

The effects of thermal and ethanolic stress in industrial strains of

Saccharomyces cerevisiae

Os efeitos do estresse térmico e etanólico em linhagens industriais de

Saccharomyces cerevisiae

Los efectos del estrés térmico y etanólico en cepas industriales de

Saccharomyces cerevisiae

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Abstract

Saccharomyces cerevisiae is exceptional microorganisms used in biotechnological processes, mainly in the ethanol production chain. Studies of the cellular responses of industrial yeasts under ethanolic and thermal stress in an association are still incipient. This study aimed to evaluate the action of thermal and ethanolic stress in industrial strains of *Saccharomyces cerevisiae* under different temperatures and concentrations of ethanol, to understand whether these factors influence ethanol production. For cytotoxicity and genotoxicity tests, yeasts were grown in 2% YPD medium incubated for 10 hours at 250 rpm. After growth, the

samples were grown in sugarcane juice in concentrations of 5, 10 and 15% ethanol and incubated at 30 and 40 °C. In Petri dishes containing the solid medium YPD 2% the yeasts were dripped and incubated for 72 hours the cytotoxic action was analyzed by cell growth and genotoxicity through the comet assay and ethanol production by gas chromatography. Cell growth occurred in all conditions, however, at 30 °C there was inhibition in 10% (v v⁻¹) of ethanol being potentiated in 15% (v v⁻¹), at 40 °C. The genotoxicity analysis showed an induction of DNA damage in yeasts, however, the FLE yeast was the one with the highest DNA damage index. The yeast Pedra-2 was more tolerant and produced more ethanol, showing to be a tolerant strain concerning the analyzed fermentative interferences.

Keywords: Fermentation; Yeasts; Stress condition.

Resumo

Saccharomyces cerevisiae são microrganismos excepcionais utilizadas nos processos biotecnológicos, principalmente na cadeia produtiva do etanol. Estudos das respostas celulares das leveduras industriais sob estresse etanólico e térmico em associação ainda são incipientes. Este estudo objetivou avaliar a ação do estresse térmico e etanólico em linhagens industriais de *Saccharomyces cerevisiae* sob diferentes temperaturas e concentrações de etanol, visando entender se estes fatores influenciam na produção de etanol. Para os testes de citotoxicidade e genotoxicidade as leveduras foram crescidas no meio YPD 2% incubadas por 10 horas a 250 rpm. Após crescimento as amostras foram cultivadas em caldo de cana nas concentrações de 5, 10 e 15% de etanol e incubadas a 30 e 40 °C. Em placas de Petri contendo o meio sólido YPD 2% foram gotejadas as leveduras e incubadas por 72 horas. A ação citotóxica foi analisada pelo crescimento celular a genotoxicidade por meio do ensaio cometa e a produção de etanol por cromatografia gasosa. Ocorreu crescimento celular em todas as condições, contudo, em 30 °C houve inibição em 10% (v v⁻¹) de etanol sendo potencializada em 15% (v v⁻¹) a 40 °C. A análise de genotoxicidade evidenciou indução de danos ao DNA das leveduras, porém a levedura FLE foi quem apresentou maior índice de lesões ao DNA. A levedura Pedra-2 foi mais tolerante e produziu mais etanol, mostrando ser uma linhagem tolerante em relação aos interferentes fermentativos analisados.

Palavras-chave: Fermentação; Leveduras; Condição de estresse.

Resumen

Saccharomyces cerevisiae son microorganismos excepcionales utilizados en procesos biotecnológicos, principalmente en la producción de etanol. Estudios de las respuestas

celulares de levaduras industriales bajo estrés etanólico y térmico en asociación son todavía incipientes. El objetivo fue evaluar la acción del estrés térmico y etanólico en cepas industriales de *Saccharomyces cerevisiae* a diferentes temperaturas y concentraciones de etanol, con el fin de comprender si estos factores influyen en la producción de etanol. Para las pruebas de citotoxicidad y genotoxicidad, las levaduras se cultivaron en medio YPD al 2% incubados durante 10 horas a 250 rpm. Las muestras se cultivaron en jugo de caña de azúcar en 5, 10 y 15% de etanol y se incubaron a 30 y 40 °C. En placas de Petri con el medio sólido YPD 2% se gotearon y se incubaron durante 72 horas, se analizó la acción citotóxica por crecimiento celular y genotoxicidad mediante el ensayo cometa y la producción de etanol mediante cromatografía de gases. El crecimiento celular ocurrió en todas las condiciones, a 30 °C hubo inhibición en 10% ($v v^{-1}$) de etanol siendo potenciado en 15% ($v v^{-1}$) a 40 °C. El análisis de genotoxicidad mostró una inducción de daño en el ADN en las levaduras. La levadura FLE fue la que presentó el mayor índice de daño en el ADN. La Pe-2 fue más tolerante y produjo más etanol, demostrando ser una cepa tolerante en relación a los interferentes fermentativos analizados.

Palabras clave: Fermentación; Levaduras; Condición de estrés.

1. Introduction

Saccharomyces cerevisiae yeasts are exceptional microorganisms that, through their dynamic cellular activities, have been used since ancient times by humans in various industrial and biotechnological processes. Mainly due to its skilful ability to convert, through the fermentative route, sugars into metabolites of industrial interest such as ethanol. This is considered a cornerstone of the biofuels sector, since it contributes significantly to the energy matrix of several countries such as Brazil and the United States, in addition to being an alternative to petroleum fuels (Gallone et al., 2016; Azhar et al., 2017).

However, throughout the ethanological processes, yeasts face several stress factors that act simultaneously inducing different responses, examples are variations in temperatures, progressive decrease in nutrients, high concentrations of ethanol, changes in pH and osmotic pressure, which require adjustments to metabolic pathways and cause changes in physiological patterns, which can result in decreased viability, cell growth, interfere with fermentative efficiency and, consequently, ethanol production (Stanley et al., 2010; Tesniere et al., 2013; Martins et al., 2017).

The shock caused by temperatures above 32 °C causes cellular disturbances 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 (Vargas-Trinidad et al., 2020). Thus, the fermentation of ethanol at high temperatures is desirable, since it has advantages such as the reduction of contamination and reduction of cooling costs (Abdel-Banat et al., 2010), although they can cause physiological changes in yeasts.

Ethanol varies approximately between 8% and 10% in the fermentation medium (Cray et al., 2015) is an alcohol formed by two carbons which, due to its short alkene chain and the hydroxyl group, is soluble in water and lipids, and thus it can cross the plasma membrane producing an increase in fluidity and alteration of the membrane permeability, in addition to modifying the biosynthesis of macromolecules and causing greater production of heat shock proteins and increased mutations (Morard et al., 2019; Hu et al., 2009). In addition, the accumulation of extracellular ethanol causes a restriction in the excretion of intracellular ethanol that causes a suppressed production effect (Cao et al., 2020).

The multiple industrial stressors to which yeasts are exposed can cause damage to deoxyribonucleic acid-DNA, cease the cell cycle, cause abnormal enzyme production, among other molecular changes (Brown & Kobor, 2019). In addition to these external agents, cell metabolism itself generates thousands of daily lesions, which alter the double helix of DNA and cause instability of the genome that can compromise replication and transcription and consequently result in mutations during the S phase, or chromosomal aberrations when there are breaks in the DNA (De Andrade Lima, 2015). Cytotoxic analyzes are essential to prevent harmful interference to yeasts in the industrial context, in addition to identifying the harmful capacity of compounds harmful to these living beings. Genotoxicity must also be taken into account since it covers processes that alter the chemical and physical structure of DNA (Santos, 2019).

The ethanol tolerance phenotype is complex and also influenced by external factors, mainly as temperature, in many studies, there is an inherent interest in the particularity of responses involving similar genes that thermal and ethanolic stress induce and some even demonstrate that tolerance to a factor is dependent on another (Fujita et al., 2006; Caspeta et al., 2019). In this sense, research is aimed at understanding the mechanisms of tolerance of these microorganisms in the face of thermal and ethanolic stress. But, most of these studies look at factors separately and not their associations (Riles & Fay, 2019). Therefore, this theme is still not completely elucidated because it is a characteristic of multiple loci and their related genes are randomly distributed in the genome (Giudici et al., 2005).

In this bias, studies are becoming important to understand the respective effects of the action of ethanol associated with high temperature and how they trigger lesions in yeast cells and genetic material. Thus, this study aimed to evaluate the action of thermal and ethanolic stress in industrial strains of *Saccharomyces cerevisiae* at different temperatures and concentrations of ethanol, aiming to understand how these stress factors can cause cellular changes and consequently influence the production of ethanol, an important biotechnological product.

2. Methodology

2.1. Research location

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

2.2. Microorganisms Used

The microorganisms used for this study were *Saccharomyces cerevisiae* FT-858 obtained from the company Fermentec located in Piracicaba - SP, Pedra-2 (Pe-2) acquired in the company LNF Biotecnologia Aplicada, located in Bento Gonçalves - RS and the yeast Fleischmann® (FLE) acquired commercially.

2.3. Pre-inoculum

The pre-inoculum consisted of 2% YPD medium containing 1.0% (w v⁻¹) of yeast extract; 1.0% (w v⁻¹) peptone; 2.0% (w v⁻¹) glucose, sterilized in an autoclave at 120 °C for 20 minutes, in which 0.10 grams of lyophilized yeasts were inoculated and incubated at 30 °C for 10 hours at 250 rpm. After this period, the cells were collected by centrifugation, resuspended and washed with sterile saline.

2.4. Fermentative Condition

For the fermentation experiment, 50 mL of the sterile cane juice was added in 125 mL Erlenmeyer flasks. After 10 hours of growth, the biomass obtained was inoculated in the

fermentation medium in the presence of the stressor ethyl alcohol in the concentrations of (5%, 10% and 15%) and the samples were incubated at 30 °C and 40 °C for 10 hours at 250 rpm . For the negative control, there was no addition of ethanol and for the positive control, in the genotoxicity test, 3% hydrogen peroxide was added.

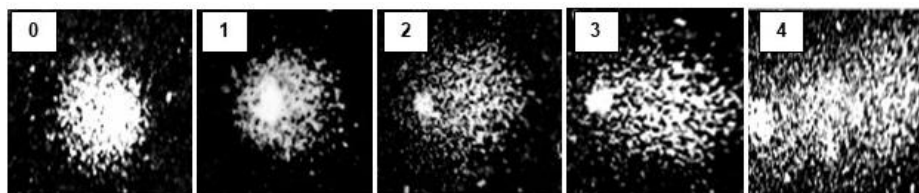
2.5. Cytotoxicity Test

The stress-induced samples, as described in item 2.4, were added in a Petri dish containing the solid medium YPD 2%, 5 µl of the samples were dripped and incubated at 30 °C for 72 hours. The results were analyzed concerning cell growth capacity in the face of different concentrations of ethanol and temperature.

2.6. Genotoxicity Test

The comet assay was conducted according to Mueller et al. (2019), 100 nucleoids were randomly selected by optical microscopy, analyzed within five classes of DNA damage (0, 1, 2, 3 and 4) according to the intensity and drag pattern of the degraded genetic material, where 0 refers to the lowest level of degradation and 4 to the highest (Figure 1). The DNA damage index (DDI) was calculated according to the methodology of Fernandes, Bustos-Obregon & Salvadori (2015).

Figure 1. Sequence of images showing yeast cells and the damage induced to DNA by stress factors classified in (0, 1, 2, 3 and 4).



Source: Authors.

2.7. Quantification of Ethanol

The analysis of the ethanol concentration was determined with a gas chromatograph (CG 3900) with flame ionization detector (Varian), using a 30m long fused silica capillary column (ZB-5). The chromatographic condition used was an injection volume of 1 µL, split ratio 1:20 and oven temperature of 90 °C. The samples were filtered through a 0.22 µm

ultrafilter and the injector and detector temperatures were 240 °C, according to the methodology of Batistote et al. (2010).

2.8. Data Treatment and statistical analysis

























The experiments analyze and data processing were performed in triplicate, treated with the aid of the programs, Microsoft Word, Excel for the construction of the descriptive text, tables and quantification. The graphs were plotted in the Origin 8 program.

3. Results and Discussion

3.1. Cytotoxicity

From the analysis of the cytotoxicity test concerning ethanolic and thermal stress, it was possible to observe that there was cell growth in all concentrations of ethanol and at both temperatures. However, at 30 °C the strains began to show an inhibition in cell growth from the concentration of (10% v v⁻¹) of ethanol and this effect was enhanced in (15% v v⁻¹). Even so, at a temperature of 40 °C, it is permissible to point out that yeasts have become more susceptible in general to the synergism of the action of ethanol and temperature, since we observed less growth among the colonies. However, it is worth noting that both at 30 °C and 40 °C, the yeast Pe-2 showed a higher tolerance profile, mainly about the higher concentration of the stressor agent, evidenced by the greater growth observed when compared to the other strains. In this sample, the FLE yeast showed greater sensitivity to the associated factors, especially in 15% ethanol. The FT-858 strain showed an intermediate growth profile with the other analyzed yeasts (Table 1). Probably, the yeasts FT-858 and Pe-2 showed a greater phenotype of tolerance to stress inducers, due to their high fermentative capacity and cellular vitality in adapting to fermentative niches, which demonstrates that such yeasts present themselves as important microorganisms to be used in biotechnological processes.

Table 1. Growth profile images of Fleischmann®, FT-858 and Pedra-2 yeasts colonies about ethanol concentrations, at 30 °C and 40 °C in 10 hours of fermentation.

Strains		Concentration of etanol			
		C	5%	10%	15%
FLE	30°C				
	40°C				
FT-858	30°C				
	40°C				
PE-2	30°C				
	40°C				

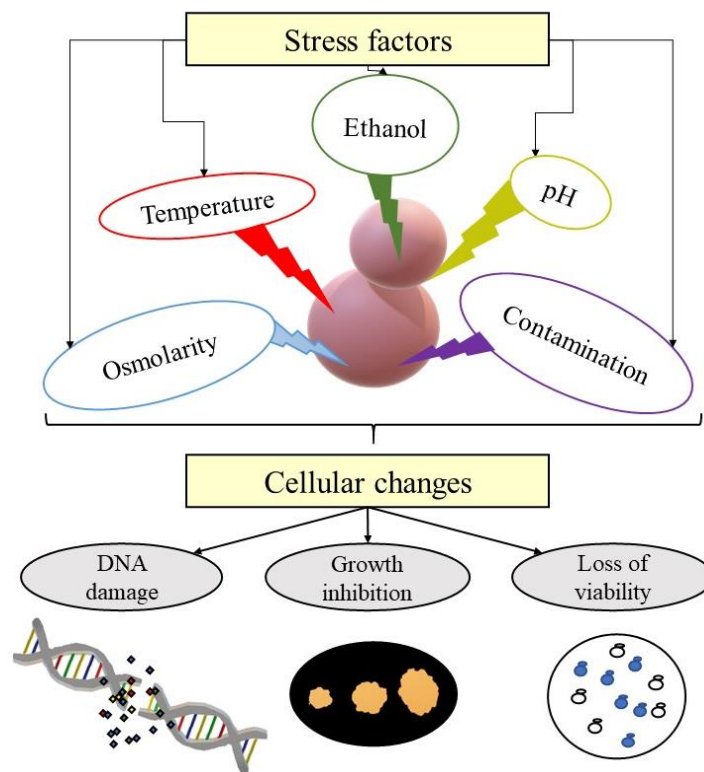
Source: Authors.

The survival of yeasts in the conditions of the industrial environment depends on a network of sensing and signal transduction pathways, which lead to the adjustment of the cell cycle, improves the profile of gene expression and as a consequence of metabolic activities. However, cellular responses are only obtained until adverse conditions become lethal (Saini et al., 2018). However, the biological design of the stress response goes far beyond just protecting cellular components but also prepares for a later harmful external factor of the same agent or of a different type (Swiecilo, 2016).

For Azhar et al. (2017) the diversity of yeast species in different niches is determined by their ability to metabolize different carbon sources and their nutritional selectivity exhibits great specialization for the habitat, which allows them to inhabit extreme environments as is the case. industrial fermentations. The increase in ethanol in the fermentation medium can cause growth inhibition and alter the cell viability of these microorganisms and this inability to grow in medium containing high alcohol content leads to the inhibition of ethanol

production, in addition to toxicity, high temperatures, pH and osmolarity are also recognized as tensions to be overcome during the process. Such an occurrence could be observed by the phenotypic profile presented by the yeast FLE, which proved to be more sensitive to the action of inhibitory agents. Figure 2 represents a schematic of how stress factors in the fermentative environment can influence yeast cell mechanisms, inducing responses to adverse conditions.

Figure 2. Scheme of stress factors present in the fermentative niche and their response mechanisms in *Saccharomyces cerevisiae*.



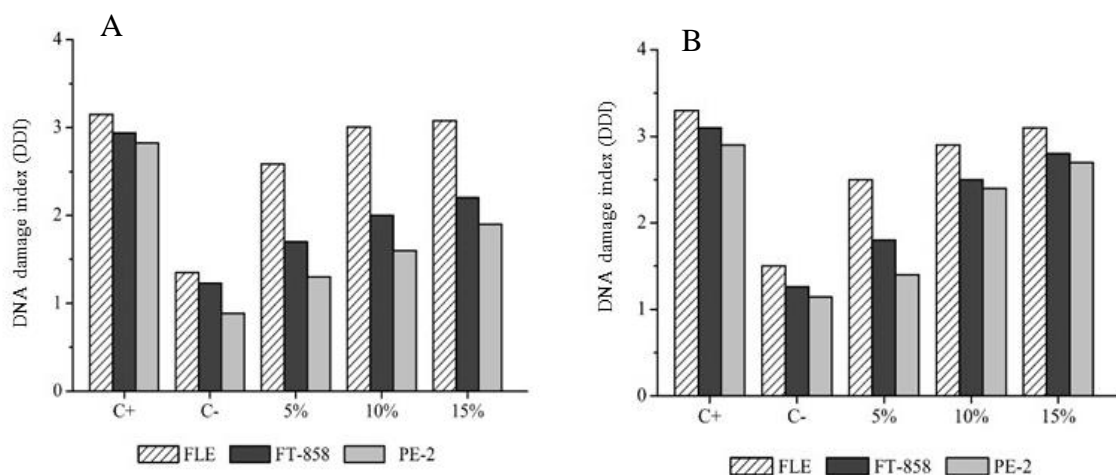
Source: Authors.

In the fermentative niche, stressors can limit the development of other microorganisms, as well as that of the yeast itself and in this context, this inhibition can be considered cytotoxic (Cray et al., 2013). Selected yeasts can tolerate an anoxic environment, with high temperatures and high alcohol content that is generated during industrial fermentations, better than wild yeasts and this fact can be considered a selective advantage. In this sense, high concentrations of ethanol in the fermentation medium can be potentially harmful (Goddard & Greig, 2015).

3.2. Genotoxicity

From the genotoxicity analyzes through the comet assay it was possible to observe that there was the induction of lesions in the deoxyribonucleic acid – DNA of the yeasts analyzed in all ethanol concentrations and at both temperatures. Despite this, at 30 °C the lowest injury rate was observed at 5% in the Pe-2 strain and the second-lowest for FT-858 at the same concentration, while the FLE yeast showed the greatest sample damage at a concentration of 15% at the same temperature. mentioned. At 40 °C yeasts showed a damage profile similar to that described for the previous temperature, however, with an increase in lesions in the concentrations of 10 and 15% mainly for the lines Pe-2 and FT-858, however, the FLE was more susceptible in relation to the other strains, especially in the highest concentration of ethanol (Figures 3A and 3B).

Figure 3. DNA damage index (DDI) of Fleischmann®, FT-858 and Pedra-2 industrial yeasts in relation to ethanol concentrations at 30 °C (A) and 40 °C (B) for 10 hours of fermentation.



Source: Authors.

Eventually, only the ethanol factor alone has already caused lesions sufficiently harmful to the cells, especially for the FLE strain, which showed greater sensitivity to this stress factor. However, the action of elevated temperature associated with a high concentration of ethanol was able to generate a higher damage index in the analyzed yeasts, which is probably related to a profile of distinct physiological responses that each yeast presents in relation to the separate damage and in synergism. It is worth mentioning that the toxic action of ethanol and the high temperature was also observed in the results presented of

the cell growth capacity in relation to the studied yeasts. The tolerance profile given to Pe-2 may be related to the fact that this strain has a lower amount of DNA-induced damage that characterizes a lesser genotoxic action of ethanol compared to this strain.

The changes that occur in the course of industrial fermentations, most of the times unfavourable, require adequate cellular responses and among the several existing mechanisms of cellular protection that are activated in response to stressful conditions, we can mention the cross-protection against different stresses, in which the cells exposed to a light dose of a stressor respond in a resistant way to larger and generally lethal doses of others (Swiecilo, 2016).

In addition, stress response elements (STREs) control responsive genes in response to different stresses, including heat shock and high concentrations of ethanol through transcriptional factors like Msn2p and Msn4p that modulate protein kinase-dependent gene expression (PKA), however, are activated in different ways and induce the transcription of a large battery of genes containing STRE, which will mediate the overall stress response in yeasts (Estruch, 2000; Saini et al., 2018). Studies by Riles & Fay (2019), analyzed the genetic basis of ethanol tolerance in *Saccharomyces cerevisiae* at elevated temperatures and showed that polymorphisms of some genes can cause sensitivity or tolerance to ethanol at high temperatures. As is the case with two amino acid substitutions in the SEC24 gene region, which underlies the locus that gives the yeast thermotolerant characteristics.

In this study, we can observe that the induction of the DNA damage index was more significant when the stress factors were associated in relation to the strains analyzed. This fact may be related to the hostile conditions that frequently occur during fermentation processes, causing yeasts to present intermittent physiological mechanisms of responses to such adverse conditions. Therefore, our data corroborate with the literature, because it was observed that yeasts had a functional profile of different inductions of genetic lesions in relation to the associated stressors. Possibly, the yeast response mechanisms are not a singular event, but the interactivity of several genes involved forming a systematic and complex network in the intracellular environment leading to cytological and genetic changes.

3.3. Ethanol Production

In the evaluation of the concentration of ethanol, it was possible to observe differences in the production profile of this compound concerning the yeasts at the analyzed temperatures. Higher production of this metabolite (9.0 v v^{-1}) was observed at a temperature of 30 °C for

yeast Pe-2, however, this strain also excelled in ethanol production at 40 °C, showing a profile of thermal and ethanol tolerance when compared other strains. The higher temperature affected the production of this by-product since there was a decrease in its concentration for all strains. However, it can be noted that the yeast FLE was the one with the lowest production (6.3 v v^{-1}), at a temperature of 40 °C. Possibly, the synergistic action of ethanol and temperature contributed negatively to the physiological profile of the production of this metabolite by the yeasts analyzed with its toxic action (Table 2).

Table 2. Concentration of ethanol from Fleischmann®, FT-858 and Pedra-2 lines in sugarcane juice during 10 hours of fermentation at temperatures of 30 °C and 40 °C.

Strains	Concentration of ethanol % (v v^{-1})	
	Temperature	
	30 °C	40 °C
FLE	7.5±0.09	6.3±0.14
FT-858	8.5±0.12	7.3±0.12
Pe-2	9.0±0.08	7.5±0.05

Average of three readings followed by \pm standard deviation of the samples. Source: Authors.

Among the yeasts selected for bioethanol production, the Catanduva-1 and Pedra-2 strains stand out, which are more resistant to fermentative recycling as well as to high concentrations of ethanol and temperature, being responsible for 60% of the Brazilian ethanol production. (Souza et al., 2018). And in this perspective, little evidence is available regarding the molecular mechanisms of ethanol tolerance (Navarro-Tapia et al., 2017) and the search for strains more tolerant to this and other stressors is a primary line of research within the area biotechnological, since more adapted strains can withstand the extreme environment imposed by industrial fermentations and dominate the fermentation process with excellence.

The yeast FLE strain has been used as an initiator of the fermentation process since it is cheaper and has a high biomass production, however, it has low viability and consequently inefficient sugar consumption of ethanol (Batistote et al., 2010). However, the yeast FT-858 has high fermentative performance, resistance to pH variations and tolerance to high concentrations of ethanol (Amorim et al., 2011), as well as its close relative Pe-2, which shows great aptitude in adapting to the harsh conditions of the vats fermentation (Amorim & Lopes, 2013). Its high fermentative profile can be related to several polymorphisms in its

homologous chromosomes, allowing great advantage in the capacity of yield and productivity (Argueso et al., 2009; Babrzadeh et al., 2012).

In this sense, our data corroborate with the literature, since it was possible to observe that the yeast FLE was the one that produced less ethanol in relation to stressors. And it obtained greater toxicity in relation to cell growth when compared to ethanol concentrations, especially at 10 and 15%. However, the Pe-2 strain showed less toxicity compared to ethanol, low DNA damage and higher production of this metabolite. In the fermentation niche, there are countless selective pressures imposed during the production of ethanol, which interposes and induce changes in cellular responses that can influence cell growth, sensitivity to the concentration of this metabolite and fermentative efficiency. Although ethanol is contradictory, it is an important industrial product, it is potentially toxic to yeasts because it induces severe cellular changes, which can limit the productivity of this biotechnological product.

4. Final Considerations

The evaluation of cytotoxicity showed that the action of the associated stress factors ethanol and high temperature acted synergistically, inhibiting cell growth. The genotoxicity test showed a higher rate of degradation in the DNA of the yeast Fleischmann®, which in turn was also more susceptible to the cytotoxic action of ethanol. The yeast Pedra-2 showed greater tolerance to the stressor and emerged as a robust strain to be used in biotechnological processes due to its response to stressors.

Regarding ethanol production, the Pedra-2 line obtained the highest production of this metabolite and Fleischmann®, the lowest. The yeast FT-858 showed fermentative performance similar to Pedra-2. From this, we can infer that such yeasts were more tolerant of fermentation interferents and have great potential to be used in fermentation processes.

The results pointed out in this study, show and encourage the relevance in using inexpensive and reproducible techniques already consolidated in other areas of science for application in the industrial scope in order to optimize the processes of ethanol production with the use of tolerant strains. In addition, these analyzes indicate that further studies are needed to identify stress mechanisms by fermentative interferents as well as their respective molecular targets that trigger cellular and physiological responses in yeasts.

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