

Cell Stress Profile During Metabolite Production by *Saccharomyces cerevisiae* Catanduva-1 in Sugarcane Wort

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Article history: Received: 29 June 2017; revised: 17 September 2017; accepted: 17 September 2017. Available online: 25 January 2018. DOI: <http://dx.doi.org/10.17807/orbital.v10i1.1029>

Abstract: During ethanol production, yeasts are subjected to a number of stress factors - among them high substrate concentrations and high temperatures - while displaying an ability to adapt to the adverse conditions found during fermentation. This study evaluated the metabolic profile exhibited by *Saccharomyces cerevisiae* Catanduva-1 during fermentation. Yeast cells were cultured in 2% YPD medium, incubated in a shaker at 30 °C and 250 rpm for 10 h, and subsequently centrifuged and washed in saline solution for biomass harvesting. Fermentation experiments were performed at 18, 22, and 25°Bx sterilized at 120 °C for 20 min. Biomass was reinoculated into wort and incubated in a shaker at 30 °C and 40 °C at 250 rpm. Evaluation of fermentation parameters revealed better fermentative performance at 30 °C for all substrate concentrations investigated. Under the test conditions, cellular stress was observed at 40 °C. A superior ethanol production profile was obtained at 22°Bx and 30 °C. Glycerol accumulation was highest at 25°Bx and 40 °C.

Keywords: ethanol; glycerol; fermentation

1. INTRODUCTION

The search for renewable sources of energy, aimed at minimizing the environmental impact of fossil fuels, has stimulated the use of ethanol as a clean energy source, in several countries [1]. In Brazil, the second-largest ethanol producer, cane juice or molasses are employed as raw materials for ethanol generation. Of more than 395 Brazilian ethanol plants currently in operation, 190 employ selected starter yeasts, particularly the Catanduva-1, Pedra-2, Santa Adélia, and Barra Grande strains [2], notable for their high fermentation capacity and viability in fermentation vats [3].

A study conducted in the midwestern state of Mato Grosso do Sul during the 2009-2010 harvest revealed a predominance of Catanduva-1, Fleischmann, Pedra-2, and Barra Grande strains cultured in sugarcane juice-based wort at 15 °Bx and 30 °C, with superior fermentation performance for Catanduva-1 (88% cell viability and 16% ethanol yield, v:v) and Pedra-2 (82% cell viability and 14% ethanol yield, v:v). Cell biomass yield (15 mg/mL),

cell viability (66%), and ethanol yield (5%, v:v) were lowest for the Fleischmann strain [4].

Mato Grosso do Sul is a major supplier of sugar to India (28.54% of imports of the product), Bangladesh (12.57%), Russia (12.28%), and the United Arab Emirates (10.74%) and of ethanol to the Netherlands (61.74%) and the United Kingdom (38.26%) [5]. Pedra-2, Santa Adélia, Reg Instan, and, most notably, Catanduva-1 are the strains most often employed (whether uncombined or in consortium) for ethanol generation in plants located in the south of São Paulo state [6].

Substrate concentration and strain selection are crucial to fermentation effectiveness [7]. Yeasts metabolize sucrose in the presence of invertase, yielding glucose and fructose monomers. Permeases carry glucose into the intracellular medium and, through a sequence of biochemical reactions, the carbon source is converted into adenosine triphosphate (ATP), essential to physiological reactions involved in cell growth, nutrient transport, viability rates, excretion, and other mechanisms [8].

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In the absence of O_2 , glucose enters the glycolytic pathway, where it is mediated by specific enzymes,

yielding pyruvate, acetaldehyde, and ethanol [9] (Figure 1).

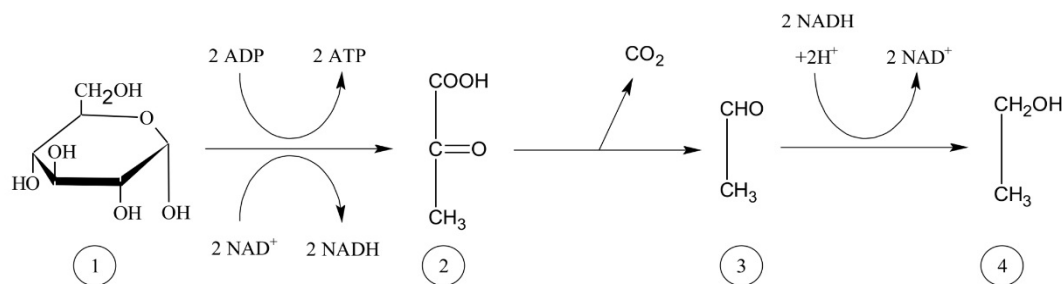


Figure 1. Fermentation stages: glucose (1), pyruvate (2), acetaldehyde (3), and (4) ethanol.

During fermentation *S. cerevisiae* undergoes cell stress, sequentially or simultaneously caused by high ethanol concentration, high temperature, osmotic pressure, contaminants, acid treatment, and low pH, among other stressors [7]. Temperature variation has a crucial effect on the metabolism of fermentation agents, influencing growth, fermentation capacity, metabolite production, and cell viability, while altering the fermentation medium with acidic compounds, soluble solids, and ethanol concentration [10].

In response to fermentative stress, yeasts produce glycerol, in a self-protective mechanism. At the industrial scale, substrate concentration and temperature are intentionally modified to cause cell stress. In *S. cerevisiae*, glycerol is metabolized in response to hyperosmolarity, which is regulated by the chymase-activated high-osmolarity glycerol (HOG) pathway [1].

Because glycerol is a fermentation inhibitor, increasing the amount of this metabolite in wort limits the generation of ethanol [11]. Glycerol production by fermentation, however, has a number of applications in the cosmetic, paint, food, pharmaceutical, textile, and paper and pulp industries [12, 13].

All 22 ethanol plants operating in Mato Grosso do Sul employ selected yeasts, including Catanduva-1, to ferment sugarcane juice. Locally, typically high summer temperatures can compromise operating conditions in alcohol plants, leading to overheating of fermentation vats, with consequent losses in ethanol yield. Monitoring operating conditions and fermentation parameters have proved decisive in ensuring yeast performance and optimizing ethanol generation.

Given the relevance of further elucidating how

yeasts can adapt to adverse conditions in the fermentative environment, the purpose of this study was to evaluate the fermentation profile of *S. cerevisiae* Catanduva-1 under fermentative stress and investigate metabolite production by this strain under a range of culture conditions.

2. MATERIAL AND METHODS

Collection and preparation of the substrate

Wort, obtained from an ethanol plant, was stored in sterilized flasks and transported at 4 °C to the Biotechnology, Biochemistry and Biotransformation Laboratory of the Centro de Estudos em Recursos Naturais (CERNA) at the Universidade Estadual de Mato Grosso do Sul, in Dourados county, Mato Grosso do Sul, where the material was filtered through cotton to remove impurities and subsequently through filter paper. With the aid of a beaker and a saccharimeter, the wort samples were adjusted to 18, 22 and 25°Bx. Sucrose was employed to concentrate the samples and distilled water was used to dilute them

Strain

Catanduva-1 is a commercial strain of *S. cerevisiae* often used as a fermentation starter in Brazilian ethanol plants. The strain investigated was obtained from LNF Latin American Applied Biotechnology (Bento Gonçalves, RS, Brazil).

Growth conditions

To develop cell biomass, a classical YPD culture medium at 2% was used, containing 1.0%

(w:v) yeast extract, 1.0% (w:v) peptone, and 2.0% (w:v) glucose. The medium was adjusted to pH 5.0 with 1 N hydrochloric acid and sterilized in an autoclave at 120 °C for 20 min. The flasks containing the yeast cells remained in a shaker at 30 °C for 10 h. Grown cells were collected by centrifugation (800 g, 20 min), suspended, and washed three times in 0.85% sterile saline solution, resulting in a biomass concentration of 10 mg mL⁻¹.

Fermentation experiment

Fermentation employed sterilized saccharine sorghum broth and sugarcane wort as the substrates, each at 18, 22, and 25°Bx, without pH correction, in 125 mL Erlenmeyer flasks, where a 50 mL volume of substrate was incubated at 30 or 40 °C in a shaker at 250 rpm. At 10, 20, and 40 h of fermentation, aliquots were collected for measurement of fermentation parameters.

Analytical methods

Cell density was measured by spectrophotometry at 570 nm and correlated with a dry weight vs. optical density calibration curve [4]. Cell viability was determined by methylene blue staining [14]. Carbohydrate analysis was performed using a colorimetric assay with 2-hydroxy-3,5-dinitrobenzoic acid [15]. An enzymatic kit for triglyceride analysis was employed to measure glycerol concentration [16]. Ethanol was analyzed on a CG 3900 gas chromatograph equipped with a flame ionization detector (Varian), using a 30 m-long fused silica capillary column (ZB-5). The chromatographic conditions adopted were 1 µL injection volume, 1:20 displacement ratio, 90 °C oven temperature, and 240 °C detector injector temperature. The samples were filtered with a 0.22 µm ultrafilter.

3. RESULTS AND DISCUSSION

Fermentation capacity proved lower at higher temperatures and greater Brix concentrations (Table 1). At 22°Bx, cell biomass yield was highest (13.0 mg mL⁻¹), with an 85% viability rate and a high conversion of substrate at 40 h of fermentation at 30°C. At 40 °C and 25°Bx, however, fermentation capacity decreased at all timepoints investigated, leaving a higher residual sugar content in the medium. Yeast cells may have suffered fermentative stress

owing to osmotic pressure and longer fermentation times.

Establishing optimal nutrient concentrations in sugarcane wort is critical, since insufficient or excessive levels can impact the effectiveness of fermentation. Increased sugar concentrations accelerate fermentation, impairing sugar transport and reducing ethanol generation [17]. Fermentation time is a stress-producing factor. Prolonged times increase the likelihood of contamination, of reduced cell viability, and excess sugar in wort [18].

In a study conducted in Minas Gerais state, Southeast Brazil [19], 66 *S. cerevisiae* strains isolated from distilleries and fruit wine fermentations were evaluated at various temperatures (30, 37, and 42 °C), osmolarities (0.5, 0.7, and 1 mol L⁻¹ NaCl), and ethanol tolerance values (6%, 12%, and 18%, v:v). Twenty-one strains proved more resistant to these conditions, while 45 exhibited higher sensitivity at 42 °C, 1 mol L⁻¹ NaCl, and 12% ethanol (v:v).

In an evaluation of fermentation parameters in industrial yeasts used in Mato Grosso do Sul ethanol plants during the 2009-2010 harvest, Catanduva-1 cells grown in 50 mL of sugarcane wort at 15°Bx and 30 °C for 16 h exhibited a biomass yield of 14 mg mL⁻¹, with 88% viability and 16% ethanol generation (v:v), while the Fleischmann strain, despite higher biomass production (16 mg mL⁻¹), showed a marked loss in cell viability (to 66%) and lower ethanol concentration (5%, v:v). The superior fermentative efficiency of Catanduva-1 cells, relative to regular baker's yeast, has led to its preference for ethanol production in Brazilian plants [3].

Monitoring metabolites, including glycerol and ethanol, during fermentation can reveal whether yeasts are efficiently converting the substrate into the desired product. Catanduva-1 cells exhibited superior performance at 18 and 22°Bx, with ethanol concentrations of 3.4% and 4.5% (v:v), respectively, at 20 h of fermentation at 30°C. At 40°C, however, a 1.0% loss (v:v) in ethanol concentration was observed for cells tested at 22°Bx and 20 h of fermentation. At 40 h, the loss proved much more pronounced for cells grown at 25°Bx (Figure 3). These values revealed that high substrate concentrations and high temperatures, associated with prolonged fermentation times, promote cell stress and ethanol volatilization.

For industrial yeasts grown in wort at 12, 15, 24, and 30°Bx for 8 h at 30 °C, ethanol production has been reported as highest (8.5%, v:v) for 15°Bx, while

losses in this metabolite were observed in more concentrated substrates [20]. In alcohol plants, CAT-1 and PE-2 strains, uncombined or in consortium, have shown higher conversion efficiency with substrates at 18°Bx in the 30-35 °C range for fermentation times of 8-10 h [6].

Table 1. Evaluation of fermentative stress on *Saccharomyces cerevisiae* Catanduva-1 cultured in sugarcane juice-based wort at selected Brix concentrations, temperatures, and fermentation times.

	Time (h)	Cell biomass (mg mL ⁻¹)	Viability (%)	Residual sugar (%)
30 °C				
18°Bx	10	10.0	90.0	80.0
	20	11.0	87.0	39.0
	40	11.0	85.0	10.0
22°Bx	10	9.0	90.0	80.0
	20	13.0	86.0	38.0
	40	13.0	85.0	10.0
25°Bx	10	8.0	85.0	80.0
	20	10.0	80.0	36.0
	40	10.0	78.0	10.0
40 °C				
18°Bx	10	9.0	85.0	80.0
	20	11.0	83.0	43.0
	40	11.0	80.0	20.0
22°Bx	10	9.0	83.0	65.0
	20	10.0	78.0	54.0
	40	10.0	75.0	22.5
25°Bx	10	9.0	73.0	62.0
	20	9.0	71.0	30.0
	40	10.0	70.0	24.0

During fermentation, stressors can alter yeast metabolism in a number of aspects, including nutrient content, temperature, substrate concentration, pH, contaminants, ethanol, glycerol, and acidity, affecting the generation of secondary metabolites [7].

Selected *S. cerevisiae* strains are widely used in the sugar-energy industry for their high fermentation efficiency and long viability, currently yielding billions of liters of ethanol. During the 2007-2008 sugarcane harvest, Catanduva-1 and Pedra-2 strains were employed in roughly 150 Brazilian plants, accounting for 60% of national bioethanol production [11].

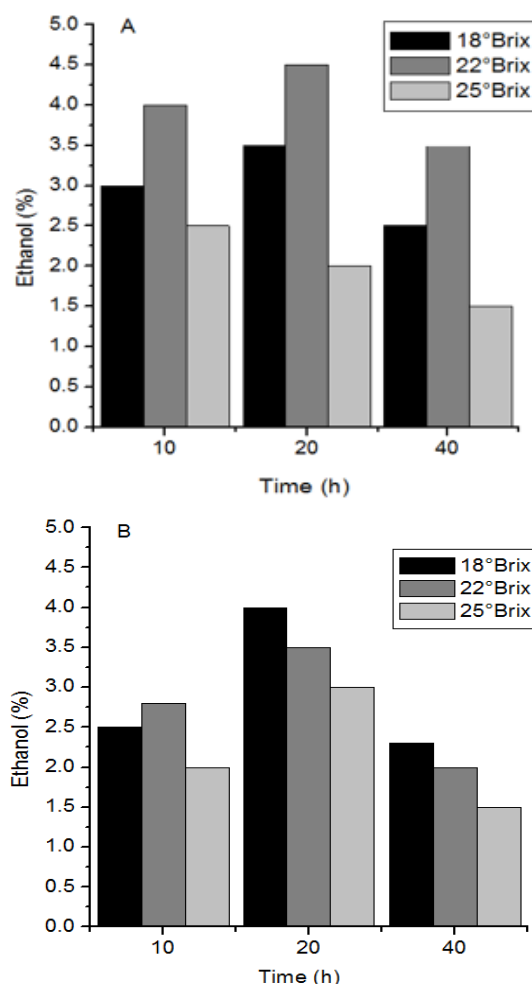


Figure 3. Ethanol concentrations obtained with *Saccharomyces cerevisiae* Catanduva-1 yeasts at 30°C (A) and 40°C (B) in sugarcane juice-based wort, for various Brix concentrations and fermentation times.

Grown at 30 °C, Catanduva-1 cells showed lower glycerol production (0.42 g L⁻¹) when employed at 18 and 22°Bx, for all timepoints investigated. Production of this metabolite was highest (0.44 g L⁻¹) at 25°Bx. At 40 °C, in contrast, increased glycerol generation was observed for all Brix concentrations and time points investigated. At 25°Bx, glycerol concentration increased by 0.5 g L⁻¹. Higher temperatures and substrate concentrations may have caused Catanduva-1 yeasts to increase glycerol generation in response to osmotic and thermal stresses (Figure 4).

During fermentation, glycerol formation may inhibit or limit the generation of ethanol, slowly and linearly decreasing alcohol productivity [11]. Glycerol is the third major by-product of alcoholic fermentation [21].

Glycerol has many functions in the physiology of yeasts, mainly in maintaining cell viability and protecting the integrity of plasmatic membranes. High sugar concentrations, however, affect glycerol production, promoting an imbalance in physiological functions in yeasts [22]. Glycerol generation plays a role in several adaptive mechanisms, allowing yeast cells to adjust to fermentative stress [23].

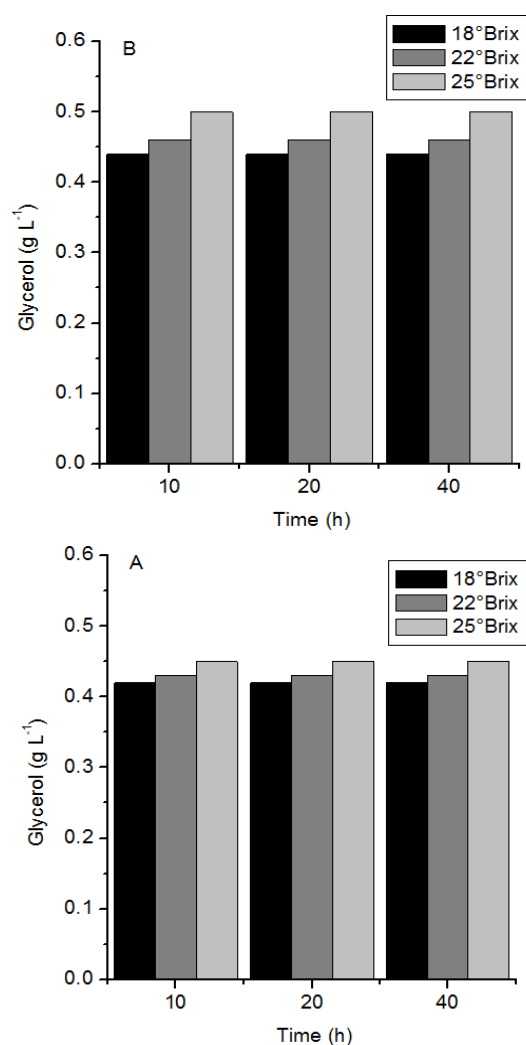


Figure 4. Glycerol concentrations obtained with *Saccharomyces cerevisiae* Catanduva-1 yeasts at 30 °C (A) and 40 °C (B) in sugarcane juice-based wort, for various Brix concentrations and fermentation times.

4. CONCLUSION

Changes in fermentation parameters were found for *Saccharomyces cerevisiae* Catanduva-1 yeasts tested at 25°Bx and 40 °C, possibly owing to cell stress and consequent changes in metabolic

profile.

Ethanol was generated at all substrate concentrations at 30 °C, with higher accumulation at 22°Bx and 20 h of fermentation. At 40 °C, ethanol production was highest at 20 h of fermentation for all substrate concentrations tested. Longer fermentation led to loss of this metabolite. At both temperatures, glycerol concentration proved higher at 25°Bx for all fermentation times investigated.

5. ACKNOWLEDGMENTS

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Apoio ao Desenvolvimento do Ensino, à Ciência e à Tecnologia do Estado do Mato Grosso Do Sul (FUNDECT).

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