SEARCHING RESISTANCE MARKERS IN *SACCHAROMYCES SP.* UNDER ETHANOL STRESS BY PROTEIN PROFILING AND ULTRASTRUCTURE ANALYSIS

Edgard Freitas de Siqueira Junior[1], Celso Duarte Carvalho Filho[2], Antônio Ferreira da Silva[3], Jéssica Guerreiro[3], Edson Delgado Rodrigues[4], Suzana Telles da Cunha Lima[5]

[1]Instituto Federal Baiano, [2]Faculdade de Farmácia e [3]Instituto de Física da Universidade Federal da Bahia, [4]Departamento de Ciências da Vida da Universidade do Estado da Bahia (UNEB) e [5]Instituto de Biologia da Universidade Federal da Bahia (UFBA),

RESUMO

Saccharomyces cerevisiae é um importante modelo para investigar a resposta de levedura ao estresse ambiental. O presente estudo objetivou analisar o perfil de proteínas de diferentes estirpes sob estresse por etanol, a identificação de peptídeos com expressão aumentada e análise de alterações morfológicas. Os métodos utilizados foram SDS_PAGE e microscopia eletrônica de varredura. Os resultados indicaram bandas mais fortes com pesos moleculares de 10 a 160 kDa, coincidindo com diferentes proteínas de choque térmico (HSP)s sintetizadas em resposta a condições de estresse. A ultraestrutura demonstrou alterações na morfologia como deformações na parede celular, dimorfismo de célula e brotamento, provavelmente como um comportamento de defesa. Apesar disso, todas as estirpes apresentaram fermentação bastante satisfatória, demonstrando serem apropriadas para bioprocessos industriais. As proteínas HSP são muito conservadas entre espécies e conhecidas por aumentarem a resistência dos diferentes organismos ao ambiente. Assim sendo, acreditamos que elas podem ser usadas como marcadores para a seleção de cepas mais apropriadas para processos de fermentação, não só em Saccharomyces, mas também entre as espécies não-saccharomyces.

Palavras-Chaves: *Saccharomyces cerevisiae*; marcadores proteicos; fermentação; etanol e HSP.

ABSTRACT

Saccharomyces cerevisiae is an important model to investigate the response of yeast cells to environmental stress. The present study aimed to analyze the protein profile of different strains under stress for ethanol, the identification of peptides with increased expression and analysis of morphological changes. The methods used were SDS_PAGE and scanning electron microscopy. The results indicated stronger bands with molecular weights of 10 to 160 kDa, coinciding with different heat shock proteins (HSP)s synthesized in response to stress conditions. The ultrastructural changes in morphology were deformation in the cell wall, cell dimorphism and

budding, probably as a defensive behavior. Nevertheless, all strains presented fairly satisfactory fermentation, demonstrating to be suitable for industrial bioprocesses. HSP proteins are very conserved between species and known to increase the resistance of different organisms to the environment. Therefore, we believe that they can be used as markers for selection of most suitable strains for fermentation processes, not only with Saccharomyces, but also using non-saccharomyces species.
Key-words: *Saccharomyces cerevisiae*; protein markers; fermentation; ethanol; HSP.

1. INTRODUCTION

The term fermentation derives from the Latin *fervere* (boil), due to the appearance of bubbles of carbon dioxide resultant from the yeast action over the fruit extract, or malt grains [1]. The alcoholic fermentation consists in the transformation of sugars into ethanol, carbon dioxide, and energy needed for maintaining vital activities [2]. Yeasts are the main organisms performing this catalytic action at industrial level. They are fungus, primarily under unicellular form, presenting vegetative reproduction by gemmulation, cell wall, spherical to ovoid or even filamentous to ellipsoid form. Many physical (temperature, osmotic pressure), chemical (pH, oxygenation, mineral and organic nutrients, inhibitors) and microbiological factors (species, stain and concentration of yeast, bacterial contamination) affect the yield of fermentation and the efficiency of converting sugar into ethanol [3].

The effective cost of ethanol production depends, among other factors, on the high yield and quick conversion of carbohydrates into ethanol. However, ethanol accumulated in the culture medium can become a significant stressor during fermentation. According to Silva and colaborators [4], during the process of fermentation a series of compounds that can act as potential inhibitors are originated, among them, secondary metabolites and contaminants, including the ethanol produced in the process. The high concentrations of ethanol have a detrimental effect on proteins, phospholipid bilayer (increasing fluidity and decreasing integrity) and other cellular components [5, 7]. As a consequence, there is limitation in growth and metabolic activity, as well as in the production of alcohol [6].

Simple fungi have evolved sophisticated mechanisms to detect and respond to environmental stimuli, enabling coordinated changes in cellular physiology, morphology and cell adhesion. Physiologically, ethanol causes an inhibition on the growth and viability of yeast because of its negative action on different transport systems (including permeases of amino acids), glucose cell signaling and activity of keys enzyme of glycolytic pathway [8] [9] [10].

Critical nutrient depletion also induces changes in morphology, including growth interruption and the formation of spores that can tolerate a wide variety of environmental stresses. Another resistance alternative is to growth dimorphic filaments, characterized by branching networks of chained cells or hyphae, forming a mycelium [11] [12]. This dimorphic switch is essentially modulated and can be activated by a wide variety of stimuli according to the lifestyle of the fungus.

The ability to quickly respond to variations in temperature, nutrients, as well as other environmental changes is essential to the general activity of the cell and its survival. The cells of *Saccharomyces cerevisiae*, when subjected to stress, develop a rapid molecular response to repair damage and protect structures [13]. These responses are characterized by the synthesis, among others, of a group of proteins called heat shock proteins or HSPs, which act as chaperones in the processes of folding after translation or denaturation for recovery of damaged proteins [14] [15].
Understanding the response of cells regarding to environmental changes is a topic of great interest because it could offer clues about the molecular apparatus that allow cells to adapt to new environments. At biotechnological level, it is an interesting topic since many molecules of financial interest increase their production under stress conditions (ex. biodiesel precursors and pigments of microalgae [16]).
The fact that *S. cerevisiae* has a completely sequenced genome allows rapid identification of genes involved in response to stress with a specific function, as well as identification of orthologs in other organisms. All these features make this yeast an excellent experimental model to study stress response.
The aim of this study was to evaluate protein expression of different *S. cerevisiae* strains under ethanol stress, identify the molecular weights of proteins with increased expression and relate them to others found in the literature. In addition to that, analyze by Scanning Electron Microscopy (SEM), the changes in the morphology of the *Saccharomyces bayanus* caused by ethanol stress.

2. MATERIALS AND METHODS

SAMPLES

Six starter strains of Saccharomyces (Table 01) were used for the fermentation of beer, wine, and bread. Dry brewer's yeast Safale S-04 Ale and Saflager S-189 Lager (LALLEMAND, Quebec) were used in this article under the code S04 and S189, respectively. Dry yeast for production of cachaça (Cenex-ICB-UFMG) was used under the code ScFC. Dry yeasts from wine production in the sub median São Francisco river (Lagoa Grande, PE, Brazil) were used under the code SbV (*Saccharomyces bayanus*) and ScVY (*Saccharomyces cerevisiae* unknown *strain "y"*). Instant Dry Yeast used in the production of bread (Dr. Oetker, São Paulo) was used under the code ScFP.
The method for preparing starter culture and stress-inducing conditions were the same as described by REIS [17] with modifications in the ethanol concentration, as described below.

Table 01 – Ethanol concentration and cell resistance for different strains of Saccharomyces. The X indicates that cells grew at the YEPD medium plate containing this concentration.

Strains	Concentration of Ethanol						
	3%	5%	7%	9%	12%	15%	21%
S04	X	X	X	X	X	X	
S189	X	X	X	X	X		
ScFC	X	X	X	X	X		
ScFP	X	X	X	X	X		
SbV	X	X	X	X	X		
ScVy	X	X	X	X	X		

ETHANOL STRESS

An yeast colony in a petri dish containing YEPD medium and 100µg of chloramphenicol was removed with a platinum loop and seeded in a chloramphenicol-free YEPD medium culture dishes containing 3%, 5%, 7%, 9%, 12%, 15%, and 21% of absolute ethanol (v/v). To make these chloramphenicol-free dishes ethanol was added to the still liquefied medium at 50-55°C and after solidification, the plates were sealed with *Parafilm*. All ethanol was incorporated and solidified with the medium. The inoculated plates were kept at 28° C for 7 days.

CELL LYSIS

The yeast samples were transferred to centrifuge tubes containing lysis buffer (100 mM PBS, 5 mM EDTA, 100 mM PMSF, pH 7.0). Glass beads (0.5 mm) were added and the tubes were vortexed by one minute to promote cell lysis and homogenization. This procedure was repeated four times at intervals of 1 minute under ice bath, followed by centrifugation at 850g for 2 minutes.

The supernatants were transferred to new tubes, identified and kept in ice bath. The lysis was repeated with the precipitate and the supernatants obtained were added to previous one. The total volume of the supernatant was homogenized, separated into aliquots and stored at -20ºC.

PROTEIN QUANTIFICATION

The concentration of proteins was determined by the Bradford Method [18]. A bovine serum albumin (BSA) standard curve was plotted, with concentration of protein as a function of absorbance obtained at 595nm. The BSA curve was used for calculating the samples´ protein concentration (mg/mL).

SDS-PAGE ANALYSIS

The SDS-PAGE gel was run at 75 volts (Gibco BRL Power Supply, Life Technologies) after preparation according to Laemmli [19] using Mini-protean cell Electrophoresis (Biorad). A standard concentration of 10 µg of protein was applied to each lane for assessment of expression levels. Many repetitions (at least five gels)

were made for each sample for confirming the peptides (bands) with increased expression. The molecular weight was evaluated according to a standard marker for proteins (Protein Ladder BenchMark, Invitrogen) after staining with *Coomassie Brilliant Blue* (Invitrogen) or silver nitrate method [20].

MORPHOLOGICAL ANALYSIS BY SCANNING ELECTRON MICROSCOPY (SEM)
Samples of *S. bayanus* grown in agar plates under different concentrations of ethanol (3%, 5%, 7%, 9%, 12%, 15%, and 21%) were attached to the surface of conductive carbon and metallized with gold using a sputter coater Desk V (Denton Vacuum, New Jersey) for SEM analysis.
The images were obtained with accelerating voltage between 5 and 20 KV in a Scanning Electron Microscopy JEOL KAL-6610LV with tungsten filament belonging to Electronic Microscopy Multi-user Laboratory (LAMUME-UFBA).

3. RESULTS AND DISCUSSION

YEAST GROWTH UNDER ETHANOL STRESS
There was no yeast growth at concentrations of 21% ethanol (v/v). The concentration of 15% (v/v) proved to be a limiting factor for the growth of most strains of Saccharomyces analyzed (Table 01). Only S04 grew at 15% ethanol (v/v) after 168 hours of culture. Apparently, lag phase adaptations to environmental stress extended the time needed to start log phase, which occurs later in these conditions, according to our observations.

PROTEIN EXPRESSION PROFILE AFTER ETHANOL STRESS
Ethanol levels starting at 3% showed increasingly expression of proteins with molecular weights between 10 and 160 kDa for all strains tested (Table 02).

Table 02 – Molecular weight of peptides (KDa) with increased synthesis during stress by ethanol by each yeast strain analyzed. X represents the presence of the peptide in SDS-PAGE.

MW (KDa)	ScFC	ScFP	SbV	ScVY	S-04	S-189
220						
160						X
120						
100	X					
90					X	X
80	X					
70		X	X			
60				X		

50	X	X	X	X	X	X
40			X			
30	X	X	X	X	X	X
25		X	X	X	X	
20	X	X				
18				X		
15			X	X	X	
10					X	

In strain S-189, used for beer fermentation, this feature can be reflected by slight increase in protein expression during stress. However, it is possible to see an upturn in proteins with 30, 50, 90 and 160 kDa (Figure 01).

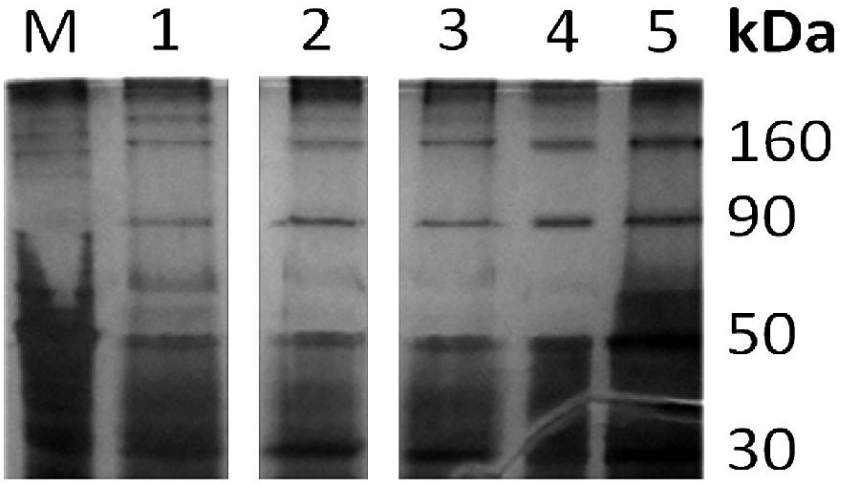

FIGURE 01 – Electrophoretic Profile (SDS-PAGE) of strain S189 under stress by ethanol (M= molecular weight marker, Lane 1= 3%, 2= 5%, 3= 7%, 4= 9%, 5= 12% of ethanol).

Strains ScVY and SbV, used in wine fermentation, presented a similar profile with increase in bands between 15 and 60-70 kDa (Figures 02 and 03).

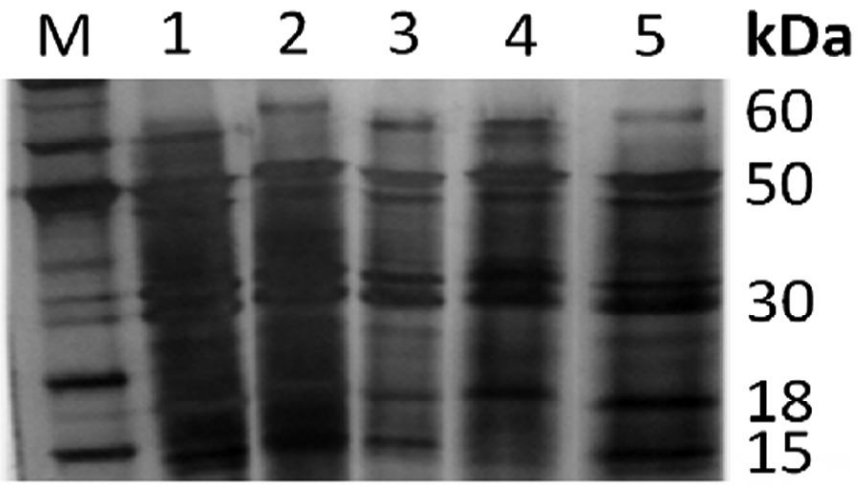

FIGURE 02 - Electrophoretic Profile (SDS-PAGE) of strain ScVY under stress by ethanol (M= molecular weight marker, Lane 1= 3%, 2= 5%, 3= 7%, 4= 9%, 5= 12% of ethanol).

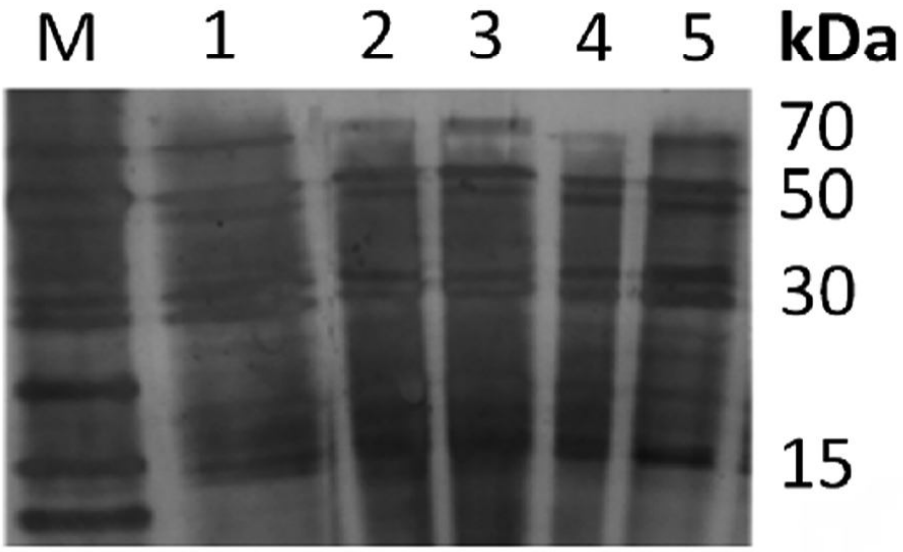

FIGURE 03 - Electrophoretic Profile (SDS-PAGE) of strain SbV under stress by ethanol (M= molecular weight marker, Lane 1= 3%, 2= 5%, 3= 7%, 4= 9%, 5= 12% of ethanol).

Ethanol is capable of destabilizing the hydrophobic interactions of the complex chaperones-HSP and activates thermal shock genes, increasing the concentration of HSP [39]. Cellular characteristics as accumulation of threalose [21] [22] [23], lipid

content of plasma membrane [28] [29] [30] [31] [32] [33] [34], HSP104 activity [35], mitochondrial stability [36] and H^+-ATPase activity [24] [25] [26] may be responsible for the variation of ethanol tolerance observed in different strains. The biosynthesis of ergosterol [37] and phospholipids [38] are other mechanisms used by yeast to develop tolerance to ethanol. A selection of genes involved in ethanol stress and related protein function is presented in Table 03. Data was gathered from http://www.ncbi.nlm.nih.gov/protein using: stress, ethanol and saccharomyces as search terms.

TABLE 03 - Relationship of some genes and proteins involved in stress by ethanol, according to protein database.

ORF	*GENE*	PROTEIN ACTIVITY	MW (Da)
YHR104W	*GRE3*	Aldose Reductase. Induced by thermal shock, osmotic (ethanol), ionic, and oxidative stress	37,188
YCR021C	*HSP30*	HSP induced by thermal shock, ethanol and organic weak acid.	37,044
YFL0144	*HSP12*	HSP induced by thermal shock, oleate, alcohol and osmotic stress.	11,693
YHR206W	*SKN7*	Transcription factor involved in osmotic regulation.	69,202
YLL026W	*HSP104*	HSP induced by thermal stress, ethanol and sodium arsenite.	102,034
YLR251W	*SYM1*	Thermal stress-induced protein used in the metabolism of ethanol.	22,915
YNL064C	*YDJ1*	Chaperonin HSP40 homolog involved in regulation of HSP70 and 90 during stress.	44,670
YBR072W	*HSP26*	HSP	23,879
YOL151W	*GRE2*	Methylglyoxal reductase induced by osmotic, ionic, oxidative, and thermal stress and heavy metals.	38,169
YPL223C	*GRE1*	Osmotic stress-induced protein	19,025
YPR006C	*ICL2*	Non-Functional Isocitrate Lyase. Ethanol-induced	64,976
YRE065C	*ICL1*	Isocitrate Lyase ethanol-induced and repressed by glucose	62,408
YHR076W	*PTC7*	Mitochondrial phosphatase 2C induced by ethanol and sustained by glucose	41,190
YPR093C	*ASR1*	Protein involved in the signaling pathway of putative response to alcohol. Accumulates in the nucleus under stress of alcohol.	33,347
YBR086C	*IST2*	Plasma membrane protein involved in osmotic tolerance.	105,856
YMR175W	*SIP18*	Osmotic stress protein	8,873

In the present work, all strains expressed a protein with molecular weight of approximately 33kDa that could be related to Asr1 (alcohol sensitive RING/PHD1). Asr1 is encoded by gene *ASR1/YPR093C* and was the first yeast protein found to be involved in a putative signaling pathway in response to alcohol. It accumulates in the nucleus when the cell is under stress [40] [41] as a signaling mechanism for cell growth in media containing high concentrations of alcohol. This protein represents a key element in tolerance to this kind of stress [40]. The HSP104, ATPase and IST2, with a molecular weight of approximately 100 kDa are expressed in response to stress by ethanol, but are also related to other stressors, such as heat, osmotic and sodium arsenate. [41] [42] [43] [44] [45] [46] [47]. This molecular weight matches the same of the peptide with increased expression in ScFC strains analyzed in our work (Table 01 and Figure 04).

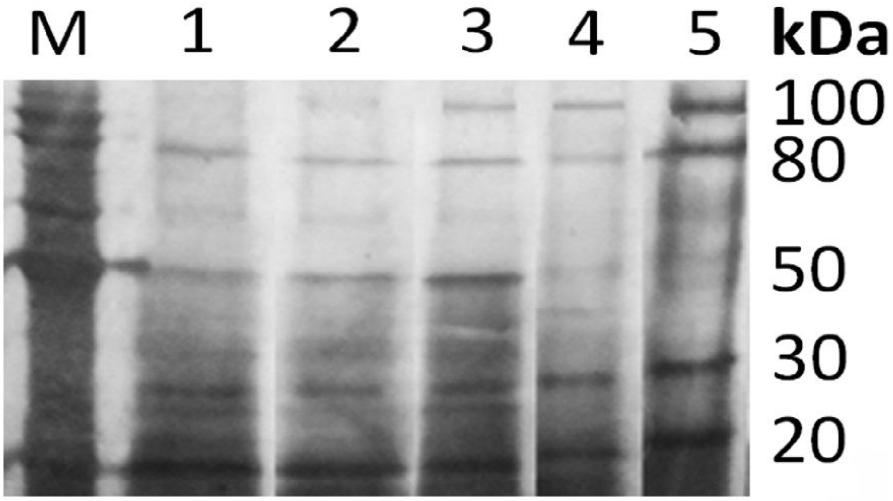

FIGURE 04 - Electrophoretic Profile (SDS-PAGE) of strain ScFC under stress by ethanol (M= molecular weight marker, Lane 1= 3%, 2= 5%, 3= 7%, 4= 9%, 5= 12% of ethanol).

Our results also show an increase in the expression of proteins with low molecular weight (Figure 02 and 03) and this finding is in agreement with recent studies that have confirmed the expression of several small HSP (sHSP) induced by ethanol. However, the low expression of at least one of these proteins, the HSP26, does not affect the tolerance for this kind of stress [48] [49]. The family of sHSP is composed of proteins with molecular weight between 12 and 43 kDa. They can form multimeric structures and display a wide range of cellular functions [50]. HSP12 on plasma membrane is induced by osmotic stress and suppressed in the presence of protein kinase A, having an important role in the maintenance of membrane organization in stress conditions [51] [52] [53]. According to PIPER et al. (1994) [54], Northern Blotting analysis of mRNA of BJ2168 strain showed increased expression of HSP26 in response to stress by ethanol. Increased tolerance to ethanol is inducible, often

taking place under the same conditions as those which lead to increased thermo tolerance [42] [55]. The toxic effect of ethanol can be evaluated by inhibition of glycolytic enzymes and in numerous biological processes, many of which are associated with cell membrane lipids [56].
Since HSP is highly conserved among many different organisms, at least for this protein it is easy to confirm identity through *western blotting* analysis using anti-bodies anti-HSP of the same molecular mass commercially available (*New England Biolab*). The extension of this work would be to confirm the identity of all proteins with increased expression under ethanol stress and use them as markers of more resistant strains from fruits and grains. This would increase the value of alcoholic beverages since the addition of Saccharomyces is implemented due to a low resistance of yeasts from grapes, sugar cane, wheat and barley.

MORPHOLOGY OF SACCHAROMYCES EXPOSED TO ETHANOL STRESS

Under normal growth conditions, without exposure to ethanol, oval and elliptical *S. bayanus* present themselves individually without the formation of budding, curls and deformations in the cell wall (Figure 05).

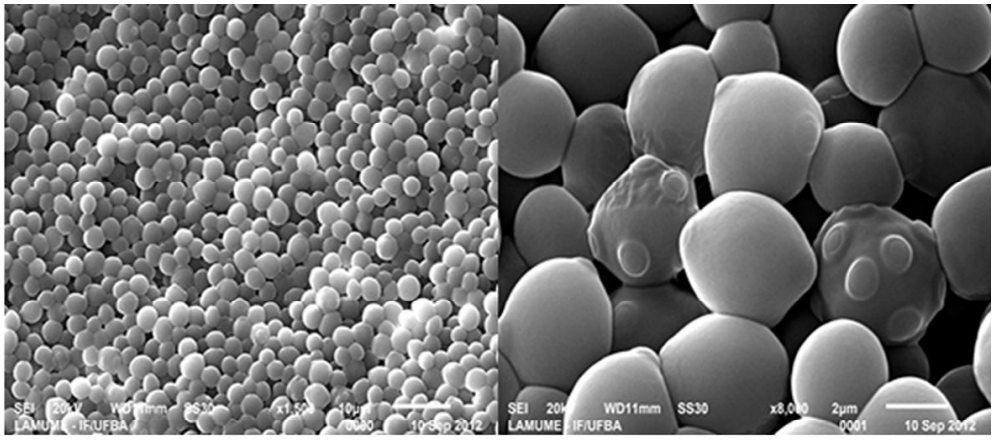

FIGURE 05 – *S. bayanus* grown in YEPD medium without ethanol. (A) Image increased 1.500 X; (B) Image increased 8.000 X

At 5% ethanol (Figure 06) it is possible to notice changes in the structure of the cell wall, cell morphology and reproduction mode. The morphology becomes more elongated and unipolar budding occurs (Figure 06B), with the daughter cells mechanically connected to the parent cell through protein interactions in the cell wall. Deformations in the yeast cell wall can be observed under these conditions.

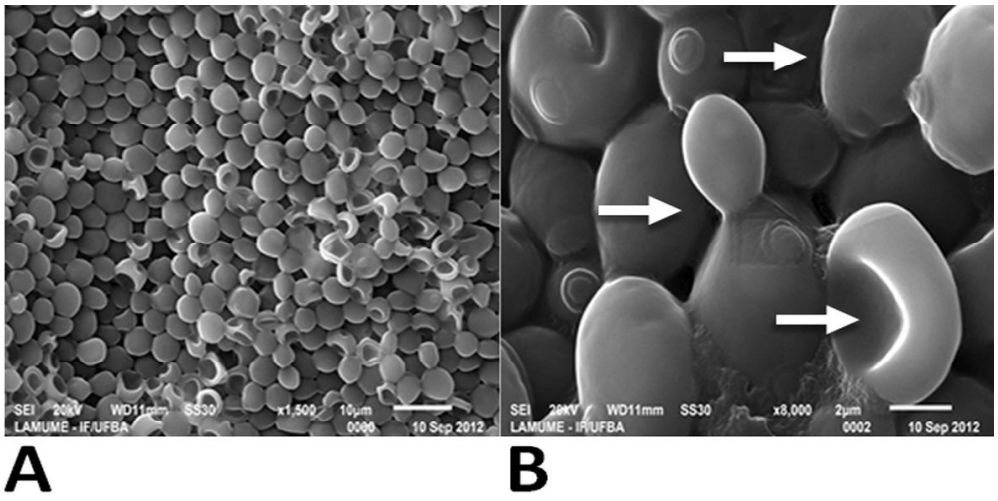

FIGURA 06 – *S. bayanus* grown in YEPD medium with 5 % ethanol. (A) Image increased 1.500 X; (B) Image increased 8.000 X

Higher concentrations of ethanol cause increasing deformations in the cell wall (figures 07A, B and 09) which can be a reaction to toxicity or a dehydration effect due to the increased concentration of alcohol.

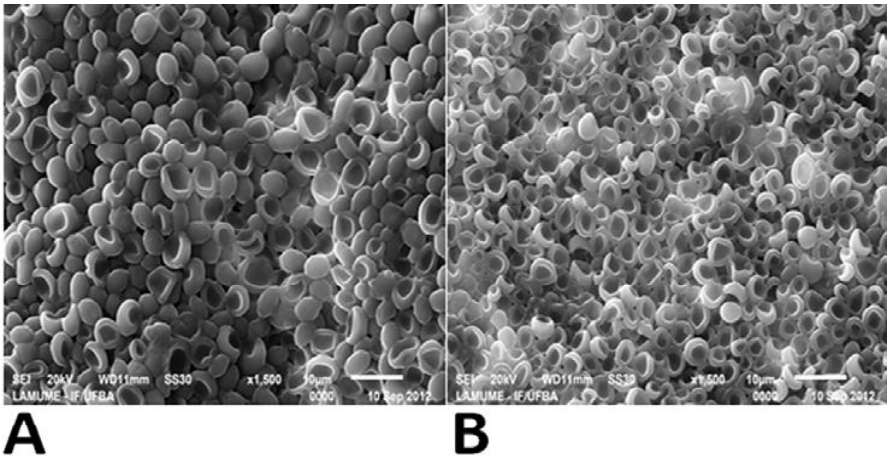

FIGURA 07 – *S. bayanus* grown in YEPD medium with (A) 7 % and (B) 12 % of ethanol. Image increased 1.500 X.

Figure 8 shows an amplified image of yeast under 9% ethanol and arrows point the formation of pseudohyphae, with aggregation of walls among cells.

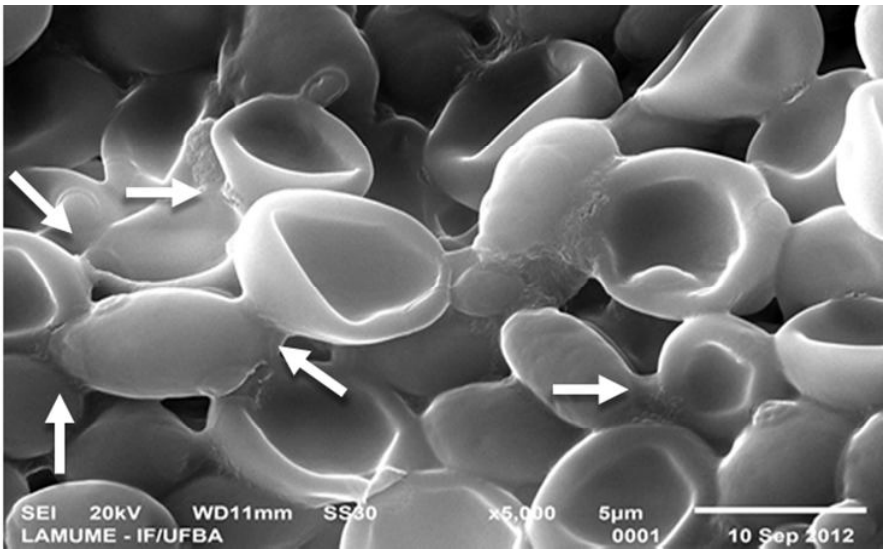

FIGURE 08 - *S. bayanus* grown in YEPD medium with 9 % ethanol concentration. Arrows indicate the formation of pseudohyphae. Image increased 5,000 X.

Although ethanol is the primary product of fermentation in *S. cerevisiae*, yeast produces a variety of other alcohols (mainly products of metabolism of branched chain amino acids) collectively known as fusel alcohols [57]. The morphology of the cells under higher concentration of ethanol found in the present study is very similar to that of cells exposed to fusel alcohols. Lorenz et al. (2000) [58] characterized the link between these morphological changes induced by alcohol and pseudohyphae. Isoamyl alcohol and butanol promote filamentous growth on solid medium, and elongated shape and filamentous in liquid medium. According to the author and also in our results, concentration of 9% ethanol (v/v) stimulated cells to a different behavior; the daughter cells do not separate from the parent cell after the budding, forming filaments or curls. Filamentous morphology of yeast can be a problem for the fermentation process. There is a tendency for the formation of cell aggregates of yeast in the form of foam at the top of the fermentation tank that reduces process efficiency [59]. According to POSSAS (2010) [60], pseudohyphae-shaped yeasts have disadvantages compared to single cells, with less ability to convert substrate into ethanol, due to a smaller area of contact with the environment. The formation in pseudohyphae is a self-protection mechanism aiming increased tolerance to formed ethanol, preventing the exposure of daughter cells to adverse factors.

4. CONCLUSION

Fermentation suffers the influence of factors such as ethanol concentration, pH, and temperature, among others. Strains of *S. cerevisiae* are characterized by good resistance to these fermentation conditions, which makes the species a good model for determining molecular markers of stress tolerance to be used in other strains. The use of molecular markers facilitates bioprospection of tolerant species, decreasing

the need for adding *S. cerevisiae* to the fermentation media. Most of these molecular markers found in our study were proteins with molecular weight equivalent to HSPs. Since those proteins are highly conserved among many different organisms, the next step of this work would be to confirm identity through *western blotting* analysis using anti-bodies anti-HSP of the same molecular mass of the peptides with increased expression under stress.

From the morphological point of view, it was observed that the increase in the concentration of ethanol in culture medium causes damage to the yeast, with dimorphism, growth of pseudohyphae, and formation of bunches. The reproduction by simple budding also changes to unipolar budding. These changes appear to be a defense response of the organism against adverse conditions, and may affect the efficiency of fermentation.

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