ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

CHARACTERIZATION OF A SALT-RESISTANT *Saccharomyces cerevisiae* MUTANT

M.Sc. THESIS

Seçil ERBİL

Department of Advanced Technologies

Molecular Biology-Genetics and Biotechnology MSc Programme

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TUZA DİRENÇLİ *Saccharomyces cerevisiae* MUTANTININ KARAKTERİZASYONU

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ABBREVIATIONS

CDW	**:** Cell Dry Weight
CPR	**:** Carbondioxide Production Rate
GRAS	**:** Generally Regarded As Safe
EMS	**:** Ethyl Methane Sulphonate
HOG	**:** High Osmolarity Glycerol
HPLC	**:** High Performance Liquid Chromatography
IPG	**:** Immobilized pH Gradient
MAPK	**:** Mitogen Activated Protein Kinase
MAT	**:** Mating Type
OUR	**:** Oxygen Utilization Rate
PCR	**:** Polymerase Chain Reaction
RQ	**:** Respiratory Quotient
STRE	**:** Stress Response Element
YMM	**:** Yeast Minimal Medium
YPD	**:** Yeast Peptone Medium

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CHARACTERIZATION OF A SALT-RESISTANT *Saccharomyces cerevisiae* MUTANT

SUMMARY

In food industry and biotechnology, plenty of processes involve Baker's yeast. In these processes yeast cells are exposed to a variety of stress conditions. To advance the yield of processes driven by yeast cells, studies on obtaining stress resistant yeast strains are necessary. Characterization and sustainabilty tests of obtained stress resistant yeasts are also important. In this study, salt resistant *Saccharomyces cerevisiae* strain *T8*, which was previously obtained by evolutionary engineering, and wild type strain were characterized and compared with each other. Characterization was made in two steps, first of which was carried out by incubating the strains in a bioreactor. Since mutant *T8* is NaCl resistant, both wild type and *T8* strains were incubated in control medium and 5% (w/v) NaCl containing medium to have an eloquent data which demonstrate difference between w/t and mutant strains in salt stress conditions. While incubating these different strains in bioreactor, both with the control and salt stress containing medium, growth physiology, internal metabolite production rates, and morphology of the strains were investigated. Additionally, their oxygen consumption and carbondioxide production rates were determined to calculate their respiratory quotients that provided detailed information on the internal metabolic activity. According to the bioreactor experiments, in 5% NaCl stress containing medium, mutant *T8* grew more and faster than the wild-type (w/t) and had higher glucose consumption rates. Moreover, the mutant had higher glycerol, acetate and ethanol production rates and yields than the wild type under stress conditions. When the off-gas analysis of carbondioxide and oxygen for *T8* and w/t were compared; it was found that in stress conditions wild type cells could not perform any metabolic activies, while they showed better activities in control conditions than the mutant *T8*. Additionally, *T8* mutant and w/t differed from each other in morphological properties. According to results of flocculation tests, *T8* cells showed much higher flocculation rates than the w/t, and their flocculation rate and floc size became higher with increasing NaCl level and time of incubation. In the second part of this study, cellular proteins of both mutant and w/t cells; which were grown in control and NaCl stress medium, were compared by 2D gel electrophoresis. According to these results, both strains' cellular proteins are similar as they were generally similar: they were mostly in the size of 30-60 kDa and had an isoelectric point between 6-10. However, there may be a difference in the cellular proteins which are smaller than 25 kDa and have an isoelectric point around 5-7, between the samples that were taken from the control medium and cultures exposed to NaCl stress for both wild -type and the mutant.

TUZA DİRENÇLİ *Saccharomyces cerevisiae* MUTANTININ KARAKTERİZASYONU

ÖZET

Gıda endüstrisinde ve biyoteknolojide bir çok üretim süreci ekmek mayası üzerinden yürütülmektedir. Bu üretim süreçlerinde maya hücreleri çok çeşitli stress koşullarına maruz kalmaktadır. Endüstriyel üretimlerindeki bu maya süreçlerinin verimini arttırmak, hücrelerin süreç boyunca maruz kalacağı stress koşullarına karşı dirençli hale getirilmesiyle sağlanabilir. Bu sebeple, hem endüstride maya kullanımını geliştirmek hem de ileri ökaryotlarda stres cevaplarına bir model sağlamak amacı ile *Saccharomyces cerevisiae* mayası evrimsel mühendislik yöntemleri ile çeşitli streslere dirençli hale getirilmeye çalışılmaktadır. Günümüze kadar bu mayanın etanol, ağır metaller, donma erime, hidrojen peroksit gibi faktörlere karşı dirençli suşları elde edilmiştir. Bu dirençli suşların endüstride kullanılabilirliğinin araştırılması için strese dirençli suşların karakterizasyonu ve sürdürülebilirlik testlerinin yapılması önemlidir. Bu çalışmada, daha önceden evrimsel mühendislik yöntemleri ile elde edilmiş NaCl tuzuna dirençli *Saccharomyces cerevisiae T8* mutantı ile yaban tip karakterize edilmiş ve birbirleriyle karşılaştırılmıştır. Çalışmada karakterizasyon işlemleri iki adımda yapılmıştır. Birinci adımda, yaban tip ve mutant hücrelerin tuzlu ortamdaki farklılıklarının anlaşılması amacıyla *T8* ve yaban tip hücreler biyoreaktörde kontrol ve %5 (w/v) NaCl içeren besiyerlerinde üretilmiştir. Biyoreaktördeki her bir üreme koşulu ve her bir suş için, üreme fizyolojisi, hücre içi metabolit üretim oranları ve morfolojik farklılıkları araştırılmıştır. Ayrıca, hücre içi metabolik aktiviteler hakkında ayrıntılı bilgi edinebilmek için suşların oksijen tüketim ve karbondioksit üretim miktarları kaydedilmiş, bu değerler ile her suşa-koşula ait solunum katsayısı hesaplanmıştır. Biyoreaktör, sıcaklık, pH, çalkalama hızı gibi inkübasyon değişkenlerini her bir suş-koşul için aynı tutabilme özelliği ile mutant hücreler ve yaban tip hücreler arasında sağlıklı karşılaştırma yapma olanağı sağlamıştır. Her bir fermentasyonda biyoreaktör koşulları 30°C sıcaklık, pH 4,5 ve 700 rpm çalkalama hızında tutularak her suş-koşul için eşit şartlar sağlanmıştır. Biyoreaktör ile yapılan inkübasyonlarda üç farklı parametreye gore büyüme eğrileri elde edilmiştir. Bunlardan birincisi spektrofotometre ile 600 nm'de hücre miktarının ölçülmesi ile, ikincisi kuru ağırlık tartımı ile, üçüncüsü ise direkt hücre sayımı ile elde edilmiştir. Spektrofotometrik ölçümler ile elde edilen eğride yüksek tuz konsantrasyonunun hem mutant hem de yaban tip hücrelerin büyümesini inhibe ettiği, ancak büyüme engellenmesinin yaban tipte daha fazla olduğu gözlemlenmiştir. Hesaplanan spesifik büyüme katsayıları da bu gözlemi doğrulamıştır. Kuru ağırlık ölçümleri ile elde edilen eğrilerde tuz konsantrasyonu yüksek olan ortamda hem yaban tip hem de mutant suşlar yeterli büyüme gösteremediğinden dolayı sadece inkübasyonun 24.saatinde ölçüm yapılabilmiş ve büyüme eğrisi elde edilememiştir. Direkt hücre sayımı ile elde edilen büyüme eğrilerinde kontrol koşullarında yaban tip ve mutant arasında, diğer büyüme eğrilerinde gözlenmeyen, beklenmedik bir farklılık gözlenmiştir. Bu farklılığın sebebi; sayımdaki hata payının, mutant hücrelerin

birbirlerine yapışma özelliğinden dolayı, muhtemel yüksekliğinden kaynaklandığı düşünülmüştür. Her ne kadar kuru ağırlık ve direkt hücre sayımları ile elde edilen büyüme eğrilerinde eksik noktalar olsa da, tuz stres koşullarında mutant hücrelerin daha hızlı ve fazla büyüdüğü her büyüme eğrisinde açıkça gözlenmiştir. Yaban tip ve mutant hücrelerin kontrol ve tuz stresi içeren ortamdaki fermentasyonlarına ait CO_2 ve O_2 ölçümleri de stresli ortamda mutant hücrenin daha iyi büyüdüğünü, hatta yaban tipin stresli ortamda hiç bir metabolik faaliyet gösteremediğini desteklemektedir. Ayrıca gaz analizlerinde, özellikle kontrol koşullarında inkübasyonun ilk 10 saatinde maya hücreleri üzerindeki Crabtree etkisi açıkça gözlenmiştir. Solunum katsayılarının bu sure içinde 1'den çok büyük olması hücrelerin aerobic ortamda, solunumla eş zamanlı olarak fermentasyon da yaptığının göstergesidir. Solunum katsayısı eğrileri, HPLC analizi ile elde edilmiş glikoz tüketim miktarı ve etanol üretim miktarı eğrileri ile karşılaştırıldığında; Crabtree etkisinin gözlendiği ilk 10 saatin glikozun tüketilip tamamen bitirildiği etanolün ise devamlı olarak üretildiği zaman dilimine denk geldiği görülmüştür. Inkübasyonun 10.saatinden sonra glikozun tükenmesiyle, önceden üretilen etanol karbon kaynağı olarak solunumda kullanılıp tüketilmeye başlanmıştır. Bu da inkübasyonun 10.saatinden sonra solunum katsayısında 1 civarına düşüşlere sebep olmuştur. Bunun yanısıra, etanol, gliserol ve asetat üretiminin ölçüldüğü HPLC analizi sonuçlarına göre, stres koşullarında mutant hücreler adı geçen her üç metabolitin üretimini en yüksek düzeyde yapmıştır. Mutant hücrede gliserol üretiminin artması; aydınlatılmış osmotik stres direnç mekanizmasının direkt sonucu olarak düşünülmüştür. Ancak etanol ve asetat üretim miktarlarındaki artış tuz stress direnci mekanizmasının sadece gliserol ile değil diğer hücre içi metabolit üretim süreçleri ile de bağlantılı daha karmaşık bir yapısı olduğunu düşündürmüştür. Suşlar arasında kontrol ve tuz stresi içeren ortamlardaki trehaloz ve glikojen üretim miktarları karşılaştırılmıştır. Mutant suşun, stres koşulları altında yaban tipten daha fazla trehaloz ve glikojen ürettiği gözlenmiştir. Trehaloz ve glikojen molekülleri stres koşulları altında hücreyi koruma mekanizmasındaki genel rolüyle bilinmektedir. Bu sebeple tuz mutantında, strese karşı direnç sağlanmasında trehaloz ve glikojen üretim süreçlerinde meydana gelmiş olası bir modifikasyonun rolünün de olabilieceği düşündüldü. Mutant ve yaban tip hücrelerinin morfolojik farklılıkları öncelikle faz contrast mikroskobu ile karşılaştırılmıştır. Mikroskop ile yapılan araştırmada hücreler arasındaki en belirgin morfolojik farklılık mutant hücrelerinde görülen flokulasyon olmuştur. Ayrıca flokulasyon oranının artan tuz konsantrasyonu ve inkübasyon süresi ile doğru orantılı olarak arttığı gözlenmiştir. Flokulasyon oranındaki bu artışın sebebi, yüksek miktarda tuz içeren ortam ile hücrelerin temas yüzeyini azaltmanın olası avantajı olarak düşünülmüştür. Morfolojik farklılık araştırmalarına, invasiv büyüme deneyi, plastik adezyon testi ve kalsiyum bağımlı flokulasyon testi ile devam edilmiştir. Bu üç deneye, yaban tip ve mutant hücrelere ek olarak, *flo11* geni PCR bazlı delesyon ile silinmiş mutant suş da (*T8 flo11:KANMX*) dahil edilmiştir. *flo11* geni maya hücrelerinde flokulasyondan sorumlu gendir. Önceki çalışmalarda tuz stresine dayanıklı mutant *T8* suşunda bu genin ifadesinin arttığı saptanmıştır. Bu sebeple *flo11* geninin mutant hücredeki morfolojik değişikliklere katkısını görmek amacıyla bu suş da çalışmaya dahil edilmiştir. Invasiv büyüme deneyinde, suşlar 5 gün boyunca YPD agar besiyerinde büyütülmüş ve daha sonra sabit hızla akan çeşme suyunun altında yıkanarak hangi suşun agar içine doğru tutunarak büyüdüğü araştırılmıştır. Bu deney sonucunda, mutant hücrenin agar içine doğru büyüme oranı yaban tipten daha fazla gözlenmiştir. Ayrıca *flo11* geni silinmiş mutant suşta agar içine doğru büyüme hiç gözlenmemiştir. Plastik adezyon testinde ise tuz mutantı en fazla adhezyonu gösteren suş olarak

belirlenmiş ve besiyerinde glikoz konsantrasyonu arttıkça mutantın adhezyon oranının da arttığı gözlenmiştir. Kalsiyum katyonuna bağımlı flokulasyon araştırmasında ise, hücrelerin flokulasyon oranı flokulasyon engelleyici madde (EDTA) varlığında ve kalsiyum klorür varlığında ölçülerek kalsiyum katyonunun maya hücrelerinin flokulasyon oranına etkisi hesaplanmıştır. Bu deney sonucunda da tuz mutantı kalsiyum katyonuna bağlı flokulasyonu en yüksek oranda gösteren suş olarak belirlenmiştir. Ancak, beklenmedik bir şekilde *flo11* geni silinmiş tuz mutantı yaban tipten daha fazla flokulasyon göstermiştir. Bu durum, maya hücrelerinde kalsiyuma bağımlı flokulasyonun sadece *flo11* genine bağlı olmadığını, *flo* gen ailesinin diğer bireylerinden de etkileniyor olabileceğini düşündürmüştür. Ayrıca tuz mutant hücrelerinin yüksek flokulasyon oranının tuz sresiyle direk bağlantılı olduğu düşünülmesine rağmen; *flo11* geni silinmiş mutant hücrelerin tuz stresi içeren koşullarda; *flo11* geni silinmemiş mutant hücreler ile yarışabilecek kadar iyi büyüyebilmiş olması, *flo* gen ailesi ile ilgili bu düşünceyi desteklemiştir. Çalışmanın ikinci bölümüde, kontrol ve NaCl stresi içeren ortamlarda üretilmiş *T8* ve yaban tip suşların hücre içi proteinleri iki boyutlu jel elektroforezi yöntemiyle karşılaştırılmıştır. Bunun için ilk once suşların hücre içi proteinleri French press uygulaması ile izole edilmiş ve Bradford tayini ile protein miktarları belirlenmiştir. Suşların hücre içi proteinleri hakkında genel bir bilgiye sahip olmak için öncelikle SDS PAGE uygulaması yapılmış ancak suşların proteinleri arasında belirgin bir farklılık gözlenmemiştir. Daha sonra bu örnekler ile 2 boyutlu jel elektroforezi yapılmış ve suşların hücresel proteinleri hem büyüklüklerine gore hem de izoelektrik noktalarına gore birbirlerinden ayrılmıştır. Protein analizi sonuçlarına göre, iki suşun da proteinleri birbirlerine benzer bir şekilde 30-60 kDa boyutları arasında yoğunlaşmış ve bu proteinlerin izoelektrik noktaları 6 ile 10 arasında değişiklik göstermiştir. Ancak, yaban tip ve mutant hücrelerin kontrol ve NaCl- stresli ortamda üretilmiş kültür örnekleri arasında kendi içlerinde 25 kDa‘dan küçük boyutlu izoelektrik noktası 5 ile 7 arasında olan proteinlerde farklılık gözlenmiştir. Ancak bu farklılığın var olduğundan emin olmak için aynı örnekler ile 2 boyutlu el elektroforezi tekrar edilmeli ve protein farklılığı araştırması için daha hassas metodlar kullanılmalıdır. Bu çalışmada analiz edilen proteinler suşların hücre içi proteinleridir. Ancak, ozmotik stres direnci mekanizmasının membran geçirgenliği ile direkt alakasından dolayı mutant ve yaban tip hücrelerin membran proteinlerinin araştırılması; bu iki suş arasındaki proteome farklılıklarının saptanmasında etkili bir yöntem olabilir. Ayrıca, *flo11* genine ek olarak *flo* ailesine ait diğer genlerin tuz mutantında delesyonu yapılarak bu çalışmada yapılan morfoloji karşılaştırma testleri yeni delesyonlu hücrelere uygulanabilir. Ayrıca bu genlerin yaban tip ve mutant hücrelerdeki ifade oranları microarray uygulaması ile araştırılabilir. Böylece, tuz mutantının gösterdiği yüksek flokulasyona sebep olan mekanizma daha iyi anlaşılabilir. Tuz stresine dirençli mutantın, tuz stres koşulları altında asetat üretim metabolizması indükleyen sebepler, bu sebeplerin diğer metabolik süreçler ve özellkle Crabtree etkisi ile ilişkisi araştırılarak asetat üretiminin ozmotik stres direnci üzerindeki etkisi araştırılabilir. Ayrıca, tuz stresinin ağır metaller, donma erime stresi, etanol, hidrojen peroksit vb. stress faktörleri ile olası çapraz dirençleri incelenebilir. *Saccharomyces cerevisiae* mayası ile yapılan bu çalışmada tuza dirençli maya suşu incelenerek, direncin fenotipik etkileri ve mekanizması anlaşılmaya çalışılmıştır. Bu çalışma, mayada başka streslere dirençli suşların direnç mekanizmaları ile birleştirilerek; *S.cerevisiae*’de ortak stres direnç mekanizmalrı aydınlatılabilir ve bu gelişmiş ökaryotik canlılardaki stres mekanizmalrı için bir temel oluşturabilir.

1. INTRODUCTION

1.1 General Information About the Yeast *Saccharomyces cerevisiae*

Yeasts are simple unicellular eukaryotic organisms, which have similarities with advanced eukaryotes. Because of their ability to adapt easily and rapidly to new environments and changes, they commonly are used in scientific studies (Adames, *et al.*, 2000).

Yeast cells are classified according to their morphological, physiological, molecular, and sexual properties (Alberts, *et al.*, 2002). According to these parameters, the taxonomic classification of *Saccharomyces cerevisiae* is as shown in Table 1.1, and its phylogenetic relationship with other yeast species whose genomic sequence have been identified is shown in Figure 1.1.

Table 1.1: Taxonomic classification of *S.cerevisiae* (Hansen, 1883).

Kingdom	**Fungi**
Phylum	Ascomycota
Class	Hemiascomycets
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	*Saccharomyces*
Species	*S. cerevisiae*

S.cerevisiae is a kind of yeast which can grow in the most simple medium easily, has cell wall and mitochondria but no chloroplast (Alberts, *et al.*, 1998). The main material which is involved in the formation of the cell is the polymer Mannan-β-glukan (Zetic, *et al.*, 2001). The largest organelle in the cell is vacuole. Generally, each cell has only one globular vacuole whose size changes according to cell type. Vacuoles have significant roles on cellular pH regulation, homeostasis, osmoregulation as well as storage of amino acids, ions and hydrolytic enzymes (Çakar, *et al.*, 2000).

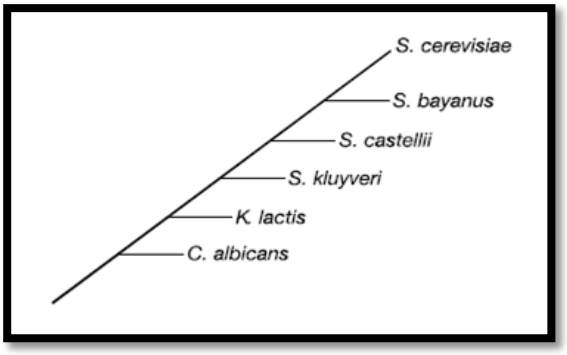

Figure 1.1: Phylogenetic relation of *S.cerevisiae* with other yeast species (Langkjær, *et al.*, 2003).

A microscopic image of *Saccharomyces cerevisiae* is shown in Figure 1.2 It has elipsoidal structure. The haploid cells have long axis approximately 4.76 and short axis 4.19m size; and for diploids 6.01 μm to 5.06 μm (Herskovitz, 1988). Moreover, their morphology and cell size can differ according to environmental factors such as nutritional conditions as well as the age of the cell.

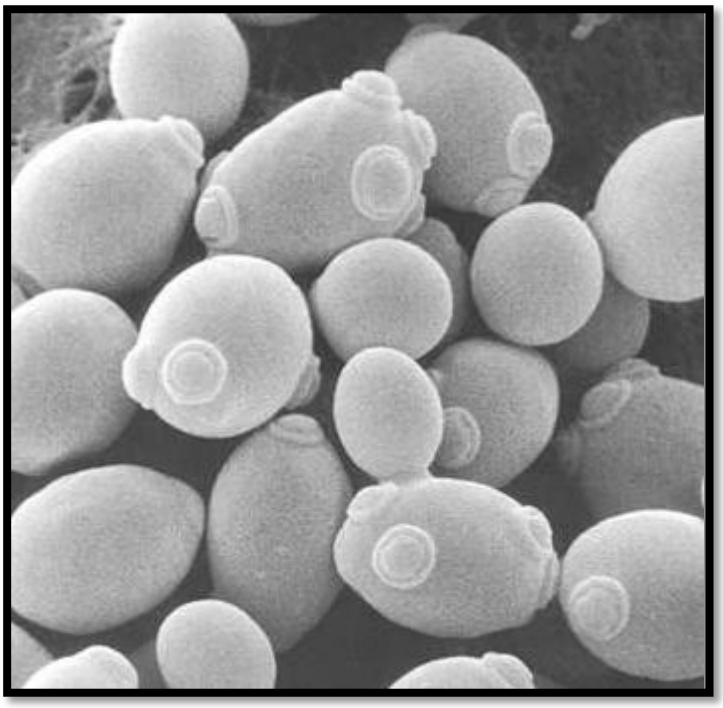

Figure 1.2: Electron microscopic image of *S. cerevisiae* (Url-1).

S.cerevisiae is commonly known as the Baker's Yeast. Under aerobic conditions, *S.cerevisiae* can make respiration while it switches from respiration to fermentation in order to produce metabolites such as glycerol, acetate, and ethanol. This property

makes *S.cerevisiae* very important and commonly used in bakery and alcoholic beverage industry (Deak, 2007). The reactions in alcoholic fermentation of *S.cerevisiae* are shown in Figure 1.3.

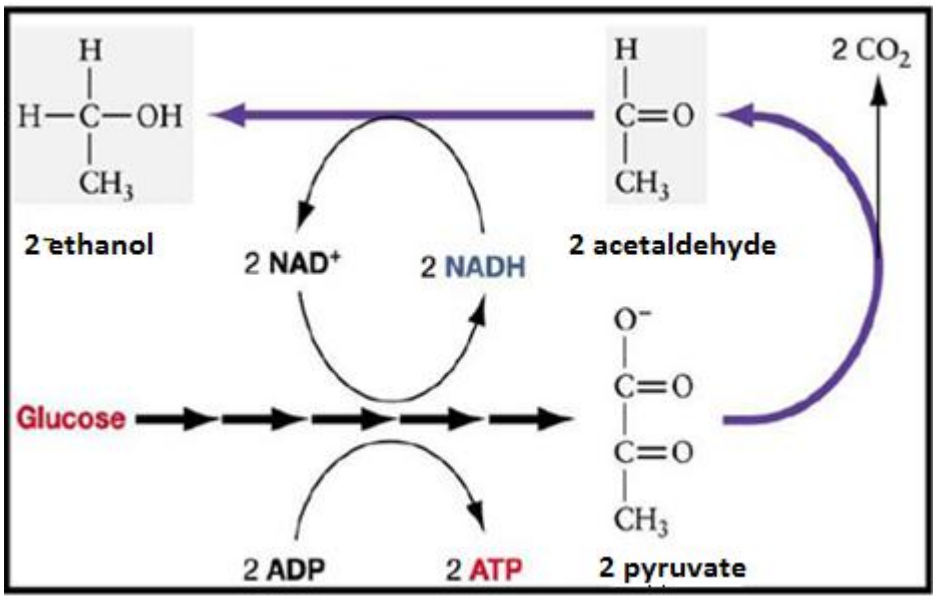

Figure 1.3 Alcohol fermentation reactions in *S.cerevisiae* (Muller, 2003).

Yeast cells reproduce in two different ways which are sexual and asexual (Herskowitz, 1988). Asexual reproduction of yeast is the result of budding. In asexual reproduction the budding yeast cell is named as mother cell, while newly occuring cell is named as daughter cell. The cell of asexual reproduction cycle starts when the mother cell starts budding and it ends when the bud separate from the mother cell (Burke, *et al.*, 2000).

However, in asexual reproduction only two types of cells are observed, in sexual production of the yeast cells three different kinds of cells are present which are MATa, MATα and MATa/α. Sexual production starts when a MATa and MATα cell contacts each other (Herskowitz, 1988). After they have an interaction with each other they fuse and form the diploid cell MATa/α. Diploid MATa/α is not able to mate, however it can produce spores and haploid yeast cells by meiosis (Spellman, *et al.*, 1998; Zetic, *et al.*, 2001).

The life-cycle of yeast *S.cerevisiae* is shown in Figure 1.4.

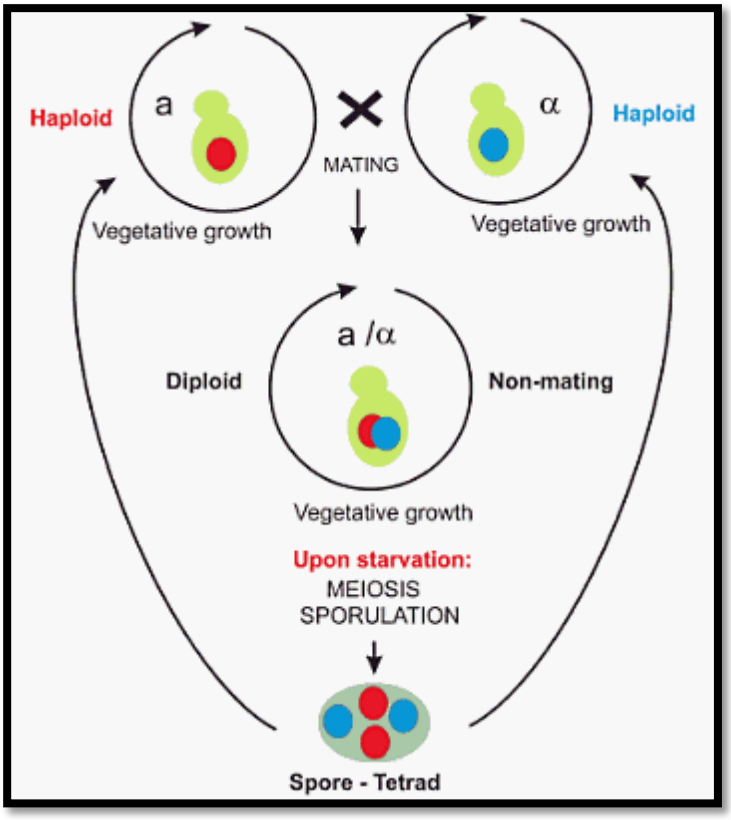

Figure 1.4: Reproductive cycle of *S.cerevisiae* (Feldmann, 2010).

There are distinct morphological and physiological differences between the cells that have role in sexual and asexual production. The sizes of diploid cells are approximately 1.3 times bigger than the haploid ones. Also, while haploid cells are circular, the diploid ones are oval. Moreover, the budding models of haploid and diploid cells are different, too. Haploid cells bud on a linear axis, attached to each other; however, diploid budding forms a polar motif (Burke, *et al.*, 2000).

In the eukaryotic research field, usage of *S.cerevisiae* is common as a result of its superiority in easy use than other eukaryotic organisms (Zetic, 2001).

1.2 Importance of *S.cerevisiae* in Industrial and Biotechnological Processes as a Model Organism

As an important industrial yeast, *S.cerevisiae* has been reported to have a market volume expressed in million dollars (Attfield, *et al.*, 1997). Its fast growth, short generation time, simplicity of its incubation and the its low costs makes it attractive

for industrial prosecces. Moreover, *S.cerevisiae* is identified as one of the GRAS organisms by FDA, which makes it safe to work with (Deak, 2007).

S.cerevisiae is commonly used in alcoholic beverage production industry and in bakery. Also, it is used in dairy, animal food production and as an additional alternative nutritional source in human diet, vitamin production such as vitB and vitD, ethanol and glycerol production and as a component in microbial medium in the form of yeast extract (Deak, 2007; Attfield, *et al.*,1992; Lewis, *et al.*,1997; Tanghe, *et al.*,2002; Madigan, *et al.*, 2003).

S.cerevisiae is used most commonly in the bakery and production of alcoholic beverages. Under low oxygen conditions yeast cells start to produce ethanol and carbondioxide as waste product of fermentation. However, ethanol is a waste product for *S.cerevisiae* and is toxic for yeast cells in concentrations higher than 14% (v/v), all of the alcoholic beverage producers use yeast cells for ethanol acquirement in the beverages such as beer, wine, cider, sake and liqueurs (Walker, 1998). This toxicity to yeast cells creates a limitation in the alcohol percentage of the beverage obtained by using yeast cells. In other words, if no distillation procedure is not going to apply to the product the alcohol percentage of it cannot exceed 16% as in the wine. If other beverages with higher alcohol concentration, such as liqueur and whiskey the fermented product must be distilled (Petro, 2010).

In addition to alcoholic beverage production, yeast has been used for centruies to make bread. As mentioned previously, in low oxygen conditions yeast cells start to carry out fermentation and produce carbondioxide as waste product. In bakery, the waste product carbondioxide is the main factor as release of it causes dough to swell and gives the bread its characteristic shape (Boekhout, *et.al.* 2003).

S.cerevisiae is a favorable model organism to work with, as it is safe, and is a simple model for complex eukaryotes (Shermann, 2002).

S.cerevisiae is commonly used in production of medically important substances such as interferon, insulin, and also proteins for animal feed, and human food. It is also used in biofuel production (Kirsop, 1988).

As schematic summary of importance and mission of yeast in biotechnology is shown in Figure 1.5.

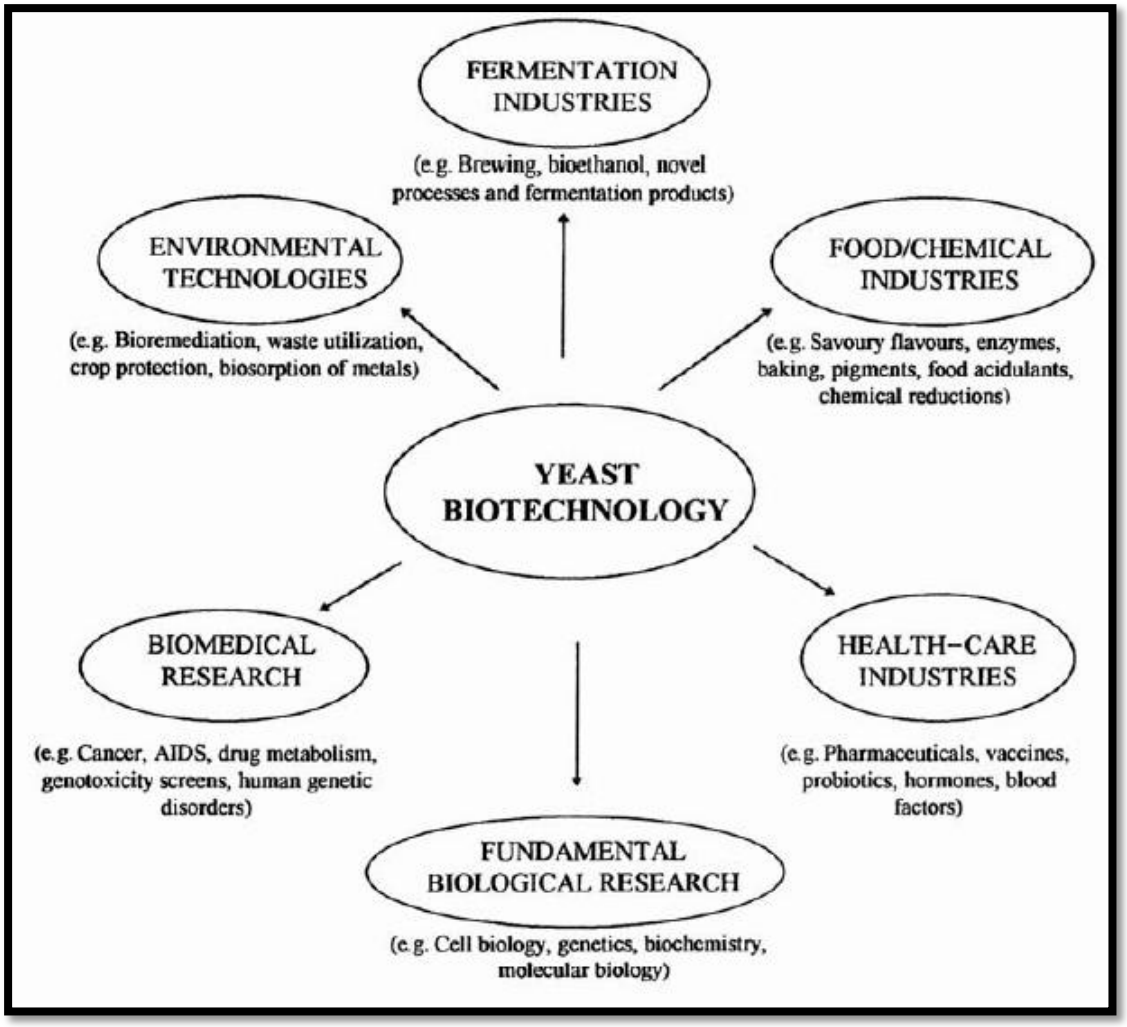

Figure 1.5: Fields of biotechnology in which yeast are used (Walker, 1998).

1.3 Stress Response Mechanisms in *S.cerevisiae*

Environmental changes are important for all living cells as they have to get adapted and get used to unsteady extrinsic conditions to survive. Rapid changes in the conditions may put cells under pressure making them change the way they used to live with complex signal cascades and web of communication systems they have. *S.cerevisiae* is one of the organisms that have several autonomous defense mechanisms evolved against stress conditions (Hohmann, *et.al.* 2003; Gasch, *et.al.*2000).

Stress can be any extreme change in the conditions which challenge all living organisms. Therefore, stress can be a rapid change in temperature or pH, starvation, osmotic changes, radiation, high metal concentrations, desiccation, oxidative stress pressure, which may result in the disruption of enzyme systems and metabolic

pathways of the cells. The general summary of stress-response mechanism in yeast is as shown in Figure 1.6 (Costa, *et al.*, 2001).

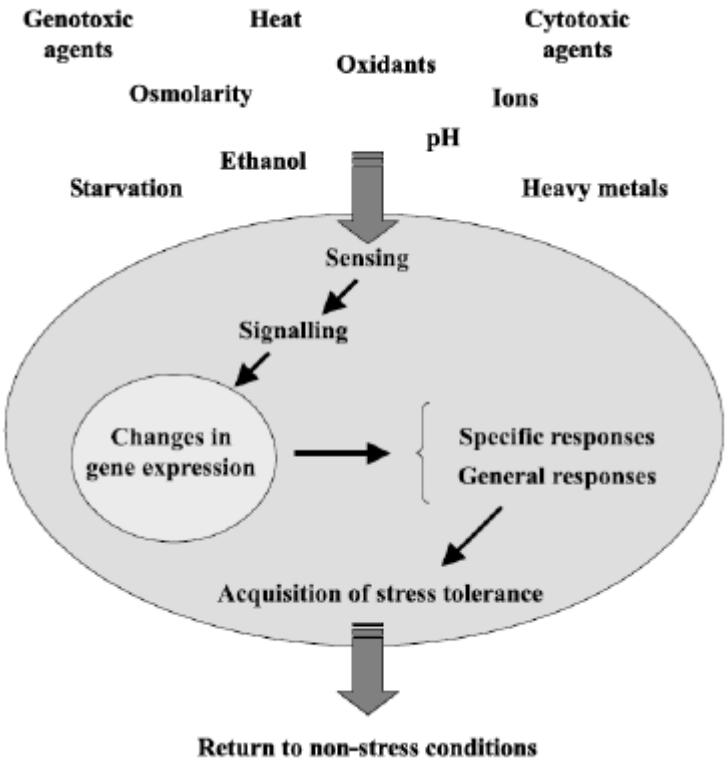

Figure 1.6: Summary of stress-response mechanisms in yeast (Costa, *et al.*, 2001).

Although stress can occur in various forms, the response mechanism against each stress in yeast is similar or has common points for all kinds of stress (Lado, *et al.*, 2002; Gasch, *et al.*, 2002). On the other hand, each kind of stress causes a change in a specific gene expression as a response in yeast (Gasch, *et al.*, 2002). In yeast nearly 900 genes, named as environmental stress response genes, found to change their expression levels under stress conditions. When the cell is under stress conditions, nearly 2/3 of these genes' expression levels decrease while the other 1/3 increase. Most of the genes whose expression levels decrease with stress are the ones that play significant roles in protein synthesis. Since protein production requires plenty of energy the cells prefer block protein synthesis in order to save energy, by decreasing the expressions of responsible genes. On the other hand, yeast cells increase the expression of other genes which are in the 1/3 part, since their expressions are essential for survival and defense (Gasch, *et al.*, 2002). A general flowchart of yeast

metabolic pathways that decrease or increase in the frequency under stress conditions, is shown in Figure 1.7.

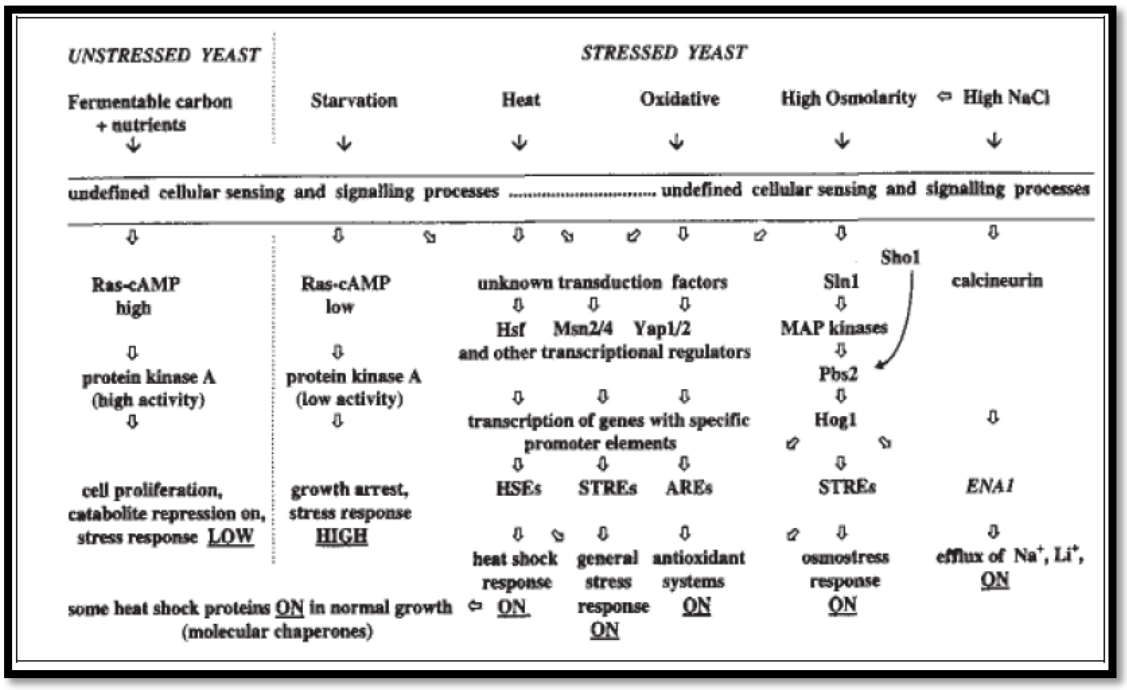

Figure 1.7: Summary of change in metabolic pathways of yeast in stress conditions (Attfield, 1997).

Studies on yeast stress response mechanisms revealed that, when a yeast strain becomes tolerant to a stress, it also becomes tolerant to some other stresses (Costa, *et al.*, 2001). For instance, heat shock genes are found to be activated not only in rapid temperature changes but also in other kinds of stresses (Gasch, *et al.*, 2000). Stress respone elements (STRE) and transciption factors *MSN2* and *MSN4* are other examples to show common steps in different stress responses. STRE is a common sequence in promoter regions of all stress-induced genes (Schmitt, *et al.*, 1996). *MSN2* and *MSN4* are the transcription factors which are activated in any stress condition for defense (Kobayashi, *et al.*, 1990).

1.4 Osmotic Stress and Responses of Yeast *S.cerevisiae*

Yeast cells are exposed to osmotic stress in the environment naturally. For example, a yeast cell living on a fruit can be exposed to hyper-osmotic stress when an erosion occurs in the crust of fruit, causing yeast cell contact in fruit content (Url-2).

However yeast cells have to survive in natural osmotic changes, they are not halo-tolerant (Cimerman, 2009).

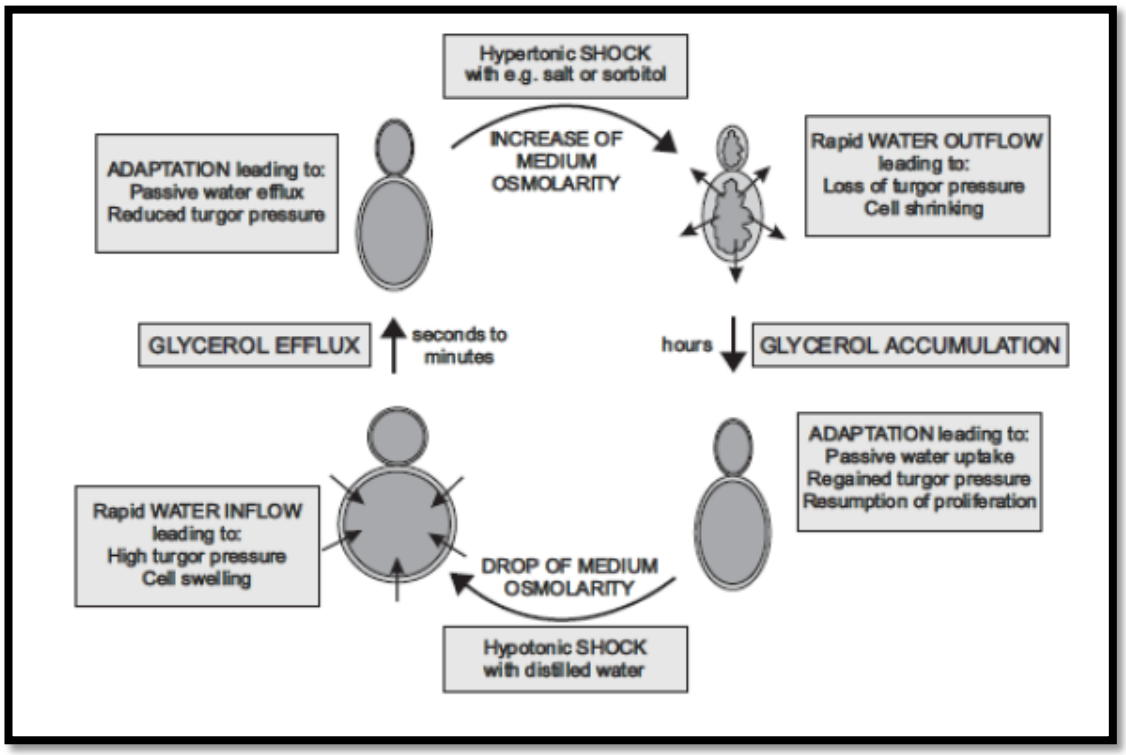

Figure 1.8: Basic osmotic stress response mechanisms in yeast (Hohmann, *et al.*, 2003).

There are plenty of studies on yeast cells' responses to osmotic stress. In Figure 1.8, the basic osmotic stress responses of yeast are shown.

For laboratory studies, osmotic stress is a problem because of the high salt concentrations in yeast growth medium. High salt concentration can damage the cell wall by decreasing the permeability and disrupting the transmembrane proteins, which may be important for osmoregulation (Mattanovich, *et al.*, 2004).

Several studies show that the osmotic stress response mechanisms of yeast cells are highly complex. In a series of researches based on osmosensitivity of different modified yeast strains, such as cytoskeleton mutants, transmembrane ATPase mutants, and vacuole mutants the complexity of the response were observed (Coury, *et al.*, 1999; Hohmann, 2002; Lippuner, *et al.*, 1996; Escanciano, 2009). These complex mechanisms involve sensing the osmotic changes and then regulation by signal transduction. Different types of signalling pathways have been revealed.

One and the best characterized pathway is the high osmolarity glycerol (HOG) pathway. HOG pathway, which can be activated in a very short time less than 1 minute, is a common osmoregulatory pathway in all eukaryotes. Overactivation of the pathway is lethal and lack of activation causes osmosensitivity in all eukaryotes (Hohmann, 2002).

In Figure 1.9, the molecules and the signal cascade between these molecules are demonstrated for HOG pathway. Sho1, Msb2, and Sln 1 proteins are the receptor proteins that activate MAPKK, and resulting in the initiation of the stress response pathway (Hohmann, 2002). As shown in Figure 1.9, activation of the MAPKK has two alternative ways, regarding to receptor protein, which had been activated with the hyperosmolarity. Depending on the first activated protein, which can be Sho1 or Sln1, the MAPKK activation pathway and molecules involved may differ.

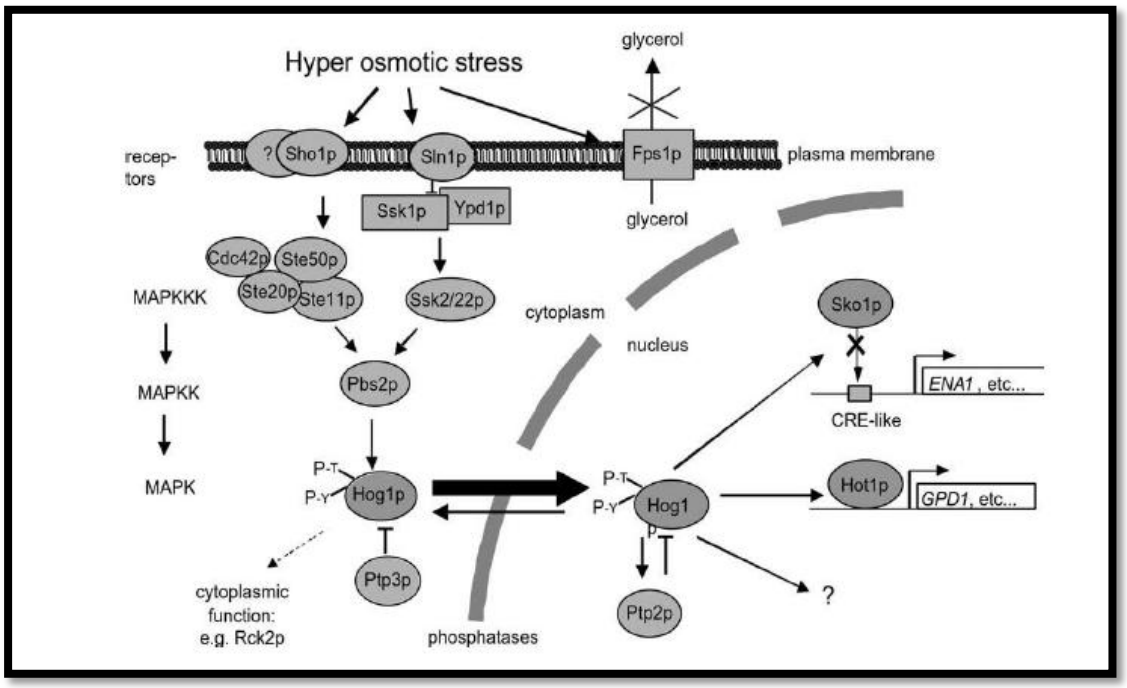

Figure 1.9: HOG pathway in *S.cerevisiae* (Mager, *et al.*, 2002).

Another pathway found to be responsible in osmotic stress response is the Calcineurin pathway. Calcineurin pathway is induced if the osmotic stress causing solute is ionizing, such as NaCl. Different from uncharged solutes, ionizing solutes create ion toxicity as well as osmotic pressure. Yeast cells undergo calcineurin pathway to get rid of the ion toxicity present in the cell, by polarization of cell

membrane in order to decrease the Na^+ uptake, and transferring and storing Na^+ ions that are already taken up to decrease the ion concentration in the cytoplasm. Calcineurin pathway is the main process, which is responsible Na^+ / K^+ homeostasis in yeast. It has a Ca^{2+} binding point, which is also regulatory region. When any Ca^{2+} ion bind to calmodulin, the calmodulin/Ca^{2+} complex binds to catalytic region of calcineurin which is not then autoinhibited. Activation of calcineurin results in interaction other molecules, which are responsible in the salt stress response (Hamilton, *et al.*, 2002). Then the salts stress signal perception, transduction and regulation in yeast is shown in the Figure 1.10.

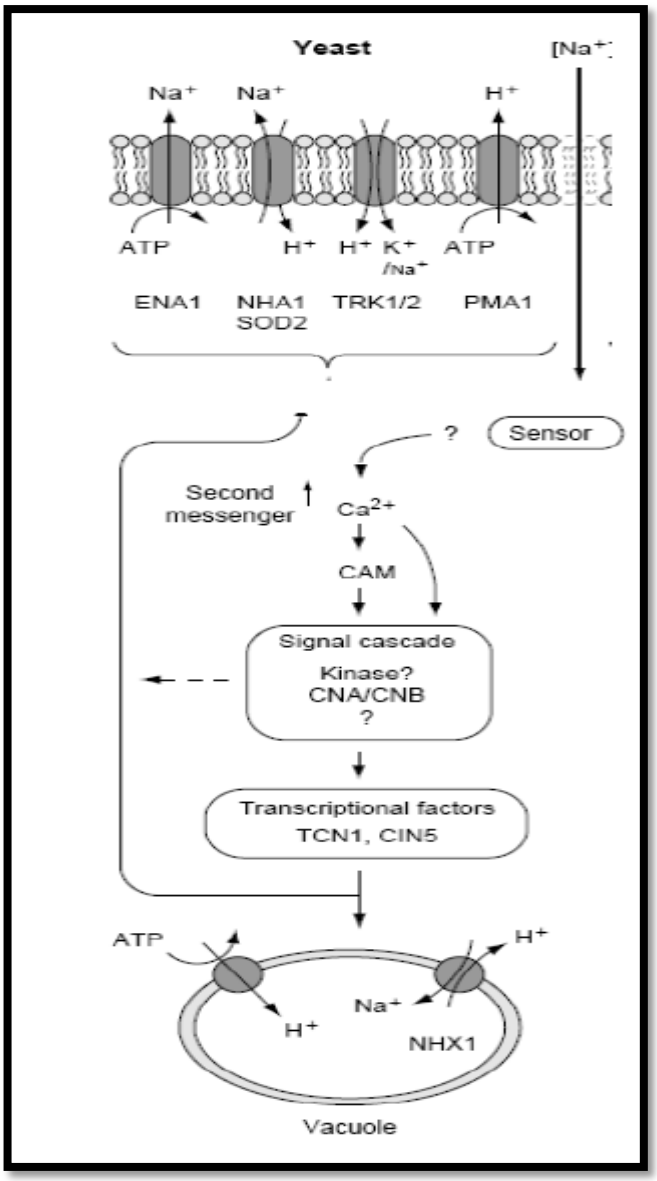

Figure 1.10: Salt sress signal perception, transduction and regulation in yeast (a hypothetical model) (Bressan, *et al.*, 1998).

In yeast, there are two main mechanisms of salt stress response, which are sequestering uptaken ions in to vacuoles and polarization of membrane to decrease influx of ion from the environment. Antiporter NHX1 is a significant molecule in transferring and storing processes of ions in to vacuoles, as shown in Figure 1.9. Also, *HAL4* and *HAL5* protein kinases that are induced by high affinity K^+ channel, *TRK1*, control the depolarization of plasma membrane (Hamilton, 2002).

1.5 Adhesion and Flocculation in *S.cerevisiae*

Microorganisms adhere to the surface they grow on to protect themselves from splitting off in a possible washing out or any threatening conditions. Yeast cells are the unicellular eukaryotic organisms which are able to adhere to abiotic surfaces and flocculate (Barrales, *et al.*, 2008). The adherence of yeast cells are quite important in industry. In fermentation processes, as the sugar is consumed and ethanol and carbondioxide are produced, yeast cells start to cell-to-cell adhesions which are called flocs. As a result of high flocculation rate, yeast cells are advantageous to be used in industry, as they are easy to get seperated from the medium after the fermentation is completed and the product needs to be exempt from yeast cells. In other words, downstream processes of the product is easier and cheaper when yeast cells are in floc forms, as they can be isolated from the product easier than single cells (Feldmann, 2005). Yeast cells' flocculation characteristics may be different in different strains. Some strains form flocs which precipitate, while some of them produce flocs that stay on the surface of the medium. The flocculation characteristics affect the trait of product. Especially, in the beverage industry it is really important to control the characteristics of flocculations during the fermentation in order to have the same taste in each fermentation (Verstrepen,2006).

Flocculins are the membrane proteins which lay out of the cell membrane, are responsible for attachment of the yeast cell to a surface or another cell. Flocculins provides attachment by binding to an amino acid or glucose molecule which is present on the surface (Claro, *et al.*, 2007). *FLO* gene family is responsible for the production of flocculins in yeast cells. There are five important genes in the family, *FLO1, FLO5, FLO9, FLO10,* and *FLO11*. These genes are responsible for the direct cell-to-cell attachment except *FLO11*, which is found to be the only *FLO* gene

expressed in some laboratory yeast strains (Govendar, *et al.*, 2008; Ishigami, *et al.*, 2008; Verstrepen, 2006). *FLO11* gene has a significant role in adhesions to a surface by sugar independent hydrophobic interactions in yeast. As a result of *FLO11* expression in *S.cerevisiae*, it can attach to agar or plastic. In this kind of sugar independent binding, flocculins usually bind peptides rather than sugar molecules. In addition to sugar independent adhesion, the second model is lectin-like adhesion. In lectin like adhesion model, in the presence of calcium ions, flocculins bind to sugar molecules present on the other cell or on the surface reversibly (Van Mulders, *et al*., 2009; Verstrepen, *et al*., 2003, Verstrepen, *et al*., 2006).

Flocculation genes are not always active in yeast cells. They are activated with the signals coming from the environment such as change in oxygen, pH, osmotic pressure, temperature, and starvation. When yeast cells adhere to each other, they separate the cells which are located in the middle from the medium enabling them to survive in unfavorable medium. Moreover, when there is a nutrient deficiency, by attaching to each other yeast cells form a bigger structure to seek for food easier. Therefore, yeast flocculation mechanism is a defense mechanism against unfavorable environmental conditions (Verstrepen, *et al.*, 2003).

1.6 Metabolic Engineering

In 1991, Bailey defined metabolic engineering as, "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology**"** (Bailey, *et al.*, 1991).

Metabolic engineering is an important tool for improvement of microbial strains used in industrial processes. Yeasts are one of the most common microorganisms in industrial production, as a result of their safety, low costs, and ease of work. On the other hand, yeast cells are very sensitive to environmental changes. Therefore; fluctuations, which can easily occur in industrial processes in temperature, pH or ion concentration can easily inactivate yeast cells and cause undesired consequences in production. To overcome these problems, metabolic engineering methodologies can be used in yeast strains for increasing metabolic activity, enhancing product rate, and extending substrate range, as well as eliminating waste products (Stephanopoulos, 1999). In other words, metabolic engineering refers to series of applications that are made to modify metabolic pathways as required.

In metabolic engineering, cells are optimized genetically with recombinant DNA technologies for altering their production rate of a specific molecule such as enzymes, metabolites; and for prividing benefit from this change (Nevoigt, 2008). Metabolic engineering consists of two parts that are analytical and synthetic parts. In analytical part, mainly chemical techniques are used for determining metabolite production levels, fermentation experiments and pathway analysis; while in synthetic part genetic engineering methods are used for transferring the gene of interest to the organism (Ostergaard, *et al.*, 2000). Different aspects of analytical and synthetic parts of metabolic engineering are shown in Figure 1.11.

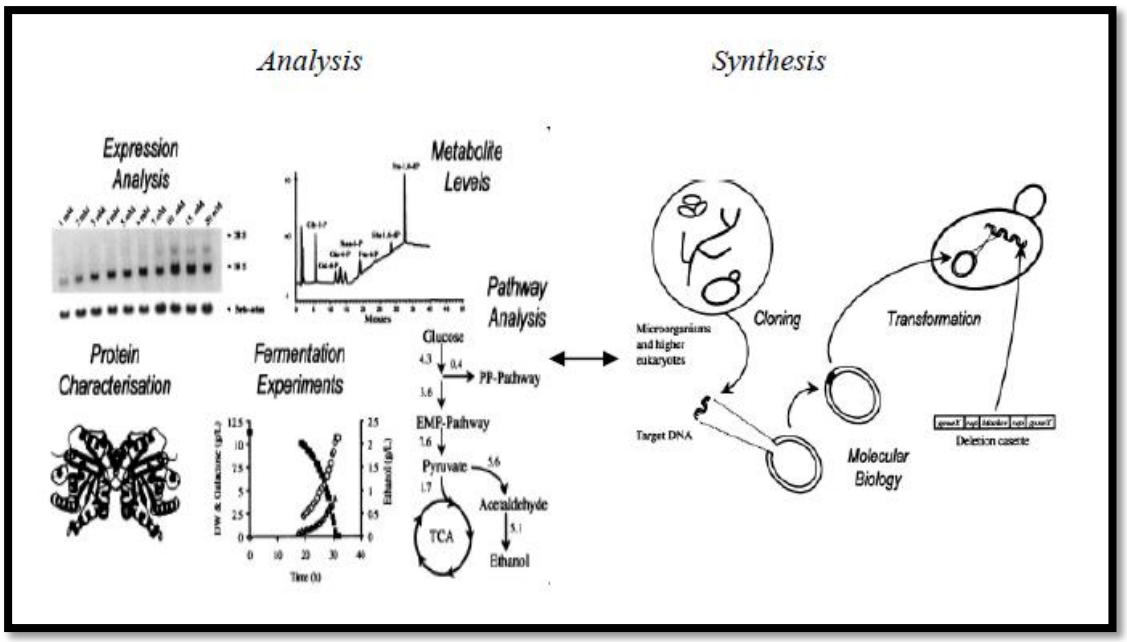

Figure 1.11: Different aspects of analytical and synthetic parts of metabolic engineering (Ostergaard, *et al.*, 2000).

Since 1980s when the metabolic engineering started to be used on strain development, several devices and techniques are developed such as chromatography, 2D gel electrophoresis, microarray (Bailey, *et al.*, 1996).

1.6.1 Inverse metabolic engineering

The common procedure of inverse metabolic engineering is as summarised in Figure 1.12.

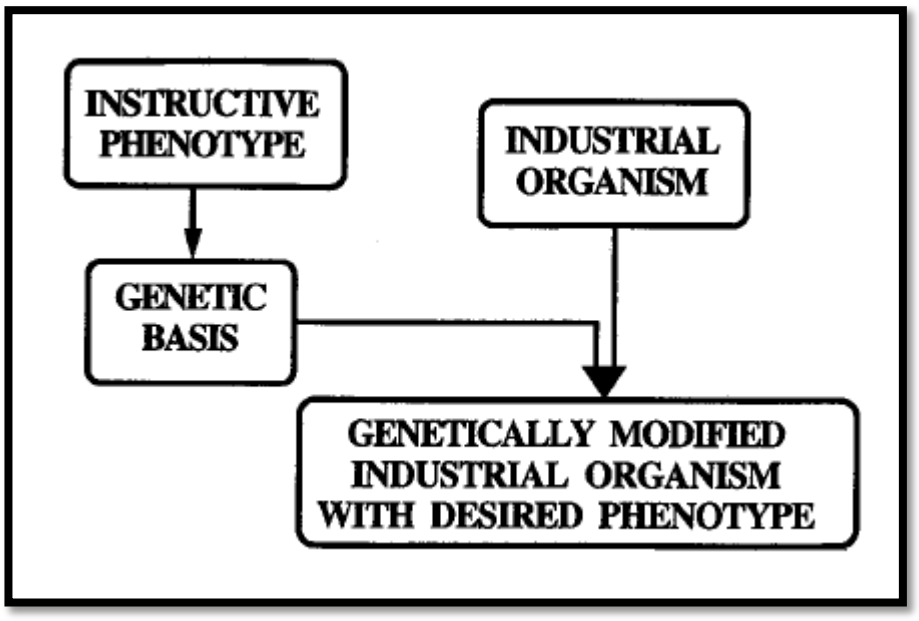

Figure 1.12: A common procedure of inverse metabolic engineering (Bailey, *et.al.*1996).

In the first step of the procedure, the desired phenotype is identified and modelled. In the second step, the genetic basis of this phenotype is determined or estimated. Then the industrial organism is granted with the desired genotype with environmental or genetic modifications to have genetically modified industrial organism with desired phenotype (Bailey *et al.*, 1996; Nevoigt, 2008).

1.6.2 Evolutionary engineering

Evolutionary engineering is a useful strategy for obtaining desired phenotypes in yeast. By using evolutionary strategies, yeast strains which are tolerant to stresses of high metal concentrations, freezing-thawing, osmotic pressure, ethanol, an temperature were succesfully obtained (Çakar, *et al.,* 2005). The main strategy used in evolutionary engineering is continuous stress selection methodology, which is applied to the randomly mutated cells. To make it more obvious, in this strategy randomly mutated cells are exposed to a stress level, in which all cells can survive. Then, going higher in the stress level thousands of generations is obtained by selecting a strain which can survive in the related level of stress (Zeyl, 2004). By

screening these selected strains, desired phenotype can be obtained by evolutionary engineering. If these obtained strains are investigated in the genetic manipulation and its relation with the phenotypic modification, they can be used in metabolic engineering strategies. Therefore, evolutionary engineering is a powerful technique that makes use of and contributes to metabolic engineering as well (Nevoigt, 2008).

1.7 2D-Gel Electrophoresis

2D-gel electrophoresis is a gel technique used for quantification of proteins according to both their masses and isoelectric points. 2D gel electrophoresis technique consists of two steps which are the 1st dimension and the 2nd dimension. In the first dimension of the gel, the proteins are separated according to their isoelectric points by isoelectric focusing. In the second dimesion the proteins that are previously separated according to their isoelectric points are diverged by SDS gels. Therefore; at the end of second dimension, the proteins are dropped into spots whose isolectric points and masses are different from each other. To make these spots visible, the gels can be stained either with Coomassie blue, or silver stain. The 2D gel software programs, such as Melanie, Delta2D, Bionumerics2D, and Progenesis make it possible to compare different protein samples on the gels. By using these softwares, spot differences between various samples and protein concentration variations in the same sample can be compared. Following 2D gel electrophoresis, mass spectrometric analysis of interested protein spots should be done to completely identify the different spots (Arora, *et al.,* 2005; Pedreschi, *et al.,* 2008).

1.8 Aim of the Study

This study was carried out to identify the phenotypic properties of salt resistant *S.cerevisiae* mutant *T8*. *T8* strain was obtained by utilising evolutionary engineering strategies in a previous study (Sezgin, 2010), in which randomly EMS (Ethyl-Methane-Sulfonate) mutated wild type cells were used as the initial population which was exposed to increasing NaCl stress concentrations. *T8* mutant was one of the most salt tolerant individual mutants derived from the final population. As well as obtaining stress resistant yeast strains, it is significant to check the trait sustainability and strain characteristics. In this study the aim was to characterize the salt resistant mutant *T8*, and to find out whether its traits are sustainable or not. In order to achieve this aim, growth performance, metabolite production and glucose consumption rates,

morphological, and proteomic properties of *T8* mutant were compared with the ones that belong to wild type.

2. MATERIALS AND METHODS

2.1 Materials and Laboratory Equipment

2.1.1 Yeast strain

Wild type *Saccharomyces cerevisiae* strain CEN-PK113-7D was kindly provided by Dr.Peter Kötter from Johann Wolfgang Goethe University, Frankfurt, Germany. Salt resistant *S.cerevisiae* mutant *T8* was obtained by Dr.Tuğba Sezgin in a previous study by evolutionary engineering based on random mutagenesis with EMS and continuous stress selection (Sezgin, 2010).

2.1.2 Yeast culture medium compositions

2.1.2.1 Yeast minimal medium (YMM)

Yeast minimal medium was used for regular growth, fermentation experiment and other tests that were applied to yeast strains. The content of this medium was shown on Table 2.1.

Table 2.1: Components of Yeast Minimal Medium (YMM).

Yeast Minimal Medium (YMM)	
Component	**Amount**
D-Glucose Monohydrate	20 gram
Yeast Nitrogen Base without Aminoacids	6.7 gram
Agar (for solid medium)	20 gram
Total Volume	1 Liter (with distilled water)

2.1.2.2 Yeast extract peptone dextrose medium (YPD)

Throughout of these studies stock cultures were transferred firstly to YPD medium to provide better conditions of growth. YPD composition was shown on the Table 2.2.

Table 2.2: Components of Yeast Peptone Dextrose Medium (YPD).

Yeast Peptone Dextrose Medium (YPD)	
Component	**Amount**
D-Glucose Monohydrate	20 gram
Yeast Extract	10 gram
Peptone	20 gram
Agar (for solid medium)	20 gram
Total Volume	1 Liter (with distilled water)

2.1.3 Laboratory equipment

Laboratory equipments used in this thesis project were demonstrated on the Table 2.3.

Table 2.3: List of Laboratory Equipment Used in Experiments.

Laboratory Equipment	Supplier
Micropipettes	Eppendorf (Germany)
Microcentrifuge	Eppendorf Microcentrifuge (Germany)
Multifuge	Heraeus (Germany)
Magnetic Stirrer	Labworld (Germany)
Autoclave	Tomy SX 700E (China)
Laminar Flow	Biolab Faster BH-EN 2003 (Italy)
UV-Visible Spectrophotometer	Amersham Biosciences (Germany)
Spectrophotometer	Beckman (USA)
Phase Contrast Microscope	OLYMPUS (Germany)
Desiccator	Krackeler Scientific Inc. (USA)
Deep Freezers and Refrigerators	LIEBHERR (Germany)
Sonicator	VWR (Germany)
Vortex mixer	Heidolph (Germany)
Gel Electrophoresis Power Supply	Amersham Biosciences (Germany)
Orbital Shaker	Certomat (Germany)
pH Meter	Reiss (Germany)
HPLC Pump	Jasco 880-PU Intelligent (USA)
HPLC Refractometer	Knauer Differential (Germany)
HPLC Column	Trännsaule Polyper OA HY Coulmn, Merck (Germany)
Bioreactor	BIOENGINEERING AG. (Switzerland)
Carbon Dioxide and Oxygen Detector	SICK MAIHAK GmBH. (Germany)
French Press	Constant Cell Disruption Systems (UK)
Shaker	B-BRAUN (Germany)
Gel Scanner	Amersham Biosciences (Germany)
IPG Phor	Pharmacia Biotech. (USA)
IPG Strips - Strip Holders	GE Healthcare Bio Sciences AB (Uppsala)
Chamber	Thoma Assistent (Germany)
Electrode Wicks	BIO-RAD (USA)
Gel Cassettes	Invitrogen (USA)

2.1.4 Chemicals, solutions, markers, inhibitors and medium components

Chemicals used in this thesis project were demonstrated on the Table 2.4.

Table 2.4: List of Chemicals through the Study.

Chemical	Supplier
Glycerol for synthesis (99%)	Applichem GmBH (Germany)
Ethanol (absolute)	Applichem GmBH (Germany)
Sodium Chloride	Applichem GmBH (Germany)
Potasyum Acetate	Birnboim & Doly (Germany)
Sulphiric Acid	Fluka (Germany)
Tris	Applichem GmBH (Germany)
SDS	Applichem GmBH (Germany)
Bromophenol Blue	Pharmacia Biotech. (USA)
Urea	Biochemica Applichem. GmBH (Germany)
DDT	Biochemica Applichem. GmBH (Germany)
Iodoacetamid	Biochemica Applichem. GmBH (Germany)
Rotiphorese	Fluka (Germany)
Ammoniumperoxodisulphate (APS)	Biochemica Applichem. GmBH (Germany)
TEMED	Biochemica Applichem. GmBH (Germany)
Acetic Acid	Applichem GmBH (Germany)
Coomassie Brillant Blue G-250	Fluka (Germany)
Ammonium Sulphate	Merck (Germany)
Methanol	Applichem GmBH (Germany)
σ - Phosphoric Acid	Fluka (Germany)
2-Butanol	Applichem GmBH (Germany)
Acrylamide	Biochemica Applichem. GmBH (Germany)
Sodium Acetate	J.T.Baker (Holland)
Sodium Hydoxide	Biochemica Applichem. GmBH (Germany)
HEPES	Carl Roth GmBH & Co. (Germany)
Bovine Serum Albumin	Biochemica Applichem. GmBH (Germany)

Medium components used in this thesis project were demonstrated on the Table 2.5.

Table 2.5: List of Medium Components used in the Study.

Component	Supplier
D-Glucose Monohydrate	Merck (Germany)
Yeast Nitrogen Base without Aminoacids	Carl Roth GmBH + Co. KG (Germany)
Yeast Extract	Merck (Germany)
Peptone	Applichem GmBH (Germany)
Agar (for solid medium)	Applichem GmBH (Germany)

Markers, inhibitors enzymes and special chemicals used in this thesis project are shown on Table 2.6.

Table 2.6: List of Markers, Inhibitors, Enzymes and Special Chemicals.

Markers	Supplier
Page Ruler Unstained Protein Ladder	Fermentas (Germany)
Page Ruler Plus Pre-stained Protein Ladder	Fermentas (Germany)
Spectro Multicolor Broad Range Protein Ladder	Fermentas (Germany)
Special Chemical	**Supplier**
Glucose oxidase/peroxidase reagent	Sigma
o-dianisidin dihydrochloride tablet	Sigma
Trehalase (from porcine kideney)	Sigma
Enzyme	**Supplier**
α-Amyloglycosidase	Roche
Inhibitor	**Supplier**
Protease Inhibitor Coctail Set III EDTA- Free	Calbiochem. (USA)

2.1.5 HPLC standards

HPLC mixture components used in this thesis project are shown on Table 2.7.

Table 2.7: Components of mixture used in HPLC Calibration.

Components	Concentration
Sodium Chloride	1 mg/ml
Na-Acetate	2 mg/ml
Glycerol	2 mg/ml
Ethanol	2 mg/ml
Glucose	1 mg/ml
	in 0.5mM Sulphuric Acid

2.1.6 French press buffer

French Press buffer components used in this thesis project are shown on the Table 2.8.

Table 2.8: Components of French Press Buffer.

Component	Concentreation
HEPES/KOH, pH :7.5	20 mM
KaC, pH :7.5	100 mM

2.1.7 SDS PAGE solutions and gel compositions

SDS PAGE solutions and gel components used in this thesis project were demonstrated on the Table 2.9.

Table 2.9: Solutions and Gels Components used in SDS PAGE.

Material	Ingredients
Seperating Gel Buffer	1.5 M Tris/HCl, pH 8.8
Stacking Gel Buffer	0.5 M Tris/HCl, pH 6.8
Running Buffer (10x)	30.3 g/L Tris-base 144 g/l Glycin 10 g/L SDS pH 8.3
Sample Buffer (2x)	4.05 ml dH_2O 1.25 ml Stacking Gel Buffer 2.5 ml Glycerin 2.0 ml 10% SDS Buffer 0.2 ml 1% Bromophenolblue Buffer 154 mg DTT
Seperating Gel (7.5 %)	4.85 ml dH_2O 2.5 ml Separating Gel Buffer 100 µl 10% SDS Buffer 2.5 ml 30% Acrylamide Buffer 100 µl 10% APS Buffer 5 µl TEMED
Stacking Gel (4 %)	6.1 ml dH_2O 2,5 ml Stacking Gel Buffer 100 µl 10% SDS Buffer 1.33 ml 30% Acrylamide Buffer 100 µl 10% APS Buffer 10 µl TEMED
Staining Buffer	0.05 % (w/v) Coomassie Brillant Blue G250 50 % (v/v) Methanol 10 % Acetic Acid
De-staining Buffer	50 % (v/v) Methanol 10 % Acetic Acid

2.1.8 2D Gel buffers and gel compositions

Components of gels and 2D gel buffers used in this thesis project are shown on Tables 2.10-2.18.

Table 2.10: Composition of Extraction Buffer (1x).

Material	Final Concentration
Urea	7M
Thiourea	2M
CHAPS	4 % (w/v)
BioLyt pH 3-10	0.5 % (w/v)
Bromophenol Blue	0.002 % (w/v)
DTT	40 mM

Table 2.11: Composition of Rehydration Buffer (1x).

Material	Final Concentration
Urea	7M
Thiourea	2M
CHAPS	2% - 4 % (w/v)
BioLyt pH 3-10	1% (w/v)
Bromophenol Blue	0.002 % (w/v)
DTT	20 mM

Table 2.12: Composition Equlibration Buffer 1.

Ingredients	Final Concentration
Tris, pH 7.5	125 mM
SDS	2.5 % (w/v)
Glycerol	30 % (v/v)
Urea	6 M
Bromophenolblue	0.002 % (w/v)
DTT	1 % (w/v)

Table 2.13: Composition of Equlibration Buffer 2.

Ingredients	Final Concentration
Tris, pH 7.5	125 mM
SDS	2.5 % (w/v)
Glycerol	30 % (v/v)
Urea	6 M
Bromophenolblue	0.002 % (w/v)
Iodacetamid	2.5 % (w/v)

Table 2.14: Composition of Tris-Glycin Running Buffer.

Material	Final Concentration
Tris- Base	25 mM
Glycin	192 mM
SDS 20%	0.1 % (v/v)

Table 2.15: Ingredients of 2nd dimension 10% Tris-Glycin Gel.

Material	Amount (for 1 gel)
dH_2O	4.0 ml
Rotiphorese	3.3 ml
1.5 M Tris pH 8.8	2.5 ml
10 % SDS	100 µl
10 % Ammonium Peroxodisulfates (APS)	100 µl
TEMED	4 µl

Table 2.16: Components of Agarose Solution.

Material	Amount
Agarose	0.1 g
1.5 M Tris/HCl, pH 8.8	1.25 ml
SDS 20 %	0.2 ml
Bromophenol Blue	a pinch with pipet tip
dH_2O	8.55 ml

Table 2.17: Composition of Fixing Solution.

Material	Final Concentration (v/v)
Methanol	50%
Acetic Acid	7%
dH_2O	43%

Table 2.18: Composition of Staining Solution.

Components	Final Concentration
Coomassie Brilliant Blue G-250	0.1 (v/v)
Ammonium Sulfate	17 % (w/v)
Methanol	34 % (v/v)
o -Phosphoric Acid	3 % (v/v)

2.2 Methods

In a previous and completed Ph.D. thesis study by Tuğba Sezgin, supervised by Prof. Dr. Zeynep Petek Çakar, sodium chloride resistant *S.cerevisiae* mutant *T8* was obtained via evolutionary engineering methods based on continuous stress selection strategy. In the current study, *T8* mutant was compared with w/t *S.cerevisiae* in the way it grows, changes morphology, produces metabolites and proteins; with an experimental procedure mainly based on cultivating both cells in different salt stress conditions in bioreactor.

2.2.1 Stock culture preparation

After obtaining *T8* mutant strain, frozen stock cultures were prepared for long term storage at -80 °C. To prepare stock cultures, 1.5 ml of each grown w/t and *T8* strain was harvested and washed with sterile YMM for twice by centrifugation at 16'363 *g* for 5 min in 2 ml sterile microfuge tubes. After the last washing step, 500 µl sterile YMM and 500 µl sterile 60% glycerol solution were added to cells to have final 30% glycerol concentration in stock cultures. Finally, they were vortexed very well and stored at -80 °C.

2.2.2 Incubation of yeast cells

Throughout this study, w/t and sodium chloride resistant mutant strain *T8* were used. Stock cultures of these strains were taken from -80 °C, vortexed very well and then a few drops of the stock culture were added into YPD agar plate and incubated at 30 °C for 48 h to revive the cells. After the cells grew on YPD agar plates, they were inoculated to liquid YMM for pre-culturing. According to further step of incubation, whether it is going to be in flask or in bioreactor, several differences in the protocols that are explained in detail below, were applied.

2.2.2.1 Incubation of yeast cells in flasks

Pre-cultures of w/t and *T8* mutant strains were prepared in 100 ml culture volume at 500 ml flask and incubated at 30ºC and 200 rpm agitation during overnight. The next day, approximetly at the 12^{th} h of their incubation, they were inoculated to 200 ml fresh 5% (w/v) NaCl containing 'YMM' and 8% (w/v) NaCl containing fresh YMM,

and as control they were also inoculated to 200 ml YMM. In all conditions the initial OD_{600} was set to 0.3 in 1-L flasks.

2.2.2.2 Incubation of yeast cells in bioreactor

Wild type and *T8* mutant strain of *S.cerevisiae* were grown in bioreactor (Bioengineering AG Wald CH 16 L bioreactor) for 24 hours. 1 L pre-culture was prepared in 4- 1L flasks each including 250 ml yeast minimal medium. Pre-cultures were incubated overnight at 30 °C at 200 rpm.

In bioreactor salt stress containing and non-stress containing forms of YMM was used as growth medium. To avoid reaction between ammonia groups and glucose which are both present in YMM, glucose was sterilized separately from the medium.

For stress-free condition, 8L YMM w/o glucose was sterilized in bioreactor and 1 L 9X glucose solution was sterilized separately in autoclave and then added to sterile medium in bioreactor. For 5% (w/v) NaCl containing condition, 6L YMM w/o glucose was sterilized in bioreactor. 1L 9X glucose and 2L 10X NaCl solutions were sterilized separately in autoclave and then they were added to sterile medium in bioreactor. Similarly for the %0.5 NaCl containing medium 7L YMM w/o glucose was sterilized in bioreactor. 1L 9X glucose and 1L 10X NaCl solutions were sterilized separately in autoclave and then added to sterile medium in bioreactor. Then, in all conditions, 1L pre-culture was added to bioreactor and 10L of end volume was obtained.

In bioreactor, cells were incubated at 30 °C, at 700 rpm. pH was fixed to 4.5 with 5 M NaOH. The oxygen, which dissolved in liquid medium, was set to 100% manually at the beginning of incubation, and the decrease in dissolved oxygen rate was observed with oxygen sensor of the bioreactor itself. Moreover, gas flow rate was set to 0.5 L/min and CO_2 and O_2 gas outputs were measured with CO_2 and O_2 sensor (MAIHAK FINOR and OXYGOR 6N), and according to output gas concentrations respiratory quotient of each strain was calculated.

Also, samples from 0^{th} and 24^{th} h of incubation and from pre-culture were incubated on YPD plates at 30 °C for 48 h to check contamination.

At the end of incubation, the bioreactor was sterilized with the whole content of it without allowing any culture to go out of bioreactor. After the sterilization step, each part of the bioreactor was washed very well with distilled water.

2.2.3 Obtaining growth curves of w/t and mutant strains

During the time of incubation of both w/t and mutant strains, once in several times samples were taken to measure OD_{600}, and cell dry weight (cdw), also to make direct microscopic cell counts in order to have growth curves according to different parameters to compare.

2.2.3.1 Obtaining growth curves according to OD_{600} measurements

During incubation of strains in bioreactor at 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} , 7^{th} , 8^{th}, 9^{th}, 10^{th},11^{th}, and 24^{th} hours of incubation samples were taken to measure OD_{600} values with spectrophotometer (Amersham Biosciences Ultraspec 3100 pro UV/visible spectrophotometer) to obtain a growth curve.

2.2.3.2 Obtaining growth curves according to cell dry weight measurements

After cultures' OD_{600} values reached ˜1.5, 1.5 ml of culture was centrifuged at 16'363 *g* for 5 minutes in pre-dried and tared microfuge tubes. (Tubes were kept empty at 100°C for 5 h for drying). Then supernatant was discarded and pellet was dried at 100 °C for 5 h and then weighted to obtain cell dry weight. Before measuring their weights both empty and full microfuge tubes used in cdw measurments were kept in desiccator overnight after they dried in 100 °C. All of the cdw measurements made 3 times and average of 3 measurements were used as data. For detailed data of cdw see Appendix A.

2.2.3.3 Obtaining growth curves according to direct cell counts

During the fermentation samples were taken at 0^{th}, 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th}, and 24^{th} hours of incubation and direct cell counts were held with chamber (Thoma Assistant 0.0025 mm^2, 0.100 mm chamber). After that, number of cells which are present in 1 ml of sample was calculated and growth curves were drawn according to these calculations.

2.2.4 Comparing morphologies of w/t and mutant strains

After sampling at 0^{th}, 3^{rd}, 6^{th}, 9^{th}, and 24^{th} h of incubation cultures were imaged under phase contrast microscope (Olympus BH-2) with 100X objective (Olympus Japan A100 PL 1.30 Oil 160/0.17) and their images were taken with camera (Canon Powershot A530) to use in checking morphological difference between w/t and mutant cells.

2.2.5 Invasive growth assay

To investigate the invasive growth variaton between w/t, mutant *T8,* and *Flo11::KANMX* (*Flo11* deleted *T8*) cells, their pre-cultures were prepared in 10 ml YPD in 50 ml test tube and incubated overnight. Then, their OD_{600} value were set to 0.1 in fresh YPD in another 50 ml test tube. After their OD_{600} reaches approximetly 1; 4µl of each culture was spotted on YPD-agar plate and incubated at 30ºC for 5 days. After the 5 days of incubation, they were washed under tap water that has a constant flow rate and their images before and after washing were compared.

2.2.6 Adherence to plastic test

To find out differences between w/t, *T8* and *Flo11::KANMX* strains, adherence to plastic test was carried out as described by Reynolds and Fink (2001). Each strain was incubated in 10 ml of 0.05%, 2%, and 4% (w/v) glucose containing YPD in 50 ml test tubes until their OD_{600} value reached 0.5-2. After that their OD_{600} values were set to 1 by centrifugation and resolving cells. Then, 100 µl of each culture was incubated in polystrene microtiler 96-well plate for 24 h at 30ºC. 1% crystal violet was added to each well and kept at room temperature for 15 min. Wells were washed with water and visualized. 100 µ of 10% SDS was added into wells and incubated for 30 min . Lastly, 100 µl distilled water was added into each well and mixed well by pipetting, then OD_{570} values were measured with microplate spectrophotometer (Benchmark Plus, *BIO-RAD*).

2.2.7 Ca^{2+} dependent flocculation assay

Wild type , *T8,* and *Flo11::KANMX* were incubated in 10 ml YMM in 50 ml test tubes at 30 ºC for 2 days. Then their initial OD_{600} were set to 0.1 in 5 ml fresh YMM and again incubated at 30 ºC for 2 days. 100 mM EDTA (pH 8) was added to each

culture obtaining the final EDTA concentration as 50 mM. After vortexing very well 100 µl of each culture was mixed with 900 µl of 100 mM EDTA and their OD_{600} values, which were named as Value A, were measured. One ml of each culture was then centrifuged at 16'500 *g* for 5 min and the supernatant was discarded. After washing the pellet with 1 ml of distilled water; 1 ml of 10 mM $CaCl_2$ was added and vortexed for 10 seconds. They were then kept at room temperature for 1 min without any intervention. Lastly their OD_{600} values, named as value B, were measured and flocculation rate of each sample was calculated according to Value A and Value B.

2.2.8 Quantitative assesment of glycogen and trehalose content

Trehalose and glycogen contents of w/t and *T8* cultures were determined upon incubation in YMM and 0.5 M NaCl containing YMM for 24 h at 30 ºC and 150 rpm shaking speed. 25 optical density unities of cells, were harvested at each sample collecting time point. They were taken either in 10 ml test tube or 1.5 ml centrifuge tubes and centrifugated at 15'000 *g* for 5 min. Following centrifugation, supernatants were discarded and glycogen and trehalose contents were measured using the pellets by the procedure previously performed and described (Parrau, *et.al.*1997). After adding 250 µl 0.25 M sodium carbonate to the pellets and vortexing well, the cells were incubated at 95 ºC for 4 h. The pH of these cell suspensions were then set to 5.2 with 150 µl 1M acetic acid and 600 µl 0.2M sodium acetate buffer and mixed well. 500 µl of the suspensions were transferred in other tubes (for trehalose determination) and incubated with 10 µl trehalase overnight at 37 ºC. 20 µl alpha-glycosidase was pipetted into other 500µl of the suspensions (for glycogen determination) and incubated on a rotary shaker at 57 ºC, overnight. After overnight incubations, in addition to glucose standards, 20 µl of all samples were distributed into different wells of the 96-well plate. After adding 200 µl glucose oxidase/peroxidase reagent into each well, the 96-well plate was incubated at 37 ºC for 30 min. The samples' absorbance at 490 nm were then measured.

2.2.9 Determination of external metabolite concentrations via HPLC

After sampling at 0^{th}, 1^{th}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} , 7^{th} , 8^{th}, 9^{th}, 10^{th},11^{th}, and 24^{th} hours of incubation, High Pressure Liquid Chromatography (Jasco 880-PU Intelligent HPLC Pump, Knauer Differential Refractometer, Tränsaule Polyper OA HY Column, Merck) analysis was held for measuring the concentrations of glucose and internal metabolites acetate, ethanol and glycerol. To prepare samples for HPLC, 1.5 ml of culture was taken to microcentrifuge tube and centrifuged at 16'353 *g* for 5 min. The supernatant was filtered with 0.2 µm filters to be ready for HPLC analysis.

HPLC was calibrated two times with the HPLC mixture (Table 2.7) and if both calibrations were parallel to each other, their mean values were accepted as the calibration curve.

0.5 mM sulphuric acid solution was used as mobile phase in HPLC. The measurements were made with a Tränsaule Polyper OA HY Coulmn, whose temperature was set to 80 °C, with the flow rate of 0.5 ml/min., and 50 kg/cm^2 pressure.

Each sample was injected into HPLC device in the volume of 100 µl manually with the special HPLC injector.

2.2.10 2D Gel electrophoresis analysis of yeast cells

Cellular proteins of w/t and *T8* mutant *S.cerevisiae* strains were compared throughout this study. 2D Gel comparisons were made in two replicas. In the first replica, w/t and mutant strains were grown in YMM, 5% (w/v) NaCl containing YMM, and 8% (w/v) NaCl containing YMM in flasks, and all of the samples were collected from cultures at the late exponential phase. In the second replica, the cells were grown in bioreactor in YMM, and 5% (w/v) NaCl containing YMM. In the second replica, samples from YMM were collected at the late exponential phase like in the previous one. However; differently from the first replica, in the second one samples from 5% (w/v) NaCl containing YMM were collected at a later phase than exponential phase.

2.2.10.1 Preparing protein samples from yeast cells

In the first replica of 2D Gel analysis, the pre-cultures of w/t and *T8* mutant strain were prepared in 100 ml culture volume at 500 ml flask and incubated at 30ºC and 200 rpm agitation during overnight. The next day they were inoculated to 200 ml fresh 5% (w/v) NaCl containing Yeast Minimal Medium 'YMM' and 8% (w/v) NaCl containing fresh YMM, and as control they were also inoculated to 200 ml YMM. In all conditions the initial OD_{600} was set to 0.3 in 1-L flasks. Their growth was monitored by measuring OD_{600}.

OD_{600} values of each culture were checked by sampling at each hour of incubation, and all of the cultures were grown through their logarithmic phase. In control medium, both w/t and *T8* strains' samples were collected when their OD_{600} value reached ~3. In 5% NaCl containing stress medium, w/t cells' sample was taken when its OD_{600} reached ~1, while *T8* sampling was performed when its OD_{600} was 1.5. Also, in 8% NaCl stress containing medium both w/t and *T8* strains were grown until their OD_{600} value reached ~1, and then their samples were collected for proteomic analysis.

In the second replica, from the fermentations in bioreactor with w/t and *T8* strains were grown in stress-free medium and 5% (w/v) NaCl stress containing medium; samples were taken to prepare new protein samples for 2D-Gel replicas. OD_{600} values of each culture were checked by sampling at each hour of incubation. In control medium, both samples of w/t and *T8* strains were collected when their OD_{600} value reached ~2.75. In 5% NaCl containing stress medium, w/t cells were grown to OD_{600} ~1.5 while *T8* was grown to OD_{600} ~3; and then their samples were taken for proteomic analysis.

For all samples came from two replicas, After all of the cultures reached to desired OD_{600} values their incubations were ended and 250 ml of each culture was centrifuged at 8500 rpm, 4 °C for 10 minutes (Heraeus Multifuge 3 S-R). After centrifugation, supernatants were discarded and pellets were washed once with French Press Buffer which was prepared according to Table 2.8, and whose pH afterwards was fixed to 7.5 with 1M KOH solution.

After the washing step, 1:200 (v/v) protease inhibitor (539134 Protease Inhibitor Coctail Set III EDTA-free Calbiochem, USA) was added to the french press buffer. The buffer with protease inhibitor was added to each pellet in the amount of three folds volume of the pellets. Pellets were dissolved in the buffer and then french press was applied to these solutions with 2 kilo bar pressure and 9 repeats for each sample. Also, the samples were checked under phase contrast microscope (Olympus BH-2) via 100x objective (Olympus Japan A100 PL 1,30 Oil 160/0,17) to be sure that all of the cells were disrupted.

After disrupting cells with french press, all of the samples centrifuged at 8500 rpm, at 4 °C for 10 min. After the centrifugation, supernatants were stored at -20 °C, and the pellets were discarded.

2.2.10.2 Bradford assay

Bradford assay was applied to determine the protein concentrations in each sample, by spectrophotometric measurements (Beckman, Du 7400 Spectrophotometer). Firstly, the spectrophotometer was calibrated with fresh Bovine Serum Albumin (BSA) solutions which were in different concentrations. For calibration, 0%, 2.5%, 5%, 7.5% , 15% and 20% BSA containing solutions were prepared. 800 µl of each concentration was put in 1ml-spectrophotometer cuvettes and 200 µl of Coomassie solution (Roti- Quant Coomassie Lösung) added on them. They were mixed well and after 3 min of reaction time their OD_{595} values were measured and the spectrophotometer was calibrated. Then, protein samples, which belong to w/t and *T8* strains grown in control and different salt stress conditions, obtained via French press were diluted in the ratio of 1:800 to have a trustable measurement with spectrophotometer. After that, 800 µl of each diluted sample was put in 1 ml-spectrophotometer cuvette and 200 µl of Coomassie blue added on them. They were mixed well and after 3 minutes of reaction time their OD_{595} values and protein concentrations were measured, and the real protein concentration of samples were calculated according to dilution factor.

2.2.10.3 SDS PAGE

SDS-PAGE gel electrophoresis was applied to all of the samples, as the first step in searching protein differences between w/t and *T8* strains which were grown in

control medium, 5% NaCl containing and 8% NaCl containing medium, to investigate differences in protein size through samples.

For SDS-PAGE gel electrophoresis protein concentrations of all samples were set to 1 mg/ml. The stacking (4%) and separating(7.5%) gels were prepared according to concentrations indicated in Table 2.9. During the time passed for polymerisation of gels, 20µl of each sample was mixed with 20 µl fresh prepared sample buffer (Table 2.9) and mixed well with pipetting. The mixture was kept at 95 °C for 3 min and then 20 µl of each mixture were loaded to wells in the gel. Moreover, 5µl of unstained marker (Fermentas Pageruler Unstained Protein Ladder) and 10µl of pre-stained marker (Fermentas Pageruler Plus Pre-stained Protein Ladder) were loaded to specific wells. After loading samples and markers, the gel was exposed to 200 Volt for 50 min. After running step is completed the gel was washed with distilled water for a few minutes. Then it was kept in staining buffer (Table 2.9) for an hour at room temperature on shaker. After that the gel was de-stained with de-staining buffer (Table 2.9) for 1 hour at room temperature on the shaker. After staining and de-staining steps the gel was imaged with scanner (ImageScanner Amersham Pharmacia Biotech).

2.2.10.4 2D gel electrophoresis

To search protein production differences between w/t and *T8* strains in control and stress containing medium, 2D gel electrophoresis was made for all of the protein samples belong to w/t and *T8* cells grown in different medium.

2D-gel electrophoresis was made in 5 steps. The first step, in other words the 1st dimension, was for separating the proteins according to their isoelectric points. The second step, in other words the 2nd dimension, of the gel was for determining the sizes of proteins which were separated according to their isoelectric points in the first dimension. In the third step the gel was stained with Coomassie Blue, and de-stained in the fourth step. Then in the last and fifth step, the gel was scanned.

Before applying 2D-gel electrophoresis to all samples, different protein amounts of w/t protein sample derived from control medium were tried on 2D-gel electrophoresis, in order to find the optimal protein amount to be applied on the gels. At this step, 33 µg and 100 µg w/t-control protein samples were run on both 1st and 2nd dimension gels and the gels were scanned. It was observed that the spots formed

by the 100 µg protein sample were clearer and stronger than the other ones formed by 33 µg sample. However, it was observed that increased protein amount resulted in better gel image; the protein amount could not be applied in the amounts of higher than 100 µg, as it is the highest protein amount that can be applied on the 1st dimension gel (GE Healthcare Immobiline™ Dry Strip pH 3-10 NL, 7 cm).

2.2.10.4.1 First dimension of 2D gel electrophoresis

For the 1st dimension of 2D-gel, first of all 120 µg protein containing volume of the sample was calculated according to protein concentrations defined by Bradford Assay. However, this step starts with 120 µg protein, 100 µg containing volume of it was transferred to 1st dimension gel since 100 µg is the highest protein amount that can be applied on the 1st dimension gel.

First of all, 120 µg protein containing volume of the sample was taken and mixed with 3 folds volume of pre-cooled 70% acetone, and the mixture was kept in the -20 °C freezer overnight. Then the mixture was centrifuged at 16'363 *g*, at 4 °C for 10 minutes. After centrifugation, the supernatants were discarded and the tubes left with their lids open until the pellets become completely dry, and the tubes do not smell like acetone. When the pellets got dry, fresh prepared 30 µl 40 mM DDT Extraction Buffer (Table 2.10) was added on the pellets and placed in sonicator for a short time. Then they were kept in room temperature for 30 minutes. After 30 minutes in extraction buffer, 120 µl 25 mM DDT rehydration buffer (1,25x) (Table 2.11) was added in tubes and centrifuged at 18'000 *g* for 4 minutes.

While the cells were being centrifuged, the IPG Strip Holders' electrodes were covered with little wet electrode wicks (Bio-Rad Electrode Wicks). After centrifugation finished, 125 µl of the supernatant was placed in strip holder between the electrodes. Then the IPG Strip (GE Healthcare Immobiline™ Dry Strip pH 3-10 NL, 7 cm) was placed in the holder, and 400 µl mineral oil was added on the strip and the lid of the holder was closed. Then the 1st dimension gel was run in IPGphor (IPGphor Pharmacia Biotech) according to program written in Table 2.19.

Table 2.19: Details of the program run on IPGphor for 1st Dimension Gel.

Steps of 1st Dimension	Program
Rehydration	0 min., 20 °C
IEF Parameter	20 °C, 50 µA/Strip
Step 1	Step-n-hold, 30 V x 12 Hours
Step 2	Step-n-hold, 300V, 4h
Step 3	Gradient, 1000V, 30 min.
Step 4	Gradient, 5000V, 1:30 h.
Step 5	Step-n-hold, 5000 V, 36 min.
Step 6	0 (stop the program)

After the program was finished, the strips were washed with distilled water for a very short time and then they were put into fresh prepared equilibration buffer 1 (Table 2.12) and kept on the shaker for 20 min. After that, equilibration buffer 1 was replaced with fresh prepared equilibration buffer 2 (Table 2.13) and the strips were kept on shaker for 20 minutes. After these equilibration steps, 1st dimension strips were washed for a short time with Tris-Glycin running buffer (Table 2.14) and then they were ready to be transferred to 2nd dimension gel.

2.2.10.4.2 Second dimension of 2D gel

For the 2nd dimension of 2D gel, 1.5 mm, 10 % Tris-Glycin-Gel was prepared with the material shown in Table 2.14. After the 2nd dimension gel was prepared and mixed well, it was poured into the gel chambers and after it was polymerized, 1st dimension strip was placed on it. Also 7 µl of the marker (Fermentas Spectra™ Multicolor Broad Range Protein Ladder SM 1841) was loaded next to the positive end of the strip. To fix the strip and the marker on the second dimension gel, agarose solution (Table 2.16) was poured on them, and when agarose got solid, the whole gel system was run first at 15 mA, 76 V for 15 minutes, and then it was run at 30 mA, 600 V for 1.5 hours. After the run was complete, the gel was ready to be stained.

2.2.10.4.3 Fixing, staining and destaining of 2D gel

After the run was completed, the strip was separated from the gel and discarded. The rest of the gel was first washed with distilled water for a few minutes and then it was put in the fixing solution (Table 2.17), and kept on shaker for 30 min. After 30 min, fixing solution was replaced with staining solution (Table 2.18) and the gel was incubated in staining solution overnight on shaker. Following the staining step, the gel was de-stained in 10 % acetic acid solution (de-staining buffer) for 30 min. Then it was washed with distilled water for 1h. After that, the gel was ready to be scanned.

2.2.10.4.4 Scanning and comparing 2D gels of different samples

The gels were scanned with Image Scanner (Amersham Pharmacia Biotech), and were viewed with LabScanner 5.0. Also, the visibility of the spots controlled and the contrast of the gel pictures were changed with SnagIt 7.0, to have more clear gel images.

After having gel images with LabScanner 5.0, the original forms of gel images were compared to detect protein spot differences between different samples using Image Master ^{TM} 2D Platinium Amersham Biosciences version 5.0.

3. RESULTS

As indicated in Section 2, bioreactor experiments were done including w/t and mutant *T8* cells in control and 5% (w/v) NaCl containing medium, as well as w/t in 0.5% (w/v) NaCl containing medium. However, in this section, the results of w/t in 0.5 (w/v) NaCl containing medium (see Appendix B) was not demonstrated since they have slightly differed from w/t cells grown in control medium.

3.1 Growth Curves of Wild Type and Mutant Strains

As indicated in Section 2.2.3, three different kinds of growth curves were obtained in this study, incuding spectrophotometric measurements (OD_{600}), cell dry weights, and direct microscopic cell counts, for both w/t and mutant cells grown in control and 5% (w/v) NaCl containing medium. The obtained growth curves are as shown in Sections 3.1.1-3.1.3.

3.1.1 Growth curves according to OD_{600} values

During the incubation at particular time intervals samples were taken from cultures and their spectrophotometric measurements were done at 600 nm to obtain growth curves shown in Figure 3.1.

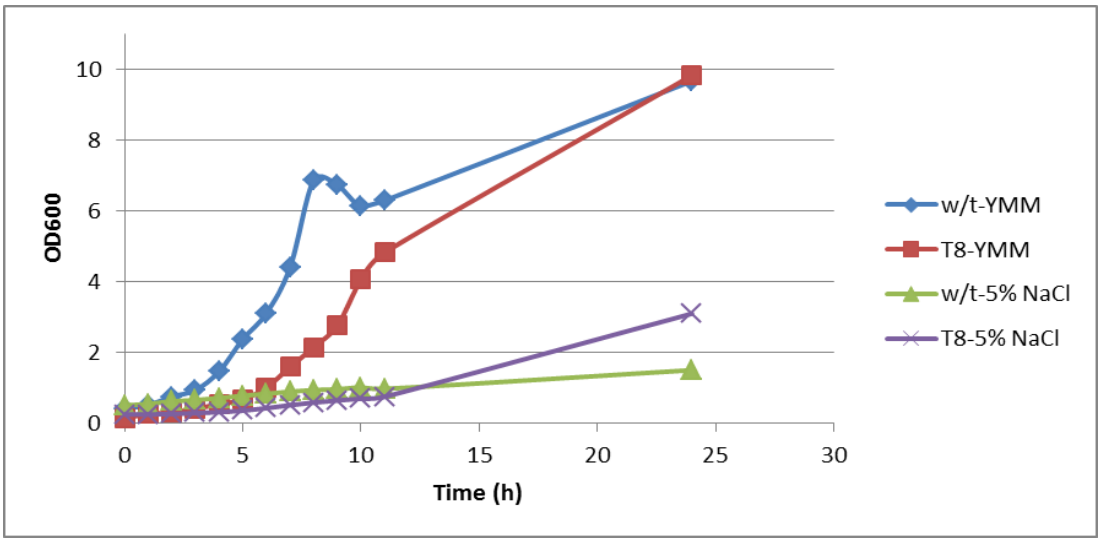

Figure 3.1: Growth curves, (OD_{600} vs. time), of w/t and *T8* strains in different media.

In stress-free conditions, w/t cells grew faster than the mutant cells during the first 11 h of incubation (Fig.3.1). However, at the end of the incubation, optical densities of both strains reached almost the same value. In 5% stress containing medium, in the first 11 h of incubation, strains seemed to have no big difference regarding their growth curves. However, at the end and 24th h of incubation, mutant cells reached more than 2 folds higher OD_{600} value than the w/t.

3.1.2 Growth curves according to cell dry weight measurements

During incubation, samples were taken to measure cdw. The growth curves obtained by cell dry weights (cdw) (Section 2.2.3.2) measurements are shown in Figure 3.2.

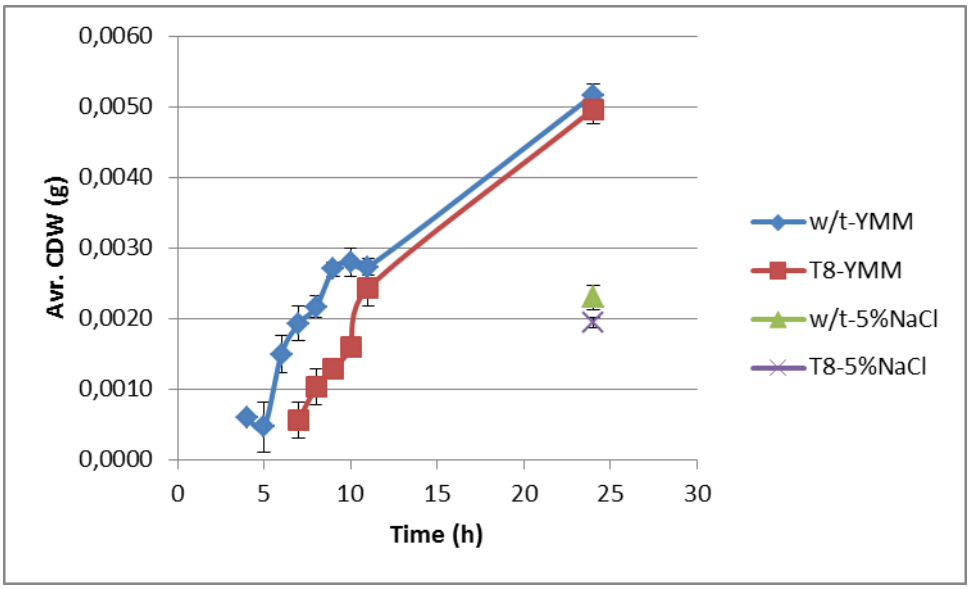

Figure 3.2: Cell Dry Weight Measurements of w/t and *T8* in control and 5% (w/v) NaCl containing medium.

For w/t cells grown in YMM and 5% NaCl and *T8* cells grown in YMM it can be generally said that their growth curves were similar to the ones obtained according to OD_{600} data. However, there were some differences at a few points of sampling. For example, in w/t YMM cdw at 5th h of incubation was lower than the previous one while it was definitely expected to be higher. Such differences might have been resulted from the low sample amount which was only 1.5 ml.

On the other hand, for w/t and *T8* cells grown in 5% NaCl it was not possible to sample for cdw during the first 11 hours of incubation, since their OD_{600} values were very smaller than 1.5. Therefore, for these two cultures it was only possible to make

cdw measurements at the 24th h of incubation. To be able to make cdw analysis in such low OD_{600} values it may be better to work in higher volumes while preparing cdw samples, too. However, at the 24th h of incubation, the average cdw of w/t in 5% (w/v) NaCl is higher than cdw of *T8* in 5% (w/v) NaCl unexpectedly.

3.1.3 Growth curves obtained by direct cell counts

During the fermantation, sampling for direct cell count was done once in two hours using the method and devices indicated in Section 2.2.3.3. The number of estimated cells per mililiter were then calculated and demonstrated as cell number versus time interval of incubation in Figure 3.3.

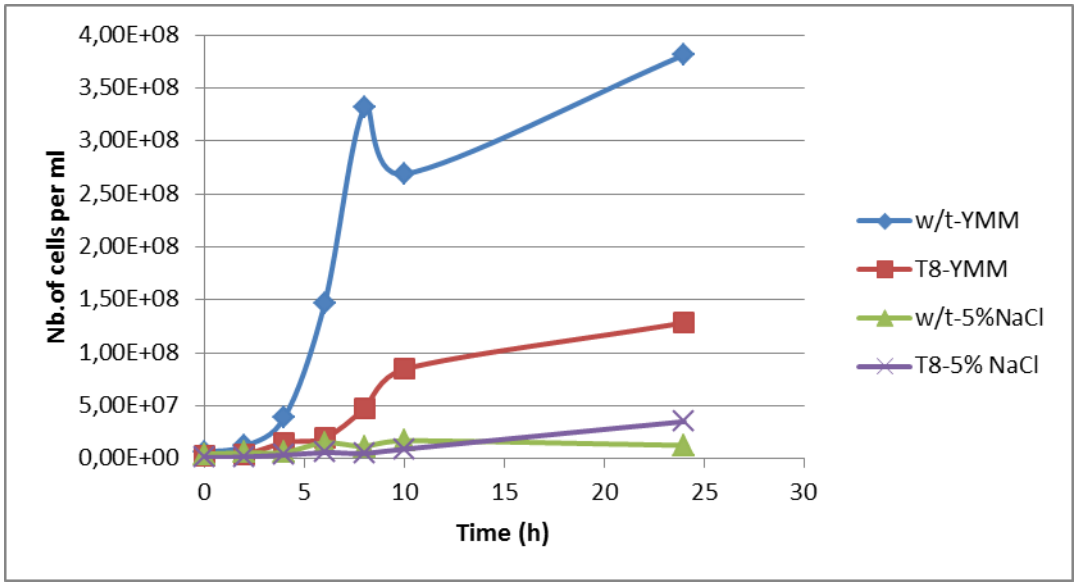

Figure 3.3: Growth Curves of w/t and *T8* strains obtained by direct cell counts.

Growth curves of strains obtained OD_{600} measurements and direct cell counts seem parallel to each other except 24th h sample of w/t-5%NaCl containing medium. However in OD_{600} measurements the last sample of w/t-5%NaCl had the highest OD_{600} value, in direct cell count it was lower than the previous one. Therefore, the curves obtained by OD_{600} values and by direct cell counts were different at the last point, in other words at 24th h of incubation for w/t cells grown in 5% NaCl.

3.2 Specific Growth Rates of Wild Type and Mutant Strains

To have a better idea about growth of strains in different media their specific growth rates (μ, h^{-1}) were calculated. Natural logarithm of cultures' OD_{600} values was used to draw a new line. The slope of the trend line was accepted as the specific growth rate for the culture (see Figure 3.4). The specific growth rates of wild type and *T8* in control medium were, 0.39, 0.33; and in 5% (w/v) NaCl containing medium 0.08 and 0.16, respectively.

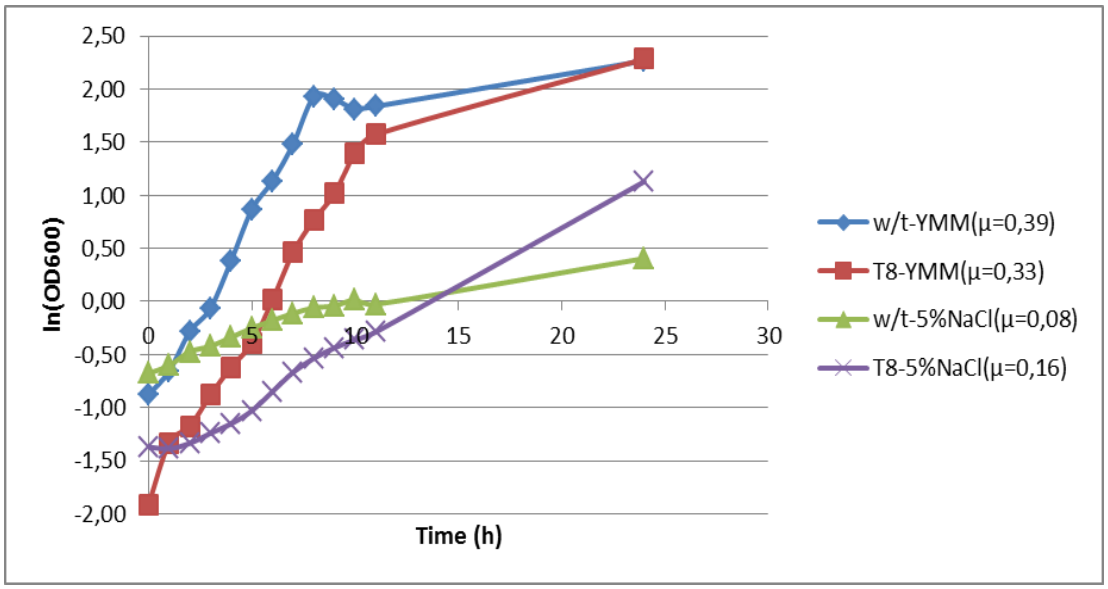

Figure 3.4: Specific Growth Rates of w/t and *T8* strain in control and 5% NaCl (w/v) Containing Medium.

In control medium, specific growth rate of *T8* is slight less than the one belongs w/t, however it was not significant. On the other hand, in 5% (w/v) NaCl containing medium, specific growth rate of *T8* is two times higher than that of the w/t.

3.3 Alteration in Dissolved Oxygen in Medium

During the fermentation changes in dissolved oxygen rate, which had been set to 100% manually at the beginning of the incubation, was recorded hourly. For w/t and *T8* strains in different media, change of dissolved oxygen rate is shown in Figure 3.5.

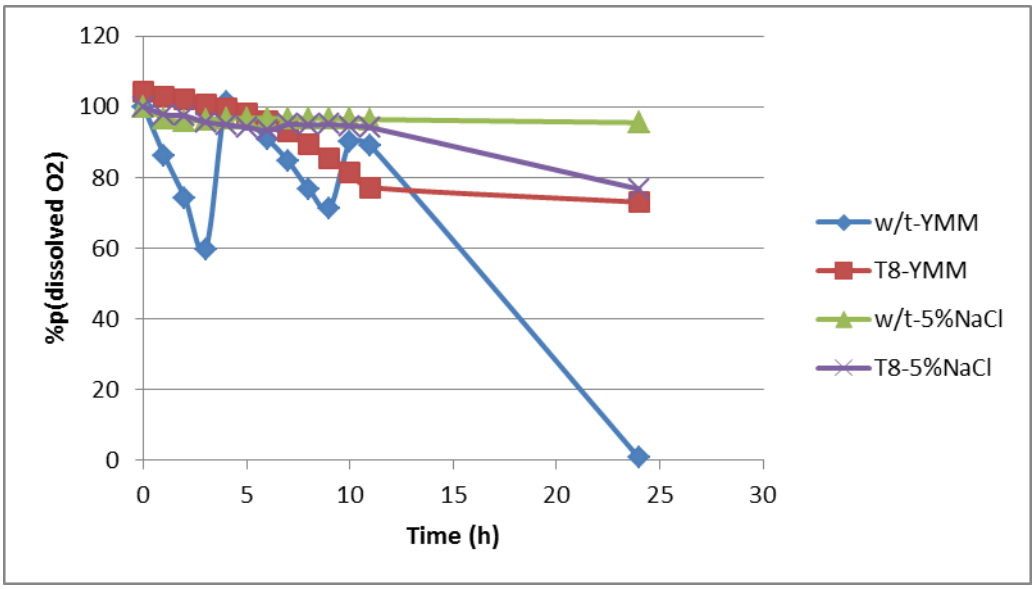

Figure 3.5: Change in oxygen dissolved in medium.

According to results indicated in Figure 3.4, it can be said that, dissolved oxygen in medium is inversely correlated with cell growth. In other words, the more the culture grows, the less dissolved oxygen is observed. However, for w/t-YMM it was different since in this incubation the agitation rate was not set to 700 rpm at all times. Incubation was started with 500 rpm, and then at the 4^{th} h of incubation it was increased to 1000 rpm, moreover it was changed to 700 rpm at 11^{th} h of incubation. That was why curve of dissolved oxygen belongs to w/t-YMM shows fluctuations at 4^{th} and 11^{th} hours of incubation. Moreover, in w/t-YMM, the dissolved oxygen percentage decreases to nearly 0 at the end of incubation. Since, these data did not show a correlation with output O_2 gas data (see Appendix C), it was thought that this big decrease results from a possible damage in oxygen sensor.

3.4 Respiratory Quotients of Wild Type and Mutant Strains

Outlet CO_2 and O_2 gases were measured during whole time for all fermentations with an additional detector (MAIHAK, FINOR OXYGOR 6N). According to these data oxygen utilization rates (OUR, mol/L.h) and carbon dioxide production rates (CPR, mol/L.h) were calculated using the formulas below **(3.1-3.3)**, to obtain respiration quotient (RQ).

$$OUR = \frac{V\alpha G,n}{VM,n * VL} * 60\frac{min}{h} * ({}_{\alpha}Y_{O2} - [(1 - {}_{\alpha}Y_{O2} - {}_{\alpha}Y_{CO2}) / (1 - {}_{\omega}Y_{O2} - {}_{\omega}Y_{CO2})] * {}_{\omega}Y_{O2}) \quad \textbf{(3.1)}$$

$$CPR = \frac{V\alpha G,n}{VM,n * VL} * 60\frac{min}{h} * ([(1 - {}_{\alpha}Y_{O2} - {}_{\alpha}Y_{CO2}) / (1 - {}_{\omega}Y_{O2} - {}_{\omega}Y_{CO2})] * {}_{\omega}Y_{CO2} - {}_{\alpha}Y_{CO2}) \quad \textbf{(3.2)}$$

$$RQ = CPR/OUR \quad \textbf{(3.3)}$$

$V^{\alpha}{}_{G,n}$ = Gas flow rate (L/min)

$V_{M,n}$ = Mol volume (22,414 L/mol)

V_L = Culture volume (10 L)

${}_{\alpha}Y_{O2}$ = Beginning concentration of O_2 (0,21-in air)

${}_{\alpha}Y_{CO2}$ = Beginning concentration of CO_2 (0,0003-in air)

${}_{\omega}Y_{O2}$ = Output concentration of O_2

${}_{\omega}Y_{CO2}$ = Output concentration of CO_2

For monitored output CO_2 and O_2 concentrations see Appendix C, and calculated OUR, CPR and RQ values were shown in Figures 3.6-3.9.

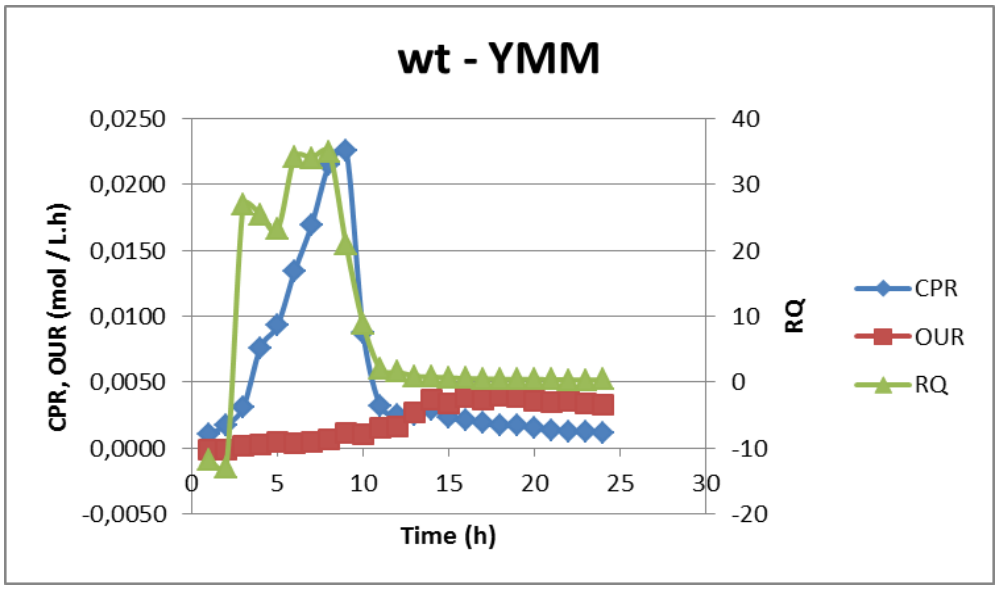

Figure 3.6: Calculated CPR, OUR, and RQ values of w/t in control medium.

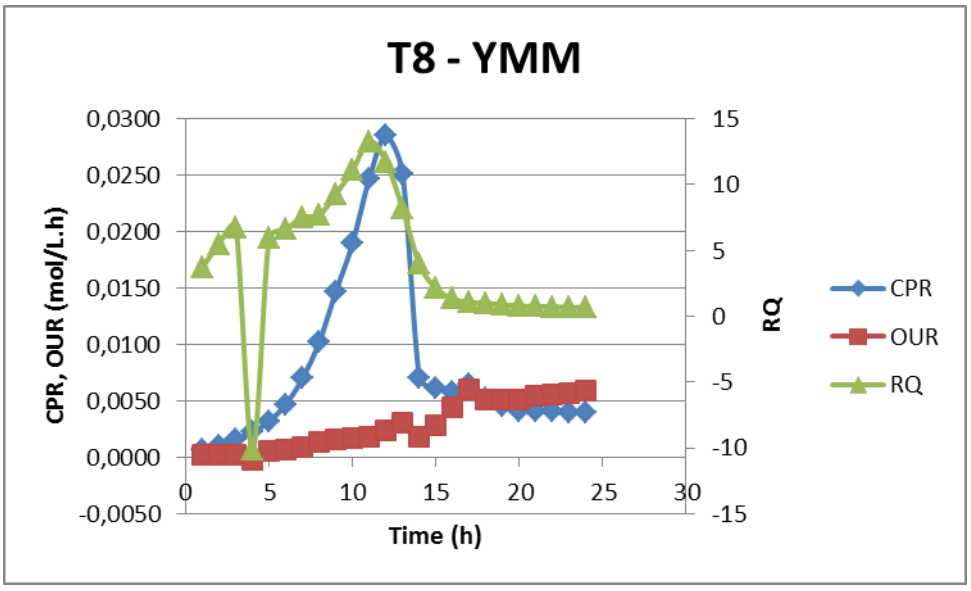

Figure 3.7: Calculated CPR, OUR, and RQ values of *T8* in control medium.

In control medium, the average RQ for w/t was about 20; however, for *T8* it was around 8. Also, while RQ values started to decrease significantly at aboout 9th h of incubation for w/t, it continued to increase until almost the 12th h of incubation for *T8*.

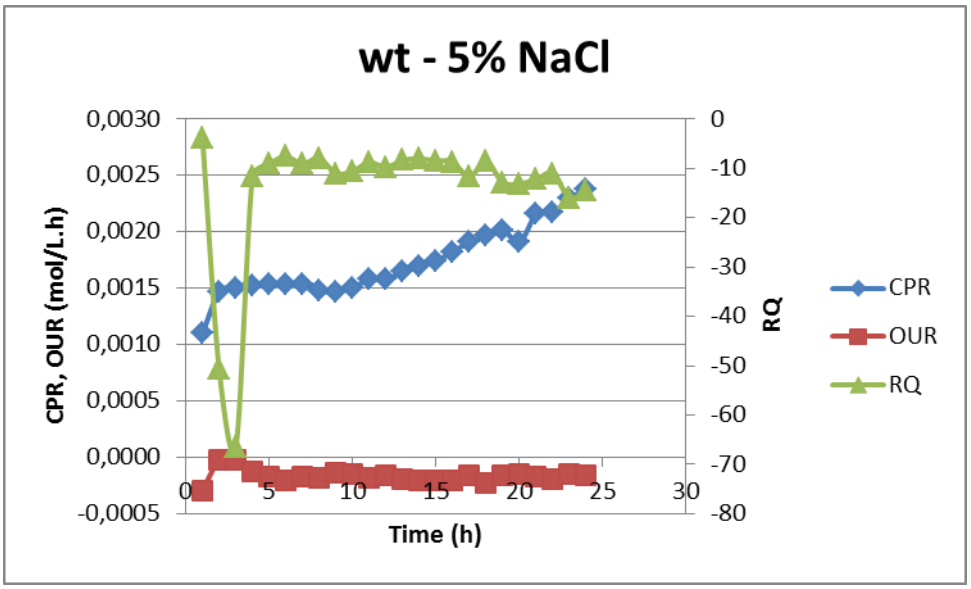

Figure 3.8: Calculated CPR, OUR, and RQ values of w/t in 5% (w/v) NaCl containing medium.

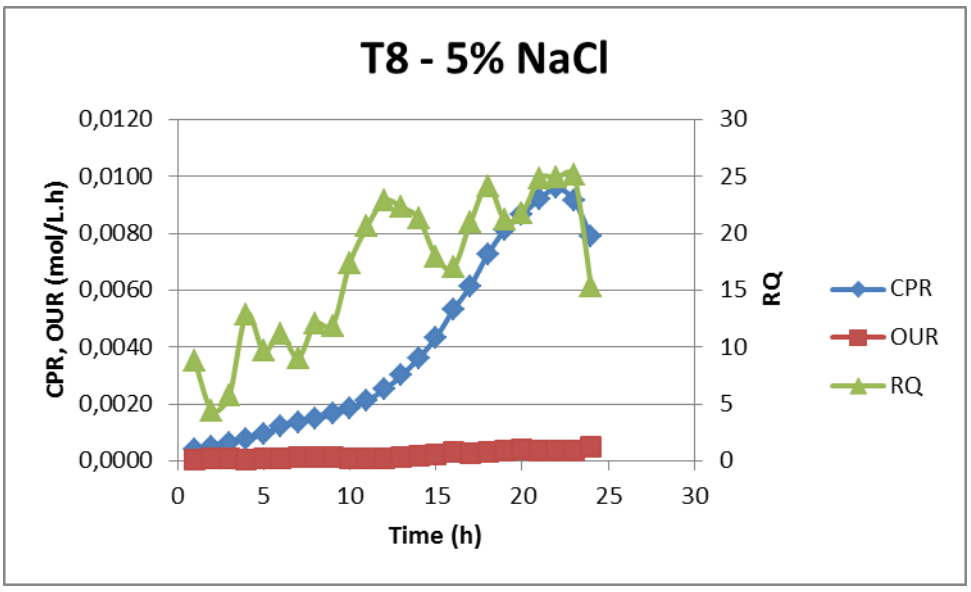

Figure 3.9: Calculated CPR, OUR, and RQ values of *T8* in 5% (w/v) NaCl containing medium.

In 5% (w/v) NaCl containing medium, the average RQ value for w/t was around -10, however for *T8* it was much higher. For *T8* the average RQ value was around 15 in 5% (w/v) NaCl containing medium. In RQ values of *T8*, there were several fluctuations. Both for w/t and *T8*, RQ values did not start to decrease regularly during the 24 h of incubation.

3.5 Differences in Morphology between Wild Type and Mutant Strains

After sampling at the times indicated in Section 2.2.4, morphological differences between w/t and mutant *T8* cells were investigated with phase contrast microscope.

For stress-free conditions, both w/t and *T8* cells were viewed as budding yeast cells. However, at the 24th h of incubation images differ in the flocculation rate of strains.

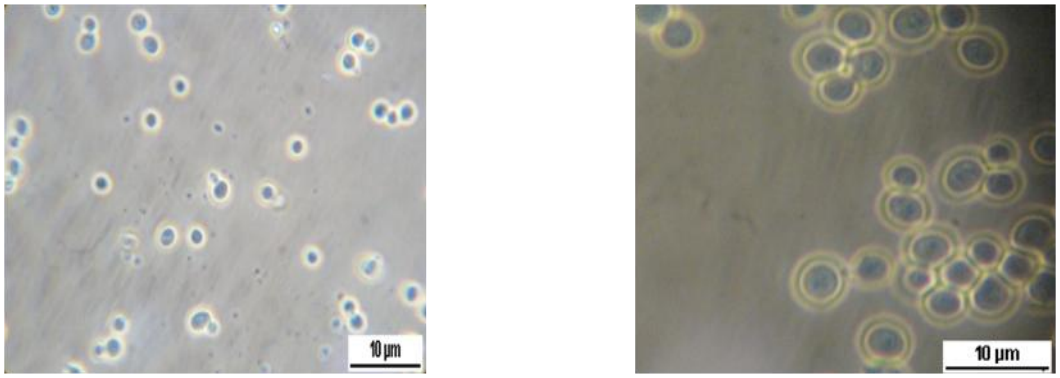

Figure 3.10: Images of w/t-YMM and *T8*-YMM on their 24th h of incubation (left w/t; right *T8*).

In Figure 3.10, images of w/t-YMM and *T8*-YMM at the end of their incubations were demonstrated. According to Figure 3.10, it was observed that while w/t cells continued to grow as individual cells after the 24th h of incubation; *T8* cells appeared as flocculated cell groups, or cell clusters.

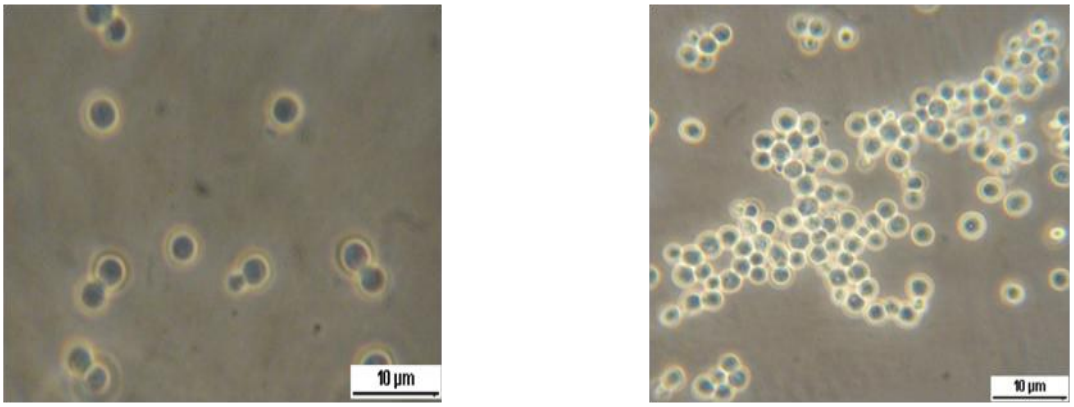

Figure 3.11: Images of w/t-5%NaCl and *T8*-5%NaCl on their 24th h of incubation (left w/t; right *T8*).

In addition to increase of flocculation clustering in *T8* cells, in high stress containing medium, while w/t cells became more individual; *T8* cells formed bigger flocs or clusters (Figure 3.11).

3.6 Invasive Growth Assay

In order to find out the invasive Growth differences between w/t, *T8* and *T8 flo11::KANMX* strains, invasive growth assay was carried out as mentioned in the Section 2.2.5. Since three replicates showed exactly the same result, only one of them was set out in Figure 3.12.

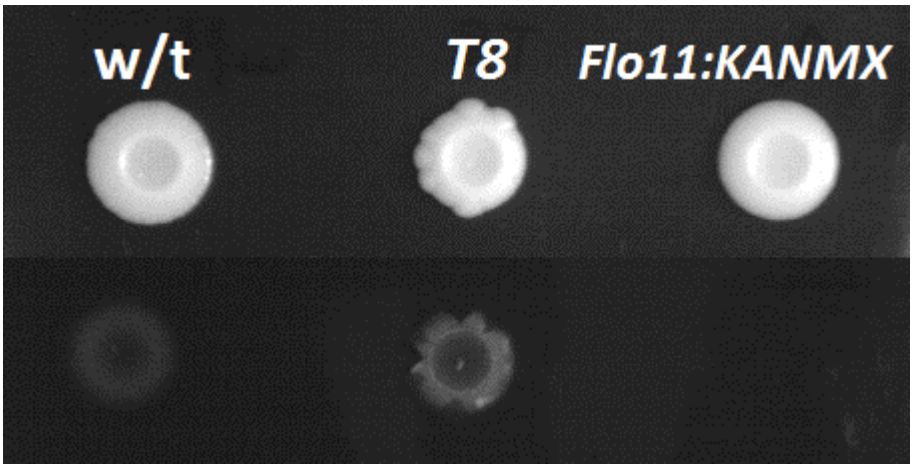

Figure 3.12: YPD plate with strains after 5 days of incubation (top; before washing, below; after washing).

As it is seen in the results of invasion growth assay, in agar plates salt mutant *Saccharomyces cerevisiae* strain *T8* shows repetitive strong invasive phenotype, however *T8 flo11::KANMX* strain could not show any invasion into agar as it easily and quickly slipped out in the first few seconds of washing while *T8* did not get apart from the agar only by water flow and needed to be scratched with finger tip.

3.7 Adherence to Plastic Test

To investigate the variation in adherence to plastic between the *S.cerevisiae* strains w/t, *T8* and *T8 flo11::KANMX* the procedure that was mentioned in Section 2.2.6

was carried out. The image of the 96-well plate after washing out crystal violet is shown in Figure 3.13.

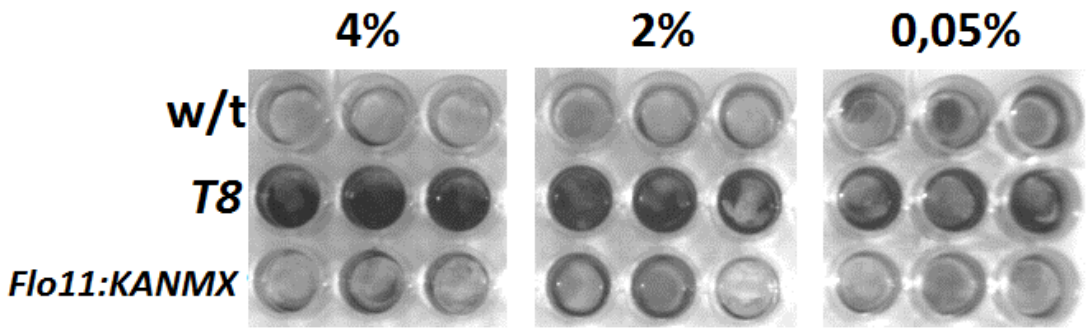

Figure 3.13: Image of 96-well plate after the removal of crystal violet with water. Each sample was inoculated for 3 times, and glucose concentrations (w/v) were shown above the images.

In all three kinds of YPD with different glucose concentrations, *T8* is the strain which showed the highest adherence to plastic (Figure 3.13). Also, it was obvious that rate of adhesion got higher as the glucose concentration was increased. In 0.05 % NaCl containing YPD, w/t was better in adhering to plastic than *T8 flo11::KANMX* strain; however, increasing glucose concentration caused *T8 flo11::KANMX* strain adhere to plastic better than the w/t. Regarding this observation, in 2% and 4% glucose containing YPD w/t showed less adhesion than *T8 flo11::KANMX T8* strain; however, in 0.05% its adherence rate was obviously better than *T8 flo11::KANMX* in adherence in glucose containing YPD. In addition to this visual result of adherence to plastic test, quantification of strains' adherence to plastic was done by multiplate spectrophotometer and the absorbance values were shown in Figures 3.14-3.16, in different glucose concentrations.

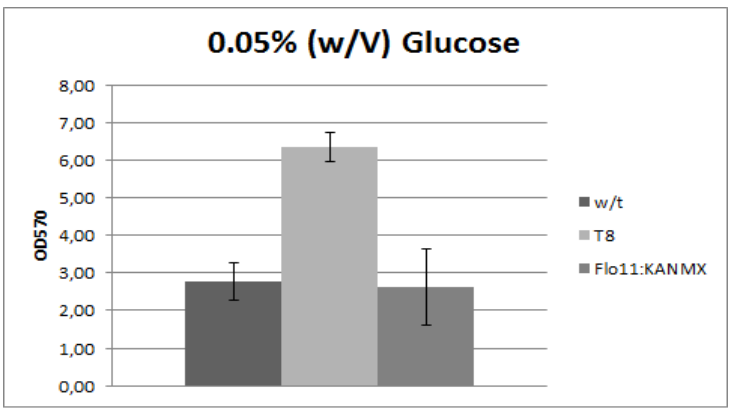

Figure 3.14: Absorbance values of strains in 0.05% (w/v) glucose containing YPD.

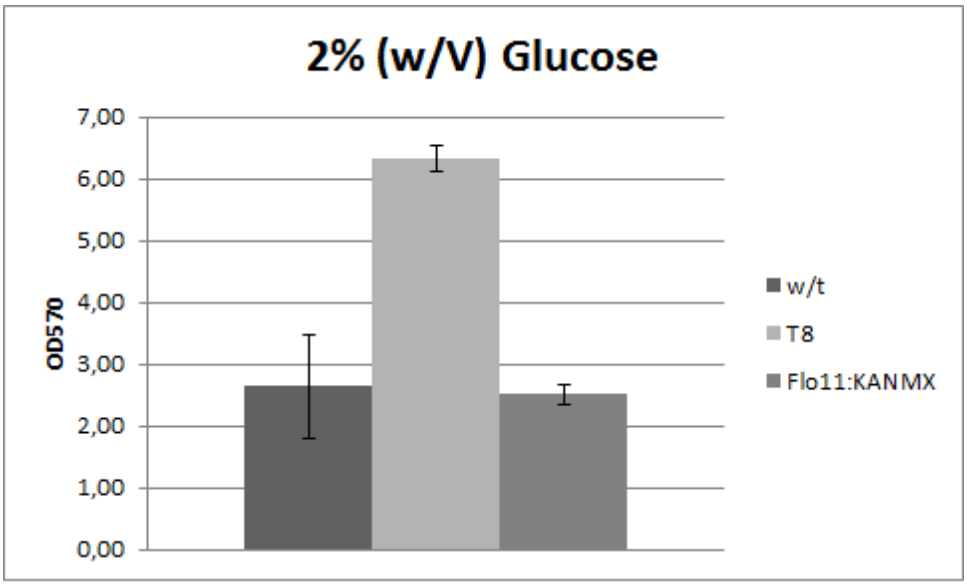

Figure 3.15: Absorbance values of strains in 2% (w/v) glucose containing YPD.

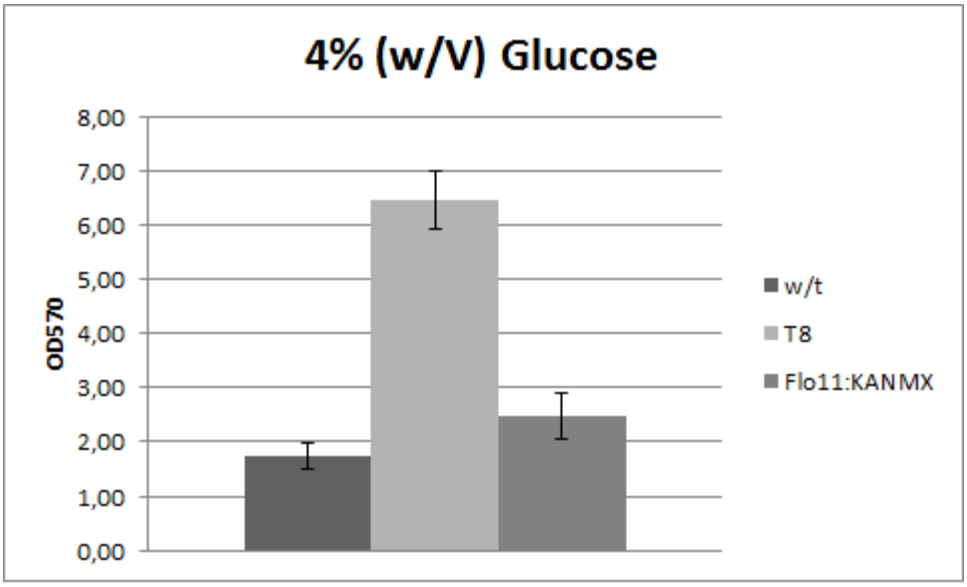

Figure 3.16: Absorbance values of strains in 4% (w/v) glucose containing YPD.

As it shown in Figures 3.14-3.16, absorbance values were similar with to visual observation results. However, in absorbance results there is not a rise in adhesion rate of *T8* with increasing glucose concentrations. Adherence to plastic test results were nearly the same in all glucose concentrations for *T8.* Moreover, in visual results in 2% glucose concentration *T8 flo11::KANMX* seems to have an adhesion rate higher than the w/t; however, in absorbance results it seems to be the opposite.

3.8 Ca^{2+} Dependent Flocculation Test

Ca^{2+} flocculation tests of the strains w/t, *T8* and *T8 flo11::KANMX* were performed according to Section 2.2.7. After obtaining Value A and Value B; flocculation rates of the strains were calculated by using Formula 3.4.

Flocculation (%) = [(Value A – Value B) / Value B] x100 **(3.4)**

The calculated flocculation percentages of the strains are shown in Figure 3.17.

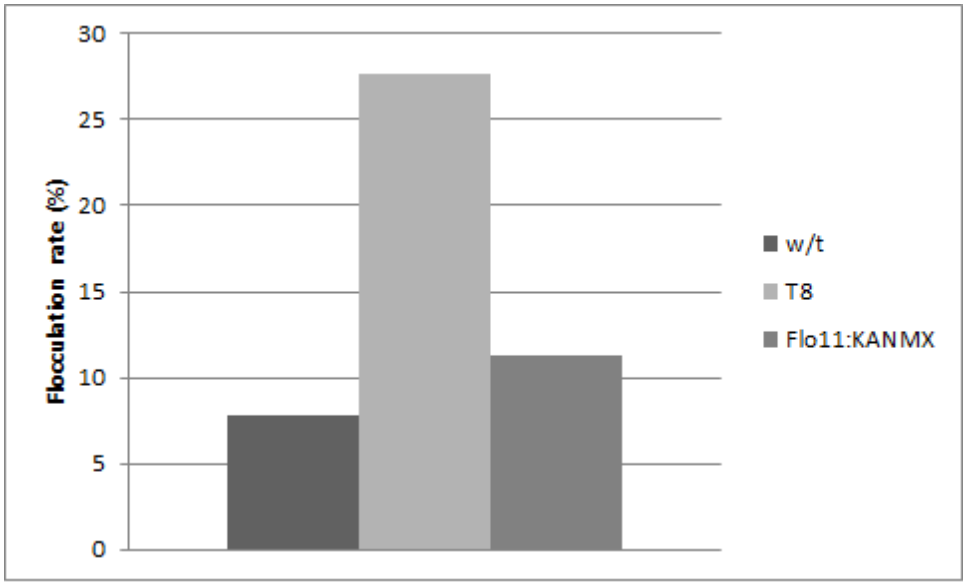

Figure: 3.17: Flocculation rates of the strains.

According to flocculation rates shown in Figure 3.13, it can be concluded that *T8* had the highest flocculation rate followed by *T8 flo11:KANMX*, and w/t respectively.

3.9 Measurement of Glycogen and Trehalose Content

Trehalose and glycogen contents of w/t and salt-mutant *T8* were measured using the experimental protocol described in Section 2.2.8. Results of the experiments areshown in Figures 3.18-21.

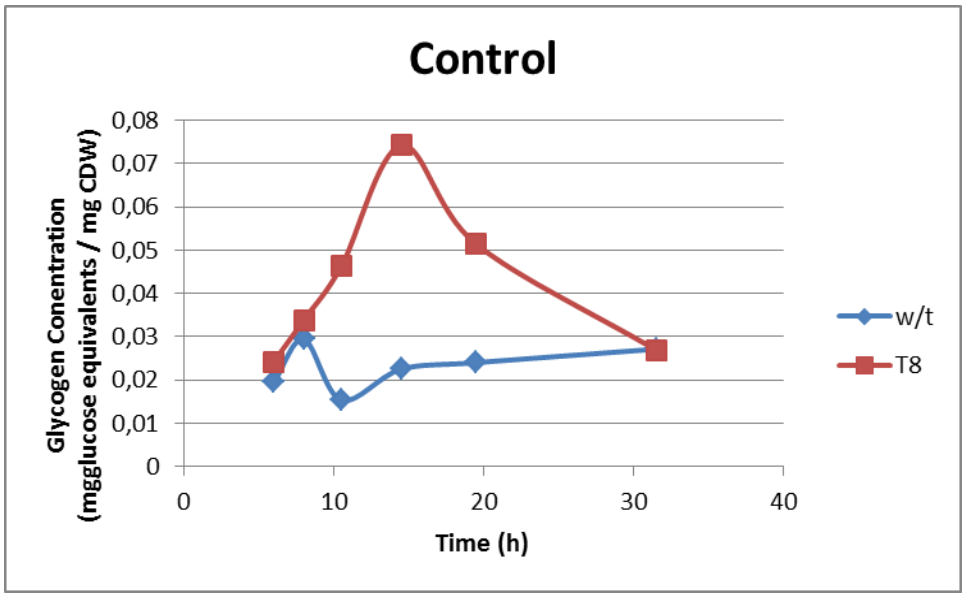

Figure 3.18: Intracellular glycogen content of w/t and *T8* in control medium.

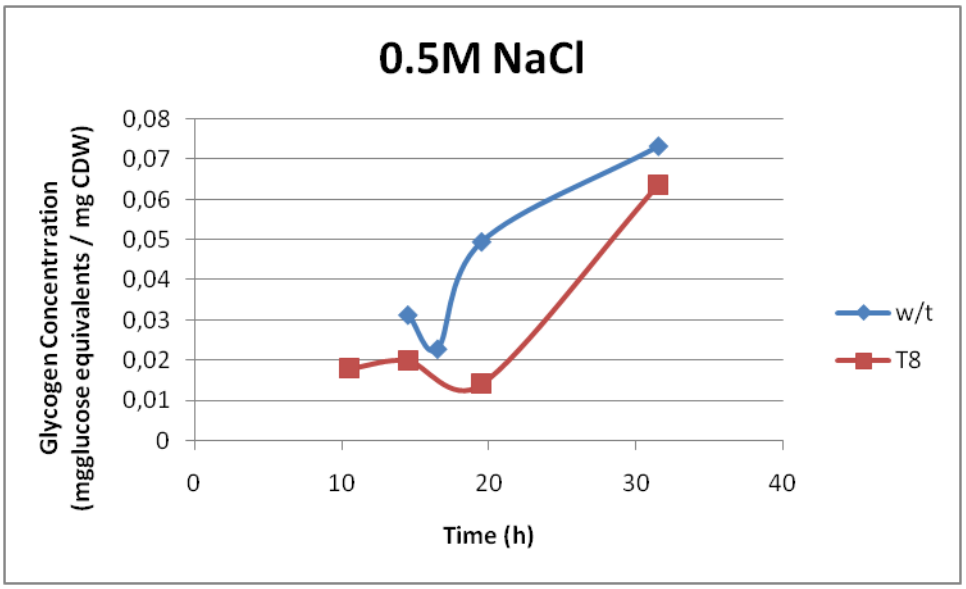

Figure 3.19: Intracellular glycogen content of w/t and *T8* in 0,5 M NaCl.

According to Figures 3.18 and 3.19, it was obvious that NaCl triggered the glycogen production in w/t; while *T8* mutant produced it well in both absence and presence of NaCl stress.

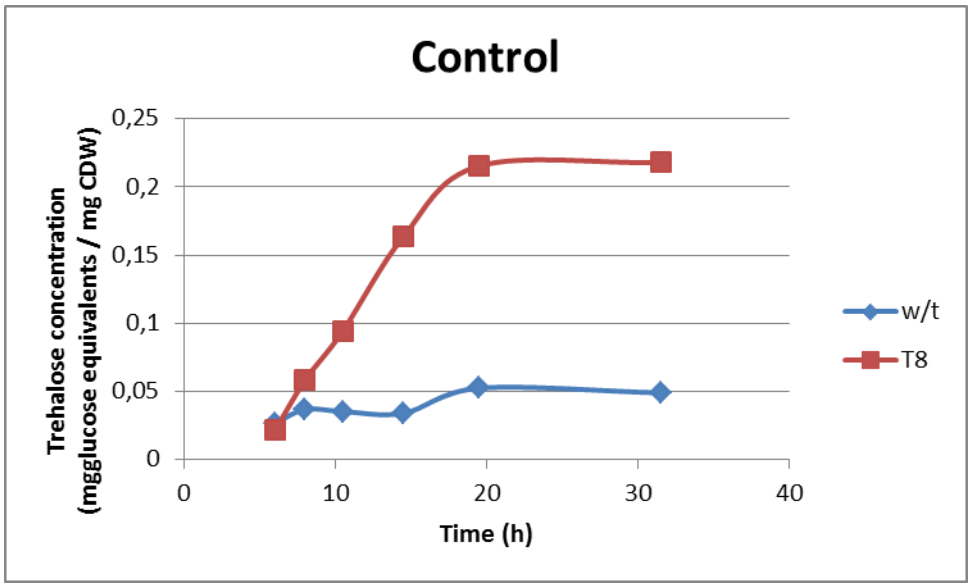

Figure 3.20: Intracellular trehalose content of w/t and *T8* in control medium.

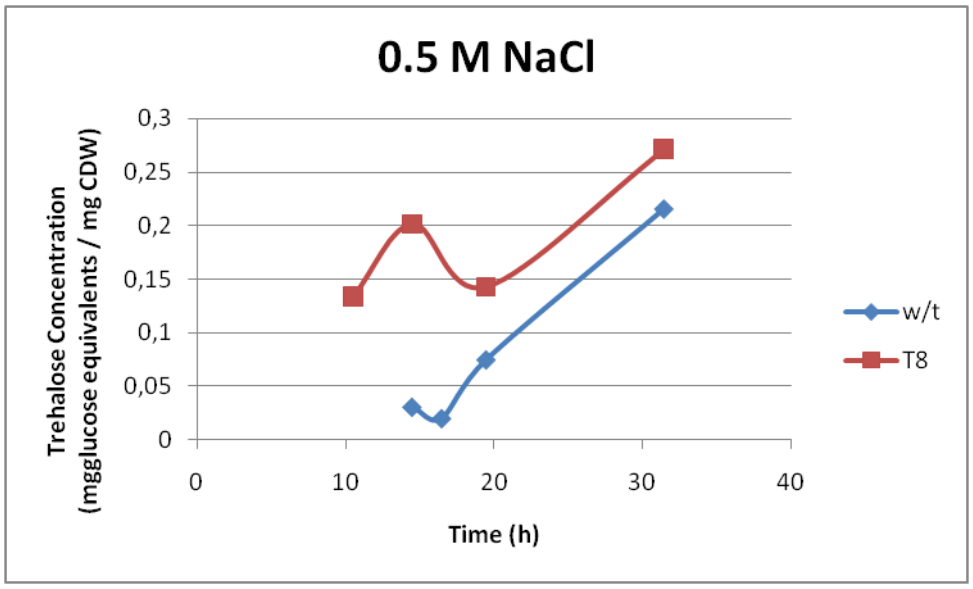

Figure 3.21: Intracellular trehalose content of w/t and *T8* in 0,5 M NaCl.

According to Figures 3.20, 3.21; NaCl stress addition increased the production of trehalose both for w/t and *T8*. However, *T8* seemed to be producing higher amounts of trehalose as its maximum trehalose concentration was five times higher than the maximum concentration produced by w/t.

3.10 Change in Glucose and External Metabolite Concentrations

Changes in concentrations of glucose and external metabolites, such as glycerol, acetate, and ethanol were measured by HPLC (Section 2.2.5). The curves which were obtained by HPLC measurements are shown in Figures 3.22-3.25.

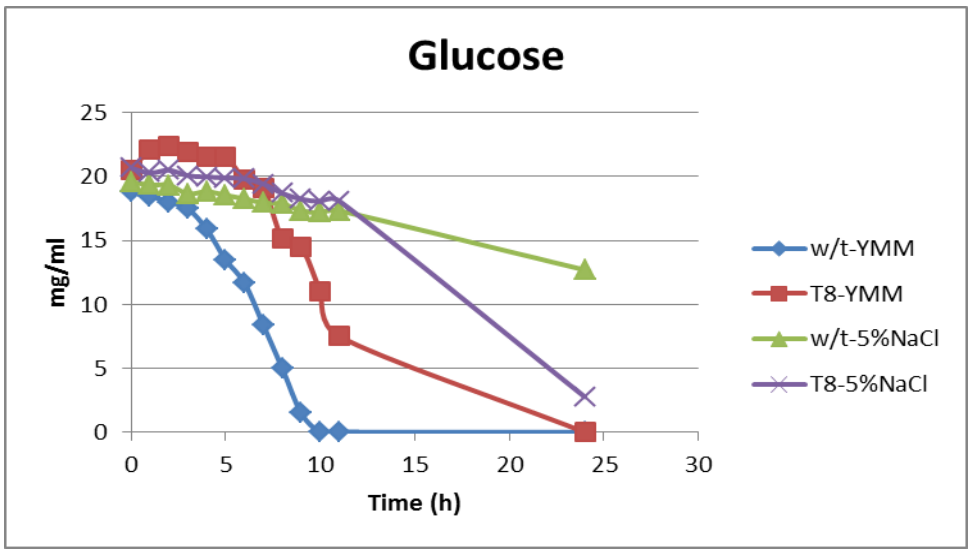

Figure 3.22: Residual glucose concentrations in the medium.

For each culture, incubation started with 20 mg/ml glucose concentration. Glucose in w/t-YMM, and *T8*-YMM cultures were completely consumed after 24th h of incubation. However, in 5% NaCl stress containing medium, strains could not consume glucose completely even after the 24th h of incubation.

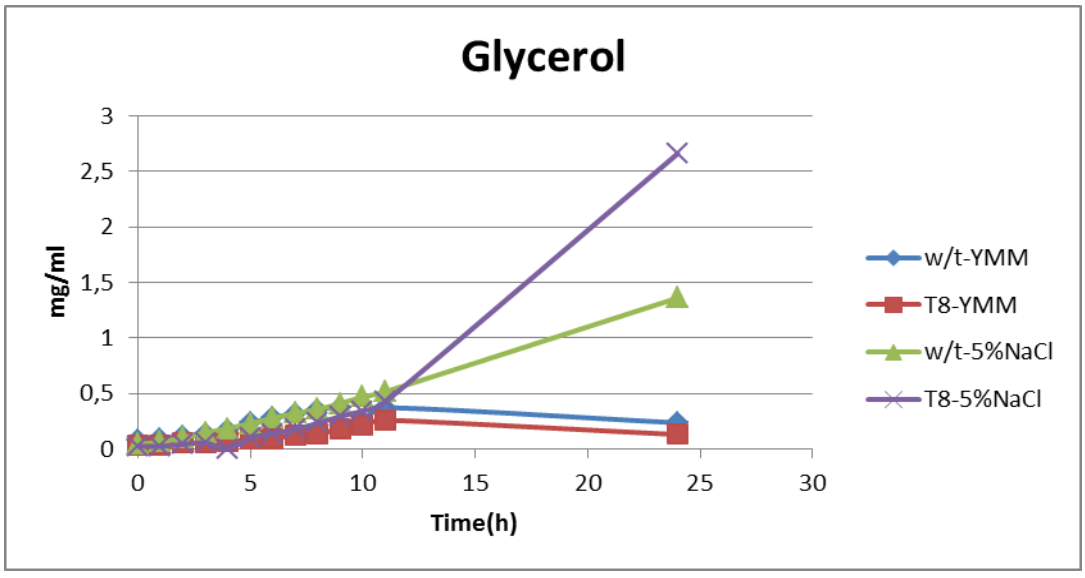

Figure 3.23: Glycerol concentration of w/t and *T8* in medium.

In high salt concentration, wild type strain produced nearly 6 folds higher glycerol than the one produced in stress-free medium; while *T8* cells produced 20 times higher glycerol in 5% (w/v) NaCl containing medium. Moreover, *T8* cells produced 2 times higher glycerol than w/t cells in high salt stress; however in normal conditions w/t cells produced more glycerol than *T8* strain.

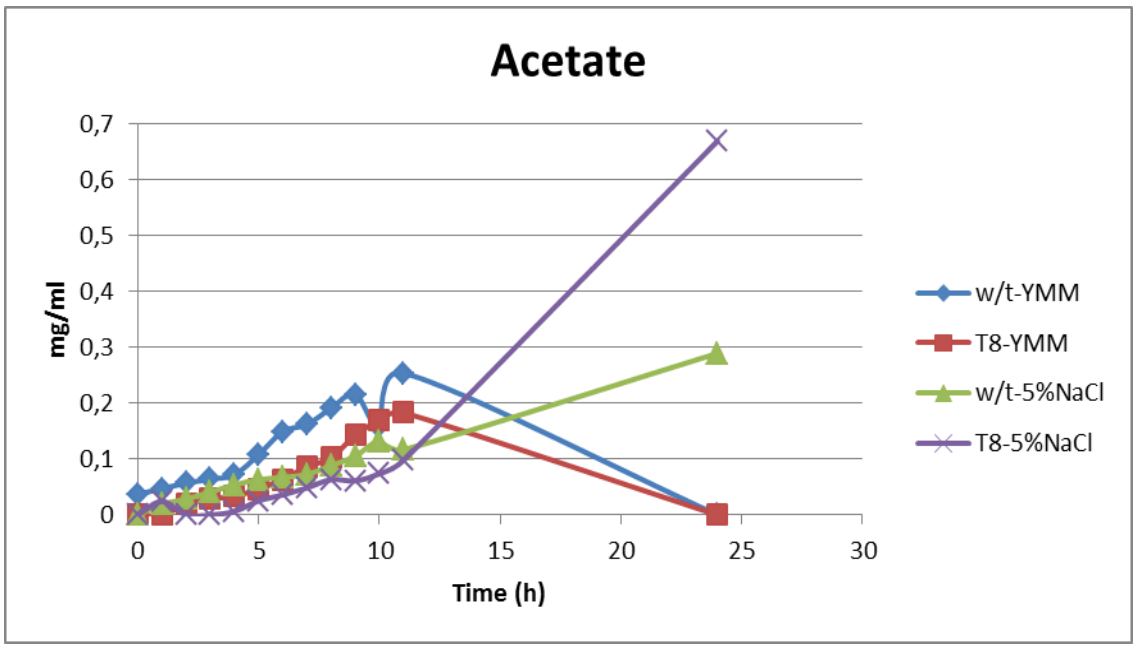

Figure 3.24: Acetate concentration of w/t and *T8* in medium.

In normal conditions, w/t cells produced more acetate than the mutant cells. In high salt concentrations, wild type cells produced as much acetate as in normal conditions; however they reached the same value during very later phases of growth. This delay was observed in *T8* strain in high salt concentration, too. However; the acetate production rate of mutant cells reached a value that was almost 4 times higher than the ones observed in normal conditions, and nearly 3 times higher than the ones observed with w/t cells with high stress.

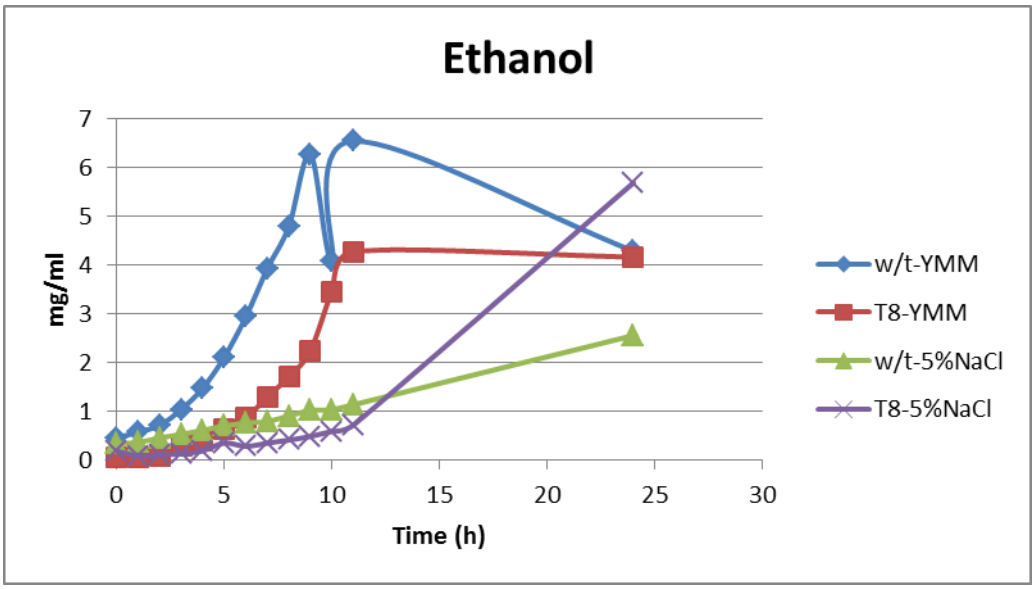

Figure 3.25: Ethanol concentration of w/t and *T8* in medium.

In normal conditions, w/t cells produced more ethanol than the mutant cells. After 11th hour of incubation w/t cells started to consume the ethanol they had produced. In high salt concentration, *T8* cells produced more than 2 times higher concentration of ethanol, however; this amount was still lower than the maximum amount of ethanol that w/t cells could produce in normal conditions.

3.11 Protein Concentrations of w/t and *T8* Samples

At the beginning of each replica of protein analysis, all of the samples' protein concentration was measured by Bradford Assay (Section 2.2.6.2). Obtained protein concentrations are shown in the Table 3.1 and 3.2 for first and second replica, respectively.

Table 3.1: Protein Concentrations of the samples collected and measured by Bradford Assay during the first replica.

Sample	Protein concentration (mg/ml)
w/t (YMM)	8.5
w/t (5% NaCl)	2.2
w/t (8% NaCl)	1.7
T8 (YMM)	7.6
T8 (5% NaCl)	5.5
T8 (8% NaCl)	1.8

In the first replica, according to protein concentrations obtained by Bradford assay (Table 3.1), wild type cells grown in control medium had the highest protein concentration followed by *T8*-control, *T8*-5% NaCl, w/t-5% NaCl and *T8*-8% NaCl. Protein concentration of w/t grown in 8% NaCl had the lowest value. However, there was only a little difference between the protein concentrations of w/t and *T8* strains which were grown in the highest salt stress condition (8% NaCl), in 5% NaCl stress containing medium, the protein concentration of *T8* was more than two folds higher than w/t.

In the second replica, in stress conditions the protein concentrations of samples were directly proportional with OD_{600} values, while in control conditions they were not. Both w/t and *T8* strains were collected at almost the same OD_{600} value, however, protein concentration of w/t sample was lower than *T8* in control conditions.

The protein concentrations of samples which were collected during the second replica of protein analysis, from the bioreactors are shown in Table 3.2.

Table 3.2: Protein Concentrations of the samples collected and measured by Bradford Assay during the second replica**.**

Sample	Protein concentration (mg/ml)
w/t (YMM)	8.0
T8 (YMM)	10.1
w/t (5% NaCl)	3.1
T8 (5% NaCl)	9.0

3.12 SDS-PAGE

At the first step of protein analysis SDS-PAGE was done, according to Section 2.2.6.3, to have a general idea about protein characteristics of all samples.

Obtained SDS-PAGE result was as shown in the Figure 3.26.

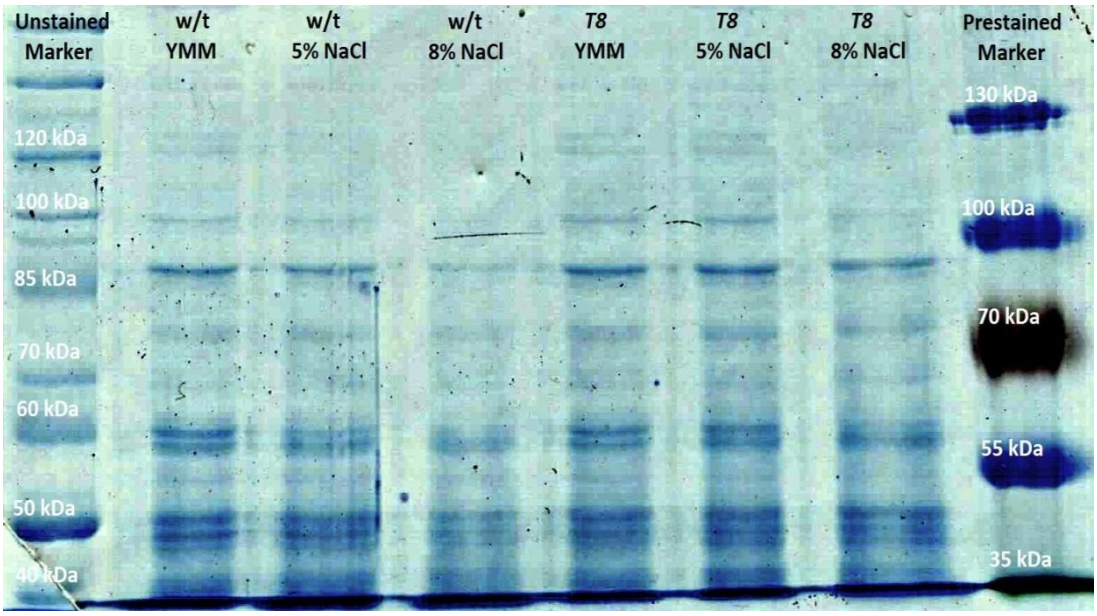

Image 3.26: SDS-PAGE image of w/t and *T8* strains in different stress containing and control medium.

As shown in Image 3.26, all of the protein samples demonstrated band formations exactly at the same sizes. In all samples, proteins condensed between the sizes of 50 – 35 kDa. Also, two band formations were observed between the sizes of 55-60 kDa, in all protein samples. However, all of the samples demonstrated a distinct band formation at the size of 90 kDa, the sample which was derived from w/t cells grown in 8% NaCl stress containing medium, only demonstrated a weak band formation at this size. Moreover, the sample which was derived from w/t cells grown in 8% NaCl stress containing medium was different from others in the way of its bands' impotency. However, the bands referring to almost the same protein size were observed and all of the samples have exactly the same initial protein concentration (1mg/ml), the bands belong to w/t cells grown in 8% NaCl stress containing medium were much weaker than the others.

3.13 2D-Gel Electrophoresis

In order to have more detailed information about differences between protein expression and search for differences in protein production between w/t and *T8* mutant cells, 2D gels of the samples were made (see section 2.2.6.4) in addition to SDS-PAGE. The results of 2D gels, for both replicas, are shown in the Figures 3.27-32.

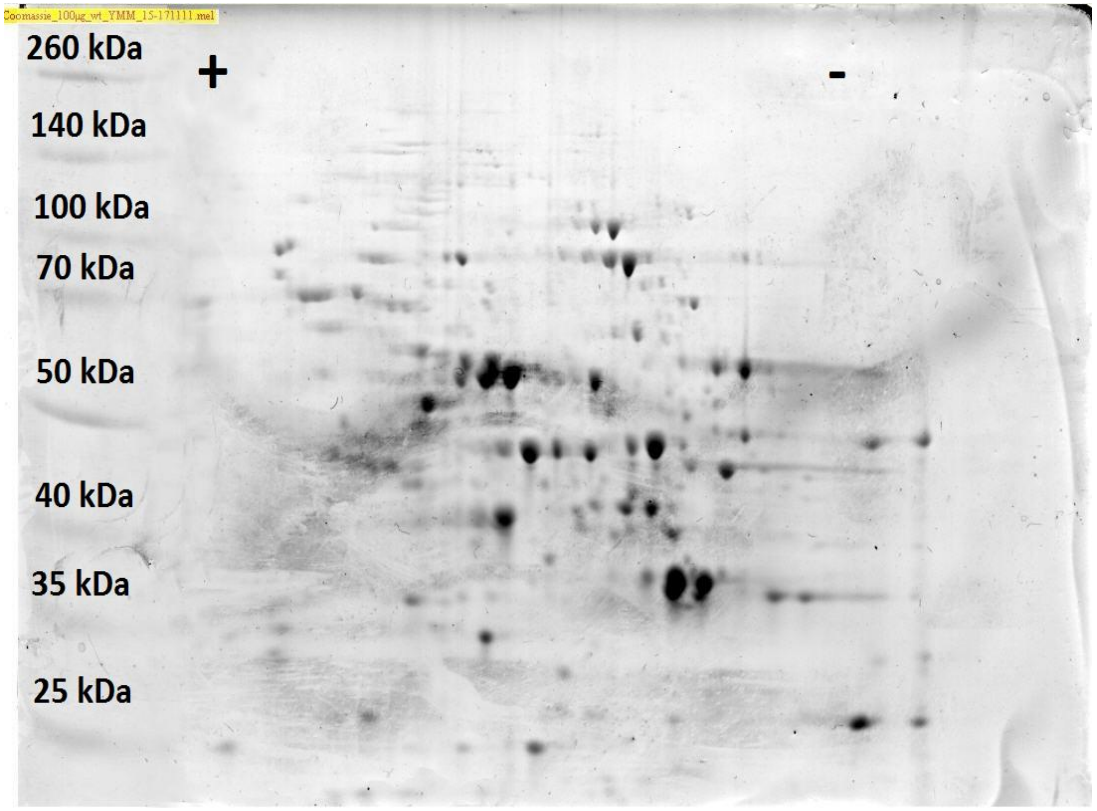

Figure 3.27: 2D gel image of w/t proteins derived from the cells grown in control medium in first replica.

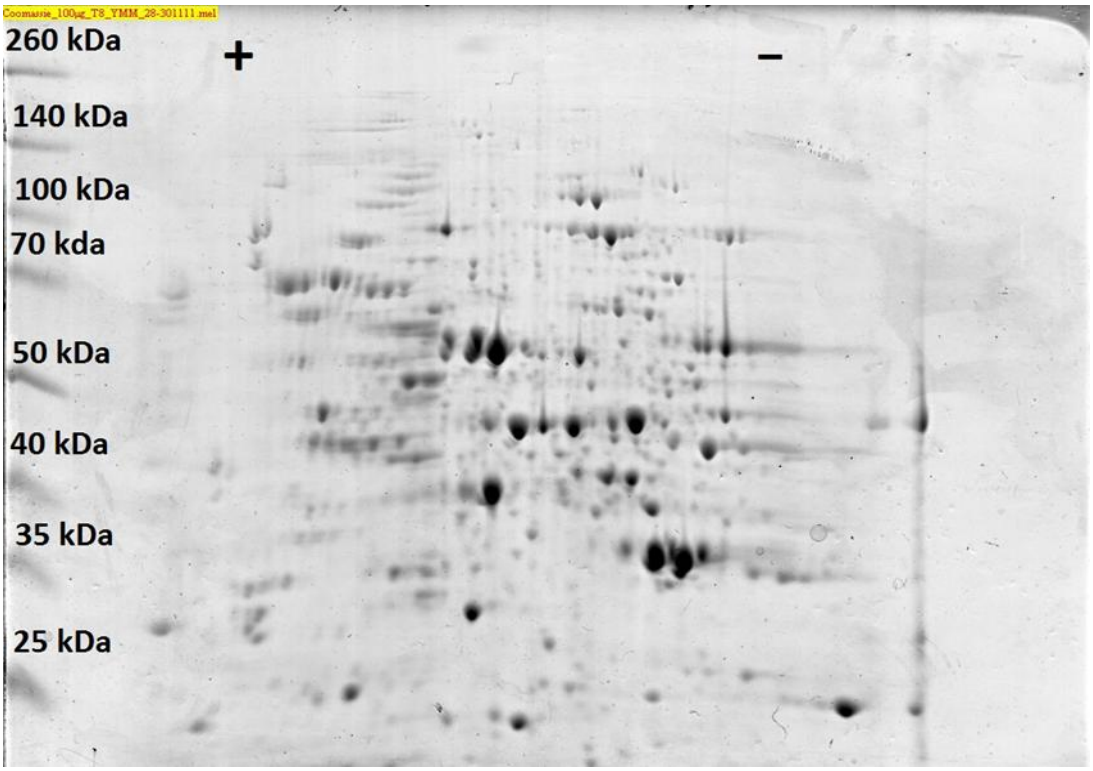

Figure 3.28: 2D gel image of *T8* proteins derived from the cells grown in control medium from the first replica.

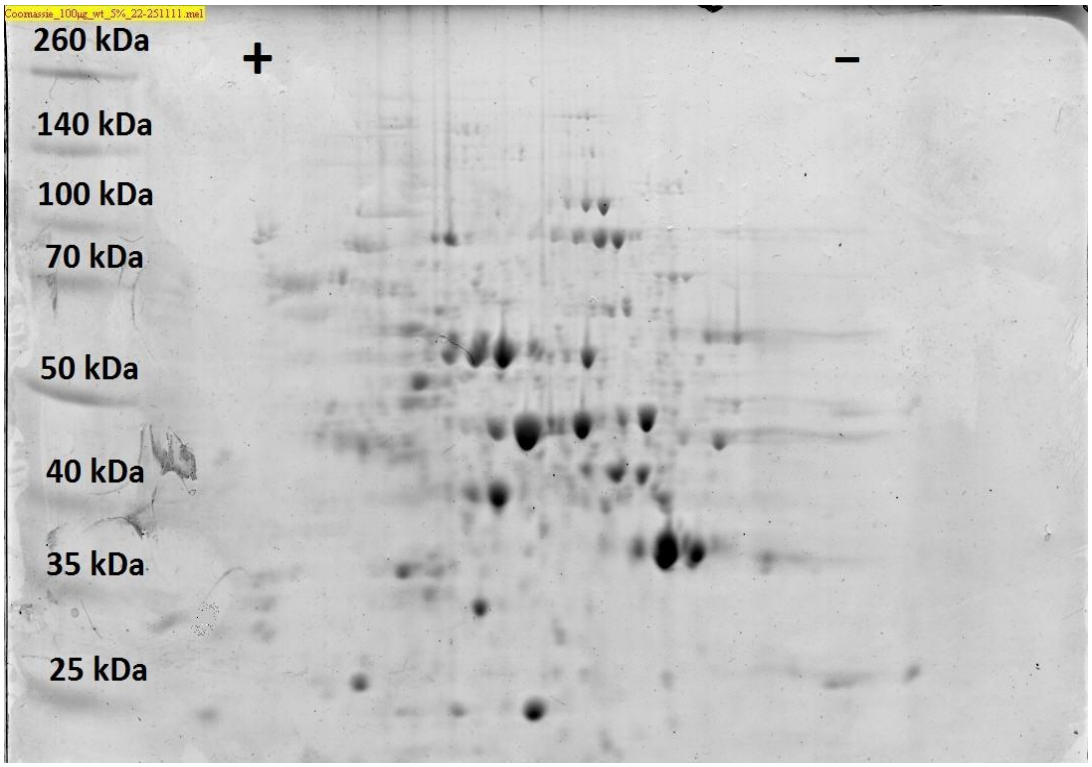

Figure 3.29: 2D gel image of w/t proteins derived from the cells grown in 5% NaCl containing YMM, from the first replica.

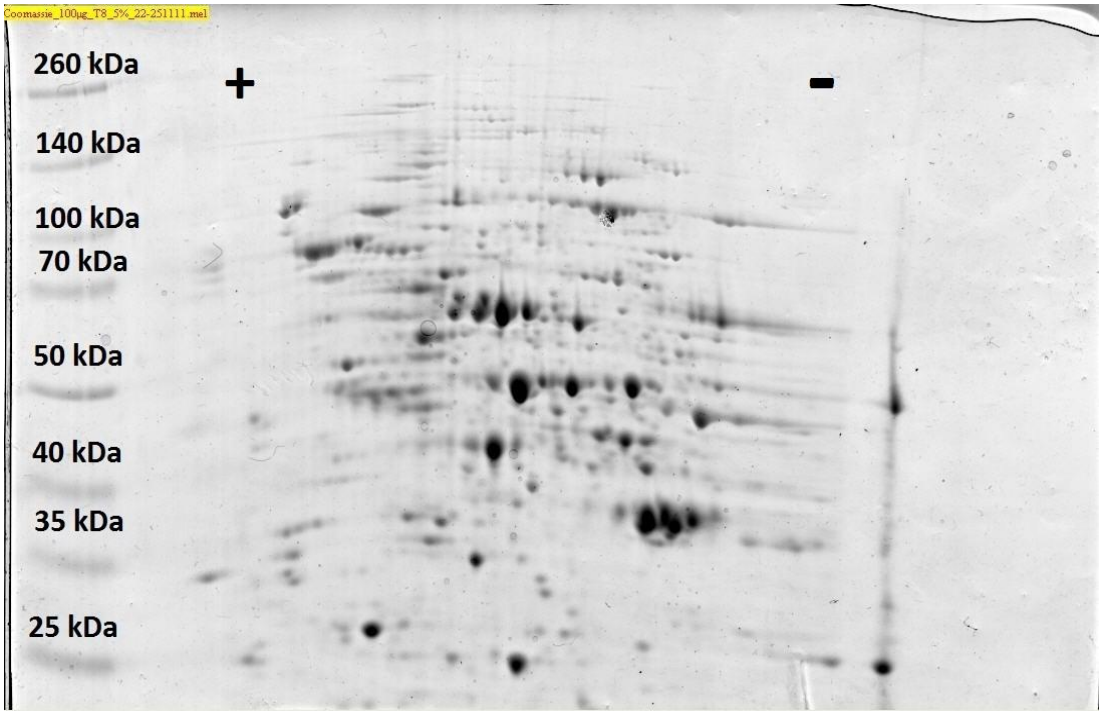

Figure 3.30: 2D gel image of *T8* proteins derived from the cells grown in 5% NaCl containing YMM from the first replica.

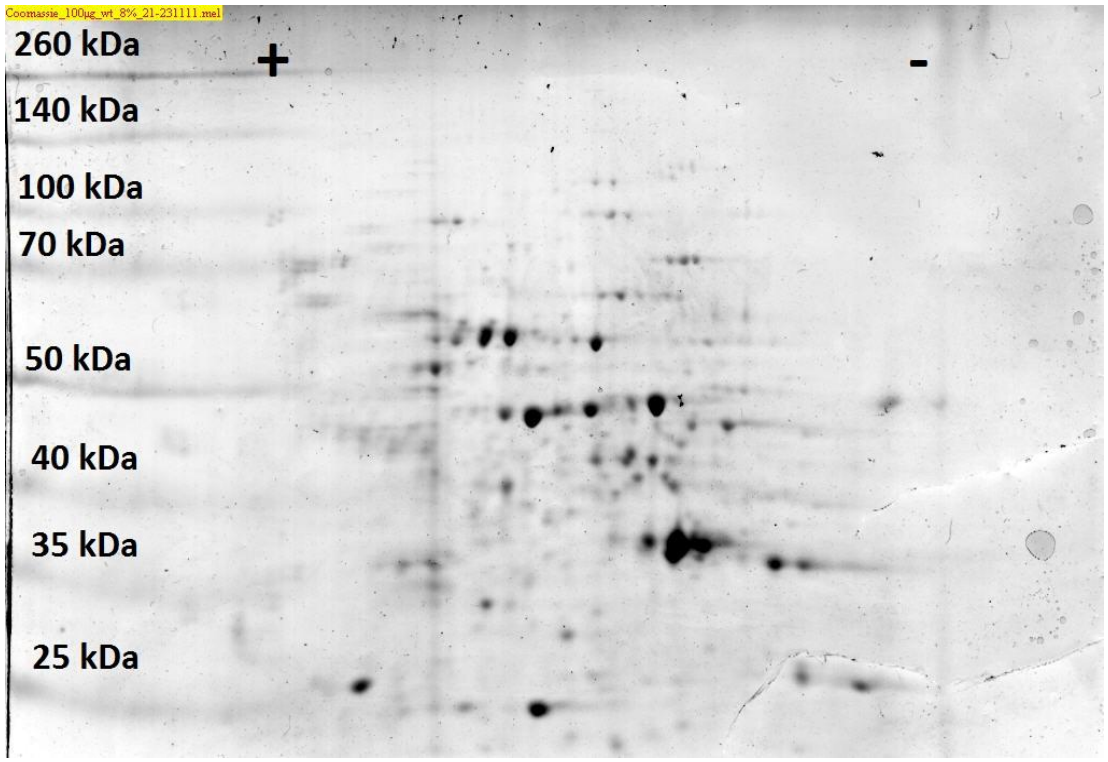

Figure 3.31: 2D gel image of w/t proteins derived from the cells grown in 8% NaCl containing YMM from the first replica.

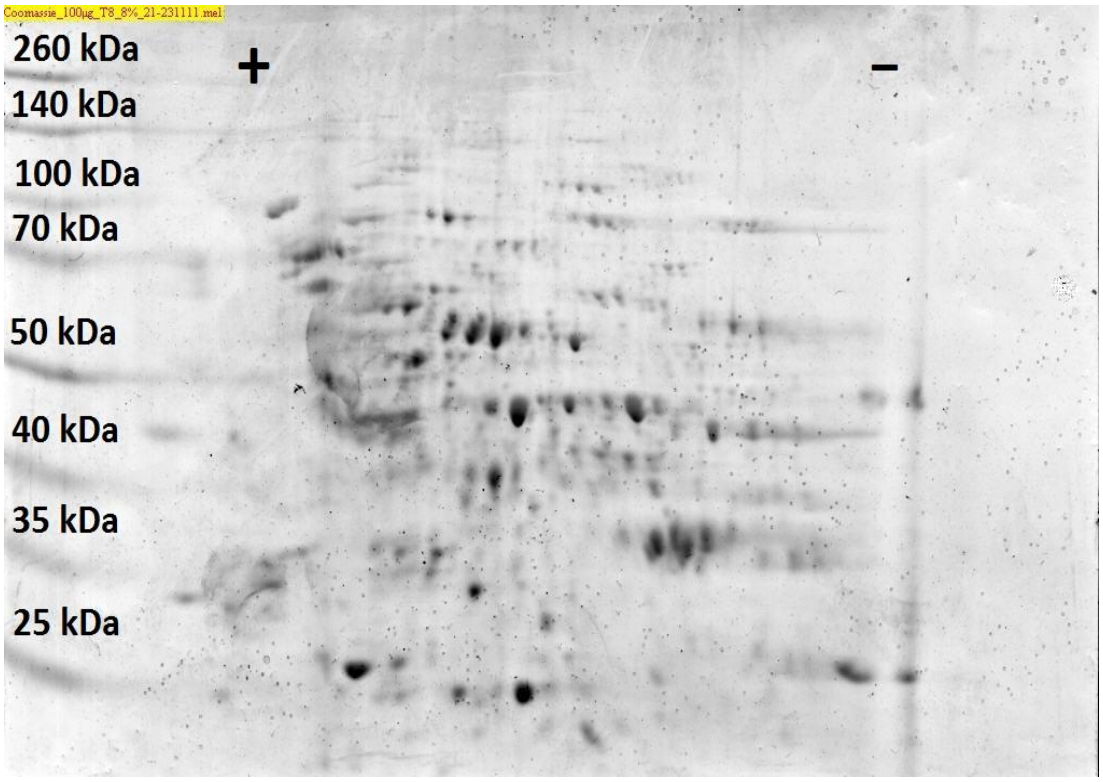

Figure 3.32 : 2D gel image of *T8* proteins derived from the cells grown in 8% NaCl containing YMM from the first replica.

In Figures 3.27-32, the protein sizes that the marker bands indicate and the positive and negative terminals of the strips were demonstrated on the images. The proteins were intensive in the sizes around 60 kDa and smaller than 60 kDa. This result was parallel with the one obtained by SDS-PAGE (see Image 3.3). Also, isoelectric points of the samples of these proteins mainly ranged from 5 to 8, in all samples from the first replica. Moreover, in images, the strength of the spots differing can be easily seen between the samples. The spots which belong to *T8* proteins were stronger than the spots which w/t proteins formed, in all of the media; control (YMM), 5% NaCl containing YMM and 8% NaCl containing YMM. However, there was no distinct spot that created a difference among the samples.

In addition to results of first replica, the scanned 2D gel images of second replica are shown in Figures 3.33-3.36.

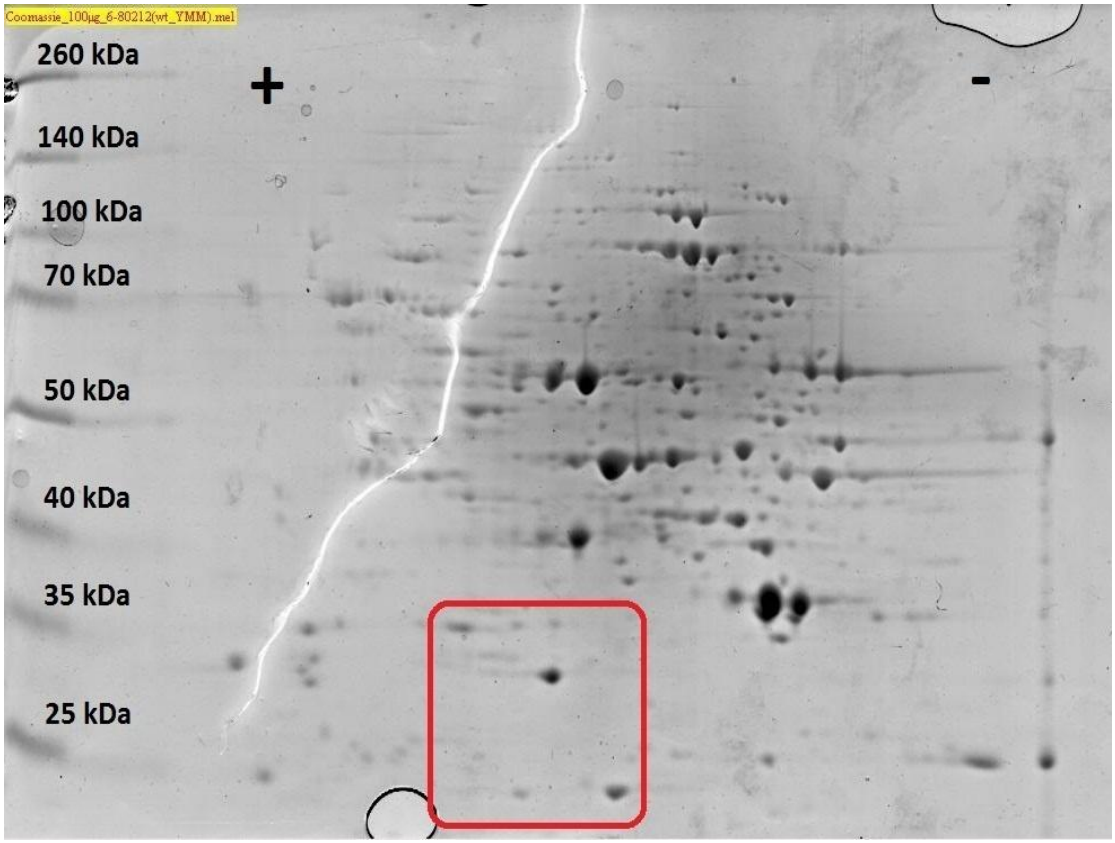

Figure 3.33 : 2D gel image of w/t proteins derived from the cells grown in YMM, from second replica.

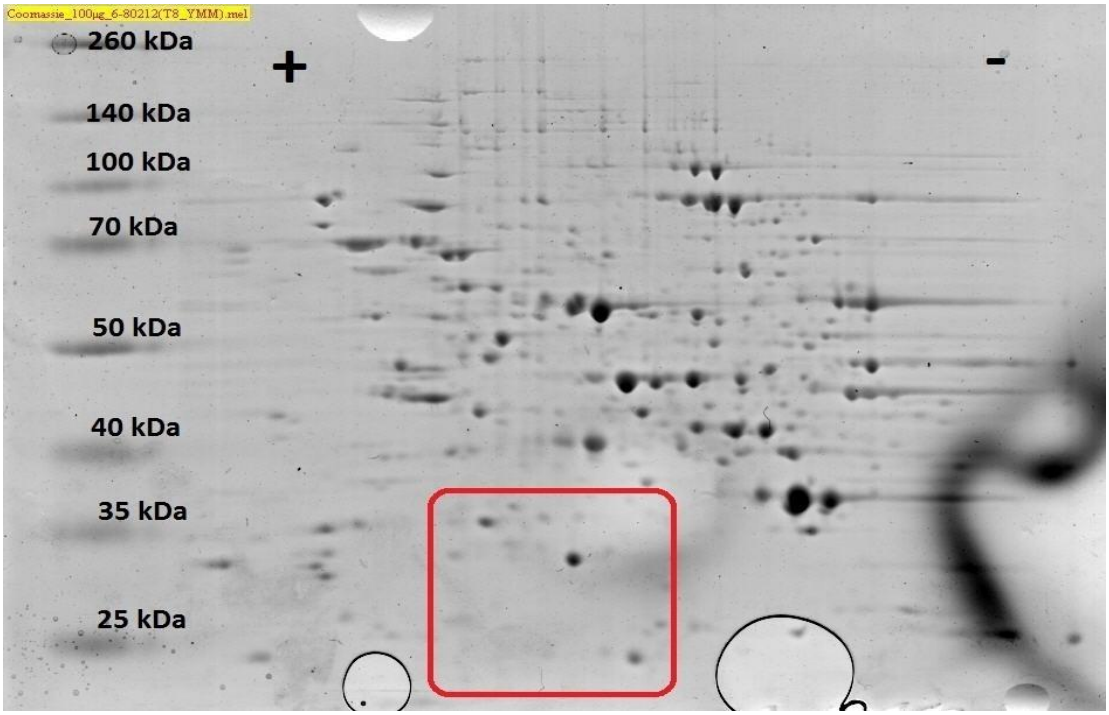

Figure 3.34 : 2D gel image of *T8* proteins derived from the cells grown in YMM, from the second replica.

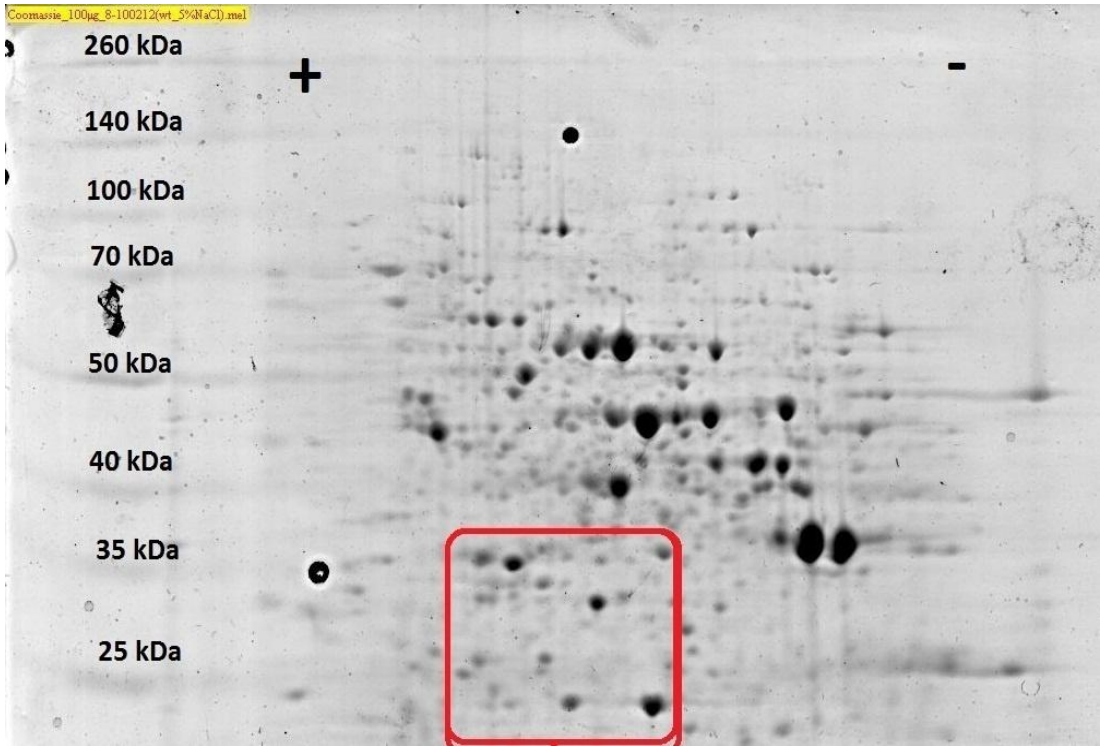

Figure 3.35 : 2D gel image of w/t proteins derived from the cells grown in 5% NaCl containing YMM, from the second replica.

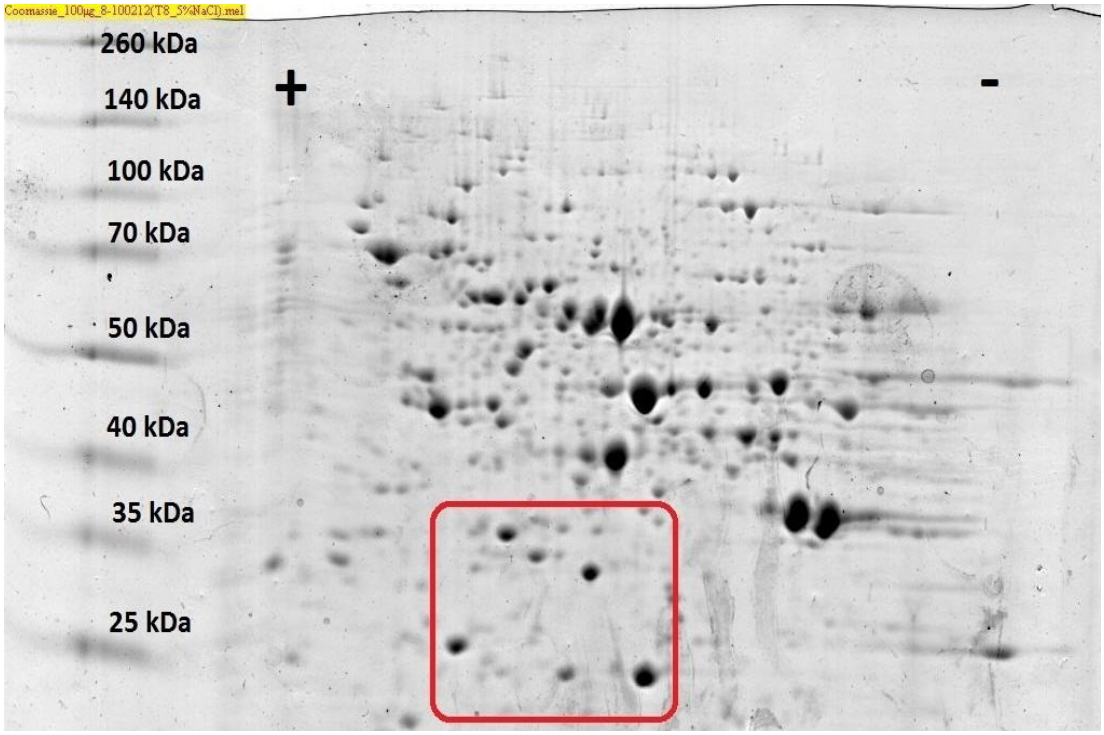

Figure 3.36 : 2D gel image of *T8* proteins derived from the cells grown in 5% NaCl containing YMM, from the second replica.

In contrast to first trial of 2D gels, in second replica some spot differences were found and they were pointed out with squares on the images. According to this difference, both for w/t and *T8* strains in high salt concentration 6 spots of proteins were detected in the red squares; however, for both of the strains only 3 of these spots could be detected in stress-free medium. However, there was no difference in these 6 spots between first and second replica of 2D gels. In other words, in first replica the 6 spots were present in all samples. Therefore; replica w/t-YMM and *T8*-YMM samples also differed from the first trial of w/t-YMM and *T8*-YMM.

4. DISCUSSION AND CONCLUDING REMARKS

In this study, salt resistant *Saccharomyces cerevisiae* mutant *T8* that was previously obtained by evolutionary engineering was investigated regarding its phenotypic, physiological and proteomic differentiations from the wild type strain. In order to achieve the goals of the study; bioreactor experiments, flocculation tests and 2D gel electrophoresis of *T8* were carried out in comparison with the wild type.

Bioreactor experiments allowed a detailed comparison of the w/t and *T8* according to their growth behaviour, metabolite production and morphology.

Based on three different growth curves obtained during bioreactor experiments by OD_{600} measurements, direct cell counts and cell dry weight analysis of w/t and mutant *T8*, the best growth curve was the one obtained by OD_{600} measurements, since the others had some fluctuations. However, there are eligible reasons for the flocculations in growth data obtained by direct cell counts and cell dry weight analysis. In direct cell counting method, yeast cells were counted one by one under phase contrast microscope. However, 40 X was the highest magnitude, which could be used with the chamber. Since, as mentioned before, yeast cells, especially *T8* mutants substantially form flocs during their growth; it was impossible to distinguish each cell and make an accurate count by eye (Barrales, *et al*, 2008). Therefore, big chambers do not fit very well with yeast cells to count them directly. On the other hand, the reason that made growth curve obtained by cell dry weight measurements less suitable than that obtained by the spectrophotometric analysis could be the slight volume of sample taken (1.5 ml) which resulted in a low dry weight with heigh errors. Therefore, cell dry weight analysis of yeast cells would be better if it could be made with larger volume of samples. Overall, it was clearly observed that *T8* mutant endured salt stress conditions more than the wild type.

Decrease in oxygen concentration of the medium was one of the most important data indicating the growth of the organism in related medium. Measured oxygene concentrations were all parallel with the growth curves; in other words, the more the

cells grew the more dissolved oxygen was consumed in the medium. However, there was an unexpected rapid decline in dissolved oxygen amount at the end of the incubation of w/t cells in control medium. The rapid decrease thought to be resulted from a damage in the oxygen sensor; since the off gas oxygen and dissolved oxygen curves should have been parallel.

Respiratory quotients calculated regarding off gas data of all samples, except w/t in 5% (w/v) NaCl salt stress containing medium, were all positive. In control medium, both w/t and *T8* cells' respiratory quotients were higher than 1 until the time they fully consumed glucose and started to deplete ethanol. In other words, both strains made fermentation dominantly than respiration until they started to consume ethanol that they formerly produced. In stress conditions, respiratory quotient of *T8* never became lower than 1, meaning that *T8* could not wind up all glucose content in the medium and did not switch to respiration until the termination of reaction in bioreactor. However, an extraordinary result was observed in 5% (w/v) salt stress containing medium for w/t cells. The respiratory quotient of w/t cells was negative during 24 h of incubation in stress conditions. In the off-gas data of w/t cells in stress conditions (Appendix C), it was obvious that the cells did not demonstrate any metabolic activities and were inhibited by high salt concentration, as they did not consumed any oxygen and produced slight amounts of carbondioxide. Moreover, the unusual negative respiratory quotient of the cells was thought to be resulted from the imbalance between oxygen consumption and carbondioxide production rates. Besides, the reason for imbalance between oxygen and carbondioxide, in other words how cells did produce carbondioxide even if in slight amounts while they did not consume any oxygen, was explained with the assumption that they might have started ethanol fermentation directly to survive in stress conditions and made no respiration.

Since *S.cerevisiae* is a Crabtree positive yeast, it can make fermentation simultaneously respiration under aerobic conditions, if the glucose concentration is high in the medium. The RQ values which were higher than 1 during the first 10 h of fermentations in control medium, shows that 20 mg/ml glucose was consumed both by fermentation and respiration during the exponential growth phase. However, after glucose was totally consumed, produced ethanol was started to be consumed by respiration. That is why RQ values decreased dramatically after glucose

consumption. These comments were parallel to HPLC results showing that ethanol was produced continuously until the 10^{th} h of incubation and then started to be consumed; moreover glucose was totally consumed around the 10^{th} of incubation.

T8 cells differed from w/t in the flocculation rate during incubation. However, w/t strain showed flocculation as well as *T8* strain, the flocculation rate of w/t was much lower than *T8*. The reason that stimulated high flocculation rate in *T8* strain thought to be resulted from apparent advantage of reducing total cell surface area in contact with the salty environment. To advance this result; invasive growth assay, calcium dependent flocculation assay and adherence to plastic test were carried out with w/t, *T8* and *T8 flo11::KANMX* strains. As it was shown in results section, *T8 flo11::KANMX* mutant could not show any invasive growth into the agar, while *T8* strain showed the highest invasion. Since it was obviously observed that *T8 flo11 : KANMX* strain, which has no *FLO11* gene expression, could not demonstrate any invasion into agar it was hypothesized that *FLO11* gene expression has a major effect on invasion. *T8* was the strain which invaded into agar mostly, therefore, it can be mentioned that in salt-resistant mutant *Saccharomyces cerevisiae T8 FLO11* gene was expressed more than w/t (Sezgin, 2010). As mentioned before there are several other genes, such as *Flo1, Flo5*, *Flo9* and *Flo10*, that are responsible for forming flocs in *S.cerevisiae* (Govendar, *et al.*, 2008). Therefore, it was thought that *FLO11* gene deletion may have caused other members of *Flo* family to be inhibited, as there were not any trace of invasion into agar in *T8 flo11::KANMX* strain. When these three strains were compared according to their adhesion rates to plastic, it was observed that, glucose concentration may alter the behavior of the cell. For instance, as indicated in results section, in 0.05 % (w/v) glucose concentration the consequences of adhesion to plastic assay were as expected. In other words, *T8* was the strain, which showed the highest adhesion rate, it was followed by w/t, and *T8 flo11::KANMX* respectively. However, in higher glucose concentrations adhesion rate of w/t was less than *T8 flo11::KANMX* strain, while *T8* still had the highest rate. These results were observed in 2% (w/v) and 4% (w/v) both by visual and spectrophotometric analysis in all triplicates. Therefore, it was thought that, plastic adhesion may have a more complex mechanism other than *FLO11* gene family and it probably has a direct relation with glucose concentration in the medium. Similar with the adhesion to plastic results, in Ca^{2+} dependent flocculation test *T8* mutant

was the strain, which had the highest flocculation rate, too. However, *T8 flo11::KANMX* strain had a higher rate than w/t. In all four different tests related with flocculation characterization of these three strains, *T8* was observed as the most flocculating strain. However, in adhesion to plastic and calcium cation dependent flocculation tests *T8 flo11::KANMX* strain observed as flocculating more than the wild type, unexpectedly. Therefore, it was thought that the mechanism that is responsible for flocculation in *S.cerevisiae* is not only related with *Flo* gene family, but also possibly involves a more elaborate pathway, which is affected by glucose and calcium cation. To have a better insight into the exact mechanism of flocculation in *S.cerevisiae,* real time PCR, sequencing and microarray studies of each strain would be carried out and compared with each other.

Glycogen and trehalose are the two carbohydrates that are present as storage molecules in yeast cells and it is known that stress conditions, especially low water availability in the environment induce metabolism of these molecules (Parrou, *et al.,* 1997). Thus, trehalose and glycogen concentrations of w/t and *T8* mutant were measured in control and 0.5 M NaCl containing medium. When glycogen concentrations are compared in control medium, it was observed that *T8* mutant had a higher glycerol concentration than the w/t; however, in 0.5M NaCl containing medium *T8* had less glycogen than w/t. It was an unexpected result for *T8* to have a lower glycogen concentration as it had much higher concentrations in control medium formerly. Trehalose concentration results were similar both in control and 0.5 M NaCl containing medium as in each case *T8* had a higher concentration of trehalose than w/t. Overall, it can be concluded that salt resistant mutant *T8* differs from w/t in the amount of trehalose and glycogen it produces. Moreover, since these two molecules are known to be related with stress directly, it can be suggested that *T8* might have gained several modifications in trehalose and glycogen metabolism.

Ethanol, glycerol and acetate are the major internal metabolites that *S.cerevisiae* strains produce. Concentrations of these metabolites as well glucose consumption rate were measured with HPLC for control and 5% (w/v) NaCl containing YMM. Glucose consumption rates of the strains in different medium were completely parallel to their growth curves. In other words, the more the strain grew the more glucose was consumed. For instance, in control medium for both strains, glucose was depleted at about 10^{th} h of incubation; however, in stress containing medium no

depletion was observed even if *T8* mutant was about the consume all glucose in the medium at the 24th h of incubation, when the fermentation was terminated. Ethanol concentrations were observed to be changing in inverse ratio with glucose concentrations. During the time glucose was consumed, ethanol was produced and when there were no traces of glucose left in the medium, ethanol that was previously produced was then utilized by yeast cells. Ethanol production rates of w/t was higher than *T8* in control medium, though *T8* was better in stress conditions and had ethanol concentrations compatible with that of the w/t in control medium. Glycerol and acetate concentrations of w/t and *T8* were almost the same in control medium and they started to be consumed at the very end of fermentation. In stress conditions, *T8* produced approximately 2 times higher concentrations of glycerol and acetate than the w/t. Glycerol is known as being very effective for survival of the cell under high osmotic pressure (Albertyn, *et al*., 1994). Therefore, it is an evident that *T8* has a modified glycerol production pathway as it produces more glycerol to handle with the high salt concentration in the environment. On the other hand, there is –to our knowledge- no pevious information in the literature indicating that acetate production rate is directly releated with osmotic stress. However, considering the result that *T8* produced acetate at concentrations 2 times higher than that of the w/t; it can be concluded that acetate metabolism might be related with osmotic stress response in *S.cerevisiae.*

In addition to phenotypic and physological characterization of *T8* strain, its preliminary proteomic analysis was also done by 2D gel electrophoresis in comparison with the wild type. There were no significant differences between the results of *T8* mutant and the w/t, but there were concentration variations in the first replicate that was carried out with the samples from late exponential phase. However, in the second trial where samples from the stationary phase of growth were present, several differences were observed. There were three spot differences both in w/t and *T8* samples derived from 5 % (w/v) NaCl containing medium, in the isoelectric point range of 5-8; and sizes between 20-30 kDa. Observed difference was not between w/t and *T8* ; but it was a protein expression variation occurred both in w/t and mutant *T8* when they were grown in stress containing medium.

In conclusion, salt-resistant *Saccharomyces cerevisiae* mutant strain *T8* was compared in this study with the wild type according to its physological, phenotypical

and proteomic properties and it was found to be different from the w/t in all cases. Morphologically, *T8* strain is distinct as it showed higher flocculation, adhesion and invasion rates than the wild type. Moreover, regarding the comparisons carried out with *Flo11::KANMX* strain, this morphological variation resulted from a possible change in *Flo* gene family expression level in *T8.* Also, *T8* mutant produced higher amounts of glycogen and trehalose as a possible result of a gained osmotic stress defence mechanism. Especially in glycerol production, *T8* strain was superior when it was grown in stress containing medium. This observation showed that *T8* strain possibly gained a modification in glycerol production metabolism, which had been clarified as one of the most common osmotic stress responses in yeast (Albertyn, *et al*., 1994). However, the increase in acetate production rate of *T8* in salt stress conditions is not a commonly encountered observation. Also, relation between acetate metabolism and osmotic stress has not been identified yet. Therefore, for future studies it can be suggested to create a *S.cerevisiae* mutant with a deficiency in acetate metabolism, to investigate the possible link between osmotic stress and acetate metabolism. In 2D gel electrophoresis carried out in comparison with w/t in different salt concentrations for the cytoplasmic proteins, three possible spot differences were found, however, to make sure that there is truly a difference, 2D gel electrophoresis must be repeated for several times with the same samples, and those spots should be identified with sophisticated techniques. For future studies regarding proteomic analysis of *T8* strain, it could be suggested to isolate membrane proteins of mutant and w/t strains, and compare them to each other since osmotic stress is known to be directly related with the permeability modifications of the membrane (Hohmann, 2002).

REFERENCES

Adames, N., R., Cooper, J., A., (2000). Microtubule Interactions with the Cell Cortex Causing Nuclear Movements in *Saccharomyces cerevisiae*, The Journal of Cell Biology, **149:**863-874**.**

Alberts, B., Johnson, A., Lewis, J., Raff, M., Bray, D., Hopkin, K., Roberts, K., Walter, P., (1998). Essential Cell Biology. An Introduction to the Molecular Biology of the Cell. *Garland Publishing.New York.*

Alberts, B., A., Johnson, J., Lewis, M., Raff, K., Walter, R., P., (2002). Molecular Biology of The Cell 4th edition. *Nex York Garland Sciences*

Albertyn, J., Hohmann, S., Thevelein, J.,M., Prior, B.,A. (1994). GPD1, Which Encodes Glycerol-3-Phosphate Dehydrogenase, Is Essential for Growth under Osmotic Stress in Saccharomyces cerevisiae, and Its Expression Is Regulated by the High-Osmolarity Glycerol Response Pathway*Mol. Cell. Biol.* 1994, **14(6)**:4135

Arora PS, Yamagiwa H, Srivastava A, Bolander ME, Sarkar G (2005). Comparative evaluation of two two-dimensional gel electrophoresis image analysis software applications using synovial fluids from patients with joint disease"**.** *J Orthop Sci* **10 (2)**: 160–6

Attfield, P., V., (1997). Stress Tolerance: The key to effective strains of industrial baker's yeast, Nature Biotech, **15**:1351-1357.

Attfield, P., V., Raman, A., Northcott, C.,J (1992). Constructiion of *Saccharomyces cerevisiae* strains that accumulate relatively low concentrations of trehalose, and their application in testing the contribution of the dissaccharide to stres tolerance, *FEMS Microbiology Letters,* **94:** 271-276.

Bailey, J. E. (1991). Toward a science of metabolic engineering. *Science* **252**: 1668-1675**.**

Bailey, J.E., Sburlati, A., Hatzimanikatis, V., Lee, K., Renner, W.A., Tsai, P.S., (1996). Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. *Biotech. and Bioeng.* **52**: 109-121**.**

Barrales, R., R., Jimenez, J., Ibeas, J., I., (2008). Identification of novel activation mechanisms for FLO11 regulation in *Saccharomyces cerevisiae*, *Genetics,* **178** : 145-156.

Boekhout, T. and V. Robert (2003). Yeasts in Food : Beneficial and Detrimental Aspects. Cambridge, Woodhead Pub. ; Boca Raton : CRC Press.

Bressan R. A., Hasegawa P. M. (1998). Plants use calcium to resolve salt stress, *Trends in Plant Science***. 3:11:**, 410-412.

Burke, D., Dawson, D., Stearns, T., (2000). Methods in Yeast Genetics. *Cold Spring Laboratory Press, New York.*

Cimerman N. G., Ramos J., Plemenitas A. (2009). Halotolerant and halophilic fungi, *Mycological Research*.. 1231-1241.

Claro, F., B., Rijsbrack, K., Soares, E., V., (2007). Flocculation onset in *Saccharomyces cerevisiae*: effect of ethanol, heat and osmotic stress, *Journal of Applied Microbiology*, **102** : 693-700.

Costa, V., Moradas-Ferreira, P., (2001). Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases, *Molecular Aspects of Medicine*. **22**: 217-246.

Coury, L. A., M. Hiller, J. C. Mathai, E. W. Jones, M. L. Zeidel, and J. L. Brodsky. (1999). Water transport across yeast vacuolar and plasma membrane-targeted secretory vesicles occurs by passive diffusion. *Journal of Bacteriology*. **181**: 4437–4440.

Çakar, Z., P., Sauer, U., Bailey, J., E., Müller, M., Stolz, M.,Wallimann, T., Schlattner, U.,(2000). Vacuolar morphology and cell cycle distribution are modified by leucine limitation in auxotrophic *Saccharomyces cerevisiae*, Biology of The Cell, **92**: 629-637.

Çakar, Z. P., U. O. Seker, Tamerler, C., Sonderegger M., Sauer, U. (2005). "Evolutionary engineering of multiple-stress resistant Saccharomyces cerevisiae." *FEMS Yeast Research* **5(6-7)**: 569-578.

Deak, T., Handbook of Food Spoilage Yeast, *CRC Press*, (2007)**.**

Escanciano A.F., Garciadeblas B., Rodriguez-Navarro A., Benito B, (2009). Role of ENA ATPase in Na+ efflux at high pH in bryophytes, *Plant Molecular Biology* .**71**, 599–608.

Feldmann, H., (2005). Yeast Molecular Biology: A short compendium on basic features and novel aspects, Adolf-Butenandt- Institute, University of 64 Munich. Retrieved 08.04.2012 from http://biochemie.web.med.uni-muenchen.de/Yeast_Biol

Feldmann, H. (2010). Yeast : Molecular and Cell Biology. Weinheim, Wiley-VCH.

Gasch, A.,P., Spellman, P., T., Kao, C., M., Carmel-Harel, O., Eisen, M., B., Sorz, G., Botstein, D., Brown, P., O, (2000). Genomic expression

programs in the response of Yeast cells to environmental changes, M*olecular Biology of the Cell*, **11**: 4241-4257.

Gasch, A., P., Washburne, M., W., (2002). The genomics of yeast responses to environmental stres and starvation, Funct Integr Genomics, **2**: 181-192.

Govender, P., Domingo, J., L., Bester, M., C., Pretorius, I., S., Bauer, F., F., (2008). Controlled expression of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*, *Applied and Environmental Microbiology*, **74 (19)**: 6041-6052.

Hamilton C.A., Taylor G. J., Good. A. G., (2002). Vacuolar H.-ATPase, but not mitochondrial F1F0-ATPase, is required for NaCl tolerance in *Saccharomyces cerevisiae. FEMS Microbiology Letters.* **208:**227-232.

Hansen, (1883), Integrated Taxonomic Information System (ITIS), *Saccharomyces cerevisiae* species name, retrieved from, http://www.itis.gov on 07.04.2012.

Herskowitz, I., (1988). Life Cycle of the Budding Yeast *Saccharomyces cerevisiae*, Microbial Reviews, **52**: 536-553.

Hohmann S. (2002). Osmotic Stress Signaling and Osmoadaptation in Yeasts, Microbiology and Molecular Biology Reviews. *American Society for Microbiology.* **66**: 300–372.

Hohmann, S., Mager, P.,W.,H., (2003). Yeast Stress Responses, *Springer*. 1.

Ishigami, M., Nakagawa, Y., Hayakawa, M., Iimura, Y., (2004). FLO11 is essential for flor formation caused by the C-terminal deletion of NRG1 in *Saccharomces cerevisiae, FEMS Microbiology Letters,* **237:** 425-430.

Kirsop, B. E. and C. P. Kurtzman, (1988). Yeasts. Cambridge, Cambridge University Press.

Kobayashi N. and McEntee K (1990). Evidence for a heat shock transcription factorindependent mechanism for heat shock induction of transcription in *Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences.* **87** : 6550-6554.

Lado, B., H., Yosef, A., E., (2002). Alternative food-preservation technologies: efficacy and mechanisms, *Microbes and Infection,* **4:** 433-440.

Langkjær, R., B., Cliften,P., F., Johnston, M., Piskur, J.,(2003). Yeast genome duplication was followed by asynchronous differentiation of duplicated genes, Nature, **421**: 848- 852.

Lewis, J., G., Learmonth, R., P., Attfield P., V., Watson, K, (1997). Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*, Journal of Industrial Microbiology & Biotechnology, **18**:30-36.

Lippuner V., Cyert M.S., Gasser C.S. (1996). Two Classes of Plant cDNA Clones Differentially Complement Yeast Calcineurin Mutants and Increase Salt Tolerance of Wild-type Yeast, *The Journal of Biological Chemistry* **.271**: 12859–12866.

Madigan, M.,T., Martinko J.,M., Parker, J., (2003). Brock Biology of Microorganisms. *Prentice Hall Inc., New Jersey.*

Mager W.H., Siderius M. (2002). Novel insights into the osmotic stress response of yeast, *FEMS Yeast Research*. **2**: 251-257.

Mattanovich D., Gasser B., Hohenblum H., Sauer M. (2004). Stress in recombinant protein producing yeasts, *Journal of Biotechnology* **113**: 121–135.

Muller M.,(2003), retrieved on 07.04.2012 from http://www.uic.edu/ classes/bios/ bios100/ lectf 03am/lect11.htm

Nevoigt, E. (2008). "Progress in metabolic engineering of Saccharomyces cerevisiae." *Microbiology and Molecular Biology Reviews*. **72(3)**: 379-412.

Ostergaard S., Olsson L., Nielsen J. (2000). Metabolic Engineering of *Saccharomyces cerevisiae. Microbiology and Molecular Biology Reviews* **64** : 34-50.

Parrou, J. L. and J. Francois, (1997). "A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells." *Analytical Biochemistry* **248(1)**: 186-188.

Pedreschi R, Hertog ML, Carpentier SC, (2008). Treatment of missing values for multivariate statistical analysis of gel-based proteomics data. *Proteomics* **8 (7)**: 1371–83.

Petro, S. (2010). "Fermentation in the Yeast Saccharomyces cerevisiae." *PDF article http://phobos.ramapo.edu/~spetro/lab_pdf/Fermlab.pdf.*

Reynolds,T., B., Fink, G., R., (2001). Baker's yeast, a model for fungal biofilm formation, *Science*, **291**, 878-881

Schmitt AP, McEntee K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences U S A.* **93**: 5777-5782.

Sezgin, T. (2010). Evolutionary Engineering of *Saccharomyces cerevisiae* for Improved Industrial Properties- PhD Thesis. *Istanbul Technical University***.**

Sherman, F. (2002). "Getting started with yeast." *Methods in Enzymology* **350**: 3-41.

Spellman, P., T., Sherlock, G., Zhang, M., Q., Iyer, V., R., Anders, K., Eisen, M., Brown, P., O., Botstein, D., Futcher, B., (1998). Comprehensive Identification of Cell Cycle–regulated Genes of the Yeast *Saccharomyces cerevisiae* by Microarray Hybridization, Molecular Biology of the Cell, **9**: 3273-3297.

Stephanopoulos G. (1999). Metabolic Fluxes and Metabolic Engineering. *Metabolic Engineering*. **1** : 1-11.

Tanghe, A., Dijck P., V.,Dumortier, F., Teunissen, A., Hohmann, S., Thevelein, J., M., (2002). Aquaporin Expression Correlates with Freeze Tolerance in Baker's Yeast, and Overexpression Improves Freeze Tolerance in Industrial Strains, Applied and Environmental Microbiology. **68**: 5981-5989.

Url-1 <*http://www.sciencenews.net.au/using-yeast-cells-to-run-calculations*>, retrieved on 07.04.2012.

Url- 2 <http*://www.britannica.com/ebarticle-66615/beer*>, accessed at 08.04.2012.

Van Mulders, S., E., Christianen, E., Saerens, S., M., G., Daenen, L,. Verbelen, P., J., Willaert, R., Verstrepen, K., J., Delvaux, F., R, (2009). Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*, *FEMS Yeast Research*, **9**: 178-190

Verstrepen, K., J., Derdelinckx, G., Verachtert, H., Delvaux., F., R., (2003). Yeast flocculation : what brewers should know, *Applied Microbiology and Biotechnology*, **61**: 197-205.

Verstrepen, K., J., Klis, F., M., (2006). Flocculation, adhesion and biofilm formation in yeasts, *Molecular Microbiology*, **60(1)**, 5-15.

Walker, G. M. (1998). Yeast Physiology and Biotechnology. Chichester ; New York, J. Wiley & Sons.

Zetic, V., G., Tomas, V., S., Grba, S., Lutilsky, L., Kozlek, D., (2001). Chromium uptake by *Saccharomyces cerevisiae* and isolation of glucose tolerance factor from yeast biomass, J. Biosci., **26**: 217- 223.

Zeyl, C. (2004). "Capturing the adaptive mutation in yeast." *Research in Microbiology* **155(4)**: 217-223.

APPENDICES

APPENDIX A: Supplement Data for Cell Dry Weight Measurements

While measuring cell dry weight, for each measurement three samples were taken in 3 different tubes. The figures demonstrated in the report were drawn by average values of measurements. The detailed values of cell dry weight analysis are as shown below.

Table A.1: Cell dry weight values including each three measurements, average cell dry weight and standard deviation for w/t – YMM.

Time (h)	Tube 1 (g)	Tube 2 (g)	Tube 3 (g)	Avr.(g)	Std.Dev.
4	0.0006	0.0006	0.0006	0.0006	0.0000
5	0.0008	0.0005	0.0001	0.0005	0.0004
6	0.0014	0.0013	0.0018	0.0015	0.0003
7	0.0022	0.0019	0.0017	0.0019	0.0003
8	0.002	0.0023	0.0022	0.0022	0.0002
9	0.0028	0.0026	0.0027	0.0027	0.0001
10	0.0026	0.003	0.0028	0.0028	0.0002
11	0.0026	0.0028	0.0028	0.0027	0.0001
24	0.0052	0.0053	0.005	0.0052	0.0002

Table A.2: Cell dry weight values including each three measurements, average cell dry weight and standard deviation for *T8* – YMM.

Time (h)	cdw 1 (g)	cdw 2 (g)	cdw 3 (g)	Avr.(g)	Std.Dev.
7	0.0008	0.0003	0.0006	0.0006	0.0003
8	0.0008	0.0010	0.0013	0.0010	0.0003
9	0.0012	0.0014	-	0.0013	0.0001
10	0.0015	0.0017	-	0.0016	0.0001
11	0.0022	0.0024	0.0027	0.0024	0.0003
24	0.0048	0.0052	0.0049	0.0050	0.0002

Table A.3: Cell dry weight values including each three measurements, average cell dry weight and standard deviation for w/t – 5% NaCl.

Time(h)	cdw1	cdw2	cdw3	Avr.(g)	Std.Dev.
24	0.0025	0.0022	0.0022	0.0023	0.0002

Table A.4: Cell dry weight values including each three measurements, average cell dry weight and standard deviation for *T8* – 5% NaCl.

Time(h)	cdw 1 (g)	cdw 2 (g)	cdw 3 (g)	Avr(g)	Std.Dev.
24	0.0020	0.0013	0.0019	0.0017	0.0004

APPENDIX B: Results obtained from the fermentation of w/t strain in 0.5% (w/v) NaCl containing medium in bioreactor.

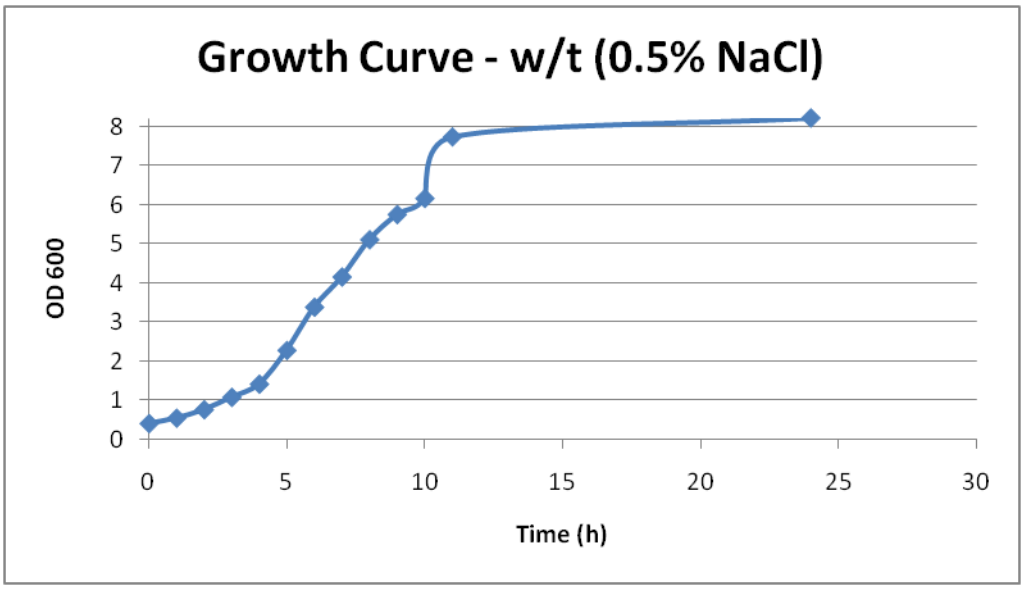

Figure B.1: Growth Curve of w/t cell which were grown in 0.5 (w/v) NaCl containing medium, in bioreactor.

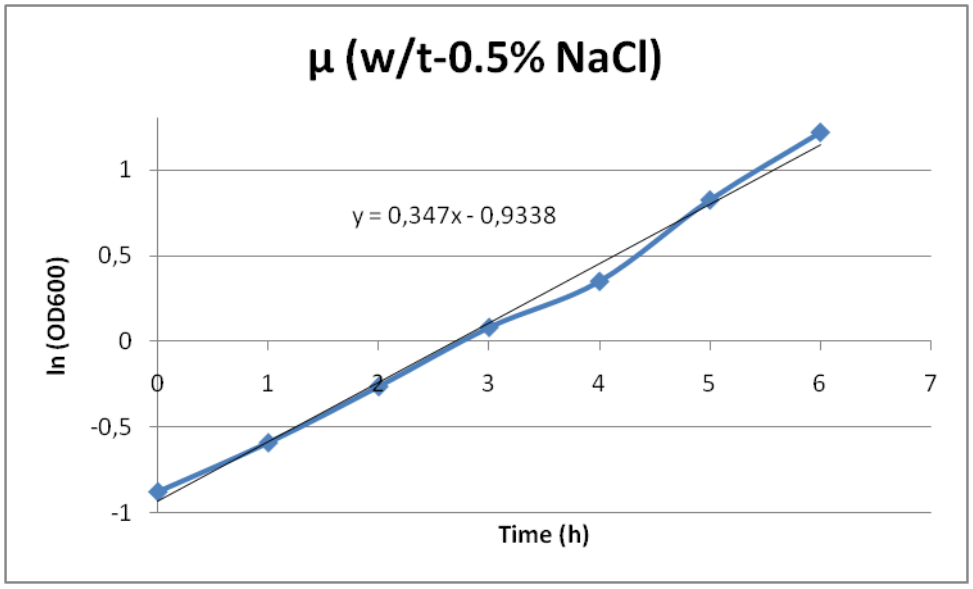

Figure B.2: Specific growth rate obtained by natural logarithm of OD_{600} values of w/t cells in 0.5% (w/v) NaCl containing medium.

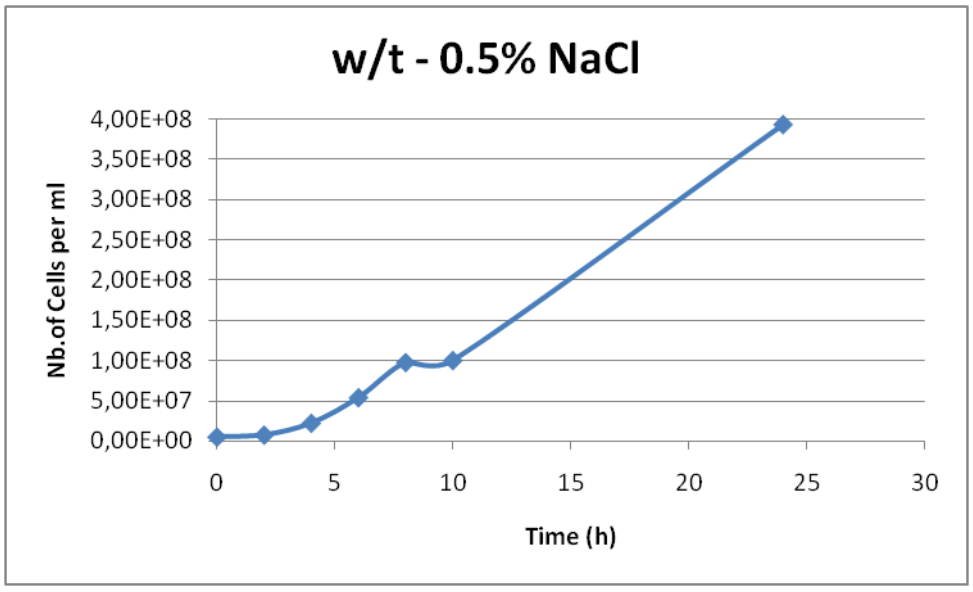

Figure B.3: Growth curve according to number of cells in one milliter of culture of w/t cells grown in 0.5% (w/v) NaCl containing medium, at specific time intervals.

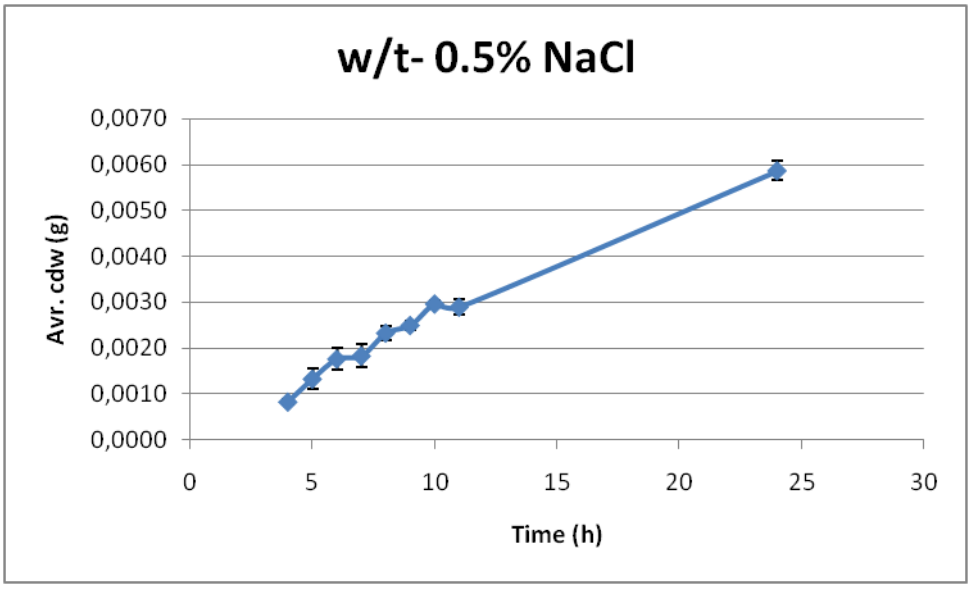

Figure B.4: Growth curve according to cell dry weight of culture of w/t cells grown in 0.5% (w/v) NaCl containing medium.

Table B.1: Cell dry weight values including each three measurements, average cell dry weight and standard deviation for w/t in 0.5 % (w/v) NaCl containing medium.

Time (h)	cdw 1 (g)	cdw 2 (g)	cdw 3 (g)	Avr. (g)	Std. Dev.
4	0.0008	0.0008	0.0009	0.0008	0.0001
5	0.0012	0.0016	0.0012	0.0013	0.0002
6	0.0019	0.0019	0.0015	0.0018	0.0002
7	0.0018	0.0021	0.0016	0.0018	0.0003
8	0.0025	0.0022	0.0023	0.0023	0.0002
9	0.0025	0.0024	0.0026	0.0025	0.0001
10	0.003	0.0029	0.003	0.0030	0.0001
11	0.003	0.003	0.0027	0.0029	0.0002
24	0.0061	0.0057	0.0058	0.0059	0.0002

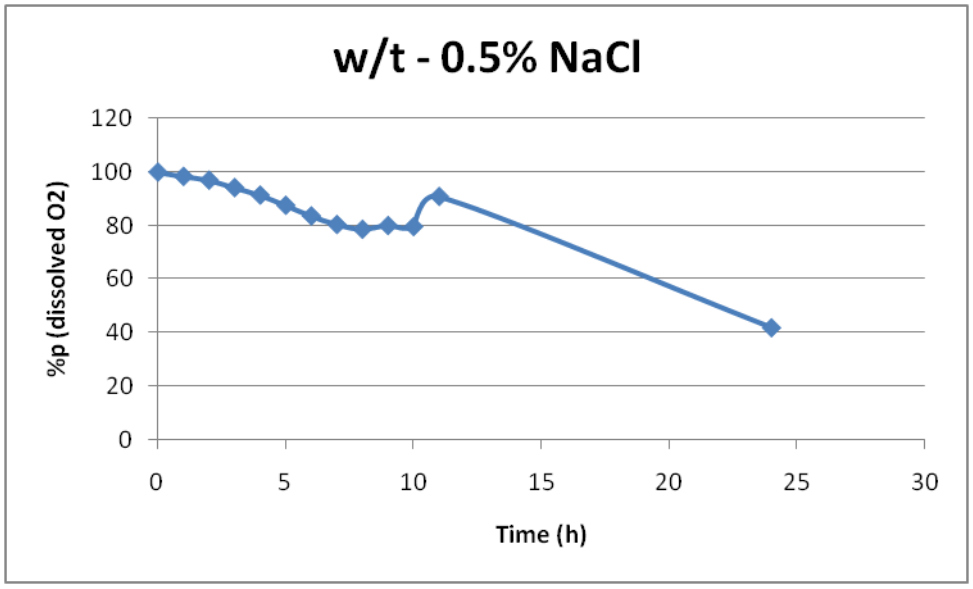

Figure B.5: Residual concentration change of dissolved oxygen in 0.5% (w/v) NaCl containing medium of w/t cells.

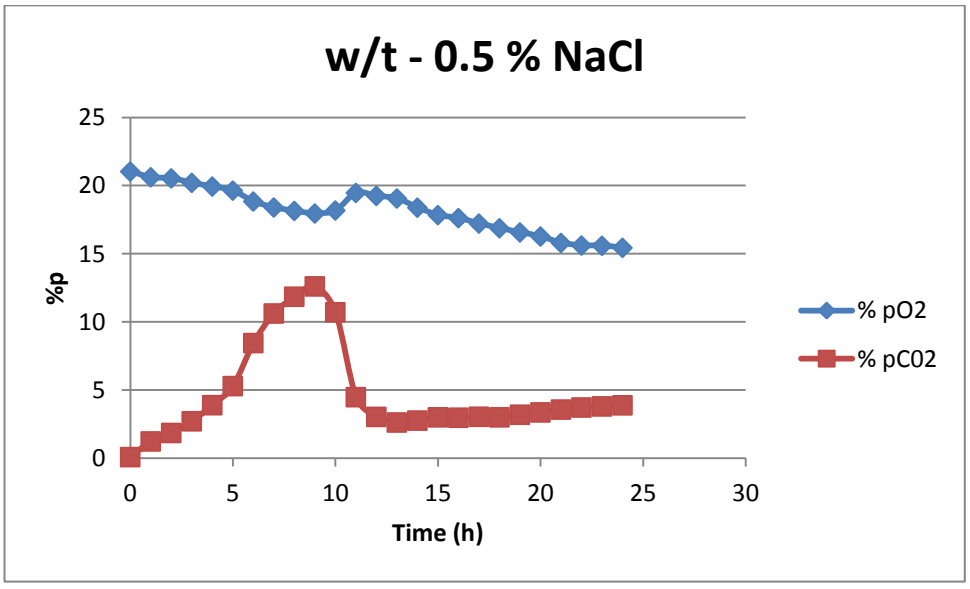

Figure B.6: Off gas data of oxygen and carbondioxide in 0.5% (w/v) NaCl containing medium of w/t cells.

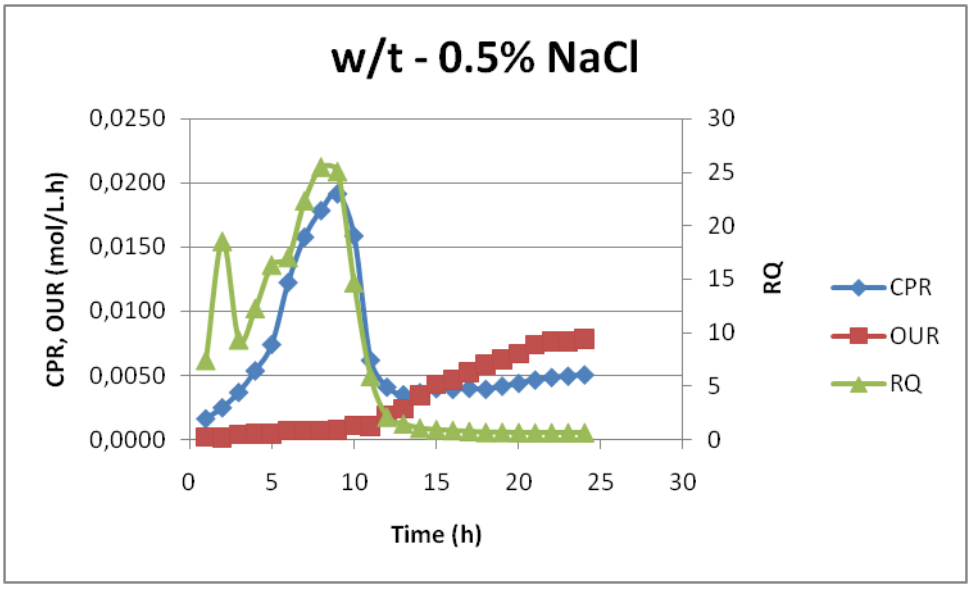

Figure B.7: Calculated carbondioxide production (CPR), oxygen uptake rates (OUR) and respiratory quotients (RQ) according to off gas results of w/t in 0.5 % (w/v) NaCl containing medium.

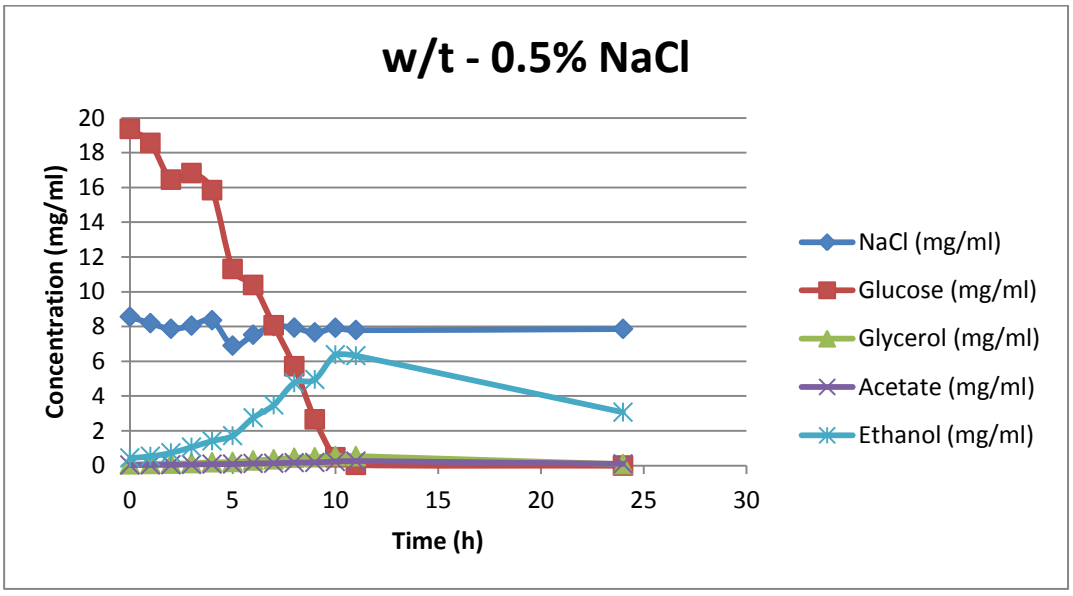

Figure B.8: HPLC results of w/t in 0.5 % (w/v) NaCl containing medium.

APPENDIX C: Monitored output O_2 and CO_2 concentrations of w/t and *T8* in control and 5% (w/v) NaCl containing medium.

Table C.1: Off-gas data and calculated CPR,OUR, and RQ values of w/t-YMM.

Time (h)	% pO2	% pCO2	CPR	OUR	RQ
0	21	0		-	
1	20.91	0.66	0.00102	0.00009	-11.758
2	20.84	1.1	0.00174	0.00013	-13.054
3	20.55	1.91	0.00308	0.00011	26.969
4	19.91	4.55	0.00761	0.00030	25.418
5	19.66	5.52	0.00933	0.00040	23.228
6	19.2	7.75	0.01344	0.00039	34.210
7	18.77	9.59	0.01697	0.00050	34.058
8	18.25	11.85	0.02151	0.00061	34.973
9	17.93	12.41	0.02260	0.00108	20.839
10	19.45	5.19	0.00871	0.00100	8.748
11	19.84	1.96	0.00314	0.00155	2.021
12	19.9	1.55	0.00246	0.00160	1.538
13	19.36	1.57	0.00248	0.00268	0.924
14	18.82	1.84	0.00290	0.00365	0.794
15	19.03	1.44	0.00225	0.00339	0.665
16	18.8	1.37	0.00213	0.00387	0.552
17	18.93	1.22	0.00189	0.00367	0.516
18	18.82	1.11	0.00171	0.00393	0.437
19	18.87	1.1	0.00170	0.00383	0.443
20	19.01	0.99	0.00153	0.00360	0.424
21	19.11	0.89	0.00137	0.00344	0.397
22	19.05	0.82	0.00125	0.00358	0.350
23	19.14	0.79	0.00121	0.00342	0.353
24	19.23	0.77	0.00118	0.00325	0.362

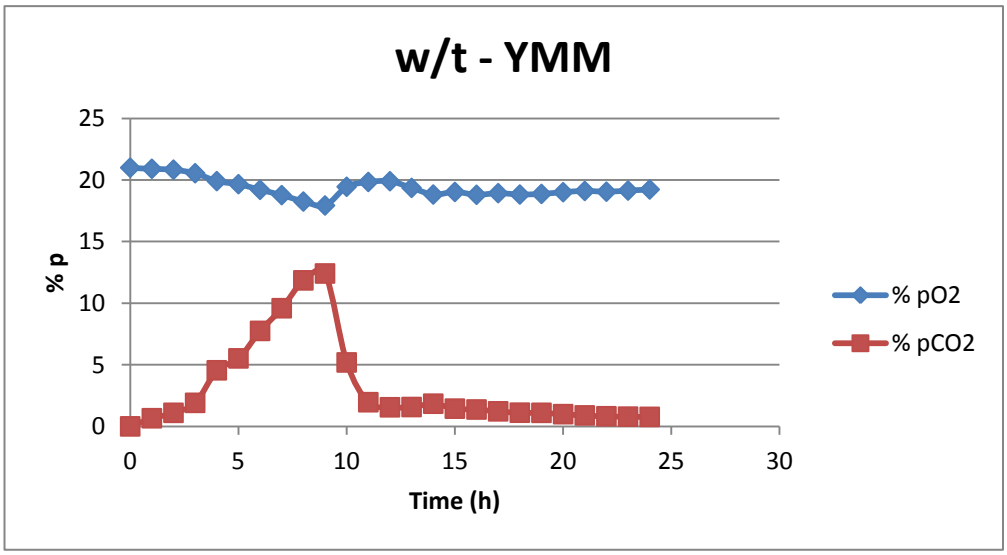

Figure C.1: Off CO_2 and O_2 concentrations of wt-YMM.

Table C.2: Off-gas data and calculated CPR,OUR, and RQ values of *T8*-YMM.

Time (h)	% pO2	% pCO2	CPR	OUR	RQ
0	20.89	0.25			
1	20.81	0.48	0.00073	0.00020	3.724
2	20.77	0.68	0.00105	0.00019	5.495
3	20.67	1.04	0.00164	0.00024	6.772
4	20.82	1.39	0.00222	0.00022	-10.169
5	20.33	1.98	0.00319	0.00054	5.917
6	20.06	2.88	0.00470	0.00071	6.598
7	19.68	4.22	0.00700	0.00093	7.527
8	19.12	6.04	0.01021	0.00133	7.690
9	18.51	8.47	0.01470	0.00158	9.300
10	18.01	10.66	0.01895	0.00171	11.095
11	17.38	13.46	0.02470	0.00186	13.284
12	16.77	15.28	0.02854	0.00243	11.738
13	16.79	13.75	0.02512	0.00308	8.164
14	19.26	4.28	0.00707	0.00178	3.963
15	18.82	3.79	0.00618	0.00289	2.137
16	18.11	3.63	0.00585	0.00439	1.333
17	17.21	4.03	0.00646	0.00603	1.071
18	17.8	3.25	0.00519	0.00514	1.008
19	17.9	2.84	0.00451	0.00510	0.884
20	17.92	2.61	0.00413	0.00514	0.803
21	17.77	2.6	0.00410	0.00544	0.755
22	17.71	2.57	0.00405	0.00557	0.728
23	17.65	2.5	0.00393	0.00571	0.689
24	17.55	2.53	0.00398	0.00589	0.675

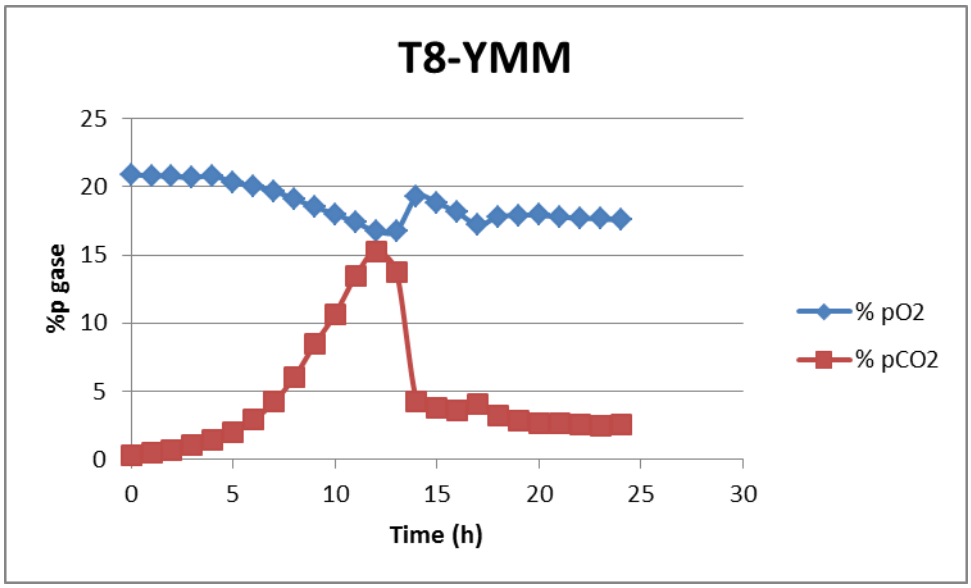

Figure C.2: Off CO_2 and O_2 concentrations of *T8*-YMM.

Table C.3: Off-gas data and calculated CPR,OUR, and RQ values of w/t-5%NaCl.

Time (h)	%p O2	%p CO2	CPR	OUR	RQ
0	20.87	0.84			
1	21	0.84	0.00110	0.00029	-3.762
2	20.79	1.11	0.00146	0.00003	-50.592
3	20.78	1.14	0.00150	0.00002	-66.598
4	20.84	1.15	0.00152	0.00013	-11.758
5	20.86	1.16	0.00153	0.00017	-9.170
6	20.88	1.16	0.00153	0.00020	-7.608
7	20.86	1.16	0.00153	0.00017	-9.170
8	20.88	1.12	0.00148	0.00019	-7.904
9	20.85	1.11	0.00146	0.00013	-11.102
10	20.85	1.14	0.00151	0.00014	-10.546
11	20.86	1.19	0.00158	0.00018	-8.842
12	20.85	1.19	0.00158	0.00016	-9.785
13	20.86	1.24	0.00164	0.00020	-8.374
14	20.86	1.28	0.00170	0.00021	-8.058
15	20.85	1.31	0.00174	0.00020	-8.508
16	20.84	1.37	0.00182	0.00021	-8.716
17	20.8	1.43	0.00191	0.00016	-11.758
18	20.83	1.47	0.00196	0.00023	-8.588
19	20.78	1.51	0.00202	0.00016	-12.867
20	20.79	1.43	0.00191	0.00014	-13.156
21	20.77	1.61	0.00215	0.00018	-12.252
22	20.78	1.62	0.00217	0.00020	-11.021
23	20.73	1.71	0.00229	0.00014	-16.012
24	20.73	1.77	0.00238	0.00017	-14.396

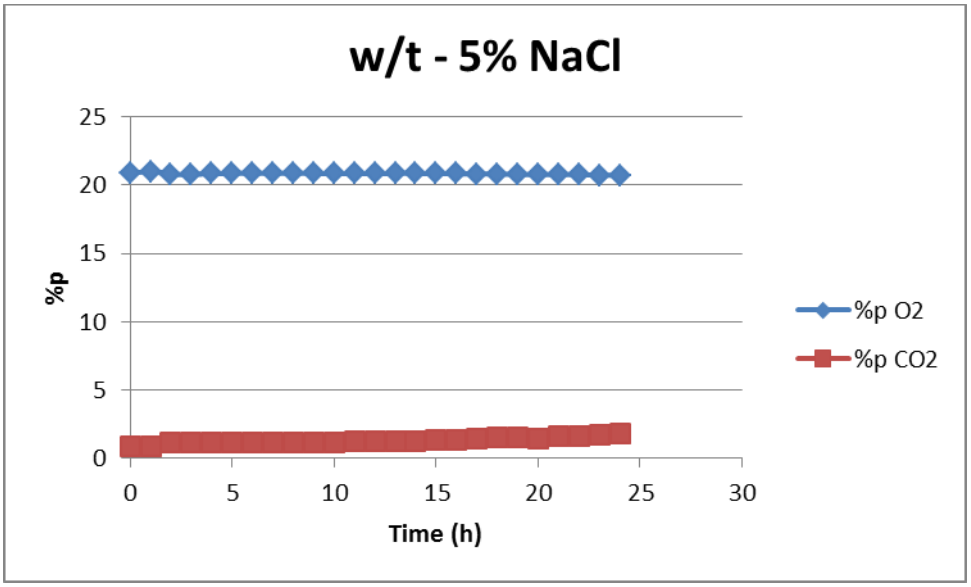

Figure C.3: Off CO_2 and O_2 concentrations of w/t-5%NaCl.

Table C.4: Off-gas data and calculated CPR,OUR, and RQ values of *T8*-5%NaCl.

Time (h)	%pO2	%pCO2	CPR	OUR	RQ
0	20.89	0.42			
1	20.91	0.33	0.00040	0.00005	8.786
2	20.86	0.39	0.00048	0.00011	4.418
3	20.84	0.49	0.00062	0.00011	5.735
4	20.84	0.62	0.00080	0.00006	12.927
5	20.79	0.75	0.00097	0.00010	9.683
6	20.75	0.92	0.00120	0.00011	11.155
7	20.7	1.04	0.00137	0.00015	9.086
8	20.7	1.12	0.00148	0.00012	12.125
9	20.66	1.26	0.00167	0.00014	11.907
10	20.65	1.4	0.00186	0.00011	17.400
11	20.61	1.6	0.00214	0.00010	20.607
12	20.55	1.87	0.00251	0.00011	22.902
13	20.46	2.23	0.00301	0.00013	22.326
14	20.35	2.66	0.00362	0.00017	21.307
15	20.2	3.18	0.00435	0.00024	17.997
16	20.02	3.85	0.00531	0.00031	16.999
17	19.91	4.43	0.00616	0.00029	20.979
18	19.75	5.18	0.00726	0.00030	24.197
19	19.58	5.77	0.00814	0.00038	21.171
20	19.5	6.12	0.00867	0.00040	21.802
21	19.44	6.48	0.00922	0.00037	24.850
22	19.38	6.73	0.00960	0.00039	24.904
23	19.45	6.45	0.00917	0.00036	25.189
24	19.54	5.61	0.00789	0.00052	15.317

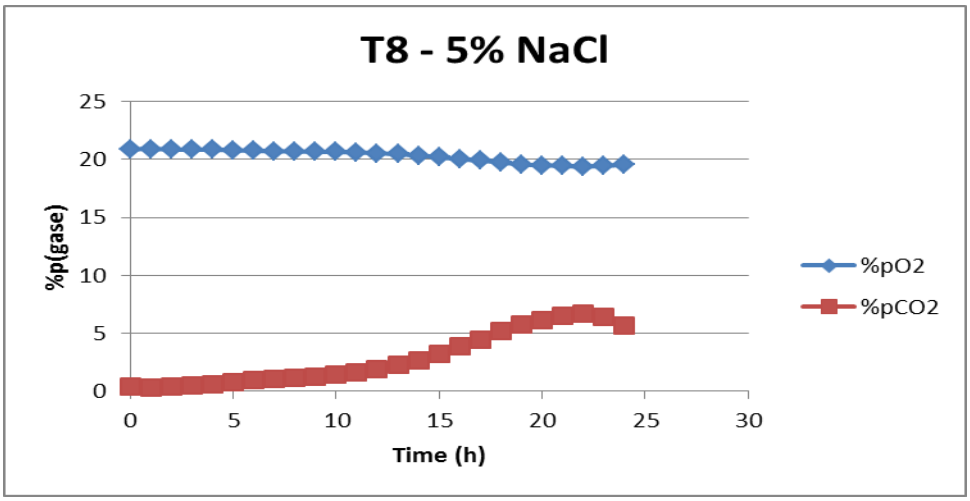

Figure C.4: Off CO_2 and O_2 concentrations of *T8*-5%NaCl.

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