HAYATI Journal of Biosciences 24 (2017) 176–181

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Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Original Research Article

Physiological Basis for the Tolerance of Yeast *Zygosaccharomyces bisporus* to Salt Stress

Akshya Sharma,[*] Sukesh Chander Sharma

Department of Biochemistry, Panjab University, Chandigarh, India.

ARTICLE INFO

Article history:
Received 4 September 2017
Received in revised form
10 October 2017
Accepted 3 November 2017
Available online 7 December 2017

KEYWORDS:
cell growth,
halotolerance,
PC,
polyols,
TAC,
TBARS

ABSTRACT

Zygosaccharomyces bisporus is a moderately halotolerant yeast isolated from highly sugary and salty foods. We performed various evident biochemical and *in vivo* experiments as first of its kind to sketch out the possible overlay of salt tolerance mechanism in this model organism. The growth and survival curve analysis revealed that 1.0 M NaCl concentration (sublethal) enacts growth inhibitory effects with prompting immediate delay in cell division cycle; however, yeast cells adopted modified stress physiologically with further stretched stress spans which was accompanied by an upsurge in the level of cellular metabolites such as trehalose (reserve carbohydrate) and chiefly glycerol (polyols) as major compatible osmolytes, suggesting their role in defense mechanism against osmotic stress. To further elucidate the relation of osmotic stress cell physiology to salinity, thiobarbituric acid reactive substances, protein carbonyl, and reduced glutathione content were measured in salt-stressed cells demonstrating positive correlation of reactive oxygen species generation in *Z. bisporus* with an elevated concentration of lipid and protein oxidation, thereby damaging cell membrane and eventually causing cell death. We assessed NaCl exposure sourcing increased intracellular reactive oxygen species concentration, by an electron transfer-based colorimetric cupric-reducing antioxidant capacity assay justifying that cellular total antioxidant capacity which uses all the combined antioxidant activities present within vitamins, proteins, lipids, and glutathione reverses these deleterious stress effects. Henceforth, performance of *Z. bisporus* MTCC 4801 mounted because of stress regime seems to be multifactorial.

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1. Introduction

It is well known that certain yeasts cause spoilage of foods genuinely rich in high sugar and high salt content such as pickles, honey, raw sugar cane, juices, jams, and jellies etc. (Deak & Beuchat, 1993; Stratford, 2006; James & Stratford) owing to their relative tolerance to either low pH, low water activity, low temperature, or presence of preservatives such as benzoate, sorbate, and acetic acid. Typical yeasts isolated from these processed foods responsible for organoleptic food spoilage correspond to the genus *Zygosaccharomyces*, the majority of which are *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus* leading a huge economic loss to food industry (Fleet, 2011). *Z. bisporus* holds a typical feature of osmotolerance and moderately halotolerance [compatibly grows in medium containing more 50% (w/v) D-glucose, whereas lacks growth above 2.0 M NaCl] entitling the cells to blend with high saline conditions through physiological alterations such as osmotic stress, ion toxicity, and transition in physical and chemical architecture of the cell wall and plasma membrane (Dakal *et al.*, 2014). High external osmolarity outrushes water from the cell impelling a higher intracellular concentration of ions and metabolites, thereby claiming arrest of the cellular activity. Under such an adverse environmental stress, the low metabolic activity of yeast instigates a highly specific, defensive cellular adaptability phenomenon for osmotic balance restoration: (1) regulation of morphological and anatomical properties of the cell wall and plasma membrane; (2) modulation of cation transport entity; (3) production, accumulation, and retention of metabolically compatible biochemical (Hohmann, 2002; Arino *et al.*, 2010).

Previously, studies on stress responses have been focused on ion homeostasis, cell wall properties, and osmotolerance in *Z. rouxii* and other spoilage yeasts (Pribylova *et al.*, 2007; Nishi & Yagi, 1995). Recent studies on the impact of *Z. rouxii* growth because of high sugar, temperature, pH, and antimicrobial compounds revealed advantageous effective microbial stability of apple juice during

* Corresponding author. Department of Biochemistry, Panjab University, Chandigarh, 160014, India.
E-mail address: akshya.msdn@gmail.com (A. Sharma).
Peer review under responsibility of Institut Pertanian Bogor.

https://doi.org/10.1016/j.hjb.2017.11.001
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desired storage (Wang *et al*., 2016). However, very little is known about *Z. bisporus* response to different environmental stresses on its morphological, physiological, and genomic characteristics. The aim of the present study was to report the effect of different initial range of salts (0.0–1.0 M) on the growth and cell survival of *Z. bisporus* MTCC 4801 emanating 1.0 M to be considered as sublethal concentration, which when exposed to log phase cells in another experiment explains the hypothesis of salt-adapted cells to grow and show surprising stability under such extreme salt stress conditions on the behalf of growth aiding factors.

The degree of ingenious factors like intracellular induction of trehalose along with another compatible osmolyte glycerol which protects enzymes and structural proteins against inactivation, inhibition, and denaturation (Brown, 1978), equipoise with the salt tolerance while correlating the treated with untreated group. Considering the generation of intracellular reactive oxygen species (ROS), we decided to compare the thiobarbituric acid reactive substances (TBARS), protein carbonyl (PC), and reduced glutathione levels between the groups, and consequently augmented intracellular total antioxidant capacity (TAC) was reported to be involved in lowering down ROS levels under salinity stress.

2. Materials and Methods

2.1. Microorganism, inoculum preparation, and culture conditions in shake flasks

The present yeast strain *Z. bisporus* MTCC 4801 was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla, maintained at 4°C on preautoclaved Yeast Peptone Dextrose Agar (YPDA) plates containing (w/v) 2% dextrose, 1% yeast extract, 2% peptone, and 2% agar. Cells were first grown on YPD agar plates (pH of the medium adjusted to 5.5) incubated at 28°C for 48 h; and from these plates, one loopful of the organism was transferred to 10 mL Yeast Peptone Dextrose (YPD) medium for inoculum preparation at 28°C for 16 h at 200 r.p.m. in rotary shaker. Cell growth was measured as an increase in optical density to ~0.5 (midlog phase cells) at 600 nm (OD600) with UV 1800 Shimadzu Spectrophotometer (Shimadzu Manufacturer, Japan). From this preculture, 1.0% (v/v) of inoculum containing 0.7×10^6 cells/mL was transferred to YPD medium in Erlenmeyer flasks under similar growth conditions to obtain exponential phase yeast cells.

2.2. Stress treatment

During the first part of the experiment, for determining the growth inhibiting stress concentration, plastic disposable loops were used to streak yeast on YPD agar plates, containing varying concentrations (0.5, 0.75, and 1.0 M) of sodium chloride, which when incubated at 28°C for 48 h, formed colonies of ~1 mm across. During the second part of the experiment, determined cells survival: (1) yeast cells from preculture were inoculated in the YPD medium containing varying concentrations of NaCl (0.5, 0.75, and 1.0 M), incubated at 28°C, 200 r.p.m. on rotatory shaker for different time intervals, i.e. 16, 32, 48, and 72 h. At these specific time intervals, 10 μL of cell sample was taken in 1.5 mL microcentrifuge tube, serially diluted (10^5 factor) and 50 μL of diluted sample was plated in triplicates on YPDA plates to check the cell survival. (b) YPD medium containing midlog phase cell culture (16 h) was subjected to 1.0 M NaCl stress, incubated at 28°C, 200 r.p.m. for different time intervals of 60, 90 and 120 minutes. At these specific time intervals, 10 μL of cell sample was taken in 1.5 mL microcentrifuge tube, serially diluted (10^5 factor) and 50 μL of diluted sample was plated in triplicates on YPDA plates. For biochemical studies, midlog phase cell culture exposed to 1.0 M sodium chloride stress was incubated at 28°C and 200 r.p.m. Samples were taken from different time intervals of 60, 90, and 120 minutes for the determination of trehalose, glycerol, as well as for lipid peroxidation (LPO), PC, and TAC estimation.

2.3. Percent cell survival measurement

Cell survival was determined by the colony count method (c.f.u./mL). Samples were serially diluted to 10^5 times and spread onto YPD agar in triplicates. Colonies were scored after 2 days of incubation at 28°C. The control c.f.u. was determined at the 0 timepoint as well as at the same timepoint as in the stressed samples from the plates, therefore calculated total number of c.f.u./mL culture for both sample types. The percent survival was calculated as ratio of c.f.u./mL of stressed sample to c.f.u./mL of control sample at a given time multiplied by 100.

2.4. Determination of trehalose

Midlog phase sample cultures with different stress exposure time spans grown at 28°C were washed twice with water. The resuspended pellet in distilled water was then placed in water bath (95°C) following denaturation and protein precipitation. After centrifugation at $20,000 \times g$ for 15 minutes, from collected supernatant trehalose (intracellular) was estimated by the anthrone method (Wyatt & Kalf, 1957; Jagdale & Grewal, 2003) under the units mg/mg protein. Sample protein estimation was achieved by the proposed method of Bradford (1976).

2.5. Total glycerol estimation

The intracellular glycerol present in the sample was estimated by Bok & Demain colorimetric procedure (1977). Briefly, from centrifuged and washed midexponential sample cultures, added 1.0 mL of 0.015 M sodium metaperiodate in 0.12 M hydrochloric acid (HCl) to 0.1 mL sample aliquot which on incubation for 10 minutes at 37°C underwent termination of periodate oxidation of alditols to formaldehyde, 2.0 mL of 0.1% L-rhamnose was added to remove extra periodate ions. Addition of 4.0 mL Nash reagent produced a yellow-colored complex in a water bath at 52°C for 15 minutes and absorbance was read spectrophotometrically at 412 nm.

2.6. LPO assay

The LPO level in the cell supernatant at 535 nm was determined by measuring TBARS content according to Buege and Aust method (1978) using TBA reagent (15% wt/vol. TAC and 0.375% wt/vol. TBA in 0.25 M HCl) and results expressed as nmol/mg protein.

2.7. Quantification of protein carbonylation

PCs being the major hallmarks of oxidative stress determine the extent of damage caused by ROS to proteins. PC is measured by the reaction of PCs with dinitrophenylhydrazine as described by Reznick and Packer (1994).

2.8. TAC (cupric-reducing antioxidant capacity assay)

TAC based on the reduction of Cu^{+2} to Cu^{+1} in bathocuproinedisulfonic acid disodium salt (chelating agent) presence measures the capacity of biological samples to scavenge the amount free radicals as described by Da Cruz (2003). Cupric-reducing antioxidant capacity assay measures both thiol containing antioxidants and other plasma antioxidants like ascorbic acid, α-tocopherol, glutathione, etc. Cell suspensions treated with 1.0 M NaCl were centrifuged, as described previously. Five microliters of each supernatant was mixed with 200 μL of 0.25 mM bathocuproinedisulfonic acid disodium salt in 10 mM phosphate-buffered saline pH 7.4 and absorbance was read at 490 nm. Then, 50 μL of 0.5 mM $CuSO_4$ was added to this reaction mixture, and incubated for 3 minutes at 37°C. Later, 50 μL of 0.01 M Ethylenediaminetetraacetic acid (EDTA) was added and again absorbance

taken at 490 nm against the deionized water as blank with vitamin-E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) taken as standard, and data were expressed in terms of mmol Trolox equivalent per liter.

2.9. Statistical analysis

Data shown as means ± SD were analyzed using statistical functions in GraphPad Prism version 7.0 (San Diego, CA, USA). Two-tailed Student's t tests were performed for significance of differences in sample means between treatment and negative control with a cutoff of $p < 0.05$.

3. Results

3.1. Increasing concentration of salt effects growth of *Z. bisporus*

Growth of the yeast cells was observed over a salt range from 0.5 to 1.0 M NaCl supplemented in YPD medium. *Z. bisporus* could tolerate salt concentration from 0.5 to 0.75 M and no growth was observed at 1.0 M. In the control without NaCl, maximum cell growth was observed and as the salt concentration increased from 0.5 to 0.75 M, the cells become sensitive to a higher concentration of NaCl and growth apparently reduced (Figure 1), suggesting 1.0 M to be considered as sublethal concentration of sodium chloride for inducing appropriate stress in liquid cell culture.

3.2. Effect of salt stress on cell survival

Here, in this experiment, the percent survival values derived for different concentrations of sodium chloride show very interesting trends. The cell survival was monitored in the culture media by increasing concentration of NaCl. As shown in Figures 2 and 3, the percent cell survival remained low under stressed condition compared with unstressed cells. Figure 2 depicted more pronounced reduction in cell survival rate in cells challenged with 1.0 M NaCl comparative with 0.5 M. The curve analysis defined apical percent survival for the negative control (0.0 M) while the lowest percent survival was for 1.0 M NaCl over a 72-h stress span. In negative control cells, the absolute values of c.f.u./mL corresponding to 100% at their respective time intervals of 16, 32, 48, and 72 h are $111.32 \pm 8.08 \times 10^6$, $240.66 \pm 6.1 \times 10^6$, $274.00 \pm 6.0 \times 10^6$, and $300.00 \pm 8.02 \times 10^6$. The maximum stress survival was 82% on 0.5 M NaCl treatment, which reduced to 49% and 38% when stressed with 0.75 and 1.0 M, respectively, relative to control at 16 h culture. With increasing stress incubation period, the survival further reduced to 72.66%, 47.77%, and 37.55% for respective salt concentrations of 0.5, 0.75, and 1.0 M NaCl correlated to control cells (100%) at 72 h illustrating notable decrease at 72 h in cell viability for 1.0 M NaCl. This is most probably due to high accumulation of sodium in *Z. bisporus*, leading to metabolic poisoning in yeast cells possibly causing defects in cell cycle, growth inhibition, and increased number of metabolically inactive cells. In other words, the result for each specified NaCl concentration is convincingly different from the control values, suggesting that the varying NaCl concentrations had a definite effect on the cells and were not just minor effects. Similar results were apparent from Figure 3 that with sudden salt shock (1.0 M), the cells immediately experienced Na^+ toxicity with significant decrease in survival by 44.46%. Afterward, stressed cells had the moderate survival of 73.37% compared with unstressed cells. In control cells, the absolute values of c.f.u./mL corresponding to 100% at their respective time intervals of 60, 90, and 120 minutes are $111.32 \pm 8.10 \times 10^6$, $114.0 \pm 8.0 \times 10^6$, $118.0 \pm 5.28 \times 10^6$, and $122.66 \pm 6.10 \times 10^6$.

3.3. Effect of salt stress on trehalose content

There was an overall significant increase ($p < 0.05$) in trehalose content in salt-treated group contrastive to control. However, with immediate stress effect in cells, 32.52% initial reduction in trehalose was noticed referring to control. Strikingly, with further increase in time to 90 and 120 minutes, trehalose content examined to rise to 11.89% and 9.5%, respectively, reaching a maximum of four-fold at 120 minutes correlated to their controls (Figure 4A). In addition, in the view of 60 minutes, the maximal trehalose content reached at 0.519 ± 0.016 mg/mg protein in stress conditioned cells after 120 minutes. These observations hinted some additional and more considerable factors are engaged in displaying exceptional salt tolerance of *Z. bisporus* in association with trehalose.

3.4. Glycerol production under salt shock

As shown in Figure 4B, *Z. bisporus* started to accumulate glycerol immediately after salt shock, and reached steady state levels after 2 h. The glycerol content produced in salt-stressed yeast cells was increased significantly maximal to 60.00% after 90 minutes, which then further increased by approximately 10.90% after 120 minutes. These estimation experiments of cellular metabolites contributed to suggestive role of glycerol more as of osmoprotectant than trehalose.

3.5. LPO status: TBARS assay (end product of LPO)

Table presents TBARS content of salt conditioned *Z. bisporus* as a measure of LPO. The TBARS content increased by 50% during 60 minutes stress, which nearly increased up to maximal percent of 197% (1.43 ± 0.027 nmol/mg protein) after 120 minutes. Otherwise, nearly lower constant MDA levels were identified in control sets of different time intervals.

3.6. Protein oxidation content

Effect of salt on the PC content of the *Z. bisporus* is presented in Figure 5. The increased oxidative protein modification due to free radicals propagation reactions is determined by overall formation of PCs. A drastic increased level of PC was seen in case of salt-

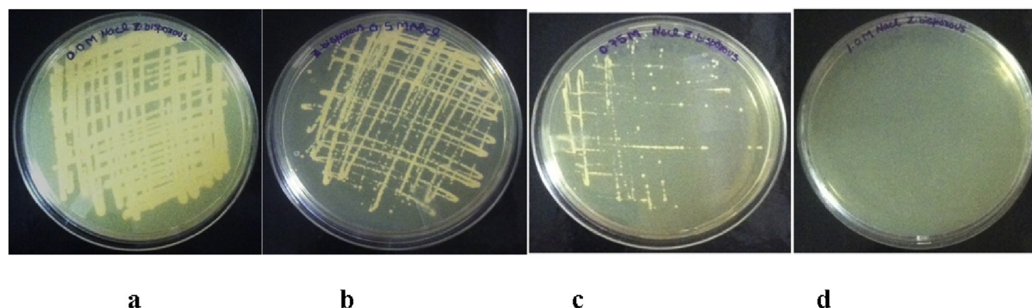

Figure 1. NaCl reduces cell growth rate and clonigenic survival in *Z. bisporus* cells grown on YPD agar plates containing 0.0 (A), 0.5 (B), 0.75 (C), and 1.0 M NaCl (D) for 48 h at 28°C. Plates were photographed showing cells in plate D hypersensitive to salt stress. Three independent biological experiments were conducted to observe the growth phenotypes.

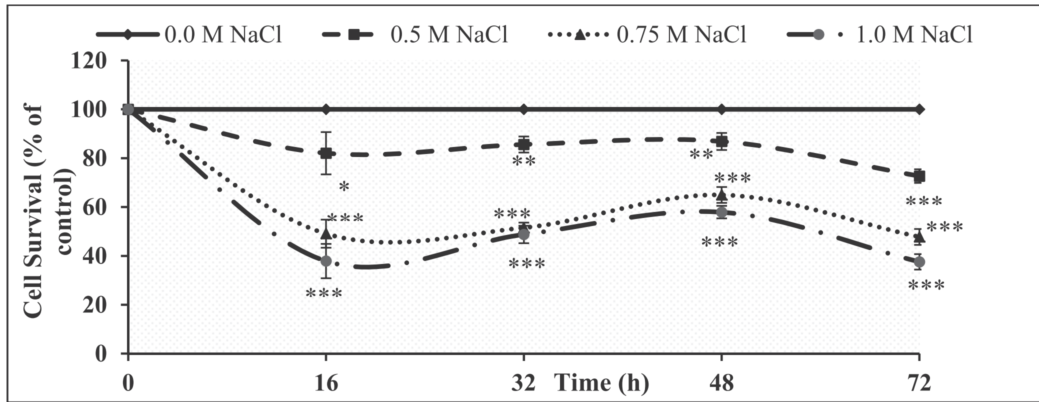

Figure 2. Percent cell survival of *Z. bisporus* cultures without addition of salt or with addition of 0.5 or 0.75 or 1.0 M NaCl (stress agent) in YPD agar plates, incubated at 28°C for 16, 32, 48, and 72 h. Error bars correspond to SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ compared with control cells.

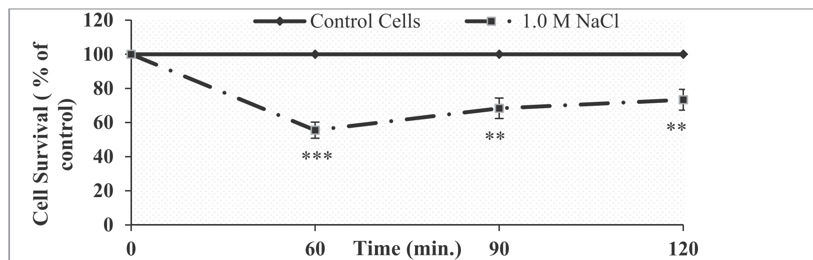

Figure 3. Percent survival of midexponential phase *Z. bisporus* cells in YPD without addition of salt (control cells) and with addition of 1.0 M NaCl stress, incubated at 28°C, 200 r.p.m. for 60, 90, and 120 minutes. Error bars correspond to SD. $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ compared with control cells.

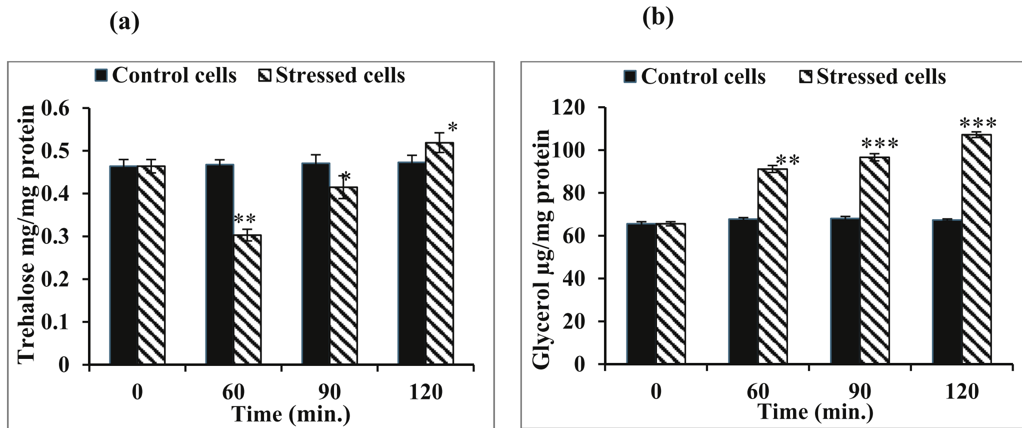

Figure 4. Histogram showing trehalose (mg/mg protein) (A) and glycerol content (µg/mg protein) (B) of salt-stressed *Zygosaccharomyces bisporus* at different time intervals. Data represented are means of three triplicates, $n = 3$; values are expressed as mean ± SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ compared with control cells. n = number of independent experiments.

Table. The TBARS content (nmol/mg protein) of salt-stressed *Zygosaccharomyces bisporus* at different time intervals

Time (min)	Control cells	Stressed cells
0	0.441 ± 0.029	0.441 ± 0.0290
60	0.480 ± 0.019	0.720 ± 0.030***
90	0.501 ± 0.115	1.030 ± 0.022***
120	0.480 ± 0.017	1.430 ± 0.027***

Error bars correspond to SD. $^{***}p \leq 0.01$ compared with control cells ($n = 3$).
TBARS = thiobarbituric acid reactive substances.

challenged cells as compared with control over the defined experimental time spans with a maximum amount of 9.38 nmol/mg protein under 120 minutes stress regime.

3.7. Assessment of effect of sodium chloride on TAC cellular levels

To determine whether ROS accumulation in NaCl-treated cells is facilitated by free radicals, we evaluated the effect of NaCl on antioxidant levels. Comparing untreated versus NaCl-treated

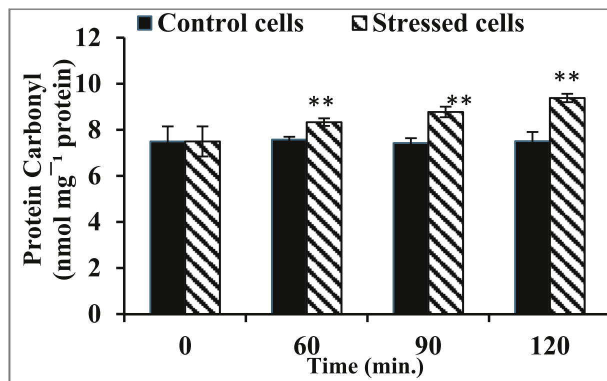

Figure 5. Histogram showing total protein carbonyl content (nmol/mg protein) of salt-stressed *Zygosaccharomyces bisporus* at different time intervals. Data represented are means of three triplicates, $n = 3$; values are expressed as mean ± SD. $^{**}p \leq 0.01$ compared with control cells. n = number of independent experiments.

samples indicated that treatment causes immediate elevation of oxidative burden whose effect is reversed on increased antioxidant levels which ranges from 0.949 ± 0.098 mg Trolox equivalent per liter at 60 minutes to 1.510 ± 0.063 mg Trolox equivalent per liter at 120 minutes stress interval. However, controls of respective stress spans experienced lesser change in TAC range was evaluated (Figure 6).

4. Discussion

The characteristic trait of competency to attune allows industrial yeasts to withstand distress environmental conditions (highly acidic, saline or sugary, and reduced water activity) conventional to industrial bioprocesses, where other microorganisms cannot (Solieri *et al.*, 2014; Bubnova *et al.*, 2014). *Z. rouxii* another most osmotolerant yeast phylogenetically related to *Z. bisporus*, able to grow at very low water activity, low pH, and can even bear the presence of best effective preservatives therefore, subject to diverse selective pressures (Marvig *et al.*, 2015; Rojo *et al.*, 2015). Similarly, *Z. bailii* exhibits extreme preservative resistance (e.g. sorbic acid) and conversely to *Z. bisporus* lesser osmotolerant probably due to prolonged lag phase (Pitt & Hocking, 2009; Stratford *et al.*, 2014). In this study, we examined few osmoadaptation strategies adopted by

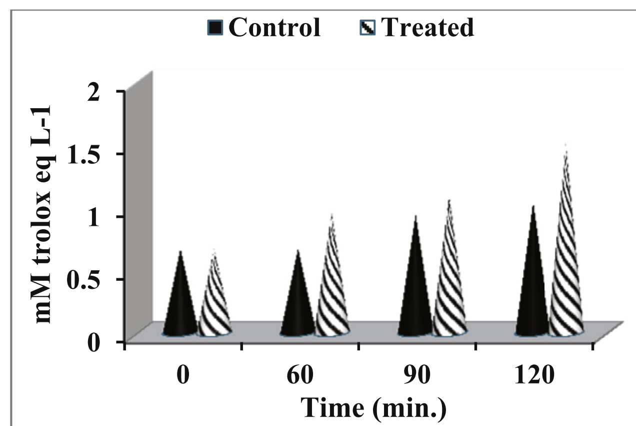

Figure 6. The conical representation showing the CUPRAC-BCS content as antioxidant status (mg Trolox equivalent per liter) of salt-stressed *Zygosaccharomyces bisporus* at different time intervals. BCS = bathocuproinedisulfonic acid disodium salt; CUPRAC, cupric-reducing antioxidant capacity.

Z. bisporus under salt stress regime. The progressively slower growth of *Z. bisporus* was observed in the presence of increasing concentration of NaCl. Interestingly, for a given salt concentration (1.0 M), again a significant growth inhibition was observed for about 1.5 h, which later revived with time. Indeed, our data showed that cell survival dropped incomparably within 60 minutes of stress. This can be analogized with deleterious metabolic poisoning of toxic intracellular Na^+ accumulation showcasing the stretching of lag phase indicating cell cycle abort with fizzled cell survival. Likewise, limited energy will be accessible for growth, beholding a negative impact on the specific growth rate and biomass production (data not shown). The results are in agreement with previous findings claiming subdued *Z. bisporus* and *Z. rouxii* growth in response to varying salt concentrations excluding *Z. bailii*, which shows almost no growth at 2.0 and 3.0 M NaCl (Van Eck *et al.*, 1993; Solieri *et al.*, 2014). Further procurement in compromised cell survival with extended time may be attributed to a lower Na^+/K^+ ratio in cells (data not shown) by active Na^+ ions extrusion out of the cell through cation-H^+ antiporters, conferring a protection against Na^+ toxicity, as Nishi & Yagi (1995) has also conveyed similar information about the entity and role of such membrane transporters in *Z. rouxii* and *Z. mellis* (Pribylova *et al.*, 2008; Dakal *et al.*, 2014).

Our data correlate and support the observations made by others that salinity burden stimulates and significantly enhances intracellular trehalose and glycerol production, where trehalose as membrane stabilizer and osmoprotectant, mitigates stress protection by strengthening protein structures and lipid membranes (Kwon *et al.*, 2003). The results are consistent with previous remarks probed in *Z. rouxii* (Wang *et al.*, 2013). Strikingly, glycerol emerged as a preferable osmoregulatory solute in lieu of trehalose, D-arabitol, or D-mannitol because it counterpoise the cytoplasmic water activity with the surrounding environment (Spencer and Spencer, 1978; Yancey *et al.*, 1982), and therefore, offers remarkable status of protection to antioxidant enzymes and macromolecular structures as reported earlier by Brown (1978). It was reported back that arabitol and mannitol are not just as osmotically responsive as glycerol (Van Eck *et al.*, 1993) considering the effective assistance of HOG1 genes responsible for glycerol production (Iwaki *et al.*, 1999; Kinclova *et al.*, 2001) as well as *Zr*STL1 and *Zr*STL2 genes, encoding glycerol transporters, thereby mediating glycerol active uptake and accumulation under stress (Duskova *et al.*, 2015).

The salt-induced ROS inflict oxidative damage on essential biomolecules such as proteins, lipids, and DNA causing LPO, protein denaturation, etc., through chain propagation reactions. Hence, ROS together with other lipid peroxides and decreased polyunsaturated fatty acids percentage within the cell membrane results in net formation of PCs and TBARS via different pathways making cells salt sensitive (Duskova *et al.*, 2015). As expected, immediate salt injury also posed a serious decline in the intracellular Glutathione (GSH) content (data not shown) suggesting possible roles of various haloprotectants in overall maintenance of redox homeostasis in salt-challenged cells.

In conclusion, our arguments sensibly unfold the obscured comment on *Z. bisporus* for its moderately halotolerance and proficiency behavior to grow and survive in high ionic (1.0–2.0 M NaCl) solutes (Dakal *et al.*, 2014). Microscopic examination along with growth curve analysis indicated the toxicity of NaCl at higher doses. Declined growth and survival rate is both dose and time dependent, enabling the cells to respond through similar general principles of osmoregulation likewise found in other halotolerant yeasts by diverting the active metabolism during glycolysis to trehalose and glycerol synthesis, with an exception laying more preference to glycerol rather trehalose as stress protectant. In addition, *Z. bisporus* cells challenged with sodium chloride led to an appreciable

increase in the level of ROS estimated by cellular TAC assay in conjugation to ROS induced oxidative damage to membrane lipids and proteins indicated by markers like PC and lipid peroxides. The comments made in this study could add more knowledge about food spoilage yeasts, to better understand the relationships between cell survival and their physiological responses against osmostress because relatively little has been explored in *Z. bisporus* with respect to molecular insight concerning food preservation while the relevance is high. A critical understanding of physiological together with genomic variations becomes valuable for industrial exploitation of this yeast, directing to design new strategies mainly to overcome spoilage of processed foods. In addition, future work on elucidating the mechanisms, sensors, and identification and rapid mapping of the candidate genes involved in biosynthesis of osmolytes or ROS scavenging enzymes or signal transduction pathways for regulation and ion homeostasis during salt stress will expand the glimpse of short- and long-term adaptation addressing cell adaptation and restoration against stresses, especially in *Z. bisporus* as much of such studies are restricted only to *Z. rouxii* (Hou *et al*., 2013; Solieri *et al*., 2013 (a, b); Stirbny *et al*., 2012; Watanabe *et al*., 2013). Considering such research will be of our great interest in agriculture biotechnology field.

References

Arino J, Ramos J, Sychrova H. 2010. Alkali metal cation transport and homeostasis in yeasts. *Microbiol Mol Biol Rev* 74:95–120.

Bok SH, Demain AL. 1977. An improved colorimetric assay for polyols. *Anal Biochem* 81:18–20.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–54.

Brown AD. 1978. Compatible solutes and extreme water stress in eukaryotic microorganisms. *Adv Microbiol Physiol* 17:181–242.

Bubnova M, Zemancikova J, Sychrova H. 2014. Osmotolerant yeast species differ in basic physiological parameters and in tolerance of non-osmotic stresses. *Yeast* 31:309–21.

Buege JA, Aust SD. 1978. Microsomal lipid peroxidation. *Methods Enzymol* 52: 302–10.

Da Cruz G. 2003. Use of bathocuproine for the evaluation of the antioxidant power in liquids and solutions. U.S. Patent No 6,613,577.

Dakal TC, Solieri L, Giudici P. 2014. Adaptive response and tolerance to sugar and salt stress in the food yeast *Zygosaccharomyces rouxii*. *Int J Food Microbiol* 185: 140–57.

Deak T, Beuchat LR. 1993. Yeasts associated with fruit juice concentrates. *J Food Prot* 56:777–82.

Duskova M, Ferreira C, Lucas C, Sychrova H. 2015. Two glycerol uptake systems contribute to the high osmotolerance of *Zygosaccharomyces rouxii*. *Mol Microbiol* 97:541–59.

Fleet GH. 2011. Yeast spoilage of foods and beverages. In: *The Yeasts, A Taxonomic Study*. pp. 53–63.

Hohmann S. 2002. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* 66(2):300–72.

Hou L, Wang M, Wang C, Wang C, Wang H. 2013. Analysis of salt-tolerance genes in *Zygosaccharomyces rouxii*. *Appl Biochem Biotechnol* 170:1417–25.

Iwaki T, Tamai Y, Watanabe Y. 1999. Two putative MAP kinase genes, ZrHOG1 and ZrHOG2, cloned from the salt-tolerant yeast *Zygosaccharomyces rouxii* are functionally homologous to the *Saccharomyces cerevisiae* HOG1 gene. *Microbiology* 145:241–8.

Jagdale GB, Grewal PS. 2003. Acclimation of entomopathogenic nematodes to novel temperatures: trehalose accumulation and the acquisition of thermotolerance. *Int J Parasitol* 33:145–52.

James SA, Stratford M. 2011. Zygosaccharomyces Barker (1901). In: Kurtzman CP, Fell JW, Boekhout T (Eds.). *Yeasts in Food Beneficial and Detrimental Aspects*. Hamburg: Behr's Verlag, Hamburg. pp. 937–47.

Kinclova O, Potier S, Sychrova H. 2001. The *Zygosaccharomyces rouxii* strain CBS732 contains only one copy of the HOG1 and the SOD2 genes. *J Biotechnol* 88:151–8.

Kwon HB, Yeo ET, Hahn SE, Bae SC, Kim DY, Byun MO. 2003. Cloning and characterization of genes encoding trehalose-6-phosphate synthase (TPS1) and trehalose-6-phosphate phosphatase (TPS2) from *Zygosaccharomyces rouxii*. *FEMS Yeast Res* 3(4):433–40.

Marvig CL, Kristiansen RM, Nielsen DS. 2015. Growth/ no growth models for *Zygosaccharomyces rouxii* associated with acidic, sweet intermediate moisture food products. *Int J Food Microbiol* 192:51–7.

Nishi T, Yagi T. 1995. Efflux of sodium ions by a $Na+/H+$ antiporter during salt stress in the salt tolerant yeast *Zygosaccharomyces rouxii*. *J Gen Appl Microbiol* 41: 87–97.

Pitt JI, Hocking AD. 2009. *Fungi and Food Spoilage*, XV, ISBN 978-0-387-92207-2. pp. 519.

Pribylova L, Farkas V, Slaninova I, De Montigny J, Sychrova H. 2007. Differences in osmotolerant and cell-wall properties of two *Zygosaccharomyces rouxii* strains. *Folia Microbiol* 52(3):241–5.

Pribylova L, Papouskova K, Sychrova H. 2008. The salt tolerant yeast *Zygosaccharomyces rouxii* possesses two plasma- membrane $Na+/K+$- antiporters (ZrNha1p and ZrSod2-22p) playing different roles in cation homeostasis and cell physiology. *Fungal Genet Biol* 45:1439–47.

Reznick AZ, Packer L. 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* 233:357–63.

Rojo MC, Arroyo López FN, Lerena MC, Mercado L, Torres A, Combina M. 2015. Evaluation of different chemical preservatives to control *Zygosaccharomyces rouxii* growth in high sugar culture media. *Food Control* 50:349–55.

Solieri L, Dakal TC, Bicciato S. 2014. Quantitative phenotypic analysis of multistress response in *Zygosaccharomyces rouxii* complex. *FEMS Yeast Res* 14(4):586–600.

Solieri L, Dakal TC, Giudici P. 2013a. *Zygosaccharomyces sapae* sp. nov., a novel yeast species isolated from Italian traditional balsamic vinegar. *Int J Syst Evol Microbiol* 63:364–71.

Solieri L, Dakal TC, Croce MA, Giudici P. 2013b. Unraveling genomic diversity of the *Zygosaccharomyces rouxii* complex with a link to its life cycle. *FEMS Yeast Res* 13: 245–58.

Spencer JFT, Spencer DM. 1978. Production of polyhydroxy alcohols by osmotolerant yeasts. *Economic Microbiol* 2:393–425.

Stirbny J, Kinclova-Zimmermannova O, Sychrova H. 2012. Potassium supply and homeostasis in the osmotolerant non-conventional yeasts *Zygosaccharomyces rouxii* differ from *Saccharomyces cerevisiae*. *Curr Genet* 58:255–64.

Stratford M, Steels H, Nebe-von-Caron G, Avery SV, Novodvorska M, Archer DB. 2014. Population heterogeneity and dynamics in starter culture and lag phase adaptation of the spoilage yeast *Zygosaccharomyces bailii* to weak acid preservatives. *Int J Food Microbiol* 181:40–7.

Stratford M. 2006. Food and beverage spoilage yeasts. In: Querol A, Fleet GH (Eds.). *Yeasts in Food and Beverages*. Berlin: Springer-Verlag, Berlin. pp. 335–80.

Van Eck JH, Prior BA, Brandt EV. 1993. The water relations of growth and polyhydroxy alcohol production by ascomycetous yeasts. *J Gen Microbiol* 139: 1047–54.

Wang H, Hu Z, Long F, Guo C, Niu C, Yuan Y, Yue T. 2016. The effects of stress factors on the growth of spoilage yeasts isolated from apple-related environments in apple juice. *J Food Safety* 36:162–71.

Wang X, Zhang Y, Wang C, Hou L. 2013. Intracellular level of trehalose in soy sauce yeasts under different stresses. *IERI Procedia* 5:321–6.

Watanabe J, Uehara K, Mogi Y. 2013. Adaptation of the osmotolerant yeast *Zygosaccharomyces rouxii* to an osmotic environment through copy number amplification of FLO11D. *Genetics* 195:393–405.

Wyatt GR, Kalf GF. 1957. The chemistry of haemolymph trehalose and other carbohydrates. *J Gen Physiol* 40:833–47.

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. 1982. Living with water stress: evolution of osmolyte systems. *Science* 217:1214–22.