3.1 Materials

3.1.1 Organism and culture conditions

Yeast strain *Pachysolen tannophilus*, Y1038 (was 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 for 15-17 hours.

3.1.2 Chemicals

Anthrone, L- cysteine, Guanidine hydrochloride were obtained from SISCO Research Laboratories (SRL), Trehalose, and Yeast extract, Peptone, Dextrose, Agar YNB and α tocopherol were purchased from Hi-Media. DEM (Diethyl maleate), (DOP) Dioctyl phthalate, (DNPH) dinitrophenylhydrazine, Xylene and Methylene blue stain solution were purchased from MERCK. (DCFH-DA )2', 7'-dichlorofluorescein diacetate, DTNB (5, 5'-dithio-bis-2-nitrobenzoic acid) were purchased from Sigma-Aldrich Co., USA. L-Glutamic acid sodium salt was purchased from The British Drug House Ltd. England. Glycine, Chloroform was purchased from Qualigens fine chemicals. The analytical reagent ethanol (99.9%) was purchased from Changshu Yangyuan Chemical China. All routine chemicals used in present study were of analytical grade and were purchased from Hi-Media, SRL, Merck, Rankem and Qualigens.

3.1.3 Growth media

3.1.3.1 YPD medium

The YPD medium for growth of yeast cells contained 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose (pH5.5).

3.1.3.2 YNB medium

YNB (0.67%, w/v) in glass containers, containing 2.0% (w/v) glucose was used.
3.2 Experimental Design

3.2.1 Growth conditions and harvesting of cells

Inoculums was prepared by transferring cells of a single colony from YPDA plate in 10ml medium and grown at 30°C for overnight at 200 rpm in 50 ml conical flasks. The 100μl of inoculums was transferred to YPD 50ml liquid medium in 250ml flasks. Cells were grown till mid log phase (~17hr) at 200rpm at 30°C. Cells were harvested by centrifugation at 3000x g for 10minutes at 4°C. The pellet was subsequently washed two times with deionized water to remove adhering metabolites and unused ingredients of the medium. The pellet was stored at -20°C for further analysis.

3.2.2 Assay of cell resistance to ethanol

To observe the sensitivity of yeast to ethanol, the ethanol was added in YPD media that contained cells of exponential phase (approximately 1-2x10^7 cells ml^-1) to make final concentration 10% (v/v), in Erlenmeyer flasks. After incubation at 30°C and 100 rpm for various time intervals, fixed aliquots were withdrawn with autopipette.

3.2.3 Incorporation of Tocopherol into yeast cells

To enrich yeast cells with the tocopherol, cells were cultured overnight with the tocopherol 200μM (final conc.) dissolved in 1ml ethanol. The 5mM stock solution of the tocopherol was prepared by dissolving appropriated amount of tocopherol in ethanol.

3.2.4 Assay of glutathione enrichment and depletion

The glutathione enrichment was studies, as described by Izawa et al. (1995). For enrichment of glutathione content cells were incubated in a mixture containing 0.5 M glucose, 0.01 M MgCl₂, 0.02 M L-glutamic acid, 0.02 M L-cysteine, 0.02 M glycine and 0.1 M potassium phosphate buffer (pH 7.4) at 30°C for 1 h with shaking. For depletion of glutathione content the methodology of McNutt-Scott and Harris
(1998) was used. Briefly, cells were incubated with the 5mM DEM (Diethyl Maleate) at 28°C for 1 h with shaking. After the incubation, cells were treated with the ethanol.

3.2.5. **Modulation of membrane lipids**

The cells treated with the (DOP) dioctylphthalate (final conc. 30μM) in YNB media to increase unsaturated fatty acids in yeast membranes, as described by the Chattejee et al. (2001).

3.2.6 Yeast and stress treatment

To see the effect of various antioxidants and unsaturated membrane lipids on the ethanol induced yeast cells, the whole study was divided into three parts.

3.2.6.1 **Role of tocopherol on the ethanol induced stress**

In first part four groups were taken

1. Group first, was a control, without any treatment.

2. In second group, cells were treated with the ethanol stress (final conc. 10%).

3. In third group, cells were treated with ethanol (1% ethanol) to study the effects of vehicle.

4. In the fourth group, the cells were enriched with tocopherol and thereafter with ethanol stress (final conc. 10%)

   To enrich yeast cells with tocopherol, cells were cultured overnight in presence of tocopherol 200μM (final conc.).

   The cells from these four groups were taken at 0, 60 and 120 min intervals

3.2.6.2 **Role of Glutathione on the ethanol induced stress**

In the second part of the study, there were also four groups.

1. The first group was the control without any treatment.
2. In second group cells were exposed to ethanol stress (final conc. 10%).

3. In third group, glutathione depleted cells were exposed to ethanol stress (final conc. 10%).

4. In fourth group, the glutathione enriched cells were exposed ethanol stress (final conc. 10%).

The cells from these four groups were taken at 0, 60 and 120 min intervals.

3.2.6.3 Role of lipids on ethanol stress tolerance in yeast

In the third part of the study, we investigated three groups.

1. First group of the study was a control group.

2. In second group, cells exposed to ethanol stress (final conc. 10%).

3. In third group, cells with increased unsaturated fatty acids, exposed to ethanol stress (final conc. 10%).

The cells from all the groups were taken at 0, 60 and 120 min intervals.

3.3 Extraction and analysis of trehalose:

For determination of trehalose, mid log phase grown cells were harvested and final pellet boiled in 1.0 ml of distilled water for 10 min and centrifuged at 3,000x g for 10 min. The supernatant was used as sample for estimation of trehalose and pellet for protein. 200μl of saturated Na₂SO₄ and 750μl of absolute ethanol were added in the sample. The samples were centrifuged at 20,800x g for 8 min at room temperature (25±1°C). The supernatants were transferred to the glass tubes and dried at 80°C. After drying, samples were dissolved in 0.2 ml of ddH₂O and 0.2 ml of (0.2N) H₂SO₄, boiled for 10 min to hydrolyze any sucrose or glucose-1-phosphate. The solution was made alkaline by addition of 0.15 ml of 0.6 N NaOH and again heated at 100°C for 10 minutes to eliminate reducing sugars. In each chilled tube, 3ml of anthrone reagent (0.05mg anthrone per 100ml of 72% concentrated H₂SO₄) was added; the contents
were boiled for 10 min for colour development and chilled again. The absorbance measurement was made at 630 nm. The trehalose concentration was calculated using a standard curve.

3.3.1 Quantification of trehalose:

   The quantification of trehalose was done by the anthrone procedure (Morris, 1948) as modified by Jagdale and Grewal (2003).

3.3.2 Principle:

   The reaction is based on the formation of furfural derivatives upon treatment with concentrated sulphuric acid. Furfural derivatives react with anthrone to form a blue-green colour. The anthrone reaction is the basis of rapid and hexuronic acids, either free or present in polysaccharides.

3.3.3 Reagents

(i) Anthrone reagent (0.05mg per 100 ml of 72% conc. H$_2$SO$_4$)

(ii) Standard trehalose (0.2 mg/ml)

3.3.4 Procedure:

   An aliquot of 0.1 ml of combined supernatant was made 1.0 ml with distilled water. Added 3.0 ml of anthrone reagent and mixed rapidly. Placed the tubes in boiling water bath for 10 minutes covered with foil or glass beads to prevent loss of water by evaporation. The tubes were cooled and read the absorbance at 630 nm against blank containing 1.0 ml of water.

   Trehalose content was calculated from the standard curve prepared by taking trehalose in the range of 20-200 μg.
3.4 Estimation of glutathione:

A known quantity of the cells was homogenized in 10 volumes (w/v) of 10% chilled TCA to deproteinize the yeast samples. The homogenate was centrifuged at 10,000x g for 30 min and clear supernatant was used to estimate reduced as well as total glutathione.

3.4.1 Estimation of total glutathione

Total glutathione was estimated by the method of Habeeb (1972).

3.4.1.1 Principle

Total glutathione content of yeast cell homogenate was estimated after reduction of GSSG to GSH with Sodium borohydride.

3.4.1.2 Reagents

i) TCA 10% (w/v)
ii) 0.3M Disodium hydrogen phosphate

iii) DTNB (1mM) in 1% (w/v) potassium Citrate

iv) Sodium borohydride 1.6%(w/v) (fresh prepared in chilled water)

v) Potassium dihydrogen phosphate (1M) in 0.6N HCl

vi) 2.0M Tris Base

3.4.1.3 Procedure

0.5 ml of the sample was taken in a tube, added 0.5 ml of 0.3M disodium hydrogen phosphate and also 1.0ml of Sodium borohydride. The test tubes were incubated at 37°C for 30 min. After incubation, the excess of borohydride was destroyed by adding 1.0ml of Potassium dihydrogen phosphate (0.3M). The tubes were kept for 10 min at room temperature and 1 ml of Tris Base was added. After shaking the contents, 1ml of DTNB was added and the absorbance was measured at 412 nm against a blank prepared by taking 0.35ml of 2% TCA in place of the glutathione sample. Total glutathione content of yeast cells was calculated from the standard curve prepared by taking 25-125 µg of glutathione in 2% TCA.
3.4.2 Estimation of reduced glutathione

The methodology of Beutler et al. (1963) was followed for the estimation of reduced glutathione.

3.4.2.1 Principle

\[
\text{DTNB} + \text{GSH} \rightarrow \text{GS-TNB} + \text{TNB (yellow colour)}
\]

Thiol groups of two GSH molecules and sulfide group of DTNB react by exchange reaction and produce two molecules of 2-nitro 5 thiobenzoate anion, which has an intense yellow colour with a molar absorptive of \(13,600 \text{M}^{-1}\text{cm}^{-1}\) at 412 nm.

3.4.2.2 Reagents:

i) TCA 10% (w/V)

ii) (0.3M) Disodium Hydrogen Phosphate

iii) DTNB (1mM) in 1% (w/v) potassium Citrate
3.4.2.3 Procedure:

A suitable amount of sample (0.1 ml) containing approximately 25μg GSH was transferred to test tubes having 2 ml of 0.3M disodium hydrogen phosphate and the content were mixed well. Then 1.0 ml of reagent (iii) was added and the absorbance was measured at 412 nm against a blank prepared by taking 1% TCA in place of the glutathione sample.

The content of GSH in yeast cells was calculated from the standard curve prepared by taking 10- 60 μg of GSH in 1% TCA.

![Standard curve for reduced glutathione](image)

**Figure 3.3: Standard curve for reduced glutathione**

3.5 Extraction and estimation of glycerol

The mid log cells used for trehalose determination were harvested and final pellet boiled in 1.0 ml of distilled water for 10 min and centrifuged at 3,000x g for 10 min. The supernatant was used for the estimation of glycerol and the pellet was further processed for protein estimation.
3.5.1 Quantification of Glycerol

Quantification of Glycerol was done according to the method of Bok and Demain (1977).

3.5.2 Principle

Acidic periodate reacts with glycerol. The unreacted periodate is reduced to iodate or iodide by the action of L-rhammanose. The formaldehyde formed thus reacts with Nash reagent forming a yellow coloured product 3,4-Diacetyl-1-4 dihydrotoludine having absorption maximum at 412 nm with colour being proportional to concentration of glycerol content in the sample.

3.5.3 Reagents:

i) 15.0 gm. of ammonium acetate + 0.2 ml glacial acetic acid + 0.2 ml acetyl acetone made to total volume of 100 ml using double distilled water (Nash reagent)

ii) Sodium metaperiodate- 0.016 M in 0.12M HCl

iii) L- Rhammanose-0.1%

iv) Glycerol standard

All reagents were prepared freshly and used.

3.5.4 Procedure:

An aliquot of 0.1 ml was withdrawn; 1 ml of sodium metaperiodate solution was added to it after proper mixing of the solution it was allowed to stand for 10 min at room temperature. 2.0 ml of L-rhammanose solution was added to remove the excess of periodate followed by the addition of 4.0 ml of Nash reagent, the tubes were incubated at 52°C for 15 minutes. After cooling the tubes, O.D. was read at 412nm.

Glycerol content was calculated from the standard curve, prepared from glycerol.
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3.6 Lipid Peroxidation

Lipid peroxidation in the yeast cells was estimated by the method of Buege and Aust (1978).

3.6.1 Principle

Lipid peroxidation is a well-defined mechanism of cellular damage in animals and plants. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA), natural biproduct of lipid peroxidation.

These aldehydic secondary products of lipid peroxidation are generally accepted as markers of oxidative stress. Thiobarbituric Acid Reactive Substances (TBARS) is a well-established assay for screening and monitoring lipid peroxidation. MDA forms a 1:2 adduct with thiobarbituric acid (Figure 3.5). The MDA-TBA adduct

Figure 3.4: Standard curve for glycerol
formed from the reaction of MDA in samples with TBA can be measured colorimetrically at 532 nm.

3.6.2 Reagent

i) Stock TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid.

This solution was mildly heated to assist in the dissolution of the thiobarbituric acid.

3.6.3 Procedure:

0.5-ml samples of cell suspension were removed and added to 1 ml of TBA reagent (15% wt./vol. TCA and 0.375% wt./vol. TBA in 0.25 M HCl). Addition of the reagent terminated lipid peroxidation and initiated the assay. Samples were heated for 15 min in a boiling water bath and after cooling centrifuged at 10,000g for 5 min in order to remove cell debris. Absorbance of the supernatant at 535 nm were measured by spectrophotometer, against a reference solution comprising 1 ml of TBA reagent with the sample replaced by an equal volume (0.5 ml) of distilled deionized water. The concentrations of TBARS in samples were calculated by using molar extinction coefficient of MDA–thiobarbituric chromophore $1.56 \times 10^5 \text{M}^{-1} \text{cm}^{-1}$.

3.7 Protein Carbonyl Assay

For assessment of Protein carbonyls, the reaction with dinitrophenylhydrazine was employed as described by the Reznick and Packer (1994).

3.7.1 Principle

Protein carbonyl assay is based on the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein
carbonyls, forming a Schiff base to produce the corresponding hydrazone (Figure 3.6), which can be analyzed spectrophotometrically at 375 nm.

\[
\text{R} \quad \text{O} \quad \text{H}_2\text{NNH} \quad \text{NO}_2 \\
\text{Protein} \quad \text{O} \quad \text{H}_2\text{NNH} \quad \text{NO}_2 + \text{H}_2\text{O}
\]

Figure 3.6 Biochemistry of the Protein Carbonyl Assay

3.7.2 Reagents

i) 10 mM dinitrophenylhydrazine in 2.5 M HCl
ii) 20% TCA
iii) Absolute ethanol/ethyl acetate (1:1) solution
iv) 6 M guanidine hydrochloride

3.7.3 Procedure:

For each determination, samples containing 2–10 mg ml\(^{-1}\) of protein were treated with 4 ml of 10 mM dinitrophenylhydrazine in 2.5 M HCl for 1 h at room temperature. One tube, used as the blank, was incubated only with 2.5 M HCl. The reaction was stopped by addition of 5 ml of 20% TCA. The pellets were washed twice with 3 ml of absolute ethanol/ethyl acetate (1:1) solution. The protein pellets were finally dissolved in 6 M guanidine hydrochloride and the absorption at 375 nm (dinitrophenylhydrazine minus sample blank) was determined. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22,000 M\(^{-1}\) cm\(^{-1}\) and expressed as nmol carbonyl/mg of protein.
3.8 Quantification of intracellular oxidative stress level

Intracellular ROS was detected by the oxidant-sensitive probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA) as described by the Davidson et al. (1996).

3.8.1 Principle

The assay employs the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2', 7'-Dichlorodihydrofluorescein (DCF) by ROS (Figure 3.7). The fluorescence intensity is proportional to the ROS levels within the cell cytosol, which can be analyzed by Spectrofluorophotometer with excitation at 502 nm and emission at 521 nm.

![Figure 3.7 The breakdown of DCFH-DA. (Courtesy of Bland et al. 2001)](image-url)
3.8.2 Reagents

i) 5 mM stock solution of 2', 7'-dichlorofluorescein diacetate (DCFH-DA)

3.8.3 Procedure:

2', 7'-dichlorofluorescein diacetate (DCFH-DA) was added from a fresh made 5 mM stock (prepared in ethanol) to a final concentration of 10 μM in 1 ml of yeast cell suspension (10^7 cells), 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 μL of water and 1.5 g of glass beads were added. Cells were lysed by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice. The supernatant was obtained after centrifugation at 25,000 g for 5 min and after appropriate dilution with water; the fluorescence was measured using a Shimadzu Spectrofluorophotometer (RF-5301PC) with excitation at 502 nm and emission at 521 nm.

ROS level was calculated by comparing the cells with the control cells. The calculation was done by assuming the normal fluorescence of the control cells one or zero.

3.9 Antioxidant enzymes assays

3.9.1 Preparation of cell lysate

For preparing yeast cell lysate for antioxidant enzymes assays, glass beads (0.5 mm) were used as described by the Favre et al. (2008). The yeast cells were harvested by centrifugation at 3,000 x g and resuspended in 1 ml of lysis buffer (50 mM of Tris-HCl, 150 mM of sodium chloride, and 50 mM of EDTA at pH 7.2). To disrupt cells, 1/2 volume of ice-chilled glass beads (0.5 mm) were added and cells were lysed by three cycles of 1 min agitation on a vortex mixer followed by 1 min on ice bath. Cellular debris was removed by centrifugation at 15,000 x for 10 min and supernatant was collected for the assay of antioxidant enzymes.
3.9.2 Superoxide dismutase [EC: 1.15.1.1] assay

SOD activity was assayed in cell lysate supernatant according to the method of Kono (1978).

3.9.2.1 Principle

Generation of superoxide anions by the autoxidation of hydroxylamine hydrochloride reduce nitroblue tetrazolium to form blue fomazan which has absorption maxima at 560 nm. SOD inhibits the reduction of NBT, which is taken as a measure of enzyme activity.

3.9.2.2 Reagents

i) Solution A: 50 mM Sodium carbonate in 0.1 mM EDTA (pH 10.8).
ii) Solution B: 96 μM Nitroblue tetrazolium in solution A.
iii) Solution C: 0.6% (v/v) Triton X-100 in solution A.
iv) Solution D: 20 mM Hydroxylamine hydrochloride (pH 5.0)

3.9.2.3 Procedure

The reaction mixture consisted of 50 mM sodium carbonate in 0.1 mM EDTA (pH 10.8), 96 μM NBT, 0.6% Triton X-100, requisite cell lysate supernatant and 20 mM hydroxylamine hydrochloride. The reference contained all the above except cell lysate. Absorbance was recorded at 560 nm for 3 minutes. The extent of inhibition was taken as a measure of enzyme activity.

The enzyme activity was expressed as units/mg protein, where one unit of enzyme activity is defined as the amount of enzyme inhibiting the rate of reaction by 50%

3.9.3 Catalase [EC: 1.11.1.6] assay

Catalase activity was assayed in the cell lysate following the method of Luck (1971).
3.9.3.1 Principle

The decomposition of hydrogen peroxide by catalase is monitored spectrophotometrically by following the decrease in absorbance at 240 nm.

3.9.3.2 Reagents

i) 0.067 M Phosphate buffer (pH 7.0)

ii) 12.5 mM H$_2$O$_2$ in 0.067 M phosphate buffer (pH 7.0)

3.9.3.3 Procedure

Appropriate amount of cell lysate was added to 12.5 mM H$_2$O$_2$ in 0.067M phosphate buffer (pH 7.0). The decrease in absorbance was followed at 240 nm for 3 minutes.

Results were expressed as µmoles of H$_2$O$_2$ decomposed/min/mg protein using molar extinction coefficient of H$_2$O$_2$ (71 M$^{-1}$ cm$^{-1}$)

3.9.4 Glutathione peroxidise [EC: 1.11.1.9] assay

Glutathione peroxidise activity was assayed in the cell lysate by the method of Pagalia and Valentine (1967).

3.9.4.1 Principle

Glutathione peroxidase catalyzes the reduction of H$_2$O$_2$ at the expense of oxidation of GSH to GSSG. GSH is maintained at constant concentration by the addition of exogenous glutathione reductase and NADPH, where oxidized GSSG is converted to GSH. The rate of GSSG formation is measured by following the absorbance of NADPH at 340 nm.

\[
\text{Glutathione Peroxidase}:
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}
\]

\[
\text{Glutathione Reductase}:
\text{GSSG} + \beta-\text{NADPH} \rightarrow \beta-\text{NADP} + 2 \text{GSH}
\]

Abbreviations used:

GSH = Glutathione, Reduced Form

GSSG = Glutathione, Oxidized Form

\[\beta\text{-NADPH} = \beta\text{-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form}\]

\[\beta\text{-NADP} = \beta\text{-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form}\]
3.9.4.2 Reagents

i) 50 mM Phosphate buffer containing 1.5 mM EDTA (pH 7.0)
ii) 30 mM Sodium azide
iii) 6mM NADPH (tetra sodium salt)
iv) 30 mM GSH
v) Glutathione reductase
vi) 2.2 mM Hydrogen peroxide

3.9.4.3 Procedure

The reaction mixture consisted of 50 mM phosphate buffer (containing 1.5 mM EDTA, pH 7.0), 30mM sodium azide, 6mM NADPH, 60 mM GSH, glutathione reductase (1 unit) and an appropriate amount of cell lysate. The contents were allowed to equilibrate at 20°C for 2 minutes. The reaction was initiated by addition of 2.2 mM H₂O₂ and the decrease in absorbance was followed at 340 nm for 3 minutes.

Results were expressed as nmoles NADPH oxidized/min/mg protein, using molar extinction coefficient of NADPH (6.22 × 10⁶ M⁻¹ cm⁻¹).

3.10 Trehalase [EC: 3.2.1.28] assay

Trehalase activities were assayed by measuring D-glucose liberated from the trehalose, using a glucose-oxidase-peroxidase system kit. The method used was a modification of that described by Dahlqvist (1964).

3.10.1 Reagents

i) 0.2M Phosphate Buffer (pH=7.0)
ii) Trehalose solution (0.1 M)
iii) Standard glucose solution
iv) Glucose-oxidase-peroxidase reagent
3.10.2 Procedure

To 0.2 ml substrate (trehalose), added 0.25 ml sample and incubated for 30 min at 37 °C. Added 2.5 ml glucostat (glucose-oxidase-peroxidase) reagent to each tube and incubated at 37 °C for 1 hour. Appropriate blank and standard glucose samples were run simultaneously. Absorbance was measured at 500 nm.

3.11 Estimation of Vitamin E

The vitamin E was determined by reaction of vitamin E with TPTZ (2,4,6-tripyridyl-s-triazine) according to the method of Martinek (1964).

3.11.1 Principle

2,4,6-tripyridyl-s-triazine (TPTZ) is a colour reagent. In oxidimetric colour reaction when TPTZ reacts with the tocopherol, release ferrous iron. This ferrous iron is used as an index of the concentration of the vitamin in sample.

3.11.2 Reagents

i) Standard α-tocopherol- 1mg/100ml

ii) TPTZ, 0.12% (w/v) - Dissolve 0.12 gm of 2,4,6-tripyridyl-s-triazine in, and dilute to 100 ml with, n-propanol. This solution is stable at room temperature. If a precipitate forms, add 0.1 ml of concentrated HCl per 100 ml of reagent.

iii) Ferric chloride, 0.12% (w/v) Dissolve 0.12 gm FeCl₃ in

Ferric chloride, 0.12% (w/v) Dissolve 0.12 gm of FeCl₃·6H₂O in, and dilute to 100 ml with, absolute ethanol. This solution is stable at room temperature.

3.11.3 Procedure

Adequate amount of sample was taken and made final volume 2 ml by admixing absolute ethanol. 500µl of Xylene was added followed by 500µl TPTZ reagent. After mixing 100µl of ferric chloride solution was added and O.D. at 600 nm was read. Tocopherol content was calculated from the standard curve of α-tocopherol and the amount of tocopherol was expressed in µg/g. dry cell weight.
3.12 Extraction of total lipids

Total lipids from yeast cells were extracted according to the method of Folch et al. (1957).

3.12.1 Reagents

i) Chloroform
ii) Methanol
iii) Mixture of chloroform and Methanol (2:1)
iv) Normal saline (0.9%)

3.12.2 Procedure

The method of Folch et al. (1957) was used with some modification for the extraction of total lipids. A known amount of the cells was homogenized using glass beads. The contents were transferred to an air tight glass stoppard 250 ml Erlenmeyer flask containing 20 ml of chloroform: methanol (2:1v/v) for each gram of material. The contents were shaken on rotary shaker for 4-5 hrs at a speed of 250 rpm and
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filtered through a sintered glass funnel (G-3 type 4.3). The residue was extracted twice with 50 ml of chloroform: methanol mixture and filtered. The filtrates were pooled and the excess solvent was evaporated under vacuum using flask evaporator then lipids were again dissolved in 2.5 ml of chloroform: methanol (2:1 v/v) per gram of material. To remove water soluble impurities, the crude lipids obtained above were mixed with normal saline (5:1 v/v). The contents were transferred to a separatory funnel, shaken vigorously and allowed to stand till the two distinct layers were formed. Free sugars, amino acids and other water soluble impurities got mixed in the upper aqueous layer. Lower chloroform layer containing lipids were separated and the upper layer was again treated with 10 ml of chloroform to remove the residual lipids. This was repeated three times for the complete recovery of lipids. The chloroform extracts were pooled and 5 gm of anhydrous sodium sulphate was added to remove the traces of moisture. The solvent was distilled off under vacuum at a temperature below 60°C and pure lipids obtained were dissolved in chloroform and stored at -4°C until used.

3.13 Estimation of phospholipids

Phospholipids were estimated by the method of Bartlett (1959) as modified by Marinetti (1962). The organic phosphorous is converted to inorganic phosphorous by digesting with perchloric acid. Total phospholipids were obtained by estimating inorganic phosphate and multiplying it with a factor of 25 (a factor calculated taking into consideration an average molecular weight of phospholipids as 775). The factor 25 was obtained by dividing 775 with 31, the mass number of phosphorous.

3.13.1 Reagents

i) Standard phosphorous

a) Stock solution (1mg/ml) was prepared by dissolving 439 mg of KH$_2$PO$_4$ in 100ml DDW.

b) Working standard was prepared by diluting 0.5 ml stock solution to 50 ml with DDW. The final concentration of standard would be 10 µg/ml.

ii) ANSA reagent
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a) Prepare by dissolving the following compounds in DDW and making the volume to 100 ml
   a. 1 - amino 2,4-napthol sulfonic acid: 200mg
   b. Sodium metasulphite : 12 gm
   c. Sodium sulfite(anhydrous) : 1.2 gm

iii) Ammonium Molybdate (2.5%)

   2.5 g ammonium molybdate was dissolved in 0.1 N H2SO4.

iv) Perchloric acid (70%)

3.13.2 Procedure

0.1ml of lipid extract was evaporated to dryness and digested with 1ml of 70% perchloric acid over a sand bath. Total volume of acid hydrolysate was made to 4ml with DDW. 0.5ml of ammonium molybdate and 0.2ml of ANSA reagent was added. Mixed well and kept in boiling water bath for 7 min OD measured at 830nm. The phosphorous content of the sample was calculated from the value of standard which run simultaneously. The amount of phospholipids was determined by multiplying the amount of P by a factor of 25.

![Absorbance at 830nm vs Phosphate(µg)](image)

Figure 3.9: Standard curve for phospholipid using potassium dihydrogen phosphate as standard (Marinetti, 1962)
3.14 Estimation of total sterol

Sterol was estimated according to the method of Zlatkis et al. (1953). Sterol in presence of concentrated sulphuric acid and glacial acetic acid forms a violet coloured complex with ferric chloride. The reaction involves initial dehydrogenation of sterol to 3, 5-diene or 2, 4- diene which polymerizes to dimer or trimer. The polymers react with FeCl$_3$-H$_2$SO$_4$ mixture to form a coloured complex, which is measured calorimetrically at 540nm.

3.14.1 Reagents

i) Glacial acetic acid

ii) Conc. H$_2$SO$_4$

iii) Ferric chloride (5%/w/v) 5g of hydrated ferric chloride was dissolved in 100ml glacial acetic acid.

iv) Colouring reagent 1 ml of ferric chloride was diluted to 100 ml with concentrated sulphuric acid.

v) Standard cholesterol (1mg/ml) dissolved 10 mg of cholesterol in 10 ml of glacial acetic acid

3.14.2 Procedure

Evaporated 0.1ml of lipid extract and added 3.0 ml of glacial acetic acid, 2.0 ml of working FeCl$_3$ and mixed well. The tubes were kept in dark for 30 min and OD read at 540nm.
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3.15 Estimation of Free sterol

Free sterol was estimated by the method of Courchaine et al. (1959).

3.15.1 Reagents

i) Acetone -95% ethanol mixture (1: v/v)

ii) Digitonin (1% in 50% ethanol) – 1 gm. digitonin was dissolved in 50% ethanol

iii) Acetone

iv) Ferric chloride (5%) – 5 gm. of ferric chloride was dissolved in 100 ml glacial acetic acid.

v) Coloring reagent - 1 ml of ferric chloride was diluted to 100 ml with concentrated sulphuric acid.
3.15.2 Procedure

1 ml of lipid extract was evaporated to dryness. To this, 1 ml of acetone - ethanol mixture and 1 ml of digitonin were added and contents were thoroughly mixed. After 10 min, the tubes were centrifuged at 3000g for 5 min. The residues was dissolved in 4 ml of acetone and centrifuged at 3000g for 5 min. After centrifugation, the residue was allowed to drain for 5 min and then re dissolved the residue in 5 ml of glacial acetic acid. To each tube, 2 ml of working FeCl₃ working reagent was added. Mixed well and OD was measured at 540nm after keeping the tubes for 30 min in the dark.

3.16 Esterified sterol estimation

Esterified sterols were estimated after fixation of free sterol by the digitonin. To 1 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 ml of petroleum ether. Tubes were heated at 60-70°C till half of the solvent gets evaporated. Tubes were cooled at 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 sterol esters were estimated using the same procedure as described above for the determination of total sterol.

3.17 Estimation of glycolipids

Glycolipids were estimated on the basis of their sugar content. The Dubois et al. (1956) method was used for the determination of sugar content of lipid sample. Monosaccharides get dehydrated to furfurals by concentrated H₂SO₄. Furfurals condense with phenols to form coloured complex having λ at 490 nm.

3.17.1 Reagents

i) Phenol solution (5%)

ii) Conc. H₂SO₄
iii) Standard Sugar solution (2.2 mM) Dissolve 10 mg of glucose in 250 ml of water. Diluted this solution 1:10 before use

3.17.2 Procedure

Appropriate aliquots of lipid solution were evaporated to dryness; to this residue, added 2 ml of water and mixed on a vortex mixture. 1 ml of 5% phenol solution was added and mixed thoroughly then added 5 ml of concentrated sulphuric acid and mixed. This was allowed to cool for 30 minutes and absorbance was recorded at 490 nm against a reagent blank. For calibration, standard containing 20, 40 and 80 µg of glucose were run simultaneously.

![Absorbance at 490nm vs Glucose (µg) graph](image)

Figure 3.11: Standard curve for glycolipids using glucose (Dubois et al., 1956)

3.18 Extraction of protein

The proteins precipitated by the 10% TCA were washed with acetone two time and centrifuged at 3,000x g for 5 minutes. The dried proteins were dissolved in 0.5ml / 1.0ml of 0.1 N NaOH and boiled for 10 minutes. The contents were centrifuged at 5,000xg for 10 minutes. The proteins were estimated in supernatant by Lowry et al. (1957) method.
Materials and Methods

3.18.1 Estimation of protein:

Protein was estimated by the method of Lowry et al. (1957) using Bovine Serum Albumin as a reference standard.

3.18.2 Principle

The basis of the reaction is the presence of aromatic amino acids (tyrosine, tryptophan and phenylalanine). Proteins react with Folin-Ciocolteau reagent to form colored complex. The color produced is due to a combination of Biuret reaction and reduction of phosphomolybdate by aromatic amino acids. The intensity of the color produced depends upon the amount of aromatic amino acids present and varies for different proteins.

3.18.3 Reagents

i) Bovine Serum Albumin (0.2 mg/ml)
ii) Folin-Ciocolteau reagent (1:1) dilution with ddH₂O
iii) Na₂CO₃ (2%) in 0.1 N NaOH
iv) 1% CuSO₄ solution
v) 2% Sodium potassium tartarate

Lowry’s reagent: It was prepared by mixing 1 ml copper sulphate solution (1 %), 1 ml sodium potassium tartarate solution (2 %) and 48 ml sodium carbonate solution (2 %) to make 50 ml Lowry’s reagent.

3.18.4 Procedure:

An appropriate volume of samples and BSA standard were taken in respective tubes and made volume to 1.0 ml with distilled water. Added 3.0 ml of Alkaline or Lowry’s reagent in all tubes and placed at 37°C for 10 minutes. This was followed by addition of 0.3 ml Folin-Ciocolteau reagent. The contents were vortexed and allowed to stand for 30 min at 37°C. Appropriate blank and standard samples were run simultaneously. Absorbance was measured at 680 nm.
The content of protein was calculated from standard curve prepared by taking 40-200 μg of BSA (0.2 mg/ml).

![Graph showing standard curve for protein estimation using Bovine serum albumin (Lowry et al., 1957)](image)

**Figure 3.12: Standard curve for protein estimation using Bovine serum albumin (Lowry et al., 1957)**

### 3.19 FTIR (Fourier Transform Infrared Spectrometry)

FTIR was done after lyophilization of yeast cells.

#### 3.19.1 Principle

FTIR spectroscopy works by shining infrared radiation on a sample and seeing which wavelength of radiation in the infrared region of the spectrum are absorbed by the sample. Each compound has a characteristic set of absorption bands in its infrared spectrum. Variation in stretching and bending modes of vibration with single functional group is normally coupled with the vibration of adjacent group as well as with the number of substitutions taking place on the molecule itself. This leads to the shifting and overlapping of the peaks of two or more functional groups in the same region of the IR spectrum.
3.19.2 Procedure:

Yeast cultures were harvested by centrifugation at 5000 rpm for 10 min. The pellet was washed thrice with distilled water and lyophilized. After lyophilization, the dried biomass was collected by gentle scrapping and powdered for FTIR analysis. KBr discs were prepared by mixing biomass with KBr in the ratio of 1:100 in pestle. The homogenized mixture loaded onto a KBr, pressure of 10 ton was applied to obtain a uniform transparent film. FTIR spectra were recorded on an ABB FTIR spectrometer (FTLA-2000, ABB Bomem Inc., Quebec, Canada) with resolution 4 cm⁻¹. The absorption spectrum between 900 and 3000 cm⁻¹ was measured by co-adding 50 scans and subtracting the background. Before data analysis, the quality of each spectrum was assessed by the FTSW100 Process Software version 2.01. The spectra were collected from the culture in triplicate and tentative assignment of the FTIR bands was done according to important FTIR signatures.

3.20 SEM (Scanning electron microscopy)

Scanning electron microscopy was performed after fixation of samples in glutaraldehyde as described by the Lee et al. (2006).

3.20.1 Reagents

i) 2.5% glutaraldehyde solution prepared in phosphate buffer saline (0.1M, pH 7.2)

ii) Phosphate buffer saline

iii) Ethanol

3.20.2 Procedure:

Yeast cells washed thrice with phosphate buffer saline than fixation was done by incubating cells in 2.5% glutaraldehyde solution for 1 hour at 22°C. After incubation, cells were rinsed 5-6 times in phosphate buffer saline. At final step gradual dehydration was carried out by washing with ethanol gradient solutions from 50-90%. Final washings were given with 100% ethanol.
In SEM centre

Cell pellet was lyophilized and mounted on a stub with silver tape. The stub was sputter coated with gold using fine coat, Jeol ion sputter, model JFC-1100. This gold coated stub was finally examined at different magnifications under the scanning electron microscope.

3.21 Cell viability study

Viability of the cells was determined by colony counting after spreading appropriate dilutions in duplicate on YPD-agar, following by incubation at 30°C for 3 days. The percent viability was calculated with respect to cells grown in control samples.