AN INTEGRATED APPROACH TO INVESTIGATE TOR SIGNALING MECHANISM IN *SACCHAROMYCES CEREVISIAE*

by

Elif Dereli

B.S., Chemical Engineering, Yıldız Technical University, 2005

M.S., Chemical Engineering, Boğaziçi University, 2007

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ABSTRACT

AN INTEGRATED APPROACH TO INVESTIGATE TOR SIGNALING MECHANISM IN *SACCHAROMYCES CEREVISIAE*

Target of rapamycin (TOR) signaling, a conserved mechanism from yeast to human, is the major regulator of cell metabolism and growth. Rapamycin, an immunosuppressive and anti-proliferative drug, and caffeine, a widely used psychoactive drug, target and inhibit TOR function. In this study the long-term administration effects of 2 nM rapamycin and 5 mM caffeine, together with oxygen availability and acidity as well as the rapamycin dosage (2 nM or 200 nM) on yeast transcriptional and metabolic responses were comparatively investigated. For this purpose batch cultivations of yeast cells were carried out in the absence and presence of rapamycin and caffeine with different levels of oxygen availability and pH. Batch cultivations were conducted under micro-aerated conditions where pH was maintained at 5.5 to investigate the effects of the rapamycin dosage. The analysis of the transcriptional responses of yeast cells to the long term presence of rapamycin revealed that long term administration of low dose of rapamycin resulted in more subtle changes in transcriptional responses of yeast causing a partial TOR inhibition than the administration of high dose. However both low and high doses of rapamycin resulted in a set of transcriptional changes mimicking nutrient starvation responses of yeast cells and they both induced autophagy and altered membrane trafficking. Low dose of rapamycin was still effective at the transcriptome level by leading metabolism to autophagy but this dose may not be enough to block the proliferation of aggressive tumors. The effects of caffeine in the long term administration resulted in a wider set of transcriptional changes resembling the high dose of rapamycin treatment; high dose of rapamycin or caffeine suppressed the growth-related processes and increased the energy requirement of the cells. pH was observed to be more decisive parameter on the transcriptional responses of the cells than the oxygen level and both oxygen availability and pH should be carefully evaluated during rapamycin or caffeine treatment.

ÖZET

SACCHAROMYCES CEREVISIAE'DA TOR SİNYAL İLETİ MEKANİZMASININ BÜTÜNLEŞTİRİLMİŞ BİR YAKLAŞIMLA İNCELENMESİ

Rapamisin hedefi (TOR) sinyal ileti mekanizması mayadan insana kadar birçok organizmada korunmuş bir yapı olup hücre metabolizması ve büyümenin en önemli düzenleyicisidir. Rapamisin bağışıklık sistemini baskılayan ve hücre çoğalmasını engelleyen bir ilaçtır. Kafein ise çoğunlukla kullanılan psikoaktif bir ilaçtır ve rapamisin gibi TOR mekanizmasını hedef almakta ve baskılamaktadır. Bu çalışmada 2 nM rapamisin ve 5 mM kafeinin uzun süreli kullanımlarının oksijen ulaşılabilirliği ve asidite ile birlikte meydana getirdiği etkilerin yanı sıra rapamisin dozunun (2 nM veya 200 nM) etkileri mayada gen anlatımı ve metabolik düzeylerde incelenmiştir. Bu amaçla maya hücreleri kesikli kültürlerde rapamisin veya kafein yokluğunda ve varlığında değişik oksijen ve pH seviyelerinde büyütülmüştür. Rapamisin dozunun etkilerini incelemek amacıyla yapılan kesikli fermentasyonlar havalandırmasız şartlarda pH 5.5'te sabit tutularak gerçekleştirilmiştir. Maya hücrelerinin uzun süre yüksek doz rapamisin varlığında büyümeye gen anlatım düzeyinde verdikleri cevaplar düşük dozdan daha belirgindir. Düşük doz rapamisin varlığının TOR işlevinin bir kısmını baskıladığı gözlemlenmiştir. Düşük ve yükek doz rapamisin mayada gen anlatım düzeyinde besin kısıtlamasına benzer etkiler göstermiştir. Rapamisin her iki dozda otofajiyi ve membran taşınımını aktif hale getirmiştir. Düşük doz rapamisin hücrede otofajiyi tetikleyerek halen etkin olmakla beraber agresif tümörlerde çoğalmayı engelleyecek kadar etkin olmayabilir. Uzun süre kafein varlığının hücrede gen anlatımında meydana getirdiği değişimler yüksek doz rapamisine benzemektedir. Rapamisin ve kafeinin büyüme ile ilgili süreçleri baskılarken hücrenin enerji ihtiyacını arttırdığı gözlemlenmiştir. Rapamisin ve kafein varlığında ve yokluğunda pH seviyesinin mayanın gen anlatım düzeyinde oksijen seviyesinden daha belirgin bir etmen olduğu gözlemlenmiştir. Bu çalışmanın sonuçları rapamisin ve kafein ile tedavi edilen bireylerde oksijen ulaşılabilirliğinin ve pH seviyelerinin dikkatli bir şekilde takip edilmesi gerekliliğini işaret etmektedir.

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dO_2	Dissolved oxygen
F1	Synthetic defined media
g	Gram
h	Hour
his	Histidine
L	Liter
leu	Leucine
met	Methionine
ura	Uracil
v/v	Volume per volume
w/v	Weight per volume
$Y_{e/s}$	Ethanol yield on glucose
$Y_{g/s}$	Glycerol yield on glucose
μ	Specific growth rate

LIST OF ACRONYMS/ABBREVIATIONS

AGC	protein kinase A, G, and C families
ANOVA	Analysis of Varience
ANP	Aerated, pH not controlled
AP	Aerated, pH controlled
aRNA	Amplified ribonucleic acid
ATP	Adenosine triphosphate
BioGRID	Biological General Repository for Interaction Datasets
caf	Caffeine
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EC	European Commission
EGO	Exit from rapamycin-induced growth arrest
eIF2	Eukaryotic initiation factor 2
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FADH	Flavin adenine dinucleotide
FC	Fold change
FDA	Food and Drug Administration
GO	Gene Ontology
HILIC	Hydrophilic intercation choromotography
LC-MS	Liquid chromatography–mass spectrometry
MANP	Micro-aerated, pH not controlled
MAP	Micro-aerated, pH controlled
MAPK	Mitogen-activated protein kinase
MAT	Mating
max	Maximum
MEP	Mid-exponential phase
MeV	Multi experiment viewer

mRNA	Messenger RNA
MRM	Multiple reaction monitoring
mTOR	Mammalian Target of Rapamycin
NADH	Reduced form of nicotinamide adenine dinucleotide
NCI	National Cancer Institute
NCR	Nitrogen catabolite repression
OD	Optical density
UPLC	Ultra Performance Liquid Chromatography
PI3K	Phosphatidylinositol 3-kinase
PIKK	Phosphatidylinositol kinase-related kinase
PKC	Protein kinase C
PP2A	Protein phosphatase 2
PP2Ac	Catalytic domain of the PP2A protein phosphatase
PPI	Protein-protein interaction
rap	Rapamycin
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SGD	Saccharomyces Genome Database
SP	Stationary phase
STRE	Stress response element
TCA cycle	Tricarboxyacidic cycle
TF	Transcription factor
TOR	Target of Rapamycin
TORC1	Target of Rapamycin Complex 1
TORC2	Target of Rapamycin Complex 2
TRN	Transcriptional regulatory network
tRNA	Transfer ribonucleic acid
YEASTRACT	Yeast search for transcriptional regulators and consensus tracking
YPD	Yeast extract-peptone-dextrose

1. INTRODUCTION

Cell growth and division are the key processes that must be carefully regulated to enable the coordinated growth of the organism, replacement of dead cells in an orderly fashion, and initiation of the repair of the cells when needed. Growth disorders occur at the cellular level and these consequently promote the subsequent events in cancer, in which a group of cells display uncontrolled growth and division, invasion and sometimes metastasis [1].

Saccharomyces cerevisiae is a single celled eukaryote and has been extensively used as a model organism in several studies in order to investigate the regulation of metabolism, cell growth, DNA repair, cell cycle and gene expression. Yeast cells divide in a similar manner to human cells and about 20% of the human disease genes have orthologs in yeast indicating a potential use of yeast to investigate the functional relationships of these genes and to test new drugs [2]. Yeast cells prefer fermentation rather than respiration like cancer cells and fermenting and rapidly proliferating yeast metabolism is very similar to the cancer cells. The uncontrolled cell growth, unbalanced activity of genes that promote and suppress cell division and the problems with the regulation of apoptosis of damaged cells are common features of cancer cells.

Target of rapamycin (TOR) is a major controller of cell metabolism and growth and is involved in many cellular processes like cell proliferation, protein synthesis, transcription, cell motility and cell survival, therefore its perturbation is implicated in many human diseases [3]. Activation of the TOR is frequently encountered in human cancers and has been found to promote glycolysis by up-regulating genes involved in glucose uptake and glycolysis. In addition to its role in cancer, the activity of TOR is also a major determinant in aging, diabetes and a number of neurological diseases like Alzheimer's disease, Huntington's disease, tuberous sclerosis complex (TSC), brain tumors, epilepsy and autism. Therefore TOR has been an attractive target for several clinical applications [4, 5].

Rapamycin is macrolide with immunosuppressive and antiproliferative properties generally used in organ transplant patients to prevent rejection and in cancer treatment. Rapamycin targets TOR and inhibits its function resulting in inhibition of transcription, translation, ribosome biogenesis and cell cycle as well as activation of autophagy, utilization of poor nitrogen sources and stress response. Caffeine is the world's most widely used psychoactive stimulant drug in the forms of coffee, tea, soft drinks and energy drinks and a component of various headache and pain remedies. Like rapamycin caffeine also targets and inhibits the function of TOR and results in a similar transcriptional response since it inhibits ribosome biogenesis, protein synthesis and transcription. However it was reported that the effects rapamycin and caffeine were not totally the same [6].

The oxygen availability and pH levels have major effects on the cell physiology and metabolism in all organisms. Furthermore they are also important in the therapeutic effectiveness of drug agents and may alter the rate of drug metabolism and thus the effective therapeutic dose [7]. The extracellular pH of tumors was reported to be significantly lower than the extracellular pH of normal tissues; however both tissues have similar intracellular pH. The effect of intracellular-extracellular pH gradient is very important on the chemotherapeutic drug efficacy [8].

The aim of this study was to investigate the effects of administration regime of rapamycin and caffeine on yeast cell population together with the environmental conditions; air supply and pH control. In this study the effects of rapamycin dosage on yeast cells were also investigated and the transcriptional and metabolomic responses of yeast cells to the presence of high dose of rapamycin were compared with those of the cells grown in the presence of low dose of rapamycin or caffeine. The transcriptional and metabolic responses of the yeast populations were studied in the absence or presence of either one of the two drugs; rapamycin or caffeine in batch cultures, in which the cultivation pH was controlled or left to take its natural course, and in which oxygen was supplied to the fermenters to saturate the culture medium or was maintained under micro-aerated conditions. The effects of the rapamycin dosage were investigated under micro-aerated and pH controlled conditions.

The experimental methods used as well as the materials are explained in detail in the Materials and Methods section. The optimization of the drug concentrations and the transcriptional and metabolomic responses of yeast cells to the presence of rapamycin or caffeine together with the environmental conditions, oxygen availability and pH control as well as the comprehensive argument of the obtained results were presented in Results and Discussion section. Results and Discussion section also comprises the comparative investigation and the comprehensive argument of the effects of rapamycin dosage on yeast cells. The study is summarized with important key points in the Conclusion section.

2. LITERATURE SURVEY

Saccharomyces cerevisiae is a unicellular eukaryotic microorganism that has extensively been regarded either as a model system for investigating cellular physiology and molecular events relevant for human cells and human disease or as a cell factory for biotechnological use. There are several well-known inherent features to *S. cerevisiae* that make it an ideal model organism. There are also practical benefits from the fact that this yeast has been used for many years and hence a lot of molecular biology tools and data have been amounted over time [9]. Much information is available at the molecular level of this organism, and a large number of studies have been undertaken to analyze the transcriptome, the proteome, the metabolome, and the interactome. Yeast and human share many orthologs and many pathways are conserved among yeast and human. Furthermore, genes that are involved in human disease can be expressed in yeast and therefore various human diseases and processes including cancer [5], heart disease [10], cholesterol metabolism [11], diabetes [12], Parkinson's, Alzheimer's and Huntington's diseases [13], DNA metabolism [14], aging [15], apoptosis [16] and cell cycle control [17] can be studied in yeast in order to contribute to the understanding of the molecular mechanisms underlying the development of human disease.

Nutrient sensing and signaling mechanisms as well as the nutrient metabolism have several roles in human diseases. In addition to their role as source of energy and building blocks for the cell, nutrients also act as regulators of metabolism, growth and development. Adaptation to changing nutritional conditions is a key process for survival, especially in microorganisms. This adaptation is regulated by a variety of signaling pathways [18].

Yeast has the notable capacity to grow under different growth conditions, and can adequately adapt to rapid and major changes in its environment. Cells adapt to the changes in the nutritional availability, by altering gene expression profile. Although limitation for any one nutrient induces a nutrient-specific transcriptional response, much of the transcriptional change upon nutrient limitation is independent of the limiting nutrient. In addition to the transcriptional pattern of the cell, nutrient availability also influences cells

growth rate. Cells have the great ability to maintain balanced growth over a wide range of growth rates [19].

The two most basic nutrient sources for *S. cerevisiae* are carbon and nitrogen. In response to the quality of carbon and nitrogen, the expression of genes involved in different metabolic pathways, particularly those involved in the utilization and transport of the available nutrients are regulated. Genetic analysis in budding yeast led to the identification of signaling pathways that detect the quality of nutrients and regulate gene expression [20].

TOR (target of rapamycin), a nutrient-sensitive protein kinase, is the major regulator of cell growth and aging, and dysregulation of TOR has been observed in many human diseases, such as cancer and diabetes. Therefore TOR has been an attractive target for several clinical applications, particularly in cancer [21].

2.1. The Target of Rapamycin (TOR)

The target of rapamycin (TOR) proteins, Tor1p and Tor2p, were first identified in a genetic screen on *S. cerevisiae* in order to investigate the effects of rapamycin [22]. The serine/threonine kinase TOR is a member of the phosphatidylinositol kinase-related kinase (PIKK) family and its carboxyl terminus is homologous to the catalytic domain of phosphatidylinositol 3-kinase (PI3K). However it displays kinase activity toward proteins, not lipids [23].

TOR is highly conserved from yeast to mouse, rat and human. 95% identity at the amino-acid level is shared by TOR proteins [22]. Its homologues have also been identified in plants (*Arabidopsis thaliana*), flies (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*). *S. cerevisiae* and other yeasts harbour two homologous proteins of this atypical serine/threonine kinase, Tor1p and Tor2p, forming two complexes TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2), whereas there is only one TOR protein in all other eukaryotes [24]. However, the single TOR protein also forms two different complexes. Many proteins in the TOR complexes are highly conserved from yeast to mammals. The mammalian TOR (mTOR) forms two complexes, the rapamycin

and nutrient-sensitive mTORC1 and insensitive mTORC2. However, both mTOR complexes were reported to be stimulated by growth factors [21].

TOR proteins are main regulators in the cell and they have a role in the control of cell growth with respect to nutrient availability and cellular stresses. They, directly or indirectly regulate many cellular processes including protein synthesis and degradation, membrane traffic, PKC signaling, ribosome biogenesis, transcriptional activation, autophagy, meiosis, cell cycle, nutrient permease sorting and turnover, and actin cytoskeleton organization in yeast [25]. Although Tor1p and Tor2p are closely related proteins with partially redundant and rapamycin sensitive functions, like early G1 progression and translation initiation in response to nutrients, Tor2p has the essential, unique and rapamycin insensitive function of organization of the actin cytoskeleton [26, 27].

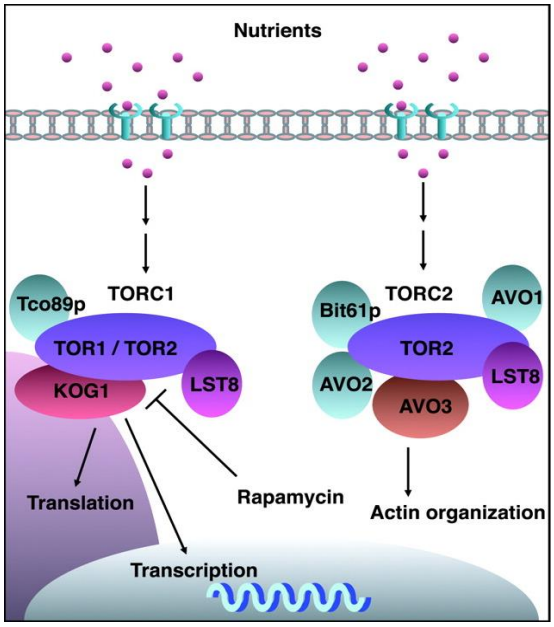

Figure 2.1. The TOR complexes in yeast [26].

TORC1 has been found to play a role in protein turnover inhibition, translation initiation, tetrad formation, and transcriptional repression of specific genes that are induced by nutrient starvation. It includes either Tor1p or Tor2p, together with Kog1p, Lst8p, and Tco89p (Figure 2.1). TORC2 has a role in the regulation of actin cytoskeleton polarization during cell cycle progression, receptor endocytosis and cell wall integrity. It consists of

only Tor2p along with Avo1p, Avo2p, Tsc11p (Avo3p), Lst8p and Bit61p. TORC2 was also reported to be involved in the regulation of sphingolipid biosynthesis [28].

Several studies reported that TORC1 is localized on the limiting membrane of the vacuole. However, other controversial results claimed that TORC1 shuttles in and out of the nucleolus to regulate rDNA expression. The vacuole is a major nutrient reservoir in yeast and the vacuolar localization of TORC1 seems logical since it regulates several processes in response to the nutrient availability [29].

2.1.1. The Upstream of TORC1 Signaling

The activity of TORC1 was regulated by several environmental and intracellular effectors. TORC1 activity was reported to be inhibited by a wide range of environmental stresses, including rapamycin, caffeine, nutrient starvation (carbon, nitrogen, phosphorous), hyperosmolarity (NaCl, KCl, sorbitol), oxidants (H_2O_2, diamide), ethanol, weak organic acids, heat shock, and SDS [30, 31]. Additionally inhibition of TORC1 activity was also reported in response to DNA damage or mitochondrial dysfunction. Furthermore signals from downstream effectors also regulate TORC1 with feedback loops: blocking translation increases TORC1 activity, possibly increasing the amino acid concentrations [32] and deletion of *SCH9* (TORC1 substrate) or *SFP1* (encodes a TORC1-regulated transcription factor) induces TORC1 function [33].

Under inappropriate environmental conditions cells stop dividing, slow down their metabolism, induce the expression of stress responsive genes and accumulate energy stocks. This state is known as G0 phase of cell cycle. Rapamycin causes yeast to arrest in a G0-like state. Escape from rapamycin-induced growth arrest (EGO) complex was identified to regulate TORC1 in re-initiation of growth upon rapamycin removal [34].

2.1.2. The Downstream of TORC1 Signaling

In favorable growth conditions TORC1 is active and it promotes growth and the accumulation of cellular mass while antagonizing the processes involved in stress response. There are two major effectors of TORC1 responsible for transmitting the

essential signals downstream of this complex: The AGC kinase Sch9p and PP2A-related protein phosphatases [33, 35]. Sch9p is required for the regulation of ribosome biogenesis in a TORC1-dependent manner, translation initiation, entry into G0 phase and transactivation of osmostress-responsive genes. It is the best characterized substrate of TORC1 and TORC1 directly phosphorylates five to six serine and threonine residues of Sch9p.

PP2A has three catalytic subunits in yeast (Pph21p, Pph22p and Pph23p) and there are several PP2A-related catalytic subunits, including Sit4p. Sit4p is an important regulator of TORC1 and Tap42p is an essential regulatory subunit of PP2Ac and Sit4p complexes. When TORC1 is active Tap42 is phosphorylated and upon TORC1 inactivation Tap42p becomes dephosphorylated and active and/or has an altered access to substrates [36].

Cell growth requires robust protein synthesis. In order meet this requirement yeast cells need to produce incredible numbers of ribosomes and tRNAs which themselves require accessory factors for their processing and assembly. Expression of the genes encoding these factors requires the concerted activities of all three nuclear RNA polymerases (RNA Pol I, II and III). This energy consuming process needs tight control mechanisms for regulation and coordination the expression of these genes [37]. TORC1 stimulates RNA Pol I activity and Pol II ribosome biogenesis genes via Sch9p and Sfp1. TORC1 also regulates Pol II ribosomal protein genes via the fork head transcription factor Fhl1p. TORC1 was reported to stimulates the RNA Pol III activity which transcribes 5S, tRNA, and other stable nonconding RNAs in a rapamycin-sensitive fashion via Sch9p and Maf1p [38].

Yeast cells start a new round of cell division only after reaching a critical cell size, which varies according to growth conditions. TORC1 regulates the cell-size threshold in response to environmental conditions. Under inappropriate growth conditions the activity of TORC1 reduces leading a subsequent decrease in the activities of Sfp1p and Sch9p. Consequently, this would decrease ribosome biogenesis and therefore cell size threshold required for cell division is reduced.

In addition to role in G1 regulation, TORC1 also mediates other phases of the cell cycle. TORC1 initiates S phase by maintaining deoxynucleoside triphosphate pools and regulates the G2/M transition via Tap42p [39]. In conjunction with its regulatory role in G2/M transition, rapamycin inhibition of TORC1 function was found to alter the phosphorylation of a number of proteins involved in DNA replication [40].

In addition to regulating transcription, rapamycin treatment was reported to alter the turnover of a large number of mRNAs likely via multiple mechanisms. Several rapamycin-sensitive phosphoproteins involved in mRNA degradation/processing and/or their altered localization upon rapamycin treatment were identified [41].

TORC1 couples environmental cues to the cell growth machinery by regulating mRNA translation initiation via Gcn2p. When active, Gcn2p was reported to phosphorylate the translation initiation factor eIF2 and this phosphorylation event resulted in a great reduction in global translation initiation rates. TORC1 represses Gcn2p activity, and thus promotes global translation, via both Sch9p and Tap42p [42].

The role of TORC1 in aging was also reported. The activity of TORC1 is regulated by nutrients and dietary restriction is a well-known procedure that slows aging. TORC1 was reported to be involved in both replicative lifespan (the number of daughter cells a single cell can produce prior to senescence) and chronological lifespan (the length of time a cell can survive in a quiescent state) through different effectors. Specifically, TORC1 appears to influence replicative lifespan via its regulation of protein synthesis and chronological lifespan via its regulation of stress-response programs [43].

TORC1 as a central regulator of cell growth it also influences the metabolism. Several expression studies revealed that in addition to diminished ribosomal protein and ribosome biogenesis gene expression TORC1 inhibition also repressed the expression of genes involved in glycolysis and induced the expression of genes involved in citric acid cycle. These findings suggest a metabolic switch from fermentation to respiration [44]. However although the altered mRNA abundances of these genes, this would not be reflected at the protein level owing to the general repression of translation.

In the presence of high quality nitrogen sources (glutamine, asparagine or ammonia) the transcription factor Gln3p is bound by its cytoplasmic anchor Ure2p. In the absence of the preferred nitrogen sources or when the TORC1 is inactive, Gln3p is released from Ure2p, translocates to nucleus and activates the expression of genes involved in the utilization of poor nitrogen sources (proline or allantoin). This phenomenon is known as nitrogen catabolite repression or nitrogen discrimination and nitrogen source quality signals are transmitted to Gln3p and Ure2p in part by TORC1 and in part by independent pathways [45].

Alterations in mitochondrial function are transmitted to nuclear gene expression by retrograde response which is a mitochondria-to-nucleus signaling pathway [46]. This pathway culminates with Rtg1p and Rtg3p, which activate genes encoding citric acid cycle and peroxisomal enzymes required for *de novo* amino acid biosynthesis in general and glutamine/glutamate homeostasis in particular. TORC1 represses Rtg1p/3p activity presumably by promoting their interaction with a cytoplasmic anchor composed of Mks1p, Bmh1p and Bmh2p. When TORC1 is inactive Mks1p associates with an inhibitor, Rtg2p, and this leads to subsequent release of Rtg1p/3p [29].

TORC1 also regulates metabolism through more direct routes that do not involve transcription. TORC1 signaling regulates the hierarchical consumption of preferred nutrients before the suboptimal ones through regulating the sorting or the activities of the nutrient permeases via Npr1p [29]. TORC1 was also reported to regulate metabolism via inhibition of Snf1p which is a stimulator of ATP-producing catabolic reactions [47].

In addition to its role as a cell growth stimulator, TORC1 is also involved in the suppressing stress-response programs. Nutrient stresses can trigger different developmental responses in yeast. For example, the absence of glucose leads to activation of filamentous/invasive growth of haploid cells whereas the absence of both fermentable carbon and high-quality nitrogen sources triggers meiosis and sporulation in diploids. The inhibition of meiosis and sporulation by fermentable carbon and high-quality nitrogen sources was reported to be mediated by TORC1 since TORC1 antagonizes the nuclear localization and stabilization of Ime1p, a transcription factor and master regulator of

developmental process. TORC1 may regulate filamentous growth by regulating the phosphorylation and the activity of the Ser/Thr kinase Ksp1p [48, 49].

Besides the nutritional stresses TORC1 also regulates environmental stress through the transcription factors Msn2p and Msn4p which bind to stress-response elements (STREs). The signals to Msn2p/4p are transmitted via Tap42p and Sch9p as well as Rim15p. Inactivation of TORC1 activates Rim15p which induces Msn2p/4p leading to induction of expression of STRE-regulated genes. The environmental stress response is also important for chronological lifespan since partial inhibition of TORC1 or deletion of *SCH9* was reported to increase chronological lifespan in a Rim15p and Msn2p/4p dependent manner [50, 51]. Activation of environmental stress response by partial inhibition of TORC1 or deletion of *SCH9* allows cells to better tolerate exposure to stress conditions and thus remain viable longer when in quiescent state. Autophagy is a catabolic process where cellular components are degraded by the lysomal/vacuolar system to liberate and thus reallocate molecular building blocks and it was reported to play an important role in chronological aging since induction of autophagy extends chronological lifespan in yeast.

2.1.3. TORC2 Signaling

While TORC1 is defined a major regulator of cell growth in *S. cerevisiae*, TORC2 also mediates cell growth through regulating processes including actin cytoskeleton organization and endocytosis. TORC2 is insensitive to rapamycin. Several members of TORC2, *TOR2*, *LST8*, *AVO1* and *AVO3* are essential genes [52].

TORC2 regulates the actin cytoskeleton organization through Pkc1p and Mpk1p-MAP kinase cascade. TORC2 activates Rom2p and activated Rom2p converts Rho1p and Rho2p to their active GTP-bound state. Then Rho1p and Rho2p bind and phosphorylate Pkc1p. Pkc1p then regulates the organization of actin cytoskeleton via Mpk1p-MAP kinase cascade [53]. This cascade was reported to be involved in the maintenance of cell integrity by regulating cell wall biosynthesis in response to stress [54]. It was stated that the depolarization of the actin cytoskeleton in response to inactivation of TORC2 could be repressed by hyperactivation of the cell wall integrity pathway which involves Pkc1p [55].

Ypk2p, a direct substrate of TORC2, is phosphorylated by TORC2. TORC2 was reported to mediate processes including actin remodeling via Ypk2p. In addition to Ypk2p, Slm1p and Slm2p were also reported as direct TORC2 substrates and these proteins can regulate actin organization independently of Ypk2p [56].

TORC2 was also reported to be involved in sphingolipid biosynthesis and mediate *de novo* ceramide biosynthesis. Sphingolipids and their metabolites are known as building components of cellular membranes, and also as signaling molecules mediating cell growth, endocytosis, actin regulation, and stress response. Deficiency in TORC2, but not TORC1 was reported to result in a decrease in sphingolipid metabolites [57].

2.2. The Inhibitors of TOR: Rapamycin and Caffeine

Rapamycin was discovered as a product of a bacterial strain *Streptomyces hygroscopicus* in a soil sample from Easter Island (also called "Rapa Nui" in local language) [58]. It was first developed as an antifungal agent, later on immunosuppressive and antiproliferative properties of rapamycin were discovered [24].

Rapamycin (Rapamune) was approved as an immunosuppressant in 1999 by the Food and Drug Administration (FDA) and in 2000 by the European Commission (EC). Due to its immunosuppressive and antiproliferative properties it is used in organ transplantation to prevent organ rejection, anticancer therapies to block the growth of tumor cells and coronary artery disease to prevent restenosis after angioplasty [59, 60]. Although antitumor activity of rapamycin has been found against several solid-tumor models in the National Cancer Institute (NCI) screening program, its development in anti-cancer therapy has been far slower yet. Stability and formulation concerns in producing a parenteral formulation are playing the main role in this slow progress [61].

Fpr1p (also known as FKBP12) is a direct cellular receptor of rapamycin. Rapamycin forms a complex with FKBP12 and this complex binds the FRB domain of TOR (Figure 2.2). Rapamycin-FKBP12 complex inhibits TORC1 activity and this mechanism of action of rapamycin is conserved from yeast to mammals suggesting that biochemical steps affected by rapamycin are conserved [63]. Although rapamycin inhibits TORC1, TORC2

is insensitive to rapamycin because the rapamycin-FKBP12 complex does not bind to Tor2p when it is present in TORC2 [24, 55, 64]. It was reported that rapamycin does not directly block the kinase activity of mTOR, it rather alters the composition of mTOR complexes in order to prevent the upstream signals to integrate with the complex or the accessibility of the kinase to downstream substrates [62].

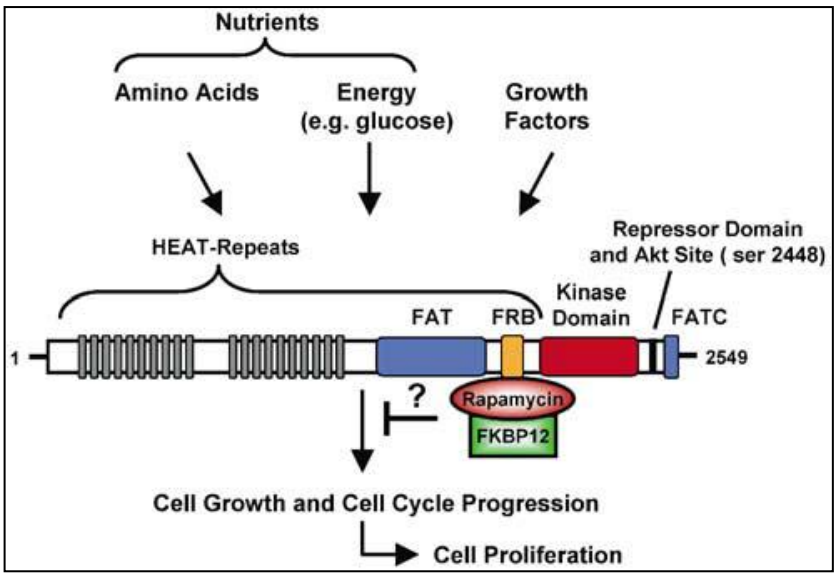

Figure 2.2. The domain structure of TOR [62].

Caffeine belongs to naturally occurring methylxanthine family and has pleiotropic effects on the respiratory, cardiovascular, nervous, and renal systems in humans. It is the most widely consumed stimulant reaching plasma concentrations of 50 μM with a moderate consumption of coffee. Plasma levels above 200 μM were reported to be toxic in humans. Caffeine is used medically in treatment of apnea of prematurity and is a component of various headache and pain remedies. In addition to rapamycin, caffeine has been reported to affect TOR signaling in many organisms including yeast, plants and mammals [65]. Earlier studies had suggested that caffeine targets cAMP phosphodiesterase. However, that TORC1, and not cAMP phosphodiesterase, was found as a major target of caffeine in later studies in yeast. Caffeine was reported to act as a low affinity ATP analog and several effects of caffeine on cell growth, DNA metabolism and cell cycle progression have been reported in budding and fission yeast [6].

High doses of caffeine (> 10 mM) was reported to result in mutagenic effects in yeast [66] and the inhibition of tumor formation and selective induction of apoptosis in existing tumors in mice [67]. In *S. cerevisiae* it was found that caffeine results in hypersensitivity in the mutants of the specific components of TORC1 (not TORC2) and this finding indicated that TORC1 is the direct target of caffeine as in the case of rapamycin [64]. Studies revealed that, the effects of caffeine were mainly on DNA repair and recombination pathways, cell morphology and integrity and intracellular calcium homeostasis [68, 69]. Although similar transcriptional responses were observed in response to rapamycin or caffeine treatments, the effects of rapamycin and caffeine were not completely the same [6]

Rapamycin treatment affects many cellular processes. Protein synthesis decreases upon rapamycin treatment since it blocks the translation initiation and alters the phosphorylation of the translation-related proteins [40, 70, 71]. A down-regulation in the expression levels of genes that are related with the protein synthesis was also reported in the caffeine treated cells [6]. TORC1 regulates ribosome biogenesis at transcriptional and post-transcriptional levels. Rapamycin represses the expression of ribosomal protein mRNAs by RNA polymerase II, inhibits ribosomal protein synthesis and results in a decrease in 5S rRNA, 35S rRNA, tRNA production and RNA Pol I-dependent transcripts. RNA Pol II and III are controlled by TORC1 via Sch9p and Maf1p proteins [30, 40, 72]. Like rapamycin, caffeine was also reported to result a down-regulation in the expressions of genes related to transcription, ribosome biogenesis and assembly [6]. TOR signaling pathway regulates the gene expression in yeast and mammals via mRNA stability. Rapamycin leads to mRNA destabilization and induces mRNA turnover [41].

Cell cycle is also regulated by TORC1. While addition of rapamycin was reported to causes G1 arrest, prolonged G2/M by inhibiting TORC1 activity [39], no arrest in any stage of cell cycle was observed in response to caffeine treatment in yeast [6]. But caffeine was reported to induce G1 delay in mammalian cells and it was shown to inhibit the G1/S progression in human fibroblast strains. Caffeine was also shown to reverse S and G2 checkpoint functions and reduce DNA repair in irradiated cells in human [73].

TOR pathway controls the nutrient signaling pathways in response to the quality of the carbon and nitrogen sources. The activity of TORC1 is regulated by nutrients but there

is no reported regulation for TORC2 by nutrient availability. TORC1 regulates the localization and the expression of permeases in response to nutritional conditions. Rapamycin affects the cell physiology in a way similar to nutrient starvation in yeast and mammalian cells. Rapamycin treatment results in a transcriptional response similar to low quality carbon source or glucose deprivation. Rapamycin represses the transcription of genes that are activated by glucose and induces the transcription of genes that are repressed by glucose [74, 75]. TORC1 regulates the transcription of nitrogen catabolite repression (NCR) genes and amino acid biosynthesis. Rapamycin treatment causes a similar response to nitrogen starvation in yeast and mammalian cells. Both caffeine and rapamycin induce the expression of genes involved in the Gln3p/Gat1p controlled NCR [6].

In addition to stimulating growth in response to nutrient availability, TOR signaling also suppresses stress response to promote growth. Rapamycin treatment activates stress genes since TOR controls the transcription factors *MSN2* and *MSN4* which induce the expression of genes that are responsive the stress conditions such as heat shock and H_2O_2 treatment [72, 76, 77]. Stress responsive genes were found to be differentially expressed in response to caffeine treatment in yeast [6].

Autophagy is also another important mechanism coordinated by TOR signaling both in yeast and mammalian cells. Rapamycin inhibition of TOR function stimulates autophagy even under nutrient rich conditions [78, 79]. Induction of macroautophagy was found to increase both replicative and chronological life span. Inhibition of TORC1 activity by rapamycin or caffeine extends chronological life span in yeast and this extension was proposed to be mediated by TORC1-Sch9p-Rim15p kinase cascade in yeast [80]. A life span extension was also reported in case of the inhibition of TORC1 activity in worms, flies and mice [30, 81–84]. The conservation of this cascade in higher eukaryotes and the correlation between coffee consumption and decreased risk of death makes this branch attractive for the lifespan extension studies in human.

Cell wall is crucial for cells to survive both under normal growth and stress conditions. Cell wall integrity pathway is responsible for the conservation of the homeostasis of this organization by regulating the expression of many cell-wall biosynthetic enzymes to remodel the cell wall according to the changing environmental

conditions [30, 54]. Both TORC1 and TORC2 have roles in maintaining the cell wall integrity pathway. Rapamycin or caffeine treatment causes the activation of cell wall integrity pathway indicating that TORC1 negatively regulates this pathway [6, 85, 86].

2.3. The Effects of Environmental Parameters; Oxygen Availability and pH

While studying the effects of drug agents on yeast metabolism the experimental conditions have great importance since the environmental conditions such oxygen availability and the pH of the growth medium have major effects on cell physiology and metabolism in all organisms.

Oxygen availability has a strong impact on yeast metabolism. Yeast has a capability to grow under fully anaerobic conditions and also ferment sugars into ethanol even when oxygen is abundant. As a result, yeast can switch its metabolism from respiratory to respiro-fermentative and fermentative mode according to the changing oxygen and nutrient availability. In aerobic respiration, TCA cycle becomes active and produces precursors for amino acid biosynthesis and cofactors NADH and $FADH_2$ for respiratory chain. In order to produce ATP, respiratory chain uses electrons from NADH and $FADH_2$. The respiratory chain is present in the presence and the absence of oxygen. Under anaerobic conditions ATP is generated through glycolysis and fermentation. In comparison to aerobic conditions, in the absence of oxygen, the plasma membrane of *S. cerevisiae* was reported to have more saturated fatty acids and less ergosterol [87]. Also the lack of oxygen results in a remodeling in the cell wall and membrane in order to adjust the porosity and import sterols and fatty acids [88]. In response oxygen levels different cell wall mannoproteins are synthesized and this process is controlled at the transcriptional level [89].

Although oxygen is essential for organism it has also harmful effects since reactive oxygen species (ROS) are produced by mitochondrial respiratory chain in the presence of oxygen. Accumulation of ROS results in oxidative stress which can damage the nucleic acids, proteins and lipids and oxidative stress affects many processes like apoptosis and aging [89, 90]. The pentose phosphate pathway has a crucial role in protection from oxidative stress [91].

The availability of oxygen results in major changes at transcriptional and post-transcriptional levels. The regulation of genes expression in response to oxygen depends also on the carbon and nitrogen sources [88]. Oxygen regulates the transcription of genes, especially those related to functions that need oxygen, such as respiration, heme, sterol and unsaturated fatty acid biosynthesis. Irrespective of the nutrient limitation, the lack of oxygen results in a response in the expression levels of genes involved in metabolism and energy, transport, cell rescue and defense, protein synthesis and cell wall organization [92]. The transcriptome and proteome analyses of aerobic and anaerobic yeast cultures showed that processes such as glycolysis, aminoacyl-tRNA, purine nucleotide and amino acid biosynthesis are regulated post-transcriptionally [93].

Not only the availability but also the level of oxygen affects different processes than the presence and absence of oxygen. Genes involved in respiratory pathway, TCA cycle, metal ion homeostasis and MAPK signaling pathways of mating and filamentous growth were found to be responsive to the intermediate levels of oxygen [94].

In addition to oxygen, regulation of internal pH is crucial for many cellular processes. In response to changing environmental conditions cells should maintain internal pH homeostasis in order to sustain processes such as transport, biosynthetic ad metabolic pathways and response to stress [95]. *S. cerevisiae* can tolerate a wide range of external pH, but it prefers acidic environment in which the cell proliferation occurs more rapidly and external alkaline pH results in a stress response. *S. cerevisiae* maintains the internal pH between 5.5 and 5.75 in an acidic environment such as pH is 3.0 or between 5.9 and 6.75 when the extracellular pH is between 6.0 and 10.0 [96]. Yeast plasma membrane H^+-ATPase plays an important role in the regulation of internal pH by creating a proton gradient. This gradient acts as a driving force for the transport of nutrients and cations [96–99]. Internal pH regulates the cAMP, the heat shock proteins and enzymes in glycolysis and gluconeogenesis [99]. Changes in pH affect protein folding, conformational stability of proteins and interactions between proteins, lipids and metabolites [100, 101]. Oxidation-reduction potentials of specific reductases [102] and dehydrogenases as well as the major components of membranes such as phospholipids, sterols, sphingolipids and glycolipids are affected by changing environmental pH [101].

While yeast cells are growing the culture media becomes acidic since the cells secrete organic acids including acetic acid which inhibits yeast growth and induces the chronological aging. It was reported that controlling the pH of the culture media at 6.0 significantly increased the chronological life span in yeast [103, 104]. Adjustment of the external pH to a value closer to the intracellular pH was reported to decrease the inhibitory effects of organic acids and the stress on the cells leading to less energy consumption for maintaining the intracellular pH at a value required for optimal growth [96].

Changes in the ambient pH result in an alteration in the expression levels of some genes. Alkaline pH was reported to induce the expression of genes involved in phosphate transport and metabolism, copper and iron transport and homeostasis. These responses resemble the transcriptional responses of the cells to the phosphate starvation or decreased copper or iron availability [97].

The pH response of the yeast is found to be regulated by a number of signaling pathways. The Rim101p pathway, Slt2p MAP kinase and calcium-mediated pathways were reported to be involved in the regulation of pH response. Rim101p is a transcriptional repressor which has roles in both positive and negative regulation. In addition to regulation of alkaline pH adaptation, it positively regulates sporulation, filamentous and invasive growth and ion homeostasis [105]. Rim101p is also involved in cell wall assembly together with the Slt2p which is activated by protein kinase C and extracellular alkaline pH [106]. Calcium signaling also plays a role in the transcriptional response to high pH, since the expressions of *ENA1* and *PHO89* are regulated by calcineurin in response to alkaline stress [97].

The processes regulated in response to changes in intra and extracellular pH were also investigated widely in higher eukaryotes. It was reported that in mammalian cells the increase of intracellular pH promotes cell proliferation, the timing for G2/M entry and transition [107] as well as cytoskeleton assemblies. The decrease in the intracellular pH was observed in the cells undergoing apoptosis [108]. Intracellular and extracellular pH affects the ability of the tumor cells to migrate and hence to metastasize [109]. These finding indicate the important role of pH in cancer and the possible usage of acidification as a therapeutic tool in treatment of cancer [110].

3. MATERIALS AND METHODS

3.1. Strain and Growth Conditions

Homozygous *ho*Δ*/ho*Δ strain of *S. cerevisiae* BY4743 (*MAT*a*/MAT*Δ *his3*Δ*1*/*his3*Δ*1 leu2*Δ*0*/l*eu2*Δ*0 lys2*Δ*0*/+ *met15*Δ*0*/+ *ura3*Δ*0*/*ura3*Δ*0*), purchased from the European *Saccharomyces cerevisiae* Archive for Functional Analysis was used in this study. The absence of the deleted gene was verified using PCR-based methods [111]. Cells were cultured overnight in YPD medium (2% (w/v) D-glucose, 2% (w/v) peptone, 1% (w/v) yeast extract) at 30 °C in an orbital shaker at 180 rpm prior to the fermentations. All other cultures were grown in defined synthetic medium [112].

3.2. Determination of the Working Concentrations of Rapamycin and Caffeine

The optimal rapamycin and caffeine concentration of the culture medium was determined by growing the cells in identical shake flasks with 1:7 working volume to total volume ratio containing a gradient of rapamycin or caffeine concentrations. The rapamycin stock solution with a concentration of 1mg/ml was prepared by dissolving rapamycin powder (Sigma Cat. No: R0395) in 90/10 (v/v) ethanol/Tween-20 solution. Rapamycin was introduced into the medium either in the beginning prior to the inoculation or during the mid-exponential phase of the culture growth to achieve final concentrations of 0.5, 2, 20 or 200 nM. Caffeine (Sigma Cat. No: C8960) was introduced to the medium prior to inoculation or during the mid-exponential phase of the culture growth to achieve final concentrations of 0.5, 5, 10 and 20 mM. The cell growth was monitored in terms of optical density at a wavelength of 600 nm using a spectrophotometer (DU730, Beckman Coulter Inc., U.S.A).

Biomass concentrations were determined gravimetrically. The longevity analysis was carried out on solid YPD and synthetic defined media by inoculating 10µl of 1:20 diluted culture samples. The extent of survival of the cells was determined based on colony formations.

3.3. Batch Cultivations, Sampling and Determination of Growth Characteristics

Batch cultivations were carried out in 2L B-Braun Biostat B Plus fermenters in duplicates for the fermentations that were carried out in order to investigate the effects of rapamycin and caffeine together with aeration and pH control with 1.5 L working volume. The batch cultivations that were carried out in order to investigate the dose effects of rapamycin were carried out in triplicates under micro-aerated and pH controlled conditions. Temperature was kept constant at 30 °C and the rate of agitation was 800rpm. The dissolved oxygen (DO) saturation was kept above 90% throughout the experiment via a constant flow of air at 1.5 L/min in aeration controlled cultivations. Micro-aerated cultivations were initially brought to a dissolved oxygen saturation of 100% and air supply was blocked at all times throughout the fermentation. pH was controlled at 5.5 with 0.5 M NaOH and HCl in pH controlled fermentations. Sampling was carried out at mid-exponential phase of growth at an OD range of 0.6-0.8. Cells were washed twice with distilled water followed by centrifugation for the gravimetric determination of biomass concentration. Samples harvested for the transcriptome analysis were immediately frozen in liquid nitrogen and were stored at -80 ^{o}C until RNA isolation.

3.4. RNA Isolation and Transcriptome Analysis

RNA was isolated in a robotic workstation, QIAcube (Qiagen, USA) using the enzymatic lysis protocol as described by Qiagen RNeasy mini kit (Cat no: 74106). The quantity and the purity (A_{260}/A_{280}) of the RNA were determined using a micro-volume UV-vis spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific Inc., U.S.A). RNA integrity was checked using a microfluidics-based platform (Bioanalyzer 2100 Agilent Technologies, USA) using RNA6000 Nanokit (Agilent Technologies, USA). First-strand cDNA was synthesized and then converted into a double-stranded DNA initially from ca.100ng of total RNA using GeneChip® 3' IVT Express Kit (Affymetrix Inc., U.S.A). This double stranded cDNA was used as a template for *in vitro* transcription and synthesis of biotin-labelled aRNA. The final product was purified using clean up kits and it was quantified using the Nanodrop spectrophotometer before fragmentation. Fragmented aRNA was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). A total of 5µg of quality checked aRNA was loaded onto 169 format

Affymetrix Yeast 2.0 arrays. The chips were then loaded into a fluidics station for washing and staining using Affymetrix Command Console® Software (AGCC) 3.0.1 Fluidics Control Module with Mini_euk2v3. All applications were performed as described in the Affymetrix GeneChip® Expression Analysis Technical Manual. Finally, the chips were loaded onto the Affymetrix GeneChip Scanner 3000**.**

3.5. Microarray Data Acquisition and Analysis

The data was processed, normalized and quality assessed using dChip software [113]. Hierarchical clustering was carried out using MultiExperiment Viewer (MeV) [114]. Pearson correlation was used as the distance metric and average linkage was used as the linkage metric.

The significance of gene expression was evaluated using analysis of variance (ANOVA) at a significance threshold of p-value=0.01. In order to analyze the effects of three factors (drug, D (R for rapamycin, C for caffeine), aeration (A) and pH control (P)) on the transcriptional response of yeast cells, 2x2x2 factorial design was used. Each of the three factors had two levels; drug presence vs. control, fully-aerated vs. micro-aerated and pH=5.5 vs. pH monitored. A three-way ANOVA model was fitted to take into consideration all possible main effects and their interaction effects on gene expression. The genes with the significant (p-value <0.01) 3-way interaction coefficient (DxAxP) were regarded as the genes that were responsive to the changes in each factor at the same time. The binary interaction effects were identified fitting a 2-way ANOVA model to the genes with insignificant 3-way interaction effects. The genes with significant 2-way interaction coefficients (DxA, DxP and AxP) were identified as the genes that were responsive to the combinatorial changes in the respective sets of those two factors. A one-way model was fitted to the expression levels of the genes with insignificant 2-way interaction coefficients in order to identify the single effects of each factor (D, A, P) on gene expression [115]. The genes with the significant drug-related interactions coefficients (DxA, DxP, DxAxP) and the significant D coefficient were pooled to identify the set of genes that were affected by the corresponding drug. The genes were classified as significant if the p-value was less than 0.01 and fold change was greater than 1.5.

The significance of gene expression was evaluated using student's t-test for the triplicate sets of batch fermentation that were carried out to investigate the effects of rapamycin dosage. The genes were classified as significant if the p-value was <0.05. The significantly enriched GO biological process terms were determined via AMIGO [116].

3.6. Metabolite Analysis, Sample Preparation, LC-MS/MS Protocol and Enzymatic Method

Extraction of extra and intracellular metabolites was carried out as described previously [117]. Samples were subjected to methanol quenching and immediate centrifugation. The supernatant was used for the analysis of extracellular metabolites. Boiling ethanol procedure was used in order to obtain intracellular metabolites. The extracts were lyophilised and stored at -20 °C until further analysis. The supernatant of the culture medium was stored at -20 ^{o}C for exo-metabolic measurements.

3.6.1. Determination of the Amino Acids Concentration

Chromatographic separation was performed using an Agilent 1260 Infinity UPLC (USA) with a 4.6 x 100 hydrophilic interaction chromatography (HILIC) column (Zorbax Rapid Resolution, particle size 3.5 µM). The initial composition of the mobile phases were 15% (A) containing 40 mM ammonium formate with 2% formic acid and 85% (B) acetonitrile followed by a linear gradient to 85% A and 15% B in 13 minutes with a 2 minutes hold and 4 minutes of re-equilibration of the column to its initial conditions prior to the next run, thus a total assay time for each assay was 19 minutes. The flow rate was 0.4 µL/min and 2 µL of sample was injected.

The amino acids standards were obtained from Sigma-Aldrich, Inc. (USA). The optimal MS/MS MRM transitions, fragmentation patterns and the retention time behaviours of each amino acid were determined using Agilent 6430 Triple Quadrupole LC-MS (USA). The MS parameters were as follows: gas temperature at 300 ^{o}C, gas flow 10 L/min, nebulizer pressure 40 psi, the capillary current at 3750 V. The ESI probe was operated at positive ion mode.

3.6.2. Determination of the TCA Cycle Metabolite Concentrations

Chromatographic separation was performed using an Agilent 1260 Infinity UPLC (USA) with a 4.6 x 150 Eclipse XDB column (Zorbax Rapid Resolution, particle size 5 µM). The initial composition of the mobile phases were 15% (A) containing 10 mM ammonium acetate adjusted to pH 8 using ammonium hydroxide and 85 % (B) acetonitrile with 0.1% formic acid, followed by a linear gradient to 85% A and 15% B in 4 minutes and back to its original composition immediately after 4 minutes with a hold run for another 6 minutes for the re-equilibration of the column prior to the next run, thus a total assay time for each assay was 10 minutes. The flow rate was 0.4 µL/min with a 2 µL sample injection.

The TCA cycle standards were obtained from Sigma-Aldrich, Inc. (USA). The optimal MS/MS MRM transitions, fragmentation patterns and the retention time behaviors of each amino acid were determined using Agilent 6430 Triple Quadropole LC-MS (USA). The MS parameters were as follows: gas temperature at 300^{o}C, gas flow 10 L/min, nebulizer pressure 40 psi, the capillary current at 3750 V. The ESI probe was operated at negative ion mode.

The quantitation of the samples was acquired using MassHunter Quantitative Analysis Software, version B.05.00 (Agilent Tech. Inc., USA) against calibration curves generated with standard solutions.

Enzymatic assay kits purchased from Boehringer-Mannheim were used to determine D-glucose, ethanol, glycerol and ammonia concentrations in the supernatant.

3.7. Identification of Reporter Transcription Factors and Proteins

In order to identify the key transcription factors responsive to the presence of rapamycin or caffeine Reporter Features Algorithm was used [118]. For this purpose the yeast transcription factors (TF) that are present in YEATRACT TF-Consensus list [119] and the proteins that are annotated with transcription factor or regulatory activity in SGD [120] were collected. The target genes known to be regulated by these TFs, with a reported

direct evidence, retrieved from YEASTRACT [119]. The constructed yeast Transcriptional Regulatory Network (TRN) includes 129 TFs and 24778 TF-gene interactions. The key TFs identified by Reporter Features Algorithm were ranked according to their p-values and the top 10 scoring TFs were regarded as key TFs, around which most transcriptional changes occur.

Regulation of key TFs was evaluated based on whether the TFs are significantly expressed or not. There are two possible cases; if the key TF is significantly expressed, it is mainly transcriptionally governed or if the key TF is not significantly expressed, it is mainly post-transcriptionally regulated.

In order to identify the key proteins around which significant transcriptional changes occur in respose to the presence of 2 nM or 200 nM rapamycin or 5 mM caffeine the yeast physical protein-protein interactions were retrieved from BioGRID and integrated with transcriptome data using reporter features algorithm [118]. The constructed yeast PPI network includes 5633 proteins and 75584 interactions. A p-value threshold of 0.05 was used to define reporter proteins.

4. RESULTS AND DISCUSSION

The target of rapamycin (TOR) signalling is an evolutionarily conserved serine/threonine kinase that regulates cell growth and proliferation in response to nutrient quality. TOR controls translation, transcription, ribosome biogenesis, cell cycle, autophagy and aging. Rapamycin is an immunosuppressive and anti-proliferative antibiotic that targets and inhibits the TOR kinase. Although rapamycin is an immunosuppressant, which is frequently used in organ transplant patients, it is also preferred in anti-tumor therapies owing to its anti-angiogenetic properties. In addition to rapamycin, caffeine which is a widely used psychoactive drug also targets and inhibits TOR function. During the treatments with these two drugs, parameters such as the oxygen levels and pH of the blood as well as the drug dosage should be carefully evaluated. In this study, the effects of long-term administration of rapamycin or caffeine on yeast growth and longevity were investigated as a function of oxygen and pH control. In addition, the effects of the dosage of rapamycin on transcriptional and metabolic responses of yeast were examined with an integrative approach.

4.1. Long-term Adaptation Characteristics of Yeast to the Presence of Rapamycin or Caffeine

In this study, the effects of rapamycin and caffeine on yeast growth and longevity were investigated. A detailed analysis was carried out to evaluate the changes in yeast metabolism under different environmental conditions incorporating the transcriptional and metabolic growth characteristics. The effect of pH and the presence of oxygen were thus investigated in response to the presence of a chemical stimulus; rapamycin or caffeine. For this purpose, batch cultures of wild type yeast cells were grown in shake flasks in order to determine the administration regime and the dosage of rapamycin and caffeine. Then the cells were grown in controlled fermentations, in the absence or presence of pre-determined doses of rapamycin or caffeine under different environmental conditions. The metabolic and the transcriptomic response of yeast cells were analyzed during mid-exponentail phase of growth along with the growth characteristics of the fermentations.

4.1.1. Investigation of the Growth Characteristics of Yeast Populations in Response to Rapamycin or Caffeine Administration

Rapamycin is a macrolide, which was used as an immune suppressant and anti-tumour agent in organ transplantation and cancer treatment. The long-term administration of rapamycin in humans was significantly associated with systemic toxicity in the metabolism [121]. Yeast is one of the simplest eukaryotic organisms but many essential cellular processes are conserved between yeast and humans and it is used to model eukaryotic cell behaviour in higher organisms and this characteristic may in fact facilitate the investigation of the response of human cell lines to rapamycin, used either as a chemotherapeutic agent or as an immunosuppressant.

The mode of action of rapamycin as an immunosuppressant involved the targeting of newly formed cell populations and their destruction via disrupting cytokine signalling, which promoted lymphocyte growth and differentiation [122]. Furthermore rapamycin and its derivatives were reported to be selectively supersensitive on tumours with specific characteristics [123–125].

The effect of the administration regime of rapamycin on yeast cell population was first investigated in batch cultures in the present study. For this purpose, the effect of rapamycin on growth and survival of wild type yeast populations was investigated in rich medium (YPD), where a complex cocktail of nutrients was provided, or in defined medium (F1), where sufficient amounts of the necessary nutrients were individually supplied in their simpler molecular forms. The effect of drug administration was investigated in yeast populations, in which the drug was introduced either during the mid-exponential phase (exp) of culture growth or prior to the inoculation of cells (t=0).

The growth profiles of the cultures were then followed and periodic sampling was carried out for longevity analysis by transferring culture samples of nearly equal population size (ca. $2x10^5$ cells) on to solid medium (YPD-agar and F1-agar plates) at regular intervals. The viability of the population was evaluated after incubation for 2 (in rich medium) or 3 (in defined medium) days.

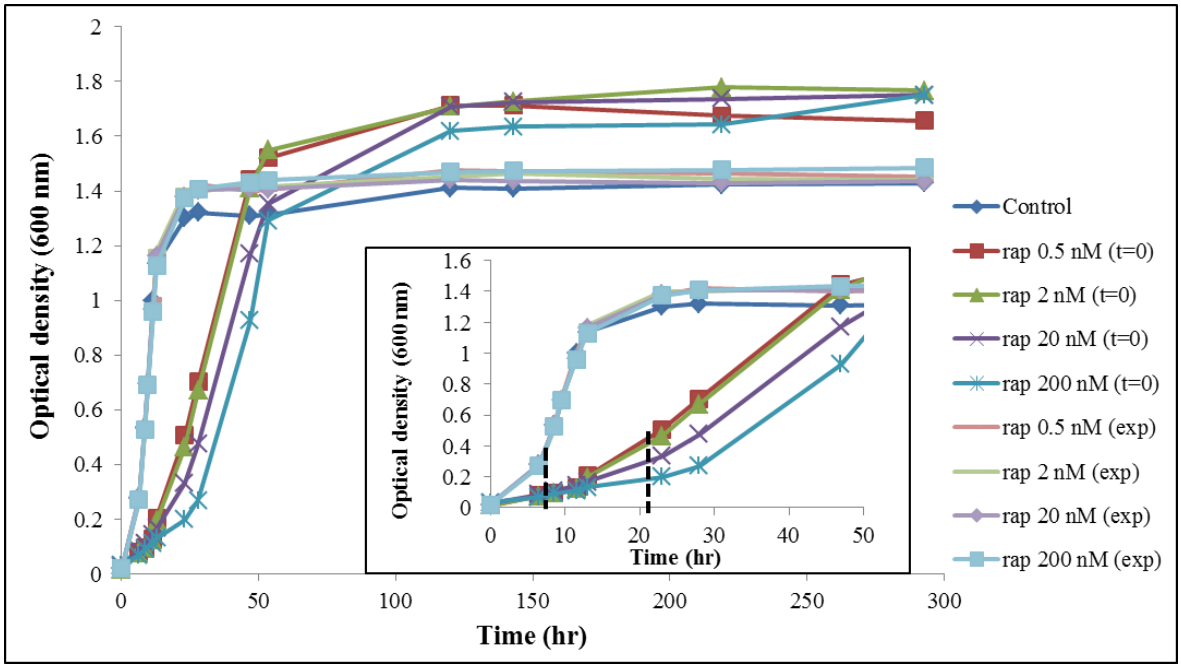

Figure 4.1. The growth profiles of the cultures in rapamycin (rap) containing media (t=0) or treated with rapamycin at the mid-exponential phase of the growth (exp).

A number of studies, investigating the effects of rapamycin on yeast metabolism were available and a range of working concentrations of rapamycin (0.1nM-10μM) was reported in the yeast literature [55, 126–128]. A stricter range of rapamycin concentrations between 0.5 nM and 200 nM was tested in the present study. The cells, which were treated with rapamycin at the mid-exponential phase of growth, displayed very similar growth profiles to the control fermentations that were lacking any drugs. The growth profiles of the cells were showed in Figure 4.1 and the separate small figure embedded in the main figure shows the first 50 hours of the cultivations (Figure 4.1). The dashed lines indicate the induction of the exponential phase. The cells that were exposed to rapamycin from inoculation onwards underwent longer lag phases (ca. 15 hours longer), had 80% lower maximum specific growth rates ($\mu_{max,avg,(t=0)}$=0.05; $\mu_{max,avg,(exp)}$=0.254) and reached significantly higher optical densities ($OD_{600,avg}$=1.73, p-value= $5.9x10^{-5}$) at stationary phase of growth than the control populations and the cells treated with rapamycin at the mid-exponential phase of growth ($OD_{600,avg}$=1.45) (Figure 4.1, Table 4.1) whereas the addition of rapamycin into the culture, during its steady phase of growth, did not cause any difference in the final cell population density. These results indicated that administration of rapamycin from early stages of population growth resulted in a lower maximum specific growth rates and allowed the culture to achieve a higher optical density.

Table 4.1. The optical density and maximum specific growth rates of the cultures grown in the absence and presence of different concentrations of rapamycin or caffeine (t=0) or treated at the mid-exponential phase of growth (exp).

Culture	**Stationary phase OD (600 nm)**	**μ_{max} (hr^{-1})**
Control	1.430	0.253
Rap 0.5 nM (t=0)	1.657	0.042
Rap 2 nM (t=0)	1.766	0.044
Rap 20 nM (t=0)	1.750	0.051
Rap 200 nM (t=0)	1.750	0.062
Caf 0.5 mM (t=0)	1.429	0.246
Caf 5 mM (t=0)	1.549	0.226
Caf 10 mM (t=0)	1.458	-
Caf 20 mM (t=0)	1.172	-
Rap 0.5 nM (exp)	1.451	0.257
Rap 2 nM (exp)	1.441	0.260
Rap 20 nM (exp)	1.436	0.247
Rap 200 nM (exp)	1.490	0.254
Caf 0.5 mM (exp)	1.460	0.257
Caf 5 mM (exp)	1.393	0.242
Caf 10 mM (exp)	1.311	0.239
Caf 20 mM (exp)	1.152	0.228

The longevity of the cell populations indicated variations with respect to the drug dosage, drug administration regime or the availability of complex nutrients in the medium. Regardless of the drug concentration or the type of medium in which cells were grown, healthy cell populations undergoing optimal growth, i.e. during exponential phase of culture growth, were observed to maintain their viability following the administration of rapamycin even during the 12^{th} day of growth (Figure A.2 and Figure A.4), which would approximately correspond to 150 generations with an approximate doubling time of 2 hours for diploid cells [129]. It was previously reported that mechanisms that would lead yeast cells to go through adaptive evolution would come in to action after 150 generations

[130]. Evolutionary response of yeast to rapamycin is beyond the scope of this study and was therefore not attempted to be investigated.

On the other hand, the viability of the cultures that were inoculated in rapamycin containing media displayed variations both with respect to the concentration of the drug that was present and to the type of medium onto which the cells were transferred (Figure A.1, Figure A.2, Figure A.3 and Figure A.4). Although the viability was maintained in control populations that were grown without rapamycin, the viability of the cultures grown in the presence of rapamycin was diminished by the 12^{th} day of growth. The only exceptions to this were the samples grown in defined medium in 0.5 nM rapamycin and the samples grown in rich medium in the presence of 200 nM rapamycin (Figure A.1 and Figure A.3). This result might indicate that in rich medium, the cells would have switched on the required mechanisms to condition themselves to survive in the presence of rapamycin at high concentrations.

This conditioning was further investigated to assess whether the adaptation could be the result of a mutation or of a genetic switch. Cells grown in the control environment or in the presence of 2 nM or 200 nM rapamycin were first tested for their survival on the 10^{th} day. Fresh control culture, 2 nM and 200 nM rapamycin containing cultures were inoculated with single colonies, which grew from the viable cells of their respective initial cultures and yet the presence of 200 nM rapamycin in the environment did not cause any shortening in the lag phase or a change in maxiumum specific growth rate. The results ruled out the possibility of mutational or genetic adaptation mechanisms playing a role in this conditioning. The consecutive use of 2 nM rapamycin in the culture medium, on the other hand, caused the lag phase to be shortened slightly, which might provide an adaptive advantage over a period of time (Figure 4.2). In Figure 4.2, Control, Rap2 and Rap200 indicate the cultures that were inoculated into fresh medium without any drug from the control culture as well as cultures grown in 2 nM or 200 nM rapamycin, respectively. Control (Rap 200) indicates the inoculation of a single colony from the control culture into a culture with 200 nM rapamycin supplement. Rap2 (Rap2) and Rap200 (Rap 200) indicates the inoculation of a single colony from the cultures that were grown in the presence of 2 nM or 200 nM rapamycin into cultures containing 2 nM or 200 nM rapamycin, respectively.

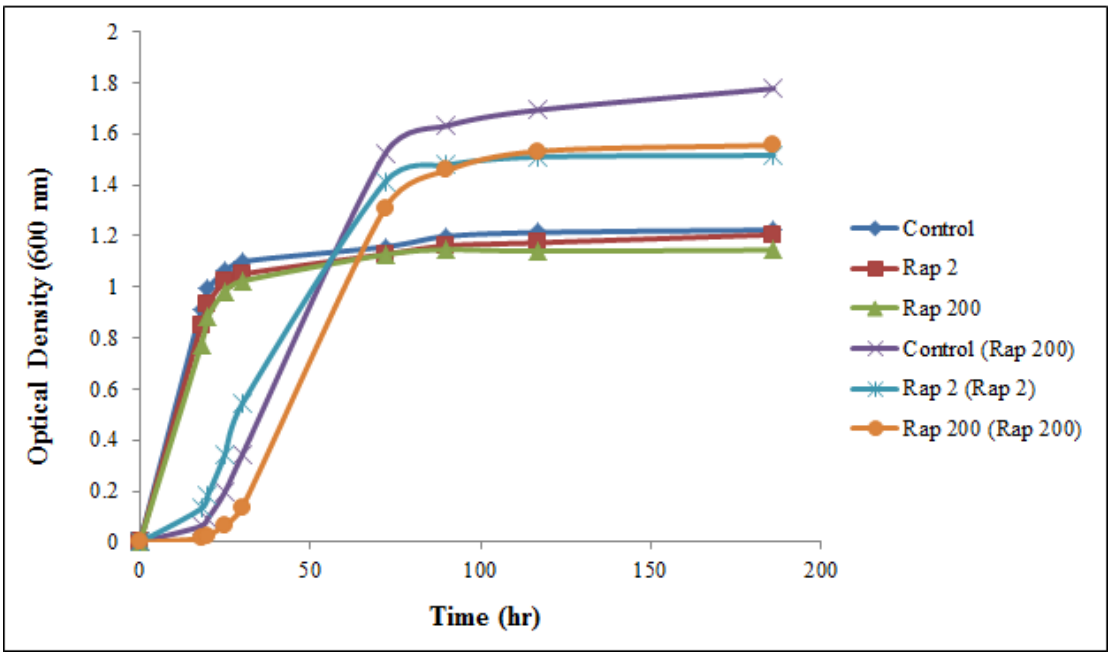

Figure 4.2. The growth profiles of the single colonies which grew from the viable cells of their respective initial cultures at the 10^{th} day of growth.

Yeast is regarded as a model organism for cancer studies since the proliferation rate and metabolisms of yeast and cancer cells were reported to be similar [5, 131]. Therefore, if a yeast population growing at a steady rate of growth (exponential phase) could be considered as being analogous to rapidly proliferating tumor cells, the administration of rapamycin was shown to affect neither the viability of the population itself nor its maximum specific growth rate during the exponential phase of growth regardless of the concentration of the administrated drug.

On the other hand, a limited number of yeast cells, which were allowed to grow and reproduce in the presence of rapamycin could be considered as an analogous population to an initiating or post-operational tumour cell population. Investigating the response of this population to the presence of varying concentrations of rapamycin in complex or defined media would be interesting from a therapeutic perspective. The exposure of cells to an optimum minimal dose of rapamycin from early stages of growth onwards was shown to be effective in reducing their viability when low drug concentrations were used. This would point towards the efficacy of rapamycin as a chemotherapeutic agent acting effectively on low tumour loads or as an immunosuppressant administered prior to the

accumulation of high antibody loads that would cause organ rejection since rapamycin was shown to impair immune reactivity at the earliest stages in mammalian cell lines [132].

Therefore a low concentration, at which rapamycin would be effective during early stages of population growth (2 nM), was considered to be of potential interest in further analyses. The transcriptional and metabolic response of yeast to the presence of 2 nM rapamycin as well as its growth characteristics were investigated under different environmental conditions.

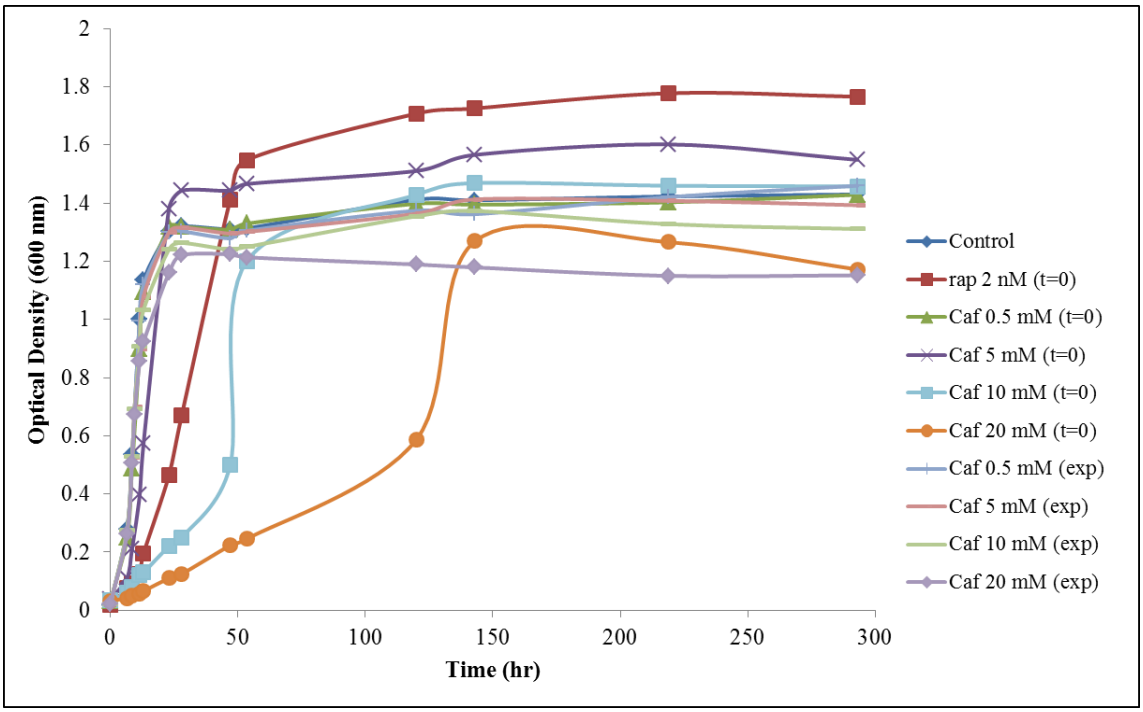

Figure 4.3. The growth profiles of the cultures in caffeine containing media (t=0) or treated with caffeine (cap) at the mid-exponential phase of the growth (exp).

An optimal caffeine dosage yielding a similar viability profile to that of the population grown in the presence of 2 nM rapamycin was determined in order to investigate the effects of caffeine on yeast population growth and longevity. The cells were exposed to 0.5 mM, 5 mM, 10 mM and 20 mM concentrations of caffeine either from the beginning of the fermentation or at the mid-exponential phase of growth in a similar manner to the investigation of the dosage effect of rapamycin. Exposing yeast cells to caffeine at the mid-exponential phase of growth did not result any changes in the maximum specific growth rates in comparison to control cells as in the case of rapamycin.

Addition of 0.5 mM caffeine to the cultures prior to inoculation did not yield any growth rate decrease and the presence 10 mM or 20 mM caffeine that were added prior to inoculation probably resulted in a toxic effect since the cells followed an unusual growth pattern. The viability of the cells treated with caffeine prior to inoculation was followed by inoculating them on synthetic defined media without any drug supplement. The viability of the cells grown in the presence of 10 or 20 mM caffeine was observed to be diminished both at the 9^{th} and 12^{th} days of growth. This may also indicate a toxic effect of high dose of caffeine. High doses of caffeine (>10 mM) was previously reported to result in mutagenic effects in yeast [66]. The presence of 0.5 mM caffeine did not result any differences in the viability of the cells. 5 mM caffeine was observed to diminish the viability of the cells at the 12^{th} day of growth (Figure A.5). The final cell densities of the caffeine-treated cultures were not as high as those of rapamycin-treated cultures. Nonetheless denser populations were achieved when the cells were grown in 5 mM caffeine. The optimal working concentration and the optimal administration regime were thus determined as 5 mM of caffeine added to growth medium prior to the inoculation of cells (Figure 4.3).

4.1.2. The Mechanism of Action of Rapamycin or Caffeine in Yeast in Response to Oxygen Availability and pH

Cellular proliferation, DNA damage response and apoptosis were reported to play significant roles in mechanisms involved in tumour formation and growth and both intracellular and extracellular pH and the availability of oxygen were reported to be key criteria in functioning of these pathways [110, 133–135]. Therefore, understanding the intracellular events in response to variations of these parameters in the presence of rapamycin or caffeine might provide clues on how the drug efficacy would be improved or whether if suggestions could be made towards achieving an optimized drug administration regime. A multi-factorial (2x2x2) design structure was used to investigate the effects of oxygen availability and pH maintenance on the growth characteristics in the presence of caffeine (5 mM) or rapamycin (2 nM) and to investigate how the transcriptional and metabolic response of yeast was altered. Batch fermentations were conducted in duplicates under different environmental conditions controlling the pH at 5.5 or only monitoring its course, saturating the culture with dissolved oxygen (dO_2 ≥85%) or leaving it micro-aerated. These environmental conditions were abbreviated as follows; AP (culture was

aerated and pH was kept at 5.5), MAP (culture was micro-aerated and pH was kept at 5.5), ANP (culture was aerated and pH was not controlled) and MANP (culture was micro-aerated and pH was not controlled). The response that was observed in the growth characteristics as well as the metabolic and the transcriptomic response of yeast cells were determined as a function of these parameters and the interactions among them in the presence of rapamycin or caffeine (Figure 4.4).

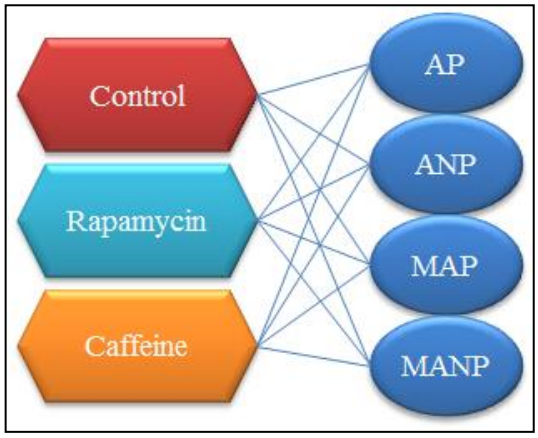

Figure. 4.4. Schematic diagram of the experimental parameters.

4.1.2.1. The Growth Characteristics of Batch Fermentations**.** The growth characteristics of the batch cultures were investigated during the exponential and stationary phases of growth taking average maximum specific growth rates and the biomass concentrations of the cultures into consideration (Table 4.2). The gravimetrically determined biomass concentrations were normalized with respect to the cell density (OD_{600}) (Figure 4.5). The maximum specific growth rates of the cultures were evaluated during exponential phase. The introduction of rapamycin or caffeine rendered it low under all conditions with the exception of MANP. The maximum specific growth rate was as low as 79% or 70% of the control populations in the presence of either rapamycin or caffeine, respectively, in aerated cultures.

The biomass density normalized with respect to cell concentration was lower for the cultures growing in the presence of rapamycin except for the case of MANP; the micro-aerated cultures without any pH control and these findings were complementary to maximum specific growth rate observations (Figure 4.5). Nakashima *et al*. reported decreased cell size in response to rapamycin treatment of yeast cells in shake flasks, which

would generally be in accordance with the present findings if cell size and cell mass would be considered as proportionate [136].

Table 4.2. Average maximum specific growth rates ($\mu_{max,avg}$) and biomass concentrations of the cultures at the mid-exponential (MEP) and stationary phases (SP) of growth (SD: standard deviation).

Conditions		**$\mu_{max,avg}$±SD (hr^{-1})**	**MEP Biomass ±SD (g/L)**	**SP Biomass ±SD (g/L)**
AP	**Control**	0.27±0.00	0.43±0.01	1.41±0.05
	Rapamycin	0.20±0.06	0.33±0.03	1.05±0.05
	Caffeine	0.08±0.01	0.55±0.05	1.61±0.09
ANP	**Control**	0.38±0.01	0.74±0.02	1.32±0.02
	Rapamycin	0.08±0.00	0.46±0.11	1.10±0.04
	Caffeine	0.12±0.00	0.38±0.08	1.46±0.04
MAP	**Control**	0.32±0.01	0.69±0.01	1.41±0.11
	Rapamycin	0.31±0.01	0.50±0.07	1.17±0.07
	Caffeine	0.10±0.01	0.53±0.03	1.29±0.14
MANP	**Control**	0.18±0.01	0.17±0.01	0.61±0.01
	Rapamycin	0.32±0.00	0.28±0.02	0.89±0.06
	Caffeine	0.19±0.04	0.64±0.06	1.25±0.05

However, the micro-aerated fermenter lacking any control of extracellular pH; the only setup in the current design, which would closely mimic the shake flask cultivations in the study of Nakashima *et al*., failed to comply reaching increased unit mass per cell in the presence of rapamycin in comparison to that of the wild type [136]. On the contrary, supply of oxygen, control of pH or the interacting effect of these two parameters change the cell mass drastically and result in a decreased mass per individual cell. In aerated cultures with extracellular pH control and in micro-aerated cultures lacking extracellular pH control, the presence of caffeine also caused an increase in normalized mass per cell measurements. These findings were in accordance with increased cell size observations regarding caffeine treated cells as previously reported [6]. Although the maximum specific growth rates of the cells cultivated in the presence of caffeine were 40% lower than those of cultivated in the presence of rapamycin in MANP cultures, the weight per cell

normalized by the OD values as well as the total cell density of this population was observed to be higher than even that of the control cultures. This might indicate that the caffeine possibly blocked the proliferation of the cells although the cellular material was replicated within the cell. Although no arrest in any stage of cell cycle was reported in response to caffeine treatment in yeast [6], previous studies investigating the effect of caffeine on tumour cells reported the inhibition of cellular proliferation accompanied by G0/G1 [137, 138] or G2/M cell cycle arrest [139].

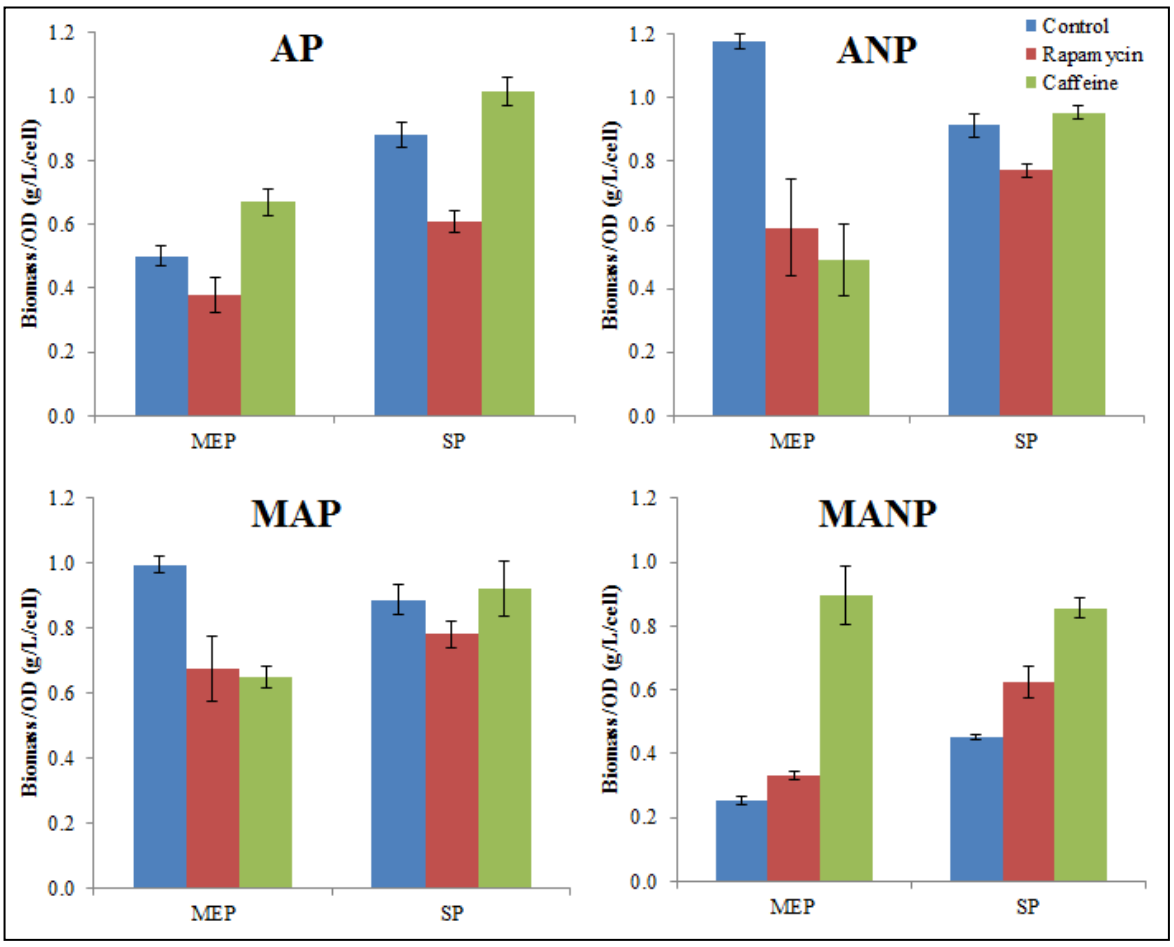

Figure 4.5. The normalized biomass concentrations of the cultures at the mid-exponential (MEP) and stationary (SP) phases.

The normalized biomass concentrations of the cultures were observed to be higher during stationary phase than their values in exponential phase indicating the presence of heavier cells, the only exceptions being caffeine treatment in MANP cultures and control fermentations in ANP and MAP cultures (Figure 4.4). The micro-aerated cultivation without controlling pH (MANP) was the environment simulating shake flask conditions

more closely. However while the extension of lag phase that was observed in the presence of either rapamycin or caffeine in shake flasks, it was not observed in the fermenters in the case of cultures grown in the presence of rapamycin. Further environmental simulations were carried out lowering the rate of agitation in the fermenter from 800 rpm to 400 rpm to approach shake flask conditions (180 rpm). The lag phase and the exponential phase were observed to spread over time and the maximum specific growth rate was observed to be lower as the rate of agitation decreased (Figure 4.6). The results indicated that the rate of agitation was an important factor in the adaptation of yeast cells in the presence of rapamycin or caffeine. The findings also established a cautionary ground in comparing and contrasting the growth characteristics in the present fermentations with previously reported shake flask experiments [6, 74, 140].

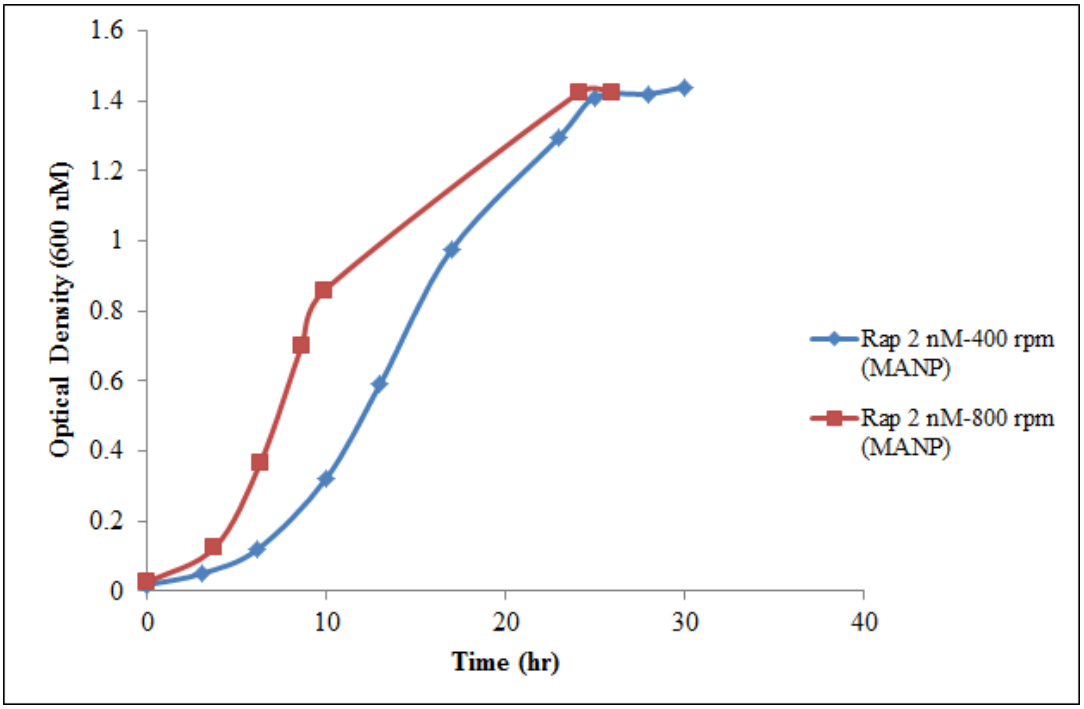

Figure 4.6. The effects of agitation speed on cell growth in the presence of rapamycin.

4.1.2.2. Analysis of the Global Transcriptional Responses of Yeast Cells to the Presence of Rapamycin or Caffeine. Exposing yeast cells to either rapamycin or caffeine (Rap; rapamycin treated, Caf; caffeine treated, C; control), controlling extracellular pH (P; pH controlled, NP; no pH control) or dO_2 levels (A; aerated, MA; micro-aerated) resulted in 12 different transcriptional profiles for the yeast cell cultures during the exponential phase of growth taking into consideration the average of the replicate data values.

The transcriptional profiles of the samples were hierarchically clustered using Pearson correlation as the distance metric and each fermentation was denoted by the experimental conditions under which the cells were cultivated; drug treatment (Rap; rapamycin treated, Caf; caffeine treated, C; control), pH control (P; pH maintained at 5.5, NP; no pH control) and aeration (A; aerated, MA; micro-aerated). The dashed line visually separates cultures grown in caffeine from those grown in rapamycin and the control cultures. The boxes distinguish the subgroups identified by controlling the pH at 5.5 (green) or without any control (red) (Figure 4.7).

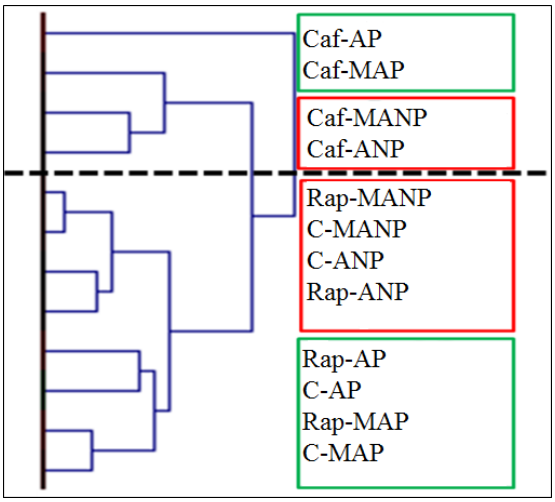

Figure 4.7. Hierarchical clustering of the experimental design parameters with regard to the transcriptional profiles.

The hierarchical clustering results revealed that the transcriptional profiles of the cells, which were treated with rapamycin, were clustered together with the corresponding control cultures that were carried out at the same environmental conditions. The addition of caffeine resulted in a set of transcriptional changes that would locate the samples in separate clusters except for the aerated cultures with pH control (AP), which was hierarchically connected as the most distant sample from all other conditions. These results indicated that the transcriptional response of yeast cultures growing in the presence of caffeine was more distinct than those of the control cultures or the cultures growing in the presence of rapamycin. The effect of oxygen availability and pH control was observed to be less decisive on the transcriptional response than the presence of the drug. The control of extracellular pH was more effective than aeration since the genome-wide transcriptional

profiles of the cells grown in the absence and presence of rapamycin or caffeine were clustered according to the pH level.

4.1.2.3. Analysis of the Metabolic Responses of Yeast Cells to the Presence of Rapamycin or Caffeine. The extracellular metabolite snapshots indicated that the yeast consumed glucose faster if they were allowed to cultivate in the presence of rapamycin and even faster in the presence of caffeine except for the culture grown in the presence of caffeine under MANP condition.

Table 4.3. Fermentation characteristics of the *S. cerevisiae* cells grown under different conditions.

Conditions	**Consumed Glucose (g/L)**	**Ethanol (g/L)**	**Glycerol (g/L)**	**Consumed Ammonia (g/L)**	**MEP Biomass ±SD (g/L)**	**$\mu_{max,avg}$ (hr^{-1})**
Control AP	6.22±0.20	1.58±0.02	0.20±0.00	0.75±0.00	0.43±0.01	0.27
Control ANP	7.41±0.34	1.43±0.04	0.12±0.01	0.75±0.00	0.74±0.02	0.38
Control MAP	7.82±0.30	2.00±0.03	0.39±0.00	0.78±0.01	0.69±0.01	0.32
Control MANP	6.32±0.22	1.15±0.02	0.27±0.00	0.74±0.01	0.17±0.01	0.18
Rap AP	9.19±0.50	1.29±0.05	0.21±0.02	0.75±0.00	0.33±0.03	0.20
Rap ANP	8.00±0.41	1.52±0.10	0.15±0.01	0.73±0.02	0.46±0.11	0.08
Rap MAP	7.91±0.34	2.05±0.21	0.43±0.03	0.76±0.01	0.50±0.07	0.31
Rap MANP	8.12±0.42	2.60±0.08	0.24±0.02	0.76±0.00	0.28±0.02	0.32
Caf AP	10.02±0.56	1.77±0.07	0.31±0.04	0.75±0.00	0.55±0.05	0.08
Caf ANP	11.45±0.43	2.59±0.21	0.27±0.03	0.72±0.03	0.38±0.08	0.12
Caf MAP	9.00±0.52	2.56±0.19	0.31±0.01	0.78±0.02	0.53±0.03	0.10
Caf MANP	5.04±0.20	1.91±0.09	0.16±0.00	0.74±0.02	0.64±0.06	0.19

The average glucose consumption and the ethanol production of the cells cultivated in the presence of rapamycin was 16% and 21% higher than the cells in the control culture, respectively. Cells grown in the presence of caffeine under AP, ANP and MAP conditions were observed to consume 42% more glucose and produce 38% more ethanol than the control cultures, in average. The only exception to this was the behaviour of the yeast cells grown under micro-aerated fermentations in which pH was not controlled (MANP). In the presence of caffeine 3.8 fold higher biomass concentration was accompanied by 20% lower glucose consumption and relatively lower ethanol production by these cells.

4.1.2.4. Analysis of the Significantly Expressed Genes in Response to Environmental Parameters. The multi-parametric nature of the experiments were studied and the effect of these parameters on gene expression response was analysed using n-way analysis of variance (ANOVA) with n=1,2,3 varying based on the number of interacting parameters under investigation. An analysis allowing the elucidation of the effect of each parameter, i.e. the factors in ANOVA (drug (D); R: rapamycin and C: caffeine, aeration (A) and pH control (P)) and their combinatorial effects was carried out. The genes that were responsive to variations (p-value <0.01 and fold change >1.5) in each factor were identified by taking into consideration main effects as well as the effect of the interaction between them on gene expression (D, DxA, DxP, DxAxP).

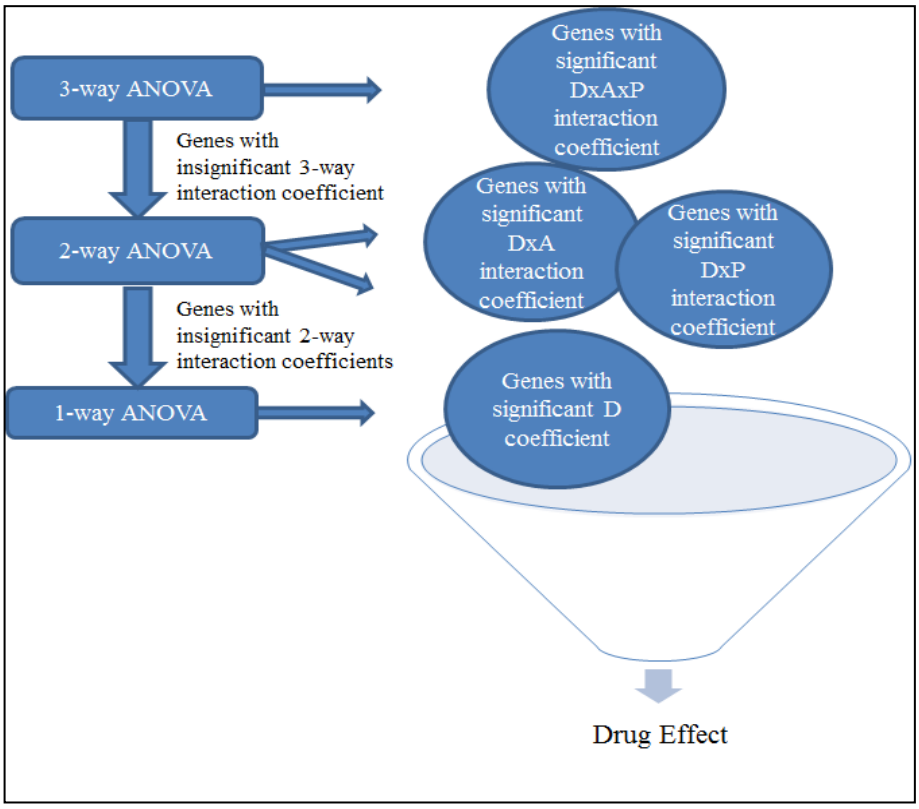

Figure 4.8. A schematic diagram of the microarray data analysis used in this study.

Drug responsive genes were determined as the total of genes that have a significant D-related coefficient (D, DxA, DxP, DxAxP). The individual effects of growing the cells in presence of a drug were also investigated, in isolation from their combined effects with other parameters (D). A strategy was developed to eliminate these combinatorial effects using a reductionist approach. Genes that were identified to have a significant change in

their expression in response to ternary and binary interaction effects were removed in order to be able to observe the single-parameter effects on the transcriptional changes (Figure 4.8).

Table 4.4. Number of significantly expressed genes in response to changing environmental conditions.

Drug	**n-way ANOVA effects**	**Number of Significantly Expressed Genes (p-value<0.01)**
Rapamycin	3-way ANOVA rapxairxpH interaction effect	636
	2-way ANOVA rapxair interaction effect	198
	2-way ANOVA rapxpH interaction effect	190
	1-way ANOVA only rap effect	157
	Total (rapamycin responsive genes)	1181
Caffeine	3-way ANOVA cafxairxpH interaction effect	1217
	2-way ANOVA cafxair interaction effect	494
	2-way ANOVA cafxpH interaction effect	327
	1-way ANOVA only caf effect	1122
	Total (caffeine responsive genes)	3160

Rapamycin was observed to affect a total of 1181 genes including the genes significantly expressed in response only to the rapamycin (R), the interactive effects of rapamycin and aeration (RxA), rapamycin and pH (RxP) and the ternary interactions of these three parameters (RxAxP). 157 genes showed significant transcriptional response only to the presence of rapamycin (R). Caffeine resulted in a more drastic change in the transcriptional reprogramming of the cells by altering the expression of 3160 genes, in response only to the presence of caffeine (C), the interactive effects of caffeine and aeration (CxA), caffeine and pH (CxP) and the ternary interactions of these three parameters (CxAxP). 1122 genes were found to be responsive only to the presence of caffeine at the transcriptional level (C) (Table 4.4). The drug responsive genes, the total of the genes having a significant D-related coefficient (D, DxA, DxP, DxAxP), were then analysed with a fold change threshold of 1.5 for each specific condition (AP, ANP, MAP

and MANP) according to their responses. The affected processes due to the presence of rapamycin or caffeine were investigated in terms of their significantly enriched GO biological process terms (p-value <0.05). A manual investigation was also carried out in order to examine the effects of long term administration of rapamycin and caffeine in conjunction with their interactions with aeration and pH control.

4.1.2.5. Analysis of the Significantly Expressed Genes in Response to Rapamycin Under Different Environmental Conditions. As a main effector regardless of its interactions with aeration or pH control, rapamycin was observed to affect 157 genes (having a significant D coefficient) that are involved in the intracellular trafficking and regulation of transcription. 95 out of the 157 genes whose expression was significantly changed only due to the presence of rapamycin were found to be up-regulated in all conditions and no GO biological process terms were found to be associated with these genes. However a manual investigation revealed that genes (*TRS130, ARF1, COG7, COG3* and *BET3*) involved in intra-Golgi vesicle-mediated transport were among these 157 genes. The altered intracellular trafficking was previously reported upon TORC1 inhibition [141]. 41 genes were observed to share a repressed transcriptional response in all conditions and no significant GO biological process terms were found to be associated with these genes. A manual investigation revealed that 2 of the 7 genes (*HIP1* and *SMF1*) involved in the manganese ion transport process were among the down-regulated 41 genes. The possible functions of manganese and calcium ions within the Golgi include roles in protein modification, regulation of sorting and vesicular traffic, and removal of toxic levels of ions. It was reported that Golgi manganese transport is required for rapamycin signaling and manganese ion in the Golgi inhibits TORC1 function. Manganese transport by Pmr1p into the Golgi was stated to be required for rapamycin sensitivity in yeast [142]. The medium used in the present study lacked any manganese supplements and the limited amount of manganese ions that the yeast cells would utilize was introduced during inoculation from the preculture grown in rich medium. Manganese starvation was reported to induce the *SMF1* although it was down-regulated in the long-term administration of rapamycin under all conditions in an environment that is scarce in terms of manganese ions. Furthermore *PMR1* expression levels did not changed in any of the conditions. The effects of rapamycin on cellular metal ion transport mechanisms may indicate a regulatory role of TOR signaling in manganese transport mechanism.

Genes involved in regulation of transcription from RNA Pol II in response to oxidative stress; *SCH9*, *AFT2*, *SKN7* and *HAA1*, were observed to be repressed only in response to the presence of rapamycin. Rapamycin treatment was reported to induce a general stress response in yeast and it was reported to induce oxidative stress responsive genes in adult stem cells [143]. However the long term treatment of relatively low dose of rapamycin was observed to repress the regulators of stress response. This may be due to the long term adaptation of yeast cells to the presence of low dose of rapamycin.

The genes responsive to the rapamycin were defined as the genes that have significant R, RxA, RxP or RxAxP coefficients. This pool of drug responsive genes consists of 1181 genes and was analysed in each condition with a fold-change threshold of 1.5 in comparison to their control counterparts.

Yeast cells were observed to respond to the presence of rapamycin in a way that mimicked their response to nitrogen starvation. The expression of *DAL1*, *DAL3* and *DUR1,2* in allantoin degradation pathway were significantly up-regulated in response to the presence of rapamycin under pH controlled and aerated conditions (AP) and under micro-aerated fermentations lacking pH control (MANP) but not in those fermentations in which only one parameter was changed. This raised the question whether if allantoin degradation were, in fact, an outcome of only a combinatorial effect of aeration level and pH control in the presence of rapamycin. An analysis of variance conducted for the genes in this 3-parameter combinatorial effect pool revealed that the presence of rapamycin induced the activation of the expression of the genes in this pathway subject to an interactive play between the oxygen level and pH of the fermentation medium. Yeast was previously reported to activate this metabolic pathway under nitrogen-starved conditions [144] and the present findings supported this finding that the cells attempt to mimic nitrogen-starved conditions.

In addition to allantoin degradation, rapamycin was observed to induce the expression of ammonium permeases (*MEP1*, *MEP2* and *MEP3*), the general amino acid permease *GAP1*, genes involved in transport and utilization of proline (*PUT1*, *PUT2* and *PUT4*) as well as glutamine biosynthetic gene *GDH2* under aerated and pH controlled conditions (AP) and micro-aerated conditions without pH control (MANP). This also

indicates that rapamycin treatment represents nitrogen starved conditions and suggests that cells undergo a transcriptional reprogramming in order to use high-quality or low-quality nitrogen sources in the presence of rapamycin indicating an increased demand in nitrogen utilization.

Nitrogen limitation was reported to initiate pseudohyphal growth in yeast under the control of TOR signaling. Although rapamycin was observed to alter mechanisms functioning under nitrogen starved conditions, the genes involved in pseudohyphal growth (in AP cultures) and meiosis (in MAP cultures); the two differentiation responses of diploid *Saccharomyces cerevisiae* to nitrogen starvation [145] were down-regulated in cultures at constant pH. Previous studies have shown that rapamycin inhibits pseudohyphal growth under nitrogen limited conditions regardless of quality of the limiting nitrogen source. Furthermore it was reported that rapamycin did not induce the pseudohyphal growth at any concentration with different levels of nitrogen source [146]. In this case rapamycin reduced the expression levels of the genes involved in the pseudohyphal growth even in the presence of high-quality nitrogen source ammonia. This may indicate a partial reduction in TOR activity in the long-term administration of low dose of rapamycin to a level supressing cellular differentiation processes.

The presence of rapamycin was observed to induce all genes (*DSS1*, *SUV3* and *CBP1*) involved in mitochondrial RNA catabolism under micro-aerated conditions when pH was maintained at 5.5 (MAP). RNA degradation was reported to be an essential process in the expression of genetic information and neccessary to control RNA abundance and thus gene expression and to eliminate atypical or malformed molecules that inevitably form during RNA synthesis and maturation [147]. It was previously demonstrated that maintaining the balance between RNA synthesis and degradation is important for the correct functioning of the genetic system of mitochondria. TOR signaling pathway was reported to control mRNA turnover in yeast. In addition rapamycin was observed to induce several catabolic processes including the degradation of mRNA and mitochondria called mitophagy [41, 148].

The presence of rapamycin was shown to significantly down-regulate genes that were involved in the regulation of cell size in aerated fermentations in which pH was left to

take its natural course (ANP). Among these genes *SCH9*, which was previously reported to be a major target of TORC1 in *S. cerevisiae* [33], was identified. Repression of TORC1 activity by rapamycin, in turn, down-regulated *SCH9* expression along with *WHI5*, *SKN7* and *YCR061W* involved in the regulation of cell size. Under this set of conditions, the biomass concentration of the fermentations normalized with respect to their OD values during growth in rapamycin containing medium had the sharpest decrease from that of the control cultivations. If cell size would be considered a rough estimate of cellular mass per a unit number of cells, alterations in the regulation of cell size was observed to directly result in changes in cell density displaying a direct phenotypic effect caused by transcriptional alterations. Although *SKN7* and *WHI5* were also down regulated when cells were cultivated in an aerated and pH-controlled environment (AP), the cell size was not as distinctively lower than the control fermentations implying that the changes in cell size was more closely related to the changes in *SCH9* and *YCR061W* expression.

Phosphate is an essential nutrient involved in biosynthesis of several cellular components, including nucleic acids, proteins, sugars and lipids. Yeast cells have developed complex responses to adapt to different phosphate levels [149]. The mammalian TOR protein, mTOR, was reported to contribute to the regulation of renal phosphate transport, and rapamycin was found to influence phosphate balance through phosphate transport. mTOR was stated to up-regulate NaPi-IIa, a major renal tubular phosphate transporter and rapamycin was shown to inhibit renal tubular phosphate transport and results in excretion of phosphate into urine in mice which caused phosphaturia [150]. Yeast was observed to down-regulate the expression of *PHO89* and *MIR1*, which are the two phosphate ion trans-membrane transporters of yeast, in aerated fermentations (AP and ANP). Although the mechanisms underlying the induction of NaPi-IIa by mTOR are not entirely clear the repression of phosphate ion trans-membrane transporter upon long-term administration of rapamycin may indicate the presence of a similar mechanism in yeast.

The interaction between the presence of rapamycin and the dissolved oxygen level in the culture affected the expression of 198 genes (RxA). These genes were not found to be enriched with any significant GO biological process terms. With a manual investigation it was found that saturation of the medium with oxygen resulted in the up-regulation of 74 genes including genes involved in mitochondrial respiratory chain complex IV assembly in

AP and ANP cultures whereas it was deterred in a micro-aerated environment (MAP and MANP) causing an impairment of the mitochondrial function. The mitochondrial respiratory chain catalyzes the oxidation of fuel molecules and the concomitant energy conversion into ATP via five complexes, which are embedded in the inner mitochondrial membrane. Complex IV (cytochrome c oxidase, COX) is the terminal oxidase of the respiratory chain catalyzing the transfer of reducing equivalents from cytochrome c to molecular oxygen [151]. Complex IV mitochondrial respiratory chain deficiency (COX deficiency) is a metabolic disorder where the body doesn't have enough cytochrome c oxidase which is needed in the process of energy production by body cells. Deficiency in cytochrome c oxidase results in muscle weakness, heart and kidney dysfunction and high blood level of lactic acid. Reduced TOR signaling was reported to activate mitochondrial function and respiration [127]. The activation of mitochondrial respiratory chain complex IV assembly in response to the presence of rapamycin under fully aerated conditions (dO_2 >95 %) was consistent with the aggravating effects of rapamycin on mitochondrial function. However the opposite effects of rapamycin under micro-aerated conditions (35%> dO_2 >55%) points to the influence of rapamycin action by oxygen levels. These results highlight the requirement of the careful evaluation of environmental parameters during drug treatment and may suggest the necessity of the maintenance of blood oxygen levels at a certain level during rapamycin treatment in order to prevent a mitochondrial dysfunction.

Cellular trafficking was also affected from a combinatorial effect created by the presence of rapamycin and the mode of aeration in the cultivation medium. Micro-aeration was observed to induce the expression of genes that were involved in protein N-linked glycosylation process if pH was maintained at 5.5 (MAP) and these genes were members of the oligosaccharyltransferase complex located in the endoplasmic reticulum. Glycosylation is required for the maintenance of protein quality in the vesicular protein trafficking pathway in eukaryotic cells and was previously reported to have potential functions relating to recognition and biological triggering in the cell and these properties would only operate in a specific biological context. Such changes in glycosylation patterns would both reflect and results in physiological changes, for instance leading to cancer and rheumatoid arthritis in higher organisms [152].

The expression levels of 190 genes with a significant RxP coefficient were observed to be altered due to the interaction effects of the presence of rapamycin and the medium pH and these genes were not found to be significantly associated with a GO biological process term. However a manual investigation revealed that the interaction effects of rapamycin and pH also altered cellular trafficking. In pH controlled fermentations the medium pH was kept at 5.5 whereas pH was decreased and the medium gets acidic during the cultivations in the fermenters without pH control. 125 genes were found to be up-regulated in the presence of rapamycin with respect to their control counterparts under constant pH regardless of the mode of aeration. These genes were significantly enriched with the protein import into nucleus GO biological process term (p-value $8.6x10^{-3}$). In addition, the expression of the genes in the arginine biosynthetic pathway, which were not involved in the *de novo* biosynthesis of pyrimidine nucleotides, were up-regulated at constant pH at 5.5 when the cells were cultivated in the rapamycin-containing medium. Furthermore, the expression of the genes involved in arginine biosynthesis and protein import into nucleus were acting in concert in yeast cultures in the presence of rapamycin and extracellular pH was a determinant of this activity. These genes were up-regulated at relatively higher pH (AP and MAP) and they were constitutively down-regulated at lower pH (ANP and MANP). It seems that at relatively high constant pH, the combinatorial effects of rapamycin and pH led to activation of intracellular protein trafficking whereas as the medium gets acidic the transport mechanisms were down-regulated. TOR signaling represses the amino acid biosynthesis genes and it was previously reported that impairment of the TOR pathway by growth with a sublethal rapamycin concentration (5 ng/ml) causes increased amino acid levels [153]. The activation of arginine biosynthesis genes and higher intra and extracellular arginine concentrations at relatively high pH support these observations. The mammalian TOR complex was previously reported to have a role in sensing variations in ambient pH and it was suggested that acidic pH inhibits mTORC1 and may serve as a signal for this protein to down-regulate energy consuming anabolic processes such as the biosynthetic pathways since low pH was associated with low nutrient resources, which necessitated taking lower energy expenditure routes in the metabolism [154]. Both the intracellular and the extracellular arginine concentration of these cultures without pH control was either very similar to or was lower than that of the control fermentations in conjunction with the down-regulation observed at the transcriptional level (Figure 4.9 and Figure 4.10).

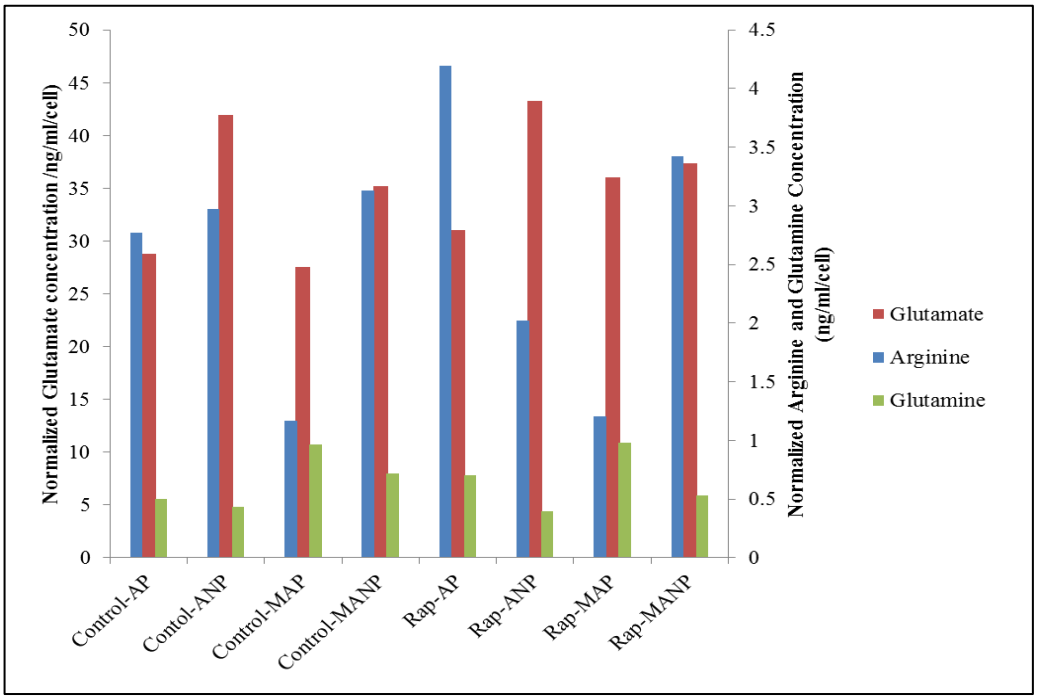

Figure 4.9. Normalized extracellular amino acid concentrations.

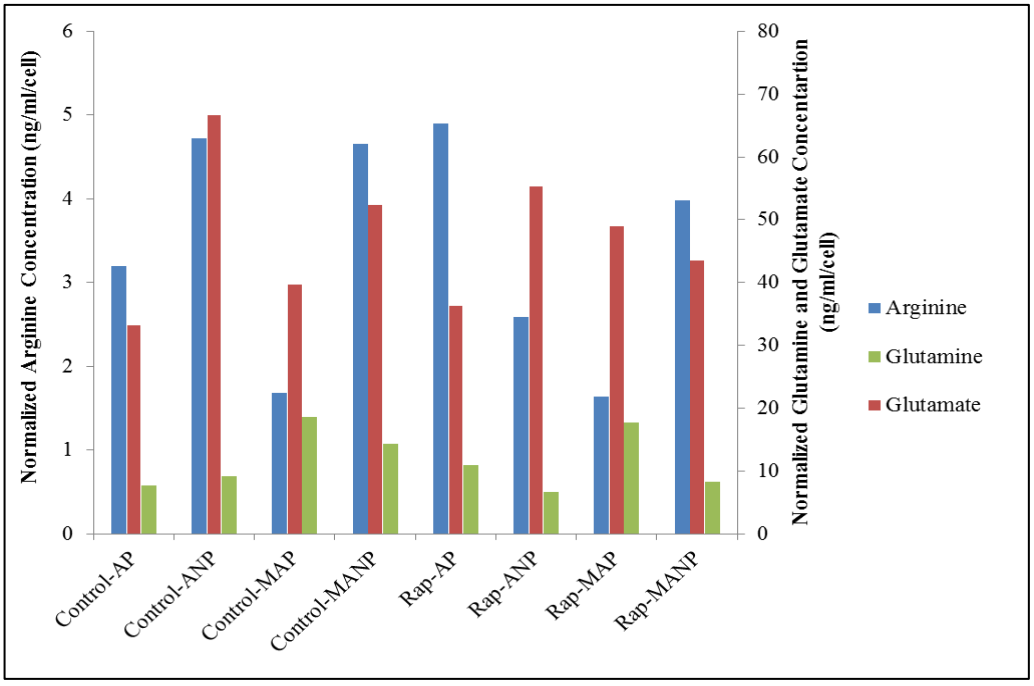

Figure 4.10. Normalized intracellular amino acid concentrations.

Similarly, the extracellular and intracellular concentrations of glutamate and glutamine also followed similar patterns. In the present scenario, the presence of rapamycin in the cultures lacking pH control first led to activation of arginine biosynthesis

to maintain high intracellular and extracellular arginine concentrations. As the medium gets acidic, the combinatorial effect of the presence of rapamycin and acidity finally overcame; cells repressed the expression of genes involved energy consuming anabolic processes such as intracellular trafficking and arginine biosynthesis.

The expression levels of the genes involved in arginine biosynthesis and inositol phosphate dephosphorylation were identified to change antagonistically in response to rapamycin treatment with respect to variations in medium pH. This dual control was possibly established through the significant up-regulation of *ARG82*, whose gene product is a transcriptional regulator of the arginine metabolism and which was also reported to be involved in the phosphorylation of inositol [155]. This may also be due to the low pH increasing the energy requirement of the cells and dual inhibition of TORC1 by rapamycin and low pH leading to activation of catabolic processes.

The global transcriptional responses of yeast cells in the presence of rapamycin were further analysed by taking the expression levels of all genes in four conditions (AP, ANP, MAP and MANP) into account. These genes were classified according to their fold changes with respect to their control counterparts. With a general point of view, the expression of the genes having roles in translation initiation was low regardless of the environmental condition. On the other hand, genes that were involved in recombinational repair and chromosome segregation were up-regulated under all conditions.

The gene expression of the ACG family protein kinase Sch9p was down-regulated under all conditions in response to the presence of rapamycin regardless of the variation in environmental conditions. Sch9p is a major target of TORC1 in *S. cerevisiae* and required for the regulation of ribosome biogenesis, translation initiation, and entry into G0 phase via TOR signalling pathway [33]. The expression of *DOT6* and *TOD6* were also down-regulated during cultivation in the presence of rapamycin. Sch9p was reported to act as a master regulator of ribosome biogenesis via direct inhibitory phosphorylation of the transcriptional repressors Stb3p, Dot6p and Tod6p. Furthermore, the deletion of *STB3*, *DOT6* and *TOD6* was reported to partially bypass the growth and cell size defects of a strain lacking *SCH9* [156]. In the present study, the yeast cells would have attempted to rescue ribosome biosynthesis despite the down-regulation of *SCH9* via also down-

regulating the inhibitors of ribosome biogenesis; *DOT6* and *TOD6*, in the presence of the repressor; rapamycin since the transcription of ribosome biosynthetic genes themselves was not affected significantly under rapamycin treatment.

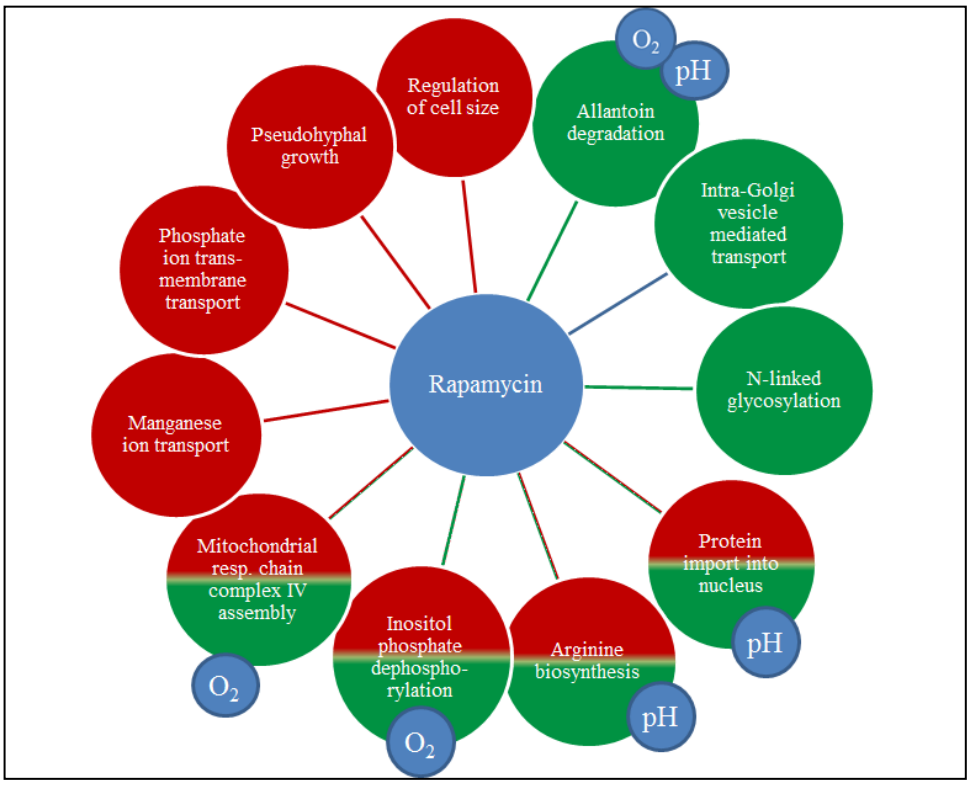

Figure 4.11. The overall view of the processes affected by the presence of rapamycin.

Previous studies on the cellular response of yeast cells to high dose rapamycin treatment reported a severe decrease in translation initiation and an arrest in the early G1 phase of the cell cycle [71, 157], rDNA chromatin remodeling and condensation [158], a down-regulation in rRNA transcription, induction of glycogen accumulation, sporulation and autophagy, involvement in partial regulation of respiro-fermentative switch, induction of vacuolar protease expression and utilization of poor nitrogen sources despite the availability of high-quality nitrogen sources [74]. The effects of the long term administration of relatively low dose rapamycin on transcriptional level were more subtle than the effects of short-term treatment of high dose that were reported in the literature. Although no decrease was observed in the growth of the cells in the fermenter cultures, cells were observed to reduce the protein synthesis by lowering the expression of genes involved in the translation initiation. However a well-accepted effect of rapamycin on inhibition of ribosome biogenesis gene expression was not observed in the long-term

adaptation of the cells while a down-regulation was observed in the expression level of the main regulator of ribosome biogenesis, Sch9p.

The overall view of the processes affected by the presence of rapamycin as well as the interaction effects of rapamycin with the environmental factors were summarized in Figure 4.11. Green circles indicate the up-regulated processes, red circles indicate the down-regulated processes and the green/red circle indicates the processes affected by the interactions of the presence of drug and oxygen or pH control.

4.1.2.6. Analysis of the Significantly Expressed Genes in Response to Caffeine Under Different Environmental Conditions.The long-term administration of caffeine caused a wider change in the transcriptional reprogramming of the cells. 1122 genes were observed to show a significant change in their expression levels (significant C coefficient) only in response to the presence of caffeine and 565 of them were found to be up-regulated only in response to the presence of caffeine. The up-regulated genes were found to be significantly associated with ubiquitin-dependent protein catabolic process (p-value $3.31x10^{-5}$) and protein targeting to vacuole (p-value $5.94x10^{-3}$). Caffeine treatment activated the catabolic processes and this may be due to the nutrient limited environment mimicked by the presence of caffeine. Further manual investigation of the significantly expressed genes revealed that genes with a role in fungal cell wall organisation and biogenesis were up-regulated regardless of the availability of oxygen or the pH of the extracellular environment. This response of cells is a well-known action of caffeine since it was reported that cell wall structure of caffeine-treated cells was significantly altered [6].

A total of 494 genes were found to be down-regulated only in response to the presence of caffeine and these genes were enriched with the mitosis ($8.44x10^{-3}$) GO biological process term. A manual investigation of the significantly expressed genes revealed that the genes responsive only to the presence of caffeine were also involved in chromatin silencing, RNA transport and ribosome biogenesis. Like high dose of rapamycin treatment long term administration of caffeine also inhibited ribosome biogenesis and this was possibly due to the inhibition of TORC1 function by caffeine [64]. The inhibitory role of caffeine on mitosis was previously reported on human lymphocytes [159]. Chromatin silencing is required for repression of genes localized in the silent chromatin regions and

maintenance of chromosome stability. It was reported that TOR pathway regulates Sir3p phosphorylation via Mpk1p MAP kinase. But this regulation was stated to be activated due to the stress conditions rather than nutrient signaling. Rapamycin treatment or stress was reported to cause Sir3p hyperphosphorylation that is accompanied by decreased silencing. Probably the long term administration of caffeine resulted in a stress response hence decreased chromatin silencing [160]. The decreased RNA transport in the long-term administration of caffeine may also be a result of inhibition of transcription and translation by caffeine.

The genes responsive to the caffeine were defined as the genes that have significant C, CxA, CxP or CxAxP coefficients. This pool of caffeine responsive genes consisted of 3160 genes and they were analysed in each condition in comparison to their control counterparts. Genes involved in transcription, translation, regulation of transcription or translation and rRNA processing were down-regulated in AP, ANP, MAP cases.

The misperception of nutrient availability in the presence of caffeine possibly led the metabolism to rearrange its transcriptional response to up-regulate the expression of genes involved in filamentous growth (AP) or sporulation (MANP). Caffeine was reported to promote sporulation and filamentous growth however this outcome of caffeine treatment was observed only AP and MANP cultures. Previous reports discussed the development of filamentous growth as a more complex and globally regulated behaviour rather than a nutrient-regulated growth response [161]. In the light of this information it should be noted that caffeine did not suppress pseudohyphal growth in nutrient rich medium in contrast to rapamycin.

The transcription level of the genes involved in catabolic processes and energy generation mechanisms were increased in response to the presence of caffeine. The presence of caffeine possibly increased the energy requirement of yeast and the cells responded by increasing the expression of genes that were involved in cofactor biosynthesis, ATPase activity and oxidation reduction in MAP case, oxidative phosphorylation and negative regulation of hydrolase activity in ANP case, amino acid catabolic process in MANP case. The increased energy expenditure upon caffeine treatment was previously reported in humans [162].

The processes up-/down-regulated in response to the interaction effects of rapamycin with aeration and pH were observed to be grouped according to the mode of aeration or pH level. However the processes identified to be altered in response to the interaction effects of caffeine with oxygen level and pH were not grouped according the mode of aeration or the level of pH as in the case of the presence of rapamycin. This indicates that caffeine affected the transcriptional responses of cells more effectively and competed with the effects of aeration and pH levels.

A total of 327 genes were found to be significantly expressed in response to the interactive effects of caffeine with pH (CxP) and no GO biological process terms were found to be associated with genes. However manual investigation of these genes revealed that the presence of caffeine was observed to down-regulate the expression of the genes involved in GMP biosynthesis under the control of an interactive effect between caffeine and pH (CxP). IMP dehydrogenase (IMPDH), catalyzes the first step of *de novo* guanine nucleotide synthesis and was reported to have a key role on growth of many cell types, including lymphocytes and rapidly proliferating cells. GMP synthesis and its regulation were reported to play an important role in cellular proliferation owing to the increased IMP dehydrogenase activity observed in rapidly proliferating cells including several types of tumour cell lines [163]. These results are consistent with the documented effects of caffeine on inhibiting cell proliferation and the usage of it as an effective drug in cancer therapies [138].

Furthermore the interaction effects of caffeine and pH was observed to render the protein glycosylation activity high in AP cultures while it was observed to be low in MAP, ANP and MANP cultures. These observations were coupled with the transport processes under the stated cases. Glycosylation is an essential modification which often occurs co-translationally for the maintenance of protein quality in the vesicular protein trafficking pathway in eukaryotic cells. The reduced glycosylation activity probably affected the transport mechanisms. These response may also be related to the catabolic processes since the susceptibility of non-glycosylated enzymes to degradation by proteinases, heat and other denaturing conditions was previously reported [164].

A total of 494 genes were found to be significantly expressed in response to the interactive effects of caffeine with oxygen (CxA) and no GO biological process terms were found to be associated with genes.

However manual investigation of these genes revealed that the interaction effects of aeration and the presence of caffeine caused the down-regulation of tRNA methylation in AP, ANP and MAP cultures. The tRNA methylases are found in all cells and tissues and the variation of their activity with the differentiation state of the cells under the influence of many internal and external factors and elevation of their activities in embryonic and cancerous tissues were reported previously [165]. The down-regulation of the tRNA methylation in response to the interaction of caffeine and aeration would support the potential uses of caffeine as an effective drug in cancer therapy.

The interaction effects of caffeine and oxygen level were resulted in an induction of fatty acid metabolism in AP, ANP and MAP cases. Together with this induction the interactive effect of caffeine and pH was observed to stimulate the expression of the genes involved in glycerolipid biosynthesis and glycerophospholipid metabolisms under aerated conditions with pH control (AP). Glycerolipids are formed by joining fatty acids and play important roles in cell signaling, membrane trafficking, and anchoring of membrane proteins in addition to membrane structure [166].

Up-regulation of lipid synthesis also coupled with the induction of cell wall genes. These observations indicate that the induction of cell wall remodelling genes in the presence of caffeine also induced the lipid biosynthesis which is a main constituent of cell wall. Yeast cells were reported to synthesize the same general classes of lipids found in other eukaryotes via pathways largely homologous to those in mammalian cells [167]. One of the similarities between cancer cell physiology and rapidly proliferating yeast in fermentative mode was that both cells displayed highly active pathways for the synthesis of fatty acids and their incorporation into complex lipids. The imbalance in this synthesis or turnover into lipids was reported to affect both growth and viability of both yeast and cancer cells [5].

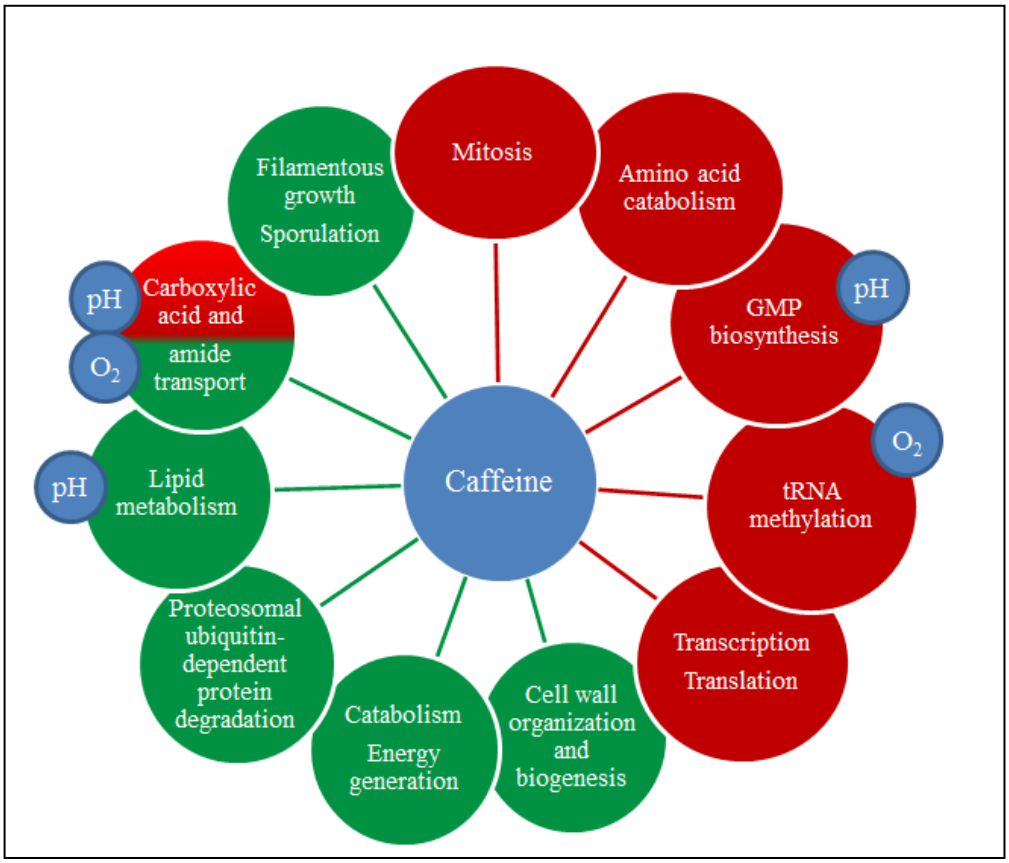

Figure 4.12. The overall view of the processes affected by the presence of caffeine.

The overall view of the processes affected by the presence of caffeine and the interaction effects of caffeine with the environmental factors were summarized in Figure 4.12. Green circles indicate the up-regulated processes, red circles indicate the down-regulated processes and the green/red circle indicates the processes affected by the interactions of the presence of drug and oxygen or pH control.

4.1.2.7. Comparison of the Effects of the Long Term Administration of Rapamycin or Caffeine Together with the Environmental Parameters. The results obtained from this study indicate that long term administration of relatively low dose of rapamycin caused a set of transcriptional responses mimicking the nutrient limited/starved conditions which is a mostly known effect of rapamycin due to the TORC1 inhibition. However the transcriptional responses of the yeast cells that were grown in the presence of rapamycin were different than the reported responses of the cells that were treated with rapamycin. The well-documented outcomes of rapamycin treatment such as inhibition of ribosome biogenesis, transcription and protein synthesis as well as the cell cycle arrest were not

observed in the long term administration of low dose of rapamycin. This may be due to the cells started growing in the presence of rapamycin and exhibited an adaptive response. However the induction of several permeases resulting in an altered intracellular trafficking and allantoin degradation as well as the inhibition of pseudohyphal growth in the presence of rapamycin indicated that it was still effective on the transcriptional responses of yeast cells, possibly resulting in a partial inhibition of TOR function.

The investigation of the interactive effects of the oxygen level and pH revealed a tight coupling between the pH level and the mode of action of rapamycin. The transcriptional responses of yeast cells to the presence of rapamycin were observed to group with respect to the pH level. Furthermore the effects of rapamycin on the yeast growth were observed to be altered by the different extracellular pH levels. The combinatorial effects of oxygen level and rapamycin point to the importance of the maintenance of the oxygen level in the long term rapamycin treatment. The low levels of oxygen during the long term usage of rapamycin may cause COX deficiency in humans.

The effects of caffeine in the long term administration resulted in a wider set of transcriptional changes resembling the documented outcomes of relatively high dose of rapamycin treatment. In contrast to the gene expression response of yeast to being cultivated in the presence of relatively low dose of rapamycin, the transcriptional response of yeast to the presence of caffeine was traceable at lower-hierarchy genes performing the necessary metabolic functions.

The well-documented processes known to be regulated by TOR such as ribosome biogenesis, transcription, chromatin silencing, translation and RNA processing were observed to be inhibited in the presence of caffeine. The long term exposure to caffeine induced the cell wall assembly and catabolic processes as well as the intracellular processes and lipid metabolism in an interactive manner with oxygen level and pH. The inhibition of processes such as GMP biosynthesis in response to the presence of caffeine and pH as well as tRNA methylation in response to oxygen levels highlight its anti-proliferative effects and the potential usage of caffeine in cancer treatment.

4.2. Investigation of the Effects of Rapamycin Dosage on Transcriptional and Metabolomic Responses of Yeast

In this study, the dose effects of rapamycin on yeast growth and transcriptional responses were investigated during long term administration. 2 nM rapamycin was used to investigate low dose rapamycin effect and 200 nM rapamycin, which is a widely used concentration in studies investigating rapamycin effects [71, 75, 168–170], was used to demonstrate high dose rapamycin effect.

In Section 4.1 the transcriptional profiles of the yeast cells to the presence of rapamycin were observed to be clustered according to the extracellular pH (Figure 4.7). This raised the question whether the effects of rapamycin is dependent on the medium pH. In order to evaluate the effects of medium pH on the action of rapamycin, shake flask cultivations of yeast cell were carried out in the absence and presence of rapamycin with different pH adjustments. Citrate-phosphate buffers were used to maintain the medium pH at the levels of 3.0, 4.2, 5.4 and 6.6 and one shake flask was used as control without pH maintenance. In the absence of rapamycin, cells were observed to grow faster and reached higher optical cell density values when pH of the medium was not controlled and at relatively higher pH such as 6.6 (Figure 4.13).

However when cells cultivated in medium containing rapamycin at different pH values the growth rate of the cultures maintained at relatively higher pH (6.6) was the lowest. Cell grew faster in relatively acidic environment and reached higher optical cell density values in the presence of rapamycin (Figure 4.14). These results indicate that the medium pH is very effective on the mechanism of action of rapamycin in yeast cell growth. Therefore the effects of rapamycin should be investigated with carefully designed experimental setups in order to eliminate the effects of environmental factors.

In the light of these results the fermentations were carried under constant pH. Batch fermentations of *S. cerevisiae hoΔ/hoΔ* strain were conducted in triplicates in the absence and presence of rapamycin (2 nM or 200 nM) under micro-aerated conditions with pH maintenance at 5.5 (MAP). Rapamycin was added into the medium prior to inoculation. The effects of low and high doses of rapamycin on metabolic and transcriptional responses

were also comparatively examined with the responses of the yeast cells to the presence of caffeine under the same environmental conditions.

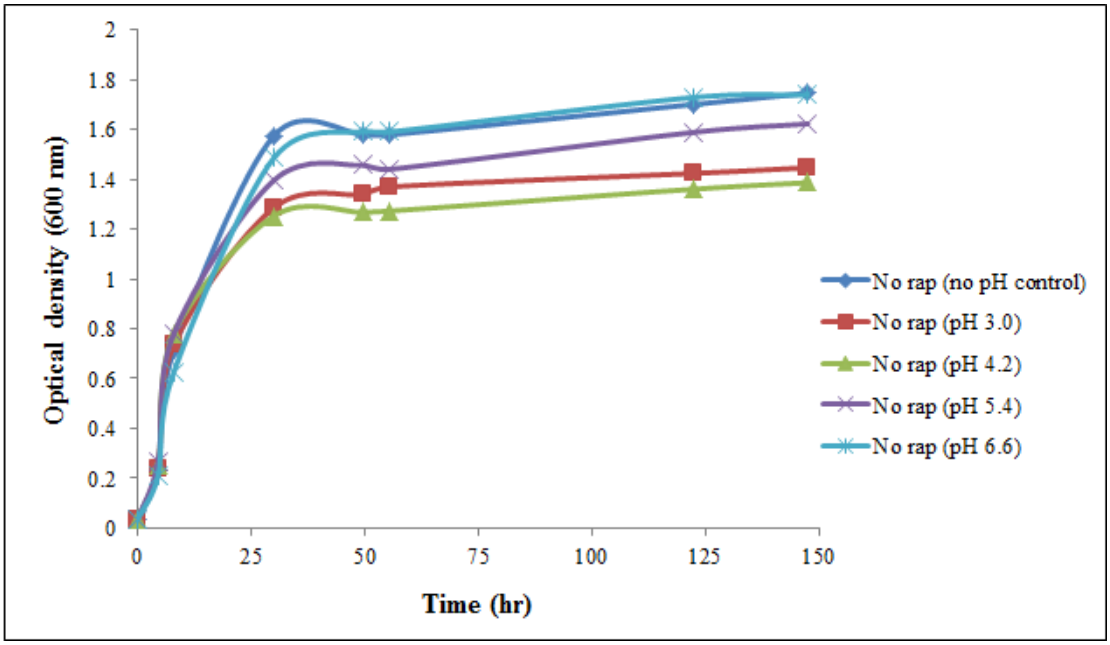

Figure 4.13. The growth profiles of the cells cultivated in the absence of rapamycin at different extracellular pH.

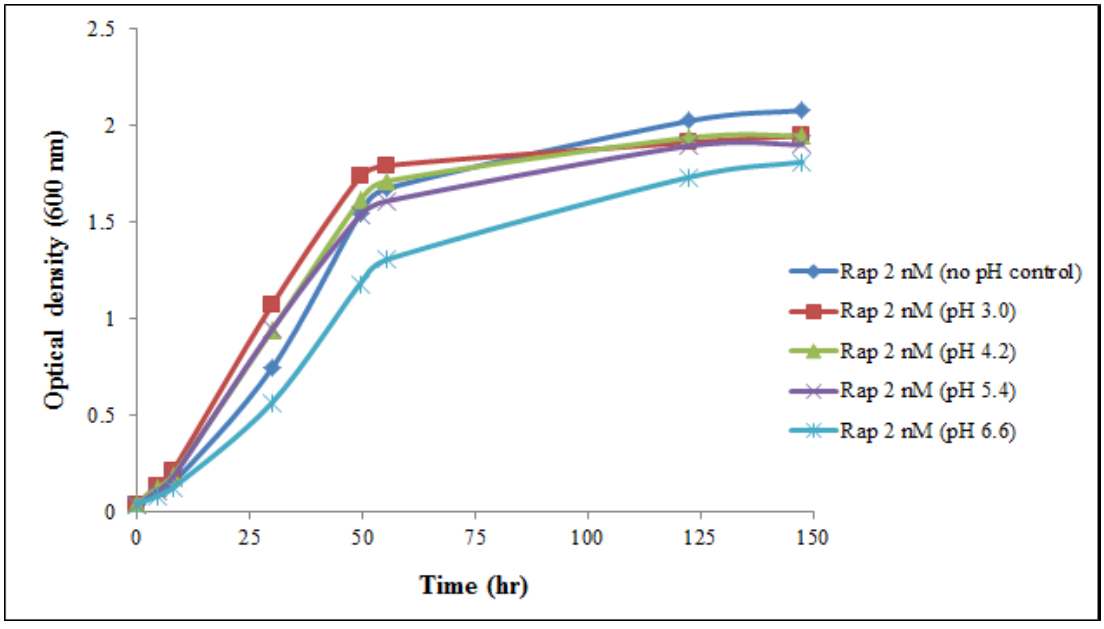

Figure 4.14. The growth profiles of the cells cultivated in the presence of rapamycin at different extracellular pH.

4.2.1. The Growth Characteristics of Batch Fermentations in the Presence of Low and High Doses of Rapamycin

The growth characteristics of the batch cultures were investigated during the exponential phase of growth taking average specific growth rates into account (Table 4.5). The growth profiles of the cells that were grown in the absence and presence of 2 nM, 200 nM rapamycin or caffeine were shown in Figure 4.15. Although the presence of rapamycin or caffeine resulted in a lower growth rate regardless of the concentration, more drastic drops in growth rates were observed in the cultures with 200 nM rapamycin or 5 mM caffeine.

Table 4.5. The maximum specific growth rates of the cultures at the mid-exponential phase of growth.

Conditions	**$\mu_{max,avg}$ ± SD (hr^{-1})**
Control	0.32±0.01
Rapamycin 2 nM	0.31±0.01
Rapamycin 200 nM	0.06±0.00
Caffeine 5 mM	0.10±0.01

The maximum specific growth rates of the cultures having 2 nM rapamycin was very close to that of the control cultures whereas the maximum specific growth rates of the cultures were 69% and 81% lower than the control cultures in the presence of caffeine or 200 nM rapamycin, respectively.

The gravimetrically determined biomass concentrations were normalized with respect to optical cell density (OD) (Figure 4.16). The presence of rapamycin or caffeine yielded a lower normalized biomass density than the control culture. If cell size and cell mass would be considered as proportionate the lower normalized biomass density may indicate lower cell size. Decrease in cell size upon rapamycin treatment were reported previously in fission yeast [136] and mammalian cell lines [171]. These results may indicate a similar impact of rapamycin or caffeine treatment on the growth and cell size of *S. cerevisiae* under micro-aerated and pH controlled conditions.

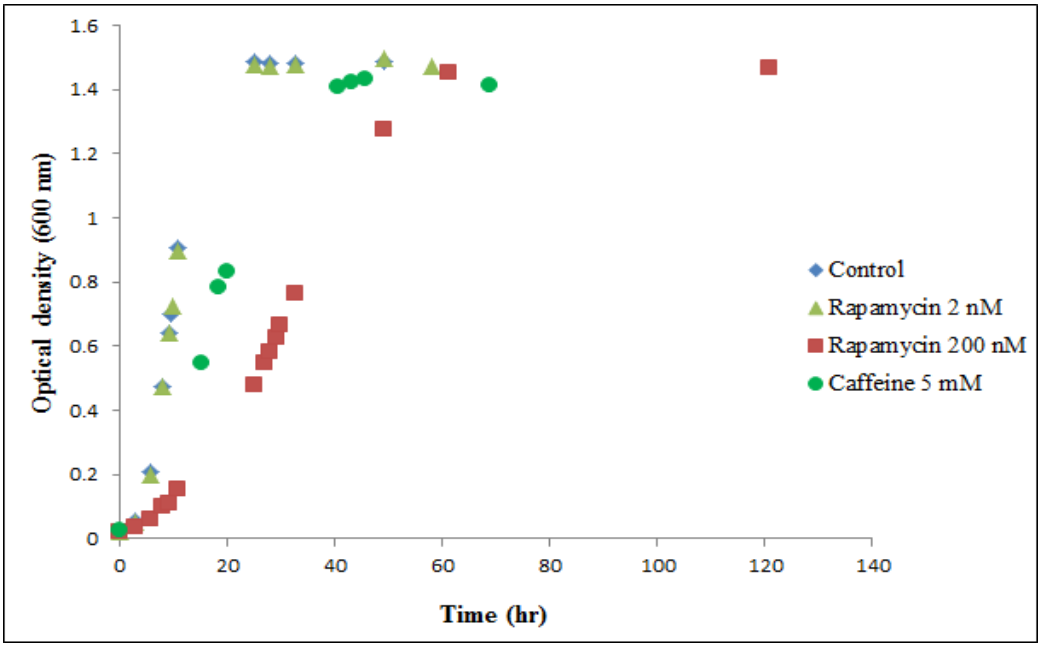

Figure 4.15. The growth profiles of the cultures in the absence of and presence of 2 nM or 200 nM rapamycin or caffeine.

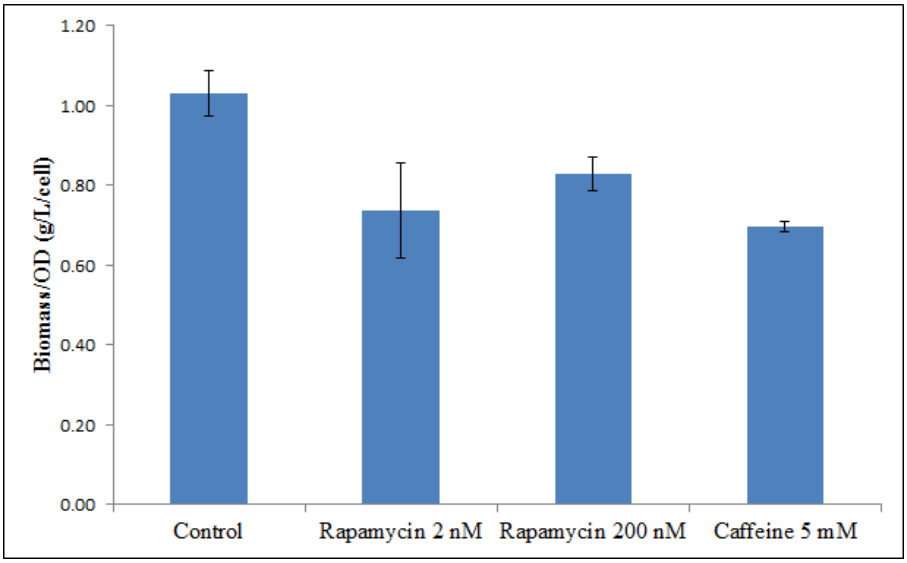

Figure 4.16. The normalized biomass densities of the cultures.

Enzymatically determined extracellular metabolite concentrations indicated that the presence of 2 nM rapamycin did not affect the glucose consumption or ethanol yield of the yeast cells. However the presence of 200 nM rapamycin caused 11% decrease in the glucose consumption of the cultures in comparison to control cultures. However the ethanol and glycerol yields based on glucose consumption were similar to that of the

control cultures. The glucose consumption of the cells grown in the presence of caffeine was increased 15%. However the ethanol yield of the cultures grown in the presence of caffeine was very similar to that of the control cultures (Table 4.6).

Table 4.6. Extracellular metabolite concentrations.

Conditions	**Control**	**Rap 2 nM**	**Rap 200 nM**	**Caf 5 mM**
Consumed Glucose (g/L) ±SD	7.82±0.11	7.91±0.08	7.20±0.10	9.00±0.06
Ethanol Yield ($Y_{e/s}$) ±SD	0.26±0.03	0.26±0.04	0.22±0.03	0.28±0.02
Glycerol Yield ($Y_{g/s}$) ±SD	0.05±0.01	0.05±0.01	0.05±0.01	0.03±0.01
Consumed Ammonia (g/L) ±SD	0.78±0.05	0.76±0.07	0.77±0.08	0.78±0.05

4.2.2. Comparative Analysis of Transcriptional Responses of Yeast Cells to the Low and High Doses of Rapamycin and the Presence of Caffeine

The multi-parametric analyses of the effects of the presence of 2 nM rapamycin or 5 mM caffeine on transcriptional responses of yeast cells were carried out under different environmental conditions in Section 4.1. The aim of these analyses was to investigate the mechanism of action of rapamycin or caffeine in response to oxygen availability and acidity. In this section the transcriptional responses of yeast cells to the low and high doses of rapamycin were investigated regardless of the environmental parameters. For this purpose the effects of the presence of low (2 nM) or high (200 nM) dose of rapamycin were investigated and compared with the effects of 5 mM caffeine on transcriptional responses of yeast.

The transcriptional responses of yeast cells to the presence of 2 nM rapamycin, 200 nM rapamycin or 5 mM caffeine during the exponential phase of growth were hierarchically clustered using Pearson correlation as the distance metric taking into consideration the average of the replicate data values. The hierarchical clustering results revealed that the transcriptional profiles of yeast cells in the presence of 2 nM rapamycin (Rap 2 nM) were clustered together with the control cultures. The transcriptional responses of cells to the presence of 5 mM caffeine (Caf 5 mM) or 200 nM rapamycin (Rap 200 nM) under micro-aerated and pH controlled conditions were very similar to each other since they fell into the same cluster (Figure 4.17).

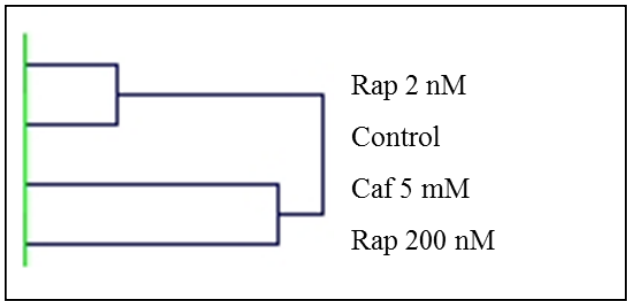

Figure 4.17. The hierarchical clustering of the transcriptional profiles of the cultures in the presence of rapamycin or caffeine.

In order to determine the significantly expressed genes in response to the long term administration of 2 nM rapamycin or 200 nM rapamycin under micro-aerated conditions with constant pH at 5.5, t-test was used with a p-value threshold of 0.05. The presence of rapamycin at low dose resulted in a significant change in the expression levels of 361 genes when compared with the control culture. 311 out of the 361 significantly expressed genes were found to be up-regulated, while the expressions of 50 genes were repressed. The long term administration of high dose of rapamycin (200 nM) was observed to result in more drastic changes in the transcriptional reprogramming of the yeast cells by affecting the expressions of 2975 genes.

Table 4.7. The number of significantly expressed genes in response to the long term administrations of low and high doses of rapamycin as well as caffeine.

Conditions	**Number of up-regulated genes**	**Number of down-regulated genes**	**Total**
Rap 2 nM	311	50	361
Rap 200 nM	1551	1424	2975
Caf 5 mM	1292	711	2003

A total of 1551 genes out of the 2975 genes were induced in response to the presence of 200 nM rapamycin while 1424 genes were down-regulated. The extent of the effects of the long term administration of caffeine was observed to be similar to that of the high dose rapamycin since it resulted in a significant change in the expression levels of 2003 genes. In the presence of caffeine 1292 genes were up-regulated and 711 genes were down-regulated (Table 4.7).

4.2.2.1. Analysis of the Significantly Expressed Genes in Response to the Presence of 2 nM Rapamycin. Investigation of the effects 2 nM rapamycin on transcriptional responses of yeast cells regardless of the environmental parameters, such as aeration or pH control, would provide detailed evidence about the transcriptional reprogramming of the cells in the presence of low dose of rapamycin. The genes that were significantly up- or down-regulated in the presence of low dose of rapamycin were not found to be significantly enriched with any GO biological process terms. However a manual investigation was carried out to analyze the response of yeast cells to the presence of low dose of rapamycin.

Low dose of rapamycin induced the expression of genes that are involved in intracellular trafficking such as protein transport, mitochondrial transport (*MDM10*, *ECM10*, *SAM35*, *TIM8*, *TIM17*, *OCT1*, *MIM2* and *TOM5*) and mitochondrial outer membrane translocase complex assembly (*MDM10*, *TOM5*, *MIM2*, *SAM35*). Cells were reported to respond to nutrient availability by modulating intracellular trafficking events [172] and TORC1 was reported to be involved in regulation of protein trafficking in response to nutritional signals [24] and inhibition of TORC1 with rapamycin was stated to alter the intracellular trafficking [141]. Microtubules play an essential role in vesicle trafficking in eukaryotic cells and act as tracks upon which organelles and vesicles are moved [173]. In addition to the transport processes, mRNA levels of the genes involved in tubulin folding pathway (*GIM4*, *CIN4*, *ALF1* and *RBL2*) were also induced in response to the presence of low dose of rapamycin. It was reported that rapamycin treatment rapidly causes microtubule instability and defects in microtubule-related functions, such as spindle elongation, orientation and chromosomal segregation. Genes that are involved in protein N-linked glycosylation (*OCH1*, *KTR7*, *OST1*, *VAN1* and *KTR1*) were found to be up-regulated. This was another response of cells to the presence of rapamycin in the context of protein trafficking since glycosylation is an essential modification which often occurs co-translationally for the maintenance of protein quality in the vesicular protein trafficking pathway in eukaryotic cells. In addition to intracellular trafficking, membrane trafficking was also altered since the presence of low dose of rapamycin induced the transcription of several permeases. Ammonium (*MEP1*, *MEP2*, *MEP3*) and amino acid (*GAP1*, *AGP1*, *MUP3*, *FUR4*, *TAT2* and *DIP5*) permeases were up-regulated in order to transport nitrogen sources into the cell despite the presence of ammonium in the medium. Another nutrient starvation related catabolic membrane-trafficking phenomenon, autophagy, (*ATG18*,

ATG20, ATG26, ATG27, COG3, YPT1, HSV2, GEA1, VPS33) was found be induced in the presence of low dose rapamycin. TOR inhibits autophagy under nutrient rich conditions and inhibition of TOR function by rapamycin treatment was reported to induce autophagy [26]. Moreover, the genes involved in glycine decarboxylation via glycine cleavage system (*GCV2* and *GCV3*) were found to be significantly induced in response to the presence of low dose of rapamycin. Glycine is required by the cell for the synthesis of proteins and the mitochondrial glycine cleavage complex has an important role in metabolism since it breaks down of glycine into ammonia. The transcriptional regulation of these genes with respect to nitrogen source was reported previously [174]. In this case the presence of rapamycin, mimicking nitrogen depleted environment, resulted in induction of the expressions of genes involved in glycine catabolism in order to produce ammonia to use as nitrogen source.

The expression levels of genes involved in chromatin remodeling (*ISW1, RSC6, NHP10, RVB1, SPT6, SNF6, VPS71, ARP9*) were also found to be significantly up-regulated in the presence of 2 nM rapamycin. Chromatin remodeling is the core mechanism of the regulation of gene expression. In addition to controlling gene expression, dynamic remodeling of chromatin imparts an epigenetic regulatory role in several key biological processes such as DNA replication and repair, apoptosis, chromosome segregation and development. It was reported that TOR controls nucleolar structure and function through a chromatin-mediated mechanism in response to nutrients and uses multiple chromatin-dependent mechanisms to control gene expression. Inhibition of TOR by nutrient starvation or rapamycin treatment was reported to result in histone H4 deacetylation, rDNA chromatin condensation and reduced nucleolar size leading to repression of rDNA transcription [158]. It seems that cells up-regulated the genes involved in chromatin re-arrangement in order to control, probably to inhibit, gene expression in response to the presence of low dose rapamycin.

The presence of 2 nM rapamycin was observed to repress the expressions of genes that have roles in glycolysis (*CDC19, PGI1, PGK1*) and carbon utilization (*PKP2* and *NCE103*). Furthermore, the genes involved in mitochondrial respiratory chain complex assembly (*MRP10, EMI1, SOM1*) were found to be up-regulated. Rapamycin was shown to cause a shift from glucose utilization to fatty acid oxidation in model muscle cells under

nutrient rich conditions [175]. In addition to this, prolonged rapamycin treatment was reported to cause insulin resistance and severe glucose intolerance in human [176]. These results indicated that the presence of low dose of rapamycin resulted in transcriptional reprogramming in order to decrease glucose utilization and increase respiratory capacity as it was previously stated. Consistent with these observations a metabolic shift from fermentation to respiration was reported previously in terms of expression levels in yeast [29]. However the effects of these transcriptional responses were not observed at the metabolic level in the cells grown in the presence of low dose of rapamycin, indicated by similar glucose consumptions and ethanol production to the control cultures. This may be due to the adaptation of the cells to the long term administration of the low dose of rapamycin.

4.2.2.2. Analysis of the Significantly Expressed Genes in Response to the Presence of 200 nM Rapamycin. The presence of high dose of rapamycin (200 nM) resulted in considerably greater changes in the transcriptional programming of the cells than the presence of low dose of rapamycin in terms of the number of significantly expressed genes. The expression levels of 1551 genes were induced in response to the presence of high dose of rapamycin. The significantly enriched GO biological process terms of these genes were represented in Table 4.8. A manual investigation was also carried out in order identify the altered processes in response to the presence of 200 nM rapamycin.

The expression levels of the genes involved in tricarboxylic acid (TCA) cycle were found to be significantly induced (p-value 2.59×10^{-6}) (Table 4.8). Parallel with the up-regulation of the expression levels of the TCA cycle genes in response to the presence of 200 nM rapamycin the extracellular concentrations of the TCA cycle intermediate metabolites were found to be higher than the control cultures (Table 4.9). In addition to this observation, ethanol yield of the cultures grown in the presence of high dose of rapamycin was lower than the control cultures (Table 4.6). These results indicate a shift from fermentation to respiration which was also previously reported upon rapamycin treatment in yeast [29].

Manual investigation of the significantly expressed genes revealed that the expressions of the 18 of the 32 genes involved in glycogen biosynthesis were found to be

up-regulated in the presence of 200 nM rapamycin as in the case of diauxic shift. A similar observation was stated previously for cells treated with 100 nM rapamycin at an OD of 1.0 [74]. A well-documented outcome of rapamycin treatment or nutrient starvation is an increased glycogen biosynthesis and accumulation in cells [177]. Therefore these results indicate that the long-term administration of high dose of rapamycin resulted in an induction of starvation-related transcriptional programs.

Table 4.8. The significantly enriched GO biological process terms of up-regulated genes in response to the presence of 200 nM rapamycin.

GO Term	**P-value**
Energy derivation by oxidation of organic compounds	1.36E-11
Cellular respiration	3.45E-08
Tricarboxylic acid cycle	2.59E-06
Aerobic respiration	2.73E-06
Oxidative phosphorylation	2.43E-04
Mitochondrial ATP synthesis coupled electron transport	7.35E-04
ATP synthesis coupled electron transport	7.35E-04
Electron transport chain	1.42E-03
Mitochondrion degradation (mitophagy)	1.83E-03
Respiratory electron transport chain	4.24E-03

Table 4.9. The extracellular concentrations of metabolites in TCA cycle.

TCA Cycle Metabolite Concentrations (ng/ml)	**Control**	**Rapamycin 2 nM**	**Rapamycin 200 nM**	**Caffeine 5 mM**
Alpha-keto glutaric acid	1.39	1.70	1.45	1.49
Cis-aconitic acid	0.03	0.04	0.03	0.28
Citric acid	0.87	0.99	1.11	0.86
Fumaric acid	1.44	1.63	2.18	1.54
Isocitric acid	5.84	5.83	6.12	5.73
Malic acid	0.92	1.20	0.97	1.74
Sodium pyruvate	0.47	0.37	2.39	0.57
Succinic acid	4.03	4.60	2.89	5.40

In the presence of high quality nitrogen sources the expressions of genes required for the utilization of poor nitrogen sources are repressed and this regulatory system is known as nitrogen discrimination pathway. The presence of high dose of rapamycin resulted in activation of nitrogen discrimination pathway despite the presence of high quality nitrogen source, ammonia.

The expression levels of genes required for glutamate metabolism (*IDP2, IDP1, GDH1, GDH2, CIT1, CIT2, GAD1, UGA2, GDH3, GLT1* and *PUT1*) were found to be up-regulated. The negative regulation of genes involved in glutamate and glutamine biosynthesis by TOR function was previously stated [178]. Furthermore all genes involved in allantoin degradation were induced in order to convert allantoin to ammonia to use as nitrogen source. Nitrogen starvation or rapamycin treatment was reported to induce autophagy by decreasing TOR function and consistent with the previous statements the long term administration of rapamycin was observed to up-regulate the expression of 46 genes involved in autophagy [26].

In addition to autophagy, genes involved in selective types of autophagy such as mitochondrion degradation (mitophagy) (p-value $1.83x10^{-3}$) and piecemeal microautophagy of nucleus were also induced by the presence of 200 nM rapamycin. In piecemeal microautophagy of nucleus, small pieces of the nucleus were reported to be seperated into invaginations of the vacuolar membrane and subsequently degraded and this offers starving cells an easy mechanism for the bulk degradation and recycling of nucleolar preribosomes that are no longer needed in the cytoplasm [179]. Mitophagy play an important role in maintaining mitochondrial function and integrity. Although the relationship between TOR and mitophagy is unclear, TOR was reported to suppress mitochondrion degradation (mitophagy) and rapamycin treatment reduces the formation of reactive oxygen species (ROS) through stimulating the mitophagy [148].

Genes involved in oxidative stress response were up-regulated in the presence of high dose of rapamycin. Rapamycin was recently shown to increase the oxidative stress response genes in adult stem cells [143]. An extensive overlap in genes affected from rapamycin treatment and oxidative stress was also reported [168].

Table 4.10. The significantly enriched GO biological process terms of down-regulated genes in response to the presence of 200 nM rapamycin.

GO Term	**P-value**
Ribosome biogenesis	6.03E-23
Cytoplasmic translation	1.62E-18
rRNA processing	7.82E-15
Gene expression	1.05E-10
Nucleocytoplasmic transport	2.40E-10
Nuclear transport	2.40E-10
RNA transport	5.89E-07
Intracellular transport	9.84E-07
Translational initiation	3.74E-06
Protein glycosylation	2.96E-05
Transcription from RNA polymerase I promoter	2.69E-04
ER to Golgi vesicle-mediated transport	2.09E-03
Retrograde vesicle-mediated transport, Golgi to ER	3.04E-03
Protein import into nucleus	4.58E-03
Mitotic cell cycle	7.62E-03
tRNA aminoacylation for protein translation	8.69E-03

A total of 1424 genes were found to be down-regulated in the presence of 200 nM rapamycin. Significantly associated GO biological process terms were presented in Table 4.10 and a further investigation of the biological processes associated with these genes was carried out manually. The presence of high dose of rapamycin repressed the expression of genes required for ribosome biogenesis (p-value 6.03×10^{-23}), processing of rRNA (p-value 7.82×10^{-5}) and tRNA (p-value 8.69×10^{-3}). Specifically genes involved in transcription from RNA Pol I (p-value 2.69×10^{-4}) and III were found to be down-regulated. The TOR dependent repression of Pol I and III transcription in response to rapamycin treatment was previously stated [38]. TOR function promotes ribosome biogenesis, transcription and translation as being the major regulator of cell growth [180]. TOR also regulates the cell growth and cell cycle progression coordinately in response to nutrient availability. The inhibition of TOR function by rapamycin treatment or nutrient starvation was reported to

cause a reduced rate of cell cycle progression and proliferation [181]. The cell cycle-related processes such as regulation of spindle pole body separation, cell cycle cytokinesis and septin ring organization were found to be suppressed by the presence of high dose of rapamycin.

Interestingly, the mRNA levels of the genes involved in several trafficking processes such as protein import into nucleus, rRNA export from nucleus and retrograde vesicle-mediated transport, Golgi to ER were significantly down-regulated together with the protein N-linked glycosylation process (Table 4.10). As mentioned above protein N-linked glycosylation and mitochondrial trafficking processes such as mitochondrial transport and mitochondrial outer membrane translocase complex assembly were found to be up-regulated in the presence of low dose of rapamycin. The regulation of membrane trafficking through modulating the expression and transport of several permeases in response to nutrient levels by TOR was reported previously. The presence of high dose of rapamycin significantly induced the expression levels of several permeases including, ammonium permeases (*MEP1*, *MEP2*, *MEP3*), amino acid permeases (*GAP1*, *BAP2*, *VBA1* and *DIP5*), allantoin and allantoate permeases (*DAL4*, *DAL5*), as well as carbon source permeases (*MAL31*, *GAL2* and *MPH3*). These results indicate that the presence of high dose of rapamycin activated membrane trafficking in order to facilitate the transport of nutrient sources into the cell although there is no nutrient limitation. In addition to membrane trafficking, the intracellular trafficking was stated to be altered in the presence of rapamycin [141]. While the presence of rapamycin activated the membrane trafficking by inducing several permeases regardless of its dosage, it seems that different doses of rapamycin alter the intracellular trafficking in different ways. Low dose of rapamycin (2 nM) facilitates the mitochondrial transport processes whereas the high concentrations (200 nM) inhibit the intracellular trafficking between Golgi and ER.

The global transcriptional responses of cells to low and high doses of rapamycin were observed to converge on nutrient-starvation like responses. Rapamycin, at both doses, induced autophagy and altered the membrane trafficking in order to facilitate the usage of alternative nitrogen sources in the presence of high quality nitrogen source ammonia. Low dose of rapamycin did not cause any growth rate decrease while the long term administration of high dose of rapamycin resulted in 81% decrease in the growth rate. The

long term administration of low dose rapamycin did not exhibit well-accepted outcomes of rapamycin treatment such as the inhibition of ribosome biogenesis, transcription, protein synthesis and cell cycle arrest while the transcriptional responses of cells to the long term administration of high dose of rapamycin were parallel to the short term treatment responses that were documented in the literature.

4.2.2.3. Analysis of the Significantly Expressed Genes in Response to the Presence of 5 mM Caffeine. The presence of caffeine induced the expressions of 1292 genes. The significantly enriched GO biological process terms of these genes were represented in Table 4.11. A manual investigation was also carried out in order identify the altered processes in response to the presence of 5 mM caffeine.

Table 4.11. The significantly enriched GO biological process terms of up-regulated genes in response to the presence of caffeine.

GO Term	**P-value**
Oxidation-reduction process	5.74E-10
Mitochondrial ATP synthesis coupled electron transport	1.85E-03
ATP synthesis coupled electron transport	1.85E-03
Oxidative phosphorylation	3.97E-03
Cofactor metabolic process	4.49E-03
Respiratory electron transport chain	8.05E-03
Carboxylic acid metabolic process	9.57E-03

The presence of caffeine caused an induction of the expression of 16 of the 29 TCA cycle genes and genes involved in mitochondrial ATP synthesis coupled electron transport ($1.85x10^{-3}$) (Table 4.11). The overexpression of the TCA cycle genes upon caffeine treatment were previously reported [6]. The effect of the induction of TCA cycle genes were also observed in the metabolite level. The extracellular concentrations of the TCA cycle intermediates in the presence of caffeine were observed to be higher than the control cultures (Table 4.9). A similar observation was also obtained in the presence of high dose of rapamycin. Therefore caffeine, as being another chemical compound targeting TORC1,

resulted in a set of transcriptional changes resembling the effects of high dose of rapamycin on the transcriptional responses of yeast cells.

In addition, the 19 of the 37 genes involved in lipid degradation and 30 of the 68 genes involved in fatty acid metabolism were found to be up-regulated by manual inspection. It was previously reported that caffeine increases energy expenditure in humans and results in lipid mobilization and oxidation [162]. Moreover caffeine activated the expression of 17 of the 32 genes involved in proteasomal ubiquitin-dependent protein degradation. This activation is a typical catabolic response due to the nutrient deprived conditions evoked by the presence of caffeine. The transcriptional responses of the cells to the presence of caffeine indicated a situation implying increased energy requirement in yeast cells driving the transcriptional programming in order to induce energy production processes.

Table 4.12. The significantly enriched GO biological process terms of down-regulated genes in response to the presence of caffeine.

GO Term	**P-value**
Regulation of gene expression	4.29E-08
Transcription, DNA-dependent	5.12E-08
Mitotic cell cycle	6.80E-08
Regulation of transcription, DNA-dependent	1.51E-05
Chromosome organization	3.76E-04
Chromatin remodeling	2.37E-03
Nucleosome organization	4.20E-03
Transcription from RNA polymerase II promoter	9.23E-03

A total of 711 genes were found to be down-regulated in the presence of caffeine. The significantly enriched GO biological process terms of these genes were represented in Table 4.12. A manual investigation was also carried out in order identify the altered processes in response to the presence of 5 mM caffeine. The presence of caffeine caused the down-regulation of genes involved in mitotic cell cycle (p-value 6.8×10^{-8}), transcription (p-value 5.12×10^{-8}), ribosome biogenesis and assembly, translation and rRNA

processing as in the case of high dose of rapamycin. In addition caffeine repressed the expression of genes involved in DNA repair mechanism. Caffeine was reported to augment the chromosomal breakages induced by UV irradiation, alkylating agents or maleic hydrazide in plants and animals [182].

4.2.2.4. Comparison of the Effects of the High Dose of Rapamycin and Caffeine. High dose of rapamycin and caffeine were observed to affect the expression of an overlapping set of gene in the same direction (Figure 4. 18). 631 genes were found to be overexpressed both in the presence of high dose of rapamycin or caffeine. These genes were significantly enriched with the mitochondrial ATP synthesis coupled electron transport (p-value $1.96x10^{-6}$) and TCA cycle (p-value $4.05x10^{-4}$) GO biological process terms. A manual investigation of the common up-regulated genes revealed that genes involved in lipid catabolism (*TGL2, YJU3, CIT3, GDE1, PGC1, PLB1, YDC1, SPO1, POX1, IDP3, PDH1, FOX2, ICL2* and *ADR1*) and fatty acid metabolism (*IZH2, ERG25, CIT3, MCT1, PEX11, CAT2, FAA4, ETR1, SCS7, POX1, IDP3, PDH1, YAT1, CRC1, FOX, ICL2* and *ADR1*) were induced both in the presence of rapamycin or caffeine.

A total of 920 genes were found to up-regulated only in response to the presence of rapamycin. These genes were significantly enriched with the mitochondrion degradation (p-value $7.98x10^{-3}$). Furthermore genes involved in glycogen biosynthesis (*PGM2, GLC8, GAC1, PSK2, GLG1, GSY2, PCL10, GLG2, GDB1, PIG2* and *PIG1*) were significantly expressed only in the presence of rapamycin. 661 genes were identified to be induced only in the presence of caffeine. These genes were significantly associated with actin cytoskeleton organization (p-value $9.1x10^{-3}$).

Manual investigation of the significantly expressed genes revealed that genes involved in proteasomal protein degradation (*PRE10, PRE8, SCL1, PRE2, PRE5, PRE4, PRE3* and *PUP3*) and membrane lipid metabolic process (*LIP1, GPI18, TLG2, DPL1, PBN1, ARV1, GPI13, PGA1, PER1, GPI15, YPC1, LAC1, GAA1, CWH43, ERI1, SUR2* and *ORM1*) were significantly up-regulated only in response to the presence of caffeine.

A total of 368 genes were repressed both during the long-term administration of 200 nM rapamycin or 5 mM caffeine. These genes were significantly enriched with the mitotic

cell cycle (p value $1.63x10^{-4}$). However manual investigation of the common down-regulated genes revealed that 46 of the 423 genes involved in ribosome biogenesis, 35 of the 321 genes involved in rRNA processing, 70 of the 747 genes involved in transcription, 14 of the 67 genes involved in translation initiation and 23 of the 163 genes involved in amino acid biosynthesis were repressed in response to both rapamycin or caffeine.

A total of 1056 genes were found to be repressed only in response to the presence of high dose of rapamycin. These genes were significantly associated with the intracellular transport (p-value $1.46x10^{-4}$), tRNA modification (p-value $4.89x10^{-4}$) and protein glycosylation (p-value $4.25x10^{-3}$). 343 genes were observed to be down-regulated only in the presence of caffeine. These genes were significantly enriched with the regulation of transcription (p-value $7.44x10^{-4}$) and RNA biosynthesis (p-value $1.84x10^{-3}$).

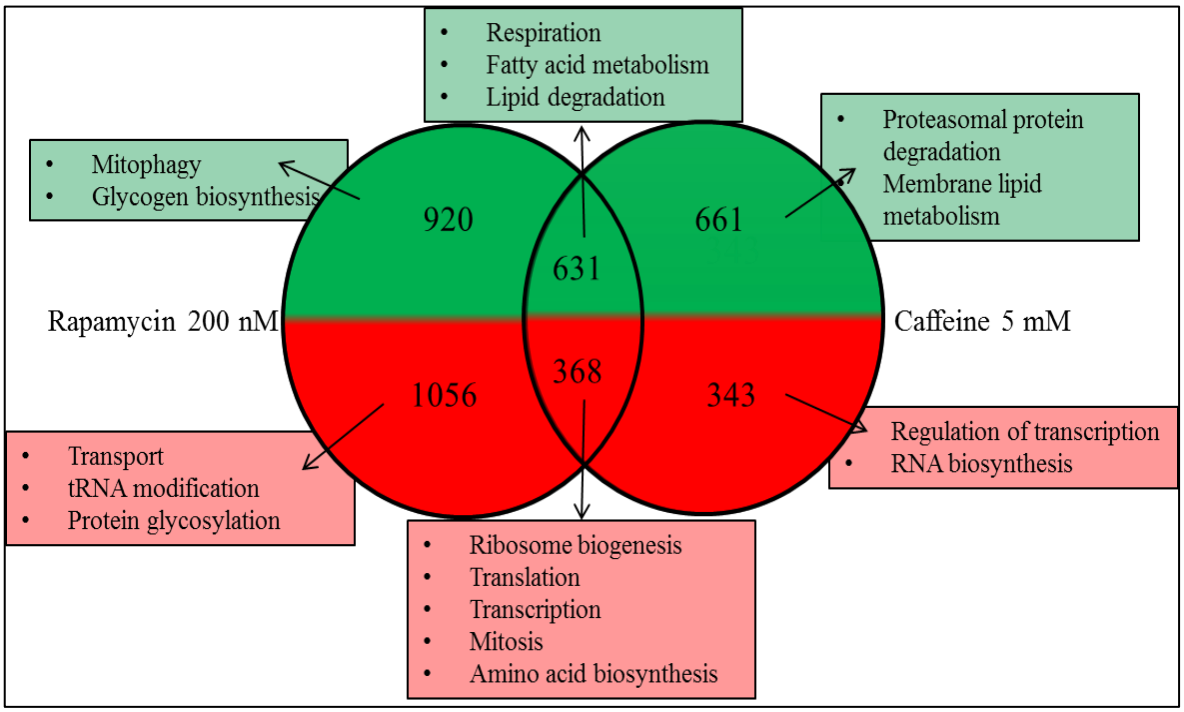

Figure 4.18. The processes that are affected from the presence of 200 nM rapamycin or 5 mM caffeine.

These results indicated that the presence of high dose of rapamycin or caffeine suppressed the growth-related processes and increased the energy requirement of the cells leading a transcriptional modulation in order to meet this requirement. Rapamycin and

caffeine both switched the metabolism from fermentation to respiration. The anabolic processes were down-regulated coupled with an increase in the catabolic processes.

Although rapamycin and caffeine were found to affect a set of similar mechanisms in yeast cells they also resulted in an alteration of a group of different processes. Rapamycin activated autophagy, specifically mitophagy, while caffeine induced proteasome-mediated degradation. These two cross-linked mechanism were reported to take part in reactive oxygen species (ROS) homeostasis and oxidative stress resistance generated due to the increased activities of TCA cycle and the mitochondrial respiratory chain [183]. The presence of rapamycin was resulted in increased expression of glycogen biosynthesis genes while caffeine induced membrane lipid metabolism. In addition to being a reserve carbohydrate and providing energy in starvation conditions, glycogen is also a constituent of cell wall. These results indicate that both rapamycin and caffeine activated cell wall stress and cells were induced the metabolism of the cell wall constituents in response to this stress.

4.3. Identification of Key Regulators and Proteins Responsive to the Presence of Rapamycin or Caffeine

In the present study the transcriptional responses of yeast cells to the presence of rapamycin or caffeine as well as rapamycin dosage (2 nM or 200 nM) were integrated with yeast transcriptional regulatory network. In order to identify and highlight the regulatory pathways affected from the presence of rapamycin or caffeine the Reporter Features Algorithm [118] was used. Yeast transcriptional regulatory network (TRN) was constructed with transcription factors (TF) that are present in the YEASTRACT TF-Consensus list and regulatory proteins that are annotated with transcription factor or regulatory activity in SGD. The target genes known to be effected by these regulatory proteins, with a reported direct evidence, retrieved from YEASTRACT [119]. The constructed yeast TRN includes 129 TFs and 24778 TF-gene interactions. The key TFs identified by Reporter Features Algorithm were ranked according to their p-values and the top 10 scoring TFs with significance count >1 were regarded as key TFs, around which most transcriptional changes occur.

4.3.1. Key Transcription Factors Responsive to the Presence of 2 nM Rapamycin

Gis1p was identified as the top scoring key TF in response to the presence 2 nM rapamycin. Gis1p activates transcriptional reprogramming of carbon metabolism and the stress response during nutrient starvation [184]. Gis1p is also involved in chronological cell aging by regulating genes that have roles in acetate and glycerol formation [185] and regulation of phospholipid biosynthesis. It was reported that the activity of Gis1p is controlled by target of rapamycin (TOR) and protein kinase A (PKA) via the Rim15p kinase [30].

Gcn4p which is a general controller of amino acid biosynthetic genes in response to amino acid starvation was identified among the key TFs in the presence of 2 nM rapamycin. Gcn4 p also regulates the expression of genes involved in purine biosynthesis, organelle biosynthesis, autophagy, glycogen homeostasis and multiple stress responses. Gcn4p was reported to play a role in the rapamycin-sensitive signaling pathway by regulating the expression of genes involved in the utilization of poor nitrogen sources and TOR pathway controls Gcn4p activity by regulating the translation of Gcn4p mRNA [186]. The presence of 2 nM rapamycin induced the expression of Gcn4p-regulated genes as well as the expression of Gcn4p. These results indicated that the regulatory activity of Gcn4p is mainly transcriptionally governed.

Aca1p and Cst6p (Aca2p), the two paralogous transcriptional activators which are members of ATF/CREB family together with the repressor Sko1p, were among the key TFs around which a collective change in the expression of the genes occur in response to the presence of 2 nM rapamycin. Aca1p is important for the carbon source utilization and Aca2p is important for growth on non-optimal carbon sources, chromosome stability as well as resistance to a variety of drugs [187]. The rapamycin treatment results in a nutrient starvation-like response since TOR is responsive to carbon and nitrogen source availability. Significant changes in the expression levels of the target genes of Aca1p and Cst6p may be due to the presence of rapamycin in the medium resembling a condition of carbon source starvation.

Crz1p, a calcineurin-responsive zinc finger TF was also among the key TFs. Crz1p activates stress response and regulates genes involved in ion transport, cell wall synthesis and maintenance, lipid and sterol metabolism, vesicle transport and protein degradation [188]. The nuclear localization of Crz1p was reported to be regulated by TOR. The differential expression of Crz1p regulated genes may be due to the activation of cell wall integrity pathway in response to the presence of rapamycin [86]. In addition to this, the pivotal role of TORC2 in the negative regulation of calcineurin-dependent stress signaling was also reported previously [189]. Although TORC2 was widely known as rapamycin insensitive, accumulation of data suggests that rapamycin can inhibit either TORC1- or TORC2-dependent functions. In mammalian cells it was reported that rapamycin inhibits TORC1 upon short exposure to the drug, but upon long exposure also causes disassembly and inhibition of TORC2 [190]. A similar mechanism may operate in yeast. Rapamycin treatment was reported to induce stress responsive genes [168] and in this case inactivation of antagonistic TORC2 function in calcineurin-dependent stress signaling by long-term rapamycin treatment would result in Crz1-dependent activation of stress response.

Yrr1p and Yrm1p, the two paralogous transcription factors acting on an overlapping set of genes, were identified as key TFs in response to the presence of 2 nM rapamycin. These transcription factors activate genes involved in multidrug resistance. Multidrug resistance is often acquired through the activation of multidrug efflux pumps, belonging to the ATP-Binding Cassette (ABC) or Major Facilitator Superfamilies (MFS). As a common target of Yrr1p and Yrm1p, the multidrug resistance transporter Flr1p was observed to be up-regulated in the presence of rapamycin. Flr1p, a member of MFS, was reported to be induced at the transcriptional level when yeast cells are exposed to the stresses this gene confers resistance to Flr1 has been shown to confer resistance to a large number of chemically and structurally unrelated xenobiotics and drugs, including cycloheximide, 4-nitroquinoline-1-oxide (4-NQO), benomyl, methotrexate, diazaborine, cerulenin, diamide, diethylmaleate, menadione, paracetamol and mancozeb [191]. Although there is no documented response of Flr1p to rapamycin, these results may indicate that Flr1p is also responsive to long term treatment with low dose rapamycin and Yrr1p and Ymr1p regulates drug response of yeast cells in the presence of rapamycin at low concentrations.

Ppr1p was found to be one of the key transcription factors responsive to the presence of 2 nM rapamycin. Ppr1p is a zinc finger transcription factor and positively regulates the genes involved in *de novo* pyrimidine biosynthesis in response to pyrimidine starvation. Together with its target genes, *PPR1* was also found to be significantly up-regulated in response to the long term administration of 2 nM rapamycin indicating that regulator activity is mainly transcriptionally governed. It was recently reported that activation of mTORC1 stimulated the *de novo* pyrimidine biosynthesis [192]. It should be noted that the significantly expressed targets of Ppr1p were not belong to the *de novo* pyrimidine biosynthesis pathway. It seems that Ppr1p induced genes that have roles in mediating cell death in response to chromosome missegregation, suppressing genes involved in regulation of pyruvate dehydrogenase activity, rather than *de novo* pyrimidine biosynthesis due to the inhibition of TOR function by rapamycin.

4.3.2. Key Transcription Factors Responsive to the Presence of 200 nM Rapamycin

The long term administration of 200 nM rapamycin was observed to affect stress responsive transcription factors. Skn7p, Cad1p, Sko1p and Hsf1p which are known as the regulators of oxidative stress were among the key TFs identified in response to the presence of 200 nM rapamycin. Skn7p and Hsf1p, which are known to interact with each other, were reported to regulate stress-responsive genes and cooperate for maximal induction of heat shock genes in response specifically to oxidative stress. In addition to the observation of rapamycin treatment to activate stress responsive genes it was also reported to affect the expression of genes at same direction with the conditions of heat/oxidative stress [193]. In addition to its regulatory role in oxidative stress conditions, Skn7p is also involved in osmoregulation and regulation of cell size. Skn7p was also reported to be activated by Rho1p in response to cell wall stress [54]. Hsf1p was reported to be involved in the negative regulation of TOR signaling cascade. Hsf1p regulates genes involved in protein folding, detoxification, energy generation, carbohydrate metabolism, cell wall integrity, spindle pole body duplication, protein transport and cell cycle progression. The expression level of *SKN7* was observed to be significantly changed indicating that its regulator activity was mainly transcriptionally governed whereas *HSF1* was not significantly expressed in response to the presence of 200 nM rapamycin suggesting its post-transcriptional regulation. In addition to Skn7p and Hsf1p, Sko1p was also reported to

be involved in osmotic and oxidative stress response and it was reported to Sko1p transcription repressor has been shown to regulate certain Hog1p-dependent genes [194].

Cad1p (Yap2p) is one of the key transcription factors known to be involved in stress response in addition to iron metabolism and pleiotropic drug resistance. It was reported to regulate a set of proteins involved in the stabilization and folding of proteins in an oxidative environment. Besides Cad1p, another transcription factor of Yap family involved in iron metabolism, Yap5p was identified as a key transcription factor responsive to the presence of 200 nM rapamycin. Yap5p regulates the expression of vacuolar iron transport in response to cytosolic iron. *YAP5* was reported to be strongly induced under amino acid starvation, nitrogen depletion and stationary and diauxic phases [195]. *YAP5* was found to be significantly up-regulated in response to the presence of 200 nM rapamycin indicating its activity was transcriptionally regulated. *CAD1* was also found to be induced but it was not among the significantly expressed genes suggesting its post-transcriptional regulation. Since rapamycin treatment is known to mimic amino acid or nitrogen starvation conditions, cells probably induced transcription factor *CAD1* and *YAP5* in response to the presence of rapamycin imitating nitrogen depletion. A specific role of iron in regulation of mammalian target of rapamycin (mTOR) signaling was shown recently [196]. Iron is also an important cofactor in amino acid biosynthesis and uptake. The induction of *CAD1* and *YAP5* was probably for regulation of iron metabolism in order to induce amino acid biosynthesis in an environment similar to amino acid starved [197].

Forkhead-like transcription factor Fhl1p was identified among the key transcription factors responsive to the presence of 200 nM rapamycin. Fhl1p is a central regulator involved in regulation of expression of ribosomal protein genes through TOR and PKA [30]. In the presence of 200 nM rapamycin, *FHL1* was found to be significantly down-regulated, in order to repress ribosomal gene transcription, indicating that the activity of Fhl1p transcriptionally regulated.

TORC1 pathway was reported to respond to nitrogen starvation and induce filamentous growth [198, 199]. Rapamycin treatment was observed to inhibit pseudohyphal growth under nitrogen limited conditions [146]. Three regulators, Ste12p, Ash1p and Phd1p known to be involved in the regulation of pseudohyphal growth were

identified as key transcription factors responsive to the presence of 200 nM rapamycin. Ste12p is activated by MAP kinase signaling and it activates genes involved in mating and pseudohyphal/invasive growth pathways. Ash1p is involved in regulation of pseudohyphal growth in diploid cells deprived of nitrogen and required during pseudohyphal growth to activate the transcription of *MUC1*/*FLO11*, which encodes a flocculin that helps cells stick together as they grow into the substratum. Phd1p is a transcriptional activator that enhances pseudohyphal growth and regulates *FLO11* expression. In the presence of 200 nM rapamycin these three transcriptional activators of pseudohyphal growth were found to be significantly repressed. These results may indicate that inhibition of TORC1 function by rapamycin hindered the filamentous growth by regulating its transcription factors at the transcription level.

Adr1p which is a carbon source-responsive transcription factor was among the key transcription factors identified in the presence of 200 nM rapamycin. Adr1p is required for transcription of the glucose-repressed genes and activates genes involved in ethanol, glycerol, lactate, and amino acid utilization, peroxisome biogenesis, and the β-oxidation of fatty acids. The activity of Adr1p was reported to be inhibited by Bmh proteins through direct binding of Bmh proteins to its regulatory domain in the presence of glucose. Bmh proteins are involved in many signaling and cell differentiation pathways, including pseudohyphal differentiation, DNA damage checkpoint, nitrogen catabolism, TOR signaling, stress response, protein degradation, retrograde signaling, exocytosis and vesicle transport, catabolite inactivation, and cell cycle regulation. Bmh1p was reported to be genetically interacting with TOR signaling pathway and it was suggested to be a downstream target of TOR [200, 201]. *ADR1* was observed to be significantly up-regulated in the presence of 200 nM rapamycin and this could be a result of regulatory role of Bmh proteins on Adr1p through TOR signaling pathway in response to the presence of rapamycin resembling glucose depleted conditions.

4.3.3. Key Transcription Factors Responsive to the Presence of 5 mM Caffeine

The long term administration of caffeine was observed to affect an overlapping set of transcription factors with high dose (200 nM) of rapamycin administration. The stress responsive transcription factors, Skn7p and Sko1 were among the key transcription factors

responsive to the presence of caffeine. Furthermore, another stress response regulator that has role in iron metabolism Cad1p (Yap2p), the iron sensing transcription factor Yap5p which is responsive to nitrogen depletion and the transcription factor Ste12p involved in the regulation of pseudohyphal growth, were among the key transcription factors around which a collective change in the expression of the genes occur in response to the presence of caffeine. In addition, another member of Yap family, Yap4p was also identified among the key transcription factors that are responsive to the presence of caffeine but not to the presence of rapamycin. Yap4p was also reported to be responsive to a broad range of stress conditions, including osmotic and oxidative stress, temperature shift and arsenic exposure [202]. In addition to rapamycin, caffeine also directly inhibits TORC1 function and the activation of stress response by inhibition of TOR was reported previously [64]. Moreover caffeine was reported to induce a starvation response that would lead significant changes in the expression of genes responsive to nitrogen depletion as well as genes involved in pseudohyphal growth [203]. It was reported that the rapamycin and caffeine display remarkably similar effects on global gene expression [64]. These results may indicate that the transcriptional responses of yeast cells to the presence of rapamycin or caffeine converged on a set of key transcription factors that are involved in regulation of stress, iron metabolism and nitrogen starvation.

In addition to common regulators that are responsive both to the presence of rapamycin or caffeine, the long term administration of caffeine also affected a distinct set of transcription factors. The key transcription factors Swi4p, Swi6p and Mbp1p, constituting SBF (Swi4p-Swi6p) and MBF (Swi6p-Mbp1p) complexes, regulate late G1-specific transcription of targets including cyclins. A second role of SBF in cell wall integrity signaling was also reported. Swi4p is the DNA binding subunit of SBF complex and Swi6p is required for binding to cell cycle-regulated promoters. In addition to Swi6p, Mpk1p, a mitogen-activated protein kinase (MAPK) of the cell wall integrity signaling pathway, forms a complex with Swi4p to activate SBF and Swi6 is recruited to this complex to activate transcription. It was reported that if Mpk1p is activated by caffeine, a typical activator of cell wall integrity pathway, it fails to bind Swi4p and cannot induce Swi4p-driven transcription [204]. Moreover, cell wall stress induces phosphorylation of Swi6p by Mpk1p, which regulates Swi6p localization [205]. Swi6p forms another complex with Mbp1p (MBF complex) which plays an important role in the periodic transcription of

genes necessary for DNA replication and repair [206]. Caffeine was reported to cause cell cycle arrest in humans [207] and cell wall stress. Caffeine also elicits the hypersensitivity to the DNA-damaging agents since it causes the disruption of DNA damage checkpoints [208]. Moreover, TORC1 signaling was reported to be involved in cell survival in response to DNA damage [209]. These results suggest that the presence of caffeine led to transcriptional modulation of the genes involved in cell cycle, cell wall stress and DNA damage through SBF and MBF complexes.

Centromere binding factor Cbf1p was identified as one of the key transcription factors responsive to the presence of caffeine. Cbf1p has a role in transcriptional activation of sulfur metabolism in yeast [210]. Cbf1p affects nucleosome positioning and it is required for efficient chromosome stability and methionine prototrophy [211]. Cbf1p protein abundance was stated to be increased in response to DNA replication stress. Chromosome segregation was reported to be altered by nutrient limitation [212]. *CBF1* was observed to be significantly down regulated in the presence of caffeine indicating that its regulatory activity is governed transcriptionally. Since caffeine treatment resembles nutrient limited conditions, both Cbf1p and its targets were significantly expressed in order to regulate chromosome stability and nucleosome positioning in response to nutrient depletion.

Table 4.13. Key transcription factors responsive to the presence of 200 nM rapamycin or 5 mM caffeine.

200 nM Rapamycin	5 mM Caffeine
SKN7 SKO1 YAP5 STE12 CAD1	
HSF1	SWI4
FHL1	SWI6
ASH1	MBP1
PHD1	CIN5
ADR1	CBF1

Five of the ten key regulators identified in the presence of caffeine were common with those of identified in the presence of 200 nM rapamycin (Table 4.13). This may indicate that the cellular differentiation processes, nitrogen starvation and stress responses of yeast cells in the presence of rapamycin and caffeine were regulated through the same transcription factors. Caffeine was observed to affect the expression of genes regulated by transcription factors involved in processes such as cell cycle, DNA damage and cell wall integrity.

4.3.4. Identification of the Reporter Proteins in Responsive to the Presence of Rapamycin and Caffeine

In this study the transcriptomic responses of yeast cells to the presence of rapamycin or caffeine were integrated with the yeast protein-protein interaction network in order to identify the key proteins around which significant transcriptional changes occur. The yeast physical protein-protein interactions were retrieved from BioGRID and integrated with transcriptome data using reporter features algorithm [118]. The constructed yeast PPI network includes 5633 proteins and 75584 interactions. A p-value threshold of 0.05 was used to define reporter proteins.

A total of 215 proteins were identified as reporter proteins whose neighbors displayed significant changes in the expression level in response to the presence of 2 nM rapamycin. These proteins significantly enriched with the mRNA splicing via spliceosome (p-value 4.92×10^{-12}), microtubule cytoskeleton organization (p-value 1.42×10^{-6}), chromosome segregation (p-value 6.59×10^{-5}), protein localization (p-value 1.37×10^{-4}), cell division (p-value 1.32×10^{-4}) establishment of cell polarity (p-value 9.1×10^{-3}) and mitosis (1.51×10^{-3}). Furthermore the top 10 scoring reporter proteins include Lea1p, Lsm3p, Prp2p, Prp45p and Cwc2p which are involved in mRNA splicing; Myo1p, Dad1p and Cik1p which are involved in different processes in cell cycle and division such as cytokinesis, chromosome segregation and mitotic spindle organization. Siz1p was also among the top 10 scoring reporter proteins, which is involved in chromosome segregation and protein sumoylation. This post-translational modification affects many processes such as transport, transcriptional regulation, apoptosis, protein stability, response to stress and progression through cell cycle [213].

A total of 506 reporter proteins were identified around which most transcriptional changes occur in response to the presence of 200 nM rapamycin. These proteins were significantly enriched with the ribosome biogenesis (p-value $2.9x10^{-42}$), rRNA processing (p-value $1.12x10^{-33}$), transcription from RNA polymerase I promoter (p-value $7.3x10^{-4}$) and translational initiation (p-value $3.18x10^{-4}$). Ubiquitin-related proteins and proteases were observed to be involved in the top 10 scoring reporter proteins in the presence of 200 nM rapamycin. The non-ATPase base subunits of 19S regulatory particle of the 26S proteasome, Rpn1p, Rpn10p and Rpn1p, as well as the ATPase of 19S regulatory particle of the 26S proteasome, Rpt5p were among the key proteins. The ubiquitin-specific protease Ubp3p and Bre5p which is a ubiquitin protease cofactor which forms deubiquitination complex with Ubp3p were ranked in the top 10 scoring reporter proteins. In addition, the ubiquitin Ubi4p which marks proteins for selective degradation via the ubiquitin-26S proteasome system and Smt3p, a ubiquitin-like protein of SUMO family were identified as reporter proteins. Fcj1p, a mitochondrial inner membrane protein and Fun12p, a GTPase required for general translation initiation were among the reporter proteins around which significant transcriptional changes occur in response to the presence of 200 nM rapamycin. It was observed that the presence of high doses of rapamycin resulted in a transcriptional reprogramming in genes involved in degradation processes and translation. The induction of ubiquitination and degradation processes upon rapamycin treatment or nutrient starvation was reported previously [214, 215]. Moreover the inhibition of translation initiation upon inhibited TOR function is a well-documented outcome of rapamycin treatment [71].

There were 245 reporter proteins around which most transcriptional changes occur in response to the presence of caffeine. These proteins were significantly enriched with the regulation of transcription from RNA polymerase I promoter (p-value $2.73x10^{-3}$) GO biological process term. Moreover, the reporter proteins were observed to be involved in processes such as TCA metabolism, glutamate metabolism, proteolysis and DNA repair. Three of the top 10 scoring reporter proteins (Fcj1p, Rpn11p and Ubi4p) identified in response to the presence of caffeine were common proteins with those identified in the presence of 200 nM rapamycin. In addition to Rpn11p and Ubi4p two other proteins, Pup2p and San1p which are also involved in ubiquitin or proteasome-dependent catabolism were identified among the reporter proteins in the presence of caffeine. Slf1p

which regulates mRNA translation and copper ion homeostasis was ranked among the top 10 scoring reporter proteins together with its paralogous protein Sro9p. Sro9p is also involved in translation and Heme regulation of Hap1p which was also identified as reporter protein. Hap1p is a transcription factor involved regulation of respiration and complex regulation of gene expression in response to levels of Heme and oxygen.

Table 4.14. The top 10 reporter proteins identified in response to presence of rapamycin or caffeine.

2 nM rapamycin	**200 nM rapamycin**	**5 mM Caffeine**
LEA1	RPN11	
LSM3	FCJ1	
SIZ1	UBI4	
MYO1	BRE5	SRO9
DAD1	FUN12	HAP1
PRP2	RPN1	NAB2
YCR051W	RPN10	YLF2
CIK1	RPT5	PUP2
PRP45	SMT3	SAN1
CWC2	UBP3	SLF1

There were no common reporter proteins identified as responsive to the low and high doses of rapamycin (Table 4.14). TOR, as a master regulator of growth, is involved in many growth related processes including, cell cycle, transcription, ribosome biogenesis and translation. Low dose of rapamycin was observed to affect cell cycle and division related processes as well as mRNA splicing. It was stated that 90% of all mRNA splicing events occur on ribosomal protein transcripts [216] and TORC1 was reported to play an active role in mRNA stability and pre-mRNA splicing [30]. The reporter protein analysis also revealed that like high dose of rapamycin low dose of rapamycin also affected the expression of genes whose products are neighbors of the protein involved in mitosis.

High dose of rapamycin was observed to affect degradation and translation-related processes. The presence of caffeine and high dose of rapamycin reflected their effects on an overlapping set of reporter proteins around which a significant transcriptional response

concentrated. These results may indicate that the responses of yeast cells to the presence of caffeine resemble the responses to the presence of high dose of rapamycin, as in the case of transcriptional regulatory responses. Like rapamycin, caffeine also resulted in an alteration of transcriptional programming of the genes involved in translation and catabolic processes. The analysis of transcriptional responses of yeast cells to the presence of rapamycin and caffeine revealed that both high dose of rapamycin and caffeine were found to induce the expression levels of the genes involved in TCA cycle and respiration. This response was also observed in reporter protein analysis of the cells grown in the presence of caffeine whereas this result was not obtained through the reporter protein analysis of the cells grown in the presence of high dose of rapamycin. The regulation of respiration by TOR signaling was reported previously through stating that reduced TOR signaling increases respiration and extends chronological life span [44]. The involvement of TORC1 in DNA damage response and the disruption of DNA damage checkpoints upon caffeine treatment were also reported previously [208, 209].

5. CONCLUSION

In the scope of this thesis the responses of the yeast cells to the long-term administration of rapamycin and caffeine were investigated in conjunction with the oxygen availability and pH of the cultivation medium at transcriptional, metabolomic and phenotypic levels. For this purpose first the working concentrations of rapamycin (2 nM) and caffeine (5 mM) which resulted in a similar growth phenotype were determined in shake flask cultivations. The batch cultivations of the yeast cells in the absence and presence of determined concentrations of rapamycin or caffeine were carried out under four different environmental conditions in fermenters. In these cultivations the fermenters were fully aerated or they were micro-aerated and the pH of the medium was kept at 5.5 or it was left to take its natural course.

The transcriptional responses of yeast cells to the long term administration of 2 nM rapamycin were different from the reported responses of yeast cell populations that were treated with rapamycin at a certain instance of population growth. The well-documented transcriptional outcomes of rapamycin treatment such as the down-regulation of the genes involved in ribosome biogenesis, protein synthesis and transcription as well as cell cycle arrest were not observed in the long term treatment of 2 nM rapamycin. This was possibly because the cellular response had already adapted to the presence of rapamycin at the transcriptional level. However the presence of rapamycin resulted in a set of transcriptional changes similar to the nitrogen starvation responses of yeast cells by inducing the expression of several ammonium and amino acid permeases and the genes involved in allantoin degradation. This may indicate that 2 nM rapamycin was still effective but caused a partial TORC1 inhibition since it is a relatively low dose. The presence of rapamycin also induced the expression of genes involved in mRNA degradation whereas repressed phosphate-ion transport and cells size regulatory genes.

pH was observed to be more decisive parameter on the transcriptional responses of the cells than the oxygen level since the transcriptional profiles of the cells in the presence of rapamycin were clustered according to the pH level. Furthermore the effects of rapamycin on yeast growth were observed to be altered by the different extracellular pH

levels. The combinatorial effects of oxygen level and rapamycin pointed to the importance of the maintenance of the oxygen level in the long term rapamycin treatment. The low levels of oxygen during the long term usage of rapamycin may cause COX deficiency in humans.

The effects of caffeine in the long term administration resulted in a wider set of transcriptional changes resembling the documented outcomes of relatively high dose of rapamycin treatment. In contrast to the gene expression response of yeast to being cultivated in the presence of relatively low dose of rapamycin, the transcriptional response of yeast to the presence of caffeine was traceable at lower-hierarchy genes performing the necessary metabolic functions. The well-documented processes known to be regulated by TOR such as ribosome biogenesis, transcription, chromatin silencing, translation and RNA processing were observed to be inhibited in the presence of caffeine. The long term exposure to caffeine induced the cell wall assembly and catabolic processes as well as the intracellular processes and lipid metabolism in an interactive manner with oxygen level and pH. The inhibition of processes such as GMP biosynthesis in response to the interaction effects of the presence of caffeine and pH level as well as tRNA methylation in response to the interaction effects of oxygen levels highlight its anti-proliferative effects and the potential usage of caffeine in cancer treatment.

In the present study the effects of rapamycin dosage on yeast transcriptional responses were also investigated and the transcriptional and metabolic responses of cells to the long term administration of high dose of rapamycin were compared with the responses of the cells to the presence of low dose rapamycin or caffeine under micro-aerated and pH controlled conditions. A severe decrease in the maximum specific growth rate of the cells was observed in high dose rapamycin treated cultures coupled with the repression of the genes involved in ribosome biogenesis, transcription and translation while low dose resulted in more subtle changes without any growth defect. The transcriptional responses of the yeast cells to the long-term administration of rapamycin were parallel to the short term treatment of high dose, indicating a non-adaptive response. However both low and high doses of rapamycin resulted in a set of transcriptional changes mimicking nutrient starvation responses of yeast cells. They both induced autophagy and altered membrane trafficking. These results may indicate that dosage of rapamycin should be carefully

evaluated especially in the long term usage as a chemotherapeutic and immunosuppressant. Cells may adapt to the presence of low dose without displaying any growth defects. However it is still effective at the transcriptome level by leading metabolism to autophagy but this dose may not be enough to block the proliferation of aggressive tumors. On the other hand high dose of rapamycin may stop many processes related to cell growth by causing severe responses at the transcriptional and metabolic levels and result much severe growth defects.

The transcriptional responses of cells to the long term administration of caffeine mimicked closely the case of a high dose rapamycin. The presence of high dose of rapamycin or caffeine suppressed the growth-related processes and increased the energy requirement of the cells leading a transcriptional modulation in order to meet this requirement. Rapamycin and caffeine both switched the metabolism from fermentation to respiration. The anabolic processes were down-regulated coupled with an increase in the catabolic processes. Caffeine induced the expression of genes involved in cell wall biogenesis and assembly. Although rapamycin did not directly alter the expression of the genes involved in cell wall architecture, it induced the genes involved in glycogen synthesis which is a reserve carbohydrate and a constituent of cell wall.

The results obtained in this study provided a better insight for the interactions of drug action mechanisms and the environmental parameters and highlighted the importance of oxygen and extracellular pH levels on the drug action. Furthermore this study pointed the necessity of the careful evaluation of the drug dosage during the long term usage of rapamycin. The results obtained from this study supported the observations that both caffeine and rapamycin alters a common set of processes however the effects of them on yeast cells were not completely the same. For a further and detailed investigation of the differences of the mechanism of actions of these two drugs the yeast cells may be cultivated in the presence of these two drugs simultaneously. The results may also provide a perspective for the usage of caffeine during the cancer treatment as a supplement to rapamycin.

APPENDIX A: SUPPLEMENTARY FIGURES

In order to determine the working concentrations of rapamycin the yeast cells grown in defined medium in shake flasks were sampled at regular intervals and diluted samples were inoculated for 48 or 72 hours on complex media (YPD) (t=0 or exp) (Figure A.1 and Figure A.2) or defined media (t=0 or exp) (Figure A.3 and Figure A.4) without any drug supplements at 30^{o}C, respectively. Rapamycin was added to the cultures prior to inoculation (t=0) or at the mid-exponential phase of growth (exp). Samples were collected from cultures that had 0.5nM, 2nM, 20 nM and 200 nM rapamycin. The colony formation from the samples taken on the 9th and the 12th days of growth in shake flasks are presented in the figure.

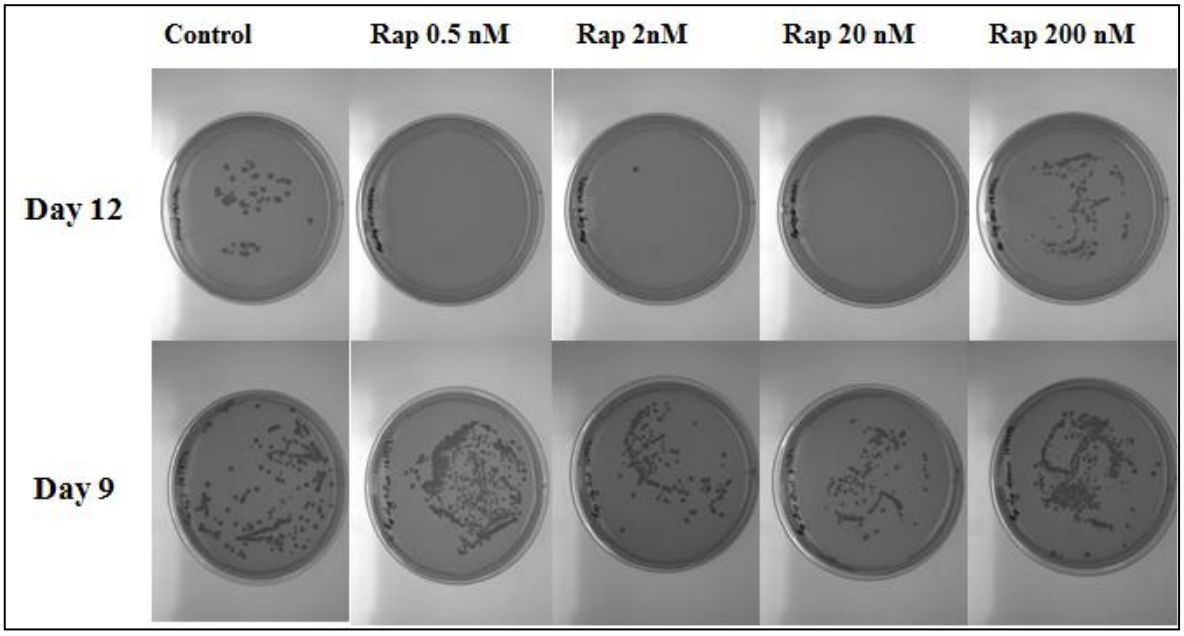

Figure A.1. The viability analysis of the cells grown in the presence of rapamycin (t=0) on complex media

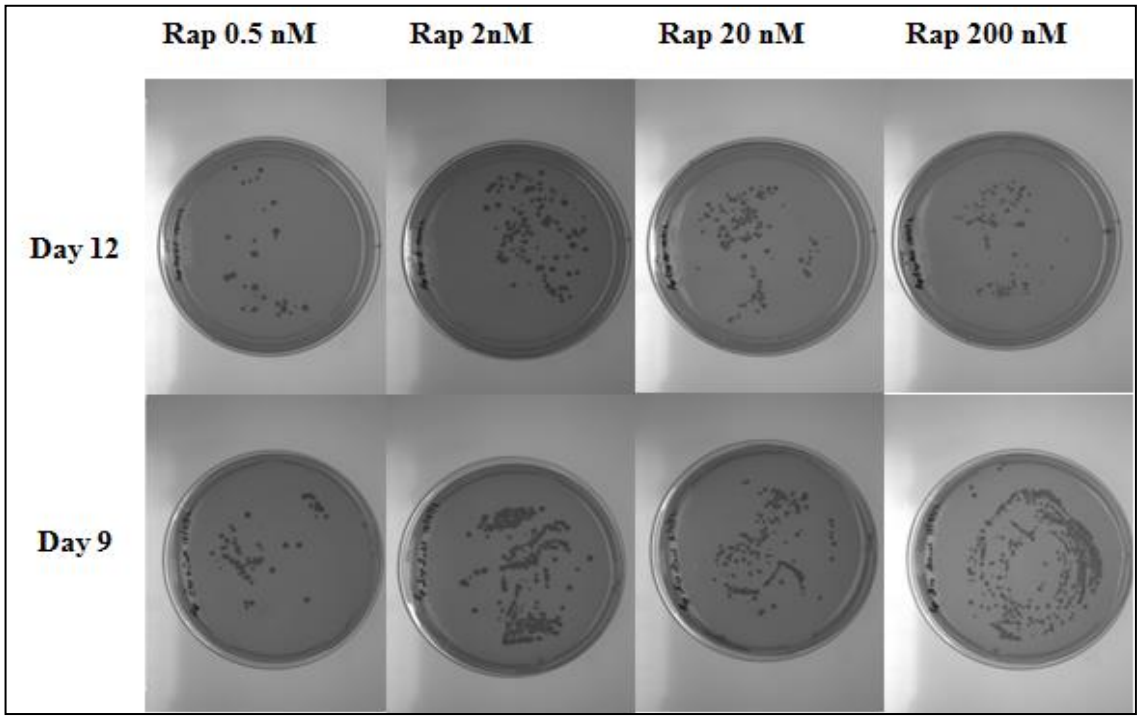

Figure A.2. The viability analysis of the cells treated with rapamycin at the mid-exponential phase of growth (exp) on complex media

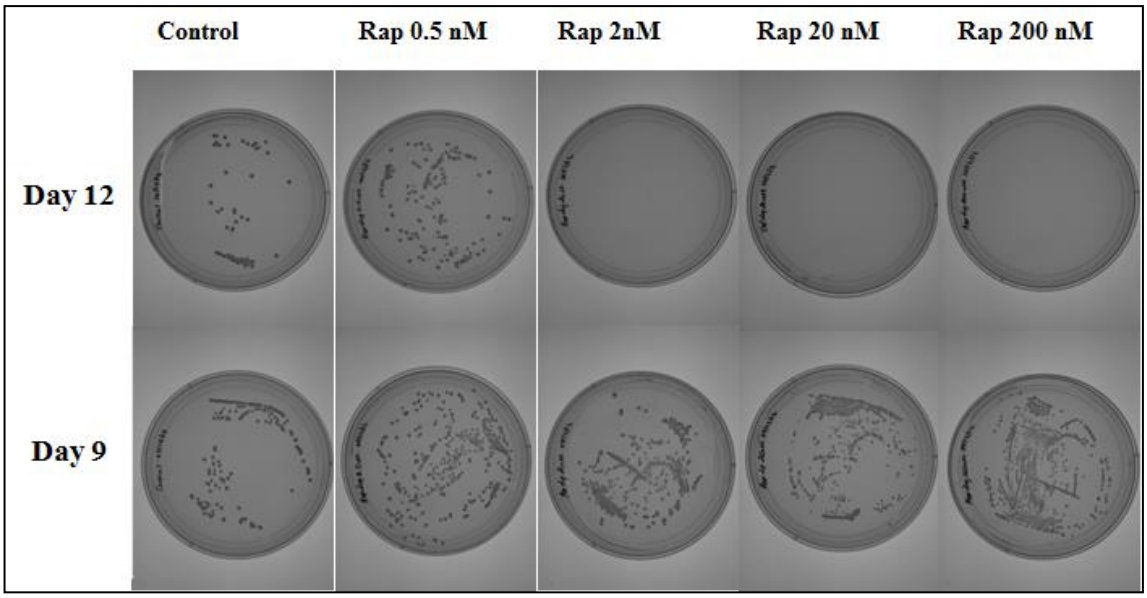

Figure A.3. The viability analysis of the cells grown in the presence of rapamycin (t=0) on synthetic defined media

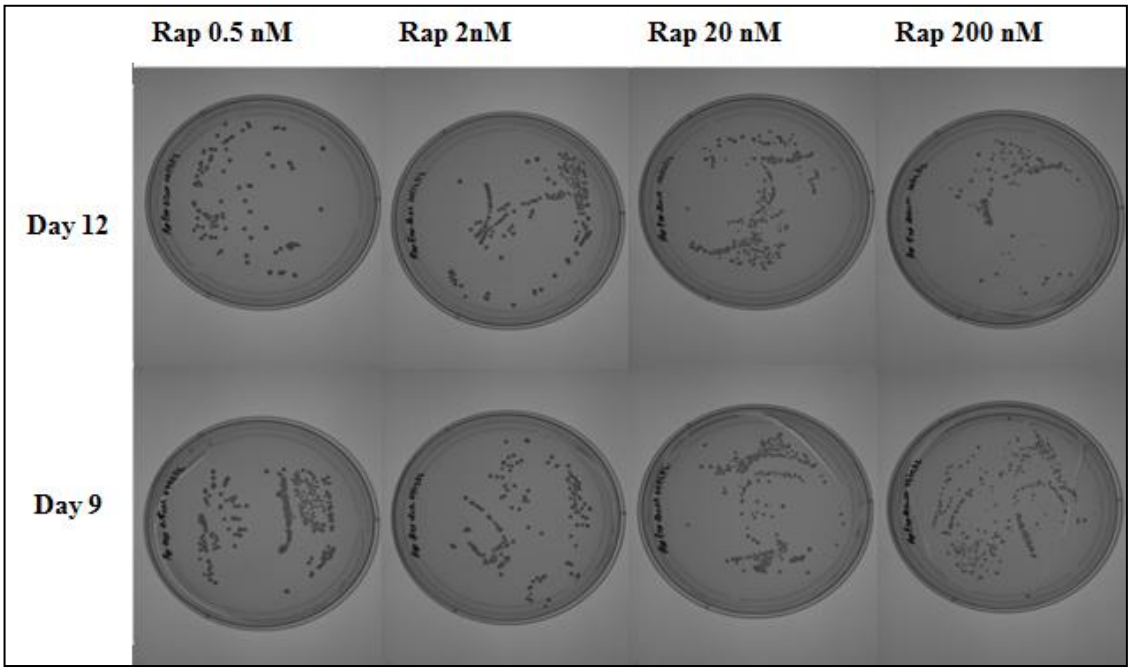

Figure A.4. The viability analysis of the cells treated with rapamycin at the mid-exponential phase of growth (exp) on synthetic defined media

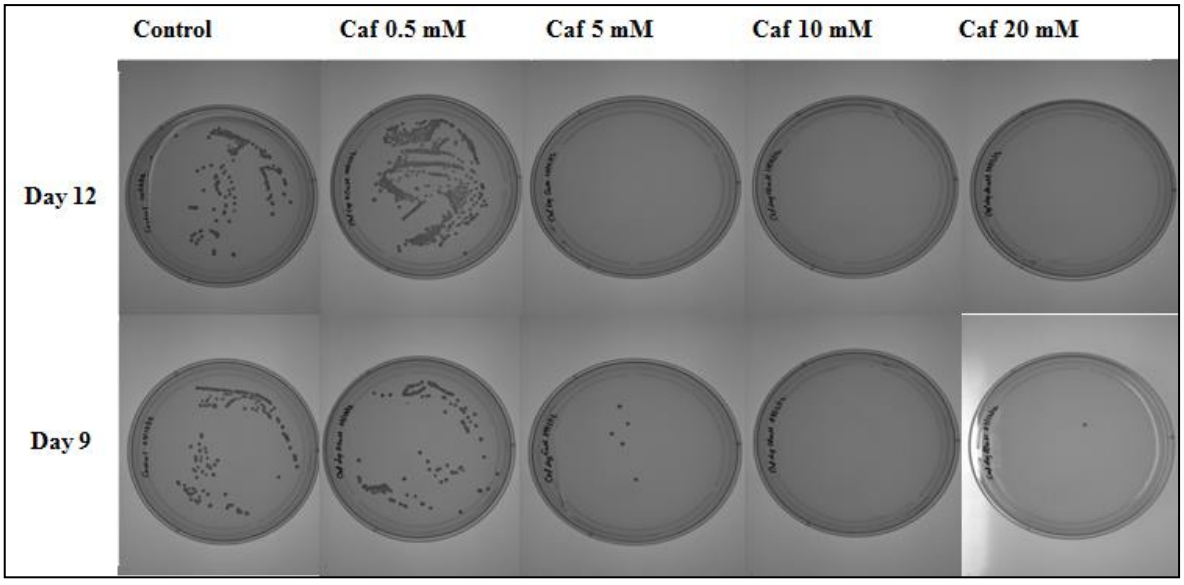

Figure A.5. The viability analysis of the cells grown in the presence of caffeine (t=0) on synthetic defined media

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