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Effect of salt and sugar osmotic stress on the viability and morphology of *Saccharomyces boulardii*

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***Abstract*— *Changes in the viability, composition and morphology of the probiotic yeast Saccharomyces boulardiiwerefollowed inhyperosmoticYPD broths withNaCland sucrosein concentration ranges from0.2 to 2M. Samples were observedbyscanning (SEM) and transmission (TEM) electron microscopy. SEM revealed changes inthe morphology (swelling) in the walls ofthe yeasts grown in high-osmolarity broths. TEM showed that, as a result of the high osmolarity, the cell wall was thickenedand vesicles were formed in the cytoplasm. The growth kinetics results indicated thatS. boulardiicould be considered as anosmotolerantyeast, since it could still grow and reach concentrations of$3.4x10^5$ and $3.6x10^7$ CFU/mL at sucrose concentrations of 2.0 M and 1.5Mrespectively. The results also suggested thatthis yeast could also be consideredhaloduric sincecell concentrationsof$3.9x10^6$ and $3.4x10^5$CFU/mLcould be maintained in broths with 0.4 and 1.4MNaCl respectively. S. boulardii was still able to produce 0.5% ethanol in the 2.0 M broth.*
Keywords*—*osmotic stress, Saccharomyces boulardii, viability, trehalose, glycerol.

I. INTRODUCTION

Probiotics are defined by the FAO/ WHO (2002) as"those live microorganisms, which when administered in adequate amounts, confer a health benefit on the host". It has been reported that the numberof viable probiotics in a product at the time of consumption should be in the range of $1x10^7$ - $1x10^9$CFU /mL, but this depends on the species and strain.It is also required that the probiotic strain used be safe and of human origin (Makinen *et. al.*, 2012).However, there are studies that show that there are probiotic strains of human intestinal and food origin, although it is not easy to maintain a permanent colonization of the intestinal tract by an exogenous strain (Gueimonde and Salminen, 2006). The most common probiotic microorganismsinclude strains of lactic acid bacteria, bifidobacteria and yeasts such as *Saccharomyces boulardii*, a non-pathogenic yeast, which is the only probiotic yeast approved by the FDA for human consumption (Zamith-Miranda *et. al.*, 2016).

Today, there is more diversity in the food matrices that are intended to be used as vehicles for probiotic microorganisms, including formulations with high amounts of ionic (mainly Na salts) and nonionics (mainly sucrose and polyols)solutes. These compounds have been used for centuries to provide taste and as food preservatives (Sunny-Roberts and Knorr, 2008), since these solutes are able to reduce thewater activity (a_w)of foods and are the major abiotic stressors that reduce the growth of yeasts in foodsby maintaining a high osmolarity in the medium(Dakal *et. al.*, 2014). Nevertheless, the growth and survival in each environmental niche, depend on the ability of each genus and strain of yeast to detect and respond to osmotic stress conditions, as this causes water to be expelled from the cell and result in an increase in the concentration of ions and metabolites and decreased cellular activity. Francois (2016)has described the way in which yeast cells reorganize the integrity and fluidity of their plasma membrane to produce changes in the cell wall nanomechanical properties.It is assessed that about 200 genes are involved in encoding enzymes involved in the biogenesis and remodelling of the cell wall in yeasts. These changes are aimed at establishinga balance by which the force pushing water across the osmotic gradient into the yeast cell is neutralized by turgor pressure against the plasma membrane and cell wall (Levin, 2011). Specific differences of each yeast strain, also have been described at the level of the inner layer of the ß-D-glucans and mannans of the cell wall. However, further research is still required to identify the factors affecting the resistance mechanisms and kinetic changes in the growth ofmicroorganisms(Jordan *et. al.*, 2008).Exposure of yeast cells to conditions of high concentrations of ionic and nonionic solutes during fermentation, involves several metabolic activities whichinduce specific cellular adaptation capacities to the sudden and severe fluctuations in water availability to restore or maintain normal biochemical and physiological functions(Guerzoni *et al*., 2007; Dakal*et al*.,2014).

In this study, morphological changes and survival of the probiotic yeast *Saccharomyces boulardii* under conditions of osmotic stress with sodium chloride and sucrose were evaluated. The results of their adaptation to this type of stress can be used to determine more accurately their technological use in the food industry.

II. MATERIALS AND METHODS

Yeast strain and growth conditions

The strain of probiotic yeast *Saccharomyces boulardii* (Hansen CBS 5926) used was isolated from Floratil®(Merk, S.A. de C.V., México). The freeze-dried content of a capsule was emptied into 100 ml of YPD broth (0.05% NaCl, 1% glucose, 0.5% peptone, and 0.5% yeast extract) and incubated over-night at 37°C. The yeast wasthen grown in YPD agar slants and kept refrigerated and the procedure repeatedapproximately every two months to keep them in a viable state (active). Cells were grownroutinely in 250 mL flasks to 37°C over a period of 16 h. Cells were kept frozen at -20°C in YPDbroth supplemented with 50% glycerol (v/v).

High osmolarity culture preparation

The high osmolarity medium was prepared by adding the necessary amount of NaCl or sucrose in a 100 mL volumetric flask to reach concentrations for 0.2 to 2 M. YPD broth was used to dissolve the solute. The modified medium was sterilized in Erlenmeyer flasks and inoculatedwith 5% of a suspension of *S. boulardii*, previously activated and adjusted to an OD of 1 at 640 nm. Subsequently, the medium was incubated at 37 ° C for 24 h andthe growth (measured as absorbance) was monitored spectrophotometrically at 640 nm (Spectrophotometer6405 UV/Vis, JENWAY)and the pH measured.

Determination of alcohol content

The distillation method described in the Mexican Standard NMX-V-043-1972 was followed. A sediment free sample (60 mL) of fermented medium was taken and its temperatureadjusted to 20 °C. It was quantitatively transferred to a distillation flask with 60 mL of water, which was connected to a refrigerant and to a condensation flask. Boiling was suspended when a condensate volume of approximately 70 mL was reached. The flask content was emptied into a graduated measuring cylinder and the reading taken with a Gay Lussac densimeter at a temperature of 15 °C.

Acetic acid determination by HPLC

The fermentedsugar osmotic stress broths were analyzed for acetic acid, following the method of Zheng *et al.* (2008). Chromatographic separation was performed on a Varian 920 LC HPLC system (Varian Inc., Palo Alto, California, USA) provided with a diode array detector (PDA) and quaternary pump. The column used a support containing C18 5 µM, 4.6 x 250 mm (XSelect® HSS T3, Waters, Milford, MA, USA) and placed in an oven at a temperature of 30 °C. The injection volume was 20 µL for all samples and the time of each run of 18 min, by passing the solvent (mobile phase) at a rate of isocratic flow of 0.8 mL min^{-1}, consisting of 5% acetonitrile and 95% 0.01 M KH_2PO_4 in water (pH 2.7). The samples were centrifuged at 12,000 x g for 15 min at 4 °C, of the supernatant was taken an aliquoted and diluted in a 1: 5 with mobile phase solution. Prior to injection, 20 µL was filtered through a 0.22 µm membrane for aqueous solvents (tetrafluoroethylene filter, Gelman/ Pall Life Sciences, Michigan, USA).

Biomass and intracellularglycerol and trehalose content.

*S. boulardii*cells cultured under osmotic stress were harvested via centrifugation at 10,000 x g for 5 min. For determination of intracellular glycerol, cell pellets were washed twice with 2 mL distilled water and resuspended inPBSto a final volume of 2 ml(Kobayashi *et. al.*, 2013). For dry cell weight determination, 1 mL of the cell suspension was kept at 80°C for 12 h. The other ml of suspension was transferred into a new tube,centrifuged and the pellet resuspended in 1 mL boiling 0.5 M Tris/ HCl buffer (pH 7.0) for 20 min. The cells debris were removed by centrifugation and the glycerol concentration was determined enzymatically in the supernatant (Cayman Chemical, Kit No. 10010755). For determination of the intracellular trehalose,the cells pellet was resuspended in 1 mL of 0.25 M $NaCO_3$, the suspension was incubated at 100 °C for 20 min, then cooled and centrifuged for 10 minutes at 15,000xg (Housa *et. al.*, 1998). An aliquot of 200 µL of the supernatant was taken and neutralized by addition of

100 µL of 1 M acetic acid and 100 µL of Buffer T (300 mM sodium acetate and 30 mM calcium chloride, pH 5.5). An aliquot of 100 µL of this mixture was incubated in the presence of 50 µL of trehalase (Sigma T-8778 No., diluted 1:3) for 6 hours at 37 ° C. The reaction was stopped by incubation of the sample at 100 °C for 10 min. The glucose released from the trehalose was estimated by the glucose oxidase and peroxidase method (Glucose PAP SL, GPSL-0507, ELI Tech, SEES–France) following the manufacturer's instructions.

TEM and SEM observations

The steps in preparing of *S. boulardii* cellsin TEM and SEM analyses, were: One milliliter of the cells pellet was washed twice with 0.1 M phosphate buffer pH 7.3 - 7.4 (Sorenson solution) to remove traces of the culture medium, centrifuged each time at 5040xg to remove the supernatant and subsequently fixed in 3 % glutaraldehyde solution in 0.1 M phosphate buffer for 12 hours. After this time, the cells were washed with 0.1 M phosphate buffer pH 7.2 to remove glutaraldehyde and centrifuged at 5000 rpm for 5 minutes. In each wash the cells were allowed to stand for 30 minutes. They were contrasted with osmium tetroxide and subsequently dehydratedwith ethyl alcohol at different concentrations (20, 30, 40, 50, 70, 80 and 100%). In the case of TEM analyses, after the ethanol treatments, two washes with ACN 100% were performed before making their inclusion in resin-acetonitrile (1: 1) for 48 h in a desiccator and then only with resin, left to stand for 48 h in the oven at 60 ° C. Ultrathin sections, were contrasted with uranylacetate for further observation in TEM. In the case of SEM analyses, ethanol was removed from the cells with the aid of a critical point dryer, and then the dry sample was placed on a copper and carbon tape covered with gold before observation under a JEOL JSM-7800F SEM. (Vazquez-Nin and Echeverria, 2000).

III. RESULT AND DISCUSSION

Yeast cell viability under osmotic stress

Survival to osmotic stress of *S. boulardii* in YPD broth, modifiedto reach concentrations ranging from 0.2 to 1.4 M NaCl,was evaluated (Fig. 1a). In the 0.2 M broth, the logarithmic phase started after 4 hours of fermentation and lasted around 8 h, reaching a population of 7.08 log CFU/mL, while in the condition of 0.4 M the population reached 6.59 log CFU/mL after 24 hours of fermentation. NaCl, in the concentration range of 0.6M to 1.4M, showed a zymostatic effect towards *S. boulardii*since the yeast had greater difficulty to withstand and adapt to the osmotic stress generated by ionic solutes. *S. boulardii*, then,could be considered as ahaloduric yeast, that is, a microorganism that can survive in high salt concentrations but cannot grow. Most bacteria and fungi use the strategy of compatible solutes buildup for keeping their intracellular concentrations of Na^+underneath toxic levels. It has been reported that the salt-sensitive yeast*Saccharomyces cerevisiae* almost exclusively uses glycerol as the compatible solute (Gunde-Cimerman *et al*., 2009). At 34°C, concentrations of 1.5 M or higher of NaCl inhibited the growth of *S. cerevisiae* (Almagro *et al*., 2000), showing a similar behavior to *S. boulardii* in this study. Papouskova and Sychrova (2007) showed that the co-action of osmotic and high temperature stresses actually results in growth improvement in *Debaryomyces hansenii* so the influence of combined stress situations must be studied also.

In the case of sucrose, the stress evaluation was performed at a concentration range of 0.2 to 2.0 M (Figure 1b).A similar trend was observed at concentrations of 0.2 to 0.6 M (7-20% sucrose) with respect to the control, with longerlag and log phases.Populations of 7.45 log CFU/mL and 7.3 log CFU/mL after 10 and 20 h of fermentation were reached in the control and in the 0.6 M concentration respectively. When the sucrose concentration was increased to 0.9 M and 1.5 M (30 and 50% sucrose)similar populations could only be reached after 24 and 30 h respectively. *S. boulardii* had a high tolerance to sugar osmotic stress and could be considered as an osmoduric yeast since it presented viability and a feeble growth even at concentrations of 1.8 and 2.0 M (> 60%), indicating that none of the concentrations evaluated in this assay managed to have a complete inhibitory effect. In general, increasing the concentration of the osmolyte resulted in an increase in the duration of the lag and log phases. The ethanol concentration (Table 1) was also measured and the highest concentrations were obtained in the 0.4 and 0.6 M sucrose broths (8% alcohol) after 24 h of fermentation.The presence of a large amount of CO_2could also be observed.Ethanol (0.5%) could still be produced even at concentrations of 1.8 M and 2.0 M after 36 h of fermentation.This behavior reflects thefact that the cells still retain their fermentation capacity even at the low concentration of 5.4 log CFU/mL. Hernandez-Lopez *et al*. (2003) found that, in the case of*Torulaspora delbrueckii* and *S. cerevisiae*,the exposure tothe hyperosmotic stress of bread dough containing 20% (0.62M) sucrose resulted in a dramatic drop of the fermentative capacity. This confirms the higher osmotolerance of the *S. boulardii* strain of this study.

Trehalose and glycerol accumulation in yeast cells

Acetate was measured in the YPD broths after 24 h of fermentation only as an indication that it was being produced and that the yeast cells could also be accumulating it intracellularly. The acetate concentrations were 1.16, 2.85, and 2.22 mg/mL in the broths with 0, 0.9, and 1.8M sucrose concentrations respectively, indicating that the high sugar stress increases the synthesis of acetate for a possible use as an osmotic protectant. Trehalose and glycerol function as potential stress protectants by preserving the integrity of the plasma membrane and stabilizing the proteins (Wang *et al.*, 2014). Under stress-free conditions, trehalose starts accumulatingwhen cells enter the stationary phase and contributes to survival in the stressful conditions of this growth phase.Recently, it has been shown that, in the case of *S. cerevisiae*under saline stress conditions, there is anincreased expression of the genes encoding the enzymes involved in trehalose, glycerol and acetate syntheses (Mahmud *et al.*, 2009). These facts support the idea that these compounds play important roles in osmotic tolerance. The presence of acetate as a stress protectant is important since organic acids produced during fermentation (lactic, acetic, and succinic acids) by yeasts account for a significant fraction of the metabolic products (Zheng *et al.*, 2009).

The intracellular trehalose and glycerol contents were determined after the cultures entered the stationary phase (see Table 2).The increased levels of trehalose detected in the case of the NaCl osmotic stress, indicates that *S. boulardii* behaves in a similar way as *S. cerevisiae* (Mahmud *et al.*, 2009) when exposed to saline stress and synthesize trehalose to be used as a protectant. On the other hand, the trehalose levels obtained in the culture media with sucrose were similar to those of the control indicating that, unlike *S. cerevisiae* (Wang et al., 2014), *S. boulardii* does not use the production of trehalose as a strategy to tolerate osmotic stress. Yoshiyama *et al.* (2015) related the amount of accumulated intracellular trehalose (0.6 mg/g) in *S. cerevisiae* with an enhanced tolerance to acetic acid. Hounsa *et al.* (1998) found, also in S. *cerevisiae* under osmotic stress with NaCl and sorbitol, values of trehalose of 2.52 and 2.56 mg/g biomass respectively in the stationary phase. Several authors (Yoshiyama *et al*, 2015; Malgorzata *et al*, 2015; Hounsa *et al*, 1998) have indicated that the concentration of intracellular trehalose is directly related to cell survival, however, this is not universal as in the case of *S. boulardii* under osmotic stress.

Glycerol, the main compatible solute in *S. cerevisiae*, is accumulated intracellularly when the yeast is exposed to osmotic stress (Nevoigt and Stahl, 1997). In this study (Table 2), the glycerol intracellular levels increased with the severity of the saline or osmotic stress.Similar results were reported by Pigeau and Inglis, (2007) where higher levels of glycerol were detected in yeast fermentations with high sugar content (1.2 M) compared to fermentations witha lower sugar content (0.6 M).An increase in glycerol intracellular concentration was also reported in the case of the yeast *Saccharomycopsis fibuligera* when subjected, as in this study, to high salt concentrations (0.5 and 1 M) in YPD media (Yan *et al*., 2008).

Effect of osmotic stress on the cell morphology of *S. boulardii*

In the SEM micrographs, the cells subjected to osmotic stress (Fig.2c-f) show no apparent change in size with respect to thestress-free cells (Fig. 2a-b). In the yeasts with salt stress treatment, cells with wrinkles and with diverse degrees of plasmolysis can be observed (Fig. 2c-d). In the cells subjected to osmotic stress with sucrose, no differences can be observed with respect to the control with the exception of a slightly rougher surface.Similar results were observed by Dakal *et al*. (2014) with the yeast*Zygosaccharomyces rouxii*. Figures3a-b show TEM micrographs of cells of *S. boulardii* untreated, where a typical structure and a regularly stained cytoplasm with a high electron density and a well-defined ultrastructure can be observed. With the increase insalt or sucrose concentration, cells have a tendency to retain nutrients in order to keep the intracellular homeostasis; this process results in the generation of reserve vesicles as shown in Figures3c and 3e. In the case of Figure 3c (1.4 M NaCl), a greater damage is observed with the presence of membranous bodies and structural disorder in the cytoplasm and a cell generally less electrodense. Figure 3d shows a more elongated cell but not too different from the control. Figure 3e (1.8M sucrose) shows the cell wall surrounded by an outer and an inner dark thick coat not observed in the other cases. This extra double barrier could help the cell to avoid the plasmolysis phenomenon observed in hypertonic media. In all stress conditions a thickened and electrondense cell wall was observed.Yeast cell walls have an inner matrix of interlinked ß-glucan and chitin to offer rigidity and tensile strength. Yeast cells are known to be able to remodel their cell walls with time in response to osmotic and other environmental stresses (Ene *et al*., 2015). Aguilar-Uscanga*et al.* (2005) observed that the variation in composition of the polysaccharides of the cell wall in *S. cerevisiae* plays an important role during cell formation.Dakal *et al.* (2014) determined that in the presence of high concentrations of extracellular solutes, cells undergo important physiological variationssuch as changes in the chemical and physical structure of the plasma membrane and cell wall and alteration in theosmotic pressure and volume of the cell wall. The first studies inZygosaccharomyces rouxii suggested that,in the presence of salt, the concentration of mannans of the cell wall decreased (Hosono, 1992).In the case of*Saccharomyces pombe* and *Saccharomyces cerevisiae*,signaling pathways known as cell wall integrity pathways have been localized for the regulation of changes in the cell wall (CWI) (Klis *et al*., 2006; Madrid *et al*., 2006; Levin, 2011).

IV. CONCLUSIONS

The above results showed that the accumulation of osmotically compatible metabolites such as trehalose and glycerol is very important for the survival of cells and for maintaining the stability and functionality of the cell wall and plasma membrane. An increased intracellular glycerol content was consistent with the presence of high concentrations of salt or sugar in the environment whereas trehalose was produced in a larger amount only in salt stress conditions. High sugar stress increased the synthesis of acetate for a possible use as an osmotic protectant. Changes at the ultrastructural level in *S. boulardii* cells were more evident in the case of NaCl, where plasmolysis could be observed, than in sucrose osmotic stress.

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FIGURES AND TABLES

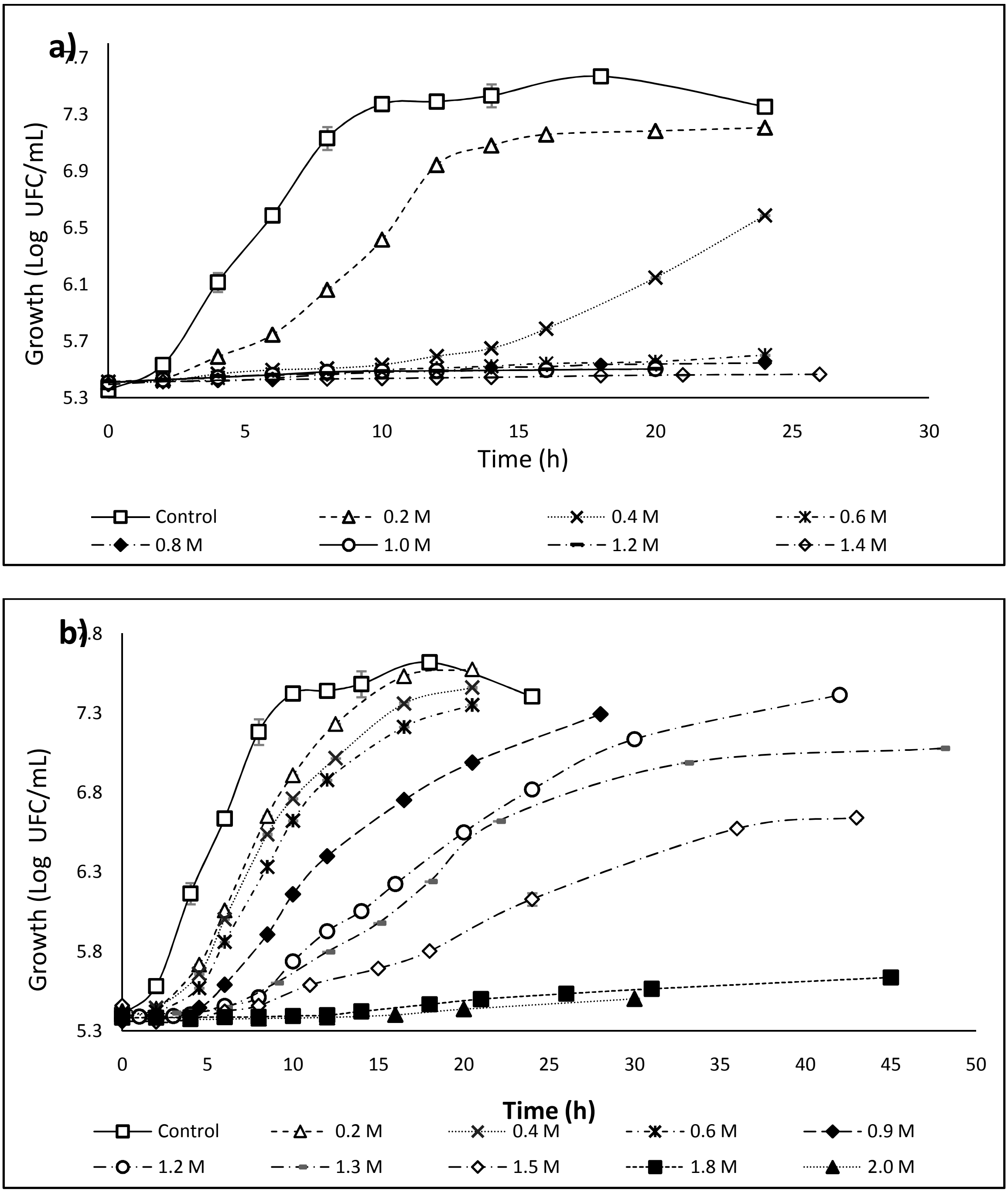

Fig.1:Saccharomyces boulardiisurvival under different conditions of osmotic stress: a) NaCl and b) Sucrose.

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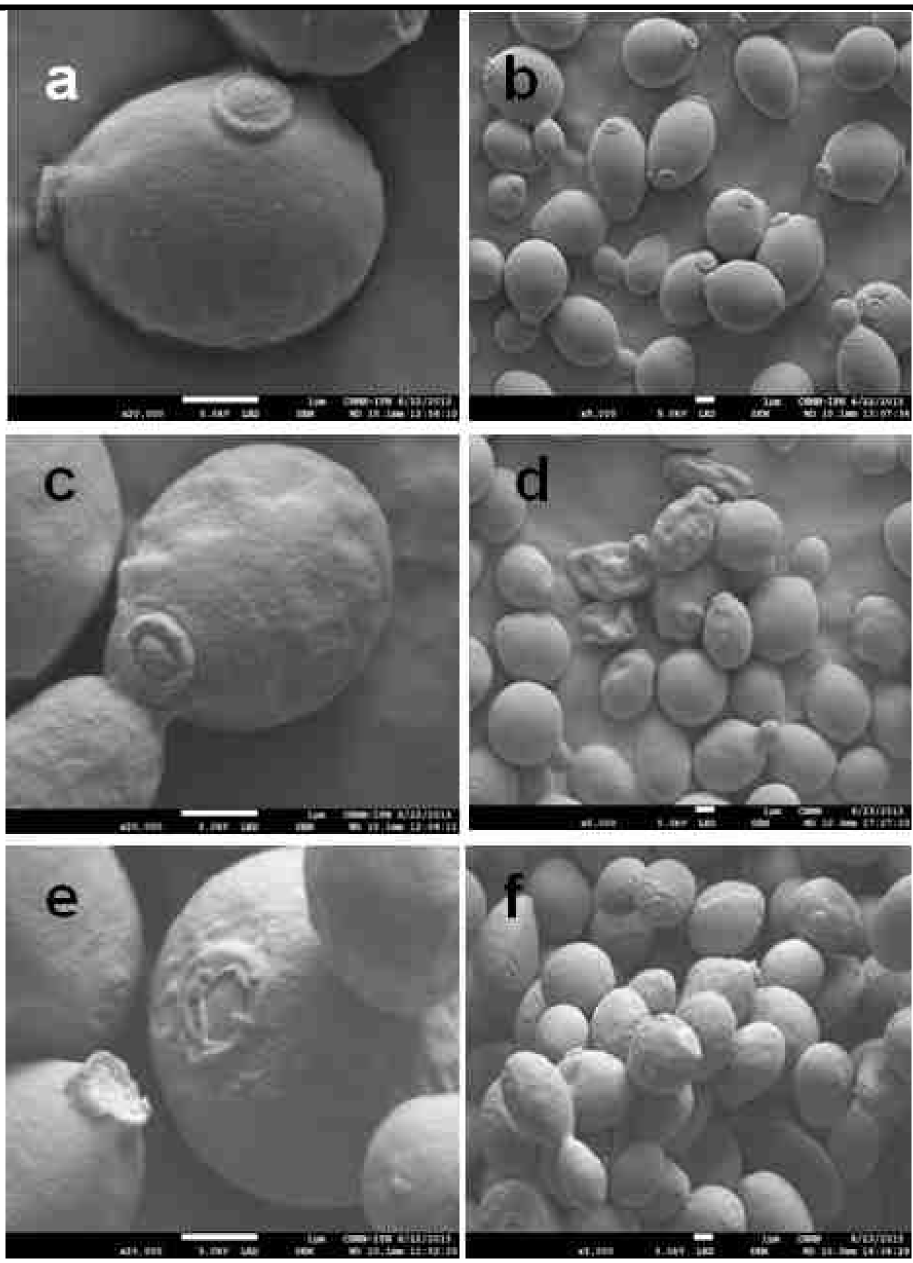

Fig.2: Scanning electron micrograph of S. boulardii, a) and b) control cells, c) and d) grown in 1.4 M NaCl YPD broth e) and f) grown in1.8 M sucrose YPD broth. Magnification is 20000x, white bar indicates 1 µm.

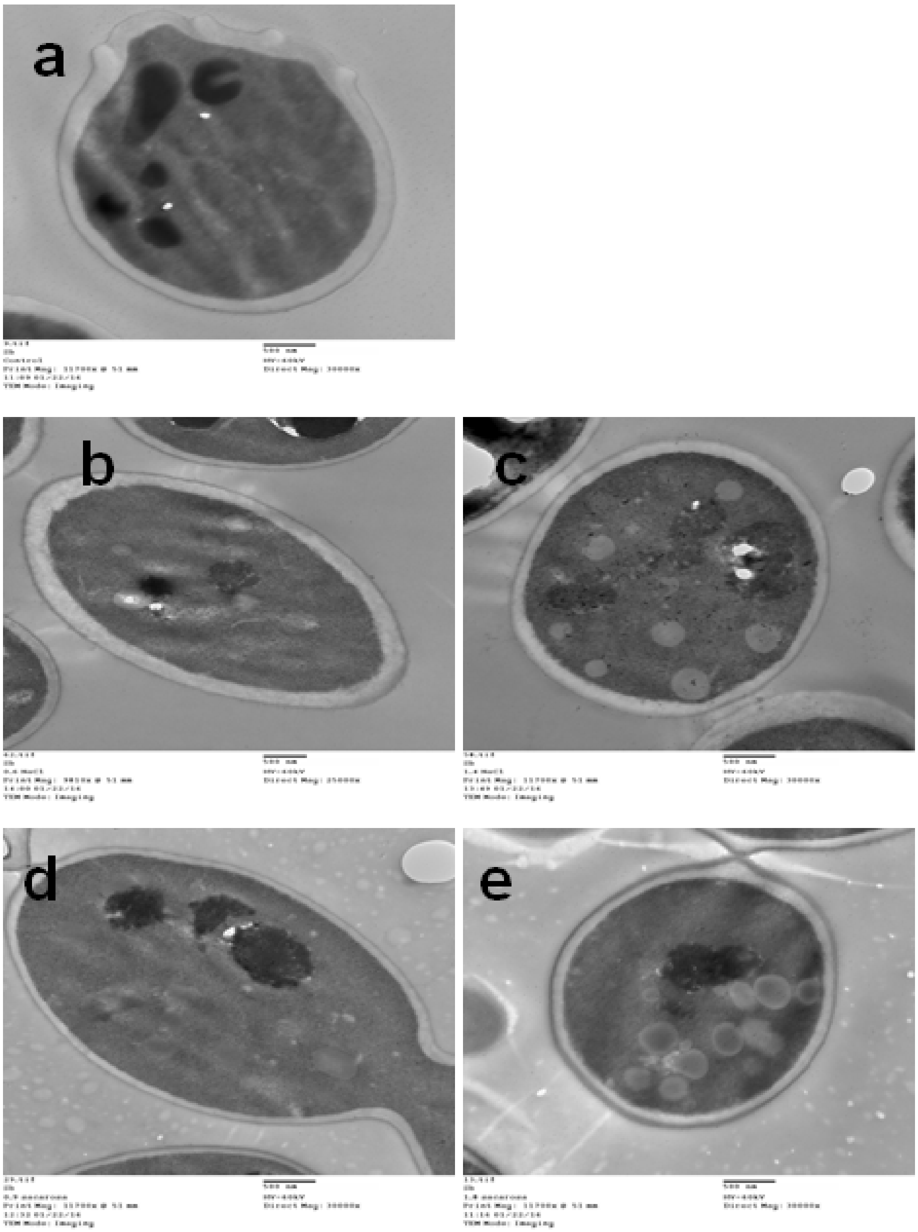

Fig.3: Transmission electron micrograph of S. boulardii a) control, b) grown in 0.6 M NaCl YPD broth (c) grown in 1.4 M NaCl YPD broth (d) grown in 0.9 M sucrose YPD broth and (e) grown in1.8 M sucrose YPD broth. Magnification is 60,000x, black bar is 500 µm.

Table.1: Alcohol content in fermented YPD broth at different sucrose concentrations

Sucrose concentration	Alcohol (mg/mL)	Alcohol (% v/v)
0.2 M	39.5	5
0.4 M	63.1	8
0.6 M	63.1	8
0.9 M	59.2	7.5
1.2 M	39.5	5

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1.3 M	35.5	4.5
1.5 M	35.5	4.5
1.8 M	11.8	1.5
2.0 M	3.9	0.5

Table.2: Mean intracellular trehalose and glycerol content in S. boulardii at different concentrations of NaCl and sucrose in YPD medium at the stationary phase

Treatment	Trehalose content (mg/g dry weight)	Glycerol content (mg/g dry weight)
Control	0.25 ± 0.007	12.35 ± 0.685
NaCl 0.6 M	0.58 ± 0.009	192.34 ± 2.102
NaCl 1.4 M	1.16 ± 0.007	872.15 ± 2.809
Sucrose 0.9 M	0.23 ± 0.002	206.87 ± 4.314
Sucrose 1.8 M	0.32 ± 0.013	791.64 ± 2.506

* Values are mean ± SD, n = 2