Integrated Master in Chemical Engineering

Impact of rapamycin on chronological life span of filamentous fungi

Master Thesis

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Abstract

Filamentous fungi have an immense impact on humanity. They can be both detrimental, causing several human infections and millions of dollars of crops loss annually, or beneficial when used to produce millions of tons of chemicals, food stuffs, and pharmaceuticals. Therefore, extension of chronological life span (CLS) in filamentous fungi may have an immense impact on industry. There is significant evidence that the cellular recycling mechanism known as autophagy has an effect on the lifespan of many organisms. To this day, most of the studies on autophagy have used the yeast *S. cerevisiae* as a model organism and relatively few have focused on filamentous fungi. *Aspergillus nidulans* is considered a model organism for fungal research studies due to its several advantages such as a short generation time, easy culture in the laboratory, and an easy storage at -80°C.

The aim of my work is to study the impact of the macrolide rapamycin, an autophagy inducer, on the CLS of filamentous fungi, specifically the *Aspergillus nidulans* GFP-*atg8* strain. In order to do so, *A. nidulans* was grown in shake flasks and, by measuring the dry cell weigh (DCW), it was possible to make a growth curve. For most microorganisms the biomass concentration is proportional to the number of viable cells. However, this is not the case in filamentous fungi because their rigid cell walls can keep cells intact long after death. Therefore to test the effect of rapamycin on lifespan we must employ an assay that directly measures metabolic activity, instead of using commonly employed biomass quantification methods, such as DCW and turbidity. A method based on the tetrazolium salt XTT is used to quantify the metabolic activity of the cultures. This salt is reduced to its colorimetric formazan derivate in the presence of metabolic active cells. This enables measurements of the metabolic activity of a microbial culture using visible spectophotometry in the 430-490 nm range. The effect of rapamycin was studied by measuring the optical density (OD) of the grown culture at 490 nm.

As expected, addition of rapamycin was shown to reduce growth rate and increase vacuolation. Fluorescent microscopy studies show that autophagy is an ATP dependent process as GFP-*atg8* cells were unable to transport autophagosomes to vacuoles in the absence of a carbon source (i.e. glucose). Induction of autophagy (in presence of trace amounts of glucose) was shown to increase life span by 300%, as the metabolic activity remains higher for a longer period time, after glucose is exhausted.

Key words: Autophagy; Rapamycin; TOR signaling Pathway; XTT assay;

Resumo

Os fungos filamentosos têm um grande impacto na humanidade. Estes tanto podem ser prejudiciais, causando diversas infecções humanas e milhões de dólares de perdas de culturas anualmente, ou, benefícios, quando utilizados para produzir milhões de toneladas de químicos, produtos alimentares e farmacêuticos. Um aumento na longevidade cronológica (CLS) nos fungos filamentosos poderá portanto ter um grande impacto na indústria.

Existe uma grande evidência de que os mecanismos de reciclagem celular têm efeito na longevidade de muitos organismos. Até hoje, a maioria dos estudos sobre autofagia utilizaram a levedura *S. cerevisiae* como organismo modelo e relativamente poucos estudos se concentraram nos fungos filamentosos. *Aspergillus nidulans* é considerado um organismo modelo para investigação sobre fungos devidos às suas diversas vantagens, tais como curto tempo de geração, fácil culturabilidade em laboratório e um fácil armazenamento a -80°C.

O objectivo deste trabalho é estudar o impacto do macrolídeo rapamicina, um indutor da autofagia, na CLS de fungos filamentosos, especificamente na estirpe *Aspergillus nidulans* GFP'*Atg8*. Para tal, *A. nidulans* foi crescido em frascos e, através da medição do peso seco (DCW), foi possível gerar a curva de crescimento. Para a maioria dos microrganismos, a concentração de biomassa é proporcional ao número de células viáveis. No entanto, este não é o caso dos fungos filamentosos porque as suas paredes rígidas podem manter as células intactas algum tempo depois de mortas. Portanto, para testar o efeito da rapamicina na longevidade, temos que aplicar um método que directamente meça a actividade metabólica em alternativa aos comummente aplicados métodos de quantificação de biomassa, como a DCW e a turbidimetria. Um método baseado no sal de tetrazólio XTT foi utilizado para quantificar a actividade metabólica das culturas. Este sal é reduzido ao ser derivado corado formazan na presença de células metabolicamente activas. Isto permite media a actividade metabólica da cultura microbiana utilizando a espectrofotometria na gama 430-490nm. O efeito da rapamicina foi estudado medindo a densidade óptica (OD) da cultira a 490nm.

Como esperado, a adição de rapamicina mostrou reduzir a velocidade de crescimento e aumentou a vacuolização. Estudos no microscópio de fluorescência mostram que a autofagia é um processo dependente do ATP uma vez que as células da estirpe GFP'*Atg8* foram incapazes de transportar os autofagosomas para os vacúolos na ausência de uma fonte de carbono (i.e. glucose) a indução da autofagia (na presença de quantidades mínimas de glucose) mostrou aumentar a longevidade em 300%, uma vez que a actividade metabólica da cultura, permanece alta por um maior período de tempo, mesmo depois de a glucose acabar.
Palavras chave: Autofagia; Rapamicina; Via de sinalização da TOR; ensaio XTT

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Notation and Glossary

Greek letters

μ	Specific growth rate	h^{-1}

List of acronyms

An∆atg13	*Aspergillus nidulans atg13* deletion strain
*An*GFP-*atg8*	*Aspergillus nidulans* GFP-*atg8* strain
ATG	Autophagy related genes
CLS	Chronological life span
DCW	Dry Cell Weight
FKBP12	FK506 binding protein
FRB	FKBP12-rapamycin binding
GFP	Green fluorescent protein
MAG	Malt extract glucose
mTOR	Mammalian TOR
UU	Uracil and Uridine
OD	Optical density
P13K	Phosphoinositide 3-kinase
PAS	Phagophore assembly site
PBS	Phosphate buffer
PCD	Programmed Cell death
PE	Phosphatidylethanolamine
PIK	Phosphatidylinositol kinase
PMSF	Phenylmethanesulfonylfluoride
R+	Presence of rapamycin
R-	Absence of rapamycin
RLS	Replicative life span
SOP	Standard operating procedure
TCA	Tricalboxylic acid cycle
Tor	Target of rapamycin
TORC1	Tor complex 1
TORC2	Tor complex 2
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl(5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide

Introduction

1.1. Theme introduction and project presentation

Humans have long been interested in how and why we age. Therefore it is relevant to understand age-related pathology that limits human lifespan as well as health. Several model organisms are instrumental in understanding aging. Often, these organisms possess conserved pathways and factors that have physiological and pathological relevance in humans[1]. Noxious and stressful events which can initiate programmed cell death (PCD), contributing to ageing [2]. To combat aging, however, cells activate multiple protective pathways in response to stressful stimuli. One such pathway is macroautophagy (hereafter referred to as autophagy) which is responsible for recycling cytoplasm, macromolecules, and organelles. This process may prolong cellular survival particularly during starvation [3].

All organisms are occasionally subjected to severe changes in the natural environments. One of the biggest challenges that cells are confronted with is the restricted availability of nutrients [4]. As a built in mechanisms to face this struggle, nutrient limited cells activate specific autophagy proteins triggering a cascade of responses. These mechanisms shut off certain non-essential metabolic pathways and mobilize new energy sources to other essential processes, thus extending survival during starvation [4].

Studies suggest that induction of autophagy might be useful for the treatment of bacterial and viral infections, as well as cancer [2]. Experiments performed in cancer cells show that autophagy is required for the acquisition of the senescent phenotype. Senescence and programmed cell death constitute the major endogenous barriers against oncogenesis. Madeo et al. described that the onset of cancer can be enhanced by deletion of several autophagy relevant-genes [5]. Kaeberlein et al. describes that some genes that define aging in yeast sometimes are defined only for yeast. However, if a given gene functions to regulate longevity in a wide variety of organisms, such as yeast, worms and mice, there is a good chance this function will be conserved in humans[6]. It remains important to investigate

autophagy in yeast, but there is significant evidence that programmed cell death pathways (e.g. apoptosis, autophagy) of fungi maybe more analogous to those of higher eukaryotes [7]. However, much less is known about autophagy in filamentous fungi. Studying autophagy in filamentous fungi is also important because they have an immense impact on humanity, being both detrimental, causing several human infections and millions of dollars of crops loss annually, or, beneficial when used on industry to produce chemicals, food stuffs and pharmaceuticals. For this reason, an extension of chronological life span (CLS) in filamentous fungi may have an immense impact on industry.

1.2. Work contribution

Due to the prevalence of autophagy inducing conditions in the health and bioprocess fields, this research project has many potential impacts. Fungi are vastly important in the pharmaceutical and food industry, and manipulation of organism lifespan could lead to improved production. One study has already applied this concept by increasing production in CHO cells [8]. My study serves as a proof of concept that similar process control may be utilized in fungi.

Autophagy has an important role in diseases such as cancer and Alzheimer’s disease. Both of these illnesses could benefit from localized manipulation of cell lifespan (i.e. decreasing the lifespan of cancerous cells or increasing the lifespan of neuronal cells) Utilizing the ability to turn autophagy on or off could make it possible to quell symptoms or even reduce the incidence of such diseases.

1.3. Thesis Organization

This thesis is organized in five main sections: introduction, state of the art, technical description, results discussion and appendixes.

The first section, introduces the thesis theme and a succinct description of important concepts. A brief discussion relating the concepts underlying my project to my work proving evidence of autophagy in *Aspergillus nidulans* is also presented.

In the next section, state of the art, a relation between works performed about this theme is displayed and interrelated. XTT assays are also introduced, explaining the main features.

The next section, technical description, shows all the methods used in this work. It presents, in particular, how growth curves were plotted, as well as how XTT assays were performed. Also, the procedure used to study the impact of glucose on autophagy is described.

After the methodology is described, results obtained are shown and discussed. Main conclusions about the theme are also given, being continued on the following chapter, conclusions, making this the most important section of this paper.

Appendixes are provided to supplement the technical description section and aid in the overall understanding of this paper. Detailed procedures are presented in Appendix I. Appendix II consists of the procedure to make agar plates to inoculate *A. nidulans* for banking purposes. Appendix II consists of the solutions used for the experiments and their preparation. Appendix IV contains a small presentation that was used for introduce my subject to the research group.

State of the art

2.1. Autophagy

In 1963, Christian de Duve (who received the 1974 Nobel Prize in Physiology and Medicine) invented the word “autophagy” with the discovery of lysosomes in mammalian cells. Duve was the first to prove the involvement of lysosomes in cellular recycling[9]. However, it was in the last decade that the concept evolved, and research increased the area[9].

Autophagy is a catabolic process that plays an important role in cell growth, development, differentiation, and amino acid homeostasis. Under certain conditions it protects against cell death [3,10]. This phenomenon occurs in all organisms from yeast to human and involves self-eating of cytoplasm, macromolecules, and organelles by sequestration within double membraned vesicles called autophagosome.

Noda et al. demonstrated in 1998 that Tor negatively regulates the induction of autophagy [11]. Also, this author showed that Atg genes, which encodes proteins that are required for macroautophagy [12], are essential for autophagy induced by starvation[11].Remarkably autophagy in fungi reflects features of both yeast and mammals and is slightly more analogous to autophagy in higher eukaryotes than S. Cerevisiae is. This is exemplified by finding that filamentous fungi lack the S. cerevisiae clade/specific Atg31 protein but contain Atg101 which is absent in this clade[13]. Also fungi, as in higher eukaryote systems like humans, *Drosophila melanogaster,* and *Caenorhabditis elegans* have only one Tor gene, while S. *cerevisiae* contains two Tor paralogs [14].

Although autophagy is called type II programmed cell death, there is evidence that it may play both causative and preventive roles in cell death[3]. Autophagy is important to cellular function, protecting from pathogens and degrading long-lived proteins and organelles. Upon starvation conditions, its up-regulation provides the cell with needed energy

by recycling molecules such as amino acids, nucleotides, and free fatty acids. This way, the cell can maintain cellular nutrients and amino acids at levels essential for survival [3,13,15]. Autophagy also reduces the accumulation of potentially toxic aggregates, i.e. defective mitochondria that produce reactive oxygen species (ROS) [5]. Fabrizio et al. proposed that as in non-dividing cells, there is no mechanism beyond autophagy to remove damaged organelles, indicating that this process is a key element in longevity extension[16]. On the other hand, when there is an excessive degradation of mitochondria or other essential cellular components due to autophagy, or if autophagy is repressed with concurrent nutrient deprivation, cell death and apoptosis may occur [3,15].

Autophagy involves more than 30 autophagy-related (ATG) genes and can be divided into six steps: a) Induction, b) nucleation, c) cargo recognition, d) expansion and completion of the autophagosome, e) fusion with the lysosome vacuole and f) digestion and recycling of the cargo (Figure 1). The target of rapamycin (TOR) signaling pathway controls induction. The protein kinase Tor negatively regulates autophagy[13], and therefore inhibition of Tor kinase activity (during nutrient starvation or exposure to rapamycin) leads to the activation of the autophagy pathway[3]. Other proteins involved in the induction process include Atg1, Atg13 and Atg17[13], which must form a complex known as “Atg1 complex” in order to induce autophagy.

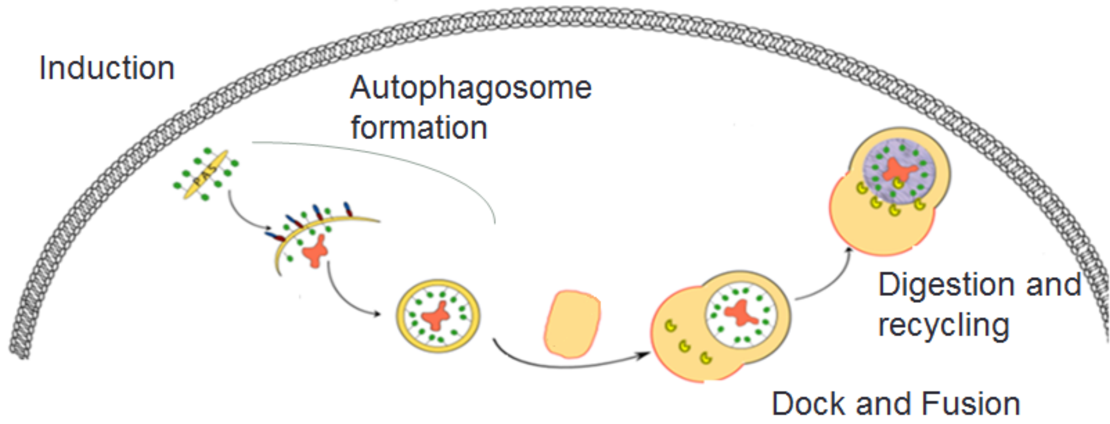

Figure 1: Schematic illustration of the autophagy process. Adapted from [13].

The second step of autophagy, nucleation, remains the least understood step [13]. The Pre-Autophagosomal Structure (PAS) is responsible for the formation of vesicles. Among the main proteins involved in this step are Atg6, Atg14, a functional complex of class III phosphatidylinositol 3 kinase (Vps34), and Vps 15.

There is no consensual information about the third step of autophagic process, i.e., about the mechanism that carries out the selection of the macromolecular complexes and

organelles to be degraded[13]. Although there is a set of proteins that are thought to be involved in this step, there is no evidence of a general mechanism that will fit all species [13]. The process of expansion and completion of the autophagosome is mediated by two main sets of conjugation complexes: The Atg12 system and the *Atg8* lipidation system. The Atg12 system consists of a complex of Atg12p, Atg5p and Atg16p. The *Atg8* lipidation system modifies the lipid phosphatidylethanolamine (PE) and consists of a complex of *Atg8*, Atg3 and Atg7. This last system is especially important because of its association with completed autophagosomes, therefore serving, as an important marker to monitor autophagy (e.g. GFP-*atg8* tagging for fluorescent microscopy studies)[13]. Pollack et al. showed that deletion of the *atg8* gene inhibits autophagy and affects growth and differentiation[3]. After completion of the autophagosome, it fuses with vacuolar membrane. The last step consists of digestion and recycling of cargo. It is characterized by vacuolar hydrolases attacking the double-membraned autophagic body and its contents [13].

2.2. Rapamycin: An Autophagy Inducer

Rapamycin is one of the best characterized pharmacological autophagy inducers, and several studies have shown that it prolongs life span in a wide variety of organisms[5]. Autophagy is required for extension of yeast chronological longevity by rapamycin. This is attributable in part to the role of autophagy in amino acid homeostasis [10]. Correspondingly, Madeo et al. described that in yeast, rapamycin only extends lifespan under conditions in which autophagy can be induced (i.e. with all autophagy genes present) [5].

Different studies show that rapamycin extends CLS in yeast. Noda et all, showed in 1998 that when rapamycin is added to cells growing in YPD, the autophagic bodies accumulate in vacuoles, showing that autophagy happens in presence of rapamycin[11]. Alvers et al. proved the same fact in 2009 showing that without rapamycin, autophagy occurred on day 1 and during days 2-5 the level of macroautophagy was very low[10]. In the presence of 10nM of rapamycin, this author describes that there is a prolongation of macroautophagy through day 12 of the CLS experiment[10]. Noda et al. also proved that disruption of *FKB1,* the gene for FKBP, result in failure of rapamycin to induce autophagy. Thus, he proved that somehow, the effect of rapamycin is mediated by FKBP [11]. Later, Power et al showed that in yeast, at concentrations of rapamycin sufficient to slow cells division, the integral of life span curve increase to 4.23 at 300 pg/mL and to 4.25 at 1ng/mL[17]. This same author also described that this antibiotic only extends life span when administrated before cells enter in stationary phase[17].

There are some other compounds that, like rapamycin, extend life span in a wide variety of organisms. Among these, resveratrol and spermidine are the best characterized. Resveratrol acts replicative life span by modulating a number of longevity-related processes (such as transcription of genes involved in key longevity pathways, stimulating p53 deacetylation, and increasing insulin sensitivity and mitochondrial number, among others)[18]. Spermidine extends chronological life span in yeast and replicative life span in nematodes, fruit flies and human PBMC[2]. It acts by inhibiting histone acetyltransferases and promoting H3 deacetylations, which activate transcription of numerous autophagy related genes [2]. Although less studied than these two compounds, there is evidence that Caffeine and Methionine sulfoximine (MSX) also extend chronological life span in yeast by reducing Tor activity[19].

2.3. TOR pathway

Chemically, autophagy can be induced with rapamycin (section 2.2), an antibiotic that down-regulates Tor activity in a wide variety of eukaryotes[10]. Tor is a conserved Ser/Thr kinase [20]. There are two functionally distinct TOR complexes in yeast, TORC1 (Tor complex 1), which is rapamycin sensitive and regulates cell growth, translation initiation, and responses to nutrients, and TORC2 (Tor complex 2), which is rapamycin insensitive and regulates cytoskeleton remodeling [21]. Fungi only possess the TORC1, known as TorA in filamentous fungi. Although the mechanism of this pathway remains unclear [22], it is known that alteration of Tor signaling affects transcription, translation, and protein degradation [3,15]. Tor also controls growth by positively regulating ribosome biogenesis and cytoplasmic translation and by negatively regulating stress response genes, autophagy, and utilization of alternate carbon and nitrogen sources [1,23]. The loss of TOR function in yeast cells results in an early inhibition of translation initiation. As a consequence of this translation defect, TOR-inhibited cells arrest in the G_1 phase of the cell cycle [24]. In filamentous fungi deletion of *torA* is lethal. In *Fusarium fujikuroi*, inhibition of *torA* by rapamycin not only affected autophagy and development, but also the expression of secondary metabolite genes [13].

TORC1 acts by sensing the nutrient availability in the media. When nutrient supplies are sufficient, Tor hyperphosphorylates Atg13 at multiple Ser residues [13], reducing its affinity for Atg1 and thereby turning off autophagy. During starvation, Tor is inactive and Atg13 becomes dephosphorylated. This increases its affinity for Atg1, allowing for the formation of the Atg1 complex and initiation of autophagy [25].

All TOR proteins have a conserved C-terminal region that has strong homology to the catalytic domains of phosphatidylinositol 3-kinase (P13K) and phosphatidylinositol 4-

kinase[26]. This feature defines the TOR-related family of kinases, termed the PIK-related kinases. Various experiments revealed that a Ser residue (Ser[1972] in TOR1 and Ser[1975] in TOR2) is important for the interaction of TOR with the FKBP12-rapamycin complex. In the N-terminal to the kinase domain, the TORs contain a region of approximately 100 amino acids, the FKBP12-rapamycin binding (FRB) domain [26], where rapamycin binds with high affinity (Figure 2).

Figure 2: Chemical structure of rapamycin. Regions where TOR and FKBP interacts with rapamycin[24].

Rapamycin acts by forming a ternary complex with Tor and the highly conserved immunophilin (family of proteins that bind immunosuppressants) FKBP12 [24,27], which results in up-regulation of autophagy related genes [3]. The crystal structure of FKBP12-rapamycin bound to the FRB domain reveals that FKBP12 and the FRB domain of mammalian TOR (mTOR) interact primarily via rapamycin[27]. Rapamycin occupies a hydrophobic binding compartment in FKBP12 and a hydrophobic compartment in the FRB domain, thus placing together FKBP12 and mTOR (Figure 3).The protein-protein contacts between FKBP12-rapamycin and mTOR may explain why rapamycin by alone cannot interact with TOR. The mechanism by which FKBP12-rapamycin inhibits TOR function is unknown. It may inhibit TOR kinase activity directly or, may alternatively block access to substrates or partner proteins[28].

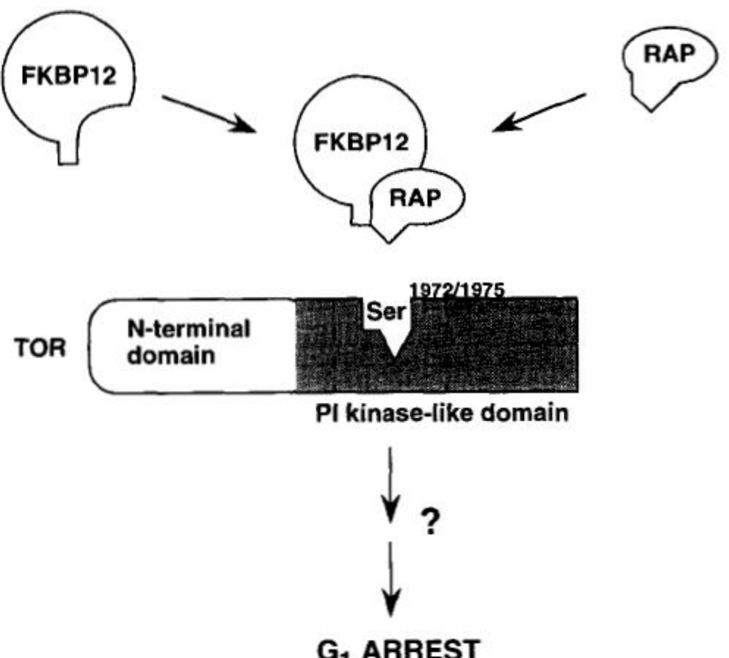

Figure 3: Model for interaction between rapamycin and FKBP12 involving Ser^{1972}[26]

Blagosklonny et al. described that in yeast, inhibition of TORC1 signaling extends both replicative and chronological life span (Figure 4) [23]. Replicative life span (RLS) is defined as the number of daughter cells that a mother cell can originate before senescence [29]. Chronological life span (CLS), on the other hand, describes the length of time that a nondividing yeast cell remains viable during stationary phase[29]. This way, both RLS and CLS model cellular aging. The aging of mitotically active cells in multicellular organisms can be studied by analyzing RLS, whereas CLS may assesses the aging of postmitotic (non-dividing) cells [2,16,29]. Filamentous fungi studies use CLS model due to the fact that they grow with hyphae, thus it is difficult to separate parent and daughter cells without causing damage.

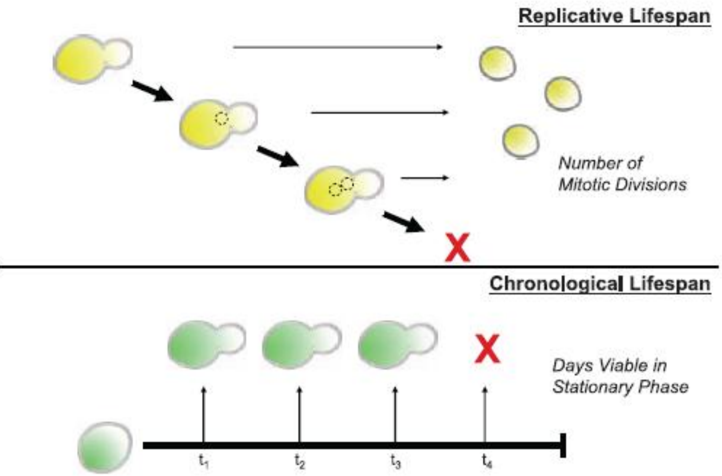

Figure 4: Schematic illustration of replicative and chronological life span models [6]

These two models for aging in yeast allow for comparisons between the aging process of both proliferating and nonproliferating cells in a single-celled organism [6].

2.4. Evidence of autophagy in *Aspergillus nidulans*

In order to determine if autophagy occurs in the *A. nidulans* GFP-*atg8* strain in the presence of rapamycin, members of Marten's lab performed growth experiments in different conditions (Figure **5**).

Atg8 is an important component of the autophagosome membrane. It can be found in the membrane of PAS (Pre autophagosomal structure), in autophagosomes, and in autophagic bodies. When tagged with the green fluorescent protein (GFP), *Atg8* plays an important role as a marker to monitor autophagy. Typically when autophagic bodies are delivered into the vacuoles, they are rapidly degraded. To delay this degradation, PMSF (phenylmethanesulfonyl fluoride) is added to the media. This causes GFP-atg8 to accumulate in vacuoles and enables the visualization and monitoring of autophagy [25]. Figure 5 shows that when A. nidulans GFP-atg8 strains were grown in complex media and subsequently transferred to a complex media with PMSF, no vacuoles or vacuolar content was observed. Conversely, when grown in media lacking a nitrogen source or in the presence of rapamycin, a greater level of vacuolation and GFP-*atg8* fluorescence in the vacuolar content was found [25]. This indicates that autophagy occurs in *A. nidulans* in a starvation environment or in the presence of rapamycin.

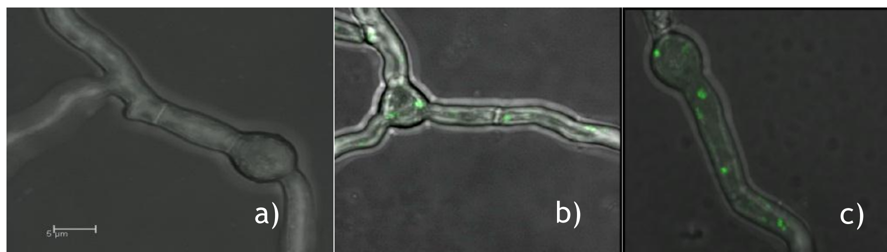

Figure 5: Analysis of autophagic bodies localization in the vacuoles of Aspergillus *nidulans* GFP-*atg8* strains under different autophagy induction conditions. A) *Aspergillus nidulans* GFP-*atg8* was transferred from complex media to complex media with PMSF; b) *Aspergillus nidulans* GFP-*atg8* was grown under nitrogen starvation and c) *Aspergillus nidulans* GFP-*atg8* was grown in the presence of rapamycin. Images were kindly provided by Usha Sripathineni [25]

2.5. The XTT salt

Assessment of viability (through metabolic activity) in filamentous fungi can be difficult as the biomass concentration is not proportional to the number of viable cells (as in yeast or bacteria). To test the effect of rapamycin on lifespan, it must be employed an assay that directly measures metabolic activity.

Tetrazolium salts are heterocyclic organic compounds that substitute the final acceptor in the biological redox process [30]. The tetrazolium salt XTT is extracellularly reduced by mitochondrial dehydrogenases to its colorimetric formazan derivate in the presence of metabolic active cells [14,31]. NADH transports electrons across the plasma membrane, reducing an electron coupling agent (for instance menadione).

$$\text{menadione} + NADH + H^+ \rightleftharpoons \text{menadiol} + NAD^+$$

Figure 6: Chemical reaction of reduction of menadione to menadiol in presence of NADH [32]

Menadione subsequently reduces XTT to its colorimetric formazan derivate (Figure 7).

XTT

Figure 7: Reduction of XTT to formazan in the presence of metabolic active cells (adapted from [33]).

Thus, the formation of XTT-formazan is in proportion to both the quantity and activity of cells, and therefore, metabolic activity of a microbial culture can be quantified using visible spectophotometry in the 430-490nm range [14]. Sensitivity of this type of assay can vary depending on the menadione concentration [31].Quantifying metabolic activity using XTT offers several advantages over other methods. Some of these advantages include a relatively rapid measurement time, the colorimetric nature of its formazan derivate, and an aqueous

solubility[14]. Also, this is an inexpensive method that can be performed in most laboratories[31].

This method also display several disadvantages, including difficulty of inter-strain comparisons, potential for nonlinearity of XTT signal, and a potentially disproportional relationship between XTT concentration and colorimetric signal. Additionally, an XTT assay is only sensitive to the TCA cycle, neglecting other forms of metabolism, such as anabolism[14]. Despite these limitations, members of Marten's lab demonstrate the XTT assay's ability to quantify viability and metabolic activity of *A. nidulans* in all phases of batch culture growth while accounting for any decrease in activity or viability caused by processing [14].

Antachopoulos et al. studied the effect of three antibiotics (amphotericin B, posaconazole and voriconazole) in 14 clinical isolates of Zygomycetes using the XTT assay. He demonstrated a significant metabolic activity before visual or spectrophotometric detection of fungal growth at the 6- and 8-h time points with OD_{450} in the range of 0.17 to 1.06, for cells treated with 200, 100 or 50 µg/mL and 25 µM menadione [31]. Moss et al. utilized the fungus *A. nidulans* to quantify metabolic activity and viability in suspension cultures, by testing the correlation between XTT-formazan production and growth performance. To do that, cells were exposed to damaging conditions to correlate extent of damage to XTT-formazan production. According to this author, XTT is a method to rapidly quantify metabolic activity and viability of *A. nidulans* in suspension culture[14].

Antachopoulos also described that for *Aspergillus* species, MICs (minimum inhibitory concentration) obtained by the XTT assay at 24 and 48h were equivalent to those obtained by the National Committee for Clinical Laboratory Standards, therefore proving the efficiency and accuracy of such method[31].

Meletadis et al. evaluated some features of the XTT assay. According to this author, XTT is not metabolized for *Aspergillus* species until an electron couple agent is added (usually, menadione or PMS). Also, conversion of XTT depends on its concentration, cultures exposure time and menadione concentrations[30].

Technical Description

3.1. Experiments

3.1.1. Preparing the growth curve

To construct a growth curve of the *Aspergillus nidulans* GFP-*atg8* strain, the dry cell weight (DCW) method was used. A 250mL baffled flask was inoculated with $1x10^6$ spores containing autoclaved MAG+UU growth medium (see Appendix III) (final volume of the culture of 50mL). The pH was adjusted to 3.3 to prevent spore aggregation. Once sterilized, 2.5mL of 20% glucose, 50µL of Hutner's solution, 50µL of vitamin solution and 250µL of $MgSO_4$ were added. The flask was placed in the shaker (250rpm and 30ºC) for 12 hours and then transferred to a 2.8L flask containing MAG+UU (final volume of culture of 1.2L). To this flask, 30 mL of 20% glucose, 1.2mL of Hutner's solution, 1.2mL of vitamin solution and 6mL of $MgSO_4$ were added. The large flask was placed in the shaker. Every four hours, for a period of at least 24 hours, 15 mL of broth was removed and filtered using vacuum filtration. The pre-weighed filter papers (glass fiber filter, diameter: 4.25 cm, 0.2 μm pore size, Fisherbrand) were placed in the oven at 90ºC for at least 24 hours. Once dried, filter papers were weighed. The amount of cells in the media was plotted as a function of time (Figure 10). For more detailed procedures, see Appendix I.

Figure 8: Illustration of filters papers at: a) 0h after inoculation; b) 8h after inoculation; c) 24h after inoculation;

Equation 1 describes how Dry Cell Weigh was calculated:

$$DCW\left(\frac{g_{cell}}{kg_{broth}}\right) = \frac{mass_{cell\,paste}\,(g_{cell})}{m_{broth}\,(kg_{broth})} \qquad 1$$

Where DCW is the Dry Cell weight $\left(\frac{g_{cell}}{kg_{broth}}\right)$, $mass_{cell\,paste}$ is the difference between the mass of the filter paper with cells (after dried) and the mass of the filter paper (g_{cell}), and m_{broth} is the mass of the broth that was used in the filtration (kg_{broth}).

For a cultures growing exponentially, the biomass concentration is described by Equation 2.

$$X = X_0\exp(\mu t) \qquad 2$$

Where X is the final biomass concentration in ($\frac{g_{biomass}}{L}$), X_0 is the initial biomass concentration ($\frac{g_{biomass}}{L}$), μ is the specific growth rate (h^{-1}) and t is the time (h).

In order to know the Biomass yield, the equation 3 was utilized:

$$Y = \frac{g\ biomass\ produced}{g\ substrate\ utiliized} \qquad 3$$

3.1.2. XTT Assays

To perform the XTT assay, cells were grown as in DCW experiment (see section 3.1.1). When cells reached the mid-exponential phase (12 hours of growth), the broth was filtered under sterile conditions, and the cake was washed with phosphate buffer (PBS) in order to remove all soluble residues. A 0.2g sample of cells (weighed in aseptic conditions) from the filter paper were added to each two baffled flasks containing 50mL of minimal media (See appendix III) and 50µL of vitamin solution, 50µL of Hutner's solution as well as 250µL of $MgSO_4$. A minimal amount of glucose was then added (7.5 µM), to create a nutrient starvation environment. Rapamycin (Sigma) dissolved in ethanol and stored at -4°C was added to one of the flasks yielding a final concentration of 2mg/L in the media.

Every four hours, a 1 mL aliquot from each flask was placed in two sterile Eppendorf tubes. As control, 1 mL of sample was filtered (0.2µm pore size Fisherbrand) using a sterile syringe and placed in another Eppendorf tube. Duplicates were performed for each 250mL flask. XTT solution was prepared to a concentration of 5.75 mg/ml in sterile PBS. Menadione was prepared to a final concentration of 0.52 mg/ml in acetone. Both of these solutions were stored at 4ºC for no longer than 10 days. 40 µL of XTT and 10 µL of menadione were added to all of the Eppendorfs (final concentration of 0.23 mg/mL of XTT and 5.2 µg/mL of

menadione). After vortexing, the tubes were placed in the incubator for 20min at 34ºC. Following the incubation, samples were centrifuged for 10 min at 4 x g in a refrigerated room. Finally, supernatants were pipetted into cuvettes and absorbance at 490 nm was measured using filtered media to blank the spec. Each experiment took at least 5 days of work.

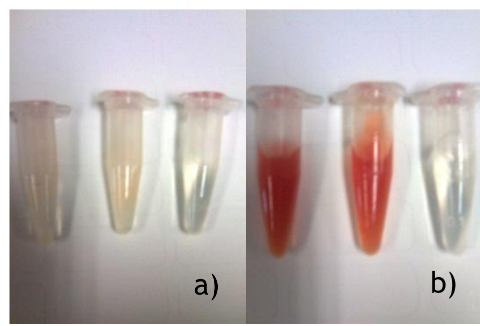

Figure 9: a) Illustration of Two Eppendorf tubes with 1mL of sample. The third Eppendorf contains the filtered media (blank). b) Illustration of Two Eppendorf tubes with 1mL of sample, XTT and menadione. The third Eppendorf contains the filtered media (blank).

3.1.3. Evidence of impact of glucose on autophagy

In order to determine the impact of glucose on autophagy, the *A. nidulans* GFP-*atg8* strain was grown in MAG+UU medium for 12 hours. The whole broth was filtered as in the XTT assays. Cells were equally separated into two 250 mL blaffled flasks containing minimal media and rapamycin. A minimal amount of 20% glucose (final concentration of 0.03g/L) was added to one of the flasks. A sample was removed from both flasks and it was analyzed in confocal microscope (Leica TCS 4D with a 63x magnification). Pictures were taken and analyzed (Figure 20 and Figure 21).

Results Discussion

4.1. Growing *A. nidulans* in shake flask with complex media

This research project is designed to study the effect of rapamycin (an autophagy inductor) on the chronological life span of the *Aspergillus nidulans* GFP-*atg8* strain of filamentous fungi. Specifically, it evaluates the growth differences of two strains, an autophagy capable GFP-*atg8* strain, and an autophagy deficient ∆*atg13* strain. To do so, the strains were grown in baffled shake flasks with MAG+UU growth media. By measuring the dry cell weight (DCW), a growth curve can be generated by plotting the concentration of cells as a function of time. Lifespan can be evaluated by measuring the cell's metabolic activity in the stationary phase. We hypothesize that cells grown in the presence of rapamycin (i.e. when autophagy is induced) will remain metabolically active longer during starvation, than cells grown in control media. For most microorganisms the biomass concentration is proportional to the number of viable cells. However, this is not the case in filamentous fungi because their rigid cell walls can keep cells intact long after death. As a result, commonly employed biomass quantification methods, such as DCW and turbidity, can provide erroneous information about culture activity and viability [14]. Therefore to test the effect of rapamycin on lifespan we must employ an assay that directly measures metabolic activity.

Metabolic activity was quantified with a method based on the tetrazolium salt, 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl) 5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), which has been shown previously to relate fungal activity to viability [14]. This salt is reduced to its colorimetric formazan derivate in the presence of metabolically active cells. This enables us to measure the metabolic activity of a microbial culture using visible spectophotometry in the 430-490 nm range. By adding small amounts of rapamycin in the media, and measuring the optical density (OD) of the grown culture at 490 nm, it was possible to draw conclusions about the effect of rapamycin on the CLS of *A. nidulans*.

Figure 10 represents the growth curve for *A. nidulans* GFP-*atg8* strain, defined from now on for DCW experiments as the control experiment.

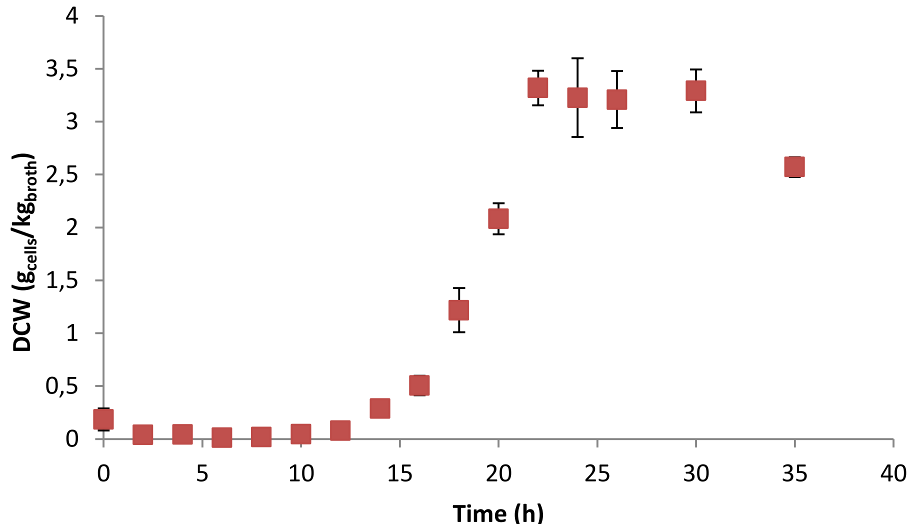

Figure 10: DCW curve generated for *An*GFP-*atg8* strain.

Thus, plotting the exponential phase of the growth curve in a logarithmic scale, as function of time (Figure 12), makes it possible to infer the specific growth rate of the strain.

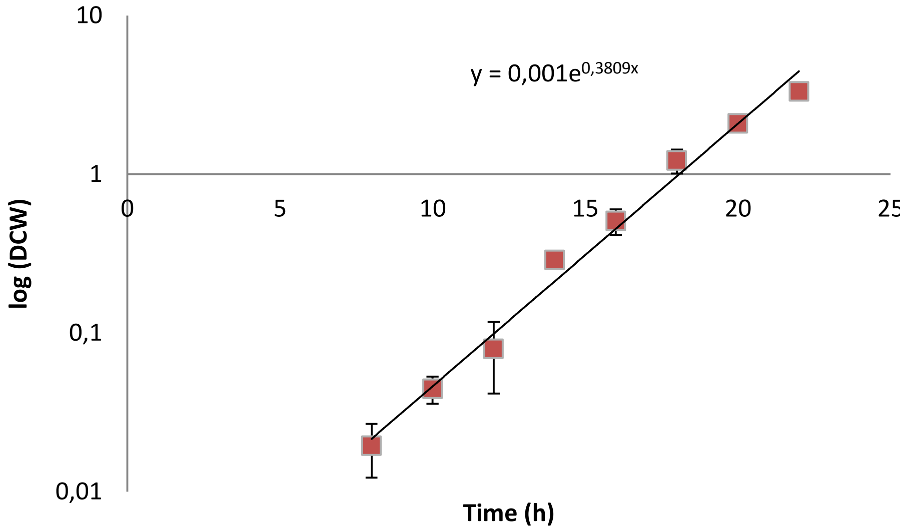

Figure 11: Dry cell weight as a function of culture time for A. nidulans in shake flask culture

The specific growth rate of the AnGFP-*atg8* strain is around 0.38 h^{-1}, and the initial DCW is around 0.01 g_{cells}/kg_{broth} as expected [14,34]. The initial time point was measured when the broth was changed from the 250ml flask to the 2.8L flask one.

The Atg13 deletion renders the cells an incapable of autophagy. This happens because the Atg1 complex that leads to autophagy cannot be formed. In order to evaluate the importance of autophagy on growth, *An*Δ*atg13* strain was grown in shake flaks with the same environmental conditions than the GFP-*atg8* strain. Figure 12 compares the growth curve for both strains of *A. nidulans,* showing that both curves are very similar, although the strain Δ*atg13* begins exponential phase earlier.

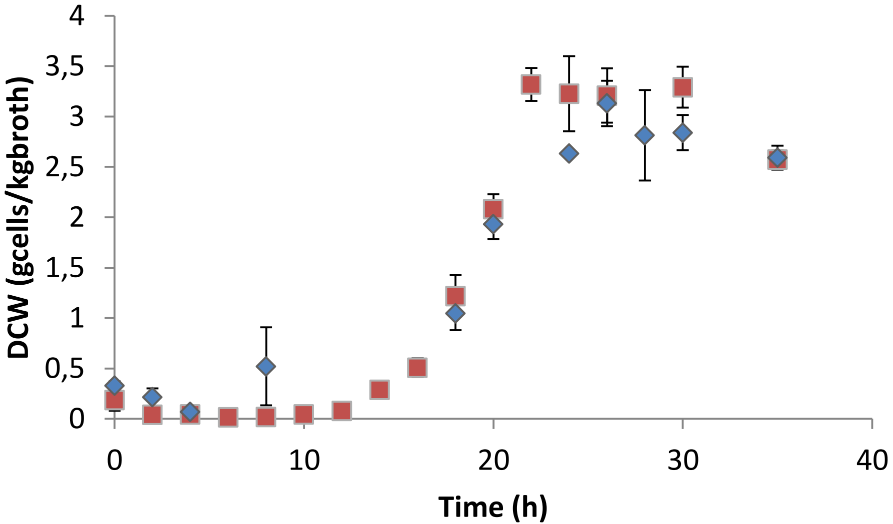

Figure 12: Dry cell weight as a function of culture time for GFP-*atg8* (■) and Δ*atg13* (◆) *A. nidulans* strain in shake flask culture.

A look in deeper to the exponential phase of both strains (Figure 13) let us know that *An*Δ*atg13 grows* slower (μ=0.1735 h^{-1}) than the strain *An*GFP-*atg8* (μ=0.3809 h^{-1}).

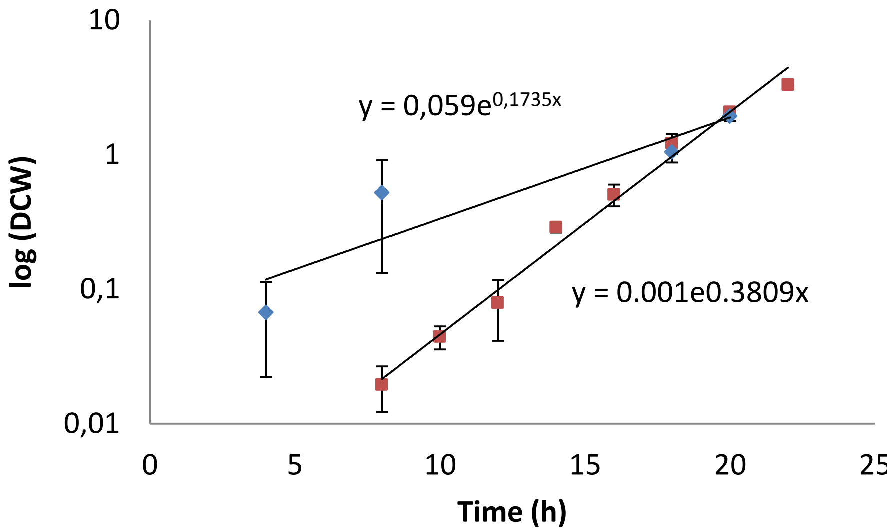

Figure 13: DCW as a function of time for the exponential phase of GFP-*atg8* ( ) and Δ*atg13* ( ).*nidulans* strain.

Based on these results, it appears that the autophagy pathway affects *A. nidulans* growth even under nutrient rich conditions. The length of the GFP-*atg8* strain's lag phase is over double that of the *An*Δ*atg13* strain. The GFP-*atg8* strain has a growth rate of 0.38 h^{-1}, while the autophagy deficient *An*Δ*atg13* strain grows much slower at 0.17 h^{-1}. Although the *An*Δ*atg13* strain grows slowly, the final DCW of both strains is approximately 3g/l. Therefore both strains have similar values of biomass yield on glucose (0.43 $g_{cells}/g_{substrate}$) (see equation 3 in section 3.1.1).

4.2. Impact of rapamycin on CLS of *A. nidulans*

The impact of rapamycin on CLS of the filamentous fungi *A. nidulans* was evaluated through measuring the metabolic activity using the XTT assay. It was previously demonstrated that metabolic activity is a strong indicator on culture viability and overall fitness[14]. Other studies [17] show that small amounts of rapamycin extend life span in yeast and other organisms. Thus, a small amount of rapamycin (2mg/L) was added to the fungal cultures. Figure 14 presents the normalized results (Metabolic activity/Metabolic activity at time 0) of the impact of rapamycin (and therefore induced autophagy) on metabolic activity of the GFP-*atg8* strain.

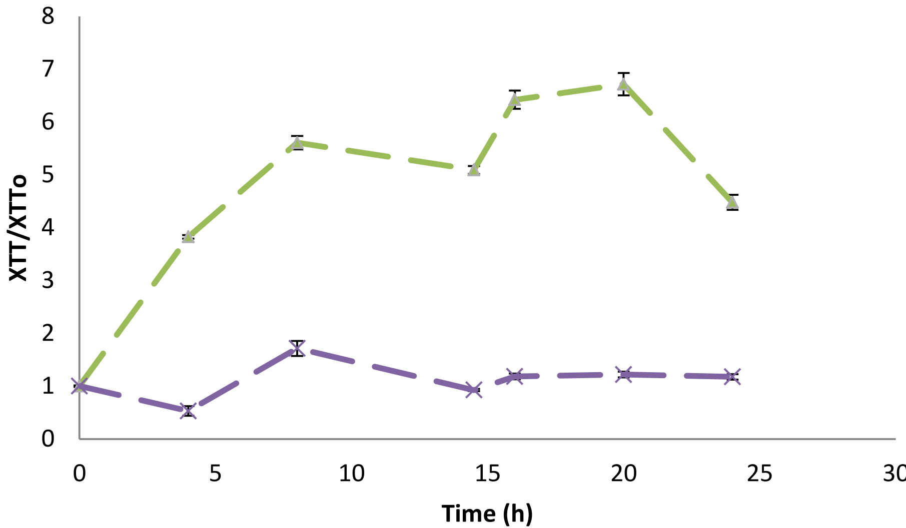

Figure 14: Normalized XTT as a function of time for *A. nidulans* GFP-*atg8* strain grown in shake flasks in the presence ( ) and in the absence ( ) of rapamycin. The error bars represent the standard deviation for 3 replicates (3 different assays, with 2 duplicates each one).

The presence of rapamycin extended the metabolic activity of the fungus. While trace amounts of glucose is present in the media, the activity increases. In order to know when the amount of glucose in the media finishes, samples were removed and analyzed. The conclusion is that glucose finishes after 8h of growth.

Comparing to the control culture, there is a higher increase of metabolic activity in the presence of rapamycin. This is fact is difficult to explain. Further studies have to be performed in order to figure out an explanation. After 8h of growth, cells experience complete starvation, metabolic activity remains high in culture with rapamycin. The presence of the rapamycin in the media extended the metabolic activity around 60% In order to evaluate whether the increase in activity is dependent on autophagic proteins (i.e. the Tor/Atg1 signaling cascade), or is the result of some other effect of rapamycin, the same experiment was done with *An*Δ*atg13* (Figure 15):

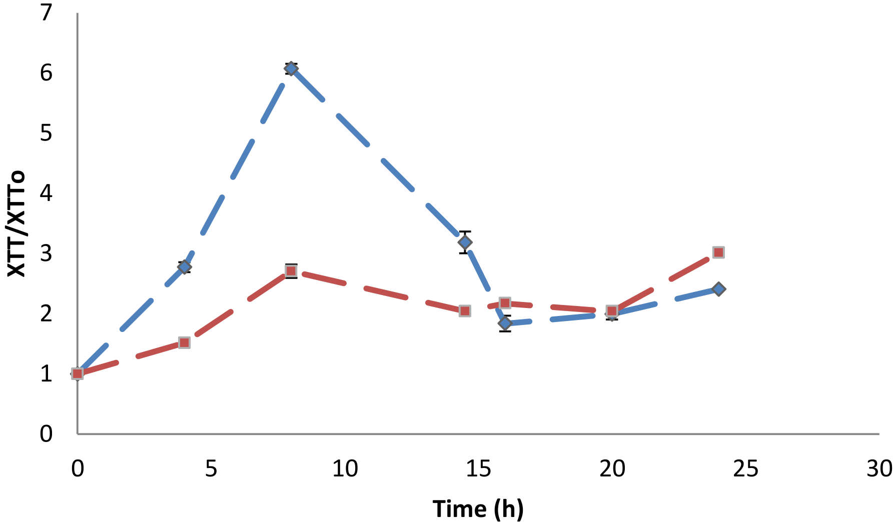

***Figure 15**: Normalized XTT as a function of time for* An⊿atg13 *strain in shake flasks with (◆) and without (■) rapamycin.* The error bars represent the standard deviation for 3 replicates (3 different assays, with 2 duplicates each one).

As in the GFP-*atg8* strain, the addition of rapamycin causes increased metabolic activity, and overall cell fitness. However, the advantage of rapamycin is soon lost when autophagy is not functional (I.e. Atg13 mutant) and the metabolic activity quickly decreases until it is nearly the level of the control (in which autophagy is not induced). This indicates that rapamycin needs autophagy to keep cells metabolically actives Figure 16 compares both strains, in both autophagy inducing, and control media.

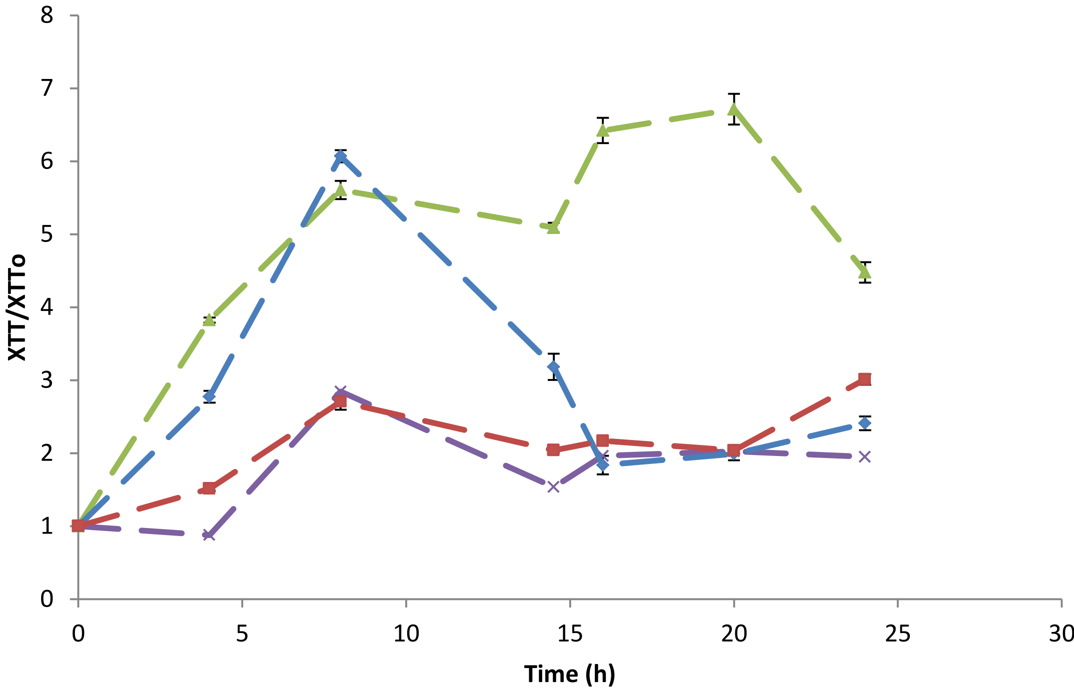

Figure 16: Normalized XTT as a function of time for *An*GFP-*atg8* and *An⊿atg13* strains grown in shake flasks in the presence and absence of rapamycin. The error bars represent the standard deviation for 3 replicates (3 different assays, with 2 duplicates each one). is for *An*GFP-*atg8* with rapamycin and is for *An*GFP-*atg8* without rapamycin. is for *An⊿atg13* with rapamycin and without rapamycin. ANOVA analysis shows that there is no significant difference between the 3 curves after 16, see Appendix IV.

For both strains there is greater metabolic activity in the presence of rapamycin while glucose remains in the media (until 8h). This occurs for both for autophagy capable GFP-*atg8* and autophagy null *An∆atg13* strain, in the presence and in the absence of rapamycin, indicating that this increase is not dependent on autophagy. This can be explained by the fact that the Tor kinase does not solely acts as an autophagy inhibitor. In addition to autophagy the downstream processes of Tor includes, growth control, translational regulation, ribosome biogenesis, transcription, and amino acids biosynthesis. In order to quantify this increase, the slope of each individual curve is plotted (Figure 18):

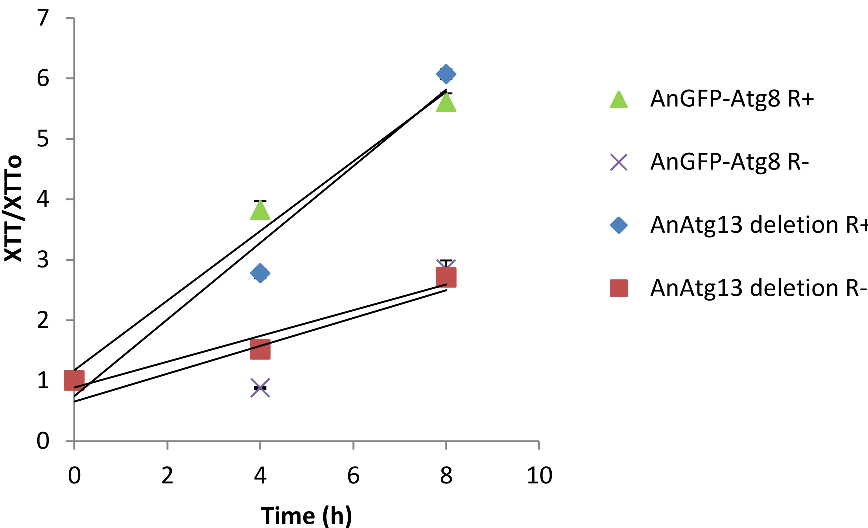

***Figure 17**: Slopes of the curves of the parent strain and the mutant in the presence and in the absence of rapamycin.* The error bars represent the standard deviation for 3 replicates (3 different assays, with 2 duplicates each one).

Table 1 allow to compare the slopes of all the four curves:

Table 1: Slopes of growth of the different strains until 8h.

Rapamycin	Strain	Slope
Present	GFP-*atg8*	0.5758
	Δ*Atg13*	0.6337
Absent	GFP-*atg8*	0.2133
	Δ*Atg13*	0.2306

The slopes of the curves are very similar, indicating that the increases in activity is autophagy dependent, rather than strain dependent.

After glucose in the media is exhausted, the strain *An*Δ*atg13* shows a rapid decrease in metabolic activity. This is indicative of an autophagy dependent (i.e. *atg13* dependent) improvement of lifespan with rapamycin. Figure 18 allows us to compare the differences between the parent strain and the mutant (AnGFP-*atg8* and AnΔ*atg13*) with rapamycin, the controls (AnGFP-*atg8* and *An*Δ*atg13* without rapamycin).

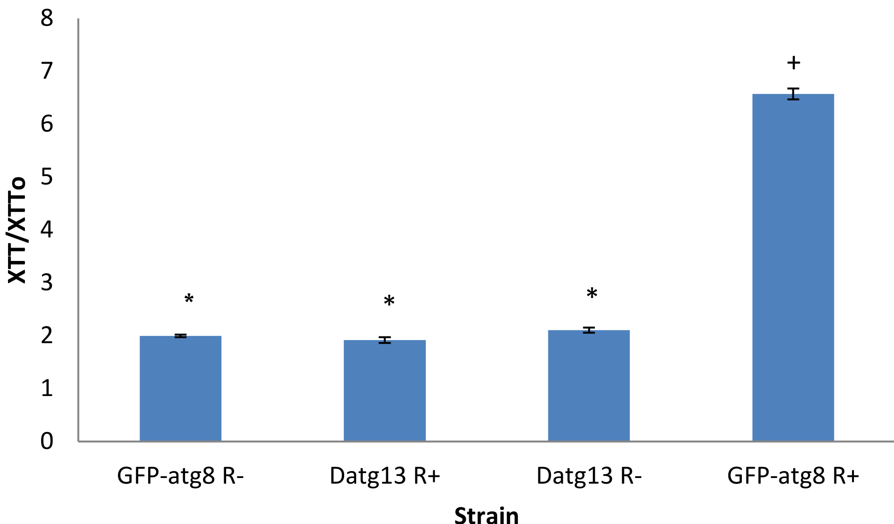

Figure 18: Relation between the metabolic activity of the cultures of *An∆atg13* and AnGFP-*atg8* for the average of 16h and 20h of grown in shake flasks, in the presence and absence of rapamycin (* are experiments not significantly different, + is an experiment significantly different from the other ones). The error bars represent the standard deviation for 3 replicates (3 different assays, with 2 duplicates each one).

The metabolic activity of AnGFP-*atg8*, grown in autophagy inducing conditions, is considerable higher than all other cultures at the same time. Figure 19 consists of the metabolic activity of both strains normalized by the highest metabolic activity (8h of growth):

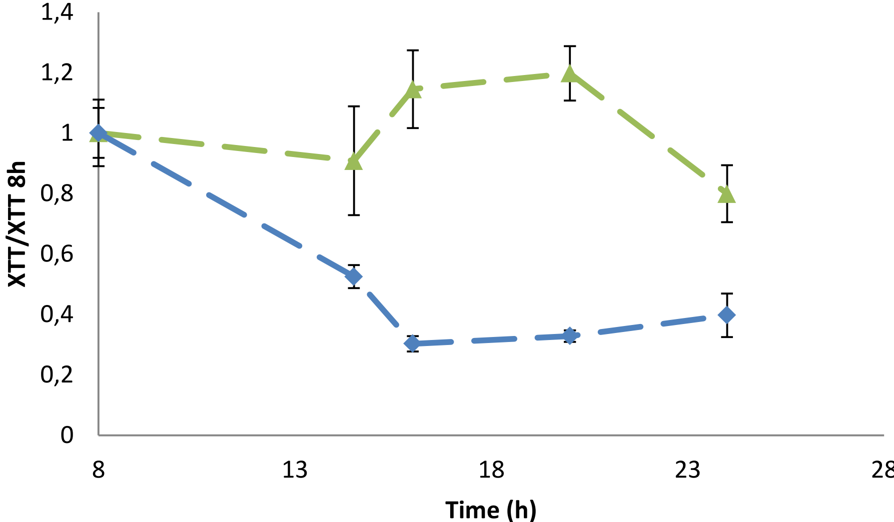

Figure 19: Normalized XTT by the highest metabolic activity for *An*GFP-*atg8* ( ) and *An∆atg13* ( ) in the presence of rapamycin after 8h of growth. The error bars represent the standard deviation for 3 replicates (3 different assays, with 2 duplicates each one).

The *An∆atg13* strain cannot undergo autophagy and after 8h of growth, the *An∆atg13* cells begin to die. It is important to observe that after 24h the decline in cellular activity is

30% for the GFP-*atg8* strain and twice (60%) for the Δ*atg13* strain. This indicates that an intact autophagy pathway is necessary for CLS extension in *A. nidulans,* and the extension of life is not due to one of Tor's other effectors.

4.3. Evidence of impact of glucose on autophagy

It is known that caloric restriction extends lifespan in filamentous fungi, as it inhibits the Tor kinase activity. To test the effect of glucose on autophagy in filamentous fungi, the GFP-*atg8 A. nidulans* strain was grown supplemented with rapamycin in either minimal or glucose free media. In the presence of a minimal amount of glucose, punctuate spots of GFP-*atg8* are indicative of autophagosomal localization to vacuoles. In absence of glucose the GFP is diffuse, indicating a defect in autophagy. This implies that autophagy is at least in part an ATP dependent process.

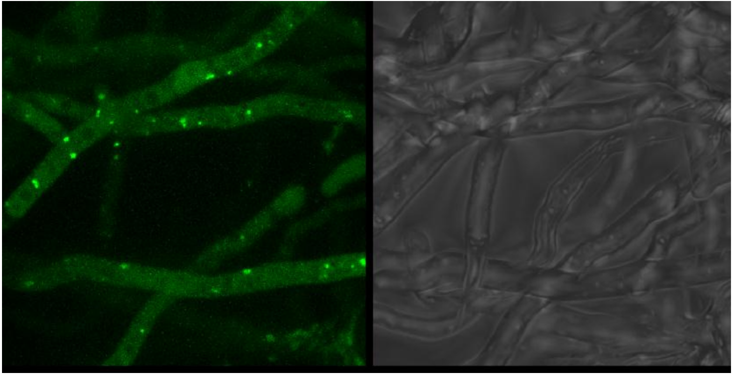

Figure 20: Visualization of the effect of glucose in the media. In the presence of glucose, it is possible to observe autophagic bodies in vacuoles. This image was taked with a confocal microscope (Leica TCS 4D with a 63x magnification).

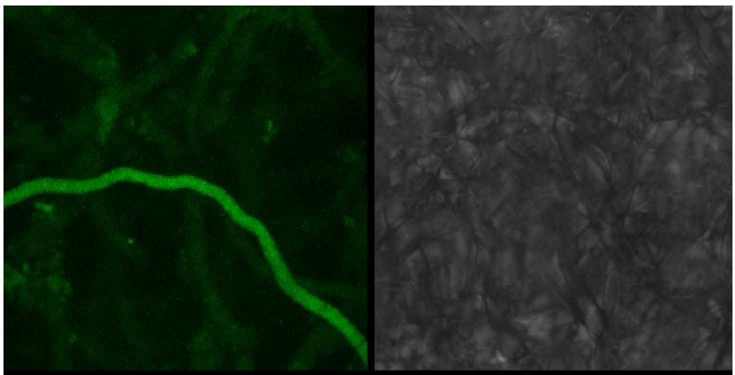

Figure 21: Visualization of the effect of the absence of glucose in the media. Here, it is not possible to observe autophagic bodies. GFP is spread throughout the hyphae and there is no

evidence of autophagy. This image was taken with a confocal microscope (Leica TCS 4D with a 63x magnification).

Conclusions

Here we show that a small amount of rapamycin (2mg/L) extends life span of the filamentous fungi *Aspergillus nidulans* by approximately 300%. This value is obtained by comparing the strain in the presence and in the absence of rapamycin. Fluorescent microscopy studies show that autophagy is an ATP dependent process as GFP-*atg8* cells were unable to transport autophagosomes to vacuoles in the absence of a carbon source (i.e. glucose).

In order to study the effect of autophagy on rapamycin a strain of *A. nidulans* without *Atg13* (and thus without autophagy) was grown and tested in the same conditions as an autophagy capable GFP-*atg8* strain. Both strains increase in metabolic activity in the presence of glucose. However, when glucose is exhausted the *Atg13* dies faster than the GFP-*atg8* strain, until the activity is close to cells grown without rapamycin. This behavior demonstrates that autophagy is necessary for increasing CLS with rapamycin. Also, the metabolic activity of the cultures that have rapamycin is higher that of those that has not. This shows that rapamycin not only extends life span, but also increases the overall metabolic activity of the strains, albeit in a non autophagy (i.e. *Atg13*) dependent manner.

Evaluation of work done

6.1. Objectives achieved

This research project had three main goal:

- Evaluate the impact of autophagy on CLS of the fungi *Atg8 A. nidulans:* This goal was completely achieved. By adding 2 microgram/liter of rapamycin to a test culture and growing simultaneously one culture without the antibiotic. it was possible to measure how rapamycin influences metabolic activity and CLS of the filamentous fungi.
- Study the optimal amount of rapamycin that extend life span: this goal was not achieved. It must be used as future work, once it is important to figure out what quantity of rapamycin best extends CLS.
- Evaluate if autophagy is needed for rapamycin effect: This goal was reached. A strain without the gene *Atg13* was used to make comparasions with the GFP-*atg8 A. nidulans* strain. The conclusion is that the effect of rapamycin does depends on autophagy.

6.2. Extra work done

Besides the work performed on impact of rapamycin on CLS, I did some agar plates, and grow *A. nidulans* GFP-*atg8* on them. To do this, a procedure in Appendix II was followed.

Also, it was asked to me to begin to write a review article named *"CLS in fungi"*, with Cheyenne Falat, an UMBC undergraduate student that recently joined Marten's lab group. I'll continue working on it after finishing this project.

6.3. Limitations and future work

The major limitation of this work is the sensitivity of the XTT assay. XTT is a pretty sensitive method that depends on the number of cells that are collected in the sample. It also depends on quantity of menadione (that defines the sensitivity level of the assay) that is added to each sample. 30mM of menadione are added to each sample, which is a really small amount, so the slightest inaccuracy in measurement leads to a large difference in XTT signal.

There was not enough time to achieve all the objectives. So, in the future, there are some aspects that should be tested. It would be interesting to test the use of MSX and caffeine to see the effect on autophagy and CLS. Also it would be prudent to try different concentrations of rapamycin, to figure out which more extend life span.

6.4. Final assessment

My personal feeling about this research project is good. I learned many new things and also, I could share some of my knowledge. I think that if I had more time, I would get more consistent results, and contribute more broadly for the theme. UMBC had all the conditions I needed to do my job, and the lab where I worked was good enough. The subject was also pretty interesting.

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Appendixes

Appendix I – Detailed Procedures

The procedure described here is for inoculating 1x106 spores (in one 2 mL vial) into 50 mL of medium supplemented with 0.5% (w/v) malt extract (in one 250 mL baffled shake flask). The 50 mL culture will then inoculate into 1.2 L of minimal media (ion one 2.8 L baffled shake flask).

1. Solution preparations

1. Prepare a small beaker (to contain 50 mL solution) and a magnetic stirrer.
2. Pipette 2.5 mL of salt mix into the beaker (use pipette gun).
3. Add 30 mL of d.i. water (from white tap).
4. Add:
    a. 250 mg of malt extract.
    b. 100 mg of Peptone.
    c. 56 mg of Uracil.
    d. 60 mg of Uradine.
5. Using HCl of H_2SO_4, adjust pH to 3.2-3.4.
6. Add d.i. water to a final volume of 50 mL.
7. Transfer entire content into a 250-mL baffled shake flask.
8. Close the flasks using pink foam caps. Cover with aluminum foil.

Note: This small flask will be later used to inoculate the large flask. If inoculating more than one large flask, multiply the amount of solutions described here appropriately.

9. Prepare a 1L beaker and a large magnetic stirrer.
10. Pippete 60mL of salt mix into a beaker.
11. Add 1110 mL of d.i. water.
12. No need to adjust pH (should be around ≈6.6)

13. Transfer the entire content into a 2.8 L baffled shake flask.
14. Add:
    a. Malt extract 2.4 g
    b. Peptone 2.4 g
    c. Uracil 1.34 g
    d. Uradine 1.44 g
15. Cover the mouth of the flask with 2 sheets of milk filter, tied with string, and cover with aluminum foil.

Note: If inoculating more than one large plask, multiply the amount of solutions described here appropriately.

16. Place both flasks (250-mL and 2.8 L flasks) in an autoclave tray. Make sure all flasks/bottles are labeled. Also stick an autoclave tape on them to indicate sterilization.
17. Set autoclave at P4 (liquid setting). Press start. The run will take about 1 hour.

2. Shake flask inoculation

1. Turn on the floor shaker. Make sure that temperature is set at 32ºC, and speed 260 rpm.
2. Take out two inculum vials from -80ºC freezer. Thaw the vials at room temperature by immersing in water, or by rubbing them with hands.
3. Spray the laminar hood with 70% ethanol solution.
4. Place in the laminar hood: the two small shake flasks, glucose stock bottle, Hutner's solution, vitamin solution and inoculums vials. Also, each of 1 mL pipette and 10 mL pipette.

Note: Everything entering into the laminar hood must be sprayed with ethanol, including gloves.

5. Add 2.5 mL of 20% glucose solution into the small shake flask using 10 mL pipette.
6. Add 50 μL of Hutner's solution into the small shake flask using 1 mL pipette.
7. Add 50 μL of Vitamin solution into the small shake flask using 1 mL pipette.
8. Add 250 μL of MgSO4 stock solution into the small shake flask using 1 mL pipette.
9. Mix the flasks by gently shaking.
10. Add one inoculum vial (≈ 1.5 mL) into the small shake flask by dumping the contents and swirling.

Note: The volume of inoculums can be adjusted so that about 1x10^6 spores are inoculated.

11. Close the cap to the flask.
12. Take out the flasks and bottles from laminar hood.
13. Place the shake flasks into the shaker. Let culture grow for 12 hours.

Note: If pellets are observed, then small flask growth will need to be repeated. Pellets imply that there is oxygen mass transfer limitation, which could occur if starting inoculums spore concentration was too high; try adding less inoculums volume.

14. When the growth in the small flask is sufficient, it is ready to be used to inoculate the large flask.
15. Take the large flask, glucose stock, Hutner's solution, Vitamin solution, 50 mL pipette, and 10 mL pipette into the laminar hood after spaying with ethanol.
16. Add 30 mL 20% glucose solution into the 2.8 L flask.
17. Add 1.2 mL Hutner's solution into the 2.8 L flask.
18. Add 1.2 mL Vitamin solution into the 2.8 L flask.
19. Add 6 mL $MgSO_4$ solution into the 2.8 L flask.
20. Mix well by gently swirling.
21. Pour the content of the small flask into the large flask (final volume: 1L). To get all of the mycelia, you can pipette some of the media from the large flask and wash the walls of the small flask, and pour into the large flask.
22. Take out the flasks and bottles from the laminar hood.
23. Place the shake flask into the shaker.

3. Dry Cell Weigh Procedure

1. Bring in two pre-weighed 15-mL tubes into the laminar hood. Clearly mark/label each tube.
2. At time t=0 (immediately after large flask inoculation), pipette 5mL broth into each tube.
3. Weigh the tubes and record the measurement.
4. Measure pH and record.
5. Take pre-dried filter papers (glass fiber), weigh and record each paper, put on aluminum dish.
6. Set up the filtration unit. Assemble the flask, base, filter paper, and glass cup. Connect unit to a vacuum pump.

7. Pour the content of one tube into the filtration unit. Wash the tube with d.i. water. Make sure that all the cells are on the filter paper (none sticking on wall).
8. Wait a few seconds with the pump on.
9. Place the filter paper back on the aluminum dish. Place in 100ºC oven overnight (at least 24h).
10. Repeat for the content of the other tube.
11. Repeat sampling process every 4-6 hours.

4. XTT assay

1. Grow cells according to regular SOP (Atg 8 in 1.2 L of MAG+UU for 12 hours).
2. Autoclave two 250 mL baffled flasks with 50 mL of minimal media, 6 Eppendorf tubes, a funnel, spatula/ scoopula, d.i. water, and one milk filter.
3. Prepare stock solutions of XTT (5.75 mg/nL in sterile PBS) and menadione (0.52 mg/mL in acetone).
4. Add sterile elements to both flasks and place autoclaved material in the hood with a bottle for filtered media:
    - 50 μL of Vitamin Solution.
    - 50 μL of Hutner's solution.
    - 250 μL of MgSO4 stock solution.
    - 4 μL of 20% glucose solution.
5. Filter the cells and wash them with d.i. water, measure out the same wet weight of cells pre-weighed BD falcon tubes and place in both 250 mL flasks.
6. Add rapamycin (5mg/L) to one of the flasks.
7. Place a 1 mL sample from each flask in a sterile Eppendorf tube every 24 hours (Take a 1mL sterile filtered sample for zeroing as well) and add 40 μL of XTT solution and 10 μL of menadione solution.
8. Vortex, then place in incubator for 20 minutes. Vortex again and place in cold room centrifuge at 130 rpm for 10 minutes.
9. Pipette supernatant from the Eppendorf tubes into cuvettes (dilute if necessary). Set absorbance to 460 nm. Use filtered broth to zero, then measure samples.

Appendix II – Detailed Procedure for produce *A. nidulans* spores on an agar plate

1. Procedures

This section contains the following subsections:

1.1. Making the agar plates (1L)

1.2. Inoculating the plates with spores

1.3. Checking plate growth

1.4. Harvesting spores from plate

2. Making the agar plates (1 L)

Prepare a 1L capped bottle and a large magnetic stirrer. Alternatively, if using less than 1L medium, can use an Erlenmeyer flask with sponge cap.

Add ingredients according to section 2.3.

Add 900 ml of deionized water (from white tap). Mark the water level and pipette about 200 ml out of the bottle.

Add malt extract and peptone.

Add Uracil and Uridine. May not fully dissolve, but will after autoclaving.

Add 15 g Agar.

Make up the volume upto the 900 ml mark with water.

No need to adjust pH (should be around ~6.4-6.6).

Immediately autoclave the solution at 121°C for 20 min (P4 mode, 1.25 hour).

Take a package of sterile (unopened) petri dishes (12 cm in diameter). Spray the bag with 70% ethanol before putting it into the laminar hood.

Immediately after autoclaving, spray and put the capped bottle into the hood. The solution should remain hot (above 55 °C) so that it does not solidify.

Add the following solutions according to Section 2.3: Hutners, Glucose, vitamin. Swirl gently or put on a stir plate to mix (20 s). Minimize air bubble.

Pour about 30 ml hot agar solution into each dish (fills half the height of the dish) for ~ 30 dishes. *Warning: Bottle is hot! Use multiple paper towels to hold.*

Close the dishes; let them sit 2 hours in the laminar hood to solidify the solution.

Note: After the agar has solidified, continue procedure 3.2. If not needed immediately, take the plates out, seal with parafilm, label, and store in cold room. If there is too much condensation in agar plates, store in incubator (30°C) for a day or so.

Inoculating the plate with spores

a. From conidial plug (master): Conidial plug is a piece of agar containing culture with spores, in an Eppendorf microfuge tube. Add 1mL phosphate buffer (2.5.1) into the tube. Vortex on high for 1 min to release the spores into the liquid phase. Use a sterile inoculum loop to gently scrape off the surface of the agar. Vortex again.

From Frozen Vial: From -80 °C freezer, take one inoculum vial (spore suspension), thaw at room temperate for **1 hour**, and vortex for 20 seconds.

In the laminar hood, aspirate the spore suspension with 1 ml pipette. Add 2 drops onto each agar plate. Will get 10-12 plates with each vial.

Using a cell scraper, gently disperse the spore suspension evenly over the agar surface for all the plates.

Close and invert the plates such that the agar layer is on top (spores facing downward into air space).

Place the plates in the incubator at **30°C** for **5 days**. This is enough time for sporulation (“conidiation”) to occur.

Note: It is important to invert the plates to prevent contamination. If there is too much liquid on the agar, wait one day before inverting.

Checking plate growth

Day 2 – There should be light white growth on agar surface, possibly some hint of color

Day 4 and 5 – colony should have green color, except for rac1 has bright yellow.

Harvesting spores from plate

Note: Wearing a mask is required for steps 2-5.

Move the plates into the laminar hood.

Add 4-5 ml of sterile 0.2 M phosphate buffer into each plate.

Using a cell scraper, gently rub over the colonies to harvest the spores. A change in color on the agar plate is observable. Spore suspension will be dark green or bright yellow (only for rac1). *Note:* the colony initially repels liquid; push liquid on colony with cell scraper (while gently scraping surface) until liquid can stay on colony surface. The darker the spore suspension, the more spores it will contain.

Using a 25 or 50 ml pipette, aspirate as much of the spore suspension as possible (~2-3 ml). Tilting the plate so that the liquid falls to a particular end is helpful. Make sure that when aspirating, that the liquid bubbles does not reach the top of the pipette.

Dispense all the spore suspension into a sterile 50-ml centrifuge tube. From 10-12 plates, will get ~30 mL total volume. Vortex 60 s to break spore chains (spores that adhere to each other).

Take a 60-ml syringe. Using sterile forceps, insert some sterile glass wool (4in. long, autoclaved) into the syringe (about the length of the syringe), and push with plunger so the glass wool sits on the inner half of the syringe.

Filter the spore suspension through the glass wool. Collect the filtered spore suspension (light yellow or light green color) into a new 50-ml centrifuge tube.

Measure the spore concentration with Coulter Counter (see SOP\20 Basic Equipments) or with **hemacytometer and microscope**. The benefit of the latter is that you get to check on the quality of the spores (smooth round=healthy, wrinkled=not so healthy).

Calculate volume of spore solution needed to have a final concentration of 4.0E6 spores/mL for working bank (or 1.0E7 for master bank) in 50 mL total solution. Then make sure that the final concentration you get is the desired final concentration. This can be done by following step 8 and then accordingly adjust the concentration to the desired final concentration. See Section 8 for calculation example.

Take a new sterile 50 mL centrifuge tube. Add the previously calculated volume of spore solution; add 12.5 mL glycerol solution (2.5.2). Add phosphate buffer (2.5.1) up to 50 mL. Mix well by inverting tube 10 times.

This is important because proper mixing of the glycerol ensures the viability of the spores when banked.

Take a pack of 50 2ml cryogenic vials. Aliquot 1 mL into each vial.

Note that we are mixing glycerol solution, phosphate buffer and spore solution together and then aliquoting 1 mL of this mix to each 2 mL vial.

Label ALL the vials with name of banker, date, strain name, and concentration. Use pre-printed labels (better than writing on each vial!).

Store in cryogenic box, put in labeled storage box in –80°C freezer, ENG 307.

Agar plates containing cultures can be stored in 4°C for ~2 months. Wrap with parafilm.

Appendix III - Solutions

MAG+UU Growth medium

Table 3.1: *Salt mix, 20x Stock, lacking* $MgSO_4$

Component	Amount per liter of distilled water
$NaNO_3$	120.0 g
KCl	10.4 g
KH_2PO_4	16.3 g
K_2HPO_4	20.9 g
H_2O (d.i.)	Up to 1L

Table 3.1: $MgSO_4$ *solution 0,4M (200x) stock*

Component	Amount per 100 mL of distilled water
$MgSO_4$. $7H_2O$	10.4 g
H_2O (d.i.)	To 100 mL

Table 3.2: *Glucose stock solution (50%)*

Component	Amount per liter of distilled water
Glucose (dextrose)	500 g
H_2O (d.i.)	To 1 L

Table 3.3: *Hutner's Solution trace elements (Short: Hutners)*

Component	Amount per liter of distilled water
$ZnSO_4$ $7H_2O$	2.2 g
H_3BO_3	1.1 g
$MnCl_2$ $4H_2O$	0.5 g
$FeSO_4$ $7H_2O$	0.5 g
$CoCl_2$ $6H_2O$	0.16 g
$(NH_4)6MO_7O_{24}$ $4H_2O$	0.11 g
EDTA	5.0 g
H_2O (d.i.)	Up to 100 mL

Table 3.4: *Vitamin Solution, 100 mL Stock solution (FGSC-Methods)*

Component	Final concentration in 1L	Amount to add
H_2O (d.i.)		Up to 100 mL
Biotin		10 mg
Pyridoxin HCl		10 mg
Thiamine HCl		10 mg
Riboflavin	2.5 μg/mL	10 mg
Para-aminobenzoic acid (PABA)	1.0 μg/mL	10 mg
Nicotinic acid (or niacin)	0.5 μg/mL	10 mg

Table 3.5: *MAG+UU*

Component	Amount per liter of distilled waster
Salt mix lacking MgSO4 stock	60 mL
H_2O (d.i.)	1110 mL
Malt extract (0,2 %)	2.4 g
Peptone	2.4 g
Uracil	1.344 g
Uradine	1.44 g

Table 3.6: *Materials added in laminar hood*

Component	Amount per liter of distilled waster
MgSO4 solution stock	6 mL
Hutner's solution	1.2 mL
Vitamin solution	1.2 mL
50 % glucose solution	6 mL

Table 3.7: *Minimal medium (MM) Solution*

Component	Amount per liter of distilled waster
Salt Mix	50 mL
H_2O (d.i.)	895 mL

Table 3.8: *Phosphate Buffer Solution*

Component	Amount per liter of distilled waster
NaH_2PO_4	2.4 g
H_2O (d.i.)	100 mL

Table 3.9: *XTT solution*

Component	Amount per liter of distilled waster
XTT	17.25 mg
PBS 0.2M	3 mL

Table 3.10: *Menadione solution*

Component	Amount per liter of distilled waster
Menadione	2.6 mg
Acetone	5 mL

Table 3.11: *Rapamycin solution*

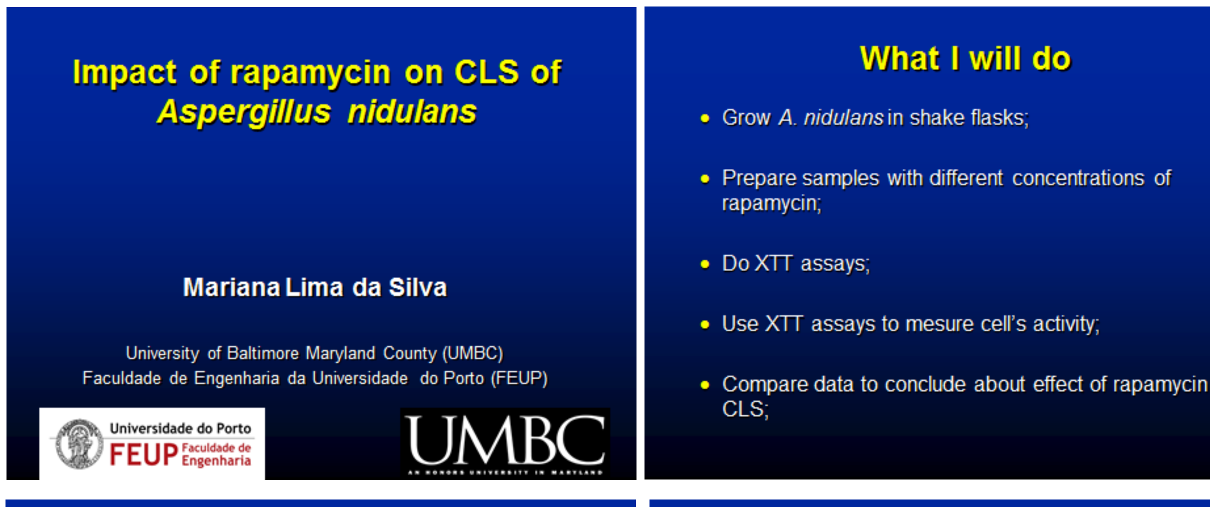
Component	Amount per liter of distilled waster
Rapamycin	10 mg
Ethanol (70%)	1 mL

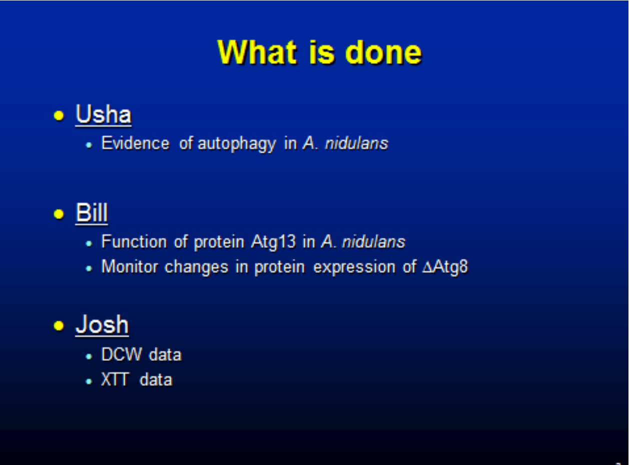
Appendix IV – ANOVA analysis

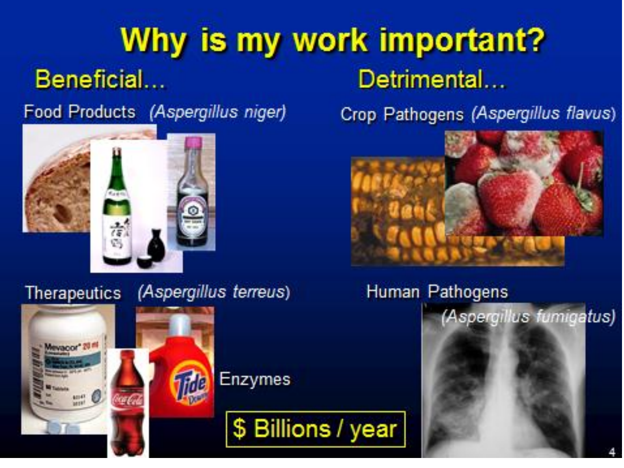
Table 4.1: *Different p-values for the curves of An*GFP-*atg8 without rapamycin and An∆atg13 with and without rapamycin, between 16h and 24h*

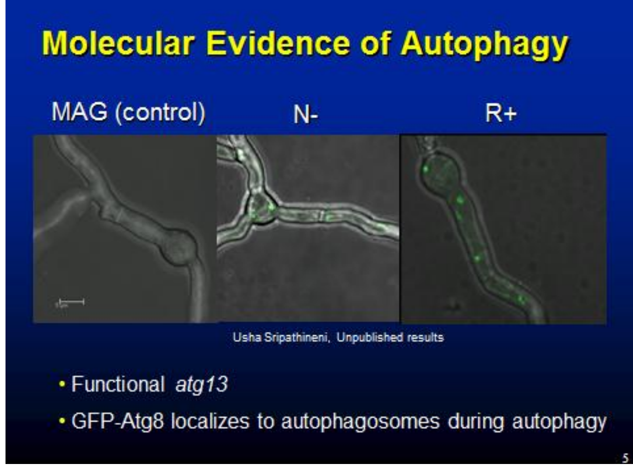
Comparison		p-value
AnGFP-*atg8* R-	*An∆atg13* R+	0.845
AnGFP-*atg8* R-	*An∆atg13* R-	0.467
An∆atg13 R+	*An∆atg13* R-	0.796

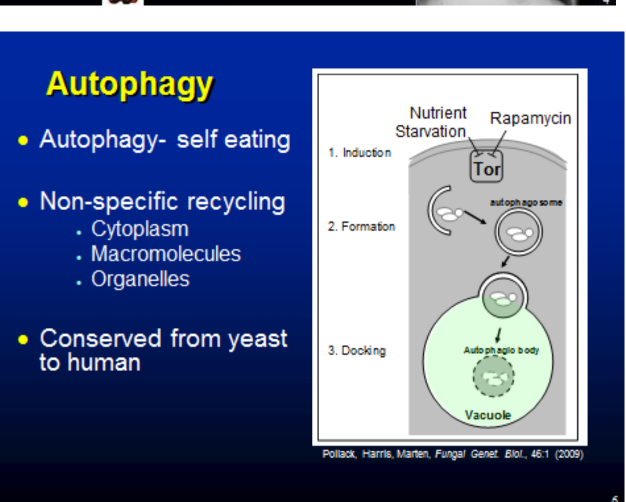
Appendix V - Presentation for introduce my project to the group

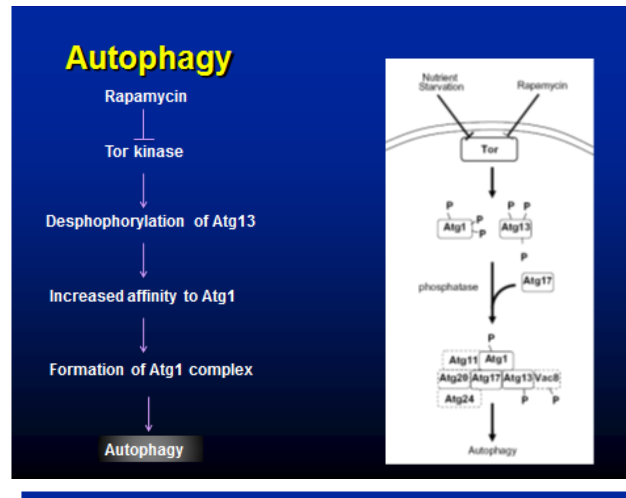


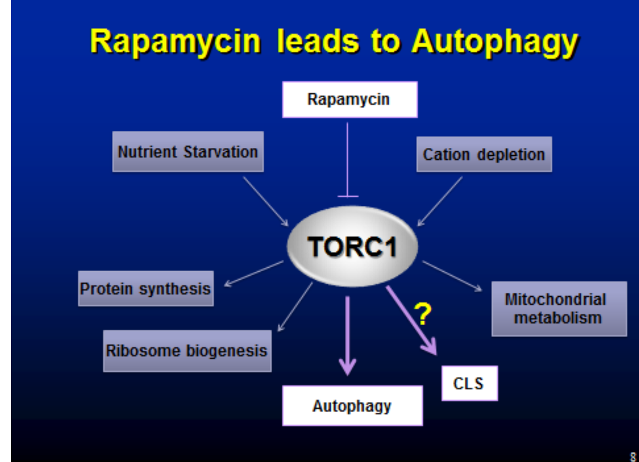


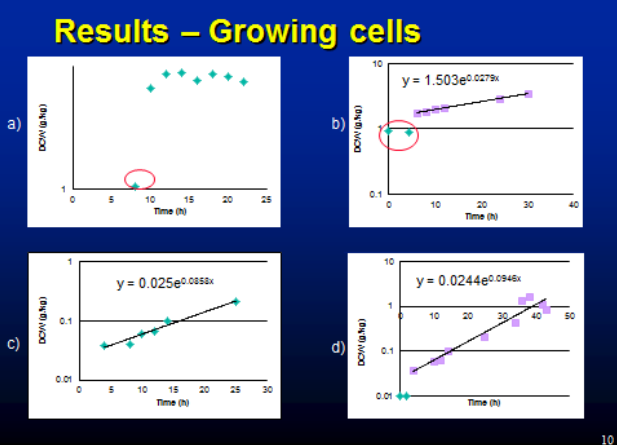












Dry Cell Weights

Starter Flask

12 hr in MAG UU

2.8 L

Shake Flask

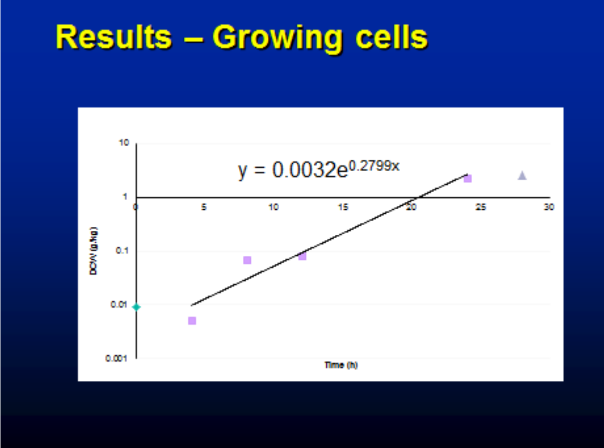
Every 4hr

15 ml Test Tube

Filter Broth

Filter Paper

0h 8h 24h



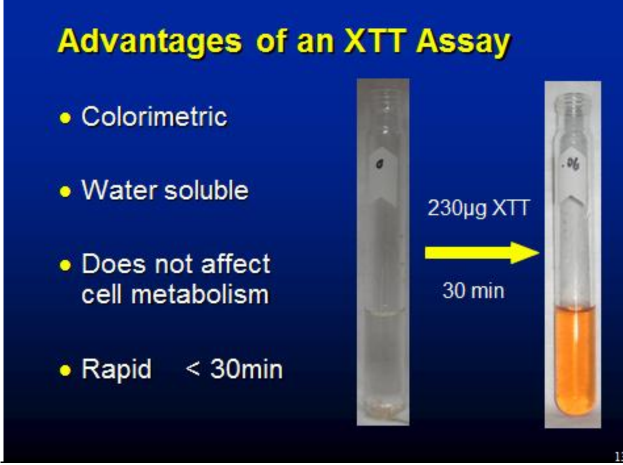
XTT

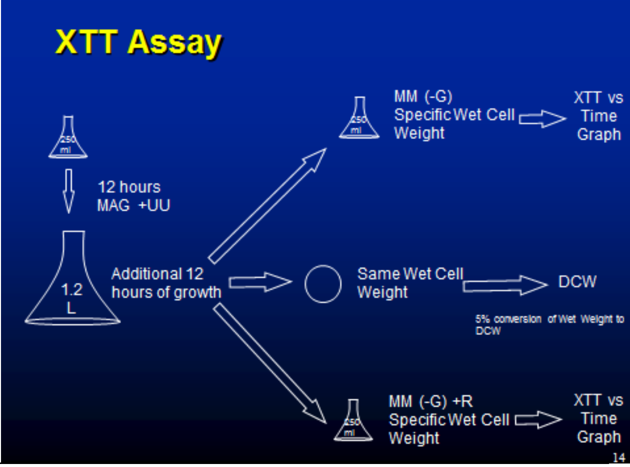
- 2,3-bis(2-methoxy-4-nitro-5-sulfophenly)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide
- XTT reduced when TCA cycle is active

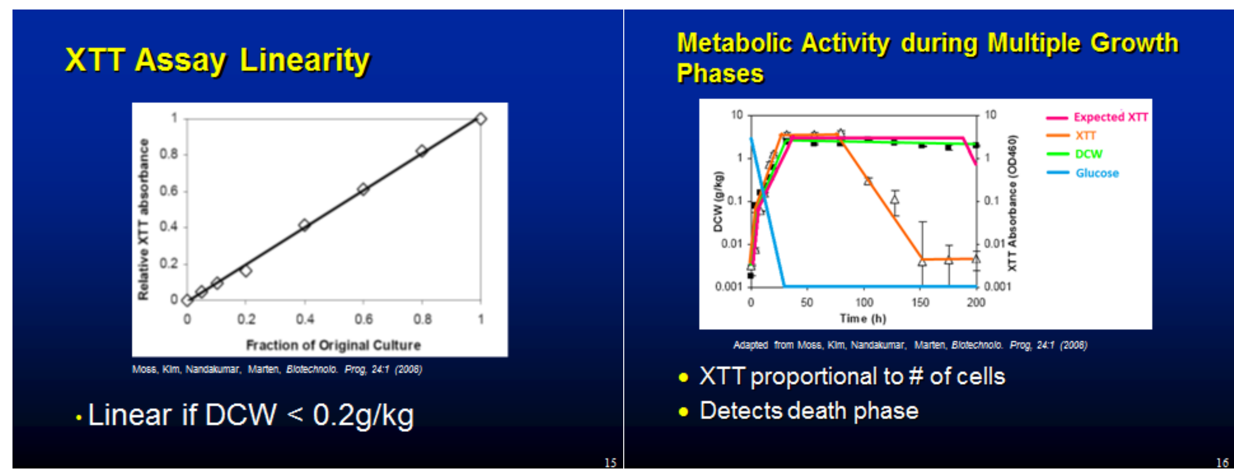
XTT

XTT-formazan

Scudiero, Dominic A., Cancer Research 48, 4827-4833,1988






XTT Assay Linearity
Relative XTT absorbance
Fraction of Original Culture
Moss, Kim, Nandakumar, Marten, Biotechnolo. Prog, 24:1 (2008)
• Linear if DCW < 0.2g/kg
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Metabolic Activity during Multiple Growth Phases
Expected XTT
XTT
DCW
Glucose
DCW (g/kg)
XTT Absorbance (OD460)
Time (h)
Adapted from Moss, Kim, Nandakumar, Marten, Biotechnolo. Prog, 24:1 (2008)
• XTT proportional to # of cells
• Detects death phase
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