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Adaptation of *Scheffersomyces Stipitis* Cells as a Strategy to the Improvement of Ethanol Production from Sugarcane Bagasse Hemicellulosic Hydrolysate

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Sugarcane bagasse, a co-product from sugar mills in Brazil, is a biomass constituted by cellulose and hemicellulose, rich in carbohydrates like pentoses (xylose and arabinose) and hexose (glucose, manose and galactose). Acid hydrolysis with diluted H_2SO_4 has been used to the release of sugars, resulting also in the generation of by-products such as acetic acid, furfural, hydroxymethylfurfural, phenols that are potential fermentation inhibitors and affect the growth rate of Scheffersomyces stipitis. The inhibition may occur by the action of multiple factors, such as interference in enzymatic activities, since these compounds can act synergistically or alone. Detoxification strategies have been evaluated to remove these inhibitory compounds, but they usually result in sugar and hydrolysate volume loss besides adding costs to the process. Therefore, the adaptation techniques of microorganisms could be used as a strategy to increase the fermentability of hydrolysates. In this context, the current study aims to evaluate the adaptation of *Scheffersomyces stipitis* cells in different ratios (25 %, 50 %, 75 % and 100 %) of non-detoxified sugarcane bagasse hemicellulosic hydrolysate and subsequently to use this adapted cells in detoxified hydrolysate for ethanol production. Inoculum adaptation was accomplished by sequential transfer of culture samples to adaptation media containing concentrations of non-detoxified hydrolysate from 25 to 100 % (25, 50, 75 and 100 %). The adapted and non-adapted cells were cultured in hydrolysate detoxified by flocculation with vegetal polymer and supplemented at 30 °C, 200 rpm for 72h in a 125 mL Erlenmeyer flasks containing 50 mL medium. The cell adaptation technique improved the bioconversion of xylose to ethanol. During the fermentation of detoxified hydrolysate using adapted cells with 50% of non-detoxified hydrolysate it can be observed a xylose consumption (98 %) and ethanol production (14.97 g L^{-1}) with values of yield ($Y_{P/S}$), productivity (Q_P) and conversion efficiency of 0.33 g g^{-1}, 0.21 g $L^{-1}h^{-1}$ and 64.38 %, respectively. These values of $Y_{P/S}$ and Q_P were about 22 and 49% higher when compared with not-adapted cells. The adapted *S. stipitis* cells in 50 % of non-detoxified hydrolysate was able to ferment the detoxified sugarcane bagasse hemicellulosic hydrolysate improving ethanol production and presenting a good strategy to overcome the problems caused by the presence of toxic compounds in the hydrolysate.

1. Introduction

Ethanol is an useful biofuel that can be used as a substitute fuel to fossil biofuel. The ethanol production from plant biomass such as sugarcane bagasse, opens up new production possibilities with a sustainable basis. Lignocellulosic materials consist of the two major cellulose and hemicellulose fractions. Ethanol production from sugarcane bagasse requires the separation of its fractions through pre-treatment methods. The acid hydrolysis process is efficient and relatively competitive, despite its associated pollutants and waste products that inhibit subsequent fermentation (Dantas et al., 2013). Hemicellulosic hydrolysate containing the mixed sugars generated after pre-treatment and *Saccharomyces cerevisiae*, traditionally used for the ethanol production, does not efficiently ferment non-glucose sugars, and even worse, it cannot metabolize xylose which is the most dominant sugar in hemicellulose (Kim et al., 2013). In addition, inhibitory compounds to microbial metabolism are also released an/or generated during the pre-treatment. Among these compounds acetic acid, furfural, hydroxymethilfurural (HMF) and phenols stand

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out (Palmqvist and Hahn-Hangerdal 2000). Some detoxification techniques to remove these inhibitors are being evaluated however they usually result in loss of sugar and in loss of hydrolysate volume which reduces the efficiency of the fermentation, increasing process costs. Numerous efforts are being made to develop metabolic pathways capable of fermenting both pentoses and hexoses with satisfactory yields in *S. cerevisiae*, however, the methods are expensive, it is difficult to obtain stable mutants, the commercial availability of this process depends on recombinant microorganisms and their compliance with bio-security standards (Laluce et al., 2012; Dantas et al., 2013). Another alternative would be the adaptation of microorganisms naturally producing ethanol from pentoses such as *Scheffersomyces stipitis*, the most exhaustively investigated organisms and most efficient xylose fermenter. Toxic compounds present in the hemicellulosic hydrolysate reduce cell growth and diminish ethanol production (Palmqvist and Hahn-Hangerdal 2000; Nigam, 2001). However, from the economic viewpoint, the development of a robust microorganism that is able to ferment hydrolysate to ethanol more efficiently would be highly important and very useful (Huang et al., 2009). Besides, adaptation is a strategy that can result in displaying evolutionary processes in a cell population due to stimulations of changes in the cell. The increased tolerance of adapted strains to inhibitors shows characteristics as growth at higher hydrolysate concentrations; significantly reduced lag phases and a shorter process time (Laluce et al., 2012). In many studies, the growth of microorganism in the non-detoxified hydrolysate is common. In this work, with the intention of increasing ethanol productivity, the adaptation of *S. stipitis* in different ratios of non-detoxified hydrolysate as well as its subsequently growth in detoxified hydrolysate was evaluated

2. Methodology

2.1 Preparation of the sugarcane bagasse hemicellulosic hydrolysates

The pre-treatment of sugarcane bagasse was performed according reported by Pessoa Júnior et al. (1997). For inoculum adaptation, this hydrolysate was submitted to a pH adjustment to 5.5 with NaOH. For the fermentations, the hydrolysate was concentrated under vacuum a 4-fold. Afterwards, it was submitted to detoxification procedure with vegetal polymer (Chaud et al. 2012). Both hydrolysates were autoclaved at 111 °C, under 0.5 atm.

2.2 Microorganism and Cell Adaptation

The experiments were performed with *S. (Pichia) stipitis* NRRL Y-7124 maintained at 4 °C on malt-extract agar slants. Five inocula were prepared: 4 different levels of adaptation to sugarcane bagasse hemicellulose hydrolysate and 1 inoculum not adapted to the hydrolysate (control). Cell adaptation was accomplished by sequential transfer culture sample to media formulated with different ratios of non-concentrated and non-detoxified hydrolysate (14.48 g L^{-1} of xylose, 0.43 g L^{-1} of glucose; 1.53 g L^{-1} of arabinose, g L^{-1} of acetic acid, 0.0275 g L^{-1} of furfural, 0.0039 g L^{-1} of HMF and 4.55 g L^{-1} of total phenolic compounds, pH ajusted to 5.5). In the first level of adaptation (i 25 %) cells were grown in medium composed of 25 % hydrolysate and 75 % distilled water supplemented with nutrients $(NH_4)_2SO_4$ (2.0 g L^{-1}), $CaCl_2.2H_2O$ (0.1 g L^{-1}), peptone (5.0 g L^{-1}) and yeast extract (3.0 g L^{-1}) in Erlenmeyer flasks (125 mL), containing 50 mL medium each, incubated on a rotary shaker (200 rpm) at 30 °C for 24 h. Afterwards, the cells were recovered by centrifugation and resuspended in sterilized water. These cells were used for the second level of adaptation (i 50 %) in medium consisting of 50 % hydrolyzed and 50 % distilled water, and subsequently to the third level (i 75 %) in medium consisting of 75 % hydrolysed and 25 % distilled water, and fourth level (i 100 %), supplemented with the same nutrients and incubated in the same conditions previously employed. For non-adapted inoculum (control), a loopful of cells grown on a malt-extract agar slant was transferred to the medium containing xylose (30.0 g L^{-1}) and the same nutrients previously employed and incubated in the same conditions.

2.3 Medium and Fermentation Conditions

Cells from 5 inocula adapted to different ratios of hydrolysate were grown in concentrated and treated sugarcane bagasse hemicellulosic hydrolysate (49.45 g L^{-1} of xylose, 3.04 g L^{-1} of glucose; 6.02 g L^{-1} of arabinose, 3.0 g L^{-1} of acetic acid, 0.0048 g L^{-1} of furfural, 0.0079 g L^{-1} of HMF and 3.10 g L^{-1} of total phenolic) supplemented with the same nutrients of inocula. The media (50 mL) were placed in 125 mL Erlenmeyer flasks and fermented at 200 rpm, for 72 h, at 30 ºC with initial pH adjusted to 5.5, initial cell concentration 1.5 g L^{-1}. Experiments were carried out in duplicate and Tukey's test was used to evaluate the statistical significance of the inoculum adaptation.

2.4 Analytical methods

Sugars, acid, ethanol, hydroxymethilfurfural and furfural concentrations were determinated HPLC analysis. Total phenolic compounds concentration was estimated by ultraviolet spectroscopy at 280 nm (Gouveia et al. 2009). Cell growth was monitored by measuring absorbance at 600 nm. Yeast cells were stained with methylene blue (1%) and observed with a digital binocular light microscope equipped with digital camera (x 100 objective) for morphology analysis.

3. Results and Discussion

Detoxification of the hydrolysate with vegetal polymer is an important alternative due to its biodegradability, resulting in a less aggressive process to the environment. However, detoxification is partial and detoxified hydrolysate contains remnants concentrations of toxic compounds, mainly phenols. Believing in effective improvement of ethanol production, the cell adaptation was evaluated and Figure 1A shows the xylose consume during fermentations of sugarcane bagasse hemicellulosic hydrolysate by *S. (Pichia) stipitis* adapted to different ratios of non-detoxified hydrolysate. In all fermentations using adapted cells, around 95 % of xylose was consumed and significant difference between them as not observed ($p<0.05$, Tukey's test). However, in fermentation employing non-adapted inoculum, xylose consumption was only 50.78 %. Another important point is that adapted cells grew and consumed xylose more quickly than non-adapted cells, with xylose consumption rate around 96 % greater than the one observed with non-adapted inoculum (Table 1). Such behaviour may be related to the tolerance of adapted cells to the hydrolysate and the reduction of toxic effects by cell adaptation due to its uptake by cells and transformation into less toxic compounds. In this study, the assimilation of HMF in all adaptation levels evaluated was observed. These results agree with those reported by Martins et al. (2007) which observed faster utilization of sugars and conversion of the furaldehydes by xylose utilizing genetically engineered strain of *S. cerevisiae* adapted to sugarcane bagasse hydrolysate, indicating the ability of this yeast to tolerate and to transform the inhibitors present in lignocellulose hydrolysate.

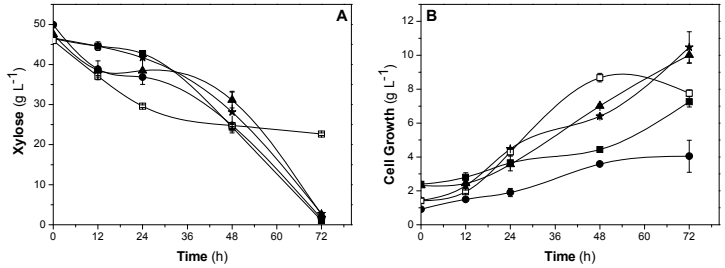

Figure 1: Xylose utilization (A) and cell growth (B) from fermentations of sugarcane bagasse hemicellulosic hydrolysate using cells of S. stipitis adapted to different ratios of hydrolysate: (●) i 25 %, (■) i 50 %, (▲) i 75 % e (*) i 100 % e non-adapted cells (□).

Table 1: Summaries of fermentation results from sugarcane bagasse hemicellulosic hydrolysate by adapted cells of S. stipitis adapted or non-adapted to hydrolysate

Fermentative Parameters*	i 25 %	i 50 %	i 75 %	i 100 %	non-adapted
Ethanol concentration ($g\ L^{-1}$)	2.32	14.97	12.13	12.63	10.41
Ethanol yield ($g_{eth}\ g_{xyl}^{-1}$)	0.254	0.329	0.268	0.287	0.450
Ethanol productivity ($g\ L^{-1}\ h^{-1}$)	0.171	0.208	0.168	0.175	0.140
Xylose consumption (%)	96.93	97.75	94.79	94.10	50.78
Xylose consumption rate ($g\ L^{-1}\ h^{-1}$)	0.671	0.631	0.626	0.609	0.324

* after 72h (maximum ethanol concentration)

Regarding the other sugars present in the hydrolysate, for all conditions evaluated, the consumption of 100% glucose and absence of arabinose assimilation were observed. The complete depletion of glucose is due to its small amount and because it is the sugar preferably used by most micro-organisms, being used to maintain cell growth in the first hours of fermentation, when xylose assimilation is poor. This behavior is

similar to the one reported for this same yeast grown in oat hulls hydrolysate (Chaud et al., 2012) and also for *Candida guilliermondii* grown in sorghum straw hemicellulosic hydrolysate (Sene et al., 2011). Co-fermentation of glucose and xylose by *S. stipitis* has been reported by other authors (Canilha et al., 2010; Gutiérrez-Rivera et al., 2011) however the utilization of arabinose is less reported. According to Verho et al. (2004), natural L-arabinose utilizing yeasts are very poorly characterized and as reported by Fonseca et al. (2007) although arabinose catabolism closely resembles that of xylose, the two sugars often make use of distinct uptake system. According to Agbogbo and Coward-Kelly (2008) *S. stipitis*, one of the most efficient pentoses-fermenting yeast does not have the ability to use arabinose. And Nigam (2001b) showed that *S. stipitis* can use arabinose in cell growth but not for ethanol production. Still among the compounds present in the hydrolysate and consumed during the fermentation, there is the acetic acid. In all conditions evaluated it was verified the consumption of 75 % of this acid by yeast, which was accomplished by raising the medium pH. However a direct relation between levels of inoculum adaptation and acetic acid assimilation was not observed (data not shown). This acid has been pointed as inhibitory to biomass growth and ethanol production depending on its concentration in the hydrolysate (Felipe et al., 1995). For the cell growth (Figure 1B), it was found that the higher the level of inoculum adaptation the greater the final cell concentration with significant difference ($p < 0.05$, Tukey's test) for cell growth only between conditions i 25 % and i 50 %. It was also observed to adapted inocula that, during fermentations, cells showed a trend to remain adhered to each other forming flocs, mainly from 72h fermentation in conditions i 25 % and i 50 % (Figure 2A and 2B). In conditions 75 % and i 100 % isolated cells or cells forming pseudohyphae were observed (Figure 2C and 2D). Such behavior was not observed in the fermentation performed with non-adapted cells (Figure 2E).

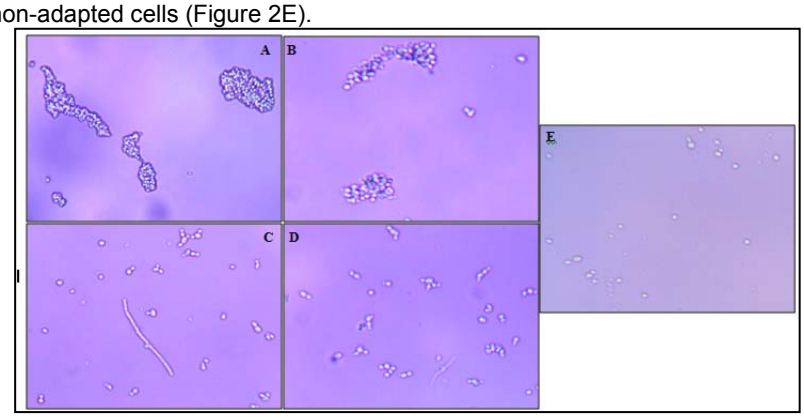

Figure 2: Cell morphology of S. stipitis after 72h fermentation of sugarcane bagasse hydrolysate. (A) i 25 %, (B) i 50 %, (C) i 75 %, (D) i 100 % and (E) non-adapted cells. Cells were photographed at 100× magnification.

According to Zhao and Bai (2009), there is no clear explanation yet regarding how the cells can survive due to inhibition. However, the morphology of the cells which stick together with high numbers of cell members (flocs) probably gives a possibility for the cells to work in concert and to protect each other against inhibitors. This means that the cells at the outer layer of the population are sacrificed and that they protect the other cells by converting toxic materials. The use of flocculent *S. stipitis* and *S. cerevisiae* in lignocellulosic hydrolysates was also evaluated by Schorr-Galindo et al. (2000), respectively, suggesting the use of flocculating yeast in non-detoxified hydrolysate for ethanol production. As reported by Zhao and Bai (2009), improvement in stress tolerance of the flocculating yeast can contribute to save energy consumption and for efficient biomass recovery for ethanol production. Figure 3A shows the ethanol production throughout the fermentation of sugarcane bagasse hemicellulosic hydrolysate by *S. stipitis* progressively adapted to hydrolysate. Contrary to the behaviour observed for xylose consumption, a significant difference was noted ($p < 0.05$, Tukey's test) for the ethanol production among condition i 50 % and the other conditions of inoculum adaptation evaluated. The highest ethanol production (14,97 g L^{-1}) was verified at 72 h of fermentation using inoculum adapted to hydrolysate at condition i 50 %, as can be found in Table 1 and Figure 3. This value was 21 % higher than the one observed for conditions i 25 %, i 50 %, i 100 % and 44 % higher than the one obtained for fermentation with non-adapted inoculum. In relation to fermentative parameters, it was observed that the use of inoculum adapted to hydrolysate at i 50 % condition results in an improvement of 22 % both $Y_{P/S}$ and Q_P in comparison with other levels of inocula adaptation (i 25%, i 75% and i 100%), highlighting that the productivity for condition i 50 % was 49

% higher than the one observed for non-adapted inoculum, corresponding to a conversion efficiency of 64.38 % (Table 1).

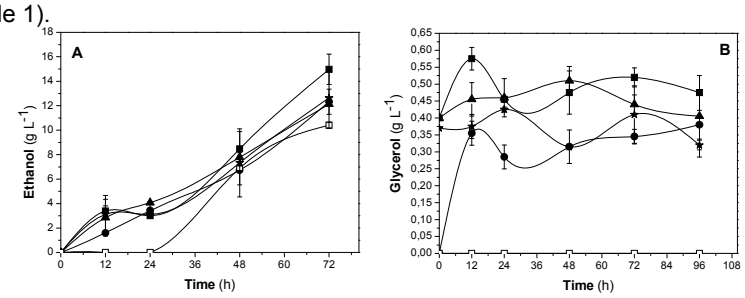

Figure 3: Ethanol (A) and glycerol (B) production during fermentations of sugarcane bagasse hemicellulosic hydrolysate by S. stipitis adapted to different ratios of hydrolysate: (●) i 25 %, (■) i 50 %, (▲) i 75 % e (*) i 100 % e non-adapted cells (□).

It is important to emphasize that the maximum productivity value was found in the fermentation with inoculum condition that provided the highest cell flocculation corroborating the reported by Zhao and Bai (2009) on improving the process of ethanol production with flocculating yeast. Other possible products of cell metabolism were evaluated. Xylitol formation was not detected in all evaluated conditions. Formic acid at concentrations around 0.25 g L^{-1} was detected at the end of all fermentations. In relation to glycerol, this was produced in all fermentations employing adapted inoculum (Figure 3B), but it was not observed a direct correlation between levels of adaptation and formation of this by-product of the metabolism of *S. stipitis*. Glycerol was most probably produced due to the adverse conditions of fermentation media caused by the presence of toxic compounds inherent to the hydrolysate. According to Vriesekoop et al. (2009), glycerol synthesis is associated with the regeneration of oxidized cofactors (NAD^+), playing a role in the control of the redox balance. On the other hand, the high production of glycerol by the yeast is also observed under conditions of osmotic stress, acting as a cell protector (Nevoigt and Stahl, 1997). Arruda and Felipe (2009) correlated the formation of glycerol with the defense mechanisms of *C. guilliermondii* due to the presence of toxic compounds in the sugarcane bagasse hemicellulosic hydrolysate used in its cultivation.

4. Conclusions

The adapted *S. stipitis* cells in 50 % of non-detoxified hydrolysate (i 50 %) was able to ferment the detoxified sugarcane bagasse hemicellulosic hydrolysate improving ethanol production and presenting a good methodology to overcome the problems caused by the presence of toxic compounds. Besides, in this condition, cells showed the characteristic of flocculation, which is an advantage for the development of technology to scaling-up the process for obtaining ethanol from lignocellulosic materials.

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