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FERMENTATION OF HEXOSES AND PENTOSES FROM HYDROLYZED SOYBEAN HULL INTO ETHANOL AND XYLITOL BY *Candida guilliermondii* BL 13

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Abstract – This work investigated the ability of a recently isolated strain of *Candida guilliermondii* to convert hexoses and pentoses obtained from acid-enzymatic soybean hull hydrolysates into ethanol and, in smaller amounts, into xylitol. Operational conditions and media formulation were optimized concerning ethanol production using experimental designs (Plackett-Burman and Central Composite Design). Results showed that *C. guilliermondii* BL 13 was capable of growing in non-supplemented, non-detoxified biomass hydrolysates, and the best culture conditions were determined to be 28 °C, pH 5.0, and 10^9 CFU mL^{-1} of inoculum size. Ethanol productivity reached 1.4 g L^{-1} h^{-1}, and maximal yields of 0.41 g g^{-1} were obtained, representing 80.4 % of the expected theoretical yields, whereas small amounts of xylitol were also produced. These results suggest that *C. guilliermondii* BL13 is a potentially useful yeast strain to be applied in second-generation ethanol production from lignocellulosic biomass based on its natural capacity to metabolize C-5 and C-6 sugars.

Keywords: Lignocellulosic Biomass; Fermentation; Ethanol production; Xylitol production; *Candida guilliermondii*.

INTRODUCTION

Lignocellulosic biomass materials represent an abundant and renewable source of carbohydrates that can be used to produce chemical specialties and biofuels of high-added value through biochemical processes, which is a promising alternative to oil-based products (Martínez, 2012). The production of ethanol from sugary substrates, such as sugarcane in Brazil and maize starch in the USA, is economically sound and has been the main technology to obtain this biofuel. However, there is a large amount of lignocellulosic biomass, consisting of cellulose, hemicellulose, and lignin, which could be used to obtain second-generation ethanol (Kuhad *et al*., 2011). Second-generation ethanol might contribute to reduce negative environmental impacts of fossil fuels, at the same time avoiding the *food versus fuel* competition for arable land (Cassman and Liska, 2007). However, in order to achieve these objectives, compatible costs of biomass preparation (i.e., the liberation of sugars from the lignocellulosic matrix), and the use of yeast strains capable of converting both hexoses and pentoses into ethanol, are necessary.

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Soybean (*Glycine max*) is the world´s most cultivated oilseed, with the United States of America and Brazil as its main producers, representing 35 % and 27 % of the global production share, respectively (USDA, 2015). Grain hulls represent the major by-product of the soybean processing industry, representing approximately 8 % to 10 % (mass fraction) of the whole seed (Gnanasambandan and Proctor, 1999). The insoluble carbohydrate fraction of soybean hull cell walls consists of 40 % of cellulose and 27 % of hemicellulose (Cassales *et al*., 2011). In a typical hydrolysis process, the hemicellulose is depolymerized into pentoses (predominantly xylose), whereas cellulose is mainly broken into glucose (Chen *et al*., 2011).

Considering that hemicellulose is the second most abundant polymer in lignocellulosic materials, the conversion of biomass to ethanol only becomes economically feasible if both pentoses and hexoses are converted into this alcohol. This conversion, in second-generation ethanol production, depends on the ability of microorganisms to ferment the different sugars resulting from hydrolysis. Pentoses cannot be fermented by wild-type strains of *Saccharomyces cerevisiae*, the most extensively used microorganism for ethanol production, whereas yeasts such as *Scheffersomyces (Pichia)*, *Candida*, and *Pachysolen* have been used for the xylose conversion. However, *Scheffersomyces* and *Pachysolen tannophilus,* for example, require a fine control of the supply of dissolved oxygen to produce ethanol or to assimilate nutrients and form biomass, which is generally difficult to control in the fermentation process (Lin *et al*., 2012; Fu and Peiris, 2008). Alternatively, other alcohols, such as xylitol and butanol, can be produced by some of these yeasts.

Xylitol is a natural five-carbon alcohol that has been highly valued for food and pharmaceutical applications because of its sweetening power, as a dental cavities reducer, and as insulin-independent carbohydrate source for diabetic-patient support (Mussatto *et al*., 2006). However, the production of xylitol by yeasts is highly depended on several parameters, such as the type of biomass used, the hydrolysis conditions, and fermentation parameters such as pH, substrate concentration, size of inoculum, and medium composition (Granström *et al*., 2001). Different species of *Candida* have been used for xylitol production. *Candida tropicalis* W103, for example, was able to convert xylose into xylitol under aerobic and anaerobic conditions in a synthetic medium (Cheng *et al*., 2014). In another study, *Candida guilliermondii* FTI 20037 was studied concerning the influence of the toxic compounds present in brewers spent grain hydrolysate on xylose-to-xylitol bioconversion (Mussatto and Roberto, 2008). The same strain was used to ferment rice straw hydrolysate with a high xylose concentration into xylitol (Mussatto and Roberto, 2004a). However, the literature concerning the use of *C. guilliermondii* for ethanol production on lignocellulosic hydrolysates as substrates is scarce. In a previous work, we tested *C. guillermondii* NRRL Y-2075, a culture-collection strain, to ferment an acid-hydrolyzed soybean hull medium, presenting high osmotic pressure (Schirmer-Michel *et al*., 2008). The strain was able to convert glucose into ethanol, showing reasonable yields ($Y_{P/S}$ = 0.46 g g^{-1}), but low productivities concerning this alcohol (Q_P = 0.24 g L^{-1} h^{-1}).

In this context, the present work aimed at evaluating the biotechnological bioconversion of sugars obtained from soybean hull hydrolysate (SHH) into ethanol, using a recently isolated strain of *Candida guilliermondii* (also known as *Meyerozyma guilliermondii*) in the fermentation process. This strain was isolated from a natural environment and has never been used in bioprocesses before. In order to optimize ethanol production, experimental designs were developed to define the important nutrients and the best environmental conditions for the fermentation using SHH as substrate. Xylitol production was also evaluated as a by-product of the fermentation, but its production was not optimized. To our knowledge, this is the first report in the literature describing the optimization of fermentation conditions for ethanol production using a *C. guilliermondii* strain on a lignocellulosic biomass hydrolysate.

MATERIALS AND METHODS

Microorganism and cell maintenance

Candida guilliermondii BL 13 was used in this research. This strain was isolated from environment-discharged piles of rotten rice hulls. This strain was selected because it has shown the ability to metabolize C-5 and C-6 sugars and to produce ethanol. The isolated strain was identified comparing the ITS1 and ITS4 amplicon DNA sequences with GenBank databases (access number JQ425356.1), producing 100 % of homology with *Candida guilliermondii* species. Stock cultures were maintained on YM agar containing (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 20. For long-term stock storage, cells were kept frozen at -20 °C in a suspension of 50 % glycerol (volume fraction) and 50 % of cultivation medium.

Soybean hull hydrolysate preparation

The Solae Company kindly supplied the soybean hulls (Esteio, Brazil). The composition of this biomass, including sugar-degradation products such as hydroxymethylfurfural and furfural, are described elsewhere (Cassales *et al*., 2011). The complete optimized procedure for acid-enzymatic hydrolysis of the soybean hulls was carried out following methodologies described by Hickert *et al*., (2014). The hulls were milled to a particle size smaller than 1 mm in diameter and the acid hydrolysate (SHH) was obtained by acid diluted hydrolysis of this material in an autoclave (Phoenix, Brazil)

at 1 atm and 121 °C, in a solution of 1 % (v/v) concentrated sulfuric acid (98 % v/v), using a solid-liquid proportion (m/m) of 1.0:8.8, and a reaction time of 40 min. This procedure was based on a previously published experimental design (Cassales *et al*., 2011). This hydrolysate was used in the Plackett-Burman and CCD designs.

After the acid hydrolysis, liquid and solid fractions were separated by filtration and the liquid fraction was vacuum-concentrated at 60 °C in order to increase the final sugar concentration. The pH was adjusted to 5.0 with NaOH. The solid fraction was washed with tap water until neutral pH was reached. Enzymatic hydrolysis of cellulose present in this solid fraction was performed using a cellulolytic enzyme complex produced by *Penicillium echinulatum* strain S1M29, which was obtained from the mutant strain 9A02S1 (Dillon *et al*., 2011). The enzymatic hydrolysis was carried out using a solid-liquid ratio (mass fraction, dry matter) of 1:20 in citrate phosphate buffer (pH 4.8) and 15 FPU g^{-1} dry matter of enzyme, incubated on an orbital shaker at 120 rpm, 50 ºC for 72 h (Hickert *et al*., 2014). Both fractions of hydrolysates were mixed, vacuum-concentrated at 70 °C in order to increase the sugar concentration, and autoclaved at 121 °C for 30 min. The amount of toxic compounds (inhibitors of microbial growth), formed during hydrolysis in the final soybean hull acid-enzymatic hydrolysate (SH-AEH), was determined by HPLC analysis. The pH was adjusted to 5.0 with solid pellets of NaOH. Both hydrolysates (SHH and SH-AEH) were used in the fermentations without any detoxification (removal of furans and acetic acid), apart from the loss during the final sterilization in the autoclave. Table 1 presents the complete composition of SHH and SH-AEH.

Inocula preparation and fermentation conditions in orbital shaker

Pre-inocula were prepared by seeding one isolated colony of yeast cells from plates into 250 mL Erlenmeyer flasks containing 50 mL of synthetic medium composed of (in g L^{-1}): yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10. Cultivations were carried out in an orbital shaker (Marconi MA 830, Brazil) at 180 rpm, 28 °C for 24 h and cells were subsequently recovered by centrifugation (3 000 *g*, 15 min). The cell pellet was washed with sterile distilled water, resuspended in culture medium and inoculated into culture flasks (10 % v/v).

The experiments using SHH as substrate were carried out in 250 mL Erlenmeyer flasks covered using cotton-wool plugs, containing 60 mL of SHH and incubated in an orbital shaker at 180 rpm for 72 h, with temperature varying according to the CCD (23 ºC to 33 ºC). Samples were collected during cultivation to determine biomass, ethanol, glycerol, and residual sugars concentrations in the broth. The experiments using SH-AEH as substrate were carried in 500 mL Erlenmeyer flasks, filled with 120 mL of this hydrolysate, and incubated in an orbital shaker under the best conditions obtained in the CCD (2.5).

Plackett-Burman design

Plackett-Burman (PB) design was used to screen and evaluate the effects of four complex nutrients (peptone, yeast extract, corn steep liquor, and Tween 80) on the bioconversion of SHH into ethanol by *C. guilliermondii* BL 13. The PB design consisted of randomized experiments of eight fermentations plus three replicates at the central point to evaluate the reproducibility of the experimental procedure. The variables were chosen because they would represent the addition of a nitrogen source (peptone); growth cofactors (yeast extract and corn steep liquor, which is an inexpensive nutrient source); and a source of unsaturated fatty acids (Tween 80) in order to avoid physiological impairments due to oxygen limitations. For each variable, the higher level (+1), the central point, and the lower level (-1) of the components were tested (Table 2). Student's t-test was performed to determine the significance of each variable. Significant positive effects were considered when the reported *p*-values were lower than 0.05.

Central composite design

A 2^3 full factorial central composite design (CCD), with four replicates at the central point and six axial points resulting in a total of eighteen experiments, was used to investigate the fermentation conditions of temperature, pH, and inoculum size for ethanol production by *C. guilliermondii* BL 13 on SHH. The coded and uncoded values are presented in Table 3 and were chosen based on the literature (Silva and Roberto, 2001; Cunha-Pereira *et al*., 2011; Mussatto and Roberto, 2004b). Experimental data were analyzed using a second-order response surface regression procedure.

Fermentation using acid-enzymatic soybean hull

In order to improve the conversion of total sugars present in the soybean hull into ethanol, an acid-enzymatic hydrolysate (SH-AEH) was used for fermentations under the conditions that were optimized in the CCD. The experimental procedure was identical to the cultivations described for SSH, except for the medium, which was SH-AEH (composition defined in the section "**Soybean hull hydrolysate preparation"**). These experiments were conducted in triplicates.

Analytical methods

Glucose, xylose, arabinose, ethanol, and xylitol concentrations were determined by HPLC (Shimadzu, Japan) equipped with a refractive index detector and

Table 1. Final composition of acid soybean hull hydrolysates (SHH) and acid-enzymatic soybean hull hydrolysates (SH-AEH) used for fermentations.

Component (g L^{-1})	SHH	SH-AEH
Glucose	17.75 ± 0.10	42.1 ± 0.69
Xylose	18.25 ± 0.22	15.3 ± 0.13
Arabinose	4.95 ± 0.52	5.83 ± 0.32
Furfural	0.02 ± 0.01	0.08 ± 0.02
HMF	0.07 ± 0.01	0.58 ± 0.01
Acid Acetic	1.10 ± 0.08	2.10 ± 0.12
Osmotic pressure (mOsm kg^{-1})	2,149	2,916

Table 2. Levels of the real and the codified values of independent variables tested in the Plackett-Burman design to test supplementation of SHH.

Independent variables (g L^{-1})	Range of levels		
	Low -1	Central point 0	High 1
Yeast Extract	0	2.0	4.0
Corn Steep Liquor	0	2.0	4.0
Peptone	0	2.5	5.0
Tween 80	0	0.2	0.4

Table 3. Levels of the real and the codified values of independent variables used in the central composite design to test fermentation on SHH.

Independent variables	Symbols	Range of levels				
		-1.68	-1	0	1	1.68
pH	X_1	4.16	4.5	5.0	5.5	5.84
Temperature (°C)	X_2	23	25	28	31	33
Inoculum (Log CFU mL^{-1})	X_3	5	6	7	8	9

Bio-Rad HPX-87H column (300 x 7.8 mm) using 5 mM sulfuric acid as eluent at 45 °C, flow rate of 0.6 mL min^{-1} and sample volumes of 20 µL. Furfural and HMF were determined by HPLC using an UV detector (at 276 nm) and a Nucleosil C18 column (250 x 4.6 mm) at 24 °C, using acetonitrile–water (2:8) containing 10 g L^{-1} acetic acid as eluent, and a flow rate of 1.1 mL min^{-1}.

Biomass was estimated as viable cells, using CFU (colony forming units, expressed as CFU mL^{-1}) plated in yeast morphology agar (YMA) medium. Results were statistically evaluated using the Statistica 7.0 software.

Calculation of fermentation parameters

Ethanol conversion yield ($Y_{P/S}$) was calculated as the ratio between the highest ethanol concentration produced and the sugars consumed (glucose and xylose; difference between the initial and residual sugar concentrations). Ethanol productivity (Q_P) was defined as the ratio between the highest ethanol concentration and the respective time of fermentation, which was at 12 h of fermentation in all experiments.

RESULTS AND DISCUSSION

Analysis of supplementation requirements using PB design

The components yeast extract, corn steep liquor, peptone, and Tween 80 were screened at the confidence level of 90 % based on their effects and the results for ethanol productivity and yields are shown in Table 4. All coefficients exhibited high *p*-values for ethanol yield and productivity (Table 5), implying that they had no significant effects on ethanol production, productivity, and yield. Similar results were observed in the fermentation of brewers spent grain hydrolysate by *Candida guilliermondii* FTI 20037 aiming at conversion of xylose to xylitol (Mussatto and Roberto, 2008). These authors reported that the hydrolysate supplementation with calcium chloride, ammonium sulfate, and rice bran extract did not show any significant effect on xylose-to-xylitol conversion.

The results obtained in this work suggest that essential nutrients are already present in the hydrolysate as this substrate is rich in nitrogen and has several minerals in its chemical composition (Cassales *et al*., 2011), fulfilling the requirements of the yeast metabolism. Thus, the addition of these nutrients could be eliminated for the subsequent experiments, at the same time showing that the use of soybean hull hydrolysate is an interesting substrate for fermentation processes because it does not require expensive supplementations.

Optimization of fermentation conditions

A central composite design (Table 6) was carried out to evaluate the effect of temperature, pH, and inoculum size on ethanol productivity and yield during the fermentation of SHH by *C. guilliermondii* BL 13. Four assays at the central

point were carried out to estimate the experimental error needed for the analysis of variance. The linear, quadratic, and interaction effects for ethanol productivity and yield were predicted using the analysis of variance (ANOVA) aiming at the lowest *p*-values for the regression model, shown in Table 7. A quadratic model was estimated and the less significant values were removed in order to obtain the lowest *p*-values for the regression model. The data provided by the model equations indicates that 74.20 % (R^2=0.7420) of the ethanol productivity, and 74.22 % (R^2=0.7422) of ethanol yield can be explained by the model, showing that the model provides good predictions of the experimental results. It was observed that the model is highly significant, both for ethanol productivity (p = 0.000004) and for yield (p = 0.003). The reduced models for ethanol productivity and yield are presented in equations (1) and (2), respectively:

where X_1, X_2, and X_3 are the coded values of pH, temperature, and inoculum size, respectively.

$$Q_P = 0.1361 - 0.0519X \tag{1}$$

$$Y_{P/S} = 0.3281 - 0.01X_1 + 0.0106X_2 - 0.0264X_2^2 - 0.0117X_3^2 - 0.0187X_2X_3 \tag{2}$$

The models suggest that, within the chosen range, ethanol productivity is independent of pH and temperature. The response surfaces described by the model (Table 7) to estimate ethanol productivity based on the independent variables temperature (X_2) and inoculum size (X_3) is shown in Figure 1. For ethanol productivity, the increase in inoculum size produced the greatest effect. Laopaiboon *et al*. (2007), who studied the effects of initial cell concentrations (10^6, 10^7, and 10^8 CFU mL^{-1}) on ethanol production in batch fermentations of *S. cerevisiae* in sweet sorghum juice, reported that, although the final ethanol concentration would remain the same, there would be a faster substrate consumption and increased ethanol productivity proportionally to higher initial cell concentrations.

The response surfaces obtained for ethanol yield described by the model are depicted in Figure 2a and 2b. *C. guilliermondii* BL 13 produced higher yields of ethanol

Table 4. Plackett-Burman design matrix showing the effects of SHH supplementation with yeast extract, corn steep liquor, peptone, and Tween 80 on ethanol productivity (Q_p) and yields ($Y_{P/S}$).

Assays	Yeast Extract ($g\ L^{-1}$)	Corn Steep Liquor ($g\ L^{-1}$)	Peptone ($g\ L^{-1}$)	Tween 80 ($g\ L^{-1}$)	$Y_{P/S}$ ($g\ g^{-1}$)	Q_P ($g\ L^{-1}\ h^{-1}$)
1	+1 (4)	-1 (0)	-1 (0)	+1 (0.4)	0.35	0.13
2	+1 (4)	+1 (4)	-1 (0)	-1 (0)	0.32	0.13
3	+1 (4)	+1 (4)	+1 (5)	-1 (0)	0.41	0.16
4	-1 (0)	+1 (4)	+1 (5)	+1 (0.4)	0.28	0.11
5	+1 (4)	-1 (0)	+1 (5)	+1 (0.4)	0.47	0.15
6	-1 (0)	+1 (4)	-1 (0)	+1 (0.4)	0.31	0.12
7	-1 (0)	-1 (0)	+1 (5)	-1 (0)	0.32	0.11
8	-1 (0)	-1 (0)	-1 (0)	-1 (0)	0.44	0.15
9	0 (2)	0 (2)	0 (2.5)	0 (0.2)	0.37	0.15
10	0 (2)	0 (2)	0 (2.5)	0 (0.2)	0.39	0.14
11	0 (2)	0 (2)	0 (2.5)	0 (0.2)	0.39	0.15

Table 5. Plackett-Burman effect estimated values of ethanol productivity (Q_p) and yields ($Y_{P/S}$).

Response variable	Factor	Effect	*p*-value
Q_P	Mean/Interaction	0.1373	0.0000
	Yeast Extract	0.0144	0.2887
	Corn Steep Liquor	-0.0036	0.7830
	Peptone	-0.0015	0.9087
	Tween 80	-0.0110	0.4081
$Y_{P/S}$	Mean/Interaction	0.3697	0.0000
	Yeast Extract	0.0483	0.2734
	Corn Steep Liquor	-0.0675	0.1429
	Peptone	0.0125	0.7657
	Tween 80	-0.0181	0.6669

Table 6. Central composite design to optimize fermentation conditions of SHH using *C. guilliermondii* BL 13 and the experimental values obtained for ethanol productivity (Q_P) and yields ($Y_{P/S}$).

Assay	pH	Temperature (°C)	Inoculum (CFU mL^{-1})	Q_P (g L^{-1} h^{-1})	$Y_{P/S}$ (g g^{-1})
1	-1 (4.5)	-1 (25)	-1 (10^6)	0.10	0.28
2	+1 (5.5)	-1 (25)	-1 (10^6)	0.10	0.26
3	-1 (4.5)	+1 (31)	-1 (10^6)	0.11	0.34
4	+1 (5.5)	+1 (31)	-1 (10^6)	0.11	0.33
5	-1 (4.5)	-1 (25)	+1 (10^8)	0.19	0.29
6	+1 (5.5)	-1 (25)	+1 (10^8)	0.18	0.26
7	-1 (4.5)	+1 (31)	+1 (10^8)	0.20	0.27
8	+1 (5.5)	+1 (31)	+1 (10^8)	0.20	0.26
9	-1.68 (4.16)	0 (28)	0 (10^7)	0.07	0.35
10	+1.68 (5.84)	0 (28)	0 (10^7)	0.11	0.36
11	0 (5.0)	-1.68 (23)	0 (10^7)	0.15	0.33
12	0 (5.0)	+1.68 (33)	0 (10^7)	0.21	0.30
13	0 (5.0)	0 (28)	-1.68 (10^5)	0.02	0.34
14	0 (5.0)	0 (28)	+1.68 (10^9)	0.24	0.30
15	0 (5.0)	0 (28)	0 (10^7)	0.12	0.25
16	0 (5.0)	0 (28)	0 (10^7)	0.12	0.27
17	0 (5.0)	0 (28)	0 (10^7)	0.12	0.27
18	0 (5.0)	0 (28)	0 (10^7)	0.12	0.30

Table 7. Regression coefficients estimated by ANOVA for ethanol productivity as a function of temperature (T), pH, and inoculum size (I) in the CCD.

Response Variable	Source	Coefficient	*p*-value	Regression		
				p-value	F	R^2
Q_P	Mean/Interaction	0.1361	0.0000	0.000004	46.014	0.7420
	Linear I	0.0519	0.000004			
$Y_{P/S}$	Mean/Interaction	0.3281	0.0000	0.003	6.9086	0.7422
	Linear pH	-0.0100	0.1097			
	Linear T	0.0106	0.0952			
	Quadratic T	-0.0264	0.0009			
	Quadratic I	-0.0117	0.0195			
	T x I	-0.0187	0.0297			

at pH below 4.5. The individual effects of the initial pH have been well documented in the literature for several yeast strains described within the genus *Candida*, and some strains of *C. guilliermondii* for xylitol production (Tamburini *et al*., 2015; Felipe *et al*., 1997), but not for ethanol. Regarding the variable temperature, Phisalaphong *et al*. (2006) studied its effect on ethanol fermentation by *S. cerevisiae* M30. The authors reported that ethanol yields were slightly higher when increasing the isothermal control from 30 ºC to 33 ºC, but above this range negative effects on cell metabolism and ethanol production were observed.

The interaction between temperature and inoculum size suggests that, at low inoculum size, higher temperatures have higher positive response and, inversely, increasing inoculum size, lower temperatures will be significantly better to increase ethanol yields. For instance, Silva and Roberto (2001) studied the combined effects of initial xylose concentration and inoculum size on xylitol production by *C. guilliermondii* growing in rice straw hydrolysate. The authors reported the optimum xylose concentration and inoculum size to be 82 and 3 g L^{-1}, respectively. The authors did not comment on ethanol production. Powchinda *et al*. (1997) demonstrated that, for *S. cerevisiae* fermentation of sugars, the increase in inoculum size increases ethanol yields up to a critical amount of cells because there is a better utilization of sugars by yeast. However, high cell densities can adversely affect mass and energy transfer in culture broths and increase cell-to-cell interactions, negatively affecting metabolism and ethanol production (Jarzebski *et al*., 1989; Laluce *et al*., 2009).

In this work, the highest ethanol productivity would be dependent of inoculum size (1×10^9 CFU mL^{-1}), independent of the pH and temperature, whereas best ethanol yields would be achieved for pH and inocula sizes around their minimal values and temperature between 28 °C and 29 °C. Although larger inoculum sizes would increase ethanol productivity, they would negatively affect ethanol yields, probably because of more energy being channeled for cell maintenance. Similar results were reported by Yamada *et al*., (2011), who evaluated the effect of the initial inoculum concentration on ethanol production from brown rice hydrolysate by cultures of *S. cerevisiae*.

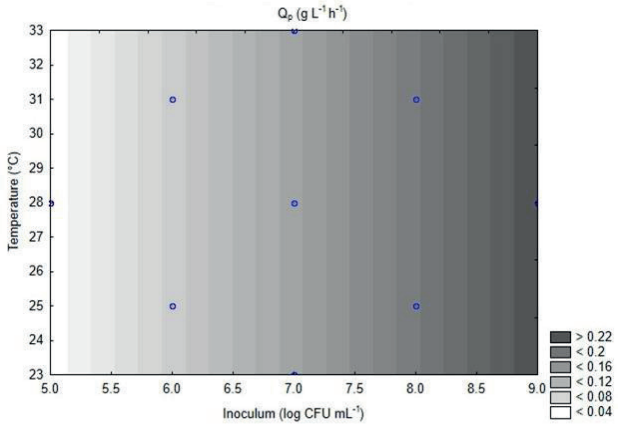

Figure 1. Response surface plots for ethanol productivity (Q_p, g L^{-1} h^{-1}) as a function of inoculum size and temperature.

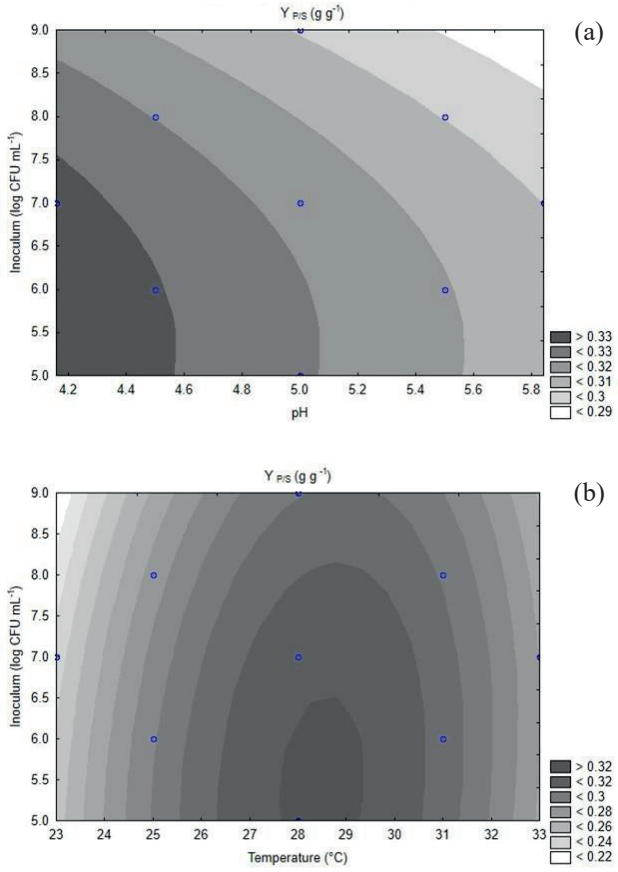

Figure 2. Response surface plots for ethanol yields ($Y_{P/S}$, g g^{-1}) as a function of (A) inoculum size and pH; and (B) inoculum size and temperature.

In order to validate the models predicted by the CCD, experiments were carried out in triplicates under the conditions representing the maximal response for ethanol productivity, which was 0.24 g L^{-1} h^{-1}, using an inoculum size of 1×10^9 CFU mL^{-1}, pH 5.0 and temperature of 28 °C. An experimental mean value of 0.21 ± 0.015 g L^{-1} h^{-1} ethanol productivity was obtained, close enough to the predicted value in the CCD, validating the response model.

Some authors reported the production of xylitol by several strains of *C. guilliermondii* (Mussatto *et al*., 2006; Silva and Roberto, 2001; Roberto *et al*., 1996). However, there are only few reports concerning ethanol production by this yeast, and in none of them the optimization of fermentation conditions was performed. This fact reflects the metabolic preference of this yeast to ferment xylose to xylitol instead of ethanol under specific conditions of oxygen limitation. Nevertheless, in this work, it was possible to show reasonable ethanol productivities (0.21 g L^{-1} h^{-1}) by *C. guilliermondii* BL 13. These results compared well with another report for this yeast, *C. guilliermondii* strain NRRL Y-2075, which showed ethanol productivities of 0.12 g L^{-1} h^{-1} when it was cultivated in non-detoxified concentrated SHH containing 0.8 g L^{-1} of glucose and 16 g L^{-1} of xylose in shaker flasks (Schirmer-Michel *et al*., 2008).

Ethanol production under optimal conditions in SH-AEH

Assuming that the results of *C. guilliermondii* BL 13 fermentation of SHH were interesting from the point of view of ethanol production, it was decided to test this yeast in a richer medium, containing sugars from the hemicellulose and cellulose fractions of soybean hull, which was designated SH-AEH, obtained by the enzymatic and acid hydrolyses of this biomass (section 2.2). The kinetics of sugar consumption, ethanol, and xylitol production for *C. guilliermondii* BL 13 growing in SH-AEH are shown in Figure 3. Fermentation of SH-AEH proceeded vigorously during the first 12 h, with all glucose consumed with a corresponding ethanol production of 16.8 g L^{-1} and a high ethanol productivity of 1.4 g L^{-1} h^{-1}, and yield of 0.41 g g^{-1}. Exponential growth lasted for about 6 to 12 h of cultivation reaching a maximum biomass formation of 1.45×10^{10} CFU

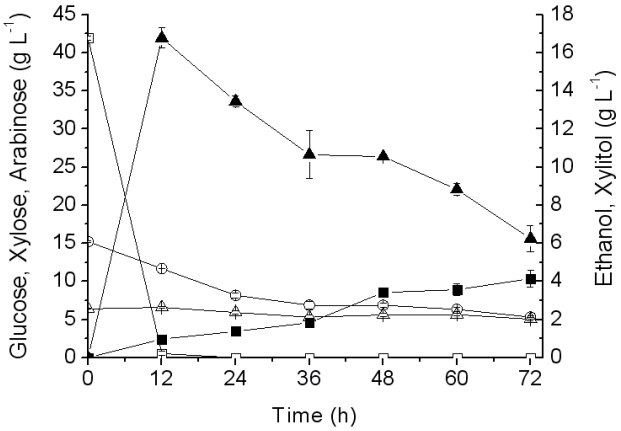

Figure 3. Time course of substrate consumption, ethanol, and xylitol production by *C. guilliermondii* BL13 cultivated on soybean hull acid-enzymatic hydrolysate. Experiments were carried out in orbital shaker at 28 °C, pH 5.0, 180 rpm. Glucose (□); xylose (○); arabinose (Δ); ethanol (▲); xylitol (■). Results are the mean of triplicates.

mL^{-1}. The xylitol productivity was 0.05 g L^{-1} h^{-1} and the xylose to xylitol conversion was 0.46 g g^{-1}. Efficiency of xylose utilization by this yeast was 67 % in SH-AEH. Comparatively, a xylose consumption rate of 69 % was observed for *Candida guilliermondii* FTI 20037 growing in semi-defined medium containing 85 g L^{-1} of xylose and supplemented with $(NH_4)_2SO_4$, $CaCl_2.2H_2O$, and 10 % of rice bran extract (Mussatto *et al*., 2006). Hickert *et al*., (2014) cultivated *S. cerevisiae, S. arborariae*, and *C. shehatae* in a similar soybean hull hydrolysate as used in this work, in a shaker, and reported that the tested yeasts lost their viability after 24 h of cultivation and only 50 % of glucose present in the medium was consumed, whereas xylose remained intact.

Because the culture conditions in a shaker, as conducted in this work, were under a limited supply of oxygen, it would be expected that the ethanol produced should be consumed along with xylose in diauxic kinetics after glucose depletion, a fact clearly observable in Figure 3. Similar fermentation profiles were reported for other yeast species cultivated in lignocellulosic biomass hydrolysates (Schirmer-Michel *et al*., 2008; Cunha-Pereira *et al*., 2011; Hickert *et al*., 2013). Using *C. guilliermondii* strain FTI 20037 in supplemented enzymatic hydrolysate of sugarcane bagasse, which was de-lignified and pretreated by hydrothermal processing, Silva et al. (2011) reported an ethanol production of 20.5 g L^{-1} in 28 h, in shaker cultures growing at 200 rpm and 30 ºC.

Roberto et al. (1996) evaluated xylitol production by *C. guilliermondii* strain FTI 20037 in rice straw hydrolysate. High initial cell density did not show a positive effect in this bioconversion, and increasing the initial cell density from 0.67 g L^{-1} to 2.41 g L^{-1} had a detrimental effect on the rate of xylose utilization and xylitol accumulation, resulting in xylitol yields of 0.47 g g^{-1} and 51 % efficiency of consumption in 72 h of cultivation. These results are consistent with results obtained in this work, where xylose was slowly converted into xylitol, even after all glucose had been metabolized, resulting in a yield of 0.46 g g^{-1} and conversion efficiency of 50 % in 72 h of cultivation.

The final composition of the enzymatic hydrolysate (SH-AEH) contained small amounts of HMF, furfural, and acetic acid, all considered toxic compounds for cell metabolism, and an osmotic pressure of 2 916 mOsm kg^{-1}. However, these factors did not influence yeast metabolism in this work. Comparatively, the growth of *C. guilliermondii* FTI 20037 was inhibited when cultivated in concentrated acid-hydrolyzed soybean hull showing osmotic pressures of 2 950 mOsm kg^{-1} and 2.8 g L^{-1} of toxic compounds combined (Schirmer-Michel *et al*., 2008).

Table 8 presents a general comparison of data for ethanol production using several agro industrial byproducts or residues, including starchy and sugary biomass for comparison, because the last-mentioned are the standard raw materials in the ethanol industry. Comparing the experiments of this work with those using starch, sweet

Table 8. Comparison among several feedstocks used for the fermentation process to obtain ethanol.

Feedstock	Pretreatment	Strains	Ethanol concentration (g L^{-1})	$Y_{P/S}$ (g g^{-1})	Q_P (g L^{-1} h^{-1})	Reference
Traditional sources of fermentation						
Starch/Sugar						
Sweet sorghum	-	*S. cerevisiae*	100	0.42	1.67	Laopaiboon *et al.* (2007)
Brown rice	-	*S. cerevisiae*	28.8	0.43	1.2	Yamada *et al.* (2011)
Cassava pulp (5 %)	Hydrothermal reaction and enzymatic hydrolysis	*S. cerevisiae* MT8-1/pGA11	18.6	0.50	0.77	Kosugi *et al.* (2009)
Second-generation ethanol						
Lignocellulose						
Soybean hull	Acid and enzymatic hydrolysis	*C. guilliermondii*	16.8	0.41	1.4	This work
Corn stalk	Steam exploded and enzymatic hydrolysis	*P. stipitis*	42.15	0.45	0.89	Yang *et al.* (2011)
Corn stover	Steam exploded and enzymatic hydrolysis	*S. cerevisiae*	43.21	0.47	0.72	Li *et al.* (2011)
Soybean hull	Acid hydrolysis	*C. guilliermondii*	5.78	0.53	0.24	Schirmer-Michel *et al.* (2008)
Rice straw	Acid hydrolysis	*P. stipitis*	18.7	0.37	0.39	Silva *et al.* (2010)
Sugarcane bagasse	OAFEX and Enzymatic hydrolysis	*C. shehatae* *S. cerevisiae*	4.83 6.6	0.28 0.46	0.20 0.47	Chandel *et al.* (2013)

sorghum, brown rice, and cassava pulp (Laopaiboon *et al*., 2007; Yamada *et al*., 2011; Kosugi *et al*., 2009), the results are promising, considering that SH-AEH was not supplemented (Laopaiboon *et al*., 2007; Yamada *et al*., 2011; Kosugi *et al*., 2009), and because genetically modified or adapted yeast strains were not used (Laopaiboon *et al*., 2007; Yamada *et al*., 2011; Kosugi *et al*., 2009) in the fermentation. Results for SH-AEH, compared with other lignocellulosic biomass such as corn stalk and stovers, soybean hull, rice straw, and sugarcane bagasse (Schirmer-Michel *et al*., 2008; Silva *et al*., 2011; Li *et al*., 2011; Yang *et al*., 2011; Chandel *et al*., 2013), show better ethanol volumetric productivity (1.4 g L^{-1} h^{-1}), even when pretreatment and enzymatic hydrolysis were used in combination with inhibitors-adapted or ethanol-tolerant strains (productivities not higher than 0.89 g L^{-1} h^{-1}) (Li *et al*., 2011; Yang *et al*., 2011). In the frame of the concept of biodistilleries, soybean hull could be considered a valuable resource.

CONCLUSION

In this work, the possibility was demonstrated of using SHH and SH-AEH as substrates for ethanol production without the addition of any synthetic nutrients to the media. When using SHH, ethanol productivity and yield could be improved by optimizing temperature, pH, and inoculum size. The strain *C. guilliermondii* BL 13 proved to be an efficient converter of hexoses to ethanol and, to a lesser extent, xylose to xylitol. Using the enzymatic hydrolysate under the optimal conditions resulted in reasonably high ethanol productivities. Therefore, further optimization of the culture conditions of *C. guilliermondii* BL 13 on lignocellulosic hydrolysates might be carried out in future experiments, at the same time testing it in co-cultures with other ethanogenic microorganisms, under different oxygen conditions. Results obtained in the present study are promising in terms of product yields and volumetric ethanol productivity to grant further scaling-up studies of such a process.

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