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Selection of inoculum size and *Saccharomyces cerevisiae* strain for ethanol production in simultaneous saccharification and fermentation (SSF) of sugar cane bagasse

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The aim of this work was to select an inoculum concentration and a *Saccharomyces cerevisiae* strain for ethanol production in the simultaneous saccharification and fermentation (SSF) of sugar cane bagasse. Three concentrations of inoculum (0.4, 4.0 and 8.0 g/L) and two strains of *S. cerevisiae* (UFPEDA 1238 and UFPEDA 1334) were used to ferment a culture medium containing glucose as the carbon source (100 g/L). Ethanol production was lower with 0.4 g/L inoculum, independent of the strain used. Experiments with 4.0 and 8.0 g/L inoculum showed no growth and higher ethanol production. Maximum ethanol concentration was obtained with UFPEDA 1238 and 8.0 g/L inoculum concentration. These conditions were selected for ethanol production from sugar cane bagasse in SSF. Maximum ethanol concentration was attained with SSF (28 g/L), enzymatic convertibility of cellulose (76%) and volumetric productivity (0.93 g/L h).

Key words: Ethanol, *Saccharomyces cerevisiae*, inoculum, efficiency.

INTRODUCTION

The amount of inoculum used is one of the most important factors that influences industrial fermentation, as well as lag phase duration, specific growth rate, biomass yield and the quality of the final product. Although the effect of a large quantity of inoculum on reducing the duration of the lag phase is well known, the relationship between product yield and inoculum has not been widely reported (Chen and Hashimoto, 1996).

Yeast inoculum size has a significant effect for ethanol production (Turhan et al., 2010). Gibbons and Westby (1986) reported that a 5% inoculum (v/v) resulted in rapid yeast and ethanol production. Higher inoculum showed no advantages. Tahir et al. (2010) using a different inoculum at 1-5% (v/v) observed that the amount of ethanol produced gradually increased with the increase in the inoculum. However, it was found that maximum ethanol production was achieved at 3% (v/v) inoculum. Results of Izmirlioglu and Demirci (2012) showed that 3%

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(v/v) inoculum was optimum for maximum ethanol concentration and production rate. Statistical analysis has revealed a significant effect of varying inoculum on ethanol production from sugarcane bagasse (Asgher et al., 2013).

Despite the reports on the increase in ethanol production by given inoculum, the initial biomass concentration was unclear. Therefore the aim of this work was to select an inoculum concentration (0.4, 4.0 and 8.0 g/L initial biomass concentration) and a *Saccharomyces cerevisiae* strain for ethanol production in the simultaneous saccharification and fermentation (SSF) of sugar cane bagasse.

MATERIALS AND METHODS

Microorganism and fermentation

Two industrial strains (UFPEDA 1238 and UFPEDA 1324) of *S. cerevisiae*, provided by the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco, Brazil, were used. These strains were maintained in a solid medium containing (in g/L) glucose (20), yeast extract (4), peptone (3) and agar (15), at pH 7.0. Inoculum was prepared by transferring cells of *S. cerevisiae* into a 500 mL flask containing 100 mL of the culture medium (20 g/L glucose, 3 g/L peptone, 4 g/L yeast extract; pH 7.0), and incubating this at 30°C for 12 h. Cells were harvested by filtration (0.45 µm filter), suspended in 10 mL sterilized water and used to inoculate the fermentation medium (Santos et al., 2012): 100 g/L glucose, 4 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4 and 0.75 g/L $MgSO_4.7H_2O$. The pH was adjusted to 5.5.

Ethanol production was carried out at 34°C without agitation and in duplicate, with 0.4 (A), 4 (B) and 8 (C) g/L of the inoculum in 250 mL flasks with a working volume of 100 mL. Samples were withdrawn after 12 h, filtered (0.45 µm filter), and the cell free supernatant was used to determine the glucose and ethanol by high performance liquid chromatography.

Steam-pretreated sugarcane bagasse and delignification

Sugarcane bagasse, pretreated by steam explosion at 200°C for 7 min on the pilot scale, was provided by the Department of Biotechnology of the Lorena Engineering College (University of Sao Paulo). A portion of the pretreated material was delignified at 100°C for 30 min and with 1% w/v NaOH. The delignification reaction took place in a 20 L rotary reactor fitted with mixing and heating systems (Regmed AUE/20, Regmed Indústria Técnica Ltda., Brazil), using a solid : liquid ratio of 1:10 w/v. The pre-treated and delignified bagasse was filtered through a cloth, and washed seven times to remove the remaining lignin and to reduce the pH. The pulp was dried at 50°C and stored for subsequent chemical analyses and simultaneous saccharification and fermentation.

Simultaneous saccharification and fermentation

Simultaneous saccharification and fermentation took place in 250 mL Erlenmeyer flasks, contained 90 mL of the fermentation medium (nutrients dissolved in a sodium citrate buffer at 50 mM and pH 4.8), 8 g of solids (pre-treated delignified bagasse) and enzyme loads of 10 FPU/g cellulose (Celluclast 1.5 L; 69.50 FPU and 13.70 CBU) and 5% v/v (of the volumetric Celluclast 1.5 L addition) β-glucosidase (Novozym 188; 1340 CBU) preparation, both from Novozymes A/S (Bagsværd, Denmark). The Erlenmeyer flasks were incubated in a rotary shaker at 50°C and 150 rpm. After a 6 h pre-hydrolysis, each Erlenmeyer flask was inoculated with yeast cells and incubated at 37°C and 80 rpm. The enzymatic convertibility of cellulose (ECC) was calculated based in ethanol concentration (Martín et al., 2008):

$$ECC = \frac{E_f - E_i}{C_i \times 0.57} \times 100\% \quad (1)$$

Where, E_f is the final ethanol concentration (g/L); E_i is the initial ethanol concentration (g/L); C_i, initial cellulose concentration (g/L). The factor 0.57 is the stoichiometric yield of ethanol from cellulose.

Analytical methods

Samples (10 mL) were filtered in a membrane (0.45 µm) for quantification of the microbial biomass by dry weight. The membrane was heated at 80°C for 24 h until constant weight. The content of the polysaccharides and lignin in the raw material was determined by two-step analytical acid hydrolysis, according to the analytical procedure validated for sugarcane bagasse by Gouveia et al. (2009).

Sugars, carboxylic acids, ethanol and furan aldehydes were quantified by HPLC (Agilent HP 1100, Germany) in an Aminex HPX-87H+ (Bio-Rad, Hercules, CA, USA) column at 60°C, using 5 mM H_2SO_4 at a flow rate of 0.6 mL/min as the mobile phase, and detected using an RI-detector (Agilent). All the experiments were conducted in duplicate. Statistical analysis was performed by analysis of variance (ANOVA) using the software Origin 6.0.

RESULTS AND DISCUSSION

Table 1 shows cell growth (∆X) obtained in fermentation medium by *S. cerevisiae* UFPEDA 1238 and UFPEDA 1324, using the three concentrations of the inoculum (0.4, 4 and 8 g/L). Higher growth was achieved in fermentations with inoculum A (0.4 g/L), independent of strain. Growth with inoculum B (4.0 g/L) and C (8.0 g/L) were much less. This was similar to when *S. cerevisiae* UFPEDA 1324 was used. On the other hand, growth with these inoculum and *S. cerevisiae* UFPEDA 1238 was less than that found for inoculum A, but higher than that found for *S. cerevisiae* UFPEDA 1334.

Glucose consumption (∆S) obtained using two strains and three concentrations of inoculum are shown in Table 1. Higher consumption coincided with lower growth. Glucose can be utilized in different ways by *S. cerevisiae*, depending on the presence of oxygen and carbon sources. In the absence of oxygen, alcoholic fermentation of sugars occurs.

Ethanol production was significantly enhanced as the amount of the inoculum was raised from 0.4 to 4 g/L for the industrial strains *S. cerevisiae* (Table 1): UFPEDA 1238 (95% increase) and UFPEDA 1324 (76% increase). On the other hand, when the amount of inoculum was raised from 4 to 8 g/L ethanol production using the UFPEDA 1324 strain did not increase (Table 1). When the amount of inoculum was raised from 4 to 8 g/L for *S.*

Table 1. Cell growth (ΔX), substrate consumption (ΔS), ethanol production (ΔP), yields ($Y_{X/S}$ and $Y_{P/S}$) and productivities (Q_P) in the fermentations with three inoculum and two industrial strains of *Saccharomyces cerevisiae.*

S. cerevisiae UFPEDA	Inoculum (g/L)	ΔX (g/L)	ΔS (g/L)	ΔP (g/L)	$Y_{X/S}$ (g/g)	$Y_{P/S}$ (g/g)	Q_P (g/L.h)
	0.4	2.31	41.52	11.33	0.06	0.28	1.04
1238	4.0	0.92	64.26	22.08	0.01	0.35	1.84
	8.0	0.57	86.24	37.16	0.00	0.43	3.10
	0.4	1.80	45.71	13.06	0.04	0.29	1.22
1324	4.0	0.12	49.86	22.07	0.00	0.46	1.91
	8.0	0.10	58.07	24.79	0.00	0.43	2.07

cerevisiae UFPEDA 1238, however, ethanol production increased by 64%. Maximum ethanol production (37.16 g/L) and productivity (3.10 g/L.h) were achieved with an initial biomass concentration of 8 g/L in the fermentation of glucose by *S. cerevisiae* UFPEDA 1238. This higher concentration of ethanol was obtained using a high concentration of glucose in the culture medium (100 g/L).

Growth (ΔX) and glucose consumption (ΔS) were used to calculate the fermentation yields ($Y_{X/S}$ and $Y_{P/S}$). Even in fermentations in which there was growth (inoculum A), yields in biomass ($Y_{X/S}$) were lower than 0.06 g/g. The higher yields in ethanol ($Y_{P/S}$) were observed for *S. cerevisiae* UFPEDA 1238 using inoculum C (0.43 g/g) and for *S. cerevisiae* UFPEDA 1324 using inoculum B (0.46 g/g). Productivity (Q_P) varied from 0.85 to 3.18 g/L.h for *S. cerevisiae* UFPEDA 1238 and 0.96 to 2.06 g/L.h for UFPEDA 1324.

Analysis of variance was performed on the productivity obtained from both strains and the three concentrations of inoculum (0.4, 4.0 and 8.0 g/L). These results were significantly different ($F = 36.30$; $\alpha = 0.05$). The analysis of variance between the 0.4 g/L inoculum for both strains, however, showed that the productivity of each was not significantly different ($F = 0.83$; $\alpha = 0.05$).

Likewise, the analysis of variance between the concentration 4.0 g/L inoculum for both strains showed that the productivity did not differ significantly ($F = 0.18$; $\alpha = 0.05$). On the other hand, the analysis of variance between 8.0 g/L inoculum for both strains showed that the productivity of each was significantly different ($F = 24.35$; $\alpha = 0.05$).

Simultaneous saccharification and fermentation of pretreated sugar cane bagasse by *S. cerevisiae* UFPEDA 1238 were carried out with 4 g/L inoculum. This concentration is equivalent to 8 g/L of glucose fermentation, since in the SSF the glucose concentration was about 50 g/L. Figure 1 shows ethanol production, glucose consumption and ECC. There was an accumulation of glucose during the first hours of the process, probably because the cells could not consume glucose at the rate that was released by the enzymes during the early phase of the SSF (Philippidis and Smith, 1995).

Maximum ethanol concentrations coincided with the disappearance of glucose. One reason for the lack of increase in ethanol, after 18 h, can be related to the residual cellulose which seems to be inaccessible to the enzyme (Philippidis and Smith, 1995). Maximum ethanol concentration, enzymatic convertibility of cellulose (ECC) and volumetric productivity were 28 g/L, 76% and 0.93 g/L.h, respectively. Initial cellulose concentration in the pretreated and delignified bagasse was 65.3 g/L. This value was used to calculate ECC according to Equation 1.

Wanderley et al. (2013) in separate hydrolysis and fermentation (SHF) of sugar cane bagasse using 4 g/L inoculum (for 50 g/L initial glucose concentration) reported about 0.9 g/L.h productivity and 24 g/L.h maximum ethanol concentration. However, total time taken was 120 h of hydrolysis and 24 h of fermentation. On other hand, Santos et al. (2012) in an SSF process of pretreated sugar cane bagasse using 1 g/L (18 g/L initial glucose concentration) inoculum found about 25 g/L ethanol, 0.7 g/L.h productivity and 72% of ECC, respectively. Increase of 12, 29 and 5% in ethanol production, productivity and ECC, respectively, was obtained in this work, as compared to that of Santos et al. (2012).

Conclusions

In this work, the concentration of 8.0 g/L of inoculum was selected when the culture medium containing 100 g/L glucose, using *S. cerevisiae* UFPEDA 1238 was used for ethanol production. These conditions favored the process, since it was faster and significantly increased productivity. This work showed that ethanol production from sugar cane bagasse can be increased by use of a high initial biomass concentration.

Conflict of Interests

The author(s) have not declared any conflict of interests.

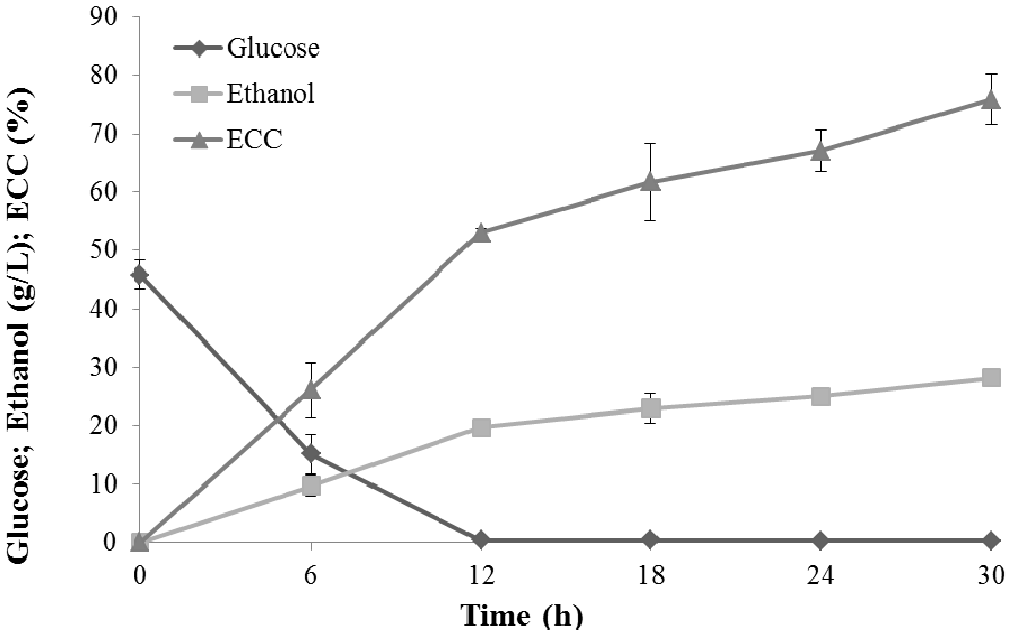

Figure 1. Time course of simultaneous saccharification and fermentation of pretreated sugar cane bagasse using 4 g/L of initial biomass concentration.

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