A study of the effect of trehalose accumulation on environmental stresses in *Saccharomyces cerevisiae*

(酵母 *Saccharomyces cerevisiae* の環境ストレスに対するトレハロース蓄積の効果に関する研究)

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**List of Publications**

**Journal papers**

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**Abstract**

The yeast *Saccharomyces cerevisiae* is widely used in brewing and fermentation industries for production of useful products such as chemicals, fuels, foods and medicine. In industrial processes it is always desirable to have useful microorganisms that will be resistant to stress conditions and also show higher growth to reduce the production cost with maximal product yield. These aspects have motivated early research into the control of the cellular content of glycerol and trehalose, low-molecular-weight compounds serving as compatible solutes to adjust intracellular water activity and to protect biomolecules from denaturation. In this research the effect of trehalose in stress tolerance in yeast is mainly investigated. This study is aimed to elaborate the role of trehalose as an effective stress tolerant metabolite and also to construct a stress resistant strain which would not only be useful industrially but also serve the purpose of revealing the stress tolerance mechanism of yeast.

This thesis contains 5 chapters. Chapter 1 contains the general introduction of this research which includes the background of current research. Chapter 2 describes the strategy for artificial trehalose accumulation in yeast by metabolic engineering by constructing yeast strains lacking the trehalose degradation genes and also overexpressing trehalose synthesis genes and testing the constructed strains under saline stress condition. The results indicate that high trehalose accumulation prior to NaCl addition rather than after NaCl addition is necessary to achieve high growth activity under stress condition. Chapter 3 investigates selected recombinants strains that are able to accumulate trehalose than their parent strain and test those under various environmental stresses to find out multiple stress resistant strains. The strains obtained

in Chapter 2 were further selected to 3 recombinant strains along with the parent strains and were tested under ethanol, heat, freezing and oxidative stress conditions, the stresses yeast commonly encounter in industrial processes. The results indicated that high trehalose accumulation can make yeast cells resistant to multiple stresses, but the importance of this accumulation before or after stress induction is varied depending on the type of stress. Chapter 4 further investigates a multiple stress tolerant strain (*TPS1-overexpressing triple trehalases deletion strain*) along with its parent strain by DNA microarray technique in order to find out the differences in gene expression between the parent and the recombinant strains. For this the two strains were subjected to no stress, ethanol and heat stress conditions, the two more common stresses yeast are exposed to during industrial fermentation and the gene expression of these strains under the above environmental stresses were determined by microarray. The data analysis revealed that the stress tolerant strain had similar gene expression response to the parent strains in non stress as well as stress conditions. GO term analysis of the differentially expressed genes in the stress tolerant strain revealed that under heat stress, this strain incorporated more glucose than the parent strain. Chapter 5 summarizes the results obtained in this research. In summary it can be confirmed that trehalose is a multiple stress protectant in yeast the role of which is varied depending on the type of stress. The comparison of multiple stress tolerant strain with its parent strain revealed stress adaptation process. This stress tolerant strain is expected to be industrially useful.

**Table of Contents**

**Chapter 1**

**General Introduction**

The budding yeast, *Saccharomyces cerevisiae*, has been used for a long time in brewing and fermentation industries for the production of useful compounds (Pretorius, 2000). These compounds range from chemicals, foods and beverages, medicines to bioethanol as fuel. This unicellular yeast is also extensively used for studying various processes in eukaryotes. During these processes, yeast cells are exposed to a variety of stresses, ranging from severe to mild, in order to get the desired product and to reduce the formation of undesirable by products. However, as a consequence, the yield of target product is often reduced as well as the growth is also compromised. Therefore initial interest in the molecular mechanisms of yeast stress tolerance is originated from the need to improve the performance of yeast strains under industrial conditions, which are often associated with rapid alterations in environment (Attfield, 1997). Additional practical aims are the improvement of food preservation methods, which require a better understanding of the impact of low water activity on yeast cells, especially in combination with other stress factors such as heat, cold, acidity, and chemical food preservatives.

These aspects have motivated early research into the control of the cellular content of glycerol and trehalose, low-molecular-weight compounds serving as compatible solutes that adjust intracellular water activity and protect biomolecules from denaturation. Among these two molecules, glycerol has been shown to confer resistance to stress conditions, as well as its concomitant increase when stress is applied to yeast cells (Lewis et al., 1995). Like glycerol, trehalose also increases during various stresses such as heat, freezing and osmotic stresses. However, whether this increase is directly

related to the stress tolerance is still obscure. Therefore this research is aimed at the clarification of the role of trehalose under common industrial stress conditions.

## 1.1 Chemical structure and properties of trehalose

Trehalose is a disaccharide consisting of two molecules of glucose bound by an α-1, 1 linkage (α -D-glucopyranosyl-[1, 1]-α -D-glucopyranoside; Fig. 1.1). It was discovered from the ergot of rye in 1832 by Wiggers. Subsequently, the French chemist Berthelot found this sugar in trehala (a desert manna from Asia Minor that is produced by the weevil *Larinus nidificans*) and called it trehalose. In yeast cells the presence of trehalose was first shown by Koch and Koch (1925).
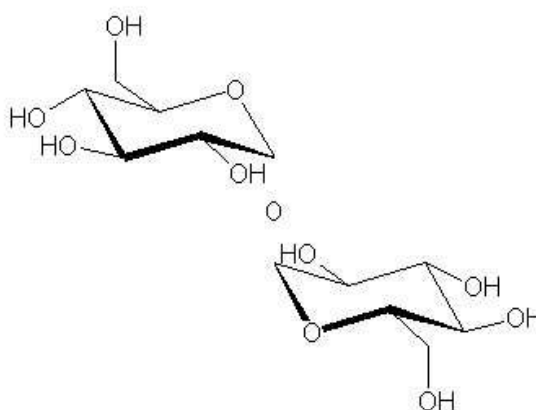


**Figure 1.1:** Chemical structure of trehalose.

Trehalose remains stable at elevated temperatures and at low pH and does not undergo the Maillard reaction with proteins (Colaco et al., 1995).

## 1.2.1 Trehalose and trehalases in bacteria, fungi and animals

Trehalose is a common sugar in both prokaryotic and eukaryotic organisms,

such as bacteria, fungi and invertebrates (Elbein, 1974; Crowe and Crowe, 1984). In these organisms, trehalose often accumulates in large quantities. Trehalose is an important storage compound in vegetative cells and spores in fungi and is most widely distributed disaccharide in fungi. In vegetative structures it is usually found together with sugar alcohols and glycogen (Blumenthal, 1976). Trehalose mainly appears to serve as a storage carbohydrate during periods of non-proliferation. This applies to the life cycle, and other conditions under which cessation of growth occur, such as starvation. During yeast life cycle, trehalose is accumulated in the reproductive stages and mobilized during germination. In *Escherichia. coli*, trehalose accumulates in response to high osmotic strength and is thought to act in this species as an active osmolyte. In *Bacillus subtilis*, trehalose is not involved in osmoprotection and serves exclusively as a carbon source (Argüelles, 2000). In some bacteria trehalose occurs as structural compound located as cell wall glycolipid. Several species of insects contain trehalose in the hemolymph, which is quickly mobilized during flight. Also in higher animals, enzymes involved in trehalose metabolism are present, although their role is not well understood. In humans, trehalase has been located both in the border membranes of epithelial cells of the small intestine. It may be involved in sugar transport across the membrane and/or hydrolysis of ingested trehalose. However, the physiological role of trehalase found in human kidney and serum remains a mystery, because trehalose is not found in blood (Muller et al., 1995; Argüelles, 2000).

## 1.3 Trehalose metabolic pathway in *Saccharomyces cerevisiae*

### 1.3.1 Trehalose synthesis and degradation

Trehalose biosynthesis is catalyzed by the sequential action of

trehalose-6-phospate synthase (Tps1p) and trehalose-6-phosphate phosphatase (Tps2p) activities called the trehalose synthase complex (Vuorio et al., 1993) using UDP-glucose (UDP-Glc) and glucose-6-phosphate (G6P) as substrates (Fig. 1.2). It is usually synthesized in two steps. First, trehalose-6-phosphate synthase (Tps1p) catalyses the synthesis of trehalose-6-  phosphate (Trehalose-P) from glucose-1-phosphate (G1P) and UDP-glucose (UDP-Glc). Subsequently trehalose-6-phosphate phosphatase (Tps2p) removes the phosphate yielding trehalose (Elbein, 1974).  A deletion of *TPS1* gene leads inability of cells to synthesize trehalose and prevents growth on glucose. The inability of the *TPS1* mutant to grow on glucose suggests that a role for this gene as a sensor for glucose influx into the cell. A *TPS3* gene encoding the 123-kDa polypeptide seems to regulate the *TPS1* activity of the trehalose synthase (Vuorio et al., 1993).

The enzyme hydrolyzing trehalose was first reported in *S. cerevisiae* by Fischer (1895). Since then trehalase (α, α-trehalose-1-D-glucohydrolase, EC 3.2.1.28) has been detected in many other organisms of the plant and animal kingdom. In the yeast *S. cerevisiae*, the catabolism of trehalose occurs by the action of these hydrolytic enzymes, yielding two glucose units. Interestingly, *S. cerevisiae* and related yeasts contain two different types of trehalase: the neutral trehalases (Nth1p and Nth2p) localized in the cytosol, and the acidic trehalase (Ath1p) that is found in the vacuole (Argüelles, 2000). Trehalose hydrolysis is believed to be an important biochemical process during the various life functions of yeast, for example, fungal spore germination, resumption of growth in resting cells and stress response.
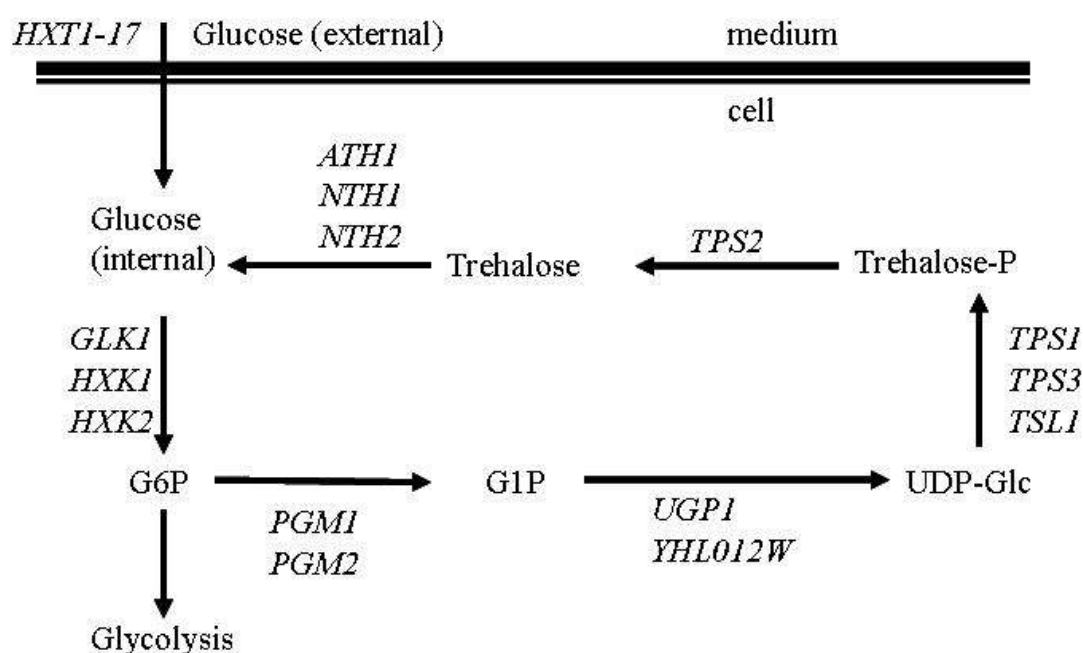
**Figure 1.2:** Trehalose metabolism in *Saccharomyces cerevisiae.* G6P, Glucose-6-Phosphate; G1P, Glucose-1-Phosphate; UDP-Glc, UDP Glucose; Trehalose-P, Trehalose-6-Phosphate.

### 1.3.2 Alignment of trehalases form various organisms

Amino acid sequence comparison of available trehalase sequences from *S. cerevisiae* neutral trehalases (Sc-Nth1p and Sc-Nth2p) , *Kluyveromyces lactis* neutral trehalase (Kl-Nth1p), *E. coli* periplasmic trehalase (E-Trea) and cytoplasmic trehalase (E-Tref) (Gutierrez et al., 1989), rabbit small intestine trehalase (R-Tre)(Ruf et al., 1990), insect trehalases from *Tenebrio moliter* (T-Tre)(Takiguchi et al., 1992), and *Bombyx mori* (B-Tre) is shown in Fig. 1.3. This alignment reveals an N-terminal extension of yeast trehalases (Sc-NTH1p, Sc-Nth2p from *S. cerevisiae* and Kl-Nth1p from *K. lactis*) in relation to the other trehalases. The N-terminal extension of the neutral trehalases from yeast contains the putative cAMP dependent protein kinase

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B-tre/1-579     .........................................................................
T-tre/1-555     .........................................................................
R-tre/1-578     .........................................................................
E-trea/1-565    .........................................................................
E-tref/1-549    .........................................................................
Sc-Nth1p/1-751  1 ------MSQVN-TSQG-------PVAQG------------RQRRLSSLSEFNDPFSNAEVYYGPPTDPRKQKQAKPAKINRTRTMSVFD------NVSPFKKTGFGKLQ-QTRRGSED85
Sc-Nth2p/1-780  1 MVDFLPKVTEINPPSEGNDGEDNIKPLSSGSEQRPLKEEGQQGGRRHHRRLSSMHEYFDPFSNAEVYYGPITDPRK--QSKIHRLNRTRTMSVFN------KVSDFKN-GMKDYT-LKRRGSED114
Kl-Nth1p/1-754  1 ----MDGKVNNN-PPRS----------------RHRRTSSLEEVVDPFSTPDVVYGPKSDPSK--LLSKNRFTRTRTFSVAEPGGGKGHSSSYTSPYFDTTVPLRRRGSED88

B-tre/1-579     1 --------MRLFLLLVGLTT---VIADDLPPTCIRPVYCNSTLLHYVQMA-----------RLYPDSKTFVDFQMRKDENATLSAFQELLDRTNHNPTKEDLQEFVVDFFDET-------S92
T-tre/1-555     1 --------MIPFLLMVAFADTVLQVSAQSQPSCDSKVYCQGKLLHVVEMS-----------RIFNDSKTFVELKMINDEQTTLENFDNFLRDTNHKRTRADLMKFVSDNFKQE-------N95
R-tre/1-578     1 ---MPGSTWELHLLLLLGLGLG---SEQALPPPCESQIYCHGELLHQVQMA-----------RLYPDDKQFVDMPLSTAPDQVLQSFAELAATYNNTVPREQLEKFVQEHFQAVG------Q99
E-trea/1-565    1 ----MKSPAPSRPQKMALIPACIFLCFAALS----VQAEETP--VTPQPPD-----------ILLGP------LFNDVQNAKLFPDQKTFADAVPNSDPLMILADYRMQQNQSG-------F88
E-tref/1-549    1 -MLNQKIQNPNPDELMIEVDLCYELDPYELKLDEMIEAEPEPEMIEGLPAS-----------DALTPADRYLELFEHVQSAKIFPDSKTFPDCAPKMDPLDILIRYRKVRRHRD-------F103
Sc-Nth1p/1-751  86 DTYSSSQGNRRFFIEDVDKTLNELLAAEDTDKNYQITIEDTGPKVLKVGTANSYGKHININRGTYMLSNLLQELTIAKSFGRHQIFLDEARINENPVNRLSRLINTQFWNSLTRRVDLNNVGEI209
Sc-Nth2p/1-780  115 DSFLSSQGNRRFYIDNVDLALDELLASEDTDKNHQITIEDTGPKVIKVGTANSNGFKHVNVRGTYMLSNLLQELTIAKSFGRHQIFLDEARINENPVDRLSRLITTQFWTSLTRRVDLYNIAEI238
Kl-Nth1p/1-754  89 DSYSASQGQRRFYIEDVDKTLKELLASEDTDGNYQITIEDTGPKVIRVGTVNSNGYKHVHIRGTYMLSNLLQELTLAKLFNRKQVILDEARLNENPVNRMTRLISGQFWKSLTRRIDSNNIAKI212

B-tre/1-579     93 ELEEWKPDDHKENPPFLAKIRDQGFREFAKALNDIWPTLARRVKPSVLEKPEQSSLVPMTHG-------------------FIVPGGRFKEIYYWDAYWIIEGLLITDMTETAKGMIEN192
T-tre/1-555     96 EFESWTPTDFTDNPTLLSRIEDKTIRQFAQDLVKIWPTLARRVKKEVLDYPEHYSLLPVDNG-------------------FIIPGGRFTEFYYWDSYWIVEGLLLSDMHETVRGMLDN195
R-tre/1-578     100 ELESWTPGDWKESPQFLQKISDPKLRAWAEQLHLLWKKLGKKIKPEVLSQPERFSLIYSQHP-------------------FIVPGGRFVEFYYWDSYWVMEGLLLSEMAETVKGMLQN199
E-trea/1-565    89 DLRHFVNVNFTLPKE-GEKYVPPEGQSLREHIDGLWPVLTRSTE----NTEKWDSLLPLPEP-------------------YVVPGGRFREVYYWDSYFTMLGLAESGHWDKVADMVAN183
E-tref/1-549    104 DLRKFVENHFWLPEVYSSEYVSDPQNSLKEHIDQLWPVLTREPQ----DHIPWSSLLALPQS-------------------YIVPGGRFSETYYWDSYFTMLGLAESGREDLLKCMADN199
Sc-Nth1p/1-751  210 AKDTKIDTPGAKNPRIYVPYDCPEQYEFYVQASQMHPSLKLEVEYLPKKITAEYVKSVNDTPGLLALAMEEHFNPSTGEKTLIGYPYAVPGGRFNELYGWDSYMMALGLLEANKTDVARGMVEH333
Sc-Nth2p/1-780  239 ARDSKIDTPGAKNPRIYVPYNCPEQYEFYIQASQMNPSLKLEVEYLPKDITAEYVKSLNDTPGLLALAMEEHVNPSTGERSLVGYPYAVPGGRFNELYGWDSYLMALGLIESNKVDVARGMVEH362
Kl-Nth1p/1-754  213 AYDTKIDTPGAKNPRIYVPYNCQDEYQQLVQWSEMDPSLQLEVNYLPKDITPEFVKSLNDKPGLLCLAMESHMDPVTGEETWVGFPYAVPGGRFNELYGWDSYFMALGLLESNKLDVARGMVEH336

B-tre/1-579     193 LIELLYKFGHIPNGSRWYYQERSQPPLLAAMIKLYYEKTKDIEFIRKYISALEKELEYWLD--------THLIAFNKDDRVYTLLRYYIPSAGPRPE---SYYEDYELAQKLDKNTDPNDIYAD305
T-tre/1-555     196 FLSIVEKYGFIPNGARVFYLNRSQPPLLTLMVSLYVSATNDMEWLAKNIRTIDTELRFWLN--------NRLVDVVKDGIVYKLAQYNSNSGSPRPE---SYYEDVTTASVFSDERDKAELYMD308
R-tre/1-578     200 FLDLVTAYGHIPNGGRVVYYLQRSQPPLLTLMMDRYVAHTGDLAFLRENIETLALELDFWAE--------NRTISVSSGGNSHTLNRYHVPYGGPRPE---SYSKDTELAHTLPEGS-WETLWAE311
E-trea/1-565    184 FAHEIDTYGHIPNGNRSYYLSRSQPPFFALMVELLAQH-EGDAALKQYLPQMQKEYAYWMDGVENLQAGQQEKRVVKLQDGTLLNRYWDDRDTPRDE---SWVEDIATA-KSNPNRPATEIYRD302
E-tref/1-549    200 FAWMIENYGHIPNGNRTYYLSRSQPPVFALMVELFEE--DGVRGARRYLDHLKMEYAFWMDGAESLIPNQAYRHVVRMPDGSLLNRYWDDRDTPRDE---SWLEDVETA-KHS-GRPPNEVYRD316
Sc-Nth1p/1-751  334 FIFEINHYGKILNANRSYYLCRSQPPFLTEMALVVFKKLGGRSNP-DAVDLLKRAFQASIK-----EYKTVWTASPRLDPETGLSRYHPNGLGIPPETESDHFDTVLLPYASKHGVTLDEFKQL451
Sc-Nth2p/1-780  363 FIFEIDHYSKILNANRSYYLCRSQPPFLTDMALLVFEKIGGKNNP-NAIQLLKRAFAAIK-----EYKEVVMWSSPRLDSLTGLSCYHSDGIGIPPETEPDHFDTILLPYAEKYNVTLEKLRYL480
Kl-Nth1p/1-754  337 FIFEIDHYGKILNANRSYYLCRSQPPFLTDMALQVCRKMGGDKNP-VAVDLLRRAFKAAIK-----EYLTVWTASPRLDEKTGLSCYHPDGIGIPPETEPGHFDSILRKYAEKYNVSIPEFRDL454

B-tre/1-579     306 LKSAAESGWDFSTRWFISESGDNSGNLTNLNTK----NVIPVDLNAIFAGALQITANFQAILKNPRR----------AAHWGYMAEQWRSSIEQALWDEEDGVWHDYDILNNKPRRYFYTSNLA415
T-tre/1-555     309 LKSAAESGWDFSSRWIVDEYGGTRGNLSALHTR----RIIPVDLNAFLCQAFQKLSEFYQTLGDYPN----------ATFWSKLVKIWQHSIEMVHYNRDDGIWYDWDNELSQHRRMFFPSNFA418
R-tre/1-578     312 LKAGAESGWDFSSRWLVGSP-NPDSLGSIRTS----KLVPVDLNAFLCQAEELLSGFYSRLGNESQ----------ATKYRNLRAQRIAALTALLWDEDKGAWFDYDLENQKKNHEFYPSNLT419
E-trea/1-565    303 LRSAAASGWDFSSRWMDNPQ-----QLNTLRTT----SIVPVDLNSLMFKMEKILARASKAAGDNAM----------ANQYETLANARQKGIEKYLWNDQQGWYADYDLKSHKVRNQLTAAALF407
E-tref/1-549    317 LRAGAASGWDYSSRWLRDTG-----RLASIRTT----QFIPIDLNAFLFKLESAIANISALKGEKET----------EALFRQKASARRDAVNRYLWDDENGIYRDYDWRREQLA-LFSAAAIV420
Sc-Nth1p/1-751  452 YNDGKIKEPKLDEFFLHDRGVRESGHDTTYRFEGVCAYLATIDLNSLLYKYEIDIADFIKEFCDDKYEDPLDHSITTSAMWKEMAKIRQEKITKYMWDDESGFFFDYNTKIKHRTSYESATTFW575
Sc-Nth2p/1-780  481 YNEGMIKEPKLDAFFLHDRAVRESGHDTTYRFEGVCAYLATIDLNSLLYKEKDIAFVIKEYFGNEYKDENDGTVTDSEHWEELAELRKTRINKYMWDEDSGFFFYYNTKLKCRTSYESATTFW604
Kl-Nth1p/1-754  455 YNSQKVHEPDLDVFFLHDRGVRESGHDTTYRFENVCAYLATIDLNSLLYKYEVDIAYVIKKYFGDNFEGLPEG-HRTSNDWEKLAEVRKERIDKYLWDEETGFYYDYNVKTEKRTSYESVTTFW577

B-tre/1-579     416 PLWMNAVEKPFLAKHGARVLEYLHESQ---ALEYPGGVPVSLVNSGEQWDFPNAWPPEVSIVVTAIQNIGSEESSKLAKELAQVWVRACKSGFTEKK-QMFEKYDALNAGKYGGGG-EYTVQ--532
T-tre/1-555     419 PLWSETFDSRNAEILGEMAAEYFITQN---MMDYHGGIPTSLSHTGEQWDYPNAWPPMQSIIVMGLDKSGSYRAKQLARELARRWVKANLIGFRQTG-EMFEKYNVEVPGQNGGGG-EYVVQ-535
R-tre/1-578     420 PLWAGCFSDP---AIADKALQYLQDSQ---ILNHRHGIPTSLQNTGQQWDFPNAWAPLQDLVIRGLAKSPSARTQEVAFQLAQNWIRTNFDVYSQRS-AMYEKYDISN-AQPGGGG-EYEVQ-532
E-trea/1-565    408 PLYVNAAAKDRANKMATATKTHLLQ--------PGGLNTTSVKSGQQWDAPNGWAPLQWVATEGLQNYGQ---KEVAMDISWHFLTNVQHTYDREK-KLVEKYDVST-TGTGGGGEYPLQ-515
E-tref/1-549    421 PLYVGMANHEQADRLANAVRSRLLT--------PGGILASEYETGEQWDKPNGWAPLQWMAIQGFKMYGD---DLLGDEIARSWLKTVNQFYLEQH-KLIEKYHIADGVPREGGGGEYPLQ-529
Sc-Nth1p/1-751  576 ALWAGLATKEQAQKMVEKALPKLEMLGGLAACTERSRGPISISRPIRQWDYPFGWAPHQILAWEGLRSYGY---LTVTNRLAYRWLFMMTKAFVDYNGIVVEKYDVTRGTDPHRVEAEYGNQGA696
Sc-Nth2p/1-780  605 SLWAGLATEEQAKITVEKALPQLEMLGGLVACTEKSRGPISIDRPIRQWDYPFGWAPHQILAWKGLSAYGY---QQVATRLAYRWLYMITKSFVDYNGMVVEKYDVTRGTDPHRVAEYGNQGA725
Kl-Nth1p/1-754  578 ALWAGMSSQEQAQRMVENALPKLEEFGGLVACTARSRGELSLDRPTRQWDYPFGWAPHQILVWDGLVRYGY---ENHTRRLAYRWLFLMTKAFVDYNGIVVEKYDVTRGTDPHRVAEYGNQGA698

B-tre/1-579     533 -------DGFGWSNGVVLEFLDRYGAVLTSVDSVDASANNGQSNEESETDSKEK---                                         579
T-tre/1-555     536 -------SGFGWTNGVVLEFINQFFTT----------------------------                                          555
R-tre/1-578     533 -------EGFGWTNGVALMLLDRYGDRLSSGTQLALLEPHCLAAALLLSFLTR----                                        578
E-trea/1-565    516 -------DGFGWTNGVTLKMLDLICPKEQPCDNVPATRPTVKSATTQPSTKEAQPTP                                        565
E-tref/1-549    530 -------DGFGWTNGVVRRLIGLYG---EP--------------------------                                          549
Sc-Nth1p/1-751  697 DFKGAATEGFGWVNASYILGLKYMNSHARRALGACIPPISFFSSLRPQERNLYGL--                                         751
Sc-Nth2p/1-780  726 DFKGVATEGFGWVNTSYLLGLKYMNNHARRALAACSPPLPFFNSLKPSEKKLYYL--                                         780
Kl-Nth1p/1-754  699 DFKGVATEGFGWVNSSYLLGMKYMNNFARRALGTCVTPKVFFGRLPPKEKKKYGLE-                                         754
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**Figure 1.3:** Alignment of the predicted amino acid sequence of eight known trehalases from various organisms. Sc-Nt1p and Sc-Nth2p represent neutral trehalase from *Saccharomyces cerevisiae*. Kl-Nth1p represents neutral trehalase from *Kluyveromyces lactis*. E-Trea and Tref represent *E. coli* periplasmic and cytoplasmic trehalases

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respectively. B-tre and T-tre represent trehalases from insects *Bombyx mori* and *Tenebrio molitor*, respectively. R-Tre represents trehalase from rabbit small intestine. Alignment was produced by ClustalW2.

recognition sites ArgArg-X-Ser (Rittenhouse et al., 1986) at two different positions. One or two phosphorylation sites may therefore mediate the cAMP-dependent phosphorylation of Nth1p and possibly also of Nth2p (Kopp et al., 1994). Such an N-terminal extension seems to be important for the regulation of *S. cerevisiae* trehalase and *K. lactis* neutral trehalase by a cAMP dependent phosphorylation process. Furthermore, the relationship of the trehalases from different sources can be seen from the dendogram of Fig. 1.4. The more closely related the organisms are, the more similar the trehalases appear to be. While the trehalases from *S. cerevisiae* and *K. lactis* share a high percentage of identity, inclusion of trehalase form any of the other organisms will reduce the identity.

### 1.3.3 Regulatory and Non-regulatory trehalases

An inactive (Zymogen) form of trehalase, which is activated by cAMP-dependent phosphorylation, was reported by Van Solingen et al (1975). In 1982, Keller and co-workers demonstrated that the phosphorylatable trehalase is localized in the cytosol, whereas a second, permanently active, trehalase was found in the vacuoles. Londesborough and Varimo (1984) separated two activities and determined optimal pH for the two enzymes. The phosphorylatable enzyme localized in the cytosol had its maximal activity at pH 7 and was therefore designated the 'neutral trehalase', while the
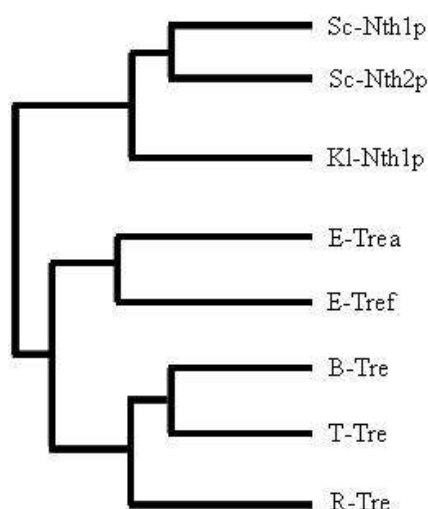
**Figure 1.4:** Dendogram of eight trehalases from various organisms. The relationship between various proteins is depicted. Sc-Nt1p and Sc-Nth2p represent neutral trehalase from *Saccharomyces cerevisiae*. Kl-Nth1p represents neutral trehalase from *Kluyveromyces lactis*. E-Trea and Tref represent *E. coli* periplasmic and cytoplasmic trehalases respectively. B-tre and T-tre represent trehalases from insects *Bombyx mori* and *Tenebrio molitor*, respectively. R-Tre represents trehalase from rabbit small intestine.

vacuolar trehalase, which has its maximal activity at pH 4.5, was designated the 'acid trehalase'. It was also demonstrated by the authors that cAMP, ATP and $Mg2^+$ activate neutral trehalase. The localization of the phosphorylated trehalase (neutral trehalase) in the cytosol complements the fact that trehalose is a cytoplasmic compound, finding that led to early speculation of a role for trehalose as a protective agent. A gene called *NTH2* (formerly called as YBR0106), was described by Wolfe and Lohan (1994). Because of the high identity of the *NTH1* and *NTH2* gene products, as well as the homology of the *NTH2* gene product to other trehalase sequences from a variety of prokaryotes and

eukaryotes, the *NTH2* gene was designated a trehalase gene. However, a role for *NTH2* in trehalose metabolism has till not been demonstrated. Recently Jules et al. (2008) has demonstrated that *NTH2* also has active trehalase activity *in vivo*.

The idea of intracellular compartmentalization of trehalase and its substrate in yeast (Myrback and Ortenblad, 1937; Avigad, 1960) came about due to the finding that starved yeast cells that contain high concentrations of trehalose also contain high trehalase activity. Further investigations after disruption and centrifugation of protoplasts indicated that trehalase is found in the soluble fraction whereas trehalose remains in the sediment (i.e. protoplast 31). From these data, it was concluded that trehalose is separated from trehalase through its binding to special sites on the cytoplasmic membrane.

The biological function of trehalase consists of control of trehalose concentration via degradation to trehalose into glucose units. Using mutants of the *nth1Δ*, Nwaka et al. (1995) has demonstrated that the neutral trehalase is the major enzyme responsible for the trehalose hydrolysis *in vivo*. The acid trehalase has so far no known trehalose-hydrolyzing activity *in vivo*, but it recognizes trehalose as a substrate *in vitro*. In a search for the role of acid trehalase in trehalose metabolism, it was found that an acid trehalase-deficient mutant (*ath1Δ*) does not grow on trehalose as a carbon source, in contrast to wild-type and *nth1Δ* mutant (Nwaka et al., 1996). This suggests a role of the acid trehalase in trehalose utilization in a manner that is different from the neutral trehalase. Furthermore, a growth defect of the acid trehalase mutant on glycerol, similar to the neutral trehalase mutant, presents possible evidence for the involvement of the two enzymes in trehalose hydrolysis *in vivo*.

**1.3.4 The acid trehalase and trehalose transport**

A trehalose carrier that is assumed to move trehalose toward both sides of the yeast cell membrane has been described (Eleutherio et al., 1993). Nothing is known about the trehalose carrier gene; however, the trehalose carrier protein is repressed by glucose, and its mutant obtained by chemical mutagenesis does not grow on trehalose as a carbon source, similar to the acid trehalase mutant. However, the trehalose carrier mutant shows high sensitivity to dehydration even in the presence of high trehalose concentration (Eleutherio et al., 1993), in contrast to the acid trehalase mutant, which results in high dehydration tolerance (Kim et al., 1996). This discrepancy between the trehalose carrier and the acid trehalase makes it unlikely that both proteins are the same. However, two trehalose transport systems have been biochemically characterized: a high-affinity $H^+$ symporter and a low-affinity transport activity (Stambuk et al., 1996). The high affinity transporter was shown to have an optimum pH of 5.0, to be repressed by glucose, and to be inhibited by uncouplers such as organic mercury compounds. Some of these characteristics are similar to what has been described for the acid trehalase (Mittenbuhler and Holzer, 1988; Alizadeh and Klionsky, 1996). These findings indicate that the acid trehalase may mediate the transport of extracellular trehalose into the cell (i.e., a second function of the acid trehalase in addition to trehalose hydrolysis).

**1.4 Stress responses in *Saccharomyces cerevisiae***

**1.4.1 Stress regulation by stress responsive elements**

In the yeast *S. cerevisiae*, stress conditions induce the expression of genes through at least two promoter elements, the heat shock element (HSE) and the stress responsive element (STRE: CCCCT) (Kobayashi and McEntee, 1990). When shifted to

high temperature, yeast cells induce the synthesis of heat shock proteins involved in the care of damaged proteins. In yeast, this response is controlled by heat shock transcription factor (Hsf1p), which binds the HSEs (Wu, 1995). This HSE also induces the synthesis of metabolic enzymes and anti-oxidants defence proteins (Boucherie et al., 1996). Several of these proteins are also induced by Msn2p and Msn4p transcription facors (Boy-Marcotte et al., 1998). The genes encoding these proteins generally have STREs in their regulatory region. In response to various stresses, the Msn2p/ Msn4p transcription factors translocate to the nucleus and activate transcription of target genes by binding to STREs (Gorner et al., 1998). STRE is known to mediate the stress induced expression of the *TPS2* gene encoding the 102-kDa subunit of the trehalose-6-phosphate synthase complex (Schmitt and McEntee, 1996). It is also present in the promoter region of other stress-induced genes. The observation of this motif in the promoter regions of the *NTH1* and *NTH2* genes, but not in the *ATH1* gene, explains the heat-stress induced expression of the genes *NTH1* and *NTH2* as well as the requirement of these genes (not of the *ATH1*) for the recovery of yeast cells after heat shock (Zahringer et al., 2000). Although the exact roles of all the stress-induced proteins are not well understood, it is evident that stress response requires the coordination of a number of gene products involved in diverse cellular functions. The regulation of the *NTH1* and *NTH2* genes by heat stress, the presence of the STRE in their respective promoter and the requirement of these genes for recovery of cells after heat shock suggests that not only trehalose biosynthesis but also its turnover as a whole may be necessary for effective stress response on yeast.

**1.4.2 Trehalose accumulation during growth under non-stress and stress conditions**

Yeast cells growing exponentially on glucose or other fermentable carbon sources (rich carbon sources), such as fructose and galactose, have very low trehalose levels as these cells exhaust their carbon source as they enter the respiratory phase of growth (diauxie). Trehalose accumulation increases until stationary phase when nutrients become limiting (Kienle et al., 1993). The extreme increase in trehalose begins after consumption of glucose. It was suggested that repression of the Tps1p by glucose and derepression of the synthesis of this enzyme after consumption of glucose might be responsible for the drastic change in concentration of trehalose from the exponential phase to the stationary phase (Francois et al., 1991).

In contrast to fermentable carbon sources, cells growing on non-fermentable carbon sources (poor carbon sources), such as glycerol, ethanol, and acetate, show high levels of trehalose during both exponential and stationary growth. (Van Dijck et al., 1995; Elliot and Futcher, 1993) Nutrient stress therefore supports the accumulation of trehalose in the cell.

In recent years, much attention has been drawn to a possible function of trehalose as a stress protectant, mainly based on the remarkable stress-protective properties *in vitro* and on the strong correlation between trehalose content and stress resistance *in vivo* (Wiemken, 1990; Van Laere, 1989). Transfer of exponentially growing cells on glucose from normal growth temperature of 30 $^{\circ}$C to heat stress temperature of 39 $^{\circ}$C for 40 to 60 min causes a rapid increase in the concentration of trehalose (Kienle et al., 1993). Also the temperature dependent increase in the concentration of trehalose is reversible: shifting the 39 $^{\circ}$C treated cells back to 30 $^{\circ}$C for 40-60 min causes a drop in the concentration back to low. This accumulation of trehalose during heat stress has

been shown to result from increase of both activity of *TPS1* (Neves and Francois, 1992) and the concentration of substrates UDP-glucose and glucose-6-phosphate (Winkler et al., 1991).

In addition to heat stress, trehalose accumulates in cells exposed to dehydration, pressure, hazardous chemicals (Hottiger et al., 1989; Hottiger et al., 1987; De Virgilio et al., 1994; D'Amore et al., 1991;). The parallelism between trehalose accumulation and tolerance to various stress conditions suggested that the major function of trehalose is not storage, but a stress protectant against adverse environmental conditions. Doubts as to the universal validity of this relationship arose when Winkler et al (1991) demonstrated that a mutant deficient in the synthesis of the heat shock protein 104 (Sanchez and Lindquist, 1990) does not exhibit thermotolerance after heat stress, even though the accumulation of trehalose was as high as in the corresponding wild-type strain suggesting that under some conditions trehalose does not mediate thermotolerance. In fact, it was shown that trehalose is important for thermoprotection in nonfermentable carbon sources and not in fermentable carbon sources (Van Dijck et al., 1995).

### 1.4.3 Regulation of the expression of genes encoding trehalases by stress conditions

Cells respond to environmental or physiological stresses by altering the expression of specific genes encoding proteins that serve a protective or adaptive role. In *S. cerevisiae*, stress induces the expression of heat shock genes, as well as genes involved in protein degradation, glycolysis, membrane function, antioxidative defense, metal homeostasis, trehalose biosynthesis (Mager and Ferreira, 1993), and trehalose hydrolysis (Nwaka et al., 1995). In contrast, the synthesis of many proteins transiently

decreases after a stress shock mainly due to cessation of RNA synthesis and degradation of preexisting mRNA. (Plesset et al., 1982). The expression of a set of the stress-induced genes is also induced when cells enter the stationary phase. This can be considered as a stress response triggered by nutrient limitation (Werner-Washburn et al., 1989). Although the exact roles of all the induced proteins are not well understood, it is evident that stress tolerance requires the coordinated activity of a number of gene products involved in diverse cellular functions.

Trehalose concentration increases during heat stress. An increase in mRNA of the *TPS1* and *TPS2* genes has been reported and these genes appear to have stress related functions (Guonalaki and Thireos, 1994). Heat and saline stress also leads to increased mRNA of *NTH1* and *NTH2* genes. This implies that these genes are regulated at both transcriptional and translational levels under stress, and their protein products can therefore be called stress proteins. The increase in neutral trehalase activity seems to result form increase in *de novo* protein synthesis due to the high *NTH1* mRNA level during heat stress and not due to a post-translational modification of existing protein. Stress may damage components of the membrane and limit the uptake of glucose and other nutrients. In this situation, in the internal supply of glucose for energy production coming from trehalose breakdown might be necessary (Nwaka et al., 1995). This situation may apply for stress conditions that involve the accumulation of trehalose and an increase in the expression of the trehalases at the same time. For some stress response-inducing chemicals, which do not favor the production of trehalose and the trehalases at the same time, other mechanisms, such as prevention of damage to cellular components after stress, might be used. This would favor a role of these proteins as chaperones.

### 1.4.4 Possible mechanism of protection of trehalose under stress

Trehalose is a non-reducing glucose disaccharide that seems to play a dual role: (1) preserving the integrity of the plasma membrane by substituting water and binding to the polar head groups of phospholipids (Crowe and Crowe, 1984), and (2) functioning as a "chaperone" by stabilizing proteins in their native state during injury as well as by suppressing the aggregation of denatured proteins (Singer and Lindquist 1998). In addition, trehalose hydrolysis provides the energy necessary for correct renaturation of proteins during stress recovery (Singer and Lindquist 1998). The protective effect of trehalose is not well understood at the molecular level. Two hypotheses (the water replacement hypothesis and the glass transition hypothesis) have been posed; Trehalose has been shown to stabilize proteins and membranes under stress conditions, especially during desiccation and heat stress (Crowe et al., 1984; Wiemken, 1990). Trehalose prevents the denaturation of proteins by replacing the water and binding via hydrogen bridges to polar residues of the protein, by the same mechanism the disaccharide prevents the fusion of membranes. In addition, trehalose forms glasses (vitrification) in the dry state, a process that may be required for the stabilization of dry macromolecules (Crowe et al., 1998).

### 1.5 Objective of this study

This study is aimed to elaborate the role of trehalose as an effective stress protectant and also to construct a stress tolerant yeast strain which will not only be useful industrially but also will serve the purpose of revealing the stress tolerance mechanism in yeast.

**1.7 Overview of the thesis**

Chapter 1 is the general introduction of this research. It contains the background of current research, and review of literature regarding trehalose, trehalases, and their stress response.

Chapter 2 describes the strategy for artificial trehalose accumulation in yeast by metabolic engineering by constructing yeast strains by removing the trehalose degradation pathway and also overexpressing trehalose biosynthesis genes and testing the constructed strains under saline stress condition. The results indicate that high trehalose accumulation prior to salt addition rather than after salt addition is necessary to achieve high growth activity under stress condition.

Chapter 3 investigates the growth and trehalose accumulation of selected recombinant strains that were able to accumulate trehalose than their parent strain under various environmental stresses to find out multiple stress resistant strains. The strains obtained in Chapter 2 were further selected to three recombinant strains along with the parent strain, and were tested under ethanol, heat, freezing and oxidative stress conditions, respectively. The results indicate that high trehalose accumulation can make yeast cells resistant to multiple stresses, but the importance of this accumulation before or after stress induction is varied depending on the type of stress.

Chapter 4 further investigates a multiple stress resistant strain along with its parent strain by DNA microarray technique in order to find out the differences in gene expression between the parent and the recombinant strains. For this, the two strains were subjected to no-stress, ethanol and heat stress conditions and the gene expression of these strains under the above three environmental conditions were determined by microarray analysis. Data analysis revealed that the recombinant strain had similar gene

expression response to the parent strains in non-stress as well as stress conditions which means that trehalose accumulation do not perturb the metabolic pathways. Moreover, the reasons for its improved growth and high trehalose content were discussed in light of microarray data comparison.

Chapter 5 summarizes the results obtained in this research and also discusses further scope of this research.

**Chapter 2**

**Effect of trehalose accumulation on response to saline stress in *Saccharomyces cerevisiae***

**2.1 Introduction**

Trehalose is a disaccharide of glucose that was formerly thought as a reserve component for a variety of organisms including yeast. Recently, evidences are accumulating that the secondary function of trehalose is a stress protective element within the cells because it has shown remarkable macromolecular protective properties *in vivo*. Under normal growth conditions, trehalose accumulates after cells enter the stationary phase (Thevelein, 1984; Van Laere, 1989; Wiemken, 1990). However, its concentration increases under several stress conditions. Recently, a whole-genome expression analysis of *S. cerevisiae* under saline stress conditions showed that the genes encoding enzymes involved in acetate, glycerol, and trehalose syntheses were upregulated under stress conditions, and that high glycerol-producing yeast strains exhibited resistance to osmotic shock (Hirasawa et al., 2006). These facts support the idea that chemically inert osmolytes such as glycerol and trehalose play important roles in osmotic tolerance.

Trehalose biosynthesis is catalyzed by the sequential action of trehalose-6-phosphate synthase (Tps1p) and trehalose-6-phosphate phosphatase (Tps2p), which form the trehalose synthase complex (Londesborough and Vuorio, 1993), using UDP-glucose and glucose-6-phosphate as the substrates. In addition, *S. cerevisiae* carries 2 types of trehalose-degradation enzymes (Argüelles, 2000): the neutral trehalases (Nth1p) localized in the cytosol and the acidic trehalase (Ath1p) found in the

vacuole. The products of *NTH1* and *ATH1* have been shown to degrade trehalose (Nwaka et al., 1994; Alizadeh and Klionsky, 1996). The *NTH2* gene product shares high homology with the *NTH1* gene product as well as trehalases from bacteria and other eukaryotes (Wolfe and Lohan, 1994). Recently, Jules et al. (2008) found and characterized *NTH2* as a trehalase-encoding gene in *S. cerevisiae*.

For effectively testing trehalose as a stress protectant, it is necessary to increase its intracellular concentration and then assess its effect. One of the strategies for intracellular accumulation of trehalose is to block trehalose degradation by deletion of the trehalase genes. In addition, overexpression of the trehalose biosynthesis genes is expected to enhance further accumulation of trehalose in deletion strains of trehalases. In this chapter, to examine the effect of trehalose accumulation on response to saline stress in *S. cerevisiae*, I constructed 7 strains with all deletion combinations of the 3 trehalase genes *ATH1*, *NTH1*, and *NTH2*. Moreover, plasmids for the overexpression of *TPS1*- and *TPS2*-encoding trehalose biosynthesis genes were separately introduced into the constructed deletion strains. Growth behavior and trehalose accumulation were observed in these strains in order to determine the combination(s) of gene deletion and overexpression that was/were effective for trehalose accumulation and good growth under high saline stress condition. It was found that the high trehalose content before addition of stress was useful for having improved growth under stress condition.

## 2.2 Materials and methods

### 2.2.1 Strains, media, and culture conditions

*S. cerevisiae* FY834 (MATα *his3-Δ200 ura3-52 leu2-Δ1 lys2-Δ202 trp1-Δ63*) was used for all experiments in this study. The strains were cultured in YPD medium

(2% glucose, 2% bacto peptone, and 1% bacto yeast extract) at 30 °C. For strain construction of *S. cerevisiae*, 2 × YPAD medium (2% glucose, 2% bacto peptone, 1% bacto yeast extract, and 0.02% adenine), YPGal medium (2% galactose, 2% bacto peptone, and 1% bacto yeast extract), SC-His medium [0.67% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), 0.192% yeast synthetic drop-out medium supplements without histidine (Sigma-Aldrich, St. Louis, MO) and 2% glucose] and SC-Ura medium [0.67% yeast nitrogen base without amino acids, 0.192% yeast synthetic drop-out medium supplements without uracil (Sigma-Aldrich) and 2% glucose] were used. For plating, 2% agar was added to the medium.

For evaluating the growth behavior of *S. cerevisiae* under high saline stress condition, 24-h-grown yeast culture was transferred into 100 ml of YPD medium in a Sakaguchi flask to achieve an optical density of around 0.1 at 600 nm, and the cells were then incubated at 30 °C with reciprocal shaking. When cell growth reached the mid-log phase (i.e., 6 h after inoculation), 25 ml of 5 M NaCl was added to the culture to achieve a final NaCl concentration of 1 M and the cells were then incubated. For calculation of the specific growth rate, values of the optical density at 600 nm obtained 7, 8, 9, and 10 h after inoculation (i.e., 1–4 h after NaCl addition) were used.

*Escherichia coli* JM109 [*e14⁻(mcr⁻) recA1 endA1 gyrA96 thi-1 hsdR17(rK⁻mK⁻) supE44 relA1 Δ(lac-proAB)/F'(traD36 proAB lacIq lacZ ΔM15)*] used for plasmid construction was cultivated in LB medium (1% bacto tryptone, 0.5% bacto yeast extract, 1% NaCl, pH 7.0) at 37 °C. If necessary, 50 µg/ml of ampicillin was added to the LB medium.

**Table 2.1.** List of primers used in this study.

| Primer name | Sequence | Purpose |
| --- | --- | --- |
| #104 | ACCTAATATTGCATCTGTTTTTACCGAAATTTCCTCATTCCAATACAGCTGAAGCTTCGTACGC | Amplification of *HIS3* for *ATH1* disruption |
| #105 | AACTTATAATATTAAAAAAAGGCTACTTATATGCATCATTTCATTGCATAGGCCACTAGTGGATCTG | Amplification of *HIS3* for *ATH1* disruption |
| #106 | GCCTGATAAACAAAAAAAGAAAAAATTAACAAAAAAAATCAGTAGACAGCTGAAGCTTCGTACGC | Amplification of *HIS3* for *NTH1* disruption |
| #107 | GACTGTACCTGGAGTATATATATATATATATATTATCTCAAGCATAGGCCACTAGTGGATCTG | Amplification of *HIS3* for *NTH1* disruption |
| #108 | GGATCAGACATTTGGTATATAAGGAGACCTGCATATACTCAAATACAGCTGAAGCTTCGTACGC | Amplification of *HIS3* for *NTH2* disruption |
| #109 | AAAGAAGAAATATAACATATATCTTATTTACCATATGTTCGTCAATAGCATAGGCCACTAGTGGATCTG | Amplification of *HIS3* for *NTH2* disruption |
| #110 | TTTCTTTCAGGTCACCTCTCAGCCG | Verification of *ATH1* disruption |
| #111 | GGTGCAAAAGACCAATTTCAAGGTC | Verification of *ATH1* disruption |
| #112 | CCTATCGTTTTCCGTAGAGT | Verification of *NTH1* disruption |
| #113 | CCTGAAGTCGAGTAAACAGA | Verification of *NTH1* disruption |
| #114 | TATTTGACGCAGGTTCGGATCAGAC | Verification of *NTH2* disruption |
| #115 | GCAAGAAGATCTGGCAAGCTACAAGG | Verification of *NTH2* disruption |
| #116 | CTTATTAAGAGGTACCACTATGACTACGG | *TPS1* amplification |
| #117 | ACGATGAGCTCATTTGCATCGGG | *TPS1* amplification |
| #118 | CACTATTTCGGTACCGAAATGACCACCAC | *TPS2* amplification |
| #119 | CTAGTCATAACGAGCTCGTTAAAAAGGG | *TPS2* amplification |
| #120 | ACTTCATTGCTTGGCGGGTCATCGC | Confirmation of integration of plasmid derived from pAURΔCENARS |
| #121 | TTCGTTTTAAAACCTAAGAGTCAC | Confirmation of integration of plasmid derived from pAURΔCENARS |
| #122 | GTTTCCATCCAAGCCGTTTTGTCC | *ACT1* amplification for probe preparation |
| #123 | AACATACGCGCACAAAAGCAGAG | *ACT1* amplification for probe preparation |

### 2.2.2 Disruption of trehalase genes in *S. cerevisiae*

Gene disruption using the disruption cassette was performed based on the method by Güldener et al. (1996). The list of primers is given in Table 2.1. The *HIS3* deletion cassette with homologous regions for each target gene was amplified by PCR from pUG6-HIS3 (Ookubo et al., 2008) using Z-Taq polymerase (Takara Bio Inc., Shiga, Japan) and the primers #104 and #105 for *ATH1*, #106 and #107 for *NTH1*, and #108 and #109 for *NTH2* (Table 2.1). After that, each amplified cassette was introduced into the FY834 strain by the lithium acetate method (Gietz and Woods, 2002) and the transformants were selected on SC-His plates.

For further deletion, a plasmid, pSH47, which carries the gene for Cre recombinase, was introduced into each disruptant, and the transformants were selected on SC-Ura medium. The Cre recombinase production was induced and HIS3 marker excision was performed by culturing the transformant in YPGal medium for 2 h at 30 °C. Finally, pSH47 was rescued from the cell by streaking the cells onto an SC-Ura plate supplemented with 0.005% uracil and 0.1% 5-fluoroorotic acid (Burke et al., 2000). Deletion of each gene was confirmed by PCR, using primers #110 and #111 for *ATH1*, #112 and #113 for *NTH1*, and #114 and #115 for *NTH2*.

### 2.2.3 Construction of *TPS1*- and *TPS2*-overexpressing strains of *S. cerevisiae*

For construction of the plasmids to overexpress the *TPS1* and *TPS2* genes, the DNA fragments were amplified by PCR from the chromosome DNA of FY834 using primers #116 and #117 for *TPS1* and #118 and #119 for *TPS2* and then cloned into the *KpnI* and *SacI* sites of a YIp-type plasmid pAURΔCENARS (Hirasawa et al., 2007); the resulting plasmids were named pAURTPS1 and pAURTPS2, respectively. Subsequently,

pAURTPS1 or pAURTPS2 was introduced into all constructed deletion strains and the transformants were selected on YPD plates containing 0.25 µg/ml of aureobacidin A (Takara Bio Inc.). In the transformants, the *TPS1* or *TPS2* gene is constitutively expressed from the *ADH1* promoter on pAURΔCENARS. Integration of the plasmids into the chromosome DNA was confirmed by PCR using primers #120 annealing to the upstream region of the *AUR1* gene on the genome of *S. cerevisiae* and #121 annealing to the ADH1 promoter region on pAURΔCENARS.

### 2.2.4 Confirmation of *TPS1* and *TPS2* genes overexpression by Northern blotting

To confirm *TPS1* and *TPS2* gene overexpression in pAURTPS1- and pAURTPS2-carrying strains, Northern blotting was performed. As a control experiment, the transcript of *ACT1* was also detected. Total RNA preparation, formaldehyde agarose gel electrophoresis, and blotting the RNA to the membrane were performed according to the method described by Hirasawa et al. (2006). For hybridization and detection of the transcripts, the Alkphos direct labelling and detection system (GE Healthcare UK, Buckinghamshire, UK) was used. For probe preparation, *TPS1* and *TPS2* gene fragments were amplified from the genomic DNA of the FY834 strain by using the primers #116 and #117 for *TPS1* and #118 and #119 for *TPS2*. For *ACT1* probe preparation, the DNA fragment was amplified by PCR using a set of primers #122 and #123.

### 2.2.5 Measurement of trehalose concentration

The trehalose concentration was measured based on the anthrone method (Spiro and Spiro, 1965). The yeast cells were quickly chilled on ice, collected by

centrifugation at 15,000 × g for 2 min at 4 °C, and washed 3 times with chilled distilled water. The cell pellets were stored at –20 °C until use. The frozen cells were suspended with 200 μl of 500 mM trichloroacetic acid (TCA), mixed vigorously, and then incubated at room temperature for 40 min to extract trehalose from the cells. Subsequently, the supernatant collected by centrifugation was transferred into new tubes, and the pellet was for re-extraction with the same amount of TCA. The supernatants from the 2 extraction procedures were mixed and used for the trehalose assay. After transferring a 200-μl aliquot of the obtained sample into a test tube, 1 ml of cold anthrone reagent [0.005% anthrone and 1% thiourea in 66% (v/v) $H_2SO_4$] was added, and then mixed vigorously, followed by incubation in boiling water for 10 min. The sample was cooled immediately in water and the absorbance of the sample was then measured at 620 nm.

It has been believed that the most soluble carbohydrate measured by the anthrone method is trehalose. For confirming the accuracy of the anthrone method for the measurement of trehalose concentration, the trehalose content in the *TPS2*-overexpressing triple deletion strain was measured by both the anthrone method and enzymatic assay method, using the trehalose measurement kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland). High correlation between the trehalose contents measured by the 2 measurement methods could be confirmed. Therefore, the data of trehalose content determined by anthrone method were presented.

**2.3 Result**

**2.3.1 Growth properties and trehalose profiles of the constructed deletion strains under non-stress condition**

The deletion strains were observed for their growth behavior and trehalose accumulation in order to find whether deletion of trehalases resulted in any altered growth or trehalose level in the cell. They were grown in YPD medium at 30 °C and samples for OD and trehalose measurements were collected in every three hours. The growth experiment and trehalose measurement were done three times for reproducibility. The results were shown in Fig. 2.1. The growth profiles and specific growth rates at the exponential phase in all the deletion strains were almost similar to that of the parent strain FY834 (Table 2.2). The log phase started 3 h after inoculation, continued for around 9 h, and the stationary phase was then observed. This similar growth pattern suggests that none of the trehalase gene deletions affect the growth of *S. cerevisiae* in YPD medium.

High trehalose accumulation was evident in the triple deletion strain (approximately 10 mg/g dry cell) at mid log phase (i.e., 9–12 h after inoculation), whereas others maintained similar trehalose levels (about 5 mg/g dry cell) to the FY834 strain (Fig. 2.1). At the late log phase, trehalose was maintained at around 5–10 mg/g dry weight in all the strains and remained low for the next few hours of the stationary phase. Increased trehalose content was observed 18 h after starting cultivation.

**2.3.2 Saline stress response in trehalase genes deletion mutants from the viewpoint of growth and trehalose accumulation**

To examine cell growth and trehalose accumulation in all deletion strains under

the saline stress condition, each strain was cultivated in YPD medium and 1 M NaCl was added to the culture at mid log phase (i.e., 6 h after inoculation). The growth of each strain was observed for 15 h and trehalose accumulation was measured from 6 to 15 h at 3-h intervals. The result of trehalose accumulation is shown in Fig. 2.2.

The trehalose content did not increase significantly immediately after NaCl addition in the parent strain, single deletion strains of *ath1Δ* and *nth2Δ*, and the double deletion strain of *nth2Δ ath1Δ* (Fig. 2.2). On the other hand, the *nth1Δ* single deletion, *nth1Δ ath1Δ* and *nth1Δ nth2Δ* double deletion, and *nth1Δ nth2Δ ath1Δ* triple deletion strains showed immediate increase in trehalose content after NaCl addition. All of these 4 strains carried the *NTH1* deletion, inferring that *NTH1* is important for trehalose degradation under conditions of stress *in vivo*.

Next, the growth of all deletion strains was compared with that of FY834. The difference in the growth activity among the strains was evaluated by comparing their specific growth rates after NaCl addition (Table 2.2). Under stress condition, the specific growth rate of the FY834 strain was 0.101 h$^{-1}$, while all other deletion strains showed a growth rate similar to the parental FY834 strain, except for the triple deletion strain, which showed a significantly higher growth rate of 0.113 h$^{-1}$. These results indicate that triple deletion might lead to improved growth due to the high accumulation of trehalose under the saline stress condition.

## 2.3.3 Effects of *TPS1* and *TPS2* overexpression on cell growth and trehalose accumulation in trehalase-deletion strains

It was reported that the deletion of *TPS1* or *TPS2* resulted in diminished trehalose accumulation (Brewster et al., 1993; Conlin and Nelson, 2007; Hounsa et al.,
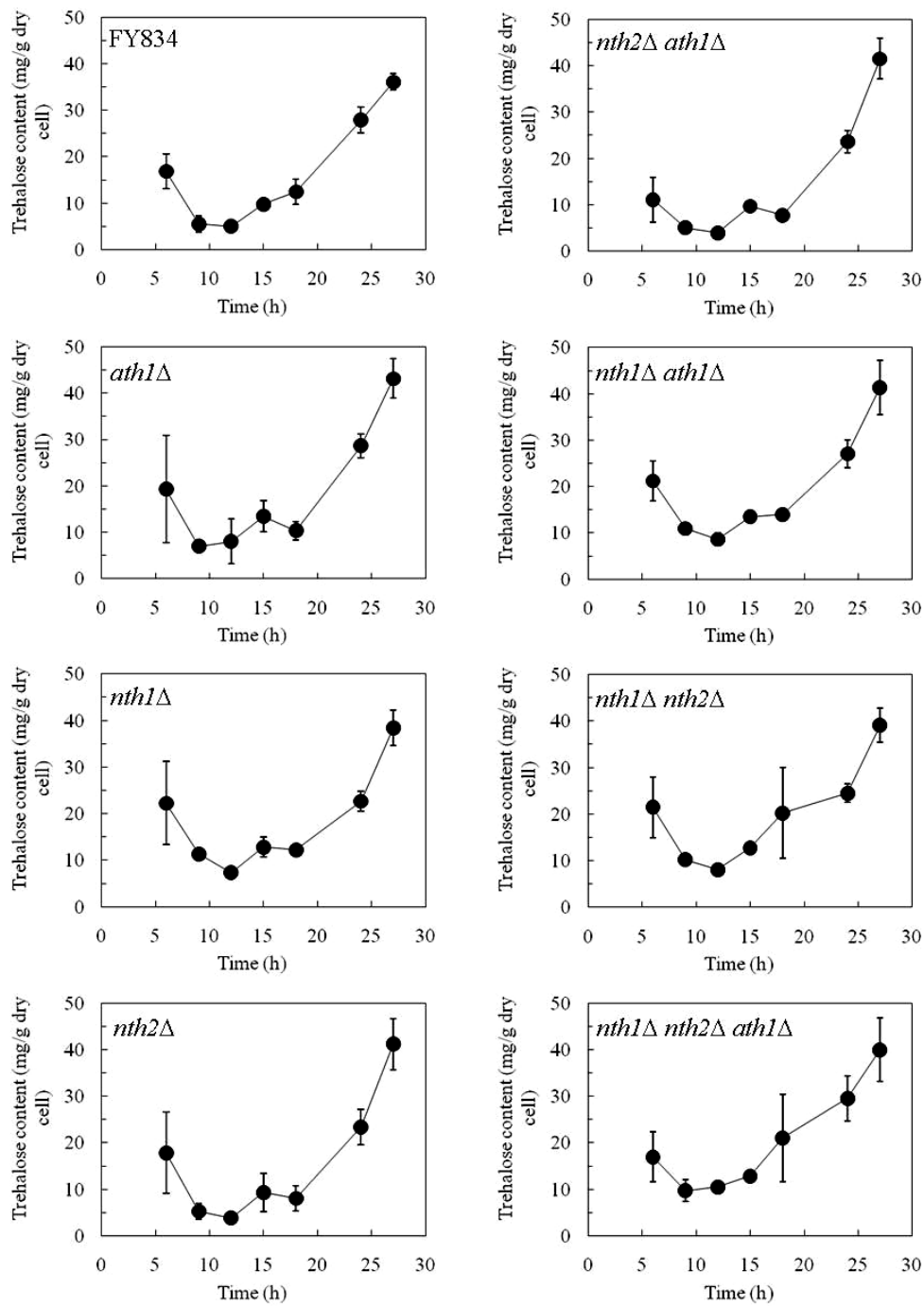
**Figure 2.1:** Trehalose content in the parental (FY834) and trehalase deletion strains under non-stress conditions. Average and standard deviation in 3 independent experiments are shown.
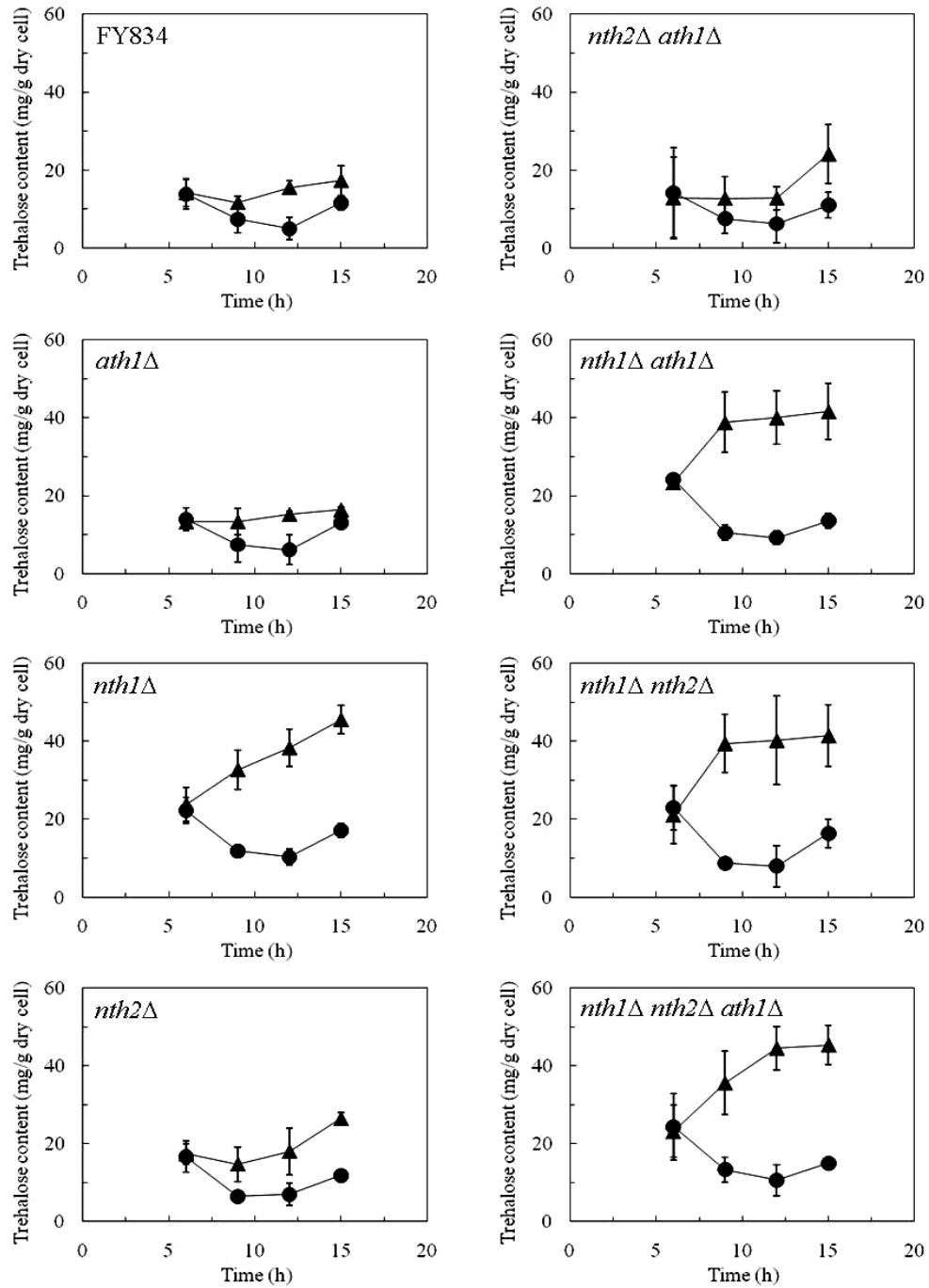
**Figure 2.2:** Trehalose accumulation in the parental (FY834) and trehalase deletion. Average and standard deviation in 3 independent experiments are shown. Circles and triangles represent the trehalose contents under non-stress and saline stress conditions, respectively.

1998). Moreover, overexpression of the *tps1* gene encoding trehalose-6-phosphate synthase could achieve high accumulation of trehalose and confer stress resistance in the fission yeast *Schizosaccharomyces pombe* (Soto et al., 1999). Thus, we next examined the effects of overexpression of *TPS1* and *TPS2* genes in all constructed trehalase gene deletion strains on further trehalose accumulation under non-stress condition. Overexpression of *TPS1* and *TPS2* genes were achieved by introducing the plasmids pAURTPS1 and pAURTPS2 carrying *TPS1* and *TPS2*, respectively, into yeast cells and were confirmed by Northern blot (Fig. 2.3).

**Table 2.2:** Specific growth rate of trehalase deletion strains under saline stress condition.

| Strain | Specific growth rate ($h^{-1}$) | |
|---|---|---|
| | Non-stress condition | Saline stress condition |
| FY834 | 0.511 ± 0.043 | 0.101 ± 0.006 |
| *ath1Δ* | 0.524 ± 0.055 | 0.089 ± 0.008 |
| *nth1Δ* | 0.499 ± 0.067 | 0.099 ± 0.009 |
| *nth2Δ* | 0.517 ± 0.070 | 0.092 ± 0.008 |
| *nth2Δ ath1Δ* | 0.528 ± 0.053 | 0.098 ± 0.011 |
| *nth1Δ ath1Δ* | 0.514 ± 0.054 | 0.098 ± 0.004 |
| *nth1Δ nth2Δ* | 0.504 ± 0.053 | 0.104 ± 0.005 |
| *nth1Δ nth2Δath1Δ* | 0.524 ± 0.056 | 0.113 ± 0.002* |

Average and standard deviation of specific growth rates in 3 independent experiments are shown. * indicates that the specific growth rate at this instance was significantly higher than that in the FY834 strain cultured under stress condition ($p < 0.05$, determined by Student's t-test).

All strains showed a growth pattern similar to the FY834 strain carrying an empty vector pAURΔCENARS under non-stress condition (data not shown), indicating that overexpression did not alter cell growth. However, several strains showed increased accumulation of trehalose at the exponential phase (Fig. 2.4). Eight strains, *TPS1*- or *TPS2*-overxpressing *nth1Δ* single deletion, *nth1Δ ath1Δ* double deletion, *nth1Δ nth2Δ* double deletion, and *nth1Δ nth2Δ ath1Δ* triple deletion strains, showed higher trehalose content throughout the exponential phase than other *TPS1*- or *TPS2*-overexpressing deletion and pAURΔCENARS-transformed FY834 strains. All of these strains showed a trehalose level of around 20–30 mg/g dry cell at the mid-log phase. This was almost twice that of the parent strain at the mid-exponential phase.

## 2.3.4 Effects of *TPS1* and *TPS2* overexpression on response to saline stress in trehalase genes deletion strains from the viewpoint of growth and trehalose content

To observe the effect of increased trehalose accumulation on the saline stress resistance, the overexpression strains were subjected to 1M NaCl at the mid-log phase and were observed until 15 hours for growth and trehalose accumulation. (Figs 2.5 and 2.6).

*TPS1*- and *TPS2*-overexpressing *ath1Δ* single deletion, *nth2Δ* single deletion, and *nth2Δ ath1Δ* double deletion strains showed similar constitutive trehalose levels to the control strain (around 10 mg/g dry cell), and the trehalose content did not increase immediately after NaCl addition (Figs 2.5 and 2.6); a similar phenomenon was also observed in their untransformed counterparts (Fig. 2.2). However, 12 h after Inoculation, a slight increase in the trehalose content occurred in these strains. As expected, these strains showed a specific growth rate similar to the control strain under the stress
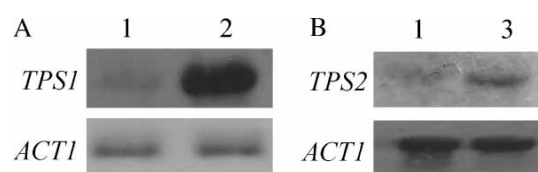
**Figure 2.3:** Overexpression of the *TPS1* and *TPS2* genes in the *nth1Δ nth2Δ ath1Δ* triple deletion strain carrying pAURTPS1 and pAURTPS2. Northern blot results for *TPS1* (A) and *TPS2* (B) transcripts in the *nth1Δ nth2Δ ath1Δ* triple deletion strain carrying pAURTPS1 and pAURTPS2, respectively, are shown as example. In addition, the result for the *ACT1* transcript in each strain is shown as control. Lane 1, pAURΔCENARS- transformed FY834; lane 2, pAURTPS1-transformed *nth1Δ nth2Δ ath1Δ* triple deletion strain; lane 3, pAURTPS2-transformed *nth1Δ nth2Δ ath1Δ* triple deletion strain.

condition (Table 2.3). On the other hand, *TPS1*-overexpressing *nth1Δ* strain showed a high trehalose content (around 25 mg/g dry cell) prior to NaCl addition and maintained almost the same trehalose level after NaCl addition (Fig. 2.5). Moreover, the specific growth rate of the *TPS1*-overexpressing *nth1Δ* strain was also higher than the FY834 strain transformed with the empty vector (Table 2.3). *TPS1*- or *TPS2*-overexpressing *nth1Δ ath1Δ* and *nth1Δ nth2Δ* double deletion strains also showed high levels of trehalose (around 20 mg/g dry cell) prior to NaCl addition (Figs 2.5 and 2.6). In addition, the *TPS1*-overexpressing *nth1Δ nth2Δ* and *nth1Δ ath1Δ* double deletion strains showed a sharp increase in trehalose content as a response to stress (Fig. 2.5).

The *TPS1*- or *TPS2*-overexpressing triple deletion strains exhibited the highest

levels of trehalose content (about 25–30 mg/g dry cell) at the mid-log phase under non-stress condition among all the strains examined (Figs 2.5 and 2.6). In the case of the *TPS1*-overexpressing triple deletion strain, there was a slight decrease in the trehalose level at 3 h after NaCl addition, followed by a slight increase immediately after NaCl addition (Fig. 2.5). On the other hand, in the *TPS2*-oeverexpressing triple deletion strain, the trehalose level was maintained constant after the slight increase in trehalose content on NaCl addition (Fig. 2.6).

Considering all these results, it is clear that a high trehalose level prior to stress exposure is important for yeast cells to grow under saline stress condition.

**Table 2.3:** Specific growth rate of *TPS1*- or *TPS2*-overexpressing trehalase deletion strains under saline stress condition. Values of the absorbance at 600 nm obtained from 7, 8, 9, and 10 h after incubation (i.e. 1-4 h after NaCl addition) were used for the specific growth rate calculation. * Growth is significantly higher than the parent strain FY834 under stress condition ($p < 0.05$, determined by Student's t-test).

| Strain | Specific growth rate ($h^{-1}$) | |
|---|---|---|
| | *TPS1*-overexpressing- | *TPS2*-overexpressing- |
| FY834 | (0.088 ± 0.007) | |
| *ath1Δ* | 0.074 ± 0.002 | 0.091 ± 0.003 |
| *nth1Δ* | 0.108 ± 0.007* | 0.095 ± 0.011 |
| *nth2Δ* | 0.089 ± 0.002 | 0.092 ± 0.004 |
| *nth2Δ ath1Δ* | 0.092 ± 0.007 | 0.066 ± 0.005 |
| *nth1Δ ath1Δ* | 0.103 ± 0.014 | 0.103 ± 0.009 |
| *nth1Δ nth2Δ* | 0.112 ± 0.009* | 0.106 ± 0.009* |
| *nth1Δ nth2Δ□ath1Δ* | 0.116 ± 0.008* | 0.109 ± 0.006* |
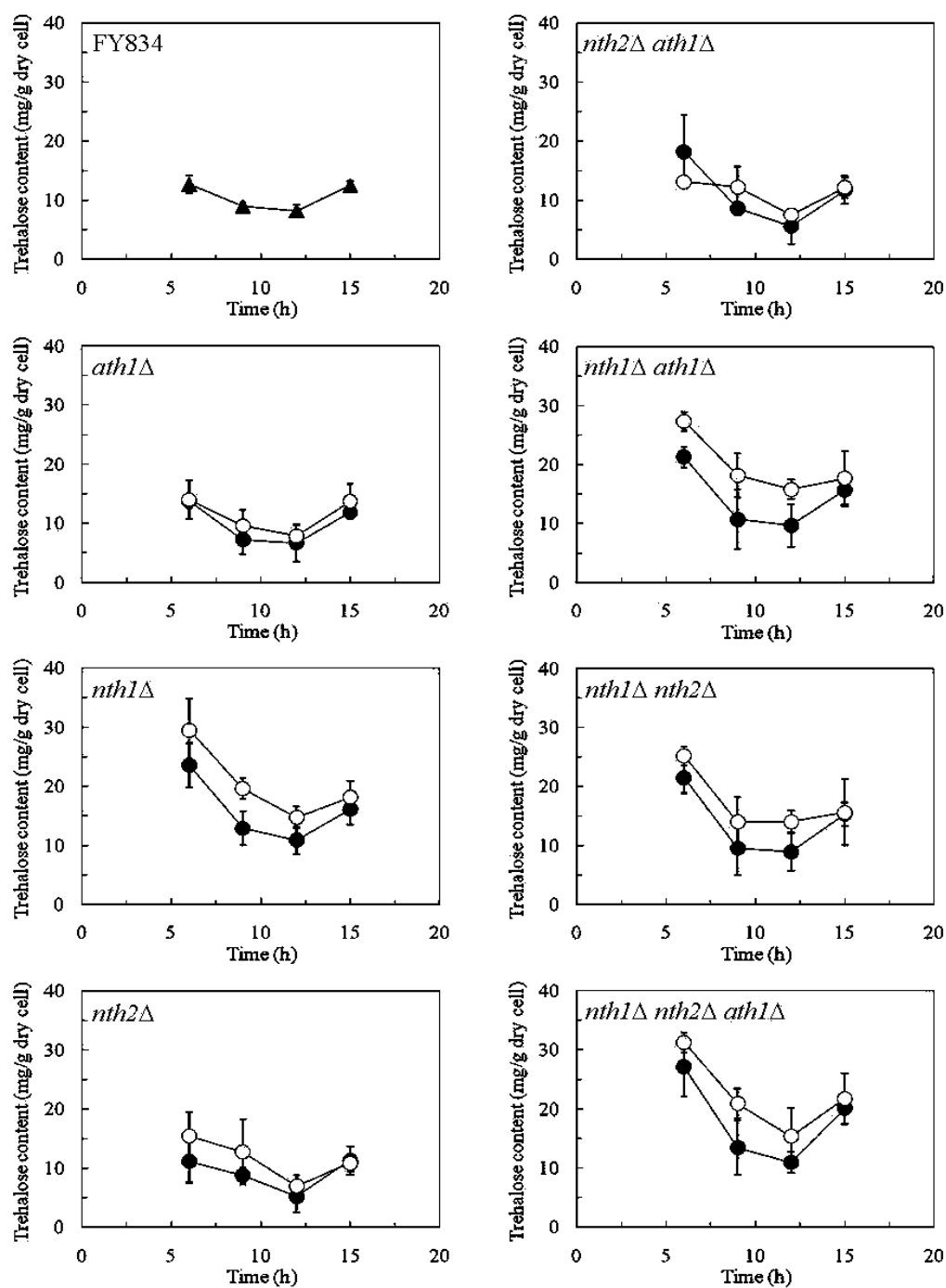
**Figure 2.4:** Trehalose contents in *TPS1*- and *TPS2*-overexpressing trehalase deletion strains under non-stress condition. Average and standard deviation in 3 independent experiments are shown. Closed and open circles represent the trehalose contents in *TPS1*- or *TPS2*-overexpressing each trehalase deletion strain, respectively. Trehalose content in FY834 strain transformed with pAURΔCENARS is shown in triangles.

**Figure 2.5:** Trehalose accumulation in *TPS1*-overexpressing trehalase deletion strains under saline stress condition. The average and standard deviation in 3 independent experiments are shown. Circles and triangles represent the trehalose contents under non-stress and saline-stress conditions, respectively. In the case of FY834, the trehalose content in the FY834 strain transformed with pAURΔCENARS is shown.

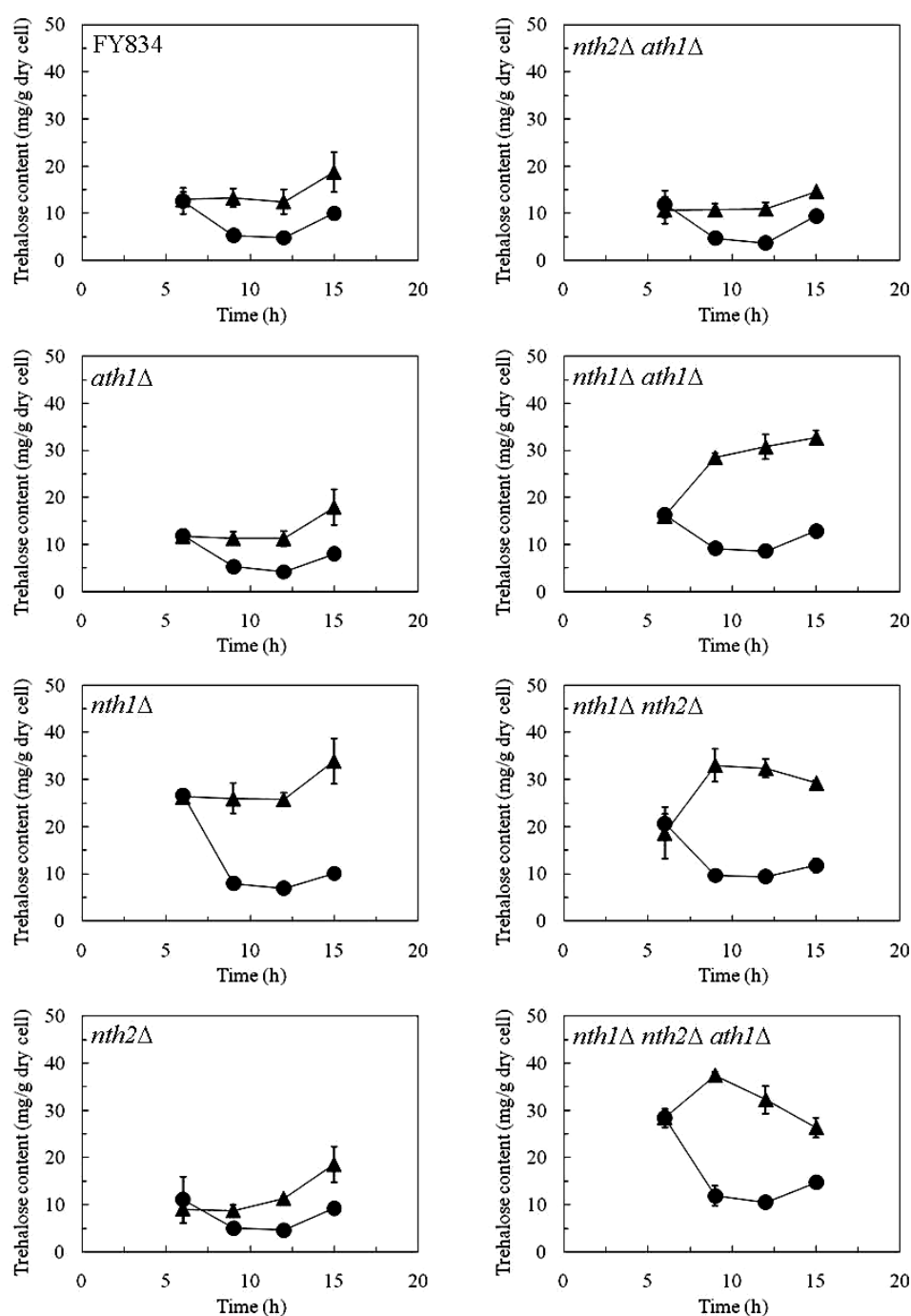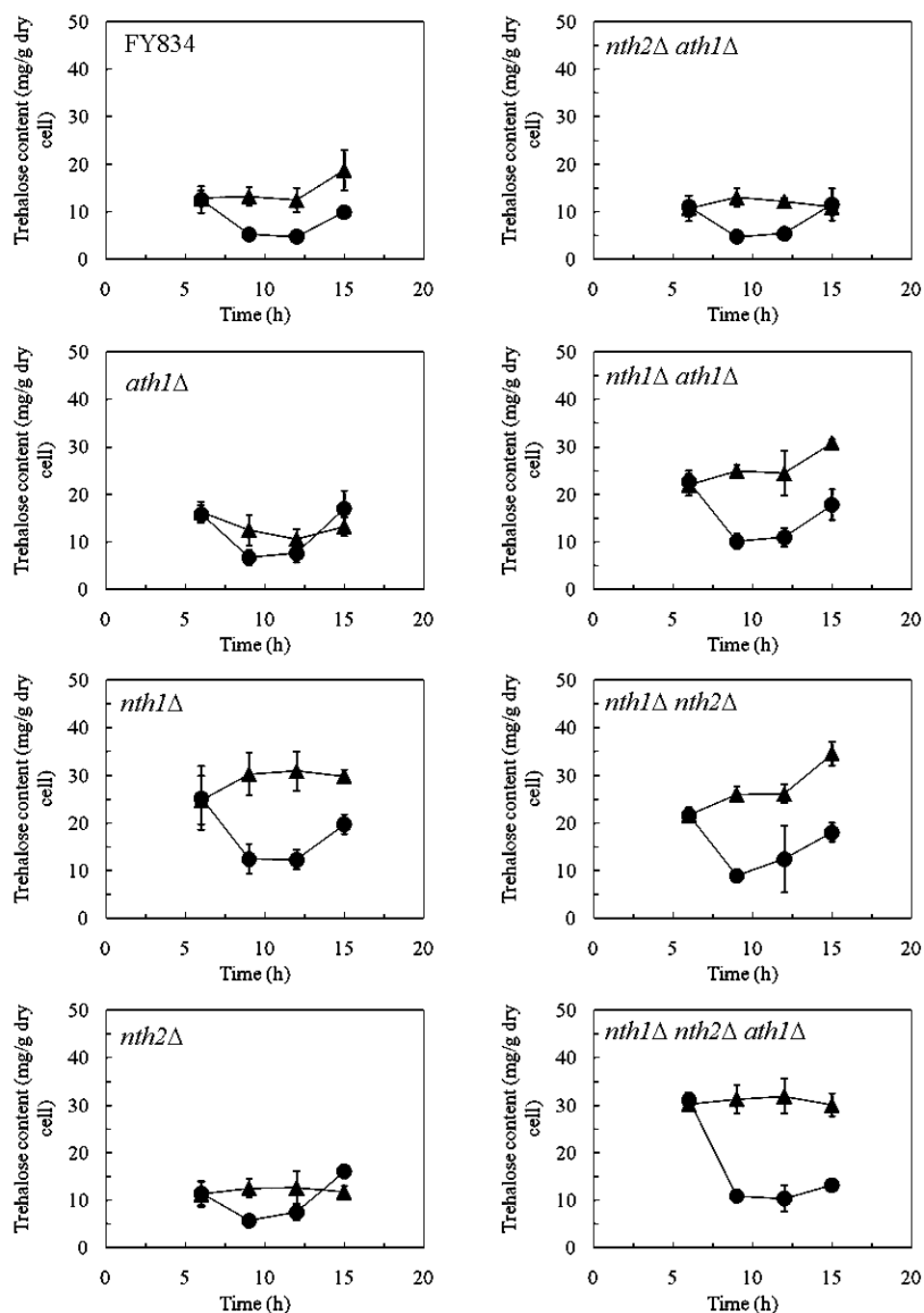**Figure 2.6:** Trehalose accumulation in *TPS2*-overexpressing trehalase deletion strains under saline stress condition. The average and standard deviation in 3 independent experiments are shown. Circles and triangles represent the trehalose contents under non-stress and saline stress conditions, respectively. In the case of FY834, the trehalose content in the FY834 strain transformed with pAURΔCENARS is shown.

**2.4 Discussion**

Trehalose is predominantly present in fungi, including yeasts, in which it is supposed to function as a reserve carbohydrate (Thevelein, 1984). In the yeast *S. cerevisiae*, trehalose levels vary depending on the environment of cell. In this chapter, we examined the effect of deletion of trehalase genes on trehalose accumulation under the saline stress condition. We constructed a set of trehalase gene deletion strains to examine the combination of trehalase gene disruptions that could achieve a high level of trehalose accumulation. Under the saline stress condition, four out of seven deletion strains, namely, a single deletion strain (*nth1Δ*), 2 double deletion strains (*nth1Δ ath1Δ* and *nth1Δ nth2Δ*), and the triple deletion strain (*nth1Δ nth2Δ ath1Δ*), showed an increased trehalose content (Fig. 2.2). Interestingly, all of these strains carry the *NTH1* deletion, supporting several reports that *NTH1* is the main trehalase in *S. cerevisiae* (Kopp et al., 1993; Nwaka et al., 1994; Nwaka et al., 1995). Moreover, trehalase gene deletion resulted in trehalose accumulation under the saline stress condition only, suggesting that the wild-type and trehalase deletion strains, except for the deletion strains carrying the *nth1Δ* deletion, perform a futile cycle of trehalose production and hydrolysis under such stress conditions, as proposed by Blomberg (2000).

Although disruption of the *ATH1* gene was previously reported to confer better survival after dehydration, freezing, and ethanol shock (Kim et al., 1996), we did not find any correlation between saline stress tolerance and *ATH1* disruption (Table 2.2). This might be due to the difference in the growth phase of the cells for evaluation of stress tolerance; in our case, we examined stress resistance during exponential growth, whereas the other study examined this at stationary phase. Cells have shown more stress tolerance in the stationary phase than during exponential growth (Hounsa et al., 1998).

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Moreover, the methods for the evaluation of stress tolerance were also different between the previous and our studies; the previous study measured remaining viability, while we measured growth activity evaluated by specific growth rate at the exponential phase.

We found that the triple deletion strains overexpressing *TPS1* or *TPS2* had the highest levels of trehalose accumulation at the exponential phase under non-stress condition (Fig. 2.4). The *TPS1*- or *TPS2*-overexpressing *nth1Δ nth2Δ* double deletion and *nth2Δ nth1Δ ath1Δ* triple deletion strains showed high trehalose accumulation at the mid-log phase and then exhibited a high specific growth rate under the saline stress condition, regardless of whether there was further increase in trehalose content after NaCl addition. On the other hand, other *TPS1*- or *TPS2*-overexpressing trehalase gene disruptants showed decreased trehalose levels, which resulted in decreased growth under the stress condition (Figs 2.4, 2.5, and 2.6 and Table 2.3). These results suggest that high trehalose accumulation before NaCl addition is important for exhibiting high growth activity under stress conditions.

In the case of NaCl stress, water is expelled from the cell and such dehydration might be encountered by accumulated trehalose to increase intracellular osmotic pressure. Intracellular accumulation of trehalose could be the reason for protecting cells from the damage caused by water dehydration and for improved growth. The protection of cellular structures and macromolecules might be the underlying reason for the greater resistance of *S. cerevisiae* under severe stress conditions. Hottiger et al. (1994) found that the physiological concentration of trehalose protects a number of enzymes against heat inactivation. Iwahashi et al. (1995) provided evidence that membrane fluidity of *S. cerevisiae* was related to trehalose accumulation, and this was related to the degree of thermotolerance.

In conclusion, we have been able to show that increased trehalose accumulation led to increased stress tolerance in *S. cerevisiae*, whereas low constitutive level of trehalose followed by sharp increase of trehalose did not make it stress resistant. Finally it is expected that even more increase of constitutional trehalose level has the potential to make strains even more tolerant to saline stress. Moreover, this property could render resistance to other stress conditions, such as heat, dehydration, ethanol and oxidative stresses.

**Chapter 3**

**Effect of trehalose accumulation on response to multiple stresses in *Saccharomyces cerevisiae***

**3.1 Introduction**

Trehalose, once thought solely to serve as a reserve carbohydrate, has recently been considered as a stress protectant in the yeast *Saccharomyces cerevisiae*. The formation of trehalose starts from glucose -6-phosphate requiring a trehalose synthetase complex in which *TPS1* and *TPS2* are the main catalysts. Trehalose is degraded by neutral trehalases, encoded by *NTH1* and *NTH2*, and also by the acidic trehalase, encoded by *ATH1*.

Under normal growth conditions, trehalose level in yeast is very low at the exponential phase and the increase is only observed at the late stationary phase which also simulates the stress condition. The increase in trehalose content is thought to be contributed to the increase in the amount of trehalose synthesis pathway enzymes. It has been shown that mRNAs corresponding to *NTH1* and *TPS2* increased during heat shock from 30 to 40 $^{o}$C (Nwaka et al., 1995; De Virgilio et al., 1993). The *TPS1* gene product also increased when yeast cells were shifted from 27 to 40 $^{o}$C, as shown by western blot analysis (Bell et al. 1992). It has been thought that increase in trehalose content after stress might be correlated with the increase of stress tolerance. However, in Chapter 2, it was shown that the increase in trehalose after addition of saline stress could not make the cells resistant to stress. On the other hand, accumulation of trehalose before addition of stress was found to be more important for stress tolerance. This time we tested several stress conditions to find out whether accumulation of stress before or after the

stress addition was important for stress tolerance. In industrial applications of yeast, the ability to show high growth activity under high stress is an important factor for bioproduction. Therefore this chapter reveals the importance of trehalose accumulation before and/ or after stress provocation. We used four kinds of stresses based on the fact that yeast cells often encounter these stresses while producing target products in bio-industries. They are heat, oxidative, ethanol and freezing stresses. Previous reports have suggested that trehalose might be important for all of the above stresses. However, the differential importance of trehalose, i.e., whether trehalose is more important before and/or after stress provocation is still obscure. This information is very important for constructing a multiple stress resistant strain. Therefore this chapter is focused on revealing the importance of trehalose under these stress conditions.

## 3.2 Materials and methods

### 3.2.1 Strains, media, and culture conditions

We used a laboratory strain of *S. cerevisiae* FY834 (MATα *his3-Δ200 ura3-52 leu2-Δ1 lys2-Δ202 trp1-Δ63*), its *nth1Δ nth2Δ ath1Δ* triple deletion strain, and *TPS1-* or *TPS2*-overexpressing triple deletion strains (Mahmud et al., 2009a) in this study. All of these strains were grown in YPD medium (2% glucose, 2% bactopeptone, and 1% yeast extract) at 30°C.

For evaluating the growth behavior of *S. cerevisiae* under ethanol, oxidative, or heat stress, 24-h-grown yeast culture was transferred into 100 ml of YPD medium in a Sakaguchi flask to achieve an optical density (OD600) of around 0.1 at 600 nm, and the cells were then incubated at 30°C with reciprocal shaking. After cell growth reached the mid-exponential phase (OD600 ≈ 1), (i) for ethanol stress, we added pure ethanol to the

culture to achieve final concentrations of 5, 6, and 8%, (ii) for oxidative stress, we added 1 M hydrogen peroxide to the culture to achieve final concentrations of 0.2 and 0.5 mM, and (iii) for heat stress, the cultures were transferred into another incubator preheated at 38, 40, and 41.5°C, and the cells were incubated. For control experiments, the cells were grown at 30 $^o$C without any additions. We evaluated the growth activities of the constructed yeast strains under the above conditions by calculating the specific growth rate. To calculate the specific growth rate, we used OD600 values obtained 7–10 h after inoculation (i.e., 1–4 h after stress induction).

For freezing stress, the cells were first grown up to the mid-exponential phase by the same method described above, and then 1 ml of culture was maintained at –20°C for 7 days. The cell samples were thawed at room temperature for 30 min. Cell viability was determined using thawed samples at appropriate dilutions, and spreading these samples on YPD agar plates (YPD medium containing 2% agar).

### 3.2.2 Measurement of intracellular trehalose content

Cell culture (1–5 ml) was taken from Sakaguchi flasks at 6, 8, 10, and 12 h after inoculation (i.e., before and 2, 4, and 6 h after stress induction), and the cells harvested by centrifugation were washed with 1 ml of water. Cells were resuspended in 0.2 ml of water and transferred to a boiling water bath, followed by incubation for 10 min to extract intracellular compounds. After centrifugation of the boiled sample, the supernatant was obtained and used for measuring the trehalose content. For the measurement of the trehalose concentration in the supernatant, we used an enzymatic assay kit purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland). The cell dry weight used for calculating the trehalose content was determined using the

OD600 value, according to the following equation:

$$\text{Cell dry weight (g/l)} = 0.2 \times \text{OD600}$$

## 3.3 Results

### 3.3.1 Trehalose accumulation enables yeast cells to grow well under ethanol stress

The strains harboring combinations of trehalase deletion and trehalose synthase gene overexpression plus their parent strain were used to investigate the quantitative response of strains with various levels of trehalose before stress provocation. Just before stress induction, trace amounts of trehalose accumulated in the FY834 strain (Fig. 3.1). As compared to Chapter 2, the amount of trehalose determined in this strain was less. This might be due to the enzymatic method used for this chapter. The method used in Chapter 2 also detects some glucose.

On the other hand, In case of non-stress condition, all of the strains gradually decreased their trehalose contents until the time of observation. In response to various concentrations of ethanol stress, FY834 did not immediately increase trehalose content until 8th hour but from 10th hour, concentrations increased gradually. For the other recombinant strains, an immediate increase of trehalose was observed just after addition of ethanol and amount of increase was directly correlated with the increasing concentration of ethanol. Moreover, the *TPS1*- and *TPS2*- overexpression strains had more increase of trehalose under all concentrations of ethanol.

When the specific growth rate was measured for all of these strains from 0 to 8% ethanol stress it was found that, under non stress condition, their growth rates were similar (Table 2.1). At low ethanol stress of 5%, all of the recombinant strains were able to show higher growth than the control. As the concentration increased, only the *TPS2*-

overexpressed triple deletion strain was able to show higher growth activity upto 8% ethanol. This indicates that under low to moderate degree of stress, initial trehalose accumulation helps in higher growth rate Also when the pattern of trehalose accumulation of before and after trehalose in *TPS2*- overexpressed triple deletion strain was highest and this strain also showed higher growth rate under all concentrations of ethanol stress. This strengthens the fact that trehalose was able to help cells for higher growth activity. Therefore the accumulation after ethanol addition was more important for ethanol stress tolerance.

**Table 3.1:** Specific growth rate of the parent and recombinant strains under ethanol stress. * Growth is significantly higher than the parent strain FY834 under stress condition ($p < 0.05$, determined by Student's t-test).

| Strain | Specific growth rate ($h^{-1}$) | | | |
|---|---|---|---|---|
| | No ethanol addition | 5% ethanol | 6% ethanol | 8% ethanol |
| FY834 | $0.507 \pm 0.002$ | $0.137 \pm 0.010$ | $0.118 \pm 0.003$ | $0.076 \pm 0.003$ |
| Triple deletion strain for trehalase genes | $0.482 \pm 0.014$ | $0.168 \pm 0.013*$ | $0.138 \pm 0.015*$ | $0.082 \pm 0.004$ |
| *TPS1*-overexpressing triple deletion strain | $0.470 \pm 0.003$ | $0.163 \pm 0.005*$ | $0.124 \pm 0.006$ | $0.074 \pm 0.004$ |
| *TPS2*-overexpressing triple deletion strain | $0.520 \pm 0.014$ | $0.169 \pm 0.007*$ | $0.135 \pm 0.003*$ | $0.090 \pm 0.001*$ |

**Figure 3.1:** Trehalose content in FY834, the *nth1Δnth2Δ ath1Δ* triple deletion, *TPS1*-overexpressing triple deletion, and *TPS2*-overexpressing triple deletion strains under ethanol stress condition. Average values with standard deviation obtained in 3 independent experiments are shown. Circles, no ethanol addition; squares, 5% ethanol addition; triangles, 6% ethanol addition; diamonds, 8% ethanol addition. Arrows indicate the time (i.e. 6 h after inoculation) when ethanol was added.

**3.3.2 Trehalose accumulation is important for improved growth under heat stress**

Heat stress was applied into the recombinant and parent strains under various temperatures shifting. The results of trehalose content under heat stress are shown in Fig. 3.2. Unlike ethanol stress, heat stress resulted in immediate increase of trehalose in all of the strains including the control FY834. This increase correlated with the increase of temperature upto 41.5 $^{o}$C. As a general characteristic, none of the strains were able to increase trehalose content gradually, i.e., they reached peak at 6 or 8 th hour and then either maintained the same level or decreased further. This meant that the strains had reached their maximum amount of trehalose level at respective temperature. The highest increase was observed at 41.5 $^{o}$C, near 175 μg/g dry cell for three recombinant strains at from 8 to 12 hour of observation. This was almost twice the concentration of trehalose at the same time point in FY834. This high increase was also important as their growth rate was higher than FY834 at temperatures 38, 40 and 41.5 $^{o}$C (Table 3.2). This phenomenon suggests that not only the trehalose accumulation before heat stress is important but also the trehalose needs to be constantly present during the whole period of heat stress. An important point to note that all of the recombinant strains had similar levels of trehalose regardless of their genetic background, i.e. even though the latter two recombinant strains had trehalose synthesis genes as overexpressions, their increase in trehalose was similar to their triple deletion only counterpart. This phenomenon suggests that heat stress enables a very strong trehalose synthesis process.
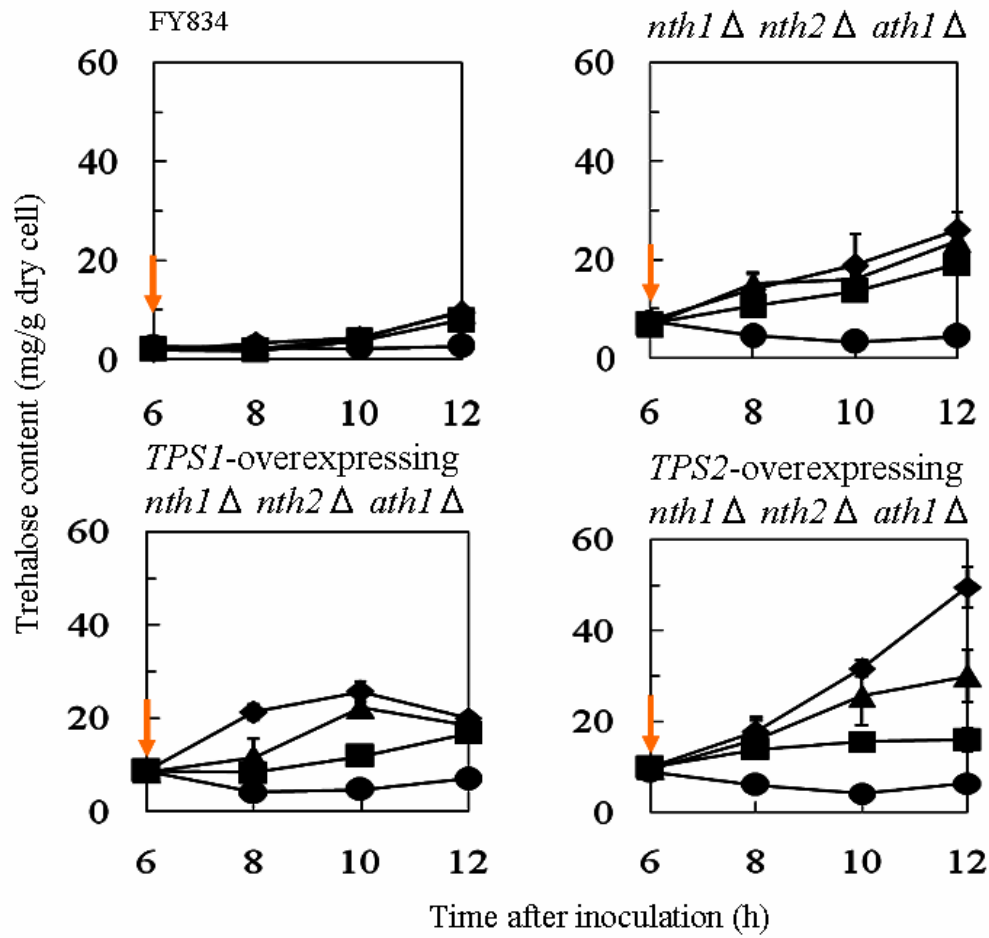
**Figure 3.2:** Trehalose content in FY834, the *nth1Δnth2Δ ath1Δ* triple deletion strain, *TPS1*-overexpressing triple deletion strain, and *TPS2*-overexpressing triple deletion strain under heat stress. Average values with standard deviation obtained in 3 independent experiments are shown. Culture temperature was changed at 6 h after inoculation. Circles, 30°C; squares, 38°C; triangles 40°C; diamonds, 41.5°C.

**Table 3.2:** Specific growth rate of the parent and recombinant strains under ethanol stress. * Growth is significantly higher than the parent strain FY834 under stress condition ($p < 0.05$, determined by Student's t-test).

| Strain | Specific growth rate ($h^{-1}$) | | | |
|---|---|---|---|---|
| | 30°C | 38°C | 40°C | 41.5°C |
| FY834 | 0.507 ± 0.002 | 0.431 ± 0.003 | 0.237 ± 0.003 | 0.064 ± 0.004 |
| Triple deletion strain for trehalase genes | 0.482 ± 0.014 | 0.456 ± 0.004* | 0.259 ± 0.012* | 0.077 ± 0.002* |
| *TPS1*-overexpressing triple deletion strain | 0.470 ± 0.003* | 0.449 ± 0.007* | 0.261 ± 0.005* | 0.114 ± 0.002* |
| *TPS2*-overexpressing triple deletion strain | 0.520 ± 0.014* | 0.447 ± 0.004* | 0.260 ± 0.003* | 0.105 ± 0.003* |

### 3.3.3 Trehalose accumulation does not confer resistance to oxidative stress

Oxidative stress, in the form of $H_2O_2$, was applied at the mid-log phase. The result of trehalose content is shown in Fig. 3.3. The results indicated that only the three recombinant strains had an increase of trehalose just after addition which was followed by decrease or remaining at the same level. In case of the parent FY834, the increase was minimal, but for the other strains increase was within 1.5 fold of their respective initial concentration which means the oxidative stress does not induce trehalose synthesis gene, as much as it had its influence in ethanol or heat stress or even saline stress (Chapter 2). The highest accumulation of trehalose was observed in *TPS1*- and *TPS2*- overexpressed triple deletion strains at 12th hour of growth which was around 25 mg/ g dry cell. Table 3.3 showed that none of the strains were able to show higher growth rate as compared to the parent strain. These results indicated that trehalose accumulation neither before nor after the oxidative stress help in higher growth rate for yeast.
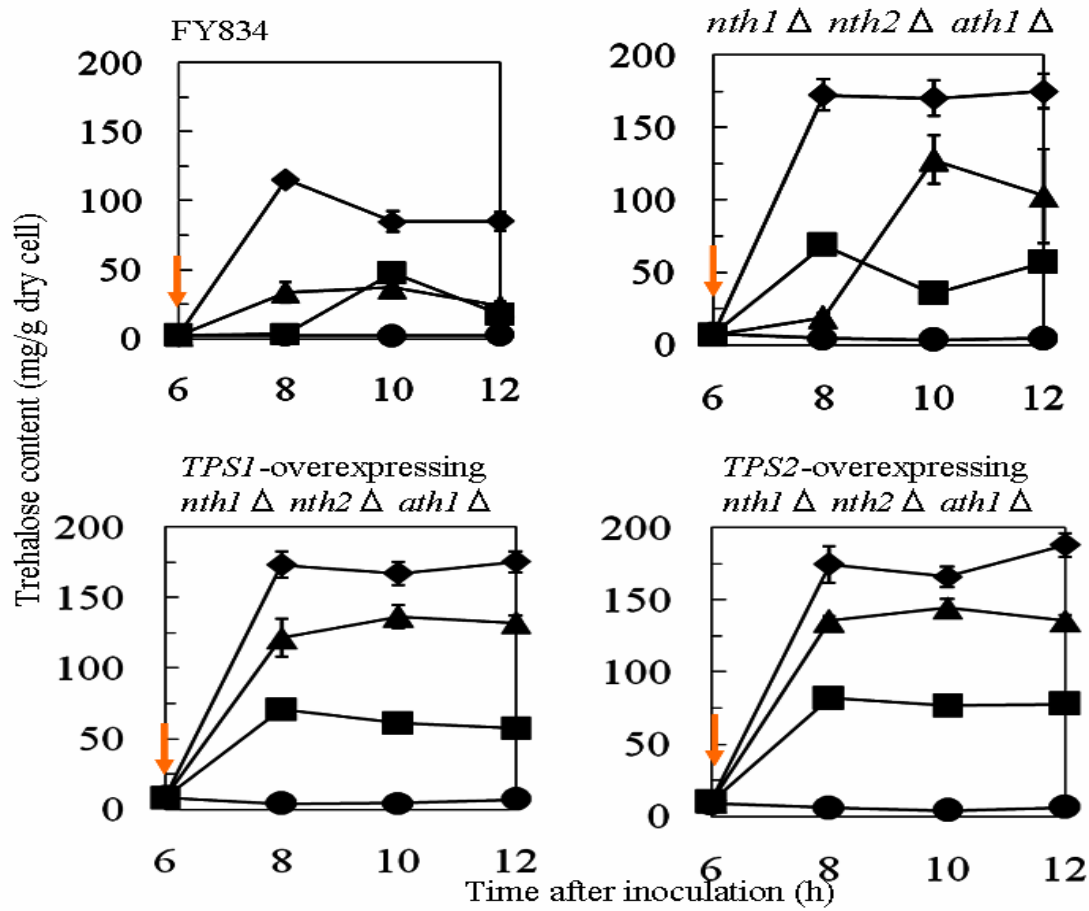
**Figure 3.3:** Trehalose content in FY834, the *nth1Δnth2Δ ath1Δ* triple deletion strain, *TPS1*-overexpressing triple deletion strain, and *TPS2*-overexpressing triple deletion strain under oxidative stress condition. Average values with standard deviation obtained in 3 independent experiments are shown. $H_2O_2$ was added to the culture at 6 h after inoculation. Circles, no $H_2O_2$ addition; squares, 0.2 mM $H_2O_2$ addition; triangles, 0.5 mM $H_2O_2$ addition.

**Table 3.3:** Specific growth rate of the parent and recombinant strains under oxidative stress. * Growth is significantly higher than the parent strain FY834 under stress condition ($p < 0.05$, determined by Student's t-test).

| Strain | Specific growth rate ($h^{-1}$) | | |
|---|---|---|---|
| | No addition | 0.2 mM $H_2O_2$ addition | 0.5 mM $H_2O_2$ addition |
| FY834 | $0.507 \pm 0.002$ | $0.389 \pm 0.003$ | $0.317 \pm 0.003$ |
| Triple deletion strain for trehalase genes | $0.482 \pm 0.014$ | $0.408 \pm 0.007$ | $0.288 \pm 0.019$ |
| *TPS1*-overexpressing triple deletion strain | $0.470 \pm 0.003$ | $0.419 \pm 0.023$ | $0.311 \pm 0.021$ |
| *TPS2*-overexpressing triple deletion strain | $0.520 \pm 0.014$ | $0.397 \pm 0.004$ | $0.322 \pm 0.030$ |

## 3.3.4 Constitutive trehalose accumulation results in increased survival under freezing stress

Finally, we examined the effect of freezing stress on the survival of trehalose-accumulating yeast strains. All strains were subjected to freezing stress for 1 week and the cell viability was measured by colony formation both before and after stress induction. As shown in Fig. 3.4, after freezing, the number of viable cells of the triple deletion strain was higher than that of the control FY834 strain. Moreover, the number of viable cells of the *TPS1*- and *TPS2*-overexpressing triple deletion strains was even higher than that of the triple deletion strain. As expected, the trehalose content in the *TPS1*- and *TPS2*-overexpressing triple deletion strains was also higher than that in the triple deletion strain. These results indicate that the gradually increasing constitutive trehalose results in high cell viability after stress induction.
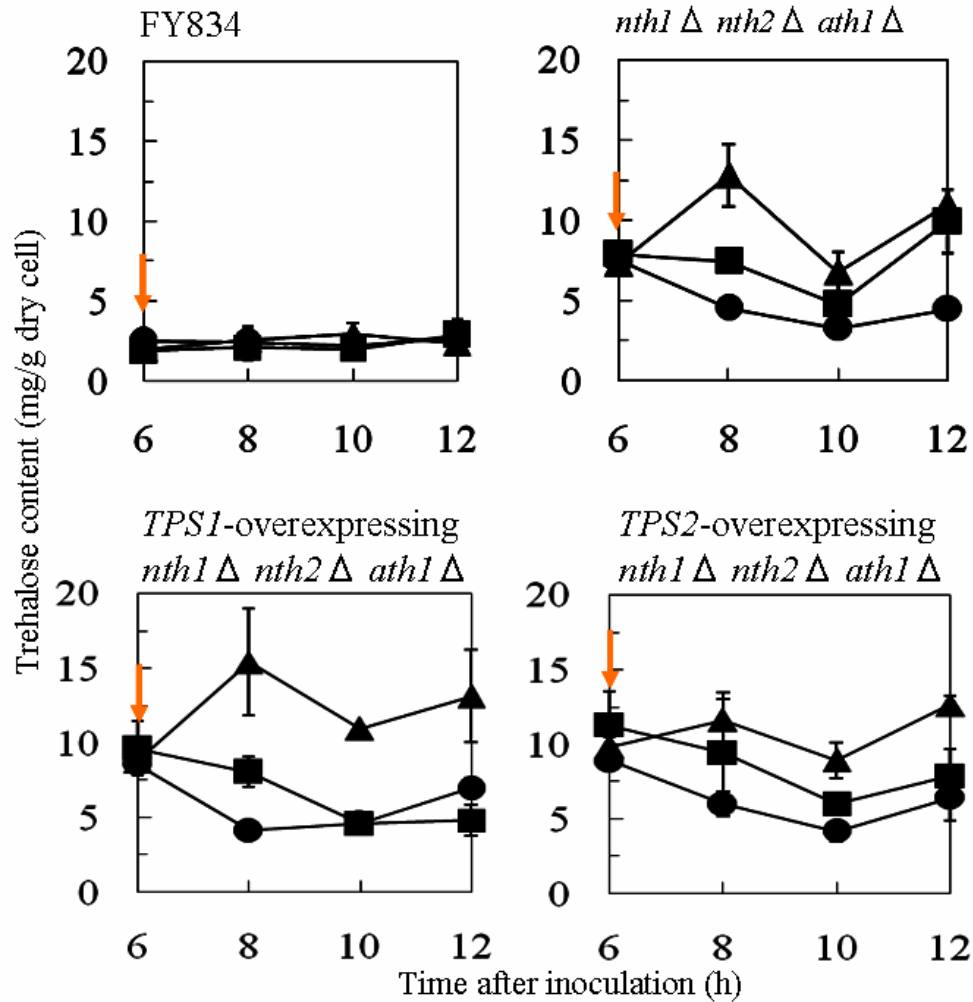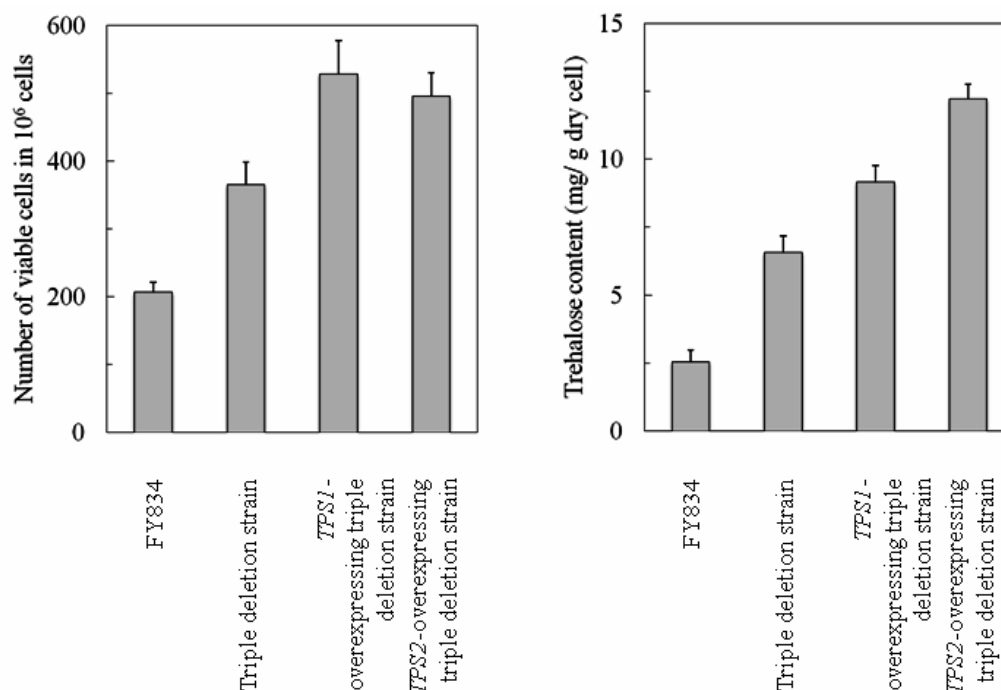
**Figure 3.4:** Cell survival (A) and trehalose contents (B) in FY834, the *nth1 Δnth2Δ ath1Δ* triple deletion strain, *TPS1*-overexpressing triple deletion strain, and *TPS2*-overexpressing triple deletion strain under freezing stress. Number of viable cells after inducing freezing stress per $10^6$ cells before stress exposure was shown. Average values with standard deviation obtained in 3 independent experiments are shown.

## 3.4 Discussion

In Chapter 2, it has been shown that trehalose accumulation before saline stress provocation might be more important than the later increase of trehalose (Mahmud et al., 2009a). Now it is important to see whether this phenomenon is also applicable to other forms of stresses. The results of chapter 2 are important in describing the stress tolerance property of yeast and the definite role of trehalose under these conditions.

From this chapter it was found that depending on the type of stress, trehalose accumulation before and/ or after stress was important for improved growth.

In our work, the futile cycling of trehalose might be active in both stresses as previously suggested by Blomberg (2000) and this was also stated in Chapter 2 where an active futile cycling was observed under saline stress. This infers that the common response of yeast under stress condition is to increase the activity of this metabolic pathway. One of the main reasons for this could be due to changing the direction metabolism towards less energy efficient pathway in order to have less ATPs. On the other hand, the level of activation of futile cycle might be different depending on the type of stress. For example, for heat stress, this cycle is more highly activated than ethanol stress which can be seen from the responses of the triple deletion strain under both stresses. Moreover, this futile cycle seemed to be dose-dependent, i.e. increasing the concentration of ethanol or increasing temperature resulted in increase amount of trehalose in the recombinant strains. Particularly, *TPS1-* and *TPS2-* overexpressed triple deletion strains had high trehalose content before the stress and their increase of trehalose after stress was also higher than their deletion only counterpart. This difference is due to the overexpressing genes inserted into their genome. The effectiveness of trehalose as protectant also varied depending on stress. In case of ethanol stress, we found that increasing concentration of ethanol resulted only *TPS2-* overexpressing strain to show higher growth rate from low to moderate stress. Conlin and Nelson (2007) also reported that increase of constitutive trehalose before heat stress enabled yeasts to have more survival rate than wild type. Previous studies also increased constitutive trehalose level by incubating yeast cells by high temperature and then transferring to other stress which resulted in increased viability (Benaroudj et al, 2001).

However for those experiments, it is thought that increasing temperature also increased other stress responsive factors which contributed to higher growth.

The relation between oxidative stress and trehalose is not straightforward as there are some contradicting reports regarding whether trehalose is really important for oxidative stress tolerance (Pedreno et al., 2002; Benaroudj et al., 2001). It has been reported that concentration of $H_2O_2$ in the range of 0.3 to 0.5 mM acts as an oxidative stress in yeast cells (Parrou et al., 1997; Zarringer et al., 1997). However, in our case, trehalose did not seem to play a role in protecting cells under various concentrations of hydrogen peroxide. da Costa Morato Nery et al. (2008) showed that trehalose can only be effective protector under oxidative stress when it is present in the both side of medium. In our case, increased accumulation of trehalose occurred only in the inner cell as the trehalose; plus the transporter responsible for export of trehalose into the medium might be inactive during exponential phase of cell growth as reported previously (Crowe et al., 1991; de Araujo et al., 1991) or might be absent due to *ATH1* deletion (Chapter 1).

Freezing stress causes osmotic shrinkage and freezing of extracellular water. According to Griffiths (1978), biological macromolecules and membranes are liable to denature due to concentration effect. Freezing also causes ice crystal formation which is harmful for cell. In our study, the increase in constitutive trehalose resulted in the increase of survival rate under freezing stress and more increase in constitutive trehalose content resulted in increased number of surviving cells. Thus it can be concluded that trehalose has a cryoprotective role.

From Chapter 2 and Chapter 3, it was possible to construct a recombinant strain that had higher concentration of trehalose both before and after stress provocation

and had higher growth rate under most of the stress conditions studied. However, we cannot say that trehalose alone was responsible for its higher growth as cells tend to tune other important metabolic pathways in order to achieve continuous growth. Therefore it is possible that the modification of trehalose metabolic pathway might activate other factors, such as, amino acids or glycerol synthesis which together give recombinant strains the growth advantage. Therefore further research is performed in Chapter 4 in order to find the differences between the multiple-stress tolerant and the parent strain.

**Chapter 4**

**Analysis of the stress tolerant yeast strain by DNA microarray**

**4.1 Introduction**

In Chapter 2, several strains were constructed that were capable of growing better than the parent strain under saline stress condition. In Chapter 3, some of those strains were further tested under several other environmental stress conditions in order to evaluate the effect of accumulation of trehalose under those stresses. The result was positive and from the results of Chapter 2 and Chapter 3, a multiple stress tolerant yeast strain was identified. This strain, a *TPS1-* overexpressing triple deletion strain, highly accumulated trehalose as compared to the parent strain under non-stress condition. Moreover, its concentration was higher than that in the parent strain under several stress conditions, such as NaCl (Chapter 2), ethanol and heat stress conditions (Chapter 3). Moreover, in all the above stresses, this strain has shown significantly higher growth rate than the parent strain. Therefore it was suggested that the trehalose accumulation is involved in better growth of this strain under stress condition. However, under stress conditions, cells undergo remodeling of entire transcriptome and translatome, which also contributes to the adaptation to stresses (Gasch et al., 2000; Causton et al., 2001). Since this tolerant strain has entire trehalose degradation pathway blocked, therefore it is expected that this accumulation of trehalose could influence the transcriptome and/ or translatome in yeast, under both non-stress and stress conditions which has the potential to uncover other novel adaptive processes. Therefore it is important to precisely elucidate the differences in transcriptional level between the parent strain and this stress tolerant strain. This will enable us to precisely understand its stress tolerance

mechanism and also will give the indication of further modification of the strain for future development. This cycle of construction and evaluation is important in understanding the overall stress response.

From the concept of central dogma, it is well-known that the flow of genetic information of cells originates from genes in nucleus, through mRNA, to proteins in cytoplasm. Proteins, in form of enzymes, combine with other components to make the metabolic network and co-operation of these factors in the cell determine its ultimate phenotype. To analyze the intracellular state under stress as well as non-stress conditions, DNA microarray is a powerful tool which allows us to carry out a comprehensive analysis in transcriptional level to identify important genes regarding to stress adaptation. This allows us to understand the important genes for stress tolerance and also gives the idea of effect of genetic perturbation in the cell. Thus in this chapter, we used DNA microarray to find out the differences between the parent and the stress tolerant strains under non-stress condition as well to identify the genes and the functional categories of the genes that could be involved in the growth under ethanol and heat stress conditions. Such analysis can provide a better understanding about the particular cellular state that enables this stress tolerant yeast to grow well under multiple stress conditions. Heat and ethanol stresses were used as the experimental stress conditions as they are the major stress factors that decrease the production of target products in the fermentation processes using yeast.

## 4.2 Materials and methods

### 4.2.1 Strains, medium and cultivation conditions

A laboratory strain of *S. cerevisiae* FY834 (MATα *his3Δ200 ura3-52 leu2Δ1*

*lys2Δ202 trp1Δ63*) and the stress tolerant strain which is a *TPS1*- overexpressing triple

trehalase deletion yeast strain (Mahmud et al., 2009a, 2009b), were used.

Yeast cells were precultured in 5 ml of YPD medium (1% bacto yeast extract,

2% bacto peptone, 2% glucose) at 30 ºC for 24 h. Then, for the main culture, the

precultured yeast cells were cultivated aerobically at 30 ºC in a 500 ml Sakaguchi flask

containing 100 ml of YPD medium. The growth of each strain was monitored by

measuring the optical density of the culture medium at 600 nm (OD600) with a

spectrophotometer, UVmini-1240 (Shimadzu Corporation, Japan). After cell growth

reached the mid-exponential phase (OD600 ≈ 1), (i) for ethanol stress, pure ethanol was

added to the culture to achieve final concentration of 5%, (ii) for heat stress, the cultures

were transferred into another incubator preheated at 40°C, and the cells were incubated

for 2 more hours before collecting samples for total RNA. For control experiments, the

cells were grown at 30 °C without any additions and the total RNA samples were

collected at the mid-log phase (i.e., after 6 h of growth).

### 4.2.2 DNA microarray experiment

Cells cultivated in YPD medium were harvested by centrifugation and were

immediately frozen in liquid nitrogen. After that, they were stored at −80 °C until

preparing the total RNA samples. Total RNA was extracted by the hot phenol method

(Kohrer and Domdey, 1991) and further purified using RNeasy Mini Kit (Qiagen, USA).

100 ng of this purifed total RNA was used to synthesize the first and second strand

cDNA. This double stranded cDNA was used to synthesize single stranded, biotin

labeled RNA strands called aRNA using Genechip 3' IVT Express Kit (Affymetrix Co.,

USA). The total RNA samples of the stress tolerant and control strains under no stress,

ethanol and heat stresses were used for this purpose. Each aRNA sample was fragmented and hybridized with cDNA probes corresponding to a part of each open reading frame of *S. cerevisiae* on a Gene Chip Microarray (Yeast genome 2 Array, ver. 2, Affymetrix, USA). The fluorescence intensity of each spot was measured using an Affymetrix scanner. The preparation of fluorescent dye-labeled aRNA targets, hybridization, and washing were carried out as described in instruction manuals supplied by the manufacturer of the DNA microarray.

### 4.2.3 Data analysis

The fluorescent signal intensities on the DNA microarray were acquired using the Affymetrix scanner software, Gene Chip Operating Software (GCOS). The signal was normalized by Quantile method (Bolstad et al., 2003). The functions of the gene products were classified by using the gene ontology (GO) terms in the *Saccharomyces* genome database (http://www.yeastgenome.org/). To evaluate whether or not a functional category was overrepresented by the genes with up- or downregulated expression, the web-implemented GO term finder (http://go.princeton.edu/ cgi-bin/GOTermFinder; Boyle et al. 2004) was used. A hypergeometric test was applied to evaluate whether or not a functional category was overrepresented by a group of selected genes from total genes in genome by the following formula:

$$f(k; N, m, n) = \frac{\binom{m}{k}\binom{N-m}{n-k}}{\binom{N}{n}}$$

Here N is the total number of genes, M is the number of genes related to a

functional category from the total genes, n is the number of genes in the selected genes, and k is the number of genes related to a functional category from among the selected genes. The data analysis was performed using Matlab ver. 2006b (Mathworks Inc., USA) and Microsoft Excel (Microsoft, USA).

### 4.2.4 Determination of glucose uptake rate

For measurement of glucose, the culture supernatant of each culture obtained by centrifugation of the culture broth was used. A glucose measurement kit (Glucose C2, Wako, Japan) was used to measure glucose in the supernatant. For calculating the glucose uptake rate, glucose concentrations obtained from 7, 8, 9, 10 and 11 h (i.e. 1-5 h after addition of heat stress) were used. The OD600 values obtained from above time points were converted into dry cell weights using the equation described in section 3.2.2.

### 4.3 Result

### 4.3.1 Transcriptional analysis of the parent and the stress tolerant yeast strains under ethanol and heat stresses

The gene expression profiles of the stress tolerant and control yeast strains under ethanol and heat stress conditions were analyzed using DNA microarray. For this, both strains were subjected to non-stress, ethanol and heat stress conditions. For each case, total RNA samples were collected and the gene expression of each strain under each condition was analyzed. Therefore for each strain, three expression data were obtained totaling to six microarray datasets to deal with.

At first all the normalized data were plotted against each other to find out the

general pattern of transcriptional perturbation upon exposure to stress. Fig. 4.1 shows the result of the scatter plot. All data represent log2 values after normalization. In each case, when ethanol and heat stress is compared within the same strain for the non-stress condition, both stress conditions resulted in large number of genes to be differentially expressed for both strains.

This figure also shows the pattern of gene expression of two organisms against each other under non-stress and stress conditions. . As shown in Chapter 3, this stress tolerant strain was able to accumulate much more trehalose before as well as after the heat and ethanol stresses, and was also able to show higher growth rate. In addition under heat stress, the stress tolerant strain showed much higher accumulation of trehalose than ethanol stress. Therefore gene expression comparison is important as it gives us the information about the transcriptional difference between two strains which helps explaining the differences in trehalose content and growth activity. For non-stress condition, it was found that stress tolerant strain did not have many differentially expressed genes under non-stress conditions as compared to the parent strain. Even though in heat stress, a large amount of trehalose accumulated in this strain (Chapter 2), the changes in transcription were unexpectedly small. This means trehalose accumulation does not affect the gene expression network under normal condition as well as stress conditions and the gene expressions of two strains behave similar way.
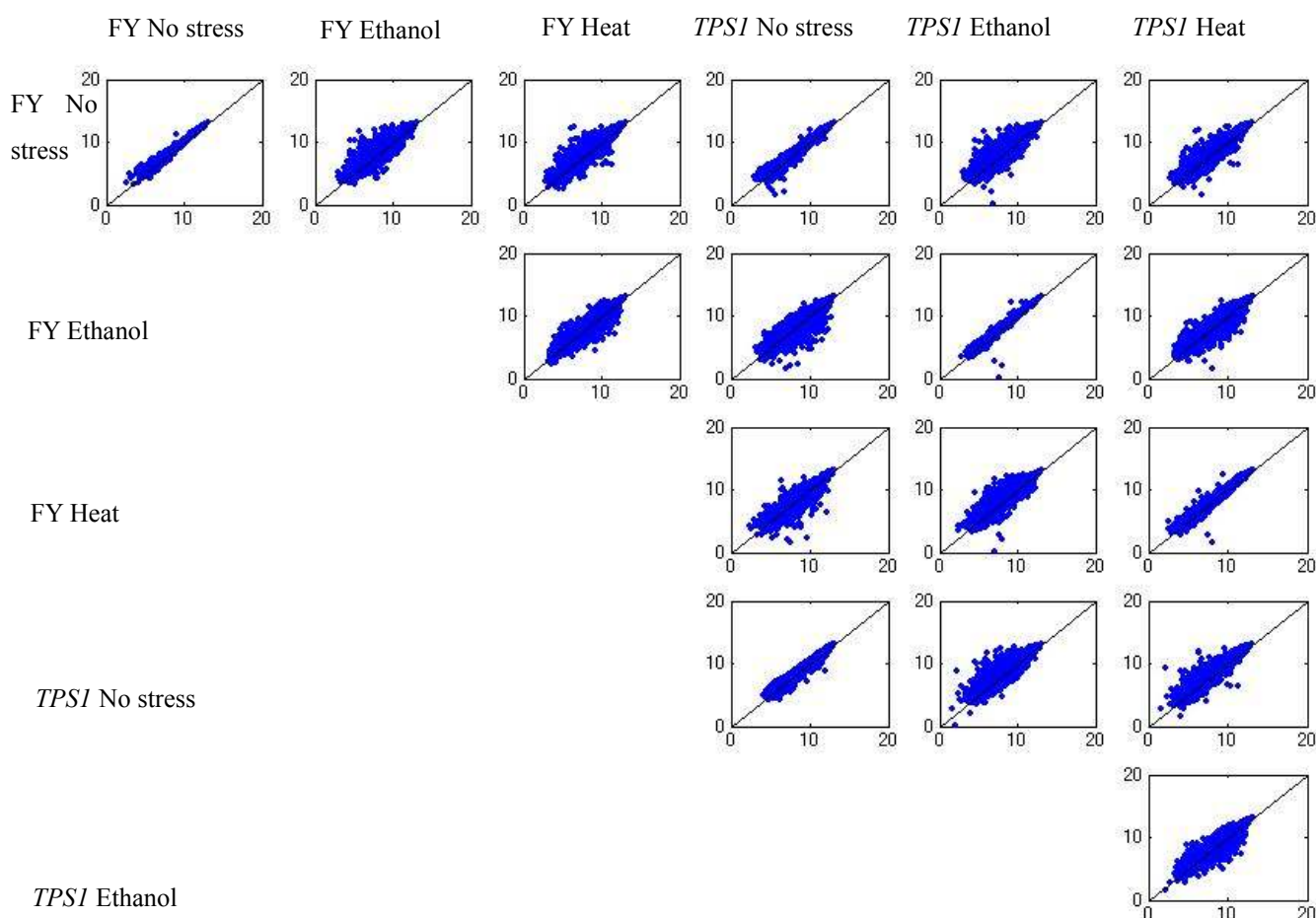
**Figure 4.1:** Scatter plot of log-transformed normalized values of gene expression of FY834 and stress tolerant strain under non-stress, heat and ethanol stress conditions. All combinations of three conditions between two strains are plotted against each other. Each dot represents the coordinates for expression values of pair of conditions. X and Y axes are the log2 transformed expression values of each array. In each graph a Y=X line is drawn to divide the up- and downregulated genes. The first figure is two separate FY834 culture under non-stress condition plotted against each other to see the reproducibility. FY, parent strain (FY834); *TPS1*, the stress tolerant strain (*TPS1*-overexpressing triple deletion strain).

**4.3.2 The response of the parent strain under ethanol and heat stress conditions**

The response of the parent and the stress tolerant strain was analyzed using the microarray data by comparing each strain to its non-stress condition and was compared with the previously published similar results. The genes which expression was increased or decreased at least two times as compared to the non-stress condition were selected for analysis. For ethanol stress, 420 and 421 genes were up and downregulated in the stress tolerant strain, respectively while for heat stress 314 and 300 genes were up and downregulated in the stress tolerant strain, respectively. The total numbers of genes that is differentially expressed more than two times for each stress conditions thus account for approximately 10% of total yeast genes. These genes were then classified using GO functional category, and the categories that were significantly overrepresented are presented from Tables 4.1 to 4.4. Table 4.1 shows functional categories of upregulated genes under ethanol stress. Functional categories of several amino acid metabolism, such as glutamate and arginine were screened. Thai et al (2009) also showed that several amino acids were upregulated in the FY834 strain under high ethanol stress. Similarly, the downregulated genes under ethanol stress were in accordance with the previous study, the genes ribosome biogenesis, translation were downregulated (Table 4.2).

Next the genes which were up- and downregulated in the FY834 under heat stress were categorized using GO biological process (Tables 4.3 and 4.4). As reported previously by Causton et al (2001) and Gasch et al (2000), the genes involved in protein folding and transport were upregulated in this strain. The induction of protein folding genes is consistent with the need to contend the widespread protein denaturation that occurs on heat stress.

**Table 4.1:** GO annotation of genes upregulated in FY834 under ethanol stress condition

as compared to non-stress condition. The cut-off value was set to be more than 2 times

or less than 0.5 times and the categories with p < 0.01 are shown.

| GO Biological process | P- value | No. of up-regulated genes | No. of total genes |
|---|---|---|---|
| organic acid metabolic process | $5.07 \times 10^{-18}$ | 64 | 304 |
| arginine biosynthetic process | $1.23 \times 10^{-12}$ | 10 | 10 |
| generation of precursor metabolites and energy | $1.75 \times 10^{-12}$ | 47 | 236 |
| urea cycle intermediate metabolic process | $2.74 \times 10^{-09}$ | 10 | 15 |
| carbohydrate metabolic process | $6.86 \times 10^{-09}$ | 40 | 231 |
| glutamine family amino acid metabolic process | $3.46 \times 10^{-08}$ | 15 | 43 |
| energy reserve metabolic process | $1.81 \times 10^{-07}$ | 13 | 36 |
| Response to stress | $3.61 \times 10^{-07}$ | 59 | 468 |
| cytochrome c oxidase complex assembly | $1.83 \times 10^{-06}$ | 6 | 8 |
| Ornithine metabolic process | $6.47 \times 10^{-06}$ | 5 | 6 |
| Aspartate family amino acid biosynthetic process | $1.20 \times 10^{-05}$ | 8 | 19 |
| lysine biosynthetic process | $1.15 \times 10^{-04}$ | 5 | 9 |
| glucan biosynthetic process | $1.73 \times 10^{-04}$ | 7 | 20 |
| alcohol biosynthetic process | $1.81 \times 10^{-04}$ | 9 | 33 |
| glucose transport | $2.38 \times 10^{-04}$ | 4 | 6 |
| Response to oxidative stress | $2.65 \times 10^{-04}$ | 13 | 66 |
| gluconeogenesis | $1.04 \times 10^{-03}$ | 7 | 26 |
| Pyruvate metabolic process | $1.80 \times 10^{-03}$ | 8 | 36 |
| serine family amino acid metabolic process | $3.60 \times 10^{-03}$ | 6 | 24 |
| regulation of carbohydrate biosynthetic process | $3.66 \times 10^{-03}$ | 5 | 17 |
| protein complex assembly | $4.15 \times 10^{-03}$ | 14 | 98 |
| glutamate catabolic process | $4.22 \times 10^{-03}$ | 2 | 2 |
| heat acclimation | $4.22 \times 10^{-03}$ | 2 | 2 |
| vitamin biosynthetic process | $4.27 \times 10^{-03}$ | 8 | 41 |
| polysaccharide biosynthetic process | $4.56 \times 10^{-03}$ | 7 | 33 |
| glycine metabolic process | $4.71 \times 10^{-03}$ | 3 | 6 |
| methionine biosynthetic process | $4.71 \times 10^{-03}$ | 3 | 6 |
| purine base biosynthetic process | $4.71 \times 10^{-03}$ | 3 | 6 |
| riboflavin biosynthetic process | $4.71 \times 10^{-03}$ | 3 | 6 |
| coenzyme metabolic process | $5.01 \times 10^{-03}$ | 17 | 132 |
| nucleobase metabolic process | $5.43 \times 10^{-03}$ | 7 | 34 |
| heterocycle metabolic process | $6.36 \times 10^{-03}$ | 11 | 72 |
| cellular protein complex assembly | $7.42 \times 10^{-03}$ | 9 | 54 |
| Aldehyde metabolic process | $7.80 \times 10^{-03}$ | 5 | 20 |
| Glycogen catabolic process | $7.85 \times 10^{-03}$ | 3 | 7 |
| homoserine metabolic process | $7.851 \times 0^{-03}$ | 3 | 7 |
| cell communication | $7.87 \times 10^{-03}$ | 24 | 219 |
| regulation of DNA replication | $7.88 \times 10^{-03}$ | 4 | 13 |
| regulation of progression through mitotic cell cycle | $7.88 \times 10^{-03}$ | 4 | 13 |

**Table 4.2:** GO annotation of genes that were downregulated in FY834 under ethanol stress condition as compared to non-stress condition. The cut-off value was set to be more than 2 times or less than 0.5 times and the categories with p < 0.01 are shown.

| GO Biological process | P- value | No. of up-regulated genes | No. of total genes |
|---|---|---|---|
| ribosome biogenesis and assembly | $7.00 \times 10^{-55}$ | 112 | 328 |
| rRNA processing | $1.81 \times 10^{-28}$ | 59 | 172 |
| 35S primary transcript processing | $4.09 \times 10^{-18}$ | 32 | 80 |
| processing of 20S pre-rRNA | $3.68 \times 10^{-14}$ | 21 | 44 |
| protein-RNA complex assembly | $2.35 \times 10^{-13}$ | 36 | 139 |
| ribosomal large subunit assembly and maintenance | $1.10 \times 10^{-08}$ | 15 | 40 |
| tRNA modification | $1.35 \times 10^{-07}$ | 15 | 47 |
| translational initiation | $3.99 \times 10^{-05}$ | 12 | 48 |
| lipid biosynthetic process | $8.99 \times 10^{-05}$ | 20 | 121 |
| Pyridine nucleotide metabolic process | $1.04 \times 10^{-04}$ | 11 | 45 |
| sulfur amino acid metabolic process | $1.40 \times 10^{-04}$ | 9 | 32 |
| oxidoreduction coenzyme metabolic process | $1.99 \times 10^{-04}$ | 12 | 56 |
| isoprenoid biosynthetic process | $2.38 \times 10^{-04}$ | 4 | 6 |
| steroid metabolic process | $3.34 \times 10^{-04}$ | 10 | 43 |
| vitamin metabolic process | $3.72 \times 10^{-04}$ | 15 | 86 |
| methionine metabolic process | $4.61 \times 10^{-04}$ | 7 | 23 |
| acetate biosynthetic process | $1.04 \times 10^{-03}$ | 3 | 4 |
| polyamine biosynthetic process | $1.04 \times 10^{-03}$ | 3 | 4 |
| protein refolding | $1.71 \times 10^{-03}$ | 4 | 9 |
| alcohol metabolic process | $2.22 \times 10^{-03}$ | 21 | 165 |
| inactivation of MAPK activity | $2.48 \times 10^{-03}$ | 3 | 5 |
| protein import into nucleus | $2.84 \times 10^{-03}$ | 9 | 47 |
| carboxylic acid biosynthetic process | $3.66 \times 10^{-03}$ | 5 | 17 |
| purine base metabolic process | $3.66 \times 10^{-03}$ | 5 | 17 |
| pentose-phosphate shunt | $4.03 \times 10^{-03}$ | 4 | 11 |
| GTP biosynthetic process | $4.22 \times 10^{-03}$ | 2 | 2 |
| alkene biosynthetic process | $4.22 \times 10^{-03}$ | 2 | 2 |
| spermine biosynthetic process | $4.22 \times 10^{-03}$ | 2 | 2 |
| terpene biosynthetic process | $4.22 \times 10^{-03}$ | 2 | 2 |
| heterocycle metabolic process | $6.36 \times 10^{-03}$ | 11 | 72 |
| protein transport | $6.36 \times 10^{-03}$ | 28 | 263 |
| acetate metabolic process | $7.85 \times 10^{-03}$ | 3 | 7 |
| polyamine metabolic process | $7.85 \times 10^{-03}$ | 3 | 7 |
| processing of 27S pre-rRNA | $7.88 \times 10^{-03}$ | 4 | 13 |

**Table 4.3:** GO annotation of genes that were upregulated in FY834 under heat stress condition as compared to non-stress condition. The cut-off value was set to be more than 2 times or less than 0.5 times and the categories with p < 0.01 are shown.

| GO Biological process | P- value | No. of up-regulated genes | No. of total genes |
|---|---|---|---|
| Protein folding | $3.27 \times 10^{-7}$ | 17 | 83 |
| Energy derivation by oxidation of organic compounds | $8.46 \times 10^{-7}$ | 27 | 198 |
| Response to stress | $9.47 \times 10^{-7}$ | 47 | 468 |
| Trehalose metabolic process | $1.69 \times 10^{-4}$ | 4 | 7 |
| glutamine family amino acid metabolic process | $1.73 \times 10^{-4}$ | 9 | 43 |
| Hexose metabolic process | $2.56 \times 10^{-4}$ | 13 | 87 |
| Amino acid catabolic process | $2.90 \times 10^{-4}$ | 7 | 28 |
| Cytochrome c oxidase complex assembly | $3.26 \times 10^{-4}$ | 4 | 8 |
| Urea cycle intermediate metabolic process | $5.23 \times 10^{-4}$ | 5 | 15 |
| Arginine biosynthetic process | $9.04 \times 10^{-4}$ | 4 | 10 |
| Non-protein amino acid metabolic process | $5.64 \times 10^{-4}$ | 4 | 9 |
| Glucose catabolic process | $1.02 \times 10^{-3}$ | 7 | 34 |
| Cellular carbohydrate catabolic process | $1.24 \times 10^{-3}$ | 11 | 78 |
| Alcohol catabolic process | $1.25 \times 10^{-3}$ | 8 | 45 |
| Coenzyme metabolic process | $1.72 \times 10^{-3}$ | 15 | 132 |
| Ornithine metabolic process | $2.03 \times 10^{-3}$ | 3 | 6 |
| Glutamate catabolic process | $2.35 \times 10^{-3}$ | 2 | 2 |
| Protein complex assembly | $2.62 \times 10^{-3}$ | 12 | 98 |
| Regulation of transcription by pheromones | $3.42 \times 10^{-3}$ | 3 | 7 |
| Response to oxidative stress | $4.27 \times 10^{-3}$ | 5 | 24 |
| Glycolysis | $5.16 \times 10^{-3}$ | 5 | 24 |
| Oxidative phosphorylation | $6.19 \times 10^{-3}$ | 7 | 46 |
| Aromatic compound catabolic process | $6.81 \times 10^{-3}$ | 2 | 3 |
| Beta-tubulin folding | $6.81 \times 10^{-3}$ | 2 | 3 |
| Methylglyoxal catabolic process | $6.81 \times 10^{-3}$ | 2 | 3 |
| Thiamin biosynthetic process | $7.83 \times 10^{-3}$ | 4 | 17 |

**Table 4.4:** GO annotation of genes downregulated in FY834 under heat stress condition as compared to non-stress condition. The cut-off value was set to be more than 2 times or less than 0.5 times and the categories with p < 0.01 are shown.

| GO Biological process | P- value | No. of up-regulated genes | No. of total genes |
|---|---|---|---|
| amino acid and derivative metabolic process | $3.84 \times 10^{-12}$ | 35 | 199 |
| nitrogen compound metabolic process | $1.79 \times 10^{-11}$ | 38 | 243 |
| carboxylic acid metabolic process | $1.15 \times 10^{-09}$ | 40 | 304 |
| sulfur amino acid metabolic process | $9.57 \times 10^{-06}$ | 9 | 32 |
| leucine biosynthetic process | $2.18 \times 10^{-05}$ | 4 | 5 |
| siderophore transport | $2.24 \times 10^{-05}$ | 5 | 9 |
| methionine metabolic process | $5.53 \times 10^{-05}$ | 7 | 23 |
| metal ion transport | $2.44 \times 10^{-04}$ | 10 | 57 |
| cell-cell adhesion | $2.93 \times 10^{-04}$ | 5 | 14 |
| cellular response to phosphate starvation | $3.81 \times 10^{-04}$ | 3 | 4 |
| sulfur amino acid transport | $9.20 \times 10^{-04}$ | 3 | 5 |
| Aspartate family amino acid metabolic process | $9.33 \times 10^{-04}$ | 8 | 45 |
| Aromatic compound metabolic process | $1.10 \times 10^{-03}$ | 9 | 57 |
| glutamine family amino acid biosynthetic process | $1.22 \times 10^{-03}$ | 6 | 27 |
| heterocycle metabolic process | $1.64 \times 10^{-03}$ | 10 | 72 |
| Response to stimulus | $1.85 \times 10^{-03}$ | 50 | 718 |
| Response to pheromone | $2.12 \times 10^{-03}$ | 11 | 87 |
| phosphatidylinositol metabolic process | $2.14 \times 10^{-03}$ | 2 | 2 |
| NAD metabolic process | $2.28 \times 10^{-03}$ | 5 | 21 |
| interphase of mitotic cell cycle | $2.79 \times 10^{-03}$ | 11 | 90 |
| glucose catabolic process to ethanol | $3.00 \times 10^{-03}$ | 3 | 7 |
| homoserine metabolic process | $3.00 \times 10^{-03}$ | 3 | 7 |
| cell division | $3.35 \times 10^{-03}$ | 26 | 319 |
| glycolytic fermentation | $4.64 \times 10^{-03}$ | 3 | 8 |
| glycerophospholipid biosynthetic process | $4.91 \times 10^{-03}$ | 6 | 35 |
| proline biosynthetic process | $6.23 \times 10^{-03}$ | 2 | 3 |
| sodium ion homeostasis | $6.23 \times 10^{-03}$ | 2 | 3 |
| regulation of transcription from RNA polII promoter | $7.99 \times 10^{-03}$ | 18 | 209 |

**4.3.3 Identification of differentially expressed genes under non-stress conditions for the stress tolerant strain**

Since the main objective of this chapter is to elucidate the differences in gene expression between the parent strain and the stress tolerant strain, it is necessary to identify the genes that are differentially expressed in the tolerant strain. From Fig. 4.1 it was clear that the difference between the parent and the tolerant strain is small under both non-stress and stress conditions, thus it is difficult to identify differentially expressed genes that could give a meaningful interpretation of the changes in phenotype. In this study, the expression ratios of more than 1.55 and less than 0.64 times were set to be differentially expressed genes. In the reproducibility experiment using FY834 under non-stress condition, these values represent 97% of the values within this range. Table 4.5 summarizes the number of genes that were identified to be differentially expressed in each strain under non-stress and stress conditions. In case of ethanol stress a large number of genes were differentially expressed than heat stress.

**Table 4.5:** Numbers of up- and downregulated genes in the stress tolerant strain compared to the parent strain under non-stress, ethanol and heat stress condition. The cut-off value was set to be more than 1.55 times or less than 0.645 times.

|  | Upregulated genes | Downregulated genes |
| --- | --- | --- |
| No stress condition | 150 | 211 |
| Ethanol stress | 75 | 58 |
| Heat stress | 227 | 151 |

Next, these genes were classified according to GO term annotations. As shown in Table 4.6, the genes related to transcription were significantly upregulated in the stress tolerant strain, which might be important for its response to stresses. This is also supported by the fact that RNA and purine biosynthetic process genes were also overexpressed in this strain. Moreover, the stress response genes were also upregulated in this strain, notably *MSN4* gene was more upregulated in the stress tolerant strain under non-stress condition which might be responsible for its improved stress response. Msn2p and Msn4p are the two important transcription factors that regulate the expression of genes that have STRE sequences in the promoter region. Therefore in order to see whether increase of *MSN4* results in the increase of genes under non-stress conditions, we selectively observed the ratios of those genes regulated by *MSN2 /MSN4*. The list of genes was retrieved from previously published papers (Treger et al., 1998). However, the expression of most of the Msn2p- and Msn4p-regulated genes remained unchanged (data not shown). This might be because the increase of the expression of *MSN4* is not so much as compared to stress conditions (approx. 2 times higher) as well as only *MSN4*, not *MSN2*, was upregulated. Previous report has stated that *MSN2*/*MSN4* both are required to activate the stress responsive factors under several stress conditions (Martinez-Pastor et al., 1996).

The downregulated genes in the stress tolerant strain mainly were amino acid synthesis and transport this means that under non-stress condition this strain does not require to synthesize amino acids like arginine and leucine.

**Table 4.6:** GO annotation of genes that were differentially expressed in the stress tolerant strain as compared to the parent strain under non-stress condition. The cut-off value was set to be more than 1.55 times or less than 0.645 times and the categories with p < 0.01 are shown.

| GO Biological processes | P-value | Genes |
|---|---|---|
| **Upregulated genes** | | |
| Transcription | $2.16 \times 10^{-5}$ | *TFC3, PDR3, PAF1, ASF2, TFG2, STB5, MCM10, FKH1, SDS3, CKA1, SPT10, ASF1, HYM1, MSN4, RRN5, MEC3, GAL80, YKU80, SIN4, RAS2, RTG1, YAP7, ESC8, HAP5, SPP1, RLF2, CSR2* |
| Chromatin silencing | $2.87 \times 10^{-5}$ | *ASF2, MCM10, FKH1, SDS3, ASF1, MEC3, YKU80, ESC8, SPP1, RLF2* |
| Developmental process | $7.00 \times 10^{-5}$ | *GIP1, BEM1, OSH2, SPR28, GIN4, RIM15, BUD13, PRS3, BNR1, FKH1, CKA1, PAN1, HYM1, MSN4, VPS51, LAS1, YKU80, RAS2, AVO1, RIM20, SPS4, BEM3* |
| Response to stimulus | $9.26 \times 10^{-5}$ | *MSN4, ASP3-4, GAL80, SIP5, RAS2,* |
| RNA biosynthetic process | $1.07 \times 10^{-4}$ | *TFC3, PDR3, PAF1, ASF2, TFG2, MCM10, FKH1, SDS3, CKA1, SPT10, ASF1, MSN4, RRN5, MEC3, GAL80, YKU80, SIN4, RAS2, RTG1, YAP7, ESC8, SPP1, RLF2, CSR2* |
| Chromatin modification | $7.70 \times 10^{-4}$ | *SHG1, PAF1, ASF2, MCM10, FKH1, SDS3, SPT10, ASF1, MEC3, YKU80, ESC8, SPP1, RLF2* |
| Response to endogenous stimulus | $9.94 \times 10^{-4}$ | *MGT1, RGT2, DIN7, CKA1, SPT10, RAD26, RAD5, MEC3, UFO1, YKU80, PSO2, RAD14, FYV6, DPB2* |
| Mitotic sister chromatid cohesion | $1.01 \times 10^{-3}$ | *MCD1, CSM3, CTF18, VIK1* |
| Heterocycle metabolic process | $1.30 \times 10^{-3}$ | *MET8, ADE3, BIO2, PRS3, DAL3, URA6, ADE4* |
| Establishment and/or maintenance of cell polarity | $1.32 \times 10^{-3}$ | *BEM1, OSH2, GIN4, BUD13, BNR1, CKA1, PAN1, LAS1, BEM3* |
| Purine ribonucleotide biosynthetic process | $1.69 \times 10^{-3}$ | *INH1, PRS3, ADE4, ATP19, ATP20* |
| Response to DNA damage stimulus | $2.06 \times 10^{-3}$ | *MGT1, DIN7, CKA1, SPT10, RAD26, RAD5, MEC3, UFO1, YKU80, PSO2, RAD14, FYV6, DPB2* |
| Mitosis | $2.23 \times 10^{-3}$ | *KIP1, DAD3, MCD1, MAD1, APC9, CSM3, CTF18, BFR1, VIK1* |
| Negative regulation of cellular metabolic process | $2.47 \times 10^{-3}$ | *ASF2, MCM10, FKH1, SDS3, ASF1, MEC3, CSM3, YKU80, ESC8, SPP1, RLF2* |
| Positive regulation of transcription by carbon catabolites | $5.10 \times 10^{-3}$ | *GAL80, RAS2* |
| Budding cell bud | $5.36 \times 10^{-3}$ | *GIN4, VPS51, LAS1, BEM3* |

| growth | | |
|---|---|---|
| Nucleobase metabolic process | $7.49 \times 10^{-3}$ | *ADE3, PRS3, URA6, ADE4* |
| Histone methylation | $7.71 \times 10^{-3}$ | *SHG1, PAF1, SPP1* |
| Regulation of cell size | $8.26 \times 10^{-3}$ | *PRS3, FKH1, HYM1, VPS51, RAS2, AVO1, RIM20, BEM3* |
| **Downregulated** | | |
| Nitrogen compound biosynthetic process | $4.86 \times 10^{-7}$ | *LYS2, LEU2, ARG5,6, MET6, STR3, MET13, TRP5, LEU1, BAT1, LYS1, ARG3, CPA2, ECM17, LEU4, ARG1, SPE2* |
| Heterophilic cell adhesion | $3.65 \times 10^{-5}$ | *FLO9, FLO1, FLO1, FLO1, AGA1* |
| Sulfur amino acid biosynthesis | $1.59 \times 10^{-4}$ | *MET6, STR3, MET13, ECM17, JLP1, JLP1, JLP1, BIO3, GSH2* |
| Arginine biosynthetic process | $1.98 \times 10^{-4}$ | *ARG5,6, ARG3, CPA2, ARG1* |
| Metal ion transport | $2.13 \times 10^{-4}$ | *ALR2, ZRT1, CCH1, SMF2, CTR2, CTR3, FET4, FRE3* |
| Leucine biosynthetic process | $3.25 \times 10^{-4}$ | *LEU2, LEU1, LEU4* |
| Glycerophospholipid metabolic process | $4.12 \times 10^{-4}$ | *CDS1, INP51, LSB6, LAS21, PLB2, ERI1, GPI2, DPM1* |
| Cofactor metabolic process | $8.71 \times 10^{-4}$ | *COQ1, ADH5, HEM1, FAU1, COQ6, SDH1, MDH1, SIS2, ADH2, BIO3, SPE2, GSH2, ISU2, FDH1* |
| Phosphoinositide metabolic process | $1.70 \times 10^{-3}$ | *INP51, LSB6, LAS21, ERI1, GPI2, DPM1* |
| Cation transport | $2.92 \times 10^{-3}$ | *PMP1, ALR2, ZRT1, CCH1, SMF2, CTR2, CTR3, FET4, FRE3* |
| Carboxylic acid transport | $5.37 \times 10^{-3}$ | *AGP2, AVT1, DAL5, MMP1, DIC1, PET8* |
| Dephosphorylation | $6.54 \times 10^{-3}$ | *PPH22, PPH21, PTP3, RTS3, INP51* |
| phospholipid metabolic process | $9.33 \times 10^{-3}$ | *CDS1, INP51, LSB6, LAS21, PLB2, ERI1, GPI2, DPM1* |
| NADH metabolic process | $9.53 \times 10^{-3}$ | *ADH5, ADH2, FDH1* |
| Copper ion import | $9.91 \times 10^{-3}$ | *CTR2, CTR3* |
| Sulfur amino acid transport | $9.91 \times 10^{-3}$ | *MMP1, PET8* |

## 4.3.4 Identification of differentially expressed genes under stress conditions for the stress tolerant strain

Tables 4.7 and 4.8 show the differentially expressed genes under ethanol and heat stresses, respectively. Since the response to ethanol stress for both strains is similar, the difference is not straightforward (Table 4.7). Some of the stress response genes were

upregulated in the tolerant strain which might explain its higher growth under ethanol stress. The two genes (*ARG81, WHI3*) were upregulated which are involved in restricting the transport of mRNA from nucleus to cytoplasm. The downregulated genes were mainly related to mitotic process which means that ethanol stress has some effect in the mitotic process of the stress tolerant strain. Therefore it seems the severity of stress is not enough to trigger change in transcriptome due to protective effect of trehalose.

Finally, functional category under heat stress is shown in Table 4.8. Here it was found that the carbohydrate transport genes containing the HXT gene family were upregulated in the stress tolerant strain under heat stress condition. Hxt proteins are involved in transport of external glucose into the yeast cytoplasm. Upregulation of these genes shows the fact that the cells could have more intracellular glucose which contributes to its faster growth. Also stress response genes were more upregulated in the recombinant strain which explains its improved response under heat stress condition.

**Table 4.7:** GO annotation of genes that were differentially expressed in the stress tolerant strain as compared to the parent strain under ethanol stress. The cut-off value was set to be more than 1.55 times or less than 0.645 times and the categories with p < 0.01 are shown.

| GO Biological processes | P-value | Genes |
|---|---|---|
| **Upregulated** | | |
| Negative regulation of transport | $3.95 \times 10^{-4}$ | *ARG81, WHI3* |
| Cell differentiation | $2.15 \times 10^{-3}$ | *UBC5, RIM4, NEM1, SGA1, RAD10, SPS18, SPS4* |
| Response to stress | $2.51 \times 10^{-3}$ | *TEL1, SCO1, TPS1, SMP1, MGT1, UBC5, RAD30, PTP3, UBA4, MGA2, ASP3-4, RAD10, HOF1* |
| Response to pheromone | $3.22 \times 10^{-3}$ | *CDC36, PTP3, MPT5, SST2, AGA1* |
| Cation transport | $4.10 \times 10^{-3}$ | *SCO1, PMA1, COX19, FRE3, ATP4* |
| Adaptation of signaling pathway | $9.52 \times 10^{-3}$ | *MPT5, SST2* |
| **Downregulated** | | |
| Coenzyme A biosynthetic process | $1.61 \times 10^{-3}$ | *SIS2, VHS3* |
| Spindle pole body duplication in nuclear envelope | $5.78 \times 10^{-3}$ | *MPS2, SPC42* |
| Transcription from RNA polymerase II promoter | $5.88 \times 10^{-3}$ | *PDR3, MED2, YAP6, SUT1, SPT10, GCR2, RTG1, AFT2* |
| Microtubule organizing center organization and biogenesis | $6.70 \times 10^{-3}$ | *MPS2, SPC42* |
| Sterol transport | $7.69 \times 10^{-3}$ | *SWH1, SUT1* |
| G1/S transition of mitotic cell cycle | $7.89 \times 10^{-3}$ | *SWI4, SIS2, VHS3* |
| Vesicle organization and biogenesis | $8.74 \times 10^{-3}$ | *MST28, VPS51* |
| Mannose inositol phosphoceramide metabolic process | $8.96 \times 10^{-3}$ | *SUR1* |
| Response to UV-B | $8.96 \times 10^{-3}$ | *WSS1* |

**Table 4.8:** GO annotation of genes that were differentially expressed in the stress tolerant strain as compared to the parent strain under heat stress. The cut-off value was set to be more than 1.55 times or less than 0.645 times and the categories with p < 0.01 are shown.

| GO Biological processes | P-value | Genes |
|---|---|---|
| **Upregulated** | | |
| Carbohydrate transport | $1.54 \times 10^{-9}$ | *MPH2, MTH1, HXT7, HXT4, HXT1, HXT5, HXT9, YMD8, HXT2, HXT11* |
| Response to abiotic stimulus | $4.73 \times 10^{-4}$ | *PTP3, SHO1, BNR1, RRD1, SIS2, VRP1, WSC2, GSH2, GAC1, GRE1, HAL1, OPY2* |
| Proline biosynthetic process | $3.59 \times 10^{-3}$ | *PRO1, PRO2* |
| Intracellular signaling cascade | $3.32 \times 10^{-3}$ | *GCN4, PTP3, RSR1, GIC1, LCB3, RHO4, WSC2, RAS2, PKH2, PKH2, BEM3* |
| Cofactor metabolic process | $4.84 \times 10^{-3}$ | *ACS1, COQ1, MET8, HEM13, FAU1, BIO2, ARN2, GND1, SIS2, ADH2, GSH2, HEM15, FDH1* |
| Response to stress | $6.75 \times 10^{-3}$ | *PHO5, TPS1, HSM3, DUN1, VID21, PRB1, GCN4, MIG3, PTP3, RAD51, SHO1, GND1, SCH9, BNR1, RRD1, XBP1, SRX1, SIS2, VRP1, HOF1, WSC2, GSH2, GAC1, GRE1, RAD53, HAL1, OPY2* |
| Cell-wall organization and biogenesis | $7.00 \times 10^{-3}$ | *KTR3, ROT2, PMI40, ECM34, CHS7, ECM27, PIR3, CWP1, SPO77, SMA2, WSC2, PKH2, PKH2, CSR2* |
| Porphyrin biosynthetic process | $9.38 \times 10^{-3}$ | *MET8, HEM13, HEM15* |
| **Downregulated** | | |
| Negative regulation of protein transport | $5.41 \times 10^{-4}$ | *WHI3* |
| Nucleobase metabolic process | $1.04 \times 10^{-3}$ | *PHO2, URA3, URA6, BAS1, ADE2* |
| Synapsis | $1.32 \times 10^{-3}$ | *ZIP1, HFM1, CST9* |
| C-terminal protein lipidation | $3.15 \times 10^{-3}$ | *ATG7, ATG3* |
| Endoplasmic reticulum organization and biogenesis | $5.16 \times 10^{-3}$ | *MYO4, SWC3* |
| Lactate metabolic process | $5.16 \times 10^{-3}$ | *DLD2, GLO4* |
| Peptide or protein carboxyl-terminal blocking | $5.16 \times 10^{-3}$ | *ATG7, ATG3* |
| Purine base metabolic process | $6.66 \times 10^{-3}$ | *PHO2, BAS1, ADE2* |

**4.3.5 Identification of the mechanism for difference in trehalose content between ethanol and heat stresses in the stress tolerant strain**

From Chapter 3, it was found that under ethanol and heat stresses the stress tolerant strain had different levels of trehalose. In case of heat stress around 175 mg/g dry cell of trehalose was accumulated which was more than 5 times than under ethanol stress. This finding is important since this could be used for further constitutive increase of trehalose in yeast. Therefore we searched our microarray data for the expression values of genes which are involved in the synthesis of trehalose from glucose. The expression values of the genes involving trehalose metabolic pathway are shown in Fig. 4.2. It was found that the expression of *PGM2*, which converts glucose-6-phosphate to glucose-1-phosphate, the first step of trehalose biosynthesis pathway, is upregulated under heat stress conditions (1.28 times higher in FY834 as compared to the stress tolerant strain). However, under both stress conditions, expression of *TPS1* is upregulated in the stress tolerant strain and in case of heat stress its value is even more that that in ethanol stress (1.51 and 1.35 times, as compared to non- and ethanol stresses, respectively). This finding also reflects the difference in trehalose content in the stress tolerant strain under ethanol and heat stresses (Chapter 3). Therefore, it is apparent that more glucose is available in the cell due to increased glucose incorporation by the Hxt proteins (Table 4.8). This might be the reason for increased trehalose content under heat stress condition.

To prove that the increase of Hxt genes expression resulted in increased glucose uptake, we measured glucose uptake rate for both the parent and the stress tolerant strain under heat stress condition (40 $^{\circ}$C). The results showed that the recombinant strain indeed had higher glucose uptake rate (1.944±0.002 g/g dry cell/ h)
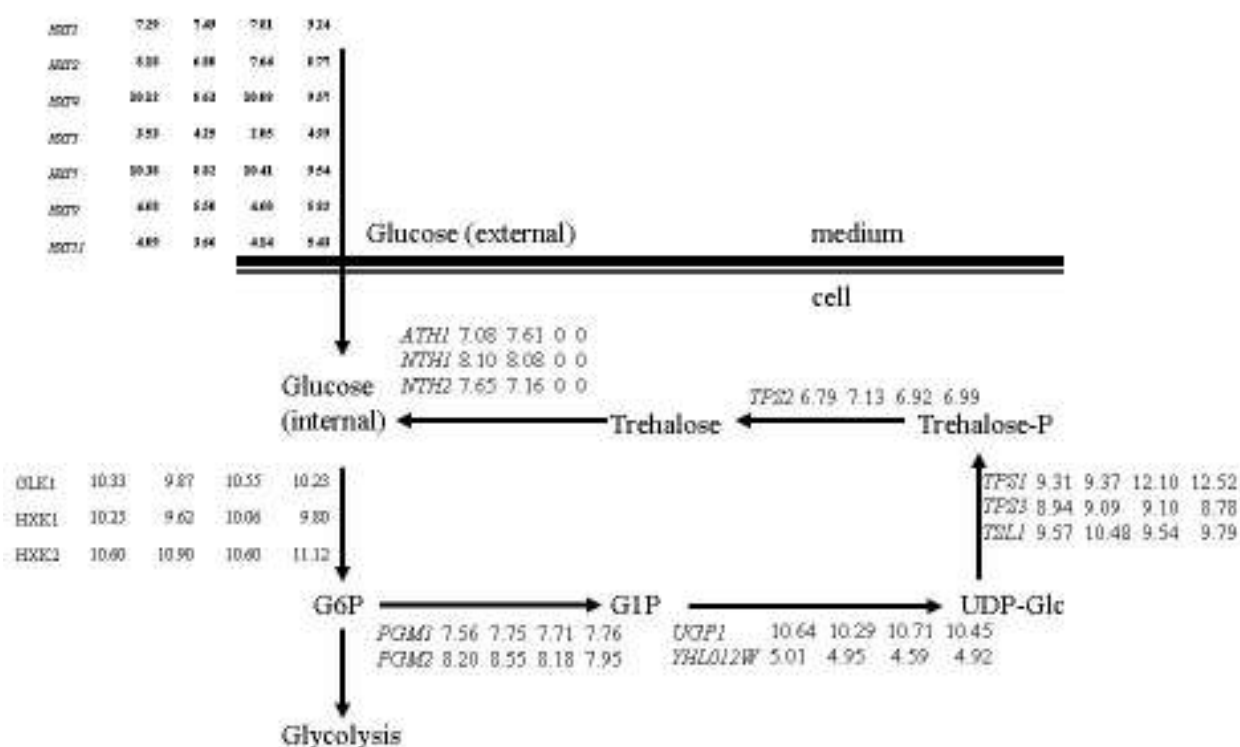
**Figure 4.2:** Expression data of the parent and the stress tolerant strains of the genes involving the trehalose metabolic pathway under ethanol and heat stress conditions. Log2 transformed expression values are shown. Values from left to right rows for each gene: FY834 under ethanol stress, FY834 under heat stress, the stress tolerant strain under ethanol stress, the stress tolerant strain under heat stress. G6P, Glucose-6-Phosphate; G1P, Glucose-1-Phosphate; UDP-Glc, UDP Glucose; Trehalose-P, Trehalose-6-Phosphate.

than the parent strain (1.784±0.079 g/g dry cell/h). Since it was also observed in Fig. 4.2 that under heat stress, trehalose synthesis gene *TPS1* is also more upregulated than that in ethanol stress, it is possible that the combination of the increased availability of glucose per cell as well as the increased ability of trehalose biosynthesis system to synthesize more trehalose per cell was responsible for more trehalose accumulation in

the heat stress as compared to ethanol stress. Other possibilities, like effect of heat in the more trehalose accumulation can be excluded by comparing with the parent strain under same heat and ethanol stress conditions. The possibility of intracellular import of trehalose from the external medium influenced by heat stress is also excluded as YPD medium used in this study does not contain trehalose. However, still there might be other possibilities for accumulation of trehalose under heat stress which cannot be explained by only observing the gene expression data as the causal relationship between the glucose uptake and the increased trehalose accumulation cannot be proved.

## 4.4 Discussion

Although not exhaustive, the genome-wide approach based on DNA microarray allows detecting changes in the transcriptional programs and functional categories in cells under variety of conditions which allows us to identify important genes. From the previous chapters, I was able to construct a stress tolerant strain that showed multiple stress resistance. In this strain, both *TPS1* overexpression and complete loss of trehalose degradation pathway resulted increase of trehalose content both before and after stress addition and ultimately rendered the yeast tolerant to several stresses. Since the tolerance to stress is a highly coordinated process involving several metabolic and signaling pathways (Causton et al., 2001; Gasch et al., 2000), it is necessary to identify any affected genes under stress conditions caused by altered trehalose content inside cells. Our result shows that the difference in gene expression between the two strains is not so much under non-stress condition. This means that elimination trehalose degradation pathway has no affect on transcriptional network of cells under non-stress condition. However, surprisingly we found that the two strains also had similar gene

expression level under heat and ethanol stress conditions; the number of differentially expressed genes in the stress tolerant strain was low, although their difference in trehalose content was large for ethanol stress and even more in case of heat stress. This affirms the fact that altered trehalose levels probably have minor effects on the overall transcriptome level even under stress conditions.

Therefore, in order to select genes that were differentially changed in the stress tolerant strain, we had to lower the threshold number in order to catch the effect of trehalose accumulation on the transcripts. One useful finding is that in the stress tolerant strain, less genes were differentially expressed than the parent strain under each stress condition (Fig. 4.1), which means the recombinant strains is less prone to environmental perturbation. Recently, Halbeisen and Garber (2009) have shown that under weak stress conditions, yeast cells can grow better than under the severe stress condition mainly because the overall transcriptome level is unaffected, or to explain in the other way, cells manage the stress conditions not to affect the mainstream cellular processes, the same phenomenon might be happening in case of the stress tolerant strain.

Although few genes were differentially changed between two strains, we were able to identify some important genes which could be used for the explanation of the higher growth rate of the stress tolerant strain than the parent strain. One useful finding was the increase in expression of the genes related to the incorporation of glucose in the cells under heat stress. Usually under stress condition glucose uptake rate is decreased as cells try to stop metabolic processes during stress. However, if cells have the option to have more glucose available in alternative way, they could grow better because they would have formed more ATPs. This seemed to be the case for the stress tolerant strain which was faster than the parent strain with more available glucose along with the help

of protective effect of trehalose.

This proves that trehalose accumulation is effective in keeping the transcription level nearly constant during stress which is enough to give the strain its growth advantage. So finally it can be said that the differences in the gene expression levels reflect the different phenotypes with respect to the growth rate under ethanol and heat stress conditions. This result of comprehensive expression analysis will be the basis for better understanding the stress tolerance and adaptation of yeast cells.

**Chapter 5**

**General conclusion**

The response to stresses is a famous field for both industrial and basic research, for a variety of organisms, from prokaryotic bacteria to eukaryotic plants. The basic survival strategy of life is to effectively tackle the stresses that organisms encounter, to ensure the survival of the fittest. Therefore to increase the fitness of the cell, one of the important criteria is the ability to tolerate stresses. The sources of stress can be multifarious; in fact any condition that does not favor normal growth activity can be regarded as stress. For laboratory and industrial studies, only a few stress conditions have widely been used along with model organism like yeast. In this research, the yeast *Saccharomyces cerevisiae*, a widely industrially used organism and an excellent laboratory model for eukaryotic study, was employed to understand the stress tolerance mechanism.

In Chapter 1, the background and significance of the research was described. General introduction mainly focused on the importance of trehalose in various stages of life cycle of yeast as well as its metabolism and occurrence. Finally the objective of thesis and outline of this research were also presented in this chapter.

In Chapter 2, the forced accumulation of trehalose in yeast cell was performed and its effect was observed under saline stress condition. Increase of trehalose was found to be only in case of strains with all the deletions of trehalase genes, and this constitutive increase resulted in increased growth activity under saline stress condition. This phenomenon was further confirmed by further increase in constitutive trehalose accumulation by overexpression of trehalose synthesis genes. Based on the observations of these overexpression strains under saline stress, it was concluded that the increased

accumulation that occurred constitutively in recombinant strain enabled the cells grow faster under saline stress. Therefore it was concluded that accumulation of trehalose before stress provocation is important of saline stress tolerance.

In Chapter 3, the conclusion obtained from Chapter 2 was further studied under other industrially important stress conditions, such as ethanol, heat, oxidative and freezing stresses. Unlike the saline stresses, the result found here was different. While for freezing stress, constitutive trehalose was indeed important for higher growth, for ethanol and heat stresses, inductive trehalose accumulation after stress provocation was found to be more effective for stress tolerance. And finally for oxidative stress, neither constitutive nor inductive trehalose accumulation could make the cells tolerant. This result shows that trehalose is important for a number of stresses, but this importance varies depending on the type of stress. Therefore considering the results of Chapter 2 and 3, I was able to find a multiple stress resistant strain which was able to grow better than the parent strain under several stress conditions, such as salt, ethanol, heat and freezing stresses. It is possible that this strain could show tolerance to more stress condition not covered in this research.

In Chapter 4, DNA microarray analysis of the stress tolerant strain was performed and this was compared with the same analysis of the parent strain. Ethanol and heat stresses, the two common stresses that yeast encounter during its industrial utilization was employed for this purpose. After analyzing the data and comparing the strains under non-stress, ethanol and heat stress conditions, differences in gene expression between the two strains were found to be smaller than expected. This meant that perturbation of trehalose pathway had no big effect on the cellular state. However, there were some minor differences which could explain the differences in growth and

trehalose content under stress conditions. For example, it was found that under heat stress condition, the stress tolerant strain was able to incorporate more glucose than the parent which could be the reason for its faster growth. Therefore it was concluded that in addition to protective effect of trehalose, other components also contributed to the increase growth of the recombinant strain under stress conditions.

In conclusion, the effect of trehalose accumulation on stress tolerance and its detail cellular affect and growth was extensively studied in this research. Accumulation of trehalose enabled yeast cell to grow faster in a number of stress conditions and its importance of accumulation before and/ after stress provocation was also clarified in this research. The difference between the stress tolerant and parent strains was identified on transcriptional dimension. The stress tolerant strain differed in number of ways from its parental counterpart which helped us explain its reason for higher growth and trehalose accumulation extensively.

To make our constructed strain useful in industry, all of the strains in this research were studied in the stress conditions that are equivalent to that yeast cells encounter during bioproduction. For example, the osmolarity of molasses, a common raw material used in fermentation industry for growing yeasts, varies form 2 to 4 mol/kg (Munene et al., 2002). In Chapter 2, the constructed strains were observed under 1M NaCl which also has an osmolarity of 2. Similarly, yeasts encounter ethanol stress at the end of industrial fermentation which range from 4% (beer fermentation) to more than 10% for bioethanol production. In our experiments, the ethanol stress conditions used was also within this range (5%, 6% and 8%). In addition, yeast cells also encounter heat stress when they are dried for preservation at the end of bioprocess which usually ranges from 38-30$^{\text{o}}$C. In our study also, we used similar temperatures to simulate industrial

heat stress conditions (38, 40 and 41.4$^{o}$C). From the evaluation of the specific growth rate, our results suggest that the multiple stress tolerant strain was able to confer resistance to a number of stresses. For the stress tolerant strain, the increase of specific growth rate was found to be as high as 1.32 times when compared to the parent strain under saline stress (Table 2.3). The same strain grew 1.22 times higher than the parent strain under 5% Ethanol stress (Table 3.2) and as high as 1.78 times under heat stress (at 41.5$^{o}$C, Table 3.2). Therefore, the cumulative effect of this multiple stress tolerance property of our constructed strain is high. In industrial fermentations using yeast, the specific growth rate is one of the most important factors for bioproduction. This is because increased specific growth rate means rapid bioconversion which results more efficient synthesis of target product. Therefore the stress tolerant strain is expected to be industrially useful.

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