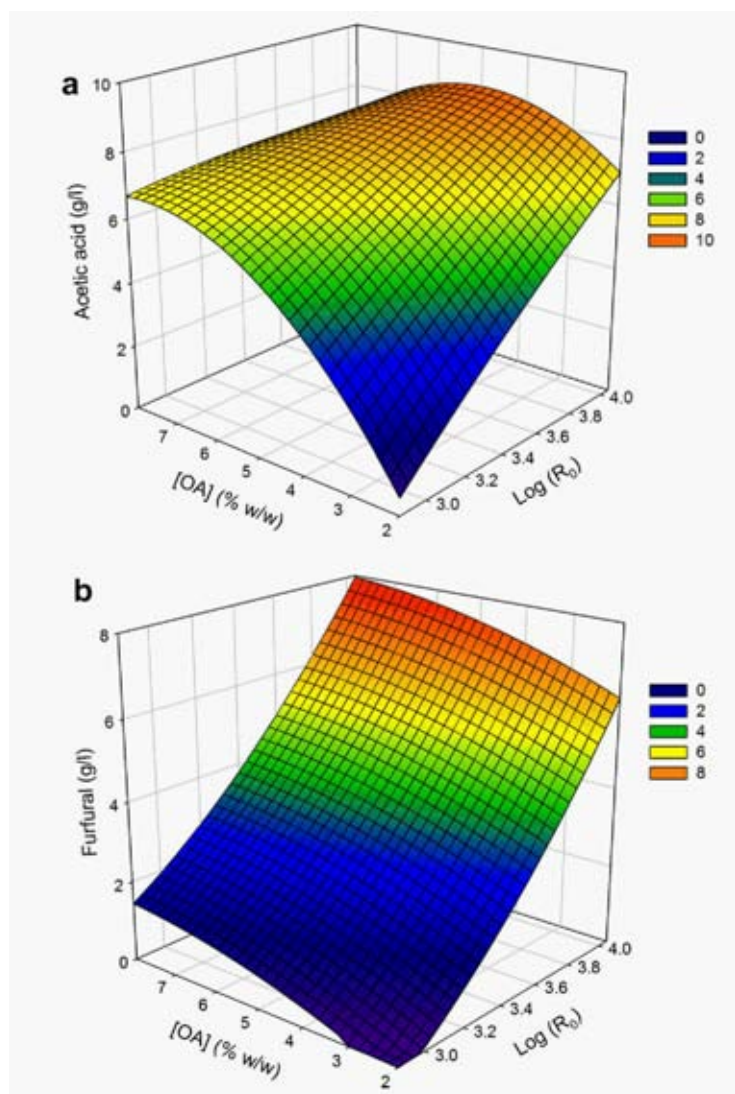


Fig. 2. Perspective plot of the fitted (a) acetic acid (g/l) and (b) furfural (g/l) response surface of severity factor $[\text{Log}(R_0)]$ versus oxalic acid concentration after dilute-OA-pretreatment of *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack.

3.4. Fermentation of hemicelluloses hydrolysate

Hemicellulosic derived sugars collected in the HF coming from dilute-OA-pretreatment at 2.93 SF and 3.21% (w/w) [OA] was used for the fermentation test.

Monomeric sugars and inhibitor compounds are shown in Table 3. The pH of the HF was increased to 5.0 with $\text{Ca}(\text{OH})_2$ and

Table 4
Fermentation at different pH using *Pichia stipitis* CBS 6054 and *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack. hemicelluloses hydrolysate after dilute-OA-pretreatment.

Fermentation Parameters	pH			
	5.0	5.5	6.0	6.5
EtOH concentration (g/l)	7.20	8.90	10.55	9.10
Fermentation time (h)	96	72	48	48
EtOH yield (g/g)	0.24	0.29	0.35	0.30
Total sugar utilized (%)	94.06	97.03	97.19	96.04
Cell growth (96 h) (g/l)	7.03	4.92	5.39	7.47
Xylitol production (g/l)	0.40	0.50	0.50	0.60
Acetic acid consumption (%)	83.3	100	100	100
Final pH (96 h)	6.92	7.68	7.78	7.84

then adjusted to 5.5, 6.0 and 6.5 with NaOH. After adding $\text{Ca}(\text{OH})_2$ a decrease in sugars and acetic acid concentration was observed, as had been elsewhere reported (Vanzyl et al., 1988; Amartei and Jeffries, 1996). Xylose and glucose decreased 9.1% and 7.7%, respectively, while acetic acid decreased of 16.6%.

At an initial pH 5.0, acetic acid is largely undissociated; this permits diffusion or transport into the cell cytoplasm, where it dissociates and decreases the intracellular pH (Sreenath and Jeffries, 2000). As a result, a long lag phase was observed at that condition. No fermentation started until 72 h after inoculation. Increasing the initial pH from 5.0 to 5.5, 6.0 and 6.5, respectively, evidently improved the fermentability of the HF (Table 4). Fig. 3 shows the performance of *P. stipitis* CBS 6054 at pH 6.0, the best condition obtained in the HF fermentation test.

After 48 h fermentation, when the ethanol concentration reached its maximum value (10.5 ± 0.35 g/l), glucose was completely consumed, while 0.85 ± 0.35 g/l of xylose still remained. Acetic acid was consumed from the beginning and continued after the ethanol peak was reached, as confirmed by an increased pH at the end of the fermentation (from 6.0 to 7.8), and in accordance with previous studies on sugar cane bagasse, wood and corn stover hemicelluloses hydrolysates (Vanzyl et al., 1988; Sreenath and Jeffries, 2000; Agbogbo and Wenger, 2007).

Ethanol yield reached 0.35 g/g, approximately 69% of the maximum theoretical value, which is comparable to the yield reported by Ferrari et al. (1992) and Nigam (2001), with *P. stipitis* NRRL Y-7124 to ferment acid hydrolysates of eucalyptus wood and wheat straw, respectively. The yield was low relative to that obtained with *P. stipitis* CBS 6054 and NRRL Y-7124 (0.41 g/g and 82% max theoretical) that had been adapted to corn cob and wheat straw hydrolysates by repeated subculturing (Amartei and Jeffries, 1996; Nigam, 2001). At the end of fermentation xylitol and cell density reached values of 0.50 g/l and 5.39 g/l, respectively, confirming that this yeast produces little xylitol (Jeffries, 2008).

3.5. Enzymatic hydrolysis (EH)

The solid fraction from dilute-OA-pretreatment was subjected to EH at a solid loading of 2% (w/v) and after minimal DI hot water washing. Enzymes employed were used as is, after filter sterilization. The enzyme loading was 0.5 ml/g cellulose, with

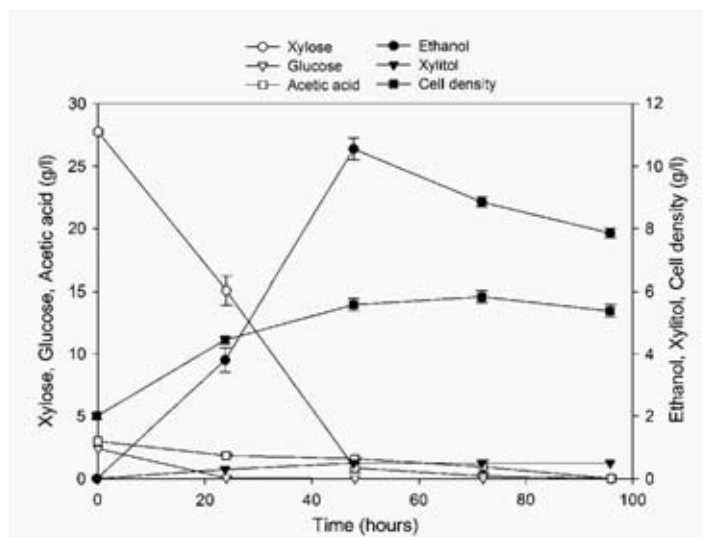


Fig. 3. Hydrolysate fraction fermentation after dilute-OA-pretreatment of *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hack. with *Pichia stipitis* CBS 6054 at 150 rpm, 30°C and pH 6.0.

endoglucanase activity of 1000 (CMC) U/g cellulose and β -glucosidase activity of 160 (pNPG) U/g cellulose, respectively.

Glucan conversion increased with SF from the lowest to the highest. Fig. 4 compares glucan hydrolysis of the SF versus [OA] after 96 h incubation at 200 rpm and 50 °C.

Glucan conversion rose from 42.4% at 2.93 SF and 3.21% (w/w) [OA] to maximum value of 89.9% at 4.05 SF and 5.0% (w/w) [OA]. The lower xylan content in the solid fraction pretreated at higher SF (0.8%) compared to the lower SF (10.8%), might have contributed to the higher saccharification rate, since it has been shown that the percentage of residual xylan appears to be a good indicator of cellulose digestibility (Jeoh et al., 2007). In fact, enzymatic digestion of cellulose in pretreated biomass increases with xylan removal (Kabel et al., 2007).

Similar results have been reported by Bura et al. (2009), in which decreased xylan content in SO_2 -pretreated corn stover and poplar biomass improved cellulose conversion.

The strongest effect on glucan conversion was given by the SF rather than [OA] because when we kept the [OA] constant at 5.0% (w/w), raising the SF from 2.87 to 4.05, converted about 43% more glucan. In comparison when we fixed SF (3.46) while increasing the [OA] from 2.0 to 8.0% (w/w), we observed an increase of only 11%. In the central point of the design the model predicted a value of 78.2% glucan conversion, compared to our result of $79.7 \pm 1.63\%$. Glucan conversion response was statistically significant ($p \geq 0.01$) only for SF main effects.

3.6. Simultaneous saccharification and fermentation (SSF)

SSF was carried out at a solid loading of 10% (w/v) and after minimal deionized hot water washing. The enzyme loading was 0.5 ml/g cellulose, and 2 g/l of *S. cerevisiae* FPL 450 strain as inoculum. In the SSF process glucose released from cellulase and β -glucosidase enzymes was simultaneously fermented to ethanol by yeasts. After 72 h incubation at 150 rpm and 30 °C ethanol production increased with treatment severity. This decreased the amount of xylan in the dilute-OA-pretreated Saccharum substrate, as discussed above. Fig. 5 shows the ethanol production after 72 h SSF of the SF versus [OA].

Ethanol production rose from 4.4 g/l at 2.93 SF and 3.21% (w/w) [OA] to maximum value of 17.8 g/l at 4.05 SF and 5.0% (w/w) [OA]. The latest value corresponds to 90% of the SSF conversion efficiency

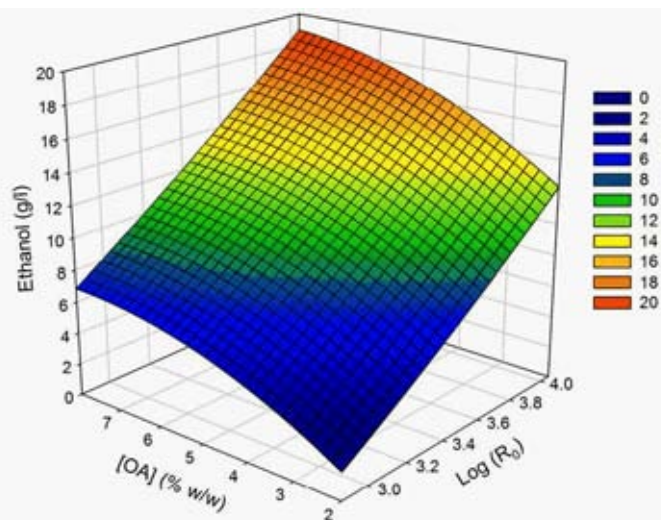


Fig. 5. Perspective plot of the fitted ethanol production (%) response surface of severity factor [Log (R_0)] versus oxalic acid concentration after 72 hours simultaneous saccharification and fermentation.

and 53% of the theoretical yield. As noted for EH, SF showed a larger influence than [OA] on ethanol production. In fact, raising SF from 2.87 to 4.05 while keeping [OA] constant at 5.0% (w/w) resulted in an increase of ethanol concentration from 4.9 to 17.8 g/l. Vice versa, at fixed SF (3.46), when increasing [OA] from 2.0 to 8.0% (w/w) ethanol production increased from 6.1 to 12.9 g/l.

In the central point of the design the model predicted a value of 10.9 g/l of ethanol, compared to our result of 10.8 ± 0.37 g/l. SF and [OA] main effects were both statistically significant ($p \geq 0.01$), while no significant both the interaction and quadratic effects.

Although it is difficult to compare results among different substrates, bioreactor configuration, nature of the acid/catalyst, enzymes employed and yeast strains used, our results are in agreement with several authors that used SSF for ethanol production (Ballesteros et al., 2004; Rudolf et al., 2008; Lee et al., 2009).

4. Conclusion

S. spontaneum ssp. *aegyptiacum* is attractive as a non-food energy crop based on its high biomass yield, relatively high carbohydrate composition, its perennial growth and its ability to grow well on marginal and nonagricultural lands. Oxalic acid can release fermentable hydrolysates from Saccharum and prepare the residual solids for enzymatic saccharification, however the optima for hydrolysate fermentation and SSF are distinctly different. To obtain a fermentable hydrolysate moderate severity and low acid concentration should be used to avoid the formation of inhibitory compounds. In contrast, to obtain an appropriate solid fraction, with low xylan content, easily digested by enzymes and consequently easily fermented by yeasts, higher severity should be used. Other pretreatment technologies might avoid such dual optima.

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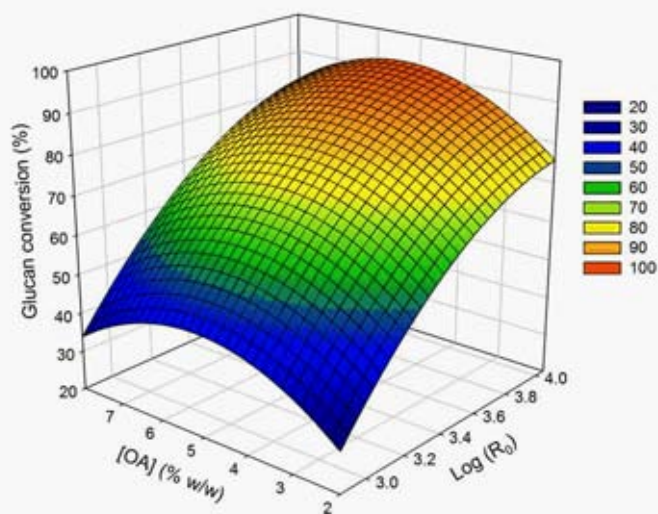


Fig. 4. Perspective plot of the fitted glucan conversion (%) response surface of severity factor [Log (R_0)] versus oxalic acid concentration after 96 hours enzymatic hydrolysis.

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