

STUDIES ON THE ROLE OF *HAP4*, *MIG1* AND *MIG2* IN TRANSCRIPTIONAL  
CONTROL NETWORK OF *SACCHAROMYCES CEREVISIAE*

by

Elif Dereli

B.S. in Ch.E., Yıldız Technical University, 2005

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

Graduate Program in Chemical Engineering  
Boğaziçi University

2007

## ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my thesis advisor Prof. Betül Kırdar for her enthusiastic supervision and endless support. Her understanding, research perspective and invaluable guidance have been of great value for me. It has been a great honor for me to work with her.

I am also grateful to the members of my thesis committee for devoting their precious time to read and comment on my thesis.

This work was financially supported by Boğaziçi Research Fund through projects 07M106, 06A505 and TUBITAK- 106M444.

I gratefully thank to Duygu Dikicioğlu for her guidance, advices and kindly answering to my endless questions. I would also like to thank to Ayça Cankorur, for her help, friendship and sharing experiment nights with me.

I would like to say a big ‘thank-you’ to all my former and present friends in KB 440 and KB 441: Esra Yücel, Saliha Durmuş Tekir, Betül Kavun, Ece Acuner, K. Yalçın Arga, Tunahan Çakır, Aylin Şanoviç, Hilal Karabıyık and Nil Çınar. I thank you for enjoyable coffee and chatting times.

I owe very special thanks to two important people: Arzu Uyar and Selin Taşdöğen. Arzu, my garfik, you are a lifesaver! Thank you for all your help, support, friendship and always being there whenever I needed. Selin, my expeditious friend, your motivation and energy was incredible. I consider myself very lucky to have you as friends.

Last, but certainly not the least, I am forever indebted to my parents. Thank you for your endless support, understanding, foresight, love and patience when it was most required. Without you I would never achieve anything in my life. This thesis is dedicated to my family.

## ABSTRACT

### STUDIES ON THE ROLE OF *HAP4*, *MIG1* AND *MIG2* IN TRANSCRIPTIONAL CONTROL NETWORK OF *SACCHAROMYCES CEREVISIAE*

In this study, transcriptional responses of *HAP4*, *MIG1*, *MIG2* and *GSM1* genes to transient perturbations under glucose and nitrogen limited conditions were investigated in order to understand glucose sensing and signaling pathways in yeast. For this purpose six sets of chemostat experiments with pulse injections were performed with three homozygous deletion strains (*hoΔ/hoΔ*, *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) of *Saccharomyces cerevisiae* BY4743. Growth characteristics and metabolite profiles for glucose, ethanol, ammonia and glycerol were compared. *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* strains produced higher biomass than *hoΔ/hoΔ* under glucose limitation. Absence of *MIG2* did not affect the glucose repression mechanism as much as *MIG1*. *mig2Δ/mig2Δ* produced the lowest biomass under nitrogen limitation. The glucose concentration was the highest in *hoΔ/hoΔ* in nitrogen limited chemostat cultivation. The expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1* were determined via real time rt-qPCR. *HAP4*, *MIG1*, *MIG2* and *GSM1* were up-regulated under glucose limitation in *hoΔ/hoΔ*. Deletion of *MIG1* or *MIG2* resulted in repression of *HAP4* and *GSM1* under glucose limitation. After the glucose pulse injection *GSM1* was activated after *HAP4* in *mig1Δ/mig1Δ* and this provided further evidence that *GSM1* is regulated by HAP complex. Absence of *MIG2* delayed the activation of *HAP4* after the glucose pulse compared with the absence of *MIG1*. *GSM1* showed similar expression levels with *HAP4* both in *hoΔ/hoΔ* and *mig2Δ/mig2Δ* throughout the glucose limited cultivations. This indicated a possible role of *GSM1* in regulation of respiratory pathway of yeast together with *HAP4*. In nitrogen limited cultivation of *hoΔ/hoΔ*, *HAP4*, *MIG1* and *GSM1* were repressed. Deletion of *MIG1* diminished the up-regulation of *MIG2* under nitrogen limitation. With the ammonium sulfate addition *HAP4*, *MIG1*, *MIG2* and *GSM1* were activated in *mig1Δ/mig1Δ*. In the absence of *MIG2*, *HAP4* was repressed after the nitrogen pulse injection. Deletion of *MIG2* also delayed the activation of *MIG1* after the addition of ammonium sulfate to the medium.

## ÖZET

### **SACCHAROMYCES CEREVISIAE’NİN TRANSKRİPSİYONEL KONTROL AĞYAPISINDA *HAP4*, *MIG1* VE *MIG2* GENLERİNİN ROLLERİ ÜZERİNE ÇALIŞMALAR**

Bu çalışmada, mayadaki glikoz algılama ve sinyal ileti yollarının anlaşılması amacıyla *HAP4*, *MIG1*, *MIG2* ve *GSM1* genlerinin geçici pertürbasyonlara yazılım aşamasında verdikleri tepkiler glikoz ve azot kaynağı sınırlı koşullarda incelenmiştir. Bu amaçla *S. cerevisiae* BY4743 suşunun 3 homozigot delesyon suşu (*hoΔ/hoΔ*, *mig1Δ/mig1Δ* ve *mig2Δ/mig2Δ*) yeni besi yeri eklemesi (darbe) uygulanarak kesiksiz şekilde büyütülmüştür. Bu suşların büyüme karakteristikleri ve metabolitleri birbirleriyle kıyaslanmıştır. Glikoz sınırlamalı (C-sınırlamalı) durumda *mig1Δ/mig1Δ* ve *mig2Δ/mig2Δ* suşlarının *hoΔ/hoΔ* suşundan daha fazla hücre ürettiği gözlemlenmiştir. *MIG2* geninin silinmesinin glikoz baskılama mekanizmasında *MIG1* geninin yokluğu kadar büyük bir etki yaratmadığı gözlemlenmiştir. Azot kaynağının eksik olduğu durumda (N-sınırlamalı) en az hücre üretimi *mig2Δ/mig2Δ* suşunda olmuştur. Aynı ortamda glikoz konsantrasyonunun *hoΔ/hoΔ* suşunda en yüksek miktarda olduğu gözlemlenmiştir. *HAP4*, *MIG1*, *MIG2* ve *GSM1*’in gen anlatımları gerçek zamanlı rt-qPCR ile saptanmıştır. C-sınırlamalı durumda *hoΔ/hoΔ* suşunda *HAP4*, *MIG1*, *MIG2* ve *GSM1* etkin durumda bulunmaktadır. *MIG1* veya *MIG2* silinmesi C-sınırlamalı durumda *HAP4* ve *GSM1*’in baskılanmasına yol açmıştır. *mig1Δ/mig1Δ* suşunda glikoz darbesi sonrası *GSM1*, *HAP4* geninden sonra etkin hale geçmiş ve bu gözlem *GSM1*’in HAP kompleksi tarafından idare edildiği bilgisine ek kanıt sağlamıştır. *MIG2* geninin yokluğunda *HAP4* glikoz darbesiyle *MIG1*’in yokluğundakinden daha geç etkin hale geçmiştir. *GSM1* ve *HAP4*, C-sınırlamalı ortamda *hoΔ/hoΔ* ve *mig2Δ/mig2Δ* suşlarında benzer gen anlatımları göstermişlerdir. Bu durum, *GSM1*’in mayanın solunum yolizinde *HAP4* ile beraber muhtemel bir rolünün olabileceğine işaret etmiştir. N-sınırlamalı durumda *MIG1* geninin silinmesi *MIG2*’nin baskılanmasına yol açmıştır. *MIG2*’nin silinmesi, *MIG1*’in amonyum sulfat darbesinden sonra daha geç etkin hale geçmesine neden olmuştur.

## TABLE OF CONTENTS

|                                                                                           |      |
|-------------------------------------------------------------------------------------------|------|
| ACKNOWLEDGMENTS .....                                                                     | iii  |
| ABSTRACT.....                                                                             | iv   |
| ÖZET .....                                                                                | v    |
| LIST OF FIGURES .....                                                                     | ix   |
| LIST OF TABLES .....                                                                      | xiii |
| LIST OF SYMBOLS/ABBREVIATIONS.....                                                        | xiv  |
| 1. INTRODUCTION.....                                                                      | 1    |
| 1.1. Glucose Metabolism in <i>Saccharomyces cerevisiae</i> .....                          | 1    |
| 1.1.1. Glucose Induction and Repression Pathways in <i>Saccharomyces cerevisiae</i> ..... | 2    |
| 1.2. <i>MIG1</i> (YGL035C) and <i>MIG2</i> (YGL209W).....                                 | 6    |
| 1.3. <i>HAP4</i> (YKL109W).....                                                           | 12   |
| 1.4. <i>GSM1</i> (YJL103C).....                                                           | 14   |
| 1.5. Reverse Transcription Quantitative Real Time Polymerase Chain Reaction ..            | 16   |
| 1.6. The Aim of This Thesis.....                                                          | 17   |
| 2. MATERIALS AND METHODS .....                                                            | 19   |
| 2.1. Microorganisms .....                                                                 | 19   |
| 2.2. Chemicals and Disposable Materials .....                                             | 19   |
| 2.2.1. Medium.....                                                                        | 19   |
| 2.2.1.1. YPD Medium.....                                                                  | 19   |
| 2.1.1.2. F1 Medium .....                                                                  | 20   |
| 2.1.1.3. Trace Element Solution 1 .....                                                   | 20   |
| 2.1.1.4. Trace Element Solution 2 .....                                                   | 20   |
| 2.1.1.5. Vitamin Stock Solution .....                                                     | 20   |
| 2.2.2. Kits .....                                                                         | 21   |
| 2.2.3. Chemicals and Buffers Required for Polymerase Chain Reaction Applications .....    | 21   |
| 2.2.4. Miscellaneous.....                                                                 | 21   |
| 2.2.5. Laboratory Equipment.....                                                          | 22   |
| 2.3. Methods.....                                                                         | 23   |

|                                                                                                                                                                                                 |    |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 2.3.1. Sterilization .....                                                                                                                                                                      | 23 |
| 2.3.2. Cultivation Conditions.....                                                                                                                                                              | 24 |
| 2.3.3. Sample Preparation and Storage .....                                                                                                                                                     | 25 |
| 2.3.4. Determination of Metabolite Concentrations via Enzymatic Analyses                                                                                                                        | 25 |
| 2.3.4.1. Determination of D-Glucose Concentration.....                                                                                                                                          | 25 |
| 2.3.4.2. Determination of Ethanol Concentration .....                                                                                                                                           | 26 |
| 2.3.4.3. Determination of Glycerol Concentration.....                                                                                                                                           | 27 |
| 2.3.4.4. Determination of Ammonia Concentration .....                                                                                                                                           | 28 |
| 2.3.5. RNA Extraction.....                                                                                                                                                                      | 29 |
| 2.2.6. Reverse Transcription Real Time Quantification Polymerase Chain<br>Reaction (RT-rtqPCR).....                                                                                             | 31 |
| 2.2.7. Relative Quantification of Gene Expression .....                                                                                                                                         | 33 |
| 3. RESULTS .....                                                                                                                                                                                | 34 |
| 3.1. Growth Characteristics of Deletion Strains.....                                                                                                                                            | 34 |
| 3.1.1. Response of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) to Glucose Pulse .....                                                                                                        | 35 |
| 3.1.2. Response of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) to Nitrogen Pulse .....                                                                                                       | 36 |
| 3.1.3. Response of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) to Glucose Pulse                                                                                                          | 38 |
| 3.1.4. Response of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) to Nitrogen<br>Pulse. ....                                                                                                | 40 |
| 3.1.5. Response of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) to Glucose Pulse                                                                                                          | 41 |
| 3.1.6. Response of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) to Nitrogen<br>Pulse. ....                                                                                                | 43 |
| 3.2. Transcriptional Responses of <i>HAP4</i> , <i>MIG1</i> , <i>MIG2</i> and <i>GSM1</i> to a Sudden<br>Release from Nutritional Limitations in Deletion Strains of <i>S. cerevisiae</i> ..... | 45 |
| 3.2.1. Transcriptional Responses of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) to<br>Glucose Pulse .....                                                                                    | 46 |
| 3.2.2. Transcriptional Responses of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) to<br>Nitrogen Pulse .....                                                                                   | 50 |
| 3.2.3. Transcriptional Responses of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) to<br>Glucose Pulse .....                                                                                | 53 |
| 3.2.4. Transcriptional Responses of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) to<br>Nitrogen Pulse .....                                                                               | 56 |
| 3.2.5. Transcriptional Responses of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) to                                                                                                       |    |

|                                                                                                                                             |    |
|---------------------------------------------------------------------------------------------------------------------------------------------|----|
| Glucose Pulse .....                                                                                                                         | 59 |
| 3.2.6. Transcriptional Responses of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) to<br>Nitrogen Pulse .....                           | 62 |
| 4. DISCUSSION .....                                                                                                                         | 66 |
| 4.1. Comparison of Groth Characteristics of the Deletion Strains .....                                                                      | 66 |
| 4.2. Comparison of Transcriptional Responses of <i>HAP4</i> , <i>MIG1</i> , <i>MIG2</i> and <i>GSM1</i><br>Pulses in Deletion Strains ..... | 70 |
| 5. CONCLUSIONS AND RECOMMENDATIONS .....                                                                                                    | 77 |
| 5.1. Conclusions .....                                                                                                                      | 77 |
| 5.2. Recommendations .....                                                                                                                  | 78 |
| REFERENCES .....                                                                                                                            | 80 |

## LIST OF FIGURES

|             |                                                                                                                                                                         |    |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1.1. | Glucose metabolism in yeast .....                                                                                                                                       | 2  |
| Figure 1.2. | Glucose induction pathway in <i>S. cerevisiae</i> .....                                                                                                                 | 3  |
| Figure 1.3. | Glucose Repression Pathway in <i>S. cerevisiae</i> .....                                                                                                                | 5  |
| Figure 1.4. | Schematic view of the mode of action of Mig1p and its regulation .....                                                                                                  | 7  |
| Figure 3.1. | Growth curve of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under glucose limited condition in chemostat culture with pulse injection.....                           | 35 |
| Figure 3.2. | Extracellular metabolite profiles of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under glucose limited condition in chemostat culture with pulse injection .....     | 36 |
| Figure 3.3. | Growth curve of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under nitrogen limited condition in chemostat culture with pulse injection.....                          | 37 |
| Figure 3.4. | Extracellular metabolite profiles of <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under nitrogen limited condition in chemostat culture with pulse injection .....    | 37 |
| Figure 3.5. | Growth curve of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under glucose limited condition in chemostat culture with pulse injection.....                       | 39 |
| Figure 3.6. | Extracellular metabolite profiles of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under glucose limited condition in chemostat culture with pulse injection ..... | 39 |

|              |                                                                                                                                                                          |    |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 3.7.  | Growth curve of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under nitrogen limited condition in chemostat culture with pulse injection.....                       | 40 |
| Figure 3.8.  | Extracellular metabolite profiles of <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under nitrogen limited condition in chemostat culture with pulse injection ..... | 41 |
| Figure 3.9.  | Growth curve of <i>S. Cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under glucose limited condition in chemostat culture with pulse injection.....                        | 42 |
| Figure 3.10. | Extracellular metabolite profiles of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under glucose limited condition in chemostat culture with pulse injection .....  | 43 |
| Figure 3.11. | Growth curve of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under nitrogen limited condition in chemostat culture with pulse injection.....                       | 44 |
| Figure 3.12. | Extracellular metabolite profiles of <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under nitrogen limited condition in chemostat culture with pulse injection ..... | 44 |
| Figure 3.13. | Expression profile of <i>HAP4</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under glucose limitation in chemostat cultures with pulse injection .....       | 48 |
| Figure 3.14. | Expression profile of <i>MIG1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under glucose limitation in chemostat cultures with pulse injection .....       | 48 |
| Figure 3.15. | Expression profile of <i>MIG2</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under glucose limitation in chemostat cultures with pulse injection .....       | 49 |
| Figure 3.16. | Expression profile of <i>GSM1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under glucose limitation in chemostat cultures with pulse injection .....       | 49 |

|              |                                                                                                                                                                         |    |
|--------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 3.17. | Expression profile of <i>HAP4</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under nitrogen limitation in chemostat cultures with pulse injection .....     | 51 |
| Figure 3.18. | Expression profile of <i>MIG1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under nitrogen limitation in chemostat cultures with pulse injection .....     | 52 |
| Figure 3.19. | Expression profile of <i>MIG2</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under nitrogen limitation in chemostat cultures with pulse injection .....     | 53 |
| Figure 3.20. | Expression profile of <i>GSM1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> ) under nitrogen limitation in chemostat cultures with pulse injection .....     | 53 |
| Figure 3.21. | Expression profile of <i>HAP4</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under glucose limitation in chemostat cultures with pulse injection .....  | 55 |
| Figure 3.22. | Expression profile of <i>MIG2</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under glucose limitation in chemostat cultures with pulse injection .....  | 55 |
| Figure 3.23. | Expression profile of <i>GSM1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under glucose limitation in chemostat cultures with pulse injection .....  | 56 |
| Figure 3.24. | Expression profile of <i>HAP4</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under nitrogen limitation in chemostat cultures with pulse injection ..... | 58 |
| Figure 3.25. | Expression profile of <i>MIG2</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under nitrogen limitation in chemostat cultures with pulse injection ..... | 58 |

|                                                                                                                                                                                      |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 3.26. Expression profile of <i>GSM1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig1Δ/mig1Δ</i> ) under nitrogen limitation in chemostat cultures with pulse injection ..... | 59 |
| Figure 3.27. Expression profile of <i>HAP4</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under glucose limitation in chemostat cultures with pulse injection .....  | 61 |
| Figure 3.28. Expression profile of <i>MIG1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under glucose limitation in chemostat cultures with pulse injection .....  | 61 |
| Figure 3.29. Expression profile of <i>GSM1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under glucose limitation in chemostat cultures with pulse injection .....  | 62 |
| Figure 3.30. Expression profile of <i>HAP4</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under nitrogen limitation in chemostat cultures with pulse injection ..... | 64 |
| Figure 3.31. Expression profile of <i>MIG1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under nitrogen limitation in chemostat cultures with pulse injection ..... | 64 |
| Figure 3.32. Expression profile of <i>GSM1</i> gene in <i>S. cerevisiae</i> BY4743 ( <i>mig2Δ/mig2Δ</i> ) under nitrogen limitation in chemostat cultures with pulse injection ..... | 65 |
| Figure 4.1. Growth characteristics of <i>S. cerevisiae</i> BY4743 strains in glucose limited chemostat culture.....                                                                  | 67 |
| Figure 4.2. Growth characteristics of <i>S. cerevisiae</i> BY4743 strains in nitrogen Limited chemostat culture .....                                                                | 69 |

## LIST OF TABLES

|            |                                                                                                                           |    |
|------------|---------------------------------------------------------------------------------------------------------------------------|----|
| Table 2.1. | RT-rtqPCR primer sequences .....                                                                                          | 31 |
| Table 3.1. | Sampling times for RNA extraction in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> )<br>under glucose limitation .....     | 47 |
| Table 3.2. | Sampling times for RNA extraction in <i>S. cerevisiae</i> BY4743 ( <i>hoΔ/hoΔ</i> )<br>under nitrogen limitation .....    | 50 |
| Table 3.3. | Sampling times for RNA extraction in <i>S. cerevisiae</i> BY4743<br>( <i>mig1Δ/mig1Δ</i> ) under glucose limitation.....  | 54 |
| Table 3.4. | Sampling times for RNA extraction in <i>S. cerevisiae</i> BY4743<br>( <i>mig1Δ/mig1Δ</i> ) under nitrogen limitation..... | 57 |
| Table 3.5. | Sampling times for RNA extraction in <i>S. cerevisiae</i> BY4743<br>( <i>mig2Δ/mig2Δ</i> ) under glucose limitation.....  | 60 |
| Table 3.6. | Sampling times for RNA extraction in <i>S. cerevisiae</i> BY4743<br>( <i>mig2Δ/mig2Δ</i> ) under nitrogen limitation..... | 63 |

**LIST OF SYMBOLS / ABBREVIATIONS**

|                |                                                                             |
|----------------|-----------------------------------------------------------------------------|
| A              | Absorbance                                                                  |
| his            | Histidine                                                                   |
| leu            | Leucine                                                                     |
| met            | Methionine                                                                  |
| mRNA           | Messenger RNA                                                               |
| N              | Cycle number                                                                |
| T <sub>m</sub> | Melting temperature                                                         |
| ura            | Uracil                                                                      |
| CF             | Cycle fluorescence                                                          |
| DEPC           | Diethylpolycarbonate                                                        |
| MAT            | Mating                                                                      |
| OD             | Optical density                                                             |
| PID controller | Proportional, integral, derivative controller                               |
| RFU            | Relative fluorescence units                                                 |
| RT-rtqPCR      | Reverse transcription real time quantification polymerase<br>chain reaction |
| TCA cycle      | Tricarboxylic acid cycle                                                    |
| YPD            | Yeast extract-peptone-dextrose                                              |

## 1. INTRODUCTION

Glucose is the most abundant sugar on earth, and living organisms have developed extensive regulation of its catabolism. One such organism, namely *Saccharomyces cerevisiae*, has been known to humankind since the dawn of human civilization for producing wine and beer and for leavening the dough for making breads. It is now extensively used as an eukaryotic model organism, as it is easily amenable for genetic manipulations, and it was also the first eukaryotic genome to be sequenced. Much information is available at the molecular level of this organism, and many studies have been undertaken to analyze the transcriptome, the proteome, the metabolome, and the interactome. Knowledge of the sugar signaling system in the simplest, unicellular, eukaryotic living organism, *S. cerevisiae*, would enable extrapolation of understanding toward multicellular organisms, as there are parallels between the *S. cerevisiae* glucose metabolism, tumor cell growth, and diseased/senescent state of eukaryotic cells, and it would eventually provide a stepping stone for gaining improved understanding of glucose metabolism-related diseases like diabetes and cancer (Raghevedran *et al.*, 2005).

### 1.1. Glucose Metabolism in *Saccharomyces Cerevisiae*

Because glucose is the principle carbon and energy source for most cells, most organisms have evolved numerous and sophisticated mechanisms for sensing glucose and responding to it appropriately. This is especially apparent in the yeast *S. cerevisiae*, where these regulatory mechanisms determine the distinctive fermentative metabolism of yeast, a lifestyle it shares with many kinds of tumor cells (Johnston and Kim, 2005).

*S. cerevisiae* has an unusual lifestyle; it prefers to ferment rather than oxidize glucose, even when oxygen is abundant. Glucose is metabolized through glycolysis to pyruvate, which has two fates. In the presence of oxygen, most organisms convert pyruvate to carbon dioxide and water (via the tricarboxylic acid cycle), generating many ATPs via oxidative phosphorylation. Only when oxygen becomes limiting do most cells resort to fermentation, because it yields only 2 ATPs per molecule of glucose. *S. cerevisiae* is one of the few organisms that prefer to ferment glucose, even when oxygen is abundant. The propensity

of *S.cerevisiae* to carry out aerobic fermentation is called ‘Crabtree effect’ (Johnston and Kim, 2005).

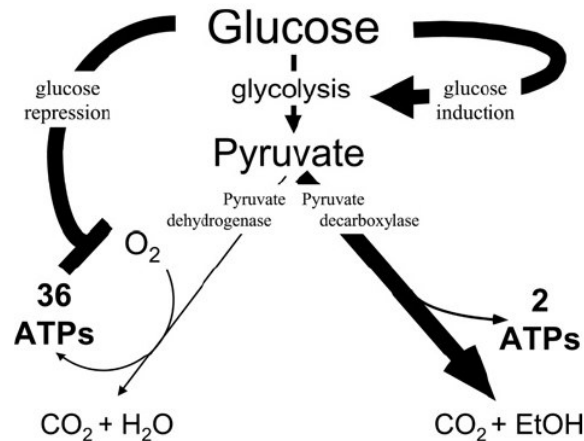


Figure 1.1. Glucose metabolism in yeast (Johnston and Kim, 2005)

### 1.1.1. Glucose Induction and Repression Pathways in *Saccharomyces Cerevisiae*

Nutrient sensing is crucial for all cells. How cells sense glucose is of great interest because defects in glucose sensing can have serious consequences for organisms (Moriya and Johnston, 2004). Glucose is known to affect a variety of processes in *S. cerevisiae* (Palomino *et al.*, 2005). *S. cerevisiae* has sophisticated glucose-sensing and –signaling mechanisms enable it to sense a wide range of glucose concentrations and utilize glucose efficiently (Kim *et al.*, 2006). *S. cerevisiae* is able to detect the glucose levels of extracellular medium and to generate intracellular signals that result in an adequate cellular response to variations in the glucose medium composition. Many of these responses involve alterations in gene expression at the transcriptional, post-transcriptional, translational and post transcriptional levels (Arga *et al.*, 2007). The majority of these alterations occur at the level of mRNA transcription by the repression or activation of the transcription of many genes that encode enzymes implicated in carbon metabolism (Moreno *et al.*, 2005).

In *S. cerevisiae*, glucose is not shown to carry out the inducer/repressor activity by directly binding to an inducer or a repressor protein. Instead, it is the products of glucose metabolism that are somehow involved in triggering the repression. There are two distinct

pathways that mediate glucose transport and metabolism. In the first pathway, known as the glucose induction pathway, glucose has to be sensed so that glucose transporters can be transcribed and translated to transport glucose inside the cell. The second pathway, called the glucose repression pathway, becomes active after the glucose has been transported inside the cell and phosphorylated. The glucose induction and repression pathway have to work in synergy to efficiently utilize the glucose (Raghevendran *et al.*, 2005).

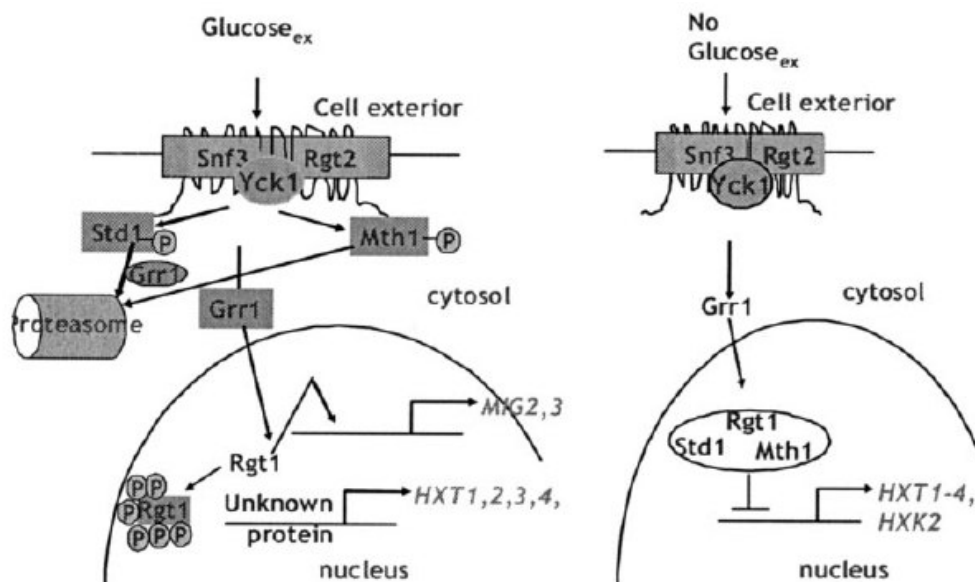


Figure 1.2. Glucose induction pathway in *S. cerevisiae* (Raghevendran *et al.*, 2005)

Yeast has two plasma membrane glucose sensors Snf3 and Rgt2 with very long cytoplasmic tails that sense low and high concentrations of glucose, respectively. When glucose binds to sensors, the sensors undergo a conformational change that activates the membrane-bound casein kinase, Yck1. Two proteins Mth1 and Std1, which interact with the cytoplasmic tails in glucose grown cells, are phosphorylated by Yck1. Phosphorylated Mth1 and Std1 are ubiquitinated by the protein Grr1. Ubiquitinated proteins are recognized by the proteasomal machinery and degraded. All the above mentioned protein-protein interactions occur in the cytoplasm, resulting in a signal being sent to the nucleus, where the transcription factor Rgt1 becomes hyperphosphorylated. Once Rgt1 becomes hyperphosphorylated, it is believed that an unknown protein activates the transcription of the HXT genes. In the absence of glucose, the proteins Mth1 and Std1 move to the nucleus,

where they interact with the active Rgt1 protein and repress the transcription of HXTs (Raghevendran *et al.*, 2005).

The importance of glucose to yeast is underscored by the large number of hexose transporters it possesses. The presence of 18 such proteins has been reported to date. At least six of these (Hxt1, 2, 3, 4, 6 and 7) are known to be glucose transporters, one (Gal2) is a galactose transporter and the others (Hxt5 and Hxt8-17) are probably transporters of other hexoses such as fructose and mannose, which are closely related to glucose. Each of the glucose transporters has different affinity and capacity for glucose. Hxt1 is a low-affinity, high-capacity transporter that is more useful when glucose is abundant (1%); Hxt2 is a high-affinity, low-capacity glucose transporter that is necessary when glucose is scarce (0.2%). The other hexose transporters have evolved for dealing with different concentrations of glucose and under different conditions. Thus, yeast cells have many different glucose transporters to deal with many different conditions.

Several of the glucose transporters are expressed only when glucose is available, and only under the appropriate conditions. The low-affinity, high-capacity Hxt1 glucose transporter is only expressed when glucose is abundant; the high-affinity, low-capacity Hxt2 glucose transporter is only expressed when glucose is scarce. *HXT3*, which encodes a glucose transporter of intermediate affinity, is expressed in cells exposed to both low (0.2%) and high (2%) levels of glucose (Johnston and Kim, 2005).

*Saccharomyces cerevisiae* and many other types of yeast may thrive on a variety of carbon sources, but glucose and fructose are the preferred ones. When one of these sugars is present, the enzymes required for the utilization of alternative carbon sources are synthesized at low rates or not at all (Gancedo, 1998). Since glucose is the most repressing sugar, the term “glucose repression” is often employed (Klein *et al.*, 1998).

A major result of glucose repression is the switching off genes responsible for mitochondrial respiration, alternate carbon source consumption, and gluconeogenesis, resulting in the production of large amounts of ethanol under aerobic growth at high glucose concentrations. Besides the interest at the fundamental level, glucose repression is also of industrial interest because it causes fermentative growth under aerobic conditions,

resulting in reduced biomass yields as in baker's yeast production and in reduced production of heterologous proteins with yeast as an expression system, e.g. human insulin and hepatitis vaccine (Raghevendran *et al.*, 2005).

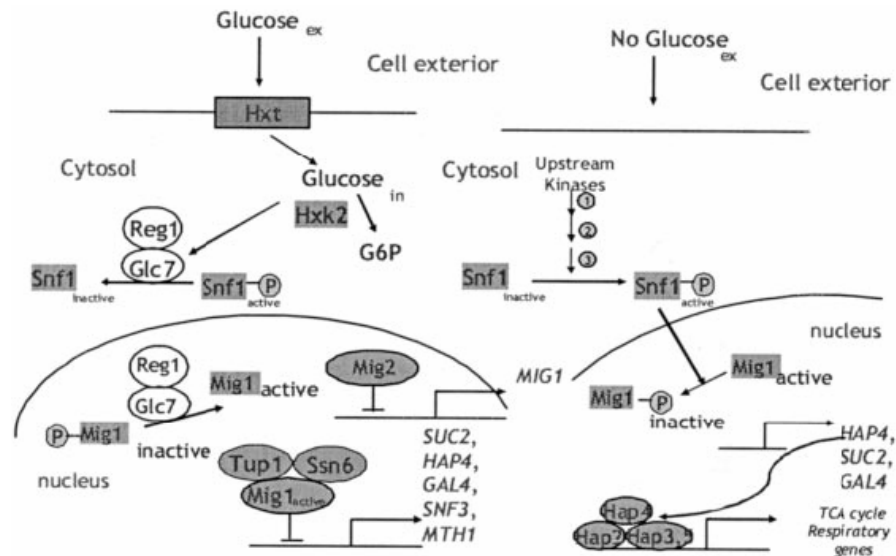


Figure 1.3. Glucose Repression Pathway in *S. cerevisiae* (Raghevendran *et al.*, 2005)

In the presence of glucose, inactive Mig1 (a DNA-binding protein with zinc finger domain) gets dephosphorylated by the Glc7 phosphatase via its regulatory subunit Reg1. It is believed that the metabolic enzyme, hexokinase 2 (Hxk2, it phosphorylates glucose in glycolysis) positively regulates this step either directly or indirectly. Active Mig1 (nonphosphorylated) remains in the nucleus and interacts with the co-repressors Ssn6 and Tup1 and binds to the promoters of various genes, e.g. genes encoding enzymes of the tricarboxylic acid (TCA) cycle, electron transport chain, alternative carbon sources consumption, and gluconeogenesis, and represses the transcription of these genes. When glucose becomes depleted, protein kinase Snf1 gets activated by three upstream kinases. Active Snf1 phosphorylates the transcriptional repressor Mig1 causing Mig1p to translocate from nucleus to cytoplasm thereby inhibiting its interaction with the co-repressors Ssn6 and Tup1 and enabling the transcription of many genes responsible for oxidative growth, like in glucose-limited chemostat. The action of the protein kinase Snf1 is regulated via Reg1 by Glc7, which dephosphorylates Snf1, thereby rendering it inactive under glucose- excess conditions.

As mentioned before, the induction and repression pathways have to function in close association for efficient utilization of glucose. Some of the genes in induction pathway, like *SNF3* and *MTH1*, which directly regulate the expression of hexose transporters, are regulated by the repression pathway (Figures 1.2 and 1.3). In the meantime, genes whose protein products function in the repression pathway like *MIG2*, *MIG3* and *HXX2*, are regulated by the induction pathway (Raghevendran *et al.*, 2005).

### 1.2. *MIG1 (YGL035C)* and *MIG2 (YGL209W)*

*MIG1* was first identified as a multicopy inhibitor of galactose gene expression (Nehlin and Ronne, 1990). Mig1p is a Zinc-finger protein, of the C<sub>2</sub>H<sub>2</sub> type, that binds specifically to DNA with a GC-rich consensus sequence and a flanking AT sequence (Lundin, *et al.*, 1994). The fingers of Mig1p are very similar to those present in the mammalian Egr finger proteins, which are induced during early growth response, and also to the finger protein encoded by a human gene that is deleted in Wilms' tumor cells (Nehlin and Ronne, 1990).

Major function of Mig1p is to repress the transcription of genes whose expression is shut off when glucose is present, such as those encoding enzymes for utilization of the sugars maltose (*MAL*), sucrose (*SUC*), or galactose (*GAL*) (Schüller, 2003; Carlson, 1999). It has also been shown that in some contexts, Mig1p functions as a transcriptional activator (Schuller, 2003; Santangelo, 2006).

DNA binding repressor Mig1 is one of the key elements of the main glucose repression pathway with those sensing hexokinase PII (Hxk2), the serine/threonine protein kinase Snf1. Kinases seem to play a crucial role in this repression cascade, such as hxk2, which phosphorylates rapidly fermentable sugars and exhibits protein kinase activity. Snf1p together with Snf4p (Cat3p), is a part of a protein kinase complex. It is activated by phosphorylation under derepressing conditions, and inactivated by dephosphorylation under repressing conditions. The gene function of *GLC7*, as well as those *REG1 (HEX2)* and *REG2* are known for their antagonism to Snf1; they code for the protein phosphatase 1 complex and are therefore assumed to dephosphorylate Snf1.

Moreover Reg1 has been reported to be involved in the signal transfer for stimulated mRNA degradation (Klein *et al.*, 1998).

A major function of Snf1 kinase is to inhibit repression by Mig1 when glucose is limiting. Snf1 appears to regulate the localization of Mig1 (Figure 1.4). At low levels of glucose, Snf1 is activated through a phosphorylation, and the active Snf1p then catalyzes the phosphorylation of Mig1, causing Mig1p to translocate from the nucleus to the cytosol. Hereby, Mig1-associated repression of several different genes is released. On the other hand, at high levels of glucose, when Snf1 is not phosphorylated, Mig1p remains in the nucleus and here it represses the transcription of genes involved in the metabolism of carbon sources other than glucose and fructose (Carlson, 1999; Westergaard *et al.*, 2007). Addition to this it has also been found that Snf1 is directly involved in nitrogen signaling. Nitrogen limitation improves the phosphorylation of Snf1 and makes it active (Orlova *et al.*, 2006)

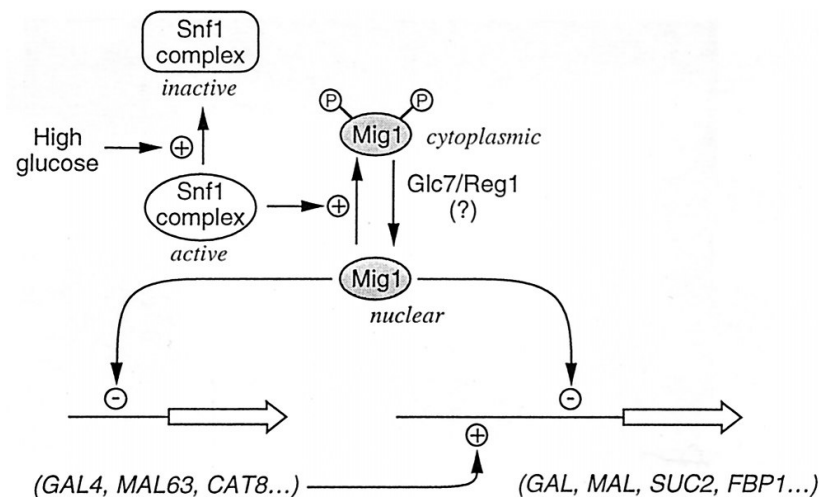


Figure 1.4. Schematic view of the mode of action of Mig1p and its regulation  
(Gancedo, 1998)

Since *MIG1* plays a pivotal role in glucose repression for many metabolic functions it is necessary to discuss the metabolic functions and genes under Mig1-mediated glucose repression. Metabolic functions can be grouped into central metabolism, which comprises glycolysis, the tricarboxylic acid cycle, fermentative and respirative functions, the

glyoxylate shunt and gluconeogenesis, and the peripheral functions such as the catabolism of sugars to the level of glucose-6-phosphate.

As mentioned above in the glucose metabolism, the gene products of *HXT1-17* and *SNF3* involve in regulation of glucose transport and in glucose sensing. *MIG1* deletion mutants have shown that *HXT2*, *HXT4* and *SNF3* are subject to strong Mig1-mediated glucose repression, whereas Mig1p has a lower repressive impact on *HXT3* expression (Özcan and Johnston, 1995). The weak glucose repression of *HXT3* stands in contrast to a general induction of *HXT3* by glucose. It could be explained by a fine-tuned Mig1-mediated repression i.e. a balance between glucose induction and repression gives the appropriate gene expression. Also, Westergaard *et al.* (2007) showed that *HXT2* and *HXT4* are significantly upregulated in the *HXK2Δ* mutant, while these two genes are repressed by Mig1p (Kaniak *et al.*, 2004). This points to a link between Hxk2p and Mig1p.

Uptake of rapidly fermentable sugars (glucose, fructose and mannose) is followed by phosphorylation by gene products of *HXK1*, *HXK2* or *GLK1*. There is evidence that Mig1p is involved in glucose repression of *HXK1* and *GLK1* (Klein *et al.*, 1998).

The disaccharides sucrose and melibiose and the trisaccharide raffinose are hydrolysed outside the cell membrane into monosaccharides, which are then taken up by the cell. Secretion of hydrolytic enzymes is essential because there are no major transport systems for melibiose and raffinose. The *SUC2* gene of the yeast *S. Cerevisiae* encodes the secreted enzyme invertase, which hydrolyses sucrose and raffinose. High levels or complete absence of glucose repress expression of the *SUC2* gene. But low levels of glucose are required for maximal transcription of *SUC2* (Özcan *et al.*, 1997). *SUC2* expression on glucose is nine times higher in the *MIG1* deleted strain than in the wild-type (Nehlin and Ronne, 1990). Nehlin and Ronne (1990) proved by Dnase I footprinting that *MIG1* binds to the *SUC2* upstream region. Besides extracellular sucrose hydrolysis, direct sucrose uptake has been reported, playing an important role under repressing conditions (Santos *et al.*, 1982; Mwesigye and Bardford, 1996).

Melibiose hydrolyses melibiose into glucose and galactose. It is encoded by the *MEL* genes, of which *MEL1* is the most thoroughly investigated. There is evidence that *MEL1*

expression is under Gal4p control, whose expression is under Mig1p control. Moreover, there are good indications that Mig1p directly binds to the *MEL1* upstream sequence, as verified for example by DNase I footprinting (Lundin *et al.*, 1994).

Maltose metabolism is important for brewing and bread-making. In contrast to the above mentioned extracellular sugar hydrolysis, maltose is first taken up via maltose permease which is encoded by *MALT*. It plays a crucial role for maltose metabolism, being essential not only for maltose uptake, but also indirectly for maltose induction of the *MAL* genes: *MAL* genes cannot be induced if maltose cannot be transported into the cell. Maltase is encoded by *MALS*. *MALR* encodes a zinc finger protein essential for maltose induction and glucose repression of *MALT* and *MALS* expression. Hu et al. (1995) identified Mig1p binding sites on promoters of *MAL* genes and quantified the glucose derepression caused by *MIG1* deletion as well as the increase in repression that is caused by *MIG1* overexpression. Glucose control was shown to be partly mediated by, and partly independent of, Mig1p.

Galactose is taken up by galactose permease (encoded by *GAL2*), phosphorylated by galactokinase and further converted to glucose-6-phosphate by enzymes of the Leloir pathway, which are encoded by *GAL7*, *GAL10* and *GAL5* (*PGM2*). *GAL4* encodes a protein that activates transcription of *GAL2*, *GAL1*, *GAL7*, *GAL10* and *GAL5* by binding to sites located upstream of each gene. The *GAL80* gene encodes a protein that binds directly upstream of each gene, preventing it from activating transcription (Johnston, 1987). *GAL4* and *GAL1* are repressed by Mig1p, as proven biochemically by Dnase I footprinting, and physiologically by *MIG1* deletion and *MIG1* overexpression (Nehlin and Ronne, 1990; Nehlin et al., 1991). *GAL1* is under both Mig1 and Gal4 control, whereas *GAL2*, *GAL7* and *GAL10* are at least under indirect *MIG1* control. Moreover, Mig1p has been shown to bind to the *GAL3* promoter and to have a consensus sequence on the *GAL5* promoter (Lundin et al., 1994; Fu et al., 1995).

*FPS1* encodes a channel protein that facilitates glycerol uptake and efflux (Luyten *et al.*, 1995). It has been proved that Mig1p binding to a consensus sequence on the *FPS1* promoter (Lundin *et al.*, 1994). This finding bears relevance for the anaerobic physiology of *S. cerevisiae* where glycerol secretion is necessary for the oxidation of excessive

NADH. A deletion of *MIG1* would allow more glycerol to be secreted if secretion via Fps1p were the rate controlling step. Two *mig1Δ/mig1Δ* strains were shown to secrete significantly higher amounts of glycerol compared to their parental strains (Klein *et al.*, 1996).

Due to the unidirectional glycolytic reactions of phosphofructokinase and pyruvate kinase, the functions of *FBP1* and *PCK1* are essential for gluconeogenesis. Both *FBP1* and *PCK1* have three consensus sequences on their promoters: for Mig1p, for the activating Hap2/ Hap3/ Hap4/ Hap5 complex and for derepressing zinc finger Cat8 protein. Cat8p is related to Gal4p, requires phosphorylation for activation and its expression is under control of Mig1p. *HAP4* has a Mig1-binding site on its promoter; moreover *HAP2* and *HAP4* have shown to be glucose-repressible. It has, however, been reported that a *MIG1* deletion did not result in a derepression of *HAP4*, suggesting a redundant repression mechanism (Klein *et al.*, 1996).

The Mig1p paralogue Mig2p, which has essentially the same binding site as Mig1p, also plays a role in the glucose repression of the expression of some genes. Glucose repression of *MIG1* expression mediated by Mig1 itself, in collaboration with Mig2 (and Mig3), constitutes a feedback regulatory circuit, whereby synthesis of the repressor is decreased under conditions in which it is active. The effect of this regulation is to dampen Mig1-mediated glucose repression, possibly to avoid overrepression, and conceivably to enable a more rapid recovery from repression when glucose becomes depleted from the medium (Kaniak *et al.*, 2004).

Mig2p was initially identified as a repressor that collaborates with Mig1p to cause glucose repression of *SUC2* expression. Mig2p contains zinc fingers very similar to those of Mig1p. These two proteins have overlapping functions: deletion of either one alone had a modest to no effect on *SUC2* glucose repression, and deletion of both almost completely relieved repression (Lutfiyya and Johnston, 1996).

The Snf3/Rgt2 glucose induction pathway contributes to glucose repression by regulating expressions of *MIG2* (and *MIG3*). Rgt1p is likely a direct regulator of these genes because it binds to their promoters in vivo. *MIG2* expression is induced by high

levels of glucose and is constitutively induced in *SNF3-1* and *RGT2-1* mutants. One of the outputs of the glucose signal that is generated by the Rgt2 and Snf3 glucose sensors is glucose repression of expression of genes that are targets of Mig2p (and Mig3) (Kaniak *et al.*, 2004).

As above mentioned Snf1 protein kinase inhibits Mig1 under nonrepressing conditions, by phosphorylating it, leading to its exit from the nucleus. Because of this, *SUC2* expression is abolished in a *SNF1Δ* mutant. Mig1p is clearly responsible for this, because deletion of *MIG1* but not *MIG2* restores expression. Snf1p appears not to inhibit Mig2p function. Nevertheless, Mig2p function is regulated in response to glucose, suggesting that the regulator of Mig2p responds to glucose (Lutfiyya *et al.*, 1998).

Regulation of Mig1p and Mig2p function occurs by different mechanisms. The nuclear localization of Mig1p is regulated by glucose, but Mig2p is located in the nucleus both in the presence and absence of glucose. Also in contrast to Mig1p, Mig2p localization does not depend on phosphorylation (Westergaard *et al.*, 2007). These are consistent with the observation that Snf1p, whose action causes Mig1p to move to the cytoplasm, does not regulate Mig2p activity. *MIG1* expression is repressed about 12-fold in the presence of glucose; *MIG2* is expressed constitutively. Mig1p, together with Mig2, represses its own expression in the presence of glucose (Lutfiyya *et al.*, 1998).

It is known that Mig1p requires for binding an AT- rich sequence directly upstream of the GC-box (Lundin *et al.*, 1994), because only 12% of the sites bound by Mig1p contain two or more G's or C's within the AT-box. In contrast, 22% of the sites bound by Mig2p contain two or more G's and C's within the AT-box. Thus, Mig2p may not have as strict a requirement as Mig1p for the AT-rich sequence for binding. It can be concluded that Mig1p and Mig2 probably recognize the same sequences. Among several genes in the yeast genome whose promoters contain potential Mig1-binding sites, no genes found that are regulated only by Mig1p. Thus, Mig2p always works in conjunction with Mig1p. However Mig1p appears to be more important than Mig2p, since it is sufficient to cause nearly full repression of most of the genes it regulates. An effect of deleting *MIG2* alone is observed only for a few genes. Perhaps Mig2p binds to DNA with lower affinity than Mig1p, causing it to repress gene expression only in the absence of Mig1p; or Mig2p may

be regulated post-transcriptionally, so that it present only at low levels in the presence of Mig1p (Lutfiyya *et al.*, 1998).

### 1.3. *HAP4* (YKL109W)

Heme is a critical cofactor for all living cells as it is involved in many biological processes, including oxidative metabolism, xenobiotic detoxification, gene regulation at the level of transcription, protein translation and targeting, and maintaining protein stability. *S. cerevisiae* makes heme in response to oxygen, and it is proposed that heme then activates the Hap complex which in turn reduces the expression of heme biosynthetic genes to dampen the expression of *HEM* genes (Raghevendran *et al.*, 2006).

In *S. cerevisiae*, the heme-activated protein (HAP) complex plays a major role in orchestrating the transcription of genes involved in the tricarboxylic acid cycle, the electron transport chain, ATP generation, and mitochondrial biogenesis by binding to the CCAAT box at the upstream activation sequence of genes encoding enzymes of these pathways (Raghevendran *et al.*, 2006).

As previously mentioned, the yeast *S. cerevisiae* has a predominantly fermentative metabolism. When grown on media containing glucose as carbon source, yeast cells repress their respiratory metabolism up to the point where all glucose has been consumed, leaving only ethanol as carbon source. In order to use ethanol, the cells reprogram its metabolism, a phase called 'diauxic shift'. The greater part of this reprogramming is under the control of the HAP complex (Buschlen *et al.*, 2003).

The HAP complex is a heteromeric transcriptional regulator composed of four proteins. Hap2p, Hap3p and Hap5p associate to form the DNA-binding part, while Hap4p contains the activation domain (Buschlen *et al.*, 2003). Hap4p has also been found to possess at least two distinct trans-activation domains, each of which has different levels of dependence on co-activation proteins (Raghevendran *et al.*, 2006). While *HAP2*, *HAP3*, *HAP5* are constitutively expressed, transcription of *HAP4* is induced during the diauxic shift. The HAP complex was originally identified as upregulating the expression of

cytochrome C and later on, of several genes encoding TCA cycle and respiratory chain enzymes (Buschlen *et al.*, 2003).

Overexpression of *HAP4* has been shown to result in a 10% increase in the respiratory capacity, implying that Hap4p activity is limiting the respiratory metabolism. The increased level of respiration because of over expression of *HAP4* has been shown further to result in an extended life span of yeast (Raghevendran *et al.*, 2006).

Although the other members of the complex are constitutively expressed, the expression of *HAP4* is regulated by the carbon source and is up-regulated manifold upon glucose exhaustion. Glucose represses the expression of *HAP4* via the Mig1 pathway and thereby activation of respiration is prevented at high glucose concentrations. However, loss of function of Mig1 alone does not result in constitutive activation of respiration and the tricarboxylic acid cycle. Under fermentative conditions, yeasts have compromised mitochondrial function, and the control of the tricarboxylic acid cycle genes might take place by a synergistic action of the retrograde genes and the Hap complex (Raghevendran *et al.*, 2006).

*HAP4* mRNA is present in carbon-limited cultivations even under anaerobic conditions where Hap4p has no obvious role. The promoter of *HAP4* has three carbon source responsive elements, which require a functional Cat8p for the activation; however, deletion of *CAT8* had no effect on the steady state level of *HAP4* expression. It has not been proven *in vivo* that Mig1p binds to the *HAP4* promoter even though it is generally accepted that *HAP4* expression is under glucose repression via Mig1 (Raghevendran *et al.*, 2006). Moreover, overexpression of *HAP4* in a  $\Delta mig1$  deletion background does not contribute to derepression of *SUC2*, but surprisingly restores the repressed state of the *SUC2* promoter (Lascaris *et al.*, 2004).

Hap4p and Mig1p have independent actions in carbon source metabolism. *HAP4* overexpression enables escape from glucose repression by up-regulation of respiratory function and deletion of *MIG1* enables escape from glucose repression by derepression of genes involved in the metabolism of sugars other than glucose (Lascaris *et al.*, 2004)

It has been found by Raghevendran *et al.* (2006) that deletion of *HAP4* had little or no effect on the specific growth rate, compared with the wild type both aerobically as well as anaerobically. Certain key transcriptional regulators has also been found to be responding to the deletion of *HAP4*, including the transcriptional repressor Mig2 that is known to be active only under glucose-repressing conditions. Even though both the wild type and the *hap4* $\Delta$  mutant were grown in glucose-derepressing conditions and expression of *MIG2* was at a low level, *MIG2* was significantly down-regulated in the mutant compared with the wild type.

The HAP complex is also known to regulate ammonia metabolism and the nitrogen catabolite repression via regulation of the activity of two major enzymes in ammonia metabolism, GDH1 and GDH3 (ter Schure *et al.*, 2000). In the yeast *S. cerevisiae*, two NADP<sup>+</sup>-dependent glutamate dehydrogenase isoenzymes encoded by *GDH1* and *GDH3* catalyze the synthesis of glutamate from ammonium and  $\alpha$ -ketoglutarate. *GDH1* expression is controlled by transcriptional activators that have been considered as exclusive of either nitrogen or carbon metabolism (HAP complex). It has been previously shown that *GDH1* expression is glucose-repressed and highly induced in the presence of nonfermentable carbon sources. Impairment of *HAP2*, *HAP3* or *HAP4* diminished *GDH1*-dependent  $\beta$ -Gal activity in ethanol-grown cells. It has been shown that Hap4p is not involved in glucose-dependent regulation, but it does play a role in *GDH1* expression in ethanol-grown cultures. In glucose-grown cells, the HAP complex operates in a Hap4p independent manner, while in ethanol the three subunits are involved in *GDH1* expression; supporting previous observations indicating that under respiratory metabolism, the  $\alpha$ -ketoglutarate yielding enzymes are HAP-controlled (Riego *et al.*, 2002).

#### 1.4. *GSM1 (YJL103C)*

*GSM1 (YJL103C)* is a yeast gene of unknown function. Gsm1p is a transcription factor that regulates mRNA degradation. It was found by Deutschbauer *et al.* (2002) that *GSM1* could inhibit meiosis and spore formation. *GSM1* may directly regulate energy metabolism which could indirectly result in modulating meiosis. It may also play a role in regulating cell growth and division (Deng *et al.*, 2004).

Gsm1p is a member of the zinc cluster family of transcription factors, whose binding sites are variations of CGG repeats. The binding site of Gsm1p is two direct repeats of CGG separated by eight or nine nucleotides. It is somewhat surprising that the spacing of the CGG repeats is variable, because the binding sites of nearly all members of this family of DNA-binding proteins have rigid spacing requirements. In fact, it is the spacing of the CGG repeats (and their orientation) that determines the specificity of DNA binding of each protein. Perhaps Gsm1p forms a complex with other proteins that modify its sequence spacing requirement (Ho *et al.*, 2006).

Analysis revealed a conserved CCAAT box motif in the putative promoters of ORFs involved in the respiratory chain process that is also found in the *GSM1* promoter. *GSM1* belongs to the energy metabolism group and more specifically, the respiratory chain subgroup. A HAP complex binding site was found in *GSM1* promoter and a cytochrome C heme-binding signature in its protein sequence, which has been conserved in the promoters or protein sequences of all other ORFs in the same subgroup. These strongly support that *GSM1* is involved in the regulation of energy metabolism, specifically oxidative phosphorylation, a process that contributes to the cellular respiratory reaction in mitochondria.

Analysis of a *GSM1* deletion mutant did not lead to obvious growth defects on a non-fermentable substrate. It is generally believed that *GSM1* is in the nucleus and probably acts as a transcription factor. It is known that the cellular respiratory chain is located in the mitochondrion. It is not known how Gsm1p participates in the energy process from nucleus. However, nuclear proteins participating in respiratory activity have been found in other studies. Judging the fact that N-terminus of the Gsm1p has repeated alternating hydrophobic and hydrophilic amino acids and the protein contains at least 4 S or T residues in its 20 N-terminal residues, these structures may allow it to be translocated from nucleus to mitochondria (Deng *et al.*, 2004).

It has been identified that several genes differentially regulated when *GSM1* is overexpressed or deleted. Gsm1p binds *in vivo* upstream of genes that contain the CCGN<sub>8</sub>CGG sequence motif. The known or predicted functions of the proteins encoded by these genes are enriched in carbon compound and carbohydrate metabolism, consistent

with the proposed role of Gsm1p in energy utilization (Ho *et al.*, 2006). *GSM1* expression was also found to be significantly downregulated by *HAP4* deletion. It can be concluded that *GSM1* should encode a protein that is involved in transcriptional regulation of the respiratory pathway of yeast (Pir *et al.*, 2006).

### **1.5. Reverse Transcription Quantitative Real Time Polymerase Chain Reaction**

Many cellular functions are regulated by changes in gene expression. Thus, quantification of transcription levels of genes plays a central role in the understanding of gene function and of abnormal alteration in regulation that may result in a disease state (Overbergh *et al.*, 2003). With the ability to measure the PCR products as they are accumulating, or in “real time”, it is possible to measure the amount of PCR product at a point in which reaction is still in the exponential range. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of the template (Ginziger, 2002). The real-time chemistries allow for the detection of PCR amplification during the early phases of the reaction. The real-time PCR system is based on the detection and quantification of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

A primer is a synthetic oligonucleotide with a sequence complementary to that of a section of a DNA (or RNA) molecule of interest. Primer anneals to DNA and is extended by a polymerase, resulting in a copy of a selected region. Primers generally should be chosen from a region where sequence error is likely to be low, should be restricted to area close to/within the region of interest, should not self-hybridize or form hairpins and should be site-specific. There are several criteria required for primer design.

The recommended primer length is 18-24 nucleotides. The length of the PCR product (amplicon) should be 100-150 base pairs or 200-250 base pairs. The GC content of the primers should range between 50 per cent and 60 per cent. The 3' ends (where amplification starts) should be free of secondary and repetitive sequences. The sequences

should lack complementarity to each other, especially at their 3' ends (so primer-dimer will not form). Repeats of G's or C's longer than 3 bases should be avoided. G's and C's should not be placed on ends of the primer.

Most primers should have melting temperatures between 50°C and 65°C where 55°C is considered to be the best. Primers that are used together should have similar  $T_m$  values unless there is contamination, mis-priming, primer-dimer artifacts, etc.

Real-time PCR systems rely upon the detection and quantification of a fluorescent reporter. The reporter signal increases in direct proportion to the amount of PCR product in a reaction. There are two main types of reporters; DNA-binding dyes such as SYBR® Green and probes such as TaqMan® and Molecular Beacons.

The quantification of gene expression is carried out in either of the two ways; absolute quantification which requires a known amount of DNA as the standard curve or relative quantification. The amount of DNA theoretically doubles with every cycle of PCR. After  $N$  cycles, there will be  $2^N$  times as much DNA. Since the reaction cannot go on forever and it eventually tails off and reaches a plateau phase. There is a linear relationship between amount of DNA and cycle number when investigated on a logarithmic scale since PCR amplification is a logarithmic reaction. On a regular scale, the linear part is the very early part of the curve.

Relative quantification requires the presence of control and housekeeping genes to be taken as reference. The housekeeping gene should not be regulated or influenced by the experimental procedure (Radonic *et al.*, 2004).

## 1.6. The Aim of This Thesis

This thesis aims to investigate the transcriptional response of *HAP4*, *MIG1*, *MIG2* and *YJL103C* (*GSM1*) genes to transient perturbations under glucose and nitrogen limited conditions in order to understand glucose sensing and signaling pathways in yeast.. For this purpose six sets of chemostat experiments with *S. cerevisiae* BY4743 strains (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) were carried out. *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) was

included into this study as a control to investigate the variations in the expression of these genes.

In this study chemostat cultivations with pulse injections were performed. Metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained as well as the growth curves. Expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1* genes were determined via reverse transcription real time quantitative polymerase chain reaction (real time RT-qPCR).

The experimental methods used as well as the materials are explained in detail in the Materials and Methods section. Results section comprises all findings that are obtained from this study. The comprehensive argument of the obtained results is given in Discussion. The study is summarized with important key points in the Conclusions section.

## 2. MATERIALS AND METHODS

### 2.1. Microorganisms

Two homozygous deletion mutants (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) of the parent strain *Saccharomyces cerevisiae*, BY4743 (MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0) were used in the experiments.

The deletion mutants were generated by European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF). Prof. Stephen G. Oliver (Faculty of Life Sciences, University of Manchester) kindly provided these strains.

### 2.2. Chemicals and Disposable Materials

#### 2.2.1. Medium

YPD (complex medium) in liquid form and F1 medium with limitations on carbon and nitrogen were used as culture media in the experiments. The compositions of the mediums were as follows:

##### 2.2.1.1 YPD Medium

|                                      |         |
|--------------------------------------|---------|
| ▪ Yeast Extract (Lab M)              | 7 g     |
| ▪ Bacteriological Peptone (Acumedia) | 14 g    |
| ▪ D-Glucose (Merck)                  | 14 g    |
| ▪ H <sub>2</sub> O                   | 665 ml. |

Glucose was added from previously prepared stocks after sterilization of the remaining of the media.

2.2.1.2. F1 Medium

|                                                           |                                                                         |
|-----------------------------------------------------------|-------------------------------------------------------------------------|
| ▪ D-Glucose (Merck)                                       | 20 g or 2.5 g in limited cases                                          |
| ▪ (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (Merck) | 3.13 g or 0.46 g in limited cases                                       |
| ▪ KH <sub>2</sub> PO <sub>4</sub> (Merck)                 | 2 g                                                                     |
| ▪ MgSO <sub>4</sub> ·7H <sub>2</sub> O (Merck)            | 0.55 g                                                                  |
| ▪ NaCl (Merck)                                            | 0.1 g                                                                   |
| ▪ CaCl <sub>2</sub> ·2H <sub>2</sub> O (Merck)            | 0.09 g                                                                  |
| ▪ Uracil (Fluka)                                          | 0.02 g                                                                  |
| ▪ Histidine (Lifco)                                       | 0.02 g                                                                  |
| ▪ Leucine (Merck)                                         | 0.1 g                                                                   |
| ▪ Trace Element Solution 1                                | 100 µl                                                                  |
| ▪ Trace Element Solution 2                                | 100 µl                                                                  |
| ▪ Vitamin Stock Solution                                  | 1.67 µl                                                                 |
| ▪ H <sub>2</sub> O                                        | 992 ml (in carbon limited case) or<br>948 ml (in nitrogen limited case) |

2.2.1.3. Trace Element Solution 1

|                                                |          |
|------------------------------------------------|----------|
| ▪ ZnSO <sub>4</sub> ·7H <sub>2</sub> O (Merck) | 0.0175 g |
| ▪ CuSO <sub>4</sub> ·5H <sub>2</sub> O (Merck) | 0.0025 g |
| ▪ H <sub>3</sub> BO <sub>3</sub> (Merck)       | 0.0025 g |
| ▪ KI (Merck)                                   | 0.0025 g |
| ▪ H <sub>2</sub> O                             | 25 ml    |

2.2.1.4. Trace Element Solution 2

|                                              |          |
|----------------------------------------------|----------|
| ▪ FeCl <sub>3</sub> ·6H <sub>2</sub> O (BDH) | 0.0125 g |
| ▪ H <sub>2</sub> O                           | 25 ml    |

2.2.1.5 Vitamin Stock Solution

|                    |           |
|--------------------|-----------|
| ▪ Inositol (Merck) | 0.062 g/L |
|--------------------|-----------|

|                            |            |
|----------------------------|------------|
| ▪ Thiamine/HCL (Sigma)     | 0.014 g/L  |
| ▪ Pyridoxine (Fluka)       | 0.004 g/L  |
| ▪ Ca-panthothenate (Fluka) | 0.004 g/L  |
| ▪ Biotin (Merck)           | 0.0003 g/L |

### **2.2.2. Kits**

RNeasy MiniKit (250) for RNA extraction and QuantiTect SYBR Green RT-PCR Kit for reverse transcription real time quantification polymerase chain reaction (one step procedure) were purchased from Quiagen. Enzymatic kits for D-glucose, ethanol, glycerol and ammonia concentration determination were purchased from BOEHRINGER MANNHEIM – Roche.

### **2.2.3. Chemicals and Buffers Required for PCR Applications**

Integrated DNA Technologies supplied all primers. Inc. Hind III, Buffer E, Taq Polymerase, MgCl<sub>2</sub> and Mg Free PCR Buffer were provided from Promega. dNTP mix was supplied by Biorad.

### **2.2.4. Miscellaneous**

Other chemicals used in various processes and applications were as follows:

- Absolute Ethanol (Sigma)
- DEPC (Sigma)
- RNase Away (Invitrogen)
- Glycerol (Merck)
- Nitric Acid (Merck)
- RNeasy – RNA Stabilization Reagent (Quiagen)

PCR tubes and disposable plasticware were provided from USA Scientific Inc. iCycler 96 well PCR Plates and Optical Tapes were purchased from BIORAD. Millex

Sterile filter units (0.22  $\mu\text{m}$ ) were supplied by Millipore. Sartopore Sterile filter units (0.45+0.2  $\mu\text{m}$ ) were purchased from Sartorius.

### 2.2.5. Laboratory Equipment

- Autoclave ALP Model CL-40M (Japan)
- Balance Precisa 80A-200M (Switzerland)
- Blower NITTO KOHKI (Japan)
- Centrifuges BECKMAN COULTER, Avanti J-26 XPI  
Superspeed (USA)  
Eppendorf 5415 C (Germany)
- Deep freezers -80°C, New Brunswick Scientific U410 (England)  
-20°C, BOSCH (Germany)
- Dismembrator Biolab Micro-Dismembrator S (New England, USA)
- Fermenter Bioflo III Batch/Continuous Fermenter,  
New Brunswick (England)
- Heating Magnetic Stirrers MR 3001, Heidolph (Germany)  
Scientifica ARE, VELP (Italy)
- Incubators NÜVE EN500 (Turkey)
- Laminar Flow Cabinet HBB 2460 LaminAir, Holten (Denmark)
- O<sub>2</sub> Probe Mettler Toledo (Switzerland)
- Orbital Shakers GFL 3032, GFL (Germany)

INNOVA 4340 Illuminated refrigerated Incubator  
Shaker, New Brunswick Scientific (USA)

- Oven Incucell, MMM Group (Germany)
- Refrigerators +4°C Ariston (Italy)  
+4°C Arçelik (Turkey)
- Spectrophotometer DU 640 Beckman (USA)
- Thermo-cyclers Thermal Reactor TR1, HYBAID (UK)  
GeneAmp PCR System 9600 Perki-Elmer Cetus (USA)  
BIORAD iCycler (USA)
- Transilluminators Reprostar II, CAMAG (Switzerland)  
Foto/uv 15, Eptodyne (USA)
- Vortex Elektromag (Turkey)
- Water Baths HETO, CB 8-30e AT<sub>110</sub> (Denmark)  
HETO DT Hetotherm (Denmark)
- Water Purification Systems Millipore, Milli Ro Plus (USA)  
Millipore, Milli-Q UF Plus (USA)

## 2.3. Methods

### 2.3.1. Sterilization

During the experiments, contamination in any form was prevented by sterilization. Steam and filter sterilization were performed. The steam sterilization was performed in an autoclave at 15 psig pressure at 121°C. Prior to the process every source of external contact that may occur after the sterilization was prevented by taking appropriate measures into account, such as sealing the necks of bottles.

The duration of sterilization was varied according to the nature of the material to be sterilized. For any sort of chemical stock solutions except for glucose, the sterilization time was set to 15 minutes. Glucose was an exception to this because of its highly susceptible nature to caramelization at elevated temperatures. Therefore its sterilization time was limited to 3 minutes. All glassware, plasticware, the fermenters and their complementary parts which were used in chemostat experiments were also sterilized for 15 minutes.

Filter sterilization was preferred for the sterilization of the medium using 0.45 (+0.2)  $\mu\text{m}$  sterile Sartorius disposable filter. The filters were placed at the entrance of the 20 L carboys from where the medium was fed into the fermenters. The medium pumped into the carboys by filter sterilizing.

### **2.3.2. Cultivation Conditions**

100 ml of YPD medium was used as the medium for preculturing process. In this volume of preculture 2 ml of frozen stocks were added for inoculation. The precultures were incubated in orbital shakers at 30°C and 180 rpm. The preculture was ready to be used at its late exponential phase when the optical density was measured to be between 0.9 and 1.1.

BIOFLO3000 fermenters with a working volume of 2 L of F1 medium with C limitation or N limitation were used in chemostat cultivation with pulse injection experiments. The temperature was kept constant at 30°C via PID controllers and the agitation was set to 800 rpm. 20 ml of preculture was used to inoculate the culture. The dilution rate was set to 0.1  $\text{hr}^{-1}$ . The pH was kept at 5.5 via automatic addition of 1M NaOH and HCL. Cultures were flushed with air at a flow rate of 1 L/min using compressor. The dissolved  $\text{O}_2$  concentration was monitored with an  $\text{O}_2$  probe.

In all experiments the same sampling procedure was applied. Until the first steady state condition was reached, only optical densities were measured. After the cells spent three residence times at steady state, in order to reserve the system to concentrations of no limitation, pulse injections of glucose or ammonium sulfate were applied to the carbon limited or nitrogen limited cultures, respectively. Prior to the pulse injection, samples were

taken for the purposes of RNA extraction and extracellular metabolite determination. Four samples were taken for RNA extraction within the first minute after the pulses were given and then for the same purpose samples were taken on 5 minute basis for the first 20 minutes. Then sampling time was extended to hourly basis for ten hours. Sampling for extracellular metabolites was carried out on hourly basis for ten hours after the injection. The last samples were collected after the cells spent three residence times at the second steady state conditions. Duration of the sampling after the pulse injections were 10 hours because at the end of ten hours more than % 95 of the fermenter volume was replaced with fresh feed.

### **2.3.3. Sample Preparation and Storage**

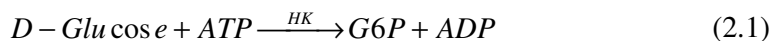
Samples for extracellular metabolite analyses were collected in 2 ml Eppendorf tubes. They were centrifuged at 8000 rpm for 6 minutes in the Eppendorf (Germany) centrifuge with rotor 5415C. The supernatant was transferred to a new Eppendorf tube and was stored at -20°C. Prior to enzymatic analyses, they were incubated at 80°C for 15 minutes to cease any possible enzymatic activity that would have remained.

For RNA extraction, samples were shock frozen in liquid nitrogen or equal volume of culture and RNA stabilization solution. They were kept at -80°C until use.

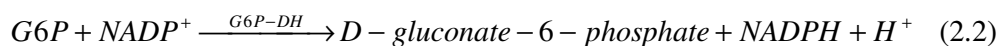
### **2.3.4. Determination of Metabolite Concentrations via Enzymatic Analyses**

Concentration profiles of extracellular D-Glucose, ethanol, glycerol and ammonia were determined. The necessary dilutions of supernatants were carried out as indicated in the protocols prior to the analyses. The metabolite concentrations were determined by enzymatic analysis kits supplied by Boehringer –Mannheim, Germany. The kits were used as described by the manufacturers.

2.3.4.1. Determination of D-Glucose Concentration D-Glucose is phosphorylated to D-Glucose-6-phosphate (G6P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP).



In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G6P is oxidized by nicotinamide adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH).



The test combination contains solution I consisting of triethanolamine buffer, pH 7.6, NADP, ATP, magnesium sulphate and stabilizers, and Solution II consisting of hexokinase and glucose-6-phosphate dehydrogenase.

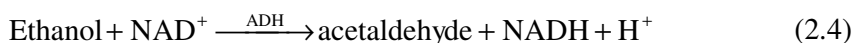
In a cuvette, 0.5 ml of solution I, 0.05 ml sample solution and 0.95 ml redistilled water were pipetted. In another cuvette as blank, 0.5 ml of solution I and 1.0 ml redistilled water were pipetted. The absorbencies ( $A_1$ ) were read at 340 nm against air. Then the reaction was initiated by the addition of 0.01 ml of Solution II. After about 10-15 minutes, the absorbences ( $A_2$ ) were read again.

Absorbance difference of the blank ( $A_2 - A_1$ ) was subtracted from the absorbance difference of the samples ( $A_2 - A_1$ ), thereby obtaining  $\Delta A_{D-glucose}$ . The concentration of D-glucose was calculated by the following equation:

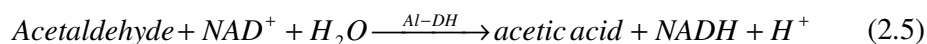
$$c_{D-glucose} = \frac{d \times 5.441}{\epsilon} \times \Delta A_{D-glucose} \quad (g / L) \quad (2.3)$$

where  $\epsilon$  was the extinction coefficient of NADH at 340 nm = 6.3 [L/(mmol x cm)] and d is the dilution factor of the sample.

2.3.4.2. Determination of Ethanol Concentration Ethanol is oxidized to acetaldehyde in the presence of the enzyme alcohol dehydrogenase (ADH) by nicotinamide-adenine dinucleotide (NAD).



Acetaldehyde is oxidized in the presence of aldehyde dehydrogenase (Al-DH) quantitatively to acetic acid.



The test combination contains Mixture I containing potassium diphosphate buffer, pH 9.0, tablets containing NAD, aldehyde dehydrogenase and stabilizers, and Solution II consisting of ADH.

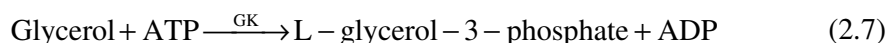
In a cuvette, 1.5 ml of mixture I and 0.05 ml sample were pipetted. In another cuvette as blank, 1.5 ml mixture I and 0.05 ml distilled water were pipetted. The absorbencies ( $A_1$ ) were read at 340 nm against air. About 10-15 minutes after the addition of 0.025 ml of Solution II the absorbences ( $A_2$ ) were read again.

Absorbance difference of the blank ( $A_2 - A_1$ ) was subtracted from the absorbance difference of the samples ( $A_2 - A_1$ ), thereby obtaining  $\Delta A_{\text{Ethanol}}$ . The concentration of the ethanol was calculated by the following equation:

$$c_{\text{Ethanol}} = \frac{d \times 0.7256}{\epsilon} \Delta A_{\text{Ethanol}} (\text{g/L}) \quad (2.6)$$

where  $\epsilon$  was the extinction coefficient of NADH at 340 nm = 6.3 [L/(mmol x cm)] and d is the dilution factor of the sample.

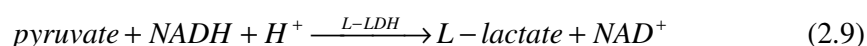
2.3.4.3. Determination of Glycerol Concentration Glycerol is phosphorylated by adenosine-5'-diphosphate (ATP) to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase (GK).



The adenosine-5'-diphosphate (ADP) formed in the above reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) in to ATP with the formation of pyruvate.



In the presence of the enzyme L-lactate dehydrogenase (L-LDH), pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the oxidation of NADH to NAD.



The test combination contains solution I consisting of glycylglycine buffer, pH 7.4, NADH, ATP, magnesium sulfate and stabilizers, solution II consisting pyruvate kinase and L-lactate dehydrogenase and solution III consisting of glycerokinase.

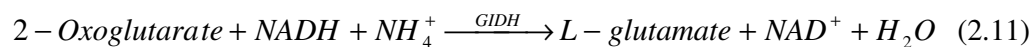
In a cuvette, 0.5 ml of solution I 0.05 ml, sample solution 0.95 ml redistilled water and 0.005 ml solution II were pipetted. In another cuvette as blank, 0.5 ml of solution I, 1.0 ml redistilled water and 0.005 ml solution II were pipetted. The absorbences ( $A_1$ ) were read at 340 nm against air after 5-7 minutes. By addition of 0.005 ml of solution III the reaction began and after about 5 to 10 minutes absorbences ( $A_2$ ) were read again at the same conditions both for sample and for the blank.

Absorbance difference of the blank ( $A_1 - A_2$ ) was subtracted from the absorbance difference of the samples ( $A_1 - A_2$ ), thereby obtaining  $\Delta A_{\text{Glycerol}}$ . The concentration of the glycerol was calculated by the following equation:

$$c_{\text{Glycerol}} = \frac{2.781}{\epsilon} \Delta A_{\text{Glycerol}} (\text{g} / \text{L}) \quad (2.10)$$

where  $\epsilon$  was the extinction coefficient of NADH at 340 nm = 6.3 [L/(mmol x cm)].

2.3.4.4. Determination of Ammonia Concentration In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia reacts with 2-oxoglutarate to L-glutamate, whereby NADH is oxidized.



The amount of NADH oxidized in the above reaction is stoichiometric to the amount of the ammonia. NADH is determined by means of its light absorbance.

The test combination contains Mixture I containing triethanolamine buffer, pH 8.0, 2-oxoglutarate, tablets containing NADH and Solution II consisting of glutamate dehydrogenase.

In a cuvette, 0.5 ml of mixture I, 0.05 ml sample and 0.95 ml distilled water were pipetted. In another cuvette as blank, 0.5 ml mixture I and 1 ml distilled water were pipetted. After 5 minutes, the absorbencies ( $A_1$ ) were read at 340 nm against air. 20 minutes after the addition of 0.01 ml of Solution II the absorbences ( $A_2$ ) were read again.

Absorbance difference of the blank ( $A_1 - A_2$ ) was subtracted from the absorbance difference of the samples ( $A_1 - A_2$ ), thereby obtaining  $\Delta A_{\text{Ammonia}}$ . The concentration of the ammonia was calculated by the following equation:

$$c_{\text{Ammonia}} = \frac{d \times 0.5143}{\epsilon} \Delta A_{\text{Ammonia}} \quad (\text{g / L}) \quad (2.12)$$

where  $\epsilon$  was the extinction coefficient of NADH at 340 nm = 6.3 [L/(mmol x cm)] and  $d$  is the dilution factor of the sample.

### 2.3.5. RNA Extraction

Due to its highly degradable nature, any work involving RNA was performed in RNase and DNase free environments and this was provided by the use of certified RNase and DNase free plasticware and the use of DEPC treated water in cleaning of every surface and glassware. DEPC treated water was prepared as follows: 1  $\mu\text{l}$  DEPC (diethylpolycarbonate) was used per 1 ml of water. It was stored overnight at 37°C and autoclaved.

“RNeasy protocol for extracting yeast via mechanical disruption” was used as described by the manufacturer (Quiagen, USA) for RNA extraction from samples. Samples were first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivated RNases to ensure isolation of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy mini column where the total RNA bound to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in 30  $\mu$ l, or more, of water. With the RNeasy procedure, all RNA molecules longer than 200 nucleotides were isolated. The procedure provided enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20 per cent of total RNA) were selectively excluded.

The kit contents were RNeasy mini spin columns, 2 ml and 1.5 ml collection tubes, Buffer RTL, Buffer RPE, Buffer RW1 and RNase free water.

To lyse the cells and release the RNA, RNeasy protocols were used for isolation of total RNA from yeast via mechanical disruption used high-speed agitation in a bead mill in the presence of glass beads and lysis buffer. The mechanical disruption protocol was suitable for time-course experiments where enzymatic incubation steps could not be tolerated. The first step in the protocol was to prepare acid-washed glass beads, 0.45–0.55 mm diameter, by soaking in concentrated nitric acid for 1 hour, washing extensively with deionized water, and drying in a baking oven. After disruption in a bead mill homogenizer, all steps of the RNeasy protocol were performed at room temperature. It was necessary to work quickly during the procedure. After harvesting the cells, all steps were performed at 20–25°C in a standard microcentrifuge.

600  $\mu$ l of acid-washed glass beads were added to an Eppendorf tube. The yeast cells were harvested by centrifuging at 6800 rpm for 5 min at 4°C. The supernatant was decanted and the remaining media was carefully removed by aspiration. 600  $\mu$ l of Buffer RLT was added to the tube and vortexed to resuspend the cell pellet. The resuspended sample was added to the Eppendorf tube containing the pre prepared glass beads. The sample was vortexed and agitated at 500 rpm for 5 minutes in a bead-mill homogenizer. The sample was removed from the bead mill, and the beads were allowed to settle. The

lysate was transferred to a new microcentrifuge tube and was centrifuged for 2 min at 10000 rpm. The supernatant was transferred to a new microcentrifuge tube. 350 µl of 70 per cent ethanol was added to the homogenized lysate, and mixed by pipetting. The sample was applied to an RNeasy mini column placed in a 2 ml collection tube which was centrifuged for 15 s at 10000 rpm and the flowthrough was discarded. 700 µl of Buffer RW1 was added to the RNeasy column and it was centrifuged for 15 s at 14000 rpm to wash the column. The RNeasy column was transferred into a new 2 ml collection tube and 500 µl of Buffer RPE was added onto the RNeasy column and it was again centrifuged for 15 s at 10000 rpm to wash the column. Another 500 µl of Buffer RPE was applied to the RNeasy column and the column was centrifuged at 14000 rpm to dry the RNeasy silica-gel membrane. For elution, the RNeasy column was transferred to a new 1.5 ml collection tube, 30–50 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane, the tube was gently closed and centrifuged for 1 min at 10000 rpm. All centrifugations were carried out using Eppendorf (Germany) centrifuge with rotor 5415 C and Sorvall RC-5B centrifuge (DuPont, USA) with rotor SS-34. Purified RNA was stored at –20°C.

### 2.3.6. Reverse Transcription Real Time Quantification Polymerase Chain Reaction (RT-rtqPCR)

Primers required to quantitatively amplify the products of *HO*, *HSP12*, *HAP4*, *MIG1*, *MIG2* and *GSM1* (*YJL103C*) genes were designed according to the previously stated criteria (Introduction Section). For each individual sample, duplicates of experiments were carried out. Negative controls containing no sample but a mixture of the primers were also included into the experiments. The forward and reverse primer sets which were used are given in Table 2.1.

Table 2.1. RT-rtqPCR primer sequences

|         |                              |
|---------|------------------------------|
| HO_F    | 5-CCGCGTCATAAATGTCAC-3       |
| HO_R    | 5-CCTACCATCAAGCGTCTG-3       |
| HSP12_F | 5-CTGACGCAGGTAGAAAAGGATTCG-3 |
| HSP12_R | 5-CGGCATCGTTCAACTTGGACTTG-3  |

Table 2.1. RT-rtqPCR primer sequences (continued)

|        |                             |
|--------|-----------------------------|
| MIG1_F | 5- AACGCGTTATCGTCCCTA-3     |
| MIG1_R | 5- TGTGCTCTGAGACTTCCA-3     |
| MIG2_F | 5- CACTGGGGAAAAACCTCA-3     |
| MIG2_R | 5- CTGGTAGGACTTGCGGTA-3     |
| HAP4_F | 5-CACCATGACGAGTTAGGTTTCAG-3 |
| HAP4_R | 5- GGTGGCAGTTGCATCATTGTTG-3 |
| GSM1_F | 5- ACACAGTACCCAAAAGCA -3    |
| GSM1_R | 5- CGCCTGATCTTCTCCAGA -3    |

For RT-rtqPCR applications within this study, Quiagen QuantiTect® SYBR® Green RT-PCR kit was used for quantitative, real-time, one-step RT-PCR. The kit included QuantiTect SYBR Green RT-PCR Master Mix; containing HotStarTaq® DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer, dNTP Mix including dUTP, SYBR Green I, ROX (passive reference dye) and 5 mM MgCl<sub>2</sub>, QuantiTect RT Mix; containing Omniscript® Reverse Transcriptase and Sensiscript® Reverse Transcriptase and lastly RNase-free water.

The reaction mixture for 25 µl total volume was as follows:

|                                         |            |
|-----------------------------------------|------------|
| QuantiTect SYBR Green RT-PCR Master Mix | 12.5 µl    |
| Primers                                 | 2X1. 25 µl |
| QuantiTect RT Mix                       | 0.25 µl    |
| RNA sample                              | 0.5 µl     |
| RNase-free water                        | 8.75 µl    |

The reaction program including the melt curve analysis which was used in the experiments is given as (Dikicioğlu, 2005):

|               |         |        |            |
|---------------|---------|--------|------------|
| Cycle 1 (1X)  | Step 1: | 50°C   | 30 minutes |
| Cycle 2 (1X)  | Step 1: | 95°C   | 15 minutes |
| Cycle 3 (40X) | Step 1: | 94°C   | 15 seconds |
|               | Step 2: | 52.2°C | 15 seconds |

|               |         |      |                             |
|---------------|---------|------|-----------------------------|
|               | Step 3: | 72°C | 30 seconds                  |
| Cycle 4 (1X)  | Step 1: | 95°C | 1 minute                    |
| Cycle 5 (1X)  | Step 1: | 50°C | 1 minute                    |
| Cycle 6 (80X) | Step 1: | 48°C | 10 seconds with ramp: 0.5°C |

### 2.3.7. Relative Quantification of Gene Expression

The experimental output files of the BioRad software are given as cycle florescence (CF) in relative fluorescence units (RFU) of the amplified regions where SYBR Green intercalated. The housekeeping gene *HO* was taken to be the reference gene in expression calculations and the relative quantification of gene expression was calculated according to the reference gene (*HO*). The average values of CF were calculated for both reference gene and the genes of interest. The average CF value of the reference gene was subtracted from the average CF value of the gene of interest.  $2^N$  was considered to be the quantification ratio of the PCR amplification.

### 3. RESULTS

In order to investigate the transcriptional responses of *HAP4*, *MIG1*, *MIG2* and *GSM1* (*YJL103C*) genes to transient perturbations under glucose and nitrogen limited conditions, four sets of chemostat experiments with *S. cerevisiae* BY4743 strains (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) were carried out.

In this study chemostat cultivations with pulse injections were performed. Metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained as well as the growth curves. Expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1* genes were determined via reverse transcription real time quantitative polymerase chain reaction (real time RT-qPCR).

#### 3.1. Growth Characteristics of Deletion Strains

The chemostat cultivations were carried out for two homozygous deletion strains of *S. cerevisiae* wild type strain BY4743, namely *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* in carbon or nitrogen limited F1 medium at 30 °C. pH was kept constant at 5.5 and dilution rate was set to 0.1 hr<sup>-1</sup>, as described in the section of Materials and Methods. *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) was included into this study as a control to investigate the variations in the expression of these genes. Samples from similar chemostat cultivations out with *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under similar conditions were provided by Ayça Cankorur and Aylin Şanoviç.

Samples were regularly taken to follow the growth characteristics of the chemostat cultures. Glucose consumption, ethanol production, ammonia and glycerol concentrations were determined to investigate the effect of the pulse injections on the growth characteristics of these three strains. Arrows indicate where the pulse injections were applied.

### 3.1.1. Response of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) to Glucose Pulse

A strain of *S. cerevisiae* BY4743 carrying a homozygous deletion of *HO* gene (*hoΔ/hoΔ*) was cultivated under glucose limited conditions. A glucose pulse was given to remove the nutrient limitation after three residence times at the steady state conditions. The experiment was terminated after three residence times at the second steady state conditions.

Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) in glucose limited chemostat culture were shown in Figures 3.1 and 3.2. At the end of 80<sup>th</sup> hour of chemostat cultivation the first steady state sample was taken and after that, the glucose pulse injection was applied. Then hourly sampling was done for 10 hours. And the second steady state sample was taken at the 146<sup>th</sup> hour of the fermentation.

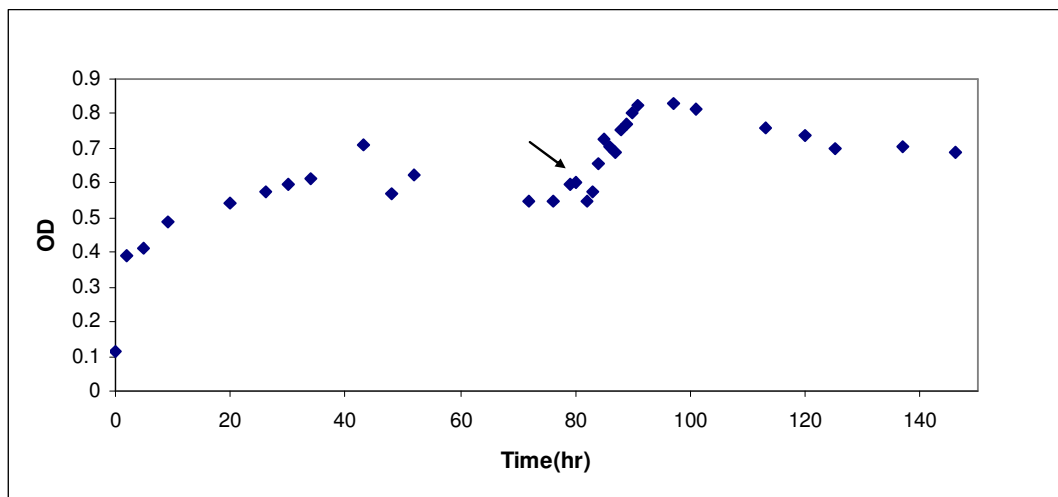


Figure 3.1. Growth curve of *S. Cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limited condition in chemostat culture with pulse injection

After the glucose pulse injection a slight decrease in the growth of the cells was observed. After decreasing for two hours, the growth of the cells was increased and cells continued to show an increasing trend in biomass formation for 13 hours. Cultivations then reached to a second steady state but remained at a higher value than those observed at the first steady state.

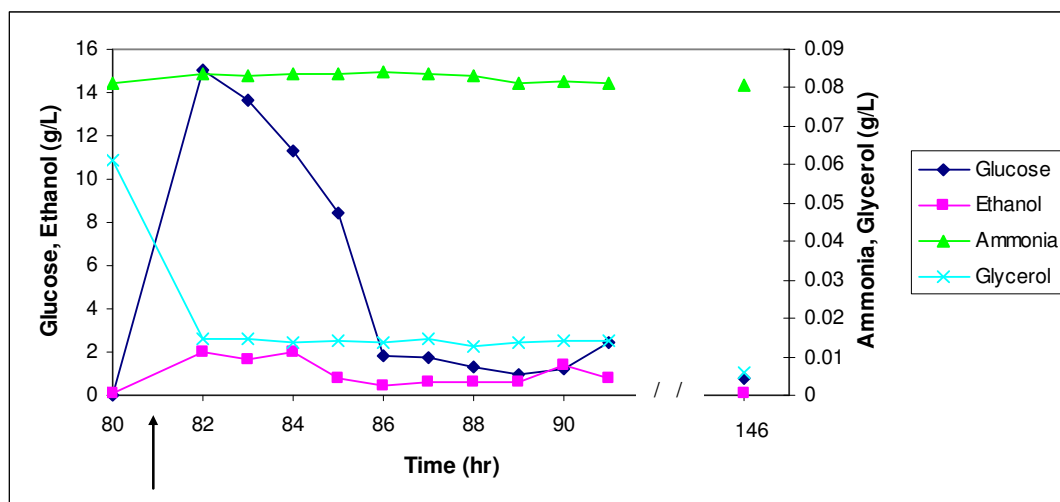


Figure 3.2. Extracellular metabolite profiles of *S. Cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limited condition in chemostat culture with pulse injection

In *S. Cerevisiae* BY4743 (*hoΔ/hoΔ*) cultivated under glucose limited condition, the glucose concentration was 0.02 g/L at the first steady state. Just after the glucose pulse the glucose concentration increased sharply to 17 g/L, then decreased to a value of approximately 2 g/L. At the second state the glucose concentration was measured as 0.7 g/L. Ethanol concentration was 0.06 g/L before the pulse and it reached to 2.4 g/L just after the pulse. It then decreased and at the second state the ethanol concentration was measured as 0.07 g/L. Ammonia concentration remained constant during the chemostat cultivation and its concentration was 0.08 g/L. Extracellular glycerol production decreased sharply soon after the application of the glucose pulse. Prior to the pulse its concentration was measured as 0.06 g/L and then decreased to 0.01 g/L after the glucose pulse injection. At the second steady state glycerol concentration was measured as 0.006 g/L.

### 3.1.2. Response of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) to Nitrogen Pulse

*HO* deleted strain of *S. cerevisiae* BY4743 was also cultivated under nitrogen limited conditions. An ammonium sulfate pulse was given to remove the nutrient limitation after passing three residence times at the steady state conditions. The experiment was terminated after three residence times at the second steady state conditions.

Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) in nitrogen limited chemostat culture were given in Figures 3.3 and 3.4. At the end of 48<sup>th</sup> hour of chemostat cultivation the first steady state sample was taken and after that the nitrogen pulse injection was applied. Then hourly sampling was done for 10 hours. And the second steady state sample was taken at the 118<sup>th</sup> hour.

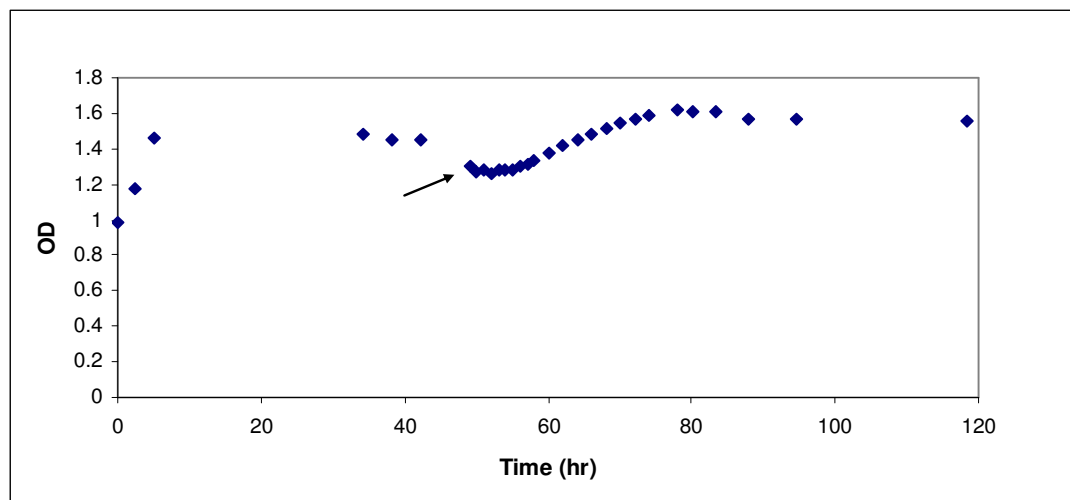


Figure 3.3. Growth curve of *S. Cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limited condition in chemostat culture with pulse injection

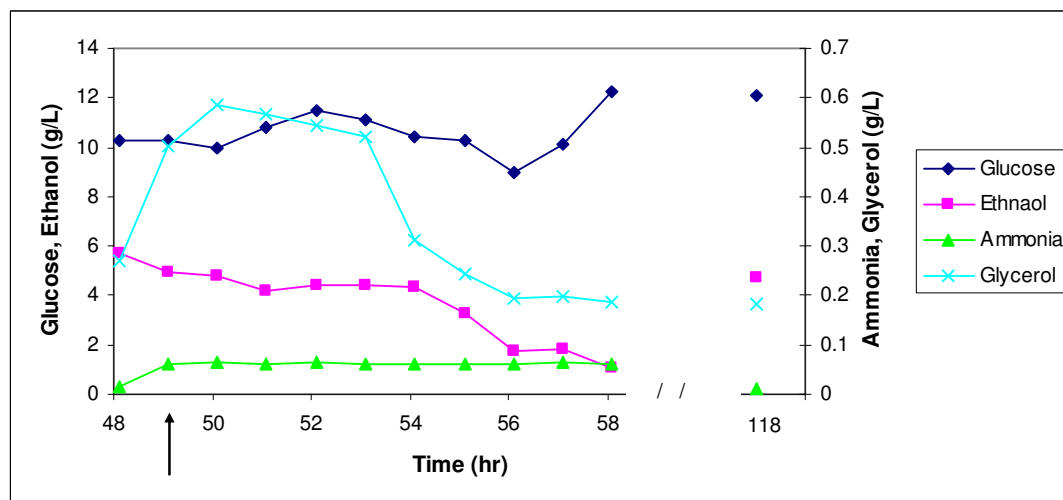


Figure 3.4. Extracellular metabolite profiles of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limited condition in chemostat culture with pulse injection

A slight decrease in the growth of the cells with the nitrogen pulse injection was observed. Biomass production reached slowly to a constant value and at the second steady state the OD values were higher with respect to the first steady state.

Under nitrogen limitation the glucose concentration was nearly constant in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) during the cultivation and varied between 10 g/L and 12 g/L. Ethanol concentration decreased after the pulse injection. Its concentration was 5.6 g/L before the pulse and decreased slowly with time to a value of 1 g/L. At the second steady state ethanol concentration was measured to be 4.7g/L. Ammonia concentration was increased from 0.01 g/L to 0.06 g/L with the pulse and remained constant for at least 9 hours. At the second steady state ammonia concentration decreased to its initial value. Glycerol concentration first increased with the pulse then it decreased and remained constant at the level of 0.2 g/L.

### **3.1.3. Response of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) to Glucose Pulse**

A homozygous deletion strain of *MIG1* (*mig1Δ/mig1Δ*) in a genetic background of wild type *S. cerevisiae* BY4743 was cultivated under glucose limited conditions. A glucose pulse was given to remove the nutrient limitation after passing three residence times at the steady state conditions. The experiment was terminated after three residence times at the second steady state conditions.

Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) in glucose limited chemostat culture were shown in Figures 3.5 and 3.6. At the end of 48<sup>th</sup> hour of chemostat cultivation the first steady state sample was taken and after that the glucose pulse injection was applied. Then hourly sampling was done for 10 hours. And the second steady state sample was taken at the 120<sup>th</sup> hour.

After the glucose pulse first a slight decrease and than an increase in the growth of the cells were observed. The growth of the cells decreased after 58<sup>th</sup> hour of cultivation for 6 hours then increased sharply and remained constant for 9 hours. After the 78<sup>th</sup> hour the growth decreased to its initial value and remained constant at this value at the second steady state.

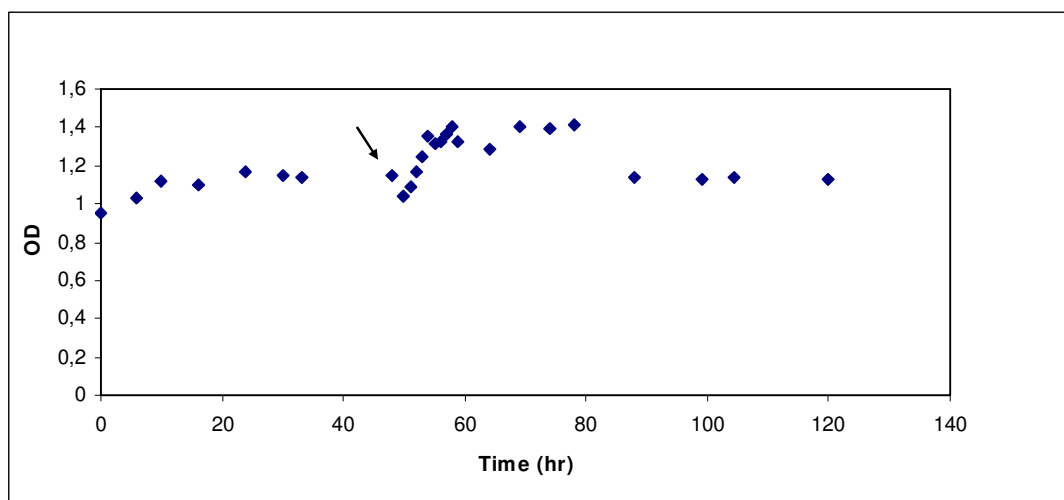


Figure 3.5. Growth curve of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under glucose limited condition in chemostat culture with pulse injection

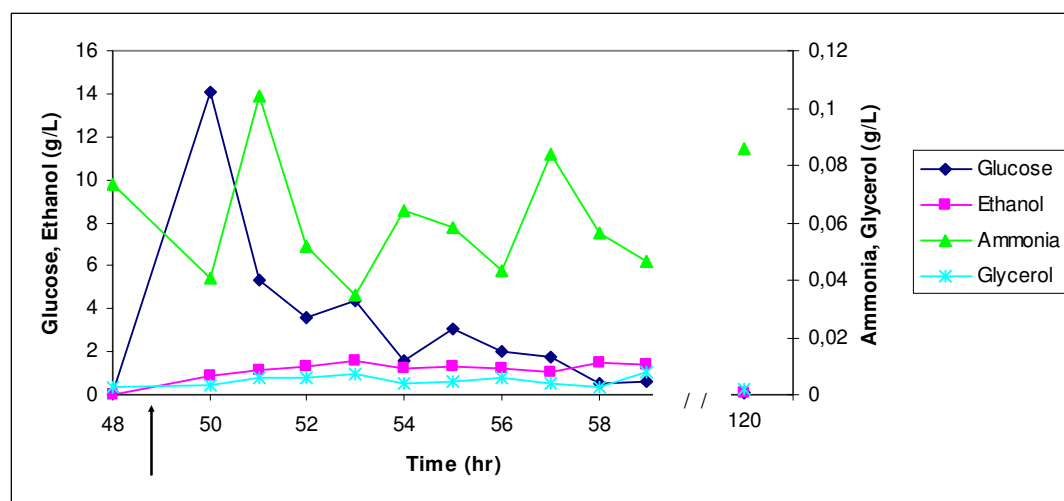


Figure 3.6. Extracellular metabolite profiles of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under glucose limited condition in chemostat culture with pulse injection

In *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) cultivated under glucose limited condition, the glucose concentration was 0.05 g/L at the first steady state. After the first hour of glucose pulse injection, the glucose concentration increased sharply to 14 g/L. At the second steady state its concentration decreased to 0.1 g/L. The concentration of ethanol was measured as 0.03 g/L before the pulse injection. Ethanol production reached to its maximum value of 1.6 g/L at the end of fourth hour and decreased to 0.09 g/L at the second steady state. Ammonia concentration changed in the range of 0.03 and 0.1 g/L and

showed an oscillatory behavior. After the application of glucose pulse, glycerol concentration first increased for 4 hours then decreased to its initial value of 0.002 g/L at the second steady state.

#### 3.1.4. Response of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) to Nitrogen Pulse

*MIG1* disrupted strain of *S. cerevisiae* BY4743 was cultivated under nitrogen limited conditions. An ammonium sulfate pulse was given to remove the nutrient limitation after passing three residence times at the steady state conditions. The experiment was terminated after three residence times at the second steady state conditions.

In Figures 3.7 and 3.8, extracellular metabolite profiles and growth curve of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) in nitrogen limited chemostat culture were depicted. At the end of 47<sup>th</sup> hour of chemostat cultivation the first steady state sample was taken. After that, the nitrogen pulse injection was applied and a sample was taken within the first minute of pulse. Then hourly sampling was done for 10 hours. And the second steady state sample was taken at the 101<sup>st</sup> hour.

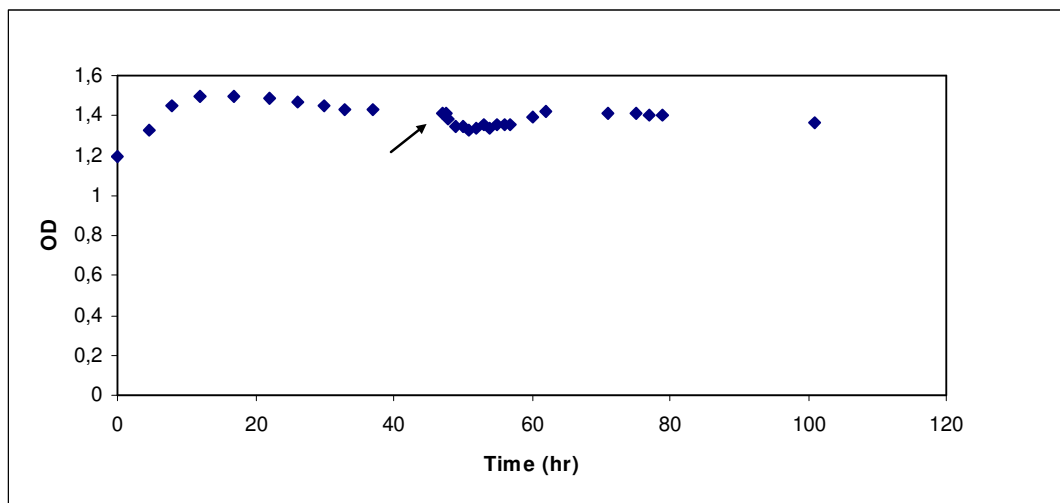


Figure 3.7. Growth curve of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under nitrogen limited condition in chemostat culture with pulse injection

After sudden addition of ammonium sulfate to the nitrogen limited medium a decrease in growth was observed for 4 hours. Then the growth of the cells increased again

and remained nearly constant at the same level until the end of second steady state.

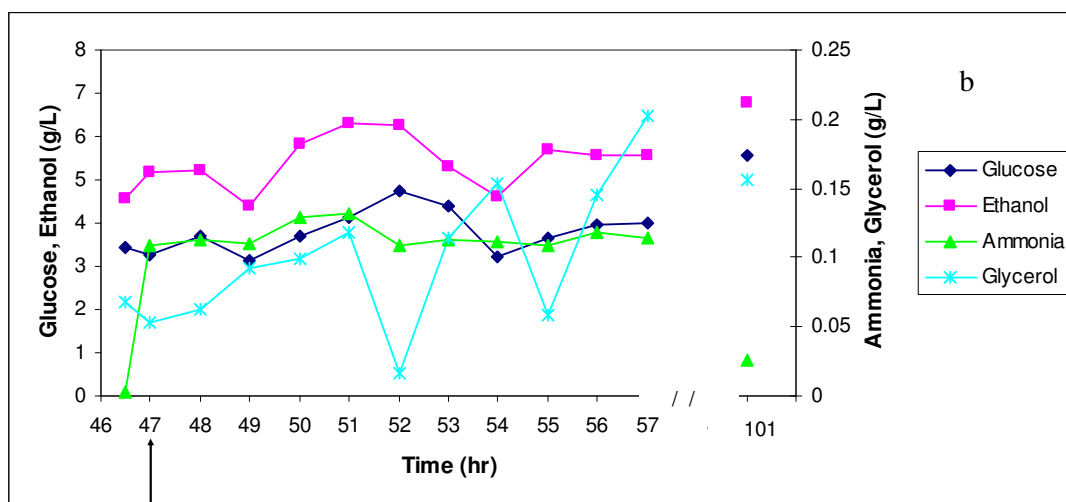


Figure 3.8. Extracellular metabolite profiles of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under nitrogen limited condition in chemostat culture with pulse injection

Under nitrogen limited conditions glucose concentration was determined as 3.4 g/L in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) just before the pulse injection was applied at steady state. It slightly increased after nitrogen pulse but it did not show much variations. At the second steady state glucose concentration was higher than the first one; it was 5.5 g/L. Ethanol concentration was 4.5 and 6.7 g/L at the first and second steady states, respectively. It showed the same profile as glucose. Ammonia concentration was 0.002 g/L at the first steady state. With ammonium sulfate addition to the medium, ammonia concentration increased sharply to 0.1 g/L and it was nearly constant for 10 hours. At the second steady state it decreased to a value of 0.02 g/L. Glycerol concentration was 0.06 g/L at the first steady state. It slightly decreased just after the nitrogen pulse and then started to increase for 4 hours. It showed an oscillatory behavior after the fourth hour. At the second steady state glycerol concentration was determined as 0.15 g/L.

### 3.1.5. Response of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) to Glucose Pulse

A strain of *S. cerevisiae* BY4743 where both copies of *MIG2* were disrupted (*mig2Δ/mig2Δ*) was cultivated under glucose limited conditions. A glucose pulse was given to remove the nutrient limitation after passing three residence times at the steady state

conditions. The experiment was terminated after three residence times at the second steady state conditions.

The growth characteristics of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) in glucose limited condition were given in Figures 3.9 and 3.10. At the end of 47<sup>th</sup> hour of chemostat cultivation the first steady state sample was taken and after that the glucose pulse injection was applied. Then hourly sampling was done for 10 hours. And the second steady state sample was taken at the 124<sup>th</sup> hour.

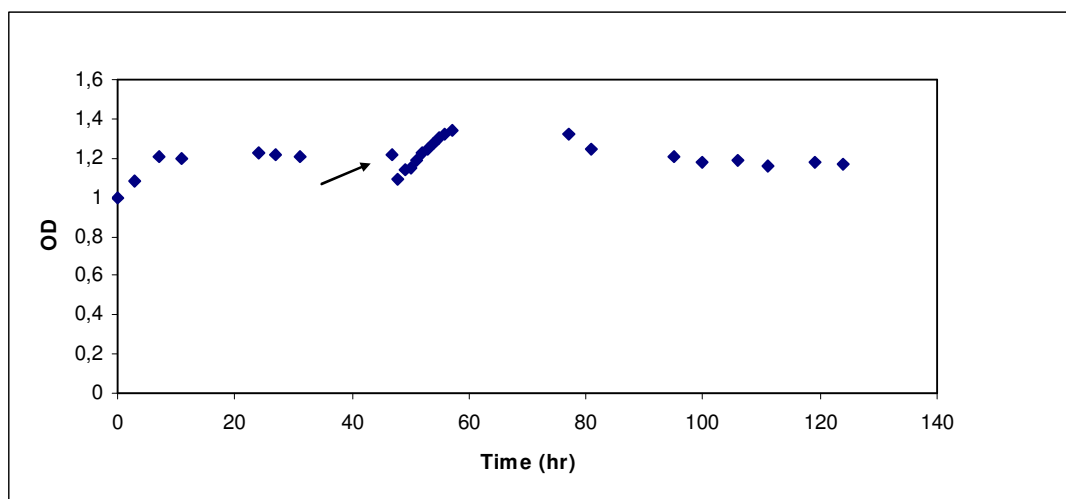


Figure 3.9. Growth curve of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limited condition in chemostat culture with pulse injection

A sharp decrease in growth was observed soon after the glucose pulse injection and after the first hour of the pulse injection it started to increase. 10 hours later from the glucose pulse injection, growth of the cells decreased. The second steady state value of the growth was slightly lower than that of the first steady state.

In *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limited condition (before the pulse was given), the initial glucose was almost completely consumed and remaining glucose concentration was measured as 0.3 g/L. After the first hour of the pulse, its concentration sharply increased to 13.6 g/L, then decreased to 0.6 g/L at the second steady state. Ethanol concentration was 0.7 g/L prior to the glucose pulse; it increased to 3.1 g/L one hour after the injection of the pulse.

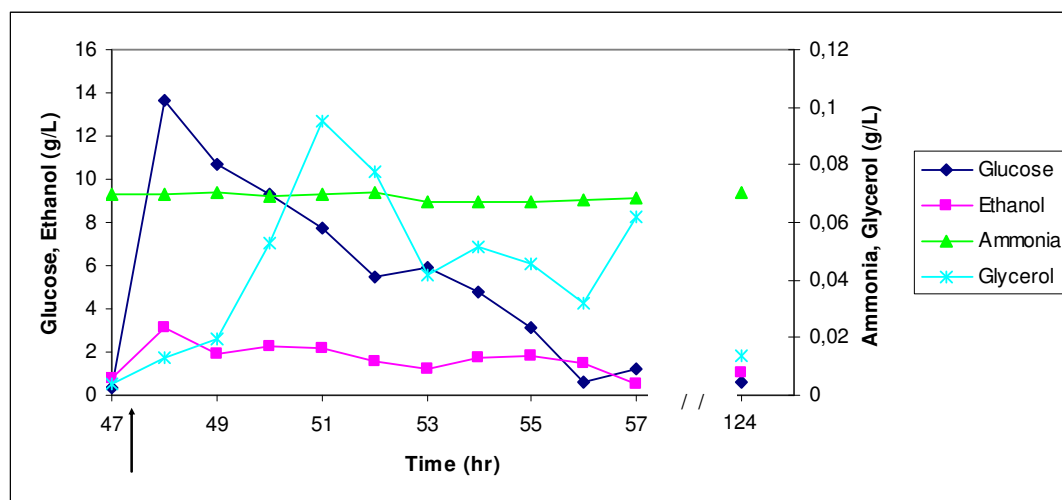


Figure 3.10. Extracellular metabolite profiles of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limited condition in chemostat culture with pulse injection

Final ethanol concentration at the second steady state was measured as 1 g/L. Ammonia concentration was nearly constant at a value of 0.07 g/L during the cultivation. The concentration of glycerol increased to maximum value of 0.09 g/L soon after the pulse and then decreased. First and second steady state values of glycerol concentration were measured as 0.004 and 0.01 g/L, respectively.

### 3.1.6. Response of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) to Nitrogen Pulse

*MIG2* disrupted strain of *S. cerevisiae* BY4743 was cultivated under nitrogen limited conditions. An ammonium sulfate pulse was given to remove the nutrient limitation after passing three residence times at the steady state conditions. The experiment was terminated after three residence times at the second steady state conditions.

The growth and metabolic concentration profiles of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limitation were presented in Figures 3.11 and 12. At the end of 96<sup>th</sup> hour of chemostat cultivation the first steady state sample was taken. After that, the nitrogen pulse injection was applied and a sample was taken within the first minute of pulse. Then hourly sampling was done for 10 hours. And the second steady state sample was taken at the 141<sup>st</sup> hour.

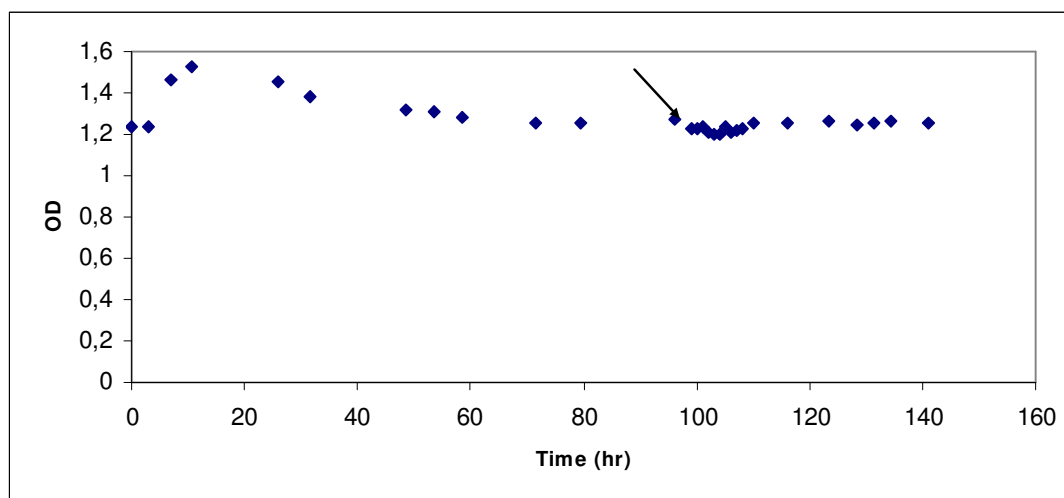


Figure 3.11. Growth curve of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limited condition in chemostat culture with pulse injection

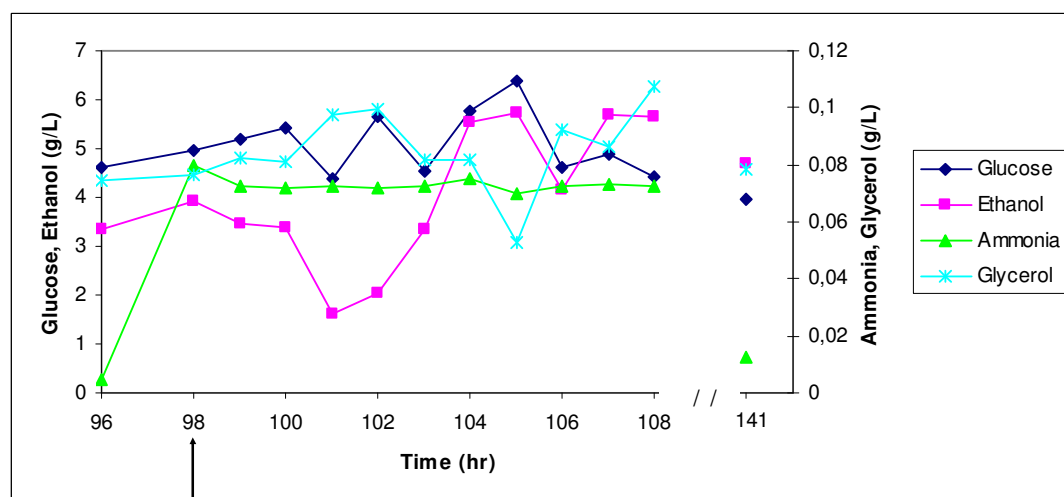


Figure 3.12. Extracellular metabolite profiles of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limited condition in chemostat culture with pulse injection

Within the four hours after the injection of ammonium sulfate to the nitrogen limited medium a decrease in the growth of the cells was observed in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) cultures. Then the growth of the cells increased and reached to the value of first steady state and remained nearly constant for three residence times at the second steady state conditions.

In *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limited condition, the initial glucose concentration was 4.6 g/L. It increased with nitrogen pulse and reached a maximum value of 6.39 g/L at the 7<sup>th</sup> hour of pulse injection. The final concentration of glucose at the second state was 3.95 g/L. Ethanol production decreased with the nitrogen pulse for 3 hours and then it started to increase. The final concentration of ethanol was determined as 4.7 g/L at the second steady state. Ammonia concentration was 0.004 g/L prior to the pulse injection. It sharply increased to a value of 0.08 g/L just after the pulse and remained constant at this value for 10 hours. At the second steady state the concentration of ammonia was measured as 0.01g/L. The glycerol concentration slightly changed during the cultivation. After pulse it increased, at the end of 4<sup>th</sup> hour it started to decrease and then again it increased. The steady state concentrations of glycerol had the same value as 0.07 g/L.

### **3.2. Transcriptional Responses of *HAP4*, *MIG1*, *MIG2* and *GSM1* to a Sudden Release from Nutritional Limitations in Deletion Strains of *S. cerevisiae* BY4743**

MIG1 encodes a C<sub>2</sub>H<sub>2</sub> zinc finger protein that binds specifically to DNA with a GC rich consensus sequence and a flanking AT sequence (Nehlin and Ronne, 1990; Lundin, *et al.*, 1994). Mig1 is a repressor responsible for glucose repression of many genes, including GAL, SUC, and MAL. Mig1p binds to the promoters of glucose-repressed genes and recruits the general repressors Ssn6 and Tup1. Subcellular localization of Mig1 is regulated by glucose. Mig1 is imported into the nucleus within minutes after the addition of glucose and is just as rapidly transported back to the cytoplasm when glucose is removed (DeVit *et al.*, 1997).

A second repressor Mig2, 71% identical to Mig1, has been shown to bind to some of the Mig1-binding sites (Klein *et al.*, 1998). Mig2p remains localized in the nucleus in response to glucose and which probably performs a less important role in glucose repression than Mig1p (Lascaris *et al.*, 2004).

In *S. cerevisiae*, the heme-activated protein complex Hap2/3/4/5 plays a major role in the transcription of genes involved in respiration. The DNA binding capability of the Hap complex is conferred by the Hap2/3/5 proteins, and Hap4p enhances their activity via its

activation domain. Hap4p has also been found to possess at least two distinct trans-activation domains, each of which has different levels of dependence on co-activation proteins. Although the other members of the complex are constitutively expressed, the expression of *HAP4* is regulated by the carbon source and is up-regulated manifold upon glucose exhaustion. Glucose represses the expression of *HAP4* via the Mig1 pathway and thereby activation of respiration is prevented at high glucose concentration (Raghevendran *et al.*, 2006).

*GSM1* (*YJL103C*) is a yeast gene of unknown function. The expression pattern of *GSM1* is most similar to the genes in the energy group and respiratory chain subgroup. It is found that *GSM1* contains a HAP2,3,4 box in its promoter region and a cytochrome C heme-binding signature in its protein sequence. The findings define a potential role for *GSM1* in the regulation of energy metabolism, specifically in the process of oxidative phosphorylation (Deng *et al.*, 2004). *GSM1* expression was also found to be significantly downregulated by *HAP4* deletion. It can be concluded that *GSM1* should encode a protein that is involved in transcriptional regulation of the respiratory pathway of yeast (Pir *et al.*, 2006).

In addition to the extracellular metabolite profiles and growth behavior, transcriptional responses of *HAP4*, *MIG1*, *MIG2* and *GSM1* to a sudden relief of the starvation in carbon or nitrogen, in the absence of *MIG1* and *MIG2* genes were investigated for the same experiments. Samples from culture were collected as previously stated and mRNA was extracted from samples as described in Materials and Methods section. The extracted mRNA was first reverse transcribed into cDNA and later amplified at the specified locations (real time RT-qPCR). *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) was also included as a control and the samples were provided by Ayça Cankorur and Aylin Şanoviç. Arrows indicate the pulse injections.

### 3.2.1. Transcriptional Responses of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) to Glucose Pulse

*S. cerevisiae* BY4743 (*hoΔ/hoΔ*) was grown in chemostat culture of glucose limited F1 media. After three residence times at steady state conditions a glucose pulse injection

was applied to remove the glucose limitation. The chemostat was terminated after three residence times at the second steady conditions.

The expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1* genes in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) for the carbon limited cultivation followed by glucose pulse injected to the system are displayed in Figures 3.13, 3.14, 3.15 and 3.16, respectively. Samples for RNA extraction were taken as presented in Table 3.1.

Table 3.1. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limitation

| Sample Number | Time (hr) |
|---------------|-----------|
| 1             | 0         |
| 2             | 0,016     |
| 3             | 0,016     |
| 4             | 0,016     |
| 5             | 0,016     |
| 6             | 0,083     |
| 7             | 0,166     |
| 8             | 0,25      |
| 9             | 0,333     |
| 10            | 1         |
| 11            | 2         |
| 12            | 3         |
| 13            | 4         |
| 14            | 5         |
| 15            | 6         |
| 16            | 7         |
| 17            | 8         |
| 18            | 9         |
| 19            | 10        |
| 20            | 72        |

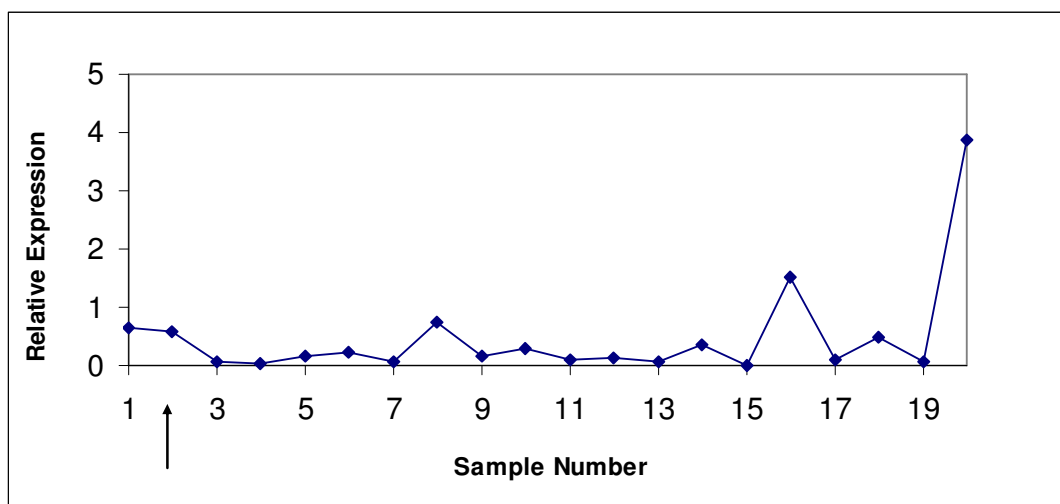


Figure 3.13. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limitation in chemostat cultures with pulse injection

The expression level of *HAP4* decreased sharply soon after the glucose pulse injection. At the end of 15<sup>th</sup> minute and 7<sup>th</sup> hour of the pulse injection increases in the expression level of *HAP4* were observed. The expression level of *HAP4* increased at the second steady state.

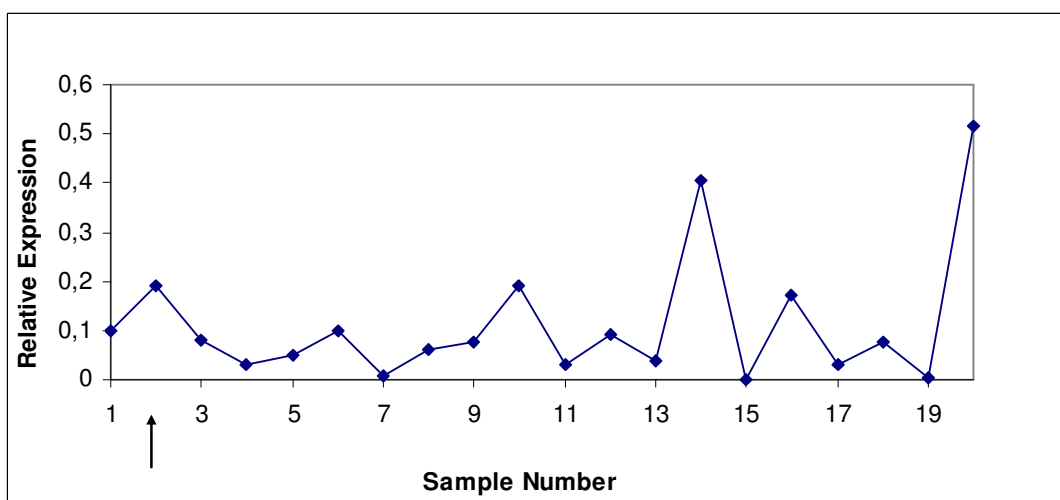


Figure 3.14. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limitation in chemostat cultures with pulse injection

The expression level of *MIG1* decreased following the glucose pulse injection to carbon limited culture of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*). *MIG1* expression showed an oscillatory behavior throughout the chemostat culture and increased at the second steady state.

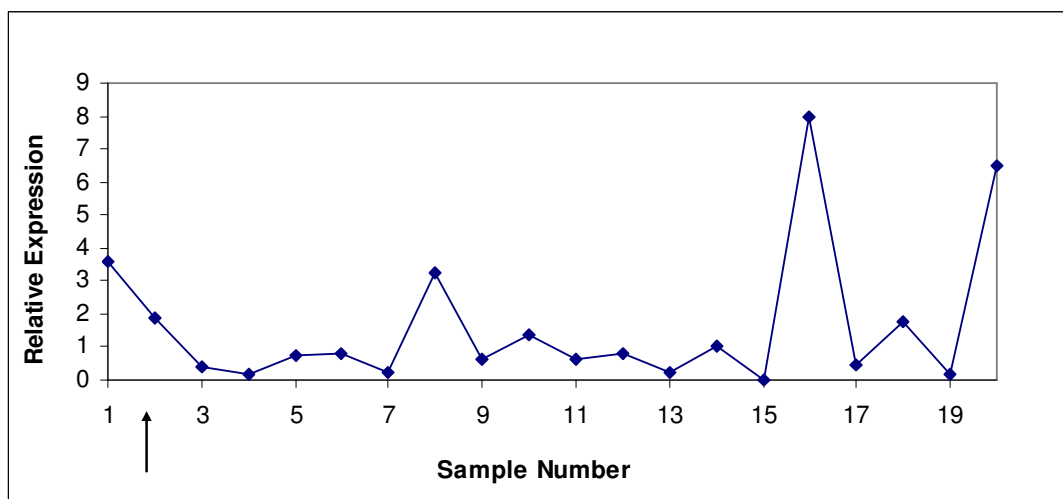


Figure 3.15. Expression profile of *MIG2* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limitation in chemostat cultures with pulse injection

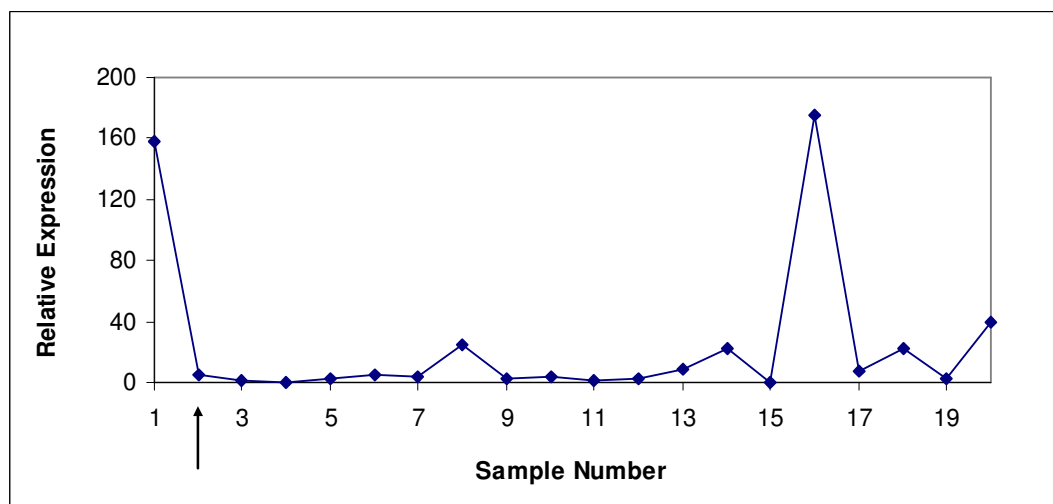


Figure 3.16. Expression profile of *GSM1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under glucose limitation in chemostat cultures with pulse injection

*MIG2* was also observed to be over-expressed in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) grown in glucose limited medium. With the glucose pulse, expression of *MIG2* decreased. After the 6<sup>th</sup> hour of glucose pulse injection *MIG2* was over-expressed; however its expression decreased in one hour. It was over-expressed at the second steady state again.

*GSM1* was observed to be over-expressed when glucose is limited in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*). With the glucose pulse, expression level of *GSM1* decreased and after 7 hours the expression level of *GSM1* increased again. This increase decayed in one hour.

### 3.2.2. Transcriptional Response of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) to Nitrogen Pulse

*hoΔ/hoΔ* strain of *S. cerevisiae* BY4743 was grown in chemostat culture under nitrogen limitation. After three residence times at steady state conditions an ammonium sulfate pulse injection was applied to remove the nitrogen limitation. The chemostat was terminated after three residence times at the second steady conditions.

The expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1* genes in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) in nitrogen limited cultivation followed by ammonium sulfate pulse injected to the system are displayed in Figures 3.17, 3.18, 3.19 and 3.20, respectively. Samples for RNA extraction were taken as presented in Table 3.2.

Table 3.2. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limitation

| Sample Number | Time (hr) |
|---------------|-----------|
| 1             | 0         |
| 2             | 0,016     |
| 3             | 0,016     |
| 4             | 0,016     |
| 5             | 0,016     |
| 6             | 0,083     |
| 7             | 0,166     |
| 8             | 0,25      |

Table 3.2. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limitation (continued)

|    |       |
|----|-------|
| 9  | 0,333 |
| 10 | 1     |
| 11 | 2     |
| 12 | 3     |
| 13 | 4     |
| 14 | 5     |
| 15 | 6     |
| 16 | 7     |
| 17 | 8     |
| 18 | 9     |
| 19 | 10    |
| 20 | 70    |

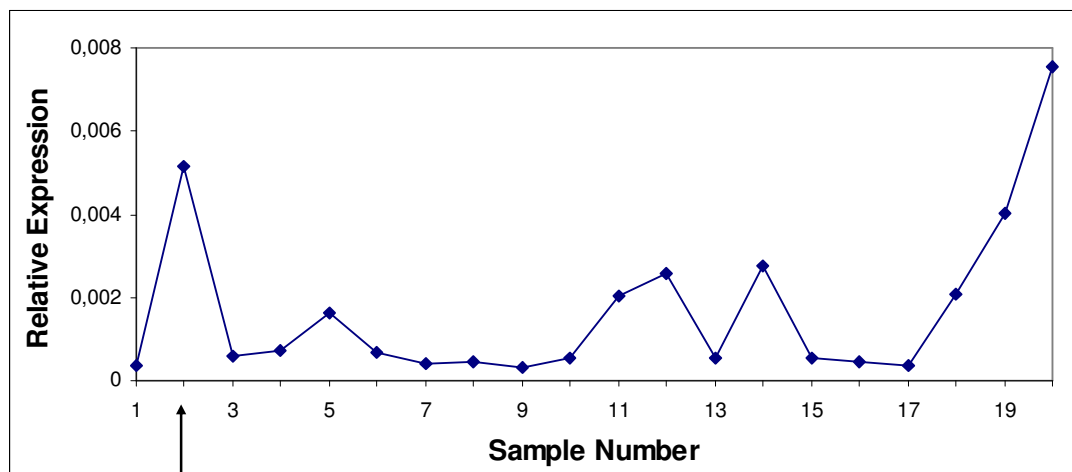


Figure 3.17. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limitation in chemostat cultures with pulse injection

The nitrogen pulse raised the expression of *HAP4* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) grown in nitrogen limited medium. The response of *HAP4* to nitrogen pulse was immediate and short. There were some fluctuations after first hour of pulse injection. After the 8<sup>th</sup> hour an increase in the expression level of *HAP4* was observed. At the second

steady state, where the culture was under nitrogen limited condition, *HAP4* was over-expressed. The glucose concentration was nearly constant during the experiment.

The expression level of *MIG1* was observed to be undetectable in nitrogen limited condition in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*). With nitrogen pulse injection, 115-fold increase in the expression level of *MIG1* was detected. The response of *MIG1* to the nitrogen pulse injection was immediate and short, after the first minute of the pulse injection there were no significant changes in the expression profile of *MIG1*.

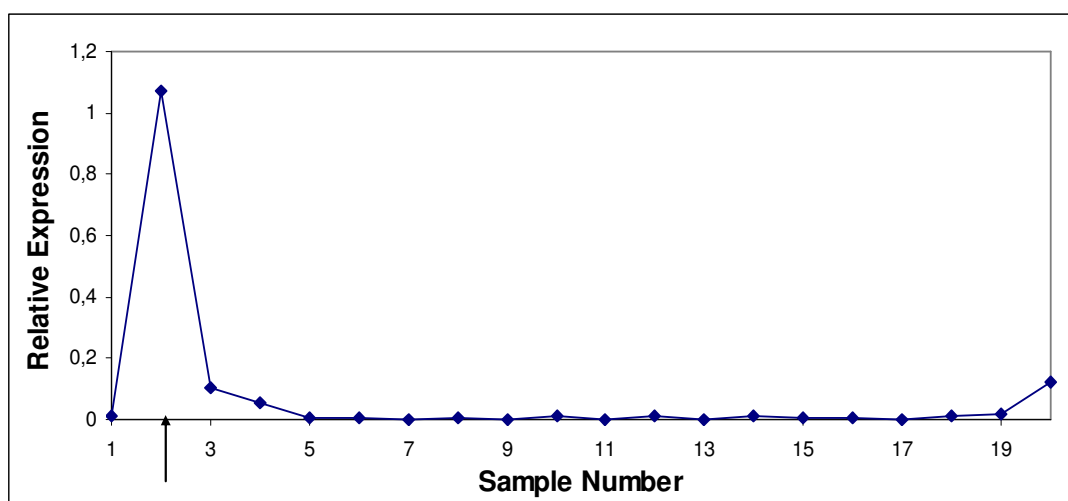


Figure 3.18. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *MIG2* showed an increase in the first minute after the nitrogen pulse injection in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) in nitrogen limited medium. Then it decreased and remained nearly constant until the end of 8<sup>th</sup> hour of the pulse injection. *MIG2* was over-expressed at the end of 8<sup>th</sup> hour and also at the second steady state. The glucose concentration was nearly constant during the experiment.

The expression level of *GSM1* was undetectable before nitrogen pulse in *S. cerevisiae* BY4743 (*Δho/hoΔ*) grown in nitrogen limited medium. After the injection of the pulse there was a slight increase in the expression level of *GSM1* but this increase diminished immediately. *GSM1* was over-expressed at the second steady state where the nitrogen is limited.

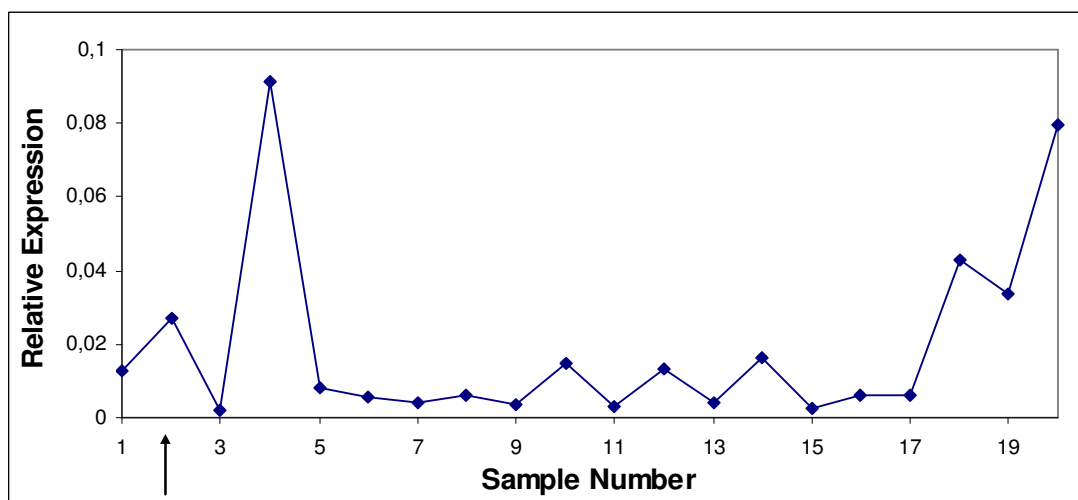


Figure 3.19. Expression profile of *MIG2* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limitation in chemostat cultures with pulse injection

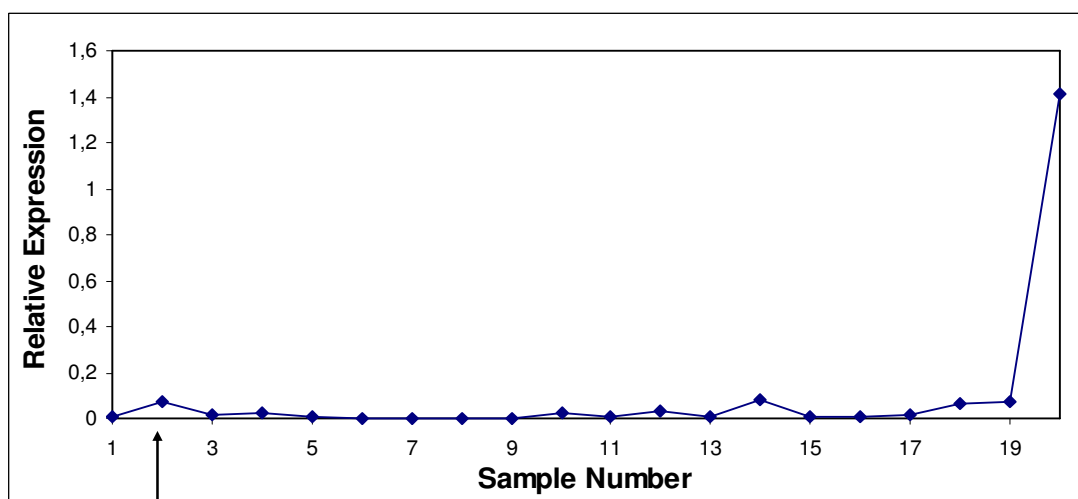


Figure 3.20. Expression profile of *GSM1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under nitrogen limitation in chemostat cultures with pulse injection

### 3.2.3. Transcriptional Response of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) to Glucose Pulse

*mig1Δ/mig1Δ* strain of *S. cerevisiae* BY4743 was grown in chemostat culture under glucose limitation. After three residence times at steady state conditions a glucose pulse injection was applied to remove the glucose limitation. The chemostat was terminated after three residence times at the second steady conditions.

The expression profiles of *HAP4*, *MIG2* and *GSM1* genes in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) in the glucose limited chemostat cultivation followed by the glucose pulse injected to the system are displayed in Figures 3.21, 3.22 and 3.23, respectively. Samples for RNA extraction were taken as presented in Table 3.3.

Table 3.3. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under glucose limitation

| Sample Number | Time (hr) |
|---------------|-----------|
| 1             | 0         |
| 2             | 0,016     |
| 3             | 0,016     |
| 4             | 0,016     |
| 5             | 0,016     |
| 6             | 0,083     |
| 7             | 0,166     |
| 8             | 0,25      |
| 9             | 0,333     |
| 10            | 1         |
| 11            | 2         |
| 12            | 3         |
| 13            | 4         |
| 14            | 5         |
| 15            | 6         |
| 16            | 7         |
| 17            | 8         |
| 18            | 9         |
| 19            | 10        |
| 20            | 72        |

The expression of *HAP4* could not be detected in glucose limited medium in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*). The increase of the expression level of *HAP4* was started after the glucose pulse.

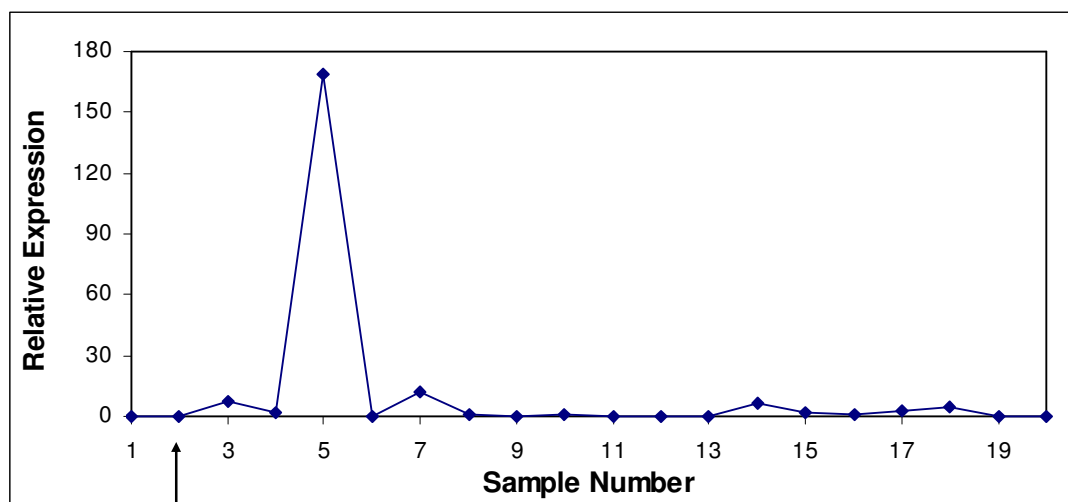


Figure 3.21. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under glucose limitation in chemostat cultures with pulse injection

There was a 78-fold increase in the expression of *HAP4* at the end of first minute of the pulse injection. After the first minute no significant change in the expression level of the *HAP4* was detected.

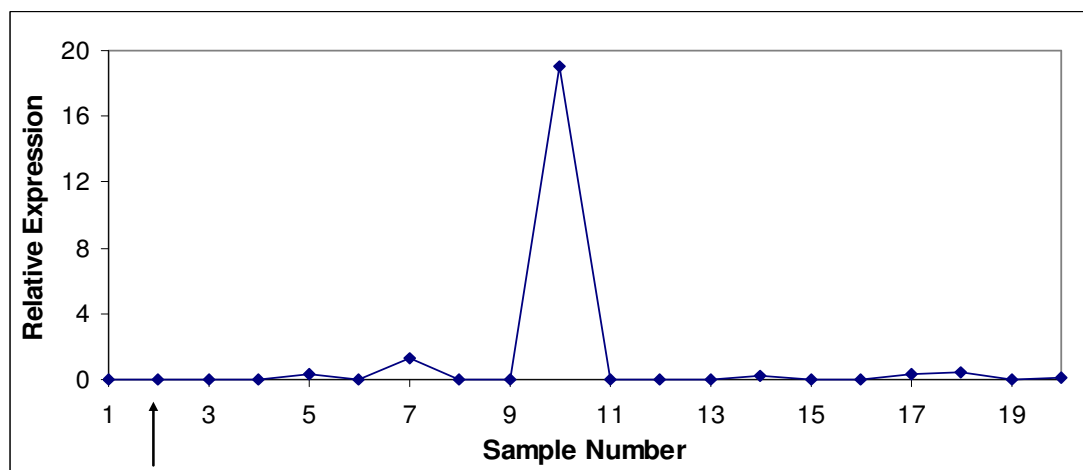


Figure 3.22. Expression profile of *MIG2* gene in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under glucose limitation in chemostat cultures with pulse injection

Under glucose limitation the expression level of *MIG2* was undetectable in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*). The only and short response of *MIG2* to glucose pulse was observed at the end of first hour and its expression level increased. After the first hour

no significant change in the expression level of *MIG2* could be observed and the expression levels of this gene remained undetectable at the second steady state.

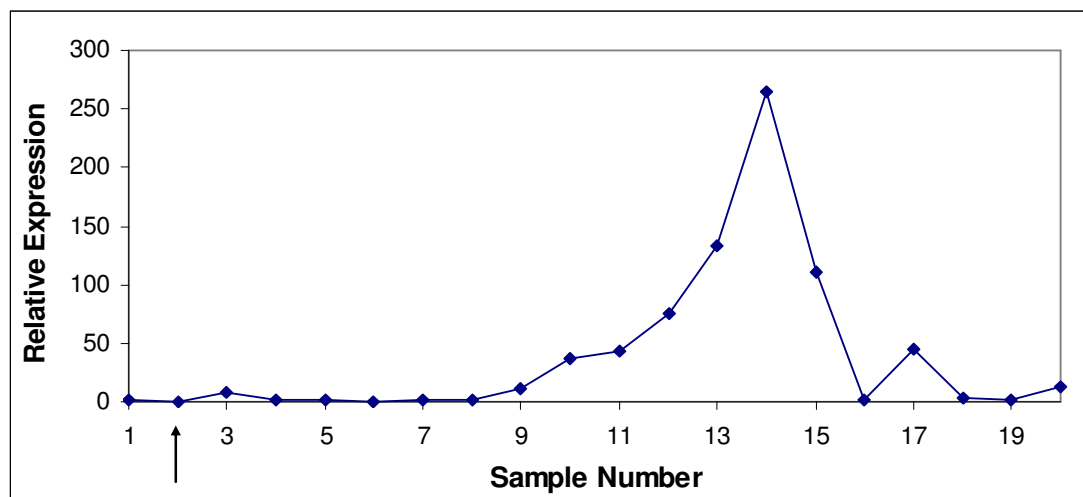


Figure 3.23. Expression profile of *GSM1* gene in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under glucose limitation in chemostat cultures with pulse injection

The expression level of *GSM1* was low when glucose is limited in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*). The increase in the expression level of *GSM1* started 15 minutes after the pulse injection and took 5 hours. After the 5<sup>th</sup> hour of the pulse injection the expression level of *GSM1* decreased and did not show any variations after the 9<sup>th</sup> hour until the end of the cultivation.

### 3.2.4. Transcriptional Response of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) to Nitrogen Pulse

*mig1Δ/mig1Δ* strain of *S. cerevisiae* BY4743 was grown in chemostat culture under nitrogen limitation. After three residence times at steady state conditions an ammonium sulfate pulse injection was applied to remove the nitrogen limitation. The chemostat was terminated after three residence times at the second steady conditions.

The expression profiles of *HAP4*, *MIG2* and *GSM1* genes in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) grown under the nitrogen limitation followed by ammonium sulfate pulse

injected to the system are displayed in Figures 3.24, 3.25 and 3.26, respectively. Samples for RNA extraction were taken as presented in Table 3.4.

Table 3.4. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under nitrogen limitation

| Sample Number | Time (hr) |
|---------------|-----------|
| 1             | 0         |
| 2             | 0,016     |
| 3             | 0,016     |
| 4             | 0,016     |
| 5             | 0,016     |
| 6             | 0,083     |
| 7             | 0,166     |
| 8             | 0,25      |
| 9             | 0,333     |
| 10            | 1         |
| 11            | 2         |
| 12            | 3         |
| 13            | 4         |
| 14            | 5         |
| 15            | 6         |
| 16            | 7         |
| 17            | 8         |
| 18            | 9         |
| 19            | 10        |
| 20            | 54        |

Under nitrogen limitation, where the concentration of glucose is not limited, the expression level of *HAP4* was undetectable and increased just after the nitrogen pulse injection. The response of *HAP4* to the pulse took nearly 10 minutes. There was a slight decrease in the glucose concentration just after nitrogen pulse and an increase after 2<sup>nd</sup> hour of the pulse.

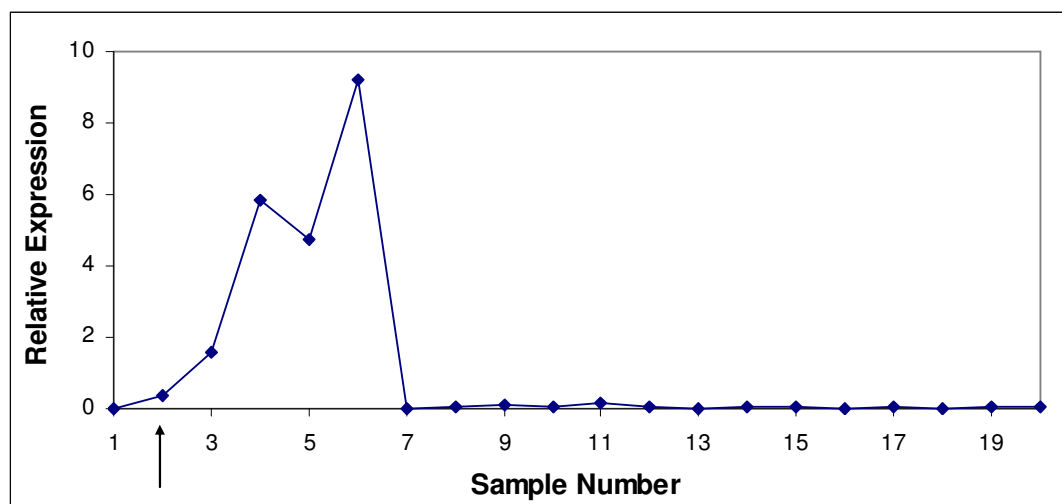


Figure 3.24. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under nitrogen limitation in chemostat cultures with pulse injection

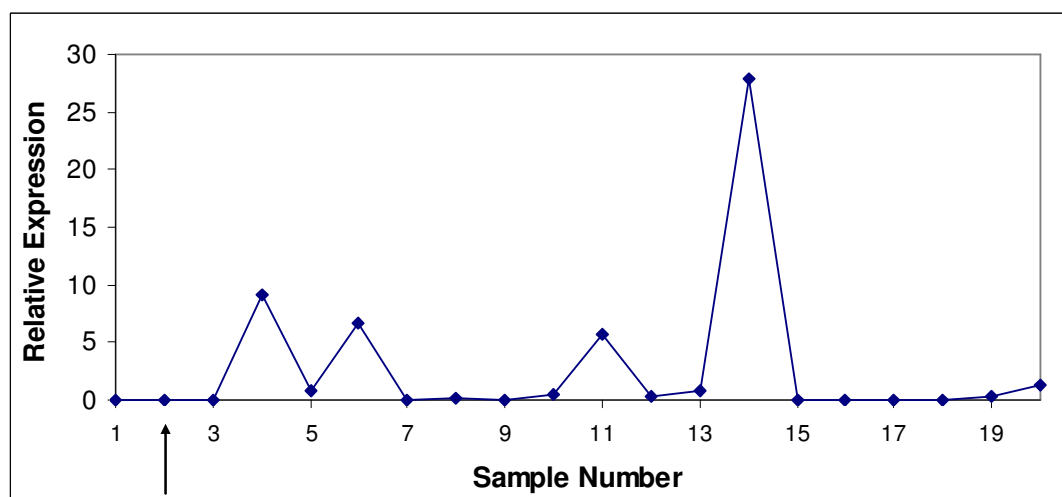


Figure 3.25. Expression profile of *MIG2* gene in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under nitrogen limitation in chemostat cultures with pulse injection

While the expression level of *MIG2* was undetectable when nitrogen is limited in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*), after the addition of ammonium sulfate to the media the expression level of *MIG2* increased. The expression of *MIG2* decreased after the 10<sup>th</sup> minute of the pulse injection. It was over-expressed 4 hours later from pulse injection. After the 5<sup>th</sup> hour of the pulse injection no significant change in the expression level of *MIG2* was observed.

When nitrogen is limited the expression level of *GSM1* was undetectable in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*). *GSM1* responded to the nitrogen pulse immediately and expression of this gene increased. However the expression level of *GSM1* had an oscillatory behavior throughout the cultivation.

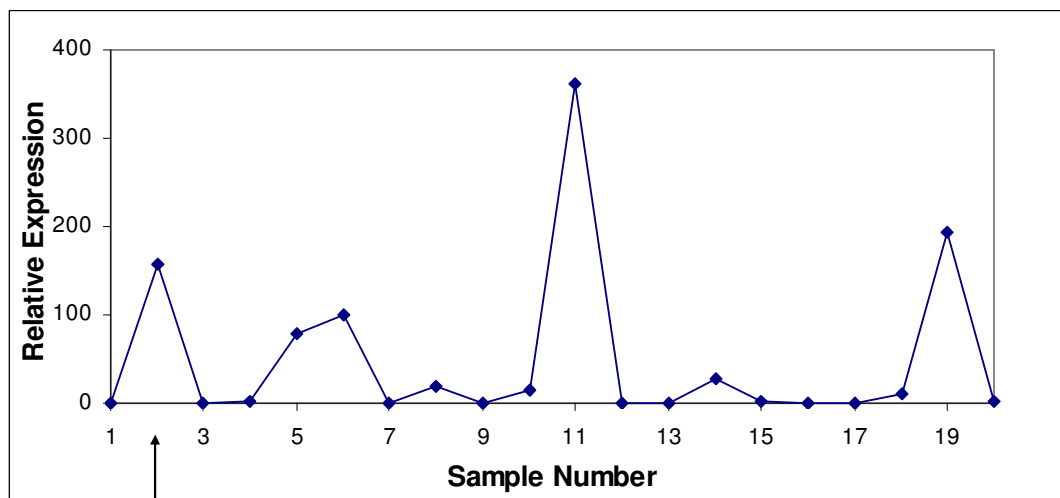


Figure 3.26. Expression profile of *GSM1* gene in *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) under nitrogen limitation in chemostat cultures with pulse injection

### 3.2.5. Transcriptional Response of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) to Glucose Pulse

*mig2Δ/mig2Δ* strain of *S. cerevisiae* BY4743 was grown in chemostat culture under glucose limitation. After three residence times at steady state conditions a glucose pulse injection was applied to remove the glucose limitation. The chemostat was terminated after three residence times at the second steady conditions.

The expression profiles of *HAP4*, *MIG1* and *GSM1* genes in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limitation followed by glucose pulse injected to the system are displayed in Figures 3.27, 3.28 and 3.29, respectively. Samples for RNA extraction were taken as presented in Table 3.5.

The expression level of *HAP4* was low under glucose limited condition in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*). Just after the glucose pulse *HAP4* was over-expressed.

At the end of the 20<sup>th</sup> minute of the pulse injection expression level of *HAP4* increased 194-fold.

Table 3.5. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limitation

| Sample Number | Time (hr) |
|---------------|-----------|
| 1             | 0         |
| 2             | 0,016     |
| 3             | 0,016     |
| 4             | 0,016     |
| 5             | 0,016     |
| 6             | 0,083     |
| 7             | 0,166     |
| 8             | 0,25      |
| 9             | 0,333     |
| 10            | 1         |
| 11            | 2         |
| 12            | 3         |
| 13            | 4         |
| 14            | 5         |
| 15            | 6         |
| 16            | 7         |
| 17            | 8         |
| 18            | 9         |
| 19            | 10        |
| 20            | 78        |

After the first hour its expression level decreased and remained without changing until the end of 7<sup>th</sup> hour. The expression level of *HAP4* increased at the end of 7<sup>th</sup> hour, decreased after the 8<sup>th</sup> hour and then no significant change was observed.

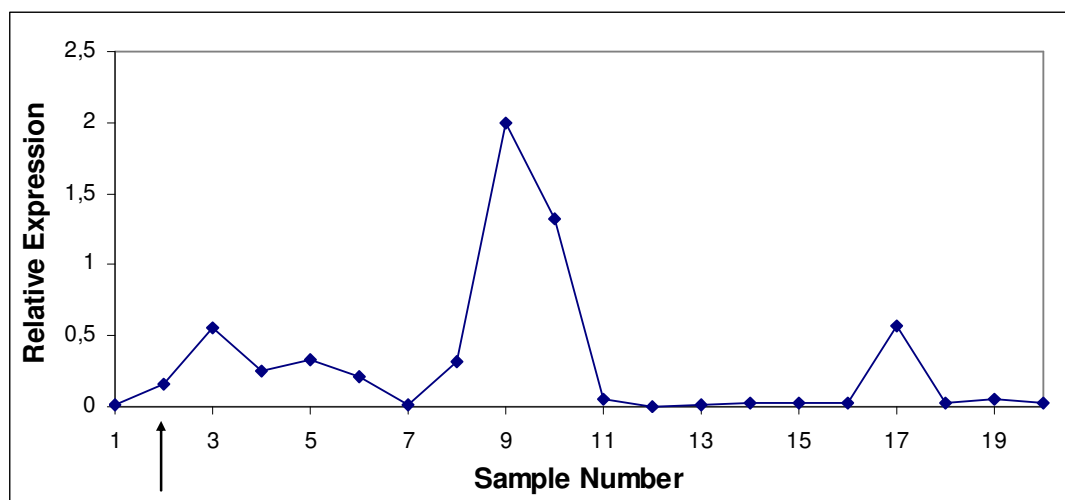


Figure 3.27. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limitation in chemostat cultures with pulse injection

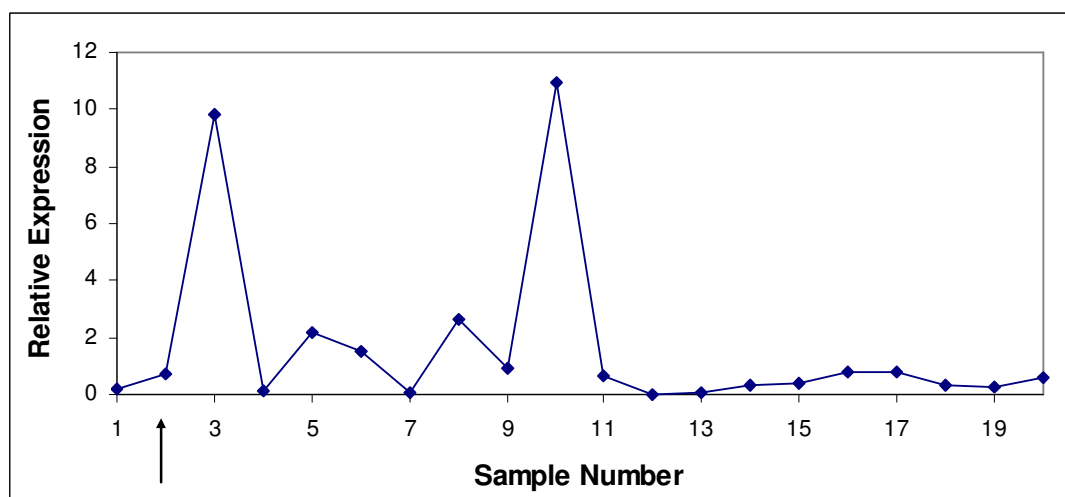


Figure 3.28. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limitation in chemostat cultures with pulse injection

In glucose limited medium the expression level of *MIG1* was observed to be low in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*). *MIG1* was over-expressed just after the glucose pulse and its expression levels showed an oscillatory behavior for two hours. The expression levels of this gene then remained almost constant.

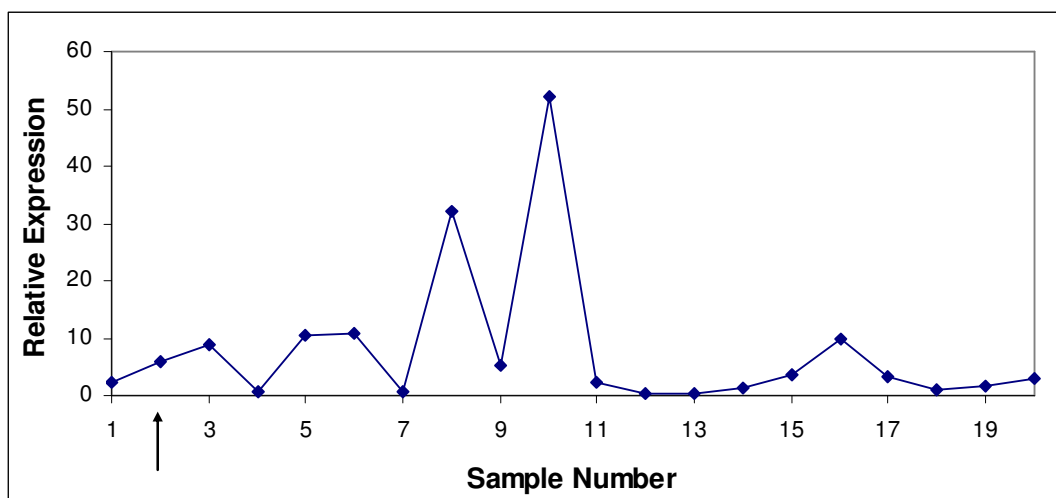


Figure 3.29. Expression profile of *GSM1* gene in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under glucose limitation in chemostat cultures with pulse injection

In the absence of *MIG2* when glucose is limited the expression level of *GSM1* was observed to be low in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*). After glucose pulse *GSM1* was over-expressed and showed an oscillatory expression profile throughout the cultivation. At the first hour of the pulse injection, expression level of *GSM1* increased 22-fold then it decreased and remained without changing until the end of 4<sup>th</sup> hour. After that the expression level of *GSM1* showed an increasing profile for 3 hours and decreased again. The expression level of the gene at the second steady state was close to that of the first steady state.

### 3.2.6. Transcriptional Response of *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) to Nitrogen Pulse

*mig2Δ/mig2Δ* strain of *S. cerevisiae* BY4743 was grown in chemostat culture under nitrogen limitation. After three residence times at steady state conditions an ammonium sulfate pulse injection was applied to remove the nitrogen limitation. The chemostat was terminated after three residence times at the second steady conditions

The expression profiles of *HAP4*, *MIG1* and *GSM1* genes in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limitation followed by ammonium sulfate pulse injected to

the system were displayed in Figures 3.30, 3.31 and 3.32. Samples for RNA extraction were taken as presented Table 3.6.

Table 3.6. Sampling times for RNA extraction in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limitation

| Sample Number | Time (hr) |
|---------------|-----------|
| 1             | 0         |
| 2             | 0,016     |
| 3             | 0,016     |
| 4             | 0,016     |
| 5             | 0,016     |
| 6             | 0,083     |
| 7             | 0,166     |
| 8             | 0,25      |
| 9             | 0,333     |
| 10            | 1         |
| 11            | 2         |
| 12            | 3         |
| 13            | 4         |
| 14            | 5         |
| 15            | 6         |
| 16            | 7         |
| 17            | 8         |
| 18            | 9         |
| 19            | 10        |
| 20            | 78        |

The expression level of *HAP4* was undetectable under nitrogen limitation in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*). No significant changes were observed in the expression level of *HAP4* during the first 5 hours after the injection of the ammonium sulfate to nitrogen limited media.

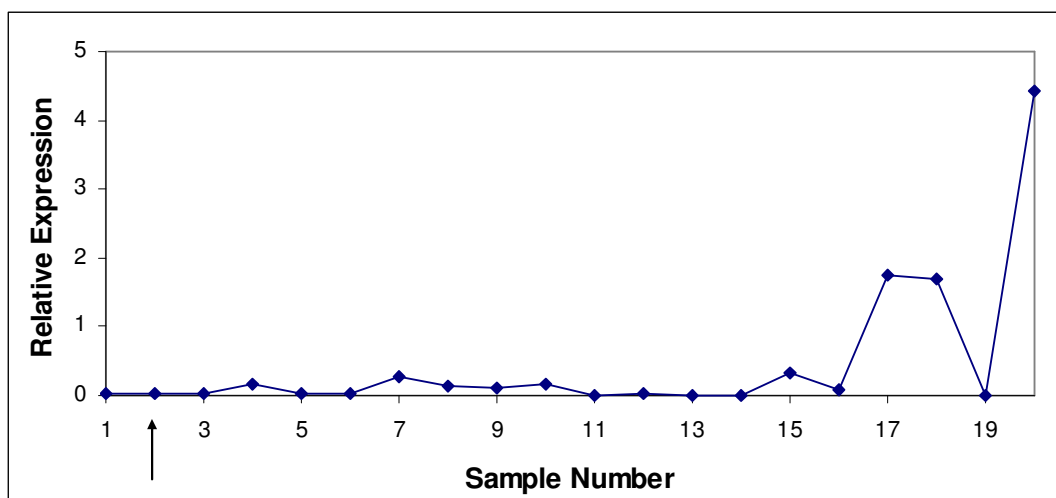


Figure 3.30. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limitation in chemostat cultures with pulse injection

After the 7<sup>th</sup> hour of pulse injection there was an increase in the expression profile and its expression level remained high for 2 hours and the expression level of *HAP4* decreased at the end of 10<sup>th</sup> hour. It was also obtained that *HAP4* was over-expressed at the second steady state where nitrogen is limited.

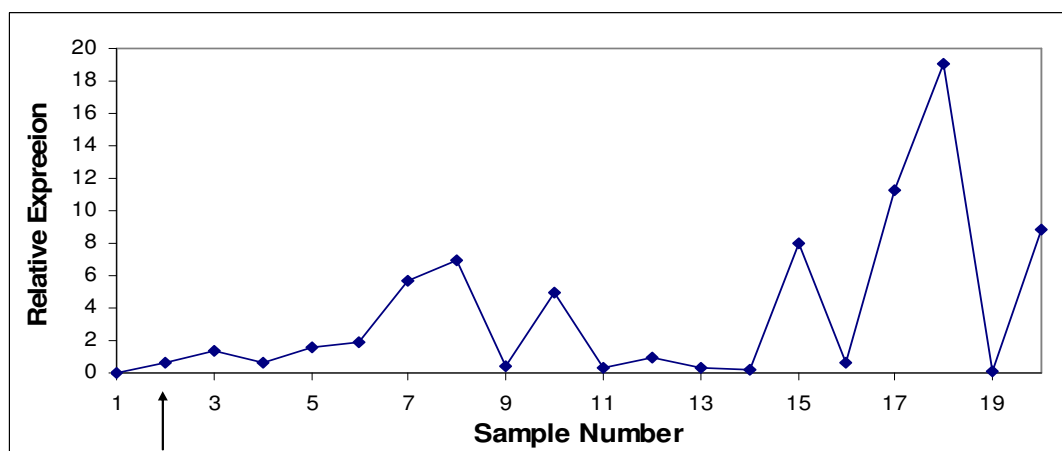


Figure 3.31. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *MIG1* under nitrogen limitation was observed to be low in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*). With the injection of ammonium sulfate pulse, the expression level of *MIG1* increased. After the 15<sup>th</sup> minute of pulse injection, the expression

level of *MIG1* showed an oscillatory profile. *MIG1* was over expressed after 7<sup>th</sup> hour of pulse injection and also at the second steady state.

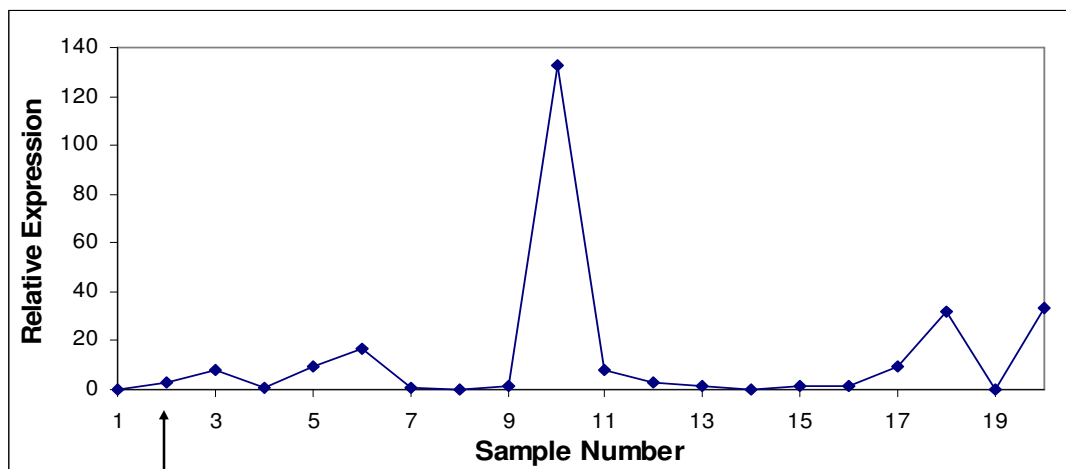


Figure 3.32. Expression profile of *GSM1* gene in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *GSM1* was undetectable in *S. cerevisiae* BY4743 (*mig2Δ/mig2Δ*) grown under nitrogen limitation. Just after the pulse a slight increase in the expression level of *GSM1* was observed. At the end of the first hour it was over-expressed and after that the expression level of *GSM1* decreased to detectable levels and remained constant for 5 hours. After the 7<sup>th</sup> hour of the pulse injection the expression level of *GSM1* increased again and decreased at the end of 9<sup>th</sup> hour. The expression level of *GSM1* was higher at the second steady state that of the first steady state.

## 4. DISCUSSION

In this study the transcriptional responses of *HAP4*, *MIG1*, *MIG2* and *GSM1* genes to transient perturbations under glucose and nitrogen limited conditions were investigated. For this purpose, glucose and nitrogen limited chemostat cultivations followed by pulse injections of either glucose or ammonium sulfate were carried out. *Saccharomyces cerevisiae* BY4743 strains (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) were used in these cultivations. *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) was selected as a control to investigate the effects of deletions Δ) on the expression of *HAP4*, *MIG1*, *MIG2* and *GSM1* (*YJL103C*) genes in *S. Cerevisiae* BY4743 (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*).

Previous studies provided the information about deletions of both copies of *HO* being indifferent to respiratory metabolism and hence making it preferential strain as reference. Therefore, BY4743 and *hoΔ/hoΔ* could safely be used interchangeably in studies related to deletion strains in chemostat cultures (Baganz *et al.*, 1997; Dikicioğlu, 2005).

### 4.1. Comparison of Growth Characteristics of the Deletion Strains

Three deletion strains of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*, *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) were cultivated under glucose and nitrogen limitation. The pulse injections of glucose or nitrogen were applied. The OD values (600nm) of the three strains at the first and second steady states were compared and shown in the Figures 4.1 and 4.2.

The two deletion strains of *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) shared the same growth behavior with *hoΔ/hoΔ*. All of the three deletion strains showed decreases in their growth just after the injection of the glucose and nitrogen pulses (Figures 3.1 (a), 3.2 (a), 3.3 (a), 3.4 (a), 3.5 (a), 3.6 (a)). The decreases in the growth of the cells with addition of glucose to the medium may be the result of glucose repression. A major result of glucose repression is the switching off genes responsible for mitochondrial respiration. Glucose repression causes fermentative growth under aerobic conditions, resulting in reduced biomass yields (Raghevendran *et al.*, 2005). Mendes-Ferreira *et al.*

(2007) also showed that with the addition of a nitrogen source to nitrogen-starved yeast cells, genes involved in growth-related functions were repressed.

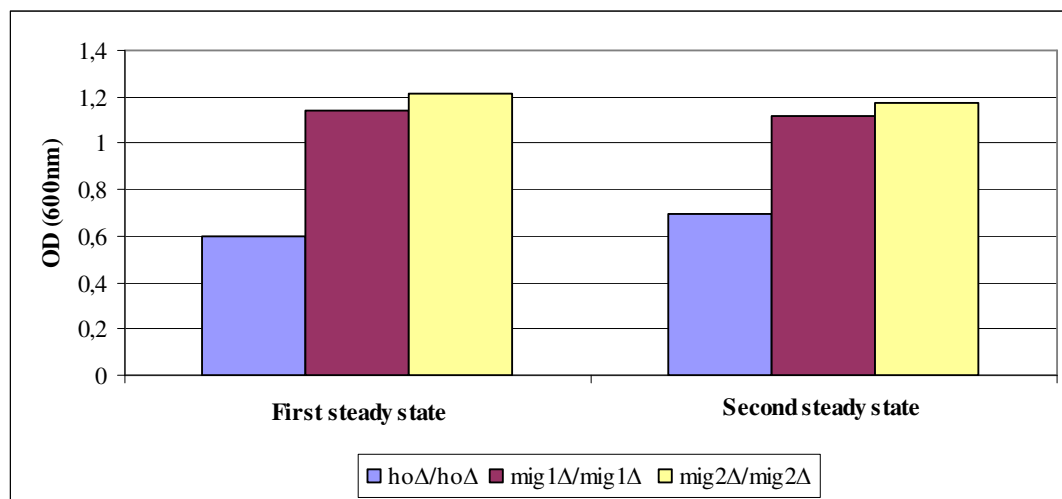


Figure 4.1. Growth characteristics of *S. cerevisiae* BY4743 strains in glucose limited chemostat culture

Under glucose limitation the growth of *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) is the lowest compared with *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*. It has been stated by van Maris *et al.* (2001) that deletion of *MIG1* and *MIG2* exhibited a higher specific growth rate than the wild type in glucose-limited cultures. The *hoΔ/hoΔ*, *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* strains produced nearly the same amount of biomass under glucose limitation both at the first and second steady states.

Glucose was found to be completely consumed in glucose limited cultures just before the pulse. In *hoΔ/hoΔ*, ethanol production was nearly zero under glucose limitation. Under glucose limitation *S. cerevisiae* BY4743 (*mig1Δ/mig1Δ*) exhibited completely respiratory growth; ethanol and glycerol formation were nearly zero. Low glycerol production and some ethanol production were observed in *mig2Δ/mig2Δ*.

Glucose consumption after the injection of glucose pulses in *S. cerevisiae* BY4743 (*hoΔ/hoΔ* and *mig2Δ/mig2Δ*) strains showed similar behaviors (Figures 3.1. (b), 3.5. (b)). Glucose was consumed slower in both strains than *mig1Δ/mig1Δ*. There was a sharp decrease in glucose concentration after the first hour of the glucose pulse injection in

*mig1Δ/mig1Δ* (Figure 3.3. (b)). Although the three strains had the same amount of glucose one hour after the injection of glucose, ethanol production was high in *mig2Δ/mig2Δ* and low in *mig1Δ/mig1Δ*. *S. cerevisiae* BY4743 (*hoΔ/hoΔ* and *mig2Δ/mig2Δ*) strains showed similar behavior in ethanol production but the produced ethanol amount was higher in *mig2Δ/mig2Δ*. In *mig1Δ/mig1Δ* ethanol concentration did not increase as sharply as in *hoΔ/hoΔ* and *mig2Δ/mig2Δ* after the injection of glucose to medium. Ethanol production increased smoothly in *mig1Δ/mig1Δ*, then remained nearly at the same value and decreased to its initial value at the second steady state. In this case, deletion of *MIG2* did not affect glucose repression after the addition of glucose to the medium. But in the absence of *MIG1* a release in glucose repression was observed. This observation is in good correlation with the literature that Mig2p has a minor role relative to Mig1p (Zhou and Winston, 2001).

Ammonia concentration was constant in *hoΔ/hoΔ* and *mig2Δ/mig2Δ* and showed an oscillatory behavior in *mig1Δ/mig1Δ* in glucose limited cultures following the application of the pulse injection. This may suggest a possible role of *MIG1* in ammonia utilization. Glycerol concentration decreased after pulse injection of glucose in *hoΔ/hoΔ*, increased in *mig2Δ/mig2Δ* and remained constant in *mig1Δ/mig1Δ*. The glycerol production was maximum in *mig2Δ/mig2Δ* and minimum in *mig1Δ/mig1Δ* after the pulse injection. It has been shown that glycerol secretion is necessary for the anaerobic physiology of *S. cerevisiae* and *FPS1* overexpression enhances glycerol production. Mig1p binds to a consensus sequence on the *FPS1* promoter. Under anaerobic conditions it has been observed that deletion of *MIG1* leads to more glycerol production (Luyten *et al.*, 1995; Klein *et al.*, 1998). According to this information, it is not surprising to observe less glycerol production in *mig1Δ/mig1Δ* under aerobic conditions.

Under nitrogen limitation the growth of *hoΔ/hoΔ* and *mig1Δ/mig1Δ* strains were at the same level and the growth of *mig2Δ/mig2Δ* was lower at the first steady state. At the second steady state the growth of *hoΔ/hoΔ* was higher than the first steady state. The increase in the growth of *hoΔ/hoΔ* may be related to the residual glucose concentration and ethanol production. At the second steady state ethanol production was lower than the first steady state. So the *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) preferred respiration instead of fermentation and as a result the biomass production increased under nitrogen limited conditions.

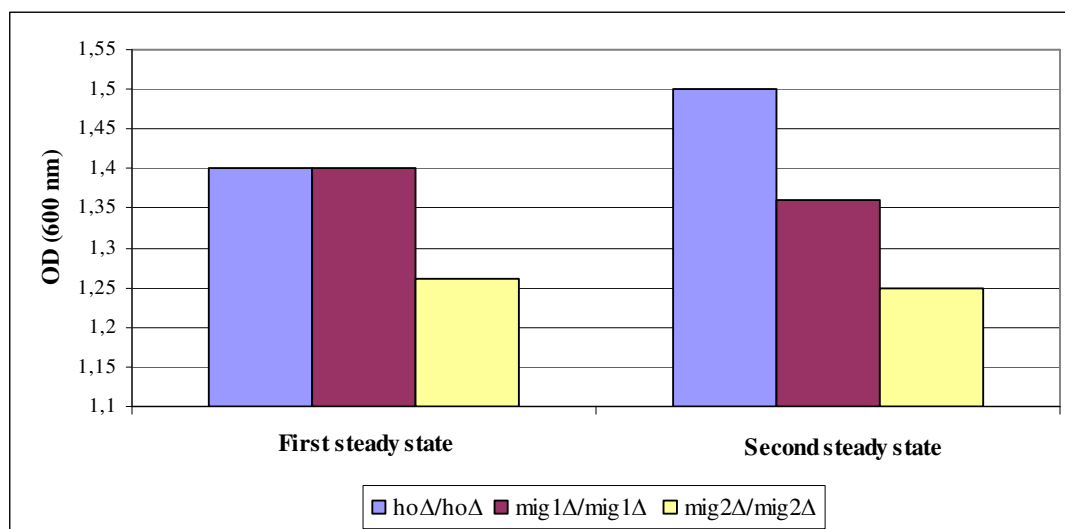


Figure 4.2. Growth characteristics of *S. cerevisiae* BY4743 strains in nitrogen limited chemostat culture

In this case, the residual glucose concentration was not enough to cause glucose repression. No significant changes were observed in the growth of *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* between the first and second steady states.

Ammonium sulfate addition to nitrogen limited medium did not affect the glucose consumption in *hoΔ/hoΔ*, *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*. Glucose concentrations in these strains were approximately constant before and after the ammonium sulfate injections. Deletion of *MIG1* or *MIG2* leads to higher consumption of glucose. After the nitrogen pulse, ammonia concentration was comparatively higher in *mig1Δ/mig1Δ*. The ammonia consumption in *hoΔ/hoΔ* and *mig2Δ/mig2Δ* showed similar behavior. Ethanol production in *hoΔ/hoΔ* showed a decreasing profile. The glycerol concentration in *hoΔ/hoΔ* first increased and then decreased and the highest formation of glycerol obtained in *hoΔ/hoΔ* until the 6<sup>th</sup> hour of the pulse injection. In *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* ethanol production profiles followed the glucose consumption profiles. The *mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* strains produced the same amount of glycerol and showed the same behavior in glycerol production.

#### 4.2. Comparison of Transcriptional Responses of *MIG1*, *MIG2*, *HAP4* and *GSM1* to Pulses in Deletion Strains

Under glucose limitation in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*), the expression level of *HAP4* was high at the first and second steady states and it decreased with the injection of glucose after the first steady state (Figure 3.13). This observation is in good correlation with the literature. It has been reported that glucose represses the expression of *HAP4* via Mig1 pathway and thereby activation of respiration is prevented at high glucose concentration (Raghevendran, 2006). In the 7<sup>th</sup> hour of the pulse injection an increase in the expression level of *HAP4* was observed. An increase in the growth of the cells was also observed at the same time. It can be explained that the activation of *HAP4* induces respiration and consequently led to an increase in biomass in the *hoΔ/hoΔ*.

Deletion of *MIG1* or *MIG2* in glucose limited chemostat culture led to repression of *HAP4* (Figures 3.21 and 3.27). In *mig1Δ/mig1Δ*, *HAP4* was up-regulated at the end of first minute of the pulse injection. It was shown by Lascaris *et al.* (2004) that *HAP4* gene was over-expressed in a *MIG1* deletion background, generating a mutant in which respiratory function is stimulated and glucose repression is diminished.

Under glucose limitation in *mig2Δ/mig2Δ* strain, *HAP4* started to be activated just after the pulse. Since Mig2p has essentially the same binding site as Mig1p and is a repressor that collaborates with Mig1p to cause glucose repression (Kaniak *et al.*, 2004, Lutfiyya and Johnston, 1996) the activation of *HAP4* in the lack of *MIG2* after the glucose pulse is consistent with the literature. In the absence of *MIG2*, *HAP4* reached the highest expression level later than in the absence of *MIG1*. In this case the activation of *HAP4* in the absence of *MIG2* was delayed. Lutfiyya *et al.* (1998) showed that no genes found, whose promoters contain potential Mig1p-binding sites, are regulated only by Mig1p. This confirms the statement of that Mig2p always works in conjunction with Mig1p.

In nitrogen limited condition (where glucose is not limited) *HAP4* expression was low and increased with the injection of ammonium sulfate pulse in *hoΔ/hoΔ* (Figure 3.17). Since no significant changes observed in the glucose concentration after the injection of the nitrogen pulse, it can be said that the changes in the expression level of *HAP4* were

independent of glucose concentration. It is known that the HAP complex regulates ammonia metabolism and the nitrogen catabolite repression via regulation of the activity of two major enzymes in ammonia metabolism, GDH1 and GDH3 (ter Schure *et al.*, 2000). It has been also shown that Hap4p is not involved in glucose-dependent regulation, but it does play a role in *GDH1* expression in ethanol-grown cultures. In glucose-grown cells, the HAP complex operates in a Hap4p independent manner (Riego *et al.*, 2002). In this experiment, ammonium sulfate addition to nitrogen-limited medium activated the *HAP4* gene in *hoΔ/hoΔ*. Up-regulation of *HAP4* with nitrogen pulse suggests a possible role of *HAP4* during the shift from nitrogen limited conditions to nitrogen excess conditions. *HAP4* gene was observed to be up-regulated significantly after the 8<sup>th</sup> hour of the pulse injection. An increase in glucose concentration and a decrease in ethanol production were also observed at the same time. At the second steady state the growth of the cells was also higher than the first steady state. In this case the, up-regulation of *HAP4* after the 8<sup>th</sup> hour may be the major cause of the direction the metabolism of *S. cerevisiae* to respiration in *hoΔ/hoΔ*.

In nitrogen limited condition *HAP4* was repressed in the absence of *MIG1*. With the injection of ammonium sulfate *HAP4* was activated for 5 minutes and then no significant changes were observed in the expression level of *HAP4* (Figure 3.24). Injection of ammonium sulfate did not effect the glucose concentration. Since the residual glucose concentration was very low and the ammonia concentration was comparatively high in the *mig1Δ/mig1Δ* the up-regulation of *HAP4* was independent of glucose concentration under this condition. This observation is consistent with previously suggested role of this gene in *hoΔ/hoΔ*. Deletion of *MIG1* diminished the oscillations in the expression profile of *HAP4* after the injection of the nitrogen pulse when compared to *hoΔ/hoΔ*. *HAP4* was also found to be repressed at the second steady state in the absence of *MIG1*. In this case the repression of *HAP4* might be due to the presence of *MIG2* or a repressive mechanism distinct from the main glucose repression pathway (Lascaris *et al.*, 2004).

At the first steady state of nitrogen limited chemostat cultivation of *mig2Δ/mig2Δ* the expression level of *HAP4* was observed to be undetectable (Figure 3.30). After the injection of nitrogen pulse the expression level of *HAP4* did not change significantly. The effect of the deletion of *MIG2* observed as repression of *HAP4* in *mig2Δ/mig2Δ* after the

injection of the nitrogen pulse. After the 7<sup>th</sup> hour of the nitrogen pulse injection, an increase in the expression level of *HAP4* was observed and ethanol concentration decreased. This behavior after the 7<sup>th</sup> hour of the pulse injection was common in the expression profiles of *HAP4*, *MIG1* and *GSM1* in *mig2Δ/mig2Δ*. In this case the increasing expression of *HAP4* might possibly lead *S. cerevisiae* directed its metabolism to respiration. At the second steady state *HAP4* was highly activated under nitrogen limitation in *mig2Δ/mig2*. This behavior of *HAP4* in *mig2Δ/mig2* at the second steady state was similar to its behavior in *hoΔ/hoΔ*. The reason of higher level of gene expression at second steady state in these strains remains to be elucidated.

In *hoΔ/hoΔ*, *MIG1* was activated with the injection of glucose pulse to glucose limited media (Figure 3.14). This observation is in good correlation with the literature. It has been reported that, at high levels of glucose Mig1p becomes active, remains in the nucleus and here it represses the transcription of genes involved in the metabolism of carbon sources other than glucose and fructose (Carlson, 1999; Westergaard *et al.*, 2007). Within the first minute of pulse injection the expression level of *MIG1* decreased. Kaniak *et al.* (2004) reported that glucose repression of *MIG1* expression mediated by Mig1p itself, in collaboration with Mig2 (and Mig3), constitutes a feedback regulatory circuit, whereby synthesis of the repressor is decreased under conditions in which it is active. The effect of this regulation is to dampen Mig1-mediated glucose repression, possibly to avoid over-repression, and conceivably to enable a more rapid recovery from repression when glucose becomes depleted from the medium. This feedback regulatory circuit may be responsible for the oscillations observed in the expression of *MIG1* gene throughout the chemostat culture in *hoΔ/hoΔ*. Raghevendran *et al.* (2006) reported also the presence of oscillations occur at low dilution rates in carbon limited chemostat, even in wild type, and are prevented by briefly imposing oxygen limitation. At the second steady state *MIG1* was activated even to a higher level. There were no significant changes in the metabolite profiles except for glycerol compared with the first steady state. Glycerol concentration was significantly lower at the second steady state. It is known that Mig1p binding to a consensus sequence on the *FPS1* promoter (Lundin *et al.*, 1994) and *FPS1* encodes a channel protein that facilitates glycerol uptake and efflux (Luyten *et al.*, 1995). The change in glycerol concentration and the up-regulation of *MIG1* at the second steady state may be related.

Under glucose limitation *MIG1* was down-regulated in the absence of *MIG2* (Figure 3.28). With the injection of glucose pulse *MIG1* was activated but it showed an oscillatory behavior at the first two hours of the pulse injection. Comparatively to wild-type (*hoΔ/hoΔ*), deletion of *MIG2* diminished the oscillations after the second hour of the pulse injection and up-regulation of *MIG1* at the second steady state.

Although the glucose concentration was high under nitrogen limited medium in *hoΔ/hoΔ*, *MIG1* expression level was undetectable (Figure 3.18). Sudden injection of ammonium sulfate to nitrogen limited chemostat culture of *hoΔ/hoΔ* activated *MIG1* immediately. In this case nitrogen limitation played a key role on the expression profile of *MIG1* rather than glucose concentration. It is known that Snf1 is active when it is phosphorylated and active Snf1p catalyzes the phosphorylation of Mig1p, causing Mig1p to be inactive (Carlson, 1999; Westergaard *et al.*, 2007). Orlova *et al.* (2006) showed that nitrogen limitation makes Snf1p active. In this case at the first steady state Snf1p was active and Mig1p was phosphorylated thus inactive under nitrogen limitation. After the pulse injection when nitrogen is not limited Snf1 was inactive and could not phosphorylate Mig1, therefore *MIG1* was up-regulated.

At the first steady state of nitrogen limited cultivation of *mig2Δ/mig2* the expression level of *MIG1* was undetectable (Figure 3.31). In the absence of *MIG2*, activation of *MIG1* after the nitrogen pulse was not immediate; in contrast to *hoΔ/hoΔ*, the expression level of *MIG1* continued to increase for 15 minutes. Since the glucose concentration did not change during the nitrogen limited cultivation of *mig2Δ/mig2*, it can be said that the expression profile of *MIG1* was independent of glucose concentration. The activation of *MIG1* with the nitrogen pulse may be related to the inactivation Snf1 when nitrogen is excess (Orlova *et al.*, 2006). After this point, *MIG1* showed an oscillatory behavior. Comparatively to *hoΔ/hoΔ*, the lack of *MIG2* in nitrogen limited cultivation leads to a more oscillatory expression profile of *MIG1*.

*MIG1* was first activated just after the glucose pulse injection and then down-regulated, however *MIG2* was down-regulated just after the glucose pulse injection in *hoΔ/hoΔ* strain grown under glucose limitation (Figure 3.15). The slight difference in the response of these genes to glucose pulse may be related to different regulatory mechanisms

in the control of gene expression of these genes. Indeed it has been reported that Mig1p, but not Mig2p is inactivated by Snf1 protein kinase in the absence of glucose (Lutfiyya *et al.*, 1998). The expression profiles of *MIG1* and *MIG2* were almost similar in *hoΔ/hoΔ* throughout the cultivation soon after the pulse. Interestingly, *MIG2* also shared the same expression profile with *HAP4* and *GSM1* in *hoΔ/hoΔ*. The peak at the 7<sup>th</sup> hour of the glucose pulse injection was common in the expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1*. A sharp increase in the growth of the cells was also observed at the same time.

The expression level of *MIG2* under glucose limitation in *mig1Δ/mig1Δ* was undetectable before and just after the glucose pulse injection (Figure 3.22). *MIG2* started to respond to glucose ten minutes after the injection. A significant up-regulation of *MIG2* was observed at the first hour of the pulse injection. This observation can lead to a conclusion that disruption of *MIG1* down-regulates the expression of *MIG2* in glucose limited conditions. The expression levels of *MIG2* showed a less oscillatory expression profile after the glucose pulse injection when *MIG1* was not present.

Under nitrogen limitation in *hoΔ/hoΔ*, *MIG2* was up-regulated just after the nitrogen pulse and showed an oscillatory behavior within the first minute and after the first hour of the nitrogen pulse injection (Figure 3.19). It was recently reported that *MIG2* was differentially expressed according to different nitrogen sources and this finding was explained that nitrogen regulation of regulatory genes allows other metabolic and signaling pathways of yeast to be modulated according to the nitrogen source (Godard *et al.*, 2007). *MIG2* was activated after the 8<sup>th</sup> hour of the pulse injection. An increase in the level of glucose concentration in medium was also observed at the same time. The expression profiles of *MIG1* and *MIG2* in *hoΔ/hoΔ* were nearly the similar throughout the cultivation before and after the pulse injection. Although *MIG1* and *MIG2* are both activated at the second steady state *HAP4* was not repressed in *hoΔ/hoΔ* under nitrogen limited.

In the absence of *MIG1* under nitrogen limited condition, the expression level *MIG2* was undetectable (Figure 3.25). Deletion of *MIG1* diminished the up-regulation of *MIG2* at the first and second steady states. Raghevendran *et al.* (2005) reported that *MIG1* expression regulated by *MIG2*. The lack of *MIG1* may be the cause of down-regulation of *MIG2* under these conditions. However, an oscillatory expression profile of *MIG2* was

observed with the injection of ammonium sulfate. At the 5<sup>th</sup> hour of the nitrogen pulse injection *MIG2* displayed higher activation levels.

Under glucose limitation, *GSM1* was up-regulated in *hoΔ/hoΔ* and after the pulse it was repressed (Figure 3.16). At the 7<sup>th</sup> hour of the pulse injection *GSM1* was activated together with *HAP4*, *MIG1* and *MIG2*. An increase in biomass was also observed at the same time. *GSM1* exactly exhibited the same expression profile with *HAP4* under these conditions. This observation is in good correlation with the literature that *GSM1* must have role in regulation of respiratory pathway of yeast (Pir *et al.*, 2006; Deng *et al.*, 2004).

Under glucose limitation, deletion of *MIG1* caused repression of *GSM1* (Figure 3.23). With the injection glucose pulse, *GSM1* had not been activated immediately. After most of the injected glucose had been consumed, *GSM1* was up-regulated. This up-regulation is possibly related to an increase in biomass and decrease in ethanol production observed under these conditions. This observation provides further evidence that *GSM1* may have a role in respiration. The presence of *MIG1* and *MIG2* led to up-regulation of *GSM1* in *hoΔ/hoΔ* under glucose limitation. Deletion of *MIG1* or *MIG2* diminished this up-regulation under the same conditions. The expression of *HAP4* gene also decreased in these strains under glucose limited conditions. *HAP4* was activated just after glucose pulse injection and *GSM1* was activated later in *mig1Δ/mig1Δ*. This observation provides further evidence that *GSM1* is regulated by HAP complex (Deng *et al.*, 2004).

Deletion of *MIG2* repressed *GSM1* when glucose is limited (Figure 3.29). With the injection of glucose pulse, *GSM1* was activated immediately. In *mig2Δ/mig2Δ* grown under glucose limitation, *GSM1* and *HAP4* shared the same expression profile before and after the pulse injection. In this case, deletion of *MIG2* caused a repression in both genes under glucose limitation and activation in glucose excess conditions. It can be concluded that, the lack of *MIG2* leads to changes in the expression profiles of both *GSM1* and *HAP4*.

In nitrogen limited cultivation of *hoΔ/hoΔ*, *GSM1* was activated with the injection of the nitrogen pulse (Figure 3.20). And it was up-regulated at the second steady state like *MIG1*, *MIG2* and *HAP4*. The reason of higher level of gene expression at second steady state in these strains remains to be elucidated. *HAP4* and *GSM1* exhibited similar

expression profiles in nitrogen limited chemostat cultivation of *hoΔ/hoΔ*. This observation may indicate a possible role of *GSM1* during the shift from nitrogen limited conditions to nitrogen excess conditions in yeast similar to that of *HAP4*.

Under nitrogen limitation, deletion of *MIG1* did not change the repressed situation of *GSM1* and the expression level of the gene remained undetectable at the second steady state in *mig1Δ/mig1Δ* (Figure 3.26). In this strain, *GSM1* and *HAP4* exhibited some similarities in their expression profiles but they did not follow the same profile exactly as in *hoΔ/hoΔ*. It can be concluded that, the lack of *MIG1* leads to changes in the expression profiles of both *GSM1* and *HAP4*.

In *mig2Δ/mig2Δ*, *GSM1* was repressed at the first steady state and up-regulated at the second steady state when nitrogen is limited (Figure 3.32). Deletion of *MIG2* had no effect on the expression profile of *GSM1* compared with the *hoΔ/hoΔ* under nitrogen limitation. *GSM1* responded to nitrogen pulse immediately as in *hoΔ/hoΔ* and it was up-regulated at the end of first hour. In *mig2Δ/mig2Δ*, *GSM1* exhibited a different expression profile than *HAP4*. It can be concluded that, the lack of *MIG2* leads to more significant changes in the expression profiles of *HAP4* than *GSM1*.

## 5. CONCLUSIONS AND RECOMMENDATIONS

### 5.1. Conclusions

In the scope of this thesis, transcriptional responses of *HAP4*, *MIG1*, *MIG2* and *GSM1* (*YJL103C*) genes to transient perturbations under glucose and nitrogen limited conditions were investigated in order to understand glucose sensing and signaling pathways in yeast. For this purpose two homozygous deletion strains (*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ*) of the parent strain *Saccharomyces cerevisiae*, BY4743 were used. *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) was included into this study as a control to investigate the variations in the expression of these genes. These strains were cultivated under glucose and nitrogen limitations and then pulse injections of either glucose or nitrogen were applied. Growth characteristics and metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained. The expression profiles of *HAP4*, *MIG1*, *MIG2* and *GSM1* were determined via real time rt-qPCR.

*mig1Δ/mig1Δ* and *mig2Δ/mig2Δ* strains produced higher biomass than *hoΔ/hoΔ* under glucose limitation. *MIG2* deleted strain of *S. cerevisiae* BY4743 produced much more ethanol than *hoΔ/hoΔ*. Deletion of *MIG1* reduced the ethanol and glycerol production and led to quick consumption of glucose after the pulse injection. Absence of *MIG2* did not affect the glucose repression mechanism as much as *MIG1*.

*mig2Δ/mig2Δ* produced the lowest biomass under nitrogen limitation. The highest ammonia concentration was observed in *mig1Δ/mig1Δ* after ammonium sulfate pulse was injected to the nitrogen limited culture. The residual glucose concentration was the highest in *hoΔ/hoΔ* in nitrogen limited chemostat cultivation. .

*HAP4*, *MIG1*, *MIG2* and *GSM1* were up-regulated under glucose limitation in *hoΔ/hoΔ*. With the glucose pulse injection, *HAP4*, *MIG2* and *GSM1* were repressed and *MIG1* was induced. Deletion of *MIG1* or *MIG2* resulted in repression of *MIG2*, *HAP4* and *GSM1* under glucose limitation. After the glucose pulse injection *HAP4* was activated immediately, *GSM1* and *MIG2* were activated later in *mig1Δ/mig1Δ*. The activation of

*GSM1* after the up-regulation of *HAP4* provided further evidence that *GSM1* is regulated by HAP complex. Absence of *MIG2* delayed the activation of *HAP4* after the glucose pulse compared with the absence of *MIG1*. *GSM1* showed similar expression levels with *HAP4* both in *hoΔ/hoΔ* and *mig2Δ/mig2Δ* throughout the glucose limited cultivations. This indicated a possible role of *GSM1* in regulation of respiratory pathway of yeast together with *HAP4*.

In nitrogen limited cultivation of *hoΔ/hoΔ*, *HAP4*, *MIG1* and *GSM1* were repressed and the expression level of *MIG2* was low. After the injection of ammonium sulfate *HAP4*, *MIG1*, *MIG2* and *GSM1* were up-regulated. Deletion of *MIG1* diminished the up-regulation of *MIG2* under nitrogen limitation. With the ammonium sulfate addition *HAP4*, *MIG2* and *GSM1* were activated in *mig1Δ/mig1Δ*. Up-regulation of *HAP4* (and *GSM1*) with nitrogen pulse suggested a possible role of *HAP4* (and *GSM1*) during the shift from nitrogen limited conditions to nitrogen excess conditions. In the absence of *MIG2*, *HAP4* was repressed after the nitrogen pulse injection. Deletion of *MIG2* also delayed the activation of *MIG1* after the addition of ammonium sulfate to the medium.

## 5.2. Recommendations

Some additional work may be useful to provide a better view of glucose sensing, signaling and repression in *Saccharomyces cerevisiae*. Addition to the investigated strains of *S. cerevisiae*, a double deletion of *MIG1* and *MIG2* should be cultivated under glucose and nitrogen limited chemostat cultivations. This strain will provide insights for the roles of *MIG1* and *MIG2* in glucose repression.

To elucidate the roles of *MIG1* and *MIG2* in the glucose sensing and signaling pathways in yeast the genes that are involved in these pathways (e.g. *CYC8*, *GRR1*, *MTH1*, *RGT1*, *RGT2*, *SKP1*, *SNF3*, *STD1*, *TUP1*, *YCK1*, *YCK2*, *ELM1*, *GLC7*, *HXK2*, *PAK1*, *REG1*, *SNF1*, *SNF4*, *TOS3*, *MBA1*) should be investigated. Additionally, the possible roles of *MIG1* and *MIG2* in the nitrogen metabolism of *S. cerevisiae* should be investigated.

Relative quantification of gene expression provides information at the transcriptional level. However, most biological processes are mediated by proteins, which are subject to

posttranscriptional regulation that is generally not observable at mRNA levels. A complete understanding of biological systems requires knowledge of protein properties. It is also known that phosphorylation of Mig1p is very important in glucose repression mechanism in *S. cerevisiae*. This process may be monitored via mass spectrometry to reveal information of the transcriptional control of this protein over yeast metabolism.

The transcription factor activities of Mig1p and Mig2p should also be estimated using the mRNA levels of downstream genes thought to be regulated by these factors in order to capture combinatorial regulation of the transcripts as described by Ronen and Botstein (2006).

## REFERENCES

- Arga K. Y., Z. İlşen Önsan, B. Kırdar, K. Ö. Ülgen and J. Nielsen, 2007, “Understanding Signaling in Yeast: Insights From Network Analysis”, *Biotechnology and Bioengineering*, Vol. 97, No. 5, pp. 1246-1258.
- Baganz, F. , A. Hayes, D. Marren, D. C. Gardner and S. G. Oliver, 1997, “Suitability of Replacement Markers for Functional Analysis Studies in *Saccharomyces cerevisiae*”, *Yeast*, Vol. 13, pp. 1563-1573.
- Buschlen, S., J-M. Amillet, B. Guidard, A. Fournier, C. Marcireau and M. Bolotin-Fukuhara, 2003, “*The S. cerevisiae* HAP Complex, a Key Regulator of Mitochondrial Function, Coordinates Nuclear and Mitochondrial Gene Expression”, *Comparative and Functional Genomics*, Vol.4, pp. 37-46.
- Carlson, M., 1999, “Glucose Repression in Yeast”, *Current Opinion in Microbiology*, Vol.2, pp. 202-207.
- Deng, Y., T. He, Y. Wu, P. Vanka, G. Yang, Y. Huang, H. Yao and S. J. Brown, 2005, “Computationally Analyzing the Possible Biological Function of YJL103C – an ORF Potentially Involved in the Regulation of Energy Process in Yeast”, *International Journal of Molecular Medicine*, Vol. 15, pp. 123-127.
- Deutschbauer, A. M., R. M. Williams, A. M. Chu and R. W. Davis, 2002, “Parallel Phenotypic Analysis of Sporulation and Postgermination Growth in *Saccharomyces Cerevisiae*”, *Proceedings of the National Academy of Sciences*, Vol. 99, No. 24, pp. 15530-15535.
- Dikicioğlu. D., 2005, *Use of Flux Balance Analysis and Metabolomics in the Analysis of the Respiratory Pathway in Saccharomyces Cerevisiae*, M.S. Thesis, Boğaziçi University.

- Fu, L., P. Bounelis, N. Dey, B. L. Browne, R. B. Marchase and D. M. Bedwell, 1995, "The Posttranslational Modification of Phosphoglutamutase Is Regulated by Galactose Induction and Glucose Repression in *Saccharomyces Cerevisiae*", *Journal of Bacteriology*, Vol. 177, No. 11, pp. 3087-3094.
- Gancedo, J.M., 1998, "Yeast Carbon Catabolite Repression", *Microbiology and Molecular Biology Reviews*, Vol. 62, No. 2, pp. 334-361.
- Ginziger, D. G., 2002, "Gene Quantification Using Real-Time Quantitative PCR: An Emerging Technology Hits Mainstream", *Experimental Hematology*, Vol. 30, pp. 503-512.
- Godard, P., A. Urrestarazu, S. Vissers, K. Kontos, G. Bontempi, J. van Helden and B. Andre, 2007, "Effect of 21 Different Nitrogen Sources on Global Gene Expression in the Yeast *Saccharomyces cerevisiae*", *Molecular and Cellular Biology*, Vol. 27, No. 8, pp. 3065-3086.
- Ho, S-W., G. Jona, C. T. L. Chen, M. Johnston and M. Synder, 2006, "Linking DNA-Binding Proteins to Their Recognition Sequences by Using Protein Microarrays", *Proceedings of the National Academy of Sciences*, Vol. 103, No. 26, pp. 9940-9945.
- Hu, Y., T. G. Cooper and G. B. Kohlhaw, 1995, "The *Saccharomyces Cerevisiae* Leu3 Protein Activates Expression of GDH1, a Key Gene in Nitrogen Assimilation", *Molecular and Cellular Biology*, Vol. 15, pp. 52-57.
- Johnston, M., 1987, "A Model Fungal Gene Regulatory Mechanism: The GAL Genes of A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*", *Microbiological Reviews*, Vol. 51, No. 4, pp. 458-476.
- Johnston, M., J. H. Kim, 2005, "Glucose as a Hormone: Receptor-Mediated Glucose Sensing in the Yeast *Saccharomyces cerevisiae*", *Biochemical Society Transactions*, Vol. 33, pp. 247-252.

- Kaniak, A., Z. Xue, D. Macool, J-H. Kim and M. Johnston, 2004, "Regulatory Network Connecting Two Glucose Signal Transduction Pathways in *Saccharomyces cerevisiae*", *Eukaryotic Cell*, Vol. 3, No. 1, pp. 221-231.
- Kim, J-H., V. Brachet, H. Moriya and M. Johnston, 2006, "Integration of Transcriptional and Posttranslational Regulation in a Glucose Signal Transduction Pathway in *Saccharomyces cerevisiae*", *Eukaryotic Cell*, Vol. 5, No. 1, pp. 167-173.
- Klein, C.J., L. Olsson, B. Ronnow, J. Dalgaard Mikkelsen and J. Nielsen, 1996, "Alleviation of Glucose Repression of Maltose Metabolism by *MIG1* Disruption in *Saccharomyces cerevisiae*", *Applied and Environmental Microbiology*, Vol. 62, No. 12, pp. 4441-4449.
- Klein, C. J. L., L. Olsson and J. Nielsen, 1998, "Glucose Control in *Saccharomyces cerevisiae*: the Role of MIG1 in Metabolic Functions", *Microbiology*, Vol. 144, pp. 13-24.
- Lascaris, R., J. Piwowarski, H. van der Spek, J. Teixeira de Mattos, L. Grivell and J. Blom, 2004, "Overexpresion of HAP4 in Glucose-Derepressed Yeast Cells Reveals Respiratory Control of Glucose-Regulated Genes", *Microbiology*, Vol. 150, pp. 929-934.
- Lundin, M., J. O. Nehlin and H. Ronne, 1994, "Importance of a Flanking AT-Rich Region in Target Site Recognition by the GC Box-Binding Zinc Finger Protein Mig1", *Molecular and Cellular Biology*, Vol. 14, No. 3, pp. 1979-1985.
- Lutfiyya, L.L., M. Johnston, 1996, "Two Zinc-Finger-Containing Repressors Are Responsible for Glucose Repression of SUC2 Expression", *Molecular and Cellular Biology*, Vol. 16, No. 9, pp. 4790-4797.
- Lutfiyya, L.L., V. R. Iyer, J. DeRisi, M. J. DeVit, P. O. Brown and M. Johnston, 1998, "Characterization of Three Related Glucose Repressors and Genes They Regulate in *Saccharomyces cerevisiae*", *Genetics*, Vol. 150, pp. 1377-1391.

- Luyten, K., J. Albertyn, W. F. Skibbe, B. A. Prior, J. Ramos, J. M. Thevelein and S. Hohmann, 1995, "Fps1, a Yeast Member of the MIP Family of Channel Proteins, is a Facilitator for Glycerol Uptake and Efflux and is Inactive Under Osmotic Stress", *The EMBO Journal*, Vol. 14, pp. 1360-1371.
- Mendes-Ferreira, A., M. del Olmo, J. Garcia-Martinez, E. Jimenez-Marti, A. Mendes-Faia, J. E. Perez Ortin and J. Leao, 2007, "Transcriptional Response of *Saccharomyces cerevisiae* to Different Nitrogen Concentrations during Alcoholic Fermentation", *Applied and Environmental Microbiology*, Vol. 73, No. 9, pp. 3049-3060.
- Moreno, F. D. Ahuatzi, A. Riera, C. A. Palomino and P. Herrero, 2005, "Glucose Sensing Through The Hxk2-dependent Signaling Pathway", *Biochemical Society Transactions*, Vol. 33, pp. 265-268.
- Moriya, H. and M. Johnston, 2004, "Glucose Sensing and Signaling in *Saccharomyces cerevisiae* Through The Rgt2 Glucose Sensor and Kasein Kinase I", *Proceedings of the National Academy of Sciences*, Vol. 101, No. 6, pp. 1572-1577.
- Mwesigye, P. K. and J. P., Barford, 1996, "Mechanism of Sucrose Utilization by *Saccharomyces cerevisiae*", *Journal of General and Applied Microbiology*, Vol. 42, pp. 297-306.
- Nehlin, J. O. and H. Ronne, 1990, "Yeast MIG1 Repressor is Related to The Mammalian Early Growth Response and Wilms' Tumour Finger Proteins", *The EMBO Journal*, Vol. 9, pp. 2891-2898.
- Orlova, M., E. Kanter, D. Krakovich and S. Kuchin, 2006, "Nitrogen Availability and TOR Regulate the Snf1 Protein Kinase in *Saccharomyces cerevisiae*" *Eukaryotic Cell*, Vol. 5, No. 11, pp. 1831-1837.
- Overbergh, L., A. Giuliatti, D. Valckx, B. Decallonne, R. Bouillon and C. Mathieu, 2003. "The Use of Real-Time Reverse Transcriptase PCR for the Quantification of

- Cytokine Gene Expression”, *Journal of Biomolecular Techniques*, Vol. 13, pp. 33-43.
- Özcan, S. M. Johnston, 1995, “Three Different Regulatory Mechanisms Enable Yeast Hexose Transporter (HXT) Genes To Be Induced by Different Levels of Glucose”, *Molecular and Cellular Biology*, Vol. 15, No. 3, pp. 1564-1572.
- Özcan, S., L. G. Vallier, J.S. Flick, M. Carlson and M. Johnston, 1997, “Expression of the SUC2 Gene of *Saccharomyces Cerevisiae* is Induced by Low Levels of Glucose”, *Yeast*, Vol. 13, pp. 127-137.
- Palomino, A., P. Herrero and F. Moreno, 2005, “Rgt1, a Glucose Sensing Transcription Factor, is Required for Transcriptional Repression of the *HXK2* Gene in *Saccharomyces Cerevisiae*”, *Biochemical Journal*, Vol. 388, pp. 697-703.
- Pir, P., K. Ö. Ülgen, A. Hayes, Z. İlsen Önsan, B. Kırdar and S. G. Oliver, 2006, “Annotation of Unknown Yeast ORFs by Correlation Analysis of Microarray Data and Extensive Literature Searches”, *Yeast*, Vol. 23, pp. 553-571.
- Radonic, A., S. Thulke, M. Mackay, O. Landt, W. Siegert and A. Nitsche, 2004, “Guideline to Reference Gene Selection for Quantitative Real-Time PCR”, *Biochemical and Biophysical Research Communication*, Vol. 313, pp. 856-862.
- Raghevendran, V., J. Nielsen and L. Olsson, 2005, “Teaching Microbial Physiology Using Glucose Repression in Baker’s Yeast as an Example”, *Biochemistry and Molecular Biology Education*, Vol. 33, No. 6, pp. 404-410.
- Raghevendran, V., K.R. Patil, L. Olsson and J. Nielsen, 2006, “Hap4 Is Not Essential for Activation of Respiration at Low Dilution Rates in *Saccharomyces Cerevisiae*”, *The Journal of Biological Chemistry*, Vol. 281, No. 18, pp. 12308-12314.
- Riego, L., A. Avendano, A. DeLuna, E. Rodriguez and A. Gonzalez, 2002, “GDH1 Expression Is Regulated by GLN3, GCN4, and HAP4 Under Respiratory Growth”,

*Biochemical and Biophysical Research Communication*, Vol. 293, pp. 79-85.

Ronen, M. and D. Botstein, 2006, "Transcriptional Response of Steady-state Yeast Cultures to Transient Perturbations in Carbon Source", *Proceedings of the National Academy of Sciences*, Vol. 103, No. 2, pp. 389-394.

Santangelo, G. M., 2006, "Glucose Signaling in *Saccharomyces Cerevisiae*", *Microbial and Molecular Biology Reviews*, Vol. 70, No. 1, pp. 253-282.

Santos, E., L. Rodriguez, M. V. Elorza and R. Sentandreu, 1982, "Uptake of sucrose by *Saccharomyces Cerevisiae*", *Arch Biochem Biophys*, Vol. 216, pp. 652-660.

Schuller, H. J., 2003, "Transcriptional Control of Nonfermentative Metabolism in the Yeast *Saccharomyces Cerevisiae*", *Current Genetics*, Vol. 43, No. 3, pp. 139-160.

ter Schure, E. G. , N. A. W. van Riel and C. T. Verrips, 2000, "The Role of Ammonia Metabolism in Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*", *FEMS Microbiology Reviews*, Vol. 24, pp. 67-83.

van Maris, A. J. A. , B. M. Bakker, M. Brandt, A. Boorsma, M. J. T. de Mattos, L. A. Grivell, J. T. Pronk and J. Blom, 2001, "Modulating the Distribution of Fluxes Among Respiration and Fermentation by Overexpression of HAP4 in *Saccharomyces cerevisiae*", *FEMS Yeast Research*, Vol. 1, pp. 139-149.

Westergaard S. L., A.P. Oliveira, C. Bro, L. Olsson and J. Nielsen, 2007, "A systems biology approach to study glucose repression in the yeast *Saccharomyces cerevisiae*", *Biotechnology and Bioengineering*, Vol. 96, No. 1, pp. 134-145.

Zhou, H. and F. Winston, 2001, "NRG1 is required for glucose repression of the SUC2 and GAL genes of *Saccharomyces cerevisiae*", *BMC Genetics*, Vol. 2, No. 5.