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Bioconversion of giant reed (*Arundo donax* L.) hemicellulose hydrolysate to ethanol by *Scheffersomyces stipitis* CBS6054

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ABSTRACT

The objective of this study was to evaluate the production of ethanol by *Scheffersomyces* (*Pichia*) *stipitis* CBS6054, a native xylose fermenting yeast, from sugars contained in the giant reed (*Arundo donax* L.) hemicellulosic hydrolysate.

A response surface methodology with two input parameters, severity factor and oxalic acid concentration ranging from 2.87 to 4.05 and from 2 to 8 (% w oxalic acid/w solid dry matter), respectively, was employed to minimize degradation products and maximize sugar release. However, at the optimum condition for sugar release (43.8 $g\,l^{-1}$), levels of toxic degradation products (acetic acid, furfural, HMF and phenolic compounds) were considered too high for yeast fermentation. The condition to minimize degradation products and maximize sugar yields was judged to be 2.87 severity factor and 5.0% oxalic acid concentration. At this condition 26.0 $g\,l^{-1}$ xylose, 5.0 $g\,l^{-1}$ glucose and 2.4 $g\,l^{-1}$ arabinose were recovered in giant reed hydrolysate fraction. Adjustment of pH to 5.0 with $Ca(OH)_2$ decreased xylose, glucose and acetic acid, 22%, 8% and 27% respectively. Increasing the initial pH from 5.0 to 5.5, 6.0 and 6.5, respectively, significantly improved the fermentability of the giant reed hemicelluloses hydrolysate; no fermentation was observed at pH 5.0 after 96 h, while 8.20 $g\,l^{-1}$ of ethanol was obtained at pH 6.0 after 48 h, with an ethanol yield of 0.33 (g_e/g_s) and a productivity of 0.17 $g\,l^{-1}\,h^{-1}$. The optimum pH of acid hydrolysate fermentation for ethanol production was 6.0–6.5.

1. Introduction

The European Union plans to increase the use of biofuels to at least 10% of gasoline and diesel transportation fuels by 2020 [1]. In the near future, much of the growth in bioethanol production is expected to come from second generation processes, which include the use of lignocellulosic plant materials, such as softwood, hardwood, agricultural residues and dedicated bioenergy crops. These raw materials contain both hexose and pentose sugars, however, xylose and other C5 sugars contained in hemicelluloses are much more difficult to ferment efficiently than glucose [2]. The most abundant hemicelluloses in nature are xylans and glucomannans. Xylans are usually available in enormous amounts as by-

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products of forest, pulp and paper industries, agriculture, agro-industries and dedicated bioenergy crops [3]. Among dedicated bioenergy crops, giant reed (*Arundo donax* L.) a perennial, herbaceous non-food crop, is one of the highest yielding species for biomass production in southern environment of Europe; it is able to grow well on marginal and nonagricultural lands, and its raw material has carbohydrate content similar to those of agricultural residues, such as corn stover and wheat straw [4]. Moreover, it has been recognized as very robust species with the potential to avoid competition with food crops for lands [5,6]. A previous study on the bioconversion of giant reed by simultaneous saccharification and fermentation of the cellulosic fraction to ethanol, following dilute oxalic acid pretreatment, has shown the potentiality of this species as feedstock for second generation bioethanol production [4]. However, to the best of our knowledge information on the fermentation of giant reed hemicellulose hydrolysate has not been reported to date.

Conversion of the hemicellulose fraction is essential to improve the overall yield of ethanol from lignocelluloses; nevertheless, efficient xylose fermenting microrganisms are required [2]. Among the xylose fermenting yeasts, *Scheffersomyces* (*Pichia*) *stipitis*, has been reported as the most promising for industrial application, because it ferments xylose to ethanol with high yields [7]. Moreover, genes from *S. stipitis* have been used to engineer xylose metabolism in *Saccharomyces cerevisiae* [8]. The natural recalcitrance of lignocellulosic cell walls to microbial and enzymatic degradation imposes a pretreatment step in order to catalyze the hydrolysis of hemicelluloses, decrystallize cellulose and displace lignin structure [4]. Several pretreatment technologies to fractionate biomass into its major components are available: alkaline or neutral pH methods remove hemicellulose as oligomers, while low pH methods, such as dilute acids pretreatment (either mineral or organic), remove hemicellulose as monomers, the ratios of which are dependent on the severity of the pretreatment (temperature, reaction time and acid concentration) [4,9]. Even though hydrolysis by dilute acids generates product streams rich in fermentable sugars, inhibitory compounds such as acetic acid, furfural, hydroxymethylfurfural, and various lignin derivatives are produced as well. Such compounds inhibit microbial metabolism, hindering the bioconversion of sugars into desired products [10]. The amount of inhibitory compounds released depends on the severity of the pretreatment, while the microbial inhibition depends on the type and concentration of the inhibiting compounds [11]. Furthermore, synergistic effects among different types of inhibitors can increase inhibition [11,12]. In order to reduce the concentrations of inhibitory compounds, and thus improve the bioconversion of sugars into desired products, different methods such as pH adjustment, active charcoal adsorption, ion-exchange resins adsorption and biological abatement, have been investigated [13,14].

A strategy to reduce the inhibitory concentration, and thus decrease the number of sub-processes, could be achieved optimizing the pretreatment process to minimize degradation products. In this respect a number of factors may play a role, including the recalcitrance and size of the feedstock, the intrinsic nature and the ratio of the acid loading for catalyst, the temperature and the reaction time.

In the present study we attempted this objective by using dilute oxalic acid (OA) pretreatment of giant reed. Dicarboxylic organic acids can hydrolyze β-(1,4)-bonds more selectively than sulfuric acid [15,16]. This can result in more efficient catalysis of the β-(1-4) linkage, while the relatively weaker ionization potential of oxalic relative to sulfuric acid can reduce subsequent dehydration reactions [4]. Furthermore, oxalic acid is less toxic to yeast and other microbes than acetic or sulfurous acids, does not inhibit glycolysis and does not produce noxious odors [17].

The aim of the present study was to evaluate the capacity of *S. stipitis* CBS6054, a native xylose fermenting yeast, to produce ethanol from the giant reed hemicellulosic hydrolysate that was obtained after a full factorial 2^n central composite design to minimize degradation products and maximize sugar release after dilute-OA pretreatment.

2. Materials and methods

2.1. Raw material

Giant reed biomass, clone Capo d'Orlando [18], was harvested in spring 2008 at the "Experimental Fields of the Facoltà di Agraria", University of Catania (10 m a.s.l., 37° 25′ N lat., 15° 30′ E long.) after one year growing season, according to the ordinary practices for perennial bioenergy crops. Moisture content of stems and leaves was stabilized to 4.0 ± 0.72%, according to the protocol provided by NREL (LAP-001) [19], milled to a particle size smaller than 2 cm (Wiley Mill Model No 2, Philadelphia, USA), homogenized and stored at room temperature until compositional analysis and pretreatment.

2.2. Pretreatment

Giant reed biomass and pretreatment solutions were placed in sealed stainless steel pressure vessels (approximately 1-L) which were mounted inside of a larger steam rotating pretreatment unit and heated externally via steam while rotating at the speed of 2 rpm.

A solution of 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).

Pretreatment temperature and residence time were combined into a single severity term through the Severity Factor (SF) formula [20]:

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

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.

After pretreatment, the vessels were placed in a water bath until the temperature reached the room value. The hydrolysates were separated by the solid materials by means of vacuum filter and stored at 4 °C. Dilute-OA-pretreatment of giant reed was optimized using a full factorial 2^n central

composite design with two input parameters with the aim to minimize the inhibitory compounds generated by hemicellulosic sugars and lignin degradations. The parameters studied were SF and OA concentration in the formation of monomeric sugars and degradation products, namely acetic acid, furfural, hydroxymethylfurfural and phenolic compounds, released in the hydrolysate fraction. 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

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

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 CBS6054, also known as *Pichia stipitis* CBS6054, was used to ferment the hemicelluloses hydrolysate. The new designation follows Kurtzman and Suzuki's (2010) taxonomic realignment [21].

Cells of *S. stipitis* CBS6054 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^{-1} 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^{-1}.

2.4. Preparation of hemicellulose hydrolysate fraction and inoculation

Giant reed hemicellulose hydrolysate was obtained after dilute-OA pretreatment. The initial pH (≈2) was increased to 5.0 with $Ca(OH)_2$, adjusted with NaOH to a final pH of 5.5, 6.0 and 6.5, centrifuged, filter sterilized, and then transferred to sterilized 125 ml Erlenmeyer flasks containing: 45 ml of hydrolysate (pH 5.0, 5.5, 6.0 or 6.5) supplemented with 3 ml of nutrients (5 g l^{-1} urea, 0.5 g l^{-1} $MgSO_4 \cdot 7H_20$ and 1 g l^{-1} KH_2PO_4) and 2 g l^{-1} of cell density as inoculum. Flasks were capped with cotton plugs and incubated at 30 °C, 150 rpm for 96 h. Samples were withdrawn after 0, 24, 48, 72 and 96 h to quantify the products consumed and generated. Before performing analysis of variance, results were previously evaluated according to Bartlett's test for homogeneity of variances. When statistical significance was observed, Student Newman Keuls test was carried out per $p \leq 0.05$ confidence level with percentage values previously arcsin $\sqrt{\%}$ transformed.

2.5. Analytical methods

Carbohydrate compositions of the original giant reed samples were analyzed according to the method of Davis (1998) [22]. Initially, samples were milled to pass 1.0 mm screen using

Table 1 – Experimental conditions and relative severity factor 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.

a Wiley mill (Thomas Scientific, Swedesboro, New Jersey) and vacuum dried at 45 °C. Primary hydrolysis of 40–60 mg subsamples was performed with 1.0 ml 72% (v/v) H_2SO_4 for 1 h at 30 °C. Hydrolysates were diluted to 4% (v/v) H_2SO_4 with distilled water, fucose was added as an internal standard, and a secondary hydrolysis performed for 1 h at 120 °C. Following filtration through 0.45 μm Teflon syringe filters (National Scientific, Lawrenceville, GA), 5 μL supernatant samples were measured by means of a high-performance anion exchange chromatography (ICS-3000, Dionex, Sunnyvale, California) with pulsed amperometric detection (HPAEC-PAD). The solids after filtration were dried in an oven at 105 °C until constant weight. After recording the dry weight the solid was transferred to a previously weighted crucible, which was allocated in a muffle furnace at 550 ± 50 °C for 8 h. The difference of weight was used to calculate the percentage of Klason lignin content. Ash content was measured before and after the two steps acid hydrolysis and referred as whole ash (before hydrolysis) and acid-insoluble lignin ash (AL ash), namely the only ash left after the primary and secondary step acid hydrolysis, respectively.

Xylose, glucose, arabinose, acetic acid, ethanol and xylitol concentration in both hydrolysate fractions and fermentation broths 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 HPX-87H column (Bio-rad Laboratories Inc., Hercules, CA) operating at 55 °C, 5 mM H_2SO_4 as mobile phase and 0.3 ml/min as flow rate. A subsequent mild post-acid hydrolysis (4% H_2SO_4, 121 °C, 1 h) was performed to estimate the monomeric forms in the hydrolysate fraction, and the resultant hydrolysate analyzed as above.

Hydroxymethylfurfural (HMF) and furfural in the hydrolysate fractions 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. Total phenol compounds were estimated colorimetrically by the Folin–Ciocalteu method via standard curve relating 760 nm adsorbance [23]. All samples were properly diluted and filtered through 0.22 μm spin-filter before analysis to remove the particle size.

3. Results and discussion

3.1. Sugar yield and inhibitory compounds

Giant reed raw material comprised the following (% of the total dry weight): 57.6% structural polysaccharides, 20.4% Klason lignin, and 5.9% ash, of which consisted of 1.7% acid-insoluble lignin ash. C6 sugars were mainly glucan (34.6%), while galactan and mannan made up of only a small fraction (less than 1%). Being a monocot crop, giant reed C5 sugars are represented by arabino-xylans with 1.81 and 20.4% total dry weight (tdw), respectively, which together with galactan, mannan and rhamnan constitute the hemicellulose fraction (Table 2). The ratio found between cellulose, hemicellulose and Klason lignin content is in agreement with a previous study on giant reed harvested in wild environment [4].

Table 2 – - Raw material composition of giant reed (*Arundo donax* L.).

Component	Dry matter (%)
Glucan	34.60 ± 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
K. Lignin	20.44 ± 0.07
Ash	5.90 ± 0.10
AL ash	1.67 ± 0.08

Mean values and standard deviation of two determinations.

Giant reed solid biomass was pretreated with dilute-OA, at a liquid to solid ratio of 4:1 (% w OA/w solid dry matter), in order to depolymerize the hemicelluloses contained in the raw material and to collect the hemicellulosic derived sugars in the hydrolysate for fermentation. A low liquid to solid ratio not only reduces thermal energy cost during pretreatment but also improves the sugar concentration in the hydrolysate reducing subsequent downstream ethanol separation and distillation cost [24].

During the pretreatment, xylan, arabinan, galactan and mannan were reduced from their levels in the raw material, while glucan and lignin contents increased. The resistance of glucan and lignin could 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 [9,24,25].

The response of monosaccharide yield released in the hydrolysate fraction ($g\,l^{-1}$) depended on the SF or the OA concentration during the pretreatment. Xylose was the major sugar detected in the hydrolysate accounting for 80% of the total sugars, in the average of all conditions tested. Glucose was found in significant amount at higher SF or OA exceeding 5%, accounting for 18% of the total sugars released, in the average of all conditions tested. This is in agreement with the recalcitrance of the glucan under the pretreatment conditions used. Arabinose was detected in negligible amounts (2%), while galactose, mannose and rhamnose were almost absent. A detailed two-dimensional contour plot was depicted to show total sugars release (xylose, glucose and arabinose) at different SF conditions versus the OA concentration (Fig. 1).

Maximum sugar yield was attained at SF ≈ 3.32 and 5% OA, corresponding to 43.8 $g\,l^{-1}$. Above that SF level, a further increase in OA concentration causes a decrease of total sugars release, while under that SF level, raising OA concentration leads an increase in total sugars release. The lowest monosaccharide concentration was found at the highest SF (4.05) and 5% OA, corresponding to 15.2 $g\,l^{-1}$. Low concentrations were also found at the lowest SF and OA; while in the former case sugar degradation products are formed due to high temperatures and long reaction time [10], the latter is related to the mild severities of the pretreatment, leading to low hydrolysis of the hemicellulose and consequently small sugars release.

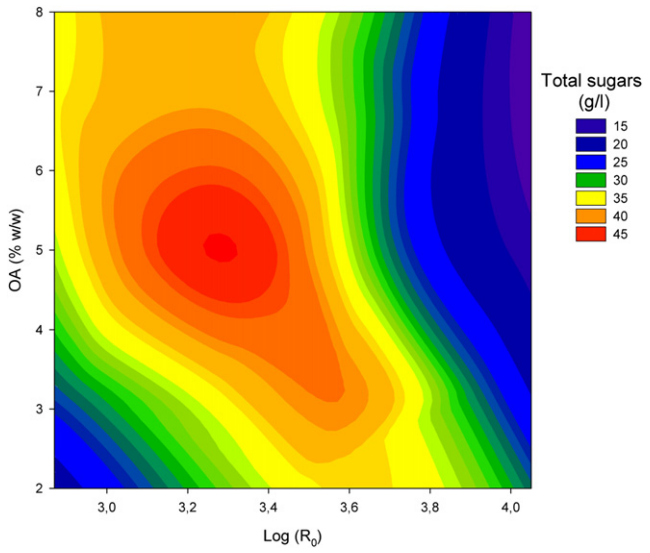

Fig. 1 – Response of total sugar as a function of severity factor [Log (R_0)] versus oxalic acid concentration (% w/w) after dilute-OA pretreatment of giant reed (*Arundo donax* L.).

The statistical analysis of the experimental data showed that the two independent variables significantly influenced the responses of total sugars during oxalic acid pretreatment of giant reed. Such influences could be successfully described by linear and quadratic models whose suitability of fit and statistical significance are illustrated in Table 3. A coefficient of determination of 0.93 in reasonable agreement with the adjusted R^2 (0.89) showed that large portion of the variance in the response was explained by the independent variables. ANOVA showed that SF is the most effective variable, followed by acid loading. When 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. *P*-values of the fitted coefficients of SF, as well as its interaction and the quadratic term showed statistical significance at 99% confidence level ($p \leq 0.001$) while OA concentration and its quadratic at 95% ($p \leq 0.05$).

The hydrolysis condition that resulted in the highest concentration of sugars is not likely suitable for fermentation due to the high content of degradation products, which after certain levels became to be very toxic for yeasts. Lohmeier-Vogel et al. (1998) [12] and Delgenes et al. (1996) [26] suggested that more that 10 g l^{-1} of acetic acid, 0.3 g l^{-1} of furfural and 0.9 g l^{-1} of hydroxymethylfurfural (HMF) are very problematic for yeasts during fermentation. This is particularly true for pentose fermenting yeasts as native *S. stipitis* or engineered *S. cerevisiae* when fermenting xylose [27,28]. Moreover, phenolic and other lignin-derived products have been recognized as the more toxic compounds [10]. In this respect, the pretreatment conditions have to be optimized for both high level of sugars and low level of inhibitors.

The main inhibitory compounds detected in giant reed hydrolysate were acetic acid from the release of acetyl groups from acetylated xylan, furfural from pentose degradation, HMF from hexose degradation, and phenolic compounds from lignin degradation. Acetic acid, furfural, HMF, and total phenolic compounds increased as the SF of the pretreatment rose, and increased with OA concentration when SF was held constant (Fig. 2a–d).

The lowest values for degradation products were found at 2.93 SF and 3.21% OA concentration with (g l^{-1}) 2.80, 0.61, 0.46 and 4.60 against the highest values at 4.05 SF and 5.0% OA with (g l^{-1}) 11.0, 7.57, 1.48 and 7.37 for acetic acid, furfural, HMF and total phenolic compounds, respectively. Acetic acid, furfural as well as HMF are strongly influenced by the SF, as they increased 2.9, 12.4 and 3.2-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 [10].

The relatively weak ionization potential of oxalic acid leaves cellulose almost unaffected. This results in a low degradation of glucose and consequently low concentrations of HMF even at the highest SF or OA concentrations. Increasing the OA concentration further at the highest level of SF decreases HMF, probably because it breaks-down to formic and/or levulinic acid. Total phenolic compounds increased 1.6-fold when SF rose from the lowest to the highest value and 1.2-fold when the OA was increased as well.

At the central point of the design (SF 3.46 and 5.0% OA), which was carried out three times, an average of (g l^{-1}) 8.0, 3.47, 1.21 and 6.26 for acetic acid, furfural, HMF and total phenolic compounds, respectively was achieved. The statistical analysis of the experimental data showed that the two independent variables significantly influenced the responses of degradation products during oxalic acid pretreatment of

Table 3 – Analysis of variance and fitting coefficients for total sugars 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
Regression	5	733.458	146.692	25.31	0.000	a_o	40.203	0.000
Residual	11	58.468	5.315			a_x	−7.094	0.000
Lack-of-fit	9	58.301	6.478	77.73	0.013	a_y	3.563	0.006
Pure error	2	0.167	0.083			a_{xy}	−9.032	0.001
Total	16	791.925				a_{xx}	−12.625	0.001
R^2	0.93	$R^2(adj)$	0.89			a_{yy}	−5.771	0.010

Degree of Freedom (DF); Sum of Square (SS); Adjusted Mean Square (Adj MS).

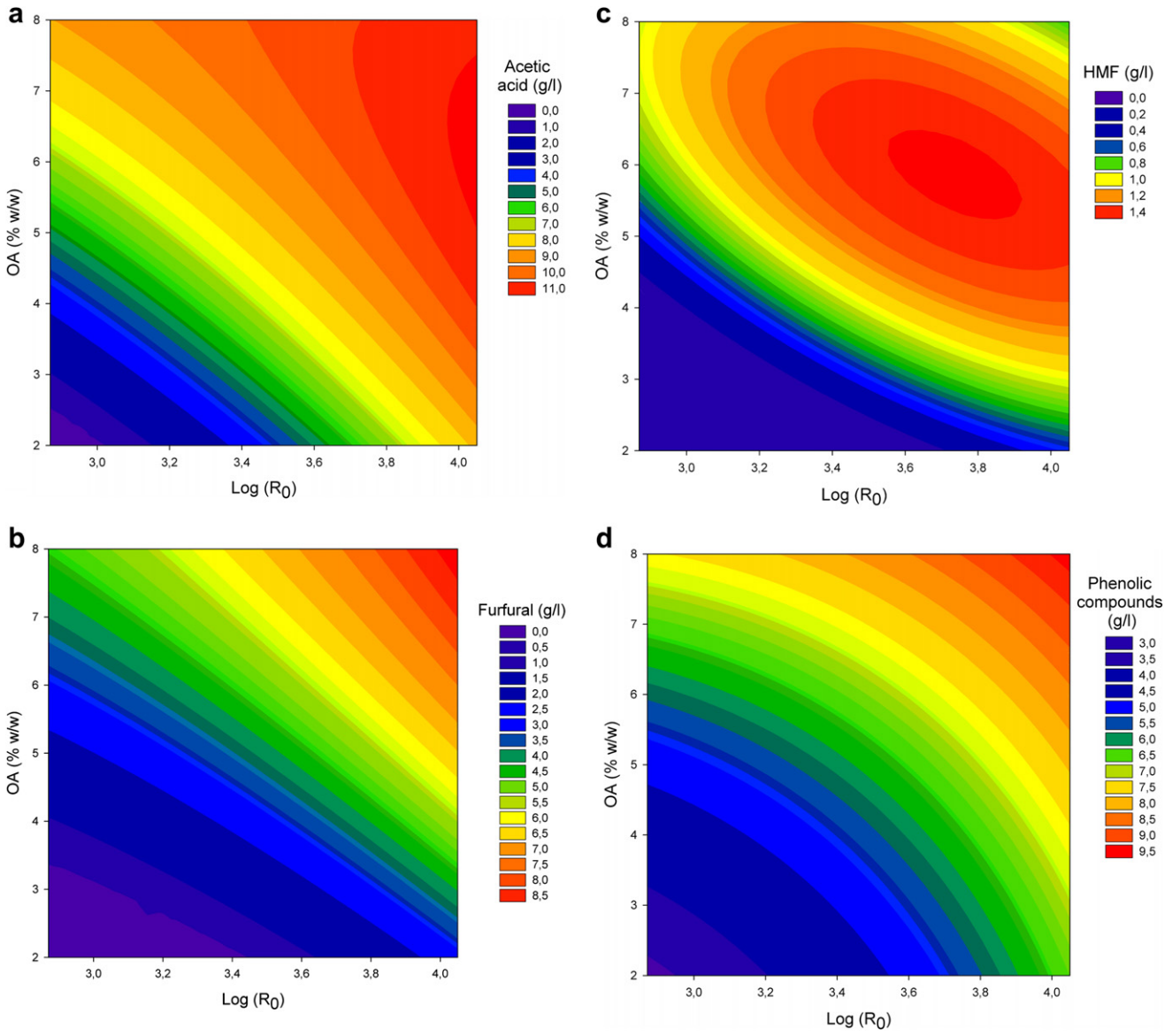

Fig. 2 – Response of (a) acetic acid, (b) furfural, (c) hydroxymethylfurfural and (d) phenolic compounds as a function of severity factor [Log (R_0)] versus oxalic acid concentration (% w/w) after dilute-OA pretreatment of giant reed (*Arundo donax* L.).

giant reed. ANOVA showed that SF was the dominant factor in obtaining high inhibitory compounds, followed by OA concentration (Table 4). P-values of the fitted coefficients of SF was zero and OA loading was close to zero ($p \leq 0.001$) while the quadratic terms were mainly not significant, therefore the responses of inhibitory compounds could be explained by a linear regression, suggesting SF and OA acted independently.

The optimal condition to maximize yield and minimize degradation products was judged to be 2.87 SF and 5.0% OA concentration. Sugar concentration was 26.0 g l^{-1}, 5.0 g $l^{-1}$and 2.4 g l^{-1} of xylose, glucose, and arabinose, respectively (Table 5). This pretreatment condition gave the source of hemicellulose hydrolysate for fermentation with *S. stipitis* CBS6054.

3.2. *Fermentation of hemicelluloses hydrolysate*

Sugars released into the hydrolysate in their monomeric forms consisted of xylose (78%), glucose (15%) and arabinose (7%) of the total sugars released. Galactose, mannose and rhamnose were detected in negligible amounts. A mild post-acid hydrolysis with H_2SO_4 (4%, 121 °C and 1 h) confirmed the presence of oligomers in the hydrolysate fraction. These were comprised of xylose, glucose and arabinose (9%, 17% and 6% respectively).

Acetic acid and total phenolic compounds accounted for 44.3%, furfural and HMF respectively for 5.6 and 5.7% of the total inhibitor compounds released in the giant reed hydrolysate fraction. The concentration was 4.80 g l^{-1} respectively for acetic acid and total phenolic compounds, 0.61 and

Table 4 – Analysis of variance and fitting coefficients for degradation products 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
Furfural								
Regression	5	74.2005	14.840	10.50	0.001	a_o	3.89505	0.000
Residual	11	15.5450	1.4132			a_x	2.21135	0.000
Lack-of-fit	9	15.5154	1.7239	116.48	0.091	a_y	3.07063	0.001
Pure error	2	0.0296	0.0148			a_{xy}	−0.08444	0.941
Total	16	89.7455				a_{xx}	0.36530	0.659
R^2	0.83	$R^2(adj)$	0.75			a_{yy}	−0.55952	0.573
Acetic acid								
Regression	5	105.789	21.1578	67.03	0.000	a_o	8.3421	0.000
Residual	11	3.472	0.3156			a_x	2.9375	0.000
Lack-of-fit	9	3.471	0.3858	528.49	0.127	a_y	3.0941	0.001
Pure error	2	0.0014	0.00073			a_{xy}	−1.9321	0.004
Total	16	109.261				a_{xx}	−0.2000	0.610
R^2	0.97	$R^2(adj)$	0.95			a_{yy}	−1.3530	0.013
HMF								
Regression	5	1.60813	0.321627	28.28	0.000	a_o	1.2182	0.000
Residual Error	11	0.12508	0.011371			a_x	0.3192	0.000
Lack-of-fit	9	0.11848	0.013165	3.99	0.216	a_y	0.4112	0.003
Pure error	2	0.00660	0.003299			a_{xy}	−0.1951	0.075
Total	16	1.73322				a_{xx}	−0.1224	0.118
R^2	0.93	$R^2(adj)$	0.90			a_{yy}	−0.2321	0.021
Phenolic compounds								
Regression	5	9.9061	1.98122	7.76	0.002	a_o	5.9614	0.000
Residual	11	2.8074	0.25521			a_x	0.88016	0.001
Lack-of-fit	9	2.7127	0.30141	6.37	0.143	a_y	1.05627	0.002
Pure error	2	0.0946	0.04732			a_{xy}	−0.13881	0.773
Total	16	12.7134				a_{xx}	0.11735	0.738
R^2	0.78	$R^2(adj)$	0.68			a_{yy}	0.06903	0.869

Degree of Freedom (DF); Sum of Square (SS); Adjusted Mean Square (Adj MS).

0.62 g l^{-1} for furfural and HMF, respectively (Table 5). Before strain inoculation, the pH of the hydrolysate fraction was increased to 5.0 with $Ca(OH)_2$ and then adjusted to a final pH of 5.5, 6.0 and 6.5 with NaOH. After adding $Ca(OH)_2$ a decrease in sugars and acetic acid concentration was observed, as had been elsewhere reported [29,30]. Xylose and glucose decreased 22% and 8%, respectively, while acetic acid decreased 27%.

At initial pH 5.0, acetic acid is largely un-dissociated; this permits diffusion into the cell cytoplasm, where it dissociates and decreases the intracellular pH below the physiological range [12], resulting in a long lag phase, cell growth inhibition and greatly decreased xylose consumption [31]. As for acetate, the mechanism by which furfural and HMF affect intracellular pH homeostasis remains poorly understood. One possibility may be through their actions on the proton-pumping plasma membrane ATPase, which is responsible for maintaining intracellular pH in yeast cell [12].

Table 5 – Concentration of monomeric sugars and inhibitory compounds in giant reed hydrolysate fraction obtained after dilute-OA pretreatment at SF 2.85 and 5% (w/w) OA concentration.

Hydrolysate fraction	Concentration (g l^{-1})
Xylose	26.0 ± 0.56
Glucose	5.00 ± 0.41
Arabinose	2.40 ± 0.34
Acetic acid	4.80 ± 0.43
Furfural	0.61 ± 0.09
HMF	0.62 ± 0.07
Phenolic compounds	4.80 ± 0.19

Mean values and standard deviation of two determinations.

The increase in the initial pH from 5.0 to 5.5, 6.0 and 6.5, respectively, significantly improved the fermentability of giant reed hemicellulose hydrolysate.

At pH 5.5, the ethanol concentration reached 6.70 g l^{-1} after 72 h fermentation, with an ethanol yield of 0.27 (g_e/g_s) and a productivity of 0.09 g l^{-1} h^{-1}. About 94% of total sugars and 74% of acetic acid were consumed. The final pH increased to 7.68 and the cell density to 5.20 g l^{-1}. Xylitol production was only 0.40 g l^{-1} (Fig. 3a). The increase in pH may be attributed to the consumption of acetic acid; a similar observation of an increase in pH during *S. stipitis* fermentation was made on sugarcane bagasse hemicellulose hydrolysate [29], wood hydrolysates [31] and corn stover hydrolysate [32]. Increasing the pH to 6.0 resulted in significant improvements. Ethanol concentration reached 8.20 g l^{-1} after 48 h fermentation, with an ethanol yield of 0.33 (g_e/g_s) and a productivity of 0.17 g l^{-1} h^{-1}. About 100% of sugars and 89% of acetic acid were taken up. The final pH increased to 7.73 and the cell density to 6.90 g l^{-1}. Only xylitol production was not significantly different than pH 5.5 (Fig. 3b).

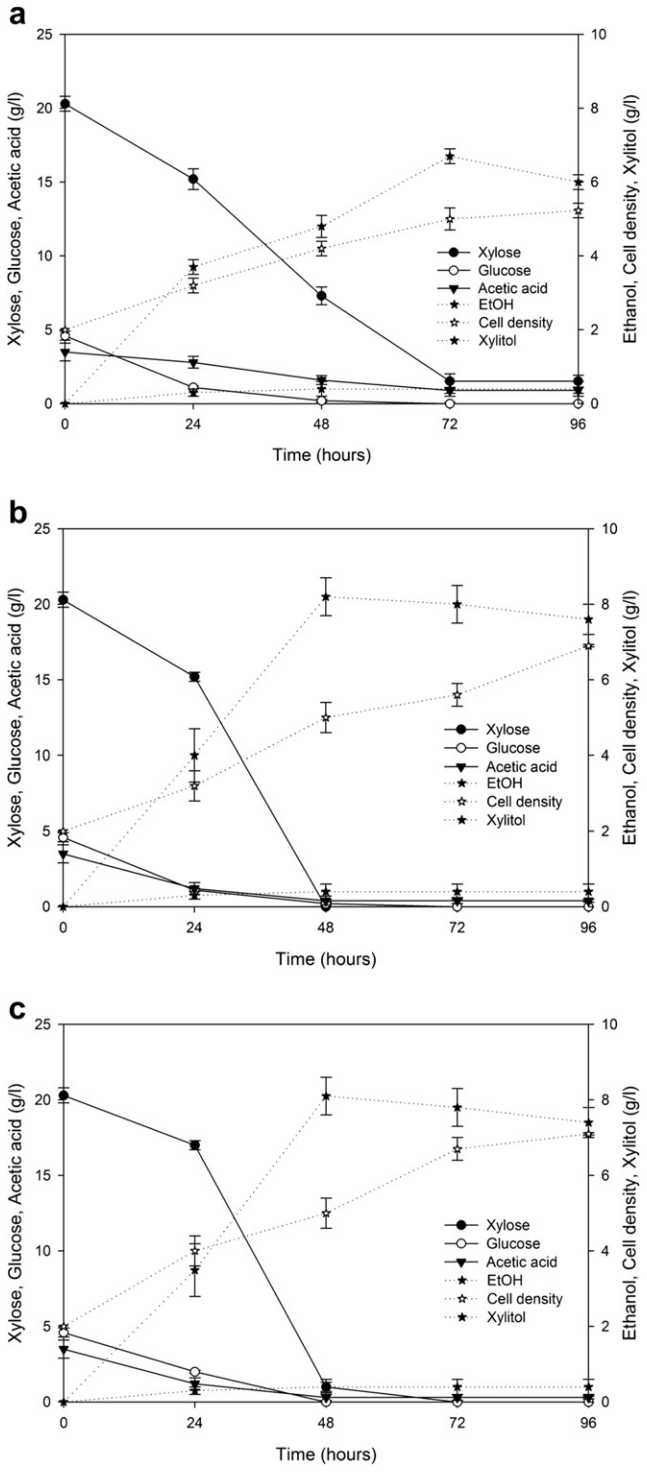

Fig. 3 – Hemicellulose hydrolysate fraction fermentation after dilute-OA pretreatment of giant reed (*Arundo donax* L.) using *Scheffersomyces stipitis* CBS6054 at 150 rpm, 30 °C and (a) pH 5.5, (b) pH 6.0 and (c) pH 6.5. Vertical bars represent the standard deviation of three replications.

At pH 6.5, a slight decrease in final ethanol concentration was observed compared to pH 6.0 experiments (Fig. 3c). However, ethanol concentration, fermentation time, ethanol yield, total sugars utilized, final pH and xylitol production were not significantly different. The specific cell growth rate, calculated at the fermentation time that gave the ethanol peak, was significantly higher at pH 6.5, 0.22 $g\,l^{-1}\,h^{-1}$, followed by pH 6.0 and pH 5.5, both undifferentiated with 0.11 and 0.09 $g\,l^{-1}\,h^{-1}$, respectively. Acetic acid consumption during fermentation was significantly higher at pH 6.5 followed by pH 6.0 and pH 5.5, while the substrate consumption rate was significantly higher and undifferentiated at pH 6.0 and 6.5, respect to pH 5.5 (Table 6).

Table 6 – Fermentation parameters at different pH using *Scheffersomyces stipitis* CBS6054 and giant reed hemicellulose hydrolysate after dilute-OA pretreatment.

Fermentation parameters	pH			
	5.0	5.5	6.0	6.5
EtOH concentration ($g\,l^{-1}$)	0.00	6.70b	8.20a	8.10a
Fermentation time (h)	96.0	72.0	48.0	48.0
EtOH yield (g_e/g_s)	0.00	0.27b	0.33a	0.33a
Total sugars utilized (%)	0.00	93.92b	100.0a	100.0a
Substrate consumption rate ($g\,l^{-1}\,h^{-1}$)	0.00	0.32b	0.52a	0.49a
Cell growth (96 h) ($g\,l^{-1}$)	2.00d	5.23c	6.90b	7.06a
Specific growth rate ($g\,l^{-1}\,h^{-1}$)	0.00	0.09b	0.11b	0.22a
Xylitol production ($g\,l^{-1}$)	0.00	0.40a	0.40a	0.40a
Acetic acid consumption (%)	0.00	74.28c	88.57b	91.42a
Final pH (96 h)	4.94b	7.68a	7.73a	7.76a

Within the same parameter, different letters indicate statistical significance at $p \leq 0.05$ confidence level, according to the SNK test. ANOVA was carried out excluding pH 5.0 values when equal to zero and fermentation time.

The yields achieved were comparable to those reported by several authors who used over liming detoxification and different microbial strains and feedstocks [33]. Chandel et al. (2010) [34] and Scordia et al. (2010) [9] obtained 0.36 g_e/g_s from *Saccharum spontaneum* hemicelluloses hydrolysate and *P. stipitis* NCIM3498 and CBS6054, respectively. Improved ethanol yields (0.41 g_e/g_s) were reached by Amartey and Jeffries (1996) [30] and Nigam (2001) [35] after adapting *P. stipitis* CBS6054 and NRRL Y-7124 by repeated subculturing to corn cob and wheat straw hydrolysates, respectively.

4. Conclusion

A central composite design was employed to maximize sugar yields and minimize degradation products. However, at the optimal condition for sugars release, acetic acid along with sugar losses and especially xylose degradation into furans exceeded the critical level for fermentation suggesting that other responses need to be optimized.

Employing mild conditions during pretreatment can obtain a hemicellulosic hydrolysate almost ready for fermentation. The results show that pH adjustment with $Ca(OH)_2$, and further adjustment to pH > 5.5, in giant reed hemicellulose hydrolysate, reduces the content of inhibitory compounds of the hydrolysate and thus can be fermented to ethanol by *S. stipitis* CBS6054. The optimum pH of acid hydrolysate fermentation for ethanol production was 6.0–6.5.

While giant reed has shown its potentiality as feedstock for second generation bioethanol production, outstanding bottlenecks to make bioethanol program successful at industrial scale include further studies for maximum recovery of pentoses with low level of inhibitor concentrations and the development of robust strains having the ability to produce ethanol from all the sugars available in lignocelluloses.

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