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QUANTIFICAÇÃO ESTEQUIOMÉTRICA DO METABOLISMO RESPIROFERMENTATIVO DE LEVEDURAS SELECIONADAS PARA A PRODUÇÃO DE ETANOL

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RESUMO: Esse trabalho visa estimar o metabolismo respirofermentativo de leveduras selecionadas para produção de etanol (CAT-1, Fermel, FT858, PE-2) por meio de cálculos estequiométricos. O meio de cultivo foi mosto de cana de açúcar (15 °Brix). A fermentação transcorreu durante 8 horas. A cada hora os fermentadores foram pesados. A diferença entre a primeira e a oitava pesagem correspondeu à massa de CO_2 produzida no processo respirofermentativo. A massa de etanol produzida na fermentação foi calculada a partir do teor alcoólico do vinho. A massa de sacarose consumida e a massa de CO_2 produzida durante a fermentação foi estimada usando a equação da fermentação alcoólica. A massa de CO_2 produzida na respiração foi calculada por diferença (massa CO_2 produzida no processo respirofermentativo - massa CO_2 produzida na fermentação). A massa de sacarose consumida na respiração foi estimada usando a equação da respiração. Somando-se as massas de sacarose consumidas na respiração e fermentação obteve-se a massa total de sacarose metabolizada pela levedura. O consumo dos açúcares pelas leveduras foi influenciado pelo Efeito Crabtree *short-term*. O metabolismo respirofermentativo mais intenso foi mensurado para as leveduras CAT-1, FT 858 e PE-2, sendo a levedura CAT-1 a que mais produziu etanol.

Palavras-chave: fermentação, respiração, *Saccharomyces cerevisiae.*

STOCHIOMETRIC QUANTIFICATION OF RESPIRO-FERMENTATIVE METABOLISM OF SELECTED YEASTS FOR ETHANOL PRODUCTION

ABSTRACT: This work aimed to estimate the respiro-fermentative metabolism of selected yeasts for ethanol production (CAT-1, Fermel, FT858, PE-2) by stoichiometric methods. The culture medium was sugarcane must (15 °Brix). Fermentation occurred for 8 hours. The fermenters were weighed every hour. The difference between the first and the eighth weighing corresponded to the CO2 mass produced in the respir-fermentation process. The mass of ethanol produced during fermentation was calculated from the wine alcohol content. The mass of sucrose consumed and the amount of CO2 produced during fermentation were estimated by the following equation for alcoholic fermentation: The CO2 mass produced during respiration was calculated by the difference (CO_2 mass produced during the respiration-fermentation process - CO_2 mass produced during fermentation). The mass of sucrose consumed during respiration was estimated by the respiration equation. Adding the sucrose mass consumed in respiration and fermentation, the total sucrose mass metabolized by the yeast was obtained. Sugar yeast consumption was influenced by short-term crab tree effects. The most intense respiratory-fermentative metabolism

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was measured for the CAT-1, FT 858 and PE-2 yeasts, with CAT-1 being the yeast that produced the most ethanol.

Keywords: fermentation, respiration, *Saccharomyces cerevisiae.*

1. INTRODUCTION

The species *Saccharomyces cerevisiae* is the main fermenting organism used in the sugar and alcohol sector. Its physiological characteristics allow it to adapt to environments with different concentrations of sugars, oxygen, ethanol, pH, salt stress, protein stress, resistance to contaminants and temperature changes. When inserted into a medium with glucose or other sources of fermentable sugars, this yeast uses alcoholic fermentation as the main metabolic pathway to obtain energy with consequent production of ethanol and CO2 (Bai; Anderson; Moo-Young, 2008; Gibson *et al.*, 2007; *et al*., 2010).

In alcoholic yeasts, respiration (aerobic metabolism) and fermentation (anaerobic metabolism) are part of their primary metabolism and occur simultaneously. Therefore, the metabolism of these microorganisms is classified as *respirofermentative.* (Gancedo and Serrano, 1989 Käppeli, 1986). Yeasts metabolize glucose in must through fermentation, producing ethanol and carbon dioxide. The oxygen present in the must, at the beginning of the fermentation process, is used by yeast to produce sterols, unsaturated fatty acids and cell growth through respiration. More than 90% of sugars are metabolized via the fermentative pathway, and the remainder are used for cell growth (Ingledew, 2009).

The aerobic and anaerobic metabolism of an alcoholic yeast can be estimated using a stoichiometric method (Venturini Filho *et al*., 2013; Venturini Filho *et al*., 2014; Venturini Filho *et al*., 2018). Stoichiometry is the calculation of the quantity of substances involved in a chemical reaction, measured with the help of the corresponding chemical equations. In chemical reactions, substances react with each other, creating products in specific proportions (Fiorotto, 2013). Knowing the amount of ethanol and carbon dioxide produced during the fermentation process, it is possible to calculate, using the stoichiometric equations of respiration and alcoholic fermentation, the amount of sugar consumed by the microorganism during the respiration and fermentation processes, as well as the reaction yield.

Theoretically, stoichiometric calculations indicate that 51.1 g of ethanol and 48.9 g of CO2 are produced from 100 g of glucose (Bai; Anderson; Moo-Young, 2008). However, under working conditions, despite the rigor of the technique, approximately 48.5 g of ethanol is obtained at 15 °C. This is because approximately 5% of sugars are reserved for cell growth and the formation of fermentation byproducts, such as glycerol and succinic acid. Furthermore, together with alcoholic fermentation, secondary reactions promote a reduction in practical yield (Menezes, 1980).

Alcoholic yeasts are living organisms with multiple metabolic abilities that are capable of altering the stoichiometry of the reaction, with a great impact on the yield of the fermentation process (Lima, *et al*., 2001). The use of selected strains for ethanol production promotes increased yield, reduced input costs, greater tolerance to variations in alcohol concentration, temperature and pH, faster harvest start-up and a higher sedimentation rate. Due to the importance of yeast in the ethanol production process, this work aimed to estimate the aerobic and anaerobic metabolism of dry yeast selected for ethanol production through stoichiometric calculations.

2 MATERIALS AND METHODS

The experimental design was completely randomized and included four treatments composed of dry yeast (*S. cerevisiae*) from the LNF brand (CAT-1 – batch 20180822C; Fermel – batch 20180501M; FT858 – batch 20190502F; PE-2 – batch 20180730) and three replications,

totaling 12 experimental plots. Each experimental plot comprised a system consisting of a 4 L open fermenter (beaker), 1 kg of sugarcane juice standardized at 15.0 °Brix, 100 g of yeast and a polypropylene rod.

The standardized wort was transferred to an open fermenter. Next, dry yeast was added and mixed with the must with the help of a polypropylene stick that was kept inside the fermenter. The initial temperature of the must was 23 °C, and fermentation occurred at an ambient temperature of 28±1 °C.

After assembling the systems, they were weighed on a precision scale (Gehaka BG 2000), and the initial mass was noted. The system weighing process was carried out every hour, totaling eight weights. The difference between the first and eighth readings corresponded to the mass of carbon dioxide produced during the respirofermentation process.

At the end of eight weighings of the fermenting must mass, the wine was distilled in a bench distiller (Buchi K355), and the alcoholic content was measured using a digital hydrometer (Mettler Toledo DM 45).

The mass of ethanol produced during alcoholic fermentation was calculated to determine the alcohol content of the wine. With this result and using the simplified stoichiometric equation of alcoholic fermentation (Equation 1), the mass of sucrose consumed and the mass of carbon dioxide produced during the alcoholic fermentation process were measured. The reference sugar used for the calculations was sucrose, as it is the predominant carbohydrate in sugarcane juice.

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4\,C_2H_5OH + 4\,CO_2 \quad (1)$$
342 18 184 176

Knowing the total mass of carbon dioxide produced during the respirofermentative process and the mass of carbon dioxide produced during the alcoholic fermentation process, the mass of CO2 produced during respiration was calculated by difference. With these data and using the simplified stoichiometric equation of respiration (Equation 2), the mass of sucrose consumed during the respiration process was calculated.

$$C_{12}H_{22}O_{11} + H_2O + 12\,O_2 \rightarrow 12\,CO_2 + 12\,H_2O \quad (2)$$
342 18 384 528 216

By adding the masses of sucrose consumed in the respiration and alcoholic fermentation processes, the total mass of sucrose metabolized by yeast was obtained.

Analyses of soluble solids were carried out on must (digital refractometer, brand Reichert, model r ^{2} i300), pol (polarimeter, brand Anton Paar, model MCP 200) and pH (pH meter, brand Tecnal, model TEC-5) instruments. The purity (pol/Brix) of the pol and soluble solids was calculated.

The alcoholic contents and amounts of sucrose consumed during respirofermentation metabolism by the yeasts CAT-1, Fermel, FT858 and PE-2 were evaluated using analysis of variance, and the means of the results were compared using the Tukey test (5% probability) (Vieira, 2006) with the MiniTab 16 program (Minitab, 2010).

RESULTS AND DISCUSSION

In a sugar and alcohol plant, the sugarcane juice obtained through the milling process, whether for the production of ethanol or sugar, must contain a sucrose concentration between 8 and 20% (m/m) and a pH in the range of 5.0 to 5.5 (Lima; Marcondez, 2002; Lopes; Gabriel; Borges, 2011). The purity of these raw materials can be classified as very low (< 80%), low (from 80% to 85%), medium (from 85% to 90%) or high (> 90%) (Lopes, Gabriel and Borges, 2011). Based on the aforementioned analyses and information from these authors, the

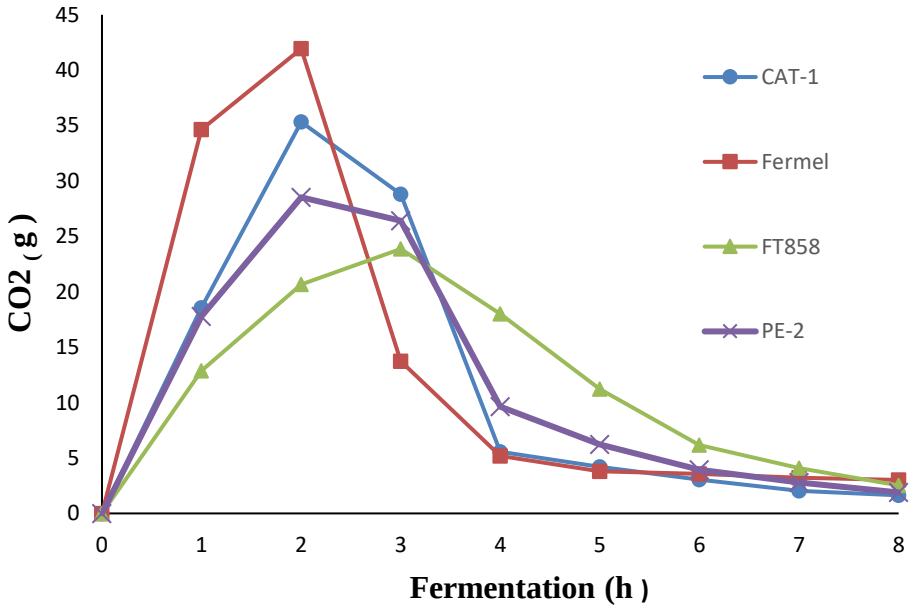
sugarcane juice used in this research presented a high degree of purity with the sucrose concentration and pH recommended for the fermentation process (Table 1).

Table 1. Composition of sugarcane juice must.

Analytics	Results
Soluble solids (°Brix)	15.00
In (% m/m)	13.51
Purity (%)	90.10
pH	5.30

The fermenter, CAT-1 and PE-2 yeasts had peak $CO_{2\ production}$ in the second hour of the fermentation process (41.94 g, 35.32 g and 28.53 g of $CO_{2,\ respectively}$). The FT 858 yeast achieved maximum $CO_{2\ production}$ in the third hour of the fermentation process (23.87 g of CO_2) (Figure 1). Several physical, chemical and microbiological factors affect yeast metabolism, notably temperature, inoculum concentration, pH, bacterial contamination, nutrients and the presence of inhibitory agents (Lima *et al*., 2001). As the physical and chemical characteristics of the substrate were the same for all yeasts, as well as the concentration of yeast added at the beginning of the fermentation process, genetic characteristics inherent to the yeasts and enhanced by the strain selection process determined different behaviors regarding the production of CO2.

Figure 1. Kinetics of carbon dioxide production from CAT-1, Fermel, FT858 and PE-2 yeasts.



For ferment yeast, the high production of CO2 in the first two hours suggested the consumption of sucrose via the aerobic route; the respiratory rate of this yeast was significantly greater than that of other yeasts. There was no significant difference in the percentage of sucrose consumed in the breath among the yeasts CAT-1, PE-2 and FT 858 (Table 2).

In an inversely proportional manner, the percentage of sucrose consumed in Fermel yeast fermentation was significantly lower than that consumed by other yeasts. CAT-1, PE-2 and FT

858 were not significantly different in terms of the percentage of sucrose consumed during fermentation (Table 2). In a study that evaluated the respirofermentative metabolism of beer, wine and bread yeast, Figueira *et al.* (2021) reported that the rate of sucrose consumed in respiration is inversely proportional to the rate of sucrose consumed in fermentation.

The rate of carbohydrate consumption during the respiration process was used by some authors as a parameter to classify yeasts. For Kocková-Kratochvílová (1990), yeasts can be classified into three groups: a) respiration: the aerobic pathway is responsible for 100% of carbohydrate catabolism (e.g., forage yeasts); b) respiration and fermentation: the aerobic route corresponds to 40 to 50% of carbohydrate catabolism (e.g., pathogenic, bakery and high-fermentation yeasts); and c) fermentation: the aerobic route accounts for 10 to 15% of carbohydrate catabolism (distillery, wine and low-fermentation yeast). Briggs *et al*. (2004) classified yeasts into two groups: a) obligate aerobes, which catabolize sugar only aerobically (e.g., *Rhodotorula*, *Lipomyces*, *Cryptococcus*, *Rhodosporidium* and *Saccharomycopsis*); and b) facultative anaerobes, which catabolize sugar simultaneously through aerobic and anaerobic pathways. Within this last group, we have the following subdivisions: b $_1$) respiratory yeasts, which catabolize more than 70% of sugars aerobically (e.g., *Candida*, *Hansenula*, *Kluyveromyces* and *Pichia*); b $_2$) fermentative yeasts, which catabolize up to 10% of sugars aerobically (e.g., *Saccharomyces*, *Brettanomyces* and *Schizosaccharomyces*). Based on information from these authors, the yeasts CAT-1, Fermel, FT 858 and PE-2 are classified as facultative anaerobes with a prevalence of fermentation in relation to respiration. However, the percentages of sucrose consumed by these yeasts aerobically were greater than the reference values proposed by the aforementioned authors (Table 2).

Boulton and Quain (2001) reported that the regulation of sugar consumption by yeast can be influenced by five types of mechanisms: a) *the short-term crab effect*, in which a sugar substrate is inserted into a sugar substrate and a reduction in the rate of respiration occurs; b) the *long-term crab* effect, in which a sugar substrate is inserted into a sugar substrate, respiratory enzymes are repressed or inactivated; c) the Pasteur effect, in which a reduction in the rate of glycolysis under aerobic conditions occurs; d) the Kluyver effect, in which a mandatory aerobic use of disaccharides occurs; and e) the cluster effect, in which an aerobic stimulation of the glucose fermentation rate occurs. As the yeasts CAT-1, Fermel, FT 858 and PE-2 showed respirofermentative metabolism with a reduction in the rate of respiration in relation to fermentation, we can conclude that the consumption of sugars in these yeasts was influenced by the *short-term crab tree effect*.

The mass of sucrose consumed during the respiration and fermentation processes makes it possible to estimate the metabolism of these yeasts. The most intense respirofermentative metabolism was measured for the yeasts CAT-1, FT 858 and PE-2. Fermel yeast had the lowest metabolic rate, which was different from that of the other yeasts (Table 2).

The mass of sucrose in a substrate can be calculated using Pol. For the must used in this research, the calculated sucrose mass was 135.10 g (Table 1). However, the mass of sucrose consumed by yeast during the respiration and fermentation processes varied from 141.07 g to 147.08 g (Table 2). In addition to sucrose, the must obtained from sugarcane juice contains other fermentable sugars, such as glucose and fructose. According to Lopes, Gabriel and Borges (2011), the composition of sugarcane juice, often called absolute juice in sugar and alcohol plants, may contain 1% glucose and 0.5% fructose.

Ethanol production occurs during the fermentation process as the sugars contained in the must are transformed into ethyl alcohol by the action of yeast (Lopes; Gabriel; Borges, 2011). The yeasts CAT-1, PE-2 and FT 858 showed statistically equal rates of sucrose consumption during fermentation. However, CAT-1 yeast produced the most ethanol, with a significant difference in relation to the others. The PE-2 and FT 858 yeasts had statistically equal ethanol

concentrations. Fermel yeast produced the least amount of ethanol because it consumed more sucrose during the respiratory process (Table 2).

Table 2. Quantification of sucrose consumed during respirofermentative metabolism by the yeasts CAT-1, Fermel, FT858 and PE-2.

	CAT-1	**Fermel**	**FT858**	**PE-2**
TA (% v/v)*	7.98 to	6.84c	7.80 b	7.85b
And (g)	66.86	56.82	65.38	65.93
CO2F (g)	63.95	54.34	62.54	63.06
SF (g)	124.27	105.60	121.53	122.54
CO_2 FR (g)	99.17	109.11	99.42	97.21
CO2R (g)	35.22	54.76	36.88	34.14
SR (g)	22.81	35.47	23.89	22.12
SRF (g)*	147.08 a	141.07b	145.41 a	144.66 a
TSF (%)*	84.49 a	74.86b	83.57 a	84.71 a
TSR (%)*	15.51b	25.14 a	16.43b	15.29b

TA = Alcoholic content; E = Mass of ethanol; CO_2F = Mass of CO_2 produced during fermentation; SF = Mass of sucrose consumed in fermentation; CO_2FR = Mass of CO_2 produced in fermentation and respiration; CO_2R = Mass of CO_2 produced in respiration; SR = Mass of sucrose consumed in respiration; SRF = Mass of sucrose consumed in respiration and fermentation; TSF = rate of sucrose consumed in fermentation; TSR = rate of sucrose consumed in breathing; *Tukey test (α=5%).

4 CONCLUSIONS

The alcoholic yeasts studied in this work exhibited respirofermentative metabolism, with fermentation predominating in relation to respiration. The consumption of sugars by these yeasts was influenced by the *short-term crab tree effect*.

The most intense respirofermentative metabolism was measured for the yeasts CAT-1, FT 858 and PE-2, with the CAT-1 yeast producing the most ethanol.

As a way of estimating the primary metabolism of yeasts, the stoichiometric method proved to be effective for this purpose. Furthermore, due to its simplicity and speed in practical development, this method can be used for teaching purposes.

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