STUDIES ON THE GENES CONTROLLING GLUCOSE SIGNALING IN YEAST

by

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ABSTRACT

STUDIES ON THE GENES CONTROLLING GLUCOSE SIGNALING IN YEAST

This project was aimed to study the phosphorylation or dephosphorylation of the proteins involved in glucose sensing, signal transduction and glucose repression pathways by transcription of genes encoding these proteins. *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were cultivated, in chemostat experiments, under carbon 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 to be investigated were of *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4, MBA1* genes. The cell densities of all four strains increased with carbon pulse injection. The highest cell density in steady state was seen in *rip1Δ/rip1Δ* strain. Prior to pulse injection, glucose concentration was nearly insignificant, which increased with pulse injection in all four strains. Ethanol concentration did not change with carbon pulse injection. The respiratory deficient strains *hap4Δ/hap4Δ* and *rip1Δ/rip1Δ* produced maximum amount of ethanol. It is seen that the expression of *HAP4* downregulated in *hoΔ/ hoΔ* strain, whereas it was upregulated in the homozygous and heterozygous deletion mutants of *RIP1* of *S. cerevisiae* showing the lack of the response of *HAP4* to glucose pulse caused by respiratory deficiency. It is also seen that partial respiratory deficiency effects *GRR1* and *SKP1* similarly. *MTH1, STD1, YCK1* and *YCK2* genes were downregulated in the strains *hoΔ/hoΔ*, *hap4Δ/hap4Δ* and *RIP1/rip1Δ*, and upregulated in the homozygous deletion mutant of *RIP1*. A similar behaviour was observed with the *PAK1* gene. In all four strains, it can be observed that *MTH1* is repressed in high concentrations of glucose. *REG1*, *GLC7*, *MIG1*, *SNF1* and *SNF4* genes are upregulated with carbon pulse suggesting their involvement in the glucose repression pathway in *S. cerevisiae. MBA1* gene expression upregulated after carbon pulse in *hoΔ/hoΔ, rip1Δ/rip1Δ* and *RIP1/rip1Δ* strains, however for the *HAP4* deletion mutant, the response to pulse injection was not immediate.

ÖZET

MAYALARDA GLUKOZ SİNYAL İLETİMİNİ KONTROL EDEN GENLER ÜZERİNDE ÇALIŞMALAR

Bu proje kapsamında glukozun algılanma, sinyal iletimi ve baskılanma mekanizmalarının yazılımında rol alan proteinlerin ve bu proteinleri şifreleyen genlerin araştırılması amaçlanmıştır. *S. cerevisia* BY 4743'nin gen delesyonu taşıyan *hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1Δ/rip1Δ ve RIP1/rip1Δ* suşları glukoz kaynağını eksik miktarda içeren F1 ortamında, sıcaklık, pH, çözünmüş oksijen ve karıştırma hızı kontrollü kesiksiz fermentasyon koşullarında büyütülmüştür. Ortama glukozun 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. *CYC8, GRR1, MTH1, RGT1, RGT2, SKP1, SNF3, STD1,TUP1, YCK1, YCK2, ELM1, GLC7,HXK2, MIG1, PAK1, REG1, SNF1, SNF4, TOS3, HAP4* ve *MBA1* genlerinin gen anlatım profilleri incelenmiştir. Glukoz darbesinden sonra dört suşda da hücre üretiminin artışı gözlenmiştir. *rip1Δ/rip1Δ* suşunda en yüksek miktarda hücre üretimi gözlenmiştir. Glukoz darbesi ile her dört suşda da glukoz derişiminde artış gözlenmiştir. Glukoz darbesi ile etanol derişiminde bir değişim gözlenmemiştir. En yüksek etanol derişimi solunum eksikligi gözlenen *hap4Δ/hap4Δ* ve *rip1Δ/rip1Δ* genlerinde belirlenmiştir. *HAP4* geninin anlatımı *hoΔ/ hoΔ* suşunda baskılanırken *RIP1* geni silinmiş suşlarda artmıştır. Bu da *HAP4* geninin glukoz darbesine yanıtının solunum eksikliği ile azaldığını göstermiştir. Kısmı solunum eksikliği ise *GRR1* ve *SKP1* genlerini benzer şekilde etkilenmiştir. *hoΔ/hoΔ*, *hap4Δ/hap4Δ* ve *RIP1/rip1Δ* suşlarında *MTH1, STD1, MTH1, STD1, YCK1* ve *YCK2* genlerinin gen anlatımları baskılanırken *rip1Δ/rip1Δ* suşunda arttığı gözlenmiştir. Benzer bir ekti *PAK1* geninde de gözlenmiştir. Her dört suşda *MTH1* gen anlatımının yüksek glukoz derişiminde baskılandığı belirlenmiştir. *REG1*, *GLC7*, *MIG1*, *SNF1* ve *SNF4*genlerinin gen anlatımlarının glukoz darbesi ile artması bu genlerin glukoz baskılanma mekanizmasında rol aldığını önermiştir.

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LIST OF SYMBOLS / ABBREVIATIONS

A	Absorbance
C_t	Threshold cycle
N	Cycle number
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
mRNA	Messenger RNA
OD	Optical density
ORF	Open reading frames
RFU	Relative fluorescence units
rRNA	Ribosomal RNA
RT-rtqPCR	Reverse transcription real time quantification polymerase chain reaction
TCA cycle	Tricarboxyacidic cycle

1. INTRODUCTION

There are many steps in the pathway leading to DNA to protein, and all of these steps 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. However, for most genes, the initiation of RNA transcription in the most important point of control. Cells can change the pattern of genes they express in reponse to changes in their environment (Alberts *et al*., 1994)

Many studies have been proceeded on the yeast *Saccharomyces Cerevisiae* to analyze the transcriptome, the proteome, the metabolome, and the interactome. Knowledge of the sugar signalling system of this organism would enable extrapolation of understanding toward multicellular organisms, as there are parallels between the *S. cerevisiae* glucose metabolism, tumor cell growth, and diseased/senescent state of eukaryotic cells, and it would eventually provide knowledge for gaining improved understanding of glucose metabolism related diseases like diabetes and cancer (Raghevendran *et al*.,2005).

1.1. Yeast Metabolism

Yeast metabolism refers to the biochemical assimilation and dissimilation of nutrients by yeast cells. The subject therefore encompasses all enzymic reactions within the yeast cell and how such reactions are regulated. Assimilatory, anabolic pathways are energy-consuming, reductive processes which lead to the biosynthesis of new cellular material. Dissimilatory, catabolic pathways are oxidative processes which remove electrons from intermediates and use these to generate energy. The reductive and oxidative processes of anabolism and catabolism are mediated by dehydrogenase enzymes which pre-dominantly use NADP and NAD, respectively, as redox cofactors. Such biosynthetic

and degradative pathways, however, do not operate in isolation and should be regarded as components of the integrated processes which are associated with the growth and survival of the yeast cell. Figure 1.1 summarizes the linkage of anabolic and catabolic processes which occur in yeasts.

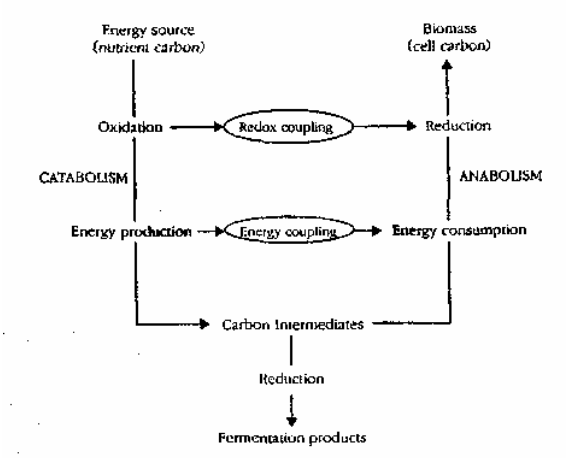


Figure 1.1**.** Simplistic overview of yeast carbon metabolism. (Wolker, G. M., 2000)

Yeasts are chemoorganotrophic microorganisms which derive their chemical energy, in the form of ATP, from the breakdown of organic compounds. However, there is metabolic diversity in the way in which yeasts generate and consume energy from carbon substrates.

1.2. Central Carbon Metabolism in *Saccharomyces Cerevisiae*

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

Most yeasts utilize sugars as their preferred carbon and energy sources. The sequence of enzyme-catalysed reactions that oxidatively convert glucose to pyruvic acid in the yeast cytoplasm is known as glycolysis. Glycolysis provides yeast with energy, together with precursor molecules and reducing power for biosynthetic pathways. The key regulatory enzymes in glycolysis are irreversible phosphofructokinases (*PFK*) and

pyruvate kinase (*PYC*) whose activity is influenced by numerous effectors, including ATP. (Walker, 1998)

With regard to fermentation, several biotechnologically important yeasts including *S. cerevisiae* are fermentative and, as such, are defined as organisms which use organic substrates anaerobically as electron donor, electron acceptor and as carbon source. During alcoholic fermentation of sugars, yeast re-oxidizes NADH to NAD in terminal step reactions from pyruvate. In this first of these reactions pyruvate is decarboxylated to acetaldehyde, catalysed by pyruvate decarboxylase (*PDC*), and then to reduced to ethanol.

S. cerevisiae is one of the few organisms that prefer to ferment glucose, even when oxygen is abundant. The propensity of this yeast to carry out aerobic fermentation is called "Crabtree Effect" (Johnston and Kim, 2005).

Glucose may affect enzyme levels by causing a decrease in the concentration of corresponding mRNA's, a decrease in their translation rate, or an increase in the degradaiton 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 transcription level (Gancedo, 1998)

1.3. Diauxic Shift and the HAP Complex

Microorganisms adapt their metabolism to environmental conditions and, particularly, to nutrient disponibility. The baker's yeast *Saccharomyces cerevisiae* has a clear preference for glucose as a carbon source, with subsequent conversion to ethanol by fermentation (Bourgarel *et. al.,* 1999). 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' (Buschlen *et. al.,* 2003). During this shift from fermentation to respiration, gene expression is largely reprogrammed. This is achieved by derepression of glucose-repressed genes and specific induction of genes involved in gluconeogenesis, metabolism of alternate carbon sources,

respiration and mitochondrial development. These transcriptional changes are largely mediated by the Hap2/3/4/5p transcription factor. Indeed, disruption of any subunits of this complex renders the cells unable to grow on non-fermentable carbon sources (Bourgarel *et. al.,* 1999).

The HAP complex is a heteromultimer composed of four subunits. Subunits 2, 3 and 5 are necessary and sufficient for binding to the target promoters. Functional complementation of HAP2, HAP3 and HAP5 mutants of *S. cerevisiae* has allowed cloning of the respective homologues from both eukaryotic microorganisms and metazoans. Each of the subunits contains a core domain that has remained highly conserved throughout evolution. This core domain is necessary for DNA binding and subunit association. Extensive studies on yeast and mammalian homologues have led to a considerable understanding of the molecular interactions between DNA and the three subunits. The situation is quite different for the Hap4p subunit. This subunit is not necessary for *in vitro* DNA binding, interacts directly with the Hap2/3/5p trimer and contains a transcriptional activation domain in its C-terminal part. Hence, the HAP complex holds an original modular structure in which DNA binding and activation domains are contained on physically distinct proteins. The activity of the complex can be modulated via HAP4 expression, HAP4 being induced under specific environmental conditions. Indeed, in *S. cerevisiae,* HAP4 transcription is induced from five to 10 times during the diauxic shift from glucose to ethanol, or by growth on non-fermentable carbon sources, whereas HAP2 and HAP3gene expression is constitutive. This suggests that Hap4p is really the key regulator of the complex activity in response to carbon sources in *S. cerevisiae* (Bourgarel *et. al.,* 1999). The HAP complex was originally identified as upregulating the expression of cytochrome *c* and later on, of several genes encoding TCA cycle and respiratory chain enzymes. During the global transcriptome analysis of yeast genes in relation to growth phase on glucose, genes are clustered according to their expression pattern and it is found that nuclear-encoded genes playing a role in respiratory chain function and TCA cycle were also transcriptionally induced at the diauxic shift, leading to the hypothesis that *HAP4* was responsible for this induction. The fact that *HAP4* plays a role in programming a respiratory metabolism is further confirmed by its metabolic effect when overexpressed and by its induction when yeast cells are placed in conditions of adaptative evolution under glucose-limiting conditions (Buschlen *et. al.,* 2003).

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 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.

1.4. Glucose limited medium

Yeast cells, like other free-living microorganisms, encounter large differences in nutrient availability. The response of haploid yeast cells to starvation for essential nutrients is a complex cellular process, which leads either to adaptation to alternative nutrients or to entry into stationary phase. In exponential cultures of *S. cerevisiae* grown on rich media, the first limiting nutrient will usually be the fermentable sugar (e.g. glucose). At this stage, the cells enter the diauxic shift, where their metabolism is shifted to the oxidative utilization of ethanol and acetate, the byproducts of fermentation. The cells then resume growth (the slow growth phase) on these carbon sources. Depletion of all carbon sources or other essential nutrients leads to entry into the stationary phase. Stationary phase entails extensive changes in the physiology of the cells, allowing for long-term survival and rapid emergence into the division cycle when nutrients become available (Jona *et. al.*, 2000).

The effects of nutrient starvation on macromolecular synthesis have been addressed in several studies. According to studies by Jona *et. al.* protein synthesis is severely affected, as is the synthesis of some classes of RNA. RNA polymerase I transcription is nearly shut off, whereas the levels of tRNA and mRNA are only mildly affected. Amino acid starvation leads to a selective decrease in the levels of mRNA encoding ribosomal proteins, suggested to be analogous to the stringent response in bacteria. On the other hand,

starvation for glucose induces transcription of a set of genes including heat shock proteins (HSPs) and the metallothionein gene (CUP1), which resembles the set of genes expressed during the entry into stationary phase. Transcription rates decline to less than 20 percent when yeast cells are transferred from glucose media to media containing galactose or lacking sugars. The decrease in transcription is rapid and sustained (Jona *et. al.*, 2000).

Prolonged chemostat cultivation under defined conditions offers an excellent approach to study evolution, and has been applied to various micro-organisms and nutrient limitations. Under nutrient-limited conditions, selection is primarily for an improved affinity for the growth-limiting nutrient. A general trend observed during prolonged chemostat cultivation is a progressive, generally hyperbolic, decrease of the residual concentration of the growth-limiting nutrient (Jansen *et. al.*, 2005).

Genome-wide transcriptome analysis, performed during prolonged chemostat cultivation of *S. cerevisiae* on glucose, revealed changes in expression of many genes, including several genes encoding proteins involved in central carbon metabolism. Furthermore, the strong selection pressure in these cultures resulted in the enrichment of mutants with one or more duplications of particular *HXT* genes, encoding high-affinity hexose transporters. Although these studies yielded important insights into the dynamics of the yeast genome under selective conditions, the changes that were observed at the transcriptome level were not correlated to enzyme levels or metabolic capacities (Jansen *et. al.*, 2005).

After prolonged glucose-limited cultivation of *S. cerevisiae*, in addition to an increased affinity for glucose, Jansen *et. al.* observed a dramatic decrease in fermentative capacity (Jansen *et. al.*, 2005).

1.5. The Glucose Induction 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 percent), 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)

1.6. The Main-Glucose Repression Pathway

1.6.1. 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).

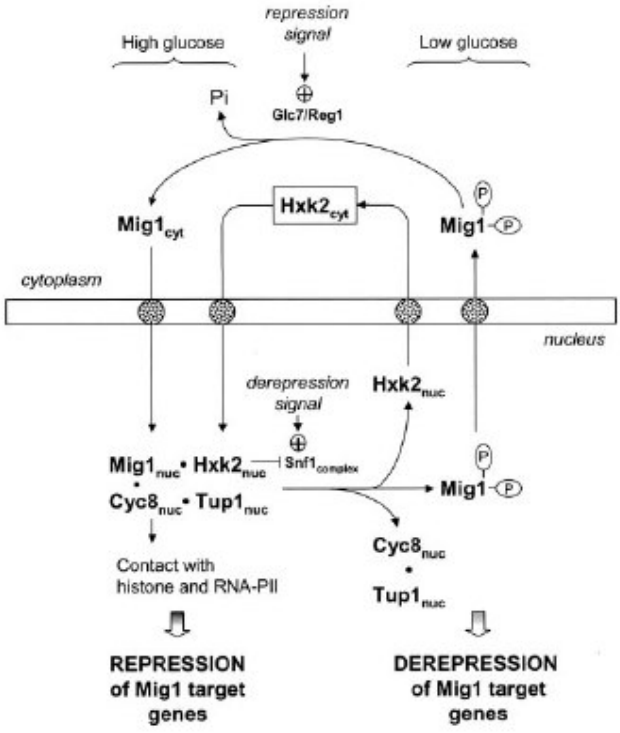


Figure 1.2. Nucleocytoplasmic Translocation of Hxk2 (Ahuatzi *et al*., 2004)

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).

1.6.2. 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).

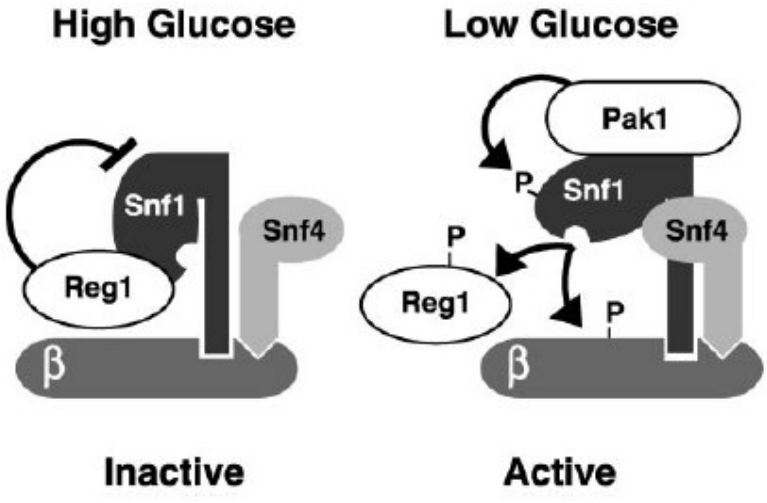


Figure 1.3. Activation and Inactivation of Snf1 Complex (Nath *et al*., 2003)

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).

1.6.3. 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).

1.6.4. 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).

1.7. Reverse Transcription Real Time Quantification PCR

The polymerize chain reaction (PCR) has proven to be a versatile tool in molecular biology. The use of this technique has generated unprecedented advances in gene discovery, diagnostics, and gene expression analysis. In addition, new techniques that build on PCR have further expanded its range of scientific applications.

Real-time is a powerful advancement of the basic PCR technique. Though the use of appropriate flourescent detection strategies in conjunction with proper instrumentation, the starting amount of nucleic acid in the reaction can be quantitated. Quantitation is achieved by measuring the increase in flourescence during the exponential phase of PCR.

Applications of real-time PCR include measurements of viral load, gene expression studies, clinical diagnostics, and pathogen detection.

Although the performance of PCR in more routine molecular biology applications can be relatively straightforward to optimize several parameters must be evaluated and optimized.

The factors that affect real-time PCR fall into 3 categories. These are general laboratory practices, template and primer design, and reaction components and conditions. When determining which conditions to optimize, the ultimate assay goal (i.e. qualitative analysis vs. quantitation) must be considered.

1.7.1. Intercalation Dyes

The introduction of real-time PCR had made it possible to accurately quantify starting amounts of nucleic acid during the PCR reaction without the need for post-PCR analyses. At the end of the amplification, the reaction mixture can be discarded without opening the tube, thus avoiding contamination of the laboratory with PCR product. In real-time PCR, a flourescent reporter can be of a nonspecific nature or of a specific nature. The flourescent of the reporter molecule increases as products accumulate with each successive round of application. In the early cycles of amplification, the change in flourescent of the reporter is usually undetectable, but at some point during amplification, the accumulation of product results in a measurable change in the flourescent of the reaction mixture. There is a linear relationship between the log of the starting amount of template and the corresponding threshold cycle. This standard curve can then be used to determine the starting amount for each unknown template, based on its threshold cycle.

The first demonstration of real-time PCR used the nonspecific reporter ethidium bromide. The flourescence of ethidium bromide increases significantly as it binds and intercalates into double stranded DNA during the extension step of the amplification cycle. SYBR Green I can also be used as a flourescent reporter in real-time PCR. Intercalating dyes bind to all double-stranded DNA products. Therefore the increase in flourescence does not necessarily accurately reflect the increase in desired product.

The PCR machine contains a sensitive camera which monitors the flourescence in each well of the 96-well plate at frequent intervals during the PCR reaction. As DNA is synthesized, more SYBR Green will bind and the flourescence will increase.

The melting temperature of a DNA double helix depends on its base composition (and its length if it is very short). All PCR products for a particular primer pair should have the same melting temperature, unless there is contamination, mispriming, primer-dimer artifacts, or some other problem.

Since SYBR Green does not distinguish between one DNA and another, an important means of quality control is to check that all samples have a similar melting temperature. After real time PCR amplification, the machine can be programmed to do a melt curve, in which the temperature is raised by a fraction of a degree and the change in flourescence in measured. At the melting point, the two strands of DNA will separate and the flourescence will rapidly decrease. The software plots the rate of change of the flourescence with temperature against the temperature and this will results in a peak at the melting temperature (Tm).

1.8. The Aim of This Thesis

This study aimed to investigate the effect of carbon 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 carbon 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

2.1.2.1. Media Used. Carbon 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 were used.

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 was purchased from Quiagen was used.

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

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^{o}C, New Brunswick Scientific U410 (England)
  -20^{o}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^{o}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^{o}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. The dissolved O_2 concentration was monitored with an O_2 probe.

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 times at steady state, pulse injections of glucose 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 percent 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^{o}C after it was transferred to a new Eppendorf

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

For RNA extraction, samples were shock frozen in liquid nitrogen then they were transferred to -80 ^{o}C. They were kept at -80^{o}C until use.

2.2.1.4. Enzymatic Analyses for Determination of Metabolite Concentrations. Concentration profiles of extra cellular ethanol, glucose, ammonia and glycerol 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).

$$D-Glu\cos e+ATP\xrightarrow{HK}G6P+ADP \qquad (2.1)$$

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).

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

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 determined 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 catalyzed 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:

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

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^{o}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. The mechanical disruption protocol was suitable for time-course experiments where enzymatic incubation steps could not be tolerated. The first step in the protocol was to prepare acid-washed glass beads, 0.45–0.55 mm diameter, by soaking in concentrated nitric acid for 1 hour, washing extensively with deionized water, and drying in a baking oven. After disruption in a bead mill homogenizer, all steps of the RNeasy protocol were performed at room temperature. It was necessary to work quickly during the procedure. After harvesting the cells, all steps were performed at 20–25°C in a standard microcentrifuge.

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). 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, and MBA1* genes were designed according to the following criteria. 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 place on ends of the primer. Most primers had melting temperatures between 50^{o}C and 65^{o}C where 55^{o}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
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

Table 2.1. RT-rtqPCR primer sequences (Continued)

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
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:

Table 2.2. Reaction mixture for PCR

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

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

Table 2.3. Reaction program for PCR

Cycle 1 (1X)	Step 1:		50°C	30 minutes		
Cycle 2 (1X)	Step 1:		95°C	15 minutes		
Cycle 3 (40X)	Step 1:		94°C	15 seconds		
			Step 2:		52.2°C	15 seconds
			Step 3:	72°C	30 seconds	
Cycle 4 (1X)	Step 1:		95°C	1 minute		
Cycle 5 (1X)	Step 1:	50°C	1 minute			
Cycle 6 (80X)	Step 1:	48°C	10 seconds with ramp: 0.5°C			

2.2.1.7. 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 this project, 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, chemostat experiments were carried out with the following four strains of *S. cerevisiae* BY4743: *hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1 Δ/rip1 Δ and RIP1/rip1Δ*. These four chemostats were carried out under glucose limited conditions with injections of glucose pulse. Growth curves and metabolite profiles for glucose, ethanol, ammonia and glycerol were obtained. Reverse transcription real time quantitative polymerase chain reaction (real time RT-qPCR) was used in order to determine the 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 under glucose limited conditions.

3.1. Growth Characteristics of Deletion Strains

The cells were grown in glucose limited F1 medium, in which cells prefer respiration instead of fermentation. Optimal growth conditions for *S. cerevisiae*, with temperature at 30^{o}C and pH within the range of 5.5 were obtained during the cultivations. *S. cerevisiae BY4743 hoΔ/hoΔ* strain were used for wild type strain, the other three respiratory deficient or partial respiratory deficient strains. Samples were collected on hourly bases for ten hours after the injection of carbon pulse. The first and second steady state samples were collected after three residence times spend in steady state both prior to and after pulse injection. External metabolite profiles and growth characteristics were monitored. The arrows on the following graphs indicate pulse injections.

3.1.1. Growth Characteristics *S.Cerevisiae BY4743 (hoΔ/hoΔ)* under Carbon Limitation

S. cerevisiae BY4743 (*hoΔ/hoΔ*) strain was used as a control and was cultivated as described in the Materials and Methods section. Growth curve and metabolite profiles of *S.*

cerevisiae BY4743 (*hoΔ/ho1Δ)* in carbon limited chemostat cultures are presented in Figures 3.1 a and b.

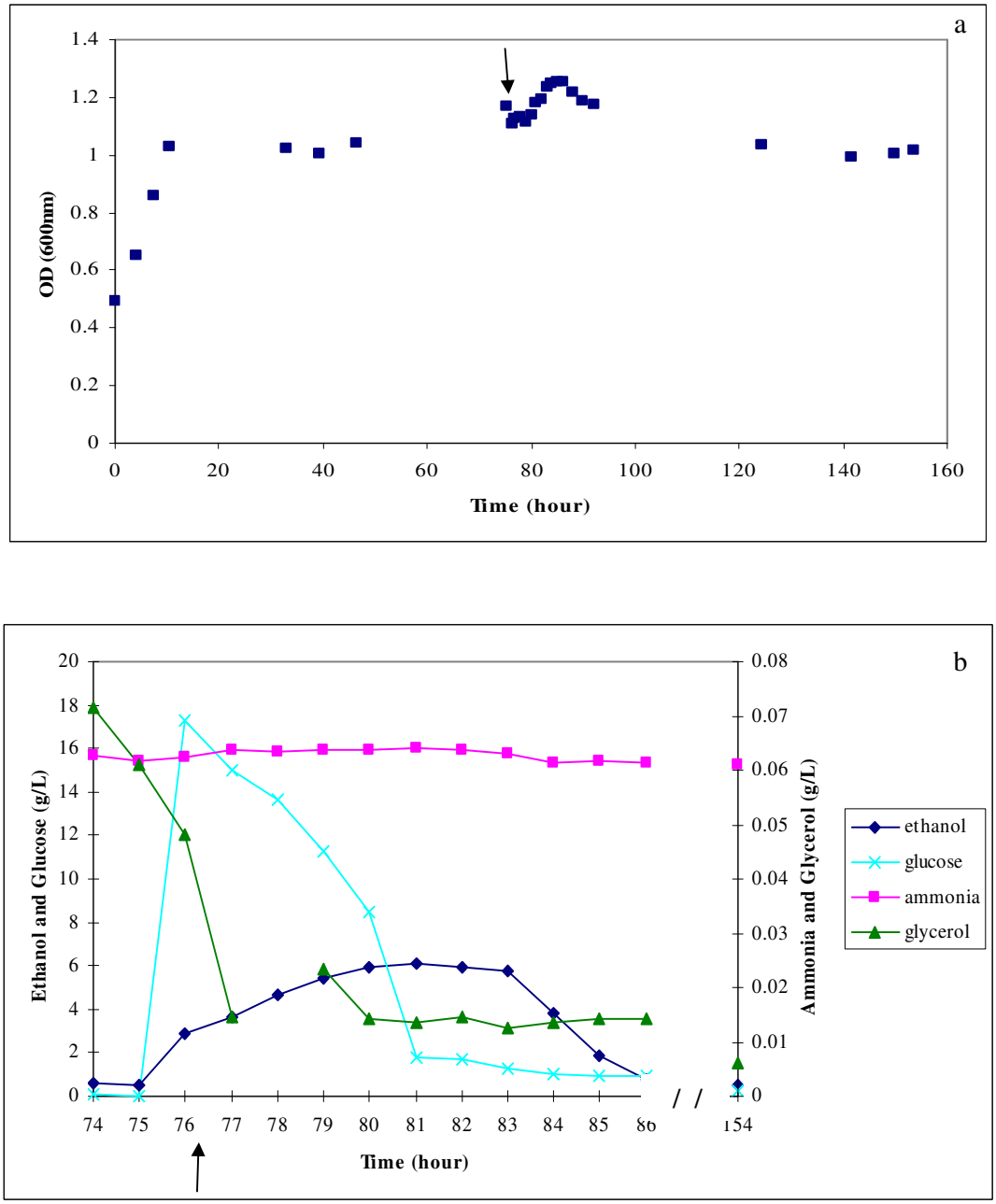


Figure 3.1. Growth curve (a) and extracellular metabolite profiles (b) of *S. Cerevisiae* BY4743 *(hoΔ/hoΔ)* under carbon limited condition in chemostat culture with pulse injection

The culture was left to operate batch wise overnight, after inoculation of the cell. The first steady state was reached within 20 hours after switching to chemostat. Glucose pulse was injected after passing three residence times at steady state at 76th hour of the chemostat cultivation. As it can be seen in Figure 3.1a, a minor decrease was observed followed by an increase in cell growth for the 10 hours after the glucose pulse injection. Then the OD decreased for 8 hours and reached the second steady state at about the same OD value as the first steady state.

Prior to the pulse injection, the glucose concentration and the ethanol concentration were nearly zero. After the pulse injection, the glucose concentration was 18 g/L and it started decreasing until it reached its second steady state value which was 0.25 g/L. The ethanol concentration also increased and reached a value approximately of 6 g/L 4 hours after pulse injection. The ethanol concentration remained constant before starting to decrease 7 hours after pulse injection and reached a value of 0.5 g/L, which is about the same as the concentration at first steady state. Decrease in glucose concentration started one hour after pulse injection, with the steepest decrease observed 4 hours later. Ammonia concentration was nearly constant throughout the cultivation with values between 0.08 g/L and 0.085 g/L. Glycerol concentration, which was 0.07 g/L at first steady state, decreased right after pulse injection and stayed constant after the 4th hour of sampling.

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

A homozygous deletion strain of *HAP4* (*hap4Δ/hap4Δ*) in a genetic background of wild type strain of *S. cerevisiae* BY4743 was cultivated under carbon limited conditions. The growth characteristics and metabolite concentrations including glucose, ethanol, glycerol and ammonia were determined throughout the chemostat cultivation of *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* before and after the pulse injections grown under carbon limited conditions (Figures 3.2 a and b.).

Before starting the chemostat, the cells were left to operate batchwise for one night right after inoculation. After chemostat started, the OD value increased slightly and reached steady state value. At the 87th hour, after three residence time had passed in steady

state, glucose pulse was injected. Right after pulse injection, a slight decrease in the OD values was observed followed by cell growth for the next 10 hours. After 10 hours, the OD decreased and second steady state value was reached, being a higher value than first steady state. Once again three residence time was spent in steady state before ending the experiment.

Prior to pulse injection, the glucose concentration was approximately 0 g/L in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ).* After the injection of glucose pulse, the glucose concentration showed a sharp increase and reached the value of 17 g/L. The concentration decreased during the cultivation and as the second steady state was reached, very little amount of glucose existed. Ethanol concentration value was also close to zero prior to pulse injection, and it increased after the injection to a value of 7 g/L. Ethanol concentration decreased as glucose concentration decreased and as second steady state was reached, the ethanol concentration was 0.9 g/L, which was the concentration before pulse injection. The ammonia concentration stayed almost constant throughout the cultivation, between 0.57 g/L and 0.62 g/L. Glycerol concentration was 0.08 g/L before the pulse injection, and decreased steadily until there was trace amount left at the end of cultivation.

3.1.3. Growth Characteristics *S. Cerevisiae BY4743 (rip1Δ/rip1Δ)* under Carbon Limitation

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

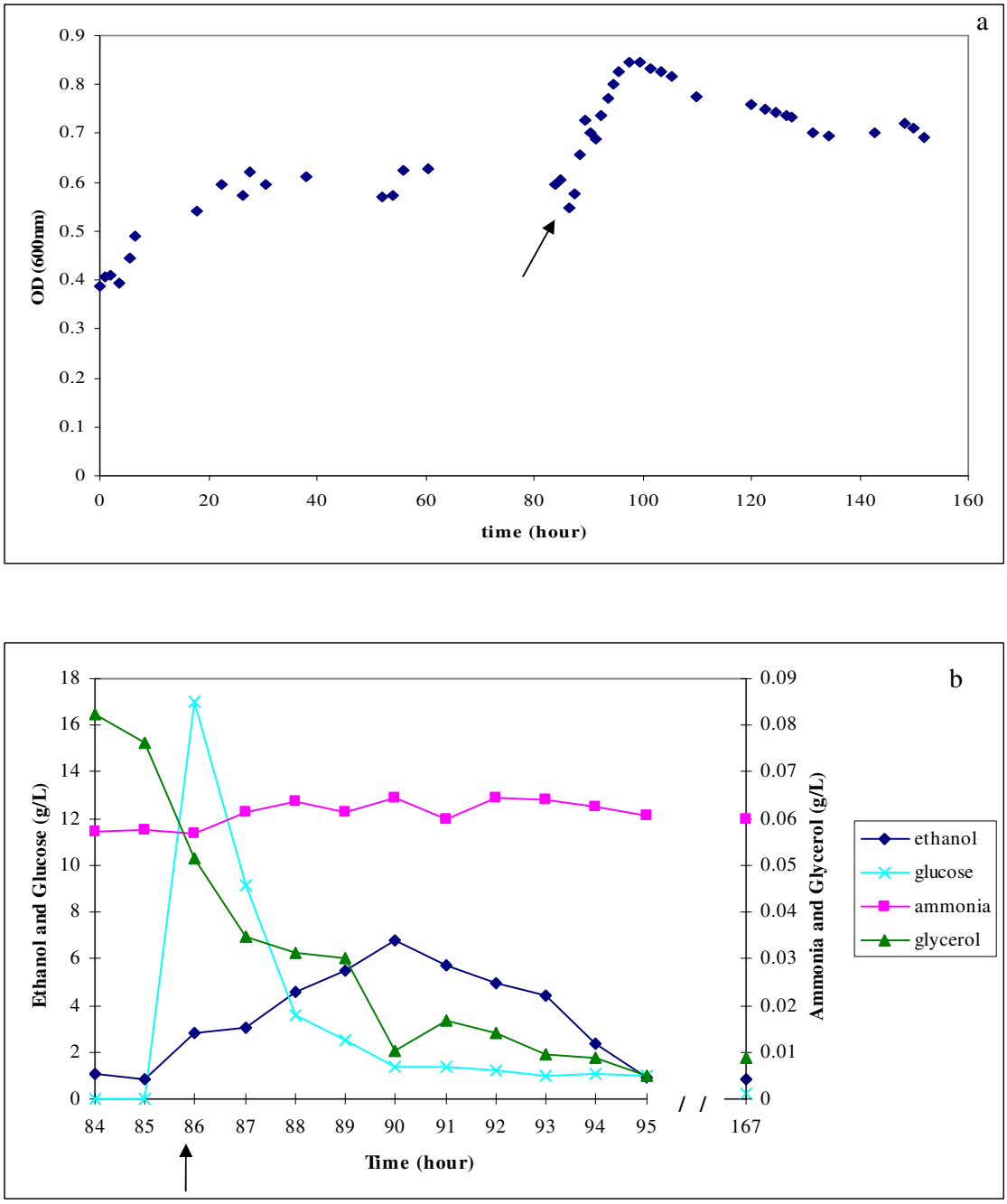


Figure 3.2. Growth curve (a) and extracellular metabolite profiles (b) of *S. Cerevisiae* BY4743 *(hap4Δ/hap4Δ)* under carbon limited condition in chemostat culture with pulse injection

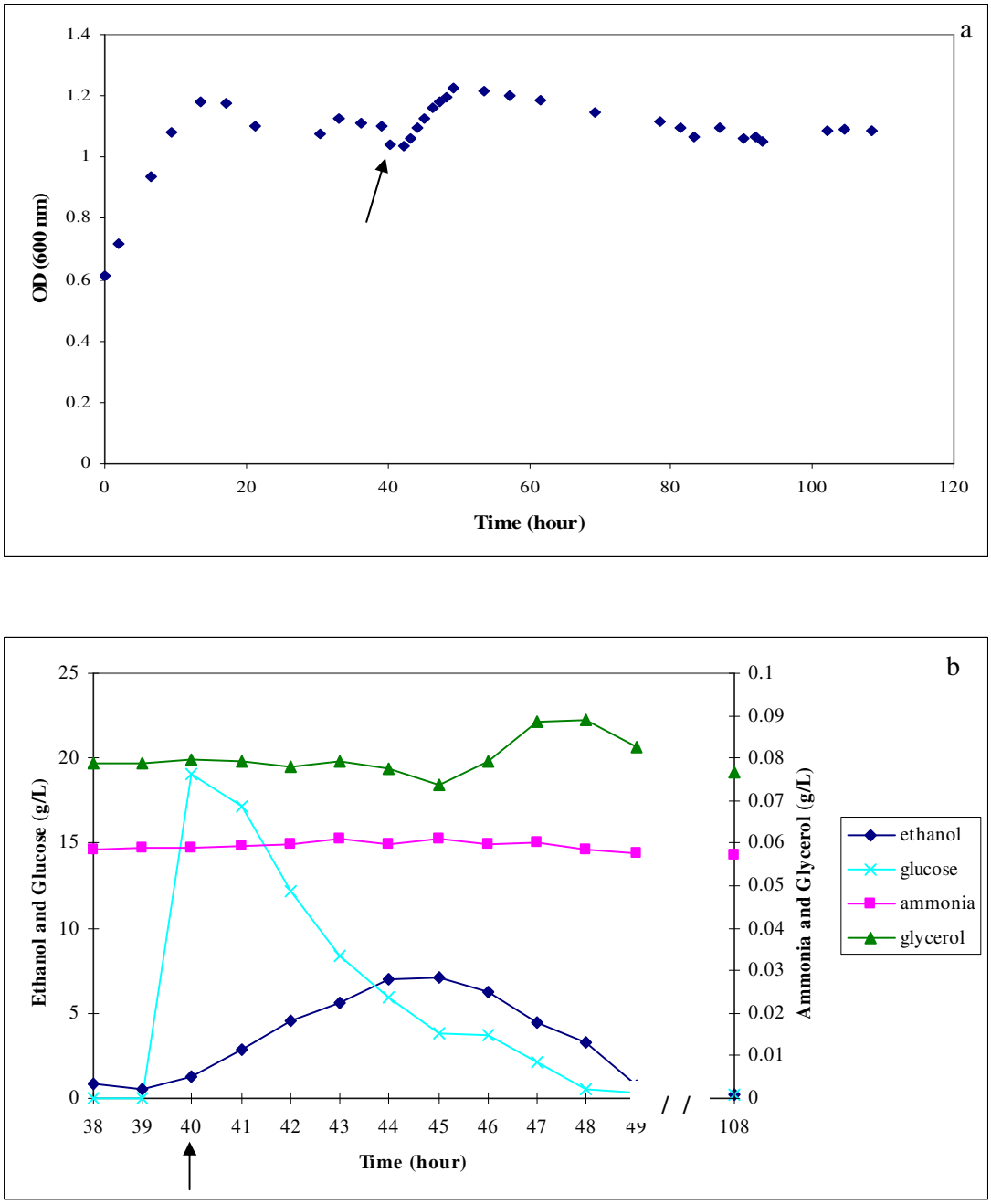


Figure 3.3. Growth curve (a) and extracellular metabolite profiles (b) of *S. Cerevisiae* BY4743 *(rip1Δ/rip1Δ)* under carbon limited condition in chemostat culture with pulse injection

After leaving the cultivation batchwise overnight, it was switched to chemostat. The OD increased for 18 hours before reaching steady state. Around the 17th hour, the OD value was higher than the steady state value seen in the 33rd hour which was around 1.1

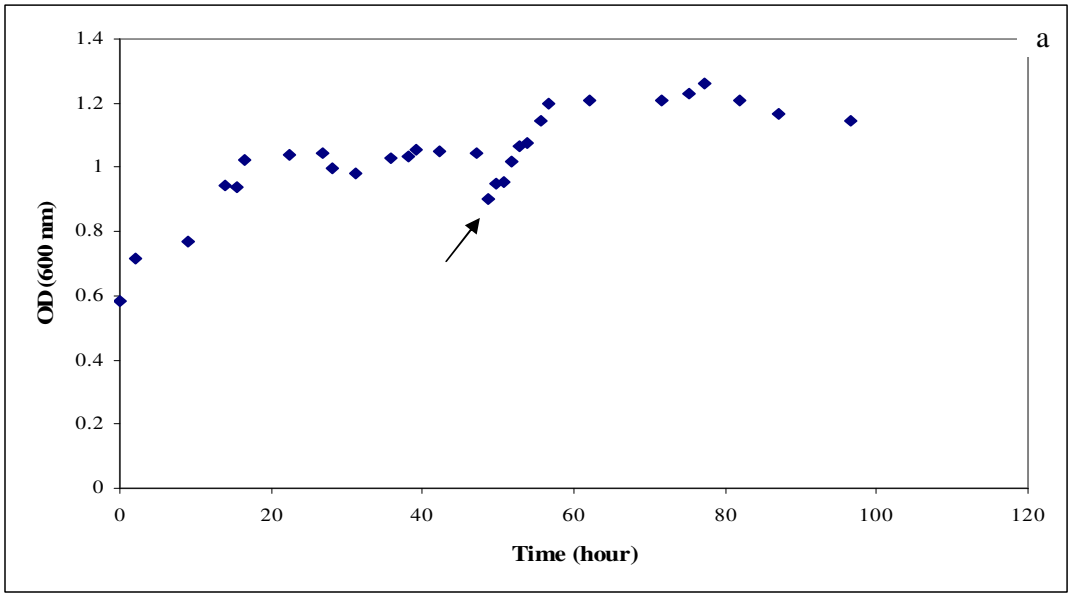
g/L. The glucose pulse injection was given at the end of three residence times. A slight decrease in the OD values was followed by the increase of OD values for the next 10 hours. After 10 hours, the OD values started decreasing until second steady state and were reached with a slightly lower value for cell growth than the first steady state. After three residence times in the second steady state, the experiment was terminated.

Glucose concentration was very little before pulse injection. Its concentration increased sharply soon after the pulse injection to 19 g/L. The concentration of glucose then decreased during the cultivation down to 0.2 g/L at the second steady state. Ethanol concentration was 0.5 g/L prior to pulse injection. After the injection the ethanol concentration increased up to 7 g/L, and then decreased to 0.2 g/L at the second steady state. Ammonia concentration was steady during the cultivation. Slight fluctuations around 0.009 g/L were observed. Glycerol concentration also stayed constant during most of the cultivation. The glycerol concentration was 0.079 g/L until the 47^{th} hour, when it increased to 0.088 g/L as ethanol concentration decreased. As the second steady state was reached, the glycerol concentration decreased to 0.76 g/L.

3.1.4. Growth Characteristics *S. Cerevisiae BY4743 (RIP1/rip1Δ)* under Carbon Limitation

A heterozygous deletion strain of *RIP1* (*RIP1/rip1Δ*) in a genetic background of wild type strain of *S. cerevisiae* BY4743 was cultivated under carbon limited conditions. Growth curve and metabolite profiles of *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* before and after the pulse injection in carbon limited chemostat culture were presented in Figures 3.4 a and b.

After overnight batch cultivation the system was switched to chemostat. The OD increased for 20 hours before reaching steady state. The glucose pulse injection was given at the end of three residence times in steady state. After the pulse injection, the OD value decreased for one hour and then cell growth increased for the next 9 hours and the steady state value was reached. The second steady state value for this cultivation was higher than the first steady state value. After three residence times in steady state, the experiment was terminated.



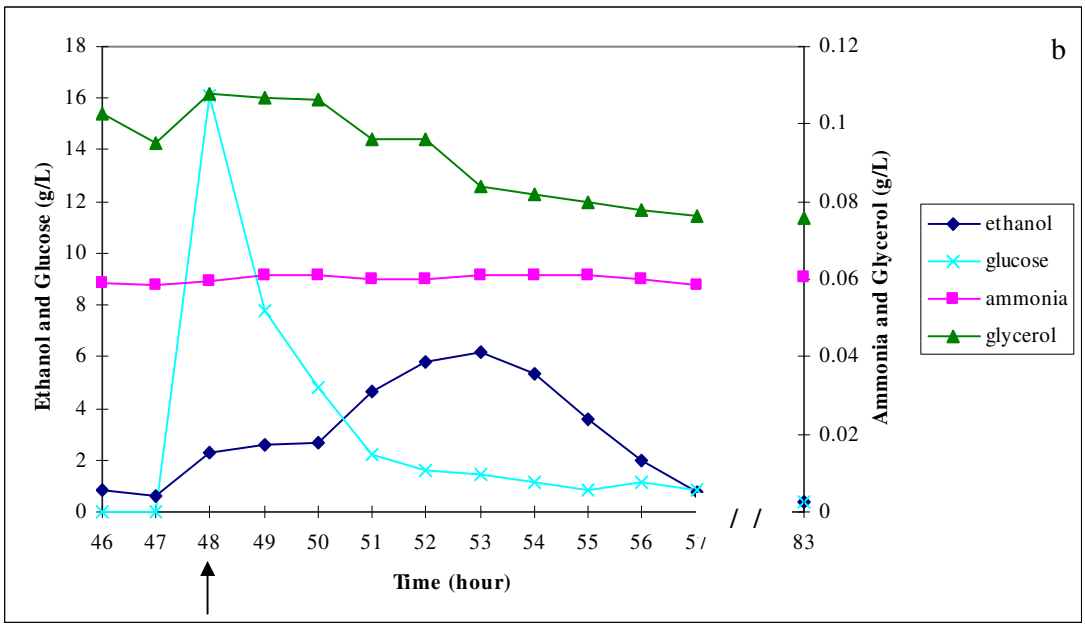


Figure 3.4. Growth curve (a) and extracellular metabolite profiles (b) of *S. Cerevisiae* BY4743 *(RIP1/rip1Δ)* under carbon limited condition in chemostat culture with pulse injection

After overnight batch cultivation the system was switched to chemostat. The OD increased for 20 hours before reaching steady state. The glucose pulse injection was given at the end of three residence times in steady state. After the pulse injection, the OD value decreased for one hour and then cell growth increased for the next 9 hours and the steady state value was reached. The second steady state value for this cultivation was higher than the first steady state value. After three residence times in steady state, the experiment was terminated.

Prior to pulse injection, the remaining glucose concentration was approximately 0.01 g/L in *S. cerevisiae* BY4743 (*RIP1/rip1Δ).* After the injection the glucose concentration increased to 16 g/L. Its concentration decreased during the cultivation and 0.3 g/L glucose was remained. Ethanol concentration was 0.58 g/L prior to pulse injection. After the pulse injection the ethanol concentration increased for 6 hours, reaching a concentration of 6 g/L. Ethanol concentration then decreased and 0.4 g/L ethanol was measured at the second steady state. Ammonia concentration was almost constant throughout the cultivation, ranging between 0.01 g/L and 0.008 g/L. Glycerol concentration was 0.1 g/L prior to pulse injection and decreased steadily until it reached 0.075 g/L at the end of cultivation.

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

Four chemostat experiments with partial and respiratory deficient mutants of *S. cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ*) were carried out in carbon limited F1 medium. Real time RT-qPCR was used to identify the variations in the expression levels of the genes involved in glucose sensing, signal transduction and glucose repression pathways. To recover the carbon limitation, glucose pulse was injected after the cells spent three residence time at steady state. Samples were collected in hourly basis, mRNA were extracted from the samples collected. RT-qPCR enabled the investigation of transcriptional responses by initially reverse transcribing mRNA into cDNA and than amplifying at specified locations. The following genes were investigated: *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 Glucose Pulse

RGT2 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, therefore it is a sensor of high 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).

For the investigation of transcriptional responses the first samples were collected just before pulse injection. In the first minute after injection, 4 samples were collected (samples 2-5). For the next 20 minutes samples were collected every 5 minutes (samples 6-9). The following samples (10-19) were collected every hour for ten hours. The last samples were collected at the end of three residence time at the second steady state.

The expression profiles of *RGT2* in deletion strains of *Saccaromyces cerevisiae* (*hoΔ/hoΔ, hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ)* were represented in Figure 3.5, 3.6, 3.7 and 3.8 respectively.

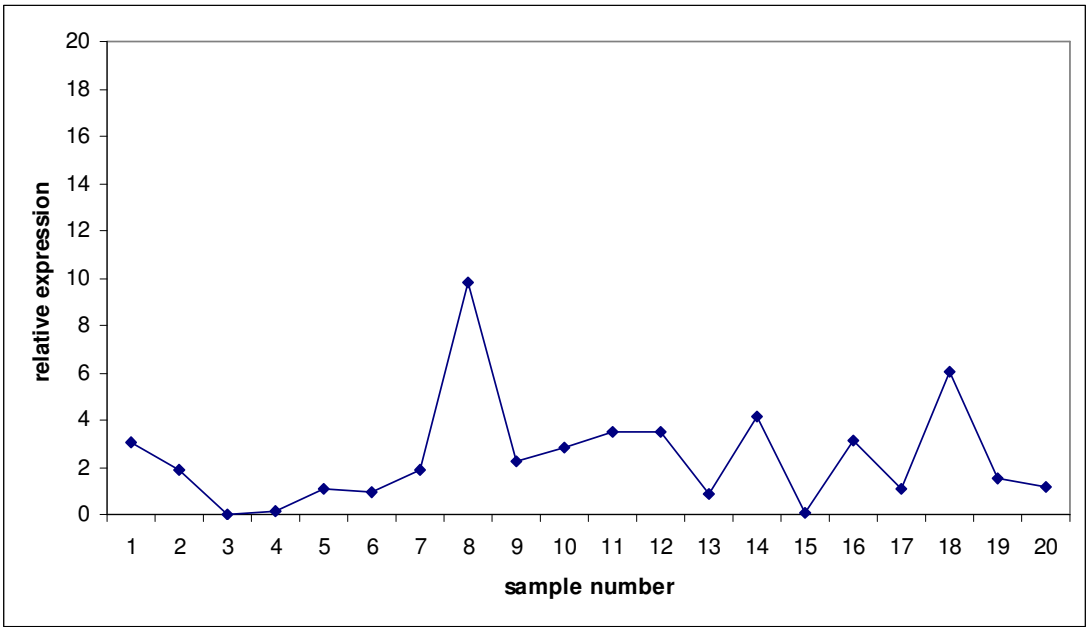


Figure 3.5. Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *RGT2* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* strain decreased right after the injection of glucose pulse. At the 15th minute after the injection the expression level increased 5 fold, and then in decreased to approximately its initial level. It showed an oscillatory behavior as cell growth was reaching steady state

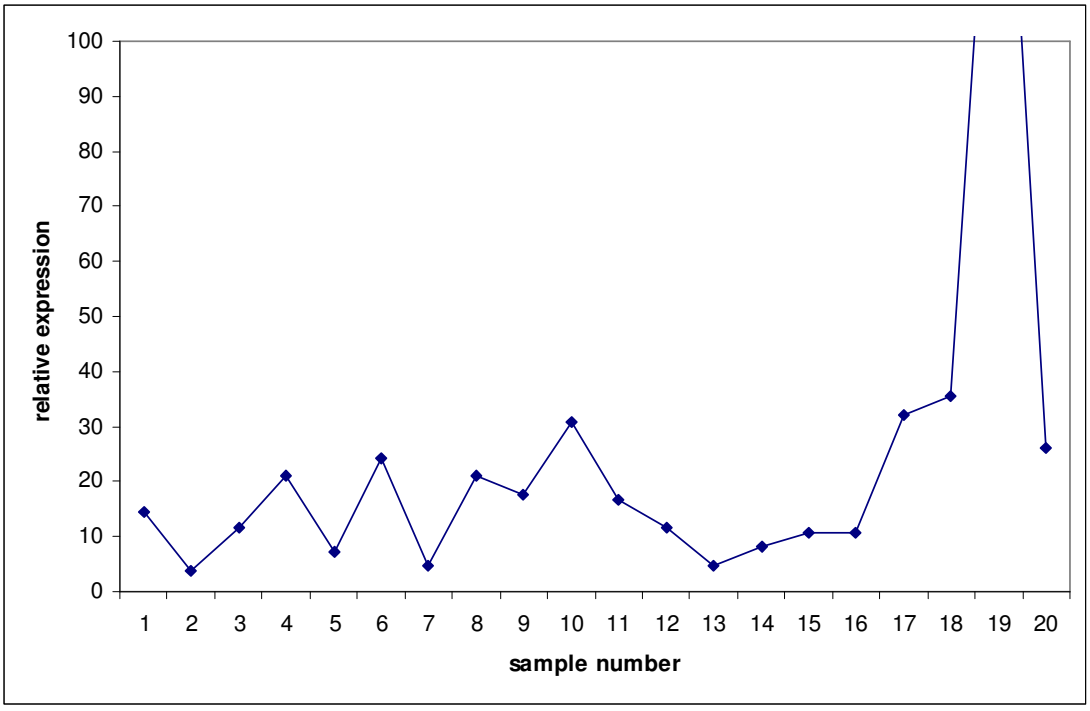


Figure 3.6. Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain, the expression level of *RGT2* decreased right after the pulse injection and showed an oscillatory behavior within the first fifteen minutes. The expression level was about 5 fold high in the 10th hour after the pulse injection. The second steady state expression level was 1.5 fold higher than the value in the first steady state.

After the injection of carbon 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* in *S. cerevisiae* BY4743 (*RIP1Δ/rip1Δ)* strain decreased right after carbon pulse injection, but it increased immediately to a value higher than the initial steady state value. At the end of the first minute the expression level decreased below the steady state value. Another peak was seen 9 hours after pulse injection. The second steady state value was nearly equal to the first steady state value.

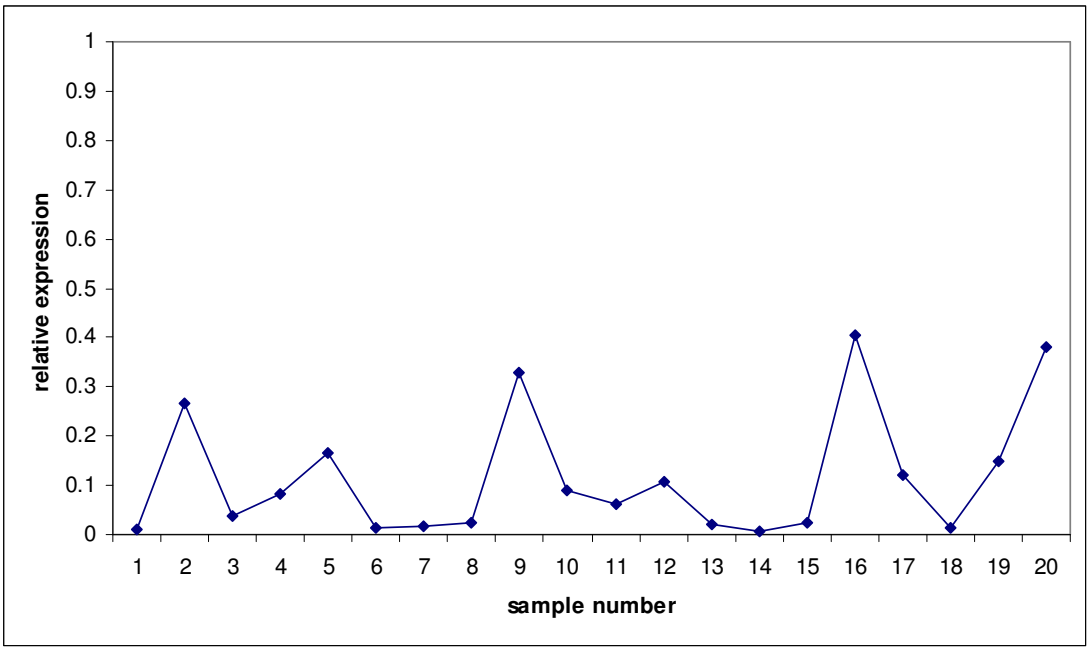


Figure 3.7. Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.2. Expression Profile of *SNF3* as a response to Glucose Pulse Injection

SNF3 is a plasma membrane glucose sensor that has 12 predicted transmembrane segments, low glucose induction of hexose transporter genes HXT2 and HXT4 is implemented by long cytoplasmic C-terminal tail. *SNF3* is also a regulator of glucose transport (Özcan *et al.,* 1996). When glucose levels are low, *SNF3* transcription is at the highest level, and it has been reported that *SNF3* expression is repressed about fivefold by high levels of glucose (Özcan *et al.,* 1999).

Samples were collected as stated previously during cultivation and expression profiles of *SNF3* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in the figures 3.9, 3.10, 3.11 and 3.12.

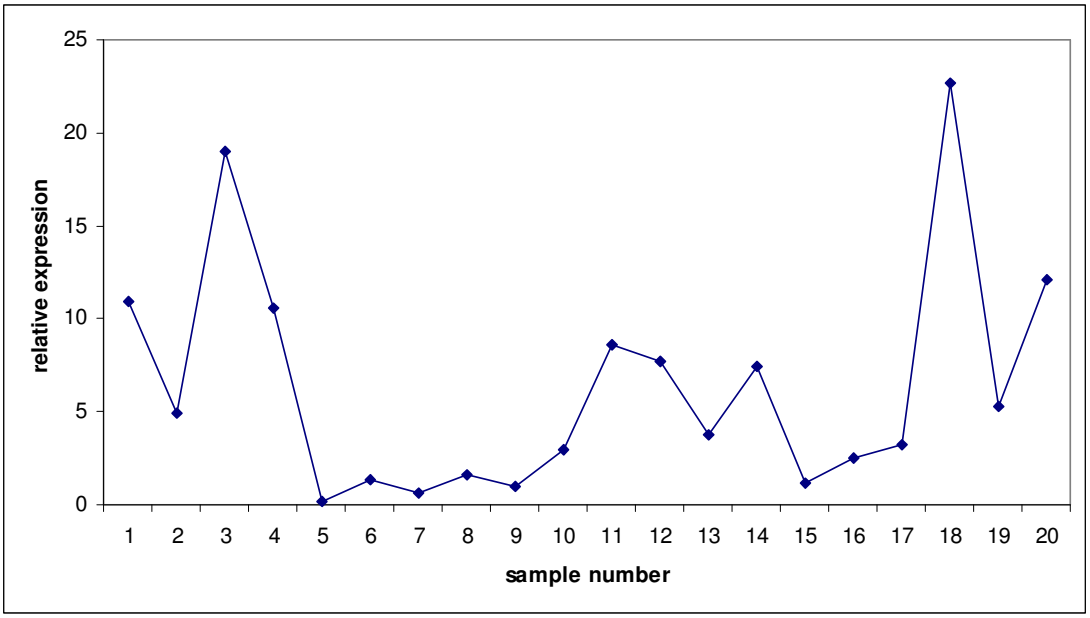


Figure 3.8. Expression profile of *RGT2* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *SNF3* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* decreased right after the pulse injection, and at the 15th minute the expression level showed a sharp increase. An oscillatory behavior was observed until the ninth hour, when the expression level started to increase reaching a two fold higher value than the first steady state

Carbon pulse resulted in an immediate decrease in *SNF3* expression level in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)*. However the third sample in the first minute showed an increase of 4 fold which rebounded immediately with the next sample. The expression level gradually decreased after the first hour. There was a very slight increase in the 10th hour, but the second steady state value was lower than the first steady state value.

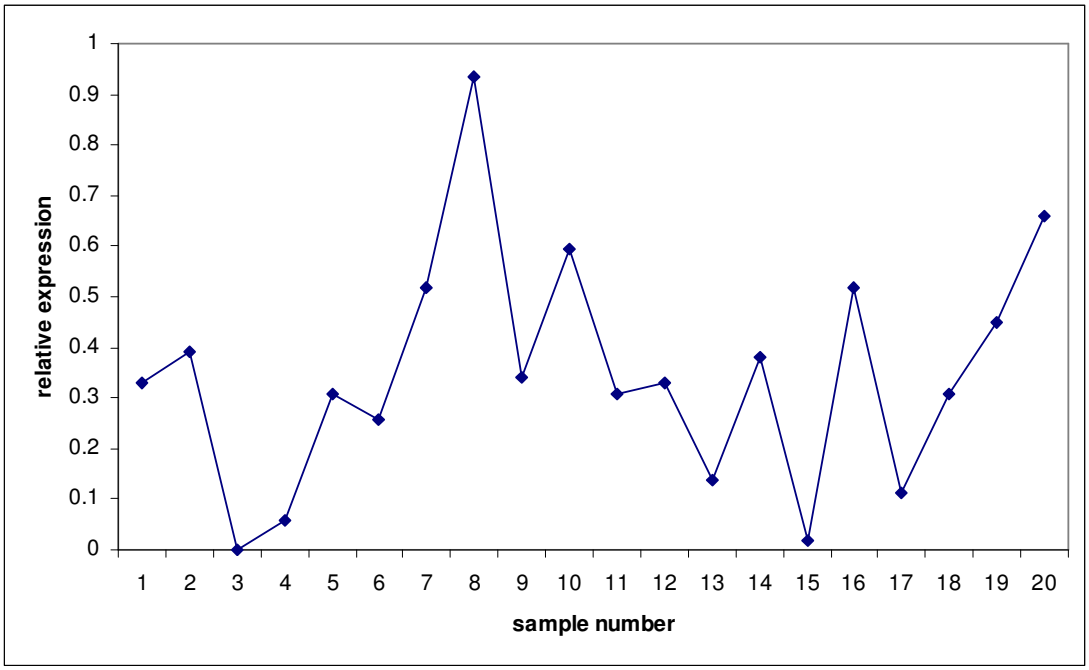


Figure 3.9. Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

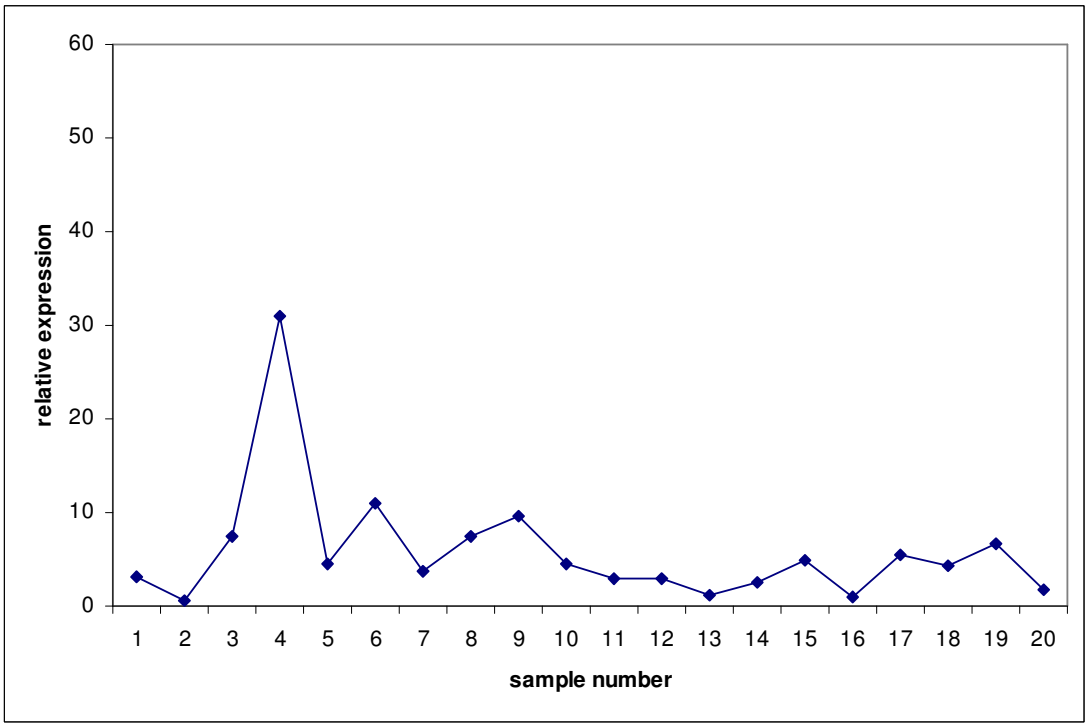


Figure 3.10. Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

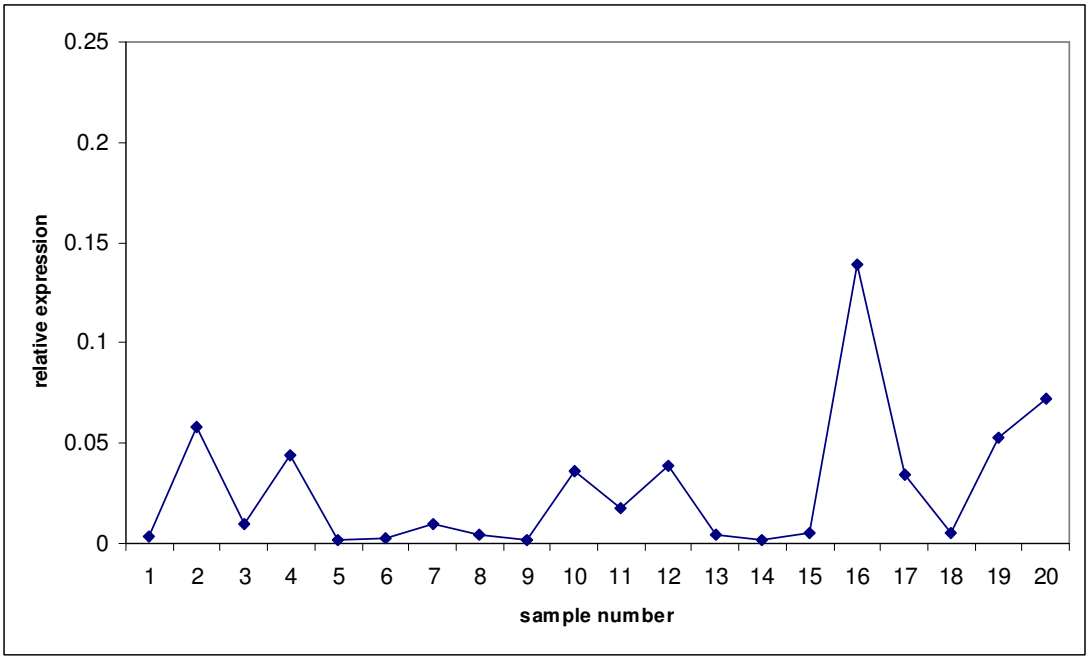


Figure 3.11. Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

Prior to the pulse injection, *SNF3* expression was nearly undetectable in *S. cerevisiae* BY4743 (*rip11Δ/rip1Δ)* strain. The expression increased immediately after the pulse injection, but it dimmed in the next 15 seconds. The level was undetectable between the end of the first minute and 20th minute after the pulse injection. A peak was seen in the 7th hour. The second steady state value was much higher than the first steady state value.

The expression level of *SNF3* was decreased soon after the injection of the carbon pulse in the case of *S. cerevisiae* BY4743 (*RIP1/rip1Δ).* The level increased 30 seconds after the pulse to a level higher than the steady state level. The level decreased once again and was steady until a high peak in the 9th hour. Compared to the first steady state value, the second steady state value was only slightly higher.

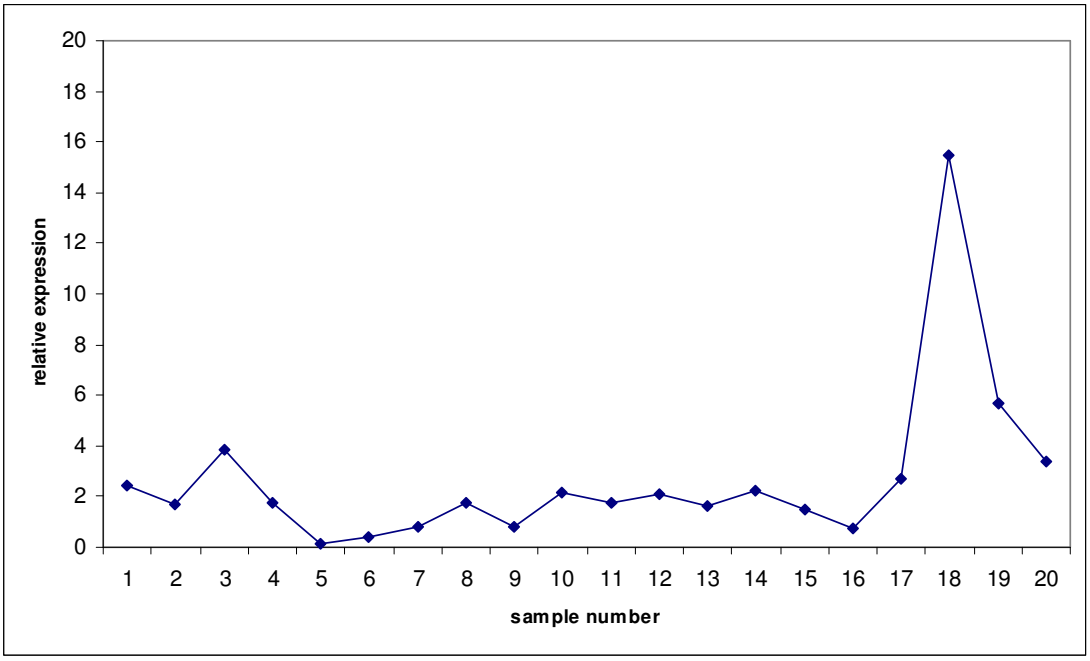


Figure 3.12. Expression profile of *SNF3* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.3. Expression Profile of *MTH1* as a response to Glucose Pulse

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

Samples were collected as stated previously during cultivation and expression profiles of *MTH1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.13-3.16.

The expression level of *MTH1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* slightly decreased after the glucose pulse injection. By the end of the 15th minute, the level reached the initial value, and the changes after that were not very significant. At the 7th hour, the expression level was undetectable, however the initial value was reached during the next hour.

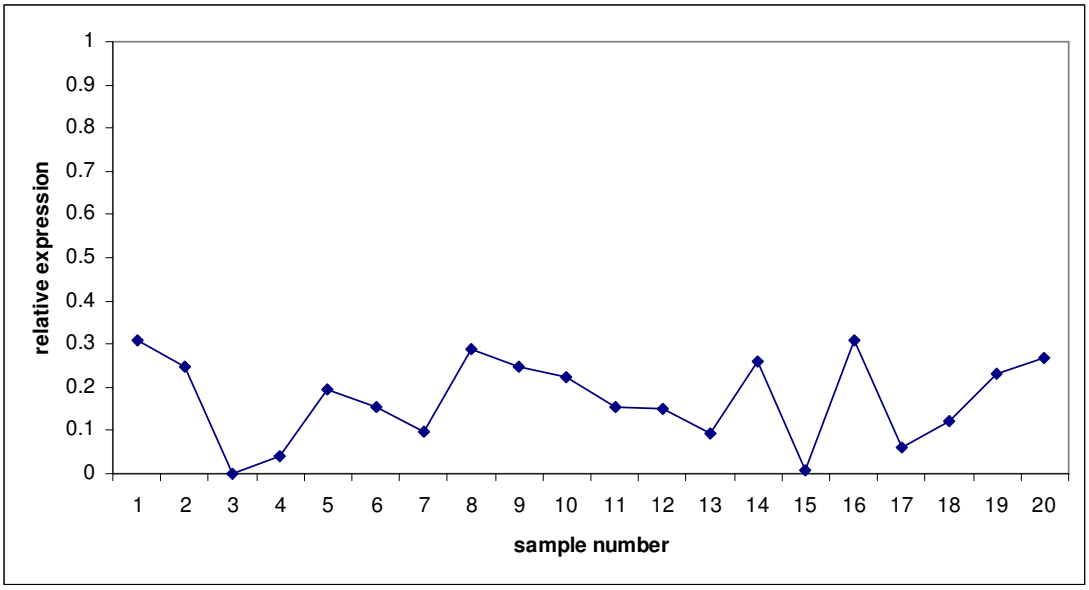


Figure 3.13. Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

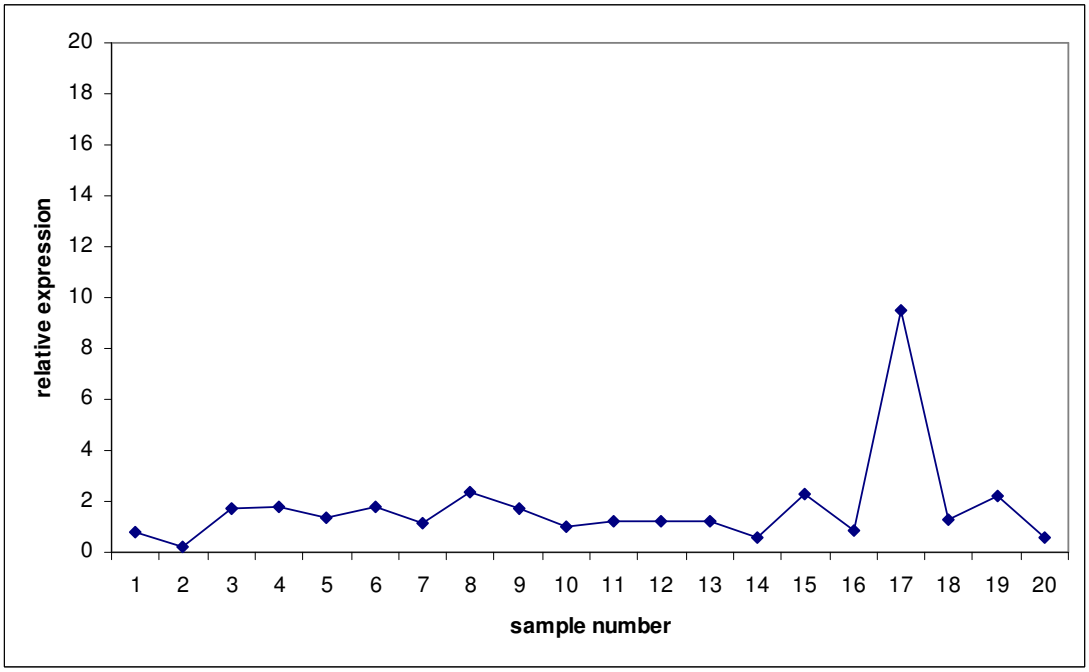


Figure 3.14. Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *MTH1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* decreased right after the pulse injection, as it can be seen from the Figure 3.14, then increased to a

value slightly higher than the first steady state value. The expression level was steady until the end of the first hour. A peak was seen in the 8th hour which rebounded immediately. The second steady state value was about the same as the first steady state value.

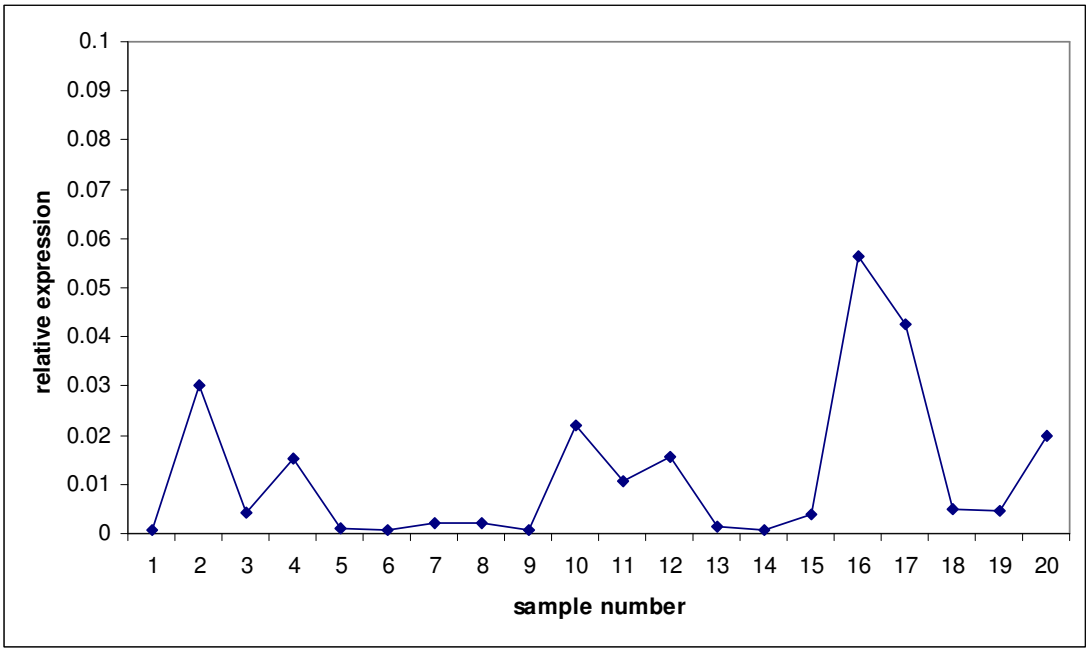


Figure 3.15. Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *MTH1* was initially not detectable in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*) strain under carbon limitation but increased immediately after the carbon pulse injection. The level dropped in the next 15 seconds. Between the end of the first minute and the 20th minute, the expression of *MTH1* was very small. A steep increase was seen 7 hours after the pulse injection, and the level stayed high for the following two hours then decreased.

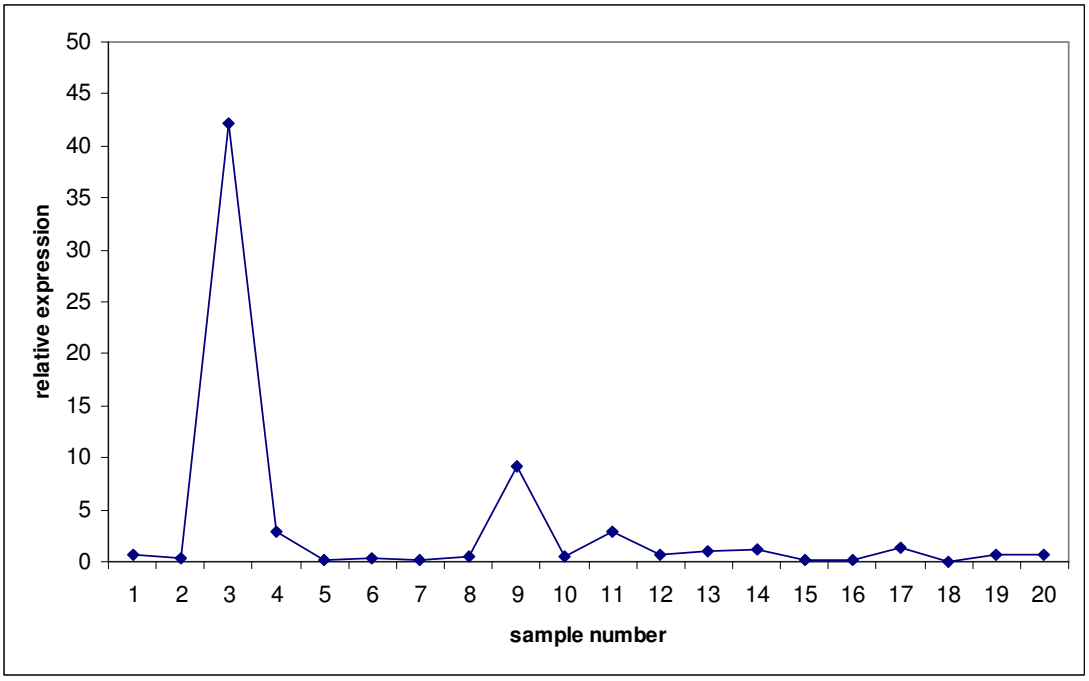


Figure 3.16. Expression profile of *MTH1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

A 2 fold decrease was observed right after pulse injection in the expression level of *MTH1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*. This decrease was followed by a very steep increase in the next 15 seconds, which rebounded immediately. The second steady state expression level was nearly equal to the first steady state value.

3.2.4. Expression Profile of *STD1* as a response to Glucose Pulse

Std1p is a protein involved in the control of glucose-regulated gene expression. It interacts with glucose sensors Snf3p and Rgt2p and with protein kinase Snf1p, and acts as a regulator of Rgt1p transcription factor. In conditions where glucose is lacking Std1p directly interacts with Rgt1. It is required for the repression of HXT gene transcription by Rgt1p (Lakshmanan *et al*., 2003). *STD1* expression is induced by glucose (Moriya and Johnson, 1004), which would be expected to counteract Std1 degradation which 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).

Samples were collected as stated previously during cultivation and expression profiles of *STD1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.17, 3.18, 3.19 and 3.20.

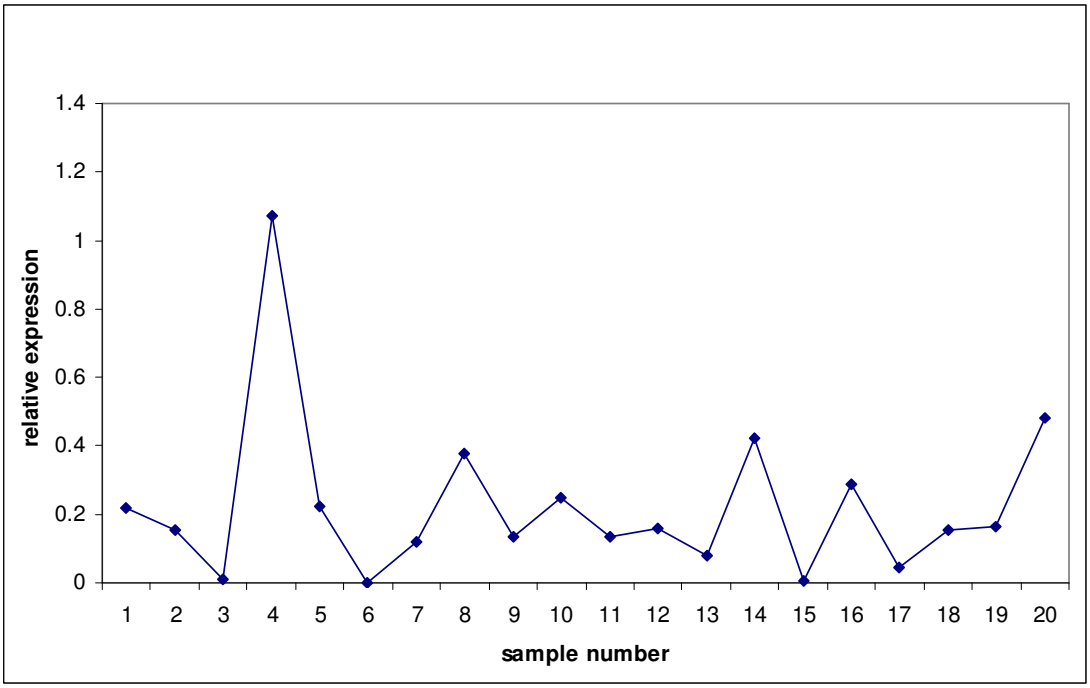


Figure 3.17. Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

After injection of glucose pulse, there was an immediate decrease in the expression level of *STD1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*, followed by a 5 fold increase in the third sample of the first minute. The expression level of this gene at second steady state was approximately the same with first steady state, however, after three residence times, an increased expression level was observed with the last sample.

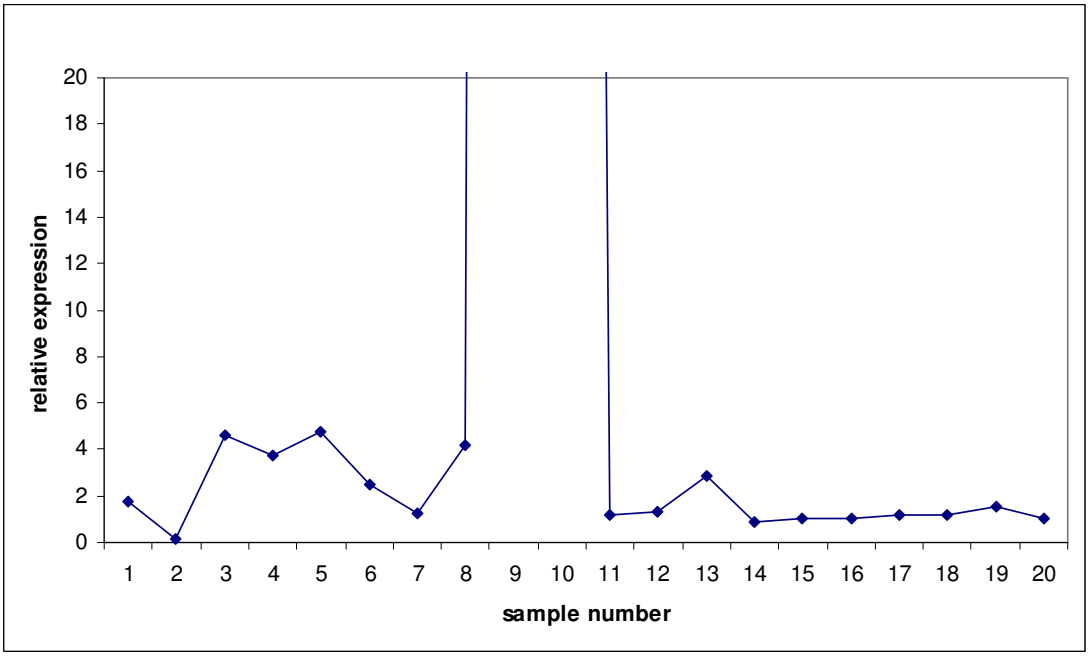


Figure 3.18. Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *STD1* decreased immediately after the injection of carbon pulse in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* and then increased to a value two times higher than the first steady state value. The highest value was observed at the twentieth minute and it did not rebound immediately, the expression level at the end of the first hour was also very high. The value decreased to its initial value in the next hour. The expression level at the second steady state was slightly lower than the first steady state value.

Expression level of *STD1* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* before the injection of carbon pulse. Although the pulse caused an increase in the expression level, it immediately decreased again. 7 hours after pulse injection a peak was seen, which rebounded within the next hour. The second steady state value was higher than the value right after the pulse injection.

After the injection of carbon pulse the expression level of the *STD1* in *RIP1/rip1*Δ mutant decreased and became nearly unnoticeable. Although the value increased slightly in the first minute, the expression level was undetectable until 1 hour after pulse injection. A

very high value was observed in the 7th hour which dimmed immediately. The second steady state value was 2 folds higher than the first steady state value.

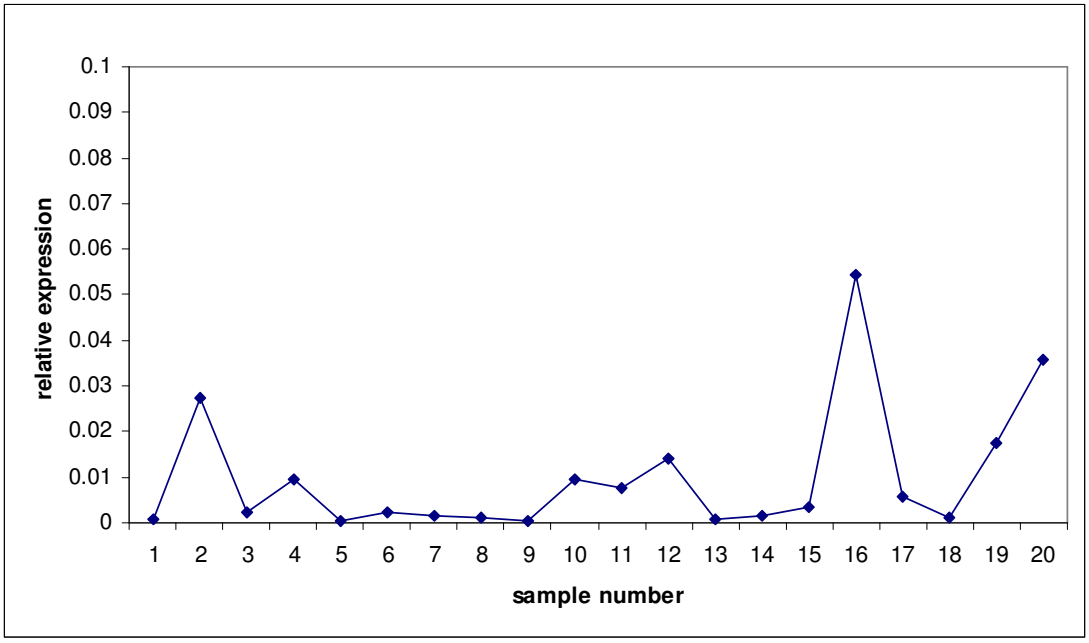


Figure 3.19. Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

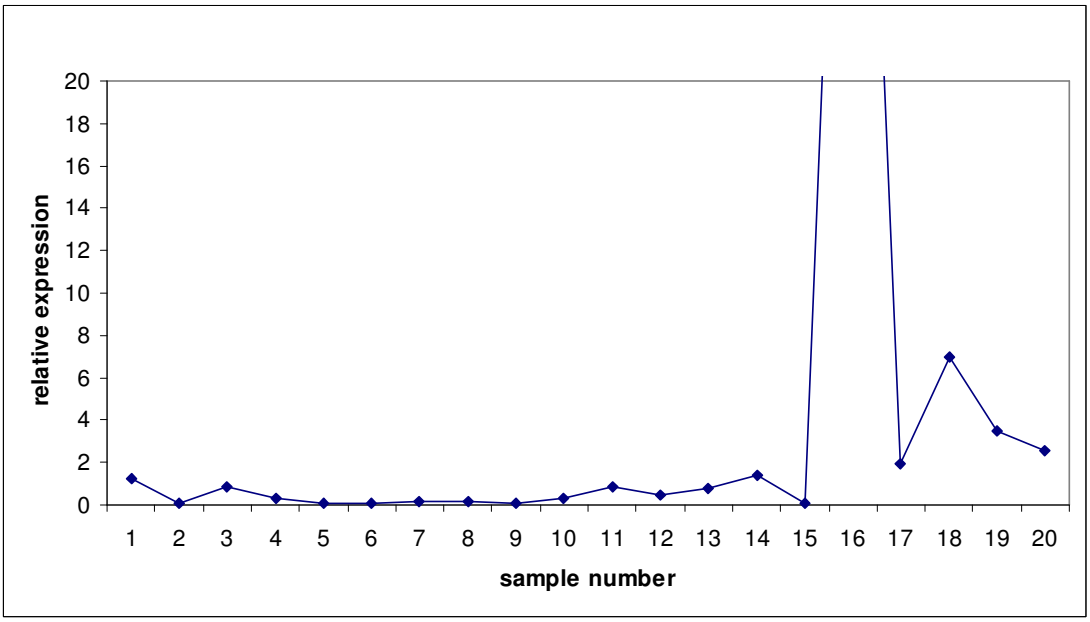


Figure 3.20. Expression profile of *STD1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.5. Expression Profile of *YCK1* as a response to Glucose Pulse

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). Plasma membrane-bound casein kinase I isoform shares redundant functions with Yck2p in morphogenesis (Flick *et. al*., 2003; Moriya and Johnson, 2004; Spielewoy *et al*,. 2004).

Samples were collected as stated previously during cultivation and expression profiles of *YCK1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.21-3.24.

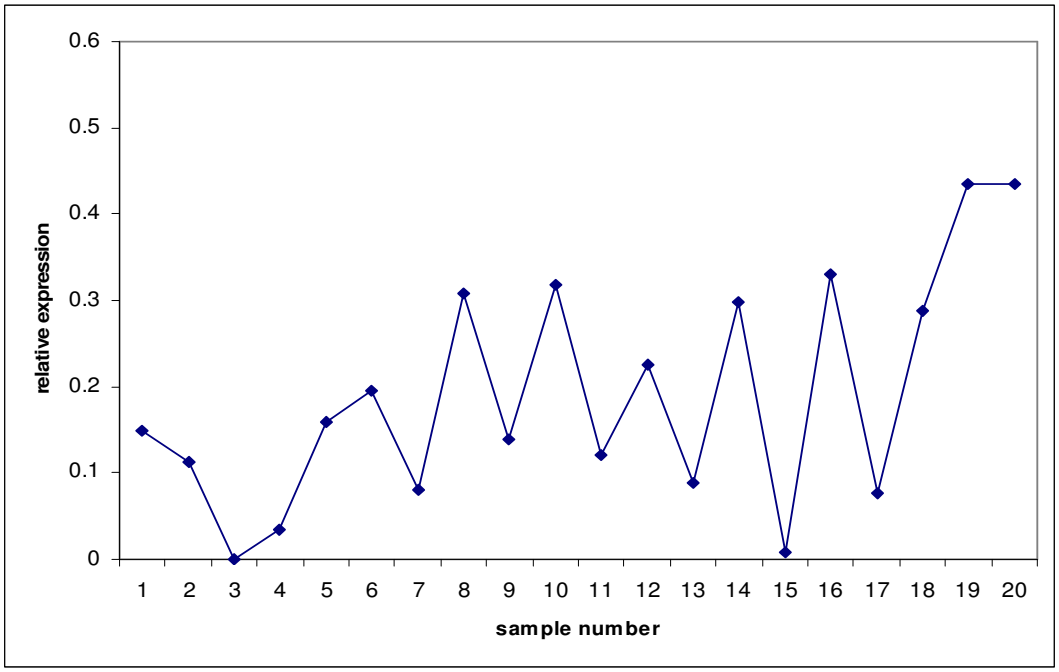


Figure 3.21. Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

Examination of the relative expression values of *YCK1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*, revealed a decrease right after injection of glucose pulse. Then the level increased to the initial value. An oscillatory behavior was seen until the second steady state

was reached. The second steady state value was approximately 4 fold higher than the first one.

The expression level of *YCK1* in *hap4Δ/hap4Δ* strain of *S. cerevisiae* decreased right after the pulse injection but reached its initial value immediately with the second sample. Until the end of the second hour, the expression level showed an oscillatory behaviour. Between the second hour and the 8th hour the behaviour was steady. The level in the ninth hour was 2 times higher than the initial value, but the second steady state value was the same with the first value.

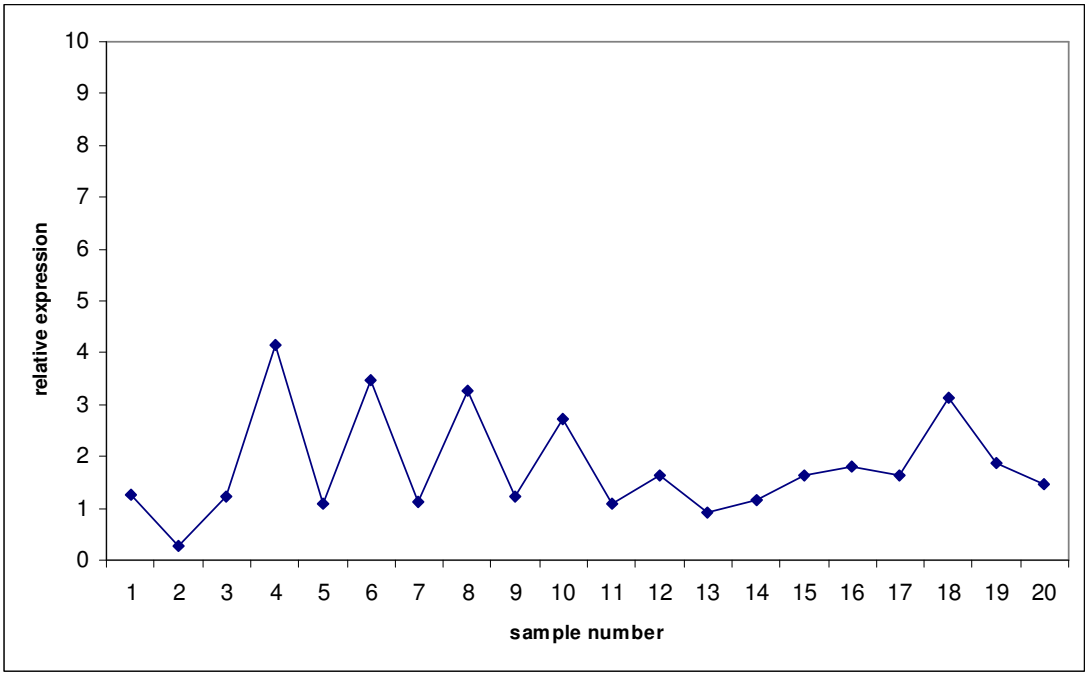


Figure 3.22. Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

As it can be seen from the figure, expression level of *YCK1* was almost zero in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* strain prior to carbon pulse injection. Fluctuation was observed in the first minute, then the expression level dropped to nearly zero again. The highest value was seen in the second steady state expression level.

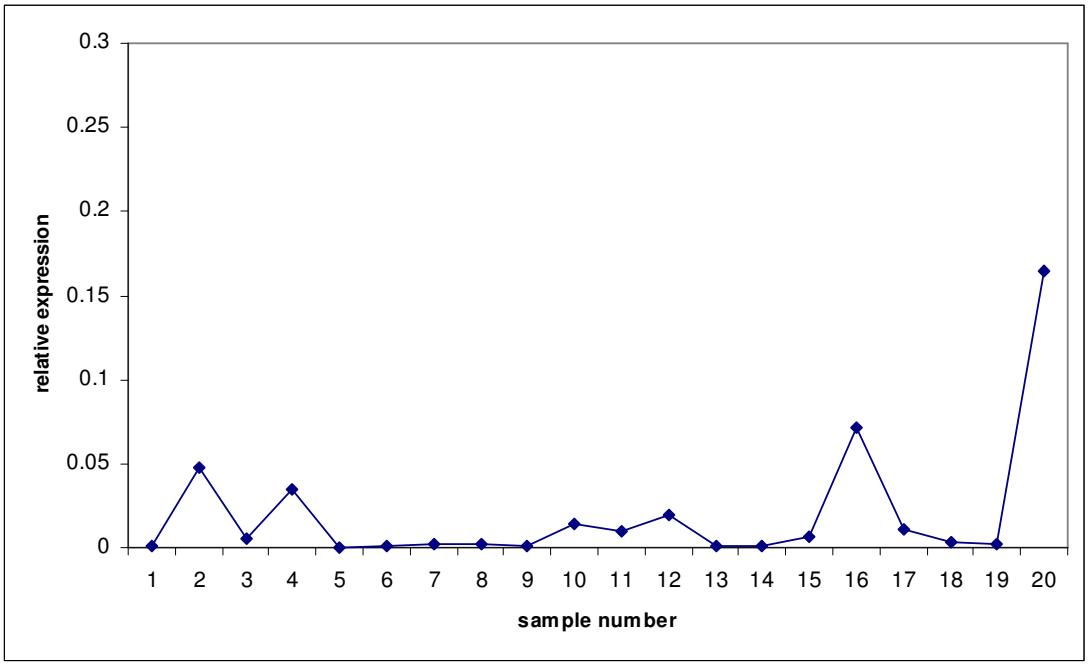


Figure 3.23. Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

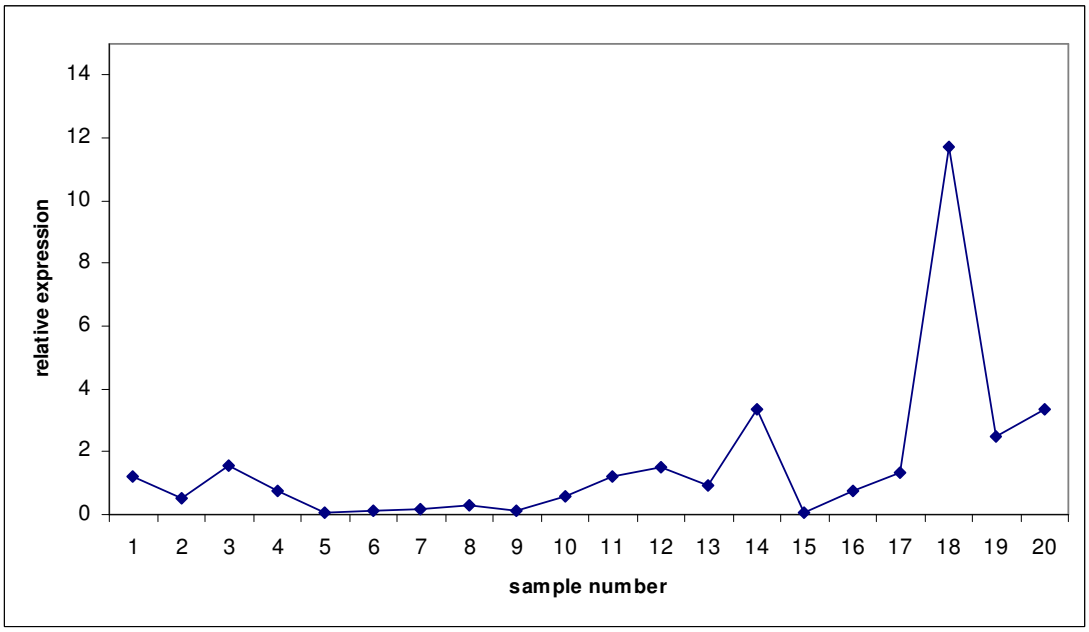


Figure 3.24. Expression profile of *YCK1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

With the pulse injection, expression level of *YCK1* in *RIP1/rip1Δ* mutant decreased 2 fold. The level increased 15 seconds later, however at the end of the first minute, the expression was unnoticeable. A high peak was seen 9 hours after pulse injection which rebounded. The second steady state value was 3 times high when compared to the first steady state value.

3.2.6. Expression Profile of *YCK2* as a response to Glucose Pulse

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

Samples were collected as stated previously during cultivation and expression profiles of *YCK2* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in the following figures (figures 3.25, 3.26, 3.27 and 3.28).

After the injection of carbon pulse, expression level of *YCK2* decreased slightly in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)*, then it increased to its initial value. At the 15th minute there was a sharp increase of 20 fold, then it rebounded. Expression level at the second steady state was approximately 2 fold higher than the first steady state value.

YCK2 expression in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain decreased 5 fold right after the pulse injection, and was nearly undetectable. However, the value increased higher than the initial value within the first minute. The highest value was seen in the 20th minute, which decreased and remained steady throughout the cultivation. The second steady state value was slightly higher than the first steady state value.

Expression level of *YCK2* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* was undetectable prior to carbon pulse injection. It increased right after the injection but dimmed immediately. The expression level was very low through most of the sampling period. A peak was seen in the 7th hour which rebounded immediately. The second steady state value was the same with the value right after pulse injection.

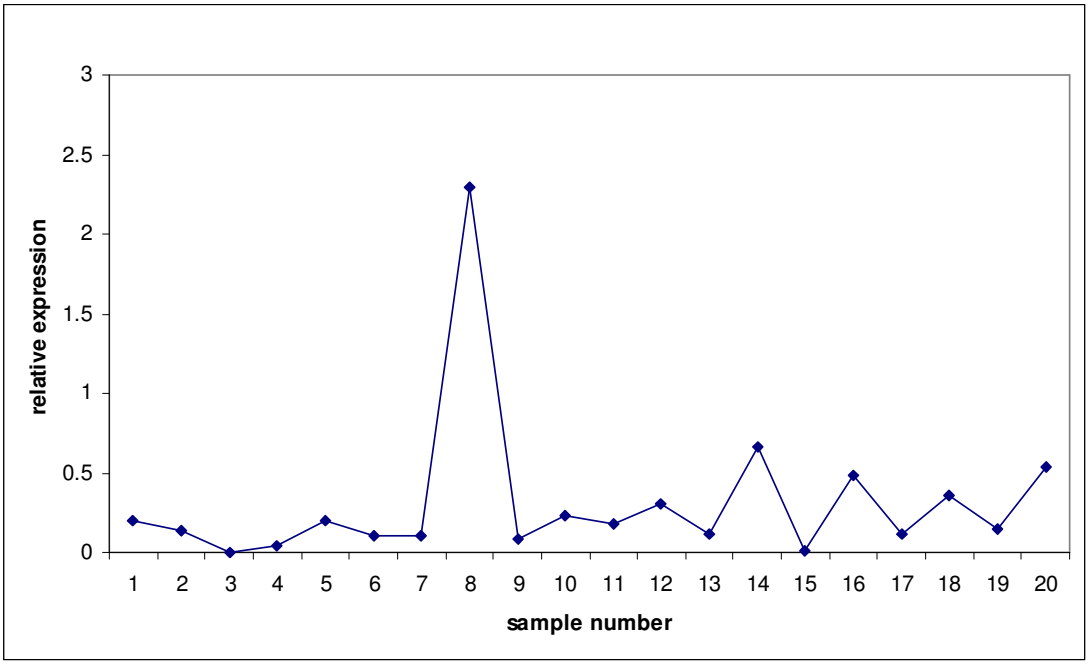


Figure 3.25. Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

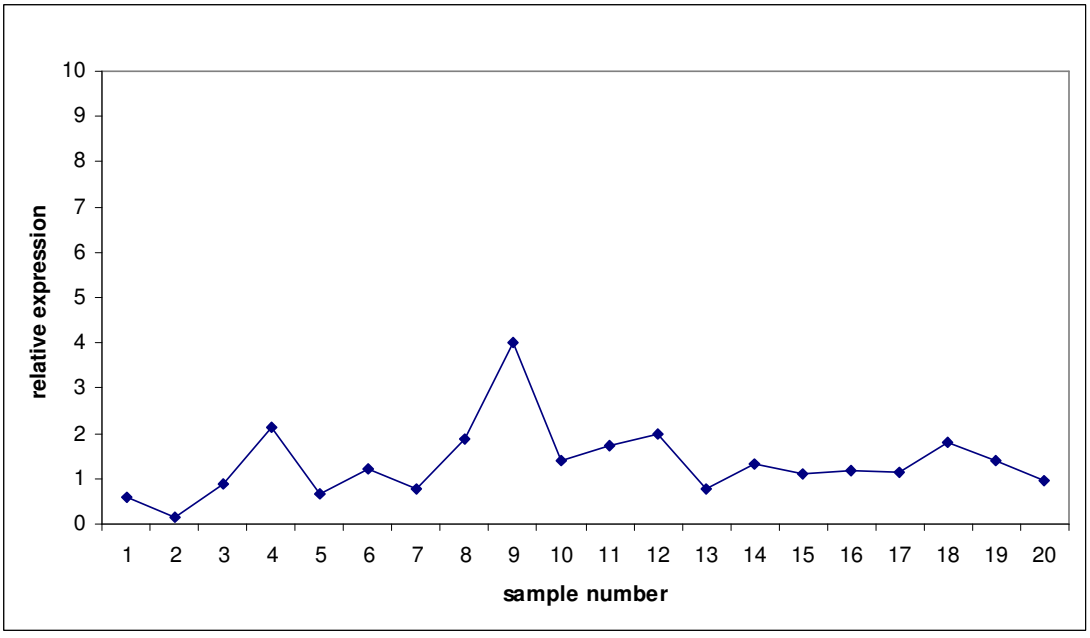


Figure 3.26. Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

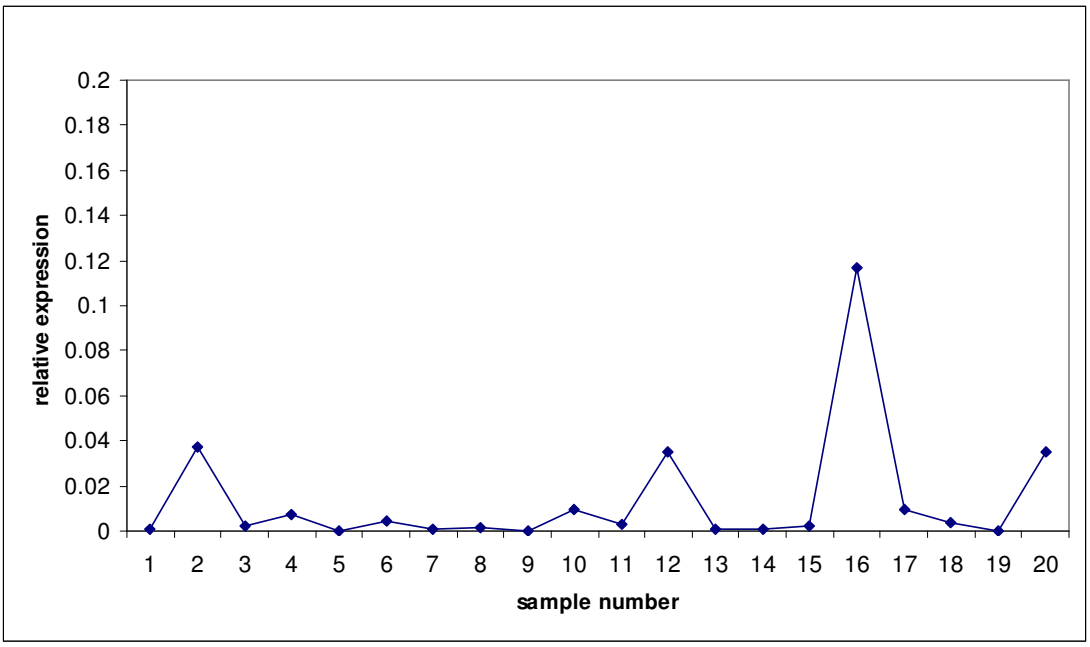


Figure 3.27. Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

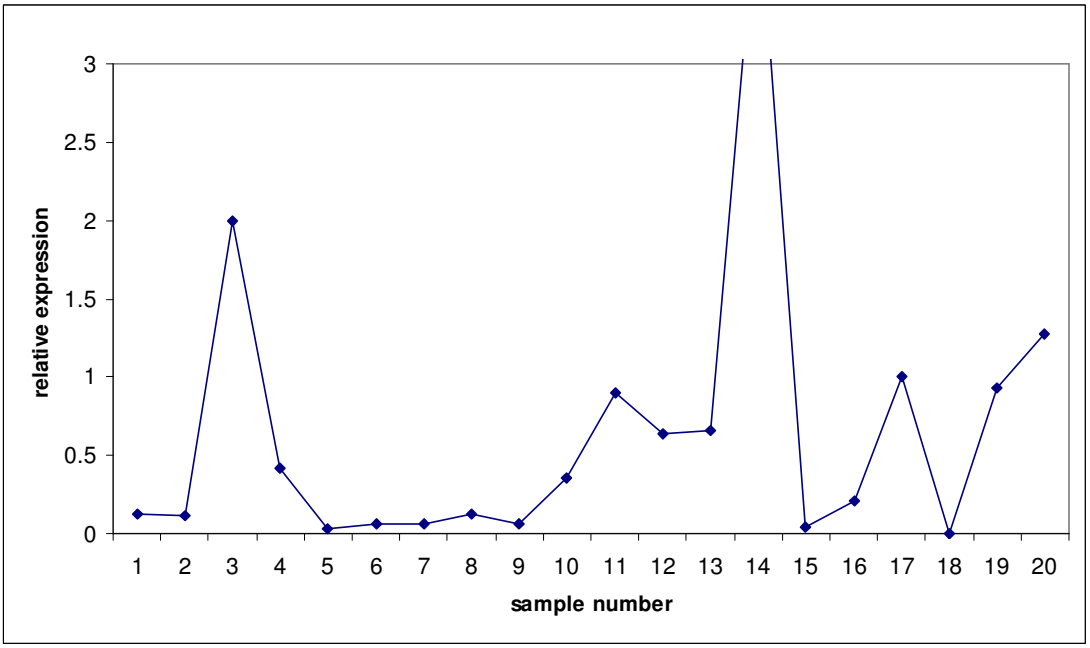


Figure 3.28. Expression profile of *YCK2* gene in *S. cerevisiae* BY4743 (*RIP1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

After the pulse injection, the expression level of the *YCK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* showed a very slight decrease, which was followed by a 15 fold increase in the next 15 seconds. This increase dimmed immediately. 5 hours after pulse injection the highest peak was seen that also rebounded immediately. The second steady state expression level was 10 times higher than the first steady state level.

3.2.7. Expression Profile of *GRR1* as a response to Glucose Pulse

Grr1p is a protein involved in carbon catabolite repression, glucose-dependent divalent cation transport, high-affinity glucose transport, and morphogenesis. It is an F-box protein component of SCF ubiquitin-ligase complex. Grr1 is required for inhibition of Rgt1 function (Ning and Johnston, 1999). Grr1 interacts with Skp1. In response to glucose, Grr1 is required to inactivate Mth1 and Std1. Glucose acts via Grr1 to regulate the abundance of Mth1 (Flick *et al*., 2003).

Samples were collected as stated previously during cultivation and expression profiles of *GRR1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.29, 3.30, 3.31 and 3.32.

A 1.1 fold decrease in the expression level of *GRR1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* was observed right after the glucose pulse injection. The expression level increased within the first minute, however the initial value was not reached. The expression level fluctuated until the second steady state was reached, and this level was 2 fold higher than the first steady state level.

Expression profile of *GRR1* gene slightly decreased immediately after the pulse injection in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain. However the value increased 7 fold in the 15^{th} second and remained higher than the initial value until the fourth hour. Another increase was seen in the 10^{th} hour, and the second steady state value was slightly higher than the first steady state value.

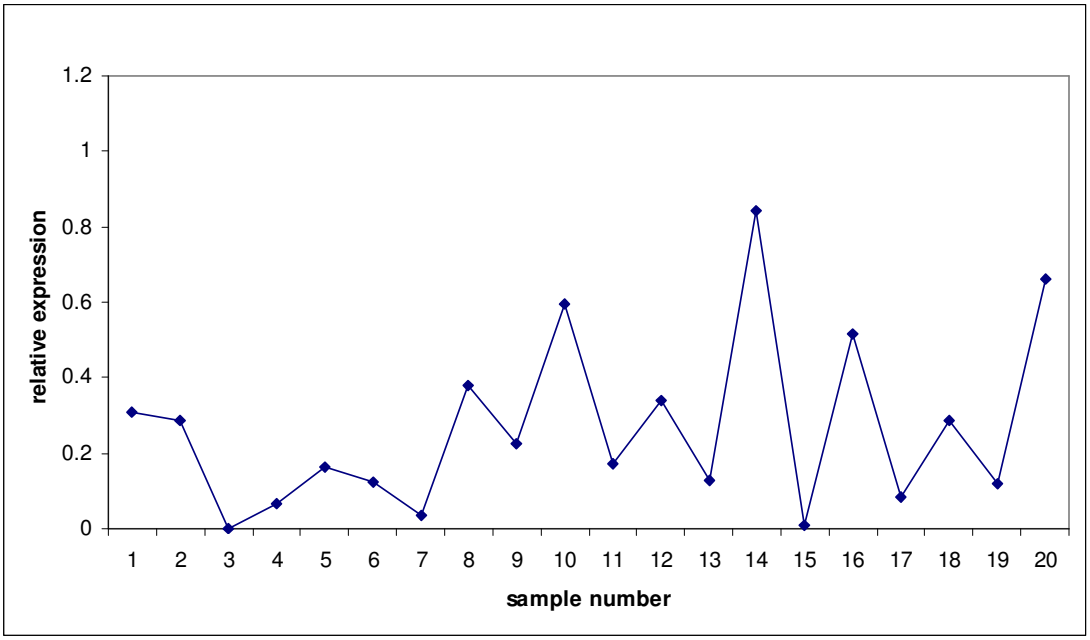


Figure 3.29. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

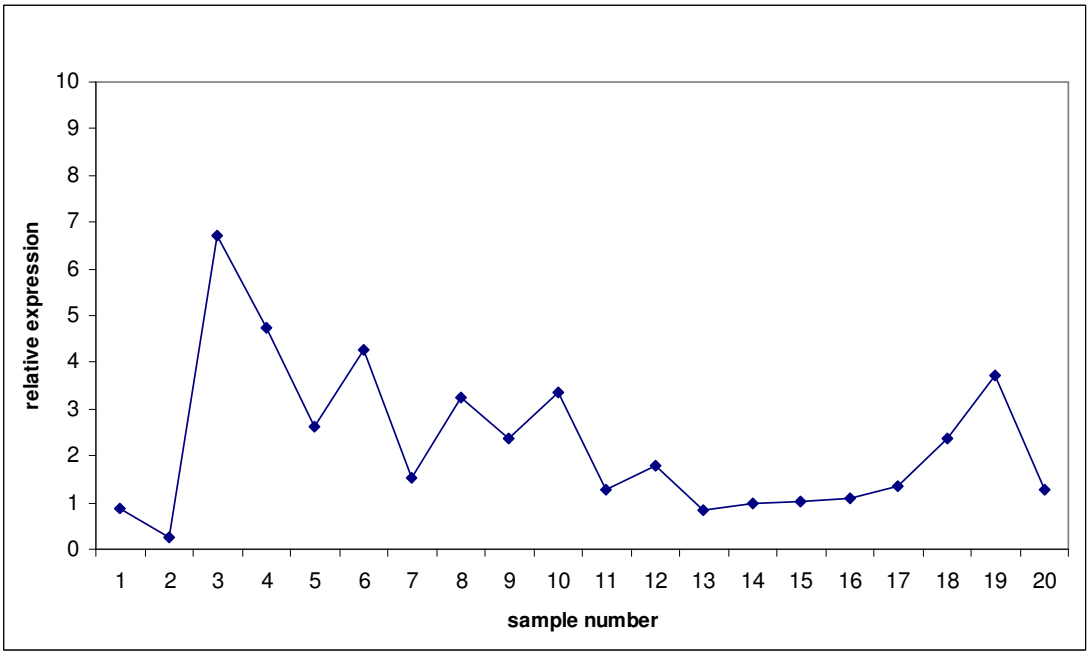


Figure 3.30. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

An increase in the expression level of *GRR1* was observed immediately after the pulse injection in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)*. During the time between the first minute and the 20th minute the expression level was very low. A peak was seen 7 hours later than the injection At the second steady state, the expression level of *GRR1* was higher than the value 5 seconds after the pulse injection.

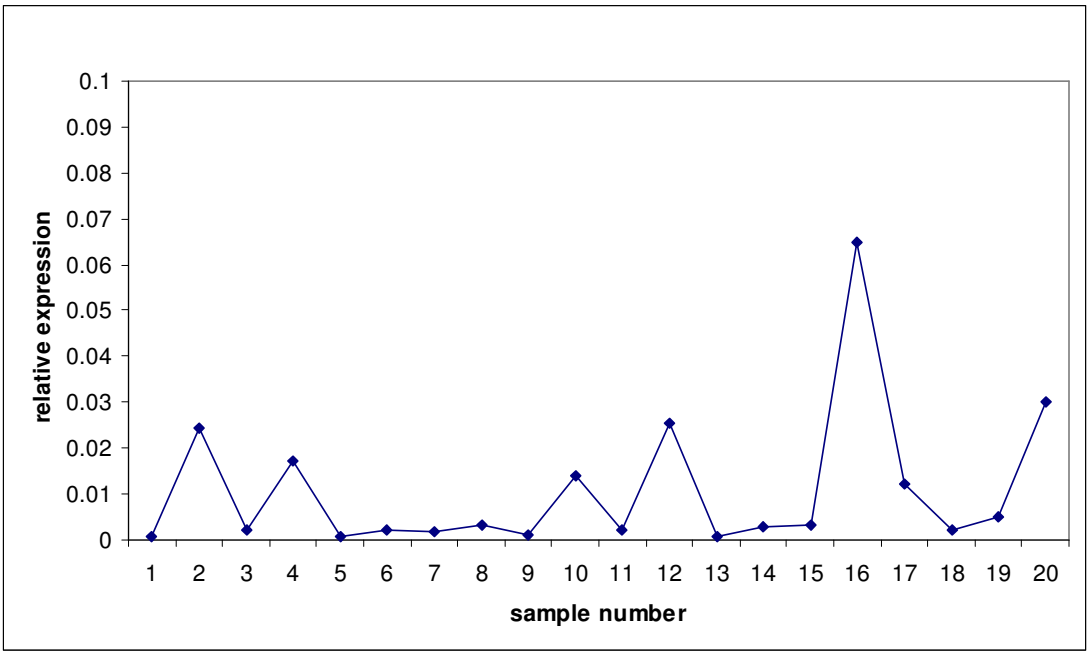


Figure 3.31. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *GRR1* did not show a significant change with the injection of carbon pulse in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)*. The value however dropped to unnoticeable levels at the end of the first minute. A peak was seen in the 9th hour which rebounded immediately. 2 fold higher expression level was obtained at the second steady state, in comparison to the first steady state.

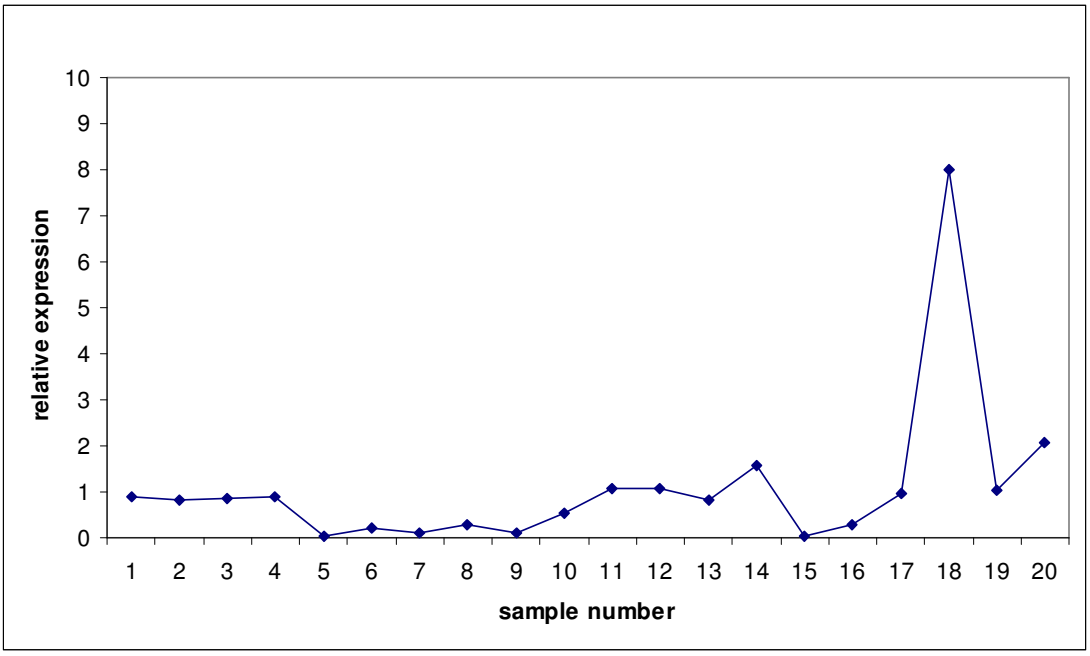


Figure 3.32. Expression profile of *GRR1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.8. Expression Profile of *SKP1* as a response to Glucose Pulse

Ubiquitin ligase complexes with distinct structures and related functions that target a wide variety of regulators to the 26S proteasome for degradation. These complexes which are named SCF; have two common components, Skp1 and the cullin *C*dc53, and a third component which is an exchangeable *F*-box protein. Grr1 is required for inhibition of Rgt1 function and interacts with Skp1. It has been reported that the Grr1-Skp1 interaction is significantly enhanced by high levels of glucose (Li and Johnson, 1997; Santangelo, 2006).

Samples were collected as stated previously during cultivation and expression profiles of *SKP1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.33, 3.34, 3.35 and 3.36.

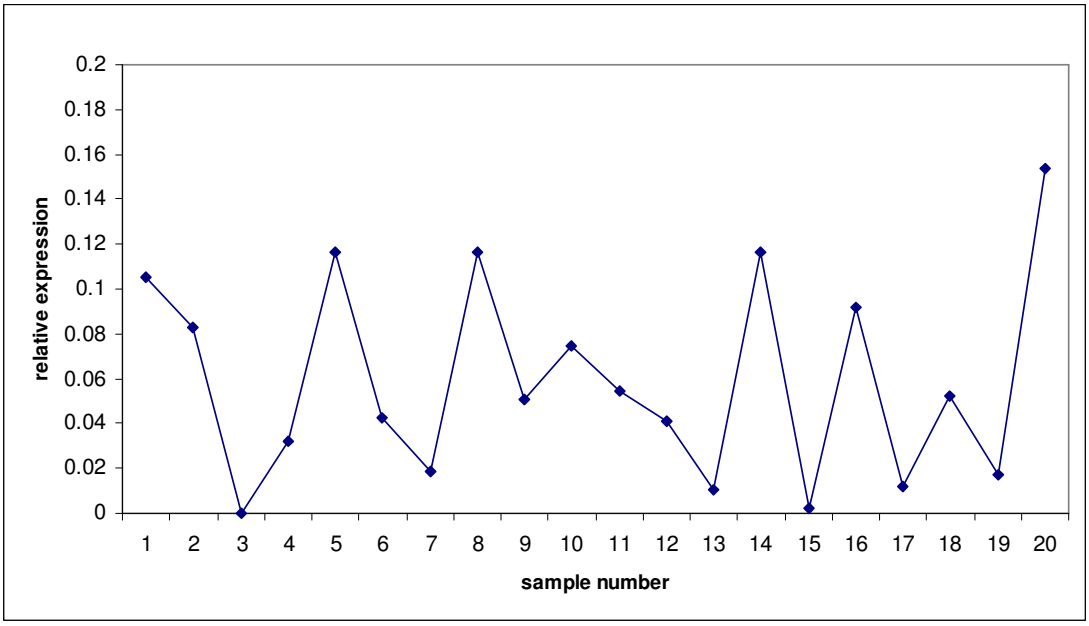


Figure 3.33. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

Glucose pulse resulted in a decrease in the expression level of *SKP1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ).* Then it increased to approximately its initial value. Until the second steady state was reached, the expression level fluctuated, without showing sharp changes. The second steady state value was slightly higher than the initial value.

The application of carbon pulse resulted in a decrease in the expression level of *SKP1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* strain and its expression level fluctuated with values higher than the initial value until after the 9th hour when the expression level decreased. The value decreased more as the second steady state was reached, the steady state values were very close.

Expression level of *SKP1* was undetectable before the application of the pulse in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ).* It increased and decreased again within the first minute after the pulse. A peak was observed 15 minutes after the injection which rebounded immediately.

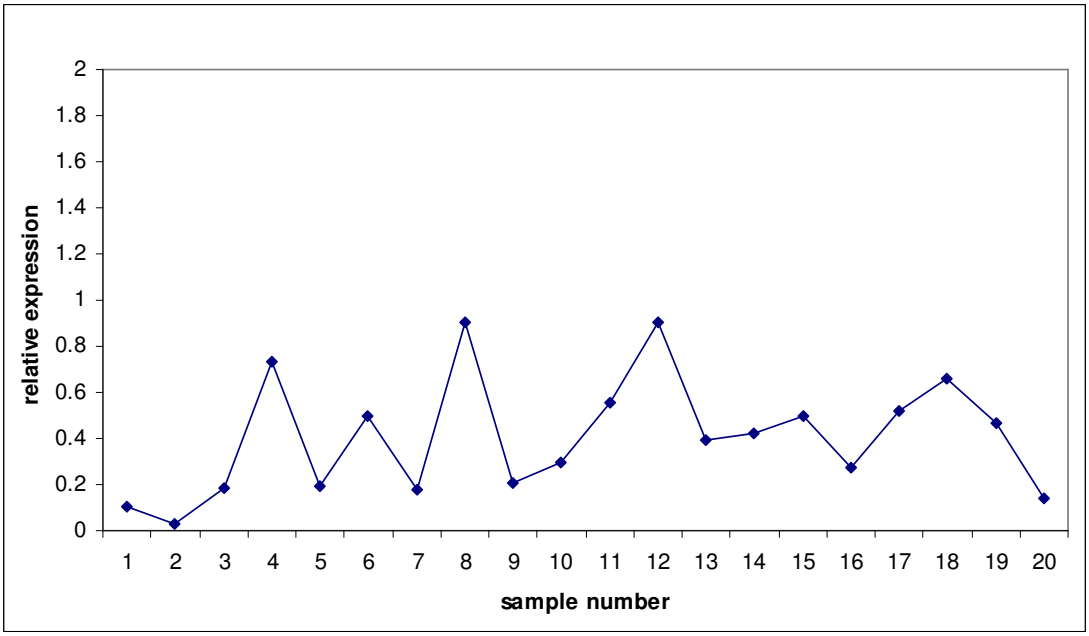


Figure 3.34..Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

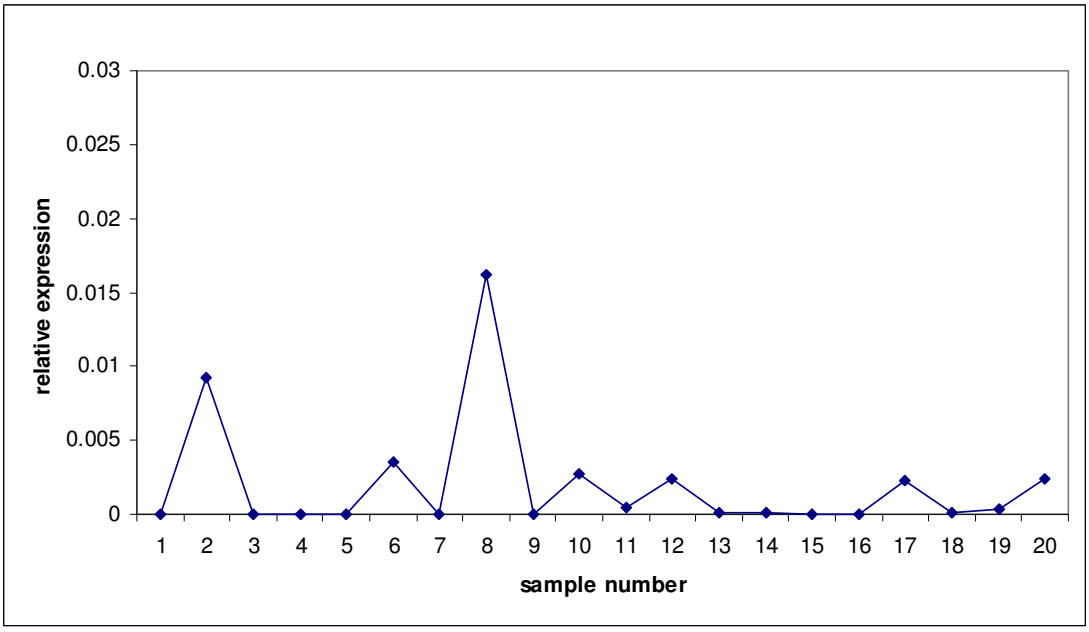


Figure 3.35. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *SKP1* did not show a significant change immediately after the carbon pulse was given in the heterozygous mutant of Rip1. a 10 fold increase was observed 30 seconds after the pulse injection. During the time between 4 hours and 10 hours after pulse injection, the expression was undetectable. A sharp increase was seen as the second steady state was reached, which was when this gene was expressed highest.

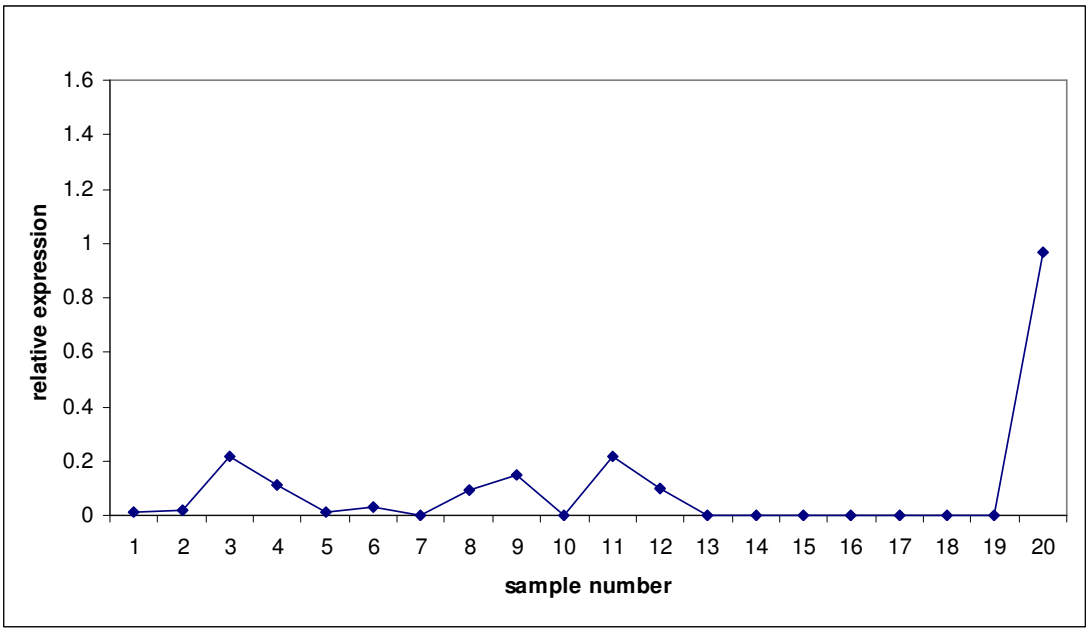


Figure 3.36. Expression profile of *SKP1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.9. Expression Profile of *RGT1* as a response to Glucose Pulse

Rgt1 is a transcription factor. It has two effects on the transcription of *HXT* genes in *S. cerevisiae* and it regulates the expression of these genes. When glucose levels are high (i.e. >2per cent), Rgt1p activates transcription of *HXT1*, whereas in the absence of glucose it represses transcription (Ozcan *et. al*., 1996; Polish *et al*., 2004). In low levels of glucose Rgt1p has no effect on transcription. 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 activation by high levels of glucose, Grr1p is necessary. As previously stated, the activity of Rgt1p is likely to be regulated post-transcriptionally since transcription of *RGT1* is not altered in response to glucose (Ozcan *et al*., 1996).

Samples were collected as stated previously during cultivation and expression profiles of *RGT1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.37, 3.38, 3.39 and 3.40.

With glucose injection pulse, the expression level of *RGT1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* immediately decreased, than a gradual increased was observed within after first minute. The level did not reach the initial expression level until the end of the first hour. Fluctuating behavior resided during sampling period. The expression level at the second steady state value was approximately same with the first one.

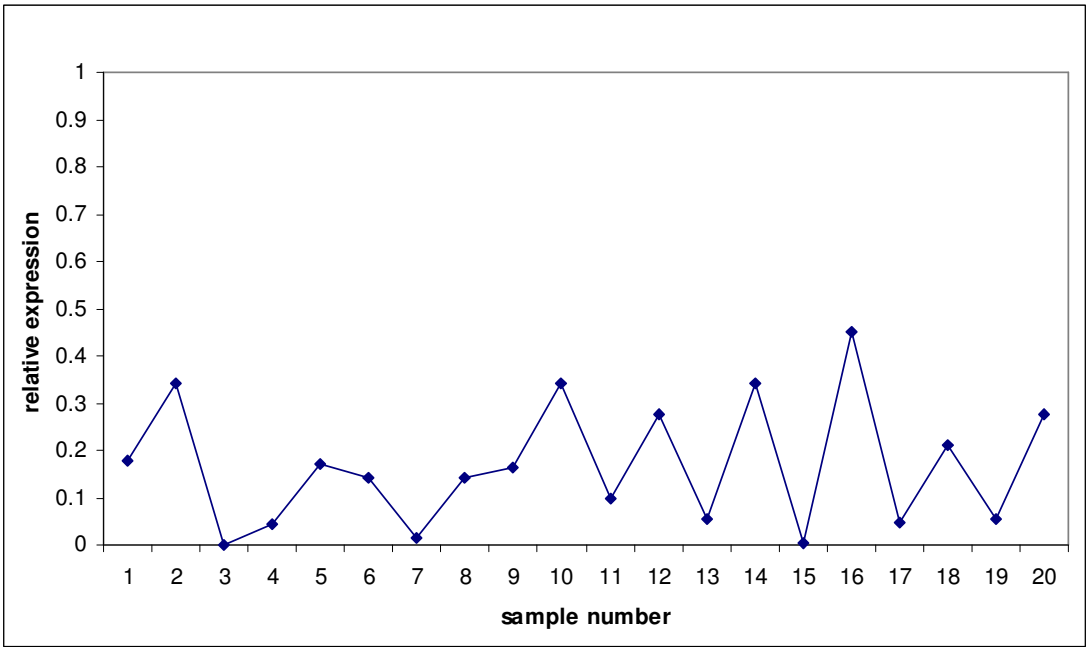


Figure 3.37. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures with pulse injection

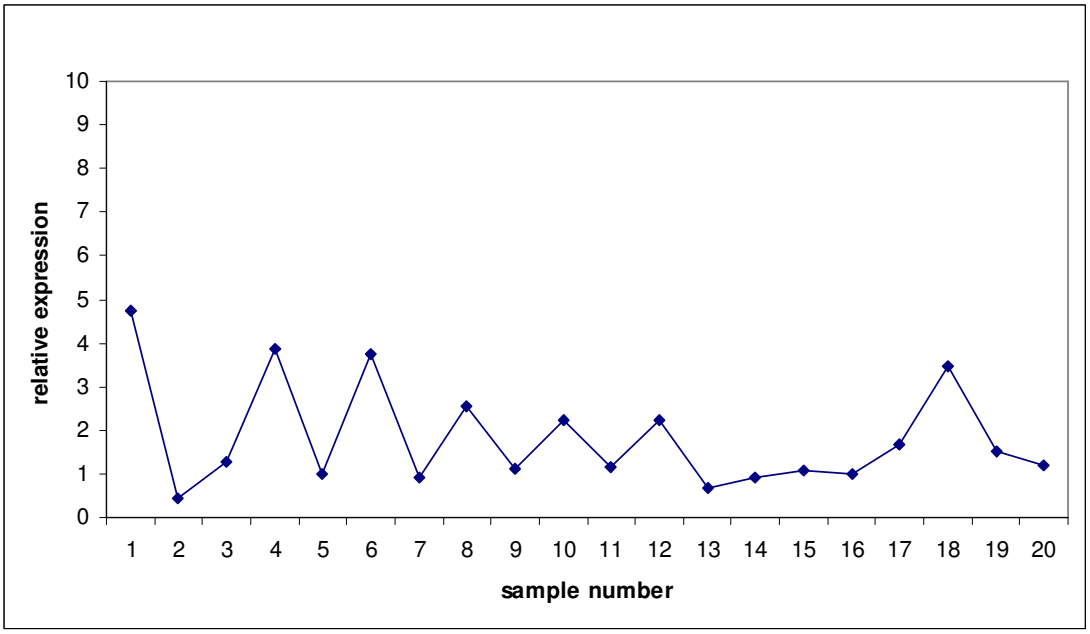


Figure 3.38. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

The first steady state expression level of *RGT1* gene in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ)* under carbon limitation in chemostat cultures was high and this value was not reached again throughout the cultivation. The expression level fluctuated until the fourth hour, and was steadier later with a peak in the 9th hour. The second steady state value was 4 fold lower than the first steady state value.

The expression level of this gene was not detectable in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures before the pulse injection but increased as soon as the carbon pulse was injected. After the 20th minute, where the level was undetectable once again, an oscillatory behavior was seen. The highest value was initially reached 7 hours after the pulse injection, which dimmed immediately, and the second steady state value was as high as this expression level.

In *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain, the relative expression of *RGT1* showed an increase of 2 fold with the application of carbon pulse. Its expression level remained high until the end of the first minute. The value was very small until 1 hour later.

A high peak was seen in the 9th hour which rebounded immediately. The second steady state value was 2 times higher than the first steady state value.

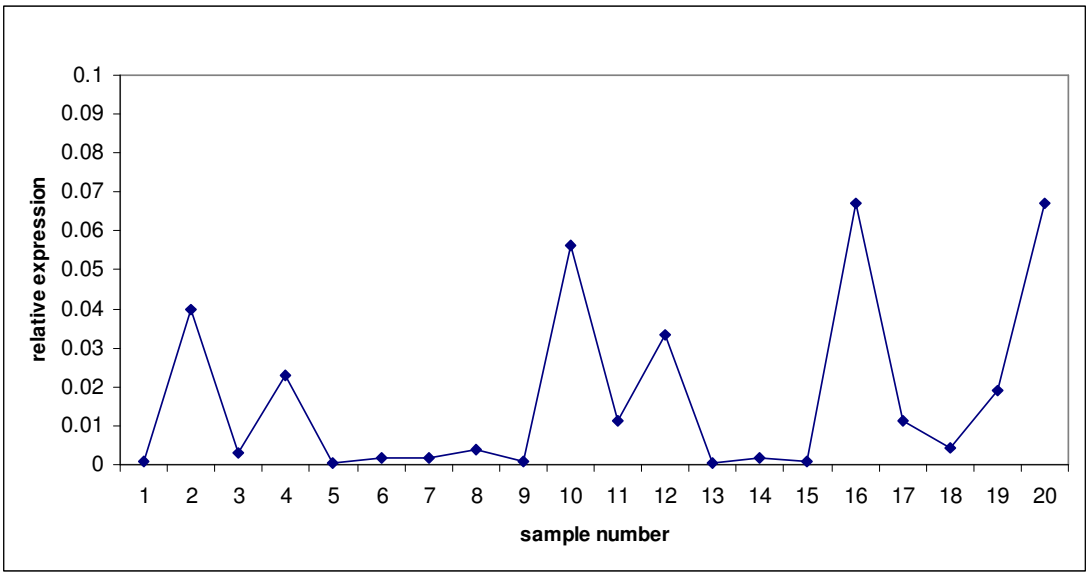


Figure 3.39. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

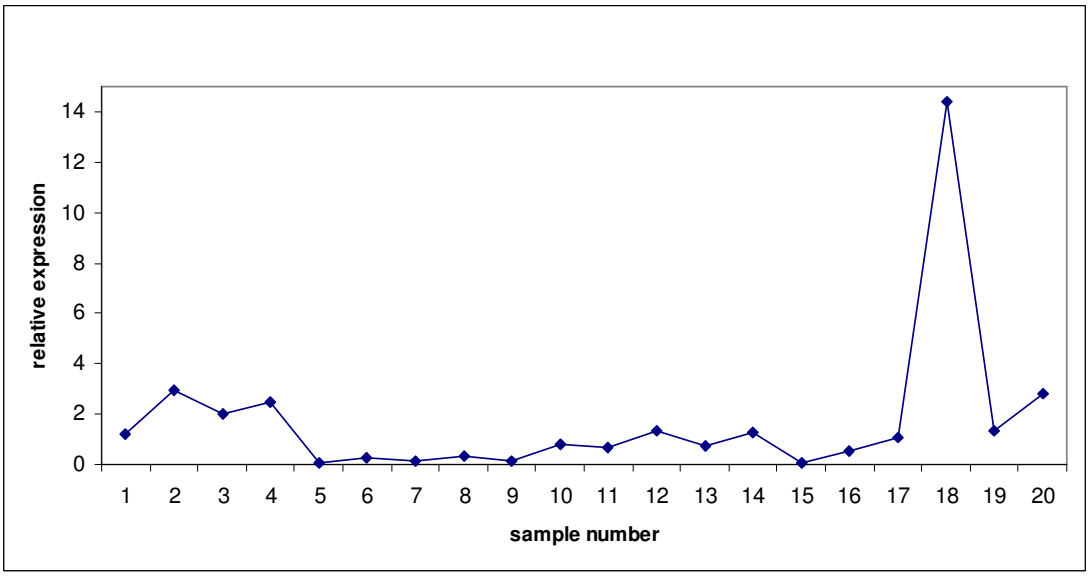


Figure 3.40. Expression profile of *RGT1* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.10. Expression Profile of *GLC7* as a response to Glucose Pulse

In high glucose conditions, Mig1 is de-phosphorylated by the Glc7-Reg1 protein phosphatase complex (Alms *et al*., 1999). By promoting the dephosphorylation of Snf1 threonine 210, the Glc7- Reg1 complex acts in opposition to the Snf1 signaling pathway (McCartney and Schmidt, 2001).

Samples were collected as stated previously during cultivation and expression profiles of *GLC7* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in the following figures; 3.41, 3.42, 3.43 and 3.44.

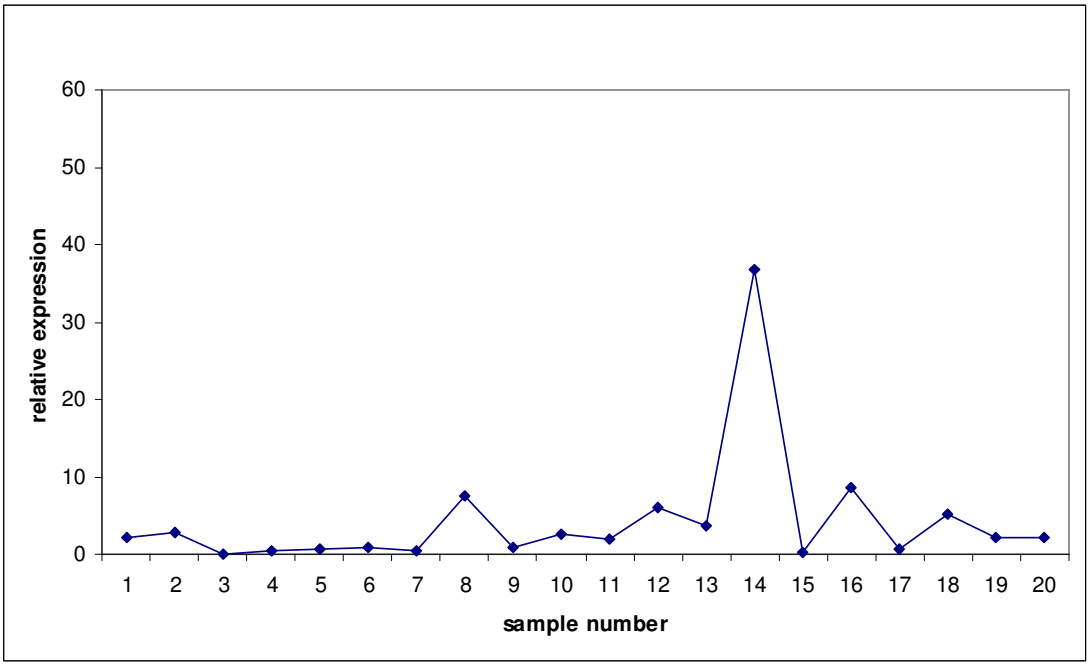


Figure 3.41. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

The injection of glucose pulse resulted in a slight decrease in the expression level of *GLC7* within the first minute in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)*. Until the 15th minute, where a sharp increase was observed, the expression was undetectable. Another increase was observed in the 5th hour of sampling, which was a 12 fold increase, however it rebounded to the initial value. The second steady state value was the same as the first steady state value.

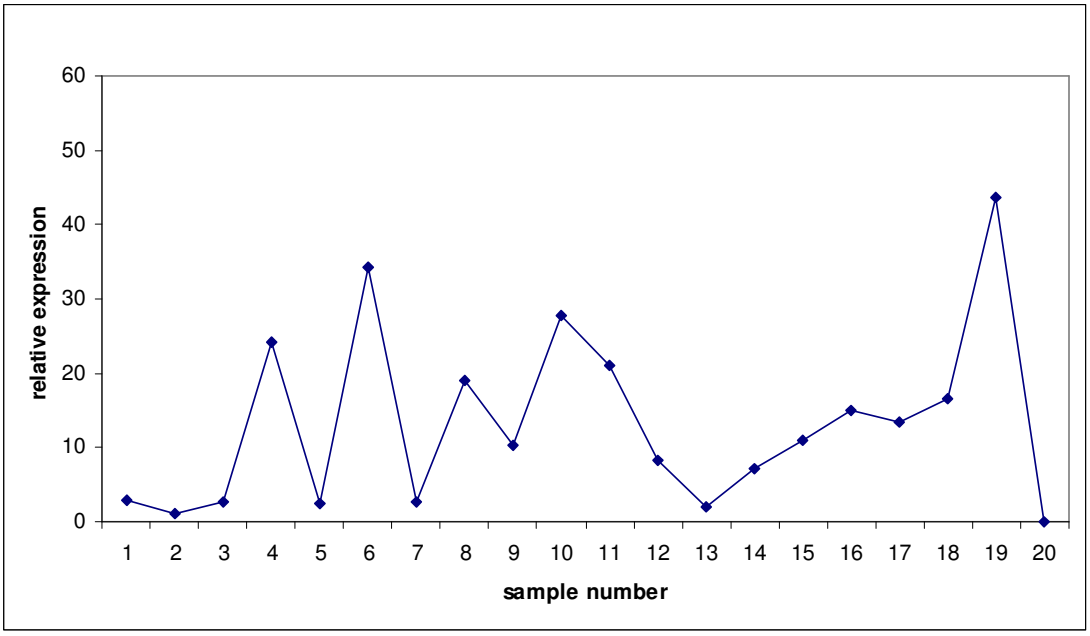


Figure 3.42. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain, the value decreased slightly after the pulse injection. The first peak was seen in the 45th second, which rebounded immediately. The second peak was seen in the 10th minute with a value higher than before. The fifth peak was seen in the 10th hour with an even higher value. However the expression level decreased steeply as the second steady state was reached; the expression of *GLC7* was undetectable.

As it can be seen in Figure 3.43, expression level of *GLC7* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation prior to the pulse. The expression level of the gene increased right after the pulse. A peak was seen at the 45th second after the pulse injection but dimmed immediately and the expression was undetectable for the next 20 minutes. The expression was also undetectable after the 8th hour but it increased in the second steady state.

In *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain, expression level of *GLC7* showed a 3 fold increase after the injection of carbon pulse and the increase continued for the next 30 seconds. The expression levels from the end of the first minute, until 2 hours after pulse

injection were lower than the first steady state value. Higher values were observed from then on until 6 hours after pulse injection. The second steady state value was 1.6 times more than the first steady state value.

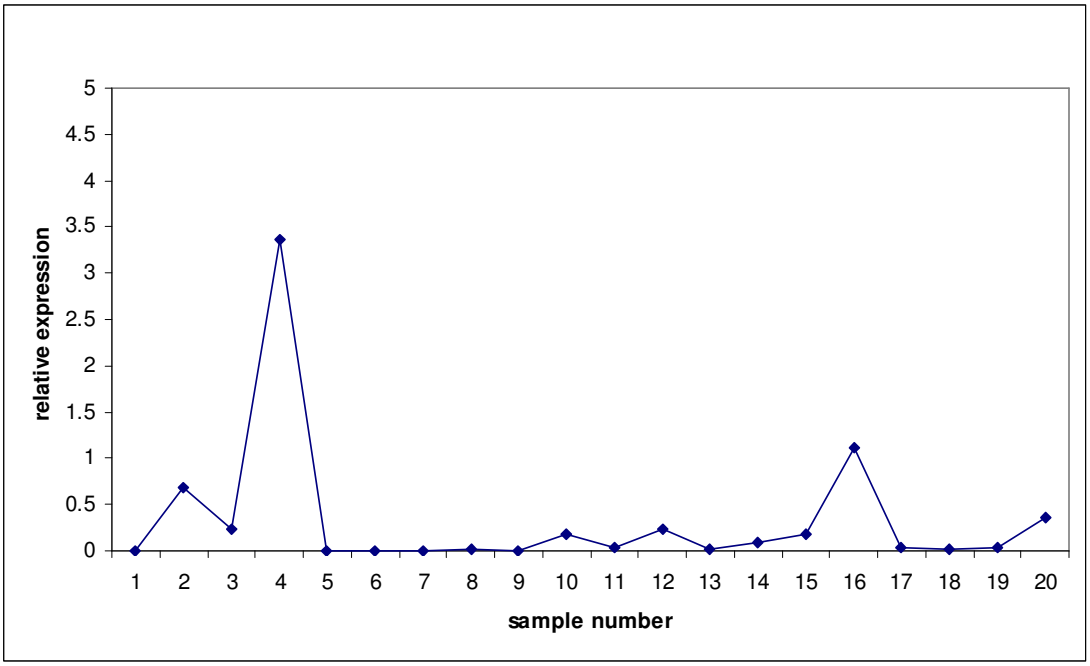


Figure 3.43. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.11. Expression Profile of *REG1* as a response to Glucose Pulse

Reg1 is phosphorylated depending on Snf1 and is associated with Snf1 (Sanz *et al*., 2000). Reg1p protein is one of the regulatory subunits of type 1 protein phosphatase Glc7 (Tu and Carlson, 1995; Nath *et al*., 2003).

Samples were collected as stated previously during cultivation and expression profiles of *REG1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in the following figures; 3.45, 3.46, 3.47 and 3.48.

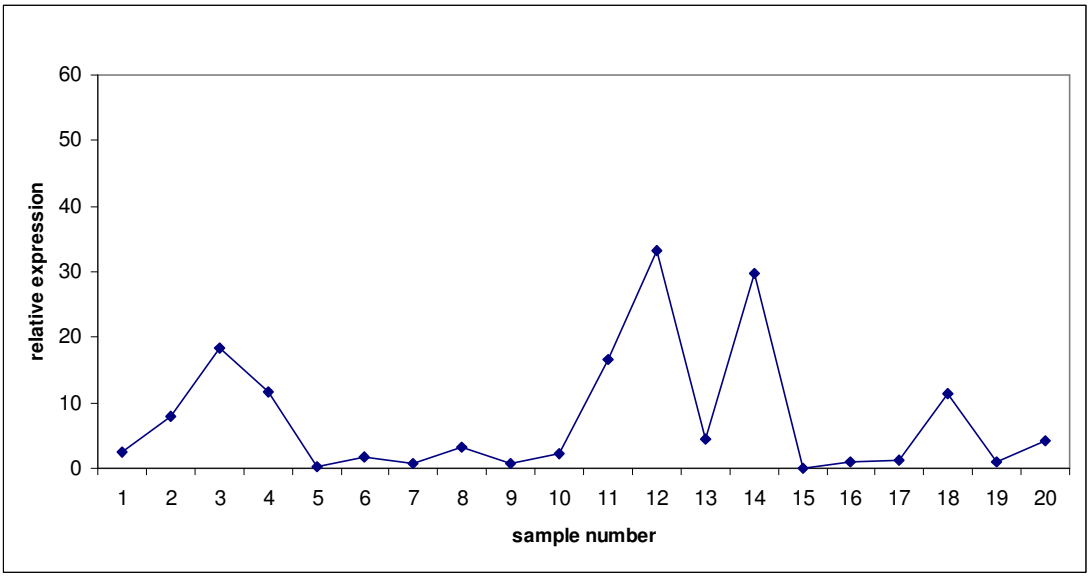


Figure 3.44. Expression profile of *GLC7* gene in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

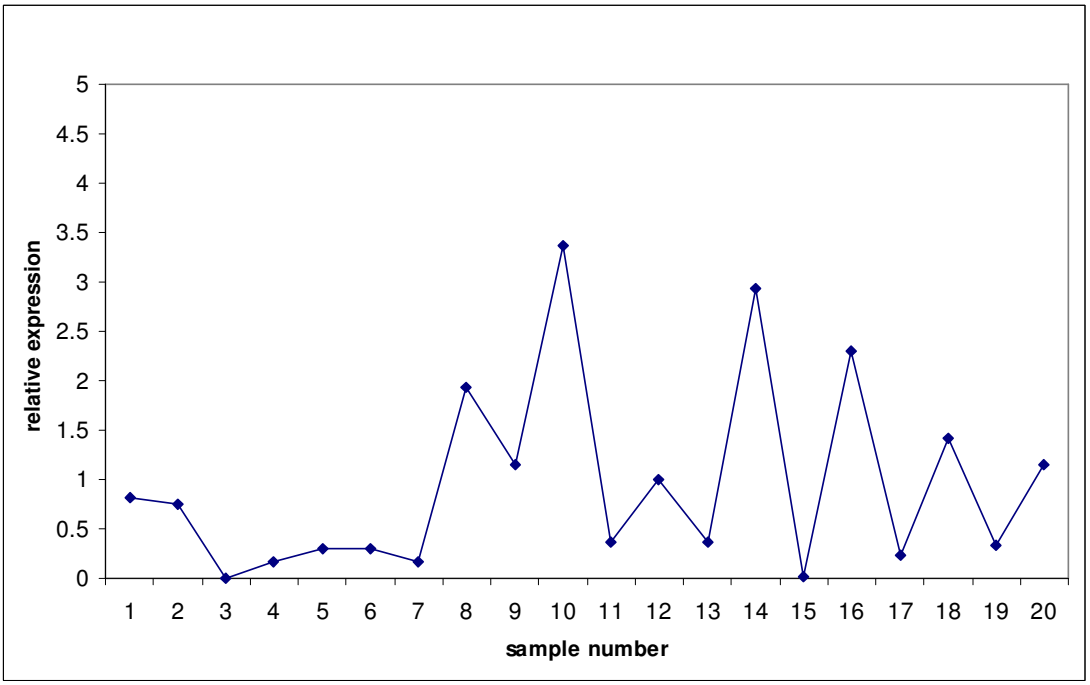


Figure 3.45. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *REG1* decreases right after the pulse injection in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures. There is no significant increase until the 15th minute, where there is a 12 fold increase. A second peak is observed at the end of the first hour. The expression level decreases to a value less than the first steady state value within the next hour. The expression level of *REG1* fluctuates until the second steady state is reached.

A fluctuating trend was observed in the relative expression of *REG1* throughout the cultivation of *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)*. The highest peak was seen in the 15th minute. The second steady state value was 2 fold lower than the first value.

Prior to the injection of carbon pulse, the expression level of *REG1* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures. After a small increase in 15 seconds, the expression decreased again and remained so until 6 hours after pulse injection. A peak was seen in the 7th hour which rebounded immediately to zero. The second steady state value was slightly higher than the value 15 seconds later than pulse injection.

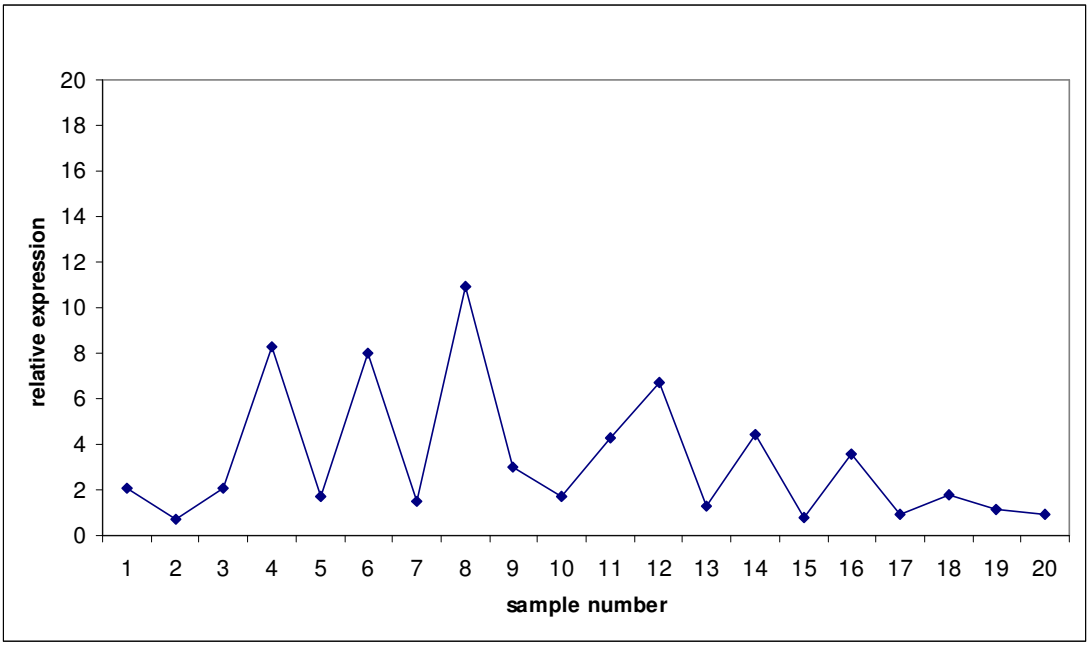


Figure 3.46. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

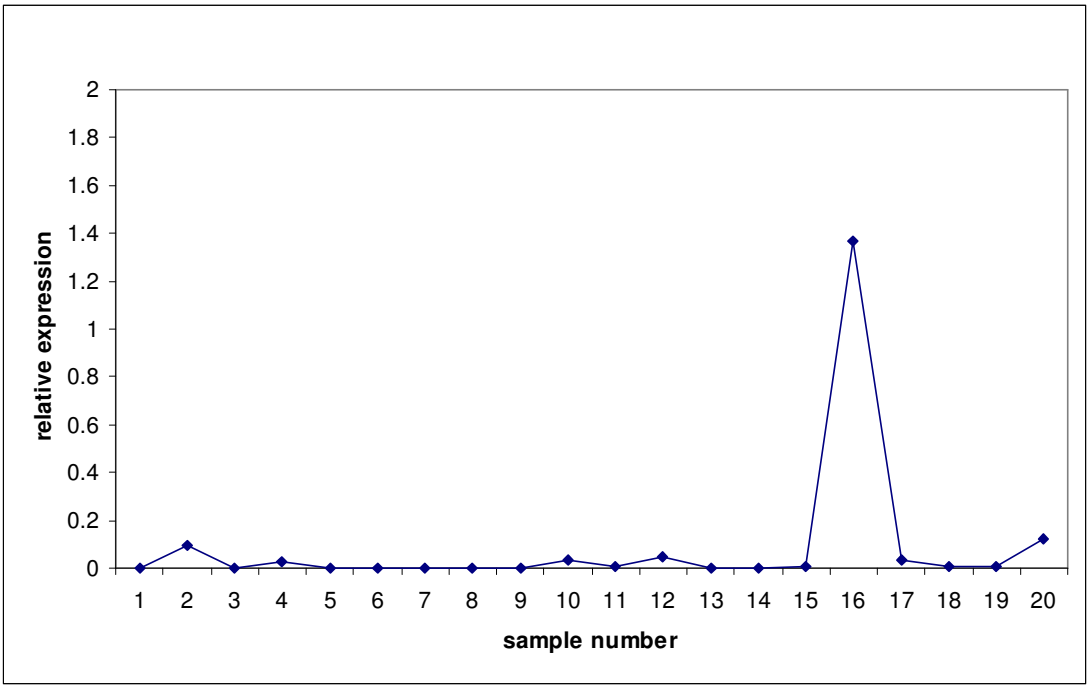


Figure 3.47. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *REG1* increased significantly 30 seconds after the injection of carbon pulse in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ).* At the end of the first minute the expression dimmed to values lower than first steady state value. Slight oscillatory behavior was seen with the highest peak being 9 hours after pulse injection. The second steady state value was 3 fold higher than the first steady state value.

3.2.12. Expression Profile of *SNF1* as a response to Glucose Pulse

Snf1 kinase is a heterotrimer that contains catalytic α-subunit Snf1, the γ-subunit Snf3 and one of the related β-subunits Gal83, Sip1 or Sip2 which regulated the subcellular location of the kinase complex (Vincent *et al.*, 2001). Snf1 protein kinase is active in glucose limited conditions and it regulates subcellular relocalizations of Mig1.

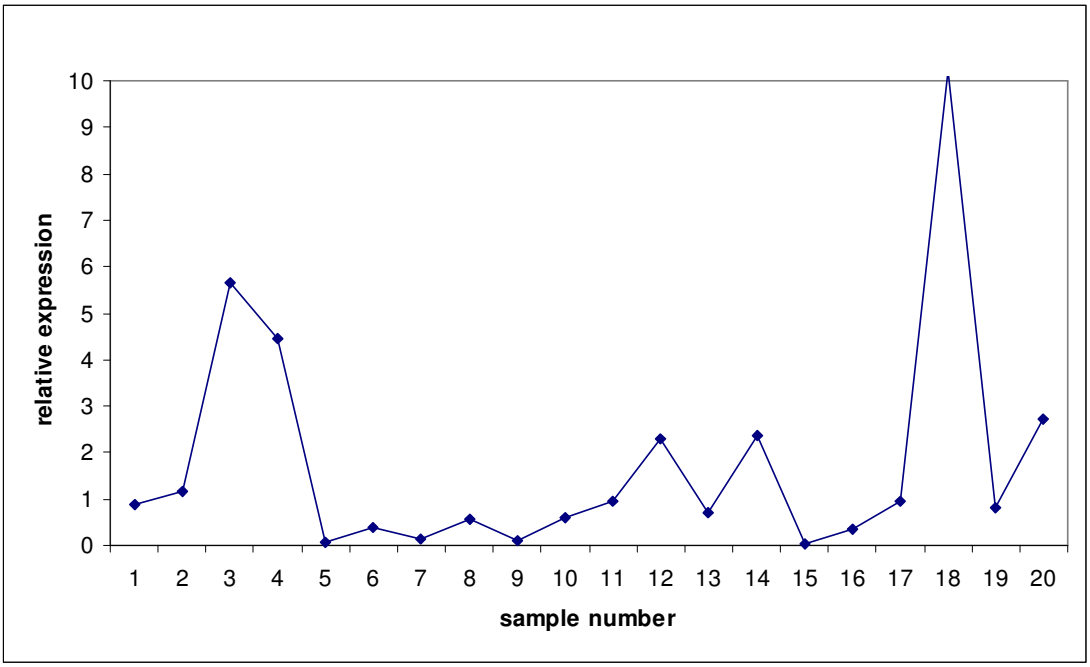


Figure 3.48. Expression profile of *REG1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

Samples were collected as stated previously during cultivation and expression profiles of *SNF1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.49, 3.50, 3.51 and 3.52.

After the injection of glucose pulse, a decrease was observed in the expression level of SNF1 in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* grown under carbon limitation in chemostat, followed immediately by an increase to a level less than the initial value. A peak was seen in the 15th minute with an 8 fold increase. The expression level decreased to the initial value in the next 5 minutes. The level increased in the 5th hour, and fluctuated in the next 4 hours. The second steady state level was undetectable.

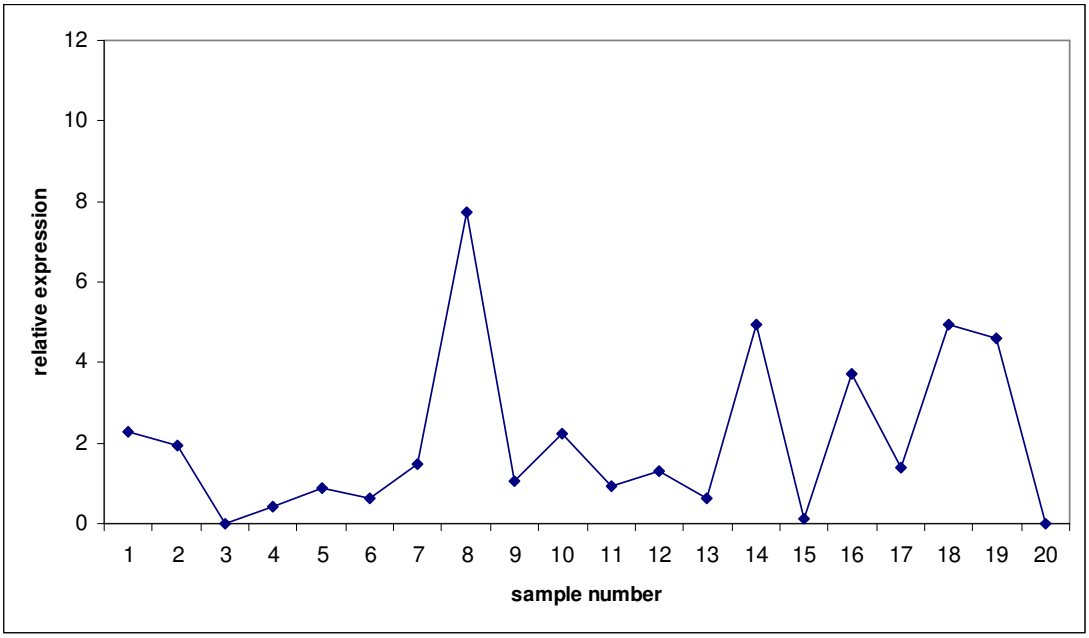


Figure 3.49. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

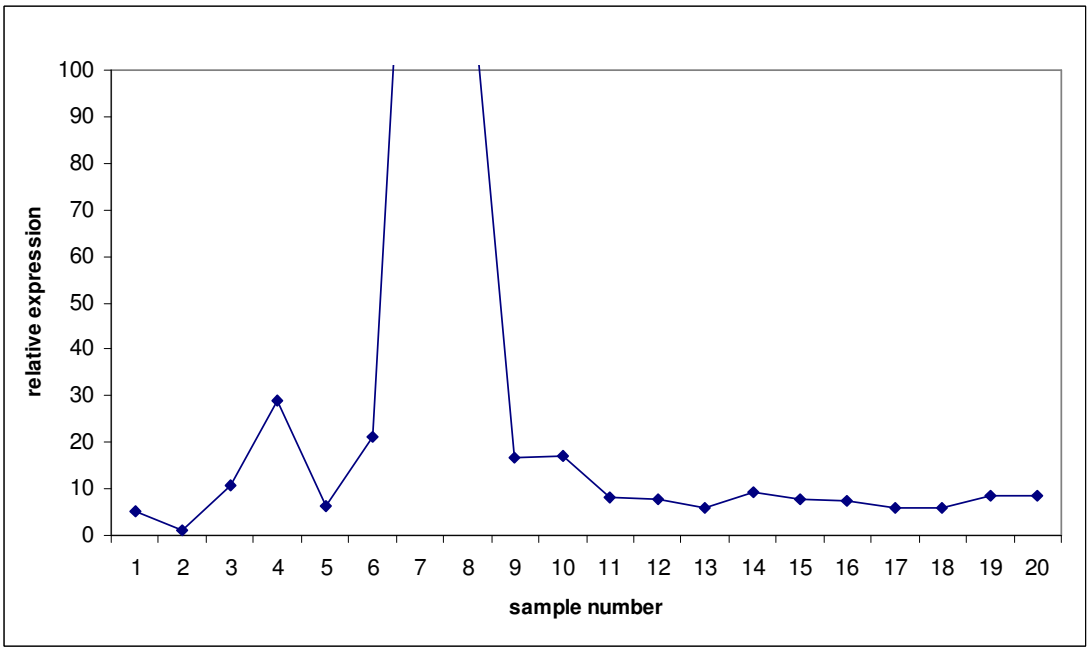


Figure 3.50. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

Expression level of *SNF1* decreased after the injection of carbon pulse in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* but reached a level higher than the initial level in the 30th second. The values in the 10th and 15th minutes were very high. The expression level was steady after the 20th minute with a value slightly more than the first steady state value.

In the case of *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)*, the expression level of *SNF1* was insignificant prior to carbon pulse injection. The expression level increased as soon as the pulse was injected and later gradually decreased. The highest peak was seen 7 hours after pulse injection, which also gradually dimmed. The value was also insignificant in the 9th hour, then a slight increase was seen as second steady state was reached.

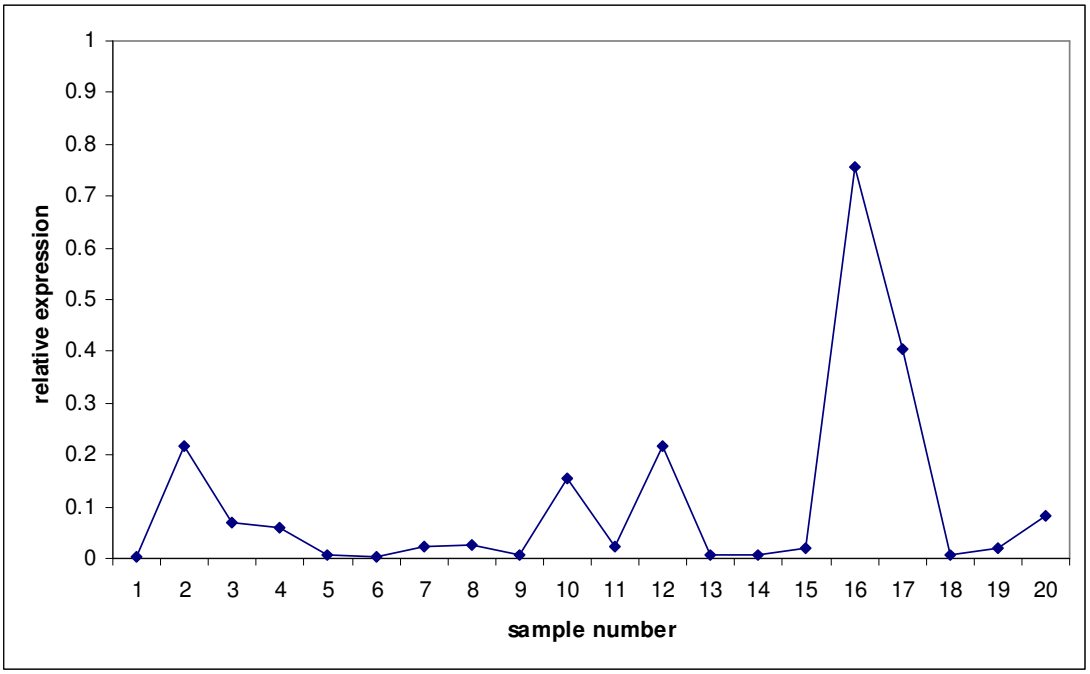


Figure 3.51. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The carbon pulse injection in chemostat cultures of *SNF1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ)* strain resulted in a 6.5 fold increase in the expression of the gene *SNF1*, 30 seconds after injection. Fluctuating behavior was seen between the 2nd and 10th hours of the sampling. The second steady state value was 2 times higher than the first steady state value.

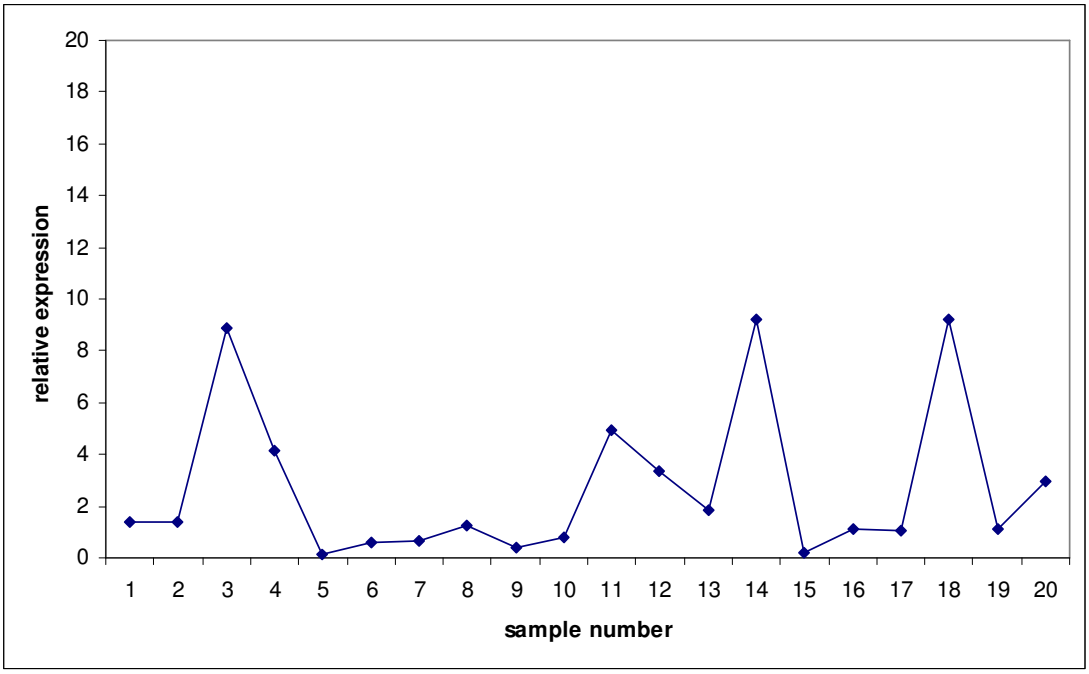


Figure 3.52. Expression profile of *SNF1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.13. Expression Profile of *SNF4* as a response to Glucose Pulse

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

Samples were collected as stated previously during cultivation and expression profiles of *SNF4* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.53, 3.54, 3.55 and 3.56.

The expression level of *SNF4* increased immediately after the pulse injection and slightly decreased for the next 15 minutes in *S. cerevisiae* BY4743 (*hoΔ/hoΔ)* under carbon limitation in chemostat cultures. A 25 fold increase was observed in the 20th minute. The expression level immediately rebounded in the 2nd hour and showed a fluctuating behavior until the second steady state was reached. At the second steady state, the expression level of *SNF4* was undetectable.

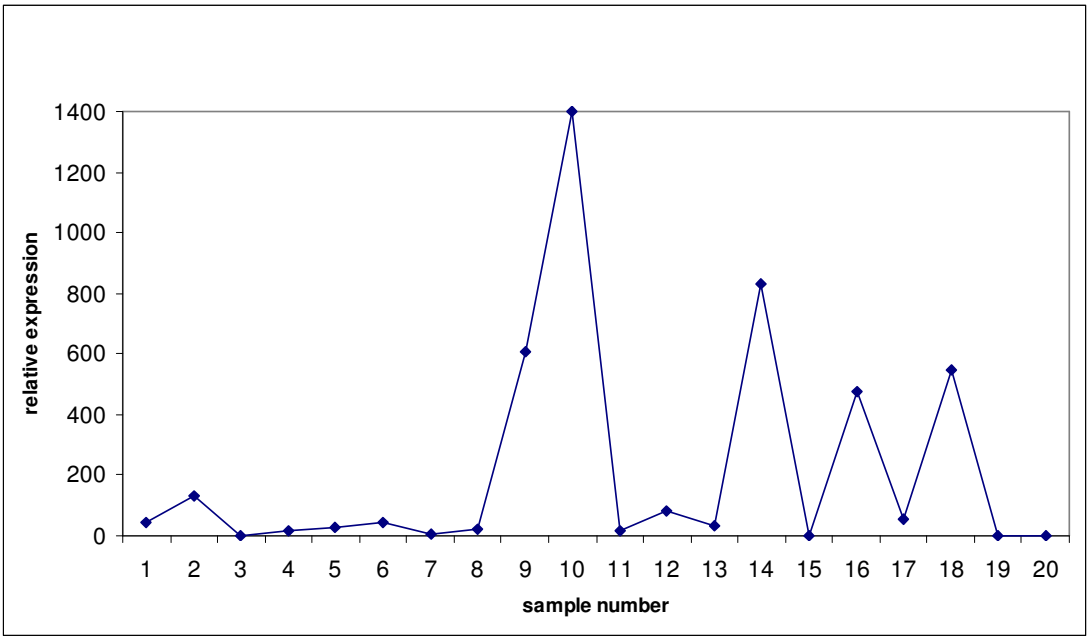


Figure 3.53. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain, a fluctuating behavior was seen after the injection of carbon pulse in the expression level of *SNF*. The highest peak was seen in the 15th minute which rebounded immediately in the next 5 minutes. The expression level was undetectable in the 4th hour, as well as in the second steady state.

In homozygous deletion mutant of *RIP1*, the expression level of *SNF4* was insignificant in the first steady state. With the pulse injection, a peak was seen, but the level returned to zero immediately. The expression level was undetectable until 1 hour after pulse injection. The highest peak was seen in the 7th hour which gradually decreased to zero until the end of the 10th hour. The expression level in the second steady state was as high as the expression level 15 seconds after pulse injection.

In *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain, *SNF4* expression level showed a fluctuating behavior. The initial response to carbon pulse was an increase of 4 fold. Expression level at the second steady state was 2 fold higher than the first steady state level.

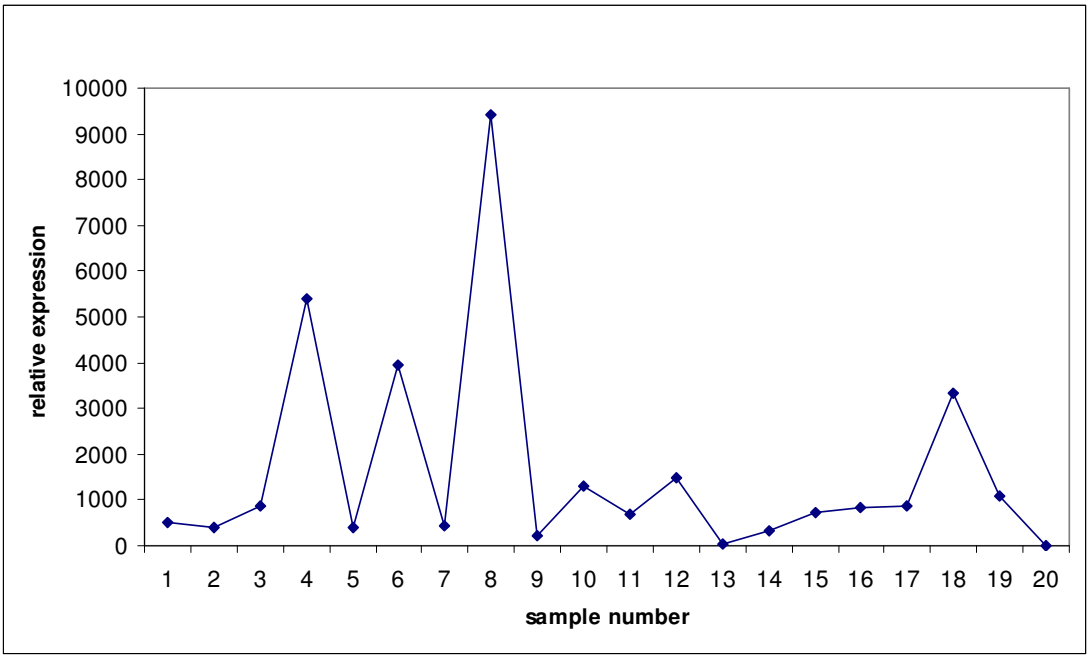


Figure 3.54. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

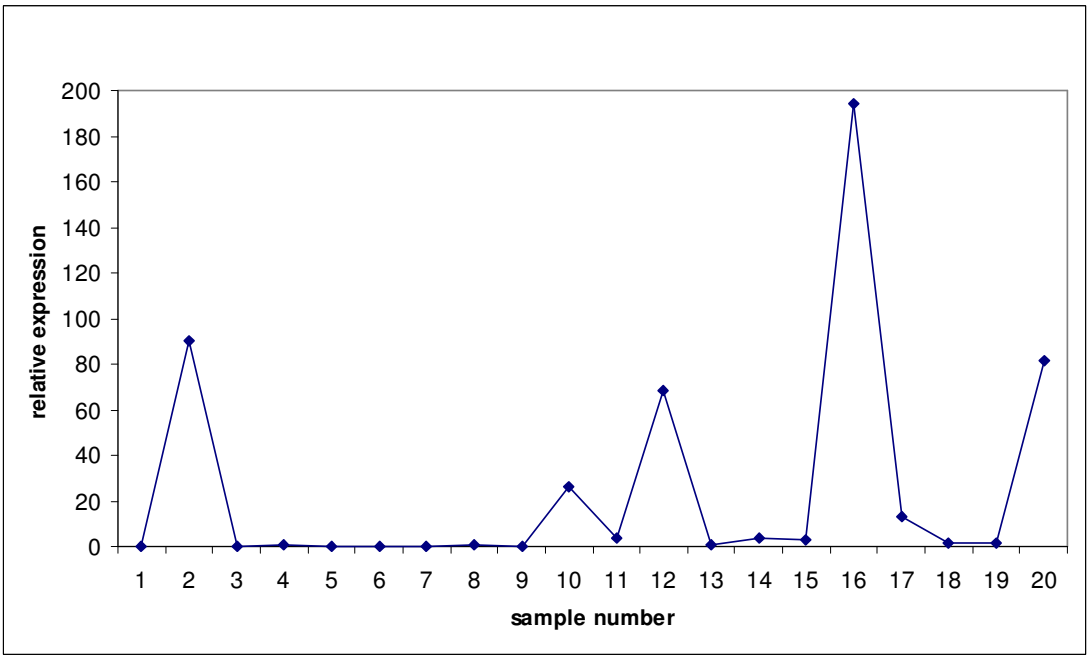


Figure 3.55. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

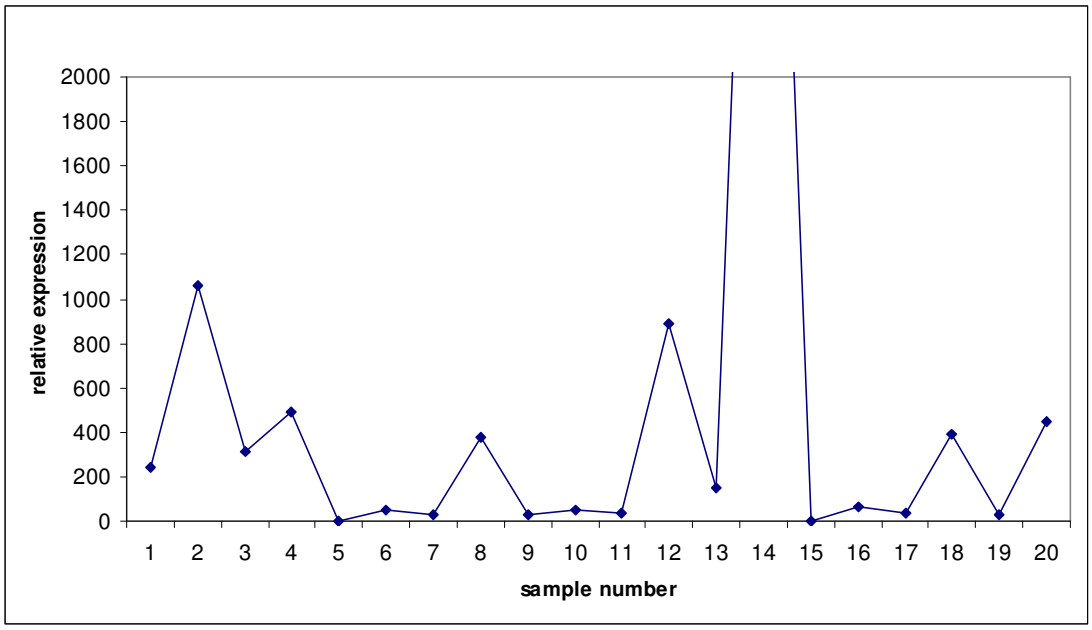


Figure 3.56. Expression profile of *SNF4* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.14. Expression Profile of *PAK1* as a response to Glucose Pulse

It is known that Pak1 is associated with either Snf1 or Snf4 (Gavin *et al*., 2002). For maximal activity, Snf1 kinase requires *PAK1*. Snf1 dependent phosphorylation of Mig1 is stimulated by Pak1 kinase (Nath *et al*., 2003).

Samples were collected as stated previously during cultivation and expression profiles of *PAK1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in the following figures; 3.57, 3.58, 3.59 and 3.60.

After the injection of glucose pulse, a decrease was observed in the expression level of *PAK1* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* grown under carbon limitation in chemostat, followed immediately by an increase to a level less than the initial value. A peak was seen in the 15th minute with a 3 fold increase which rebounded very quickly. Expression level at the second steady state was approximately the same as the initial level.

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)*, the expression level of *PAK1* was nearly undetectable right after the injection of carbon pulse. However, the value in the 30th second was 25 times higher than the initial steady state value. A very high peak was seen in the 15th minute which rebounded immediately. The expression level after this was very low and steady and the second steady state value was slightly lower than the first value.

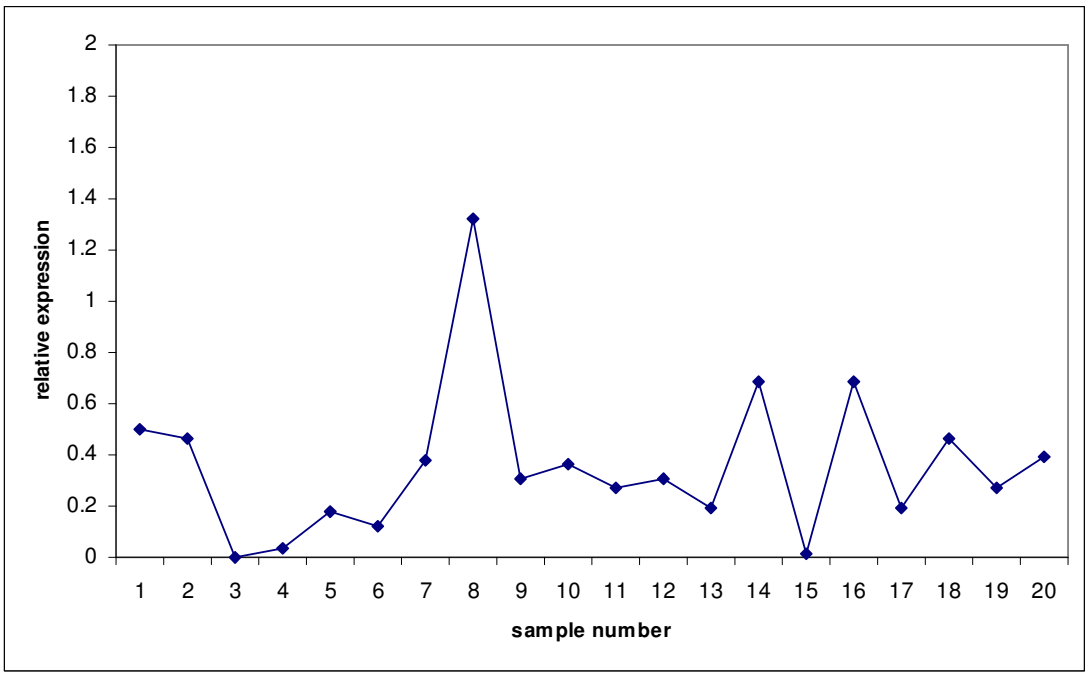


Figure 3. 57. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*) under carbon limitation in chemostat cultures with pulse injection

The injection of carbon pulse caused a steep increase in the expression of *PAK1* which was very small prior to pulse injection to chemostat cultures of *S. cerevisiae* BY4743 (*rip1Δ/ rip1Δ*). The increase rebounded immediately. Fluctuating behavior in the expression level of this gene was observed during the period of time between 20 minute and 9 hour later than the pulse injection. The expression level of *PAK1* at the second steady state increased.

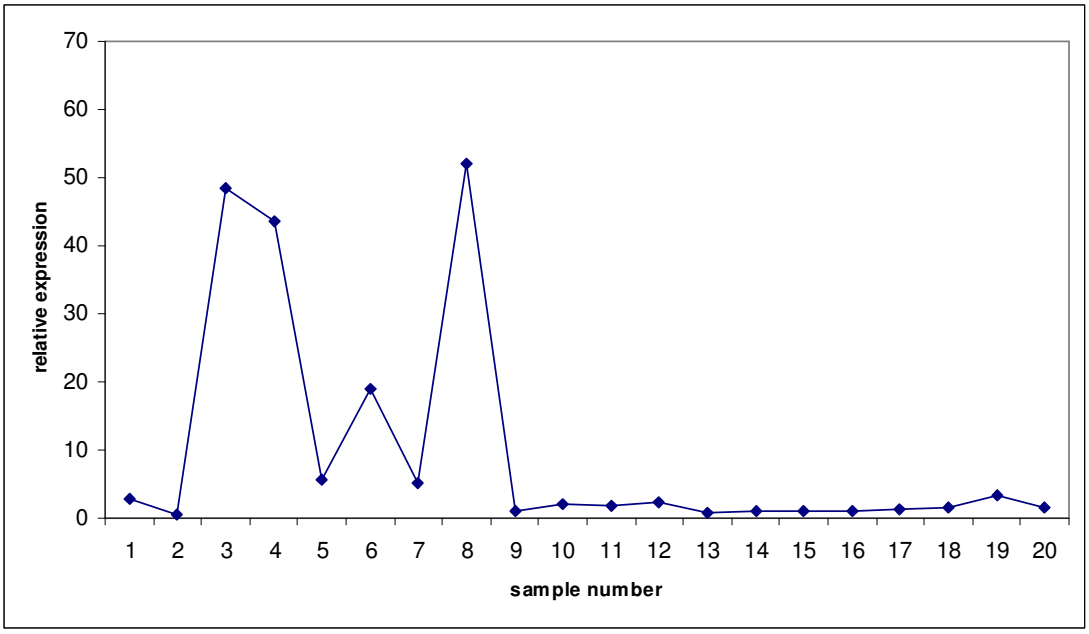


Figure 3.58. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

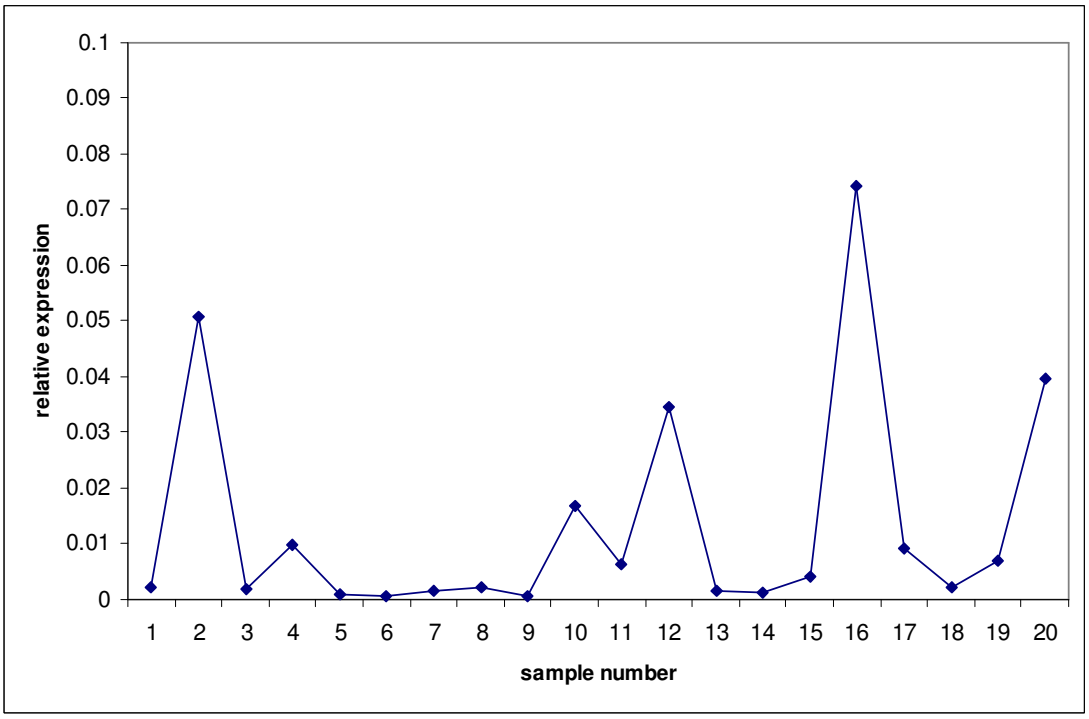


Figure 3.59. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

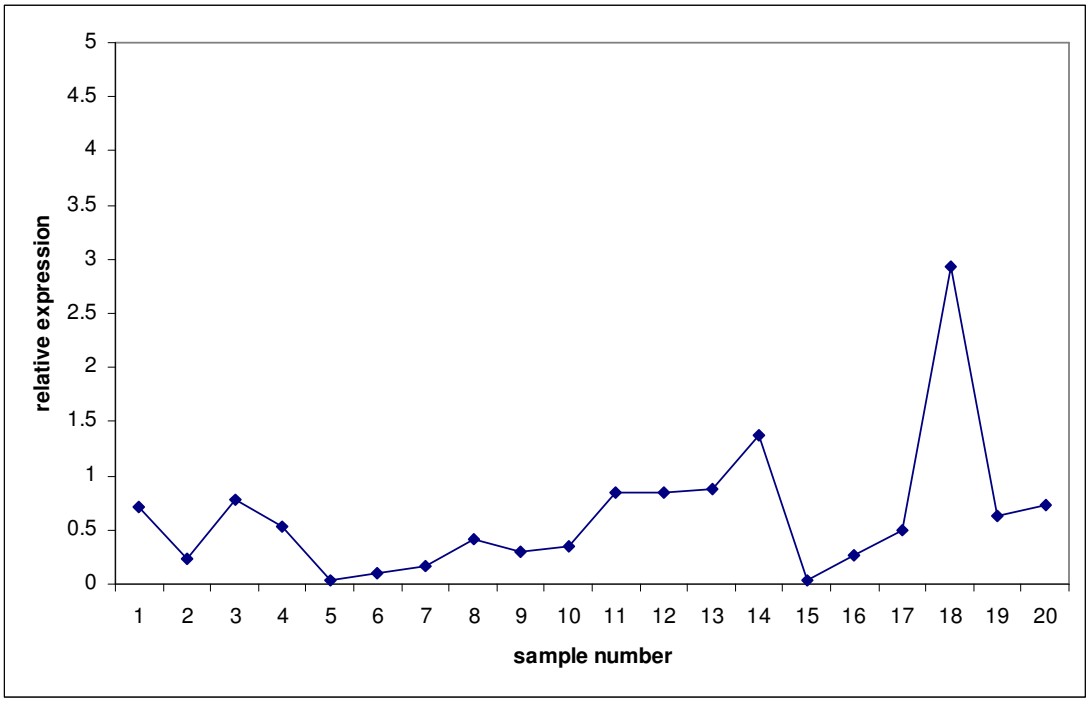


Figure 3.60. Expression profile of *PAK1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

In the case of heterozygous mutant of *RIP1*, a 3 fold decrease was observed in the expression level of *PAK1* soon after the injection of carbon pulse. The value recovered to its initial value in the next 15 seconds. The second steady state expression level not significantly different when compared to the first steady state level.

3.2.15. Expression Profile of *TOS3* as a response to Glucose Pulse

For the activation of *SNF1* complex in glucose limited conditions, at least one of the proteins Elm1p, Pak1p or Tos3p, which are members of a subfamily of yeast kinases that can phosphorylate Thr210 on Snf1p, is required (Sutherland *et al*., 2003).

Samples were collected as stated previously during cultivation and expression profiles of *TOS3* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.61, 3.62, 3.63 and 3.64.

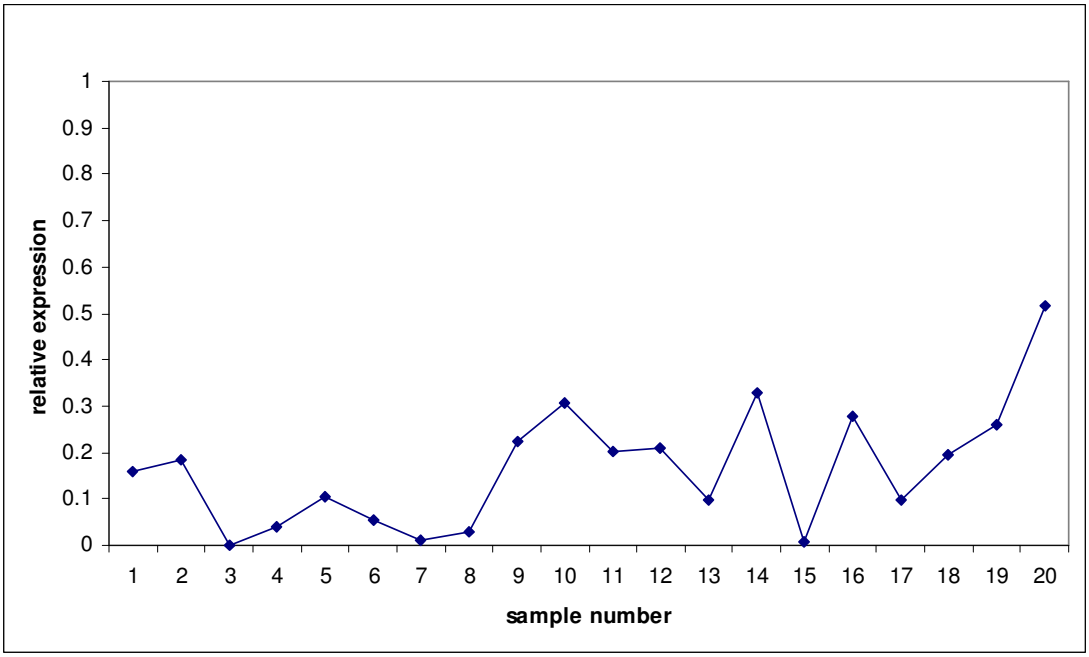


Figure 3.61. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

In *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* strain, after the injection of glucose pulse the expression levels of *TOS3* first decreased, then increased gradually. The expression level of this gene at the second steady state was 5 fold higher than the first steady state level.

In *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain, the expression level of *TOS3* decreased right after the injection of carbon pulse and increased in the next 30 seconds to values higher than the initial value. After the 20th minute the expression level dropped to a value lower than the first steady state value and stayed constant. The second steady state value was slightly lower than the first steady state value.

Prior to the pulse injection, the expression of *TOS3* was not detectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* grown under carbon limitation in chemostat cultures. With the injection of carbon pulse an immediate increase was observed. The expression of this gene could not be detected 1minute after the pulse injection, until the first hour. Peaks were seen in the first hour, third hour and 7th hour, the last being the highest. The second steady state expression level was nearly as high as the expression level immediately after pulse injection.

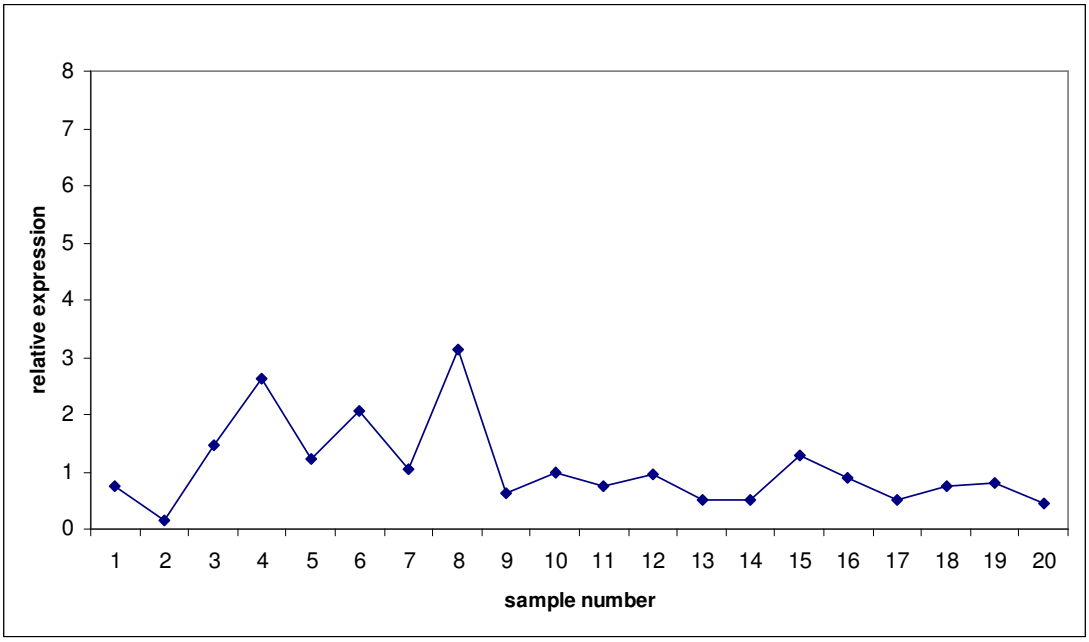


Figure 3.62. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

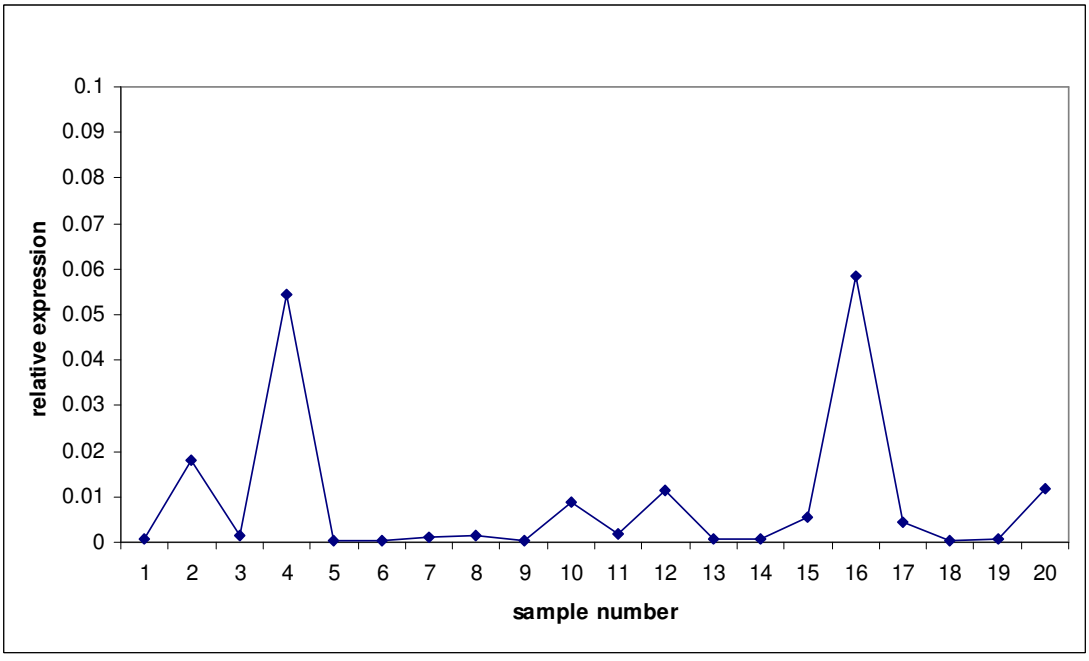


Figure 3.63. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

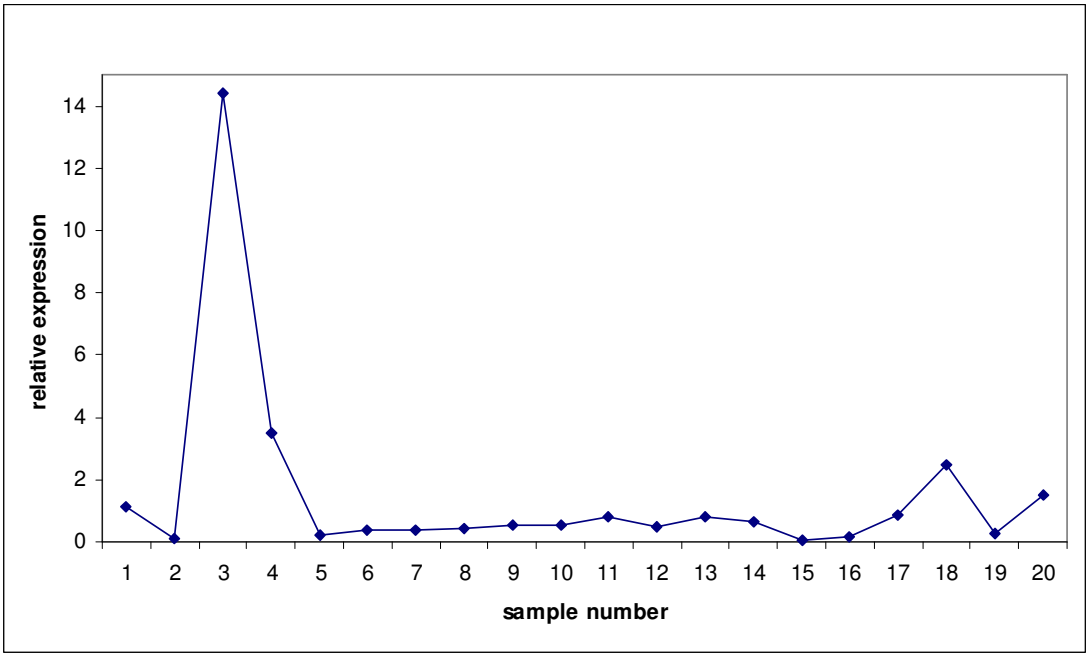


Figure 3.64. Expression profile of *TOS3* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *TOS3* decreased to an unnoticeable level right after the carbon pulse injection in *S. cerevisiae* BY4743 (*RIP1/rip1Δ).* However the level showed a very sharp increase 30 seconds after the injection. The level between the time period of the end of first minute and the end of the 7th hour was lower than the first steady state value and was steady. The second steady state value was not much higher than the first steady state value.

3.2.16. Expression Profile of *ELM1* as a response to Glucose Pulse

As mentioned earlier, Elm1p is also a member of a subfamily of yeast kinases that phosphorylate Thr210 on Snf1p. Besides this, Elm1p also has regulatory functions that involve cell morphology, filamentous invasive growth that may have no connection to the Snf1 pathway (Hong *et al*., 2003).

The expression level of *ELM1* was insignificant prior to and after glucose pulse injection throughout cultivations of *hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ* strains of *S. cerevisiae*.

3.2.17. Expression Profile of *MIG1* as a response to Glucose Pulse

Mig1 is a repressor responsible for glucose repression of many genes such as *GAL, SUC,* and *MAL.* In response to glucose, Mig1 repressor moves between the nucleus and cytoplasm in the yeast (Nehlin and Fonne, 1990; De Vit *et al*., 1997; Ostling and Ronne, 1998, Papamichos-Chronakis *et al*., 2004). With the addition of glucose to the growth medium, Mig1 is imported into the nucleus, and when glucose is removed, it is moved back to the cytoplasm. *MIG1* encodes a C_2H_2 zinc finger protein that binds to the DNA with a GC rich consensus sequence and a flanking AT sequence (Nehlin and Ronne, 1990; Lundin *et al*., 1994).

Samples were collected as stated previously during cultivation and expression profiles of *MIG1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in the following figures; 3.65, 3.66, 3.67 and 3.68.

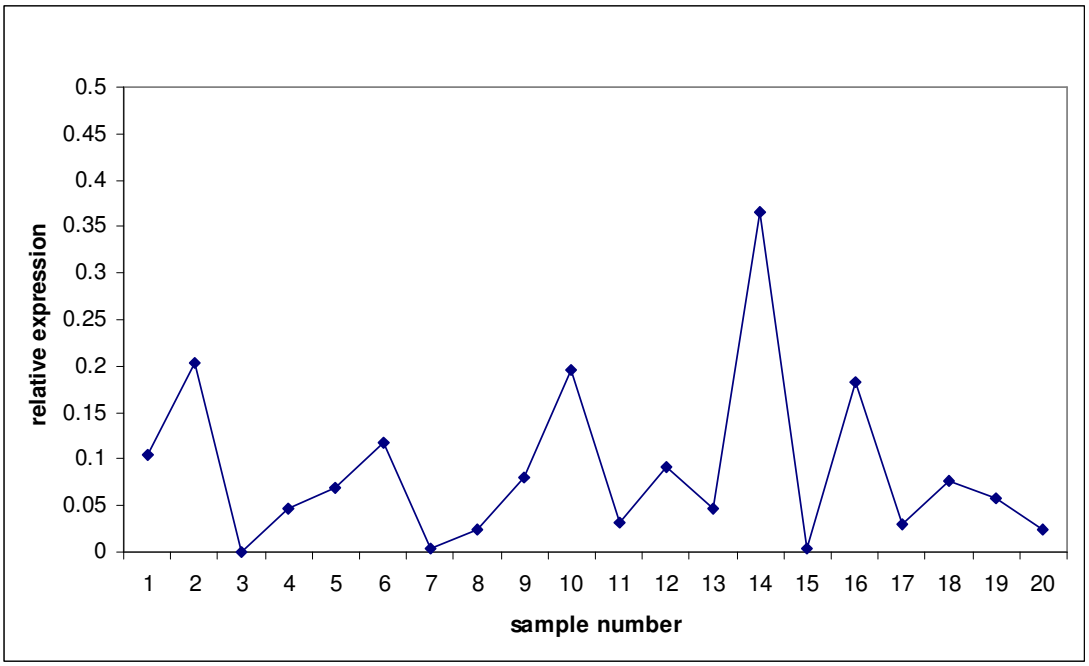
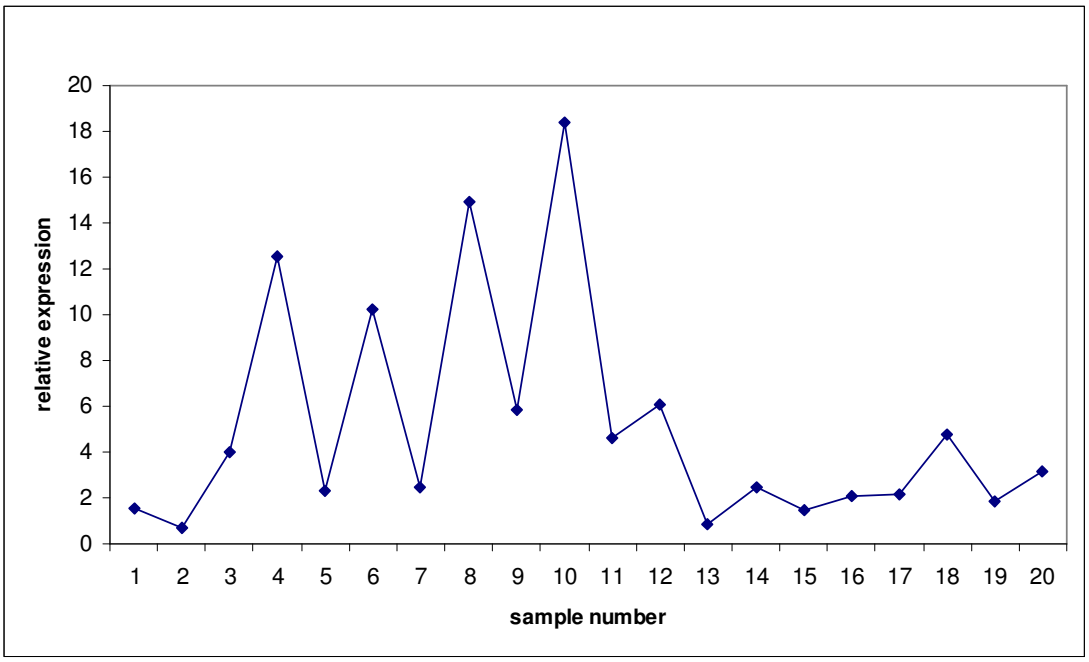


Figure 3.65. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

Injection of the glucose pulse resulted in decrease in the expression level of *MIG1* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse

injection. The level increase immediately within the first minute. The expression level showed an oscillating behavior throughout the cultivation ending with a second steady state value less than the first value. Comparison of the first and second steady state values indicated that expression level at the first steady state was almost 4 fold higher than the second steady state level.



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

The carbon pulse resulted in the initial two fold decrease in the expression level of the *MIG1* in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* grown under carbon limitation. In the 45th second the expression level was 12 fold higher than the value right after the pulse injection. A fluctuation was observed until the end of 2nd hour. The expression level of this gene at second steady state was 2 fold higher than the first steady state.

In *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* the expression level of *MIG1* showed an increase soon after the injection of carbon pulse. The expression levels were usually undetectable with higher levels of expression observed during the period of time between 20 minutes and 7 hours later than the pulse injection. Its expression level at the second steady state increased to a higher value than that of the first steady state.

After the carbon pulse, a gradual increase was observed in the expression level of *MIG1* in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ).* However at the end of the first minute the expression level had dropped to an insignificant value. A high peak was seen 9 hours after pulse injection. The expression level at the second steady was 10 fold higher than the expression level at the first steady state.

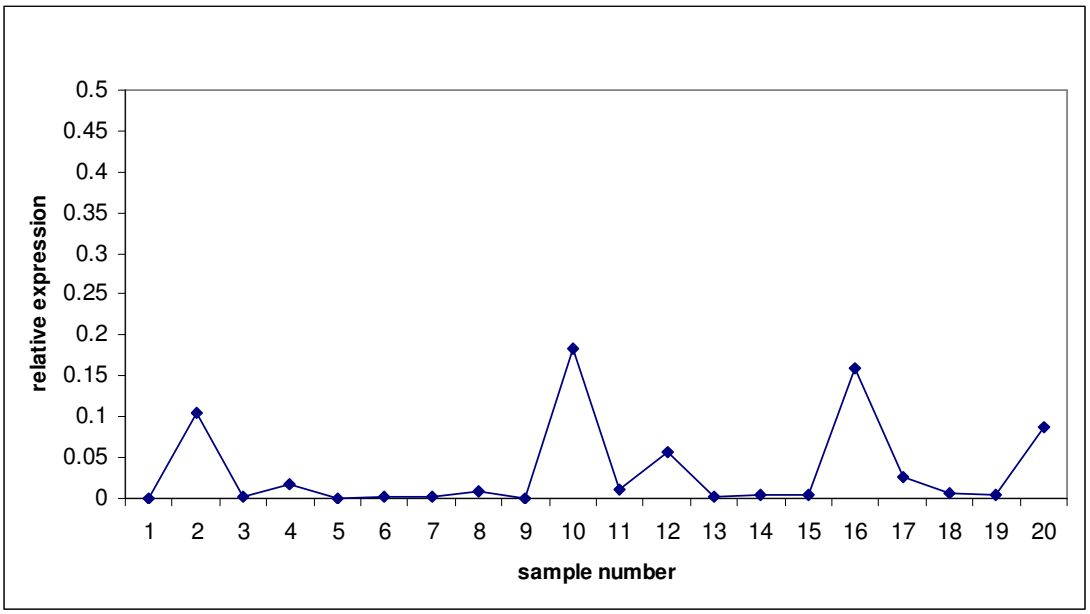


Figure 3.67. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.18. Expression Profile of *CYC8* as a response to Glucose Pulse

CYC8 is a transcriptional co-repressor, acts together with Tup1p and also acts as part of a transcriptional co-activator complex that recruits the SWI/SNF and SAGA complexes to promoters. By recruiting the general co-repressor complex Cyc8 (Ssn6)-Tup1, Mig1 is believed to inhibit transcription (Nehlin *et al.*, 1991; Treitel and Carlos, 1995; Tzamarias and Struhl, 1995; Papamichos-Chronakis *et al.*, 2004). Cyc8 interacts with the non-phosphorylated form of Mig1 and in poor glucose concentration conditions Snf1- dependent phosphorylation of Mig1 releases its interaction with Cyc8-Tup1.

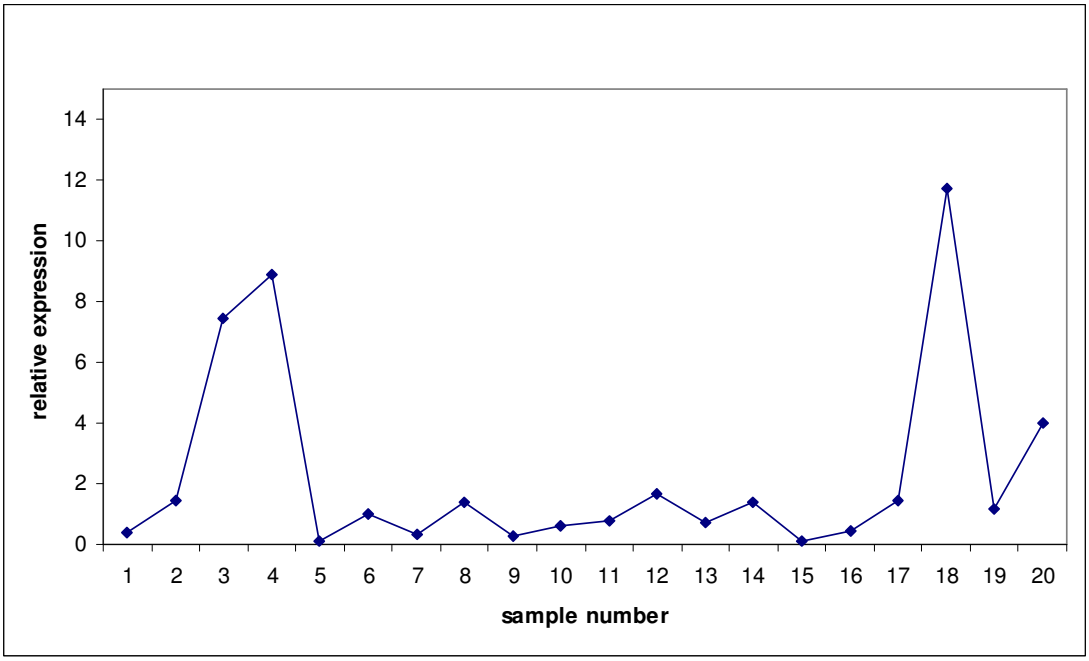


Figure 3.68. Expression profile of *MIG1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

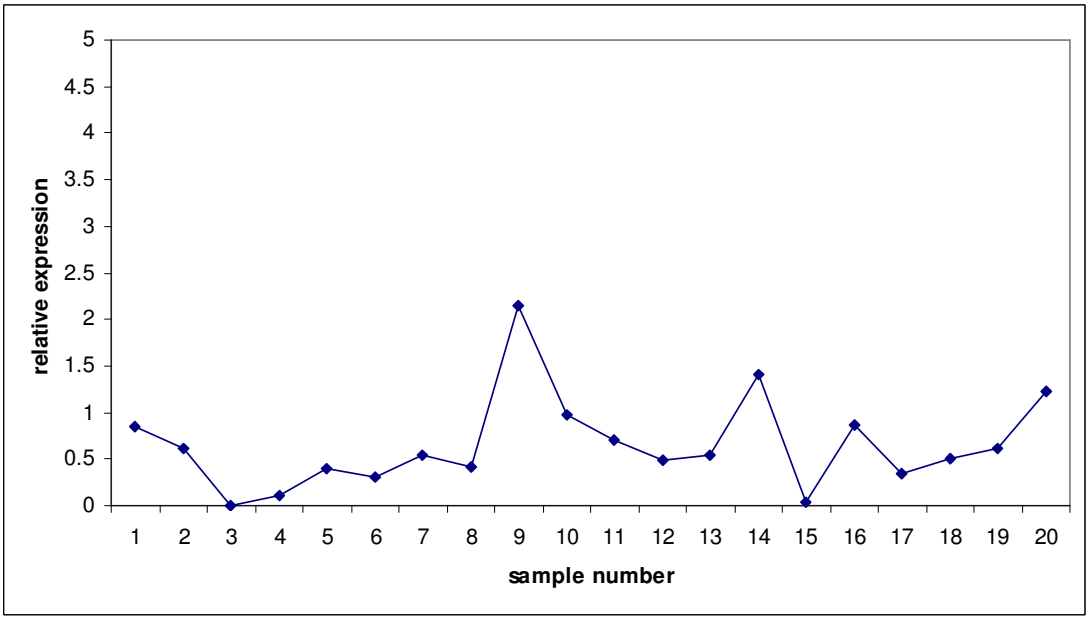


Figure 3.69. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

Samples were collected as stated previously during cultivation and expression profiles of *CYC8* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.69, 3.70, 3.71 and 3.72.

As it can be seen from the Figure 3.69, carbon pulse resulted in an immediate decrease in the expression level of *CYC8* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures. The level increased slightly until the 20th minute where a 4 fold increase was observed. The expression level decreased during the cultivation and the second steady state value was reached as a value approximately the same as the first value.

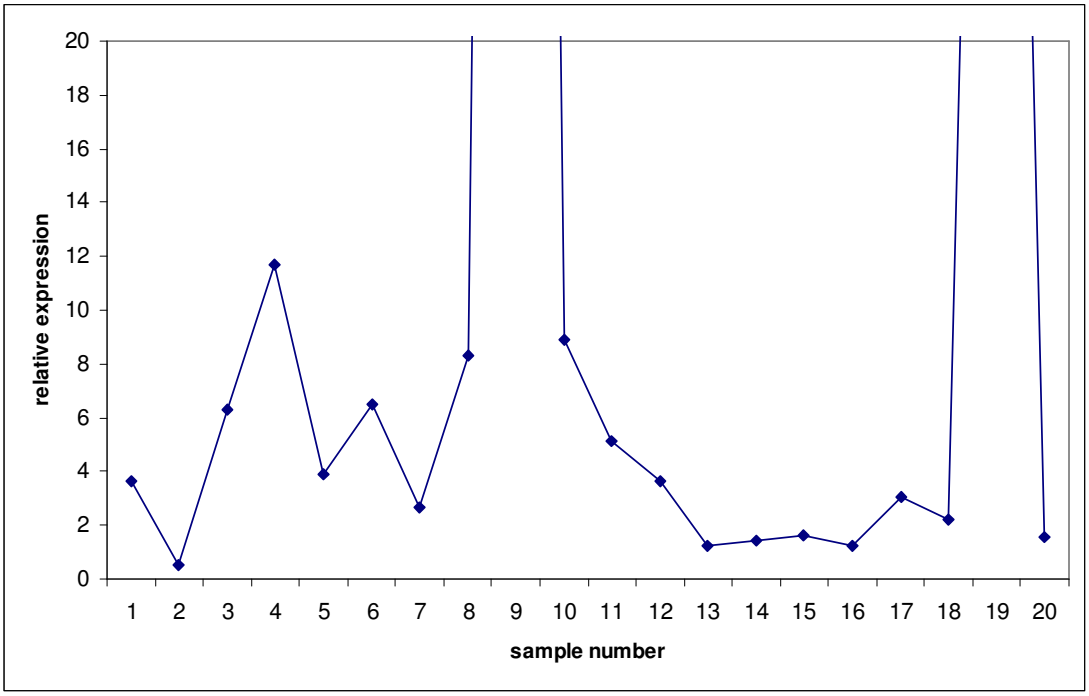


Figure 3.70. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *CYC8* showed a six fold decrease soon after the injection of carbon pulse in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* grown under carbon limitation in chemostat cultures. Then within the first minute after the injection, the expression level of *CYC8* increased above its initial level. There was a peak in the 20th minute where the expression level was 19 fold higher than the level prior to it, it then rebounded

immediately. Another peak was seen in the 10th hour which also dimmed immediately. The expression level at the second steady state was 2 fold lower than the initial level.

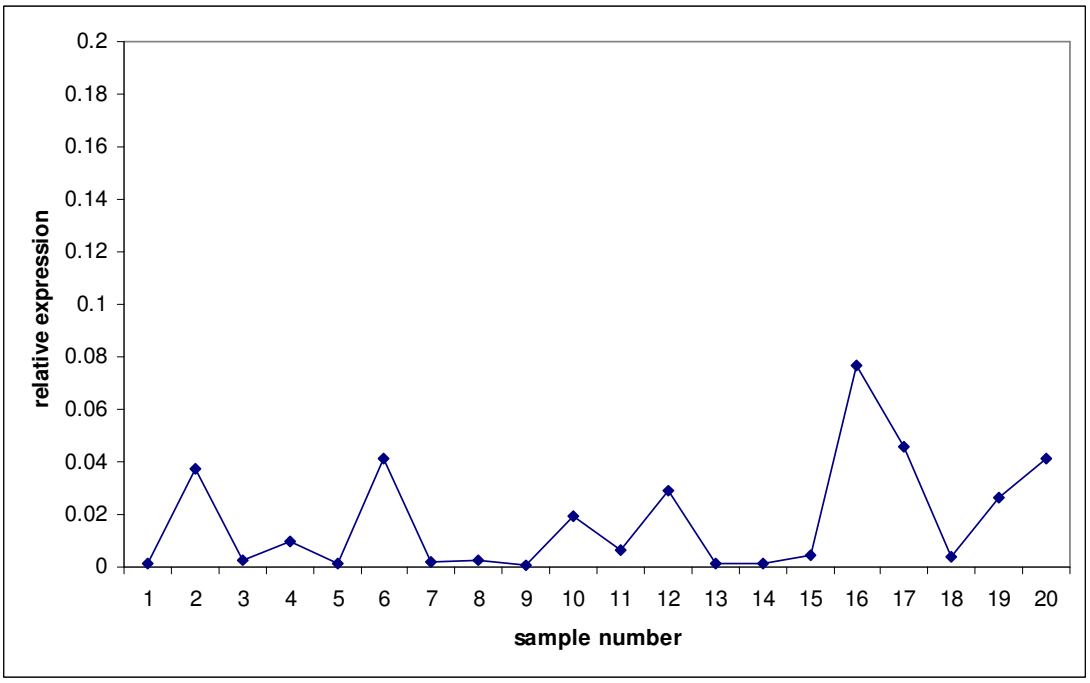


Figure 3.71. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *CYC8* increased sharply within the 15 seconds later than the pulse injection in chemostat cultures of *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* strain and decreased again. Fluctuating behavior was seen throughout the cultivation with the highest expression being 7 hours later than pulse injection. The level decreased again within the next 2 hours, then increased as the second steady state was reached.

A decrease in the expression level of *CYC8* in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* was followed by an increase after carbon pulse injection. Lower expression levels were observed until one hour after the injection. The highest level was seen 9 hours later, which rebounded immediately. The second steady state value was twice the first steady state value.

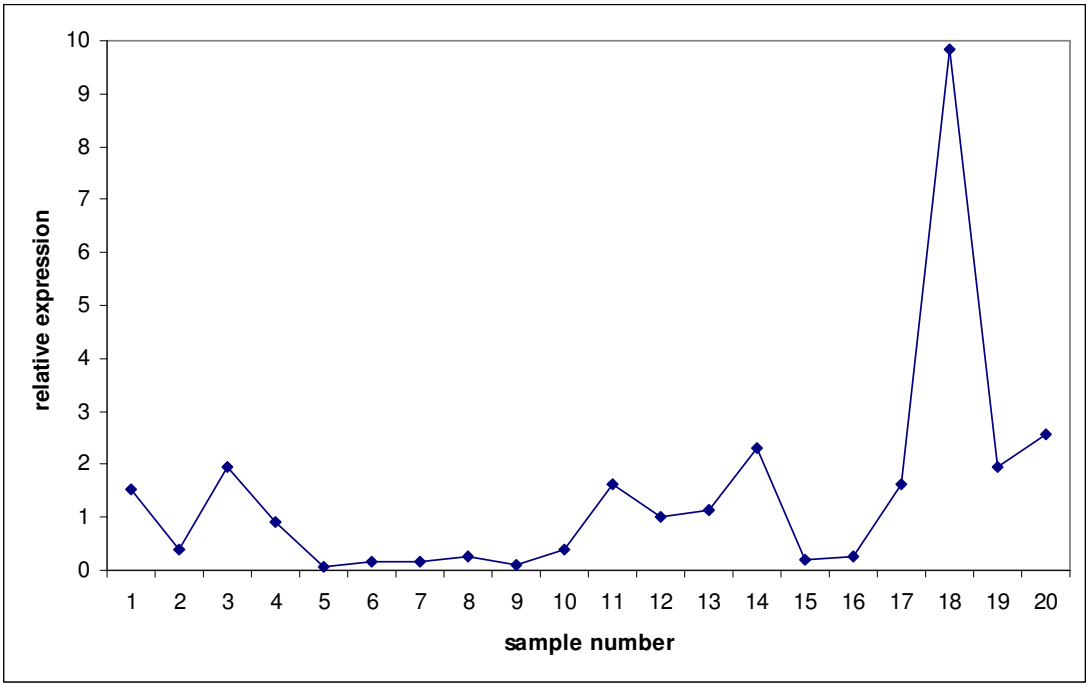


Figure 3.72. Expression profile of *CYC8* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.19. Expression Profile of *TUP1* as a response to Glucose Pulse

Tup1p is a general repressor of transcription. It forms complex with Cyc8p, involved in the establishment of repressive chromatin structure through interactions with histones H3 and H4 (Nehlin *et al*., 1991; Treitel and Carlos, 1995; Tzamarias and Struhl, 1995; Papamichos-Chronakis *et al*., 2004).

Samples were collected as stated previously during cultivation and expression profiles of *TUP1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.73, 3.74, 3.75 and 3.76.

After the injection of glucose pulse, the expression level of *TUP1* decreased in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* strain. The expression level increased after five minutes until the 20th minute, and the level decreased for the following 9 hours. There was a sharp increase in the 10th hour, which rebounded as the second steady state was reached with a value slightly higher than the first value.

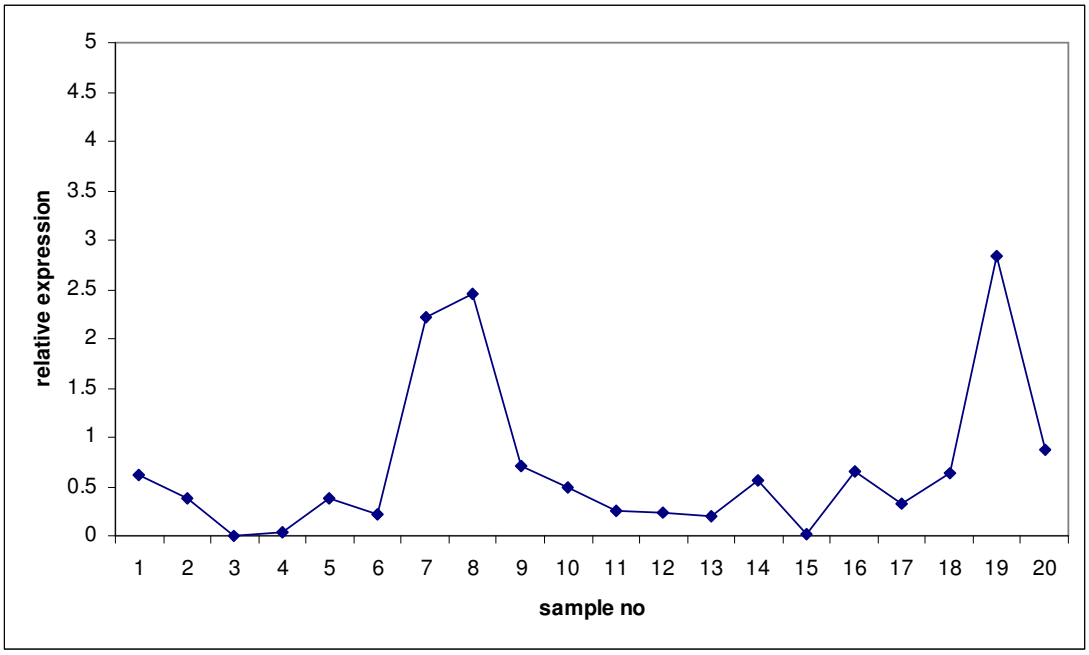


Figure 3.73. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

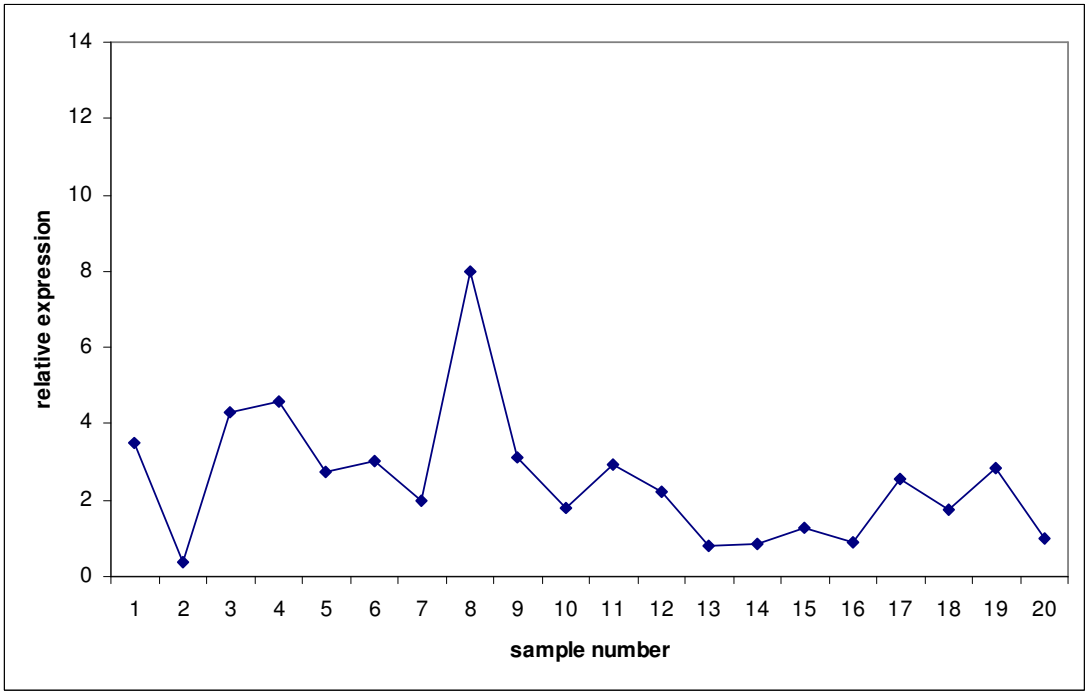


Figure 3.74. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

After the injection of carbon pulse, the expression level of *TUP1* decreased 10 fold in strain which had a homozygous deletion of *HAP4*. However, within the next 15 seconds, the level increased to a value higher than the initial steady state level. A peak was seen in the 15th second. During the time duration between the fourth and seventh hours the expression level was lower than the initial value which then increased slightly. The second steady state value was 3.5 fold lower than the first steady state value.

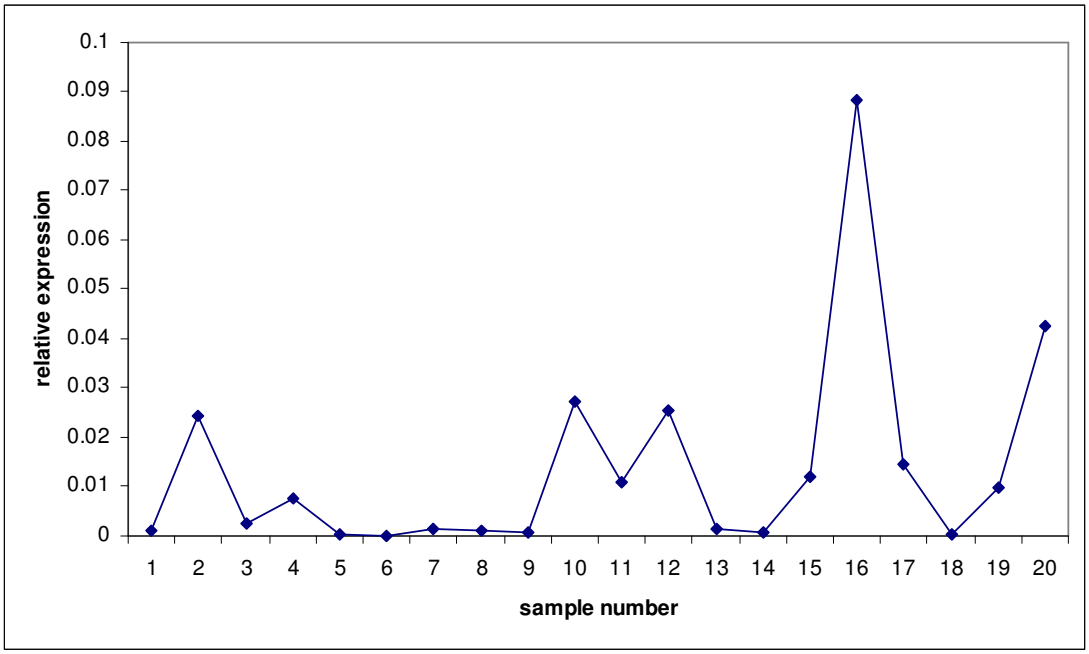


Figure 3.75. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

Prior to the carbon pulse, expression level of *TUP1* was undetectable in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* grown under carbon limitation in chemostat cultures. A sharp increase was observed as soon as the pulse was injected. The expression was undetectable again between the end of the first minute and the first hour. A sharp increase was seen 7 hours after pulse injection. The second steady state value was higher than the value 15 seconds after pulse injection.

A slight decrease was observed after the injection of carbon pulse in the expression level of *TUP1* in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain. The level recovered in the next 15 seconds. The level was undetectable between the ends of the first minute until the

first hour. At the 9th hour a sharp increase were observed however it immediately rebounded. The expression level at the second steady state was 2.4 fold higher than the initial level.

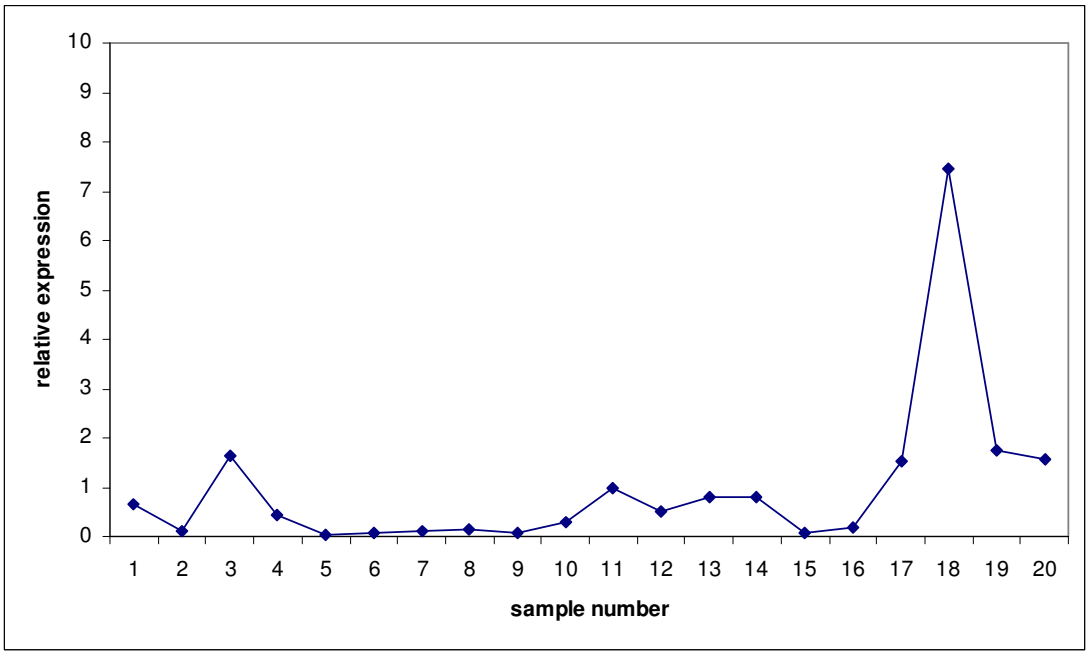


Figure 3.76. Expression profile of *TUP1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

3.2.20. Expression Profile of *HXK2* as a response to Glucose Pulse

By phosphorylation at C-6, the protein Hxk2p initiates the intracellular metabolism of glucose. It also plays an important role in glucose repression (Moreno and Herrero *et al*., 2002). One of the major functions of Hxk2 is to inhibit Snf1 protein kinase activity by blocking Mig1 phosphorylation at nuclear level. Glucose regulates the nuclear localization of Hxk2. This is also dependent on Mig1 since there is a direct correlation between the level of Mig1 in the cell and the amount of Hxk2 in the nucleus (Ahuatzi *et al*., 2004).

Samples were collected as stated previously during cultivation and expression profiles of *HXK2* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.77, 3.78, 3.79 and 3.80.

The expression level of *HXK2* in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* strain decreased after the glucose pulse injection, and increased immediately. A fluctuating pattern was observed throughout the cultivation until the second steady state value was reached, which when compared to the first steady state value, had not changed.

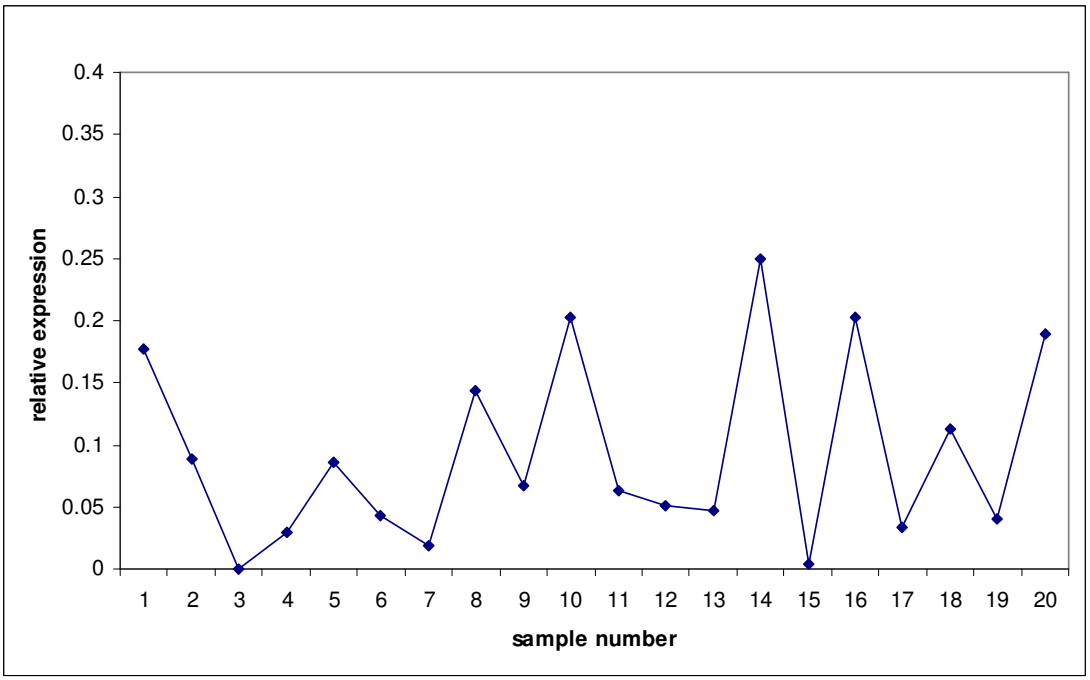


Figure 3.77. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

The expression level of *HXK2* showed a 2-fold increase right after the injection of carbon pulse in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ*) grown under carbon limitation in chemostat cultures. In the next 15 seconds an increase of 32 fold was seen. The level showed an oscillatory behaviour during the time between the ends of the first minute until the end of the second hour after the pulse. For the next 5 hours, the expression level was steady. The value increased in the 8^{th} hour after the pulse, which decreased in the following two hours. The second steady state value was 23 fold higher than the first steady state value.

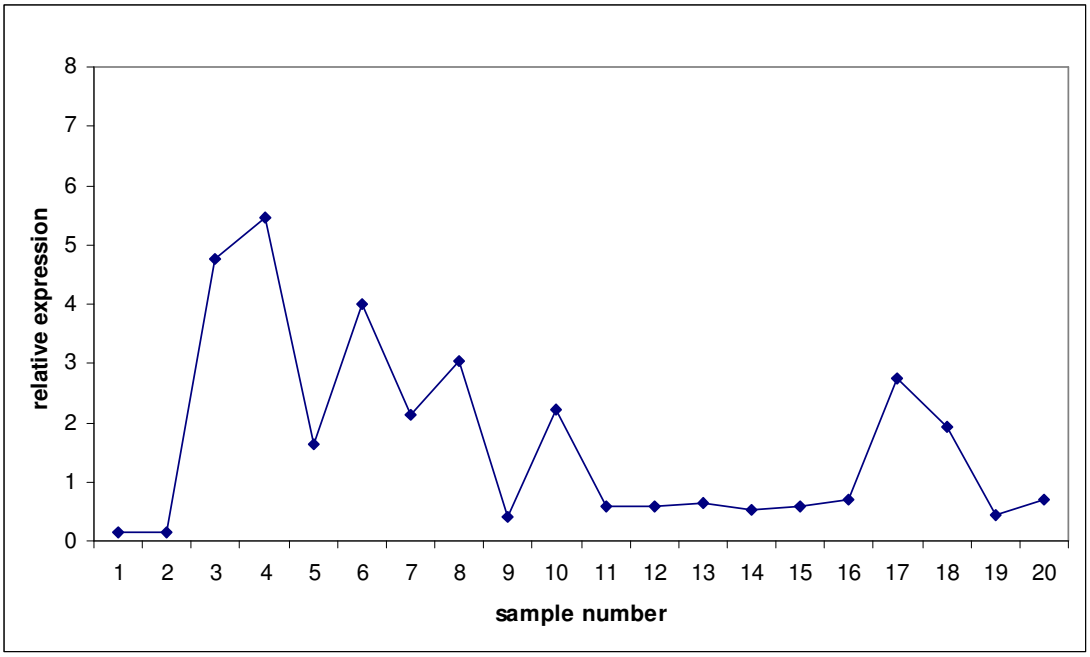


Figure 3.78. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

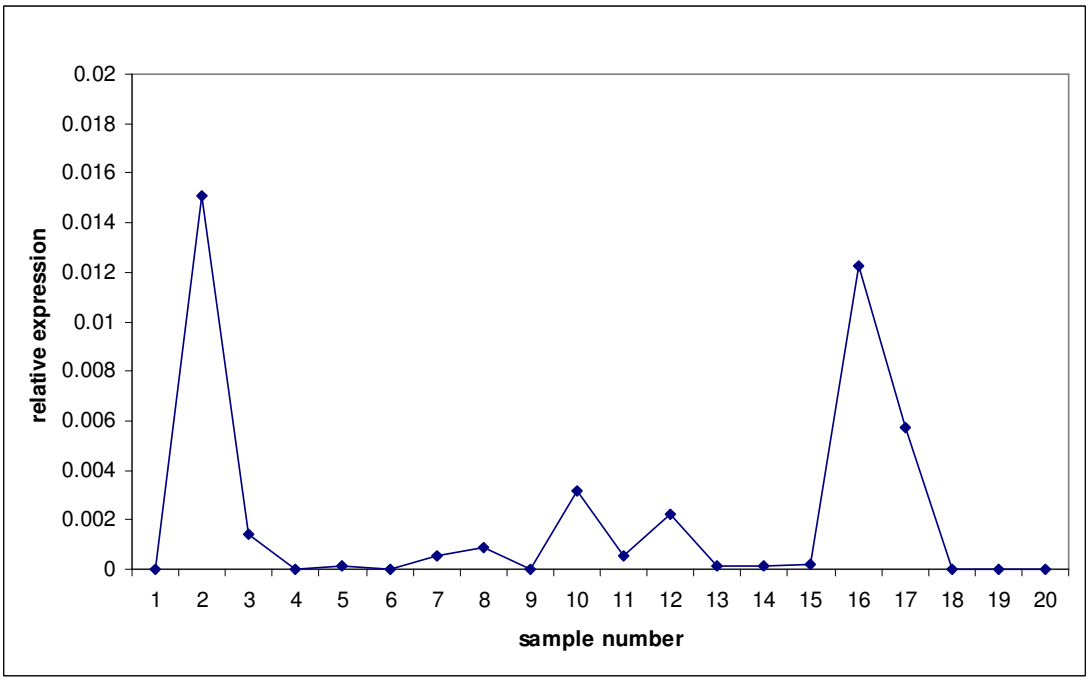


Figure 3.79. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

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. Higher values were seen in the 7th and 8th hours. The value then dropped to zero and remained so until the second steady state.

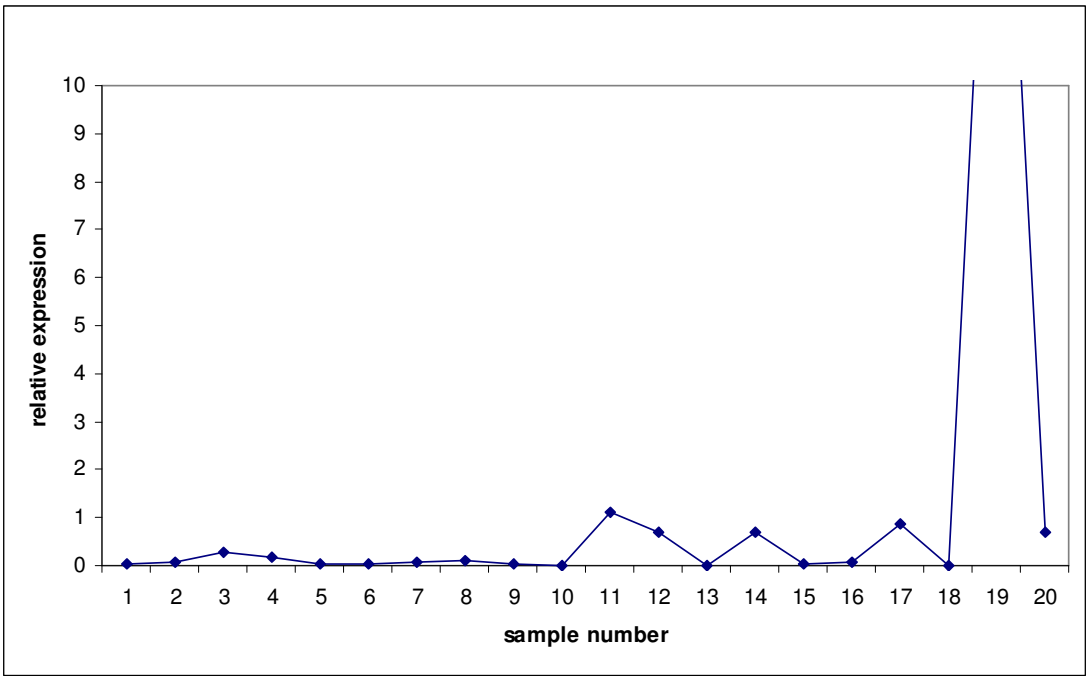


Figure 3.80. Expression profile of *HXK2* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

Also in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ*) strain expression level of *HXK2* was undetectable before the injection of carbon pulse and increased only slightly 30 seconds after the pulse injection. After the first hour fluctuations in the expression level of the gene was observed, with a very high peak 9 hours later than the pulse injection. The second steady state value was high when compared to the initial value and the value within the first hour of pulse injection.

3.2.21. Expression Profile of *HAP4* as a response to Glucose Pulse

The heme-activated protein complex Hap2/3/4/5 plays a major role in the transcription of genes involved in respiration in *S. cerevisiae*. Hap4p enhances the activity of the Hap2/3/5 proteins via its activation domains and the DNA binding capability of the Hap complex is also conferred by these. Hap4p possesses at least two distinct trans-activation domains, each of these domains have different levels of dependence on co-activation proteins. The expression of *HAP4* is regulated by the carbon source even through the other members of the complex are constitutively expressed, and it is up-regulated with glucose exhaustion. Activation of respiration is prevented at high glucose concentrations because glucose represses the expression of *HAP4* via the Mig1 protein (Raghevendran *et al*., 2006).

Samples were collected as stated previously during cultivation and expression profiles of *HAP4* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.81, 3.82 and 3.83.

A decrease in the expression level of *HAP4* was observed after the glucose pulse injection in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* grown under carbon limitation in chemostat cultures. However a sharp increase was observed with the next sample in the same minute. This increase rebounded immediately and the level decreased to a lower value than the initial value. An oscillating behavior was seen for the next 10 hours, until second steady state was reached. The expression level at the second steady state was lower 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 prior to carbon pulse injection. A small increase was observed with pulse injection. The expression level was very low until 7 hours after pulse injection which gradually decreased. In the 9^{th} hour, the level was undetectable once again. The value increased slightly as the second steady state was reached.

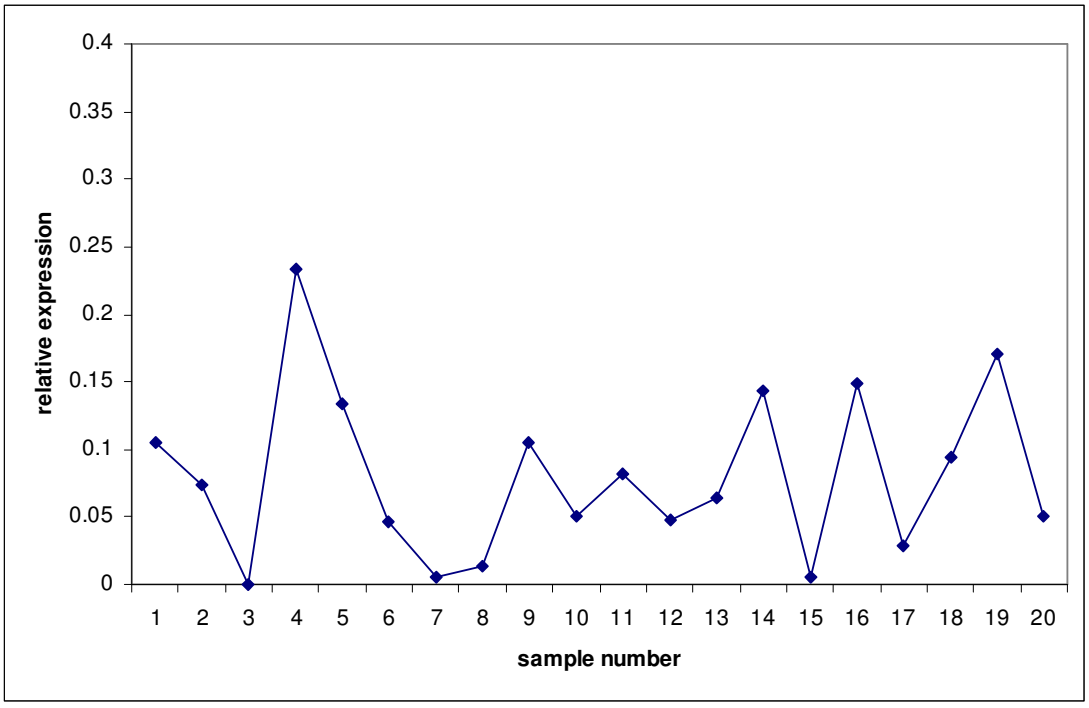


Figure 3.81. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

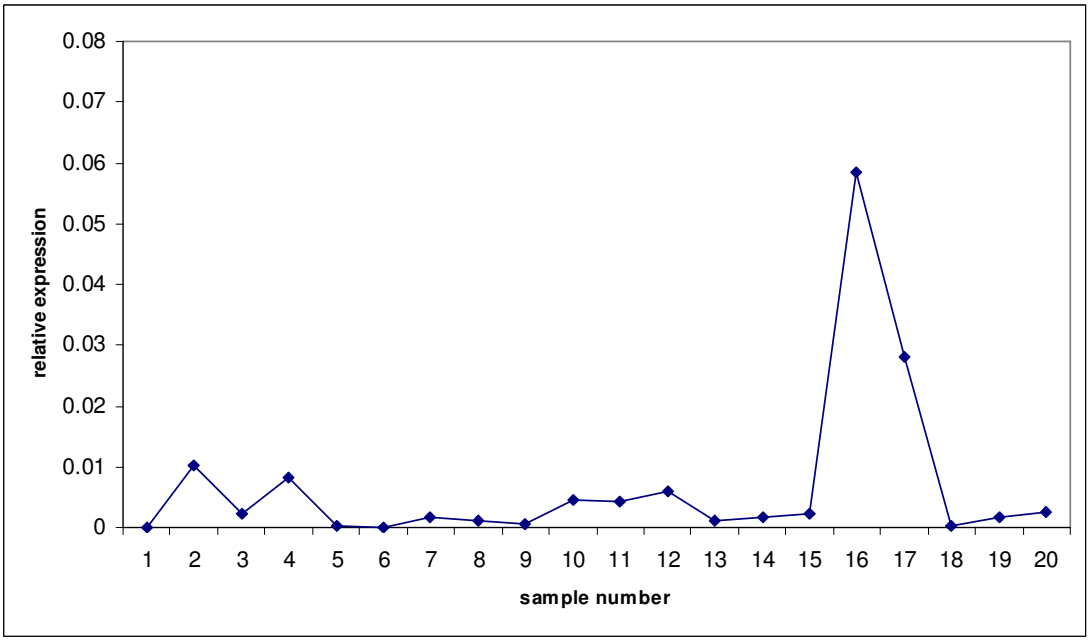


Figure 3.82. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

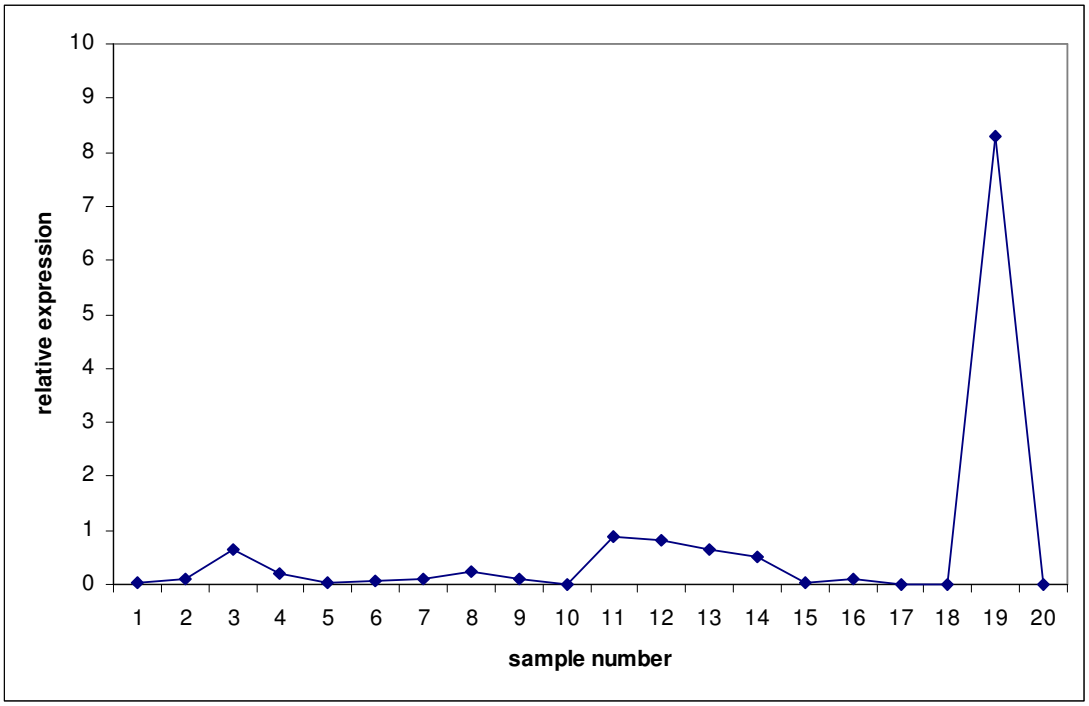


Figure 3.83. Expression profile of *HAP4* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

In the case for the heterozygous deletion mutant of *RIP1*, expression level of *HAP4* increased slightly as the pulse was injected and this was followed by a more considerable increase in the next 30 seconds. There was also an increase 2 hours after pulse injection which decreased slowly in the next 4 hours. At the second steady state, the relative expression of *HAP4* was not at detectable levels.

3.2.22. Expression Profile of *MBA1* as a response to Glucose Pulse

Mba1p is involved in the assembly of mitochondrial respiratory complex and it may act as a receptor for proteins that export from the mitochondrial matrix to the inner membrane (Herrmann *et al*., 2001).

Samples were collected as stated previously during cultivation and expression profiles of *MBA1* in *S. cerevisiae* (*hoΔ/ hoΔ, hap4Δ/ hap4Δ, rip1Δ/ rip1Δ, RIP1/rip1Δ)* are presented in figures 3.84, 3.85, 3.86 and 3.87.

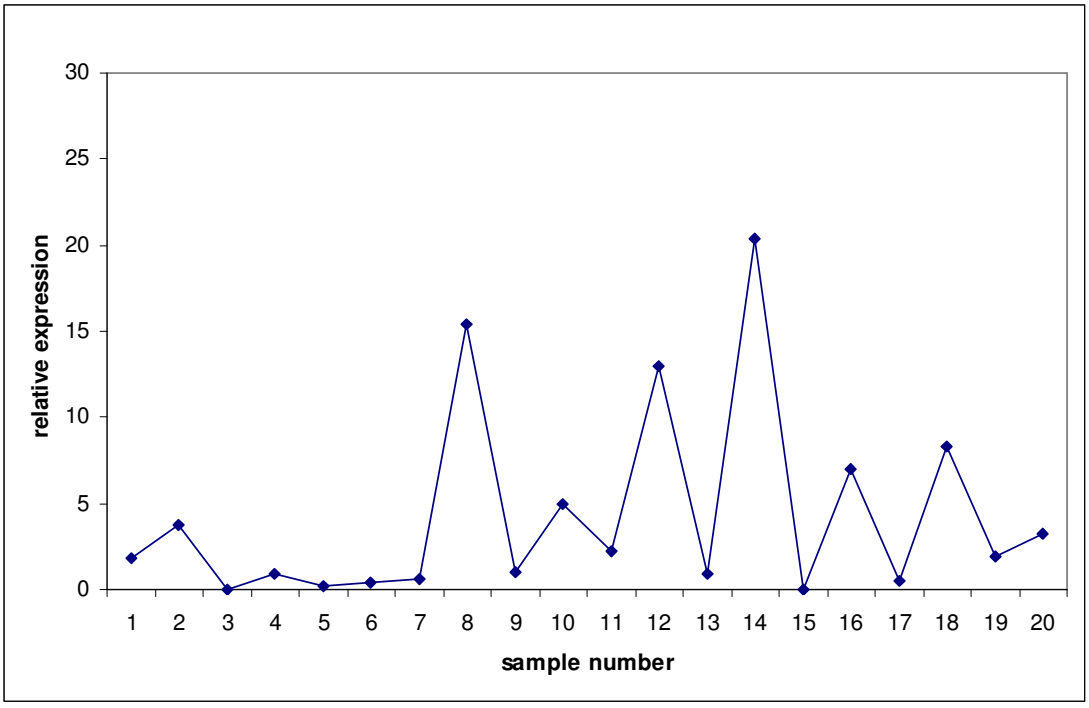


Figure 3.84. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* under carbon limitation in chemostat cultures with pulse injection

Investigation of the effect of carbon pulse on the *MBA1* expression level in *S. cerevisiae* BY4743 (*hoΔ /hoΔ)* revealed the presence of a decrease in the expression of this gene following the pulse injection. For the next 10 minutes the level was insignificant. At the 15th minute there was a 15 fold increase in the expression of *MBA1* which rebounded immediately in the next 5 minutes. Another sharp increase was observed in the 5th hour of sampling, a 20 fold increase which also rebounded in the next hour. The second steady state expression level was approximately equal to the initial steady state level.

No significant change was observed in the expression level of *MBA1* in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* strain after the carbon pulse injection. Between the 45th second and the 2nd hour after the pulse injection, a fluctuation was seen. The highest peak was seen in the 10th minute. After the second hour the expression level returned to the initial, steady state value and remained unchanged.

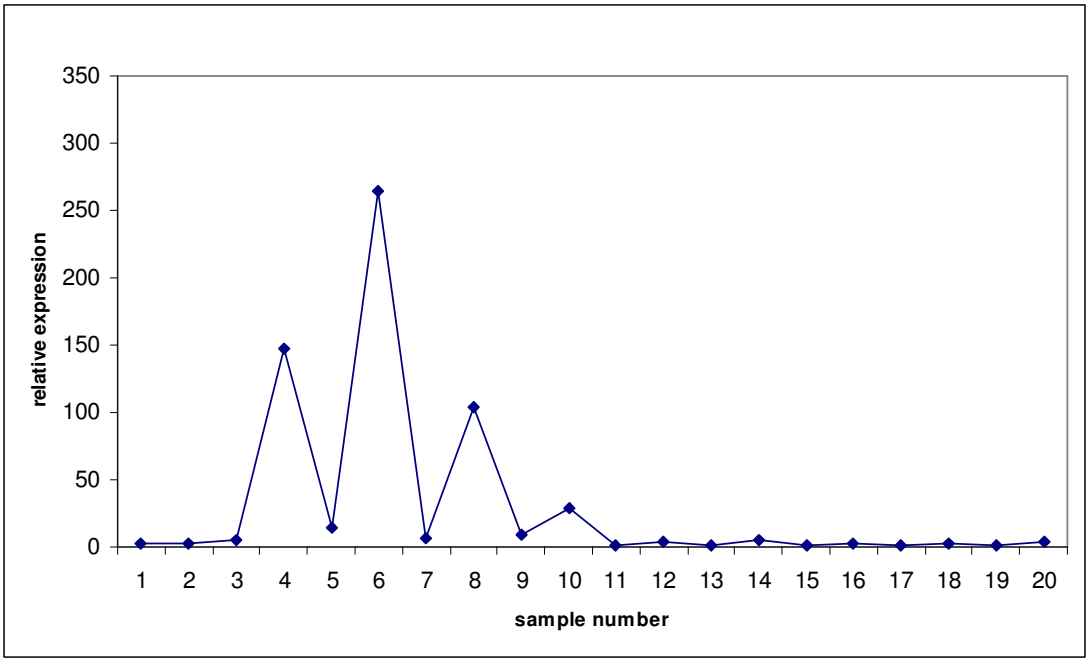


Figure 3.85. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*hap4Δ /hap4Δ)* under carbon limitation in chemostat cultures with pulse injection

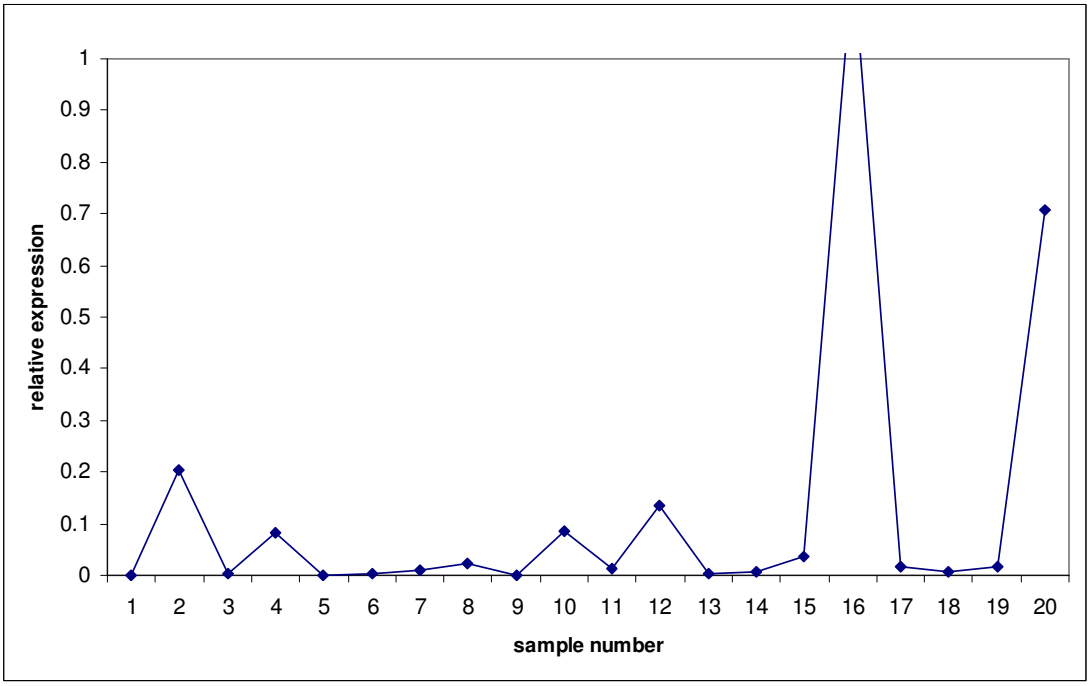


Figure 3.86. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

After the injection of carbon pulse, a slight increase was seen in the expression level of *MBA1* in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ)* which was undetectable in the first steady state. A very sharp increase was seen 7 hour after pulse injection which rebounded immediately. The second steady state level was also very high.

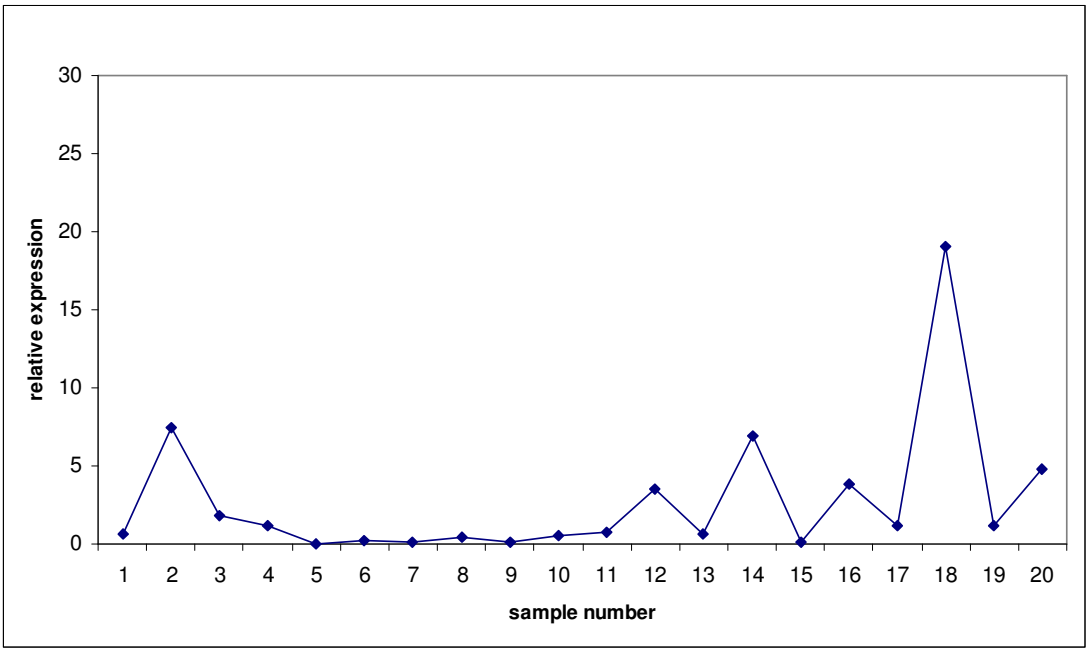


Figure 3.87. Expression profile of *MBA1* gene in *S. cerevisiae* BY4743 (*RIP1 /rip1Δ)* under carbon limitation in chemostat cultures with pulse injection

In *S cerevisiae* BY4743 (*RIP1 /rip1Δ)* strain, the effect of the carbon pulse on the expression level of *MBA1* was a sharp increase of 11 fold. The level however dropped to unnoticeable levels by the end of the first minute and remained steady until 3 hours after pulse injection. Fluctuation was observed after this with the highest value being seen in the 9^{th} hour. Second steady state value was 7 fold higher than its initial value.

4. DISCUSSION

The aim of this study was to investigate the effect of glucose limitation on glucose sensing and signaling mechanism in yeast and to understand whether the signature of post-transcriptional events can be observed in the transcription of genes encoding these proteins.

In order for this goal to be achieved, chemostat cultivations with pulse injections were performed in the present study. After the yeast cells spent three residence times at steady state, carbon pulse was injected in order to recover glucose limitation. Sampling started just before pulse injection, and continued for the next 10 hours. At the end of 10 hours more than 95 percent of the fermentor volume was replaces with fresh, i.e. glucose limited feed. Second steady state samples were collected once again after thress residence times spent at steady state.

Four sets of chemostat experiments with *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1Δ/rip1Δ and RIP1/rip1Δ)* under glucose limited conditions were carried out in this study in order to identify the variations in the expression levels of genes involved in glucose sensing, signal transduction and glucose repression pathway as a response to system level perturbations. The parent strain, *hoΔ/hoΔ* was selected as reference strain since researches showed this strain as being indifferent to respiratory metabolism (Baganz *et. al.,*1997; Dikicioglu, 2005).

4.1. Growth Characteristics of Deletion Strains

In this study the cells were grown in F1, glucose limited medium, in which the optimal growth conditions for *S. cerevisiae* were obtained throughout the cultivations. The temperature was kept at 30^{o}C and pH was controlled within the range of 5.5. The cells were left to grown batch overnight before switching to chemostat. For all four strains of *S. cerevisiae* BY4743, cell growth continued after switching to chemostat, before reaching steady state. Carbon pulse was injected after cells spent three residence times at steady state.

When comparing growth profiles of the strains at first steady state, it was observed that the *S. cerevisiae* BY4743 strain *rip1Δ/rip1Δ* strain reached to the highest densities. It can also be seen that *hap4Δ/hap4Δ* strain had the lowest cell density at the first steady state. Cell densities of *hoΔ/hoΔ* and *RIP1Δ/rip1Δ* were similar to each other.

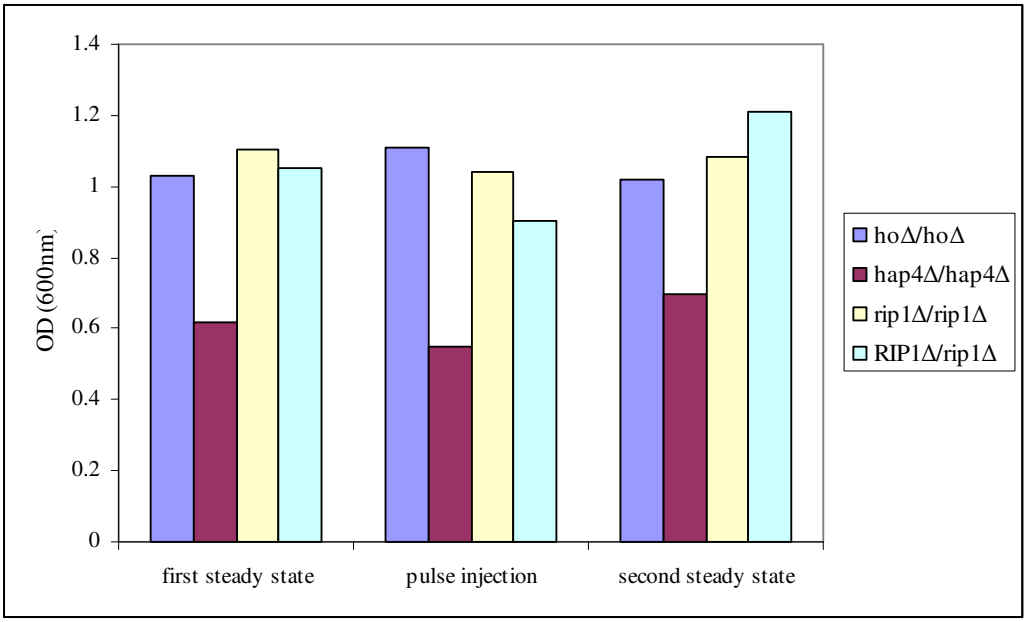


Figure 4.1. Comparison of growth characteristics of *S. cerevisiae* BY4743 strain in glucose limited conditions

As can be seen in Figure 4.1, with the injection of glucose pulse, the first reaction of all strains except *hoΔ/hoΔ* strain was with a slight decrease. However, in all cases cell densities recover and cell growth continues as second steady states are reached. For the *hoΔ/hoΔ* strain, 6 hours after pulse injection, cell density had reached a value higher than the first steady state.

For all four strains of *S. cerevisiae* glucose, which was nearly completely consumed at the first steady state, increased with the injection of carbon pulse. In *hoΔ/hoΔ* strain, a sharp decrease was observed in glucose concentration 5 hours later than the pulse whereas in *hap4Δ/hap4Δ* strain this sharp decrease was 2 hours later than the pulse. During the 10 hour sampling period, the glucose concentration gradually decreased in *rip1Δ/rip1Δ* strain

On the other hand in *RIP1/rip1Δ* strain, most of the glucose in the medium was consumed within the first 4 hours after the pulse injection. In all strains at the second steady state the glucose concentrations were very small.

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

The highest glycerol production at the first steady state was observed in *RIP1/rip1Δ* strain whereas the double deletion of *RIP1* resulted in the decrease of glycerol production. In *hoΔ/hoΔ* and *hap4Δ/hap4Δ* strains, glycerol concentration decreased with the injection of the pulse. Since it is reported that elimination of glycerol production increases ethanol yield (Nissen *et al*., 2000) the decrease in the glycerol concentration in these strains could be considered in conjunction with the increase in the ethanol production. Moreover in *S. cerevisiae* BY4743 *hap4Δ/hap4Δ* strain, increase was observed in biomass after the injection of carbon pulse. Since the *hap4Δ/hap4Δ* strain is completely respiratory deficient, the increase in the glycerol production was plausible. 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).

On the other hand, the glycerol production increased slightly in *rip1Δ/rip1Δ* mutant. The different respond to carbon pulse indicate a possible effect of *RIP1* gene on the glycerol production.

As glucose concentration decreases, an increase in the ethanol production is observed as an expected results. In the *hoΔ/hoΔ* strain a deep decrease between the 4^{th} and 5^{th} hours of sampling period can be seen in the glucose concentration, and this is when ethanol production is highest. Maximum ethanol concentration was obtained 5 or 6 hours later than the pulse injection in all mutants. *rip1Δ/rip1Δ* strain produced the maximum ethanol., this was followed by *hap4Δ/hap4Δ* strain. Since these strains are respiration deficient, it is plausible that they produce more ethanol than the strains which show respiro-fermentative behavior.

4.2. Expression Levels of Glucose Sensors

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 (Özcan *et al.,* 1996, 1998). Transcription of *SNF3* is maximal when glucose levels are low, it was indicated that *SNF3* expression is repressed about fivefold by high levels of glucose (Özcan and Johnston, 1999). On the other hand *RGT2* 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).

As can be seen in Figure 4.2, in *S. cerevisiae* BY4743 *hoΔ/hoΔ* strain, *SNF3* was expressed lower right after pulse injection, where glucose level is highest. This result agrees with previous results indicated by (Özcan and Johnston, 1999). The expression of this gene was again higher in the second steady state, where the glucose level was low. The *RGT2* gene was downregulated after pulse injection, but the expression levels during the 10 hour sampling period, where the glucose levels are relatively high, are higher than the steady state expression levels. The expression levels of these genes showed an oscillatory behavior after the pulse injection which may be explained with the oscillatory dynamics observed in the majority of metabolites in yeast cells grown continuously at high cell density (Murray *et al.,* 2006).

Expression levels of these genes in the *hap4Δ/hap4Δ* and *RIP1/rip1Δ* strains also agree with the previous results indicated, as seen in Figure 4.3 and Figure 4.4. It is observed that *SNF3* was downregulated after the glucose pulse was injected and when the glucose levels were lower, *SNF3* expression was higher. *RGT2* gene was also downregulated after the pulse injection.

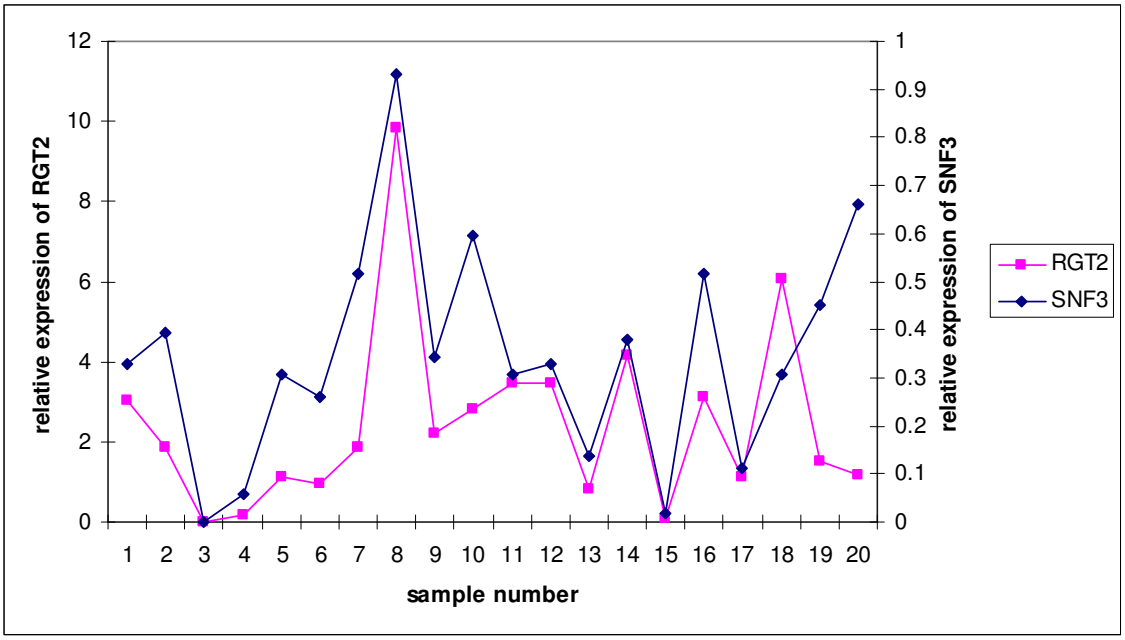


Figure 4.2. Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

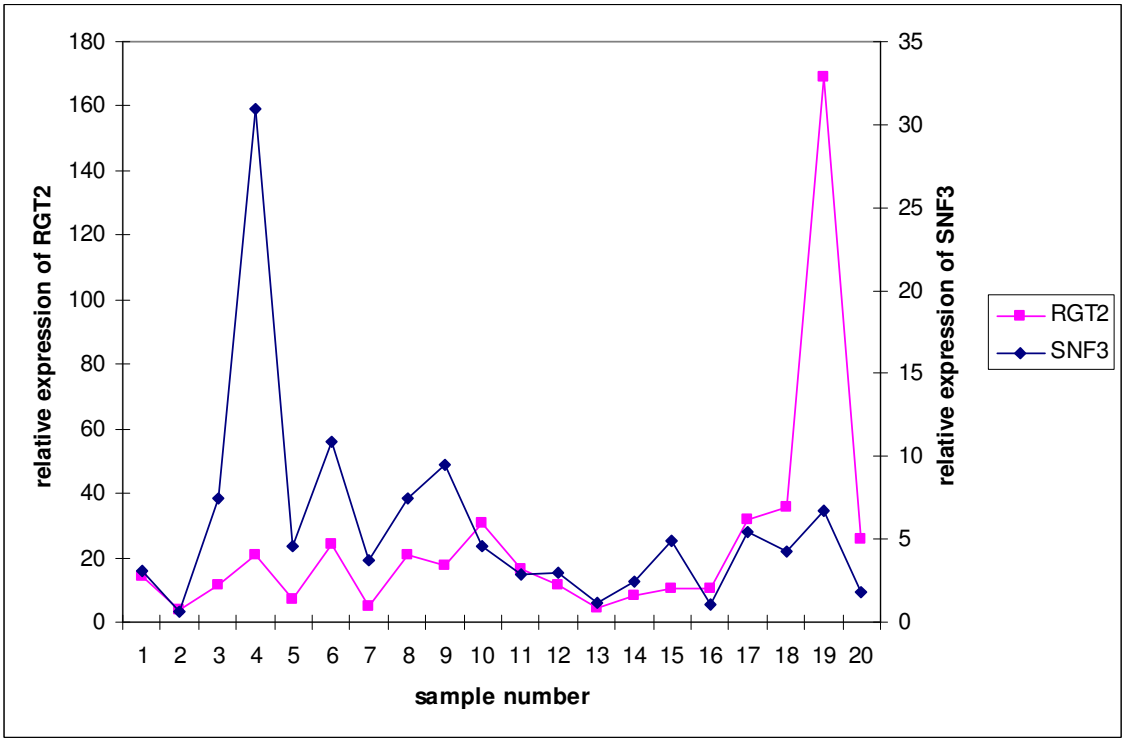


Figure 4.3. Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

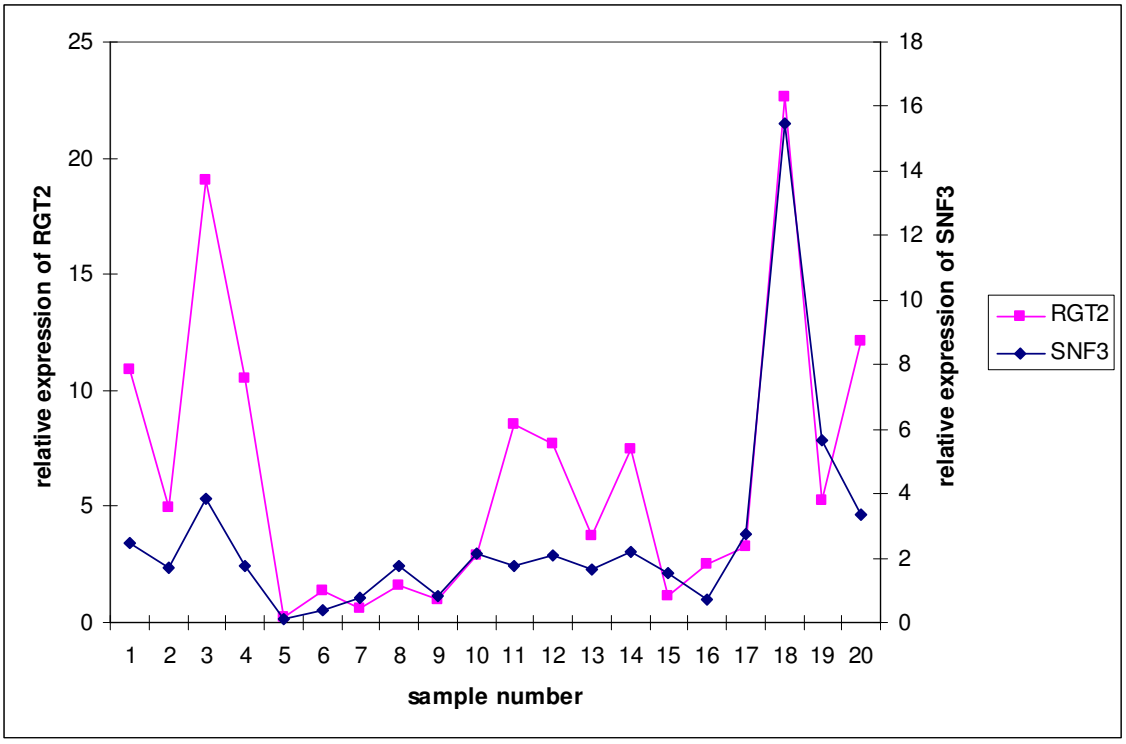


Figure 4.4. Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

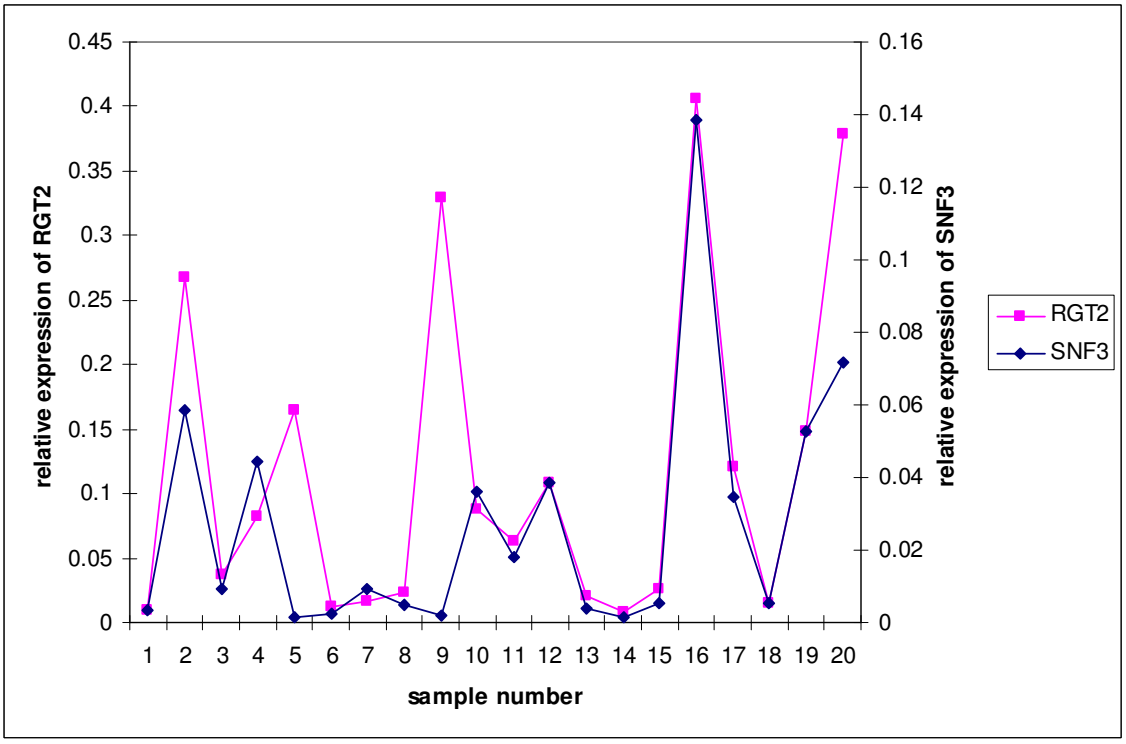


Figure 4.5. Expression level of *RGT2* and *SNF3* in *S. cerevisiae* BY4743 (*rip1Δ /rip1Δ*)

SNF3 and *RGT2* were both upregulated right after pulse injection in the homozygous deletion mutant *rip1Δ/rip1Δ* but the response here was a faster response when compared to the other strains. The expressions of these genes were higher in the second steady state where glucose level was very low, also agreeing with previous results.

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

It is known that the transcription of the *MTH1* gene is repressed at high concentrations of glucose (Lakshmanan *et al.*,2003). 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 (Moriya and Johnston, 2004).

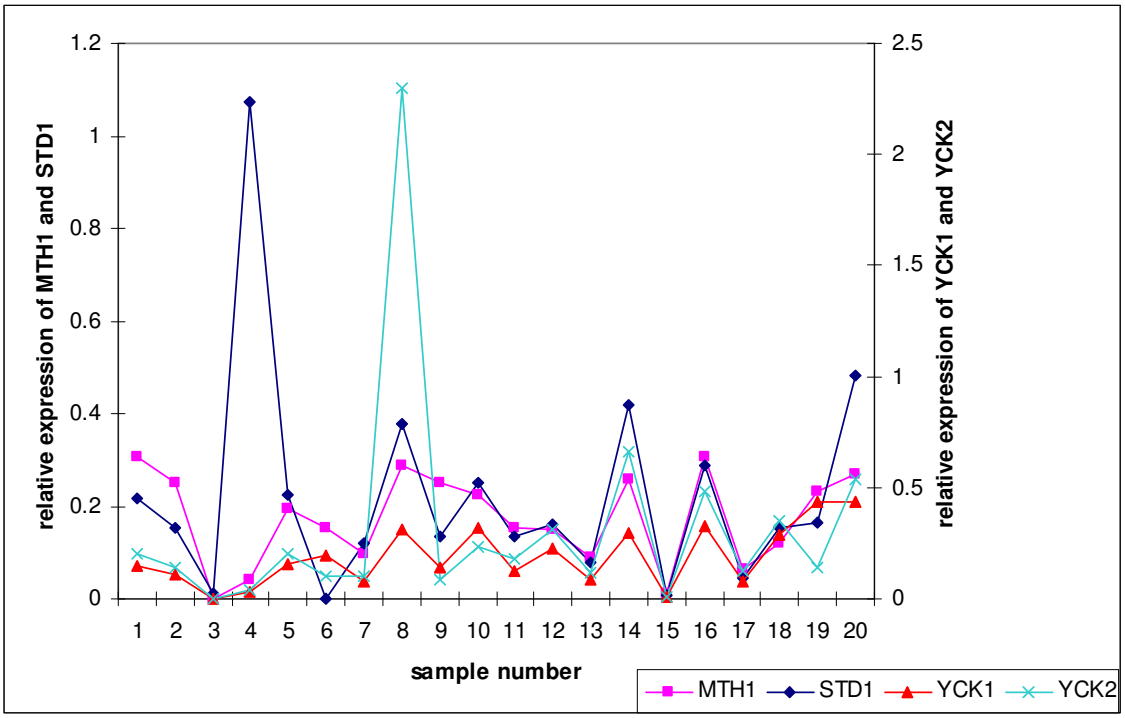


Figure 4.6. Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

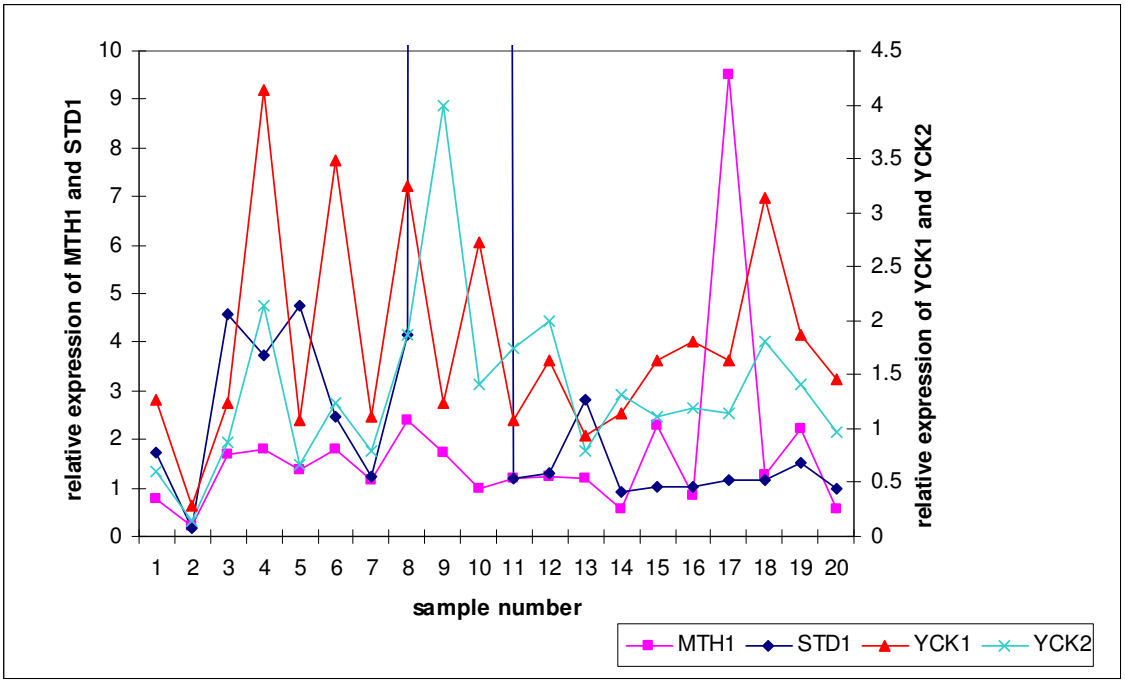


Figure 4.7. Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

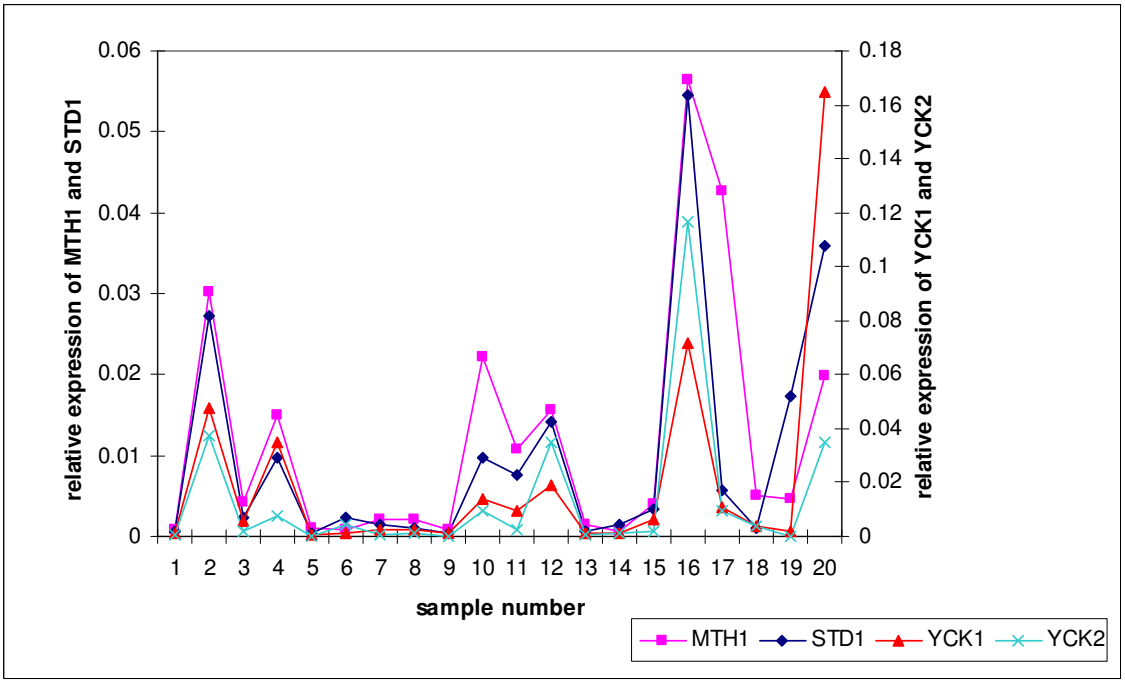


Figure 4.8. Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

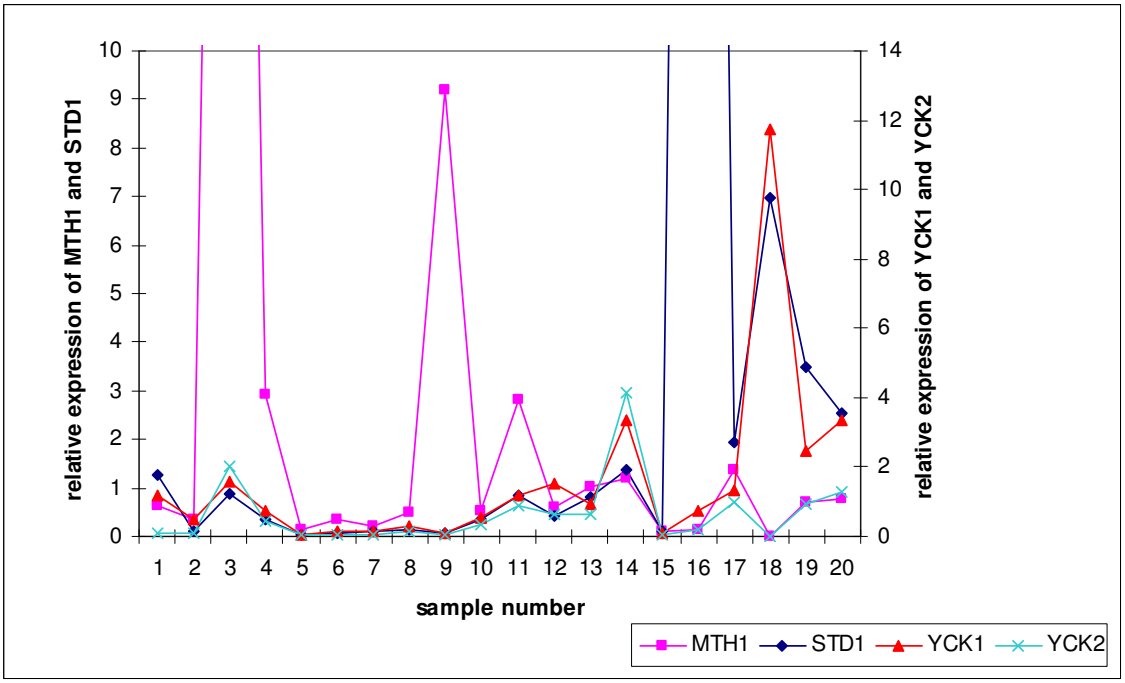


Figure 4.9. Expression level of *MTH1, STD1, YCK1* and *YCK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

As can be seen in figures 4.6, 4.7 and 4.9 the expression levels of *MTH1, STD1, YCK1* and *YCK2* in *hoΔ/hoΔ, hap4Δ/hap4Δ* and *RIP1/rip1Δ* strains become downregulated with the pulse injection. The expression level of *MTH1* is higher in steady state values where the glucose concentration is low. This agrees with previous results where it is observed that the transcription of *MTH1* gene is repressed at high concentrations of glucose. It is also previously stated that *STD1* expression is induced by glucose which would be expected to counteract Std1 degradation (Moriya and Johnson, 2004). In *hoΔ/hoΔ* strain in this study *STD1* expression increases in the first minute after pulse injection, which agrees with previous studies. In *hoΔ/hoΔ* strain, the expression of *YCK1* does not show significant changes, whereas the expression of *YCK2* show an important increase 15 minutes after pulse injection. The expression levels of these two genes in the *hoΔ/hoΔ, hap4Δ/hap4Δ* and *rip1Δ/rip1Δ* strains are higher than first steady state expression level 1 minute after the injection of glucose pulse. The signal generated by the glucose sensors in response to glucose activates casein kinase I (Yck1), encoded by *YCK1* and *YCK2* (Johnston and Kim, 2005), therefore this is an expected result.

In the *hap4Δ/hap4Δ* strain, these four genes show a very similar expression profile right after pulse injection. In the later samples, *MTH1* expression does not change significantly, where as the other three genes continue to show similar expression. However, in the homozygous and heterozygous deletion mutants of *RIP1* these four genes have similar profiles throughout the cultivation.

In the homozygous deletion mutant *rip1Δ /rip1Δ* the initial response to glucose pulse injection for all four genes is to be upregulated. Also the second steady state expressions are high. It can be assumed that *RIP1* gene effects the expression of *MTH1, STD1, YCK1* and *YCK2*, but since the same effect is not observed with the *HAP4* deletion mutant, respiratory deficiency is not the reason for the upregulation but the mutation of the complex containing the *RIP1* gene.

4.4. Expression profiles of *GRR1* and *SKP1* under Glucose Limited Conditions

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

In the previously reported data it was 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 Johnston, 1997; Santangelo, 2006). As can be seen in figures 4.10, 4.11 and 4.12 *GRR1* and *SKP1* genes have similar transcriptional trends as a response to pulse injection in all strains except in the heterozygous deletion mutant of *RIP1*, so it can be said that partial respiratory deficiency effects the similarity of these two genes.

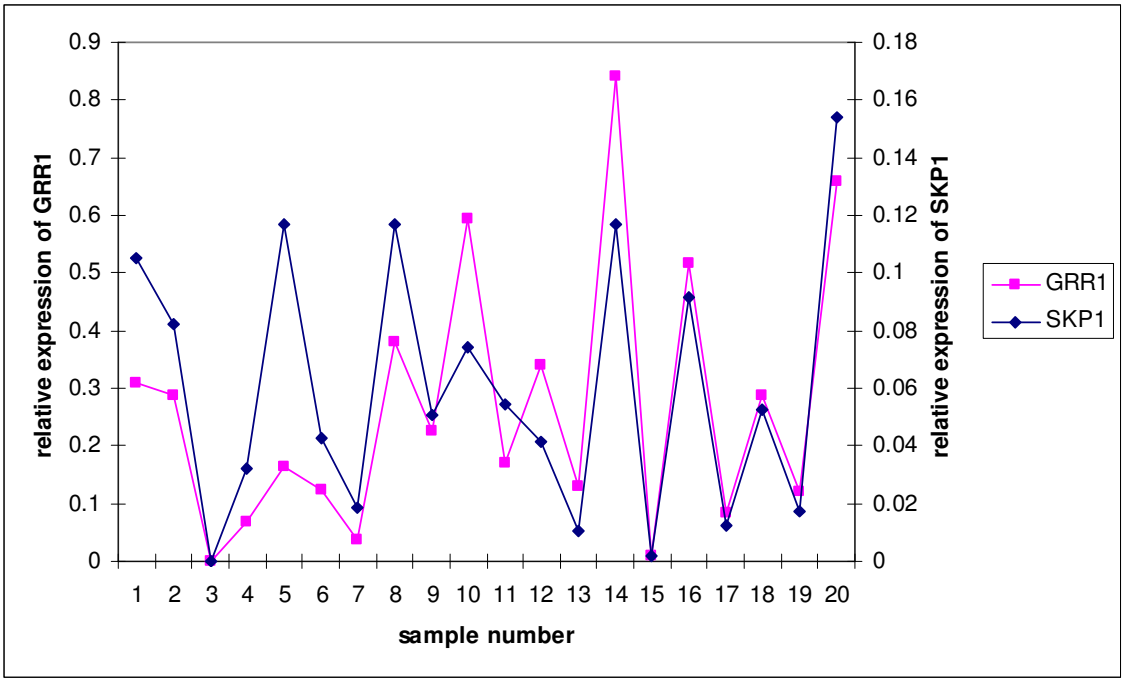


Figure 4.10. Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

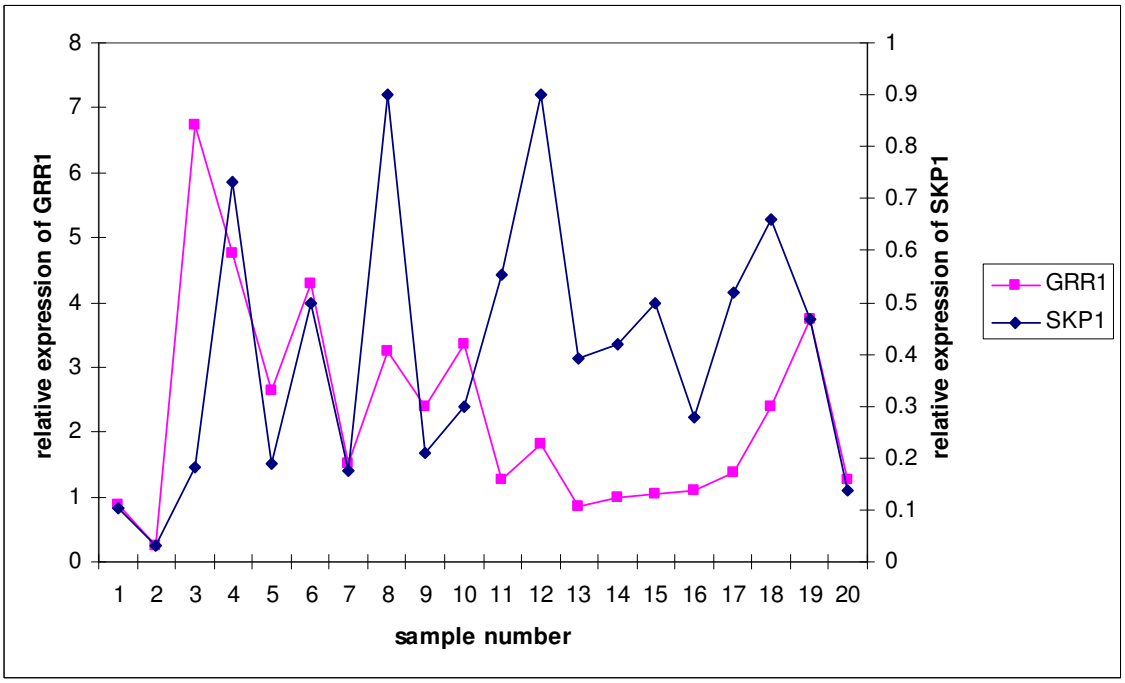


Figure 4.11. Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

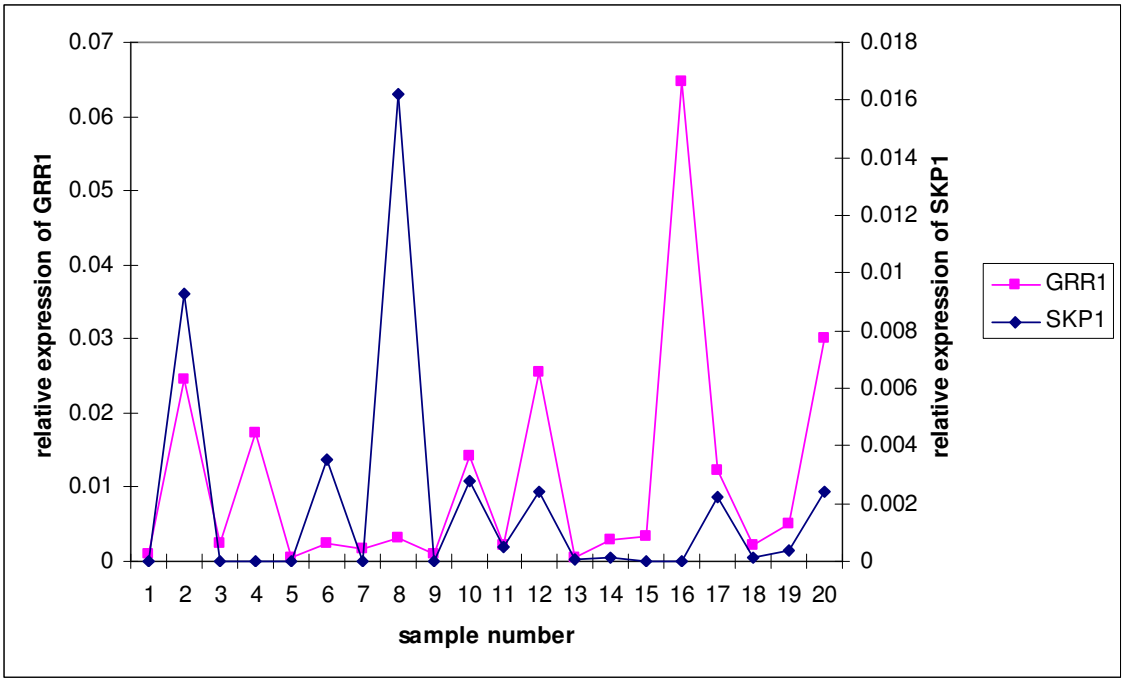


Figure 4.12. Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

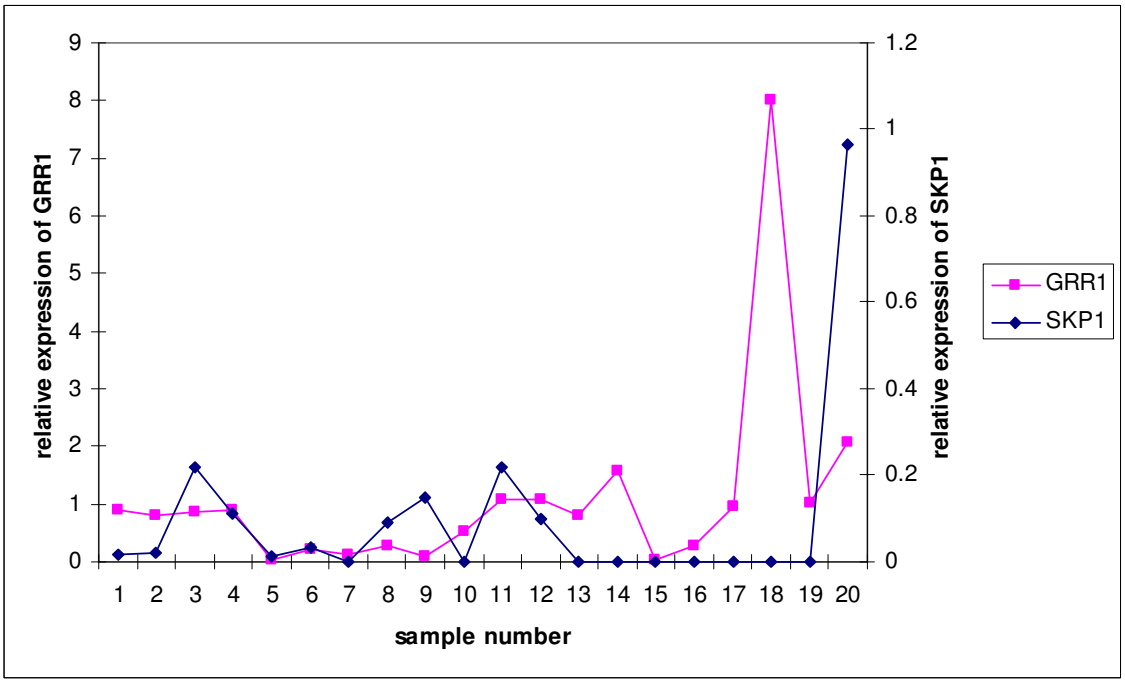


Figure 4.13. Expression level of *GRR1* and *SKP1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

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

Rgt1 is a transcriptional factor which acts as a repressor when glucose is absent (Özcan *et al*.,1996; Mosley *et al*., 2003). It represses transcription of *HXT1-4* and *HXK2* in the absence of glucose. On the other hand, when glucose levels are high (>2 percent), Rgt1p activates transcription of *HXT1* (Özcan *et al*., 1996; Polish *et al*., 2004). It was also stated that, since transcription of *RGT1* is not altered in response to glucose, the activity of Rgt1p is likely to be regulated posttranscriptionally (Özcan *et al*., 1996).

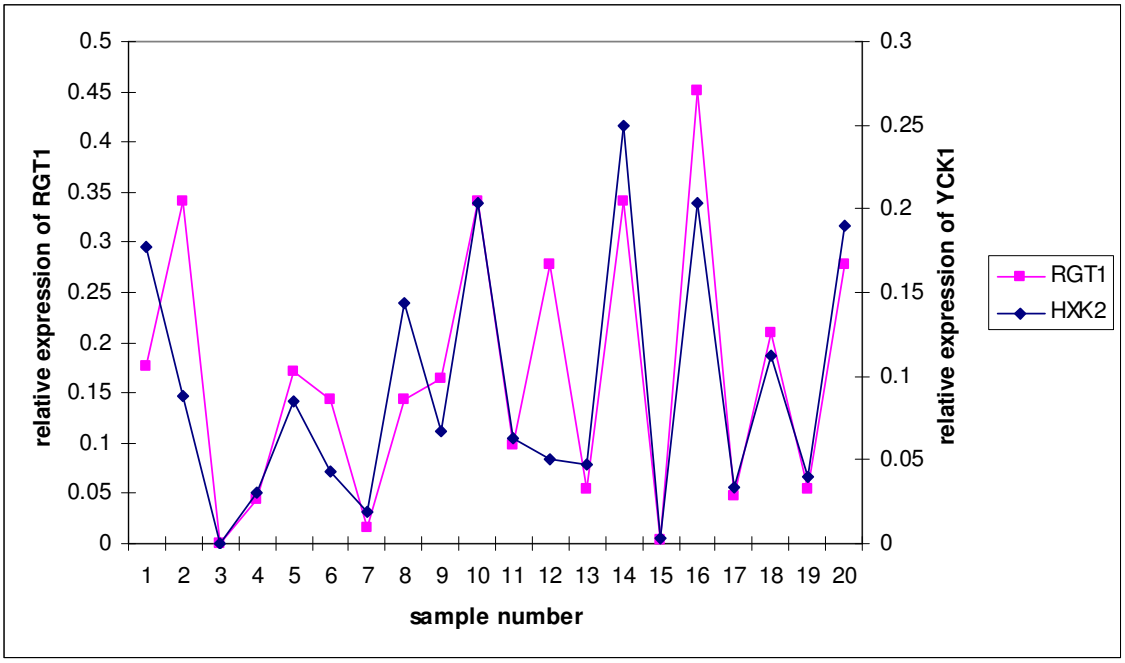


Figure 4.14. Expression level of *RGT1* and *HXK2* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

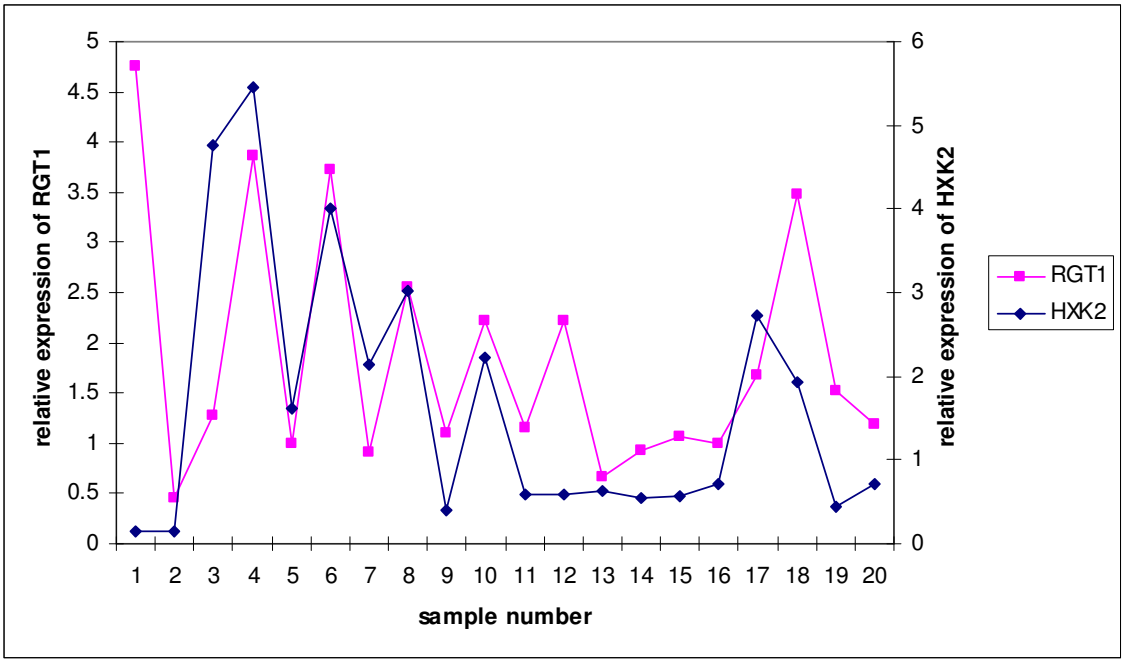


Figure 4.15. Expression level of *RGT1* and *HXK2* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

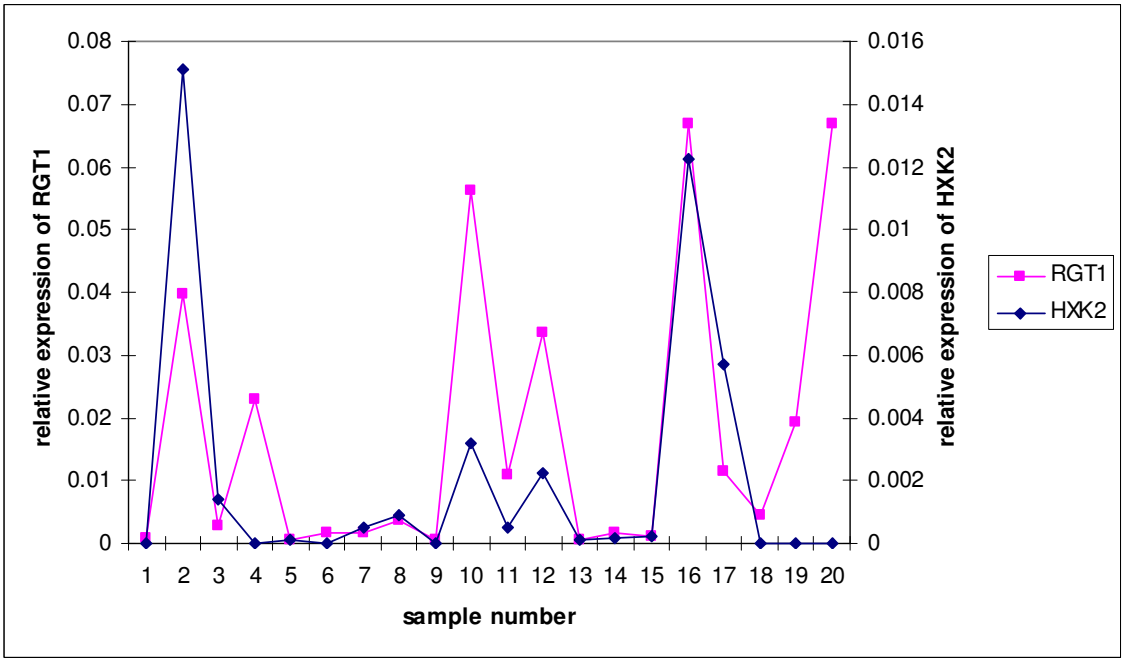


Figure 4.16. Expression level of *RGT1* and *HXK2* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

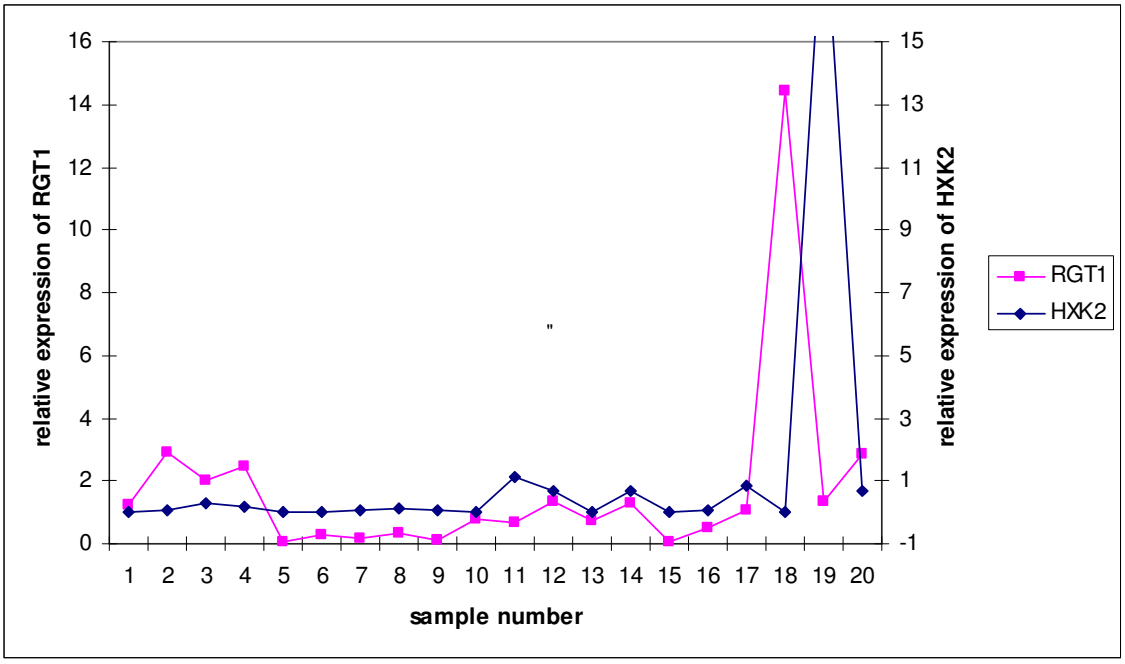


Figure 4.17. Expression level of *RGT1* and *HXK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

Even though previous data state that *RGT1* gene is not altered by glucose, when glucose pulse was injected, this gene was upregulated in *S. cerevisiae* BY4743 *hoΔ/hoΔ, rip1Δ/rip1Δ* and *RIP1/rip1Δ* strains and downregulated in the *hap4Δ/hap4Δ* strain. It can be also observed that *HXK2* gene downregulated with the pulse injection in the *hoΔ/hoΔ* strain. However, as glucose concentration decreases these two genes are similarly transcripted.

The expression profiles of *RGT1* and *YCK1* were similar in all four strains as can be observed in figures 4.18, 4.19, 4.20 and 4.21. Only in the *RIP1/rip1Δ* strain, a more slight response to pulse injection is observed when compared to the other three strains. 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) and the degradation of Mth1p is accompanied with casein kinase 1, encoded by *YCK1* and *YCK2* (Johnston and Kim, 2005), this results is in good agreement.

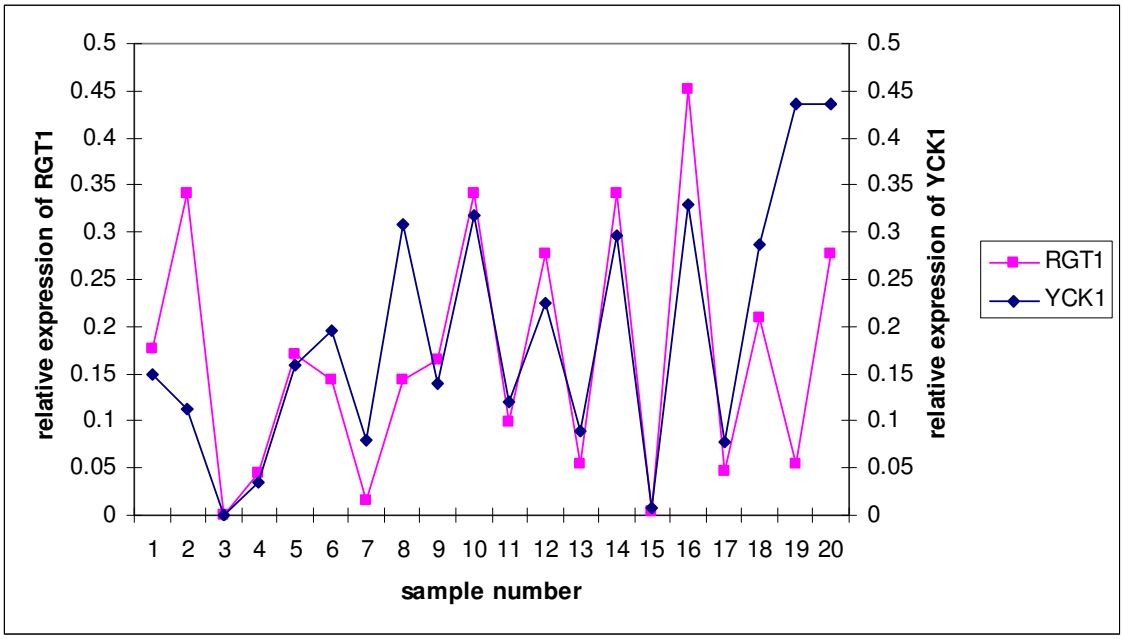


Figure 4.18. Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

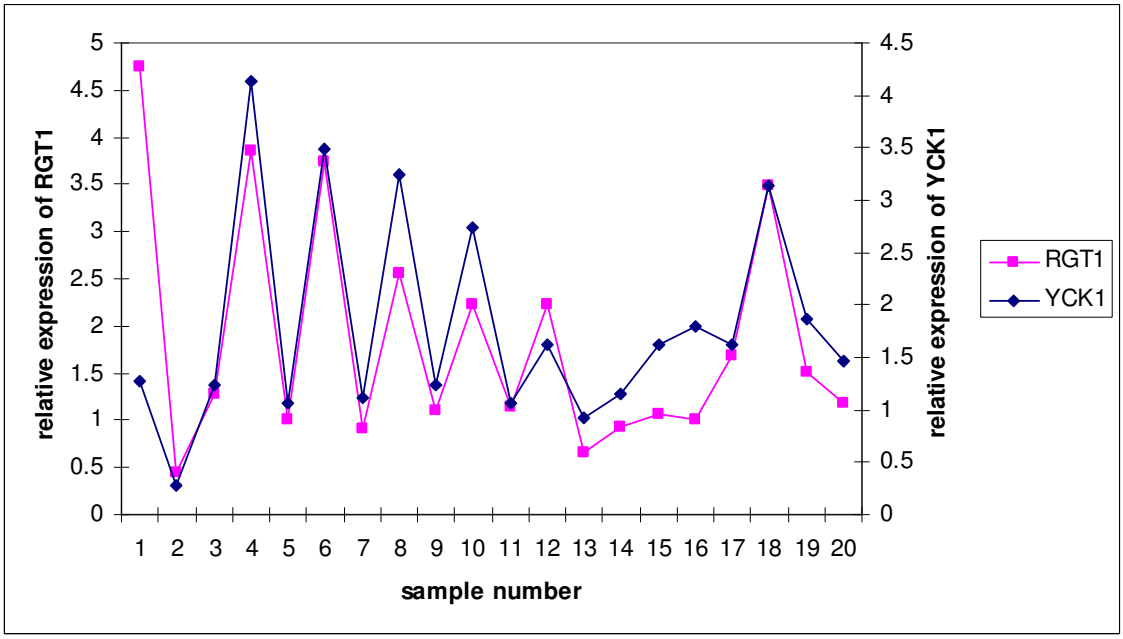


Figure 4.19. Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

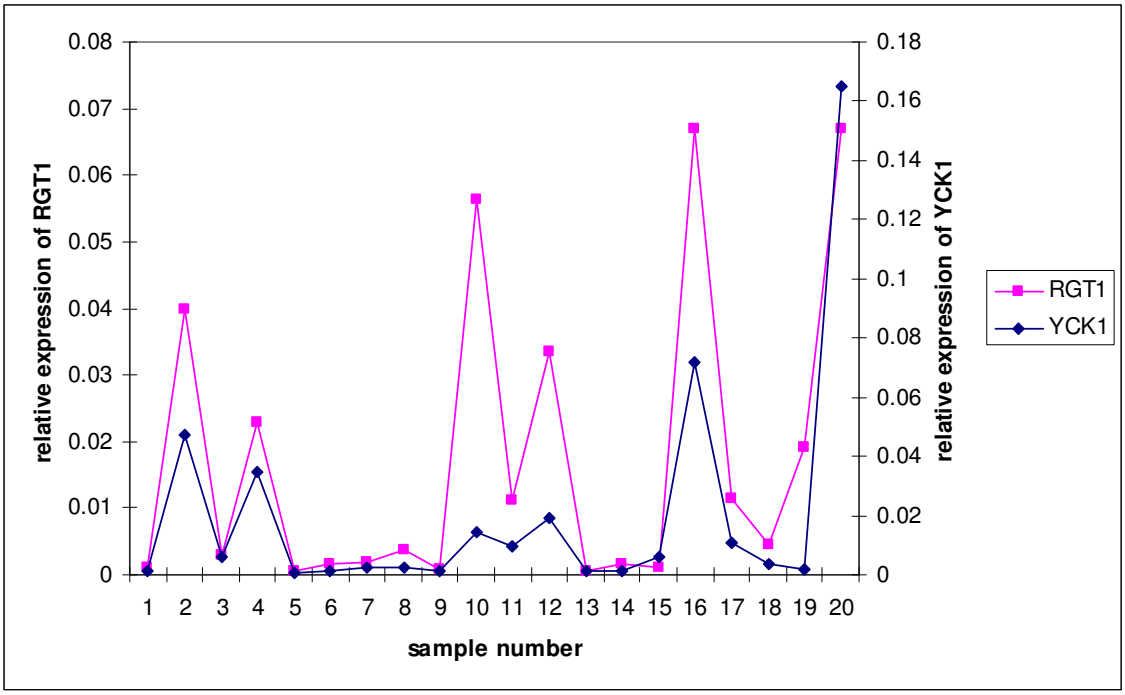


Figure 4.20. Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

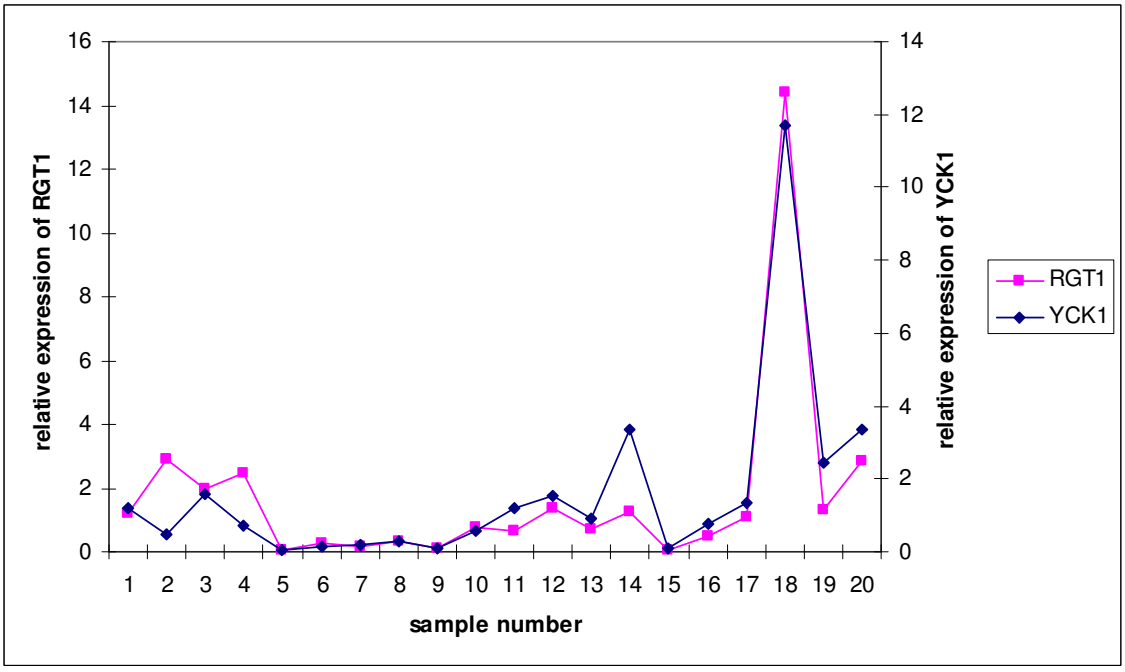


Figure 4.21. Expression level of *RGT1* and *YCK1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

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

REG1, *GLC7*, *MIG1*, *SNF1* and *SNF4* are the genes that are involved in glucose repression pathway in *S. cerevisiae*. The Snf1 protein kinase regulates the subcellular relocalization of Mig1 (DeVit & Johnson, 1999). In high levels of glucose, Mig1 is de-phosphorylated by the Glc7-Reg1 protein phosphatase complex (Alms *et al*., 1999) and is located in the nucleus where it can repress the transcription. As the glucose is removed, Mig1 is phosphorylated by Snf1 protein kinase complex and translocated into the cytoplasm (DeVit *et al*., 1997, Ahuatzi *et al*., 2004). Snf4 is required for the Snf1 kinase activity, and thus must be considered as a positively acting regulatory subunit of Snf1 (Schüller, 2003).

In the *S. cerevisiae* BY4743 *hoΔ/hoΔ* strain, *GLC7* and *REG1* shown similar transcription levels (Figure 4.22). This is an expected result since the proteins encoded by these two genes form a protein phosphatase complex. Since this complex activates Mig1p at transcriptional level (Alms *et al*.,1999), the expression profiles of *MIG1* gene showing similarity to the complex is also expected. This results suggests that *MIG1* is transcriptionally regulated by this complex.

In the *hap4Δ/hap4Δ, rip1Δ/rip1Δ* and *RIP1/rip1Δ* strains the similar transcription levels can also be observed. The three genes are observed to be upregulated with pulse injection which agrees with previously states results.

Expression profiles of *SNF1* and *SNF4* were observed to be similar as glucose concentration decreased (Figure 4.26, 4.27, 4.28, 4.29). Since *SNF1* is activated in low concentrations of glucose and Snf4 is required for the activity of Snf1 kinase, this result is expected.

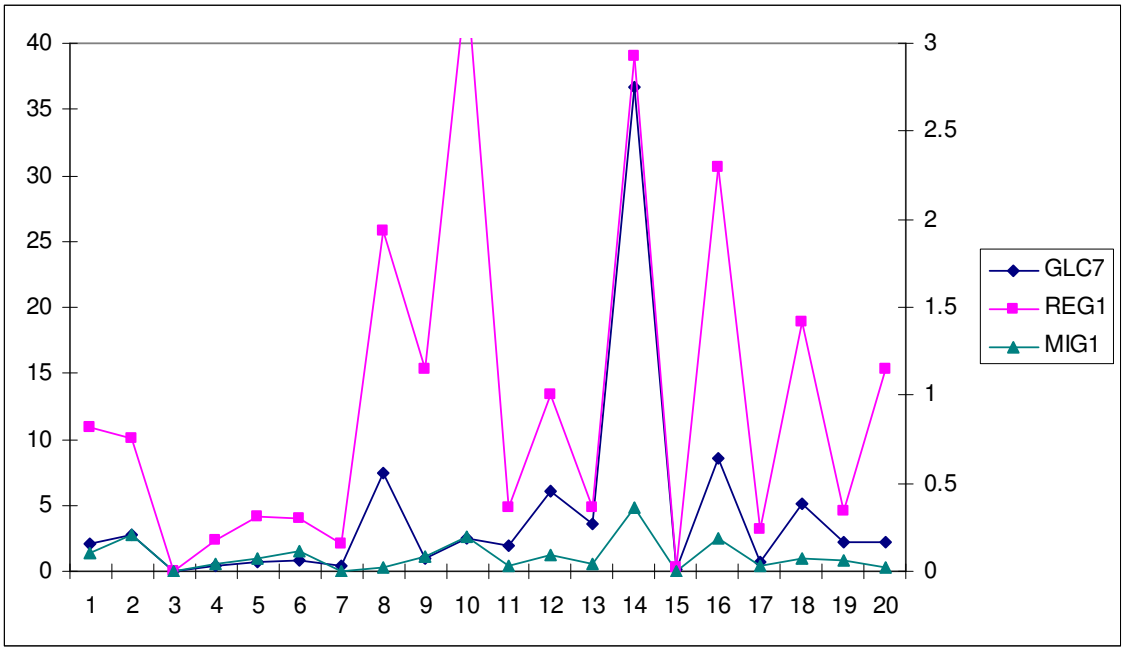


Figure 4.22. Expression level of *GLC7*, *REG1* and *MIG1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

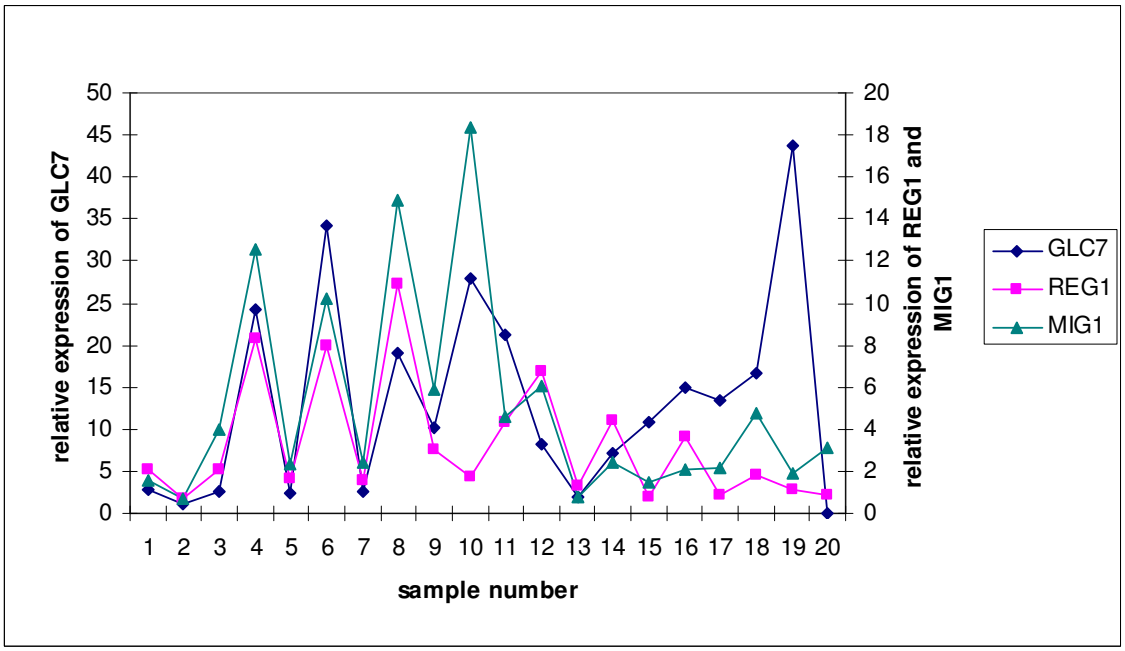


Figure 4.23. Expression level of *GLC7*, *REG1* and *MIG1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

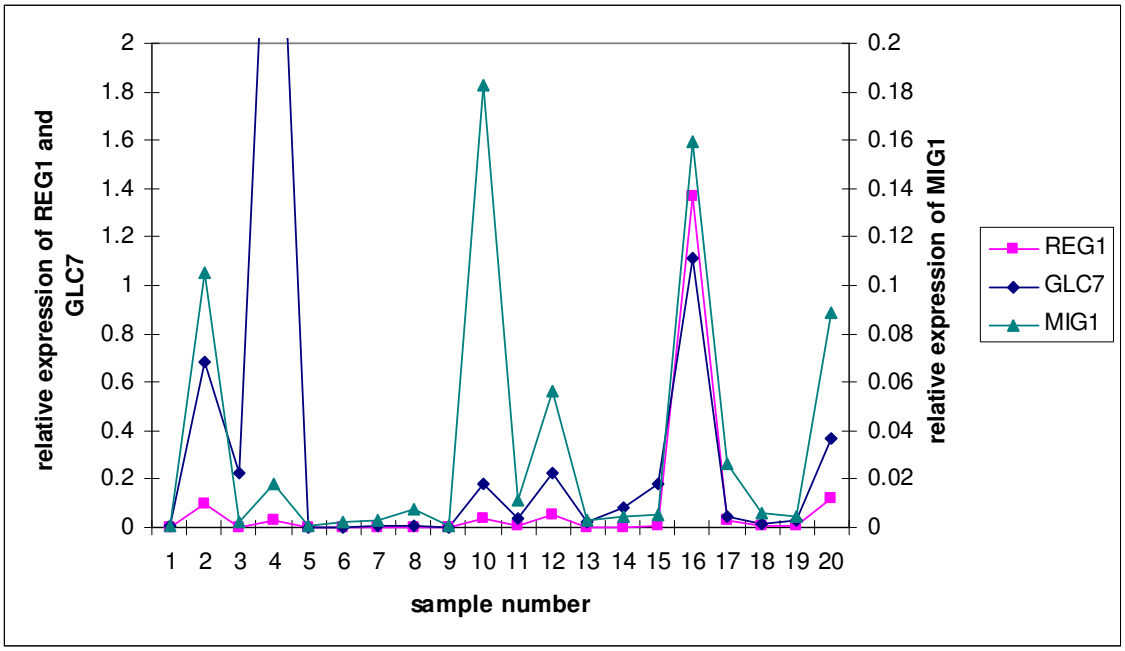


Figure 4.24. Expression level of *GLC7*, *REG1* and *MIG1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

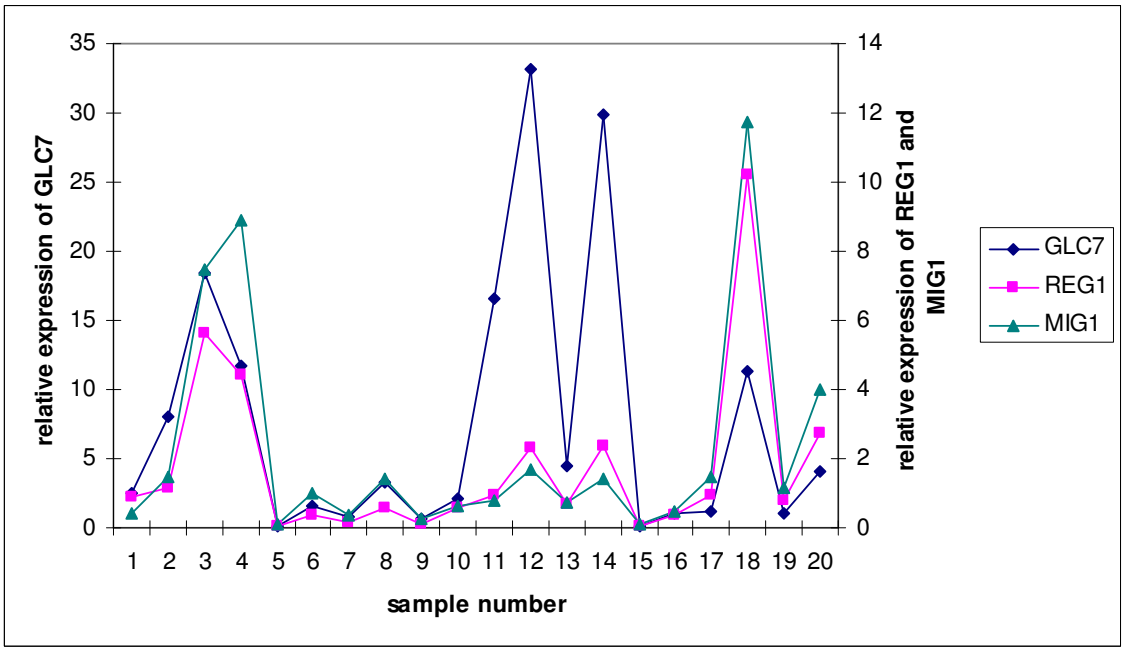


Figure 4.25. Expression level of *GLC7*, *REG1* and *MIG1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

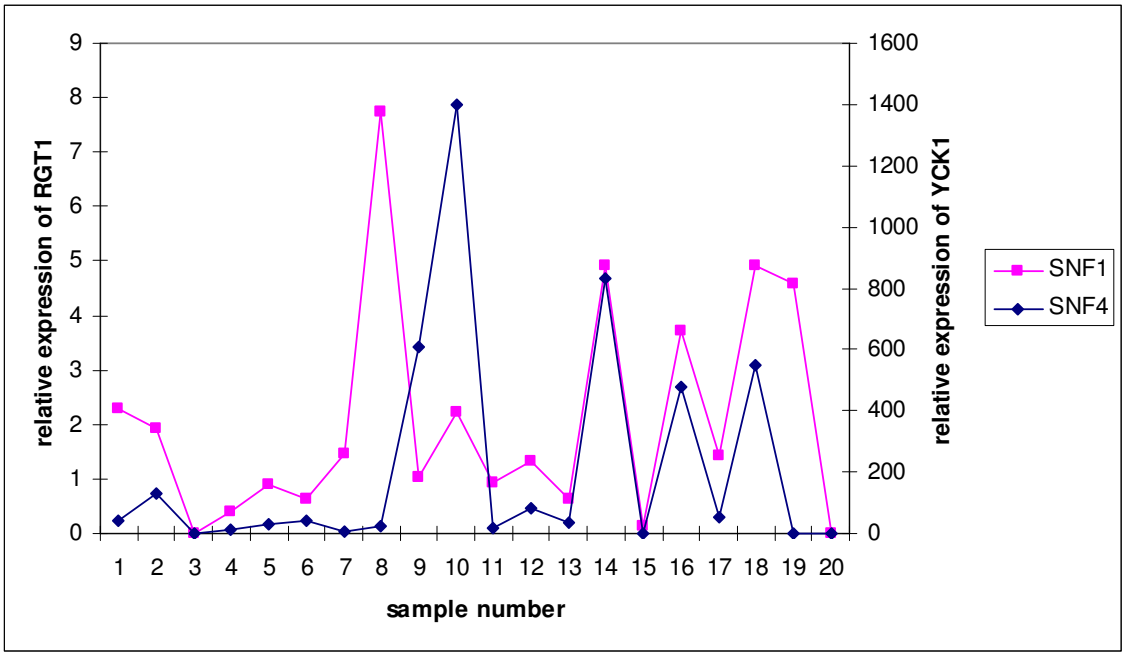


Figure 4.26. Expression level of *SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

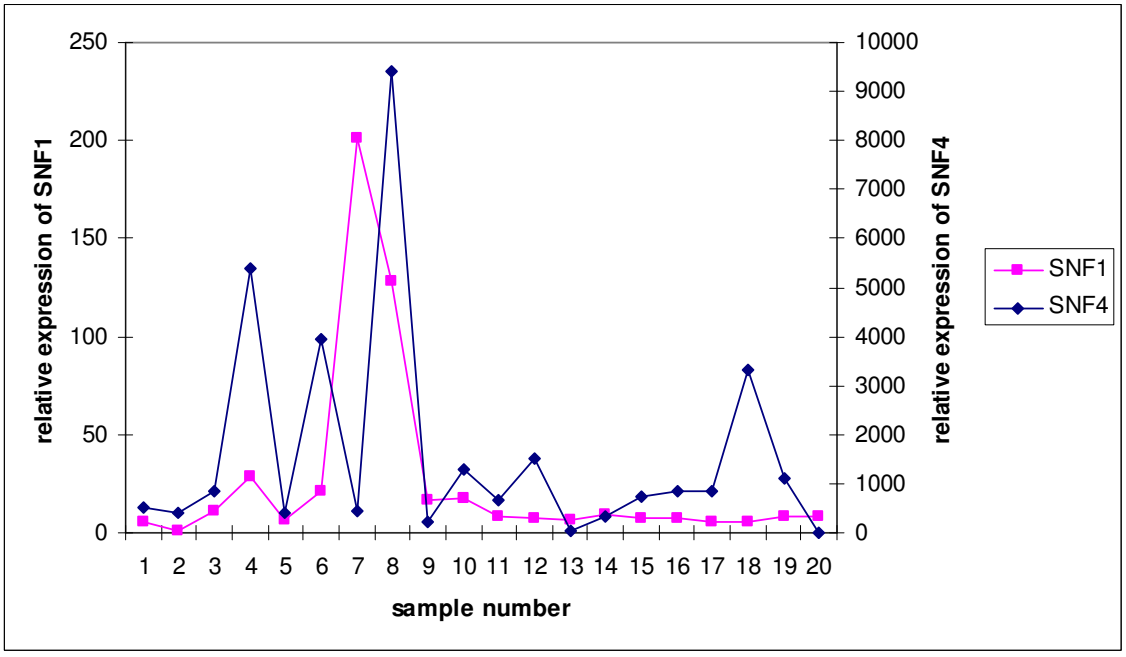


Figure 4.27. Expression level of *SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

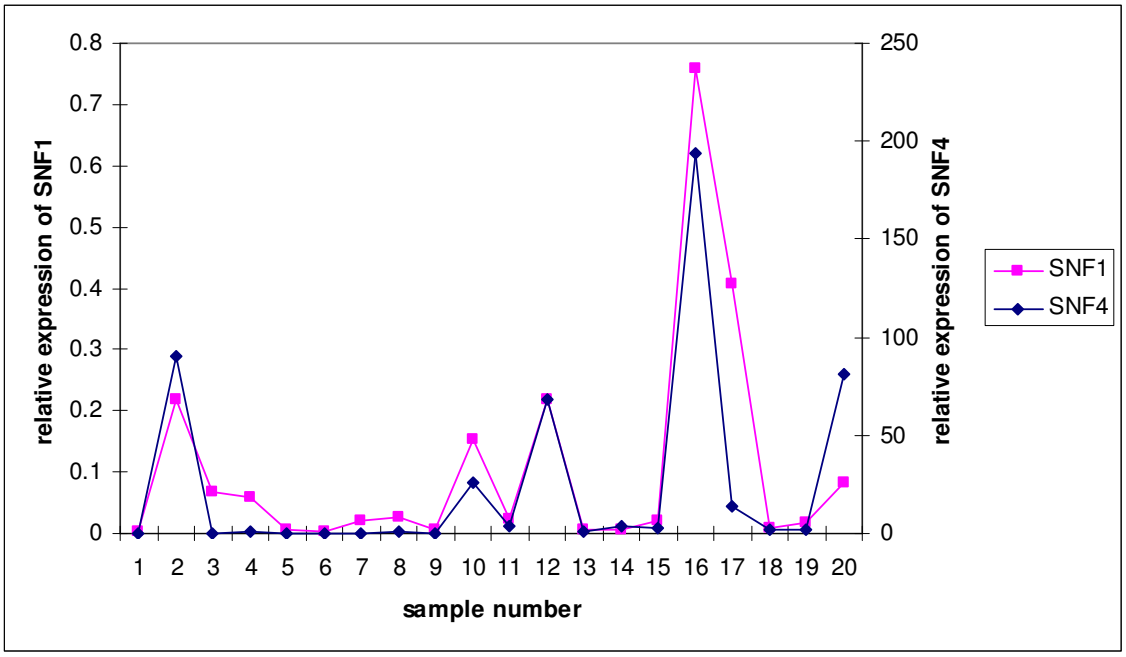


Figure 4.28. Expression level of *SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

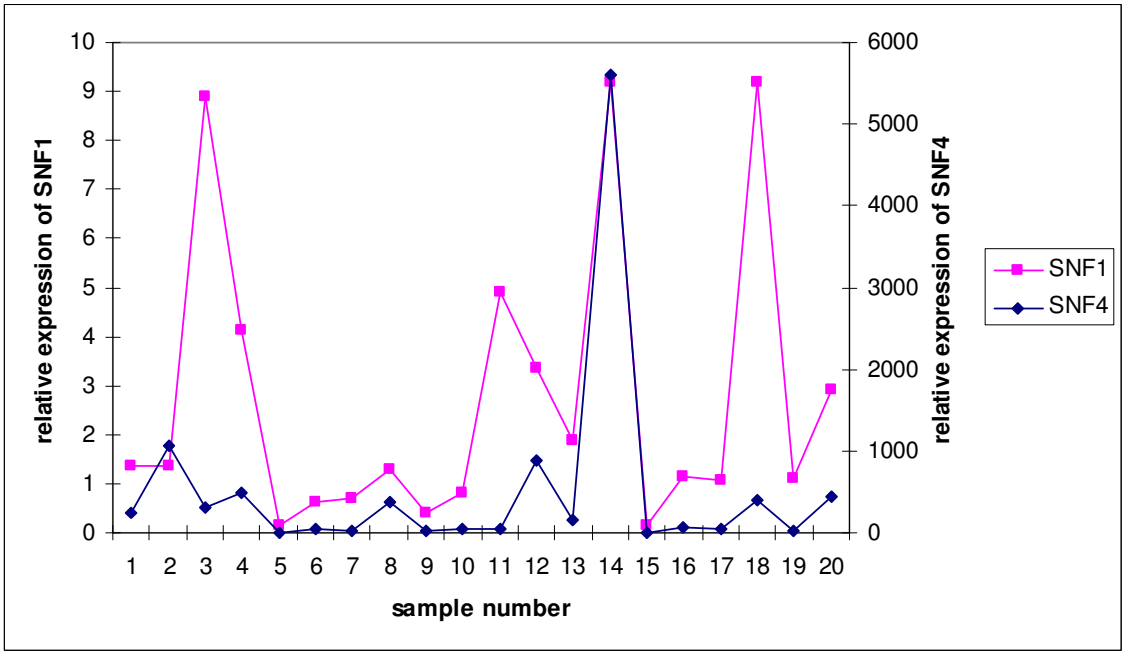


Figure 4.29. Expression level of *SNF1* and *SNF4* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

In the *hap4Δ/hap4Δ* strain, one hour after pulse injection the *SNF1* transcription level does not show significant changes. This result is due to the glucose concentration

level during the cultivation. Since the glucose level does not change much during this time, *SNF1* transcription is nearly constant.

Although the Reg1-Glc7 complex acts in opposition to the Snf1 signaling pathway (McCartney and Schmidt, 2001), the expression profiles of the complex and *SNF1* showed similar trends. (Figures 4.30, 4.31, 4.32, 4.33)

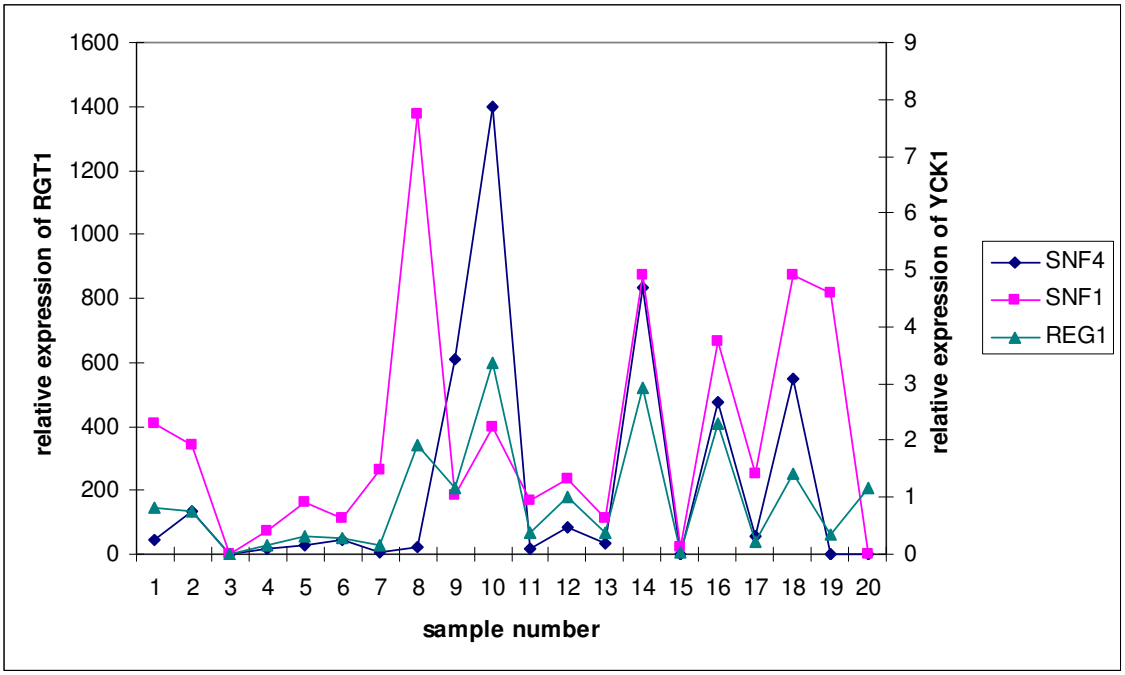


Figure 4.30. Expression level of *SNF4*, *SNF1*, and *REG1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

Previous results stated that expression of *REG1* is not regulated by glucose (Gancedo, 1998). If the response to glucose pulse injection is observed in terms of fold change, it can be seen that the response is not a significant one. This result is in good agreement with earlier studies.

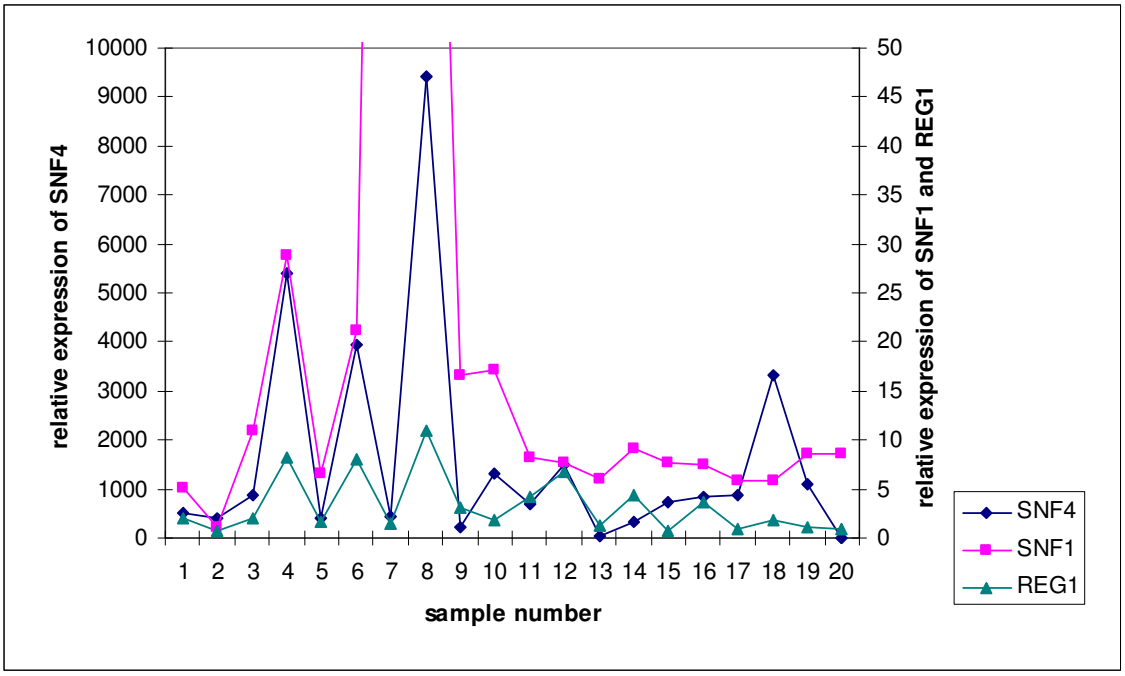


Figure 4.31. Expression level of *SNF4*, *SNF1*, and *REG1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

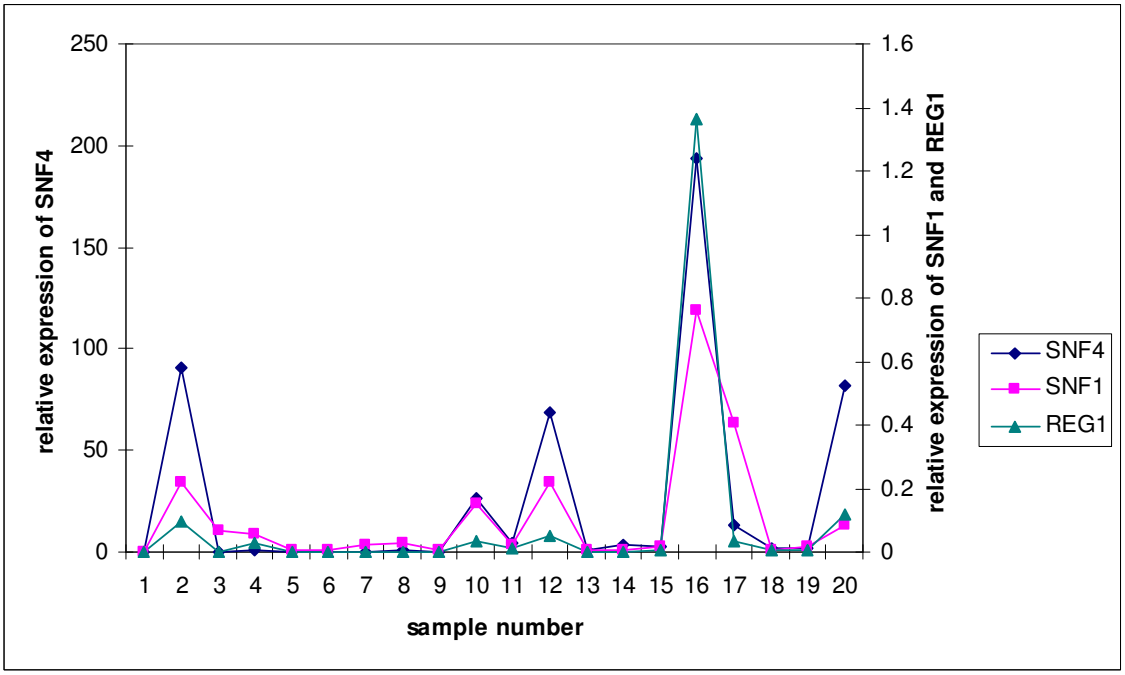


Figure 4.32. Expression level of *SNF4*, *SNF1*, and *REG1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

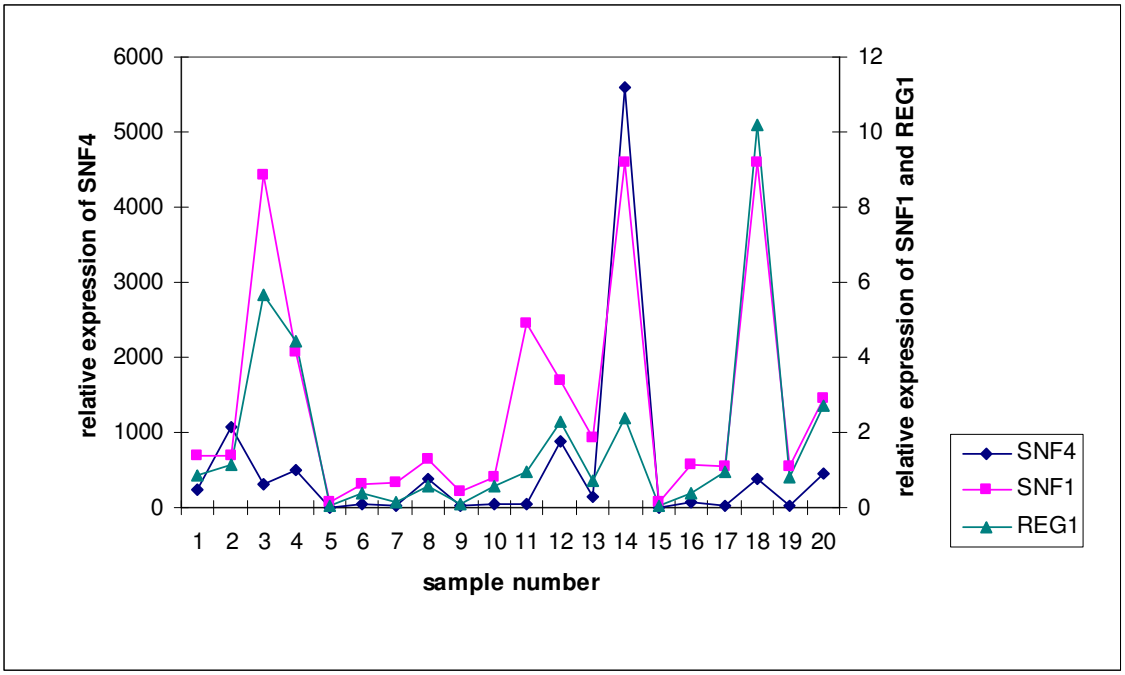


Figure 4.33. Expression level of *SNF4*, *SNF1*, and *REG1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

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

Although it is reported that Pak1 may be posttranscriptionally regulated (DeRisi *et al*., 1997; Nath *et al*., 2003), it is observed in the *hoΔ/hoΔ, hap4Δ/hap4Δ* and *RIP1Δ/rip1Δ* strains of the yeast *S. cerevisiae* that it is downregulated with the injection of glucose pulse (figures 4.34, 4.35, 4.37). Similar profiles are observed for *PAK1* and *TOS3* genes. In the *rip1Δ/rip1Δ* strain, with the effect of the *RIP1*, these three genes were upregulated with pulse injection (figure 4.36).

Undetectable transcription level of *ELM1* both prior to and after pulse injection in all strains may indicate the unresponsiveness of the transcription, or may be caused by a technical problem in the amplification of this gene.

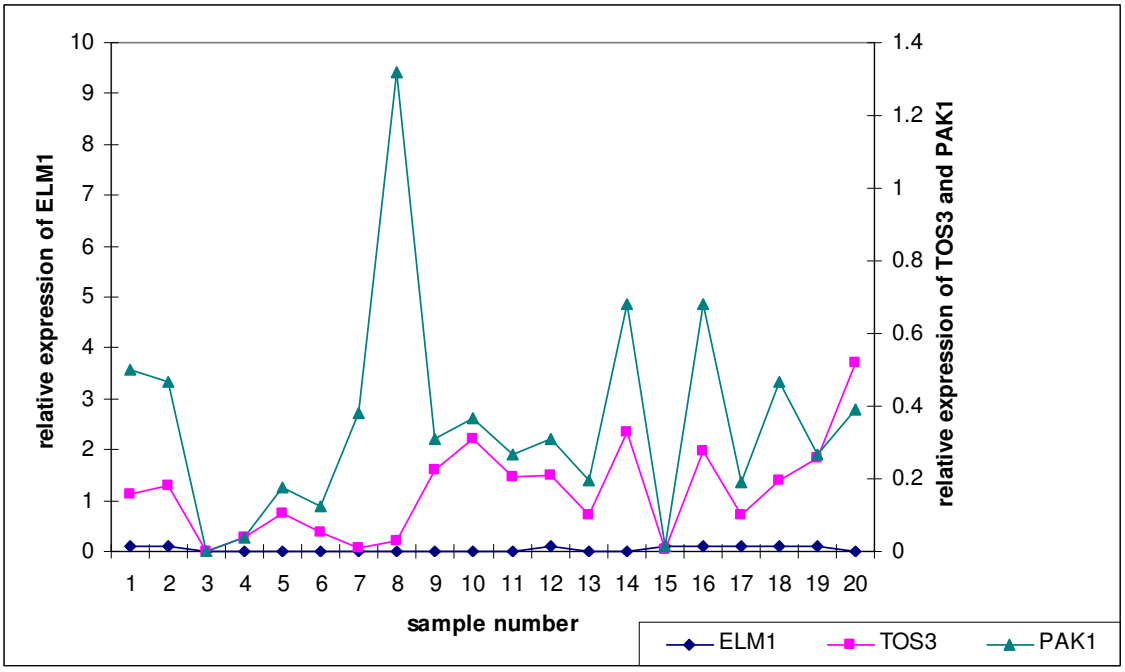


Figure 4.34. Expression level of *ELM1*, *TOS3*, and *PAK1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

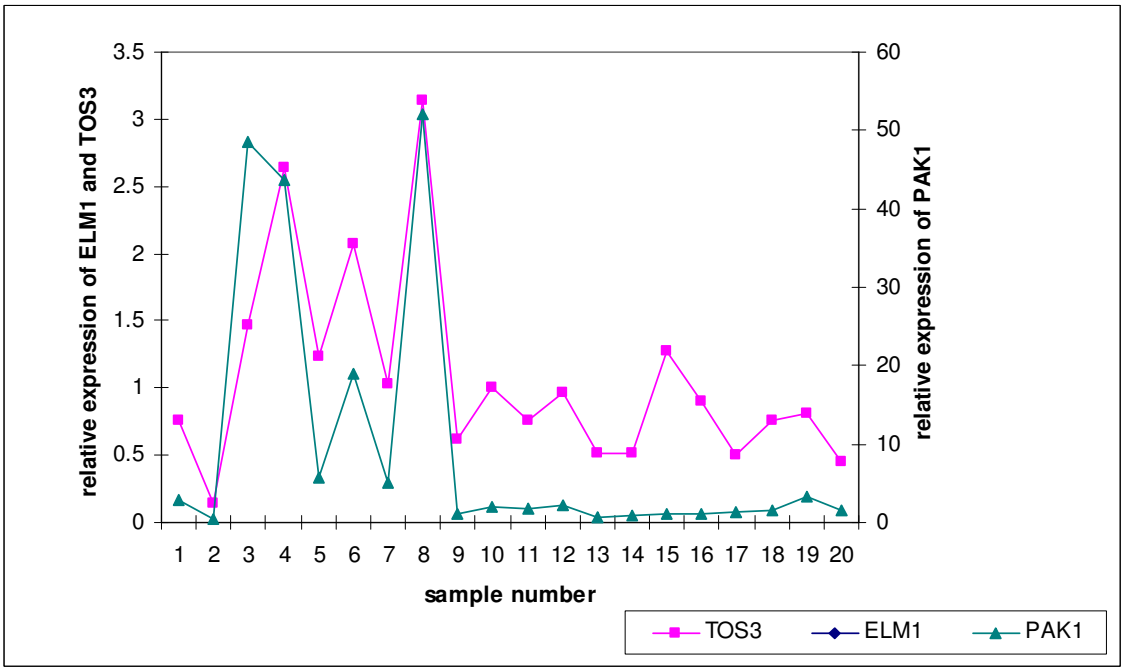


Figure 4.35. Expression level of *ELM1*, *TOS3*, and *PAK1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

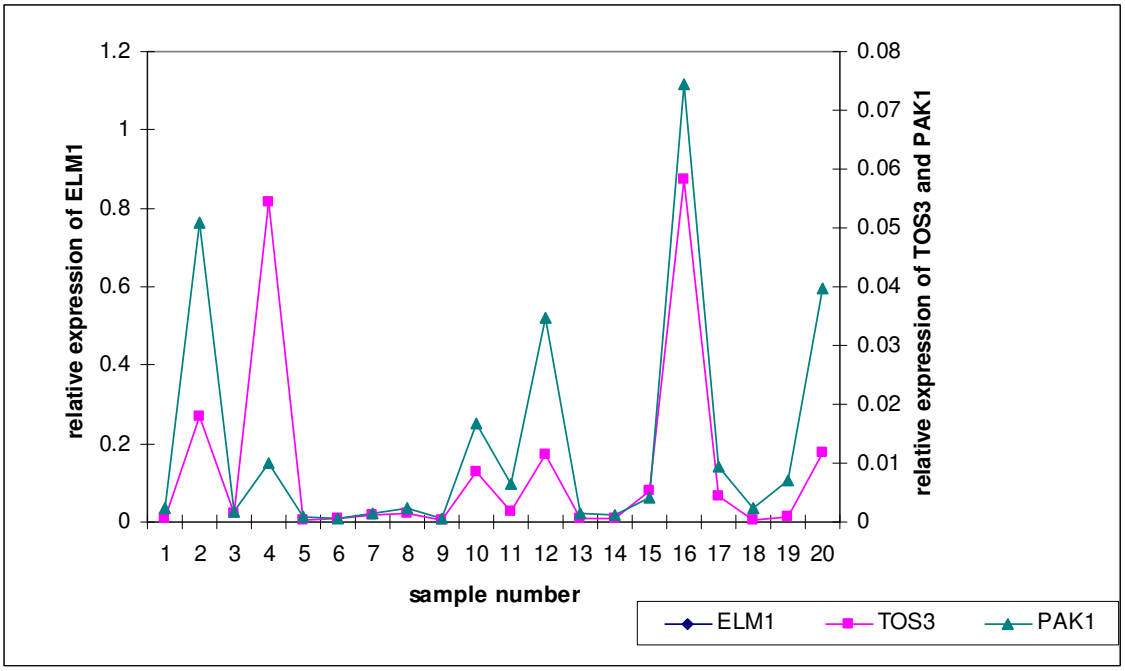


Figure 4.36. Expression level of *ELM1*, *TOS3*, and *PAK1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

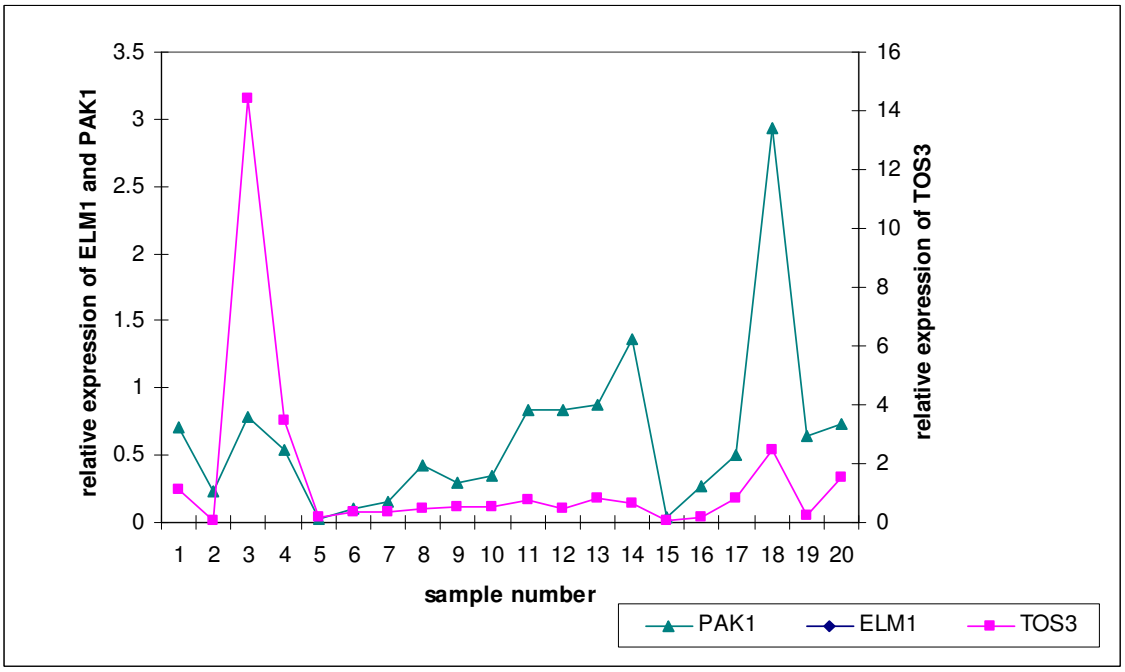


Figure 4.37. Expression level of *ELM1*, *TOS3*, and *PAK1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

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

It is reported that Mig1 inhibits transcription by recruiting the general co-repressor complex Cyc8 (Ssn6) – Tup1 (Papamichos-Chronakis *et al.*, 2004). It can be expected that these three genes have similar expression profiles. However, as seen in figure 4.38, in the strain *hoΔ/hoΔ* these genes do not show very similar trends. Similar trends is observed in the other three mutants (figures 4.39, 4.40, 4.41). *MIG1* is upregulated with the injection of glucose pulse in all four strains, as an expected result.

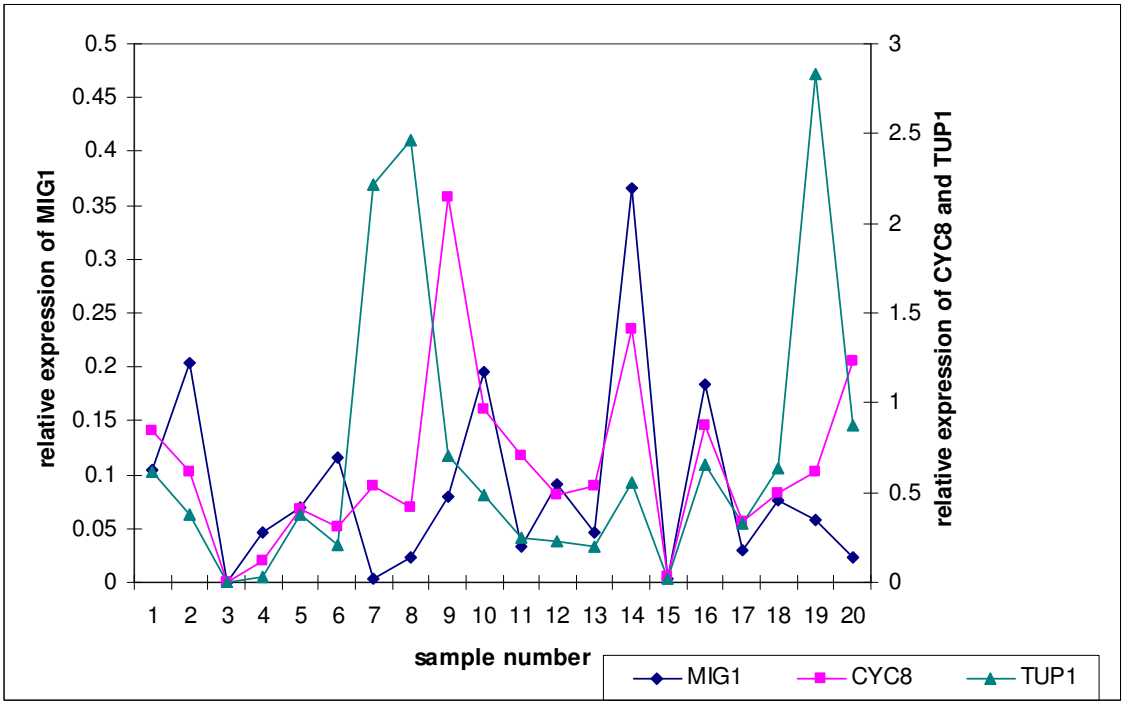


Figure 4.38. Expression level of *MIG1*, *CYC8*, and *TUP1* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

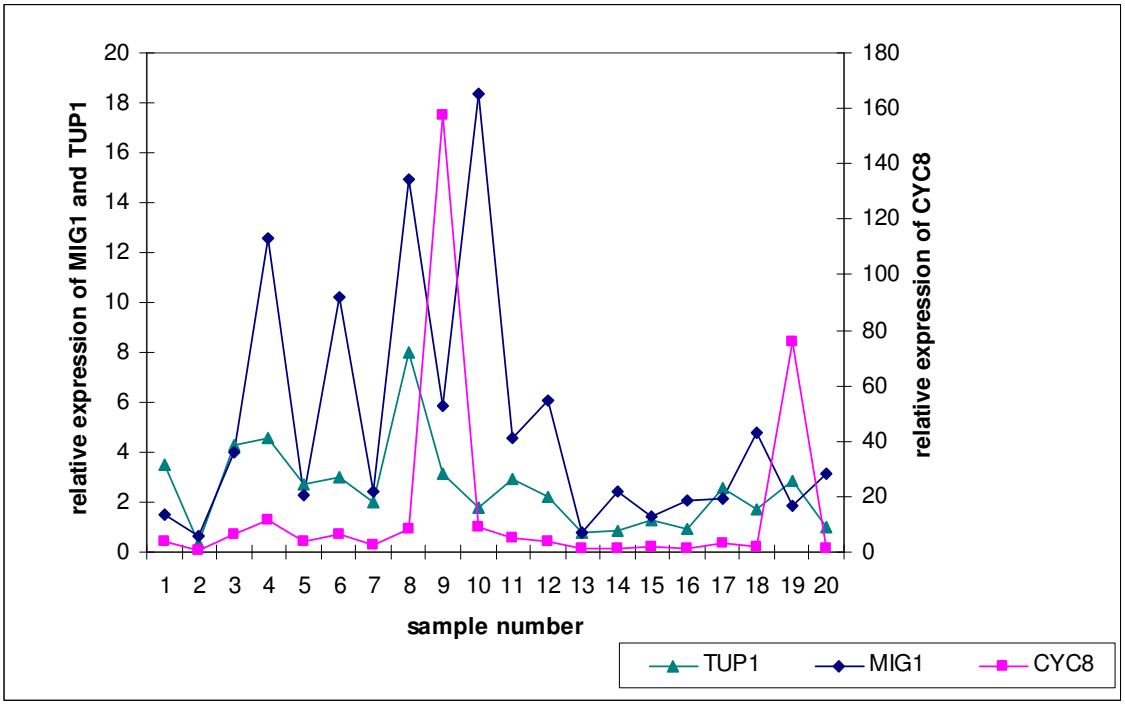


Figure 4.39. Expression level of *MIG1*, *CYC8*, and *TUP1* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

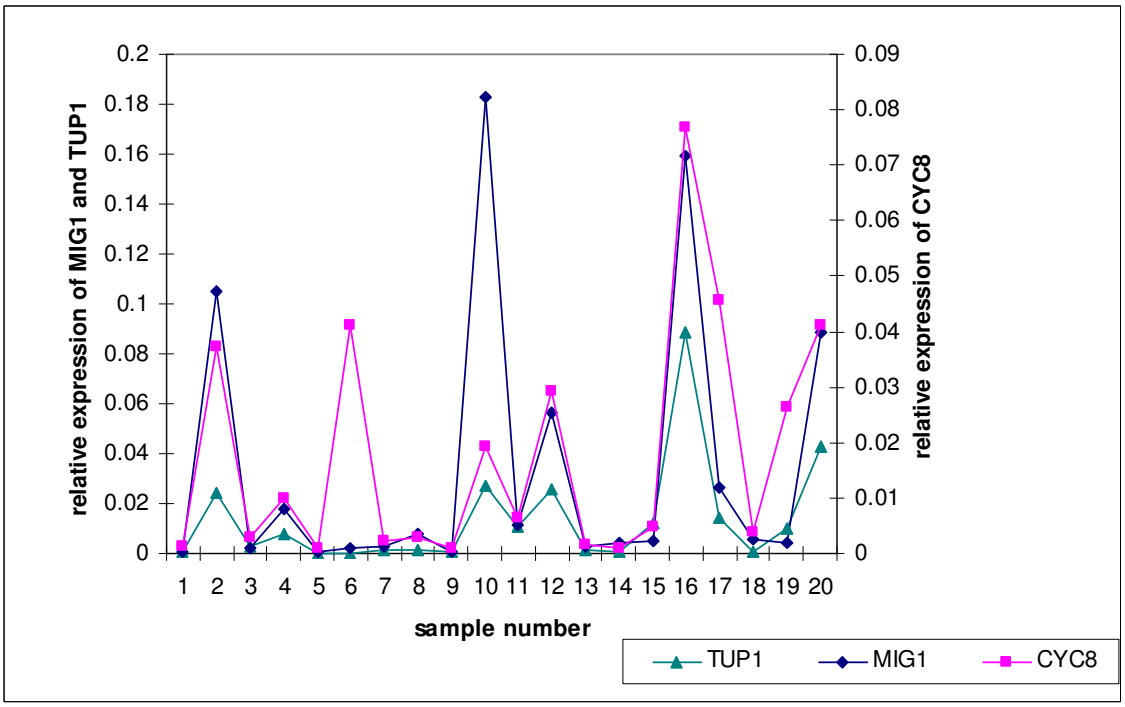


Figure 4.40. Expression level of *MIG1*, *CYC8*, and *TUP1* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

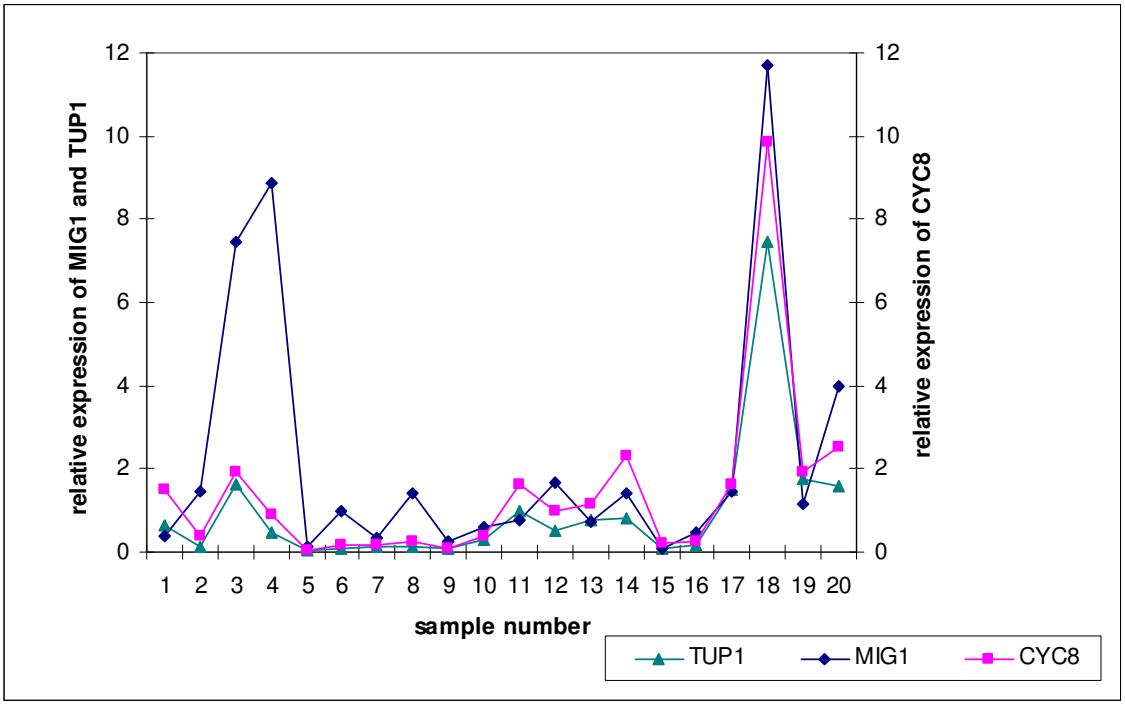


Figure 4.41. Expression level of *MIG1*, *CYC8*, and *TUP1* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

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

It is known that there is a direct correlation between the amount of Hxk2p located in the nucleus and the level of Mig1p in the cell (Ahuatzi *et al*., 2004).

As can be seen in figures 4.42, 4.43, 4.44 and 4.45 although an oscillatory behavior is seen in the expression of *HXK2*, higher levels can be observed in the lack of glucose. The expression is downregulated with the injection of glucose pulse. The similarity of the expression of the two genes can also be observed in the following figures.

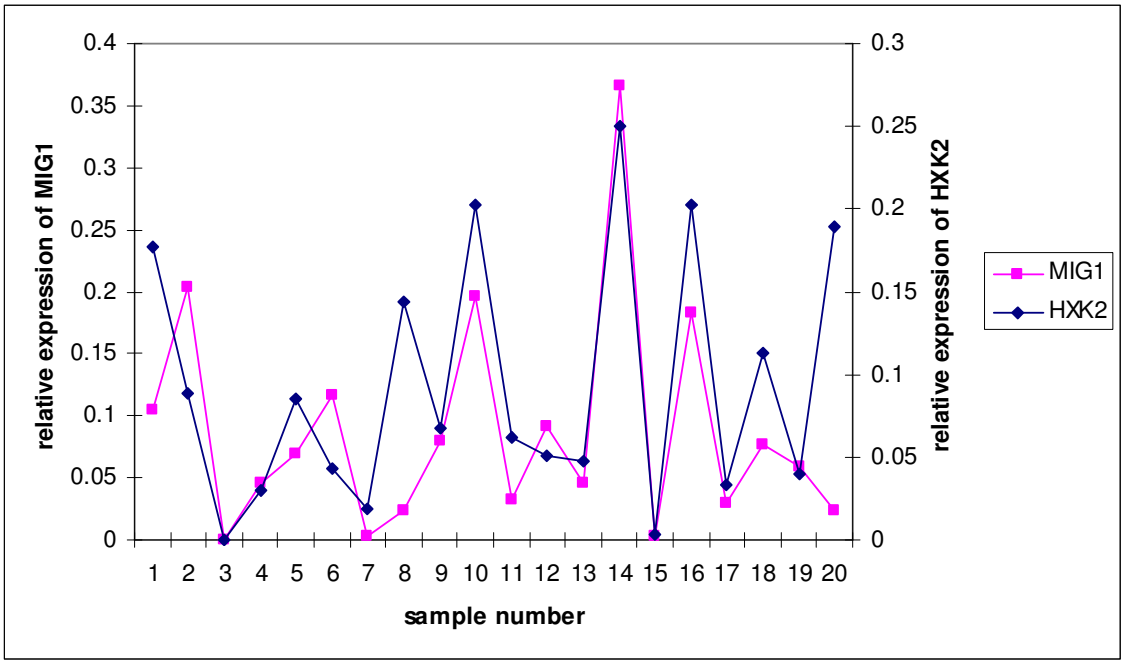


Figure 4.42. Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*hoΔ/hoΔ*)

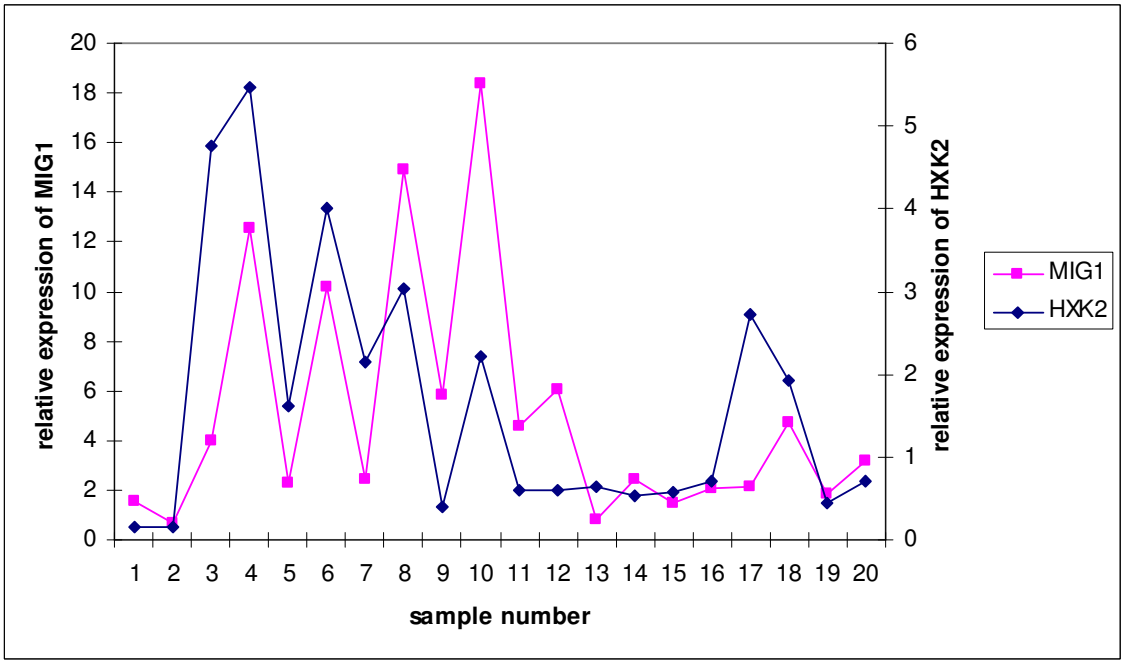


Figure 4.43. Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*hap4Δ/hap4Δ*)

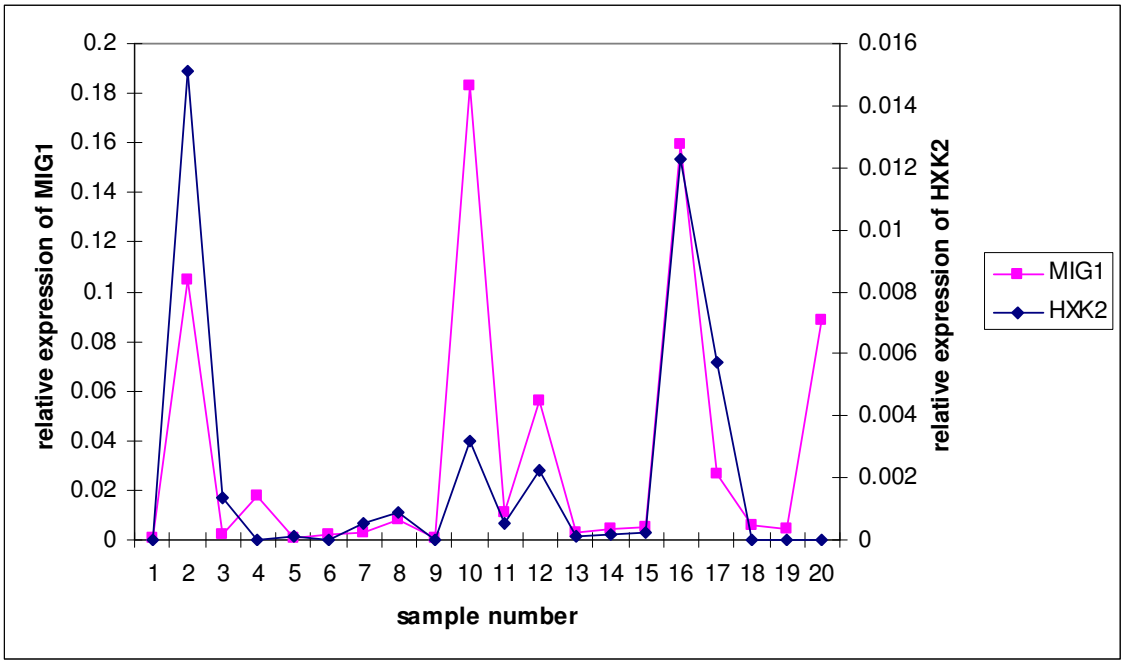


Figure 4.44. Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*rip1Δ/rip1Δ*)

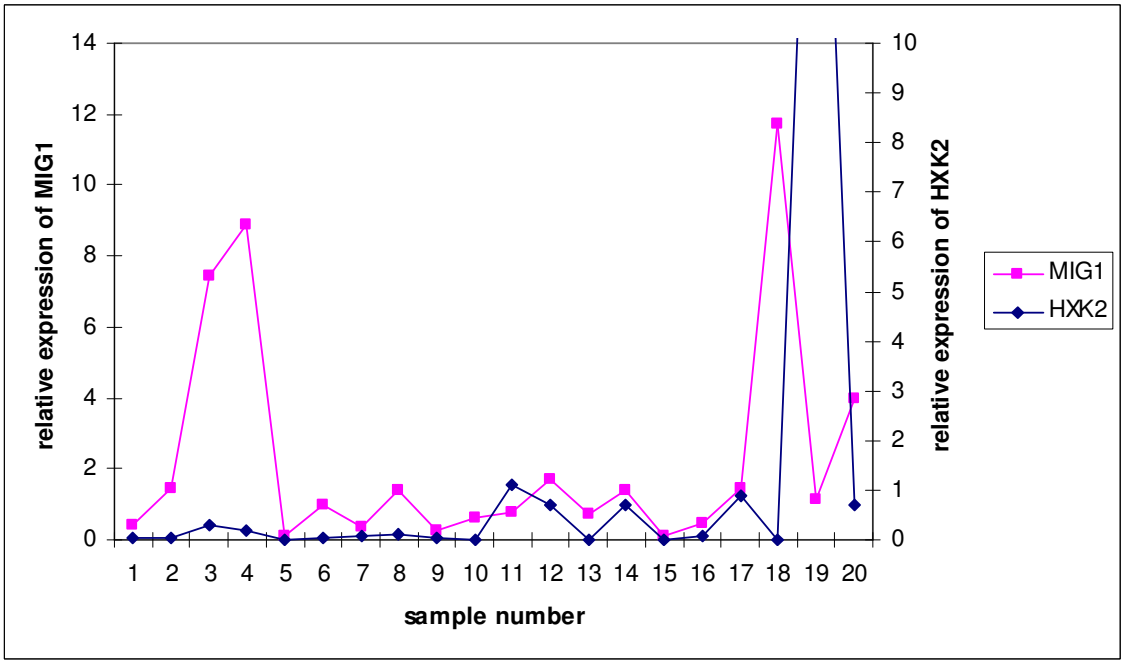


Figure 4.45. Expression level of *MIG1* and *HXK2* in *S. cerevisiae* BY4743 (*RIP1/rip1Δ*)

4.10. Expression Profile of *MBA1* under Glucose 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 transcripted in higher levels under glucose limitation for the three strains of *S. cerevisiae* (*hoΔ/hoΔ, rip1Δ/rip1Δ* and *RIP1/rip1Δ*) and upregulated after glucose pulse injection. For the *HAP4* deletion mutant, the response of *MBA1* to pulse injection was not an immediate one and the transcription under glucose limitation was low. Expression levels displayed a fluctuating behavior in all four cases in Figure 4.46.

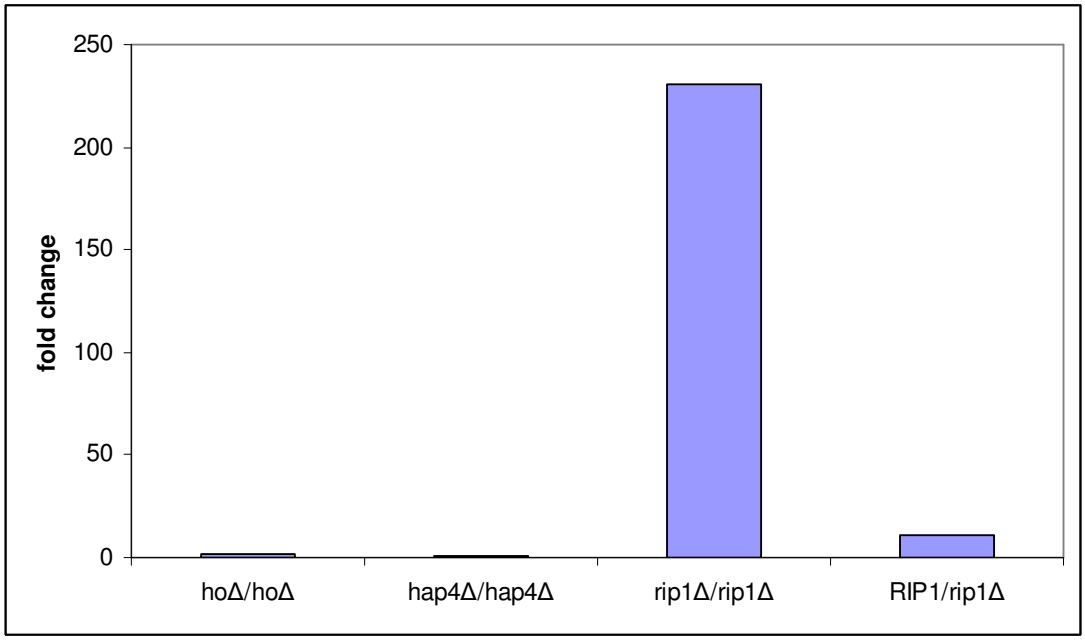


Figure 4.46. Fold change in the expression level of *MBA1* after glucose pulse injection

4.11. Expression Profile of *HAP4* under Glucose Limited Conditions

In *S. cerevisiae* BY4743 *hoΔ/hoΔ* strain, the expression of *HAP4* was downregulated with the injection of glucose pulse. This is a results that agrees well with the literature. It is known that glucose represses expression of *HAP4* via Mig1 pathway and thereby activation of respiration is prevented at high glucose concentration (Raghevendran, 2006). As the glucose level decreases, *HAP4* gene is upregulated as expected.

In the homozygous and heterozygous deletion mutants of *RIP1* of *S. cerevisiae* *HAP4* was upregulated with glucose 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 glucose 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 carbon catabolite repression in yeast.

5. CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

The aim of this study was to investigate the effect of carbon limitation on 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.

In the study, four sets of chemostat experiments with *S. cerevisiae* BY4743 strains *(hoΔ/hoΔ*, *hap4Δ/hap4Δ, rip1Δ/rip1Δ and RIP1/rip1Δ)* were carried our under carbon limited F1 medium. After the cells reached steady state and remained three residence times in steady state, glucose was injected into the medium, eliminating carbon 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).

The highest cell density was seen in the *, rip1Δ/rip1Δ* strain. Deletion of *HAP4* resulted in low cell density when compared to the *hoΔ/hoΔ* strain.

Prior to pulse injection, the glucose concentration in all four strains were nearly insignificant and increase with pulse injection. Ethanol production was highest in the respiratory deficient *rip1Δ/rip1Δ* strain. Glycerol concentration decreased in the *hoΔ/hoΔ* and the *hap4Δ/hap4Δ* strains as glucose concentration decreased throughout the cultivation, whereas with the *RIP1* deletion, the same effects was not seen. Ammonia concentrations remained nearly constant for all four strains throughout the cultivation.

In *hoΔ/hoΔ* strain, all genes were downregulated with pulse injection with the exceptions of *RGT1, MIG1, SNF4* and *MBA1*, which seem to be the genes involved in the glucose repression pathway of *S. cerevisiae.*

In the *HAP4* deletion mutant, all genes were downregulated in response to glucose pulse injection.

Deletion of both allele of *RIP1* resulted in the upregulation of all genes in response to glucose pulse injection. Partial deletion of this gene has a repressing effect on *TUP1, YCK1, MTH1, STD1, RGT2, SNF3, PAK1* and *TOS3.* Partial respiratory deficiency seems to effect *GRR1* and *SKP1* genes similarly.

5.2. Recommendations

The chemostat experiments could be repeated for more accurate data. Statistical tools may be useful in analyzing the data. Clustering methods may be used in order to determine genes that show similar trends.

During 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.

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