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CIÊNCIA e NATURA

ISSN 2179-460X
OPEN ACCESS

Ci. e Nat., Santa Maria, v. 46, e83730, 2024 • https://doi.org/10.5902/2179460X83730
Submitted: 10/05/2023 • Approved: 14/03/2024 • Published: 06/09/2024

Biology Genetics

Stress factors and cytotoxic and genotoxic actions of ethanol in *Saccharomyces cerevisiae*

Fatores de estresse e ação citotóxica e genotóxica do etanol em *Saccharomyces cerevisiae*

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ABSTRACT

In industrial fermentation, Saccharomyces cerevisiae are exposed to different stress conditions. In this sense, the aim of this study was to evaluate the toxic action of ethanolic stress on Saccharomyces cerevisiae. Exploratory research was carried out on the stress factors that cause injuries in yeast. Fermentation tests were conducted with the Fleischmann® and Pedra-2 strains, cultivated in sugarcane juice at 22º Brix and pH 5.0, adding concentrations of 5, 10, and 15% of ethyl alcohol, and incubated at 30°C at 250 rpm for 10 hours. For the cytotoxic tests, 100 µl of samples were collected for evaluation of cell growth by spectrophotometric measurements at 570 nm, and 5 µl were dripped into Petri dishes containing 2% YPD solid medium and incubated at 30ºC for 72 hours for colony growth. For the genotoxicity test, the comet test was used with 0.5 µl of the samples added to slides previously prepared and subjected to electrophoretic running and subsequently stained in a 0.1% silver nitrate solution. 100 random nucleotides were evaluated, evaluating five DNA damage classes (0, 1, 2, 3, and 4) according to the intensity and pattern of genetic material entrainment. The results show that stress factors interfere with yeast performance. Fleischmann® showed sensitivity to ethanolic stress.

Keywords: Yeasts; Fermentation; Deoxyribonucleic acid

RESUMO

Na fermentação industrial, Saccharomyces cerevisiae são expostas a diferentes condições de estresse. Nesse sentido, o objetivo deste estudo foi avaliar a ação tóxica do estresse etanólico em Saccharomyces cerevisiae. Foi realizada uma pesquisa exploratória sobre os fatores de estresse que causam injúrias nas leveduras. Os ensaios de fermentação foram conduzidos com as linhagens Fleischmann® e Pedra-2, cultivadas em caldo de cana-de-açúcar a 22º Brix e pH 5,0, adicionando as concentrações de 5, 10 e 15% de álcool etílico e incubadas a 30ºC a 250 rpm por 10 horas. Para os testes citotóxicos foram

coletadas 100 µl das amostras para avaliação do crescimento celular por medidas espectrofotométricas a 570 nm e 5 µl foram gotejados em placas de Petri contendo meio sólido YPD 2% e incubadas a 30ºC por 72 horas para o crescimento das colônias. Para o teste de genotoxicidade foi utilizado o teste do cometa com 0,5 µl das amostras adicionadas a lâminas previamente preparadas e submetidas a corrida eletroforética e posteriormente foram corados em solução de nitrato de prata a 0,1%. Foram avaliados 100 nucleotídeos aleatórios avaliando cinco classes de dano ao DNA (0, 1, 2, 3 e 4) segundo a intensidade e padrão de arraste do material genético. Os resultados mostram que os fatores de estresse interferem no desempenho das leveduras. A Fleischmann® apresentou sensibilidade ao estresse etanólico.

Palavras-chave: Leveduras; Fermentação; Ácido desoxirribonucleico

1 INTRODUCTION

Saccharomyces cerevisiae has aroused interest for use in different experimental models. These are yeast-like fungi with a high capacity for the production of numerous compounds and applicability. These microorganisms have been used by mankind since ancient times in fermentative processes for the production of feed, baking, and beverage manufacturing, among others (Parapouli et al., 2020). The versatility and adaptation to biotic and abiotic environmental conditions presented by these microorganisms (Bernardi & Wendland, 2020) are being widely explored and are crucial for their use in different biotechnological processes.

The characteristics present in these microorganisms include rapid cell duplication, easy adaptation to the conditions of the culture medium, it also has efficiency in sporulation and the possibility of cross-hybridization, being, in this sense, ease to sort and genetically transform. According to Opalek and Wloch-Salamon (2020), this microorganism has been extensively studied, and there is a wealth of biological information about it available in web databases. This information covers its functional aspects and transcriptional regulation, facilitating the analysis of gene expression. Yeastract (2023) also provides valuable insights into transcriptional regulators and consensus tracking in this organism.

The genome of *S. cerevisiae* is formed by S288C composed of 12.07 Mb of chromosomal DNA, 85 kb of mitochondrial DNA and 6.3 kb of plasmids. This genome has 6604 open reading frames (ORFs) with 79% of the ORFs verified, 11%

uncharacterized and 10% considered doubtful, with 1786 of the ORFs still annotated for unknown function, as highlighted by Pretorius and Boeke (2018) and Belda et al. (2019). This microorganism can be easily transformed through basic and molecular genetic techniques or even changes in metabolic pathways, due to its budding capacity and cell viability, the size and compactness of its genome favour the distinction of natural or artificially evolved populations by sequencing medium (Gopalakrishnan and Winston, 2019). These are promising microorganisms to be used in different industrial processes such as fermentation.

According to Mavrommati et al. (2022), industrial fermentation is considered an unfavourable environment for the physiological conditions of yeasts, as mismatches can occur in the fermentative environment, resulting in several disturbing factors that are widely known as stress factors. These factors intersperse and induce physiological cell responses and metabolic changes.

However, changes that occur in fermentation vats, such as high temperature, osmotic pressure, pH variations, cell recycles and high alcohol content, can also induce cellular responses and interfere with the final yield of the product, in addition to causing toxicity in these microorganisms (Gomes et al., 2021; Grellet et al., 2022).

It is a fact that temperature is one of the most severe stress factors for yeast cells, especially when it comes to the fermentation process. These microorganisms have an ideal temperature range ranging from 28 to 32ºC (Santos et al., 2022). Yeasts respond to stress factors in different ways, at higher temperatures, for example, the synthesis of heat shock proteins occurs, which alters the composition of the cell membrane (Plante et al., 2023).

The stress factors influence the modulation activity of ion exchange processes, inducing the production of glutathione enzymes and superoxide dismutase, resulting in the production of trehalose and glycerol to the detriment of ethanol (Naghshbandi et al., 2019; Eigenfeld et al., 2021), notably the presence of high concentrations of ethanol or other chemical compounds in the fermentation medium can lead to loss of

yeast fermentation efficiency or even cell death (Eardley and Timson, 2020).

The association of high levels of ethanol and temperatures accelerate toxicity in yeast leading to loss of viability, induction of flocculation and increased mutations (Walker & Basso 2020). To maintain cellular integrity, concerning these exogenous, yeast cells use response pathways, which include activation of proteins and enzymes related to protein folding and degradation, deoxyribonucleic acid repair, antioxidants, alcohol metabolism, trehalose synthesis, as well as other metabolic pathways (Auesukaree, 2017).

Some studies report the action of multiple stress factors on *S. cerevisiae* and their bioactivities, discussing their influence on the metabolism of these microorganisms. However, few reports discuss the influence of stress factors in isolation. Given the above, the study aims to evaluate the action of stress factors and the toxicity of ethanol in strains of *Saccharomyces cerevisiae*.

2 MATERIALS AND METHODS

2.1 Study location

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

2.2 Major stress factors for yeast

Exploratory research was carried out regarding stress factors in yeast, mainly in fermentation processes. Articles published between the years 2018-2023 were used. The search used the keywords “Yeast AND Stress”. The articles were evaluated for their content and relevance to the present study. After sorting, articles that discussed specifically *S. cerevisiae* yeasts were considered.

2.3 Yeasts used, cultivation and sample preparation

The yeasts used throughout this study were *S. cerevisiae* Fleischmann® acquired commercially and Pedra-2, acquired from the company LNF Biotecnologia Aplicada, located in Bento Gonçalves – RS.

These microorganisms were activated with a pre-inoculum with 2% YPD liquid medium based on yeast extract (1% p v^{-1}), peptone (2% p v^{-1}), dextrose (2% p v^{-1}), which was added in 125 mL Erlenmeyer flasks. Flasks were inoculated with 0.10 grams of yeast and incubated for 10 hours at 30°C at 250 rpm. After the incubation period, the cells were recovered by centrifugation at 800 rpm for 20 minutes, resuspended and washed three times with sterile saline solution (0.85% NaCl). The obtained biomass was promptly inoculated in the fermentation medium that was previously prepared with sugarcane juice at a concentration of 22º Brix and pH 5.0, which was added in 125 mL Erlenmeyer flasks. Concentrations of 5, 10 and 15% ethyl alcohol (99.5% PA) were added to the flasks. The flasks were incubated at 30°C for 10 hours at 250 rpm. After this period, 100 µl samples were collected and washed twice consecutively with cold ultrapure water, resuspended in Sorbitol-S buffer and stored in a freezer until use.

2.4 Cytotoxicity Test

Evaluation of biomass production and cell growth analyses were performed using spectrophotometric measurements at 570 nm, correlated with a calibration curve according to the method by (Batistote et al., 2010).

The samples submitted to the fermentation assays were collected with the aid of a micropipette and 5 µL dripped into Petri dishes containing the solid medium 2% YPD based on yeast extract (1% p v^{-1}), peptone (2% p v^{-1}), dextrose (2% p v^{-1}), agar (2% p v^{-1}), and incubated at 30ºC for 72 hours. The data were analyzed concerning the cell growth capacity in the plates at different ethanol concentrations and temperatures.

2.5 Genotoxicity Test

Analysis of damage to deoxyribonucleic acid cells were carefully collected and 0.5 µl was added to Eppendorf, which was resuspended in ice-cold S buffer (1M Sorbitol and 25mM KH_2PO_4), 70 µl of 0.5% low melting point agarose (LMP) and 2 mg mL^{-1} of Liticase enzyme (Sigma-Aldrich) were added to the Eppendorf. Then, aliquots of this suspension were dispensed onto the slides (previously coated with 1% normal melting point agarose (NMP) and dried at room temperature, the second layer was in 0.5% NMP agarose solidified on ice) which were covered coverslips and incubated at 30°C for 1 hour and 30 min. After this period, the slides were placed at a low temperature (4ºC) for enzyme inactivation, and the coverslips were removed. The slides were then immersed in 0.5% NMP agarose and subsequently incubated in ice-cold lysis solution (30mM NaOH, 1M NaCl, 0.1% N-lauroylsarcosine, 100mM DMSO, 1% Triton-X100) for 1 hour in the absence of light. The slides were immersed in the running buffer in an electrophoretic tank for 30 min, 25V, and 300 mA. After electrophoresis, the slides were incubated in a neutralization buffer (400 mM Tris-HCl, pH 7.5) for 15 min, washed and dried at room temperature, fixed in a fixative solution (15% acetic acid, 5% zinc sulfate and 5% glycerol) and stained in a staining solution (5% calcium carbonate, 0.1% ammonium nitrate, 0.1% silver nitrate, 0.25% tungstosilicic acid and 0.15% formaldehyde). Then, washed with distilled water and submerged for 5 min in a stop solution (1% acetic acid), and 100 nucleoids were randomly selected by optical microscopy and analyzed within five DNA damage classes (0, 1, 2, 3 and 4) according to the intensity and pattern of carryover of the degraded genetic material where 0 refers to the lowest degradation level and 4 the highest. All analyses were performed in triplicate.

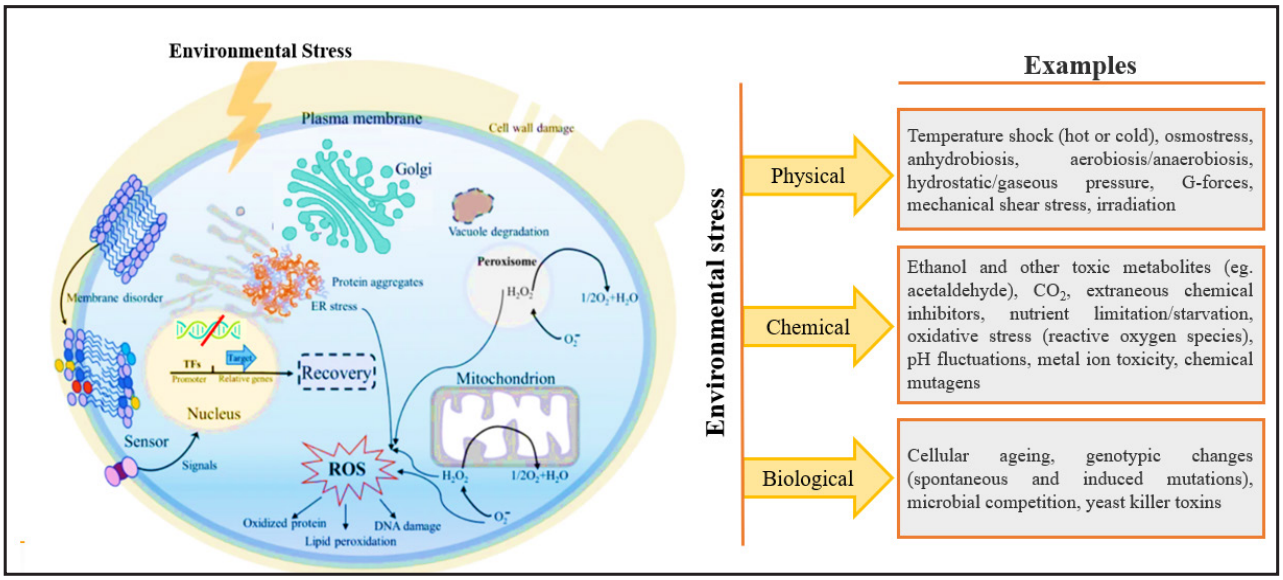
2.6 Statistical analyzes

The results were analyzed with Excel 2016 software with mean followed by standard deviation and graphs plotted in OriginLab 8.

3 RESULTS AND DISCUSSION

Industrial fermentation processes are susceptible to several intrinsic and extrinsic factors that can act on yeasts and interfere with their fermentation performance. Stress factors can cause different responses, considering that each yeast strain has peculiar characteristics and even being of the same genus, they can have different behaviour depending on the synergy, intensity and time of exposure to these factors. In industrial fermentation, the factors that can negatively influence the fermentative performance of yeasts are physical, chemical and biological (Figure 1).

Figure 1 – Stress factors that directly affect the physiology of industrial yeasts



Source: Adapted from Lin et al. (2022) and Walker and Walker (2018)

According to Lin et al. (2022), in the fermentation process for the production of beverages or biofuels, the factors related to stress to yeasts are ethanol toxicity, mistress and pH and temperature oscillations. These factors can negatively affect yeast growth and metabolic activities, either as individual stresses or when combined. Walker and Basso (2020) point out that even yeasts are acidophilic, the pH should be between 5.0 to 5.5 at the beginning of the fermentation process, if it is lower it can lead to a slower process susceptible to contamination.

High concentrations of sugars can affect the yield of fermentation by the yeast as it causes osmotic pressure to the cells (Tse et al., 2021), the same problem occurs when there is a shortage of nutrients in the fermentation medium as well as low concentrations of fermentable sugars and deficiency complementary nutrients such as amino acids (Zazulya et al., 2020). According to Ceccato-Antonini (2018), the presence of contaminants such as bacteria and wild yeasts is also considered a stress factor, since there is competition for nutrients and the formation of compounds that can inhibit both the growth and the functional metabolism of the plants. yeasts.

The synergism between stress factors can lead to the accumulation of reactive oxygen species (ROS) in yeast cells, causing oxidation, which influences their viability. The accumulation of ROS can occur during cell propagation or even be induced by the presence of environmental and chemical contaminants, or by metabolites generated by the yeast itself. Oxidation is among the main causes of cellular dysfunctions (Wang et al., 2022). The presence of reactive radicals interacts directly with organelles and membranes, causing cell death (Câmara Jr & Sant'Ana, 2021).

During the process of industrial production or propagation of yeasts, under normal physiological conditions, they have an intracellular homeostatic balance that regulates the level of free radicals resulting in a stable intracellular redox environment (Cui et al., 2020). Under adverse conditions in the presence of stress factors, homeostasis is disturbed, with oxidative stress occurring, which induces the activation of defence mechanisms to maintain basal ROS levels, which interact at different levels, being grouped according to enzymatic and non-enzymatic pathways, leading them to the transcription of genes that encode heat stress proteins, in addition to expressing genes responsible for activating glutathione and thioredoxin biosynthesis (Picazo & Molin, 2021; Moreno et al., 2019).

Enzymatic pathways constitute the primary defences that facilitate the removal or promote repair of oxidative damage. The main enzymes are catalase (CAT), superoxide dismutase (SOD), the glutaredoxin family and glutathione peroxidase

according to Harre et al. (2018). The non-enzymatic comprise small molecules that sequester radicals neutralizing them (Faulkner, Maksimovic & David, 2021). ROS cause several problems in *S. cerevisiae*, such as changes in the composition of the lipid bilayer resulting in lipid peroxidation, which is correlated with membrane disintegration and cell death (Sunyer-Figueres et al., 2020).

The analysis of the cytotoxic action of different concentrations of ethanol in the production of biomass and cell growth demonstrated that the yeast Fleischmann® has a lower tolerance to this compound, considering that with the increase in the concentration of ethanol, there was a decrease in the production of biomass of this yeast strain to the Pedra-2 strain (Figures 1 A and B). Possibly the yeasts suffered the toxic action of ethanol concentrations and showed different responses, the Fleischmann® yeast showed significantly inhibited cell growth in the presence of the highest concentrations of ethanol. Ethanol tolerance is an important factor to be considered in *S. cerevisiae*.

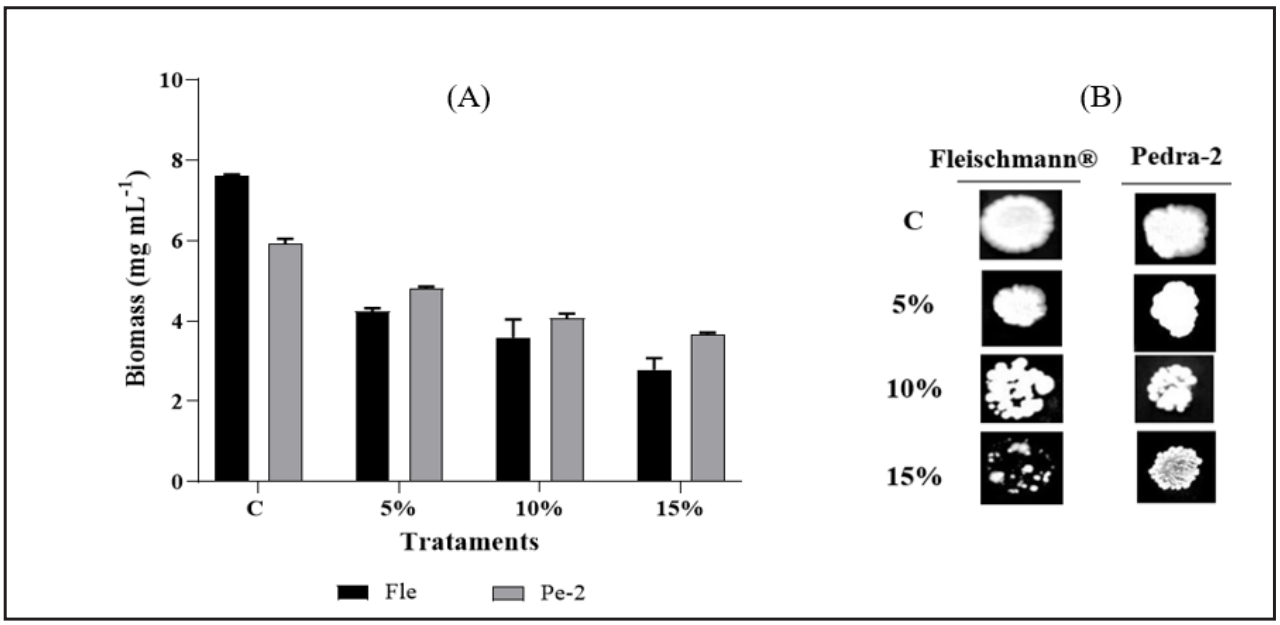
Resistance to stress factors varies among these microorganisms and is related to the type of stress, synergism and exposure time (Lin et al., 2022). These factors act in isolation or association, as a rule, they trigger different levels of response in *S. cerevisiae* to maintain their cellular functions, with this, the fermentative performance and cell viability of these microorganisms are affected. According to Vamvakas and Kapolos (2020), yeast strains show expressive responses to ethanol tolerance.

Although the fermentation process for the production of fuel ethanol is very simple with high productivity rates, it is highly disadvantageous for yeast due to the stress factors throughout the process, which can induce changes in its numerous metabolic pathways, leading to significant loss of fermentation efficiency. However, the stress response mechanisms and their interactions need to be better understood to ensure the cellular integrity of the yeast and therefore better ethanol production.

There are countless studies regarding the exploitation of *S. cerevisiae*, and its applicability in biotechnological processes. Some are related to physiology, proteomics,

metabolism, genetic manipulation and more recent synthetic biology approaches. Such information supports the knowledge and development of more robust strains tolerant to stress factors to be used in biotechnological processes such as ethanol production.

Figure 3 – Evaluation of the cytotoxic effect on biomass production and cell growth, of yeast Fleischmann® (A) and Pedra-2 (B), under the action of different concentrations of ethanol at a temperature of 30°C for 10 hours of fermentation



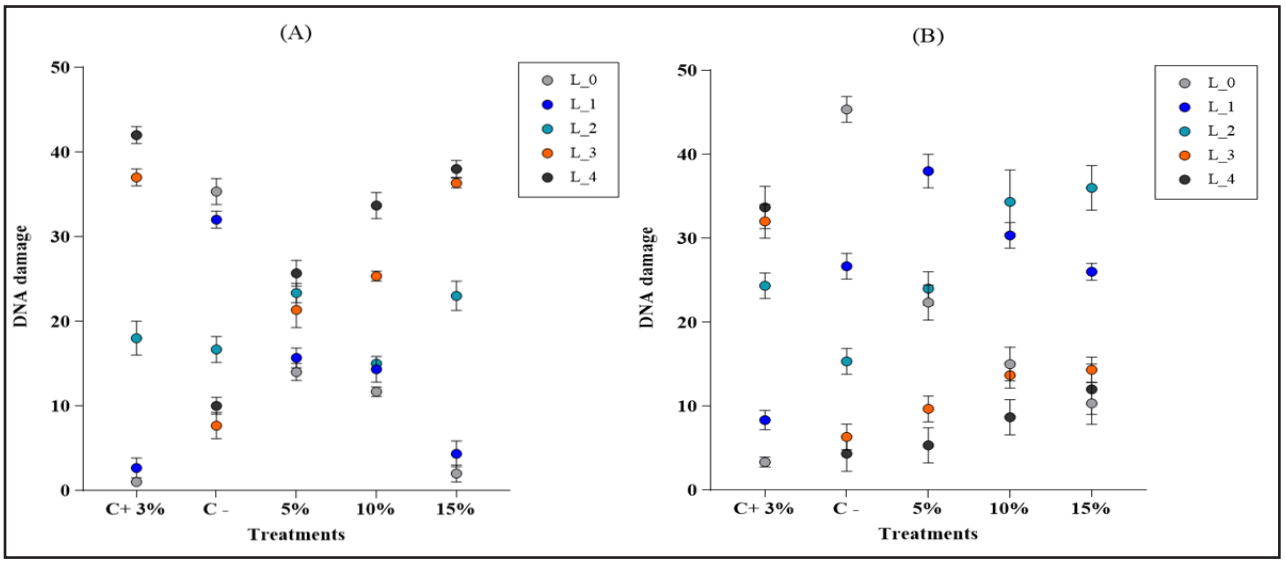
Source: Survey data. Mean of three readings followed by ± sample standard deviation

In this study, the analysis of the damage caused to deoxyribonucleic acid by different concentrations of ethanol in industrial strains showed that the yeast Fleischmann® presented a greater number of damages to the DNA, demonstrating less tolerance to ethanol. However, the yeast Pedra-2 showed a smaller number of damages to the DNA concerning the used concentrations (Figures 4A and 4B). The adverse environmental fluctuations of the fermentation process require the use of robust and adequate yeasts that resist the action of ethanol, aiming to guarantee productivity and cellular integrity.

Studies conducted by Mascarenhas et al. (2022), evaluating the genotoxic effect of cell recycling in the industrial S. cerevisiae strains Fleischmann, Pedra-2, and

FT858, which were cultivated in sugarcane juice at 30°C and exposed to consecutive fermentative cycles, observed that the Fleischmann yeast showed greater sensitivity to recycling. The data showed that fermentative cycles can affect these microorganisms and cause damage to genetic material. These authors emphasize that this may have occurred due to the phenotypic characteristics of the yeasts.

Figure 4 – Analysis of genotoxicity concerning the action of different concentrations of ethanol on deoxyribonucleic acid in the Fleischmann® (A) and Pedra-2 (B) strains, at a temperature of 30°C for 10 hours of fermentation



Source: Elaborated with the data obtained in the research. The DNA damage levels expressed and followed by the damage intensity (L_0; L_1; L_2; L_3 and L_4)

It is a fact that for the industrial fermentation process, more robust strains are required that have high tolerance to stress factors. *S. cerevisiae* are the most used yeasts in different bioprocesses, particularly these microorganisms are attractive due to their characteristics regarding safety, not being pathogenic, and easy manipulation (Mitsui, Yamada and Ogino, 2019). The result showed that the genotoxic test can be a valuable tool for measuring the damage caused to cells during the industrial process by the action of stress factors. The comet test can help elucidate the molecular response

mechanisms to stress tolerance in *S. cerevisiae*.

In addition to this test, there are other techniques that explore the genetic diversity of industrial yeasts and that can lead to the construction of new strains through synthetic engineering, metabolic engineering, and genetic manipulation, contributing to the development and improvement of potent and adapted yeast strains for ethanol production. One of these techniques is the CRISPR system, which according to Mitsui, Yamada and Ogino (2019), has the possibility of inserting multiple genes.

These same authors point out that this system has been relevant and can be employed in *S. cerevisiae*, in which conventional methods of genetic recombination are already well established. This technology has been an alternative, used in the genetic modification of bacteria and yeasts for the production of bioethanol, biobutanol, biodiesel, among others (Shanmgam et al, 2020; Arias et al., 2021). Amidst various environmental problems, there is a growing need for the use of microorganisms, such as *S. cerevisiae*, which are considered eco-friendly biofactories of compounds.

However, stress factors during the fermentative process are complex and reflect the synergistic action of many genes, making it difficult to design yeasts with stress tolerance. In this sense, our data may corroborate with molecular biology techniques, especially regarding studies using selected yeasts with high fermentative performance that can be used in bioprocesses.

4 CONCLUSIONS

The mechanisms of responses to stress factors vary between strains, as each has specific responses. Yeasts of the same genus can react in different ways depending on the synergy, intensity and duration of exposure to these factors. In industrial processes, stress factors impair the fermentative performance of yeasts.

The Fleischmann® yeast is more susceptible to the action of ethanol since there was a reduction in the biomass concentration in the presence of higher concentrations of ethanol. Cell growth was also impaired. However, the Pedra-2

yeast showed the best response under the analyzed conditions.

The evaluation of deoxyribonucleic acid damage caused by different ethanol concentrations revealed that the Fleischmann® yeast showed less tolerance to this compound than the Pedra-2 strain.

The data demonstrate that the comet test can be used to assess damage to deoxyribonucleic acid caused by stress factors in yeast strains during the industrial process. Such information may help to unravel the complexity of the physiological and molecular mechanisms of response in S. cerevisiae to stress factors.

ACKNOWLEDGEMENTS

The Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul/FUNDECT, Financiadora de Inovação e Pesquisas/FINEP, Conselho Nacional de Desenvolvimento Científico e Tecnológico/CNPq; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior –Brasil/CAPES.

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How to quote this article

Mascarenhas, M, S., Mueller, L. P., & Batistote, M. (2024). Strees factors and cytotoxic and genotoxic actions of ethanol in *Sacharomyces cerevisiae*. *Ciência e Natura*. 46, e83730. https://doi.org/10.5902/2179460X83730