Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.7426

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **7 (1):** 545-552 (2019)

Research Article

Physiological and Biochemical Analyses Revealing the Protective Role of Glutathione in Resistance of *Zygosaccharomyces bisporus* to Salt Stress

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Received: 9.01.2019 | Revised: 14.02.2019 | Accepted: 20.02.2019

ABSTRACT

Studies on food spoiler yeasts, recognized Zygosaccharomyces, Debaryomyces, and Yarrowia as commercial players referring their biochemistry and food spoilage activities. However, the impact of salinity on physiology and biochemical traits is largely unknown in leading organoleptic halotolerant yeast Zygosaccharomyces bisporus. Interestingly, within 1 h in vitro sub-lethal salt stress in Z. bisporus MTCC 4801, a considerable variation in growth was visible in early phase (minimum cell viability) synchronically with observed ($P<0.05$) reduced biomass yield, intracellular glutathione content, and K^+/Na^+ ratio. Immediate glutathione depletion addresses its essentiality in scavenging reactive species attributable to overlap between osmotic and oxidative stresses initiated under salinity. Though prolonged 2 h stress duration ameliorated these reductions together with rapid efflux of toxic sodium ions, which strongly suggest that Na^+ in Z. bisporus is toxic and strategically excluded, while cellular GSH performs growth restoration preparing metabolic systems to be high salt-resistant. From the lipid profiling studies, we further confirmed consequential lipid alterations contribute to higher membrane fluidity. Hence, assist the cells in osmo-adaptation against deleterious effects of NaCl allied to expulsion of Na^+ ions and GSH protection. These data have an agricultural and food industrial application in assessing the spoilage potential of new/ existing fermented product formulations.

Key words: *Food spoilage; Salt stress; K^+/Na^+ ratio; Growth and biomass yield; GSH.*

INTRODUCTION

Yeasts generally known for their beneficial activities in food fermentation and beverage industry, often goes unrecognized for their undesirable spoilage activity, posing huge loss to the food industry and illness to consumers. Comparatively, the study of diverse and unparalleled spoilage flora emphasized their identification and biodiversity in wide range of food types, physiological and genetic history to stress responses describing regulation of their metabolic activities, and adopted effective strategies for their prohibition/ inactivation[7,6].

Cite this article: Sharma, A. and Sharma, S.C., Physiological and Biochemical Analyses Revealing the Protective Role of Glutathione in Resistance of *Zygosaccharomyces bisporus* to Salt Stress, *Int. J. Pure App. Biosci.* **7(1):** 545-552 (2019). doi: http://dx.doi.org/10.18782/2320-7051.7426

Pitt & Hocking[14] suggested nearly 10 non-conventional organoleptic species which includes *Z. bailli*, *Z. rouxii*, *D. hansenii*, *Y. lipolytica* and many more as they possess homeostatic and osmo-protective mechanisms allowing them to perform better in stressful conditions such as low pH, low water activity and even in the presence of some chemical preservatives. However, salinity is a severe abiotic stress categorizing both ionic and osmotic stress. In view of low water potential, osmotic stress is negatively correlated to growth and biomass yield. Viewing the intracellular accumulation of Na^+ over time, the subsequent ionic imbalance instigates membrane injury and ion toxicity[21,15]. As *Z. bailii* under osmotic stress induce alterations in various fatty acids of membrane phospholipids, comprising changes in their composition, chain length and intermittent branching in addition to sterol content, thus mediate necessary role in sustaining proper membrane fluidity[2]. Resultantly, reduction in yield conjointly with other important affected cellular processes, such as cell growth and division, lipid metabolism, and glutathione (abundant antioxidant) synthesis etc. is considered a significant metabolic effort aided for yeast adaptation, growth maintenance, and positive stress response. Potentially, another strategy adopted by most halotolerant yeasts such as *D. hansenii*[12] and *Z. rouxii*[22], involves equipoised efflux- influx regulation of intracellular concentration of cations (Na^+ and K^+) via transporters like Na^+-ATPases, Na^+/H^+-antiporters and PM-ATPase[6] in lieu of salt assaults. Therefore, halotolerant strains are of utmost biotechnological importance for production of compatible osmolytes (such as glycerol, trehalose, etc.), halo-enzymes, food and alcoholic beverages, and in biological waste treatment.

Accordingly, it is of priority to classify and recognize efficiently the halotolerant characteristics of food borne yeasts, as reflected by Stratford[20] isolation of yeast *Zygosaccharomyces bisporus* from spoiled soft drinks and wine experiencing a high salt tolerance up to 11 % (w/v). However, there are no insight reports comprehend its inherent halotolerant property. These facts prompted us to investigate the osmotic restorations of Na^+ and K^+ allied to membrane fluidity established by lipid profiling, in this halotolerant yeast at different stress intervals in an attempt to understand the physiological and biochemical basis of halotolerance. In addition, we examined the stress effect on growth, biomass yield in relation to glutathione content. Based on our results, we proposed a hypothesis to explain the halo-tolerant ability of *Z. bisporus* under high salinity.

MATERIALS AND METHODS

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

The study strain *Z. bisporus* MTCC 4801 was procured from the Department of Biotechnology, Himachal Pradesh University, Shimla, maintained at 4 °C on YPDA plates (w/v): Yeast (1%) Peptone (2%) Dextrose (2%) Agar (2%) pH-5.5. From plates, one loopful organism was transferred to 5.0 mL Yeast Peptone Dextrose (YPD) medium preparing inoculum at 28 °C, 16 h and 200 r.p.m. in rotary shaker. Cell growth was measured at optical density OD_{600} ~0.5 (mid-log phase) with UV 1800 Shimadzu Spectrophotometer (Shimadzu Manufacturer, Japan). From this pre-culture, 1.0% (v/v) of inoculum (0.7×10^6 cells/mL) was transferred and grown in YPD medium to obtain exponential phase yeast cells.

2.2 Stress treatment

To determine growth inhibition, mid-log phased 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. From where, 1.0 mL of cell sample was taken in 1.5 mL micro centrifuge tube, serially diluted (10^5 factor). 0.05 mL of diluted sample was plated onto YPDA plates, incubated at 28 °C for 48 h.

2.3 Ion analyses by MPAES (Microwave Plasma Atomic Emission Spectroscopy) method

In Erlenmeyer flasks, 10.0 ml of cell suspension (control/ treated) was mixed with same volume of conc. HNO_3 (strong oxidizing

agent) for acid digestion of samples into ionic form. Further heating made final mixture to half of the initial reaction mixture volume. The cooled, syringe filtered sample extracts were then quantified for the concentration (mM) of an element in a sample against the known element concentrations, plotted on a calibration curve.

2.4 Glutathione Determination

After centrifugation (10,000 × g, 30 min), reduced (GSH) and total glutathione content (GSH+GSSG) were estimated by Beutler *et al*.[4] and Habeeb's method[9] respectively from supernatant. The difference in their values yields oxidized glutathione (µg/mg protein).

2.5 Lipid analyses

Total lipids from yeast cells were extracted according to the procedure of Bligh and Dyer[5]. Phospholipids, glycolipids and sterols **(**total and free) were estimated using the Ames[1] and Dubois *et al*.[8] and Sperry and Webb procedure[19] respectively.

2.6 Statistical Analysis

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

RESULTS

3.1 Effect of salt concentrations on the growth of *Z. bisporus*

Z. bisporus incubated in maximum salinity level (1.0 M NaCl) for 2 h, showed most notable reduced growth in 60 min. As appeared, beyond this threshold period, the cells were able to cope with stressful agent gradually by mounting appropriate protective cellular response (Fig. 1).

3.2 Effect of salinity on biomass production in *Z. bisporus*

The highest biomass (Total Dry Weight) was measured in control set of 120 min. (w/o NaCl), and lowest in salt treated group at 60 min. (4.33 ± 0.25 mg). However, the biomass estimated at the end of stress imposition almost approaches near to control values correlating these alterations more towards salinity tolerance (Fig. 2).

3.3 Effect on intracellular levels of inorganic ions shortly after salt stress

Determination of intracellular Na^+ and K^+ content in *Z. bisporus* cells during various stress imposition intervals (Fig. 3) stated that in either absence/ presence of salt, both Na^+ and K^+ accumulated in cells. The stressed cells accounted for immediate uptake and accumulation of Na^+ up to 25 mM at 60 min. However, later this parameter decreased at a constant rate in 90 and 120 min. by 127.75 % and 65.70 %, respectively in comparison to 60 minutes stress interval. Likewise, a non-linear change in intracellular K^+ concentration was obtained with an immediate, maximum degree of 50 % reduction within 60 min. stress interval (71.09 ± 0.51). Contrary, this rapid K^+ efflux was soon stabilized around 120.76 ± 0.79 mM at the end of the stress course comparatively to control set. Hence, it is evident that cells accumulated high Na^+ and moderate K^+ concentration at the start of stress experiment majorly due to immediate efflux of K^+ ions.

3.4 Salt stress effect in relation to antioxidant status (Glutathione content) of *Z. bisporus*

The salt induced oxidative stress resulted in 40.8 % decrease in reduced glutathione (GSH) concentration at 60 min. stress period compared to control (43.67 ± 0.641 µg mg protein^{-1}). In further stress stretch of 90 minutes, concomitant more decrease (16.64 ± 0.803 µg mg protein^{-1}) with reverse drastic upsurge pattern in GSH level (34.10 %) was seen after 120 min. owing to cope with oxidative stress in comparison to 60 min. stress treated group. This indicated symbolic outcomes of the study that with time, cells reserved to normal redox status (Table 1).

3.5 Effect of salinity on lipid composition of *Z. bisporus*

3.5.1 Estimation of phospholipids

Experimentally salt shocked cells experienced changes in the phospholipid make-up (Fig. 4a), where regular sufficient level has increased ($p \leq 0.001$) compared to control group.

However, on comparing at different stress intervals within the treated sample, the level reduced to 4.50 ± 0.18 mg mg dry wt^{-1}during 60 min. While later experimental times of 90 and 120 min. depicted a significant level upturn (8.35 ± 0.124, 9.51 ± 0.09) restoring back the cells to near normalcy.

3.5.2 Estimation of glycolipids

The cells administered with 1.0 M salt, showed drastically increased glycolipids content compared to those in control sample at varied time-spans (Fig. 4b). The treated group exhibited considerable increase (19.67 %) over 60 min. comparing to control, whereas this glycolipid percent increase was further elaborated over 90 and 120 min. time, which in concordance with the phospholipids experimental data maintains cell membrane consistency with stress overload.

3.5.3 Estimation of sterols

Lipid extracts containing sterols have been categorically estimated as free and esterified sterols in both control and treated groups with their free/ esterified sterol ratio demonstrated in Table 2.

Free Sterols: On the onset of stress, the cells experienced rapid increase in free sterol content than in the later periods of 90 and 120 min. suggesting continual synthesis of free sterols throughout stress incubation with significant increase (53.50 %) in 120 min. when compared to its respective control group. While no marked difference was noticed in control sets with time.

Esterified Sterols: The esterified sterol content of salt burdened cells was lowered in 60 min. stress duration, which was considerably decreased (0.133 ± 0.020 mg mg dry $weight^{-1}$) after 120 min. salt incubation. Subsequently, in control sets, significant rise in esterified sterols was seen throughout the stress experiment though always lower than their respective free sterol content in control group, and also to their corresponding esterified sterol concentration in treated group.

DISCUSSION

In this study, possible multifarious role of GSH in response of *Z. bisporus* to salt shock was discussed. Being a stress simulator, sodium chloride (NaCl) causes both osmotic and ionic stress[15]. The growth profile of *Z. bisporus* was affected differentially under specified stress exposure intervals. It is believed that participation of intracellular GSH in growth resumption is interrelated to K^+ accumulation. Intracellular transition in the GSH level was biphasic: the glutathione level rapidly decreased during the initial stage of response by its increased consumption, whilst increased during the later stage. Similar observed biphasic switch in intracellular level of K^+, suggest GSH deficiency leads to higher K^+ leakiness in the medium with little K^+ concentration. Though, an increase in osmolarity caused an increase in GSH level fostering its involvement in turning off the K^+ efflux channels, while positively affects the Trk influx channels for K^+. Therefore, under hyperosmotic shock (1.0 M NaCl) the intracellular GSH accumulation stimulates and contributes to the re-accumulation of K^+ for proper functioning of log phase cells growing in low K^+ medium. Some previous studies on osmotic stress had found similar relation of GSH with K^+ accumulation in *E. coli*[13,18,23]. Notably, initial loss in GSH rendered the cells susceptible to oxidative stress marking growth rate and biomass yield during the 1st hour of stress exposure much lower than that of unstressed cells. This justifies cellular stability and integrity be better retained in the GSH presence when surviving a strong osmotic shock. It is deduced that salinity induce upregulation of key glycolytic enzymes accompanied with retarded growth as an early response to funnel energy and carbon skeletons for primary and secondary metabolite (osmoprotectants) synthesis as late antioxidant defenses thus, elaborating yeast osmo-adaptation as an energy consuming phenomenon, principally depending on oxidative phosphorylation. Smirnova[18] Likewise, stressed *Z. bisporus* following GSH synthesis and accumulation also expected to derive energy from oxidative phosphorylation, as hinted from greater carbohydrate metabolism of small molecular metabolites

(osmolytes) such as trehalose, glycogen[16]. This strongly affirms the GSH presence to cope with osmotic stress, increasing the overall fitness of the stressed cells; restoring dry biomass near to unstressed condition. Additionally, the oxidative stress prompted reactive species like LOOHs in biological membranes might be involved in membrane damage[11]. Wherefore, a marked overall increase in membrane lipid content of *Z. bisporus* against toxic salt-shock certified the established importance of glutathione in redox biochemistry and oxidative stress tolerance as a defense line. Our data indicated significant decrease in phospholipids as a primarily stress effect during the 1st hour, with pleiotropic positive effects of glutathione pronounced over 2–hour stress period. Initial loss of phospholipids/ increase in free fatty acids suggests activation of phospholipases during early stress stage, where after GSH induction plays a dual role in significant restoration of the redox status (GSH/GSSG) attenuating lipid peroxidation[17], and might downregulated the phospholipases or initiated phospholipid synthesis amid stress recovery period. This endogenous restoration of phospholipids allied with increased glycolipid and sterol content pin-downs that other mechanisms are also involved in the membranous protection as illustrated from advances in lipidomics. The relative compositional change in free sterols particularly, the increase in ergosterol was paralleled to its 'planar' structure theory suggesting moderate packing with phospholipids acyl chains owing to its rigid, lesser planar structure. However, further increase in such bulky side chains-sterols would add to lessen its condensing effect on biological membranes[10]. Bernsdorff & Winter[3] found that high Na^+/K^+ ratio resulted in more planar free sterols which might be advantageous in enhancing ion exclusion as again apparent from this study.

Table 1: The estimated glutathione content (total, reduced and oxidized) in salt stressed *Z. bisporus* cells at different time intervals

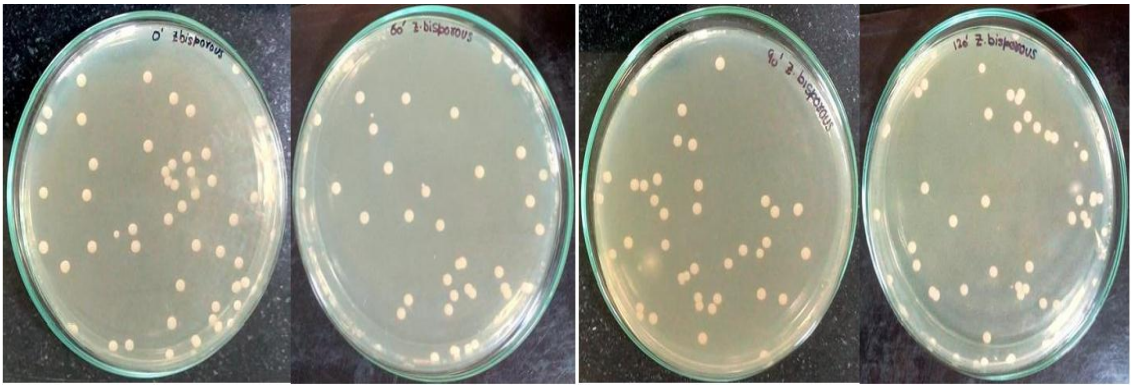
	Total GSH (µg mg^{-1} protein)		Reduced GSH (µg mg^{-1} protein)		Oxidized GSH (µg mg^{-1} protein)	
Time	**Control**	**Stressed**	**Control**	**Stressed**	**Control**	**Stressed**
0	52.64 ± 0.040	52.64 ± 0.040	43.64 ± 0.187	43.64 ± 0.187	9.003 ± 0.147	9.003 ± 0.147
60	52.65 ± 0.036	53.92 ±	43.67 ± 0.641	25.85 ±	8.649 ± 0.198	28.071 ± 0.928***
90	52.66 ± 0.040	53.94 ±	43.70 ± 0.795	16.64 ±	8.967 ± 0.778	37.298 ± 0.788***
120	52.66 ± 0.042	55.73 ±	43.78 ± 0.596	20.40 ±	8.946 ± 0.698	35.330 ± 0.673***

Data represented are means of three triplicates, n=3; values are expressed as mean ± SD. ***p ≤ 0.001 compared to control cells.

Table 2: Free to esterified sterol ratio as membrane fluidity hallmark in salt treated *Z. bisporus* cells at different time intervals

	Free Sterols/ Esterified Sterols			
Time (min.)	**0**	**60**	**90**	**120**
Control	2.76 ± 0.40	2.84 ± 0.163	3.04 ± 0.23	3.14 ± 0.16
Stressed	2.76 ± 0.40	5.70 ± 0.936**	7.55 ± 1.17*	10.24 ± 1.95***

Data represented are means of three triplicates, n=3; values are expressed as mean ± SD. **p ≤ 0.01, ***p ≤ 0.001 compared to control cells.



a **b** **c** **d**

Fig. 1: Effect of NaCl at different time intervals: (a) 0 min (b) 60 min (c) 90 min (d) 120 min on the growth of *Z. bisporus*. Mid- exponential phase cell culture exposed to 1.0 M NaCl stress was incubated at 28 °C for specified time intervals at 200 r.p.m. At these respective intervals, the serially diluted culture was spotted on YPDA plates and incubated at 28 °C. Plates were photographed after 2 days. Three independent biological experiments were conducted to observe the growth phenotypes.

Fig. 2 **Fig. 3**

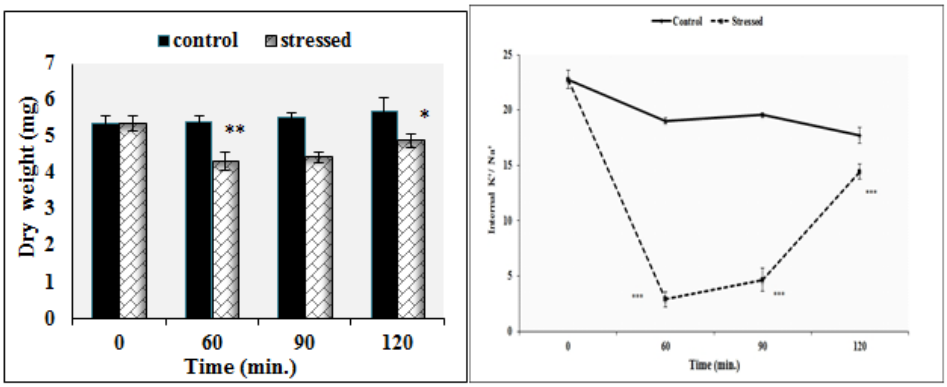


Fig. 2: Dry weights of exponential phase *Z. bisporus* cells, respectively after incubation growth under salinity stress conditions at different time intervals. Data represented are means of three triplicates, n=3; values are expressed as mean ± SD. *p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 compared to control cells.

Fig. 3: Line graph illustrating the intracellular ratio of $K^{+/}$ Na^{+} in cells grown in YPD and then incubated with 1.0 M NaCl for specified stress interval of 2 h compared to control. Data represented are means of three triplicates, n=3; values are expressed as mean ± SD. *** p ≤ 0.001 compared to control cells.

Fig. 4 a **Fig. 4 b**

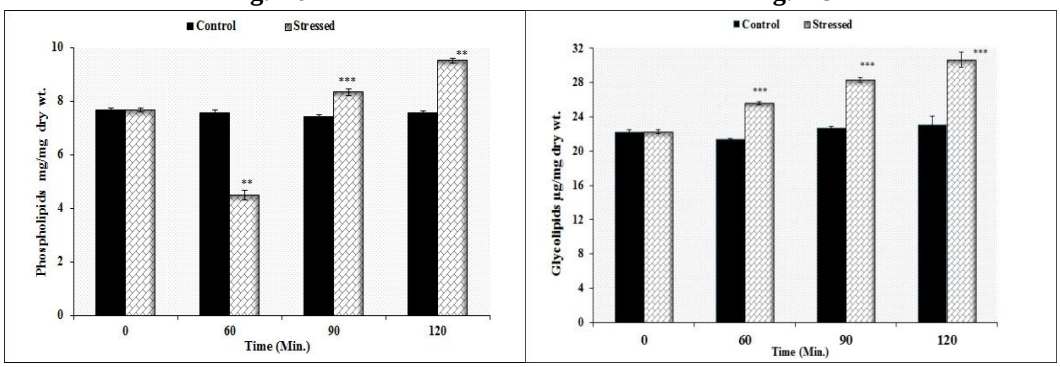


Fig. 4: Histogram showing (a) phospholipid and (b) glycolipid content of salt stressed *Z. bisporus* at different time intervals. Data represented are means of three triplicates, n=3; values are expressed as mean ± SD. **p ≤ 0.01, *** p ≤ 0.001 compared to control cells.

CONCLUSION

The classic viewpoint regarding food spoiler *Z. bisporus* osmo-behavior is its ability to sense the environmental salt insult in due course deputing cell membrane as prime target of damage, which triggers activation of multiple stress responses discussed in this study with pleiotropic effects on both cell membrane structure essentially biosynthesis and conformational-compositional remodeling of lipid profile to a higher level, and on key shift to higher reduced GSH level for active extrusion of ions. Hence, voicing cell's adaptation and restoration against stress timely, whereby ensuring cell growth and survival.

Acknowledgement

Authors are thankful to the Indian Council of Medical Research, New Delhi for financial help. Grant number: 3/1/3/JRF-2013/HRD-134 (10040).

REFERENCES

1. Ames, B. N., Methods in Enzymology, *Edited by Neufield, E. F. and Ginsburg, V.,* **8:** Academic Press, New York, pp. 115– 118 (1966).
2. Baleiras Couto, M. M. and Huis in't Veld, J. H. J., Influence of ethanol and temperature on the cellular fatty acid composition of *Zygosaccharomyces bailii* spoilage yeasts, *J. Appl. Bacteriol*, **78:** 327- 334 (1995).
3. Bernsdorff, C. and Winter, R., Differential properties of the sterols cholesterol, ergosterol, sitosterol, trans-7-dehydrocholesterol and lanosterol on DPPC bilayer order, *J. Phys. Chem*, **107:** 10658 (2003).
4. Beutler, E., Duron, O. and Kelly, B. M., Improved method for the determination of blood Glutathione, *J. Lab. Clin. Med*, **61:** 882 – 888 (1963).
5. Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and quantification, *Can. J. Biochem. Physiol*, **37:** 911– 917 (1959).
6. Dakal, T. C., Solieri, L. and Giudici, P., Adaptive response and tolerance to sugar and salt stress in the food yeast *Zygosaccharomyces rouxii*, *Int. J. Food. Microbiol*, **185:** 140- 57 (2014).
7. Deak, T. and Beuchat, L. R., Yeasts associated with fruit juice concentrates, *J. Food. Prot*, **56:** 777- 82 (1993).
8. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., Colorimetric method for determination of sugars and related substances, *Anal. Chem*, **28:** 350-356 (1956).
9. Habeeb, A. F. S. A., *Methods in Enzymology*, **25:** Hirs, C. H. W. and Timasheff, S. N., eds., Academic, New York. pp. 457– 464 (1972).
10. Hosono, K., Effect of salt stress on lipid composition and membrane fluidity of the salt-tolerant yeast *Zygosaccharomyces rouxii*, *J. Gen. Microbiol*, **138:** 91- 96 (1992).
11. Inoue, Y., Matsuda, T., Sugiyama, K., Izawa, S. and Kimura, A., Genetic Analysis of Glutathione Peroxidase in Oxidative Stress Response of *Saccharomyces cerevisiae*, *J. Biol. Chem*, **274(38):** 27002– 27009 (1999).
12. Kumar, S., Randhawa, A., Ganesan, K., Singh Raghava, G. P. and Mondal, A. K., Draft genome sequence of salt-tolerant yeast *Debaryomyces hansenii* var. hansenii MTCC 234, *Eukaryot. Cell*, **11:** 961– 962 (2012).
13. Ohwada, T. and Sagisaka, S., Role of Glutathione in the Accumulation of Potassium Ions in *Escherichia coli* under Osmotic Stress, *Biosci. Biotechnol. Biochem*, **56(4):** 640- 643 (1992).
14. Pitt, J. I. and Hocking, A. D., Fungi and Food Spoilage, XV, ISBN 978-0-387-92207-2. pp. 519 (2009).
15. Pribylova, L., Farkas, V., Slaninova, I., De Montigny, J. and Sychrova, H., Differences in osmotolerant and cell-wall properties of two *Zygosaccharomyces rouxii* strains, *Folia. Microbiol*, **52(3):** 241- 245 (2007).

16. Sharma, A. and Sharma, S. C., Physiological Basis for the Tolerance of Yeast *Zygosaccharomyces bisporus* to Salt Stress. *HAYATI. J. Biosci*, **24:** 176- 181 (2017).
17. Sharma, A. and Sharma, S. C., The importance of antioxidants and plasma membrane ion transporters which play role in cellular response against salt induced oxidative stress, *Ecological Genetics and Genomics*, **9:** 16-22 (2018).
18. Smirnova, G. V., Krasnykh, T. A. and Oktyabrsky, O. N., Role of glutathione in the response of *Escherichia coli* to osmotic stress, *Biochemistry (Mosc)*, **66(9):** 973-978 (2001).
19. Sperry, W. and Webb, M., A revision of Schoenheimer–Sperry method for cholesterol estimation, *J. Biol. Chem*, **187:** 97– 106 (1950).
20. Stratford, M., Food and beverage spoilage yeasts. In: Querol A, Fleet GH (Eds.), Yeasts in Food and Beverages, Springer-Verlag, Berlin. pp. 335-380 (2006).
21. Turk, M., Mejanelle, L., Sentjure, M., Grimalt, J. O., Gunde-Cimerman, N. and Plemenitas, A., Salt- induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi, *Extremophiles*, **8:** 53- 61 (2004).
22. Watanabe, Y., Oshima, N. and Tamai, Y., Co-expression of the Na^+/H^+-antiporter and H^+-ATPase genes of the salt-tolerant yeast *Zygosaccharomyces rouxii* in *Saccharomyces cerevisiae*, *FEMS. Yeast. Res*, **5:** 411– 417 (2005).
23. Zhang, Y., Zhu, Y., Mao, S. and Li, Y., Proteomic Analyses to Reveal the Protective Role of Glutathione in Resistance of *Lactococcus lactis* to Osmotic Stress, *Appl. Environ. Microbiol*, **76(10):** 3177– 3186 (2010).