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ORIGINAL ARTICLE

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Role of Heat Shock Proteins and Plasma Membrane on Thermotolerance in *Saccharomyces cerevisiae*-VS$_3$ Strain

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Aim: Study of HSPs synthesis after heat and cold shock and explanation of thermotolerance by the transport of HSPs to the plasma membrane.
Methods and Results: Physical (cold and heat shock) and chemical (lignocaine) damage to plasma membrane was achieved in thermotolerant and mesophilic strains of *Saccharomyces cerevisiae*. In shocked yeasts K^+ ion efflux, leakage of UV_{280} absorbing material, HSP expression profile and viability at 25 and 45°C were studied. Physical/chemical shock was given for 30 minutes and subsequently yeasts were incubated at 25°C to avoid further membrane damage by stress. In thermotolerant strain, membrane damage increased up to 70 minutes (30 min of shock and 40 min at 25°C) and reduced thereafter. *De-novo* HSPs in membrane were noted at 60 minutes and reached maximum at 80 minutes in thermotolerant strain. In mesophilic yeast, *de-novo* HSPs were not synthesized and leakage was continuous up to the studied period (100 minutes).
Conclusion: These *de-novo* HSPs are transported to the membrane for restoring the membrane integrity and to prevent the leakage. The thermotolerant strain can grow at higher temperatures compared to mesophilic strain due to more production of HSPs and HSP associated membrane damage reversal.
Significance and Impact of the Study: Several reports established the role of HSPs in thermotolerance but their mode of action is not well characterized. The current method explains the mechanism for acquiring thermotolerance in yeast.

Key words: *Saccharomyces cerevisiae, VS3, Heat shock proteins, HSP 104, Thermotolerance, Cold shock*

Severe heat stress causes protein denaturation in various cellular compartments. This necessitates conformational repair of vital proteins for survival because gene expression is transiently blocked after thermal insult (Mühlhofer *et al*., 2019; Hanninen *et al.,* 1999).

Yeast that grows at 40°C and above is considered as thermo tolerant (Sree *et al.,* 2000). Fermentation using thermotolerant yeasts at such temperatures also result in faster fermentation rates, cuts the over all fermentation and cooling costs, so that ethanol can be made at cheaper rates (Prado *et al.,* 2020). There are very few reports on the selection of yeasts that are able to grow and ferment at higher temperatures (Banat *et al.,* 2000). The *Saccharomyces cerevisiae* is an invaluable model for research of regulatory features of stress response (Le Breton and Mayer, 2016). In addition, budding yeast has been an outstanding model organism to elucidate the role of chaperones and correlating their functions (Verghese *et al.,* 2012).

A substantial number of genes undergo repression after stress, including many genes associated with growth and cell division, such as actin, alpha and beta tubulin whereas stress induces more HSPs (Trinklein *et al.,* 2004). The classical view of the HS response is that, stressing agents cause the accumulation of denatured proteins in the cell with a concomitant induction of the genes responsible for HSPs synthesis (Parsell and Lindquist, 1993; Kumar *et al.,* 2020). Furthermore the temperature sensing mechanism is thought to be intimately associated with membrane structure and function (Chatterjee *et al.,* 1997). The signals leading to HSF (Heat Shock Factor) activation and HSP synthesis i.e., the cellular thermometer is still a matter of debate (Castells-Roca *et al.,* 2011). Among the proposed hypotheses are the presence of abnormal, unfolded or misfolded proteins, alteration in physical state of cell membrane, second messenger induction and generation of reactive oxygen species (ROS) have been implicated in this process (Polla *et al.,* 1997). Lignocaine (a local anaesthetic & membrane fluidizer) depresses the membrane lipid phase transition (Mizogami *et al.,* 2002) and fluidize the lecithin membrane by unsaturable nonspecific binding (Ueda *et al.,* 1977).

Studies have shown that an efflux of potassium ions is a first indication of membrane damage (Fujita, 2002; Heipeiper *et al.* 1996). Potassium ion is a major cytoplasmic cation in growing yeast cells, and is involved in several key functions (Fujita and Kubo, 2002). Proton influx (Sikkema *et al.,* 1995) and UV_{280} absorbing material leakage also follows K^+ ion leakage. In addition to leakage of ions upon damage to cell membrane several other components are excreted.

In the present study, thermotolerant and mesophilic strains of *Saccharomyces cerevisiae* were used to investigate the effect of temperature (cold and heat shock) and chemical (lignocaine) shocks on HSP expression profile (by western blotting and autoradiography) and the damage, caused if any to the yeast plasma membrane (by efflux of potassium ions and UV_{280} absorbing material). HSP expression studies and cell viability assays were used to understand the mechanism of thermotolerance.

MATERIALS AND METHODS

Microorganism

Saccharomyces cerevisiae - VS_3 was isolated from soil samples collected from hot regions near the Kothagudem Thermal Power Plant located in Khammam District, A.P, India. The organism was isolated, mutated by UV and identified as *Saccharomyces cerevisiae -VS_3* strain in our lab (Sree *et al.,* 2000). It was maintained on Yeast extract Peptone Dextrose agar medium (YEPD) (1% Yeast Extract, 2% Peptone, 2% Glucose, 2% Agar-Agar, pH 6.0). Mesophilic yeast *Saccharomyces cerevisiae* MTCC 3205 was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India, grown and maintained as described above at 25°C.

Anti-Hsp 104 & anti Hsp 30 polyclonal antibodies and anti-Hsp 90, anti-Hsp 70, anti Hsp 60 & Anti-Beta Actin monoclonal antibodies & horse radish peroxidase conjugated secondary antibodies were obtained from Sigma Chemicals. Different chemicals used were obtained from Himedia and Sigma chemicals.

Radiolabelled methionine was obtained from BARC (Baba Atomic Research Center) Mumbai, India.

Membrane damage, HSP profile and viability of thermotolerant and mesophilic strains of Saccharomyces cerevisiae following temperature stress

Preliminary experiments were carried out to determine whether the two yeast strains could withstand severe cold shock (-20^{0}C) or heat shock (45^{0}C) for 30 minutes. For this purpose thermotolerant and mesophilic yeast strains were grown in YEPD broth medium of pH 6.0 for 48 hours at 150-rpm and 25°C and cultures of OD 0.3 were aliquoted, treated with lignocaine (0.1065 mg ml^{-1}) and then subjected to the above temperatures for 30 min (since longer time periods count high mortality). After heat/cold stress, yeast was incubated at 25 ^{o}C. Cells treated in similar fashion in the absence of lignocaine served as controls and cells grown at 25°C served as control for shocked cells. The samples were aliquoted at 0 minutes to 70 minutes of incubation at 25 ^{o}C after heat/cold shock and analyzed for K^+ ion efflux, UV_{280} absorbing material leakage, viability and membrane HSP profile. HSPs were detected both by autoradiography and western blot analysis.

Optimization of heat and cold shock temperature effect on membrane damage, HSP profile and viability

In an attempt to determine the temperatures at which cold and heat shock would be maximum, the thermotolerant yeast was subjected to temperatures ranging from -20° to 50° C for 30 minutes in the presence and absence of lignocaine (0.1065 mg ml^{-1}). Subsequently the cultures were shifted to 25° C and after 70 minutes the cells were analyzed for K^+ ion efflux, UV_{280} absorbing material leakage, viability and membrane HSP profile.

Cell viability assay

The effect of heat shock on yeast survival rate was studied by the plate colony count method. Yeast was given heat shock as described above and, diluted sample (0.1 ml of 10^{-4}) was plated on YEPD agar plates in triplicates. After 2 days of incubation at 25 and 45°C, colony-forming units were recorded.

[^{35}S] Methionine labelling, protein extraction, and electrophoresis

Thermotolerant yeast culture (10 ml) was washed and resuspended in 2 ml of YNB medium (0.67% yeast nitrogen base, 0.3% KH_2PO_4, 2% glucose, pH 6.0) without amino acids. After subjecting cells to thermal stress, 10 mCi of [^{35}S] methionine (specific activity, 1,150 Ci mM) was added to control and shocked samples and the preparations were incubated at 25°C temperatures for 70 minutes. Cells were pelleted and protein was extracted by cell disruption in an ultrasonicator for detection of total cell HSPs.

In thermotolerant and mesophilic yeast, after stress induction and [^{35}S] methionine labelling, samples were collected at 0, 10, 20, 30, 40, 50,60 and70 minute intervals from the set incubated at 25°C for detection of membrane HSPs. Protein concentrations of cell lysate and membrane were determined using Lowry method (Lowry *et al.,* 1951). Fifty mg of extracted protein sample was resolved using 10 % SDS-PAGE. The gels were stained with Coomassie blue, dried and exposed to Hyper- film-MP (Amersham) at -70°C for 5 days before being developed.

Yeast plasma membrane isolation

Yeast plasma membrane isolation was carried out according to Santos *et al.* (1978). The membrane pellet equivalent to 50 mg of protein was resolved by electrophoresis and the gels were processed for autoradiography according to Sambrook *et al.* (1989).

Western immunoblot analysis

Following extraction and electrophoresis, proteins were transfered to Hybond-C super nitrocellulose membranes (Amersham). All the antibodies were used at a dilution of 1:10000 in phosphate buffered saline (PBS) with 0.1% Triton X 100. The appropriate secondary antibody with conjugated horse radish peroxidase was used at a dilution of 1:1000. Prior to detection, the final membranes were washed 3 times for 5 minutes each in PBS containing 0.1% Triton X100. Membranes were developed using 3,3-diaminobenzidine

in 30 ml of PBS buffer containing 30 ml of H_20_2 and developed by keeping them in darkness for a few minutes.

Membrane damage studies: Potassium efflux

Plasma membrane damage of yeast cells was monitored based on efflux of potassium ions by flame photometry. Yeast cells grown overnight in YEPD broth at 25°C as described earlier, were harvested, washed with 10 mM EDTA, and then with distilled water twice and finally re-suspended in saline to an OD_{450} nm equivalent to 2.0. Lignocaine treated and untreated cell suspensions of thermotolerant yeast was exposed to -20, -10, 0, 25, 40, 45 and 50°C for 30 minutes and subsequently incubated at 25 ^{o}C. From this sample the potassium ion concentration in the cell free supernatant was determined up to 70 minutes at an interval of 10 minutes. In another set, shock was given to thermotolerant and mesophilic strains at -20°, 25°, 45°C and potassium ion efflux was studied at different time intervals as explained above.

Leakage of UV $_{280}$ absorbing material

The method of Williams *et al.* (1991) was followed to determine the leakage of UV $_{280}$ absorbing material. The cell suspension was prepared as described above and the absorbance of the cell free supernatant was determined at 280 nm using Spectronic UV spectrophotometer.

Statistical Analysis

Statistical analysis was carried out using Two-way ANOVA (Table 1).

RESULTS

Membrane damage

Thermotolerant and mesophilic strains of yeast following exposure to –20 and 45°C showed time dependent increase in membrane damage as judged by K^+ ion efflux and leakage of UV_{280} absorbing material. Membrane damage appeared to be more severe in the presence of lignocaine than the absence of lignocaine. The increase in membrane damage observed in 25°C cultured cells can be attributed to the presence of lignocaine. From the results of Table 1 it is clear that –20, 45 and 50°C caused appreciable membrane damage. In an attempt to ascertain the temperatures which would cause maximum membrane damage the thermotolerant strain was subjected to temperatures ranging from –20 to 50°C. The results indicate that –20 and 50°C caused maximum damage to the membrane irrespective of the presence or absence of lignocaine. In thermotolerant strain, membrane leakage increased with time up to 70 minutes (including 30 min shock time) but decreased thereafter. Whereas in mesophilic yeast, membrane leakage was continuously increased up to the studied period of 100 minutes (Table 1).

Induction of thermotolerance after heat and cold shocks

Yeast cells were grown at 25°C and then given shock at different temperatures ranging from -20 to 50°C for 30 minutes. Subsequently the cultures were shifted to 25°C for 70 minutes and their viability was recorded following plating and incubation at 25 and 45°C respectively. The results indicate that culture subjected to cold shock is comparatively more thermotolerant than culture subjected to heat shock. Further when cells treated with lignocaine were compared with cells in the absence of lignocaine it appeared that heat shock at 30, 40°C made the cells more thermotolerant in the presence of lignocaine. In contrast to thermotolerant strain, increase in thermotolerance was not observed following cold and heat shock in mesophilic yeast strain. The experiments were replicated thrice in triplicates and mean values varied by no more than $\pm$ 5% except values otherwise indicated (Table 2 & Fig 1).

Induction of heat shock proteins after cold and heat shock in thermotolerant Saccharomyces cerevisiae-VS$_3$ strain

Autoradiographic and SDS-PAGE analysis after total proteins of thermotolerant yeast following cold (0 to –20°C) and heat (25 to 50°C) shock for 30 min and subsequent incubation at 25°C for 70 min indicated the synthesis of a number of proteins. Following cold shock, increase in the synthesis of proteins of molecular weight 30, 60, 70, 90, 104 KD was observed whereas following

heat shock only proteins of molecular weight 60, 70 KD increased in levels up to 45°C.

Table 1a: Potassium efflux (ppm) and leakage of UV_{280} absorbing material (OD) following heat and cold shock of thermotolerant *Saccharomyces cerevisiae- VS₃* yeast and mesophilic *Saccharomyces cerevisiae* MTCC 3205 strains in the absence or presence of lignocaine*.

Time (min)	*Saccharomyces cerevisiae* (VS_3) **						Mesophilic *S.cerevisiae* (MTCC 3205)**					
	Potassium efflux (ppm)			Leakage of UV_{280} absorbing material (OD)			Potassium efflux (ppm)			Leakage of UV_{280} absorbing material(OD)		
	-20°C	25°C	45°C	-20°C	25 °C	45 °C	-20°C	25°C	45°C	-20°C	25 °C	45 °C
30	1.2± 0.01	1.3± 0.03	1.7± 0.01	0.11± 0.03	0.09± 0.01	0.19± 0.01	1.4± 0.01	1.3± 0.02	1.6± 0.02	0.13± 0.01	0.10± 0.02	0.20± 0.02
40	1.8± 0.01	1.4± 0.02	2.0± 0.01	0.26± 0.04	0.15± 0.02	0.28± 0.04	1.8± 0.02	1.6± 0.02	2.2± 0.01	0.27± 0.04	0.15± 0.02	0.30± 0.04
50	2.5± 0.01	1.6± 0.02	2.3± 0.02	0.39± 0.05	0.18± 0.02	0.37± 0.05	2.1± 0.02	1.9± 0.03	2.4± 0.02	0.39± 0.05	0.18± 0.02	0.41± 0.04
60	2.9± 0.02	1.7± 0.01	2.6± 0.02	0.49± 0.04	0.21± 0.04	0.41± 0.04	2.5± 0.03	2.3± 0.03	2.7± 0.01	0.51± 0.03	0.26± 0.02	0.47± 0.04
70	3.3± 0.02	1.9± 0.01	2.9± 0.02	0.50± 0.03	0.22± 0.02	0.43± 0.04	2.8± 0.03	2.4± 0.02	3.2± 0.03	0.54± 0.03	0.29± 0.04	0.49± 0.04
80	3.0± 0.01	1.6± 0.01	2.7± 0.03	0.46± 0.04	0.14± 0.03	0.40± 0.04	3.2± 0.03	3.0± 0.04	3.4± 0.04	0.59± 0.03	0.32± 0.05	0.54± 0.03
90	2.5± 0.02	1.3± 0.01	2.0± 0.03	0.30± 0.01	0.11± 0.04	0.31± 0.02	3.5± 0.03	3.1± 0.01	3.5± 0.04	0.62± 0.03	0.39± 0.03	0.56± 0.01
100	1.8± 0.03	1.1± 0.02	1.4± 0.03	0.15± 0.02	0.09± 0.02	0.20± 0.01	4.1± 0.02	3.2± 0.01	3.9± 0.04	0.70± 0.03	0.46± 0.02	0.62± 0.02

* Both strains in the absence of lignocaine showed time dependent increase in K^+ ion efflux and UV_{280} absorbing material leakage. But, the values are significantly lower compared to cells treated with lignocaine (data not shown).
** All the experiments were repeated thrice using three replicates each time (n=9) and values are represented as ppm ± SD for K^+ ion efflux and OD_{280} for UV absorbing material. For two way ANOVA analysis three average results of replicates was taken (n=3).

Table 1b: Two-way ANOVA of Potassium efflux (ppm) following heat and cold shock of thermotolerant *Saccharomyces cerevisiae- VS₃* yeast and mesophilic *Saccharomyces cerevisiae* MTCC 3205 strains in the presence of lignocaine.

Source of Variation	Sum of Squares	Total (n)	d.f.	Mean Square	F Ratio	$F_{0.95}$
Total	51.9	144	143			
Between-means of Products	617.49	8	7	88.21	19.38	2.09
Between-means of judges	165	18	17	9.70	2.13	1.75
Error	542.19		119	4.55		

For Error d.f. = 143-17-7= 119
Note : Potassium efflux (ppm) following heat and cold shock of thermotolerant and mesophilic yeast strains in the presence of lignocaine is significant at 5% level.

Table 1c: Two-way ANOVA of leakage of UV_{280} absorbing material following heat and cold shock of thermotolerant *Saccharomyces cerevisiae- VS₃* yeast and mesophilic *Saccharomyces cerevisiae* MTCC 3205 strains in the presence of lignocaine.

Source of Variation	Sum of Squares	Total (n)	d.f.	Mean Square	F Ratio	$F_{0.95}$
Total	5.25	144	143			
Between-means of Products	11.98	8	7	1.71	57	2.09
Between-means of judges	12.94	18	17	0.76	25.33	1.75
Error	4.29		119	0.036		

For Error d.f. = 143-17-7= 119

Note: Leakage of UV_{280} absorbing material following heat and cold shock of thermotolerant and mesophilic yeast strains in the presence of lignocaine is significant at 5% level.

After 30 minutes shock at -20°C, extra cellular K^+ ion& UV_{280} absorbing material content was equal to un shocked yeast and it was more in heat shocked yeast. In cold shocked yeast, extracellular K^+ ion & UV_{280} absorbing material content increased after 50minutes (includes 30 minutes of shock and 20 minutes of incubation at 25°C). But in heat-shocked yeast, leakage is time dependent and increased with time. In thermotolerant strain at 80th minute, extracellular K^+ ion & UV_{280} absorbing material decreased than the value at 70th minute, indicating their intake inside the cell. In mesophilic yeast there was no intake and only efflux was noted up to the studied period. There was more K^+ ion efflux and leakage of UV_{280} absorbing material in lignocaine treated compared to untreated samples (data not shown).

Table 2: Potassium efflux, UV $_{280}$ absorbing material leakage and viability at 25 and 45 °C of shocked *VS_3* strain.

Yeast	Lignocaine untreated † *Saccharomyces cerevisiae-VS_3* Strain				Lignocaine treated † *Saccharomyces cerevisiae-VS_3* Strain			
Parameter	K^+ ion efflux	UV_{280}	Viability CFU		K^+ efflux	UV_{280}	Viability CFU	
Temp.(°C)			25 °C	45 °C			25 °C	45 °C
-20	1.75± 0.018	0.15± 0.003	96	98	2.0± 0.012	0.16± 0.001	92	72
-10	2.1± 0.022	0.217± 0.029	102	104	2.4± 0.019	0.228± 0.013	103	105
0	1.5± 0.029	0.145± 0.019	97	98	1.7± 0.014	0.142± 0.019	94	96
25	1.1± 0.036	0.057± 0.015	105	80	1.1± 0.017	0.081± 0.022	92	102
30	1.3± 0.041	0.181± 0.023	104	90	1.4± 0.015	0.117± 0.025	92	98
35	1.2± 0.044	0.145± 0.041	101	92	1.5± 0.030	0.179± 0.036	91	97
40	1.3± 0.042	0.225± 0.012	90	98	1.4± 0.032	0.281± 0.033	94	96
45	1.2± 0.011	0.213± 0.026	98	93	1.5± 0.039	0.225± 0.046	92	97
50	3.1± 0.015	0.360± 0.024	22	17	3.5± 0.041	0.414± 0.029	11	08

†: All the experiments were repeated thrice using three replicates each time (n=9) and values are represented as ppm ± SD for K^+ ion efflux , OD_{280} for UV absorbing material and colony forming units (CFU) for viability.

There was more K^+ ion efflux, leakage of UV_{280} absorbing material in lignocaine treated compared to untreated yeast. There was more leakage at 50°C and -20° C shock.. Viability (growth at 25° C) & thermotolerance (growth at 45° C) were reduced in the yeast shocked at -20° C& 50° C. There was maximum thermotolerance in yeast, shocked at -10° C followed by 45° C.

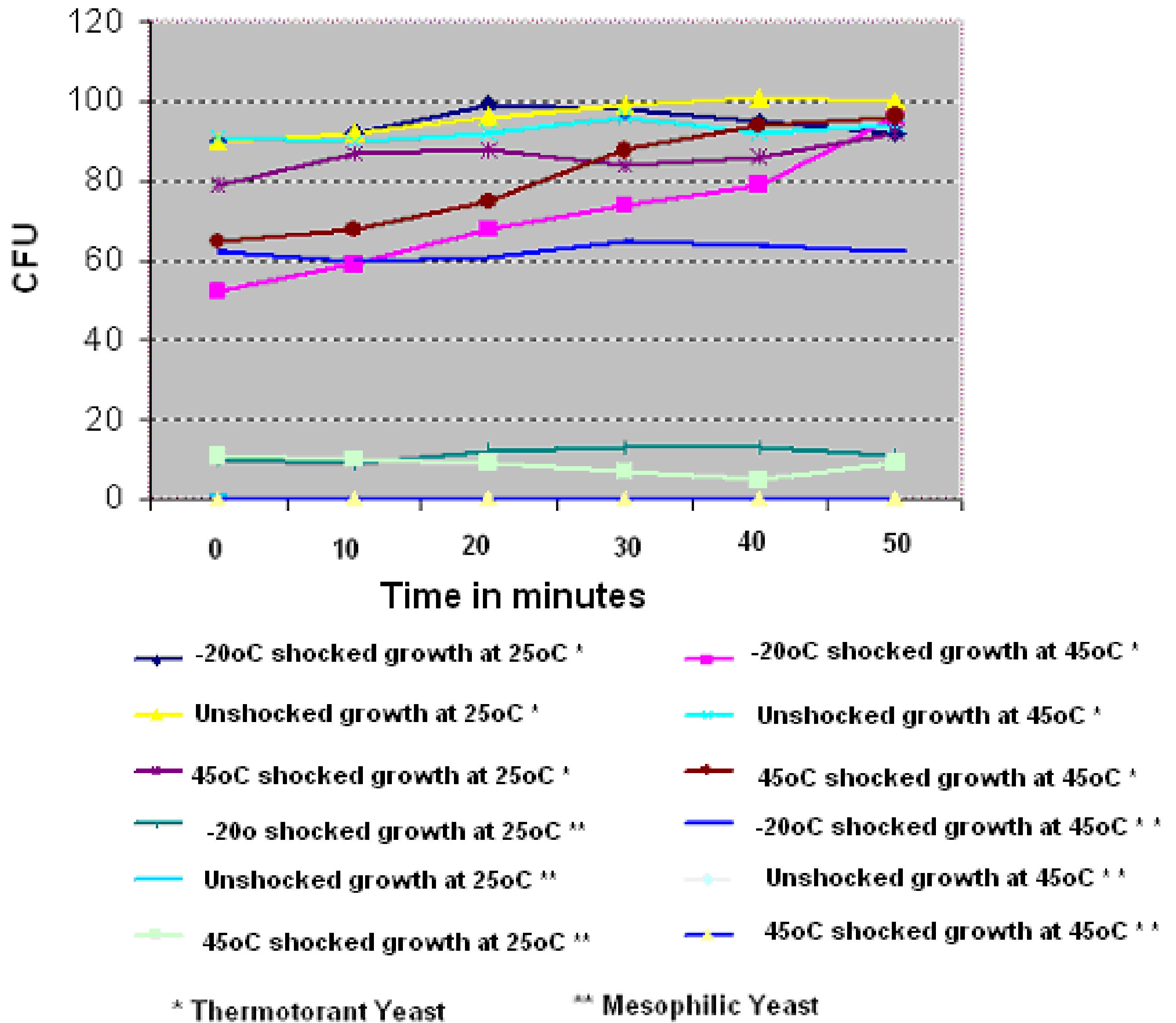

Figure 1: Colony forming units per ml at 10^{-4} dilution of heat and cold shocked yeast with lignocaine treatment at various time intervals of post shock incubation at 25°C.

Lignocaine treated yeasts showed less colony forming units (CFU) than untreated yeasts at 25°C. In thermotolerant strain after cold shock at -20° C, few cells were killed initially (low count), but the CFU were gradually increased with the post shock incubation at 25° C and attained the maximum at 80th minute. In mesophilic yeast, heat and cold shock has decreased the initial viable cells and there are no CFU at 45° C, indicating that there is no increase in thermotolerance.

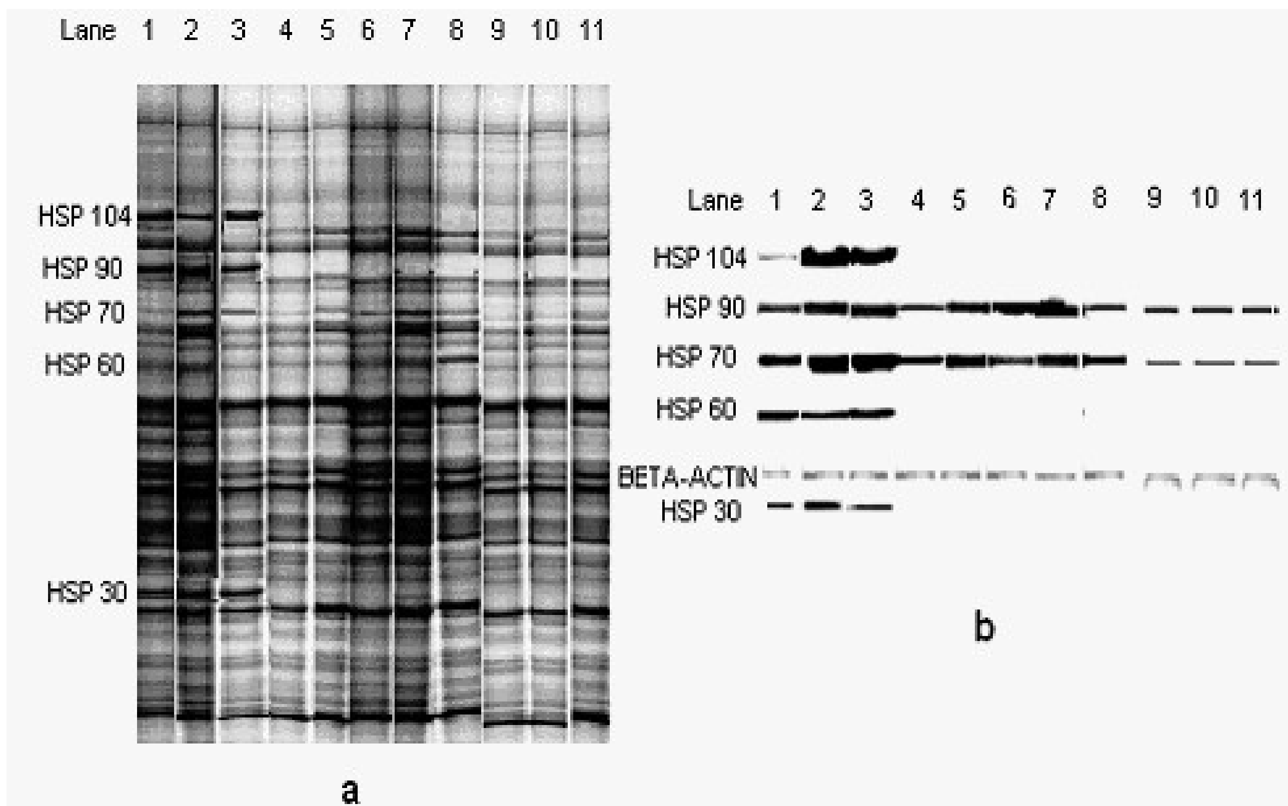

Figure 2:Total cell HSP profile of Thermotolerant yeast

(a) Autoradiograph (b) Western blot

Lane 1-8: Heat and cold shocked samples of thermo tolerant yeast at -20, -10, 0, 25, 35, 40, 45 and 50° C temperatures Lane 9-11 mesophilic yeast shocked at-20, 25 and 45 ^{o} C

a) In thermotolerant yeast de-*novo* proteins were produced after heat and cold shock. *De-novo* proteins of molecular weight 104, 90, 70, 60, and 30 were detected in cold shocked yeast. Their levels are more in -10° C shocked yeast. Proteins of molecular weight 70 and 60 were produced by heat shock. Apart from these proteins, many other proteins were also produced but their levels were not increased with the intensity of shock temperature. In mesophilic yeast presence of *de-novo* proteins in the molecular weight range of HSPs was not noted.

b) Five types of HSPs (104,90,70,60 &30) were produced by cold shock and maximum production was observed at -10° C shock. HSPs 70 & 60 were synthesized by heat shock and their levels increased up to 45° C shock and there after decreased. In 50° C heat shocked sample, many of the yeasts were killed, which may be leading to the decreased HSP levels (Table 2). Lignocaine treated and shocked (heat and cold) yeast produced HSPs similar to particular shocked yeast, but HSP levels were increased. In lignocaine treated and unshocked yeast (25° C), HSP 70 and 60 were noted and the levels were more than the base line (unshocked yeast). Induction of HSP 70, 60 by lignocaine resembles the results of heat shock. In stressed mesophilic yeast only base-line Hsps were noted as seen in un shocked yeast.

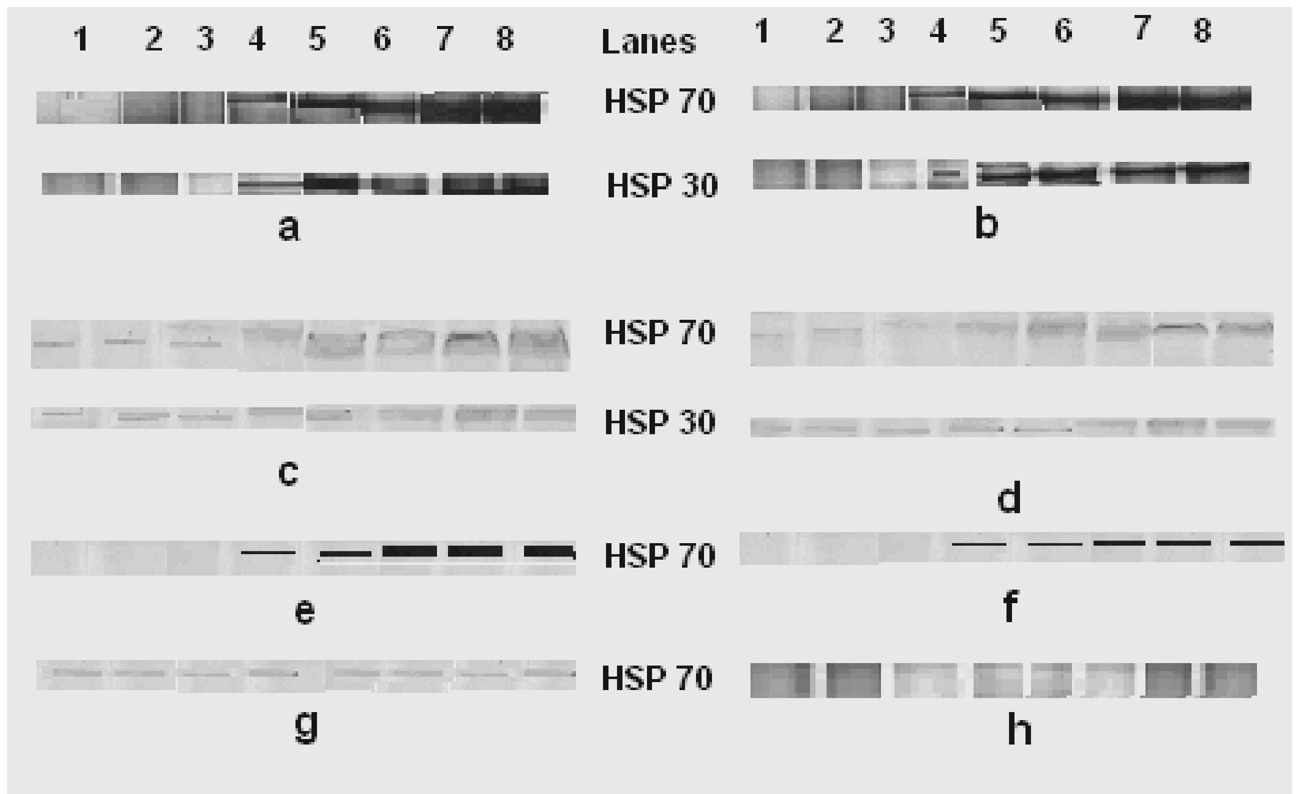

Fig: 3 Cell membrane HSP profile of Thermotolerant yeast

(a) Autoradiograph of lignocaine treated,

(b) Autoradiograph of lignocaine untreated,

(c) Western blot of lignocaine treated,

(d) Western blot of lignocaine untreated and cold shocked(–20°C)

(e) Autoradiograph of lignocaine treated ,

(f) Autoradiograph of lignocaine untreated and heat shocked (45°C)

(g) Western blot of lignocaine treated and heat shocked (45°C) mesophilic yeast

(h) Autoradiograph of lignocaine treated and heat shocked (45°C) mesophilic yeast

Lanes 1-8: Protein sample from membrane extracted at 0, 10, 20, 30, 40 ,50,60 and 70 minutes of 30 minutes post shock incubation at 25°C after shock.

De-novo HSPs (HSP 70, 30) in plasma membrane were noted only after 30 minutes of incubation at 25° C after the shock at -20° C (a). In lignocaine treated and -20° C shocked yeast HSP levels are more than lignocaine untreated yeast (b). In western blot analysis increase of HSPs from base line was noted similar to autoradigraph (c, d). In 45°C heat shocked yeast, only *de-novo* HSP 70 was detected in the membrane after 30 minutes of incubation at 25°C. The level of this HSP was more in lignocaine treated (e) compared to lignocaine untreated yeast (f). In stressed mesophilic yeast only base-line Hsps were noted in membrane and de-nova Hsps were not found as seen in un-shocked yeast.

However when the cells were subjected to 50°C heat shock both the above proteins decreased in their intensity. Western blot analysis confirmed the presence of above molecular weight proteins as respective HSPs (Fig 2). Lignocaine treated and shocked yeast cells showed HSP profile similar to that of heat shocked yeast. In mesophilic yeast *de-novo* HSPs were not synthesized after heat and cold shock and their levels were maintained constant like the un shocked yeast. Beta–Actin levels were maintained stable in heat/cold shocked yeasts at all studied periods (Fig 2).

Membrane stress proteins

In thermotolerant yeast, after giving cold and heat shock only baseline levels of HSPs were found in the

membrane and no *de-novo* HSPs were detected up to 50 minutes (shock time and time at 25°C). Thereafter gradual increase in HSPs was found up to 80 minutes, after this it was stable (Fig 2). In mesophilic yeast, baseline HSP levels were maintained up to 100 minutes indicating that no more HSPs were produced. In lignocaine treated and unshocked thermotolerant strain, HSP expression was similar to that in heat shocked yeast. *De-novo* HSP expression observed in thermotolerant strain was not found in mesophilic yeast. Membrane western blots and autoradiographs (Fig 3) indicated that *de-novo* HSP 70 and 30 are transported to the membrane from 60 minutes.

DISCUSSION

Exposure to a mild non-lethal heat shock renders cells resistant to subsequent challenge at higher temperatures in all organisms (Sanchez *et al.,* 1993). Experiments were carried out to determine the effect of different heat shock temperatures on inducing thermotolerance. The thermotolerant strain of *Saccharomyces cerevisiae (*VS_3*)* survives, at 45°C but grows very poorly. However prior heat or cold shock confers thermotolerance as judged by increased number of colony forming units compared to the control (Table 2 & Fig 1). Though earlier studies have indicated improvement in thermotolerance following heat shock (Piper *et al.,* 1997; Silva *et al.,* 1994; Trinklein *et al.,* 2004) this study demonstrates first time that the cold shock also is capable of improving thermotolerance in yeast.

Increased thermotolerance may occur due to synthesis of HSPs, which are known to confer protection to high temperature (Hanninen *et al.,* 1999; Lindquist and Kim, 1996; Sanchez *et al.,* 1993) but their exact involvement and mode of action was not studied. *Saccharomyces cerevisiae* cells exposed to 43°C (normal being 30°C) exhibit synthesis of heat shock proteins (HSPs). Time course studies indicated that the major HSPs (97, 85 and 70 kDa family) are induced within 10 minutes of heat shock attaining maximum levels within two hrs of treatment (Kaul *et al.,* 1992). In the present study yeasts were shocked at various temperatures for 30 minutes and subsequently incubated at 25 °C for 70 minutes (Total HSP expression time was 100 minutes) in order to observe the membrane damage reversal at different time intervals. Yeasts were incubated at 25°C to block further damage of membrane by heat or cold shock. In our study, an increase in the synthesis of Heat shock proteins HSP 60 & 70 was observed, confirming earlier published results (Kaul *et al.,* 1992). However it is also observed that following cold shock a number of heat shock proteins such as HSP 30,60,70,90 & 104 were produced. Heat/cold shock is known for down regulation of several genes including beta actin (Trinklein *et al.,* 2004). As beta–Actin is heat/cold stable, its expression is reduced/blocked after heat shock therefore, it was used for normalization of western blots. Thus implying that HSPs may also have a role to play in protecting the cells during cold shock and subsequently during high temperature tolerance. Earlier reports have indicated a role of HSP30 & 104 in yeast thermotolerance (Hanninen *et al.,* 1999; Lindquist and Kim, 1996; Patriarca and Maresca, 1990; Piper *et al.,* 1997; Silva *et al.,* 1994). Though the exact mechanism as to how HSPs confer thermotolerance is not known, it could however be attributed to the ability of HSPs to control conformational repair of both cold and heat damaged proteins (Hanninen *et al.,* 1999). The importance of HSPs with reference to thermotolerance has clearly demonstrated by experiments in which cells failed to grow at high temperatures in the absence of HSP 70 and HSP 104 (Lindquist and Kim, 1996; Suutari *et al.,* 1990]. In the present study absence of mesophilic yeast growth at higher temperatures can be explained due to non synthesis of HSP 70 & 104 and thermotolerance can be explained by synthesis of HSPs and their subsequent transport in to the membrane.

HSP 30 has been identified earlier as the only plasma membrane integrated HSP in *Saccharomyces cerevisiae.* This protein is induced by several physical and chemical stress (Piper *et al.,* 1997) and has been implicated in preventing denaturation of membrane bound enzymes (Patriarca and Maresca, 1990). In thermotolerant yeast strain in addition to HSP 30 another Heat shock protein HSP 70 has also been identified in the membrane.

Increase in heat shock proteins following heat shock is probably a consequence of a change in membrane physical state, which in turn would induce the expression of heat shock genes (Polla *et al.,* 1997; Vigh *et al.,* 1998; Lloyd *et al.,* 1993; Sanchez. *et al.,* 1993). Therefore it would be logical to assume that a membrane perturbing agent like lignocaine, which is known to fluidizing the membrane should also induce changes similar to heat shock (Mizogami *et al.,* 2002; Steels *et al.,* 1994). In fact, in the present study it is observed that in the thermotolerant strain, lignocaine does increase the levels of HSPs. Lignocaine treated and shocked yeast showed HSP profile corresponding to the type of shock, but the HSP levels are more than the lignocaine untreated and shocked yeast (Fig 2 & 3). The study also confirms similar changes in HSP levels following cold shock probably due to membrane damage. It may be worth while to mention that in addition to HSP 60 & 70 which are induced both following cold and heat shock, a few other proteins such as HSP 30, 90, 104 are induced only during cold shock. Therefore it is tempting to postulate that the later 3 proteins may be associated with the cold shock.

Damage to the cell membrane following cold and heat shock was confirmed by the efflux of K^+ ions, leakage of UV_{280} absorbing material from the two strains. This confirms earlier observation of Fujitha and Kubo (2002) in yeast and Williams *et al.* (1991) in *Staphylococcus aureus.* It was also observed that in thermotolerant strain the above two (efflux of K^+ ions, leakage of UV_{280} absorbing material) processes slowed down after 60 minutes coinciding with the synthesis of HSPs in the membrane. Therefore it appears that membrane integrity is recovering with time and probably the heat shock proteins are facilitating the recovery.

Cold shock at -10°C caused optimal membrane damage, induced more HSPs and gave maximum thermotolerance in thermotolerant yeast. It indicates that the membrane damage up to some extent can be reversed by blocking the damage with the help of HSPs. Further damage (by –20 & 50°C) to the membrane caused the loss of viability of cells (Table 2).

Mechanism of HSPs and plasma membrane in thermotolerance

Physical (cold and heat shock) or chemical (lignocaine) damage which alters the physical state of the membrane stimulates the cellular thermometer (Polla *et al.,* 1997). Changed electro potential after stress due to intracellular acidification by influx of protons and efflux of K^+ and other ions or cell protein content leakage may be stimulating the cellular thermometer (Coss *et al.,* 2003). Cellular thermometer activates HSF and HSPs are synthesized (Polla *et al.* 1997). Cellular thermometer of a particular organism may be genetically determined. In thermotolerant strain, little stimulation may be enough to synthesize HSPs where as more stimulation may be needed in mesophilic yeast. HSP production is effected, if the stimuli are less or more than the optimum. In thermotolerant strain, HSPs 70 & 30 produced are transported to the membrane to recover the damage created by physical or chemical stress. This causes the decrease of leakage and increased intake of ions for achieving the balance. In mesophilic yeast, the sensitivity levels may be more and HSPs may be produced at a later period. Meanwhile heat shock causes irreversible damage of proteins and DNA, which leads to the death of the organism.

CONCLUSION

In present study using a mesophilic and a thermotolerant yeast it is clearly demonstrated that both cold and heat shock confer thermotolerance. The mechanism underlying this survival process is closely associated with the induction in the synthesis of heat shock proteins. These HSPs in turn stabilize the damage caused in the membrane and thus facilitate the thermotolerance. It is interesting that the subset of HSPs that are involved in conferring thermotolerance following cold and heat shock are different. This indicates that the involvement of Heat shock proteins along with plasma membrane are associated with the increased thermotolerance in thermotolerant *Saccharomyces cerevisiae* strain. In mesophilic yeast as *de-novo* HSPs were not produced after shock, it was unable to grow at high temperature.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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