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A study on morphology and membrane ultrastructure of lipid mutant of *S. cerevisiae* under hypertonic conditions

Kanika Sachdeva
Dev Samaj College for Women, Ferozepur City, Punjab, India

Abstract
Saccharomyces cerevisiae is a characteristic eukaryote with membranous organelles, as well as the lipids that form its membranes. Since principles of osmoadaptation are conserved across eukaryotes, yeasts are ideal model systems with which to study the protective role played by membranes. A wild type strain of *S. cerevisiae* and its lipid mutant defective in the synthesis of phosphatidylcholine and phosphatidylethanolamine, have been used in this study. Cells were tested for viability with colony count method as well as through EM following salt treatment.
In wild type cells the cell surface is quite smooth. In comparison, the mutant cells show wrinkled surface. This shows that lipids have some important role in cell wall biogenesis also. Mutant cells seem to be more rounded as compared to the typical ovoid nature of wild type cells. Change in the shape may be due to the cytosolic disruption indirectly or directly due to phospholipid changes; because cytosolic proteins are known to be interacting with phospholipids especially negatively charged lipids. EM studies show that cell wall is more sensitive to salt in mutant cells. Not only plasma membrane but cell wall is also affected, leading to more lysis in the mutant strain.
The loss of viability of *S. cerevisiae* after hypertonic stress superimposes the fact that plasma membrane is the primary site of osmotic injury.

Keywords: salt stress, *S. cerevisiae*, phospholipids, osmotolerance

1. Introduction
Micro-organisms have evolved several mechanisms to cope with sudden unfavorable changes viz. temperature, pH, osmolarity, free radicals, lack of availability of nutrients, etc., encountered in their environment. In order to survive the aforesaid conditions, cells have to sense changes in their microniche, respond to them and act favourably by a process called inducible response [1, 2].
Similarly, there have been attempts to study the behavior of yeast in all of the above conditions. Yeasts are genetically well characterized, simple to handle, inexpensive to grow, complete a cycle in about 90 minutes and therefore yield quick results [3], thus making it a suitable eukaryotic model organism for biological research.
Talking about the yeast *S. cerevisiae* (name created for yeast strain observed in malt 1837), it generally strikes that this group most likely is the oldest cultivated creature. Yeast is a saprophytic organism that lives on decaying flora and fauna and metabolises organic molecules for survival [4].
Applications of yeast in recent years have gained importance from their uses in diverse industries such as bakery, brewing, nutraceuticals, pharmaceuticals and bioremediation [5, 6]. Genetically modified yeasts are used in the production of chemicals like isoprenoids, phenolics, alkaloids and polyketides [7]. Biopharmaceuticals like insulin, hepatitis vaccine and human serum albumin are produced using yeast [8]. Yeast are also important in the sense that they may cause a high level spoilage of canned or potted food material mainly because of their ability to grow in the presence of the preservatives present in food and brew. Some species are pathogenic to plants, animals and humans [9, 10]. In their natural habitats, yeasts are exposed to wide range of varying environmental conditions like nature of food available, heat, acidity, radioactivity, aerobics and osmolarity [11].

1.1 Membrane studies with respect to yeast
Membrane is the most important structure in the cell and is the foremost target organelle after interaction of the cell with its environment. Membranes are a large part of cell structure. It is because of this widespread recognition of the membranous nature of cells and the functional role of membranes associated with living state that an increasing amount of research is being devoted to membrane study. "Membranes are involved in ion accumulation or active transport, nerve impulse conduction, conversion of light into electrical or chemical energy, protein synthesis, phagocytosis and pinocytosis. Membranes also serve as permeability barrier of ions and molecules, site of immunological reactions, oxidative and photosynthetic phosphorylation" [12].
All biological membranes possess the common structural blueprint as projected by Davson and Danielli in 1952. The strongest investigational proof in this regard is provided by the EM studies of biological membranes and organelles which led to the unit membrane hypothesis by Robertson [13].
Saccharomyces cerevisiae is a rather characteristic eukaryote with reverence to its membranous organelles, as well as the lipids that form its membranes. Like other eukaryotic organisms, "it synthesizes and incorporates sterol into its

membranes". In addition, its membranes contain an asymmetric distribution of phospholipids, including sphingolipids, cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine, which is typical of eukaryotes [14, 15].

The pathways for the synthesis of yeast membrane lipids are increasingly well characterized. In addition a number of mutations affecting these pathways have been characterized and reliable methodologies for isolation of some subcellular membrane fractions are being developed. As result, *S. cerevisiae* is an attractive organism in which to conduct studies on the roles of specific lipids in membrane biogenesis and membrane-mediated processes [6].

Membrane is the site for quite a number of vital functions; and these are influenced by membrane fluidity [16]. Changes in membrane fluidity may cause membrane perturbations in the stress induced gene expression [17]. Modification in the lipid content of membranes of an organism is significant in response to environmental stress [18].

In the plasma membrane, the lipid composition plays a crucial role in controlling membrane physical properties. Changes in the lipid composition of plasma membrane may contribute to control of membrane packing properties and also be of importance concerning lipid protein interaction due to induced changes in lipid micro environment surrounding the protein, thereby affecting membrane function and its efficiency as a selective barrier.

Since *S. cerevisiae* is a eukaryotic organism, yeast lipid mutants can be used for probing the role of lipids in the biogenesis and functioning of variety of membranous organelles.

1.2 Salt stress in yeast

NaCl is a frequently used laboratory reagent that increases osmolarity of the medium. The osmotic responses elicited by NaCl are fundamentally the same as for other solutes at concentrations causing similar osmolarity [19, 20, 21].

Yeast cells have evolved ways to regulate, to some extent, the inside-directed driving force for water in environments of high external osmolarity [19]. Research inclined towards molecular mechanisms involved in yeast osmoadaptation from the industrial requirement to improve the economic yield from yeast strains, since industrial processes are often associated with sudden changes in water activity and especially with high solute concentration [22, 23, 24]. This driving force to improvise fermentation performance in winemaking still continues and recent studies by proved that salt "preconditioning" of yeast manifested higher capability to ferment high-sugar containing media with increased cell viability and with higher ethanol production compared to controls [25].

Other motivation to conduct salt studies in yeasts was the need to improvise food preservation methods, which need an enhanced insight of the interaction of yeast cells in hypertonic environment, complexed with other stress factors such as extremes of temperature, pH and chemical food preservatives [26]. Other studies have been carried out by Dhar et al to characterize evolutionary adaptation owing to salt stress in yeast [27]. Attempts have also been made to assess the effect of high concentrations of NaCl on glucose uptake in marine yeasts [28].

Eventually, it was concluded that the accumulation of chemically inert cellular protectants, such as glycerol, plays a central role in osmoadaptation [19, 29, 30, 31].

Further trends were set on studying intracellular glycerol and trehalose concentrations, using low-molecular-weight solutes to adjust intracellular water activity within limits of viability [31, 56, 32]. It was also observed that the principles underlying osmoadaptation are well conserved [27, 32] Thus, yeast Saccharomyces cerevisiae, emerged as an excellent model system with which to study the molecular biology and physiology of osmoadaptation [19]

1.3 Osmotolerance in yeast

Yeasts often face sudden changes in the water activity in vivo, e.g., when ripe fruits open up, or substrate starts drying up, they are subjected to hyperosmotic shock, along with water outflow along the concentration gradient by passive diffusion, increase in concentration of cellular contents and cell shrinking, eventually resulting in an arrest of cellular activity: a phenomena called high osmolarity or hyperosmotic stress.

On the other hand, cells adapted to high sugar levels on drying fruits or flowers may be washed away in rainfall into distilled water. Such a hypo-osmotic shock increases the water concentration gradient and leads to rapid influx of water, cell swelling, and hence increased turgor pressure. Within broad limits, the cell wall keeps the cell from bursting [33]. This variation in tolerance to salt stress is not clear at the molecular level [24].

Water flows out of the cell during a hypo osmotic shock. This increases the concentration of cellular contents. The higher concentration of some ions is supposed to act as a signal [34]. The binding of such a ligand (ion in this case) may cause a sensor protein to undergo a conformational change and thereby elicit a response. Such responses, that may alter metabolism, could be regulated via changed concentration of low-molecular-weight compounds in a withered cell.

For favourable biochemical activity, it is very important that osmolarity of cytoplasm is slightly higher than surrounding medium, i.e an optimum cell volume and and a "ratio of free to bound water" [19]. This leads water to constantly flow forcefully into the cell along through diffusion. Turgor pressure is exerted against this force because plasma membrane and cell wall in particular, have limited abilities to expand [32].

Osmoadaptation is an active process based on sensing of osmotic changes through osmosensors and appropriate signal transduction and translation aimed at maintaining biological activity. Adaptation may continue for many hours after a hyperosmotic shock [32]. Therefore, yeast cells can survive and flourish over a range of water activities. This range of osmotolerance varies according to species [32, 35]. Beyond the range of osmotolerance where cellular activity exists, yeasts have the ability to survive almost complete dehydration, a property that is used for the production of dry yeast cake [22]. Yeast cakes contain approximately 10% water.

A number of studies attempted to decipher the underlying molecular mechanisms for survival of a hyperosmotic shock and osmoadaptation had a common consensus: cells adapted to moderately high osmolarity survive a severe osmotic shock

better than nonadapted cells [36, 37, 38].

The discovery of highly conserved eukaryotic signal transduction pathway has paved way for much wider scientific interest in osmoadaptation. Present knowledge confirms that many principles of osmoadaptation are conserved across eukaryotes, and therefore yeasts are ideal model systems with which to study the underlying mechanisms. Osmoadaptation is part of cellular osmoregulation, which plays an important yet not fully appreciated role in cell growth and morphogenesis.

When yeast cells are shifted to high osmolarity medium, there is quick stimulation of a mitogen-activated protein (MAP) kinase cascade, the high-osmolarity glycerol (HOG) pathway, [39, 40] leading to production of cellular protectants, which is in sync with expression of other genes. Similar pathways have been discovered in other organisms [41, 34].

Osmoadaptation, regulation of cell surface properties, cellular morphogenesis, development, and multiplication are highly harmonized events. The role of Skn7p regulator is suspected in synchronising these events [19].

Since osmotic changes can be closely monitored and regulated, many researchers have preferred osmoadaptation as attempt to study principles of cell biology and molecular functioning. Hyperosmotic stress leads to a prominent expression of around 10% of the yeast genome [20, 42, 43, 21, 44].

The present investigation has been carried out on two strains of *S. cerevisiae* to understand their adaptation to hypertonic stress. Scanning Electron microscopy has been used as a tool to visualise this adaptation.

2. Methodology

The wild type strain of *S. cerevisiae* (6210) and its lipid mutant (RY200T) defective in the synthesis of phosphatidylcholine and phosphatidylethanolamine due to absence of the enzyme, serine decarboxylase have been used in this study. These were obtained from Prof. M. Opekavova, Institute of Microbiology, Prague, Czech Republic. Cultures were maintained on Yeast extract-Peptone-Dextrose (YPD) Agar slants at 4℃.

Tris base was purchased from Sisco Research Laboratories Pvt Ltd, Mumbai, India. Peptone, yeast extract and glucose were purchased from Hi-Media Lab, India. All other chemicals used in the study were of analytical grade and were procured from local commercial sources.

The YPD medium for growth of yeast cells contained:

Yeast Extract	1%w/v
Peptone	2%w/v
Dextrose	2%w/v
pH	5.5

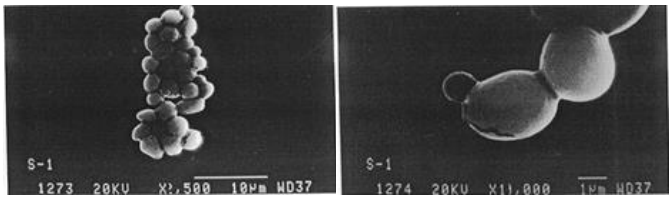
The cultures of *Saccharomyces cerevisiae* (6210 and RY200T) were maintained on slants of YPD medium, prepared as described above. Inoculum was prepared by transferring a single colony of cells from slant to 2 ml of YPD medium in 10ml tube and incubating it overnight at 30 degree at 200 rpm. The 25 microlitre of inoculum was transferred to 50 ml YPD in 250ml flask. Cells were grown till mid log phase (approx.16 hrs) at 200 rpm at 30℃. Cells were harvested by centrifugation at 2000 x g for 10 minutes and pellet was subsequently washed three times to remove adhering metabolites and unused ingredients of medium. The pellet was used for further analyses.

Cells of both strains were suspended in 1.2M NaCl under shaking conditions of 100 rpm for 30 minutes. Cells were tested for viability with colony count method as well as through EM. These were studied with respect to their controls.

3. Results and Discussion

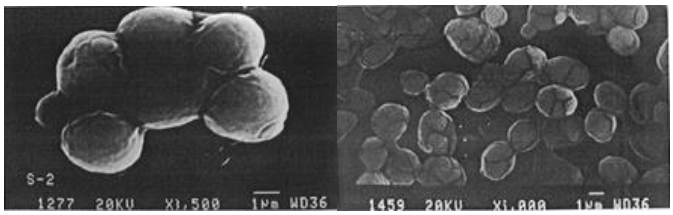
Electron microscopic observation of the mutant strain cells show that the bud separation from the mother cell might be delayed. This may be due to the altered lipid composition, suggesting that lipid composition might be playing some role in separating the bud from the mother cell (Fig 2(a) and (b).

Scanning electron micrographs of control cells



(a) (b)

Fig 1(a) and (b): Wild type strain of *S. cerevisiae*



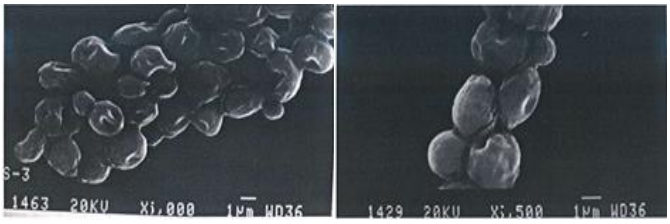
(a) (b)

Fig 2(a) and (b): Mutant strain of *S. cerevisiae* at different resolution

In wild type cells the cell surface is quite smooth. In comparison, the mutant cells show wrinkled surface. This shows that lipids have some important role in cell wall biogenesis also (Fig 1 and 2). Other studies on the structure of the mycobacterial cell wall and its lipids but also support this concept [39].

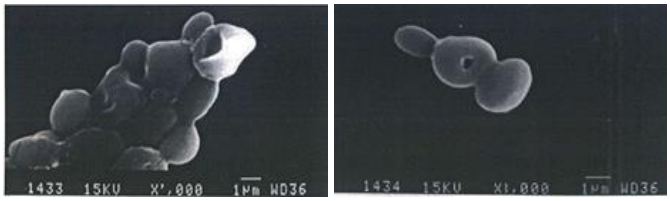
Mutant cells seem to be more rounded as compared to the typical ovoid nature of wild type cells. (Plates1and 2). Change in the shape may be due to the cytosolic disruption indirectly or directly due to phospholipid changes; because cytosolic proteins are known to be interacting with phospholipids especially negatively charged lipids [45].

Scanning electron micrographs of salt treated *S.cerevlsiae*



(a) (b)

Fig 3 (a) and (b): Wild type strain



(a) (b)

Fig 4 (a) and (b): Mutant strain, at different resolutions

EM studies show that cell wall is more sensitive to salt in mutant cells. Not only plasma membrane but cell wall is also affected, leading to more lysis in the mutant strain (Plates 3 and 4).

The loss of viability of *S. cerevisiae* after hypertonic stress was directly related to the reduction in cell volume in the shrunken state. This is in accordance with the well accepted fact that plasma membrane is the primary site of osmotic injury [28].

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