STUDIES ON THE CONTROLLING NETWORK OF *S. CEREVISIAE* VIA EXPRESSION PROFILING

by

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ABSTRACT

STUDIES ON THE CONTROLLING NETWORK OF S. CEREVISIAE VIA EXPRESSION PROFILLING

This study aimed to identify whether the signature of phosphorylation or dephoshorylation of the proteins involved in glucose sensing, signal transduction and glucose repression pathways can be observed at the transcription of the genes encoding those proteins. *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1Δ/rip1Δ and RIP1/rip1Δ)* were cultivated under nitrogen limited condition in order to identify the variations in the expression levels of genes involved in glucose sensing, signal transduction and glucose repression pathways as a response to system level perturbations. Metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained as well as the growth curves. Expression profiles of *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4, MBA1* genes were determined. The cell densities of the *hoΔ/hoΔ*, *rip1Δ/rip1Δ and RIP1/rip1Δ* mutants decreased whereas growth of *hap4Δ/hap4Δ* mutant increased in response to nitrogen pulse. Highest cell density was obtained in *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains at the second steady state. The ethanol production was higher in respiratory deficient *hap4Δ/hap4Δ* and *rip1Δ/rip1Δ* mutants. Glycerol production was significantly lower in *RIP1* mutations in comparison to the other studied strains. The expression level of *MIG1, MBA1, GRR1* and *REG1* increased in response to nitrogen pulse in all studied mutants. *RIP1* deletion resulted in the repression of *HAP4*, *MTH1*, *SKP1 RGT1, MBA1* and *YCK1* genes. Respiratory deficiency seems to affect the expression of *RGT1, MBA1* and may be the reason for the dissimilarity observed between the expression profiles of *MIG1-HXK2, GRR1-SKP1* and *MIG1-CYC8-TUP1.* The deletion of *HAP4* does not seem to affect significantly the expression patterns of *REG1, GLC7, MIG1, SNF1* and *SNF4. HAP4* seems to plays an important role in nitrogen catabolite repression in yeast. Deletion of *RIP1* seems to affect the transcriptional response of *HAP4* in nitrogen catabolite repression in yeast.

ÖZET

GEN ANLATIM PROFİLİ İLE *SACCHAROMYCES CEREVISIAE*’NIN KONTROL AĞI ÜZERİNE ÇALIŞMALAR

Azot kaynağını eksik miktarlarda içeren F1 kullanılarak kontrollü (sıcaklık, pH, çözünmüş oksijen, karıştırma hızı) kesiksiz fermentasyon koşullarında (kemostat) *S. cerevisiae* BY4743’nin gen delesyonu taşıyan *hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1Δ/rip1Δ ve RIP1/rip1Δ* suşları büyütüldü. Ortama amonyum sülfatın darbe (pulse) şeklinde eklenerek besin sınırlandırılmasının kaldırılması sonucu glukoz algılama, sinyal iletimi ve glukoz baskılama yolizinde görev alan genlerin gen anlatımındaki değişimler gözlenmiştir. Suşların büyütülmesi sırasında belirli zaman aralıklarında alınan örneklerde biyokütle, etanol, glukoz, gliserol ve amonyak derişimleri belirlenmiştir. Gen anlatımı profilleri incelenen genler *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4* ve *MBA1’* dır. Amonyum sülfat darbe (pulse) şeklinde eklendikten sonra *hoΔ/hoΔ*, *rip1Δ/rip1Δ* ve *RIP1/rip1Δ* suşlarının hücre üretimlerinde azalma gözlenirken, *hap4Δ/hap4Δ* suşunda artış gözlenmiştir. *hoΔ/hoΔ* ve *hap4Δ/hap4Δ* suşları daha yüksek miktarda hücre üretmiştir. En yüksek etanol derişimi, solunum eksikliği gözlenen *hap4Δ/hap4Δ* ve *rip1Δ/rip1Δ* suşlarında belirlenmiştir. *RIP1* geni silinen suşlarda gliserol derişiminde azalma saptanmıştır. *MIG1, MBA1, GRR1* ve *REG1* genlerinin anlatımı çalışılan bütün suşlarda azot eklenmesinden sonra artış göstermiştir. *RIP1* geni silinen suşlarda, *HAP4, MTH1, SKP, RGT1, MBA1* ve *YCK1* genlerinin anlatımının baskılandığı gözlenmiştir. *RGT1, MBA1* genlerinin anlatımının ve *MIG1-HXK2, GRR1-SKP1* ve *MIG1-CYC8-TUP1* genlerinin anlatımları arasındaki benzerliğin solunum eksikliğinden etkilendiği saptanmıştır. *hap4Δ/hap4Δ* suşunun *REG1, GLC7, MIG1 SNF1* ve *SNF4* genlerinin anlatımında etkili olmadığı saptanmıştır. HAP4 geninin azot metabolizmasında etkili bir rol oynadığı düşünülmektedir. *RIP1* geni silinmiş suşlarda, *HAP4* geninin azot metabolizmasındaki etkisinde farklılık belirlenmiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

A	absorbance
C_t	Threshold cycle
mRNA	Messenger RNA
N	Cycle number
rRNA	Ribosomal RNA
T_m	Melting temperature
v/v	Volume per volume
w/v	Weight per volume

EUROSCARF	European *Saccharomyces cerevisiae* Archive for Functional Analysis
FRET	Fluorescence resonance energy transfer
OD	Optical density
ORF	Open reading frames
RFU	Relative fluorescence units
RT-rtqPCR	Reverse transcription real time quantification polymerase chain reaction
TCA cycle	Tricarboxyacidic cycle
YPD	Yeast extract-peptone-dextrone

1. INTRODUCTION

The differences between the various cell types of an organism depend on the particular genes that the cells express, the level of the control of gene expression exercised. There are many steps in the pathway leading from DNA to protein and all of them can be regulated. A cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed, (2) controlling how the primary RNA transcript is spliced or processed, (3) selecting which completed mRNAs in the cell nucleus are exported to the cytoplasm, (4) selecting which mRNAs in the cytoplasm are translated by ribosome, (5) selectively the destabilizing certain mRNA molecules in the cytoplasm or selectively activating, inactivating or compartmentalizing specific protein molecules after they have been made. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control. Cell can change the pattern of genes they express in response to changes in their environment (Alberts *et al*., 1994)

Glucose may affect enzyme levels by causing a decrease in the concentration of corresponding mRNAs, a decrease in their translation rate, or an increase in the degradation rate of the protein. In turn, mRNA levels would depend on both on the rate of transcription of the corresponding gene and on the stability of the mRNA. The main effect of glucose takes place at the transcriptional level (Gancedo, 1998).

1.1. The Glucose Induction Signaling Pathway

For yeasts, like for many other micro-organisms, glucose is the preferred carbon and energy source. It is therefore not surprising that glucose is an important primary messenger molecule, signaling optimal growth conditions to the cellular machinery. Accordingly, glucose also affects many of the yeasts' commercially important traits such as growth rate, fermentation capacity and stress resistance (Rolland *et al*., 2002). In the presence of glucose, *Saccharomyces cerevisiae* increase rate of transcription of some genes encoding glycolytic enzymes and represses the transcription of genes encoding enzymes necessary for utilization of alternative carbon sources.

Among the many genes induced by glucose is a family of hexose transporters encoded by *HXT* genes. The *HXT* family consists of 17 genes encoding proteins that are closely related but subject to distinct pattern of regulation by glucose. The best characterized members of the family include *HXT1*, which is induced in high but not low glucose; *HXT2* and *HXT4*, which are induced in low but not high glucose, and *HXT3*, which is induced in both low and high glucose. Repression of *HXT1* gene expression in the absence of glucose is known to require *RGT1*. In contrast, the transcriptional repression of *HXT2* and *HXT4* observed in high glucose is apparently mediated via a separate mechanism involving *MIG1* (Flick *et al*., 2003).

SNF3 and *RGT2* are membrane receptors that bind glucose outside the cell and generate a signal inside the cell for activation of gene expression. Transcription of *SNF3* is maximal when glucose levels are low (*SNF3* expression is repressed about fivefold by high levels of glucose) (Özcan and Johnston, 1999). *RGT2*, on the other hand, appears to be a sensor of high levels of glucose, because it is required for maximal induction of *HXT1* expression by high concentrations of glucose but not for induction of *HXT2* and *HXT4* expression by low levels of glucose (Özcan and Johnston, 1999). It is appropriate, then, that *RGT2* is expressed in cells growing on high levels of glucose (it is expressed constitutively, being neither repressed nor induced by glucose) (Özcan *et al*., 1996)

The sensor proteins Snf3p and Rgt2p assess the glucose availability in the surrounding medium and transmit this information to the internal cellular machinery (Ozcan *et al*, 1998). This signal is transmitted through the SCF-Grr1 ubiquitination complex (Li and Johnson, 1997) and finally modulates the activity of Rgt1, a transcription factor which acts as a repressor when glucose is absent (Ozcan *et al*., 1996; Mosley *et al*., 2003). Std1p and Mth1p which are the additional components of the glucose induction pathway bind Rgt1p and modulate negatively *HXT1* expression (Ozcan and Johnson, 1999; Lakshmanan *et al*., 2003). Also recent studies show that Std1 and Mth1 may also interact with C-terminal tails of the glucose sensors Rgt2p and Snf3p (Schmidt *et al*., 1999; Lafuente *et al*., 2000) and that yeast protein kinase I (Yck1/Yck2) phosphorylates these components (Flick *et al*., 2003; Moriya and Johnson, 2004; Spielewoy *et al*., 2004). The signal generated by the glucose sensors in response to glucose activates casein kinase I (YckI), encoded by *YCK1* and *YCK2* (Johnston and Kim, 2005). The phosphorylation of

Mth1p and Std1p is necessary for their recognition by the SCF-Grr1 complex, which signal them for degradation (Flick *et al*., 2003; Moriya and Johnson, 2004; Spielewoy *et al*., 2004). The degradation of Std1p and Mth1p leads into the glucose-induced dissociation of Rgt1p from the *HXT1* promoter and its activation (Flick *et al*., 2003; Moriya and Johnson, 2004; Spielewoy *et al*., 2004). Std1p and Mth1p directly interact with Rgt1p when glucose is absent and are required for the repression of *HXT* gene transcription by Rgt1p (Lakshmanan *et al*., 2003).

Rgt1 is a transcription factor that regulates expression of *HXT* genes in *Saccaharomyces cerevisiae*. Rgt1p has two effects on transcription. Rgt1 represses transcription in the absence of glucose. This requires the corepressors Cyc8 and Tup1 (Ozcan and Johnson 1995; Thomas Cobos and Sanz 2002) an at least one of the paralogous proteins Mth1 and Std1 (Schmidt *et al*., 1999; Lakshmann *et al*., 2003; Mosley *et al*., 2003). Glucose inhibits Rgt1p function by stimulating degradation of Mth1p (Flick *et al*., 2003, Moriya and Johnson 2004). When glucose level are high (>2 per cent), Rgt1p activates transcription (Ozcan *et al*., 1996; Polish *et al*., 2004). Rgt1 becomes phosphorylated in response to glucose, which interferes with its ability to bind to the *HXT* gene promoters and to repress transcription (Lakshmanan *et al*., 2003). It has no effect on transcription in the presence of low levels of glucose. Grr1p is required both for inhibition of Rgt1p repressor function in response to low levels of glucose and for conversion of Rgt1p from a repressor to an activator by high levels of glucose. It is previously stated that, since transcription of *RGT1* is not altered in response to glucose, the activity of Rgt1p is likely to be regulated posttranscriptionally (Ozcan *et al*., 1996).

Grr1 mutataions exhibit multiple abnormalities including cell elongation, slow growth on glucose, increased sensitivity to osmotic stress and nitrogen starvation, decreased divalent cation transport, and enhanced filamentous growth, defects in sporulation, and slow growth or inviability when combined with amino acid biosynthetic mutants. Although the requirement for Grr1 in those processes has been well documented, its targets in most of those pathways are poorly characterized (Spielewoy *et al*., 2004).

Grr1 protein on the yeast *Saccharomyces cerevisiae* is a central component of a glucose signal transduction mechanism responsible for glucose-induced gene expression.

Mutations in *GRR1* relieve repression of many glucose-repressed genes. Grr1 is required for glucose stimulated regulation of Rgt1, a repressor of several glucose induced *HXT* genes. *GRR1* acts at an early stage of glucose signal transduction to inhibit the function of Rgt1, thereby causing de-repression of *HXT* gene expression (Li and Johnston, 1997). Instead, Grr1 is required to inactivate Mth1 and Std1 in response to glucose. Mth1 inactivation seems to occur at the level of degradation. Based upon these data, it is concluded that glucose acts via Grr1 to regulate the abundance of Mth1. Inactivation of Mth1 leads to hyperphosphorylation of Rgt1 and dissociation from *HXT* promoters (Flick *et al*., 2003).

Skp1 is a component of several structurally distinct but functionally related ubiquitin ligase complexes that target a wide variety of regulators to the 26S proteasome for degradation. These complexes are termed SCF to indicate their two common components, *S*kp1 and the cullin *C*dc53, and a third exchangeable component, the *F*-box protein. Grr1 interacts with Skp1 and is required for inhibition of Rgt1 function. Grr1 degradation of Mth1 and Std1 appear to require in addition to Skp1 interaction with the Grr1 Fbox. The Grr1-Skp1 interaction is significantly enhanced by high levels of glucose. (Li and Johnson, 1997 ; Santangelo, 2006).

It is also observed that the transcription of the *MTH1* gene is repressed at high concentration of glucose (Lakshmanan *et al*., 2003). The regulation of *MTH1* expression constitutes feedforward regulation: glucose reduces *MTH1* expression at the same time it stimulates proteasome-mediated degradation of Mth1 (Johnston and Kim, 2005). On the other hand *STD1* expression is induced by glucose which would be expected to counteract Std1 degradation (Moriya and Johnson, 2004). This is feedback regulation: glucose inhibits Std1 function by stimulating its degradation while at the same time inducing *STD1* expression through the Snf3/Rgt2-Rgt1 signaling pathway (Johnston and Kim, 2005). This opposing transcriptional regulation of *MTH1* and *STD1* expression provides for rapid induction of *HXT* gene expression in response to glucose and for prompt establishment of the repression of *HXT* gene expression when available glucose has been exhausted (Johnston *et al*., 2006).

1.2. The Main-Glucose Repression Pathway

1.2.1. Mig1 Complex

Not all glucose-repressible genes are repressed in the same way but isolation and characterization of repression and derepression mutants has identified general glucose repression machinery involved in the regulation of expression of a large number of glucose-repressed genes. Its central components are the Mig1 transcriptional repressor complex, the Snf1-protein kinase complex and protein phosphatase 1.

The yeast Mig1 repressor shuttles between the nucleus and cytoplasm in response to glucose (De Vit *et al*., 1997; Ostling& Ronne, 1998; Papamichos-Chronakis *et al*., 2004). It is imported into the nucleus within the minutes after the addition of glucose to the growth medium and is just as rapidly transported back to the cytoplasm when glucose is removed. The Snf1 protein kinase regulates this subcellular relocalization of Mig1. Msn5 mediates nuclear export of Mig1 after its phosphorylation by Snf1 in the nucleus (DeVit & Johnson, 1999). It is believed that Mig1 inhibits transcription by recruiting the general co-repressor complex Cyc8 (Ssn6)-Tup1 (Papamichos-Chronakis *et al*., 2004). It is shown that Cyc8 interact specifically with the non-phosphorylated form of Mig1 and, upon glucose depletion, Snf1-dependent phosphorylation of Mig1 releases its interaction with Cyc8-Tup1. Regulation of Mig1 interaction with Cyc8-Tup1 through Snf1-mediated phosphorylation is a key molecular switch that controls the repression (Papamichos-Chronakis *et al*., 2004). In the high glucose, Mig1 is de-phosphorylated by the Glc7-Reg1 protein phosphatase complex (Alms *et al*., 1999) is located in the nucleus where it can repress the transcription; upon glucose removal, Mig1 is rapidly phosphorylated by Snf1 protein kinase complex and translocated into the cytoplasm (DeVit *et al*., 1997; Ahuatzi *et al*., 2004).

Cyc8-Tup1 plays diverse role in transcriptional regulation. Besides the well established repression activity, which is performed by Tup1p, the Cyc8-Tup1 protein complex can also act as a transcriptional co-activator, and this function is predominantly mediated by the Cyc8 protein. Cyc8-Tup1 activates *CIT2* transcription in response to mitochondrial dysfunction. Transcription of *CIT2* is controlled by Rtg3 and Rtg1, both

members of the bHLH/Zip family of DNA-binding proteins. Recombinant Rtg3 and Rtg1 bind as a heterodimer at two sites within an upstream activation sequence of the *CIT2* gene termed UASr. Heterologous promoters bearing a UASr respond to mitochondrial dysfunction in a Rtg1/Rtg3-dependent manner indicating that UASr is sufficient to mediate *CIT2* regulation (Conlan *et al*., 1998). Also genetic studies have indicated that Rtg2 acts upstream of Rtg1 and Rtg3 in the regulation of *CIT2* (Pray-Grant *et al*., 2002). An other study shows that the intracellular ammonium concentration induces the activity of Rtg2 (Usaite *et al*., 2006). Several lines of evidence suggest that Cyc8-Tup1 is directly involved in the activation of *CIT2* transcription and that this function is performed by the Cyc8 subunit. First, Cyc8-Tup1 specifically associates with the activation domain of Rtg3. This region of Rtg3 has been shown to be the major activation domain of the Rtg1/Rtg3 heterodimer because Rtg1, which does not possess independent transactivation properties, functions to recruit Rtg3 to its binding site. Thus, a possible role of the Rtg1/Rtg3 activation domain is to simply contact the Cyc8- Tup1 complex. Moreover activation of the *CYC1* gene transcription by the Hap1 transactivator and maximal induction of *SUC2* gene both require functional Cyc8 protein (Conlan *et al*., 1998).

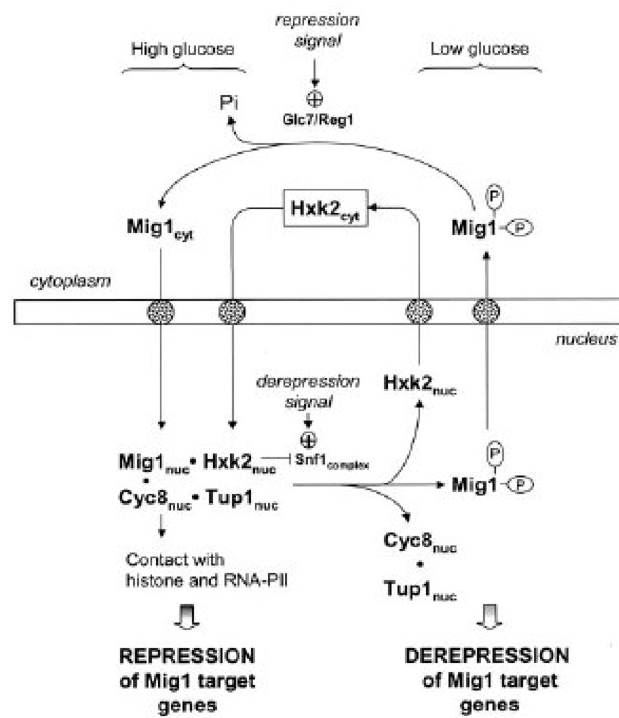


Figure 1.1. Nucleocytoplasmic Translocation of Hxk2 (Ahuatzi *et al*., 2004)

1.2.2. Snf1 Complex

Snf1 kinase is a heterotrimer. It contains catalytic α-subunit Snf1, the α-subunit Snf4 and one the related β-subunits Gal83, Sip1 or Sip2, which regulates the subcellular localization of the kinase complex (Vincent *at al*., 2001). Snf4 is required for the Snf1 kinase activity and, thus, must be considered as a positively acting regulatory subunit of Snf1 (Schüller, 2003). Also two protein kinases, Pak1 and Cka1 are associated with either Snf1 or Snf4. Snf1 kinase requires PAK1 for maximal activity. Pak1 kinase is able to stimulate Snf1 dependent phosphorylation of Mig1 (Nath *et al*., 2003). Pak1p associates with Snf1p in vivo and the association is greatly enhanced under low-glucose conditions when Snf1p is activated (Nath *et al*., 2003). Furthermore, genetic and biochemical studies have shown that Reg1 associated with Snf1 and is phosphorylated in a Snf1-dependent manner (Sanz *et al*., 2000). The Reg1 protein is one of the regulatory subunits of type 1 protein phosphatase Glc7 (Nath *et al*., 2003). The Reg1-Glc7 complex acts in opposition to the Snf1 signaling pathway by promoting the dephosphorylation of Snf1 threonine 210 (McCartney and Schmidt, 2001). On the other hand, it is not yet clear whether glucose can regulate the activity of the Glc7-Reg1 comlex. Expression of *REG1* is not regulated by glucose (Gancedo, 1998). In the presence of low glucose concentration, Pak1 associates with the Snf1 kinase complex and phosphorylates Snf1 on threonine 210. It is found that Pak1 accumulation increases under condition of glucose limitation. However a similar increase in Pak1 mRNA was not detected in microarray experiments (DeRisi *et al*., 1997; Nath *et al*., 2003). This finding suggests that activity of Pak1 may be regulated by a posttranscriptional modification.

In addition to above mechanism for regulation of Snf1 kinase activity, the homologous proteins encoded by the *STD1* and *MTH1* genes have been implicated in regulation of Snf1 function. When cells are grown in high glucose, Std1p is thought to bind glucose sensors, primarily Rgt2p; when cells are grown in low or no glucose, Std1p is released and is free to interact with other proteins. Hence, when expressed from its native promoter, Std1p positively modulates Snf1 kinase activity only when cells are grown in low or no glucose (Kuchin *et al*., 2002).

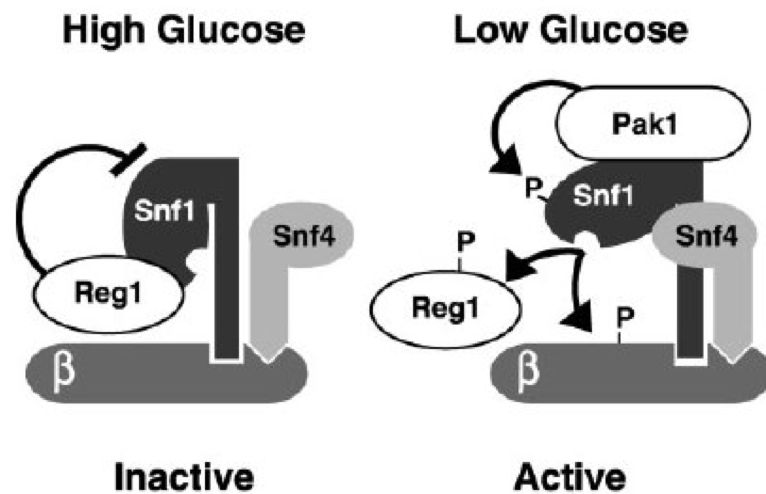


Figure 1.2. Activation and Inactivation of Snf1 Complex (Nath *et al.*, 2003)

Snf1 is also required for coping with a number of stresses unrelated to glucose limitation. Orlova *et al.* found that Snf1 is required for diploid pseudohyphal (PH) differentiation, a filamentous-growth response to nitrogen limitation. The requirement of Snf1 for a nitrogen regulated phenotype suggested a role in nitrogen signaling. Maximal catalytic activation of Snf1 requires phosphorylation of its conserved activation loop Thr210 residue. Orlova *et al.*, presented the evidence that Snf1 is directly involved in nitrogen signiling. First, nonphosphorylable Snf1-T210A does not support PH differentiation. Second, nitrogen limitation leads to improved Thr210 phosphorylation, indicating that Snf1 responds to a nitrogen signal. Furthermore, Thr210 phosphorylation is negatively regulated by the rapamicin-sensitive TOR kinase, which plays essential roles in signaling nitrogen and amino acid availability (Orlova *et al.*, 2006).

1.2.3. Elm1-Pak1-Tos3

Elm1p, Pak1p and Tos3p are a subfamily of yeast kinases that can phosphorylate Thr210 on Snf1p. At least one of these is required for the activation of the Snf1 complex in the glucose limited condition (Sutherland *et al.*; 2003). On the other hand Elm1p has regulatory functions involving cell morphology, filamentous invasive growth that may have no connection to the Snf1 pathway (Hong *et al.*; 2003).

1.2.4. Mig1-Hxk2

Hxk2p is a protein that initiates the intracellular metabolism of glucose by phosphorylation at C-6, but in addition it plays a vital role in glucose repression (Moreno and Herrero *et al.*, 2002). One major function of Hxk2 may be to inhibit Snf1 protein kinase activity by blocking Mig1 phosphorylation at nuclear level. Upon glucose depletion, Snf1 protein kinase is activated (Ahuatzi *et al.*, 2004) and phosphorylates Mig1 (DeVit and Johnson, 1999). Phosphorylation induces Mig1 nuclear export (DeVit *et al.* 1997), sequestering the protein in the cytoplasm together with the Hxk2 (Ahuatzi *et al.*, 2004). Nuclear localization of Hxk2 is regulated by glucose and depends on Mig1. There is a direct correlation between the amount of Hxk2 located in the nucleus and the level of Mig1 in the cell (Ahuatzi *et al.*, 2004). Transcription of *HXK2* is regulated by Rgt1p in the absence of glucose (Palomino *et al.*, 2005).

1.3. Nitrogen Metabolism

In its natural habitat the yeast *Saccharomyces cerevisiae* encounters a wide variety of nitrogen sources. Growth on good nitrogen sources yields relatively higher growth rates than poor nitrogen sources. Good nitrogen sources are ammonia, glutamine and asparagines whereas proline and urea are quantified as poor nitrogen sources. Nitrogen regulation (nitrogen catabolic repression) is the mechanism designed to prevent or reduce the unnecessary divergence of the cell' synthetic capacity to the formation of enzymes and permeases for the utilization of compounds that are non-preferred sources of glutamate and glutamine when a preferred nitrogen source is available (Magasanik and Kaiser, 2002). Yeast cells have to convert a molecule into glutamate and glutamine in order to use the molecule as a nitrogen source. Both glutamate and glutamine can be synthesized directly using ammonia as the ammonia group donor. The NADPH dependent glutamate dehydrogenase (NADPH-GDH), product of *GDH1*, converts ammonia and α-ketoglutarate into glutamate. Glutamine synthetase (GS), product of *GLN1* produces glutamine out of ammonia and glutamate. All nitrogen sources are degraded to finally yield ammonia and α-ketoglutarate by NAD dependent glutamate dehydrogenase (NAD-GDH).

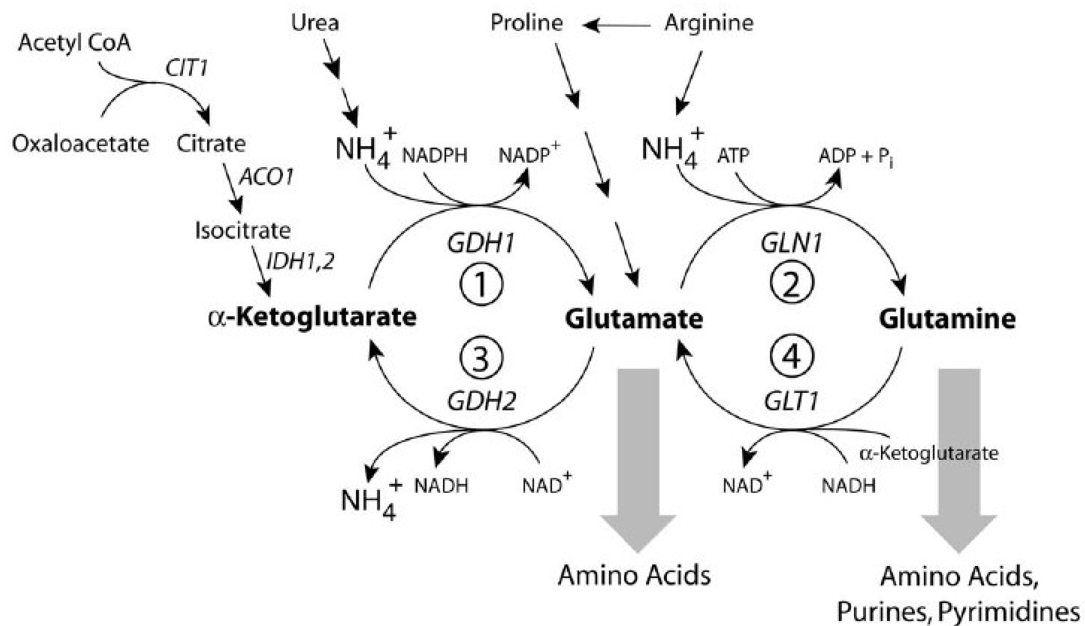


Figure 1.3. Central Pathways for Nitrogen Metabolism (Magasanik and Kaiser, 2002)

During growth on ammonia, ammonia and α-ketoglutarate are converted to glutamate by NADPH-GDH. Isoforms of this enzyme are encoded by *GDH1* and *GDH3*. The transcription of *GDH1* has been shown to be Gln3p dependent. The expression of *GDH1* was decreased with the increasing residual ammonia concentration in the growth medium. *GDH1* expression is also regulated by CCAAT box. The CCAAT box binding factor is required for optimal expression of *GDH1*. The CCAAT box is recognized by the HAP complex. *GDH1* transcription is activated by the HAP complex. (Verrips *et al*., 2000) Hap4p play a role in *GDH1* expression in ethanol grown cultures, but it is not involved in glucose-dependent regulation. Thus, in glucose grown cells, the HAP complex operates in a Hap4p independent manner (Riego *et al*., 2002).

1.3.1. TOR Kinase Pathway

TOR kinase pathway is another process that controls growth and metabolism in response to nutrient availability (Beck and Hall, 1999; Cardenas *et al*., 1999; Schmelzle and Hall, 2000; Crespo *et al*., 2002). This pathway is inhibited by nitrogen starvation. Recently it has been proposed that the TOR kinase pathway regulates the transcriptional

response related to changes in both nitrogen and carbon source availability. For example, the TOR kinase pathway affects the subcellular localization of Msn2, a transcriptional activator of genes regulated by stress, by regulating its interaction with Bmh2, a yeast member of the 14-3-3 protein family (Beck and Hall, 1999). Moreover 14-3-3 proteins have been regarded as positive regulators of the TOR kinase pathway (Crespo *et al*., 2002). One of the roles of the TOR kinase pathway is the regulation of phosphatase activity. Type 2A protein phosphatase catalytic subunits Pph21 and Pph22 and type 2A related phosphatase Sit4 are components of the TOR signaling pathway. Activation of the TOR kinases leads to the inhibition of the corresponding phosphatase, whereas inactivation of TOR kinase pathway results in its activation (Scmelzle and Hall, 2000; Crespo *et al*., 2002; Cooper, 2002).

TOR kinase pathway also plays role in the process of induction of *HXT1* by glucose. Glucose activates the TOR kinase pathway, allowing glucose induction. It is suggested that in the absence of a proper TOR kinase pathway, the Cdc55-PP2A phosphatase would be active and this might interfere with Yck1/2 activity or could dephosphorylate Std1 and Mth1, in this way preventing *HXT1* glucose induction. 14-3-3 proteins can also play a role in the initial steps of *HXT1* glucose induction, perhaps assisting SCF-Grr1 in recognizing its corresponding phosphorylated substrates (Tomas-Cobos *et al.*, 2005).

1.4. Glycerol Production

During respiratory glucose dissimilation, eukaryotes produce cytosolic NADH via glycolysis. This NADH has to be reoxidized outside the mitochondria, because the mitochondrial inner membrane is impermeable to NADH. In *Saccharomyces cerevisiae*, this may involve external NADH dehydrogenase (Nde1p or Nde2p) and/or a glycerol-3-phosphate shuttle consisting of soluble (Gpd1p or Gpd2p) and membrane –bound (Gut2p) glycerol-3-phosphate dehydrogenases (Overkamp *et al*., 2000). Synthesis of one mole of glycerol, the second major by-product of glucose fermenting cells of *S. cerevisiae*, results in consumption of one mole of NADH while other by-products like acetate lead to the production of cytosolic NADH. However, the largest part of excess cytosolic NADH formation is connected to biomass formation (Rigoulet *et al*., 2004). In anaerobic condition, the additional NADH originating from biomass production can be reoxidized via

glycerol production. Under aerobic conditions, glycerol production is not necessary, because the reoxidation of cytosolic NADH can be coupled to the mitochondrial respiratory chain (Pronk *et al.*, 2000). It seems clear that the external NADH dehydrogenase and the glycerol-3-phosphate shuttle are the most important systems maintaining a cytosolic redox balance during aerobic growth of *S. cerevisiae*. High glucose concentration will render the glycerol-3-phosphate shuttle inactive since one of its components, the Gut2p, is subjected to glucose repression. To large extent cytosolic redox balance is restored by ethanol and glycerol formation under such condition (Rigoulet *et al.*, 2004). Respiration is only partly repressed by high glucose concentration. Glycerol production often serves a redox sink, when excess NADH formed in anabolic pathways cannot be regenerated by respiratory chain at a sufficient high rate (van Maris *et al.*, 2001)

Glycerol is quantitatively the most important fermentation product after ethanol and carbon dioxide. It is also involved in osmotic cell regulation. During growth under osmotic stress conditions glycerol is formed and accumulates inside the cell where it works as an efficient osmolyte that protects the cell against lysis (Nissen *et al.*, 1999).

1.5. Regulation of Respiration

The yeast *Saccharomyces 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 cell has to reprogram its metabolism, a phase called “diauxic shift”. This reprogramming is under the control of the *HAP* complex. The *HAP* complex is a heteromeric transcriptional regulator composed of four proteins. *HAP2, HAP3* and *HAP5* associate to form the DNA-binding part, while *HAP4* contains the activation domain (Buschlen *et al.*, 2003).

Expression of *HAP4* is repressed to a low level in the presence of glucose and induced when only non-fermentable carbon sources are available, while *HAP2* and *HAP3* are expressed constitutively. This suggests that *HAP4* is necessary for activity of the Hap2/3/4/5 complex (van Maris *et al.*, 2001). The *HAP* complex controls the complete TCA cycle and related pathways. All subunits of the respiratory chain complex III possess

the CCAAT binding site required for *HAP4* and their expressions are reduced even down to 29 per cent in the absence of this gene (Buschlen *et al*., 2003). The *HAP* complex is originally identified as up-regulating the expression of cytochrome c and later on of several genes encoding TCA cycle and respiratory chain enzymes (e.g. Qcr8p and Cyt1p of the bc_1 complex). The elevated level of Hap4p in glucose-containing medium leads to derepression of the transcription of *QCR8* (Blom *et al*., 2000). The expressions of several hundred genes are controlled directly or indirectly by the *HAP* complex (Buschlen *et al*., 2003). Presence of a fermentable substrate like glucose inhibits the expression of *HAP4* via the Mig1 pathway, and hereby the activation of respiration is prevented at high glucose concentrations (Raghevendran *et al*., 2006). Even though the name of the gene suggest that Hap4 is activated by heme neither heme nor the oxygen regulation of the Hap4 complex is clearly understood. Tai *et al.* reported that *HAP4* mRNA is present in carbon-limited cultivations even under anaerobic conditions where Hap4 has no obvious role.

Hap4 play a role in activating respiration only for specific growth rates between 0.08 and 0.3 h^{-1}. Hap4 is not essential for complete on/off expression of tricarboxylic acid cycle genes, as deletion of *HAP4* does not result in complete shutting down the expression of tricarboxylic acid cycle and respiration genes. Moreover, deletion of *HAP4* has little or no effect on the specific growth rate, compared with the wild type both aerobically as well as anaerobically (Raghevendran *et al*., 2006).

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). *GDH1* transcription is activated by the *HAP* complex. (Verrips *et al*., 2000) Hap4p play a role in *GDH1* expression in ethanol grown cultures, but it is not involved in glucose-dependent regulation. Thus, in glucose grown cells, the *HAP* complex operates in a Hap4p independent manner (Riego *et al*., 2002).

The *HAP* complex interacts with *STD1;* a protein kinase activator, *TUP1*; a stress response and DNA damage regulator, *SSN6*; repressor of *SUC2* at high glucose levels, LYS14; a lysine pathway transcriptional activator, *MTH1*; a negative regulator of *HXT* gene expression, *SNF3*; the sensor of low external glucose concentrations, *RGT1*; a

transcriptional activator, *RGT2*; the sensor of high external glucose concentrations and *SUC2*; which is involved in molecular hydrolysis.

1.6. Regulation of Mitochondrial Respiration

The mitochondrial respiratory chain consists of multisubunit enzyme complexes that are embedded in the inner mitochondrial membrane. Electron transport through the ubiquinol-cytochrome c reductase and cytochrome oxidase complexes in *S. cerevisiae* is coupled to vectorial H^+ translocation into the intermembrane space, resulting in the establishment of a H+ gradient and subsequent membrane potential. The energy from this gradient is then used as the driving force for ion translocation, protein import into mitochondria, and ATP synthesis, which is catalyzed by ATPases (Malaney *et al*., 1997).

The mitochondrial electron transport chain complex is composed of four complexes, complex I (NADH-ubiquinone oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinone-cytochrome *c* reductase or cytochrome *bc*1 complex), and complex IV (cytochrome *c* oxidase). In contrast to most eukaryotes, the yeast *Saccharomyces cerevisiae* does not possess a complex I but rather contains two NADH dehydrogenases associated with the inner membrane (Stuart *et al*., 2000).

The mitochondrial cytochrome bc1 complex, a multisubunit membrane protein, is one of the fundamental components of the respiratory chain. It catalyzes electron transfer from ubiquinol to cytochrome c, while the process is coupled to electrogenic translocation of protons across inner mitochondrial membrane. Cytochrome bc1 complex is composed of 10 proteins in *S. cerevisiae*. It accepts electrons from Rieske iron-sulfur protein and transfer electrons to cytochrome c.

The catalytic subunits of bc1 complex are *COB, CYT1* and *RIP1*. These proteins, together with the other non-catalytic subunits (*QCR1, QCR2, QCR6, QCR7, QCR8, QCR9, QCR10*), assemble to form an enzymatically active complex. The analysis of the steady state levels of these subunits has suggested that the assembly pathway of this complex occurs in a coordinated fashion, involving the formation of specific assembly intermediates. According to this model, cytochrome b initially forms a subcomplex with

QCR7 and QCR 8, which subsequently joins with the *QCR1* and *QCR2* proteins. Cytochrome c1, on the other hand, is proposed to form another subcomplex with *QCR6* and *QCR9*. Formation of each of these subcomplexes ensures stability against proteolytic attack for the individual subunits contained within them. The cytochrome b and cytochrome c1 subcomplexes subsequently unite to form a 'cytochrome bc1 precomplex' prior to the assembly of the Rieske FeS protein and, presumably, the non-essential subunit *QCR10*. (Stuart *et al*., 1999).

QCR7 is the ubiquinol-cytochrome-c reductase subunit 7. It forms the core subcomplex together with QCR8 and cytochrome c. It is an essential component of the complex and its absence causes complete respiratory deficiency. *QCR7* forms complex III with *QCR6, 8, 9, 10, RIP1, COR1, COR2, CYT1 and COB*. Transcription of *QCR8* (11 kDa) needs HAP2/3/4 complex for rapid induction during transition from repressed to derepressed conditions. *ABF1* may act in coordination with *HAP2/3/4* complex while *CPF1* is a negative regulator modulating the induction response. *QCR8* also has an upstream binding site for *MIG1* (Güldener *et al*., 2005).

RIP1 is the ubiquinol--cytochrome-c reductase iron-sulfur protein precursor. It is involved in electron transport and membrane associated energy conservation. Its disruption causes complete respiratory deficiency. It is located in the respiratory chain complex III as *QCR7* and it interacts with the RNA export mediators *GLE1* and *GLE2* other than its role in complex III (Güldener *et al*., 2005)

1.7. The Aim of This Thesis

This study aimed to investigate the effect of nitrogen limitation on glucose sensing and signaling mechanism in yeast and identify whether the signature of post- translational events can be observed in the transcription of the genes encoding those proteins.

In order to achieve this goal, chemostat cultivations with pulse injections with *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ, hap4Δ/hap4Δ, rip1 Δ/rip1 Δ and RIP1/rip1Δ)* were performed. Metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained as well as the growth curves. Expression profiles of *CYC8, GRR1, MTH1, RGT1, RGT2,*

SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4, MBA1 genes were determined under nitrogen limited condition via reverse transcription real time quantitative polymerase chain reaction (real time RT-qPCR).

The experimental methods pursued as well as the materials are explained in detail in the Materials and Methods section. Results section covers all experimental findings that are obtained. The comprehensive argument of the obtained results is given in Discussion. The study is summarized with important key points in the Conclusions and Recommendation section. Some new techniques and additional work to improve the study is suggested also in the same section.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Strains

In this study three homozygous deletion mutants of BY4743 namely; *hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ*, and one heterozygous deletion mutant of BY4743 namely; *RIP1/rip1Δ* were used. Prof. Stephen G. Oliver (Faculty of Life Sciences, University of Manchester) kindly provided these strains. The deletion mutants were generated by European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF).

2.1.2. Chemicals and Disposable Materials

<u>2.1.2.1. Media Used</u> Nitrogen limited F1 medium were used as culture media in the experiments. Complex medium (YPD) in liquid forms was used as preculture. The compositions were as follows in (v/v) for liquids:

- F1

D-Glucose 2 per cent (Merck), $(NH_4)_2SO_4$ 0.046 per cent (Merck), KH_2PO_4 0.2 per cent (Merck), $MgSO_4.7H_2O$ 0.055 per cent (Merck), NaCl 0.01 per cent (Merck), $CaCl_2.2H_2O$ 0.009 per cent (Merck), Uracil 0.002 per cent (Fluka), Histidine 0.002 per cent (Lifco), Leucine 0.01 per cent (Merck), Trace Element Solution 1 0.01 per cent, Trace Element Solution 2 0.01 per cent, Vitamin Stock Solution 0.17 per cent.

- Trace Element Solution 1

$ZnSO_4.7H_2O$ 0.07 per cent (Merck), $CuSO_4.5H_2O$ 0.01 per cent (Merck), H_3BO_3 0.01 per cent (Merck), KI 0.01 per cent (Merck)

- Trace Element Solution 2

$FeCl_3.6H_2O$ 0.05 per cent (BDH)

- Vitamin Stock Solution

Inositol 3.72 per cent (Merck), Thiamine / HCl 0.84 per cent (Sigma), Pyridoxine 0.24 per cent (Fluka), Ca-panthothenate 2.4 per cent (Fluka), Biotin 0.018 per cent (Merck)

- YPD

Yeast Extract 1 per cent (Lab M), Bacteriological Peptone 2 per cent (Acumedia), D-Glucose 2 per cent (Merck) – for solid media Agar-Agar 1.8 per cent (Merck). Glucose was added from previously prepared stocks after sterilization of the remaining of the media.

2.1.2.2. Kits For determination of ammonia, D-glucose, ethanol and glycerol concentrations, enzymatic kits which were purchased from BOEHRINGER MANNHEIM – Roche.

For RNA extraction RNeasy MiniKit (250) and for reverse transcription real time quantification polymerase chain reaction (one step procedure) QuantiTect SYBR Green RT-PCR Kit which were purchased from Quiagen were used.

2.1.2.3. Buffers and Chemicals Used for Polymerase Chain Reaction Applications All primers were provided from Integrated DNA Technologies. Promega supplied Inc. Hind III, Buffer E, Taq Polymerase, $MgCl_2$ and Mg Free PCR Buffer and Biorad supplied dNTP mix.

2.1.2.4. Miscellaneous RNAlater – RNA Stabilization Reagent (Quiagen), RNase Away (Invitrogen), Absolute Ethanol (Sigma), DEPC (Sigma), Glycerol (Merck), Nitric Acid (Merck), were the other chemicals used in various processes and applications.

- Disposable plasticware as well as the PCR tubes were supplied by USA Scientific Inc.
- iCycler 96 well PCR Plates and Optical Tapes were purchased from BIORAD.
- Millex Sterile filter units (0.22 μm) were provided from Millipore
- Sartopore Sterile filter units (0.45+0.2 μm) were purchased from Sartorius.

2.1.3. 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)
Scientifca ARE, VELP (Italy)
- Incubators — NÜVE EN500 (Turkey)
- Laminar Flow Cabinet — HBB 2460 LaminAir, Holten (Denmark)
- O_2 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 — BIORAD iCycler (USA)
- Transilluminators — Reprostar II, CAMAG (Switzerland)
Foto/uv 15, Fptodyne (USA)
- Vortex — Elektromag (Turkey)
- Water Baths — HETO, CB 8-30e AT_{110} (Denmark)
HETO, CB 8-30e DT_1 (Denmark)
HETO DT Hetotherm (Denmark)
- Water Purification Systems — Millipore, Milli Ro Plus (USA)
Millipore, Milli-Q UF Plus (USA)

2.2. Methods

2.2.1. Experimental Methods

2.1.1.1 Sterilization In order to prevent contamination equipments and medium used in the experiments were sterilized. Steam and filter sterilization were preferred. The steam sterilization was performed in an autoclave at 15 psig pressure at 121°C.

The medium were sterilized by filter sterilization using 0.45 (+0.2) µm sterile Sartorious disposable filter. The filters were placed at the entrance of the 20L carboys from where the medium was fed into the fermenters. The medium pumped into the carboys by filter sterilizing.

All glassware, plasticware, fermenters and their complementary parts which were used in chemostat that were to be used were steam sterilized for 15 minutes. The sterilization time of glucose was limited to 3 minutes. It was an exception because of its highly susceptible nature to caramelization at elevated temperatures.

2.2.1.2. Cultivation Conditions All precultures were prepared as 100 ml of YPD medium. They were harvested with a single colony of cells from frozen stocks. 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 after overnight batch cultivation.

Chemostat cultivations were carried out in BIOFLO 3000 fermenters. The working volume was 2 L of F1 medium. The temperature was kept constant at 30°C via PID controllers and the agitation was set to 800 rpm. The pH is kept at 5.5 via automatic addition of 1M NaOH and HCL. 20 ml of preculture was used to inoculate the culture. The dilution rate was set to 0.1 hr^{-1}. Cultures were flushed with air at a flow rate of 1 L/min using blower. O_2 probe was used to monitor the dissolved O_2 concentration.

2.2.1.3. Sampling and Storage In all experiments same sampling procedure was applied., Until the steady state was reached, optical densities were monitored. After the cells spent three residence time at steady state, pulse injections of ammonium sulfate were injected in order to reserve the system to concentrations of no limitation. Prior to the pulse injection, samples for RNA extraction purpose and extracellular metabolites were collected. Four samples were taken for RNA extraction purposes in 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 hourly sampling was applied for ten hours. Sampling for extracellular metabolites was carried out on hourly basis for the ten hours after the injection. The last samples were collected after the cells spent three residence time at the second steady state. Duration of the sampling after the pulse injection were 10 hours because at the end of ten hours more than % 95 of the fermentor volume was replaced with fresh feed. 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 stored at -20°C after it was transferred to a new Eppendorf

tube. Prior to enzymatic analyses, they were incubated at 80°C for 10 minutes to stop any possible enzymatic activity that would have remained.

Samples were shock frozen in liquid nitrogen then they were transferred to -80 °C for RNA extraction. They were kept at -80°C until use.

2.2.1.4. Enzymatic Analyses for Determination of Metabolite Concentrations

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

- Enzymatic Analysis for 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).

$$D-Glucose + ATP \xrightarrow{HK} G6P + ADP \quad (2.1)$$

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

$$G6P + NADP^{+} \xrightarrow{G6P-DH} D-gluconate-6-phosphate + NADPH + H^{+} \quad (2.2)$$

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 constitute the test combination.

0.5 ml of solution I, 0.05 ml sample solution and 0.95 ml redistilled water were pipetted in a cuvette. In another cuvette, 0.5 ml of solution I and 1.0 ml redistilled water were pipetted which was used as blank. 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 $\Delta A_{D\text{-glucose}}$. The concentration of D-glucose was calculated by the following equation:

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

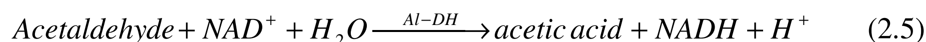
where ε was the extinction coefficient of NADH at 340 nm = 6.3 [L/(mmol x cm)] and d is the dilution factor of the sample.

- Enzymatic Analysis for Determination of Ethanol Concentration

In the presence of the enzyme alcohol dehydrogenase (ADH), ethanol is oxidized to acetaldehyde by nicodinamide-adenine dinucleotide (NAD).

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{acetaldehyde} + \text{NADH} + \text{H}^+ \qquad (2.4)$$

In the presence of aldehyde dehydrogenase (Al-DH), acetaldehyde is oxidized quantitatively to acetic acid.



$$Acetaldehyde + NAD^+ + H_2O \xrightarrow{Al-DH} acetic\ acid + NADH + H^+ \qquad (2.5)$$

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

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

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

$$c_{Ethanol} = \frac{d \times 0.7256}{\varepsilon} \Delta A_{Ethanol} (g/L) \qquad (2.6)$$

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

- Enzymatic Analysis for Determination of Ammonia Concentration

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

$$2 - Oxoglutarate + NADH + NH_4^+ \xrightarrow{GIDH} L - glutamate + NAD^+ + H_2O \qquad (2.7)$$

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

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

0.5 ml of mixture I, 0.05 ml sample and 0.95 ml distilled water were pipetted in a cuvette. In another cuvette, 0.5 ml mixture I and 1 ml distilled water were pipetted in order to use as blank. The absorbencies (A_1) were read at 340 nm against air after 5 minutes.

Then 0.01 ml of Solution II was added. The absorbences (A_2) were read again 20 minutes after the addition.

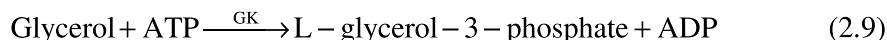
Absorbance difference of the blank (A_1-A_2) was subtracted from the absorbance difference of the samples (A_1-A_2), thereby obtaining $\Delta A_{Ammonia}$. The concentration of the ammonia was calculated by the following equation:

$$c_{Ammonia} = \frac{d \times 0.5143}{\varepsilon} \Delta A_{Ammonia} (g/L) \tag{2.8}$$

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

- Enzymatic Analysis for Determination of Glycerol Concentration

In the reaction catalysed by glycerokinase (GK), glycerol is phosphorylated by adenosine-5'-diphosphate (ATP) to L-glycerol-3-phosphate.



$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{L} - \text{glycerol} - 3 - \text{phosphate} + \text{ADP} \tag{2.9}$$

The adenosine-5'-diphophate (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.

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate} \tag{2.10}$$

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.

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L} - \text{lactate} + \text{NAD}^+ \tag{2.11}$$

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 constitute the test combination.

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 a cuvette,. In another cuvette, 0.5 ml of solution I, 1.0 ml redistilled water and 0.005 ml solution II were pipetted in order to use as blank. 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. Absorbences (A_2) were read again at the same conditions both for sample and for the blank after about 5 to 10 minutes.

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

$$c_{Glycerol} = \frac{2.781}{\varepsilon} \Delta A_{Glycerol} (g/L) \tag{2.12}$$

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

2.2.1.5. RNA Extraction Any work involving RNA was performed in RNAse and DNAse free environments. 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 µl DEPC (diethylpolycarbonate) was used per 1 ml of water. It was stored overnight at 37°C and autoclaved.

The RNA was extracted from samples using “RNeasy protocol for extracting yeast via mechanical disruption” as described by the manufacturer (Quiagen, USA). In the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivated RNases to ensure isolation of intact RNA; samples were first lysed and homogenized. To provide appropriate binding conditions ethanol was added. 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 µl, 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. RNeasy mini spin columns, 2 ml and 1.5 ml collection tubes, Buffer RTL, Buffer RPE, Buffer RW1 and RNase free water constitute the kit content.

RNeasy protocols 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 to lyse the cells and release the RNA. In the protocol the first step 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. All steps of the RNeasy protocol were performed at room temperature after disruption in a bead mill homogenizer,. 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. Approximately 600 µl 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 µl 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

2.2.1.6. Reverse Transcription Real Time Quantification Polymerase Chain Reaction (RT-rtqPCR) The criteria for designing the primers required to quantitatively amplify the products of HO, HSP12, *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4, MBA1* genes were as follows:

The primer length was selected to be 18-24 nucleotides. The length of the PCR product (amplicon) was 100-150 base pairs or 200-250 base pairs. The GC content of the primers ranged between 50 per cent and 60 per cent. Since the 3' ends (where amplification starts) should be free of secondary and repetitive sequences and 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 was avoided. G's and C's did not placed on ends of the primer. Most primers had melting temperatures between 50°C and 65°C where 55°C is considered to be the best. Since the primers that were used together should have similar T_m values, close melting temperature tried to be selected.

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
RGT2_F	5-TTGGTGTTTCCGAGGGAA-3

Table 2.1. RT-rtqPCR primer sequences (Continues)

RGT2_R	5-AGTGGGTACAGTTCAGCA-3
SNF3_F	5-TGTGTCACCTTCTCTGCA-3
SNF3_R	5-TGAGTCGCTTGAGAGTGA-3
MTH1_F	5-TTATTGGCCTCCTCACCTA-3
MTH1_R	5-ATGCTTCATCCAGGCTCA-3
STD1_F	5-TAAATACCCCGGACAGTCA-3
STD1_R	5-TGCTGGATCAGACATCAGA-3
YCK1_F	5-TCAAGGCTTACCTGTGCA-3
YCK1_R	5-TGGATTTGGATGGCCGTA-3
YCK2_F	5-ACTCCTGATTATGAGGGCTA-3
YCK2_R	5-ATGGTGATGATCGGGTGA-3
SNF1_F	5-TTATAGAGTACGCCGGGAA-3
SNF1_R	5-AGCCGCATAATTGGGAGA-3
SNF4_F	5-AAATGACCACTCCGGTCA -3
SNF4_R	5-TCAAGCCTAGGACATCGTA-3
MIG1_F	5-AACGCGTTATCGTCCCTA-3
MIG1_R	5-TGTGCTCTGAGACTTCCA-3
CYC8_F	5-TACATGGTACCATCTCGGTA-3
CYC8_R	5-AGGCGTCTCTGTATTGAGA-3
TUP1_F	5-ACAGGTGCTGAAGACAGA-3
TUP1_R	5-ATACGAACGGTACGGTCA-3
GRR1_F	5-TCGGCTGTTTTGAGAGGA-3
GRR1_R	5-ACATTCCTTGCCTGAGGA-3
SKP1_F	5-AGACTCGGAAACGAACCA-3
SKP1_R	5-TCGTCGTCATCTTCGTCA-3
RGT1_F	5-TTATTGCCCCCACTGACA-3
RGT1_R	5-TGGTAGGGCACTTTCCAA-3
GLC7_F	5-ATATGTCGAGATAGCCGAGA-3
GLC7_R	5-ATGCCTTCTGCAAATCCA-3
REG1_F	5-AGAAGAGAACCGTCACAGA-3

Table 2.1. RT-rtqPCR primer sequences (Continues)

REG1_R	5-TTCTCCTACAGGATTCGGTA-3
HXK2_F	5-AGGACATTTACGGCTGGA-3
HXK2_R	5-ACGGACTTACCTTCAGCA-3
TOS3_F	5-AATGACCCTGAGAGCACA -3
TOS3_R	5-TGCGTTATCCCTTGGGAA-3
PAK1_F	5-AATCAGGCACCAGTAGCA-3
PAK1_R	5-TTAGAACTCCCGCTCGAA-3
ELM1_F	5-ACTGACAGAAGCGAGTCA-3
ELM1_R	5-ACTCGGTTTGTCCTGTGA-3
HAP4_F	5-CACCATGACGAGTTAGGTTCAG-3
HAP4_R	5- GGTGGCAGTTGCATCATTGTTG-3
MBA1_F	5-ACTAGTCTCAGTGCAACCA-3
MBA1_R	5-TCCTCGTAGCTTTTCGGTA-3

Quiagen QuantiTect® SYBR® Green RT-PCR kit was used for quantitative, real-time, one-step RT-PCR for RT-rtqPCR applications within this study. 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 $MgCl_2$, QuantiTect RT Mix; containing Omniscript® Reverse Transcriptase and Sensiscript® Reverse Transcriptase and RNase-free water constitute the kit content.

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 was given previously by Dikicioğlu (2005). The reaction program consists of 6 cycles. In the first cycle the temperature was 50 ^{o}C and the duration was 30 minutes whereas in the second cycle the temperature was 95 ^{o}C and the duration was 15 minutes. The third cycle consists of 3 steps which are repeated 40 times. First step is at 94°C for 15 seconds, second step is at 52.2^{o}C for 15 seconds and the third step is at 72°C for 30 seconds. The duration of fourth and fifth steps are 1 minute at 95°C and 1 minute at 50°C, respectively. The last sixth cycle is repeated 80 times and the temperature is 48°C and ramps 0.5^{o}C at every 10 seconds.

2.2.1.7. Relative Quantification of Gene Expression Cycle florescence (CF) in relative fluorescence units (RFU) of the amplified regions where SYBR Green intercalated were given as the experimental output files of the BioRad software. The reference gene in expression calculations was chosen as the housekeeping gene *HO*. 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. The quantification ratio of the PCR amplification was considered to be 2^{N}.

3. RESULTS

In order to identify the variations in the expression levels of genes involved in glucose sensing, signal transduction and glucose repression pathways as a response to system level perturbations under nitrogen limited condition four sets of chemostat experiments with *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1 Δ/rip1 Δ and RIP1/rip1Δ)* 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 *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4, MBA1* genes were determined under nitrogen limited condition via reverse transcription real time quantitative polymerase chain reaction (real time RT-qPCR).

3.1. Growth Characteristics of Deletion Strains

External metabolite profiles and growth characteristics were monitored in normal, respiratory deficient and partial respiratory deficient strains of *S. Cerevisiae. S. cerevisiae BY4743* (*hoΔ/hoΔ*) was used as wild type whereas the other mutants were reported to have an increased ethanol production when compared to wild type (Pir *et al.*, 2006; Duygu Dikicioğlu, 2005). The cells were grown in nitrogen limited F1 medium in which it was expected that cells prefer fermentation instead of respiration. The optimal growth conditions for *S. Cerevisiae* were used in the experiments with temperature kept at 30°C and the pH controlled within the range of 5.5.

Steady state samples for extracellular metabolites were collected after passing three residence times at the steady states prior to and after the pulse injection. Samples were taken on hourly bases and growth characteristics of the cultures were followed for ten hours after the injection of nitrogen pulse. Arrows indicate the time points of pulse injections.

3.1.1. Growth Characteristics *S. cerevisiae BY4743 (hoΔ/hoΔ)* under Nitrogen Limitation

S. cerevisiae BY4743 (*hoΔ/hoΔ*) strain was cultivated as described in the Materials and Methods section. This strain was used as a control. Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*hoΔ/ho1Δ)* in nitrogen limited chemostat culture were presented in Figures 3.1 a and b.

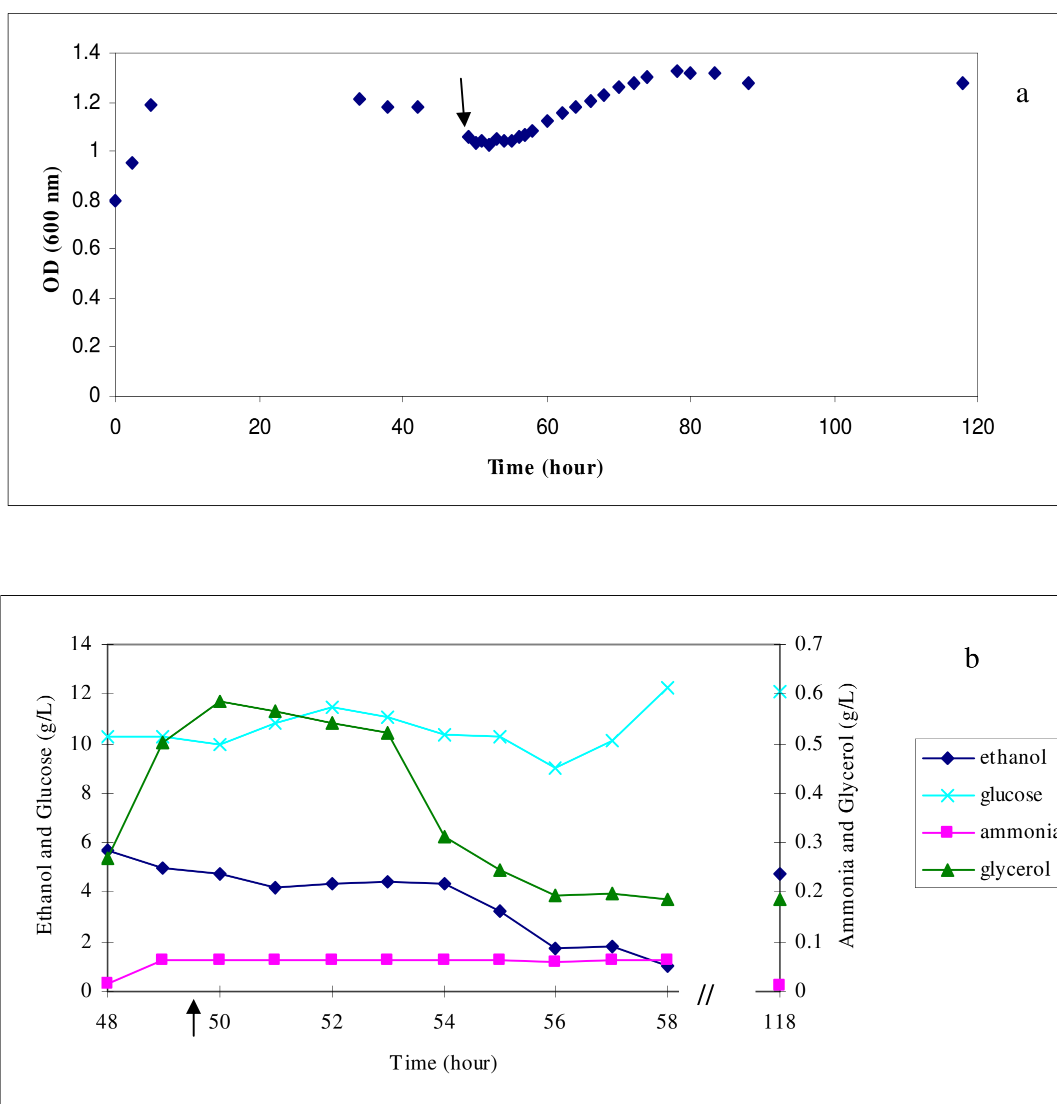


Figure 3.1. Growth curve (a) and extracellular metabolite profiles (b) of *S. Cerevisiae* BY4743 *(hoΔ/hoΔ)* under nitrogen limited condition in chemostat culture with pulse injection

After inoculation of the cell, the culture was left to operate batch wise overnight. The first steady state was reached within 20 hours after switching to chemostat. Nitrogen pulse was injected after passing three residence times at steady state at 49th hour of the chemostat cultivation. As it can be seen in Figure 3.1a, after the nitrogen pulse injection a decrease in the growth of the cells was observed. The OD remained constant for ten hours. Then the OD increased for 14 hours and reached the second steady state at a higher OD value then the first steady state.

The glucose concentration was nearly constant in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* during the cultivation which was measured 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 injection 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.

3.1.2. Growth Characteristics *S. cerevisiae BY4743 (hap4Δ/hap4Δ)* under Nitrogen Limitation

A homozygous deletion strain of *HAP4* (*hap4Δ/hap4Δ*) in a genetic background of wild type strain of S. cerevisiae BY4743 was cultivated under nitrogen limited conditions. Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* before and after the pulse injections in nitrogen limited chemostat culture were presented in Figures 3.2 a and b.

The culture was left to operate batchwise overnight after inoculation of the cell. After switching to chemostat, the OD value remained constant. At the end of the three residence time ammonium sulfate pulse was injected. After the nitrogen pulse injection a slight increase in the growth of the cells was observed for 27 hours. Then the OD decreased to the value of the first steady state. The experiment was terminated after three residences time in steady state.

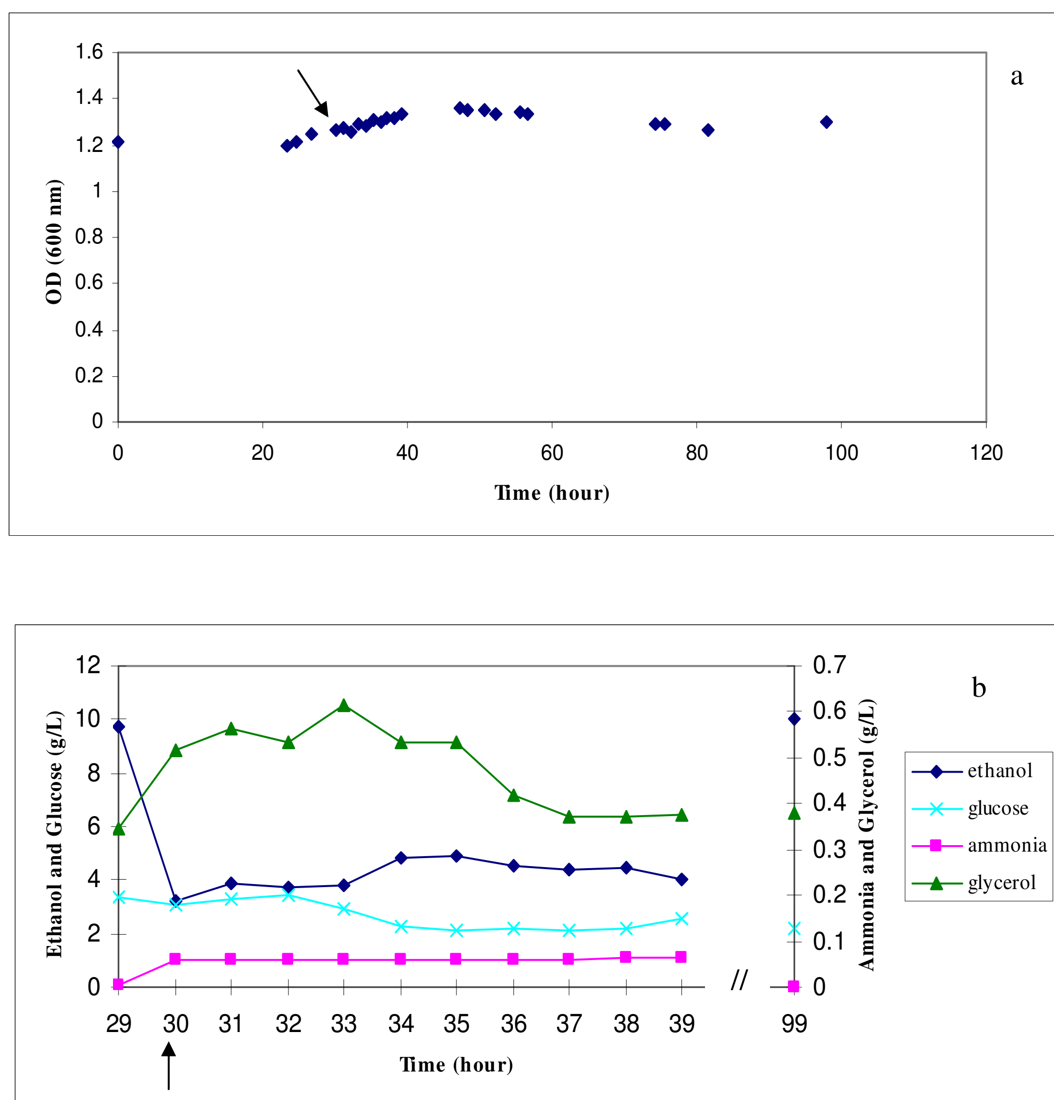


Figure 3.2. Growth curve (a) and extracellular metabolite profiles (b) of *S. cerevisiae* BY4743 *(hap4Δ/hap4Δ)* under nitrogen limited condition in chemostat culture with pulse injection

Prior to pulse injection, the glucose concentration was approximately 3.3 g/L in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ).* After the injection of nitrogen pulse, the glucose concentration did not change for three hours, and then it slightly decreased to 2.2 g/L during the cultivation. Also at the second steady the glucose concentration remained constant approximately at 2 g/L. Ethanol concentration showed a sharp decrease after the pulse injection. Its concentration was 9.7 g/L before the pulse and decreased with time to a value of 3 g/L in an hour. Within the ten hours following the pulse injection, the ethanol

concentration remained almost constant between 3 and 4 g/L. At the second steady state ethanol concentration was measured to be 10g/L. Ammonia concentration was increased from 0.006 g/L to 0.06 g/L with the pulse injection and remained constant for at least 9 hours. At the second steady state ammonia concentration decreased to 0.0002 g/L. Glycerol concentration first increased with the pulse then it decreased and remained constant at the level of 0.35 g/L.

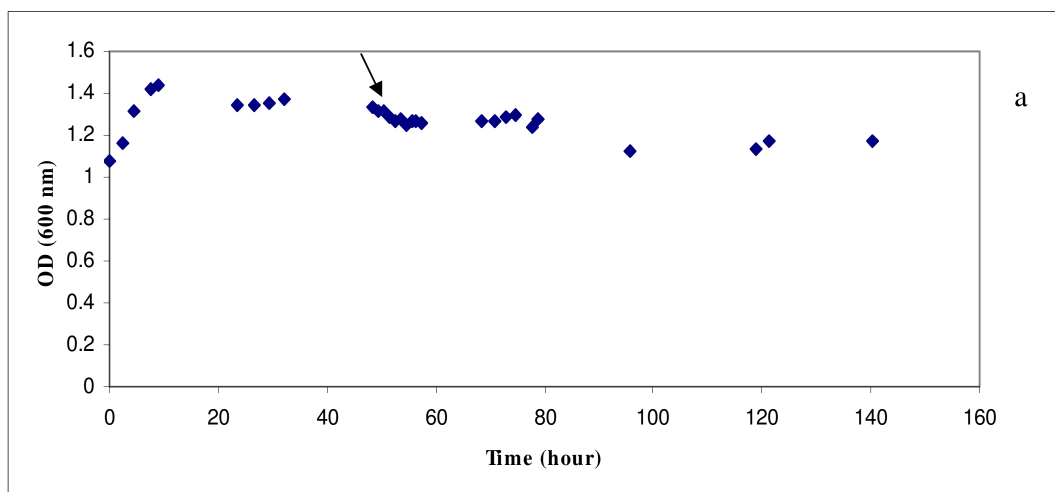
3.1.3. Growth Characteristics *S. cerevisiae BY4743 (rip1Δ/rip1Δ)* under Nitrogen Limitation

A homozygous deletion strain of *RIP1* (*rip1Δ/rip1Δ*) in a genetic background of wild type strain of S. cerevisiae BY4743 was cultivated under nitrogen limited conditions. Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* in nitrogen limited chemostat culture were presented in Figures 3.3 a and b.

After switching to chemostat following the overnight batch cultivation, OD increased for 9 hours. Then it slightly decreased and reached steady state at 19th hour of the chemostat. At the end of the three residence time ammonium sulfate pulse was injected. After the nitrogen pulse injection a slight decrease in the growth of the cells was observed for 3 hours. Then the OD remained constant for 26 hours. Then the OD decreased and cell growth reached a steady state at a lower OD value then the first steady state. After passing three residences time the experiment was terminated.

Prior to pulse injection, the glucose concentration was approximately 4 g/L in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ).* After the injection of nitrogen pulse, the glucose concentration slightly increased and remained constant approximately at 5 g/L during the cultivation. At the second steady the glucose concentration increased to 6 g/L. Ethanol concentration was approximately 6 g/L prior to the pulse injection. Ethanol concentration changed between 4.5 and 5.5 g/L within the ten hours after the pulse injection. At the second steady state it was 4.5 g/L. Ammonia concentration was increased from 0.001 g/L to 0.06 g/L with the pulse and remained constant for 9 hours. At the second steady state ammonia concentration decreased to 0.01 g/L. After the injection of nitrogen pulse,

glycerol concentration decreased. At the both steady state, the glycerol concentration was 0.09 g/L.



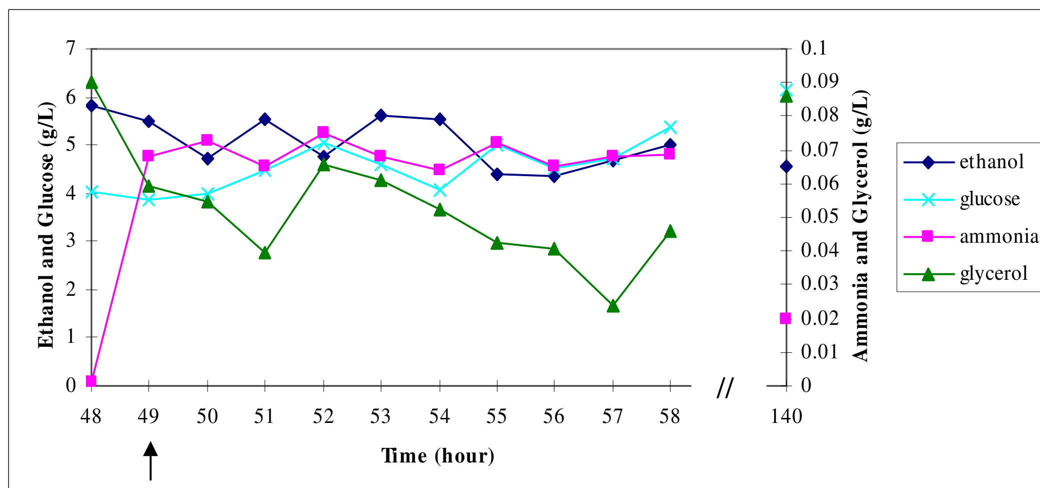


Figure 3.3. Growth curve (a) and extracellular metabolite profiles (b) of *S. cerevisiae* BY4743 *(rip1Δ/rip1Δ)* under nitrogen limited condition in chemostat culture with pulse injection

3.1.4. Growth Characteristics *S. cerevisiae BY4743 (RIP1/rip1Δ)* under Nitrogen Limitation

A heterozygous deletion strain of *RIP1* (*RIP1/rip1Δ*) in a genetic background of wild type strain of S. cerevisiae BY4743 was cultivated under nitrogen limited conditions.

Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* before and after the pulse injection in nitrogen limited chemostat culture were presented in Figures 3.4 a and b.

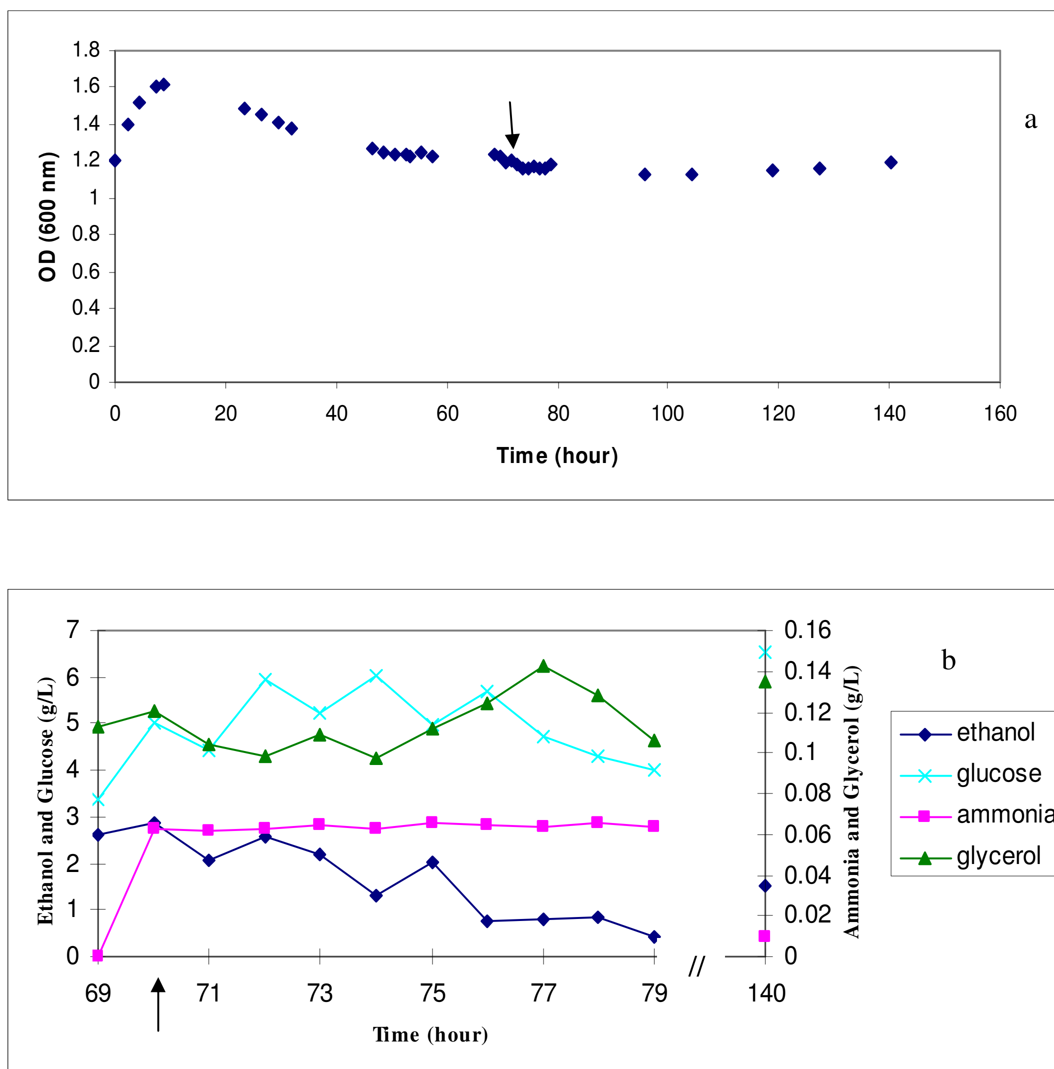


Figure 3.4. Growth curve (a) and extracellular metabolite profiles (b) of *S. cerevisiae* BY4743 *(RIP1/rip1Δ)* under nitrogen limited condition in chemostat culture with pulse injection

After overnight batch cultivation, the system was switched to chemostat. OD increased for 9 hours but then it slightly decreased and reached steady state at 40th hour of the chemostat. After the injection of the ammonium sulfate pulse at the end of the three residence time at steady state, a slight decrease in the growth of the cells was observed for

4 hours. Then the cell growth reached a steady state at a lower OD value then the first steady state. After passing three residences time the experiment was terminated.

Prior to pulse injection, the glucose concentration was approximately 3 g/L in *S. cerevisiae* BY4743 (*RIP1/rip1Δ).* After the injection of nitrogen pulse, the glucose concentration slightly increased and remained constant approximately at 5 g/L during the cultivation. At the second steady the glucose concentration increased to 6 g/L. Ethanol concentration was approximately 2.5 g/L prior to the pulse injection. Ethanol concentration decreased up to 0.5 g/L within the ten hours after the pulse injection. At the second steady state it was 1.5 g/L. Ammonia concentration was increased from 0.0002 g/L to 0.06 g/L with the pulse and remained constant for 9 hours. At the second steady state ammonia concentration decreased to 0.009 g/L. Glycerol concentration did not change during the experiment, its concentration remained approximately at 0.1 g/L.

3.2. Transcriptional Responses of Genes to a Sudden Release from Nitrogen Limitation in Deletion Strains of *S. cerevisiae* BY4743

In order to identify the variations in the expression levels of genes involved in glucose sensing, signal transduction and glucose repression pathways by real time RT-qPCR as a response to system level perturbations, four chemostat experiments were conducted with partial and respiratory deficient mutants of *S. cerevisiae*. *hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ* strains were cultivated in nitrogen limited F1 medium. After three residence time at steady state, ammonium sulfate pulses were injected in order to recover the nitrogen limitation.

Samples from cultures 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 using real time RT-qPCR.

The genes that the transcriptional responses were investigated, were *CYC8, GRR1, MTH1,RGT, RGT2, SKP1 SNF3, STD1,TUP, ,YCK1,YCK2,ELM1,GLC7, HXK2, MIG1, PAK1,REG1,SNF1, SNF4, TOS3, HAP4* and *MBA1.*

3.2.1. Expression Profile of *RGT2* as a Response to Nitrogen Pulse

RGT2 is a sensor of high levels of glucose, because it is required for maximal induction of *HXT1* expression by high concentrations of glucose but not for induction of *HXT2* and *HXT4* expression by low levels of glucose (Özcan and Johnston, 1999). *RGT2* is expressed in cells growing on high levels of glucose (it is expressed constitutively, being neither repressed nor induced by glucose) (Özcan *et al.*, 1996).

The expression profiles of *RGT2* in deletion strains of *Saccaromyces cerevisiae* (*ho*Δ*/ho*Δ*, hap4*Δ*/hap4*Δ*, rip1*Δ*/rip1*Δ and *RIP1/rip1*Δ*)* were represented in Figure 3.5, 3.6, 3.7 and 3.8 respectively. The first samples were collected prior to the pulse injection. The sample numbers 2-5 were the samples that were taken within the first minute after the pulse injection. The sample numbers 6 to 9 were the samples that were collected for 20 minutes at every 5 minute. Then the samples 10-19 were collected at every hour for ten hours. The last samples were taken at the end of three residence time at the second steady state.

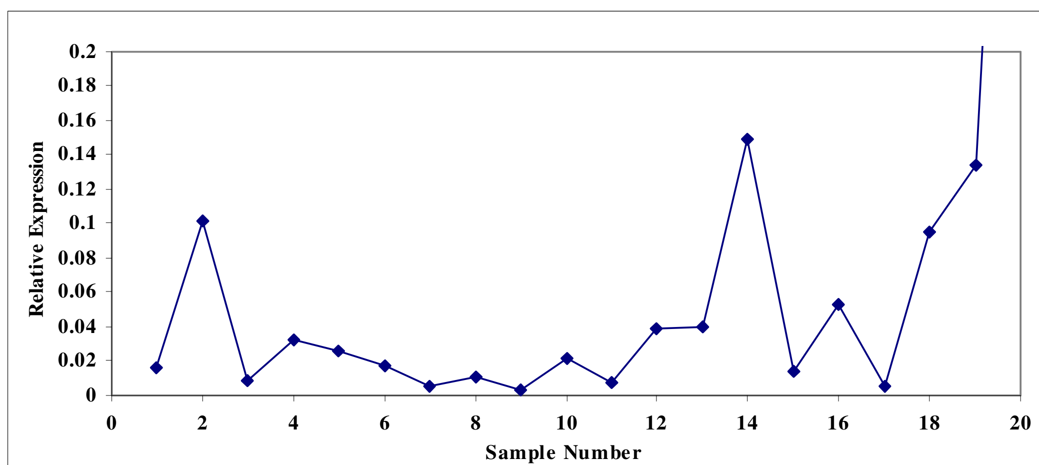


Figure 3.5.Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *RGT2* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* strain increased 6 fold after the injection of ammonium sulfate pulse. Then it decreased to its initial level. In

the last three samples which correspond to 9th, 10th hour and the second steady state data, expression level of *RGT2* increased.

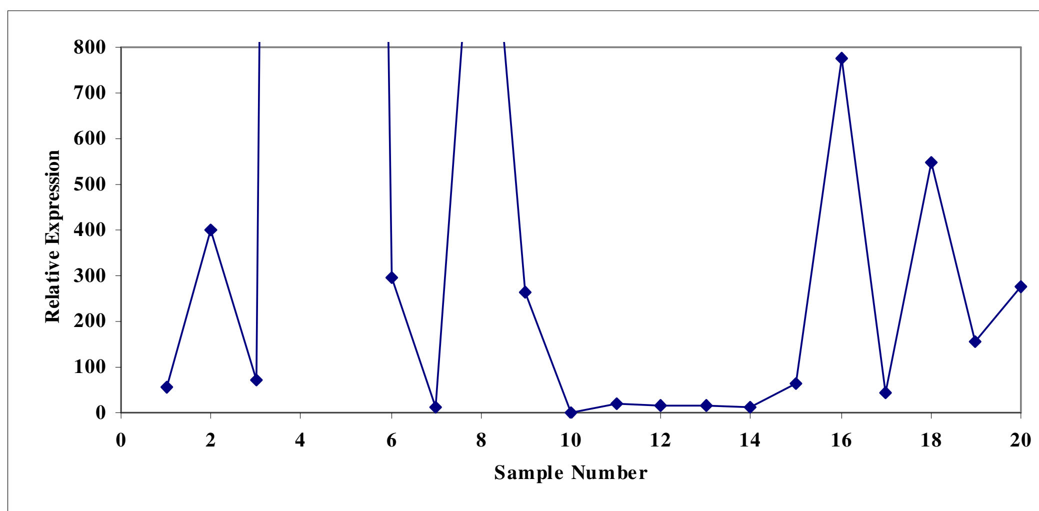


Figure 3.6.Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain, the expression level of *RGT2* increased just after the pulse injection and showed an oscillatory behavior within the first fifteen minute. Between the first and the seventh hour, the expression level of *RGT2* was below its initial value. After seven hours later than the pulse injection, an increase in the expression level of the gene was observed. Its expression level remained higher at the second steady state compared to that of the first steady state

After the injection of nitrogen pulse, a 4 fold decrease in the gene expression level of *RGT2* gene was observed in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)*. Increases were observed in the expression level of *RGT2* within the first hour after the pulse injection. The expression level of *RGT2* was zero after one hour following the pulse injection and remained unnoticeable at the second steady state.

The expression level of *RGT2* decreased in response to nitrogen pulse in *S. cerevisiae* BY4743 (*RIP1Δ/rip1Δ)* strain. Then it showed a fluctuating behavior during the whole cultivation. The expression level of *RGT2* at the second steady state is lower than the initial level.

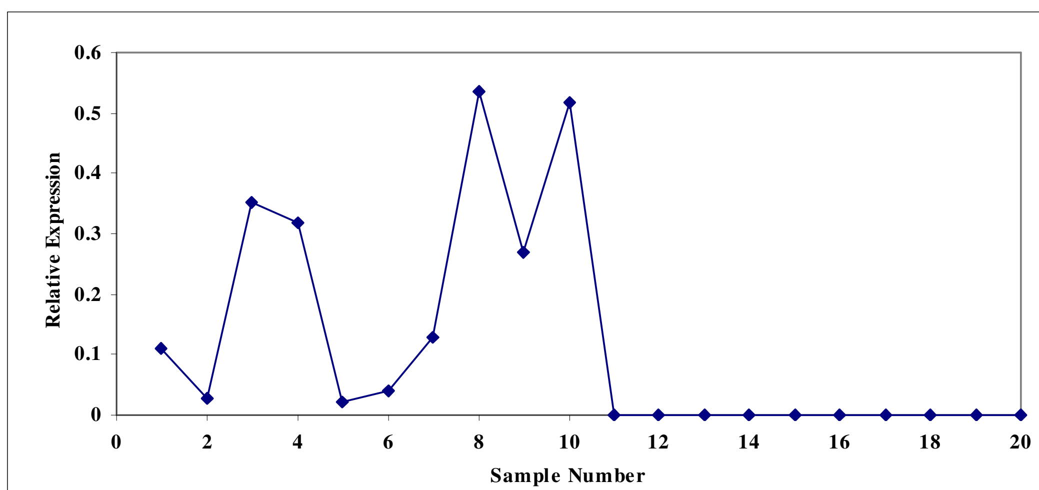


Figure 3.7.Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

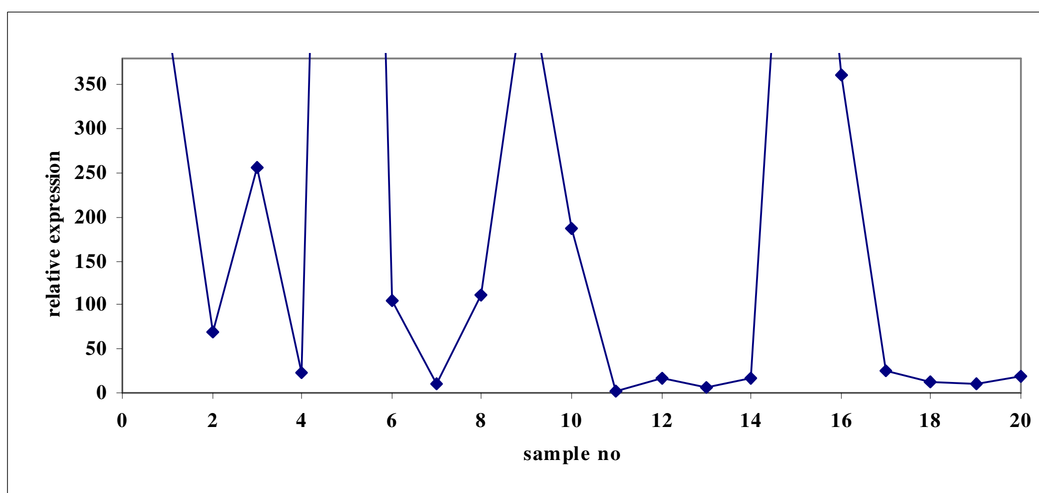


Figure 3.8.Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.2. Expression Profile of *SNF3* as a Response to Nitrogen Pulse Injection

SNF3 is plasma membrane glucose sensor that regulates glucose transport. It has 12 predicted transmembrane segments; long cytoplasmic C-terminal tail is required for low glucose induction of hexose transporter genes HXT2 and HXT4 (Özcan *et al.*, 1996). Transcription of *SNF3* is maximal when glucose levels are low and it has been *reported*

that SNF3 expression is repressed about fivefold by high levels of glucose (Özcan *et al.*, 1999).

The expression profiles of *RGT2* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were represented in Figure 3.9, 3.10, 3.11 and 3.12 respectively. Samples were collected as previously stated.

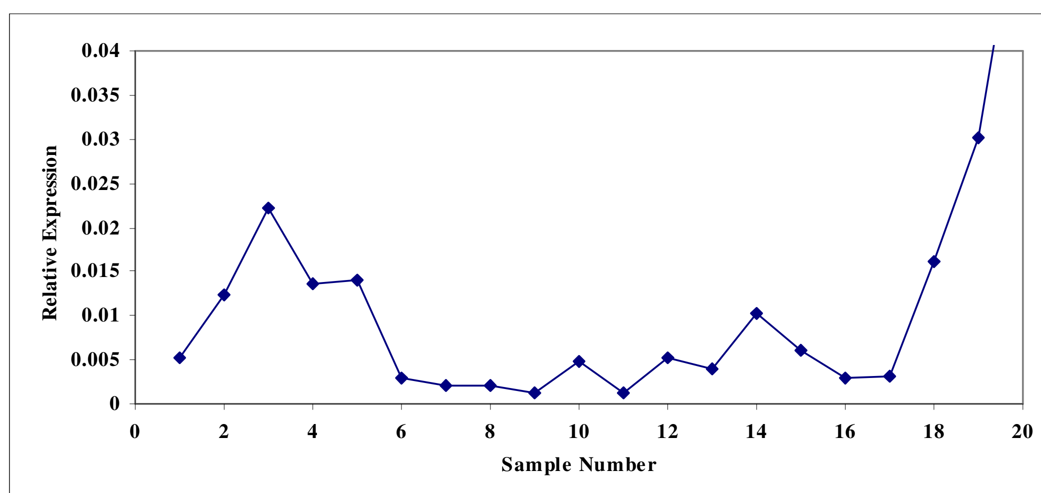


Figure 3.9.Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *SNF3* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* showed an increasing trend within the first minute after the injection of the pulse. Until the ninth hour, the values remained lower or close to initial level. Beginning with the ninth hour an increasing trend was observed. Also the second steady state value was 11 fold higher than the initial case.

Nitrogen pulse resulted in an immediate increase in *SNF3* expression level in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)*. Fluctuating behavior was seen at the other data points. The expression levels of the gene were low at the first and second steady state and are close to each other.

Prior to the pulse injection, *SNF3* expression was undetectable in *S. cerevisiae* BY4743 (*rip11Δ/rip1Δ)* strain. Within the first hour, *SNF3* was observed to be expressed at

higher levels. However, 1 hour later than the pulse injection, the expression of *SNF3* became undetectable. At the second steady state, a slight increase was observed at the expression level of *SNF3*.

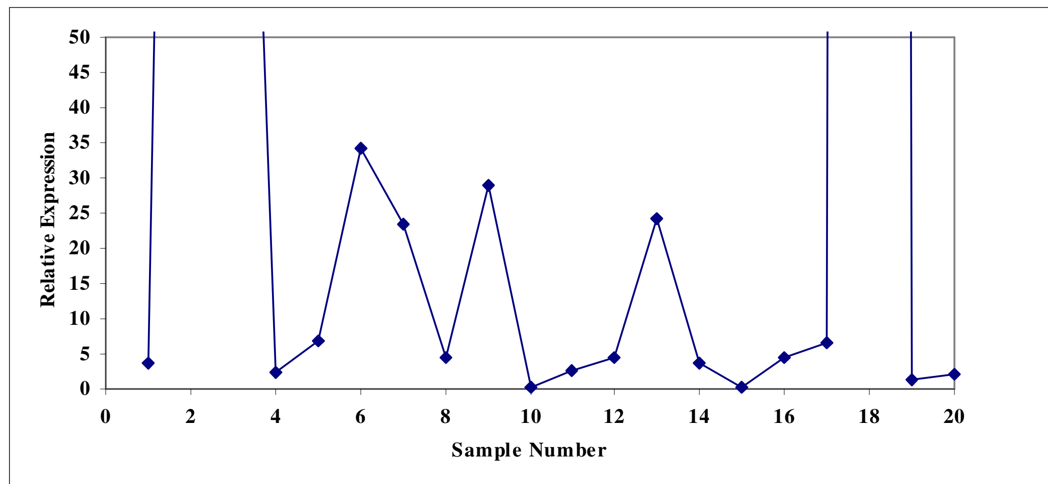


Figure 3.10.Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

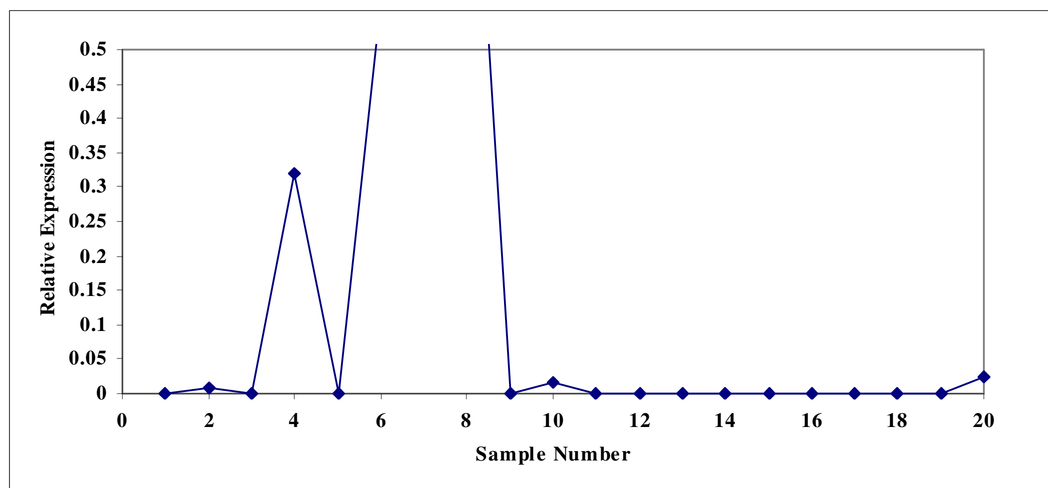


Figure 3.11.Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *SNF3* was decreased soon after the injection of the pulse in the case of *S. cerevisiae* BY4743 (*RIP1/rip1Δ).* An oscillatory behavior was observed in

the expression levels of this gene throughout the cultivation. The expression level of *SNF3* at the first steady state was six fold higher than the second steady state level.

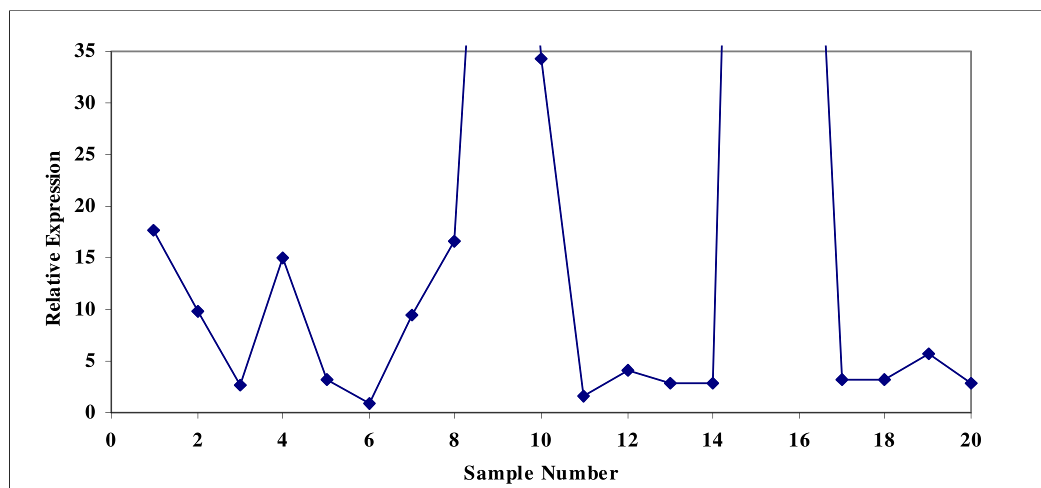


Figure 3.12.Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.3. Expression Profile of *MTH1* as a Response to Nitrogen Pulse

MTH1 is a negative regulator of the glucose-sensing signal transduction pathway. It is required for the repression of transcription by Rgt1p. Mth1p interacts with Rgt1p, Snf3p and Rgt2p (Schmidt *et al.*, 1999; Lafuente *et al.*, 2000) and it is phosphorylated by Yck1p (Flick *et al.*, 2003; Moriya and Johnson, 2004; Spielewoy *et al.*, 2004). It is observed that the transcription of the *MTH1* gene is repressed at high concentration of glucose (Lakshmanan *et al.*, 2003).

The expression profiles of *MTH1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.13, 3.14, 3.15 and 3.16 respectively. Samples were collected as previously stated.

Nitrogen pulse resulted in 6 fold increase of the expression level of *MTH1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ).* Moreover, 12 fold increase was observed one hour later than the pulse injection. Beginning with the 8th hour, an increasing trend was observed.

The expression level at the second steady state was 12 fold higher than its value at the first steady state.

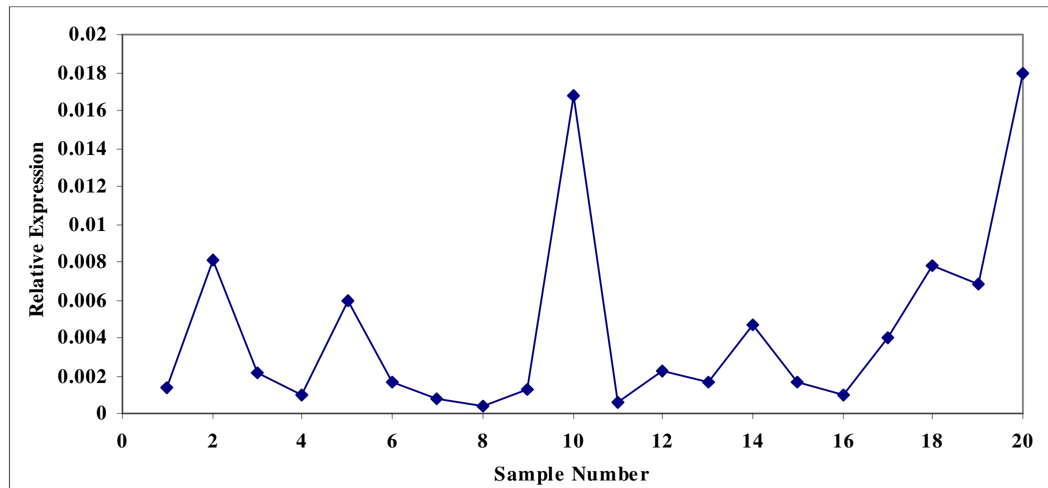


Figure 3.13.Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

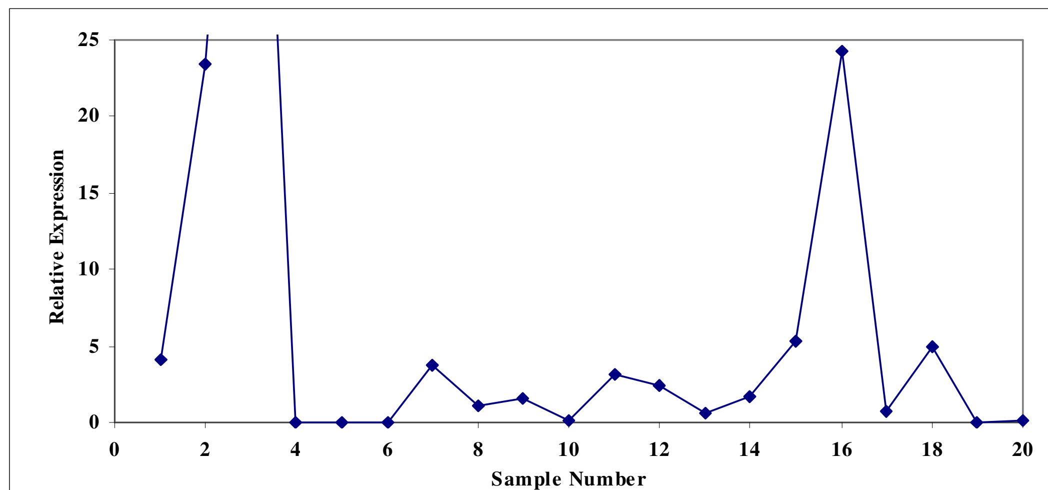


Figure 3.14.Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

As it can be seen from the Figure 3.14, the expression level of *MTH1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* increased within the 30 second after the pulse injection and then, it decreased. The expression level prior to the pulse injection was 60 times higher than the second steady state value.

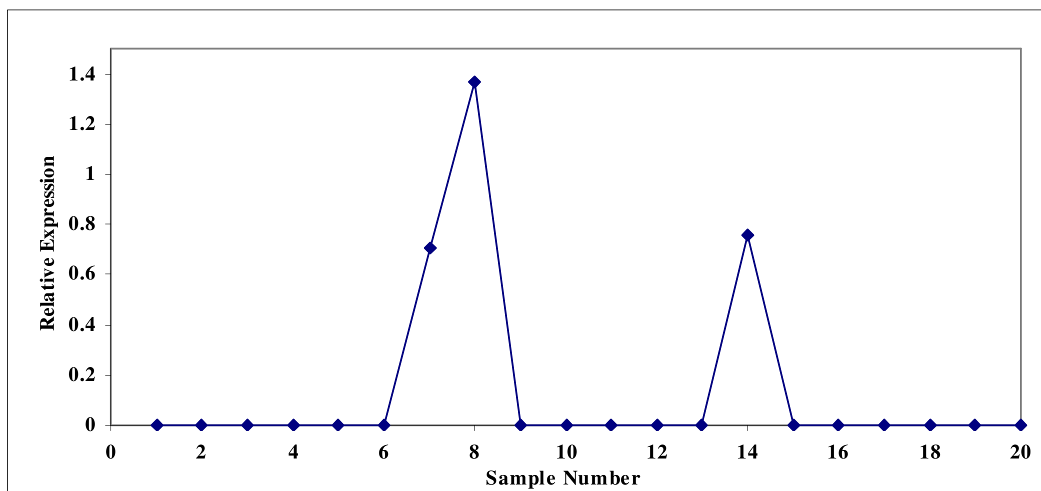


Figure 3.15.Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *MTH1* was not detectable in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*) strain under nitrogen limitation and remained unchanged after the pulse injection in the first five minutes. Only during the time duration between five and fifteen minutes later than the pulse injection and five hours later than the injection, *MTH1* was expressed.

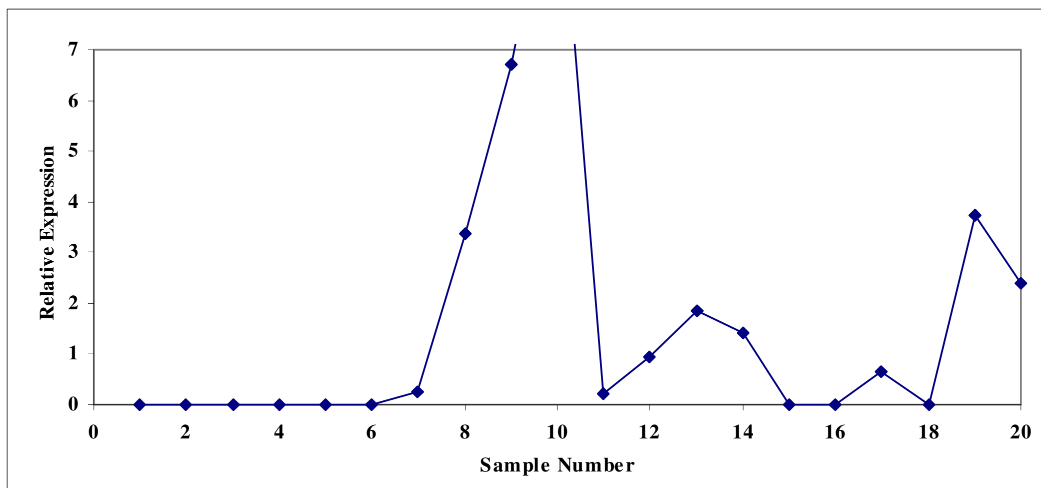


Figure 3.16.Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Expression level of *MTH1* was also undetectable *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* prior to the pulse injection and remained unchanged after the pulse injection. The expression level of the gene had reached to a maximum value 10 minutes later than the pulse injection. Higher expression level was detected at the second steady state.

3.2.4. Expression Profile of *STD1* as a Response to Nitrogen Pulse

Std1p is a protein involved in the control of glucose-regulated gene expression. It interacts with protein kinase Snf1p, glucose sensors Snf3p and Rgt2p and acts as a regulator of the transcription factor Rgt1p. Std1p directly interact with Rgt1p when glucose is absent and are required for the repression of HXT gene transcription by Rgt1p (Lakshmanan *et al.*, 2003). The degradation of Std1p leads into the glucose-induced dissociation of Rgt1p from the HXT1 promoter and its activation (Flick *et al.*, 2003; Moriya and Johnson, 2004; Spielewoy *et al.*, 2004).However *STD1* expression is induced by glucose which would be expected to counteract Std1 degradation (Moriya and Johnson, 2004).

The expression profiles of *STD1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.17, 3.18, 3.19 and 3.20 respectively. Samples were collected as previously stated.

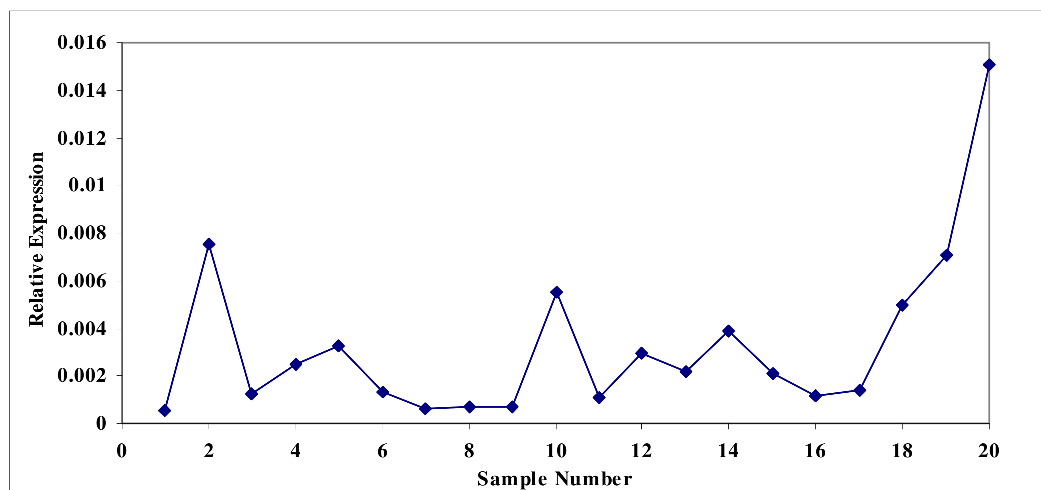


Figure 3.17.Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

15 fold increase was observed in the expression level of *STD1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* after the injection of nitrogen pulse. An oscillatory behavior was observed in the expression levels of this gene throughout the cultivation. The expression level of this gene was quite high at the second steady state compared to the first steady state value.

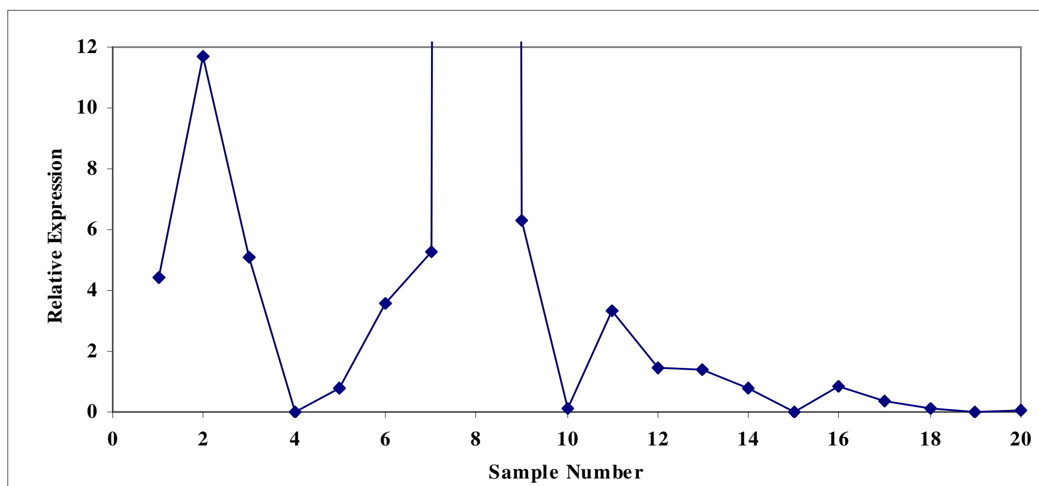


Figure 3.18.Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *STD1* increased immediately after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* and then dipped below the starting level. The highest peak was observed at the fifteenth minute and then it decreased gradually below the initial value. Also the expression level at the second steady state was lower than the first steady state value.

Expression level of *STD1* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* before the injection of nitrogen pulse. Although the pulse caused an increase in the expression level, it immediately decreased to zero again. Between the 15th minute and the 3rd hour and at the second steady state, it is seen that *STD1* is expressed.

After the injection of nitrogen pulse the expression level of the *STD1* in *RIP1/rip1*Δ mutant did not changed. Only at the 20th minute and 6th hour, relatively higher peaks were

observed. The expression levels at the first and second steady states were close to each other.

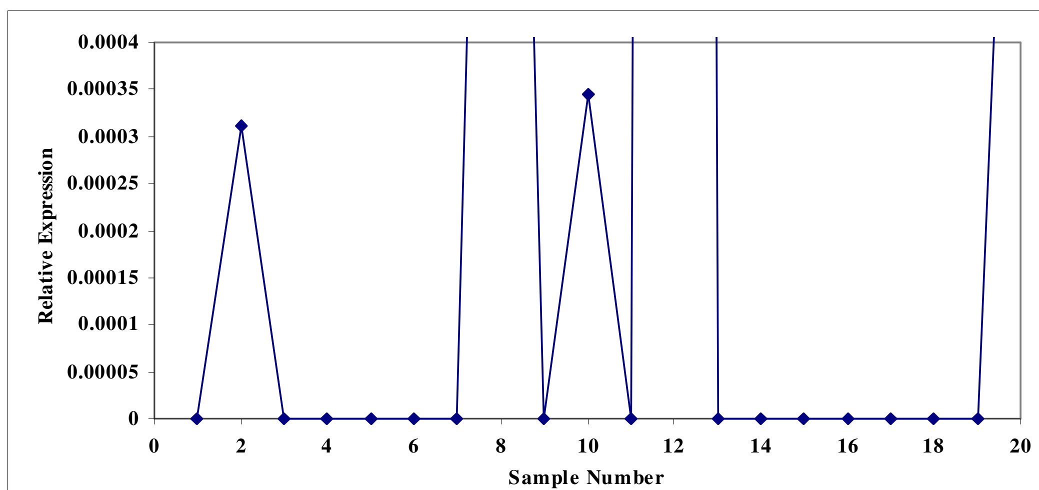


Figure 3.19.Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

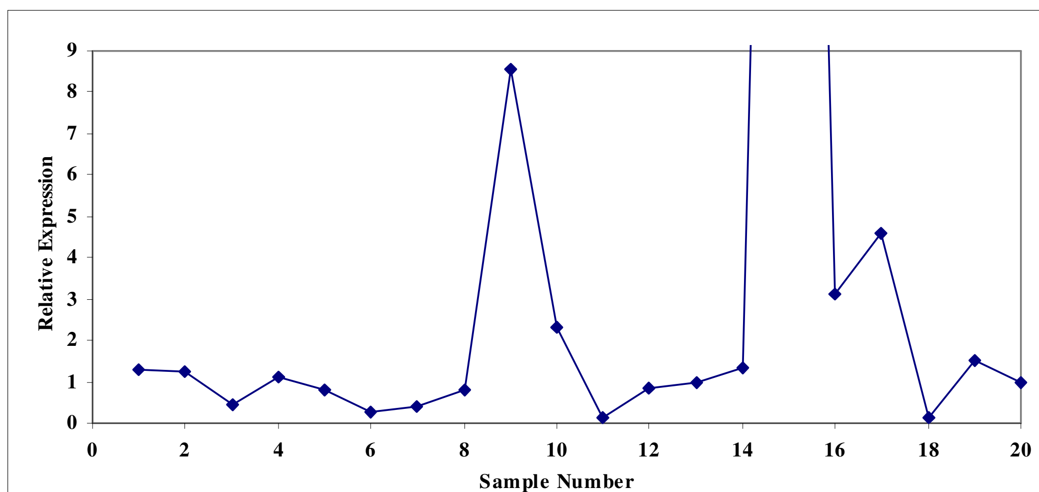


Figure 3.20.Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.5. Expression Profile of *YCK1* as a Response to Nitrogen Pulse

Plasma membrane-bound casein kinase I isoform shares redundant functions with Yck2p in morphogenesis. Yeast protein kinase I (Yck1/Yck2) phosphorylates Std1p and

Mth1p (Flick *et al.*, 2003; Moriya and Johnson, 2004; Spielewoy *et al.*, 2004). The phosphorylation of Mth1p and Std1p is necessary for their recognition by the SCF-Grr1 complex, which signal them for degradation (Flick *et al.*, 2003; Moriya and Johnson, 2004; Spielewoy *et al.*, 2004).

The expression profiles of *YCK1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.21, 3.22, 3.23 and 3.24 respectively. Samples were collected as previously stated.

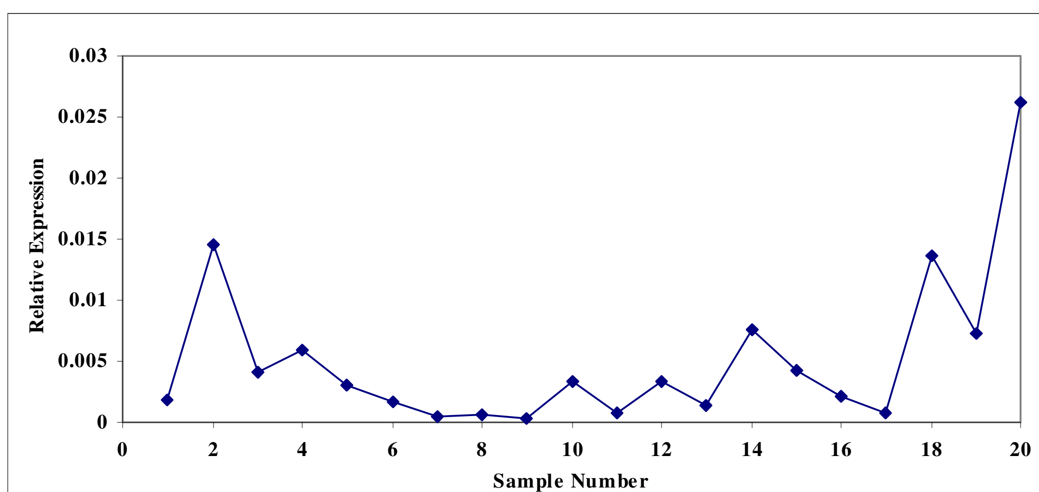


Figure 3.21.Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

Examination of the relative expression values of *YCK1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*, revealed an increase of 7 fold after the injection of nitrogen pulse. Then until the third hour, a slight decreasing trend was observed. The second steady state value was 14 fold higher than the initial one.

In the case of *hap4Δ/hap4Δ* strain of *S. cerevisiae*, the expression level of *YCK1* rose to the highest peak and then dipped below its initial level within the first minute following the pulse injection. During the time duration between the 20th minute and 4th hour, higher expression levels were obtained in comparison to the initial value. 6 hours later than the pulse injection expression level of *YCK1* decreased below its initial value.

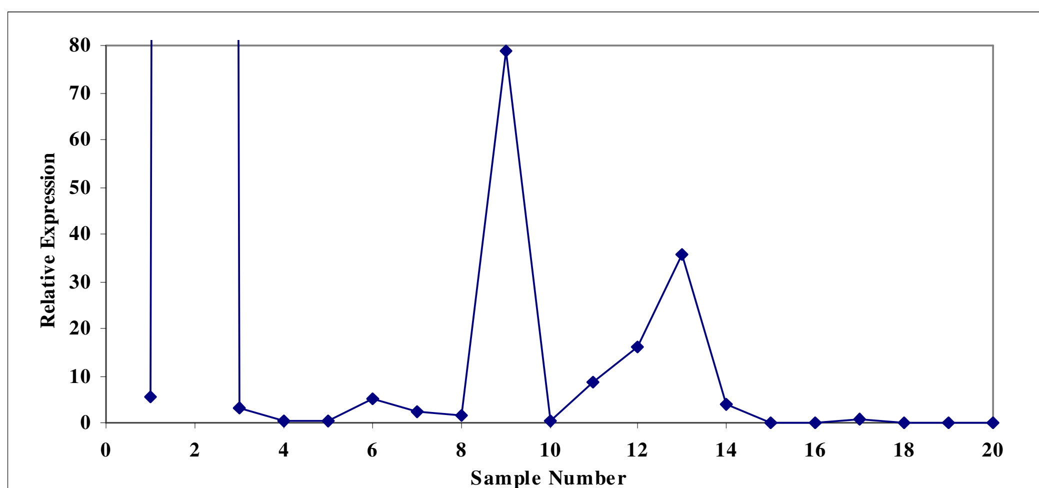


Figure 3.22.Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

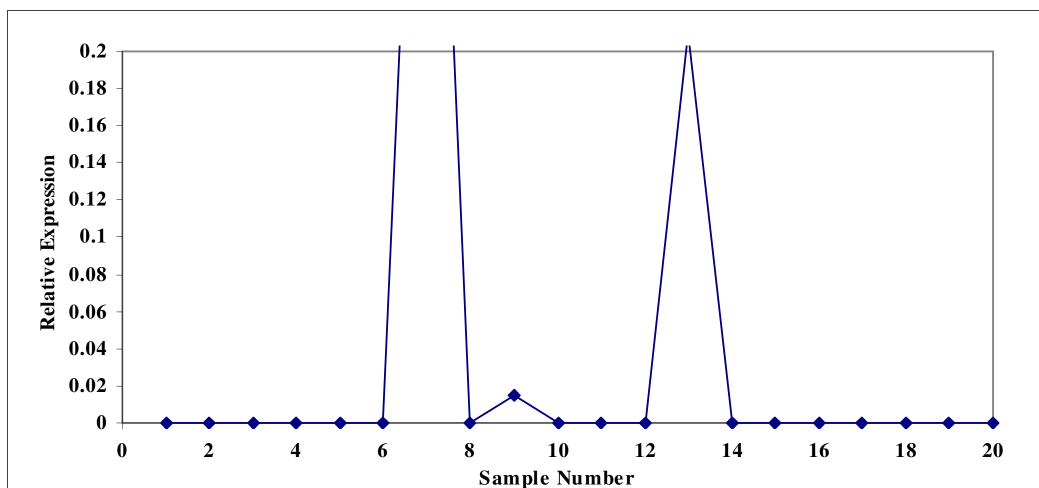


Figure 3.23.Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

As it can be seen from the figure, expression level of *YCK1* was almost zero in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* strain. 10 minutes later than the pulse injection, *YCK1* began to be expressed. However four hours later than the injection, expression level of *YCK1* decreased to zero.

Prior to the pulse injection, expression level of *YCK1* was also unnoticeable in *RIP1/rip1Δ* mutant. However, the effect of pulse was observed earlier, in comparison to

the homozygous mutant. The expression value reached a peak between the 6th and 7th hour. It is also observed that, at the second steady state, *YCK1* was expressed.

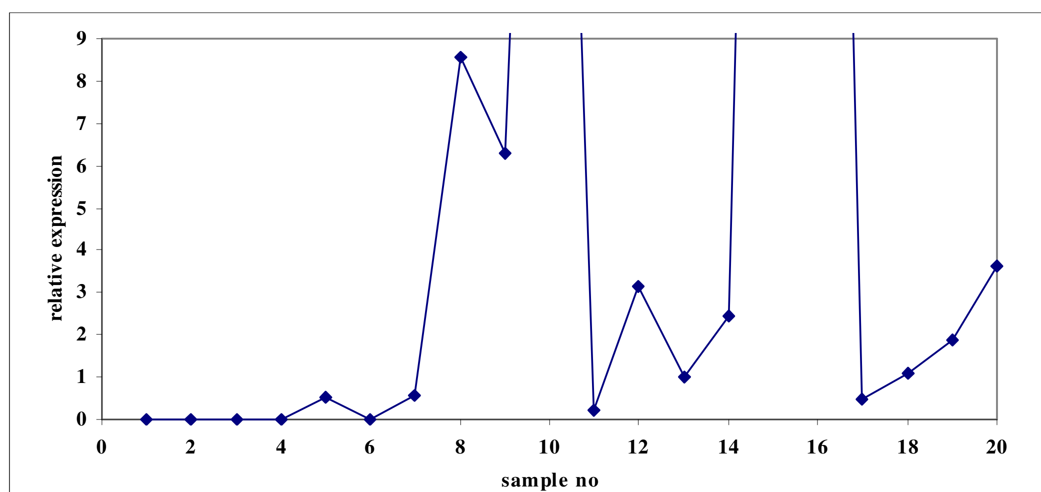


Figure 3.24.Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.6. Expression Profile of *YCK2* as a Response to Nitrogen Pulse

Yck2p is a paralogue of Yck1p. Yeast protein kinase I (Yck1/Yck2) phosphorylates Std1p and Mth1p (Flick *et al.*, 2003; Moriya and Johnson, 2004; Spielewoy *et al.*, 2004).

The expression profiles of *YCK2* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.25, 3.26, 3.27 and 3.28 respectively. Samples were collected as previously stated.

After the injection of nitrogen pulse, expression level of *YCK2* increased 12 fold in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* but then it rebounded. One hour later than the pulse injection, fluctuating behavior began to be observed in the expression of *YCK2*. Expression level at the second steady state was 12 fold higher than the first steady state value.

YCK2 expression in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain increased immediately after the injection of nitrogen pulse and then rebounded quickly. Then a

fluctuating behavior was observed. Expression level of *YCK2* was undetectable at the second steady state.

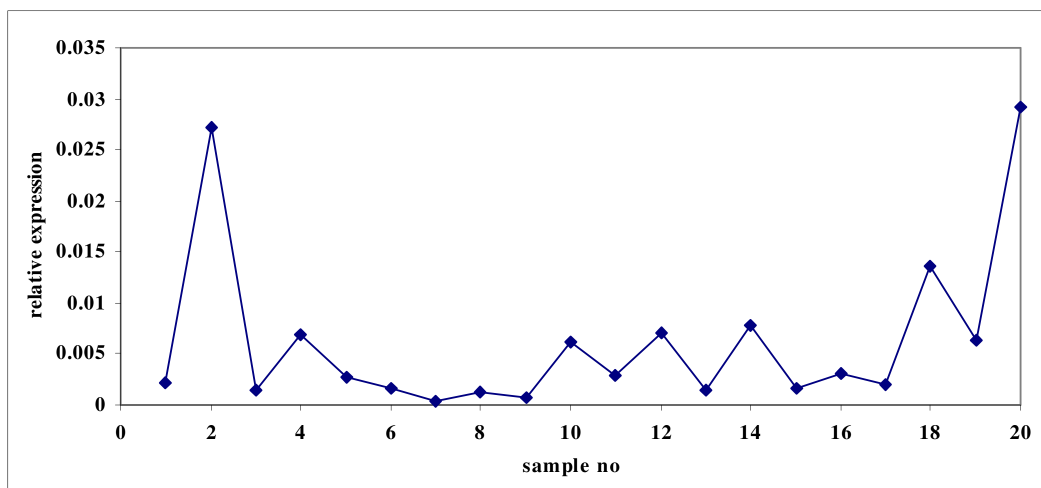


Figure 3.25.Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

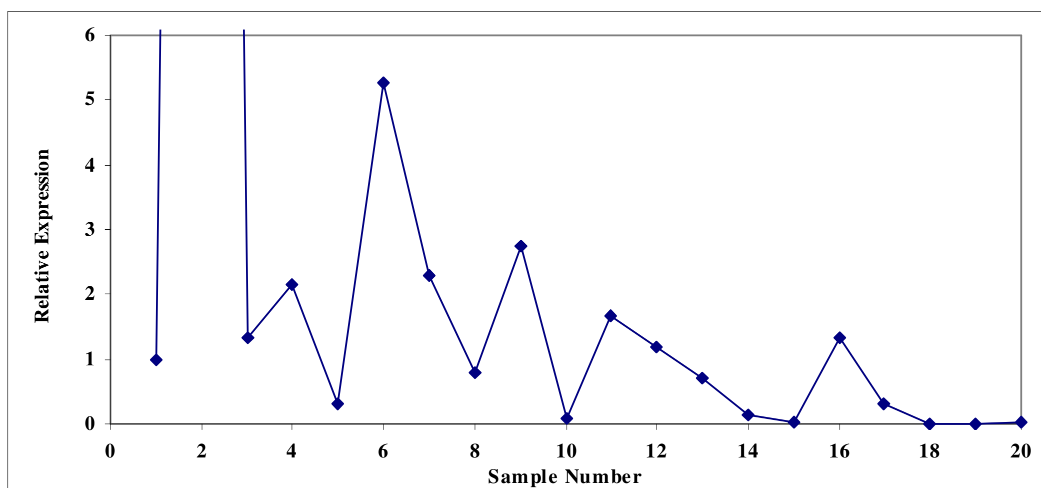


Figure 3.26.Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Expression level of *YCK2* slightly increased in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* after the injection of nitrogen pulse. Two sharp increases were observed in the expression level of *YCK2* at the 10th minute and 4th hour. After the sharp increase at the 4th hour,

expression level of *YCK2* decreased to zero. Comparing the steady state values, it was seen that, second steady state value was 3 fold higher than the initial one.

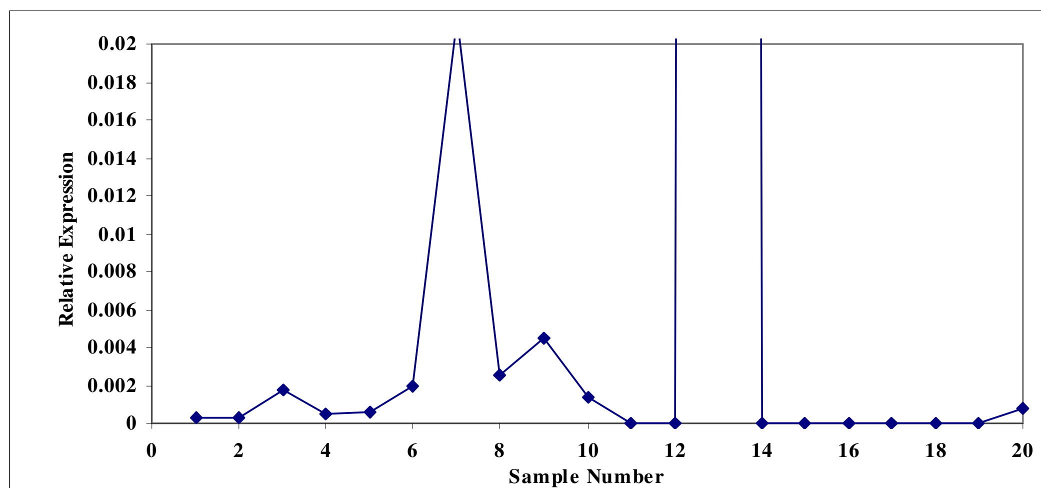


Figure 3.27. Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

After the pulse injection, the expression level of the *YCK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* did not show a significant change at the first 20 minutes. At the 20th minute it showed an increasing trend but then it dipped below its initial value. Second steady state value was 8.5 fold higher than the initial value.

3.2.7. Expression Profile of *GRR1* as a Response to Nitrogen Pulse

Grr1p is F-box protein component of the SCF ubiquitin-ligase complex. It is involved in carbon catabolite repression, glucose-dependent divalent cation transport, high-affinity glucose transport, and morphogenesis. Grr1 interacts with Skp1 and is required for inhibition of Rgt1 function (Ning and Johnton, 1999).

Grr1 is required to inactivate Mth1 and Std1 in response to glucose. Glucose acts via Grr1 to regulate the abundance of Mth1. Inactivation of Mth1 leads to hyperphosphorylation of Rgt1 and dissociation from *HXT* promoters (Flick *et al.*, 2003).

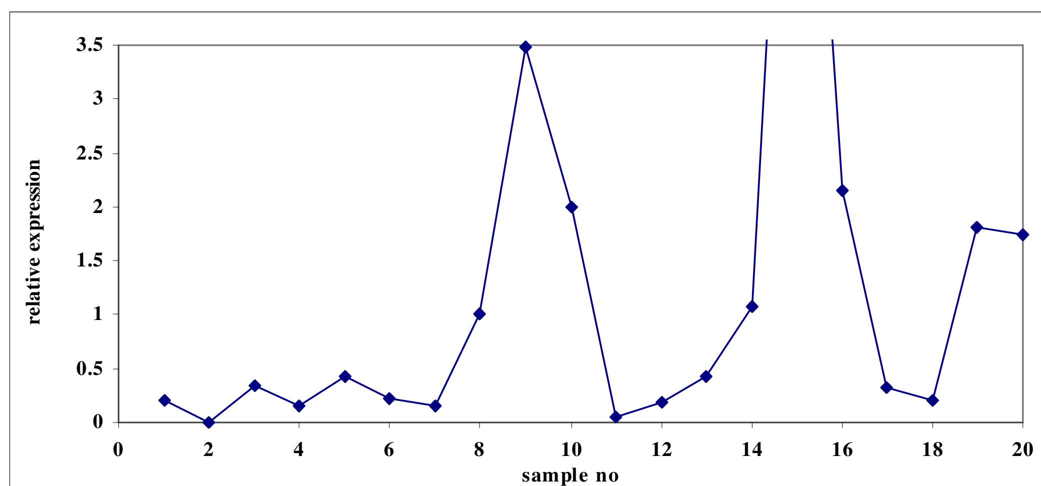


Figure 3.28. Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression profiles of *GRR1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.29, 3.30, 3.31 and 3.32 respectively. Samples were collected as previously stated.

Nitrogen pulse resulted in the immediate 16 fold increase in the expression level of *GRR1* in *S. cerevisiae* BY4743. Then it rebounded and remained close to its initial value. Beginning with the fifth hour, again an increasing trend was observed. Expression level at the second steady state was 20 fold higher than the first steady state level.

Expression profile of *GRR1* gene showed a fluctuating behavior in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain. After the injection of nitrogen pulse, expression level of *GRR1* increased 4 fold. Expression levels at the steady states were close to each other.

An increase in the expression level of *GRR1* began to be observed 45 seconds later than the pulse injection in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)*. 6 hour later than the injection, the expression level of *GRR1* decreased to zero. At the second steady state, the expression level of *GRR1* was higher than the first steady state value.

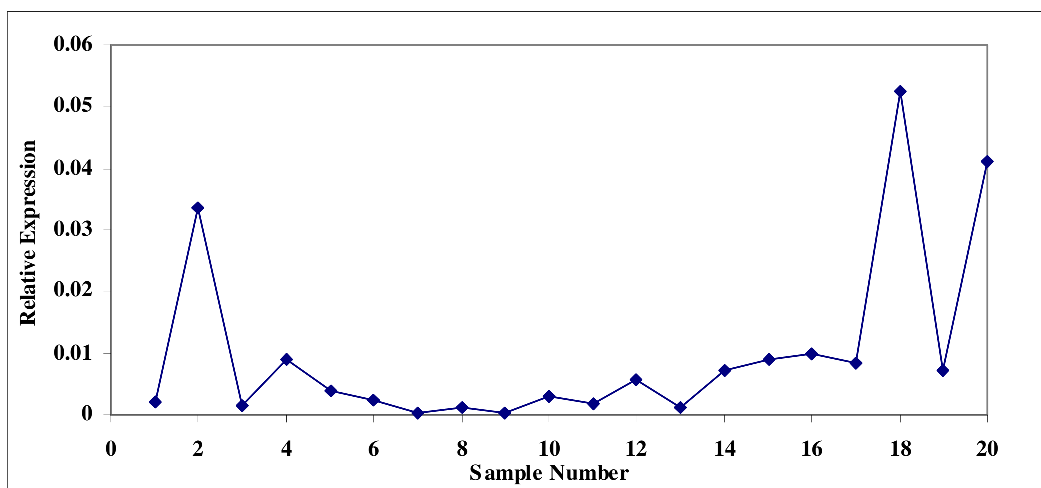


Figure 3.29. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

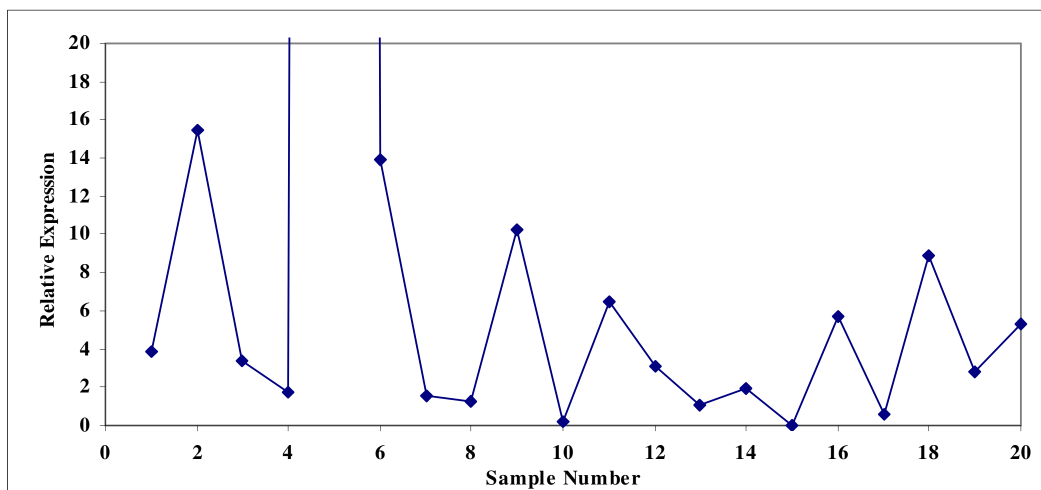


Figure 3.30. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *GRR1* increased three fold after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*. Then it showed a fluctuating behavior during the whole cultivation.

3 fold higher expression level was obtained at the second steady state, in comparison to the first steady state.

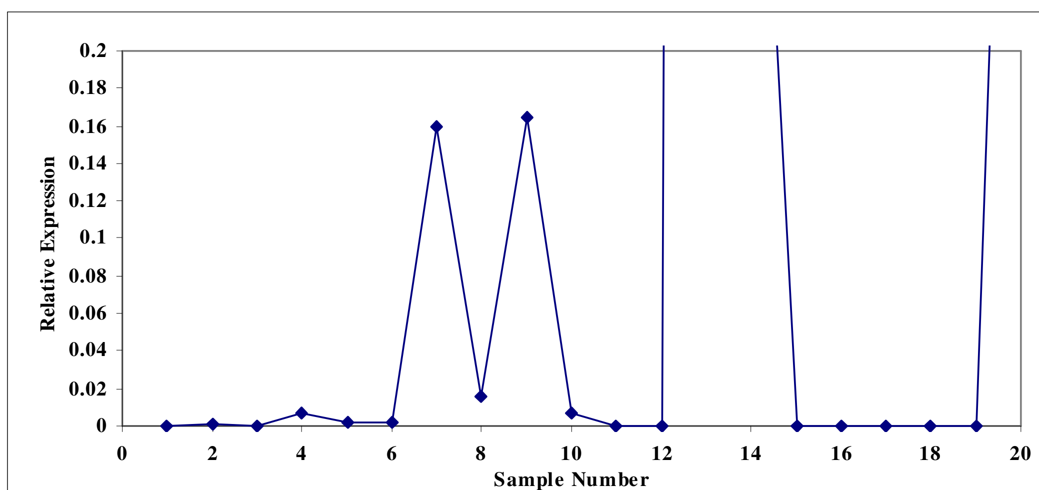


Figure 3.31. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

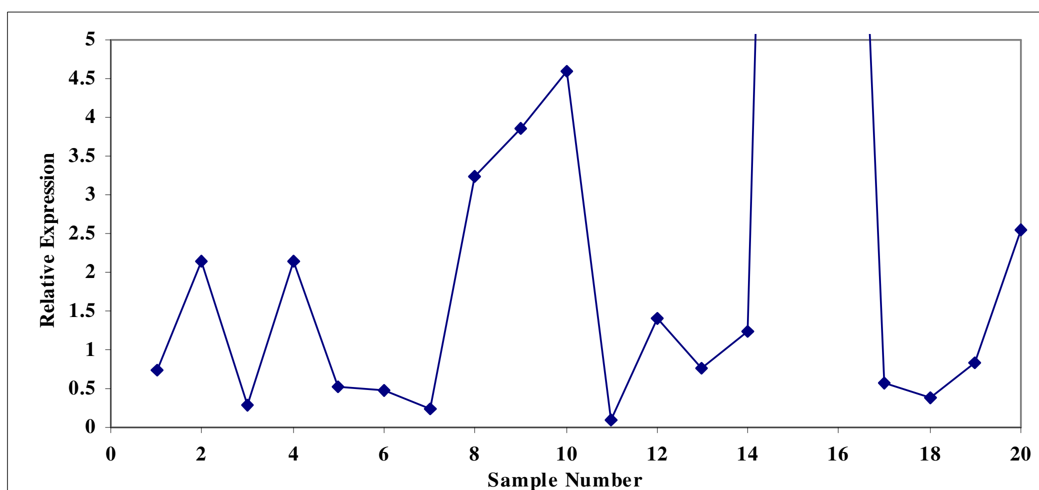


Figure 3.32. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.8. Expression Profile of *SKP1* as a Response to Nitrogen Pulse

Skp1 is a component of several structurally distinct but functionally related ubiquitin ligase complexes that target a wide variety of regulators to the 26S proteasome for degradation. These complexes are termed SCF to indicate their two common components, *S*kp1 and the cullin *C*dc53, and a third exchangeable component, the *F*-box protein. Grr1 interacts with Skp1 and is required for inhibition of Rgt1 function. The Grr1-Skp1

interaction is significantly enhanced by high levels of glucose (Li and Johnson, 1997; Santangelo, 2006).

The expression profiles of *SKP1* in deletion strains of *Saccaromyces cerevisiae* (*ho*Δ*/ho*Δ, *hap4*Δ*/hap4*Δ, *rip1*Δ*/rip1*Δ and *RIP1/rip1*Δ*)* were presented in Figure 3.33, 3.34, 3.35 and 3.36 respectively. Samples were collected as previously stated.

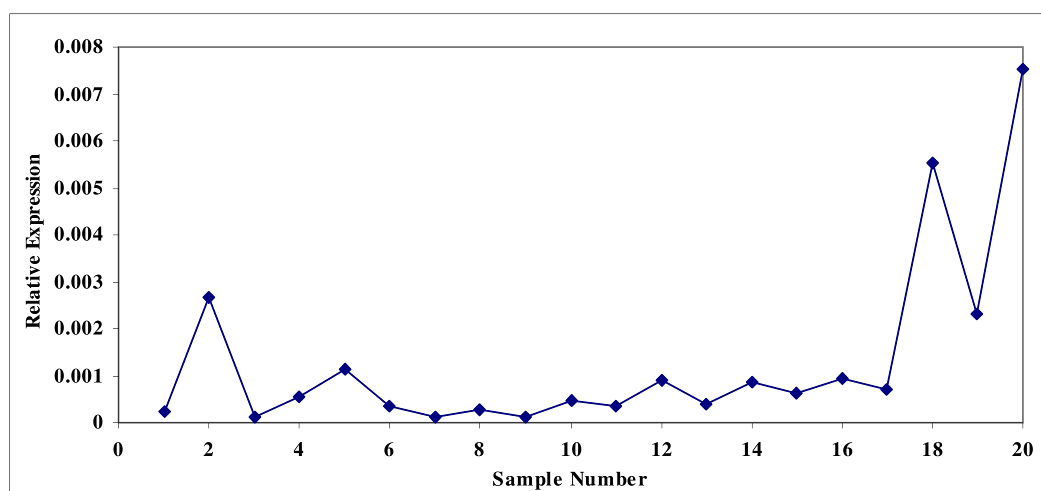


Figure 3.33. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*ho*Δ*/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

Nitrogen pulse resulted in an initial 10 fold increase in the expression level of *SKP1* in *S. cerevisiae* BY4743 (*ho*Δ*/hoΔ).* Then it immediately decreased below its initial value. Then until the nine hour later than the injection, no significant change was observed in the expression level of *SKP1*. The second steady state value was 30 times higher than the initial value.

The application of nitrogen pulse resulted in a sudden increase in the expression level of *SKP1* in *S. cerevisiae* BY4743 (*hap4*Δ*/hap4Δ)* strain and its expression level lowered below its initial value within 45th second after the pulse injection. No observable expression levels could be detected at second steady state.

Expression level of *SKP1* was undetectable before the application of the pulse in *S. cerevisiae* BY4743 (*rip1*Δ*/rip1Δ)* and remained unchanged after the pulse injection.

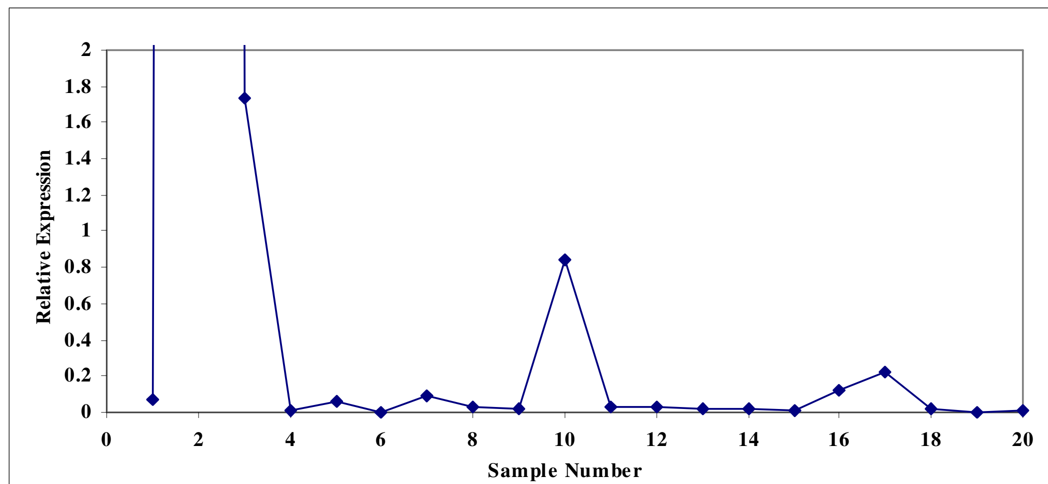


Figure 3.34. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

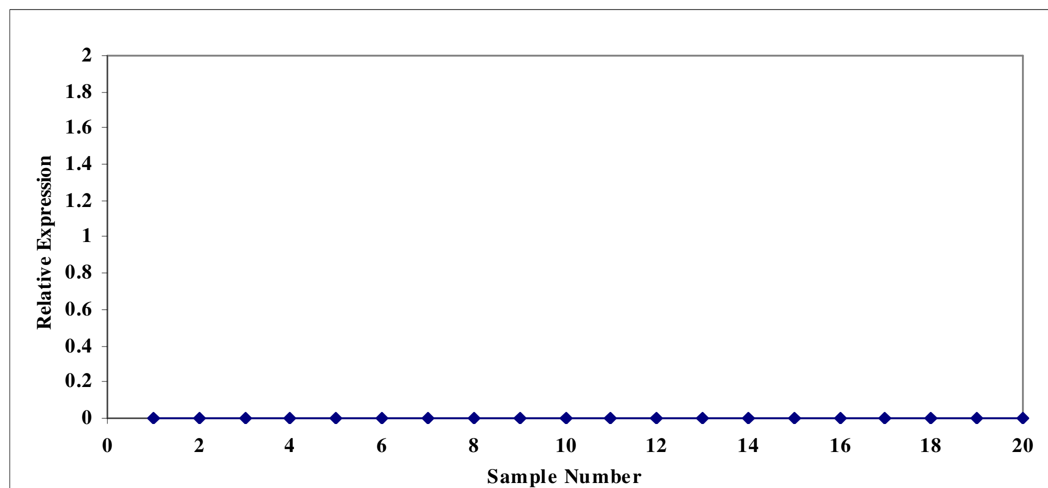


Figure 3.35. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *SKP1* was also not detectable in the heterozygous mutant of Rip1 prior to and after the pulse. The expression level of *SKP1* remained unchanged after the pulse injection.

Only at the fifth hour, tenth hour and the second steady state, *SKP1* was seen to be expressed whereas it was undetectable at the first steady state.

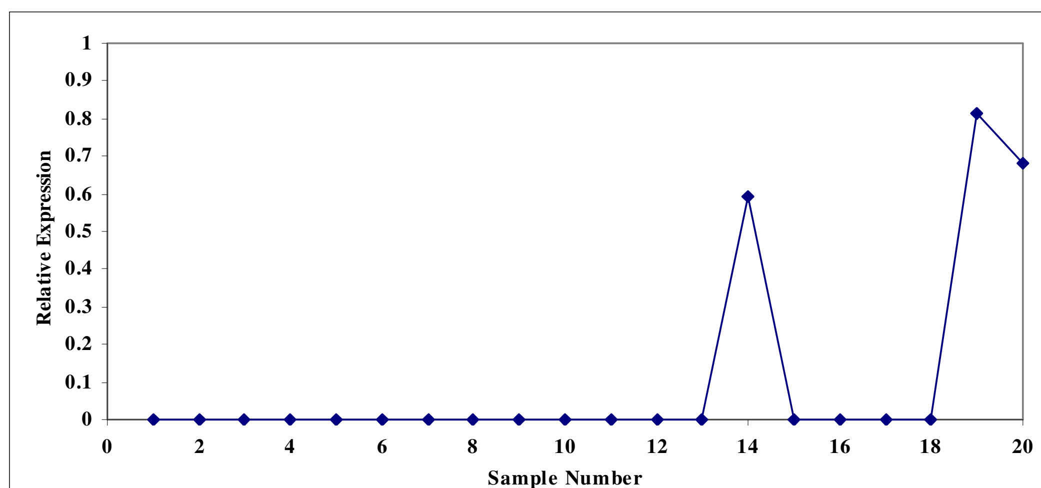


Figure 3.36. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.9. Expression Profile of *RGT1* as a Response to Nitrogen Pulse

Rgt1 is a transcription factor that regulates the expression of *HXT* genes in *Saccaharomyces cerevisiae* and has two effects on the transcription of these genes. Rgt1 represses transcription in the absence of glucose while when glucose level are high (>2 per cent), Rgt1p activates transcription of *HXT1* (Ozcan *et al.*, 1996; Polish *et al.*, 2004). It has no effect on transcription in the presence of low levels of glucose. Grr1p is required both for the inhibition of Rgt1p repressor function in response to low levels of glucose and for conversion of Rgt1p from a repressor to an activator by high levels of glucose. It is previously stated that, since transcription of *RGT1* is not altered in response to glucose, the activity of Rgt1p is likely to be regulated post-transcriptionally (Ozcan *et al.*, 1996).

The expression profiles of *RGT1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.37, 3.38, 3.39 and 3.40 respectively. Samples were collected as previously stated.

With injection pulse, three fold increase was observed in the expression level of *RGT1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ).* Fluctuating behavior resided during sampling period. The expression level at the second steady state value was 8 fold higher than the first one.

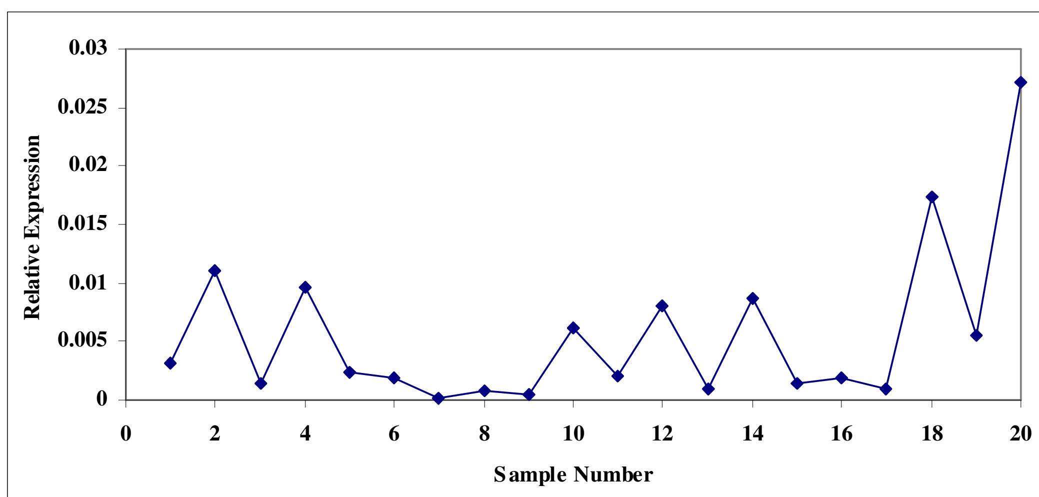


Figure 3.37. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*ho*Δ*/hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

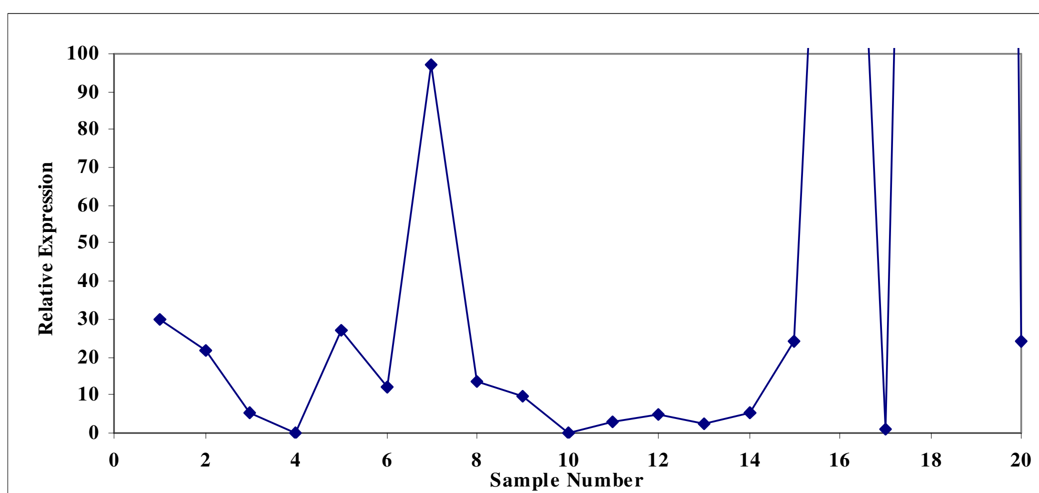


Figure 3.38. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*hap4*Δ*/hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Expression level of *RGT1* gene in *S. cerevisiae* BY4743 (*hap4*Δ*/hap4Δ)* under nitrogen limitation in chemostat cultures displayed a decreasing trend within the first minute after the pulse injection. 10 minutes later than the injection, a sharp increase was observed in the expression level of *RGT1*. 7 hours later than the injection, a fluctuating behavior was began to be observed in the expression level of the gene. Its expression level was restored to its initial value at the second steady state.

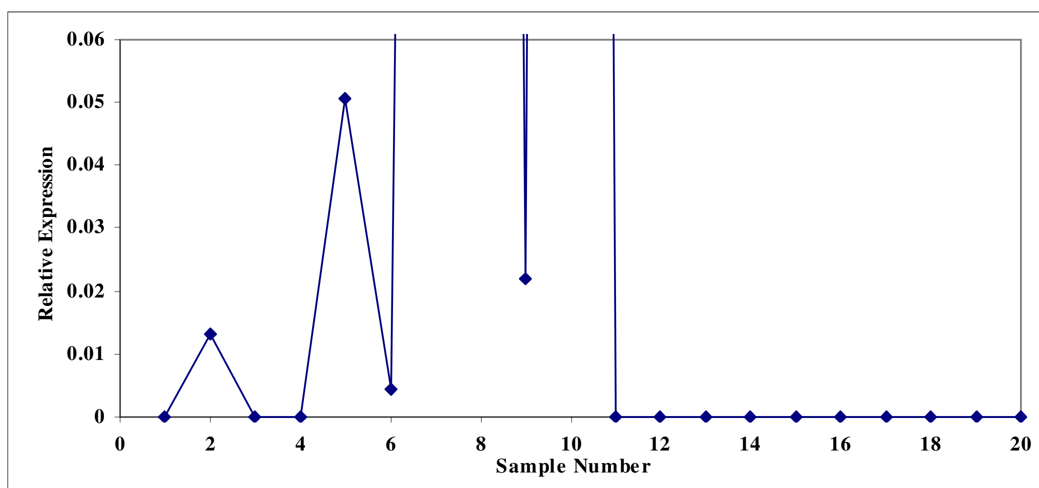


Figure 3.39. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of this gene was not detectable in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under nitrogen limitation in chemostat cultures before the pulse injection and at the second steady state. With the injection of nitrogen pulse, the expression of *RGT1* displayed an oscillatory behavior within the first hour after the application of the pulse and then remained at its initial value throughout the cultivation.

In *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain, the relative expression of *RGT1* showed a slight decrease with the application of nitrogen pulse. Its expression level displayed a fluctuating behavior and reached to a value at the second steady state which was four fold higher than the initial value.

3.2.10. Expression Profile of *GLC7* as a Response to Nitrogen Pulse

The Reg1-Glc7 complex acts in opposition to the Snf1 signaling pathway by promoting the dephosphorylation of Snf1 threonine 210 (McCartney and Schmidt, 2001). In the high glucose, Mig1 is also de-phosphorylated by the Glc7-Reg1 protein phosphatase complex (Alms *et al.*, 1999). The expression profiles of *GLC7* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.41, 3.42, 3.43 and 3.44 respectively. Samples were collected as previously stated.

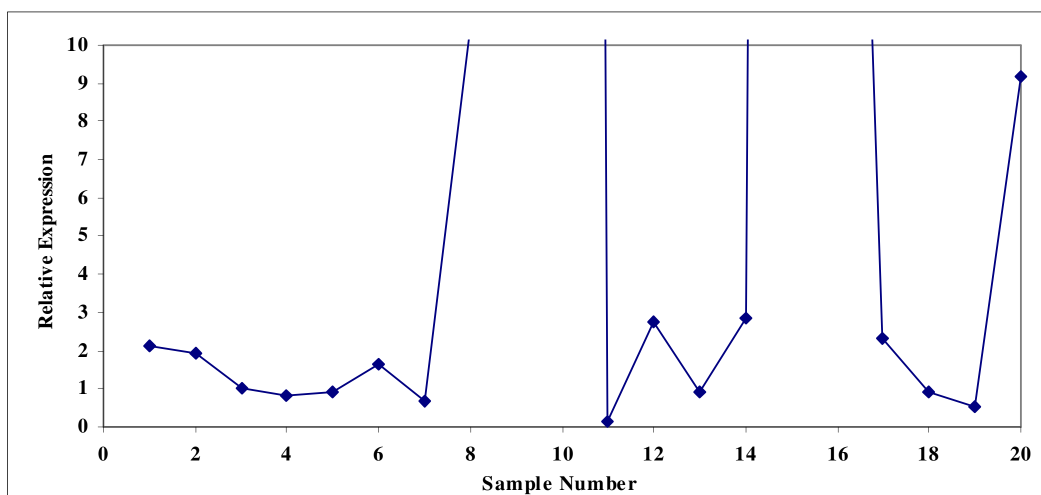


Figure 3.40. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

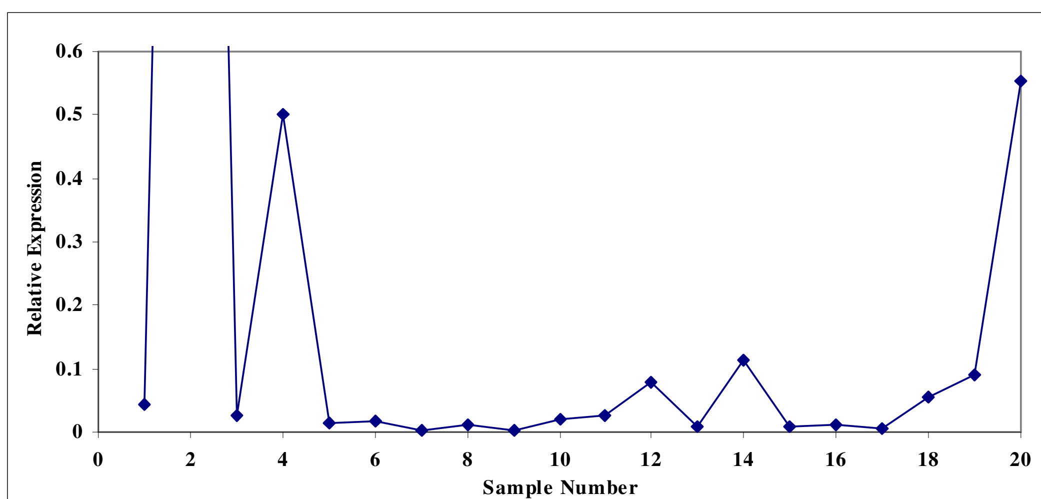


Figure 3.41. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

The injection of nitrogen pulse resulted in an immediate increase in the expression level of *GLC7* within the first minute in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)*. However the effect of pulse diminished after the first minute and then no significant change was observed. The expression level of *GLC7* at the second steady state was 12 fold higher than the initial one.

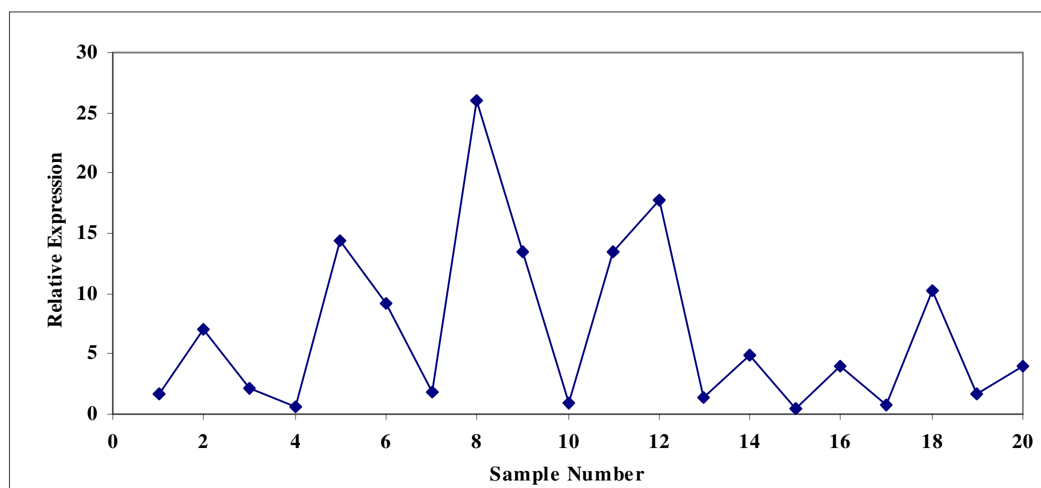


Figure 3.42. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain, a fluctuating behavior was observed in the expression level of the *GLC7* after the injection of nitrogen pulse. Comparison of the expression levels of this gene at two steady states showed that at the second steady state expression level was approximately 2 fold higher than the first steady state.

As it can be seen in Figure 3.43, expression level of *GLC7* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation prior to the pulse. The expression level of the gene increased at the 15th minute after the pulse injection and displayed an increasing trend in the following 7 hours. Seven hours later than the injection, the expression level of the *GLC7* decreased to undetectable values.

In *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain, expression level of *GLC7* showed a slight increase after the injection of nitrogen pulse. Then it showed a fluctuating behavior during the whole cultivation.

When the steady state levels were compared in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain, it was observed that the second steady state value was higher than the expression level at the first steady state.

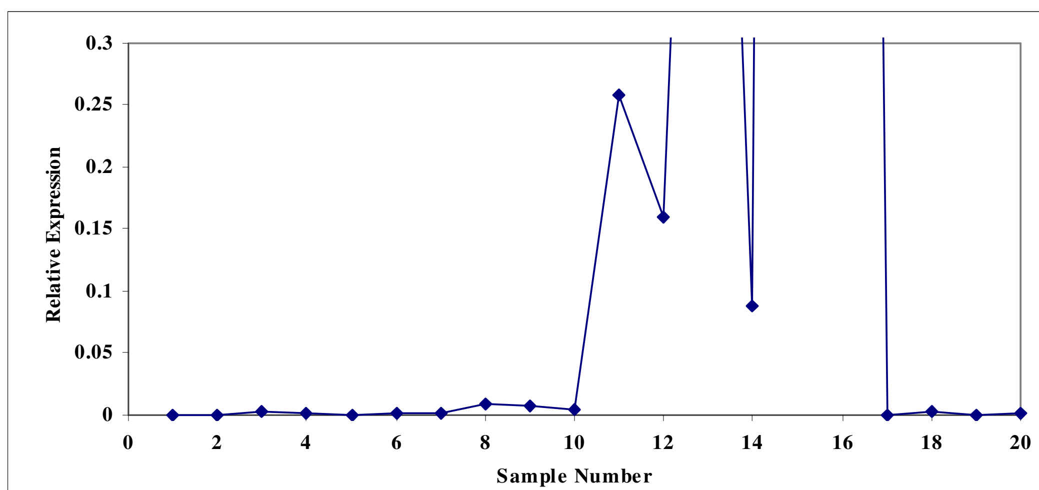


Figure 3.43. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

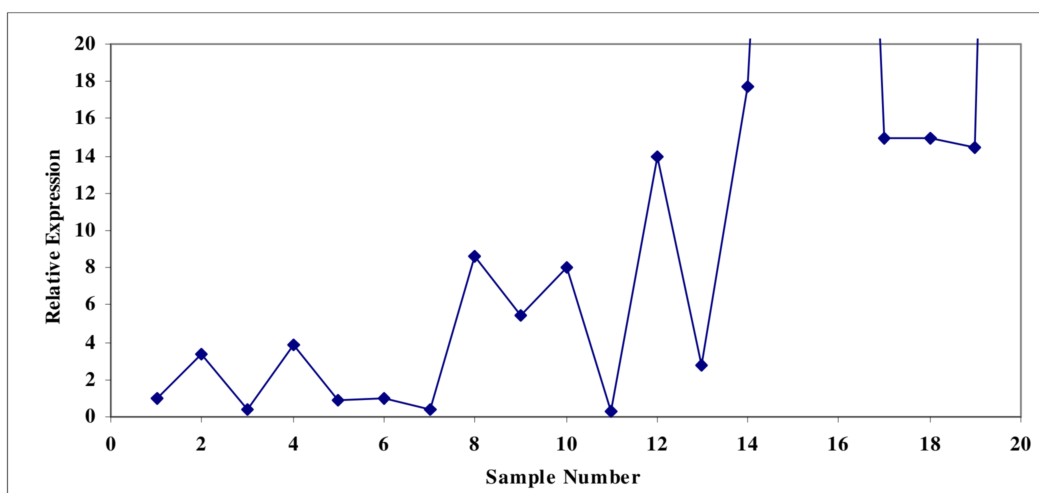


Figure 3.44. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.11. Expression Profile of *REG1* as a Response to Nitrogen Pulse

Reg1 is associated with Snf1 and is phosphorylated in a Snf1-dependent manner (Sanz *et al.*, 2000). The Reg1p protein is one of the regulatory subunits of type 1 protein phosphatase Glc7 (Tu and Carlson., 1995; Nath *et al.*, 2003). The expression profiles of *REG1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ*

and *RIP1/rip1*Δ*)* were presented in Figure 3.45, 3.46, 3.47 and 3.48 respectively. Samples were collected as previously stated.

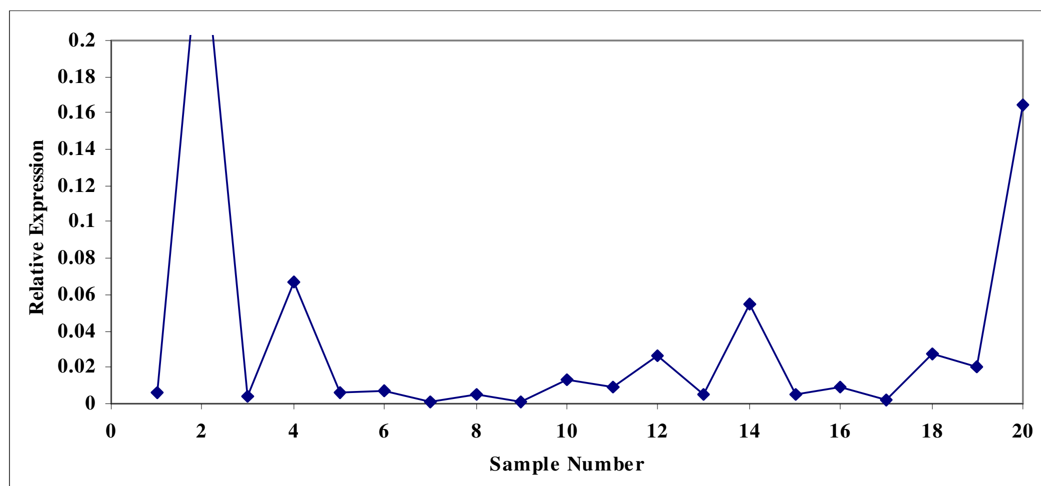


Figure 3.45. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *REG1* increased and reached a peak and rebounded very quickly within 30 seconds later than the injection in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection. Then no significant change was observed. At the second steady state 25 fold higher expression level was obtained in comparison to the initial case.

An increasing trend was observed in the relative expression of *REG1* soon after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)*. Fluctuating behavior in the expression level of this gene was observed during the sampling period. Steady state expression levels were close to each other.

Prior to the injection of nitrogen pulse, the expression level of *REG1* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures. An increase in the expression level of the gene could be detected after 15 minutes later than the pulse injection and this effect diminished within 5 hours. Relatively higher expression level was obtained at the second steady state in comparison to the initial level.

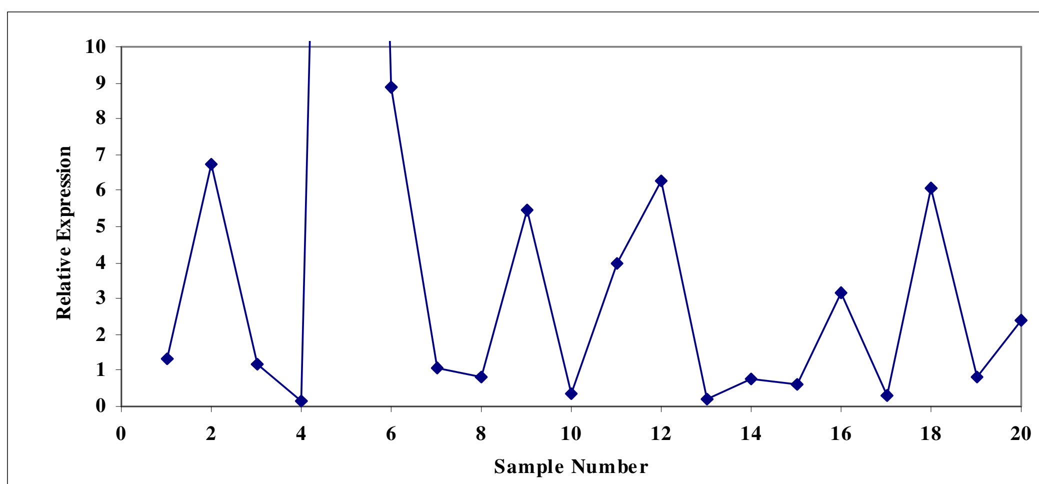


Figure 3.46. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

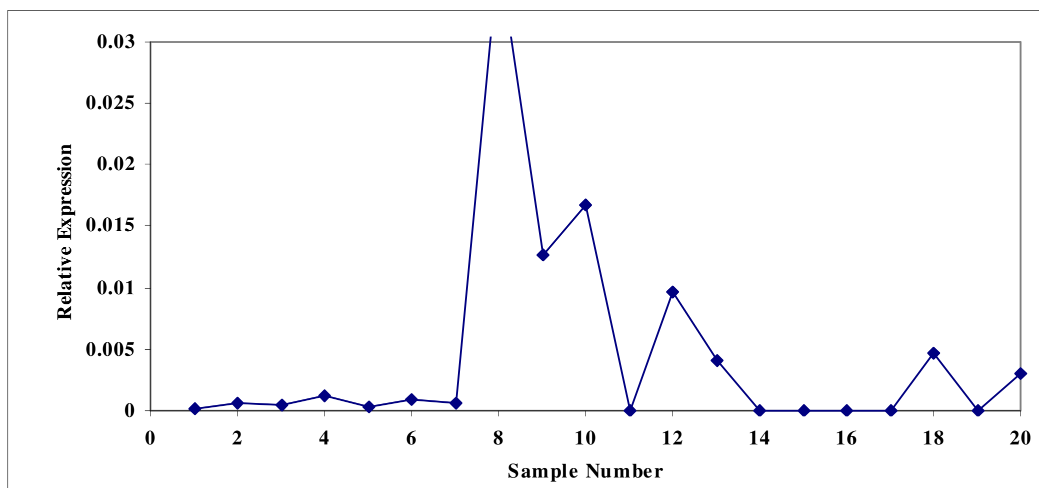


Figure 3.47. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *REG1* did not change significantly after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ).*

A fluctuated behavior was observed in the expression level of the gene and the second steady state expression level was higher than the initial level.

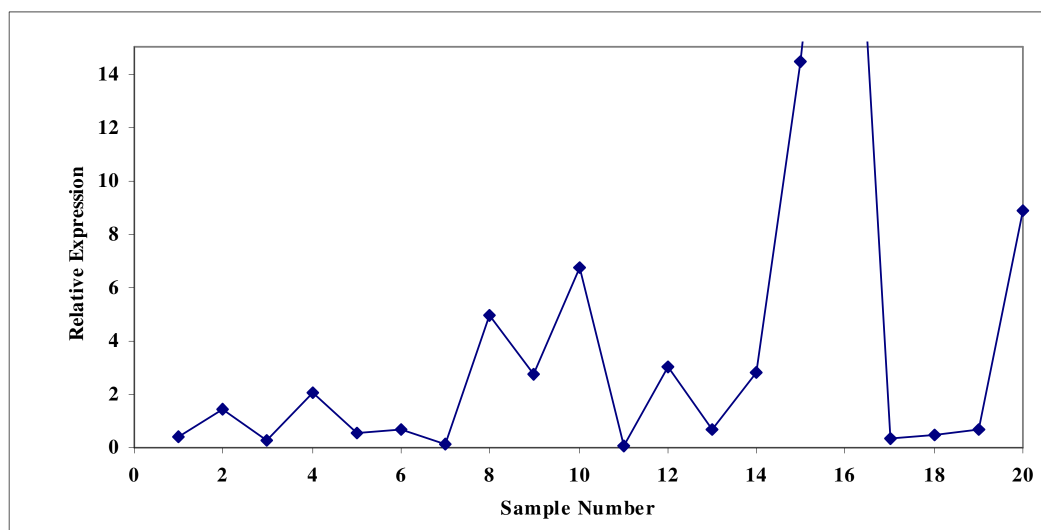


Figure 3.48. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.12. Expression Profile of *SNF1* as a Response to Nitrogen Pulse

Snf1 kinase is a heterotrimer. It contains catalytic α-subunit Snf1, the α-subunit Snf4 and one the related β-subunits Gal83, Sip1 or Sip2, which regulates the subcellular localization of the kinase complex (Vincent at al., 2001). The Snf1 protein kinase regulates this subcellular relocalization of Mig1 and it is active in glucose limited conditions.

The expression profiles of *SNF1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.49, 3.50, 3.51 and 3.52 respectively. Samples were collected as previously stated.

After the injection of nitrogen pulse, a fluctuating behavior was observed within the first minute in the expression level of SNF1 in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* grown under nitrogen limitation in chemostat. Then within the 20 minutes, no significant change was observed. Again a fluctuating behavior was seen after 20th minute. In comparison to the first steady state, expression level of this gene at the second steady state was 23 fold higher.

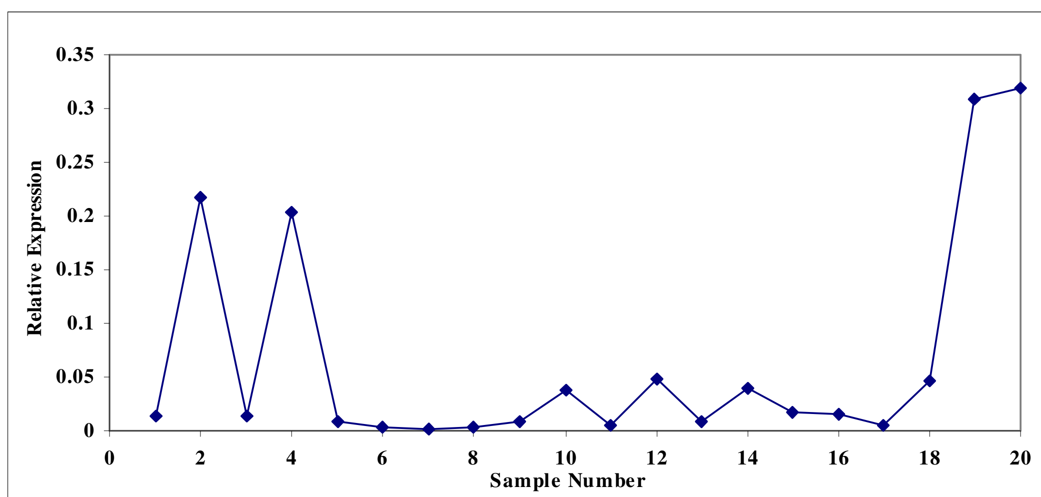


Figure 3.49. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

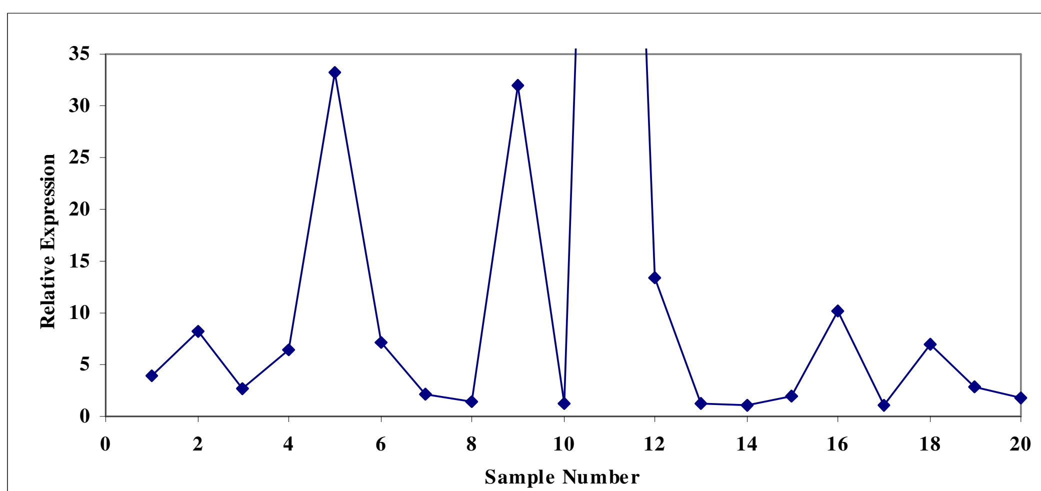


Figure 3.50. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Expression level of *SNF1* increased after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)*. The expression level at the second steady state was lower than the first steady state expression level.

In the case of *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)*, the expression level of *SNF1* did not change significantly until the fifth minute after the injection of nitrogen pulse.

Comparing the initial and second steady state values, it is seen that, second steady state value was much higher.

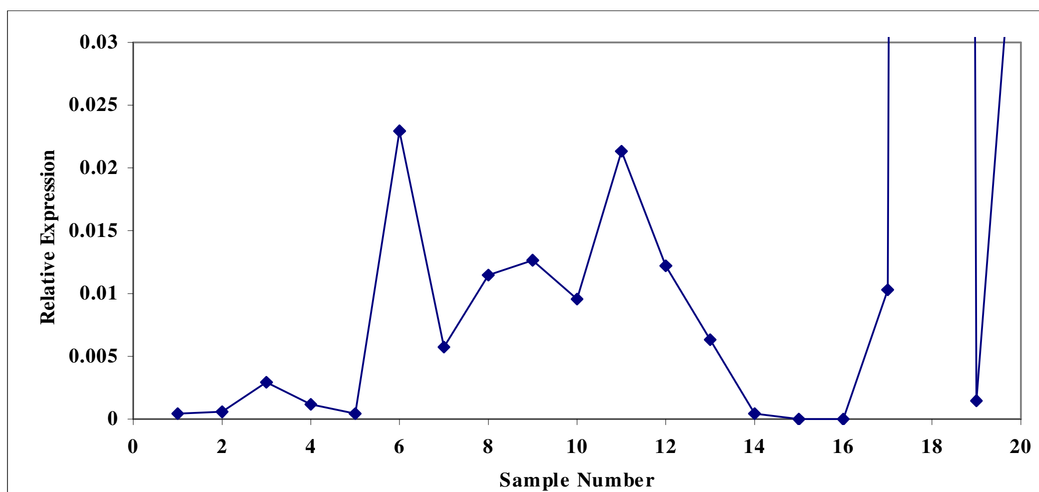


Figure 3.51. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

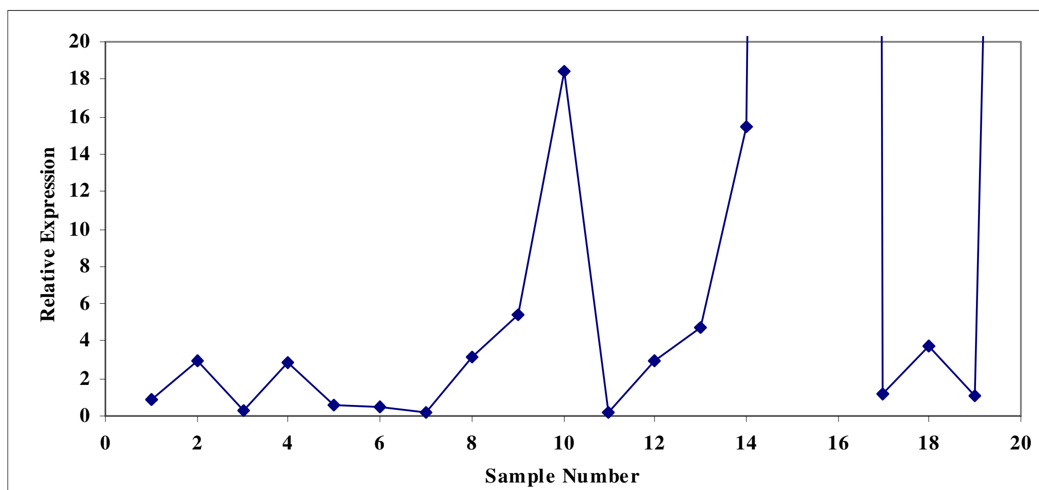


Figure 3.52. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The respond of *SNF1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain showed similarities with homozygous deletion mutant of *RIP1*. The increasing trend in the expression levels began at the 15th minute. The second steady state value was also much higher than the first steady state value.

3.2.13. Expression Profile of *SNF4* as a Response to Nitrogen Pulse

Snf4p is activating gamma subunit of the AMP-activated Snf1p kinase complex. It activates glucose-repressed genes and represses glucose-induced genes.

The expression profiles of *SNF4* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.53, 3.54, 3.55 and 3.56 respectively. Samples were collected as previously stated.

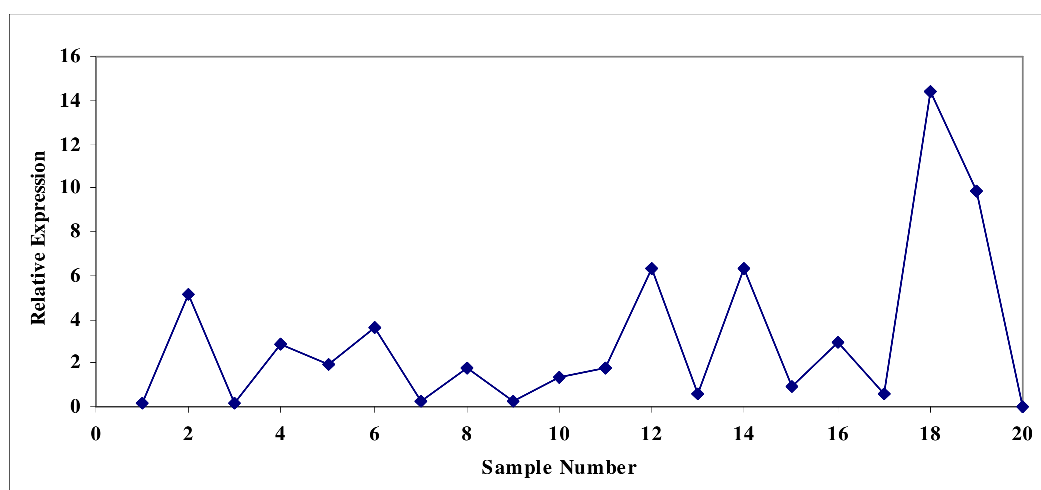


Figure 3.53. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

SNF4 expression showed a fluctuating behavior after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under nitrogen limitation in chemostat cultures. At the second steady state, the expression level of *SNF4* was undetectable.

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain, after the injection of nitrogen pulse an increase in the expression level of *SNF4* was observed. Comparing the steady state expression levels, it is seen that, initial and the last values were close to each other.

In homozygous deletion mutant of *RIP1*, the expression level of *SNF4* showed a continuous and slight increase until the fourth hour. After a decrease at the fifth hour, the

expression value reached a peak at 9th hour. At the second steady state, the expression level of *SNF4* was four fold higher than the first steady state level.

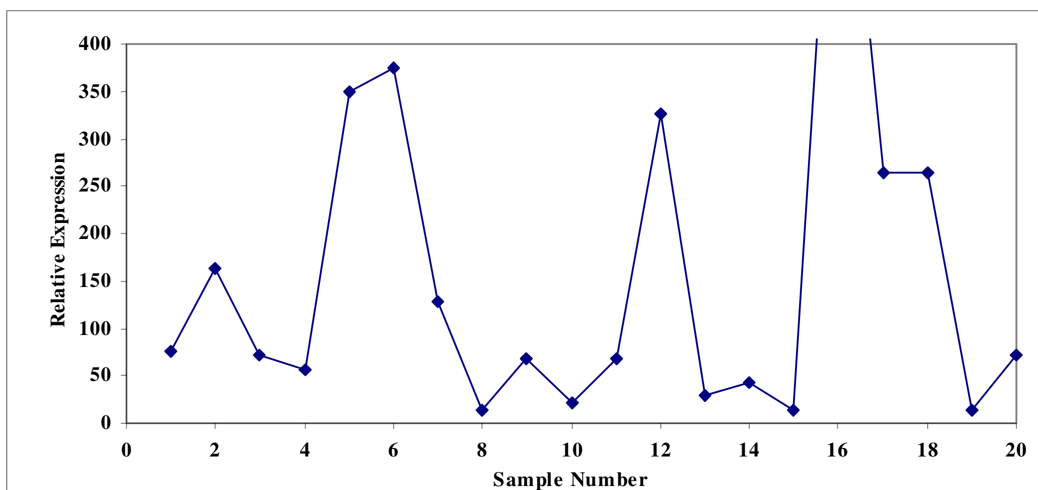


Figure 3.54. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

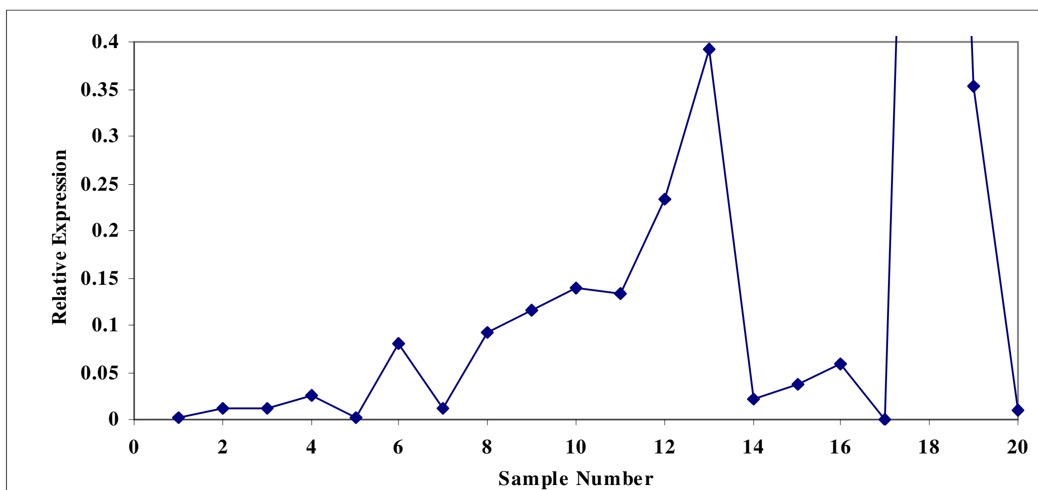


Figure 3.55. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain, *SNF4* expression level showed a fluctuating behavior. Expression level at the second steady state was 18 fold higher than the first steady state level.

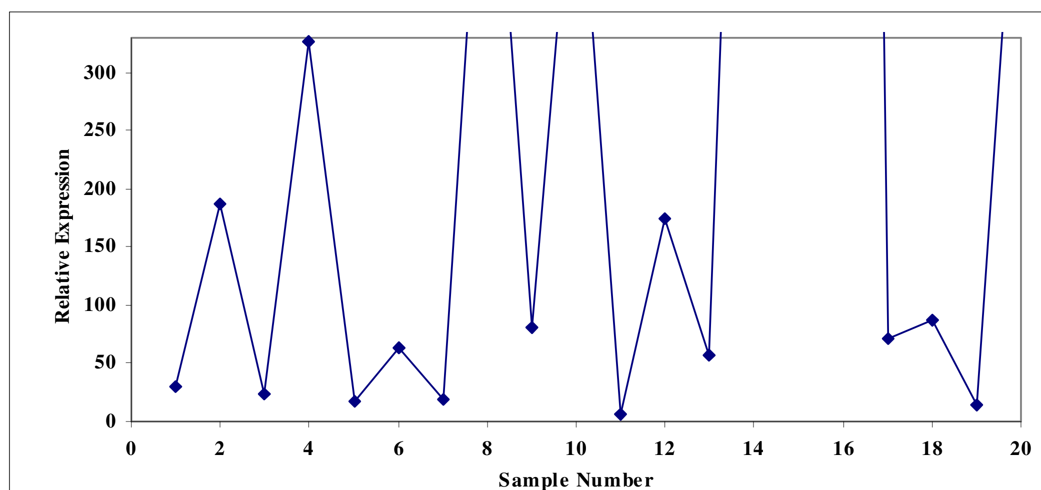


Figure 3.56. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.14. Expression Profile of *PAK1* as a Response to Nitrogen Pulse

Pak1 is associated with either Snf1 or Snf4 (Gavin *et al.*, 2002). Snf1 kinase requires *PAK1* for maximal activity. Pak1 kinase is able to stimulate Snf1 dependent phosphorylation of Mig1 (Nath *et al.*, 2003).

The expression profiles of *SNF4* in deletion strains of *Saccharomyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.57, 3.58, 3.59 and 3.60 respectively. Samples were collected as previously stated.

Nitrogen pulse resulted in a six fold initial increase in the expression level of *PAK1* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures. However, it immediately decreased to its initial value and remained close to it until the second hour. But also this sharp increase at the second hour rebounded very quickly. Expression level at the second steady state was 12 fold higher than the initial level.

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)*, a three fold increase in the expression level of *PAK1* was observed after the injection of nitrogen pulse. No significant change was observed in the expression level of *PAK1* after 7 hours later than the pulse injection. The expression levels after that time were lower than the initial level.

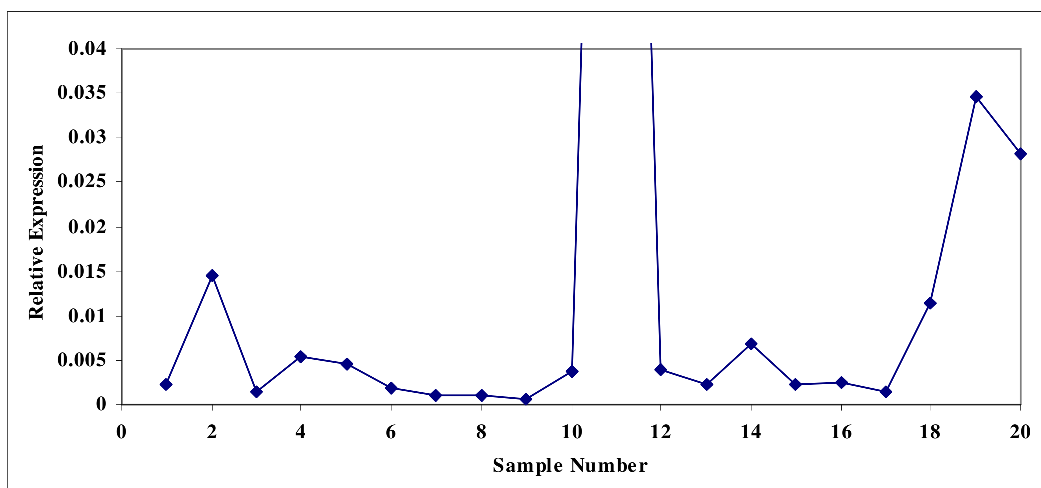


Figure 3.57. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

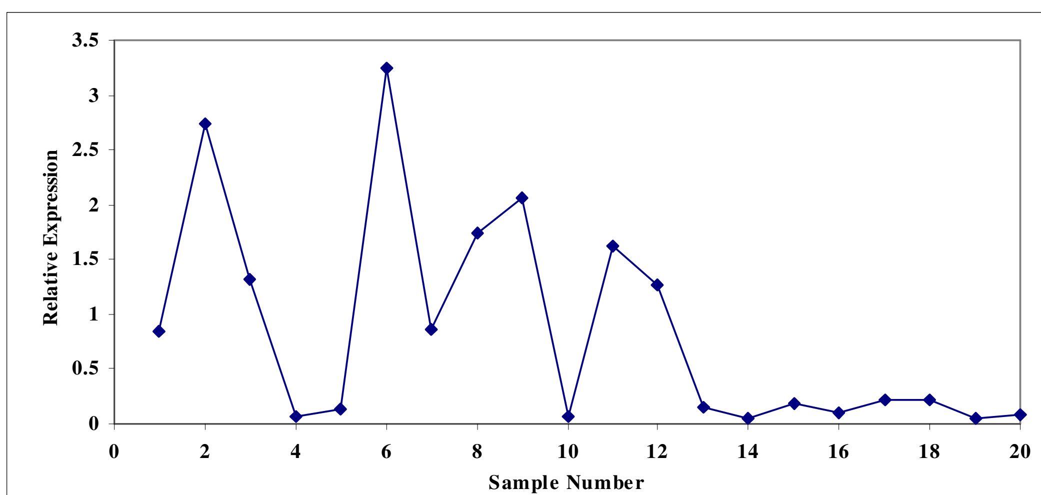


Figure 3.58. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The injection of nitrogen pulse caused no significant change in the expression of *PAK1* in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain within the first 10 minutes after the pulse injection. Fluctuating behavior in the expression level of this gene was observed during the period of time between 20 minute and 7 hour later than the pulse injection. The expression level of *PAK1* at the second steady state was undetectable as it was the case for first steady state.

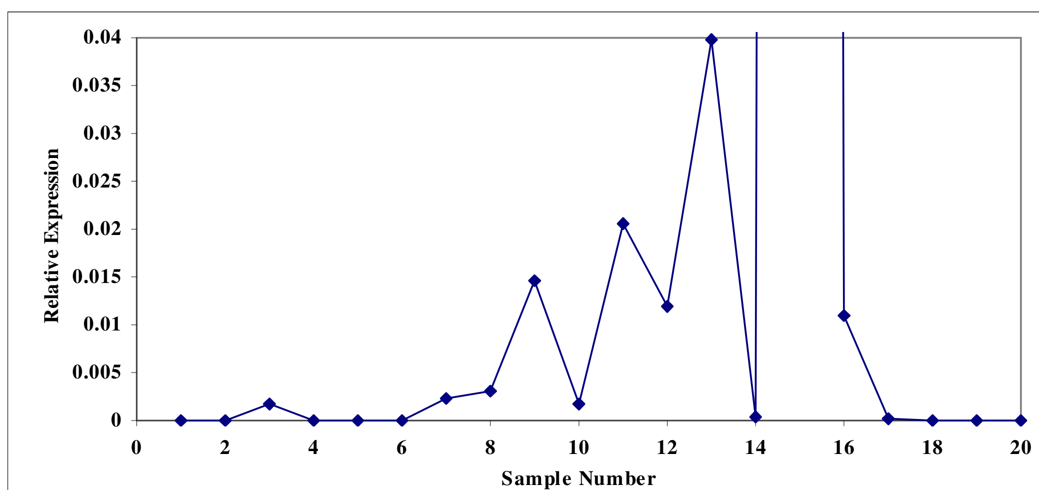


Figure 3.59. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

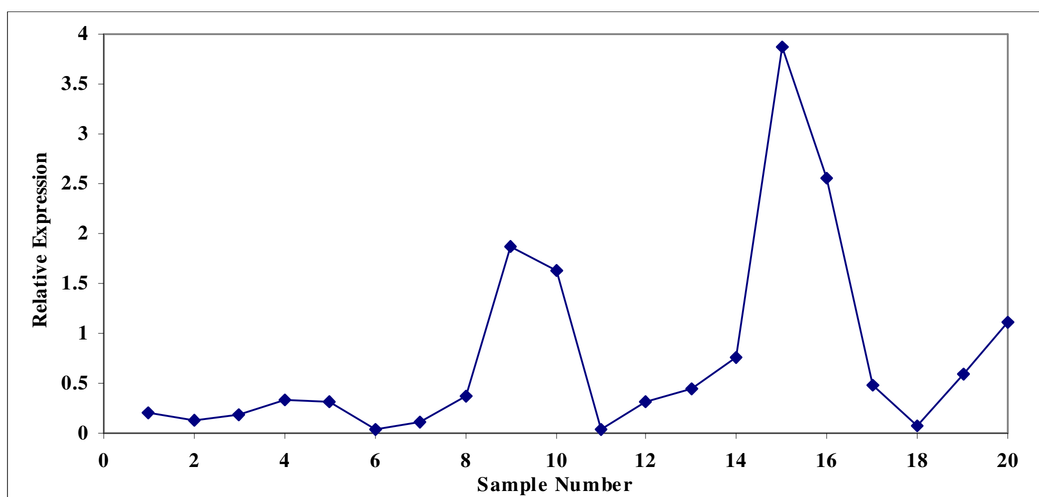


Figure 3.60. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

In the case of heterozygous mutant of *RIP1*, no significant change was observed in the expression level of *PAK1* soon after the injection of nitrogen pulse. One hour later than the pulse injection, increases were begun observed in the expression of *PAK1*. When the first and the second steady state expression levels were compared it was observed that the second steady state expression level was 5 fold higher than the initial value.

3.2.15. Expression Profile of *TOS3* as a Response to Nitrogen Pulse

Elm1p, Pak1p and Tos3p are members of a subfamily of yeast kinases that can phosphorylate Thr210 on Snf1p. At least one of these proteins is required for the activation of the *SNF1* complex in the glucose limited condition (Sutherland *et al.*; 2003).

The expression profiles of *TOS3* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.61, 3.62, 3.63 and 3.64 respectively. Samples were collected as previously stated.

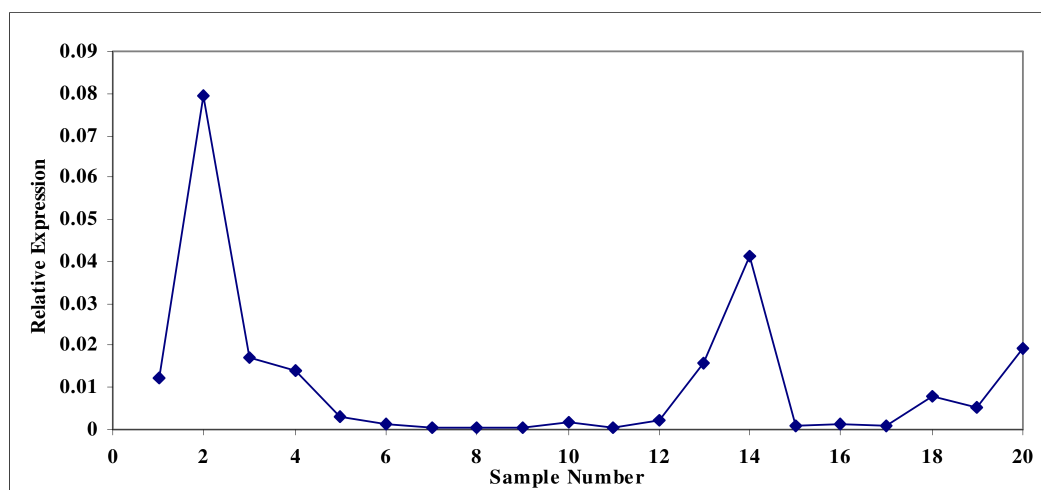


Figure 3.61. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

Investigating the expression level of *TOS3* resulted in the observation that after the injection of nitrogen pulse, the expression level reached a peak and immediately rebounded. The expression level of this gene at the second steady state was close to that of the first steady state level.

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain, after the injection of nitrogen pulse, fluctuating behavior was observed in the expression levels of *TOS3*. In the second steady state, the expression level of *TOS3* decreased in comparison to the initial value.

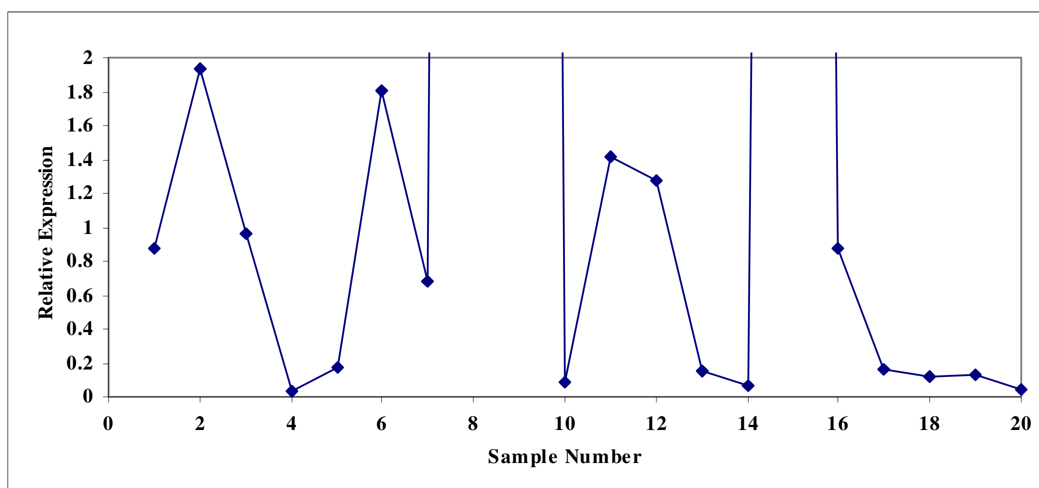


Figure 3.62. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

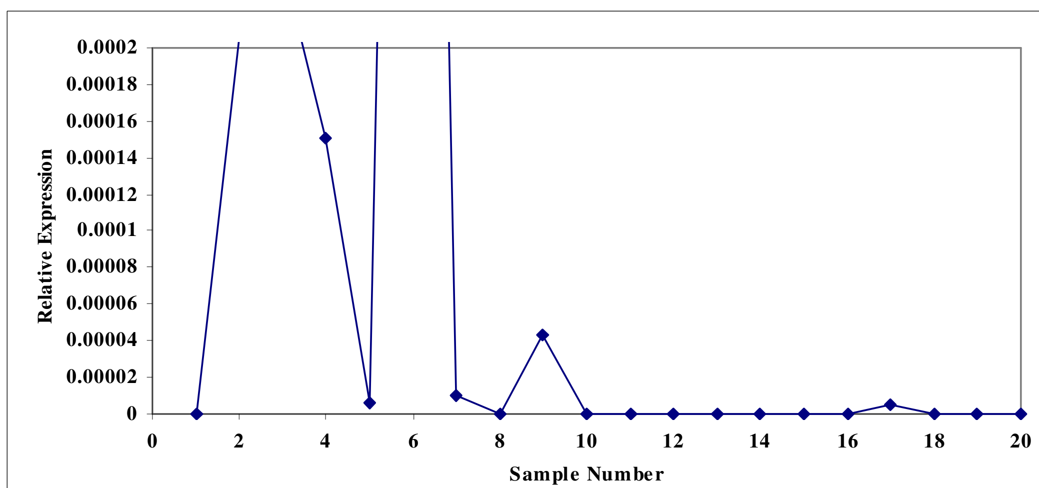


Figure 3.63. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Prior to the pulse injection, the expression of *TOS3* was not detectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* grown under nitrogen limitation in chemostat cultures. With the injection of nitrogen pulse an immediate increase was observed.

The expression of this gene could not be detected 1 hour later than the pulse injection and remained undetectable at the second steady state.

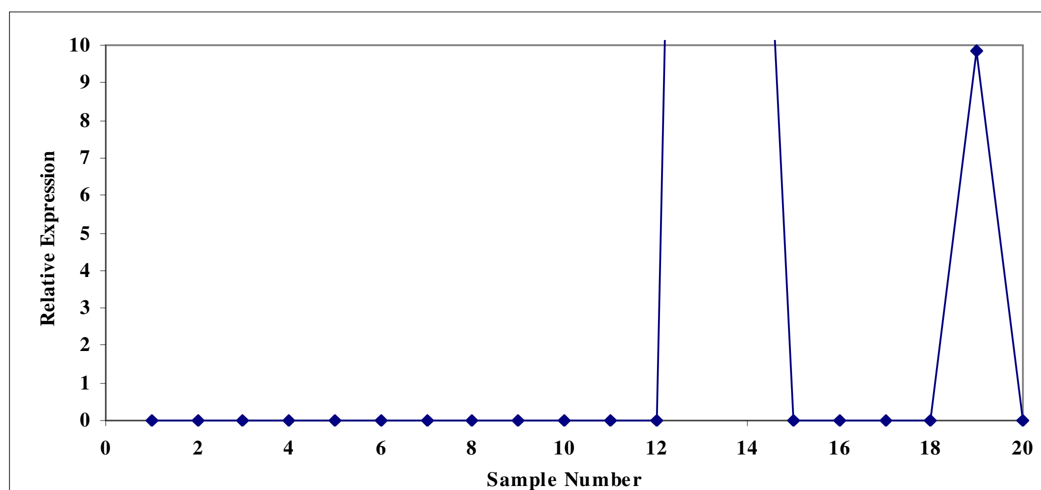


Figure 3.64. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *TOS3* was similarly undetectable prior to the pulse injection in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*. After the injection of ammonium sulfate pulse, its expression level remained unchanged until the 3 hours later than the pulse injection. Higher levels of expressions were observed at 4, 5 and 10 hour later than the injection. The expression level of *TOS3* decreased to undetectable levels again at the second steady state.

3.2.16. Expression Profile of *ELM1* as a Response to Nitrogen Pulse

Elm1p, is also members of a subfamily of yeast kinases that can phosphorylate Thr210 on Snf1p. On the other hand Elm1p has regulatory functions involving cell morphology, filamentous invasive growth that may have no connection to the Snf1 pathway (Hong *et al.*; 2003).

In all deletion strains of *Saccaromyces cerevisiae* that are studied (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ*) the expression level of *ELM1* was undetectable prior or after the nitrogen pulse injection throughout the cultivation.

3.2.17. Expression Profile of *MIG1* as a Response to Nitrogen Pulse

MIG1 encodes a C_2H_2 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*. The yeast Mig1 repressor shuttles between the nucleus and cytoplasm in response to glucose (Nehlin and Ronne, 1990; De Vit *et al.*, 1997; Ostling& Ronne, 1998; Papamichos-Chronakis *et al.*, 2004). It is imported into the nucleus within the minutes after the addition of glucose to the growth medium and is just as rapidly transported back to the cytoplasm when glucose is removed.

The expression profiles of *MIG1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.65, 3.66, 3.67 and 3.68 respectively. Samples were collected as previously stated.

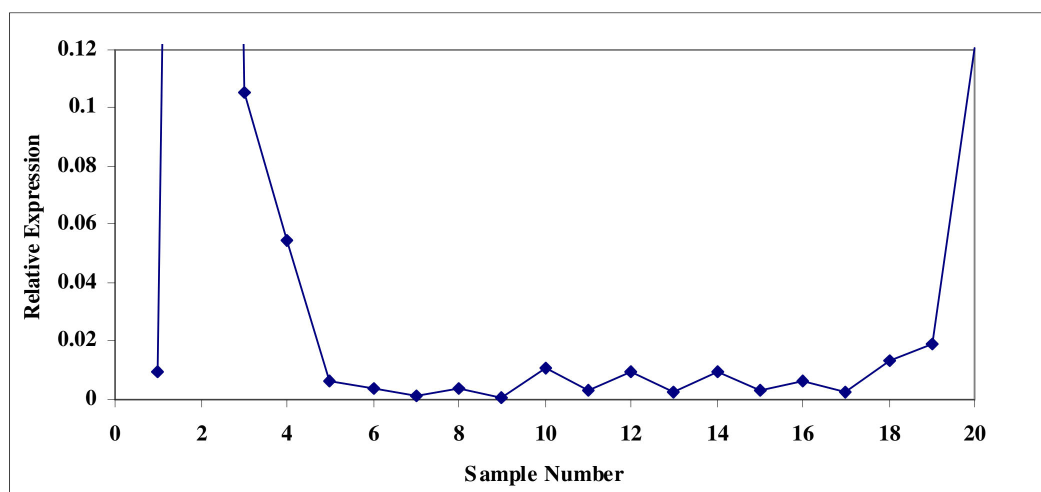


Figure 3.65. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

Injection of the nitrogen pulse resulted in a sharp increase in the expression level of *MIG1* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection. The expression level of the gene decreased sharply within the first minute, remained constant within the 10 hours later than the pulse injection. Comparison of the first and second steady state values indicated that expression level at the second steady state was almost 13 fold higher than the first steady state level.

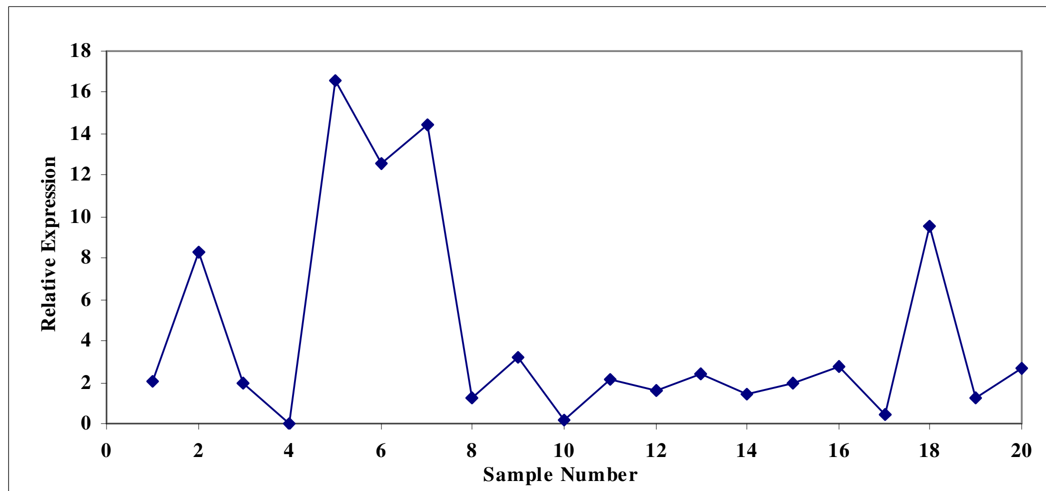


Figure 3.66. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The nitrogen pulse resulted in the initial four fold increase in the expression level of the *MIG1* in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* grown under nitrogen limitation. The expression levels of this gene at the first and second steady states were close to each other.

In *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* the expression level of *MIG1* showed a slight increase soon after the injection of nitrogen pulse, Higher levels of expression were observed during the period of time between10 minutes and 5 hours later than the pulse injection. Its expression level at the second steady state was also higher in comparison to the first steady state.

After the nitrogen pulse, a fluctuating behavior was observed in the expression level of *MIG1* in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ).* The expression level at the second steady value was 3 fold higher than the expression level at the first steady state.

3.2.18. Expression Profile of *CYC8* as a Response to Nitrogen Pulse

It is a general transcriptional co-repressor and acts together with Tup1p. It also acts as part of a transcriptional co-activator complex that recruits the SWI/SNF and SAGA complexes to promoters. It is believed that Mig1 inhibits transcription by recruiting the general co-repressor complex Cyc8 (Ssn6)-Tup1 (Nehlin *et al.*, 1991; Treitel & Carlos,

1995; Tzamarias& Struhl, 1995; Papamichos-Chronakis *et al.*, 2004). It is shown that Cyc8 interact specifically with the non-phosphorylated form of Mig1 and, upon glucose depletion, Snf1-dependent phosphorylation of Mig1 releases its interaction with Cyc8-Tup1.

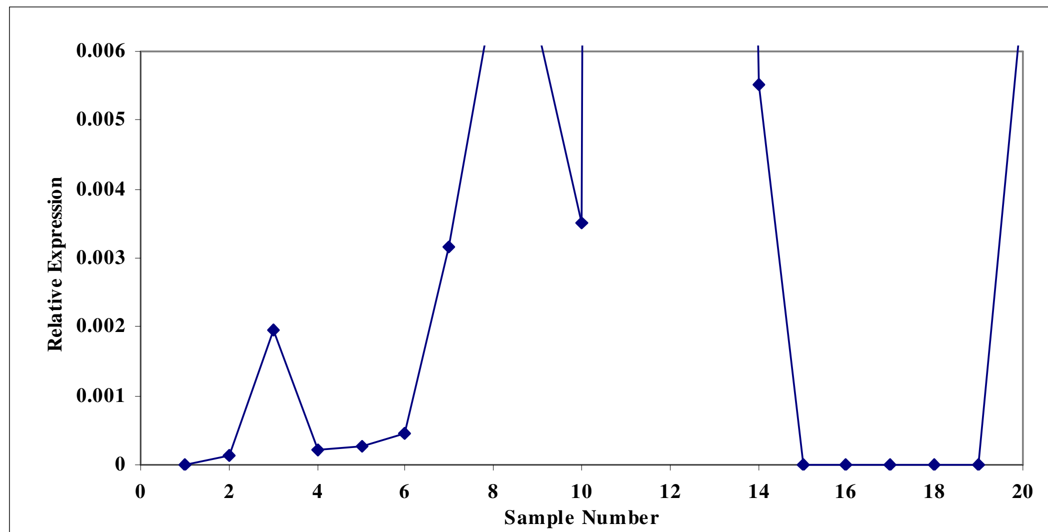


Figure 3.67. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

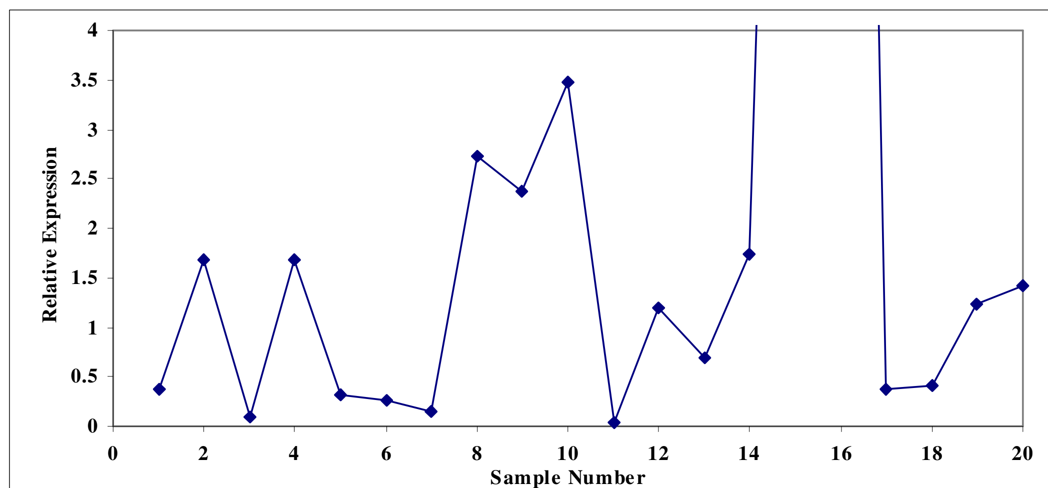


Figure 3.68. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression profiles of *CYC8* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.69, 3.70, 3.71 and 3.72 respectively. Samples were collected as previously stated.

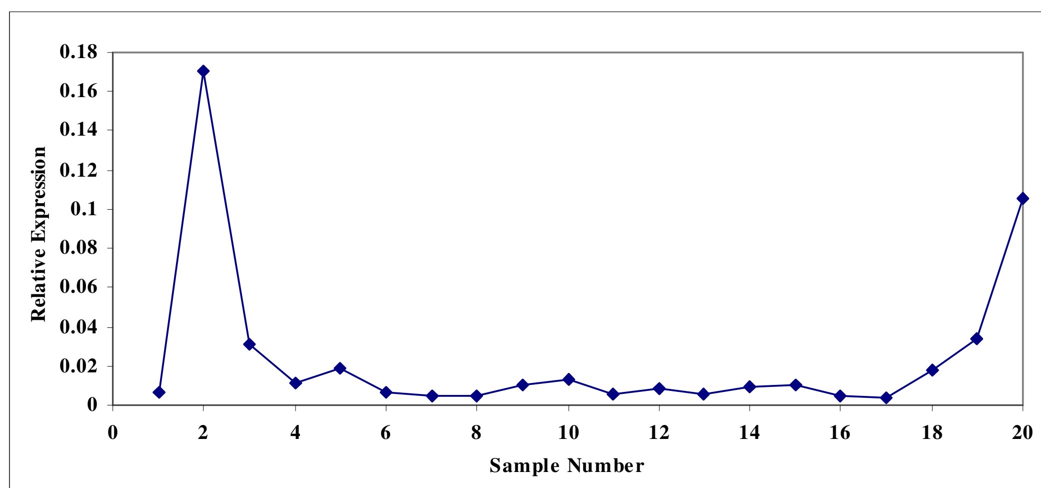


Figure 3.69. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

As it can be seen from the Figure 3.69, nitrogen pulse resulted in an immediate increase in the expression level of *CYC8* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures. Then it rebounded to its initial value and did not show a significant change throughout the experiment. However, its expression level at the second steady state was 15 fold higher than the initial level.

The expression level of *CYC8* showed a two fold increase after the injection of nitrogen pulse in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection. Then within the first minute after the injection, the expression level of *CYC8* decreased below its initial level.

During the period of time between the 1 minute and 2 hours later than the injection, fluctuating behavior was observed. The expression level at the second steady state was 10 fold lower than the initial level.

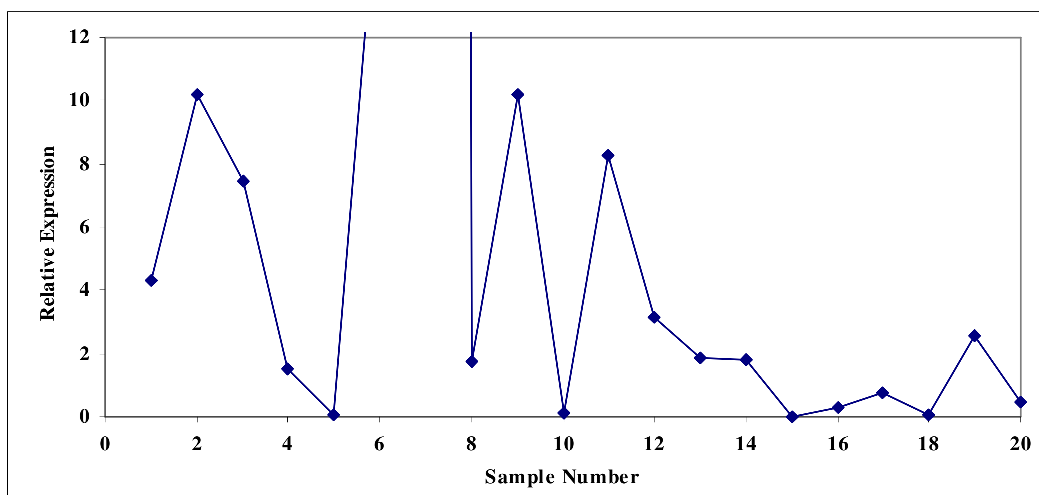


Figure 3.70. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

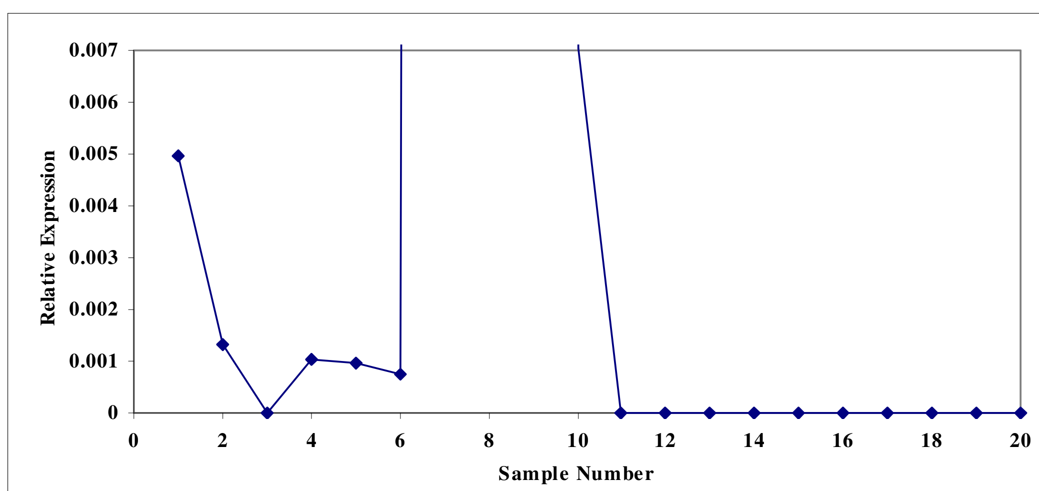


Figure 3.71. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression level of *CYC8* decreased sharply within the 30 seconds later than the pulse injection in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain and increased again during the time duration between the 10 and 20 minutes later than the injection. The expression level of the gene then remained constant and undetectable until the end of the experiment.

Prior to the injection of nitrogen pulse, the expression level of *CYC8* was undetectable. It increased within 30 seconds later than the pulse injection. Expression level

of *CYC8* showed a fluctuating behavior throughout the experiment. A high level of expression was observed at the second steady state.

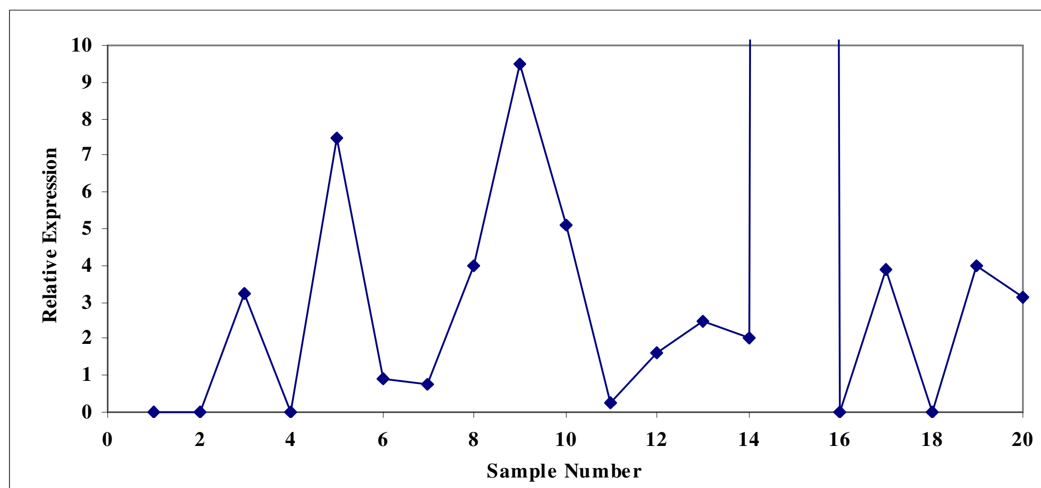


Figure 3.72. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.19. Expression Profile of *TUP1* as a Response to Nitrogen Pulse

Tup1p is general repressor of transcription and it forms complex with Cyc8p, involved in the establishment of repressive chromatin structure through interactions with histones H3 and H4. It is believed that Mig1 inhibits transcription by recruiting the general co-repressor complex Cyc8 (Ssn6)-Tup1 (Nehlin *et al.*, 1991; Treitel & Carlos, 1995; Tzamarias& Struhl, 1995; Papamichos-Chronakis *et al.*, 2004).

The expression profiles of *TUP1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were presented in Figure 3.73, 3.74, 3.75 and 3.76 respectively. Samples were collected as previously stated.

After the injection of nitrogen pulse, the expression level of *TUP1* increased two fold in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* strain. During the period of time between the five minutes and the four hours later than the injection, lower expression levels were observed in comparison to its initial value. After nine hours later than the injection, an increasing

trend was observed in the expression level of *TUP1*. At the second steady state, *TUP1* was overexpressed.

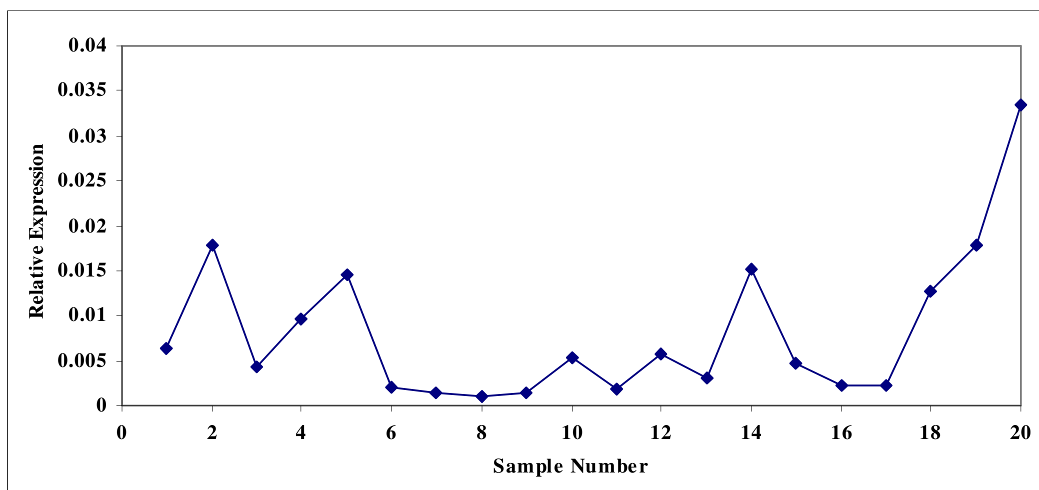


Figure 3.73. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

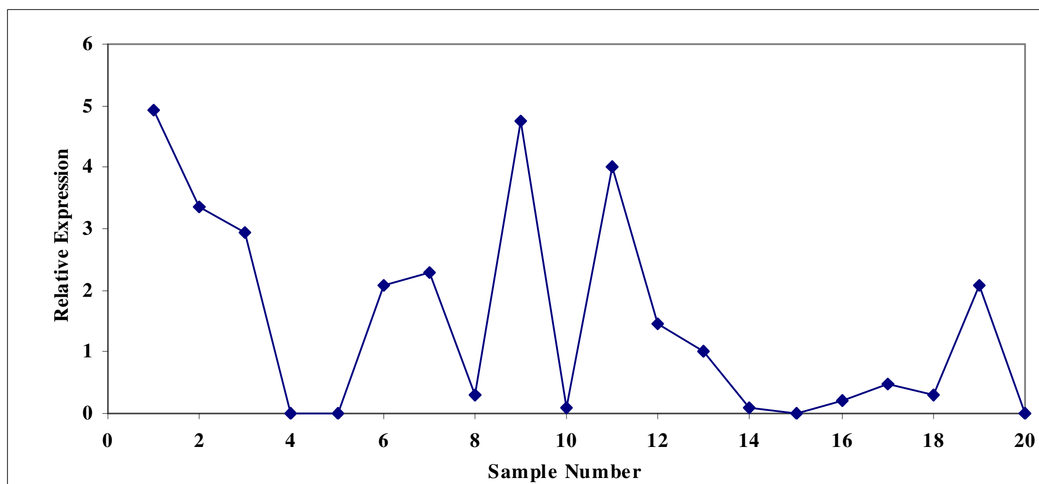


Figure 3.74. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

After the injection of nitrogen pulse, the expression level of *TUP1* decreased in strain which had a homozygous deletion of *HAP4*. At the end of the first minute, expression level decreased to undetectable levels. During the time duration between the five minutes and 2

hours later then the pulse injection fluctuating behavior was observed. At the second steady state, expression level of *TUP1* decreased again to undetectable levels.

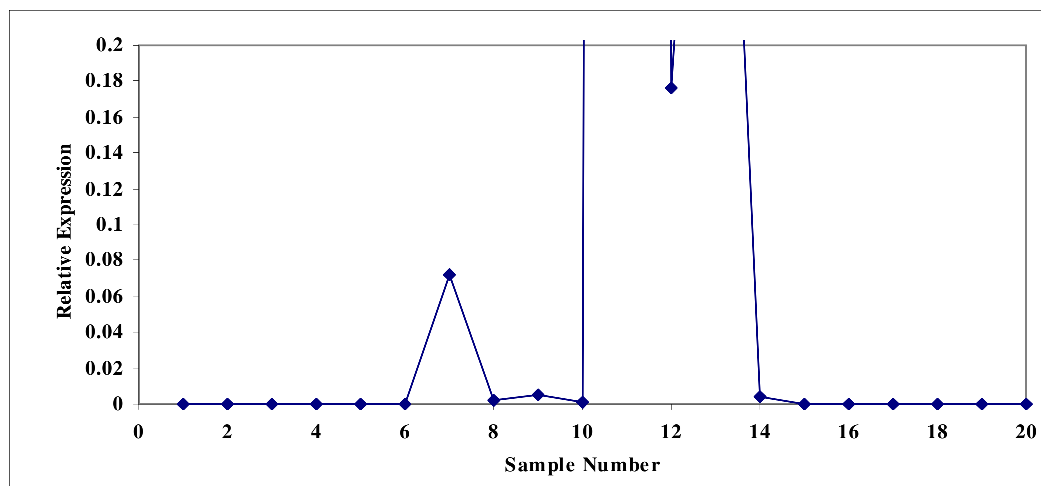


Figure 3.75. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Prior to the nitrogen pulse, expression level of *TUP1* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* grown under nitrogen limitation in chemostat cultures. Ten minutes later than the pulse injection, increase was observed in the expression level of *TUP1*. However 6 hours later than the injection expression level decreased back to undetectable levels. Also at the second steady state, expression level of *TUP1* was undetectable.

A slight decrease was observed after the injection of nitrogen pulse in the expression level of *TUP1* in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain. At the 20th minute and the 6th hour two sharp increases were observed however they immediately rebounded. The expression level at the second steady state was 2.5 fold higher than the initial level.

3.2.20. Expression Profile of *HXK2* as a Response to Nitrogen Pulse

Hxk2p is a protein that initiates the intracellular metabolism of glucose by phosphorylation at C-6, but in addition it plays a vital role in glucose repression (Moreno and Herrero *et al.*, 2002). One major function of Hxk2 may be to inhibit Snf1 protein

kinase activity by blocking Mig1 phosphorylation at nuclear level. Nuclear localization of Hxk2 is regulated by glucose and depends on Mig1. There is a direct correlation between the amount of Hxk2 located in the nucleus and the level of Mig1 in the cell (Ahuatzi *et al.*, 2004).

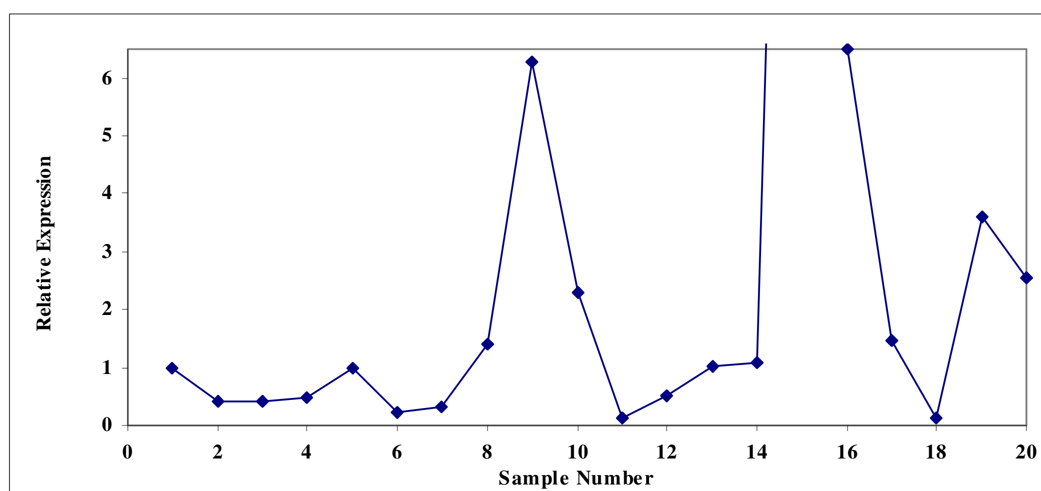


Figure 3.76. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression profiles of *HXK2* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were represented shown in Figure 3.77, 3.78, 3.79 and 3.80 respectively. Samples were collected as previously stated.

Injection of nitrogen pulse resulted in the 13 fold increase in the expression level of *HXK2* but it immediately dipped below its initial level in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* strain. No significant change was observed in this strain until 1 hour later than the pulse injection. During the time duration between the 1 and 6 hours later than the injection, fluctuating behavior was observed in the expression levels of this gene. After seventh hour an increasing trend was observed. The highest expression level of *HXK2* was observed at the second steady state.

After the injection of nitrogen pulse, the expression level of *HXK2* had displayed a sharp decrease within the first minute. 20 minutes later than the pulse injection a sharp increase was observed. Also 2 hours later than the injection, the expression level increased

sharply and decreased to undetectable levels within three hours. At the second steady state, the expression level of *HXK2* was undetectable.

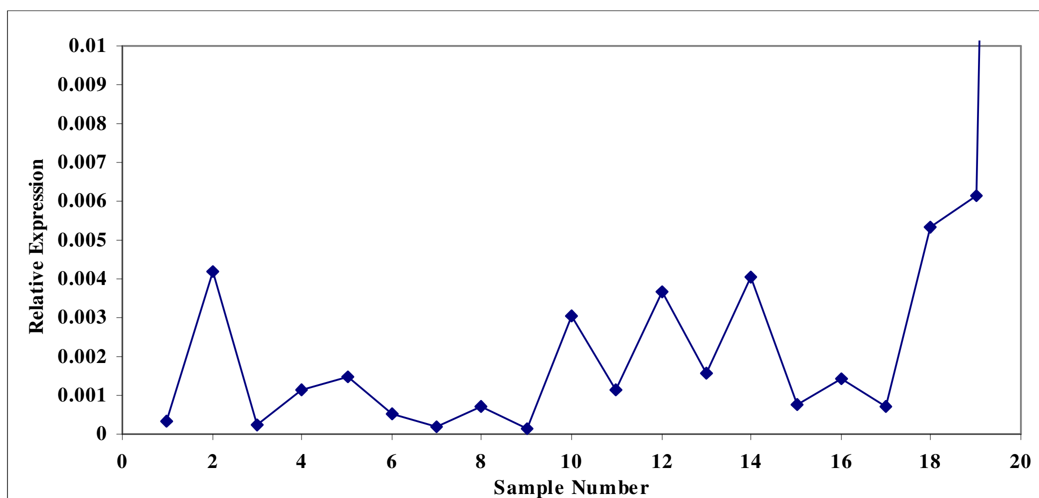


Figure 3.77. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

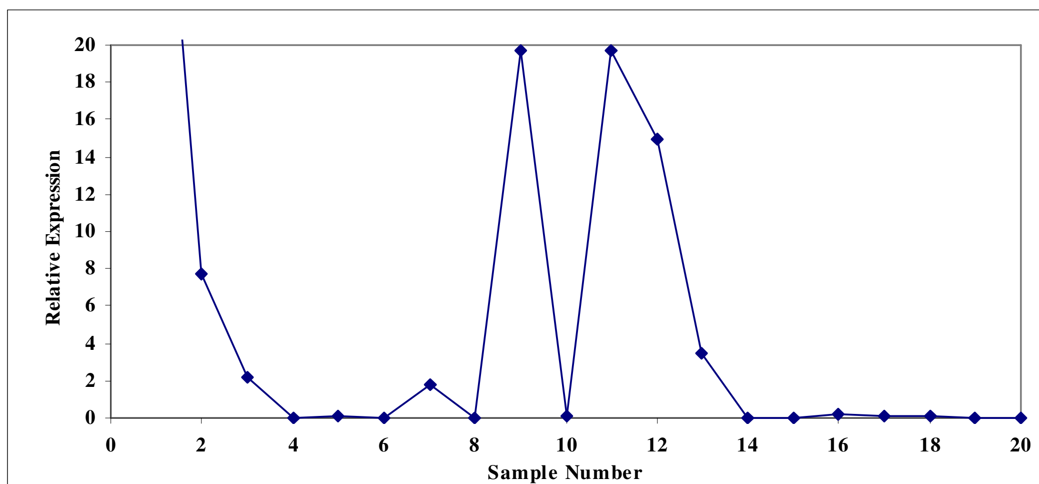


Figure 3.78. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

As it can be seen from Figure 3.79, the expression level of *HXK2* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain before the pulse injection. A sharp increase in the expression level of this gene could be detected after 15 seconds of the injection.

However its expression level dropped to unnoticeable levels and remained constant until the second steady state.

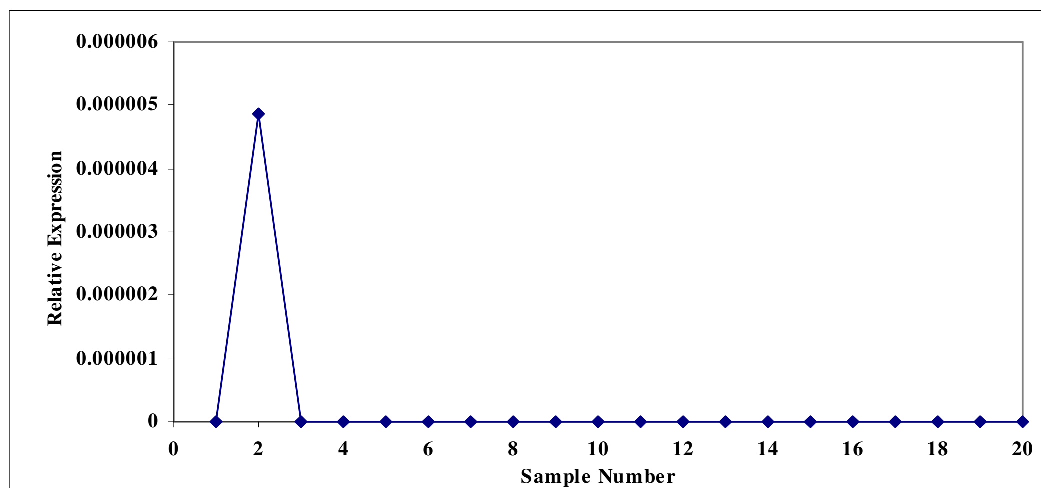


Figure 3.79. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

Also in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ*) strain expression level of *HXK2* was undetectable before the injection of nitrogen pulse and remained constant soon after the pulse injection. An increase in the expression level could be observed only one hour later than the pulse injection. Higher level of expression of *HXK2* was observed at the second steady state.

3.2.21. Expression Profile of *HAP4* as a Response to Nitrogen Pulse

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).

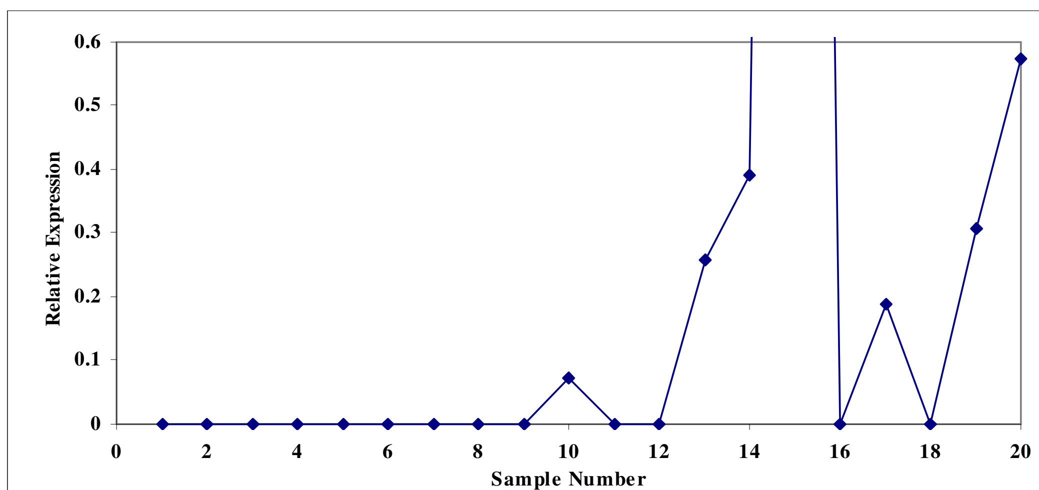


Figure 3.80. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

The expression profiles of *HXK2* in deletion strains of *Saccaromyces cerevisiae* (*ho*Δ/*ho*Δ, *rip1*Δ/*rip1*Δ and *RIP1/rip1*Δ*)* were represented in Figure 3.81, 3.82 and 3.83 respectively. Samples were collected as previously stated.

An increase in the expression level of *HAP4* was observed after the nitrogen pulse injection in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* grown under nitrogen limitation in chemostat cultures. However this effect diminished within the first minute. The expression level at the second steady state was higher in comparison to the first steady state level.

As it can be seen in the Figure 3.82, in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain, the expression level of *HAP4* was undetectable and no change in the expression level of this gene was observed with the injection of nitrogen pulse. Only at the seventh sample, which corresponded to ten minutes after the pulse, *HAP4* was expressed and then decreased again to undetectable levels and remained constant throughout the cultivation.

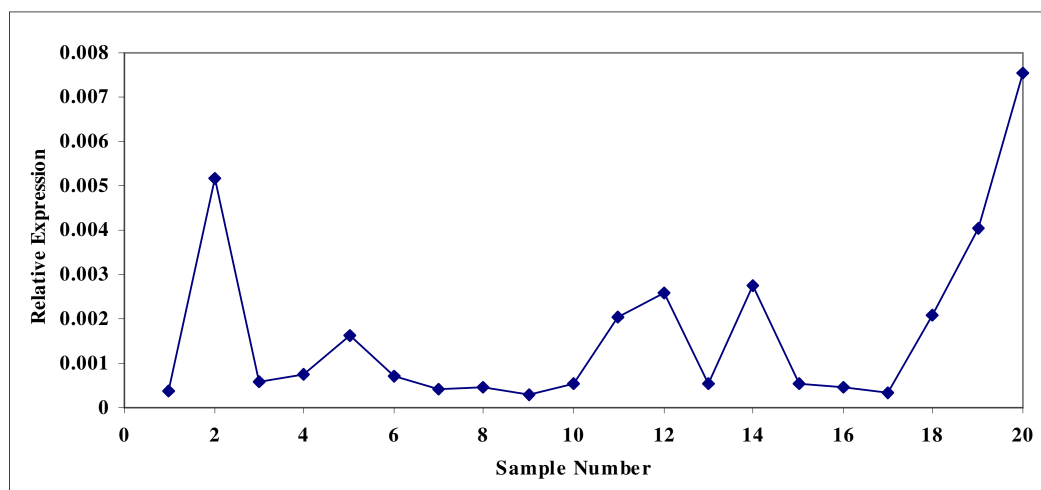


Figure 3.81. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

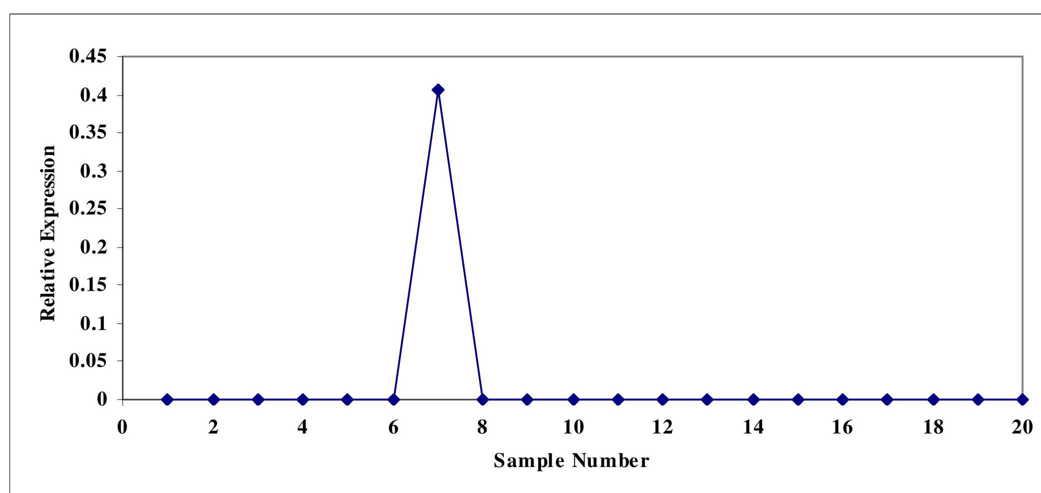


Figure 3.82. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

As it was the case for the homozygous deletion mutant of *RIP1*, expression level of *HAP4* was not detectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain. 5 minutes later than the pulse injection, the expression level of this gene increased to detectable levels. However, 4 hours later than the injection, the expression level of *HAP4* decreased back to undetectable levels. At the second steady state, the relative expression of *HAP4* was similarly not at detectable levels.

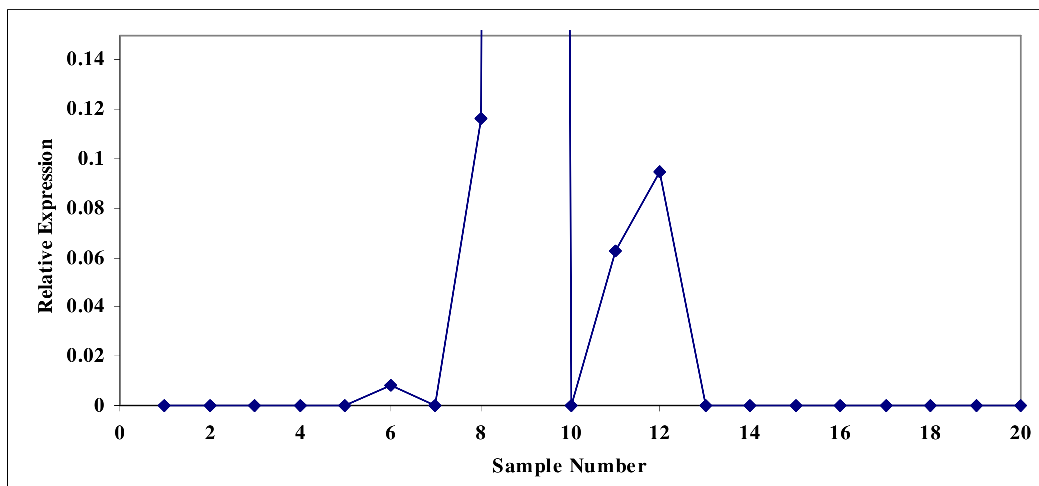


Figure 3.83. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

3.2.22. Expression Profile of *MBA1* as a Response to Nitrogen Pulse

Mba1p is a protein involved in assembly of mitochondrial respiratory complexes. It may act as a receptor for proteins destined for export from the mitochondrial matrix to the inner membrane (Herrmann *et al.*, 2001).

The expression profiles of *MBA1* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were shown in Figure 3.84, 3.85, 3.86 and 3.87 respectively. Samples were collected as previously stated.

Investigation of the effect of nitrogen pulse on the *MBA1* expression level in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* revealed the presence of an immediate increase in the expression of this gene following the pulse injection. The second steady state expression level of *MBA1* was 27 fold higher than the initial value.

An immediate increase was also observed in the expression level of *MBA1* in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain after the injection of the nitrogen pulse. The second steady state expression level was lower than the first one.

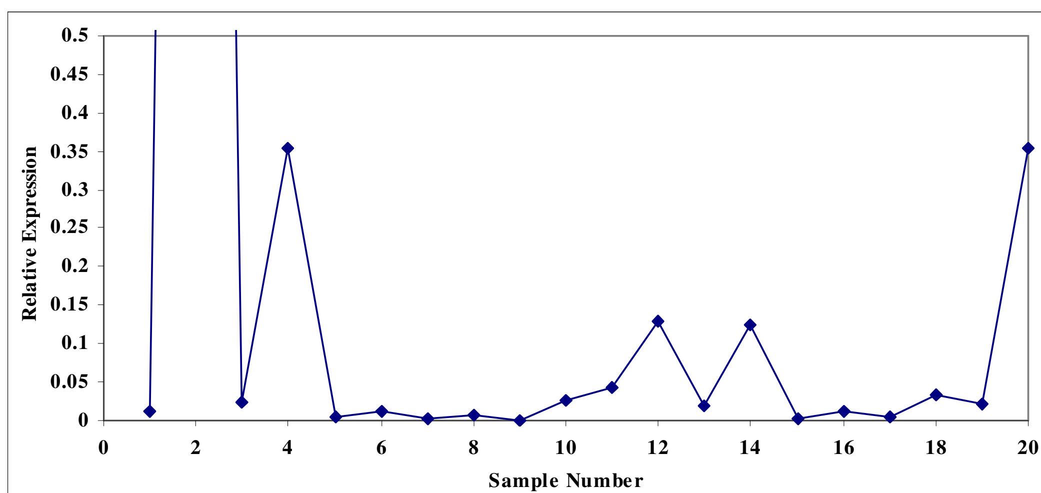


Figure 3.84. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under nitrogen limitation in chemostat cultures with pulse injection

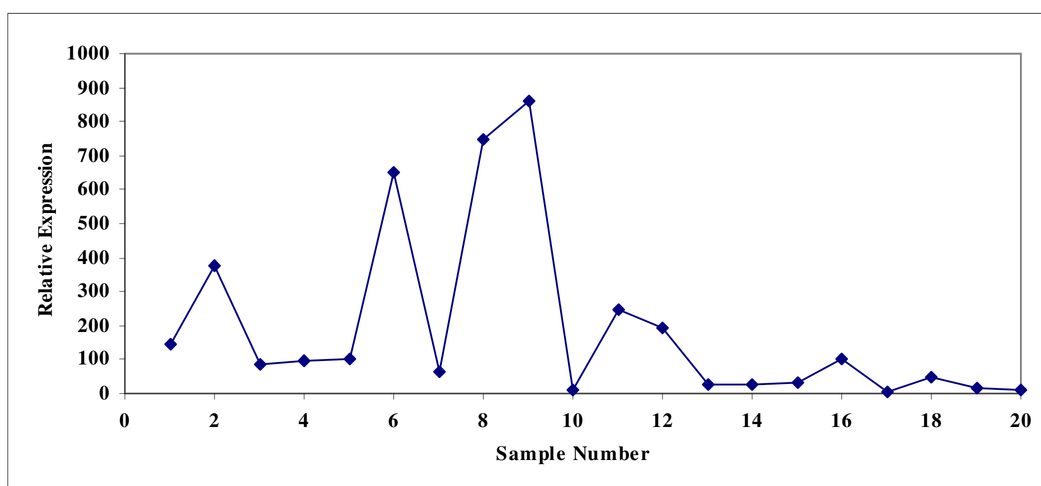


Figure 3.85. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under nitrogen limitation in chemostat cultures with pulse injection

After the injection of nitrogen pulse, a slight increase was seen in the expression level of *MBA1* in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ).* 12 fold higher expression level was obtained at the second steady state in comparison to the first one.

In *S cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain, the effect of the nitrogen pulse on the expression level of *MBA1* was a sharp increase. Then a fluctuating behavior was observed. Second steady state value was 3 fold higher than its initial value.

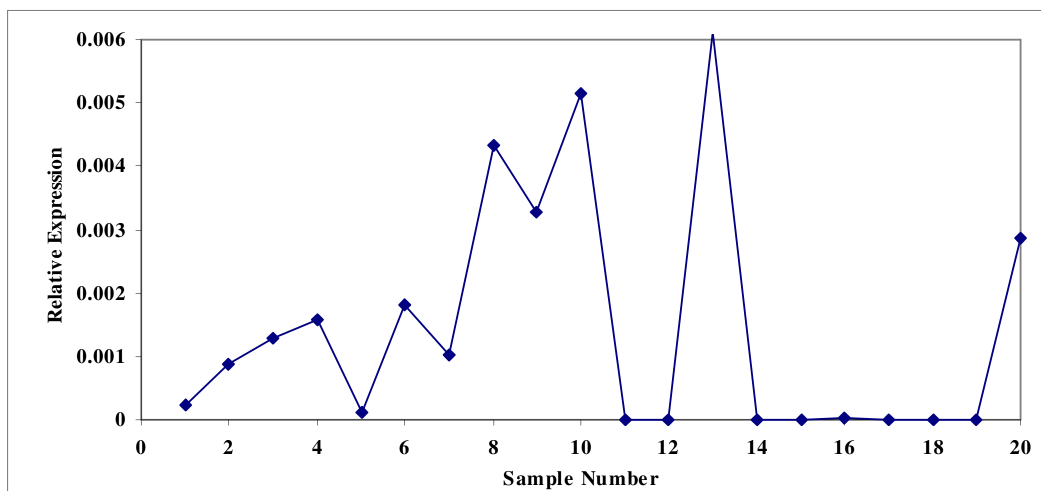


Figure 3.86. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

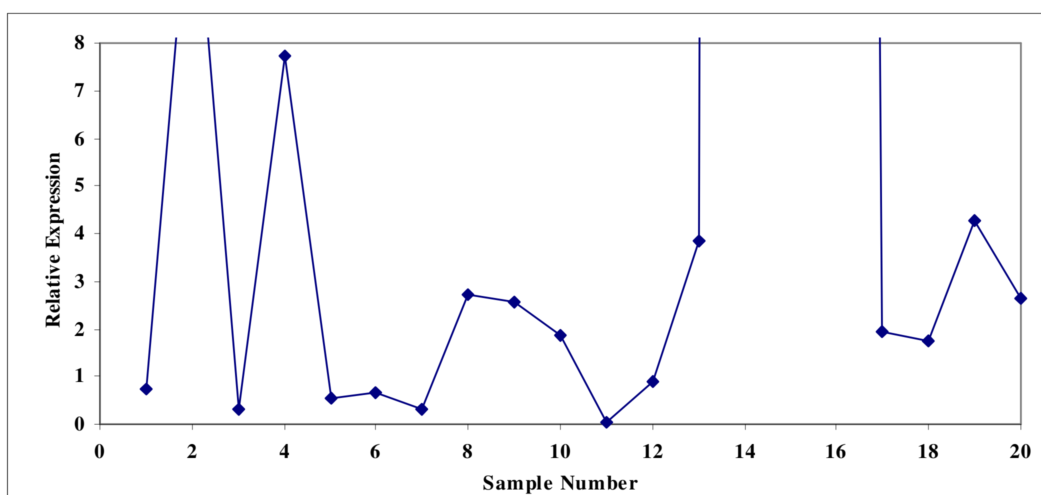


Figure 3.87. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under nitrogen limitation in chemostat cultures with pulse injection

4. DISCUSSION

In this study, four sets of chemostat experiments with *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1 Δ/rip1 Δ and RIP1/rip1Δ)* under nitrogen limited condition were carried out in order to identify the variations in the expression levels of genes involved in glucose sensing, signal transduction and glucose repression pathways as a response to system level perturbations.

The parent strain or *hoΔ/hoΔ* was selected as reference strain in studies related to the investigation of deletion strains of *S. cerevisiae* regarding to the respiratory deficiency. Researches revealed information about *hoΔ/hoΔ* being indifferent to respiratory metabolism and hence making it a preferential strain as reference (Baganz *et al.,* 1997). Therefore, *hoΔ/hoΔ* was selected as reference strain in this study.

This study aimed to investigate the effect of nitrogen limitation on glucose sensing and signaling mechanism in yeast and identify whether the signature of post- translational events can be observed in the transcription of the genes encoding those proteins.

In order to achieve this goal, chemostat cultivations with pulse injections were performed in the present study. After the cells spent three residence times at the steady state, ammonium sulfate pulse was injected in order to recover the nitrogen limitation. Sampling just prior to the pulse injection and 10 hours period of time after the injection were applied. Second steady state samples were collected again after cells spent three residence times at steady state. Duration of the sampling after the pulse injection were 10 hours because at the end of ten hours more than 95 per cent of the fermentor volume was replaced with fresh feed.

4.1. Growth Characteristics of Deletion Strains

The cells were grown in F1, nitrogen limited growth medium in which it is expected that cells prefer fermentation in state of respiration. The optimal growth conditions for *S. Cerevisiae* were used in the experiments with temperature kept at 30°C and the pH

controlled within the range of 5.5. The system switched to chemostat after operating overnight batch. In *S. cerevisiae* BY4743 strains *hoΔ/hoΔ*, *rip1Δ/rip1Δ and RIP1/rip1Δ* the growth of the cells showed increase after switching to chemostat. After these initial increases, growth showed slight decrease and the cells reached to the steady state. However in *S. cerevisiae* BY4743 strains of *hap4Δ/hap4Δ,* the cells had been reached to the steady state when the system switched to chemostat.

When growth profiles of the strains at the steady state were compared, it was observed that prior to the pulse injection; the *S. cerevisiae* BY4743 *rip1Δ/rip1Δ* strain reached to the highest densities when compared with other strains included into this study. Cell densities in case of *hap4Δ/hap4Δ* and *RIP1/rip1Δ* strains were similar at the first steady state. At the second steady state, the cell densities of *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains were higher and close to each other. The deletion of *HAP4* was reported to have little or no effect on the specific growth rate, compared with the wild type both aerobically as well as anaerobically (Nielsen *et al.*, 2005). Considering the *hoΔ/hoΔ* strain as wild type, it seemed to be in agreement with this report that *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains showed similar growth. At the second steady state growth of respiratory deficient strains, namely *rip1Δ/rip1Δ* and *RIP1/rip1Δ* strains were lower than that of *hoΔ/hoΔ* and *hap4Δ/hap4Δ.* It is indeed an expected result since there are reports stating the poor growth of respiratory deficient cells in the literature (Panoutsopolou *et al.,* 2001; van Maris et al., 2001).

The highest ethanol production was observed in completely respiratory deficient *hap4*Δ/*hap4*Δ strain under nitrogen limited conditions prior to the pulse injection. hap4Δ/hap4Δ strain was reported to produce higher amount of ethanol in comparison to the *hoΔ/hoΔ* strain at steady state in chemostat experiments conducted with rich medium as well as with nitrogen and glucose limited medium (Dikicioğlu, 2005; Pir, 2005). 100 per cent respiratory deficient nuclear petites exhibit higher rates of fermentation resulting in the higher ethanol production (Panoutsopolou *et al.,* 2001). Higher ethanol production in hap4Δ/hap4Δ strain is in agreement with these previous reports. In *rip1Δ/rip1Δ* strain, lower ethanol production in comparison to the hap4Δ/hap4Δ strain and higher ethanol production in comparison to the *RIP1*/rip1Δ and hoΔ/hoΔ mutants was observed. This result was also in agreement with the reports stating that respiratory deficient nuclear

petites exhibit higher rates of fermentation. The ethanol production was lowest in *RIP1*/rip1Δ and *ho*Δ/*ho*Δ mutants prior to the pulse injection. Since *RIP1*/rip1Δ and *ho*Δ/*ho*Δ mutants show respiro-fermentative behavior, lower ethanol production in comparison to the completely respiratory deficient mutants was also reported in strains displaying a respiro-fermentative behavior (Otterstedt *et al*., 2004).

A decrease in the production of ethanol after the injection of nitrogen pulse was observed in all strains included in this study. This decrease in the production of ethanol could possibly be explained by the fact that, with the injection of nitrogen pulse, the cells preferred to respire or produce biomass instead of fermentation. In *S. cerevisiae* BY4743 *hap4Δ/hap4* strain, the decrease in the ethanol production was more significant. The increase in the growth of cells with the injection of nitrogen pulse also indicated that, the cells preferred to produce biomass instead of fermentation after nitrogen pulse.

No significant change in residual glucose concentrations throughout the cultivation was observed before and after the pulse injections in all strains.

The ammonia concentration increased with the injection of ammonium sulfate as nitrogen pulse as expected and remained constant during the 10 hour sampling period. At the second steady state, the ammonia concentration decreased to its initial value.

Glycerol production often serves a redox sink, when excess NADH formed in anabolic pathways cannot be regenerated by respiratory chain at a sufficient high rate (Blom *et al*., 2001). The additional NADH originating from biomass production can be reoxidized via glycerol production in respiratory deficient conditions (Pronk *et al*., 2000).The highest glycerol production at the first steady state was observed in *RIP1/rip1Δ* strain. On the other hand double deletion of *RIP1* resulted in the decrease of glycerol production. Glycerol production prior to the pulse injection was higher in *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains in comparison to the *rip1*Δ*/rip1*Δ. In *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains, glycerol concentration increased with the injection of nitrogen. In *S. cerevisiae* BY4743 *hap4Δ/hap4Δ* strain, increase was observed in biomass after the injection of nitrogen pulse. Since the *hap4Δ/hap4Δ* strain is completely respiratory deficient, the increase in the glycerol production was plausible. Moreover, the increase in the glycerol

production could be explained by the decrease in the ethanol production. Since it is reported that elimination of glycerol production increases ethanol yield (Nissen *et al*., 2000) the increases in the glycerol concentration in *hoΔ/hoΔ* could be considered in conjunction with the decrease in the ethanol production.

On the other hand, the glycerol production decreased in *rip1Δ/rip1Δ* mutant and did not change significantly in *RIP1/rip1Δ* mutant. The lower glycerol production and the different respond to nitrogen pulse indicate a possible effect of *RIP1* gene on the glycerol production.

4.2. Expression Levels of Glucose Sensors under Nitrogen Limited Conditions

SNF3 and *RGT2* are membrane receptors that bind glucose outside the cell and generate a signal inside the cell for activation of gene expression. The sensor proteins Snf3p and Rgt2p assess the glucose availability in the surrounding medium and transmit this information to the internal cellular machinery (Ozcan *et al*., 1996a, 1998). Transcription of *SNF3* is maximal when glucose levels are low (*SNF3* expression is repressed about fivefold by high levels of glucose) (Özcan *et al.*, 1999). *RGT2*, on the other hand, appears to be a sensor of high levels of glucose, because it is required for maximal induction of *HXT1* expression by high concentrations of glucose but not for induction of *HXT2* and *HXT4* expression by low levels of glucose (Özcan and Johnston, 1999). It is appropriate, then, that *RGT2* is expressed in cells growing on high levels of glucose (it is expressed constitutively, being neither repressed nor induced by glucose) (Özcan *et al.*, 1996)

Expression levels of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* strains were low under nitrogen limited conditions. These genes were upregulated in *hoΔ/hoΔ, hap4Δ/hap4Δ,* whereas downregulated rip1*Δ/rip1Δ* and *RIP1/rip1Δ* after the pulse injection (Figure 4.1, 4.2, 4.3 and 4.4). Since the glucose concentrations did not change significantly, nitrogen concentration seemed to affect the expression level of *RGT2* and *SNF3.* However the expression levels of these genes displayed an oscillatory behavior after the pulse injection independent of glucose and nitrogen concentrations in the cultivation medium which may

be considered to be constant. Oscillatory dynamics observed in the majority of metabolites in yeast cells grown continuously at high cell density (Murray *et al.*, 2006) may explain the oscillatory behavior observed in the transcription of the genes.

RGT2 and *SNF3* became downregulated one hour after the pulse in *rip1Δ/rip1Δ* and 8 hours after the *pulse in RIP1/rip1Δ.* Therefore the deletion of one or both copies of *RIP1* seems to have an effect on the proper function of these sensors but the molecular mechanism behind this observation remains to be elucidated.

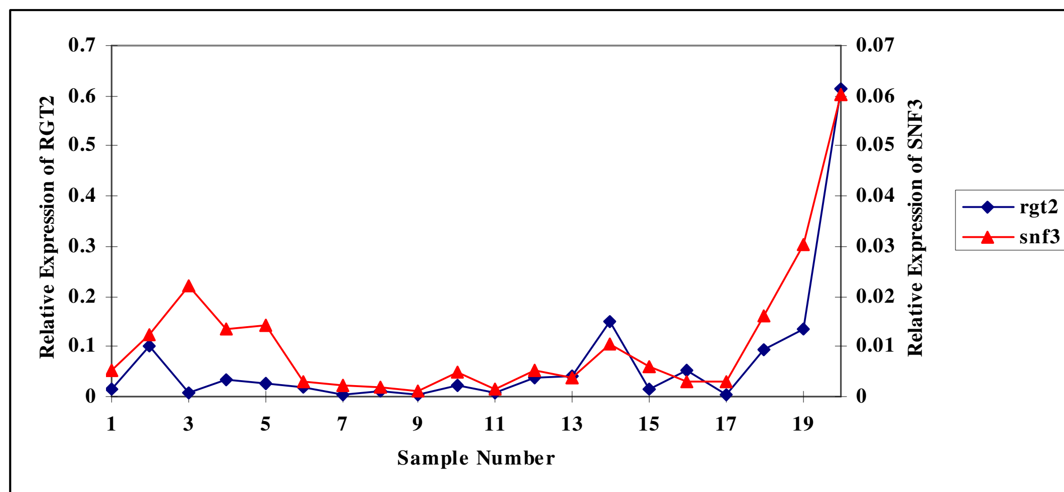


Figure 4.1 Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*

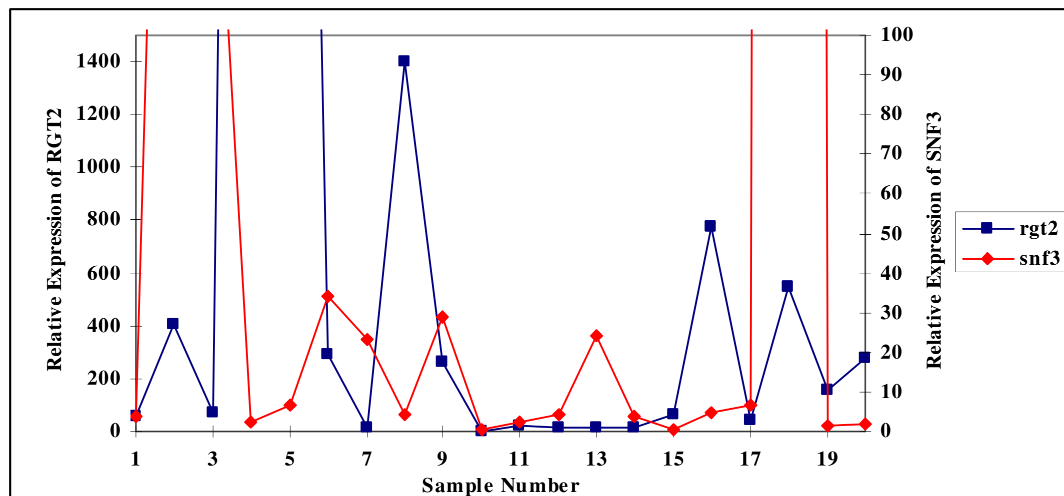


Figure 4.2 Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)*

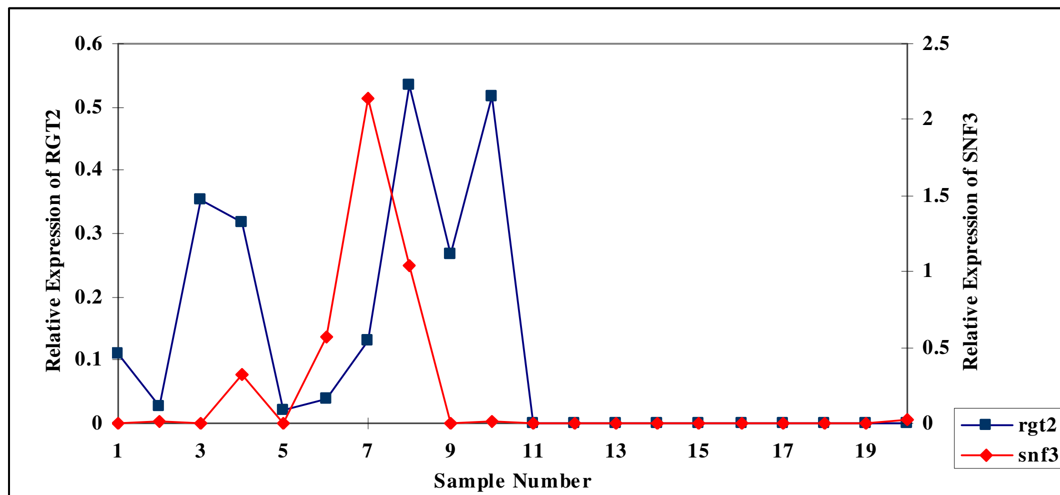


Figure 4.3 Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

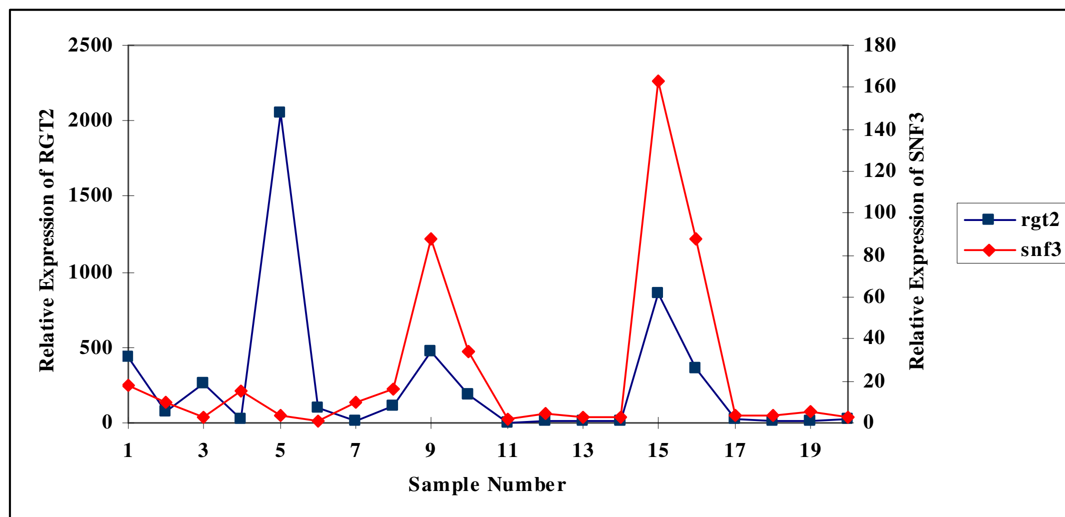


Figure 4.4 Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

4.3. Expression Profiles of *MTH1, STD1, YCK1* and *YCK2* under Nitrogen Limited Conditions

The expression levels of *MTH1, STD1, YCK1* and *YCK2* in *hoΔ/hoΔ* strain were downregulated under nitrogen limitation and they become upregulated just after the pulse injection (Figure 4.5). In nitrogen limitation Yck1/2 activity is interfered and Std1 and

Mth1 are suggested to be dephosphorylated due to the absence of proper TOR kinase pathway (Tomas-Cobos et al., 2005). TOR kinase pathway is inhibited by nitrogen starvation (Beck and Hall, 1999). Inhibition of TOR kinase pathway by nitrogen limitation may thus be related to the down regulation of *MTH1, STD1, YCK1* and *YCK2* prior to the pulse injection. *MTH1* and *STD1* will not be recognized by proteosomal degradation and move to the nucleus. Their expression may not need to be upregulated. *MTH1, STD1, YCK1* and *YCK2* were upregulated after the nitrogen pulse and the increase in the expression levels could be due to the activation of TOR kinase pathway with the elimination of nitrogen limitation. Therefore the effect of nitrogen limitation on the activity of Yck1/2, Mth1, and Std1 could be observed at transcriptional level in hoΔ/hoΔ strain. Oscillatory behavior was observed throughout the cultivation after the injection of nitrogen pulse.

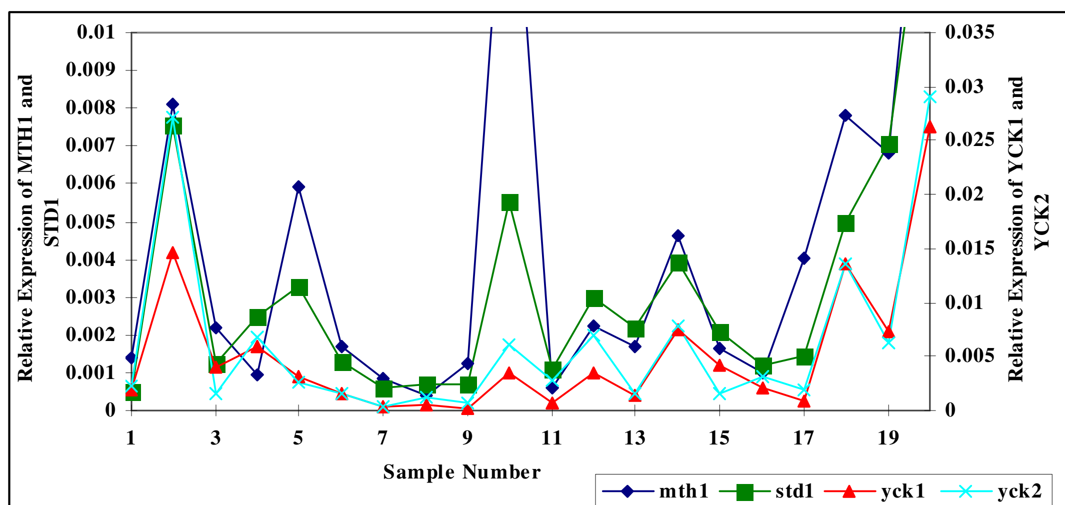


Figure 4.5 Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*ho*Δ*/hoΔ)*

The expression of *MTH1, STD1, YCK1* and *YCK2* was also downregulated in hap4Δ/hap4Δ mutant before the pulse injection and upregulated with the nitrogen pulse as in the case of *ho*Δ*/hoΔ* (Figure 4.6). Oscillatory behavior was observed throughout the cultivation after the injection of nitrogen pulse. The second steady state expression levels were similar to the first steady state profile.

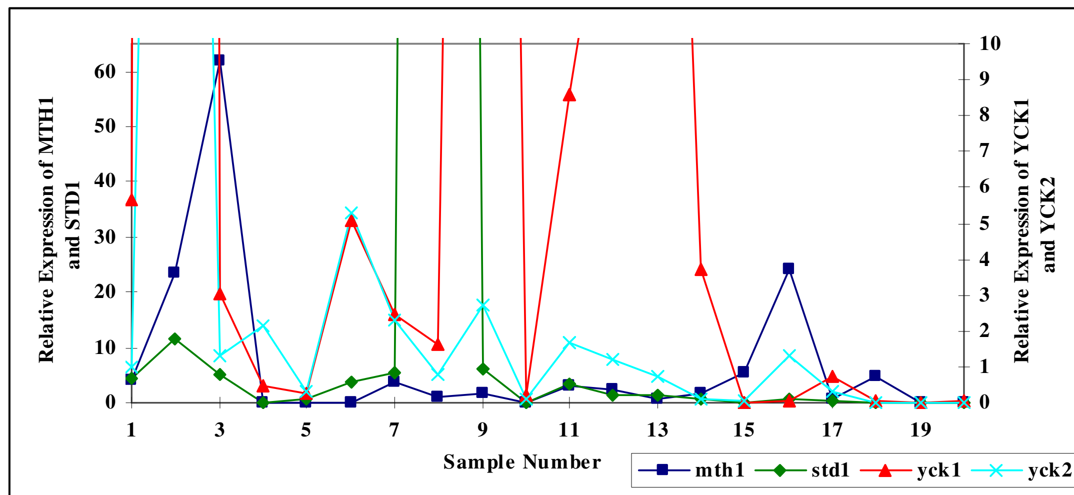


Figure 4.6 Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)*

The expression of *MTH1* and *YCK1* were repressed in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ and RIP1/rip1Δ)* strains prior and after the nitrogen pulse injections (Figure 4.7 and Figure 4.8). Expression levels of *STD1* and *YCK2* increased in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* strain just after the pulse whereas, they remained approximately constant in the *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain. It can be concluded that *RIP1* mutation have repression affect on the expression of *MTH1* and *YCK1* and the effect of homozygous or heterozygous deletions of *RIP1* have different effects on the expression of *STD1* and *YCK2*. It was also previously reported that, deletion of the one or two allele of *RIP1* affects the expression of the genes in the glucose signaling pathway differently (Pir, 2005).

4.4. Expression Profiles of *GRR1* and *SKP1* under Nitrogen Limited Conditions

Grr1p protein on the yeast *Saccharomyces cerevisiae* is a central component of a glucose signal transduction mechanism responsible for glucose-induced gene expression. Grr1p is required for glucose stimulated regulation of Rgt1, a repressor of several glucose induced *HXT* genes (Li and Johnston, 1997). Instead, Grr1 is required to inactivate Mth1 and Std1 in response to glucose.

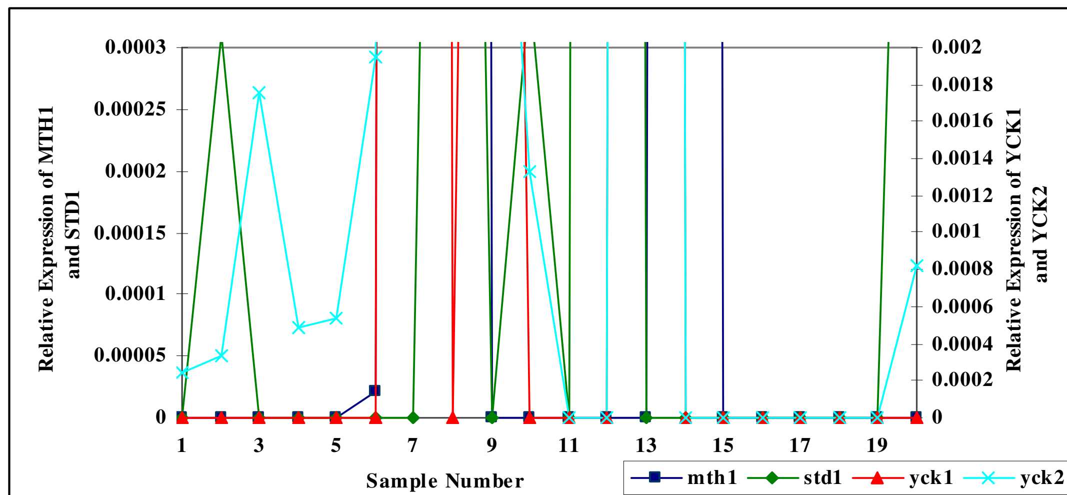


Figure 4.7 Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)*

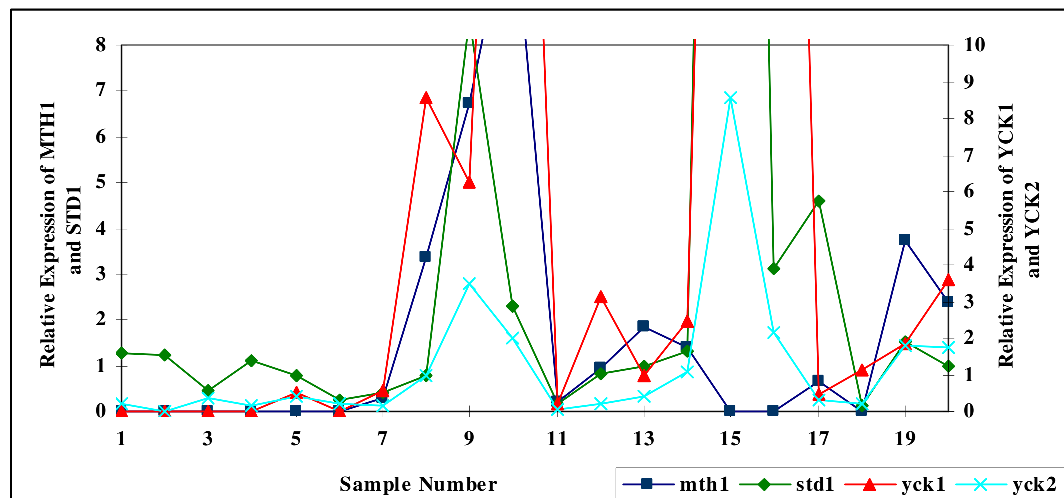


Figure 4.8 Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*

In hoΔ/hoΔ mutant, the expression level of *GRR1* and *SKP1* showed similar trends throughout the cultivation (Figure 4.9). This observation is in good agreement wit the previously reported data. It is stated that Grr1 degradation of Mth1 and Std1 appear to require Skp1 and Grr1-Skp1 interaction is significantly enhanced by high levels of glucose (Li and Johnson, 1997; Santangelo, 2006). *GRR1* and *SKP1* were downregulated prior to the pulse injection and upregulated after the elimination of the nitrogen limitation in hoΔ/hoΔ mutant. It is known that, the activated TOR kinase pathway with the injection of

nitrogen pulse could result in the inactivation of Mth1 and Std1. This could explain the increase in the expression level of the *GRR1* which is required for the ubiquitylation and degradation of these phosphorylated proteins after the injection of nitrogen pulse.

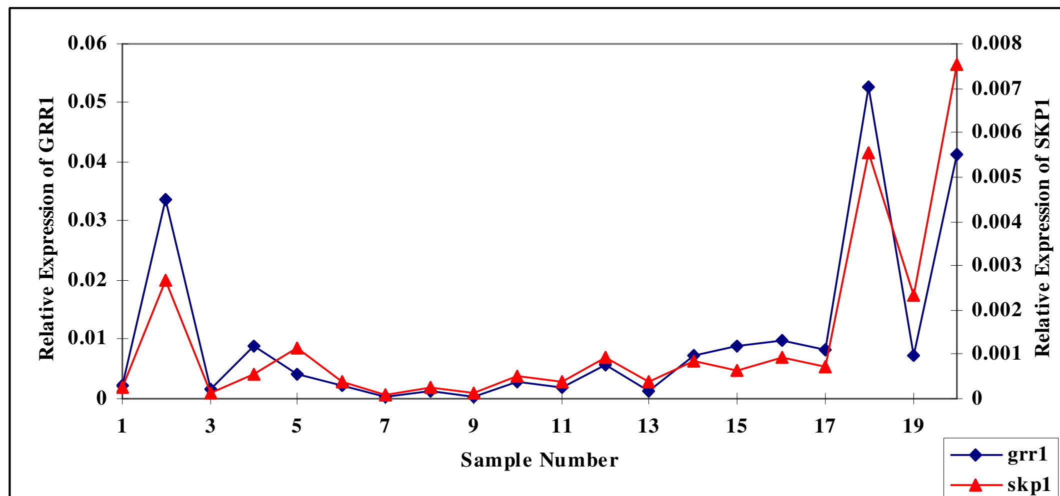


Figure 4.9 Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*ho*Δ/*ho*Δ)

The similarity between the expression profiles of *GRR1* and *SKP1* throughout the cultivation was not observed in the other mutants included into this study.

In *hap4*Δ/*hap4*Δ mutation, the expression of *GRR1* and *SKP1* was downregulated prior to the pulse injection and upregulated with the injection of the pulse as it was the case in *ho*Δ/*ho*Δ strain (Figure 4.10).

The expression of *SKP1* was repressed in *RIP1* mutations prior to and after the nitrogen pulse. *GRR1* was downregulated before the nitrogen pulse and only slight increases were observed in the expression levels of *GRR1* (Figure 4.11 and Figure 4.12). We also observed that *YCK1* which phosphorylates Std1p and Mth1p was repressed in the absence of one or both copies of *RIP1*, Since the phosphorylation of Std1p and Mth1p is necessary for their recognition by SCF-Grr1 complex (Flick *et al*., 2003; Moriya and Johnson, 2004; Spielewoy *et al*., 2004), they are not possibly be degraded in strains with *RIP1*deletions. Therefore the downregulation observed in the expression of Grr1 after the pulse may be explained by the fact that its synthesis is not necessary under this condition in *rip1*Δ/*rip1*Δ and *RIP1*/*rip1*Δ.

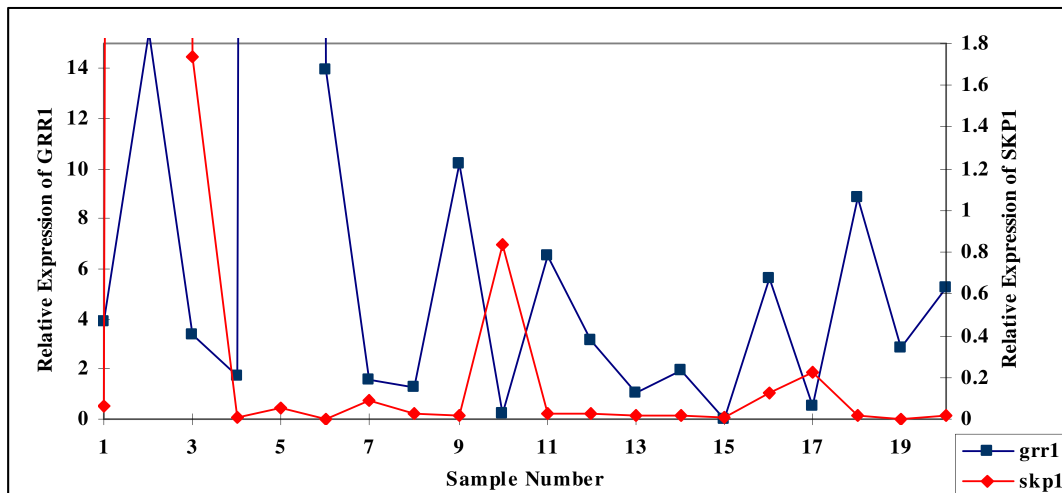


Figure 4.10 Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

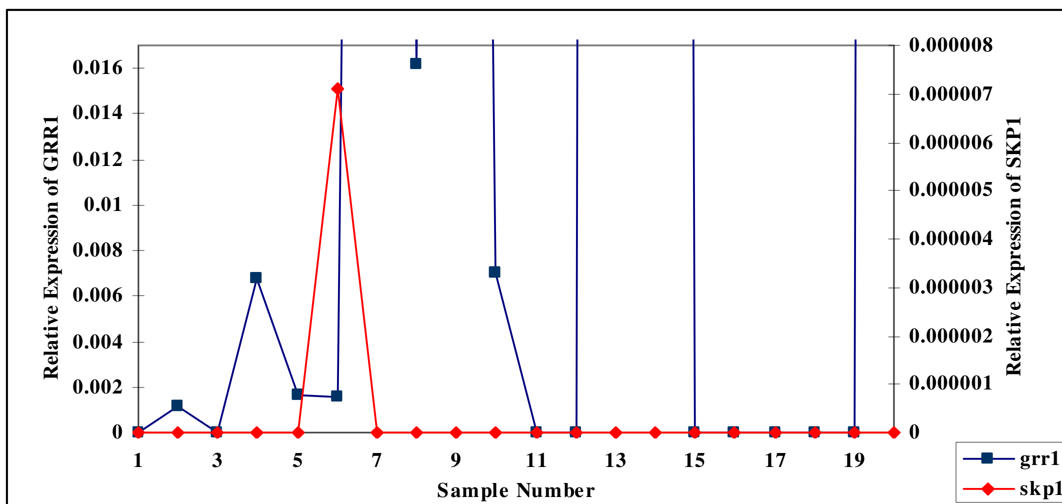


Figure 4.11 Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

4.5. Expression Profiles of *RGT1* under Nitrogen Limited Conditions

Rgt1p has a dual role in glucose signal transduction pathway. Rgt1 represses transcription of *HXT1-4* and *HXK2* in the absence of glucose. On the other hand when glucose level are high (>2 per cent), Rgt1p activates transcription *HXT1* (Ozcan et al., 1996; Polish et al., 2004). Moreover it was previously stated that, since transcription of

RGT1 is not altered in response to glucose, the activity of Rgt1p is likely to be regulated posttranscriptionally (Ozcan et al., 1996).

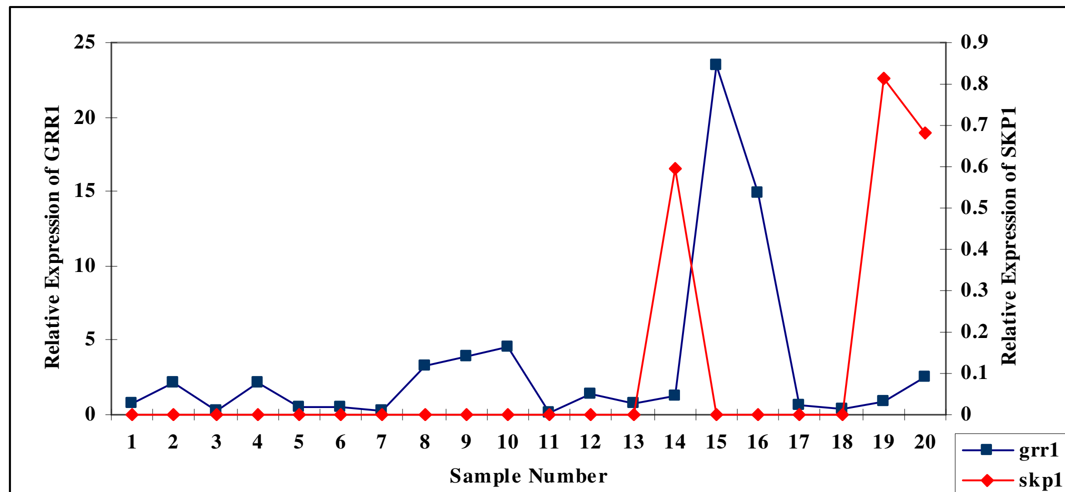


Figure 4.12 Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*

However response of *RGT1* gene was observed when ammonium sulfate pulse injection was introduced into the system in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* (Figure 4.13). *RGT1* was observed to be expressed in all strain under nitrogen limited conditions except (*rip1 Δ /rip1Δ)* whereas it was upregulated just after the pulse only in *hoΔ/hoΔ*. This response might be due to the conversion of Rgt1p from a repressor to an activator by the degradation of Mth1p with the injection of nitrogen pulse since the degradation of Std1p and Mth1p leads into the glucose-induced dissociation of Rgt1p from the *HXT1* promoter and its activation (Flick *et al.*, 2003; Moriya and Johnson, 2004; Spielewoy *et al.*, 2004). The expression profile of *RGT1* was quite similar to that of *YCK1* in this strain. Since the degradation of Mth1p is accompanied with casein kinase I, encoded by *YCK1* and *YCK2* (Johnston and Kim, 2005) this observation is in good agreement.

In *S. cerevisiae* BY4743 (hap4Δ/hap4Δ) strain, the expression level of *RGT1* showed only a slight decrease with the injection of nitrogen pulse (Figure 4.14). Thus the deletion of *HAP4* which is the activator of respiration has a decreasing effect on the transcription of *RGT1* under nitrogen limitation. Activation of the gene expression at later

stages after the pulse injection needs further experiments to be explained. The expression profile of *RGT1* is not similar to that of *YCK1* in this strain.

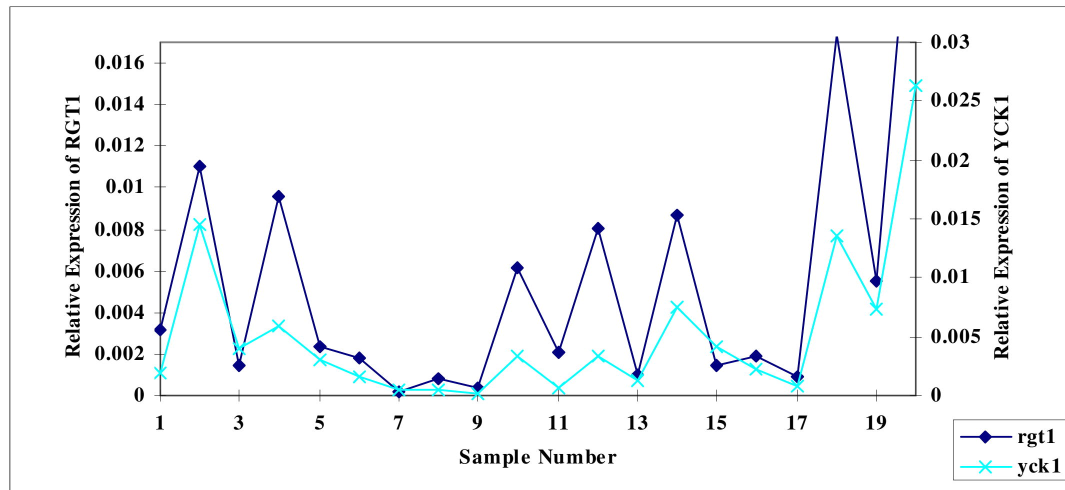


Figure 4.13 Expression level of *RGT1*and *YCK1* in *S. cerevisiae* BY4743 (*ho Δ /hoΔ)*

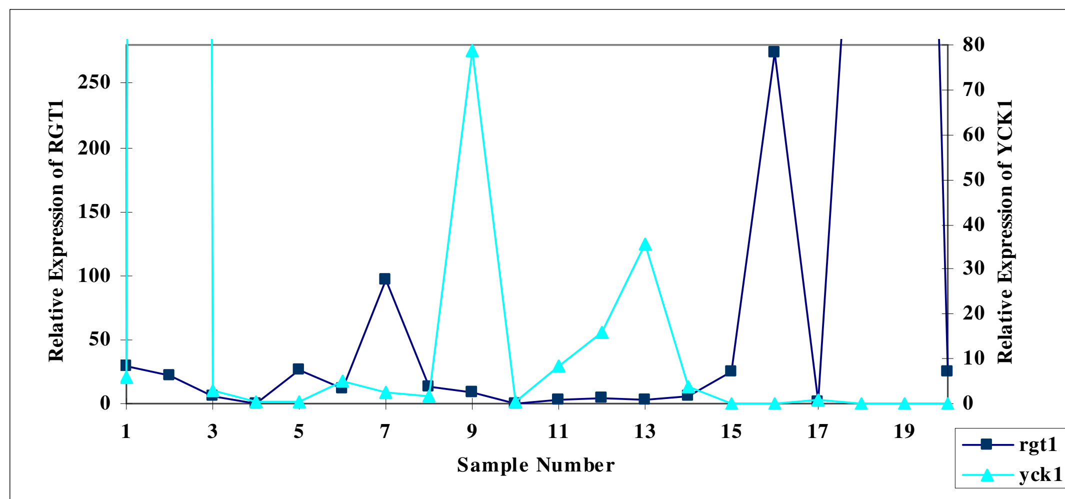


Figure 4.14 Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*hap4 Δ /hap4Δ)*

In respiratory deficient *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain expression of *RGT1* was repressed before and after the pulse injection (Figure 4.15). In partially respiratory deficient *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain, the expression of *RGT1* was downregulated prior to the pulse and did not change just after the injection of nitrogen

pulse (Figure 4.16). The homozygous or heterozygous deletion of *RIP1* seems to have an inactivating effect on the transcription of *RGT1* under nitrogen limited conditions. However oscillations were observed after the pulse injection. Respiratory deficiency seems to affect the expression of *RGT1* since the response of *RGT1* gene to nitrogen pulse in respiratory deficient mutants is different from that of *hoΔ/hoΔ* strain.

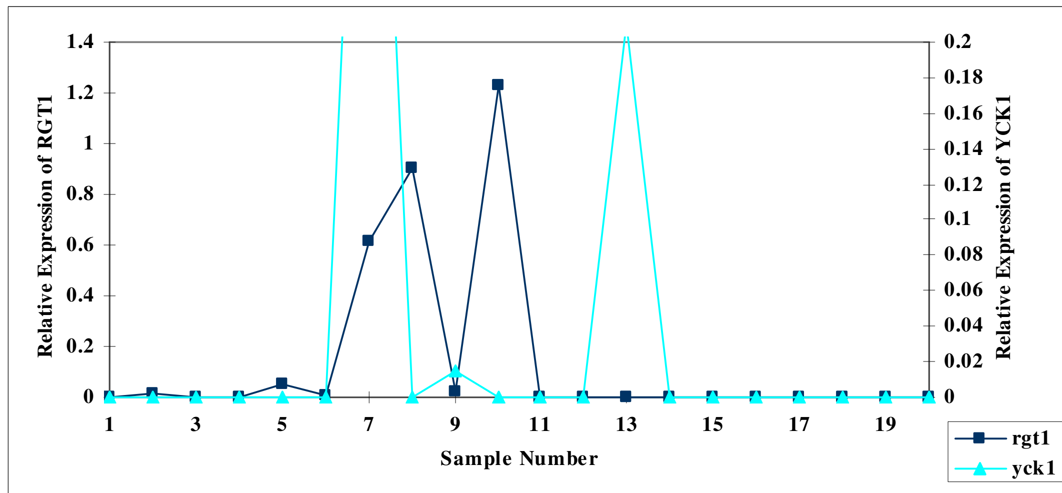


Figure 4.15 Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ*)

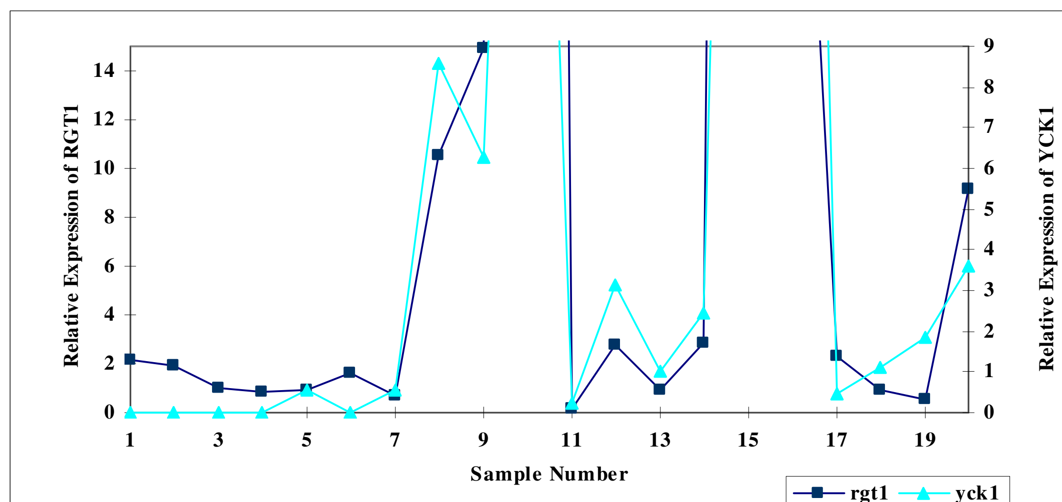


Figure 4.16 Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

4.6. Expression Profiles of *REG1, GLC7*, *MIG1*, *SNF1* and *SNF4* under Nitrogen Limited Conditions

REG1, GLC7, *MIG1*, *SNF1* and *SNF4* are the genes involved in glucose repression pathway in *S. cerevisiae* were downregulated in all strains under nitrogen limitation and upregulated after the pulse (Figure 4.7-11). The expression profiles of *REG1* and *GLC7* were similar to each other in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* strain. The proteins encoded by these two genes form a protein phosphatase complex; therefore it was plausible to observe similar trends. Nitrogen limitation leads to improved Snf1 Thr210 phosphorylation (Orlova *et al*., 2006). Injection of nitrogen pulse could lead to the de-phosphorylation of the Snf1 Thr210 which is promoted by Reg1-Glc7 complex. This could be an explanation of the increase in the expression level of *REG1* and *GLC7*. The expression profiles of *MIG1* and the complex also showed similar trends. This similarity could be the signature of the activating effect of Glc7-Reg1 protein phosphatase complex on Mig1p at transcriptional level. Mig1 is de-phosphorylated by the Glc7-Reg1 protein phosphatase complex in high glucose condition (Alms et al., 1999). Although there was no change in the glucose concentration, *MIG1* expression is upregulated and also possibly dephosphorylated (thus active) after the injection of nitrogen pulse. This could be due to the increased *REG-GLC7* expression. The expression level of MIG1 remained almost constant after an initial response to nitrogen pulse. However other genes displayed an oscillatory behavior in their expression levels. Although the Reg1-Glc7 complex acts in opposition to the Snf1 signaling pathway (McCartney and Schmidt, 2001), the expression profiles of the complex and the *SNF1* showed the similar trends. The increase in the expression levels of *SNF1* might be compensated by post-transcriptional regulation on the Snf1p.

The expressions of *REG1, GLC7, MIG1, SNF1* and *SNF4* were downregulated prior to the pulse in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain (Figure 4.18) and were upregulated after the pulse injection. Expression profiles of *REG1* and *GLC7* showed also similar trends throughout the cultivation after the injection of nitrogen pulse. The response of the *REG1*, *GLC7* and *MIG1* genes to the nitrogen pulse in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain was quite similar to the responses in *S. cerevisiae* BY4743 (*hoΔ/ho4Δ)* strain. The second steady state expressions were in consistence with the first

steady state levels of expression of these genes. The deletion of *HAP4* does not seem to affect significantly the expression patterns of these genes.

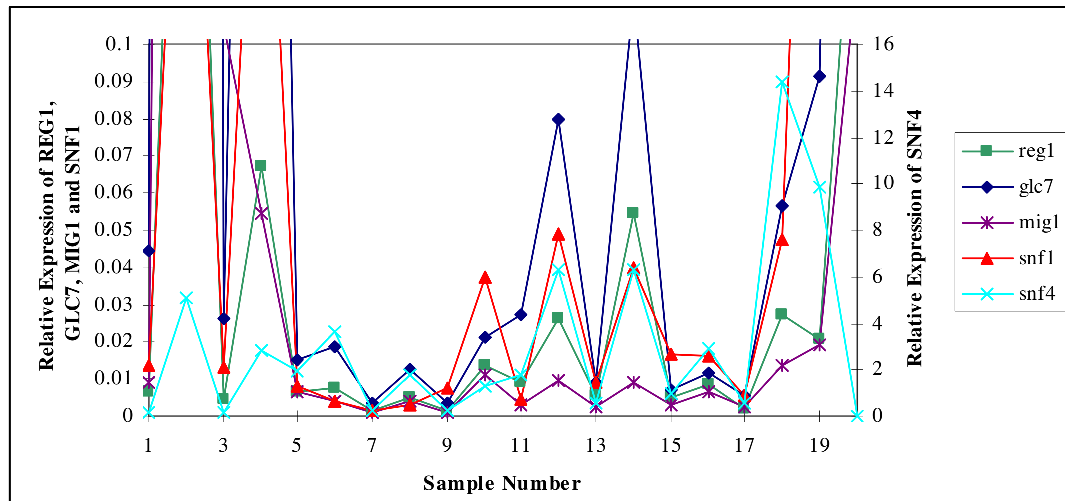


Figure 4.17 Expression level of *REG1, GLC7, MIG1, SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*

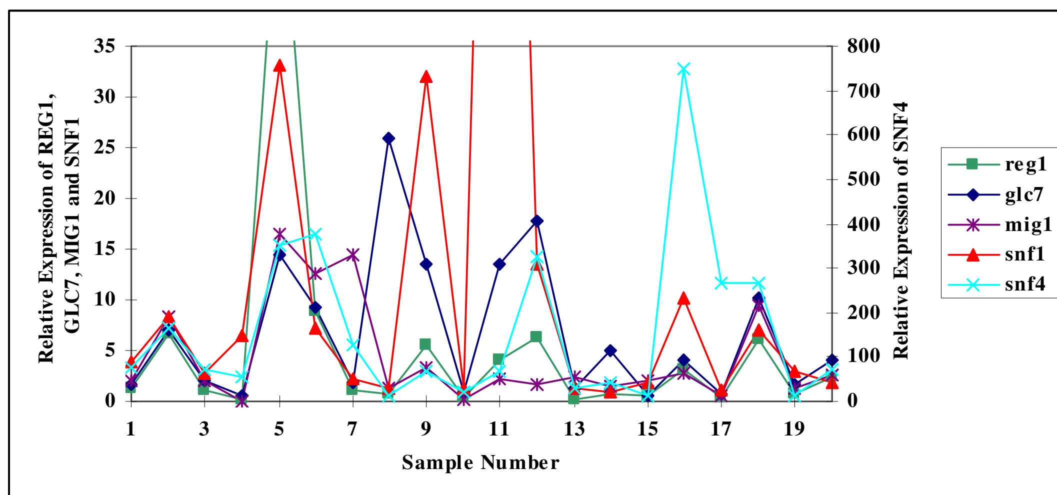


Figure 4.18 Expression level of *REG1, GLC7, MIG1, SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)*

Although the presence of a similarity between the expression profiles of *REG1*, *GLC7* and *MIG1* could not be detected in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* strain these genes were downregulated under nitrogen limited conditions and upregulated after the release of this limitation and display a fluctuating behavior throughout the experiment

(Figure 4.19). The fact that the expression level of the *SNF1* remains almost constant during the cultivation in this strain may indicate also a possible effect of Rip1p on the expression level of *SNF1* after the pulse.

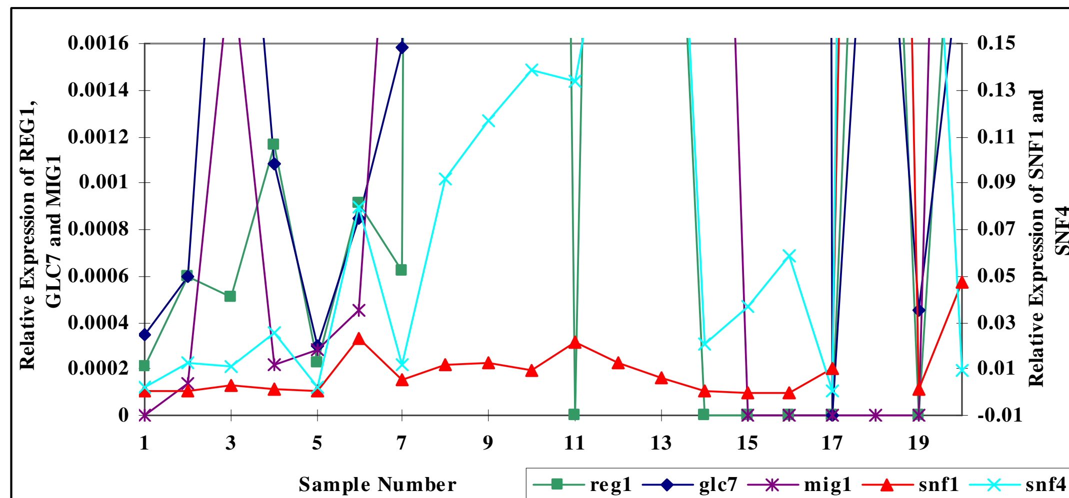


Figure 4.19 Expression level of *REG1, GLC7, MIG1, SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)*

The expression profiles of *MIG1, REG1* and *GLC7* was approximately same in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* (Figure 4.20). Nitrogen pulse had an increasing effect on the expression of these genes.

The fact that the expression level of the *SNF1* does not remain constant during the cultivation in this strain may indicate that the presence of at least one allele of *RIP1* may be effective on the expression level of *SNF1* after the pulse.

4.7. Expression Profiles of *TOS3*, *PAK1* and *ELM1* under Nitrogen Limited Conditions

The expression levels of *PAK1* and *TOS3* were low under limitation and upregulated upon release from nutrient limitation in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* and *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* (Figure 4.21 and 4.22). Therefore the deletion of *HAP4* resulting in respiratory deficiency had no effect on the transcriptional behavior of these genes. Whereas deletion of *RIP1* homozygously or heterozygously resulted in the

repression of the transcription of *PAK1* just after the nitrogen pulse and the gene was switched on later after the pulse (Figure 4.23 and 4.24).

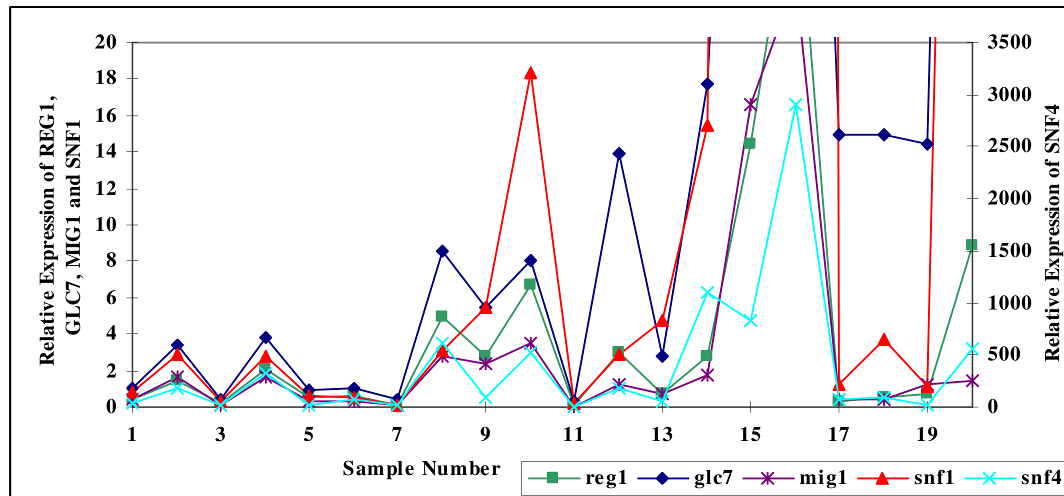


Figure 4.20 Expression level of *REG1, GLC7, MIG1, SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*

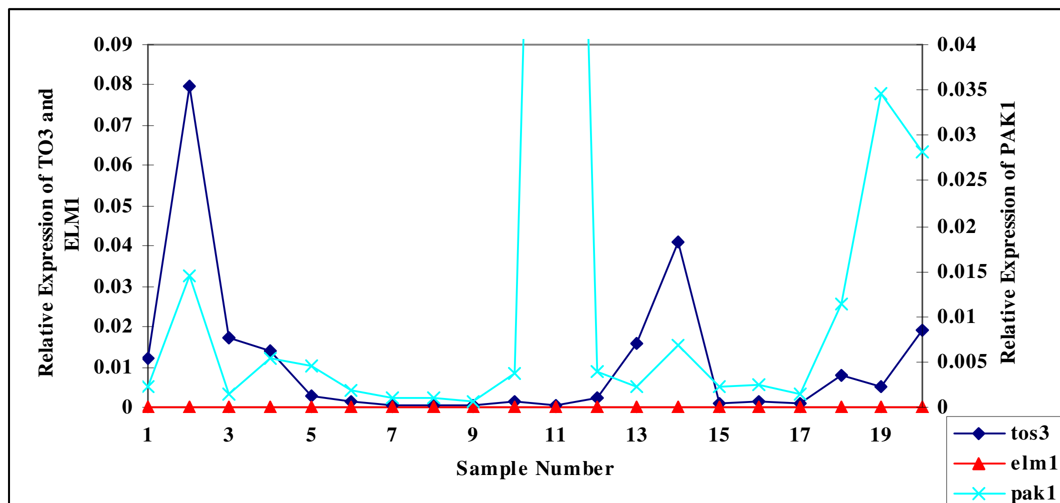


Figure 4.21 Expression level of *TOS3, ELM1* and *PAK1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*

Undetectable transcription level of *ELM1* before and after the pulse injection in all strains may indicate the unresponsiveness of the transcription under the present conditions or may be caused by a technical problem in the amplification of this gene.

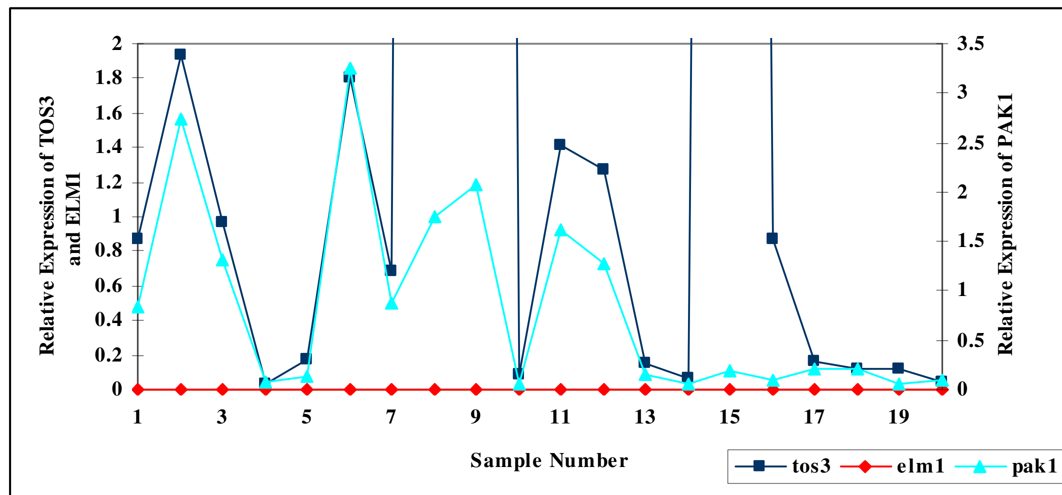


Figure 4.22 Expression level of *TOS3, ELM1* and *PAK1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

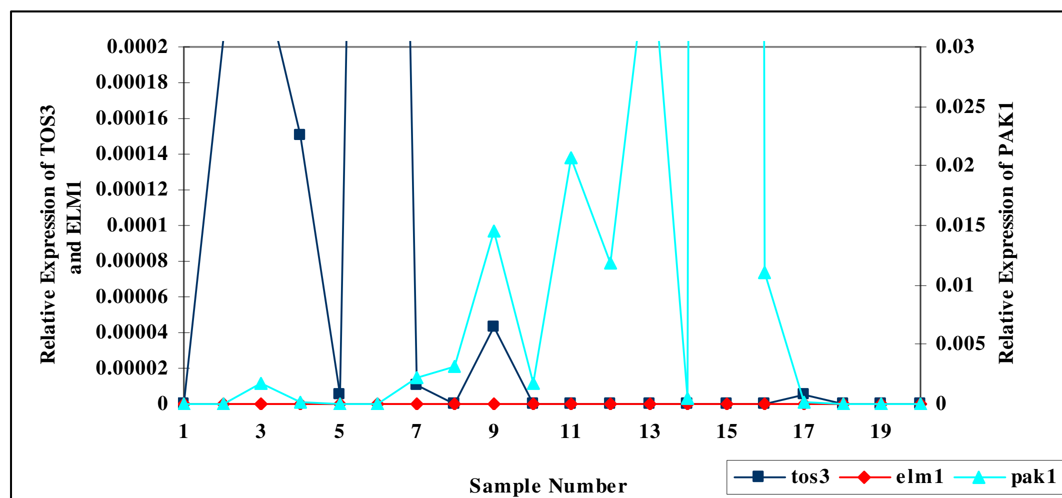


Figure 4.23 Expression level of *TOS3, ELM1* and *PAK1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

4.8. Expression Profiles of *CYC8*, *TUP1* and *MIG1* under Nitrogen Limited Conditions

Since Mig1 inhibits transcription by recruiting the general co-repressor complex Cyc8 (Ssn6)-Tup1 (Nehlin et al., 1991; Treitel & Carlos, 1995; Tzamarias& Struhl, 1995; Papamichos-Chronakis et al., 2004), therefore it seems to be plausible to examine the expression profiles of these three genes together. The expression profiles of *CYC8*, *TUP1*

and *MIG1* were indeed observed very similar after the nitrogen pulse in *S. cerevisiae* BY4743 (hoΔ/hoΔ) strain (Figure 4.25). All the genes were downregulated in nitrogen limited condition and upregulated after the elimination of the limitation and displayed an oscillatory behavior throughout the cultivation. However the same similarity could not be observed in respiratory deficient strains.

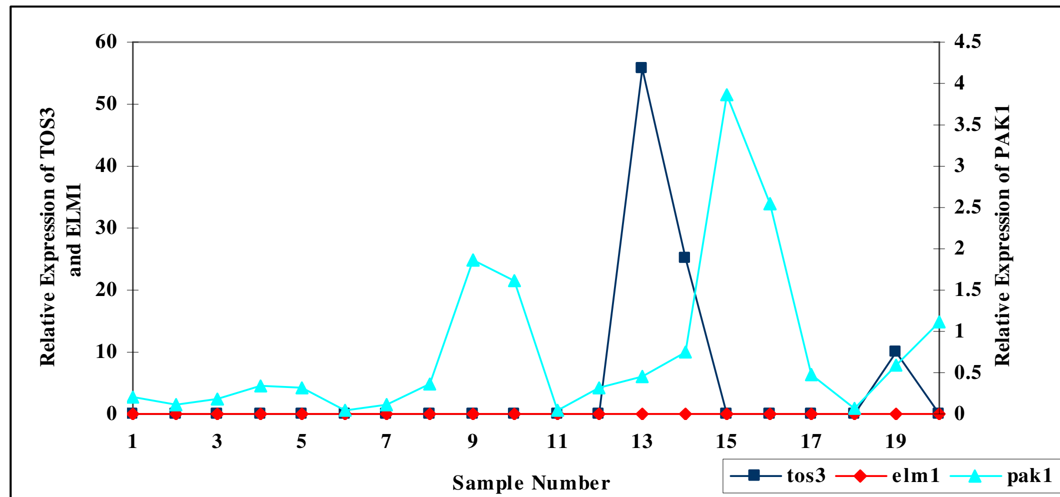


Figure 4.24 Expression level of *TOS3, ELM1* and *PAK1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

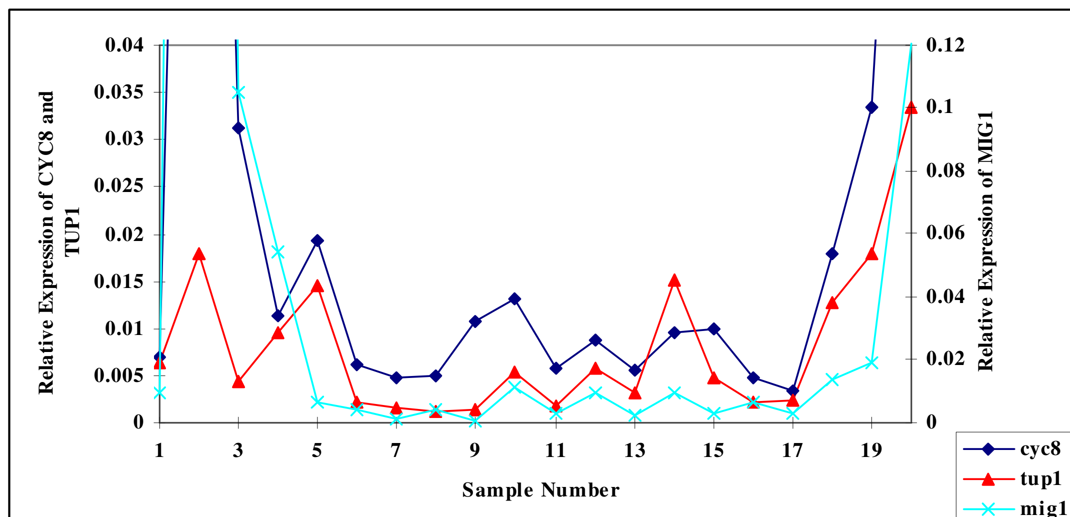


Figure 4.25 Expression level of *CYC8, TUP1* and *MIG1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

In *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain, expression profiles of *CYC8* and *MIG1* are downregulated under nitrogen limited conditions and upregulated after the pulse as in the case of *hoΔ/hoΔ* whereas *TUP1* is downregulated just after the nitrogen pulse (Figure 4.26). Cyc8-Tup1 protein complex may act both as transcriptional activator and repressor. This complex activates *CIT2* transcription in response to mitochondrial dysfunction or respiratory deficiency. This stimulatory effect is predominantly mediated by the Cyc8 protein and the repression function of this complex is performed by a specific domain of Tup1p. The molecular mechanism by which Cyc8-Tup1 is converted from a co-repressor to a co-activator of *CIT2* is not yet understood, one model predicts that upon the induction of *CIT2*, Tup1 dissociates from the complex thus unmasking Cyc8 activation potential (Conlan et al., 1999). Transcription of *CIT2* is also regulated by the intracellular ammonium concentration (Usaite *et al*., 2006). Activation of *CIT2* transcription in response to mitochondrial dysfunction may lead to the dissociation of Tup1 from the complex. This may explain the difference in the responses of *TUP1* and *CYC8* after the injection of nitrogen pulse.

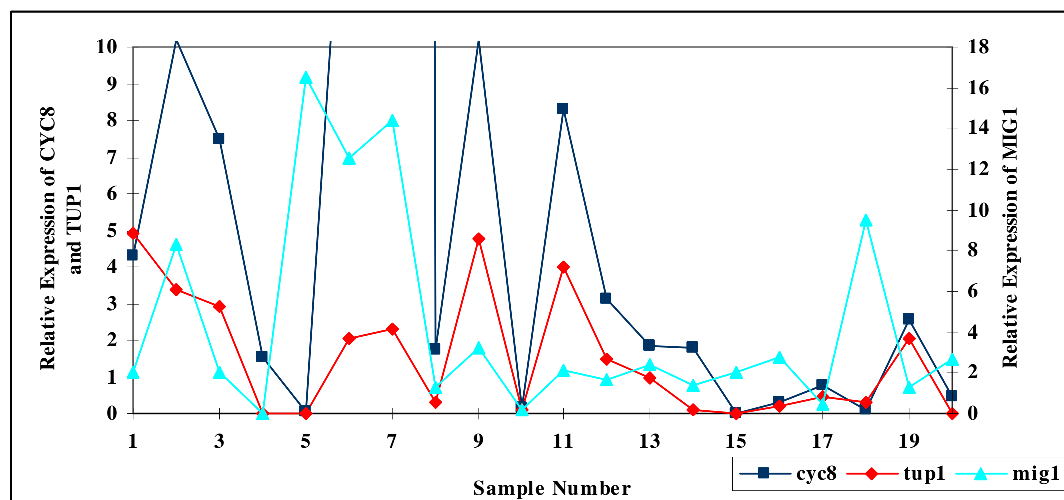


Figure 4.26 Expression level of *CYC8, TUP1* and *MIG1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)*

In *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* strain which is completely respiratory deficient, it was observed that *CYC8* expression was downregulated after the pulse injection whereas *TUP1* was repressed prior to and after the pulse injection (Figure 4.27).

The difference in the effect of deletion on these genes in case of *rip1Δ/rip1Δ* and *hap4Δ/hap4Δ* might be due to different molecular functions of *HAP4* and *RIP1*.

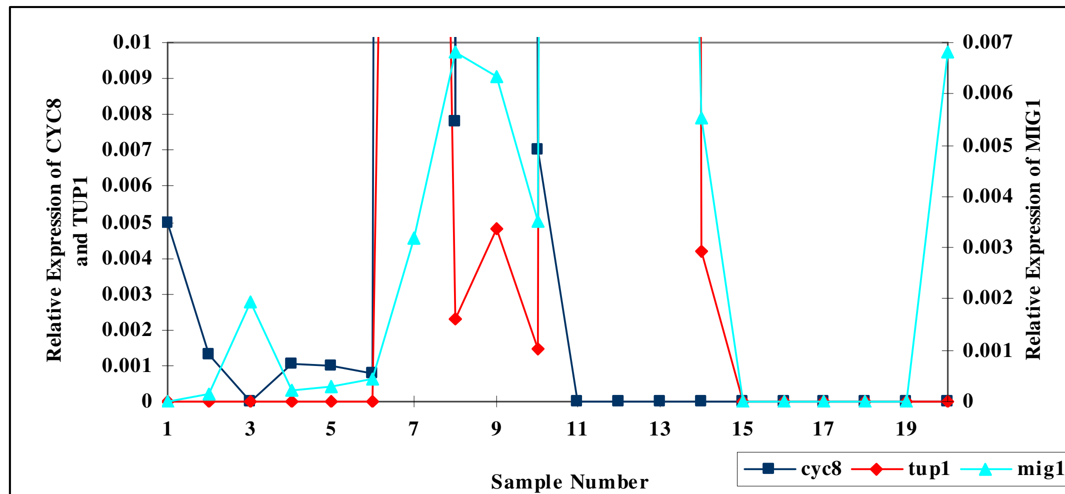


Figure 4.27 Expression level of *CYC8, TUP1* and *MIG1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)*

In *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain, the expression of *CYC8* was upregulated after the injection of nitrogen pulse whereas only a slight decrease was observed in the expression of *TUP1* with the injection of nitrogen pulse (Figure 4.28). A fluctuating behavior was observed in the expression profile of the both genes after the pulse. It could be concluded that homozygous and heterozygous mutations of *RIP1* has not the same effect on the regulation of the expression of the *CYC8*. This result is in agreement with the previous reports indicating the difference between the expression profiles of the genes involved in the glucose signaling, glycolysis and galactose pathways in *rip1Δ/rip1Δ* and *RIP1/rip1Δ* strains (Pir, 2005).

4.9. Expression Profiles of *HXK2* and *MIG1* under Nitrogen Limited Conditions

A direct correlation between the amount of Hxk2p located in the nucleus and the level of Mig1p in the cell was already reported (Ahuatzi et al., 2004). In *S. cerevisiae* BY4743 (*ho*Δ*/hoΔ)* strain, the expression profiles of *MIG1* and *HXK2* showed similar trends (Figure 4.29). Both genes are expressed at undetectable levels when nitrogen limited

and become upregulated and then fluctuated after the pulse injection. This similarity could be the signature of the importance of the transcriptional regulation of these genes.

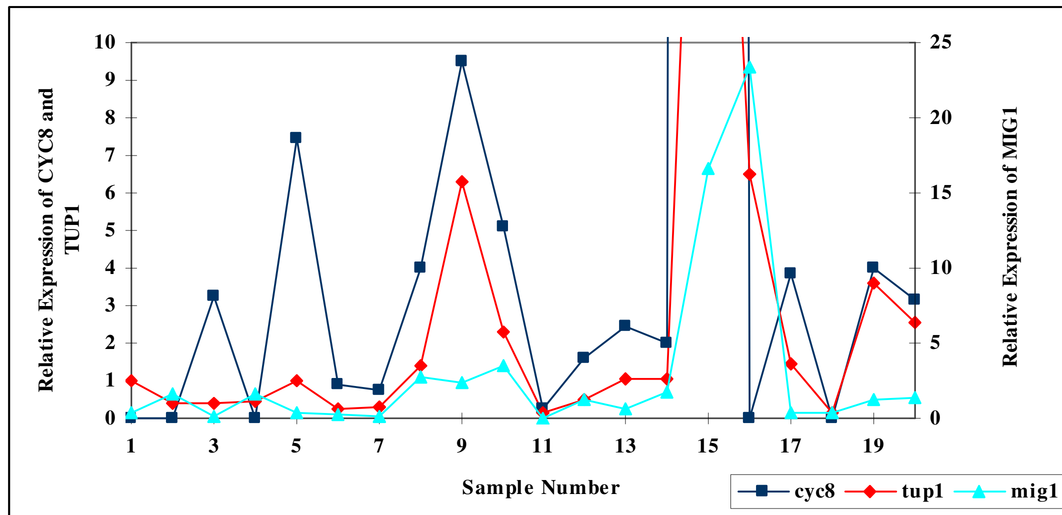


Figure 4.28 Expression level of *CYC8, TUP1* and *MIG1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*

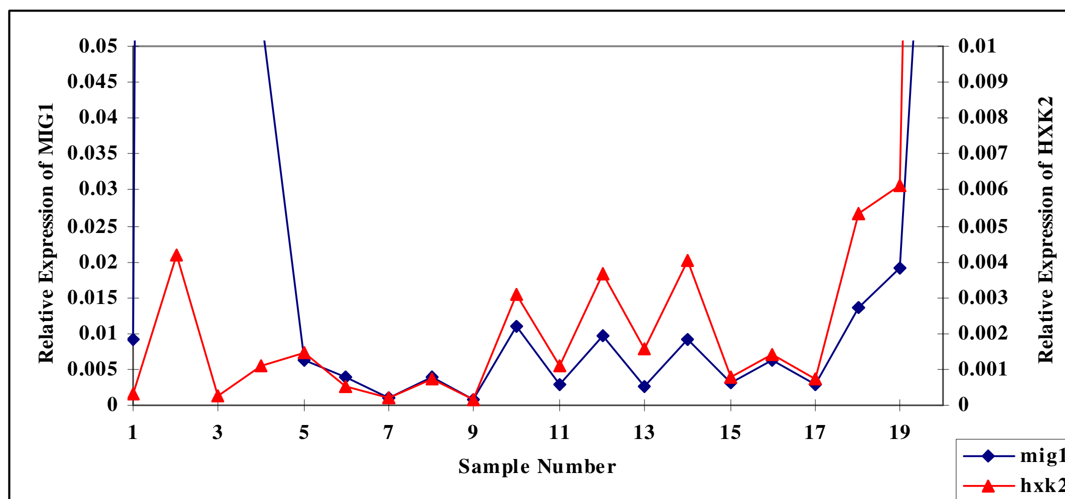


Figure 4.29 Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*

However, such a similarity in the expression profiles of *MIG1* and *HXK2* is lacking in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ, rip1Δ/rip1Δ, RIP1/rip1Δ)* (Figure 4.30, 4.31 and 4.32). In *S. cerevisiae* BY4743 hap4Δ/hap4Δ strain *HXK2* and *MIG1* displayed an

antagonistic response to the nitrogen pulse. *RIP1* deletion seems to cause repression of *HXK2*. In *rip1Δ/rip1Δ* strain, the expression of the *HXK2* was upregulated just after the pulse and then rebounded and remained repressed throughout the cultivation whereas in *RIP1/rip1Δ* strain, the upregulation of *HXK2* after the injection of nitrogen pulse was observed one hour later than the injection.

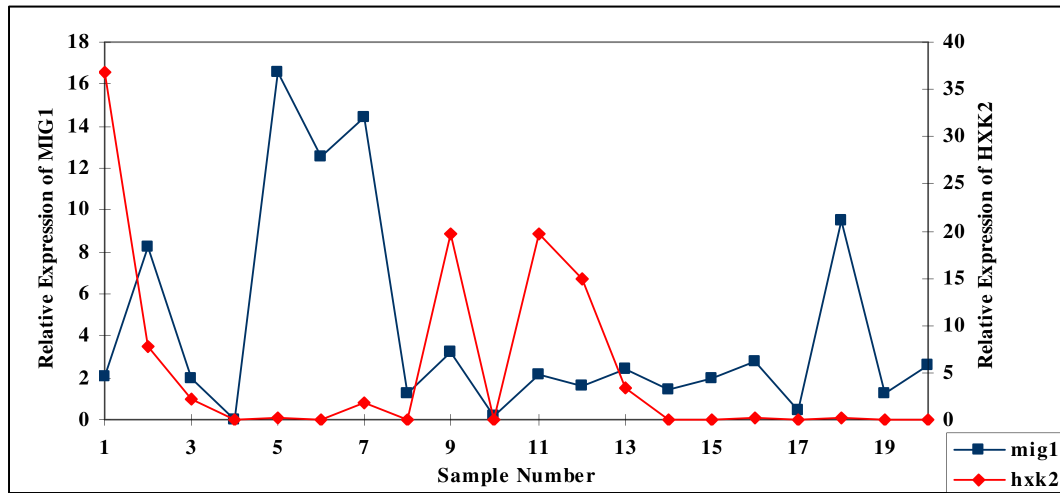


Figure 4.30 Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

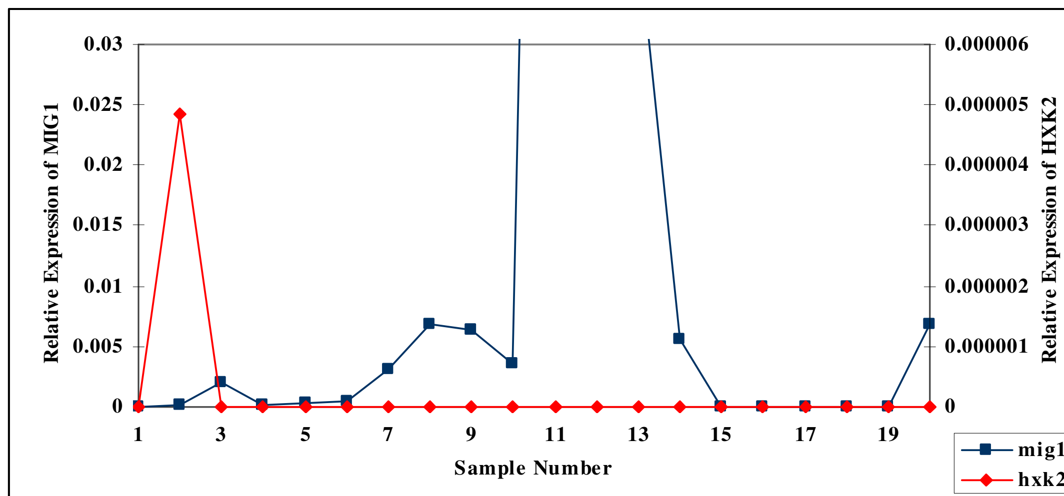


Figure 4.31 Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

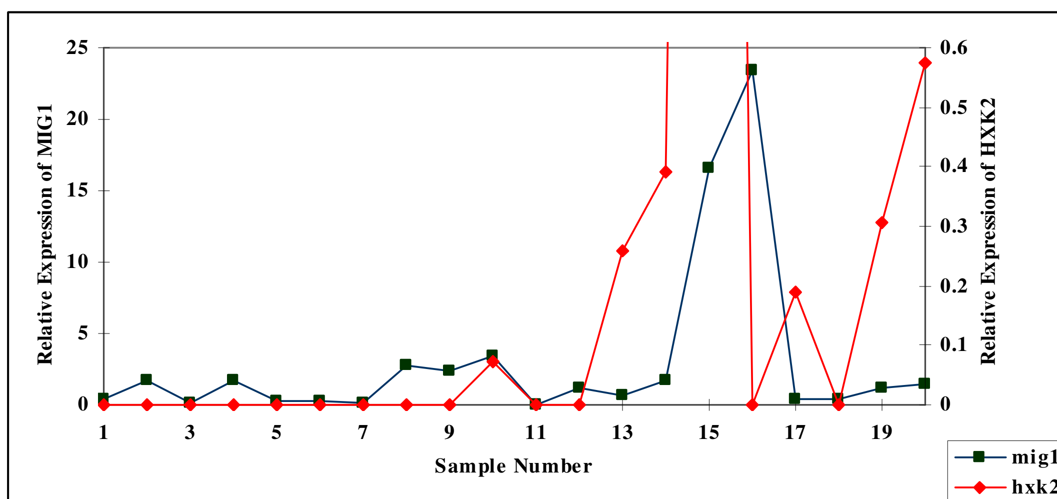


Figure 4.32 Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

4.10. Expression Profiles of *MBA1* under Nitrogen Limited Conditions

Mba1p is a protein involved in assembly of mitochondrial respiratory complexes. It may act as a receptor for proteins destined for export from the mitochondrial matrix to the inner membrane. This gene was downregulated under nitrogen limitation and upregulated after the release of limitations in all strains included into the present study. Expression levels displayed a fluctuating behavior in all cases.

Genes encoding components of the respiratory chain are regulated by *HAP4*. Buschlen *et al.* (2003) reported that a coordinated increase in expression of about two or three fold in the wild-type as compared to the *hap4Δ/hap4Δ* mutant was observed for nuclear genes that code for the different components of the five respiratory chain complexes in yeast. In the same study, it was reported that the median expression of *MBA1* in wild type was 1.6 times the median expression of *MBA1* in *hap4Δ/hap4Δ* mutant. When the expression levels of *MBA1* in *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains were compared, it was seen that the fold change in the expression level of *MBA1* in response to nitrogen pulse was 13 fold higher in *ho/ho4Δ* strain. Moreover, in *rip1Δ /rip1Δ* strain, the effect of nitrogen pulse on the *MBA1* expression was less significant, whereas RIP1/*rip1Δ* strains showed similar response with *hap4Δ/hap4Δ* strain (Figure 4.33). Therefore the effect of the

nitrogen pulse to induce the expression of *MBA1* is less pronounced in respiratory deficient strains.

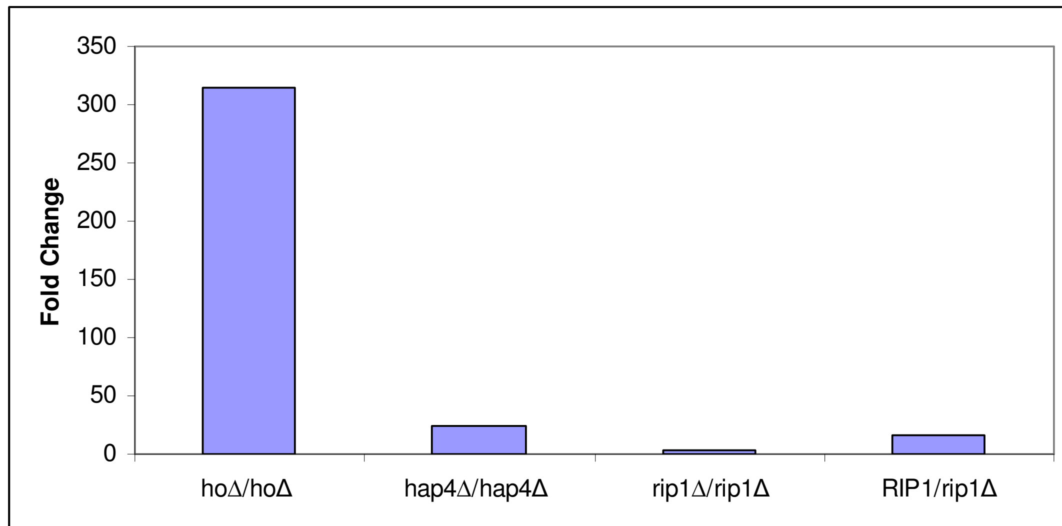


Figure 4.33 Fold change in the expression level of *MBA1* after nitrogen pulse injection

4.11. Expression Profiles of *HAP4* under Nitrogen Limited Conditions

In *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* strain, the fact that that the expression of *HAP4* was upregulated after the injection of nitrogen pulse and glucose concentration did not change indicate the possibility that *HAP4* plays an important role in nitrogen catabolite repression in yeast. It was previously reported that Tor proteins which are inactivated by nitrogen limitation, may be upstream of the Hap2,3,4,5p complex (Hardwick *et al*., 1999). 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). Thus elimination of the nitrogen limitation can effect the expression of *HAP4* in a TOR pathway dependent manner.

On the other hand, in *RIP1* deletion strains *HAP4* was downregulated in nitrogen limitation and remained repressed after the pulse injection. Thus respiratory deficiency caused by the deletion of one or two copies of *RIP1* may be responsible for the lack of the response of *HAP4* to nitrogen pulse. The *HAP* complex is originally identified as

upregulating the expression of cytochrome c and later on of several genes encoding TCA cycle and respiratory chain enzymes (e.g. Qcr8p and Cyt1p of the bc_1 complex) (Bloom *et al*., 2000). *RIP1* is the catalytic subunit of bc1 complex and its deletion may severely affect the transcriptional response of *HAP4* in nitrogen catabolite repression in yeast.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

This study aimed to investigate the effect of nitrogen limitation on glucose sensing and signaling mechanism in yeast and identify whether the signature of post- translational events can be observed in the transcription of the genes encoding those proteins.

In order to achieve this goal, four sets of chemostat experiments with *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1 Δ/rip1 Δ and RIP1/rip1Δ)* under nitrogen limited condition were carried out in order to identify the variations in the expression levels of the related genes as a response to system level perturbations. After the cells reached and remained three residence times at steady state, ammonium sulfate was injected into the cultivation medium to eliminate the nitrogen limitation. Metabolite and expression profiles were investigated during the 10 hours following the pulse injection and also at the steady states prior to and after the injections. Metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained as well as the growth curves. Expression profiles of *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4, MBA1* genes were determined via reverse transcription real time quantitative polymerase chain reaction (real time RT-qPCR).

Deletion of *HAP4* had no effect on the cell density in comparison to the *hoΔ/hoΔ* strain whereas deletion of one or two allele of *RIP1* resulted in the lower cell density under nitrogen limitation.

The highest ethanol production was obtained in *hap4Δ/hap4Δ* mutant and it was followed by *rip1Δ/rip1Δ* mutant. After the injection of ammonium sulfate pulse, ethanol concentration decreased in all studied strains. *RIP1* mutation resulted in a lower glycerol production.

All genes were upregulated in *hoΔ/hoΔ* strain in response to nitrogen pulse.

RGT1, *TUP1* and *HXK2* were downregulated in *hap4Δ/hap4Δ* strain after the injection of nitrogen pulse, whereas the other genes were upregulated. The deletion of *HAP4* does not seem to affect significantly the expression patterns of *REG1, GLC7, MIG1, SNF1* and *SNF4.*

Deletion of one or two allele of *RIP1* has a repressing effect on the expression of *HAP4*, *MTH1*, *SKP1, GRR1, PAK1, RGT1, MBA1* and *YCK1* prior to and after the nitrogen pulse. Deletion of one or two allele of *RIP1* has different effect on the expression of *STD1, YCK2, SNF1* and *CYC8.*

Respiratory deficiency seems to affect the expression of *RGT1, MBA1* and may be the reason for the dissimilarity observed between the expression profiles of *MIG1-HXK2 GRR1-SKP1* and *MIG1-CYC8-TUP1.*

HAP4 seems to plays an important role in nitrogen catabolite repression in yeast. Deletion of *RIP1* seems to affect the transcriptional response of *HAP4* in nitrogen catabolite repression in yeast.

The oscillatory behavior observed in the expression of genes after the injection of nitrogen pulse can be due to the feedback mechanisms taking part in the regulations of these genes. These mechanisms need further experimental study to be elucidated.

5.2. Recommendations

During the chemostat cultivations, CO_2 content of the exhaust gas may be measured in order to have better idea about the respiration preference of the cells.

Several more genes may be investigated for their expression levels in the cultivations under nutritional stress. In order the gain more insight about the linkage between the carbon and nitrogen metabolisms, nitrogen catabolite repression genes and the genes related to TOR kinase pathway may be investigated. Moreover, determination of protein levels in addition to the transcriptome analyzes may be necessary to determine the posttranscriptional effect.

After the repetitions of the experiments, application of some statistical tools may be useful in analyzing the data. Clustering methods may be used in order to determine genes that show similar trends.

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