ARTICLE

DAMAGE TO DEOXYRIBONUCLEIC ACID - DNA AND ITS INFLUENCE ON ETHANOL PRODUCTION IN INDUSTRIAL LINES OF *SACCHAROMYCES CEREVISIAE* IN RELATION TO FERMENTATIVE CYCLES

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Abstract: In industrial processes, more robust and tolerant *Saccharomyces cerevisiae* strains are needed, as stress conditions can affect these microorganisms and cause cellular changes. Thus, this study aimed to evaluate the genetic and physiological effect of fermentation cycles in industrial strains of *S. cerevisiae*. A pre-inoculum was performed by inoculating 0.10 g of Pedra-2, FT858 and Fleischmann yeasts in YPD 2% liquid medium and incubated at 30°C for 10 hours at 250 rpm. The cells were recovered and the biomass obtained was inoculated in sugarcane juice with 22°Brix at a temperature of 30°C at 250 rpm for 10 hours being conducted with cell recycling. Aliquots were taken at each cycle for genotoxicity analysis by the comet test and physiological stress by ethanol quantification. THE yeast Fleischmann showed lower tolerance to the fermentation cycle, showing greater damage to deoxyribonucleic acid (DNA). The ethanol productivity of the Fleischman strain, in the first fermentation cycle, was similar to the Pedra-2 and FT858 yeasts. However, during the cycles, there was a reduction in the content of this metabolite.

Keywords: Deoxyribonucleic acid, metabolites, *Saccharomyces cerevisiae*.

DANOS AO ÁCIDO DESOXIRIBONUCLEICO - DNA E SUA INFLUÊNCIA NA PRODUÇÃO DE ETANOL EM LINHAGENS INDUSTRIAIS DE *SACCHAROMYCES CEREVISIAE* EM RELAÇÃO A CICLOS FERMENTATIVOS

Resumo: Em processos industriais, cepas de *Saccharomyces cerevisiae* mais robustas e tolerantes são necessárias, pois condições de estresse podem afetar esses microrganismos e causar alterações celulares. Assim, este trabalho teve como objetivo avaliar o efeito genético e fisiológico dos ciclos fermentativos em linhagens industriais de *S. cerevisiae*. Um pré-inóculo foi realizado inoculando 0,10 g das leveduras Pedra-2, FT858 e Fleischmann em meio líquido YPD 2% e incubadas a 30 °C por 10 horas a 250 rpm. As células foram recuperadas e a biomassa obtida foi inoculada em caldo de cana

com 22 °Brix a uma temperatura de 30 °C a 250 rpm por 10 horas, sendo realizada a reciclagem das células. Alíquotas foram retiradas a cada ciclo para análise de genotoxicidade pelo teste do cometa e estresse fisiológico pela quantificação de etanol. A levedura Fleischmann mostrou menor tolerância ao ciclo fermentativo, apresentando maior dano ao ácido desoxirribonucleico (DNA). A produtividade em etanol da linhagem Fleischman, no primeiro ciclo fermentativo, foi semelhante às leveduras Pedra-2 e FT858. No entanto, durante os ciclos, houve redução no conteúdo desse metabólito.

Palavras-chave: Ácido desoxirribonucleico, metabólitos, *Saccharomyces cerevisiae*.

Introduction

Over the last few decades, the development of technologies for the production of biofuels has been improved, mainly with a view to the imminent need to reduce greenhouse gas emissions into the atmosphere (Liu et al., 2021). Thus, ethanol is gaining a prominent role as an alternative energy source that has, among other attributes, being produced from renewable sources, such as biomass or energy crops. This biofuel can positively assist in energy security, as it provides diversity in the energy matrix in addition to reducing dependence on fossil fuels (Zhang et al., 2015; Liu et al., 2021).

Brazil has been standing out in the production of biofuels, mainly in the production of ethanol. The process in this country has been perfected over time to the point of being considered the most profitable when compared to the ethanol production process in other countries since Brazilian ethanol is produced from sugarcane, rich biomass in fermentable sugars and which has a good yield in productivity when compared to other crops, such as starch (Manochio et al., 2017; Santos et al., 2018). The production process of this biofuel occurs through direct fermentation and is known as E1G first-generation ethanol (Garcia et al., 2022).

The main innovations inserted in this process were in relation to the development of more productive sugarcane varieties with a higher concentration of sugars (Terradas-Cobas & Céspedes-Payret, 2015), and obtaining yeasts with high fermentative performance made this process more efficient and economically viable (Lopes et al., 2016). The yeasts used are of the specie *Saccharomyces cerevisiae* (Meyen ex E. C. Hansen, 1883), which, given its characteristics, are used in other processes such as the production of bread yeast, beer yeast, food additives, heterologous proteins, the biosynthesis of products of the pharmaceutical interest and through breeding genetically, these microorganisms have become excellent producers of natural compounds with high economic potential such as biofuels, especially ethanol (Santos et al., 2021; Jach et al., 2022).

S. cerevisiae has wide and valuable application in numerous industries, due to its life cycle and the fact of "make-accumulate-consume", this is due to the Crabtree effect according to Thomson et al. (2005). For these authors, this occurs when the yeasts are under aerobic conditions and do not use the respiratory tract to assimilate saccharides and produce biomass, but produce ethanol which, as a consequence, is excreted into the fermentation medium. *S. cerevisiae* is one of the most studied and used organisms in biotechnological processes with numerous applications acting as a biocatalyst, so understanding its biochemistry and genetics is a way to optimize the production of various value-added molecules (Cristobal-Sarramian & Atzmüller, 2018; Batistote & Santos, 2020). According to Eardley & Timson (2020), this microorganism is considered a pillar of biotechnology.

In the ethanol production process, strains of *S. cerevisiae* with characteristics aimed at this type of process are necessary. Selected yeasts are usually used that are tolerant of stress conditions in the vat environment, such as pH oscillation, osmotic pressure, temperature instability, and contamination, among others (Lopes et al., 2016). However, the stress conditions during the fermentation process and the synergism between them can cause changes in the biochemical, genetic and physiological mechanisms of these microorganisms, resulting in the loss of fermentative capacity and, consequently, inhibiting the production of metabolites (Lin et al., 2021). Such stress conditions can cause cytological and genetic alterations.

A good example is the high concentrations of ethanol that cause toxic effects for yeasts, Saini et al. (2018), explain that the yeast response to ethanolic stress is multifaceted and affects the cell, interfering with its self-protection functions or even causing the loss of its viability. Such a situation may be due to associated stress conditions, which leads to the choice of more tolerant yeasts. According to Mueller et al. (2020), some industrial yeasts used by ethanol production plants have a high tolerance to the stress factors of the fermentation medium, indicating an adaptation originated throughout their evolutionary histories of use in the industrial environment.

According to Święciło (2016), the synergism between stress factors and intensity results in pressure on the yeasts resulting in an adaptation to the fermentation medium. However, they occur in response to the induction of mechanisms related to gene regulation, which act in the biosynthesis of metabolites such as trehalose and heat shock proteins responsible for the protection mechanism (Unrean et al., 2018). *S. cerevisiae*, under such conditions, regulate their biochemical machinery to maintain cellular integrity (Deparis et al., 2017). Some studies report that the deletion of a specific gene favours the generation of new strains tolerant to different types of fermentation stress, including ethanol, thermal, and osmotic (Peetermans et al., 2021; Mavrommati et al., 2021; Martínez - Matías et al., 2021).

Ethanol production in Brazil uses sugarcane juice and recycled yeasts, which, despite being adapted to the stress conditions of the fermentation medium, like any living organism, are exposed to the effects of the environmental conditions. Although these microorganisms are considered excellent fermenting agents, there are numerous biochemical adjustments to maintain both their cellular integrity and the response to the production of metabolites, which can be quantified and taken as a response to stress conditions. Studies focused on yeast responses at genomic and physiological levels, mainly in relation to its fermentative performance, the ability to produce important molecules and its applicability in industrial products is important given the need for new energy sources. Thus, the study aims to evaluate the genetic and physiological effects of fermentation cycles in industrial strains of *S. cerevisiae*.

Material and methods

Study location

The study was developed at the Laboratory of Biotechnology, Biochemistry and Biotransformation of the Center for the Study of Natural Resources - CERNA of the State University of Mato Grosso do Sul, Dourados/MS.

Microorganisms, pre-inoculum and fermentative culture

The microorganisms used were *S. cerevisiae* Pedra-2 strains, acquired at LNF Biotecnologia Aplicada, in Bento Gonçalves-RS; the FT858 yeast obtained from the company Fermentec, in Piracicaba –SP, and the Fleischmann acquired in the local market.

For the pre-inoculum, a YPD 2% liquid medium was used, containing 1.0% (pv^{-1}) yeast extract, 1.0% (pv^{-1}) peptone and 2.0% (pv^{-1}) glucose, sterilized in an autoclave at 120 ºC for 20 minutes, in which 0.10 grams of the lyophilized yeasts were inoculated and incubated at 30 ºC for 10 hours at 250 rpm. After this period, the cells were washed three consecutive times in sterile saline solution (0.85%) and recovered by centrifugation, resulting in 10 $mg.mL^{-1}$ of wet biomass.

The fermentation medium was prepared with 50 mL of sterile sugarcane juice in 125 mL Erlenmeyer flasks, the obtained biomass was inoculated and incubated at 30 °C at 250 rpm. At each cell cycle, it consisted of recovering the biomass from the fermentation medium and inoculating it again into a new fermentation medium for ten hours of fermentation, in four cycles. At each cycle, samples were collected for analysis.

Comet Test

The comet test was performed according to the methodology adapted from Lah et al. (2004); Da Silva (2007) and Mueller et al. (2019). The 5.0 µl aliquots of cells were resuspended in buffer solution (1M Sorbitol and 25mM KH2PO4), mixed with 70 µl of Low agarose. Melting Point 0.5% and 2 $mg.mL^{-1}$ of Liticase enzyme. This solution was placed on slides covered with coverslips that were incubated at 30 °C for 2 h. After this period the reaction was inactivated at a low temperature (4 °C). The slides were then immersed in 0.5% NMP agarose, and in an ice-cold lysis solution (30mM NaOH, 1M NaCl, 0.1% N - lauroylsarcosine, 100mM DMSO, 1% Triton-X100) for 1 h in the dark. Then, they were washed and immersed in solution (30mM NaOH and 2mM EDTA, pH 13) in the electrophoresis tank for 20 min to denature the deoxyribonucleic acid-DNA. The run was performed on an ice-cold surface in the absence of light and the slides were subsequently neutralized in buffer (400 mM Tris-HCl, pH 7.5) for 15 min, washed and dried at room temperature and submerged in solution (15% acetic acid, zinc sulfate 5% and glycerol 5%), and stained with (calcium carbonate 5%, ammonium nitrate 0.1%, silver nitrate 0.1%, tungstosililic acid 0.25% and formaldehyde 0.15%), being washed with distilled water and placed in a solution (1% acetic acid) for 5 min. A quantitative analysis was performed using 100 randomly selected nucleoids and evaluated by light microscopy. The result was expressed as DNA changes.

Ethanol quantification

The analysis of ethanol concentration was determined according to the methodology described by Batistote et al. (2010), using a gas chromatograph (GC) 3900 with a flame ionization detector (Varian), with a 30 m long fused silica capillary column (ZB-5).

Results and Discussion

Studies carried out to compare strains of *S. cerevisiae*, showed the high potential of these microorganisms, given that they have an already decoded genome, are easy to manipulate and can produce numerous molecules with high added value (Navarrete et al., 2020). Due to these characteristics, this microorganism has been successfully used in biotechnological processes and has emerged as a biological system that has high productivity on an industrial scale, being used for the production of ethanol and other metabolites in smaller amounts such as higher alcohols, glycerol among others.

The evaluation of the action of the fermentative cycles showed that there was interference in a differentiated way in the expression of the genic and metabolic mechanisms of the yeasts. This fact can be seen in Fig. (1A, 1B and 1C), in which the Fleischmann yeast showed lower tolerance to recycling, resulting in greater damage to DNA deoxyribonucleic acid. Perhaps this response is related to the phenotype of the yeast, since it is a bakery strain, possibly less adapted to the action of fermentation stress. On the other hand, Pedra-2 and FT858 yeasts showed greater tolerance to genetic material damage in relation to fermentation cycles. Possibly, this result is due to the characteristics of these strains, such strains have greater fermentative robustness, high rate of cell viability, with

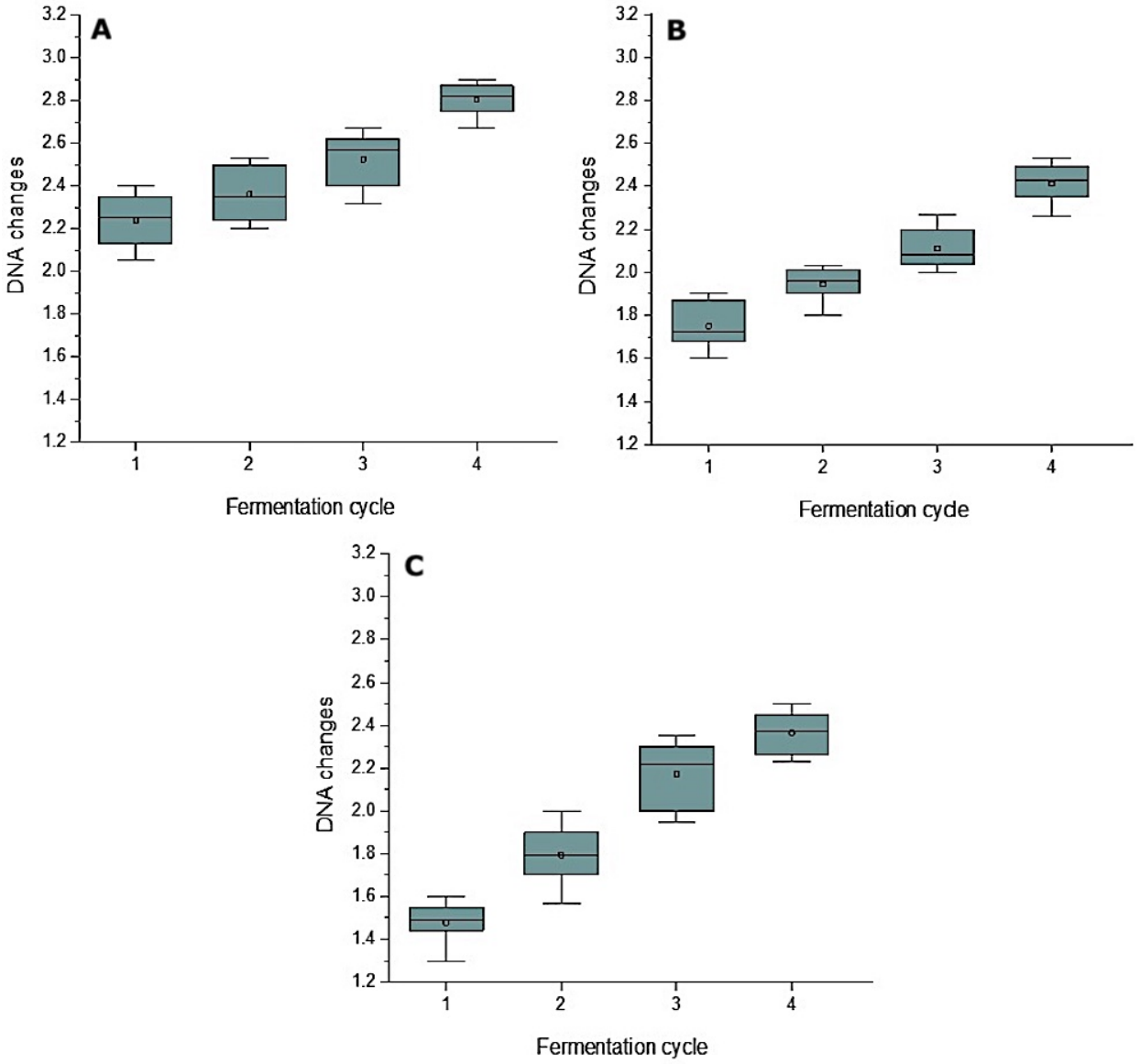

Fig. 1. Deoxyribonucleic acid - DNA degradation profile as a function of fermentation cycles with Fleischmann (A), Pedra-2 (B) and FT858 (C) strains cultivated in sugarcane juice at 30 °C.

high fermentative performance, being the most used to ferment sugarcane juice-based must in the production of ethanol in mills from Brazil.

According to Olsson et al. (2022), *S. cerevisiae* is a preferred microorganism for industrial alcoholic fermentation processes due to its high ability to convert sugars into alcohol, being widely used in the production of biofuels such as ethanol. This yeast has phenotypic stability, being responsible for high production rates and product yield, although it is in an environment with several disturbances (Gong et al., 2017; Tse et al., 2021).

Yeasts have different physiological behaviour in the face of each type of stress, which can be chemical, physical or biological. This fact implies, for industrial-scale production, the use of yeasts with high fermentative performance and a high degree of gene diffusion in order to guarantee cell vitality and viability and to overcome the stress conditions imposed in the fermentation vats (Dzialo et al., 2017; Moscoviz et al., 2018; Oh & Jin, 2020).

Studies focused on the metabolic, genomic and proteomic profile in *S. cerevisiae*, in relation to its application in different processes, have led this microorganism to be an example to evaluate the conditions of adaptation to stress factors (Taymaz -Nikerel et al., 2016; Deparis et al., 2017; Marsit et al., 2017). In fact, the metabolic adjustments of yeast, in the face of changes in the medium, are obtained through the stabilization of cellular mechanisms, gene expression, the profile of protein levels and the production of metabolites (Geng et al., 2017; Šoštaric et al., 2021; Nagamatsu et al., 2021).

Cell recycling possibly induced Pedra-2 and FT858 yeasts to adjust their biochemical machinery, which ensured their cell vitality and viability, guaranteed survival and influenced DNA stability throughout the fermentation cycles. This suggests that the response of less damage to genetic material in these microorganisms is possibly related to the type and amplitude of stress factors present in the fermentation medium and that tolerance to these factors induces a rapid adaptation as a function of the readjustment of the metabolism in these microorganisms. industrial strains.

In the fermentation medium, normally, there are numerous oscillations that are considered stress conditions, which induce yeasts to adverse responses in relation to ethanol production. Understanding how these responses occur is an important condition to ensure the production of this metabolite. It can be observed that the ethanol productivity of the Fleischmann strain, in the first fermentation cycle, showed similarity in relation to the Pedra-2 and FT858 yeasts. However, throughout the cycles there was a loss of ethanol, probably this yeast has suffered more from the action of fermentation stress, leading to adverse biochemical changes and non-maintenance of metabolite production (Tab. 1).

In the ethanol production process, yeasts are constantly exposed to stress conditions in the medium, which can interfere with their metabolism and affect the production of the metabolite (Favaro et al., 2019). In this study we can observe that the Fleischman strain throughout the cycles showed a marked loss in the accumulation of ethanol in relation to the Pedra-2 and FT858 yeasts, which showed better ethanol productivity throughout the fermentation cycles, thus highlighting the importance of choosing yeast to be used in the biofuel production process.

The stress of the fermentation medium imposes extreme conditions on the yeasts that can affect the fermentation performance and interfere with the final production of ethanol (Favaro et al., 2019). This occurs, either as a function of the associated stress factors or their intensity, causing the activation of the stress response mechanisms of these microorganisms, triggering the interaction of several genes, heat shock proteins (HSP), osmotic regulators and enzymes. oxidative (Auesukaree, 2017).

Thus, the choice of yeast is important, since ethanol production depends solely on its tolerance and response to the stress factors

Tab. 1. Ethanol production from industrial yeasts under stress conditions by fermentation cycles.

Fermentation cycle	Ethanol production % (v.v^{-1}) ±SD		
	Fleischmann	Pedra-2	FT858
1	7.7 ± 0.1	9.0 ± 0.1	8.8 ± 0.2
2	4.5 ± 0.2	6.7 ± 0.2	6.5 ± 0.2
3	2.7 ± 0.1	3.9 ± 0.2	3.8 ± 0.2
4	0.6 ± 0.1	1.2 ± 0.1	1.0 ± 0.1

Standard Deviation: SD.

present in the fermentation medium. However, in the industrial process for the production of ethanol, the choice of a robust yeast is important, because it must stand out from the variations of the medium, without altering the fermentative characteristics, since recycling is a routine in industrial processes, where the yeasts are reused throughout the season. And the advancement of the 1EG first-generation ethanol production process undoubtedly requires yeast strains that are able to survive adverse conditions in the fermentation medium, maintaining the genetic and physiological integrity of these microorganisms as well as guaranteeing high ethanol productivity.

In this study, strains of *S. cerevisiae* industries were compared, which showed different responses in relation to the profile of DNA alteration and ethanol production, in the face of the action of fermentation cycles. However, during the fermentation process numerous stress factors are present and understanding the cellular mechanisms and the production of metabolites of industrial yeasts is an important tool to be analyzed and applied in biotechnological processes, because through this knowledge it is possible to select yeasts with characteristic more robust, to ensure the production of biomolecules with high added value.

Conclusions

The yeast Fleischmann presented a lower tolerance to the action of the fermentation cycles since he presented a greater amount of alterations in the deoxyribonucleic acid. The Pedra-2 and FT858 yeasts showed a smaller change in damage to the genetic material, showing better adaptation to the conditions of the fermentation medium and to the effect of the stress used.

The ethanol production of the analyzed yeasts, in the first fermentation cycle, was similar. However, there was a loss in the production of this metabolite in subsequent cycles, mainly for Fleischman yeast. The cell recycling process induced different responses in the strains, showing the importance of choosing the yeast to be used in industrial processes.

S. cerevisiae has several mechanisms of response to the stress factors of the industrial process, which induce cells to acquire tolerance and different physiological responses to each type of stress. Understanding which factors represent stress to this microorganism and how they adjust their cellular machinery in the face of these can bring improvements in relation to both the environmental and economic sustainability of biofuel plants.

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