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REGULAR ARTICLE

Ability of the *Saccharomyces cerevisiae* Y904 to tolerate and adapt to high concentrations of selenium

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Autor contribution
LAM and ASB: conceptualization; LAM and APMS: Methodology; LAM, GMFLL and EAS: Formal analysis; LAM, APMS, RPC, EAS and: GMFLL: Investigation; LAM, APMS, RPC and EAS: data curation; LAM: writing-original draft preparation; RPC, ASB, APMS, LAM: writing-review and editing; ASB: visualization, supervision and funding acquisition, resources.

Abstract

The alcoholic fermentation industry generates a large surplus of yeasts, which, in turn, have the ability to bioaccumulate minerals and enable their bioavailability after cell autolysis. Among these minerals, selenium (Se) stands out, which participates in the formation of antioxidant enzymes. The objectives of the work were to define the minimum and maximum concentration of Se that yeasts (*Saccharomyces cerevisiae* – Y904) support and the concentrations that they tolerate once adapted. To this end, a test of tolerance to Se was carried out, using treatments with different concentrations of Se. The adaptive process started at the maximum concentration obtained in the tolerance test of 60 μg mL^{-1}, with increasing addition of 6 μg mL^{-1}, reaching up to 246 μg mL^{-1} of Se. The macromorphological characteristics and number of colony forming units (CFU) were evaluated. It was identified that yeasts without adaptation grew on substrate containing up to 60 μg mL^{-1} of Se and those adapted, up to 246 μg mL^{-1} of Se. In addition to the reduction in yeast growth speed, from the concentration of 84 μg mL^{-1} of Se in the medium, morphological changes in colony color were observed. It is concluded that non-adapted yeasts support up to 60 μg mL^{-1} of Se and, after the adaptive process, they support 246 μg mL^{-1} of Se in the medium after the adaptive process, which adds value to the final product, and makes yeasts suitable for human nutrition as a supplement or even in the formulation of probiotics.

Keywords

Cultivation in high selenium; organic selenium; organominerals; selenium tolerance

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Introduction

Brazil is currently the second-largest producer of fuel ethanol in the world. The country generates approximately 33 billion liters per year (Pereira et al., 2020). In the ethanol production process, at the end of each fermentation cycle, there is a large surplus of yeasts of the *Saccharomyces cerevisiae* species, around 20 kg of yeasts per m^3 of ethanol produced, which generates about 660,000 tons (Desmonts, 1996). These single-celled microorganisms can transform sugars into ethanol, carbon dioxide, energy, and other by-products. In the ethanol production process, yeasts are used only as agents of biotransformation of sugar into ethanol, and after this biochemical reaction, they can be reused for other purposes, such as enrichment in nutrients of interest (Mussatto et al., 2010). In some situations, while the yeast cell recycling, after yeast treatment, part of them can be removed from the process and enriched with minerals, such as Se (Suhajda et al., 2000; Basso et al., 2008). As they also have the ability to bioaccumulate many chemical elements, yeasts are used as sources of micro and macronutrients for human supplementation. Among these nutrients, Se can be highlighted, among these nutrients, as a micronutrient that participates in several antioxidant metabolic routes in human body (Riaz and Mehmood, 2012).

According to Abedi et al. (2018) and Tinggi (2003), Se participates in the conversion of the hormone triiodothyronine into thyroxine, exerts action against toxic metals and xenobiotics, has evidence in the regression of cancers, acts in the prevention of chronic and non-communicable diseases. It also participates in important biological processes such as

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ubiquinone biosynthesis. Furthermore, Se is an essential nutrient for animals and humans, which can be a source of yeast enrichment (Hou et al., 2020).

The yeast *S. cerevisiae* used in alcoholic fermentation can transform inorganic Se into organic compounds, which facilitates its bioavailability in the body and, depending on its growing conditions, it can accumulate remarkable amounts of Se in the form of selenomethionine and selenocysteine (Pedrero and Madrid, 2009). The organic forms of Se are part of the active site of important selenoproteins, such as glutathione peroxidase, which act to contribute to cell homeostasis by fighting free radicals (Rocha et al., 2020).

For these reasons, the objectives of this study were to evaluate the tolerance of yeast *S. cerevisiae* to high Se concentrations; to carry out the evolutionary adaptation of yeasts to this mineral and investigate the morphological variations of yeasts colonies / cells according to the evolutionary adaptation.

Materials and methods

Testing location

The tests were carried out at the Sugarcane and Bioenergy Technology Laboratory (LTSBio), Sugar and Alcohol Sector, Department of Agribusiness, Food and Nutrition, of Luiz de Queiroz College of Agriculture – University of São Paulo (ESALQ-USP).

Tolerance study of Saccharomyces cerevisiae to Se

The tolerance tests of the yeast *S. cerevisiae* Y904 to Se, as sodium selenite (Na_2SeO_3) ACS QM® with 99.0% purity, were performed in Petri dishes, containing YEPDA culture medium (0.5% Yeast Extract, 1% Peptone, 2% Dextrose and 2% Agar) with and without the addition of Se. The treatments were: 0 µg mL^{-1} (T1); 30 µg mL^{-1} (T2); 60 µg mL^{-1} (T3); 120 µg mL^{-1} (T4) and 240 µg mL^{-1} of Se (T5). In addition, an intermediate treatment of 70 µg mL^{-1} (T6) of Se was performed, so that the limit concentration tolerated by the yeast cell could be ensured. Cultivation was performed in quadruplicates, with 10 mL of the substrate and 100 µL of inoculum, under two serial dilutions of 10^{-5} and 10^{-6} CFU mL^{-1}, incubated under 30 ± 2˚C, from 24 to 48 hours, depending on the appearance of colonies (Assunção, 2011).

As a criterion for analyzing tolerance, yeast growth was considered up to 48 hours of incubation, after inoculation in a Se-rich medium. Thus, yeasts growing under these conditions were considered tolerant and those that did not grow were considered susceptible to Se.

Adaptation study os Saccharomyces cerevisiae in a medium enriched with sodium selenite

From the results obtained in the tolerance study, the maximum dose of the nutrient in which the yeasts managed to grow was selected. Then the adaptation process of the yeast *S. cerevisiae* Y904 was started in a culture medium enriched with sodium selenite (Na_2SeO_3) ACS QM®, 99.0% purity.

The first adaptive cycle was performed with YEPDA enriched with 60 µg mL^{-1} of Se. Subsequently, gradual increases in Se concentrations of 6 µg mL^{-1} per cycle were performed during 32 consecutive culture cycles. The adaptation process started with 60 µg mL^{-1} (D1) and in cycle 32, the concentration of Se in the culture medium was 246 µg mL^{-1} (D32).

Petri dishes contained 10 mL of the substrate and received 100 µL of inoculum, with dilutions of 10^{-5} and 10^{-6} CFU mL^{-1}. The incubation was carried out at 30 ± 2 ˚C, for 24 to 48 hours, according to the colonies growth and to the methodology described by Assunção (2011). As the colonies grew, those that grew within the 48-hour period were considered adapted and those that did not grow were considered susceptible. The analyzed macromorphological characteristics of the colonies / cells were: color, size, odor, and roughness.

Scanning electron microscopy (SEM) analysis

After the yeast adaptation tests to the minimum, average and maximum concentrations of sodium selenite equivalent at T1 (60 µg mL^{-1}), T11 (120 µg mL^{-1}), and T31 (240 µg mL^{-1}), respectively, SEM analysis was performed in the treatments.

The biomass of each treatment was stored in a 0.5 mL microtube containing the modified Karnovisky reagent, composed of 2.5% gluraldehyde, 2.5% formaldehyde 0.05M, sodium cacodylate buffer solution at pH 7.2, and $CaCl_2$ 0.001 M. The samples preparation followed the protocol of Kitajima and Leite (1999), in which a drop of poly-L-lysine was added to the coverslip and this was placed to rest for 15 to 20 minutes, then a sample drop was added in suspension keeping at rest for more 30 minutes in the coverslip. The coverslips with separators between one sample and another were placed in the "cage" to dehydrate inside the "cage" in a beaker in increasing concentrations of acetone: 30%, 50%, 70%, and 90% for 30 minutes at each concentration, and 100%, three times of 30 minutes each, to then be dried to the critical point, using CO_2. Finally, the coverslips were fixed in the *stubs* submitted to the metallization process, so that the samples could be observed and analyzed in the SEM with an increase of 10,000 times.

Results and discussion

Tolerance of Saccharomyces cerevisiae to Se

In the treatments used to define the dose of tolerance to Se, concentrations of 0 µg mL^{-1} (T1), 30 µg mL^{-1} (T2), 60 µg mL^{-1} (T3), 120 µg mL^{-1} (T4), and 240 µg mL^{-1} (T5) of sodium selenite were added to the medium. The results obtained showed that the cultivation of yeasts under the conditions of the T1 (control), T2, and T3 treatments obtained colonies growth within 48 hours after inoculation. The composition of the T3 medium was the maximum Se concentration that yeasts managed to grow. On the other hand, no colonies growth was observed within 48 hours after incubation, on substrates subjected to treatments T4 and T5. In the T1 treatment (without the addition of sodium selenite) with 10^{-5} CFU mL^{-1} dilution of the inoculum, the largest number of colonies was obtained per unit volume of substrate, with 3.9 x10^2 CFU mL^{-1}. These colonies showed white color, circular shape, not rough and sizes varying between 0.8 and 5 mm in diameter.

Under the conditions of treatments T2 and T3, smaller increases in the number of colonies were observed, with approximately 2.8 and $0.1x10^2$ CFU mL^{-1}, respectively. It was also identified that these colonies showed white color, circular shape, and not rough. However, ranging from 2 and 5 mm in diameter. In the conditions of treatments T4 and T5, no growth of colonies was observed within 48 hours of incubation, as can be seen in Figure 1, with the images of the treatments of tolerance.

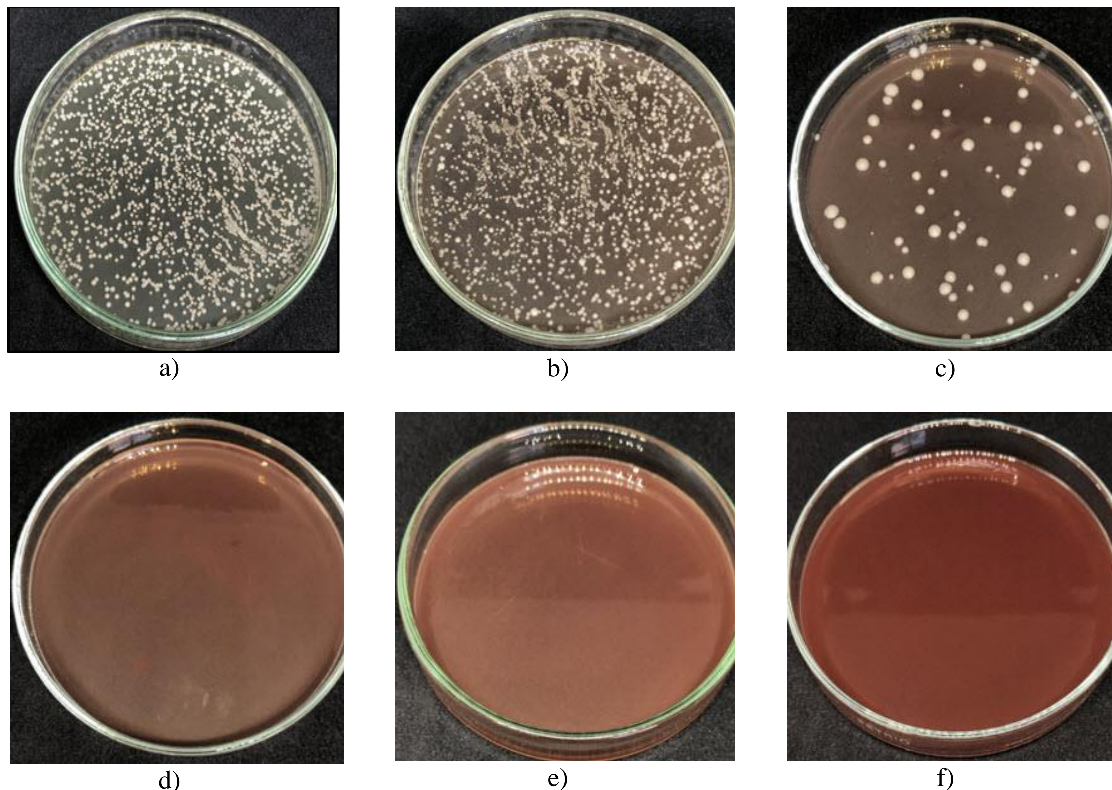


Figure 1. Yeast growth in YEPD medium, dilution 10^{-5} under 30˚C for 48 hours. a) Treatment T1, b) Treatment T2; c) Treatment T3; d) Treatment T6; e) Treatment T4; f) Treatment T5.

As no colony growth was identified at a concentration of 120 µg mL^{-1} (T4) in the YEPDA culture medium, it was necessary to define the tolerance interval between 60 (T3) and 120 (T4) µg mL^{-1}. However, at the concentration of 70 µg mL^{-1} of sodium selenite (T6), there was no growth of colonies. Therefore, it was defined that 60 µg mL^{-1} (T3) was the maximum concentration with cell growth without the need for an adaptive process. Pankiewicz et al. (2017) who also used selenium for enrichment in *S. cerevisiae*, reported that this bioaccumulation occurs in two steps. The first is called biosorption and is related to the accumulation of cations on the outer surface of the cell wall, while the second is called bioaccumulation, it is metabolism-dependent intracellular uptake and involves the penetration of metal ions inside the cell using specific membrane transporters and the metabolic cycles of cells.

Similar results were found in the works of Assunção (2011) and Rajashree and Muthukumar (2013), using the same species of yeast, but from another strain. According to Assunção (2011), the objective was to evaluate the inhibitory effect of 0.0 concentrations; 5.6; 34.8; 49.7 and 94.0 µg mL^{-1} of sodium selenite in YEPDA in the growth of the yeast *S. cerevisiae* EVN 166, in 24 h. As a result, the colony-forming unit (CFU mL^{-1}) was the same for all Se concentrations after 24 h of growth; however, a slightly lower value of CFU mL^{-1} was observed for the 94.0 µg mL^{-1} of sodium selenite.

The study by Rajashree and Muthukumar (2013) aimed to analyze the toxicity of Se (0, 10, 20, 30, 40, 50, 75, 100, 125, 150 µg mL^{-1} of sodium selenite) in yeast cells *S. cerevisiae* NCYC 1026 and the effects on biomass production, in sterile Sabouraud Dextrose, under aseptic conditions and incubation for 72 hours at 30˚C. As a result, it obtained the highest concentration of 75 µg mL^{-1} with no change in biomass production, and 50 µg mL^{-1} taking into account the bioaccumulation of Se by the cell.

According to the studies by Kieliszek and Dourou (2021), Kaur and Rasconi (2006), Stabnikova et al. (2008), and Marinescu, Stoicescu and Teodorof (2011), the decrease in the number of yeast cells is directly related to the increase in the concentration of sodium selenite in the culture medium, because the higher amounts of sodium selenite in the culture medium has a strong inhibitory effect on the growth of yeast. As explained by Kieliszek et al. (2019a), the slowdown in yeast growth may be a result of the occurrence of oxidative stress caused by the presence of high concentrations of Se in the culture medium, which can lead to another phenomenon called the level of lipid peroxidation.

Adaptation of Saccharomyces cerevisiae to Se

The adaptive process carried out for 64 days in 32 consecutive cultivation cycles made expansion of tolerance capacity of yeasts to Se from 60 µg mL^{-1} to up to 246 µg mL^{-1} possible. As the concentration was increased, changes in the color of the yeast colonies were observed (Figures 2).

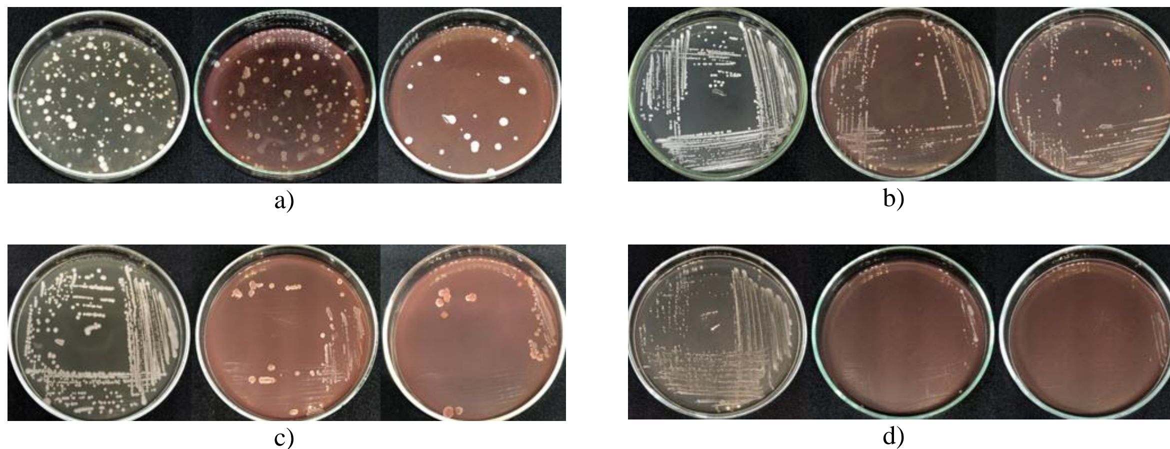


Figure 2. Some treatments of the yeast adaptation process in YEPDA medium at 30˚C after 48 hours in the control group, dilutions 10^{-5} and 10^{-6} respectively, according to the image of each letter. (a) T1 treatment, 60 µg mL^{-1} of sodium selenite was added to the culture medium; (b) T5 treatment, 84 µg mL^{-1} of sodium selenite was added to the culture medium; (c) T15 treatment, 144 µg mL^{-1} of sodium selenite was added to the culture medium; (d) Treatment 32, 246 µg mL^{-1} of sodium selenite was added to the culture medium.

At the beginning of the adaptation process, the colonies were light beige and shiny, as the doses of Se in the medium were increased. Then the yeast colonies began to show a darker color so that, after 32 cultivation cycles, the colonies had an intense reddish-brown color.

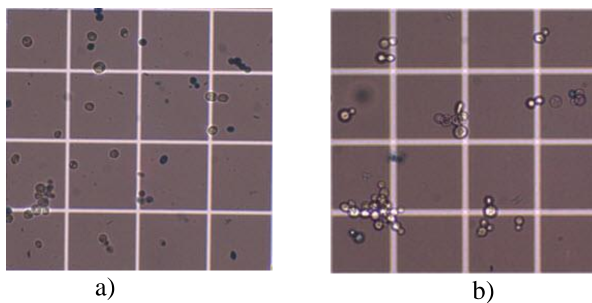
In Figure 3a it is possible to clearly see the color change from beige to orange-red, when the yeasts were grown at 96 µg mL^{-1} medium. In Figure 3b, colonies with intense orange red coloring and roughness at the edges were found when the yeasts were grown at 150 µg mL^{-1}. These changes in cell staining can be explained by the biotransformation that happens inside the cell when the selenite (transparent coloring) is reduced to Se amorphous (reddish coloring) (Konetzka, 1977). In addition, there was a reduction in the number of colonies and an increase in the roughness of the colonies on the entire surface, but mainly at the edges. Changes in the odor of the colonies were also observed, which began to show similarity to the smell of garlic.

The strong garlic-like odor is attributed to the volatile metabolite dimethylselenide (Nuttall, 2006). According to Ohta and Suzuki (2008), the accumulation of Se by the microorganism can convert a part into selenoproteins and another, to a lesser extent, into dimethylselenide (DMSe), a volatile gas that is released from the tissues and is non-toxic (Neumann et al., 2003). In the work of Ståhl, Anundi, and Högberg (1984), they explained that sodium selenite added to the culture medium chemically reacts with sulfhydryl compounds. The reaction with glutathione (GSH) is the first reaction in the metabolic pathway that leads to the formation of volatile metabolites. The first stable intermediate, selenogluthione (GSSeSG), is a substrate for glutathione disulfide (GSSG) reductase and is reduced to selenopersulfide (GSSeH) at the expense of NADPH. GSSeH can be further reduced to selenide by the same enzyme. Selenide is methylated under physiological conditions but can evaporate at low pH. The dimethylselenide formed in vivo is excreted and is responsible for the "garlic odor".

Figure 3. Adaptation of yeast cells *Saccharomyce cerevisiae*. a) Treatment T7 e b) Treatment T16.

Although yeasts grow in concentrations from 222 to 246 µg mL^{-1}, a reduction in the growth speed of colonies that started to develop after 24 or 36 hours of incubation was observed. While at concentrations of 60, 66 and 72 µg mL^{-1}, yeast colonies could be observed in the first 12 hours of incubation.

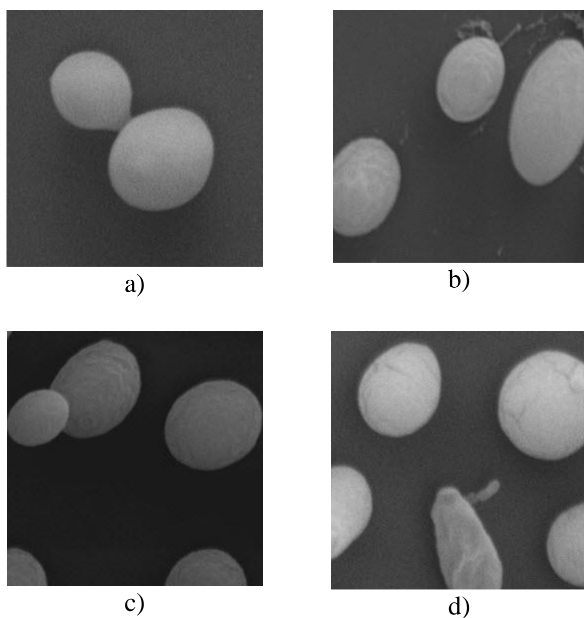
With the presence of high concentrations of Se in the substrate, morphological changes were observed in the yeast cells. At T6 (90 µg mL^{-1} of sodium selenite in the culture medium) was possible to verify cell clusters, increase in size and transverse area of the cells, using an optical microscope (Figure 4).



a) b)

Figure 4. Optical microscopy of yeasts grown in YEPD medium at 30˚C after 48 hours, with a 400x magnification. (a) Treatment T1- Enrichment of the medium with 60 µg mL^{-1} of sodium selenite; (b) Treatment T16 – Enrichment of the medium with 156 µg mL^{-1} of sodium selenite.

The changes promoted in the yeast cell wall were observed by means of scanning electron microscopy, through which it was possible to observe the wrinkling of the surface of the yeast cells and changes in the shape of the cells when they were subjected to concentrations of 60, 120 and 240 µg mL^{-1} of sodium selenite (Figure 5), once the characteristic of yeast without the presence of Se is smooth.



a) b) c) d)

Figure 5. Scanning electron microscopy of *Saccharomyces cerevisiae* yeasts grown in YEPD medium at 30˚C after 48 hours, (a) Control Treatment - No enrichment 0 µg mL^{-1} of sodium selenite; (b) Treatment T1 – Enrichment of the medium with 60 µg mL^{-1} of sodium selenite, (c) Treatment T10 – Enrichment of the medium with 120 µg mL^{-1} of sodium selenite e (d) Treatment T31 - Enrichment of the medium with 240 µg mL^{-1} of sodium selenite.

Also in the work of Biringer et al. (2002), it was found that Se causes morphological changes in yeast, possibly altering the structure of the cell wall and membrane complex. In the work by Kieliszek et al. (2019b), Se supplementation increased the participation of unsaturated acids, such as linoleic acid and linolenic acid, in the biomass of *Candida utilis* ATCC 9950 and *S. cerevisiae* MYA-2200. As the biosynthesis of these acids may be associated with increased desaturase activity and lipid peroxidation, these processes may be directly linked to changes in yeast cell morphology. Some changes already mentioned in the literature are: increase in the size of cells, shrinkage of yeasts, thickening of the cytoplasm, or changes in the structure of the vacuole (Kieliszek, 2016).

The morphological change in yeasts according to the increase in the concentration of sodium selenite was also found in the work of Rajashree and Muthukumar (2013), where the control group without the addition of sodium selenite had yeast cells with a smooth edge surface, while the treatments with sodium selenite acquired roughness on the surface. Damage to the cell wall was observed in yeast cells, which was caused by high concentrations of sodium selenite, resulting in a reduction in the number of yeast cells. According to Kieliszek et al. (2016), the addition of sodium selenite (salt) to the medium causes osmotic stress in the yeast cells, and as a response, the formation of grooves in the cell wall and consequently the wrinkling occurs, thus being the identified roughness.

The results of the optical microscopy images (Figure 4) corroborate with those observed in the study conducted by Kieliszek et al. (2016), where the analysis of microscopic images of yeasts of the species *C. utilis* TCC 9950, demonstrated that the concentrations of 20, 30 and 40 µg mL^{-1} of sodium selenite in the substrate caused a significant increase in size and cross-sectional area of cells due to agglomerations and vacuoles among them when compared to cells without the addition of Se. Comparing with other similar results, Rajashree and Muthukumar (2013) identified the change in the surface of the yeasts using SEM, observing that the smooth surface of *S. cerevisiae* without the addition of sodium selenite, contrasted yeast cells with rough surfaces when grown in a medium enriched with 50 µg mL^{-1} of sodium selenite, while those grown in 100 µg mL^{-1} were partially damaged (with small cracks).

Despite being of the same species, *S. cerevisiae*, and having the ability to bioaccumulate several elements and, as a result, tolerate higher concentrations in the medium, the different strains show different behaviors to stress and specific variations. There are studies with yeasts of the same species that tolerate different concentrations of Se (White and Gadd, 1987). As in the results obtained by Wang, Zhang and Tan (2010), the addition of 90 µg mL^{-1} in the late exponential development phase of the yeast of lineage GS2, was the highest tolerated concentration taking into considering the decrease in biomass production.

In the present work, the adaptive process allowed the yeast to tolerate up to 246 µg mL^{-1} of sodium selenite in the culture medium. Due to the gradual increase of Se in the medium and the metabolic interactions caused by Se, the yeasts presented different characteristics, such as, the reduction of the cell multiplication speed, roughness, intense garlic odor and increase in the intensity of the reddish brown color. Such results were similar to those cited in the literature. Suhajda et al. (2000), enriched *S. cerevisiae* also using sodium selenite, obtained a reddish color in the yeast cells.

The adaptive ability of yeast can be acquired when exposed to the appropriate selection pressure (White and Gadd, 1987). According to Bronzetti et al. (2001), the addition of high concentrations of sodium selenite to the culture medium was demonstrated to have a mutagenic effect in the yeast cells of the species *S. cerevisiae* D7, generating a 70% decrease in cell survival when compared to the control group.

The change in all these characteristics (change in color, roughness, odor, size, and the number of cells) with the increase in the concentration of sodium selenite, demonstrates how the yeasts have undergone modifications and / or adaptations in order to survive the stress caused by the enrichment of the culture medium with Se, which may induce to believe that adaptive evolution of yeast cells has occurred.

A yeast adapted to higher Se concentrations in the growth medium induces higher Se concentrations to be absorbed or adsorbed by the cell. As yeast has enzymes capable of metabolizing this absorbed Se into organic Se (which is Se linked to an amino acid, such as methionine or cysteine), capable of generating antioxidant enzymes (Ogra et al., 2018), such as glutathione peroxidase, o final product would have a greater added value.

According to Meena et al. (2020) mineral-enriched yeast emerged as a potential solution for mineral deficiency in the population. According to Martiniano et al. (2020) yeasts are widely used as food additives due to their high content of proteins and micronutrients, in addition to being sources of important nutraceutical compounds present in the cell wall, acting as prebiotics and probiotics.

Thus, these yeasts adapted to high concentrations of Se, could be used to fortify staple foods or even in probiotic formulations to combat mineral deficiencies and even strengthen the immune system.

Conclusions

The yeasts of the species *Saccharomyces cerevisiae* – Y904, after a tolerance test, were able to grow in a medium enriched with Se at a concentration of 60 µg mL^{-1}. After the adaptive process of 32 cycles in increasing additions of 6 µg mL^{-1} of sodium selenite, the yeasts were able to grow up to 246 µg mL^{-1} of sodium selenite in the culture medium. During the adaptive process, there were some color changes, roughness on the cell surface, changes in the speed of development, and in the number of cells, which demonstrates how these microorganisms underwent adaptations in order to be able to develop in a stressful environment with a high concentration of Se. Yeasts undergoing changes to force their development, lead us to believe in greater adsorption and absorption of the cell by the micronutrient. This increase in the concentration of Se absolved by the cell infers to the increase of Se in its organic form, capable of generating antioxidant enzymes, which would originate a yeast cell with high added value and which could be destined for human nutrition as a supplement or even in probiotic formulations.

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