Industrial Crops and Products 49 (2013) 392–399

Contents lists available at SciVerse ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Enzymatic hydrolysis, simultaneous saccharification and ethanol fermentation of oxalic acid pretreated giant reed (*Arundo donax* L.)

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ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form 17 May 2013
Accepted 27 May 2013

Keywords:
Arundo donax
Giant reed
Pretreatment
SSF
Bioethanol production
Enzymatic hydrolysis

ABSTRACT

Giant reed was evaluated for enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) using a commercial cellulase/β-glucosidase and *Scheffersomyces* (*Pichia*) *stipitis* CBS 6054 for ethanol production following dilute-oxalic acid pretreatment. A response surface methodology with two input parameters – severity factor (SF) and oxalic acid concentration (OA) – was employed to optimize both enzymatic hydrolysis and SSF. Xylan content after dilute-OA pretreatment decreased with increasing SF and OA; almost complete hydrolysis was observed when the harsher pretreatment conditions were used. Glucan and lignin content showed an opposite trend with respect to xylan content after dilute-OA pretreatment. Accordingly, enzymatic hydrolysis and ethanol production reached 95% of glucan conversion and 18 g l^{-1} (75.3% of the maximum theoretical ethanol yield), respectively, with the pretreatment condition 4.05 SF and 5% OA w/w.

Dilute-OA mediated pretreatment of giant reed followed by coupled saccharification and fermentation can be considered a promising methodology for second generation bioethanol production.

1. Introduction

The European Council endorsed a mandatory target of a 20% share of energy from renewable sources in overall Community energy consumption by 2020 and a mandatory 10% minimum target to be achieved by all Member States for the share of biofuels in transport petrol and diesel consumption by 2020 (2009/28/EC). This target, however, has to be achieved after taking into account sustainability criteria, both for raw material production and bioconversion technologies. To meet these mandated targets, alternative crops with low energy input requirements that are able to be grown on marginal and/or underutilized lands must be identified.

In general, perennial grasses are high yielding crops that have been attracting growing interest due to their extensive environmental benefits at both global- and agricultural community-scales (Rettenmaier et al., 2010; Fernando et al., 2010). Compared to traditional row crops, perennial grasses generally require lower energy inputs (e.g. fertilizers, pesticides and herbicides), can be grown on marginal cropland and provide benefits in terms of soil structure and stability (e.g. reduced soil loss, erosion and runoff), soil quality (e.g. increase in soil fertility, organic matter and nutrient retention) and biodiversity (e.g. cover for native wildlife) (Sims et al., 2006; Zegada-Lizarazu et al., 2010).

In addition to weedy cellulosic species, as *Lantana camara*, *Prosopis juliflora*, *Saccharum spontaneum*, among others, which are able to grow on agriculturally, degraded lands (Chandel et al., 2009; Chandel and Singh, 2011), giant reed (*Arundo donax* L.), a lignocellulosic, rhizomatous C3 perennial crop has been recognized as a potential energy crop for southern temperate environments, thanks to its high biomass yield, positive energy balance with respect to the ratio and to the net gain (Mantineo et al., 2009), but also for its significant potential as feedstock for second generation bioethanol production (Scordia et al., 2011, 2012). It is currently widespread in India, China, USA, Australia, Southern Africa and in the Mediterranean regions (Rossa et al., 1998). Even though it is a warm-temperate or subtropical species, it is able to survive short period frost; it prefers soils with abundant moisture but also presents high resistance to drought due to its vigorous roots that penetrate deeply into soil (Lewandowski et al., 2003).

The most critical component in a bioenergy chain is to secure reliable feedstock supplies, which becomes complicated by the fact that resources are geographically dispersed, maturity and yield are time and weather-dependent, there are short-time sowing and harvesting windows and there may be potential competitive demand for resources from non-energy users (Bauen et al., 2009). Moreover, bioconversion technologies are not still optimized for lignocellulosic feedstocks. Second generation bioethanol technologies consist

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http://dx.doi.org/10.1016/j.indcrop.2013.05.031

of a (i) pretreatment step to remove hemicelluloses, disrupt or rearrange lignin structure and make cellulose more available for (ii) enzymatic hydrolysis by cellulase/β-glucosidase to free sugars and (iii) ferment the free sugar to ethanol. All those steps need to be optimized to achieve maximum yields and/or lower energy consumption. Various methods of pretreatment can be used, including mechanical, steam explosion, ammonia fiber explosion, alkali, sulphite and dilute acid, either inorganic or organic (Mosier et al., 2005; Zhu et al., 2010) with different degree of strength and weakness (Chandel and Singh, 2011). Enzymatic hydrolysis carried out by enzyme complexes known as cellulases are involved in cellulose digestibility after pretreatment enhancing glucose yield, even though end products as cellobiose and glucose act as inhibitors for exo-1,4-β-glucanase and β-glucosidase, respectively (Philippidis et al., 1993). One of the most successful methods to improve enzymatic hydrolysis was the simultaneous saccharification and fermentation (SSF). In this process, the glucose produced by the hydrolyzing enzymes is consumed immediately by the fermenting microorganisms present in the media, minimizing the inhibitory effect of cellobiose and glucose and increasing ethanol yields (Eklund et al., 1995).

In the present manuscript, a response surface methodology was adopted to optimize dilute oxalic acid pretreatment of giant reed. Glucan, xylan and lignin concentration in the solid residues after pretreatment were studied. We examined subsequent enzymatic saccharification and the simultaneous saccharification and ethanol fermentation of the solid fractions using *Scheffersomyces* (*Pichia*) *stipitis* CBS 6054 yeast strain.

2. Materials and methods

2.1. Raw material

Giant reed biomass, clone Capo d'Orlando, was provided by the collection of Cosentino et al. (2006), established in Catania plain (10 m a.s.l., 37°25′ N lat., 15°30′ E long). Raw material had an initial moisture content of 4.0 ± 0.72% and composed of (%, w/w) glucan 34.6 ± 0.14, xylan 20.41 ± 0.02, arabinan 1.81 ± 0.03, galactan 0.66 ± 0.06, mannan 0.12 ± 0.01, rhamnan 0.06 ± 0.00, Klason lignin 20.44 ± 0.07 and acid insoluble lignin ash 1.67 ± 0.08 (Scordia et al., 2012).

2.2. Pretreatment

A solution of oxalic acid (hereinafter referred to OA) and water was loaded together with 100 g of material (dry weight) at a solvent/solid ratio of 4:1 (%, w OA/w solid dry matter), according to the method of Scordia et al. (2012). Pretreatment temperature and residence time were combined into a single severity, following the severity factor [Log(R_0)] formula (hereinafter referred to SF) proposed by Overend and Chornet (1987).

$$\mathrm{Log}(R_0) = \mathrm{Log}\left[t \cdot \exp\left(\frac{T_\mathrm{p} - T_\mathrm{ref}}{14.75}\right)\right]$$

where t is the time (min), T_p the pretreatment temperature (°C) and T_ref the reference temperature, which is usually set to 100 °C.

Heat treatment was performed at temperatures ranging from 150 to 190 °C, from 10 to 40 min of residence time and from 2 to 8 (%, w/w) of diluted-OA concentration. It is worth to mention that the ramping time to reach the target temperature ranged between 8 and 11 min (150 and 190 °C, respectively), however, these values were not included in the SF calculation.

Dilute-OA-pretreatment of giant reed was optimized using a full factorial 2^n central composite design with two input parameters (SF and OA concentration) with the aim to optimize enzymatic hydrolysis and simultaneous saccharification and fermentation. SF ranged from 2.87 to 4.05 while OA concentration from 2 to 8% (%, w OA/w solid dry matter).

Quadratic models were fitted to obtain predict values at any points within the experimental design, according to the following equation

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

where z is the response, x is the SF values [(condition of the run − condition at the central point)/step change of the factor], y is the OA concentrations [(condition of the run − condition at the central point)/step change of the factor] and a's are model coefficients that were estimated by fitting the z's to the predictor variables via least squares.

The response surface methodology analysis of variance was conducted using Minitab® 15.0 software, with a confidence level of 99% ($p \leq 0.001$). The experimental conditions, run in full randomized order, are reported in Table 1.

2.3. Microorganisms and growth conditions

S. stipitis CBS 6054 was used for simultaneous saccharification and fermentation experiments. Cells of *S. stipitis* CBS 6054 were cultivated in 1000 ml Erlenmeyer flasks containing 400 ml of YPD (10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 20 g l^{-1} glucose) in an orbital shaker incubator at 30 °C, shaken at 200 rpm (New Brunswick Scientific, Innova 4400). Following 24 h growth, cells were harvested by centrifugation (10,000 rpm, 10 min, 4 °C), washed with sterile deionized water and adjusted to a calculated concentration of 30 g l^{-1} dry cell weight (DCW) using standard curves that related 600 nm absorbance to DCW/l concentration (Agilent 8453, UV–visible Spectroscopy system). An aliquot was transferred to fresh fermentation medium for an initial cell concentration of 2.0 DCW/l.

2.4. Enzymatic hydrolysis (EH)

Dilute-OA-pretreated giant reed was subjected to EH in 125 ml Erlenmeyer flask, each containing 10% (w/v) dry substrate in 50 ml (5 mM) sodium citrate buffer solution (pH 4.8) at 50 °C using an orbital shaking incubator (New Brunswick Scientific, Innova 4400) at 200 rpm. A commercial preparation of Accelerase 1000 enzymes (Genencor Inc., A Danisco division), with a loading of 0.50 ml (g glucan)$^{-1}$ (corresponding to an endoglucanase activity of 1000 carboxymethycellulose (CMC) U/g cellulose and β-glucosidase activity of 160 para-nitrophenyl-β-D-glucopyranoside (pNPG) U/g cellulose, respectively) was used.

Glucan conversion (%) was calculated according to the following expression

$$\frac{C \times V}{M \times \mathrm{Gn} \times (162/180)} \times 100$$

where C is the glucose concentration expressed as g l^{-1}, V is the volume of medium, M is the substrate loading expressed as g and Gn is the glucan content of the substrate expressed as g glucan/g substrate.

2.5. Simultaneous saccharification and fermentation (SSF)

SSF experiments were carried out in 125 ml Erlenmeyer flasks, each containing 10% (w/v) dry dilute-OA-pretreated solid substrates, 50 ml (5 mM) sodium citrate buffer solution (pH 6.0), 0.50 ml (g glucan)$^{-1}$ of Accelerase 1000 enzymes and 5 ml of nutrients containing: 5 g l^{-1} yeast extract, 5 g l^{-1} urea, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 1 g l^{-1} KH_2PO_4. The initial cells density used as inoculum was 2 g l^{-1} of *S. stipitis* CBS6054. Flasks were closed with

Table 1
Experimental conditions and relative severity factor [Log(R_0)] for dilute-OA-pretreatment of giant reed (*Arundo donax* L.), according to the central composite design.

Experiment	Codes			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

X_1, X_2 and X_3 represent the coded values of temperature (°C), oxalic acid concentration (%, w OA/w solid dry matter) and reaction time (min), respectively. $\text{Log}(R_0) = \text{Log}[t \cdot \exp((T_p - T_{ref})/14.75)]$, where t is the time (min), T_p the pretreatment temperature (°C) and T_{ref} the reference temperature, which is usually set to 100 °C (Overend and Chornet, 1987).

cotton plug and incubated at 30 °C, 150 rpm for 96 h. Samples were withdrawn at 0, 24, 48, 72 and 96 h.

Theoretical ethanol yield (%) was calculated according to the following expression

$$\frac{E}{(\text{Gn}/(162/180)) \times 0.511} \times 100$$

where E is the ethanol concentration expressed as g l^{-1}, Gn is the glucan content of the substrate after pretreatment expressed as g glucan/g substrate and 0.511 is the stoichiometric ethanol yield for yeasts.

2.6. Analytical methods

Carbohydrate compositions of the original and pretreated giant reed samples were analyzed according to the method of Davis (1998).

Xylose, glucose and ethanol concentration after enzymatic hydrolysis and simultaneous saccharification and fermentation were determined by HPLC (Gilson 307 system, Villiers-le-Bel, France) equipped with a refraction index detector (Hitachi High Technologies Corporation model L-2490, Japan), using a Bio-Rad HPX-87H (300 mm × 7.8 mm) column. The samples were diluted with deionized water, filtered through 0.22 μm spin-filter before analysis to remove the particle size and thus injected in the chromatograph under the following conditions: column temperature of 55 °C, 5 mM sulfuric acid as mobile phase at a flow rate of 0.3 ml/min, and an injection volume of 20 μl. The concentration of these compounds was calculated using calibration curves obtained from standard solutions.

3. Results

3.1. Structural carbohydrates and lignin content after dilute-OA pretreatment

The residual solid fraction, mainly composed of enriched-glucan, lignin and part of hemicelluloses was analyzed and results are depicted in Fig. 1.

Fig. 1a shows the residue glucan contents (%, w/w) after dilute-OA-pretreatment in the range of 2.87–4.05 SF and 2–8% OA concentration; glucan content increased when the severity of the pretreatment increased as well. The initial content in the original dry biomass was 34.6% of the total dry weight, which means that it increased of about 15% at the mild-high severities (SF > 3.4 and OA > 5%).

As in the case of other monoct crops, the larger fraction of giant reed hemicelluloses is represented by xylans. Its original content of 20.4%, relative to the original dry weight, was reduced only by 5% under mild SF (2.93) and low OA concentration (3.21%, w/w), while almost complete hydrolysis was shown under the most severe treatments (Fig. 1b). At the harsher condition tested (4.05 SF and 5% OA concentration) the residue xylan after dilute-OA pretreatment was reduced of 99% compared to the original untreated content.

As for glucan content, the concentration of the lignin in the insoluble solid fraction increased at high severity factors. At conditions of lower severity its concentration was unaffected, while values close to 40% were observed at the highest level of SF and 5% OA concentration, as shown in Fig. 1c.

A statistical analysis of xylan, glucan and lignin content after dilute-OA pretreatment is shown in Table 2. Experimental data showed that the two independent variables significantly influenced the responses of residual structural carbohydrates and lignin during dilute-OA pretreatment. Such influences could be successfully described by linear and quadratic models whose suitability of fit and statistical significance is reported. A coefficient of determination of 0.95–0.99 in reasonable agreement with the adjusted R^2 (0.92–0.98) showed that large portion of the variance in the response was explained by the independent variables. ANOVA showed that SF and OA (a_x and a_y, respectively) were both statistically significant at a 99% confidence level ($p \leq 0.001$). When SF was analyzed separately, temperature had a much more significant effect compared to time, which is reflected in the SF where temperature is raised to an exponential value. The interactions and quadratic terms of the fitted coefficients resulted statistically significant ($p \leq 0.001$ and $p \leq 0.05$) except for the quadratic terms of glucan and lignin (a_{yy} and a_{xx}, respectively).

3.2. Enzymatic hydrolysis (EH)

The solid fractions from dilute-OA-pretreatment were subjected to EH at a solid loading of 10% (w/v) after minimal hot water washing. Glucan conversion increased with SF from the lowest to the highest level. Fig. 2 compares glucan hydrolysis of the SF versus OA concentration after 96 h incubation at 200 rpm and 50 °C.

Table 2
Analysis of variance and fitting coefficients for glucan, xylan and lignin residues in the solid fraction as a function of severity factor (a_x), and oxalic acid concentration (a_y), used during dilute-OA pretreatment of giant reed (*Arundo donax* L.).

Source	DF	SS	Adj MS	*F*-value	*P*-value	Coefficient	Value	*P*-value
Xylan (%, w/w)								
Regression	5	347.932	69.586	68.92	0.000	a_o	6.261	0.000
"Linear"	2	318.344	146.145	144.75	0.000	a_x	−6.036	0.000
"Square"	2	10.124	5.030	4.98	0.029	a_y	−4.280	0.000
"Interaction"	1	19.464	19.464	19.28	0.001	a_{xy}	4.201	0.001
Residual	11	11.106	1.010			a_{xx}	1.829	0.021
Lack-of-fit	9	10.388	1.154	3.22	0.260	a_{yy}	1.889	0.042
Pure error	2	0.718	0.359					
Total	16	359.038						
R^2	0.97	R^2(adj)	0.95					
Glucan (%, w/w)								
Regression	5	209.346	41.8692	40.38	0.000	a_o	51.843	0.000
"Linear"	2	179.169	78.8220	76.01	0.000	a_x	4.130	0.000
"Square"	2	23.146	11.5553	11.14	0.002	a_y	3.609	0.000
"Interaction"	1	7.031	7.0308	6.78	0.025	a_{xy}	−2.525	0.025
Residual	11	11.406	1.0369			a_{xx}	−3.249	0.001
Lack-of-fit	9	11.394	1.2660	209.84	0.005	a_{yy}	−1.356	0.131
Pure error	2	0.012	0.0060					
Total	16	220.752						
R^2	0.95	R^2(adj)	0.92					
K. Lignin (%, w/w)								
Regression	5	158.107	31.6214	177.15	0.000	a_o	31.4274	0.000
"Linear"	2	150.787	73.9204	414.13	0.000	a_x	4.6924	0.000
"Square"	2	4.001	1.9935	11.17	0.002	a_y	2.2147	0.000
"Interaction"	1	3.319	3.3193	18.60	0.001	a_{xy}	−1.7349	0.001
Residual	11	1.963	0.1785			a_{xx}	0.4153	0.175
Lack-of-fit	9	1.879	0.2088	4.96	0.179	a_{yy}	−1.3654	0.002
Pure error	2	0.084	0.0421					
Total	16	160.070						
R^2	0.99	R^2(adj)	0.98					

DF, Degree of Freedom; SS, Sum of Square; Adj MS, Adjusted Mean Square; R^2(adj), adjusted R^2.

Glucan conversion rose from 5% at 2.93 SF and 3.21% (w/w) OA to maximum value of 95% at 4.05 SF and 5.0% (w/w) OA. The lower xylan content in the solid fraction pretreated at higher SF (∼1%) compared to the lower SF (∼13%), might have contributed to the higher saccharification rate. The strongest effect on glucan conversion was given by the SF respect to OA concentration because when we kept the OA concentration constant at 5.0% (w/w) and time constant at 25 min, raising the temperature from 150 to 190 °C (2.87–4.05 SF), converted about 65% more glucan. In comparison when we fixed both temperature and time (170 °C and 25 min, respectively, corresponding to 3.46 SF) while increasing the OA from 2.0 to 8.0% (w/w), an increase of 45% was observed in glucan conversion. Time had the weakest effect on glucan conversion. Conversion increased from 35 to 75% as time increased from 10 to 40 min when both temperature and OA were fixed (170 °C, 5% OA). At level of SF higher than 3.8 increasing the OA concentration further than 7% a depression in glucan conversion was observed probably due to the effect of inhibitors generated with those harsher conditions. In the central point of the design (3.46 SF and 5% OA) the model predicted a value of 60.6% glucan conversion, compared to the observed result of 58.9 ± 1.53%.

Experimental data showed that the two independent variables significantly influenced the responses of glucan conversion (Table 3). A coefficient of determination of 0.90 in reasonable agreement with the adjusted R^2 (0.85) showed that large portion of the variance in the response was explained by the independent variables. However, a lack-of-fit test suggested that limiting the analysis to a second order approximation often resulted in models which could not describe the experimental data. Indeed, SF and OA were both statistically significant at 99% confidence level, however, the quadratic terms of the fitted coefficients were not significant, therefore the responses of glucan conversion could be explained by a linear regression, suggesting SF and OA acted independently.

3.3. Simultaneous saccharification and fermentation (SSF)

Fig. 3 shows the ethanol production after 72 h SSF of the SF versus OA concentration.

Ethanol production rose from 0.80 g l^{-1} at 2.93 SF and 3.21% (w/w) OA concentration to maximum value of 18.0 g l^{-1} at 4.05 SF and 5.0% (w/w) OA.

As noted for enzymatic hydrolysis, SF showed a larger influence than OA on ethanol production. Raising the SF from 2.87 to 4.05, while keeping OA constant at 5.0% (w/w) resulted in an increase of ethanol concentration (from 4.0 to 18.0 g l^{-1}). Vice versa, at fixed SF (3.46), when increasing OA concentration from 2.0 to 8.0% (w/w), ethanol production rose from 2.0 to 12.0 g l^{-1}. Time had the weakest effect on ethanol production. Ethanol increased from 4.0 to 10.0 g l^{-1} after as time increased from 10 to 40 min when both temperature and OA were fixed (170 °C, 5% OA). In the central point of the design (3.46 SF and 5% OA) the model predicted a value of 6.0 g l^{-1} of ethanol concentration, compared to the observed result of 5.9 ± 0.21 g l^{-1}.

The theoretical ethanol yield, calculated on the basis of the maximum ethanol production from the glucan content after pretreatment, is shown in Table 4. As expected the lowest value was achieved at 2.93 SF and 3.21% (w/w) OA concentration (14.3%), while a maximum value of 75.3% at 4.05 SF and 5.0% (w/w) OA concentration.

As noted previously, experimental data showed that the two independent variables significantly influenced the responses of ethanol production (Table 5). A coefficient of determination of 0.93

Table 3
Analysis of variance and fitting coefficients for glucan conversion as a function of severity factor (a_x), and oxalic acid concentration (a_y), used during dilute-OA pretreatment of giant reed (*Arundo donax* L.).

Source	DF	SS	Adj MS	*F*-value	*P*-value	Coefficient	Value	*P*-value
Glucan conversion (%, w/w)								
Regression	5	4774.63	954.93	19.38	0.000	a_o	60.7014	0.000
"Linear"	2	4460.98	2145.30	43.53	0.000	a_x	23.4284	0.000
"Square"	2	158.61	78.93	1.60	0.245	a_y	15.8701	0.000
"Interaction"	1	155.05	155.05	3.15	0.104	a_{xy}	−11.8574	0.104
Residual	11	542.06	49.28			a_{xx}	0.3189	0.948
Lack-of-fit	9	541.75	60.19	395.93	0.003	a_{yy}	−9.7795	0.115
Pure error	2	0.30	0.15					
Total	16	5316.69						
R^2	0.90	R^2(adj)	0.85					

DF, Degree of Freedom; SS, Sum of Square; Adj MS, Adjusted Mean Square; R^2(adj), adjusted R^2.

Table 4
Theoretical ethanol yield as a function of severity factor [Log(R_0)] and oxalic acid concentration [OA (%, w/w)] after dilute-OA pretreatment of giant reed (*Arundo donax* L.).

Run	Log(R_0)	OA (%, w/w)	Theoretical ethanol yield (%)
1	2.93	3.21	14.34
2	3.59	3.21	38.87
3	2.93	6.79	32.61
4	3.59	6.79	54.67
5	3.24	3.21	23.74
6	3.93	3.21	49.18
7	3.24	6.79	32.08
8	3.93	6.79	63.95
9	2.87	5.00	18.56
10	4.05	5.00	75.28
11	3.46	2.00	18.83
12	3.46	8.00	43.90
13	3.06	5.00	30.59
14	3.66	5.00	36.25
15	3.46	5.00	33.66
16	3.46	5.00	34.01
17	3.46	5.00	33.14

$\text{Log}(R_0) = \text{Log}[t \cdot \exp((T_p - T_{ref})/14.75)]$, where t is the time (min), T_p the pretreatment temperature (°C) and T_{ref} the reference temperature, which is usually set to 100 °C (Overend and Chornet, 1987).

in reasonable agreement with the adjusted R^2 (0.91) showed that large portion of the variance in the response was explained by the independent variables. However, a lack-of-fit test suggested that limiting the analysis to a second order approximation often resulted in models which could not describe the experimental data. Indeed, SF and OA were both statistically significant at 99% confidence level, however, the quadratic terms of the fitted coefficients were not significant, therefore the responses of ethanol production could be explained by a linear regression, suggesting SF and OA acted independently.

4. Discussion

In this study giant reed biomass was subjected to dilute-OA-pretreatment in order to catalyze the hydrolysis of hemicelluloses, decrystallize cellulose and displace lignin structure for subsequent enzymatic hydrolysis and simultaneous saccharification and fermentation using a response surface methodology to optimize pretreatment conditions of giant reed.

As in the case of other monoct crops, the larger fraction of giant reed hemicelluloses is represented by xylans, while galactan, arabinan, mannan and rhamnan make-up the remaining fraction of hemicelluloses (Scordia et al., 2010; Chandel and Singh, 2011).

Results showed that the increase in glucan content could be ascribed to the hydrolysis of the xylan, the compound of hemicelluloses more susceptible to acid pretreatment. Furthermore, the other constituents of hemicelluloses, namely galactan, arabinan, mannan and rhamnan were completely hydrolysed after pretreatment contributing to the increase in glucan content.

Xylan removal has been reported to be a good indicator of the effectiveness of the pretreatment (Kabel et al., 2007) and dilute-OA pretreatment looks a promising approach in this respect. However, the xylan removed from the solid and collected in the soluble hydrolysates did not completely cover the amount of xylan released from the residues (data not shown). This was observed by Scordia et al. (2012), who studied dilute-OA pretreatment of giant reed for xylose release and subsequent fermentation to ethanol by *S. stipitis* CBS 6054. Common observations were made for several raw materials pretreated in a similar way, since high temperatures and long residence times lead to xylose degradation to furfural and other inhibitors (Palmqvist and Hahn-Hägerdal, 2000).

As for glucan content, the concentration of the lignin in the insoluble solid fraction increased at high severity factors. During pretreatment an almost simultaneous depolymerization and repolymerization of lignin takes place due to the acid environment

Table 5
Analysis of variance and fitting coefficients for ethanol production as a function of severity factor (a_x), and oxalic acid concentration (a_y), used during dilute-OA pretreatment of giant reed (*Arundo donax* L.).

Source	DF	SS	Adj MS	*F*-value	*P*-value	Coefficient	Value	*P*-value
Ethanol production ($g\,l^{-1}$)								
Regression	5	286.030	57.206	33.52	0.000	a_o	5.962	0.0001
"Linear"	2	264.440	137.323	80.46	0.000	a_x	3.419	0.0001
"Square"	2	21.572	10.786	6.32	0.015	a_y	2.179	0.0001
"Interaction"	1	0.019	0.019	0.01	0.919	a_{xy}	−0.040	0.9188
Residual	11	18.774	1.707			a_{xx}	0.948	0.0127
Lack-of-fit	9	18.634	2.070	29.58	0.033	a_{yy}	−0.360	0.2793
Pure error	2	0.140	0.070					
Total	16	304.805						
R^2	0.93	R^2(adj)	0.91					

DF, Degree of Freedom; SS, Sum of Square; Adj MS, Adjusted Mean Square; R^2(adj), adjusted R^2.

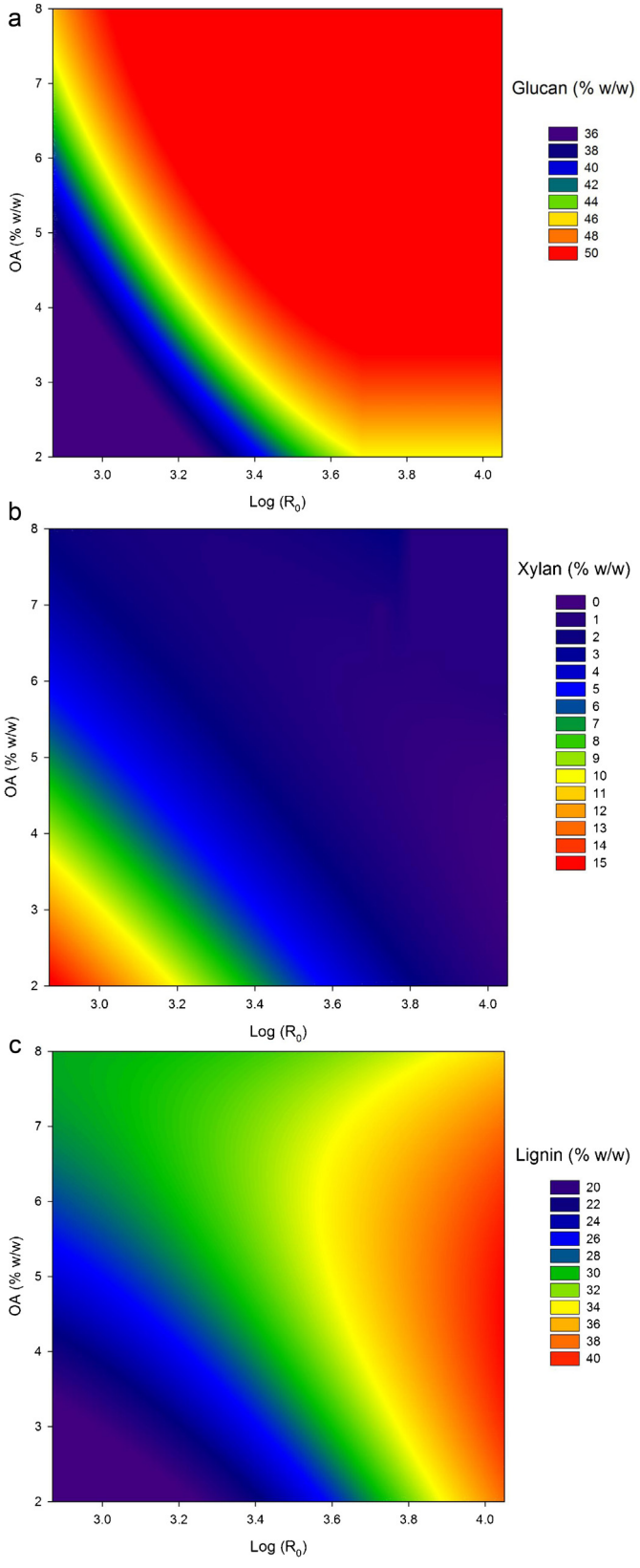

Fig. 1. Response of (a) glucan, (b) xylan and (c) lignin content as a function of severity factor [Log(R_0)] versus oxalic acid concentration (OA%, w/w) after dilute-oxalic acid pretreatment of giant reed (*Arundo donax L*).

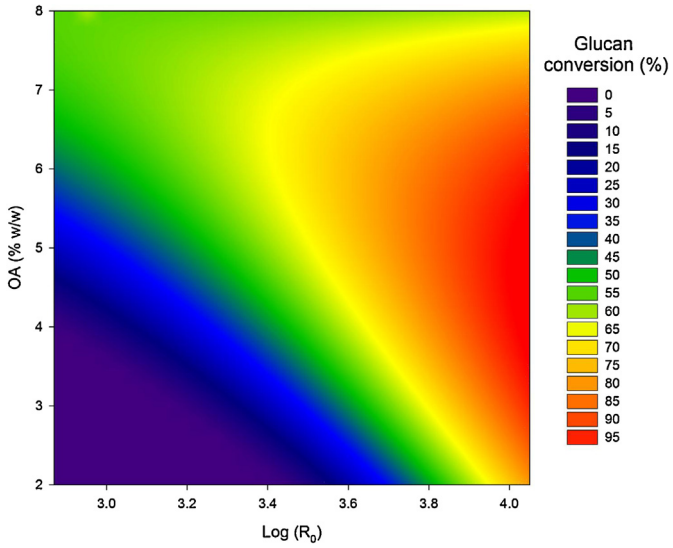

Fig. 2. Response of glucan conversion as a function of severity factor [Log(R_0)] versus oxalic oxalic acid concentration (OA%, w/w) after dilute-oxalic acid pretreatment of giant reed (*Arundo donax* L.).

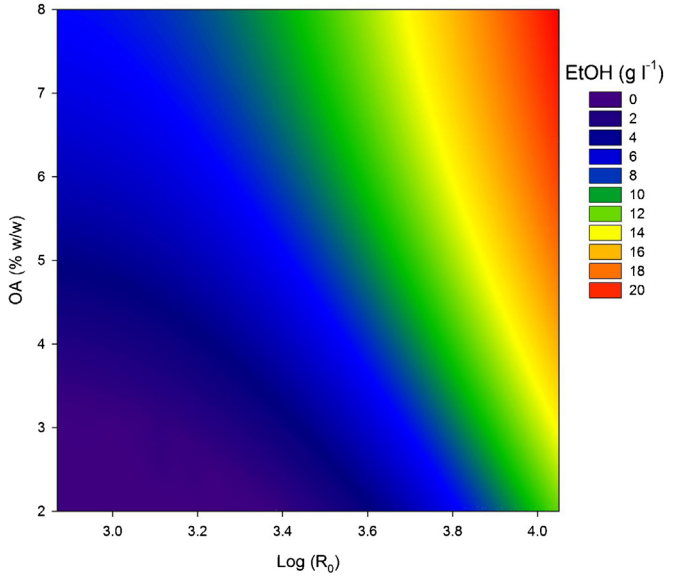

Fig. 3. Response of ethanol production as a function of severity factor [Log(R_0)] versus oxalic oxalic acid concentration (OA%, w/w) after dilute-oxalic acid pretreatment of giant reed (*Arundo donax* L.).

generated by the oxalic acid. This is strengthened by the release of acetic acid from the acetylated xylan of the hemicelluloses (Scordia et al., 2012). Li et al. (2007) found that a gradual increase in molecular weight was observed in pretreated aspen wood at a severity factor of 3.2–4.5 due to the cleavage of β-O-4′ linkages in the lignin reacting with cleavage of ether bonds, lignin fragmentation, hemicelluloses derived and degraded sugars, resulting in an increase in molecular size and a more heterogeneous lignin structure. Under harsher conditions, repolymerization of lignin seems to dominate and its precipitation on the fiber surface results in an increased lignin content (Li et al., 2007; Negro et al., 2003).

Moreover, the resistance of glucan and lignin compared to the xylan may be related to the recalcitrance of both cellulose and lignin under the pretreatment conditions used, as had been already reported on studies with dilute-OA pretreatment of different lignocellulosic feedstocks (Scordia et al., 2010; Lee et al., 2011; Kim et al., 2011).

Chandel et al. (2013) conducted a microscopy and spectroscopic research to study chemical change, at molecular level, of sugarcane bagasse after oxalic acid fiber expansion (OAFEX) pretreatment. The scanning electron microscopy, atomic force microscopy, X-ray diffraction, Raman spectroscopy, Fouriers transform infrared spectroscopy and near infrared spectroscopy techniques, showed that OAFEX pretreatment markedly disrupt hemicellulose and simultaneously re-localizes lignin moieties, aiding the increased exposure of cellulose to cellulases. After enzymatic hydrolysis a complete disruption of cellulose aggregates into glucose was observed, suggesting that re-localization of lignin moieties did not affect enzymatic action toward the saccharification of cellulose.

The predictive equations and the ANOVA results listed in Table 2 showed that the two independent variables significantly influenced the responses of residual structural carbohydrates and lignin during dilute-OA pretreatment with a coefficient of determination of 0.95–0.99 in reasonable agreement with the adjusted R^2 (0.92–0.98).

The solid fractions from dilute-OA-pretreatment were subjected to EH at a solid loading of 10% (w/v) after minimal hot water washing. A minimal liquid to solid ratio for washing (4:1%, w/w) is more meaningful for commercial applications in terms of energy and resource savings. It was shown that glucan conversion (%) increased with SF from the lowest to the highest level; the lower xylan content in the solid fraction pretreated at higher SF (~1%) compared to the lower SF (~13%), 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).

RSM analysis predicted a value of 60.6% glucan conversion in the central point of the design (SF 3.46 and 5% OA), which was carried out three times, similar to the experimental results (58.9 ± 1.53%), suggesting that large portion of the variance in the response was explained by the independent variables (R^2 0.90 and adjusted R^2 0.85). However, a lack-of-fit test suggested SF and OA acted independently in the responses of glucan conversion; a linear regression could better describe the experimental data than a second order approximation.

As for EH, SSF was carried out at a solid loading of 10% (w/v) and after minimal hot water washing. 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 giant reed substrate, as discussed above. As noted for enzymatic hydrolysis, SF showed a larger influence than OA on ethanol production.

The theoretical ethanol yield, calculated on the basis of the maximum ethanol production from the glucan content after pretreatment achieved a maximum value of 75.3% at 4.05 SF and 5.0% (w/w) OA concentration.

RSM analysis predicted a value of 6.0 g l^{-1} ethanol concentration in the central point of the design (SF 3.46 and 5% OA), which was carried out three times, similar to the experimental data (5.9 ± 0.21 g l^{-1}), suggesting that large portion of the variance in the response was explained by the independent variables (R^2 0.93 and adjusted R^2 0.91). However, a lack-of-fit test suggested SF and OA acted independently in the responses of ethanol production; a linear regression could better describe the experimental data than a second order approximation.

5. Conclusion

Giant reed showed its high potential for bioconversion to second generation bioethanol production. Dilute-OA pretreatment almost completely removed the xylan content from the raw material at high severities, while glucan and lignin content increased mostly for the resistance or recalcitrance of these structural compounds under the pretreatment condition employed. This is a notable aspect for subsequent glucan conversion and ethanol production.

Indeed, glucan conversion, ethanol production and ethanol yield attained maximum values at the most severe pretreatment condition employed in this study (4.05 SF and 5% OA). However, a yield reduction between glucan conversion (95%) and theoretical ethanol yield (75.3%) following the same pretreatment condition may be related to the different operation temperature for enzymes and yeast (50 and 30 °C, respectively) during SSF.

Important goals for future improvements in the second generation biotechnology include, among others, the exploitation and identification of new biomass resources (improved varieties or genetically modified), pretreatment technologies to fractionate the biomass into the three main components (hemicellulose, cellulose and lignin), reduction of cost, hydrolysis time and loading of enzymes, fermenting microorganisms improved for C5 and C6 sugars and resistant to high level of inhibitors. Finally, development of high efficient consolidated bioprocessing (CBP).

Acknowledgements

EdeniQ supported this work under USDA CRADA 08-RD-11111126-007. The authors gratefully acknowledge Frederick J. Matt of the Analytical Chemistry and Microscopy Laboratory of the USDA Forest Products Laboratory (FPL) for carrying out the structural carbohydrate determinations.

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