Integrated Masters in Chemical Engineering

# *Study of Autophagy Induced Changes in Fungal Mycelia*

## Master's Thesis

by

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**Developed in the field of Dissertation**

conducted in

**University of Baltimore Maryland County**

UMBC
AN HONORS UNIVERSITY IN MARYLAND

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U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

**Chemical Engineering Department**

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Mestrado Integrado em Engenharia Química

# *Estudo de Modificações Induzidas Por Autofagia em Micélio de Fungos*

## Tese de Mestrado

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**Desenvolvida no âmbito da disciplina de Dissertação**

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## Resumo

Fungos filamentosos são um dos organismos mais importantes para o homem. Estes têm um grande impacto para o homem como um agente patogénico tanto para humanos como colheitas. São também um dos organismos mais importantes usados em bioprocessos. A autofagia é um fenómeno omnipresente em células eucariotas. É um processo onde as células reciclam os seus conteúdos citoplasmáticos, activado quando as células estão privadas de uma fonte externa de nutrientes. Estudos demonstraram que a autofagia pode causar modificações nos fungos que por sua vez levam a consequências positivas em parâmetros de bioprocessos como a transferência de massa. Outros estudos demonstraram que, quando privados de uma fonte externa de carbono, os fungos sofrem um processo de modificações morfológicas, como consequência directa da indução de autofagia.

O objectivo deste projecto é o estudo das modificações morfológicas causadas por autofagia e o seu impacto nas propriedades mecânicas da parece celular dos fungos.

O ensaio de simulação de alimentação por pulsos revelou algumas consequências da privação de carbono externo na taxa de crescimento do micélio, no entanto não foi conclusivo se esta alteração é ou não definitiva. Os ensaios de AFM não produziram quaisquer dados, foi então impossível identificar quaisquer diferenças nas propriedades materiais da parede celular dos fungos.

Estes resultados dão algumas indicações interessantes que podem vir a ser exploradas em trabalhos futuros.

Palavras-chave: autofagia, microscópio de força atómica, micélio de fungo, *Aspergillus oryzae*.

## Abstract

Filamentous fungi are one of the most important microorganisms to man. They have a major impact as a pathogenic agent to humans and crops, but also are one of the most used organisms used in bioprocesses. Autophagy is a phenomenon omnipresent in eukaryotic organisms. It is a process in which the cells recycle their own cytoplasm constituents when deprived of an exterior nutrient feed. Studies have shown that autophagy can lead to some changes fungi that lead to positive consequences in bioprocess parameters such as mass transfer. Other studies have shown that when deprived of an exterior carbon source, fungi undergo a process of morphological changes due to induction of autophagy.

The objective of this project was to study the morphological changes caused by autophagy, and the impact of these on the cell wall of fungi.

The pulse feeding simulation assay revealed that there were some consequences of the carbon deprivation on the fungi on the growth rate, though there were no conclusions on if these were or not definitive. AFM assays gave no data, so it was impossible to assess any differences in cell wall material properties.

These results provide some interesting views that can be explored in different ways in the future.

Key-words: Autophagy, Atomic Force Microscopy, fungal mycilia, *Aspergillus oryzae*.

# Index

# Glossary

| | | |
|---|---|---|
| E | Elastic modulus | Pa |
| h | Cell wall thickness | m |
| $K_{canti}$ | Cntilever spring constant | Pa.m |
| $K_{CW}$ | Spring constant of the cell wall | Pa.m |
| m | Slope | $N.m^{-1}$ |
| R | Radius of the hyphae | m |

**List of acronyms**

| | |
|---|---|
| AFM | Atomic Force microscopy |
| EM | Electron Microscopy |
| *A. nidulans* | *Aspergillus nidulans* |
| *A. oryzae* | *Aspergillus oryzae* |
| G- | Carbon deprived medium |
| TOR | Target of rapamycin |
| YG | Rich medium |

# 1. Introduction

## 1.1. Background and presentation of the project

### 1.1.1. Mycology

Mycology is the study of the fungi kingdom, one of the organisms with most impact on the bioengineering industry.

Looking at the history of classification of organisms, the earlier classifications defined all organisms to either plants or animals, as so fungi were included in the plant kingdom due to their immotility and having a cell wall. In 1866 this increasingly outdated approach was changed. It was then proposed a system that separated microscopic organisms such as fungi, algae and bacteria into a group called protist or protista. The modern classification system, designated as the Five kingdoms approach. It recognizes two domains: prokaryote, the bacteria domain; and the eukaryote domain for the plant, animals, fungi and protists.

In terms of growth fungi show some diversity. Most of fungi are mycelia, growing a network of hyphae and are commonly referred as moulds. There are also some more primitive fungi that often have single rounded cells or dichotomously branched cells, attached to a food source by narrowing rhizoids. Others grow as unicellular yeasts and reproduce either by budding or binary fission. The main differences in fungal growth can be explained by the different environments where these fungi grow. Some fungi show different types of growth depending on the environment they found themselves in. Examples of these can be foung in fungal pathogens of higher animals, these fungus grow as yeasts for spread in water films or body fluids, and as hyphae for invasion the tissues (Deacon 1980).

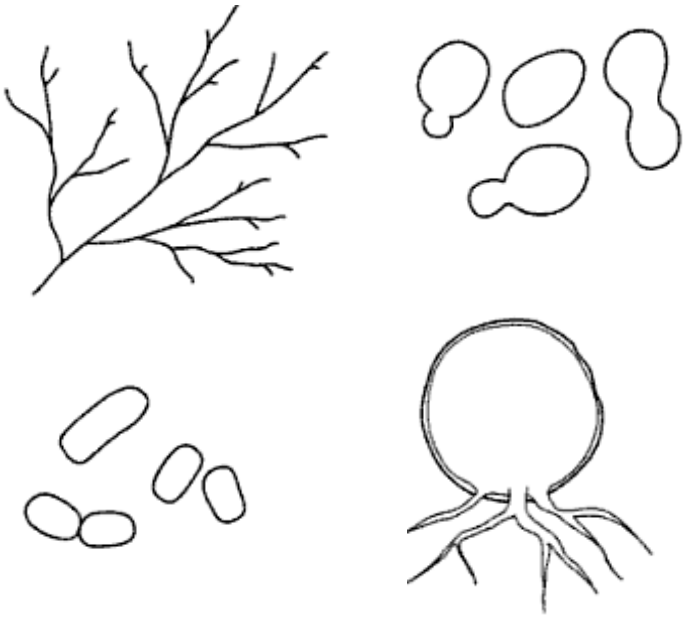

**Figure 1- Forms of fungal growth. From the top right: mycelial, budding, fission and chytridiaceous growth with rhizoids.**

In industry yeasts and moulds are widely used in the food industry, pharmaceutical industry. Some examples are *Saccharomyces cerevisiae* is used in the baking and brewing industry, while *Penicilium* strains are used in the production of the famous camembert cheese and in pharmaceutical industry to produce some antibiotics, most famous of which the penicillin.

The fungal impact on human life is not all of a positive manner, millions of dollars worth in crops are lost due to fungal infection.

In bioprocess involving filamentous fungi mass transfer is one of the main challenges due to the poor mixing of the highly viscous medium. (Wang et al., 2007)

### 1.1.2. Autophagy

Autophagy is a non-selective catabolic process ubiquitous to eukaryotic cells, in which the cells recycle their own cytoplasmic constituents. Autophagy plays numerous roles in cellular regulation such as nutrient recycling, cellular degradation, cell death and cellular differentiation. Autophagy can be induced by nutrient starvation or by certain compounds such as the drug rapamycin.

Most of the molecular understanding of autophagy comes from studies employing the yeast *Saccharamyces cerevisiae*, where it has been showed that autophagy is negatively regulated by TOR kinase.(Kim, Islam et al. 2011)

Numerous studies have shown that autophagy induces changes in the behavior of fungal growth, leading to morphological changes. Previous studies have showed that carbon starvation can induce fragmentation due to cell wall degradation((Lahoz et al., 1986);(McNeil, Berry et al. 1998)) or due to physiological changes such as increased expression of hydrolitical enzymes(McIntyre, Berry et al. 2000) or increased vacuolation(Cox, Paul et al. 1998). Segmented deprivation of carbon source have been shown to improve important parameters on bioreactor growth of filamentous fungi, such as viscosity, (Bhargava, Nandakumar et al. 2003) oxygen mass transfer(Bhargava, Wenger et al. 2003) and even productivity(Bhargava, Wenger et al. 2003).

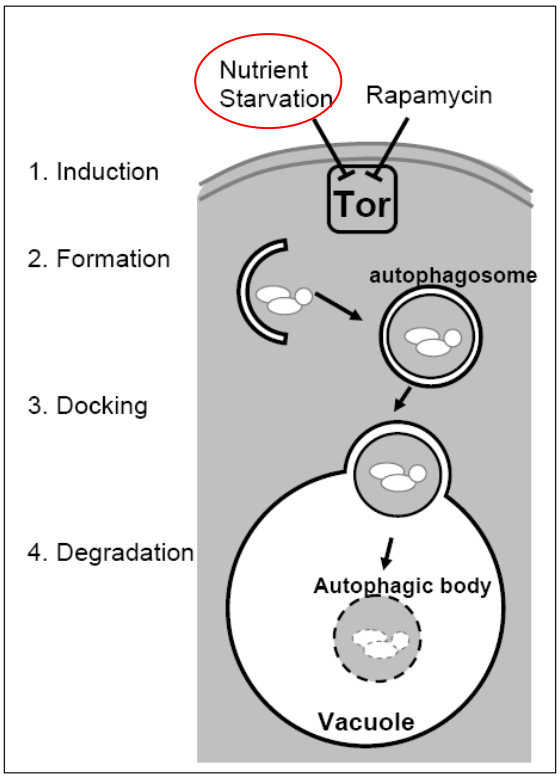


**Figure 2- Representation of the autophagy general mechanism(Pollack, Harris et al. 2009)**

Nutrient starvation inhibits the Tor kinase activity, inducing the formation of a double-membrane vesicle, sequestering the target molecule and cytosol, forming an autophagosome. Then the autophagosome couples on the vacuole, and the outer membrane fuses with the vacuolar membrane. Once inside the autophagic body is degradated by the vacuolar enzymes.

Most of the information available on autophagy came from studies on yeast. Yeast is one of the more simple organisms and is of major interest for industry, so it makes a perfect object for study. But due to some findings on relations between autophagy, infection resistance (Hayward, Tsao et al. 2009) and aging process(Vellai 2009) has triggered interest on many other cell types, such as plant and mammalian cells.

Autophagy has been studied in diverse number of organisms from yeast to human cells.

Recent studies have found that other types of stress to the cells, other than nutrient starvation and rapamycin trigger autophagy.(Yorimitsu and Klionsky 2007) Other studies have found that rapamycin and nutrient starvation have different effects on cells. (Kim, Islam et al. 2011) The knowledge on autophagy is growing by the day and more interesting ways to study autophagy as well.

The study of the behavior of organisms and the adaptations made by these when presented with variations to its environment conditions is of outmost

importance. In industry for example, mass transfer in a bioreactor is one of the most difficult problems in a production process. Understanding how an organism adapts when it is deprived of an external food source may help find new ways to respond to these problems.

Pollack et al., 2008 through the use of a flow chamber, studied morphological changes in fungi during a deprivation of an exterior source of carbon. They observed different phases of the organism transformation. First the cells stop growing and during this period the vacuoles grow then the cell starts to consume the content of these vacuoles and restarts its growth, but with reduced cell growth rate.

**Figure 3- Fungal hyphae with increased vacuolation and newly grown tip on carbon deprived medium (Pollack, Li et al. 2008)**

Bhargava et al. describes a work where a step feeding was performed on a bioreactor to study the influence of autophagy on the viscosity of the medium. As was mentioned before the broth of a filamentous fungi bioprocess is characterized as having a high viscosity due to the mycelium clusters formed naturally by these organisms. They verified that when the step feeding was performed, the viscosity of the broth decreased. This suggested that carbon induced autophagy influenced the cell wall properties.

### 1.1.3. Atomic Force Microscopy

Atomic Force Microscopy (AFM) or scanning force microscopy (SFM) is a technique that allows nanoscale analysis of material properties. AFM can be included in a wider group of techniques named scanning probe microscopies (SPM). The basis of these techniques is that the surface of the sample is scanned by a probe, following parallel lines, measuring local interactions between the probe and the sample in the near-field region, and recording the value for each position.(Santos and Castanho 2004) The atomic force microscope (AFM) was invented as a new application of the scanning tunneling microscope (STM) in 1985 by Binning, Quate and Gerber. Their concern was the measurement of forces that entered the dimension of atoms. To respond to this problem they proposed that the STM would be used to monitor the movement of a cantilever beam. This would allow obtaining a level of sensibility on the regime of inter-atomic forces between single atoms. (Binnig, Quate et al. 1986)

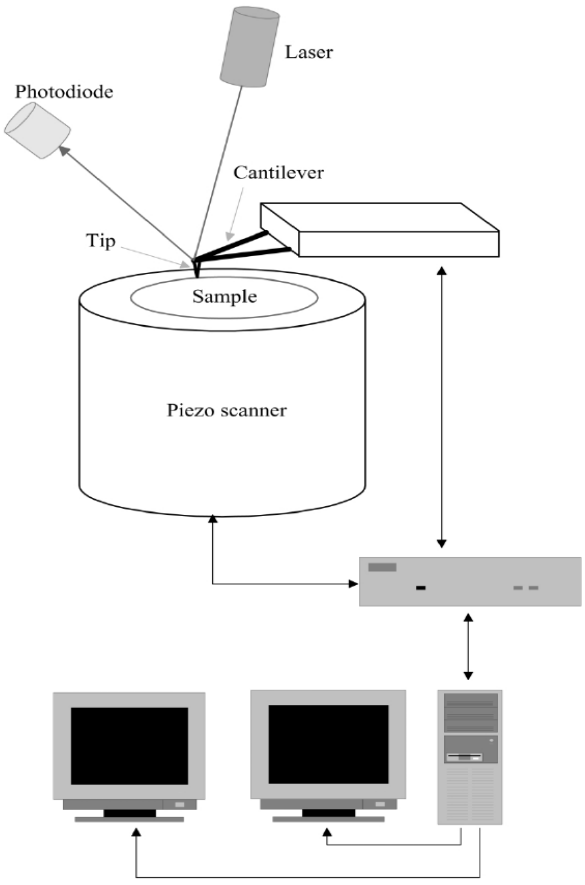


**Figure 4- Typical assembly of the AFM apparatus (Santos and Castanho 2004)**

The AFM has been used for a wide variety of applications in areas such as electronics, mechanics and biological. These applications can be divided into two main groups, topological and non-topological. The non-topological consist on the use of the tip not only as a probe but also as tool for sample manipulation capable of cutting, dragging, dissection or conformational alteration. This characteristic makes the AFM a versatile tool of great value. The AFM was originally developed to study the morphology of electronic conductors (Binnig, Quate et al. 1986), but due to its sensibility made its way into biological studies. AFM also allows to perform studies of cells in their biological state.

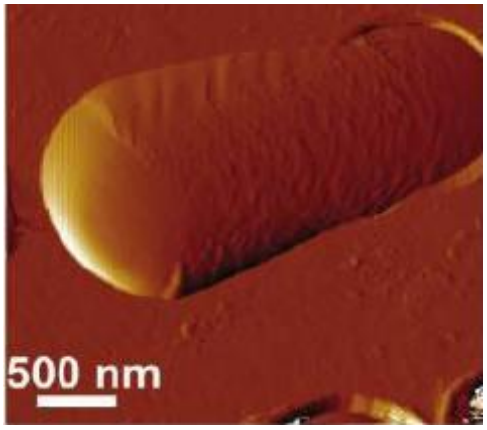


**Figure 5- AFM image of *Lactobacillus plantarum* (Andre, Deghorain et al. 2011)**

One of the most interesting applications of the AFM is in the usage in force spectroscopy mode. This allows the AFM to register the cantilever deflection as a function of the vertical displacement. This results in a Force vs. scanner displacement curve, which can be transformed into a force-distance curve.(Dufrene 2008)

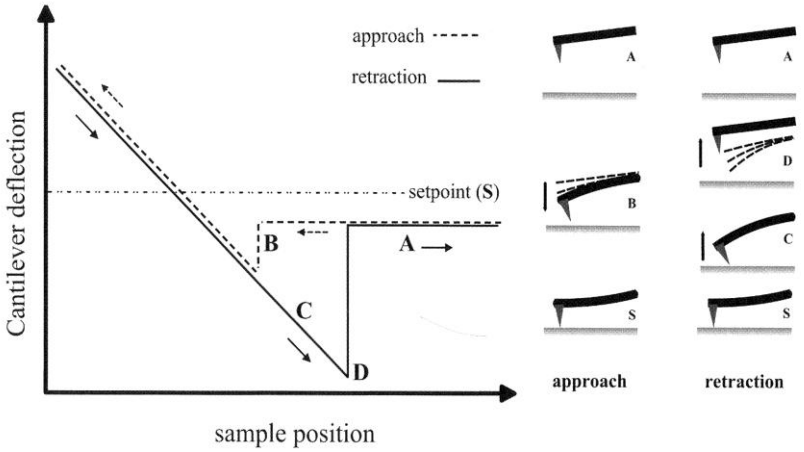


Figure 6- Typical cantilever deflection vs. sample position curve (Ricci and Braga 2004)

## 1.2. AFM for biological applications

The application of AFM to biological studies has was a natural step due to the sensibility it offers in studies on a nanoscale level as well as the possibility of study cells in their biological active state. It has been widely applied in studies of topography of cellular wall on a different number of cells. ((Andre, Deghorain et al. 2011); (Gad, Itoh et al. 1997); (Morris, Gunning et al. 1997); (Zhao, Schaefer et al. 2005);(Shilo, Ravichandran et al. 2004);(van Der Mei, Busscher et al. 2000);(Dufrene, Boonaert et al. 1999)) But the AFM does not limit itself to topological application, during its evolution, other non-topological applications have been developed. Cutting, dragging, dissection or conformational alterations have already been successfully applied in studies of cells, virus, proteins, lipoproteins and planar lipid membranes.(Santos and Castanho 2004)

The main challenge with the usage of AFM for studies on cells on their physiological state is the development of a support for the cells. The cells must be adhered to the support such that they don't move when the tip probes them.

$$E = 0.8\frac{K_{CW}}{h}\left(\frac{R}{h}\right)^{1.5}$$ (Zhao, Schaefer et al. 2005)

This is the equation for assessing the elastic modulus (E), where $K_{CW}$ is the spring constant of the cellular wall, h is the thickness of the cellular wall and R is the

hyphae radius. The $K_{CW}$ constant can be obtained through the graph of Force vs. Vertical displacement obtained through AFM tests. The R and h can be obtained by electron microscopy (EM).

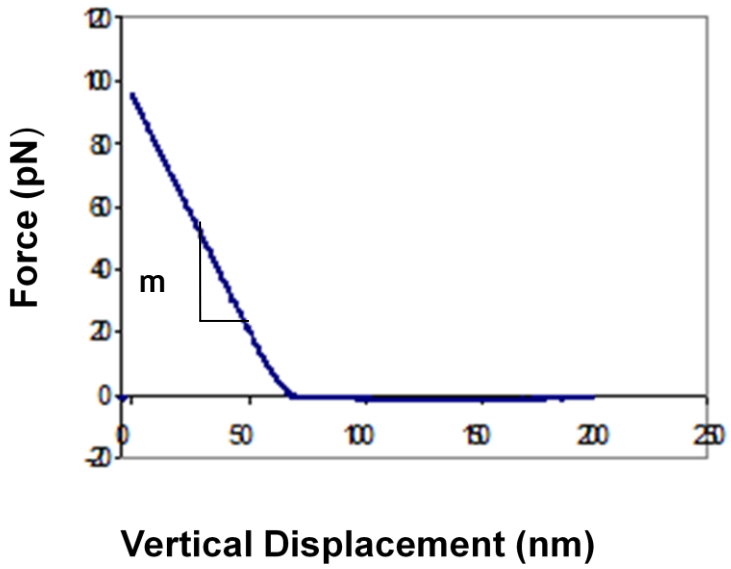


**Figure 7- Schematic of a Force vs. displacement plot obtained through AFM measurements**

$$K_{CW} = \frac{(K_{Canti} \times m)}{(1-m)}$$ (Zhao, Schaefer et al. 2005)

$K_{Canti}$ corresponds to the spring constant of the cantilever and the m is the slope of area of the graph corresponding to the contact with the sample.

## 1.3. Contributions of the work

The work conducted gives some contribution to the study of autophagy in fungi, mainly on the carbon starvation induced autophagy. Growth curves of cellular mycelium were made in order to understand the consequences of carbon deprivation on fungal growth.

Improvement of a standard protocol for adhesion of spores to cover slips for AFM experiments was done.

## 1.4. Thesis Organization

The thesis is organized in several sections.

In the first chapter a brief introduction to autophagy and AFM is presented. These help understand he interest and importance of the study conducted during the project. Some examples of studies conducted on autophagy and other interesting works involving AFM are referred.

In the following chapter, the technical description, the preparation of the work conducted is described.

In the Results and discussion chapter, the main results of the work are presented, as well as the main conclusion from the results obtained and some indications on future work to be done on the matter.

Finally the last chapter is the evaluation of the work, where personal notes of the work conducted are given.

# 2. Technical Description

## 2.1. Materials and Methods

An industrially relevant *Aspergillus oryzae* strain (AMG #13), obtained from J. Lehmbeck (Novo Nordisk A/S, Bagsvaerd, Denmark), is a transformant of IFO4177 (Institute for Fermentation, Osaka, Japan). This strain contains the *Aspergillus niger* glucoamylase gene, under control of the *A. oryzae* TAKA a-amylase promoter on a pBoel-777-like construct (Christensen et al., 1988), and has been the subject of previous studies (Amanullah et al., 1999; Bhargava et al., 2003a,b,c, 2005; Bocking et al., 1999).

The AFM and imaging of fungi requires the spores to be adhered to a surface. The surface chosen was glass and for the spores to adhere to the surface of the glass concanavalin-A was chosen. The glass had to be treated with sodium hydroxide in order to the concanavalin-A to coat the cover slips. The NaOH reacts in the surface of the glass to form Si-OH which increases the glass surface affinity to hydrophilic compounds. The concanavalin-A is a protein extracted from the plant *Canavalia ensiformis* and it bonds specifically to carbohydrates through hydrogen bond.(Edelman and Wang 1978) Concanavalin-A type IV, lyophilized powder from *Canavalia ensiformis* obtain from Sigma-Aldrich™.

## 2.2. Cell Growth Curves

**- Cover slip preparation**

The cover slips were prepared by first cleaning them with filter sterilized alcohol (C2H5OH), and then with filter sterilized sodium hydroxide (NaOH). This treatment with sodium hydroxide will cause a chemical modification in the surface of the glass allowing the protein Concanavalin-A to bind to the glass. Then the cover slips were taken to the laminar hood, there they were washed with filter sterilized water (H2O) by dipping those 2 times. Concanavalin-A coating was done by bathing the cover slips on a solution for 15 min. Place the sterile Teflon strips on a Petri dish. Take the cover slips, wash them again in filter sterilized water by dipping them 3

times. Place the cover slips on the Teflon stripes and place the oven at 60 ºC for 1 hour. The cover slips were taken from the oven and on the laminar hood the spores were adhered by … with spore solution. Place the cover slips with the adhered spores on the incubator for 1 hour. Transfer the cover slips into a Petri dish filed with Medium solution and proceed with the planed experiment.

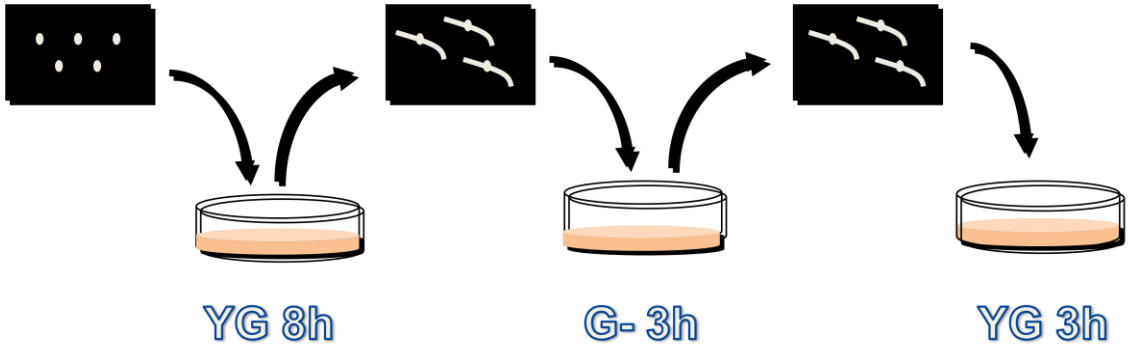


**Figure 8- Schematic of the imaging experience**

The YG medium was prepared by adding 50 ml 20x salt mix, 2 g yeast extract to 930 ml d.i. water. The pH of the solution was then corrected to 6.5 through addition of HCl and then autoclaved. In the hood add 5 ml of MgSO4, 1 ml of trace solution (Hutner's), 15 ml of 20%m/V glucose solution (final concentration 3 g/L of glucose).

The G- medium was prepared by adding 50 ml of 20x salt mix to 954 ml of d.i. water. The pH of the solution was then corrected to 6.5 by adding HCl and then autoclaved. In the hood was added 5 ml of MgSO4, 1 ml of trace solution (Hutner's).

For mycelia morphology assessment the cells were grown in YG medium for 8h, then transferred to G- for 3h and finaly to YG again for 3h. Pictures of the samples were taken hourly since the 8h time point.

The control was performed by growing the spores in YG medium for 14h, taking hourly samples from 8h time point. The pulse feeding simulation was done by growing the cells for 8h in YG medium. At that point the cover slips were transfered to G- medium and growing the cells for 3h, and then tranfered again to YG medium and growing them in there for 3h. The samples were taken hourly from the 8h time point.

Cell growth curves were prepared by taking between 15 and 20 images of individual mycelia on an optic microscope with 16x magnification at different time

points. The projected area was the measured magnitude used for growth representation. The images were then processed using Image Pro. The program then counts the number of pixels on the area of the mycelia. With an hemocytometer image, on the same magnification, we get an correlation between pixels and area.

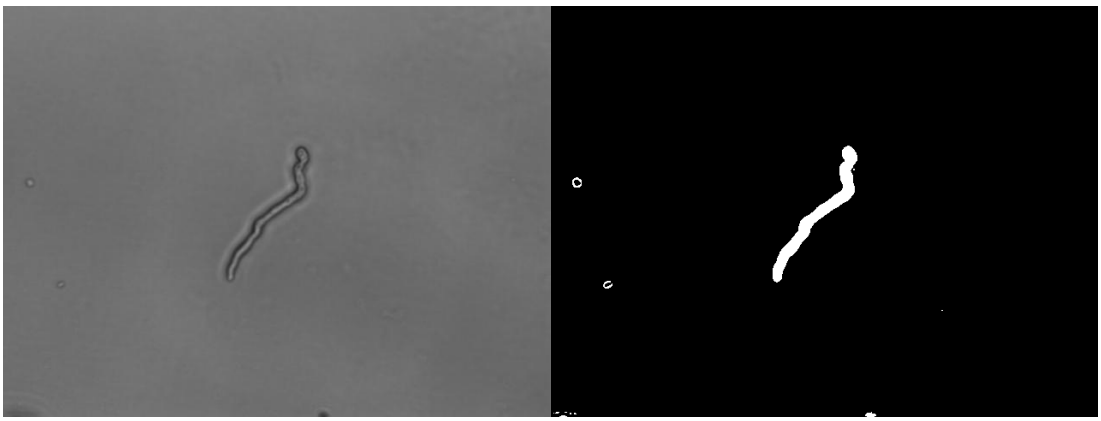

**Figure 9- fungal mycelia image before and after processing(16x magnification)**

## 2.3. AFM experiments

For the AFM experiments the preparation of the spore solutions is described in appendix, the growth mediums used are descreibed in point 2.2.

Cover slips were prepared by first cleaning it with filter sterilized alcohol ($C_2H_5OH$), and then with filter sterilized sodium hydroxide (NaOH). Take the cover slips and wash them with filter sterilized water ($H_2O$) by dipping those 2 times. Bathe them in Concanavalin-A solution for 15 min. Place the sterile Teflon strips on a Petri dish. Take the cover slips, wash them again in filter sterilized water by dipping them 3 times. Place the cover slips on the Teflon stripes and leave them to dry at room temperature for 48h. Take the cover slips to the laminar hood and adhere the spores onto the cover slips. Place the cover slips with the adhered spores on the incubator for 1 hour. Transfer the cover slips into a Petri dish pre-filed with Medium solution and proceed with the planed experiment. After the 8h, transfer the cover-slips to the G- media Petri dish. After 2 hours take the cover-slips carefully from the Petri dish and place them on to Teflon slips. Leave them to dry at room temperature. Cover the top of the cover-slips with sodium azide.

The cover slips were then taken to the Veeco Bioscope™ II Atomic Force Microscopy microscope where the force measurements were performed. For this the topography of the mycelia was needed. For that an analysis area, tip velocity and resolution is needed to be defined. The analysis area was around 20 $\mu m^2$, 0.1 m/s for tip velocity and 128 passages was defined as resolution parameter.

# 3. Results and discussion

## 3.1. Morphological changes in fungal mycelium

Images of fungal mycelium were taken to observe the morphological changes when autophagy was induced by carbon starvation. The cells were grown on rich medium for 6h, when the first image was taken, and then transferred to a poor medium in carbon content. After 2h on this medium, new images were taken. The results are shown in figure 4 A and B.

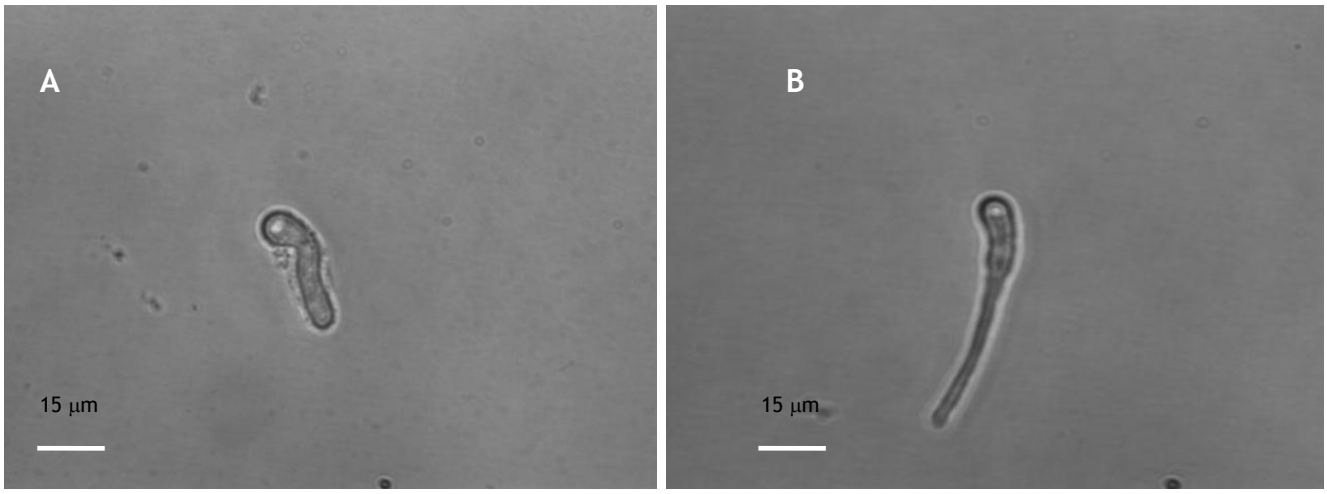


**Figure 10- *A.oryzae* microscope images(64x magnification) at 6h in rich medium growth (A) and at 6h YG medium growth plus 2h G- growth (B)**

The morphological changes observed corresponded to the ones reported on Pollack et al., the increased vacuolation with a no growth stage, followed by re-growth of a thinner mycelium.

Then growth curves of *A.oryzae* were traced. Samples were taken on an hourly fashion. The mycelium growth can be best described as an exponential curve. Duplicates were made to assess if the assay was reproducible.

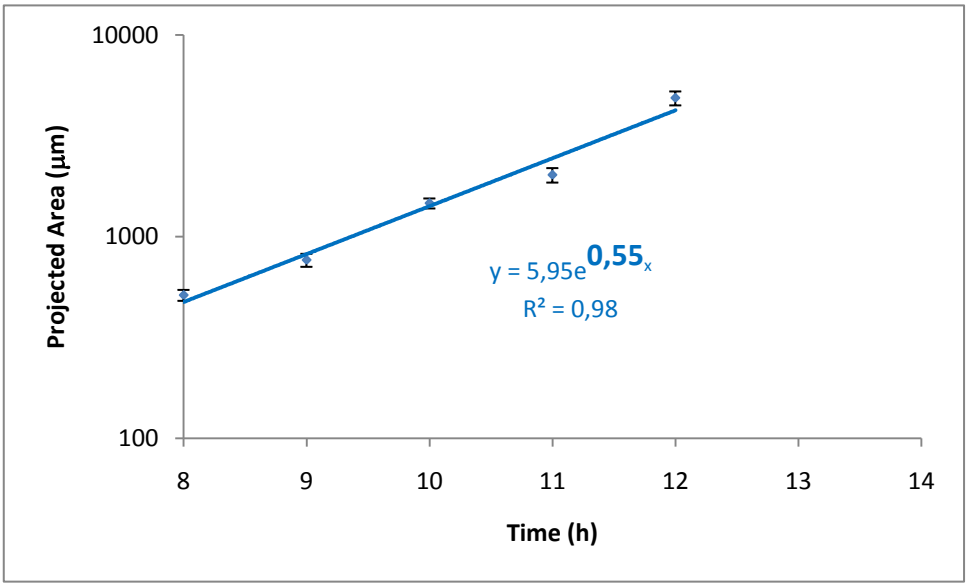


**Figure 11- Growth curve of *A. oryzae* on YG medium**

The growth rate of *A. oryzae* mycelia under favorable conditions was found to be ~0,55 $h^{-1}$.

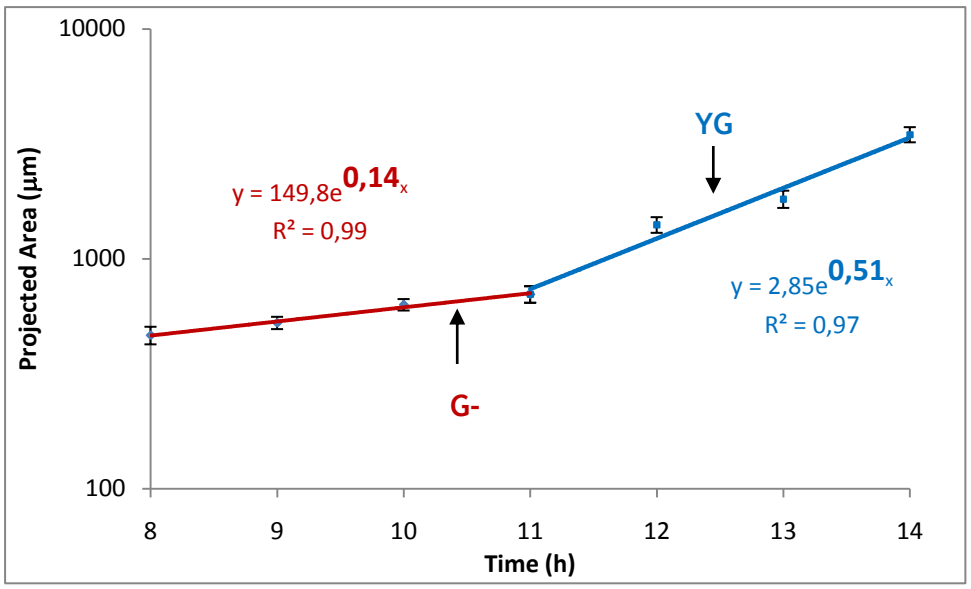


**Figure 12- Growth curves of *A. oryzae* of simulated step feed**

First we looked at the change in growth rate when the mycelium was transferred to the G- medium. In comparison with the control assay the growth curve decreased dramatically. This result was no surprise as it was previous evidenced on Pollack et al.(2008). The growth during this period indicates that the cells were using endogenous carbon.

The cells were then transferred to YG medium again to assess if the carbon starvation had a permanent effect on the fungi. The fungi had an increased growth rate form their previous state, though it was still below their original growth rate. This indicates that the fungi are recovering their typical growth, though it is not safe to assume that full recovery is achieved.

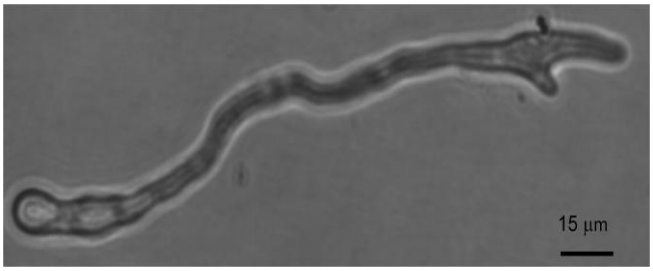


**Figure 13- A.oryzae grown in the sequence: YG 6h, G- 3h and YG 3h.(64x magnification)**

## 3.2. Force Measurements on AFM

The purpose of this study was to help figure if autophagy, in addition to induce morphological in the fungi, it would cause changes in material properties of the fungi cell wall, and if so quantify those differences.

As discussed previously in AFM applications in biotechnology, one of the main limitations of this equipment is the sample preparation(Santos and Castanho 2004). The AFM requires the sample fixed to a solid support, in addition to its sensitivity to any vibrations, which can lead to a bad reading by the equipment. The protocol used for the AFM experiments was similar to the one used by the laboratory for the imaging experiments. The major difference was on the drying time and temperature of the cover slip posterior to the treatment with Concanavalin-A. This should strengthen the bond between the Concanavalin-A and the glass, which would improve the bond between the spore and the cover slip, thus making the fungi less susceptible to movement when the probe contacts with it.

After the observation that the protocol was inefficient for the assays that were conducted, alterations were made and new assays were conducted to see if these would improve the quality of the adhesion.

On the first set of assays there were not enough spores adhered to the cover slips to do the assays. An increase of the spore concentration was used and it worked.

Then using poly lysine instead of concanavalin-A. This compound was used in Zhao et al., 2005 for the assessment of elastic properties of the fungal wall of *Aspergillus nidulans*. This was proven ineffective due to the lack of adhered spores on the cover slip.

Then it was decided to increase the concentration of concanavalin-A to double its original value. This alteration could be responsible for a phenomenon observed during the assays that prevented the assay to work, which was upon engagement the cover slip would stick to the tip holder of the AFM. The increase in concentration of concanavalin-A could be responsible in the sense that it increased the affinity of the cover slip to other materials.

Even though all these changes were performed still it was not possible to obtain any useful data that would help respond to the objective proposed.

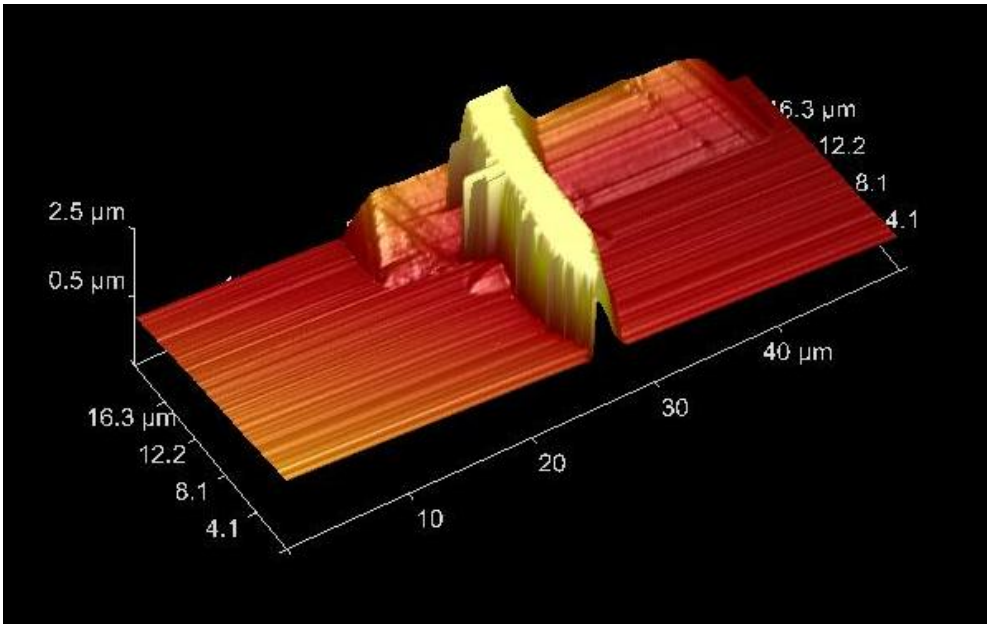


**Figure 14- 3D image of hyphae tip on AFM**

The stretching that is visible on the hyphae image is due to the drag caused by the tip.

### 3.2.1. Future work

There is plenty of ways to explore the findings reported here. One could prolong the period of the assay and find exactly if and when the cells recover their prior growth rate. Also the growth rate prior to the change to G- medium should be studied.

Pollack et al.(2008) describes a method to study an individual mycelia through a period of time using a flow chamber. In the article mentioned it is showed that certain information is not evident when looking at averages of large samples. This indicates that a flow chamber assay could give other important information on the matter.

On this work the focus was on modifications to the protocol for the possibility to acquire data of the AFM. The AFM equipment offers the possibility of tuning of certain analysis parameters to be made. On future work this side could be explored for some improvement of the analysis.

Other possibility would be the use of another cellular strain like *A. nidulans*. Zhao et al., 2005, has already made AFM assessment of *A. nidulans* cell wall mechanical properties. But it was not clear if the morphological changes were as apparent as in *A. oryzae*. In Appendix 2 it is presented images of *A. nidulans* after autophagy was induced by carbon deprivation. The differences in the newly grown mycelium were not as apparent as in A. *oryzae* though the thickness of the newly grown mycelium was notably distinct.

# 4. Evaluation of the work

## 4.1. Objectives accomplished

The changes in mycelium morphology and growth rate were successfully studied, and some conclusions were taken from the studies conducted.

Differences in mycelium material properties were not studied due to lack of data on the mater.

## 4.2. Limitations and future work

There were several limitations during the accomplishment of the objectives of the work. The main limitation of the project was time as t seemed to be a very ambitious project for the limited time disposed for its execution. The AFM and imaging experiments were very time consuming which limited the amount of experiments that were conducted.

The future work was as well mostly discussed on the main text.

There is another technique that would be needed to implement for the retrieval of an elastic modulus, as described in Zhao et al., 2005. For the elastic modulus there would be needed a force curve on the AFM and cell wall thickness data through EM. EM was not considered for usage in this project due to the lack of time.

## 4.3. Final appreciation

The objectives of this project were not all accomplished, which was somewhat disappointing.

The struggle to get the AFM assays running were so much time consuming that there was little time left to do anything else. Three months of the project were dedicated to this, the last month was dedicated to the imaging assays in an effort to get some results that would be interesting to present.

The overall experience was useful as a learning process. It served as a learning experience in an intellectual level as several useful techniques were learned, and on a personal level how to deal with problems in a work environment.

Although sometimes extremely frustrating, the experience was quite enjoyable and would recommend to anyone interested in doing the same.

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# Appendix 1

## Preparation of spore solution

**- Plate preparation**

Potato dextrose agar solution 40g/L was prepared on a 1L capped bottle with a large magnetic stirrer. No need to adjust pH (should be around ~6.4-6.6). The solution was immediately autoclaved (liquid cycle on autoclave). A package of sterile (unopened) Petri dishes was taken to the laminar hood following the measures of conduct for using this. Immediately after autoclaving, spray and put the capped bottle into the hood. The solution should remain hot (above 55 ºC) so that it does not solidify. 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. Close the dishes. Let them sit 2 hours in the laminar hood to solidify the solution.

**- Inoculating plates**

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 the solution on high for 1 min for releasing of the spores. Use a sterile inoculum loop to gently scrape off the surface of the agar. Vortex it again.

From Frozen Vial: From -80 ºC 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.

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 ºC for 5 days. This is enough time for sporulation to occur. Sporulation can be

confirmed by observing a white fluffy layer on top of the plate for *Aspergillus oryzae*.

**- Harvesting spores**

After sporulation occurs plates can be stored in the freezer for later utilization. For harvesting plates, these were moved the plates into the laminar hood. 4-5 ml of sterile 0.2 M phosphate buffer was added 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. Using a 25 or 50 ml pipette, aspirate as much of the spore suspension as possible (~2-3 ml). 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. 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 into a new 50-ml centrifuge tube. Measure the spore concentration with hemocytometer and microscope. According to the spore concentration dilute the spore solution to a concentration of 5E05 spore/L for AFM assay or 1E05 for microscopy assay.

**Medium preparation**

Solution A consisted of a solution of 12g of Monosodium Phosphate Monohydrate in 100mL of water. Solution B consisted of 14g of Disodium Phosphate Heptahydrate in 100mL of water. Phosphate Buffer was prepared by adjusting the pH of Solution A to 6.0 (initial pH ~4.8) with Solution B. For 20mL of Concanavalin-A solution was prepared by mixing 10.7 mg of Concanavalin-A powder, with 0.1mL of 1mM $CaCl_2$ solution and 20mL of Phosphate Buffer. Concentration of the solution around 0.5 g/L of Concanavalin-A.

20x salt mix solution were prepared by adding 120.0g of $NaNO_3$, with 10.4g of KCl, 16.3g of $KH_2PO_4$ and 20.9g of $K_2PO_4$ to 1L of de-ionized water. Mixture was then autoclaved (liquid cycle on autoclave) and stored at 4 ºC.

$MgSO_4$ solution was prepared by adding 10.4g of $MgSO_4.7H_2O$ to 100mL of de-ionized water. Mixture was then autoclaved and stored at 4ºC.

20% glucose solutions were prepared by adding 200g of glucose to 1L of de-ionized water. Mixture was then filter sterilized and stored at 4 ºC.

Hutners (Hutner's trace elements) solutions were prepared by adding 2.2g of $ZnSO_4.7H_2O$, 1.1g of $H_3BO_3$, 0.5g of $MnCl_2.4H_2O$, 0.5g of $FeSO_4.7H_2O$, 0.16g of $CoCl_2.6H_2O$, 0.16g of $CuSO_4.5H_2O$, 0.11g of $(NH_4)_6Mo_7O_{24}.4H_2O$ and 5.0 g of EDTA to 100 mL of de-ionized water. Heat till boiling occurs to assist dissolving and let it cool to 60 ºC. The pH was adjusted to 6.5-6.8. Solution was then filtered sterilized using 0.22 μm filter and stored at 4 ºC.

# Appendix 2

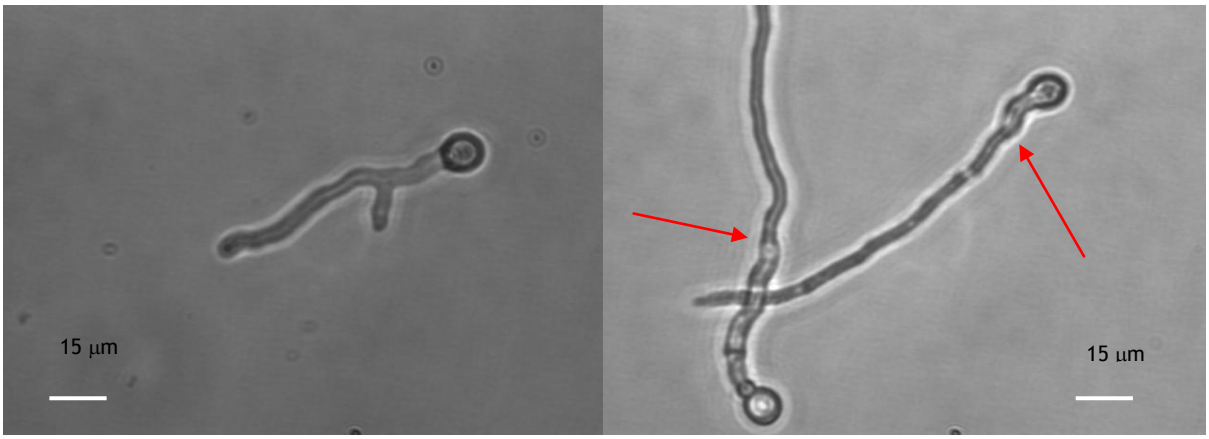


**Figure 15- *Aspergillus nidulans* grown 8h in MAG UU (A) plus 3h in MM (B)**

The changes were observed on *Aspergillus nidulans*, regrowth is marked by the red pointers.