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FULL PAPER

# Evaluation of Metabolites and Amino Acids Assimilation by Yeast FT-858 in Saccharine Substrates for the Production of Bioethanol

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## Abstract:

Yeasts are exposed to stressing agents in the fermentation process. The study aims to analyze the production of ethanol and intracellular glycerol, as well as to evaluate the consumption of amino acids by yeast FT-858 in different culture conditions. The sugarcane and sorghum broth were used, at 22ºBx and pH of 5.0. A 2% YPD medium was pre-inoculated, and 0.10 g of lyophilised yeast were inoculated, incubated at 30 °C for 12 hours at 250 rpm. Cells were recovered by centrifugation and reinoculated in the fermentation medium at 30 °C and 40 °C at 250 rpm, this procedure was conducted with cell recycles, and 4 mL aliquots were withdrawn for analysis. Quantification of glycerol was accomplished by cell lysis and enzymatic kit of triglycerides, ethanol by gas chromatography and amino acid analysis by high performance liquid chromatography. The results showed that intracellular glycerol production and ethanol concentration were inversely proportional due to stress factors. The highest amount of amino acids available was in sorghum broth, and the most assimilated by the yeast were: serine, arginine, alanine and tryptophan and the high temperature accelerated their consumption of them.

**Keywords:** biosynthesis; fermentation; *Saccharomyces cerevisiae*

## 1. Introduction

Growing concern about environmental issues and the continuing scarcity of fossil fuel sources has been driving research into the use of renewable natural resources for energy purposes [1]. These studies are being carried out both in developed countries and in Brazil with the aim of encouraging the use of clean energy, as it has a lower impact in relation to greenhouse gases. In this sense, Brazil plays a prominent role in the development of clean technologies, such as the improvement of the ethanol production process [2, 3]. Brazil has a prominent role in the development of technologies for the sugar-energy sector, especially for the production of first-generation ethanol [2]. This country is considered as the second largest producer of ethanol and the first producer of sugarcane, given its climatic conditions and large areas of arable land [3], being the primary raw material for production of this biofuel, since it is rich in sucrose, a soluble sugar, formed of glucose and fructose [4].

Thus, the challenges now lie in increasing the production of ethanol with lower costs and inserting new renewable sources of biomass rich in sugars [5], since ethanol can be produced from other raw materials containing a fermentable sucrose content [6]. In this way, the sorghum (*Sorghum bicolour* (L.) Moench) has the potential to be used in the production of fuel ethanol, since it has a high yield of biomass and fermentable sugars [7], processed at the same sugarcane production plant [8].

Studies by Masson et al. [9], comparing the sorghum broth with the sugarcane juice in the

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fermentation process, observed that this culture could be used for the ethanol production, since it showed a yield and fermentative efficiency similar to sugarcane, such factors are essential for a process with quality and productivity. A process with quality and efficacy becomes possible with the choice of custom industrial yeasts, such as the FT-858 lineage that presents high fermentation performance, resistance to pH variations and tolerance to high concentrations of ethanol [10]. Studies by Batistote et al. [11] using selected yeasts such as Catanduva-1, Pedra-2 and Barra Grande in the 2008-2009 harvest in plants located in the south of Mato Grosso do Sul, observed the analyzed strains presented an efficient variation in ethanol production from 14% to 16% (v $v^{-1}$).

Yeasts are exposed to several stressors throughout the fermentation process, such as high concentrations of ethanol, osmotic pressure, thermal instability, pH, contaminating agents, among others. These conditions may alter the physiological patterns of these microorganisms and influence cell viability and growth, fermentative efficiency and metabolite formation [12, 13]. Thus, some yeast strains have characteristics that make them more adapted to the numerous stress factors they are subjected to during fermentation [14].

According to Cruz et al. [15] temperature oscillation is one of the factors that directly affect the cellular metabolism, interfering in the substrate bioconversion and consequently in the formation of the secondary compounds. Ethanol and glycerol are examples of such compounds synthesized in response to this factor in order to establish cell balance. Thus, increased glycerol synthesis occurs under stress conditions and can be considered a limiting factor for fermentation, since the gradual accumulation of this compound may inhibit the production of ethanol [16].

During fermentation, nutrients are assimilated by yeast to maintain cell growth and viability rate. Compounds such as carbon and nitrogen are essential for this process. Among the carbon sources are monosaccharides and disaccharides [17]. Nitrogen compounds may be present in the substrate in the form of amino acids, and their availability varies according to culture. Moreover, for *Saccharomyces cerevisiae*, the assimilation of these can cause changes in the fermentation profile and prevent slow or interrupted fermentations [18, 19]. Considering the above, this study aims to analyze the production of ethanol and glycerol, as well as to evaluate the consumption of amino acids by FT-858 yeast in different culture conditions.

## 2. Results and Discussion

Intracellular glycerol accumulated by the yeast cultured in cane and sorghum broth presented a gradual increase throughout the cellular recycles at both temperatures. However, the loss of ethanol concentration occurred. The highest accumulation of glycerol occurred in the third recycle of 0.17 g $L^{-1}$ at 40 °C in cane juice (Figures 1A and B). In the first recycle, the highest ethanol production of 2.5% (v/v) occurred in both saccharine substrates at the temperatures analyzed. It can be observed that the sorghum broth at 40 ºC presented a higher concentration of this metabolite throughout the fermentative cycles about the sugarcane juice (Figures 1C and D). Possibly the cellular and high temperature recycles allowed the yeast to adapt its physiological mechanism accumulating intracellular glycerol to the detriment of ethanol production. In this study, the results obtained corroborate with the literature where the associated stress factors led the FT-858 yeast to produce the metabolites inversely proportional.

The synthesis of glycerol and ethanol in yeasts is often related to the conditions under which the fermentation process occurs, and the biochemical reactions of these microorganisms are aimed at maintaining cellular integrity and adaptive capacity concerning the production of these metabolites. Increased intracellular glycerol may occur when yeast cells are exposed to high substrate concentrations, elevated temperatures and exposures to contaminants. Ethanol originates in the same biochemical pathway and the increase in the accumulation of glycerol culminates in a decrease in ethanol and, therefore, glycerol can be considered a factor of inhibition or limitation of fermentation [20, 17]. Therefore, the production of these metabolites in yeasts is inversely proportional, and the stress factors contribute effectively to the synthesis of these same ones [14].

Yu et al. [21], consider that sorghum is a

viable crop for ethanol production, because it has high fermentable sugar content in its composition, favors direct fermentation and thus can be used in the off season to produce ethanol [22]. Studies performed by Santos et al. [23] evaluated the fermentative efficiency of yeast FT-858 in cane juice and sorghum observed the production of 7.5% (v $v^{-1}$) and 7.0% (v $v^{-1}$) respectively. However, sorghum has great potential, which makes its use feasible to produce ethanol.

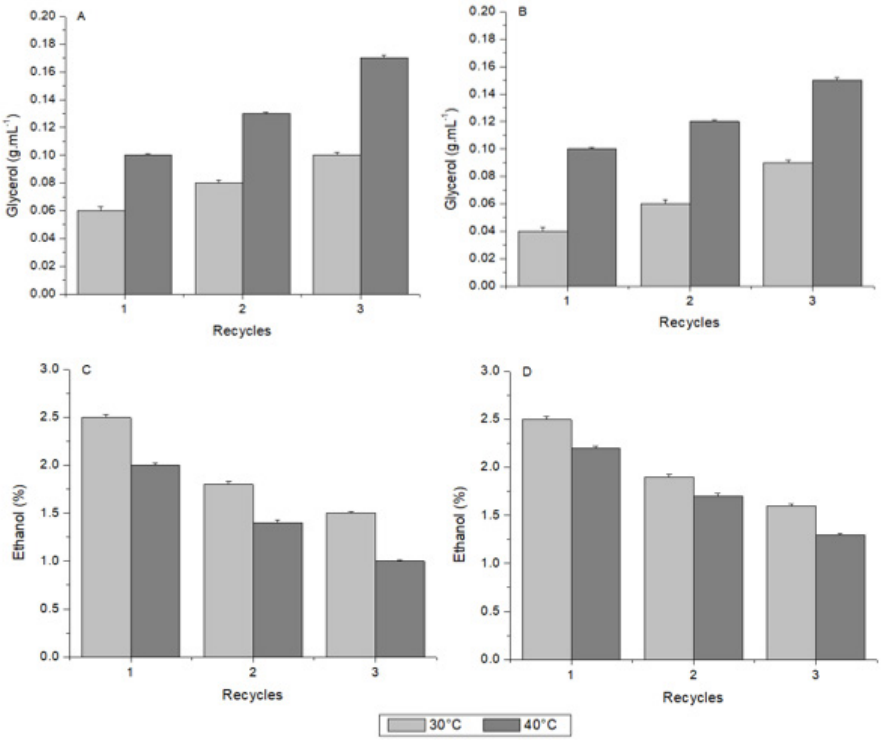


**Figure 1-** Intracellular glycerol accumulation of FT-858 yeast in cane broth (A) and sorghum broth (B) and ethanol concentration in cane broth (C) and sorghum broth (D) of yeast FT- 858. Average of three readings followed by ± standard deviation of the samples.

Analysis of the amino acids present on the saccharine substrates showed that a higher concentration of these compounds was in sorghum broth. Since the amino acid serine, arginine, alanine and tryptophan were the most assimilated by yeast in both substrates. Besides, it was observed that in the highest temperature the highest consumption of these compounds by the yeast occurred (Table 1).

Amino acids have essential functions in the cellular metabolism of yeasts during fermentation. They can act as structural components, be incorporated directly, or participate in the formation of proteins. Thus, the nutrient availability of the substrates as well as the assimilation of these by the yeast is of paramount importance as they influence the physiological mechanisms as well as the fermentative yield [24, 25].

In the studies by Molina et al. [26] during wine fermentation using *Saccharomyces cerevisiae*, the highest temperature had a direct influence on the central metabolism of these microorganisms. Also, the nutrient intake was higher in the temperature of 28 ºC compared to 15 ºC. Analysis of gene expression related to tryptophan biosynthesis in yeast showed that the overexpression of the TRP1-3 and TRP5 genes confer higher resistance to ethanolic stress [27, 28]. For Godin et al. [29] depletion of this amino acid in the substrate influences cell growth and renders them more susceptible to damage to deoxyribonucleic acid. Microorganisms adapted to tolerate stress conditions by ethanol are desirable by the industry since these characteristics can be selected and used to optimize the fermentation process [28].

**Table 1.** Analysis of the assimilation of amino acids by yeast FT-858 on saccharine substrates for 10 hours of fermentation at 30 °C and 40 °C in 22ºBx.

| Amino acid | Saccharine sorghum | | | Sugarcane | | |
|---|---|---|---|---|---|---|
| | *Initial (μg $L^{-1}$) | **30°C (μg $L^{-1}$) | **40°C (μg $L^{-1}$) | * Initial (μg $L^{-1}$) | **30°C (μg $L^{-1}$) | **40°C (μg $L^{-1}$) |
| Serine | 78.89 ± 0.03 | 40.89 ± 0.03 | 34.18 ± 0.03 | 30.89 ± 0.03 | 24.76 ± 0.02 | 22.23 ± 0.02 |
| Threonine | 14.57 ± 0.03 | 9.58 ± 0.03 | 8.98 ± 0.03 | 10.62 ± 0.03 | 8.59 ± 0.01 | 5.54 ± 0.01 |
| Alanine | 42.39 ± 0.04 | 28.34 ± 0.04 | 28.02 ± 0.04 | 25.33 ± 0.02 | 14.41 ± 0.02 | 9.78 ± 0.01 |
| Valine | 3.03 ± 0.01 | 1.73 ± 0.01 | 1.65 ± 0.01 | 2.12 ± 0.01 | 1.98 ± 0.01 | 1.66 ± 0.01 |
| Methionine | 2.37 ± 0.01 | 1.56 ± 0.01 | 1.45 ± 0.01 | 2.07 ± 0.01 | 2.01 ± 0.01 | 1.87 ± 0.01 |
| Isoleucine | 6.76 ± 0.01 | 3.54 ± 0.01 | 3.45 ± 0.01 | 3.82 ± 0.01 | 3.56 ± 0.01 | 2.71 ± 0.01 |
| Tryptophan | 10.03 ± 0.03 | 5.82 ± 0.03 | 4.15 ± 0.03 | 7.23 ± 0.02 | 4.48 ± 0.02 | 3.87 ± 0.02 |
| Arginine | 50.69 ± 0.02 | 37.75 ± 0.04 | 34.67 ± 0.04 | 20.34 ± 0.02 | 12.23 ± 0.02 | 9.79 ± 0.02 |
| Proline | 2.59 ± 0.01 | 1.43 ± 0.01 | 1.35 ± 0.01 | 2.13 ± 0.01 | 1.63 ± 0.01 | 1.44 ± 0.01 |
| Cysteine | 2.59 ± 0.01 | 1.78 ± 0.01 | 1.67 ± 0.01 | 2.04 ± 0.01 | 1.87 ± 0.01 | 1.71 ± 0.01 |

* Substrates before fermentation; ** Substrates after fermentation. M ± SD* means followed by the standard deviation.

In this study, the higher temperature allowed greater assimilation of the amino acids by the yeast. It can be observed that the tryptophan present in the sorghum broth had a higher availability of the assimilation of this amino acid that confers to *Saccharomyces cerevisiae* a better resistance to the stress factors. In this way, it may suggest the use of the sorghum broth for ethanol production.

## 3. Material and Methods

### 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 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 for the Study of 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 Bx was concentrated at 22°Bx by evaporation and accompanied by a portable refractometer, and the pH was adjusted to 5.0 with 1 N hydrochloric acid through the use of pH meter.

### Strain used

The yeast used for this study was *Saccharomyces cerevisiae* FT-858, obtained from Fermentec located in Piracicaba, São Paulo.

### 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.

### Fermentation condition

The fermentation was carried out on a substrate based on cane juice and sorghum at 22°Bx 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 with cell recycles which consisted of a fermentation process every 10 hours and recovered the cells by centrifugation and being inoculated again in fermentative medium with the same characteristics as the initial one. This procedure was performed for three consecutive times, aliquots of 4 mL of each recycle were removed for analysis of intracellular glycerol and ethanol concentration. All experiments were performed in triplicate.

### Analytical Methods

***Quantification of glycerol***

In order to quantify intracellular glycerol, cell lysis was performed with the obtained biomass, in which 2 mL of lysis buffer solution (0.01 mM Tris / HCL pH 7.2) and 10 g of glass beads were added to a tube 15 mL falcon. The procedure consisted of 10 minute vortex stirring steps interspersed for 2 minutes in an ice bath and another 10 minutes in ultrasonic tub. The process was monitored by an optical microscope to verify the cellular disruption. The concentration of glycerol was determined from the enzymatic kit for triglyceride analysis (Laborlab®) and consisted of adding 10 µL of sample and 1000 µL of the enzymatic reagent to a test tube which was then incubated in a 37 °C water bath for 10 minutes. The samples were read in a spectrophotometer at 505nm. The analysis and data processing was based on a standard curve obtained with solutions of glycerol in the concentration range of 0.05 to 0.80 g $L^{-1}$ [30].

***Quantification of ethanol***

Ethanol was analyzed by gas chromatography CG 3900 with flame ionization detector (Varian), using a 30m long fused silica capillary column (ZB-5). The chromatographic condition used was: 1µL injection volume, 1:20 displacement ratio and 90 ºC oven temperature. The detector injector temperatures were 240 °C. The samples were filtered in a 0.22 µm ultrafilter [11].

***Amino acids analysis***

The samples for amino acids analysis were prepared employing five hundred µL of the sample, 1.5 mL of borate buffer (pH = 9), 0.1 mol $L^{-1}$, and 1 µL of diethyl ethoxymethyl-enemalonate (DEEM). The solution was stirred and incubated at 50 °C for 50 minutes. Subsequently, it was filtered with 0.20 µm ultrafiltration for HPLC analysis. The samples were analyzed in an analytical HPLC (LC6AD, Shimadzu, Kyoto, Japan) system with a binary solvent a diode array detector (DAD) monitored at λ = 200-800 nm. The HPLC column was a C-18 (15 cm × 4.6 mm; particle size, 5 µm; Luna, Thermo Electron Corporation, Torrance, CA, USA). In each analysis, the flow rate and the injected volume were set as 0.9 mL $min^{-1}$ and 20 µL, respectively. All chromatographic analyses were performed at 23 ºC. Elution was carried out using the following solvent-gradient programs: Mobile phase A consisting of a 25 mM solution of acetic acid and 0.02% in ultrapure water, adjusted for pH 6 and mobile phase B was acetonitrile. Elution was carried out using 0 min 96% A and 4% B, 3 min 88% A and 12% B by 10 min, 17 min 69% A and 31% B by 5 min and 5 min 96% A and 4% B. A 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 the stock solution. The stock solution was dissolved in ultrapure water in order to obtain solutions in the range of 0.1-100 µg $L^{-1}$ for analysis by HPLC. Identification of the free amino acids was performed by comparing retention times and spectra of the amino acid standards, in the region of 200 to 800 nm, with the peaks obtained in real samples. The analysis was performed in triplicate [31].

## 4. Conclusions

The elevated temperature and associated cellular recycles led to alterations in the biosynthesis of the glycerol and ethanol metabolites since there was an increase in the accumulation of glycerol and the decrease of the ethanol in FT-858 yeast.

The sorghum broth presented the highest amount of available amino acids and the most assimilated by the yeast were: serine, arginine, alanine and tryptophan, because of this substrate, it shows great potential to be used in the production of ethanol.

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