International Journal of Scientific Research and Engineering Development-– Volume 5 Issue 1, Jan-Feb 2022

Available at www.ijsred.com

RESEARCH ARTICLE OPEN ACCESS

Influence of Temperature on the Effect of Multiple Stressors in *Debaryomyces Hansenii*

Camille A. Berrocal*, Brent Petersen**, Dr. Govind Nadathur ***

*(Department of Marine Science, University of Puerto Rico at Mayaguez, Mayaguez, PR 00681, Puerto Rico, Email: camille.amaro1@upr.edu)

** (Department of Marine Science, University of Puerto Rico at Mayaguez, Mayaguez, PR 00681, Puerto Rico, Email: brent.petersen@upr.edu)

*** (Department of Marine Science, University of Puerto Rico at Mayaguez, Mayaguez, PR 00681, Puerto Rico, Email: govind.nadathur@upr.edu)

----------**********----------

Abstract:

Stress-induced gene expression has been significant for understanding the effect of organisms' biological responses to environmental stressors. The ascomycete marine yeast *Debaryomyces hansenii* is considered to be an extremophile capable of living in a wide range of salinities (3% – 12% w/v), temperatures (6°C – 37°C), and in environments loaded with heavy metals like Cobalt and Zinc. In this study, *D. hansenii*, was used to identify the coactive effects of osmotic, oxidative, and thermal stresses to develop scenarios for engineering this robust yeast for industrial biomolecule production. This study found physiological variations caused by the prophylactic effect of some stressors, such as low temperature, which seems to protect the organism against osmotic and oxidative stresses. At higher temperatures it seems to be the stressors that help the organism endure the thermal fluctuation.

Keywords **—Synergistic stressor effect, thermic stress, oxidative stress, osmotic stress.**

----------**********----------

I. INTRODUCTION

Debaryomyces hansenii J6 is an ascomycete marine yeast originally isolated from a Swedish estuary [1,2]. Like all organisms, it must adjust its metabolism to accommodate an ever-changing environment. *D. hansenii* has been shown to tolerate much higher sodium chloride (NaCl) concentrations than those typical of seawater [3]. This yeast can grow in a wide pH range and in the presence of heavy metals, such as Cobalt [4,5]. Moreover, *D. hansenii* is known to grow at temperatures ranging from 11°C to 37°C (24°C being optimal), a broader range than those reported for more familiar yeasts [6] (unpublished data- Camille Amaro Berrocal & Dr. Govind S. Nadathur – University of Puerto Rico – Department of Marine Sciences).

Temperature is known to alter the response of *D. hansenii* toward other stressors [7,8]. These changes can enhance or diminish the organism's fitness in various environmental conditions. For example, in Prista et al. 2005 [9], 1M NaCl combined with high temperatures (~34°C) stimulated growth beyond the recorded maximum for either individual stressor. A study into the effect of concurrent stressors (temperature and NaCl) on the production of glycerol and trehalose in Saccharomyces cerevisiae found a similar synergistic effect in that organism [10,11]. According to Babazadeh et al. 2017 [12], this enhanced response can vary depending on the carbon source utilized by the yeast. Sorensen and Jacobson performed a similar study in 1997, which used flow cytometry to detect changes in the growth patterns of yeasts, including *Debaryomyces*, under the combined effects of pH, temperature, and

NaCl concentrations. In that work, higher tolerances for additional stressors were observed at lower temperatures [13]. In contrast, Papouskova et al. 2007 [8] reported only a temperature effect on *D. hansenii*, in a multiplexed investigation into its response to various combinations of sorbitol, NaCl, and KCl along a temperature gradient.

Debaryomyces spp can grow in a range of heavy metal concentrations e.g., cobalt (<5mM), zinc (<4mM), copper (<0.5mM), cadmium (<180uM) [5, 14-15]. Some strains produce riboflavin when exposed to metallic substances [14, 16-17], while others are spontaneous riboflavin producers (unpublished data- Dr. Govind S. Nadathur – University of Puerto Rico- Department of Marine Sciences).

Previous stress studies of *Debaryomyces* were often conducted at a single temperature to observe the isolated effect of the stressor of interest [5, 11]. The analysis of stressor interdependence yields a more complete understanding of cell resilience. Since *D. hansenii* tolerates a wide range of temperatures and can thrive in varied NaCl and heavy metals concentrations, this organism constitutes an ideal model for studying gene interactions under different stress conditions. Further research in this area will be fundamental to determining the actual genes involved in specific stress responses versus those that are part of a global 'rescue reaction'. The complete genome of the *D. hansenii* strain (J6) used in this study has been sequenced [18]. However, it is necessary to apply transcriptomics to understand the genetic component behind the biological response to temperature fluctuations. This study utilizes cell viability as a proxy for identifying physiological changes caused by multiplexed stressors. Further gene expression analysis will be performed to evaluate the influence of temperature variation in the stress response of strain J6 to the presence of heavy metals (Cobalt) and osmotic stress (NaCl) for. This study will serve as a base to proceed with further transcriptomic research on the different conditions.

II. Materials and Methods

A. Yeast strain, growth conditions, and growth curve

D. hansenii strain J6 (originally isolated from a Swedish estuary [2] (gifted by Dr. Adler) was cultured in 250mL of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) for 72 hrs in a shaking incubator set at 24oC and 150rpm (New Brunswick Scientific, Edison, USA). The culture was sampled every 24 hrs to determine a growth curve. A growth curve was generated from sample cell count viability readings collected with a ThermoFisher Cell Counter Countess II FL, utilizing the standard 0.4% trypan blue reagent (1:1). This curve was used to establish the late log phase (1E+08 cell/mL density) to avoid glucose interactions when adding the stressors.

B. Multiple Stressors and Cell viability

The same basal conditions from the growth curve study were employed to grow a fresh culture of *D. hansenii*to a cell density of 1E+08 cell/mL. 100mL aliquots from this master batch were measured into Erlenmeyer flasks which were sorted into four treatment sets nested in four temperature sets. Stressors were added and the culture flasks were transferred to different shaking incubators according to their assigned temperatures (11°C, 24°C, 30°C, and 37°C). At each temperature there were three flasks per stressor plus three control flasks.

The factors tested at each temperature were osmotic stress (2M NaCl) [17], oxidative stress (5mM Co (II)) [5], and a combination of the two (2M NaCl + 5mM Co (II)). Each flask was sampled in triplicate at 0 hr, 0.5 hr, 1.5 hr, and 3.0 hrs after stress induction. All samples were assessed for cell viability and total population with a ThermoFisher Cell Counter Countess II FL, utilizing the standard 0.4% trypan blue reagent (1:1). When necessary, dilutions were made to keep cell densities within the accurate range of the instrument. The 24°C treatment was used as a control group.

III. RESULTS

C. D. hansenii Growth Curve

Grown at its putative optimal temperature of 24°C (Figure 1), *D. hansenii* J6 took 30 hours to reach the predetermined induction point of 1E+08 cell/mL. In the first 24 hours, the population increased 100-fold. It took 72 hours to achieve the maximum population level, which was 1.8E+10 cell/mL. Growth variation due to the influence of Co (II), NaCl, and Co (II)+NaCl at all investigate temperature can be found in Figure 2.

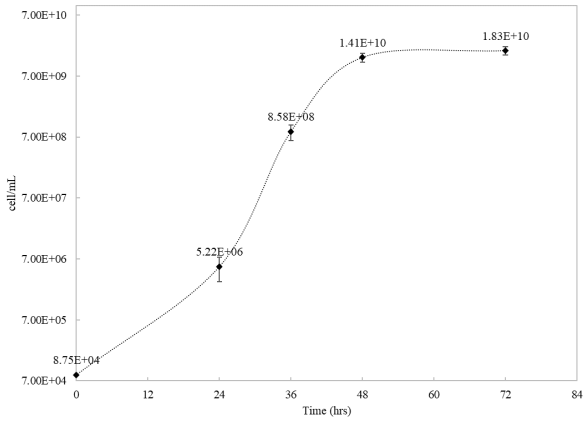

Figure 1 Growth curve of *D. hansenii* J6 at 24°C without stressors.

D. Thermally Induced Physiological Changes

Thirty minutes after shifting *D. hansenii* cultures from 24°C to 11°C the impact on population expansion became apparent as all treatment populations contracted while the colonies adjusted to the changed environment. At the 90-minute point both the control and 5mM Co (II) cultures had become sufficiently acclimatized to begin a second population growth phase. The populations for the 2M NaCl and 5mM Co II + 2M NaCl cultures continued to decline for the 180-minute duration of the study. These last two samples showed cell mortality (Table 1) of 30% at the 1.5hr mark and 27% at the 3hr mark. Decreasing populations were observed in both sets, as represented by their negative slopes (Fig. 2 a). The sample set for 5mM Co (II) appears to run parallel to that of control, and its cell death rate decreased from an initial 15% to 9%.

When cultures were kept at 24°C the control samples behaved predictably like those used to generate the initial growth curve. Increasing medium NaCl molarity to 2M resulted in an immediate population boom (7.5X higher when compared to control) that peaked and crashed (30% mortality rate) at 30 minutes for those samples. This was followed by modest population growth after 90 minutes, though mortality remained high (27%.) The 5mM Co (II) sample set experienced a delayed population dip after 30 minutes followed by resumed population growth after 90 minutes. This behaviour was also noticeable on the combined sample (5mM Co II + 2M NaCl), where it had proportional mortality of 37% at 1.5hr time point. This temperature treatment yielded a positive slope at the 1.5hr to 3.0hr time point interval suggesting population increase (Table 1).

As is evident in Fig. 2c, when temperature was shifted from 24°C to 30°C the control samples had an initial 6% mortality rate and exhibited continued recovery at all subsequent time points, surpassing the 24°C control maximum cell count at the 3hr mark. Likewise, the 5mM Co (II) and 2M NaCl had high initial mortality rates (26% and 56% respectively after 30 minutes). Of all treatments in this group the 2M NaCl samples had the most precipitous population crash upon induction. However, the cultures impinged with both 5mM Co II and 2M NaCl experienced an immediate population boom of 5x with a comparably low mortality rate of 22%. This population spike was followed by a crash at 30 minutes. All sample were recovering after 90 minutes, with the control samples showing the most robust recovery.

Shifting temperature from 24°C to 37°C elicited a similar response from the control sample as did the shift to 11°C. Wherein both cases cells seemed to

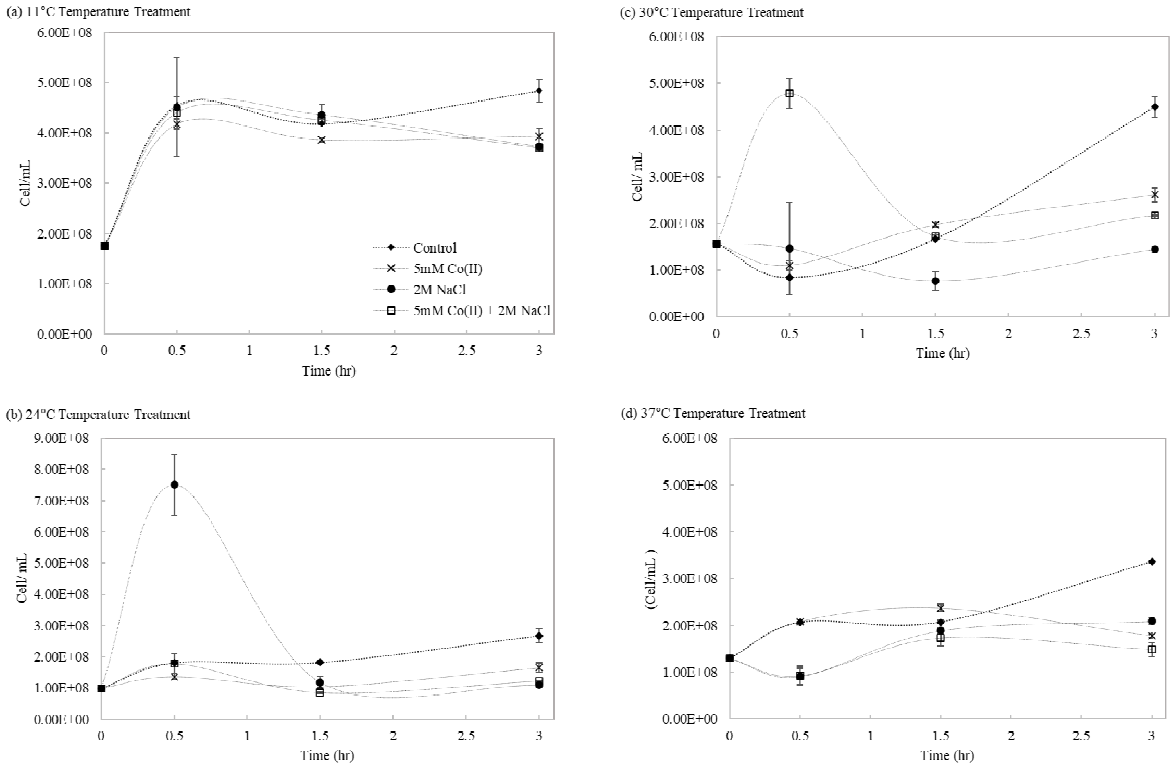

Figure 2a, 2b, 2c and 2d Influence of multiples stressors on yeast cell growth.Each represents the yeast population growth under oxidative (5mM Co II) and osmotic stress (2M NaCl) at 11°C (2a), 24°C (2b), 30°C (c), and 37°C (d) temperature treatments. The thick dash line shows the control of *D. hansenii* at each temperature without added stressors.

TABLE I
CELL VIABILITY PER MULTIPLE STRESSOR TREATMENT

	Time Point/ % Cell mortality		
Treatments	**0.5hr**	**1.5hr**	**3.0hr**
11°C Control temperature	1.7%	7.4%	5.2%
11°C 2M NaCL	14.5%	30.3%	27.6%
11°C 5mM Co(II)	15.9%	14.7%	9.2%
11°C 2m NaCl + 5mM Co(II)	12.9%	37.3%	27.1%
24°C Control temperature	1.8%	7.5%	5.3%
24°C 2M NaCL	14.6%	30.4%	27.6%
24°C 5mM Co(II)	16%	14.8%	9.2%
24°C 2m NaCl + 5mM Co(II)	13%	37.3%	27.1%
30°C Control temperature	6%	3.3%	9%
30°C 2M NaCL	**56.9%**	36.4%	25.9%
30°C 5mM Co(II)	26.3%	3.5%	19.7%
30°C 2m NaCl + 5mM Co(II)	22.3%	28.8%	7.9%
37°C Control temperature	0.8%	2%	0.7%
37°C 2M NaCL	24.2%	10.9%	15.5%
37°C 5mM Co(II)	5.8%	5.9%	1.8%
37°C 2m NaCl + 5mM Co(II)	**41.5%**	19.2%	26%

be assimilating to the initial shock between the 0.5hr and 1.5hr time points and later recovering cell division at the 3hr mark. While the 5mM Co (II) samples, had comparatively low mortality rates throughout the study, their cell growth seems to have been inhibited, and by the 3hr mark, their populations were in decline. The 2M NaCl samples had a 24% mortality rate at the 0.5hr time point but were experiencing recovery after 90 minutes. The negative impacts of oxidative and osmotic stresses seemed to be additive at this temperature with 5mM Co II + 2M NaCl sample having a 41% mortality rate (Table 1).

IV. Discussion and Conclusions

At lower temperatures, a delayed response to stressors was observed. For example, the 11°C Temperature Treatment samples, with or without added stressors, exhibited similar initial responses, indicating that temperature was the steering stressor. Acclimatization to the thermal change was the priority, while the low cell death rate (<15%) indicates that the effect was not critical to survival. The initial growth increase from 0hr to 0.5hr is consistent with first-stage cold response of enhanced phospholipid synthesis and fatty-acid desaturation [19]. The control sample reinitiated cell growth 1.5 hr -3.0hr. Since the organism is endemic to a Swedish estuary where the temperatures range from 4°C-19°C, it is preadapted to this condition.

At 11°C and 24°C, NaCl seems to have a negative delayed effect on cell growth. Interestingly 2M NaCl seemed to increase cell death, > 30%, over time. Being a marine yeast, *D.hansenii's* typical habitat possesses 3.5% salinity that equates to ~ 0.75M NaCl; here, it was exposed to 3.3X higher concentration. In this scenario, the salinity is the driving stressor since the same mortality % pattern is reflected in all NaCl samples at 11°C and 24°C. Although preadapted to moderate salinity [20], this organism shows difficulty in recovering from more extreme salinity. Nevertheless, J6 showed higher resilience to increased osmolarity at low temperatures than has been observed in *D. hansenii* strain CBS 767 [21,22]. It would appear that *D.hansenii* J6 is more tolerant to osmotic stress than other strains. NaCl seems to be mildly disadvantageous, while Co (II) seems to be a stressor they overcome easily. The yeast did not react critically to subtoxic levels of Cobalt in the media; initial death rate was 15% of cell population and recovery continued. It is known after 4-12hrs S. cerevisiae, enters the second phase of the cold general response, which brings the upregulation of glycogen and trehalose metabolism genes, detoxifying reactive oxygen species (ROS), and defense against oxidative stress [19,23]. This behaviour was likewise identified in *D. hansenii* by Guma-Cintron et al. 2015 [5], where the fast-acting assembly of DNA synthesis and repair genes within 30 mins and cell growth recovery 3hrs later, under 24°C temperatures was observed. Although *D. hansenii* is not preadapted to heavy metal oxidative stress, lower temperatures seem to accelerate its adaptive response.

At 30°C samples behave differently than at other temperatures. All other temperatures show a driving trend, and this is clear in the multiplexed stressor samples (5mM CoII +2M NaCl) across all temperatures, where cell mortality and cell growth seem parallel to but shifted higher than the more deleterious individual stressor. However, in the 30°C environment the multiplexed stressor samples showed an initial 5X increase in cell population and only a 22% cell death rate. At this temperature, the single 2M NaCl as a lone stressor caused a mortality rate of 56% (Table 1), which indicates a non-additive effect of combined stressors. The control sample required 3hr to reach the same high point in population size that the 5mM CoII +2M NaCl samples achieved in 30 minutes. This is the only case suggesting a positive synergistic effect of

multiple stressors, where the effect seemed to stimulate growth and recovery. Observing the individual stressors in this treatment, cell death oscillated over time, and recovery seemed more difficult than in the combined sample.

At the highest temperature investigated (37°C), the detrimental effects of the individual stressors on cell death seemed to be subdued, although cell growth was stagnant for some samples. In this instance 5mM Co (II) affected cell growth, but not cell death; with the population plateauing after an initial 0.5hrs of cell growth. The cultures subjected to 2M NaCl had a 24% mortality rate, but had begun recovering by the 3hr mark. This stress response was lower than expected considering the increased cell deaths from low to high temperature (Table 1). Previous studies have shown growth impedance at the same temperature for *D. hansenii* CBS767 at ~0.4M NaCl, an even lower concentration of the osmolyte than studied here [22]. Here the multiplexed stressor samples did not appear to benefit from synergism. They suffered 41% mortality at the first time point, and their recovery appeared to be slow or static.

In an overall assessment, lower temperatures seemed to mitigate the effects of stress, while the stressors appeared to be prophylactics against temperature swings. It is of note that although 2M NaCl seemed to have detrimental effects from 30°C -37°C it performed better than other *D. hansenii* strains like CBS767 or CBS1793, it is genuinely remarkable in its ability to not only survive but thrive [8]. This means that this yeast can endure far more intense stress than previously expected. A future area of research would be to determine the cold response for *D. hansenii* J6 and identify potential phases marked by specific metabolic pathways. Also, it would be of interest to research the transcriptional response at all these temperature points since it seems to be an essential aspect in assimilating other environmental stressors. Finally, it would be useful to determine why different strains of this organism react differently to the various stressors.

ACKNOWLEDGMENT

This work was financed by the Molecular Marine Biology Laboratory from the Department of Marine Sciences, the University of Puerto Rico at Mayaguez, Mayaguez, Puerto Rico.

REFERENCES

[1] Norkrans B 1966. Studies on marine occurring yeasts: growth related to pH, NaCl concentration, and temperature. Archiv für Mikrobiologie, 54(4), 374-392.

[2] Adler L 1986. Physiological and biochemical characterization of the yeast Debaryomyces hansenii in relation to salinity. In: The Biology of Marine Fungi. Moss, S. T. (ed.). Cambridge Univ. Press, New York, pp. 81-90

[3] Norkrans B, Kylin A 1969. Regulation of the potassium to sodium ratio and of the osmotic potential in relation to salt tolerance in yeasts. Journal of Bacteriology, 100(2), 836-845.

[4] Converti A, Domínguez JM 2001. Influence of temperature and pH on xylitol production from xylose by Debaryomyces hansenii. Biotechnology and Bioengineering, 75(1), 39-45. doi:10.1002/bit.1162

[5] Gumá-Cintrón Y, Bandyopadhyay A, Rosado W, Shu-Hu W, Nadathur GS 2015. Transcriptomic analysis of cobalt stress in the marine yeast Debaryomyces hansenii. FEMS yeast research, 15(8)

[6] Breuer U, Harms H 2006. Debaryomyces hansenii — an extremophilic yeast with biotechnological potential. Yeast, 23(6), 415-437. doi:10.1002/yea.1374

[7] Gasch AP, Werner-Washburne M 2002. The genomics of yeast responses to environmental stress and starvation. Functional & Integrative Genomics, 2(4-5), 181-192. doi:10.1007/s10142-002-0058-2

[8] Papouskova K, Sychrova H 2007. The co-action of osmotic and high temperature stresses results in a growth improvement of Debaryomyces hansenii cells. International journal of food microbiology, 118(1), 1-7.

[9] Prista C, Loureirodias M, Montiel V, Ramos J 2005. Mechanisms underlying the halotolerant way off. FEMS Yeast Research, 5(8), 693-701. doi:10.1016/j.femsyr.2004.12.009

[10] Carvalheiro F, Roseiro J, Gírio F 1999. Interactive effects of sodium chloride and heat shock on trehalose accumulation and glycerol production by Saccharomyces cerevisiae. Food Microbiology, 16(6), 543-550. doi:10.1006/fmic.1999.0274

[11] Michán C, Martínez JL, Alvarez MC, Turk M, Sychrova H, et al. 2013. Salt and oxidative stress tolerance in Debaryomyces hanseniian d Debaryomyces fabryi. FEMS Yeast Research, 13(2), 180-188. doi:10.1111/1567-1364.12020

[12] Babazadeh R, Lahtvee P, Adiels CB, Goksör M, Nielsen JB, et al. 2017. The yeast osmostress response is carbon source dependent. Scientific Reports,7(1). doi:10.1038/s41598-017-01141-4

[13] Sørensen BB, Jakobsen M 1997. The combined effects of temperature, pH, and NaCl on growth of Debaryomyces hansenii analyzed by flow cytometry and predictive microbiology. International journal of food microbiology, 34(3), 209-220.

[14] Gadd G, Edwards S 1986. Heavy-metal-induced flavin production by Debaryomyces hansenii and possible connexions with iron metabolism. Transactions of the British Mycological Society, 87(4), 533-542. doi:10.1016/s0007-1536(86)80094-8

[15] Navarrete C, Siles A, Martã-nez JL, Calero F, Ramos J 2009. Oxidative stress sensitivity in Debaryomyces hansenii. FEMS Yeast Research, 9(4), 582-590. doi:10.1111/j.1567-1364.2009.00500.x

[16] Anderson FB, Harris G 1963. The Production of Riboflavin and D-Arabitol by Debaryomyces subglobosus. Journal of General Microbiology, 33(1), 137-146. doi:10.1099/00221287-33-1-137
[17] Seda-Miró JM, Arroyo-González N, Pérez-Matos A, Govind NS 2007. Impairment of cobalt-induced riboflavin biosynthesis in a Debaryomyces hansenii mutant. Canadian Journal of Microbiology, 53(11), 1272-1277. doi:10.1139/w07-098
[18] Berrocal CA, Rivera-Vicens RE, Nadathur GS 2016. Draft Genome Sequence of the Heavy-Metal-Tolerant Marine Yeast Debaryomyces hansenii J6. Genome Announcements, 4(5). doi:10.1128/genomea.00983-16
[19] Aguilera J, Randez-Gil F, Prieto JA 2007. Cold response in saccharomyces cerevisiae: New functions for old mechanisms. FEMS Microbiology Reviews, 31(3), 327-341. doi:10.1111/j.1574-6976.2007.00066.x
[20] Gonzalez NA, Vázquez A, Zuazaga HG, Sen A, Olvera HL, et al. 2009. Genome-wide expression profiling of the osmoadaptation response of Debaryomyces hansenii. Yeast, 26(2), 111-124. doi:10.1002/yea.1656
[21] Almagro A, Prista C, Castro S, Quintas C, Madeira-Lopes A, et al. 2000. Effects of salts on Debaryomyces hansenii and Saccharomyces cerevisiae under stress conditions. International Journal of Food Microbiology, 56(2-3), 191-197. doi:10.1016/s0168-1605(00)00220-8
[22] Bubnová M, Zemančíková J, Sychrová H 2014. Osmotolerant yeast species differ in basic physiological parameters and in tolerance of non-osmotic stresses. Yeast, 31(8), 309-321. doi:10.1002/yea.3024
[23] Homma T, Iwahashi H, Komatsu Y 2003. Yeast gene expression during growth at low temperature. Cryobiology, 46(3), 230-237. doi:10.1016/s0011-2240(03)00028-2

