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Original Research Article

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

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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.

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 majorly 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

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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 × *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