A SYSTEM BASED RATIONAL APPROACH TO IMPROVE FIRST AND SECOND GENERATION BIOETHANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE*

by

Ceyda Kasavi

B.S., Chemical Engineering, Istanbul Technical University, 2003

M.S., Chemical Engineering, Istanbul Technical University, 2006

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Graduate Program in Chemical Engineering

Boğaziçi University

2013

ACKNOWLEDGEMENTS

This thesis has been seen through to completion with the support and encouragement of numerous people. At the end of past six years, it is a pleasant task to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

First of all, I would like to express my sincere gratitude to my advisors, Prof. Betül Kırdar and Assoc. Prof. Ebru Toksoy Öner, for their everlasting support, encouragement, and excellent guidance during my study. I have learned a lot from them and I could not have finished my dissertation successfully without their help.

I also would like to express my appreciation to Prof. Stephen G. Oliver for his invaluable advises besides making this study possible. I also thank to Ilaria Finore for her contribution and sharing her experience.

I would like to thank the members of my thesis committee, Prof. Zeynep İlsen Önsan, Prof. Zeynep Petek Çakar, and Assist. Prof. Kazım Yalçın Arga for the time they have devoted to reading and commenting on my thesis.

The financial supports of Boğaziçi University Research Fund through project 6530, TÜBİTAK through projects 106M444, 106T756 and 110M692, Turkish State Planning Organization through project DPT-09K120520, British Council UK-Turkey Higher Education and Industry Partnership Programme through project TR/012012/KP17 are gratefully acknowledged. I also acknowledge the doctoral scholarship provided by TÜBİTAK-BİDEB 2211. BÜVAK, TÜBİTAK, DPT and FEBS are acknowledged due to financial contributions regarding conferences.

My special thanks are due to former and present members of Biosystems Engineering Research Group at Boğaziçi University; Elif Dereli, Ayça Cankorur Çetinkaya, Duygu Dikicioğlu, Esra Börklü Yücel, Şebnem Öç, Erkan Karabekmez, Saliha Durmuş Tekir and Büşra Gürses for providing me a very nice and friendly working environment in

the last six years. I owe very special thanks to Dr. Serpil Eraslan for her contribution, support and guidance besides making this study possible. I feel lucky to be a member of this group.

I am deeply indebted to my parents for their everlasting support, patience and also for creating me a perfect family. I would not have made it this far without them.

ABSTRACT

A SYSTEM BASED RATIONAL APPROACH TO IMPROVE FIRST AND SECOND GENERATION BIOETHANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE*

There is a growing need for the use of renewable energies due to the steady increase in energy consumption, current dependence on fossil fuels to meet the growing energy demand, and the instability of the oil economy. Ethanol is mainly of interest because of its proven record as a fuel for land transportation. *Saccharomyces cerevisiae* is the preferred microbial cell factory for ethanol production. Bioethanol production by *S. cerevisiae* is currently the largest fermentation process in industrial biotechnology, and continuous efforts have been made to improve this process. Further progress could be achieved by developing optimized cell factories that can be used in industrial ethanol production. Moreover, the identification of sustainable substrates that can be used in productive and cost-effective processes is still a challenge in industry. The aim of this thesis was to develop new strategies to enhance first and second generation bioethanol production by *S. cerevisiae*. For this purpose, firstly, the suitability of five industrial *S. cerevisiae* strains for strain improvement studies were evaluated for their potential use in ethanol production processes. Moreover, utilization of by-products from agri-food industry by these strains was also investigated. Then, studies focused on ethanol stress, which is considered as one of the major stresses that yeast cells are exposed to during industrial ethanol production. For this, a novel network based approach was developed with the aim to find novel gene targets for rational design of ethanol tolerant *S. cerevisiae* strains, and the systems based information on transcriptome level was used for the identification of molecular mechanisms related to ethanol tolerance in *S. cerevisiae*. Finally, the global transcriptional response of a genetically engineered *S. cerevisiae* strain was also investigated with the aim of understanding the effect of the genetic modification, which confers wild-type cells with the ability of starch utilization. This study provides further evidence that system based approaches are powerful tools to understand and improve bioethanol production processes.

ÖZET

SACCHAROMYCES CEREVISIAE İLE BİRİNCİ VE İKİNCİ KUŞAK BİYOETANOL ÜRETİMİNİN SİSTEM BAZLI AKILCI BİR YAKLAŞIMLA İYİLEŞTİRİLMESİ

Sürekli olarak artan enerji tüketimi, enerji talebini karşılayabilmek için fosil yakıtlara olan bağımlılık ve petrol ekonomisindeki kararsızlık yenilenebilir enerji kullanımını gerektirmektedir. Kara taşımacılığında yakıt olarak kullanılabilme özelliği nedeni ile etanol büyük ölçüde ilgi çekmektedir. Etanol üretiminde tercih edilen mikrobiyal hücre fabrikaları *Saccharomyces cerevisiae*'dır. Günümüzde endüstriyel biyoteknoloji alanındaki en büyük fermentasyon süreci maya ile biyoetanol üretimidir ve bu prosesin iyileştirilmesi için çalışmalar devam etmektedir. İyileştirmeler ancak endüstriyel ölçekte etanol üretiminde kullanılabilecek optimize hücre fabrikalarının tasarımıyla mümkündür. Ayrıca, verimli ve uygun maliyetli proseslerde kullanılabilecek sürdürülebilir hammaddelerin belirlenmesi endüstriyel açıdan önemini korumaktadır. Bu tezin amacı, *S. cerevisiae* ile birinci ve ikinci kuşak biyoetanol üretimini arttıracak yeni stratejiler geliştirmektir. Bu amaçla, ilk olarak beş endüstriyel *S. cerevisiae* suşunun, etanol üretim proseslerinde kullanılabilecek yeni suşlar geliştirmek için uygunlukları değerlendirilmiştir. Ayrıca bu suşların, gıda endüstrisinin yan ürünlerini kullanarak etanol üretebilme kapasiteleri araştırılmıştır. Daha sonra çalışmalar endüstriyel etanol üretim süreçlerinde maya hücrelerini etkileyen temel stres olan etanol stresine odaklanmıştır. Etanole toleranslı maya suşlarının akılcı tasarımı için hedef genlerin belirlenmesi amacıyla ağyapı bazlı bir yaklaşım geliştirimiş ve mayada etanol toleransıyla bağlantılı moleküler mekanizmaların aydınlatılmasında transkriptom düzeyindeki sistem bazlı bilgi kullanılmıştır. Son olarak, yabanıl tip maya hücrelerine nişasta kullanabilme yetisi kazandıran genetik değişikliğin etkisini anlamak amacıyla genetik yapısı değiştirilmiş *S. cerevisiae* suşunun gen anlatımları incelenmiştir. Bu çalışma sistem bazlı yaklaşımların biyoetanol üretim süreçlerinde oluşan değişimleri anlama ve geliştirmede güçlü bir yaklaşım olduğunu göstermektedir.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ……..…….…..…. iii
ABSTRACT …….…… v
ÖZET …….……... vi
LIST OF FIGURES …….…… xii
LIST OF TABLES …….…….. xvi
LIST OF SYMBOLS ……..…….…… xix
LIST OF ACRONYMS / ABBREVIATIONS …… xx
1. INTRODUCTION …….…….. 1
2. BIOETHANOL PRODUCTION BY INDUSTRIAL *SACCHAROMYCES CEREVISIAE* STRAINS …...…….…….. 4
2.1. Comparison of the Fermentation Performances of Industrial *Saccharomyces cerevisiae* Strains ……... 4
2.1.1. Background Aspects ……...……..... 4
2.1.2. Methods ……...…… 7
2.1.2.1. Strains and Growth Conditions ……..... 7
2.1.2.2. Cultivation Conditions …….……..…….. 7
2.1.2.3. Determination of Maximum Specific Growth Rate and Dry Cell Weight …….…….……..…….. 7
2.1.2.4. Extracellular Metabolite Analysis …….…….. 8
2.1.2.5. Statistical Analysis ……...…… 8
2.1.2.6. Determination of Ethanol Tolerance …… 8
2.1.2.7. Identification of Nucleotide Variations Present in Ethanol Tolerances Genes of Industrial Yeast Strains …….. 8
2.1.3. Results and Discussion .……... 9
2.1.3.1. Fermentation Parameters of *S. cerevisiae* Strains 9
2.1.3.2. Ethanol Tolerance of *S. cerevisiae* Strains ..……..…….. 13
2.1.3.3. Identification of Nucleotide Variations Present in Ethanol Tolerances Genes of Industrial Yeast Strains ..……..…….. 15
2.1.4. Concluding Remarks ....……... 19

2.2. Evaluation of Industrial *Saccharomyces cerevisiae* Strains for Ethanol Production from Biomass 20
2.2.1. Background Aspects 20
2.2.2. Methods 22
2.2.2.1. Strains 22
2.2.2.2. Cultivation Conditions 22
2.2.2.3. Preparation of Biomass Substrates 22
2.2.2.4. Determination of Maximum Specific Growth Rate and Dry Cell Weight 23
2.2.2.5. Extracellular Metabolite Analysis 23
2.2.2.6. Statistical Analysis 23
2.2.2.7. Sugar Assays 23
2.2.3. Results and Discussion 23
2.2.3.1. Bioconversion of biological residues into ethanol 23
2.2.4. Concluding Remarks 26
3. INVESTIGATION OF THE ETHANOL TOLERANCE AND STRESS MECHANISMS IN *SACCHAROMYCES CEREVISIAE* 28
3.1. Identification of Novel Gene Targets for Improved Ethanol Tolerance in *Saccharomyces cerevisiae* through a Network-Based Approach 28
3.1.1. Background Aspects 28
3.1.2. Methods 30
3.1.2.1. Network Reconstruction 30
3.1.2.2. Network Tuning 31
3.1.2.3. Determination of Network Topology 32
3.1.2.4. Module Identification and Functional Enrichment 32
3.1.2.5. Strains and Media 33
3.1.2.6. Determination of Ethanol Tolerance 34
3.1.2.7. Determination of Maximum Specific Growth Rate and Dry Cell Weight 35
3.1.2.8. Extracellular Metabolite Analysis 35
3.1.2.9. Statistical Analysis 35
3.1.3. Results and Discussion 35
3.1.3.1. Network Reconstruction and Tuning 35

3.1.3.2. Topological Analysis of the Network ……..……..…….. 37
3.1.3.3. Biological Processes Related to Ethanol Tolerance ..…….. 41
3.1.3.4. Modular Analysis …….……..... 43
3.1.3.5. Ethanol Tolerances of *S. cerevisiae* Strains ……..…….. 45
3.1.4. Concluding Remarks ....……... 50
3.2. Investigation of the Transcriptional Response to Ethanol Stress in *Saccharomyces cerevisiae* …..……... 51
3.2.1. Background Aspects ……...……..... 51
3.2.2. Methods ……...…… 53
3.2.2.1. Strains and Media ..……..……..... 53
3.2.2.2. Cultivation Conditions and Sampling …….…….. 53
3.2.2.3. RNA Isolation and Microarray Analysis ……..…… 54
3.2.2.4. Microarray Data Acquisition and Analysis ...…..…….. 55
3.2.2.5. Clustering of Conditions ……...……...…… 55
3.2.2.6. Reporter Features Analysis ……...…… 55
3.2.3. Results and Discussion .……... 56
3.2.3.1. Experimental Design to Investigate the Global Transcriptional Response of *S. cerevisiae* Strains to *PMT7* and *YHL042W* Gene Deletions and Ethanol Stress …….. 56
3.2.3.2. Clustering of Conditions ……... 56
3.2.3.3. Global Transcriptional Response to *PMT7* and *YHL042W* Gene Deletions ……...…….. 58
3.2.3.4. Global Transcriptional Response to Ethanol Stress ...…….. 68
3.2.3.5. Analysis of Transcriptional Response to Ethanol Stress in Context of Reporter Features …….…….. 82
3.2.4. Concluding Remarks ....……... 84
4. INVESTIGATION OF THE ETHANOL PRODUCTION VIA ONE STEP CONVERSION OF STARCH BY *SACCHAROMYCES CEREVISIAE* ……. 86
4.1. Investigation of the Global Transcriptional Response of A Genetically Engineered *Saccharomyces cerevisiae* Strain for One Step Bioconversion of Starch to Ethanol …….……... 86
4.1.1. Background Aspects ……...……..... 86
4.1.2. Methods ……...…… 88

4.1.2.1. Strains and Growth Media 88
4.1.2.2. Cultivation Conditions and Sampling 88
4.1.2.3. Determination of Amylase Activity 89
4.1.2.4. RNA Isolation and Microarray Analysis 90
4.1.2.5. Microarray Data Acquisition and Analysis 90
4.1.2.6. Clustering of Conditions 90
4.1.2.7. Reporter Features Analysis 91
4.1.3. Results and Discussion 91
4.1.3.1. The Growth Characteristics of Batch Fermentations 91
4.1.3.2. Global Transcriptional Response of *S. cerevisiae* Strains 93
4.1.3.3. Clustering of Conditions 93
4.1.3.4. The Identification of Significantly Expressed Genes 94
4.1.3.5. Transcriptional Response to the Presence of Plasmid 95
4.1.3.6. Transcriptional Response to the Aeration 96
4.1.3.7. Transcriptional Response to the Interactive Effects of Factors 97
4.1.3.8. Analysis of Transcriptional Response to the Presence of Plasmid and Oxygen Limitation in the Context of Reporter TFs 98
4.1.4. Concluding Remarks 100
4.2. Fermentation Characteristics of *Saccharomyces cerevisiae* WTPB-G in Ethanol Production from Raw Starch 100
4.2.1. Background Aspects 100
4.2.2. Methods 102
4.2.2.1. Strains and Growth Media 102
4.2.2.2. Cultivation Conditions 102
4.2.2.3. Determination of Maximum Specific Growth Rate and Dry Cell Weight 102
4.2.2.4. Extracellular Metabolite Analysis 102
4.2.2.5. Determination of Starch Concentration 102
4.2.3. Results and Discussion 103
4.2.3.1. Fermentation Parameters of WTPB-G Strain 103
4.2.3.2. Optimization of Starch Content 104

4.2.4. Concluding Remarks ....……... 105
5. CONCLUSIONS AND RECOMMENDATIONS …….…. 107
5.1. Conclusions …….…….……... 107
5.2. Recommendations ……...…….……... 110
APPENDIX A: SUPPLEMENTARY TABLES FOR CHAPTER 3 ……..…. 112
REFERENCES ……..…… 124

LIST OF FIGURES

Figure 2.1. *S. cerevisiae* strains with clean lineages highlighted in grey; name color indicates source and dot color indicates geographic origin. ... 6

Figure 2.2. Variations in biomass (■), glucose (●) and ethanol (▲) concentrations. ..……... 10

Figure 2.3. Determination of the maximum specific growth rates. ...……..... 11

Figure 2.4. PCA plot of (a) growth (b) production related parameters; the 80% of the total variance was explained by first and second principle components; Red: BC187, Black: L-1374, Green: L-1528, Blue: K11, Pink: Y9. ...……..……... 12

Figure 2.5. Colony forming ability of undiluted (C), 10 fold (1/10) and 100 fold (1/100) diluted *S. cerevisiae* Y9 cultures grown in YPD containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol after 24 h, 48 h and 72 h. 14

Figure 2.6. Colony forming ability of *S. cerevisiae* L-1528 cultures grown in YPD containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol after 24 h, 48 h and 72 h. ...……. 14

Figure 2.7. Colony forming ability of *S. cerevisiae* BC187 cultures grown in YPD containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol after 24 h, 48 h and 72 h. ...……... 15

Figure 2.8. Pairwise nucleotide sequence comparison of ethanol tolerance genes of two sets of strains: whereas the former set included two ethanol tolerant strains (Y9 and BC187), the latter one embraced a tolerant strain (Y9) and the least tolerant strain (L-1528). 17

Figure 2.9. Multiple ClustalW2 alignments of Dog1p amino acid sequences in five industrial *S. cerevisiae* strains. ...…… 18

Figure 2.10. Yield comparisons of industrial *S. cerevisiae* strains grown on different carbon sources containing 1 gL^{-1} total carbohydrate. 26

Figure 3.1. Schematic illustration of the network reconstruction algorithm, SPA. …..……. 33

Figure 3.2. Connectivity distribution for the reconstructed networks (a) ETN (b) tETN. ……... 38

Figure 3.3. Average clustering coefficient distribution of (a) ETN (b) tETN. … 39

Figure 3.4. Average clustering coefficient distribution of Biogrid network. 40

Figure 3.5. Schematic illustration of the significantly enriched processes occurred in ETN and tETN. ..……... 42

Figure 3.6. The clusters of four gene-products (*YDR307W*, *YHL042W*, *YMR215W*, and *YPL264C*) whose first neighbors were enriched with ethanol tolerance related terms. ..……... 45

Figure 3.7. Colony-forming ability of undiluted (C), 10 to 10^6 fold diluted *S. cerevisiae* cultures grown in YPD and then spotted on YPD agar containing 10% (v/v) ethanol. .....…… 46

Figure 3.8. Growth profiles of the wild-type strain in the presence of increasing concentrations ethanol. 47

Figure 3.9. Relative viability (ratio of CFU at time t to the initial CFU at the time of ethanol treatment) of *S. cerevisiae* strains. ……. 48

Figure 3.10. Growth profiles of *S. cerevisiae* strains in media containing 8% (v/v) ethanol. ..…… 49

Figure 3.11. PCA plot of transcriptome data; the 75% of the total variance was explained by first and second PCs; star, diamond, circle represent wild-type, *pmt7*Δ/*pmt7*Δ, yhl042wΔ/yhl042wΔ strains; blue, magenta, red represent M, E0, E8 data, respectively. ..…… 57

Figure 3.12. Hierarchical clustering of conditions obtained using HCE 3.5. ...… 57

Figure 3.13. The distribution of (a) up- and (b) down-regulated genes together with their enriched GO processes in response to *PMT7* and *YHL042W* gene deletions. ..……...……. 59

Figure 3.14. Regulatory TFs around which genes were affected following (a) *PMT7* and (b) *YHL042W* single gene deletions. ……... 62

Figure 3.15. The distribution of (a) up- and (b) down-regulated genes and their enriched GO process terms in response to *PMT7* and *YHL042W* gene deletions 2 h after ethanol treatment (E8). ...…… 65

Figure 3.16. Regulatory TFs around which genes were affected following (a) *PMT7* and (b) *YHL042W* single gene deletions under stressed condition. ..……. 68

Figure 3.17. Expression profiles of genes related to lipid metabolism. ..……... 71

Figure 3.18. Distribution of significantly up-regulated genes in response to ethanol stress and their enriched GO process terms. ..……. 74

Figure 3.19. Distribution of significantly down-regulated genes in response to ethanol stress and their enriched GO process terms. ..……. 80

Figure 3.20. Regulatory TFs around which genes were affected in response to ethanol stress (a) wild-type strain (b) *pmt7*Δ/*pmt7*Δ and (c) yhl042wΔ/yhl042wΔ strains. ..…….…… 83

Figure 4.1. Growth profiles of FY23 (□, ■) and WTPB-G (▲, △) strains under aerated (filled symbols) and micro-aerated (open symbols) conditions. …… 92

Figure 4.2. Hierarchical Clustering of Conditions (M and A represent the micro-aerated and aerated conditions, respectively). ..……. 94

Figure 4.3. Starch concentrations of WTPB-G cultures. ...……. 104

LIST OF TABLES

Table 2.1.	Comparison of fermentation parameters of *S. cerevisiae* strains.	9
Table 2.2.	Ethanol tolerance of *S. cerevisiae* strains. ...……..	13
Table 2.3.	Fermentation parameters of industrial *S. cerevisiae* strains in different carbon sources containing 1 gL^{-1} total carbohydrate.	24
Table 2.4.	Maximum ethanol concentrations (gL^{-1}) reached by *S. cerevisiae* strains in different carbon sources containing 1 gL^{-1} total carbohydrate. …..…….	25
Table 3.1.	Core proteins of the network.	31
Table 3.2.	Yeast strains used in this study. ...…….....	34
Table 3.3.	Topological properties of protein interaction networks. ...…….	37
Table 3.4.	The maximum specific growth rates of the wild strain BY4743 in media containing 0-10% ethanol (v/v). ..……	46
Table 3.5.	Fermentation parameters of *S. cerevisiae* strains. ...……	50
Table 3.6.	GO enrichments of SEGs which were significantly up-regulated in response to ethanol stress in wild-type strain. ...……..	69
Table 3.7.	GO enrichments of SEGs which were significantly up-regulated in response to ethanol stress in *pmt7*Δ/*pmt7*Δ strain. ...……..	70

Table 3.8. GO enrichments of SEGs which were significantly up-regulated in response to ethanol stress in yhl042wΔ/yhl042wΔ strain. ……..... 73

Table 3.9. Functional categories of up-regulated SEGs in *pmt7*Δ/*pmt7*Δ strain only. ...……...… 75

Table 3.10. Functional categories of up-regulated SEGs in yhl042Δ/yhl042Δ strain only. ...……... 76

Table 3.11. Functional categories of up-regulated SEGs in *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains. ……. 78

Table 3.12. GO enrichment categories of SEGs which were significantly down-regulated in response to ethanol stress. ...…..…… 79

Table 3.13. Functional categories of down-regulated SEGs in wild-type strain. .. 81

Table 4.1. Fermentation parameters of *S. cerevisiae* strains. …….. 91

Table 4.2. Biological processes affected in genetically engineered *S. cerevisiae* cells from the presence of the plasmid or aeration. ..…….. 97

Table 4.3. Significantly expressed genes in response to interactive effect in *S. cerevisiae* WTPB-G and the reference strain (number of genes was provided in parenthesis). ...……. 98

Table 4.4. Significantly expressed genes in response to interactive effect under aerated and micro-aerated conditions in *S. cerevisiae* WTPB-G (number of genes was provided in parenthesis). ...……. 99

Table 4.5. Fermentation parameters of WTPB-G in different starch substrates. 103

Table 4.6 Fermentation parameters of WTPB-G in corn starch with varying amounts of initial starch content. ..…….. 105

Table A.1 Annotation collection table. ..……... 112

Table A.2 Hub proteins of ETN and tETN. ..…… 113

Table A.3 Bingo Results for hub proteins of ETN. ..……. 114

Table A.4 Bingo Results for hub proteins of tETN. ..……... 115

Table A.5 Bingo Results for ETN. ..…….. 116

Table A.6 Bingo Results for tETN. ..……. 120

LIST OF SYMBOLS

g	gram
h	hour
L	litre
min	minute
nm	nanometer
rpm	Revolutions per minute
v/v	Volume per volume
vvm	Volume per volume per minute
w/v	Weight per volume
μ_{max}	Maximum specific growth rate
Yps	Product yield on substrate
Ypx	Product yield on biomass

LIST OF ACRONYMS / ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BH	Benjamini - Hochberg
BLAST	Basic Local Alignment Search Tool
C	Carbon
CC	Clustering coefficient
CFU	Colony forming unit
CPL	Characteristic path length
d	Diameter
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DNS	3-5 dinitrosalycilic acid
DO	Dissolved oxygen
ETN	Ethanol Tolerance Network
EtOH	Ethanol
EVC	Eigen vector centrality
FDR	False discovery rate
GO	Gene Ontology
k	connectivity
MIPS	Munich Information Centre for Protein Sequences
mRNA	Messenger ribonucleic acid
OD	Optical density
ORF	Open reading frames
PC	Principal Component
PCA	Principal Component Analysis
PLS	Partial least squares
PPI	Protein - protein interaction
RNA	Ribonucleic acid
SGD	*Saccharomyces* Genome Database

SGRP	*Saccharomyces* Genome Resequencing Project
SPA	Selective Permissibility Algorithm
RNA	Ribonucleic acid
tETN	Tuned Ethanol Tolerance Network
tRNA	Transfer ribonucleic acid
YMM	Yeast minimal media
YP	Yeast extract - peptone
YPD	Yeast extract - peptone - dextrose
YPDS	Yeast extract - peptone - dextrose - starch

1. INTRODUCTION

The use of fossil fuels has been questioned, by many nations, from the economic, ecology and environmental point of views all over the world [1]. Currently, the world is confronting serious challenges such as global warming due to greenhouse gas emission and the depletion of oil reserves causing limitation of energy [1–4]. The use of renewable energy is considered as one of the possible solutions to these global challenges, and this has basically led to increased efforts in developing routes for producing biofuels [2,3]. Of the liquid biofuel alternatives, bioethanol is expected to play the major role in expanding the global biofuel economy due to its comparatively low production costs, and its proven performance as an extender or replacement for gasoline as a fuel for land transportation [5]. The growing awareness of the urgent need to reduce oil imports, enhance rural economies and improve air quality, has increased fuel ethanol production considerably in many countries over the last few years. According to the Renewable Fuel Association 2012 statistics, the world ethanol production reached about 100 billion of liters in 2011. The United States is the world's largest bioethanol producer, accounting for more than half percent of global bioethanol production. With all of the new government programs in America, Asia, and Europe, total global bioethanol demand could grow to exceed 125 billion liters by 2020 [6].

Industrial microbial fermentation has been used for bioethanol production and strain development is required for not only improving yield, titer, and productivity, but also utilizing cheap raw materials efficiently [7]. *Saccharomyces cerevisiae* is the most preferred microbial cell factory for bioethanol production because it gives high ethanol yield, high productivity and is able to withstand relatively high ethanol concentrations in order to keep distillation costs low [8,9]. Although, ethanol is the major product of yeast sugar fermentation, a high level of ethanol is one of the major environmental stressor for yeast during industrial fermentations. Therefore, yeast cells that have high growth ability under high ethanol concentrations, are desired substantially for industrial production processes [10–12]. The underlying causes responsible for decreasing the performance of ethanol stressed cells are yet to be identified to construct strains with enhanced tolerance to

ethanol. In addition to strain development, extending the substrate range of *S. cerevisiae* becomes a thrilling area of interest. Recently, there have been growing concerns about the diversion of food and fodder-grade feed-stocks from the food chain to bioethanol production and the impact of this might have resulted in increasing food prices and reducing biodiversity [1,13,14]. Therefore, significant efforts have focused on ranging substrates that can be utilized by *S. cerevisiae* to produce bioethanol. The conversion of biomass into bioethanol has become an important industry for improved productivity.

Microbial production of biofuels, including ethanol, is a result of complex network of interacting biological processes. Efforts directed to the improvement of production rate and/or yield of microbial bioethanol production processes should consist of studies on gene, enzyme, reaction and pathway levels, and therefore, there is an increasing need for systems biology approaches, methods, and tools. Industrial systems biotechnology is the application of a systems biology perspective to bioprocesses that involve the conversion of biomass to chemicals, materials, and/or energy via biocatalysis using microbial fermentation or enzyme catalysis [15]. The ultimate objective of the industrial systems biotechnology is the development of optimized cell factories that can speed up existing bioprocesses and hence bring the final product to the market faster. The tools of systems biology are also used to identify sustainable raw materials that can be used in robust, productive and cost-effective processes which are constant challenges in industry [15,16].

In addition to data available both at the global level and the molecular level for *S. cerevisiae*, the vast amount of knowledge about its physiology, biochemistry, and genetics extends the use of this yeast as a model for systems-based studies. Most of the systems biology tools, such as high-throughput genome sequencing, transcriptional profiling, metabolomics, fluxomics, proteomics, in silico modeling at genome-scale, and bioinformatics driven data integration have first been applied to *S. cerevisiae*. These high-throughput experimental techniques are invaluable for the phenotypic characterization of different mutants. Integration of different types of data will be helpful to obtain quantitative understanding of the cellular processes and interactions within the cell [16].

Application of systems biology principles to biofuel technologies is still a challenging research area. Hence, in this study considering the importance of next-

generation biofuel technologies in the sustainable development, various *S. cerevisiae* strains were assessed for their potential use in bioethanol production processes.

In the first part of the study, five industrial *S. cerevisiae* strains, selected from *Saccharomyces* Genome Resequencing Project (SGRP), were evaluated for ethanol production from biomass. The effect of various biological residues as carbon source on bioethanol production was analyzed. The ethanol tolerances of industrial *S. cerevisiae* strains were examined, and the difference in the observed ethanol tolerance of strains was investigated using the known genome sequences of these strains. The detected relationship between strain's ethanol tolerance and ethanol production yields showed the importance of the isolation of ethanol tolerant mutants to improve productivity and yield in bioethanol production processes.

In the second part of the study, ethanol tolerance and toxicity mechanisms in *S. cerevisiae* were investigated. A novel network-based modular approach was developed to identify genes which may have potential roles in ethanol tolerance in *S. cerevisiae*. The identified genes were tested experimentally via deletion mutant assays, and *S. cerevisiae* strains carrying deletions of two identified genes were found to exhibit improved tolerance to ethanol. These ethanol tolerant mutants were subjected to ethanol and transcriptional responses of these strains to ethanol stress were investigated with the aim of understanding the molecular mechanism underlying ethanol tolerance. The results indicated the major biological processes involved in ethanol stress response in *S. cerevisiae*.

In the last part of the study, the genome-wide transcriptional response of the previously constructed amylolytic *S. cerevisiae* strain was investigated in order to understand the effect of plasmid presence together with the effect of aeration. Moreover, considering the need for extending the substrate range for bioethanol production, the amylolytic *S. cerevisiae* strain was assessed for its capability to produce ethanol from raw starch substrates. This study provides further evidence that system-based approaches are powerful tools to understand and improve bioethanol production processes.

2. BIOETHANOL PRODUCTION BY INDUSTRIAL *SACCHAROMYCES CEREVISIAE* STRAINS

This chapter of the study deals with the ethanol production processes by industrial *S. cerevisiae* strains. In the first part of the study, the suitability of five industrial *S. cerevisiae* strains, selected from *Saccharomyces* Genome Resequencing Project [17] strain collection, on strain improvement were evaluated for future use in ethanol production processes. The growth and production performances of these strains were assessed. Since strains' tolerances to high concentrations of ethanol affect the ethanol yields during industrial bio-production processes, the ethanol tolerances were also compared. In the second part of the study, considering the importance of bioethanol production from different types of biomass, the growth and ethanol production performances of these strains on various agro-industrial wastes, including sugar beet pulp, starch and sugar beet molasses, and biological residues, including carrot, tomato and potato peels, are presented.

2.1. Comparison of the Fermentation Performances of Industrial *Saccharomyces cerevisiae* Strains

2.1.1. Background Aspects

Yeasts are mainly utilized in food and beverage industries. Latest developments in science and biotechnology made significant contributions to the industries related to bioethanol production and the technologic importance of yeast passed beyond the brewing and food industries. *S. cerevisiae* has been serving as the dominant species for a large portfolio of industrial products, such as fuel ethanol, industrial enzymes, pharmaceuticals, and chemicals [18]. It has long been a favored organism due to the vast amount of knowledge about its physiology, biochemistry, and genetics.

Particular genetic attributes of *S. cerevisiae* are its ease and rapidity of growth, ready isolation and selection of mutants, its small genome and the existence of both haploid and diploid life cycles. Moreover, the technological attributes that make yeast attractive can be

listed as well understood fermentation and downstream processing techniques; straight forward research to production scale up technology; and wide range of carbon sources applicable to yeast [19].

S. cerevisiae strains are very attractive and promising for bioethanol production and the development of yeast strains that efficiently ferment sugars and resistant to multiple stresses will improve bioethanol production processes. Numerous strains of *S. cerevisiae* have been isolated since the discovery of yeast as ethanol producing organism [20–22], and several efforts have been undertaken to improve yeast strains aiming to enhance the efficiency of the fermentation processes together with the product quality [23]. Despite their different roles, industrial yeast strains all share the general ability to grow and function under the stressful conditions in comparison to non-industrial strains. In addition, there are numerous differences within the industrial yeast strains and all these differences highlights the genetic diversity of *S. cerevisiae* species [24].

Identification of the complete genome sequences of *S. cerevisiae* strains has been increased in order to understand the genetic differences. As a part of the *Saccharomyces* Genome Resequencing Project (SGRP), near-complete sequences of over seventy *S. cerevisiae* isolates were reported and the phylogenetic tree presented in Figure 2.1 was generated [17]. The investigation of *S. cerevisiae* population genomics revealed genomic and phenotypic variations between these isolates and Wine/European and Sake strains were found to have improved fermentation properties [17].

Yeast cells are exposed several environmental changes and stresses during fermentation in industrial production processes. Accumulation of ethanol is one of the main environmental changes that blocks the whole fermentation process [10]. Ethanol stress results in reduced cell growth, diminished viability, consequently decreased ethanol yield and productivity. Therefore, yeast cells that are tolerant to high concentrations of ethanol, are desired in ethanol production processes [12].

In the present study, five industrial *S. cerevisiae* strains, namely BC187, L-1374, L-1528, K11 and Y9, were selected from the Wine/European and Sake strains group of the *Saccharomyces* Genome Resequencing Project [17] strain collection and assessed for their

potential use in ethanol production processes. In order to compare the performances of the selected industrial strains, the ethanol tolerances were also determined by following their colony-forming ability on agar plates as well as their growth rates in liquid cultures in the presence of different concentrations of ethanol.

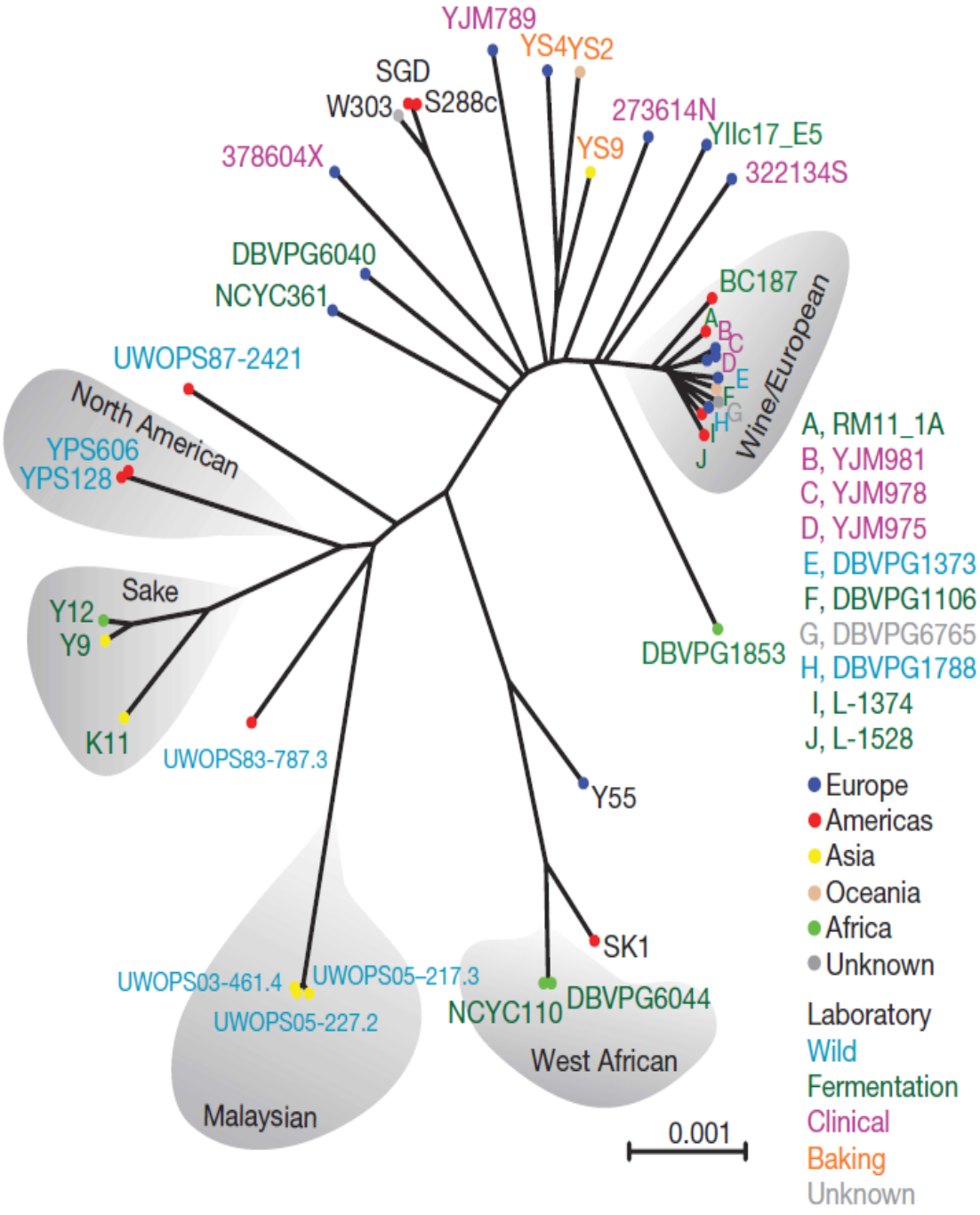

Figure 2.1. *S. cerevisiae* strains with clean lineages highlighted in grey; name color indicates source and dot color indicates geographic origin [17].

2.1.2. Methods

2.1.2.1. Strains and Growth Conditions. Five industrial strains of *S. cerevisiae*, namely BC187, L-1374, L-1528, K11 and Y9 were selected from the *Saccharomyces* species collection of SGRP [17] and used in this study. BC187 was isolated from a wine barrel in California [25], L-1374 and L-1528 were isolated from Chile [17], Kyokai no. 11, an ethanol-tolerant saké yeast [22], was obtained from Japan [20], and Y9, a wine yeast [26], was obtained from Indonesian ragi [20]. *S. cerevisiae* strains were kindly provided by Ed Louis from University of Nottingham.

Precultures were inoculated with a single colony of cells taken from yeast extract-peptone-glucose (YPD) agar plates and incubated in YPD medium (20 gL^{-1} D-glucose, 20 gL^{-1}peptone, 10 gL^{-1} yeast extract) at 30ºC and 180 rpm.

2.1.2.2. Cultivation Conditions. *S. cerevisiae* strains were grown in YPD medium. A preculture at a volume fraction of 1% was used to inoculate the culture. Cultures were kept with vigorous shaking at 30°C and 180 rpm. Optical densities (OD_{600}) were monitored by spectroscopic measurements at 600 nm wavelength until the steady-state was reached. All experiments were carried out in duplicate.

Samples taken from the culture at regular intervals were centrifuged at 8000 rpm for 6 min (Eppendorf 5415C; Germany) to determine substrate utilization, extracellular product formation, and metabolite concentrations.

2.1.2.3. Determination of Maximum Specific Growth Rate and Dry Cell Weight. For maximum specific growth rate (μ_{max}) determination, OD_{600} of samples collected during the exponential phase of growth were used.

Dry cell weights (DCW) of steady state cultures were determined gravimetrically by first recovering cells from 1 ml culture samples through centrifugation at 8000 rpm for 6 min. Cells were washed with distilled water three times and then dried at 70°C until constant weight was achieved. Reported values are averages of five biological replicates for each data point.

2.1.2.4. Extracellular Metabolite Analysis. In order to determine substrate utilization, extracellular product formation, extracellular glucose and ethanol concentrations during exponential phase of growth were determined by using enzymatic analysis kits (Sigma) as described by the manufacturer.

2.1.2.5. Statistical Analysis. Principal Component Analysis (PCA) was carried out with the PLS Toolbox of MATLAB 7.4 by using the measured growth related and production related parameters as variables and the strains as the objects, as described by Wold et al. [27].

To statistically describe the differences between strains, comparisons of the fermentation parameters were carried out using Analysis of Variance (ANOVA) computed in MS Excel.

2.1.2.6. Determination of Ethanol Tolerance. 5 ml of YPD medium containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol, was inoculated with a final OD_{600} of 0.05 and was incubated at 30ºC under 180 rpm shaking for 72 h. In order to test the colony forming ability of the cells, every 24 hours a sample from the liquid culture was spotted onto a YPD agar plate by use of a Singer Instruments Rotor HDA and cultured at 30ºC for 48 h. After 72 h, OD_{600} were measured [28].

2.1.2.7. Identification of Nucleotide Variations Present in Ethanol Tolerances Genes of Industrial Yeast Strains. The difference in the observed ethanol tolerance of industrial *S. cerevisiae* strains was investigated using the known genome sequences of these strains. 15 ethanol tolerance genes that are thought to be related with ethanol stress, including *URA7, LAP3, YOR139C, CYB5, SFL1, HSP26, RTC3, OLE1, EDE1, TPS1, ELO1, MSN2, DOG1, INO1, HAL1* [29–34] were selected and compared to reveal differences which might be associated with increased tolerance in industrial strains. The whole-genome sequences of the industrial *S. cerevisiae* strains were obtained from the *Saccharomyces* Genome Resequencing Project at the Sanger Institute [17].

The DNA sequence of each gene obtained from SGD (*Saccharomyces* Genome Database) was used to define the precise chromosomal localization of ethanol tolerance genes in each strain by BLAST. Sequence variations in each gene were determined by the pairwise comparison of each strain using MATLAB 7.4. Protein sequences were identified from nucleotide sequences by ORF Finder tool [35], and compared by the multiple sequence alignment tool ClustalW2 [36].

2.1.3. Results and Discussion

2.1.3.1. Fermentation Parameters of *S. cerevisiae* Strains. The fermentation performances of the five industrial *S. cerevisiae* strains, grown on YPD medium, were investigated and compared.

Table 2.1. Comparison of fermentation parameters of *S. cerevisiae* strains.

Parameter	**Strains**				
	BC187	**L-1374**	**L-1528**	**K11**	**Y9**
μ_{max} (h^{-1})	0.521 ±0.001	0.525 ±0.004	0.558 ±0.011	0.540 ±0.009	0.581 ±0.006
Final DCW (gL^{-1})	1.44±0.32	1.50±0.21	1.21±0.18	1.59±0.09	1.18±0.41
Max. Ethanol Concentration (gL^{-1})	9.16±0.28	9.46±0.36	9.47±0.07	10.70±0.13	11.23±0.16
Glucose Utilization Rate ($gL^{-1}h^{-1}$)	0.89	0.82	1.22	1.04	1.16
Volumetric productivity ($gL^{-1}h^{-1}$)	0.66	0.82	0.82	0.93	0.98
Spesific productivity ($gDCW^{-1}h^{-1}$)	0.46	0.55	0.68	0.59	0.83
Y_{ps} (g ethanol g^{-1} glucose)	0.46	0.48	0.49	0.50	0.52
Y_{px} (g ethanol g^{-1} biomass)	6.37	6.31	7.83	6.73	9.52

The biomass and metabolite concentrations were determined from the samples collected throughout the exponential and stationary phase of growth. Variations in the OD_{600} together with the glucose and ethanol concentrations that were enzymatically determined throughout the fermentations are provided in Figure 2.2.

Maximum biomass and ethanol concentrations reached by the cultures, as well as their glucose utilization rates and maximum specific growth rates were measured (Table 2.1). Maximum specific growth rates of the strains were determined from the OD_{600} of samples collected during the exponential phase of growth (Figure 2.3). In addition the volumetric and specific ethanol productivities, ethanol yield on glucose (Y_{ps}) and biomass (Y_{px}) were also determined for each strain (Table 2.1).

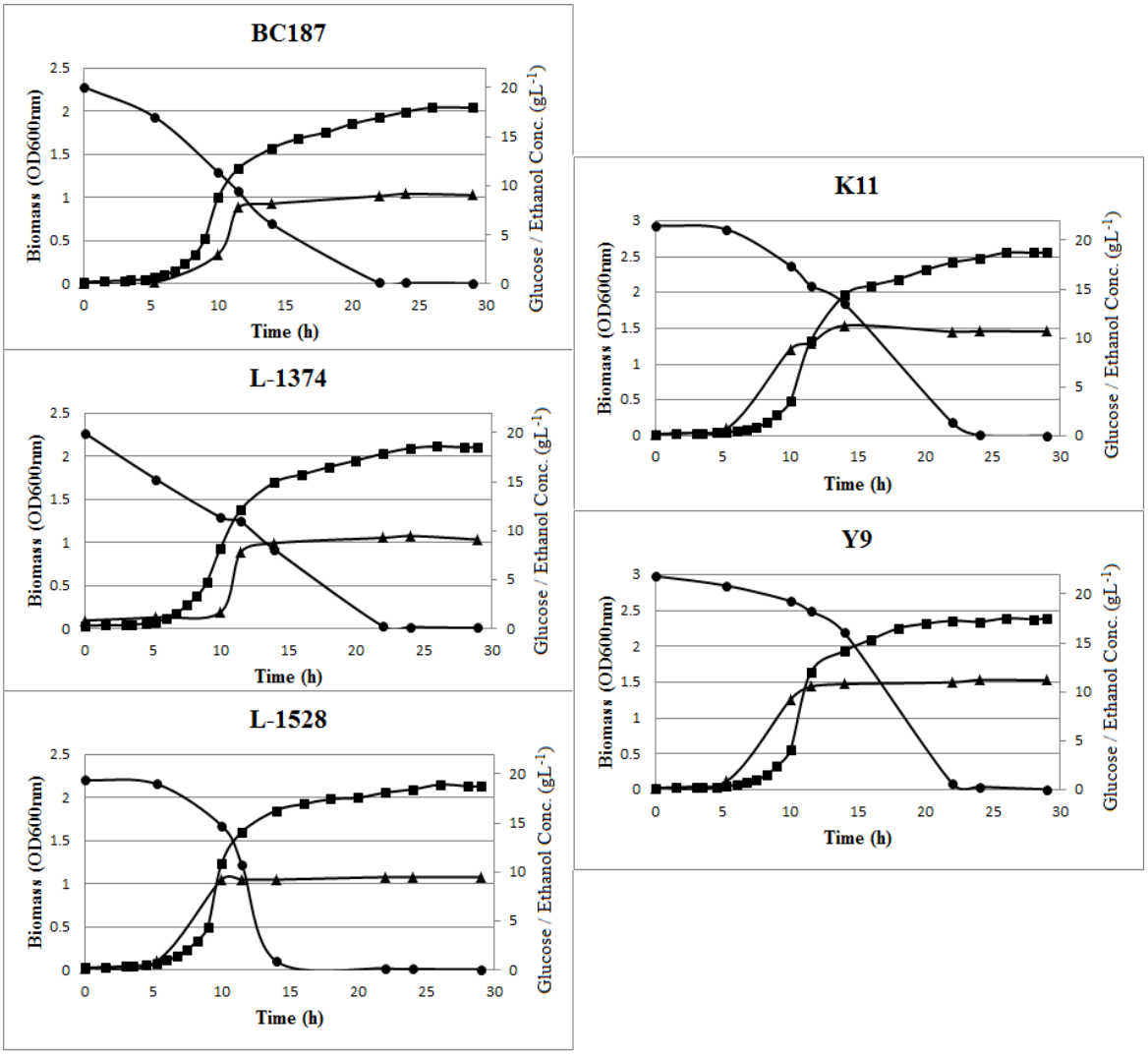

Figure 2.2. Variations in biomass (■), glucose (●) and ethanol (▲) concentrations.

To statistically describe the differences between strains, comparisons of the fermentation parameters were carried out using ANOVA computed in MS Excel and highly significant differences in the fermentation parameters were observed between the strains ($p < 0.0005$).

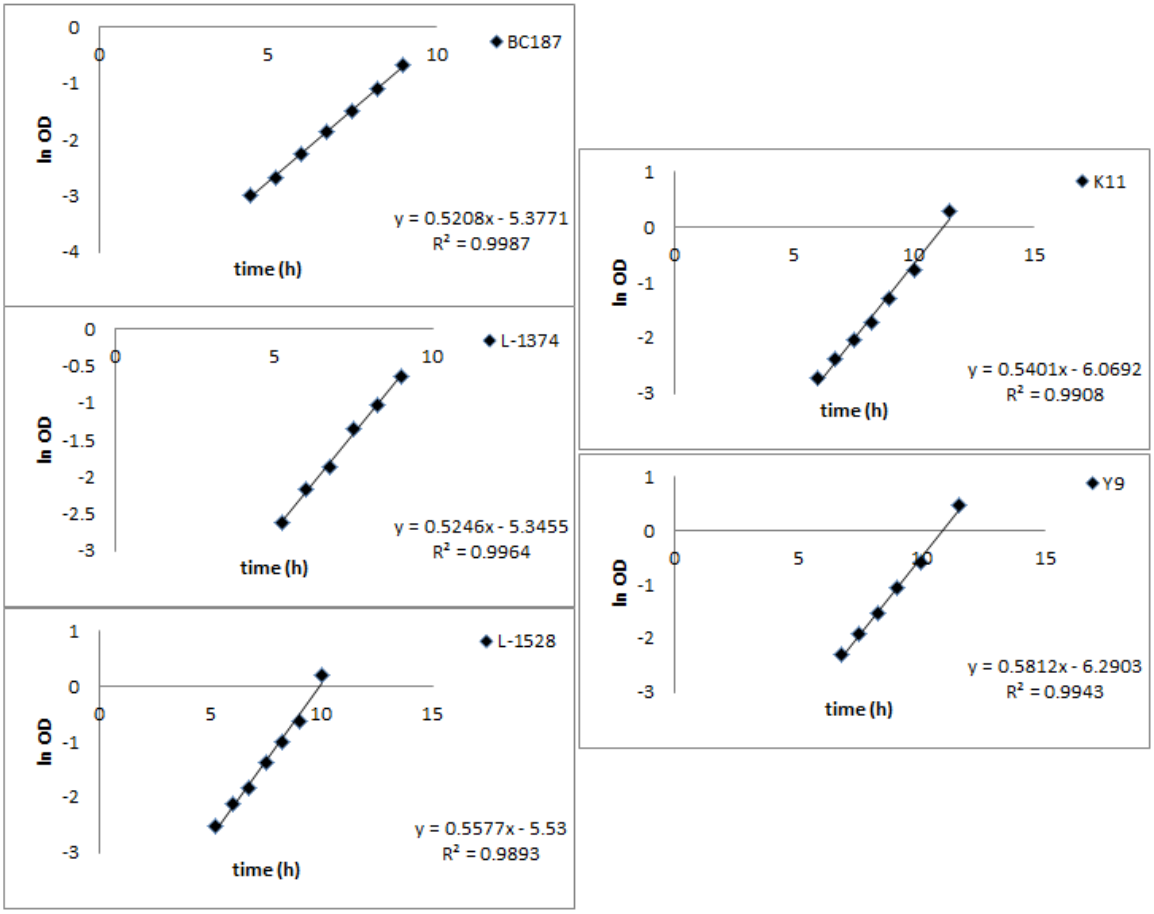

Figure 2.3. Determination of the maximum specific growth rates.

PCA of growth-related (Figure 2.4A) and production-related (Figure 2.4B) parameters resulted in two different plots. Growth patterns revealed two distinct groups of strains with similar growth profiles, namely, BC187 and L-1374 strains and L-1528 and Y9 strains with K11 forming a separate group. Whereas glucose consumption rates within the groups did not vary significantly, the 30% lower utilization rate of the former group was also reflected in their lower specific growth rates (Table 2.1).

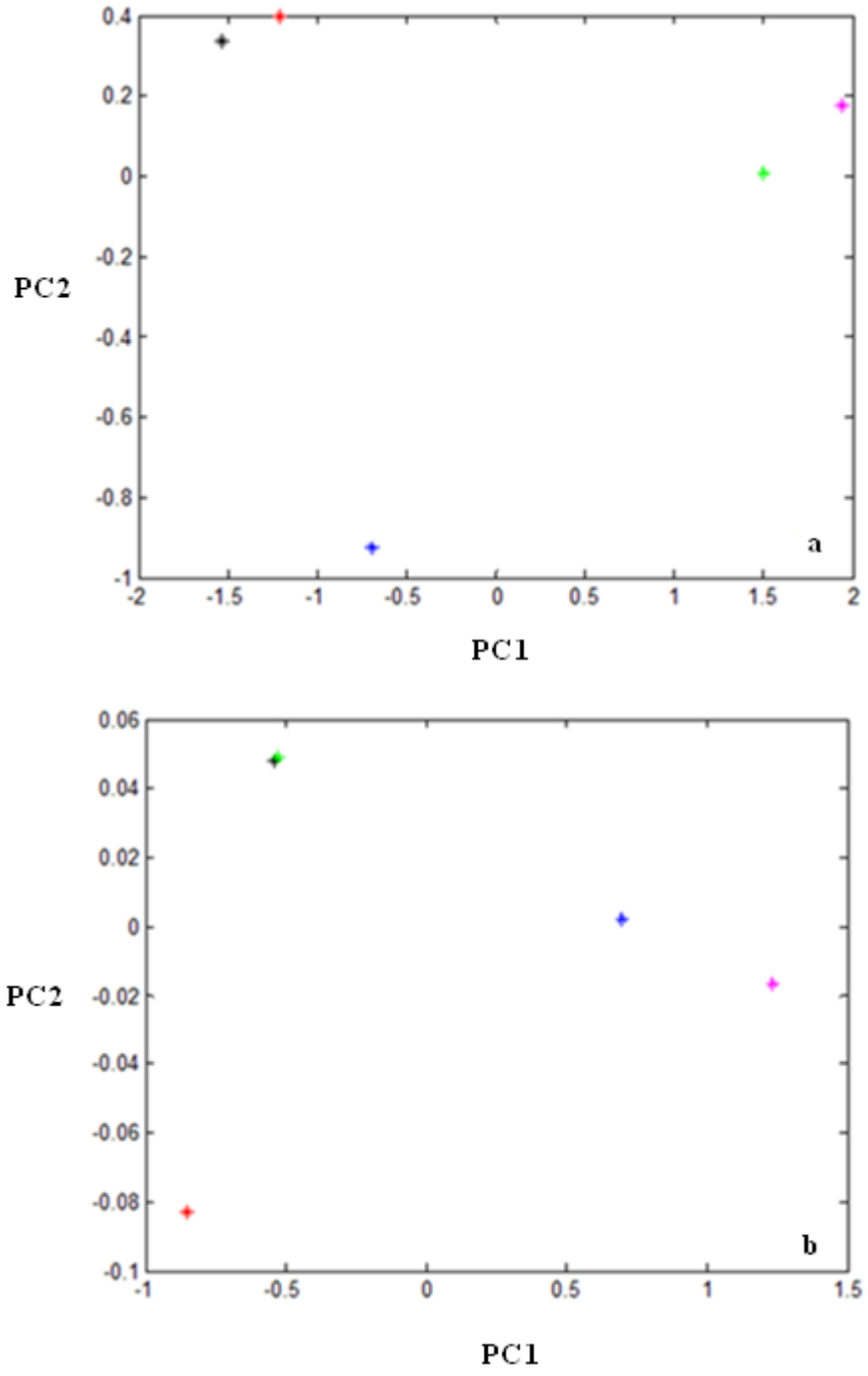

Figure 2.4. PCA plot of (a) growth (b) production related parameters; the 80% of the total variance was explained by first and second principle components; Red: BC187, Black: L-1374, Green: L-1528, Blue: K11, Pink: Y9.

Based on the production related parameters, L-1374 and L-1528 strains and Y9 and K11 strains were mutually comparable. Higher ethanol concentration values were reached by Y9 and K11 strains, with the volumetric and specific ethanol productivities being

highest in Y9 and lowest in the BC187 cultures. Hence generally, the Y9 strain was found to stand out with its rapid growth and the highest ethanol yields in terms of both cell mass of the producer and glucose consumed.

2.1.3.2. Ethanol Tolerance of *S. cerevisiae* Strains. The ethanol tolerances of the five *S. cerevisiae* strains were evaluated by growing the five industrial strains in the presence of varying amounts of ethanol and then comparing their cell yields with those of the control cultures without ethanol (Table 2.2).

Table 2.2. Ethanol tolerance of *S. cerevisiae* strains.

% volume fraction of EtOH	**EtOH (gL^{-1})**	Growth % = [OD_{600} (x% EtOH) / OD_{600} (0% EtOH)] *100				
		BC187	**L-1374**	**L-1528**	**K11**	**Y9**
0	0	100	100	100	100	100
3	23.64	99.55	86.53	85.44	98.79	87.33
5	39.40	98.63	84.16	80.35	95.04	78.31
6	47.28	98.22	81.48	78.15	85.38	77.26
8	63.04	95.40	77.99	71.10	76.87	73.29
10	78.80	67.70	57.53	59.56	53.44	65.36
12	94.56	4.66	1.94	1.72	2.07	2.07
15	118.20	2.03	1.26	1.11	1.29	1.82

All strains showed 77% or higher yield of cell biomass in media containing between 0-6% (v/v) ethanol. Growth ability of strains showed a distinct decrease with increasing ethanol concentration and strains showed only 1-2% growth in media containing 15% (v/v) ethanol. The colony-forming ability of all strains in the presence of varying amounts of ethanol 3-15% (v/v) were tested by culture on solid media as described in Methods section. Figure 2.5 shows the colony-forming ability of *S. cerevisiae* Y9 strain grown in YPD media containing 0-15% (v/v) ethanol. For all strains, no significant changes in colony-forming patterns could be detected up to 10% (v/v) ethanol for 72 hours. The L-1528 strain displayed slow growth at 12% (v/v) and no growth at 15% (v/v) ethanol (Figure 2.6). In contrast at 15% (v/v) ethanol, the Y9 (Figure 2.5) and BC187 (Figure 2.7) strains showed significant colony-forming ability, while K11 and L-1374 formed few colonies.

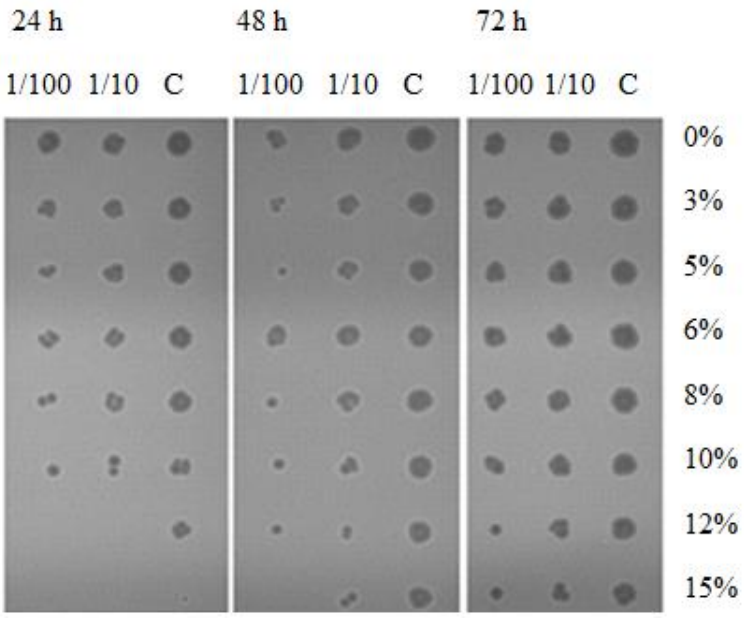

Figure 2.5. Colony forming ability of undiluted (C), 10 fold (1/10) and 100 fold (1/100) diluted *S. cerevisiae* Y9 cultures grown in YPD containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol after 24 h, 48 h and 72 h.

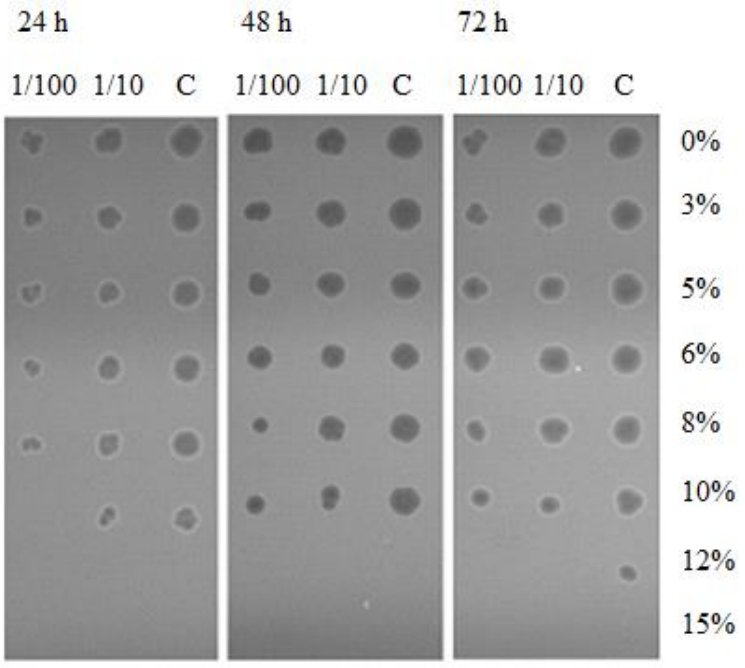

Figure 2.6. Colony forming ability of *S. cerevisiae* L-1528 cultures grown in YPD containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol after 24 h, 48 h and 72 h.

Among yeast species, *S. cerevisiae* is known to tolerate high ethanol concentrations [20] and, indeed, the industrial yeast strains used in this study were all highly tolerant up to 10% (v/v) ethanol for 72 hours. The observed ethanol tolerance of K11 is in agreement with earlier studies where K11, the ethanol-tolerant mutant of yeast strain K7, was reported to produce ethanol at high titers at the final stage of fermentation [22].

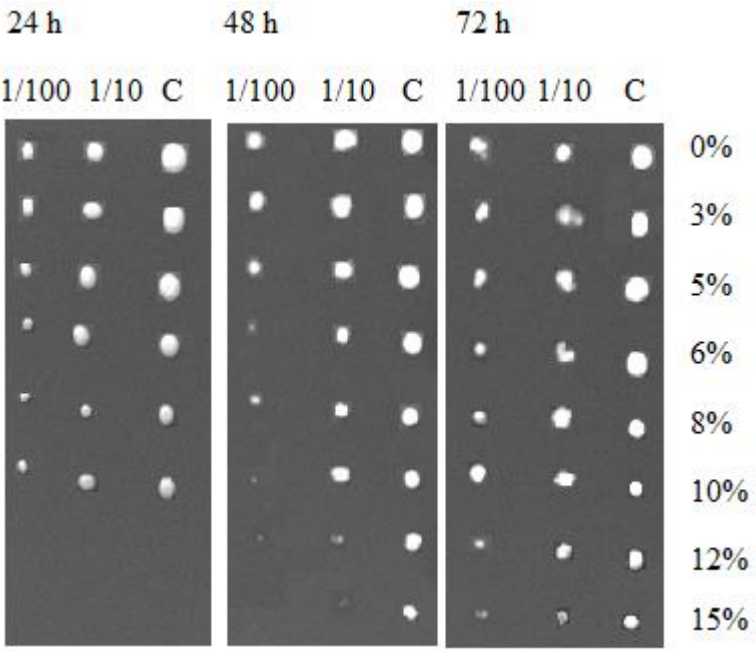

Figure 2.7. Colony forming ability of *S. cerevisiae* BC187 cultures grown in YPD containing 0, 3, 5, 6, 8, 10, 12, and 15% (v/v) ethanol after 24 h, 48 h and 72 h.

2.1.3.3. Identification of Nucleotide Variations Present in Ethanol Tolerances Genes of Industrial Yeast Strains. There are several reports in literature on finding gene targets for the construction of ethanol tolerant *S. cerevisiae* strains. By screening the collection homozygous diploid deletants (representing 4741 non-essential genes), two null mutants (*ura7*Δ and *gal6*Δ) that grew faster than the wild type in medium containing 8% (v/v) ethanol were identified [29]. By similar means, strains carrying the *CYB5, SFL1*, and *YOR139C* deletions were identified as conferring ethanol tolerance [30]. The ethanol tolerance and fermentative performance of wine yeasts were improved through modifying the expression levels of the *HSP26* and *YHR087W* (*RTC3*) genes [31]. In order to understand the effect of fatty acid composition on ethanol tolerance in *S. cerevisiae*, the ethanol tolerance of yeast cells that contained various levels of unsaturated fatty acids were examined and improved tolerance in cells overexpressing *OLE1* was observed which, in

turn, indicated the importance of the total unsaturated fatty acid content (rather than the degree of unsaturation) for ethanol tolerance [32]. Using strain-specific differences in the transcriptional response to ethanol by identifying gene expression differences between strains with and without the ability to acquire increased ethanol tolerance after ethanol pre-treatment, new genes (*EDE1*, *ELO1*, *MSN2*, *TPS1*) were identified that increased ethanol tolerance when overexpressed [33]. By an inverse metabolic engineering approach, *S. cerevisiae* cells transformed with a genomic library for higher ethanol tolerance were screened and *INO1*, *DOG1*, *HAL1* and *MSN2* were identified as target genes whose overexpression resulted in increased volumetric ethanol productivities and specific growth rates of the cultures [34].

Within the scope of this study, genes that have been reported to be associated with ethanol tolerance, including *URA7, LAP3 (GAL6), YOR139C, CYB5, SFL1, HSP26, RTC3, OLE1, EDE1, TPS1, ELO1, MSN2, DOG1, INO1, HAL1* were selected for genomic comparison of industrial yeast strains. Nucleotide variations in these genes were systematically analyzed by pairwise comparisons to the most (Y9) and least (L-1528) ethanol-tolerant strains characterized in this study.

The *Saccharomyces* Genome Resequencing Project at the Sanger Institute reported the near-complete genome sequences of *S. cerevisiae* from different sources and locations and identified single nucleotide polymorphisms and nucleotide insertions and deletions in the *S. cerevisiae* nuclear genome. The report pointed out the presence of five lineages exhibiting the same phylogenetic relationship across their entire genome in the *S. cerevisiae* population [17]. Based on this classification, Y9 and K11 are in the Saké lineage, whereas BC187, L-1374 and L-1528 are in the Wine/European lineage.

In this study, significant nucleotide variations (quantified as percent nucleotide differences) in ethanol tolerance genes between strains were identified (Figure 2.8). Nucleotide variations in *HSP26*, *TPS1*, *INO1*, *CYB5* and *LAP3* genes appear to be same between Y9 and BC187 strains and Y9 and L-1528 strains. These differences could be explained by the different lineages to which these strains belong. On the other hand, in the remaining genes some nucleotide variations were found between Y9 and L-1528 strains that are different from the variations between the Y9 and BC187 strains. These variations

in genes previously associated with ethanol tolerance could be responsible for the observed ethanol tolerances of industrial yeast strains. Remarkably, 41 nucleotide variations were identified in the *DOG1* gene between the Y9 and L-1528 strains, 13 of these variations were also found between Y9 and BC187 strains; the other 28 variations were not seen between Y9 and BC187 strains. Therefore, these 28 variations in *DOG1* gene could be the reason for the decreased ethanol tolerance of L-1528 strain.

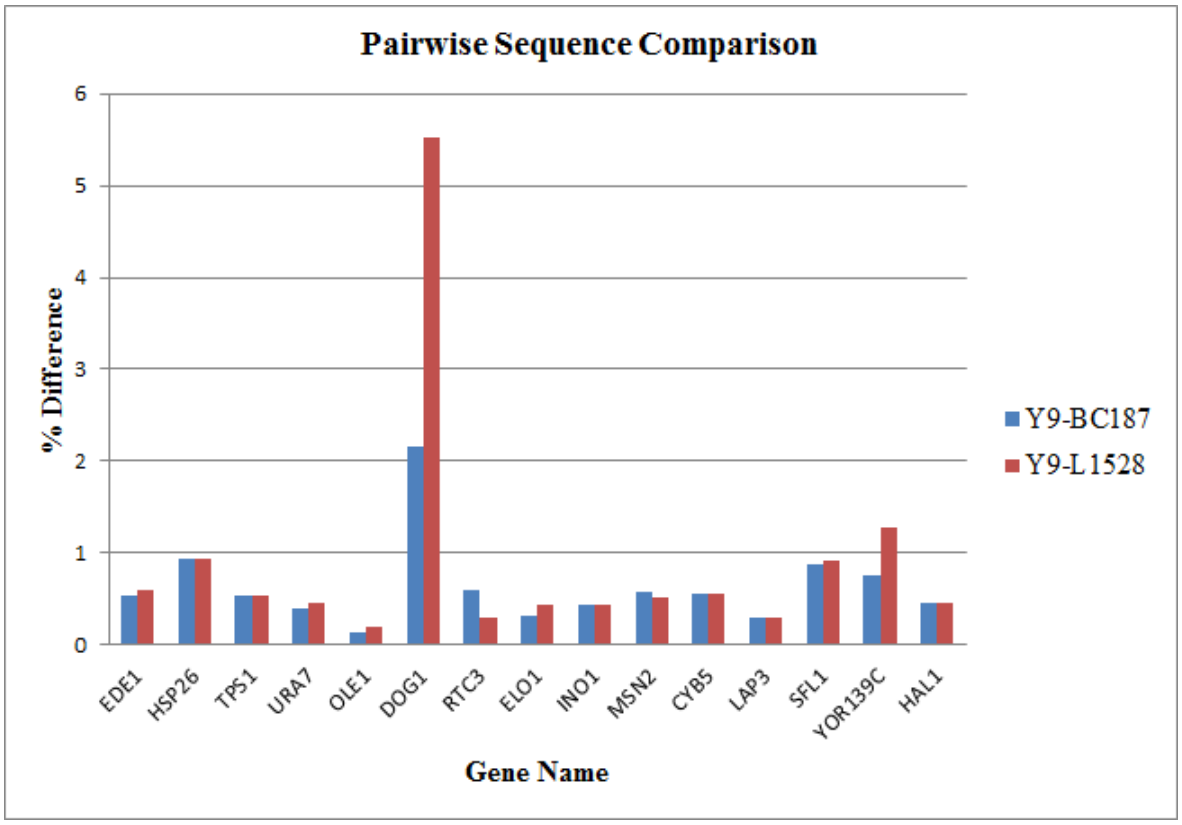

Figure 2.8. Pairwise nucleotide sequence comparison of ethanol tolerance genes of two sets of strains: whereas the former set included two ethanol tolerant strains (Y9 and BC187), the latter one embraced a tolerant strain (Y9) and the least tolerant strain (L-1528).

Dog1p (2-deoxyglucose-6-phosphate phosphatase) catalyzes the hydrolysis of 2-deoxy-D-glucose-6-phosphate to 2-deoxy-D-glucose and phosphate. It physically interacts with two protein kinases (Kin2p and Rck1p), a protein related with cell division (Cdc12p), a protein required for mRNA splicing (Snp1p), and an essential gene involved in the assembly of cytosolic and nuclear iron-sulfur proteins (Cia1p) [37,38], and has genetic interactions with 18 genes [39,40]. There are no unique domains/motifs, transmembrane domains, and signal peptides predicted for Dog1p, but it has 5 domains that it shares with

known protein families: the Gene3D homologous superfamily: 3.40.50.1000, the phosphatase panther protein class, the HAD-like superfamily, the haloaciddehalogenase-like hydrolases, and HAD-superfamily hydrolases [41].

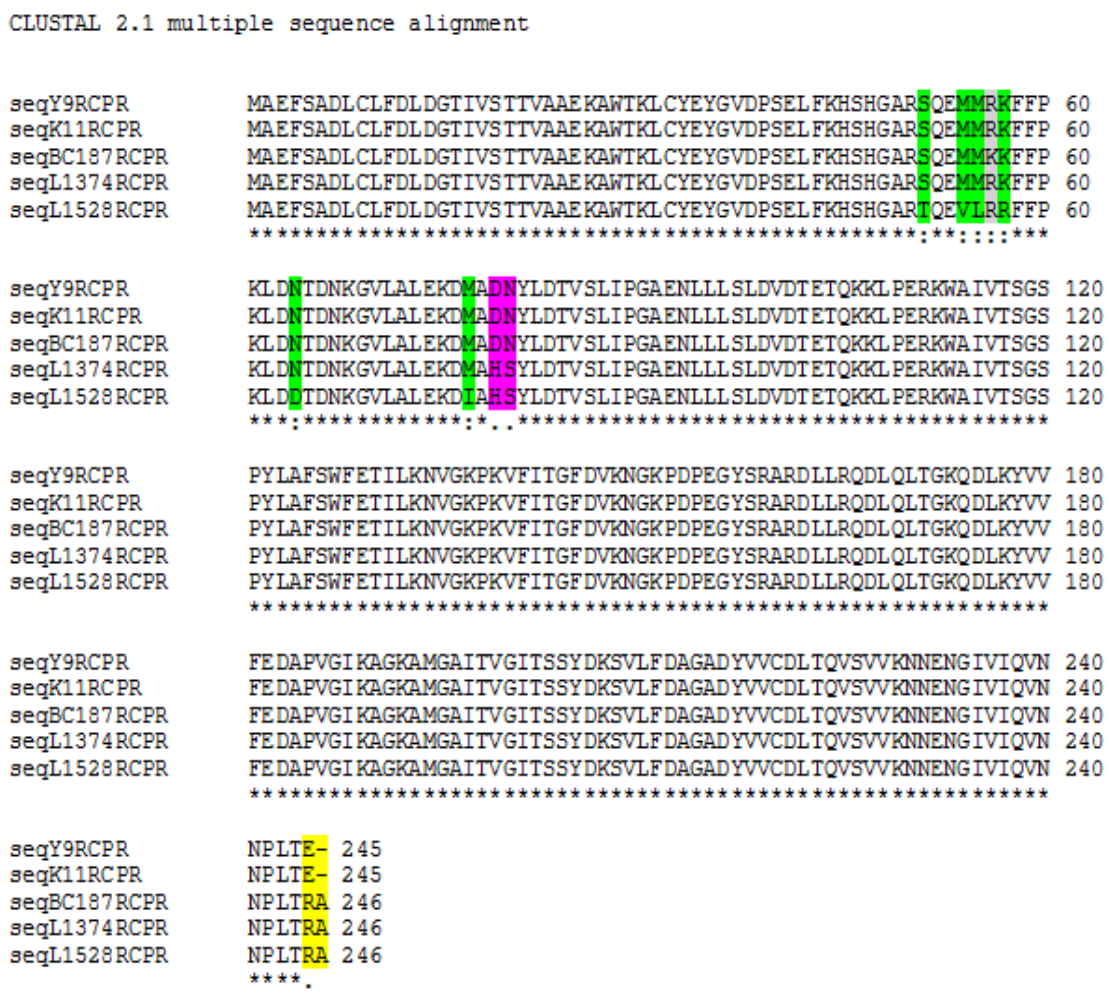

Figure 2.9. Multiple ClustalW2 alignments of Dog1p amino acid sequences in five industrial *S. cerevisiae* strains.

In order to understand the possible effects of nucleotide sequence variations on phenotype, amino-acid sequences of the *DOG1* protein products were compared between all 5 strains and 11 amino-acid differences were identified (Figure 2.9). Two of these differences were seen between the saké yeasts (Y9 and K11) and the European wine yeasts (BC187, L1374 and L1528). Therefore, these differences could be explained by the different lineages to which these strains belong. On the other hand, the amino-acid sequence of the L-1528 Dog1p was different from that of all other strains at 6 positions Thr_{51}, Val_{54}, Leu_{55}, Arg_{57}, Asp_{64}, Ile_{77}, and the amino-acid sequences of L-1528 and L-

1374 were different from all other strains at positions His_{79} and Ser_{80}. Since the ethanol tolerances of the L-1528 and L-1374 strains were less than those of Y9, BC187 and K11, these 8 amino-acid differences, which were located in the known domains of Dog1p, might be involved in the decreased ethanol tolerances of L-1528 and L-1374.

2.1.4. Concluding Remarks

Bioethanol is a promising alternative to fossil fuels as a commercially sustainable and environmentally favorable energy. Therefore, five industrial *S. cerevisiae* strains were assessed for their potential use in bioethanol production processes. Both the fermentation properties and the ethanol tolerances of five industrial *S. cerevisiae* strains indicated the importance of choosing the appropriate strain to be used for bioethanol production. Investigation of the growth and production related parameters separately showed differences not only between industrial strains, but also between strains used within the same industry. For instance, saké yeasts (Y9 and K11) used in this study showed similar characteristics regarding to ethanol production, whereas growth related properties were significantly different.

Industrial yeast strains have ability to grow under the stressful conditions in comparison to non-industrial strains. Since high concentration of ethanol is one of the major stresses that yeast cells are exposed to during industrial ethanol production processes, the ethanol tolerances of these strains were compared. Although the industrial yeast strains had the ability to highly tolerate up to 10% ethanol, testing the colony forming abilities revealed that BC187 and Y9 had the highest tolerance to ethanol. The difference in ethanol tolerances of strains were also analyzed by comparing the genome sequences of genes previously associated with ethanol tolerance and the nucleotide variations in *URA7, YOR139C, SFL1, RTC3, OLE1, EDE1, ELO1, MSN2, DOG1, HAL1* genes were found to be a possible reason for the observed ethanol tolerances of industrial yeast strains.

In conclusion, both ethanol production yields and growth ability under high ethanol concentrations made these industrial strains valuable for bioethanol production processes. Considering the importance of providing sustainable energy without compromising food security and the environment, further studies were conducted in order to investigate the

potential use of these industrial *S. cerevisiae* strains in second generation ethanol production processes.

2.2. Evaluation of Industrial *Saccharomyces cerevisiae* Strains for Ethanol Production from Biomass

2.2.1. Background Aspects

Biomass appears to be the most feasible feedstock for current routes to the production of biofuels since it is renewable, economical, has low sulfur content, involves no net release of carbon dioxide, and so has a high potential to become economically feasible in near future [6,42]. The main biomass sources are currently represented by dedicated crops, in particular sugar, starch and oil crops. Biodiesel can be produced from waste edible oils and fats by transesterification processes or cracking [43]; whereas biological residues, manure, and grasses can be suitable for biogas production [44]. On the other hand, biological organic materials with considerable amounts of starch or cellulose that can be converted into sugar can be used to produce bioethanol. Sugarcane, sugar beetroot, and sugar sorghum are examples of raw materials that contain sugar; whereas wheat, barley and corn are starch crops. A significant part of the wood of trees and herbs is composed of cellulose, which can be converted into sugar by using available technologies. However, since conversion of cellulose into glucose is more complicated than the conversion of starch, ethanol production is currently based on wheat and corn in the USA, sugar cane in Brazil, and wheat, barley, and sugar beet in Europe [6,9,45,46].

The conventional ethanol production processes involve a pretreatment step where the sugar in the raw material is separated and fed as substrate to the fermenter where it is converted to ethanol. These processes are based on traditional brewing techniques and utilize *S. cerevisiae* strains because they give a high ethanol yield, a high productivity, and their ability to withstand high ethanol concentrations keeps distillation costs low. In the conventional ethanol production process when the raw materials employed are grains, then only the germ of the corn and barley (i.e. the starch-containing parts) are used. Since these parts represent only a small portion of the total mass of the plant, a significant amount of fiber residue is generated [9,45]. Although these ethanol technologies are mature and play

a significant role in generating liquid fuels for the transportation sector, they rely on food-grade biomass resources. Recently, there have been growing concerns about the diversion of food and fodder-grade feedstocks from the food chain to biofuel production and therefore, much research has focused on technologies which use non-food crops, the non-food parts of edible crops, or the residues from wood-based or food-based industries such as wood chips, and the skins and pulp from fruit pressing [1,6,13,14].

These ethanol technologies, which provide sustainable energy without compromising food security or the environment, do not involve additional energy expenditures for substrate production and collection. Depending on the biomass source and product employed, they include cellulose hydrolysis followed by fermentation, pyrolysis, gasification, and anaerobic digestion, as well as their various combinations depending duct [9]. Such ethanol production processes are not yet in extensive commercial use; however, a number of pilot and demonstration plants have been set up recently with considerable active research programs being carried out in North America, Europe, Brazil, China, India and Thailand [42].

In general, ethanol processes start with the hydrolysis of the biomass followed by the yeast-based fermentation of the resulting sugars. Co-fermentation of C5 and C6 sugars has been quite challenging and, as with traditional ethanol production processes, recombinant *S. cerevisiae* strains are the preferred microorganisms for the fermentation step. In constructing recombinant *S. cerevisiae* strains for ethanol production processes utilizing biomass resources, the choice of the host strain is crucial. This choice does not only depend on a strain's fermentation parameters and ethanol tolerance but also on its ability to utilize carbon sources available in agro-industrial residues.

Considering the importance of the utilization of different types of substrates, five industrial *S. cerevisiae* strains were used in order to evaluate their potential use in second generation ethanol production processes. For this, important fermentation parameters of the strains were investigated on different agro-industrial residues like sugar beet pulp, starch molasses and sugar beet molasses as well as biological residues like carrot, tomato and potato peel wastes.

2.2.2. Methods

2.2.2.1. Strains. Five industrial *S. cerevisiae* strains, namely BC187, L-1374, L-1528, K11 and Y9, were used in this study.

2.2.2.2. Cultivation Conditions. *S. cerevisiae* strains were grown in YP medium (20 gL^{-1} peptone, 10 gL^{-1} yeast extract) including biological residues as a carbon source in order to test the capability of bioconversion of different typologies of biomass substrates into ethanol. A preculture at a volume fraction of 1% was used to inoculate the culture. Sugar beet pulp, carrot, tomato and potato peel, starch and sugar beet molasses at 1 gL^{-1} total carbohydrate content were added as carbon sources. The medium, containing 20 gL^{-1} glucose as carbon source, was used to grow the cells as control. Cultures were kept with vigorous shaking at 30°C and 180 rpm. OD_{600} were monitored until the steady-state was reached. All experiments were carried out in duplicate.

2.2.2.3. Preparation of Biomass Substrates. From the different biomass resources used in this study, carrot, tomato and potatoes were obtained from a local market. Sugar beet molasses (with average mass composition of 48-51% sucrose, 15-18% moisture, 11-13% ash, 8% betaine, 3.61% potassium, 0.9% chloride, 0.53% calcium, 0.45% sodium, 0.27% sulfate, 0.09% nitrate, 3% carbohydrate and 7.5% other organic compounds) and sugar beet pulp (with average mass composition of 10.4% crude protein, 0.9% crude fat/ether extract, 18–30% dry matter and 4–9% total ash) were obtained from Kütahya Sugar Factory (Kütahya, Turkey) as by-products of the manufacture of sucrose from sugar beet. Starch molasses containing a mass fraction of 50.6% total sugars and 20% glucose was a by-product of dextrose manufacture from corn and it was obtained from Akmaya yeast factory (Avcilar, Turkey).

Whereas molasses and sugar beet pulp were used directly, peel from the carrots, tomatoes and potatoes were first washed with tap water and then with distilled water in order to remove surface dust particles. After washing, a blenching operation was carried out by immersing them into hot water at 75-80°C for 20 min, followed by a drying step in an oven at 45°C [47]. The dried material was ground, then sterilized at 121°C for 20 min, and stored at 4°C before further use.

2.2.2.4. Determination of Maximum Specific Growth Rate and Dry Cell Weight. Maximum specific growth rates and the dry cell weights of cultures were determined as described in Section 2.1.2.4.

2.2.2.5. Extracellular Metabolite Analysis. Extracellular ethanol concentrations were determined by using enzymatic analysis kits as described by the manufacturer (Sigma).

2.2.2.6. Statistical Analysis. The fermentation parameters were compared using ANOVA to statistically describe the differences between strains.

2.2.2.7. Sugar Assays. Reducing sugar concentration was assayed by 3-5 dinitrosalycilic acid (DNS) method [48]. In order to determine reducing sugar concentrations, 100 μl of samples taken from the culture were mixed with three volumes of DNS solution and immediately placed in boiling water for 10 minutes. Then, the reaction was stopped by incubating on ice for 5 minutes. After adding 800 μl of distilled water to the samples, ODs were monitored at 540 nm wavelength against blank containing 300 μl DNS solution and 900 μl distilled water treated in the same way as the samples.

Starch concentration was monitored colorimetrically [49]. In order to determine residual starch concentrations, samples taken from the culture at regular intervals were centrifuged at 10000 rpm for 1 min. The sample supernatant was mixed with 1.5 ml of iodine solution. OD's were monitored at 550 nm wavelength against blank containing 1.5 ml of iodine solution. OD measurements were converted to starch concentration by using starch calibration curve prepared with known amounts of starch.

2.2.3. Results and Discussion

2.2.3.1. Bioconversion of biological residues into ethanol. The ability of the five strains to produce ethanol from a variety of agricultural residues, including carrot, tomato and potato peels and industrial wastes like sugar beet pulp, starch, and sugar beet molasses, was evaluated.

Table 2.3. Fermentation parameters of industrial *S. cerevisiae* strains in different carbon sources containing 1 gL^{-1} total carbohydrate.

***S. cerevisiae* strain**	**BC187**	**Y9**	**K11**	**L-1374**	**L-1528**
Waste Source	**μ_{max} (h^{-1})**				
Starch molasses	0.328 ±0.027	0.390 ±0.059	0.379 ±0.068	0.331 ±0.003	0.348 ±0.002
Sugar beet molasses	0.258 ±0.037	0.385 ±0.007	0.372 ±0.059	0.323 ±0.002	0.410 ±0.029
Tomato peel waste	0.221 ±0.001	0.343 ±0.048	0.323 ±0.024	0.286 ±0.005	0.201 ±0.059
Carrot peel waste	0.245 ±0.015	0.273 ±0.038	0.350 ±0.051	0.222 ±0.001	0.344 ±0.084
Potato peel waste	0.064 ±0.019	0.078 ±0.015	0.071 ±0.074	0.029 ±0.026	0.075 ±0.015
Sugar beet pulp	0.313 ±0.026	0.378 ±0.050	0.324 ±0.037	0.417 ±0.017	0.466 ±0.022
	OD_{600} at steady state				
Starch molasses	1.18	1.27	1.23	1.14	1.08
Sugar beet molasses	1.20	1.30	1.30	1.19	1.11
Tomato peel waste	0.84	0.95	0.94	0.97	0.82
Carrot peel waste	1.06	1.14	1.20	1.13	0.90
Potato peel waste	0.62	0.85	0.86	0.71	0.65
Sugar beet pulp	1.15	1.25	1.30	1.20	1.03
	% reducing sugar utilized				
Starch molasses	60.69	59.87	67.82	63.87	60.12
Sugar beet molasses	57.98	70.83	67.88	63.90	67.26
Tomato peel waste	54.66	48.24	54.75	42.31	45.36
Carrot peel waste	63.00	55.56	60.00	72.95	65.97
Potato peel waste	39.37	26.30	37.84	32.95	26.12
Sugar beet pulp	54.69	44.24	50.33	49.65	46.51

Growth and sugar composition and ethanol production profiles of the batch cultures were followed. μ_{max} and the percentages of reducing sugars utilized were summarized in Table 2.3, and maximum ethanol concentrations reached by the strains were provided in Table 2.4. These results were analyzed by ANOVA and highly significant differences in fermentation parameters were observed between the strains ($p < 0.0001$).

Sugar analysis of culture supernatants at intervals during the fermentations showed that most of the fermentable sugars were exhausted within the first 8 hours of incubation. All the strains grown on sugar beet and starch molasses were found to utilize (60-70)% of the reducing sugar present in the fermentation media. The maximum yeast biomass concentrations reached by the strains as well as their μ_{max} values on both types of molasses were found to be very similar. However, higher ethanol concentrations were reached with sugar beet molasses. The Y9 strain was found to be superior to the others not only in its utilization of the reducing sugars present in sugar beet molasses, but also in the conversion of these sugars into ethanol (Table 2.4). After molasses, carrot peel was also found to be efficiently utilized by all strains, reaching comparable ethanol concentrations (Figure 2.10).

Table 2.4. Maximum ethanol concentrations (gL^{-1}) reached by *S. cerevisiae* strains in different carbon sources containing 1 gL^{-1} total carbohydrate.

***S. cerevisiae* strain** / **Waste Source**	**BC187**	**Y9**	**K11**	**L-1374**	**L-1528**
Starch molasses	0.22±0.07	0.29±0.04	0.37±0.05	0.35±0.09	0.35±0.02
Sugar beet molasses	0.32±0.09	0.49±0.11	0.43±0.07	0.40±0.03	0.45±0.09
Tomato peel waste	0.25±0.01	0.27±0.03	0.24±0.04	0.16±0.01	0.18±0.01
Carrot peel waste	0.33±0.04	0.33±0.05	0.34±0.06	0.37±0.04	0.36±0.04
Potato peel waste	0.12±0.02	0.17±0.02	0.16±0.02	0.12±0.03	0.13±0.01
Sugar beet pulp	0.34±0.05	0.32±0.05	0.31±0.03	0.19±0.01	0.35±0.07

This work was performed on vegetable substrates of unknown provenance, for which the chain of custody is not known. The species and the cultivars cannot be specified. However, a single batch of each vegetable substrate has been used in all fermentation experiments in order to prevent errors associated with batch-to-batch, geographical, or seasonal variations in the chemical composition of the substrates. The current study mainly

demonstrates the differences in the fermentation performances of different industrial yeast strains.

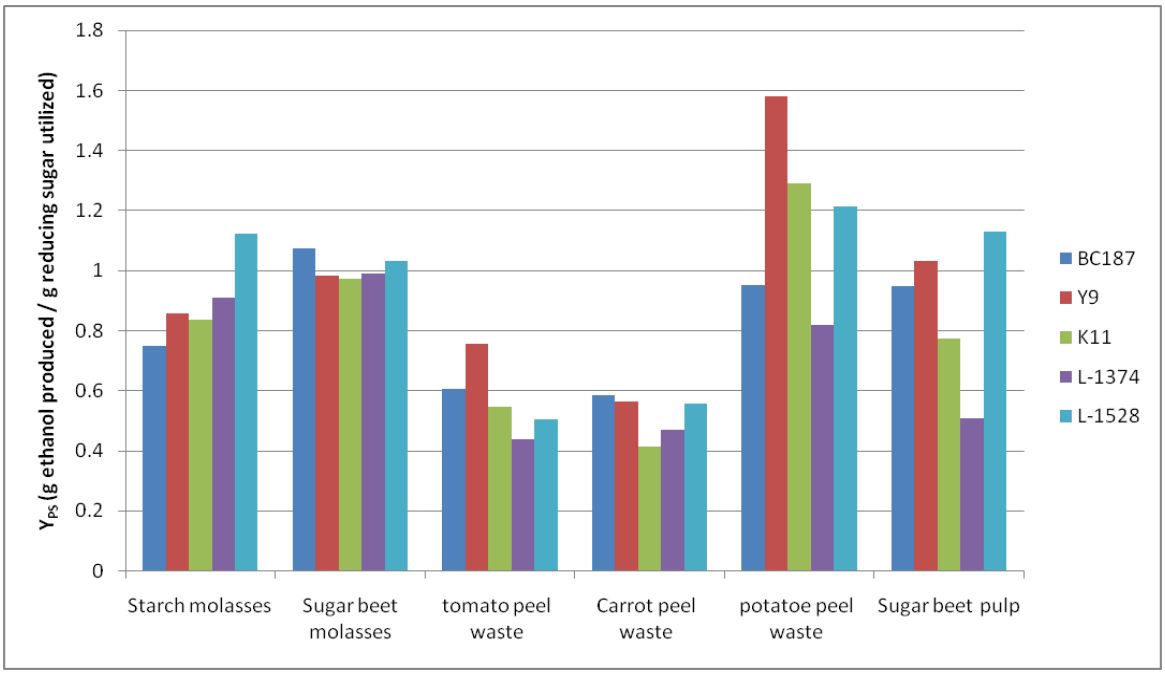

Figure 2.10. Yield comparisons of industrial *S. cerevisiae* strains grown on different carbon sources containing 1 gL^{-1} total carbohydrate.

2.2.4. Concluding Remarks

The search for clean technologies, using alternative feedstocks is economically advantageous and has been encouraged by environmental issues during the last years. The abundant availability of various types of biomass provides a good justification to develop bioethanol processes. Considering the importance of next-generation bioethanol technologies for sustainable development, this study was carried out to assess the possibility of increasing ethanol yields from alternative and available feedstocks, and revealed that choosing the appropriate *S. cerevisiae* strain to be used for second generation bioethanol production will not only depend on a strain's ethanol tolerance but also on its ability to utilize carbon sources available in agri-food residues.

Both types of molasses were found to be the best source to produce yeast biomass followed by carrot wastes. Higher ethanol concentrations were achieved with sugar beet molasses depicting its probable use in bioethanol production. Although, the lowest initial

reducing sugar contents were detected for media based on potato peels, fermentable sugars were converted to ethanol at high yields. However, less than 40% of the reducing sugar content of this media was utilized by the strains.

This study revealed that strain selection to improve ethanol productivity is very dependent on the biomass source and the appropriate pre-treatment processes could help to improve the utilization of sugars on media based on these biomass sources. The information derived from this study can be used to understand the link between these industrial strains and the agro-industrial wastes.

3. INVESTIGATION OF THE ETHANOL TOLERANCE AND STRESS MECHANISMS IN *SACCHAROMYCES CEREVISIAE*

This chapter of the study is concerned with how yeast copes with the high concentrations of ethanol occurring during fermentations. In the first part of the study, a network based modular approach was developed with the aim to find novel gene targets for rational design of ethanol tolerant *S. cerevisiae* strains. For this, firstly, a protein-protein interaction network that has the potential to predict candidate proteins related to ethanol tolerance in *S. cerevisiae* was reconstructed by integrating protein-protein interaction data with gene ontology vocabulary. Then, its modular topology was analyzed in order to elucidate the biological processes related to ethanol tolerance, to identify candidate proteins associated with ethanol tolerance mechanism, and to generate hypothesis for further studies to design ethanol-tolerant mutant strains. The modular investigation of the tuned network revealed four gene-products having potential association with ethanol tolerance. The hypotheses was tested experimentally through homozygous single gene deletion mutant assays and strains carrying *YDR307W* and *YHL042W* deletions were found to exhibit improved tolerance to ethanol. In the second part of the study, the global transcriptional response of *YDR307W* and *YHL042W* gene deletion mutants to ethanol stress was investigated to understand the molecular mechanisms underlying their improved tolerance to ethanol.

3.1. Identification of Novel Gene Targets for Improved Ethanol Tolerance in *Saccharomyces cerevisiae* through a Network-Based Approach

3.1.1. Background Aspects

S. cerevisiae can produce high concentrations of ethanol [29,50,51]. Therefore, it is commonly used for alcohol related brewing and fermentation technologies such as the production of alcoholic beverages, fuel ethanol, and other products in food and chemical industries [21,50,52].

During industrial bio-production processes, yeast cells are exposed to various environmental stresses, such as high temperature [53], ethanol accumulation [10,12,50–52,54], and high amount of sugar [55]. Among these stresses ethanol accumulation is considered to be the major stress that obstructs the complete fermentation and ethanol production [52]. Even though it is a conventional ethanol-producing microorganism, *S. cerevisiae* is also sensitive to higher concentrations of ethanol [52,56].

The increase in ethanol level acts as an inhibitor of microorganism growth and viability [8,11,57,58]. Therefore, yeast cells that have high growth ability under high ethanol concentrations, are preferred in ethanol production processes [12]. Ethanol accumulation not only affects different transport systems, including uptake of glucose, maltose, ammonium and amino acids [59]; but also reduces final ethanol titer and productivity [60]. In addition, accumulation of ethanol affects the integrity of the cell membrane [51,54,61]; causes leakage of intracellular components [59]; decreases fluidity and damages permeability of plasma membrane [62] leading to increased proton influx [51], which in turn acidify intracellular and vacuolar conditions [51,63], and hence perturb protein conformation causing protein denaturation and dysfunction [64,65]. Moreover, the mechanisms underlying ethanol tolerance and ethanol toxicity are still not well known [51,52,54]. The mechanism of ethanol tolerance involves many genes having a broad range of functional categories and biological processes suggested that ethanol tolerance is associated with interplay of complex networks at the genome level [51]. Therefore, to elucidate and understand the tolerance and stress response mechanisms in yeast, it is important to capture the global picture via a network perspective [51,52].

Significant efforts have been made to study ethanol stress response of *S. cerevisiae* in past decades using different approaches including evolutionary engineering [66], deletion library screening [29,30], and investigation of the response to ethanol at various omics levels [12,33,67]. These efforts resulted in the identification of the ethanol tolerance genes. However, a novel network approach that would reduce the number of genes to be analyzed would facilitate the identification of target genes involved in ethanol tolerance.

The protein-protein interaction (PPI) network analysis elicits rich system level information to understand the changes in cellular functions. There are several algorithms

used to construct functional sub-networks from the large PPI data. Netsearch constructs sub-networks whose members' expression profiles are highly correlated [68]. The Selective Permissibility Algorithm (SPA) was developed to reconstruct sub-networks having functionally linked protein groups [69]. Zhao *et al.* proposed a method for the identification of signaling networks based on an integer linear programming [70]. When dealing with complex biological systems, modular approach helps to understand the cellular processes that can be divided into subsystems with interacting molecules. Since each module performs a particular function independent from the context, the cellular process could be elucidated by analyzing the processes that modules involved [71]. Therefore, modular analysis of PPI networks may reveal insight to the multi-functional characteristics of the cellular proteins [72].

The ultimate goal of the present study was to find novel gene targets for rational design of ethanol tolerant strains and we developed a novel network based modular approach to identify the genes which may have potential roles in ethanol tolerance in *S. cerevisiae*. For this aim, firstly, a PPI network that has the potential to predict candidate proteins related to ethanol tolerance in *S. cerevisiae* was reconstructed by integrating PPI data with Gene Ontology (GO) terms. Then, the network was tuned statistically to reduce the effect of false positives. Modular topology analysis of the tuned network revealed four gene-products with no previously reported experimental evidence on their relevance to ethanol tolerance. Through testing experimentally via deletion mutant assays, *S. cerevisiae* strains, deleted by two of these selected genes were found to exhibit improved tolerance to ethanol. Hence the network-based approach used in this study has proven to be highly successful in the identification of novel gene targets for strain improvement studies.

3.1.2. Methods

3.1.2.1. Network Reconstruction. To reconstruct a protein interaction network associated with the ethanol tolerance mechanism in *S. cerevisiae*, SPA [69] was recruited in a non-iterative manner (Figure 3.1). Initially, proteins, whose mutations were reported to increase the resistance to ethanol, were selected from *Saccharomyces* Genome Database (SGD) and used as core proteins (Table 3.1) of the network to be reconstructed. Then the annotation collection table was created by pooling the GO annotations of core proteins in terms of

cellular component, molecular function and biological process. The annotation collection table (Table A.1) covers 130 GO annotations extracted out of a total of 4189 annotations (about 3%). In the reconstruction phase, a candidate protein was included into the network, if all of three GO annotations (component/function/process) of the protein match to those in the annotation collection table. Then, the network was expanded by extracting physical interactions between the selected proteins (Figure 3.1). BioGrid [73] database release 3.1.73 was used to collect physical interactions between proteins included into the network. The resulting network (ETN – Ethanol Tolerance Network) was obtained by eliminating self-loops, the duplicated edges, and significantly small connected components.

Table 3.1. Core proteins of the network.

Core Proteins	Description	Reference
URA7	CTP synthase isozyme	[29]
LAP3 (*GAL6*)	Cysteine aminopeptidase	[29]
EDE1	Endocytic protein	[33]
ELO1	Elongase I (fatty acid elongation)	[33]
TPS1	Trehalose-6-phosphate synthase	[33]
MSN2	Transcriptional activator	[33,34]
DOG1	2-deoxyglucose-6-phosphate phosphatase	[34]
HAL1	Cytoplasmic protein involved in halotolerance	[34]
INO1	Inositol-3-phosphate synthase	[34]
OLE1	Delta(9) fatty acid desaturase	[32]
CYB5	Cytochrome b5	[30]
SFL1	Repression of flocculation-related gene	[30]
HSP26	Heat shock protein	[31]
RTC3	Involved in RNA metabolism	[31]

3.1.2.2. Network Tuning. The reconstructed network was statistically tuned using the eigenvector centrality (EVC) which is a topological metric representing the importance of a node in the network [74,75]. EVC, like degree centrality, identifies nodes that have high interactions with many other nodes in the network. However, in contrast to degree centrality, EVC specifically detects nodes that are connected to central nodes within the network. Thus, it takes into account the entire pattern of the network [74].

100 random networks were generated by preserving the degree of each node: Initially, two edges, (u, v) and (s, t), were randomly selected such that u, v, s, t are different nodes and (u, t), (s, v) are not currently edges. Then, these two edges, (u, v), (s, t), were deleted and (u, t), (s, v) were inserted to the network. So that, in 100 random networks, the topology in ETN was preserved in terms of node degrees, whereas the interacting protein pairs were not. EVC values of ETN and random networks were computed in MATLAB 2010a (MathWorks Inc.). For randomized networks, average values of EVC corresponding to each node were computed and a hypothesis testing was carried out for all nodes using two-tailed t-test with a confidence level of 99.99%. The null hypothesis was the EVC value of a node in ETN is equal to the average EVC value of a node in 100 random networks. This hypothesis represents that the information taken from the protein in ETN is the same as that in random networks and the protein is included to the network randomly. The alternative hypothesis was the EVC value of a node in ETN is not equal to the average EVC value of a node in 100 random networks. This hypothesis represents that the information taken from the protein in ETN is significantly different from that in random networks. Consequently, the proteins in ETN, which are significantly different from those in random networks, were identified.

3.1.2.3. Determination of Network Topology. Topological properties of the networks, such as degrees, betweenness centralities, diameter, average shortest path length and clustering coefficients were examined by Network Analyzer [76] plug-in of Cytoscape [77]. In order to define hub proteins, all nodes were ranked based on their degrees. Cummulative percentages determined from cumulative histogram of degrees of proteins were used to identify the hubs. The degree corresponding to 1% of difference between cumulative percentages was selected as threshold and proteins displaying degrees above this threshold were identified as hubs.

3.1.2.4. Module Identification and Functional Enrichment. The highly connected protein subgroups of ETN, tETN and the BioGrid network were identified via MCODE [78] plug-in of Cytoscape. In MCODE, loops were not included while scoring the networks and the degree threshold was set to 2. The node score threshold, K-core threshold, and maximum

depth were set to 0.2, 2 and 100, respectively. The fluff parameter was turned off and the hair-cut parameter was turned on.

The GO enrichment analysis of modules, having at least 5 members, were performed via BINGO [79] plug-in (v2.44) of Cytoscape [77]. The enrichment was evaluated by hypergeometric test using whole annotation as reference set; the multiple testing correction was made by Benjamini and Hochberg's false discovery rate (FDR) correction; and the significance level was chosen to be 0.0001.

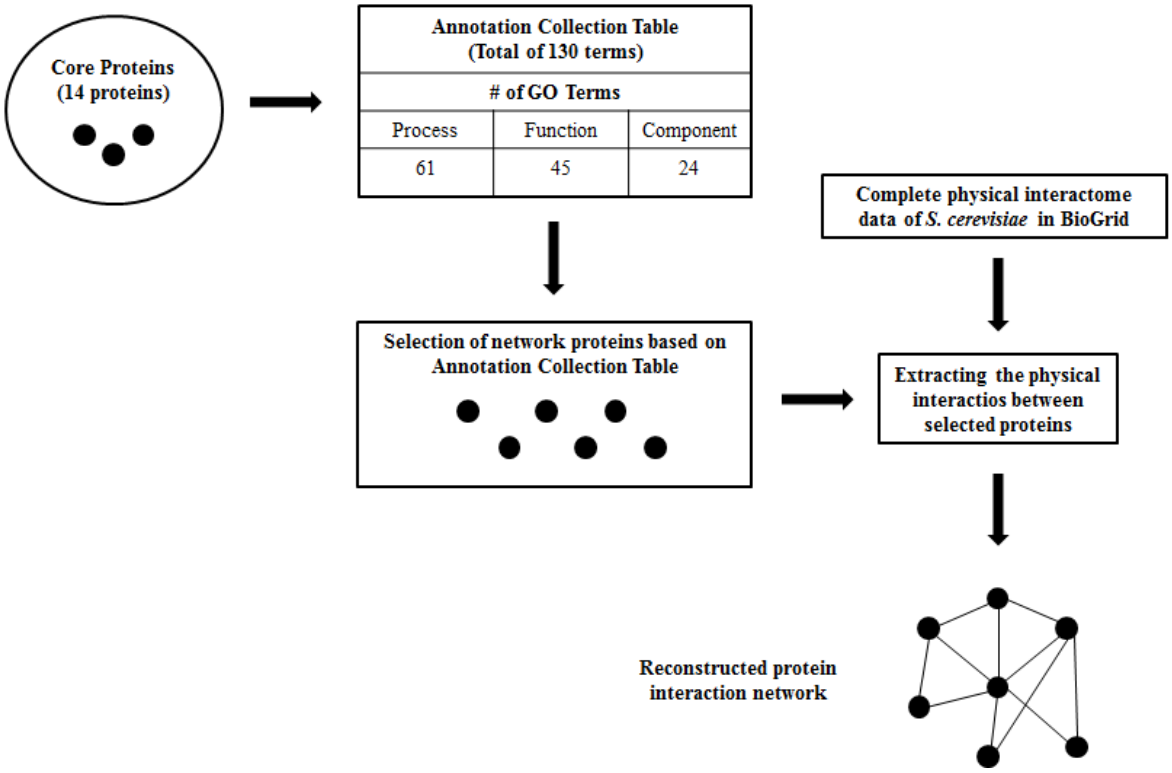

Figure 3.1. Schematic illustration of the network reconstruction algorithm, SPA.

3.1.2.5. Strains and Media. The homozygous single gene deletion strains used in this study (Table 3.2) were derived from *S. cerevisiae* BY4743, and obtained from EUROSCARF collection.

Precultures were inoculated with a single colony of cells taken from YPD agar plates and incubated in YPD medium at 30ºC and 180 rpm.

3.1.2.6. Determination of Ethanol Tolerance. The ethanol tolerances of deletion strains together with the wild type strain were determined by means of colony-forming ability and viability. 5 ml of YPD medium was inoculated with a final optical density at 600 nm of 0.05 (OD_{600} = 0.05) and was incubated at 30ºC under 180 rpm shaking for 24 h. In order to test the colony-forming ability of the cells, samples were taken from the liquid cultures, serially diluted and 5 µl of diluted samples were spotted onto a YPD agar plate containing 5, 7, 10% (v/v) ethanol at 30ºC for 48 h. All experiments were carried out in duplicate.

Table 3.2. Yeast strains used in this study.

Strain	Genotype
BY4743	MATa/MATα his3Δ 0/his3Δ 0; leu2Δ /leu2Δ 0; met15Δ 0/MET15; LYS2/lys2Δ 0; ura3Δ 0/ura3Δ 0
ydr307wΔ/ydr307wΔ	BY4743; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YDR307w::kanMX4/YDR307w::kanMX4
yhl042wΔ/yhl042wΔ	BY4743; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YHL042w::kanMX4/YHL042w::kanMX4
ymr215wΔ/ymr215wΔ	BY4743; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YMR215w::kanMX4/YMR215w::kanMX4
ypl264cΔ/ypl264cΔ	BY4743; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YPL264c::kanMX4/YPL264c::kanMX4
ydl123wΔ/ydl123wΔ	BY4743; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YDL123w::kanMX4/YDL123w::kanMX4

Viability of cells was determined by colony-forming unit (CFU) method [80]. *S. cerevisiae* strains were grown in 200 ml YPD medium. A preculture at a volume fraction of 1 % was used to inoculate the culture. After 10 h of incubation at 30ºC under 180rpm shaking, cells were treated with ethanol to have 8% (v/v) final ethanol concentration. A sample from the liquid culture was 1000 fold diluted and spread onto YPD agar plates

(four replicates: two biological and two technical) before ethanol treatment and 2, 4, 6, 8 h after ethanol treatment. Then, colony formation was monitored after 48 h incubation at 30ºC.

The effect of ethanol on the growth rates of ydr307wΔ/ydr307wΔ and yhl042wΔ/yhl042wΔ strains were analyzed by inoculating overnight cultures into YPD media supplemented with 8% ethanol. Maximum specific growth rates having 95% confidence intervals were determined from the optical densities of samples collected during the exponential phase of growth were used.

3.1.2.7. Determination of Maximum Specific Growth Rate and Dry Cell Weight. Maximum specific growth rates and the dry cell weights of cultures were determined as described in Section 2.1.2.4.

3.1.2.8. Extracellular Metabolite Analysis. Extracellular glucose and ethanol concentrations were determined by using enzymatic analysis kits (Sigma) as described by the manufacturer.

3.1.2.9. Statistical Analysis. A two-tailed t-test was performed for network tuning. A confidence level of 99.99% was used and all parameters were computed in MS Excel.

For the GO enrichment analysis, a hypergeometric test was performed via BINGO. Benjamini and Hochberg method was used to control the false discovery rate and the 0.0001 p-value treshold was maintained for the detection of enriched GO process terms.

To statistically describe the differences between strains, multiple comparisons of the viability profiles were carried out using ANOVA computed in MS Excel. 95% confidence intervals, calculated in MS Excel, were provided for the growth parameters.

3.1.3. Results and Discussion

3.1.3.1. Network Reconstruction and Tuning. SPA [69] was recruited in a non-iterative manner to reconstruct ETN. This algorithm was already used to integrate physical protein

interaction data with GO annotations, for the reconstruction of protein interaction networks related to a specific pathway, biological process or phenotype. Sub-networks related to various signaling pathways such as, glucose-induction signaling and high osmolarity signaling [69], sphingolipid metabolism [81], chronological aging [82] in *S. cerevisiae*, Wnt and Hedgehog signaling in *Drosophila melanogaster* [83], and insulin signaling [84] and Wnt signaling [85] in *Homo sapiens* were constructed via SPA.

A set of 14 proteins, (*URA7, LAP3, CYB5, SFL1, HSP26, RTC3, OLE1, EDE1, TPS1, ELO1, MSN2, DOG1, INO1, HAL1*) [29–34], were used as the seed for network reconstruction. Then, the network was expanded as described in the Methods section by using strict criteria based on GO annotation terminology. Two core proteins (*DOG1* and *HAL1*) were eliminated from the network due to the lack of physical interactions. The resulting network, having 1962 nodes and 7585 edges, was composed of 14 disconnected sub-networks. The largest connected component, having 1933 nodes and 7569 edges, was further investigated as the final network ETN.

Accuracy of PPI data is often criticized, since interactome data obtained from high-throughput experiments is mainly thought to have a large number of false positives, i.e. the interactions that are spurious and do not occur in the cell [69,86,87]. Therefore, in this study, the reconstructed network was further tuned statistically using a topological parameter, EVC. EVC is an indicator of the importance of a node within the topological arrangement in a graph [74,75]. For the randomized networks, in which the node degrees were preserved, average values of EVC corresponding to each node were computed and statistical tests were carried out for all nodes as described in the Methods Section 3.1.2.2. Briefly, a hypothesis testing was carried out to check whether the EVC value of the protein in ETN is significantly different from that in random networks. Following the hypothesis testing, 8% of the nodes in ETN were considered as statistically insignificant and eliminated from the network together with their interactions (7% of the interactions in ETN). 1783 proteins (among 1933 proteins) with differential EVC values were extracted and the resulted network (tETN) consisted of 7037 physical interactions between these proteins.

3.1.3.2. Topological Analysis of the Network. The topological analysis of the resultant networks (ETN and tETN) indicated that they have scale-free degree distributions following nearly a power law model, $P(k) \approx k^{-\gamma}$, having $\gamma = 1.57$ and $R^2 = 0.88$ for ETN and $\gamma = 1.59$ and $R^2 = 0.88$ for tETN (Figure 3.2). Since the distance measures, such as the network diameter (d) and characteristic path length (CPL), are orders of magnitude significantly smaller than the number of proteins (Table 3.3), both ETN and tETN have small-world properties characteristic to biological networks. Moreover, their topological parameters, such as diameter, characteristic path length and clustering coefficient (CC) were in consistence with other protein interaction networks published in literature (Table 3.3).

Table 3.3. Topological properties of protein interaction networks.

Model	**node #**	**edge #**	**d**	**CC**	**CPL**	**Reference**
ETN (*S. cerevisiae*)	1933	7569	10	0.166	3.7	this study
tETN (*S. cerevisiae*)	1783	7037	10	0.166	3.7	this study
Biogrid (*S. cerevisiae*)	5517	56035	6	0.245	2.7	this study
CAN (*S. cerevisiae*)	2359	12314	9	0.157	3.4	[25]
tCAN (*S. cerevisiae*)	1736	8458	9	0.167	3.4	[25]
Signalling (*S. cerevisiae*)	1363	3649	9	-	6.8	[23]
Wnt signalling (*H. sapiens)*	3489	10092	15	-	4.4	[28]

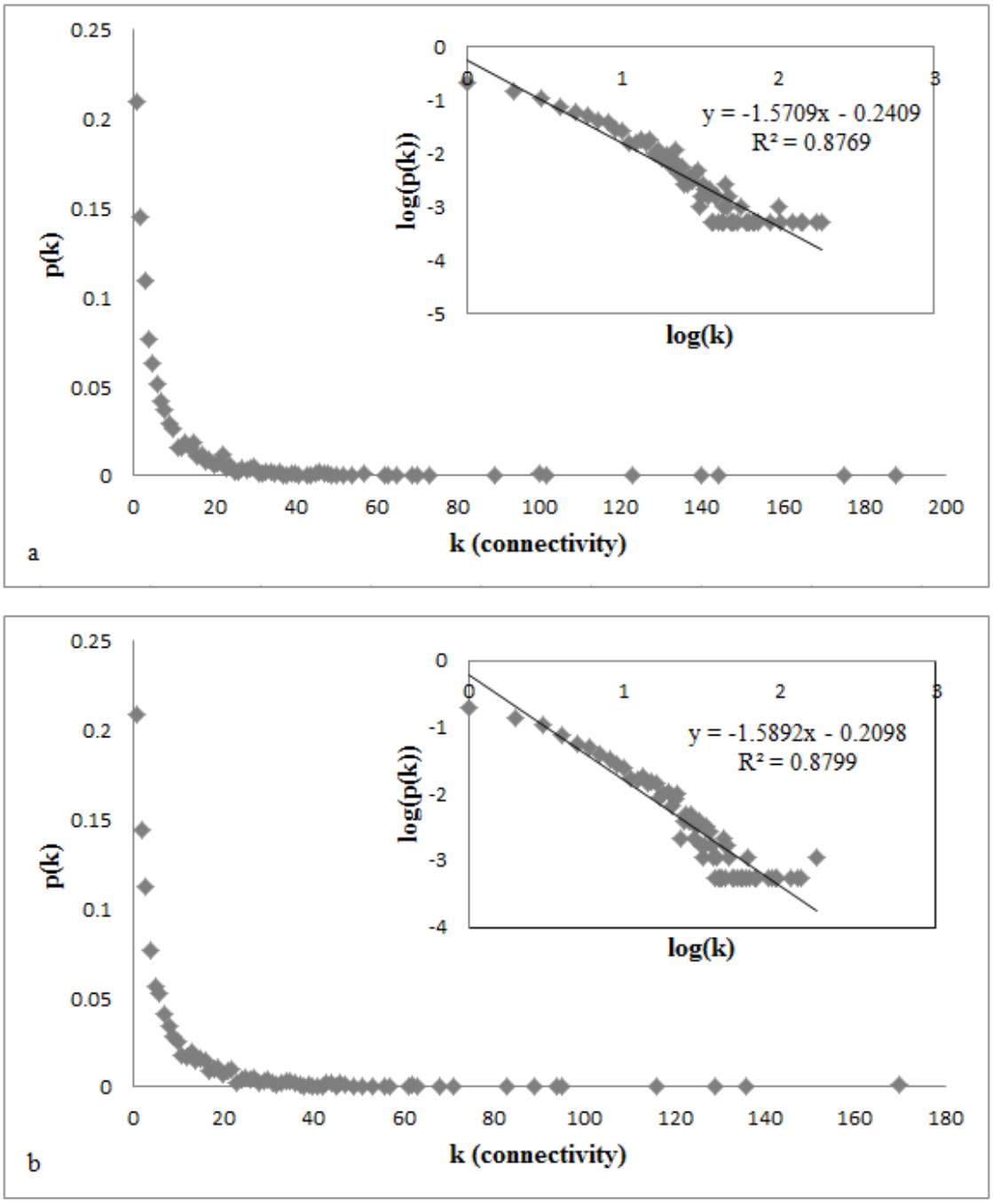

Figure 3.2. Connectivity distribution for the reconstructed networks (a) ETN (b) tETN.

In order to understand the difference between the reconstructed networks (ETN and tETN) and the BioGrid network that contained the whole PPI's in *S. cerevisiae*, the topological analysis of the BioGrid network was also carried out. The topological properties of BioGrid network were significantly different from ETN and tETN; the diameter and characteristic path length were 40% and 27% smaller, and the clustering coefficient was 48% higher than those of the ETN and tETN (Table 3.3).

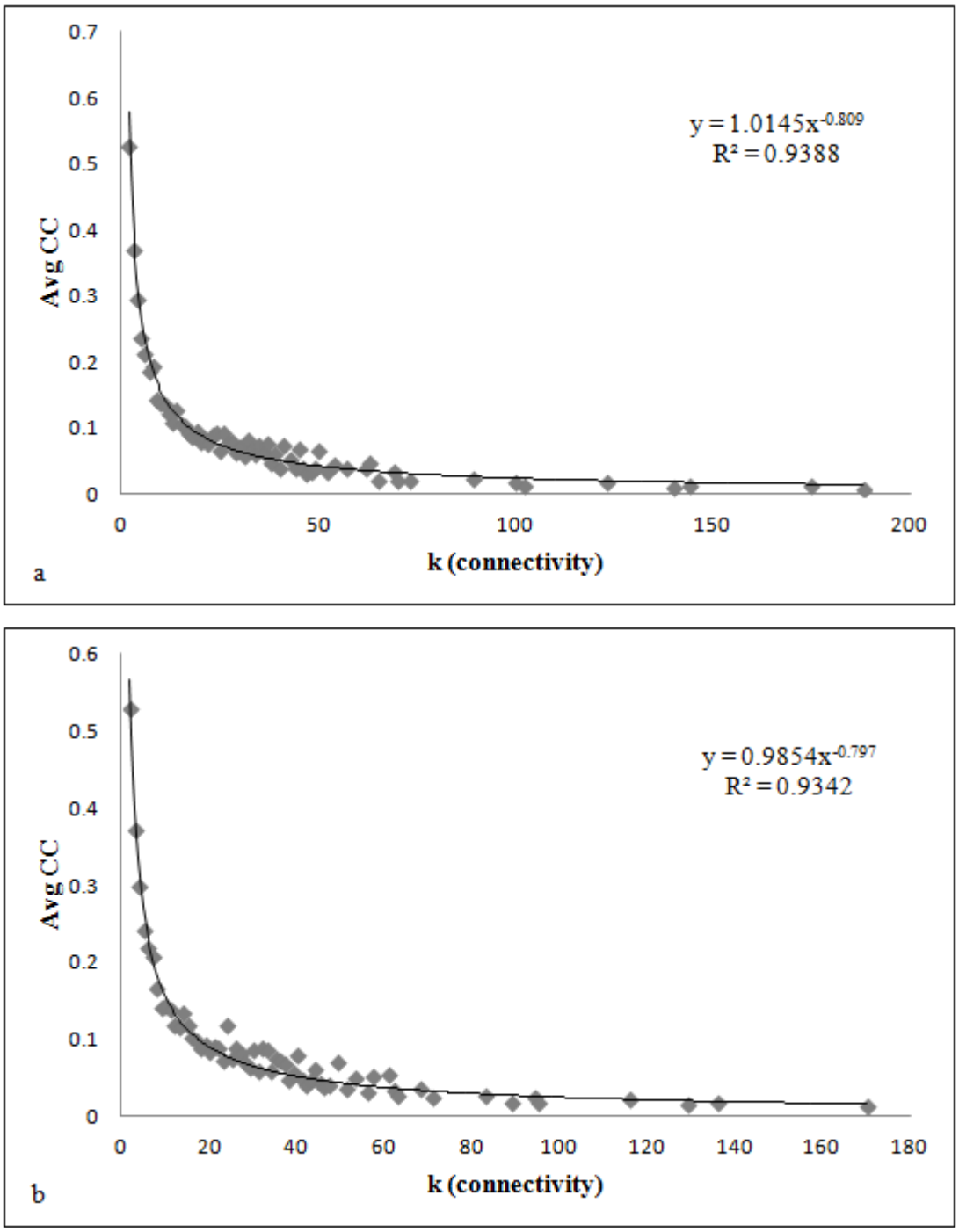

Figure 3.3. Average clustering coefficient distribution of (a) ETN (b) tETN.

Since the average clustering coefficient measures the possible modularity of the network [88–90], the average clustering coefficient curves were analyzed for ETN, tETN (Figure 3.3) and BioGrid network (Figure 3.4). For all reconstructed networks, the average clustering coefficient versus degree followed a power law distribution with $C(k) \approx k^{-w}$. The

analysis revealed that ETN and tETN were hierarchical networks having w = 0.80 which in turn was higher than those of the BioGrid network (w = 0.65). The hierarchical nature of ETN and tETN indicate that highly interconnected small clusters combine to form a larger group [89].

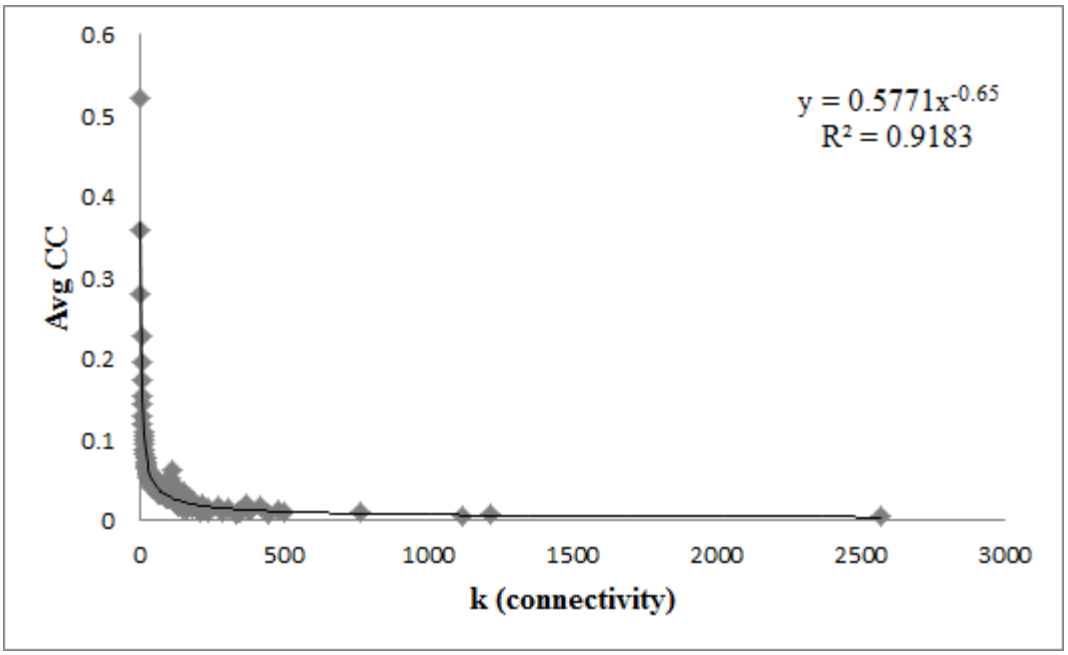

Figure 3.4. Average clustering coefficient distribution of Biogrid network.

Analysis of the topological properties of both ETN and tETN indicated that the network tuning prevented the inclusion of proteins into the network by chance alone and reduced the number of the proteins (Table 3.3) without changing the overall topology.

The first 48 and 39 of the highly connected nodes were identified as the hub proteins of the reconstructed ETN and tETN (Table A.2), respectively. Although their rankings were slightly different, identical hubs were identified both in ETN and tETN. Hubs identified in ETN (Table A.3) and tETN (Table A.4) were found to be significantly enriched with stress response, and regulation of biological and metabolic processes terms. Since the activation of several pathways including general stress response is required to survive under high concentrations of ethanol [91], the existence of stress response terms in the related processes of tETN hubs was not surprising. The hubs identified in the BioGrid network were only 36% identical to the hubs of tETN and they were significantly enriched with protein and phosphate processes.

3.1.3.3. Biological Processes Related to Ethanol Tolerance. Employment of genomic screens identified a huge number of genes associated with ethanol tolerance involving a broad range of functional categories including protein biosynthesis, amino acid metabolism, nucleotide metabolism, transport, cell cycle and growth, lipid metabolism, fatty acid and ergosterol metabolism, membrane and cell organization, proline biosynthesis, and tryptophan biosynthesis [51,92–96]. The GO enrichment analysis ($p < 0.0001$) of the ETN (Table A.5) and tETN (Table A.6) indicated that this complex picture has been fully reflected in the reconstructed network, tETN (Figure 3.5).

The GO enrichment analysis indicated significant association between ethanol tolerance and metabolic processes related to membrane stability. During fermentation, the accumulation of ethanol leads to growth inhibition and finally cell death by affecting the integrity of cell membrane and increasing the membrane fluidity. Since ethanol has relatively lower affinity for the cell membrane than other toxic alcohols, it accumulates at highest concentration and affects both membrane fluidity and functions. Studies showed that elevated ethanol tolerance may be reached by changing cell membrane composition to increase the membrane stability [54,97]. In order to antagonize the effect of ethanol by maintaining the membrane fluidity, the lipid content of plasma membrane changes in yeast. Especially, the changes in unsaturated fatty acids and ergosterol were found to be important in terms of membrane fluidity [32,54,98]. Since ergosterol increases the membrane rigidity, yeast strains having high ergosterol content in membranes are more tolerant to ethanol [99]. Genes involved in vacuolar function, membrane and cell wall composition, phospholipid biosynthesis, ergosterol biosynthesis and fatty acid metabolism were commonly observed in association with ethanol tolerance [51,63,96,100,101].

The observed relationship between ethanol tolerance and trehalose metabolic process (Figure 3.5) can also be explained by the role of trehalose on membrane stability. During ethanol stress, increase in trehalose prevents the aggregation of the misfolded proteins on the membrane and ultimately prevents the damage in the structure and functions of the membrane [54]. Under ethanol stress, the accumulation of trehalose was observed and yeast cells that cannot accumulate trehalose display retarded growth [51]. In the presence of ethanol the intracellular trehalose amount was found to be significantly higher in an ethanol tolerant strain than that of its parent strain [102]. Several studies comparing the

transcriptome profiles of wild type, ethanol tolerant and ethanol sensitive mutants also showed that the genes annotated to trehalose metabolism were differentially expressed under ethanol stress [50,101,103].

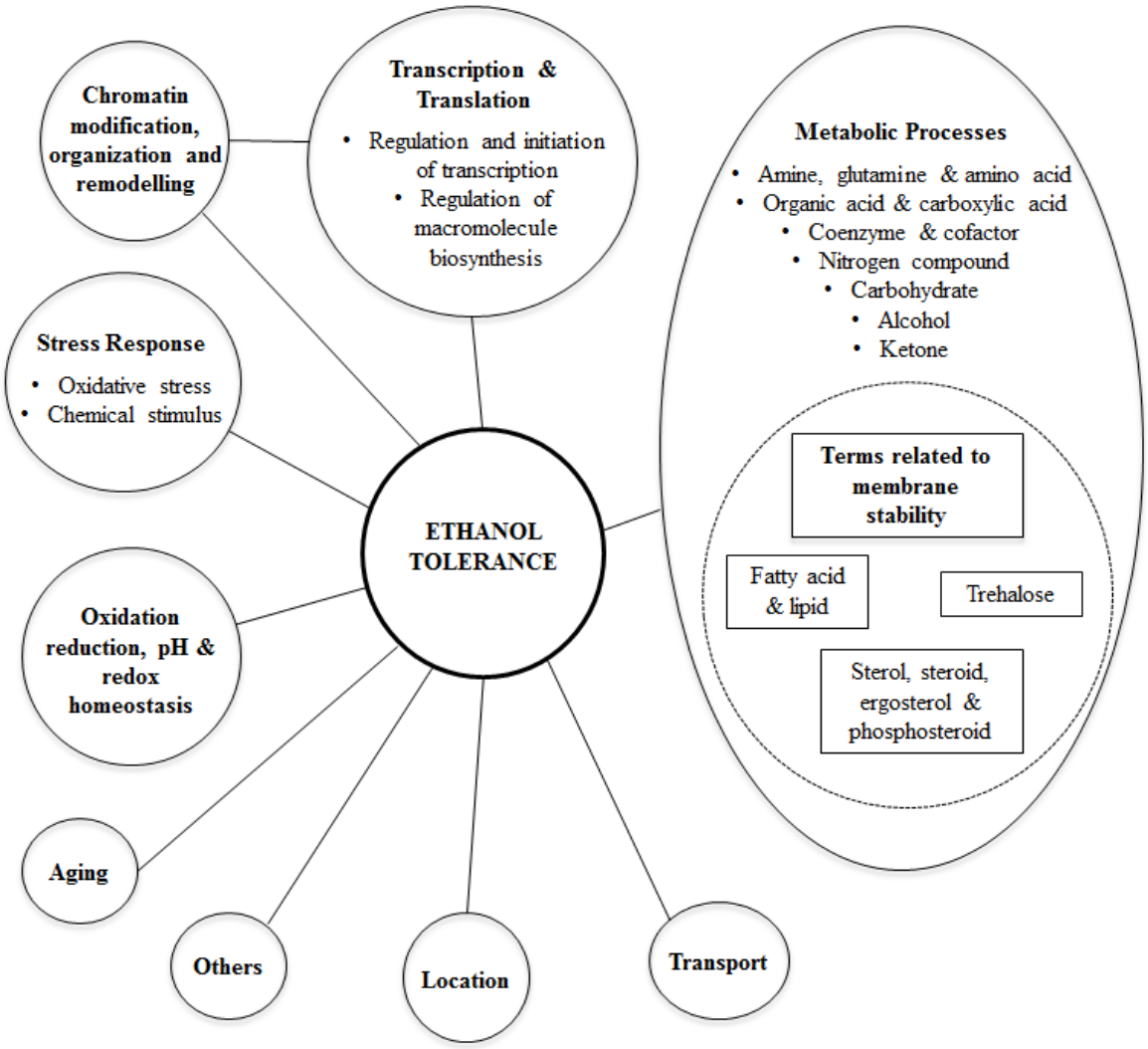

Figure 3.5. Schematic illustration of the significantly enriched processes occurred in ETN and tETN.

The observed link between ethanol tolerance and pH homeostasis in tETN may be explained by the increase in the proton influx and the intracellular acidification due to increase in membrane permeability under ethanol stress. Therefore, in order to neutralize the effect of ethanol and to maintain pH homeostasis, intracellular H^+ transport into vacuoles by H^+V-ATPase becomes important for yeast. The translocation of H^+ is

considered as a response to ethanol [51]. Genes encoding structural components of V-ATPase were observed in association with ethanol stress [30,51,63,96,100,101].

The GO enrichment analysis of the constructed network pointed to a relationship between coenzyme/cofactor metabolisms, redox and ethanol tolerance (Figure 3.5). In yeast metabolism, cofactors NADH and NADPH play important roles and they are used in many reactions involved in biosynthesis of amino acids, lipids, and nucleotides [51,104]. Most genes involved in NADH/NADPH regeneration reaction steps were up-regulated under ethanol stress [51].

Several studies comparing the transcriptome profiles of wild type, ethanol tolerant and ethanol sensitive mutants showed that the expressions of genes varied because of using different strains and ethanol concentrations. However, the stress response related GO terms was found to be affected by ethanol stress [50,103]. Stress response related GO terms were also found to be significantly enriched in the reconstructed network, tETN (Figure 3.5).

Transport, that was found to be significantly enriched in tETN, is one of the GO categories that is reported to be affected by ethanol stress [103]. Deletion of 17 genes involved in “cellular transport mechanism” caused ethanol sensitivity in yeast [101]. 15 mutations related to “vacuolar function and vesicular transport to the vacuole” showed growth deficiency under ethanol stress [63]. 254 genes, whose requirement for yeast resistance to ethanol was found by chemogenomics approach, were categorized based on GO Process terms and terms involved in transport were found to be among the most significant terms ($p < 0.01$) [96]. Moreover, 45 genes, associated with protein transport and vacuole, were found to be important for cell growth in the presence of 11% ethanol [100].

3.1.3.4. Modular Analysis. A modular approach was used for the prediction of the genes involved in ethanol tolerance and in order to understand the effect of network tuning both ETN and tETN were analyzed. 32 and 35 modules were identified within ETN and tETN, respectively.

Eighteen clusters of ETN, having at least five members, were further investigated and significantly enriched GO process terms were identified. No significantly enriched

term could be observed in six clusters (Cluster 11, 18, 19, 20, 21, and 22) among these 18 clusters. Moreover, seven clusters (Cluster 3, 5, 6, 7, 10, 12, and 23) were found to be significantly enriched with terms related to ethanol tolerance mechanism. The significantly enriched terms were found to be related to sterol, ergosterol, steroid and lipid processes in Cluster 3; histone and chromatin modification in Cluster 5; membrane, transport, localization, and lipid metabolism in Cluster 6; vitamin and glutamine metabolic processes in Cluster 7; transport, localization, pH reduction and regulation in Cluster 10; trehalose processes in Cluster 12; coenzyme, cofactor, and glutathione processes in Cluster 23. Within these seven clusters, a total of 19 proteins with unknown GO process term, were further analyzed by subjecting their first neighbors to GO enrichment analysis. Out of these, first neighbors of five gene products, namely, *YDR307W*, *YHL042W*, *YMR215W*, *YPL264C*, and *YDL123W*, were found to have significantly enriched terms related to ethanol tolerance.

Sixteen clusters of tETN, having also at least five members, were further investigated by means of enriched GO process terms. No significantly enriched GO terms could be assigned to three of these clusters (Cluster 11, 12, and 23). Moreover, seven clusters (Cluster 4, 5, 6, 7, 10, 13, and 24) were found to be significantly enriched with terms related to ethanol tolerance mechanism. The significantly enriched terms were found to be related to sterol, ergosterol, steroid, lipid processes, and transport in Cluster 4; histone and chromatin modification in Cluster 5; chromatin modification and repair in Cluster 6; vitamin and glutamine metabolic processes in Cluster 7; transport, localization, pH reduction and regulation in Cluster 10; trehalose processes in Cluster 13; coenzyme, cofactor, and glutathione processes in Cluster 24. Further GO enrichment analysis of the first neighbors of 17 proteins with unknown GO process term within these seven clusters pointed to four gene-products (*YDR307W*, *YHL042W*, *YMR215W*, and *YPL264C*) whose first neighbors were enriched with ethanol tolerance related terms (Figure 3.6). *YDR307W* (*PMT7*) is a putative mannosyltransferase similar to Pmt1p with a potential role in protein O-glycosylation [105]; *YHL042W* is a putative protein of unknown function, member of the DUP380 subfamily [106,107]; *YMR215W* (*GAS3*) is a low abundance, possibly inactive member of the GAS family of GPI-containing proteins, a putative 1,3-beta-glucanosyltransferase with similarity to other GAS family members [108–111]; *YPL264C* is a putative membrane protein of unknown function [112,113]. The identified four gene

products were novel, and they have no reported experimental evidence indicating that they are associated with ethanol tolerance before this study.

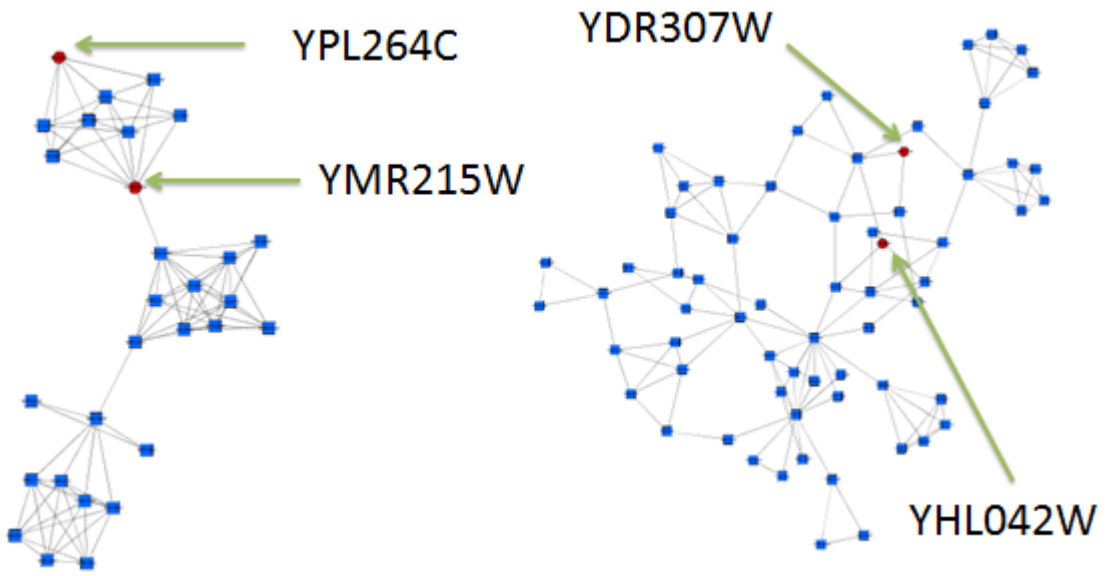

Figure 3.6. The clusters of four gene-products (*YDR307W*, *YHL042W*, *YMR215W*, and *YPL264C*) whose first neighbors were enriched with ethanol tolerance related terms.

3.1.3.5. Ethanol Tolerances of *S. cerevisiae* Strains. As a result of the modularity analysis of tETN, four gene-products (*YDR307W, YHL042W, YMR215W, and YPL264C*) were identified as candidate gene targets to study ethanol tolerance. In order to test the possible unwilling elimination of proteins during network tuning, the ydl123wΔ/ydl123wΔ strain, carrying the deletion for an eliminated gene-product, was also used as control besides the wild-type strain.

The ethanol tolerances of deletion strains together with the wild type strain were evaluated as described in Methods Section 3.1.2.6. The colony-forming ability of all strains in the presence of varying amounts of ethanol was tested by culture on solid media. For all strains, no significant changes in colony-forming patterns could be detected in media containing 5% (v/v) ethanol. However, deletion strains showed significant colony-forming ability in media containing 7 and 10% (v/v) ethanol. 10^6 fold diluted cultures of strains carrying the *YDR307W* deletion, and 10^5 fold diluted cultures of strains carrying the *YHL042W* deletion showed colony formation, whereas only 10^4 fold diluted cultures of

wild-type strains and strains carrying the *YPL264C*, *YDL123W*, and *YMR215W* deletions showed colony formation in media containing 10 % (v/v) ethanol (Figure 3.7).

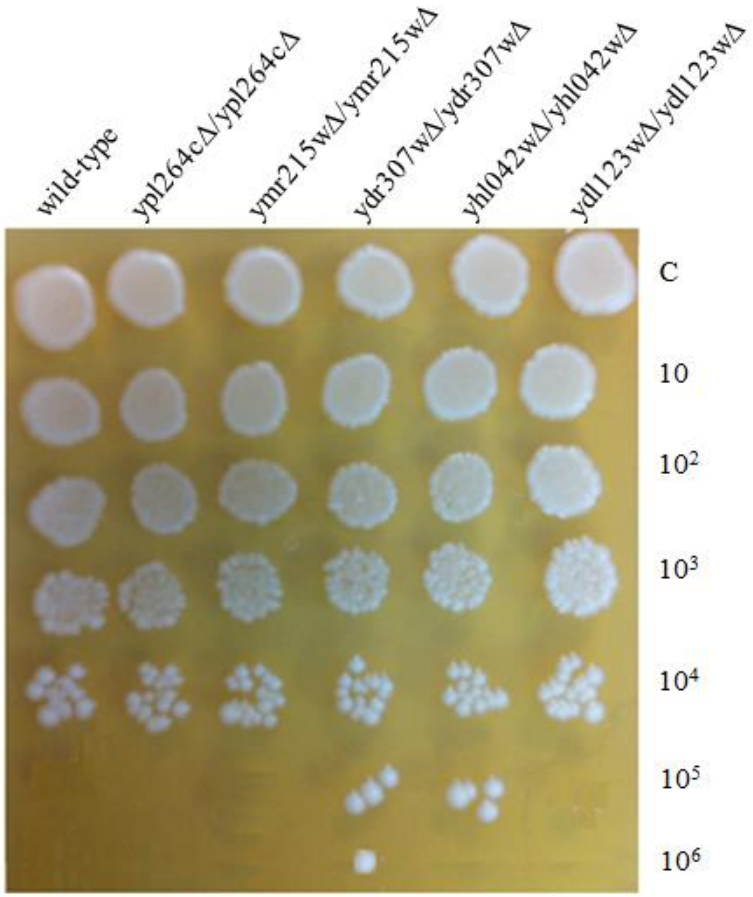

Figure 3.7. Colony-forming ability of undiluted (C), 10 to 10^6 fold diluted *S. cerevisiae* cultures grown in YPD and then spotted on YPD agar containing 10% (v/v) ethanol.

Table 3.4. The maximum specific growth rates of the wild strain BY4743 in media containing 0-10% ethanol (v/v).

% volume fraction of ethanol in YPD	**μ_{max} (h^{-1})**
0	0.4381±0.0025
5	0.2196±0.0042
6	0.1372±0.0071
8	0.0778±0.0082
10	0.0036±0.0005

In order to determine the maximum allowable ethanol concentration, growth profiles of the wild-type strain BY4743 were investigated in YPD media supplemented with varying amounts of ethanol. Increasing ethanol concentrations resulted in an expected decrease in the growth rate ($p = 5x10^{-5}$) so that the maximum specific growth rates were 0.4381 ± 0.0025 h^{-1} and 0.0778 ± 0.0082 h^{-1} in media containing 0% (v/v) and 8% (v/v) ethanol, respectively (Table 3.4). Since the growth was very poor and at undetectable levels in the presence of 10% (v/v) ethanol (Figure 3.8), 8% (v/v) ethanol was selected as the best ethanol concentration to test its toxic effect on the cultures.

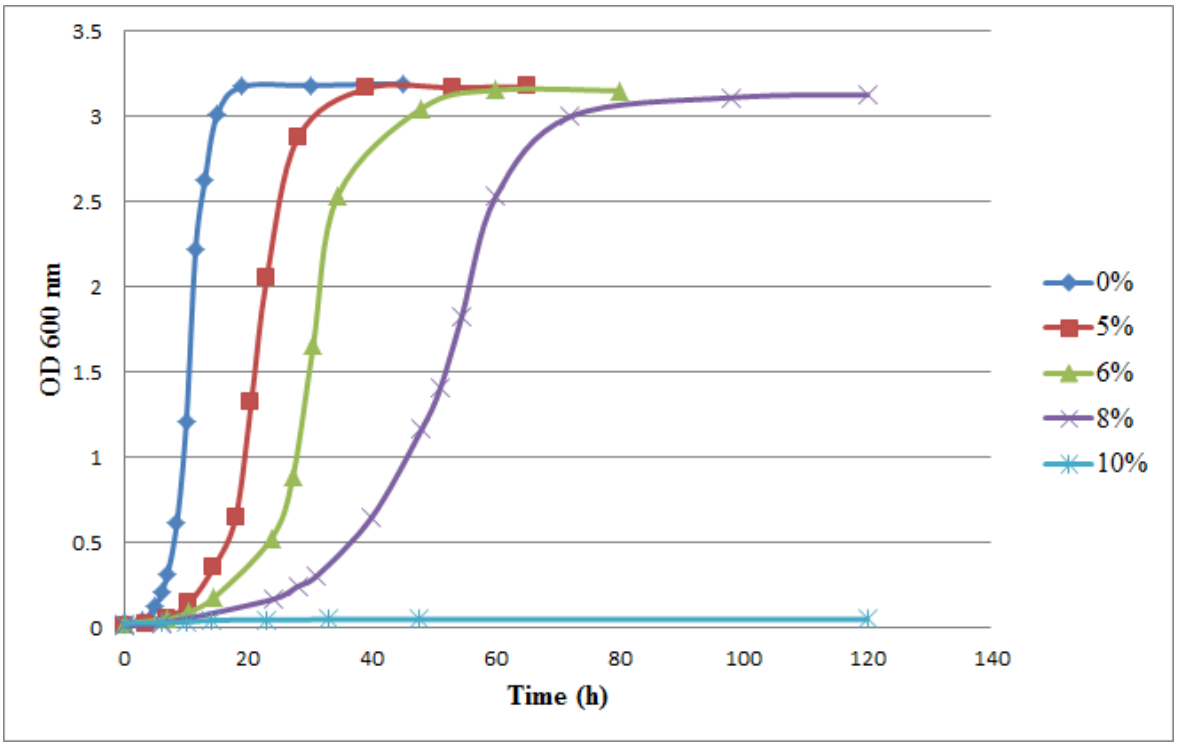

Figure 3.8. Growth profiles of the wild-type strain in the presence of increasing concentrations ethanol.

In this study, the viability of cells was investigated by CFU method after ethanol treatment. Relative viability (Figure 3.9) was calculated as the ratio of CFU at time t to CFU at time 0 (just before ethanol treatment). All deletion strains showed 1.2 - 2.5 fold higher viability than that of the wild-type strain 2 h after ethanol treatment, but strains carrying the *YPL264C*, *YMR215W* and *YDL123W* deletions showed 0.5 - 0.9 fold less viability than the wild-type strain in other time points. On the other hand, strains carrying the *YDR307W*, and *YHL042W* deletions had 1.3 – 1.9 fold higher viability than that of the

wild-type strain in all time points. The viability profiles of wild-type strain, and *YDR307W* and *YHL042W* deletion mutants (Figure 3.9) were analyzed by ANOVA and highly significant differences in viability were observed between the strains ($p < 0.005$). These results clearly showed that the gene products *YDR307W* and *YHL042W* were indeed associated with ethanol tolerance in *S. cerevisiae*.

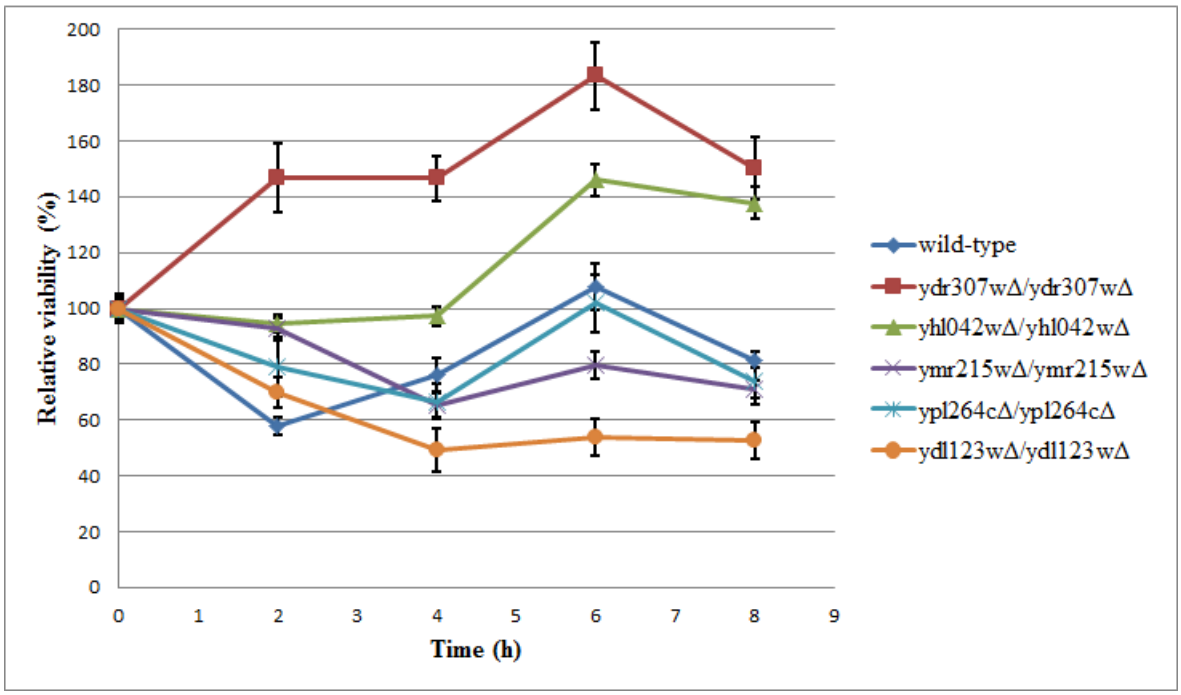

Figure 3.9. Relative viability (ratio of CFU at time t to the initial CFU at the time of ethanol treatment) of *S. cerevisiae* strains.

Improved viability of the mutant strains was further supported by comparing the growth profiles of the suspension cultures with those of the wild type strain. In the presence of 8% (v/v) ethanol, the maximum specific growth rate of strains carrying *YDR307W* and *YHL042W* deletions were 0.0859±0.0076 h^{-1} and 0.0824±0.0032 h^{-1}, respectively. Although, no significant differences were observed in growth rates, the mutants reached about 12-14% higher biomass concentrations ($p < 0.001$) than the wild-type strain at the stationary phase (Figure 3.10).

In a recent relevant study on breeding ethanol-tolerant *S. cerevisiae* strains via genome reconstruction, loss of an artificial chromosome containing *YHL042W* and fourteen other genes was found to result in ethanol-tolerant phenotype [56]. This and

similar reports definitely call for further and more detailed investigations on the role of each gene in the observed phenotype. By reporting these two genes as targets for the first time in literature, this study could also provide significant information in developing strategies for such strain improvement efforts.

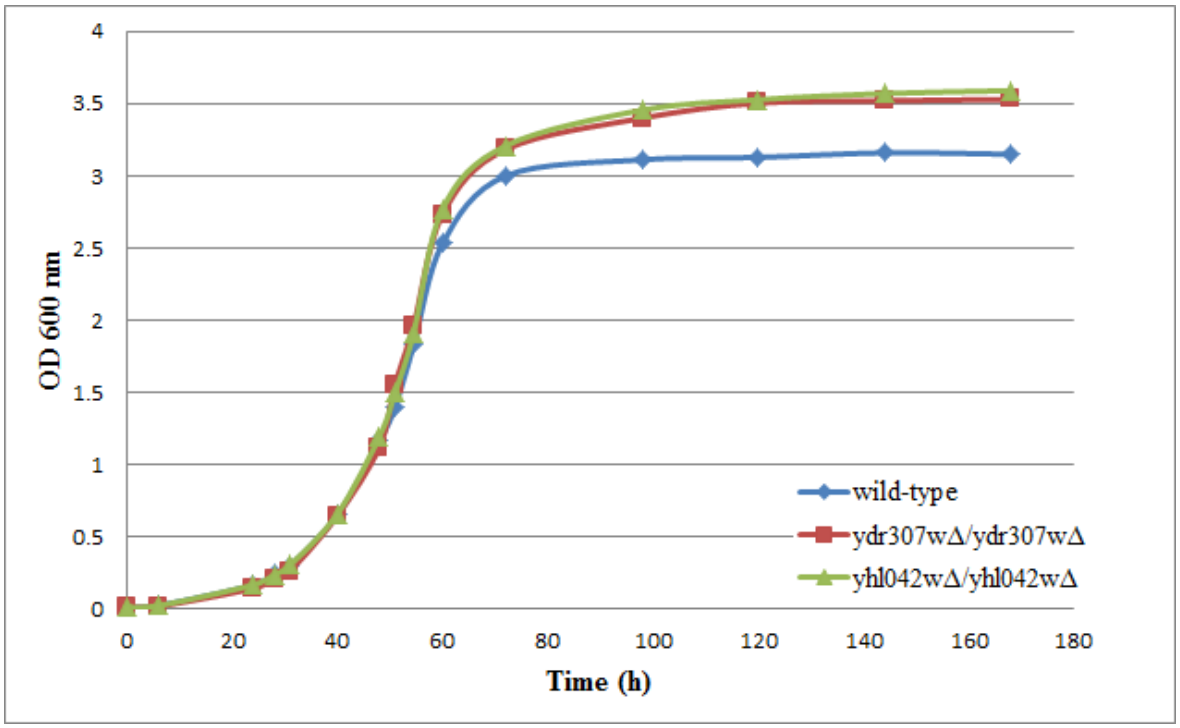

Figure 3.10. Growth profiles of *S. cerevisiae* strains in media containing 8% (v/v) ethanol.

The fermentation performances of the mutant strains together with the wild-type strain, grown on YPD medium, were investigated and compared to understand the effect of ethanol tolerance phenotype in ethanol productivity and yield. The biomass and metabolite concentrations were determined from the samples collected throughout the exponential and stationary phase of growth.

Maximum biomass and ethanol concentrations reached by the cultures, as well as utilized glucose amount were measured and ethanol yield on glucose (Y_{ps}) and biomass (Y_{px}) were also determined for each strain (Table 3.5). Improved ethanol tolerances of mutants resulted in an expected increase in ethanol concentration values, so that the maximum ethanol concentrations reached by ydr307wΔ/ydr307wΔ and yhl042wΔ/yhl042wΔ strains were 7.76 gL^{-1} ($p = 3.6x10^{-3}$) and 8.08 gL^{-1} ($p = 4x10^{-4}$),

respectively. Hence generally, the both mutants were found to stand out with their higher ethanol yields in terms of glucose consumed (Table 3.5).

Table 3.5. Fermentation parameters of *S. cerevisiae* strains.

Parameter	**Strains**		
	wild-type	**ydr307wΔ/ydr307wΔ**	**yhl042wΔ/yhl042wΔ**
Final DCW (gL^{-1})	3.02±0.22	3.21±0.34	3.58±0.17
Max. Ethanol Conc. (gL^{-1})	7.15±0.61	7.76±0.47	8.08±0.52
Total Glucose Utilized (gL^{-1})	18.52	18.72	18.96
Y_{ps} (g ethanol g^{-1} glucose)	0.384	0.415	0.426
Y_{px} (g ethanol g^{-1} biomass)	2.383	2.425	2.244

3.1.4. Concluding Remarks

There is an ever-growing interest in research towards understanding the response of *S. cerevisiae* to ethanol stress. However, due to its highly complicated nature, ethanol tolerance mechanism has still not been well understood and hence, PPI network associated with ethanol tolerance may greatly facilitate to gain an insight on the roles of genes under stressed conditions. Therefore, in the current study, a network based modular approach was developed to identify candidate genes associated with ethanol tolerance by estimating their relative importance in the ethanol tolerance related modules of the network. The modular analysis of the network revealed the presence of four gene-products having potential association with ethanol tolerance. The homozygous single gene deletion strains were used to understand the effect of these gene products on ethanol tolerance. The strains carrying the *YDR307W* (a putative mannosyltransferase similar to Pmt1p) and *YHL042W* (a putative protein of unknown function) deletions displaying a higher viability than that of the wild type strain to ethanol treatment indicated that these genes may possibly have a role in the response mechanism to ethanol in *S. cerevisiae*. Moreover, ydr307wΔ/ydr307wΔ and yhl042wΔ/yhl042wΔ strains showed significantly improved colony-forming abilities in

media containing 7 and 10% (v/v) ethanol. The ethanol tolerant phenotypes of these mutants also resulted in increased ethanol production yields. Although deletion mutants of the other two genes did not display any ethanol tolerance, the effect of the overexpression of these genes needs to be further investigated. An integrative system level investigation would possibly shed light into the molecular mechanism of the response to ethanol and the precise role of these genes in ethanol tolerance in *S. cerevisiae*.

3.2. Investigation of the Transcriptional Response to Ethanol Stress in *Saccharomyces cerevisiae*

3.2.1. Background Aspects

There is an escalating urge for ethanol production from renewable biomass as an alternative to fossil fuels [51,114]. Since *S. cerevisiae* is an efficient ethanol producer among various fermentative microorganisms, it has been extensively used in industrial ethanol production processes. However, *S. cerevisiae* is sensitive to high concentrations of ethanol [51]. During alcoholic fermentation, ethanol concentration can achieve levels that may lead to fermentation arrest and reduced ethanol yields [52]. Ethanol accumulation inhibits microorganism growth and viability, affects the fluidity of cell membrane by changing membrane lipid composition [51,54,61], disturbs cellular proteins, nucleic acids, and ATPase activity [115], changes vacuolar morphology [116], inhibits glucose and maltose uptake, amino acid metabolism [59] and hence the product formation. Although the impact of ethanol stress has been commonly reported, the molecular mechanisms of ethanol tolerance and toxicity still remain unclear [50]. Therefore, investigation of the response to ethanol at different omic levels may provide a better understanding of the molecular mechanisms underlying yeast tolerance to ethanol.

In order to tolerate stress conditions, yeast cells respond to these conditions by counteracting the deleterious effects to avoid growth disadvantage or even death. The ability of yeast cells to defend and adapt themselves to the environmental changes is important for cell survival [114,117]. During the course of evolution, yeast has developed particular stress response machinery consisting of various repair and protection

mechanisms. This response can be followed by measuring for example transcript, protein or metabolite abundances, metabolic activity, growth or cell morphology.

DNA microarray has been extensively used to investigate the global transcriptional changes after exposing yeast cells to ethanol [93,95,102]. Several studies have revealed changes in the expression of genes associated with general stress response, transport, energy, lipid metabolism, fatty acid and ergosterol metabolism, trehalose metabolism, ion homeostasis, protein synthesis, tryptophan biosynthesis, cell wall organization and certain amino acid biosynthesis upon ethanol stress [12,51,93,95,100,102,103,118].

The ethanol stress response mechanism of *S. cerevisiae*, that involves many genes, proteins and metabolites, is very complicated [52,54,67,96]. Hundreds of genes having a broad range of functional categories and biological processes were identified related to ethanol tolerance using different approaches including inverse metabolic engineering [34], deletion library screening [29,30], and investigation of the response to ethanol at various omics levels [12,33,51]. However, multi-functionality of the identified genes complicates the establishment of the relationship between the gene function and its effect on ethanol tolerance [51]. Although these studies provided vast knowledge into the molecular mechanism of ethanol tolerance, further investigations on the genome-wide transcriptional response of ethanol tolerant mutants together with their parental strains is needed in order to improve our understanding of yeast tolerance to ethanol [103].

Within the framework of this thesis we have used a network approach to identify candidate genes associated with ethanol tolerance by estimating their relative importance in the ethanol tolerance related modules of a protein-protein interaction network that was reconstructed by integrating protein-protein interaction data with gene ontology vocabulary. The modular analysis of the network identified *PMT7* and *YHL042W* as ethanol tolerance genes and mutants carrying *PMT7* and *YHL042W* deletions were found to exhibit improved tolerance to ethanol. In the light of this, further studies were initiated to systematically investigate the transcriptional response to gene deletions of two formerly identified ethanol tolerance genes, *PMT7* and *YHL042W*.

The aim of this study was to shed light into the molecular mechanism of yeast response to ethanol and to understand the precise role of these genes in ethanol tolerance. For this aim, the responses of two ethanol tolerant mutants carrying *PMT7* and *YHL042W* deletions and their parental strain to ethanol stress were investigated at genome scale. Gene expression profiles of *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains, with their parent, *S. cerevisiae* BY4743, were compared in the presence and absence of ethanol stress.

3.2.2. Methods

3.2.2.1. Strains and Media. The strains used in this study were the diploid laboratory strain *S. cerevisiae* BY4743 (MATa/MATα his3Δ 0/his3Δ 0; leu2Δ /leu2Δ 0; met15Δ 0/MET15; LYS2/lys2Δ 0; ura3Δ 0/ura3Δ 0), and two homozygous single gene deletion mutant strains (*pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ) derived from parent BY4743. All strains were obtained from EUROSCARF collection.

Precultures were inoculated with a single colony of cells taken from yeast extract-peptone-glucose (YPD) agar plates and incubated in YPD medium at 30ºC and 180 rpm.

3.2.2.2. Cultivation Conditions and Sampling. Identification of the ethanol tolerant mutants used in this work was described in Section 3.1. *S. cerevisiae* strains were grown in YPD medium with working volumes of 250 ml in 1 L flasks having screw caps. A preculture at a volume fraction of 1% was used to inoculate the culture. Cultures were kept with continuous shaking at 30ºC and 180 rpm up to the mid-exponential phase of growth ($OD_{600} \approx 0.8$-0.9). Then the exponential cultures were divided into two 500 ml flasks and cells in the first flask were grown without ethanol, whereas cells in the latter flask were treated with ethanol to have 8% (v/v) final ethanol concentration. All experiments were carried out in triplicate. Preliminary screening studies conducted within the framework of this study showed that 8% (v/v) was a non-lethal ethanol concentration that maintained the yeast cell growth and hence this level, defined as the maximum allowable ethanol concentration, was chosen for the treatments.

In order to analyze the transcriptional response of cells to *PMT7* and *YHL042W* gene deletions, samples taken at the mid-exponential phase of growth before ethanol treatment,

were used. On the other hand, samples were collected 2 h after ethanol treatment from both treated and untreated cultures to identify the transcriptional response of each strain to ethanol stress. Samples harvested for the transcriptome analysis were immediately frozen in liquid nitrogen and were stored at -80^{o}C until RNA isolation. M represents samples taken at the mid-exponential phase of growth from cultures grown in YPD without ethanol. E0 and E8 represent samples taken 2 h after ethanol treatment from untreated and treated cultures, respectively.

3.2.2.3. RNA Isolation and Microarray Analysis. RNA isolation was carried out in a robotic workstation, QIAcube (Qiagen, USA) using the enzymatic lysis protocol as described by Qiagen RNeasy mini kit (Cat no: 74106). The quality and quantity of the isolated RNA were checked via spectrophotometric analysis using UV-vis spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific Inc., USA). RNA samples were subjected to second quality check step before used in microarray analysis. RNA integrity number (RIN) values were checked using a microfluidics-based platform (Bioanalyzer 2100 Agilent Technologies, USA) using RNA6000 Nanokit (Agilent Technologies, USA) and samples with RIN values 7-10 were processed.

First-strand cDNA was synthesized and then converted into a double-stranded DNA initially from 100 ng of total RNA using GeneChip® 3' IVT Express Kit (Affymetrix Inc., USA). This double stranded cDNA was used as a template for *in vitro* transcription and synthesis of biotin-labelled aRNA. The final product was purified and quantified using the Nanodrop spectrophotometer before fragmentation. The purification and fragmentation steps were carried out using GeneChip reagents. Fragmented aRNA was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Affymetrix Yeast 2.0 arrays were prepared for hybridization using the reagents supplied in the GeneChip® Hybridization, Wash, and Stain Kit. A total of 5 µg of aRNA was loaded onto 169 format arrays and hybridized for 16 hours. The chips were then loaded into a fluidics station for washing and staining using Affymetrix Command Console® Software (AGCC) 3.0.1 Fluidics Control Module with Mini_euk2v3. Finally, the chips were loaded onto the Affymetrix **GeneChip Scanner 3000**. All applications were performed as described in the Affymetrix GeneChip®Expression Analysis Technical Manual.

3.2.2.4. Microarray Data Acquisition and Analysis. For the analysis of transcriptomics data, CEL files were normalized via quantile normalization using RMA [119] as implemented in the *affy* package [120] of R/Bioconductor suite of tools [121]. Significantly expressed genes were identified from the normalized log-expression values using the multiple testing option of LIMMA (linear models for microarray data) [122]. Benjamini Hochberg's (BH) method was used to control the false discovery rate (FDR) and the 0.005 adjusted p-value (q-value) threshold was maintained to identify significantly expressed genes. Statistically significant genes were used as inputs for gene set enrichment analysis based on GO annotations. All gene ontology analysis was performed using the Amigo software [123]. The Gene Ontology (GO) enrichment analysis was performed via GO Term Enrichment tool in Amigo by using *Saccharomyces* Genome Database (SGD) filter, with a maximum p-value of 0.01 and minimum number of gene products of 2. Functional categories of significantly expressed genes were determined using MIPS database [124], with a maximum p-value of 0.05.

3.2.2.5. Clustering of Conditions. The normalized microarray data was used to cluster the conditions. PCA was carried out with the PLS Toolbox of MATLAB 2010a by using the transcriptome data as variables and the conditions as the objects, as described by Wold et al. [27]. The hierarchical clustering of the conditions was carried out using Hierarchical Clustering Explorer (HCE) 3.5 [125] with the distance metric and the linkage metric selected as the Pearson correlation and the average linkage, respectively.

3.2.2.6. Reporter Features Analysis. The transcriptional response of *S. cerevisiae* strains to both gene deletions and ethanol stress was analyzed using Reporter Features analysis [126,127] implemented in BioMet Toolbox [128]. Reporter Feature analysis was carried out in the context of both regulatory networks and PPI networks. To reconstruct yeast regulatory network, the transcription factors (TFs) were connected to the genes known to be effected by these TFs. The TF-gene interactions were extracted from Yeastract [129–131] within the interactions considering documented regulations supported by direct evidence, and used to identify Reporter TFs. tETN, the PPI network that has the potential to predict candidate proteins related to ethanol tolerance in *S. cerevisiae*, was used to identify Reporter proteins. The 0.01 p-value threshold was maintained to determine both Reporter TFs and Reporter Proteins.

3.2.3. Results and Discussion

3.2.3.1. Experimental Design to Investigate the Global Transcriptional Response of *S. cerevisiae* Strains to *PMT7* and *YHL042W* Gene Deletions and Ethanol Stress. The aim of this study was to provide a further insight into the mechanisms of ethanol tolerance and stress in *S. cerevisiae*. A 3 x 2 factorial design was used to reveal the genome-wide transcriptional response to ethanol stress. The two parameters of interest were ethanol at two levels, 0% (v/v) and 8% (v/v); and strain at three levels, the reference strain BY4743, *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains. The experimental setup also enabled to understand the global transcriptional response of cells to gene deletions of *PMT7* and *YHL042W*. For this purpose, samples taken at the mid-exponential phase of growth from untreated cultures, and samples from ethanol treated and untreated cultures 2 h after the ethanol treatment, were used.

3.2.3.2. Clustering of Conditions. PCA clustered the conditions into three major groups according to both growth and ethanol level (Figure 3.11). Under conditions that represented the mid-exponential phase of growth in media without ethanol, all strains clustered together indicating an insignificant effect of *PMT7* and *YHL042W* gene deletions at transcriptional level. Under conditions that represented the growth of untreated cells taken from cultures 2 h after the mid exponential phase, wild-type and *pmt7*Δ/*pmt7*Δ strain clustered together, whereas yhl042wΔ/yhl042wΔ strain formed a separate cluster. This indicated that yhl042wΔ/yhl042wΔ strain showed a different transcriptional response from both other strains under unstressed growth conditions. On the other hand, ethanol treatment led wild-type strain, *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains to form separate clusters, indicating the different transcriptional responses of *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains to ethanol stress (Figure 3.11).

In order to identify the differences of *S. cerevisiae* strains in response to both gene deletions and ethanol stress, hierarchical clustering of the transcriptome of the control strain and of the mutant strains; *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains were comparatively investigated. The hierarchical clustering of conditions resulted in three major groups according to both growth and ethanol level (Figure 3.12) as revealed with PCA.

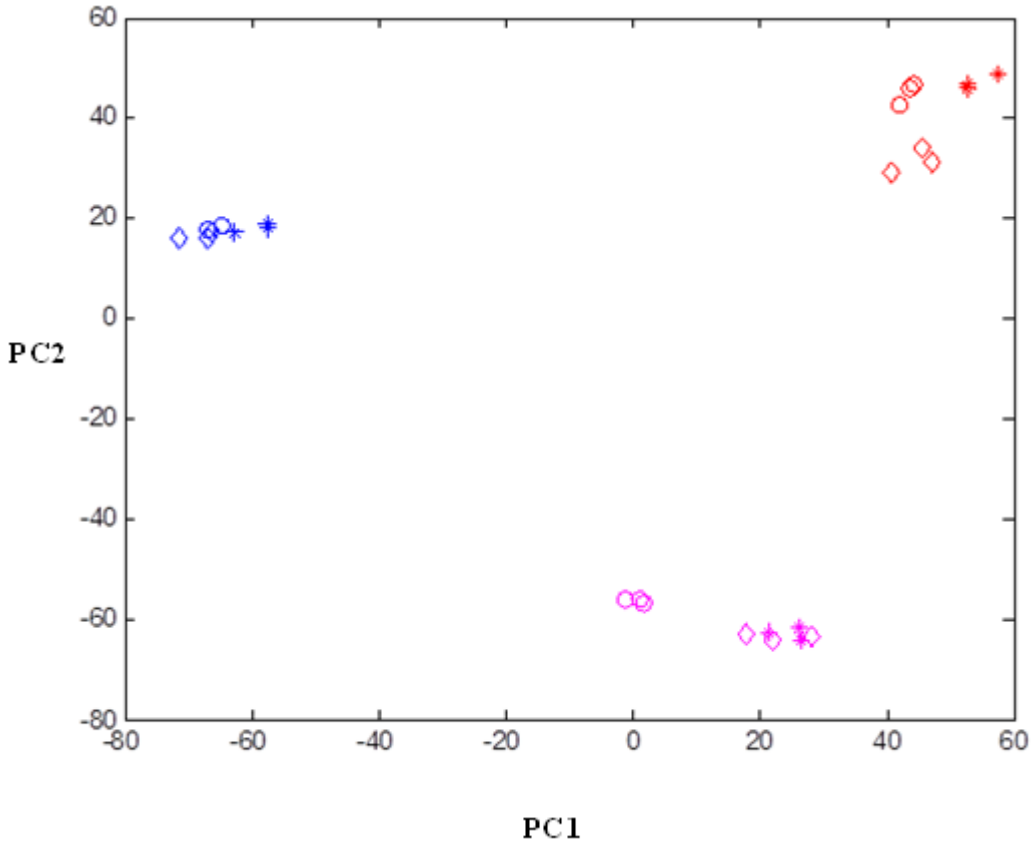

Figure 3.11. PCA plot of transcriptome data; the 75% of the total variance was explained by first and second PCs; star, diamond, circle represent wild-type, *pmt7*Δ/*pmt7*Δ, yhl042wΔ/yhl042wΔ strains; blue, magenta, red represent M, E0, E8 data, respectively.

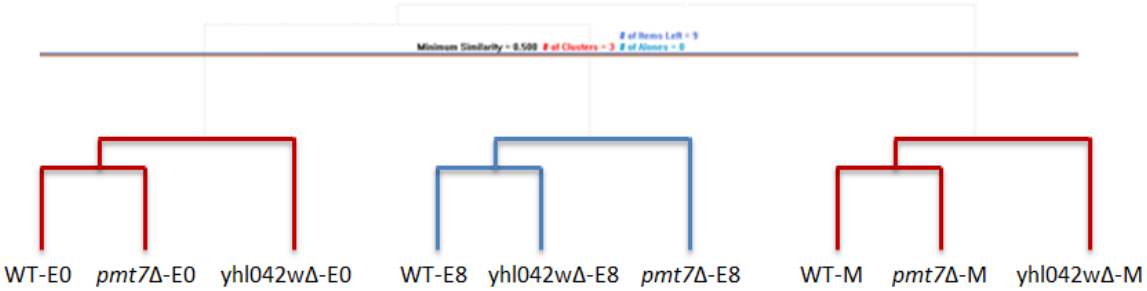

Figure 3.12. Hierarchical clustering of conditions obtained using HCE 3.5.

Under non-stressed growth conditions (M and E0), *pmt7*Δ/*pmt7*Δ strain was clustered together with the wild-type strain, whereas yhl042wΔ/yhl042wΔ strain was then connected to this sub-cluster, which in turn indicated a different transcriptional response by the yhl042wΔ/yhl042wΔ strain. On the other hand, under stressed conditions (E8), yhl042wΔ/yhl042wΔ strain was in the same cluster with wild-type strain, while

*pmt7*Δ/*pmt7*Δ strain formed a separate sub-cluster, indicating a different transcriptional response of *pmt7*Δ/*pmt7*Δ strain to ethanol stress.

3.2.3.3. Global Transcriptional Response to *PMT7* and *YHL042W* Gene Deletions. Genes whose expression levels were significantly up-regulated and down-regulated in response to *PMT7* and *YHL042W* gene deletions were screened using microarray data obtained from the samples taken at the mid-exponential phase of growth from cultures grown in YPD without ethanol and regarded as strain specific genes (SSGs). Although, all strains clustered together based on the transcriptome data, a total of 73 (30 up, 43 down) and 293 (215 up, 78 down) genes displayed a significantly altered expression levels (q-value < 0.005) in response to *PMT7* and *YHL042W* gene deletions, respectively when compared to the reference strain. Significantly enriched categories of the up- and down-regulated SSGs were analyzed using Amigo software. The distribution of the up-regulated and down-regulated genes in response to *PMT7* and *YHL042W* gene deletions and the enriched GO process terms were provided in Figure 3.13.

In response to *PMT7* gene deletion, the up-regulated genes were significantly enriched for growth associated processes including ribosome biogenesis and RNA processing (Figure 3.13A). On the other hand, the down-regulated genes were significantly enriched with energy, oxidation-reduction, carbohydrate, trehalose, and glucan metabolisms, electron transport, ATP synthesis and oxidative phosphorylation (Figure 3.13B).

PMT7 is a putative mannosyltransferase similar to Pmt1p with a potential role in protein *O*-glycosylation [105]. The secretory proteins are commonly mannosylated by mannosyltransferase proteins (PMT) in the endoplasmic reticulum; and glycosylated by several glycosyltransferases in the Golgi apparatus to form glycoproteins. Lack of one or more PMTs significantly affects the functions of *O*-glycosylated proteins [132]. Since glycosylation affects protein stability, secretion of proteins and the maintenance of the fungal morphology [132]; the deletion of one of the PMTs might have effects in some cellular functions, which is in consistence with the down-regulated genes associated with various metabolic processes.

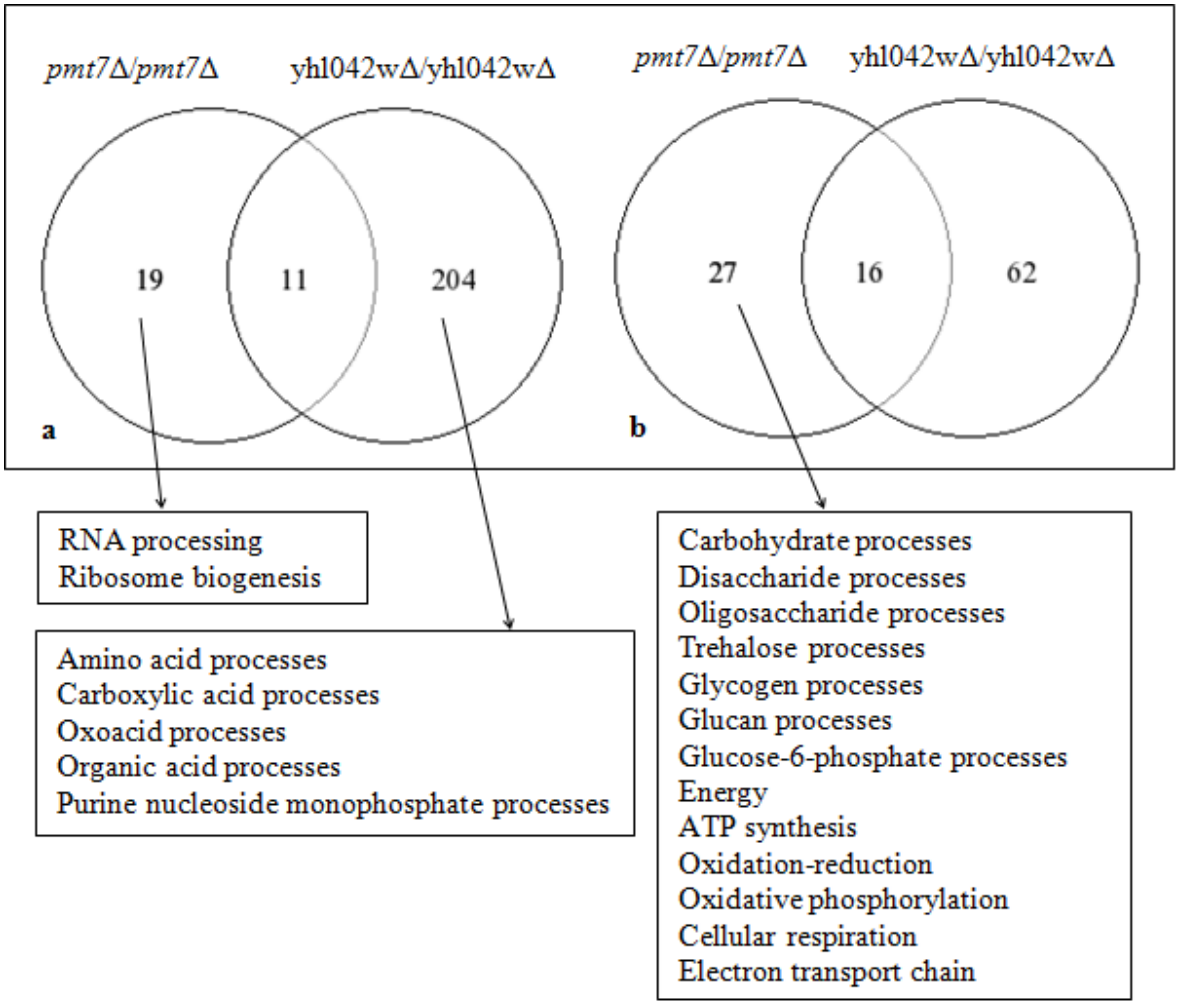

Figure 3.13. The distribution of (a) up- and (b) down-regulated genes together with their enriched GO processes in response to *PMT7* and *YHL042W* gene deletions.

In response to *YHL042W* gene deletion, expression of genes related to amino acid, carboxylic acid, oxoacid, and organic acid metabolisms were up-regulated (Figure 3.13A). Although the down-regulated genes were not significantly enriched with any biological process terms and biological processes of 15 down-regulated genes were unknown, the remaining down-regulated genes involved in carbohydrate metabolic process, protein folding, response to chemical stimulus, response to oxidative stress, cellular amino acid metabolic process, lipid metabolic process, response to osmotic stress, mitochondrion organization, nucleobase-containing small molecule metabolic process, generation of precursor metabolites and energy, DNA replication, response to DNA damage stimulus, sporulation, protein complex biogenesis, mitotic cell cycle, RNA catabolic process, protein phosphorylation, protein targeting, oligosaccharide metabolic process, response to heat,

endosomal transport, signaling, regulation of protein modification process, cellular respiration, regulation of DNA metabolic process, nuclear transport, cofactor metabolic process, meiotic cell cycle, and transmembrane transport.

YHL042W is a putative protein of unknown function and member of the DUP380 subfamily. This subfamily is composed of eleven genes and two pseudogenes which encode proteins consisting of ~380 amino acids [106]. The deletion of one or more member genes of DUP family could be responsible for the change in expressions of genes involved in protein and amino acid metabolisms.

Eleven up-regulated genes both in *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains include; *YMC2*, which encodes a putative mitochondrial inner membrane transporter having role in oleate metabolism and glutamate biosynthesis; *SSK22*, which encodes MAP kinase of the HOG1 mitogen-activated signaling pathway; *ATO3*, which encodes a plasma membrane protein having a possible role in export of ammonia from the cell; *OLE1*, which encodes delta(9) fatty acid desaturase; *UTP8*, which encodes a nucleolar protein required for export of tRNAs from the nucleus; *EFM1*, which encodes lysine methyltransferase; *UTP18*, which encodes possible U3 snoRNP protein involved in maturation of pre-18S rRNA; *DPH2*, which encodes a protein required for synthesis of diphthamide; *GRC3*, which encodes polynucleotide kinase; *FAP1*, which encodes a protein that binds to Fpr1p and conferring rapamycin resistance; *NAR1*, which encodes a component of the cytosolic iron-sulfur protein assembly machinery. Although the commonly up-regulated genes were not significantly enriched with any term, the up-regulation of genes involved in transmembrane transport, lipid metabolic process, and amino acid metabolism in response to both *PMT7* and *YHL042W* deletions might enhance the stress tolerance mechanism in *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains.

Five of the down-regulated genes (*YBR285W*, *YET3*, *FMP16*, *RTC3*, and *TMA10*) both in *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains encode proteins with unknown function. The remaining down-regulated genes include; *PRX1*, which encodes mitochondrial peroxiredoxin with thioredoxin peroxidase activity; *SSE2*, which encodes a heat shock protein that may be involved in protein folding; *OM14*, which encodes integral mitochondrial outer membrane protein; *HSP42*, which encodes small heat shock protein

with chaperone activity; *CTT1*, which encodes cytosolic catalase T; *SOL4*, which encodes 6-phosphogluconolactonase; *TFS1*, which encodes a protein that interacts with and inhibits carboxypeptidase Y and Ira2p; *YGP1*, which encodes a cell wall-related secretory glycoprotein; *DDR2*, which encodes a multi-stress response protein; *DCS2*, which encodes a non-essential, stress induced regulatory protein; and *GDB1*, which encodes a glycogen debranching enzyme. The down-regulation of genes related to stress mechanisms in response to both *PMT7* and *YHL042W* deletions indicate that *PMT7* and *YHL042W* may possibly have a role in stress mechanism in *S. cerevisiae*.

In order to highlight the regulatory pathways affected by *PMT7* and *YHL042W* deletions, Reporter TFs showing a significant change in the expression of the genes regulated by them were identified. The Reporter TF analysis identified 14 and eight TFs (p-value < 0.01) for *pmt7*Δ/*pmt7*Δ (Figure 3.14A) and yhl042wΔ/yhl042wΔ (Figure 3.14B) strains, respectively. Reporter TFs involved in stress response (Sko1, Hot1, and Cad1) together with a carbon source-responsive TF involved in regulation of pathways that metabolize non-fermentable sugars (Adr1) were identified in common for both *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains. Reporter TFs for *pmt7*Δ/*pmt7*Δ strain include TFs involved in invasive growth (Mss11), regulation of drug response (Pdr1), regulation of stress elements (Msn2 and Msn4), oxidative stress (Ixr1), activation of multiple genes in response to stresses (Hsf1), regulation of respiration (Hap2, Hap4 and Hap5), and activation of retrograde and TOR pathways (Rtg2p). Reporter TFs for yhl042wΔ/yhl042wΔ strain include a basic leucine zipper iron sensing TF involved in vacuolar iron storage (Yap5) and TFs involved in regulation of amino acid metabolism (Gcn4, Bas1, and Met4).

The identification of Hap2/4/5 regulators suggests that the single gene deletion of *PMT7* leads to transcriptional changes in respiratory genes. Although the biological process categories of *PMT7* have not been identified yet, *PMT7* might have possible roles on cellular respiration in *S. cerevisiae*. These results were also consistent with the down-regulation of genes related to cellular respiration in *pmt7*Δ/*pmt7*Δ strain (Figure 3.13B). Moreover, the oxygen-related DNA damage caused by the alteration in respiration level [126], might be responsible for the identification of Reporter TFs mainly associated with stress response in *pmt7*Δ/*pmt7*Δ strain.

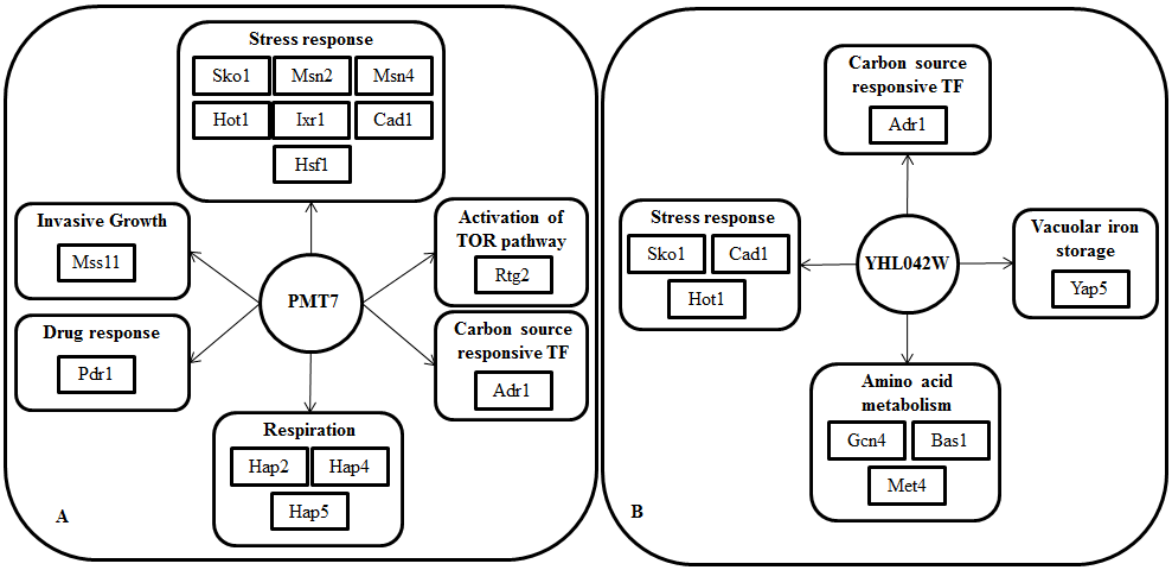

Figure 3.14. Regulatory TFs around which genes were affected following (a) *PMT7* and (b) *YHL042W* single gene deletions.

The cellular regulatory machineries affected by *YHL042W* deletion include stress response, amino acid metabolism, metabolism of non-fermentable carbon sources, and vacuolar iron storage. The deletion of *YHL042W* caused the up-regulation of genes involved in amino acid processes (Figure 3.13A). This result together with the identification of Reporter TFs associated with amino acid metabolism indicates the possible role of *YHL042W* in amino acid metabolism in *S. cerevisiae*.

As many cellular processes, ethanol tolerance and stress mechanisms are associated with the interaction of complex networks in *S. cerevisiae* [51]. To investigate the possible effects of *PMT7* and *YHL042W* gene deletions on ethanol tolerance and stress mechanisms in *S. cerevisiae*, the Reporter Proteins were identified using tETN, the PPI network related to ethanol tolerance. The Reporter Protein analysis identified 17 and 10 proteins for *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains, respectively.

For *pmt7*Δ/*pmt7*Δ strain, the identified Reporter Proteins are Sdh1p, the flavoprotein subunit of succinate dehydrogenase; Opi1p, transcriptional regulator of a variety of genes; YEL043w, a predicted cytoskeleton protein; Aim46p, Tbs1p, YJL218w and YHR097c, putative proteins of unknown function; Tfg1p, the TF II largest subunit; Sis2p, the negative regulatory subunit of protein phosphatase 1; Vid28p, protein involved in proteasome-

dependent catabolite degradation of fructose-1,6-bisphosphatase; Lat1p, the dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA; Cox6p, the subunit VI of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain; Aur1p, the protein phosphatidylinositol; Spt23p, ER-membrane protein involved in regulation of *OLE1* transcription; Mcm6p, the protein involved in DNA replication; Sch9p, the protein kinase involved in transcriptional activation of osmostress-responsive genes; and Trs85, the subunit of transport protein particle, a multi-subunit complex involved in targeting and/or fusion of ER-to-Golgi transport vesicles with their acceptor compartment. These Reporter Proteins were found to be related to respiration and electron transport chain (Sdh1p, Cox6p), lipid metabolism (Opi1p, Aur1p, Spt23p), protein phosphorylation (Tfg1p, Sis2p), gluconeogenesis (Vid28p), vacuolar transport (Vid28p, Trs85p), cell cycle (Mcm6p, Sis2p), DNA processing (Mcm6p), and response to stress (Sis2p, Sch9p). The first neighbors of these Reporter Proteins have significant association with transcription, response to stress, and regulation of cellular processes. The information coming from the Reporter Proteins was also consistent with the findings of Reporter TF and GO enrichment analysis. Reporter Proteins again show that the deletion of *PMT7* has a major impact on the transcription of genes related to respiration and stress response mechanisms.

For yhl042wΔ/yhl042wΔ strain, the identified Reporter Proteins are Atg26p, sterol glucosyltransferase involved in synthesis of sterol glucoside membrane lipids; Tda10p, the ATP-binding protein of unknown function; Sop4p, ER-membrane protein; Mga2p, ER-membrane protein involved in regulation of OLE1 transcription; Ald6p, the protein cytosolic aldehyde dehydrogenase required for conversion of acetaldehyde to acetate and involved in NADPH regeneration; Mcm6p, the protein involved in DNA replication; Cmr3p, a putative zinc finger protein; Erg13p, the 3-hydroxy-3-methylglutaryl-CoA synthase protein; Sch9p, the protein kinase involved in transcriptional activation of osmostress-responsive genes; and Nop1p, a nucleolar protein required for processing of pre-18S rRNA. These Reporter Proteins are related to lipid metabolism (Atg26p, Mga2p, and Erg13p), ATP binding (Tda10p), vacuolar transport (Sop4p), stress response (Ald6p, Sch9p), cell cycle (Mcm6p) and RNA processing (Nop1p). These results are in good agreement with the increased ethanol tolerance observed for this mutant. The GO

enrichment analysis of the first neighbors of these Reporter Proteins revealed significant association with nitrogen compound, aromatic compound, macromolecule and nucleic acid processes, transcription, DNA and RNA processing, ribosomal biogenesis, and protein phosphorylation. These results suggest that the Reporter Proteins have key roles on connecting the main processes throughout the cell.

In order to understand the response to *PMT7* and *YHL042W* gene deletions under ethanol stressed condition, the up- and down-regulated SSGs with respect to wild-type strain were identified using microarray data obtained from the samples taken 2 h after ethanol treatment (E8 samples). A total of 1358 (520 up, 838 down) and 67 (50 up, 17 down) genes displayed a significantly altered expression levels in response to *PMT7* and *YHL042W* gene deletions, respectively. The distribution of the up-regulated and down-regulated genes in response to *PMT7* and *YHL042W* gene deletions together with the enriched GO process terms were provided in Figure 3.15.

GO enrichment analysis indicated that the response of *S. cerevisiae* cells to *PMT7* deletion under ethanol stressed condition was different from the response under non-stressed condition. Under stressed condition, genes significantly enriched for respiration associated processes (Figure 3.15A) were up-regulated. Since the yeast cells may become more tolerant to ethanol stress when they shift from fermentative to respiratory growth [59], the up-regulation of genes involved in respiration might be the reason for enhanced ethanol tolerance of *pmt7*Δ/*pmt7*Δ strain. Moreover, genes significantly enriched for protein associated processes (Figure 3.15B) were down-regulated under stressed condition. Since *PMT7* has a role in glycosylation that affects protein stability and secretion of proteins, deletion of *PMT7* might be responsible for the repression of genes related to protein metabolism.

In response to *YHL042W* gene deletion under stressed condition, both up- and down-regulated genes were not significantly enriched with any biological process term. Genes involved in transport (*FRE2*, *LST4*, *TIM13*, *VBA4*, *VMR1*, and *YCR023C*), amino acid metabolism (*LST4*, *VBA4*), transcription and translation (*NUP120*, *PDR8*, *SIP3*, *VPS15*, *RPS0B*, and *RPS18B*), RNA processing (*RPS0B*, *RPS18B*), lipid metabolism (*TCB1*, *TCB3*), cell cycle (*ESP1*), DNA damage and DNA repair (*NUP120*), and response to

chemical stimulus (*VMR1*) were up-regulated; whereas genes involved in transcription (*GAT1*), protein targeting (*VPS21*), vacuole organization (*VPS21*), response to chemical stimulus (*GAT1*), organelle inheritance (*VPS21*) and transmembrane transport (*SEO1*) were down-regulated.

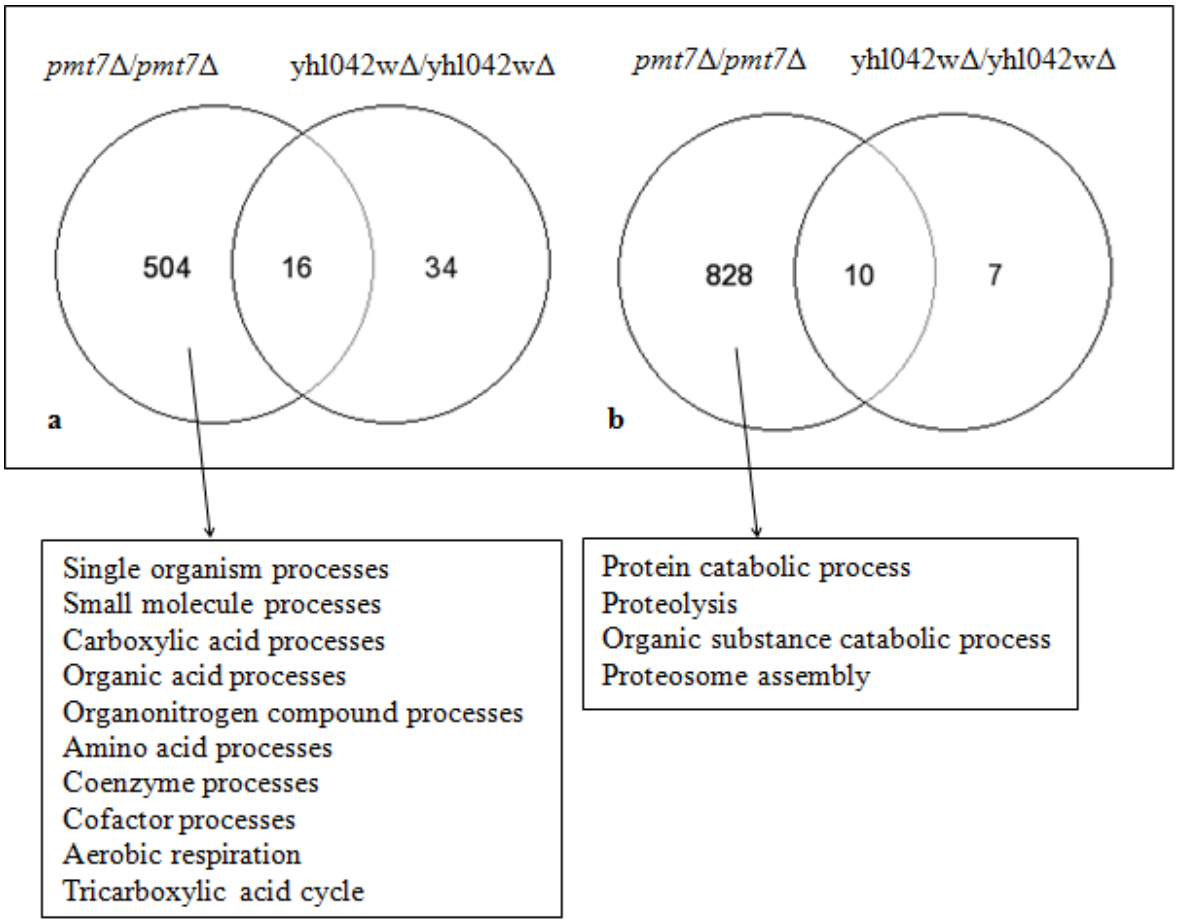

Figure 3.15. The distribution of (a) up- and (b) down-regulated genes and their enriched GO process terms in response to *PMT7* and *YHL042W* gene deletions 2 h after ethanol treatment (E8).

Sixteen genes were commonly up-regulated in response to both *PMT7* and *YHL042W* deletions under stressed condition. The commonly up-regulated genes include *ADE1*, which encodes N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase, required for 'de novo' purine nucleotide biosynthesis; *LYS2*, which encodes alpha aminoadipate reductase and involved in the biosynthesis of lysine; *ARO1*, which encodes a pentafunctional arom protein and acts in the biosynthesis of chorismate that is a precursor to aromatic amino acids; *HOM2*, which encodes aspartic beta semi-aldehyde

dehydrogenase and acts in methionine, threonine and homoserine biosynthesis; *HXT4*, which encodes high-affinity glucose transporter; *QDR2*, which encodes a plasma membrane transporter of the major facilitator superfamily, required for resistance to quinidine, barban, cisplatin, and bleomycin, and involved in copper ion export; *FRE1*, which encodes ferric reductase and cupric reductase, and involved in copper and iron ions import; *IMD3*, which encodes inosine monophosphate dehydrogenase, catalyzes the first step of GMP (guanosine monophosphate) biosynthesis; *LEU4*, which encodes alpha-isopropylmalate synthase, the main isozyme responsible for the first step in the leucine biosynthesis pathway; *ERG24*, which encodes a sterol reductase and acts in ergosterol biosynthesis; *CAR1*, which encodes arginase and is responsible for arginine degradation; *RPS0A*, which encodes ribosomal 40S subunit protein S0A required for maturation of 18S rRNA; *RPL15A*, which encodes ribosomal 60S subunit protein L15A; *RPL16B*, which encodes ribosomal 60S subunit protein L16B; *RPS7A*, which encodes protein component of the 40S ribosomal subunit; and *YLR281C*, which encodes a protein with unknown function. The majority of these commonly up-regulated genes were composed of genes encoding ribosomal proteins and genes involved in amino acid processes. These findings were consistent with the relationship between amino acid metabolisms and *PMT7* and *YHL042W* genes.

Under ethanol stressed condition, 10 genes were commonly down-regulated in response to both *PMT7* and *YHL042W* deletions. The commonly down-regulated genes include *SSA1*, which is a member of the heat shock protein 70 (HSP70) family, encodes ATPase involved in protein folding and the response to stress; *SSA4*, which is also a member of the HSP70 family, encodes a heat shock protein that is highly induced upon stress; *MDJ1*, which is a member of the HSP40 family of chaperones, encodes co-chaperone that stimulates the ATPase activity of the HSP70 protein Ssc1p, involved in protein folding/refolding in the mitochodrial matrix and required for proteolysis of misfolded proteins; *TIP1*, which encodes major cell wall mannoprotein with possible lipase activity; *AGP1*, which encodes low-affinity amino acid permease with broad substrate range, and involved in uptake of asparagine, glutamine, and other amino acids; *HSP30*, which encodes hydrophobic plasma membrane localized, stress-responsive protein that induced by heat shock, ethanol treatment, weak organic acid, glucose limitation, and entry into stationary phase; *HTB1*, which encodes histone H2B, a core histone protein required

for chromatin assembly and chromosome function; *MXR1*, which encodes methionine-S-sulfoxide reductase, involved in the response to oxidative stress and the regulation of lifespan; *PHD1* and *SCN2*, which encode a transcriptional activator that enhances pseudohyphal growth. The down-regulation of genes associated with pseudohyphal growth were in agreement both with the high levels of ethanol in media and the decreasing amount of the preferred carbon source glucose after the mid-exponential phase of growth. Since the protein conformation was disturbed by high levels of ethanol and this causes the accumulation of denatured proteins, chaperons involved in protein folding and refolding seem to be critical for yeast tolerance to ethanol [51]. Although the genes encoding two chaperons (*SSA1* and *SSA4*) were repressed in response to deletion of *PMT7* and *YHL042W* genes under stressed condition, both mutants showed enhanced tolerance to ethanol. The induction of heat shock proteins and chaperons can be dependent to concentration, strain, and time [51]. Therefore, the expressions of these genes might be induced at earlier or later time points. Since the reprogramming of the metabolism for cell survival and productivity under ethanol stress might be explained by not only the functions of individual genes, but also their interactions with other relevant genes, the induction of genes encoding proteins involved in mechanisms related to ethanol tolerance in response to ethanol stress could support the improved tolerance of these mutants.

In order to shed light into the regulatory pathways affected by *PMT7* and *YHL042W* deletions under ethanol stressed condition, Reporter TFs showing a significant change in the expression of the genes regulated by them were identified. The Reporter TF analysis identified nine and 12 TFs (p-value < 0.01) for *pmt7*Δ/*pmt7*Δ (Figure 3.16A) and yhl042wΔ/yhl042wΔ (Figure 3.16B) strains, respectively.

Two common Reporter TFs involved in amino acid metabolism (Gcn4) and stress response (Hsf1) were identified in both *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains. The remaining Reporter TFs for *pmt7*Δ/*pmt7*Δ strain include TFs involved in amino acid metabolism (Leu3), stress response (Rpn4), growth (Ash1, Sok2), unfolded protein response (Hac1), sporulation (Sum1), and regulation of histone gene transcription (Hir2). Since ethanol accumulation perturbs the protein conformation, the existence of a TF related to regulation of unfolded proteins was found to be important for tolerance to ethanol. Reporter TFs for yhl042wΔ/yhl042wΔ strain include TFs involved in amino acid

metabolism (Bas1), stress response (Cad1), growth (Ste12), protein polyubiquitination (Mot2), telomeric silencing (Rap1), vacuolar iron storage (Yap5), DNA binding (Gat3), and transcription of ribosomal proteins (Ifh1, Fhl1, and Sfp1). Reporter TFs analysis revealed the existence of different regulatory machineries in response to *PMT7* and YHL042W deletions under ethanol stressed condition when compared to non-stressed condition. This difference between the affected regulatory pathways could be explained by the increased ethanol level acting as the major stress, rather than gene deletions.

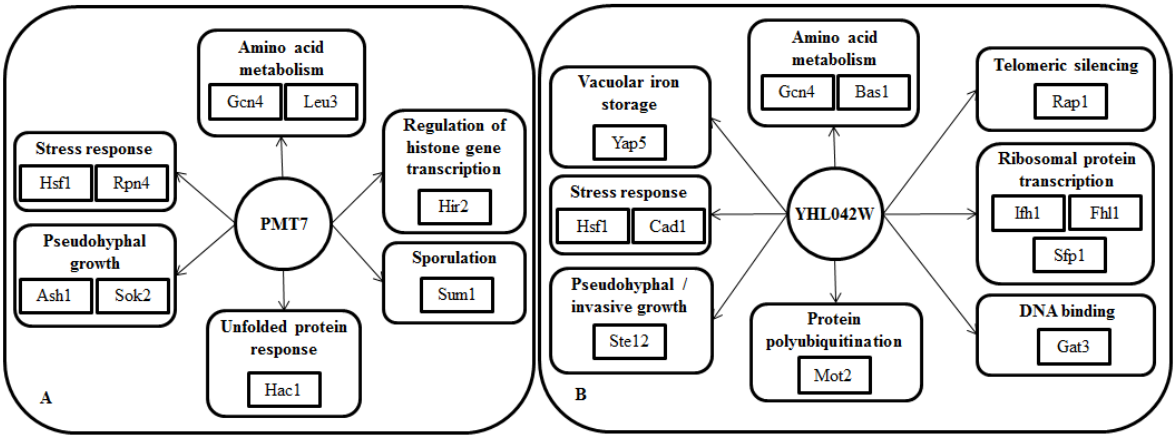

Figure 3.16. Regulatory TFs around which genes were affected following (a) *PMT7* and (b) *YHL042W* single gene deletions under stressed condition.

3.2.3.4. Global Transcriptional Response to Ethanol Stress. Genes whose expression levels were significantly up-regulated and down-regulated under ethanol stress condition in comparison with the non-stressed condition were screened using microarray data obtained from samples taken 2 h after ethanol treatment from untreated and treated cultures. Genes showing statistical significant difference in their expression levels (q-value < 0.005) between the untreated (E0) and 8% (v/v) ethanol treated (E8) conditions were regarded as significant ethanol genes (SEGs) and classified according to their up- and down-regulation in each strain. When the ethanol treated and untreated conditions were compared 3929 (1871 up, 2058 down), 3800 (1774 up, 2026 down), 3967 (1981 up, 1986 down) SEGs were identified in wild-type, *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains, respectively. Significantly enriched categories of the up- or down-regulated SEGs were analyzed using Amigo software. Analysis of the global transcriptional response of ethanol tolerant *S. cerevisiae* mutants to ethanol stressed condition in comparison to the unstressed condition indicated that several metabolic processes including carbohydrate, lipid, and phosphorous

processes, cell wall organization and biogenesis, and amino acid metabolism were affected from increased ethanol concentration.

Table 3.6. GO enrichments of SEGs which were significantly up-regulated in response to ethanol stress in wild-type strain.

Enriched GO Biological Process Terms	P-value
mitochondrion organization	3.77E-07
mitochondrial translation	4.73E-05
single-organism transport	6.00E-05
localization	2.04E-03
establishment of localization	3.42E-03
establishment of protein localization to mitochondrion	3.77E-03
protein localization to mitochondrion	3.77E-03
transport	3.94E-03
transmembrane transport	5.52E-03

A huge number of genes, involved in wide-ranging functional categories, associated with ethanol tolerance mechanism in *S. cerevisiae* were identified. The GO enrichment analysis revealed that the up-regulated SEGs were significantly enriched with mitochondria, localization and transport in both *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ and the wild-type strains (Table 3.6 -3.8). Previous reports also indicated the up-regulation of similar processes including mitochondria, transport and localization in response to ethanol stress. Genes that were involved in multiple mitochondrion functions have previously been reported to be expressed at a higher level in an ethanol tolerant mutant [67]. Moreover, mutations related to transport mechanisms was reported to be associated with ethanol sensitivity and growth deficiency in response to ethanol stress [63,101] and several genes required for yeast resistance to ethanol were reported to be significantly enriched with terms involved in transport [96,100]. Although, no significantly enriched terms related to localization were identified via the genome-wide investigation of ethanol stress, a stress related protein Asr1p was reported to change its subcellular localization upon ethanol stress [39], and GO enrichment analysis of the ethanol tolerance network (tETN) revealed significant association between ethanol tolerance and localization.

The significantly enriched terms detected in the up-regulated SEGs of *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains would enlighten the mechanisms underlying ethanol tolerance and stress since these mutants showed improved tolerance to ethanol. The GO enrichment analysis revealed significantly enriched terms related to phosphorus and phosphate-containing compound metabolic processes in up-regulated SEGs of both deletion mutants (Table 3.7 and 3.8). These results were in accordance with the previous reports identifying synthase and dehydrogenase of phosphate containing compounds in the functional categories of candidate and key genes for ethanol tolerance as revealed by quantitative transcription dynamic analysis [67].

Table 3.7. GO enrichments of SEGs which were significantly up-regulated in response to ethanol stress in *pmt7*Δ/*pmt7*Δ strain.

Enriched GO Biological Process Terms	**P-value**
mitochondrion organization	4.14E-07
phosphorus metabolic process	7.99E-07
localization	2.21E-06
phosphate-containing compound metabolic process	3.21E-06
mitochondrial translation	3.80E-06
establishment of localization	1.09E-04
transport	2.25E-04
small molecule metabolic process	3.24E-04
organophosphate metabolic process	1.06E-03
lipid metabolic process	2.07E-03
cellular lipid metabolic process	5.38E-03
ion transport	8.38E-03

Genes significantly enriched with lipid processes in *pmt7*Δ/*pmt7*Δ strain were also up-regulated (Table 3.7) in response to ethanol stress. Preceding reports indicated that improved tolerance to ethanol may be reached by changing cell membrane composition to increase the membrane stability [54,97]. In response to ethanol stress, alterations in lipid content of the plasma membrane have been observed in order to maintain the membrane fluidity in yeast [32,54,93,98].

The strong relevance of *INO1* gene with ethanol tolerance phenotype has been previously identified. Inositol-1-phosphate synthase, a protein encoded by *INO1* gene, participates in the synthesis of inositol-1-phosphate. Inositol-1-phosphate is a precursor for the synthesis of inositol containing glycerophospholipids. In ethanol stressed condition, the inositol limitation causes a reduced H^+-ATPase activity in the yeast plasma membrane because of the reduced synthesis of inositol containing glycerophospholipids [34,62]. In response to ethanol stress, the expression of INO1 gene increased 56 fold in *pmt7*Δ/*pmt7*Δ strain, whereas 35 and 33 fold increases were observed in wild-type strain and yhl042wΔ/yhl042wΔ strain, respectively.

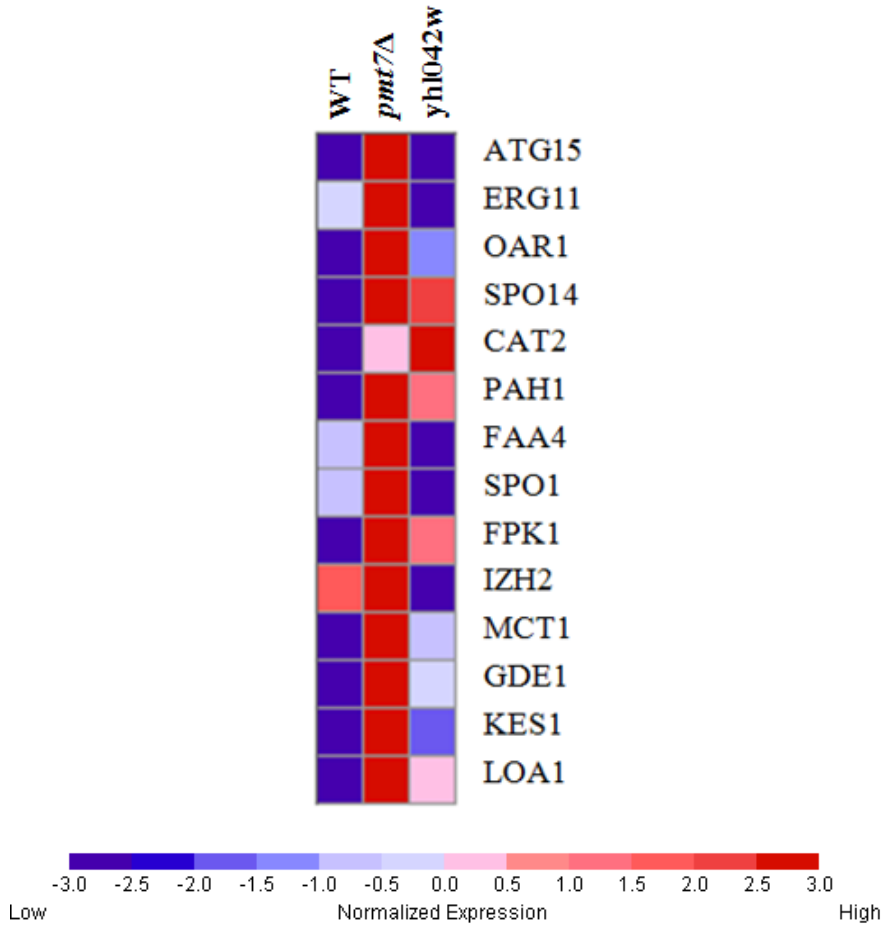

Figure 3.17. Expression profiles of genes related to lipid metabolism.

Fourteen genes involved in lipid processes were up-regulated in *pmt7*Δ/*pmt7*Δ strain (Figure 3.17), whereas the expressions of these genes were down-regulated or not significantly changed in wild-type strain and yhl042wΔ/yhl042wΔ strain. These genes include *ERG11*, which encodes lanosterol 14-alpha-demethylase; *FPK1*, which encodes a

Ser/Thr protein kinase that regulates the putative phospholipid translocases Lem3p-Dnf1p/Dnf2p; *LOA1*, which encodes lysophosphatidic acid acyltransferase; *KES1*, which encodes one of seven members of the yeast oxysterol binding protein family involved in lipid binding; *MCT1*, which encodes predicted malonyl-CoA:ACP transferase; *SPO14*, which encodes phospholipase D; *IZH2*, which encodes a plasma membrane protein involved in zinc homeostasis and osmotin-induced apoptosis; *FAA4*, which encodes long chain fatty acyl-CoA synthetase; *PAH1*, which encodes Mg^{2+}-dependent phosphatidate phosphatase; *SPO1*, which encodes meiosis-specific prospore protein involved in phospholipase activity; *CAT2*, which encodes carnitine acetyl-CoA transferase; *GDE1*, which encodes glycerophosphocholine phosphodiesterase; *ATG15*, which encodes lipase required for intravacuolar lysis of autophagic bodies and Cvt bodies; and *OAR1*, which encodes mitochondrial 3-oxoacyl-[acyl-carrier-protein] reductase. The up-regulation of genes involved in lipid processes in *pmt7*Δ/*pmt7*Δ strain supported its elevated tolerance to ethanol.

GO enrichment analysis revealed that genes involved in carbohydrate processes in yhl042wΔ/yhl042wΔ strain were significantly up-regulated (Table 3.8) in response to ethanol stress. This result was in accordance with reports indicating that genes involved in multiple functions of carbohydrate metabolism were enhanced in an ethanol tolerant mutant in response to ethanol stress [67]. Storage carbohydrates such as trehalose can prevent cell dehydration and influx of excess salts into the cell [67]. Moreover, trehalose helps to stabilize or repair denatured proteins caused by ethanol [54]. Therefore, enhanced expression of genes related to trehalose and subsequently carbohydrate metabolism are expected in order to obtain a stable intracellular environment for survival under ethanol stress condition.

Trehalose-6-phosphate, a phosphate containing compound, is necessary for trehalose synthesis. The protein encoded by *TPS1* gene participates in the synthesis of trehalose-6-phosphate and *TPS2* encodes trehalose-6-phosphate phosphatase [54,97]. *TPS1* is induced in both *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains and the wild-type strain, whereas *TPS2* is induced only in yhl042wΔ/yhl042wΔ strain in response to ethanol. *TPS3* and *TSL1* genes involved in trehalose synthase and *ATH1* gene involved in degradation of trehalose are also up-regulated in yhl042wΔ/yhl042wΔ strain. The improved ethanol tolerance of

yhl042wΔ/yhl042wΔ strain is consistent with the fact that the transcriptional changes in genes involved in trehalose synthase under ethanol stress condition lead to the production of the protective substance trehalose [54,97]. The induced expression of a gene involved in trehalose degradation also agreed with the mutants' increased ethanol tolerance, since the enhanced expression of this gene is needed to adjust the trehalose concentration [51,93].

Table 3.8. GO enrichments of SEGs which were significantly up-regulated in response to ethanol stress in yhl042wΔ/yhl042wΔ strain.

Enriched GO Biological Process Terms	**P-value**
mitochondrial translation	1.48E-06
mitochondrion organization	2.52E-06
single-organism transport	4.80E-05
phosphorus metabolic process	3.20E-04
phosphate-containing compound metabolic process	5.11E-04
carbohydrate biosynthetic process	5.40E-04
fungal-type cell wall organization or biogenesis	1.39E-03
carbohydrate metabolic process	4.00E-03
localization	7.66E-03
cellular carbohydrate biosynthetic process	7.95E-03
cell wall organization or biogenesis	8.80E-03
single-organism carbohydrate metabolic process	9.06E-03

Ethanol accumulation leads to cell death by affecting the integrity of cell membrane and increasing the membrane fluidity [51]. In association with ethanol tolerance phenotype; genes involved in cell wall composition and biogenesis, were observed to display up-regulation in yhl042wΔ/yhl042wΔ strain. Under the ethanol stressed condition, yeast cells have to maintain the cell wall integrity by changing its composition for increased cell viability [54,63,96]. The up-regulation of genes involved in cell wall organization or biogenesis in yhl042wΔ/yhl042wΔ strain would elucidate its elevated tolerance to ethanol.

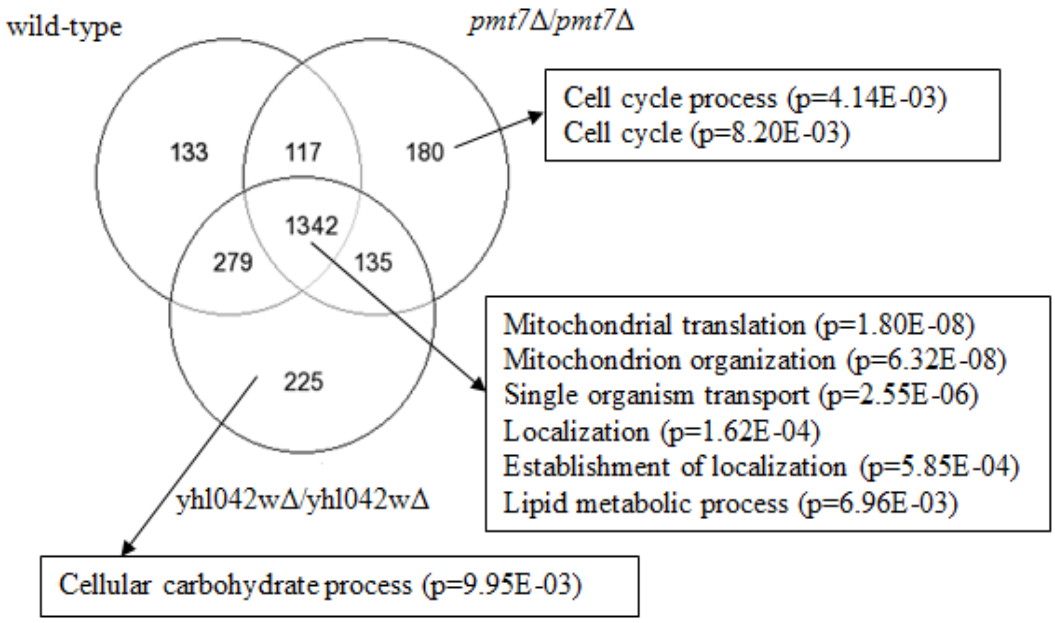

Figure 3.18. Distribution of significantly up-regulated genes in response to ethanol stress and their enriched GO process terms.

The distribution of the up-regulated genes for each strain was provided in Figure 3.18. Up-regulated SEGs detected in all strains could be important in understanding the ethanol tolerance and stress mechanisms. On the other hand, based on the improved ethanol tolerances of both deletion mutants, the up-regulated SEGs identified in *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains rather than the wild-type strain were specifically thought to be crucial for survival under high ethanol concentrations. These SEGs were classified into three classes as follows: up-regulated SEGs in (1) both *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains (135 genes), (2) *pmt7*Δ/*pmt7*Δ strain only (180 genes), and (3) yhl042wΔ/yhl042wΔ strain only (225 genes). In order to characterize each group, the up-regulated SEGs were classified into functional categories using MIPS database [124]. The list of these functional categories with p-value < 0.05 was provided in Table 3.9-3.11.

SEGs which are up-regulated in the *pmt7*Δ/*pmt7*Δ and/or yhl042wΔ/yhl042wΔ strains only, the significantly concentrated categories related to lipid, fatty acid, phosphate, and carbohydrate metabolisms were identified (Table 3.9-3.11). Phosphate, carbohydrate, and lipid metabolisms are closely related and have important roles in maintaining plasma membrane integrity in the cell during ethanol stress. The phosphate-containing compounds,

such as trehalose-6-phosphate and inositol-1-phosphate, are needed for the synthesis of storage carbohydrates and phospholipids to enhance the viability of cells in the presence of ethanol. The changes in unsaturated fatty acids were found to be important in terms of stabilizing the membrane fluidity to antagonize the effect of ethanol [32,54,98]. Genes involved in fatty acid metabolism were widely reported in association with ethanol tolerance in various studies [51,54,94]. These results were also in agreement with the GO term enrichment analysis.

Table 3.9. Functional categories of up-regulated SEGs in *pmt7*Δ/*pmt7*Δ strain only.

Description	**Functional Categories**
Metabolism	- C-2 compound and organic acid metabolism - lipid, fatty acid and isoprenoid metabolism - regulation of lipid, fatty acid and isoprenoid metabolism
Energy	- energy conversion and regeneration
Cell Cycle and DNA Processing	- cell cycle - mitotic cell cycle - chromosome condensation
Protein Synthesis	- ribosome biogenesis - ribosomal proteins - translation termination
Protein with Binding Function or Cofactor Requirement	- structural protein binding - nucleotide/ nucleoside/ nucleobase binding - GTP binding - FAD/FMN binding
Cell Rescue, Defense and Virulence	- electromagnetic waves resistance
Biogenesis of Cellular Components Cell Type Differentiation	- eukaryotic plasma membrane - fungal/microorganismic cell type differentiation - fungal and other eukaryotic cell type differentiation - budding, cell polarity and filament formation

Table 3.10. Functional categories of up-regulated SEGs in yhl042Δ/yhl042Δ strain only.

Description	Functional Categories
Metabolism	- phosphate metabolism - sugar, glucoside, polyol and carboxylate anabolism
Energy	- metabolism of energy reserves
Cell Cycle and DNA Processing	- cell cycle arrest
Protein Fate	- protein modification - modification by phosphorylation, dephosphorylation, autophosphorylation
Protein with Binding Function or Cofactor Requirement	- cyclic nucleotide binding
Regulation of Metabolism and Protein Function	- guanyl-nucleotide exchange factor
Cellular Communication / Signal Transduction Mechanism	- cellular signaling - enzyme mediated signal transduction - G-protein mediated signal transduction - small GTPase mediated signal transduction
Cell Rescue, Defense and Virulence	- stress response - heat shock response
Interaction with the Environment	- cellular sensing and response to external stimulus - osmosensing and response
Cell Fate	- directional cell growth - apoptotic mitochondrial changes
Cell Type Differentiation	- fungal/microorganismic cell type differentiation - fungal and other eukaryotic cell type differentiation - development of asco- basidio- or zygospore

Furthermore, the significantly concentrated categories associated with energy, protein, transcription, signaling, transport, biogenesis of cellular components (especially cell membrane), and cell rescue were identified (Table 3.9-3.11) in the functional

categories of SEGs up-regulated in *pmt7*Δ/*pmt7*Δ and/or yhl042wΔ/yhl042wΔ strains. The significance of genes related to "energy conversion and regeneration" might have resulted in increased ATP synthesis during ethanol stress which led to higher energy consumption for the synthesis of compounds to survive [59,133]. Also, the transcriptional changes in genes connected to energy pathways were identified for ethanol tolerant mutants [95,103,118]. Moreover, high levels of ethanol inhibits several transport systems, including the general amino acid permease and the glucose transport system [11,93]. Therefore, the up-regulation of transport and energy related genes suggested the enhanced viability of these mutants under the ethanol stressed condition.

The fact that SEGs which are up-regulated specifically in *pmt7*Δ/*pmt7*Δ strain were significantly associated with "ribosomal proteins" suggested the enhanced activity of this mutant in protein synthesis in response to ethanol stress. Yeast cells regulate ribosomal proteins to get used to the presence of ethanol and reprogramme several metabolic pathways, including ribosome biogenesis, to adapt ethanol stress [134]. The role of ribosomal proteins on improving the activity of protein synthesis were reported to be important for ethanol adapted and ethanol tolerant strains grown under the ethanol stressed conditions [95,134].

SEGs which are up-regulated specifically in yhl042wΔ/yhl042wΔ strain are significantly associated with functional categories of "stress response" and "heat shock response" (Table 3.10). The genes involved in the environmental stress response are proposed to protect critical cell functions during a transition to a stressful environment. The stress response related GO terms were found to be affected by ethanol stress in several studies comparing the transcriptome profiles of wild type, ethanol tolerant and ethanol sensitive mutants [50,103]. Genes induced under ethanol stress exhibited a broad overlapping with genes associated with heat stress response [51], which support the existence of genes related to heat shock response in response to ethanol.

The GO enrichment analysis revealed that genes involved in amino acid biosynthetic processes were repressed significantly in all strains (Table 3.12). Since amino acid synthesis is an energy-cost activity in the cell, the down-regulation of genes related to these pathways might help to survive by saving energy in the presence of ethanol [134].

Furthermore, lipid synthesis, that cells need to antagonize the effect of ethanol in the plasma membrane, is an energy requiring process [134]. Therefore, the energy saved from the inhibition of amino acid synthesis, might be used for the synthesis of lipid and fatty acids in the *pmt7*Δ/*pmt7*Δ strain, in which genes associated with lipid and fatty acid metabolisms were up-regulated under ethanol stress.

Table 3.11. Functional categories of up-regulated SEGs in *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains.

Description	**Functional Categories**
Metabolism	- degradation of tryptophan - nitrogen, sulfur and selenium metabolism - regulation of nitrogen, sulfur and selenium metabolism - regulation of C-compound and carbohydrate metabolism
Energy	-Energy
Cell Cycle and DNA Processing	- cell cycle - mitotic cell cycle and cell cycle control - cell cycle dependent cytoskeleton reorganization
Transcription	- transcription activation - transcription repression
Regulation of Metabolism and Protein Function	- regulator of G-protein signaling
Cellular Transport, Transport Facilitation and Transport Routes	- RNA transport - antiporter - nuclear transport
Cellular Communication / Signal Transduction Mechanism	- cellular signalling - second messenger mediated signal transduction - cAMP/cGMP mediated signal transduction - fatty acid derivatives mediated signal transduction
Biogenesis of Cellular Components	- nuclear membrane

Table 3.12. GO enrichment categories of SEGs which were significantly down-regulated in response to ethanol stress.

Strain	GO Enrichments
Wild-type	- RNA processing - regulation of transcription and gene expression - nitrogen / organic cyclic / aromatic compound processes - nucleobase-containing compound processes - amino acid / nucleic acid processes - regulation of biological and cellular processes - DNA repair - macromolecular complex subunit organization
*pmt7*Δ/*pmt7*Δ	- RNA processing - nitrogen / organic cyclic / aromatic compound processes - amino acid / nucleic acid processes - regulation of transcription and gene expression - macromolecular complex subunit organization - nucleobase-containing compound processes - amine biosynthetic process
yhl042wΔ/yhl042wΔ	- RNA processing - nitrogen / organic cyclic / aromatic compound processes - amino acid / nucleic acid processes - nucleobase-containing compound processes - ribosome biogenesis - ribosome location - macromolecular complex subunit organization - ribonucleoprotein complex localization - organelle localization - DNA replication

The down-regulated SEGs (Table 3.12) were also significantly enriched with RNA processing in all strains. Although transcription of genes related to ribosome biogenesis was up-regulated in response to ethanol stress for *pmt7*Δ/*pmt7*Δ strain (Table 3.9), the

inhibition of RNA processing might be explained by a defect in the mRNA export in the presence of ethanol [116,134,135].

To deal with environmental stresses, yeast cells evolved specific mechanisms, including the repair of certain damages [54]. Different from *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains, the repression of genes associated with DNA repair specifically in the wild-type strain would be responsible for reduced viability of the in the presence of ethanol stress.

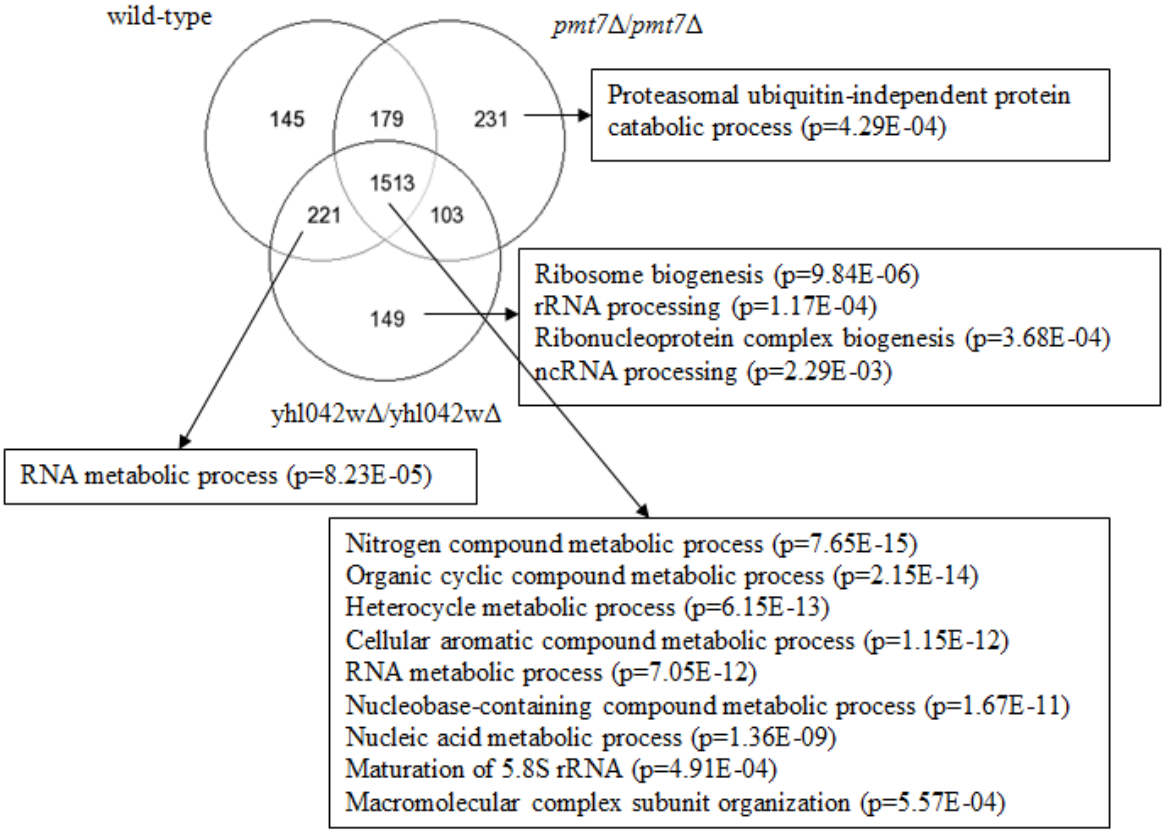

Figure 3.19. Distribution of significantly down-regulated genes in response to ethanol stress and their enriched GO process terms.

The distribution of the down-regulated genes for each strain was shown in Figure 3.19. Since the wild-type strain showed growth deficiency in the presence of ethanol, the processes associated with the down-regulated SEGs in the wild-type strain only (145 genes) were thought to be crucial to survive under high ethanol concentrations. Therefore,

these 145 genes were classified into functional categories using MIPS database [124]. The list of these functional categories having p-value < 0.05 is shown in Table 3.13.

Table 3.13. Functional categories of down-regulated SEGs in wild-type strain.

Description	**Functional Categories**
Metabolism	- phosphate metabolism - S-adenosyl-methionine - homocysteine cycle
Energy	- conversion to kinetic energy (e.g. movement)
Cell Cycle and DNA Processing	- DNA processing - DNA restriction or modification - DNA conformation modification (e.g. chromatin)
Protein Fate (folding, modification, destination)	- protein targeting, sorting and translocation - protein modification - modification by phosphorylation, dephosphorylation, autophosphorylation
Protein With Binding Function or Cofactor Requirement (Structural or Catalytic)	- thiamine pyrophosphate binding
Regulation of Metabolism and Protein Function	- regulation by modification - regulation of protein activity
Cellular Transport, Transport Facilitation and Transport Routes	- intra Golgi transport - vacuolar/lysosomal transport - vesicular cellular export - exocytosis
Cellular Communication / Signal Transduction Mechanism	- cellular signalling - enzyme mediated signal transduction - protein kinase
Biogenesis of Cellular Components	- cytoplasm

The functional categories of 145 SEGs which are down-regulated in wild-type strain revealed that ethanol affected the most important cellular processes that obstruct the growth and viability of the cells. The categories related to phosphate metabolism, energy,

DNA processing, protein modification and targeting, transport and cellular signaling were significantly concentrated (Table 3.13). The importance of these processes in ethanol stress response was already established in literature. Among these categories; phosphate metabolism and transport were detected in the functional categories of the SEGs that are up-regulated in *pmt7*Δ/*pmt7*Δ and/or yhl042wΔ/yhl042wΔ strains in response to ethanol stress. Such a difference might verify the important roles of phosphate metabolism and phosphate containing compounds together with the transport mechanisms for survival under high ethanol concentration conditions. Moreover, these results also revealed the difference between *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains, and the wild-type strain in response to ethanol stress.

3.2.3.5. Analysis of Transcriptional Response to Ethanol Stress in Context of Reporter Features. The transcriptional response of *S. cerevisiae* strains to ethanol stress was analyzed in the context of regulatory networks using Reporter Features Analysis to shed light into the regulatory machineries affected from ethanol stress. Microarray data obtained from samples taken 2 h after ethanol treatment from treated cultures (E8) were compared with the data obtained from samples taken 2 h after ethanol treatment from untreated cultures (E0). The Reporter TF analysis identified six, seven, and 12 TFs for the wild-type strain (Figure 3.20A) and *pmt7*Δ/*pmt7*Δ (Figure 3.20B) and yhl042wΔ/yhl042wΔ (Figure 3.20C) strains, respectively. Regulatory pathways, including stress response, amino acid metabolism, and respiration, were commonly affected in the presence of ethanol stress in all strains. Since the regulation of stress response and amino acid metabolism play the key role to adapt elevated amounts of ethanol, their existence is important for cell survival in the presence of ethanol.

Reporter TFs identified in wild-type strain in a response to the presence of extracellular ethanol include TFs involved in regulation of phosphate metabolism (Pho4), and chromatin remodeling (Abf1). Although regulation of these pathways are critical for cell survival under ethanol stress condition, genes related to phosphate metabolism, DNA repair and DNA processing were down-regulated in response to ethanol stress in wild-type strain. Therefore, the negative regulation of these pathways in the wild-type strain might result in decreased viability under ethanol stress.

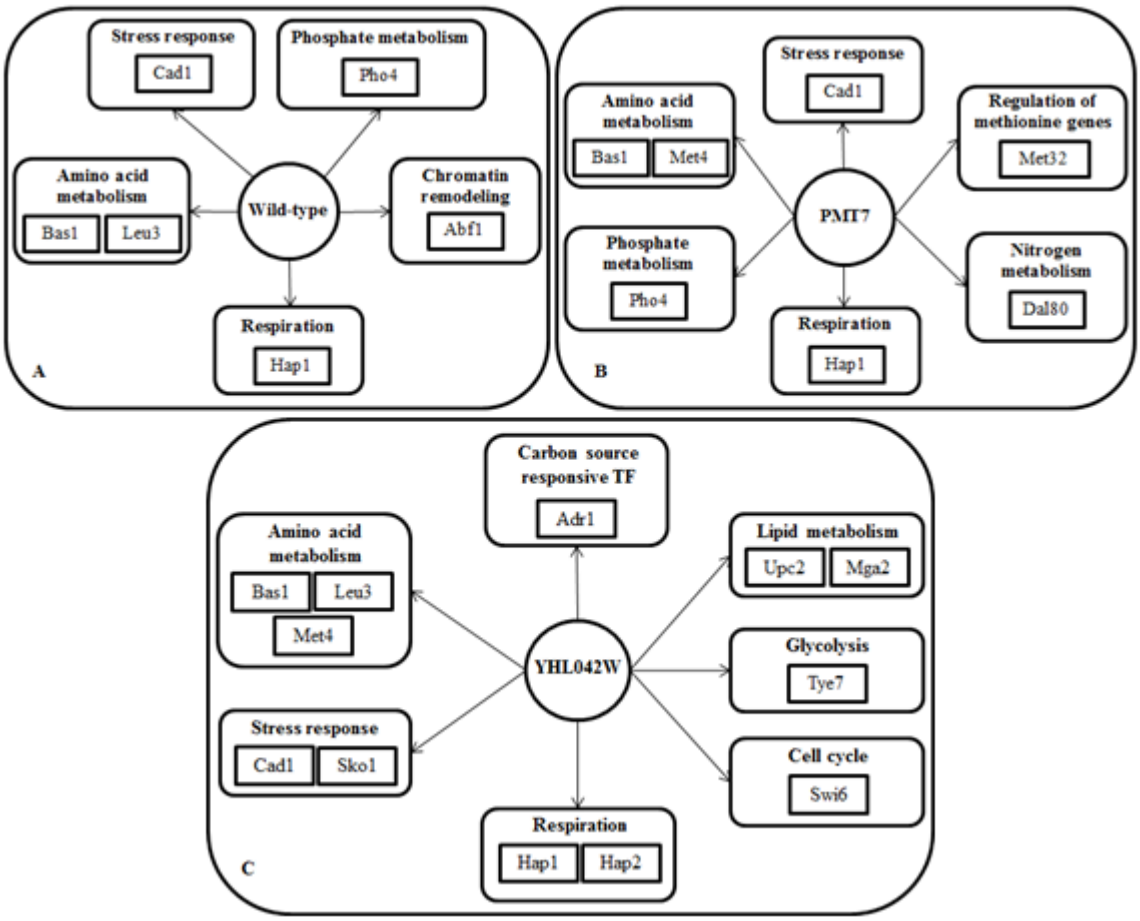

Figure 3.20. Regulatory TFs around which genes were affected in response to ethanol stress (a) wild-type strain (b) *pmt7*Δ/*pmt7*Δ and (c) yhl042wΔ/yhl042wΔ strains.

Reporter TFs for *pmt7*Δ/*pmt7*Δ strain include TFs involved in regulation of amino acid metabolism (Met4, Bas1), phosphate metabolism (Pho4), nitrogen metabolism (Dal80), stress response (Cad1), regulation of methionine gene (Met32), and respiration (Hap1). Reporter TFs for yhl042wΔ/yhl042wΔ strain include TFs involved in regulation of cell cycle (Swi6), glycolysis (Tye7), lipid metabolism (Upc2, Mga2), amino acid metabolism (Bas1, Leu3, and Met4), stress response (Cad1, Sko1) and pathways that metabolize non-fermentable sugars (Adr1). Since phosphate and lipid metabolisms are crucial in maintaining plasma membrane integrity in the cell during ethanol stress, the regulation of these pathways support the improved tolerance of both mutants.

In *S. cerevisiae*, nitrogen source utilization and amino acid biosynthetic pathways function in parallel [136] and yeasts are capable of utilizing varying sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell, such as

amino acids and consequently peptides and proteins, nucleic acids and vitamins. Therefore, the detection of regulators involved in nitrogen metabolism in *pmt7*Δ/*pmt7*Δ strain also supports the alterations in amino acid metabolism during ethanol stress.

The Reporter Proteins around which genes were affected by ethanol stress were also identified using tETN. When the p-value threshold was maintained as 0.01, only one protein, Frk1p, was identified for the wild type, *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains. Frk1p is a protein kinase of unknown cellular role. Frk1p have interactions with 34 other proteins in tETN. Seven of the interacting proteins of Frk1p are putative proteins of unknown function and the remaining proteins are rRNA transcription and ribosome biogenesis factors involved in protein folding, RNA processing, DNA replication, chromatin organization, response to stress, response to DNA damage, DNA repair, lipid metabolic process, response to chemical stimulus, and transmembrane transport. Since the Reporter Protein analysis led to the same protein for wild-type, *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains, this analysis could not facilitate to understand the difference between ethanol stress responses of these strains. However, the strong link between the functions of the interacting proteins of Frk1p and the mechanisms affected by ethanol stress revealed the importance of Frk1p in ethanol tolerance mechanism in *S. cerevisiae*.

3.2.4. Concluding Remarks

An integrative system-level investigation was carried out to shed light into the molecular mechanism of ethanol stress response and the precise roles of *PMT7* and *YHL042W* genes in ethanol tolerance of *S. cerevisiae*. The transcriptional changes were most frequently identified in energy related mechanisms and the respiratory mechanism following *PMT7* deletion, which indicated that *PMT7* may possibly have a role in cellular respiration mechanism in *S. cerevisiae*. The deletion of *YHL042W*, a member of DUP380 subfamily, which composed of genes encoding proteins consisting of 380 amino acids, caused the transcriptional changes of genes involved in the amino acid metabolism. The regulation of stress response pathways were also influenced by both gene deletions.

The reprogramming of gene expression in *S. cerevisiae* in response to ethanol stress has been examined and the observation of the genome-wide response revealed different

cellular regulatory machineries of yeast cells to protect and recover from the stressed condition. Analysis of the transcripts that were significantly responsive to ethanol stress revealed changes in the expression of genes involved in mitochondria, transport, localization, RNA processing, amino acid and nitrogen metabolisms for all strains. Following the ethanol treatment, different from the wild-type strain, alterations in the expression levels of genes that take part in carbohydrate, phosphate, nitrogen and lipid metabolisms were detected in both *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains. The present study indicated a strong link between these mechanisms with the ethanol tolerance phenotype.

4. INVESTIGATION OF THE ETHANOL PRODUCTION VIA ONE STEP CONVERSION OF STARCH BY *SACCHAROMYCES CEREVISIAE*

This chapter of the study investigates one step bioconversion of starch into ethanol. In the first part of the study, the global transcriptional response of a genetically engineered *S. cerevisiae* strain WTPB-G was investigated with the aim of understanding the effect of genetic modification which gained the ability of starch utilization to wild-type cells by the introduction of a plasmid harboring gene encoding a fusion protein. In the second part of the study, considering the importance of bioethanol production from different types of substrates, the amylolytic strain was assessed for its capability to produce ethanol from both raw starch substrates.

4.1. Investigation of the Global Transcriptional Response of A Genetically Engineered *Saccharomyces cerevisiae* Strain for One Step Bioconversion of Starch to Ethanol

4.1.1. Background Aspects

Depletion of fossil fuels and environmental problems make biomass attractive as a source of renewable energy [137]. One step conversion of biomass to ethanol, is considered the most cost-effective route to renewable fuels [7]. Besides the fermentation substrate, selection of appropriate production organism is also important for the economic viability of fuel ethanol. *S. cerevisiae* is the major microbial species that has been exploited for ethanol production due to its high fermentation rate and ethanol tolerance [138–140]. After cellulose, starch is the second most abundant plant polysaccharide and hence is an important renewable resource for bioethanol production.

Starch is a biopolymer and defined as a homopolymer consisting only one monomer, D-glucose and these D-glucose structures are linked together via α-1,4 and α-1,6 glycosidic linkages. The α-1,4 linkages produce linear chains that primarily comprise molecules

called amylose, whereas the α-1,6 linkages serve as branching points to produce branched chain molecules called amylopectin. Amylose forms the minor part (20-30%) and amylopectin forms the major part (70-80%) of starch [141]. In order to produce ethanol from starch, it is necessary to break down the chains of this carbohydrate into six carbon sugars, which can be converted into ethanol by yeasts [21,142,143]. The starch-based bioethanol industry has been commercially viable for about 30 years and corn and wheat are the most utilized sources of starch employed for ethanol production [4,142,143].

The conventional multi-step processes of ethanol production from starch by yeasts involve high-cost processes, such as gelatinization of raw starch by cooking, its liquefaction by *α*-amylase, and its saccharification to glucose by glucoamylase [144]. Hydrolyzation of starch is catalyzed by α-amylase and glucoamylase. At the first step, a thermostable α-amylase enzyme is used to produce soluble dextrins by hydrolyzing α-1,4 bonds. Then, in the saccharification step, glucoamylase is used to convert the liquefied starch into C6 sugars [145,146] and then in the fermentation step, C6 sugars are converted into ethanol [147]. When starch is used as a raw material, the amylases are strongly inhibited by hydrolysis products, such as glucose. This problem can be overcome by a simultaneous saccharification and fermentation (SFF) process, which combines enzymatic hydrolysis with fermentation [146,148].

S. cerevisiae can ferment only certain mono- and disaccharides such as glucose, fructose, maltose and sucrose, but lacks the ability to utilize starch, hence to synthesize and secrete necessary amylolytic enzymes which are required for the bioconversion of starch to ethanol. Therefore, consolidated bioprocessing of starchy materials to bioethanol by *S. cerevisiae* has required significant strain improvement efforts to introduce this ability to *S. cerevisiae* strains [144]. A plasmid-based system, WTPB-G [147], was developed by transforming a standard laboratory strain FY23 with the pPB-G plasmid [149] that contains the *Bacillus subtilis* α-amylase and the *Aspergillus awamori* glucoamylase coding sequences expressed under the control of the constitutive PGK1 promoter as an excreted bifunctional fusion protein [147]. Although significant improvements were thereby achieved with the amylolytic *S. cerevisiae* strain WTPB-G in ethanol yield from soluble starch by optimizing fermentation conditions, a time dependent loss of amylolytic activity was observed [49].

The aim of this study was to investigate the effect brought about to a wild-type strain by the presence of an extrachoromosomal self-replicating element in the form of a recombinant multicopy plasmid. For this, the fermentation performances of wild-type FY23 and the plasmid-bearing strain WTPB-G were comperatively examined in batch cultures, in which the cultivation temperature and pH were controlled, under aerated and micro-aerated conditions. The investigation of the genome-wide gene expression profiles revealed that the presence of a plasmid encoding a fusion protein caused changes in the expression levels of genes associated with cell wall biogenesis, ribosome biogenesis and RNA processing, whereas oxygen limitation resulted in the significant expression of genes involved in protein modification, respiration, and energy mechanisms.

4.1.2. Methods

4.1.2.1. Strains and Growth Media. *S. cerevisiae* WTPB-G strain [147], generated by transforming the parental haploid FY23 strain with the pPB-G plasmid [149] that contains *B. subtilis* α-amylase and *A. awamori* glucoamylase genes expressed under the control of the constitutive *PGK1* promoter as an excreted bifunctional fusion protein was used in this study.

S. cerevisiae strains were kept in glycerol stock solutions at -80ºC. Frozen glycerol cultures were used by streaking on selective yeast minimal media, YMM (20 gL^{-1} D-glucose, 6.7 gL^{-1} yeast nitrogen base without amino acids, 0.1 gL^{-1} tryptophan, 0.1 gL^{-1} uracil) agar plates for the preparation of master plates. Single colonies formed at 30ºC were transferred to YPDS (4 gL^{-1} D-glucose, 20 gL^{-1} peptone, 10 gL^{-1} yeast extract, 10 gL^{-1} soluble starch) and YMM plates and incubated at 30ºC to select for amylolytic activity. YPDS plates were stained with iodine vapor until the formation of large visible zones around colonies, indicating the utilization of starch. Colonies with larger haloes were picked from replica YMM plates and used for inoculum preparation. Agar plates were prepared by adding 18 gL^{-1} agar to the media.

Precultures were inoculated with a single colony of cells taken from YMM agar plates and incubated in YMM medium at 30ºC and 180 rpm.

4.1.2.2. Cultivation Conditions and Sampling. For batch bioreactor cultivations, cells were cultivated in fully controlled 2 L B-Braun Biostat B Plus fermenters, with a working volume of 1.5 L. The temperature was kept constant at 30°C and agitation was set to 400 rpm at all times throughout the fermentation. pH was kept constant at 5.6 by controlling with 0.5 M NaOH and HCl. The dissolved oxygen (DO) saturation was preserved above 90% throughout the experiment via a constant flow of air at 0.75 L/min in aeration controlled cultivations. To provide micro-aerated conditions, cultivations were initially brought to a dissolved oxygen saturation of 100% and air supply was turned off throughout the fermentation. ODs were monitored by spectroscopic measurements at 600 nm wavelength until the steady-state was reached. All experiments were carried out in duplicate.

Sampling was carried out during the exponential phase of growth for the determination of μ_{max}, extracellular glucose and ethanol concentrations. 95% confidence intervals were provided for μ_{max}. Extracellular metabolite concentrations were determined by using enzymatic analysis kits (Sigma) as described by the manufacturer. Samples were provided at the stationary phase to gravimetrically determine the DCW of cultures as described in Section 2.1.2.3. For the transcriptome analysis, samples were taken at the mid-exponential phase of growth ($OD_{600} \approx 0.7$-0.9), immediately frozen in liquid nitrogen and stored at -80^{o}C until RNA isolation.

4.1.2.3. Determination of Amylase Activity. Protein purification was performed with culture supernatants diluted with two volumes of ice-cold acetone and centrifuged at 10000 rpm for 20 minutes at 4ºC to recover extracellular precipitates [150]. To assay for α-amylase activity, pellets were dissolved in 50 mM NaAc buffer (pH 5.9) and 0.5 g of soluble starch was added to 100 ml boiling 50 mM NaAc buffer (pH 5.9). 100 µl appropriately diluted enzyme solutions were incubated with 500 µl starch solution at 40^{o}C for 10 minutes. 200 µl of this reaction mix was mixed with 5 ml of iodine solution (10 mM KI and 2 mM I_2). Degradation of starch was measured at 620 nm against 200 µl water in 5 ml of iodine solution as blank. One unit of α-amylase activity was defined as the quantity of enzyme required to hydrolyze 0.1 mg starch in 10 minutes at 40^{o}C.

For the glucoamylase activity assay, pellets were dissolved in 50 mM NaAc buffer (pH 4.5) and 0.5 g of soluble starch was added to 100 ml boiling 50 mM NaAc buffer (pH 4.5). 50 µl enzyme solution was mixed with 500 µl starch solution and incubated at 40^{o}C for 10 minutes. An aliquot (200 µl) of this reaction mix was used to assay for glucose. Glucose concentration was determined using enzymatic analysis kits. One unit of glucoamylase activity was defined as the amount of enzyme that released 1 mM of glucose per minute from starch.

4.1.2.4. RNA Isolation and Microarray Analysis. RNA isolation and microarray analysis were carried out as described in Section 3.2.2.3.

4.1.2.5. Microarray Data Acquisition and Analysis. For the analysis of transcriptomics data, CEL files were normalized via quantile normalization using RMA [119] as implemented in the *affy* package [120] of R/Bioconductor suite of tools [121]. Significantly expressed genes were identified from the normalized log-expression values using a two-way ANOVA implemented in MultiExperiment Viewer (MeV) [151] and 0.001 p-value threshold was maintained. 2 x 2 factorial design was used to investigate the effect of two different factors, strain (S) and aeration (A), on the transcriptional response of yeast cells. Both factors had two levels; plasmid-bearing strain vs. control, aerated vs. micro-aerated. A two-way ANOVA revealed both possible main effects and their interactive effect on gene expression. The genes, that were identified to be significantly expressed in response to interactive effect (S x A), were responsive to the changes in both factors at the same time. Statistically significant genes identified for all factors were used as inputs for gene set enrichment analysis based on GO annotations. All gene ontology analysis was performed using the Amigo software [123]. The Gene Ontology (GO) enrichment analysis was performed via GO Term Enrichment tool in Amigo by using *Saccharomyces* Genome Database (SGD) filter, with a maximum p-value of 0.01 and minimum number of gene products of 2.

4.1.2.6. Clustering of Conditions. Conditions were hierarchically clustered using the normalized microarray data. The hierarchical clustering of the conditions was carried out via MeV with the distance metric selected as the Pearson correlation and the average linkage as the linkage metric.

4.1.2.7. Reporter Features Analysis. The transcriptional responses to both the presence of plasmid and oxygen limitation were analyzed using Reporter Features analysis as described in Section 3.2.2.6.

4.1.3. Results and Discussion

4.1.3.1. The Growth Characteristics of Batch Fermentations. The growth characteristics of the batch cultures were investigated during the exponential and stationary phases of growth. The fermentation performances of the plasmid-bearing strain WTPB-G, together with the wild-type strain FY23, grown on YMM, were investigated and compared under both micro-aerated and aerated conditions (Figure 4.1). Maximum biomass, ethanol and glycerol concentrations reached by the cultures, as well as their glucose utilization rates and maximum specific growth rates were measured (Table 4.1).

Table 4.1. Fermentation parameters of *S. cerevisiae* strains.

Parameter	**Aerated**		**Micro-aerated**	
Strains	**WT (FY23)**	**WTPB-G**	**WT (FY23)**	**WTPB-G**
Biomass (gL^{-1})	2.59	2.69	2.26	2.12
μ_{max} (h^{-1})	0.2696 ±0.0042	0.2143 ±0.0084	0.2358 ±0.0055	0.2026 ±0.0068
Max. Ethanol Conc. (gL^{-1})	4.93±0.33	4.79±0.46	4.26±0.62	3.91±0.24
Glucose Utilized (gL^{-1})	17.61	17.58	18.52	18.14
Y_{ps} (g ethanol g^{-1} glucose)	0.28	0.27	0.23	0.22
Y_{px} (g ethanol g^{-1} biomass)	1.90	1.78	1.89	1.85
Max. Glycerol Conc. (gL^{-1})	0.085	0.092	0.152	0.174
$Y_{gly/s}$ (g glycerol g^{-1} glucose)	0.003	0.003	0.008	0.010

Under aerated conditions, both strains reached higher biomass concentrations. Aeration had more profound effect on the growth properties of the WTPB-G strain than the wild-type strain. Oxygen limitation caused 21% and 34% decreases in biomass concentrations of FY23, and WTPB-G cultures, respectively. Similar effect was also observed in μ_{max} values of both cultures. *S. cerevisiae* physiology was affected by aeration

which is a significant control factor during alcoholic fermentation. It has been reported that aeration improved fermentation performances of *S. cerevisiae* [152–154] and also, in this study, higher ethanol concentrations were obtained under aerated conditions with both strains. Furthermore, oxygen limitation caused approximately 3-fold increase in glycerol concentrations and yields of both strains, which is also consistent with the formation of glycerol at much higher yield under anaerobic conditions than that of the aerobic conditions. Under oxygen limited conditions, glycerol is formed to modulate NADH and NADPH levels to restore the redox balance in the cell [153,155].

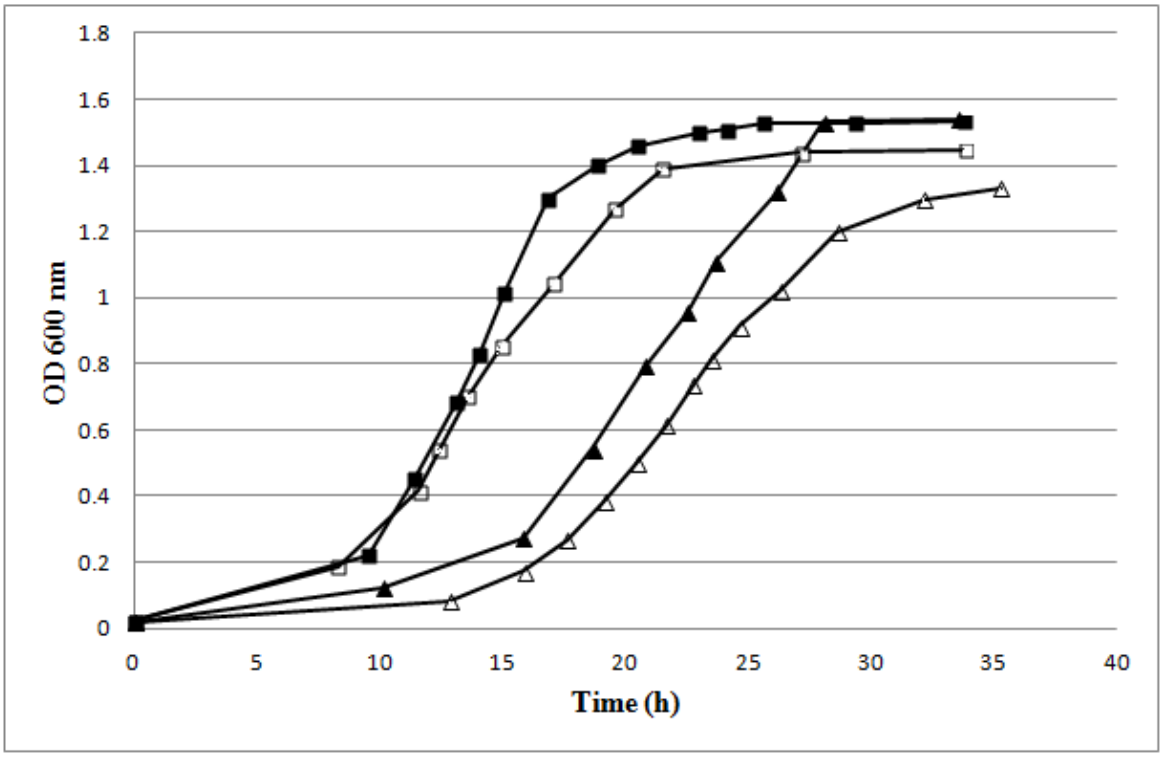

Figure 4.1. Growth profiles of FY23 (□, ■) and WTPB-G (▲, △) strains under aerated (filled symbols) and micro-aerated (open symbols) conditions.

At the end of each fermentation, that were carried out under aerated and micro-aerated conditions with WTPB-G strain, enzymes in the cell-free media were precipitated with acetone and crude enzyme solutions were subjected to amylase activity assays. The α-amylase activities were 20 U/ml culture and 22 U/ml culture, and glucoamylase activities were 227 U/L culture and 278 U/L culture under micro-aerated and aerated conditions, respectively. Since fermentations were carried out in media containing only glucose as a carbon source, the α-amylase activities of WTPB-G in both conditions were found to be

very low when compared to earlier reports. Glucoamylase and α-amylase activities were reported to be within the range of 300 to 9000 U/ml and 700 to 1200 U/ml, respectively in media containing 5% starch [147].

4.1.3.2. Global Transcriptional Response of *S. cerevisiae* Strains. The aim of this study was to provide a further insight into the effect of plasmid presence and the secretion of fusion proteins in the recombinant amylolytic *S. cerevisiae* strain WTPB-G. Since higher ethanol concentrations were reached by WTPB-G and its parent strain FY23 cultures under aerated conditions when compared to micro-aerated conditions, the mechanisms affected by aeration was also investigated. The two parameters of interest were strain at two levels, the reference strain FY23 and WTPB-G; and aeration at two levels, aerated and micro-aerated conditions. 2 x 2 factorial design enabled the investigation of the effect of these factors on the transcriptional response of yeast cells. For these purposes, samples collected at the mid-exponential phase of growth were used. The genes showing significant changes in their expression levels in response to each factor were identified via two-way ANOVA analyses. The transcriptional changes were assessed according to three factors; the effect of plasmid presence, the effect of aeration, and their interactive effect.

4.1.3.3. Clustering of Conditions. The hierarchical clustering of conditions using the normalized microarray data revealed strains as the key determinant for the arrangement of different clusters. Analysis resulted in the clustering of the conditions into two major clusters (Figure 4.2). The transcript profiles of wild-type cultures were clustered together under micro-aerated and aerated conditions; whereas the transcript profiles of WTPB-G cultures under micro-aerated and aerated conditions formed a different cluster. Since the micro-aerated and aerated conditions were clustered together for both strains, the effect of aeration was found to be less determinative on the transcriptional response than the effect of plasmid harboring a genetically engineered plasmid which produces a bifunctional enzyme to catalyze the one step conversion of starch to ethanol. Moreover, the replicate fermentations were clustered together for each strain under similar conditions.

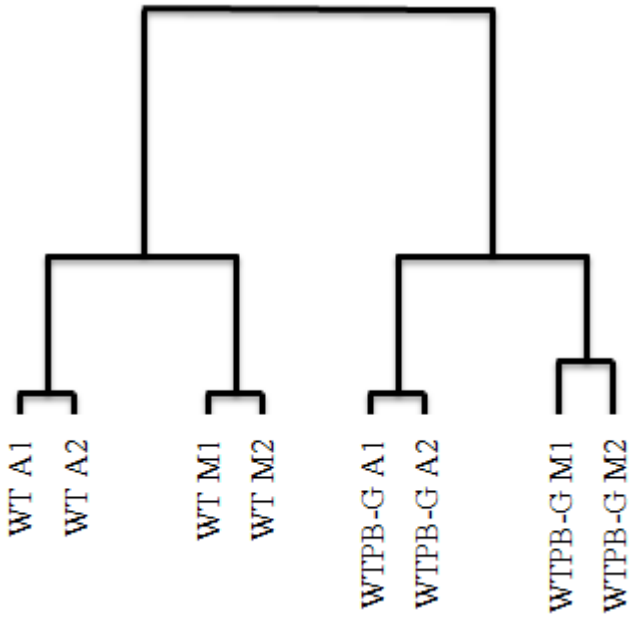

Figure 4.2. Hierarchical Clustering of Conditions (M and A represent the micro-aerated and aerated conditions, respectively).

4.1.3.4. The Identification of Significantly Expressed Genes. The genes showing significant changes (p-value < 0.001) in their expression levels were identified via two-way ANOVA analyses. When WTPB-G was compared to the reference strain, a total of 950 (524 up, 426 down) genes displayed significantly altered expression levels in response to the presence of a plasmid synthesizing a bifunctional protein which may catalyze the one step conversion of starch to ethanol. Genes whose expression levels were significantly up- and down-regulated under oxygen limitation were screened using microarray data obtained from samples collected at the mid-exponential phase of growth and 579 (309 up, 271 down) genes showed statistical significant difference in their expression levels when the micro-aerated condition was compared to the aerated condition. Moreover, 72 genes were significantly expressed in response to the interactive effect of the presence of plasmid and oxygen limitation.

Genes, whose expression levels were significantly changed in response to the presence of plasmid, were defined as strain significant genes; and genes, whose expression levels were significantly changed in response to oxygen limitation, were defined as aeration significant genes. Significantly enriched categories of the up- and down-regulated strain significant and aeration significant genes were analyzed using Amigo software. The

enriched GO process terms of strain and aeration significant genes are provided in Table 4.2.

4.1.3.5. Transcriptional Response to the Presence of Plasmid. In response to plasmid-bearing, the up-regulated genes were significantly enriched for cell wall organization and biogenesis and the down-regulated genes were significantly enriched for growth associated processes including ribosome biogenesis and RNA processing (Table 4.2). Ribosome biogenesis is an essential cellular process and the inhibition of this process may result in a terminated cell growth even under optimal growth conditions [156]. Although the genes associated with ribosome biogenesis were down-regulated, the growth performances of WTPB-G strain were not inhibited. There are approximately 420 known genes involved in ribosome biogenesis in *S. cerevisiae*, and only 60 of them were down regulated in WTPB-G strain. Therefore, the remaining genes enable this yeast to grow under controlled conditions and produce ethanol. Moreover, protein synthesis is an energy-cost activity in the cell, the down-regulation of genes related to ribosome biogenesis and RNA processing might result in reduced protein synthesis that are not essential for the cell to grow and help to save energy.

The manual investigation of genes related to DNA replication revealed that 5 genes (*POL1*, *POL2*, *RFA1*, *CDC9*, and *RNH201*) encoding proteins related to DNA replication in *S. cerevisiae* were down-regulated in response to the presence of plasmid. *POL1* is an essential gene, which encodes the largest subunit of the DNA polymerase (I) alpha and it is required for the initiation of DNA replication. *POL2* is also an essential gene encoding a catalytic subunit of DNA polymerase (II) epsilon, which is a chromosomal DNA replication polymerase that exhibits proofreading exonuclease activity. *POL2* is also involved in DNA synthesis during DNA repair. *RFA1* encodes a subunit of heterotrimeric Replication Protein A (RPA), which is a highly conserved single-stranded DNA binding protein. *RFA1* plays a key role in DNA metabolic pathways such as DNA replication, repair, and recombination. *CDC9*, an essential gene, encodes a DNA ligase found in the nucleus and mitochondria. Cdc9p is an essential enzyme that joins Okazaki fragments during DNA replication, and it also acts in nucleotide excision repair, base excision repair, and recombination. *RNH201* encodes a ribonuclease H2 catalytic subunit that removes RNA primers during Okazaki fragment synthesis. Furthermore, the presence of the plasmid

resulted in the up-regulation of 42 genes encoding proteins whose abundance increases in response to DNA replication stress.

4.1.3.6. Transcriptional Response to Aeration. Clustering of conditions indicated that aeration caused a less effective change than plasmid harboring on the transcriptional response. In response to oxygen limitation, up-regulated genes were found to be enriched with catabolic processes (Table 4.2). The GO enrichment analysis of the down-regulated aeration significant genes did not reveal any significantly enriched biological process terms. 29 genes (*RNR4*, *YIM1*, *COQ10*, *YPR1*, *CIR1*, *MET3*, *TSC10*, *FRD1*, *UGA2*, *TDH1*, *CYB5*, *RSF1*, *PEX11*, *COQ9*, *HYR1*, *AAC3*, *SUR2*, *BMH2*, *URA1*, *JAC1*, *ERG11*, *COX5B*, *MDH2*, *PDX3*, *SCS7*, *ECJ1*, *HEM13*, *RIB3*, *PHO85*) involved in oxidation-reduction processes, 5 genes (*VMA2*, *VMA3*, *VMA4*, *VMA7*, *VMA8*) involved in pH reduction, 11 genes involved in cofactor metabolic process (BUD17, CAB2, COQ10, COQ9, FAA1, HEM13, PDX3, RNR4, SAM1, SAM2, YMR178W), 8 genes (*SAM2*, *COQ10*, *BUD17*, *COQ9*, *SAM1*, *YMR178W*, *PDX3*, *CAB2*) involved in coenzyme biosynthetic process were down-regulated in response to oxygen limitation. Furthermore, oxygen limitation resulted in the down-regulation of genes involved in lipid and fatty acid metabolic processes (*BUD32*, *CAX4*, *CDS1*, *CMD1*, *CYB5*, *ECI1*, *EEB1*, *ETH1*, *ERG11*, *FAA1*, *FEN1*, *IDI1*, *PDR17*, *PDX3*, *PEX11*, *PGA1*, *PIS1*, *RER2*, *SCS2*, *SCS7*, *SEC14*, *SPO7*, *SUR2*, *TSC10*, *URA8*, *YMR210W*), carbohydrate metabolic process (*BMH2*, *BUD32*, *CAX4*, *GAS5*, *HXK1*, *IMP2*, *MDH2*, *MIG2*, *MNN10*, *MNN11*, *PFK27*, *PHO85*, *RER2*, *RHO1*, *SCW4*, *SVP26*, *TDH1*, *TYE7*, *VID24*, *YPR1*), and phosphate-containing compound metabolic process (*HOM3*, *PIL1*, *MOB2*, *SPO7*, *CPA1*, *VMA2*, *PRS2*, *CAF16*, *CDS1*, *PPH3*, *URA10*, *TPK3*, *RNA1*, *IDI1*, *CMD1*, *BUD32*, *URA4*, *SCS7*, *CAB2*, *AAH1*, *RTR1*, *YVH1*, *RAS1*, *COX5B*, *APA1*, *PDX3*, *SCS2*, *YMR178W*, *CKB2*, *YFH7*, *URA1*, *GCS1*, *PGA1*, *PFK27*, *ADE12*, *URA8*, *PIS1*, *FAP7*, *RNR4*, *PDR17*, *YER134C*, *YNL010W*, *CDC21*, *ADE1*, *YOR283W*, *BUD17*, *HXK1*, *PHO85*, *TUB1*, *SEC14*, *MCK1*). *S. cerevisiae* requires maintained cellular redox balance in order to sustain metabolism and growth. Redox cofactors participate in biochemical reactions involving oxidation-reduction [157]. The down regulation of genes related to oxidation-reduction in response to oxygen limitation is consistent with the decreased growth of both WTPB-G and reference strain cultures under micro-aerated conditions. Moreover, the down-regulation of oxidation-reduction, and coenzyme processes possibly decreases the amount of generated energy in the cells and this might

result in the repression of energy required metabolic processes, such as phosphate-containing compound metabolic process, lipid and fatty acid metabolic process.

Table 4.2. Biological processes affected in genetically engineered *S. cerevisiae* cells from the presence of the plasmid or aeration.

Enriched GO Process Terms		
	Strain Significant Genes	**Aeration Significant Genes**
UP	• cell wall organization or biogenesis • catabolic process • external encapsulating structure organization	• catabolic process • cellular catabolic process • organic substance catabolic process
DOWN	• ribosome biogenesis • ribonucleoprotein complex biogenesis • RNA processing • RNA phosphodiester bond hydrolysis • cellular component biogenesis • nitrogen compound process • endonucleolytic cleavage involved in rRNA processing • transmembrane transport	-

4.1.3.7. Transcriptional Response to the Interactive Effect of Factors. 72 genes were significantly expressed in response to interactive effect of plasmid bearing and oxygen limitation and described as interactive significant genes. When aerated condition was compared to micro-aerated condition, 32 genes were up-regulated and 40 genes were down-regulated in the reference strain. Under similar conditions, 25 genes were up-regulated and 47 genes were down regulated in WTPB-G. When the significantly expressed genes in WTPB-G in comparison to the reference strain were investigated, 32

genes were up-regulated and 40 genes were down-regulated under aerated conditions; and 37 genes were up-regulated and 35 genes were down-regulated in micro-aerated conditions.

Table 4.3. Significantly expressed genes in response to interactive effect in *S. cerevisiae* WTPB-G and the reference strain (number of genes was provided in parenthesis).

Effect of aeration	**WTPB-G**	**Reference strain**
UP	- Lipid metabolic process (4) - Golgi vesicle transport (4)	- Biological process unknown (7) - Lipid metabolic process (5) - Mitochondrion organization (5)
DOWN	- Biological process unknown (11) - Mitochondrion organization (5) - Carbohydrate metabolic process (4) - Generation of precursor metabolites and energy (4) - Ion transport (4) - Transmembrane transport (4)	- Biological process unknown (7) - Transmembrane transport (5) - Ion transport (4)

The significantly expressed genes in response to interactive effect were not found to be significantly enriched with any particular GO biological process term for each group. A total of 14 genes (*FMP21*, *GTT3*, *NQM1*, *REE1*, *YBL029W*, *YBR071W*, *YCL049C*, *YER053C-A*, *YKR075C*, *YNL040W*, *YNL058C*, *YNL234W*, *YOR131C*, and *YRO2*) had unknown GO biological process term. The processes in which these genes involved were summarized in Table 4.3 and Table 4.4 for each group.

4.1.3.8. Analysis of Regulation of Transcriptional Response to the Presence of Plasmid and Oxygen Limitation in genetically engineered *S. cerevisiae* cells. The transcriptional response of *S. cerevisiae* strain to the presence of pPB-G plasmid and oxygen limitation was analyzed in the context of regulatory networks using Reporter TF Analysis to shed light into the affected regulatory machineries.

Table 4.4. Significantly expressed genes in response to interactive effect under aerated and micro-aerated conditions in *S. cerevisiae* WTPB-G (number of genes was provided in parenthesis).

Effect of plasmid presence	**Aerated condition**	**Micro-aerated condition**
UP	- Biological process unknown (6) - Golgi vesicle transport (4)	- Biological process unknown (9) - Ion transport (6) - Lipid metabolic process (5) - Golgi vesicle transport (4) - Transmembrane transport (4) - Protein complex biogenesis (4)
DOWN	- Biological process unknown (8) - Mitochondrion organization (5) - Carbohydrate metabolic process (4) - Generation of precursor metabolites and energy (4) - Lipid metabolic process (4) - Ion transport (4) - Transmembrane transport (4)	- Biological process unknown (5) - Carbohydrate metabolic process (4) - Generation of precursor metabolites and energy (4)

Reporter TF analysis identified 3 TFs (Rts2, Hac1, Fkh1) around which genes were affected in response to the presence of plasmid. Rts2 is a basic zinc-finger protein, and it is similar to human and mouse Kin17 proteins, which are chromatin-associated proteins involved in UV response and DNA replication. Hac1 is a basic leucine zipper TF, which regulates the unfolded protein response. The protein abundance of Hac1 increases in response to DNA replication stress. Fkh1 has a minor role in the expression of G2/M phase genes, negatively regulates transcriptional elongation, and has a positive role in chromatin silencing. The existence of TFs involved in DNA replication is consistent with the presence of the self-replicating plasmid.

Reporter TF analysis identified 2 TFs (Mot3 and Rox1) around which genes were affected in response to oxygen limitation. Mot3 is a transcriptional repressor involved in repression of a subset of hypoxic genes by Rox1p. Rox1 is a repressor of hypoxic genes, and mediates aerobic transcriptional repression of hypoxia induced genes such as *COX5* and *CYC7*. The identification of Reporter TFs which regulates expression of hypoxic genes, is in good agreement with the effect of oxygen limitation during fermentations.

4.1.4. Concluding Remarks

In this study, the genome-wide transcriptional responses of the genetically engineered *S. cerevisiae* strain (WTPB-G) and its parental strain (FY23) were investigated to understand the mechanisms underlying the effects of the presence of plasmid together with the secretion of fusion protein and oxygen limitation during fermentations. WTPB-G was grown in media containing only glucose as a sole carbon source, in order to compare with its wild-type FY23.

The analysis of the significantly expressed genes revealed that plasmid replication induced cell wall organization and biogenesis, and repressed approximately 14% of the genes involved in ribosome biogenesis and RNA processing. Oxygen limitation was found to be less effective in the transcriptional changes. A link between oxygen limitation and genes involved in oxidation-reduction, pH reduction, phosphate-containing compound, lipid and fatty acid processes, was observed.

4.2. Fermentation Characteristics of *Saccharomyces cerevisiae* WTPB-G in Ethanol Production from Raw Starch

4.2.1. Background Aspects

The development of improved bioethanol production technologies will remain be of increasingly important issue as the world moves to the use of renewable resources for fuels. *S. cerevisiae* is an attractive host organism having the ability to express foreign genes through its secretory pathway. Therefore, the recombinant *S. cerevisiae* strains have been using to extend the substrate range of this microorganism to produce industrially important

goods. Extending the substrate range of *S. cerevisiae* for ethanol production is an important area of interest for not only utilizing new raw materials, such as agricultural wastes, but also reducing the waste amount coming from industrial processes [158].

Starchy food crop residues are costly to preserve; therefore, their use for biofuel production is considered as a more efficient way to utilize them and, indeed, has led to a general preference for starchy feedstocks for bioethanol production [150]. Although there are several studies on ethanol production from soluble starch using recombinant amylolytic *S. cerevisiae* strains, a few yeast strains were reported to utilize raw starch substrates. In some studies of particular importance, several recombinant *S. cerevisiae* strains were reported to be capable of fermenting raw corn starch by co-displaying amylolytic enzymes on their cell surface [159–161]. However, this system was found to be effective only in solid-state fermentation conditions, rather than the conventionally used liquid fermentations [162]. On the other hand, strains secreting *Streptococcus bovis* α-amylase and having *Rhizopus oryzae* glucoamylase/α-agglutinin fusion protein anchored on their surface were improved by switching from plasmid-based expression to multi-copy integration of the amylolytic genes followed by mating of the two haploid integrants [141]. The efficiency of the system was further improved by polyploidization through cell fusion and the constructed tetraploid yeast strain was reported to produce ethanol from raw starch efficiently [137,163].

The *S. cerevisiae* WTPB-G [147] was developed by transforming a standard laboratory strain FY23 with the pPB-G plasmid. The fermentation characteristics of *S. cerevisiae* WTPB-G were assessed in media containing soluble starch [147]. Although WTPB-G was succeeded in one step conversion of starch into ethanol, the stability of the pPB-G plasmid was found to drop less than 20% after 100 h of fermentation [147]. Then, fermentation conditions were optimized in order to prevent the observed decrease in the time dependent amylolytic activity and this resulted in significant improvements in ethanol yield from soluble starch [49].

The ultimate aim of the present study was to assess the performance of the genetically engineered amylolytic *S. cerevisiae* strain WTPB-G, for the ethanol production by direct fermentation of raw starch substrates. The results indicated that WTPB-G

produced considerably higher concentrations of ethanol from soluble starch rather than raw starch. However, the ethanol yield reached with corn starch was found to be comparable with the yield reached with soluble starch.

4.2.2. Methods

4.2.2.1. Strains and Growth Media. The amylolytic *S. cerevisiae* strain WTPB-G was used in this study. Selection for amylolytic activity of WTPB-G strain and preculture preparation were carried out as described in Section 4.1.2.1.

4.2.2.2. Cultivation Conditions. WTPB-G strain was grown in complex YPDS medium with 1% (w/v) initial starch content. Besides soluble starch, unmodified wheat starch (Sigma S5127) and unmodified corn starch (Sigma S4126) were also used as substrates. A preculture at a volume fraction of 1% was used to inoculate the culture.

Shake flask cultures were kept with vigorous shaking at 30°C and 180 rpm. Samples taken from the culture at regular intervals were centrifuged at 8000 rpm for 6 min to determine substrate utilization, extracellular product formation, and metabolite concentrations.

4.2.2.3. Determination of Maximum Specific Growth Rate and Dry Cell Weight. Maximum specific growth rate and dry cell weight were determined as described in Section 2.1.2.3.

4.2.2.4. Extracellular Metabolite Analysis. Extracellular metabolite concentrations were determined using enzymatic analysis kits as described by the manufacturer (Sigma).

4.2.2.5. Determination of Starch Concentration. In order to determine residual starch concentrations, samples taken from the culture at regular intervals were centrifuged at 10000 rpm for 1 min. The sample supernatant was mixed with 1.5 ml of iodine solution. ODs were monitored at 550 nm wavelength against blank containing 1.5 ml of iodine solution. OD measurements were converted to starch concentration by using starch calibration curve prepared with known amounts of starch.

4.2.3. Results and Discussion

4.2.3.1. Fermentation Parameters of WTPB-G Strain. The fermentation performances of WTPB-G strain were evaluated by analyzing the growth, starch utilization, ethanol and glycerol production of shake-flask cultures. Maximum biomass, ethanol and glycerol concentrations reached by the cultures, as well as their maximum specific growth rates are provided in Table 4.5. Ethanol production rates were determined from ethanol concentrations during the exponential phase of growth. In addition the ethanol yield on biomass (Y_{px}) was also determined for WTPB-G grown in various types of starch containing media (Table 4.5).

Growth associated properties indicated that WTPB-G culture grown in soluble starch were found to produce 4.9 and 1.5 fold higher biomass than that of WTPB-G culture grown in wheat and corn starch, respectively. Moreover, growth on both wheat and corn starch were slower than that of soluble starch. Variations in soluble starch content of cultures are presented in Figure 4.3. WTPB-G cultures could not consume total starch in media, residual starch was found even after 135 hours of fermentation.

Table 4.5. Fermentation parameters of WTPB-G in different starch substrates.

Parameters	Soluble starch	Wheat Starch	Corn Starch
μ_{max} (h^{-1})	0.0619 ±0.0035	0.0453 ±0.0022	0.0565 ±0.0018
Final DCW	4.80±0.59	0.98±0.35	3.12±0.64
Max. Ethanol Conc. (g L^{-1})	2.292±0.211	0.123±0.328	1.222±0.257
Max. Glycerol Conc. (g L^{-1})	0.048	0.003	0.011
Ethanol production rate (g $L^{-1}h^{-1}$)	0.041	0.021	0.030
Y_{px} (g ethanol/ g biomass)	0.478	0.126	0.391

When ethanol productivities were compared, WTPB-G grown in soluble starch was found to produce ethanol at considerably higher concentration. However, the ethanol yields and rates revealed that WTPB-G had the ability to produce ethanol from corn starch, reaching comparable ethanol concentrations reached with soluble starch. On the other hand, wheat starch could not be used effectively to produce ethanol by WTPB-G strain.

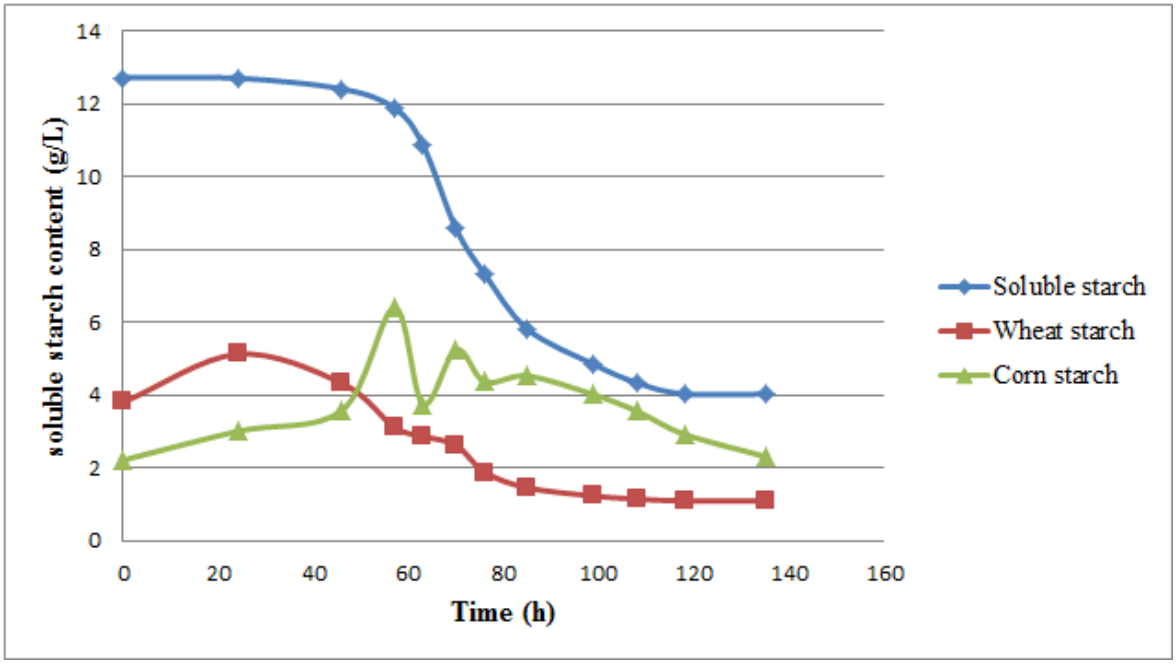

Figure 4.3. Starch concentrations of WTPB-G cultures.

4.2.3.2. Optimization of Starch Content. In order to optimize the starch content for improved ethanol yield, starch concentrations were varied while the concentrations of yeast extract and glucose were kept constant in YPDS media. It was reported that the plasmid stability depends on the starch concentration, such that high starch concentrations led to plasmid loss [150]. Therefore, fermentations were carried out by using 2, 3, and 5% (w/v) initial corn starch and the growth and production parameters were compared and presented in Table 4.6.

Increasing the initial starch concentration from 1% to 2%, 3%, and 5% (w/v) caused 32%, 96%, and 61% increase in the maximum ethanol concentration reached by WTPB-G, respectively. Ethanol yield on biomass increased up to 3% (w/v) starch content. However, ethanol yield decreased when corn starch concentration increased to 5%. These results showed the importance of the initial starch concentration in the improvement of ethanol production from WTPB-G. It has been reported that higher maximum specific growth rates caused higher plasmid stability [150]. The lower growth rate of WTPB-G in media containing 5% (w/v) might possibly cause plasmid loss and resulted in a lower ethanol yield. Previous reports indicated that the stability of pPB-G plasmid was also dependent to the starch concentration in media, and high starch concentrations led plasmid loss [150]. Furthermore, residual starch was found even after 140 h fermentation. The residual starch

percentages were similar for the initial starch concentrations of 2 and 3%. However, WTPB-G could not utilize 27% of the initial starch in media containing 5% corn starch, which resulted in 2 fold decreased ethanol yield on substrate.

Table 4.6. Fermentation parameters of WTPB-G in corn starch with varying amounts of initial starch content.

Parameters	**Initial corn starch content**		
	2% (w/v)	**3% (w/v)**	**5% (w/v)**
μ_{max} (h^{-1})	0.0592 ±0.0017	0.0605 ±0.0028	0.0563 ±0.0023
Final DCW	4.09±0.78	4.91±0.33	5.93±0.35
Max. Ethanol Conc. (g L^{-1})	1.619±0.124	2.395±0.198	1.962±0.157
Max. Glycerol Conc. (g L^{-1})	0.055	0.071	0.063
Ethanol production rate (g $L^{-1}h^{-1}$)	0.041	0.045	0.034
Y_{px} (g ethanol/ g biomass)	0.395	0.488	0.331
Residual starch after 140 h. fermentation (g L^{-1})	4.126	6.237	13.569
Residual starch (%)	20.6	20.8	27.1
Y_{ps} (g ethanol/ g starch)	0.102	0.101	0.054

4.2.4. Concluding Remarks

The fermentation characteristics of the amylolytic *S. cerevisiae* WTPB-G in one step conversion of raw starch substrates to ethanol were investigated in the present study. Shake flask cultures of WTPB-G grown in soluble starch and raw starch indicated that superior growth and ethanol production was associated with media containing soluble starch. Although WTPB-G could not utilize wheat starch effectively, the ethanol concentration reached by the utilization of corn starch was found to be comparable with the ethanol concentration reached with soluble starch. The ethanol production of WTPB-G from corn starch increased with the increasing initial starch concentration. However, ethanol yields of WTPB-G cultures decreased when 5% initial starch concentration was used for fermentations. These results revealed the importance of medium composition for efficient ethanol production from raw starch substrates by WTPB-G. Studies involving the

optimization of all components of the fermentation medium need to be carried out to increase productivity. Moreover, the high level expression of amylolytic enzymes by multi-copy integration of their coding sequences into the genome of a wild-type strain should result in stable extracellular amylolytic activities against raw starch substrates.

5. CONCLUSIONS AND RECOMMENDATIONS

Access to renewable energy is a requirement, not only for economic growth and sustainable development, but also for energy security and climate change alleviation. The proven performance of bioethanol as a fuel for transportation emphasizes its importance among liquid biofuels and microbial production of ethanol becomes an important issue for the whole world. The present study is a part of extensive research devoted to improve first and second generation bioethanol production that is currently needed in sustainable development.

5.1. Conclusions

In the first part of the study, in order to analyze the effect of different fermentation substrates on bioethanol production processes, ethanol production from biomass by five industrial *S. cerevisiae* strains, selected from *Saccharomyces* Genome Resequencing Project, were evaluated. This study revealed the importance of choosing the appropriate strain to be used for bioethanol production. One of the most important dependencies for strain selection is the ability of yeast cells to withstand stressful conditions that they might come up with during industrial fermentation processes. Therefore, performances of the industrial strains were assessed in the presence of ethanol, which is one of major stressor for yeast. All strains were found to tolerate up to 10% ethanol, which is in consistence with the fact that industrial yeast strains have the ability to grow under the stressful conditions in comparison to non-industrial strains. Among five strains, BC187 and Y9 had the highest tolerance to ethanol based on the colony-forming abilities and the ethanol production yields of Y9 were found to be superior to the others. Both ethanol production yields and growth ability under high ethanol concentrations made these strains valuable for bioethanol production processes. In order to assess the ethanol production from alternative and available feedstocks, fermentations were carried out on industrial by-products including as sugar beet pulp, starch and sugar beet molasses as well as biological residues like carrot, tomato and potato peel wastes. Among these feedstocks, both types of molasses were found to be the best source to produce yeast biomass and higher ethanol concentrations

were achieved with sugar beet molasses depicting its probable use in bioethanol production. Hence, the results of this part of the study indicated that the ability to utilize carbon sources available in agro-industrial residues is as important as strains' enhanced growth performances under stressful conditions for improved ethanol yields in industrial production processes.

The second part of the study deals with how yeast cells handle the increasing concentrations of ethanol during fermentations. A novel network based modular approach was developed to identify genes, which may have potential roles in ethanol tolerance in *S. cerevisiae* and to elucidate the biological processes related to ethanol tolerance mechanism. GO enrichment analysis of the constructed network revealed an association between ethanol tolerance and metabolic processes related to membrane stability, trehalose metabolic process, pH homeostasis, coenzyme/cofactor metabolisms, redox, stress response and transport. The modular analysis pointed four gene products having potential association with ethanol tolerance phenotype. Testing experimentally via homozygous single gene deletion mutant assays indicated that strains carrying *YDR307W* (*PMT7*) and *YHL042W* deletions were found to exhibit improved tolerance to ethanol. The ethanol tolerant phenotypes of these mutants also resulted in increased ethanol production and yield. Hence, the network based approach used in this study has proven to be highly succesful in the identification of novel gene targets for strain improvement studies.

In microbial production of ethanol, a better understanding of the ethanol tolerance and toxicity mechanisms is a significant issue for improving productivity. The reprogramming of yeast metabolism in response to ethanol stress was analyzed by monitoring changes at transcriptome level using a systems based approach. The transcriptional responses of wild-type, *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains were investigated after ethanol treatment to shed light into the molecular mechanism of the response to ethanol stress and the precise role of *PMT7* and *YHL042W* genes in ethanol tolerance in *S. cerevisiae*. A tight link between ethanol tolerance phenotype and lipid, fatty acid, ergosterol, trehalose, phosphate, and carbohydrate metabolisms, transport, energy, mitochondria, protein synthesis, tryptophan biosynthesis, and certain amino acid biosynthesis was observed. Results pointed to a possible role of *PMT7* in cellular

respiration mechanism whereas the *YHL042W* deletion caused transcriptional changes of genes involved in the amino acid metabolism.

In the last part of the study, one-step bioconversion of starch into ethanol was investigated using a plasmid-bearing *S. cerevisiae* strain WTPB-G. Investigating the changes of WTPB-G at transcriptional level indicated that the presence of plasmid synthesizing a bifunctional protein which may catalyze the one step conversion of starch to ethanol caused up-regulation of cell wall organization and biogenesis and down-regulation of ribosome biogenesis and RNA processing. Reporter TFs around which genes were affected in response to the presence of plasmid, were found to be associated with DNA replication which was reported to be important for plasmid stability. Furthermore, the effect of aeration was investigated by conducting fermentations under aerated and micro-aerated conditions with the reference strain and WTPB-G. Higher ethanol concentrations were obtained under aerated conditions with both strains. The transcriptional investigation revealed an association between oxygen limitation and genes involved in oxidation-reduction, pH reduction, phosphate-containing compound, lipid and fatty acid processes.

The ability of WTPB-G to produce ethanol from soluble starch was previously reported. In this study, one step conversion of raw starch substrates to ethanol was investigated. WTPB-G was found to produce ethanol at different rates with raw wheat and corn starch substrates. Ethanol production from corn starch was 10 fold higher than the ethanol production from wheat starch. The studies conducted to investigate the effect of initial starch concentration on ethanol production of WTPB-G from corn starch resulted in improved ethanol yields up to 5% (w/v) starch concentration; ethanol yields of WTPB-G cultures decreased when 5% initial starch concentration was used for fermentations. These results revealed the importance of medium composition for efficient ethanol production from raw starch substrates.

In conclusion, the intrinsic instability of the oil economy, as well as environmental concerns, have encouraged the investigation of alternative platforms for the sustainable production of fuels from renewable sources. Much research has focused on the next generation of biofuel technologies which use non-food crops, the non-food parts of edible crops, or wastes from agriculture or agro-industries. The application of systems biology

principles to biofuel technologies is still a challenging research area and this study provides further evidence that system based approaches are powerful tools to understand and improve bioethanol production processes.

5.2. Recommendations

This study focused on improving first and second generation bioethanol production by *S. cerevisiae*. Gained knowledge on the bioconversion of agro-industrial wastes into ethanol by industrial yeast strains, identification of novel gene targets for enhanced ethanol tolerance, the molecular mechanisms underlying ethanol tolerance, and the molecular mechanisms underlying one step conversion of starch to ethanol in *S. cerevisiae* might facilitate the rational design of genetically engineered yeast strains with improved bioethanol yields.

For further development of next generation ethanol production processes from various types of biomass, the high level expression of hydrolytic enzymes by multi-copy integration of their coding sequences into the genome of an appropriate industrial *S. cerevisiae* strain used in this study may result in stable extracellular amylolytic and cellulotytic activities against substrates of variable origin, including agricultural residues.

A detailed investigation of the ethanol tolerant *pmt7*Δ/*pmt7*Δ and yhl042wΔ/yhl042wΔ strains at the transcriptional level was carried out to understand the molecular mechanisms underlying ethanol tolerance in *S. cerevisiae*. A further detailed investigation of similar integrative systems-based analyses, especially at metabolome and proteome levels, may provide additional information on the reprogramming of yeast cells to high levels of ethanol.

One step conversion of starch was investigated using a plasmid-bearing *S. cerevisiae* WTPB-G. WTPB-G has the ability to synthesize a bifunctional protein which may catalyze the one step conversion of starch to ethanol. The investigation of the global transcriptional response of WTPB-G grown in starch containing media may facilitate to understand the molecular mechanisms underlying the plasmid presence and the expression and secretion of α-amylase and glucoamylase genes. Moreover, in order to distinguish the effect of

plasmid presence and secretion of the fusion protein, the transcriptional organization of the *S. cerevisiae* cells harbouring a plasmid not synthesizing any proteins need to be investigated. Furthermore, the high level expression of amylolytic enzymes by multi-copy integration of their coding sequences into the genome of a wild-type strain may result in stable extracellular amylolytic activities against raw starch substrates.

APPENDIX A: SUPPLEMENTARY TABLES FOR CHAPTER 3

Table A.1. Annotation collection table.

Cellular Component	Molecular Function		Biological Process	
GO:0000228	GO:0000166	GO:0008270	GO:0000122	GO:0008152
GO:0005575	GO:0003674	GO:0009055	GO:0000462	GO:0008610
GO:0005576	GO:0003676	GO:0009922	GO:0001302	GO:0008654
GO:0005622	GO:0003677	GO:0015662	GO:0001324	GO:0009405
GO:0005634	GO:0003700	GO:0016491	GO:0001682	GO:0009651
GO:0005655	GO:0003704	GO:0016563	GO:0005992	GO:0010286
GO:0005737	GO:0003824	GO:0016566	GO:0005995	GO:0010551
GO:0005739	GO:0003825	GO:0016717	GO:0005998	GO:0015828
GO:0005783	GO:0003850	GO:0016740	GO:0006006	GO:0016070
GO:0005786	GO:0003883	GO:0016757	GO:0006021	GO:0016126
GO:0005789	GO:0004197	GO:0016787	GO:0006221	GO:0019368
GO:0005792	GO:0004512	GO:0016853	GO:0006241	GO:0019575
GO:0005829	GO:0004526	GO:0016874	GO:0006350	GO:0019856
GO:0005886	GO:0004557	GO:0020037	GO:0006355	GO:0022900
GO:0005934	GO:0004564	GO:0030528	GO:0006457	GO:0030968
GO:0005935	GO:0004768	GO:0043130	GO:0006508	GO:0034599
GO:0005946	GO:0005048	GO:0043565	GO:0006526	GO:0034965
GO:0009277	GO:0005302	GO:0046526	GO:0006541	GO:0042149
GO:0016020	GO:0005488	GO:0046872	GO:0006562	GO:0043418
GO:0016021	GO:0005506	GO:0046941	GO:0006617	GO:0043618
GO:0030176	GO:0005509	GO:0051082	GO:0006629	GO:0044262
GO:0030479	GO:0005524		GO:0006633	GO:0045047
GO:0030678	GO:0008233		GO:0006636	GO:0045449
GO:0043332	GO:0008234		GO:0006810	GO:0045892
			GO:0006814	GO:0045944
			GO:0006897	GO:0046677
			GO:0006950	GO:0050826
			GO:0006970	GO:0051599
			GO:0007155	GO:0055085
			GO:0008033	GO:0055114
			GO:0008150	

Table A.2. Hub proteins of ETN and tETN.

Hubs of ETN		**Hubs of tETN**	
YPL031C	YFR034C	YDL229W	YMR308C
YDL229W	YDR174W	YPL031C	YGR060W
YPL240C	YDL014W	YPL240C	YPL106C
YHR135C	YPL235W	YHR135C	YDR448W
YNR051C	YLL024C	YNR051C	YOR244W
YNL154C	YGR060W	YNL154C	YPL235W
YBR160W	YPL106C	YAL005C	YFR034C
YAL005C	YDR448W	YBR160W	YLL024C
YDR381W	YBR154C	YDR381W	YBR154C
YNL189W	YHR079C	YNL189W	YGR252W
YGL180W	YML012W	YDL140C	YML012W
YDL140C	YHR114W	YGL180W	YFL039C
YBL007C	YFL039C	YER148W	
YBR106W	YJR010C-A	YBL007C	
YER148W	YGR252W	YBR106W	
YGL137W	YDR190C	YPL082C	
YPL082C	YLR372W	YGL137W	
YBR159W	YOL012C	YBR159W	
YMR186W	YGL112C	YMR186W	
YHR007C	YGL200C	YHR007C	
YPR110C	YGR274C	YNL209W	
YDR477W		YPR110C	
YNL209W		YGL120C	
YOR244W		YDR477W	
YMR308C		YJL095W	
YJL095W		YDL014W	
YGL120C		YDR174W	

Table A.3. Bingo Results for hub proteins of ETN.

Description	**p-value**
response to stress	1.80E-09
regulation of macromolecule biosynthetic process	4.21E-09
regulation of cellular biosynthetic process	6.20E-09
regulation of biosynthetic process	6.94E-09
biological regulation	1.10E-08
response to stimulus	2.28E-08
regulation of biological process	3.44E-08
regulation of macromolecule metabolic process	4.93E-08
chromatin organization	1.02E-07
regulation of gene expression	1.03E-07
regulation of cellular metabolic process	1.11E-07
regulation of primary metabolic process	1.11E-07
chromatin modification	1.55E-07
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2.56E-07
regulation of nitrogen compound metabolic process	2.68E-07
regulation of metabolic process	3.93E-07
transcription	4.38E-07
positive regulation of cellular process	8.75E-07
regulation of transcription	9.90E-07
'de novo' protein folding	9.98E-07
positive regulation of biological process	1.58E-06
positive regulation of cellular metabolic process	2.02E-06
positive regulation of metabolic process	2.27E-06
cellular macromolecule metabolic process	2.79E-06

Table A.4. Bingo Results for hub proteins of tETN.

Description	**p-value**
response to stress	6.58E-09
response to stimulus	3.23E-08
positive regulation of cellular process	7.07E-08
positive regulation of biological process	1.31E-07
positive regulation of cellular metabolic process	2.06E-07
positive regulation of metabolic process	2.33E-07
regulation of macromolecule biosynthetic process	3.02E-07
regulation of cellular biosynthetic process	4.09E-07
'de novo' protein folding	4.25E-07
regulation of biosynthetic process	4.47E-07
biological regulation	1.04E-06
regulation of biological process	1.25E-06

Table A.5. Bingo Results for ETN.

Description	**p-value**
transcription	6.84E-86
regulation of transcription	4.24E-71
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.92E-56
regulation of nitrogen compound metabolic process	5.44E-56
regulation of gene expression	1.93E-48
regulation of transcription, DNA-dependent	2.00E-48
regulation of macromolecule biosynthetic process	8.85E-47
regulation of RNA metabolic process	4.59E-46
regulation of cellular biosynthetic process	5.28E-46
regulation of biosynthetic process	4.09E-45
oxidation reduction	1.08E-36
regulation of transcription from RNA polymerase II promoter	3.93E-36
regulation of macromolecule metabolic process	1.60E-34
regulation of cellular metabolic process	1.57E-33
positive regulation of transcription	1.37E-32
positive regulation of gene expression	3.43E-32
positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4.88E-31
positive regulation of nitrogen compound metabolic process	4.88E-31
regulation of primary metabolic process	2.65E-30
regulation of metabolic process	4.35E-28
positive regulation of transcription, DNA-dependent	2.21E-27
positive regulation of biosynthetic process	4.44E-27
positive regulation of cellular biosynthetic process	4.44E-27
positive regulation of macromolecule biosynthetic process	1.25E-26
positive regulation of RNA metabolic process	8.67E-26
positive regulation of cellular metabolic process	7.45E-25
positive regulation of metabolic process	4.61E-24
positive regulation of macromolecule metabolic process	1.81E-23
protein transport	2.16E-22
small molecule metabolic process	1.18E-21
establishment of protein localization	3.55E-21
positive regulation of cellular process	3.82E-20
positive regulation of transcription from RNA polymerase II promoter	5.49E-20
chromatin modification	7.02E-20
response to oxidative stress	3.06E-19
nitrogen compound metabolic process	4.18E-19
cellular nitrogen compound metabolic process	5.47E-19
cellular response to oxidative stress	7.05E-19
positive regulation of biological process	8.55E-19
RNA biosynthetic process	3.13E-18
transcription, DNA-dependent	3.63E-18

Table A.5. Bingo Results for ETN (cont.).

Description	**p-value**
chromatin organization	6.10E-18
regulation of cellular process	6.71E-18
protein folding	1.14E-17
small molecule biosynthetic process	5.34E-16
protein localization	9.25E-16
regulation of biological process	2.25E-15
organic acid metabolic process	1.03E-13
cellular ketone metabolic process	1.09E-13
biological regulation	1.17E-13
oxoacid metabolic process	1.70E-13
carboxylic acid metabolic process	1.70E-13
transcription from RNA polymerase III promoter	3.07E-13
organic acid biosynthetic process	8.44E-13
carboxylic acid biosynthetic process	8.44E-13
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	9.14E-13
macromolecule localization	9.53E-13
transcription initiation	9.88E-13
transcription from RNA polymerase II promoter	8.94E-12
cell aging	1.74E-11
replicative cell aging	1.85E-11
transport	2.40E-11
alcohol metabolic process	5.67E-11
glutamine family amino acid metabolic process	9.72E-11
cellular amino acid biosynthetic process	2.73E-10
negative regulation of transcription	2.94E-10
establishment of localization	3.55E-10
vesicle-mediated transport	3.83E-10
chromatin remodeling	4.01E-10
cellular nitrogen compound biosynthetic process	4.24E-10
amine biosynthetic process	4.45E-10
negative regulation of gene expression	5.20E-10
cellular amine metabolic process	1.36E-09
aging	1.74E-09
cellular response to chemical stimulus	1.81E-09
intracellular transport	3.45E-09
amine metabolic process	4.79E-09
negative regulation of macromolecule biosynthetic process	8.88E-09
response to stress	1.02E-08
cellular amino acid metabolic process	1.44E-08
nucleotide metabolic process	1.58E-08
nucleoside phosphate metabolic process	1.58E-08

Table A.5. Bingo Results for ETN (cont.).

Description	**p-value**
response to chemical stimulus	1.65E-08
glutamine metabolic process	2.24E-08
transcription initiation from RNA polymerase III promoter	2.29E-08
intracellular protein transport	2.32E-08
cellular amino acid and derivative metabolic process	2.74E-08
negative regulation of cellular biosynthetic process	3.20E-08
nucleic acid metabolic process	3.47E-08
ergosterol biosynthetic process	3.99E-08
phytosteroid biosynthetic process	3.99E-08
negative regulation of transcription, DNA-dependent	3.99E-08
negative regulation of RNA metabolic process	5.59E-08
negative regulation of biosynthetic process	5.66E-08
negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	5.88E-08
negative regulation of nitrogen compound metabolic process	5.88E-08
ergosterol metabolic process	6.18E-08
phytosteroid metabolic process	6.18E-08
lipid metabolic process	6.67E-08
negative regulation of cellular metabolic process	8.93E-08
negative regulation of transcription from RNA polymerase II promoter	8.95E-08
RNA elongation	1.07E-07
localization	1.58E-07
lipid biosynthetic process	1.85E-07
cellular carbohydrate catabolic process	2.67E-07
establishment of localization in cell	3.03E-07
nucleobase, nucleoside and nucleotide metabolic process	3.07E-07
sterol metabolic process	3.87E-07
response to stimulus	3.95E-07
steroid biosynthetic process	3.97E-07
sterol biosynthetic process	3.97E-07
histone modification	5.47E-07
covalent chromatin modification	5.47E-07
alcohol catabolic process	5.93E-07
Golgi vesicle transport	6.09E-07
monocarboxylic acid metabolic process	7.12E-07
cellular lipid metabolic process	7.15E-07
transcription initiation from RNA polymerase II promoter	9.72E-07
monosaccharide catabolic process	1.53E-06
protein refolding	1.57E-06
acetyl-CoA metabolic process	2.11E-06
protein targeting	2.26E-06
coenzyme catabolic process	2.29E-06

Table A.5. Bingo Results for ETN (cont.).

Description	**p-value**
cellular protein localization	2.46E-06
regulation of gene-specific transcription	2.46E-06
trehalose metabolic process	2.51E-06
steroid metabolic process	3.05E-06
negative regulation of macromolecule metabolic process	3.10E-06
RNA elongation from RNA polymerase II promoter	3.94E-06
carbohydrate catabolic process	4.03E-06
cofactor catabolic process	5.16E-06
fatty acid metabolic process	5.23E-06
cell redox homeostasis	5.34E-06
mitochondrial transport	5.63E-06

Table A.6. Bingo Results for tETN.

Description	**p-value**
transcription	1.20E-79
regulation of transcription	6.69E-66
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	8.55E-53
regulation of nitrogen compound metabolic process	2.18E-52
regulation of gene expression	1.05E-45
regulation of macromolecule biosynthetic process	3.53E-45
regulation of transcription, DNA-dependent	5.07E-45
regulation of cellular biosynthetic process	1.26E-44
regulation of biosynthetic process	8.37E-44
regulation of RNA metabolic process	5.92E-43
oxidation reduction	2.18E-34
regulation of macromolecule metabolic process	4.28E-33
regulation of cellular metabolic process	3.62E-32
regulation of transcription from RNA polymerase II promoter	3.67E-32
positive regulation of transcription	1.78E-30
positive regulation of gene expression	4.02E-30
regulation of primary metabolic process	1.16E-29
positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2.36E-29
positive regulation of nitrogen compound metabolic process	2.36E-29
regulation of metabolic process	9.77E-27
positive regulation of macromolecule biosynthetic process	2.43E-25
positive regulation of biosynthetic process	2.72E-25
positive regulation of cellular biosynthetic process	2.72E-25
positive regulation of transcription, DNA-dependent	1.67E-24
positive regulation of cellular metabolic process	1.24E-23
positive regulation of RNA metabolic process	2.95E-23
positive regulation of metabolic process	6.46E-23
positive regulation of macromolecule metabolic process	1.04E-22
chromatin modification	2.72E-21
RNA biosynthetic process	3.92E-21
transcription, DNA-dependent	4.81E-21
protein transport	2.42E-20
small molecule metabolic process	3.14E-20
chromatin organization	1.04E-19
positive regulation of cellular process	1.62E-19
cellular nitrogen compound metabolic process	2.25E-19
nitrogen compound metabolic process	2.57E-19
establishment of protein localization	6.60E-19
positive regulation of transcription from RNA polymerase II promoter	2.03E-18
positive regulation of biological process	2.14E-18
regulation of cellular process	4.98E-18

Table A.6. Bingo Results for tETN (cont.).

Description	**p-value**
protein folding	4.91E-17
response to oxidative stress	5.10E-17
cellular response to oxidative stress	3.45E-16
regulation of biological process	8.36E-16
biological regulation	3.04E-14
transcription from RNA polymerase II promoter	4.22E-14
small molecule biosynthetic process	5.05E-14
protein localization	8.59E-14
transcription initiation	2.77E-13
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.19E-13
transcription from RNA polymerase III promoter	5.37E-13
organic acid metabolic process	6.28E-13
oxoacid metabolic process	7.16E-13
carboxylic acid metabolic process	7.16E-13
cellular ketone metabolic process	7.37E-13
organic acid biosynthetic process	1.00E-11
carboxylic acid biosynthetic process	1.00E-11
transport	1.90E-11
glutamine family amino acid metabolic process	2.65E-11
macromolecule localization	3.07E-11
cell aging	1.43E-10
chromatin remodeling	3.70E-10
establishment of localization	4.38E-10
alcohol metabolic process	4.77E-10
replicative cell aging	5.81E-10
vesicle-mediated transport	8.18E-10
negative regulation of transcription	1.19E-09
negative regulation of gene expression	1.87E-09
response to stress	1.94E-09
cellular amino acid biosynthetic process	3.47E-09
amine biosynthetic process	4.12E-09
cellular amine metabolic process	5.06E-09
cellular nitrogen compound biosynthetic process	6.13E-09
aging	6.61E-09
RNA elongation	9.03E-09
nucleic acid metabolic process	1.14E-08
cellular response to chemical stimulus	1.43E-08
intracellular transport	1.68E-08
response to stimulus	1.75E-08
cellular carbohydrate catabolic process	1.77E-08
amine metabolic process	1.79E-08

Table A.6. Bingo Results for tETN (cont.).

Description	**p-value**
response to chemical stimulus	2.14E-08
histone modification	2.79E-08
covalent chromatin modification	2.79E-08
negative regulation of macromolecule biosynthetic process	3.58E-08
nucleotide metabolic process	4.13E-08
nucleoside phosphate metabolic process	4.13E-08
glutamine metabolic process	4.82E-08
alcohol catabolic process	5.27E-08
cellular amino acid and derivative metabolic process	7.86E-08
cellular amino acid metabolic process	8.22E-08
negative regulation of cellular biosynthetic process	9.60E-08
negative regulation of transcription, DNA-dependent	1.04E-07
ergosterol biosynthetic process	1.11E-07
phytosteroid biosynthetic process	1.11E-07
negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.17E-07
negative regulation of nitrogen compound metabolic process	1.17E-07
intracellular protein transport	1.23E-07
ergosterol metabolic process	1.38E-07
phytosteroid metabolic process	1.38E-07
negative regulation of RNA metabolic process	1.41E-07
transcription initiation from RNA polymerase II promoter	1.58E-07
negative regulation of biosynthetic process	1.61E-07
monosaccharide catabolic process	1.62E-07
localization	1.88E-07
sterol metabolic process	2.51E-07
transcription initiation from RNA polymerase III promoter	2.61E-07
carbohydrate catabolic process	3.09E-07
lipid metabolic process	3.33E-07
negative regulation of cellular metabolic process	3.36E-07
monocarboxylic acid metabolic process	3.85E-07
steroid biosynthetic process	4.47E-07
sterol biosynthetic process	4.47E-07
RNA elongation from RNA polymerase II promoter	5.01E-07
establishment of localization in cell	7.18E-07
lipid biosynthetic process	8.21E-07
nucleobase, nucleoside and nucleotide metabolic process	8.23E-07
hexose catabolic process	9.03E-07
trehalose metabolic process	1.03E-06
Golgi vesicle transport	1.31E-06
acetyl-CoA metabolic process	1.77E-06
steroid metabolic process	1.84E-06

Table A.6. Bingo Results for tETN (cont.).

Description	**p-value**
fatty acid metabolic process	2.24E-06
coenzyme catabolic process	2.53E-06
negative regulation of transcription from RNA polymerase II promoter	2.74E-06
protein targeting	3.06E-06
cellular lipid metabolic process	3.72E-06
small molecule catabolic process	4.36E-06
tRNA transcription	4.74E-06
tRNA transcription from RNA polymerase III promoter	4.74E-06
cofactor catabolic process	5.38E-06

REFERENCES

1. Naik, S.N., V.V. Goud, P.K. Rout, and A.K. Dalai, "Production of First and Second Generation Biofuels: A Comprehensive Review", *Renewable and Sustainable Energy Reviews*, Vol. 14, No. 2, pp. 578–597, 2010.

2. González-García, S., M.T. Moreira, and G. Feijoo, "Comparative Environmental Performance of Lignocellulosic Ethanol from Different Feedstocks", *Renewable and Sustainable Energy Reviews*, Vol. 14, No. 7, pp. 2077–2085, 2010.

3. Cherubini, F., "GHG Balances of Bioenergy Systems – Overview of Key Steps in the Production Chain and Methodological Concerns", *Renewable Energy*, Vol. 35, No. 7, pp. 1565–1573, 2010.

4. Goyal, H.B., D. Seal, and R.C. Saxena, "Bio-Fuels from Thermochemical Conversion of Renewable Resources: A Review", *Renewable and Sustainable Energy Reviews*, Vol. 12, No. 2, pp. 504–517, 2008.

5. Sims R. and M. Taylor, *From 1st to 2nd Generation Biofuel Technologies*, International Energy Agency, Paris, 2008.

6. Balat, M., and H. Balat, "Recent Trends in Global Production and Utilization of Bio-Ethanol Fuel", *Applied Energy*, Vol. 86, No. 11, pp. 2273–2282, 2009.

7. Elkins, J.G., B. Raman, and M. Keller, "Engineered Microbial Systems for Enhanced Conversion of Lignocellulosic Biomass", *Current Opinion in Biotechnology*, Vol. 21, No. 5, pp. 657–662, 2010.

8. Bai, F.W., L.J. Chen, Z. Zhang, W. A. Anderson, and M. Moo-Young, "Continuous Ethanol Production and Evaluation of Yeast Cell Lysis and Viability Loss Under Very

High Gravity Medium Conditions", *Journal of Biotechnology*, Vol. 110, No. 3, pp. 287–293, 2004.

9. Escobar, J.C., E.S. Lora, O.J. Venturini, E.E. Yáñez, E.F. Castillo, and O. Almazan, "Biofuels: Environment, Technology and Food Security", *Renewable and Sustainable Energy Reviews*, Vol. 13, pp. 1275–1287, 2009.

10. Attfield, P., "Stress Tolerance: The Key to Effective Strains of Industrial Baker's Yeast", *Nature Biotechnology*, Vol. 15, No. 13, pp. 1351–1357, 1997.

11. Alexandre, H., and C. Charpentier, "Biochemical Aspects of Stuck and Sluggish Fermentation in Grape Must", *Journal of Industrial Microbiology and Biotechnology*, Vol. 20, No. 1, pp. 20–27, 1998.

12. Hirasawa, T., K. Yoshikawa, Y. Nakakura, K. Nagahisa, C. Furusawa, Y. Katakura, H. Shimizu, and S. Shioya, "Identification of Target Genes Conferring Ethanol Stress Tolerance to *Saccharomyces cerevisiae* Based on DNA Microarray Data Analysis", *Journal of Biotechnology*, Vol. 131, No. 1, pp. 34–44, 2007.

13. Koh, L.P., P. Levang, and J. Ghazoul, "Designer Landscapes for Sustainable Biofuels", *Trends Ecology and Evolution*, Vol. 24, No. 8, pp. 431–438, 2009.

14. Chen, J., W. Zhang, L. Tan, Y. Wang, and G. He, "Optimization of Metabolic Pathways for Bioconversion of Lignocellulose to Ethanol Through Genetic Engineering", *Biotechnology Advances*, Vol. 27, No. 5, pp. 593–598, 2009.

15. Otero, J.M., and J. Nielsen, "Industrial Systems Biology", *Biotechnology and Bioengineering*, Vol. 105, No. 3, pp. 439–460, 2010.

16. Nielsen, J., and M.C. Jewett, "Impact of Systems Biology on Metabolic Engineering of *Saccharomyces cerevisiae*", *FEMS Yeast Research*, Vol. 8, No. 1, pp. 122–131, 2008.

17. Liti, G., D.M. Carter, A.M. Moses, J. Warringer, L. Parts, S.A. James, R.P. Davey, I.N. Roberts, A. Burt, V. Koufopanou, I.J. Tsai, C.M. Bergman, D. Bensasson, M.J.T. O'Kelly, A. van Oudenaarden, D.B.H. Barton, E. Bailes, A.N. Nguyen, M. Jones, M.A. Quail, I. Goodhead, S. Sims, F. Smith, A. Blomberg, R. Durbin, and E.J. Louis, "Population Genomics of Domestic and Wild Yeasts", *Nature*, Vol. 458, No. 7236, pp. 337–341, (2009).

18. Roehr, M., *The Biotechnology of Ethanol: Classical and Future Applications*, Wiley VCH, Weinheim, 2001.

19. Tuite, M.F. and S.G. Oliver, *Saccharomyces: Biotechnology Handbooks*, Springer, New York, 1991.

20. Fay, J.C., and J.A. Benavides, "Evidence for Domesticated and Wild Populations of *Saccharomyces cerevisiae*", *PLoS Genetics*, Vol. 1, No. 1, pp. 66–71, 2005.

21. Bai, F.W., W.A. Anderson, and M. Moo-Young, "Ethanol Fermentation Technologies from Sugar and Starch Feedstocks", *Biotechnology Advances*, Vol. 26, No. 1, pp. 89–105, 2008.

22. Watanabe, M., K. Tamura, J.P. Magbanua, K. Takano, K. Kitamoto, H. Kitagaki, T. Akao, and H. Shimoi, "Elevated Expression of Genes Under the Control of Stress Response Element (STRE) and Msn2p in an Ethanol-Tolerance Sake Yeast Kyokai No. 11", *Journal of Bioscience and Bioengineering*, Vol. 104, No. 3, pp. 163–170, 2007.

23. Albertin, W., P. Marullo, M. Aigle, C. Dillmann, D. de Vienne, M. Bely, and D. Sicard, "Population Size Drives Industrial *Saccharomyces cerevisiae* Alcoholic Fermentation and is Under Genetic Control", *Applied and Environmental Microbiology*, Vol. 77, No. 8, pp. 2772–2784, 2011.

24. Borneman, A.R., B.A. Desany, D. Riches, J.P. Affourtit, A.H. Forgan, I.S. Pretorius, M. Egholm, and P.J. Chambers, "Whole-Genome Comparison Reveals Novel Genetic

Elements that Characterize the Genome of Industrial Strains of *Saccharomyces cerevisiae*", *PLoS Genetics*, Vol. 7, No. 2, pp. e1001287, 2011.

25. Gerke, J.P., C.T.L. Chen, and B.A. Cohen, "Natural Isolates of *Saccharomyces cerevisiae* Display Complex Genetic Variation in Sporulation Efficiency", *Genetics*, Vol. 174, No. 2, pp. 985–997, 2006.

26. Gardner, N., N. Rodrigue, and C.P. Champagne, "Combined Effects of Sulfites, Temperature, and Agitation Time on Production of Glycerol in Grape Juice by *Saccharomyces cerevisiae*", *Applied and Environmental Microbiology*, Vol. 59, No. 7, pp. 2022–2028, 1993.

27. Wold, S., K. Esbensen, and P. Geladi, "Principal Component Analysis", *Chemometrics and Intelligent Laboratory Systems*, Vol. 2, pp. 37–52, 1987.

28. Hu, X.H., M.H. Wang, T. Tan, J.R. Li, H. Yang, L. Leach, R.M. Zhang, and Z.W. Luo, "Genetic Dissection of Ethanol Tolerance in the Budding Yeast *Saccharomyces cerevisiae*", *Genetics*, Vol. 175, No. 3, pp. 1479–1487, 2007.

29. Yazawa H., H. Iwahashi, and H. Uemura, "Disruption of URA7 and GAL6 Improves the Ethanol Tolerance and Fermentation Capacity of *Saccharomyces cerevisiae*", *Yeast*, Vol. 24, No. 7, pp. 551–560, 2007.

30. Yoshikawa K., T. Tanaka, C. Furusawa, K. Nagahisa, T. Hirasawa, and H. Shimizu, "Comprehensive Phenotypic Analysis for Identification of Genes Affecting Growth Under Ethanol Stress in *Saccharomyces cerevisiae*", *FEMS Yeast Research*, Vol. 9, No. 1, pp. 32–44, 2009.

31. Jiménez-Martí, E., A. Zuzuarregui, I. Ridaura, N. Lozano, and M. del Olmo, "Genetic Manipulation of *HSP26* and *YHR087W* Stress Genes May Improve Fermentative Behaviour in Wine Yeasts Under Vinification Conditions", *International Journal of Food Microbiology*, Vol. 130, No. 2, pp. 122–130, 2009.

32. Kim, H.S., N.R. Kim, and W. Choi, "Total Fatty Acid Content of the Plasma Membrane of *Saccharomyces cerevisiae* is More Responsible for Ethanol Tolerance Than the Degree of Unsaturation", *Biotechnology Letters*, Vol. 33, No. 3, pp. 509–515, 2011.

33. Lewis, J.A, I.M. Elkon, M.A McGee, A.J. Higbee, and A.P. Gasch, "Exploiting Natural Variation in *Saccharomyces cerevisiae* to Identify Genes for Increased Ethanol Resistance", *Genetics*, Vol. 186, No. 4, pp. 1197–1205, 2010.

34. Hong, M.E., K.S. Lee, B.J. Yu, Y.J. Sung, S.M. Park, H.M. Koo, D.H. Kweon, J.C. Park, and Y.S. Jin, "Identification of Gene Targets Eliciting Improved Alcohol Tolerance in *Saccharomyces cerevisiae* Through Inverse Metabolic Engineering", *Journal of Biotechnology*, Vol. 149, pp. 52–59, 2010.

35. "ORF Finder: The Sequence Manipulation Suite", http://www.bioinformatics.org/sms/index.html, accessed at March 2011.

36. "ClustalW2: Multiple Sequence Alignment", http://www.ebi.ac.uk/Tools/msa/clustalw2/, accessed at March 2011.

37. Ho, Y., A. Gruhler, A. Heilbut, G.D. Bader, L. Moore, S. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A.R. Willems, H. Sassi, P.A. Nielsen, L.H. Hansen, H. Jespersen, A. Podtelejnikov, J. Matthiesen, R.C. Hendrickson, F. Gleeson, T. Pawson, M.F. Moran, D. Durocher, M. Mann, C.W.V Hogue, D. Figeys, and M. Tyers, "Systematic Identification of Protein Complexes in *Saccharomyces cerevisiae* by Mass Spectrometry", *Nature*, Vol. 415, No. 6868, pp. 180–183, 2002.

38. Ptacek, J., G. Devgan, G. Michaud, H. Zhu, X. Zhu, J. Fasolo, H. Guo, G. Jona, A. Breitkreutz, R. Sopko, R.R. McCartney, M.C. Schmidt, N. Rachidi, S.J. Lee, A.S. Mah, L. Meng, M.J.R. Stark, D.F. Stern, C. De Virgilio, M. Tyers, B. Andrews, M. Gerstein, B. Schweitzer, P.F. Predki, and M. Snyder, "Global Analysis of Protein Phosphorylation in Yeast", *Nature*, Vol. 438, No. 7068, pp. 679–684, 2005.

39. Costanzo, M., A. Baryshnikova, J. Bellay, Y. Kim, E.D. Spear, C.S. Sevier, H. Ding, J.L.Y. Koh, K. Toufighi, S. Mostafavi, J. Prinz, R.P. St Onge, B. VanderSluis, T. Makhnevych, F.J. Vizeacoumar, S. Alizadeh, S. Bahr, R.L. Brost, Y. Chen, M. Cokol, R. Deshpande, Z. Li, Z.Y. Lin, W. Liang, M. Marback, J. Paw, B.J. San Luis, E. Shuteriqi, A.H.Y. Tong, N. van Dyk, I.M. Wallace, J. Whitney, M.T. Weirauch, G. Zhong, H. Zhu, W. Houry, M. Brudno, S. Ragibizadeh, B. Papp, C. Pál, F.P. Roth, G. Giaever, C. Nislow, O.G. Troyanskaya, H. Bussey, G.D. Bader, A.C. Gingras, Q.D. Morris, P.M. Kim, C. Kaiser, C.L. Myers, B.J. Andrews, and C. Boone, "The Genetic Landscape of a Cell", *Science*, Vol. 327, No. 5964, pp. 425–431, 2010.

40. Fiedler, D., H. Braberg, M. Mehta, G. Chechik, G. Cagney, P. Mukherjee, A.C. Silva, M. Shales, S.R. Collins, S. van Wageningen, P. Kemmeren, F.C.P. Holstege, J.S. Weissman, M.C. Keogh, D. Koller, K.M. Shokat, and N.J. Krogan, "Functional Organization of the *S. cerevisiae* Phosphorylation Network", *Cell*, Vol. 136, No. 5, pp. 952–963, 2009.

41. "SGD: *Saccharomyces* Genome Database", 2010, http://www.yeastgenome.org/, accessed at January 2011.

42. Eisentraut E., *Sustainable Production of Second-Generation Biofuels: Potential and Perspectives in Major Economies and Developing Countries*, International Energy Agency, Paris, 2010.

43. Tsai, W.T., C.C. Lin, and C.W. Yeh, "An Analysis of Biodiesel Fuel from Waste Edible Oil in Taiwan", *Renewable and Sustainable Energy Reviews*, Vol. 11, No. 5, pp. 838–857, 2007.

44. Berglund, M. and P. Börjesson, "Assessment of Energy Performance in the Life-Cycle of Biogas Production", *Biomass and Bioenergy*, Vol. 30, No. 3, pp. 254–266, 2006.

45. das Neves, M.A., T. Kimura, N. Shimizu, and M. Nakajima, "State of the Art and Future Trends of Bioethanol Production", *Dynamic Biochemistry, Process Biotechnology and Mol Biology*, Vol. 1, pp. 1–14, 2.

46. İçöz, E., K. Mehmet Tuğrul, A. Saral, and E. İçöz, "Research on Ethanol Production and Use from Sugar Beet in Turkey", *Biomass and Bioenergy*, Vol. 33, pp. 1–7, 2009.

47. Mukherjee, A.K., H. Adhikari, and S.K. Rai, "Production of Alkaline Protease by a Thermophilic *Bacillus subtilis* Under Solid-State Fermentation (SSF) Condition Using Imperata Cylindrica Grass and Potato Peel as Low-Cost Medium: Characterization and Application of Enzyme in Detergent Formulation", *Biochemical Engineering Journal*, Vol. 39, No. 2, pp. 353–361, 2008.

48. Bernfeld, P., "Amylases α and β", *Methods in Enzymology*, Vol. 1, pp. 149–158, 1955.

49. Toksoy Öner, E., "Optimization of Ethanol Production from Starch by an Amylolytic Nuclear Petite *Saccharomyces cerevisiae* Strain", *Yeast*, Vol. 23, No. 12, pp. 849–856, 2006.

50. Stanley, D., A. Bandara, S. Fraser, P.J. Chambers, and G.A. Stanley, "The Ethanol Stress Response and Ethanol Tolerance of *Saccharomyces cerevisiae*", *Journal of Applied Microbiology*, Vol. 109, pp. 13–24, 2010.

51. Ma, M., and Z.L. Liu, "Mechanisms of Ethanol Tolerance in *Saccharomyces cerevisiae*", *Applied Microbiology and Biotechnology*, Vol. 87, No. 3, pp. 829–845, 2010.

52. Li, H., M.L. Ma, S. Luo, R.M. Zhang, P. Han, and W. Hu, "Metabolic Responses to Ethanol in *Saccharomyces cerevisiae* Using a Gas Chromatography Tandem Mass Spectrometry-Based Metabolomics Approach", *International Journal of Biochemistry and Cell Biology*, Vol. 44, No. 7, pp. 1087–1096, 2012.

53. Kim, I.S., H.S. Yun, I.S. Park, H.Y. Sohn, H. Iwahashi, and I.N. Jin, "A Knockout Strain of CPR1 Induced During Fermentation of *Saccharomyces cerevisiae* KNU5377 is Susceptible to Various Types of Stress", *Journal of Bioscience and Bioengineering*, Vol. 102, No. 4, pp. 288–296, 2006.

54. Ding, J., X. Huang, L. Zhang, N. Zhao, D. Yang, and K. Zhang, "Tolerance and Stress Response To Ethanol in the Yeast *Saccharomyces cerevisiae*", *Applied Microbiology and Biotechnology*, Vol. 85, No. 2, pp. 253–263, 2009.

55. Carrasco, P., A. Querol, and M. del Olmo, "Analysis of the Stress Resistance of Commercial Wine Yeast Strains", *Archieves of Microbiology*, Vol. 175, No. 6, pp. 450–457, 2001.

56. Park, A.H., M. Sugiyama, S. Harashima, Y.H. Kim, "Creation of an Ethanol-Tolerant Yeast Strain by Genome Reconstruction Based on Chromosome Splitting Technology", *Journal of Microbiology and Biotechnology*, Vol. 22, No. 2, pp. 184–189, 2012.

57. Fischer, C.R., D. Klein-Marcuschamer, and G. Stephanopoulos, "Selection and Optimization of Microbial Hosts for Biofuels Production", *Metabolic Engineering*, Vol. 10, No. 6, pp. 295–304, 2008.

58. Kasavi, C., I. Finore, L. Lama, B. Nicolaus, S.G. Oliver, E. Toksoy Oner, and B. Kirdar, "Evaluation of Industrial *Saccharomyces cerevisiae* Strains for Ethanol Production from Biomass", *Biomass and Bioenergy*, Vol. 45, pp. 230–238, 2012.

59. Piper, P., "The Heat Shock and Ethanol Stress Responses of Yeast Exhibit Extensive Similarity and Functional Overlap", *FEMS Microbiology Letters*, Vol. 134, pp. 121–127, 1995.

60. Pina, C., J. António, T. Hogg, "Inferring Ethanol Tolerance of *Saccharomyces* and Non-*Saccharomyces* Yeasts by Progressive Inactivation", *Biotechnology Letters*, Vol. 26, No. 19, pp. 1521–1527, 2004.

61. Salgueiro, S.P., I. Sá-Correia, and J.M. Novais, "Ethanol-Induced Leakage in *Saccharomyces cerevisiae*: Kinetics and Relationship to Yeast Ethanol Tolerance and Alcohol Fermentation Productivity", *Applied Environmental Microbiology*, Vol. 54, No. 4, pp. 903–909, 1988.

62. Furukawa, K., H. Kitano, H. Mizoguchi, and S. Hara, "Effect of Cellular Inositol Content on Ethanol Tolerance of *Saccharomyces cerevisiae* in Sake Brewing", Journal of Bioscience and Bioengineering, Vol. 98, No. 2, pp. 107–113, 2004.

63. van Voorst, F., J. Houghton-Larsen, L. Jønson, M.C. Kielland-Brandt, and A. Brandt, "Genome-Wide Identification of Genes Required for Growth of *Saccharomyces cerevisiae* Under Ethanol Stress", *Yeast*, Vol. 23, No. 5, pp. 351–359, 2006.

64. Pascual, C., A. Alonso, I. Garcia, C. Romay, and A. Kotyk, "Effect of Ethanol on Glucose Transport, Key Glycolytic Enzymes, and Proton Extrusion in *Saccharomyces cerevisiae*", *Biotechnology and Bioengineering*, Vol. 32, No. 3, pp. 374–378, 1988.

65. Millar, D., K. Griffiths-Smith, E. Algar, and R. Scopes, "Activity and Stability of Glycolytic Enzymes in the Presence of Ethanol", *Biotechnology Letters*, Vol. 4, No. 9, pp. 601–606, 1982.

66. Cakar, Z.P., U.O. Seker, C. Tamerler, M. Sonderegger, and U. Sauer, "Evolutionary Engineering of Multiple-Stress Resistant *Saccharomyces cerevisiae*", *FEMS Yeast Research*, Vol. 5, pp. 569–578, 2005.

67. Ma, M., and L.Z. Liu, "Quantitative Transcription Dynamic Analysis Reveals Candidate Genes and Key Regulators for Ethanol Tolerance in *Saccharomyces cerevisiae*", *BMC Microbiology*, Vol. 10, p. 169, 2010.

68. Steffen, M., A. Petti, J. Aach, P. D'haeseleer, and G. Church, "Automated Modelling of Signal Transduction Networks", *BMC Bioinformatics*, Vol. 3, p. 34, 2002.

69. Arga, Y.K., Z.İ. Önsan, B. Kırdar, K.Ö. Ülgen, and J. Nielsen, "Understanding Signaling in Yeast: Insights from Network Analysis", *Biotechnolgy and Bioengineering*, Vol. 97, No. 5, pp. 1246–1258, 2007.

70. Zhao, X.M., R.S. Wang, L. Chen, and K. Aihara, "Uncovering Signal Transduction Networks from High-Throughput Data by Integer Linear Programming", *Nucleic Acids Research*, Vol. 36, No. 9, p. e48, 2008.

71. Alberghina, L., G. Mavelli, G. Drovandi, P. Palumbo, S. Pessina, F. Tripodi, P. Coccetti, and M. Vanoni, "Cell Growth and Cell Cycle in *Saccharomyces cerevisiae*: Basic Regulatory Design and Protein-Protein Interaction Network", *Biotechnology Advances*, Vol. 30, pp. 52–72, 2012.

72. Mihalik, A., and P. Csermely, "Heat Shock Partially Dissociates the Overlapping Modules of the Yeast Protein-Protein Interaction Network: A Systems Level Model of Adaptation", *PLoS Computational Biology*, Vol. 7, No. 10, p. e1002187, 2011.

73. Stark, C., B.J. Breitkreutz, T. Reguly, L. Boucher, A. Breitkreutz, and M. Tyers, "BioGRID: A General Repository for Interaction Datasets", *Nucleic Acids Research*, Vol. 34, pp. D535–539, 2006.

74. Lohmann, G., D.S. Margulies, A. Horstmann, B. Pleger, J. Lepsien, D. Goldhahn, H. Schloegl, M. Stumvoll, A. Villringer, and R. Turner, "Eigenvector Centrality Mapping for Analyzing Connectivity Patterns in FMRI Data of the Human Brain", *PLoS One*, Vol. 5, No. 4, p. e10232, 2010.

75. Wang, J., G. Chen, M. Li, and Y. Pan, "Integration of Breast Cancer Gene Signatures Based on Graph Centrality", BMC Systems Biology, Vol. 5, p. S10, 2011.

76. Assenov, Y., F. Ramírez, S.E. Schelhorn, T. Lengauer, and M. Albrecht, "Computing Topological Parameters of Biological Networks", *Bioinformatics*, Vol. 24, No. 2, pp. 282–284, 2008.

77. Shannon, P., A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker, " Cytoscape : A Software Environment for Integrated Models of Biomolecular Interaction Networks", *Genome Research*, Vol. 13, No. 11, pp. 2498–2504, 2003.

78. Bader, G.D., and C.W.V Hogue, "An Automated Method for Finding Molecular Complexes in Large Protein Interaction Networks", *BMC Bioinformatics*, Vol. 4, No. 2, pp. 1-27, 2003.

79. Maere, S., K. Heymans, and M. Kuiper, "BiNGO: A Cytoscape Plugin to Assess Overrepresentation of Gene Ontology Categories in Biological Networks", *Bioinformatics*, Vol. 21, No. 16, pp. 3448–3449, 2005.

80. Brajtburg, J., S. Elberg, G. Medoff, and G.S. Kobayashi, "Increase in Colony-Forming Units of *Candida Albicans* After Treatment with Polyene Antibiotics", *Antimicrobial Agents and Chemotherapy*, Vol. 19, pp. 199–200, 1981.

81. Yücel, E., A. Cankorur, E. Dereli, F. Alazi, I. Aslandağ, B. Kavun, S. Durmus Tekir, K. Arga, and K. Ülgen, "Deciphering the Cross-Talk between Sphingolipid Mechanism and Signaling Pathways in Baker's Yeast", *Sigma*., Vol. 26, pp. 162–174, 2008.

82. Borklu Yucel, E. and K.O. Ulgen, "A Network-Based Approach on Elucidating the Multi-Faceted Nature of Chronological Aging in *S. cerevisiae*", *PLoS One*, Vol. 6, No. 12, p. e29284, 2011.

83. Eren Toku, A., S. Durmuş Tekir, F.B. Kavun Özbayraktar, and K.Ö. Ülgen, "Reconstruction and Crosstalk of Protein-Protein Interaction Networks of Wnt and Hedgehog Signaling in *Drosophila melanogaster*", *Computational Biology and Chemistry*, Vol. 35, No. 5, pp. 282–292, 2011.

84. Durmuş Tekir, S., P. Ümit, A. Eren Toku, and K.Ö. Ülgen, "Reconstruction of Protein-Protein Interaction Network of Insulin Signaling in *Homo sapiens*", *Journal of Biomedicine and Biotechnology*, Vol. 2010, pp. 1–7, 2010.

85. Nalbantoglu, B., S. Durmuş Tekir, and K.Ö. Ülgen, "Wnt Signaling Network in *Homo sapiens*", *Cell Metabolism: Cell Homeostasis and Stress Response*, pp. 163–190, 2012.

86. Mrowka, R., A. Patzak, and H. Herzel, "Is There a Bias in Proteome Research?", *Genome Research*, Vol. 11, No. 2, pp. 1971–1973, 2001.

87. von Mering, C., R. Krause, B. Snel, M. Cornell, S. Oliver, S. Fields, and P. Bork, "Comparative Assessment of Large-Scale Data Sets of Protein-Protein Interactions", *Nature*, Vol. 417, No. 6887, pp. 399–403, 2002.

88. Ravasz, E., A.L. Somera, D.A. Mongru, Z.N. Oltvai, and A.L. Barabási, "Hierarchical Organization of Modularity in Metabolic Networks", *Science*, Vol. 297, No. 5586, pp. 1551–1555, 2002.

89. Ravasz, E. and A.L. Barabási, "Hierarchical Organization in Complex Networks", *Physical Review E, Statistical, Nonlinear, and Soft Matter Physics*, Vol. 67, pp. 1–7, 2003.

90. Pavlopoulos, G.A., M. Secrier, C.N. Moschopoulos, T.G. Soldatos, S. Kossida, J. Aerts, R. Schneider, and P.G. Bagos, "Using Graph Theory to Analyze Biological Networks", *BioData Mining*, Vol. 4, p. 10, 2011.

91. Avrahami-Moyal, L., D. Engelberg, J.W. Wenger, G. Sherlock, and S. Braun, "Turbidostat Culture of *Saccharomyces cerevisiae* W303-1A under Selective Pressure Elicited by Ethanol Selects for Mutations in SSD1 and UTH1", *FEMS Yeast Research*, Vol. 12, No. 5, pp. 521–533, 2012.

92. Gasch, A.P., P.T. Spellman, C.M. Kao, O. Carmel-Harel, M.B. Eisen, G. Storz, D. Botstein, and P. O. Brown, "Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes", *Molecular Biology of the Cell*, Vol. 11, No. 12, pp. 4241–4257, 2000.

93. Alexandre, H., V. Ansanay-Galeote, S. Dequin, and B. Blondin, "Global Gene Expression During Short-Term Ethanol Stress in *Saccharomyces cerevisiae*", *FEBS Letters*, Vol. 498, pp. 98–103, 2001.

94. Chandler, M., G.A. Stanley, P. Rogers, and P. Chambers, "A Genomic Approach to Defining the Ethanol Stress Response in the Yeast *Saccharomyces cerevisiae*", *Annals of Microbiology*, Vol. 54, No. 4, pp. 427–454, 2004.

95. Dinh, T.N., K. Nagahisa, K. Yoshikawa, T. Hirasawa, C. Furusawa, and H. Shimizu, "Analysis of Adaptation to High Ethanol Concentration in *Saccharomyces cerevisiae* Using DNA Microarray", *Bioprocess and Biosystems Engineering*, Vol. 32, No. 5, pp. 681–688, 2009.

96. Teixeira, M.C., L.R. Raposo, N.P. Mira, A.B. Lourenço, I. Sá-Correia, "Genome-Wide Identification of *Saccharomyces cerevisiae* Genes Required for Maximal Tolerance to Ethanol", *Applied and Environmental Microbiology*, Vol. 75, No. 18, pp. 5761–5772, 2009.

97. Alexandre, H., L. Plourde, C. Charpentier, and J. François, "Lack of Correlation between Trehalose Accumulation, Cell Viability and Intracellular Acidification as Induced by Various Stresses in *Saccharomyces cerevisiae*", *Microbiology*, Vol. 144, pp. 1103–1111, 1998.

98. Dinh, T.N., K. Nagahisa, T. Hirasawa, C. Furusawa, and H. Shimizu, "Adaptation of *Saccharomyces cerevisiae* Cells to High Ethanol Concentration and Changes in Fatty Acid Composition of Membrane and Cell Size", *PloS One*, Vol. 3, No. 7, p. e2623, 2008.

99. Aguilera, F., R.A. Peinado, C. Millán, J.M. Ortega, and J.C. Mauricio, "Relationship between Ethanol Tolerance, H^+-ATPase Activity and the Lipid Composition of the Plasma Membrane in Different Wine Yeast Strains", *International Journal of Food Microbiology*, Vol. 110, pp. 34–42, 2006.

100. Kubota, S., I. Takeo, K. Kume, M. Kanai, A. Shitamukai, M. Mizunuma, T. Miyakawa, H. Shimoi, H. Iefuji, and D. Hirata, "Effect of Ethanol on Cell Growth of Budding Yeast: Genes That are Important for Cell Growth in the Presence of Ethanol", *Bioscience Biotechnology and Biochemistry*, Vol. 68, No. 4, pp. 968–972, 2004.

101. Fujita, K., A. Matsuyama, Y. Kobayashi, and H. Iwahashi, "The Genome-Wide Screening of Yeast Deletion Mutants to Identify the Genes Required for Tolerance to Ethanol and Other Alcohols", *FEMS Yeast Research*, Vol. 6, No. 5, pp. 744–750, 2006.

102. Ogawa, Y., A. Nitta, H. Uchiyama, T. Imamura, H. Shimoi, and K. Ito, "Tolerance Mechanism of the Ethanol-Tolerant Mutant of Sake Yeast", *Journal of Bioscience and Bioengineering*, Vol. 90, No. 3, pp. 313–320, 2000.

103. Stanley, D., P.J. Chambers, G.A. Stanley, A. Borneman, and S. Fraser, "Transcriptional Changes Associated with Ethanol Tolerance in *Saccharomyces cerevisiae*", *Applied Microbiology and Biotechnology*, Vol. 88, pp. 231–239, 2010.

104. Hou, J., N.F. Lages, M. Oldiges, and G.N. Vemuri, "Metabolic Impact of Redox Cofactor Perturbations in *Saccharomyces cerevisiae*", *Metabolic Engineering*, Vol. 11, pp. 253–261, 2009.

105. Gentzsch, M., and W. Tanner, "The PMT Gene Family: Protein O-glycosylation in *Saccharomyces cerevisiae* is Vital", *EMBO Journal*, Vol. 15, No. 21, pp. 5752–5759, 1996.

106. Despons, L., B. Wirth, V.L. Louis, S. Potier, and J.L. Souciet, "An Evolutionary Scenario for One of the Largest Yeast Gene Families", *Trends in Genetics*, Vol. 22, pp. 10–15, 2006.

107. Giaever, G., A.M. Chu, L. Ni, C. Connelly, L. Riles, S. Véronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. André, A.P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K.D. Entian, P. Flaherty, F. Foury, D.J. Garfinkel, M. Gerstein, D. Gotte, U. Güldener, J. H. Hegemann, S. Hempel, Z. Herman, D.F. Jaramillo, D.E. Kelly, S.L. Kelly, P. Kötter, D. LaBonte, D.C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S.L. Ooi, J.L. Revuelta, C.J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D.D. Shoemaker, S. Sookhai-Mahadeo, R.K. Storms, J.N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Wang, T.R. Ward, J. Wilhelmy, E.

Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J.D. Boeke, M. Snyder, P. Philippsen, R.W. Davis, and M. Johnston, "Functional Profiling of the *Saccharomyces cerevisiae* Genome", *Nature*, Vol. 418, No. 6896, pp. 387–391, 2002.

108. Popolo, L., and M. Vai, "The Gas1 Glycoprotein, a Putative Wall Polymer Cross-Linker", *Biochimica et Biophysica Acta*, Vol. 1426, No. 2, pp. 385–400, 1999.

109. Hamada, K., H. Terashima, M. Arisawa, N. Yabuki, and K. Kitada, "Amino Acid Residues in the Omega-Minus Region Participate in Cellular Localization of Yeast Glycosylphosphatidylinositol-Attached Proteins", *Journal of Bacteriology*, Vol. 181, No. 13, pp. 3886–3889, 1999.

110. Mouyna, I., M. Monod, T. Fontaine, B. Henrissat, B. Léchenne, and J.P. Latgé, "Identification of the Catalytic Residues of the First Family of β(1–3) Glucanosyltransferases Identified in Fungi", *Biochemistry Journal*, Vol. 347, pp. 741–747, 2000.

111. Rolli, E., E. Ragni, J.M. Rodriguez-Pena, J. Arroyo, and L. Popolo, "GAS3, A Developmentally Regulated Gene, Encodes a Highly Mannosylated and Inactive Protein of the Gas Family of *Saccharomyces cerevisiae*", *Yeast*, Vol. 27, No. 8, pp. 597–610, 2010.

112. Kim, H., K. Melén, and G. von Heijne, "Topology Models for 37 *Saccharomyces cerevisiae* Membrane Proteins Based on C-Terminal Reporter Fusions and Predictions", *The Journal of Biological Chemistry*, Vol. 278, No. 12, pp. 10208–10213, 2003.

113. Millson, S.H., A.W. Truman, V. King, C. Prodromou, L.H. Pearl, and P.W. Piper, "A Two-Hybrid Screen of the Yeast Proteome for Hsp90 Interactors Uncovers a Novel Hsp90 Chaperone Requirement in the Activity of a Stress-Activated Mitogen-Activated Protein Kinase, Slt2p (Mpk1p)", *Eukaryotic Cell*, Vol. 4, No. 5, pp. 849–860, 2005.

114. Benjaphokee, S., D. Hasegawa, D. Yokota, T. Asvarak, C. Auesukaree, M. Sugiyama, Y. Kaneko, C. Boonchird, and S. Harashima, "Highly Efficient Bioethanol

Production by a *Saccharomyces cerevisiae* Strain With Multiple Stress Tolerance to High Temperature, Acid and Ethanol", *New Biotechnology*, Vol. 29, No. 3, pp. 379–386, 2012.

115. Ding, J., X. Huang, N. Zhao, F. Gao, Q. Lu, and K. Zhang, "Response of *Saccharomyces cerevisiae* to Ethanol Stress Involves Actions of Protein Asr1p", *Journal of Microbiology and Biotechnology*, Vol. 20, No. 12, pp. 1630–1636, 2010.

116. Izawa, S., T. Kita, K. Ikeda, and Y. Inoue, "Heat Shock and Ethanol Stress Provoke Distinctly Different Responses in 3-Processing and Nuclear Export of HSP mRNA in *Saccharomyces cerevisiae*", *Biochemistry Journal*, Vol. 414, No. 1, pp. 111–119, 2008.

117. Causton, H.C., B. Ren, S.S. Koh, C.T. Harbison, E. Kanin, E.G. Jennings, T. I. Lee, H.L. True, E.S. Lander, and R. Young, "Remodeling of Yeast Genome Expression in Response to Environmental Changes", *Molecular Biology of the Cell*, Vol. 12, No. 2, pp. 323–337, 2001.

118. Caba, E., D.A. Dickinson, G.R. Warnes, and J. Aubrecht, "Differentiating Mechanisms of Toxicity Using Global Gene Expression Analysis in *Saccharomyces cerevisiae*", *Mutation Research*, Vol. 575, pp. 34–46, 2005.

119. Bolstad, B.M., R. Irizarry, M. Astrand, and T.P. Speed, "A comparison of Normalization Methods for High Density Oligonucleotide Array Data Based on Variance and Bias", *Bioinformatics*, Vol. 19, pp. 185–193, 2003.

120. Gautier, L., L. Cope, B.M. Bolstad, and R. Irizarry, "Affy-Analysis of Affymetrix Genechip Data at the Probe Level", *Bioinformatics*, Vol. 20, No. 3, pp. 307–315, 2004.

121. Gentleman, R.C., V.J. Carey, D.M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A.J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J.Y.H. Yang, and J. Zhang, "Bioconductor : Open Software Development for Computational Biology and Bioinformatics", *Genome Biology*, Vol. 5, No. 10, p. R80, 2004.

122. Smyth, G.K., "Linear Models and Empirical Bayes Methods for Assessing Diferential Expression in Microarray Experiments", *Statistical Applications in Genetics and Molecular Biology*, Vol. 3, p. 3, 2004.

123. Carbon, S., A. Ireland, C.J. Mungall, S. Shu, B. Marshall, and S. Lewis, "AmiGO: Online Access to Ontology and Annotation Data", *Bioinformatics*, Vol. 25, No. 2, pp. 288–289, 2009.

124. Ruepp, A., A. Zollner, D. Maier, K. Albermann, J. Hani, M. Mokrejs, I. Tetko, U. Güldener, G. Mannhaupt, M. Münsterkötter, and H.W. Mewes, "The FunCat, a Functional Annotation Scheme for Systematic Classification of Proteins from Whole Genomes", *Nucleic Acids Research*, Vol. 32, No. 18, pp. 5539–5545, 2004.

125. Seo, J., M. Bakay, Y.W. Chen, S. Hilmer, B. Shneiderman, and E.P. Hoffman, "Interactively Optimizing Signal-to-Noise Ratios in Expression Profiling: Project-Specific Algorithm Selection and Detection P-Value Weighting in Affymetrix Microarrays", *Bioinformatics*, Vol. 20, No. 16, pp. 2534–2544, 2004.

126. Oliveira, A.P., K.R. Patil, and J. Nielsen, "Architecture of Transcriptional Regulatory Circuits is Knitted Over the Topology of Bio-Molecular Interaction Networks", *BMC Systems Biology*, Vol. 2, No. 17, pp. 1–16, 2008.

127. Patil, K.R., and J. Nielsen, "Uncovering Transcriptional Regulation of Metabolism by Using Metabolic Network Topology", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 102, No. 8, pp. 2685–2689, 2005.

128. Cvijovic, M., R. Olivares-Hernández, R. Agren, N. Dahr, W. Vongsangnak, I. Nookaew, K.R. Patil, and J. Nielsen, "BioMet Toolbox: Genome-Wide Analysis of Metabolism", *Nucleic Acids Research*, Vol. 38, pp. W144–149, 2010.

129. Abdulrehman, D., P.T. Monteiro, M.C. Teixeira, N.P. Mira, A.B. Lourenço, S.C. dos Santos, T.R. Cabrito, A.P. Francisco, S.C. Madeira, R.S. Aires, A.L. Oliveira, I. Sá-Correia, and A.T. Freitas, "YEASTRACT: Providing a Programmatic Access to Curated

Transcriptional Regulatory Associations in *Saccharomyces cerevisiae* Through a Web Services Interface", *Nucleic Acids Research*, Vol. 39, pp. D136–140, 2011.

130. Monteiro, P.T., N.D. Mendes, M.C. Teixeira, S. D'Orey, S. Tenreiro, N.P. Mira, H. Pais, A.P. Francisco, A.M. Carvalho, A.B. Lourenço, I. Sá-Correia, A.L. Oliveira, and A.T. Freitas, "YEASTRACT-DISCOVERER: New Tools to Improve the Analysis of Transcriptional Regulatory Associations in *Saccharomyces cerevisiae*", *Nucleic Acids Research*, Vol. 36, pp. D132–136, 2008.

131. Teixeira, M.C., P. Monteiro, P. Jain, S. Tenreiro, A.R. Fernandes, N.P. Mira, M. Alenquer, A.T. Freitas, A.L. Oliveira, and I. Sá-Correia, "The YEASTRACT Database: A Tool for the Analysis of Transcription Regulatory Associations in *Saccharomyces cerevisiae*", *Nucleic Acids Research*, Vol. 34, pp. D446–451, 2006.

132. Goto, M., "Protein O-Glycosylation in Fungi: Diverse Structures and Multiple Functions", *Bioscience, Biotechnology and Biochemistry*, Vol. 71, No. 6, pp. 1415–1427, 2007.

133. Ding, M.Z., B.Z. Li, J.S. Cheng, and Y.J. Yuan, "Metabolome Analysis of Differential Responses of Diploid and Haploid Yeast to Ethanol Stress", *Omics : A Journal of Integrative Biology*, Vol. 14, No. 5, pp. 553–561, 2010.

134. Li, B. Z., J.S. Cheng, M.Z. Ding, and Y.J. Yuan, "Transcriptome Analysis of Differential Responses of Diploid and Haploid Yeast to Ethanol Stress", *Journal of Biotechnology*, Vol. 148, No. 4, pp. 194–203, 2010.

135. Izawa, S., "Ethanol Stress Response in the mRNA Flux of *Saccharomyces cerevisiae*", *Bioscience, Biotechnology and Biochemistry*, Vol. 74, pp. 7–12, 2010.

136. Ljungdahl, P.O., and B. Daignan-Fornier, "Regulation of Amino Acid, Nucleotide, and Phosphate Metabolism in *Saccharomyces cerevisiae*", *Genetics*, Vol. 190, pp. 885–929, 2012.

137. Yamada, R., S.I. Yamakawa, T. Tanaka, C. Ogino, H. Fukuda, and A. Kondo, "Direct and Efficient Ethanol Production from High-Yielding Rice Using A *Saccharomyces cerevisiae* Strain That Express Amylases", *Enzyme and Microbial Technology*, Vol. 48, pp. 393–396, 2011.

138. Choi, G.W., H.W. Kang, and S.K. Moon, "Repeated-Batch Fermentation Using Flocculent Hybrid, *Saccharomyces cerevisiae* CHFY0321 for Efficient Production of Bioethanol", *Applied Microbiology and Biotechnology*, Vol. 84, No. 2, pp. 261–269, 2009.

139. Inderwildi, O.R., and D. King, "Quo Vadis Biofuels?", *Energy and Environmental Science*, Vol. 2, No. 4, p. 343, 2009.

140. Kim, J.H., H.R. Kim, M.H. Lim, H.M. Ko, J.E. Chin, H. B. Lee, I.C. Kim, and S. Bai, "Construction of a Direct Starch-Fermenting Industrial Strain of *Saccharomyces cerevisiae* Producing Glucoamylase, Alpha-Amylase and Debranching Enzyme", *Biotechnology Letters*, Vol. 32, No. 5, pp. 713–719, 2010.

141. Yamada, R., Y. Bito, T. Adachi, T. Tanaka, C. Ogino, H. Fukuda, and A. Kondo, "Efficient Production of Ethanol from Raw Starch by a Mated Diploid *Saccharomyces cerevisiae* with Integrated Alpha-Amylase and Glucoamylase Genes", *Enzyme and Microbial Technology*, Vol. 44, No. 5, pp. 344–349, 2009.

142. Gray, K.A., L. Zhao, and M. Emptage, "Bioethanol", *Current Opinion in Chemical Biology*, Vol. 10, No. 2, pp. 141–146, 2006.

143. Balat, M., H. Balat, and C. Öz, "Progress in Bioethanol Processing", *Progress in Energy and Combustion Science*, Vol. 34, No. 5, pp. 551–573, 2008.

144. Favaro, L., M. Basaglia, M. Saayman, S.H. Rose, W.H. van Zyl, and S. Casello, "Engineering Amylolytic Yeasts For Industrial Bioethanol Production", *Chemical Engineering Transactions*, Vol. 20, pp. 97–102, 2010.

145. Bothast, R.J., and M.A. Schlicher, "Biotechnological Processes for Conversion of Corn into Ethanol", *Applied Microbiology and Biotechnology*, Vol. 67, pp. 19–25, 2005.

146. Sánchez, O.J., and C. Cardona, "Trends in Biotechnological Production of Fuel Ethanol from Different Feedstocks", *Bioresource Technology*, Vol. 99, No. 13, pp. 5270–5295, 2008.

147. Toksoy Öner, E., S.G. Oliver, and B. Kırdar, "Production of Ethanol from Starch by Respiration-Deficient Recombinant *Saccharomyces cerevisiae*", *Applied and Environmental Microbiology*, Vol. 71, No. 10, pp. 6443–6445, 2005.

148. Shen, Y., Y. Zhang, T. Ma, X. Bao, F. Du, G. Zhuang, and Y. Qu, "Simultaneous Saccharification and Fermentation of Acid-Pretreated Corncobs with a Recombinant *Saccharomyces cerevisiae* Expressing Beta-Glucosidase", *Bioresource Technology*, Vol. 99, No. 11, pp. 5099–5103, 2008.

149. de Moraes, L., S. Astolfi-Filho, and S. Oliver, "Development of Yeast Strains for the Efficient Utilisation of Starch: Evaluation of Constructs That Express Alpha-Amylase and Glucoamylase Separately or as Bifunctional Fusion Proteins", *Applied Microbiology and Biotechnology*, Vol. 43, No. 6, pp. 1067–1076, 1995.

150. Birol, G., Z.İ. Önsan, B. Kırdar, and S.G. Oliver, "Ethanol Production and Fermentation Characteristics of Recombinant *Saccharomyces cerevisiae* Strains Grown on Starch", *Enzyme and Microbial Technology*, Vol. 22, No. 8, pp. 672–677, 1998.

151. Saeed, A.I., N.K. Bhagabati, J.C. Braisted, W. Liang, V. Sharov, E.A. Howe, J. Li, M. Thiagarajan, J.A. White, and J. Quackenbush, "TM4 Microarray Software Suite", *Methods of Enzymology*, Vol. 411, pp. 134–193, 2006.

152. Sanchez-Gonzalez, Y., X. Cameleyre, C. Molina-Jouve, G. Goma, and S. Alfenore, "Dynamic Microbial Response Under Ethanol Stress to Monitor *Saccharomyces cerevisiae* Activity in Different Initial Physiological States", *Bioprocess and Biosystems Engineering*, Vol. 32, No. 4, pp. 459–466, 2009.

153. Alfenore, S., X. Cameleyre, L. Benbadis, C. Bideaux, J.L. Uribelarrea, G. Goma, C. Molina-Jouve, and S.E. Guillouet, "Aeration Strategy: A Need For Very High Ethanol Performance in *Saccharomyces cerevisiae* Fed-Batch Process", *Applied Microbiology and Biotechnology*, Vol. 63, pp. 537–542, 2004.

154. Guimarães, P.M.R., J.A. Teixeira, and L. Domingues, "Fermentation of High Concentrations of Lactose to Ethanol by Engineered Flocculent *Saccharomyces cerevisiae*", *Biotechnology Letters*, Vol. 30, No. 11, pp. 1953–1958, 2008.

155. Costenoble, R., H. Valadi, L. Gustafsson, C. Niklasson, and C.J. Franzén, "Microaerobic Glycerol Formation in *Saccharomyces cerevisiae*", *Yeast*, Vol. 16, No. 16, pp. 1483–1495, 2000.

156. Li, Z., I. Lee, E. Moradi, N.J. Hung, A.W. Johnson, and E.M. Marcotte, "Rational Extension of the Ribosome Biogenesis Pathway Using Network-Guided Genetics", *PLoS Biology*, Vol. 7, No. 10, p. e1000213, 2009.

157. Heux, S., R. Cachon, and S. Dequin, "Cofactor Engineering in *Saccharomyces cerevisiae*: Expression of a H_2O-Forming NADH Oxidase and Impact on Redox Metabolism", *Metabolic Engineering*, Vol. 8, No. 4, pp. 303–314, 2006.

158. Ostergaard, S., L. Olsson, and J. Nielsen, "Metabolic Engineering of *Saccharomyces cerevisiae*", *Microbiology and Molecular Biology Reviews*, Vol. 64, No. 1, pp. 34–50, 2000.

159. Shigechi, H., Y. Fujita, J. Koh, M. Ueda, H. Fukuda, and A. Kondo, "Energy-Saving Direct Ethanol Production from Low-Temperature-Cooked Corn Starch Using a Cell-Surface Engineered Yeast Strain Co-Displaying Glucoamylase and α-amylase", *Biochemical Engineering Journal*, Vol. 18, No. 2, pp. 149–153, 2004.

160. Khaw, T.S., Y. Katakura, J. Koh, A. Kondo, M. Ueda, and S. Shioya, "Evaluation of Performance of Different Surface-Engineered Yeast Strains for Direct Ethanol Production

from Raw Starch", *Applied Microbiology and Biotechnology*, Vol. 70, No. 5, pp. 573–579, 2006.

161. Khaw, T.S., Y. Katakura, K. Ninomiya, Y. Bito, S. Katahira, A. Kondo, M. Ueda, and S. Shioya, "Effect of Flocculation on Performance of Arming Yeast in Direct Ethanol Fermentation", *Applied Microbiology and Biotechnology*, Vol. 73, No. 1, pp. 60-66, 2006.

162. Khaw, T.S., Y. Katakura, K. Ninomiya, C. Moukamnerd, A. Kondo, M. Ueda, and S. Shioya, "Enhancement of Ethanol Production by Promoting Surface Contact between Starch Granules and Arming Yeast in Direct Ethanol Fermentation", *Journal of Bioscience and Bioengineering*, Vol. 103, No. 1, pp. 95–97, 2007.

163. Yamada, R., T. Tanaka, C. Ogino, H. Fukuda, and A. Kondo, "Novel Strategy for Yeast Construction Using δ-Integration and Cell Fusion to Efficiently Produce Ethanol from Raw Starch", *Applied Microbiology and Biotechnology*, Vol. 85, No. 5, pp. 1491–1498, 2010.