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Evaluation of Thermal Stress in *Saccharomyces cerevisiae* Concerning Ethanol Production and Assimilation of Amino Acids in Saccharine Substrate

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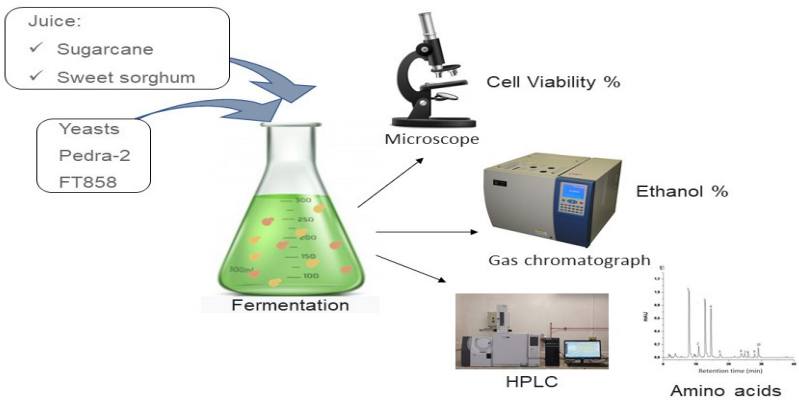
Fuel ethanol has been consolidated in view of the current energy matrix. However, for an efficient process, the yeast and the quality of the substrate used must be taken into account. Thus, this study aims to evaluate sugarcane juice and sweet sorghum and analyze cell viability, ethanol production and the assimilation of amino acids present in these substrates as a function of temperature. The Industrial yeasts *Saccharomyces cerevisiae* FT858 and Pedra-2 (Pe-2) were activated with pre-inoculum of 2% YPD. The biomass obtained was inoculated on the substrates and incubated at 30 and 40°C at 250 rpm. Aliquots were collected for feasibility analyses with methylene blue, ethanol by gas chromatography and amino acids by high performance liquid chromatography. Yeasts showed better performance in sorghum broth at 30°C. The viability of 89% for Pe-2 and 85% for FT858 and ethanol concentration of 10% (v v^{-1}) for both yeasts. At 40°C there was a reduction in these parameters. Sorghum had the highest amount of amino acids and serine, arginine, alanine and tryptophan were effectively assimilated by yeasts. The temperature of 40°C interfered in the metabolic capacity of the yeasts, causing thermal stress, inducing a greater consumption of amino acids.

Graphical abstract



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1. Introduction

The depletion of reserves of fossil sources has motivated the search for other clean and sustainable forms of energy, with a view to the need to reduce the impacts caused to the environment, especially concerning the emissions of gases

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that directly imply climate change [1]. Thus, the search for new sources that can meet current energy needs and that are in line with the principles of sustainable development are being widely researched [2]. In this context, biofuels are consolidating and presenting themselves as a promising alternative to fossil fuels, since they can be produced from renewable biomass. A good example is an ethanol, a biofuel produced from renewable energy sources known as biomass. This bioproduct can originate from different raw materials through a first or second-generation process [3].

The benefits of using biofuels can be present in different development chains and the productivity impacts of this sector can influence economic spheres with the diversification of energy sources, in the development of agriculture, in increasing investments in different areas of research and sustainability [4], and in the environmental ones, since it allows the reduction of greenhouse gases, reduction of pollutants in the air and carbon sequestration [5], and also social ones that include the generation of jobs both in the field and in industries and the transport sector [6].

According to the Ministério of Agricultura, Pecuária and Abastecimento [7], Brazil is emerging as the second-largest producer of ethanol in the world and also as a pioneer in the insertion of this biofuel in its energy matrix. In this country, fuel alcohol is produced from sugar cane by a process called the first generation according to Souza et al. [8]. This crop has good productivity with the cultivated area, low cost of production with a positive energy balance [9]. It should be noted that although only one-third of the sugarcane juice is used for the production of sugar and alcohol, the process is considered to be sustainable since solid residues are liable to be used mainly for energy cogeneration [10,11].

Another biomass that can be used in fermentation processes for the production of ethanol is sweet sorghum (*Sorghum bicolour* (L) Moench). A crop that has characteristics similar to sugarcane and that also has a maturation cycle that lasts from 90 to 120 days after planting, being considered easy to adapt and undemanding to cultural treatments [12]. This culture has carbohydrate levels that are essential for fermentation [13]. Studies using sweet sorghum and sugarcane in the fermentation process showed that the sorghum juice showed higher yield and fermentative efficiency than the sugarcane juice. This finding leads to the conclusion that the composition of the substrates provides essential elements for a process with quality and productivity [14,15].

During the fermentative process, some factors directly affect the fermentative yield, such as pH, contamination, however, the temperature variation is a limiting factor, it acts on the viability rate, on the cellular metabolism interfering in the bioconversion of the substrate quantitatively in the formation of secondary compounds such as ethanol and glycerol [16]. Furthermore, temperatures above 32 ºC cause cellular changes such as a reduction in the viability rate, which in turn leads to slow fermentations and the accumulation of toxic by-products that induce biological responses and alter productivity throughout the industrial process [17]. In this way, the temperature can interfere with the assimilation of nutrients during the fermentation process, and the interaction of the carbon and nitrogen source are essential to guarantee the production of ethanol.

The assimilation of nutrients in yeasts is a complex mechanism and involves the expression of glucose transporters and amino acids that is regulated by countless genes with membrane transporters and, by countless signalling pathways providing a specific response to the quantity and availability of nutrients [18]. Such compounds are important for cell and physiological integrity as well as alcoholic performance [19]. In the studies developed by Mueller et al. [20], evaluating the consumption of amino acids during the fermentation process using the sweet substrates of sorghum and cane juice and the yeast *Saccharomyces cerevisiae* FT858, observed that in the sorghum juice there was a greater availability of the amino acids serine, arginine, alanine and tryptophan in both substrates and that these were the most assimilated by yeast. Amino acids such as proline, tryptophan and arginine act in the cellular protection of yeasts with ethanolic stress [16].

Thus, knowledge about the substrates used for fermentation as to their composition is necessary for the composition, since the presence of nutrients such as carbon and nitrogen sources, help efficiently and are considered essential for the production of ethanol. *S. cerevisiae* need nutrients for their metabolism. In addition, understanding the dynamics of the fermentative capacity of these microorganisms can support the choice of the microorganism to be used in the fermentation process. In this sense, the present study aims to evaluate the substrates based on sugarcane juice and sweet sorghum, as well as to analyze cell viability, ethanol production and the assimilation of amino acids present in these substrates as a function of temperature.

2. Results and Discussion

The viability rate of yeasts showed the best indexes at 30 °C, in the sugarcane juice of 79% for Pe-2 and FT858 76%. These yeasts, when grown in sweet sorghum juice, the viable cell rate was 89% for Pe-2 yeast and 85% for FT858, values higher than those observed for sugarcane juice. At the highest temperature, 40 °C, a drop in the rate of viable cells was observed, as shown (Table 1). Possibly the high temperature has caused stress on the microorganisms, triggering the drop in budding and loss of cell viability.

In evaluating the ethanol concentration, the yeasts studied exhibited similar behaviour. The microorganisms showed a lower production of this metabolite depending on the substrate and temperature. When the yeast ethanol concentration is compared to the sugarcane juice and sweet sorghum, it can be observed that the best percentages were found for fermentation with the sorghum juice at a temperature of 30 °C at a rate of 10% (v v^{-1}) for both yeasts. At 40 °C, yeasts showed a loss of fermentation capacity, possibly at this temperature the microorganism has undergone the action of thermal stress, since the ethanol concentration has reduced, on average, to 7.0% (v v^{-1}) for the fermentation in sugarcane juice and 7.5% (v v^{-1}) for sorghum. Although the yeasts used in this study are industrial strains, the evaluation of the data suggests that the temperature of 40 °C affected the fermentative performance of the yeasts (Table 1).

Thus, it can be inferred that the higher temperatures are shown to be a limiting factor in the conversion of the substrate and that possibly influenced the loss of fermentative efficiency caused by the residual sugar as well as causing cellular stress. Santos et al. [15], studied the physiological response of FT858 yeast grown in sugarcane juice and sweet sorghum and observed that this strain showed the best viability results at a temperature of 30 °C in both substrates.

Table 1. Evaluation of cell viability and ethanol concentration of *Saccharomyces cerevisiae* Pe-2 and FT858 yeasts grown in sugarcane juice and sorghum for 10 hours of fermentation at 30 and 40 ºC.

Yeasts	Temperature (ºC)	Juice Cane		Sorghum juice	
		Viability (%)	Ethanol % (v v^{-1})	Viability (%)	Ethanol % (v v^{-1})
Pe-2	30	79 ± 0.15	9.0 ± 0.20	89 ± 0.12	10 ± 0.22
	40	68 ± 0.10	7.2 ± 0.14	65 ± 0.09	7.5 ± 0.15
FT858	30	76 ± 0.08	9.0 ± 0.05	85 ± 0.10	10 ± 0.09
	40	63 ± 0.20	7.0 ± 0.13	61 ± 0.16	7.8 ± 0.34

Source: Authors. Values expressed as means ± Standard Deviation.

The temperature fluctuation can interfere in the rate of viable cells and also in the yeast sprouting cycle [24]. This assumption was also described by Vargas-Trinidad et al. [17], they point out that temperatures above 32 ºC cause metabolic changes in these microorganisms, determining cell survival and influencing the production of metabolites throughout the industrial process. Pe-2 yeast has a high fermentative capacity and an ethanol production, on average, of 10 to 12% (v v^{-1}) in addition to the easy adaptability to the fermentative environment converging to a longer permanence in the process [25]. This strain is widely required in fermentation processes because it presents low glycerol accumulation and high viability during cell recycling that occurs during the harvest [26].

In the fermentative environment of ethanol production, different factors can occur that cause changes in the physiological behaviour of yeasts, such as the increase in temperature, the availability of nutrients, the concentration of substrates, so the choice of strains for this process is extremely important because these disturbances that occur in the fermentation niche can directly influence the production of ethanol [27]. In addition, these factors both acting in isolation and in synergism can cause changes in the metabolic pathway of microorganisms and influence the mechanisms of adaptation of cells to the fermentative medium [28].

The analysis of amino acids presents in sugarcane and sweet sorghum juice showed similarities in relation to the profile of these compounds. However, differences were observed regarding its initial availability, however, in the sorghum broth, it presented the highest concentrations of amino acids. It is observed that the assimilation of amino acids at a temperature of 30º C was serine, arginine, alanine, tryptophan and threonine, being the most consumed by yeast on both substrates (Figure 1A and B).

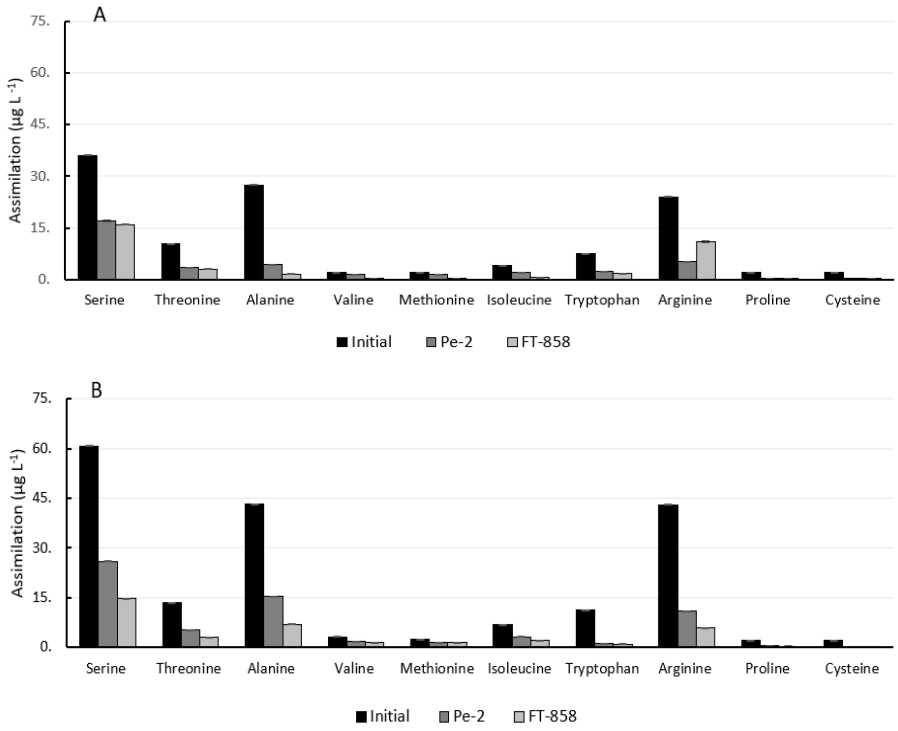

Fig. 1. Amino acid consumption by the yeasts Pedra-2 (Pe-2) and FT858 grown in sugarcane juice (A) and sweet sorghum (B) at a concentration of 22 °Brix and temperature of 30 °C and 10 hours of fermentation. Values expressed as means ± Standard Deviation. *Source*: Authors.

The amino acids proline, tryptophan and arginine contribute to the increased protective effect on yeast cells in relation to ethanolic stress [16]. *Saccharomyces cerevisiae* has a versatile metabolism, surviving on substrates with different nutrient availability, in which there may be variation in the composition of the nutrient source, as for Wenger et al. [28] and Gray and Goddard [29], these microorganisms are known as generalists. Still, according to Góes-Favani et al. [30], the quality of the substrates and the availability of nutrients directly imply the quantity and quality of bioethanol produced.

The assimilation of the same amino acids occurred more

effectively at a temperature of 40 °C on both substrates when compared to 30 °C. You can see that the yeast FT858 showed greater assimilation of amino acids when compared to Pe-2. Possibly the yeast FT858 assimilated the amino acids more quickly because it suffered more the action of thermal stress, needing these compounds to maintain its cellular vitality than Pe-2, since this yeast has a high fermentative strength (Figure 2A and B).

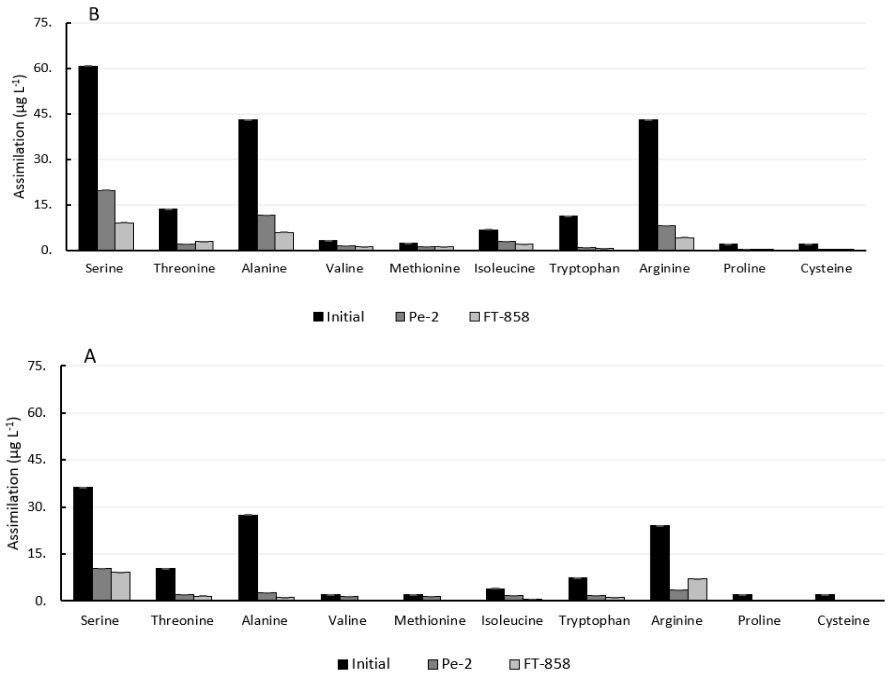

Fig. 2. Assimilation of amino acids by the yeasts Pedra-2 (Pe-2) and FT858 grown in sugarcane juice (A) and sweet sorghum (B) with a concentration of 22 °Brix and a temperature of 40 °C and 10 hours of fermentation. Values expressed as means ± Standard Deviation**.** *Source***:** Authors.

Possibly the higher temperature accelerated the transport of amino acids due to the cells being able to maintain their physiological integrity. It was also observed in this study that cell viability and ethanol concentration were altered as a result of thermal stress. In this way it can be inferred that temperature has been an extremely relevant factor with regard to the consumption of nutrients by yeasts in saccharine substrates and the way they assimilate them, we can suggest that yeasts can assimilate nitrogen sources from different ways during the fermentation process.

Amino acids influence the metabolic functions of yeast cells during fermentation, as these components act mainly on protein synthesis and the maintenance of metabolic routes. Thus, the presence and availability of free nutrients in the medium and their easy assimilation are important, as they imply the good fermentative performance of yeasts [31,32]. During fermentation, yeasts assimilate nutrients, carbon and nitrogen sources being essential to maintain cell growth [20].

3. Material and Methods

3.1. Collection and preparation of the substrate

The cane juice was obtained directly from the Bunge plant process and the sorghum broth with Embrapa Agropecuária Oeste-Dourados, and its extraction performed by milling in a conventional mill. They were packaged in sterile bottles and transported at 4 ºC to the Biotechnology, Biochemistry and Biotransformation Laboratory of the Center of Studies on Natural Resources-CERNA of the State University of Mato Grosso do Sul - UEMS/Dourados-MS. This material was filtered in cotton and on filter paper aiming at the maximum removal of the impurities. The Brix was concentrated at 22 °Brix by evaporation and accompanied by a portable refractometer, and the pH was adjusted to 5.0 with 1 mol L^{-1} hydrochloric acid through the use of pH meter.

3.2. Strain used

In this study, the following yeast strains were used *Saccharomyces cerevisiae* FT858 and Pedra-2.

3.3. Pre-inoculum

For the pre-inoculum, 2% YPD medium containing 1.0% (w v^{-1}) of yeast extract was used; 1.0% (w v^{-1}) of peptone; 2.0% (w v^{-1}) glucose and sterilized by autoclaving at 120 °C for 20 minutes in which 0.10 grams of lyophilized yeast were inoculated and incubated at 30 °C for 12 hours at 250 rpm. After this period the cells were collected by centrifugation (800 g, 20 min), resuspended and washed three consecutive times in sterile saline (0.85%), with a final concentration of 10 mg mL^{-1} wet mass which was used to fermentative experiments.

3.4. Ethanol Fermentation condition

The fermentation was carried out on a substrate based on cane juice and sorghum at 22 °Brix concentration. The bottles were filled with sterile broths containing 50 mL of sterile broth in which the biomass was inoculated and incubated at temperatures of 30 ºC and 40 ºC at 250 rpm. The experiment was conducted in a fermentation process of 10 hours. All experiments were performed in triplicate.

3.5. Analytical methods

3.5.1. Cell viability

Cell viability was assessed using the methylene blue dye. An aliquot was placed in a Neubauer chamber and examined under an optical microscope. Viability was determined by counting dead cells stained in blue and expressed as the percentage of viable cells in each culture [22].

3.5.2. Quantification of ethanol

Ethanol was analyzed by gas chromatography with a flame ionization detector [23]. The samples were filtered in a 0.45 µm ultrafilter.

3.5.3. Amino acids analysis

The samples for amino acid analysis were prepared as described by Torres et al. [24]. 500 µL of the samples, 1.5 mL of borate buffer (pH = 9), 0.1 mol L-1 and 1 µL of diethyl ethoxymethylene malonate (DEEM) were prepared. The solution was shaken and incubated at 50 °C for 50 minutes. In each analysis, the flow rate and the injected volume were set at 0.9 mL min-1 and 20 µL, respectively. All chromatographic analyzes were performed at 23 °C. Elution was performed using the following solvent gradient programs: the mobile phase A consisting of a solution of 25 mM acetic acid and 0.02% in ultrapure water, adjusted to pH 6 and the mobile phase B with acetonitrile. Elution was performed using 0 min 96% A and 4% B, 3 min 88% A and 12% B in 10 min, 17 min 69% A and 31% B in 5 min and 5 min 96% A and 4% B the sample of each amino acid (alanine, arginine, cysteine, isoleucine, methionine, proline, serine, threonine, tryptophan and valine, Sigma, ⩾ 97%) was dissolved in ultrapure water, filtered through a 0.45 µm Millex filter resulting in stock solution. The standard solution was dissolved in ultrapure water to obtain solutions in the range of 0.1-100 µg L-1 for analysis by HPLC. It was performed comparing retention times and spectra of the amino acid patterns, in the region of 200 to 800 nm. The analysis was performed in triplicate.

3.6. Statistical analysis

The results were analyzed with the Excel software version 2016.

4. Conclusions

Direct fermentation substrates, such as sugarcane juice and sweet sorghum are important for maintaining yeasts and ensuring efficiency and productivity, contribute efficiently to the increase in fuel ethanol production and also provide the identification of yeasts more resistant to the process with a view to gains in substrate conversion.

Yeasts showed better cell viability when grown at 30 °C in sweet sorghum broth. The fermentative capacity was affected at a temperature of 40 °C, caused by the end stress-causing loss in ethanol production. The sorghum broth showed the highest availability of amino acids and the most assimilated by the yeasts were serine, arginine, alanine, tryptophan and threonine, and the temperature of 40 °C directly affected the transport of these nutrients by the yeasts causing thermal stress.

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Author Contributions

Maria do Socorro Mascarenhas Santos (Conceptualization; Methodology; Investigation; Writing – review & editing); Larissa Pires Mueller (Methodology; Investigation), Margareth Batistote (Conceptualization; Methodology; Investigation; Writing – review & editing), and Claudia Andrea Lima Cardoso (Conceptualization; Methodology; Writing – review & editing).

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