

## Correlation Studies of Trehalose with Oxidative Stress in Ethanol Stressed Yeast *Pachysolen tannophilus*

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**Abstract:** The aim of the study was to investigate the role of trehalose in ethanol induced oxidative stress condition in yeast *Pachysolen tannophilus*. The laboratory experiments were carried out during 2009 and 2010 at the Yeast Stress Response Study Laboratory of Biochemistry Department in Panjab University at Chandigarh, India. Resistance of the yeast *Pachysolen tannophilus* to ethanol stress was studied under 10% (v/v) ethanol concentrations for different time periods (0, 60 and 120 min). After treating cells with ethanol, samples were taken to study the level of trehalose, ROS, Protein carbonyl content, lipid peroxidation and glutathione. A markedly increase in trehalose content after ethanol stress was observed. The increased level of protein carbonyl content, ROS generation and lipid peroxidation and decreased reduced/total glutathione ratio, viability in ethanol stressed cells shows the roles of oxidative stress in ethanol toxicity in yeast. The positive correlation between oxidative damage and trehalose shows the protective role of trehalose in oxidative stress conditions generated by the ethanol.

**Key words:** Ethanol stress, glutathione, oxidative stress, *Pachysolen tannophilus*, protein carbonyl, trehalose

### INTRODUCTION

The yeast has been used in the fermentation of various kinds of alcoholic beverages from hundreds of years. Although ethanol is a final product of anaerobic fermentation of sugars by yeast, it is toxic to yeast and induce stress responses (Piper, 1995). Ethanol toxicity is associated with its ability to suppress the biosynthesis of macromolecules, denaturing of the cytoplasmic proteins, reduction in the activity of glycolytic enzymes (Costa *et al.*, 1997; Biriukova *et al.*, 2007), disturbance in the transport of ions and metabolites across the plasma membrane (Monteiro and Sa-Correia, 1998) and alteration in the membrane's lipid composition (Dinh *et al.*, 2008). Ethanol also induces apoptosis and increase the ROS production in mitochondria (Kitagaki *et al.*, 2007), Yeast cells have developed appropriate mechanisms to deal with several types of damages caused by increased ethanol concentration. Factors that stabilize or repair denatured proteins in yeast cells, such as trehalose and Heat Shock Proteins (HSPs), have been shown an augmentation in ethanol stressed yeast cells (Hu *et al.*, 2010). Since trehalose was found to be a multifunctional molecule (Mahmud *et al.*, 2009), many approaches to investigate its role have been undertaken. In yeast, accumulation of the non-reducing disaccharide trehalose has been convincingly demonstrated as one of the main defence mechanisms against different stress conditions, such as heat shock, nutrient starvation, dehydration, toxic chemicals and oxidative stress (Pereira *et al.*, 2003; Elbein *et al.*, 2003). Trehalose seems to act by stabilizing

membranes and native proteins as well as by suppressing the aggregation of denatured proteins (Jain and Roy, 2009). Furthermore, trehalose lowers the levels of lipid peroxidation during dehydration (Pereira *et al.*, 2003).

Role of trehalose in the ethanol stress tolerance is well studied but there is not much information available about the role of trehalose in ethanol induced oxidative stress.

The yeast *Pachysolen tannophilus* was taken in the study due to its ability in conversion of biomass sugars into ethanol (Schneider *et al.*, 1981), although this ability has yet to be exploited commercially. The trouble in working with this strain is that its ethanol tolerance capacity is very limited (Barbosa *et al.*, 1990). Therefore, the aim of this study was to investigate the correlation between oxidative stress and trehalose level in ethanol stressed *Pachysolen tannophilus*, which may have further implications in better understanding of ethanol toxicity and its management during fermentation process.

### MATERIALS AND METHODS

The experiments for the study were carried out during the academic session 2009-2010 at Yeast Stress Response Study Laboratory of Biochemistry Department in Panjab University at Chandigarh, India.

**Organism and culture condition:** Yeast strain *Pachysolen tannophilus*, Y1038 (procured from IIT Delhi) was maintained at 4°C on YPDA (2% dextrose, 1%

yeast extract, 2% peptone and 2% agar). The cells were grown in YPD media (pH 5.5) in Erlenmeyer flasks with liquid-to-air volume ratio of 1:5 at 200 rpm and 30°C.

**Assay of cell resistance to ethanol:** To observe the tolerance of yeast to ethanol, the ethanol was added in YPD media that contains cells of exponential phase (approximately  $1-2 \times 10^7$  cells/ml) to make final concentration 10% (v/v), in Erlenmeyer flasks. After incubation at 30°C and 100 rpm for various time intervals, aliquots were withdrawn after appropriate durations.

**Trehalose extraction and determination:** Samples taken from the cultures were washed twice with ice-cold water. Then suspended in the distilled water and transferred to a water bath (95°C) for 10 minutes to denature and precipitate proteins. After centrifugation at  $20,000 \times g$  for 15 min. Trehalose was estimated in supernatant by the Anthrone method as described previously (Wyatt and Kale, 1957; Jagdale and Grewal, 2003). The pellet was used for the protein estimation.

**Glutathione determination:** For determination of glutathione, a known quantity of cells was homogenised in 10% TCA. After centrifugation at  $10,000 \times g$  for 30 min supernatant was used to estimate reduced glutathione (GSH) using DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) according to the method of Beutler *et al.* (1963). Total glutathione content was estimated by the method of Habeeb (1972). After reduction of GSSG to GSH with sodium borohydride.

**Evaluation of lipid peroxidation:** Lipid peroxidation was quantified by determination of thiobarbituric acid (TBA)-reactive substances (TBARS) according to the method of Beuge and Aust (Beuge and Aust, 1978). The amount of TBARS formed was measured by the reaction with thiobarbituric acid at 532 nm. The results were expressed as TBAR species (nM/mg protein) using molar extinction coefficient of MDA-thiobarbituric chromophore  $1.56 \times 10^5$  per M.cm.

**Quantification of protein bound carbonyls:** For assessment of carbonyls the reaction with dinitrophenylhydrazine was employed (Reznick and Packer, 1994). Protein-bound carbonyl concentrations were calculated via their reaction with 2,4-dinitrophenylhydrazine (DNPH) to give the corresponding hydrazone. This (yellow) product was quantified spectrophotometrically at 375 nm using an extinction coefficient of 22 m/Mcm and expressed as nM carbonyl/mg of protein.

**Measurement of intracellular oxidation level:** Intracellular ROS was detected the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Davidson *et al.*, 1996). According to this procedure

2',7'-dichlorofluorescein diacetate (DCFHDA) was added from a fresh 5 mM stock (prepared in ethanol) to a final concentration of 10 mM in one millilitre of yeast cell culture ( $10^7$  cells) then incubated at 28°C for 20 min. Finally, cells were cooled on ice, harvested by centrifugation and washed twice with distilled water. The pellet was resuspended in 500  $\mu$ L of water and 1.5 g of glass beads were added. Cells were lysed by 3 cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. The supernatant was obtained after centrifugation at  $25,000 \times g$  for 5 min. The fluorescence of supernatant was measured by using a Shimadzu Spectrofluorophotometer (RF-5301PC) with excitation at 502 nm and emission at 521 nm.

**Viability measurement:** Viability of the cells was determined by colony counting after spreading appropriate dilutions in duplicate on YPD-agar, following incubation at 30°C for 3 days. The percent viability was calculated with respect to cells grown without ethanol.

**Protein estimation:** The pellet obtained after trehalose and glutathione extraction was solubilised by boiling for 5 min with 2.0 mL of 0.1 M NaOH. The clear supernatant was used for protein estimation by Lowry *et al.* (1951) method using bovine serum albumin as standard.

**Statistical analysis:** Statistically significant differences were determined by using the one way analysis of variance (ANOVA). Values with  $p < 0.05$  were considered as statistically significant. Analyses were performed using GraphPad Prism v.5.00.288 for Windows (GraphPad Software, San Diego, CA).

## RESULTS

**Trehalose content:** Yeast cells were first cultured for 14 h to reach log phase under optimum conditions. We incubated the cells with 10% ethanol for a period of 60 and 120 min and determined the trehalose content. The trehalose level significantly increased from 252  $\mu$ g/mg protein to 373 after 60 min and finally it was increased up to 570  $\mu$ g/mg protein after 120 min (Fig. 1).

**Glutathione content:** The amount of reduced glutathione was decreased gradually after treated with the ethanol, it decreased from 16  $\mu$ g/mg protein to 10 and finally it was observed 7  $\mu$ g/mg protein after 120 min ethanol stress. The ratio of reduced and oxidised glutathione also decreased on ethanol stress for 60 and 120 min. (Fig. 2, Table 1)

**Lipid peroxidation:** The content of thiobarbituric acid substances (TBARS) significantly increased from 0.268 nM/mg proteins to 0.302 for first 60 min and after 120 min it was observed 0.350 nM/mg protein (Table 1). This

Table 1: Effect of ethanol stress on Lipid peroxidation, and glutathione content in *Pachysolen tannophilus*

Ethanol (10%) stress	Lipid peroxidation (TBAR species nM/ mg protein)	Glutathione level (µg/mg protein)		
		Reduced GSH	Total GSH	OxidisedGSH
0 min.	0.26750±0.006	16.167±0.753	26.333±0.816	10.167±.983
60 min.	0.30200±0.012	11.000±0.894	26.333±0.816	15.333±1.211
120 min.	0.35067±0.008	07.000±0.894	25.667±0.516	18.667±0.816

The results represent means ± SD of three independent experiments.

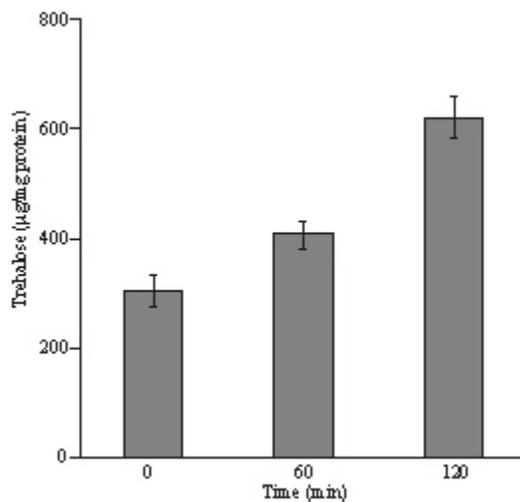


Fig. 1: Effect of ethanol stress on trehalose content of *Pachysolen tannophilus* on different time intervals. The value is mean of three independent experiments

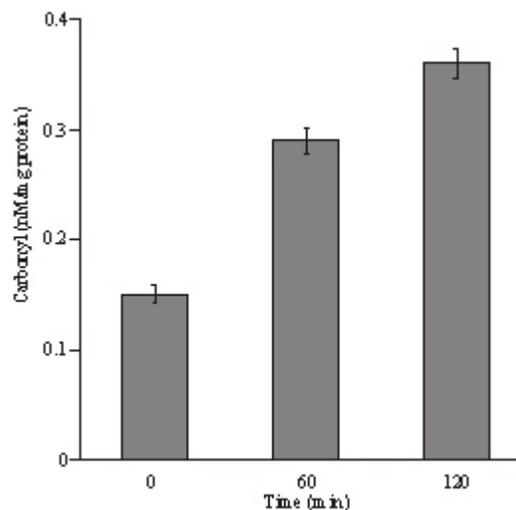


Fig. 3: Effect of ethanol induced oxidative stress on the protein carbonyl content of *Pachysolen tannophilus* on different time intervals. The value is mean of three independent experiments

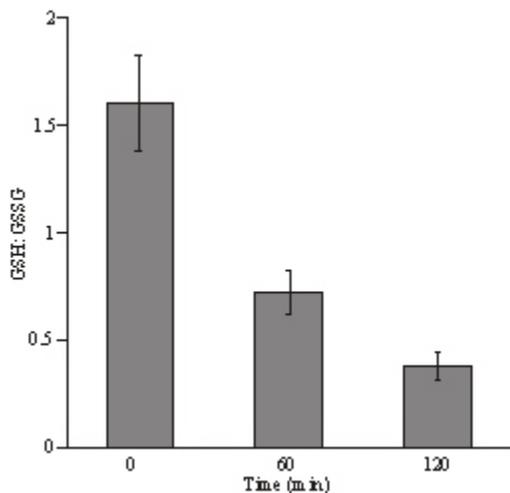


Fig. 2: Effect of ethanol stress on the ratio of reduced glutathione/ oxidised glutathione of *Pachysolen tannophilus* on different time intervals. The value is mean of three independent experiments

we determined the overall formation of protein bound carbonyls in *Pachysolen tannophilus*. In Fig. 3 results are presented which document the increase carbonyl production after 60 min from 0.15 nM/mg protein to 0.29 nM/mg protein and finally after 120 min it was observed 0.36 nM/mg protein.

**Intracellular oxidation level:** In an attempt to understand the level of oxidative stress in the ethanol stressed conditions, free radicals formations were studied in different groups. As shown in Fig. 4 significant increases in the level of ROS were found in ethanol stressed cells compared to control cells.

**Viability of *Pachysolen tannophilus*:** When yeast cells were subjected to ethanol stress, the viability was decreased 42 to 21% after 60 and 120 min, respectively as compared with the cells under similar conditions but without ethanol stress (Fig. 5).

shows increase in lipid peroxidation due to oxidative stress caused by ethanol.

**Oxidative protein modification:** To confirm the oxidative modification of proteins during ethanol stress,

**Correlation studies:** To evaluate together the role of oxidative stress and trehalose in the ethanol stress response study, a correlation among oxidative stress markers and trehalose was carried out.

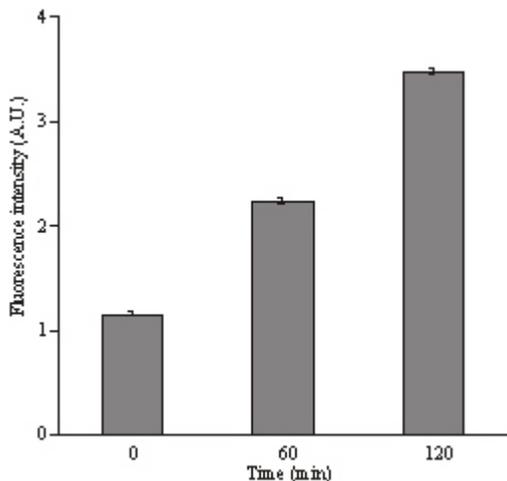


Fig. 4: The time courses of the formation of ROS species under ethanol stress. The bars represent the fluorescence intensity in arbitrary units (A.U.). Each experiment was done at least in triplicate

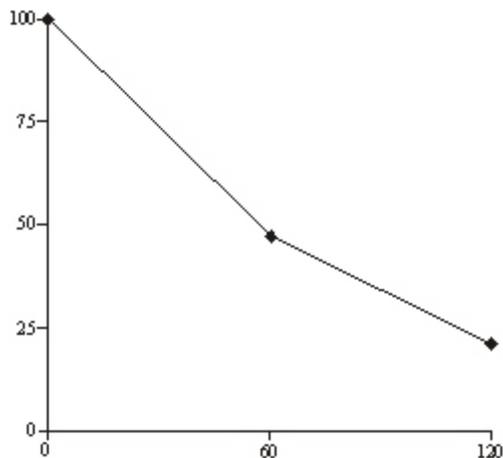


Fig. 5: Percentage viability after ethanol stress of *Pachysolen tannophilus*. The value is mean of three independent experiments

As shown in Table 2, significant positive correlation was observed between MDA and trehalose level ( $r = 0.909$ ), Protein carbonyl and trehalose ( $r = 0.848$ ) in ethanol stressed conditions while a notable negative correlation was found between MDA and GSH ( $r = -0.897$ ), trehalose and reduced GSH level ( $r = -0.891$ ) and trehalose and viability ( $r = -0.934$ ) in ethanol stress conditions.

### DISCUSSION

Ethanol constitutes the main stress factor during fermentation processes. The inability of the anti-oxidant

Table 2: Correlation among oxidative stress parameters in *Pachysolen tannophilus* with ethanol stress and control conditions

Parameters	Spearman's rank correlation coefficient (r)
Trehalose vs. MDA	0.909*
Trehalose vs. Protein carbonyl	0.848*
MDA vs. GSH	-0.897*
Trehalose vs. GSH	-0.891*
Trehalose vs. Viability	-0.934*

Values are expressed as Spearman's rank correlation coefficient (r). \*:  $p < 0.001$

defence system to cope up with ethanol stress is considered as a possible cause of ethanol toxicity. Wu and Cederbaum (2003) showed that oxidative stress may be involved in the ethanol toxicity.

Ethanol trigger the free radical generation in yeast. If these free radical species are not scavenged, they lead to the lipid peroxidation. In present study, a significantly elevated level of MDA was observed in ethanol stressed yeast cells. Literature provides a great variety of studies focused on yeast responses to unusual ethanol concentrations, which are represented mainly by synthesis of cellular trehalose (Piper, 1995; D'Amore *et al.*, 1991). We tried to elucidate the role of trehalose in lipid peroxidation, glutathione and oxidative stress. As shown in Table 2 a positive correlation between MDA level and trehalose suggests that lipid peroxidation is involved in the enhancement of cell damage in ethanol stress. This positive correlation suggest that trehalose work as a membrane stabilizing agent it is possible that during ethanol stress condition trehalose try to minimize the lipid peroxidation by the membrane stabilizing process. Pereira *et al.* (2003) shown that trehalose lowers the levels of lipid peroxidation during dehydration, ethanol stress also cause dehydration condition by lowering the water activity that may be responsible for the increased lipid peroxidation. *In vitro* studies on trehalose (Oku *et al.*, 2003) show that one trehalose molecule stoichiometrically interacts with one cis-olefin double bond of a unsaturated fatty acid, forming a stable complex and leading to significant reduction in oxidation level (Herdeiro *et al.*, 2006). During treatment of yeast cells with ethanol, the plasma membrane as its preferential target, trehalose was shown to be accumulating in the lipid bilayer for protection of the membrane. In previous studies level of trehalose also shown to increase during the oxidative stress conditions (Alvarez-Peral *et al.*, 2002) which proves the protective role of trehalose in oxidative stress conditions. Under physiological conditions, when ROS production is low, oxidative stress in controlled by the combined activities of various anti-oxidants present in the yeast. However, in the event of excessive ROS production, as is the case in ethanol stress conditions, this protection may be inadequate. Generation of ROS can lead to the oxidation of thiol (Penninckx, 2000). Yeast cells possess the ability to scavenge  $O_2$  and  $H_2O_2$  by glutathione. Redox ratio in the yeast is a significant parameter for the oxidative stress (Izawa *et al.*, 1995). In

ethanol stress, there is a change in redox ratio in the favour of oxidized glutathione (Fig. 2). GSH is a main antioxidant defence in the yeast but the decreased level of reduced glutathione in ethanol stressed condition may be attributing by the excess trehalose synthesis.

Sebollela *et al.* (2004) reported that excess trehalose cause inhibition in the glutathione reductase an enzyme that is involved in glutathione synthesis. During ethanol stress increased trehalose level may cause inhibition in the synthesis of the glutathione and by depletion of glutathione the cellular free radicals may be increase the oxidative stress. The negative correlation between the ethanol stress and viability can also be explain by the decreased glutathione level as Madeo *et al.* (1999) reported the essential role of glutathione in the yeast viability. GSH is associated with many essential functioning to the yeast cells, therefore decreased GSH level may be responsible for the reduced cell viability during ethanol stress.

The present study shows the increased levels of ROS and protein carbonyls during ethanol stress. These results are similar to the study of Benaroudj *et al.* (2001) and suggested that increased protein carbonyl formation and trehalose may be closely associated with increased oxidative stress.

Interestingly, positive correlations were obtained between trehalose and MDA level, trehalose vs. Ros level and trehalose vs. Protein carbonyl in ethanol stressed cells. Since ROS can affects the synthesis of trehalose by the transcriptory regulatory mechanism. They could be responsible for the increased trehalose synthesis during ethanol stress. Our study depicts that imbalance between oxidant and antioxidant defence may trigger severe complications in ethanol stress yeast cells. The correlation of trehalose with the oxidative stress marker suggest important role of trehalose in the protection of yeast cells from oxidation during ethanol stress conditions. Enzymatic defence mechanism can also work in the increase oxidative stress conditions so future studies might help in characterizing the mechanisms involved in ethanol toxicity.

### CONCLUSION

Our study concludes that trehalose shows a multifunctional role in yeast in ethanol stress. Increased oxidative stress during the ethanol stress conditions enhances the production of trehalose to protect the yeast cells from ethanol toxicity.

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