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Second-generation ethanol from non-detoxified sugarcane hydrolysate by a rotting wood isolated yeast strain

Suzana F. Bazoti[a,b], Simone Golunski[b], Diego Pereira Siqueira[b], Thamarys Scapini[b], Évelyn T. Barrilli[c], Diego Alex Mayer[a], Katharina O. Barros[d], Carlos A. Rosa[d], Boris U. Stambuk[e], Sérgio L. Alves Jr.[c], Alexsandra Valério[a], Débora de Oliveira[a], Helen Treichel[b,*]

[a] Federal University of Santa Catarina, Department of Chemical and Food Engineering, Brazil
[b] Federal University of Fronteira Sul – Campus Erechim – RS, Laboratory of Microbiology and Bioprocesses, Brazil
[c] Federal University of Fronteira Sul – Campus Chapecó – SC, Research Group of Enzymatic and Microbiological Processes, Brazil
[d] Federal University of Minas Gerais, Department of Microbiology, Brazil
[e] Federal University of Santa Catarina, Department of Biochemistry, Brazil

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ABSTRACT

This work aims to evaluate the production of second-generation ethanol from sugarcane bagasse hydrolysate without acetic acid (inhibitor) detoxification. Three isolated yeast strains from lignocellulosic materials were evaluated, and one strain (UFFS-CE-3.1.2), identified using large subunit rDNA sequences as *Wickerhamomyces* sp., showed satisfactory results in terms of ethanol production without acetic acid removal. A Plackett-Burman design was used to evaluate the influence of hydrolysate composition and nutrients supplementation in the fermentation medium for the second-generation ethanol production. Two fermentation kinetics were performed, with controlled pH at 5.5, or keeping the initial pH at 4.88. The fermentation conducted without pH adjustment and supplementation of nutrients reported the best result in terms of second-generation ethanol production. *Wickerhamomyces* sp., isolated as UFFS-CE-3.1.2, was considered promising in the production of second-generation ethanol by using crude (non-detoxified) sugarcane hydrolysate.

1. Introduction

The search for renewable energy sources has attained relevance in academic research and industry area. The interest in the production of compounds, with high value, leads the researchers to find new substrates from different organic residues such as lignocellulosic biomass, agricultural products such as cassava roots, cashew, hazelnut, apple, and other residues rich in nutrients, with the goal to obtain a wide range of products by biotechnology processes (Parmar and Rupasinghe, 2012; Thuy et al., 2017; Hosgun et al., 2017; Barros et al., 2017).

Among renewable energy sources, bioethanol contributes to the reduction of greenhouse gases (GHG) by the reuse of residues from sugarcane industry (sugarcane bagasse), expanding studies related to the lignocellulosic biomass hydrolysis, alcoholic fermentation, and new microorganism isolation (Siqueira et al., 2008; Rabelo et al., 2011). The processing of sugarcane bagasse consists in several steps: pre-treatment, solid-liquid separation, detoxification of the liquid fraction, cellulose and hemicellulose hydrolysis, fermentation of sugars, separation, products purification, and effluents treatment (Gámez et al., 2006; Balat et al., 2008; Sánchez and Cardona, 2008; Ramos et al., 2015; Fonseca et al., 2011; Zhang et al., 2013).

The second-generation ethanol is obtained by the fermentation of sugarcane bagasse (constituted by 50% of cellulose, 25% of hemicellulose and 25% of lignin) hydrolysates, after pre-treatment of the recalcitrant biomass, showing some advantages when compared with residues such as rice straw and wheat straw (Pandey et al., 2000; Fonseca et al., 2011). In addition to sugar production, in the pre-treatment step inhibitory compounds are formed, such as furfural and hydroxymethylfurfural (HMF) from the degradation of pentose and hexoses, respectively, and acetic acid from acetyl radical hydrolysis present in hemicellulose (Palmqvist and Hahn-Hägerdal, 1999). Therefore, the use of yeasts such as *Saccharomyces cerevisiae* can lead to a reduced fermentation yield (ethanol), due to the presence of inhibitors as acetic acid. An alternative to this problem is the use of new microorganisms with resistance to extreme conditions of pH, temperatures, substrate concentration and inhibitory compounds (Poleto et al., 2015).

In face of this scenario, in this work sugarcane hydrolysate was

* Corresponding author.
E-mail address: helentreichel@gmail.com (H. Treichel).

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used, without detoxification, to produce second-generation ethanol by using a yeast strain of a new species of *Wickerhamomyces* isolated from lignocellulosic sources.

2. Material and methods

The sugarcane bagasse hydrolysate was obtained after enzymatic hydrolysis using a Cellic CTec3 enzyme (Novozymes) of pre-treated bagasse by steam explosion, and kindly donated by Sugarcane Technology Center of São Paulo.

2.1. Yeast strains isolation

The yeast strains used in this work (UFFS-CE-3.1.2, UFFS-CE-3.6, and FLONA-CE-3.4) were isolated from rotting wood samples collected in the Park of Chapecó National Forest and in the *Campus* Chapecó of Federal University of Fronteira Sul, following the methodology described by Cadete et al. (2009). Samples were stored in sterile plastic bags and transported in isothermal boxes under refrigeration. One gram of each sample was inoculated in flaks with 20 mL synthetic minimal media (yeast nitrogen base 6.7 g/L, pH 5.0) containing 10 g/L of cellobiose and 0.2 g/L of chloramphenicol. Flasks were incubated at 25 °C on a shaker at 145 rpm until growth be detected by turbidity. After that, one loopful of each tube was streaked on plates containing the same media described above added with 20 g/L of agar. Plates were incubated at 25 °C, and the different yeast morphotypes were purified by repeated streaking on YMA plates (D-glucose 1%, peptone 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 2%) and preserved at −80 °C for later identification. The yeasts were characterized using standard methods (Kurtzman et al., 2011). Species identification were performed by analysis of the gene encoding the ITS-5.8S region and the D1/D2 variable domains of the large subunit of rRNA (White et al., 1990; O'Donnell, 1993; Kurtzman and Robnett, 1998; Lachance et al., 1999). The amplified DNA was concentrated, cleaned and sequenced in an ABI 3130 Genetic Analyzer automated sequencing system (Life Technologies, California, USA) using BigDye v3.1 and POP7 polymer. The sequences were assembled, edited, and aligned with the program MEGA6 (Tamura et al., 2013). The sequences obtained were compared with those included in the GenBank database using the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov).

2.2. Alcoholic fermentation procedure

Yeast maintenance was carried out in yeast extract peptone dextrose medium (YPD – 1% yeast extract, 2% peptone, 2% glucose and 2% agar). The pre-growth occurred at 30 °C for 72 h in a bacteriological oven and after inoculation in 10 mL of a liquid medium containing 1% yeast extract, 2% peptone and 2% glucose for 24 h at 30 °C. The inoculum was then poured into the hydrolysate previously sterilized and supplemented.

For the fermentation studies, the sugarcane bagasse hydrolysate was diluted 1:3 (v/v) with water, and 90 mL of this diluted hydrolysate in a 250 mL Erlenmeyer was autoclaved at 120 °C for 15 min. The fermentations were carried out in a shaker at 30 °C, 50 rpm in anaerobic condition. The diluted hydrolysate was supplemented with 4.5 g/L ammonium sulfate, 0.4 g/L magnesium sulfate, 5 g/L dibasic potassium phosphate, and 3 g/L yeast extract. Fermentation medium was sampled in 24, 48 and 72 h of fermentation for high-performance liquid chromatography (HPLC) analysis. For the kinetic reactions two fermentations conditions were evaluated, with the initial hydrolysate pH (4.88), or with an adjusted pH to 5.5 (adjusted with a 0.1 mol/L sodium hydroxide solution, slowly added under stirring). Samples were taken every 6 h for sugars, acetic acid and ethanol analyses in HPLC. Yeast strain growth, determined by gravimetric method, and HMF and furfural concentrations (determined as described below) were monitored every 24 h.

Table 1
Plackett-Burman design matrix of experimental design for screening medium composition.

	Level		
	(−1)	(0)	(+1)
X_1 (hydrolysate dilution v/v)	1:3	1:1	Crude
X_2 ($(NH_4)SO_4$) g/L	0.00	4.50	9.00
X_3 ($MgSO_4$) g/L	0.00	0.40	0.80
X_4 (KH_2PO_4) g/L	0.00	5.00	10.00
X_5 (yeast extract) g/L	0.00	3.00	6.00

The fermentation parameters optimization for ethanol production was investigated using a Plackett-Burman experimental design (Table 1), through the influence of the nutrients in the supplementation medium and the dilution factor of the sugarcane hydrolysate.

2.3. Analytical methodology

Glucose, xylose, acetic acid, cellobiose, arabinose, and ethanol concentrations during sugarcane bagasse hydrolysate fermentation were quantified by HPLC (Shimadzu chromatograph) equipped with a refractive index detector RID 10-A and an AMINEX® BIORAD HPX87H column. Samples of 20 μL were chromatographed at 45 °C, with 5 mM H_2SO_4 as mobile phase, and flow rate of 0.6 mL/min. Furfural and HMF compounds (samples of 20 μL) were determined using a PDA 10-A detector operated with a C18 column, eluted with 1:8 acetonitrile/water and 1% acetic acid, at 30 °C, and a flow rate of 0.8 mL/min. Before HPLC analyses, the samples were pre-filtered and diluted appropriately. The compounds concentration was determined by using calibration curves for each compound (data not shown).

3. Results and discussion

3.1. Yeast identification

Three yeast strains (UFFS-CE-3.1.2, UFFS-CE-3.6, and FLONA-CE-3.4) were isolated from the rotting wood samples. Analyses of the sequences of the D1/D2 domains of the rRNA gene showed that strains UFFS-CE-3.6, and FLONA-CE-3.4 belong to the species *Candida pseudointermedia* (Nakase et al., 1976). Strain UFFS-CE-3.6 presented a sequence identical to the type species of *C. intermedia* (GenBank accession number U44816), while the sequence of strain FLONA-CE-3.4 differs from this species by two base substitutions. Strain UFFS-CE-3.1.2 represents a new species of *Wickerhamomyces* (GenBank accession numbers MF538579 and MF538580), and this specie differs by one base in the sequences of the D1/D2 domains from an undescribed species isolated in rotten rice straw in Thailand deposited in GenBank (*Candida* sp. KU-Xs34, accession number AB557798), suggesting that these strains represent the same species. However, the Brazilian strain differs by 12 nucleotide substitutions and 24 indels in the sequences of the ITS region. These nucleotide differences in the ITS sequences suggest that the Brazilian and the Thailand strains could represent two different species. Analyses of the large subunit rDNA sequences of strain UFFS-CE-3.1.2 show that this yeast is phylogeneticly related to *C. yuanshanica*