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Effect of halo-tolerance gene Hal5 on ethanol tolerance of *Saccharomyces cerevisiae*

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ABSTRACT

Hal5 gene is involved in halo-tolerance of *Saccharomyces cerevisiae* during high salt stress. Ethanol stress and high salt stress have similarities, as both decrease the availability of water for cells and strain the osmotic homeostasis across the cell membrane. The Hal5 over-expression strain of yeast has more ethanol tolerance, but the Hal5 null mutant strain also has more ethanol tolerance than the wild-type strain. Hal5 over-expression in this yeast strain may help in adaptation to ethanol stress by way of directly stabilizing the proteins (trk1-trk2) that are responsible for maintaining osmotic homeostasis. Dysfunction of Hal5 in the null mutant may result in increased trehalose, which also stabilizes proteins and increases ethanol tolerance in comparison to wild type, although not as much as over-expression of Hal5. In biochemical assays and FTIR, we observed an increase in trehalose in Hal5 mutant in comparison to the wild-type, as well as a further increase in response to ethanol stress. The ethanol stress increases ROS, protein carbonylation, and lipid peroxidation in all strains, but the Hal5 over-expression and Hal5 null mutation mitigate these adverse effects of ethanol stress.

Introduction

Eukaryotic cells face many stressful situations during their life cycle. Throughout growth and development, various stress response mechanisms are used at the level of the genome, cytoplasm as well plasma membrane of the yeast cell. Yeast (*Saccharomyces cerevisiae)* is one of the simplest eukaryotic models to study various parameters of ethanol stress tolerance. During ethanol fermentation, yeast cells are exposed to various oxidative and osmotic stress, and ethanol-induced growth inhibition, which may decrease ethanol production during industrial fermentation [1–3]. A low level of ethanol stress reduces yeast cells' growth by slowing down cell division and later on by decreasing specific growth rate and cell volume [4]. Whereas, a high level of ethanol stress causes cell death, which results in decreased fermentation. Ethanol stress has many adverse effects on cells, such as an increase in the fluidity of the cell membrane [5,6], a decrease in $H+$-ATPase activity [7], a decrease in cell vitality [8], suppression of transport progressions [9], decrease in electrochemical gradients, decrease in proton-motive force [10], decrease in glycolysis enzyme activity due to protein denaturation [10], distortion in vacuole morphology [11], inhibition of endocytosis [12], decrease in mRNA levels, and decrease in protein concentrations [13].

Yeast cells have evolved mechanisms to counter numerous types of deterioration induced by the elevated ethanol concentration during the fermentation of sugars. These mechanisms include, stimulation of heat shock proteins [14], activation of unfolded protein response [15], increase in unsaturated fatty acids and ergosterol level in the plasma membrane [16], and intracellular trehalose accumulation [12]. Cellular ionic homeostasis disturbance triggered by ethanol stress can lead to a decline in metabolic activity and ultimately cell death [17]. Some monovalent and divalent cations, for instance, potassium, calcium, and magnesium play an important role inside yeast cells during ethanol stress [17]. Each of these ions has to be retained in a controlled range of concentrations to prevent cell toxicity [18]. Internal ion concentrations are maintained by complex homeostatic pathways against fluctuating extracellular environments. One of the major cellular cations includes potassium, aggressively retained in cells at very high concentrations. Physiological considerations such as cell volume, turgor pressure, and cytoplasmic ionic strength are primarily determined by Potassium ion concentrations present in the cell [19].

While various ion transport systems have been recognized, the approaches that regulate their activity remain undetermined. Hal5, which

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encodes homologous protein kinases, is associated with the regulation of cation uptake. Intracellular potassium ion homeostasis is essential for several enzymatic activities, stabilization of several proteins, maintenance of membrane potential, and cytosol pH. In *Saccharomyces cerevisiae,* the major high-affinity *K+* uptake pumps are encoded by partially redundant transporter genes TRK1 and TRK2 [20]. The potassium transport is triggered through potassium ion starvation and salt stress [21]. The plasma membrane stability of the Trk1-Trk2 transport system is positively regulated by Hal4 and Hal5 protein kinases. Activation of the Trk1-Trk2 potassium transport system increases the inflow of *K+* inside the cell, resulting in a decrease in the membrane potential and maintaining cellular ion homeostasis during ethanol stress. Therefore, it would be tempting to speculate about the role of Hal5 protein kinase during ethanol stress in yeast. Hence, the present study has been designed to evaluate the ethanol tolerance of yeast strains with wild type Hal5, dysfunctional Hal5 and a strain that is overexpressing Hal5. These 3 strains of yeast are compared under ethanol stress in terms of their viability, changes in carbohydrates, oxidative stress parameters, and lipid composition.

Materials and methods

Yeast strains and growth conditions

Yeast *Saccharomyces cerevisiae* wild-type (924), *Hal4,5* mutant (1300), and Hal5 over-expressed (Ep) strains used in the present study were obtained from Prof. Ramón Serrano, IBMCP, Polytechnic University of Valencia, Spain. Cultures were maintained at 4 °C on YPDA medium (2 % dextrose, 1 % yeast extract, 2 % peptone, 2 % agar).

Yeast wild-type (924), Hal5 over-expressed (Ep), and *Hal4,5* mutant (1300) strains were cultured from an inoculum prepared by transferring cells of a single colony from YPDA plate to 3.0 ml YPD broth in the 10 ml conical flasks and grown at 30 °C for overnight with shaking at 200 rpm. The inoculum of 0.05 OD_{600} was transferred to a fresh YPD liquid media (pH 5.5) in Erlenmeyer flasks with a liquid-to-air volume ratio of 1:5 and the culture was grown at 30° C in an orbital shaker at 200 rpm, till mid-log phase (16 h). Around 1/4th of the culture was harvested. The pellet was washed twice with distilled water and used as control at time 0 (h) and then 10 % ethanol was added to the rest 3/4th of the culture and harvested after 1 and 2 (h). The pellet was washed twice with distilled water and used for FTIR and analysis of trehalose and glutathione. For the estimation of protein carbonyl and lipid peroxidation, the cultures were harvested after 2 and 4 (h).

Stress tolerance assay

Spot dilution assay on solid YPDA media was performed to compare the tolerance of yeast strains to different ethanol concentrations. Cells were pre-grown in the YPD liquid medium overnight at 30 °C. The cell titer of different strains was adjusted and then 1:10 serial dilution was spotted on YPD agar plates supplemented with ethanol (6 %, 8 %, and 10 %, v/v). 6 % and 10 % ethanol are respectively sub-lethal and lethal dose in the conditions studied here. The growth was recorded after 48 h at 30 °C.

Ethanol tolerance was also examined by growth curve assay in the liquid YPD medium. Yeast cells pre-cultured in YPD medium at 30 °C with 200 rpm agitation for 16 h were inoculated into 100 ml of fresh YPD to a final cell density equal to 0.05 of OD_{600} with 8 %, 10 % (v/v) ethanol and without ethanol as control. Cells were cultivated at 30 °C with 200 rpm agitation. The cell growth was examined after each 2 (h) periodically for 48 h, as described earlier [22]

Cell survival assay was also carried out to determine the ethanol tolerance of different yeast strains. From an overnight grown culture, a 1 % (v/v) inoculum was added to 10 ml of fresh YPD medium and incubated at 30 °C for 16 h Yeast cells were harvested by centrifugation at 3000× *g* for 5 min, the pellet was washed twice with distilled water and resuspended in 100 mM phosphate buffer saline (PBS, pH 7.4) to a final cell concentration equivalent to 1.0 of OD_{600}. Ethanol was added to the cell suspension to a final concentration of 15 % (v/v), and incubated at 30 °C with an agitation of 200 rpm. Serially diluted yeast cells were spread on YPDA plates. After incubation at 30 °C for 48 h, colony-forming units (CFU) were calculated.

Morphological analyses

Assessment of cell morphology for apoptosis was done using DAPI (4,6-Diamidino-2-phenylindole) and PI (Propidium Iodide) Staining. Stain solution were prepared in Phosphate Buffer Saline (pH= 7.2–7.4). For DAPI staining, exponentially growing yeast cells were harvested, followed by DAPI (1.0 μg/ml) and PI (1.0 μg/ml) staining each for 20 min in dark [23]. Later, images were recorded at 100X with Nikon fluorescence microscope. Blue fluorescence is observed for both viable and dead cells while red fluorescence is observed for both viable and dead cells while red fluorescence for cells showing apoptotic characters.

Determination of trehalose levels

The quantification of trehalose was done from the supernatant using the Anthrone method modified by Jagdale and Grewal [24].

Estimation of oxidative stress

Intracellular ROS were determined by using oxidant-sensitive fluorescent probe, 2′,7′-dichloro fluorescein di acetate (DCFH-DA) (Sigma-Aldrich), as described by Davidson et al. [25]. The total glutathione level was determined as described by Habeeb [26]. Reduced glutathione was estimated according to the method of Beutler et al. [27]. The method for measurement of protein carbonyl is based on the binding of 2,4-dinitrophenylhydrazine (DNP) with the protein-bound carbonyl (Reznick and Packer 1994). Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) as described by Buege and Aust with some modifications [28].

Antioxidant enzymes assays

For preparing yeast cell lysate for antioxidant enzyme assays, glass beads (0.5 mm) were used. The yeast cells were extracted using a 3000 x g centrifuge and resuspended in 1.0 ml of lysis buffer (50 mM of Tris–HCl, 150 mM of sodium chloride and 50 mM of EDTA at pH=7.2). 1/2 vol of ice-cold glass beads (0.5 mm) were added to disrupt cells, and cells were lysed in three cycles of one-minute agitation on a vortex mixer followed by one minute in an ice bath. Centrifugation at 15,000 rpm was done for 10 min to remove cellular debris, and supernatant was collected for antioxidant enzyme testing. SOD activity was measured in cell lysate supernatant using Kono's (1978) technique [29]. Catalase activity was measured in cell lysate using Luck's (1971) method [30]. Glutathione Peroxidase activity was measured in cell lysates using the Paglia and Valentine (1967) technique [31]. Glutathione Reductase activity was determined in the sample using the Carlberg and Mannervik (1985) method [32].

Extraction of lipids and phospholipids

The of Folch method (1957) was used with some modification for the extraction of total lipids [33]. However, some modifications were made to Folch method for complete recovery of lipids. Lipids were extracted two more times from the solid residue using chloroform methanol (2:1) and pooled together. At washing step of removing water-soluble impurities also the upper aqueous layer was treated with chloroform two more for complete recovery of lipids. Phospholipids were calculated using the Bartlett method, as modified by Marinetti [34]. Total phospholipids were estimated by estimating inorganic phosphate and

multiplying it by a factor of 25 (a factor calculated using a phospholipid average molecular weight of 775). The factor 25 was obtained by dividing 775 with 31, the molar mass number of phosphorous. The phosphorous content of the sample was calculated from the value of the standard, carried out simultaneously. The amount of phospholipids was determined by multiplying the amount of Pi (inorganic phosphorous) by a factor of 25.

Estimation of glycolipids and sterol

Glycolipids were estimated based on their sugar content. The method of Dubois et al. (1956) was used for the determination of sugar content of a lipid sample [35]. The sterol content was estimated using the method proposed by Zlatkis and Zak [36]. Esterified sterols were estimated after fixation of free sterols by the digitonin. To 1.0 ml of lipid sample, 0.25 ml of digitonin was added, both of these were mixed thoroughly and evaporated to dryness. To this added 3.0 ml of petroleum ether. Tubes were heated at 60–70 °C till half of the solvent gets evaporated. Tubes were cooled to room temperature and ether extract was collected in another tubes. This step was repeated several times. Pooled ether was evaporated and sterols were collected in chloroform. Then sterols esters were estimated using the same procedure as described above for the determination of total sterol. Esterified sterols were estimated after fixation of free sterol by the digitonin.

FTIR analysis

Yeast cells were harvested by centrifugation at 5000 rpm for 10 min. The pellets were washed twice with distilled water and resuspended in 0.1 ml of distilled water and immediately kept at −80 °C and lyophilized. After lyophilization, the dried powdered cellular biomass pellets were used for FTIR analysis. The FTIR spectra were recorded using the FTIR Spectrophotometer Model RZX (PerkinElmer) with a resolution of 4 cm^{-1}. The transmission spectrum from 750 cm^{-1} to 4500 cm^{-1} was measured by co-adding 64 scans and subtracting the background. The spectrum quality assessment and data analysis were done using irAnalyze-RAMalyze Spectroscopic Solution trial version software according to the study of Saharan and Sharma [37].

Results

Effect of biochemical stress on viability of yeast strains differing in Hal5 gene

The three Hal5 yeast strains were grown in YPD liquid media to equal density and then serially diluted by 1:10 before spotting on YPD agar plates containing one of stress factor i.e., ethanol (6 %, 8 %, and 10 %, v/v). As shown in Fig. 1, all three strains of yeast have decreased, when grown under various stress conditions, in comparison to the control having no stress factor. Wild type yeast (924) is most sensitive to ethanol stress, whereas Hal5 over-expression strain (Ep) as well as Hal5 mutant strain (1300) both are less sensitive to ethanol in comparison to wild type (924). Slightly bigger colonies are observed for Ep strain in comparison to 1300 strain at all the cell dilution spots on YPDA plates containing ethanol at concentrations 6, 8, and 10 %. Therefore, Ep strain is slightly more ethanol tolerant than 1300 strain.

The cellular responses were assessed using DAPI / PI staining to evaluate DNA integrity and cell viability, respectively. The effects of ethanol stress on yeast Hal5 overexpressed strain (Ep), wild type (924), and Hal4,5 mutant strains were investigated under 10 % ethanol (supplementary figure 1a, 1b, and 1c respectively). The wild type (924) strain displayed a higher PI penetration as compared to the Ep and Hal4,5 mutant (1300) strain after 2 hr ethanol stress. DAPI staining revealed that all three strains had intact nuclear structures, indicating that ethanol stress did not significantly affect DNA integrity. This was expected, as DAPI staining is primarily used to visualize DNA and does not necessarily correlate with cell viability or stress resistance. On the other hand, PI staining results demonstrated differences in cell viability among the strains. The wild type (924) strain had a higher percentage of PI-positive cells, indicating decreased cell membrane integrity and increased cell death, compared to the Ep and 1300 strains. In contrast, the Hal5 overexpressed (Ep) and Hal4,5 mutant (1300) strains exhibited lower PI penetration, suggesting enhanced tolerance to ethanol stress.

Effects of ethanol stress on trehalose contents in yeast strain EP, 1300, and 924

Trehalose content were increased in all strains after exposure to ethanol stress (Table 1). The Hal5 over-expressed strain (Ep) exhibited

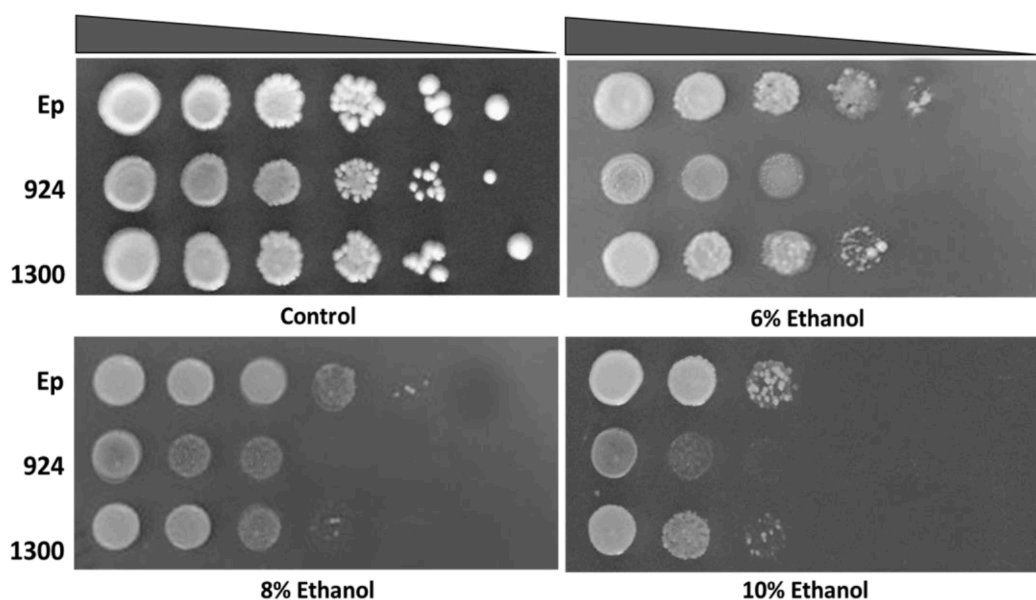

Fig. 1. Spot dilution assay for comparison of cell Growth phenotypes of yeast cells on solid YPD media, in response to different stress conditions. Cells were pre-grown in the YPD liquid medium overnight at 30 °C. The cell titers of different strains were adjusted and then 1:10 serial dilution was spotted on YPD agar plates supplemented with different stress. The growth was recorded after 48 h at 30 °C.

Table 1
Trehalose content (µg/mg protein) of *Saccharomyces cerevisiae,* Hal5 overexpressed strain (Ep), wild type strain (924) and Hal4,5 mutant strain (1300) under control without ethanol at time 0 hr and 10 % ethanol stress for 1hr and 2 hr.

	Time (hours)		
	Control	10 % ethanol stress	
Strains	0 hr	1 hr	2 hr
Ep	210.44 ± 33.09	548.31 ± 29.69**	626.32 ± 22.36**
924	180.32 ± 18.04	445.25 ± 24.03*	449.87 ± 36.29**
1300	187.29 ± 95.82	521.15 ± 74.58	586.18 ± 122.44**

Data are ± SD ($n = 3$). The error bars indicate the standard deviation.
* $P < 0.05$.
** $P < 0.01$, indicate statistically significant differences as compared to their respective control (Tuckey's multiple comparison test).

the highest percentage increase in both trehalose content under ethanol stress, followed by the Hal4,5 (Table 1). The higher increase in trehalose content in the Hal5 over-expressed strain (Ep) suggests that the over-expression of Hal5 could enhance the stress tolerance of yeast cells by promoting the accumulation of these storage carbohydrates. Previous studies have shown that trehalose accumulation is a common response in yeast cells when exposed to various environmental stresses, including ethanol stress [38]. Trehalose is a non-reducing disaccharide that has been reported to protect yeast cells from multiple stress conditions by stabilizing proteins and membranes [38].

The response of yeast cells to ethanol stress has implications in industrial applications such as bioethanol production and brewing [39]. One of the primary responses of yeast cells to ethanol stress is the accumulation of storage carbohydrates, including trehalose, which have been shown to play a role in maintaining cellular integrity and enhancing stress tolerance [40,41].

Oxidative stress caused by ethanol stress in yeast strain EP, 1300, and 924

Lipid peroxidation, the ratio of reduced and oxidized glutathione (GSH/GSSG), ROS (reactive oxygen species), and the formation of protein-bound carbonyl groups were studied to determine the oxidative stress caused by ethanol stress in the yeast strains differing in Hal5 gene. The lipid peroxidation is a key marker of oxidative stress, which is measured by formation of TBARS. TBARS form as a result of free radical-induced lipid peroxidation in cells. Ethanol stress increases the unsaturated fatty acids contents in the cell membrane, and unsaturated fatty acids are more sensitive to free radical-induced peroxidation [18]. The oxidation of such unsaturated fatty acids further increase TBARS formation [42].

Overall, the ethanol stress increases oxidative stress in all these yeast strains, as indicated by increased protein carbonylation, increased lipid peroxidation, increased ROS, and decreased ratio of reduced to oxidized glutathione (Supplementary Table 1 a-d). In all the strains, these Oxidative stress parameters increases from 0 hour to at 1 hour and further more at 2 h of ethanol stress (Supplementary Table 1 a-d). Ethanol stress caused the least oxidative stress in the Ep strain, followed by in the 1300 strain, and followed by in the 924 strain.

Antioxidant enzyme activity in response to ethanol stress in yeast strain ep, 1300, and 924

Ethanol stress in yeast cells can lead to the generation of reactive oxygen species (ROS), which are highly reactive molecules capable of causing oxidative damage to cellular components, such as lipids, proteins, and DNA. Antioxidant enzymes play a crucial role in the defense against ethanol stress by neutralizing ROS and preventing cellular damage. Some key antioxidant enzymes in yeast cells include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and Glutathione reductase. Estimation of these enzymes' activity in *Saccharomyces cerevisiae* Hal5 overexpressed (Ep), wild-type (924), and Hal4,5 mutant (1300) strains were performed under 10 % ethanol stress for 1 and 2 h (Fig. 2). All these 4 enzymes studied showed increased activity in response to ethanol stress in all the strains, the wild type showing the highest increase.

SOD is an enzyme that catalyzes the conversion of superoxide radicals ($O_2^{\bullet-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). The wild type (924) strain demonstrated the highest increase in SOD activity across the time points, followed by the Hal4,5 mutant strain (1300) and the Hal5 overexpressed strain (Ep) (Fig. 2). In the Hal5 overexpressed strain (Ep), SOD activity exhibited a substantial increase of 237.56 % after 1 hr of ethanol stress, following 2 hr of ethanol stress, the SOD activity further increased to 280.75 % compared to the control . The wild type (924) strain demonstrated even greater increases in SOD activity upon exposure to ethanol stress. After 1 hr of ethanol stress, the SOD activity increased 372.21 % from the control, whereas after 2 hr ethanol stress the SOD activity further increased by 556.34 % as compared to the control. The Hal4,5 mutant (1300) displayed a 283.89 % increase in SOD activity after 1 hr of ethanol stress, following 2 hr of ethanol stress, the SOD activity further increased to 378.51 % as compared to the control.

Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen, effectively neutralizing the toxic effects of H_2O_2. Among the strains, the wild type exhibited the greatest increase in catalase activity, followed by the Hal4,5 mutant and the Hal5 overexpressed (Fig. 2). For the Hal5 overexpressed strain (Ep), after 1 hr of ethanol stress, the catalase activity increased by 133.33 % compared to the control. This increase continued to the 2 hr ethanol stress, with a total increase of 196.30 %. In the wild type strain (924) the catalase activity after 1 hr ethanol stress increased by 207.44 % as compared to the control. By the 2 hr ethanol stress, the catalase activity further increased by 290.23 %. In the Hal4,5 mutant strain (1300), the catalase activity increased by 181.94 % after 1 hr ethanol stress as compared to the control. The catalase activity further increased by 257.41 % after 2 hr ethanol stress.

Glutathione peroxidases (GPx) are a family of enzymes that catalyze the reduction of hydrogen peroxide and organic hydroperoxides using reduced glutathione (GSH) as a substrate. Following exposure to 10 % ethanol stress, the wild type strain exhibited the most pronounced increase, followed by the Hal4,5 mutant and the Hal5 overexpressed strain (Fig. 2). For the Hal5 overexpressed strain (Ep), the Glutathione peroxidase activity exhibited a 114.87 % increase at 1 hr and a 160.16 % increase at 2 hr of ethanol stress. In the wild type strain (924), the Glutathione peroxidase activity showed a substantial 198.25 % increase

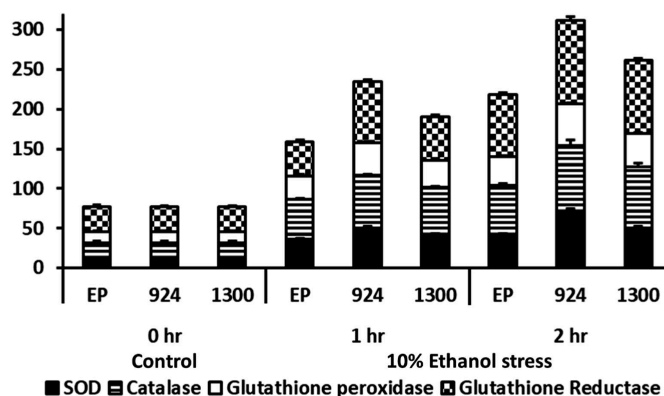

Fig. 2. Antioxidant enzymes' activity of Hal5 overexpressed strain (Ep), wild type strain (924) and Hal4,5 mutant strain (1300) under control without ethanol at time 0 hr and 10 % ethanol stress for 1hr and 2 hr. Data are means ± SD ($n = 3$). The error bars indicate the standard deviation. ** $p \leq 0.01$ indicate statistically significant differences as compared to their respective control (Tukey's multiple comparisons test).

at 1 hr and a 284.57 % increase at 2 hr of ethanol stress. For the Hal4,5 mutant strain (1300), the Glutathione peroxidase activity increased by 147.97 % at 1 hr and by 200.58 % at 2 hr of ethanol stress.

Glutathione reductase (GR) enzyme catalyzes the reduction of oxidized glutathione (GSSG) to its reduced form (GSH) using NADPH as a cofactor. GSH, in turn, serves as an essential antioxidant, participating in the detoxification of reactive oxygen species (ROS). The wild type strain (924) showed the highest increase in glutathione reductase activity in response to ethanol stress (Fig. 2.). For the Hal5 overexpressed strain (Ep), a 38.81 % increase in Glutathione Reductase activity was observed after 1 hr of ethanol stress, and a 150.37 % increase was noted after 2 hr ethanol stress. In the wild type strain (924), the enzyme activity significantly increased by 146.38 % after 1 hr and by 237.53 % after 2 hr of ethanol stress. The Hal4,5 mutant strain (1300) exhibited a 75.11 % increase in Glutathione Reductase activity after 1 hr and 197.13 % increase after 2 hr ethanol stress.

Effect of ethanol on lipid composition of Saccharomyces cerevisiae

Changes in the lipid content of yeast under ethanol stress, including alterations in phospholipids and sterols, are part of the cellular response to maintain membrane integrity and function. Sterols, such as ergosterol, are involved in maintaining membrane fluidity, permeability, stabilizing membrane proteins and modulating membrane-associated processes, which could be crucial for yeast adaptation to ethanol stress [16,43].

Total sterols (mg g^{-1} dry wt.) of three strains of *Saccharomyces cerevisiae* were assessed following exposure to 10 % ethanol stress for 1 and 2 hr. Results are described in Fig. 3. Upon exposure to ethanol stress, the Hal5 overexpressed strain (Ep) exhibited an increase in total sterols content to 18.52 % after 1 hr ethanol stress, and 56.79 % after 2 hr ethanol stress as compared to the control. In the wild type (924) strain, the total sterols content increased by 37.80 % after 1 hr ethanol stress and displayed a more substantial increase of 118.29 % after 2 hr ethanol stress. The Hal4,5 mutant (1300) strain showed an increase in total sterols content of 30.00 % after 1 hr ethanol stress and a 97.50 % increase after 2 hr ethanol stress. These results indicate differential responses in total sterols content among the all three strains of *Saccharomyces cerevisiae* upon exposure to 10 % ethanol stress. The wild type (924) strain demonstrated the highest increase in total sterols content after 2 hr of ethanol stress, followed by the Hal4,5 mutant (1300) strain, and finally the Hal5 overexpressed strain (Ep).

Upon following exposure to 10 % ethanol stress for 1 and 2 hr, the wild type strain exhibited the highest increase in esterified sterols content, followed by the Hal4,5 mutant strain, and finally the Hal5 overexpressed strain (Fig. 3). Similarly, increase in free sterols (mg g^{-1} dry wt.) was also observed the highest in the wild type (924) strain followed by the Hal4,5 mutant (1300) strain, and lastly, the Hal5 overexpressed strain (Ep) (Fig. 3). The ration of esterified sterols to free sterols' ratio decreased the most in Hal5 overexpressed strain (Ep) followed by Hal4,5 mutant and then the wild type strain (924) (Fig. 3). Glycolipids content (mg g^{-1} dry wt.) increased in the Hal4,5 mutant strain the most at 2 hr of stress exposure, whereas the wild type strain showed the highest increase after 1 hr of ethanol stress exposure. The Hal5 overexpressed strain displayed a consistent increase in glycolipid content at both 1 hr and 2 hr of ethanol stress (Fig. 4). Phospholipid content increased the highest in the wild type (924) strain, followed by the Hal4,5 mutant (1300) strain and the Hal5 overexpressed (Ep) strain (Fig.5). These results may indicate varying responses and adaptations to ethanol stress among the different *Saccharomyces cerevisiae* strains.

Observation of biochemical changes using vibrational spectroscopy

When yeast cells are exposed to ethanol stress, their FTIR spectral changes can be analyzed to understand the alterations in their biomolecules, such as lipids, proteins, and carbohydrates (Fig. 6). In the FTIR spectra, the absorbance peaks at 2926 cm-1 and 2855 cm-1 correspond to the asymmetric and symmetric stretching vibrations of the CH_2 groups, respectively, which are primarily found in fatty acids

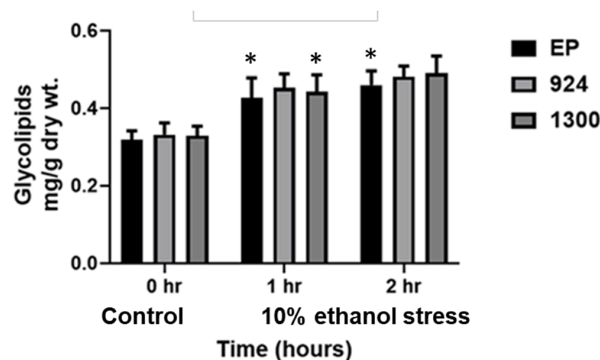

Fig. 4. Glycolipids content of Hal5 overexpressed strain (Ep), wild type strain (924) and Hal4,5 mutant strain (1300) under control without ethanol at time 0 hr and 10 % ethanol stress for 1hr and 2 hr. Data are means ± SD ($n = 3$). The error bars indicate the standard deviation. $^{*}P \leq 0.05$, indicate statistically significant differences as compared to their respective control (Tukey's multiple comparisons test).

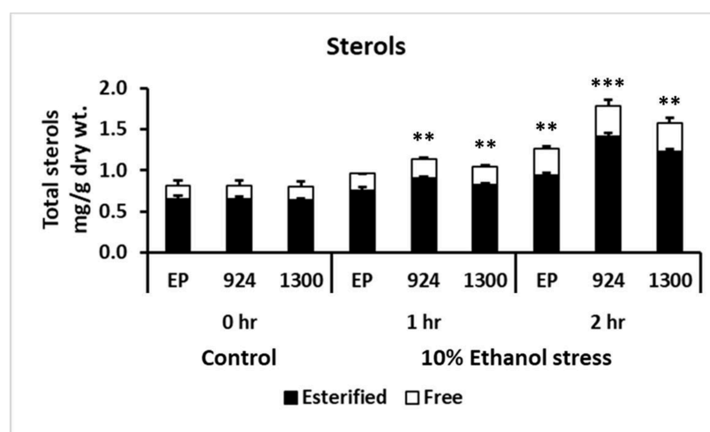

Fig. 3. Sterols content of Hal5 overexpressed strain (Ep), wild type strain (924) and Hal4,5 mutant strain (1300) under control without ethanol at time 0 hr and 10 % ethanol stress for 1hr and 2 hr. Data are means ± SD ($n = 3$). The error bars indicate the standard deviation. $^{**}P \leq 0.01$, $^{***}p \leq 0.001$ indicate statistically significant differences as compared to their respective control (Tukey's multiple comparisons test).

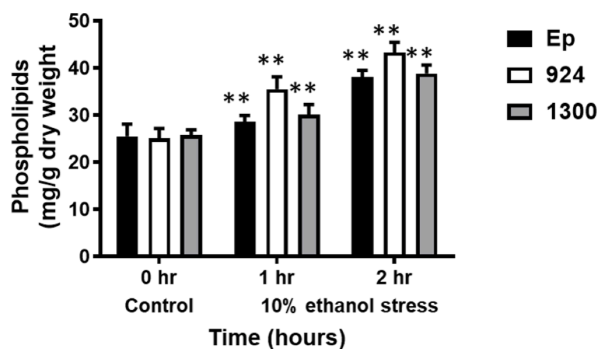

Fig. 5. Phospholipids content of Hal5 overexpressed strain (Ep), wild type strain (924) and Hal4,5 mutant strain (1300) under control without ethanol at time 0 hr and 10 % ethanol stress for 1hr and 2 hr. Data are means ± SD (n = 3). The error bars indicate the standard deviation. ** $p \leq 0.01$ indicate statistically significant differences as compared to their respective control (Tukey's multiple comparisons test).

[44]. The increase in the peak height at these wavelengths from the control to the 1-hour ethanol stress and further increase for the 2-hour ethanol stress suggest that there might be a change in the lipid composition and/or membrane fluidity of the yeast strains under ethanol stress.

Spectral region 1500–900 cm^{-1}: This region is mainly associated with carbohydrate-related vibrations, such as C—O stretching, C—O-H bending, and C—C stretching. In response to ethanol stress, yeast cells increases trehalose production as a protective mechanism, which has a stabilizing effect on proteins and cellular membranes [45,46]. The FTIR spectrum in the 1500–900 cm^{-1} region is an indicator of changes in the intracellular trehalose content, which is related to the C—O stretching, C—O-H bending, and C—C stretching vibrations. The increased peak height at around 1060 cm^{-1} for all strains under ethanol stress corresponds to C—O-C or C—O-P vibrations. This spectral region contains carbohydrate-related vibrations, therefore an increase in this region is an indicator of increase in storage carbohydrates, such as trehalose [37].

The increase in peak height at 1740 cm^{-1} for all strains under ethanol stress corresponds to the C = O stretching vibration of ester functional groups [47]. These changes indicate modifications in the cell wall or membrane composition, such as an increase in esterified fatty acids or changes in glycerophospholipids, as a response to ethanol stress. Spectral region 1760–1520 primarily contains amide I and amide II bands, which are sensitive to protein secondary structures. Ethanol-induced protein denaturation, aggregation, or unfolding can lead to alterations in protein secondary structures, as seen through FTIR spectroscopy [48]. The decrease in peak heights around 1640 cm^{-1} and 1535 cm^{-1} for strains 924 and 1300, while remaining the same for the Ep strain, under ethanol stress, is related to amide I (C = O stretching) and amide II (N—H bending and C—N stretching) vibrations, respectively, which represent proteins in the sample [49]. This suggests that protein content in strains 924 and 1300 decreases under ethanol stress, while the Ep strain maintains its protein content. This difference may indicate a greater ability of the Ep strain to maintain cellular functions under stress

Fig. 6. FTIR spectrum of different strains showing the change in trehalose, protein, and lipid under ethanol stress for 1, 2 (h), and 0 (h) as the control for no ethanol stress.

by maintain the ion homeostasis, potentially contributing to higher ethanol tolerance [50].

Spectral region 3000–2800 cm^{-1} is mainly associated with C—H stretching vibrations of lipids, especially fatty acids. Ethanol stress may lead to changes in membrane lipid composition and structure, with modifications in the unsaturation levels of fatty acids [51,52]. The increase in absorbance peaks at 2926 cm^{-1} and 2855 cm^{-1} for all strains under ethanol stress suggests an increase in the lipid content of the yeast cell membrane. These peaks correspond to C—H stretching vibrations, specifically methylene (CH2) and methyl (CH3) groups found in fatty acids that make up the lipids in cell membranes [53,54]. An increase in lipid content has been reported as a response to ethanol stress in yeast, as it may help maintain membrane integrity and fluidity [8]. Enhanced lipid production can contribute to increased ethanol tolerance, as observed in some yeast strains [55].

Discussion and conclusion

Ethanol stress has also been shown to trigger the production of reactive oxygen species (ROS) in yeast cells, which can cause oxidative damage to cellular components, including lipids [56]. The lipid peroxidation process can further exacerbate membrane damage and impair cellular functions. *Saccharomyces cerevisiae* possesses various antioxidant defense mechanisms, such as the up regulation of superoxide dismutase (SOD) and catalase (CAT) activities, to mitigate the harmful effects of ROS [57].

Antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase, have shown increased activity in response to ethanol stress in *Saccharomyces cerevisiae* strains. Ethanol stress is known to generate reactive oxygen species (ROS), which can cause oxidative damage to proteins, lipids, and DNA [58]. Therefore, the increased activity of antioxidant enzymes under ethanol stress could be an adaptive response of the yeast cells to protect themselves against the detrimental effects of ROS [59].

SOD enzymes are the first line of defense against oxidative stress, as they detoxify superoxide radicals into hydrogen peroxide and molecular oxygen [60]. The increased SOD activity in yeast cells under ethanol stress might be a compensatory mechanism to counteract the elevated production of superoxide radicals. Similarly, the observed increase in catalase activity across all strains under ethanol stress is in agreement with previous studies [61]. The increase in glutathione peroxidase activity under ethanol stress is also in line with earlier research [62]. The elevated glutathione reductase activity observed in all strains upon exposure to ethanol stress is consistent with earlier reports [63]. Glutathione reductase regenerates reduced glutathione, which is required for the activity of glutathione peroxidase and other antioxidant enzymes [32]. The increased activity of glutathione reductase in yeast cells under ethanol stress might be a response to maintain the cellular redox balance and support the efficient functioning of other antioxidant enzymes.

The Hal5 overexpressed strain (Ep) consistently demonstrated an increase in antioxidant enzyme activity, although to a lesser extent compared to the wild type strain (924). Hal5 is a protein kinase involved in the regulation of monovalent cation transport and tolerance [64]. Overexpression of Hal5 has been previously reported, enhancing tolerance to various stresses, including salt and oxidative stress [65]. Therefore, the overexpression of Hal5 in strain Ep may contribute to a lesser antioxidant response due to less oxidative damage. This observation highlights the potential interplay between different stress response pathways and the antioxidant defense system in yeast.

Effects of ethanol stress on the yeast's lipid composition is essential for understanding the cellular response to ethanol stress. In particular, sterols play a critical role in maintaining the integrity and fluidity of the plasma membrane, which is crucial for the proper functioning of the cell [66]. In the presence of ethanol, the lipid composition of the cell membrane is altered, potentially affecting cell growth, viability, and stress resistance [67]. Upon exposure to 10 % ethanol stress, all the three strains of *Saccharomyces cerevisiae* showed differential responses in total sterols content. These results highlight the importance of sterols in the yeast's adaptation to ethanol stress, as increased sterols content may improve membrane stability and fluidity, counteracting the disruptive effects of ethanol on membrane properties [55,67]. The esterified and free sterols' ratio is another essential parameter in understanding the effect of ethanol on the lipid composition of yeast cells. You et al., (2003) observed a reduction in the esterified and free sterols' ratio in all three strains upon ethanol stress [67]. A decrease in this ratio implies that yeast cells tend to accumulate more free sterols under ethanol stress, which may enhance membrane fluidity and facilitate the proper functioning of membrane proteins, thereby improving stress resistance [55].

You et al., observed that exposure to ethanol resulted in increased levels of ergosterol, a major sterol component in the yeast plasma membrane [67]. Similarly, in the present study, we observed a significant increase in total sterols, esterified sterols, and free sterols content in all three strains upon ethanol stress. Furthermore, the increase in glycolipids and phospholipids content upon ethanol stress observed in our study is consistent with previous reports that supported changes in the lipid composition of yeast cells in response to ethanol stress [55]. It is important to note that the differential responses observed among the three strains of *Saccharomyces cerevisiae* suggest that the genetic background of the yeast strain plays a crucial role in determining the extent of changes in lipid composition upon ethanol stress. The wild type (924) strain demonstrated the most pronounced increase in total sterols, esterified sterols, and free sterols content after ethanol stress, followed by the Hal4,5 mutant (1300) strain, and finally the Hal5 overexpressed strain (Ep). This implies that the Hal5 overexpression might modulate intracellular potassium ion homeostasis, which is fundamental to several essential cellular processes together with stimulation of several enzymatic activities, stabilization of vital proteins, membrane potential and maintenance of cytosolic pH that makes this strain more resistant to the disruptive effects of ethanol on cellular membranes [68].

Furthermore, the differential responses to ethanol stress among different strains of *Saccharomyces cerevisiae* may be attributed to the genetic variations and regulatory mechanisms that govern lipid metabolism. For example, the overexpression of Hal5, a protein kinase involved in the regulation of ion homeostasis, may modulate lipid metabolism and thereby impact the strain's response to ethanol stress. Understanding the genetic basis of ethanol tolerance in yeast strains could potentially lead to the development of more robust strains for industrial bioethanol production.

Ethanol is slightly increasing the FTIR peak at 900–1200 cm^{-1} (trehalose) but it is sharply decreasing the peak at 1400–1500 cm^{-1} due to protein unfolding and degradation [37]. 2800–3000 cm^{-1} FTIR peak is for unsaturated lipids which is slightly increasing in response to ethanol for each type of yeast, but among different type of yeast there is big difference such that they make clusters. These results are also consistent with our biochemical estimation of trehalose and unsaturated fatty acids in different yeast strains. In FTIR, the Hal5 overexpression strain have less trehalose, but its ethanol tolerance is more due to increased stability of trk1, caused by more Hal5. The trehalose peak is more in Hal5 mutant, compared to Hal5 over-expression. Accordingly, the protein secondary structure peak is also more in Hal5 mutant, compared to Hal5 over-expression because trehalose stabilizes all proteins in general.

Over-expression of Hal5 increases ethanol tolerance of yeast by stabilizing directly the trk1 [69]. The stability of trk1 potassium pump will help in maintaining the electric potential across cell membranes by uptake of potassium ions [70]. Whereas the null mutant has increased synthesis of carbohydrate trehalose which will increase ethanol tolerance as trehalose is also a general protein stabilizer [71]. The deletion of Hal5 gene causes up regulation of trehalose biosynthesis [70]. Over-expression of Hal5 shall be better than Hal5 null mutant for industrial production of ethanol because null mutant makes less ethanol

because of diversion of metabolism towards carbohydrate synthesis [69]. The Hal5 over-expression strain shows more ethanol tolerance than the Hal5 null mutant, although both are more ethanol tolerant than the wild type strain. Expression of Hal5 prevents degradation of trk1 and increases ethanol tolerance, but trehalose also increases the stability of proteins and thereby increases ethanol tolerance. Hal5 mutant has decreased glucose uptake and increased respiratory gene expression [69]. Ethanol will be oxidized by the citric acid cycle in mitochondria and this mutant will be producing less ethanol, although it is ethanol tolerant. The over-expression of Hal5 may be better than the deletion of Hal5 for increasing ethanol tolerance of yeast strains for industrial ethanol production, although over expression and deletion both increase the ethanol tolerance of yeast.

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Compliance with ethical standards

This article does not contain any studies with animals or human participants performed by any of the authors.

CRediT authorship contribution statement

L. Singh: Investigation, Methodology, Writing – original draft. **J. Rai:** Data curation, Formal analysis, Writing – review & editing. **S.C. Sharma:** Conceptualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbadva.2024.100123.

Data availability

Data will be made available on request.

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